The Role of Ciliary Neurotrophic Factor and TRKB Signaling in Neuroblastoma

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THE ROLE OF CILIARY NEUROTROPHIC FACTOR AND TRKB SIGNALING IN NEUROBLASTOMA

A Dissertation Presented

by

John DeWitt

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Specializing in Neuroscience

October, 2012
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Neuroscience.

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ABSTRACT

Neuroblastoma is the most common pediatric cancer in infants, arising from the sympathoadrenal lineage of the neural crest. Despite recent advances in other pediatric cancers, long term survival in high risk cases of neuroblastoma remains below 40%. Therefore, to develop successful therapeutics targeting high risk tumors, further research into the mechanisms involved in high risk tumor formation is necessary. Prognosis in neuroblastoma is determined by a number of factors, including certain genetic and biological variables. The genetic variable correlated most with high risk disease is amplification of the MYCN gene, which is present in ~25% of tumors. Additionally, ~70% of these MYCN-amplified tumors express the neurotrophin receptor TrkB, and its ligand, brain-derived neurotrophic factor (BDNF), with concurrent expression of these proteins correlated with high risk disease independent of MYCN-amplification status. To better understand factors influencing MYCN-amplified tumor cell phenotype, and the role of TrkB signaling in high risk neuroblastoma, the experiments in this dissertation examined growth factor effects on MYCN-amplified tumor cells from the TH-MYCN mouse model of neuroblastoma, as well as the creation, and expression of a constitutively active TrkB receptor in a neural crest derived cell line.

Overexpression of MYCN targeted to the sympathoadrenal lineage by the tyrosine hydroxylase (TH) promoter is sufficient to cause neuroblastoma in 100% of mice homozygous for the transgene (TH-MYCN mice). Screening growth factors, in vitro treatment of tumor cells from dissociated TH-MYCN tumors with ciliary neurotrophic factor (CNTF) was found to promote differentiation marked by increased neurite outgrowth, and withdrawal of actively dividing cells from the cell cycle. These effects were both concentration dependent, and specific to CNTF, as all other neurotrophic factors tested had no effect on differentiation. Furthermore, TH-MYCN tumor cells were found to highly express the receptor for CNTF, CNTFRα both in vitro and in vivo. Testing the ability of CNTF to affect tumor growth in vivo, CNTF treatment attenuated subcutaneous tumor growth of the TH-MYCN tumor-derived cell line NHO1S in wild type 129/SvJ mice. Therefore, CNTF signaling may be a potential therapeutic target in MYCN-amplified neuroblastoma.

In addition to being significantly correlated with a poor prognosis in neuroblastoma, the presence of activated TrkB signaling promotes a more aggressive phenotype in established neuroblastoma cell lines. However, whether TrkB signaling is sufficient to transform neural crest derived cells had not been established. To determine the role of TrkB signaling in malignant transformation, the two immunoglobulin-like (Ig) ligand binding domains were removed from a full length rat TrkB receptor. Expression of this receptor, termed ΔIgTrkB, leads to elevated phosphorylated Erk levels in the absence of ligand, indicating the receptor is constitutively active. When expressed in the neural crest derived cell line NCM-1, constitutive TrkB signaling confers a highly transformed phenotype characterized by enhanced proliferation, anchorage-independent cell growth, anoikis resistance, and matrix invasion. Furthermore, ΔIgTrkB NCM-1 cells upregulate transcripts for a number of cancer promoting genes, in addition to the poor prognosis marker MYCN. In vivo, ΔIgTrkB NCM-1 cells form highly aggressive tumors, requiring euthanasia of mice by 15 days following injection, while wild type cells fail to grow. Thus, TrkB signaling is sufficient to transform cells derived from the neural crest.
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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Introduction

Neuroblastoma is a pediatric cancer arising from the developing tissues of the sympathoadrenal lineage of the neural crest. Although neuroblastoma is the most common extracranial solid tumor in children, and the most common cancer in infants, little is known about the etiology of the cancer, and disease free survival for stage 3 or 4 disease with current therapies remains extremely poor. Neuroblastoma is a uniquely heterogeneous cancer in which, in rare cases patients can present with widespread metastatic disease that will regress with support treatment alone, while those with high risk disease often relapse following intensive chemotherapy. Thus, prognosis is determined less by the size and spread of the tumor, as with most cancers, but more by other factors such as, age of diagnosis, tumor histology, as well as the presence or absence of genetic and biological variables in the tumor. To improve patient outcomes in the high risk category we can not rely simply on earlier detection, rather, research efforts need to focus on alternative strategies, such as identifying factors that can influence tumor cell phenotype, and understanding the mechanisms involved in the pathogenesis that occurs in the developing sympathetic nervous system that gives rise to high risk disease. These efforts will allow the development of therapeutics that directly target these derangements.
High risk neuroblastoma tumors often lack differentiation, while more favorable tumors are typically more differentiated, more closely resembling the neuronal lineage from which they originated. Therefore, one strategy to treat high risk disease is to identify factors which can promote differentiation of these poorly differentiated tumors, making them less aggressive, and more amenable to surgical interventions or chemotherapeutics. One of the goals of this dissertation was to identify such factors.

Additionally, although expression of the neurotrophin receptor TrkB and its ligand brain-derived neurotrophic factor (BDNF) is correlated with a poor prognosis in neuroblastoma, it is not known whether TrkB signaling itself is sufficient to give rise to these aggressive tumors. TrkB regulated tumorigenesis would support this signaling pathway as an important drug target in TrkB-expressing neuroblastoma. Therefore, another goal of this dissertation was to determine if TrkB signaling would be sufficient to transform neural crest cells.

**Neuroblastoma**

*The Disease*

Neuroblastoma represents approximately 8-10% of pediatric cancers but is responsible for 15% of all pediatric cancer deaths (Gurney et al., 1997). Although improvements have been made in patient outcomes, long-term survival in those with high risk disease remains below 40% (Matthay et al., 1999; De Bernardi et al., 2003). Neuroblastoma
tumors arise from the developing sympathoadrenal lineage. Although they can be found anywhere in the sympathetic nervous system, approximately 50% develop in the adrenal medulla, with the rest generally arising in paraspinal ganglia of the abdomen, chest, or pelvis (Friedman and Castleberry, 2007). Presenting symptoms can vary greatly depending on primary tumor site and the presence or absence of metastatic disease. However, with most tumors arising in the abdomen (65%), symptoms are often vague, including fatigue, loss of appetite, and a swollen abdomen. Clinical presentation for neuroblastoma is separated into the three categories of localized, metastatic, and the unique case of 4S disease.

Patients have localized disease in 40% of cases of neuroblastoma, with tumors ranging from incidental findings on prenatal ultrasonography or chest radiographs, to highly invasive local tumors (Maris et al., 2007). Paraspinal tumors arising along the sympathetic chain can present as Horner’s syndrome when found in the cervical region (Mahoney et al., 2006), or extend into spinal foramina, causing symptoms of spinal cord and nerve root compression (De Bernardi et al., 2005). Overall, 5-15% of all neuroblastoma patients will present with paraspinal tumors, and 5% of all patients will present with signs of spinal cord compression, such as motor weakness or sensory loss (De Bernardi et al., 2001). In addition to symptoms of mass effect from tumor growth, patients with localized tumors can also present with paraneoplastic syndromes due to tumor cell secretion of various factors, or autoimmune responses against tumor cell antigens. These include tumor secretion of vasoactive intestinal peptide (VIP) leading to
watery diarrhea and failure to thrive (Kaplan et al., 1980), as well as opsoclonus-myoclonus syndrome, which can lead to long-term neurological deficits in 70-80% of patients (Mitchell et al., 2002).

Unfortunately, as high as 50% of neuroblastoma patients will present with widespread metastatic disease, and unlike those with localized disease, are typically very ill due to extensive tumor burden. Metastases are often found in cortical bone, causing bone pain, or the bone marrow, where marrow replacement can occur with symptoms of marrow failure such as anemia (Quinn and Altman, 1979). Additionally, for unknown reasons, neuroblastoma can often metastasize to the bony orbit, with patients in these cases often presenting with periorbital ecchymoses (bilateral black eyes).

Interestingly, in about 5% of cases, patients will present with metastatic disease that will spontaneously regress and be cured by supportive treatment alone. These tumors, termed 4S (special), typically occur in younger patients and consist of small primary tumors with metastasis to the liver, bone marrow, and skin (D'Angio et al., 1971). Although typically these patients have a favorable prognosis, extensive hepatic tumor growth can lead to hepatomegaly and respiratory failure in patients younger than 4 weeks of age (van Noesel et al., 1997).

Factors Affecting Prognosis
Although, as in other neoplasms, the presence of localized versus metastatic disease at the time of diagnosis is important in determining prognosis, in neuroblastoma prognosis is largely dependent on additional factors including International Neuroblastoma Staging System (INSS) stage, age at diagnosis, tumor pathology, \textit{MYCN} amplification status, as well as other genetic and biological variables such as Trk receptor expression. Because of the extreme heterogeneity of disease these many different variables create, neuroblastoma prognosis is currently separated into three risk categories: low, intermediate, and high (Castleberry et al., 1997). In general, those with higher risk disease have higher stage disease, are diagnosed later in life, and have tumors with \textit{MYCN}-amplification and unfavorable (less differentiated) tumor histology (Figure 1-1).
Table 1-1: Neuroblastoma prognosis risk groups

*adapted from (Brodeur, 2003)

<table>
<thead>
<tr>
<th>INSS Stage</th>
<th>Low Risk</th>
<th>Intermediate Risk</th>
<th>High Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>2A, 2B</td>
<td>Age &lt; 1 yr, or age 1-21 yrs and MYCN non-AMP, or age 1-21 yrs and MYCN AMP + FH</td>
<td>None</td>
<td>Age 1-21 yrs and MYCN AMP + UH</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>Age &lt; 1 yr and non-MYCN AMP, or age 1-21 yrs and MYCN non-AMP + FH</td>
<td>Age 0-21 yrs and MYCN AMP, or age 1-21 yrs and MYCN non-AMP + UH</td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>Age &lt; 1 yr and MYCN non-AMP</td>
<td>Age &lt; 1 yr and MYCN AMP, or age 1-21 yrs</td>
</tr>
<tr>
<td>4S</td>
<td>MYCN non-AMP and FH</td>
<td>MYCN non-AMP and UH</td>
<td>MYCN AMP</td>
</tr>
</tbody>
</table>

AMP = amplified, UH = unfavorable histology (undifferentiated, higher mitosis-karyorrhexis index, MKI) FH = favorable histology (differentiated, lower MKI)

Similar to many other malignancies, surgical staging, as defined by the International Neuroblastoma Staging System (INSS) (Brodeur et al., 1993), is an important prognostic indicator in neuroblastoma. Staging ranges from Stage 1 consisting of fully resectable localized disease with negative ipsilateral lymph nodes, to stage 4 patients who present with widespread metastatic disease to the bone marrow or other organs. Although those with the unique 4S subtype of neuroblastoma generally have a good prognosis despite metastatic disease, typically, those with higher stage disease have less favorable outcomes.
In addition to surgical staging, patient age at diagnosis is important in determining neuroblastoma prognosis. Those patients whom are diagnosed at 18 months of age or less tend to have much better outcomes, with less aggressive forms of the disease (London et al., 2005). Additionally, age might be an even better predictor of outcomes than stage, as infants have much better outcomes than older patients with the same stage disease. These outcomes are not simply dependent on delayed diagnoses, as studies on mass-screening at the age of 6 months in Japan detected a 50-100% increase in the prevalence of the neuroblastoma, but did not decrease the prevalence of the disease in patients over one year of age (Sawada et al., 1984; Woods et al., 1996). Therefore, if anything, mass screening leads to detection of tumors that would have spontaneously regressed, subjecting children to unnecessary, and potentially harmful testing, surgery, and chemotherapy. These outcomes suggest the existence of two subtypes of neuroblastoma, a biologically favorable form of the disease arising in younger patients (the screened population), and biologically unfavorable neuroblastoma forming in older patients. Therefore, the context of sympathetic neural development in which neuroblastoma tumorigenesis occurs may be important in determining tumor phenotype.

In addition to stage and age, by evaluating the pathological features of neuroblastoma tumors, and relating those features to patient’s clinical outcomes, favorable versus unfavorable histology has been developed as another prognostic tool (Shimada et al., 1999). In general, those tumors that are more differentiated, and therefore more closely resemble the tissue of the sympathetic nervous system from which
they have transformed, possess a more favorable prognosis. In addition to neuroblast differentiation, other histological factors are taken into consideration when deeming a tumor favorable versus unfavorable. These include the amount of Schwann cells present in the stroma of the tumor (more being favorable), and the number of mitotic figures and degenerating nuclei (mitosis-karyorrhexis index, MKI; lower MKI more favorable). Additionally, tumors with a favorable prognosis typically express the neurotrophin receptor TrkA, which may promote differentiation in the presence of nerve growth factor (NGF), or tumor cell apoptosis in its absence.

Similar to the prognostic significance of age, the genetic features of neuroblastoma tumors also suggest the presence of the two differently behaving subsets of the disease. The first type consists of tumor cells with mitotic dysfunction, resulting in hyperdiploid or near triploid karotypes with few cytogenic abnormalities such as deletions, duplications, or amplifications. Children with these tumors tend to have more favorable outcomes (Kaneko et al., 1987). On the other hand, poor prognosis tumors typically have a near diploid karotype, but gross chromosomal aberrations. Typically these aberrations consist of either 11q and 14q deletions, or, in the most aggressive form of neuroblastoma, 1p loss of heterozygosity (LOH) and the presence of MYCN amplification as either double minutes (DM), or homogeneously staining regions (HSRs) (Brodeur et al., 1997). In fact, the presence of MYCN amplification alone is one of the strongest prognostic variables for a poor prognosis in neuroblastoma (Brodeur et al., 1984). It is currently unknown what leads to amplification, however it is thought that
**MYCN** might be copied many times to form extrachromosomal circular DNA elements (the DMs), which can then be integrated back into a particular locus in a chromosome to form the HSRs. This genetic abnormality is found in approximately 22% of patients (Brodeur et al., 1997), and is associated with a rapid tumor progression and a poor prognosis even in infants, and in those with lower stages of disease (Seeger et al., 1985). Furthermore, TrkB and BDNF are predominantly expressed in MYCN amplified tumors (Nakagawara et al., 1994), where they may confer an autocrine survival pathway, while TrkA is often expressed in non-MYCN amplified biologically favorable tumors, promoting differentiation or apoptosis (Nakagawara and Brodeur, 1997). Therefore, one of the focuses of neuroblastoma research needs to be identifying new strategies and treatments for children with MYCN amplified tumors to improve these patient’s outcomes.

All of these prognostic variables help categorize patients into the three risk groups discussed above, which are based on the probability of death from the disease. These risk groups then dictate the intensity of therapy a patient receives, from surgery alone in low risk cases, to dose-intensive chemotherapy, surgery, radiation therapy, bone marrow transplant, as well as adjuvant immunotherapy in patients diagnosed with high risk neuroblastoma. Although, risk stratification is fairly clear for those with very low risk, or very high risk disease with respect to age and stage, predicting outcomes in patients who fall in between has been more difficult. Recently, a new International Neuroblastoma Risk Group (INRG) classification system, separating risk groups into very low, low,
intermediate, and high risk, has been proposed to try to better stratify patients to the
proper treatment strategies (Cohn et al., 2009). Despite advances in risk stratification,
these risk groups are simply surrogates for different underlying biological subtypes of
neuroblastoma. Therefore, understanding the pathogenesis that gives rise to biologically
unfavorable neuroblastoma, and developing new therapeutic strategies to treat the
disease, are crucial to improving outcomes in this patient population.

*Genetic Factors Contributing to Disease*

Unlike some cancers, where a single genetic mutation is characteristic of the disease,
genetic factors leading to neuroblastoma are much more complicated and not well
known. Although most cases of neuroblastoma are sporadic, 1-2% of cases do show a
family history with autosomal dominant inheritance (Shojaei-Brosseau et al., 2004).
Recently, it was discovered that activating mutations in the anaplastic lymphoma kinase
(*ALK*) gene are responsible for the majority of cases of familial neuroblastoma (George et
al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008), as well as mutated in 5-15%
of sporadic cases (Chen et al., 2008; George et al., 2008). The exact function of the wild
type ALK protein is yet to be fully determined, but it is expressed in the developing
nervous system (Iwahara et al., 1997; Trochet et al., 2004), and can influence
proliferation of developing sympathetic precursors (Reiff et al., 2011). Disruptions of
another gene, *PHOX2B*, less common than mutations in *ALK*, have also been linked to
familial neuroblastoma. These missense or frame shift mutations result in loss-of-
function of the Phox2b protein (Mosse et al., 2004; Trochet et al., 2004). Similar loss-of-
function mutations have been identified in a small number (2.3%) of sporadic tumors as well (van Limpt et al., 2004).

Although mutations of the cell cycle genes CDK6 (Easton et al., 1998) and CYCLIND1 (Easton et al., 1998; Molenaar et al., 2003) in single cases have been reported, other than ALK and PHOX2B, additional inheritable genetic causes of neuroblastoma have been limited to the identification of polymorphisms predisposing to an increased risk of disease. Genome-wide association studies (GWAS) have begun to uncover these genetic variations that, along with enumerable other variables, such as environmental influences, lead to sporadic neuroblastoma tumorogenesis. Single nucleotide polymorphisms (SNPs) at the putative genes FLJ22536 (Maris et al., 2008) and BARD1 (Capasso et al., 2009), and the gene LMO1 (Wang et al., 2011) have been identified to be significantly enriched among neuroblastoma patients. Additionally, in the cases of BARD1 and LMO1, these polymorphism code for proteins leading to enhanced oncogenic potential (Bosse et al., 2011), confirming their likely role in neuroblastoma formation.

Neuroblastoma – a Neural Crest Derived Tumor

Neural Crest Development

Neuroblastoma is a malignancy arising from the developing sympathoadrenal (SA) lineage of neural crest. The neural crest is a transitory structure that arises from the
neural tube early in development following neural tube closure. Soon after specification from the neural tube, trunk neural crest cells undergo an epidermal to mesenchymal transition (EMT), becoming a migratory, multipotent population of precursor cells that have the ability to differentiate into a number of cell types including glia, melanocytes, sensory neurons, and SA precursors (Baroffio et al., 1988; Dupin et al., 1990). Since neuroblastoma can form in the adrenal medulla, in addition to anywhere in the sympathetic nervous system, it is thought to arise from this SA precursor population, which has the ability to give rise to sympathetic neurons, and the chromaffin cells of the adrenal medulla (Anderson et al., 1991).

Specification of neural crest cells to the SA lineage involves the exposure of developing neural crest cells to specific growth factors, as well as the expression of particular transcription factors (Figure 1-1). The main family of growth factors specifying commitment to the SA lineage is the bone morphogenic protein (BMP) family. As shown by inhibition studies, BMPs, which are expressed in the dorsal aorta where sympathoadrenal progenitors coalesce following their migration from above the neural tube (Shah et al., 1996), are essential for initiation of differentiation to the sympathetic lineage (Schneider et al., 1999). The first pro-neural gene expressed in this progenitor population is ASCL1, encoding the basic helix loop helix (bHLH) transcription factor hASH1 (or mASH1 and cASH1 in the mouse and chicken, respectively). Rather than induce expression of ASCL1, which is in fact expressed in SA progenitor cells around the dorsal aorta prior to BMP expression, BMPs are thought to maintain expression of
ASCL1 in SA precursors (Lo et al., 1997). Following expression ASCL1, further specification of SA progenitors involves the coordinated gene expression of ASCL1, PHOX2A, PHOX2B, and HAND2 (Hirsch et al., 1998; Stanke et al., 1999; Howard et al., 2000).

PHOX2B codes for a homeodomain DNA binding protein that plays an important role in the development of the SA lineage. Although hASH1 induces expression of PHOX2B, Phox2b can reciprocally induce expression of ASCL1. Furthermore, Phox2b, but not hASH1, is able to induce expression PHOX2A and HAND2. In turn, Hand2, another bHLH transcription factor, then reciprocally upregulates expression of ASCL1 and PHOX2B, forming an autocrine loop of transcription factor expression promoting neural crest progenitor cells to the SA lineage (Bertrand et al., 2002). Following commitment to the SA lineage by this coordinated transcription factor expression, SA progenitor cells begin to express neuronal and catecholaminergic specific genes such as tyrosine hydroxylase (TH), dopamine beta-hydroxylase (DBH), neurofilament (NF), and SCG10, and then migrate from the dorsal aorta to form the secondary sympathetic ganglia and adrenal medulla, where further specification to sympathetic neurons and chromaffin cells occurs (Huber, 2006). Further specification of SA progenitors to a sympathetic neuronal fate involves expression of a number of proteins including the neuroblastoma poor prognosis marker MYCN, another DNA binding transcription factor.
Late stage and terminal differentiation to mature sympathetic neurons requires subsequent neurotrophin signaling. Following commitment to sympathetic neurons, SA begin to express TrkC and become dependent on the TrkC ligand neurotrophin-3 (NT-3) for survival (Birren et al., 1993). In addition to TrkC, sympathoblasts simultaneously transiently express TrkB (Straub et al., 2007). However, TrkB’s role in sympathetic development remains unclear, as there is no apparent sympathetic phenotype in TrkB or BDNF-knockout mice (Schober et al., 1997). Following TrkC expression, sympathoblasts begin to express TrkA and their trophic dependence shifts as they differentiate to mature sympathetic neurons, with nerve growth factor (NGF) becoming the predominant factor supporting their survival (Birren et al., 1993).
Neuroblastoma and the Neural Crest Lineage

In addition to the simple fact that neuroblastoma can arise anywhere in the distribution of the sympathoadrenal lineage, additional lines of evidence from the differentiation of the SA from the neural crest confirm neuroblastoma as a malignancy of the developing sympathetic nervous system. *ASCL1*, the first transcription factor expressed in neural crest cells destined for the SA lineage, is expressed at high levels in many neuroblastomas (Soderholm et al., 1999; Ichimiya et al., 2001). This expression suggests
that in some cases transformation may occur at this early stage of SA development, or alternatively these tumor cells may be reverting back to a less differentiated state. Despite this persistence of ASCL1 expression, these cells are able to respond to differentiation cues, as differentiation of hASH1 positive neuroblastoma cells by retinoic acid treatment leads to down regulation of ASCL1 expression (Soderholm et al., 1999; Ichimiya et al., 2001).

Phox2b is also important in both SA development and neuroblastoma. As mentioned above, mutation of the PHOX2B locus is one of the few known causes of familial neuroblastoma (Mosse et al., 2004; Trochet et al., 2004; Bourdeaut et al., 2005). Interestingly, although mutation in these familial cases leads to loss of function of the gene, suggesting PHOX2B to be a tumor suppressor, tumor samples and cell lines harboring similar PHOX2B mutations express the downstream target gene DBH, suggesting these mutations are not dominant negative (van Limpt et al., 2004; Longo et al., 2008). In any case, disruption of PHOX2B is clearly sufficient to drive neuroblastoma formation, although the mechanism involved is yet to be determined. Hand2, whose expression is initiated by Phox2b during SA progenitor development, is also frequently expressed in neuroblastoma. However, its expression does not correlate with clinical stage (Gestblom et al., 1999), indicating Hand2, unlike Phox2b, is unlikely to be a driver of tumorigenesis in neuroblastoma.
Expression of MYCN in neuroblastoma, most often in the form of amplification of the gene, is well established as a marker of biologically unfavorable tumors. Like MYC, the MYCN protein is known to bind MAX, with this heterodimer activating transcription of genes that lead to progression through the G1 phase of the cell cycle, which in the brain is important in expansion of the neuronal progenitor cell population (Knoepfler et al., 2002). However, in addition to regulating proliferation, studies in mycn knockout mice suggest the transcription factor is important differentiation as well (Sawai et al., 1993). Although, at this point it is not clear whether MYCN is predominantly involved in proliferation or differentiation during SA development from the neural crest, transformation occurring at the MYCN-expressing stage seems to lead to an aggressive MYCN amplified form of neuroblastoma. Furthermore, the importance of MYCN in neuroblastoma tumorigenesis is highlighted by the fact that MYCN overexpression alone is sufficient to initiate tumor formation, as transgenic mice that express MYCN under the tyrosine hydroxylase promoter (TH-MYCN mice) form tumors in 100% of mice homozygous for the transgene (Weiss et al., 1997). Although penetrance of tumor formation is highly strain specific, suggesting other factors are likely involved in MYCN-mediated transformation, TH-MYCN mice provide a model to study primary MYCN amplified neuroblastoma tumor cells in culture, as well as in vivo. Experiments described in this dissertation employ the use of this model in an effort to discover growth factors that can promote a more differentiated phenotype in MYCN amplified tumor cells.

Trk Signaling
The Role of Trks in Sympathetic Development

As mentioned above, neurotrophin signaling through Trk receptors is important in late stage sympathetic neuron development; and the role of Trks in neuroblastoma affords a more in-depth discussion of neurotrophin signaling in sympathetic development.

Interestingly, prior to being found to be the high affinity receptor for NGF, the Trk gene was first discovered as an oncogene fused to the tropomyosin gene promoting continuous proliferation in colon cancer cells. There are now known to be three members of the tropomyosin receptor kinase (Trk) family, TrkA, TrkB, and TrkC to which neurotrophins differentially bind (Snider, 1994). TrkA preferentially binds NGF, TrkB binds BDNF and neurotrophin-4/5 (NT-4/5), and TrkC binds neurotrophin-3 (NT-3). In addition to the Trk family of neurotrophin receptors, all members of the neurotrophin family also bind the p75, or low affinity nerve growth factor receptor (LNGFR), a member of the tumor necrosis factor receptor (TNFR) family (Snider, 1994). During development, each receptor / ligand combination has specific functions. Signaling through the NGF / TrkA ligand-receptor complex supports survival and differentiation of sympathetic neurons and sensory neurons that respond to pain and temperature (Smeyne et al., 1994). BDNF / TrkB and NT-4/5 / TrkB signaling supports sensory neurons responsible for relaying tactile stimuli and motor neurons respectively, while NT-3 / TrkC signaling supports early sympathetic progenitors (Birren et al., 1993) and sensory neurons responsive to limb movement and position (Klein et al., 1993; Klein et al., 1994).
TrkA expression is abundant throughout the sympathetic nervous system. As mentioned above, TrkA supports survival and differentiation of developing SA progenitors into terminally differentiated sympathetic neurons. During development, TrkA expression is detected in most cells by embryonic day (E) 16, and intense labeling occurs by E18 in the rat (Ernfors et al., 1992). In mice, TrkA mRNA expression is detected by *in situ* hybridization starting at E13.5 and increasing through E15.5 until birth (Fagan et al., 1996). Expression is similar in the chick, with TrkA initially seen at E5 followed by all neurons appearing immunoreactive by E8 (Straub et al., 2007).

Furthermore, TrkA expression is not restricted to the developmental period, as TrkA is highly expressed in the adult sympathetic nervous system of rats (prevertebral, paravertebral, and superior cervical ganglia [SCG]), as well as humans (paravertebral ganglion) (Wetmore and Olson, 1995; Garcia-Suarez et al., 1996; Schmidt et al., 1998).

Functionally, TrkA signaling is very important in early sympathetic neuron survival and development. This has been established by a number of studies involving NGF treatment, and TrkA mutant mice. Injection of NGF in postnatal mice causes a two- to three-fold increase in the size and cell number of SCG and thoracic sympathetic ganglia (Levi-Montalcini and Booker, 1960b; Black et al., 1972). On the other hand, treatment with anti-NGF antiserum leads to massive neuronal loss in the postnatal mouse SCG (Levi-Montalcini and Booker, 1960a). These effects are not limited to the postnatal period, as transuterine NGF injection leads to an increase in sympathetic neurons, and treatment of pregnant mice with antiserum leads to an 80% loss of neurons in the SCG.
and stellate ganglion of offspring (Kessler and Black, 1980). In TrkA mutant mice, innervation of the submaxillary gland by tyrosine hydroxylase (TH) positive sympathetic fibers is disrupted throughout development (Fagan et al., 1996). Therefore, TrkA signaling is critical for the survival of developing sympathetic precursors, and the proper development of the sympathetic nervous system.

As mentioned above, like TrkA, TrkC is expressed in the sympathetic nervous system, however the majority of this expression is limited to early developmental period when TrkC signaling can support early sympathoblast survival. In the rat, TrkC is first expressed at E13, before the onset of TrkA expression at E16 (Ernfors et al., 1992). After E17.5 TrkC expression decreases rapidly to the point that it is reduced by an order of magnitude by birth (DiCicco-Bloom et al., 1993). A similar pattern of expression occurs in both the mouse, with the onset of expression at E11.5 increasing to E13.5 and then restricted to only a few cells by birth (Fagan et al., 1996), and the chick, with expression seen at E6.5 downregulated by E8 (Straub et al., 2007). In humans, TrkC is expressed in only 10% of cells in adult sympathetic ganglia (Garcia-Suarez et al., 1996). However, despite this consistent expression pattern across species, and the ability of TrkC to support the survival of early sympathoblasts (Birren et al., 1993), TrkC expression does not appear necessary for normal sympathetic development, as TrkC knockout mice show no loss of SCG neurons (Tessarollo et al., 1997). This might be explained by the ability of NT-3 to activate TrkA in the absence of TrkC expression. Indeed, unlike TrkC mutants, NT-3 mutant mice have a 35-50% reduction in the number of SCG neurons.
(Francis et al., 1999). Thus, although TrkC is consistently expressed in during sympathetic development, its expression is not required.

Compared to TrkC, the expression of TrkB in the sympathetic nervous system is even more limited. In the mouse, TrkB expression has been observed in embryonic sympathetic ganglia by in situ hybridization (Schecterson and Bothwell, 1992), and immunohistochemically at E13 and E15, co-expressed with tyrosine hydroxylase (Straub et al., 2007). Although, in the SCG of 3 day to 4 week old rats, TrkB mRNA is barely detectable, it is seen in low but significantly higher levels in the prevertebral ganglia (Dixon and McKinnon, 1994). In the chick, TrkB immunoreactivity has been observed transiently in embryonic sympathetic ganglia starting at E5 and was lost by E8 (Straub et al., 2007). Interestingly, these TrkB-expressing sympathoblasts proliferate in response to BDNF in culture. In the adult, unlike TrkA and TrkC, TrkB immunoreactivity has not been detected in human sympathetic ganglia, and very few neurons express TrkB mRNA in adult rat SCG (Wetmore and Olson, 1995; Garcia-Suarez et al., 1996). Thus, TrkB appears to be expressed in a limited capacity in the developing mammalian and avian sympathetic nervous system.

Current evidence suggests TrkB / BDNF signaling has a limited functional role in sympathetic nervous system development. In either BDNF or TrkB mutant mice, there is no significant change in SCG size or neuron number (Ernfors et al., 1994; Fagan et al., 1996). Although, transgenic mice overexpressing BDNF under the α-mysosin heavy
chain promoter show a hyperinnervation of TH-positive neurons in the arrector pili muscle in the skin (Botchkarev et al., 1998), whether this effect is mediated through the few existing TrkB expressing sympathetic neurons or the low affinity neurotrophin receptor p75 has not been established.

The Significance of Trk Receptor Expression in Neuroblastoma

From the above discussion it is clear that TrkA is highly expressed and instrumental in sympathetic nervous system development, TrkC is consistently expressed and appears to play an early role, while TrkB expression is limited with no apparent function in normal sympathetic development. These expression patterns and functions in sympathetic nervous system development may partly explain the differences in tumor phenotype observed in neuroblastoma tumors expressing Trk receptors and their ligands. Expression of TrkA or TrkC and their respective ligands are important in sympathetic development and differentiation, and there expression in neuroblastoma leads to a less aggressive and more differentiated phenotype. On the other hand, expression of TrkB, which has no role in sympathetic differentiation, but can promote proliferation (Straub et al., 2007), is correlated with an aggressive and highly proliferative tumor cell phenotype, possibly representing cells stuck in a transient sympathoadrenal precursor stage, lacking the proper Trk receptor signaling to properly differentiate.

TrkA is expressed in biologically favorable tumors. High expression of the receptor is associated with favorable clinical features, inversely correlated MYCN
amplification (Nakagawara et al., 1992), and strongly predictive of a favorable outcome (Nakagawara et al., 1993). TrkA expression is a marker of biologically favorable tumors, and likely also contributes to this phenotype, as TrkA expressing tumor cells are able to respond to NGF treatment with enhanced survival and terminal differentiation like normal sympathetic neurons (Nakagawara and Brodeur, 1997). Furthermore, consistent with the hypothesis of trophic dependence in sympathetic neurons, when these same TrkA expressing cells are cultured in the absence of NGF they undergo apoptosis within 7 days. The ability of TrkA expressing neuroblastoma to appropriately respond to the presence or absence of NGF by differentiating or undergoing apoptosis may explain the ability of some favorable neuroblastomas, especially 4S tumors, to spontaneously undergo differentiation and regression. Recently, a developmentally regulated TrkA splice variant (TrkAIII) that splices out exons 6, 7, and 9 has been identified (Tacconelli et al., 2004). Interestingly, this splice variant, expressed exclusively in undifferentiated neuronal precursors, is constitutively active. Although, TrkAIII appears to be expressed more often in higher stage neuroblastomas, its presence is consistent with the typically undifferentiated phenotype of these tumors.

Like TrkA, expression of TrkC is found in biologically favorable neuroblastomas. The receptor is expressed in about 25% of primary neuroblastomas, and these tumors essentially represent a subset of TrkA-expressing tumors (Yamashiro et al., 1996). Furthermore, TrkC-expressing tumors are correlated with younger age, lower stage, and a lack of MYCN amplification (Ryden et al., 1996). NT-3 does not appear to be
appreciably expressed in primary neuroblastomas (Yamashiro et al., 1997). Therefore, TrkC, like TrkA in the absence of NGF, may promote apoptosis and regression in the more favorable tumors that express the receptor.

Unlike TrkA and TrkC, expression of TrkB and its ligand, BDNF, is a strong predictor of an aggressive tumor phenotype and poor prognosis in neuroblastoma. Understanding how TrkB, a receptor normally only transiently expressed in the developing sympathetic nervous system, contributes to neuroblastoma tumorigenesis is crucial to developing successful targeted therapies for poor-prognosis neuroblastoma. TrkB and BDNF are predominantly expressed in aggressive, treatment resistant neuroblastoma, where this signaling pathway can act in an autocrine manner to promote metastasis (Matsumoto et al., 1995; Brodeur et al., 2009). Tyrosine kinase activated signaling pathways are required for TrkB-mediated aggressiveness in neuroblastoma tumors, as a truncated TrkB isoform which lacks the tyrosine kinase signaling domain, is found in more differentiated tumors such as ganglioneuroblastomas (Nakagawara et al., 1994). In these tumors, truncated TrkB can act as a dominant negative inhibitor of full length TrkB signaling (Haapasalo et al., 2001)

TrkB signaling promotes an aggressive tumor cell phenotype in neuroblastoma by mediating cell growth, survival, adhesion, and metastasis through pathways activated normally by Trk signaling; these are the RAS, PI3K, and Rho signaling pathways. The primary signaling pathway involved in promoting cell growth is the Ras-MAPK/Erk
signaling cascade. Upon receptor activation, phosphorylation of the tyrosine residue Y490 is followed by phosphorylation of Shc, and recruitment of the adapter protein Grb2 complexed to the RAS exchange factor SOS. This complex stimulates RAS by promoting activation of the RAS-Raf-MEK-MAPK-Erk pathway, resulting in cell growth and proliferation (Kao et al., 2001). TrkB-mediated cell survival is primarily regulated by activation of the PI3K pathway (Yao and Cooper, 1995), which can be both RAS-dependent, and -independent. Independent of upstream RAS activation, PI3K can be activated through Shc, Grb2, or Irs1 (Reichardt, 2006). PI3K activation leads to the activation of the serine threonine kinase Akt that modulates a number of factors important in cell death pathways including Bad, Caspase-9, GSK3β, and IkB. TrkB activation can also modify adhesion molecules, such as cadherin (Zhou et al., 1997), membrane chondroitin sulfate proteoglycan (MCSP) (Schulte et al., 2005), and signaling pathways, such as the Cdc42-Rho-Rac-actin pathway (Miyamoto et al., 2006) that are important in the regulation of adhesion and metastasis.

A number of *ex vivo* studies have further elucidated how TrkB signaling promotes a biologically unfavorable phenotype in neuroblastoma. In the TrkB-expressing neuroblastoma cell line, SMS-KCN, treatment with BDNF enhances cell survival in serum-free media (Nakagawara et al., 1994; Feng et al., 2001). Furthermore, BDNF treatment of TrkB-expressing neuroblastoma cells enhances their invasiveness (Matsumoto et al., 1995; Cimmino et al., 2009), and renders them less sensitive to cytotoxic drugs (Scala et al., 1996; Ho et al., 2002; Jaboin et al., 2002; Li et al., 2005; Li
et al., 2007; Baj and Tongiorgi, 2009). In addition, TrkB activation by BDNF in SY5Y neuroblastoma cells increases production of the pro-angiogenic molecule VEGF (Nakamura et al., 2006), while expression of TrkA down-regulates expression and function of angiogenic stimulator in this same cell line (Eggert et al., 2002). Therefore, concomitant expression of both full length TrkB and BDNF likely forms an autocrine or paracrine survival pathway in the associated aggressive poor prognosis tumors.

Furthermore, an active TrkB signaling pathway may be important in the propensity of a high risk patient population to succumb to relapse following chemotherapy. It has been shown that neuroblastoma cells that survive repeated exposures to cytotoxic drugs express increasing levels of BDNF; this may help confer survival and multidrug resistance in these cells (Matsumoto et al., 1995). The increases in BDNF levels, coupled with hypoxia, cause increases in TrkB expression (Martens et al., 2007), and may provide a situation in which the microenvironment within the tumor is able to respond to environmental insults (such as chemotherapeutics) to promote survival of tumor cells, and eventual relapse. These survival effects are mediated in part by activation of PI-3kinase (Jaboin et al., 2002) and Akt (Li et al., 2005), and inactivation of GSK3β (Li et al., 2007), as in normal Trk expressing cells. Additionally, even in tumor cells in which TrkB expression is low, local environments rich in BDNF may be able to support the survival of these cells and protect them from chemotherapeutics, setting the stage for later relapse. For instance, in multiple myeloma, bone marrow-
derived BDNF is able to activate TrkB-expressing multiple myeloma cells and promote drug resistance via the PI-3kinase / Akt pathway (Pearse et al., 2005).

As well as increasing survival in neuroblastoma cells, activation of the TrkB signaling pathway also promotes migration, invasiveness, and metastatic potential. In SY5Y and SK-N-AS neuroblastoma cells, TrkB signaling enhances tumor cell invasiveness in an autocrine manner, increasing the expression of hepatocyte growth factor (HGF) and its receptor, c-MET (Hecht et al., 2005). Furthermore, in vitro, SY5Y cells transfected with TrkB, but not those transfected with TrkA, display increased invasiveness mediated by upregulation of galectin-1 (Gal-1) (Cimmino et al., 2009). Thus, research with neuroblastoma cell lines demonstrates the ability of TrkB signaling to promote a more aggressive tumor cell phenotype, suggesting the expression of TrkB / BDNF is causal in the aggressive and biologically unfavorable nature of tumors that express the receptor and ligand.

Despite evidence demonstrating the role of TrkB signaling in neuroblastoma cell lines, there has been little in vivo work showing how aberrant TrkB signaling might contribute to tumorigenesis, or whether TrkB signaling is sufficient to transform normal neural crest precursors. Developing sympathoadrenal precursors transiently express TrkB in vivo, and are able to proliferate in response to BDNF ex vivo (Straub et al., 2007), suggesting that aberrant TrkB signaling in vivo may be sufficient to cause transformation in neuroblastoma. By examining the effects of expression of a constitutively active TrkB
receptor in a neural crest derived cell line, experiments presented in this dissertation suggest TrkB signaling can be an initiator of tumor formation in neuroblastoma.

**Summary**

Taking into account the development of the sympathoadrenal lineage from the neural crest, the heterogeneity of disease seen in neuroblastoma tumor phenotypes is likely a result of tumors arising from different points in the developmental program. It seems likely that biologically unfavorable tumors arise from an earlier point in development, and like early precursors, are undifferentiated, highly proliferative, and express TrkB. On the other hand, biologically favorable tumors appear to arise later in development, and more closely resemble normal sympathetic neurons by virtue of expressing TrkA and TrkC, and responding appropriately to differentiation / apoptotic cues transmitted by these receptors. Ultimately, the development of new strategies that can shift poor prognosis tumors to the more differentiated state of biologically favorable tumors may be of therapeutic benefit. Furthermore, determining what aspects of biologically unfavorable tumors – such as MYCN amplification or TrkB signaling – are sufficient to drive neuroblastoma formation, will be important in targeting specific therapeutics for these derangements.

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CHAPTER 2: Ciliary neurotrophic factor reduces proliferation and promotes differentiation of tumor cells from a mouse model of neuroblastoma

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Abstract
Neuroblastoma is a pediatric malignancy arising in tissues of the sympathoadrenal lineage. Elevated expression of the transcription factor MYCN in tumor tissue is associated with poor clinical outcomes, and MYCN overexpression targeted to the neural crest lineage by the tyrosine hydroxylase (TH) promoter is sufficient to cause neuroblastoma in transgenic mice (TH-MYCN). Ciliary neurotrophic factor (CNTF) is a neuropoietic cytokine known to influence survival, differentiation, and proliferation in developing sympathetic neurons; therefore we determined whether TH-MYCN tumors respond to CNTF by differentiating. We found that TH-MYCN tumors predominantly arise in the celiac ganglia and many cells express CNTFR alpha. In cell culture, CNTF increases process outgrowth and decreases proliferation of cells derived from TH-MYCN tumors with an EC50 of approximately 0.4 ng/mL. CNTF promotes differentiation by first inducing cell cycle withdrawal rather than by causing a non-mitotic population to extend neurites. In vivo, CNTF treatment attenuated subcutaneous tumor growth of the TH-MYCN tumor-derived cell line NHO1S in wild type 129/SvJ mice. Thus, activation of the CNTF receptor overrides the proliferative effect of N-MYC, suggesting a possible therapeutic target for poor prognosis, MYCN amplified tumors.

Introduction
Neuroblastoma is the most common extracranial solid tumor in children, and outcomes remain poor for many patients who present with the cancer at 18 months or older. Prognosis is stratified into low, intermediate, and high risk categories (Maris et al., 2007)
based on the biological heterogeneity of the disease, with the most consistent genetic aberration correlating with high risk disease being amplification of the \textit{MYCN} gene, which occurs in approximately one third of primary tumors (Maris and Matthay, 1999).

These poor prognosis \textit{MYCN} amplified tumors are typically stroma-poor and lack differentiation, while non-amplified tumors, tend to be stroma-rich, less aggressive, and more differentiated, suggesting factors in the tumor microenvironment may be important in determining tumor phenotype (Shimada et al., 1984). Furthermore, \textit{MYCN} expression can disrupt responses to normal developmental cues, as sympathetic neurons overexpressing the \textit{MYCN} protein reenter S phase and resist cell death induced by nerve growth factor (NGF) withdrawal (Wartiovaara et al., 2002). Therefore, agents promoting differentiation of \textit{MYCN} amplified tumors may improve outcomes in high risk neuroblastoma.

Previously, a mouse model of neuroblastoma was generated by targeting expression of \textit{MYCN} to the neural crest lineage using the rat tyrosine hydroxylase (TH) promoter (Weiss et al., 1997). 100\% of mice homozygous for the TH-\textit{MYCN} transgene develop tumors, however tumor penetrance is strain specific, as mice on the C57B6/J background develop tumors by 16 weeks of age (Hansford et al., 2004), as opposed to only 6.5 weeks in 129/SvJ mice carrying the transgene. These tumors show typical histopathological features of human neuroblastoma (Moore et al., 2008), as well as genetic aberrations similar to those found in the human disease (Weiss et al., 2000; Hackett et al., 2003). Therefore, TH-\textit{MYCN} mice represent a mouse model for studying
MYCN expressing primary tumor development, as well as the properties of MYCN expressing primary tumor cells in culture.

In addition to the presence or absence of MYCN amplification, the degree of tumor differentiation, which in both normal neuronal development and neuroblastoma is influenced by growth factor signaling, is another important factor in determining patient prognosis. Children diagnosed with tumors with increased differentiation have a better probability for disease-free survival (Shimada et al., 1984). Differentiation is influenced predominantly by the NGF/TrkA and BDNF/TrkB growth factor signaling pathways, with TrkA expressed primarily in more differentiated biologically favorable tumors and an active TrkB pathway often found in poor prognosis MYCN-amplified cases (Nakagawara et al., 1993; Nakagawara et al., 1994). Furthermore, transfection of SY5Y neuroblastoma cells with the TrkA receptor confers on these cells the ability to differentiate in response to NGF (Lavenius et al., 1995), while conversely, transfection with the TrkB receptor renders these cells resistant to chemotherapeutics (Ho et al., 2002). In addition to TrkA and TrkB, other growth factor pathways have been shown to be important in determining neuroblastoma tumor cell phenotype. Activation of the epidermal growth factor receptor (EGFR) promotes proliferation in SY5Y and NLM cells (Ho et al., 2005), and SMS-KCNR cells differentiated by retinoic acid express transforming growth factor beta 1 (TGF-beta 1) and its receptors (Cohen et al., 1995). Little is known about the effects of other factors known to be trophic in sympathetic neurons.
Here we report that the neuropoietic cytokine, ciliary neurotrophic factor (CNTF), promotes differentiation and cell cycle withdrawal in tumor cells isolated from TH-MYCN mice. Furthermore, CNTF treatment attenuates growth of the TH-MYCN tumor-derived cell line NHO1S in vivo. These results suggest the CNTF signaling pathway may represent an important target for differentiating poor prognosis MYCN-expressing tumors.

Materials and methods

Ethics Statement: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont (Animal Welfare Assurance Number, A3301-01; IACUC protocol number 07-114). Every effort was made to minimize suffering of the animals used.

Mice: TH-MYCN mice homozygous for the human MYCN cDNA under the rat tyrosine hydroxylase promoter (Weiss et al., 1997) on a 129/SvJ genetic background were used for all experiments involving TH-MYCN tumor removal and growth in culture. WT 129/SvJ mice were used for in vivo experiments with NHO1S cells, a cell line derived from TH-MYCN mouse tumors.
**Immunostaining:** Tumors were removed and immersion fixed overnight in Zamboni’s fixative (4% (w/v) paraformaldehyde, 15% (v/v) picric acid in 0.1 M sodium phosphate buffer, pH 7.4). Tumors were washed with PBS to remove fix and equilibrated in 15%, and then 30% sucrose overnight at 4°C. Tissue sections of 20 mm thickness were cut using a Microm HM 560 cryostat (Thermo Scientific) and mounted on SuperFrost Plus slides (Fisher Scientific). Sections were post-fixed 15 minutes in Zamboni’s fixative and placed into blocking solution (1x PBS consisting of 10% (v/v) heat-inactivated horse serum (Invitrogen), 0.5% Triton X-100 (Sigma), and 0.1% sodium azide (Fisher Scientific)) overnight at 4°C. Sections were stained with primary antibodies overnight at 4°C, followed by secondary antibodies for three hours at room temperature (see below for specific antibodies). Slides were then incubated in PBS + Hoechst (1:2000, Invitrogen) for five minutes at room temperature to stain nuclei and mounted with Mowiol (EMD Millipore). After staining, coverslips were mounted on slides with Permafluor (Thermo Scientific) containing Hoechst (1:2000, Invitrogen). Images were captured using a Nikon C1 confocal mounted on a Nikon Eclipse E800 microscope with a 60× Plan Apo (NA 1.4) oil objective lens, E7-C1 software, and UV, Argon, and He/Ne lasers exciting at 408, 488, and 543 nm and emitting at 404–500, 555–615 nm, respectively.

**Antibodies:** Primary antibodies used were as follows. Chicken anti-tyrosine hydroxylase (1:250, Aves), rabbit anti-ki67 (1:200, Novacastra), rabbit anti-CNTFRa (1:30, a generous gift from Dr. Hermann Rohrer of the Max Planck Institute for Brain Research, Frankfurt, Germany), mouse anti-3A10 (neurofilament associated antigen, 1:1,
Development Studies Hybridoma Bank), sheep anti-BrdU (1:100, Biodesign). Secondary antibodies were donkey anti-rabbit alexa 488 (1:1666, Invitrogen), donkey anti-mouse cy3 (1:1000, Jackson), donkey anti-rabbit cy3 (1:1000, Jackson), and donkey anti-goat alexa 488 (1:1000, Invitrogen).

Cell culture: All cells were grown at 37°C in 5% CO₂. To obtain TH-MYCN tumor cells, tumors were removed from homozygous TH-MYCN mice at 35 days of age and mechanically dissociated by triturating in warm Modified Puck’s solution with glucose (a Ca²⁺, Mg²⁺-free balanced salt solution) to produce a single cell suspension. Cells were then 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 (Fisher Scientific) at 50,000 cells per well in a 24-well tissue culture plates (Falcon). Cells were grown in 20 U/mL penicillin, 20 mg/mL streptomycin, 2mM L-glutamine, and 6 mg/mL glucose in modified L15CO₂ supplemented with B27 (1:50, Invitrogen). Growth factors were added upon plating and consisted of the following – CNTF (5, 1, 0.5, and 0.25 ng/mL, Alomone), BDNF (10 ng/mL, R&D), LIF (10 ng/mL, R&D), 7s NGF (1 μg/mL, Alomone), NT-3 (30 ng/mL, R&D), and GDNF (10 ng/mL, Peprotech). NHO1S cells (a generous gift from Dr. Michelle Haber of the Children’s Cancer Institute Australia (Cheng et al., 2007)) were maintained in 10% fetal calf serum, 20 U/mL penicillin, 20 mg/mL streptomycin, 2mM L-glutamine, and 6 mg/mL glucose in modified L15CO₂. For CNTF experiments, NHO1S cells were plated on poly-D-lysine and
laminin coated coverslips at 10,000 cells per well in a 24-well plates. Cells were treated in culture with CNTF (5 ng/mL).

Quantification of process outgrowth and proliferation: Following staining, process outgrowth and proliferation were quantified with the use of a Nikon Eclipse E800 microscope connected to a computer equipped with StereoInvestigator software (MBF Bioscience, Williston, VT) in the COBRE Molecular/Cellular Core Facility. The percentage of ki67 stained cells was quantified using the fractionator program of StereoInvestigator. Briefly, after creating a defined counting area, the program selected random views within the area to quantify staining and obtain an estimate of the total population. Process outgrowth was measured using Neurolucida (MBF Bioscience, Williston, VT). For each coverslip, 16 random views were selected in which to measure process length. In order to derive the process length per cell value for each condition, the total process length from the 16 random views was divided by the cell population (as determined with StereoInvestigator).

RNA extraction and qPCR: For RNA extraction from NHO1S cells, cells were grown to confluence in 6-well plates, lysed directly into TRI Reagent (Molecular Research Center), and RNA was isolated using the manufacturer’s protocol. RNA (1µg) was transcribed to cDNA using superscript III (Invitrogen) and random hexamer priming (Invitrogen). cDNA was then diluted 1:5 and CNTFRa and 18S RNA expression were quantified by qPCR with the use of FAM/BHQ labeled primer/Taqman probe mixes
(ABI) and an ABI 7700 machine in the Molecular Cellular Core operated by the Center of Biomedical Research Excellence (COBRE) in the Neuroscience department at the University of Vermont. RNA samples from at least three separate cell cultures were quantified.

In vivo CNTF treatment and NHO1S tumor growth: NHO1S cells, generously provided by Dr. Michelle Haber, Children's Cancer Institute Australia for Medical Research, Randwick, Australia, were grown to confluence in T75 culture dishes. Cells were injected subcutaneously into the flank of WT 129/SvJ mice (N=12) at 1 million cells per mouse in 200 μL of Matrigel (BD Biosciences). When tumors became palpable, in vivo treatment with CNTF was begun. Mice received an intraperitoneal injection of either saline or CNTF (0.2 mg/kg, in saline), daily for 7 days (6 mice per condition). Tumor growth was quantified by length and width caliper measurement daily by an observer blinded to the treatment condition. Tumor volume was estimated from length and width measurements using the formula $v = 1.58(\pi/6)(L \times W)^{3/2}$ (Feldman et al., 2009). The day following the final CNTF or saline injection, final tumor dimensions were measured and mice were euthanized.

Results

Tumor development in homozygous TH-MYCN mice

Similar to previous observations of tumor formation in TH-MYCN mice on the 129/SvJ background, 100% of homozygous mice examined formed palpable tumors by an average
of 49.5 ± 0.84 days of age (Table 1). Tumors were large, highly angiogenic, and formed predominantly in the area of the celiac prevertebral ganglion, as in hemizygous mice (Chesler et al., 2007). In 2 of 14 mice observed for tumor formation, asymmetrical enlargement of the superior cervical ganglion was found in addition to abdominal tumor formation. In one mouse, along with prevertebral tumor formation, a separate tumor developing along the sympathetic chain was also observed.

**CNTF promotes differentiation of TH-MYCN tumor cells in vitro**

In order to investigate whether growth factor treatment could influence the phenotype of tumor cells from TH-MYCN mice, tumors were isolated from 35 day old homozygous mice, and dissociated tumor cells were treated with brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), nerve growth factor (NGF), neurotrophin 3 (NT-3), or a combination of NGF and NT-3. After 4 days in culture, treatment with CNTF and LIF, but not other growth factors promoted approximately a 4-fold increase in process outgrowth (Fig. 2-1A, grey bars, p < 0.01), as well as a 15% reduction in proliferation, as measured by ki67 staining (Fig. 2-1A, black bars, p < 0.05). After 3 days in culture, CNTF-treated tumor cells were visibly more differentiated than untreated cells (Fig. 2-1B and C). Whereas both CNTF and LIF signaling involves the LIFRb and GP130 receptor signaling subunits, CNTF signaling requires the additional presence of a CNTFRa subunit, a glycosyl-phosphoinositide-linked co-receptor commonly expressed in cells of both the
peripheral and central nervous system (Heller et al., 1996), which binds ciliary neurotrophic factor (CNTF) (Ip et al., 1993). Therefore, the differentiating effects of CNTF rather than LIF on TH-MYCN tumor cells were investigated further.

When quantifying CNTF-induced process outgrowth and inhibition of proliferation after 1, 2, and 3 days of CNTF treatment, we found that CNTF-induced effects reached significance after 3 days of treatment (Fig. 2-2A and B, p < 0.0001). No significant changes in cell number were observed over the same time course of CNTF treatment (Fig. 2-2C), indicating that the effect of CNTF on differentiation and proliferation was not the result of a selective survival advantage. The effects of CNTF were concentration-dependent, (p < 0.01), with an EC50 of approximately 0.4 ng/mL for the effects on neurite outgrowth (Fig. 2-2D) and proliferation (Fig. 2-2E), similar to the EC50 observed in CNTF-induced decreases in proliferation of embryonic chick sympathetic neurons (Ernsberger et al., 1989). In addition, we did not observe a concentration-dependent effect of CNTF on cell number (Fig. 2-2F).

The decrease in cell proliferation observed in the presence of CNTF could result from either a selective survival advantage provided to non-proliferating cells by CNTF, or as a result of CNTF promoting actively dividing cells to withdraw from the cell cycle. In order to distinguish between these two possibilities, we identified dividing cells before and after CNTF treatment (Fig. 2-3A). Upon plating, bromodeoxyuridine (BrdU) was added to the culture medium, and cells were treated with or without CNTF. After 24
hours, the BrdU was washed out, and the media and CNTF were replaced. BrdU incorporated into the DNA of tumor cells undergoing S-phase during this first 24-hour period. After an additional 48 hours in culture, tumor cells were fixed and stained for BrdU (Fig. 2-3B and D), and Ki67, which stains cells dividing at the time of fixation (Fig. 2-3C and E). Indicating CNTF promotes actively dividing cells to withdraw from the cell cycle, CNTF treatment caused a ~30% decrease in the percentage of BrdU positive cells that stained positive for Ki67 (Fig. 2-3E, p < 0.0001).

**CNTFRα is expressed in tumor cells in vitro and in vivo**

In order to distinguish the extent of CNTFRα expression in the population of tumor cells we were treating, we examined CNTFRα expression in cultured tumor cells treated with or without CNTF. CNTFRα was highly expressed in cells stained immediately after plating (Fig. 2-4A and B), and in cells treated with or without CNTF for 3 days (Fig. 2-4B), with an increase in expression after 3 days in culture. Suggesting the majority of tumor cells have the ability to differentiate in response to CNTF, CNTFRα was expressed in greater than 70% of cultured tumor cells in all conditions (Fig. 2-4A and B). Furthermore, despite arising from tissue of the sympathetic nervous system, the majority of cultured tumor cells lacked staining for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (Fig. 2-4A).

We determined that CNTFRα was highly expressed in cultured TH-MYCN tumor cells. To determine if the receptor is expressed in TH-MYCN tumors in vivo, and
therefore may respond to *in vivo* CNTF treatment, we examined expression of the receptor, as well as other markers, in tumors from TH-*MYCN* mice. As in cell culture experiments, tumors were isolated from homozygous mice at 35 days of age. Consistent with our *ex vivo* results, CNTFRα was also expressed in the majority of tumor cells *in vivo* (Fig. 2-4C). Furthermore, qPCR of mRNA isolated from homozygous TH-*MYCN* tumors revealed a level of CNTFRα expression equivalent to that of the superior cervical ganglion (Fig. 2-4D). Also consistent with *in vitro* TH-*MYCN* tumor cells, as well as a previous study following tumor development in hemizygous TH-*MYCN* mice (Chesler et al., 2007), the majority of TH-*MYCN* tumors stained strongly for Ki67 *in vivo*, indicating a high rate of proliferation in homozygous TH-*MYCN* tumors (Fig. 2-4E and F).

Furthermore, as we observed *in vitro*, TH expression was limited *in vivo*, and found only in small Ki67-negative clusters throughout the tumor (Fig. 2-4E and F).

**Efficacy of CNTF on tumor growth in vivo**

After investigating the differentiating effects of CNTF *in vitro*, we next determined if CNTF could inhibit TH-*MYCN* tumor cell growth *in vivo*. To measure tumor growth over the course of CNTF treatment, we used a syngenic *in vivo* tumor growth model using subcutaneous injection of a cell line derived from TH-*MYCN* tumors into wild type 129/SvJ mice. In this model, tumor growth can be easily controlled and observed, as it eliminates waiting for tumor development in homozygous mice, and tumors can be easily monitored as they grow subcutaneously rather than in the abdominal cavity. Previously, cell lines had been derived from TH-*MYCN* tumors by Cheng et. al. (Cheng et al., 2007).
Screening a few of these cell lines for responsiveness to CNTF, we identified the line NHO1S to be most representative of primary TH-MYCN tumor cells; it abundantly expresses the CNTFRα receptor and the proliferation marker ki67, expresses low levels of TH (Fig. 2-5A and B), and differentiates in response to in vitro CNTF treatment (Fig. 2-5C-E). Therefore, NHO1S cells are an appropriate cell line to model in vivo CNTF treatment of TH-MYCN tumor cells.

To assess the ability of CNTF to inhibit in vivo tumor growth, 1 million NHO1S cells suspended in 200 µL of Matrigel were injected subcutaneously into 12 wild type 129/SvJ mice. When tumors first became palpable, mice began to receive a daily intraperitoneal injection for 7 days of either saline, or CNTF at 0.2 mg/kg in saline. Tumor size was monitored daily by an observer blinded to treatment. CNTF treatment attenuated tumor growth over the course of the experiment. This was marked by a significant reduction (Fig. 2-5F, p < 0.01) in the fold tumor size (tumor size divided by starting tumor size) in mice injected with CNTF compared to saline injected mice 7 days following the first injection. Therefore, in addition to the ability to promote differentiation in vitro, CNTF is able to attenuate TH-MYCN tumor growth in vivo. Furthermore, as CNTF is known to cause weight loss in mice (Henderson et al., 1994; Lambert et al., 2001), mouse weight was measured daily to ensure that CNTF treatment did not result in an unhealthy reduction in weight. Mice receiving CNTF were observed to lose a modest 15% of their starting body weight over the course of the experiment compared with saline injected mice (Fig. 2-5G, p < 0.0001).
Discussion

The principal finding of this study is that CNTF reduces TH-\textit{MYCN} tumor cell growth by reducing cell proliferation and promoting cell differentiation. Of the neurotrophic factors tested, only the neuropoietic cytokines CNTF and LIF produced this effect. Furthermore, systemic treatment of animals with CNTF attenuated subcutaneous TH-\textit{MYCN}-derived tumor cell growth. Taken together, our data suggest that a further understanding of the molecular mechanisms involved in neuropoietic cytokine signaling could be beneficial in developing adjuvant differentiating therapies in neuroblastoma.

Since the discovery of CNTF as a growth factor with the ability to support the survival of chick embryonic ciliary ganglion neurons (Varon et al., 1979; Lin et al., 1989; Stockli et al., 1989; Eckenstein et al., 1990), it has been appreciated that CNTF can have a variety of effects on cells of the nervous system. CNTF treatment promotes survival of cultured embryonic motor neurons (Arakawa et al., 1990; Oppenheim et al., 1991), sympathetic (Eckenstein et al., 1990), and sensory neurons (Eckenstein et al., 1990; Thaler et al., 1994). Treatment of neuroblasts isolated from the sympathetic ganglia of E7 chicken embryos with CNTF promotes the early expression of vasoactive intestinal peptide (VIP), which is not normally expressed until E10 (Ernsberger et al., 1989). Furthermore, CNTF induces choline acetyltransferase (ChAT) immunoreactivity in E12 cultured sympathetic neurons, but not at E7 (Ernsberger et al., 1997). In the rat, CNTF has similar effects, inducing ChAT expression of neonatal superior cervical ganglion
neurons. In addition, it simultaneously diminishes the expression of tyrosine hydroxylase (TH) (Saadat et al., 1989). In the central nervous system (CNS), CNTF can support the survival of cultured hippocampal neurons (Ip et al., 1991), prevents the degeneration of medial septal neurons (Hagg et al., 1992), and promotes neurogenesis in the forebrain by increasing neural stem cell proliferation (Emsley and Hagg, 2003).

Similar to the effect on cultured E7 chicken sympathetic neuroblasts, we see that CNTF inhibits proliferation of primary cultured TH-MYCN neuroblastoma cells. Although CNTF is able to lead to a ~30% reduction in the number of proliferating cells in both embryonic sympathetic neurons and TH-MYCN tumor cells (see figure 2-3), many TH-MYCN tumor cells remain dividing following CNTF treatment (40%) compared to CNTF treatment in embryonic sympathetic neurons (7%) (Ernsberger et al., 1989). In the sympathoadrenal progenitor cell line (MAH), which is able to differentiate into chromaffin-like and sympathetic neuron-like cells, CNTF promotes terminal differentiation into a neurofilament positive sympathetic phenotype in vivo (Doering et al., 1995), similar to what we have observed here. Interestingly, in neuroblastoma, patients whose tumors express high mRNA levels of either CNTF or CNTFRα have improved survival over patients whose tumors have low expression of the two transcripts (http://home.ccr.cancer.gov/oncology/oncogenomics/).

While tumor cells from the TH-MYCN mouse model of neuroblastoma respond to CNTF treatment by differentiating, responses of CNTFRα-expressing human
neuroblastoma cells to CNTF remain hard to predict. Consistent with what we observed, the MYCN-amplified neuroblastoma cell lines LA-N-2 and SK-N-BE, respond to CNTF treatment with enhanced neurite outgrowth marked by an increased number of tumor cells with neurites, and increased neurite length (Rossino et al., 1995). However, in the neuroblastoma cell line SY5Y, from which the CNTFRα receptor was originally cloned (Squinto et al., 1990), treatment with CNTF activates the JAK-STAT pathway downstream of CNTFRα, but does not cause tumor cell differentiation (Rossino et al., 1995; Halvorsen et al., 1996). Furthermore, although CNTF treatment induces expression of the neurotrophin receptor TrkA in both GOTO and HTLA230 neuroblastoma cell lines, subsequent treatment with the TrkA ligand NGF results in proliferation in GOTO cells, but differentiation in HTLA230 cells (Bogenmann et al., 1998).

We used the TH-MYC N tumor-derived cell line NHO1S to determine whether CNTF can attenuate TH-MYC N tumor cell growth in vivo. For several reasons, we believe this cell line to be representative of the primary TH-MYC N tumor cells used in our in vitro experiments. This cell line is one of 6 cell lines that have been derived from TH-MYC N tumors (Cheng et al., 2007), and is one of four of these 6 cell lines that have been derived from homozygous TH-MYC N mice, similar to the primary cells used here. Furthermore, this cell line, and not other cell lines tested, like primary TH-MYC N tumor cells, abundantly expresses CNTFRα, and differentiates in response to CNTF treatment. Finally, similar to primary TH-MYC N tumor cells, the majority of NHO1S cells divide in
culture as indicated by abundant Ki67 staining, while expression of the sympathetic marker tyrosine hydroxylase is limited to rare cells. Therefore, we feel that NHO1S cells are an appropriate cell line to model the effect of CNTF on TH-MYCN tumor growth \textit{in vivo}.

Despite the encouraging prospect of CNTF as a novel differentiating agent to limit growth of neuroblastoma tumors, the idea of CNTF as a therapeutic must be approached with caution. Previously, a recombinant human CNTF (rhCNTF) was tested in the treatment of amyotrophic lateral sclerosis (ALS) following the discovery of the ability of CNTF to support the survival of motor neurons \textit{in vitro} (Sendtner et al., 1990) and \textit{in vivo} (Mitsumoto et al., 1994). Unfortunately, in addition to proving ineffective at treating ALS (Bongioanni et al., 2004), CNTF treatment had the adverse side effect of inducing anorexia and doubling the death rate in those treated at higher doses (Miller et al., 1996). Consistent with the observed ability of CNTF to cause weight loss in both human (Ettinger et al., 2003) and animal (Lambert et al., 2001) studies, in the current study, CNTF-treated mice consistently lost 15\% of their body weight over the course of the experiment, suggesting that CNTF may not be a realistic therapy for neuroblastoma. Interestingly however, recently a rhCNTF fusion protein was developed (TAT-CNTF), which is able to rescue rat motor neurons from cell death following sciatic nerve transection without the typical 20-30\% decrease in body weight seen in rhCNTF treated animals (Rezende et al., 2009). Although the work presented here implicates CNTF as a novel differentiating agent in neuroblastoma, elucidating the mechanisms involved in
CNTF-induced differentiation might be of greater therapeutic benefit in the future treatment of poor prognosis tumors.

Acknowledgements
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References


### Table 2-1: Tumor development in homozygous TH-MYCN mice

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Figure 2-1: Growth factor effects on process outgrowth and proliferation of *TH-MYCN* tumor cells in culture

(A) Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF), which both activate the CNTF receptor, significantly increase process outgrowth (grey bars, p < 0.01, ANOVA, n=5) and inhibit proliferation (black bars, p < 0.05, ANOVA, n=5) in tumor cells derived from *TH-MYCN* mice after 4 days in vitro compared to control, brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT3), and NGF and NT3 (see materials and methods for treatment concentrations). (B) Confocal image (20x) demonstrating CNTF-induced process outgrowth after 3 days in *TH-MYCN* tumor cells stained for 3A10, a
neurofilament associated antigen. Image brightness enhanced to highlight cell processes.

Scale bar = 100mm. Error bars = SEM.

Figure 2-2: CNTF effects are time and concentration dependent

(A) CNTF (closed circles) significantly increases process outgrowth and (B) inhibits proliferation over control (open circles) after 3 days in culture (p < 0.0001, ANOVA, n=4). (C) CNTF treatment has no effect on total cell number over the 3 day culture period. (D) CNTF-induced process outgrowth (p < 0.01, ANOVA, n=4) and (E) decrease
in proliferation (p < 0.001, ANOVA, n=4) is concentration dependent at 3 days \textit{ex vivo}. EC50 for both effects is \~ 0.4 ng/mL. \textit{(F)} There is no concentration-dependent effect of CNTF on tumor cell number at 3 days \textit{ex vivo}. Error bars = SEM.
Figure 2-3: CNTF promotes *TH-MYCN* tumor cells that are actively dividing to withdraw from the cell cycle

(A) Experimental design. On day 1, tumor cells were plated in the presence of bromodeoxyuridine (BrdU) and treated with or without CNTF. BrdU was washed out and fresh media and CNTF were added 24 hours later. Tumor cells were fixed and stained for BrdU and Ki67 after another 48 hours in culture. A similar number of untreated (B) and CNTF-treated (D) tumor cells are dividing at 1 day in culture indicated
by incorporation of BrdU. At the 4 day time point a difference in proliferation is observed between untreated (C) and CNTF-treated (E) tumor cells, indicated by Ki67 staining. A number of CNTF treated tumor cells withdraw from the cell cycle shown by staining for BrdU, but not Ki67 (arrows in D and E), while the majority of untreated cells remain dividing at both time points. Scale bar = 10μm. (F) Quantification of the percentage of BrdU cells that stain positive for Ki67 shows a significant decrease in Ki67 staining in CNTF treated tumor cells (p < 0.0001, Student’s t-test, n=4, error bars = SEM).
Figure 2-4: Ciliary neurotrophic factor receptor alpha (CNTFRα) expression in TH-MYCN tumor cells in vitro and in vivo

(A) Confocal image (60x) of cultured TH-MYCN tumor cells stained for CNTFRα and TH. Scale bar = 10μm. (B) Quantification of CNTFRα expression in acutely isolated cultured tumor cells and tumor cells treated with or without CNTF for 3 days in vitro. CNTF treatment has no effect on CNTFRα expression. (C) Confocal image (60x) of a tumor section stained for ciliary neurotrophic factor receptor alpha (CNTFRα red) and tyrosine hydroxylase (TH, green). Similar to cultured tumor cells, tumor cells abundantly
express the CNTF receptor in vivo, while few cells express TH. Scale bar = 10μm. (D) mRNA expression of CNTFRα in superior cervical ganglion (SCG) and tumor tissue. CNTFRα is expressed in tumor tissue at levels equivalent to that of normal sympathetic tissue. (E) Confocal image (20x) of a tumor section stained for Ki67 (red) and tyrosine hydroxylase (TH, green) isolated from a 35-day old homozygous TH-MYC N mouse. Scale bar = 100μm. (F) Confocal image (60x) shows abundant proliferation, indicated by Ki67 staining, with isolated non-proliferating TH-expressing tumor cells scattered throughout the tumor, consistent with Ki67 and TH expression in vitro. Scale bar = 100μm.
Figure 2-5: NHO1S cells differentiate in response to CNTF in vitro and CNTF treatment attenuates NHO1S tumor cell growth in vivo

(A-B) Similar to primary TH-MYCN tumor cells, NHO1S cells abundantly express CNTFRα (red stain in A) and Ki67 (red stain in B), while TH expression is more limited (green stain in A and B). (C-E) Like primary TH-MYCN tumor cells, NHO1S cells differentiate in response to in vitro CNTF treatment (p < 0.01, Student’s t-test, n=4).

Scale bar = 100µm and applies to all images. (F) Intraperitoneal injection of CNTF (0.2 mg/kg daily, black squares) attenuates subcutaneous tumor growth compared to saline injected mice (black circles) as measured by change in fold tumor size over the course of the experiment (p < 0.01, ANOVA, n=6). (G) Confirming CNTF was biologically active in vivo, CNTF treated mice lost an average of 15% of their starting weight over the course of the experiment (p < 0.0001, ANOVA, n=6). Error bars = SEM.
CHAPTER 3: 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. 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. This construct (termed ΔIgTrkB) activates phosphorylated Erk1/2 in the absence of the TrkB ligand BDNF. 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 upregulation of many transcripts encoding cancer-associated genes including cyclind1, twist1, and hgf, as well as downregulation 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 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.

**Introduction**

Neuroblastoma, a pediatric malignancy arising from sympathoadrenal precursors from the neural crest (Hoehner et al., 1996), 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 (Maris et al., 2007).

This heterogeneity in disease course is correlated with a number of different factors including Trk receptor expression (Brodeur, 2003). 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 (Miller and Kaplan, 2001; Reichardt, 2006). Following this model of development, neuroblastoma tumors that express TrkA have a favorable prognosis (Nakagawara, 1998). In contrast, many MYCN amplified, poor prognosis neuroblastomas express TrkB, resulting in tumors that are often highly aggressive and eventually fatal (Schramm et al., 2005). There is also a developmental basis for this: TrkB is transiently expressed in sympathetic progenitors prior to the onset of TrkA expression (Straub et al., 2007), and when stimulated with BDNF, the TrkB
expressing cells proliferate in cell culture (Reiff et al.; Straub et al., 2007) 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 (Matsumoto et al., 1995). Conversely, a truncated TrkB isoform lacking the tyrosine kinase domain is commonly expressed in more benign and differentiated tumors such as ganglioneuroblastomas (Brodeur et al., 1997). Furthermore, treatment of TrkB-expressing SMS-KCN neuroblastoma cells with BDNF enhances cell survival in serum free media (Nakagawara et al., 1994). 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 (Matsumoto et al., 1995; Kim et al., 1999), increased resistance to chemotherapeutics (Scala et al., 1996; Ho et al., 2002; Jaboin et al., 2002), increased production of angiogenic factors (Eggert et al., 2002; Nakamura et al., 2006), and enhanced invasion (Matsumoto et al., 1995). 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.
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. We stably transected this construct into the neural crest-derived cell line NCM-1 to investigate its transforming capacity. Constitutive TrkB signaling proved to be highly transforming in NCM-1 cells in vitro, and strongly tumorigenic in vivo. Our results suggest aberrant TrkB signaling alone is sufficient to give rise to the highly aggressive and fatal tumors seen in TrkB-expressing neuroblastoma.

Results

Removal of two immunoglobulin-like domains renders TrkB constitutively active

To better understand the role of TrkB signaling in neuroblastoma pathogenesis we created a constitutively active form of the TrkB receptor. 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 (Arevalo et al., 2000). Therefore, we created this construct (ΔIgTrkB; Fig. 3-1) and stably transected HEK293 cells. Additionally, a separate stably transected HEK293 line was created by transfection with a wild type (WT) TrkB receptor construct. Expression of the receptor in both TrkB expressing cell lines was confirmed, with ΔIgTrkB displaying a lower molecular weight than WT TrkB (Fig. 3-2A). As expected, a marked increase of phosphorylated Erk 1/2 was observed only when WT TrkB HEK293 cells were treated with BDNF. In contrast, elevated p-Erk 1/2 was observed in
both BDNF treated and untreated ΔIgTrkB HEK293 cells (Fig. 3-2A), albeit at significantly lower levels when compared to the BDNF-stimulated WT TrkB (Fig. 3-2B). Therefore, we tested whether this level of constitutive signaling by ΔIgTrkB was sufficient to promote downstream biological effects.

\[ \text{ΔIgTrkB promotes neurite outgrowth in PC12 cells} \]

PC12 cells are a well-known model of nerve growth factor (NGF) induced neuronal differentiation via TrkA (Greene and Tischler, 1976; Kaplan et al., 1991), and TrkB transfected PC12 cells differentiate in response to BDNF (Jian et al., 1996). 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 had a 6-fold increase in the number of neurite bearing cells over GFP transfected cells in the absence of NGF (Fig. 3-3A-C). ΔIgTrkB-induced neurite outgrowth is equivalent to that of NGF through TrkA, as there was no difference in the number of neurite bearing cells between ΔIgTrkB or GFP transfected cells in the presence of NGF (Fig. 3-3D).

\[ \text{ΔIgTrkB enhances proliferation in the neural crest derived cell line NCM-1} \]

NCM-1 is an immortalized, multipotent cell line with the ability to generate sympathoadrenal precursors (Lo et al., 1991). To determine if constitutive TrkB signaling promotes proliferation or differentiation in NCM-1 cells, we transfected cells with ΔIgTrkB (Fig. 3-S2). Stable ΔIgTrkB NCM-1 transfectants had a morphology
marked by more rounded cell bodies compared with the bipolar morphology of non-transfected NCM-1 cells (Fig. 3-4A-F). Furthermore, ΔIgTrkB NCM-1 cells grew to confluency more quickly than WT NCM-1 cells (Fig. 3-4A-F).

In order to quantify the apparent increase in proliferation, cells were counted using the vital fluorescent dye calcein AM (Saulnier Sholler et al., 2006). We noted a 2.5-fold increase in cell number after 4 days in ΔIgTrkB NCM-1 cells compared with WT NCM-1 cells (p < 0.0001, Fig. 3-4I). Further supporting enhanced proliferation, western blot analysis for phosphorylated histone H3, a protein preferentially expressed in dividing cells, demonstrated in ΔIgTrkB NCM-1 cells a 1.5-fold increase compared with WT cells (p < 0.05, Fig. 3-4G-H). To confirm that this phenotype did not arise from a random insertion of our construct disrupting a tumor suppressor, five additional parent lines were established. All showed a significant increase in cell number after 4 days in vitro (Fig. 3-S3, p < 0.001). Additionally, treatment with the pan-Trk kinase inhibitor K252a abolished ΔIgTrkB-mediated proliferation, resulting in calcein AM fluorescence equivalent to WT NCM-1 cells (Fig. 3-4J). This demonstrated that TrkB kinase activity is required for the observed enhanced proliferation. Therefore, constitutive signaling through ΔIgTrkB promotes a higher rate of proliferation in the neural crest-derived NCM-1 cell line.

ΔIgTrkB transforms NCM-1 cells

Although ΔIgTrkB signaling increases the rate of growth of NCM-1 cells, enhanced
proliferation alone is not sufficient to consider a cell transformed. For example, healthy cells require attachment to a surface to divide, and the loss of this requirement in transformed cells is known as anchorage-independent cell growth. To determine if ΔIgTrkB expression confers anchorage independent cell growth, cells were cultured suspended in soft agar. After one week, there was a 25-fold increase in colony formation in ΔIgTrkB transfected cells (p < 0.001, Fig. 3-5A-C).

Normally, when cells grown in culture detach from the culture surface they undergo anoikis, or detachment-induced apoptosis; however, cancer cells escape anoikis. 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. We observed a 4-fold increase in the number of live cells in the media from ΔIgTrkB NCM-1 cultures compared with WT NCM-1 (p < 0.05, Fig. 3-5D), 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’ (McKenzie et al., 2011). In this assay, cells are limited to a restricted area by 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 (Fig. 3-6A-C, E-G, J). However, there was a significant increase in the area
within which migrated cells could be found, indicating that in our assay ΔIgTrkB NCM-1
cells migrated farther compared with WT NCM-1 cells (p < 0.0001, Fig. 3-6D, H-I).

Although neural crest cells are intrinsically migratory, they are not intrinsically
invasive. Invasion requires the ability of cells to degrade extracellular matrix in order to
migrate. To investigate invasion, a layer of matrigel was overlaid on the cells in a new
set of experiments. Addition of matrigel led to a reduction in the number of cells that
traveled outside the originally confined area for both cell types (Fig. 3-6J vs. 3-6T). We
observed that ΔIgTrkB NCM-1 cells had an enhanced ability to invade extracellular
matrix that was marked by a 2.5-fold increase in the number of invading cells after 24
hours (p < 0.05, Fig. 3-6K-M, O-Q, T). Furthermore, ΔIgTrkB NCM-1 cells invaded
across a greater distance into the matrigel as indicated by a 2.5-fold increase in total area
invaded by ΔIgTrkB NCM-1 cells compared with WT (p < 0.05, Fig. 3-6N, 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 for cancer pathway genes. This
analysis revealed that expression of ΔIgTrkB increases transcript levels for a number of
cancer promoting genes (Table 3-1), as well as decreases the expression of tumor
suppressor genes (Table 3-2). Consistent with the enhanced proliferation of ΔIgTrkB NCM-1 cells compared with WT cells, we detected a 436-fold increase in transcripts levels (p < 0.0001) as well as significantly enhanced protein levels (p < 0.05, Fig. 3-S4) for the cell cycle regulatory gene cyclind1. Furthermore, upregulation of twist1 (39-fold, p < 0.0001) and hepatocyte growth factor (hgf, 29-fold, p < 0.0001), two genes known to play important roles in promoting invasion and metastasis (Li et al.; Hecht et al., 2005), 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, p<0.001) and rb1 (-1.77-fold, p<0.001). Therefore, mRNA expression in ΔIgTrkB NCM-1 cells is consistent with the highly transformed phenotype of the cells.

c-Met inhibition abolishes enhanced proliferation in ΔIgTrkB NCM-1 cells

Previous work has implicated the hepatocyte growth factor (HGF) / c-Met signaling pathway in promoting neuroblastoma cell invasiveness (Hecht et al., 2005). In ΔIgTrkB NCM-1 cells hgf levels were upregulated 29-fold. Additionally, c-Met was also upregulated 1.5-fold (p < 0.0001), suggesting that this pathway may be important in the transformed phenotype mediated by constitutive TrkB signaling. Furthermore, while K252a was first characterized as a pan-Trk kinase inhibitor, it has also been shown to inhibit a number of other tyrosine kinases including c-Met (Morotti et al., 2002). This suggested that our K252a proliferation inhibition results may have been due to c-Met inhibition. In order to determine if the c-Met signaling pathway mediates ΔIgTrkB-
induced proliferation, WT and ΔIgTrkB NCM-1 cells were grown in the specific c-Met inhibitor SU11274 and proliferation was assessed. Treatment with SU11274 (1 µM) abolished the increased proliferation of ΔIgTrkB NCM-1 cells, resulting in a 4 day growth curve that mirrored that of WT NCM-1 cells treated with, or without inhibitor (Fig. 3-7). Treatment with vehicle (DMSO) alone had no effect on the enhanced proliferation of ΔIgTrkB NCM-1 cells (Fig. 3-7, p < 0.0001), verifying that this effect was specific to the inhibitor. These results show that c-Met signaling is required for the enhanced proliferation of ΔIgTrkB NCM-1 cells.

ΔIgTrkB upregulates expression of the poor prognosis marker mycn in NCM-1 cells

To determine if the transformation of NCM-1 cells by ΔIgTrkB influences the expression of mycn, a marker of poor prognosis in neuroblastoma that was not included in the cancer pathway array, we compared transcript levels in WT and ΔIgTrkB NCM-1 cells by qPCR. We found a 21-fold increase in mycn levels in ΔIgTrkB NCM-1 cells compared with WT NCM-1 cells (p < 0.01, Table 3-1). Therefore, aberrant TrkB signaling in the neural crest derived NCM-1 cell line is sufficient to upregulate expression of the neuroblastoma poor prognosis marker MYCN.

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 1 million ΔIgTrkB or WT NCM-1 cells suspended in matrigel. One week following NCM-1 cell injection,
tumors became palpable in mice injected with ΔIgTrkB cells (Fig. 3-8A, p < 0.01), and all ΔIgTrkB NCM-1 injected mice were sacrificed by 15 days post-injection due to tumor burden (Fig. 3-8B, p< 0.01).WT NCM-1 injected mice remained tumor free throughout the experiment (Fig. 3-8). Monitoring tumor size daily, ΔIgTrkB NCM-1 tumors grew rapidly, measuring an estimated 8 cm³ by 2 weeks after injection, while WT NCM-1 cells failed to grow (Fig. 3-8C, p < 0.0001). Upon removal, ΔIgTrkB NCM-1 cell tumors were large and vascularized with an average wet weight of 4.5 grams (Fig. 3-8E-F).ΔIgTrkB tumors grew at a rapid pace and were also highly invasive, invading the vertebrae and compressing the spine in one mouse. This resulted in bilateral hind limb paralysis by 10 days following injection (Fig. 3-8G-H). Tumor tissue showed a histology that consisted of many closely packed cells with scant cytoplasm and little extracellular stroma, reminiscent of aggressive, poor prognosis neuroblastoma (Fig. 3-8D). Furthermore, a separate injection of only 100 cells formed tumors in 3/3 mice within 21 days, demonstrating that ΔIgTrkB NCM-1 cells are highly tumorigenic. Therefore, constitutive TrkB signaling is sufficient to transform the neural crest derived cell line NCM-1 into highly aggressive tumor cells in vivo.

Discussion

We provide evidence that constitutive TrkB signaling is sufficient to transform a neural crest cell line into a carcinogenic phenotype marked by enhanced proliferation, anchorage independent cell growth, anoikis resistance, migration and invasion, and tumorigenesis. Furthermore, we noted transcriptional deregulation as a consequence of
aberrant TrkB signaling, such as upregulation of MYCN, a gene whose amplification is significantly correlated with both the expression of TrkB/BDNF in neuroblastoma, as well as a more aggressive tumor phenotype and poor prognosis. Taken together, our data suggest that aberrant TrkB signaling in the developing sympathoadrenal lineage may be sufficient to promote neuroblastoma formation.

Although Trk receptors are now known to be the high affinity receptors for the neurotrophin family of growth factors, the first Trk receptor (TrkA) was discovered as an oncogene. Named the “tropomyosin-receptor kinase” (Trk), the gene was isolated from a colon carcinoma, and resulted from the fusion of a truncated tropomyosin and the tyrosine kinase domain of TrkA that rendered the kinase constitutively active (Martin-Zanca et al., 1986). Subsequently, the TrkB receptor was discovered and found to be the high affinity receptor for BDNF, and when co-expressed in 3T3 fibroblasts with BDNF, receptor activation promoted proliferation and anchorage-independent cell growth (Glass et al., 1991; Klein et al., 1991). In epithelial cells, TrkB has been shown to promote anoikis resistance by conferring cells with the ability to grow in suspension (Douma et al., 2004), whereas TrkB signaling enhances invasion in a number of cell lines including neuroblastoma (Matsumoto et al., 1995; Hecht et al., 2005; Cimmino et al., 2009), colon cancer (Yu et al., 2006), head and neck squamous cell carcinoma (Kupferman et al., 2010), non-small cell lung cancer (Zhang et al., 2010), and epithelial (Geiger and Peeper, 2007). TrkB signaling in vivo promotes tumor growth in neuroblastoma (Eggert et al., 2002), epithelial (Douma et al., 2004), and transitional cell carcinoma (Huang et al., 2004).
However, none of these studies determined whether TrkB signaling in neural crest-derived cells is sufficient to promote an aggressive, fully transformed phenotype.

Transformation of rat NCM-1 cells by ΔIgTrkB activates the expression of genes also seen in poor prognosis neuroblastoma including *cyclind1, twist1, hgf, and mycn*. *CYCLIND1* is known to be selectively amplified in poor prognosis neuroblastoma tumors (Molenaar et al., 2011), and this heightened expression is not simply a general characteristic of any cancer, but is more elevated in neuroblastoma compared to many other tumors (Molenaar et al., 2011). Further supporting ΔIgTrkB NCM-1 cells as a model for poor prognosis neuroblastoma, *TWIST1* has been shown to be expressed in 100% (7/7) of *MYCN* amplified tumors, but only 11% (2/18) of non-*MYCN* amplified tumors (Valsesia-Wittmann et al., 2004). In *MYCN* amplified neuroblastoma cell lines, *TWIST1* expression ranged from 16-164 fold that of non-*MYCN* amplified lines (Valsesia-Wittmann et al., 2004), consistent with the 39-fold increase in *twist1* expression we observed in ΔIgTrkB NCM-1 cells. This suggests a specific cooperation of TWIST1 and MYCN in neuroblastoma, TWIST1 inhibits expression of the tumor suppressor P53 (Valsesia-Wittmann et al., 2004), 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 noted a 29-fold upregulation of *hgf*, and a 1.5-fold upregulation of the HGF receptor *c-met* in ΔIgTrkB NCM-1 cells. Although increased c-Met signaling is a common occurrence in many types of cancer (Liu et al., 1992; Furukawa et al., 1995; Ramirez et al., 2000; Tokunou et al., 2001; Lengyel et al., 2005), the only other study to
demonstrate TrkB-mediated upregulation of c-Met signaling was in neuroblastoma cells, where TrkB expression led to 5- to 10-fold increases in HGF, and 40- to 400-fold increases in c-Met mRNA levels. This same study found TrkB-expressing primary neuroblastoma tumor samples to have coordinated expression of HGF and c-Met (Hecht et al., 2005).

ΔlgTrkB NCM-1 cells display an 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 (Lo et al., 1991). Although v-myc expression itself can be transforming (Ramsay et al., 1990), this is not the case in NCM-1 cells because of their ability to differentiate (Lo et al., 1991), and because they do not grow in soft agar, or form tumors in vivo. We observed that the addition of constitutive TrkB signaling resulted in dramatically transformed cells that form large invasive tumors. Similar in vivo tumor growth was seen in a v-myc immortalized rat fibroblast cell line expressing oncogenic BCR-ABL (Lugo and Witte, 1989), however this study injected 50% more cells to initiate tumorigenesis (1.5 million as opposed to 1 million injected here). In another study, expression of the oncogene BCL2 led to in vivo tumorigenesis in a rat L6 myoblast cell line expressing v-myc, but tumors formed only after 10 weeks (Screaton et al., 1997). Recently, Schulte et al. (2012) found that JoMa1 neural crest progenitor cells (maintained in an undifferentiated state by inducible c-myc expression) can be transformed by an oncogenic variant of the ALK^{F1174L}, and in 2 out of 6 mice were able
to form tumors \textit{in vivo} that were lethal to the mouse by 48 days following injection of 20 million cells (Schulte et al., 2012). Thus, ΔIgTrkB is considerably more oncogenic \textit{in vivo} than ALK^{F1174L}. ΔIgTrkB NCM-1 tumors were also highly invasive. In one mouse, tumor cell invasion of the spine, caused spinal cord compression and bilateral paralysis. This observation is reminiscent of cases of human neuroblastoma, where cancer can extend into the spinal foramina causing nerve root and spinal cord compression in patients with paraspinal tumors (Plantaz et al., 1996; De Bernardi et al., 2001; De Bernardi et al., 2005). In total, 5% of all neuroblastoma patients present with signs related to cord impingement (Plantaz et al., 1996; De Bernardi et al., 2001).

The highly aggressive behavior observed in ΔIgTrkB NCM-1 cells is not likely due solely to upregulation of \textit{MYCN}. Because \textit{MYCN} amplification is the most consistent genetic alteration seen in poor prognosis neuroblastoma (Seeger et al., 1985), it has been hypothesized that \textit{MYCN} overexpression is largely responsible for the aggressive phenotype of these tumors. The 21-fold upregulation of \textit{mycn} mRNA observed in ΔIgTrkB NCM-1 cells is consistent with the increased mRNA expression observed in \textit{MYCN}-amplified neuroblastoma tumors and cell lines, which has been observed to range anywhere from 20- to 80-fold (Schwab et al., 1984). Furthermore, while \textit{MYCN} amplification is present in only 25% of neuroblastomas (Brodeur et al., 1986), 70% of these tumors express TrkB and BDNF (Nakagawara et al., 1994). Therefore, in the proper developmental context, active TrkB signaling may drive \textit{MYCN} amplification in \textit{MYCN}/TrkB/BDNF expressing neuroblastomas. While other studies suggest \textit{MYCN}
overexpression itself is sufficient to drive neuroblastoma formation, the subsequent
tumors formed are slower growing and more spatially-confined than the tumors we
observed from ΔIgTrkB NCM-1 cells. For example, in the TH-\textit{MYCN} transgenic mouse,
targeted expression of \textit{MYCN} to the neural crest lineage by driving expression with the
tyrosine hydroxylase promoter is tumorigenic (Weiss et al., 1997). However, unlike the
human form of the disease where TrkB and BDNF are highly expressed in the majority of
\textit{MYCN} amplified tumors, TH-\textit{MYCN} tumors highly express BDNF, but lack TrkB
expression (DeWitt and Nishi, unpublished data). Also, \textit{MYCN} amplification is unlikely
to be the initiating event in human neuroblastoma, as amplification of this gene is not
known to occur in normal mammalian cells (Tlsty, 1990; Wright et al., 1990; Fischer et
al., 2012). In addition, JoMa1 neural crest cells overexpressing \textit{MYCN} form highly
variable, slow growing tumors with mice surviving anywhere from 43-123 days (Schulte
et al., 2012). Thus our results suggest that TrkB upregulates MYCN in parallel with
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) (Minturn et al., 2011).

\textbf{Materials and methods}

\textit{Constructs:} Using a full-length rat \textit{trkb} (WT \textit{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 \textit{pstI} site. Both Ig-like domains could then
be removed by \textit{pstI} (New England Biolabs, Ipswich, MA) digestion due to another \textit{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) and transfected into an rTTa-expressing PC12 Tet-on cell line (Clontech, Mountain View, CA). For NCM-1 experiments ΔIgtrkb was cloned into a piggyBAC transposon-transposase vector (pmyGENIE-3) containing a DsRed tag and hygromycin selection gene (Urschitz et al., 2010). NCM-1 cells were transfected with a GFP-expressing control piggyBAC vector (pmtGENIE-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 (Mains and Patterson, 1973). 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.

Western blotting: 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 100 µL of 1x SDS sample buffer + 5% β-Mercaptoethanol (βMe). Samples were run on an 8% polyacrylamide gel and 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, washed 4x for 10 minutes, and then incubated in 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 with 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 ΔIgtrkb construct and allowed to recover for 48 hrs prior to treatment with doxycycline (1 µg/mL, Sigma) and 7s NGF (1 µg/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 (Hruska and Nishi, 2007). 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. A neurite was defined as a process with 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 100 µL of media and viability was assessed using 2 µM 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) treatments, 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 the 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 0.5 mL of media to the top of each well every 3 days. Colony number was quantified after 7 days.
**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 (McKenzie et al., 2011). Cells (10,000) were plated on poly-D-lysine- and laminin-coated coverslips in a 10 µL volume. Initial images were acquired through a 2X PlanApo objective on a Nikon Eclipse TE-2000E inverted microscope. A second set of images acquired after 24 hours were compared and analyzed using the default settings of a custom written ImageJ macro (McKenzie et al., 2011). With the 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 was performed at the VT Cancer Center DNA Analysis Facility using a RT² First Strand kit (SABiosciences, Valencia, CA). Three arrays each testing an independently isolated RNA sample were analyzed for each cell
line. *Mycn* expression transcription levels were evaluated by reverse transcription of 1 µg 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 10^6 cells per mouse in 200 µL of matrigel (BD Biosciences). Four mice were injected per cell line. When tumors became palpable, tumor growth was quantified initially every other day, and then daily when it became apparent TrkB tumors were fast growing. Tumor volume was estimated from length and width measurements by the formula \( v = 1.58(\pi/6)(L*W)^{3/2} \) (Feldman et al., 2009). 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 that were then monitored for tumor formation.

**Conflict of interest**

The authors declare no conflict of interest.

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

References


Table 3-1: Tumor promoters upregulated in ΔIgTrkB NCM-1 cells

ΔIgTrkB and WT NCM-1 cells were grown to confluence in 6 well plates and RNA was isolated using TRI Reagent. Gene expression was determined using a Cancer Pathway qPCR array plate (SABiosciences). Mycn expression (in bold) was determined separately by Taqman-based qPCR (ABI). Data is the average of n=3 RNA samples from 3 different wells of a 6 well plate for each cell line. p-value determined by Student’s t-test.
<table>
<thead>
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<th>Symbol</th>
<th>Gene</th>
<th>Fold Regulation</th>
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<tr>
<td>ccnd1</td>
<td>Cyclin D1</td>
<td>436.1</td>
<td>0.000069</td>
<td>Promotes cell cycle progression</td>
</tr>
<tr>
<td>twistl</td>
<td>Twist homolog 1 (Drosophila)</td>
<td>38.57</td>
<td>0.000015</td>
<td>Promotes epithelial-mesenchymal transition (EMT), invasion, and metastasis</td>
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<td>hgf</td>
<td>Hepatocyte growth factor</td>
<td>28.86</td>
<td>0.008818</td>
<td>Promotes mitogenesis, cell motility, and matrix invasion</td>
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<tr>
<td>ccnd2</td>
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<td>Promotes cell cycle progression</td>
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<tr>
<td>mycn</td>
<td>N-Myc</td>
<td><strong>21.00</strong></td>
<td><strong>0.010000</strong></td>
<td>Marker of aggressive neuroblastoma</td>
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<tr>
<td>fgf2</td>
<td>Fibroblast growth factor 2</td>
<td>19.88</td>
<td>0.001753</td>
<td>Promotes angiogenesis</td>
</tr>
<tr>
<td>angptl</td>
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<td>12.25</td>
<td>0.003952</td>
<td>Promotes angiogenesis</td>
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<tr>
<td>abcg2</td>
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<td>6.385</td>
<td>0.000792</td>
<td>Mediates multidrug resistance</td>
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<tr>
<td>muc1</td>
<td>Mucin 1, cell surface associated</td>
<td>4.884</td>
<td>0.000030</td>
<td>Inhibits p53-mediated apoptosis, and promotes EMT through b-catenin stabilization</td>
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<td>Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1</td>
<td>4.661</td>
<td>0.000027</td>
<td>Promotes invasion and metastasis</td>
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**Table 3-2: Tumor suppressors downregulated in ΔIgTrkB NCM-1 cells**

ΔIgTrkB and WT NCM-1 cells were grown to confluence in 6 well plates and RNA was isolated using TRI Reagent. Gene expression was determined using a Cancer Pathway qPCR array plate (SABiosciences). Data is the average of n=3 RNA samples from 3 different wells of a 6 well plate for each cell line. p-value determined by Student’s t-test.

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<tr>
<th>Symbol</th>
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<th>Fold Regulation</th>
<th>P-value</th>
<th>Description</th>
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<tr>
<td>thbs1</td>
<td>Thrombospondin 1</td>
<td>-2.118</td>
<td>0.000563</td>
<td>Promotes cell adhesion</td>
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<td>pik3r1</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
<td>-1.913</td>
<td>0.000261</td>
<td>Inhibitor of PI3K signaling</td>
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<td>Retinoblastoma 1</td>
<td>-1.771</td>
<td>0.000464</td>
<td>Inhibits cell cycle progression</td>
</tr>
<tr>
<td>tgfbr1</td>
<td>Transforming growth factor, beta receptor 1</td>
<td>-1.755</td>
<td>0.001248</td>
<td>Inhibits cell growth</td>
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<tr>
<td>pten</td>
<td>Phosphatase and tensin homolog</td>
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<td>Inhibits cell proliferation</td>
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<td>bad</td>
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<td>0.004340</td>
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<td>cdkn1a</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
<td>-1.514</td>
<td>0.000064</td>
<td>Inhibits cell cycle progression</td>
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</tbody>
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Figure 3-1: Construction of a constitutively active TrkB receptor by removal of the two immunoglobulin-like ligand binding domains

(a) The 193 amino acid sequence coding for the two Ig-like ligand binding domains (shown in grey italics) of the 629 amino acid TrkB protein was removed by restriction digest. (b) The full-length wild type TrkB protein consists of two cysteine-rich domains (CRD) surrounding a leucine-rich domain (LRD), two immunoglobulin-like domains (IGD), and the intracellular tyrosine kinase domain (TKD). In the △IgTrkB construct the two Ig-like domains have been removed.
Figure 3-2: 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 3-3: 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 3-4: ΔIgTrkB promotes proliferation in the neural crest derived cell line NCM-1

(a-f) Phase contrast images of ΔIgTrkB and WT NCM-1 cell growth in a 12-well plate. Times indicated are hours after plating, 100,000 cells plated at time 0. (g) Calcein AM uptake reveals a highly significant increase (p < 0.0001, ANOVA) in growth in ΔIgTrkB NCM-1 cells (closed triangles, points represent mean calcein AM fluorescence, n=16, error bars = SEM) compared with wild type cells (closed squares) over a 4 day period. (h-i) ΔIgTrkB NCM-1 cells also have a two-fold increase in expression of the proliferation marker phosphorylated histone H3 (p < 0.05, Student’s t-test, n=3, error bars = SEM). (j) 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 with 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). Scale bar is equivalent to 100 µm and applies to all images.

Figure 3-5: ΔIgTrkB promotes anchorage-independent cell growth and anoikis resistance in NCM-1 cells

Soft agar assay in 6-well plates. (a) Wild type NCM-1 cells have little ability to grow in soft agar, (b) while ΔIgTrkB NCM-1 cells form numerous colonies. (c) Quantification of colony growth shows a 25-fold increase in colony formation in ΔIgTrkB NCM-1 cells (p < 0.001, Student’s t-test, n=6, error bars = SEM). (d) 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.
Figure 3-6: ΔIgTrkB enhances migration and invasion of NCM-1 cells

(a-d) Migration of wild type 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 wild type 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 1 mm and applies to all images.

Figure 3-7: c-Met inhibition abolishes ΔIgTrkB-mediated proliferation in NCM-1 cells

ΔIgTrkB and WT NCM-1 cell proliferation was quantified over a 4 day time period by calcein AM uptake. Treatment with the c-Met inhibitor SU11274 (1 mM) abolishes increased proliferation (p < 0.0001, ANOVA, n=8, error bars = SEM) in ΔIgTrkB NCM-1 cells (open triangles) compared with DMSO vehicle control treated ΔIgTrkB NCM-1 cells (closed triangles). ΔIgTrkB proliferation in the presence of the c-Met inhibitor is equal to WT NCM-1 proliferation in the presence of either inhibitor (open squares) or DMSO (closed squares).
Figure 3-8: 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 WT NCM-1 cells (solid line), or ΔIgTrkB NCM-1 cells (dotted line). No mice injected with WT 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 WT 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 1 mm and also applies to (h). Scale bar in (d) is equivalent to 100 mm.
pmhyGENIE-3 +CAG-IgTrkB + IRES + dsRED + Hygromycin

Figure 3-S1: The pBAC-ΔIgTrkB-dsRED construct

The ΔIgTrkB gene was cloned into the first multiple cloning site (MCS) of a bicistronic construct driven by the CMV-IE Enhancer, chicken β-actin promoter and β-globin intron (CAG) promoter that was previously inserted into the recombineering region of an Invitrogen pENTR1a vector. The second MCS of the bicistronic construct after the IRES2 sequence contained a dsRed Express2 gene. Due to restriction enzyme site limits the thymidine kinase promoter driven hygromycin gene and polyA was placed in reverse orientation between the end of the dsRed Express2 gene and its SV40 polyA site. The pENTR1a vector containing the above described bicistronic construct architecture was subsequently inserted into a pmhyGENIE-3 plasmid containing a hyperactive piggyBac gene, by Gateway recombineering as described previously (Urschitz et al., 2010). The
pmGENIE-3 plasmid containing a mammalian selection cassette expressing an EGFP gene was constructed as described previously (Urschitz et al., 2010).

Figure 3-S2: \( \Delta \)IgTrkB is constitutively active in NCM-1 cells

The \( \Delta \)IgTrkB construct was cloned into a piggyBAC transposon-transposase vector. NCM-1 cells were then stably transfected with this construct by hygromycin selection. \( \Delta \)IgTrkB is constitutively active in the piggyBAC vector (\( p < 0.01 \), Student’s t-test, \( n=3 \), error bars = SEM).

Figure 3-S3: Proliferation in NCM-1 cell lines

\( \Delta \)IgTrkB and WT NCM-1 cells, plus 5 additional NCM-1 cell lines stably transfected with \( \Delta \)IgTrkB were grown in culture. All \( \Delta \)IgTrkB transected cell lines have enhanced
proliferation shown by enhanced Calcein AM fluorescence compared to wild type cells after 4 days in vitro (p < 0.01, ANOVA, n=8, error bars = SEM).

**Figure 3-S4: Cyclin D1 protein is upregulated in ΔIgTrkB NCM-1 cells**

(a) Western blot of cyclin D1 protein in ΔIgTrkB NCM-1 cells and wild type NCM-1 cells. (b) Quantification of cyclin D1 protein expression reveals a significant 6-fold upregulation of cyclin D1 protein in ΔIgTrkB NCM-1 cells (p < 0.01, Student’s t-test, n=3, error bars = SEM).
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The experiments presented in this dissertation have aimed to better understand some of the factors that influence the phenotype of biologically unfavorable neuroblastoma. We identified ciliary neurotrophic factor signaling to promote differentiation in MYCN amplified tumors, as well as implicated TrkB signaling as possibly being sufficient to give rise to neuroblastoma from developing neural crest precursors. We found that the efficacy of CNTF as an in vivo treatment to reduce growth of MYCN amplified NHO1S subcutaneous tumors was limited. Due to concerns of weight loss resulting from intraperitoneal CNTF treatment, animals received only one week of CNTF treatment. Follow-up studies administering CNTF treatment every other day, and following tumor growth for up to two weeks may result in a more significant effect on NHO1S tumor growth. Additionally, the use of the newly engineered TAT-CNTF fusion protein (Rezende et al., 2009), which does not have the anorexic side effects of CNTF, would allow for greater flexibility in dosing schedules, assuming that this fusion protein would have similar differentiating effects in vitro.

We reported that TrkB signaling is sufficient to transform the neural crest derived cell line NCM-1, suggesting that aberrant TrkB signaling alone may be sufficient to initiate tumor formation in neuroblastoma. To formally confirm this hypothesis, the development of a transgenic mouse will be necessary. Using the ΔIgTrkB construct presented in this dissertation, a transgenic mouse expressing this construct
targeted to the neural crest lineage by the tyrosine hyrdoxylase (TH) promoter, as in the TH-MYCN mouse, would help to determine if TrkB signaling can be a driver of neuroblatoma tumor formation. Furthermore, the utilization of an inducible promoter, such as an rtTA / TRE tet-On system, would allow the determination of a critical period for tumorigenesis, or whether or not tumor formation at different points in development results in different tumor cell phenotypes.

**TrkB and ALK – Different Means to the Same End?**

It was recently discovered that mutations in ALK, a gene coding for another tyrosine kinase receptor, were found to be present in the majority of familial cases of neuroblastoma (Mosse et al., 2008), as well as 5-15% of sporadic cases (Chen et al., 2008). Like TrkB, gain of function ALK mutations signal through the MAPK and AKT pathways. In these ALK expressing tumors, constitutively active ALK forms a complex with hyperphosphorylated ShcC, rendering cells unresponsive to MAPK activating growth factors such as NGF (Miyake et al., 2002). Furthermore, inhibition of ALK binding to ShcC results in apoptosis in mutant ALK expressing neuroblastoma cells (Osajima-Hakomori et al., 2005). Like ALK, TrkB also binds ShcC (Liu and Meakin, 2002). Interestingly, mutations rendering ALK constitutively active are not necessary for the protein to have oncogenic activity in neuroblastoma. Overexpression of the wild type (WT) receptor to a critical threshold level leads to constitutive phosphorylation / activation irrespective of whether cells harbor the mutated or WT receptor (Passoni et al., 2009). Furthermore, primary tumors with elevated WT ALK expression have similar
clinical and molecular phenotypes as tumors with activating point mutations (Schulte et al., 2011).

Is it possible that the oncogenic properties of ALK and TrkB are mediated by the same mechanism? Both of these tyrosine kinases require high levels of activation of their receptors, either by overexpression / activating mutations (ALK), or simultaneous expression of receptor and ligand (TrkB). Furthermore, these receptors activate the same downstream intracellular pathways in neuroblastoma, utilizing the same ShcC adapter protein. It would be interesting to determine if disruption of TrkB binding to ShcC would promote apoptosis in TrkB / BDNF-expressing neuroblastoma cells, as in tumor cells with elevated ALK activation. If so, this might provide a common therapeutic target for biologically unfavorable tumor cells harboring these derangements. Alternatively, both ALK and TrkB signaling converge on the mammalian target of rapamycin (mTOR) pathway, and mTOR inhibition might reduce the malignant phenotype of both TrkB (Nakamura et al., 2006) and ALK (Berry et al., 2012) expressing neuroblasotma cells. Therefore, the further development of mTOR inhibitors as therapeutics in neuroblastoma will likely have broad efficacy across tumors expressing either TrkB, and mutated or elevated levels of ALK. The mTOR inhibitor temsirolimus is currently in phase II clinical trials for refractory neuroblastoma (Geoerger et al., 2012).

CNTF and TrkB Signaling as Personalized Medicine?
Ultimately, the experiments presented here will not be applicable to all patients with neuroblastoma. However, as the ability and ease of detection of molecular markers and genomic signatures is ever increasing, cancer treatments are becoming more and more personalized to individual patients (Awada et al., 2012). Therefore, in patients who have failed conventional treatments, yet express the CNTFRα receptor in their tumors, activation of this signaling pathway might be a legitimate alternative therapeutic option to promote the differentiation of tumor cells.

Furthermore, the sufficiency of TrkB signaling alone to transform neural crest-derived cells strengthens the argument for targeted therapies designed to inhibit TrkB signaling in TrkB / BDNF-expressing neuroblastomas. Lestaurnitinib (CEP-701) is a tyrosine kinase inhibitor that has shown potential efficacy in phase I clinical trials for neuroblastoma. Lestaurnitinib inhibits Trk activation by ligand at nanomolar concentrations, and preclinical studies suggest it may be most effective in combination with conventional chemotherapeutic agents where it renders cells more susceptible to cytotoxic drugs (Evans, Kisselbach et al. 2001; Ho, Eggert et al. 2002). Encouragingly, dose-limiting toxicities from Lestaurnitinib treatment seem to be limited to nausea, gastrointestinal irritation, and dyspepsia, which are fairly well tolerated (Marshall, Kindler et al. 2005). It will be interesting to monitor the progress of this drug through clinical trials, as therapies specifically targeting the derangements of high risk neuroblastoma will be necessary to improve upon the current 40% overall survival in the patient population.
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APPENDIX A: Expression of the α5 nicotinic acetylcholine receptor subunit in neuroblastoma

Despite surgery and chemotherapy, outcomes in high risk neuroblastoma remain poor (De Bernardi, Nicolas et al. 2003). Many of these patients will initially respond to chemotherapy, but inevitably relapse. One possibility for these outcomes is the presence of a cancer stem cell in the bone marrow that, after surviving chemotherapy treatment, is able to reseed the body and initiate new tumor development. Such ‘tumor initiating cells’ or TICs, have been isolated from the bone marrow of relapsed neuroblastoma patients (Hansford, McKee et al. 2007). Therefore, in order to prevent relapse in high risk neuroblastoma, new therapeutics must be developed which target these tumor initiating cells.

In an effort to identify compounds with the ability to kill TICs, but not normal cells, the lab of Dr. Kaplan at the Hospital for Sick Children in Toronto, Canada performed a candidate drug screen (Smith, Datti et al. 2010). This screen identified the drug MG 624, a nicotinic acetylcholine receptor antagonist, to kill TICs, but not normal skin-derived neural crest precursor cells (SKPs) (Fig. A-1, unpublished data). Therefore, we became interested in identifying what nicotinic acetylcholine receptor subunits may be expressed in neuroblastoma TICs, and whether this subunit expression contributes to the tumorigenic phenotype of these cells.
To determine nicotinic acetylcholine receptor (CHRN) subunit expression in TICs, I isolated total RNA from one TIC line from the lab of Dr. Kaplan (NB12), and from one TIC line isolated by Dr. Giselle Sholler (BO11SC). RNA was converted to cDNA and I performed quantitative PCR for the CHRN subunits α3 (CHRNA3), α4 (CHRNA4), α5 (CHRNA5), α7 (CHRNA7), β2 (CHRNB2), and β4 (CHRNB4). Expression was compared to RNA isolated from a normal pediatric sympathetic ganglion (Fig. A-2). While CHRNA3, CHRNA4, and CHRNB4 are not expressed in the TIC lines, interestingly, CHRNA5 is highly upregulated in the TIC lines compared with normal sympathetic control. CHRNA7 and CHRNB2 are expressed in the TIC lines, but at relatively low levels compared with control. Therefore, we reasoned CHRNA5 expression may be important in the ability of MG624 to kill TICs.

To determine if the observed upregulation of CHRNA5 would be consistent with other TIC lines, as well as compared to other controls, such as the normal skin derived neural crest precursor cells (SKPs) used in the candidate drug screen, Dr. Kaplan and Dr. Miller generously provided us with RNA isolated from a number of different TIC and SKP lines. The same quantitative PCR was performed, and consistent with the first TIC lines, CHRNA5 is highly upregulated in all TIC lines tested (purple bars) compared with both SKP lines (aqua bars), and the normal sympathetic tissue control (yellow bar) (Figure A-3). Furthermore, as in the first TIC lines, CHRNA3, CHRNA4, and CHRNB4 are not expressed, while CHRNA7 and CHRNB2 are lowly expressed in all TIC lines.
Next we examined whether upregulation of CHRNA5 was a characteristic specific to neuroblastoma TICs, or whether CHRNA5 is highly expressed in established neuroblastoma cell lines as well. RNA was isolated from two MYCN-amplified (KCN and KCNR) and one single-copy MYCN (SY5Y) neuroblastoma cell lines, and mRNA transcript levels were determined for CHRNA5 and CHRNA7 by quantitative PCR (Fig. A-4). As in the TICs, CHRNA5 is highly upregulated in all three neuroblastoma cell lines tested compared with normal sympathetic control, indicating CHRNA5 overexpression is not exclusive to neuroblastoma tumor initiating cells. CHRNA7 was also expressed in the neuroblastoma cell lines. However, unlike in the TICs, where CHRNA7 expression was well below that of the sympathetic control, CHRNA7 expression levels in the neuroblastoma cell lines were equivalent to levels in the sympathetic control tissue.

We observed upregulation of CHRNA5 in both neuroblastoma tumor initiating cells, and in established neuroblastoma cell lines. However, both of these groups of cells are grown in culture, and therefore do not reveal whether or not CHRNA5 is upregulated in vivo. To determine if CHRNA5 is overexpressed in the tumors of neuroblastoma patients in vivo, RNA from a number of patient’s tumors was generously provided by the Children Oncology Group (COG). This RNA was converted to cDNA and CHRNA5 transcript levels were determined by quantitative PCR as above. Consistent with expression in both the TICs, and established neuroblastoma cells, CHRNA5 was upregulated in every tumor sample tested, up to 400-fold, compared with normal sympathetic tissue (Fig. A-5).
This data provides evidence that the nicotinic acetylcholine receptor subunit $\alpha 5$ is upregulated in both tumor initiating cells of neuroblastoma, and neuroblastoma patient tumors. Additionally, high expression of CHRNA5 also correlates with poor patient survival (http://home.ccr.cancer.gov/oncology/oncogenomics/). Interestingly, the $\alpha 5$ subunit itself is unable to form a functional nicotinic acetylcholine receptor, requiring the presence of another alpha, and a beta subunit to be functionally active. However, in addition to CHRNA5 overexpression, the TIC lines express, albeit at significantly lower levels, both CHRNA7 and CHRN B2, which together, can form a rarely expressed $\alpha 5\alpha 7\beta 2$ nicotinic acetylcholine receptor (Girod, Crabtree et al. 1999). Future studies will need to confirm upregulation of the $\alpha 5$ subunit at the protein level. Furthermore, to determine if CHRNA5 overexpression is physiologically relevant in neuroblastoma cells, or can be transforming in normal cells, RNAi knockdown of the gene should be performed, as well as overexpression of the gene in non-transformed cell lines.

References


Figure A-1: MG 624 kills neuroblastoma TICs, but not skin-derived neural crest precursors

MG 624 reduces the growth rate of and/or kills neuroblastoma tumor initiating cells (TICs, yellow bars), but promotes the growth of normal skin-derived precursors (SKPs, blue bars) that are neural crest stem cells. The EC50 for this effect is approximately 100 nM. Data courtesy of Dr. D. Kaplan.
Figure A-2: Nicotinic acetylcholine receptor (CHRN) subunit expression in neuroblastoma tumor initiating cells (TICs) compared with normal sympathetic tissue

Transcript levels of the nicotinic acetylcholine receptor subunits $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_7$, $\beta_2$, and $\beta_4$ were quantified by qPCR using FAM/BHQ labeled Taqman based probes (ABI) relative to human 18s (house keeping gene). CHRNA5 is highly upregulated in the two TIC lines compared with normal sympathetic tissue, while CHRNA3, CHRNA4, and CHRN4 expression is undetectable. CHRNA7 and CHRN2 is lowly expressed in the TIC lines.
Figure A-3: CHRNA5 is upregulated in TICs compared with skin derived stem cells and normal sympathetic tissue

Total RNA from four independently isolated tumor initiating cell lines from the bone marrow of neuroblastoma patients (purple bars), three neural crest precursor cell lines from human skin follicles (aqua bars), and normal sympathetic ganglia obtained from a non-cancerous pediatric case at autopsy (yellow bar) were reverse transcribed into cDNA. CHRNA3, CHRNA4, CHRNA5, CHRNA7, CHRNB2, and CHRNB4 transcript levels were quantified by qPCR relative to human 18S using FAM/BHQ labeled Taqman based probes (ABI). As in the previous TIC lines tested, CHRNA3, CHRNA4, and CHRNB4 were undetectable. CHRNA5 was upregulated 5-40 fold compared with normal sympathetic ganglia and skin derived neural crest stem cells.
Figure A-4: CHRNA5 is upregulated in established human neuroblastoma cell lines compared with normal sympathetic tissue

Transcript levels of CHRNA5 and CHRNA7 in neuroblastoma cell lines were quantified by qPCR using FAM/BHQ labeled Taqman based probes (ABI) relative to human 18s (house keeping gene). Consistent with CHRN expression in the TIC lines, CHRNA5 is highly upregulated in the established neuroblastoma cell lines SY5Y, KCN, and KCN-R compared with normal sympathetic tissue. Unlike in the TICs, CHRNA7 expression in the cell lines is equivalent to normal sympathetic tissue.
Figure A-5: CHRNA5 is highly expressed in patient tumor samples compared with normal sympathetic tissue

Transcript levels of CHRNA5 in neuroblastoma tumor samples were quantified by qPCR using FAM/BHQ labeled Taqman based probes (ABI) relative to human 18s (housekeeping gene). Transcript levels of CHRNA5 in all tumor samples run together (blue bars) and a select number of tumor samples run at the same time as cDNA from normal sympathetic tissue (red bars). All transcript values are expressed as fold expression relative to CHRNA5 expression in normal sympathetic tissue. Samples are ordered from highest to lowest fold expression from left to right on the x-axis. CHRNA5 mRNA expression is higher in all tumor samples compared with normal sympathetic tissue. Tumor sample RNA was graciously provided by the Children’s Oncology Group (COG).