Type III TGF-β receptor promotes FGF2-mediated neuronal differentiation in neuroblastoma

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Growth factors and their receptors coordinate neuronal differentiation during development, yet their roles in the pediatric tumor neuroblastoma remain unclear. Comparison of mRNA from benign neuroblastic tumors and neuroblastomas revealed that expression of the type III TGF-β receptor (*TGFBR3*) decreases with advancing stage of neuroblastoma and this loss correlates with a poorer prognosis. Patients with *MYCN* oncogene amplification and low *TGFBR3* expression were more likely to have an adverse outcome. In vitro, TβRIII expression was epigenetically suppressed by MYCN-mediated recruitment of histone deacetylases to regions of the *TGFBR3* promoter. TβRIII bound FGF2 and exogenous FGFR1, which promoted neuronal differentiation of neuroblastoma cells. TβRIII and FGF2 cooperated to induce expression of the transcription factor inhibitor of DNA binding 1 via Erk MAPK. TβRIII-mediated neuronal differentiation suppressed cell proliferation in vitro as well as tumor growth and metastasis in vivo. These studies characterize a coreceptor function for TβRIII in FGF2-mediated neuronal differentiation, while identifying potential therapeutic targets and clinical biomarkers for neuroblastoma.

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Type III TGF-\(\beta\) receptor promotes FGF2-mediated neuronal differentiation in neuroblastoma

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Introduction
Neuroblastoma (NB), the most common cancer in infancy (1), arises from developing neurons in the sympathetic ganglia or adrenal gland. While early-stage tumors are treated effectively and may regress spontaneously, survival in patients with advanced-stage tumors is below 40% (2, 3). Clinical heterogeneity and treatment morbidity (4, 5) have driven the development of genetic and molecular screening approaches to identify children who may be spared intensive therapy (6–8).

MYCN oncogene amplification occurs in 20% of NB cases and portends a poor prognosis (7, 9, 10). MYCN epigenetically activates and represses target genes to promote NB cell proliferation and forestall neuroblast differentiation (11). While MYCN-targeted therapies have proven disappointing, the oncogene’s pleiotropic actions have generated interest in manipulating downstream transcriptional targets, either directly or by inhibiting the epigenetic modifications (33), which form ternary complexes with FGFs and FGF receptors in neuronal development (27). TGF-\(\beta\)RIII functioning to suppress tumor growth and metastasis (35). Previous reports suggest a decrease in TGF-\(\beta\)RIII expression is decreased in NB compared with human fetal neuroblasts (21). TGF-\(\beta\)RIII binding ligands that are known to promote neuronal differentiation of neuroblasts (22–26), but the function of TGF-\(\beta\)RIII in NB is unknown.

FGFs have important roles in neuronal development (27), yet their role in NB has not been explored. FGF2 has been shown to promote neuronal differentiation of neural-crest tumor cells via the Erk MAPK pathway (26, 28–30). Erk signaling is also critical to retinoic acid– and \(\alpha\)-lipoic acid–induced neuroblast differentiation (31, 32), suggesting a broader involvement for this pathway in NB differentiation.

TGF-\(\beta\)RIII is able to bind FGF2 via glycosaminoglycan (GAG) modifications (33), which form ternary complexes with FGFs and FGF receptors in neuronal development (27). TGF-\(\beta\)RIII has been shown to modulate FGF2 signaling in cardiomyocytes (34). However, the effects of TGF-\(\beta\)RIII on FGF signaling and biology in NB have not been explored. Here, we investigate the role of TGF-\(\beta\)RIII in NB pathogenesis, uncovering novel clinically relevant roles in FGF signaling and FGF-mediated biology.

Results
TGF-\(\beta\)RIII expression is decreased in NB. TGF-\(\beta\)RIII expression is decreased in many cancers, with TGF-\(\beta\)RIII functioning to suppress tumor growth and metastasis (35). Previous reports suggest a decrease in TGF-\(\beta\)RIII expression in NB (16, 20, 21). To explore a potential role for TGF-\(\beta\)RIII in NB, we determined mRNA expression in a normalized microarray...
data set (n = 213; Figure 1A). Compared with that in benign neuroblastic tumors, TGFBR3 mRNA expression was decreased in NB, with an additional significant decrease in advanced-stage NB compared with early-stage disease (Figure 1A). We performed TβRIII immunohistochemistry in 60 primary tumor samples (Figure 1B), demonstrating a decrease in TβRIII protein expression in advanced-stage tumors (Figure 1C).

As decreased TβRIII expression is a frequent event in NB, we sought to determine the prognostic significance of TβRIII expression using publicly available data sets (36, 37). Low TβRIII expression was significantly associated with decreased event-free survival (Figure 1D and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI69657DS1). TβRIII expression further stratified patients with early-stage disease (Figure 1E and Supplemental Figure 1B), selecting a subpopulation with high TβRIII expression and an excellent prognosis. Based on these data, we proceeded to identify model systems for further study of the role of TβRIII in NB. Compared with the neural crest–derived S16 Schwann cell line, NB cell lines compared with S16 Schwann cell line. (D and E) Numbers in parentheses indicate the number of samples. Background and β-actin–normalized integrated density for TβRIII are shown as percent control.
The Journal of Clinical Investigation

MYCN suppresses TβRIII expression. MYCN oncogene amplification occurs in a subset of patients with NB and confers a poor prognosis (ref. 38 and Figure 2A). Previous work by Iolascon et al. suggested a correlation between MYCN amplification and TβRIII protein expression (16). A survival analysis showed that patients with MYCN amplification and low TβRIII expression had the worst prognosis (Figure 2A and Supplemental Figure 1B). In our meta-analysis of microarray data sets, TβRIII expression was decreased in NB with MYCN amplification (Figure 2B). Consistent with this decrease, TGFBR3 mRNA expression inversely correlated with MYCN mRNA expression (Figure 2C).

To investigate whether MYCN suppresses TβRIII expression in NB cells, we used complementary inducible and repressible cell systems (39). MYCN induction decreased TβRIII expression (Figure 2D), while MYCN repression increased TβRIII expression (Figure 2E). Further, as doxycycline-mediated repression of MYCN waned,
TβRIII suppression returned (Figure 2E). Interestingly, expression of the neuronal differentiation marker β3-tubulin paralleled the rise of TβRIII expression, suggesting that neuronal differentiation may be linked to MYCN-suppressed TβRIII expression. Together, these data demonstrate that TβRIII expression is suppressed by MYCN in NB tumors and cell lines.

MYCN represses gene transcription via recruitment of HDACs and DNA methyltransferases, specifically at sites of Miz1/Sp-1 transcription (11). Since TβRIII has 4 Sp-1 binding sites in its promoter region and 2 more downstream of the transcriptional start site, we hypothesized that MYCN suppresses TβRIII expression via epigenetic silencing. ChIP demonstrated a direct interaction between MYCN and the 4 Sp-1 sites in the promoter region of TβRIII (Figure 2F). MYCN also directly bound an Sp-1 site 2 kbp downstream of the transcriptional start site but failed to bind an Sp-1 site 20 kbp downstream as well as a negative control site 90 kbp downstream (Figure 2F). Furthermore, treatment of MYCN-amplified NB cells with the HDAC inhibitors, trichostatin A and valproic acid, increased TβRIII expression (Figure 2G), suggesting that TβRIII expression could be rescued from MYCN/HDAC-mediated epigenetic silencing.

TβRIII promotes neuronal differentiation. Since TβRIII expression paralleled expression of a differentiation marker (Figure 2E) and TβRIII binds known differentiating ligands (22–26), we sought to determine whether TβRIII expression promotes neurite outgrowth and expression of differentiation marker proteins in NB cells. Neurite outgrowth was enhanced in cells with increased TβRIII expression (Figure 3, A and B, and Supplemental Figure 3A). GAG chains on cell surface receptors can bind both FGF ligands and receptors in neurons (27), we investigated whether TβRIII could interact with GAG attachment sites on FGF receptors. Indeed, exogenous TβRIII coinmunoprecipitated exogenous FGFRI in a GAG-dependent manner (Figure 4E and Supplemental Figure 4E). Furthermore, endogenous TβRIII coinmunoprecipitated exogenous FGFRI, this interaction was abrogated by TβRIII knockdown (Supplemental Figure 4E). We also observed an interaction among endogenous proteins that increased with FGF2 treatment (Supplemental Figure 4E). Treatment with an FGF2 inhibitory antibody failed to abrogate the differentiating effects of TβRIII (Supplemental Figure 3B), supporting the potential for a ligand-independent receptor crosstalk mechanism in addition to the potentiation of ligand effects by TβRIII. These results support a functional interaction among TβRIII, FGF2 ligand, and FGFR1 in NB cells.

TβRIII enhances FGF2 signaling to promote neuronal differentiation. Consistent with a coreceptor role, TβRIII enhanced both short-term (minutes to hours) and long-term (days) FGF2-mediated Erk phosphorylation in a GAG-dependent manner (Figure 5A and Supplemental Figure 5A). Silencing of TβRIII expression decreased basal Erk phosphorylation and blunted the response to FGF2 treatment (Figure 5A). To investigate the contribution of FGF signaling pathways to TβRIII/FGF2-induced neuronal differentiation, we blocked FGF receptor kinase activity with pharmacologic inhibitors (PD-173074, SU-5402) or a dominant-negative FGFR1 construct (ref. 42; Figure 5, B and C; and Supplemental Figure 5, B and D). In all cases, inhibition of FGF receptor tyrosine kinase function attenuated the differentiating effects of TβRIII expression in the presence and absence of exogenous FGF2. Similarly, pharmacologic inhibition of downstream MEK/Erk MAPK signaling with U0126 and CI-1030 attenuated the differentiating effects of TβRIII expression in the presence and absence of ligand (Figure 5B and Supplemental Figure 5, C and D). These results demonstrate that TβRIII and its GAG chains promote neuronal differentiation and enhance FGF2-induced differentiation in NB cells via FGF receptors and downstream Erk MAPK signaling.

TβRIII and FGF2 cooperate to induce Id1 expression. Similar to previous work demonstrating that FGF2 promotes differentiation of neural crest–derived cells via Erk MAPK and the transcription factor inhibitor of DNA binding 1 (Id1) (30), we found that FGF2 induced Id1 protein expression in NB cells within 1 hour of treatment, followed by a gradual decrease in expression (Figure 6A). Interestingly, TβRIII knockdown completely abrogated FGF2-induced Id1 expression. We also observed increases in Id1 protein levels in response to FGF2 over the longer time course of neuronal differentiation; this increase was inhibited by TβRIII knockdown and could be rescued by restoring TβRIII expression with GAG modifications (Figure 6B). Likewise, basal Id1 expression and FGF2-induced increases in Id1 expression were enhanced by TβRIII overexpression in a GAG-dependent manner (Supplemental Figure 5E). TβRIII- and FGF2-induced Id1 expression changes were abro-
gated by treatment with FGFR and Erk MAPK inhibitors (Figure 6C). Consistent with a downstream role for Id1 in the differentiation pathway, Id1 knockdown attenuated the differentiating effects of TβRIII expression in the presence of FGF2 treatment (Figure 6D). Moreover, in specimens from patients with NB, ID1 mRNA expression positively correlated with TGFBR3 mRNA expression (Figure 6E). These results demonstrate that TβRIII and FGF2 cooperate to induce Id1 expression. Furthermore, Id1 expression is critical to the differentiating effects of TβRIII/FGF2 and correlates with TβRIII expression in specimens from patients with NB.
TβRIII promotes neuronal differentiation via FGF2 signaling. To determine the long-term effects of altering TβRIII expression in NB cells, we used lentivirus to stably express or knockdown TβRIII (Supplemental Figure 2B). Consistent with our previous findings, stably increasing TβRIII expression promoted neuronal differentiation, while stable TβRIII knockdown decreased differentiation (Supplemental Figure 6A). Stable high TβRIII expression also enhanced FGF2-induced differentiation in a GAG-dependent manner (Supplemental Figure 6A).

Since neuronal differentiation is associated with cell-cycle arrest and tumor regression, we investigated whether stable changes in TβRIII expression affected the proliferation of NB cells. We observed...
a 35% decrease in the proliferation index of cells with stable high \( \text{T} \beta \text{RIII} \) expression (Figure 7A and Supplemental Figure 6, B and C). Conversely, stable \( \text{T} \beta \text{RIII} \) knockdown increased proliferation 2-fold (Figure 7A and Supplemental Figure 6B). Microarray and Western blot analysis demonstrated that NB tumors and cell lines with low \( \text{T} \beta \text{RIII} \) expression had increased expression of cell-cycle genes that promote proliferation (Supplemental Figure 1D and Supplemental Figure 6, D and I). Conversely, expression of the cell-cycle regulatory gene \( \text{P21} \) was decreased in tumors and cell lines with low \( \text{T} \beta \text{RIII} \) and increased in tumors and cell lines with high \( \text{T} \beta \text{RIII} \) (Figure 7B). Cells with stable high \( \text{T} \beta \text{RIII} \) expression displayed an enhanced \( \text{p21} \) response to FGF2 treatment in a GAG-dependent manner, while cells with stable \( \text{T} \beta \text{RIII} \) knockdown exhibited a dramatic attenuation of increased \( \text{p21} \) expression following FGF2 treatment (Figure 7B). While \( \text{p21} \) expression did not change with NB stage in our meta-analysis of microarray data sets (Supplemental Figure 6E), it correlated with improved prognosis in the Oberthuer data set (ref. 36 and Supplemental Figure 6F). To determine whether \( \text{T} \beta \text{RIII} \) expression affected NB cell proliferation in vivo, we implanted NB cells with stable \( \text{T} \beta \text{RIII} \) knockdown or overexpression (Supplemental Figure 6, G and H) in the mouse adrenal gland (43). As observed in vitro, \( \text{T} \beta \text{RIII} \) overexpression increased tumor cell differentiation marker expression in a GAG-dependent manner (Figure 7C), whereas tumor cells expressing the \( \text{T} \beta \text{RIII} \) knockdown construct displayed low levels of differentiation markers (Figure 7D). \( \text{T} \beta \text{RIII} \) overexpression dramatically suppressed tumor growth in a GAG-dependent manner (Figure 7C), whereas \( \text{T} \beta \text{RIII} \) knockdown accelerated tumor growth (Figure 7E), leading to earlier mortality (Figure 7F). \( \text{T} \beta \text{RIII} \) knockdown also accelerated metastasis to the contralateral adrenal gland and lungs (Figure 7G and Supplemental Table 2). These results demonstrate that \( \text{T} \beta \text{RIII} \) expression enhances neuronal differentiation to suppress NB cell proliferation, tumor growth, and metastasis.

**Discussion**

Here, we present in vitro, in vivo, and clinical data revealing a novel differentiation pathway in NB cells mediated by \( \text{T} \beta \text{RIII} \) coreceptor activity in FGF signaling. Neuronal differentiation represents a validated treatment strategy for NB, yet the growth factor signaling that promotes neuroblast differentiation remains unclear. Dissection of clinically relevant differentiation pathways provides opportunities for therapeutic advances in NB.
Consistent with prior reports (16, 20, 21), we demonstrate that TβRIII expression is decreased at both the message and protein level in NB. As low TβRIII expression is associated with poor event-free survival (Figure 1D and Supplemental Figure 1A), TβRIII could be used as a prognostic biomarker. Moreover, TβRIII expression can risk stratify both early-stage and MYCN-amplified NB (Figure 1E and Figure 2A), further supporting its utility as a prognostic biomarker.

As demonstrated here, epigenetic suppression of TβRIII is a novel downstream effector for the MYCN oncogene, which is itself a poor therapeutic target. While the precise mechanism of epigenetic suppression remains to be defined, MYCN-mediated TβRIII suppression can be reversed by inhibition of HDACs (Figure 2G). Thus, restoring TβRIII, either through HDAC inhibition or potentially through the administration of recombinant soluble TβRIII, may be beneficial in the clinical treatment of NB. To this end, we have demonstrated that soluble TβRIII can also induce differentiation in NB models (Erik Knelson, unpublished observations). These studies also urge caution in the clinical development of nonspecific tyrosine kinase inhibitors for the treatment of NB. Off-target inhibition of neuroblast differentiation, which has been observed in preclinical models (46), could lead to drug resistance and disease progression.

Contrary to well-established in vitro markers of neuroblast differentiation, identification of in vivo markers has been challenging. Expression of individual genes is often unreliable (47), and some in vitro differentiation markers, such as tyrosine hydroylase...
and neuron-specific enolase, are markers of metastasis (48) and poor prognosis (49, 50) when used clinically. In our meta-analysis of a microarray data set, none of the in vitro differentiation markers we used elsewhere correlated with stage of disease or TGFBR3 expression (data not shown). However, elevated levels of the transcription master regulator SOX10 in NB tumors correlated with high TβRIII expression (Figure 3H). As SOX10 expression is critical to neuronal differentiation in development (40) and is associ-
ated with more differentiated NB (41), the investigation of SOX10 and TGFBR3 expression may provide useful biomarkers for differentiation therapy response. In addition, we show that TGFBR3 expression tracks with a 9-gene signature of differentiated NB cells previously shown to predict response to differentiating agents (Supplemental Figure 1C and ref. 47).

Based on Scatchard analysis of binding, the TβRIII binding affinity for FGF2 (Kd ~100 pm) is on the same order of magnitude for binding of FGF2 to FGFR receptors (51), suggesting high-affinity FGF2 binding to TβRIII (Supplemental Figure 4C). While the ability of TβRIII to bind FGF2 via GAG chains has been previously reported (33) and TβRIII has been shown to promote FGF2-mediated biology in epicardial cells (34), this is the first demonstration of TβRIII effects on FGF signaling and biology in NB. Additionally, we demonstrate for the first time an interaction between TβRIII and FGFR1, which can induce Erk MAPK signaling and promote differentiation in the absence of ligand (Figure 4E, Supplemental Figure 3B, Supplemental Figure 4-E, and Supplemental Figure 5, C and D). Given the ubiquity of TβRIII expression and FGF signaling, it is likely this coreceptor activity occurs in other contexts where TβRIII and FGF2 have demonstrated roles. Based on the mechanism of signaling crosstalk via GAG chains, it is also possible that other proteoglycan coreceptors, including the glypicans and syndecans, could have similar activity to that of TβRIII in NB. The role of other proteoglycan coreceptors in NB is currently being explored.

In conclusion, we demonstrate a novel and clinically relevant mechanism for neuroblast differentiation. Further, these studies identify TβRIII expression as a prognostic biomarker for patients with early-stage and MYCN-amplified NB, while providing mechanistic support for the use of HDAC inhibitors and recombinant soluble TβRIII in clinical trials. More generally, our work provides preclinical rationale for targeting differentiating growth factors and receptors in the treatment of NB.

Methods

Microarray data set analysis. To generate our microarray data set, we downloaded 5 publicly available NB data sets from GEO (GSE12460, GSE16237, GSE13141, GSE12713, and GSE27608), which include data generated on multiple Affymetrix platforms. Microarray data were RMA preprocessed (52, 53), and all data were log2-transformed. Human Exon 1.0 ST array gene level probes were matched to their best-match HG-U133 Plus 2.0 probe set as described previously (54). To minimize batch effects from each of the 5 separate data sets, we used ComBat software as described previously (55). We then queried our data set using the gene probes listed in Supplemental Table 1. Survival analysis was conducted using the oncogenomics website (http://home.ccr.cancer.gov/oncology/oncogenomics/), specifically the Oberthuer (36) and NB prognosis (37) data sets.

TβRIII immunohistochemistry. NB tissue samples were obtained from the Children’s Oncology Group (COG) Biorepository with approval from the COG Neuroblastoma Biology Subcommittee. TβRIII immunohistochemistry was conducted using a birin-free protocol from BioCare Medical according to the manufacturer’s instructions. Briefly, patient sample slides were deparaffinized, rehydrated, and blocked with Peroxidized 1 (PX968G, Biocare Medical) and Background Punisher (BP974G, Biocare Medical), before incubation with a custom-made rabbit antibody to the cytoplasmic domain of TβRIII, as described previously (56, 57). This was followed by sequential treatment with an alkaline phosphatase polymer system and the Warp Red chromogen (M3R533G and WRR806H, respectively; Biocare Medical). Semi-quantitative analysis was performed independently by 2 blinded investigators using a 3-tiered scoring system (0, no staining; 1, staining present but minimal; 2, moderate to dark staining). Discrepancies between the 2 investigators were discussed and reconciled (<10 samples).

Cell culture and reagents. SK-N-SH-SYSY (5Y; CRL-2266) and SK-N-BE (2) (BE2; CRL-2271) cells were purchased from ATCC and grown in a 1:1 mixture of Eagle minimal essential medium and Ham’s F12 with 10% fetal bovine serum. SK-N-SH-SHEP (SHEP, gift of M.A. Armstrong, Duke University, Durham, North Carolina, USA), SK-N-AS (ATCC CRL-2137), SK-N-SH (ATCC HTB-11), S16 (ATCC CRL-2941), and SK-N-AS-MYCNER and SHEP-21N (gifts of Linda Valentijn, University of Amsterdam, Amsterdam, The Netherlands; ref. 39) were grown in Dulbecco modified Eagle minimal essential medium with 10% fetal bovine serum. All cells were grown at 37 °C in 5% CO2. Human basic fibroblast growth factor (no. 8910) and the MEK 1/2 inhibitor U0126 (no. 9903) were purchased from Cell Signaling. The MEK 1/2 inhibitor CI-1040 (S1020) was purchased from Selleck Chemicals. The FGFR inhibitor SU5402 (sc-204308) was purchased from Santa Cruz Biotechnology Inc. The FGFR inhibitor PD-173074 (P2499), the p38 inhibitor SB203580 (S8307), and the Alk 4/5/7 inhibitor SB431542 (S4317) were purchased from Sigma-Aldrich. The neutralizing TGF-β1 antibody 1D11 (MAB 1835) was purchased from R&D Systems. The neutralizing FGFR2 antibody (catalog no. 05-117) was purchased from Millipore and used at a concentration of 5 μg/ml per manufacturer’s instructions. The BMP inhibitor dorsomorphin (catalog no. 3093) was purchased from Tocris. The Alk 2/3 inhibitor LDN193189 was a gift from Paul Yu (Massachusetts General Hospital, Boston, Massachusetts, USA; ref. 58).

DNA constructs. All TβRIII and TβRIII shRNA constructs used in this study have been described previously (57, 59–63). TβRIII-HA consists of the full-length human TβRIII sequence with the HA sequence at the N terminus, within the pcDNA 3.1 vector (62). TβRIII-GFP consists of the full-length human TβRIII sequence inserted in the bicistronic pEGFP vector (61). TβRIII consists of the rat TβRIII sequence with HA tag in the pcDNA 3.1 vector (57). TβRIII-AGAG consists of TβRIII-HA, with serine-to-alanine point mutations at amino acids 534 and 545 to prevent GAG attachment (33, 59, 61, 62). TβRIII-cyto consists of TβRIII-HA with a truncation of the cytoplasmic domain (59, 63). Adenoviral constructs were used at a MOI of 10 particles per cell. TβRIII adenoviral shRNA constructs were used at an MOI of 50 particles per cell. Lentiviral vectors consisted of the same construct as used in adenoviral vectors cloned into a pSMUPE-Neo backbone (TβRIII constructs) or a pLKO.1-puro backbone (TβRIII shRNA construct and nontargeted control). Transient DNA transfections were performed using lipofectamine (Invitrogen) according to the manufacturer’s instructions.

Id1 siRNA (sc29356) and control siRNA (sc37007) were purchased from Santa Cruz Biotechnology Inc. and used according to the manufacturer’s instructions. pWZL Neo Myr Flag FGFR1 (Addgene plasmid no. 20486) was a gift of Jean Zhao and William Hahn (Dana-Farber Cancer Institute, Boston, Massachusetts, USA) (64). The dnFGFR1 plasmid with a GFP reporter (pCCALL2 dominant-negative FGFR1 IRES EGFP) was a gift of Margaret Kirby and Harriett Stadt (Duke University) (42).

Neurite analysis. Neurites were measured from phase-contrast images taken with a Nikon inverted microscope at x10 magnification using the NIH Image/Imagel plug-in NeuronJ (65). Three images were taken of each condition at each time point, and all visible neurites (thin shafts extending outward from the cell body) were measured (70–150 neurites per field).

Immunoprecipitation, Western blotting, and flow cytometry. Immunoprecipitation and Western blotting were performed using standard techniques as described previously (66, 67). Each experiment was conducted at least 3 separate times. Antibodies for differentiation and signaling markers were purchased from Cell Signaling: neurofilament 160 kDa (NF160) (no. 2838), β3-tubulin (no. 5568), tyrosine hydroxylase (no. 2792), neuron-specific enolase (no. 9536), GAP43 (no. 5307), phospho-Erk 1/2 (pErk) T202/
Y204 (no. 9101), Erk 1/2 (no. 4695), p21 (no. 2946), MYCN (no. 9405), acetyl lysine (no. 9441), and cyclin D1 (no. 2926). Id1 antibody (sc-488) was purchased from Santa Cruz Biotechnology Inc.

The lysis buffer for coimmunoprecipitation experiments contained 0.75% NP40 and 2 mM EDTA (0.1% NP40 for endogenous protein experiments). The HA antibody (HA.11 clone 16B12 MMS-101P) was purchased from Covance, and the FLAG antibody (F3165, clone M2) was purchased from Sigma-Aldrich. Both antibodies were used at a concentration of 10 μg/ml for immunoprecipitation, as per manufacturer’s instructions. For endogenous immunoprecipitation, TβRII antibody (AF-242-PB, R&D Systems) and FGFR1 antibody (9740, Cell Signaling) were used. Lysates were precleared in PGS beads (PGS for the goat TβRII antibody) for 2 hours and incubated overnight with beads and pull-down antibody.

TβRIII flow cytometry was conducted using the R&D Systems antibody following the manufacturer’s instructions and using a 488-GFP fluorophore-tagged anti-goat secondary antibody and Accuri C6 flow cytometer.

Iodinated ligand binding and crosslinking. Iodinated TGF-β1 binding and crosslinking was conducted with TβRIII pull down using a goat antibody to the extracellular domain (AF-242-PB, R&D Systems) in order to identify functional surface receptor expression as described previously (56, 59). Iodinated FGF2 binding and crosslinking were conducted as with TGF-β1, with the following changes: 0.5% NP40 lysis buffer was used instead of RIPA and 30 minutes of crosslinking with 0.02% DSS was used instead of 15 minutes with 0.1% DSS. Both iodinated TGF-β1 (NEX2670) and iodinated FGF2 (NEX268) were purchased from Perkin Elmer.

ChIP. ChIP analysis was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer’s instructions. Briefly, chromatin was sheared (~500 bp average length) by sonication with a Branson Sonifier 250 (output control 1.5; duty cycle 25%; 10 cycles of 20-second pulses at 30-second intervals). Sheared cross-linked chromatin was rotated at 4°C overnight with protein G magnetic beads and MYCN (OP13, Calbiochem) or mouse IgG (015-000-003, Jackson Immunoresearch Laboratories Inc.). Following chromatin elution, cross-link reversal, and proteinase K digestion, samples were purified using the QIAquick PCR Purification Kit (28104, Qiagen). PCR products were analyzed by quantitative RT-PCR using iQ SYBR Green Supermix (170-8882, Bio-Rad) and normalized to control = 1.0) were calculated and averaged for each of 3 individual experiments at different cell densities in order to examine proliferation differences across a range of cellular confluence. Cells were plated in a 96-well plate at a concentration of 400 to 5,000 cells per well (SHEP cells) or 5,000 to 10,000 cells per well (SK-N-AS cells). Each condition was plated in triplicate overnight prior to a 4-hour [3H]thymidine pulse (1 μCi; Amersham Biosciences/GE Healthcare). Cells were washed with PBS and 5% trichloroacetic acid prior to lysis with 0.1 N NaOH. Incorporation of [3H]thyidine was determined by scintillation counting.

Orchotopic xenograft. Antibiotic-selected stable cell lines were implanted orthotopically (2 million cells per mouse in 20 μl DMEM) in the left adrenal capsule of 8-week-old female beige/SCID mice (Charles River Laboratories) as described previously (43). Mice were housed under pathogen-free conditions on a 12-hour-light/dark cycle. Animals were monitored closely for tumor growth and signs of illness and sacrificed at humane end points. For the surgical procedure, anesthetized mice underwent left subcostal laparotomy. Gentle retraction of the spleen exposed the adrenal gland for injection using a 23-gauge needle (7804-07, Hamilton Company; 2-inch PT2) on a 25-μl syringe (no. 702, Hamilton Company). Peritoneal and cutaneous incisions were closed in 2 layers with 4.0 silk suture (Sharpoint 18 mm DA-2187N; Surgical Specialties Corp.).

Statistics. All clinical and xenograft data were analyzed using nonparametric statistics (Kruskal-Wallis global test with Mann-Whitney post-hoc tests) and presented as median, upper, and lower quartile. Survival curves were analyzed with log-rank statistics. In vitro experiments were analyzed using parametric statistics (ANOVA global test with Bonferroni-corrected 2-tailed Student’s t tests as post-hoc tests) and presented as mean ± SEM. In cases in which data were normalized to control, 1-sample Student’s t test was used with an expected value of 1 or 100% in order to decrease the likelihood of a type I error. To examine the statistical interaction between receptor expression and ligand treatment, 2-way ANOVA was performed with specific interest in the interaction term. The isolated effect of each individual variable (represented by an ANOVA P value) was also noted in the figures and referred to as main effect receptor or main effect FGF2. For all experiments, significance was set at P < 0.05. Linear regression was performed on selected microarray data, with the slope and P value for the line of best fit reported as well as the R² value for the relationship. All statistical analyses were conducted with GraphPad Prism version 6.00 (GraphPad Software).

Study approval. All patient samples were deidentified, and the project was exempted by the Duke University Health System Institutional Review Board (protocol #ID 00034541). All animal procedures were performed by the Duke University Institutional Animal Care and Use Committee (protocol A278-11-11).

At current time we thank Michael Hogarth, the Children’s Oncology Group Neuroblastoma Biology Subcommittee, Wendy London, and Evan Plunkett for providing patient tissue and serum samples. We thank Linda ValentiJ, Paul Yu, Harriettded Stad, Mary Hutson, Margaret Kirby, and Lisa Crose for providing reagents. We thank Lindsey Morgan and Terri Lucas for coordinating our animal facility use. We thank Julie Fuller for tissue processing. We are grateful to Tam How, Catherine Garza, Alison Meyer, Alisha Holzhausen, Catherine Lavau, Rebekah Moehring, Jennifer Elderbroom, Rachel Harshler, and Jasmine Nee for technical assistance and Cheryl Alles for superior clerical assistance. We are grateful to Daniel Wechsler, Dona Chikaarishi, Christopher Kontos, and Julio Ramirez for invaluable mentoring throughout this project. This work was supported in part by NIH grants F30 CA168043-01 (to E.H. Knelson), R01-CA136786 (to G.C. Blobe), and R01-CA135006 (to G.C. Blobe). Received for publication March 1, 2013, and accepted in revised form August 8, 2013.

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