Constitutively active TrkB confers an aggressive transformed phenotype to a neural crest derived cell line

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Abstract

Neuroblastoma arises from sympathoadrenal progenitors of the neural crest and expression of the neurotrophin receptor TrkB and its ligand, brain-derived neurotrophic factor (BDNF) is correlated with poor prognosis. Although activated TrkB signaling promotes a more aggressive phenotype in established neuroblastoma cell lines, whether TrkB signaling is sufficient to transform neural crest derived cells has not been investigated. To address the role of TrkB signaling in malignant transformation, we removed two immunoglobulin-like domains from the extracellular domain of the full length rat TrkB receptor to create a ΔIgTrkB that is constitutively active. In the pheochromocytoma-derived cell line PC12, ΔIgTrkB promotes differentiation by stimulating process outgrowth; however, in the rat neural crest derived cell line NCM-1, ΔIgTrkB signaling produces a markedly transformed phenotype characterized by increased proliferation, anchorage-independent cell growth, anoikis resistance, and matrix invasion. Furthermore, expression of ΔIgTrkB leads to up-regulation of many transcripts encoding cancer-associated genes including cyclind1, twist1, and hgf, as well as down-regulation of tumor suppressors such as pten, and rb1. In addition, ΔIgTrkB NCM-1 cells show a 21-fold increase in mRNA for MYCN, the most common genetic marker for a poor prognosis in neuroblastoma. When injected into NOD SCID mice, control GFP NCM-1 cells fail to grow while ΔIgTrkB NCM-1 cells form rapidly growing and invasive tumors necessitating euthanasia of all mice by 15 days post injection. In summary, these results indicate that activated TrkB signaling is sufficient to promote the formation of a highly malignant phenotype in neural crest derived cells.

Keywords

Neuroblastoma; neural crest; transformation; TrkB; MYCN

Conflict of interest

The authors declare no conflict of interest.

Supplementary information is available at the Oncogene website (http://www.nature.com/onc)
Introduction

Neuroblastoma, a pediatric malignancy arising from sympathoadrenal precursors from the neural crest, is a cancer typified by its heterogeneity of disease. Disease course in neuroblastoma can range anywhere from patients presenting with metastatic disease that will spontaneously regress with support treatment alone (stage 4S), to localized favorable tumors, to cases of aggressive neuroblastoma, in which children will often relapse following treatment despite the most intensive chemo- and adjuvant therapy (1).

This heterogeneity in neuroblastoma tumor properties is correlated with a number of different factors including Trk receptor expression (2). Trk receptors are important in normal sympathetic development; for example, TrkA, the high affinity receptor for nerve growth factor (NGF), promotes target-dependent survival of sympathetic neurons by preventing programmed cell death (3, 4) and neuroblastoma tumors that express TrkA have a favorable prognosis (5). In contrast, many MYCN amplified, poor prognosis neuroblastomas express TrkB, resulting in tumors that are often highly aggressive and eventually fatal (6). Because TrkB promotes plasticity, differentiation and survival of primary neurons, the aggressive phenotype correlated with TrkB expression was puzzling; however, we discovered that TrkB is transiently expressed in sympathetic progenitors prior to the onset of TrkA expression, and when stimulated with BDNF, the TrkB expressing cells proliferate in cell culture (7, 8) and in vivo (Straub and Nishi, unpublished observations).

An important question is whether TrkB expression is a marker of poor prognosis, or whether active TrkB signaling is directly responsible for the aggressive nature of poor prognosis neuroblastoma. Supporting a causal role for TrkB signaling, concomitant expression of full length TrkB and BDNF leads to autocrine signaling enhancing tumor cell survival and invasiveness (9), while expression of a truncated TrkB isoform lacking the tyrosine kinase domain is commonly found in more benign and differentiated tumors such as ganglioneuroblastomas (10). Furthermore, treatment of TrkB-expressing SMS-KCN neuroblastoma cells with BDNF enhances cell survival in serum free media (11). Similarly BDNF treatment of SH-SY5Y cells either transfected with TrkB or induced to express TrkB by retinoic acid have enhanced survival in conditions of limited growth factors (9, 12), increased resistance to chemotherapeutics (13–15), increased production of angiogenic factors (16, 17), and enhanced invasion (9). Therefore, TrkB signaling contributes to the aggressiveness of poor prognosis neuroblastoma, but it is still unknown whether TrkB signaling alone can transform cells of the neural crest lineage.

In order to determine if constitutively active TrkB signaling is sufficient to transform cells, we created a mutant form of the TrkB receptor by removal of two immunoglobulin-like ligand binding domains in the extracellular portion of the receptor. This construct is constitutively active and, when stably transfected into a normal neural crest-derived cell line NCM-1, promotes a highly malignant phenotype in vitro and in vivo.
Results

Removal of two immunoglobulin-like domains renders TrkB constitutively active

Previous studies have shown the TrkA tyrosine kinase receptor can be rendered constitutively active by removal of the two immunoglobulin-like (Ig-like) domains in the extracellular region of the receptor (18). Therefore, we created a similar construct (ΔIgTrkB; Figure S1) and stably transfected HEK293 cells with ΔIgTrkB and full length wild type (WT) TrkB. Expression of both types of receptor were confirmed (Figure 1a). A marked increase of phosphorylated Erk 1/2 is observed when WT TrkB HEK293 cells are treated with BDNF and ΔIgTrkB HEK293 cells (Figure 1a) albeit at significantly lower levels when compared to the BDNF-stimulated WT TrkB (Figure 1b). Therefore, we tested whether this level of constitutive signaling by ΔIgTrkB was sufficient to promote downstream biological effects.

ΔIgTrkB promotes neurite outgrowth in PC12 cells

PC12 cells are a well-known model of nerve growth factor (NGF) induced neuronal differentiation via TrkA (19, 20), and TrkB transfected PC12 cells differentiate in response to BDNF (21). PC12 cells were transiently transfected with ΔIgTrkB or GFP control construct, and two days later treated with or without 7sNGF (1μg/mL). After 6 days, cells were fixed and stained for either TrkB or GFP to identify transfected cells. Cells transfected with ΔIgTrkB show a 6-fold increase in the number of neurite bearing cells over GFP transfected cells in the absence of NGF (Figure 2a–c). The ΔIgTrkB-induced neurite outgrowth is equivalent to that of NGF through TrkA as there is no difference in the number of neurite bearing cells between ΔIgTrkB or GFP transfected cells in the presence of NGF (Figure 2d).

ΔIgTrkB enhances proliferation in the neural crest derived cell line NCM-1

NCM-1 is an immortalized, but normal multipotent cell line with the ability to generate sympathoadrenal precursors (22). To determine if constitutive TrkB signaling promotes proliferation or differentiation in NCM-1 cells, we transfected cells with ΔIgTrkB using a PiggyBac transposase/transposon vector (pmGenie 3) that integrates the desired insert into the host chromosome followed by inactivation of the transposase (23). Stable ΔIgTrkB NCM-1 transfectants grow to confluency more rapidly than untransfected (CONT) NCM-1 cells. To quantify this apparent increase in proliferation, cells were counted after various times in cell culture by measuring uptake of the vital fluorescent dye calcein AM (24). We noted a 2.5-fold increase in cell number after 4 days in ΔIgTrkB NCM-1 cells compared to CONT NCM-1 cells (Figure 3a). While constitutively active TrkA is transforming, full length (WT) TrkA plus NGF promotes differentiation (25); therefore, we compared the growth rate of WT TrkB NCM-1 cells with or without BDNF. As seen in Figure 3b, the growth rate of WT TrkB NCM-1 cells is enhanced by the presence of BDNF. Thus, ligand induced activation of WT TrkB also enhances proliferation as does the ΔIgTrkB. Additionally, treatment with the pan-Trk kinase inhibitor K252a abolished ΔIgTrkB-mediated proliferation, resulting in calcein AM fluorescence equivalent to CONT NCM-1 cells (Figure 3c) suggesting that TrkB kinase activity is required for the observed enhanced proliferation. In order to confirm that the enhanced proliferation did not arise from an
insertion of our construct into a tumor suppressor, five additional parent lines were established that all show a significant increase in cell number after 4 days in vitro (Figure S2). Further supporting the enhanced proliferation, western blot analysis for the proliferation markers, phosphorylated histone H3 and cyclin D1 kinase show significant increases in ΔIgTrkB NCM-1 compared to CONT NCM-1 cells (Figure S3). Therefore, constitutive signaling through ΔIgTrkB promotes an enhanced rate of growth in CONT NCM-1 cells.

ΔIgTrkB transforms NCM-1 cells

Enhanced proliferation alone is not sufficient to consider a cell transformed. Another feature common to transformed cells is the loss of requirement for attachment to a surface to divide, a property known as anchorage independent cell growth (26). To determine if ΔIgTrkB expression confers anchorage independent cell growth to NCM-1 cells, cells were cultured suspended in soft agar (Figure 4). While CONT NCM-1 cells failed to grow colonies in soft agar regardless of the presence of BDNF (Figure a,b), ΔIgTrkB NCM-1 cells grow many colonies whether or not BDNF is present (Figure 4c,d,g). In contrast very few colonies are formed by WT TrkB cells in the absence of BDNF (Figure 4e,g) but many are formed when WT TrkB NCM-1 cells are stimulated by BDNF (Figure 4f,g). Interestingly the number of colonies formed mirrored the level of phospho-ERK activation (compare Figure 1b to 4g). Furthermore, although ΔIgTrkB NCM-1 cells formed fewer colonies, the colonies that formed appeared larger than those formed by WT TrkB in the presence of BDNF (compare Figure 4d to 4f).

Another feature related to anchorage independent growth is the ability of transformed cells to survive when detached from the cell surface (27). Normally, when cells grown in culture detach, they undergo anoikis, or detachment induced apoptosis. To determine if ΔIgTrkB allows NCM-1 cells to become anoikis resistant, the number of live cells in the medium collected from transfected and untransfected cells was quantified (Figure 4h). We observed a 4-fold increase in the number of live cells in the medium collected from transfected and untransfected cells was quantified (Figure 4h). We observed a 4-fold increase in the number of live cells in the media from ΔIgTrkB NCM-1 cultures compared to CONT NCM-1 (p < 0.05, Figure 4d), indicating ΔIgTrkB promotes anoikis resistance in NCM-1 cells.

Another important characteristic of malignant transformation is the ability of cells to migrate to and invade surrounding tissues and blood vessels. To investigate whether ΔIgTrkB expression enhances migration and invasion we used a radial migration assay known as ‘the donut assay’ (28). In this assay, cells are limited to a restricted area by a silicone donut. Following donut removal, the number of cells migrating radially from the confined area are quantified. Neural crest cells are intrinsically migratory, as during development they must migrate from the neural tube to their final locations throughout the body. In light of this, we did not find any significant difference in the total number of migrating cells outside the originally confined area after 24 hours (Figure 5a–c, e–g, j). However, there is a significant increase in the area within which migrated cells could be found, indicating ΔIgTrkB NCM-1 cells migrate farther compared to WT NCM-1 cells (Figure 5d, h–i).

To investigate invasion, a layer of crude extracellular matrix (matrigel) was overlaid on the cells. Although addition of matrigel leads to a reduction in the number of cells traveling outside the originally confined area for both cell types (Figure 5j vs. 5t), ΔIgTrkB NCM-1
cells have an enhanced ability to invade the extracellular matrix marked by a 2.5-fold increase in the number of cells invading after 24 hours (Figure 5k–m, o–q, t). Furthermore, ΔIgTrkB NCM-1 cells invade farther into the matrigel indicated by a 2.5-fold increase in the total area invaded by ΔIgTrkB NCM-1 cells compared to CONT NCM-1 (Figure 5n, r, s).

**ΔIgTrkB enhances cancer related gene expression in NCM-1 cells**

To identify genes contributing to the transformed phenotype in ΔIgTrkB NCM-1 cells, we analyzed transcripts using a targeted qPCR array of cancer pathway genes (full table of genes analyzed is in the Supplemental Materials). This analysis revealed that ΔIgTrkB increases transcript levels for a number of tumor promoting genes (Table 1), as well as decreases in expression of tumor suppressors (Table 2). Consistent with the enhanced proliferation of ΔIgTrkB NCM-1 cells, we detected a 436-fold increase in transcripts levels as well as significantly enhanced protein levels (Figure S3) for the cell cycle regulatory gene cyclind1. Furthermore, upregulation of twist1 (39-fold) and hepatocyte growth factor (hgf, 29-fold), two genes known to play important roles in promoting invasion and metastasis (29, 30), is consistent with the enhanced invasive capacity of ΔIgTrkB NCM-1 cells. Moreover, expression of ΔIgTrkB in NCM-1 cells significantly downregulates expression of the tumor suppressors pten (−1.71-fold) and rb1 (−1.77-fold). Therefore, the RNA expression profile of ΔIgTrkB NCM-1 cells is consistent with the highly transformed phenotype of the cells.

An important marker of poor prognosis in human neuroblastoma tumors is the amplified expression of mycn. To determine if the transformation of NCM-1 cells by ΔIgTrkB influences mycn, we compared transcript levels in CONT and ΔIgTrkB NCM-1 cells by qPCR. We found a 21-fold increase in mycn levels in ΔIgTrkB NCM-1 cells compared to CONT NCM-1 cells (p < 0.01). In contrast, although NCM-1 cells were immortalized by the use of a retroviral vector carrying vmyc, the levels of myc observed with the qPCR array were very low and did not differ between CONT- and ΔIgTrkB NCM-1 cells (see supplemental material regarding the gene list and qPCR array signals observed for each gene).

**ΔIgTrkB NCM-1 cells form rapidly growing and aggressive tumors in vivo**

To determine if ΔIgTrkB expression would enhance the ability of NCM-1 cells to form tumors in vivo, NOD-SCID mice were injected subcutaneously with 10^6 ΔIgTrkB or GFP NCM-1 cells suspended in matrigel. One week following injection, tumors became palpable in mice injected with ΔIgTrkB NCM-1 cells (Figure 6a, p < 0.01), and all ΔIgTrkB NCM-1 injected mice were sacrificed by 15 days post-injection due to tumor burden (Figure 6b). GFP NCM-1 injected mice remained tumor free throughout the experiment (Figure 6). Monitoring tumor size daily, ΔIgTrkB NCM-1 tumors grew extremely rapidly, measuring an estimated 8 cm^3 by 2 weeks after injection, while GFP NCM-1 cells failed to grow (Figure 6c). Upon removal, ΔIgTrkB NCM-1 cell tumors were extremely large and heavily vascularized with an average wet weight of 4.5 grams (Figure 6e–f). Not only do ΔIgTrkB tumors grow at a rapid pace, these tumors are also highly invasive, invading the vertebrae and compressing the spine resulting in bilateral hind limb paralysis in one mouse only 10 days following injection (Figure 6g–h). Tumor tissue contains many closely packed cells with scant cytoplasm and little extracellular stroma, reminiscent of aggressive, poor
Discussion

In this study we provide evidence that constitutive TrkB signaling is sufficient to transform a neural crest cell line into a carcinogenic phenotype marked by an enhancement of proliferation, anchorage independent cell growth, anoikis resistance, migration and invasion, and upregulation of tumor promoter genes. The enhanced rate of proliferation and anchorage independent cell growth was also observed when full length TrkB was stimulated with BDNF in the same cell line. The isolation of the ΔIgTrkB NCM-1 cell line allowed us to test the behavior of these cells in vivo, and they displayed highly aggressive, tumorigenic behavior when injected subcutaneously. Taken together, our data suggest that aberrant TrkB signaling in the developing sympathoadrenal lineage may be sufficient to promote neuroblastoma formation.

The involvement of Trk receptors in cancer is complex. The first Trk, for “tropomyosin-receptor kinase”, was isolated from a colon carcinoma, and resulted from the fusion of a truncated tropomyosin with the tyrosine kinase domain of a receptor that rendered the kinase constitutively active (31). This kinase domain was subsequently discovered to belong to TrkA, the high affinity receptor for nerve growth factor (NGF; (20). In neuroblastomas, activation of full length TrkA slows the rate of proliferation and promotes differentiation, and shorter forms of TrkA have been identified that are constitutively active and antagonize the signaling between NGF and the full length TrkA (25). In contrast, the TrkB receptor, the high affinity receptor for BDNF, enhances proliferation and anchorage-independent cell growth in many cell lines (32–34) and enhances invasion in a number of cancer derived lines including neuroblastoma (9, 29, 35), colon cancer (36), head and neck squamous cell carcinoma (37), and non-small cell lung cancer (38). TrkB also enhances in vivo tumor growth in neuroblastoma (39) and transitional cell carcinoma (40). However, none of these studies have determined whether TrkB signaling in normal neural crest-derived cells is sufficient to promote an aggressive, fully transformed phenotype.

Constitutively activated TrkB in NCM-1 cells highly upregulates many genes also seen in poor prognosis neuroblastoma. CYCLIND1 is selectively amplified in poor prognosis neuroblastoma tumors (41), as is TWIST1, which is expressed in 100% (7/7) of MYCN amplified tumors, but only 11% (2/18) of non-MYCN amplified tumors (42). In MYCN amplified neuroblastoma cell lines, TWIST1 expression ranged from 16–164 fold that of non-MYCN amplified lines, levels consistent with the 39-fold increase in twist1 expression we observe here in ΔIgTrkB NCM-1 cells. Suggesting a specific cooperation of TWIST1 and MYCN in neuroblastoma, TWIST1 inhibits expression of the tumor suppressor P53, which allows MYCN-amplified tumors, and in the case of our study, ΔIgTrkB NCM-1 cells, to escape P53-dependent apoptosis. In this study we also observed a 29-fold upregulation of hgf together with a 1.5-fold upregulation of the HGF receptor c-met in ΔIgTrkB NCM-1
cells. Increased c-Met signaling is a common occurrence in many types of cancer (43–47), and it has also been observed in neuroblastoma (29).

ΔIgTrkB NCM-1 cells display a greatly enhanced ability to form rapidly growing and invasive tumors compared to oncogenes expressed in other cell lines. NCM-1 cells were isolated from rat embryos and immortalized by transduction with a v-myc-containing replication-deficient retrovirus (22). Although v-myc expression itself can be transforming (48), this is not the case in NCM-1 cells because of their ability to differentiate (22), and because they do not grow in soft agar, or form tumors in vivo. This is confirmed by our qPCR array analysis, which showed very low, barely detectable levels of myc in CONT NCM-1 cells as well as in the ΔIgTrkB NCM-1 cells. ΔIgTrkB NCM-1 cells form large tumors prompting euthanasia of mice two weeks after a subcutaneous injection of 1 million cells; when only 100 cells are injected, 100% of the mice form tumors by 21 days. Similar in vivo tumor growth was seen in a v-myc immortalized rat fibroblast cell line expressing oncogenic BCR-ABL, however this study injected 50% more cells to initiate tumorigenesis (49). In another study, expression of the oncogene BCL2 in a rat L6 myoblast cell line expressing v-myc caused tumors formed only after 10 weeks (50). Recently, Schulte et. al. found JoMa1 neural crest progenitor cells (which are maintained in an undifferentiated state by inducible c-myc expression) can be transformed by an oncogenic variant of the ALKF1174L, and 2 out of 6 mice were able to form tumors in vivo that were lethal to the mouse by 48 days following injection of 20 million cells (51). Thus, ΔIgTrkB is considerably more oncogenic in vivo than ALKF1174L. Not only did ΔIgTrkB NCM-1 tumors grow at a rapid pace, but they are also highly invasive. In one mouse, tumor cell invasion of the spine, caused spinal cord compression, and bilateral paralysis. This spinal cord invasion mimics human neuroblastoma, where the cancer can extend into spinal foramina causing nerve root and spinal cord compression in patients with paraspinal tumors (52–54). In total, 5% of all neuroblastoma patients will present with signs related to cord impingement.

It is not clear whether the TrkB signaling is directly responsible for this transformed phenotype or if TrkB is acting through upregulation of mycn. Regardless, it is likely that these changes result in alterations of other genes that contribute to the aggressive phenotype of these tumors. MYCN amplification is the most consistent genetic alteration seen in poor prognosis neuroblastoma (55) and as evidence that MYCN is sufficient to drive neuroblastoma formation, the TH-MYCN transgenic mouse forms neuroblastoma-like tumors spontaneously (56). However, the TH-MYCN derived tumors are slower growing and more confined than the tumors we observed from ΔIgTrkB NCM-1 cells. In addition, TH-MYCN tumors highly express BDNF, but lack TrkB expression (DeWitt and Nishi, unpublished data). JoMa1 neural crest cells overexpressing MYCN also form highly variable, slow growing tumors with mice surviving anywhere from 43–123 days (51). Thus, the activation of TrkB signaling likely contributes to the aggressive behavior of some tumors. On the other hand, constitutive TrkB signaling in NCM-1 cells induces a 21-fold upregulation of mycn mRNA in ΔIgTrkB NCM-1 cells that is comparable to the 20- to 80-fold MYCN levels observed in neuroblastoma tumors and cell lines (57).
Our studies suggest that one plausible initiating event in forming aggressive neuroblastoma is the failure of TrkB expression to be down-regulated early in development. Our previous studies showed that TrkB is transiently expressed during a developmental period when sympathoblasts are commencing differentiation and hence likely downregulating mycn. However, when BDNF is introduced, these TrkB positive progenitors are stimulated to divide again (7). Thus, aberrant activation of TrkB could trigger upregulation of MYCN together with the activation of additional pathways that contribute to a highly aggressive, carcinogenic phenotype. This underscores the importance of the development of therapies targeting TrkB signaling, such as lestaurtinib (CEP-701) (58).

Materials and methods

Constructs

Using a full-length rat trkb (WT trkb) construct generously provided by Dr. Moses Chao, New York University, NY, NY, we used site-directed mutagenesis to convert a single base at base pair 1814 into a pstI site. Both Ig-like domains could then be removed by pstI (New England Biolabs, Ipswich, MA) digestion due to another pstI site at base pair 1233. For HEK293 experiments ΔIgtrkb and WT trkb were cloned into pcDNA3.1 (Invitrogen, San Diego, CA). For PC12 experiments ΔIgtrkb was cloned into an inducible vector (pTRE-tight, Clontech, Mountain View, CA) and transfected into an rTTa-expressing PC12 Tet-on cell line (Clontech). For NCM-1 experiments ΔIgtrkb was cloned into a piggyBAC transposon-transposase vector (pmhyGENIE-3) containing a DsRed tag and hygromycin selection gene (61). NCM-1 cells were transfected with a GFP-expressing control piggyBAC vector (pmGENIE-3) to establish a control cell line for in vivo experiments.

Cell Culture

Cells were grown at 37°C in 5% CO2. HEK293 and NCM-1 cells were maintained in 10% (v/v) fetal bovine serum, 20 U/mL penicillin, 20 mg/mL streptomycin, 2mM L-glutamine, and 6 mg/mL glucose in modified L15CO2 (62). Serum for PC12 cells was 5% fetal bovine serum and 5% heat inactivated horse serum.

Transfections

HEK293 and PC12 cells were transfected using JetPEI (Polyplus transfection, Illkirch, France). Stably transfected HEK293 cells were established by G418 (Sigma, St. Louis, MO) selection. NCM-1 cells were transfected using X-tremeGENE 9 (Roche, Indianapolis, IN) and stable cells were established by hygromycin (Sigma) selection.

Westerns

Cells were seeded at 250,000 per well in 6-well plates. For HEK293 experiments cells were serum starved for 24 hours, treated with, or without BDNF (100ng/mL, R&D Systems) for 1 hour and then collected for SDS PAGE by direct lysis into 100μL 1x SDS sample buffer+β–Mercaptoethanol (βMe). Samples were run on an 8% polyacrylamide gel, then transferred to a nitrocellulose membrane (Osmonics, Inc., Minnetonka, MN) overnight at 4°C at 30 volts (Hoefer Scientific Instruments, San Francisco, CA). Blots were incubated with primary antibodies overnight at 4°C followed by appropriate secondary antibodies for 1 hour at room
temperature. Primary antibodies used were: goat anti-TrkB (1:1000, R&D Systems); rabbit anti-p-Erk1/2 (1:500, Cell Signaling, Boston, MA); goat anti-β actin (1:1000, Santa Cruz, Santa Cruz, CA); rabbit anti-Phospho-Histone H3 (1:500, Cell Signaling); and mouse anti-Cyclin D1 (1:1000; Cell Signaling). Secondary antibodies used were donkey anti-goat 700 (Rockland, Gilbertsville, PA); donkey anti-rabbit 800 (Rockland); and donkey anti-mouse 800 (Rockland) all at 1:10 000. Blots were analyzed using an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

**PC12 neurite outgrowth**

PC12 cells were plated on poly-D-lysine (0.5 mg/mL, Sigma) and laminin (0.02 mg/mL, purified in the Nishi lab from EHS tumors grown subcutaneously in C57Bl6 mice) coated coverslips at 50,000 cells per well. The day after plating, cells were transfected with either an inducible GFP or the inducible ΔIγtrkb construct and allowed to recover for 48 hrs prior to treatment with doxycycline (1μg/mL, Sigma) and 7s NGF (1μg/mL, Alomone, Jerusalem, Israel). Coverslips were fixed 30 min in Zamboni’s fixative (4% (w/v) paraformaldehyde, 15% (v/v) picric acid in 0.1 M sodium phosphate buffer, pH 7.4) and processed for immunocytochemistry as previously described (63). Primary antibodies were: goat anti-TrkB (1:1000, R&D Systems); chicken anti-GFP (1:1000, Aves, Tigard, OR). Secondary antibodies were: donkey anti-goat alexa 488 (1:1000, Invitrogen) and goat anti-chicken alexa 488 (1:1000, Invitrogen). A Nikon Eclipse E800 microscope connected to a computer equipped with StereoInvestigator software (MBF Bioscience, Williston, VT) was used to count neurite positive PC12 cells (at least one process of a length at least twice the cell’s soma size).

**Calcein AM**

NCM-1 cells were plated on poly-D-lysine coated 96 well plates at 200 cells per well in 100μL of media and viability assessed using 2μM calcein AM (Molecular Probes, Eugene, OR) with a FLUOstar Galaxy (BMG, Cary, NC) fluorescent microplate reader. Each condition was replicated in a minimum of 8 wells on the same plate. For K252a (Merck, Darmstedt, Germany) and c-Met inhibitor (SU11274, Merck) experiments, inhibitors (50 nM and 1 μm, respectively) were added at the time of plating.

**Soft agar assay**

6-well plates were coated with 0.5% agar (Affymetrix, Santa Clara, CA) in growth medium. After this base layer had solidified, NCM-1 cells suspended in 0.35% agar were plated on top of the base layer at 1000 cells per well. Cells were fed by adding 0.5mL of media to the top of each well every 3 days. After 10 days, cultures were fixed overnight with 4% paraformaldehyde in PBS, then stained with 0.005% crystal violet.

**Anoikis assay**

To quantify anoikis, medium was collected 3 days after cultures achieved confluence and the number of live cells growing in the media was quantified by trypan blue (0.08%, Sigma) exclusion and a hemocytometer.
Migration and invasion

The ‘donut assay’ for migration and invasion was used as described (28). 10,000 cells were plated on poly-D-lysine and laminin coated coverslips in a 10μL volume. Initial images were acquired through a 2X PlanApo objective on a Nikon Eclipse TE-2000E inverted microscope. A second set of images acquired at 24 hours were compared and analyzed using the default settings of a custom written ImageJ macro. Area migrated/invaded was quantified by measuring the area between the outer bound of the farthest migrating/invading cells after 24 hours, and the bound of the cells directly after gasket removal.

RNA extraction and qPCR array

Cells were grown to confluence in 6-well plates, lysed directly into TRI Reagent (Molecular Research Center, Cincinnati, OH), and RNA was isolated using the manufacturer’s protocol. RNA quality and genomic DNA contamination were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription and Cancer Pathways qPCRArray plate (SABiosciences, Valencia, CA) analysis were performed at the UVM Vermont Cancer Center DNA Analysis Facility using RT² First Strand kit (SABiosciences, Valencia, CA). Data shown in Tables 1 and 2 represent the mean of three independently isolated RNA samples from 3 different wells of a 6 well plate for each cell line. Mycn expression transcription levels were evaluated by reverse transcription of 1μg of RNA transcribed to cDNA using (Superscript III, Invitrogen) and subsequent Taqman-based qPCR (ABI).

In vivo

Mice were housed in an NIH and AALAC approved animal facility at UVM and treated following an approved IACUC protocol. Cells were injected subcutaneously into flanks of NOD-SCID mice at 10⁶ cells per mouse in 200μL of matrigel (BD Biosciences). Four mice injected per cell line. When tumors became palpable, tumor growth was quantified every other day at first, and then daily when it became apparent TrkB tumors were fast growing. Tumor volume estimated from length and width measurements using the established formula $v=1.58(\pi/6)(L^*W)^{(3/2)}$ (59). To examine the lower limits of the tumorigenic potential of ΔIgTrkB NCM-1 cells, 100 cells in 200 μL of matrigel were injected into 3 NOD-SCID mice, which were monitored for tumor formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Felix Eckenstein, Andy McKenzie, and Nourine Ahmed for technical assistance. We also thank Alan Howe for helpful comments on our manuscript and for assistance with the “donut” assay. Portions of this work were performed in the DNA Analysis Facility at the Vermont Cancer Center and the Neuroscience COBRE Molecular Cellular Core at the University of Vermont. This work was funded by Alex’s Lemonade Foundation (RN): R21NS25788 (RN); P30RR032135 (COBRE); P30GM103498 (COBRE); 5P20RR024206 (SM); and R01GM083158-01A1 (SM).
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ΔIgTrkB is expressed and is constitutively active. HEK293 cells were stably transfected with either a WT or ΔIgTrkB construct. Cells were then treated with or without BDNF (100 ng/mL) and protein was isolated. (a) Western blot for TrkB and phosphorylated Erk 1/2 demonstrates that ΔIgTrkB is expressed, and signals in the absence of the TrkB ligand BDNF. (b) Quantification of phosphorylated Erk 1/2 protein expression reveals a significant increase in phospho Erk 1/2 in ΔIgTrkB transfected cells treated with (black bars), or without (white bars) BDNF, compared to untreated WT TrkB transfected cells (p < 0.0001, ANOVA, n=3, error bars = SEM) or untransfected HEK293 cells. Constitutive ΔIgTrkB activity is two-fifths that of the WT TrkB receptor treated with BDNF.
Figure 2.
ΔIgTrkB promotes process outgrowth in PC12 cells. Transfected cells were assessed for process outgrowth 6 days following transfection. (a) PC12 cells transfected with GFP have minimal neurite outgrowth, (b) while ΔIgTrkB transfection stimulates neurite outgrowth. (c) Quantification of process outgrowth in PC12 cells reveals a 6-fold increase in the number of cells bearing neurites when transfected with ΔIgTrkB as opposed to a GFP control plasmid (p < 0.0001, Student’s t-test, n=3, error bars = SEM). (d) In the presence of NGF the number of cells bearing neurites is equivalent in the two transfection conditions. Scale bar is equivalent to 25 μm and applies to both images.
Figure 3.
ΔIgTrkB and full length TrkB promote proliferation in the neural crest derived cell line NCM-1. Cultures in 96 well plates were seeded with the same number of cells on day 0, then grown for the indicated periods of time up to 4 days and the number of cells were quantified using Calcein AM uptake. (a) stably transfected ΔIgTrkB NCM-1 cells (closed triangles) grow faster than the parent CONT NCM-1 cell line (closed squares; p < 0.0001, ANOVA; n=16). (b) full length TrkB expressing cells exhibit an enhanced rate with BDNF (open circles) over the same cell line grown in the absence of BDNF (closed circles; p< 0.0001,
ANOVA, n=8) The pan-Trk inhibitor K252a (50 nM) abolishes increased proliferation (p < 0.0001, ANOVA, n=8, error bars = SEM) in ΔIgTrkB NCM-1 cells (open triangles) compared to DMSO treated ΔIgTrkB NCM-1 cells (closed triangles). ΔIgTrkB proliferation in the presence of K252a is similar to WT NCM-1 proliferation in the presence of either K252a (open squares) or DMSO (closed squares).
ΔIgTrkB promotes anchorage-independent cell growth and anoikis resistance in NCM-1 cells. (a, b) The CONT NCM-1 cells have little ability to grow in soft agar, even when BDNF is added (b). (c,d) ΔIgTrkB NCM-1 cells form numerous colonies even in the absence of BDNF (c). (e,f) NCM-1 cells stably transfected with WT TrkB form colonies only when BDNF is added (f). (g) Quantification of cultures shown in a–f (p < 0.001, Student’s t-test, n=6, error bars = SEM). (h) ΔIgTrkB NCM-1 cells are also resistant to detachment-induced apoptosis marked by a significant increase (p < 0.05, Student’s t-test, n=4, error bars = SEM) in the number of live cells in suspension as determined by trypan blue exclusion from media taken from confluent cultures.
ΔIgTrkB enhances migration and invasion of NCM-1 cells in donut migration assay. (a–d) Migration in CONT NCM-1 and (e–h) ΔIgTrkB NCM-1 cells. (i) Quantification of migration shows a significant increase in the area migrated (p < 0.0001, Student’s t-test, n=8, error bars = SEM) in ΔIgTrkB NCM-1 cells (h compared to d), (j) but no difference in the total number of cells migrated. For invasion assay, cells were overlayed with matrigel. (k–n) Matrigel invasion in CONT NCM-1 and (o–r) ΔIgTrkB NCM-1 cells. (s) ΔIgTrkB significantly enhances both area invaded (r compared to n), and (t) the total number of invading NCM-1 cells (p < 0.05, Student’s t-test, n=3, error bars = SEM). Scale bar is equivalent to 1mm and applies to all panels.
ΔIgTrkB NCM-1 cells form highly aggressive tumors in vivo. (a) Kaplan-Meier plot of tumor free survival in NOD-SCID mice subcutaneously injected with GFP NCM-1 cells (solid line), or ΔIgTrkB NCM-1 cells (dotted line). No mice injected with GFP NCM-1 cells formed tumors over the course of the experiment (p < 0.01, log-rank (Mantel-Cox) test, n=4). (b) Kaplan-Meier plot of overall survival. All ΔIgTrkB NCM-1 cell injected mice had to be sacrificed by 15 days after initial cell injection due to tumor burden (p < 0.01, log-rank (Mantel-Cox) test, n=4). (c) Estimated tumor volume over the course of the experiment. ΔIgTrkB NCM-1 cell injected mice formed rapidly growing tumors starting at 1 week following initial cell injection, with a significant difference in tumor volume versus matrigel plug volume by 11 days (p < 0.0001, ANOVA, n=4, error bars = SEM). Removed tumors had an average wet weight of 4.5 grams. (d) Hematoxylin and Eosin staining of tumor tissue reveals densely packed cells with scant cytoplasm and absent extracellular stroma reminiscent of poor prognosis neuroblastoma. (e) Example of mouse injected with GFP NCM-1 cells, and (f) ΔIgTrkB NCM-1 cells (matrigel (e) and tumor (f) are outlined in black). (g–h) In one mouse, the tumor invaded the spinal cord causing bilateral hind limb paralysis. (g) Normal thoracic spinal cord (labeled SC) surrounded by vertebrae, rostral to tumor invasion. (h) The lower thoracic spinal cord (labeled SC) is compressed in the vertebrae by invading tumor cells (labeled T). Scale bar in (g) is equivalent to 1mm and also applies to (h). Scale bar in (d) is equivalent to 100 μm.
### Table 1

Tumor promoters upregulated in ΔIgTrkB NCM-1 cells

| Symbol | Gene                                                      | Fold Regulation | P-value      | Description                                                                 |
|--------|-----------------------------------------------------------|-----------------|--------------|-----------------------------------------------------------------------------|
| ccnd1  | Cyclin D1                                                 | 436.1           | 0.000069     | Promotes cell cycle progression                                             |
| twist1 | Twist homolog 1 (Drosophila)                             | 38.57           | 0.000015     | Promotes epithelial- mesenchymal transition (EMT), invasion, and metastasis|
| hgf    | Hepatocyte growth factor                                  | 28.86           | 0.008818     | Promotes mitogenesis, cell motility, and matrix invasion                    |
| ccnd2  | Cyclin D2                                                 | 26.40           | 0.000137     | Promotes cell cycle progression                                             |
| fgf2   | Fibroblast growth factor 2                                | 19.88           | 0.001753     | Promotes angiogenesis                                                       |
| angpt1 | Angiopoietin 1                                            | 12.25           | 0.003952     | Promotes angiogenesis                                                       |
| abcg2  | ATP-binding cassette, subfamily G (WHITE), member 2       | 6.385           | 0.000792     | Mediates multidrug resistance                                               |
| muc1   | Mucin 1, cell surface associated                           | 4.884           | 0.000030     | Inhibits p53-mediated apoptosis, and promotes EMT through β-catenin stabilization |
| vega   | Vascular endothelial growth factor A                      | 4.816           | 0.000115     | Promotes angiogenesis                                                       |
| serpin1| Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 4.661           | 0.000027     | Promotes invasion and metastasis                                             |
**Table 2**

Tumor suppressors downregulated in ΔIgTrkB NCM-1 cells

| Symbol | Gene                                      | Fold Regulation | P-value     | Description                    |
|--------|-------------------------------------------|-----------------|-------------|--------------------------------|
| thbs1  | Thrombospondin 1                          | −2.118          | 0.000563    | Promotes cell adhesion         |
| pik3r1 | Phosphoinositide-3-kinase, regulatory subunit 1 (alpha) | −1.913          | 0.000261    | Inhibitor of PI3K signaling    |
| rb1    | Retinoblastoma 1                          | −1.771          | 0.000464    | Inhibits cell cycle progression|
| tgfbr1 | Transforming growth factor, beta receptor 1 | −1.755          | 0.001248    | Inhibits cell growth           |
| pten   | Phosphatase and tensin homolog            | −1.710          | 0.000285    | Inhibits cell proliferation    |
| bad    | BCL2-associated agonist of cell death     | −1.572          | 0.004340    | Promotes apoptosis             |
| cdkn1a | Cyclin-dependent kinase inhibitor 1A      | −1.514          | 0.000064    | Inhibits cell cycle progression|