TUBEDOWN IN NEUROBLASTIC TUMORS

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Tubedown in Neuroblastic Tumors

by

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Abstract

Tubedown (Tbdn), a developmentally regulated gene, is a subunit of the N-terminal acetyltransferase Arrest Defective-1 (Ard1) complex that is conserved from yeast to human. The role of the Tbdn gene product was examined in the development and progression of pediatric neuroblastic tumors (ganglioneuroma, ganglioneuroblastoma, and neuroblastoma) that result from a blockage during normal sympathetic development.

Currently, very few markers have been proven useful to distinguish the various states of neuroblastic differentiation. Therefore, the use of a neuroblastic tissue microarray was essential in determining the importance of Tbdn expression as a novel biomarker. High levels of Tbdn expression correlated with advanced tumor stages (stage 3 and 4), high-risk group status, unfavorable histology, and poor outcome.

In addition, the functional relationship between the NatA complex (Tbdn and Ard1) and the MycN gene product was investigated. *MYCN* gene amplification strongly correlates with advanced tumor stage and treatment failure. Although, *MYCN* gene amplification usually results in high MycN mRNA and protein expression there are subsets of neuroblastomas that have high MycN expression without *MYCN* gene amplification. I have demonstrated a co-regulation between Tbdn, Ard1, and MycN, using an *in vitro* human neuroblastoma LA-N-5 cell model. Subsequently, a conditional *in vitro* MycN-inducible system demonstrated an over-expression of MycN that resulted in increased

expression of both Tbdn and Ard1. These results suggested that MycN may regulate the NatA complex. Promoter region analysis of *TBDN* and *ARD1* revealed a MycN consensus binding site within the *TBDN* promoter region, indicating a possible direct target for MycN. However, no MycN consensus sequence was detected within the *ARD1* promoter. The binding of MycN to *TBDN* was confirmed by chromatin immunoprecipitation potentially providing a mechanistic role for poor outcome in neuroblastoma. Finding functional links between MycN and *TBDN* will provide further validation for Tbdn as a novel biomarker that may possibly be used in diagnosis and/or prognosis.

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Five screws + One exercise ball + One laptop (minus 8 broken keys and 3 dismantles) + 14 binders of data = one priceless journey

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Abbreviations

ALK = Anaplastic lymphoma kinase

ATRA = All-trans retinoic acid

BDNF = Brain-derived neurotrophic factor

bHLH = Basic helix-loop-helix

BMP = Bone morphogenetic protein

ChIP = Chromatin Immunoprecipitation

CRABP-II = Cellular retinoic acid binding protein II

CT = Computed tomography

DM = Double-minutes

GFP = Green Fluorescent Protein

HASH-1 = Human achaete-scute homologue-1

HSR = Homogeneously staining regions

INPC = International Neuroblastoma Pathology Classification

INSS = International Neuroblastoma Staging System

IRES = Internal Ribosome Entry Site

NMDA = N-methyl-D-aspartate

mARD1 = Mouse arrest defective 1 protein

MASH-1 = Mammalian achaete-scute homologue-1

MAX = Myc-associated factor x

MCM 2 - 7 = Mini-chromosomal maintenance proteins 2 – 7

MIBG = Meta-iodo-benzyl guanidine

MKI = Mitosis-karyorrhexis index

mNAT1 = Mouse *N*-acetyltransferase 1 protein

MRI = Magnetic resonance imaging

MRP1 = Multi-drug resistance-associated protein

MYCC (c-myc) = cellular myc oncogene

MYCL (L-myc) = Lung carcinoma derived myc oncogene

MYCN (N-myc) = Neuroblastoma derived myc oncogene

MYCV (v-myc) = v-myc myelocytomatosis viral related oncogene (avian)

NAG = Neuroblastoma-amplified gene

NB = Neuroblastoma

NGF = Nerve growth factor

NT-3 = Neurotrophin-3

NT-4/5 = Neurotrophin-4/5

NTs = Neuroblastic tumors

SBRCT - Small blue round cell tumors

Tbdn - Tubedown

Tet2 (SHEPTet2) - Empty vector

Tet2N (SHEPTet2/Nmyc)- MYCN-inducible SHEP cell line

UTR - untranslated region

WNT - Wq (wingless) + INT

1.1 Cancer

Cancer is a multi-stage process arising from many genetic alterations (Hanahan and Weinberg, 2000). Cancer is the result of cumulative mutations, affecting two types of genes, oncogenes (stimulate growth), and tumor suppressor genes (inhibit growth). The activation of oncogenes or the deactivation of tumor suppressor genes will result in tumorigenesis, or the formation of cancer (Gibbs, 2003). The transformation of a normal cell into a malignant cell usually results in sporadic or induced cancers (Hanahan and Weinberg, 2000). Many factors contribute to tumorigenesis which include physical carcinogens (UV radiation, ionizing radiation), chemical carcinogens (nickel, cadmium, arsenic) or biological carcinogens (Human papilloma virus B, *Helicobacter pylori*) (Schulz, 2007).

The majority of adult cancers result from an accumulation of mutations over many years (Pahlman et al., 2004). In contrast, pediatric cancers appear earlier. Therefore, it is dubious that pediatric malignancies occur from just an accumulation of mutations. Pediatric malignancies occur from developmental abnormalities during the normal differentiation process (Figure 1-1; Maris and Denny, 2002; Pahlman et al., 2004). These developmental abnormalities are the result of both sporadic and familial cancers. In particular, many pediatric cancers are thought to involve Knudson's two-mutation hypothesis, which states the first mutation is inherited while the second is acquired

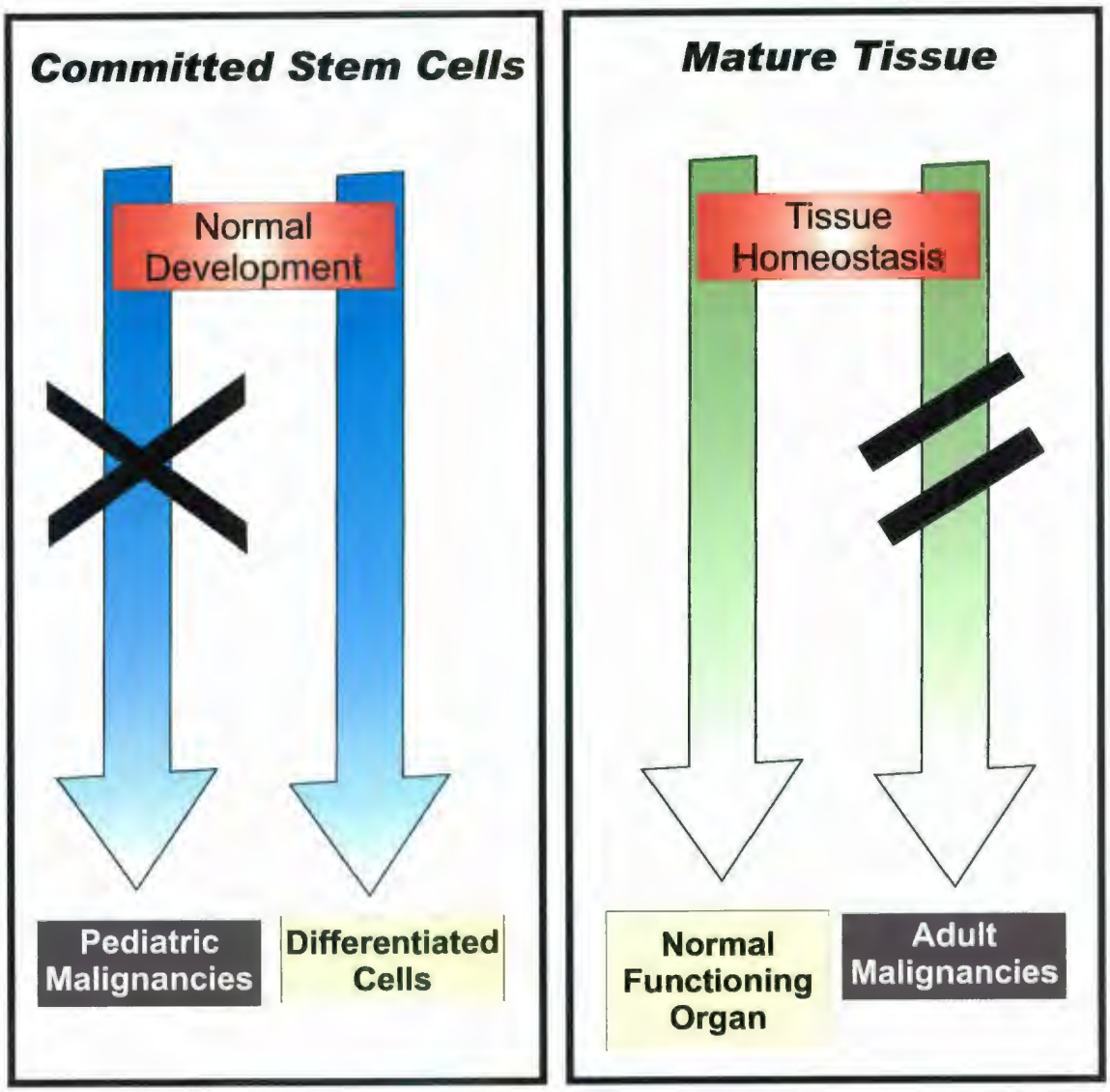


Figure 1-1. Mechanisms leading to pediatric and adult malignancies.

In children, committed stem cells undergo normal development, resulting in terminally differentiated cells. However, during normal growth and differentiation, a developmental blockage (represented by 'X') occurs resulting in the formation of pediatric malignancies. Conversely, in adults, mature tissues maintains normal homeostasis resulting in normal organ function. However, during normal homeostasis, cumulative mutations due to genetic mutations and/or environmental exposures (denoted by a double line) will result in adult malignancies.

somatically (Knudson, 1971; Knudson and Strong, 1972). Although there is an obvious difference between adult and pediatric cancers, they still share many similar underlying properties. Six key hallmark features result in malignant growth. They include self-sufficiency in growth capabilities, unresponsive to antigrowth signals, circumvention of apoptotic signals, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000; Abbott et al., 2006).

1.2 Neuroblastic Tumors

Neuroblastic tumors (NTs) are the most common extra-cranial tumors occurring in children (Mora and Gerald, 2004). NTs are composed of a combination of components including undifferentiated neuroblasts, neuropil, differentiating neuroblasts, ganglion cells, neuritic processes, Schwann cells, and fibrous tissue (Joshi, 2000). NTs are far from a uniform disease as they have varying levels of immature and mature cells. These immature neural crest cells are referred to as neuroblasts whereas the mature differentiated forms are referred to as ganglion and Schwann cells (Lonergan et al., 2002). NTs were classified into four groups (ganglioneuroma; ganglioneuroblastoma, intermixed; ganglioneuroblastoma, nodular; neuroblastoma), which vary according to their range of differentiation (Shimada et al., 1999a). Ganglioneuromas are (Schwannian stroma dominant) benign tumors composed of mature ganglion and Schwann cells. The mature ganglion cells are embedded in the stroma which contain the Schwann cell sheaths encompassing neural elements (Shimada et al., 1984). Ganglioneuroblastoma – intermixed (Schwannian stroma-rich) are considered benign tumors composed mainly of ganglion cells with the presence of microscopic neuroblast cells which are present at different stages of differentiation. Conversely, ganglioneuroblastoma – nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor) contains more aggressive and malignant composite of neuroblast clones. Neuroblastomas are (Schwannian stroma-poor) tumors consist mainly of immature, undifferentiated cells (Shimada et al., 1999a).

1.3 Neuroblastoma

In 1864, Rudolf Virchow originally described neuroblastoma as a glioma (Joyner and Lopushnyan, 2007). However, it was not until 1910 that James Homer Wright officially introduced the term 'neuroblastoma'. Wright demonstrated that neuroblastoma originated from embryonic neuroblasts or neural crest cells (Joyner and Lopushnyan, 2007). Neuroblastoma is a disease of the trunk region of the neural crest which gives rise to the sympatheticoadrenal linkage of neural crest cells (Dyer, 2004; Maris et al., 2007). Due to the high migratory nature of the neural crest cells they can arise anywhere along the sympathetic nervous system during embryogenesis (Figure 1-2; Maris, 2005; Howman-Giles et al., 2007; Bowen and Chung, 2009). The sympathetic nervous system is responsible for involuntary actions of the body, such as increasing heart rate, decreasing urine secretion, and dilating the pupils of the eye (Sherwood, 1997). During normal development, neural crest stem cells migrate to many areas within the embryo. The Trunk region of the neural crest stem cells give rise to sympathetic tissues which include sympathetic neurons, sympathetic ganglia, Schwann cells and adrenomedullary cells (Dyer, 2004; Mora and Gerald, 2004). However, a developmental blockage of the neural crest stem cells will disturb the normal proliferation and migration of these cells, resulting in pediatric embryonal malignancies.

Although greater than fifty percent of neuroblastomas occur in the adrenal medulla, other primary sites of neuroblastoma formation include the chest, neck and pelvis (Maris et al., 2007). Prior to a neuroblastoma diagnosis, fifty percent of the cases will present

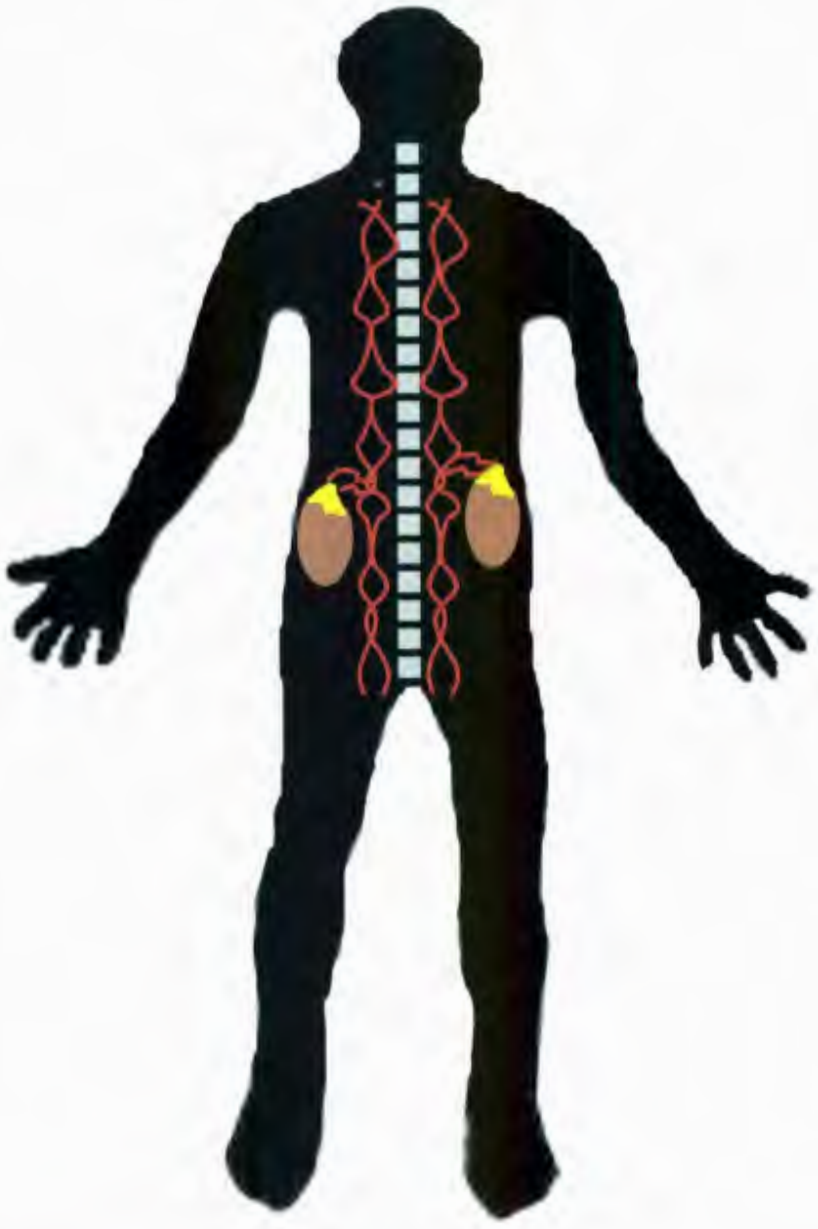


Figure 1-2. Potential neuroblastic sites.

Neuroblastic tumors can arise anywhere along the sympathetic nervous system. The chains of sympathetic ganglia (red) run along the spinal chord (light gray) and the occurrence of NTs can invade the spine including the spinal nerves. The adrenal glands (yellow) site on top of each kidney (brown) and are the source of catecholamines, epinephrine and norepinephrine.

metastasis to areas such as the bone, bone marrow, liver and/or lungs (Castel et al., 2007; Howman-Giles et al., 2007; Ishola and Chung, 2007). Neuroblastoma is one of the most complex yet intriguing diseases for scientists and clinicians. Half of the patients diagnosed with neuroblastoma, die from the disease (Pahlman et al., 2004). Paradoxically, neuroblastoma also has one of the highest rates of spontaneous regression (Pritchard and Hickman, 1994; Castel et al., 2007).

1.3.1 Spontaneous Regression & Apoptosis

In the mid 1980s, a mass screening detection program was initiated in Japan to improve the discovery of neuroblastoma (Sawada et al., 1984). Japan began screening six-month old infants for catecholamine metabolites (produced by neuroblastoma) found in the urine. Subsequently, similar efforts were taken by Canada and Germany to determine the practicality and effectiveness of these screening programs (Schilling et al., 1994; Woods et al., 1996). The screening program in Japan was terminated in 2004 after studies from Canada and Germany revealed that there was no reduction in the mortality rate (Tsubono and Hisamichi, 2004). In fact, there was an increase in the incidence of neuroblastoma since the induction of the mass screening program. The program proved unsuccessful due to an over-detection of the biologically favorable type of neuroblastomas, ultimately leading to unnecessary treatments. The treatments were deemed unnecessary, as this class of tumors would have likely undergone spontaneous regression before any clinical signs were present (Brodeur, 2003; van Noesel and Versteeg, 2004). In addition to adding a greater understanding about spontaneous regression, other important observations came from the screening program. The idea that neuroblastoma may not necessarily be derived from a common precursor and in fact may arise as separate entities (favorable and unfavorable). There is now a greater awareness that spontaneous regression occurs in neuroblastomas, and at a higher rate than any other human tumor. However, the reason for such a high rate of spontaneous regression is still unclear.

Neuroblastoma has the highest rate of spontaneous regression (10 to 100 times) of any cancer (D'Angio et al., 1971; Pritchard and Hickman, 1994; Castel et al., 2007). Two hypotheses have been suggested for this occurrence, including immunological attack on the tumor (Hellstrom et al., 1968) and spontaneous maturation (Rangecroft et al., 1978). Hellstrom and colleagues demonstrated that there was an inhibition of neuroblastoma cell growth *in vitro* using lymphocytes from children with neuroblastoma. In addition, using lymphocytes from a mother who had given birth to a child with neuroblastoma also inhibited neuroblastoma growth *in vitro* (Hellstrom et al., 1968). The problem with the first theory is the lack of immunological evidence for immune surveillance (or the lack of constant monitoring by the immune system) against the development of neuroblastomas (Pritchard and Hickman, 1994; Ochsenbein et al., 1999). In addition, neuroblastomas are not highly immunogenic, meaning that they express low levels of surface antigens that would be required for proper immune surveillance (Brodeur, 2003). Other reasons that spontaneous regression is not supported by the immunological theories is that children do not develop neuroblastoma from inherited immune deficiencies or after receiving immunosuppressant therapy (Pritchard and Hickman, 1994).

The second theory regarding spontaneous maturation also seemed implausible. Rangecroft et al. reported a case in which a stage 4S neuroblastoma spontaneously matured with minimal treatment (Rangecroft et al., 1978). Usually during spontaneous regression of a stage 4S neuroblastoma, the tumor disappears completely (the liver and

bone marrow biopsy specimens are tumor-free). In addition, there should be no traces of a treated tumor ever existing. In other words, no ganglion (or differentiated) cells would be left behind. However, in this case spontaneous maturation to ganglioneuroma had occurred leaving residual mature ganglion cells (Pritchard and Hickman, 1994). Pritchard and Hickman suggested that the most likely explanation for the spontaneous regression observed in neuroblastoma is a delay in the time-switch for apoptosis during normal development (Pritchard and Hickman, 1994) and after a period, the apoptotic pathway becomes activated. Massive cell death of sympathetic neurons occur during the perinatal period (Oppenheim, 1991; Nakagawara, 2005). The same controlled cell death mechanism appears to occur in neuroblastoma, resulting in the regression of the tumor. Oue and colleagues used *in situ* detection of DNA fragmentation and Bcl-2 expression to demonstrate a relationship between apoptosis and spontaneous regression in neuroblastoma (Oue et al., 1996). High Bcl-2 expression has been shown to block apoptosis in the advanced stages of neuroblastoma (Oue et al., 1996). Previously, Bcl-2 expression was reported to correlate with poor prognosis, unfavorable histology, and *MYCN* amplification (Castle et al., 1993). They suggested that Bcl-2 might have a role in neuroblastoma progression. However, Bcl-2 levels were decreased in children less than one year of age (Oue et al., 1996). The reports regarding the prognostic significance of Bcl-2 levels in primary tumors are varied. Therefore, the role that Bcl-2 plays in spontaneous regression still remains elusive. Moreover, Bcl-2 may have a more significant role in acquired resistance to chemotherapy (Maris and Matthay, 1999). Over eighty percent of neuroblastoma specimens analyzed post-chemotherapy treatment

expressed Bcl-2 (Castle et al., 1993). Dole et al. demonstrated that over-expression of Bcl-2 in neuroblastoma clones inhibited apoptosis, even in the presence of chemotherapy treatment (Dole et al., 1994). Their results suggested that Bcl-2 promoted tumor resistance to chemotherapy agents.

Caspase-8 is an integral part of the death receptor (extrinsic) apoptotic pathway (van Noesel and Versteeg, 2004). In general, many human cancers, including neuroblastoma, evade apoptosis through inactivation of Caspase-8. The function of Caspase-8 can be impaired due to epigenetic mechanisms or genetic deletions (Fulda, 2009). However, Eggert and colleagues demonstrated through demethylation experiments, the restoration of Caspase-8 expression using neuroblastoma cell lines. Their results suggested that the mechanism leading to the loss of Caspase-8 expression was likely due to DNA methylation as opposed to deletion of *CASPASE-8* (Eggert et al., 2000). In advanced neuroblastomas, the inactivation of *CASPASE-8* occurs via promoter hypermethylation. This represents a defective hallmark for apoptosis, suggesting that Caspase-8 may serve as a tumor suppressor (Teitz et al., 2000; Teitz et al., 2001). Unfortunately, the regulatory region of the *CASPASE-8* promoter does not contain classical CpG islands or even have promoter activity as was displayed using neuroblastoma cell line and primary tumor samples (Fulda, 2009). Nevertheless, reports show that demethylation (using 5-Aza-Cytidine) restores Caspase-8 expression (Eggert et al., 2000; Banelli et al., 2002; Fulda, 2009).

The loss of Caspase-8 expression has been reported to be as low as 25-35%, and as high as 75% in neuroblastomas (van Noesel and Versteeg, 2004; Fulda et al., 2006). In addition, there is much controversy between Caspase-8 expression and *MYCN* amplification. Some studies suggest that the loss of Caspase-8 strongly associate with *MYCN* amplification (Teitz et al., 2000), whereas other reports show no such correlation (Fulda et al., 2006). In addition, loss of Caspase-8 did not associate with the aggressiveness, prognosis or staging of neuroblastoma (Fulda et al., 2006).

Similar to Caspase-8, Survivin is also involved in the apoptotic pathway. However, *SURVIVIN* is an anti-apoptotic gene that is located on the long arm of chromosome 17. The Survivin protein is a member of the inhibitor of apoptosis, and is a negative regulator of Caspase-9 (van Noesel and Versteeg, 2004). Over-expression of Survivin is associated with high-risk neuroblastomas (Adida et al., 1998; Nakagawara, 2005).

As mentioned, neuroblastoma can also undergo spontaneous maturation (Castel and Grau, 2006). In the late 1920s, Cushing and Wolbach described that malignant neuroblastoma, consisting of immature neuroblastic cells, underwent spontaneous maturation into benign ganglioneuroma, consisting of mature ganglion cells (Cushing and Wolbach, 1927; Brodeur, 2003). A limited number of cases have been reported involving the complete maturation and regression of neuroblastoma (Stage IVS) without surgery or chemotherapy (Rangecroft et al., 1978; Haas et al., 1988). Conversely, other studies have reported that even minimal treatment have resulted in spontaneous regression of neuroblastoma (Evans et al., 1976; Howman-Giles et al., 2007).

1.3.2 Epidemiology

Neuroblastoma is one of the most common extra-cranial solid tumors occurring in children. During the first year of life, neuroblastoma accounts for fifty percent of all solid tumors (Castel et al., 2007). Only accidents precede neuroblastoma as the most common cause of death among children (Castel et al., 2007). Ninety percent of patients are diagnosed during the first 5 years of life while the median age for diagnosis is approximately 18 months (Brodeur, 2003; Castel et al., 2007; Howman-Giles et al., 2007). In Canada, there are 65 new cases diagnosed each year compared to 700 new cases reported in the United States. The prevalence of neuroblastoma is about 1 in 7000 live births and it accounts for approximately 15 percent of all childhood cancers (Brodeur, 2003; Maris et al., 2007). A more staggering statistic is that fifty percent of all children diagnosed with neuroblastoma will die from the disease (Pahlman et al., 2004).

Several epidemiologic studies have focused on the many potential risk factors for neuroblastoma. There are theories such as maternal use of illicit or recreational drugs such as marijuana (Bluhm et al., 2006) and maternal use of hair dye (McCall et al., 2005) before or during pregnancy, both increasing the risk for neuroblastoma. Conversely, activities such as maternal vitamin usage (Olshan et al., 2002) and breast-feeding (Daniels et al., 2002) suggested a decrease in the risk for neuroblastoma. However, proving the aforementioned theories will require further investigation.

A retrospective population-based cross-sectional study was completed at the Hospital for Sick Children in Toronto, Canada that examined pre-folic acid fortification (from January 1985 to December 1997) and post-folic acid fortification (from January 1998 to October 2000) food (French et al., 2003). French et al. suggested that the recent decline in neuroblastoma (from 1.57 cases per 10 000 births to 0.67 cases per 10 000 births) was attributed to folic acid fortified food (French et al., 2003). The decline observed in this study may be due to the relatively short follow-up time (25 months) for children conceived after folic acid fortification. In addition, Barone & Bunin estimated that 37% of the children would still get neuroblastoma even after folic acid fortification (Barone and Bunin, 2004). Interestingly, the incident rate comparing children conceived before and after fortification is 40%, very close to the predicative value. Although folic acid supplementation appear to have little effect on neuroblastoma prevention, more studies including longer follow-up times would be required to fully develop this relationship.

1.3.3 Genetic Predisposition

Neuroblastoma is predominantly a sporadic cancer resulting from a somatic cell mutation, which is restricted to the affected individual (Schwab, 1997). Dodge and Brenner first clinically described familial neuroblastoma in 1945 (Maris and Brodeur, 2005). Familial neuroblastoma occurs in one to two percent of neuroblastoma cases and results from a germ line mutation. Germ line mutations are not restricted to the affected individuals but passed to the offspring (Schwab, 1997; Maris and Matthay, 1999; Maris

et al., 2007). Like familial retinoblastomas, familial neuroblastomas is a multi-stage process consistent with Knudson's two-mutation hypothesis (Knudson, 1971; Knudson and Strong, 1972; Schwab, 1997; Maris et al., 2007). One of the hallmarks of familial neuroblastoma is multiple primary tumor sites (Kushner et al., 1986), which again is in accordance with Knudson's two-mutation model (Knudson and Strong, 1972).

Hereditary neuroblastoma is an autosomal dominant disease with reduced penetrance (Brodeur, 2003; Maris et al., 2007). In 2000, a hereditary neuroblastoma predisposition locus (*HNB1*) was mapped to the short arm of chromosome 16, 16p12-13 (Weiss et al., 2000; Maris et al., 2002). However, no causal gene for hereditary neuroblastoma was found to be associated with the 16p12-13 region (Maris et al., 2007). In 2003, Amiel et al. reported neuroblastoma cases containing mutations of the *PHOX2B* gene (Amiel et al., 2003). However, no such evidence for a hereditary linkage to the short arm of chromosome 4 was found (Maris et al., 2002; Maris and Brodeur, 2005). In 2004, Trochet and colleagues reported that mutations to the *PHOX2B* gene had a hereditary predisposition to neuroblastoma (Mosse et al., 2004; Trochet et al., 2004). Recently, Mosse and colleagues identified the anaplastic lymphoma kinase (*ALK*) gene as a key factor in familial neuroblastoma patients. Heritable mutations to the *ALK* gene are found in a subset of neuroblastoma cases (Mosse et al., 2008). In 2000, the presence of ALK expression in neuroblastoma was first described (Lamant et al., 2000). However, ALK expression was not limited to neuroblastoma but was also present in cancers such as breast and rhabdomyosarcoma (Chiarle et al., 2008).

1.3.4 Neural Crest Development

Wnt [Wg (Wingless) and Int] signaling is one of the earliest events in the transition of ectodermal cells into neural crest cells. Bone morphogenetic proteins (BMPs) help trigger and maintain the differentiation process of neural crest cells (Pahlman et al., 2004). Differentiation into sympathetic neurons involves many factors including basic helix-loop-helix (bHLH), homeobox genes, and tumor suppressor genes (Nakagawara, 2005).

In 1991, Lo et al. demonstrated that the bHLH factor mammalian achaete-scute homologue-1 (*MASH-1*; *HASH-1* in humans) had transient expression in neural crest cells (Lo et al., 1991). *MASH-1* null mice were used to prevent the generation of sympathetic neurons (Guillemot et al., 1993). In addition, Mash-1 (or Hash-1) is essential for the proper formation of the sympathetic nervous system (Axelson, 2004). Meanwhile, Hash-1 is present in primary neuroblastomas and neuroblastoma cells indicating the derivation of neuroblastoma from immature neural crest cells of the sympathetic nervous system (Axelson, 2004; Pahlman et al., 2004).

1.3.5 Biological Pathways

Neurotrophins are a family of proteins that bind to a receptor on a nerve cell (Brodeur, 2003). The neurotrophin family consists of four main ligands including nerve growth

factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), each binding to specific receptors. NGF binds preferentially to Trk A, BDNF to TrkB, NT-3 to TrkC and NT-4/5 prefers TrkB but also weakly binds to TrkA (van Noesel and Versteeg, 2004; Nakagawara, 2005). The p75 neurotrophin receptor ($p75^{NTR}$) is a member of the tumor necrosis factor receptor that can bind all four neurotrophins but at a lower affinity than the Trks. The neurotrophin receptors and their corresponding ligands are important regulators for neuronal cell survival, growth, and differentiation (Maris et al., 2007).

High expression of TrkA receptor is found in mature sympathetic ganglia (van Noesel and Versteeg, 2004), as well as in neuroblastoma tumors with favorable prognosis (Nakagawara et al., 1993). Moreover, high expression of TrkA receptor is found in young neuroblastoma patients with a low stage tumor that is *MYCN* non-amplified (van Noesel and Versteeg, 2004). Neuroblastoma cells expressing TrkA can undergo differentiation or apoptosis depending on the microenvironment of the tumors (Brodeur et al., 2009). In the presence of NGF, neuroblastoma cells expressing TrkA will undergo differentiation. However, in the absence of NGF, neuroblastoma cells expressing TrkA will undergo apoptosis (Nakagawara et al., 1993). Conversely, low TrkA receptor expression is found in neuroblastoma tumors associated with *MYCN* amplification and poor outcome (Kogner et al., 1993; Nakagawara et al., 1993; Brodeur, 2003). Similar to TrkA, TrkC is expressed in the lower-stage tumors and is absence in *MYCN*-amplified neuroblastomas (Ryden et al., 1996; Yamashiro et al., 1996; van Noesel and Versteeg,

2004). Also, $p75^{NTR}$ expression is, in general, associated with a favorable outcome for neuroblastoma patients (Brodeur, 2003). Conversely, TrkB expression is associated with unfavorable neuroblastomas (Maris et al., 2007). In particular, the full-length TrkB and corresponding BDNF ligand are associated with *MYCN*-amplified high-risk neuroblastomas (Nakagawara et al., 1994). *MYCN* amplification contributes to the aggressiveness of neuroblastomas (Seeger et al., 1985).

1.3.6 Myc Family

The *v-MYC* oncogene was originally identified as a transforming determinant of avian acute leukemia virus MC29 (Bister et al., 1977; Duesberg et al., 1977; Vita and Henriksson, 2006). *c-MYC* (also referred to as *MYCC* or *MYC*), the cellular homologue of *v-MYC*, was first described in 1982 (Vennstrom et al., 1982). In 1983, the *MYCN* gene was reported to share homology with the *MYC* cellular oncogene (Kohl et al., 1983; Schwab et al., 1983). In 1985, *L-MYC* (*MYCL*) was first reported by Nau and colleagues (Nau et al., 1985). *MYC*, *MYCL*, and *MYCN* are localized to 8q24, 1p32 and 2p23-24, respectively (Schwab, 2004). The *MYCL* and *MYCN* genes are 35% and 38% identical to the amino acid sequence of *MYC* (Ryan and Birnie, 1996).

The *MYC* genes encode transcription factors that are involved in a wide range of processes including but not limited to cell proliferation, differentiation and apoptosis (Vita and Henriksson, 2006). The expression pattern of the transcription factors is

distinct throughout embryogenesis (Nesbit et al., 1999). During development, there is a high expression pattern noted by Myc, MycL, and MycN, which upon maturation their expression is down-regulated (Hatton et al., 1996). Myc is expressed mainly in rapidly proliferating cells whereas MycL is primarily expressed in the developing kidney, lung, brain, and neural tube. MycN is highly expressed in the pre-B cells, kidney, forebrain, hindbrain, and intestine (Nesbit et al., 1999).

Knockout models of the *MYC* family loci helped to further differentiate the functions of the genes (Nesbit et al., 1999). Homozygous null *MYC* mice are embryonic lethal on or after 10.5E with abnormalities including reduced embryo size, dilated pericardia, enlarged hearts, delay or failure in forming the neural tube and in the axial rotation of the embryo (Davis et al., 1993). Homozygous null knockout of *MYCN* survived past 11.5E but reached embryonic lethality at 12.5E. The major challenge with these mice were the developmental defects in tissues expressing *MYCN*. Contradictory to the outcomes for both *MYC* and *MYCN*, Hatton and colleagues reported *MYCL* knockout mice to be viable. The mice lacking *MYCL* did not reveal any phenotype even though the expression of *MYCL* was abundant during the development of the central nervous system (Hatton et al., 1996).

The *MYC* and *MYCN* genes are highly conserved and share many common features. Both genes are involved in oncogenesis and the knockout of either gene results in embryonic lethality. They encode transcription factors that dimerize with Max, a

ubiquitously expressed bHLH family member. Both Myc/Max and MycN/Max can bind the same DNA target sequence, CACGTG (Lu et al., 2003). In addition, an *in vivo* study demonstrated that *MYC* and *MYCN* share similar targets. The endogenous Myc coding sequence was functionally replaced with a MycN coding sequence, which resulted in viable mice (Malynn et al., 2000). Interestingly, the *MYC* promoter was used to drive the synthesis of the *MYCN* transcript instead of the *MYC* transcript. Nevertheless, the homozygous mice for the *MYCN* mutation were able to survive into adulthood and were capable of reproducing (Malynn et al., 2000).

The Myc family of proto-oncogene such as *MYC*, *MYCL* and *MYCN* are widely known to be deregulated in many human cancers (Nesbit et al., 1999). Their deregulation can range from gene amplification to over-expression, giving this family strong oncogenic potential (Mukherjee et al., 1992; Vita and Henriksson, 2006). Deregulation is often associated with aggressive and poorly differentiated tumors, which can assume various forms including gene amplification, over-expression, or translocation (Vita and Henriksson, 2006). Myc over-expression and *MYC* amplification have been linked to solid tumors such as breast and colon whereas hematological malignancies such as Burkitt's lymphoma possess over-expression and translocation (Vita and Henriksson, 2006). *MYCL* amplification is primarily associated with small-cell lung carcinoma whereas ovarian cancers contain both over-expression and gene amplification (Vita and Henriksson, 2006). *MYCN* amplification has been mainly associated with neuroblastoma

(Vasudevan et al., 2005) and is considered to be one of the most consistent genetic aberrations associated with neuroblastoma (Maris et al., 2007).

The *MYCN* gene is located on the short arm of chromosome 2. However, during gene amplification, *MYCN* can be found as double-minutes (DMs) or homogeneously staining regions (HSRs) (Schwab et al., 1984). DMs are extrachromosomal pieces containing multiple copies of the same gene whereas HSRs are uniformly stained regions of a chromosome, which contain multiple copies of the same gene. Amplification of the *MYCN* gene refers to an increase in gene copy-number of 50 - 400 copies per cell (Maris and Matthay, 1999; Schwab, 2004). As previously mentioned, *MYCN* was originally characterized as a *MYC* related gene (Kohl et al., 1983; Schwab et al., 1983). The amino acid sequences that encode exons 2 and 3 are 27 and 39 percent identical, respectively (Stanton et al., 1986).

MYCN gene amplification, found frequently in neuroblastoma, was initially thought to be specific only for neuroblastoma (Schwab, 2004). *MYCN* gene amplification is also present in other neuronal origin tumors such as glioblastoma, small cell lung cancer, peripheral neuroectodermal tumors, and retinoblastoma. However, the incidence rate in the later tumors are much lower than in neuroblastoma (Schwab, 2004; Vita and Henriksson, 2006).

MYCN amplified neuroblastoma tumors usually result in over-expression of the MycN protein (Tang et al., 2006). However, neuroblastoma tumors that lack *MYCN* amplification can still express the *MYCN* oncogene (Nisen et al., 1988; Seeger et al., 1988; Slavc et al., 1990). The controversy arises regarding the clinical significance with respect to MycN expression in *MYCN* non-amplified tumors.

Chan and colleagues demonstrated that MycN expression could be utilized as a predicator for neuroblastoma prognosis (Chan et al., 1997). Immunohistochemistry was performed on 57 non-localized neuroblastoma specimens. Chan and colleagues used two monoclonal antibodies specific for the MycN protein. Seeger and colleagues performed immunohistochemistry on 126 neuroblastoma specimens with a polyclonal MycN antibody. Their results showed that *MYCN* amplified and *MYCN* non-amplified tumors expressed significant amounts of the MycN protein. Their results suggested that the MycN protein has no prognostic significance (Seeger et al., 1988). Therefore, aggressive tumors without *MYCN* amplification but high protein expression may rely on an alternative mechanism to transform neuroblastoma cells. Bordow and colleagues showed that a high level of MycN expression was predictive for poor outcome in older children with neuroblastoma. However, such a prognostic value was not present in infants with neuroblastoma (Bordow et al., 1998). Cohn et al. reported that MycN expression does not have prognostic significance with respect to adverse outcome in patients with advanced-stage *MYCN* non-amplified neuroblastomas (Cohn et al., 2000). Irrespective of *MYCN* amplification status of the neuroblastoma tumor, the probability of death was

unaffected as long as the tumors did not over-express MycN. Patients with *MYCN* non-amplified tumors that over-expressed MycN showed a low survival. Moreover, *MYCN* amplification was not related to clinical outcome in patients that did not over-express MycN (Alaminos et al., 2005).

As mentioned, some authors find a correlation between MycN over-expression and clinical outcome (Bordow et al., 1998; Lasorella et al., 2002; Vandesompele et al., 2003), whereas others fail to find any correlation (Cohn et al., 2000). The consensus with all of the above-mentioned studies (Bordow et al., 1998; Brodeur, 2003) is that in order to rectify the MycN controversy a large prospective study using standardized methods of treatment and detection will be required.

In general, having *MYCN* amplification / MycN over-expression is not favorable for the patient. However, could having *MYCN* amplification / MycN over-expression make neuroblastoma easier to treat? *MYCN* amplification / MycN over-expression may actually serve as a weakness for neuroblastoma during chemotherapy treatments (Ishola and Chung, 2007). MycN over-expressing tumors respond more favorably to chemotherapy treatment than tumors lacking MycN expression. Paffhausen and colleagues used a well-established *MYCN*-inducible cell line model (SHEP/MycN) to demonstrate that upon exposure to anti-tumor agents, *MYCN* induced cells underwent apoptosis (Paffhausen et al., 2007). These results suggest that MycN expression makes neuroblastoma cells more susceptible to chemotherapy treatments.

Brodeur and colleagues demonstrated a correlation between *MYCN* gene amplification and advanced neuroblastoma disease (Brodeur et al., 1984). *MYCN* gene amplification occurs in one third of advanced neuroblastoma cases, and rarely occurs in low stage cases indicating that amplification happens later in the tumorigenesis process (Kohl et al., 1983; Brodeur et al., 1984). *MYCN* gene amplification was reported to be associated with rapid disease and poor outcome (Seeger et al., 1985). Weiss and colleagues performed a hallmark experiment regarding the role of *MYCN* in neuroblastoma development (Weiss et al., 1997). In particular, Weiss et al. demonstrated that targeted over-expressed of *MYCN* in a mouse model can contribute to the transformation of neuroblasts (Weiss et al., 1997). The transgenic mice were under the control of a tyrosine hydroxylase promoter that targeted neuronal tissue. The targeted expression of *MYCN* ultimately led to the development of neuroblastoma (Weiss et al., 1997). Weiss and colleagues demonstrated that their murine model mirrored the human form of neuroblastoma. Specifically, the location of the tumors, the dosage of the *MYCN* gene affecting tumorigenesis, the histological characteristics, the positive staining for synaptophysin and neuron-specific enolase, and the syntenic gains and losses of chromosomes (Weiss et al., 1997).

Knocking down or silencing MycN expression inhibits neuroblastoma cell growth, as well as induces apoptosis and differentiation (Negroni et al., 1991; Whitesell et al., 1991; Nara et al., 2007). Particularly, inhibition of MycN expression with antisense

oligodeoxynucleotides resulted in a decrease in the proliferation rate of the LA-N-5 neuroblastoma cells *in vitro* (Negroni et al., 1991).

RA (retinoic acid) treatment of neuroblastoma cells leads to a decrease in MycN expression prior to undergoing differentiation (Thiele et al., 1985). RA treatment of neuroblastoma cells negatively regulates MycN expression and decreases growth while inducing differentiation. Constitutive over-expression of MycN in a RA treated environment resulted in cellular proliferation (Peverali et al., 1996). Conversely, constitutive over-expression of Max in a differentiated environment led to the growth arrest and differentiation of neuroblastoma cells. This study suggests that the levels of MycN to Max appears to be a critical factor in neuroblastoma growth and differentiation (Peverali et al., 1996).

Members of the Myc family encode nuclear proteins which serve as transcription factors (Schwab, 2004). The Myc family of proteins contains a transcriptional activation domain in the N-terminus. In the C-terminus there is a transcriptional regulation domain, including a basic helix-loop-helix / leucine zipper (bHLHZip) domain (Lu et al., 2003). The bHLHZip region is capable of binding other proteins with a bHLHZip domain, as well as mediating DNA-binding. To activate transcription the MycN protein must first heterodimerize with the Max protein. Max is a ubiquitously expressed nuclear protein that also contains a bHLHZip domain (Maris and Matthay, 1999). Max can homodimerize to repress transcription or heterodimerize with MycN to activate

transcription (Blackwood et al., 1991; Lu et al., 2003). The MycN/Max complex recognizes and binds to canonical E-box motifs (CACGTG or CATGTG) with high affinity resulting in transcriptional activation of target genes (Vasudevan et al., 2005). In addition, Max also heterodimerizes with Mxi1 or Mad to repress transcription. However, with an increase in MycN nuclear protein production usually from genomic amplification (Tang et al., 2006), the MycN/Max dimer complex is favored over the formation of Max/Mxi1, Max/Mad or Max/Max (Figure 1-3; Wenzel and Schwab, 1995; Maris and Matthay, 1999; Lu et al., 2003). The MycN/Max dimer will target an E-box sequence located within the 5'-UTR / promoter region of the *MYC*-targeted genes.

For over 20 years, *MYCN* gene amplification remains prognostically relevant (Cohn and Tweddle, 2004) and is the most widely accepted predictive parameter for clinical diagnosis of neuroblastoma. Currently, *MYCN* remains the only clinical relevant amplified oncogene in neuroblastoma cells. *MYCN* gene amplification usually leads to high MycN protein and RNA expression in neuroblastoma tumors (Tang et al., 2006). Amplification of the *MYCN* gene and over-expression of MycN protein is indicative of increased tumor growth and tumorigenicity (Seeger et al., 1985; Weiss et al., 1997; Cohn and Tweddle, 2004).

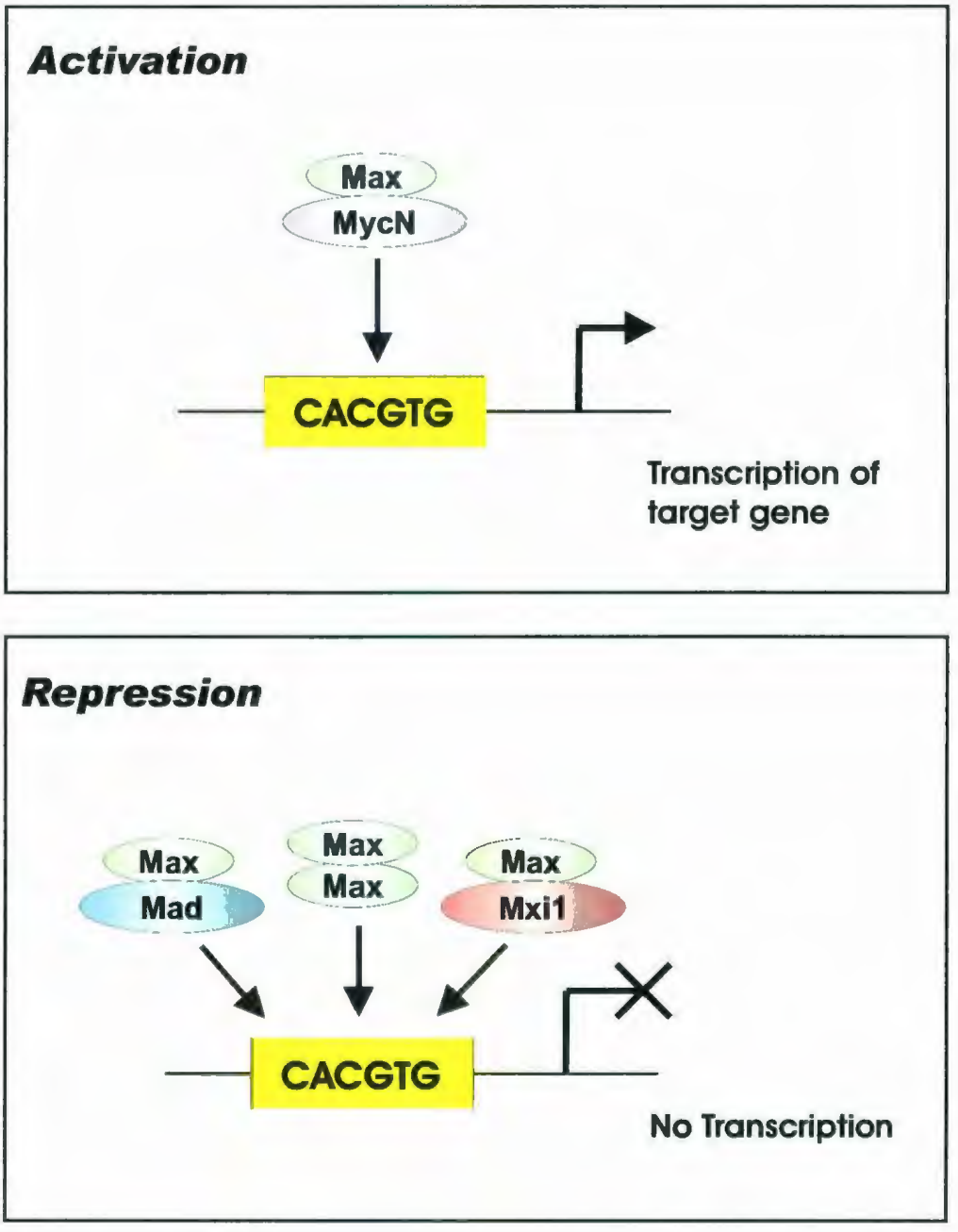


Figure 1-3. Model of MycN interactions.

The MycN nuclear protein heterodimerize with Max forming a complex that will recognize the E-box motif in the promoter region of the target gene and activate transcription. However, in the absence of MycN, Max can heterodimerize with Mxi1 or Mad leading to transcriptional repression of the target gene. In addition, Max can also homodimerize which will also lead to transcriptional expression.

1.3.7 Chromosomal Aberrations

In addition to *MYCN* gene amplification, cytogenetic aberrations are also associated with poor outcome in neuroblastoma. Neuroblastoma tumors with chromosome aberrations have a near-diploid karyotype, resulting in chromosomal rearrangements and unbalanced translocations. These tumors tend to be more aggressive and have a more adverse outcome than tumors with mitotic dysfunction. Neuroblastoma tumors with mitotic dysfunction are less aggressive and are associated with whole chromosome gains or losses (Brodeur, 2003; Maris et al., 2007).

Brodeur reported that a deletion in the short arm of chromosome 1 (containing a yet unidentified tumor suppressor) is found in patients with advanced stages of neuroblastoma (Brodeur, 2003). In addition to the allelic loss of chromosome 1p, there is a high association with *MYCN* gene amplification. The majority of neuroblastoma cases that have *MYCN* gene amplification also have the allelic loss of chromosome 1p. However, the reverse is not necessarily true, suggesting that 1p deletion may occur before *MYCN* gene amplification (Brodeur, 2003). A deletion of chromosome 1p occurs in 25 – 35% of neuroblastomas and correlates with unfavorable outcome (Caron et al., 1996b; Maris et al., 2007). In addition, deletion in chromosome 1p correlates with amplification of the *MYCN* oncogene. Although the loss of chromosome 1p may predict disease progression, it does not appear to decrease the overall survival of the neuroblastoma patients (Maris et al., 2000; Maris et al., 2007).

Gains in the long arm of chromosome 17 (17q 23-qter) is one of the most common genetic abnormalities found in primary neuroblastomas. Caron et al. described the gain in 17q as an indicator of poor prognosis (Caron, 1995). Later reports suggested 17q as an independent predicator of unfavorable outcome in neuroblastoma patients (Lastowska et al., 1997). Ninety percent of high-risk neuroblastoma patients have a gain in chromosome 17q (Vasudevan et al., 2005).

Another common genetic aberration found in 35 – 45% of neuroblastomas involves chromosome 11. An unbalanced deletion of chromosome 11 usually results in an aggressive phenotype. However, this aberration is rarely associated with *MYCN* gene amplification despite being associate with other high-risk factors (Brodeur, 2003; Maris et al., 2007).

1.3.8 Differentiation

Neuroblastoma arises in the sympathetic nervous system from a defect during normal neuro-ectodermal development (Brodeur, 2003; van Noesel and Versteeg, 2004; Maris, 2005; Vasudevan et al., 2005). A down-regulation of *HASH-1* mRNA expression occurs upon induced differentiation of human neuroblastoma cells (Soderholm et al., 1999). This down-regulation of *HASH-1* was not cell-type specific and was irrespective of the differentiate agent used (Axelson, 2004). In addition, Ichimiya et al. used neuroblastoma

cells that did not differentiate and did not express *HASH-1* (Ichimiya et al., 2001). These studies provide additional evidence for the transformation of neuroblastic cells into more mature differentiated cells. Morphological differentiation can be observed in many neuroblastoma cells lines (Sidell et al., 1983; Abemayor and Sidell, 1989). In particular, the LA-N-5 human neuroblastoma cell line is one of the most well-established models for studying differentiation (Sidell et al., 1983; Hill and Robertson, 1997). Untreated LA-N-5 cells are small and round with few protruding neurites. However, upon RA-induced differentiation, LA-N-5 cells undergo a morphological transformation. The cells produce a large number of neurites that form a strong network and the cells tend to clump and roundup (Robson and Sidell, 1985). In addition, molecular markers such as neurofilament and vimentin confirm the immature form from the well-differentiated form (Hill and Robertson, 1997; Pahlman et al., 2004).

1.3.9 Histology

Small blue round cell tumors (SBRCT) include tumors such as Ewing's sarcoma, Wilms' tumor, retinoblastoma, and neuroblastoma that have a tendency to arise in children. In addition, SBRCT share common histological characteristics upon hematoxylin and eosin (H & E) staining (Pisick et al., 2003). SBRCT are undifferentiated, small round cells with large nuclei that stain dark blue upon H & E staining.

In particular, neuroblasts are uniformly sized cells that appear blue, due to their large dark nuclei and scant cytoplasm. Homer-Wright rosettes are a characteristic feature of neuroblastoma. However, rosettes may not always be present. Rosettes are formed using a circular pattern of neuroblast cells that surround neuropil, which are neurites that protrude the cell (Figure 1-4; Lonergan et al., 2002).

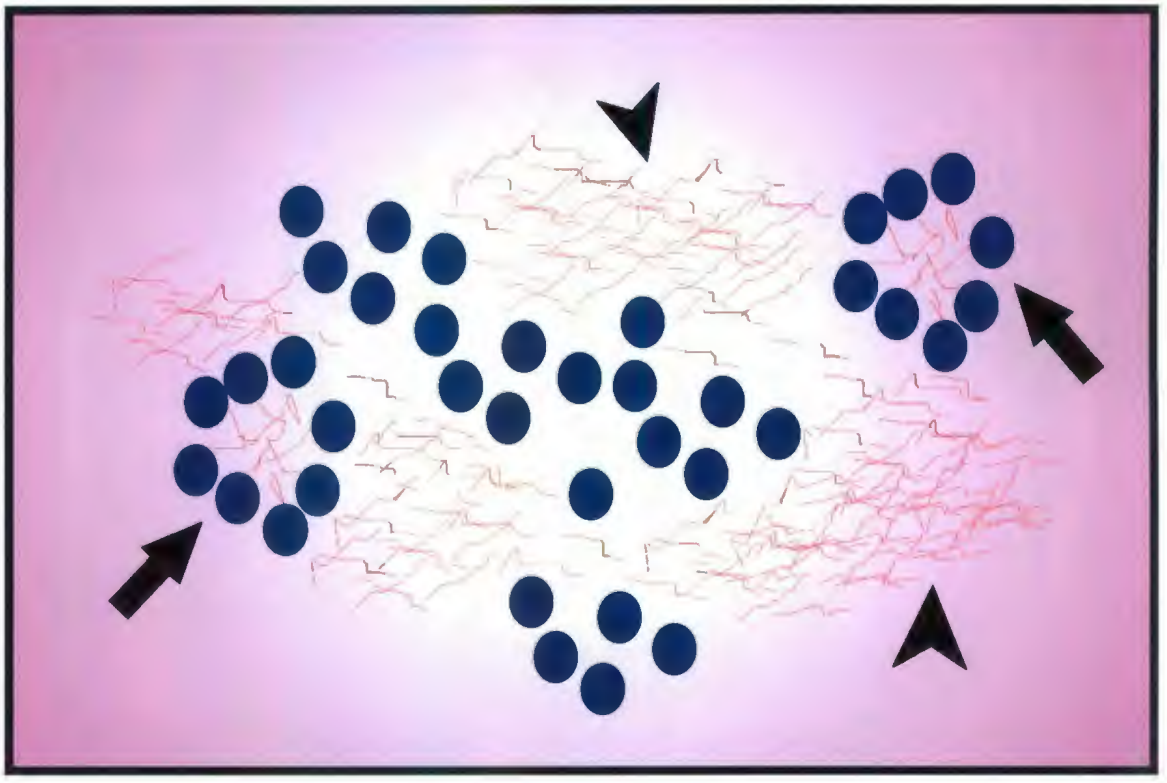


Figure 1-4. Schematic of Homer-Wright rosettes.

Homer-Wright rosettes are a cluster of neuroblasts (small uniform cells) that form a circular arrangement (arrow) around neurophil or neuritic processes (arrowhead) that are stained in red. Homer-Wright rosettes are characteristic of neuroblastomas and as such are used as a diagnostic tool.

1.3.10 Diagnosis

Although understanding the histology of neuroblastomas is an invaluable tool for evaluating tumors, it is not the first step during diagnosis. The standard routine for diagnosing neuroblastoma usually begins with a physical exam accompanied by a urine test. Neuroblastomas produce elevated levels of catecholamines, which are small molecules such as dopamine and norepinephrine that function as the main neurotransmitters of the sympathetic nervous system. Catecholmaines are broken down into urinary metabolites (vanyllylmandelic acid and homovanillic acid) and measured in the urine (Brodeur, 2003). Ninety to ninety-five percent of neuroblastomas produce high levels of urinary metabolites (Kline and Sevier, 2003).

Medical imaging also plays a pivotal role in the initial assessment of the neuroblastoma, indicating the precise location of the tumor (Howman-Giles et al., 2007). Computed tomography (CT) is an imaging method that generates two-dimensional images of the patient's body producing a cross-sectional view that when combined gives a three-dimensional view. CT is mainly utilized for assessing tumors within the abdomen, pelvis, or mediastinum (Maris et al., 2007). Another imaging technique used to examine the structure and function of the body is magnetic resonance imaging (MRI). MRI is more effective than CT in providing contrast between the soft tissues of the body. MRI is the preferred method for determining the spread of the tumor into the spinal canal (Ishola

and Chung, 2007). Alternatively, CT can define the site and extent of the tumor. In addition, CT can provide evidence of regional invasion, vascular encasement, adenopathy, and calcification (Howman-Giles et al., 2007). CT scans can determine if the tumor is neuroblastoma based on calcification evidence which occurs in over 80% of the patients (Howman-Giles et al., 2007).

Another method for the clinical assessment of tumors is metaiodobenzylguanidine (MIBG) scintigraphy. MIBG is actively absorbed by catecholamine producing cells such as neuroblastoma. MIBG is a highly specific method of detection as it occurs in more than 90% of neuroblastomas (Maris et al., 2007).

1.3.11 Classification

Following initial tumor diagnosis, the histological features of the tumor are used to classify the tumor. In 1984, Shimada and colleagues developed a histopathological classification system that focused on NTs and related this information to the tumors clinical behavior (Castel et al., 2007; Maris et al., 2007). This age-linked classification system focused on the stromal development, the degree of neuroblast differentiation, and the nuclear morphology. Stromal development focused on the organizational pattern of the connective tissue. NTs divide into stroma-poor and stroma-rich groups (Shimada et al., 1984). The degree of neuroblast differentiation was evaluated by microscopic assessment and refers to the level of neuroblastic cells maturity. An 'undifferentiated

population' contains less than 5% differentiating cells whereas a 'differentiated population' contains 5% or more differentiating cells (Shimada et al., 1984). The nuclear morphology is represented by the mitosis-karyorrhexis index (MKI). The MKI is defined as the number of mitoses and karyorrhexis (fragmentation of a cell's nucleus) per 5000 cells. The MKI is divided into 3 classes: Low, less than 100 mitotic and/or karyorrhectic cells per 5000; Intermediate, 100 - 200 mitotic and/or karyorrhectic cells per 5000; and High, greater than 200 mitotic and/or karyorrhectic cells per 5000 (Shimada et al., 1984). The Shimada histopathologic classification can stratify tumors into either favorable or unfavorable categories based on age of the patient and subtype of NT (Joshi, 2000).

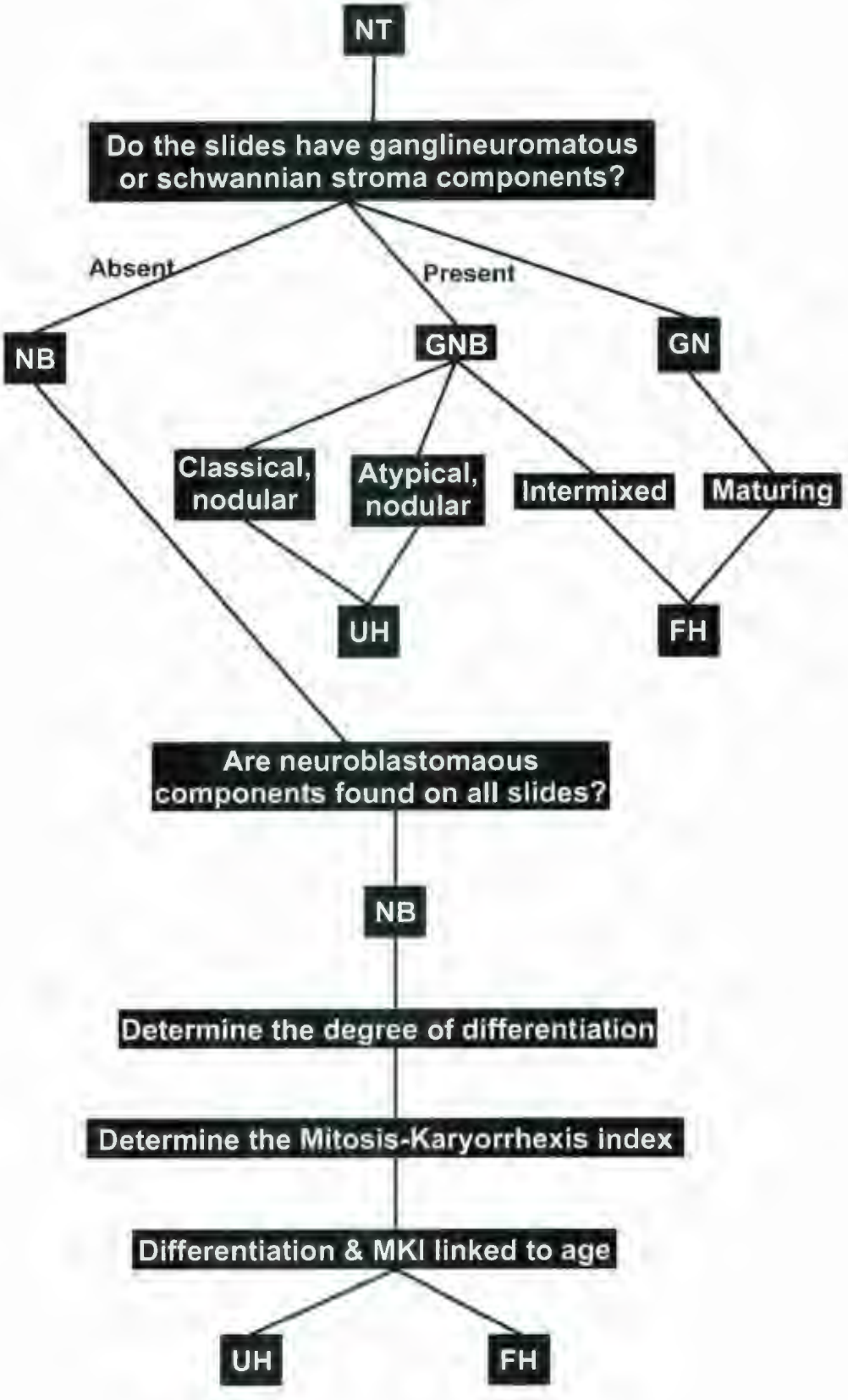
In 1994, there was a consensus among six pathologists to create an International Neuroblastoma Pathology Committee. The intention of this committee was to standardize the terminology, as well as to create a morphological classification for the NTs. The committee used a modified version of the Shimada Classification system to develop the International Neuroblastoma Pathology Classification (INPC) which was proposed in 1999 (Shimada et al., 1999b). The INPC subdivided the stroma-rich group into ganglioneuroma, ganglioneuroblastoma-intermixed and ganglioneuroblastoma-nodular. In addition, an age component was added to the stroma-poor / favorable group consisting of a <1.5 yrs and 1.5 – 5 years (Shimada et al., 1999b; Vasudevan et al., 2005). The MKI index is expressed as a percentage of mitosis and karyorrhexis where the low, intermediate and high MKI represent <2%, 2-4%, >4% respectively (Joshi, 2000). The INPC does not take into consideration the location of the tumor or the progression of the

disease during the time of diagnosis. Figure 1-5 outlines the various steps needed to classify and categorize the NTs according to INPC (Joshi, 2000).

The International Neuroblastoma Staging System (INSS) was created in 1988 but underwent modifications and was not fully developed until 1993. The INSS is currently used as a part of the diagnosis and treatment of neuroblastoma patients (Brodeur et al., 1993; Castel et al., 2007). The INSS consists of four stages (one through four) including two subsets (2B and 4s) of neuroblastoma. The INSS represents the various ways NB tumors are diagnosed and treated. Simplistically, Stage 1 tumors are defined as localized and confined to the area of origin. Stage 1 tumors are usually removed by surgery alone. Stage 2A, the tumor is confined to one side of the body and cannot be completely removed by surgery alone. Also, lymph nodes enclosed within the tumor may contain neuroblastoma but lymph nodes outside the tumor are negative for neuroblastoma. Stage 2B, the tumor is confined to one side of the body and cannot by completely removed by surgery alone. In addition, nearby lymph nodes are positive for neuroblastoma. Stage 3, the cancer cannot be completely removed by surgery alone as it has infiltrated across the midline. The surrounding lymph nodes may or may not be positive for neuroblastoma.

Figure 1-5. Brief outline of the pathological prognostic categorization of NTs.

NT classification is a complex process that requires a specific prognostic categorization protocol. Initially, the type of NT (neuroblastoma, NB; ganglioneuroblastoma, GNB; ganglioneuroma, GN) is defined. Then, subtypes of the tumor are identified. For GNB and maturing GN, age is not considered. However, age and subtypes of NB are considered when defining favorable histology (FH) or unfavorable histology (UH). The FH and UH classifications are then used in defining the neuroblastoma risk groups (see Table 1-1).


NT
Do the slides have ganglineuromatous or schwannian stroma components?
Absent
Present
NB
GNB
GN
Classical, nodular
Atypical, nodular
Intermixed
Maturing
UH
FH
Are neuroblastomaous components found on all slides?
NB
Determine the degree of differentiation
Determine the Mitosis-Karyorrhexis index
Differentiation & MKI linked to age
UH
FH

Stage 4, describes cancer that has metastized to the distant lymph nodes, bone, liver, skin, bone marrow or other organs. Stage 4S also called 'special' neuroblastoma occurs in children less than one year of age and is usually localized to one area of the body. However, the cancer may spread to the other side of the body or may spread to the liver, skin, and/or bone marrow (no greater than 10% of the marrow cells are cancerous).

The Risk Group Classification system is an extension of the INSS that focuses on tumor location and progression (*Children's Oncology Group [COG]*). Risk Group Classification is used to determine the most effective treatment for patients. This classification encompasses the stage of the disease (or INSS), age at diagnosis, *MYCN* status, DNA ploidy, and tumor histopathology (Table 1-1; Brodeur, 2003; Goldsby and Matthay, 2004; Vasudevan et al., 2005; Maris et al., 2007; Bowen and Chung, 2009). Children with neuroblastoma will be assigned into either the low-risk, intermediate-risk or high-risk group.

In an attempt to gain global solidarity, a working group was assembled in 2005 to develop an International Neuroblastoma Risk Group (INRG) classification system. The INRG represented members from the major pediatric oncology group worldwide (Maris et al., 2007). The INRG schema will include the International Neuroblastoma Risk Group staging system (INRGSS), *MYCN* status, Ploidy, Histology, age at diagnosis (increased to 18 months) and Risk group (Castel et al., 2007; Maris et al., 2007).

		Risk Groups		
		Low	Intermediate	High
INSS (Stage)	1	0-21 years	None	None
	2A/2B	Age <1 year **or** Age 1-21 years and MNA **or** age 1-21 year and MA + FH	None	Age 1-21 years and MA + UH
	3	None	Age <1 year and MNA **or** age 1 – 21 years and MNA + FH	Age 0-21 years and MA **or** age 1 – 21 years and MNA + UH
	4	None	Age <1 year and MNA	Age <1 year and MA **or** age 1 – 21 years
	4S	Age <1 year and MNA + FH and DI>1	Age <1 year and MNA and DI=1 **or** Age <1 and MNA + UH	Age <1 and MA

Table 1-1. Neuroblastoma risk groups.

The low, intermediate, and high risk groups are based on based stage, age, MYCN status, histological categories, and DNA ploidy.
MA, *MYCN* amplified; MNA, non-*MYCN* amplified; DI, DNA index (ploidy); FH, favorable histology; UH, unfavorable histology.

As mentioned earlier, the INSS is currently still in use. The INRG is also developing the INRGSS which will now use image-defined risk factors along with bone marrow morphology to aid in the definition of the disease (Castel et al., 2007; Maris et al., 2007). The new staging system will be classified into four divisions representing Stage L1, Stage L2, Stage M and Stage MS. Stage L1 represents localized disease without image-defined risk factors whereas stage L2 represents localized disease with image-defined risk factors (Cohn et al., 2009; Monclair et al., 2009). Stage M represents tumors that are widely metastatic whereas stage MS represents tumors that have a 4S INSS pattern (Maris et al., 2007). As mentioned earlier, the INRG and INRGSS classification systems are a work in progress with a final outline of the criteria yet to be determined. Nevertheless, further refinement of these risk group will ultimately improve clinical interpretation (Maris et al., 2007).

1.3.12 Treatment

As mentioned, before treatment strategies are considered the patients are assigned a risk group classification (Howman-Giles et al., 2007). The low-risk group includes localized or stage 4S neuroblastomas can regress spontaneously or differentiate into benign ganglioneuroblastoma or ganglioneuroma tumors. The regression response by certain subsets of neuroblastoma is approximately ten to one hundred times more than any other cancer (Castel et al., 2007). Treatment for the low-risk group would usually involve minimal therapy such as surgery. Treatment for the intermediate-risk group would likely

contain a combination of surgery and chemotherapy. The high-risk group contains stage 3 or 4 neuroblastoma tumors that are widely metastatic and have poor outcomes despite aggressive therapy (Brodeur, 2003). These tumors are fatal in approximately 60% of the high risk cases (Castel et al., 2007). It is also worth mentioning that fifty percent of all neuroblastoma cases are classified as high-risk for recurrence (Maris et al., 2007). Treatment for the high-risk group usually consists of a combination of chemotherapy, radiation therapy, surgery, stem cell transplant and biological based approaches (Joshi, 2000; Brodeur, 2003; Castel and Canete, 2004). Aggressive chemotherapy in young infants can lead to severe mental and physical impairments and poor quality of life that adds further burden to patients, families, and health care systems. Despite this intensive multi-modal approach, approximately 70% of those neuroblastoma cases still remain incurable (Wei et al., 2005; Maris et al., 2007). New approaches involving cytotoxic agents, immunotherapy, retinoids, angiogenesis inhibitors, and tyrosine kinase inhibitors are currently being evaluated (Maris et al., 2007).

In 2008, the International Society of Pediatric Oncology (SIOP) group completed a phase III clinically trial for high-risk neuroblastoma patients. The focus of this trial was to test the effects of the Ch14.18 antibody on high-risk neuroblastoma patients[1]. Ch14.18 is a chimeric mouse / human monoclonal antibody that binds tumors over-expressing ganglioside GD2 and induces cell-mediated cytotoxicity[2]. The outcome of the antibody-

[1] www.cancer.gov/search/ViewClinicalTrials.aspx?cdrid=69191&version=HealthProfessional&protocolsearchid=4015135 (retrieved on February 17, 2009)
[2] www.cancer.gov (retrieved on February 17, 2009)

based immunotherapy was very effective for the high-risk neuroblastoma patients. The progression-free survival and overall survival at 2 years were 20 percent and 11 percent, respectively, more effective than standard treatments[3].

Presently, standard treatments for neuroblastoma include the use of retinoids because of their role in normal neural crest development. A randomized clinical trial has previously shown that 13-cis RA, an isomer of all-trans RA (ATRA), improved the survival rates while reducing toxicity in high-risk patients (Matthay et al., 1999). The ability of the aggressive neuroblastoma tumors to differentiate into benign ganglioneuromas is a vital part of the treatment. Consequently, 13-cis RA has been included as part of the treatment regiment for high-risk neuroblastoma cases. The naturally occurring active form of RA, ATRA, has been extensively studied in culture. The ability to transform neuroblastoma cells into a more differentiated state is observed with differentiation markers such as neurofilaments (Hill and Robertson, 1997; Ross et al., 2002).

Despite advancements in treatment, neuroblastoma still accounts for 15% of cancer deaths in children (Maris et al., 2007). More effective diagnostic tools and treatments are needed to improve cure rates, reduce toxicity, and long-term effects of current therapies. Discovering novel treatments for NTs will require new insight into the interaction between existing molecular pathways and new molecular markers. New markers will provide better tools for selecting the best treatment for the neuroblastic patient.

[3] www.asco.org (retrieved on August 13/09)

1.4 Tubedown

Our laboratory isolated and characterized a developmentally regulated gene named *TUBEDOWN*. *TUBEDOWN* is found on chromosome 4 and is located at 4q31.1. There are two protein isoforms of *TUBEDOWN*, Tubedown-1 (Gendron et al., 2000) and Tubedown-100 (Fluge et al., 2002; Paradis et al., 2008). Both forms are similar to the Yeast N-terminal acetyltransferase subunit Nat1 (28% Tubedown-1 & 25% Tubedown-100). The Tubedown-100 protein harbors two different types of conserved domains including 3 full tetratricopeptide repeat (TPR) motifs (TPR1: 11-112aa, TPR2: 115-213aa; TPR3: 378-470aa) and a coiled-coil domain homologous to a syntaxin 18 conserved domain (503-676aa) (Figure 1-6A; H. Paradis, personal communication). The TPR domains mediate protein-protein interactions (Blatch and Lassle, 1999) and their presence in Tubedown suggests that this protein may be part of a multi-protein complex (Gendron et al., 2000; Fluge et al., 2002). Syntaxin-18 is a member of the syntaxin family, which are involved in membrane trafficking (Hatsuzawa et al., 2000). Recently, the neuroblastoma-amplified gene (NAG) and ZW10 were reported to be components of the syntaxin-18 complex (Aoki et al., 2009). NAG has been shown to be associated with poor prognosis (Kaneko et al., 2007). However, there is controversy surrounding the association of NAG with tumor progression and outcome (Aoki et al., 2009). In addition, ZW10 plays a role in turning off spindle checkpoint (Williams et al., 2003). Collectively, these studies suggest a role for the syntaxtin-18 complex in cell proliferation.

Fluge and colleagues used a nested RT-PCR approach to show that alternative splicing did not affect the ORF of Tubedown-100 (Fluge et al., 2002). Therefore, Tubedown-1 and Tubedown-100 are likely derived from the same transcript (Fluge et al., 2002; Willis et al., 2002; Sugiura et al., 2003). In addition, our laboratory obtained evidence that both Tubedown isoforms can be derived from the same transcript (H. Paradis, personal communication). At the protein level, Tubedown-1 has been suggested to result from an alternative translation initiation (Fluge et al., 2002). Tbdn-100 may contain an alternative start site such as an Internal Ribosome Entry Site (IRES). IRES is a nucleotide sequence that allows for translation initiation of a specific mRNA sequence. IRES acts in a cap-independent manner and are commonly located within the 5'-UTR (Baird et al., 2006). Tubedown-100 encodes an 866aa protein (Figure 1-6A) with a molecular weight of 100kDa (Fluge et al., 2002) whereas Tubedown-1 encodes a 594aa protein (Figure 1-6B) with a molecular weight of 69kDa (Gendron et al., 2000).

Tubedown-100 is a functional protein that binds to the Ard1 protein (Park and Szostak, 1992; Sugiura et al., 2003; Arnesen et al., 2005a; Arnesen et al., 2008). Recently, Tubedown-100 was shown to be present in a complex with cortactin, an actin binding protein (Paradis et al., 2008). Tubedown-100 was reported to co-localize with cortactin, which is important for endothelial permeability, vesicular transport, and cell migration (Buday and Downward, 2007; Paradis et al., 2008). In addition, cortactin has a possible role in tumor invasion through cell motility structures as lamellipodia and invadopodia

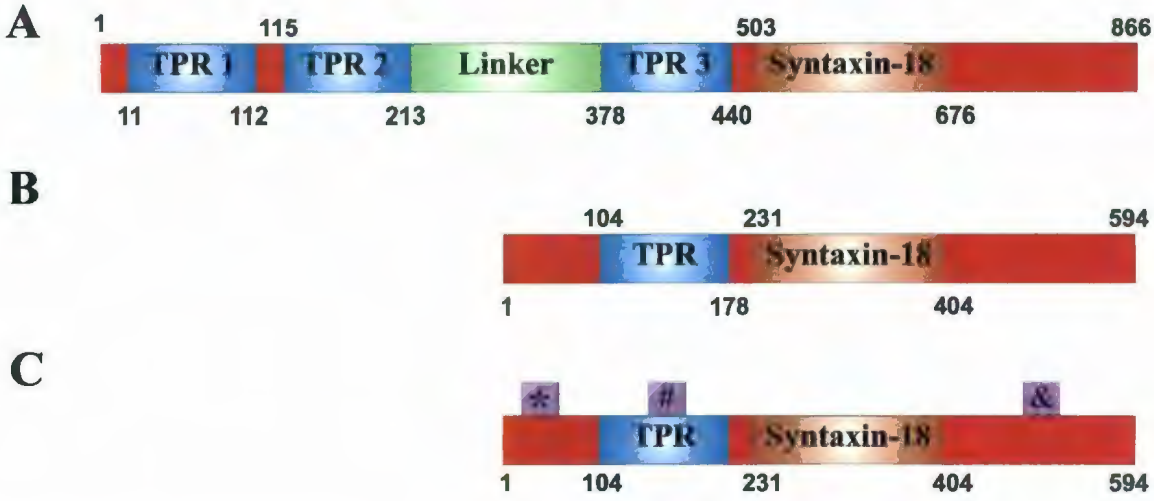


Figure 1-6. Tubedown-1 and Tubedown-100 domains.

(A) Tubedown-100 encodes a protein (866aa) whereas Tubedown-1 (B) encodes a protein of 594aa. The TPRs represent tetratricopeptide repeat motifs whereas the syntaxin domain represents a coiled-coil motif. Various antibodies were utilized in assessing Tubedown function and a schematic representing the location of the antibodies are shown (C). The asterisks (*) corresponds to the aa sequence recognized by the OE5 antibody (10-20aa), the pound symbol (#) corresponds to the aa sequence denoted by the Ab1272 antibody (160-170aa), and the ampersand symbol (&) corresponds to the aa sequence represented by the Misty antibody (483-494aa).

(Weaver, 2008). Also, over-expression of cortactin has been reported in many human cancers including head and neck squamous cell carcinomas, colorectal, gastric, breast and ovarian cancers (Buday and Downward, 2007; Weaver, 2008).

Tbdn-100 (Tbdn, also known as NATH and NARG1) and Ard1 form a functional acetyltransferase NatA complex that is evolutionarily conserved from yeast to human. Mutagenesis studies completed in yeast have previously shown that this functional Nat1-Ard1 complex is involved cell cycle control, cell growth, and sporulation (Mullen et al., 1989). Park and Szostak showed in yeast that both Nat1 and Ard1 are required to form a functional acetyltransferase complex (Park and Szostak, 1992). Sugiura and colleagues used a murine model to demonstrate the involvement of Tbdn and Ard1 during brain development as well as during neuronal tissue development (Sugiura et al., 2003). In 2005, Arnesen et al. identified and characterized the human Nat-Ard1 acetyltransferase complex (Arnesen et al., 2005a). In yeast, the NatA complex appears to have a role in ribogenesis as it binds the nascent polypeptide-associated complex and associates with ribosomes (Gautschi et al., 2003; Arnesen et al., 2005a; Raue et al., 2007; Polevoda et al., 2008). Recently, Arnesen and colleagues demonstrated that yeast and human NatA subunits are phenotypically similar. As well, the human *ARD1* and *NAT1* genes complement the yeast *Ard1* and *Nat1* genes. However, heterologous combinations (such as hArd1 and yNat1) between the species proved non-functional in yeast. Although the proportion of acetylation varied between yeast and human, NatA was still able to acetylate the same substrates in both species (Arnesen et al., 2009).

A third subunit of the yeast NatA complex, Nat5, was identified (Polevoda and Sherman, 2003). Gautschi and colleagues demonstrated that the association of the Nat1-Ard1-Nat5 complex with the ribosome depended on the presence of Nat1 and not Ard1 or Nat5 (Gautschi et al., 2003). In particular, Nat1 appears to have a role in the binding and/or positioning of the complex to the ribosomes (Gautschi et al., 2003). In 2006, the human homologue of the yeast Nat5p was described as hNAT5 (Arnesen et al., 2006a). The NatA complex has been shown to be located within the cytoplasm (Gautschi et al., 2003; Arnesen et al., 2006a). In other mammalian cells, homologues for human TBDN (mNAT2, 70% identity) and ARD1 (ARD2, 81% identity) were reported (Sugiura et al., 2003; Arnesen et al., 2006b).

Both yeast and mammalian Tbdn/Ard1 protein complexes appear to possess factor acetyltransferase (FAT) activity, which target cytoplasmic proteins, as opposed to histone acetyltransferases (HATs). Although the *in vivo* acetylated substrates of the Tbdn complex in cells have not yet been identified, Tbdn was found to associate with the actin binding protein cortactin in retinal endothelial cells, a cell type in which Tbdn exerts a homeostatic influence (Wall et al., 2004; Paradis et al., 2008). Cortactin is important for cellular permeability (Mehta and Malik, 2006) and knockdown of Tbdn expression both *in vitro* and *in vivo* leads to an increase in endothelial cell permeability (Paradis et al., 2008). To study the functional importance of Tbdn, our laboratory generated three different Tbdn antibodies (Figure 1-6C). The Misty and Ab1272 antibodies were used to

detect protein levels by Western blot analysis whereas the OE5 antibody was used to detect the level of protein expression by immunohistochemistry.

Tbdn is most highly expressed during embryonic development (mid to late gestation in mesenchymally derived cells such as endothelial, chondrocytic, hematopoietic and osteoblastic cells) and in neuronal cells (Sugiura et al., 2003; Ohkawa et al., 2008). However, in adult, high levels of Tbdn is restricted to very few tissues including the cells of bone marrow, ocular blood vessels, endothelial choroid plexus, and blood vessels of regressing ovarian follicles (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002; H. Paradis, personal communication). In humans, a low level of Tbdn expression was present in most adult tissues.

Tbdn is transiently expressed during the developing vasculature tissue with high levels of Tbdn expression found postnatally in the ocular endothelium and blood vessels of regressing ovarian follicles (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002). Tbdn has been extensively characterized as a negative regulator of angiogenesis in endothelial cells (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002). In addition, decreased Tbdn levels were demonstrated in the retinal blood vessels of patients with proliferative diabetic retinopathy (Gendron et al., 2001). A bitransgenic mouse model was used to suppress Tbdn levels within the endothelial cells, resulting in retinal neovascular pathology (Wall et al., 2004). The abovementioned studies suggest that at

least in the retinal endothelial cells, Tbdn participates in promoting vascular homeostasis in the retina.

In addition, there is evidence that Tbdn could have different functions in other cellular contexts such as the developing neuronal tissue (Sugiura et al., 2003). Sugiura and colleagues showed that both Tbdn and Ard1 demonstrated a spatio-temporal up-regulation during brain development (Sugiura et al., 2003). Additionally, a 2-fold decrease in Tbdn and Ard1 levels occurred upon RA-induced differentiation of mouse P19 embryonic carcinoma cells (Sugiura et al., 2003).

The Tbdn-Ard1 (NatA) complex also appears to have a role in tumorigenesis (Arnesen et al., 2006c). siRNA knockdown of Tbdn and Ard1 expression in HeLa cells reduced cell viability. The decrease in cell viability was attributed to an increase in apoptosis and not a reduction in cell proliferation.

In 2002, the original connection between Tbdn and cancer was established. Tbdn was up-regulated in gastric cancer and over-expressed in thyroid carcinoma tissue when compared to non-neoplastic thyroid tissue (Fluge et al., 2002; Line et al., 2002). Moreover, high expression of the Tbdn transcript was linked to the clinically aggressive thyroid tumors which contained poorly differentiated or undifferentiated areas (Fluge et al., 2002; Arnesen et al., 2008). In the same report, a Burkitt lymphoma cell line, characterized by a high proliferation rate, was shown to exhibit elevated levels of Tbdn

expression compared other adult tissues including the human brain, the heart and the normal thyroid (Fluge et al., 2002).

The second part of the NatA complex, Ard1, plays a role in cellular viability (Arnesen et al., 2008). In 2005, RNAi was used to knock-down hArd1 expression in human hepatocellular carcinoma (HepG2) cells, resulting in a reduction in cell division (Fisher et al., 2005). A decrease in cell viability and an increase in apoptosis occurred during siRNA-knockdown of hArd1 and Nath in human cervical (HeLa) cells (Arnesen et al., 2006c). In addition, induction of G1 arrest and inhibition of cell proliferation occurred in lung cancer cells (H1299 and A549) upon treatment with hArd1-silencing RNA (Lim et al., 2006). Recently, ARD1 was reported differentially expressed in stage 4 neuroblastomas compared to stage 4S neuroblastomas (Lavarino et al., 2009).

As mentioned, Tbdn expression is linked to gastric cancer and thyroid carcinoma. The more aggressive thyroid cancers have higher levels of Tbdn expression. In addition, Tbdn expression is tightly down-regulated following embryonic neural development. Therefore, Tbdn may play an important role in neural crest cancers such as neuroblastoma, which are thought to result from derangements in the normal differentiation process. Previously, our laboratory had shown that Tbdn was expressed in neuroblastoma cells (H. Paradis, personal communication). However, the role of Tbdn in neuroblastic cells remains elusive.

1.5 Hypothesis & Objectives

Tbdn expression is tightly down-regulated following embryonic neural development. Therefore, my central hypothesis is that the persistence of Tbdn is an important factor in neural crest tumors such as ganglioneuroma, ganglioneuroblastoma, and neuroblastoma, which are thought to result from derangements in the normal differentiation process.

My first objective was to determine if Tbdn expression is linked to the differentiation status and aggressiveness of NTs. Amplification and over-expression of MycN have been widely known to contribute to the growth (Weiss et al., 1997) and the aggressiveness (Brodeur, 2003; Wei et al., 2008) of neuroblastoma. My second objective focused on the regulation of Tbdn. I wanted to determine if there was a correlation between MycN and Tbdn. The 5'-UTR / promoter region of *TBDN* contained a canonical E-box motif which was a binding site for MycN. Therefore, my focus was on determining if MycN binds directly to the *TBDN* promoter region. If so, then is it possible that this relationship between MycN and *TBDN* could be responsible for contributing to the aggressiveness of neuroblastoma?

2.1 Tubedown expression correlates with the differentiation status and aggressiveness of neuroblastic tumors

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2.1.1 Authors' Contribution

Darryl Martin participated in the experimental design, performed the majority of the experimental work, carried out the analysis and interpretation of the data. Drs. Hélène Paradis and Robert L. Gendron participated in the experimental design, interpretation of the data, and provided financial support for the majority of the experiments and equipment. Dr. Ewa Miskiewicz aided in the immunohistochemisty experiments. Dr. Jason A. Jarzembowski created the neuroblastic microarrays while Dr. Valerie P. Castle provided the financial support for the development of the microarrays. Snap-frozen tissues and other paraffin-embedded sections were provided by Margaret H. Collins, Dr. Chitra Pushpanathan and the late Dr. Desmond Robb. Dr. Arie Perry performed the fluorescence in situ hybridization experiments. Dr. Thomas Inge supplied the neuroblastoma cell lines.

2.1.2 Abstract

Purpose: The discovery and validation of new prognostic factors and further refinement of risk group stratification are needed to improve clinical interpretation of neuroblastoma. Our laboratory isolated and characterized a developmentally regulated gene named *TUBEDOWN* against which we have raised a monoclonal antibody (OE5). Tubedown becomes downregulated postnatally yet remains strongly expressed in some neuroblastomas. The purpose of this study is to define the utility of Tubedown expression as a new measure of the differentiation status and aggressiveness of neuroblastic tumors.

Experimental Design: Tubedown protein expression was quantitatively assessed in neuroblastic tumors (neuroblastomas, ganglioneuroblastomas, and ganglioneuromas) and normal adrenal tissues using Western blot and OE5 immunohistochemistry. Regulation of Tubedown expression during retinoic acid-induced neuronal differentiation in neuroblastoma cell lines was assessed by Western blotting.

Results: High levels of Tubedown expression are observed in tumors with significant neuroblastic component, unfavorable histopathology, advanced stage, high-risk group and poor outcome. In contrast, more differentiated subsets of neuroblastic tumors, ganglioneuroblastomas with favorable

histopathology and ganglioneuromas, express low levels of Tubedown. *In vitro*, marked retinoic acid-induced neuronal differentiation responses of neuroblastoma cells are associated with a significant decrease in Tubedown expression while limited neuronal differentiation responses to retinoic acid were associated with little or no decrease in Tubedown expression.

Conclusions: Our results indicate that the levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastic tumors and represent an independent prognostic factor for neuroblastoma. Tubedown expression may be useful to more accurately define different neuroblastic tumor subsets and ultimately provide more adequate assessment and treatment for neuroblastoma patients. *[Clin Cancer Res 2007;13(5)1480-1487]*

2.1.3 Introduction

Neuroblastoma is one of the most common solid tumors occurring in children (Castel and Canete, 2004). Although the etiology of NB is still not clear, evidence suggests that these neuro-ectodermal tumors arise in the sympathetic nervous system from a defect occurring during normal developmental processes (Brodeur, 2003; van Noesel and Versteeg, 2004; Maris, 2005; Vasudevan et al., 2005). Neuroblastomas exhibit heterogeneity with respect to differentiation and tumor progression, making diagnosis and treatment a challenge (Brodeur, 2003; Browne et al., 2006). In young infants, localized and stage 4S neuroblastoma can spontaneously undergo complete regression or differentiate into benign ganglioneuroma requiring minimal treatment (Brodeur, 2003). Conversely, children with advanced metastatic neuroblastoma have poor outcomes despite intensive multimodality therapies (Castel and Canete, 2004; Laverdiere et al., 2005). In recent years, choice of treatment for neuroblastoma has relied on a range of prognostic factors (age at diagnosis, International Neuroblastoma Staging System, histopathological features [International Neuroblastoma Pathology Classification], DNA ploidy, and *MYCN* amplification status) that have been used to stratify these tumors into low-, intermediate-, or high-risk categories (Maris, 2005; Vasudevan et al., 2005). However, both the validation of these prognostic factors and further refinement of risk group stratification are needed to improve clinical interpretation (Maris, 2005; Vasudevan et al., 2005). Despite advancements in treatment, neuroblastoma still accounts for 15% of cancer

deaths in children (Maris, 2005). Therefore, more effective diagnostic tools and treatments are needed to improve cure rates, reduce toxicity and long-term effects of current therapies (Brodeur, 2003; Castel and Canete, 2004; Maris, 2005).

Neuroblastoma differentiation is likely an important biological process that can impact tumor outcome (Shimada et al., 1999a; Shimada et al., 1999b). The use of differentiating agents is effective in improving the survival rate of high-risk neuroblastoma patients while limiting toxicity in normal host cells (Castel and Canete, 2004). One such commonly used differentiation agent is retinoic acid. Retinoic acid is one of the most powerful *in vitro* differentiating agents of neuroblastoma cells (Reynolds et al., 2003). The monitoring of neuroblastoma differentiation using specific differentiation markers could be useful in predicting neuroblastoma outcome.

Our laboratory isolated and characterized a developmentally regulated gene named *TUBEDOWN* (Gendron et al., 2000). Tubedown-1 has been defined as a protein of 69kDa with homology to yeast Nat1, a subunit of the yeast acetyltransferase NatA (Gendron et al., 2000). A longer 100kDa variant of Tubedown-1, Tubedown-100, appears to be derived from the same transcript (Fluge et al., 2002; Willis et al., 2002; Sugiura et al., 2003). Tubedown is transiently expressed during embryogenesis in several tissues while in adults high levels are found in only a few tissues including bone marrow, ocular endothelial cells, atrial endocardium, and blood vessels of regressing ovarian follicles (Gendron et al., 2000; Gendron et al., 2001). *TUBEDOWN* transcript is also

expressed in neuronal cells during brain development but suppressed during neuronal maturation (Sugiura et al., 2003). *TUBEDOWN* is also highly expressed in papillary thyroid carcinoma cells while adult tissues except testis express low levels (Fluge et al., 2002; Arnesen et al., 2005b). We have extensively characterized Tubedown as a negative regulator of angiogenesis in endothelial cells (Paradis et al., 2002; Wall et al., 2004). However, Tubedown could have different functions in other cellular contexts (Willis et al., 2002; Arnesen et al., 2006c). Since *TUBEDOWN* expression is tightly down-regulated following embryonic neural development, I hypothesized that the persistence of Tubedown may be an important factor in pediatric tumors such as neuroblastoma which are thought to result from derangements in normal differentiation processes. The present study addresses a hypothesis that levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastic tumors and could represent an independent prognostic factor for neuroblastomas.

2.1.4 Materials & Methods

Tumor Specimens –Snap frozen tissues and paraffin embedded sections from neuroblastoma, ganglioneuroblastoma and ganglioneuroma were obtained from the Cincinnati Children's Hospital Medical Center (Cincinnati, OH), the Brain Tumor Tissue Bank (London, ON, Canada) and the Janeway Child Health Centre (St. John's, NL, Canada). Two tissue microarrays were constructed using triplicate 1.0mm cores taken from 45 paraffin-embedded, formalin-fixed neuroblastic tumors (30 neuroblastoma, 7

ganglioneuroblastoma, 7 ganglioneuroma, and 5 composite/mixed histology tumors), 5 normal adrenal glands, and 20 other neural crest-derived and unrelated neoplasms obtained from archival material at the University of Michigan and the Cooperative Human Tissue Network (National Cancer Institute). Specimens from the tissue microarrays had previously been classified according to International Neuroblastoma Pathology Classification criteria (Shimada et al., 1999a; Shimada et al., 1999b) and clinical data (age at diagnosis, sex, stage, treatment protocol, initial response to treatment, event–free survival, time to relapse, and time to death) was made available. All human specimens were obtained and studied under the approval of the Institutional Review Boards of the author's institutions.

***MYCN* fluorescence in situ hybridization** – Analyses were performed on representative sections (Bridge et al., 2006). A commercial probe cocktail was used, consisting of a Spectrum OrangeTM-labed centromere enumerating probe 2 paired with a Spectrum GreenTM-labeled *MYCN* probe on 2p24 (Vysis, Inc., Downers Grove, IL). The probes were diluted 1/50 with tDenHybTM hybridization buffer (Insitus Biotechnologies, Albuquerque, NM). For each hybridization, 100 non-overlapping nuclei were enumerated for *MYCN* and centromere enumerating probe 2 signals. Cells with *MYCN* to centromere enumerating probe 2 ratios >4 or innumerable *MYCN* signals were considered amplified. In non-amplified cases, those with >10% cells containing >2 copies of centromere enumerating probe 2 were defined as having polysomy 2.

Cell Cultures – LA-N-5 (Seeger et al., 1982a) and IMR-32 (Tumilowicz et al., 1970) cell lines were gifts from Dr. Thomas Inge (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The LA-N-5 and IMR-32 cells were respectively maintained in RPMI 1640 and in Dulbecco's Modified Eagle Media supplemented with 10% heat-inactivated fetal bovine serum and 2mM glutamine. The SK-N-DZ and RF/6A (rhesus macaque choroid-retina endothelial) cell lines were acquired from the American Type Culture Collection and maintained, respectively, in RPMI 1640 and in Dulbecco's Modified Eagle Media supplemented with 10% fetal bovine serum, 0.1mM non-essential amino acids and 4mM glutamine. The EWS-96 cell line was derived from a tumor specimen obtained from a patient with Ewing's sarcoma and will be described elsewhere. Exponentially growing cells were used for every experiment.

All-trans retinoic acid (Sigma) was dissolved in 5% dimethylsulfoxide and added to cells at an optimal concentration of 4μM. The media (with or without retinoic acid) was changed every 3 days. Cells were photographed using a Leica DMIRE2 microscope system with a QImaging RETIGA Ex*i* camera and Openlab software. Viability was assessed using trypan blue dye exclusion. All cell growth assays were performed in triplicate.

Western Blot – Cell lysates were prepared from snap frozen tumor specimens and cell lines as previously described (Gendron et al., 2000). Protein lysates were quantified

using albumin as standard and analyzed by SDS-PAGE. Western blotting was performed by standard procedures using ECL Plus chemiluminescence detection reagent (Amersham Biosciences) for anti-NF-M (Zymed Laboratories), vimentin (Ab-1) (Oncogene Research Products), c-Myb (C-19) (Santa Cruz Biotechnology) and anti-MycN (EMD Biosciences) antibodies. Ab1272 anti-Tubedown Western blot analyses were performed as described (Gendron et al., 2000; Gendron et al., 2001). All Western blots were stripped and reprobed with either ERK-1 (Santa Cruz Biotechnology) or alpha-Tubulin (Sigma) antibodies.

Tubedown Immunohistochemistry – Sections from paraffin embedded tissues were deparaffinized, post-fixed in 4% paraformaldehyde and incubated overnight with anti-Tubedown mouse monoclonal OE5 supernatant or negative control supernatant supplemented with control isotype match IgG2a antibody (Dakocytomation) in 3% non-fat milk in Tris buffer saline with 0.05% Tween 20. The mouse monoclonal OE5 hybridoma (Wall et al., 2004) recognizes Tubedown recombinant protein. Sections were developed using alkaline phosphatase and Vector Red substrate kit (Vector Laboratories), and photographed in triplicate using a Leica DMIRE2 microscope system with a QImaging RETIGA Ex*i* camera and Openlab software.

Northern Blotting and Real-Time RT-PCR – Northern blotting was performed as previously described (Gendron et al., 2000). Blots were hybridized with a 693bp ^{32}P-labeled *MYCN* cDNA probe (Image Clone ID 5502743) (American Type Culture

Collection) and reprobed with a mouse 18S ribosomal cDNA fragment in order to confirm loading equivalency and RNA integrity. Densitometric measurements of band intensity were completed using OptiQuant software.

For RT-PCR analysis, cDNA was prepared from total RNA using Roche Reverse Transcription kit. The cDNA was then amplified by Real-Time PCR with primers **5'-AAC CCC AAT GAT GAT GGA AA-3'** and **5'- CCA AAG CAA TAG ATG GCT GA-3'** specific for human Tubedown (GeneBank accession no. BC039818) using a 7000 sequence detector system (Applied Biosystems). EWS-96 cells were used as a standard for the experiments. All experiments were done in at least triplicate.

Data and Statistical Analyses – To stratify neuroblastoma patients into low-, intermediate- and high-risk groups, prognosis parameters including age at diagnosis, International Neuroblastoma Staging System, histopathology (International Neuroblastoma Pathology Classification), and *MYCN* amplification status were used as previously described (Maris, 2005).

Tubedown immunostaining was quantified as previously described (Gendron et al., 2006). Tubedown staining levels for each specimen were averaged and the average background levels subtracted. To standardize single tumors and tumors from the tissue microarrays, paraffin embedded sections from a Ewing's sarcoma xenograft tumor were used as a control in every experiment. Tubedown relative level of expression was

calculated from a ratio of expression of each specimen over that of the Ewing's sarcoma tumor.

Quantitative analyses were compared using the two-tailed Student's t-Test with a Microsoft Excel program. Tests for homogeneity of variance were performed as described[4]. The data was considered to be statistically significant if the *p*-value was less than or equal to 0.05.

[4] http://helios.bto.ed.ac.uk/bto/statistics/table8.html#Test%20for%20homogeneity

2.1.5 Results

2.1.5.1 High levels of Tubedown expression in neuroblastic tumors correlates with advanced stages and neuroblastic unfavorable histopathology

Western blotting showed that Tubedown is expressed in the LA-N-5 human neuroblastoma cell line and in several neuroblastoma tumor specimens (Figure 2-1). Much higher levels of Tubedown were detected in the stage 2 and 3 neuroblastoma specimens compared to stage 1 neuroblastoma specimens and ganglioneuroblastoma specimens (Figure 2-1). Additional neuroblastic tumor specimens were next analyzed for Tubedown expression by quantitative immunohistochemistry using anti-Tubedown OE5 monoclonal antibody (Wall et al., 2004) (Figure 2-2). In neuroblastoma, the cells that stained most intensely for Tubedown were of neuroblastic appearance (small round cells) (Figure 2-2). Tubedown staining was not detected in blood cells and blood vessels

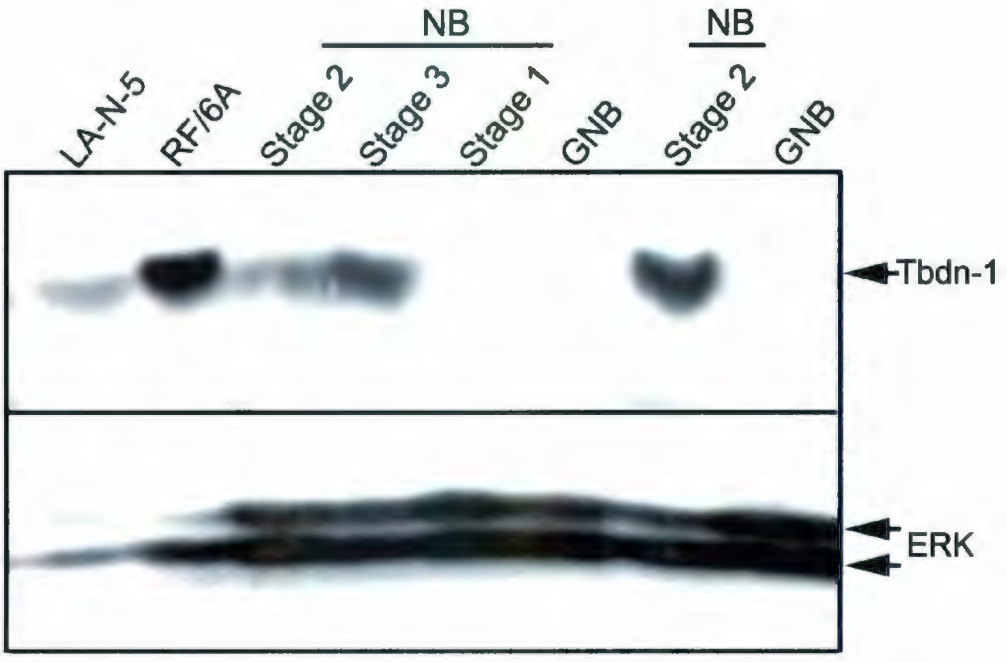


Figure 2-1. Western blot analysis of Tubedown-1 expression in cell lines and primary neuroblastic tumors.

Western blot analysis using Ab1272 anti-Tubedown (Tbdn-1) antibody(69 kDa) of LA-N-5 and RF/6A cell lines, and neuroblastoma and ganglioneuroblastoma pathology specimens. ERK (44/42 kDa) western blot analysis was used to show loading equivalency and protein integrity (Bottom panel). A representative experiment is shown.

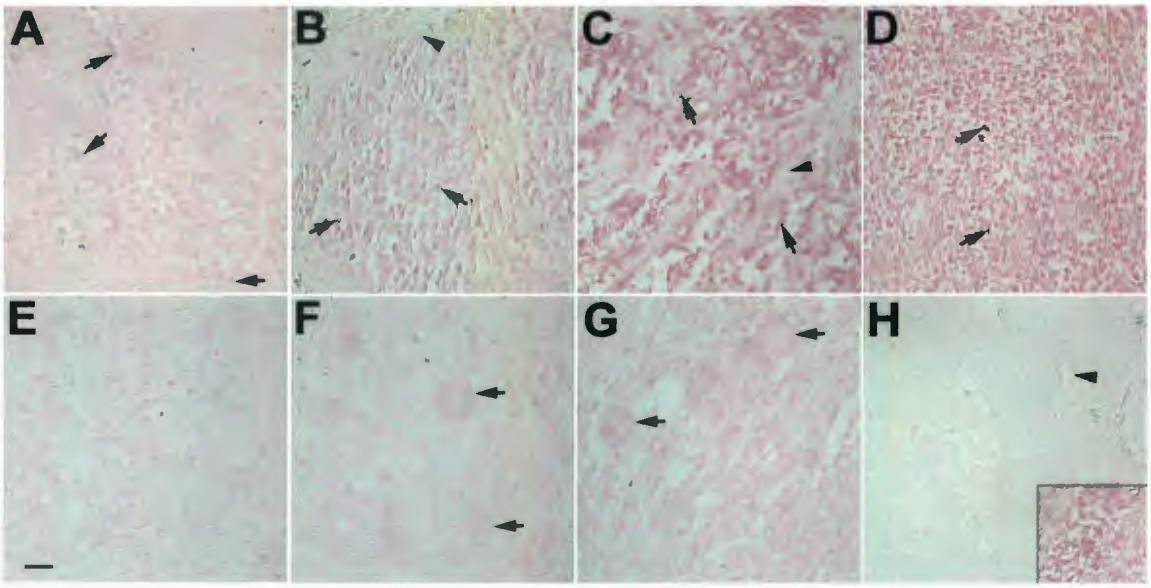


Figure 2-2. Immunohistochemistry analysis of Tubedown expression in neuroblastic tumors and normal tissues.

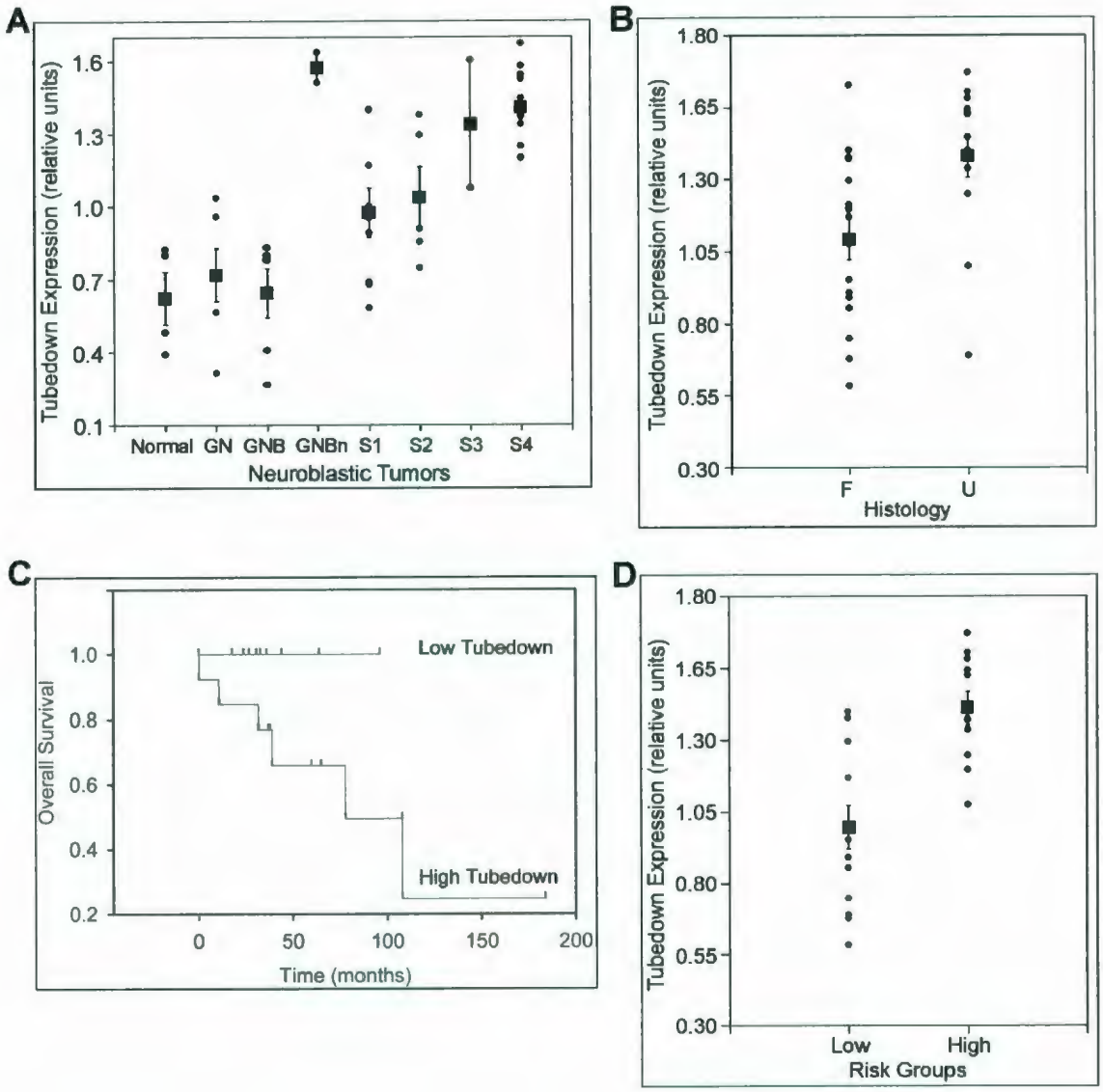
Tubedown immunohistochemistry on primary neuroblastoma tumors (stage 1 [A], stage 2 [B], stage 3 [C] and stage 4 [D and H]), stroma rich (favorable histopathology) primary ganglioneuroblastoma tumor (F), primary ganglioneuroma tumor (G) and normal adrenal tissues (E). Specimens were stained with anti-Tubedown OE5 monoclonal antibody (A-G and inset from panel H) or control with isotype matched negative control antibody (H) and developed using alkaline phosphatase (bright red). Representative experiments are shown at 400X magnification. Arrows indicate positively stained cells while arrowheads indicate absence of staining in structures such as blood vessels. The scale bar represents 100μm.

(Figure 2-2). Normal adrenal tissues contained significantly lower Tubedown staining (0.62 +/- 0.11 relative units) than stage 2, 3 and 4 neuroblastoma tumors (p=0.047, p=0.036 and p=3.4 x 10^{-6} respectively) (Figure 2-2 and 2-3A). Moreover, stage 1 and 2 neuroblastoma exhibited significantly lower levels of Tubedown immunostaining (0.97 +/- 0.10 and 1.04 +/- 0.13 relative units, respectively) than the advanced stage 3 and 4 neuroblastoma tumors (1.34 +/- 0.26 and 1.41 +/- 0.05, respectively; p=6.8 x10^{-14}) (Figure 2-2 and 2-3A).

In the more differentiated neuroblastoma subtypes (ganglioneuroblastoma and ganglioneuroma), both ganglion-like and stromal (Schwann-like) cells faintly stained for Tubedown at levels lower than the neuroblastic–like cells observed in neuroblastoma specimens (Figure 2-2 and 2-3A). Interestingly, two out of two nodular ganglioneuroblastoma specimens with unfavorable histopathology and significant neuroblastic component expressed much higher levels of Tubedown in neuroblastic-like cells (1.57 +/- 0.06 relative units) than all favorable ganglioneuroblastoma specimens with minimal neuroblastic component (0.65 +/- 0.10) (Figure 2-2 and 2-3A). Significantly lower levels of Tubedown expression in the ganglioneuroma specimens (0.72 +/- 0.11 relative units) and ganglioneuroblastoma specimens with favorable histopathology and minimal neuroblastic component were observed compared to the advanced stage 4 neuroblastoma tumors (p=6.0 x 10^{-6} and p=0.0011, respectively) (Figure 2-3A). Although the more differentiated ganglioneuroma and ganglioneuroblastoma tumors with minimal neuroblastic component express lower levels

Figure 2-3. Quantitative analysis of Tubedown expression in neuroblastic tumors.

A) Levels of Tubedown (Tbdn) expression in normal adrenal tissues (Normal, n=4) and neuroblastic tumors (ganglioneuroma, n=6; ganglioneuroblastoma with favorable histopathology: ganglioneuroblastoma, n=6; ganglioneuroblastoma with unfavorable histopathology and significant neuroblastic component: ganglioneuroblastoma(n), n=2; neuroblastoma tumors stage 1: S1, n=9; neuroblastoma tumors stage 2: S2, n=5; neuroblastoma tumors stage 3: S3, n=2; and neuroblastoma tumors stage 4: S4, n=11). B) Significantly higher levels of Tubedown (Tbdn) expression were present in the unfavorable neuroblastoma specimens (n=13) compared to the favorable neuroblastoma (n=17). C) Kaplan-Meier analysis displaying the correlation between low Tubedown (Tbdn) expression in neuroblastoma specimens and overall survival (p=0.038 at 3 years). Low expression levels (<1.3 relative units, n=9) versus high expression levels (≥1.3 relative units, n=13). D) Significantly lower levels of Tubedown (Tbdn) expression were present in the low-risk group neuroblastoma specimens (n=14) compared to the high-risk group neuroblastoma (n=11). In panels A, B and D the relative Tubedown expression units for each specimen is represented by a small dot, while the averages of Tubedown levels for each category are represented by large squares. The bars represent standard error of the mean.


A
Tubedown Expression (relative units)
1.6
1.3
1.0
0.7
0.4
0.1
Normal GN GNB GNBn S1 S2 S3 S4
Neuroblastic Tumors
B
Tubedown Expression (relative units)
1.80
1.65
1.30
1.05
0.80
0.55
0.30
F U
Histology
C
Overall Survival
1.0
0.8
0.6
0.4
0.2
Low Tubedown
High Tubedown
0 50 100 150 200
Time (months)
D
Tubedown Expression (relative units)
1.80
1.65
1.30
1.05
0.80
0.55
0.30
Low High
Risk Groups

of Tubedown than neuroblastoma specimens, the differentiation status within neuroblastoma specimens, categorized according to the International Neuroblastoma Pathology Classification system, was not associated with a significant difference in Tubedown levels. The undifferentiated neuroblastoma specimens expressed 1.18 +/- 0.13 relative units of Tubedown, while the differentiated and poorly differentiated neuroblastoma specimens expressed respectively 1.12 +/- 0.09 and 1.18 +/- 0.13 relative units of Tubedown.

There was a direct correlation between unfavorable histopathology of neuroblastoma specimens and a high level of Tubedown expression. High levels of Tubedown expression (1.38+/- 0.08 relative units) were present in the unfavorable tumors whereas low levels of Tubedown expression (1.09 +/- 0.07 relative units) were observed in the favorable tumors (p=0.009) (Figure 2-3B). High levels of Tubedown (≥1.3 relative units) were observed in 77% of unfavorable neuroblastoma tumors compared to 24% of favorable tumors. There was no correlation between the levels of Tubedown expression and whether or not the patient was diagnosed at an early age (less than 12 or 18 months of age). Tubedown expression did not correlate with *MYCN* amplification status of neuroblastoma tumors. Similarly, the treatment status of the neuroblastic tumors did not show significant correlation with Tubedown staining.

2.1.5.2 High levels of Tubedown in neuroblastoma tumors correlate with high-risk and low survival rate

Classification of our neuroblastoma cohort into three risk groups, low, intermediate and high, revealed a significant positive correlation between high Tubedown expression and the high-risk neuroblastoma patients (Figure 2-3D). Lower levels of Tubedown expression (1.00 +/- 0.08 relative units) were detected in the low-risk neuroblastoma patients compared to the intermediate- (1.32 +/- 0.12) and high-risk neuroblastoma patients (1.41 +/- 0.06; p=0.0004). High levels of Tubedown expression ($\geq$1.3 relative units) were observed in 73% of high-risk neuroblastoma cases compared to 21% in low-risk cases. Kaplan Meier analysis (Kaplan & Meier, 1958) revealed significantly better outcomes for patients with low Tubedown expression compared to high expression (3-year overall survival, p=0.038) (Figure 2-3C). Patients with low Tubedown expression had no notable events whereas 46% of patients with high Tubedown expression relapsed or died. Moreover, high Tubedown expression (1.50 +/- 0.05 relative units) was found in the patients who relapsed or succumbed to the disease (n=8) whereas a low level of Tubedown expression (1.01 +/- 0.10 relative units) was observed in patients who survived without relapse (n=14).

Analysis of our neuroblastoma cohort revealed that the event-free survival rate of patients within the cohort depended on the International Neuroblastoma Staging System (stage 4: relative risk =6.1), patient's age at diagnosis (greater than or equal to 1 year: relative risk =2.6), undifferentiated status (relative risk =2.5), and *MYCN* amplification (relative risk

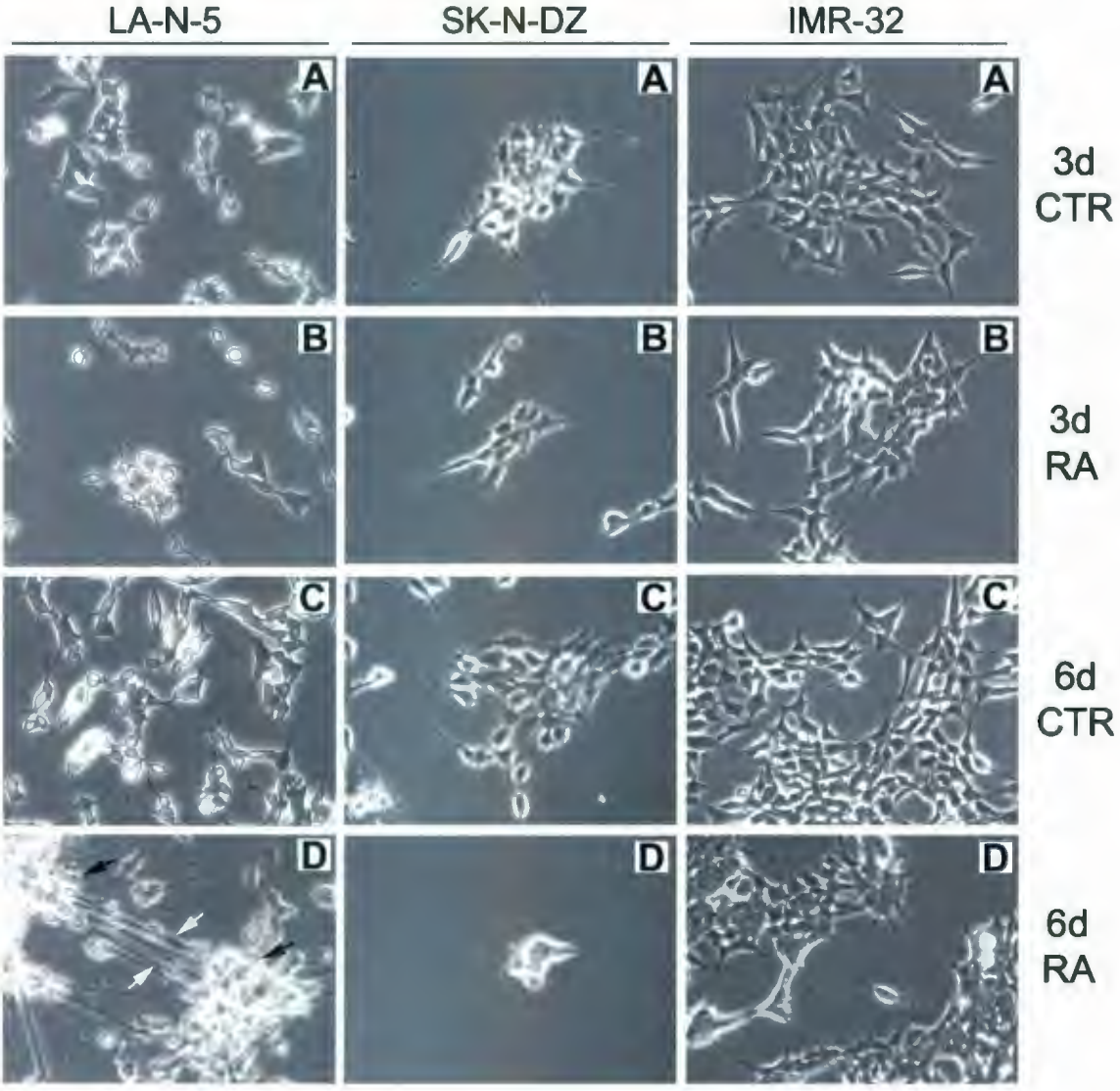
=1.1). The relative risk factors for our neuroblastoma cohort were comparable to other published cohorts (Krams et al., 2004; Hsu et al., 2005; London et al., 2005). The median age of the patient at diagnosis was 24 months (range: 0.1 to 288 months) and the percentage of *MYCN* amplified tumors was 29%.

2.1.5.3 Tubedown is down-regulated in neuroblastoma cells undergoing marked neuronal differentiation

Since Tubedown levels in the differentiated neuroblastic tumor subtypes (ganglioneuroma and ganglioneuroblastoma with minimal neuroblastic component) are low and since the state of neuroblastoma tumor cell differentiation is likely an important biological factor for the clinical outcome (Shimada et al., 1999a; Shimada et al., 1999b), I examined Tubedown expression as a function of the differentiation of neuroblastoma cell lines *in vitro*. Tubedown expression was analyzed in neuroblastoma cell lines induced to undergo differentiation by treatment with retinoic acid. The extent of neuronal differentiation in response to retinoic acid-treatment was first analyzed in 3 neuroblastic type neuroblastoma cell lines (LA-N-5, IMR-32 and SK-N-DZ). Upon treatment with retinoic acid, LA-N-5 cells developed significantly more neurite-like processes (increased length and thickness) than controls (Figure 2-4). Retinoic acid treatment of SK-N-DZ and IMR-32 cells did not significantly induce process development compared to controls (Figure 2-4). Retinoic acid treatment resulted in

Figure 2-4. Morphological differentiation of neuroblastoma cells upon retinoic acid treatment.

LA-N-5, SK-N-DZ, and IMR-32 neuroblastoma cell lines grown for 3 or 6 days in the absence (panels A and C, respectively) or presence of retinoic acid [RA] (panels B and D, respectively). LA-N-5 cells (panel D) exhibit an increase in the length and the thickness of neurites (white arrows) and cellular clustering (black arrows) in response to retinoic acid treatment as compared to control (panel C). Representative experiments are shown at 400X magnification.


LA-N-5
SK-N-DZ
IMR-32
A
B
C
D
3d CTR
3d RA
6d CTR
6d RA

moderate inhibition of cell growth in LA-N-5 while more significant growth inhibition was observed in IMR-32 and SK-N-DZ cells (Figure 2-5). The retinoic acid-induced differentiation in LA-N-5 cells was accompanied with a marked up-regulation of neurofilament-160 kDa (NF-M) expression (Figure 2-6A). The SK-N-DZ and IMR-32 cells displayed a modest or no increase in NF-M expression upon retinoic acid treatment (Figure 2-6A). All three neuroblastoma lines treated with retinoic acid showed down-regulation of vimentin (Figure 2-6B) and c-Myb expression (Figure 2-6C). LA-N-5 and IMR-32 lines expressed high levels of *MYCN* transcript and protein. However, only LA-N-5 cells significantly down-regulated *MYCN* transcript (data not shown) and MycN protein (Figure 2-6D) expression upon retinoic acid treatment.

Analysis of Tubedown levels upon retinoic acid-induced differentiation in these neuroblastoma cells lines revealed a marked reduction of expression only in LA-N-5 cells (Figure 2-6E). The limited retinoic acid-induced differentiation of SK-N-DZ and IMR-32 cells was associated with a modest or no decrease of Tubedown expression, respectively (Figure 2-6E). Real-time RT-PCR analysis of *TUBEDOWN* transcript expression during retinoic acid-induced differentiation of LA-N-5 and IMR-32 cells revealed the same pattern of down-regulation observed for the protein expression (data not shown). These results indicate that significant reduction in Tubedown expression only takes place upon induction of extensive neuronal differentiation in LA-N-5 cells.

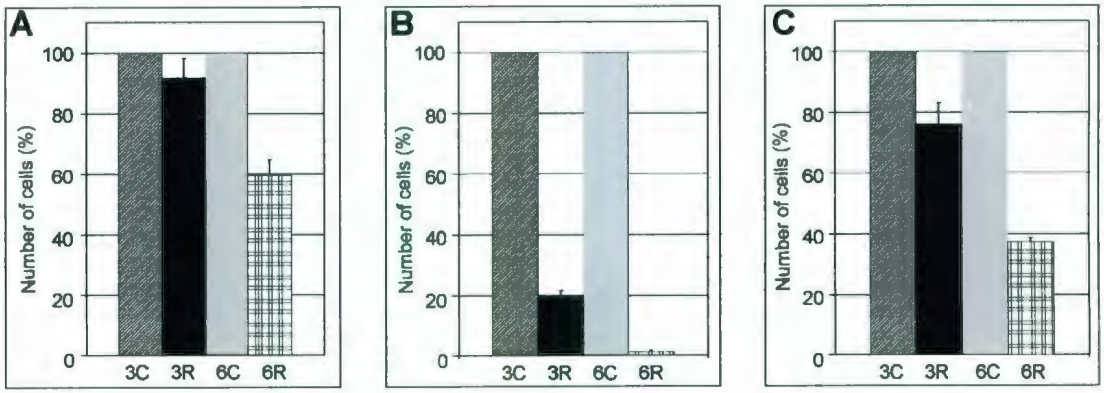
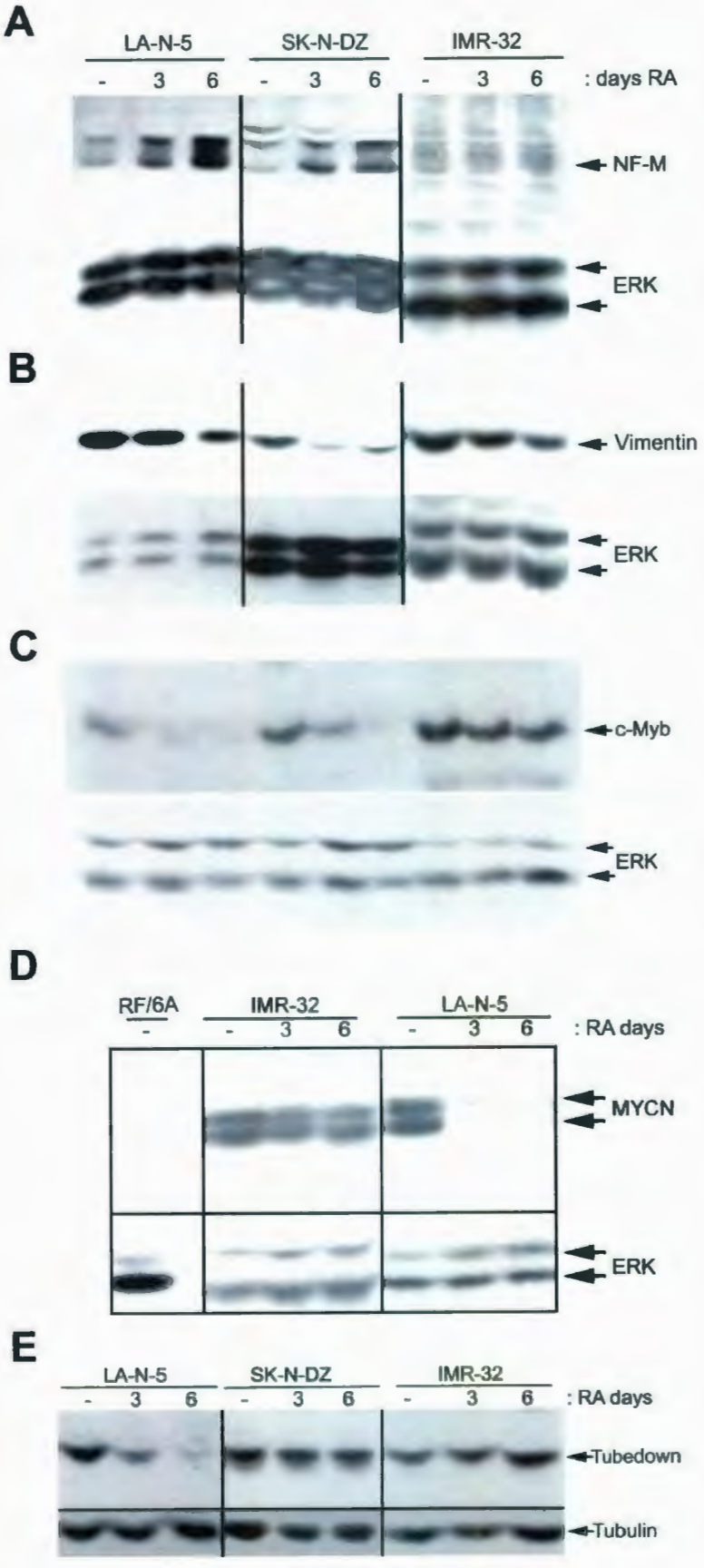


Figure 2-5. Cell growth of neuroblastoma cells upon retinoic acid-induced differentiation.

Decreases in viable cell numbers are observed for LA-N-5 (A), SK-N-DZ (B) and IMR-32 (C) cell lines when treated with retinoic acid for 3 days (3R) and 6 days (6R) compared to 3 (3C) and 6 (6C) days controls. Values are expressed as the mean percentage of the control +/- standard error of the mean. Results represent the average of at least three experiments.

Figure 2-6. Expression dynamics of Tubedown, intermediate filaments, c-Myb and MYCN in neuroblastoma cells upon retinoic acid-induced differentiation.

NF-M [160 kDa (A)], vimentin [58 kDa (B)] and c-Myb [75 kDa (C)] Western blot analyses of untreated (-), 3 days (3) and 6 days (6) retinoic acid (RA)-treated LA-N-5, SK-N-DZ and IMR-32 cell extracts. ERK (44/42 kDa) Western blot analysis was used as a loading control. D, MycN (67/64 kDa) Western blot analysis of untreated (-), 3 days (3) and 6 days (6) retinoic acid (RA)-treated LA-N-5 and IMR-32 cell extracts. RF/6A cell line represents a negative control for MycN expression and ERK Western blot analysis was used as a loading control. E, Western blot analysis with Ab1272 anti-Tubedown antibody (69 kDa) of untreated (-), 3 days (3) and 6 days (6) retinoic acid (RA)-treated LA-N-5, SK-N-DZ and IMR-32 cell extracts. Tubulin (55 kDa) was used as a loading control. Representative experiments are shown.


A
LA-N-5
SK-N-DZ
IMR-32
- 3 6 - 3 6 - 3 6 : days RA
NF-M
ERK
B
Vimentin
ERK
C
c-Myb
ERK
D
RF/6A
IMR-32
LA-N-5
- - 3 6 - 3 6 : RA days
MYCN
ERK
E
LA-N-5
SK-N-DZ
IMR-32
- 3 6 - 3 6 - 3 6 : RA days
Tubedown
Tubulin

2.1.6 Discussion

Tubedown is highly expressed in neuroblastoma cell lines and certain subsets of neuroblastic tumors. In normal adrenal tissues Tubedown expression is almost undetected. High Tubedown levels are detected in tumors presenting significant neuroblastic component and unfavorable histopathology such as advanced stage neuroblastoma and some nodular ganglioneuroblastoma. Conversely, low levels were detected in low stages neuroblastoma, ganglioneuroblastoma with favorable histopathology and ganglioneuroma. High levels of Tubedown expression also correlated with high-risk group and significantly reduced overall survival rate. These results suggest that Tubedown may be a useful marker for unfavorable neuroblastic histopathology of neuroblastic tumors. This may be especially true for the ganglioneuroblastoma subsets as a marked difference was observed in the levels of expression of Tubedown in ganglioneuroblastoma with favorable versus unfavorable histopathology. Tubedown may offer a means to assess the state of differentiation in cases of ganglioneuroblastoma, which are difficult to assess. The state of differentiation of ganglioneuroblastoma may be critical to clinical outcome of the disease (Joshi, 2000). Our results also indicate that Tubedown is a useful marker for neuroblastoma tumors within the high-risk group and advanced stages as well as for predicting poor outcome. However, since the range of Tubedown expression overlapped to a certain degree in low-risk versus high-risk patients and in tumors with favorable versus unfavorable histological features, the determination of Tubedown levels in neuroblastic tumors may be more useful in multivariate analyses with other parameters (age at diagnosis,

International Neuroblastoma Staging System, histopathological features, DNA ploidy, and *MYCN* amplification status).

The more differentiated subset of neuroblastic tumors (ganglioneuroblastoma with favorable histopathology and ganglioneuroma) express lower levels of Tubedown. However, the differentiation status within neuroblastoma specimens was not associated with significant differences in Tubedown expression. Since the International Neuroblastoma Pathology Classification system (Shimada et al., 1999a; Shimada et al., 1999b) categorizes neuroblastoma tumors as differentiating if 5% or more of the cells appear differentiated while poorly differentiated if less than 5% of cells appears differentiated (Joshi, 2000), a 5% more or less of differentiated cells would not affect the overall levels of Tubedown expression within a given specimen. Tubedown levels may only be reduced if the tumor specimen presents significant levels of differentiation as seen in ganglioneuroblastoma and ganglioneuroma. Moreover, *in vitro,* reduction in Tubedown levels only occurs upon extensive differentiation of neuroblastoma cells. *In vitro*, extensive neuronal differentiation was characterized by Tubedown suppression, increases in neurite-like formation and NF-M expression but a marked decrease in MycN expression. Reductions in growth, vimentin and c-Myb expression do not appear to be sufficient to lead to Tubedown suppression. Our *in vitro* analyses are in agreement with the pattern of expression of Tubedown in neuroblastic tumors and indicate that Tubedown expression correlates with the differentiation status of neuroblastic tumors.

Tubedown levels did not correlate with *MYCN* amplification status of neuroblastoma tumors. *MYCN* amplification is a poor prognosis factor that is observed in approximately 25% of neuroblastoma tumors (Brodeur, 2003). In our neuroblastoma cohort *MYCN* was found amplified in 29% of neuroblastoma tumors while high levels of Tubedown were found in 46% of neuroblastoma tumors. The relative risk of our neuroblastoma cohort for *MYCN* amplification was slightly low compared to the cohorts presented by others (Krams et al., 2004; Hsu et al., 2005). However, it is still clear that within our neuroblastoma cohort, amplification of *MYCN* leads to a higher relative risk compared to tumors that are non-*MYCN* amplified. At least one other factor, the allelic loss of 11q chromosomal locus, was associated with high-risk features (such as advanced stage, older age at diagnosis, and unfavorable histopathology) and did not correlate with *MYCN* amplification (Maris, 2005). Both MycN and Tubedown protein expression are reduced upon marked retinoic acid-induced differentiation of the LA-N-5 neuroblastoma cell line (Figure 2-6). However, although several studies have established a link between *MYCN* RNA and/or protein overexpression and poor outcomes, there is still some controversy surrounding this issue (Matthay, 2000; Tang et al., 2006).

To date very few markers have been proven to represent an independent prognostic value for neuroblastic tumors (Brodeur, 2003; Krams et al., 2004; Maris, 2005). Higher levels of Tubedown expression in advanced stage, histologically unfavorable and high-risk group neuroblastoma could be the consequence of a more aggressive tumor phenotype or could indicate that Tubedown itself somehow drives the aggressiveness of neuroblastic

tumors. *In vitro*, only marked differentiation of neuroblastoma cell lines is associated with reduced expression of Tubedown. In primary tumors, lower levels of Tubedown are observed in the more differentiated subsets of neuroblastic tumors. These results could indicate that Tubedown is involved in blocking differentiation. At present the role of Tubedown in aggressive neuroblastic tumors is not clear. However, in yeast the Tubedown homologue Nat1 acts in a complex with the acetyltransferase Ard1 to regulate a wide range of cellular processes including cell growth and differentiation (Gautschi et al., 2003; Polevoda and Sherman, 2003; Wang et al., 2004). Similarly to the yeast system, Tubedown forms a complex with a mouse homolog of the yeast Ard1 acetyltransferase (mARD1) (Sugiura et al., 2003) and has been shown to be involved in cellular processes that regulate cell growth and differentiation in different cellular contexts (Willis et al., 2002; Wall et al., 2004; Arnesen et al., 2006c).

My results indicate that the levels of Tubedown expression are linked to the differentiation status and aggressiveness of neuroblastic tumors. Tubedown expression may be useful to more accurately define different neuroblastic tumor subsets and to provide a new independent prognostic indicator of neuroblastoma.

2.1.7 Acknowledgements

The authors acknowledge the late Dr. Desmond Robb (Memorial University of Newfoundland), Hematology/Oncology Division Tumor Bank (Cincinnati Children's Hospital Medical Center, Cincinnati, OH), the Brain Tumor Tissue Bank (London, ON, Canada), and the Cooperative Human Tissue Network (National Cancer Institute) for providing neuroblastic tumor specimens; Dr. Thomas Inge (Cincinnati Children's Hospital Medical Center) for providing neuroblastoma cell lines; and Karina Leblanc for technical assistance.

3.1 Tubedown is up-regulated by MYCN in neuroblastoma

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[In preparation]

3.1.1 Authors' Contribution

Darryl Martin participated in the experimental design, performed all of the experimental work, carried out the analysis and interpretation of the data. Drs. Hélène Paradis and Robert L. Gendron participated in the experimental design, interpretation of the data, and provided financial support for the experiments and equipment. Dr. Manfred Schwab provided the *MYCN*-inducible SHEP cell system and Dr. Thomas Inge provided the neuroblastoma cell line.

3.1.2 Abstract

Background: Tubedown is a developmentally regulated subunit of the N-terminal acetyltransferase Ard1 that is highly expressed in a subset of neuroblastoma tumors. High levels of Tubedown expression in neuroblastoma correlate with high risk, low survival, and elevated levels of the transcription factor MycN. *MYCN* amplification occurs in approximately 20% of neuroblastomas, associates with poor prognosis and usually results in high *MYCN* expression. In the present study, I further characterized the link between Tubedown and MycN expression in neuroblastoma.

Methods: Patterns of expression of Tubedown, Ard1, and MycN during neuronal differentiation of neuroblastoma cells were analyzed by Western blot. The role of MycN on Tubedown and Ard1 expression was assessed using an inducible MycN expression neuroblastoma cell system. Chromatin Immunoprecipitation was used to determine if MycN binds to the promoter region of *TUBEDOWN*.

Results: I demonstrate a co-regulation between Tubedown, Ard1, and MycN expression during the neuronal differentiation of neuroblastoma cells. Moreover, conditional over-expression of MycN in neuroblastoma cells increased both Tubedown and Ard1 expression. *TUBEDOWN* promoter region was found to harbour a consensus E-box binding motif for MycN. Chromatin immunoprecipitation assays in neuroblastoma cells

confirmed that MycN directly binds the 5'-untranslated / promoter region of *TUBEDOWN* containing this E-box motif.

Conclusions: The regulation of *TUBEDOWN* by MycN could play a mechanistic role leading to poor outcome for neuroblastoma. Moreover, our work provides further insight into understanding the biology of neuroblastic tumors. *[In preparation]*

3.1.3 Background

Tubedown (Tbdn, also designated NATH, Narg1, hNat1) is associated with an acetyltransferase activity and is homologous to the yeast N-terminal acetyltransferase subunit Nat1p. Tbdn forms a functional acetyltransferase NatA complex with Ard1 which is conserved from yeast to humans (Park and Szostak, 1992; Sugiura et al., 2003; Arnesen et al., 2005a; Arnesen et al., 2008). *TBDN* is transiently expressed during embryogenesis in the developing vasculature and diminishes in adult during vascular maturity (Gendron et al., 2000). Similarly, a spatio-temporally specific expression pattern of *TBDN* and *ARD1* has been reported in neuronal tissue during embryogenesis. The expression of both genes decrease throughout brain development and is lowest in adult (Sugiura et al., 2003). There is evidence that the Tbdn/Ard1 complex is involved in a wide range of processes including cellular growth and differentiation (Park and Szostak, 1992; Paradis et al., 2002; Willis et al., 2002; Sugiura et al., 2003; Arnesen et al., 2005a; Arnesen et al., 2006b). For example, a decrease in Tbdn levels occur during retinoic acid (RA) induced differentiation of neuroblastic tumor cells and mouse embryonic carcinoma cells (Sugiura et al., 2003; Martin et al., 2007). Tbdn and Ard1 expression has been shown to be co-regulated and the knockdown of Tbdn in diverse cellular systems is associated with a reduction in Ard1 expression (Sugiura et al., 2003; Arnesen et al., 2006c; Hou et al., 2007; Paradis et al., 2008; Polevoda et al., 2008). Recently, new nomenclature has been introduced to rename *NAT1* as *NAA15* and *ARD1* as *NAA10* (Arnesen et al., 2009).

The NatA complex also plays a role in ribogenesis as it binds the nascent polypeptide-associated complex and associates with ribosomes in yeast (Gautschi et al., 2003; Arnesen et al., 2005a; Raue et al., 2007; Polevoda et al., 2008). N-terminal acetylation of nascent polypeptides by NatA occurs at specific substrate residues (Polevoda et al., 2008). A third component of the NatA found at ribosomes is the putative acetyltransferase Nat5, which was described in both yeast and human but has yet to be studied at the functional level (Gautschi et al., 2003; Arnesen et al., 2006a; Arnesen et al., 2008). Recently, our laboratory demonstrated that Tbdn is present in a complex with Cortactin (Paradis et al., 2008). Cortactin modulates the Actin cytoskeleton, is important for a variety of processes including cellular permeability (Mehta and Malik, 2006) and has been shown to be involved in tumor progression (Weaver, 2008).

Tbdn is also involved in tumorigenesis (Arnesen et al., 2006c). Tbdn is highly expressed in aggressive neuroblastoma tumors (Martin et al., 2007), thyroid carcinomas (Fluge et al., 2002), and gastric cancer (Line et al., 2002). Knockdown of Tbdn in tumor cells is associated with reduced viability (Arnesen et al., 2006c). In neuroblastoma, high levels of Tbdn expression have been linked to high-risk and poor outcome (Martin et al., 2007). This down-regulation in Tbdn expression is accompanied by suppression of the transcription factor MycN (also referred to as N-Myc) (Martin et al., 2007). *MYCN* is amplified in over 20% of neuroblastoma tumors (Brodeur, 2003). *MYCN* amplification usually results in high MycN mRNA and protein expression (Tang et al., 2006).

Amplification and over-expression of MycN contributes to neuroblastoma aggressiveness and can lead to increased tumor growth and tumorigenicity (Seeger et al., 1985; Weiss et al., 1997; Cohn and Tweddle, 2004). The Myc family of proteins (c-Myc, MycN and L-Myc) have been considered to have growth-promoting properties (Vita and Henriksson, 2006). Deregulation of Myc family members such as MycN can result in the development of neuroblastoma (Weiss et al., 1997). The Myc family of proteins belongs to the basic helix-loop-helix leucine (bHLH)-zipper class of transcription factors (Grandori and Eisenman, 1997) that dimerize with other bHLH family members such as Max, a ubiquitously expressed transcription factor (Lu et al., 2003). The MycN/Max complex recognizes and binds to the E-box motif (CACGTG) with high affinity resulting in transcriptional activation of target genes (Vasudevan et al., 2005). To date, there are a limited number of MycN/Max target genes known to be expressed in neuroblastomas such as cellular RA binding protein II (CRABP-II) (Gupta et al., 2006), mini-chromosomal maintenance proteins 2 - 7 (MCM2 - 7) (Koppen et al., 2007), and multi-drug resistance-associated protein (MRP1) (Manohar et al., 2004). However, the MycN target genes responsible for neuroblastoma tumor progression are still unclear (Manohar et al., 2004). Consequently, the co-regulation of Tbdn and MycN in neuroblastoma cells may indicate that Tbdn is involved in a dependent or independent mechanism with MycN, which may play a role in regulating cancer growth. Based upon previous data in which I found that Tbdn expression was correlated with neuroblastoma risk, I undertook the present study to determine if MycN regulates Tbdn expression. My results

demonstrate that MycN is regulating both Tbdn and Ard1 expression. In addition, MycN can bind directly to the *TBDN* promoter region.

3.1.4 Methods

Cell Cultures – The LA-N-5 (Seeger et al., 1982b) neuroblastoma cell line was a gift from Dr. Thomas Inge (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The neuroblastoma SHEPTet2 (Tet2) and SHEPTet2/Nmyc (Tet2N) cell lines were gifts from Dr. Manfred Schwab (German Cancer Research Center, Heidelberg, Germany) and have been described previously (Lutz et al., 1996). In brief, the Tet2 cells were generated by co-transfection of SHEP cells (a subclone of SK-N-SK human neuroblastoma cells) with pUHD15-1 containing tTA coding sequence under the control of the human cytomegalovirus promoter and a plasmid conferring neomycin resistance. Tet2N cells were generated by co-transfection of Tet2 cells with a hygromycin resistance plasmid and pUHC10-3 containing human *MYCN* coding sequence under the control of seven copies of the tet operator and a cytomegalovirus minimal promoter. Upon removal of tetracycline, MycN expression is induced in the Tet2N cell line (Lutz et al., 1996).

The LA-N-5 cells were maintained in RPMI 1640 Media supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. The Tet2 cells were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 4 mM glutamine, and 200 μg/ml G418. The Tet2N cells were maintained in the same media as

Tet2 with the addition of 90 μg/ml hygromycin B. In order to induce MycN suppression in the Tet2N cells, tetracycline was added to a final concentration of 1 μg/ml. The LA-N-5 cells were treated with all-trans RA (Sigma, Oakville, Ontario, Canada) to induce differentiation at an optimal concentration of 4 μM. The culture media with or without RA was changed every 3 days. Exponentially growing cells were used for every experiment.

Antibodies – Anti-chicken Ard1 antibody OHO20 was generated by immunizing chickens with a KLH-conjugated 11 mer peptide (ENKVESKGNSP) corresponding to the amino acids sequence of human Ard1 positions 177-187 onto which a cysteine residue was added at the N-terminus to facilitate coupling (Covance Research Products, Denver, PA). Other antibodies used in this study include ARD1 (E-16) (Santa Cruz Biotechnology, Santa Cruz, CA), Max (C-17) (Santa Cruz Biotechnology), N-Myc (C-19) (Santa Cruz Biotechnology), MycN(2) [sc:142] (Santa Cruz Biotechnology), Tbdn C755-766 (Paradis et al., 2008), extracellular signal-regulated kinase (ERK)-1 (K-23) (Santa Cruz Biotechnology), alpha-Tubulin (Sigma) and negative control isotype match mouse IgG_{2a} (Dako, Glostrup, DK).

Western Blotting – Cell lysates were prepared from cell lines as previously described (Gendron et al., 2000). Protein lysates were quantified using Albumin as standard and analyzed by SDS-PAGE. Western blotting was performed using standard procedures as previously described (Gendron et al., 2000; Gendron et al., 2001). Western blot

chemiluminescence signal detection varied depending on the antibody used. ECL advance (Amersham Biosciences, Piscataway, NJ) was used for detection of Tbdn C755-766, OHO20, ARD1, and N-Myc antibodies; Lumiglo (KPL, Gaithersburg, MD) was used for the detection of the Max antibody; and ECL plus chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ) was used for the detection of the ERK-1 and alpha-Tubulin antibodies. Densitometric analyses were performed using the Kodak Gel Logic Imaging System (Eastman Kodak Company, Rochester, NY) with Kodak Molecular Imaging Software (Version 4.0, Eastman Kodak Company, Rochester, NY).

Chromatin Immunoprecipitation (ChIP) – ChIP assays were based on modified versions of published protocols (Boyd and Farnham, 1999; Strieder and Lutz, 2003) (Farnham laboratory, http://genomecenter.ucdavis.edu/farnham/farnham/protocols /chips.html). The Tet2N (MycN inducible) cell line was cultured in the presence or absence of 1 μg/ml of tetracycline. Cultures were cross-linked with the addition of formaldehyde to a final concentration of 1% for 10 min at room temperature. To terminate the cross-linking reaction glycine was added to a final concentration of 125 mM. Cells were washed and scraped into cold phosphate-buffered saline, centrifuged, resuspended in swelling buffer (5 mM HEPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, and protease inhibitors such as 1 mM Phenylmethanesulfonyl fluoride [PMSF], 0.3 U/ml aprotinin, 10 μg/ml leupeptin) and incubated on ice. Cells were next homogenized and centrifuged. The pellets were resuspended in sonication buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, and 500 μM PMSF) and incubated on ice.

Lysed nuclear extracts were then sonicated at an amplitude of 15% maximum power using a Virsonic Cell Disrupter (The Virtis Company, Gardiner, NY) for two 10 seconds pulses on ice to obtain DNA fragments of 500-1000 bp. The chromatin solutions were cleared for 10 min. at 10000X*g*. Chromatin pre-clearing was performed using pre-blocked (with 1 μg of sheared herring sperm DNA per ml and 1 μg of Bovine Serum Albumin per ml for at least 4 hrs at 4°C) protein-G sepharose for 15 min. at 4°C on a rotating platform then centrifuged at 10000X*g*. Immunoprecipitations were performed overnight at 4°C with a mouse monoclonal MycN(2) [sc:142] or the control isotype matched IgG_{2a}. The samples were eluted, reversed cross-linked and Real-time qPCR was performed. For each ChIP assay, a standard control was used. A ratio was obtained using the standard control with both the MycN immunoprecipitation and the IgG_{2a} immunoprecipitation. For each primer pair, the difference in threshold cycle (delta CT) was calculated.

Real-time PCR (qPCR) – DNA was amplified by real-time PCR using a 7000 sequence detector system (Applied Biosystems, Foster City, CA). The area of interest included an E-box site located within the *TBDN* promoter region [GenBank: NM057175] with the forward primer, 5'-CAAGCTCCGAAACCCAGAG-3' (Figure 3: -116 to -98), and the reverse primer, 5'-CGTAGCTGCTTCAGCCTTG-3' (Figure 3: +161 to +179). The positive control contained an E-box sequence located within the *MCM2* promoter [GenBank: NM004526] with the forward primer, 5'-CTCCGTGTCCCTTCTGGTC-3' (nucleotide positions from the predicted transcription start site: -91 to -73), and the

reverse primer, 5'-GATCCTCTCCGCCACTACAG-3' (nucleotide positions from the predicted transcription start site: +27 to +46) (Koppen et al., 2007). The negative control lacked an E-box sequence within the *MCM10* promoter region [GenBank: NM182751] with the forward primer, 5'-CCTCGCAGGCTTTGTAGATT-3' (nucleotide positions from the predicted transcription start site: +728 to +747), and the reverse primer, 5'-GCCACGGTAATCTTCCCTTT-3' (nucleotide positions from the predicted transcription start site: +833 to +852) (Koppen et al., 2007).

Sequence Analysis of TBDN 5'-Untranslated region (UTR) / Promoter – The transcriptional start site for the *TBDN* gene was determined using the Database of Transcriptional Start Sites (DBTSS) (http://dbtss.hgc.jp) in combination with the NCBI database (http://www.ncbi.nlm.gov/sites/entrez?db=pubmed). The transcriptional start site and 5'-UTR for *TBDN* was located on chromosome 4 (GenBank: NM_057175) using the Entrez Gene component of the NCBI database. Examination of the 5'-UTR / promoter region was completed using manual scanning, along with, the Transcription Element Search System (TESS) software (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and the TFSEARCH software (http://www.cbrc.jp/research/db/TFSEARCH.html).

3.1.5 Results

3.1.5.1 Tbdn and Ard1 are co-regulated with MycN in neuroblastoma cells undergoing marked neuronal differentiation

To assess patterns of expression of Tbdn, Ard1, MycN and MycN binding partner Max during differentiation of neuroblastoma cells, levels of these three proteins were co-examined upon RA-induced differentiation of LA-N-5 cells by Western blot analyses (Figure 3-1). Tbdn expression was down-regulated upon RA-induced differentiation of LA-N-5 cells with a 2.1-fold decrease at day 3 (Figure 3-1). Similarly, RA-induced differentiation in LA-N-5 cells was accompanied by a marked down-regulation of Ard1 protein expression with a 4.2-fold decrease at day 3 (Figure 3-1). MycN expression was decreased by 5-fold upon 3 days of RA treatment of human neuroblastoma LA-N-5 cells. On the other hand, the level of Max, a transcriptional binding partner of MycN, was decreased by 1-fold compared to untreated cells following 3 days of RA treatment of LA-N-5 neuroblastoma cells (Figure 3-1).

3.1.5.2 Conditional MycN over-expression leads to up-regulation of Tbdn and Ard1

To further investigate the relationship between MycN and Tbdn/Ard1, a conditional MycN expression system (Lutz et al., 1996) was used to determine the effect of MycN over-expression on Tbdn and Ard1 expression in a neuroblastoma cell line. For

Figure 3-1. Tbdn co-regulation with MycN/Ard1 during cell differentiation.

Western blot analyses of untreated (-) and 3 days of RA-treated human neuroblastoma LA-N-5 cells was performed. RA treatment of LA-N-5 cells induces a significant decrease in the levels of Tbdn (101 kDa), Ard1 (28 kDa), and MycN (67 kDa) expression whereas only a minimal change in Max (21/20 kDa) expression was detected. ERK1/2 (44/42 kDa) Western blot analysis was used as a loading control. The results are from representative experiments.

these analyses I used a MycN inducible cell line (Tet2N) previously derived by Lutz et al. (1996) based on the SHEP neuroblastoma cell line (as it lacks endogenous MycN expression). In the Tet2N cells MycN expression is suppressed upon tetracycline treatment (Figure 3-2; Lutz et al., 1996). The conditional over-expression of MycN is accomplished in the absence of tetracycline by the induction of a *MYCN* cDNA construct under the control of the tetracycline responsive element (TRE) and the tetracycline-regulated transactivator (tTA) (Lutz et al., 1996; Zhu et al., 2002). Western blot analyses showed that removal of tetracycline from the Tet2N cell line resulted in an over-expression of MycN (Figure 3-2) as previously described (Lutz et al., 1996). The Tet2 (empty vector) cells did not express MycN upon removal of tetracycline. Parallel Western blot analyses showed that Tbdn was up-regulated 2.2-fold and Ard1 was up-regulated 1.7-fold after tetracycline withdrawal from Tet2N cell line but not from Tet2 control cells (Figure 3-2).

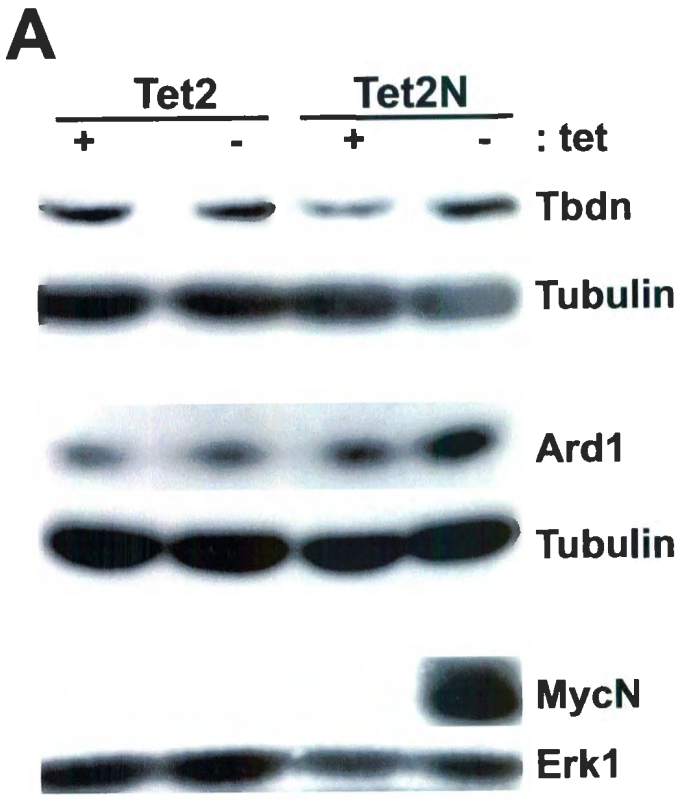
3.1.5.3 MycN binds a TBDN promoter region

Based on the co-regulation of Tbdn and Ard1 with MycN and the up-regulation of Tbdn and Ard1 upon conditional over-expression of MycN, the genomic sequence of *TBDN* promoter region was examined for a Myc-responsive element, an E-box binding motif. An E-box binding motif was located within the 5'-UTR / promoter region of *TBDN*. Figure 3-3 illustrates that the E-box motif is located after the transcriptional start site (at position +12) within the beginning of the 5'-UTR of *TBDN* transcript. The *TBDN* promoter appears to be a TATA-less promoter similar to the *MYCN* promoter (Inge et al.,

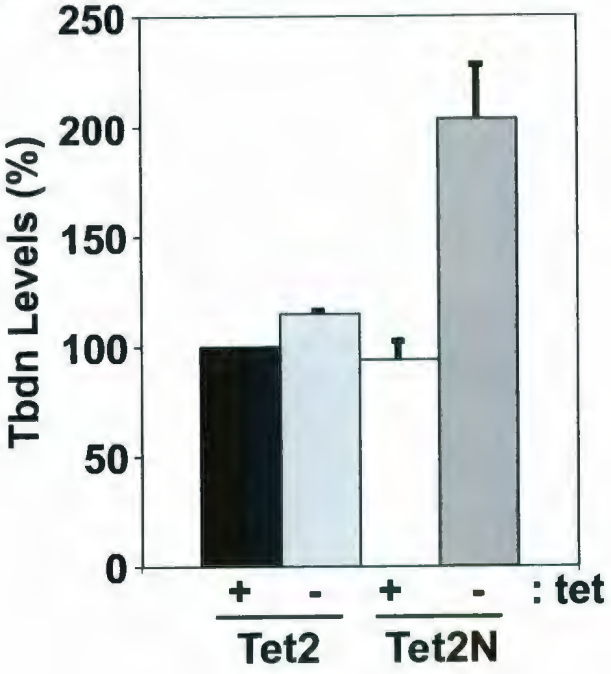
2002). In addition, the *TBDN* promoter is GC rich and includes a GC-box element at position -177 to -172 (Figure 3-3) which is known to bind SP1. SP1 is a transcription factor involved in early development, binding sites for which are also seen within the *MYCN* promoter region (Inge et al., 2002). The *TBDN* promoter also contains three Hypoxia responsive elements (HRE) harboring the consensus sequences 5'-(A/G)CGTG-3'. Two of these HRE motifs are found within the promoter region while a third motif overlaps with the E-box motif located within the 5'-UTR (Figure 3-3). To determine if MycN binds the 5'-UTR and promoter region of *TBDN* encompassing the Myc-

Figure 3-2. MycN over-expression induces Tbdn and Ard1 expression.

*(**A**) Western blot analyses revealed that removal of tetracycline from the in vitro MYCN-inducible expression system, Tet2N, resulted in an over-expression of MycN and an up-regulation of Tbdn and Ard1 expression. Graphical representation of Tbdn (**B**) and Ard1 (**C**) levels are shown. Each Western blot is paired with the respective loading control Tubulin or ERK. Results represent the average of at least three experiments. The results in **A** are from representative experiments. The error bars in **B** and **C** represent the standard error.*


A
Tet2
Tet2N
+
-
+
-
: tet
Tbdn
Tubulin
Ard1
Tubulin
MycN
Erk1

B


250
200
150
100
50
0
Tbdn Levels (%)
+
-
+
-
: tet
Tet2
Tet2N

C

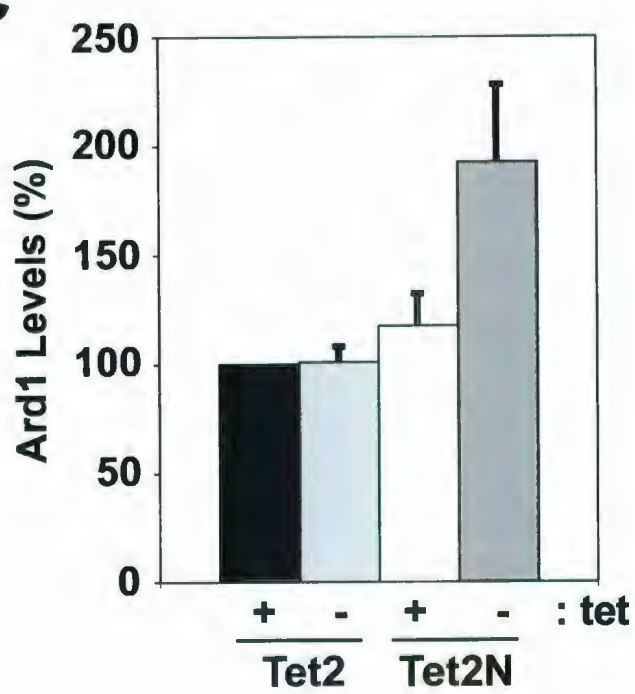
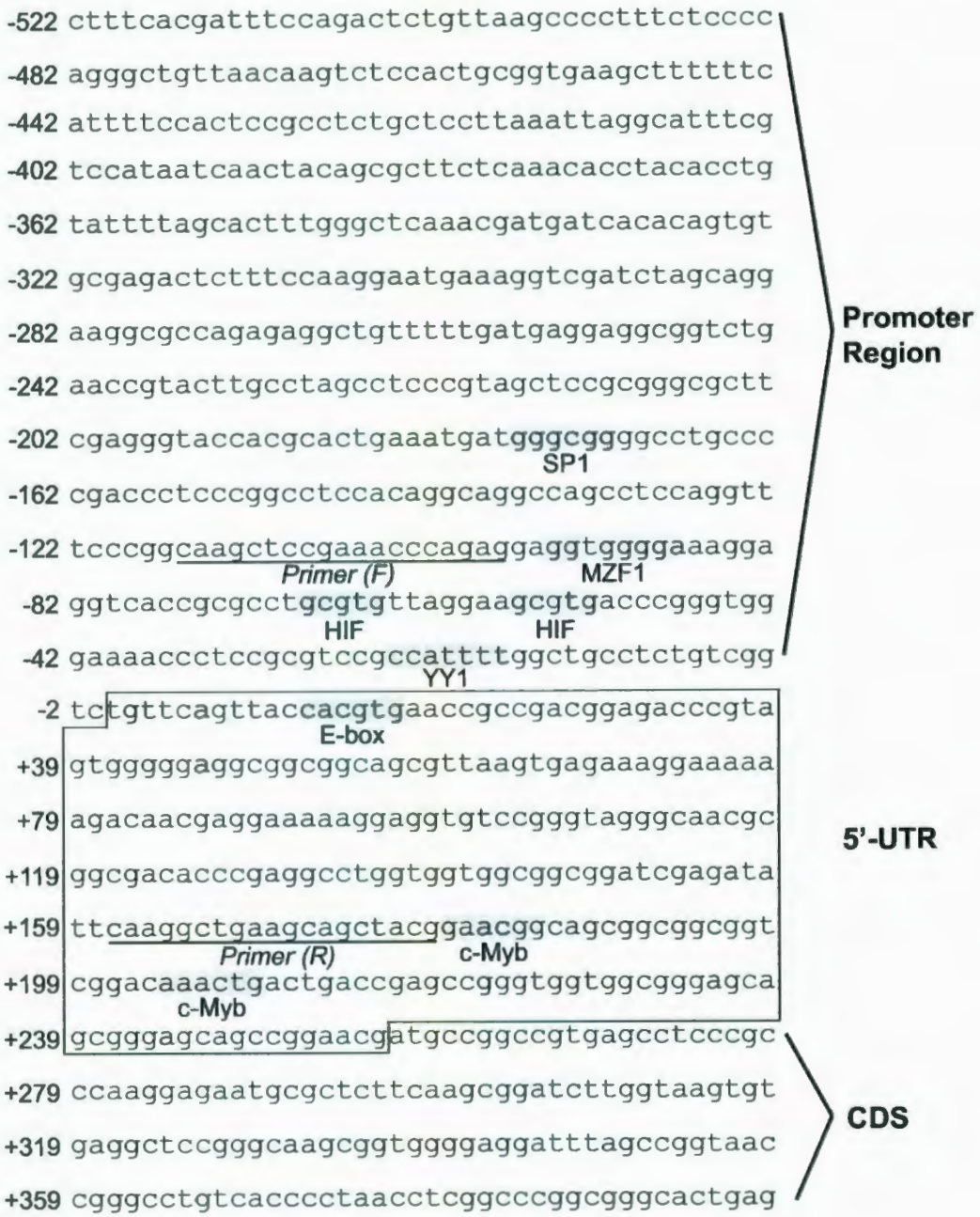

250
200
150
100
50
0
Ard1 Levels (%)
+
-
+
-
: tet
Tet2
Tet2N

Figure 3-3. The nucleotide sequence of TBDN promoter region contains a canonical E-box motif.

The illustration depicts the nucleotide sequence of TBDN promoter region and TBDN transcript [GenBank:NM057175]. The nucleotide sequence contains 522 bp of the proximal promoter, 256 bp of the 5'-UTR and 142 bp of the coding sequence (CDS). Binding sites for known transcription factors are highlighted in gray whereas the forward (F) and reverse (R) primers are underlined.


-522 ctttcacgatttccagactctgttaagccccttctcccc
-482 agggctgttaacaagtctccactgcggtgaagctttttc
-442 attttccactccgcctctgctccttaaattaggcatttcg
-402 tccataatcaactacagcgcttctcaaacacctacacctg
-362 tattttagcactttgggctcaaacgatgatcacacagtgt
-322 gcgagactctttccaaggaatgaaaggtcgatctagcagg
-282 aaggcgccagagaggctgtttttgatgaggaggcggtctg
-242 aaccgtacttgcctagcctcccgtagctccgcgggcgctt
-202 cgagggtaccacgcactgaaatgatgggcgggcctgccc
SP1
-162 cgaccctcccggcctccacaggcaggccagcctccaggtt
-122 tcccggcaagctccgaaacccagaggaggtggggaaagga
Primer (F)
MZF1
-82 ggtcaccgcgcctgcgtgttaggaagcgtgacccgggtgg
HIF
HIF
-42 gaaaaccctccgcgtccgccattttggctgcctctgtcgg
YY1
-2 tctgttcagttaccacgtgaaccgccgacggagacccgta
E-box
+39 gtgggggaggcggcggcagcgttaagtgagaaaggaaaaa
+79 agacaacgaggaaaaaggaggtgtccgggtagggcaacgc
+119 ggcgacacccgaggcctggtggtggcggcggatcgagata
+159 ttcaaggctgaagcagctacggaacggcagcggcggcggt
Primer (R)
c-Myb
+199 cggacaaactgactgaccgagccgggtggtggcgggagca
c-Myb
+239 gcgggagcagccggaacgatgccggccgtgagcctcccgc
+279 ccaaggagaatgcgctcttcaagcggatcttggtaagtgt
+319 gaggctccgggcaagcggtggggaggatttagccggtaac
+359 cgggcctgtcacccctaacctcggcccggcgggcactgag
Promoter Region
5'-UTR
CDS

responsive element (E-box) a ChIP assay was performed. The Tet2N cell line (which expressed MycN in the absence of tetracycline) was used to analyze the *TBDN* 5'-UTR and promoter region. Throughout the ChIP procedure various confirmatory steps were required. The cells were monitored under the microscope during the addition of the swelling buffer and during the lysis of the nuclei. In addition, samples from the sonication step were verified on a gel to ensure correct fragment size (500 – 1000 bp). Quantitative Real-time PCR (qPCR) was then performed on DNA immunoprecipitated with either an anti-MycN antibody or an anti-IgG_{2a} negative isotype matched control antibody. The variation in threshold cycle (delta CT) of the qPCR was determined for each sample. qPCR using *TBDN* primers flanking the E-box motif in *TBDN* 5'-UTR / promoter region and ChIP DNA revealed approximately a 6-fold increase in the delta CT of MycN-ChIP over IgG_{2a}-ChIP (Figure 3-4). *MCM2* primers flanking an E-box motif in *MCM2* promoter (positive control promoter region regulated by MycN; Koppen et al., 2007) region showed approximately an 8-fold increase in the delta CT between the MycN-ChIP samples and the negative control IgG_{2a}-ChIP (Figure 3-4). *MCM10* primers flanking a region in *MCM10* promoter devoid of an E-box (negative control promoter region not regulated by MycN; Koppen et al., 2007) presented no increase in the delta CT between the MycN-ChIP samples and the IgG_{2a}-ChIP (Figure 3-4). The qPCR products for all experiments were verified on a gel to ensure that only one product was present.

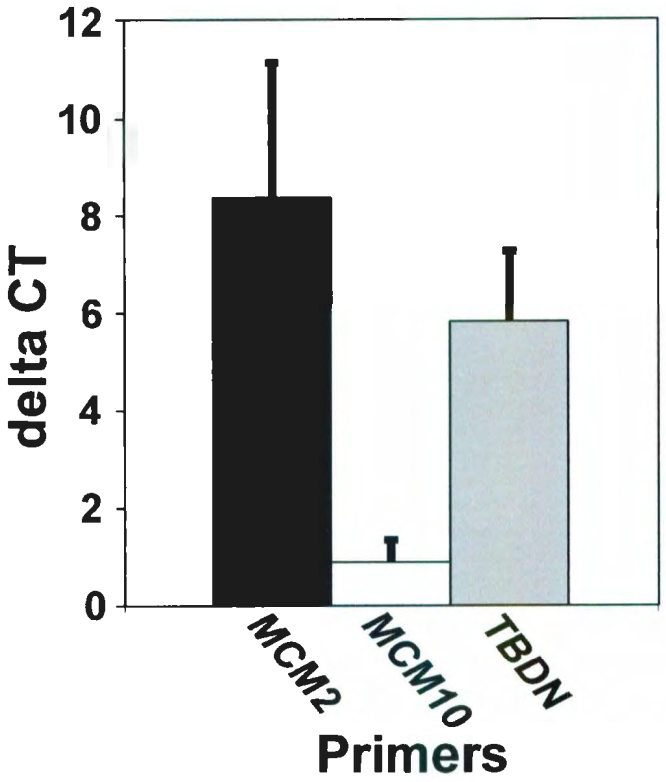


Figure 3-4. MycN binds to the TBDN promoter.

Delta Ct of qPCR on ChIP analysis of MycN binding to the E-box motif region in the MCM2 (black bar) or TBDN (gray bar) promoter and absence of binding of MycN to MCM10 (white bar) promoter region devoid of E-box motif. The error bars represent the standard error.

3.1.6 Discussion

Previously, I showed that high levels of Tbdn expression are linked with aggressive and high-risk group neuroblastoma and are associated with poor survival (Martin et al., 2007). The aggressiveness of neuroblastoma has also been linked to *MYCN* amplification (Thiele et al., 1985; Wei et al., 2008) but the reason for such an association is still unclear (Brodeur, 2003). *MYCN* amplification is associated with poor prognosis in neuroblastoma patients (Pession and Tonelli, 2005). There is a correlation between *MYCN*-amplified neuroblastoma tumors and MycN expression (Tang et al., 2006). Over-expression of human MycN under the control of a tyrosine hydroxlyase promoter in a transgenic mouse model leads to the development of neuroblastoma (Weiss et al., 1997). It is also worth mentioning that there was a co-regulation between Tbdn and MycN upon RA-induced differentiation of neuroblastoma cells (Martin et al., 2007). Based upon these findings, I sought to determine here if Tbdn expression and MycN are in any way functionally linked.

In this report, I demonstrate that Tbdn and Ard1 protein expression correlate with MycN expression during RA-induced differentiation of neuroblastoma cells. Our group as well as others have reported a significant down-regulation of MycN expression upon RA treatment of neuroblastoma cells (Thiele et al., 1985; Martin et al., 2007). In addition, a significant down-regulation of both Tbdn and Ard1 expression accompanied this RA-

induced differentiation. Although MycN heterodimerizes with Max to activate transcription, I have observed only a moderate decrease in Max expression upon RA-induced differentiation. Nevertheless, our results are in agreement with Cetinkaya et al. (2007) who demonstrated a modest reduction in Max expression upon RA-induced LA-N-5 cells (Cetinkaya et al., 2007). To verify my results, additional neuroblastoma cell line with and without *MYCN* amplification would be required.

The well-established MycN-induced SHEP model (Lutz et al., 1996) has been widely used to investigate a number of target genes in neuroblastoma (Manohar et al., 2004; Slack et al., 2005; Gupta et al., 2006; Koppen et al., 2007; Dasgupta et al., 2009). The MycN-inducible model was used to study the effects of both Tbdn and Ard1 in neuroblastoma cells. The parental SHEP cell line is an epithelial-like cell line (S-type) and not a neuronal derived cell line (N-type) (Ross et al., 1983). The SHEP cell line was used by Lutz and colleagues because it lacked MYCN amplification and MYCN expression. My results show an increase in Tbdn and Ard1 protein expression upon conditional over-expression of MycN in neuroblastoma cells. These observations indicate that MycN regulates Tbdn and Ard1 expression in neuroblastoma. In addition, our previous data demonstrated a correlation between MycN, Tbdn, and neuroblastoma differentiation status (Martin et al., 2007). To verify my MYCN-inducible data, an alternative approach targeting the knockdown of MYCN through siRNA or antisense technology could have been used.

To further explore if the regulation of Tbdn expression by MycN was direct or indirect, the promoter / 5'-UTR region of *TBDN* was examined for potential MycN binding sites. The identification of a MycN canonical E-box motif, CACGTG, within the *TBDN* promoter / 5'-UTR region suggests that MycN could potentially regulate Tbdn expression directly through up-regulation of transcription. Conversely, no canonical E-box motif could be identified within the *ARD1* promoter region for MycN to bind and regulate *ARD1* expression. It is possible that MycN regulates Ard1 expression indirectly through the action of another transcription factor. Alternatively, Tbdn levels have been shown to regulate Ard1 in systems other than neuroblastoma such as thyroid carcinoma (Arnesen et al., 2005b), cervix adenocarcinoma HeLa cells (Arnesen et al., 2006c), and endothelial cells (Paradis et al., 2008). Therefore, Tbdn up-regulation itself could be responsible for the up-regulation of Ard1 in neuroblastoma. However, the mechanism for such regulation remains to be elucidated.

Our results further show that MycN binds to the promoter / 5'-UTR region of *TBDN* that contains the identified canonical E-box motif. These results suggest that MycN directly regulates Tbdn expression through regulation of gene expression. It is not uncommon for E-box elements to be located within the 5'-UTR. Miltenberger et al. (1995) mapped an E-box motif to the 5'-UTR of *Cad* which was responsible for growth-dependent transcription of this gene (Miltenberger et al., 1995). Similarly, Hayama et al. (2007) reported the presence of an E-box motif within the 5'-UTR of *Polyserase-1* as a required element for maximal promoter activity (Hayama et al., 2007).

An E-box motif within the promoter / 5'-UTR of *TBDN* indicate a potential binding site for not only Myc/Max heterodimer but as well as other factors such as Max homodimer, Max/Mad heterodimer, Max/Mnt heterodimer, and USF and TFE3 (Facchini and Penn, 1998; Vasudevan et al., 2005). In addition to having a canonical core E-box binding motif, other characteristics may determine which factor is capable of binding to this site. Previous studies have shown that nucleotides flanking the E-box motif determine the most likely factor to bind the consensus sequence. Halazonetis & Kandil (1991) tested a number of oligonucleotides varying the sequences flanking the E-box and reported that the sequence with the highest affinity for Myc/Max was GAC-CACGTG-GTC (Halazonetis and Kandil, 1991). However, the flanking 5' nucleotides sequence T(-3)A(-2)C(-1) also provided an increase in the affinity of the tested promoter region for the Myc/Max heterodimer. The numbers represent the position of the nucleotide from the flanking core sequence. For example, -1 at the 5' (or +1 at the 3') would represent the position closest to the core sequence whereas -3 (or +3) would be located 3 positions from the core sequence. A previous study suggested that purines in either the -2 or the -3 positions are favored whereas nucleotides such as 5'-T at position -1 (or 3'-A at position +1) would not be preferred residues for Myc/Max (Solomon et al., 1993). However, Swanson and Yang (1999) demonstrated that there can be some variations in the 5' and 3' flanking sequences. In particular, the 5'-A/C/T and 3'-G/A/T can be found in the -1 and +1 positions, respectively (Swanson and Yang, 1999). The Myc/Max consensus binding sequence has been defined as 5'- RH**CACGTG**DY-3' (Swanson and Yang,

1999). The nucleotides flanking the high affinity E-box motif in *TBDN* consist of TAC in the 5' region whereas the nucleotides in the 3'- region consist of AAC. These nucleotide arrangements would indicate a preferred target for Myc/Max in the *TBDN* promoter / 5'-UTR region.

The aforementioned criteria are important in determining potential transcription factor binding sites. However, the primary criterion for determining the ability of a transcription factor to bind directly to a gene is the actual binding of the factor to the regulatory DNA element in the chromatin *in situ* (Fernandez et al., 2003). One approach to assess transcription factor binding sites is the ChIP assay, which can be performed in live cells (Fernandez et al., 2003). Using ChIP methodology, I show here that MycN binds to the *TBDN* promoter / 5'-UTR region encompassing the high affinity E-box motif. It is well known that Tet2N cells can be induced to express MycN upon withdrawal of tetracycline. However, in the presence of tetracycline MycN expression is suppressed in Tet2N cells to a minimum (Figure 2A). Lutz et al. (1996) showed that *MYCN* expression is not completely repressed in Tet2N cells in the presence of tetracycline (Lutz et al., 1996). Accordingly, MycN specific ChIP assays performed on tetracycline treated Tet2N cells showed very minimal binding of MycN to the *TBDN* promoter -5'UTR region encompassing the E-box with less than 1-fold delta CT (data not shown).

Members of the Myc family of transcription factors have been linked to many important cellular processes including proliferation which is known to provide a growth advantage to breast cancer cells, small cell lung carcinomas, and neuroblastoma cells (Ben-Yosef et al., 1998). Conditional over-expression of MycN in Tet2N cells has been associated with an increase in cell growth (Lutz et al., 1996; Koppen et al., 2007). In this report, I show that the conditional over-expression of MycN in Tet2N cells is accompanied by an increase in Tbdn and Ard1. Moreover, in neuroblastoma cells I previously showed that reduced Tbdn expression is accompanied with a decrease in cell growth and increased differentiation (Martin et al., 2007). Arnesen and colleagues (2006) reported that the knockdown of Tbdn expression in epithelial cervical adenocarcinoma (HeLa) cells using siRNA results in apoptosis (Arnesen et al., 2006c). Ard1 has also previously been shown to play a role in cellular viability (Fisher et al., 2005; Arnesen et al., 2006c; Lim et al., 2006; Arnesen et al., 2008). It is worth mentioning that cervical carcinoma cells such as HeLa cells have been shown to over-express c-Myc (Macville et al., 1999; Henriksson et al., 2001). Based on these observations it is temping to speculate a relationship between Tbdn and Myc family of transcription factors in other models as well as neuroblastoma. These studies collectively suggest that Tbdn may play a role in the growth of cancers.

A possible explanation for the association of Tbdn and MycN with a growth advantage for cancer cells may be based on ribosome biogenesis. Boon and co-workers (2001) demonstrated that MycN enhanced the expression of a large group of genes involved in ribogenesis supporting a role for MycN in ribosome biogenesis and in protein synthesis

(Boon et al., 2001). Gautschi et al. (2003) reported in yeast that the Tbdn orthologue Nat1p, is required to anchor Ard1p and Nat5p to the ribosome, and interacts with the nascent polypeptide-associated complex (Gautschi et al., 2003). Arnesen et al. (2005) demonstrated the association of Tbdn and Ard1 with ribosomal subunits (Arnesen et al., 2005a). It is well known that the ribosome is essential for cell growth. However, deregulation of ribogenesis can lead to oncogenic transformation (Bilanges and Stokoe, 2007). One example of the link between deregulation of a product of ribogenesis and oncogenesis is the ribosomal protein RPL35a. RPL35a has anti-apoptotic activity and has been shown to be over-expressed in glioblastoma, a type of malignant brain tumor that has high resistance to chemotherapy (Lindstrom, 2009). Whether the links between Tbdn and MycN impact upon ribosome biogenesis or are in any way responsible for dysfunction of growth control via ribogenesis in neuroblastoma deserves further investigation.

3.1.7 Conclusions

The work herein demonstrates a co-regulation between Tubedown, Ard1, and MycN expression during the neuronal differentiation of neuroblastoma cells. In addition, I demonstrate an increase in Tubedown and Ard1 expression upon conditional over-expression of MycN in an inducible neuroblastoma cellular system. Finally, I show that MycN binds to the *TBDN* promoter. Collectively, these results suggest a new role for MycN in regulating Tbdn expression.

3.1.8 List of abbreviations

Tbdn (NATH and Narg1), Tubedown; RA, retinoic acid; PMSF, Phenylmethanesulfonyl fluoride; UTR, untranslated region; CRABP-II, cellular retinoic acid binding protein II; MCM 2 – 7, mini-chromosomal maintenance proteins 2 – 7; MRP1, multi-drug resistant protein; Tet2, SHEPTet2; Tet2N, SHEPTet2/Nmyc; ChIP, Chromatin Immunoprecipitation; qPCR, Quantitative PCR; DBTSS, Database of Transcriptional Start Sites; TESS, Transcription Element Search System; HRE, Hypoxia responsive elements; Hif, Hypoxia-induced transcription factor.

3.1.9 Acknowledgements

The authors acknowledge Dr. Manfred Schwab (German Cancer Research Center) for providing the MYCN inducible cell system and Dr. Thomas Inge (Cincinnati Children's Hospital Medical Center) for providing a neuroblastoma cell line. This work was supported by operating grants from Canadian Institutes of Health Research and infrastructure grants from Canada Foundation for Innovation to R.L.G and H.P.

4.1 Suppression of Tubedown Expression

4.1.1 Introduction

RA treatment of neuroblastoma cells lead to a more differentiated phenotype associated with reduced cell growth *in vitro* (Sidell et al., 1983). My previous data demonstrated that RA treatment of human neuroblastoma LA-N-5 cells induced differentiation and resulted in a decrease in Tbdn expression (Martin et al., 2007). In addition, Arnesen and colleagues showed that knocking down Tbdn levels in human cervical (HeLa) cells resulted in reduced cell viability (Arnesen et al., 2006c). Therefore, the functional importance of Tbdn had to be investigated in neuroblastoma cells. In particular, suppressing Tbdn levels may play a role in inhibiting growth and/or promoting differentiation in neuroblastoma cells.

4.1.2 Materials and Methods

Cell culture – LA-N-5 (Seeger et al., 1982a) cells were a gift from Dr. Thomas Inge (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). The LA-N-5 cells were maintained in RPMI 1640 media and supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. For all transfection experiments, LA-N-5 cells were passaged one day before and grown to 80% confluency.

siRNA – Three hundred to 1000 nM of gene-specific siRNA (Dharmacon, Lafayette, CO, USA) were used for each nucleofection. The following siRNA duplex target sequences were used: HS-TBDN611 (positive control): 5'- GGGACCUUUCCUUACUACA-3' (Arnesen et al., 2006c); SC47 (negative scramble control): 5'-GAUCCGUUCAUCGUCACUA-3'; HS-TBDN1138: 5'-GAAGGCUGCCGUUAAACUU-3.

Nucleofection – Nucleofections were completed according to the supplier's adherent cell line manual (Amaxa, Cologne, Germany). In brief, LA-N-5 cells were washed with sterile phosphate-buffered saline, trypsinized and counted to determine cell density. The cells were centrifuged at room temperature for 10 min at 90 g. Following media aspiration, the cell pellet was resuspended in 100 ul of nucleofector solution V (4.5U) containing the supplement solution (1U). The final concentration of the samples were $4X10^6$ cells/100 ul and $1X10^6$ cells/100 ul. One thousand nanomolar siRNA and 2 ug pmax Green Fluorescent Protein (GFP; for monitoring cell transfection efficiencies) were added directly to the cell suspension (100 ul /cuvette). The nucleofector device was set to program V-001 or U-014. Post nucleofection, the cells were returned to the humidified 37°C / 5% CO_2 incubator.

Microinjection – The Femtoject (Eppendorf, Mississauga, ON, Canada) and InjectMan NI 2 (Eppendorf) were used to inject affinity purified anti-Tbdn rabbit antibody C10-20 (Paradis et al., 2008) into LA-N-5 cells. Prior to experiment, a tracer such as GFP (100

ug/ml) or Hoechst (0.15 ug/ml) were mixed with the Tbdn antibody and loaded directly into the Femtotip. Subsequently, LA-N-5 cells were monitored using a Leica DMIRE2 microscope system.

Liposome-mediated transfection – Liposome transfections were completed according to the supplier's manual (Invitrogen, Burlington, ON, Canada). First, the host cell line, LA-N-5, was incubated with 10 ug regulatory plasmid (pTet-on) and 0.5 – 5 ul of lipofectamine (or lipofectin) per ug of DNA. After 48 hrs, 100 ug/ml of G418 was added to each plate. G418-resistant clones were selected and analyzed by Northern blot to determine which clone had the highest fold-induction (i.e. highest expression with the lowest amount of background). Secondly, the response plasmid (pTRE-ASTBDN) was co-transfected with a selection agent (pTK-Hyg) at a ratio of 10:1 or 20:1. For every 1 ug of DNA, 0.5 – 5 ul of lipofectamine (or lipofectin) was added. The mixture was placed at room temperature for 20 min before adding to the LA-N-5 cells. After 48 hrs, 50 ug/ml of hygromycin B (HygroB) was added to each plate. HygroB-resistant and G418-resistant clones were selected and analyzed by Southern blot for the presence of the pTRE-ASTBDN construct and then by Northern and Western blot to determine if the levels of Tbdn have been diminished.

Adenofection – LA-N-5 and Ewing's sarcoma cells were infected with adenovirus at varying levels of MOI (7.5, 10, 12.5). The recombinant adenoviral vector transducing

ASTBDN-1 under the control of the CMV promoter (Ad5-CMV/ASTBDN-1) was compared to the control vector transducing β-galactosidase (Ad5-CMV/Lac Z).

4.1.3 Results & Discussion

Our laboratory was interested in understanding the function of the *TBDN* gene. Therefore, several transfection approaches were carried out to knockdown Tbdn expression. Transfection is the process of introducing foreign DNA into eukaryotic cells. Methods of transfection can range from viral-based (e.g. adenofection) to physical (e.g. microinjection, electroporation) to chemical (e.g. liposome-mediated).

Adenofection technology was originally evaluated in an attempt to knockdown Tbdn expression. However, I experienced problems infecting our β-Galactosidase plasmid (pcmv β-Gal) into neuroblastoma cell lines. I did not experience any problems with our control (Ewing's sarcoma) cell line. After several condition changes, I concluded that this technique was ineffective for infecting neuroblastoma cells. Similarly, Skog and colleagues also experienced difficulty and could not effectively infect neuroblastoma cell lines with adenofection due to a low binding capacity (Skog et al., 2002).

The microinjection approach was used to block the activity of the Tbdn protein by injecting a Tbdn antibody into the cell. The Femtojet system was used to inject the antibody. The major technical issue with this approach was the limited number of cells

that could be injected in a short period. In addition, this technique is more effective when utilizing flat cells. Unfortunately, LA-N-5 cells tend to grow as clumps adding to the difficulty of the injections. Consequently, constant resetting of the stage to a new plane of focus had to be completed. As a result, this method of injection also proved ineffective for our purposes.

The liposome method of transfection is one of the most commonly used techniques. The antisense approach was utilized because it is a well-established way of interfering with the expression of the endogenous sense gene. Various transfection reagents such as lipofectamine and lipofectin were used to transfect the antisense Tbdn (*ASTBDN*) cDNA into human LA-N-5 neuroblastoma cells. The *ASTDBN* construct was under the control of a tetracycline responsive element (TRE) and reverse tetracycline regulated transactivator (rtTA) (Figure 4-1). A few caveats with this system and model included a very low efficiency rate and very slow growing clones. In addition, the non-induced *ASTBDN* cell line appeared to be deregulated or leaky and was no longer modulated by rtTA activation.

During the initial Southern blot screen of 30 derived LA-N-5/rtTA/TRE-ASTBDN clones, three clones (LTAS#8, LTAS#10, LTAS#17) had incorporated the pTRE-ASTBDN construct. Western blot analysis revealed that clone LTAS#8 showed no suppression of Tbdn expression in response to Doxycycline treatment whereas clone LTAS#10 showed suppression of Tbdn expression. Tbdn levels for clone LTAS#17 were

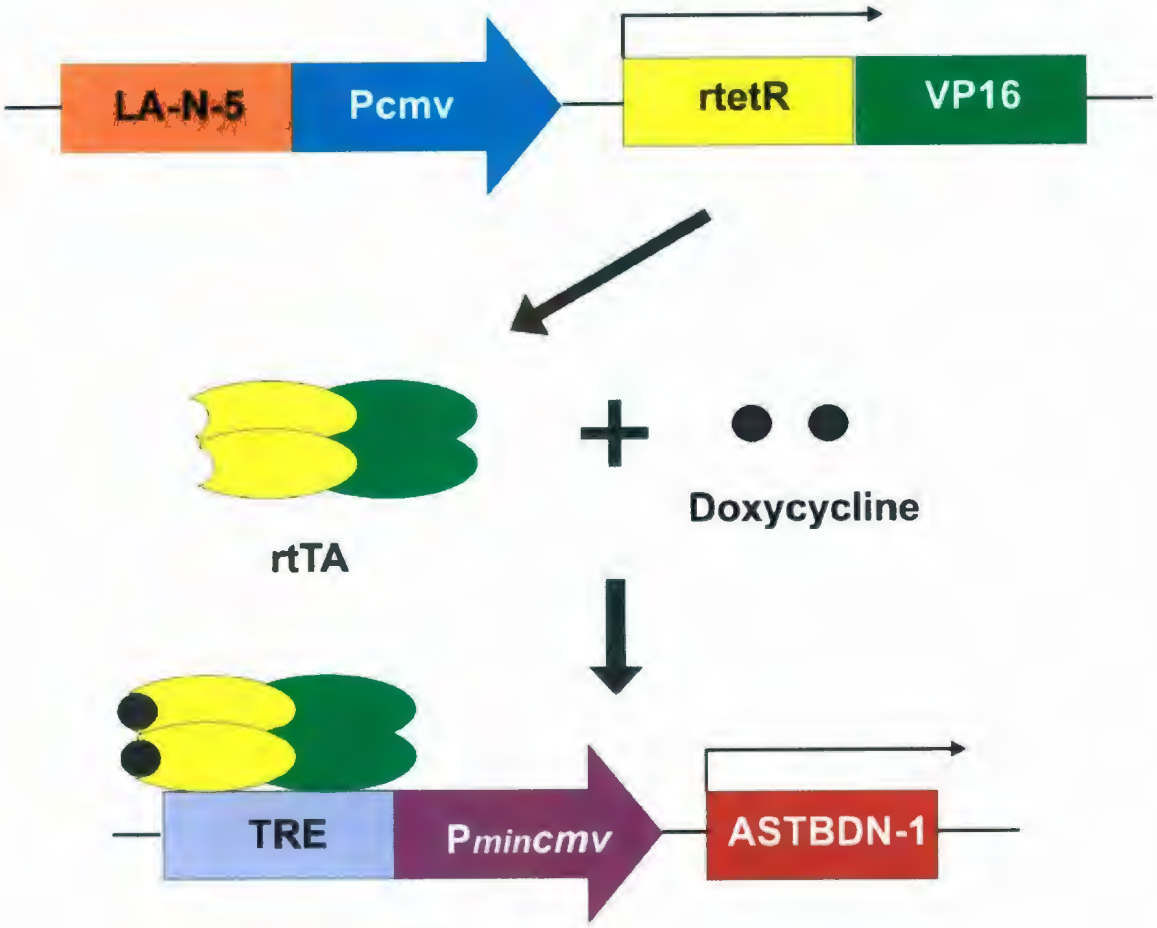


Figure 4-1. 'Tet-on' inducible antisense Tbdn system.

rtTA binds to the teto sequence of the tet-responsive element (TRE), which controls the ASTBDN-1 gene thereby activating transcription in the presence of an inducing agent, Doxycycline. *ASTBDN-1 cDNA interferes with the expression of the endogenous sense Tbdn-1 thereby decreasing Tbdn-1 levels.*

decreased with and without Doxycycline treatment indicating that the TRE-ASTBDN construct in this clone is deregulated (Figure 4-2). Upon further analysis, clone LTAS#15 (which did not contain the pTRE-ASTBDN construct) also displayed similar cell cycle results as clones LTAS#8, 10 and 17 indicating that Tbdn is likely not regulated by the inducible tetracycline-based system (data not shown).

The next transfection approach evaluated was nucleofection. I utilized nucleofection technology from Amaxa to introduce siRNA Tbdn oligonucleotides into LA-N-5 cells with the expectation of silencing Tbdn expression. Nucleofection technology is based on electroporation properties in that voltage is used to introduce a substance into a cell (Hagemann et al., 2006). This method was very effective in delivering siRNA to LA-N-5 neuroblastoma cells. Nucleofection technology is based on electrical parameters, as well as cell type specific nucleofector solutions. These solutions differed in their buffer capacity, ionic strength, and composition. A major disadvantage of this technology is that Amaxa holds the electrical parameters and nucleofector solutions in strict confidence (Hagemann et al., 2006). Another disadvantage is that every cell line has to be optimized. Amaxa has generated a database which contains optimized conditions for many commonly used cell lines. Unfortunately, optimized conditions were not available for the LA-N-5 cells. I determined that solution V along with nucleofector programs V-001 and U-014 provided optimal nucleofection rates for the LA-N-5 neuroblastoma cell line. I reached nucleofection efficiencies of approximately 60% using the control green

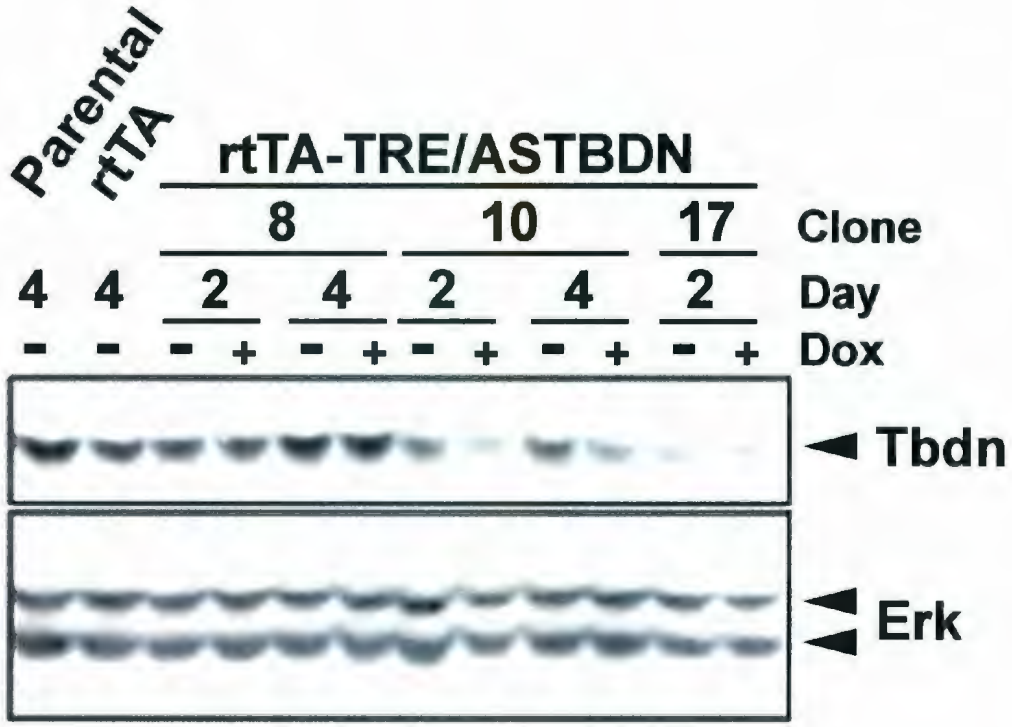


Figure 4-2. Doxycycline effects on antisense Tbdn clones.

Western blot analysis of Tbdn-1 (69 kDa) expression with and without Doxycycline. Parental (LA-N-5), rtTA (LA-N-5 TET4, empty vector) and rtTA/TRE-ASTBDN *represents the LTAS#8, 10, 17 clones. ERK (44/42 kDa) represents the loading control.*

fluorescence protein also known as maxGFP (Figure 4-3). After extensive design, the optimal Tbdn siRNA was selected. Levels of Tbdn expression were then assessed by Western blot analysis. Unfortunately, I was unable to produce a significant knockdown of Tbdn expression. In theory, designing the optimal siRNA is a relatively simple process. Design an siRNA of approximately 21 nucleotides that is complementary to your sequence of interest to get the desired degraded mRNA. However, in practicality, a far better strategy would be to design and test at least ten siRNAs. The next step should be to continue testing potential Tbdn siRNAs since an effective transfection approach (nucleofection) was found.

Several transfection approaches were utilized in determining the functional importance of Tbdn in neuroblastoma cells. However, due to the many technical problems, I was unable to knockdown Tbdn expression in the neuroblastoma cells. Nevertheless, initial studies suggested that the nucleofection approach would be an effective method to transfect human LA-N-5 neuroblastoma cells. Furthermore, suppressing Tbdn levels will determine the importance of Tbdn with respect to growth and differentiation in neuroblastoma cells.

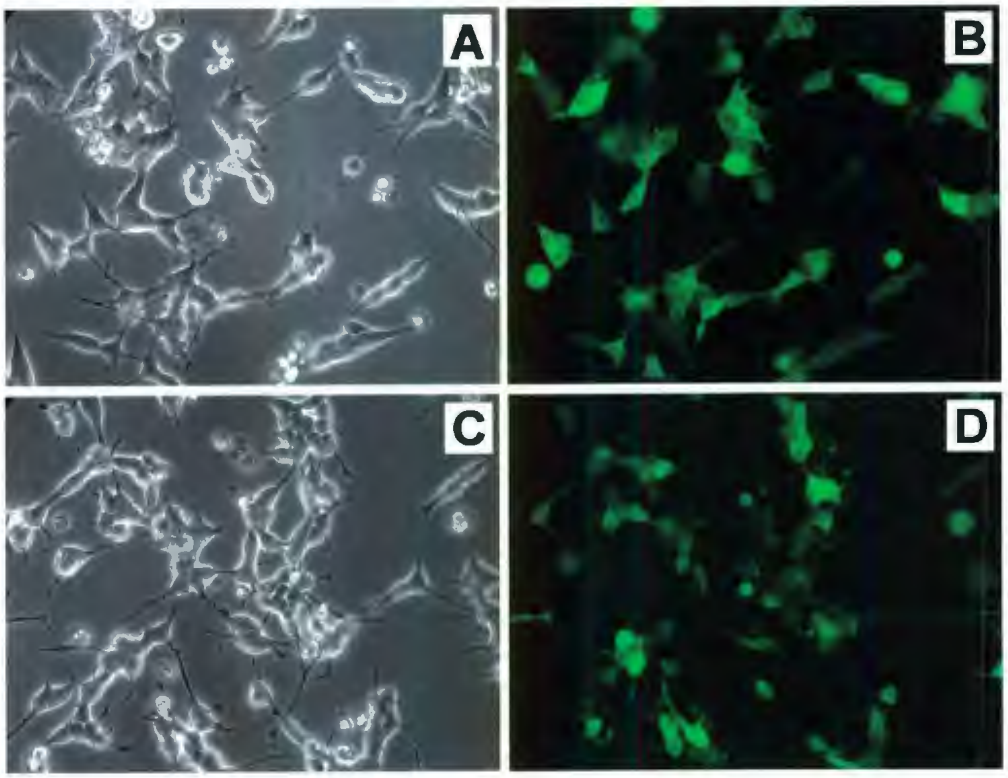


Figure 4-3. Nucleofection of LA-N-5 cells.

LA-N-5 cells were transfected with maxGFP using the U-014 (A & B) and the V-001 (C & D) Amaxa programs. Panels A & C were analyzed by phase-contrast microscopy whereas panels B & D were analyzed by immunofluorescene in which the observed green fluorescence was due to GFP expression. Magnification, x400.

5.1 Summary

5.1.1 Martin et al., 2007

Immunohistochemistry was performed on tissue microarrays, snap-frozen tissues and other paraffin-embedded sections using a Tbdn mouse monoclonal OE5 antibody. My data demonstrated that Tbdn expression maybe a novel factor that enhances the stratification of NT risk groups. Additionally, Dr. Arie Perry performed the fluorescence *in situ* hybridization experiment to show *MYCN* amplification (tumors containing more than 5 extra copies of the *MYCN* gene) or *MYCN* gains (tumors containing 1-4 extra copies of the *MYCN* gene). Corroboration of the results did not reveal any correlation between Tbdn expression and *MYCN* amplification (Martin et al., 2007). However, a potential correlation between Tbdn and MycN protein expression may still exist according to my *in vitro* data. MycN immunohistochemistry experiments were completed on paraffin-embedded section. However, a definitive conclusion could not be reached by immunohistochemistry because antibodies available for the MycN protein were not specific enough.

In addition to MycN, Tbdn expression was also compared and correlated with the differentiation status of NTs. High levels of Tbdn expression were found in the less differentiated NTs (such as the neuroblastomas) whereas low levels of Tbdn expression were present in the more differentiated NTs (such as the ganglioneuromas). Previously,

in vitro data showed that Tbdn expression correlated with a subset of differentiated neuroblastoma cells (Figure 2-6). Morphological changes in a neuroblastoma cell line treated with RA led to a decrease in Tbdn expression where the majority of the cells are undergoing differentiation upon RA treatment. The differentiated neuroblastoma cells had less Tbdn expression compared to the non-differentiated neuroblastoma cells. Based on the aforementioned *in vitro* data, Tbdn expression was compared to the differentiated status of the neuroblastoma specimens. Unfortunately, the differentiation status within the neuroblastoma specimens from the tissue array did not correlate with Tbdn expression. The reason for the discrepancy, as alluded to in chapter 2, was that the INPC system considers neuroblastoma specimens as differentiated if 5% or more of the tumor contains differentiated cells. However, a population with 5% or less of differentiated cells may not affect the overall expression levels of Tbdn for a specific specimen. In other words, 5% of the neuroblastoma tumor specimen had to be differentiated for it to be classified within the differentiated category.

Clinically, the more differentiated a tumor, the greater the chance of a response to treatment. RA treatment of acute promyelocytic leukemia has been shown to induce terminal differentiation of malignant myeloid cells into mature neutrophils (Soignet et al., 1998). Similarly, RA-induced differentiation can affect malignant neuroblastoma cells by mimicking the differentiation of neurons in culture (Robson and Sidell, 1985). *In vitro*, different forms of RA have been shown to induce differentiation and decrease the growth of neuroblastoma cells (Sidell et al., 1983; Thiele et al., 1985; Reynolds et al.,

1994; Brodeur, 2003). Currently, 13-cis RA is being used in clinical trials with high-risk neuroblastoma patients (Matthay et al., 1999) and is becoming standard practice in the treatment of high-risk neuroblastoma (Brodeur, 2003).

As stated, Tbdn is involved in neuroblastic differentiation, suggesting that it maybe an attractive target for cancer treatment. However, according to Arnesen et al., Tbdn would not be useful as a cancer drug target, since decreasing Tbdn would lead to an increase in capillary formation (Arnesen et al., 2008). This statement by Arnesen et al. was likely based on previous data by our group, showing that a down-regulation of Tbdn in an endothelial cell model resulted in an accumulation of capillary-like structures (Paradis et al., 2002). Firstly, I did not observe or report any Tbdn staining of the blood vessels within the neuroblastic specimens analyzed. As stated in chapter 2, Tbdn staining was not detected in blood cells or vessels (see Figure 2-2C). Secondly, I was looking at Tbdn in a different model system than was reported previously. There is a strong possibility that Tbdn may have different roles depending on its cellular context. For example, Stat3 activation is associated with proliferation and promoting apoptosis (Bromberg and Darnell, 2000). Activated Stat3 prevents apoptosis and increases proliferation in head and neck squamous cell carcinoma. Conversely, Stat3 activation increases mammary epithelial apoptosis (Chapman et al., 1999; Leeman et al., 2006). Additionally, the expression of the *nm23-H1* gene can vary with tumor type. High expression of nm23-H1 is associated with a favorable outcome in breast cancer and malignant melanomas (Hennessy et al., 1991; Florenes et al., 1992). Conversely, an up-regulation of nm23-H1

is linked to poor outcome in neuroblastoma and non-Hodgkin lymphomas (Niitsu et al., 2001; Godfried et al., 2002).

Although a great deal of progress has been made in creating a more efficient risk group classification system, effective treatments for various subsets of patients require new markers to aid in advancing the stratification of tumors. Tbdn is highly expressed in the most malignant and aggressive subset of neuroblastic tumors. Therefore, additional markers will enhance the neuroblastic classification system thereby providing clinicians an array of resources for assessing and treating neuroblastic patients.

5.1.2 Martin et al., 2009

Our group, as well as others, showed that MycN expression was down-regulated in human neuroblastoma LA-N-5 cells upon RA-induced differentiation (Thiele et al., 1985; Martin et al., 2007). I showed that MycN expression correlated with both Tbdn and Ard1 expression. In addition, I showed that MycN regulated Tbdn and Ard1 expression using the *MYCN*-inducible SHEP cell line. One advantage of this well-established cell system is that it is inducible which means that the same cell line can be used to over-express or suppress MycN expression. One potential drawback of our experimental setup was that I did not have access to a second *MYCN*-inducible cell line to ensure that the regulation of Tbdn by MycN was not an artifact of the cell line. However, I did demonstrate that the decrease in both Tbdn and Ard1 was not cell line dependent. Treatment of the LA-N-5

cells with RA, a differentiation agent, resulted in a co-downregulation of Tbdn, Ard1, and MycN.

The link between aggressiveness of neuroblastoma and *MYCN* amplification is still unclear (Thiele et al., 1985; Brodeur, 2003; Wei et al., 2008). However, *MYCN* amplification usually leads to high MycN protein and RNA expression in neuroblastomas (Tang et al., 2006). My previous work showed that high levels of Tbdn expression are linked with aggressive and high-risk group neuroblastoma and are associated with poor survival (Martin et al., 2007). Our present evidence supports a hypothesis that the MycN/Tbdn/Ard1 pathway may be regulated in an aberrant manner in neuroblastomas with poor outcome. Besides their roles in cancer, Tbdn, Ard1, and MycN are also involved in normal development. Throughout brain development there is a spatio-temporal up-regulation of Tbdn and Ard1 (Sugiura et al., 2003). Similarly, Grady and colleagues (1987) showed that MycN was highly expressed in the early fetal cerebral germinal layer and the primordial cortex during human fetal brain development (Grady et al., 1987). Therefore, understanding the relationship between *TBDN*, *ARD1* and MycN may provide new insight for regulation and treatment of neuroblastoma.

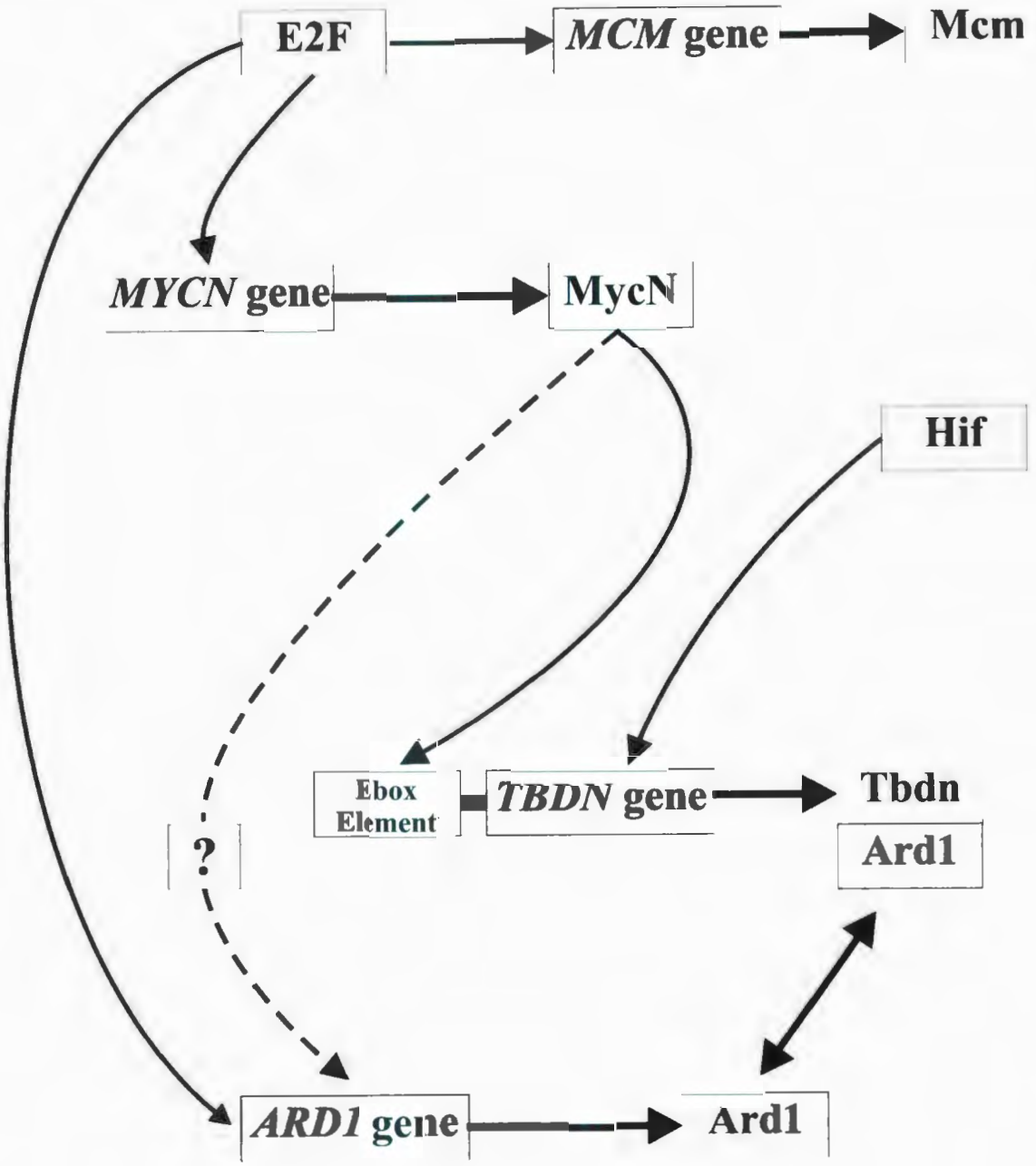
The *MYCN*-inducible SHEP cell line was an invaluable tool for studying the potential binding of MycN to the *TBDN* promoter. From our ChIP analysis, I showed that MycN binds the *TBDN* gene (Figure 5-1). The next step would be to verify our results. I could have accomplished this by mutating various nucleotides of the E-box within the promoter

region. Another method would be to delete nucleotides within the E-box region. However, this would change the size of the E-box region, ultimately affecting basal transcription. Therefore, the former option would be the preferred method. Furthermore, to determine whether MycN activates transcription of the *TBDN* gene, luciferase assays could have been performed with a number of *TBDN* promoter / reporter constructs containing various point mutations or E-box deletions. This would ultimately determine if the *TBDN* promoter activity directly correlates with MycN expression. In addition, I also used the *MYCN*-inducible system to demonstrate that MycN can regulate Ard1 expression. The decrease in Ard1 expression may result from MycN binding directly to Tbdn or MycN may directly bind *ARD1* through an unknown transcription factor (Figure 5-1).

Tbdn is expressed in the Tet2 *MYCN*-inducible cell line which lacks MycN expression upon tetracycline treatment (see Figure 3-2). The Tet2N *MYCN*-inducible system cannot be completely shut off, even in the presence of tetracycline. Therefore, the Tbdn expression present may be a direct result of the low level of MycN expression still being expressed. However, an alternative explanation may suggest that other factors could be

Figure 5-1. Tbdn, Ard1, and MycN pathway interaction.

Solid lines represent confirmed interactions whereas dotted lines represent hypothetical interactions.


E2F
MCM gene
Mcm
MYCN gene
MycN
Hif
Ebox Element
TBDN gene
Tbdn
Ard1
?
ARD1 gene
Ard1

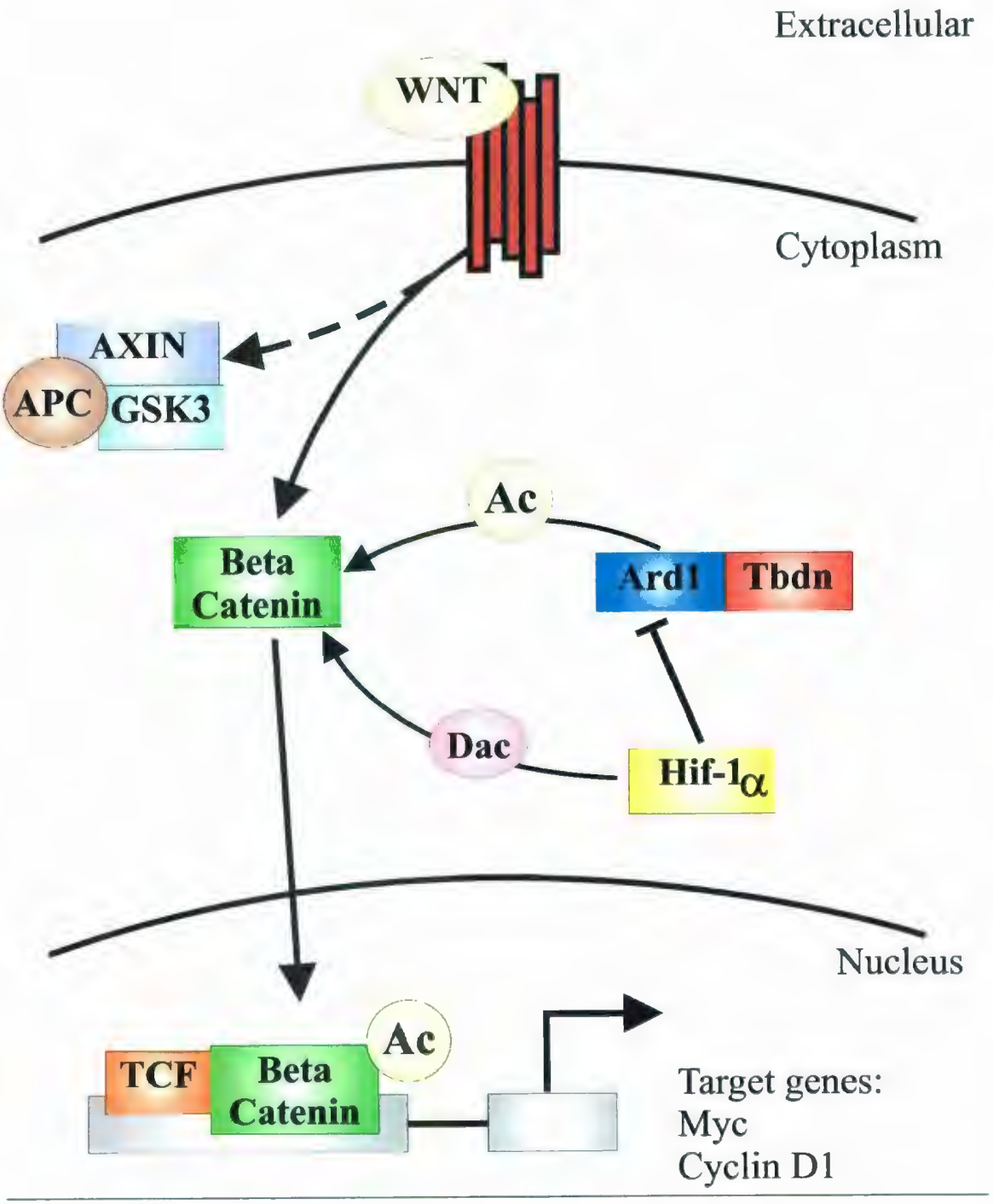
involved in regulating Tbdn such as E2F or Hypoxia-inducible factor (Hif). Therefore, I suggested a hypothetical model to explain the potential interactions between Tbdn, Ard1, and MycN (Figure 5-1).

Hypoxia can drive solid tumors towards an immature phenotype (Axelson et al., 2005). Poorly oxygenated environments express transcription factors such as Hif-1 and Hif-2 (Nilsson et al., 2005; Lofstedt et al., 2007). Before discussing the role of Hifs with the Tbdn/Ard1 complex, it is important to first describe the relationship between the Tbdn/Ard1 complex, in particular, Ard1 and the Wnt signaling pathway.

The NatA complex appears to target the Wnt signaling pathway. As mentioned, during normal neuro-ectodermal development, an anomaly results in the formation of neuroblastoma (Brodeur, 2003; van Noesel and Versteeg, 2004; Maris, 2005; Vasudevan et al., 2005). One of the earliest pathways involved in neuro-ectodermal development is the Wnt signaling pathway. Recently, Lim and colleagues demonstrated that Ard1 is responsible for acetylating and activating the β-Catenin pathway and promoting cancer proliferation (Figure 5-2; Lim et al., 2006). In addition, Wai et al. also reported an up-regulation of wingless-type mouse mammary tumor virus integration site family member 11 (WNT11) in neuroblastoma cells (Wai et al., 2002). Acetylation of β-Catenin induces the binding of TCF4 to the cyclin D1 promoter. hArd1-silencing RNA was used to treat lung cancer cells, resulting in inhibition of cell proliferation and induction of G1 arrest (Lim et al., 2006). A ChIP assay was used to demonstrate that silencing of hArd1

Figure 5-2. Possible mechanism of NatA acetylating Beta-Catenin.

An increase in the activity of Beta-Catenin through NatA acetylation results in an increase in cellular proliferation through Myc and Cyclin D1.


Extracellular
WNT
Cytoplasm
AXIN
APC
GSK3
Ac
Beta Catenin
Ard1
Tbdn
Dac
Hif-1α
Nucleus
Ac
TCF
Beta Catenin
Target genes:
Myc
Cyclin D1

inhibited the recruitment of β-Catenin/TCF complex on the cyclin D1 promoter. Since p300 was also known to active β-Catenin by acetylation, Lim et al. wanted to demonstrate that p300 did not affect the association between β-Catenin and Ard1. siRNA-p300 knockdown revealed an attenuation of p300 binding for β-Catenin but did not affect the association between Ard1 and β-Catenin.

Therefore, p300 knockdown was shown not to be necessary for the interaction between hArd1 and β-Catenin. However, both the siRNA-Ard1 and the siRNA-p300 knockdowns inhibited β-Catenin acetylation independently of each other (Lim et al., 2006). Collectively, the above mentioned studies indicate that Ard1 has a role in cellular growth.

Under hypoxic conditions, Hif-1α binds hArd1 and in the process dissociates hArd1 from β-Catenin thereby preventing hArd1 from acetylating β-Catenin (Figure 5-2; Lim et al., 2008). Specifically, the deacetylation of β-Catenin, through hArd1 removal, will ultimately repress that transcriptional activity of the β-Catenin/TF complex and down-regulate c-Myc by disrupting the β-Catenin/TF complex. Moreover, knocking down Hif-1α augments β-Catenin acetylation, resulting in activation of target genes such as c-Myc (Lim et al., 2008). In addition, Hif-1α has been reported to bind directly to β-Catenin and down-regulate c-Myc expression (Figure 5-2; Kaidi et al., 2007; Lim et al., 2008).

During prolonged hypoxia there is a notable difference between Hif-1α and Hif-2α in neuroblastoma. The Hif-2α protein levels tend to accumulate whereas Hif-1α protein

levels are reduced or disappear over time (Holmquist-Mengelbier et al., 2006; Lofstedt et al., 2007). The Hif-2 accumulation in neuroblastoma correlates with advanced stages and low patient survival. Poorly vascularized solid tumors such as neuroblastomas become de-differentiated and more aggressive under hypoxic conditions (Nilsson et al., 2005). In addition, neuroblastoma tumor evaluation revealed a strong correlation between high Hif-2 levels and unfavorable patient outcome (Holmquist-Mengelbier et al., 2006). Holmquist-Mengelbier and colleagues used a xenograft model to demonstrated that knocking down Hif-2 resulted in decreased growth of neuroblastoma (Holmquist-Mengelbier et al., 2006). Since the *TBDN* promoter region contains three HREs (Figure 3-3), and is up-regulated in the less differentiated cancers, it becomes tempting to speculate that Hif-2 would bind HRE and induce Tbdn expression, resulting in an increase in the growth of neuroblastoma.

5.1.3 Tbdn, Ard1, Myc, and Cancer

As mentioned, neuroblastoma has a wide range of heterogeneity with respect to differentiation, possibly caused by hypoxic conditions (Jogi et al., 2002; Brodeur, 2003). In addition, factors such as MycN also play a role in neuroblastoma growth and differentiation (Vita and Henriksson, 2006). Why then is it important to study Tbdn? Tbdn is involved in tumorigenesis (Arnesen et al., 2006c). Knocking down Tbdn expression in HeLa cells by siRNA technology resulted in a decrease in cell viability by increasing the rate of apoptosis (Arnesen et al., 2006c). Tbdn is highly expressed in

cancers such as neuroblastoma, papillary thyroid carcinomas, gastric cancer, and Burkitt lymphoma. A commonality between these tumor types is their high rates of proliferation. Tbdn is up-regulated in neoplastic thyroid tissue versus non-neoplastic thyroid tissue indicating that the higher levels of the Tbdn protein may provide a growth advantage for the cancer cells (Arnesen et al., 2005b). In addition, our laboratory showed that high Tbdn levels are found in the advanced stages of neuroblastoma specimens compared to the less aggressive stages (Martin et al., 2007). Possible mechanisms for such regulation of cellular growth and differentiation in cancer cells may involve the action of Nat1/Ard1 as a factor acetyltransferase, or uncharacterized activities of Tbdn protein. In particular, I have reported that MycN can bind and regulate the expression of Tbdn (Figure 3-2 & 3-4).

In addition to MycN, Myc also appears to be linked to Tbdn expression. Tbdn has been shown to regulate cell growth in cervical (HeLa) cells that over-express both Tbdn and c-Myc (Arnesen et al., 2006c). Similar to HeLa cells, Ewing sarcoma cells also over-express both Tbdn and c-Myc (Parham, 1996; Macville et al., 1999; Henriksson et al., 2001; Rorie et al., 2004; H. Paradis, personal communication). Knocking down Tbdn in this cell model resulted in a decrease in cell growth (*in vitro*) and tumor growth (*in vivo*) (H. Paradis, personal communication).

c-MYC and *MYCN* are conserved genes belonging to the same family. Knockout models of either gene results in embryonic lethality (Nesbit et al., 1999). In addition, c-Myc and

MycN are transcription factors that dimerize to Max and bind to the same E-box (CACGTG) consensus sequence (Blackwell et al., 1990; Blackwood and Eisenman, 1991). Although both c-Myc and MycN have oncogenic potential, they are involved in different tumor types. c-Myc is generally involved in tumors such as epithelial cervix adenocarcinoma whereas MycN is involved mainly with neuroblastoma (Ben-Yosef et al., 1998). Furthermore, based on the results from our group and others (Arnesen et al., 2008), it is tempting to speculate that Myc may regulate Tbdn and/or Ard1 in other cancer models.

Tbdn and Cytogenetic Aberrations

In addition to MycN, other factors such as cytogenetic aberrations may explain the pathobiology of neuroblastomas. In particular, the allelic loss of chromosome 4p was found in 20% of neuroblastomas irrespective of disease stage, patient age, loss of heterozygosity 1p or *MYCN* amplification (Caron et al., 1996a). Therefore, the allelic loss of 4p may impact upon neuroblastoma pathobiology (McGowan-Jordan et al., 1994; van Noesel and Versteeg, 2004). More recently, Gisselsson et al. completed a retrospective study using karyotypes from the Mitelman Database of Chromosome Aberrations in Cancer. Gisselsson and colleagues demonstrated whole chromosome 4 loss in low-stage and low-risk neuroblastoma tumors (Gisselsson et al., 2007). Tbdn is located at 4p31.1 and our laboratory reported that low levels of Tbdn expression are found in the low-stage and low-risk group (Martin et al., 2007). It is tempting to speculate that loss of chromosome 4 may delete Tbdn .

Recently, Tbdn was found in a complex with the actin binding protein cortactin in retinal endothelial cells (Paradis et al., 2008). Our laboratory has previously shown that Tbdn exerts a homeostatic influence in retinal endothelial cells although we have not yet identified *in vivo* acetyltransferase substrates of the Tbdn complex (Wall et al., 2004; Paradis et al., 2008). While these findings provide insight into understanding the nature of the Tbdn complex, they also raise the intriguing question of whether Tbdn associates with cortactin in neuroblastoma cells. Cortactin is also involved in the process of tumor progression and metastasis formation. Cortactin has been reported to be over-expressed in breast cancers as well as head and neck cancers (Buday and Downward, 2007). In addition, amplification of cortactin has been found to occur in aggressive head and neck squamous cell carcinoma (Clark et al., 2008), as well as a subgroup of neuroblastoma (Michels et al., 2007). Therefore, it is tempting to speculate that the association between cortactin and the Tbdn complex may contribute to the aggressiveness of the NTs.

5.1.4 Pitfalls & Future Directions

Although I was unable to silence Tbdn expression in neuroblastoma (LA-N-5) cells using siRNA technology, I knocked-down Tbdn expression in other cell models such as rhesus macaque choroid-retina endothelial (RF/6A) cells. In addition, Arnesen and colleagues silenced Tbdn expression in epithelial cervical adenocarcinoma (HeLa) cells (Arnesen et al., 2006c). However, there are many reasons for not getting a decrease in Tbdn expression in the LA-N-5 cells using siRNA technology. Firstly, the optimal siRNA

sequence may not have been selected. Again, the only way to truly determine the optimal siRNA sequence is by experimental testing. Secondly, suppression of Tbdn alone may not show an effect on neuroblastoma cells since the co-interacting Ard1 acetyltransferase may be limiting. In addition, our laboratory was also interested in over-expressing Tbdn to determine if neuroblastoma cells would become more malignant with increased levels of Tbdn expression. Many unsuccessful attempts to over-express Tbdn have been made by our laboratory. Again, over-expression of Tbdn alone may not show an effect on neuroblastoma cells since the co-interacting Ard1 acetyltransferase may be limiting. Over-expression of either Tbdn or Ard1 alone is not sufficient to result in an increase in acetyltransferase activity. Therefore, both Tbdn and Ard1 must be over-expressed to attain an increase in the acetyltransferase activity (Mullen et al., 1989; Arnesen et al., 2006c; Arnesen et al., 2008).

A decrease in Tbdn and MycN expression was observed upon RA-induced differentiation of neuroblastoma cells (Thiele et al., 1985; Martin et al., 2007). To confirm the link between Tbdn, MycN, and differentiation, a MycN inducible system could be utilized. Unfortunately, the *MYCN*-inducible SHEP system generated by Lutz et al. (1996) would not be an effective model to study the putative effects of *MYCN* on differentiation (Edsjo et al., 2004). The problem with using the MycN-induced SHEP model is that the SHEP cell line is an S-type cell line and not an N-type. Treatment of the S-type cells with a differentiation agent such as RA would induce apoptosis instead of neuronal elongation (Voigt and Zintl, 2003). Subsequent to RA treatment, N-type cells elongate their

neuronal processes, reminiscent of axons or dendrites. However, S-type of cells lose their adherence to substratum and become apoptotic (Voigt and Zintl, 2003). Edsjo et al. over-expressed MycN in a SK-N-SH (N-type) cell line resulting in a SKMYC2 cell line that constitutively expressed MYCN (Edsjo et al., 2004). However, the downfall with this system is the lack of inducibility or regulation . Similarly, Slack et al. cloned MycN cDNA into a subclone of the SHEP cell line but again the cell line was epithelial-like (Slack et al., 2005). Ideally, generating an inducible MycN system, in an N-type of cell that has the potential to differentiate would be the preferred model.

As alluded earlier, I generated an *in vitro* conditional knockdown system to examine the effects of Tbdn knockdown on neuroblastoma growth and differentiation. I planned on using these knockdown clones to complete preclinical work. A xenograft mouse model would have been used to determine if decreased Tbdn levels would have an effect on tumor growth or tumor differentiation. Three different approaches include giving Doxycycline to cells *in vitro* then injecting the cells into the nude mice; injecting the non-induced cells into the mice then letting the tumor grow before giving the mice Doxycycline to determine if the tumor decreased in size; and removing Doxycycline from the diet of the mouse to determine if the tumor reappears. The expected outcome would be that knockdown of Tbdn expression may decrease or stabilize tumor growth and shift the level of tumor differentiation toward a more differentiate form. Unfortunately, there were many issues regarding the leakiness with this inducible system. Therefore, further experiments using this model system could not be completed.

Another focus of my work involves MycN, as alluded to earlier, is an important factor surrounding the aggressivity of neuroblastoma. Since my *in vitro* data suggests that MycN regulates Tbdn expression, I would be interested in determining *in vivo* if varying levels of MycN expression affects Tbdn expression. Could this outcome influence tumor progression? Before determining the effects of MycN on Tbdn expression *in vivo*, I will need an inducible MycN mouse model. William Weiss and colleagues have generated a transgenic mouse model that over-expresses MycN in the neuroectodermal cells, resulting in development of neuroblastoma (Weiss et al., 1997). To target *MYCN* expression to the neural crest cells, Weiss and colleagues used a tyrosine hydroxylase promoter. The tyrosine hydroxylase promoter is active in migrating cells and targets catecholamine neurons during early development (Banerjee et al., 1992). Weiss et al. demonstrated that tumor formation is dependent on *MYCN* gene dosage. Homozygotes were reported to have increased incidence and decreased latency of tumor formation (Weiss et al., 1997).

I would be interested in determining if there is a relationship between *MYCN* gene dosage, Tbdn activation, and tumor formation. By varying the levels of MycN expression I would expect to get varying levels of Tbdn activation. This would ultimately result in the generation of neuroblastoma tumors that varied in size and complexity. I would expect that high levels of MycN would drive Tbdn activation and result in the

generation of poorly differentiate tumors whereas low MycN expression would only weakly activate Tbdn and result in no tumor or a more differentiated tumor.

These future experiments will help define the functional role that Tbdn plays in NT progression/development. In particular, the above mentioned experiments will determine whether Tbdn expression drives neuroblastoma growth and whether MycN regulates neuroblastoma growth through Tbdn expression using *in vivo* models.

5.1.5 Potential Tubedown Mechanism

My hypothetical Tbdn mechanism (Figure 5-3) is based on a deregulated Wnt/β-Catenin pathway that is found in high risk neuroblastoma without *MYCN* amplification (Liu et al., 2008). Wnt/β-Catenin signaling may be of particular relevance to neuroblastomas, arising from highly migratory neural crest stem cells (Dyer, 2004; Mora and Gerald, 2004). There are subsets of high-risk neuroblastomas without *MYCN* amplification that have high MycN expression (Suenaga et al., 2009). *MYCN* mRNA is expressed at high levels in some subsets of neuroblastoma with only a single copy of the *MYCN* gene. This is accomplished by positive auto-regulation of MycN in human neuroblastomas. MycN protein enhances its own promoter activity through direct recruitment onto the intron 1 region of the *MYCN* gene which contains two putative E-box sites (Suenaga et al., 2009).

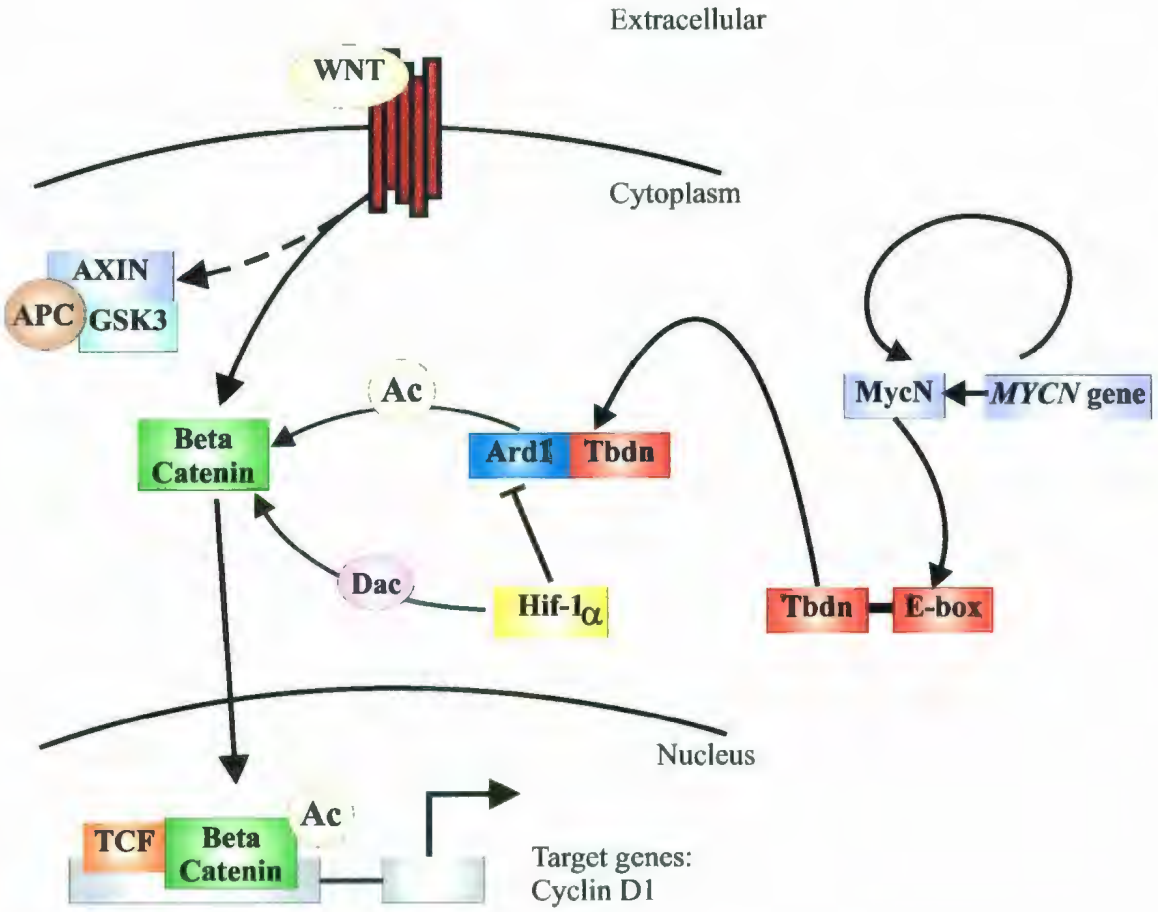


Figure 5-3. Possible mechanism of Tubedown.

Subsets of high-risk neuroblastomas without MYCN amplification can have high MycN expression that will potentially drive the expression of the Tbdn complex thereby acetylating Beta-Catenin and resulting in an increase in cellular proliferation.

High expression of the MycN protein will bind to the E-box located in the *TBDN* promoter region. Increased expression of the Tbdn/Ard1 complex will drive the acetylation of β-Catenin thereby providing a survival advantage for the cancer cells to grow.

6.1 References

Abbott, R.G., Forrest, S. and Pienta, K.J.: Simulating the hallmarks of cancer. Artif Life 12 (2006) 617-34.

Abemayor, E. and Sidell, N.: Human neuroblastoma cell lines as models for the in vitro study of neoplastic and neuronal cell differentiation. Environ Health Perspect 80 (1989) 3-15.

Adida, C., Berrebi, D., Peuchmaur, M., Reyes-Mugica, M. and Altieri, D.C.: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 351 (1998) 882-3.

Alaminos, M., Gerald, W.L. and Cheung, N.K.: Prognostic value of MYCN and ID2 overexpression in neuroblastoma. Pediatr Blood Cancer 45 (2005) 909-15.

Amiel, J., Laudier, B., Attie-Bitach, T., Trang, H., de Pontual, L., Gener, B., Trochet, D., Etchevers, H., Ray, P., Simonneau, M., Vekemans, M., Munnich, A., Gaultier, C. and Lyonnet, S.: Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. Nat Genet 33 (2003) 459-61.

Aoki, T., Ichimura, S., Itoh, A., Kuramoto, M., Shinkawa, T., Isobe, T. and Tagaya, M.: Identification of the neuroblastoma-amplified gene product as a component of the syntaxin 18 complex implicated in Golgi-to-endoplasmic reticulum retrograde transport. Mol Biol Cell 20 (2009) 2639-49.

Arnesen, T., Anderson, D., Baldersheim, C., Lanotte, M., Varhaug, J.E. and Lillehaug, J.R.: Identification and characterization of the human ARD1-NATH protein acetyltransferase complex. Biochem J 386 (2005a) 433-43.

Arnesen, T., Anderson, D., Torsvik, J., Halseth, H.B., Varhaug, J.E. and Lillehaug, J.R.: Cloning and characterization of hNAT5/hSAN: an evolutionarily conserved component of the NatA protein N-alpha-acetyltransferase complex. Gene 371 (2006a) 291-5.

Arnesen, T., Betts, M.J., Pendino, F., Liberles, D.A., Anderson, D., Caro, J., Kong, X., Varhaug, J.E. and Lillehaug, J.R.: Characterization of hARD2, a processed hARD1 gene duplicate, encoding a human protein N-alpha-acetyltransferase. BMC Biochem 7 (2006b) 13.

Arnesen, T., Gromyko, D., Horvli, O., Fluge, O., Lillehaug, J. and Varhaug, J.E.: Expression of N-acetyl transferase human and human Arrest defective 1 proteins in thyroid neoplasms. Thyroid 15 (2005b) 1131-6.

Arnesen, T., Gromyko, D., Pendino, F., Ryningen, A., Varhaug, J.E. and Lillehaug, J.R.: Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-alpha-acetyltransferase complex. Oncogene 25 (2006c) 4350-60.

Arnesen, T., Thompson, P.R., Varhaug, J.E. and Lillehaug, J.R.: The protein acetyltransferase ARD1: a novel cancer drug target? Curr Cancer Drug Targets 8 (2008) 545-53.

Arnesen, T., Van Damme, P., Polevoda, B., Helsens, K., Evjenth, R., Colaert, N., Varhaug, J.E., Vandekerckhove, J., Lillehaug, J.R., Sherman, F. and Gevaert, K.: Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci U S A 106 (2009) 8157-62.

Axelson, H.: The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1. Cancer Lett 204 (2004) 171-8.

Axelson, H., Fredlund, E., Ovenberger, M., Landberg, G. and Pahlman, S.: Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. Semin Cell Dev Biol 16 (2005) 554-63.

Baird, S.D., Turcotte, M., Korneluk, R.G. and Holcik, M.: Searching for IRES. Rna 12 (2006) 1755-85.

Banelli, B., Casciano, I., Croce, M., Di Vinci, A., Gelvi, I., Pagnan, G., Brignole, C., Allemanni, G., Ferrini, S., Ponzoni, M. and Romani, M.: Expression and methylation of CASP8 in neuroblastoma: identification of a promoter region. Nat Med 8 (2002) 1333-5; author reply 1335.

Banerjee, S.A., Hoppe, P., Brilliant, M. and Chikaraishi, D.M.: 5' flanking sequences of the rat tyrosine hydroxylase gene target accurate tissue-specific, developmental, and transsynaptic expression in transgenic mice. J Neurosci 12 (1992) 4460-7.

Barone, B.B. and Bunin, G.R.: Folic acid and neuroblastoma: too soon to tell. Clin Pharmacol Ther 75 (2004) 489; author reply 490.

Ben-Yosef, T., Yanuka, O., Halle, D. and Benvenisty, N.: Involvement of Myc targets in c-myc and N-myc induced human tumors. Oncogene 17 (1998) 165-71.

Bilanges, B. and Stokoe, D.: Mechanisms of translational deregulation in human tumors and therapeutic intervention strategies. Oncogene 26 (2007) 5973-90.

Bister, K., Hayman, M.J. and Vogt, P.K.: Defectiveness of avian myelocytomatosis virus MC29: isolation of long-term nonproducer cultures and analysis of virus-specific polypeptide synthesis. Virology 82 (1977) 431-48.

Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N. and Weintraub, H.: Sequence-specific DNA binding by the c-Myc protein. Science 250 (1990) 1149-51.

Blackwood, E.M. and Eisenman, R.N.: Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science 251 (1991) 1211-7.

Blackwood, E.M., Luscher, B., Kretzner, L. and Eisenman, R.N.: The Myc:Max protein complex and cell growth regulation. Cold Spring Harb Symp Quant Biol 56 (1991) 109-17.

Blatch, G.L. and Lassle, M.: The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays 21 (1999) 932-9.

Bluhm, E.C., Daniels, J., Pollock, B.H. and Olshan, A.F.: Maternal use of recreational drugs and neuroblastoma in offspring: a report from the Children's Oncology Group (United States). Cancer Causes Control 17 (2006) 663-9.

Boon, K., Caron, H.N., van Asperen, R., Valentijn, L., Hermus, M.C., van Sluis, P., Roobeek, I., Weis, I., Voute, P.A., Schwab, M. and Versteeg, R.: N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. Embo J 20 (2001) 1383-93.

Bordow, S.B., Norris, M.D., Haber, P.S., Marshall, G.M. and Haber, M.: Prognostic significance of MYCN oncogene expression in childhood neuroblastoma. J Clin Oncol 16 (1998) 3286-94.

Bowen, K.A. and Chung, D.H.: Recent advances in neuroblastoma. Curr Opin Pediatr 21 (2009) 350-6.

Boyd, K.E. and Farnham, P.J.: Coexamination of site-specific transcription factor binding and promoter activity in living cells. Mol Cell Biol 19 (1999) 8393-9.

Bridge, R.S., Rajaram, V., Dehner, L.P., Pfeifer, J.D. and Perry, A.: Molecular diagnosis of Ewing sarcoma/primitive neuroectodermal tumor in routinely processed tissue: a comparison of two FISH strategies and RT-PCR in malignant round cell tumors. Mod Pathol 19 (2006) 1-8.

Brodeur, G.M.: Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 3 (2003) 203-16.

Brodeur, G.M., Minturn, J.E., Ho, R., Simpson, A.M., Iyer, R., Varela, C.R., Light, J.E., Kolla, V. and Evans, A.E.: Trk receptor expression and inhibition in neuroblastomas. Clin Cancer Res 15 (2009) 3244-50.

Brodeur, G.M., Pritchard, J., Berthold, F., Carlsen, N.L., Castel, V., Castelberry, R.P., De Bernardi, B., Evans, A.E., Favrot, M., Hedborg, F. and et al.: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 11 (1993) 1466-77.

Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E. and Bishop, J.M.: Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science 224 (1984) 1121-4.

Bromberg, J. and Darnell, J.E., Jr.: The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19 (2000) 2468-73.

Browne, M., Kletzel, M., Cohn, S.L., Seshadri, R. and Reynolds, M.: Excellent local tumor control regardless of extent of surgical resection after treatment on the Chicago Pilot II protocol for neuroblastoma. J Pediatr Surg 41 (2006) 271-6.

Buday, L. and Downward, J.: Roles of cortactin in tumor pathogenesis. Biochim Biophys Acta (2007).

Caron, H.: Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma. Med Pediatr Oncol 24 (1995) 215-21.

Caron, H., van Sluis, P., Buschman, R., Pereira do Tanque, R., Maes, P., Beks, L., de Kraker, J., Voute, P.A., Vergnaud, G., Westerveld, A., Slater, R. and Versteeg, R.: Allelic loss of the short arm of chromosome 4 in neuroblastoma suggests a novel tumour suppressor gene locus. Hum Genet 97 (1996a) 834-7.

Caron, H., van Sluis, P., de Kraker, J., Bokkerink, J., Egeler, M., Laureys, G., Slater, R., Westerveld, A., Voute, P.A. and Versteeg, R.: Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. N Engl J Med 334 (1996b) 225-30.

Castel, V. and Canete, A.: A comparison of current neuroblastoma chemotherapeutics. Expert Opin Pharmacother 5 (2004) 71-80.

Castel, V. and Grau, E.: Advances in neuroblastoma research. Clin Transl Oncol 8 (2006) 701-2.

Castel, V., Grau, E., Noguera, R. and Martinez, F.: Molecular biology of neuroblastoma. Clin Transl Oncol 9 (2007) 478-83.

Castle, V.P., Heidelberger, K.P., Bromberg, J., Ou, X., Dole, M. and Nunez, G.: Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am J Pathol 143 (1993) 1543-50.

Cetinkaya, C., Hultquist, A., Su, Y., Wu, S., Bahram, F., Pahlman, S., Guzhova, I. and Larsson, L.G.: Combined IFN-gamma and retinoic acid treatment targets the N-Myc/Max/Mad1 network resulting in repression of N-Myc target genes in MYCN-amplified neuroblastoma cells. Mol Cancer Ther 6 (2007) 2634-41.

Chan, H.S., Gallie, B.L., DeBoer, G., Haddad, G., Ikegaki, N., Dimitroulakos, J., Yeger, H. and Ling, V.: MYCN protein expression as a predictor of neuroblastoma prognosis. Clin Cancer Res 3 (1997) 1699-706.

Chapman, R.S., Lourenco, P.C., Tonner, E., Flint, D.J., Selbert, S., Takeda, K., Akira, S., Clarke, A.R. and Watson, C.J.: Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev 13 (1999) 2604-16.

Chiarle, R., Voena, C., Ambrogio, C., Piva, R. and Inghirami, G.: The anaplastic lymphoma kinase in the pathogenesis of cancer. Nat Rev Cancer 8 (2008) 11-23.

Clark, E.S., Brown, B., Whigham, A.S., Kochaishvili, A., Yarbrough, W.G. and Weaver, A.M.: Aggressiveness of HNSCC tumors depends on expression levels of cortactin, a gene in the 11q13 amplicon. Oncogene (2008).

Cohn, S.L., London, W.B., Huang, D., Katzenstein, H.M., Salwen, H.R., Reinhart, T., Madafiglio, J., Marshall, G.M., Norris, M.D. and Haber, M.: MYCN expression is not prognostic of adverse outcome in advanced-stage neuroblastoma with nonamplified MYCN. J Clin Oncol 18 (2000) 3604-13.

Cohn, S.L., Pearson, A.D., London, W.B., Monclair, T., Ambros, P.F., Brodeur, G.M., Faldum, A., Hero, B., Iehara, T., Machin, D., Mosseri, V., Simon, T., Garaventa, A., Castel, V. and Matthay, K.K.: The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J Clin Oncol 27 (2009) 289-97.

Cohn, S.L. and Tweddle, D.A.: MYCN amplification remains prognostically strong 20 years after its "clinical debut". Eur J Cancer 40 (2004) 2639-42.

Cushing, H. and Wolbach, S.B.: The transformation of a malignant paravertebral sympathicoblastoma into a benign ganglioneuroma. Am J Pathol 3 (1927) 203-216.

D'Angio, G.J., Evans, A.E. and Koop, C.E.: Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet 1 (1971) 1046-9.
Daniels, J.L., Olshan, A.F., Pollock, B.H., Shah, N.R. and Stram, D.O.: Breast-feeding and neuroblastoma, USA and Canada. Cancer Causes Control 13 (2002) 401-5.
Dasgupta, A., Peirce, S.K. and Findley, H.W.: MycN is a transcriptional regulator of livin in neuroblastoma. Oncol Rep 22 (2009) 831-5.
Davis, A.C., Wims, M., Spotts, G.D., Hann, S.R. and Bradley, A.: A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes Dev 7 (1993) 671-82.
Dole, M., Nunez, G., Merchant, A.K., Maybaum, J., Rode, C.K., Bloch, C.A. and Castle, V.P.: Bcl-2 inhibits chemotherapy-induced apoptosis in neuroblastoma. Cancer Res 54 (1994) 3253-9.
Duesberg, P.H., Bister, K. and Vogt, P.K.: The RNA of avian acute leukemia virus MC29. Proc Natl Acad Sci U S A 74 (1977) 4320-4.
Dyer, M.A.: Mouse models of childhood cancer of the nervous system. J Clin Pathol 57 (2004) 561-76.
Edsjo, A., Nilsson, H., Vandesompele, J., Karlsson, J., Pattyn, F., Culp, L.A., Speleman, F. and Pahlman, S.: Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation. Lab Invest 84 (2004) 406-17.
Eggert, A., Grotzer, M.A., Zuzak, T.J., Wiewrodt, B.R., Ikegaki, N. and Brodeur, G.M.: Resistance to TRAIL-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. Med Pediatr Oncol 35 (2000) 603-7.
Evans, A.E., Gerson, J. and Schnaufer, L.: Spontaneous regression of neuroblastoma. Natl Cancer Inst Monogr 44 (1976) 49-54.
Facchini, L.M. and Penn, L.Z.: The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. Faseb J 12 (1998) 633-51.
Fernandez, P.C., Frank, S.R., Wang, L., Schroeder, M., Liu, S., Greene, J., Cocito, A. and Amati, B.: Genomic targets of the human c-Myc protein. Genes Dev 17 (2003) 1115-29.
Fisher, T.S., Etages, S.D., Hayes, L., Crimin, K. and Li, B.: Analysis of ARD1 function in hypoxia response using retroviral RNA interference. J Biol Chem 280 (2005) 17749-57.
Florenes, V.A., Aamdal, S., Myklebost, O., Maelandsmo, G.M., Bruland, O.S. and Fodstad, O.: Levels of nm23 messenger RNA in metastatic malignant melanomas: inverse correlation to disease progression. Cancer Res 52 (1992) 6088-91.
Fluge, O., Bruland, O., Akslen, L.A., Varhaug, J.E. and Lillehaug, J.R.: NATH, a novel gene overexpressed in papillary thyroid carcinomas. Oncogene 21 (2002) 5056-68.
French, A.E., Grant, R., Weitzman, S., Ray, J.G., Vermeulen, M.J., Sung, L., Greenberg, M. and Koren, G.: Folic acid food fortification is associated with a decline in neuroblastoma. Clin Pharmacol Ther 74 (2003) 288-94.
Fulda, S.: Inhibitor of apoptosis proteins in hematological malignancies. Leukemia 23 (2009) 467-76.

Fulda, S., Poremba, C., Berwanger, B., Hacker, S., Eilers, M., Christiansen, H., Hero, B. and Debatin, K.M.: Loss of caspase-8 expression does not correlate with MYCN amplification, aggressive disease, or prognosis in neuroblastoma. Cancer Res 66 (2006) 10016-23.

Gautschi, M., Just, S., Mun, A., Ross, S., Rucknagel, P., Dubaquie, Y., Ehrenhofer-Murray, A. and Rospert, S.: The yeast N(alpha)-acetyltransferase NatA is quantitatively anchored to the ribosome and interacts with nascent polypeptides. Mol Cell Biol 23 (2003) 7403-14.

Gendron, R.L., Adams, L.C. and Paradis, H.: Tubedown-1, a novel acetyltransferase associated with blood vessel development. Dev Dyn 218 (2000) 300-15.

Gendron, R.L., Good, W.V., Adams, L.C. and Paradis, H.: Suppressed expression of tubedown-1 in retinal neovascularization of proliferative diabetic retinopathy. Invest Ophthalmol Vis Sci 42 (2001) 3000-7.

Gendron, R.L., Good, W.V., Miskiewicz, E., Tucker, S., Phelps, D.L. and Paradis, H.: Tubedown-1 (Tbdn-1) suppression in oxygen-induced retinopathy and in retinopathy of prematurity. Mol Vis 12 (2006) 108-16.

Gibbs, W.W.: Untangling the roots of cancer. Sci Am 289 (2003) 56-65.

Gisselsson, D., Lundberg, G., Ora, I. and Hoglund, M.: Distinct evolutionary mechanisms for genomic imbalances in high-risk and low-risk neuroblastomas. J Carcinog 6 (2007) 15.

Godfried, M.B., Veenstra, M., v Sluis, P., Boon, K., v Asperen, R., Hermus, M.C., v Schaik, B.D., Voute, T.P., Schwab, M., Versteeg, R. and Caron, H.N.: The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2. Oncogene 21 (2002) 2097-101.

Goldsby, R.E. and Matthay, K.K.: Neuroblastoma: evolving therapies for a disease with many faces. Paediatr Drugs 6 (2004) 107-22.

Grady, E.F., Schwab, M. and Rosenau, W.: Expression of N-myc and c-src during the development of fetal human brain. Cancer Res 47 (1987) 2931-6.

Grandori, C. and Eisenman, R.N.: Myc target genes. Trends Biochem Sci 22 (1997) 177-81.

Guillemot, F., Lo, L.C., Johnson, J.E., Auerbach, A., Anderson, D.J. and Joyner, A.L.: Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75 (1993) 463-76.

Gupta, A., Williams, B.R., Hanash, S.M. and Rawwas, J.: Cellular retinoic acid-binding protein II is a direct transcriptional target of MycN in neuroblastoma. Cancer Res 66 (2006) 8100-8.

Haas, D., Ablin, A.R., Miller, C., Zoger, S. and Matthay, K.K.: Complete pathologic maturation and regression of stage IVS neuroblastoma without treatment. Cancer 62 (1988) 818-25.

Hagemann, C., Meyer, C., Stojic, J., Eicker, S., Gerngras, S., Kuhnel, S., Roosen, K. and Vince, G.H.: High efficiency transfection of glioma cell lines and primary cells for overexpression and RNAi experiments. J Neurosci Methods 156 (2006) 194-202.

Halazonetis, T.D. and Kandil, A.N.: Determination of the c-MYC DNA-binding site. Proc Natl Acad Sci U S A 88 (1991) 6162-6.

Hanahan, D. and Weinberg, R.A.: The hallmarks of cancer. Cell 100 (2000) 57-70.

Hatsuzawa, K., Hirose, H., Tani, K., Yamamoto, A., Scheller, R.H. and Tagaya, M.: Syntaxin 18, a SNAP receptor that functions in the endoplasmic reticulum, intermediate compartment, and cis-Golgi vesicle trafficking. J Biol Chem 275 (2000) 13713-20.

Hatton, K.S., Mahon, K., Chin, L., Chiu, F.C., Lee, H.W., Peng, D., Morgenbesser, S.D., Horner, J. and DePinho, R.A.: Expression and activity of L-Myc in normal mouse development. Mol Cell Biol 16 (1996) 1794-804.

Hayama, M., Okumura, Y., Takahashi, E., Shimabukuro, A., Tamura, M., Takeda, N., Kubo, T. and Kido, H.: Identification and analysis of the promoter region of the type II transmembrane serine protease polyserase-1 and its transcript variants. Biol Chem 388 (2007) 853-8.

Hellstrom, I.E., Hellstrom, K.E., Pierce, G.E. and Bill, A.H.: Demonstration of cell-bound and humoral immunity against neuroblastoma cells. Proc Natl Acad Sci U S A 60 (1968) 1231-8.

Hennessy, C., Henry, J.A., May, F.E., Westley, B.R., Angus, B. and Lennard, T.W.: Expression of the antimetastatic gene nm23 in human breast cancer: an association with good prognosis. J Natl Cancer Inst 83 (1991) 281-5.

Henriksson, M., Selivanova, G., Lindstrom, M. and Wiman, K.G.: Inactivation of Myc-induced p53-dependent apoptosis in human tumors. Apoptosis 6 (2001) 133-7.

Hill, D.P. and Robertson, K.A.: Characterization of the cholinergic neuronal differentiation of the human neuroblastoma cell line LA-N-5 after treatment with retinoic acid. Brain Res Dev Brain Res 102 (1997) 53-67.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K., Poellinger, L. and Pahlman, S.: Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell 10 (2006) 413-23.

Hou, F., Chu, C.W., Kong, X., Yokomori, K. and Zou, H.: The acetyltransferase activity of San stabilizes the mitotic cohesin at the centromeres in a shugoshin-independent manner. J Cell Biol 177 (2007) 587-97.

Howman-Giles, R., Shaw, P.J., Uren, R.F. and Chung, D.K.: Neuroblastoma and other neuroendocrine tumors. Semin Nucl Med 37 (2007) 286-302.

Hsu, W.M., Hsieh, F.J., Jeng, Y.M., Kuo, M.L., Chen, C.N., Lai, D.M., Hsieh, L.J., Wang, B.T., Tsao, P.N., Lee, H., Lin, M.T., Lai, H.S. and Chen, W.J.: Calreticulin expression in neuroblastoma--a novel independent prognostic factor. Ann Oncol 16 (2005) 314-21.

Ichimiya, S., Nimura, Y., Seki, N., Ozaki, T., Nagase, T. and Nakagawara, A.: Downregulation of hASH1 is associated with the retinoic acid-induced differentiation of human neuroblastoma cell lines. Med Pediatr Oncol 36 (2001) 132-4.

Inge, T.H., Casson, L.K., Priebe, W., Trent, J.O., Georgeson, K.E., Miller, D.M. and Bates, P.J.: Importance of Sp1 consensus motifs in the MYCN promoter. Surgery 132 (2002) 232-8.

Ishola, T.A. and Chung, D.H.: Neuroblastoma. Surg Oncol 16 (2007) 149-56.

Jogi, A., Ora, I., Nilsson, H., Lindeheim, A., Makino, Y., Poellinger, L., Axelson, H. and Pahlman, S.: Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A 99 (2002) 7021-6.

Joshi, V.V.: Peripheral neuroblastic tumors: pathologic classification based on recommendations of international neuroblastoma pathology committee (Modification of shimada classification). Pediatr Dev Pathol 3 (2000) 184-99.

Joyner, B.D. and Lopushnyan, N.: Neuroblastoma, eMedicine, 2007.

Kaidi, A., Williams, A.C. and Paraskeva, C.: Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. Nat Cell Biol 9 (2007) 210-7.

Kaneko, S., Ohira, M., Nakamura, Y., Isogai, E., Nakagawara, A. and Kaneko, M.: Relationship of DDX1 and NAG gene amplification/overexpression to the prognosis of patients with MYCN-amplified neuroblastoma. J Cancer Res Clin Oncol 133 (2007) 185-92.

Kline, N.E. and Sevier, N.: Solid tumors in children. J Pediatr Nurs 18 (2003) 96-102.

Knudson, A.G., Jr.: Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68 (1971) 820-3.

Knudson, A.G., Jr. and Strong, L.C.: Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet 24 (1972) 514-32.

Kogner, P., Barbany, G., Dominici, C., Castello, M.A., Raschella, G. and Persson, H.: Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res 53 (1993) 2044-50.

Kohl, N.E., Kanda, N., Schreck, R.R., Bruns, G., Latt, S.A., Gilbert, F. and Alt, F.W.: Transposition and amplification of oncogene-related sequences in human neuroblastomas. Cell 35 (1983) 359-67.

Koppen, A., Ait-Aissa, R., Koster, J., van Sluis, P.G., Ora, I., Caron, H.N., Volckmann, R., Versteeg, R. and Valentijn, L.J.: Direct regulation of the minichromosome maintenance complex by MYCN in neuroblastoma. Eur J Cancer 43 (2007) 2413-22.

Krams, M., Parwaresch, R., Sipos, B., Heidorn, K., Harms, D. and Rudolph, P.: Expression of the c-kit receptor characterizes a subset of neuroblastomas with favorable prognosis. Oncogene 23 (2004) 588-95.

Kushner, B.H., Gilbert, F. and Helson, L.: Familial neuroblastoma. Case reports, literature review, and etiologic considerations. Cancer 57 (1986) 1887-93.

Lamant, L., Pulford, K., Bischof, D., Morris, S.W., Mason, D.Y., Delsol, G. and Mariame, B.: Expression of the ALK tyrosine kinase gene in neuroblastoma. Am J Pathol 156 (2000) 1711-21.

Lasorella, A., Boldrini, R., Dominici, C., Donfrancesco, A., Yokota, Y., Inserra, A. and Iavarone, A.: Id2 is critical for cellular proliferation and is the oncogenic effector of N-myc in human neuroblastoma. Cancer Res 62 (2002) 301-6.

Lastowska, M., Cotterill, S., Pearson, A.D., Roberts, P., McGuckin, A., Lewis, I. and Bown, N.: Gain of chromosome arm 17q predicts unfavourable outcome in neuroblastoma patients. U.K. Children's Cancer Study Group and the U.K. Cancer Cytogenetics Group. Eur J Cancer 33 (1997) 1627-33.

Lavarino, C., Cheung, N.K., Garcia, I., Domenech, G., de Torres, C., Alaminos, M., Rios, J., Gerald, W.L., Kushner, B., LaQuaglia, M. and Mora, J.: Specific gene expression profiles and chromosomal abnormalities are associated with infant disseminated neuroblastoma. BMC Cancer 9 (2009) 44.

Laverdiere, C., Cheung, N.K., Kushner, B.H., Kramer, K., Modak, S., LaQuaglia, M.P., Wolden, S., Ness, K.K., Gurney, J.G. and Sklar, C.A.: Long-term complications in survivors of advanced stage neuroblastoma. Pediatr Blood Cancer 45 (2005) 324-32.

Leeman, R.J., Lui, V.W. and Grandis, J.R.: STAT3 as a therapeutic target in head and neck cancer. Expert Opin Biol Ther 6 (2006) 231-41.

Lim, J.H., Chun, Y.S. and Park, J.W.: Hypoxia-inducible factor-1alpha obstructs a Wnt signaling pathway by inhibiting the hARD1-mediated activation of beta-catenin. Cancer Res 68 (2008) 5177-84.

Lim, J.H., Park, J.W. and Chun, Y.S.: Human arrest defective 1 acetylates and activates beta-catenin, promoting lung cancer cell proliferation. Cancer Res 66 (2006) 10677-82.

Lindstrom, M.S.: Emerging functions of ribosomal proteins in gene-specific transcription and translation. Biochem Biophys Res Commun 379 (2009) 167-70.

Line, A., Stengrevics, A., Slucka, Z., Li, G., Jankevics, E. and Rees, R.C.: Serological identification and expression analysis of gastric cancer-associated genes. Br J Cancer 86 (2002) 1824-30.

Liu, X., Mazanek, P., Dam, V., Wang, Q., Zhao, H., Guo, R., Jagannathan, J., Cnaan, A., Maris, J.M. and Hogarty, M.D.: Deregulated Wnt/beta-catenin program in high-risk neuroblastomas without MYCN amplification. Oncogene 27 (2008) 1478-88.

Lo, L.C., Johnson, J.E., Wuenschell, C.W., Saito, T. and Anderson, D.J.: Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev 5 (1991) 1524-37.

Lofstedt, T., Fredlund, E., Holmquist-Mengelbier, L., Pietras, A., Ovenberger, M., Poellinger, L. and Pahlman, S.: Hypoxia inducible factor-2alpha in cancer. Cell Cycle 6 (2007) 919-26.

London, W.B., Castleberry, R.P., Matthay, K.K., Look, A.T., Seeger, R.C., Shimada, H., Thorner, P., Brodeur, G., Maris, J.M., Reynolds, C.P. and Cohn, S.L.: Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol 23 (2005) 6459-65.

Lonergan, G.J., Schwab, C.M., Suarez, E.S. and Carlson, C.L.: Neuroblastoma, ganglioneuroblastoma, and ganglioneuroma: radiologic-pathologic correlation. Radiographics 22 (2002) 911-34.

Lu, X., Pearson, A. and Lunec, J.: The MYCN oncoprotein as a drug development target. Cancer Lett 197 (2003) 125-30.

Lutz, W., Stohr, M., Schurmann, J., Wenzel, A., Lohr, A. and Schwab, M.: Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene 13 (1996) 803-12.

Macville, M., Schrock, E., Padilla-Nash, H., Keck, C., Ghadimi, B.M., Zimonjic, D., Popescu, N. and Ried, T.: Comprehensive and definitive molecular cytogenetic characterization of HeLa cells by spectral karyotyping. Cancer Res 59 (1999) 141-50.

Malynn, B.A., de Alboran, I.M., O'Hagan, R.C., Bronson, R., Davidson, L., DePinho, R.A. and Alt, F.W.: N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation. Genes Dev 14 (2000) 1390-9.

Manohar, C.F., Bray, J.A., Salwen, H.R., Madafiglio, J., Cheng, A., Flemming, C., Marshall, G.M., Norris, M.D., Haber, M. and Cohn, S.L.: MYCN-mediated regulation of the MRP1 promoter in human neuroblastoma. Oncogene 23 (2004) 753-62.

Maris, J.M.: The biologic basis for neuroblastoma heterogeneity and risk stratification. Curr Opin Pediatr 17 (2005) 7-13.

Maris, J.M. and Brodeur, G.M.: Genetics. In: Cheung, N.-K.V. and Cohn, S.L. (Eds.), Pediatric Oncology - Neuroblastoma. Springer, 2005, pp. 21-26.

Maris, J.M. and Denny, C.T.: Focus on embryonal malignancies. Cancer Cell 2 (2002) 447-50.

Maris, J.M., Hogarty, M.D., Bagatell, R. and Cohn, S.L.: Neuroblastoma. Lancet 369 (2007) 2106-20.

Maris, J.M. and Matthay, K.K.: Molecular biology of neuroblastoma. J Clin Oncol 17 (1999) 2264-79.

Maris, J.M., Weiss, M.J., Guo, C., Gerbing, R.B., Stram, D.O., White, P.S., Hogarty, M.D., Sulman, E.P., Thompson, P.M., Lukens, J.N., Matthay, K.K., Seeger, R.C. and Brodeur, G.M.: Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. J Clin Oncol 18 (2000) 1888-99.

Maris, J.M., Weiss, M.J., Mosse, Y., Hii, G., Guo, C., White, P.S., Hogarty, M.D., Mirensky, T., Brodeur, G.M., Rebbeck, T.R., Urbanek, M. and Shusterman, S.: Evidence for a hereditary neuroblastoma predisposition locus at chromosome 16p12-13. Cancer Res 62 (2002) 6651-8.

Martin, D.T., Gendron, R.L., Jarzembowski, J.A., Perry, A., Collins, M.H., Pushpanathan, C., Miskiewicz, E., Castle, V.P. and Paradis, H.: Tubedown expression correlates with the differentiation status and aggressiveness of neuroblastic tumors. Clin Cancer Res 13 (2007) 1480-7.

Matthay, K.K.: MYCN expression in neuroblastoma: A mixed message? J Clin Oncol 18 (2000) 3591-4.

Matthay, K.K., Villablanca, J.G., Seeger, R.C., Stram, D.O., Harris, R.E., Ramsay, N.K., Swift, P., Shimada, H., Black, C.T., Brodeur, G.M., Gerbing, R.B. and Reynolds, C.P.: Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N Engl J Med 341 (1999) 1165-73.

McCall, E.E., Olshan, A.F. and Daniels, J.L.: Maternal hair dye use and risk of neuroblastoma in offspring. Cancer Causes Control 16 (2005) 743-8.

McGowan-Jordan, I.J., Speevak, M.D., Blakey, D. and Chevrette, M.: Suppression of tumorigenicity in human teratocarcinoma cell line PA-1 by introduction of chromosome 4. Cancer Res 54 (1994) 2568-72.

Mehta, D. and Malik, A.B.: Signaling mechanisms regulating endothelial permeability. Physiol Rev 86 (2006) 279-367.

Michels, E., Vandesompele, J., De Preter, K., Hoebeeck, J., Vermeulen, J., Schramm, A., Molenaar, J.J., Menten, B., Marques, B., Stallings, R.L., Combaret, V., Devalck, C., De Paepe, A., Versteeg, R., Eggert, A., Laureys, G., Van Roy, N. and Speleman, F.: ArrayCGH-based classification of neuroblastoma into genomic subgroups. Genes Chromosomes Cancer 46 (2007) 1098-108.

Miltenberger, R.J., Sukow, K.A. and Farnham, P.J.: An E-box-mediated increase in cad transcription at the G1/S-phase boundary is suppressed by inhibitory c-Myc mutants. Mol Cell Biol 15 (1995) 2527-35.

Monclair, T., Brodeur, G.M., Ambros, P.F., Brisse, H.J., Cecchetto, G., Holmes, K., Kaneko, M., London, W.B., Matthay, K.K., Nuchtern, J.G., von Schweinitz, D., Simon, T., Cohn, S.L. and Pearson, A.D.: The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report. J Clin Oncol 27 (2009) 298-303.

Mora, J. and Gerald, W.L.: Origin of neuroblastic tumors: clues for future therapeutics. Expert Rev Mol Diagn 4 (2004) 293-302.

Mosse, Y.P., Laudenslager, M., Khazi, D., Carlisle, A.J., Winter, C.L., Rappaport, E. and Maris, J.M.: Germline PHOX2B mutation in hereditary neuroblastoma. Am J Hum Genet 75 (2004) 727-30.

Mosse, Y.P., Laudenslager, M., Longo, L., Cole, K.A., Wood, A., Attiyeh, E.F., Laquaglia, M.J., Sennett, R., Lynch, J.E., Perri, P., Laureys, G., Speleman, F., Kim, C., Hou, C., Hakonarson, H., Torkamani, A., Schork, N.J., Brodeur, G.M., Tonini, G.P., Rappaport, E., Devoto, M. and Maris, J.M.: Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 455 (2008) 930-5.

Mukherjee, B., Morgenbesser, S.D. and DePinho, R.A.: Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and trans-acting dominant mutants. Genes Dev 6 (1992) 1480-92.

Mullen, J.R., Kayne, P.S., Moerschell, R.P., Tsunasawa, S., Gribskov, M., Colavito-Shepanski, M., Grunstein, M., Sherman, F. and Sternglanz, R.: Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. Embo J 8 (1989) 2067-75.

Nakagawara, A.: Molecular and Developmental Biology of Neuroblastoma. In: Cheung, N.-K.V. and Cohn, S.L. (Eds.), Pediatric Oncology - Neuroblastoma. Springer, 2005, pp. 41-53.

Nakagawara, A., Arima-Nakagawara, M., Scavarda, N.J., Azar, C.G., Cantor, A.B. and Brodeur, G.M.: Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med 328 (1993) 847-54.

Nakagawara, A., Azar, C.G., Scavarda, N.J. and Brodeur, G.M.: Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 14 (1994) 759-67.

Nara, K., Kusafuka, T., Yoneda, A., Oue, T., Sangkhathat, S. and Fukuzawa, M.: Silencing of MYCN by RNA interference induces growth inhibition, apoptotic activity and cell differentiation in a neuroblastoma cell line with MYCN amplification. Int J Oncol 30 (2007) 1189-96.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F. and Minna, J.D.: L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318 (1985) 69-73.

Negroni, A., Scarpa, S., Romeo, A., Ferrari, S., Modesti, A. and Raschella, G.: Decrease of proliferation rate and induction of differentiation by a MYCN antisense DNA oligomer in a human neuroblastoma cell line. Cell Growth Differ 2 (1991) 511-8.

Nesbit, C.E., Tersak, J.M. and Prochownik, E.V.: MYC oncogenes and human neoplastic disease. Oncogene 18 (1999) 3004-16.

Niitsu, N., Okabe-Kado, J., Okamoto, M., Takagi, T., Yoshida, T., Aoki, S., Hirano, M. and Honma, Y.: Serum nm23-H1 protein as a prognostic factor in aggressive non-Hodgkin lymphoma. Blood 97 (2001) 1202-10.

Nilsson, H., Jogi, A., Beckman, S., Harris, A.L., Poellinger, L. and Pahlman, S.: HIF-2alpha expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia. Exp Cell Res 303 (2005) 447-56.

Nisen, P.D., Waber, P.G., Rich, M.A., Pierce, S., Garvin, J.R., Jr., Gilbert, F. and Lanzkowsky, P.: N-myc oncogene RNA expression in neuroblastoma. J Natl Cancer Inst 80 (1988) 1633-7.

Ochsenbein, A.F., Klenerman, P., Karrer, U., Ludewig, B., Pericin, M., Hengartner, H. and Zinkernagel, R.M.: Immune surveillance against a solid tumor fails because of immunological ignorance. Proc Natl Acad Sci U S A 96 (1999) 2233-8.

Ohkawa, N., Sugisaki, S., Tokunaga, E., Fujitani, K., Hayasaka, T., Setou, M. and Inokuchi, K.: N-acetyltransferase ARD1-NAT1 regulates neuronal dendritic development. Genes Cells 13 (2008) 1171-83.

Olshan, A.F., Smith, J.C., Bondy, M.L., Neglia, J.P. and Pollock, B.H.: Maternal vitamin use and reduced risk of neuroblastoma. Epidemiology 13 (2002) 575-80.

Oppenheim, R.W.: Cell death during development of the nervous system. Annu Rev Neurosci 14 (1991) 453-501.

Oue, T., Fukuzawa, M., Kusafuka, T., Kohmoto, Y., Imura, K., Nagahara, S. and Okada, A.: In situ detection of DNA fragmentation and expression of bcl-2 in human

neuroblastoma: relation to apoptosis and spontaneous regression. J Pediatr Surg 31 (1996) 251-7.
Paffhausen, T., Schwab, M. and Westermann, F.: Targeted MYCN expression affects cytotoxic potential of chemotherapeutic drugs in neuroblastoma cells. Cancer Lett 250 (2007) 17-24.
Pahlman, S., Stockhausen, M.T., Fredlund, E. and Axelson, H.: Notch signaling in neuroblastoma. Semin Cancer Biol 14 (2004) 365-73.
Paradis, H., Islam, T., Tucker, S., Tao, L., Koubi, S. and Gendron, R.L.: Tubedown associates with cortactin and controls permeability of retinal endothelial cells to albumin. J Cell Sci 121 (2008) 1965-72.
Paradis, H., Liu, C.Y., Saika, S., Azhar, M., Doetschman, T., Good, W.V., Nayak, R., Laver, N., Kao, C.W., Kao, W.W. and Gendron, R.L.: Tubedown-1 in remodeling of the developing vitreal vasculature in vivo and regulation of capillary outgrowth in vitro. Dev Biol 249 (2002) 140-55.
Parham, D.M.: Ewing's sarcoma, peripheral neuroepithelioma, and related tumors. , Pediatric Neoplasia: Morphology and Biology. Lippincott Williams & Wilkins, 1996, pp. 65-85.
Park, E.C. and Szostak, J.W.: ARD1 and NAT1 proteins form a complex that has N-terminal acetyltransferase activity. Embo J 11 (1992) 2087-93.
Pession, A. and Tonelli, R.: The MYCN oncogene as a specific and selective drug target for peripheral and central nervous system tumors. Curr Cancer Drug Targets 5 (2005) 273-83.
Peverali, F.A., Orioli, D., Tonon, L., Ciana, P., Bunone, G., Negri, M. and Della-Valle, G.: Retinoic acid-induced growth arrest and differentiation of neuroblastoma cells are counteracted by N-myc and enhanced by max overexpressions. Oncogene 12 (1996) 457-62.
Pisick, E., Skarin, A.T. and Salgia, R.: Recent advances in the molecular biology, diagnosis and novel therapies for various small blue cell tumors. Anticancer Res 23 (2003) 3379-96.
Polevoda, B., Brown, S., Cardillo, T.S., Rigby, S. and Sherman, F.: Yeast N(alpha)-terminal acetyltransferases are associated with ribosomes. J Cell Biochem 103 (2008) 492-508.
Polevoda, B. and Sherman, F.: Composition and function of the eukaryotic N-terminal acetyltransferase subunits. Biochem Biophys Res Commun 308 (2003) 1-11.
Pritchard, J. and Hickman, J.A.: Why does stage 4s neuroblastoma regress spontaneously? Lancet 344 (1994) 869-70.
Rangecroft, L., Lauder, I. and Wagget, J.: Spontaneous maturation of stage IV--S neuroblastoma. Arch Dis Child 53 (1978) 815-7.
Raue, U., Oellerer, S. and Rospert, S.: Association of protein biogenesis factors at the yeast ribosomal tunnel exit is affected by the translational status and nascent polypeptide sequence. J Biol Chem 282 (2007) 7809-16.
Reynolds, C.P., Matthay, K.K., Villablanca, J.G. and Maurer, B.J.: Retinoid therapy of high-risk neuroblastoma. Cancer Lett 197 (2003) 185-92.

Reynolds, C.P., Schindler, P.F., Jones, D.M., Gentile, J.L., Proffitt, R.T. and Einhorn, P.A.: Comparison of 13-cis-retinoic acid to trans-retinoic acid using human neuroblastoma cell lines. Prog Clin Biol Res 385 (1994) 237-44.

Robson, J.A. and Sidell, N.: Ultrastructural features of a human neuroblastoma cell line treated with retinoic acid. Neuroscience 14 (1985) 1149-62.

Rorie, C.J., Thomas, V.D., Chen, P., Pierce, H.H., O'Bryan, J.P. and Weissman, B.E.: The Ews/Fli-1 fusion gene switches the differentiation program of neuroblastomas to Ewing sarcoma/peripheral primitive neuroectodermal tumors. Cancer Res 64 (2004) 1266-77.

Ross, R.A., Hein, A.M., Braca, J.A., 3rd, Spengler, B.A., Biedler, J.L. and Scammell, J.G.: Glucocorticoids induce neuroendocrine cell differentiation and increase expression of N-myc in N-type human neuroblastoma cells. Oncol Res 13 (2002) 87-94.

Ross, R.A., Spengler, B.A. and Biedler, J.L.: Coordinate morphological and biochemical interconversion of human neuroblastoma cells. J Natl Cancer Inst 71 (1983) 741-7.

Ryan, K.M. and Birnie, G.D.: Myc oncogenes: the enigmatic family. Biochem J 314 (Pt 3) (1996) 713-21.

Ryden, M., Sehgal, R., Dominici, C., Schilling, F.H., Ibanez, C.F. and Kogner, P.: Expression of mRNA for the neurotrophin receptor trkC in neuroblastomas with favourable tumour stage and good prognosis. Br J Cancer 74 (1996) 773-9.

Sawada, T., Kidowaki, T., Sakamoto, I., Hashida, T., Matsumura, T., Nakagawa, M. and Kusunoki, T.: Neuroblastoma. Mass screening for early detection and its prognosis. Cancer 53 (1984) 2731-5.

Schilling, F.H., Erttmann, R., Ambros, P.F., Strehl, S., Christiansen, H., Kovar, H., Kabisch, H. and Treuner, J.: Screening for neuroblastoma. Lancet 344 (1994) 1157-8.

Schulz, W.A.: An introduction to human cancers, Molecular Biology of Human Cancers: An advanced student's textbook. Springer, 2007, pp. 1-23.

Schwab, M.: MYCN Amplification in Neuroblastoma: a Paradigm for the Clinical Use of an Oncogene. Pathol Oncol Res 3 (1997) 3-7.

Schwab, M.: MYCN in neuronal tumours. Cancer Lett 204 (2004) 179-87.

Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J.: Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305 (1983) 245-8.

Schwab, M., Varmus, H.E., Bishop, J.M., Grzeschik, K.H., Naylor, S.L., Sakaguchi, A.Y., Brodeur, G. and Trent, J.: Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc. Nature 308 (1984) 288-91.

Seeger, R.C., Brodeur, G.M., Sather, H., Dalton, A., Siegel, S.E., Wong, K.Y. and Hammond, D.: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313 (1985) 1111-6.

Seeger, R.C., Danon, Y.L., Rayner, S.A. and Hoover, F.: Definition of a Thy-1 determinant on human neuroblastoma, glioma, sarcoma, and teratoma cells with a monoclonal antibody. J Immunol 128 (1982a) 983-9.

Seeger, R.C., Siegel, S.E. and Sidell, N.: Neuroblastoma: clinical perspectives, monoclonal antibodies, and retinoic acid. Ann Intern Med 97 (1982b) 873-84.

Seeger, R.C., Wada, R., Brodeur, G.M., Moss, T.J., Bjork, R.L., Sousa, L. and Slamon, D.J.: Expression of N-myc by neuroblastomas with one or multiple copies of the oncogene. Prog Clin Biol Res 271 (1988) 41-9.

Sherwood, L.: The Peripheral Nervous System: Efferent Division, Human Physiology: From Cells To Systems. Wadsworth Publishing Company, 1997, pp. 203-220.

Shimada, H., Ambros, I.M., Dehner, L.P., Hata, J., Joshi, V.V. and Roald, B.: Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer 86 (1999a) 349-63.

Shimada, H., Ambros, I.M., Dehner, L.P., Hata, J., Joshi, V.V., Roald, B., Stram, D.O., Gerbing, R.B., Lukens, J.N., Matthay, K.K. and Castleberry, R.P.: The International Neuroblastoma Pathology Classification (the Shimada system). Cancer 86 (1999b) 364-72.

Shimada, H., Chatten, J., Newton, W.A., Jr., Sachs, N., Hamoudi, A.B., Chiba, T., Marsden, H.B. and Misugi, K.: Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst 73 (1984) 405-16.

Sidell, N., Altman, A., Haussler, M.R. and Seeger, R.C.: Effects of retinoic acid (RA) on the growth and phenotypic expression of several human neuroblastoma cell lines. Exp Cell Res 148 (1983) 21-30.

Skog, J., Mei, Y.F. and Wadell, G.: Human adenovirus serotypes 4p and 11p are efficiently expressed in cell lines of neural tumour origin. J Gen Virol 83 (2002) 1299-309.

Slack, A., Chen, Z., Tonelli, R., Pule, M., Hunt, L., Pession, A. and Shohet, J.M.: The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. Proc Natl Acad Sci U S A 102 (2005) 731-6.

Slavc, I., Ellenbogen, R., Jung, W.H., Vawter, G.F., Kretschmar, C., Grier, H. and Korf, B.R.: myc gene amplification and expression in primary human neuroblastoma. Cancer Res 50 (1990) 1459-63.

Soderholm, H., Ortoft, E., Johansson, I., Ljungberg, J., Larsson, C., Axelson, H. and Pahlman, S.: Human achaete-scute homologue 1 (HASH-1) is downregulated in differentiating neuroblastoma cells. Biochem Biophys Res Commun 256 (1999) 557-63.

Soignet, S.L., Maslak, P., Wang, Z.G., Jhanwar, S., Calleja, E., Dardashti, L.J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D.A., Pandolfi, P.P. and Warrell, R.P., Jr.: Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. N Engl J Med 339 (1998) 1341-8.

Solomon, D.L., Amati, B. and Land, H.: Distinct DNA binding preferences for the c-Myc/Max and Max/Max dimers. Nucleic Acids Res 21 (1993) 5372-6.

Stanton, L.W., Schwab, M. and Bishop, J.M.: Nucleotide sequence of the human N-myc gene. Proc Natl Acad Sci U S A 83 (1986) 1772-6.
Strieder, V. and Lutz, W.: E2F proteins regulate MYCN expression in neuroblastomas. J Biol Chem 278 (2003) 2983-9.
Suenaga, Y., Kaneko, Y., Matsumoto, D., Hossain, M.S., Ozaki, T. and Nakagawara, A.: Positive auto-regulation of MYCN in human neuroblastoma. Biochem Biophys Res Commun 390 (2009) 21-6.
Sugiura, N., Adams, S.M. and Corriveau, R.A.: An evolutionarily conserved N-terminal acetyltransferase complex associated with neuronal development. J Biol Chem 278 (2003) 40113-20.
Swanson, H.I. and Yang, J.H.: Specificity of DNA binding of the c-Myc/Max and ARNT/ARNT dimers at the CACGTG recognition site. Nucleic Acids Res 27 (1999) 3205-12.
Tang, X.X., Zhao, H., Kung, B., Kim, D.Y., Hicks, S.L., Cohn, S.L., Cheung, N.K., Seeger, R.C., Evans, A.E. and Ikegaki, N.: The MYCN enigma: significance of MYCN expression in neuroblastoma. Cancer Res 66 (2006) 2826-33.
Teitz, T., Lahti, J.M. and Kidd, V.J.: Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. J Mol Med 79 (2001) 428-36.
Teitz, T., Wei, T., Valentine, M.B., Vanin, E.F., Grenet, J., Valentine, V.A., Behm, F.G., Look, A.T., Lahti, J.M. and Kidd, V.J.: Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med 6 (2000) 529-35.
Thiele, C.J., Reynolds, C.P. and Israel, M.A.: Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313 (1985) 404-6.
Trochet, D., Bourdeaut, F., Janoueix-Lerosey, I., Deville, A., de Pontual, L., Schleiermacher, G., Coze, C., Philip, N., Frebourg, T., Munnich, A., Lyonnet, S., Delattre, O. and Amiel, J.: Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet 74 (2004) 761-4.
Tsubono, Y. and Hisamichi, S.: A halt to neuroblastoma screening in Japan. N Engl J Med 350 (2004) 2010-1.
Tumilowicz, J.J., Nichols, W.W., Cholon, J.J. and Greene, A.E.: Definition of a continuous human cell line derived from neuroblastoma. Cancer Res 30 (1970) 2110-8.
van Noesel, M.M. and Versteeg, R.: Pediatric neuroblastomas: genetic and epigenetic 'danse macabre'. Gene 325 (2004) 1-15.
Vandesompele, J., Edsjo, A., De Preter, K., Axelson, H., Speleman, F. and Pahlman, S.: ID2 expression in neuroblastoma does not correlate to MYCN levels and lacks prognostic value. Oncogene 22 (2003) 456-60.
Vasudevan, S.A., Nuchtern, J.G. and Shohet, J.M.: Gene profiling of high risk neuroblastoma. World J Surg 29 (2005) 317-24.

Vennstrom, B., Sheiness, D., Zabielski, J. and Bishop, J.M.: Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J Virol 42 (1982) 773-9.

Vita, M. and Henriksson, M.: The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 16 (2006) 318-30.

Voigt, A. and Zintl, F.: Effects of retinoic acid on proliferation, apoptosis, cytotoxicity, migration, and invasion of neuroblastoma cells. Med Pediatr Oncol 40 (2003) 205-13.

Wai, D.H., Schaefer, K.L., Schramm, A., Korsching, E., Van Valen, F., Ozaki, T., Boecker, W., Schweigerer, L., Dockhorn-Dworniczak, B. and Poremba, C.: Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. Int J Oncol 20 (2002) 441-51.

Wall, D.S., Gendron, R.L., Good, W.V., Miskiewicz, E., Woodland, M., Leblanc, K. and Paradis, H.: Conditional knockdown of tubedown-1 in endothelial cells leads to neovascular retinopathy. Invest Ophthalmol Vis Sci 45 (2004) 3704-12.

Wang, X., Connelly, J.J., Wang, C.L. and Sternglanz, R.: Importance of the Sir3 N terminus and its acetylation for yeast transcriptional silencing. Genetics 168 (2004) 547-51.

Weaver, A.M.: Cortactin in tumor invasiveness. Cancer Lett 265 (2008) 157-66.

Wei, J.S., Song, Y.K., Durinck, S., Chen, Q.R., Cheuk, A.T., Tsang, P., Zhang, Q., Thiele, C.J., Slack, A., Shohet, J. and Khan, J.: The MYCN oncogene is a direct target of miR-34a. Oncogene (2008).

Wei, J.S., Whiteford, C.C., Cenacchi, N., Son, C.G. and Khan, J.: BBC3 mediates fenretinide-induced cell death in neuroblastoma. Oncogene 24 (2005) 7976-83.

Weiss, M.J., Guo, C., Shusterman, S., Hii, G., Mirensky, T.L., White, P.S., Hogarty, M.D., Rebbeck, T.R., Teare, D., Urbanek, M., Brodeur, G.M. and Maris, J.M.: Localization of a hereditary neuroblastoma predisposition gene to 16p12-p13. Med Pediatr Oncol 35 (2000) 526-30.

Weiss, W.A., Aldape, K., Mohapatra, G., Feuerstein, B.G. and Bishop, J.M.: Targeted expression of MYCN causes neuroblastoma in transgenic mice. Embo J 16 (1997) 2985-95.

Wenzel, A. and Schwab, M.: The mycN/max protein complex in neuroblastoma. Short review. Eur J Cancer 31A (1995) 516-9.

Whitesell, L., Rosolen, A. and Neckers, L.M.: Antisense suppression of N-myc expression inhibits the transdifferentiation of neuroectoderm tumor cell lines. Prog Clin Biol Res 366 (1991) 45-54.

Williams, B.C., Li, Z., Liu, S., Williams, E.V., Leung, G., Yen, T.J. and Goldberg, M.L.: Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions. Mol Biol Cell 14 (2003) 1379-91.

Willis, D.M., Loewy, A.P., Charlton-Kachigian, N., Shao, J.S., Ornitz, D.M. and Towler, D.A.: Regulation of osteocalcin gene expression by a novel Ku antigen transcription factor complex. J Biol Chem 277 (2002) 37280-91.

Woods, W.G., Tuchman, M., Robison, L.L., Bernstein, M., Leclerc, J.M., Brisson, L.C., Brossard, J., Hill, G., Shuster, J., Luepker, R., Byrne, T., Weitzman, S., Bunin, G.

and Lemieux, B.: A population-based study of the usefulness of screening for neuroblastoma. Lancet 348 (1996) 1682-7.

Yamashiro, D.J., Nakagawara, A., Ikegaki, N., Liu, X.G. and Brodeur, G.M.: Expression of TrkC in favorable human neuroblastomas. Oncogene 12 (1996) 37-41.

Zhu, Z., Zheng, T., Lee, C.G., Homer, R.J. and Elias, J.A.: Tetracycline-controlled transcriptional regulation systems: advances and application in transgenic animal modeling. Semin Cell Dev Biol 13 (2002) 121-8.