



Published in final edited form as:

Curr Top Dev Biol. 2011 ; 94: 77–127. doi:10.1016/B978-0-12-380916-2.00004-8.

The Connections Between Neural Crest Development and Neuroblastoma

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Abstract

Neuroblastoma (NB), the most common extracranial solid tumor in childhood, is an extremely heterogeneous disease both biologically and clinically. Although significant progress has been made in identifying molecular and genetic markers for NB, this disease remains an enigmatic challenge. Since NB is thought to be an embryonal tumor that is derived from precursor cells of the peripheral (sympathetic) nervous system, understanding the development of normal sympathetic nervous system may highlight abnormal events that contribute to NB initiation. Therefore, this review focuses on the development of the peripheral trunk neural crest, the current understanding of how developmental factors may contribute to NB and on recent advances in the identification of important genetic lesions and signaling pathways involved in NB tumorigenesis and metastasis. Finally, we discuss how future advances in identification of molecular alterations in NB may lead to more effective, less toxic therapies, and improve the prognosis for NB patients.

1. Clinical and Biological Characteristics of Neuroblastoma (NB)

NB is the most common extracranial solid tumor in childhood, accounting for approximately 7–10% of pediatric cancers and 15% of all pediatric cancer deaths in patients less than 15 years old (Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999). NB is an extremely heterogeneous disease both biologically and clinically (Brodeur, 2003; Evans *et al.*, 1971; Maris *et al.*, 2007). NB is thought to be an embryonal tumor that is derived from precursor cells of the peripheral (sympathetic) nervous system (Brodeur, 2003; Grimmer and Weiss, 2006; Nakagawara and Ohira, 2004). The tumor can arise anywhere along the sympathetic chain but is most frequently in the adrenal medulla and paraspinal ganglia (Fig. 4.1; Johnsen *et al.*, 2009; Nakagawara and Ohira, 2004).

Historically, NB is clinically classified into five different stages (1–4 and 4S) according to the International Neuroblastoma Staging System (INSS; Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999; van Noesel and Versteeg, 2004). Early stage NB tumors (i.e., stages 1, 2) do not metastasize to bone or bone marrow and are treatable with chemotherapeutic drugs and irradiation. Advanced-stage NB tumors (stages 3 and 4) are highly metastatic and usually respond positively to initial treatment. However, they often become resistant to chemotherapy and irradiation. A fifth stage of NB tumors (stage 4S) undergoes spontaneous regression with minimum treatment or even without medical intervention.

Ninety percent of the children with this disease are diagnosed before the age of 5 years and in those patients older than 1 year, 75% of the patients present with stage 3 or 4 metastatic diseases (Brodeur, 2003; Maris *et al.*, 2007). Metastatic disease remains a major clinical challenge in the treatment of NB since greater than 50% of all NB patients are diagnosed with metastatic diseases (Maris, 2005; Maris *et al.*, 2007). In contrast, infants with this

disease tend to be at lower stages (stage 1, 2, and 4s) and to have a better prognosis (Brodeur, 2003; Maris, 2005; Maris *et al.*, 2007; van Noesel and Versteeg, 2004).

In addition to classification by stage, NB tumors are also classified into three risk groups (low, intermediate, and high risk) according to age, MYCN status, and histology (Table 4.1). A new system for tumor staging has recently been implemented by the International Neuroblastoma Risk Group Staging System (INRGSS; Cohn *et al.*, 2009; Monclair *et al.*, 2009). This system, which is based on a combination clinical and imaging data, classifies patients as L1 (localized disease without imaging risk factors), L2 (localized disease with imaging risk factors), M (metastatic tumors), and Ms (metastatic disease with metastasis only in skin, liver, and/or bone marrow; Cohn *et al.*, 2009; Monclair *et al.*, 2009). NB tumors are also graded in terms of histology using the International Pathology Classification System (INPC) which is based on the Shimada histology grading system (Shimada *et al.*, 1999). This system distinguishes good and poor prognosis tumors based on the degree of differentiation, the Schwannian stromal content, the mitotic-karyorrhexis index (MKI), and the age at diagnosis (Shimada *et al.*, 1999). Unfavorable tumors tend to include undifferentiated tumors with high MKI of any age, poorly differentiated tumors or intermediate MKI in patients older than 18 months, and differentiated tumor or low MKI in patients 5 years old or older. In contrast all other cases, including those with ganglioneuroma or tumor with regions of ganglioneuroma intermixed, have a good prognosis.

In the remainder of this review, we will discuss the development of the peripheral neural crest with a focus on how developmental factors may contribute to NB tumorigenesis and metastasis, and highlight the current understanding of other genetic changes related to NB and their importance in NB diagnosis and treatment.

2. Neural Development and NB

2.1. Neural crest contribution to sympathetic ganglia and adrenal gland

The majority of NB tumors appear to arise from neural crest-derived cells in the abdomen adjacent to the aorta in the region of the kidney or in the medullary region of the adrenal gland (Brodeur, 2003; Maris *et al.*, 2007). Thus, NB is a sympathoadrenal lineage neural crest-derived tumor. The neural crest arises from the dorsal region of the closing neural tube beneath the ectoderm (Le Dourin and Kalcheim, 1999). This transient population of cells produces multipotential progenitor cells that give rise to the peripheral nervous system, the enteric nervous system, pigment cells, Schwann cells, adrenal medullary cells, and cells of the craniofacial skeleton (Le Dourin and Kalcheim, 1999). This process is regulated by both extrinsic and intrinsic factors. The Hedgehog and Wnt signaling pathways are especially crucial for proper neural crest development (Dupin *et al.*, 2007; Le Dourin and Kalcheim, 1999; Morales *et al.*, 2005). Lineage studies in the developing embryo have shown that neural crest cells within the trunk region generate multiple neural crest derivatives such as melanocytes, Schwann cells, glia, and neurons of the dorsal root ganglia (Fontaine-Perus *et al.*, 1982; Lallier and Bronner-Fraser, 1988; Teillet *et al.*, 1987; Weston, 1963). A subset of these trunk crest cells, commonly referred to as the sympathoadrenal lineage, contributes to the sympathetic ganglia and medullary region of the adrenal gland (Anderson and Axel, 1986; Anderson *et al.*, 1991). This lineage of cells is thought to be the origin of NB (Brodeur, 2003; Maris *et al.*, 2007). However, given the fact that NB can develop anywhere along the sympathetic axis, it is likely that NB can also arise from earlier crest derivatives, before development of the sympathoadrenal lineage but after the initial fate specification (Brodeur, 2003; Maris *et al.*, 2007). This could contribute to the heterogeneous histology and pathology of NB.

Neural crest cells develop in response to extracellular signals (Bronner-Fraser, 1993). The signals that induce formation of the crest (BMP/Shh) appear to be similar along the dorsal/ventral axis of the embryo. In contrast, different factors appear to confer cell fate along the anterior/posterior axis of the embryo. Heterotopic crest cell transplantation studies indicate that the positional identity of the cells is based on their location during development, rather than the characteristics of cells in original locations (Bronner-Fraser *et al.*, 1980; Ruffins *et al.*, 1998). Although NB can arise anywhere along the developing sympathetic axis, the majority of cases arise in the abdomen (65%), frequently in the adrenal medulla, where the sympathoadrenal lineage is specified; while others are found in the paraspinal sympathetic ganglia in places such as the neck (5%), chest (20%), and pelvis (5%) (Maris *et al.*, 2007). Therefore, it is reasonable to postulate that the positional identity of the cell along the anterior posterior axis of the embryo, and factors that specify this region, likely contribute to the oncogenic potential of the crest derivatives in this region. As such, developmental signals fating the proper development, migratory pathways, and regulated cell death will be examined here in the context of NB.

2.2. Neural crest migratory pathways

Neural crest-derived cells are highly migratory. Shortly after induction the crest cells undergo an epithelial to mesenchymal transition (EMT). This EMT transition results in acquisition of enhanced migratory abilities and decreased requirements for intercellular contact which allows the neural crest cells to leave the dorsal neural tube (Le Dourin and Kalcheim, 1999). They then migrate either between the dermatome and epidermis in a dorsolateral pathway or delaminate from the neural tube via a ventrolateral pathway (Le Dourin and Kalcheim, 1999). Importantly, a similar EMT transition may also play a role in NB metastasis as described below in Section 6.1.

Neural crest migration pathways are determined by signal from the mesoderm which develops prior to the arrival of the crest cells. Trunk region neural crest cells either migrate ventrolaterally and remain in the sclerotome form the dorsal root ganglia or continue migrating to a more ventral position to form sympathetic ganglia. N-myc, an oncogene which plays a role in aggressive NB, appears to be required for the migration, survival and/or differentiation of cells that migrate to the dorsal aorta since N-myc deficient mouse embryos have decreased numbers of mature cells in the both the dorsal root ganglia and sympathetic ganglia (Charron *et al.*, 1992; Sawai *et al.*, 1993; Stanton *et al.*, 1992). Hedgehog, Wnt, and additional growth factors all play a role in inducing N-myc expression in the neural crest (Grimmer and Weiss, 2006). More detailed information on the role of N-myc in NB is present below in Sections 3.2.1 and 5.2.3.

The most complete studies on neural crest cell migration and development have been carried out in birds. These studies revealed that the avian trunk crest, which is located between somites 6 and the tail, gives rise to sympathetic neurons and that a subset of these (between somites 18 and 24) contribute to the adrenal medulla (Le Dourin and Kalcheim, 1999). The crest cells that contribute to the adrenal medulla, the site where the majority of NB tumors are found, follow the ventrolateral migratory pathway. Upon arrival to the proper target tissue, the cells then undergo final differentiation, and regulated cell death.

2.3. Sympathoadrenal lineage development

The sympathoadrenal lineage is thought to derive from a common progenitor that aggregates at the dorsal aorta after migrating from the crest utilizing the ventral pathway (Anderson, 1993). During the migration to the dorsal aorta, the crest cells encounter signals from the somites, the ventral neural tube, and the notochord. Local signals from the dorsal aorta, such as BMPS, specify the future differentiation of the crest cell as either a catecholaminergic/

adrenal chromaffin cell or sympathetic neuron (Ernsberger *et al.*, 2005; Howard *et al.*, 2000; Reissmann *et al.*, 1996; Schneider *et al.*, 1999; Shah *et al.*, 1996). The sympathoadrenal lineage is specified by a tightly regulated set of transcription factors. Trunk crest cells can first be identified by the expression of Sox10 (Betters *et al.*, 2010; Huber *et al.*, 2008). As the crest cells migrate along the ventral pathway, they are exposed to BMP signals (e.g., BMP2, 4, and 7) that induce the expression of Mash1, a helix-loop-helix transcription factor expressed throughout the autonomic progeny (Huber, 2006). Shortly thereafter Phox2b expression occurs in sympathoadrenal lineage cells. Phox2b is required for the maintenance of Mash1 and recent evidence has shown that temporal difference in expression of these two factors may separate the sympathoadrenal lineage into separate sympathetic and adrenal lineages earlier in development than thought (Huber, 2006). Mash 1 induces the expression of Phox2a which is required for the production of the biosynthetic enzymes, dopamine beta-hydroxylase (DBH) and tyrosine hydroxylase (TH) in noradrenergic cells. Phox2b also appears to be important in the development of NB since PHOX2b mutations have been found in a subset of familial NB patients (see Section 3.1.1). Shortly after the migrating cells reach the dorsal aorta, they begin to acquire their respective sympathetic and adrenal cell fates and undergo a secondary migration to the presumptive prevertebral ganglia, the adrenal medulla, and secondary sympathetic ganglia where they complete their differentiation (Fig. 4.2; Huber, 2006; Le Dourin and Kalcheim, 1999; Morales *et al.*, 2005).

Although the complete mechanisms for proper chromaffin cell development are unknown, evidence indicates a highly intrinsic program. In animals that lack an adrenal cortex, chromaffin cells migrate to the suprarenal region, downregulate neuronal markers and contain large chromaffin granules. However, the generation of proper numbers of chromaffin cells and the expression of secretogranin II and PNMT requires intact glucocorticoid signaling. The human adrenal gland is remodeled throughout fetal development, infancy, and into adulthood in a process that is greatly affected by perturbations in levels of IGF II, FGF, and epidermal growth factor (EGF) levels. Importantly, as discussed below several of these growth factors are involved in cellular proliferation and signaling in NB (Section 4.2).

3. Genetic Lesions in NB

3.1. Familial genetic lesions

Hereditary NB is both rare and heterogeneous, accounting for less than 5% of all NBs (Maris *et al.*, 2002). In addition to the known hereditary mutations that are described below, hereditary NB predisposition loci have been mapped to chromosomes 16p12–13 and 4p16 indicating other familial predisposition mutations may exist, but no genes have been shown to be inactivated or mutated in these regions, to date (Maris *et al.*, 2002; Perri *et al.*, 2002).

3.1.1. Phox2b—Germline mutations in the paired-like homeobox 2B (*PHOX2b*) gene on chromosome 4p13 are the first predisposition mutations identified in NB (Mosse *et al.*, 2004; Trochet *et al.*, 2004). As mentioned earlier, Phox2b, as well as to MASH1, are expressed early in the developing sympathoadrenal progenitors (Alenina *et al.*, 2006; Nakagawara, 2004; Nakagawara and Ohira, 2004). Shortly after expression of MASH1 and Phox2b in the sympathoadrenal lineage, Hand2, Phox2a, and GATA2/3 appear (Alenina *et al.*, 2006; Nakagawara, 2004; Nakagawara and Ohira, 2004). Phox2b has also been shown to be essential for the expression of the glial family ligand tyrosine kinase coreceptor RET (REarranged during Transfection) and for the specification of noradrenergic fates, particularly the biosynthetic enzymes TH and DBH (Alenina *et al.*, 2006; Nakagawara, 2004; Nakagawara and Ohira, 2004).

NB patients with PHOX2b mutations also have familial disorders of the neural crest such as Hirschsprung's disease (HSCR) and congenital hypoventilation syndrome (CCHS; Mosse *et al.*, 2004; Trochet *et al.*, 2004). It is unclear that the mutations in PHOX2b found in familial NB result in gain or loss of function, although many PHOX2b mutations stabilize the Phox2b protein and decrease or eliminate the ability of Phox2b to transactivate the DBH promoter (Raabe *et al.*, 2008; Trochet *et al.*, 2005). The findings that Phox2b is necessary for the differentiation of autonomic neurons and overexpression of Phox2b inhibits proliferation in neuron progenitors and cell lines suggests Phox2b is a tumor suppressor (Raabe *et al.*, 2008; Trochet *et al.*, 2005, 2009). However, the absence of tumors with loss of heterozygosity (LOH) or mutation in second allele suggests gain-of-function, dominant-negative effect, or haploinsufficiency (Benailly *et al.*, 2003; Bourdeaut *et al.*, 2005).

3.1.2. Anaplastic lymphoma kinase (ALK)—ALK is a member of receptor tyrosine kinases (RTK) and was first identified as a part of the fusion gene nucleophosmin (NMP)–ALK in anaplastic large cell lymphoma via chromosome translocation of t(2;5)(p23;q25) (Morris *et al.*, 1994, 1997). ALK is thought to play a role in the normal development of the central and peripheral nervous system since ALK mRNA is expressed throughout the nervous system in mouse and rat, but is not present in normal hematopoietic cells (Degoutin *et al.*, 2009; Hurley *et al.*, 2006; Iwahara *et al.*, 1997; Morris *et al.*, 1997; Vernersson *et al.*, 2006). More detailed studies in chick embryos have shown a similar pattern of ALK expression in the developing central nervous system in which ALK localizes primarily to the spinal motor neuron, the sympathetic ganglia, and the dorsal root ganglia. In mice, expression of ALK in the nervous system decreases after birth but is maintained at low levels in adults. Similar patterns of expression are observed in humans although additional ALK transcripts of differing size, most likely due to alternative splicing, have been observed in colon, prostate, testis, small intestine, and brain of adults (Iwahara *et al.*, 1997; Palmer *et al.*, 2009).

Full-length ALK protein is comprised of an extracellular region and an intracellular region containing a RTK domain, linked by a transmembrane (TM)-spanning segment, whereas the NMP–ALK fusion protein generated as a result of the t(2;5)(p23;q25) translocation contains the N-terminal of NMP and C-terminal kinase domain of ALK. Translocation of the *ALK* gene is also found in other tumors, such as inflammatory myofibroblastic tumor (IMT), and nonsmall cell lung cancer (NSCLC), but not in NB (Palmer *et al.*, 2009). Overexpression of wild-type ALK has also been observed in thyroid carcinoma, breast cancer, NB, melanoma, small cell lung carcinoma, glioblastoma, astrocytoma, retinoblastoma, Ewing sarcoma, and rhabdomyosarcomas NB (Cheng and Ott, 2010; Mosse *et al.*, 2009; Palmer *et al.*, 2009).

During 2008, at least five papers described ALK point mutations in 8–12% of all NB patient (both hereditary and sporadic) and some NB cell lines as well (Caren *et al.*, 2008; Chen *et al.*, 2008; George *et al.*, 2008; Janoueix-Lerosey *et al.*, 2008; Mosse *et al.*, 2008). With one exception, all the point mutations identified to date occur in the kinase domain and result in the constitutive activation of ALK. Two of these activating ALK mutants were able to transform NIH3T3 fibroblasts and induce tumor formation in nude mice (Chen *et al.*, 2008). In addition, knockdown of ALK or small molecular ALK inhibitors could reduce cell proliferation and induce apoptosis (George *et al.*, 2008; Janoueix-Lerosey *et al.*, 2008). Amplification of the *ALK* gene and/or overexpression of the ALK protein is seen in as many as 77% of all NB tumors (Passoni *et al.*, 2009) suggesting that overexpression of the ALK protein may also contribute to NB.

The downstream effects of ALK in NB need to be defined. Current data suggest that ALK may function through the Shc and MAP kinase pathways (Motegi *et al.*, 2004; Osajima-Hakomori *et al.*, 2005; Souttou *et al.*, 2001). More recent studies also suggest that activation

of ALK enhances RAP1 activity via interaction with C3G, a Crk-binding protein and Crk-like protein (CRKL), and that this complex contributes to NB tumor cell growth and neurite outgrowth (Schonherr *et al.*, 2010).

3.2. Chromosome gain and oncogene activation

Many genetic abnormalities have been identified in nonfamilial NB tumors, including amplification of the MYCN proto-oncogene (25–33% of patients) and consistent areas of chromosomal deletion and rearrangement that result in loss of 1p36 (25–35%), 11q23 (35–45%), and 14q23 (16–27%), as well as unbalanced gain of 17q22 (~50%) (Table 4.2; Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999). In contrast, known tumor suppressor genes (TSGs) such as p16^{INK4a}, pRb, p53, and p14^{ARF} are not frequently deleted or mutated in NB, although the nuclear localization of the p16^{INK4a} and p53 proteins has been reported to be altered in some tumor cell lines (Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999; Teitz *et al.*, 2001; van Noesel and Versteeg, 2004). Many of these abnormalities are powerful prognostic markers and are highly related to clinical outcome. For example, amplification of MYCN in NB patients is correlated with chromosome 1p36 LOH. NB tumors which harbor 1p36 LOH and MYCN amplification are usually advanced-stage (stages 3 and 4) aggressive tumors that are frequently metastatic and generally respond poorly to chemotherapy/irradiation (Brodeur, 2003; Maris *et al.*, 2007). In the recent years, clinical trials are increasingly based on the tumor genetic characteristics.

3.2.1. Amplification of MYCN and the 2p24 locus—In 1983, Schwab *et al.* found that a novel myc homolog gene was amplified in several NB cell lines and one NB tumor (Schwab *et al.*, 1983). Later, several papers termed this gene as *MYCN* based on homology to c-myc and expression pattern in the developing nervous system, and identified its location at chromosome 2p24 (Kohl *et al.*, 1983; Schwab *et al.*, 1984). Additional studies have shown that N-myc protein is a nuclear phospho-protein that is a member of the myc family of helix-loop-helix transcription factors (Pelengaris *et al.*, 2002). Amplification of the *MYCN* gene in patient tumors ranges from 10-fold to more than 500-fold, although the majority of tumors exhibit 50- to 100-fold *MYCN* gene amplification levels. The amplified DNA typically contains a large region of chromosome 2 ranging from 100 kb to 1 Mb which includes the entire *MYCN* gene and varying amounts of adjacent DNA. Although other genes may be coamplified with *MYCN*, *MYCN* is only consistent amplified gene from this region (Reiter and Brodeur, 1996, 1998).

MYCN amplification is rarely observed on chromosome 2p24 in primary tumors but is found to be at homogeneously staining regions (HSRs) on different chromosomes or, more frequently, as double minutes (DMs; which are small fragments of extrachromosomal DNA; Emanuel *et al.*, 1985; Schwab *et al.*, 1984). During cell culture, the amplification unit frequently integrates into chromosomes to become HSRs. The reason for the differences in the location of the amplicon in primary tumors and cultured cells remains unclear.

Amplification of *MYCN* is highly associated with aggressive NB tumors and poor outcome. Although the entire role of *MYCN* in NB is still being uncovered, amplification of the *MYCN* gene is usually accompanied by overexpression of the N-myc protein. Studies on N-myc regulation suggest that the transcription factor and signaling pathways responsible for the upregulation of N-myc are dependent on cell type (Hurlin, 2005). These factors include IL-7 and Pax-5, NF- κ B in pre-B cells, and insulin-like growth factors I and II (IGFI and IGFI) in NB cells (Strieder and Lutz, 2003). In contrast, N-myc transcription is repressed by retinoic acid (RA) in association with E2F binding, nerve growth factor (NGF) binding to TrkA receptor, the iron chelator deferoxamine mesylate, and transforming growth factor-beta 1 (TGF- β 1; Strieder and Lutz, 2003; Wada *et al.*, 1992).

Myc proteins form heterodimers with the Max protein. These heterodimers bind to E-box elements (CACGTG) to activate transcription. However, Myc–Max dimers can also associate with other transcription factors such as Miz-1 and Smad and bind to Inr (initiator) element to repress transcription. Max can also form homodimers or heterodimers with Mad to compete or suppress Myc–Max binding (Pelengaris *et al.*, 2002; Thompson, 1998; Fig. 4.3). The targets of Myc–Max are involved in various cellular processes, including cell growth, proliferation, loss of differentiation, and apoptosis (Adhikary and Eilers, 2005; Pelengaris *et al.*, 2002; Thompson, 1998), and include proteins such as MASH1 and important molecules in the normal development of sympathoadrenal lineage cells, such as the multidrug resistance protein 1 (MRP1), α -prothymosin, telomerase, Id2, MCM7; leukemia inhibitory factor, activin A, Pax-3, and MDM2 (Breit and Schwab, 1989; Haber *et al.*, 1999; Harris *et al.*, 2002; Hatzi *et al.*, 2002; Lasorella *et al.*, 2002; Mac *et al.*, 2000; Pelengaris *et al.*, 2002; Shohet *et al.*, 2002; Slack *et al.*, 2005). Many other putative N-myc targets with E-boxes in or near the promoter have also been identified although studies are still ongoing to determine which E-boxes actually bind Myc. Overexpression of N-myc is also reported to influence the expression of IL-6, NDRG1, MHC class I genes, and integrins (Chambery and Mohseni-Zadeh, 1999; Lutz *et al.*, 1996; Mac *et al.*, 2000), although the mechanisms responsible for these effects are unknown.

The transgenic mouse model demonstrates that MYCN overexpression is an initial event in NB tumorigenesis. In this model, overexpression of the human MYCN is driven by the rat TH promoter, which is expressed in migrating cells of the neural crest early in development (Banerjee *et al.*, 1992), causes the formation of NB tumors in transgenic mice (Weiss *et al.*, 1997). These tumors recapitulate most of the histological and pathological aspects of the human disease, including tumor localization, positive staining for neuronal markers, and gains and losses of chromosomes in regions syntenic with those observed in human NB (Weiss *et al.*, 1997). However, other factors are also likely to be involved in the early stages of tumor formation since amplification of the MYCN oncogene occurs in only about one-third of NBs. In addition, the tumors in these transgenic mice rarely exhibit significant metastasis despite the presence of high levels of N-myc protein suggesting that other genetic alterations and/or epigenetic changes are needed for tumor formation and metastasis. These and other studies suggest that N-myc regulates neural progenitor cell proliferation, nuclear size and differentiation (Knoepfler *et al.*, 2002). Importantly, studies using chick/quail chimera reveal that overexpression of N-myc in the early neural crest induces premature ventral migration of neural crest cells and promotes the differentiation of these cells (Wakamatsu *et al.*, 1997). In addition, other studies have shown that high level N-myc mRNA or protein expression in NB cells accelerate cell cycle progression (Lutz *et al.*, 1996), and that overexpression of N-myc in postmitotic sympathetic neurons causes quiescent cells to reenter the cell cycle and enhances the survival of these cells upon NGF withdrawal (Wartiovaara *et al.*, 2002).

The high level expression of N-myc in NB is also consistent with the hypothesis that NB arises during development. N-myc is normally expressed at the beginning of the preimplantation stage of development. As the embryo develops, N-myc expression is observed in the central nervous system and neural crest. By embryonic day 9.5, relatively high levels of N-myc are observed in the fetal brain, kidney, and in the neural crest and early stage migrating neural crest cells (Zimmerman *et al.*, 1986). During later stages of neural crest migration N-myc expression is only observed in cells undergoing neuronal migration. Even in these cells, N-myc expression is gradually downregulated as cells differentiate and become quiescent (Lee *et al.*, 1984; Zimmerman *et al.*, 1986). These data are consistent with studies using N-myc knockout mice that demonstrate that the loss of N-myc results in embryonic death at day 10.5 of gestation due to defects in the nervous system, limb, heart, liver, lung, gut, mesonephros, and genital ridge (Sawai *et al.*, 1991).

3.2.2. Gain of 17q—Gain of chromosome 17q was first identified by G-banded cytogenetic analyses in early 1980s (Gilbert *et al.*, 1984). However, researchers paid little attention to these observations since their interests focused on the genetic abnormalities of *MYCN* amplification and 1p LOH at that time. In the middle 1990s, NB scientists realized the importance of 17q abnormalities since FISH technology indicated that translocation of this chromosome arm occurs in about half of the NB primary tumors. The translocation results in unbalanced gain of one to three copies of 17q (Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999). Although the breakpoint of 17q varies, the frequent gain of regions from 17q22 suggests that increased dosage of one or more genes from this region may confer a selective survival advantage for NB tumor cells. It is estimated that as much as 20 Mb of the 17q chromosome fragment, which could include more than 200 genes, can be translocated in NB tumors. Therefore, it is difficult to identify the genes responsible for the selective advantages (Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999). Several genes in this area are considered good candidate oncogenes or tumor suppressors based on correlations between expression levels and unbalanced gain of 17q. These include survivin, *NM23A*, and *PPM1D* (Godfried *et al.*, 2002; Islam *et al.*, 2000; Saito-Ohara *et al.*, 2003). Notably, survivin is a member of apoptosis inhibiting protein family and is frequently overexpressed in many tumor types, including NB where expression has been correlated with late stage disease and poor prognosis; whereas *NM23A* is metastasis-related gene and *PPM1D* a phosphatase that suppresses stress induced apoptosis (Almgren *et al.*, 2004; Bown *et al.*, 1999).

Unbalanced gain of 17q correlates with other chromosomal deletions. The most frequent deletion site is the short arm of chromosome 1, followed by 11q. At least 30 translocation sites on 20 different chromosomes have been identified in various patient samples and cell lines (Bown *et al.*, 1999; Lastowska *et al.*, 1997; Meddeb *et al.*, 1996). Nevertheless, NB tumors harboring unbalanced gain of 17q exhibit a more aggressive phenotype and a poorer prognosis than those without this abnormality.

3.2.3. Amplification and chromosome gain of other loci—In addition to the amplification of *MYCN* gene, several other regions of gene amplifications have been identified in small groups of NB cases. These include amplification of the *MDM2* gene at 12q13, the *DDX1* gene at 2p24, the *MYCL* gene at 1p32, and unidentified DNA from chromosome 2p22 and 2p13 (Corvi *et al.*, 1995a, b; Jinbo *et al.*, 1989; Van *et al.*, 1995). The *MDM2* gene was initially found to be amplified in three NB cell lines and one primary tumor (Corvi *et al.*, 1995a). Like the *MYCN* gene amplification, the *MDM2* amplification unit first developed within DMs and then integrates into a different chromosome to form HSRs (Corvi *et al.*, 1995b). The *DDX1* gene, which encodes a RNA helicase, was found to be coamplified with *MYCN* in 4/6 NB cell lines and 6/16 tumors with *MYCN* amplification; however, *DDX1* amplification was not found without *MYCN* amplification (George *et al.*, 1996). One paper also indicated that *MYCL* gene is coamplified with *MYCN* in NB cell lines. *MYCL*, another member of myc gene family, is frequently overexpressed in small cell lung carcinoma (Jinbo *et al.*, 1989). In addition to gain of 17q, other chromosome gains have been identified on 1q, 4q, 5q, 6p, 7q, 18q using comparative genomic hybridization (CGH) methodology, although their biological and clinical significance remain unclear (Hirai *et al.*, 1999; Lastowska *et al.*, 1997; Meltzer *et al.*, 1996; Takita *et al.*, 2000; Vandesompele *et al.*, 1998).

3.3. Chromosome loss and tumor suppressor genes (TSGs)

In addition to mutation, gene amplification and increased chromosome copy number, NB tumors also experience loss of genetic material and deletion of putative TSGs.

3.3.1. LOH of chromosome 1p and CHD5, miR-34, KIF1B β —Loss of the short arm of chromosome 1 occurs in about 25–35% NB tumors. 1p LOH is correlated with amplification of MYCN in NB patients. As mentioned above, loss of 1p correlates with and may be a result of unbalanced gain of 17q, however, the exact mechanism that is responsible for these two events is not clear. The importance of 1p LOH is highlighted by studies showing that transferring chromosome 1p material into human NB cells *in vitro* led to differentiation and suppression of tumorigenicity (Bader *et al.*, 1991). In search of potential TSGs that reside in this region, extensive efforts have been made to identify the smallest region of overlap (SRO) that would include the TSG candidate. These studies delineate the 1p36.1–36.3 as the SRO. Several candidate genes, such as p73, reside in this area. However, further studies failed to demonstrate a correlation between p73 loss and NB development (Ichimiya *et al.*, 1999).

Whereas patients with 1p36 abnormalities without MYCN amplification have been identified, the reverse situation virtually never occurs suggesting either that 1p36 LOH provides a permissive environment for MYCN amplification or that tumors with these two associated genetic defects have a high degree of genomic instability (Brodeur, 2003). NB tumors with 1p36 LOH and MYCN amplification are usually aggressive tumors that are frequently metastatic and generally resistant to chemotherapy/irradiation. Although the chromosomal regions described above are known to be important in NB, the TSGs that reside within these regions have not been definitively identified.

Recent studies have identified three new putative tumor suppressors on chromosome 1p36: the chromodomain helicase DNA-binding domain 5 (CHD5), microRNA-34a (mir-34a), and the kinesin superfamily protein 1B beta (KIF1B β ; Bagchi *et al.*, 2007; Munirajan *et al.*, 2008; Welch *et al.*, 2007). All three of these proteins have effects on cell growth. For example, Bagchi *et al.* demonstrated that the effects of CHD5 on cell growth were dependent on p53 and that CHD5 positively regulates p53 via effects on p19^{ARF} expression. Thus, overexpression of CHD5 results in enhanced apoptosis and senescence, increased p53 and p19^{ARF} levels, and sequestration of MDM2, the negative regulator of p53, by p19^{ARF}. Conversely, cells lacking CHD5 exhibit decreased p16 and p19^{ARF} expression. This decrease in p19^{ARF} was mirrored by a decrease in p53 levels and enhanced cellular proliferation. Thus, CHD5 appears to function as a tumor suppressor that controls proliferation, apoptosis, and senescence via effects on the p19^{ARF}/p53 pathway. These effects are most likely due to changes in the accessibility of the p16/p19^{ARF} gene locus resulting from the chromatin remodeling function of CHD5 (Bagchi *et al.*, 2007).

In addition to CHD5, Chen *et al.* found that mir-34a was expressed at very low levels in unfavorable primary tumors and NB cell lines. This group further showed that introduction of this microRNA (miRNA) into cell lines resulted in decreased cell proliferation and caspase-dependent apoptosis. They also found that mir-34a directly targeted the E2F3 mRNA and repressed its expression (Chen and Stallings, 2007; Welch *et al.*, 2007). E2F3 is a transcription factor that induces the expression of many genes with roles in cellular proliferation.

Finally, overexpression of KIF1B β induced cell death while decreased KIF1B β levels correlated with cell proliferation and enhanced tumor formation in nude mice, suggesting that KIF1B β is also a potential TSG candidate (Munirajan *et al.*, 2008). Kaelin's group also found that KIF1B β is a downstream target of prolyl hydroxylase EglN3 and induced apoptosis in neuronal progenitor cells or NB cells when NGF is limited. In addition, they identified missense mutations of KIF1B β in inherited NBs and pheochromocytomas (Schlisio *et al.*, 2008), supporting the hypothesis that KIF1B β is a potential TSG candidate.

3.3.2. Loss of 11q and TSLC1—Loss of the long arm of chromosome 11 has been identified in 35–45% NB primary tumors with a single copy *MYCN* gene. Two large patient studies analyzed 295 NB primary tumors. These studies found loss of 11q in 44% cases, and common regions of LOH located at 11q23, suggesting there are TSGs residing in this area (Guo *et al.*, 1999; Maris and Matthay, 1999). Loss of 11q correlated with adverse clinical features including late stage disease, older age of disease onset and unfavorable histology, although it is strikingly inversely correlated with *MYCN* amplification and 1p loss. Therefore, 11q loss is a useful and important marker in determining the clinical prognosis for those advanced-stage tumors without *MYCN* amplification. Transfer of chromosome 11 induced differentiation in NB cell lines supporting the importance of loss of 11q in tumorigenesis (Bader *et al.*, 1991).

One putative tumor suppressor, the *IGSF4* (immunoglobulin superfamily 4) gene, was first localized to the common 11q23 LOH region in 1999 (Gomyo *et al.*, 1999). This gene which is also known as TSLC1/CADM1 (Tumor suppressor in lung cancer 1/cell adhesion molecule 1), is considered as a potential TSG for lung cancers. A recent CGH study which examined 236 primary tumor samples found TSLC1 LOH locus in 35% tumors. Importantly, the level of TSLC1 expression correlated with tumor stage, histological classification, *MYCN* and TrkA expression levels. Reduced expression of TSLC1 was found in unfavorable tumors. Further, introduction of TSLC1 decreased cell proliferation in NB cell lines (Ando *et al.*, 2008). These results indicated that TSLC1 is a good NB tumor suppressor candidate. Interestingly, a recent study indicates that expression of both KIF1B β and TSLC1 is controlled by the polycomb protein Bmi1, whose expression is regulated by N-myc (Ochiai *et al.*, 2010).

3.3.3. LOH of 14q—Loss of the long arm of chromosome 14 is also commonly found in NB primary tumors (~16–27% of the patients). LOH on chromosome 14q was first identified in 1989 using a polymorphic DNA marker which detected allelic deletion at specific 14q23 loci (Suzuki *et al.*, 1989). LOH analysis of 14q in a large number of primary tumors using 11 polymorphic DNA markers found 14q LOH in 83 of 372 tumors (22%) (Thompson *et al.*, 2001). 14q LOH was highly correlated with 11q loss and had an inverse relationship with 1p loss and *MYCN* amplification (Thompson *et al.*, 2001). However, LOH for 14q was present in tumors from all clinical stages, suggesting this abnormality may be a universal early event during tumor development.

In addition, to the genetic changes described above, there have also been reports of LOH and/or allelic imbalance at chromosome arms 2q, 3p, 4p, 9p, and 19q (Caron, 1996; Ejeskar *et al.*, 1998; Marshall *et al.*, 1997; Mora *et al.*, 2001; Takita *et al.*, 2001), however, the significance of these genetic changes is not clear.

4. The Role of Neurotrophins and Growth Factors in the Development of the Sympathetic Nervous System and in NB

As the neural crest cells migrate to the aorta but prior to reaching the adrenal medulla they begin to express TH which in turn controls the expression of other enzymes needed for catecholamine biosynthesis as described above in Section 2.3. Since NB appears to arise from cells that are transformed at various times during this migration, the majority of NB tumors secrete catecholamines. Indeed, the presence of high levels of catecholamines in patient urine samples is used as one of the diagnostic criteria for the disease (LaBrosse *et al.*, 1976). Based on this data, Sawada began to screen the urine of 6-month-old infants for increased catecholamine metabolites from 1984 and found that the incidence of *in situ* NB was much higher than the number of sporadic cases that had been observed previously (Sawada, 1992). These data agree with a previous hypothesis of Beckwith and Perrin who

postulated that during the development of sympathetic neurons the incidence of *in situ* NB is higher than the incidence of sporadic cases (Beckwith and Perrin, 1963). Most of these *in situ* NBs spontaneously regress as the child ages (Brodeur, 2003; Maris, 2005; van Noesel and Versteeg, 2004), suggesting they are resolved using normal developmental programs. Developmental studies and studies from knockout mice suggest the TrkA is crucial for the development of many sympathetic lineage cell types (Brodeur *et al.*, 2009). This is consistent with data indicating the NGF is required for the differentiation and survival of many sympathetic lineage cells (Nakagawara, 2004; Nakagawara and Ohira, 2004). In addition to NGF and TRKs several other growth factors also play roles in the development of the sympathoadrenal lineage cells. These include EGF which is expressed in neural crest cells and is thought to contribute to the formation of neuron and melanocytes at later point during neural crest migration (Erickson and Turley, 1987), vascular endothelial growth factor (VEGF) which is expressed by surrounding cells (McLennan *et al.*, 2010), and IGF1 and IGFII which is expressed in the neural crest, the dorsal root, the sympathetic ganglia, and the adrenal medulla (Coppola *et al.*, 2009; D'Ercole *et al.*, 1996).

4.1. Neurotrophin receptors

Although the steps in the transformation of sympathetic neuroblasts to NB cells is not clear, extensive evidence suggests that neurotrophin receptors are involved in NB tumorigenesis and in development of the nervous system and sympathoadrenal lineage cells. The neurotrophin receptors TrkA, TrkB, and TrkC, are encoded by the *NTRK1, 2, 3* genes, respectively. Upon binding to their ligands, NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3, respectively, these receptors regulate proliferation, survival, and differentiation in normal neuronal cells (Brodeur *et al.*, 2009; Maris and Matthay, 1999; Maris *et al.*, 2007; Straub *et al.*, 2007). These receptors all associate with p75, a low affinity receptor that may enhance the binding of ligand to the Trk proteins or alter the function of the Trk receptors (Brodeur *et al.*, 2009; Maris and Matthay, 1999; Maris *et al.*, 2007; Straub *et al.*, 2007). High levels of TrkA, in association with very low/no N-myc expression, are detected in favorable NB tumors which often spontaneously regress (Nakagawara, 1993; Nakagawara *et al.*, 1992). These favorable NB tumors cells usually express a small amount of NGF as do some of the surrounding cells (Nakagawara, 1993). Cells that express the most NGF are thought to undergo differentiation, while those that express less NGF undergo apoptosis (Nakagawara, 1993). TrkA expression is dramatically decreased in MYCN amplified NB tumors (Nakagawara *et al.*, 1992). Thus, the TrkA/NGF pathway could play an important role in determining the ability of these favorable NB tumors to differentiate or to regress in response to the microenvironment. While in general TrkA expression is correlated with a good prognosis, a novel TrkA splice variant has been found in advanced-stage tumors with adverse biological features (Tacconelli *et al.*, 2004). This TrkA isoform is constitutively active and promotes cell survival and angiogenesis independently of NGF expression. NGF signaling may also be linked to IGFII expression since a study in which SHSY5Y cells were transfected with TrkA found increased expression of IGFII in response to NGF binding to the transfected receptor (Kim *et al.*, 1999).

In contrast, TrkB is preferentially expressed in clinically unfavorable NB tumors and expression of TrkB strongly correlates with MYCN amplification (Nakagawara *et al.*, 1994). The TrkB ligand, BDNF, is also highly expressed in these tumors. Coexpression of ligand and receptor may form an autocrine loop to enhance survival, metastasis, and drug resistance (Douma *et al.*, 2004; Nakagawara, 1994). Interestingly, a truncated form of TrkB which lacks tyrosine kinase activity is expressed in some favorable NB tumors (Ho *et al.*, 2002).

Finally, TrkC is commonly expressed in favorable NB tumors. These tumors also express very limited amount of the TrkC ligand neurotrophin-3 and coexpress TrkA (Svensson *et al.*, 1997; Yamashiro *et al.*, 1996).

4.2. Other growth factors and growth factor receptors

In addition to NGF and BDNF several other growth factors also play roles in the development of the sympathoadrenal lineage cells and NB tumorigenesis. These include EGF, VEGF, and insulin-like IGFI and IGFII. EGF receptor 1 (EGFR1) expression is found on both primary NB tumors and tumor-derived cell lines (Ho *et al.*, 2005). Binding of EGF to EGFR1 causes receptor autophosphorylation and increases proliferation via effects on the MAPK and PI3K/AKT pathways (Henson and Gibson, 2006). Exogenous VEGF also stimulates the PI3K/AKT pathway and increases expression of survivin, an antiapoptotic gene in NB cells (Beierle *et al.*, 2005). Endocrine-derived VEGF has also been shown to play a role in the proliferation and differentiation of neural crest cells during development and to promote NB cell growth. IGF1 receptors (IGF1Rs) are expressed in the majority of NB primary tumors (Martin *et al.*, 1992). This receptor binds both IGFI and IGFII suggesting that these growth factors are important for NB tumorigenesis. Expression of IGF1R activates the PI3K/AKT and MAPK pathways and enhances cellular proliferation, cell survival, migration, and invasion, and induces chemotherapeutic resistance and reduced response to other apoptotic stimuli (Valentinis and Baserga, 2001). IGFII has been reported to be upregulated upon TRKA activation and downregulated upon TRKA overexpression suggesting potential feedback loops between these proteins (Kim *et al.*, 1999). In addition, IGF1R is transcriptionally activated by N-myc and in turn high IGF1R levels induce N-myc protein and mRNA expression suggesting the presence of an amplification loop that enhances the ability of these proteins to promote tumorigenesis (Chambery and Mohseni-Zadeh, 1999). Inhibition of IGF1R signaling has also been shown to increase N-myc phosphorylation by GSK-3 β which inactivates N-myc and enhances N-myc turnover resulting in decreased cell growth both in culture and in mice model systems (Coulter *et al.*, 2009). IGF1R has also been shown to enhance NB metastasis to bone most likely due to its ability to enhance migration and invasion and to the presence of IGF ligand in bone marrow (van Golen *et al.*, 2006). In addition, IGF increases cellular survival under hypoxic conditions via increased expression of hypoxia-inducing factor 1 α (HIF1 α) and VEGF (Treins *et al.*, 2005).

5. Programmed Cell Death (PCD; Apoptosis) in Development of NB

5.1. The role of cell death in development

Another important process during development of the peripheral nervous system is PCD, also known as apoptosis. This process is used during development to eliminate redundant cells, control cell number, and for remodeling and repair. Cell death also occurs in the developing peripheral nervous system in response to loss of essential growth factors and cytokines (De Zio *et al.*, 2005). Neural crest development therefore is a balance between proliferation, cell death, migration, and differentiation. Errors in any of these processes may leave the cell more prone to transformation and potentially increase tumorigenesis. This is especially true of pediatric cancers, such as NB, since these cancers develop during normal development. Indeed, stage 4S NB spontaneously regresses with little to no intervention (Blaschke *et al.*, 1998; De Zio *et al.*, 2005; Johnsen *et al.*, 2009). Tumors of this stage are present in younger children and the prognosis is good. It is unknown why these tumors suddenly die or cease to grow, although it is thought that apoptosis is likely to be involved in the disappearance of these tumors (Brodeur, 2003; Maris *et al.*, 2007; Schor, 1999). Several hypotheses have been suggested to explain the regression of these tumors: (1) these tumors are dependent on growth factors or other proteins that are present in low levels and that once a tumor reaches a certain size the factors are depleted and the tumor undergoes apoptosis, (2) as the child develops, the tumor is recognized by the immune system and destroyed, and/or (3) that the cells become responsive to the environmental cues and developmental regulatory apoptotic pathways as they mature which triggers apoptosis. Though any of these

possibilities may be the case, there is sparse literature investigating apoptotic pathways in the developing trunk crest as well as the peripheral nervous system. Although it is known that crest cells from rhombomeres 3 and 5 in the chicken and mammals undergo PCD (Kulesa *et al.*, 2004; Morales *et al.*, 2005) and that expression of Snail in the early migratory crest cells protects the migratory crest cells from apoptosis (Vega *et al.*, 2004), a more detailed investigation regarding specific cell death programs in crest cells contributing to the developing sympathoadrenal lineage requires more characterization.

5.2. The role of apoptosis genes in NB

As mentioned above, apoptosis or PCD in multicellular organisms is a tightly regulated process required for normal growth, development, and cellular specialization (Danial and Korsmeyer, 2004; Hengartner, 2000). Defective expression of proteins and aberrant function of constituents in the apoptotic cascade have been implicated in oncogenesis, tumor progression, and treatment resistance (Fesik, 2005; Kaufmann and Vaux, 2003; Reed and Tomaselli, 2000). There are two major apoptotic pathways in mammalian cells: the death receptor (or extrinsic) pathway and the mitochondrial (or intrinsic) pathway. Caspases (cysteine proteases) are the central components of the apoptotic machinery. At least 14 distinct caspases have been identified in mammals and are grouped into three categories (nonapoptotic, initiators, and effectors; Danial and Korsmeyer, 2004; Shi, 2004). Importantly for this review, alterations in caspase-8 expression have been observed in NB.

5.2.1. Caspase-8—Human caspase-8 (also known as FLICE) is encoded by the *CASP8* gene which is located on chromosome 2q33–2q34 (Grenet *et al.*, 1999). Caspase-8 exists as a monomer in the cell and requires dimerization or oligomerization for its activation (Boatright and Salvesen, 2003; Salvesen and Abrams, 2004). Subsequent cleavage events, although not essential for activity, further stabilize the activated protein (Boatright and Salvesen, 2003; Boatright *et al.*, 2003; Salvesen and Abrams, 2004). Activated caspase-8 is an important initiator caspase in the death receptor-mediated apoptotic pathway. The death receptor pathway is triggered by members of the death receptor superfamily (FasR, TNFR1, DR5, etc.; Hengartner, 2000). Binding of the ligand (e.g., FasL) to its receptor (FasR) induces the formation of the death-inducing signaling complex (DISC) containing death receptors, adaptor proteins and procaspase-8 (Danial and Korsmeyer, 2004; Hengartner, 2000). Procaspase-8 then dimerizes, resulting in activation and cleavage (Danial and Korsmeyer, 2004; Hengartner, 2000; Shi, 2004). The active enzyme subsequently cleaves downstream effector caspases, resulting in their activation and leading to apoptosis (Danial and Korsmeyer, 2004; Hengartner, 2000; Shi, 2004). Active caspase-8 can also cleave Bid, which is a Bcl2 family member. Cleaved Bid, termed t-Bid, translocates to the mitochondria and promotes cytochrome c release, thereby activating the mitochondrial pathway (Danial and Korsmeyer, 2004; Hengartner, 2000; Shi, 2004).

Our laboratory first found that caspase-8 is deleted or more commonly, silenced in most NB cell lines and patient tumor samples. We further identified a region within the *CASP8* gene which is methylated (Teitz *et al.*, 2000). This finding has subsequently been verified by several groups (Fulda and Debatin, 2006; Fulda *et al.*, 2001; Teitz *et al.*, 2000; Yang *et al.*, 2007). Several additional genes with roles in tumorigenesis are also hyper-methylated in NB including the PCDHB gene family, BLU, TSP-1, RASSF1A, TIG1, HIN-1, DcR1, DcR2, DcR4, etc. (Abe *et al.*, 2005; Astuti *et al.*, 2001; van Noesel *et al.*, 2002; Yang *et al.*, 2003, 2007). Among these, *CASP8*, *PCDHB*, *BLU*, *DcR2*, and *HIN-1* are found to be associated with high-risk factors and poor outcome in NB patients (Abe *et al.*, 2007; Yang *et al.*, 2007). Methylation of this region of the *CASP8* gene has been correlated with decreased caspase-8 protein expression in many (Fulda *et al.*, 2001; Hopkins-Donaldson *et al.*, 2000; Teitz *et al.*, 2000), but not all studies (Banelli *et al.*, 2002; van Noesel *et al.*, 2002). However, even in

studies where methylation has not been correlated with expression, decreased caspase-8 protein levels are observed in 50–70% of NB patient tumors. Since our initial discovery in NB, loss of caspase-8 expression has been found in many other tumors, including peripheral neuroectodermal tumors, medulloblastoma, glioblastoma multiforme, rhabdomyosarcoma, retinoblastoma, small cell lung carcinoma, and Wilms tumors (Gonzalez-Gomez *et al.*, 2004; Harada *et al.*, 2002; Hopkins-Donaldson *et al.*, 2003). Caspase-8 mutations in adult gastric and colorectal tumors have also been reported (Kim *et al.*, 2003; Soung *et al.*, 2005). Finally, loss of caspase-8 expression has been correlated with poor prognosis in medulloblastoma (Pingoud-Meier *et al.*, 2003) and with relapse and aggressive metastatic disease in glioblastoma multiforme (Martinez *et al.*, 2007). A role for caspase-8 in tumorigenesis is also supported by recent studies demonstrating increased transformation of caspase-8 null SV40 immortalized mouse embryo fibroblasts (Krelin *et al.*, 2008). In addition, we have demonstrated that the loss of caspase-8 expression has biological significance in NB cell metastasis (Lahti *et al.*, 2006; Stupack *et al.*, 2006; Teitz *et al.*, 2006). Loss of caspase-8 increases metastasis by blocking integrin-mediated death, a caspase-8-dependent process (Lahti *et al.*, 2006; Stupack *et al.*, 2006; Teitz *et al.*, 2006). The role of caspase-8 in metastasis will be discussed in more detail in the Section 6.2 describing proteins that play a role in NB metastasis.

Multiple groups have shown that caspase-8 deficient NB cells are resistant to death receptor signals and some chemotherapeutic drugs. Importantly, these defects can be corrected by reexpression of caspase-8 via demethylating agents, IFN- γ treatment and caspase-8 expressing retroviral vectors. However, in a few instances secondary defects in death receptor proteins and/or signaling pathway or increased expression of FLIP prevented restoration of the extrinsic cell death pathway (Fulda *et al.*, 2001; Johnsen *et al.*, 2004; Muhlethaler-Mottet *et al.*, 2003; Teitz *et al.*, 2000; Tekautz *et al.*, 2006; Yang *et al.*, 2003).

5.2.2. Bcl-2 family—Another group of proteins that play a role in NB tumorigenesis and metastasis are the Bcl-2 family members. This family of proteins plays a central role in the intrinsic apoptotic pathway. There are more than 20 genes of Bcl-2 family identified in mammals, including antiapoptotic members (Bcl-2, Bcl-XL, Bcl-W, Bcl-G, Bfl1, Mcl-1) and proapoptotic members (Bcl-XS, Bcl-B, Bax, Bak, Bad, Bid, Bik, Bok, Bim, Puma, Noxa, Nix, Nip3, Hrk, Mtd). They usually regulate apoptosis by controlling mitochondrial outer membrane permeabilization (MOMP) to promote or prevent the release of cytochrome c (Green and Kroemer, 2004; Johnsen *et al.*, 2009; Reed, 2006).

The antiapoptotic members, such as Bcl-2, Bcl-XL, Mcl-1, are highly expressed in neural progenitor cells, indicating their protection role in neuron development. Some proapoptotic regulators of the intrinsic pathway, such as Bid and caspase-9, are expressed preferentially in favorable tumors, whereas antiapoptotic regulators such as Mcl-1 were expressed at high levels in unfavorable tumors (Abel *et al.*, 2005), suggesting an imbalance between anti- and proapoptotic factors in NB tumors with favorable or unfavorable biology. Bcl-2 is highly expressed in the majority of NB cell lines and primary tumors and inversely correlates with the degree of cell differentiation (Ramani and Lu, 1994). Although conflicting data exists about the correlation between Bcl-2 levels and MYCN amplification or poor prognosis (Castle *et al.*, 1993; Ikegaki *et al.*, 1995; Mejia *et al.*, 1998), high Bcl-2 level is considered as an important reason for chemoresistance and transfection of Bcl-2 or Bcl-XL into NB cells conferred drug resistance (Dole *et al.*, 1994; Dole *et al.*, 1995).

5.2.3. Regulation of apoptosis-related genes by MYCN—Although the *Myc* gene was originally identified as an oncogene, it is involved in various cellular processes, including cell growth, proliferation, loss of differentiation, and apoptosis (Adhikary and Eilers, 2005; Pelengaris *et al.*, 2002; Thompson, 1998). N-myc has been found to sensitize

NB cells to death receptor induced apoptosis in the absence of cytokines, growth factors or other conditions of stress (Cui *et al.*, 2005; Fulda *et al.*, 1999; Lutz *et al.*, 1998). Cell death in response to MYCN results in caspase-8 cleavage which can be blocked using the caspase inhibitor zVAD-fmk (Cui *et al.*, 2005; Fulda *et al.*, 1999). Upregulation of p53, a direct target of MYCN, is an important mechanism of sensitization of the cell to apoptosis by N-myc (Chen *et al.*, 2010). These data suggest that resistance of NB cells with MYCN amplification to chemotherapy requires additional dysfunction in apoptotic pathways, such as silencing of caspase-8 or inhibiting the p53 pathway. Currently, there are conflicting data on a possible direct relationship between N-myc and caspase-8. Genome wide studies looking for E-box binding sites have identified caspase-8 as a target of both N-myc and c-myc (Fernandez *et al.*, 2003; Perini *et al.*, 2005). Perini and coworkers further demonstrated that the E-box sites in the caspase-8 promoter are methylated in NB preventing binding of N-myc/Max heterodimers. Furthermore, when these NB cells were treated with the demethylating agent 5-azacytidine, N-myc/Max binding was restored and caspase-8 expression was increased (Perini *et al.*, 2005). In contrast to this data which suggests a direct relationship, Fulda and coworkers failed to observe any changes in caspase-8 expression upon overexpression of N-myc or after downregulation of N-myc with antisense RNA; however, since these authors did not examine the methylation state of the E-box sequences in caspase-8, this study is somewhat difficult to interpret (Fulda *et al.*, 1999). In addition to data suggesting a possible direct relationship between N-myc and caspase-8, several studies have reported that myc expression promotes activation or priming of the mitochondrial pathway which increases the sensitivity of cells to death receptor signaling. Once the pathway is activated, caspase-8 cleavage of Bid provides a further amplification loop (Klefsstrom *et al.*, 2002; Nieminen *et al.*, 2007). Additionally others have shown that the death receptor DR5 contains two E-box sequences that bind N-myc resulting in increased expression of this receptor and further augmentation of the extrinsic pathway (Cui *et al.*, 2005). Some proapoptotic Bcl-2 family members such as Bax and puma are transactivated by MYC, which play an important role in switching p53 downstream effects from G1 arrest to apoptosis (Seoane *et al.*, 2002).

Although p53 is a transcriptional target of N-myc, N-myc also inhibits its function through increased levels of p53 negative regulators, such as MDM2 and TWIST. MDM2, the essential inhibitor of p53, is a direct target of N-myc and inhibition of N-myc results in decreased MDM2, stabilized p53, and apoptosis. TWIST, a transcription factor of bHLH family with antiapoptotic activity, is frequently overexpressed in MYCN amplified NB (Valesia-Wittmann *et al.*, 2004). Furthermore, TWIST overexpression inhibits ARF/p53 preventing apoptosis in response to N-myc overexpression (Valesia-Wittmann *et al.*, 2004).

6. The Role of Epithelial to Mesenchymal Transition (EMT) in Development and Metastasis

6.1. EMT in development

Although greater than 50% of all NB patients present with metastatic disease, little is known of the process and the mechanism. Microarray mRNA expression analysis studies comparing highly metastatic human NBs (stage 4) to nonmetastatic human tumors (stage 1 and 2) have provided information on some of the proteins that are involved in NB metastasis (Scaruffi *et al.*, 2005). Of note, transcripts encoding proteins related to the developmental program of the epithelial to mesenchymal transition or EMT, are expressed at higher levels in metastatic human NB (Scaruffi *et al.*, 2005). As mentioned above, early in development, as the neural folds join to form the neural tube, the epidermal ectoderm expresses BMP signals and the floorplate of the neural tube produces Shh. These gradients interact and result in the formation of the neural crest at the dorsal aspect of the neural tube, between the

epidermal ectoderm and the dorsal neural tube. The epithelial-like cells of the former neural folds undergo the EMT program. EMT is characterized by: (1) loss of epithelial morphology, (2) downregulation of junctional complexes (E-cadherin, cytokeratin, occludins, and claudins), (3) upregulation of intracellular migratory proteins (RhoB), (4) increased expression of matrix modulators (collagenase, matrilysin, urokinase, heparanase, matrix metalloproteinases—MMP), and (5) upregulation of matrix recognition molecules (N-cadherin; Thiery *et al.*, 2009). Metastatic cells exhibit many of these features and evidence is accumulating that several regulators of EMT are misregulated in NB (Shimono *et al.*, 2000; Valsesia-Wittmann *et al.*, 2004; Vitali *et al.*, 2008).

The transcription factors Snail, Twist, and SIP1/ZEB2 are considered to be master regulators of the EMT transition. Snail is induced primarily by TGF β signaling and directly represses E-cadherin by binding of the E-boxes in the E-cadherin promoter (Kang and Massague, 2004). The repression of E-cadherin is a hallmark of the induction of the EMT program, allowing for the motility of the crest cells. Similarly Twist, a basic helix-loop-helix transcription factor developmentally regulated via the NF- κ B pathway, represses E-cadherin expression via the E-boxes within the E-cadherin promoter, although this interaction has not been shown to be direct (Sosic and Olson, 2003). Snail also induces the expression of ZEB factors (inhibitors of E-cadherin expression) which are involved in an EMT regulation loop with miRNA-200 family (Bracken *et al.*, 2008). The miRNA-200 family inhibits the expression of the ZEB family that relieves repression of E-cadherin (Bracken *et al.*, 2008).

6.2. Metastasis-related genes

Although greater than 50% of all NB patients present with metastatic disease, little is known of the process and the mechanism. The data from our group and collaborators first indicated that loss of caspase-8 increases metastasis by blocking integrin-mediated death. Loss of caspase-8 facilitates survival in foreign environments both during development and during metastasis. This data may also explain the observation that at least 50% of NB patients present with metastatic disease (Lahti *et al.*, 2006; Stupack *et al.*, 2006; Teitz *et al.*, 2006).

Another protein that is involved in NB metastasis is CD44, a cell surface glycoprotein that plays a role in cell adhesion and metastasis. CD44 is highly expressed in colon tumors where it affects tumor invasion, however, CD44 expression variable in NB tumors (Shtivelman and Bishop, 1991; Tanabe *et al.*, 1993). There are conflicting data about the correlation between CD44 expression and MYCN amplification, however, high expression of CD44 always correlates with favorable tumors and with the presence of more differentiated cells (Combaret *et al.*, 1996; Munchar *et al.*, 2003). The observation that CD44 is only expressed in favorable tumors is consistent with its functions as a metastasis inhibitor (Munchar *et al.*, 2003; Shtivelman and Bishop, 1991).

The Nm23A protein (nucleoside diphosphate kinase A) encoded by *NM23-H1* gene mapped to 17q22 locus is highly expressed in unfavorable NB tumors. A point mutation (S120G) was identified in a subset of NB tumors with elevated NM23A expression (Chang *et al.*, 1996). In addition, NM23A promotes NB metastasis in the xenograft NB animal model and S120G mutation reduced cell adhesion and increased cell migration (Almgren *et al.*, 2004). In contrast, NM23A expression is very low in melanoma, breast, colon tumors and NM23A appears to serve as metastasis suppressor in these tumors (Bown *et al.*, 1999). The reason for the opposite expression pattern may be due to the different regulatory mechanisms in specific tissues (Okabe-Kado *et al.*, 2005).

The matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, are highly expressed in advanced-stage NB tumors. MMP-2 and MMP-9 promote cell invasion and metastasis by degrading extracellular matrix including type IV, V, VII, and X collagens as well as

fibronectin. However, there is no correlation between these MMPs and MYCN status (Ribatti *et al.*, 2004; Sugiura *et al.*, 1998).

Twist1, which is originally identified as a key inducer of mesoderm formation in *Drosophila*, is found to play an essential role during metastasis in many types of tumors. First evidence of Twist1 involvement in metastasis came from human breast cancers. High levels of Twist1 are associated with invasive carcinoma and loss of EMT. Suppression of Twist1 inhibits the ability of cancer cells to metastasize, while overexpression of Twist1 increases cell motility and decreases cell adhesion (Yang *et al.*, 2004). Further study demonstrated Twist1 may promote metastasis through direct induction of microRNA-10b, which inhibits homeobox D10, resulting in upregulation of a well-characterized prometastatic gene, RHOC (Ma *et al.*, 2007). Later studies found that Twist1 also promotes migration and invasion in bladder and prostate cancer, hepatocellular carcinoma and colorectal cancer (Matsuo *et al.*, 2009; McConkey *et al.*, 2009; Valdes-Mora and Gomez Test, 2009). Twist1 is also overexpressed in N-myc amplified NB, where it inhibits N-myc induced apoptosis by inhibiting the p53 pathway in part via downregulation of p19^{ARF} (Valsesia-Wittmann *et al.*, 2004).

7. The Role of miRNA in Development and NB

miRNAs are endogenous small noncoding RNAs of ~22 nucleotides in length that negatively regulate gene expression by mRNA cleavage or translational repression of the target mRNA (Bartel, 2004). miRNAs play an important role in regulating most cellular processes, and contribute to the process of tumorigenesis and metastasis (Zhang *et al.*, 2010). miRNA expression profiles have been correlated with prognosis, differentiation, and apoptosis in NB tumors (Chen and Stallings, 2007), suggesting that miRNAs could function as TSGs or oncogenes in NB. As mentioned above, miR-34a, which is located at 1p36, is a good TSG candidate (Welch *et al.*, 2007). Mir-34a is a direct target of p53 and knockdown of mir-34a reduced p53-dependent apoptosis (He *et al.*, 2007). In addition, one study found that N-myc is a direct target of mir-34a (Wei *et al.*, 2008), although this data is contrary to a previous study (Welch *et al.*, 2007). The discrepancy between these two studies may reflect the slightly different systems that were used for these two studies.

Recent data showed that seven miRNAs including miR-17 to -92 cluster were induced by N-myc *in vitro*, and their high expression correlates with MYCN amplification in tumors (Schulte *et al.*, 2008), suggesting this cluster is a potential oncogene in NB. This cluster, which is transcribed as a polycistronic transcript containing miR-17, -18, -19, -20, and -92, was first found to be a target of c-myc, a potential oncogene in B-cell lymphoma (He *et al.*, 2005). The miR-17 to -92 cluster is also overexpressed in lung cancers where it has been shown to enhance cell proliferation (Hayashita *et al.*, 2005). miRNAs also mediate tumor metastasis, for example, miR-10b was shown to initiate tumor invasion and metastasis in breast cancer (Ma *et al.*, 2007).

One very recent paper compared miRNA expression patterns between primary and metastatic NB tumors and found significant changes of 54 miRNAs in metastatic samples, among which 35 miRNAs were upregulated and 19 miRNAs were downregulated (Guo *et al.*, 2010). Some miRNAs, such as miR-10b, miR-29a/b, miR-335, which are known to promote metastasis were upregulated in metastatic NB tumors. In contrast, miR-7, miR-338-3p, and the let-7 family were the three of the top 10 downregulated miRNAs in the metastatic group. These miRNAs have been shown to play antimetastatic roles in other tumors (Zhang *et al.*, 2010). The authors also analyzed the predicted miRNA targets of these miRNAs and found that many of these targets are related to metastasis. For instance, both caspase-8 and integrin beta1 are the predicted targets of miR-29a and miR-29b, miRNAs

that promote metastasis in breast cancer (Gebeshuber *et al.*, 2009). However, the actual function of these identified miRNAs in metastasis needs to be investigated.

8. Other Important Genes in NB

8.1. Telomerase

Telomerase is a specialized ribonucleoprotein polymerase that synthesizes the TTAGGG telomeric repeats found at the end of chromosomes to maintain the length of the telomere. This enzyme is expressed in germ line cells but not in the majority of somatic cells. Thus, telomeres in somatic cells undergo progressive shortening and eventually lose the ability to protect chromosome ends, resulting in cell senescence and/or death. Increased telomerase expression, which results in unlimited cell replication and repression of cell senescence, is found in many tumors (Bodnar *et al.*, 1998; Hahn *et al.*, 1999). Telomerase activity was detected in most NBs, but not in ganglioneuromas or normal adrenal tissue (Hiyama *et al.*, 1997). In addition, high telomerase activity usually correlated with MYCN amplification and poor outcome, suggesting telomerase could be a prognostic marker for poor survival (Hiyama *et al.*, 1997; Ohali *et al.*, 2006; Reynolds *et al.*, 1997).

8.2. MDR1 and MRP gene family

Most NBs exhibit a strong initial response to chemotherapy followed by the appearance of multidrug resistance (MDR) in more than half of cases with remission/remaining tumor (Keshelava *et al.*, 2001; Tweddle *et al.*, 2003; Xue *et al.*, 2007). Although p53 seems to have an important role, the MDR1 gene (multidrug resistance gene 1) and the MRP (multidrug transporter MDR-associated protein) gene family also contribute to this resistance. MDR1 expression was found to be increased after treatment of NB tumors (Bourhis *et al.*, 1989; Chan *et al.*, 1991). In one report, MDR1 expression was inversely correlated with MYCN amplification and poor outcome (Chan *et al.*, 1991), while other groups did not find this correlation (Dhooge *et al.*, 1997; Kutlik *et al.*, 2002). Likewise, some studies found that expression of MRP gene family members such as MRP1, MRP4, strongly correlated with MYCN expression and chemoresistance in NBs (Haber *et al.*, 2006; Norris *et al.*, 1996, 2005), while other studies failed to find a correlation (De Cremoux *et al.*, 2007; Goto *et al.*, 2000).

8.3. GD2 and Bmi-1

Several other genes are also abnormally expressed in NB tumors such as GD2 and Bmi-1. The ganglioside GD2 is a glycolipid that is most commonly expressed in the majority of NB tumors, thus representing a good diagnostic marker and therapeutic target. Indeed recent studies using an anti-GD2 antibodies in association with GMC-SF and IL2 and RA results in a significant increase in event free survival (2-year estimates of 66% \pm 5% vs. 46% \pm 5%), and preliminary data suggest in overall survival (86% \pm 4% vs. 75% \pm 5% at 2 years $p = 0.0223$) (Yu *et al.*, 2009). Bmi-1, a polycomb ring finger gene, was found to be strongly expressed in primary NB tumors in 2006 and very recent data indicated that it is a MYCN target gene that promotes tumorigenesis through inhibition of two potential TSGs (KIF1B β and TSCL1) in NB cells (Nowak *et al.*, 2006; Ochiai *et al.*, 2010).

9. Clinical Treatment Overview

Current treatment for NB consists of surgery, chemotherapy, radiation, and biotherapy. The clinical strategy usually depends on a patient's risk stratification (Table 4.1). For examples, exposure to chemotherapy is generally limited for low risk group patients, whereas, high-risk group patients are treated with multiagent chemotherapy to reduce the overall burden of

the disease before the surgical removal of the primary tumor (Haase *et al.*, 1999; Park *et al.*, 2008).

9.1. Low risk group and intermediate risk group

Low risk group encompasses diseases at stages 1, 2, and 4S with favorable characteristics (Brodeur, 2003; Castleberry *et al.*, 1997; Matthay, 1995). Most low risk group patients at stages 1 or 2 are successfully treated with surgery alone and complete resection of the tumor is the goal. The chances of these tumors recurring or progressing to advanced-stage NB are very low and chemotherapy is reserved for those patients with recurrences. NBs at stage 4S without MYCN amplification almost always spontaneously regress and these patients can be safely observed without any treatment. The survival rate of patients of this group is greater than 95% (Alvarado *et al.*, 2000; Park *et al.*, 2008; Simon *et al.*, 2004).

Intermediate risk group patients include stage 3 patients of any age with favorable features, stage 4 infants with favorable features, and 4S patients without MYCN amplification and with unfavorable histology (Brodeur, 2003; Castleberry *et al.*, 1997; Matthay, 1995). For intermediate risk group patients, surgery, and moderate dose of multiagent chemotherapy are the basic therapeutic strategies. Sometimes radiation is also used to remove the residual tumors. Aggressive chemotherapy is utilized for patients who respond poorly to initial treatment or experience recurrence. Current treatments for intermediate risk group patients have a cure rate about 70–90% (Matthay *et al.*, 1998; Park *et al.*, 2008; Schmidt *et al.*, 2000).

9.2. High-risk group patients

High-risk group consist of patients with unfavorable biological features at stages 2, 3, 4, and those with MYCN amplification at stage 4S (Brodeur, 2003; Castleberry *et al.*, 1997; Matthay, 1995). The standard clinical strategy for this group is comprised of 4 steps: initial induction chemotherapy, local control, consolidation, and biology therapy (Haase *et al.*, 1999; Park *et al.*, 2008). Initial induction chemotherapy consists of combinations of chemotherapeutic agents such as cisplatin, etoposide, doxorubicin, cyclophosphamide, topotecan/ironotecan, and vincristine. Following the completion of induction therapy, local control is achieved by aggressive surgical resection of the primary tumor followed by external beam radiation. After that, consolidation is provided by high-dose chemotherapy and autologous hematopoietic stem cell rescue, using stem cells that are prepared during the induction therapy. The purpose of consolidation therapy is to eliminate any remaining tumors. Consolidation therapy typically employs agents such as carboplatin, etoposide, and melphalan. Sometime focal radiotherapy is also applied to the primary tumor sites. After recovery from the consolidation, patients receive biological therapy to eradicate minimal residual disease because relapse is a frequent occurrence after autologous transplantation in this group of patients. The most common used biological agent is *cis*-retinoic acid (*cis*-RA), which is a noncytotoxic differentiation inducer. The use of monoclonal antibodies against tumor-specific antigens, such as GD2, provides an alternative and promising therapy to eliminate minimal residual tumor cells as described above (Matthay *et al.*, 1999; Pearson *et al.*, 2008; Zage *et al.*, 2008).

Even with these complicated and aggressive treatments, the overall cure rate for high-risk patients is only about 30% during the last two decades (Matthay *et al.*, 1999; Pearson *et al.*, 2008; Zage *et al.*, 2008). Therefore, the development of new agents and methods to more effectively treat these patients is underway. These therapeutic approaches include immunotherapy aimed at NB-specific antigens, targeted delivery of radioactive molecules to NB, or new chemotherapy such as retinoids for inducing differentiation, tyrosine kinase inhibitors targeted at ALK, demethylating agents and HDAC inhibitors for inducing

reexpression of caspase-8 or other epigenetically silenced genes (Kelleher and McDermott, 2010; Schor, 2009; Wagner and Danks, 2009; Witt *et al.*, 2009).

10. Conclusion

The investigation and identification of genomic abnormalities and gene expression changes has improved the understanding of the molecular basis of biological and clinical characteristics of NBs (summary in Tables 4.2 and 4.3). Although great progress has been made in recent 20 years, much work needs to be done to identify tumor-specific targets for therapy. Also, deeper understanding of the development of normal sympathetic nervous system will help us find the important abnormal events that initiate NB development. In conclusion, more precise identification of molecular alterations should allow more effective and less toxic therapies with improved cure rate.

Acknowledgments

We apologize to all of our colleagues whose work was either not cited or was cited in a review article. We thank the members of the Lahti lab, especially Judith Hyle, for their input. This work was funded by NIH grants R01 CA067938 to JML, Comprehensive Cancer Center Support Grant P01 CA021765 and the American Syrian Lebanese Associated Charities ALSAC.

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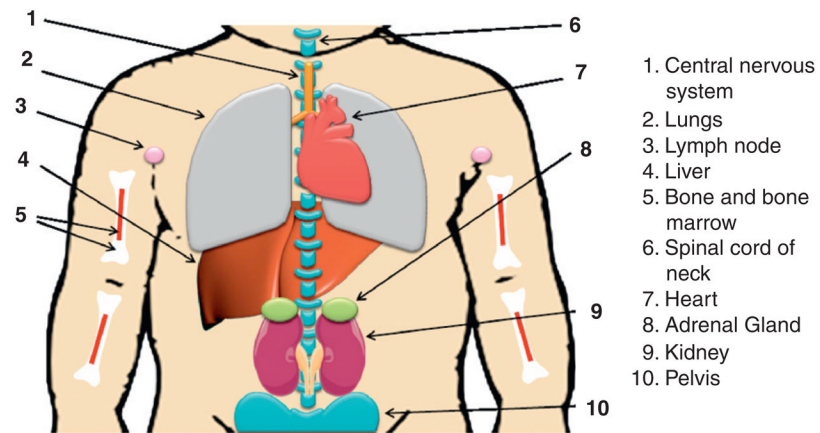


Figure 4. 1.

Neuroblastoma localization. Neuroblastoma primary tumors derive from precursor cells of the peripheral (sympathetic) nervous system and can arise anywhere along the sympathetic chain, most frequently in the adrenal gland (position 8 as shown). Neuroblastoma may also develop from spinal cord of neck (position 6) and pelvis (position 10). Neuroblastomas mainly metastasize to lymph nodes (position 3), liver (position 4), bone and bone marrow (position 5), and also spread to central nervous system (position 1) and lungs (position 2) in infants.

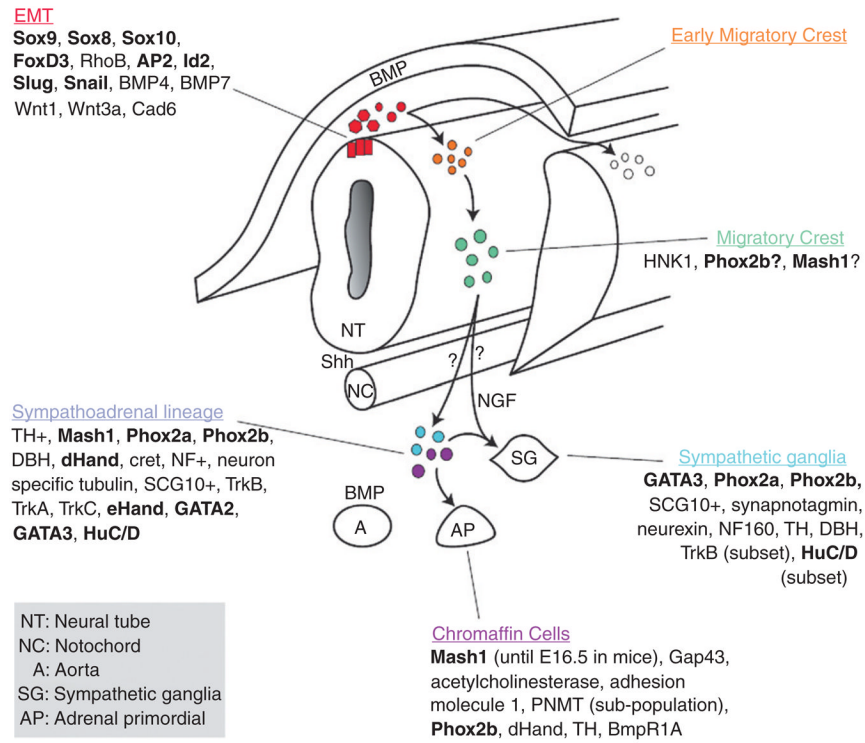


Figure 4. 2. General schema of the development of chromaffin cells and sympathetic ganglia. Cells at the dorsal region of the neural tube undergo EMT (red population), delaminate from the neural tube (orange), and migrate ventrally to the aorta (green) where they are commonly referred to as the sympathoadrenal progenitors (blue and purple). From the aortic region, the cells then migrate to the developing adrenal gland (AP) to become chromaffin cells or differentiate to become sympathetic ganglia (SG). As cells begin to differentiate as sympathetic ganglia they upregulate neural markers while chromaffin cells upregulate proteins found in the adrenal gland. Recent studies suggest that the chromaffin cell and sympathetic ganglia may come from divergent lineages rather than a common sympathoadrenal lineage. The question marks between the migrating crest and the sympathoadrenal progenitors address this possibility. A more detailed discussion on the temporal expression of the transcription factors in the sympathoadrenal lineage (including developmental stages) can be found in (Howard *et al.*, 2000) and details about the distinct chromaffin and sympathetic lineages can be found in (Ernsberger *et al.*, 2005). Additional of neurotrophins and their receptors can be found in (Straub *et al.*, 2007). Transcription Factors are shown in bold and factors implicated in neuroblastoma have been underlined. Abbreviations: NT, Neural Tube; NC, notochord; A, Aorta; SG, sympathetic ganglia; AP, adrenal primordial.

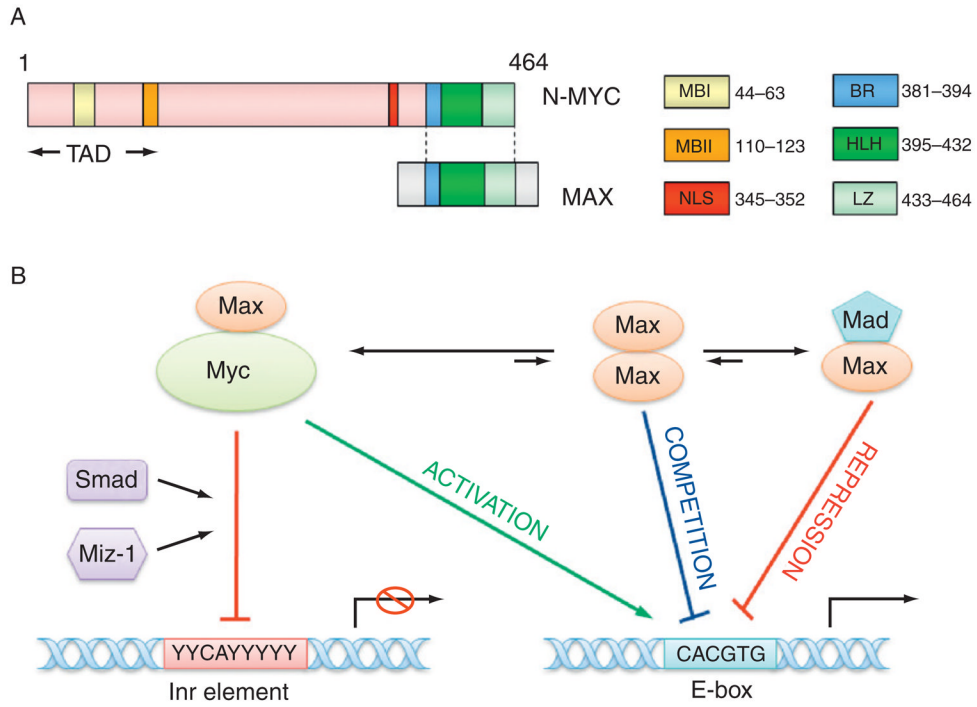


Figure 4. 3. The structure of N-myc protein and transcription regulation by N-myc. (A) The structure of N-myc protein. The N-terminal transactivation domain (TAD) contains two conserved Myc box I and II (MBI and MBII), which are essential for DNA binding. The C-terminal domain (CTD) harbors basic region (BR), helix-loop-helix (HLH) motif, and leucine zipper (LZ) for dimerization with Max. There is a nuclear localization signal (NLS) before CTD. (B) The model for the transcription regulation by N-myc. Myc–Max heterodimer may bind to E-box element (CACGTG) to activate transcription, however, Myc–Max dimer can associate with other transcription factors such as Miz-1, Smad, and bind to Inr (initiator, weak consensus) element to repress transcription. Max can also form homodimers or heterodimers with Mad to compete or suppress Myc–Max binding to E-box.

Table 4. 1

Neuroblastoma risk stratification based on age, MYCN status and histology

INSS stage	Age	MYCN status	Histology	Risk group	3-year survival rate
1	0-21 years	Any	Any	Low	>90%
2	0-21 years	Non-Amp	Any	Low	70-90% in this stage
	1-21 years	Amp	Favorable	Low	
	1-21 years	Amp	Unfavorable	High	
3	<1 year	Non-Amp	Any	Intermediate	30-50% in this stage
	1-21 years	Non-Amp	Favorable	Intermediate	
	0-21 years	Amp	Any	High	
	0-21 years	Non-Amp	Unfavorable	High	
4	<1 year	Non-Amp	Any	Intermediate	<30% in this stage
	0-21 years	Amp	Any	High	
4S	<1 year	Non-Amp	Favorable	Low	50-80% in this stage
	<1 year	Non-Amp	Unfavorable	Intermediate	
	<1 year	Amp	Any	High	

INSS, International Neuroblastoma Staging System; Amp, amplified; Non-Amp, not amplified.

Table 4. 2

Frequent chromosomal region abnormalities in neuroblastoma

Chromosomal region	Status	Frequency	Relation with MYCN amplification	Involved genes	Clinical group
1p36	Loss	25–35%	Correlation	<i>CHD5, miR-34a, KIF1Bβ</i>	Unfavorable
11q23	Loss	35–45%	Inversed correlation	<i>TSLC1/IGSF4</i>	Unfavorable
14q23	Loss	16–27%	Inversed correlation		All groups
17q22	Gain	~50%	Correlation	<i>Survivin, NIM23A</i>	Unfavorable

Table 4. 3

Summary of genetic changes in neuroblastoma

Gene symbol	Gene name	Chromosomal locus	Gene function	Gene alterations	References
<i>ALK</i>	Anaplastic lymphoma receptor tyrosine kinase	2p23	Receptor tyrosine kinase	Mutation/amplification	Caren <i>et al.</i> (2008), Chen <i>et al.</i> (2008), George <i>et al.</i> (2008), Janoueix-Lerosey <i>et al.</i> (2008), Mosse <i>et al.</i> (2008), Passoni <i>et al.</i> (2009)
<i>Bcl2</i>	B-cell CLL/lymphoma 2	18q21.3	Apoptosis suppression	High expression	Abel <i>et al.</i> (2005), Ramani and Lu (1994), Castle <i>et al.</i> (1993), Ikegaki <i>et al.</i> (1995), Mejia <i>et al.</i> (1998)
<i>Bmi1</i>	BMI1 polycomb ring finger oncogene	10p11.23	Oncogene	Overexpression	Ochiai <i>et al.</i> (2010), Nowak <i>et al.</i> (2006)
<i>Casp8</i>	Caspase 8	2q33-q34	Apoptosis/metastasis	Silence/deletion	Teitz <i>et al.</i> (2000, 2006), Yang <i>et al.</i> (2007), Fulda <i>et al.</i> (2001), Fulda and Debatin (2006), Lahiri <i>et al.</i> (2006), Stupack <i>et al.</i> (2006)
<i>CD44</i>	CD44 molecule	11p13	Integrin/antimetastasis	Expression in favorable tumors	Munchar <i>et al.</i> (2003), Combaret <i>et al.</i> (1996)
<i>CHD5</i>	Chromodomain helicase DNA-binding protein 5	1p36.31	Helicase/tumor suppressor	Deletion/low expression	Bagchi <i>et al.</i> (2007)
<i>DDX1</i>	ATP-dependent RNA helicase DDX1	2p24	RNA helicase/Oncogene	Amplification	George <i>et al.</i> (1996)
<i>B4GALNT1</i>	GM2/GD2 synthase	12q13.3	Ganglioside	High expression	Yu <i>et al.</i> (2009)
<i>KIF1b</i>	Kinesin family member 1beta	1p36.2	Kinesin/tumor suppressor	Deletion/low expression	Munirajan <i>et al.</i> (2008), Schlisio <i>et al.</i> (2008)
<i>MDM2</i>	Mdm2 p53-binding protein homolog	12q14.3-q15	Oncogene	Amplification	Corvi <i>et al.</i> (1995a)
<i>ABCB1</i>	Multidrug resistance protein 1 (MRP1)	7q21.12	Multidrug resistance	High expression	Bourhis <i>et al.</i> (1989), Chan <i>et al.</i> (1991), Dhooze <i>et al.</i> (1997), Kutlik <i>et al.</i> (2002)
<i>Mir34a</i>	MicroRNA 34a	1p36.22	Micro RNA/tumor suppressor	Deletion/low expression	Weich <i>et al.</i> (2007), Chen and Stallings (2007)
<i>Mir17-92</i>	MicroRNA cluster 17-92 (mir17, 18, 19, 20, 92)	13q31.3	Micro RNA/Oncogene	High expression	Schulte <i>et al.</i> (2008)
<i>MMP2, MMP</i>	Matrix metalloproteinase 2	16q13-q21	Proteinase/metastasis	High expression	Sugiura <i>et al.</i> (1998), Ribatti <i>et al.</i> (2004)
<i>MMP9</i>	Matrix metalloproteinase 9	20q11.2-q13.1	Proteinase/metastasis	High expression	(Sugiura <i>et al.</i> (1998), Ribatti <i>et al.</i> (2004))
<i>ABCC1</i>	Multidrug resistance-associated protein 1	16p13.1	Multidrug resistance	High expression	Norris <i>et al.</i> (1996, 2005), Haber <i>et al.</i> (2006), Goto <i>et al.</i> (2000), De Cremoux <i>et al.</i> (2007)
<i>MYCL</i>	Myc-related gene from lung cancer	1p34.2	Oncogene	Amplification	Jimbo <i>et al.</i> (1989)
<i>MYCN</i>	Neuroblastoma MYC oncogene	2p24.1	Oncogene	Amplification	Schwab <i>et al.</i> (1983, 1984), Kohl <i>et al.</i> (1983), Pelengaris <i>et al.</i> (2002), Reiter and Brodeur (1996, 1998)

Gene symbol	Gene name	Chromosomal locus	Gene function	Gene alterations	References
<i>NME1</i>	Metastasis inhibition factor NM23	17q22	Nucleoside kinase/antimetastasis	Overexpression	Godfried <i>et al.</i> (2002), Almgren <i>et al.</i> (2004), Chang <i>et al.</i> (1996)
<i>PHOX2B</i>	Paired-like homeobox 2b	4p13	Neuron development	Mutation	Trochet <i>et al.</i> (2004), Mosse <i>et al.</i> (2004)
<i>TERT</i>	Telomerase reverse transcriptase	5p15.33	Telomere maintenance	High expression	Hiyama <i>et al.</i> (1997), Reynolds <i>et al.</i> (1997), Ohali <i>et al.</i> (2006)
<i>NTRK1</i>	Neurotrophic tyrosine kinase receptor 1 (TrkA)	1q21-q22	Receptor tyrosine kinase	Inverse correlation with Myc	Nakagawara <i>et al.</i> (1992), Nakagawara (1993), Tacconelli <i>et al.</i> (2004), Kim <i>et al.</i> (1999)
<i>NTRK2</i>	Neurotrophic tyrosine kinase receptor 2 (TrkB)	9q22.1	Receptor tyrosine kinase	Strong correlation with Myc	Nakagawara <i>et al.</i> (1994), Nakagawara (1994), Douma <i>et al.</i> (2004), Ho <i>et al.</i> (2002)
<i>NTRK3</i>	Neurotrophic tyrosine kinase receptor 3 (TrkC)	15q25	Receptor tyrosine kinase	Coexpression with TrkA	Yamashiro <i>et al.</i> (1996), Svensson <i>et al.</i> (1997)
<i>CADMI</i>	Tumor suppressor in lung cancer 1 (TSLC1)	11q23.2	Cell adhesion/Tumor suppressor	Deletion/low expression	Gomyo <i>et al.</i> (1999), Ando <i>et al.</i> (2008)
<i>TWIST1</i>	Twist homolog 1	7p21.2	Apoptosis/metastasis	High expression	Valsesia-Wittmann <i>et al.</i> (2004)