Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TrkC-induced apoptosis

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Tropomyosin-related kinase receptor C (TrkC) is a neurotrophin receptor with tyrosine kinase activity that was expected to be oncogenic. However, it has several characteristics of a tumor suppressor: its expression in tumors has often been associated with good prognosis; and it was recently demonstrated to be a dependence receptor, transducing different positive signals in the presence of ligand but inducing apoptosis in the absence of ligand. Here we show that the TrkC ligand neurotrophin-3 (NT-3) is upregulated in a large fraction of aggressive human neuroblastomas (NBs) and that it blocks TrkC-induced apoptosis of human NB cell lines, consistent with the idea that TrkC is a dependence receptor. Functionally, both siRNA knockdown of NT-3 expression and incubation with a TrkC-specific blocking antibody triggered apoptosis in human NB cell lines. Importantly, disruption of the NT-3 autocrine loop in malignant human neuroblasts triggered in vitro NB cell death and inhibited tumor growth and metastasis in both a chick and a mouse xenograft model. Thus, we believe that our data suggest that NT-3/TrkC disruption is a putative alternative targeted therapeutic strategy for the treatment of NB.

Introduction
According to the classic neurotrophic theory, neuronal survival is dependent on neurotrophic factors, such as neurotrophins (1, 2). These proteins are crucial to the development of the nervous system, especially as they control the massive developmental loss of excess neurons that fail to adequately connect to their targets. However, over the years, neurotrophins — that include NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5 (2) — and their tropomyosin-related kinase receptors — TrkA, TrkB, and TrkC — have been shown to play an important role in cancer (reviewed in ref. 3).

The overall view is that, as is the case with classic tyrosine kinase receptors, dysregulation of kinase activities through various mechanisms generates survival signals via the PI3K/Akt and Ras/MEK/MAPK pathways, which in turn promote tumor progression (4).

However, TrkC does not seem to fit this picture: (a) its expression is a good-prognosis factor in different types of cancer and more specifically in melanomas (5), medulloblastomas (6, 7) and neuroblastomas (NBs) (8–12); (b) its expression and activation have been shown to trigger apoptosis in medulloblastoma cells (13); and (c) as opposed to TrkA and TrkB, it was recently proposed to act as a dependence receptor (14). Such receptors are able to initiate 2 completely opposite signaling pathways, depending on ligand availability. In the presence of ligand, a positive differentiation, guidance, or survival signal is transduced. In the absence of ligand, however, they induce an active process of apoptotic cell death. These dependence receptors also include p75

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and consequently, it is key to identify new targets for drug development. We show here that NT-3 expression in NB cells is a selective advantage for tumor cell survival and propose the disruption of the NT-3/TrkC interaction as a novel anticancer strategy to our knowledge in NBs with high NT-3 levels.

**Results**

We focused on stage 4 NBs, with a specific interest in comparing the expression levels of NT-3 and its receptor TrkC. We first analyzed the expression of NT-3 and TrkC using quantitative RT-PCR (Q-RT-PCR) in a panel of 106 stage 4 NB tumors. A substantial fraction of stage 4 NBs displayed high NT-3 levels (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI41013DS1). Thirty-eight percent of tumors showed at least a 2-fold increase in NT-3 expression compared with the median value, and more than 20% of tumors displayed a 5-fold increase (Figure 1A). Tumors with high NT-3 levels showed a high NT-3/TrkC ratio, suggesting the view of a gain of NT-3 expression in tumors (Figure 1A). We then compared NT-3 levels to the prognosis within the different subcategories of stage 4 NBs — stage 4 either diagnosed before 1 year of age or later and stage 4S. No significant differences were observed, suggesting that NT-3 upregulation is a selective gain that occurs independently of tumor aggressiveness and dissemination in a large fraction of stage 4 NBs. Similar results were obtained on stage 1, 2, or 3 NBs (Supplemental Figure 1A). Expression of NT-3 was not only detected at the mRNA level but also at the protein level by immunohistochemistry (Figure 1B).

NT-3 overexpression was seen in 38% of stage 4 NBs but also in a fraction of NB cell lines mainly derived from stage 4 NB tumor material (Table 1). Four human NB cell lines, i.e., CLB-Ge2, CLB-VolMo, SHEP-CLB, and IMR32, were studied further. All 4 cell lines express TrkC (data not shown), but CLB-Ge2, CLB-VolMo, and SHEP-CLB express high levels of NT-3, whereas NT-3 was barely detected in IMR32 cells both at the messenger level (Table 1) and at the protein level (Figure 1C). Interestingly, NT-3 immunostaining performed on CLB-Ge2 and SHEP-CLB cells, in the absence of cell permeabilization, showed a clear membrane staining. This suggests that the high NT-3 content observed in aggressive NB is associated with an autocrine expression of NT-3 in NB cells. We then started investigating the mechanisms allowing NT-3 a high level of expression in these cells. We failed to detect Ntf3 gene amplification or enhanced transcriptional activity of the NT-3 promoter in these cells (data not shown). However, we noticed that inhibition of methyltransferases with the inhibitor 5-aza-2’-deoxycytidine in either CLB-Ge2 or IMR32 cells spe-
As a second approach, we used a blocking TrkC antibody described before (14) to prevent NT-3 from binding to endogenous TrkC. As shown in Figure 2, D and E, and Supplemental Figure 2D, the addition of anti-TrkC triggered CLB-Ge2 and CLB-VolMo apoptotic cell death, as measured by caspase-3 activity assay (Figure 2D) and TUNEL staining (Figure 2E and Supplemental Figure 2D). This effect was specific for NT-3/TrkC inhibition, since the anti-TrkC antibody had no effect on IMR32 cells. Similar CLB-Ge2 cell death induction was observed when, instead of using a blocking TrkC antibody, a recombinant ectodomain of TrkC was used to trap NT-3 (Supplemental Figure 2E).

To determine whether the NB cell death associated with inhibition of the TrkC/NT-3 interaction can be extended to fresh tumors, a surgical biopsy from a tumor and the corresponding bone marrow was semi-dissociated and further incubated with the anti-TrkC antibody. This primary tumor and the disseminated neoplasia expressed both NT-3 and TrkC (Supplemental Figure 2F), and an increased cell death, measured by caspase activation, was detected in response to the anti-TrkC antibody (Figure 2F).

There are 2 different paradigms for interpreting cell death associated with the interference of the NT-3/TrkC interaction. According to the classic neurotrophic view, the observed cell death could be a death by “default” that results from the loss of survival signals triggered by the NT-3/TrkC interaction — i.e., MAPK or PI3K pathways activated through TrkC’s kinase activity. The dependence receptor method offers a different perspective, more compatible with the fact that TrkC expression is usually a good prognosis factor. In this scenario, blocking the interaction between NT-3 and TrkC leads to unbound TrkC actively triggering apoptosis. As a first approach to discriminate between these 2 possibilities, NB cell death was induced via anti-TrkC antibody treatment, after CLB-Ge2/CLB-VolMo transfection with a dominant-negative mutant for TrkC. This dominant-negative mutant, TrkC-IC D641N, has been shown to specifically inhibit the proapoptotic signaling of unbound TrkC, without affecting its kinase-dependent signaling (14). Expression of the dominant-negative mutant fully blocks anti-TrkC-mediated CLB-Ge2 and CLB-VolMo cell death (Figure 3A and Supplemental Figure 3, A and B). To further support this observation, we assessed the extent of cell death associated with NT-3 siRNA in settings of downregulation of TrkC by siRNA. As shown in Figure 3, B and C, and Supplemental Figure 3, C–E, while downregulation of TrkC in all 3 NT-3–expressing cell lines is not associated with cell death as would be expected by the classic loss of survival signaling pathways, this downregulation fully blocks cell death induced by NT-3 siRNA. This supports further the hypothesis that NT-3 upregulation observed in CLB-Ge2, CLB-VolMo, and SHEP-CLB cells inhibits the proapoptotic signaling triggered by TrkC itself. Along this line, addition of the blocking TrkC antibody to CLB-Ge2 failed to be associated with a decrease in the classic survival pathways, as exemplified here by measurement of ERK or Akt phosphorylation (Figure 3D). Moreover, TrkC caspase cleavage was enhanced by the TrkC blocking antibody (Figure 3E). Indeed, as previously described, TrkC and dependence receptors in general are cleaved by caspase, and this cleavage is a prerequisite for their proapoptotic activity (14, 16). As shown in Figure 3E, while a basal level of TrkC cleavage was detected in control conditions, addition of the blocking antibody was associated with increased TrkC cleavage, a cleavage blocked by addition of the general and potent caspase inhibitor, Boc-Asp(Ome) fluoromethyl cifically restored NT-3 expression in IMR32 cells (Supplemental Figure 1B). Together with the general hypomethylation associated with cancer, this observation suggests that NT-3 high expression is associated with inhibition of NT-3 promoter methylation.

To investigate whether the NT-3 autocrine expression observed in CLB-Ge2, CLB-VolMo, and SHEP-CLB cells provides a selective advantage for tumor cell survival, as would be expected from the dependence receptor paradigm, cell death was analyzed in response to the disruption of this autocrine loop. As a first approach, NT-3 was downregulated by RNA interference. In this approach, we used 2 different paradigms for interpreting cell death associated with the interference of the NT-3/TrkC interaction. According to the classic neurotrophic view, the observed cell death could be a death by “default” that results from the loss of survival signals triggered by the NT-3/TrkC interaction — i.e., MAPK or PI3K pathways activated through TrkC’s kinase activity. The dependence receptor method offers a different perspective, more compatible with the fact that TrkC expression is usually a good prognosis factor. In this scenario, blocking the interaction between NT-3 and TrkC leads to unbound TrkC actively triggering apoptosis. As a first approach to discriminate between these 2 possibilities, NB cell death was induced via anti-TrkC antibody treatment, after CLB-Ge2/CLB-VolMo transfection with a dominant-negative mutant for TrkC. This dominant-negative mutant, TrkC-IC D641N, has been shown to specifically inhibit the proapoptotic signaling of unbound TrkC, without affecting its kinase-dependent signaling (14). Expression of the dominant-negative mutant fully blocks anti-TrkC-mediated CLB-Ge2 and CLB-VolMo cell death (Figure 3A and Supplemental Figure 3, A and B). To further support this observation, we assessed the extent of cell death associated with NT-3 siRNA in settings of downregulation of TrkC by siRNA. As shown in Figure 3, B and C, and Supplemental Figure 3, C–E, while downregulation of TrkC in all 3 NT-3–expressing cell lines is not associated with cell death as would be expected by the classic loss of survival signaling pathways, this downregulation fully blocks cell death induced by NT-3 siRNA. This supports further the hypothesis that NT-3 upregulation observed in CLB-Ge2, CLB-VolMo, and SHEP-CLB cells inhibits the proapoptotic signaling triggered by TrkC itself. Along this line, addition of the blocking TrkC antibody to CLB-Ge2 failed to be associated with a decrease in the classic survival pathways, as exemplified here by measurement of ERK or Akt phosphorylation (Figure 3D). Moreover, TrkC caspase cleavage was enhanced by the TrkC blocking antibody (Figure 3E). Indeed, as previously described, TrkC and dependence receptors in general are cleaved by caspase, and this cleavage is a prerequisite for their proapoptotic activity (14, 16). As shown in Figure 3E, while a basal level of TrkC cleavage was detected in control conditions, addition of the blocking antibody was associated with increased TrkC cleavage, a cleavage blocked by addition of the general and potent caspase inhibitor, Boc-Asp(Ome) fluoromethyl...
ketone (BAF). Together, these data demonstrate that NT-3 upregulation observed in NB cells inhibits the proapoptotic signaling triggered by the dependence receptor TrkC.

We next assessed whether in vivo interference with NT-3/TrkC could be used to limit/inhibit NB progression and dissemination. A chicken model was developed in which grafts of NB cells in the chorioallantoic membrane (CAM) of 10-day-old chick embryos recapitulate both tumor growth at a primary site, within the CAM, as well as tumor invasion and dissemination at a secondary site, metastasis to the lung (ref. 30 and Figure 4A). In a first approach, CLB-Ge2 or IMR32 cells were loaded in 10-day-old CAM, and embryos were treated on day 11 and day 14 with anti-TrkC or an unrelated antibody. Seventeen-day-old chicks were then analyzed for primary tumor growth and metastasis to the lung. As shown in Figure 4, B and D, treatment with the anti-TrkC antibody significantly reduced primary tumor size, specifically in CLB-Ge2–grafted CAM, while an unrelated isotopic antibody had no effect. Seventeen-day-old chicks were then analyzed for primary tumor growth. As shown in Figure 4F, NT-3 siRNA treatment was associated with a significant antitumor effect. Of interest, when TrkC siRNA treatment was assessed instead of NT-3 siRNA, no effect on tumor size was observed, further suggesting that the antitumor effect seen with the blocking antibody or with NT-3 siRNA is not associated with a “classic” loss of the survival signals promoted by the tyrosine kinase activity of TrkC. On the other hand, we show that a dual treatment with NT-3 siRNA and TrkC siRNA reversed the anticancer effect observed in NT-3 siRNA–treated CAM (Figure 4F). This result thus demonstrates that NT-3 inhibition is associated with tumor growth inhibition and cell death, because it reengages TrkC-induced apoptosis.

To further provide a proof of concept that NT-3 interference may represent a putative alternative therapeutic approach, classic xeno-
grafts of NB cells in nude mice were performed. SHEP-CLB were xenografted subcutaneously in nude mice. When the engrafted tumors reached 100 mm$^3$, mice were treated intraperitoneally 3 times a week, either with NT-3 siRNA or with scrambled siRNA as successfully performed previously (31, 32). As shown in Figure 5, the growth of tumors engrafted in mice treated with NT-3 siRNA was strongly reduced compared with the ones engrafted in scrambled siRNA–treated mice. This tumor growth inhibition was associated with a reduction in tumor weight. Together, these data further support the antitumor effect associated with NT-3 interference in vivo.

Discussion

We show here that a fraction of NBs show an autocrine production of NT-3 associated with an increased NT-3/TrkC ratio. This elevated NT-3/TrkC ratio likely confers a selective advantage acquired by the cancer cells in settings of limited/no NT-3. Interestingly, this dependence on NT-3 presence appears specific for TrkC and does not involve other Trk receptors (i.e., TrkA or TrkB), as a dominant negative of TrkC is sufficient to down this dependence (Figure 3A).

Both NT-3 and TrkC have been shown to be expressed in various cancers (5, 33–47). However, most of the field has concentrated its attention on the expression of TrkC, considered as a favorable outcome factor in NB and medulloblastoma (6–8, 10–12). All these studies view NT-3 as an accessory compound that complements TrkC autoactivation and induces proliferation through TrkC kinase–dependent signaling. Along this line, NT-3 was shown to induce survival and differentiation of primary cultures derived from NB (48) and further to activate heparanase activity through TrkC and be involved in invasion during metastasis (49–51).

Here, we show that autocrine NT-3 expression is a mechanism developed by a large fraction of tumor cells to bypass TrkC-induced cell death that would occur in regions of limited NT-3 concentrations. This selective advantage is, however, only 1 of 3 possible means a tumor can use to inactivate TrkC-induced apoptosis, the 2 others being either a loss of TrkC expression or an inactivation of the downstream proapoptotic signaling. Along this later line, IMR32 cells are NT-3–negative cells and undergo apoptosis through forced expression of Bax, a classic cell death inducer.
Nevertheless, they fail to die after TrkC overexpression (Supplemental Figure 4), thus suggesting that in IMR32 cells, the loss of TrkC proapoptotic signaling has been selected. As a consequence, NT-3 high expression constitutes a new marker for NB patients that could putatively respond to a treatment based on cell death induction via disruption of the NT-3/TrkC interaction. One may wonder, what are the mechanisms leading to NT-3 upregulation. Our preliminary results suggest that NT-3 expression in cancer cells may be regulated by epigenetic mechanisms, but further work will be needed to address this important point.

The in vitro cell death effect and the in vivo antitumor effect of NT-3 interference on NT-3–expressing tumor cells support the view that NT-3 is an exciting target for drug development. So far, the data obtained are still limited in term of the number of models tested and also on the amplitude of the anticancer effect observed (Figures 4 and 5). This, however, could be explained by the fact that the tools used here (siRNA or polyclonal antibody) were proof-of-concept tools and definitely not drug candidate tools. Drug development has now to be explored. However, our data calls for a larger screen of cancers that could be responsive to such a therapeutic approach. Indeed, the therapeutic strategy presented here is fundamentally different from the now classic strategies for cancer therapy based on inhibitors of tyrosine kinase activity. In our view, inhibiting the kinase activity-dependent survival signals triggered by TrkC should only have poor tumor death effect, i.e., cytostatic, if the drug developed does not also target the activation of the proapoptotic activity of TrkC. We propose here that a treatment based on inhibition of the interaction between NT-3 and its dependence receptor TrkC, by blocking either NT-3 or TrkC, could potentially provide benefit, as a first line treatment or in combination with standard chemotherapy, to the large fraction of the patients suffering from aggressive NB and possibly other cancers with high NT-3 levels.
Figure 5
Disruption of NT-3 autocrine loop inhibits NB tumor growth. (A) The volume of SHEP-CLB engrafted tumors was measured during treatment with either NT-3 siRNA or scrambled siRNA. Mice were injected intraperitoneally with 3 μg siRNA 3 times per week. Mean tumor volume is indicated. (B) SHEP-CLB tumors were dissected after 18 days of treatment. Final tumor weight and representative pictures are shown. Scale bar: 2 mm. Data represent mean ± SEM. **P < 0.01, ***P < 0.001, 2-sided Mann-Whitney test, compared with scrambled siRNA–treated mice.

Methods
Human NB and breast tumors samples and biological annotations. After the consent of parents, surgical human NB tumor material was immediately frozen. Materials and annotations were obtained from the Biological Resources Centers of both national referent institutions for NB treatment, i.e., Centre Léon Bérard and Institut Gustave Roussy. Approval of care and use of these samples was given by the Biological Resources Center Ethical and Steering committees of Centre Léon Bérard and Institut Gustave Roussy.

Cell lines, transfection procedure, and reagents. Human NB cell lines were from the tumor banks at Centre Léon Bérard and at Institut Gustave Roussy. CLB-Ge2, CLB-VolMo, SHEP-CLB, and IMR32 cell lines were cultured in RPMI 1640 Glutamax medium (Gibco) containing 10% fetal bovine serum. Olfactory neuroblasts 13.S.24 were cultured and transfected with either NT-3 siRNA or scrambled siRNA. Mice were injected intraperitoneally with 3 μg siRNA 3 times per week. Mean tumor volume is indicated. (15) For all 3 couples of primers, polymerase was activated at 25°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds. Data represent mean ± SEM. **P < 0.01, ***P < 0.001, 2-sided Mann-Whitney test, compared with scrambled siRNA–treated mice.

Q-RT-PCR. To assay NT-3 and TrkC expression in NB samples, total RNA was extracted from histologically qualified tumor biopsies (>60% immature neuroblasts), using the NucleoSpin RNAII Kit (Macherey-Nagel), and 200 ng were reverse transcribed using 1 U SuperScript II Reverse Transcriptase (Invitrogen), 1 U RNase inhibitor (Roche Applied Science), and 250 ng random hexamer (Roche Applied Science). Total RNA was extracted from human cell lines using the NucleoSpin RNAII Kit (Macherey-Nagel), and 1 μg was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time Q-RT-PCR was performed on a LightCycler 2.0 apparatus (Roche), using the Light Cycler FastStart DNA Master SYBER Green I Kit (Roche). Q-RT-PCR was performed using the following primers: TrkC, forward 5′-AGCTCAACAGCCGACACTGGCAAAACAATGCA-3′ and reverse 5′-TGACACTGGGAAAAACATGCA-3′ and reverse 5′-GGTCCTTTTCACGAGC TTC-3′; NT-3, forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. The ubiquitously expressed human HPRT gene, showing the least variable expression in NB, was used as an internal control, using the following primers: forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds.

Immunohistochemistry and immunoblot. 8 × 10⁴ cells were cytospun on coverslips and fixed in 4% PFA. The slides were then incubated at room temperature for 1 hour with an antibody recognizing human NT-3 (1:300; Invitrogen). Cells were transfected with siRNA or TrkC constructs, using Lipofectamine 2000 (Invitrogen) for IMR32 cells (Invitrogen). Cell death was analyzed 24 hours after treatment/transfection either by trypan blue exclusion as described previously (15) or with ToxiLight Bio Assay kit (Lonza). Apoptosis was monitored by measuring caspase-3 activity as described previously (15), using the ApoAlert CPP32 Kit from Clontech (USA). For detection of DNA fragmentation, CLB-Ge2 cells were grown in poly-L-lysine–coated slides and fixed with 4% PFA 24 hours after treatment/transfection. IMR32 and CLB-VolMo–transfected cells were cytospun before PFA fixation. TUNEL was performed with 300 U/ml TUNEL enzyme and 6 μM biotinylated dUTP (Roche Diagnostics), as described previously (52). Q-RT-PCR was performed using the following primers: TrkC, forward 5′-AGCTCAACAGCCGACACTGGCAAAACAATGCA-3′ and reverse 5′-AACAGGGTTGTACCCCTCTC-3′; NT-3, forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds. The ubiquitously expressed human HPRT gene, showing the least variable expression in NB, was used as an internal control, using the following primers: forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds. The ubiquitously expressed human HPRT gene, showing the least variable expression in NB, was used as an internal control, using the following primers: forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds. The ubiquitously expressed human HPRT gene, showing the least variable expression in NB, was used as an internal control, using the following primers: forward 5′-GAAACGCGATGTAAGGAAGC-3′ and reverse 5′-CCAGCCCCAGGTGTTTATTGT-3′. For all 3 couples of primers, polymerase was activated at 95°C for 10 minutes, followed by 35 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 5 seconds.
Expression of TrkC constructs and endogenous TrkC cleavage were monitored by Western blot with anti-TrkC antibody (catalog sc-11, Santa Cruz Biotechnology Inc.), and an anti-α-actin (13E5; Cell Signaling Technology) was used as loading control as previously described (14).

Phospho-Akt and phospho-Erk levels of CLB-Ge2 cells were measured by Western blot with anti–phospho-Akt (4058, Cell Signaling Technology) and phospho-Erk1 and Erk2 (E7028, Sigma-Aldrich) after 16 hours of culture on serum-free medium with 2 μg/ml anti-TrkC antibody (catalog AF1404, R&D Systems), 20 nM Ly29402 (Sigma-Aldrich), 100 nM U0126 (Sigma-Aldrich), or 100 ng/ml NT-3 (Abcsy).

ELISA determination of NT-3 level. For NT-3 determination in cell culture supernatants, 13.S.24 cells were transfected with a human NT-3 construct and scrambled siRNA or NT-3 siRNA. Supernatants were collected 24 hours later. A white 96-well plate (CORNING) was coated with a monoclonal human NT-3 antibody (MAB267, R&D systems), blocked with bovine serum albumin, and incubated with the samples, followed by detection of NT-3 by using a biotinylated anti-human NT-3 antibody (BAP267, R&D systems), a streptavidin-peroxidase polymer (S2438, Sigma-Aldrich), and a chemiluminescent substrate (Pierce ECL Western Blotting Substrate). The luminescence was read on a Tecoan Infinite P5000 lumimeter. Human recombinant NT-3 (P450-03, Abcsy) was used as standard in the ELISA.

Chicken model for NB progression and dissemination. 10° NB cells suspended in 40 μl complete medium were seeded on 10-day-old (day 10) chick CAM. 2 μg anti-TrkC antibody or an isotopic unrelated antibody (catalog sc-1290, Santa Cruz Biotechnology Inc.) were injected in the tumor on day 11 and day 14. For siRNA treatment, 3 μg of scrambled, TrkC, or NT-3 siRNA were injected in a chorioallantoic vessel on days 11 and 14. On day 17, tumors were resected and area was measured with Axiolimage Release 4.6 software (Zeiss). To monitor apoptosis on primary tumors, they were fixed on 4% PFA, cryoprotected by overnight treatment with 30% sucrose, and embedded in Cryomount (Histolab). TUNEL staining was performed on tumor cryostat sections (Roche Diagnostics), and nuclei were stained with Hoechst (Sigma-Aldrich). Nuclei were visualized with Hoechst (Sigma-Aldrich). TUNEL staining (Sigma-Aldrich).

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