ALMA MATER STUDIORUM

UNIVERSITA' DEGLI STUDI DI BOLOGNA

PhD Program in Cell Biology and Physiology – XXI Cycle
Scientific area code Bio/18

COMPLEXITY OF N-MYC TRANSCRIPTIONAL FUNCTION IN CHILDHOOD NEUROBLASTOMA

PhD candidate: **Nunzio Iraci**

PhD Program Coordinator:

Prof.ssa Michela Rugolo

Supervisor:

Prof. Giovanni Perini

Final Exam - Bologna, April 2009

Abstract

Myc is a transcription factor that can activate transcription of several hundreds genes by direct binding to their promoters at specific DNA sequences (E-box). However, recent studies have also shown that it can exert its biological role by repressing transcription. Such studies collectively support a model in which c-Myc-mediated repression occurs through interactions with transcription factors bound to promoter DNA regions but not through direct recognition of typical E-box sequences. Here, we investigated whether N-Myc can also repress gene transcription, and how this is mechanistically achieved. We used human neuroblastoma cells as a model system in that N-MYC amplification/over-expression represents a key prognostic marker of this tumour. By means of transcription profile analyses we could identify at least 5 genes (TRKA, p75NTR, ABCC3, TG2, p21) that are specifically repressed by N-Myc. Through a dual-step-ChIP assay and genetic dissection of gene promoters, we found that N-Myc is physically associated with gene promoters *in vivo,* in proximity of the transcription start site. N-Myc association with promoters requires interaction with other proteins, such as Sp1 and Miz1 transcription factors. Furthermore, we found that N-Myc may repress gene expression by interfering directly with Sp1 and/or with Miz1 activity (i.e. TRKA, p75NTR, ABCC3, p21) or by recruiting Histone Deacetylase 1 (Hdac1) (i.e. TG2). In vitro analyses show that distinct N-Myc domains can interact with Sp1, Miz1 and Hdac1, supporting the idea that Myc may participate in distinct repression complexes by interacting specifically with diverse proteins.

Finally, results show that N-Myc, through repressed genes, affects important cellular functions, such as apoptosis, growth, differentiation and motility.

Overall, our results support a model in which N-Myc, like c-Myc, can repress gene transcription by direct interaction with Sp1 and/or Miz1, and provide further lines of evidence on the importance of transcriptional repression by Myc factors in tumour biology.

Table of Contents

INTRODUCTION 4
Neuroblastoma: an overview 5
Genetic anomalies in neuroblastoma 9
N-MYC and neuroblastoma 9
The Myc/Mad/Max network and the transcriptional control of cell behaviour 11
Biological functions of N-MYC 17
N-MYC as an activator 19
N-MYC as a repressor 24
Amplification of other loci 28
HRAS and oncogene activation 28
Specific tumour-suppressor genes 29
Chromosome deletion or allelic loss at 1p 30
Allelic loss of 11q 30
Allelic loss of 14q 31
17q gain 31
DNA content 31
Abnormal patterns of gene expression 33
Expression of neurotrophin receptors 33
Expression of ABC drug transporter genes 38
Expression of tissue transglutaminase (TG2) 42
Treatment 42
Final remarks 44
RESULTS 45
N-MYC expression inversely correlates with that of TRKA, p75NTR, ABCC3 and TG2 46
The core promoters of TRKA, p75NTR, ABCC3 and TG2 are required for N-Myc-mediated repression 49
Repression of the neurotrophin receptors TRKA and p75NTR 50
Dissecting the core promoter regions of TRKA and p75NTR 50
Sp1 and Miz1 are activator factors of TRKA and p75NTR 53
N-Myc is physically associated with the core promoter of TRKA and p75NTR in vivo 54
N-Myc interacts with Sp1 and Miz1 through distinct domains 56
N-Myc, Sp1 and Miz1 together are necessary to mediate transcriptional repression 58
NGF treatment increases apoptosis rate after re-expression of both TRKA and p75NTR 60

Repression of the ATP-binding cassette transporter ABCC3 62
N-Myc represses the ABCC3 promoter by interacting with Sp1 transcriptional factor 63
ABCC3 expression levels affect multiple neuroblastoma cell characteristics 64
Repression of tissue transglutaminase (TG2) 66
TG2 transcriptional activation is mediated by HDAC inhibitor 66
N-Myc represses TG2 transcription by directly recruiting the HDAC1 protein to the Sp1-binding site of the TG2 gene core promoter 67
N-Myc can interact with HDAC1 68
Transcriptional activation of TG2 contributes to HDAC inhibitor-induced growth inhibition in human neuroblastoma cells 70
Repression of TG2 expression by N-Myc is required for neuritic differentiation arrest in human neuroblastoma cells. 71
DISCUSSION 73
Repression of TRKA and p75NTR neurotrophin receptors 75
Repression of ABCC3 78
Repression of TG2 80
Final remarks 82
MATERIALS AND METHODS 85
CELL CULTURES 86
RNA EXTRACTION 86
THERMOSCRIPT RT-PCR SYSTEM 87
SYBR GREENER qPCR 87
ChIP- CHROMATIN IMMUNOPRECIPITATION 89
DUAL-STEP CHROMATIN IMMUNOPRECIPITATION 91
LUCIFERASE ASSAY 93
CO-IMMUNOPRECIPITATION AND GST PULL-DOWN ASSAYS 95
IMMUNOBLOTTING ANALYSIS 95
SOUTHERN BLOT 96
HYBRDIZATION ANALYSIS OF DNA BLOTTED 97
GENE SILENCING, TRANSFECTION AND CELLULAR ASSAYS 98
BIBLIOGRAPHY 99

INTRODUCTION

Neuroblastoma: an overview

Neuroblastoma is the most common extracranial solid tumour in childhood and the most frequently diagnosed neoplasm during infancy. It accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all paediatric oncology deaths. The overall incidence is about one case in 7,000 live births, and there are about 700 new cases per year in the United States. This incidence is fairly uniform throughout the world, at least for industrialized nations. The median age at diagnosis for neuroblastoma patients is about 18 months; so about 40% are diagnosed by 1 year of age, 75% by 4 years of age and 98% by 10 years of age. [1]

Neuroblastomas are tumours of the sympathetic nervous system. Due to their neural crest cell lineage, neuroblastomas may occur anywhere along the sympathetic ganglia. Most primary tumours (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla. Other common sites of disease include the neck, chest, and pelvis. The disease is remarkable for its broad spectrum of clinical behaviour. Presenting signs and symptoms are highly variable and dependent on site of primary tumour as well as the presence or absence of metastatic disease. Almost half of all patients presenting with neuroblastoma have disease dissemination at diagnosis [1] [2].

Unfortunately, neuroblastoma has not shown the same remarkable progress in cure rate that has been achieved in most other childhood malignancies, and this is one of the reasons why this tumour has been studied so extensively by paediatric oncologists worldwide. Although substantial improvement in outcome of certain well-defined subsets of patients has been observed during the past few decades, the outcome for children with a high-risk clinical phenotype has improved only modestly, with long-term survival still less than 40% [3]; [4].

From a histological point of view, neuroblastomas can be classified into:

- immature, consisting of a large population of small neuroblasts, highly undifferentiated, with little cytoplasm (neuroblastoma, malignant).
- partially mature, consisting of ganglion cells (ganglioneuroblastoma, with reduced malignancy but capable of to metastasize)
- fully mature ganglion cells in clusters surrounded by a dense stroma of Schwann cells (ganglioneuroma, benign)

(Figure 1) [5] [6]

The differentiation state of the tumour has some prognostic significance, but a more sophisticated histopathological classification has been developed to help predict outcome and select therapy.

The generally accepted method is the International Neuroblastoma Staging System (Table 1) [7].

Taken together, the stage of disease, the age of the patient at diagnosis and the site of the primary tumour are the most important clinical variables in predicting patient outcome [8] [1]. The 2-year disease-free survival of patients with stage 1, 2 and 4S is 80–90%, whereas those with stages 3 and 4 have a range of 40–50%. The outcome of infants who are less than 1 year of age is substantially better than older patients with the same stage of disease, particularly those with more advanced stages of disease. Patients with primary tumours in the adrenal gland seem to do worse than patients with tumours originating at other sites.

However, these clinical features are imperfect predictors of tumour behaviour, so further prognostic markers are needed. Advances in understanding of neuroblastoma came from cytogenetics and molecular biological approaches. Integration of biological and clinical data is crucial to facilitate predictions about neuroblastoma, and in many instances biological parameters seem to be more important than traditional clinical features as predictors of outcome [9] [2].

D'Angio and colleagues [10] first described the striking clinical phenotype of stage 4S (S=special) disease that occurs in about 5% of cases. These infants have small localised primary tumours with metastases in liver, skin, or bone marrow that almost always spontaneously regress. Neuroblastoma has the highest rate of spontaneous regression or differentiation (i.e. into a benign ganglioneuroma) observed in human cancers: the actual frequency of neuroblastomas that are detected clinically and subsequently regress without treatment is 5–10% [9]. However the frequency of true asymptomatic neuroblastomas that regress spontaneously is probably much higher, and might be equal to the number detected clinically. These clinical observations lead to considerable interest in understanding the mechanisms underlying spontaneous regression or differentiation, which in turn may lead to therapeutic approaches to stimulate these phenomena [9].

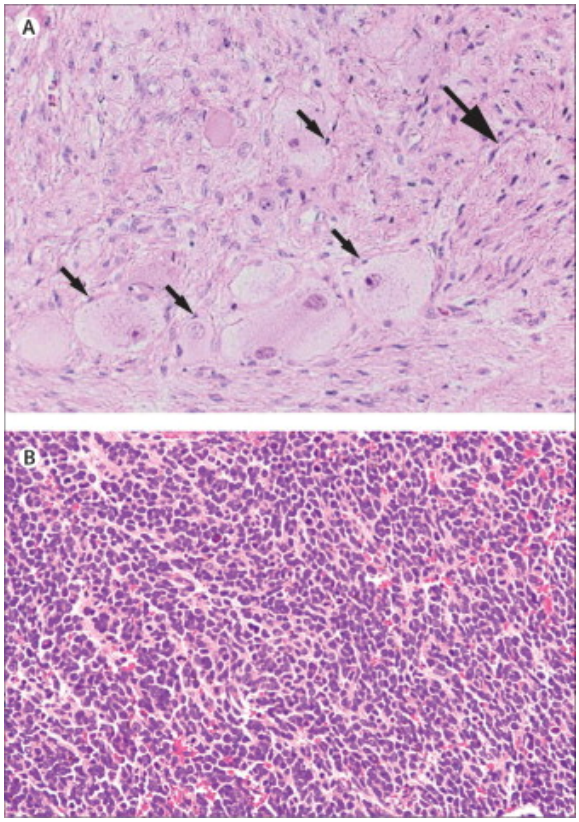

Figure 1. Degree of differentiation in neuroblastoma. **A,** Schwann cells and ganglion cells (indicated by arrows) are prominent in stroma-rich neuroblastoma. **B,** Stroma-poor neuroblastoma consists of densely packed small round blue cells with scant cytoplasm. [2]

The aetiology of neuroblastoma is still unknown. A subset of patients with neuroblastoma shows a predisposition to develop this disease, and this predisposition follows an autosomal dominant pattern of inheritance with incomplete penetrance. A family history of neuroblastoma is identified in 1–2% of cases [11] [12]. Regression analysis of neuroblastoma data was consistent with Knudson's two-mutation hypothesis for the origin of childhood cancer [13]. The median age at diagnosis of patients with familial neuroblastoma is 9 months, which contrasts with a median age of ~18 months for neuroblastoma in the general population; these patients often have bilateral adrenal or multifocal primary tumours [14] [15]. The concordance for neuroblastoma in twins during infancy indicates that hereditary factors might be predominant, whereas the discordance in older twins indicates that random mutations or other factors might also be important [16].

Table 1. International Neuroblastoma Staging System [17]

Stage	Definition
1	Localized tumour with grossly complete resection with or without microscopic residual disease; negative ipsilateral lymph nodes
2A	Localized tumour with grossly incomplete resection; negative ipsilateral nonadherent lymph nodes
2B	Localized tumour with or without grossly complete resection with positive ipsilateral nonadherent lymph nodes; negative contralateral lymph nodes
3	Unresectable unilateral tumour infiltrating across the midline with or without regional lymph node involvement, OR Localized unilateral tumour with contralateral regional lymph node involvement, OR Midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement
4	Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin or other organs (except as defined for stage 4S)
4S	Localized primary tumour (as defined for stages 1, 2A or 2B) with dissemination limited to skin, liver and bone marrow (limited to infants <1 year age)

Traditional genetic analyses have identified the short arm of chromosome 16 (16p12-13) as a likely predisposition locus, though no causal gene has been identified [18].
At the present time, it is unclear if this is the only predisposition locus, or if there are multiple loci, but this locus could account for most high-risk families. Neuroblastoma has also been seen in several patients with constitutional

chromosomal rearrangements, including deletions overlapping putative tumour suppressor loci at chromosome bands 1p36 and 11q14-23 [19] [20].
Remarkable disease heterogeneity exists within pedigrees considering affected individuals share the same predisposing genetic lesion, suggesting that the pattern of acquired secondary genetic alterations ultimately defines the tumour phenotype [9].

Genetic anomalies in neuroblastoma

Although some patients with neuroblastoma have a predisposition to the disease, most neuroblastomas occur spontaneously. Somatic changes, such as gain of alleles and activation of oncogenes, loss of alleles or changes in tumour-cell ploidy have been shown to be important in the development of sporadic neuroblastomas. Taken together, the multiplicity of potential initiating events suggests that neuroblastoma is a complex genetic disease in which interaction of effects from multiple genetic alterations might be needed for tumourigenesis.

N-MYC and neuroblastoma

Schwab and colleagues first identified a novel MYC-related proto-oncogene, N-MYC, that was amplified in a panel of neuroblastoma cell lines [21]. N-MYC, a transcription factors that can lead to deregulated growth and proliferation when overexpressed (see below), is normally located on the distal short arm of chromosome 2 (2p24): a large region from this site becomes amplified and the N-MYC locus is copied to form an extrachromosomal circular element or DM (double-minute chromatin bodies), with retention of the normal copies of N-MYC at 2p24. DMs might accumulate by uneven segregation during mitosis; however, in some cases, the amplified DNA integrates into a chromosomal locus to form an HSR (homogeneously staining regions) (Figure 2) [22] [23] [24] [25]. Other genes might be co-amplified with N-MYC in a subset of cases, but N-MYC is the only gene that is consistently amplified from this locus [26].

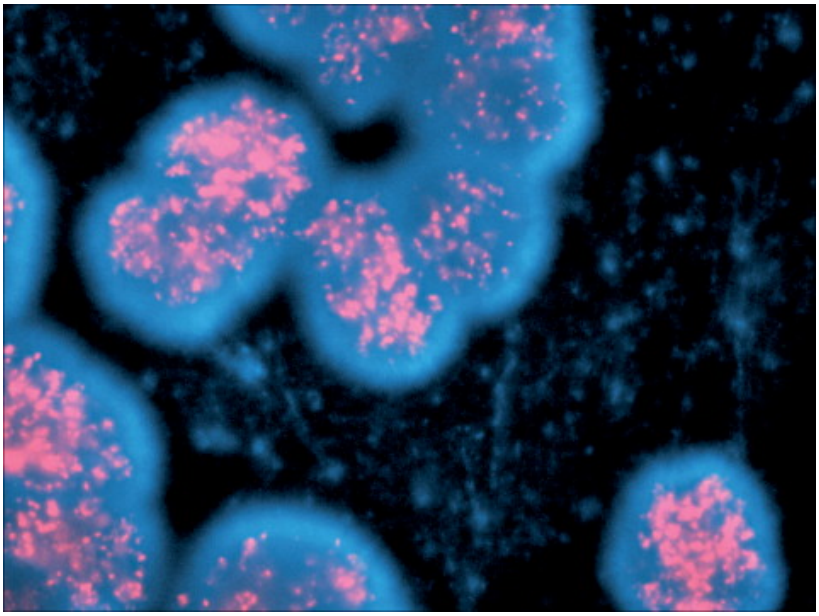

Figure 2. N-MYC amplification demonstrated by fluorescence in situ hybridisation (FISH) using a labelled N-MYC probe. [2]

Currently it is well known that the genetic aberration most consistently associated with poor outcome in neuroblastoma is genomic amplification of N-MYC [27] [28]. N-MYC amplification occurs in roughly 25% of primary tumours and is strongly correlated with advanced stage disease and treatment failure (Figure 3) [29] [30]. Its association with poor outcome in patients with otherwise favourable disease patterns such as localised tumours or INSS stage 4S disease underscores its biological importance [31] [32] [33].

Thus, amplification of N-MYC remains the most important genetically based independent prognostic factor for neuroblastoma and represents an intrinsic biological property of a subset of aggressive neuroblastomas: tumours without amplification at diagnosis rarely, if ever, develop this abnormality [9].

N-MYC amplification is also associated with other poor prognostic indicators such as chromosome 1p deletion and increased expression of multidrug resistance-associated protein, while counter-correlates with favourable markers such as TrkA and p75NTR expression (see below) [34] [35] [36] [37] [38] [39].

Activation of the MYC genes by amplification is commonly detected in solid human tumours. In neuroblastoma N-MYC is amplified from 5- to 300-fold per haploid genome, and all the copies seem to be transcriptionally active [40] [41]. Therefore tumours with N-MYC amplification result in an elevated expression of the N-Myc protein and this provides selective advantage to the cells through various

mechanisms that affect the role of N-Myc as a part of the Myc/Mad/Max transcriptional factor network (see below) [42] [40].

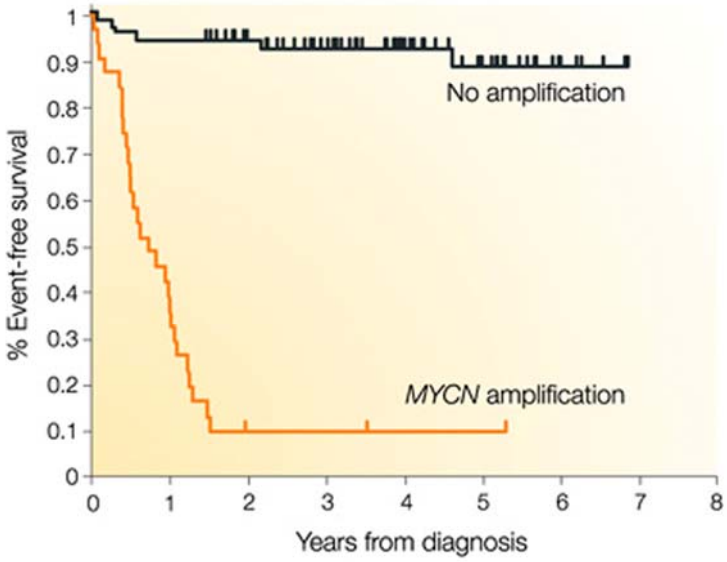

Figure 3. Survival of infants with metastatic neuroblastoma based on N-MYC status. A Kaplan–Meier survival curve of infants less than 1 year of age with metastatic neuroblastoma. The 3-year event-free survival (EFS) of infants whose tumours lacked N-MYC amplification was 93%, whereas those with tumours that had N-MYC amplification had only a 10% EFS [9].

The Myc/Mad/Max network and the transcriptional control of cell behaviour

The Myc/Mad/Max network comprises a group of nuclear transcription factors whose functions profoundly affect cell behaviour [43].These factors are a subset of the larger class of proteins containing basic-region/helix–loop–helix/leucine-zipper (BR/HLH/LZ) motifs. BR/HLH/LZ domain is known to mediate protein-protein interactions and DNA binding [43].

No monomeric Myc proteins have been found in vivo. Instead, Myc is bound to a partner protein, Max (MYC-associated factor-X), itself a small BR/HLH/LZ protein [44]. Max, physiologically, is present in stoichiometric excess to Myc, and can homodimerize and bind DNA, but such Max homodimers appear to be transcriptionally inert [45, 46]. Max can also form heterodimers with several related proteins, known as Mad1, Mxi1 (also known as Mad2), Mad3, Mad4 and Mnt (also known as Rox) (Figure 4). These four proteins, considered to compose the Mad protein family, behave much like Myc in that they have only weak homodimerization

and DNA-binding capacities but readily heterodimerize with Max and bind the DNA [43].

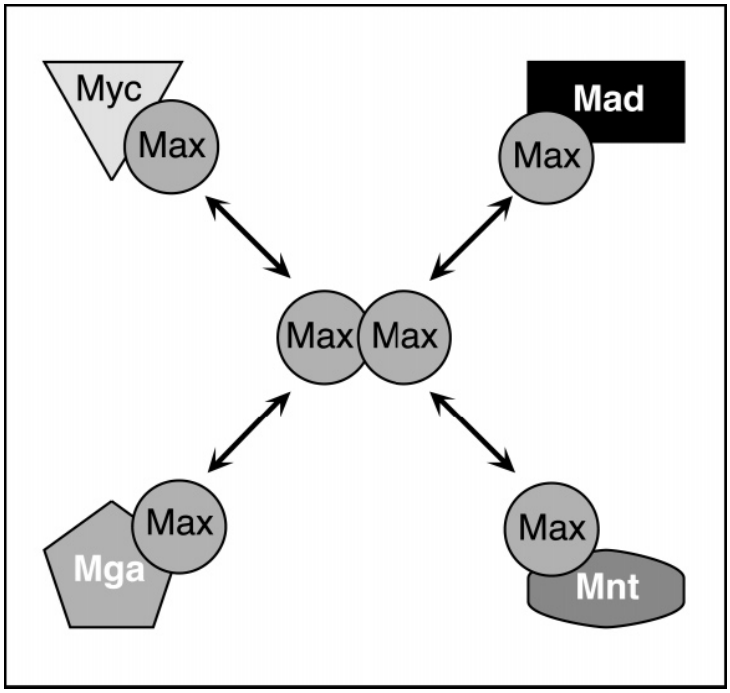

Figure 4. Max-interacting proteins. Max forms heterodimers with members of the Myc and Mad protein families as well as with the Mnt (or Rox) and Mga proteins. Each of these proteins interacts with Max through its BR/HLH/LZ domain.

Association with Max results in the formation of heterocomplexes that recognize the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG) with transcriptional activity (Figure 5) [47-49]. However, in contrast to Myc, which activates transcription at promoters proximal to E-box sites, the Mad-Max heterodimers act as transcriptional repressors at the same binding sites [45] [50] [51].

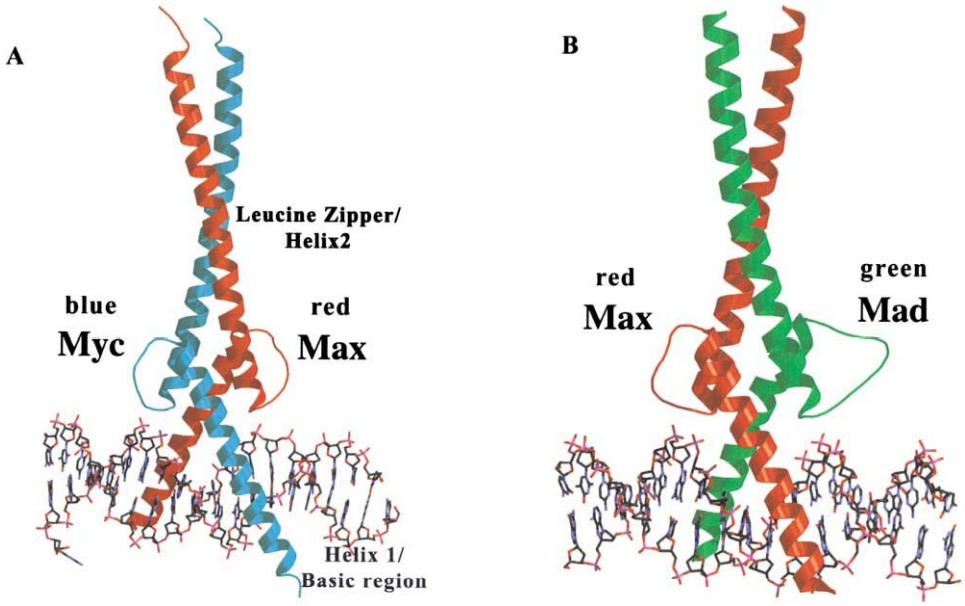

Figure 5. A, Structure of heterodimer Myc-Max bound to DNA; **B,** Structure of heterodimer Mad-Max bound to DNA.

The ability to modulate transcription is derived from specific domains within the Max interacting factors which, in turn, appear to mediate associations with specific coactivators or corepressors, resulting in the formation of higher-order complexes. Furthermore, the different complexes may have antagonistic properties whose functions play out at the level of chromatin structure (see below). The transcription activation function of Myc involves at least in part the recruitment of a histone acetyltransferase (HAT) mediated by TRRAP (transactivation/transformation-associated protein), whereas Mad–Max complexes act as transcriptional repressor by recruiting histone deacetylases (HDACs) through the adaptor protein SIN3 (Figure 6) [52] [53].

In general, Max interacting proteins have short half-lives (on the order of 20–30 min) and their biosynthesis is highly regulated [54]. Max, on the other hand, is stable and constitutively expressed, suggesting that the regulation of the network is largely dependent on the abundance of the Max associated transcription factors that must be tightly regulated [44, 55]. In vivo, Myc–Max complexes are often predominant in proliferating cells, whereas Mad–Max or Mnt–Max complexes are predominant in resting or differentiated cells (see below) [56].

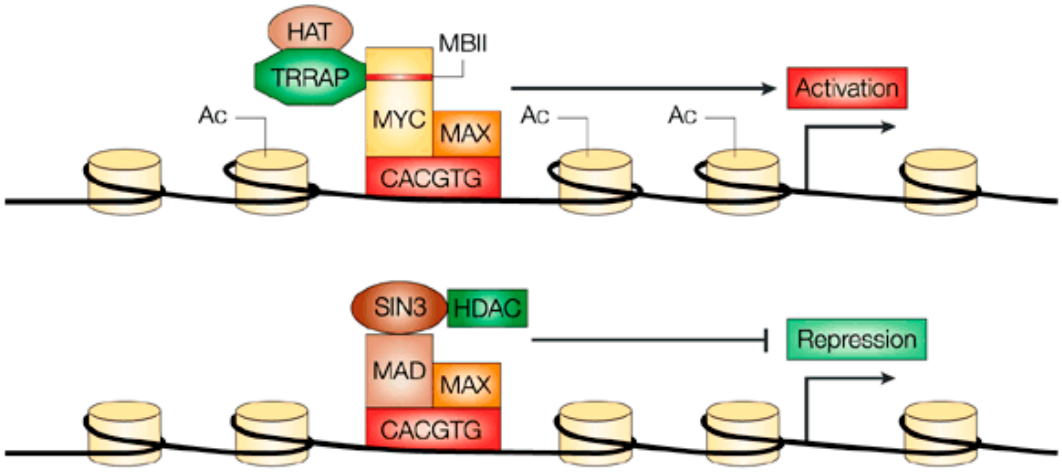

Figure 6. Transcriptional regulation by Myc/Mad/Max network through E-box elements.

Our understanding of the Myc/Max/Mad network grew out of research on the MYC oncogene family. MYC was originally defined as an oncogene (v-MYC) transduced by a number of avian retroviruses capable of potently inducing neoplastic disease [57]. Subsequently c-MYC, the cellular homolog of v-MYC, was identified and eventually shown to be a member of a family of proto-oncogenes comprising c-MYC, N-MYC, and L-MYC (Figure 7). These genes are considered proto-oncogenes in the sense that alterations in their structure and expression have been linked to a wide variety of human and other animal cancers [58-62].

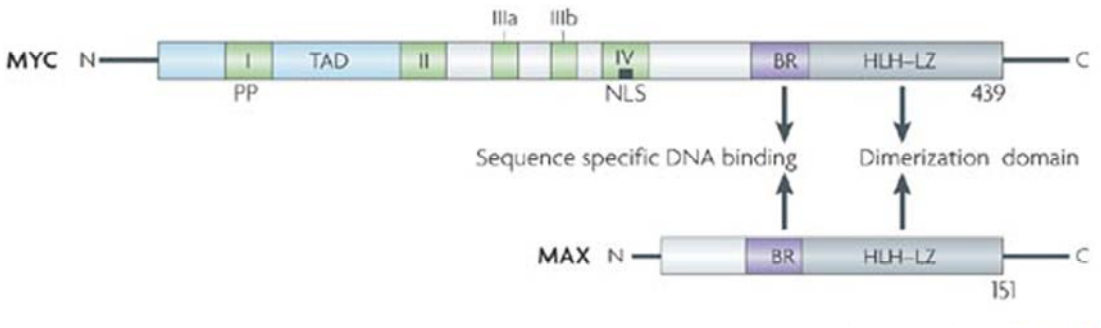

Figure 7. Structural domains of Myc oncoproteins. From N-term to C-term:
Transactivation domain, TAD (amino acids (aa) 1–143): the TAD can confer activation of gene transcription to a heterologous DNA-binding domain.
MYC homology box I, MB I (aa 44–63): MBs are regions highly conserved between MYC, N-MYC and MYCL1; within MB I, stability and activity of MYC are highly regulated through phosphorylation of Thr58 and Ser62.
MB II (aa 128–143): this domain is important for transcriptional repression and activation, region of interaction with TRRAP and other cofactors involved in transformation.
MB IIIa (aa 188–199): this domain is conserved in MYC and N-MYC but not in MYCL1 and shows intermediate transforming potential compared with the activity of the wild type MBII in vivo.
MB IIIb (aa 259–270): this domain is conserved, but no specific function has yet been assigned to it.
MB IV (aa 304–324): this domain is required for full MYC transforming activity and apoptosis.
Primary nuclear localization signal, NLS (aa 320–328): subcellular localization to the nucleus is encoded primarily by this region.
Basic region, BR (aa 355–369): this region is essential for full transformation of primary and immortal cells, and is responsible for specific binding of canonical and non-canonical MYC E-boxes to DNA, with MAX.
Helix–loop–helix–leucine zipper, HLH–LZ (aa 370–439): this domain is essential for full transformation of primary and immortal cells, and is responsible for interaction with MAX [63].

The first compelling idea about MYC was that it functions to drive cell growth and proliferation in response to diverse signals. In fact, MYC family genes are broadly expressed during embryogenesis, and targeted deletions of c-MYC or N-MYC genes in mice lead to lethality in mid-gestation embryos [64] [65]. Moreover, there is a strong correlation between MYC expression and proliferation. [66-72]. In cells with activated MYC, G1 phase is often shortened as cells enter the cell cycle, and MYC is essential for G0/G1 to S phase progression [73] [74] [75].

It is now clear that MYC expression is induced in many cell types by a wide range of growth factors, cytokines, and mitogens [76-78].The increase in MYC levels occurs through both transcriptional and post-transcriptional mechanisms and appears to occur as an immediate early response (about 2 hours) to most mitogenic factors [79].

On the contrary, anti-proliferative signals trigger rapid downregulation in MYC expression [63]. MYC downregulation is required for cells to exit the cell cycle and undergo differentiation. This important point of regulation is further enforced by the

induction and function of the Mad family members in response to differentiation cues [80].

It is important to note, however, that in situations where commitment to a specific lineage is closely linked to an increase in proliferation, Myc can promote differentiation [81].

Clearly, these data indicated that MYC is a nexus for multiple growth signal response pathways. Therefore MYC expression, and MYC activity, is tightly regulated in non-transformed cells and designed to respond quickly to proliferative cues from the extracellular milieu.

The ability of overexpressed Myc to facilitate proliferation and inhibit terminal differentiation fits well with the fact that tumours of diverse origins contain genetic rearrangements involving MYC family genes, such as genomic amplification in neuroblastoma. Indeed, many of the genomic alterations in MYC result in increased MYC mRNA levels through increased transcription initiation, decreased transcription attenuation, and augmented stability of the MYC messenger RNA [79]. Moreover Myc degradation is carried out through the ubiquitin-mediated proteosome pathway that involves phosphorylation of Thr58 and Ser62 in MB I: many tumour-related mutations in Myc result also in significant stabilization of the protein [82, 83].

One of the most striking findings of the past years has been the discovery that the enhanced expression of Myc proteins contributes to almost every aspect of tumour cell biology [84]. Whereas the ability of Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had long been recognized, more recent work shows that deregulated expression of Myc can drive cell growth and vasculogenesis, reduce cell adhesion, promote metastasis and genomic instability. Conversely, the loss of Myc proteins not only inhibits cell proliferation and cell growth, but can also accelerate differentiation, increase cell adhesion and lead to an excessive response to DNA damage [85].

This reflects the surprisingly high number of target genes regulated by Myc, as emerged in large-scale analyses of MYC-regulated genes. Indeed, in normal cells, Myc proteins appear to integrate environmental signals in order to modulate a wide, and sometimes opposing, group of biological functions, including proliferation, growth, apoptosis, energy metabolism, and differentiation (see below).

Biological functions of N-MYC

The vast majority of functional investigations have focused on the c-Myc protein, the most studied member of the MYC family. Thus, many of the functions of N-Myc were considered common to those of c-Myc. This assumption was based on various experimental evidences:

- the high level of relatedness between N-Myc and c-Myc protein structure;
- while c-MYC and N-MYC homozygous knockout mice are embryonic lethal, transgenic mice in which N-MYC replaced c-MYC showed a gross normal development, indicating that both proteins have largely overlapping functions [64] [65] [86];
- c-Myc and N-Myc share >40% of their target genes [87]

Nonetheless, since the late 80s, differences between the two members of the MYC family appeared to be important and seem more pronounced in recent years of research: actually N-Myc and c-Myc appear to bind different subsets of genes with different affinities in neuroblastoma tumours and in hematopoietic stem cells [88] [89].

Furthermore, it is well known the existence of direct negative cross-regulation between N-Myc and c-Myc [90] [91] [92].

Indeed, by analogy with c-Myc, N-Myc has related functions in regulating cell growth and proliferation, but in a more specialized context. N-Myc is subjected to a strict, rather similar temporal and spatial expression pattern, as shown by comparisons of fetal and adult human brain cells [93] and by analysis of tissues from different stages of the fetal and the developing mouse embryo [94] [95]. These results suggest that N-Myc may play an important role during mammalian development. The overall expression of the c-MYC gene, in contrast, is virtually constant during embryonal development and occurs in all proliferating cells of an organism.

Altogether, the N-MYC and c-MYC genes, in spite of their structural relationship, have distinctly different functions. The c-MYC gene plays a more ‘basic’ role, whereas N-MYC has more specialized functions.

During murine development N-MYC mRNA can be detected as early as day 7.5 in the primitive streak [96]. A peak of expression is reached between days 9.5 and 11.5, followed by a sharp decrease after day 12.5 [95]. During this time N-MYC mRNA is

present in many tissues including heart, limb buds, and neural tube [94]. N-MYC mRNA has also been detected during organogenesis in tissues such as hair follicles, lung, liver, and stomach [97]. Within a tissue N-MYC expression is not homogeneous. For example, in the heart, expression is restricted to the myocardium; in the liver it occurs mainly in the peripheral layer; in the neural crest it is initially expressed homogeneously, but after colonization of ganglion areas becomes restricted to those cells undergoing neuronal differentiation; in the human fetal kidney it is observed exclusively in the epithelially differentiating mesenchyme; in the somites it is stronger in the posterior than in the anterior half; and in the brain it is observed in the neural precursor cells but becomes more restricted after lineage commitment [98]. Thus N-MYC expression is dynamic not only in space but also in time. At birth N-MYC is still expressed in the brain, kidney, intestine, lung, and heart but then becomes down-regulated within several days or weeks depending on the tissue. In adults, N-MYC expression has mainly been detected at early stages of B-cell development. However, weak expression may be maintained in the adult brain, testis and heart [95]. The lack of N-Myc in any tissue where it is normally expressed results in developmental defects [98].

Taken together, these results reveal a complex expression pattern of N-MYC which is reflected in a correspondingly complex promoter with multiple tissue-specific, stage-specific, and signal-dependent regulatory elements. As the different expression patterns would lead one to expect, the regulatory regions of c-MYC and N-MYC are divergent [98]. Among the candidate pathways involved in differential regulation of Myc proteins are the Sonic hedgehog pathway (Shh) for N-MYC activation [99] and the Wnt/beta-catenin pathway for c-MYC activation [100]. Sonic hedgehog (Shh) signalling upregulates expression of the proto-oncogene N-MYC in cultured cerebellar granule neuron precursors. The temporal-spatial expression pattern of N-MYC, but not other MYC family members, precisely coincides with regions of hedgehog proliferative activity in the developing cerebellum [101].

These findings are paralleled by studies of human tumour cells. N-Myc normally inhibits neuronal differentiation by driving germinal cells to maintain their early embryonic short-duration cell cycles and, therefore, favours self-renewal, genetic instability and cancer [98].

Consistent with a critical role of N-MYC in the development, overexpression of N-MYC within a specific developmental window may cause several paediatric tumours

of mostly, but not exclusively, neuroectodermal origin, including neuroblastoma, rhabdomyosarcoma, medulloblastoma, retinoblastoma, astrocytoma, glioblastoma, Wilms' tumours and small cell lung carcinoma [102] [103].

These data are supported by in vivo experiments: transgenic mice overexpressing N-MYC in neural crest-derived tissues show frequent development of neuroblastomas [104].

Deregulation, for example as the result of amplification in neuroblastoma, could disrupt the intrinsic control of N-MYC expression and contribute to the neoplastic phenotype. It is possible that the cellular mechanism which evolved to regulate expression of a single copy of N-MYC is incapable of efficiently controlling the expression of multiple gene copies.

It is clear that although neuroblastomas without N-MYC amplification show heterogeneity in the level of N-MYC expression, they never produce as much N-Myc as tumours with amplification do. In fact, several studies failed to find a correlation between the expression of N-MYC in non-amplified tumours and an unfavourable outcome, suggesting that a threshold level of N-MYC expression has to be exceeded for an unfavourable outcome to occur [105].

N-MYC as an activator

N-MYC encodes a phosphoprotein of apparent molecular weight of 65/67 kDa that is localized in the nucleus and binds to DNA [106] [107].

When introduced into cells, N-MYC can activate transcription of synthetic reporter genes containing promoter proximal E-boxes in both yeast and mammalian cells [45, 46]. In addition, N-MYC stimulates natural E-box-containing promoters or sequences derived from putative Myc target genes [49, 108-110].

Myc-Max heterodimers have relatively weak transactivation activity both endogenously and in transient assays [46]. A plethora of microarray studies published recently have concurred that Myc proteins activate the majority of target genes by two-fold (generally ranging from 3- to 10-fold transactivation) [111]. Although Myc is now firmly ensconced as a transcription factor, it is certainly feeble compared to other transcription factors.

In general, transactivation domains of N-Myc function by facilitating recruitment of the basal transcription machinery either directly or indirectly. In nearly all cases, TAD function involves interactions with other proteins.

The prevailing model of MYC-mediated transcription postulates, as mentioned above, that N-Myc increases local histone acetylation at promoters [85]. N-Myc binds to histone acetyltransferase complexes including TRRAP (transformation/transcription-domain-associated protein) and either general control of amino-acid-synthesis protein-5 (GCN5) or TIP60, which preferentially acetylate histones H3 or H4, respectively [112] [53]. Myc also binds to the p300/CBP (CREB-binding protein) acetyltransferases [113].

The histone acetylation that results then opens the chromatin and provides docking sites for acetyl-histone-binding proteins, including GCN5 and the SWI/SNF chromatin-remodelling complex, both of which correlate with increased transcription (Figure 8a) [114] [115]. Transcription-factor-mediated recruitment of histone acetyltransferases is now recognized to be a major mechanism of transactivation, and many other transcription factors, including TCF (T-cell factor), E2F, the tumour suppressor p53 and Gal4, have been subsequently found to use this mechanism [116]. The region of chromatin opened through Myc would permit subsequent binding and activation by constitutive transcription factors.

Most of the genes whose expression is induced by Myc are transcribed by RNA polymerase II.

Among others, target genes include the cyclin-dependent kinase CDK4 [117], the Cdc25A phosphatase that activates CDKs [118], cyclin D2 [119] [120] and the E2F family [121]. In addition to target genes involved in cell cycle progression, Myc has been found to stimulate expression of multiple genes that control cell size and growth, including those encoding ribosomal proteins, translation factors, and metabolic enzymes [122]. These findings are consistent with the evidence that recruitment of TRRAP and associated acetylation activity is also present in vivo at both RNA polymerase III (RNA pol III)-dependent genes and RNA-pol-I-dependent and ribosomal RNA genes [123] [124] [125] [126].

Recently Myc has been shown to promote oxidative phosphorylation as well as glycolysis through coordinate transcriptional control of the mitochondrial metabolic network [127] [128].

Regulation of transcription also occurs at the level of transcriptional elongation and not just at transcriptional initiation. RNA pol II undergoes a cycle of phosphorylation and dephosphorylation during transcription and, with its C-terminal domain (CTD) in a hypophosphorylated form, RNA pol II is recruited to promoters. Phosphorylation of the CTD occurs during transcription initiation and elongation, whereas the CTD must be dephosphorylated to allow RNA pol II to be recycled for another round of transcription. RNA pol II has been found to pause on most promoters after transcribing approximately 20–40 bases. This model fits well with the finding that Myc stimulates the release of paused RNA pol II from the promoter and stimulates subsequent transcriptional elongation [129]. This correlates with a Myc-dependent increase in RNA pol II phosphorylation: Myc transactivation domain (TAD) binds directly CTD kinases (Figure 8b) [130] [131].Myc induction of RNA pol II phosphorylation occurs globally throughout the nucleus; it can be detected in the total cellular pool of RNA pol II rather than simply at MYC target-gene promoters [132].

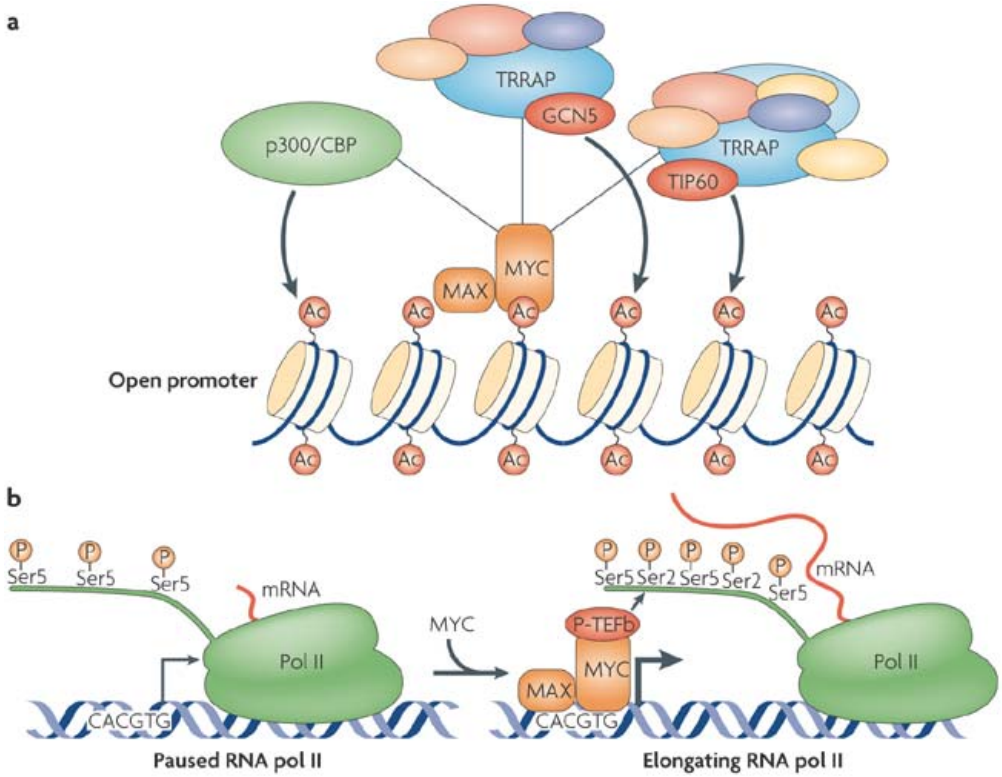

Figure 8. Mechanisms of MYC-induced transcription. **A,** Myc recruits histone acetyltransferases, which promote localized modification of chromatin through acetylation of nucleosomes. **B,** Myc recruits basal transcription factors and promotes the clearance of promoters through RNA polymerase (pol) II. The Myc protein can promote a paused RNA pol to continue transcription of the mRNA by

recruiting the P-TEFb (positive transcription-elongation factor-b) complex, which phosphorylates the CTD on Ser2 and promotes transcriptional elongation [133].

Moreover Myc can control protein expression through mRNA translation by promoting the methylation of the 5' mRNA guanine or 'cap', which is an essential step for protein-coding gene expression. As native levels of Myc regulate the expression of their targets without changes in mRNA abundance, this transcription-independent activity has the potential to influence all aspects of MYC biology in both normal and tumour cells [132].

Along with transcription, the most important nuclear function is DNA replication. The genome must be faithfully replicated each cell cycle and the chromosomes must be segregated to the daughter cells. Disruption of any step in this process, such as a stalled replication fork or DNA damage incurred during S phase, activates a checkpoint that halts the cell cycle until the lesion can be repaired. Failure to correct this damage leads to a mutation and/or genomic instability.

Previous studies have provided a link between MYC and genomic instability, but it was postulated that this was an indirect consequence of transcriptional activity [134]. A recent study describes a direct, non-transcriptional role for MYC in the initiation of DNA replication. Myc was found to bind to numerous components of the pre-replicative complex, and localize to early sites of DNA replication. These observations suggested that MYC might directly control the initiation of S phase and that the MYC effects on genomic instability might not depend on the transcriptional induction of S-phase-promoting genes [135].

Furthermore much excitement has been generated in the past years about the role of non-coding, regulatory RNAs. The first oncogenic microRNA polycistron is shown to be regulated by MYC [136] [137].

Taken together, these findings bring into question the definition of MYC just like a traditional transcription factor. It seems to exist an apparent disconnect between MYC's dramatic effects on multiple cellular functions and its molecular characterization as a relatively weak transcriptional activator.

Indeed, the notion that Myc is a general chromatin regulator, while to our knowledge unprecedented for an oncoprotein, is nonetheless consistent with several recent observations concerning MYC function. First, a series of independent expression microarray studies have collectively identified an unexpectedly large group of potential genes that are transcriptionally regulated by Myc [138]. Second, recent

experiments directly assessing genomic binding by Myc suggest binding to thousands of sites throughout the genome encompassing approximately 15% of genes as well as intergenic regions [139] [140] [141] [111]. Potentially, therefore, Myc regulates a significant proportion of all genes in an organism. The number of in vivo binding sites exceeds the number of Myc molecules in proliferating cells, which indicates that each site is bound by Myc only temporarily. Most probably, therefore, transcriptional regulation by Myc occurs by a 'hit-and-run' mechanism whereby the relatively brief binding of Myc triggers longer-lasting changes in the chromatin organization at the bound loci [63].

Ultimately there are many evidences that N-Myc has a robust role in the human genome in regulating global cellular euchromatin, including that of intergenic regions. Strikingly, N-Myc maintains 90% to 95% of total genomic euchromatic marks histone H3 acetylated at lysine 9 and methylated at lysine 4 modifications in human neuroblastoma, with enhancer-like function [142]. In neural stem cells, loss of N-Myc is sufficient to cause nuclear condensation, most likely due to a global spread of heterochromatin [142]. Myc's recruitment of histone acetyltransferases such as GCN5 and TIP60, as well as its regulation of histone acetylation at a number of genic loci, suggests that the regulation of euchromatin through histone acetylation is involved. Additional evidence suggests that the Myc-regulated chromatin program involves also methylation of lysine 4 of histone H3, possibly through the demethylase LID [143]. Furthermore Myc may regulate chromatin at a distance such that Myc binding at one location can influence chromatin at another through higher order chromatin structure.

Nonetheless, it is intriguing that intergenic binding sites for N-Myc are not enriched for E-boxes. Although E-box–independent binding has been reported and may be fairly widespread [144] such binding may be of particular importance for Myc intergenic function.

A specific global hyperactive chromatin state could be regulated by Myc and locked in place during tumorigenesis such as neuroblastoma genesis.

Furthermore, Myc has been shown to possess another feature outside the context of E-boxes: surprisingly Myc can act as well as a transcriptional repressor at certain target promoters (see below) [145].

N-MYC as a repressor

One of the first indicators that MYC might also function as a transcriptional repressor came from studies published in the 1980s that suggested that MYC participates in a negative feedback loop [146] [147]. After this, genome-wide analyses demonstrate that MYC represses at least as many targets as it activates, further emphasizing the role of repression in MYC function, including transformation [63].

Moreover recent findings show that Myc plays a fundamental role as part of the "magic quartet" of transcription factors that can reprogram somatic cells to induced pluripotent stem (iPS) cells. Ectopic expression of Myc augments the ability of Oct4, Sox2, and Klf4 to induce the formation of pluripotent cells from mouse and human fibroblasts, liver cells, and mature B cells by a factor of twofold to 10-fold, depending on the cell type [148] [149] [150] [151]. Silencing of the somatic cell expression program appears to be an important initial step required for the induction of the ES-like expression program, and it is evident a major contribution of Myc to this first step [152].

The basic mechanism underlying MYC's activation of transcription is well understood, but the mechanisms through which MYC negatively regulates or represses transcription are far less understood. Once again, several mutant analyses have pointed to the importance of Myc BoxII and the BR/HLH/LZ region in both activation and repression by MYC.

Initially, no simple consensus sequence for transcriptional repression by Myc had emerged. This opened the possibility that transcriptional repression is simply an indirect consequence of the altered physiological (e.g., transformed) state of a cell that is induced by Myc. Indeed, there is evidence in the literature for such indirect mechanisms of gene repression by Myc [153] [154].

One argument against the notion that all repression is similarly indirect was the identification of mutants of Myc that distinguish transcriptional activation from repression and the detailed analysis of the resulting phenotypes [155] [156]. Furthermore DNA elements required for Myc-mediated repression has been demonstrated to lie within the promoters of repressed target genes, indicating that Myc repression is likely mediated at the transcriptional level [145].

The repressed genes, like induced genes, fall into multiple functional classes: the first class of genes encodes proteins that are selectively expressed in quiescent cells or that directly or indirectly inhibit cell proliferation. This group encompasses the cell cycle inhibitors p21Cipl [156] [157] [158] [159] [160], p27kipl [161], pl5ink4b [159] [162] [163], pl8ink4c [122], and p57kip2 [164], as well as the differentiation-inducing proteins C/EBP-a [165] [166], the growth-arrest proteins gas1 and gas2 [155], the growth arrest and DNA damage proteins gadd34, gadd45, gadd153 [167] [168], and the Myc-antagonist Mad4 [169]. This long list points to a role for Myc-mediated gene repression in the control of cellular differentiation and in the response to growth arrest signals. It appears clear that the repression of individual genes could significantly contribute to the phenotype of MYC-transformed cells.

A second class of genes that is often repressed by Myc encodes proteins involved in cell adhesion, including a number of integrins: these mRNAs include those encoding cell surface proteins such as the class I HLA molecules in melanoma cells, the $\alpha 3\ \beta 1$ integrin in neuroblastomas, and the LFA-1 (αL $\beta 2$ integrin) cell adhesion protein in transformed B cells as well as [43, 170] [171]. Altered cell adhesion is a hallmark of many Myc-transformed cells and has been observed in different cell types [172].

Finally, genes involved in metabolic pathway such as thrombospondin and H-ferritin [173] [174]. Suppression of thrombospondin plays a causative role in the induction of angiogenesis by Myc.

Taken together, these data indicate that MYC has a powerful combination of functions that, when deregulated, may drive the limitless replicative potential characteristic of nearly all tumours.

Understanding of MYC repression is significantly advanced with the identification of both the specific DNA sequence and the specific MYC-binding proteins that are required for repression.

Recent studies show that not all genes repressed by Myc are silenced by the same mechanism.

A number of Myc-repressed targets contain a subclass of initiator elements (INRs; consensus, YYCAYYYYY, where Y is a pyrimidine base) which are usually, but not invariably, present at TATA-less promoters. INR elements are recognized by TFIID as well as a number of regulatory proteins, such as the transcription initiation factor TFII-I, YY-1, and the Myc-interacting zinc-finger protein 1 (Miz-1). Interestingly, the last three proteins have been reported to associate with the BR/HLH/LZ region of

Myc [43]. While there has been little follow-up on the initial reports involving Myc interaction with TFII-I or YY-1, the association of Miz-1 with Myc has been recently confirmed and shown to promote stabilization of Myc by inhibiting its ubiquitin-dependent degradation [83]. Miz1 contains 13 zinc fingers and, at its amino-terminus, carries a BTB/POZ-domain, which is a protein/protein interaction domain found in multiple zinc-finger proteins. Miz1 binds to the 'outside' of the helix–loop–helix domain of Myc, but does not interact with Max, Mad or Mnt proteins [156] [175].

Perhaps a stable Myc-Miz1 interaction blocks the ability of Miz1 to initiate transcription at INR-containing and other promoters [175].

Some Myc-repressed genes, such as gadd45, do not contain INR sequences; rather, repression appears to be mediated by a GC-rich region that is potentially recognized by WT1 and p53 [167]. Another GC-rich binding protein that seems to be important for Myc-mediated repression is the basal transcription factor Sp1 (specificity protein 1), a zinc-finger protein involved in gene expression in the early development of an organism. Other results show that repression by Myc happens through the Smad and the NF-Y binding sites due to direct protein/protein interactions between Myc and Smad2 and nuclear factor Y (NFY), respectively [176] [177].

The data clearly support the notion that several pathways of repression exist. Finally the present mechanistic model is that Myc–Max complexes interact with transcriptional activators that are bound directly to DNA through enhancer or initiator elements [178] [179]. These multi-protein complexes are thought to displace co-activators and recruit co-repressors [180].

Indeed some genes are repressed by MYC through a mechanism that does not involve the Max protein [74] [158]. In fact it was shown that Myc recruits a DNA methyltransferase, DNMT3a, to the Myc-Miz1 complex (without Max) on the promoter of p21, indicating that Myc-dependent gene repression could at least partly be mediated by methylation of its target promoters (Figure 9) [181]. Since Dnmt3a is complexed with histone deacetylases, its recruitment by Myc might lead to local histone deacetylation and inhibition of transcription [182]. Recruitment of Dnmt3a by Myc is an attractive mechanism for repression, since it might provide an explanation of the aberrant DNA methylation of some tumour suppressor genes that is observed in human tumours.

That Myc can form complexes with proteins outside the Max/Mnt context has been recently shown by Orian and colleagues who have found that, in Drosophila, dMyc can directly interact with the co-repressor Groucho without Max to control neuronal development [183].

Taken together these findings reveal another level of complexity to the mechanisms of Myc mediated repression.

As mentioned above, despite further support for the provocative idea of Myc as a repressor, knowledge of the molecular mechanism lagged behind that of MYC as a transactivator. Nonetheless, it seems likely that both activation and repression are required for Myc biological function. To date still less is known about N-MYC-mediated repression. Only two genes have been found to be repressed by N-Myc: the differentiation-related NDRG1 and -2 genes (N-Myc Downstream-Regulated Genes) via Miz-1-dependent interaction with their core promoter [184] [185].

Whereas the above, this thesis will be focussed to the study of the mechanism(s) underlying N-MYC-mediated repression.

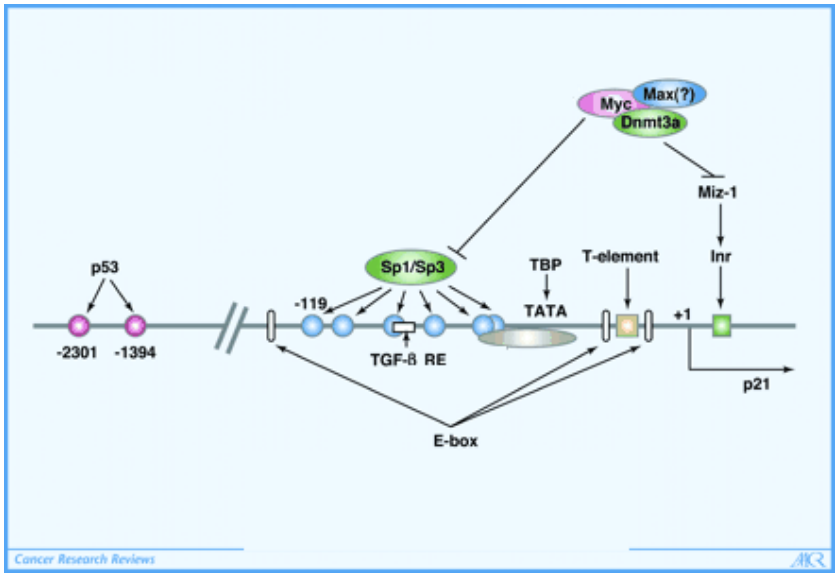

Figure 9. Multiple factors employ distinct mechanisms to repress the p21 promoter. Myc exerts their action through the proximal promoter region. Myc oncoproteins tend to interfere with positive regulators of p21 transcription, such as Sp1 and Miz1.

Amplification of other loci

Amplification of at least six regions that are nonsyntenic with the N-MYC locus at 2p24 has been shown in neuroblastoma cell lines or primary tumours. These include amplification of DNA from chromosome 2p22 and 2p13, the MDM2 gene on 12q13 and the MYCL gene at 1p32 [186] [187] [188] [189]. However, no neuroblastoma has been shown to amplify another gene that did not also amplify N-MYC. Allelic gain or amplification of other loci, including 4q, 6p, 7q, 11q and 18q, and other sites, have been identified using comparative genomic hybridization (CGH) approaches [190] [191] [192], but they mainly occur concurrently with N-MYC amplification, so their prevalence, as well as biological and clinical significance, is unclear.

HRAS and oncogene activation

Although NRAS was first identified as the transforming gene of a human neuroblastoma cell line, subsequent studies of primary neuroblastomas indicate that activating mutations of RAS proto-oncogenes are rare [193] [194]. However, there is evidence that high expression of HRAS in neuroblastomas is associated with a lower stage of disease and a better outcome [195]. RAS protein activation is a frequent consequence of activating tyrosine kinase receptors (such as TrkA, see below), which, in turn, are associated with neural differentiation. So, RAS activation or overexpression could mimic activation of this aspect of the signal-transduction pathway. However, the ultimate clinical use of the analysis of oncogene expression in neuroblastomas remains to be determined. Activation of other oncogenes by amplification, mutation or other mechanisms has not been found except for a few rare examples seen primarily in established cell lines. So, other than N-MYC amplification, which occurs in only a subset of tumours, there is no consistent evidence for activation of any other oncogene in human neuroblastomas.

Specific tumour-suppressor genes

The TP53 gene, which encodes the p53 protein, is one of the most commonly mutated genes in human neoplasia. p53 is a key regulator of cell-cycle control, and so inactivation of p53 function can contribute to malignant transformation. However, mutations are rarely found in primary neuroblastomas [196] [197]. There is recent evidence that the TP53 gene might be mutated more commonly in cell lines that are derived from patients at relapse [198] [199], but there is still controversy about the involvement of this gene in neuroblastomas. Some reports have shown cytoplasmic sequestration in undifferentiated neuroblastomas, so impairing the normal G1 checkpoint after DNA damage [200] [201].

Recently, a critical negative regulator of the p53 tumour suppressor, MDM2, has been characterized in neuroblastoma cell lines as a transcriptional target of N-MYC. These data suggest the possibility that N-MYC-driven expression of MDM2 might play a role in counterbalancing the p53-dependent apoptotic pathways concurrently stimulated by overexpression of MYC proteins [202].

Furthermore, there are evidences that N-Myc cooperates with Twist-1 (a highly conserved transcription factor that belongs to the family of basic helix–loop–helix proteins) in the development of neuroblastoma. N-Myc promotes cell proliferation whereas Twist-1 counteracts its pro-apoptotic properties by knocking-down the ARF/p53 pathway [203].

These observations provide a mechanistic explanation for the rarity of p53 mutations in neuroblastomas.

Other examples of suppressor-gene inactivation are deletions or mutations in the CDKN2A gene (which encodes INK4A, also known as p16, important in cell-cycle control and frequently inactivated in various cancers) and NF1 gene (a negative regulator of the ras signal transduction pathway). These alterations have been found in neuroblastoma cell lines, but it seems to be uncommon in primary tumours [204] [205].

Chromosome deletion or allelic loss at 1p

Deletions of the short arm of chromosome 1 (1p) is a common abnormality that can be identified in 25–35% of neuroblastomas [206] [207] [208] [209]. Deletions of chromosome 1 are found more commonly in patients with advanced stages of disease, and 1p allelic loss is highly associated with N-MYC amplification [34].

Most studies indicate that there is a single site of deletion on distal 1p36 in neuroblastomas, but there might be more than one. Indeed, there is not agreement as to the exact site, as studies by different groups have identified at least three discrete regions.

These regions are being mapped intensively to identify potential candidate genes for the putative tumour-suppressor gene that has been deleted from this region. However, the gene or genes within chromosome 1p involved in the pathogenesis of neuroblastoma have not been identified despite intensive investigation. Whether the loss of heterozygosity due to deletion of alleles from 1p is an independent indicator of prognosis remains controversial. However, evidence suggests that allelic loss at 1p36 predicts an increased risk of relapse in patients with localised tumours [210] [211] [212] [213].

Allelic loss of 11q

Allelic loss of 11q has been detected by analysis of DNA polymorphisms and by CGH techniques in 35–45% of primary tumours [190] [191] [192] [214] [215]. Deletion of 11q was directly associated with 14q deletion (see below), but it was inversely correlated with 1p deletion. Notably, this genomic aberration is rarely seen in tumours with N-MYC amplification, yet remains highly associated with other high-risk features. Therefore, loss of 11q might prove to be a useful predictor of outcome in clinically high-risk patients without N-MYC amplification [216].

Allelic loss of 14q

Loss of heterozygosity (LOH) also occurs for the long arm of chromosome 14 with [217] [218] [219]. A recent study of 280 neuroblastomas found allelic loss in 23%, and a consensus region of deletion was found in 14q23-32 [220]. There was a strong correlation with 11q allelic loss and an inverse relationship with 1p deletion and N-MYC amplification. However, no correlation was found with other biological or clinical features or outcome.

17q gain

A gain of 1–3 additional 17q copies, often through unbalanced translocation with chromosome 1 or 11, can also correlate with a more aggressive phenotype. Allelotyping and CGH studies have indicated that this abnormality might occur in more than half of all neuroblastomas [221] [222] [223]. The 17q breakpoints vary, but gain of a region from 17q22-qter suggests that a dosage effect of one or more genes provides a selective advantage rather than interruption of a gene [224] [225]. Candidate genes include BIRC5 (survivin), NME1, and PPM1D, which are overexpressed in this subset of tumours [226] [227] [228]. Gain of 17q is associated with more aggressive neuroblastomas, although its prognostic significance relative to other genetic and biological markers awaits a large prospective trial and multivariate analysis.

DNA content

The DNA content of neuroblastomas fall into two broad categories: near-diploid or hyperdiploid (often near triploid). Genetic models of neuroblastoma suggest that less aggressive tumours have a fundamental defect in mitosis associated with whole chromosome gains and losses, which could explain why near-triploidy seems to be favourable. Conversely, more malignant neuroblastomas have a fundamental defect in genomic stability, resulting in chromosomal rearrangements, unbalanced

translocations, and maintenance of a near-diploid DNA content [229]. DNA index is a prognostic marker for patients younger than 2 years who have disseminated disease. This is probably because hyperdiploid and near-triploid tumours from infants generally have whole chromosome gains without structural rearrangements, whereas hyperdiploid/near-triploid tumours in older patients also have several structural rearrangements [230] [231] [232].

However karyotype analysis is frequently unsuccessful in predicting outcome.

Finally, deletion or allelic loss has been shown at various other sites by genome-wide allelotyping or by CGH, but their biological or clinical significance is unclear (Figure 10).

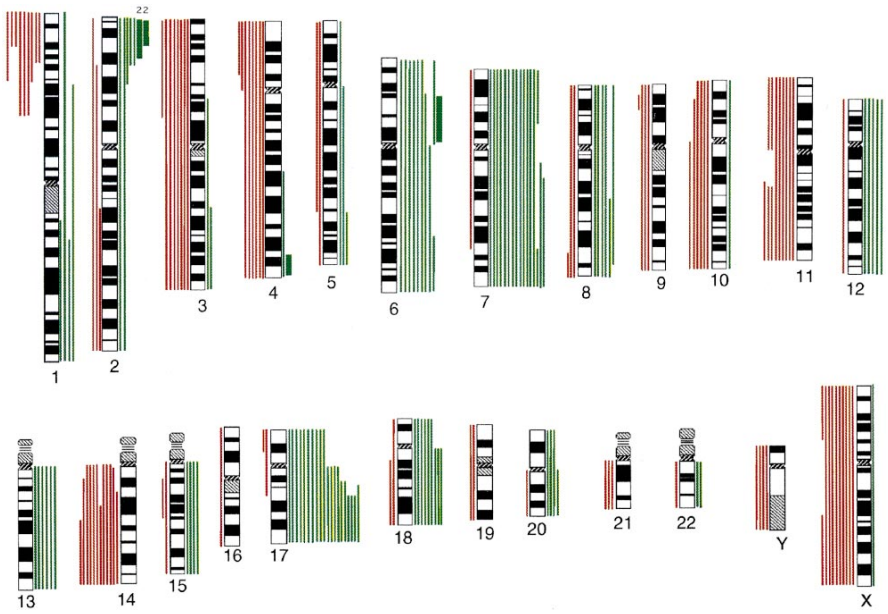

Figure 10. Genetic abnormalities in neuroblastoma: in green are represented the "gain of function" and in red the "loss of function".

Abnormal patterns of gene expression

Expression of neurotrophin receptors

The factors that are responsible for regulating the malignant transformation of sympathetic neuroblasts to neuroblastoma cells are not well understood, but they probably involve one or more neurotrophin-receptor pathways that signal the cell to differentiate.

The neurotrophin receptors (NTRK1, NTRK2, and NTRK3 encoding TrkA, TrkB, and TrkC) and their ligands (NGF, BDNF, and neurotrophin-3, respectively) are important regulators of survival, growth, and differentiation of neural cells [233]. All three neurotrophins also bind with a lower affinity to another receptor known as p75NTR, a member of the tumour necrosis factor receptor (TNFR)/Fas death-receptor super-family (Figure 11).

The Trk receptors are tyrosine kinases and derives its name from the oncogene that resulted in its discovery [234]. This oncogene consists of the first seven exons of nonmuscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase. Consequently, the proto-oncogene was named tropomyosin-related kinase (TRK) and is now commonly referred to as TRKA. The TRKB and TRKC genes were identified because of their high homology to TRKA. Comparisons of their sequences to those of other transmembrane tyrosine kinases indicated that they constitute a novel family of cell surface receptor tyrosine kinases.

Specific patterns of expression within the nervous system suggested roles in neuronal development and function: thus the neural development and maintenance of the neural network are spatiotemporally controlled by neurotrophin signalling in both peripheral and central nervous systems [235].

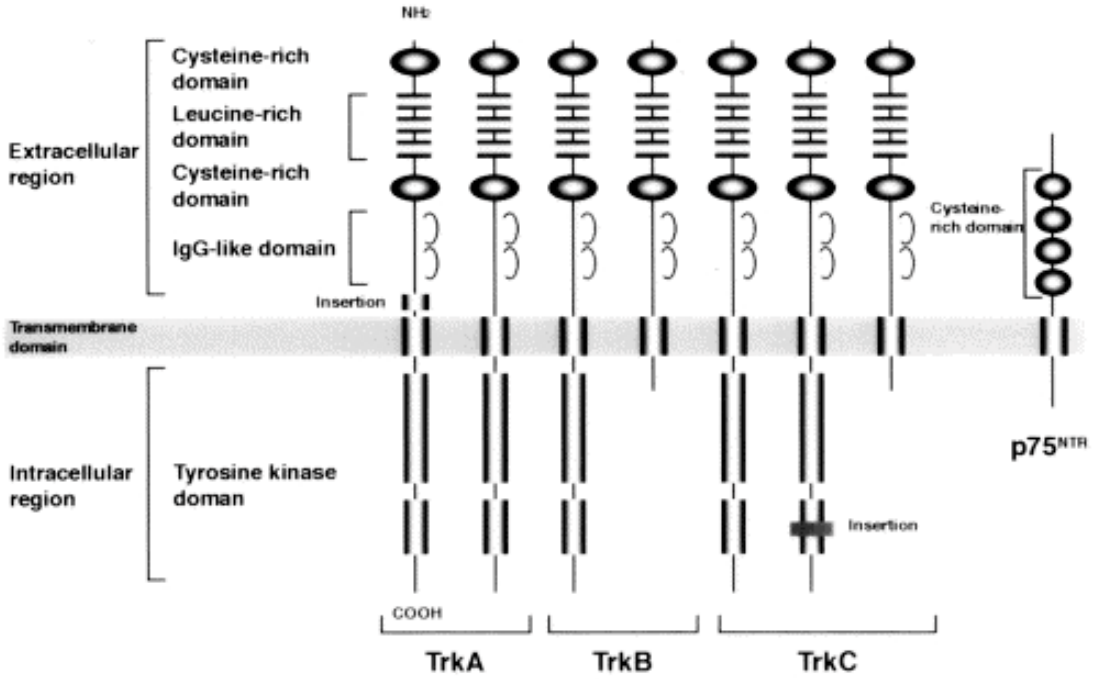

Figure 11. Schematic representation of neurotrophin family receptors. (A) Trks and p75NRT are high-affinity and low-affinity receptors for their cognate ligands, respectively. Each Trk has a single transmembrane domain and a single cytoplasmic tyrosine kinase domain. The second immunoglobin-like domain (Ig2) of TrkA and TrkB is the major ligand-binding interface.The neuronal form of TrkA receptor has a short insertion in the juxtamembrane region of its extracellular domain. Both TrkB and TrkC have truncated isoforms without the kinase domain. An isoform of TrkC has been identified with a kinase insert domain. p75NRT consists of four cysteine-repeat domains (CR). Both CR2 and CR3 have been implicated in neurotrophin-binding interactions. p75NTR has single transmembrane and cytoplasmic domains. The latter contains a "death domain" similar to those identified in TNF receptors. [233].

TrkA is a transmembrane receptor that functions as a homodimer. Binding of TrkA to a homodimer of NGF activates autophosphorylation of the receptor, docking of signalling proteins, signal transduction and induction of gene transcription (Figure 12).

TRKA is physiologically expressed at high levels in most neurons during the last stages of embryological development of the sympathetic nervous system, as a result of a "switch" expression by TRKB or C [236].

Explanted neuroblastoma cells with high TrkA expression differentiate when exposed to NGF or undergo apoptosis in the absence of NGF [37]. Thus, NGF/TrkA signalling could provoke differentiation or regression in favourable neuroblastomas depending on the particular microenvironment.

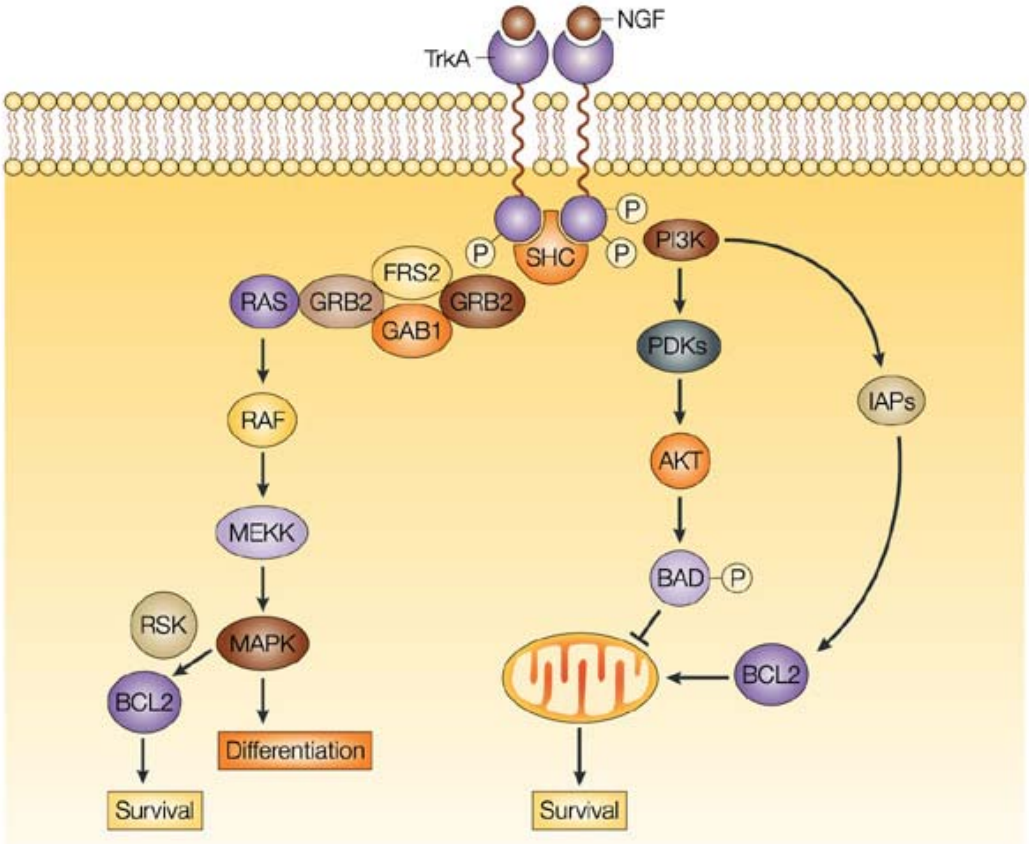

Figure 12. Signal-transduction pathway of the TrkA tyrosine kinase receptor. Binding of nerve growth factor (NGF) to the ligand-binding domain of TrkA leads to TrkA autophosphorylation and activation of various signalling cascades. Proteins that are thought to interact directly with the Trk intracellular domain are SHC, PLC 1, SH2B and IAPs, some of which are shown here. Binding of a ligand to TrkA can also trigger the RAS signalling pathway, leading to survival and differentiation, and an alternative survival signalling pathway through phosphatylinositol 3-kinase (PI3K) [9].

Indeed TRKA expression represents a powerful prognostic factor in neuroblastoma. The highest levels of TrkA are expressed in tumours with good prognosis which often showed spontaneous regression. Such tumours usually occur in patients under one year of age, with lower stage and their DNA ploidy is aneuploid. On the other hand, TrkA expression is strongly down-regulated in neuroblastomas with aggressive behaviour which usually have amplification of the N-MYC oncogene as well as allelic loss of the region of chromosome lp36. The combination of TrkA expression and N-MYC amplification provided even greater prognostic power (Figure 13). [237] [37] [9] [38] [39] [238].

A limited amount of NGF may be supplied from the stromal cells such as schwannian cells and fibroblasts, that, like normal sympathetic neurons, at least partly regulates differentiation and programmed cell death of the NBL cells [239].

A **B**

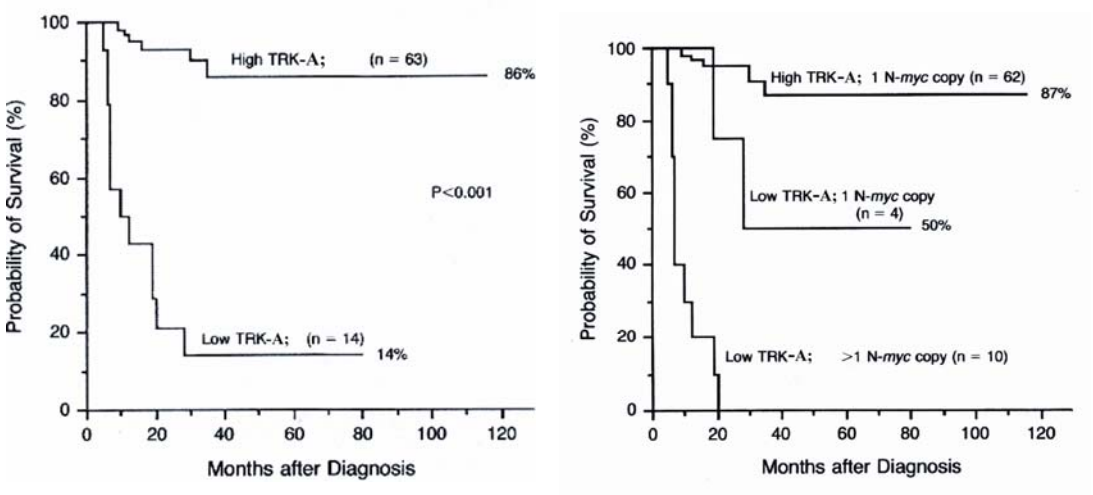

Figure 13. Probability of survival of patients with human neuroblastoma in accordance only with levels of expression of TrkA **(A)** and according to the relationship between expression of TrkA and amplification of N-Myc **(B)**.

TRKB, conversely, occurs at an early stage compared TrkA in neurons of the sympathetic system, and it is commonly expressed in biologically unfavourable neuroblastomas.

Although a truncated isoform lacking the catalytic tyrosine kinase domain could be expressed in favourable tumours, full-length TrkB is expressed along with its ligand, BDNF, predominantly in tumours with N-MYC amplification [240]. Thus, this might represent an autocrine or paracrine loop, thereby providing some survival or growth advantage that additionally promotes chemotherapy resistance, angiogenesis and metastases [241] [242] [243] [244] [245].

This scenario is very similar to that observed in normally developing sympathetic neurons which survive and differentiate by the target-derived supplement of neurotrophins (a trophic theory). However, aggressive neuroblastoma cells shut off TrkA signals by down-regulating its expression and disturbing the downstream signalling cascades, whereas they utilize BDNF /TrkB autocrine system to grow much efficiently [233].

By contrast, the expression of TRKC was found predominantly in lower-stage tumours, and, like TrkA, TrkC was not expressed in N-MYC-amplified tumours [246] [247]. Like TrkB, however, TrkC is expressed in the early stages of development of the sympathetic nervous system [236].

p75NTR activates a distinct set of signalling pathways within cells that are in some instances synergistic and in other instances antagonistic to those activated by Trk receptors. Several of these are proapoptotic, as can be expected from a member of the tumour necrosis factor receptor (TNFR)/Fas death-receptor super-family, but are suppressed by Trk receptor-initiated signalling.

p75NTR also regulates the responsiveness of TrkA receptors to NGF: its presence increases the rate of NGF association with TrkA [248] [249] [250] [251]. Thus, the specificity of neuronal responses to neurotrophins can be modulated by the type of receptor, differential splicing, and the absence or presence of p75NTR.

Theoretically, p75NTR could lead to either cell death or differentiation in response to ligand, depending on whether or not TrkA receptors were co-expressed [252] [253]. However, although Trk receptors suppress p75NTR -mediated signalling, Trk receptors are not always completely efficient at preventing p75NTR -mediated apoptosis [254] [255].

As for TRKA, the expression of p75NTR is downregulated in neuroblastomas with amplification of N-MYC and is therefore generally associated with a favourable outcome of this tumour [37] [38] [39]. Recently, furthermore, it has been shown that the expression of TRKA p75NTR is specifically silenced during tumour progression driven by the amplification of N-MYC in a model of transgenic mice for N-MYC [256]. Thus, taken together, these data suggest that there may be a direct involvement of N-MYC in the repression of TRKA and p75NTR, and that this mechanism may play a pivotal role in the malignancy of neuroblastoma.

Expression of ABC drug transporter genes

As mentioned above, amplification of the N-MYC oncogene is present in about 25% of primary untreated neuroblastomas, which is associated with advanced stage disease, rapid progression and unfavourable prognosis [21]. Moreover, this patient subgroup often demonstrates a multiple drug resistant (MDR) phenotype that develops from exposure to chemotherapeutic agents and increases with intensity of the therapy accommodated.

Cancer cells become resistant to anticancer drugs by several mechanisms. One way is to pump drugs out of cells by increasing the activity of efflux pumps, such as ATP-dependent transporters [257].

The ATP-binding cassette (ABC) genes represent the largest family of transporter genes: 48 genes in H. Sapiens, organized in seven subfamilies and highly conserved between species. Many of these genes are implicated in disease processes and/or drug resistance [258-261]. The prototype ABC protein binds ATP and uses this energy to transport molecules across cell membranes (Figure 14).

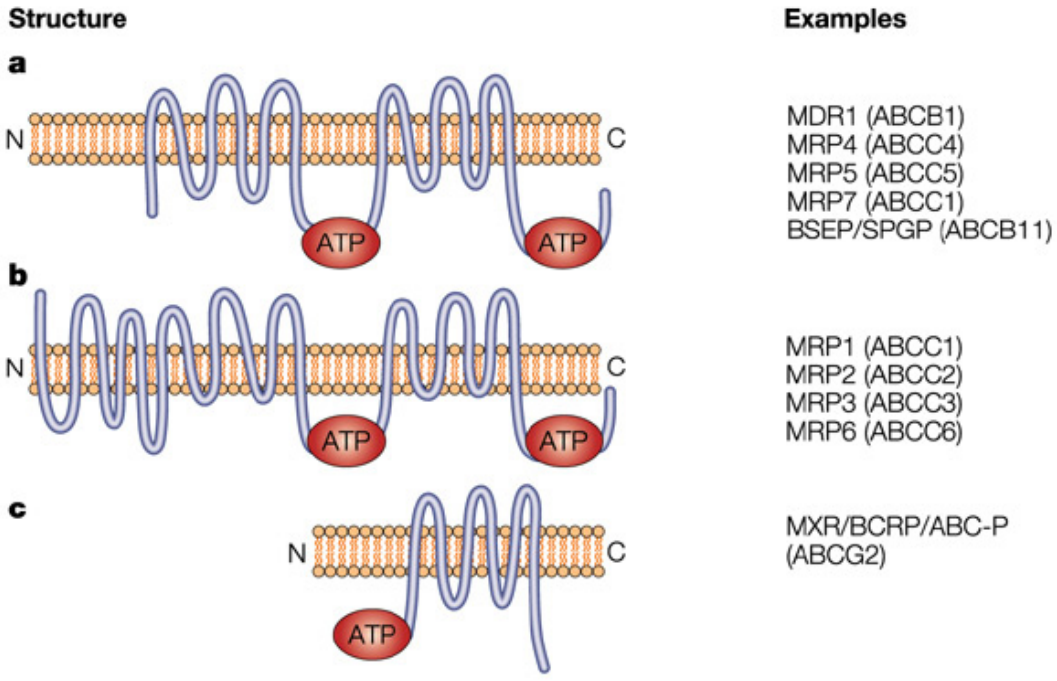

Figure 14. The structure of three categories of ABC transporters.

The genes generally associated with this phenomenon are the multidrug resistance gene 1 (MDR1, also know as ABCB1), the gene for multidrug resistance-related protein (MRP1, also know as ABCC1) and other members of these families. Most of the investigation of these genes and their encoded proteins has been done in vitro, but their expression and potential clinical significance in neuroblastomas has been addressed recently [262] [263] [35].

ABCB1 gene is the most studied ABC drug pump, and the first human ABC transporter cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells that had developed resistance to chemotherapy drugs [264].

As not all multidrug-resistant cells express ABCB1, a search for other efflux pumps was initiated, leading to the discovery of ABCC1 [265]. The discovery of ABCC1 stimulated, in turn, a genomic search for homologues, leading to the discovery of 12 additional members of the ABCC subfamily of transporters [266]. Like ABCC1, many ABCC family members have the potential, in model systems, to confer drug resistance, according to the theory that cancer cells may use several different types of ABC transporters to gain drug resistance [267].

Treatment of neuroblastoma includes induction chemotherapy, maintenance of high dose chemotherapy, radiotherapy, tumour surgery and consolidation therapy. Chemotherapeutic protocols combine alkylating agents with microtubule active drugs, topoisomerase inhibitors and antibiotics. Following initial treatment with cytotoxic drugs, tumours are highly chemoresponsive, displaying significant partial or complete remission in about 80% of tumours, even those with unfavourable prognostic outcome [268]. Although many high-risk neuroblastomas initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses.

The role of ABCB1 gene in mediating multidrug resistance in neuroblastoma is still unclear, and recent evidences suggest that ABCB1 gene expression fail to predict for outcome in this tumour. Conversely ABCC1 gene expression is a powerful prognostic indicator for children with neuroblastoma. High levels of ABCC1 expression are strongly associated with reductions in both survival and event-free survival (Figure 15). [35]. Although available evidence strongly suggests that ABCC1 is critically associated with the drug-resistant behaviour of primary

neuroblastoma, this drug efflux pump does not appear to mediate resistance to either alkylating agents or platinum compounds [269]. Members of both these classes of compounds, such as cisplatin and cyclophosphamide, are commonly used in the treatment of neuroblastoma.

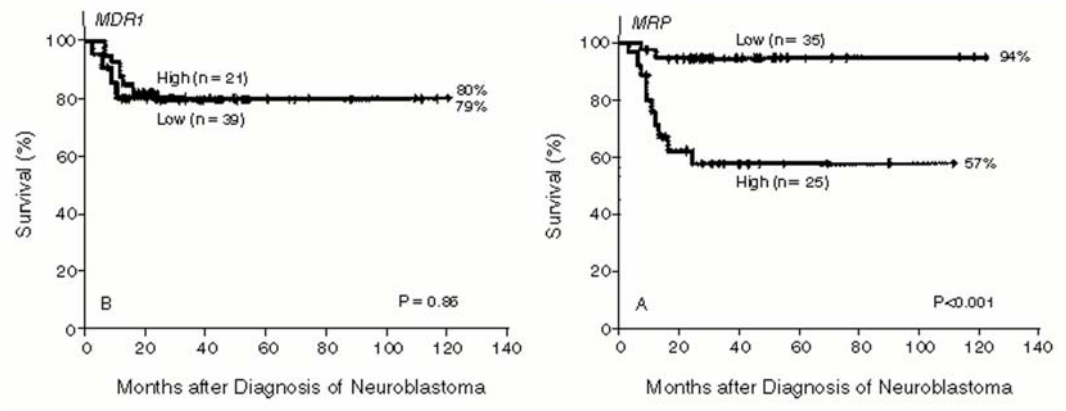

Figure 15. Expression of the MDR1 (ABCB1) and MRP (ABCC1) genes and cumulative survival in 60 patients with neuroblastoma. The survival of patients whose tumours expressed high levels of ABCC1 was significantly worse than that of patients whose tumours expressed low levels, but ABCB1 expression was not predictive of survival [35].

Thus, despite the high levels of ABCC1 observed in many aggressive tumours at diagnosis, the use of these non-ABCC1 substrate drugs may explain why the majority of neuroblastomas do initially respond to chemotherapy. Nevertheless, over half of these previously responsive tumours will eventually relapse with chemoresistant disease, suggesting the development of additional drug-resistance mechanisms [270].

Also ABCC4 is expressed in primary neuroblastoma and also its overexpression is significantly associated with N-MYC amplification and ABCC1 expression. The drug resistance phenotype of ABCC4 has to date been thought to encompass primarily nucleoside analogues (including antiretroviral agents) and methotrexate. ABCC4 is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38. Thus, like ABCC1, also high ABCC4 expression correlate with poor clinical outcome in neuroblastoma (Figure 16) [271].

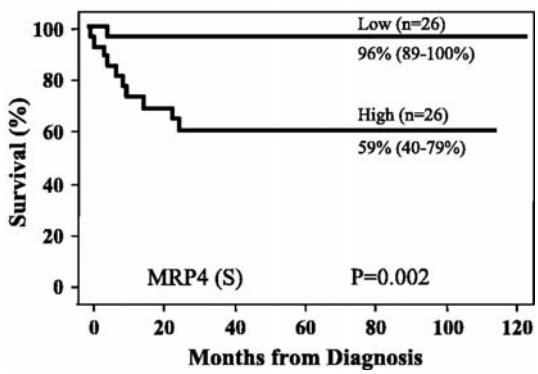

Figure 16. Survival in 52 neuroblastoma patients according to expression of the MRP4 (ABCC4) gene [271].

The laboratory of Dr. Giovanni Perini, where I am spending my Ph.D. studentship, in collaboration with the group of Dr. Michelle Haber (Children's Cancer Institute Australia, Sidney), was able to demonstrate that N-Myc in neuroblastoma and c-Myc in other cancer cell types, transcriptionally regulate a distinct set of ATP-binding cassette (ABC) transporter genes. Amongst the ABCC subfamily high levels of ABCC1 and ABCC4 are driven by N-Myc, through direct binding on their promoter. Moreover specific silencing of the ABCC1 or ABCC4 genes leads to growth inhibition, increased morphological differentiation and impaired motility of N-MYC-amplified neuroblastoma cells. Thus, these Myc-dependent changes in ABC protein levels directly affected the malignant behaviour of neuroblastoma cells in vitro and tumour aggressiveness in vivo (unpublished data).

On the contrary, in primary untreated neuroblastoma tumour samples, the group of Dr. Michelle Haber showed that low levels of ABCC3 expression are strongly predictive of poor outcome in patients with this disease (unpublished data). Indeed, although a number of reports have associated ABCC3 expression with outcome in certain cancers, there is no evidence linking this transporter with clinical drug resistance in any malignancy.

So, as well as TRKA and p75NTR, the relationship between N-MYC and ABCC3 remains to be defined.

Expression of tissue transglutaminase (TG2)

Tissue transglutaminase (TG2) is a multifunctional protein that has structural and functional homology to both transglutaminases and BH3-only protein families, and that catalyzes transamidation and multimerization of proteins in many type of normal tissues. TG2 plays an important role in many biological processes, such as blood coagulation, skin-barrier formation, hardening of the fertilization envelope, extracellular-matrix assembly, that are dependent on the rapid generation of covalent crosslinks between proteins [272] [273].
Significantly one of its functions is to promote programmed cell death by inducing a proapoptotic conformational change in the Bax protein and activation of the mitochondrial apoptosis pathway [274].
Furthermore the group of Dr. Glenn M. Marshall (Children's Cancer Institute Australia, Sidney), with which my laboratory is collaborating, has found from microarray analysis that TG2 is the gene most significantly repressed by N-Myc in neuroblastoma cells (unpublished data).
Finally, together with those already mentioned, TG2 is another example of a gene whose expression is generally inversely correlated with that of N-MYC, and for which it was not yet defined a possible mechanism underlying this repression.

Treatment

The current treatment of neuroblastoma may involve surgery, chemotherapy, radiation therapy, bone marrow rescue and/or innovative biological approaches. Localized tumours, especially those with favourable biology, are curable by surgery alone, as residual tumour tissue is prone to spontaneous regression or differentiation.
An intermediate group may be curable with chemotherapy of moderate intensity. However, patients over one year of age and with metastatic disease, as well as those with biologically unfavourable regional tumours, seem to have a benefit of a treatment with intensive multiagent chemotherapy with bone marrow rescue. Radiation therapy is useful for local control, but is of limited utility overall because of the propensity of this tumour to disseminate.

Although there are highly effective salvage therapies for patients with low-risk and intermediate-risk disease who have local relapses, recurrent disease in patients with high-risk neuroblastoma remains a clinical challenge. In the last years innovative approaches to treatment offer hope of cure with limited toxicity, such as immunotherapy, retinoids, angiogenesis inhibitors and tyrosine kinase inhibitors.

Among all possible targets, there are three features that, taken together, make N-Myc the most attractive target for tumour therapy. Firstly, there is a clear association between N-Myc amplification on the one hand and tumour aggressiveness and poor prognosis on the other. Secondly, two mouse models of tumorigenesis with experimentally controlled, reversible c-Myc expression suggest that a tumour requires continuous Myc expression and that down-regulation of Myc expression results in tumour regression [275] [276]. Thirdly, due to its restricted expression pattern after birth, side effects of even systemic down-regulation of N-Myc expression can be expected to be moderate.

However, because it is a nuclear transcription factor, it is difficult to directly target N-MYC with small-molecule inhibitors. On the other hand, gene silencing is an effective method to downregulate N-MYC activity: siRNA targeted to N-MYC resulted in increased differentiation and apoptosis, with concurrent growth inhibition [277] [278].

Finally, in the last years, it has attracted much interest the use of histone deacetylase in therapy.

Regulation of the epigenome includes DNA methylation and histone acetylation. In cancer, the balance can be pathologically altered especially in favour of histone deacetylation and DNA hypermethylation [279]. Histone deacetylase (HDAC) inhibitors are currently being tested in various clinical trials [280]. Interestingly, HDAC-1 is correlated with the multidrug resistance phenotype in neuroblastoma cells [281]. In a preclinical study of embryonal tumours, including neuroblastomas, an array of HDAC inhibitors increased the level of apoptosis in a time and dose-dependent manner [282].

Final remarks

Neuroblastomas are enigmatic, multifaceted tumours that predominantly affect children under 5 years of age. For over two decades, therapeutic advances have failed to significantly increase the 5-year survival rates of children with aggressive, advanced-stage neuroblastomas. As heterogeneity is a hallmark of neuroblastoma, current biomedical research is focused on addressing the various pathogenic intricacies of this tumour (Figure 17). Understanding the various biological and molecular components regulating tumour progression in neuroblastoma is necessary to successfully improve survival rates.

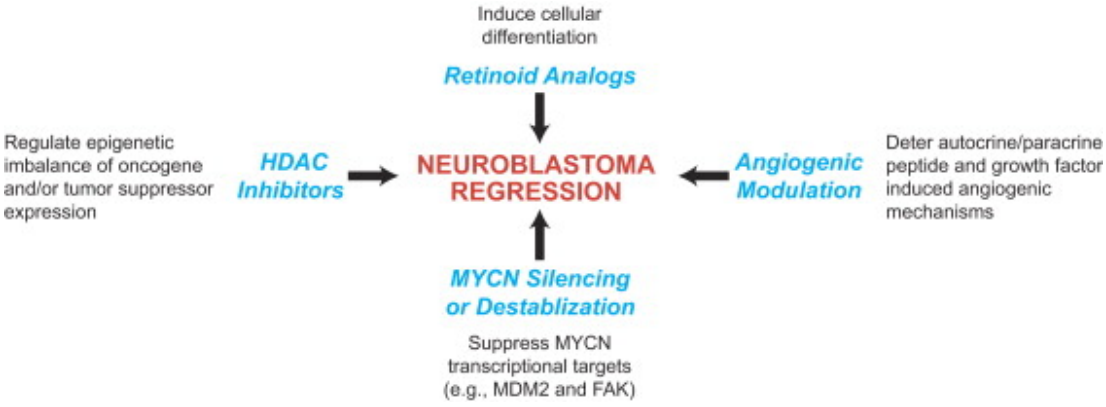

Figure 17. Advances in neuroblastoma research. Potential pathways to target for adjunct therapy include retinoid-induced differentiation, stimulators of angiogenic pathways, histone deacetylase, and N-MYC along with its transcriptional targets [17].

RESULTS

N-MYC expression inversely correlates with that of TRKA, p75NTR, ABCC3 and TG2

As mentioned above, there exists a set of genes, such as the neurotrophin receptors TRKA and p75NTR, the ATP-binding cassette transporter gene ABCC3, and the tissue transglutaminase (TG2), whose expression is strongly downregulated in neuroblastoma when N-Myc is overexpressed. These genes may potentially play an important role in differentiation and/or apoptosis of neuroblastoma cells, since their expression is correlated with lower-stage tumours and favourable outcome.

In order to determine whether N-Myc can regulate the expression of TRKA, p75NTR, ABCC3 and TG2, we analysed their expression profile in different cellular systems.

First, we used Tet-21/N cells, a human neuroblastoma cell line, in which N-Myc expression can be transcriptionally shut off by adding tetracycline in the culture medium [283]. Expression of TRKA, p75NTR, ABCC3 and TG2 genes was measured by real-time PCR as a function of tetracycline treatment and correlated with that of N-Myc. As shown in Figure 1A-B, all the analysed genes increased significantly their expression upon silencing of N-Myc expression. Second we determined the expression of TRKA, p75NTR, ABCC3 and TG2 in human neuroblastoma SK-N-BE 2C and SK-N-BE 9N cells, in response to treatment with retinoic acid. As it is well established from literature, retinoic acid treatment induces cellular differentiation of neuroblastoma cells and turns off N-Myc expression. SK-N-BE 9N derives from SK-N-BE but has been engineered to express a N-Myc recombinant construct under the control of a viral promoter which makes it unsensitive to retinoic acid. SK-N-BE 2C cells, on the contrary, carry the same construct without N-Myc coding sequence and were used as control. Results show that the expression of our genes significantly increased only in SK-N-BE 2C cells, where N-Myc expression can be downregulated by retinoic acid but not in SK-N-BE 9N cells where N-Myc expression remains elevated (Figure 1C-D). Furthermore we obtained several cell clones by stably transforming SH-SY-5Y, another human neuroblastoma cell line which expresses a low level of N-Myc, with a N-Myc expression vector. The Figure 1E-F shows that each cellular clone overexpressing N-Myc, downregulates TRKA, p75NTR, ABCC3 and TG2 as compared to parental

SH-SY-5Y cells and to a clone transfected with the empty vector. Finally we treated SK-N-BE and LAN-1, two human neuroblastoma cell lines carrying amplified MYCN, with a specific siRNA targeting MYCN mRNA. As shown in Fig 1F-G, RNAi mediating silencing of N-MYC correlated with upregulation of TRKA, p75NTR, ABCC3 and TG2.

APEX1, a gene positively regulated by N-Myc, was used as a control in every cellular system tested and, as expected, its expression paralleled that of N-Myc.

Taken together these findings support the initial hypothesis regarding a direct role of N-Myc in repressing this specific set of genes.

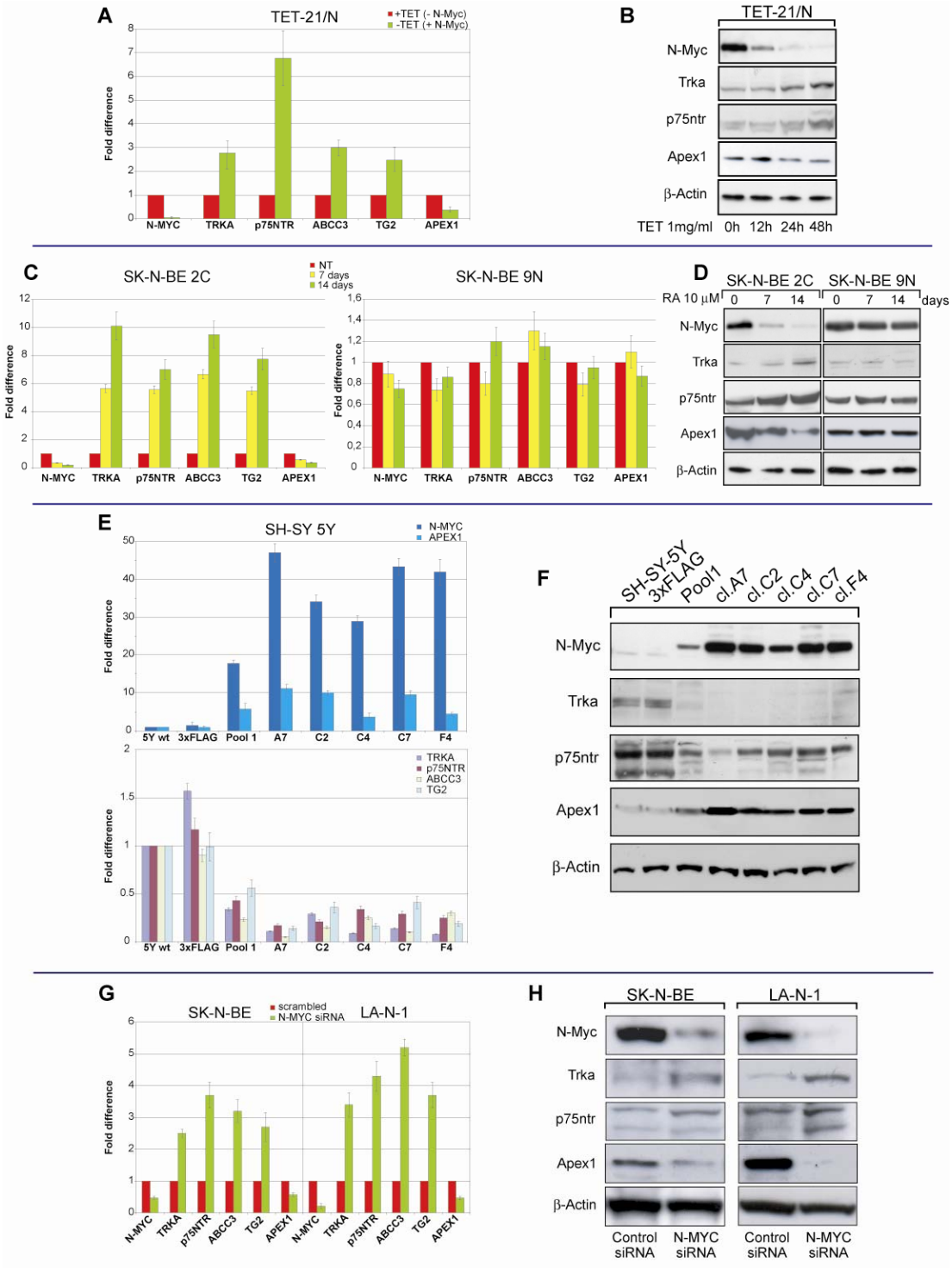

Figure 1. N-MYC expression inversely correlates with that of TRKA, p75NTR, ABCC3 and TG2 in neuroblastoma cell lines. **A,** quantification of gene transcripts by Real Time PCR as a function of N-MYC expression in TET-21/N cells. **B,** western blotting. **C,** different response to Retinoic Acid treatment between SK-N-BE 2C and 9N cells depending on N-MYC expression. **D,** western blotting. **E,** expression of tested genes in SH-SY 5Y neuroblastoma cell clones selected to overexpress N-MYC. **F,** western blotting. **G,** effect of RNAi knock-out of N-MYC in SK-N-BE and LAN-1 neuroblastoma cells on transcription of tested genes. **H,** western blotting. APEX1 is a gene activated by N-Myc and was used as a control.

The core promoters of TRKA, p75NTR, ABCC3 and TG2 are required for N-Myc-mediated repression

In order to formally confirm the functional role of N-Myc in repression of TRKA, p75NTR, ABCC3 and TG2 genes, we cloned the promoter regions of these genes upstream of a luciferase reporter gene (Figure 2). The luciferase activity of the recombinant reporters was determined in different cellular systems as a function of N-Myc expression:

- in TET-21/N as a function of tetracycline treatment;
- in SH-SY-5Y MYCN clones as a function of the level of N-Myc overexpression;
- in HeLa cells as a function of the amount of the pCMV-N-MYC expression vector co-transfected along with the luc-reporter; HeLa cells were used in order to extend these findings to a model diverse from neuroblastoma.

As shown in Figure 2, the presence of N-Myc significantly reduced the luciferase activity driven by TRKA -860/+60, p75NTR -900/+100, ABCC3 -1462/+754 and TG2 -1603/+378 reporters. On the contrary expression of N-Myc did not affect the activity of a viral promoter, such as *Cytomegalovirus,* and positively modulated the APEX1 -1000/+443 construct, used as a negative and positive control, respectively.

Finally, through a deletion analysis of the repressed promoters, we could map the region necessary for N-Myc-mediated repression within the *core promoters* of TRKA, p75NTR, ABCC3 and TG2. Deleted promoters were tested in the same conditions as described above and, as shown in Figure 2, they lost their responsiveness to N-Myc expression.

Overall these results demonstrate that the *core promoter* regions of the TRKA, p75NTR, ABCC3 and TG2 genes are required for N-Myc-mediated repression.

These findings prompted us to further investigate the mechanisms by which N-Myc mediate transcription repression.

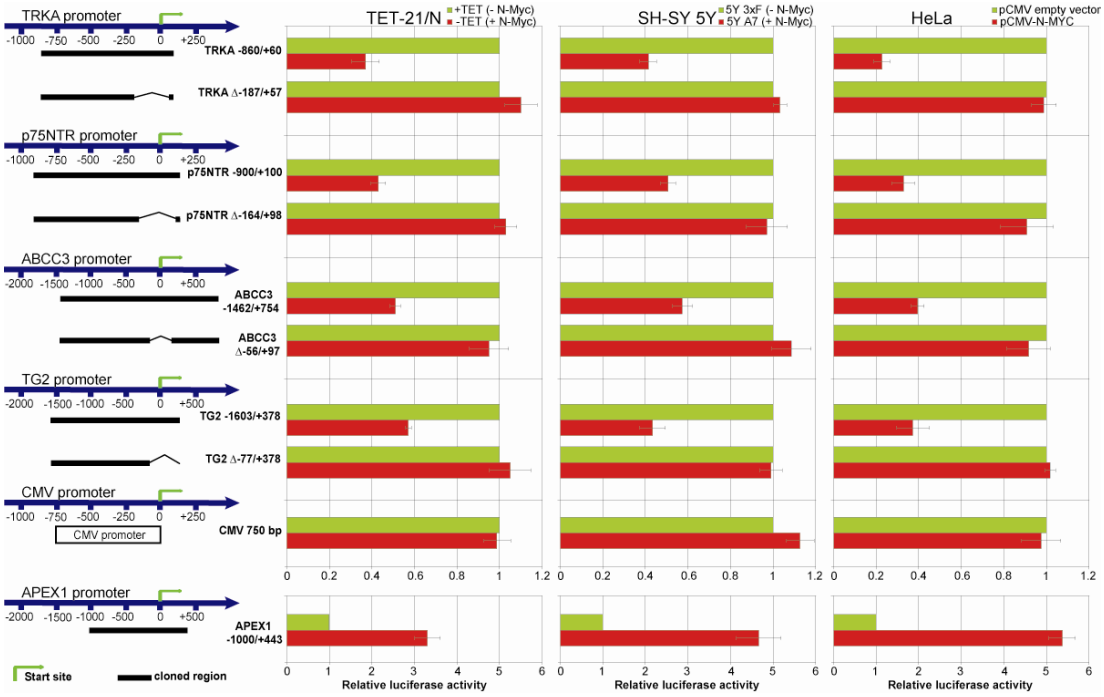

Figure 2. The core promoter region of tested genes is required to mediate repression by N-MYC. Whole promoters and mutated derivatives were cloned into a Firefly Luciferase reporter vector. Luciferase activity was tested as a function of N-MYC expression levels in distinct cell systems (TET-21/N, SH-SY 5Y clones and HeLa cells). Luciferase activity was normalized to that of Renilla Luciferase. APEX1 and CMV promoters were used as a positive and negative control respectively.

Repression of the neurotrophin receptors TRKA and p75NTR

Dissecting the core promoter regions of TRKA and p75NTR

As shown in the transient luciferase assays, we identified the regions critical for N-Myc-mediated repression in TRKA and p75NTR promoters. In order to better address this point, we had bioinformatically analysed these *core promoters* and we found several consensus sequences for Sp1 and Miz1 transcription factors. Sp1 and Miz1 binding sequence are present also in the *core promoter* of the cell cycle inhibitor p21 that is repressed by c-Myc in limphoblastoid cells (see Introduction, Figure 9) (Figure 3).

Brenner and colleagues have demonstrated that c-Myc recruits a DNA methyltransferase, DNMT3a, to the Myc-Miz1 complex on the promoter of p21, indicating that Myc-dependent gene repression could partly be mediated by methylation of its target promoters [181].

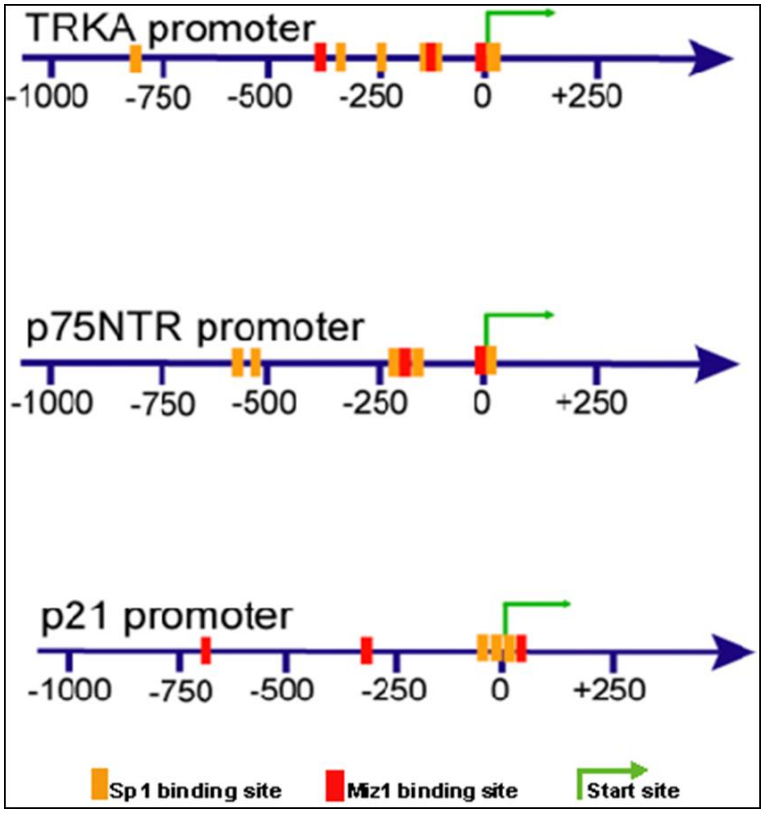

Figure 3. Schematic representation of TRKA, p75NTR and p21 promoters. The distribution of Sp1 and Miz1 binding sites is very similar among the three promoters.

To verify whether this mechanism may be extended to TrkA and p75NTR we analysed the methylation status of TRKA and p75NTR promoters by performing a Southern Blot analysis. We digested genomic DNA with MspI, which recognizes and cleaves the CCGG sequences independently of their methylation status and with HpaII, which instead cleaves the same sequence only when demethylated. The results showed that, in different human neuroblastoma cell lines, both TRKA and p75NTR promoters were not methylated (Figure 4).After excluding methylation as a mechanism of repression mediated by N-Myc, another possibility is that N-Myc, likewise c-Myc with p21, may repress TRKA and p75NTR expression, by targeting the activator functions of Sp1 and/or Miz1.

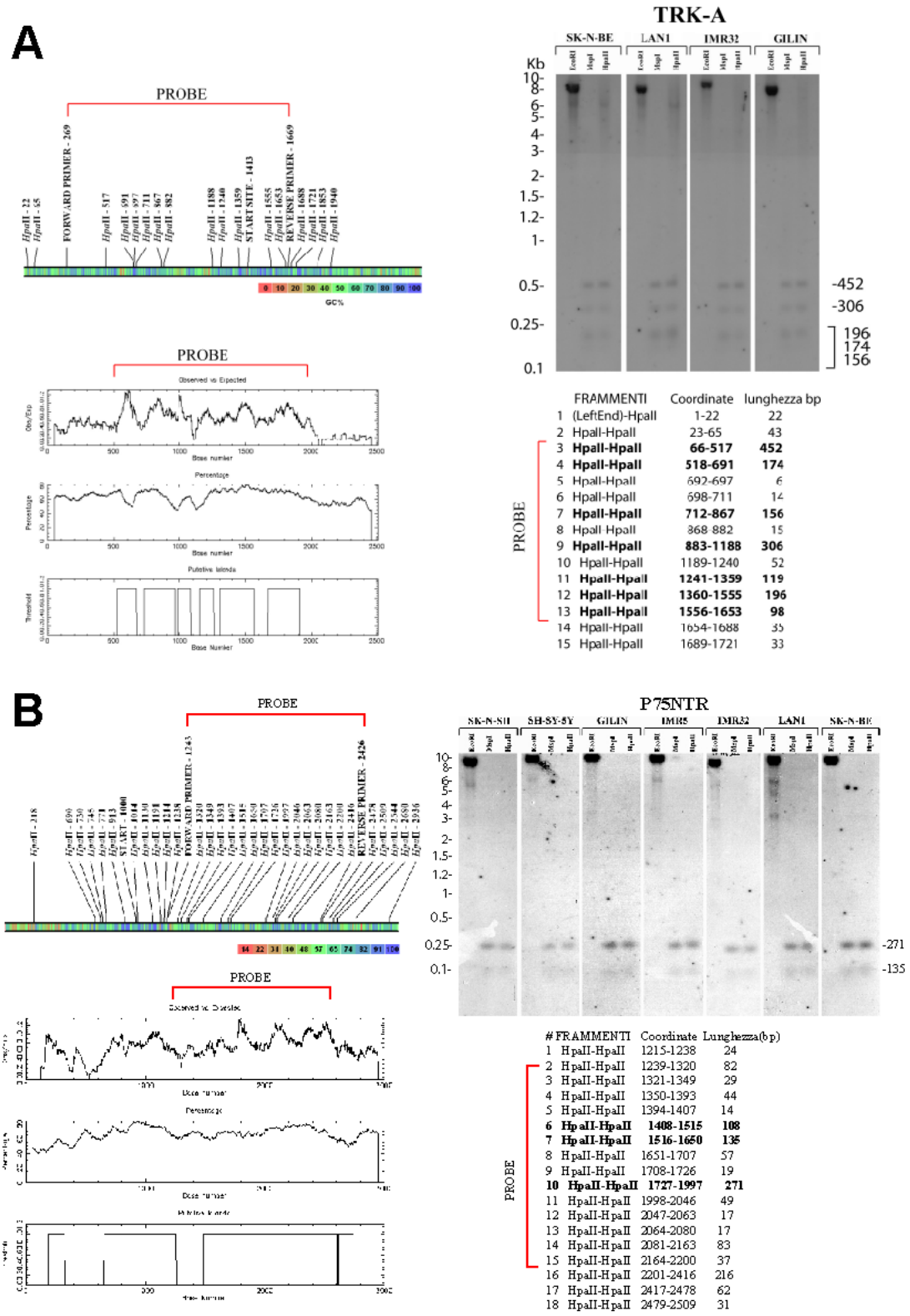

Figure 4. Southern Blot analysis of TRKA (**A**) and p75NTR (**B**) promoters. Genomic DNA (15 µg) digested with the indicated enzymes was electrophoresed on an agarose gel and analyzed by Southern blot hybridization. The probes extend from the promoter region to within the coding sequence. The positions of the HpaII/MspI sites are shown schematically over the diagrams. The digestion profile of genomic DNA with MspI was the same compared to that of HpaII, indicating that the CpG sites are not methylated.

Sp1 and Miz1 are activator factors of TRKA and p75NTR

To address this point we investigated the role of Sp1 and Miz1 transcriptional factors in the regulation of TRKA and p75NTR expression. First, we generated, two SK-N-BE stable cell clones by transfecting either Sp1 or Miz1 coding sequences under the control of a ponasterone inducible promoter. After the treatment with ponasterone, and the consequent induction of the Sp1 or Miz1 expression, we tested the expression of endogenous TrkA and p75NTR mRNA by qRT-PCR and observed a significant increase in the endogenous levels of TRKA, p75NTR. p21, which also increased was used as positive control (Figure 5A-B). Furthermore we also tested the reporter constructs of TRKA and p75NTR promoters in these two cell lines, by evaluating the luciferase activity as a function of ponasterone treatment. As shown in Figure 5C the activity of both reporters increased upon induction of Sp1 or Miz1 expression. Furthermore, the treatment with ponasterone had no effect on the activity of the *CMV* reporter, while it positively modulated the p21 -850/+120 construct, used as a negative and positive control, respectively. Moreover, the *core promoter* region of either TRKA or p75NTR had the ability to confer responsiveness to both Sp1 and Miz1 when cloned in the *CMV* reporter between the CMV promoter and the coding sequence of the luciferase (Figure 5C).

These findings support the idea that Sp1 and Miz1 play an important role in the transcriptional activation of the TRKA and p75NTR *core promoters*.

In order to establish if N-Myc could interfere with the transcription activation mediated by Sp1 and Miz1, we measured the luciferase activity of TRKA and p75NTR reporter constructs as a function of co-transfection with either a Sp1 or a Miz1 expression vector, in two different cellular context: with or without N-Myc expression (TET-21/N without and with tetracycline, respectively).

As expected, both Sp1 and Miz1 mediated upregulation of the reporters was significantly reduced in presence of N-Myc (Figure 5D). Once again the deleted constructs lost responsiveness to N-Myc, Sp1 and Miz1 while the reporters with the *core promoters* cloned downstream the CMV promoter, were sensitive to it. Furthermore, we found that, like c-Myc, even N-Myc affect the luciferase activity of p21 promoter. These transient luciferase assays were repeated and the results confirmed also in SH-SY 5Y clones and in HeLa cells (not shown).

Taken together these findings show that N-Myc could repress transcription of TRKA and p75NTR possibly by interfering with the positive regulation mediated by SP1 and Miz1 transcription factors at the level of *core promoter*.

N-Myc is physically associated with the core promoter of TRKA and p75NTR in vivo

According to the model generally accepted for Myc-mediated repression, N-Myc does not bind directly DNA (and in fact we could not find putative E-boxes within the *core promoters* of TrkA and p75NTR), but rather through interactions with proteins that bind DNA directly. So, in order to demonstrate that N-Myc contacts the *core promoters* of TRKA and p75NTR we employed the dual-crosslinking chromatin immunoprecipitation assay (dual ChIP), a variant of the standard ChIP in which two distinct crosslinking agents are used: first Di (N-succinimidyl) glutarate (DSG) that causes links between proteins and then formaldehyde that generates links between proteins and DNA and assures the recovery of proteins not in direct contact to DNA [284]. So, when dual ChIP assay was performed on SK-N-BE cells, we found that N-Myc together with its partner Max, Sp1 and Miz1 can specifically co-occupy the *core promoters* of TRKA, p75NTR and p21 *in vivo*. For Miz1 dual ChIP was performed using the inducible clone derived from SK-N-BE, which expresses a Miz1-HA protein after ponasterone treatment, as shown above. In this way we used an anti-HA antibody for immunoprecipitation, considering the lack of an anti-Miz1 available for ChIP assay.

These results strongly support the existence of a multi-proteic complex of repression involving N-Myc on the *core promoters* of TRKA, p75NTR and p21, too (Figure 6).

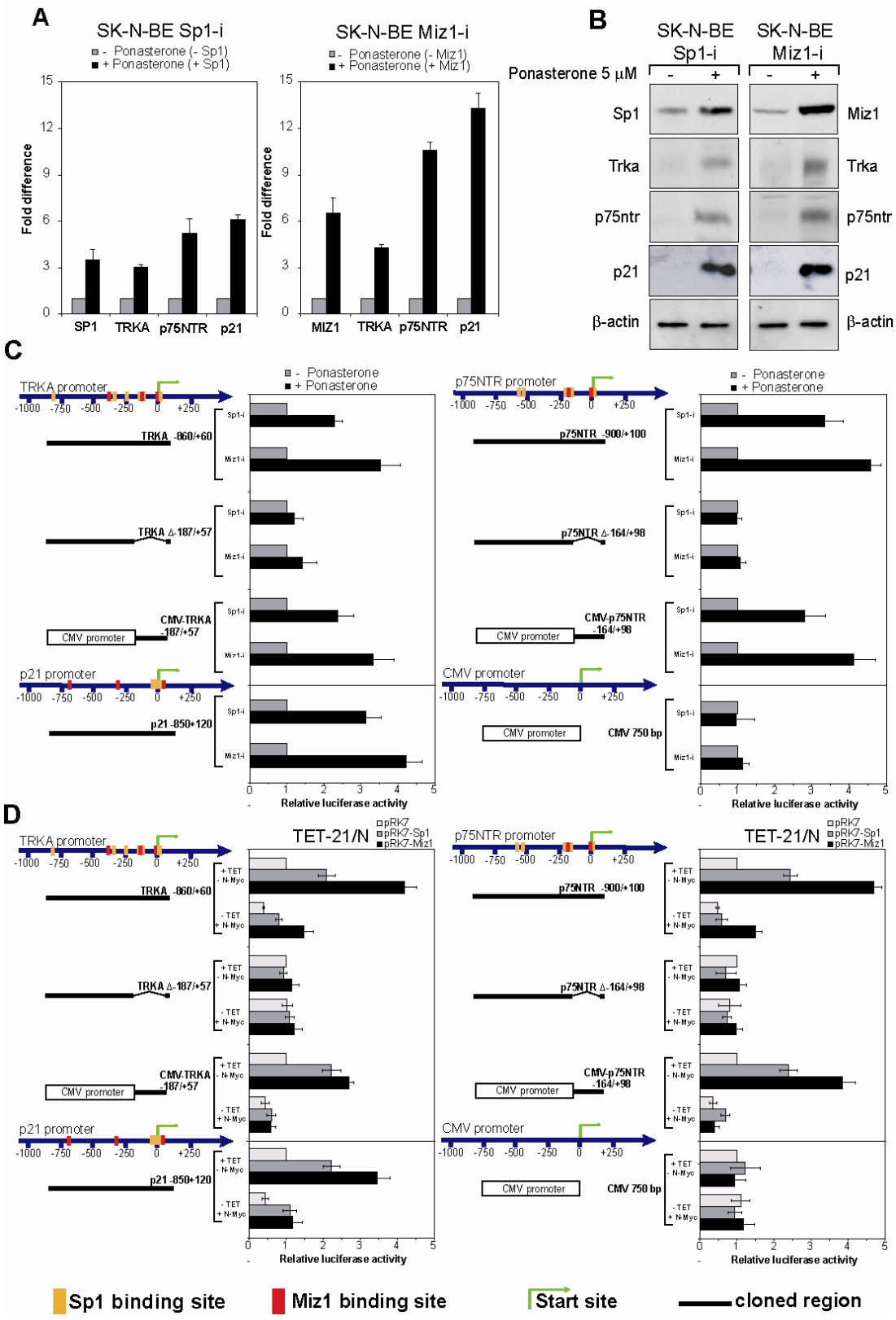

Figure 5. N-Myc represses transcription through Sp1 and Miz1 transcription factors. **A,** induced transcription of either Sp1 or Miz1 increases expression of tested genes in neuroblastoma cells. **B,** western blotting. **C,** relative luciferase activity of reporter vectors in the inducible cell lines. **D,** relative luciferase activity of reporter vectors as a function of Sp1 and Miz1 in the absence or presence of N-Myc. p21 and CMV promoters were used as controls.

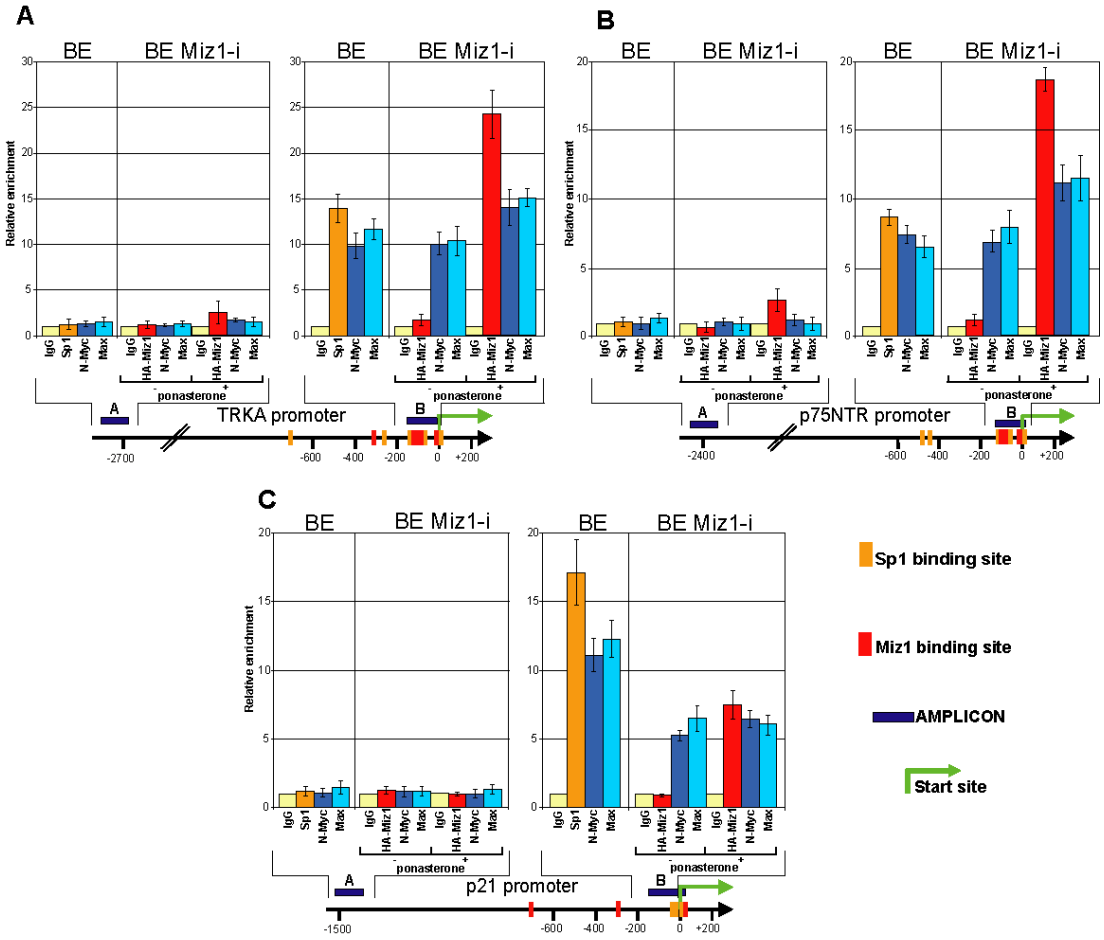

Figure 6. Dual Chromatin Immunoprecipitation analysis reveals that N-MYC is physically associated with the *core promoter* regions of repressed genes in SK-N-BE human neuroblastoma cell line. Relative enrichments were calculated as the ratio between the enrichment obtained with the specific antibody and that obtained with the pre-immune serum (IgG). Amplification of a distal region was used as negative controls for each promoter. Results represent the average of three independent experiments in which each region was amplified by qPCR in triplicate. Standard error is indicated.

N-Myc interacts with Sp1 and Miz1 through distinct domains

To confirm whether N-Myc can interact with Sp1 and Miz1 we performed a co-immunoprecipitation assay. We co-transfected HEK293 cells with an expression vector for N-Myc with a FLAG tag and an expression vector for Sp1-HA or for Miz1-HA tag proteins.

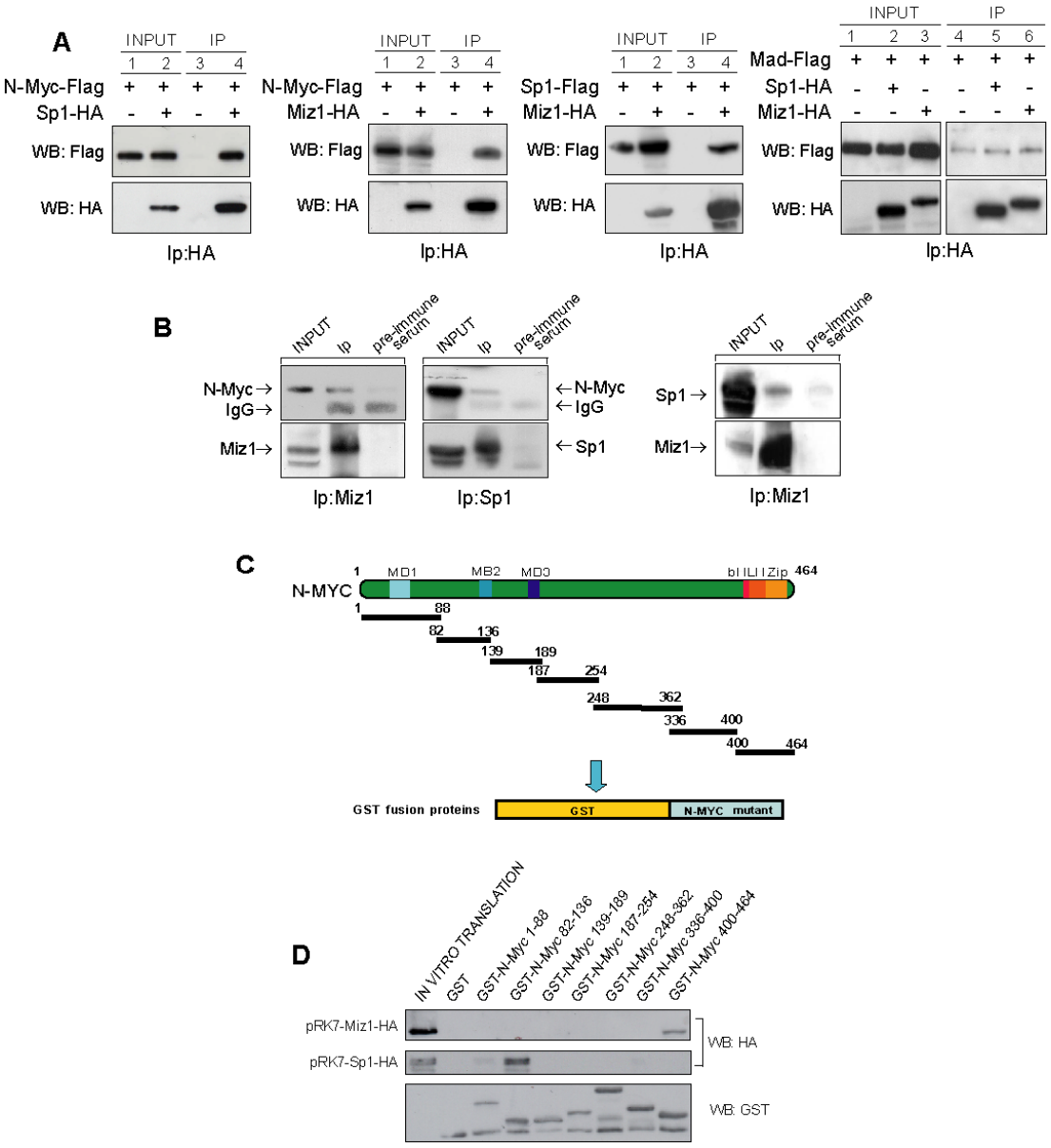

Figure 7. Co-Immunoprecipitation and GST pull-down assays show that distinct N-Myc domains can contact Sp1 and Miz1. **A,** tagged proteins were expressed in HEK293 cells. Immunoprecipitation was performed using anti-HA antibodies. Western Blotting was performed using anti-FLAG antibodies. The Mad protein was used as a negative control of the assay. **B,** association of endogenous N-Myc with endogenous Miz1 or Sp1 was determined by Co-IP using specific nuclear extracts from SK-N-BE neuroblastoma cell line. Antibodies against Sp1 or Miz1 (Immunoprecipitation) or N-Myc (western blotting) were used. **C,** schematic representation of N-Myc-GST constructs. **D,** GST pull-down assays were performed by incubating in vitro translated Sp1 or Miz1 proteins with GST (as negative control) and with N-MYC-GST constructs for 1h at 4°C followed by incubation with glutathione beads for 1h. Bound proteins were subjected to SDS/PAGE followed by immunoblotting with anti-HA antibody.

When N-Myc-FLAG was immunoprecipitated with an anti-FLAG antibody, we specifically recovered both Sp1 and Miz1 proteins, as determined by western blotting using an anti-HA antibody. We obtained the same results when Sp1-HA or Miz1-HA was immunoprecipitated with an anti-HA antibody: in summary we found that the three proteins present in the repression complex may interact with each other.

Moreover, to confirm the specificity of these interactions, we showed that Mad-FLAG, another member of the Myc/Mad/Max network that does not interact with N-Myc (see introduction), did not co-immunoprecipitate with Sp1 and Miz1 (Figure 7A). The importance of these interactions was highlighted by co-immunoprecipitation assays with endogenous proteins in SK-N-BE confirming the existence of such interactions in vivo (Figure 7B).

Finally we used a GST pull-down assay in order to determine which N-Myc region(s) could contact Sp1 or Miz1. For this purpose several N-Myc mutants, designed to cover all the length of the onco-protein, was expressed as GST derivatives and incubated with in vitro translated Sp1-HA or Miz1-HA proteins (Figure 7C). Interaction between GST-N-Myc proteins and in vitro translated ones was determined by western blotting. As shown in Figure 7D, two distinct regions of N-Myc, one containing the MB II transactivation domain and the other the BR/HLH/LZ domain, can interact with Sp1 and Miz1, respectively.

N-Myc, Sp1 and Miz1 together are necessary to mediate transcriptional repression

To formally demonstrate a functional role for the two interacting-domains of N-Myc, we generated three N-Myc mutants:

- N-Myc delta 82-136 without the domain of interaction with Sp1;
- N-Myc delta 400-464 without the domain of interaction with Miz1;
- N-Myc delta 248-362 as negative control;

By performing a transient luciferase assay we found that only the mutants without the interacting-domains have lost the ability to repress both TRKA and p75NTR reporters when co-transfected in SH-SY 5Y human neuroblastoma cell line (Figure 8A-B).

Furthermore, silencing of each component of the putative repression complex (with specific siRNA targeting N-Myc, Sp1, or Miz1), is sufficient to re-activate the endogenous expression of both the neurotrophin receptors (Figure 8C-D).

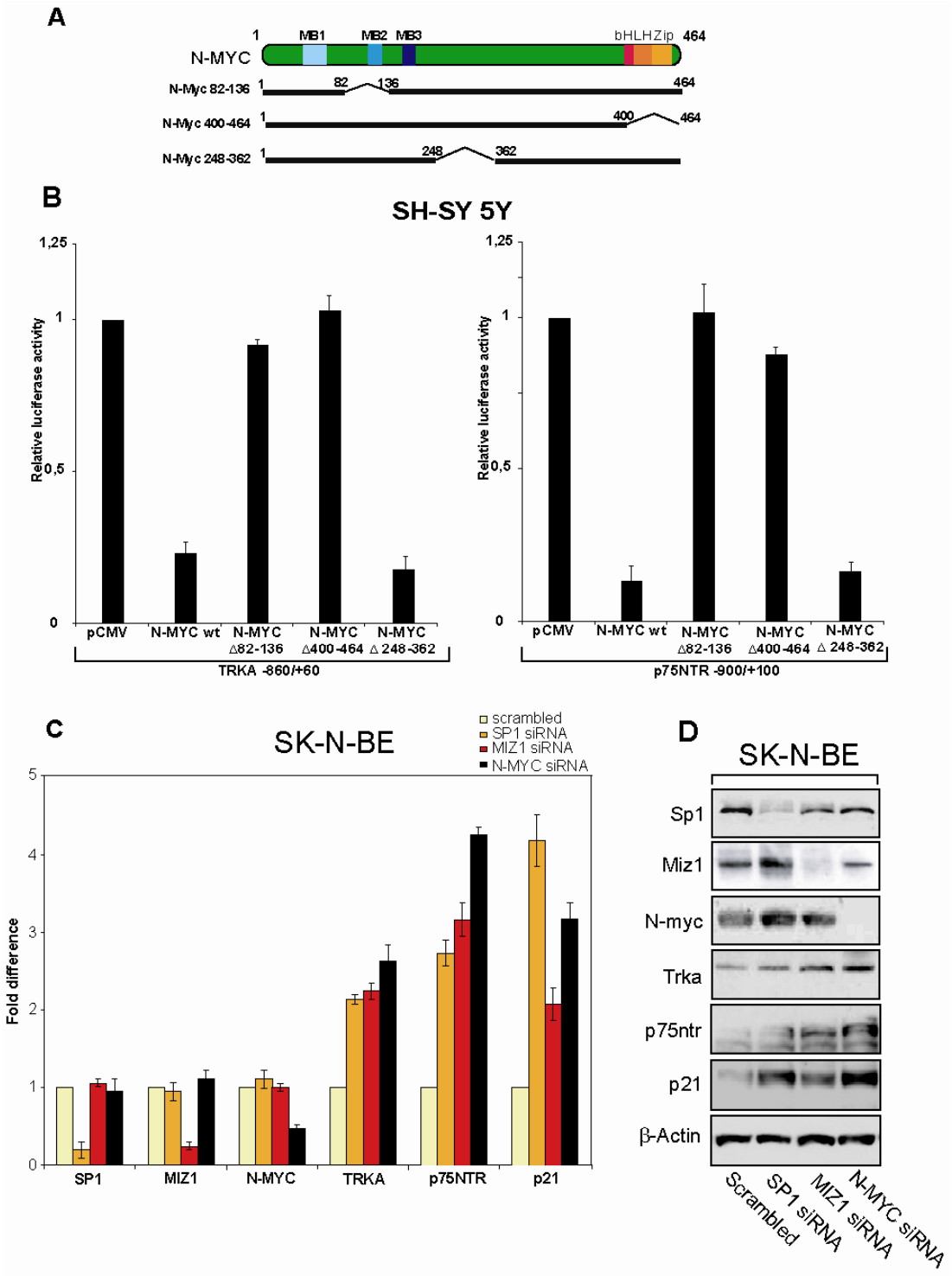

Figure 8. Functional role of N-Myc, Sp1 and Miz1 in TRKA and p75NTR repression. **A,** schematic representation of N-Myc mutants. **B,** luciferase activity was measured when each N-Myc mutant, cloned into an expression vector, were co-transfected in SH-SY 5Y with both TRKA and p75NTR reporter. The empty vector and the full-length coding sequence were used as negative and positive control, respectively. **C,** quantification of tested gene transcripts by Real Time PCR as a function of RNAi knock-out of N-Myc, Sp1 and Miz1 in SK-N-BE neuroblastoma cells. Once again p21 was used as positive control of the repression. **D,** western blotting.

From this analysis we obtained strong evidences that the two interacting domains of N-Myc may play an important role in mediating repression in complex with Sp1 and Miz1 transcription factor. Furthermore, considering that all the proteins of the complex contact each other (from Co-IP experiments) and that each protein of the complex is necessary to mediate repression (from RNAi knock-out experiments) we can speculate on the existence of a stable ternary complex (plus Max) on the *core promoter* regions of TRKA and p75NTR, responsible for their repression in neuroblastoma cells (Figure 9).

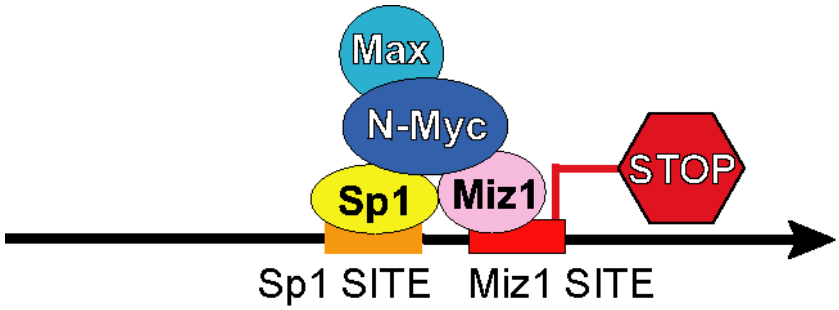

Figure 9. Plausible model of a ternary complex (plus Max) through which N-Myc may contact gene promoters *in vivo* to repress transcription.

NGF treatment increases apoptosis rate after re-expression of both TRKA and p75NTR

In order to explore the biological significance of TRKA and p75NTR re-expression, N-Myc, Sp1 and Miz1 siRNAs were used again to inhibit the repressive action of these genes in N-MYC-amplified human neuroblastoma SK-N-BE cell line. We found a significant increase in the percentage of cells undergoing apoptosis only when cells treated with each RNAi against N-Myc, Sp1 or Miz1, were also treated with NGF (Figure 10A). This finding was obtained by a BrdU incorporation assay, in which we noticed an evident increment of the sub-G1 peak, typical of apoptotic cells. To better address this point we directly assessed apoptosis using an anti-PARP rabbit polyclonal antibody on protein extracts from SK-N-BE treated as shown in Figure 10B.

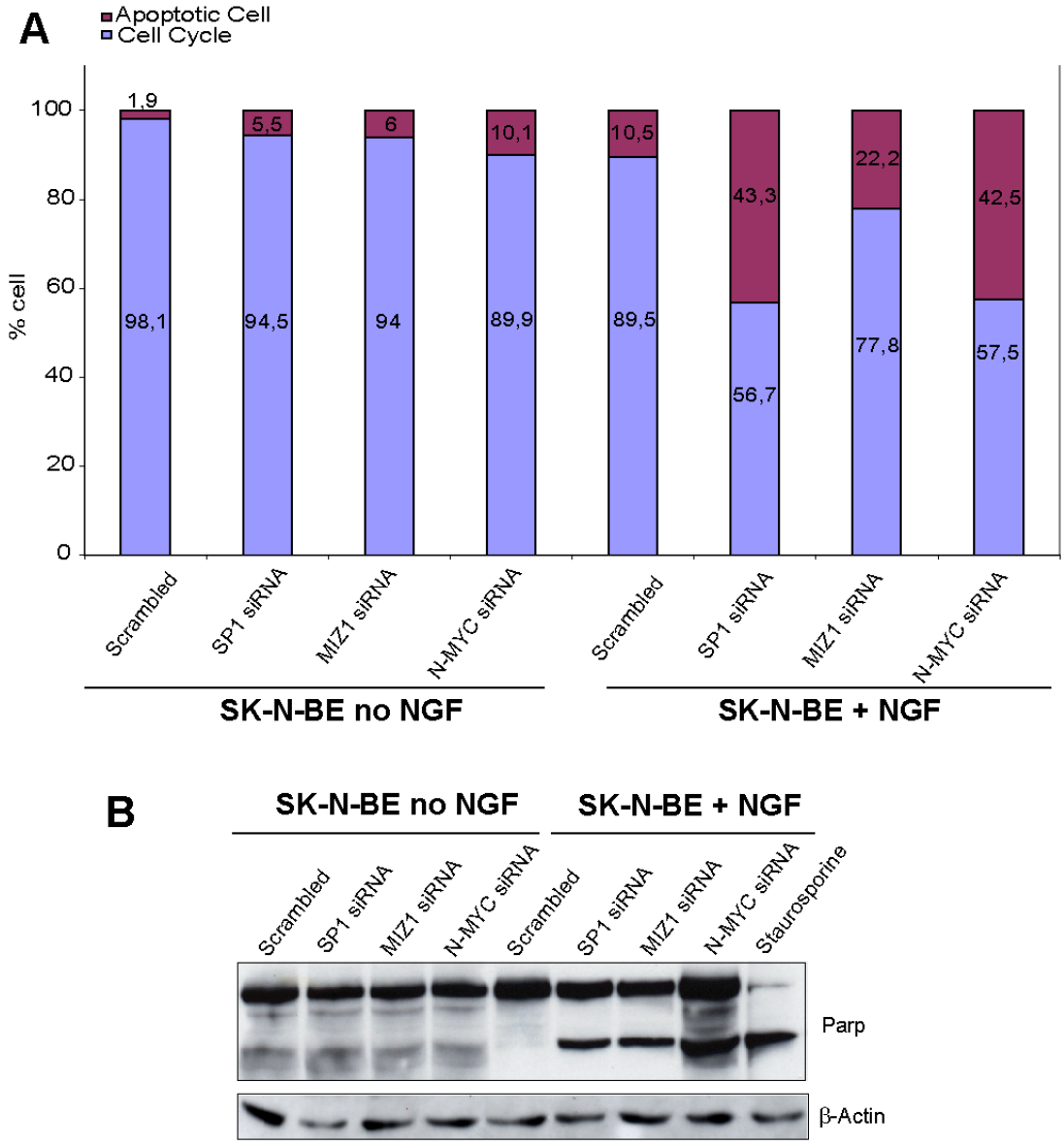

Figure 10. TRKA and p75NTR re-expression influences the rate of apoptosis upon treatment with NGF 50 ng/ml. **A,** BrdU incorporation assay was performed to monitor modification of cell cycle. **B,** Western blot assay with anti-PARP antibody. Both assays showed an increment of apoptosis in response to NGF treatment, depending on the re-expression of the endogenous levels of neurotrophin receptor.

Anti-PARP recognizes Poly-ADP-Ribose-Polymerase (PARP), a 113 kD protein that binds specifically at DNA strand breaks. PARP is also a substrate for certain caspases (for example, caspase 3 and 7) activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 89 kD and 24 kD. Thus, detection of the 89 kD PARP fragment with Anti-PARP in a Western Blot assay serves as an early marker of apoptosis.

As expected, the PARP fragment of 89 kD appeared clearly only in the presence of the RNAi knock-out of N-Myc, Sp1 or Miz1, followed by treatment with NGF (Figure 10B).

Finally these data suggest that the re-expression of the neurotrophin receptors, mediated by the inhibition of each component of the ternary repression complex (i.e. N-Myc/Sp1/Miz1), can induce apoptosis in response to NGF in human neuroblastoma cells. Overall, results support the idea that N-Myc may play a pivotal role in tumour progression by inhibiting the apoptotic pathway mediated by TrkA/p75NTR signalling.

Repression of the ATP-binding cassette transporter ABCC3

Similarly to the study we have applied to TRKA and p75NTR, we analyzed ABCC3 promoters for the presence of N-Myc, Sp1 and Miz1 binding sites. Like neurotrophin receptors, ABCC3 promoter does not contain E-Boxes in close proximity to its transcriptional start site, whereas GC boxes (that bind Sp1) are located around its start site. On the contrary we did not find any putative Miz1 binding site (Figure 11).

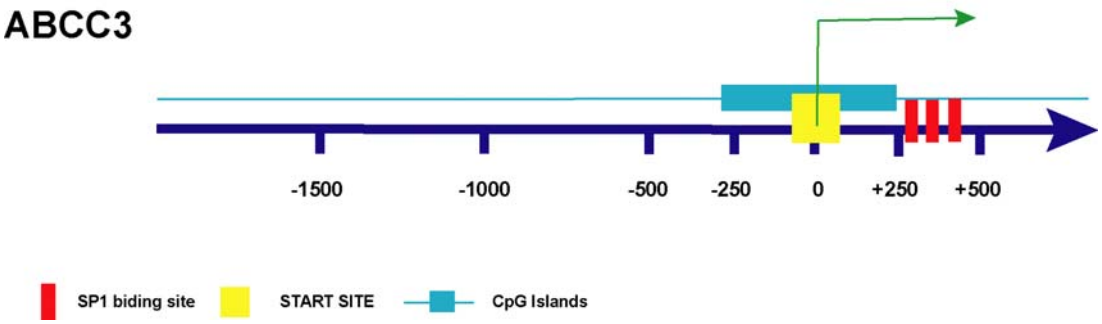

Figure 11. Schematic representation of ABCC3 gene promoter. The localization of the CpG island is indicated by sky-blue line, while Sp1 binding sites are represented by the vertical red lines.

As expected, we found that the reporter construct of ABCC3 promoter responded to the levels of Sp1 but not to those of Miz1 in a transient luciferase assay. Furthermore this construct required the Sp1 *core region* for N-Myc-mediated repression (not shown).

N-Myc represses the ABCC3 promoter by interacting with Sp1 transcriptional factor

Thus, we evaluated the binding of Sp1 to its cognate sites and checked if also N-Myc could bind ABCC3 promoter through specific interaction with Sp1. We tested this hypothesis in a Dual ChIP assay.

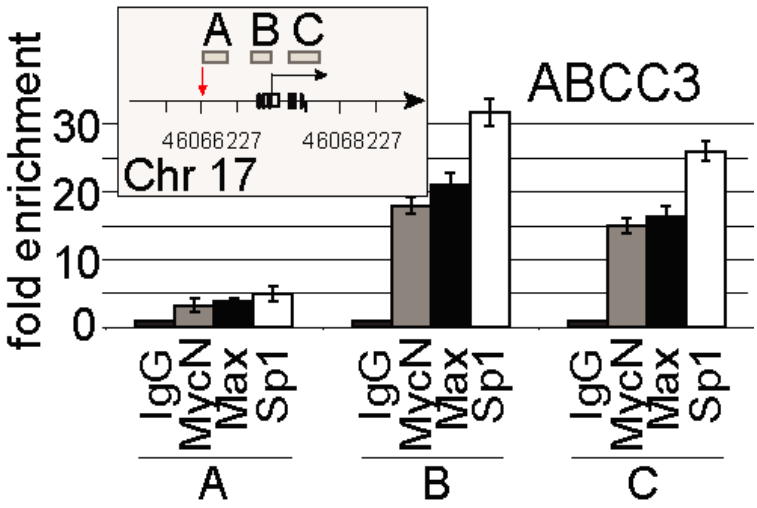

Figure 12. N-Myc is physically associated with the *core promoter* of ABCC3. Dual ChIP and quantitative PCR were applied to Tet-21/N- cell line. Fold enrichment is relative to the pre-immune serum. Results represent the mean ± SE of three independent ChIP experiments. Promoter diagram: bent arrow, transcription start site; red arrow, canonical E-box; black arrow, non-canonical E-box; open boxes, amplicons indicated with a capital letter; chromosome and coordinates (bp) are also given.

We found that either Sp1 or N-Myc (and its partner Max) bind ABCC3 promoter on a specific region containing the three Sp1 binding sites (Figure 12). Therefore, N-Myc can repress ABCC3 transcription through a direct interaction with Sp1, even in absence of Miz1. This finding supports the hypothesis that N-Myc may repress gene expression through distinct mechanisms.

ABCC3 expression levels affect multiple neuroblastoma cell characteristics

As mentioned in the introduction, ABCC1, ABCC3 and ABCC4 were the only genes from the entire ABCC family that were shown to be directly regulated by N-Myc and these were also the only genes that exhibited prognostic significance in primary neuroblastoma.

In collaboration with the group of Dr. Michelle Haber (Children's Cancer Institute Australia, Sidney), we were able to demonstrate that high levels of ABCC1 and ABCC4 are driven by N-Myc, through direct binding on their promoter. Moreover specific silencing of the ABCC1 or ABCC4 genes leads to growth inhibition, increased morphological differentiation and impaired motility of N-MYC-amplified neuroblastoma cells. Thus, these Myc-dependent changes in ABC protein levels directly affected the malignant behaviour of neuroblastoma cells in vitro and tumour aggressiveness in vivo (unpublished data).

To investigate the significance of suppressed ABCC3 expression in neuroblastoma, SK-N-BE cells, which display low endogenous levels of ABCC3, were induced to constitutively express ABCC3 (Figure 13A). Cell clones expressing ABCC3 were investigated for several cellular parameters. For example, expression of ABCC3 at significant levels induced neurite formation (Figure 13B). The enhanced morphological differentiation was accompanied by impaired clonogenic ability and decreased cell proliferation, as measured either by increase in cell number over time or by incorporation of BrdU over time (Figure 13C), and reduced migratory activity (Figure 13D).

Taken together these data indicate a potential critical role for ABCC3 in multiple aspects of tumour cell phenotype.

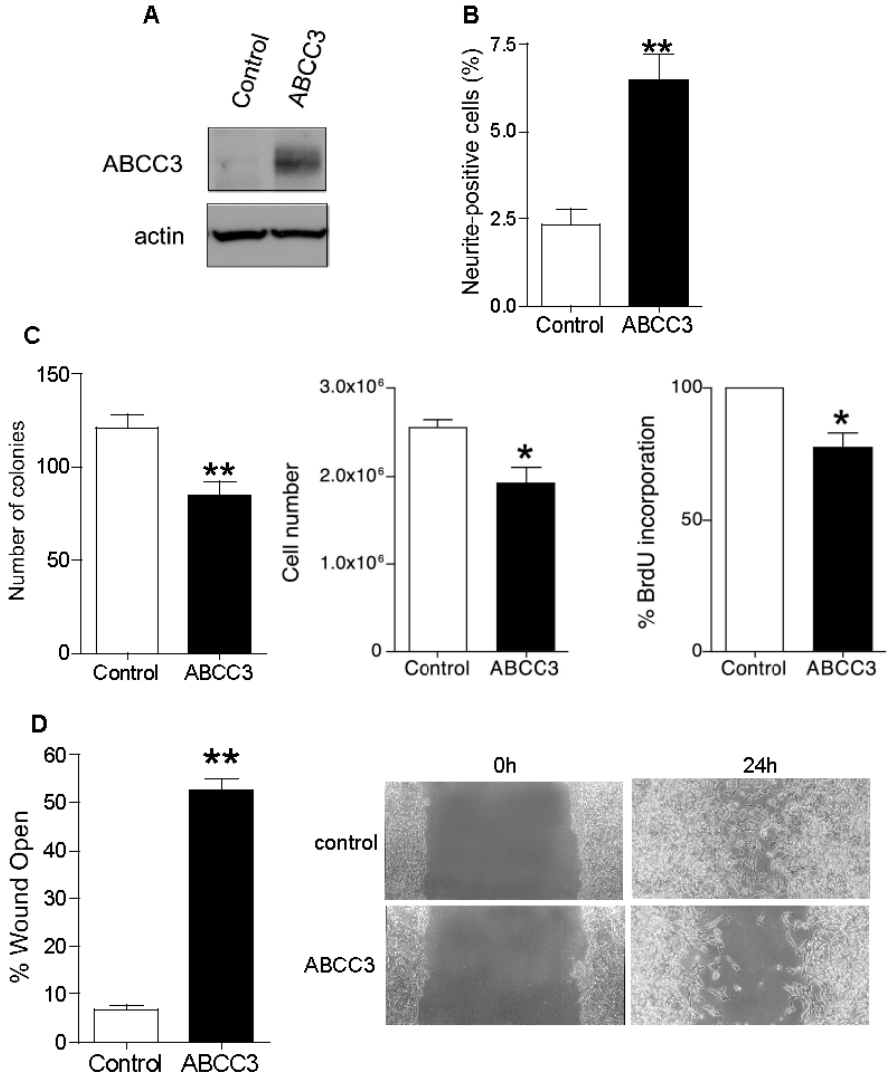

Figure 13. ABCC3 gene expression levels influence multiple properties of human neuroblastoma cells. **A,** Western blot analysis of ABCC3 protein expression following stable transduction of SK-N-BE cells with either empty vector or pCMV-ABCC3. **B,** Expression of ABCC3 enhanced neurite extension in SK-N-BE cells; **P<0.0001. **C,** Clonogenic capacity (left panel), total cell numbers (centre panel) and BrdU incorporation (right panel) were significantly reduced in cells overexpressing ABCC3; *p<0.05, **p<0.02. **D,** Quantification (left panel) and representative images (right) displaying impaired motility of SK-N-BE cells overexpressing ABCC3. **P<0.0001. In parts A-D, data is presented as mean ± SE from at least three separate experiments.

Repression of tissue transglutaminase (TG2)

TG2 transcriptional activation is mediated by HDAC inhibitor

A recent collaboration of our lab with that of Dr. Glenn Marshall (Children's Cancer Institute Australia, Sidney) led to the finding that the TG2 gene is one of the most significantly repressed genes by N-Myc in neuroblastoma cells (unpublished data). Surprisingly, reviewing published cDNA microarray gene profiling studies, TG2 results to be also commonly up-regulated by HDAC inhibitors, such as Trichostatin A (TSA), SAHA, and butyrate, in cancer cells of various organ origins, such as leukaemia and liver, renal, nasopharyngeal, and breast cancer [285] [286] [287].

To understand how TSA revert N-Myc mediated repression of TG2, we performed semiquantitative RT-PCR analysis of TG2 gene expression in neuroblastoma cells. As shown in Figure 14A, treatment with 0.1 μM TSA for 6 h reactivated TG2 gene expression in BE(2)-C, IMR-32, SHEP S1, and LAN-5 neuroblastoma cell lines. Immunoblot analysis with an antibody that identified chromatin histone H4 revealed a marked increase in histone acetylation 3 h after TSA treatment, confirming that TSA acetylated histones in the cells (Figure 14B). In contrast, TSA did not have an effect on TG2 transcription in normal nonmalignant cells (not shown). These data suggested that TG2 gene transcription is repressed by HDAC activity across cancer cells but not in normal nonmalignant cells.

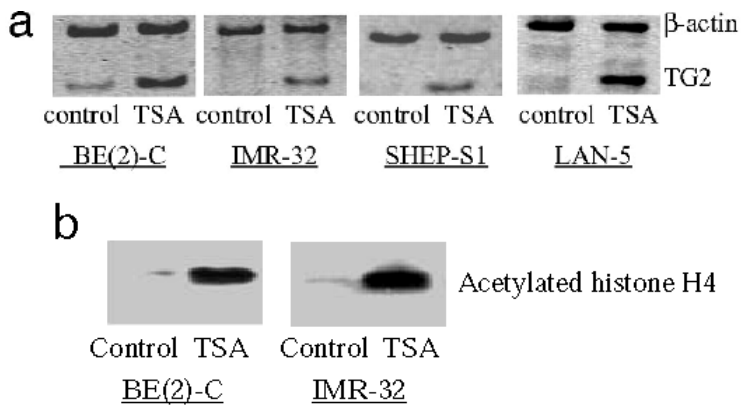

Figure 14. Up-regulation of TG2 by the HDAC inhibitor TSA. **A,** BE(2)-C, IMR-32, SHEP S1, and LAN-5 neuroblastoma cells were treated with control or 0.1 μM TSA for 6 h, followed by RNA extraction and semiquantitative competitive RT-PCR. **B,** Treatment with HDAC inhibitor acetylates histone H4 protein. Chromatin histone protein was extracted from BE(2)-C and IMR-32 cells after 3 h of treatment with control or 0.1 mM TSA, and subject to immunoblot analysis with an anti-acetylated histone H4 antibody.

N-Myc represses TG2 transcription by directly recruiting the HDAC1 protein to the Sp1-binding site of the TG2 gene core promoter

Considering the presence of several Sp1 binding sites in the *core promoter* of TG2, and based on previous findings, we tested the hypothesis that N-Myc might repress TG2 transcription through a direct mechanism involving a specific interaction with Sp1.

Dual ChIP was applied to N-Myc-amplified neuroblastoma cells (IMR-32 and LAN-1), using specific antibodies against N-Myc, Sp1, and HDAC1 proteins. A preimmune serum was used as a negative control to determine the baseline of the nonspecific background. As shown in Figure 15A, all tested antibodies could efficiently immunoprecipitate the TG2 *core promoter* that contained the Sp1-binding sites (Amplicon B). A DNA region (Amplicon A) located ≈1.6 kb from the TG2 *core promoter*, was tested in ChIP as a negative control. We obtained similar results with a second neuroblastoma cell line, IMR-32 (not shown). Once again the N-Myc repression complex appeared to have distinct protein components compared to the others analysed above: specific antibodies did not identify Max, the partner of heterodimerization with N-Myc. Conversely, HDAC1 was not present neither on the promoters of neurotrophin receptors nor on ABCC3.

To understand the dynamics of how N-Myc, Sp1, and HDAC1 contributed to TG2 repression, dual cross-linking ChIP was performed on LAN-1 neuroblastoma cells treated with TSA for 24 h. Our results showed that TSA dramatically reduced the association of HDAC1 with the TG2 promoter ($P < 0.01$), but not that of Sp1 and N-Myc (Figure 15B). Because TSA induced reactivation of TG2 transcription, this result suggested that N-Myc required HDAC1 to repress TG2 transcription, possibly by direct interaction.

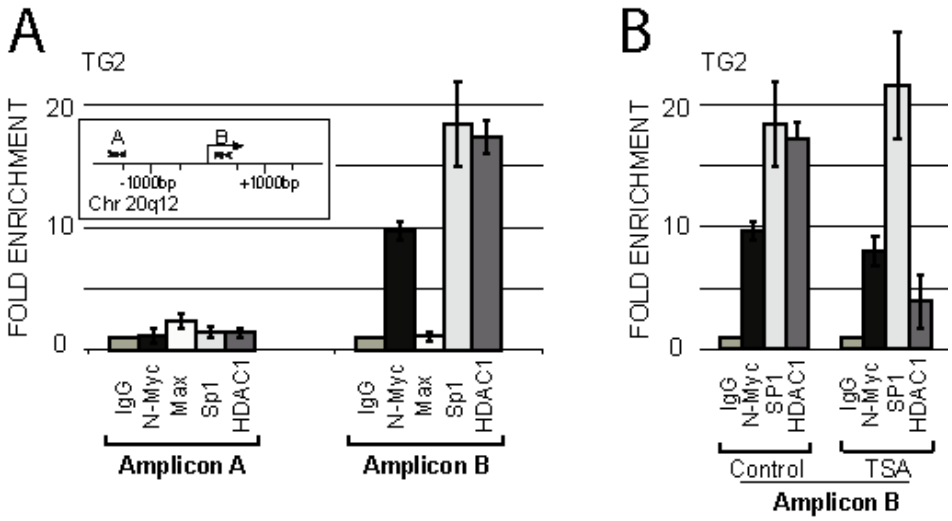

Figure 15. N-Myc represses TG2 gene transcription by recruiting HDAC1 to the TG2 gene core promoter. **A,** Dual ChIP and quantitative PCR were applied to LAN-1 cells. Quantitative PCR with primers targeting the Sp1-binding site (Amplicon B) or Amplicon A, 1.6 kb up-stream of TG2 gene transcription start site, was performed in triplicate. Results were the average of three independent dual cross-linking ChIP experiments. **B,** Dual ChIP was performed on LAN-1 cells treated with control or TSA for 24 h, when a maximal transcriptional reactivation of TG2 was observed. Error bars indicate standard error.

N-Myc can interact with HDAC1

In order to prove that N-Myc and HDAC1 can interact in situ, we performed a Co-IP assay. Nuclear extracts obtained from LAN-1 cells were incubated with specific anti-N-Myc antibodies or with preimmune IgG used as a negative control. The IP-complexes were subsequently separated in an SDS/PAGE and analyzed by Western blot, using antibodies that recognized Sp1, HDAC1 and Max. Results shown in Figure 16A showed that the anti-N-Myc antibodies co-immunoprecipitated Sp1, HDAC1 and Max, thus confirming that N-Myc was directly bound in all repression complexes identified by our dual ChIP experiments. However, when the same

nuclear extracts were incubated first with an anti-HDAC1 antibody, only Sp1 and N-Myc were identified in the repressor complex.

Next, in order to determine which domain of the N-Myc protein directly interacts with HDAC1, we performed a GST pull-down assay. We found that HDAC1 bound only the C-terminal N-Myc DNA binding domain, interestingly the same bound by Max in the repression complex of TRKA and p75NTR.

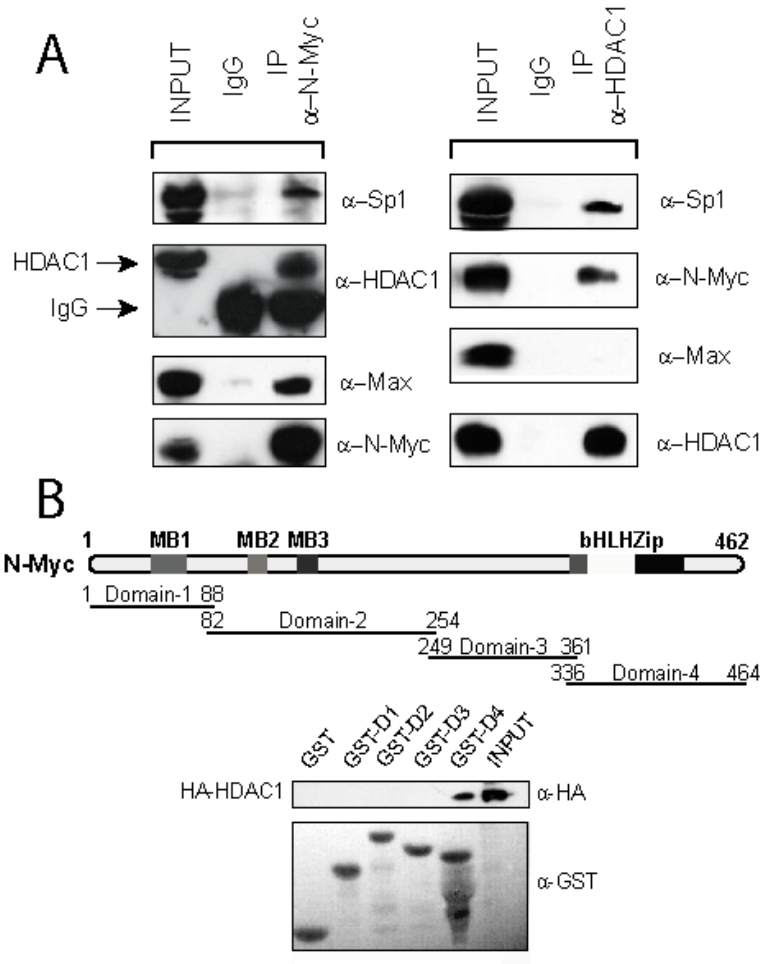

Figure 16. N-Myc directly interacts with Sp1 and HDAC1 through its carboxyl-terminal domain. **A,** Protein coimmunoprecipitation (IP) of N-Myc or HDAC1. One milligram of nuclear protein extract from LAN-1 cells was incubated with either a preimmune serum, or an anti-N-Myc antibody (Left) or an anti-HDAC1 antibody (Right). The purified IP-complex was analyzed by Western blot, using antibodies for the following proteins: Sp1, HDAC1 and Max. Lane 1, input; lane 2, preimmune serum IgG IP; lane 3, anti-N-Myc or anti-HDAC1 antibody IP. **B,** GST-N-Myc fusion proteins carrying different N-Myc domains were incubated with nuclear extracts expressing HA-HDAC1. GST pull-down complexes were analyzed by Western blot analysis, using an anti-HA monoclonal antibody.

Transcriptional activation of TG2 contributes to HDAC inhibitor-induced growth inhibition in human neuroblastoma cells

In order to assess the role of TG2 gene up-regulation in HDAC inhibitor effects, we used neuroblastoma SHEP S1, which expresses a very low basal level of TG2. In the absence of TSA, TG2 siRNA transfection had no effect on cell proliferation in SHEP S1. Although TSA suppressed cell proliferation, TG2 siRNA partly blocked this effect in the cell line tested, indicating that TG2 reactivation is required for the growth arrest induced by HDAC inhibitors in the cancer cells (Figure 17A).

TG2 has been reported to induce apoptosis by activating a BAX conformational change leading to BAX-mediated mitochondrial apoptosis [273], one of the main pathways through which HDAC inhibitors induce apoptosis [288]. We therefore tested whether up-regulation of TG2 could be responsible for HDAC inhibitor-induced apoptosis. Treatment with 0.1 μM TSA induced significant cell death in BE(2)-C cells. As shown in Figure 17B, compared with scrambled siRNA, TG2 siRNA did not affect the proportion of BE(2)-C cells stained with TUNEL 48 h after TSA treatment ($P > 0.05$). These results did not support a role for TG2 in HDAC inhibitor-induced apoptosis.

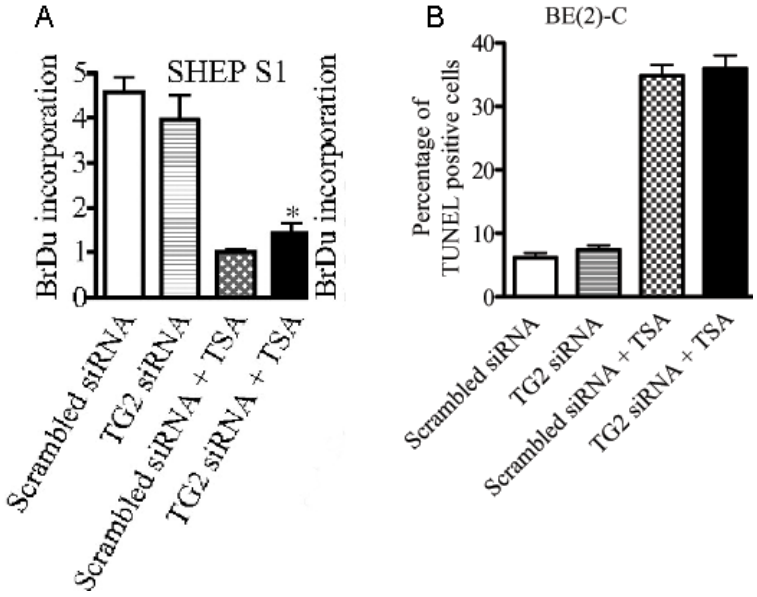

Figure 17. A, Up-regulation of TG2 by the HDAC inhibitor TSA and its role in cell proliferation in neuroblastoma cells. SHEP S1 cells were transfected with control or TG2 siRNA for 8 h, followed by treatment with control or TSA for 48 h and incubation with BrDu for the last 6 h. BrDu incorporation was measured as OD units of absorbance. *, $P < 0.05$ indicates a statistically significant increase in BrDu incorporation. Error bars indicate standard error. **B,** Up-regulation of TG2 did not contribute to HDAC inhibitor-induced apoptosis. BE(2)-C cells were transfected with scrambled or TG2 siRNA for 8 h, followed by treatment with 0.1 mM TSA for 48 h. After fixation, cells were stained with the TUNEL reagent, examined under fluorescence microscope, and the percentage of TUNEL positive cells was quantified.

Repression of TG2 expression by N-Myc is required for neuritic differentiation arrest in human neuroblastoma cells.

N-Myc-induced malignant transformation has been associated with arrest of differentiation and subsequent indefinite cell proliferation [289]. To test whether suppression of TG2 gene expression is responsible for N-Myc-induced neuroblastoma cell differentiation arrest, we transfected scrambled siRNA, TG2 siRNA, N-Myc siRNA, or TG2 siRNA plus N-Myc siRNA into N-Myc-amplified BE(2)-C cells and IMR-32 neuroblastoma cells. Although scrambled siRNA and TG2 siRNA alone did not show a significant effect on cell morphology, N-Myc siRNA alone induced neurite outgrowth within 72 h of transfection, and neurite formation was more dramatic 48 h later. In contrast, cotransfection of TG2 siRNA blocked N-Myc siRNA-induced neuritic differentiation (Figure 18).

Overall we demonstrated that TG2 is a common target of the N-Myc-mediated repression in neuroblastoma cells and that transcriptional activation of TG2 contributes to HDAC inhibitor-induced cell growth inhibition.

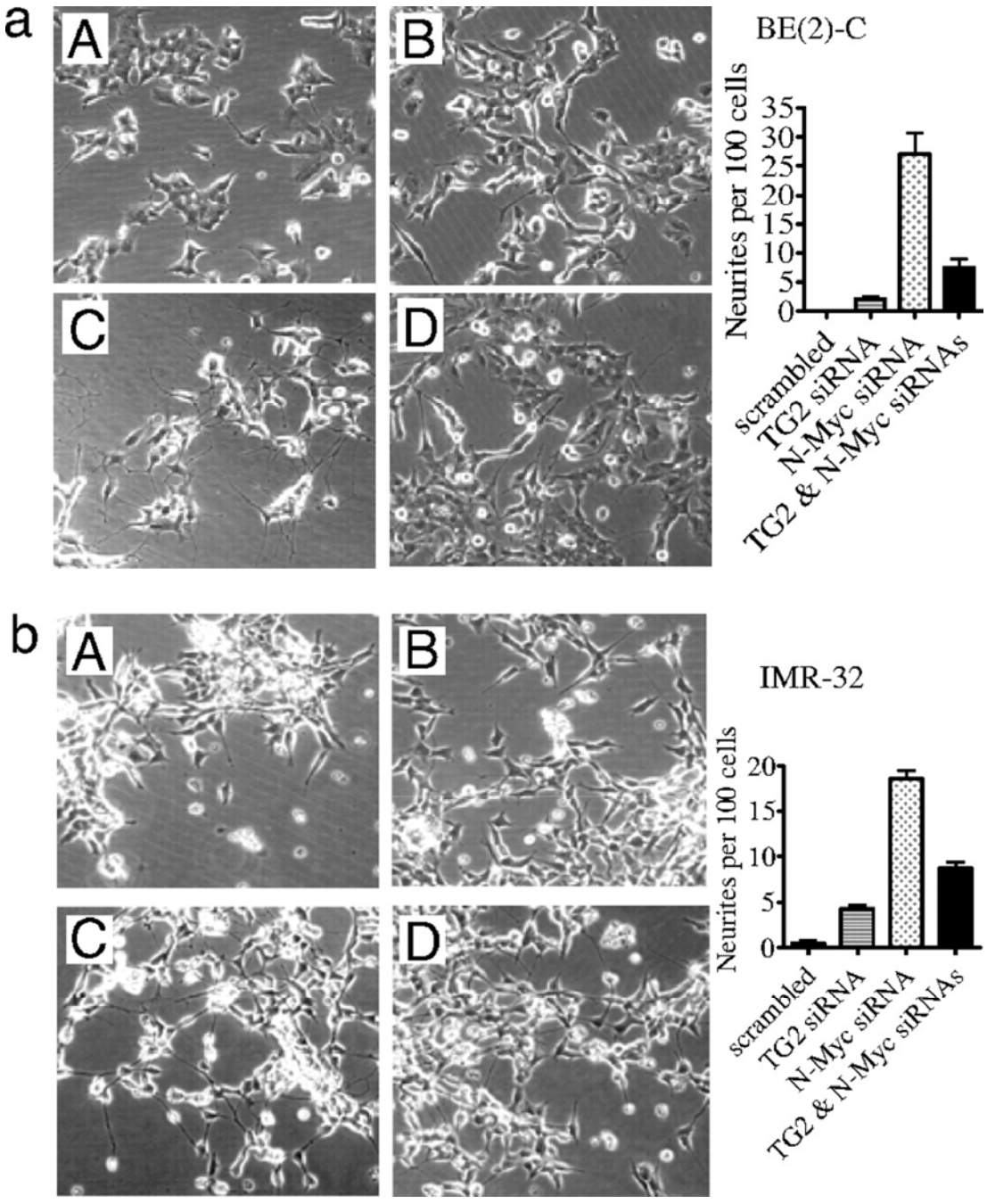

Figure 18. N-Myc blocks neuroblastoma cell differentiation by suppressing TG2 gene transcription. BE(2)-C **(a)** and IMR-32 **(b)** cells were transfected with scrambled control siRNA (A), TG2 siRNA (B), N-Myc siRNA (C), or N-Myc siRNA plus TG2 siRNA (D). Five days after transfection, cell differentiation was assessed by analyzing neurite outgrowth under phase contrast microscopy. Cell images were captured and stored, and neurite outgrowth was quantified. Error bars indicate standard error.

DISCUSSION

N-MYC belongs to the MYC oncogene family characterized by transcription factors that share a conserved BR/HLH/LZ DNA binding motif capable of dimerizing with proteins of the MAX subfamily and binding a specific unmethylated DNA sequence, called E-box [43] [49]. Altered expression and/or amplification of the N-MYC oncogene are often found in human neuroblastoma, one of the most common solid tumours in childhood originating from the sympathetic nervous systems. N-MYC amplification/overexpression has been long proposed as the most critical predictor of neuroblastoma outcome [21] [30], although a few other genetic markers have been identified as important for prognosis such as ploidy status, loss of chromosome 1p [30], expression of ABCC1 [35] and neurotrophin receptors such as TRKA and p75NTR [9].

Surprisingly, neuroblastoma has the highest rate of spontaneous regression or differentiation observed in human cancers. These clinical observations lead to considerable interest in understanding the mechanisms underlying spontaneous regression or differentiation, which in turn may lead to therapeutic approaches to stimulate these phenomena [9].

Lately, genome-wide analyses demonstrate that MYC represses at least as many targets as it activates, further emphasizing the role of repression in MYC function, including transformation [63]. Precisely how Myc switches from being activator to "repressor" is an open question; similarly, the chromatin-modifying events triggered by Myc in gene repression are not so well definite. In contrast to activation, which appears mediated by binding of Myc/Max complexes to E-box elements, several pathways of repression exist, due to the interaction of Myc with different transcription factors. Most of the information in our possession about MYC-mediated repression concern c-MYC, the principal member of the MYC family and, till now, very little is known about N-MYC-mediated repression.

Nonetheless, according to several studies, N-MYC amplification/overexpression correlates with transcription silencing of many genes that may play a key role in the mechanisms underlying tumour regression/differentiation, typical of neuroblastoma. Furthermore, it has been reported recently that overexpression and amplification of the MYCN transgene in mice result in the repression of TRKA and p75NTR, the receptors of NGF that could mediate apoptosis and/or cellular differentiation in neuroblastoma [256].

We have confirmed these findings in several neuroblastoma cellular models for a subset of 5 genes:

- TRKA and p75NTR, mentioned above;
- the ATP-binding cassette transporter gene ABCC3;
- the tissue transglutaminase TG2;
- the cell cycle inhibitor p21, already found repressed by c-Myc, but never by N-Myc.

Moreover we have found that the *core promoter* of each gene in analysis is required for N-Myc-mediated repression.

Taken together these findings suggest that N-Myc may be directly involved in mechanisms of gene transcription repression.

Repression of TRKA and p75NTR neurotrophin receptors

Neuroblastoma is a tumour that retains the genetic program of its ancestor cells. Thus it originates in the neural crest precursors that are committed to differentiate into cells comprising sympathetic ganglia or the adrenal medulla. During normal development of the nervous system, a large number of neurons die via apoptosis. This large-scale cellular suicide may be phenomenologically akin to the spontaneous and quantitative regression of favourable neuroblastoma. This unusual tumour behaviour has led to the hypothesis that neuroblastoma cells may be susceptible to the death signal. Conversely the same characteristic also generate the hypothesis that the apoptotic signalling may be defective in aggressive neuroblastoma.

In this context it has been suggested that an altered function of the signals mediated by neurotrophins/receptors may have a role.

TrkA, the receptor of NGF, is physiologically expressed at the later stages of embryonic development of the sympathoadrenal lineage, and induces differentiation or apoptosis depending on the presence or absence of its ligand, respectively. Recent molecular analyses have suggested that a similar mechanism may be functioning in neuroblastomas with favourable prognosis, of which TRKA expression is an important marker. On the contrary the amplification of N-MYC, typical of aggressive neuroblastoma and inversely correlated with TRKA expression, may be involved

directly in the repression of TrkA, and thus in the arrest of the differentiation process.

The role of p75NTR in neuroblastoma is still less understood; however, its expression in the tumour correlates with good prognosis and with normal N-Myc expression.

As mentioned above, N-Myc-mediated repression is a new field of research and in many ways still unknown. When c-Myc functions as a repressor, it does not bind DNA directly, instead, it associates with gene promoters by docking the promoter-bound Sp1 and/or Miz1 transcription factors, perhaps by interfering with their activation functions or by recruiting other co-repressors like Dnmt3a [157] [290] [291] [181]. However we did not find the TRKA and p75NTR promoters methylated in any of the human neuroblastoma cell lines analysed, suggesting that recruitment of DNA CpG methyltransferase 3a (Dnmt3a) may not be involved in the repression of the neurotrophin receptors.

Sp1 and Miz1 binding sites are indeed present in the *core promoters* of TRKA and p75NTR, and we have showed that both transcriptional factors can mediate upregulation of the endogenous levels of the neurotrophin receptors. These data may provide a mechanistic explanation to the study of Ikegaki and colleagues, who proposed that Miz1 is a new favourable gene in neuroblastoma and found it to correlate with TRKA expression [292].

Importantly, we have also found that N-Myc can interfere with Sp1 and Miz1 transcriptional activity by interacting with both of them, through different domains, at the level of TRKA and p75NTR *core promoters*.

We have achieved these results through diverse experimental approaches.

First, we have set up a new technique, named Dual ChIP, through which we could overcome some limits of normal ChIP, regarding the weak retrieval of protein not directly bound to DNA, such as N-Myc in the repression complex. To improve the formation of covalent links between proteins and stabilize the association of protein complex to DNA, we used Di (N-succinimidyl) glutarate (DSG) cross-linking agent in addition to formaldehyde [284]. Thus we were able to demonstrate that N-Myc with its partner Max, Sp1 and Miz1 are together present, *in vivo*, on TRKA and p75NTR *core promoters*.

Second, we have confirmed the physical interaction of each component of the repression complex in Co-IP assay, both in transiently transfected cells and with endogenous protein extracts from neuroblastoma cells.

Third, through GST pull-down assays, we have demonstrated that MB II transactivation domain and the BR/HLH/LZ domain of N-Myc can interact with Sp1 and Miz1, respectively.

In support of our model, in which N-Myc (with Max), Sp1 and Miz1 take part in a stable ternary repression complex, we have found that the deletion of each interacting domain of N-Myc causes the loss of TRKA and p75NTR repression. Moreover we have shown that N-Myc, Sp1 and Miz1 together are required to repress transcription, since the silencing of each component of the repression complex results in the re-expression of the endogenous levels of the neurotrophin receptors.

Although TrkA has been much studied over the years, and it is well known for its activities in mediating differentiation or apoptosis depending on the presence or absence of its ligand, in recent years the role played by p75NTR has emerged with even greater force.

After many years of study, p75NTR emerges as a unique receptor species, capable of both signalling independently and modifying the binding and signalling capabilities of its coreceptors, members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases [253].

Numerous examples of p75NTR-mediated cell death have been well characterized: what emerges from *in vivo* and *in vitro* studies is that this response is frequently cell-type specific, and restricted to distinct developmental stages or pathologic states [293] [294] [295] [296]. Thus, p75NTR activates a distinct set of signalling pathways within cells that are in some instances synergistic and in other instances antagonistic to those activated by Trk receptors. Several of these are proapoptotic but are suppressed by TrkA receptor-initiated signalling. p75NTR also influences the conformations of TrkA receptors: presence of p75NTR enhances the specificity of TrkA for NGF. Although TrkA receptor suppress p75NTR-mediated signalling, it is not always completely efficient at preventing p75NTR-mediated apoptosis. NGF, for example, increases apoptosis of cultured motor neurons from wild-type, but not from p75NTR-/- embryos [254].

Interestingly we have found that human neuroblastoma cells undergoes to apoptosis after re-expression of TrkA and p75NTR, mediated by silencing of both N-Myc and

Sp1 and Miz1, only upon treatment with NGF. A point to be stressed is that the induced levels of p75NTR have been shown to be slightly larger than those of TrkA, suggesting that p75NTR signalling might have the upper hand on TrkA.

However, still much remains to be clarified on the complexity of the signals mediated by TrkA and p75NTR in neuroblastoma tumours. Indeed, in a recent study, also TrkA expression has been shown to mediate apoptosis in neuroblastoma cells [297].

Finally, these findings indicate that transcription repression mediated by N-Myc/Sp1/Miz1 (plus Max) complex may play a pivotal role in promoting tumour progression by inhibiting the proapoptotic functions of TRKA and p75NTR. Thus, the neurotrophin receptors may be eligible as a further possible target for the design of new drugs capable of inducing their expression in the treatment of neuroblastoma.

Repression of ABCC3

Resistance to chemotherapeutic agents is a major obstacle for successful treatment of cancer. The failure of the curative treatment of cancer patients often occurs as a result of intrinsic or acquired drug resistance of the tumours to chemotherapeutic agents. The resistance of tumours occurs not only to a single cytotoxic drug used, but also occurs as a cross-resistance to a whole range of drugs with different structures and cellular targets. This phenomenon is called multiple drug resistance (MDR). Multidrug resistance (MDR) severely limits the effectiveness of chemotherapy in a variety of common malignancies and is responsible for the overall poor efficacy of cancer chemotherapy [298]. Therefore, understanding how chemoresistance develops and eventually how it can be contrasted becomes crucial to fight cancer effectively.

Chemoresistance of cancer cells is in part caused by misregulation of the activity of membrane proteins, named ATP-binding cassette transporters, responsible for the efflux of chemotherapeutic agents in cancer cells [260] [299]. The human genome codes for forty-eight functional ABC transporter genes, which can be grouped into seven subsets (from A to G) based on their degree of sequence homology [300].

Although many high-risk neuroblastoma tumours initially respond to the first cycles of intensive chemotherapy, they frequently become refractory to treatment as the disease progresses. Multidrug resistance in neuroblastoma is particularly apparent in

patients whose tumours exhibit amplification or over-expression of the N-MYC oncogene. N-MYC clearly contributes to the drug resistance phenotype of neuroblastoma and as mentioned above, it represents one of the most powerful indicators of poor outcome in this disease. The N-Myc oncoprotein is associated with increased growth potential and tumorigenicity [301] and appears to act as a transcriptional regulator, perhaps influencing the transcription of critical genes involved in multidrug resistance phenomenon, such as ABC transporter genes.

Establishing how ABC genes are regulated at transcription level and which transcription factors concur to such a control, is crucial to understanding their in physiological as well as in pathological contexts, such as cancer. Although many studies have focused on the transcriptional regulation of the ABCB1 gene, which encodes the P-glycoprotein [302], yet very little is known about the molecular mechanisms underlying transcription of the large family of ABC transporter genes.

In neuroblastoma cells, the ABCC1, ABCC3 and ABCC4 genes were among the most strongly regulated of the ABC transporters. The importance of this regulation is reinforced by the analysis of ABCC subfamily gene expression done by the group of Dr. Michelle Haber (Children's Cancer Institute Australia, Sidney) in primary untreated neuroblastoma tumour samples, revealing that high levels of ABCC1 and ABCC4, but low levels of ABCC3 expression were strongly predictive of poor outcome in patients with this disease. These genes are the only ones in the entire ABCC subfamily to demonstrate prognostic significance in neuroblastoma and also the only members found to be directly regulated by N-Myc. Furthermore, their combined expression stratify neuroblastoma patients into groups having excellent, intermediate or poor outcome, suggesting that this combination represents one of the most powerful independent prognostic markers yet identified for this disease.

In collaboration with Dr. Haber's group we found that N-Myc in neuroblastoma, and c-Myc in other cancer cell types, transcriptionally activate ABCC1 and ABCC4 through direct binding to E-boxes on their promoter. Moreover specific silencing of the ABCC1 or ABCC4 genes led to growth inhibition, increased morphological differentiation and impaired motility of N-MYC-amplified neuroblastoma cells (unpublished data).

In this thesis I focused on the regulation of the ABCC3 transcription by N-Myc. Although a number of reports have associated ABCC3 expression with outcome in certain cancers, there is no evidence linking this transporter with clinical drug

resistance in any malignancy. Now we find that specific over-expression of ABCC3 as well as silencing of the ABCC1 and ABCC4 genes leads to growth inhibition and increased morphological differentiation in neuroblastoma.

Thus, these Myc-dependent changes in ABC protein levels directly affected the malignant behaviour of neuroblastoma cells in vitro and tumour aggressiveness in vivo.

In particular, we provide the first evidence showing that the human ABCC3 gene is a real N-Myc down-stream regulated gene.

ABCC3 promoter contains three Sp1 binding sites, whereas no E-Box is found in close proximity to the transcriptional start site. We tested the binding of N-Myc in the region where Sp1 binding sites are located. Through dual-ChIP, we show that either Sp1 or N-Myc binds ABCC3 *core promoter* region containing multiple Sp1 binding sites. The dual-ChIP data show that, even in this case, Max co-occupies the same ABCC3 promoter region, indicating that not only N-Myc, but the heterodimer N-Myc/Max, may be required for repression.

Thus, we hypothesize that ABCC3 may be silenced during the development of neuroblastoma tumour at the same time with N-MYC amplification or overexpression. Furthermore, non steroidal anti-inflammatory drugs induce ABCC3 expression in colorectal cancer and seem to be involved in the suppression of tumorigenesis [303]. These evidences support the hypothesis that ABCC3 may act as tumour suppressor gene and for this reason it might be necessary to silence its expression during tumour development.

Repression of TG2

As mentioned in the introduction, more than a dozen HDAC inhibitors are currently in clinical trials for the treatment of malignancies of almost all organ origins, and the HDAC inhibitor SAHA is already in clinical use for the treatment of cutaneous lymphoma. In this study, we demonstrated that TG2 is a common transcriptional target of a HDAC inhibitor in neuroblastoma but not in normal nonmalignant cells and that transcriptional activation of TG2 contributes to HDAC inhibitor-induced cell growth inhibition.

TG2 promotes programmed cell death by inducing a proapoptotic conformational change in the BAX protein and activation of the mitochondrial apoptosis pathway [273] [274], which has been defined as one of the main pathways through which HDAC inhibitors exert their cytotoxic effects [288]. However, our results show that up-regulation of TG2 does not contribute to HDAC inhibitor-induced apoptosis.

This study demonstrated that TG2 is commonly repressed by the N-Myc oncoprotein in neuroblastoma cells that the neuritic differentiation of neuroblastoma cells induced by N-Myc siRNA depends on transcriptional activation of TG2. The transamidation activity of TG2 has been confirmed to be essential for the neuroblastoma and leukaemia cell differentiation response to retinoid therapy, and TG2 overexpression alone induces neuritic differentiation in neuroblastoma cells [304] [305] [306]. Therefore, we conclude that suppression of TG2 is essential for the differentiation block in N-Myc overexpressing neuroblastoma cells. Moreover, HDAC inhibitor therapy alone reverses the action of N-Myc on the transcriptional suppression of TG2.

Furthermore our data suggest a general mechanism by which Myc oncoproteins affect the malignant phenotype and highlight the importance of HDAC inhibitors for the treatment of cancer types overexpressing Myc oncoproteins.

In particular, we found that N-Myc can recruit the HDAC1 protein to the TG2 *core promoter* at the Sp1-binding site and that HDAC inhibitor treatment reactivates TG2 gene transcription without affecting N-Myc and Sp1 binding to the Sp1-binding site. This suggests that N-Myc and HDAC1 are contemporaneously bound to Sp1, which is bound to DNA at its consensus binding site, and that recruitment of HDAC1 is essential for N-Myc-induced transcriptional suppression of TG2.

Contrary to neurotrophin receptors and ABCC3, the dual-ChIP analysis shows that Max does not co-occupy the *core promoter* region of TG2.

That Myc can form complexes with proteins outside the Max/Mnt context has been recently shown by Orian and colleagues who have found that, in Drosophila, dmyc can directly interact with the co-repressor Groucho without Max to control neuronal development [183].

The latter result is also consistent with a recent study by Margolis and colleagues who have shown that c-Myc contributes to HIV-1 proviral latency by recruiting Hdac1 to the HIV-1 promoter [307].

Taken together these findings indicate that transcription repression mediated by a Myc/Hdac complex is a much broader phenomenon than expected.
Finally our findings highlight TG2 as a potential drug development target for the treatment of cancers overexpressing Myc oncoproteins, such as neuroblastoma: therapies that augment the expression or function of TG2 may have a synergistic therapeutic effect on cancer when combined with HDAC inhibitors.

Final remarks

Overall our study contributes to highlight the mechanisms that underlie the N-Myc-mediated repression, and how these may contribute to the tumour progression. As shown, N-Myc, through repressed genes, affects important cellular functions, such as apoptosis, growth, differentiation and motility.
Although this thesis has been focused on neuroblastoma, we have obtained evidence demonstrating that c-Myc can also regulate transcription of the same set of ABCC membrane transporter genes as N-Myc, raising the possibility that ABCC1, ABCC3 and ABCC4 may also have prognostic significance in a range of other cancers in which c-Myc dysregulation occurs. Importantly, we also have found that c-Myc can form a repression complex with Hdac1 at the TG2 promoter gene in breast cancer cell lines providing again a more general significance to our original observation.
Taken together these findings support the idea that Myc may participate in distinct repression complexes by interacting specifically with diverse proteins.
In normal condition, one of the principal functions of Myc proteins, together with their partner Max, is to promote the cell cycle progression: thus physiologically Myc expression is tightly regulated and restricted during the G1/S transition. Furthermore Myc proteins have short half-lives (on the order of 20–30 min), whereas Max, on the other hand, is stable and constitutively expressed (Figure 1A). We hypothesize that deregulation of Myc expression could contribute to the neoplastic phenotype through the interaction with novel partner outside the usual E-box context.
When Myc is dysregulated and expressed during the whole cell cycle, the Myc/Max heterodimers may become overabundant and available to bind the *core promoter* of repressed gene and interfere with other transcriptional factors such as Sp1 and Miz1. Moreover, when significantly overexpressed, for example as the result of

amplification in neuroblastoma or in other tumours, Myc may establish interactions even outside the Max context, such as with Dnmt3a and Hdac1 (Figure 1B and C). Interestingly, we found that the same domain of N-Myc involved in the interaction with Hdac1 also interacts with Max, suggesting that, at saturating levels of Max, Myc is available to interact with Hdac1 at lower affinity.

Finally our work, consistent with the latest findings, confirms the centrality and complexity of Myc function in cell fate, whose full comprehension, despite the thousand of studies published in the past 25 years remains a great challenge for all of the field.

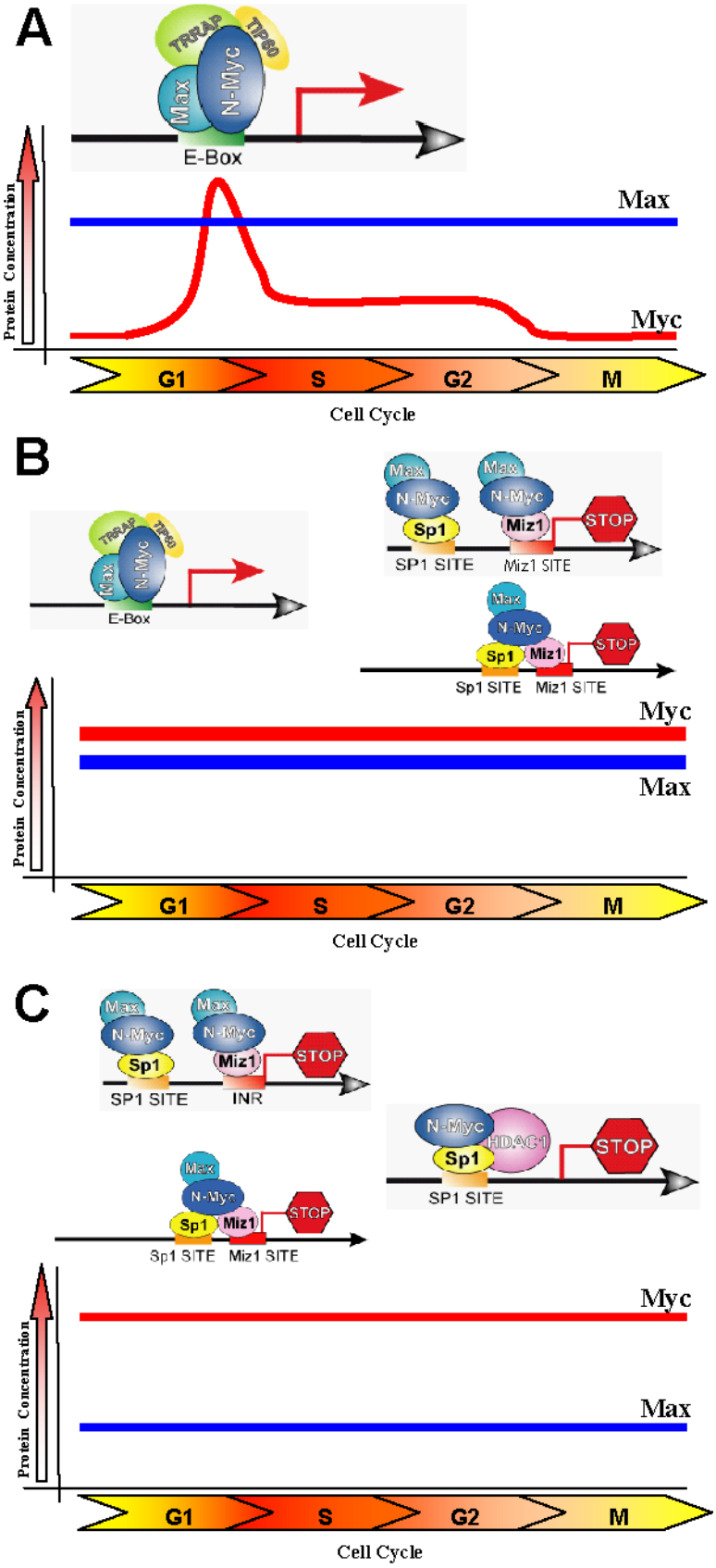

Figure 1. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in physiological **(A)** and pathological **(B-C)** conditions.

MATERIALS AND METHODS

CELL CULTURES

Human neuroblastoma Tet-21N, SHEP and IMR-32 cells were cultured in DMEM containing 10% heat-inactivated FBS and 50 mg/ml gentamycin. Human neuroblastoma SK-N-BE(2)C, SH-SY5Y, LAN-1 and SK-N-SH cells were cultured in RPMI medium 1640 containing 20% FBS and 50 mg/ml gentamycin. Tet-21N cells were treated with tetracycline as described (Lutz et al., 1996; Schuhmacher et al., 2001).

RNA EXTRACTION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Remove the medium and add slowly 1ml of PBS1X. Wash and remove. Harvest the cells using trypsin treatment and when the cells detach from the culture dish, add 1 volume of fresh medium and transfer the sunspension to a tube. Centrifuge for 5 minutes at 1000 rpm, and then remove the supernatant. Add 1-1,5 ml of TriReagent (Sigma). Pipet gently up and down and incubate for 5 minutes at room temperature. Add 300 μl of chloroform and vortex for 10 seconds. Incubate 5-10 minutes at room temperature. Centrifuge fo 5 minutes at 12000rpm at 4°C. Transfer acqueous phase in a new tube and add 750 μl of isopropyl alcohol. Mix gently and incubate for 5-10 minutes at room temperature. Centrifuge at 12000rpm for 10 minutes at 4°C. Remove the supernatant and wash the pellet with 1,5 ml EtOH 75% treated with DEPC and centrifuge at 12000 rpm for 5 minutes at 4°C. Remove the supernatant and dry the pellet. Then, resuspend the pellet in 30-50 μl of DEPC-treated water and heat the sample at 55°C for 10 minutes.

THERMOSCRIPT RT-PCR SYSTEM

The ThermoScript RT-PCR was designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step process. ThermoScript RT, an avian reverse transcriptase with reduced RNase H activity, was engineered to have higher thermal stability, produces higher yields of cDNA, and produce full-length cDNA. cDNA synthesis was performed using total RNA with oligo(dT).

In a 0,2-ml tube, combine primer (oligo(dT)), 2μg total RNA and dNTP 10mM mix, adjusting volume to 12 μl with DEPC-treated water. Denature RNA and primers by incubating at 65°C for 5 min and then place on ice. Vortex the 5X cDNA Synthesis buffer for 5 sec just prior to use. Prepare a master reaction mix on ice, with 5X synthesis buffer, 0,1M DTT, RNaseOUT (40U/ μl), DEPC-treated water and ThermoScript RT (15units/ μl). Vortex this mix gently. Pipet 8 μl of master reaction mix into each reaction tube on ice. Transfer the sample to a thermal cycler preheated to the appropriate cDNA synthesis temperature and incubate for 100 min at 50°C. Terminate the reaction by incubating at 85°C for 5 min. Add 1 μl of RNase H and incubate at 37°C for 20min. Add 80 μl of MQ-water for each reaction and store at -20°C or use for qPCR immediately. Use only 2-5 μl of the cDNA synthesis reaction for qPCR.

SYBR GREEN qPCR

SYBR GreenER qPCR SuperMix (Invitrogen) for ICycler is a ready to use cocktail containing all components, except primers and template, for real-time quantitative PCR (qPCR) on ICycler BioRad real time instruments that support normalization with Fluoresceina Reference Dye at final concentration of 500nM. It combines a chemically modified "hot-start" version of *Taq*DNA polymerase with integrated uracil DNA glycosilase (UDG) carryover prevention technology and a novel fluorescent dye to deliver excellent sensitivity in the quantification of target sequences, with a linear dose response over a wide range of target concentrations. SYBR GreenER qPCR SuperMix for ICycler was supplied at a 2X concentration and contains hot-start *Taq*DNA polymerase, SYBR GrenER fluorescent dye, 1 μM

Fluorescein Reference Dye, $MgCl_2$, dNTPs (with dUTP instead of dTTp), UDG, and stabilizers. The SuperMix formulation can quantify fewer than 10 copies of a target gene, has a broad dynamic range, and is compatible with melting curve analysis. The *Taq*DNA polymerase provided in the SuperMix has been chemically modified to block polymerase activity at ambient temperatures, allowing room-temperature set up and long term storage at 4°C. Activity is restored after a 10-minutes incubation in PCR cycling, providing an automatic hot start for increased sensitivity, specificity and yield. UDG and dUTP in the SuperMix prevent the reamplification of carryover PCR products between reactions. dUTP ensures that any amplified DNA will contain uracil, while UDG removes uracil residues from single or double-stranded DNA. A UDG incubation step before PCR cycling destroys any contaminating dU-containing product from previous reactions. UDG is then inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine target sequences. Fluorescein is included at a final concentration of 500nM to normalize the fluorescent signal on instruments that are compatible with this option. Fluorescein can ajust for non-PCR-related fluctuations in fluorescence between reactions and provides a stable baseline in multiplex reactions. Program real time instrument for PCR reaction as shown following: 50°C for 2 minutes hold (UDG incubation), 95°C for 10 minutes hold (UDG inactivation and DNA polymerase activation), 40 cycles of: 95°C for 15 seconds and 60°C for 60 seconds. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then the unique reaction components (e.g. template, forward and reverse primers at 200nM final concentration). Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate, centrifuge briefly and place reactions in a pre-heated real-time instrument programmed as described above.

ChIP- CHROMATIN IMMUNOPRECIPITATION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Two 100-mm dishes are used for each immunoprecipitation. In the specific case the protocol is intended for human neuroblastoma cells growing adhesively. Minor adjustments have to be introduced for other cell types especially for those growing in suspension. Based on our experience, one of the most critical steps in performing ChIP regards the conditions of chromatin fragmentation, which need to be empirically set up for each cell types employed.

In each plate add 270 µl of formaldehyde from a 37% stock solution and mix immediately. Incubate samples on a platform shaker for 10 minutes at room temperature. In each plate add 500 ml glycine from a 2,5 M stock solution and mix immediately. Incubate on a platform shaker for 10 minutes at room temperature. Transfer the plates in ice and remove the medium. Harvest the cells with a scraper and then centrifuge at 1500 rpm for 4 minutes in cold centrifuge, then keep samples on ice. Remove the supernatant and wash pellet 3 times with 10 ml ice-cold PBS1X/ 1 mM PMSF. After each washing centrifuge at 1500 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 500 µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 600 µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Sonication of crosslinked cells is performed in two distinct steps. First, cells are sonicated with a Branson Sonifier 2 times for 15 seconds at 40% setting. Next, cell samples are further sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and pre-clear lysate by incubating it with 50 µl of Immobilized Protein A [141] for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A [141] and incubate by constant rotation for 30 minutes at

room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer. Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.

Extract once with phenol/chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 100 µl TE buffer to the remaining phenol/chlorophorm fraction and re-extract DNA. Recover the aqueous phase and add it to the previous one. Extract once with chlorophorm/iso-amyl-alcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 1 µl glycogen (Glycogen is 20 mg/ ml stock solution), 10 µg Salmon Sperm, 1/10 volumes Na-acetate 3M pH 5.2, and 2.5 volumes of cold ethanol100% Vortex and precipitate at -80°C for 40 minutes. Centrifuge at 14000 rpm for 30 minutes at 4°C. Remove the supernatant and wash pellet with 200 µl EtOH 70%. Resuspend IP-DNA and INPUT samples in 50-100 µl 10 mM TrisHCl pH 8. Use 2-4 µl of IP-DNA for Real Time PCR analysis.

Cell Lysis Buffer:		**RIPA Buffer**
Washing buffer		
5 mM PIPES pH 8	150mM NaCl	100mM TrisHCl pH 8
85 mM KCl	1% NP40	500mM LiCl
0,5% NP40	0,5% NaDoc	1% NP40
1 mM PMSF	0,1% SDS	1% NaDoc
Protease inhibitor cocktail [308]	50 mM TrisHCl pH 8	
	1 mM PMSF	
	Protese inhibitor cocktail [308]	

DUAL-STEP CHROMATIN IMMUNOPRECIPITATION

The step by step protocol is described for cultured cells grown in two 100-mm dishes, containing 1-1,5 x 107 cells per dish. Two 100-mm dishes are used for each immunoprecipitation. In the specific case the protocol is intended for human neuroblastoma cells growing adhesively. Minor adjustments have to be introduced for other cell types especially for those growing in suspension. Based on our experience, one of the most critical steps in performing ChIP regards the conditions of chromatin fragmentation, which need to be empirically set up for each cell types employed.

Remove medium and add 2 ml PBS 1X/ 1 mM PMSF to each plate and scrape cells at room temperature. Pool together the cells from two plates and centrifuge at 1500 rpm for 5 minutes at room temperature. Wash cell pellet with 20 ml PBS1X/ 1 mM PMSF at room temperature and centrifuge at 1500 rpm for 5 minutes. Repeat this step 3 times. Resuspend pellet in 20 ml PBS1X/ 1 mM PMSF. Add disuccinimidyl glutarate (DSG) to a final concentration of 2mM and mix immediately. DSG is prepared as a 0.5 M stock solution in DMSO. (Note1) Incubate for 45 minutes at room temperature on a rotating wheel at medium speed (8-10 rpm). At the end of fixation, centrifuge the sample at 1500 rpm for 10 minutes at room temperature. Wash cell pellet with 20 ml PBS1X/ 1 mM PMSF at room temperature and centrifuge at 1500 rpm for 5 minutes. Repeat this step 3 times. Resuspend pellet in 20 ml PBS1X/ 1 mM PMSF. Add 540 µl formaldehyde from a 37% stock solution and mix immediately. Incubate samples on a rotating wheel for 15 minutes at room temperature. Add 1 ml glycine from a 2,5 M stock solution and mix immediately. Incubate on a rotating wheel for 10 minutes at room temperature. Centrifuge samples at 1500 rpm for 4 minutes in cold centrifuge, then keep samples on ice. Remove the supernatant and wash pellet 3 times with 10 ml ice-cold PBS1X/ 1 mM PMSF. After each washing centrifuge at 1500 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 500 µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 600 µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Sonication of crosslinked cells is performed in two distinct steps. First, cells are sonicated with a Branson Sonifier 2 times for 30 seconds at 40% setting. Next, cell samples are further

sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. (Note 3) Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and preclear lysate by incubating it with 50 µl of Immobilized Protein A [141] for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A [141] and incubate by constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer. Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.

Extract once with phenol/chlorophorm/isoamylalcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 100 µl TE buffer to the remaining phenol/chlorophorm fraction and re-extract DNA. Recover the aqueous phase and add it to the previous one. Extract once with chlorophorm/iso-amyl-alcohol. Recover the aqueous phase (200 µl) and transfer to a new tube. Add 1 µl glycogen (Glycogen is 20 mg/ ml stock solution), 10 µg Salmon Sperm, 1/10 volumes Na-acetate 3M pH 5.2, and 2.5 volumes of cold ethanol100% Vortex and precipitate at -80°C for 40 minutes. Centrifuge at 14000 rpm for 30 minutes at 4°C. Remove the supernatant and wash pellet with 200 µl EtOH 70%. Resuspend IP-DNA and INPUT samples in 50-100 µl 10 mM TrisHCl pH 8 Use 2-4 µl of IP-DNA for Real Time PCR analysis.

Notes

1).We have tested several crosslinking agents including DSG (disuccinimdyl glutarate), EGS [ethylene glycol bis(succinimidylsuccinate], DMA (dimethyl

adipimidate) and DSS (disuccinidimyl suberate). In our conditions, DSG was the one that worked best, although we also obtained good results with EGS.

2) Sometimes, insoluble aggregates form when DSG is added to cells resuspended in PBS 1X . However, this seems not to preclude the efficiency of the crosslinking reaction.

3) Through this procedure we could efficiently fragment chromatin in a range between 500 and 200 bp. As stated above, this is a critical step that must be empirically set up for each cell line tested. For example, HL-60 cells that grow in suspension, are sonicated with a Branson Sonifier 4 times for 30 seconds at 40% setting and subsequently with the Biogene Bioruptor at a full power for 30 minutes. This procedure allows fragmentation of HL-60 chromatin to a size range of 1000-500 bp.

Cell Lysis Buffer:		**RIPA Buffer**
Washing buffer		
5 mM PIPES pH 8	150mM NaCl	100mM
TrisHCl pH 8		
85 mM KCl	1% NP40	500mM
LiCl		
0,5% NP40	0,5% NaDoc	1% NP40
1 mM PMSF	0,1% SDS	1%
NaDoc		
Protease inhibitor cocktail [308]	50 mM TrisHCl pH 8	
	1 mM PMSF	
	Protese inhibitor cocktail [308]	

LUCIFERASE ASSAY

The Dual-Luciferase® Reporter (DLR.) Assay System (Promega) provides an efficient means of performing dual-reporter assays. In the DLR. Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly

luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. In the DLR. Assay System, both reporters yield linear assays with subattomole sensitivities and no endogenous activity of either reporter in the experimental host cells. Furthermore, the integrated format of the DLR. Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

Note: The LAR II, Stop & Glo® Reagent and samples should be at ambient temperature prior to performing the Dual-Luciferase® Assay. Prior to beginning this protocol, verify that the LAR II and the Stop & Glo® Reagent have been warmed to room temperature.

The assays for firefly luciferase activity and *Renilla* luciferase activity are performed sequentially using one reaction tube. The following protocol is designed for use with a manual luminometer or a luminometer fitted with one reagent injector.
Predispense 100µl of LAR II into the appropriate number of luminometer tubes to complete the desired number of DLR. Assays. Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times. **Do not vortex**. Place the tube in the luminometer and initiate reading.

Note: We do not recommend vortexing the solution at Step 3. Vortexing may coat the sides of the tube with a microfilm of luminescent solution, which can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.
If using a manual luminometer, remove the sample tube from the luminometer, add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.Discard the reaction tube, and proceed to the next DLR. Assay.

CO-IMMUNOPRECIPITATION AND GST PULL-DOWN ASSAYS

The interaction between different proteins is assessed by immunoprecipitation and Western blotting. Cells are washed two times in PBS 1X+ PMSF (0,1%) and lysed in the following buffer for isolation of nuclei: Hepes 10mM, NaCl 50 mM, EDTA 1mM, DTT 1mM, NaPirophosphate 1 mM, NaOrtovanadate 1 mM, Nafluorophosphate 1 mM, PMSF 1 mM, protease inhibitor (Complete, ROCHE). Nuclei are lysed in Tris-Cl pH 7,5 50 mM, NaCl 150 Mm, EDTA 10 mM, DTT 1 mM, protease inhibitors. Nuclear lysate (1 mg) is immunoprecipitated with antibody to HDAC (Upstate), N-Myc, SP1 (Upstate) overnight at 4°C. The day after, specific immunoprecipitated material is incubated with 40µl of slurry-beads protein A, allowing the link between our specific antibody and protein A. The beads with immunocomplexes are washed five times with nuclear lysis buffer + NP40 0,25% and boiled in Laemmli sample buffer for 5 min at 100°C. Eluted proteins are separated by SDS-PAGE and analyzed by Western blot.

For GST pull-down assay HEK293 cells are transfected with pRK7-SP1-HA construct and harvest 48 hrs after transfection. Cell lysates are pre-cleared by incubation with GST-saturated glutathione beads for 1 hr. lysates are incubated with GST-N-Myc 1-88, GST-N-Myc 82-254, GST-N-Myc 249-361 and GST-N-Myc 336-644 for 1 hr at 4°C followed by incubation with glutathione beads for 1hr. bound protein are eluted with sample buffer and subjected to SDS/PAGE and analyzed by Western blot.

IMMUNOBLOTTING ANALYSIS

Western blots were performed according to Invitrogen procedures for NuPAGE Novex 4-12% Bis-Tris Gel Electrophoresis system, using 100 µg of whole-cell extracts.

SOUTHERN BLOT

Southern blotting is the transfer of DNA fragments from an electrophoresis gel to a membrane support. The transfer or a subsequent treatment results in immobilization of the DNA fragments, so the membrane carries a semipermanent reproduction of the banding pattern of the gel. After immobilization, the DNA can be subjected to hybridization analysis, enabling bands with sequence to a labeled probe to be identified. The blotting is performed onto a positive charged nylon membrane with an alkaline buffer. The advantage of this combination is that no post-transfer immobilization step is required, as the positively charged membrane binds DNA irreversibly under alkaline transfer conditions. The method can also be used with neutral nylon membranes but less DNA will be retained. Digest the DNA samples with appropriate restriction enzymes, run in a agarose gel with appropriate DNA size markers, stain with ethidium bromide, and photograph with a ruler laid alongside the gel so that bend positions can later be identified on the membrane. The gel should contain the minimum agarose concentration needed to resolve bands in the area of interest and should be < 7mm thick. The amount of DNA that must be loaded depends on the relative abundance of the target sequence that will subsequently be sought by hybridization probing. Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of 0,25 M HCl. Shake slowly on a platform shaker for 30 min at room temperature. This step results in a partial depurination of the DNA fragments, which in turn leads to strand cleavage. The length reduction improves the transfer of longer molecules.pour off the HCl and rinse the gel with distilled water. Add ~10 vol of 0,4M NaOH into the dish and shake slowly on a platform shaker for 20 min. This is the denaturation step. Set up the transfer via downward capillary transfer in a glass dish filled with enough 0,4M NaOH solution. The transfer pyramid is composed of 2-3 cm of paper towels, Whatman 3MM nylon membrane and gel. An O/N transfer is sufficient for most purposes. Make sure that the reservoir of 0,4M NaOH does not run dry during the transfer. At the end of the transfer remove the paper towels and filter paper and recover the membrane. Rinse the membrane in 2XSSC, place on a sheet of Whatman 3MM filter paper, and allowto air dry. Baking or UV crosslinking is not neede with a positevely charge membrane; in fact UV crosslinking is detrimental. Store the membranes dry between sheets of Whatman 3MM paper for several months at room temperature.

HYBRDIZATION ANALYSIS OF DNA BLOTTED

Heat pre-hybridization buffer at 65°C and equilibrate the membrane in 50ml of this buffer. Incubate on a rotor for 1hr. Then eliminate this solution and incubate with 10ml of pre-hybridization buffer for 3hrs, adding 1mg of placental DNA previously denatured at 100°C for 5 min. Labell the probe. The Megaprime (Biosciences) systems allow DNA from a variety of sources to be labelled *in vitro* to high specific activity with 32P and other radionuclides. Dissolve the probe to be labelled to a concentration of 2,5-25ng/µl in TE buffer. Place the rquired tubes from the Megaprime system, with the exception of the enzyme, at room temperature to thaw. Leave the enzyme at -15°C to -30°C until required, and return immediately after use. Place 25ng of template DNA into a microcentrifuge tube and to it add 5 µl of primers and the appropriate volume of water to give a total volume of 50µl in the final reaction. Denature by heating to 95-100°C for 5 minutes in a boiling water bath. Spin briefly in a microcentrifuge to bring the contents to the bottom of the tube. Keeping the tube at room temperature and add the nucleotides and reaction buffer followed by radiolabelled dNTPs and enzyme. Mix gently by pipetting up and down and cap the tube. Spin for a few seconds in a microcentrifuge to bring the contents to the bottom of the tube. Incubate at 37°C for 10 minutes and then stop the reaction by the addition of 5µl of 0,2M EDTA. Denature the labelled DNA by heating to 100°C for 5 min, then chill on ice. At the end of pre-hybridization remove the buffer and add 10 ml of hybridization buffer with the denatured probe. Incubate O/N at 65°C and then wash 2-3 times the membrane with 50ml of washing buffer at 65°C for 45 minutes for each washing. Place the membrane in a x-ray film cassette with a sheet of autoradiography film on top of themembrane. Close the cassette and expose at -80°C for 1 week.

GENE SILENCING, TRANSFECTION AND CELLULAR ASSAYS

Lipofectamine RNAiMAX reagent (Invitrogen) was used to deliver short interfering RNAs (siRNA) according to the manufacturer's instructions. Stable clones expressing N-Myc were generated by transfection of SH-SY 5Y cells with p3XFLAG-CMV-14-N-MYC, with C-terminal FLAG tag (Sigma) followed by neomycin selection. Stable clones expressing ABCC3 were generated by transfection of SK-N-BE cells with p3XFLAG-CMV-14-ABCC3, with C-terminal FLAG tag (Sigma) followed by neomycin selection.

To quantify neurite outgrowth cells with one or more neuritic extensions of at least twice the length of the cell body, were scored as positive. 100 cells were counted per random field, and at least 5 fields were taken per treatment in each of 3 separate experiments.

Colony forming assays were performed as previously described (Verrills et al., 2006).

For wound closure assays, a pipette tip was used to remove cells from 5 separate areas of the growth substrate. Medium was replaced and the wound areas photographed at regular intervals. Wound size was quantified by averaging six measurements per wound.

For viable cell counts, cells were plated in 6-well plates at a cell density of 100,000 cells per well and counted after five days in culture using trypan blue exclusion method.

BrdU incorporation was measured using a Cell Proliferation ELISA (Roche Diagnostics). Transduced SK-N-BE cells were plated in 96-well plates at a cell density of 15,000 cells per well with 100 ml DMEM medium and cultured for 48h before addition of BrdU for 2h followed by assay according to the manufacturer's protocol.

BIBLIOGRAPHY

1. Brodeur, G.M. and J.M. Maris, *Neuroblastoma.* In: PA Pizzo and DG Poplack, Editors, Principles and practice of pediatric oncology (5th edn.), 2006: p. 933–970.
2. Maris, J.M., et al., *Neuroblastoma.* Lancet, 2007. **369**(9579): p. 2106-20.
3. De Bernardi, B., et al., *Disseminated Neuroblastoma in Children Older Than One Year at Diagnosis: Comparable Results With Three Consecutive High-Dose Protocols Adopted by the Italian Co-Operative Group for Neuroblastoma*
10.1200/JCO.2003.05.191. J Clin Oncol, 2003. **21**(8): p. 1592-1601.
4. Matthay, K.K., et al., *Treatment of High-Risk Neuroblastoma with Intensive Chemotherapy, Radiotherapy, Autologous Bone Marrow Transplantation, and 13-cis-Retinoic Acid*
10.1056/NEJM199910143411601. N Engl J Med, 1999. **341**(16): p. 1165-1173.
5. Hicks, M.J. and B. Mackay, *Comparison of ultrastructural features among neuroblastic tumors: maturation from neuroblastoma to ganglioneuroma.* Ultrastructural Pathology, 1995. **19**: p. 311–322.
6. Peuchmaur, M., et al., *Revision of the International Neuroblastoma Pathology Classification.* Cancer, 2003. **98**(10): p. 2274-2281.
7. Brodeur, G.M., et al., *Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment.* J Clin Oncol, 1993. **11**(8): p. 1466-77.
8. Maris, J.M. and K.K. Matthay, *Molecular Biology of Neuroblastoma.* J Clin Oncol, 1999. **17**(7): p. 2264-.
9. Brodeur, G.M., *Neuroblastoma: biological insights into a clinical enigma.* Nat Rev Cancer, 2003. **3**(3): p. 203-16.
10. D'Angio, G., A. Evans, and C.E. Koop, *SPECIAL PATTERN OF WIDESPREAD NEUROBLASTOMA WITH A FAVOURABLE PROGNOSIS.* The Lancet, 1971. **297**(7708): p. 1046-1049.
11. Maris, J. and G. Brodeur, *Genetics*, in *Neuroblastoma*. 2005. p. 21-26.
12. Taraneh Shojaei-Brosseau, A.C., Anne Abel, Florent de Vathaire, Marie-Anne Raquin, Laurence Brugières, Jean Feunteun, Olivier Hartmann, Catherine Bonaïti-Pellié,, *Genetic epidemiology of neuroblastoma: A study of 426 cases at the Institut Gustave-Roussy in France.* Pediatric Blood & Cancer, 2004. **42**(1): p. 99-105.
13. Knudson, A.G., Jr. and L.C. Strong, *Mutation and cancer: neuroblastoma and pheochromocytoma.* Am J Hum Genet, 1972. **24**(5): p. 514-32.

14. Maris, J.M., et al., *Molecular genetic analysis of familial neuroblastoma.* European Journal of Cancer, 1997. **33**(12): p. 1923-1928.
15. Kushner, B.H., F. Gilbert, and L. Helson, *Familial neuroblastoma. Case reports, literature review, and etiologic considerations.* Cancer, 1986. **57**(9): p. 1887-1893.
16. Kushner, B.H. and L. Helson, *Monozygotic siblings discordant for neuroblastoma: etiologic implications.* J Pediatr, 1985. **107**(3): p. 405-9.
17. Ishola, T.A. and D.H. Chung, *Neuroblastoma.* Surgical Oncology

Pediatric Surgical Oncology, 2007. **16**(3): p. 149-156.

18. Maris, J.M., et al., *Evidence for a Hereditary Neuroblastoma Predisposition Locus at Chromosome 16p12-13.* Cancer Res, 2002. **62**(22): p. 6651-6658.
19. Satgé, D., et al., *Abnormal constitutional karyotypes in patients with neuroblastoma: a report of four new cases and review of 47 others in the literature.* Cancer Genetics and Cytogenetics, 2003. **147**(2): p. 89-98.
20. Mosse, Y., et al., *Identification and high-resolution mapping of a constitutional 11q deletion in an infant with multifocal neuroblastoma.* The Lancet Oncology, 2003. **4**(12): p. 769-771.
21. Schwab, M., et al., *Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour.* Nature, 1983. **305**(5931): p. 245-8.
22. Schwab, M., et al., *Chromosome localization in normal human cells and neuroblastomas of a gene related to c-myc.* Nature, 1984. **308**(5956): p. 288-91.
23. Brodeur, G.M. and C.T. Fong, *Molecular biology and genetics of human neuroblastoma.* Cancer Genet Cytogenet, 1989. **41**(2): p. 153-74.
24. Schneider, S.S., et al., *Isolation and structural analysis of a 1.2-megabase N-myc amplicon from a human neuroblastoma.* Mol Cell Biol, 1992. **12**(12): p. 5563-70.
25. Corvi, R., et al., *MYCN is retained in single copy at chromosome 2 band p23-24 during amplification in human neuroblastoma cells*

Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(12): p. 5523-5527.

26. Reiter, J.L. and G.M. Brodeur, *High-resolution mapping of a 130-kb core region of the MYCN amplicon in neuroblastomas.* Genomics, 1996. **32**(1): p. 97-103.
27. Brodeur, G.M. and R.C. Seeger, *Gene amplification in human neuroblastomas: basic mechanisms and clinical implications.* Cancer Genet Cytogenet, 1986. **19**(1-2): p. 101-11.

28. Brodeur, G.M., et al., *Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients.* Cancer Res, 1987. **47**(16): p. 4248-53.
29. Seeger, R.C., et al., *Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas.* N Engl J Med, 1985. **313**(18): p. 1111-6.
30. Brodeur, G.M., et al., *Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage.* Science, 1984. **224**(4653): p. 1121-4.
31. Cohn, S.L., et al., *Lack of correlation of N-myc gene amplification with prognosis in localized neuroblastoma: a Pediatric Oncology Group study.* Cancer Res, 1995. **55**(4): p. 721-6.
32. Perez, C.A., et al., *Biologic variables in the outcome of stages I and II neuroblastoma treated with surgery as primary therapy: a children's cancer group study.* J Clin Oncol, 2000. **18**(1): p. 18-26.
33. Katzenstein, H.M., et al., *Prognostic significance of age, MYCN oncogene amplification, tumor cell ploidy, and histology in 110 infants with stage D(S) neuroblastoma: the pediatric oncology group experience--a pediatric oncology group study.* J Clin Oncol, 1998. **16**(6): p. 2007-17.
34. Fong, C.T., et al., *Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification*

Proceedings of the National Academy of Sciences of the United States of America, 1989. **86**(10): p. 3753-3757.

35. Norris, M.D., et al., *Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma.* N Engl J Med, 1996. **334**(4): p. 231-8.
36. Bordow, S.B., et al., *Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma.* Cancer Res, 1994. **54**(19): p. 5036-40.
37. Nakagawara, A., et al., *Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma.* N Engl J Med, 1993. **328**(12): p. 847-54.
38. Suzuki, T., et al., *Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas.* J Natl Cancer Inst, 1993. **85**(5): p. 377-84.
39. Kogner, P., et al., *Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis.* Cancer Res, 1993. **53**(9): p. 2044-50.

40. Lutz, W. and M. Schwab, *In vivo regulation of single copy and amplified N-myc in human neuroblastoma cells.* Oncogene, 1997. **15**(3): p. 303-15.
41. Schwab, M., *MYCN in neuronal tumours.* Cancer Lett, 2004. **204**(2): p. 179-87.
42. Hogarty, M.D. and G.M. Brodeur, *Wild-type sequence of MYCN in neuroblastoma cell lines.* Int J Cancer, 1999. **80**(4): p. 630-1.
43. Grandori, C., et al., *The Myc/Max/Mad network and the transcriptional control of cell behavior.* Annu Rev Cell Dev Biol, 2000. **16**: p. 653-99.
44. Blackwood, E.M., B. Luscher, and R.N. Eisenman, *Myc and Max associate in vivo.* Genes Dev, 1992. **6**(1): p. 71-80.
45. Amati, B., et al., *Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max.* Nature, 1992. **359**(6394): p. 423-6.
46. Kretzner, L., E.M. Blackwood, and R.N. Eisenman, *Myc and Max proteins possess distinct transcriptional activities.* Nature, 1992. **359**(6394): p. 426-9.
47. Blackwood, E.M. and R.N. Eisenman, *Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc.* Science, 1991. **251**(4998): p. 1211-7.
48. Prendergast, G.C. and E.B. Ziff, *Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region.* Science, 1991. **251**(4990): p. 186-9.
49. Perini, G., et al., *In vivo transcriptional regulation of N-Myc target genes is controlled by E-box methylation.* Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12117-22.
50. Ayer, D.E., L. Kretzner, and R.N. Eisenman, *Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity.* Cell, 1993. **72**(2): p. 211-22.
51. Hurlin, P.J., C. Queva, and R.N. Eisenman, *Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites.* Genes Dev, 1997. **11**(1): p. 44-58.
52. McArthur, G.A., et al., *The Mad protein family links transcriptional repression to cell differentiation.* Cold Spring Harb Symp Quant Biol, 1998. **63**: p. 423-33.
53. McMahon, S.B., M.A. Wood, and M.D. Cole, *The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc.* Mol Cell Biol, 2000. **20**(2): p. 556-62.
54. Hann, S.R. and R.N. Eisenman, *Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells.* Mol Cell Biol, 1984. **4**(11): p. 2486-97.
55. Berberich, S., et al., *max encodes a sequence-specific DNA-binding protein and is not regulated by serum growth factors.* Oncogene, 1992. **7**(4): p. 775-9.
56. Ayer, D.E. and R.N. Eisenman, *A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation.* Genes Dev, 1993. **7**(11): p. 2110-9.

57. Vennstrom, B., et al., *Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29.* J Virol, 1982. **42**(3): p. 773-9.
58. Cole, M.D., *The myc oncogene: its role in transformation and differentiation.* Annu Rev Genet, 1986. **20**: p. 361-84.
59. Cory, S., *Activation of cellular oncogenes in hemopoietic cells by chromosome translocation.* Adv Cancer Res, 1986. **47**: p. 189-234.
60. DePinho, R.A., N. Schreiber-Agus, and F.W. Alt, *myc family oncogenes in the development of normal and neoplastic cells.* Adv Cancer Res, 1991. **57**: p. 1-46.
61. Magrath, I., *The pathogenesis of Burkitt's lymphoma.* Adv Cancer Res, 1990. **55**: p. 133-270.
62. Nesbit, C.E., J.M. Tersak, and E.V. Prochownik, *MYC oncogenes and human neoplastic disease.* Oncogene, 1999. **18**(19): p. 3004-16.
63. Meyer, N. and L.Z. Penn, *Reflecting on 25 years with MYC.* Nat Rev Cancer, 2008. **8**(12): p. 976-90.
64. Davis, A.C., et al., *A null c-myc mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice.* Genes Dev, 1993. **7**(4): p. 671-82.
65. Stanton, B.R., et al., *Loss of N-myc function results in embryonic lethality and failure of the epithelial component of the embryo to develop.* Genes Dev, 1992. **6**(12A): p. 2235-47.
66. Sawai, S., et al., *Embryonic lethality resulting from disruption of both N-myc alleles in mouse zygotes.* New Biol, 1991. **3**(9): p. 861-9.
67. Moens, C.B., et al., *A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung.* Genes Dev, 1992. **6**(5): p. 691-704.
68. Charron, J., et al., *Embryonic lethality in mice homozygous for a targeted disruption of the N-myc gene.* Genes Dev, 1992. **6**(12A): p. 2248-57.
69. Davis, A. and A. Bradley, *Mutation of N-myc in mice: what does the phenotype tell us?* Bioessays, 1993. **15**(4): p. 273-5.
70. Moens, C.B., et al., *Defects in heart and lung development in compound heterozygotes for two different targeted mutations at the N-myc locus.* Development, 1993. **119**(2): p. 485-99.
71. Sawai, S., et al., *Defects of embryonic organogenesis resulting from targeted disruption of the N-myc gene in the mouse.* Development, 1993. **117**(4): p. 1445-55.
72. Hatton, K.S., et al., *Expression and activity of L-Myc in normal mouse development.* Mol Cell Biol, 1996. **16**(4): p. 1794-804.

73. Roussel, M.F., et al., *Myc rescue of a mutant CSF-1 receptor impaired in mitogenic signalling.* Nature, 1991. **353**(6342): p. 361-3.
74. Facchini, L.M. and L.Z. Penn, *The molecular role of Myc in growth and transformation: recent discoveries lead to new insights.* Faseb J, 1998. **12**(9): p. 633-51.
75. de Alboran, I.M., et al., *Analysis of C-MYC function in normal cells via conditional gene-targeted mutation.* Immunity, 2001. **14**(1): p. 45-55.
76. Kelly, K., et al., *Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor.* Cell, 1983. **35**(3 Pt 2): p. 603-10.
77. Armelin, H.A., et al., *Functional role for c-myc in mitogenic response to platelet-derived growth factor.* Nature, 1984. **310**(5979): p. 655-60.
78. Morrow, M.A., et al., *Interleukin-7 induces N-myc and c-myc expression in normal precursor B lymphocytes.* Genes Dev, 1992. **6**(1): p. 61-70.
79. Spencer, C.A. and M. Groudine, *Control of c-myc regulation in normal and neoplastic cells.* Adv Cancer Res, 1991. **56**: p. 1-48.
80. Rottmann, S. and B. Luscher, *The Mad side of the Max network: antagonizing the function of Myc and more.* Curr Top Microbiol Immunol, 2006. **302**: p. 63-122.
81. Eilers, M. and R.N. Eisenman, *Myc's broad reach.* Genes Dev, 2008. **22**(20): p. 2755-66.
82. Ciechanover, A., et al., *Degradation of nuclear oncoproteins by the ubiquitin system in vitro.* Proc Natl Acad Sci U S A, 1991. **88**(1): p. 139-43.
83. Salghetti, S.E., S.Y. Kim, and W.P. Tansey, *Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc.* Embo J, 1999. **18**(3): p. 717-26.
84. Pelengaris, S., M. Khan, and G.I. Evan, *Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression.* Cell, 2002. **109**(3): p. 321-34.
85. Adhikary, S. and M. Eilers, *Transcriptional regulation and transformation by Myc proteins.* Nat Rev Mol Cell Biol, 2005. **6**(8): p. 635-45.
86. Malynn, B.A., et al., *N-myc can functionally replace c-myc in murine development, cellular growth, and differentiation.* Genes Dev, 2000. **14**(11): p. 1390-9.
87. Boon, K., et al., *N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis.* Embo J, 2001. **20**(6): p. 1383-93.
88. Westermann, F., et al., *Distinct transcriptional MYCN/c-MYC activities are associated with spontaneous regression or malignant progression in neuroblastomas.* Genome Biol, 2008. **9**(10): p. R150.

89. Laurenti, E., et al., *Hematopoietic stem cell function and survival depend on c-Myc and N-Myc activity.* Cell Stem Cell, 2008. **3**(6): p. 611-24.
90. Dildrop, R., et al., *Differential expression of myc-family genes during development: normal and deregulated N-myc expression in transgenic mice.* Curr Top Microbiol Immunol, 1988. **141**: p. 100-9.
91. Rosenbaum, H., et al., *N-myc transgene promotes B lymphoid proliferation, elicits lymphomas and reveals cross-regulation with c-myc.* Embo J, 1989. **8**(3): p. 749-55.
92. Breit, S. and M. Schwab, *Suppression of MYC by high expression of NMYC in human neuroblastoma cells.* J Neurosci Res, 1989. **24**(1): p. 21-8.
93. Grady, E.F., M. Schwab, and W. Rosenau, *Expression of N-myc and c-src during the development of fetal human brain.* Cancer Res, 1987. **47**(11): p. 2931-6.
94. Jakobovits, A., et al., *Expression of N-myc in teratocarcinoma stem cells and mouse embryos.* Nature, 1985. **318**(6042): p. 188-91.
95. Zimmerman, K.A., et al., *Differential expression of myc family genes during murine development.* Nature, 1986. **319**(6056): p. 780-3.
96. Downs, K.M., G.R. Martin, and J.M. Bishop, *Contrasting patterns of myc and N-myc expression during gastrulation of the mouse embryo.* Genes Dev, 1989. **3**(6): p. 860-9.
97. Hirning, U., et al., *A comparative analysis of N-myc and c-myc expression and cellular proliferation in mouse organogenesis.* Mech Dev, 1991. **33**(2): p. 119-25.
98. Strieder, V. and W. Lutz, *Regulation of N-myc expression in development and disease.* Cancer Lett, 2002. **180**(2): p. 107-19.
99. Hatton, B.A., et al., *N-myc Is an Essential Downstream Effector of Shh Signaling during both Normal and Neoplastic Cerebellar Growth*
10.1158/0008-5472.CAN-06-1621. Cancer Res, 2006. **66**(17): p. 8655-8661.
100. Liu, X., et al., *Deregulated Wnt//[beta]-catenin program in high-risk neuroblastomas without MYCN amplification.* 2007. **27**(10): p. 1478-1488.
101. Kenney, A.M., M.D. Cole, and D.H. Rowitch, *Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors.* Development, 2003. **130**(1): p. 15-28.
102. Kohl, N.E., C.E. Gee, and F.W. Alt, *Activated expression of the N-myc gene in human neuroblastomas and related tumors.* Science, 1984. **226**(4680): p. 1335-7.
103. Nau, M.M., et al., *Human small-cell lung cancers show amplification and expression of the N-myc gene.* Proc Natl Acad Sci U S A, 1986. **83**(4): p. 1092-6.

104. Weiss, W.A., et al., *Targeted expression of MYCN causes neuroblastoma in transgenic mice.* Embo J, 1997. **16**(11): p. 2985-95.
105. Brodeur, G.M., *Genetics of embryonal tumours of childhood: retinoblastoma, Wilms' tumour and neuroblastoma.* Cancer Surv, 1995. **25**: p. 67-99.
106. Ramsay, G., et al., *Human proto-oncogene N-myc encodes nuclear proteins that bind DNA.* Mol Cell Biol, 1986. **6**(12): p. 4450-7.
107. Slamon, D.J., et al., *Identification and characterization of the protein encoded by the human N-myc oncogene.* Science, 1986. **232**(4751): p. 768-72.
108. Benvenisty, N., et al., *An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence.* Genes Dev, 1992. **6**(12B): p. 2513-23.
109. Bello-Fernandez, C., G. Packham, and J.L. Cleveland, *The ornithine decarboxylase gene is a transcriptional target of c-Myc.* Proc Natl Acad Sci U S A, 1993. **90**(16): p. 7804-8.
110. Gaubatz, S., A. Meichle, and M. Eilers, *An E-box element localized in the first intron mediates regulation of the prothymosin alpha gene by c-myc.* Mol Cell Biol, 1994. **14**(6): p. 3853-62.
111. Patel, J.H., et al., *Analysis of genomic targets reveals complex functions of MYC.* Nat Rev Cancer, 2004. **4**(7): p. 562-8.
112. McMahon, S.B., et al., *The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins.* Cell, 1998. **94**(3): p. 363-74.
113. Vervoorts, J., et al., *Stimulation of c-MYC transcriptional activity and acetylation by recruitment of the cofactor CBP.* EMBO Rep, 2003. **4**(5): p. 484-90.
114. Lee, K.K. and J.L. Workman, *Histone acetyltransferase complexes: one size doesn't fit all.* Nat Rev Mol Cell Biol, 2007. **8**(4): p. 284-95.
115. Li, B., M. Carey, and J.L. Workman, *The role of chromatin during transcription.* Cell, 2007. **128**(4): p. 707-19.
116. Roth, S.Y., J.M. Denu, and C.D. Allis, *Histone acetyltransferases.* Annu Rev Biochem, 2001. **70**: p. 81-120.
117. Hermeking, H., et al., *Identification of CDK4 as a target of c-MYC.* Proc Natl Acad Sci U S A, 2000. **97**(5): p. 2229-34.
118. Galaktionov, K., X. Chen, and D. Beach, *Cdc25 cell-cycle phosphatase as a target of c-myc.* Nature, 1996. **382**(6591): p. 511-7.
119. Bouchard, C., et al., *Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27.* Embo J, 1999. **18**(19): p. 5321-33.

120. Perez-Roger, I., et al., *Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1).* Embo J, 1999. **18**(19): p. 5310-20.
121. Leone, G., et al., *Myc requires distinct E2F activities to induce S phase and apoptosis.* Mol Cell, 2001. **8**(1): p. 105-13.
122. Knoepfler, P.S., P.F. Cheng, and R.N. Eisenman, *N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation.* Genes Dev, 2002. **16**(20): p. 2699-712.
123. Grandori, C., et al., *c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I.* Nat Cell Biol, 2005. **7**(3): p. 311-8.
124. Arabi, A., et al., *c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription.* Nat Cell Biol, 2005. **7**(3): p. 303-10.
125. Gomez-Roman, N., et al., *Direct activation of RNA polymerase III transcription by c-Myc.* Nature, 2003. **421**(6920): p. 290-4.
126. Poortinga, G., et al., *MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation.* Embo J, 2004. **23**(16): p. 3325-35.
127. Zhang, H., et al., *HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity.* Cancer Cell, 2007. **11**(5): p. 407-20.
128. Morrish, F., et al., *The oncogene c-Myc coordinates regulation of metabolic networks to enable rapid cell cycle entry.* Cell Cycle, 2008. **7**(8): p. 1054-66.
129. Eberhardy, S.R. and P.J. Farnham, *c-Myc mediates activation of the cad promoter via a post-RNA polymerase II recruitment mechanism.* J Biol Chem, 2001. **276**(51): p. 48562-71.
130. Eberhardy, S.R. and P.J. Farnham, *Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter.* J Biol Chem, 2002. **277**(42): p. 40156-62.
131. Bouchard, C., et al., *Myc-induced proliferation and transformation require Akt-mediated phosphorylation of FoxO proteins.* Embo J, 2004. **23**(14): p. 2830-40.
132. Cowling, V.H. and M.D. Cole, *The Myc transactivation domain promotes global phosphorylation of the RNA polymerase II carboxy-terminal domain independently of direct DNA binding.* Mol Cell Biol, 2007. **27**(6): p. 2059-73.
133. Cole, M.D. and V.H. Cowling, *Transcription-independent functions of MYC: regulation of translation and DNA replication.* Nat Rev Mol Cell Biol, 2008. **9**(10): p. 810-5.
134. Felsher, D.W. and J.M. Bishop, *Transient excess of MYC activity can elicit genomic instability and tumorigenesis.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3940-4.

135. Dominguez-Sola, D., et al., *Non-transcriptional control of DNA replication by c-Myc.* Nature, 2007. **448**(7152): p. 445-51.
136. O'Donnell, K.A., et al., *c-Myc-regulated microRNAs modulate E2F1 expression.* Nature, 2005. **435**(7043): p. 839-43.
137. He, L., et al., *A microRNA polycistron as a potential human oncogene.* Nature, 2005. **435**(7043): p. 828-33.
138. Zeller, K.I., et al., *An integrated database of genes responsive to the Myc oncogenic transcription factor: identification of direct genomic targets.* Genome Biol, 2003. **4**(10): p. R69.
139. Fernandez, P.C., et al., *Genomic targets of the human c-Myc protein.* Genes Dev, 2003. **17**(9): p. 1115-29.
140. Li, Z., et al., *A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8164-9.
141. Orian, A., et al., *Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network.* Genes Dev, 2003. **17**(9): p. 1101-14.
142. Cotterman, R., et al., *N-Myc regulates a widespread euchromatic program in the human genome partially independent of its role as a classical transcription factor.* Cancer Res, 2008. **68**(23): p. 9654-62.
143. Secombe, J., et al., *The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth.* Genes Dev, 2007. **21**(5): p. 537-51.
144. Guccione, E., et al., *Myc-binding-site recognition in the human genome is determined by chromatin context.* Nat Cell Biol, 2006. **8**(7): p. 764-70.
145. Li, L.H., et al., *c-Myc represses transcription in vivo by a novel mechanism dependent on the initiator element and Myc box II.* Embo J, 1994. **13**(17): p. 4070-9.
146. Cleveland, J.L., et al., *Negative regulation of c-myc transcription involves myc family proteins.* Oncogene Res, 1988. **3**(4): p. 357-75.
147. Penn, L.J., et al., *Negative autoregulation of c-myc transcription.* Embo J, 1990. **9**(4): p. 1113-21.
148. Okita, K., T. Ichisaka, and S. Yamanaka, *Generation of germline-competent induced pluripotent stem cells.* Nature, 2007. **448**(7151): p. 313-7.
149. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
150. Wernig, M., et al., *In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state.* Nature, 2007. **448**(7151): p. 318-24.

151. Hanna, J., et al., *Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency.* Cell, 2008. **133**(2): p. 250-64.
152. Sridharan, R., et al., *Role of the murine reprogramming factors in the induction of pluripotency.* Cell, 2009. **136**(2): p. 364-77.
153. Tanaka, H., et al., *E2F1 and c-Myc potentiate apoptosis through inhibition of NF-kappaB activity that facilitates MnSOD-mediated ROS elimination.* Mol Cell, 2002. **9**(5): p. 1017-29.
154. Crescenzi, M., D.H. Crouch, and F. Tato, *Transformation by myc prevents fusion but not biochemical differentiation of C2C12 myoblasts: mechanisms of phenotypic correction in mixed culture with normal cells.* J Cell Biol, 1994. **125**(5): p. 1137-45.
155. Lee, T.C., et al., *Myc represses transcription of the growth arrest gene gas1.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 12886-91.
156. Herold, S., et al., *Negative regulation of the mammalian UV response by Myc through association with Miz-1.* Mol Cell, 2002. **10**(3): p. 509-21.
157. Gartel, A.L., et al., *Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4510-5.
158. Claassen, G.F. and S.R. Hann, *A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9498-503.
159. Seoane, J., H.V. Le, and J. Massague, *Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage.* Nature, 2002. **419**(6908): p. 729-34.
160. Wu, S., et al., *Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter.* Oncogene, 2003. **22**(3): p. 351-60.
161. Yang, W., et al., *Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc.* Oncogene, 2001. **20**(14): p. 1688-702.
162. Staller, P., et al., *Repression of p15INK4b expression by Myc through association with Miz-1.* Nat Cell Biol, 2001. **3**(4): p. 392-9.
163. Warner, B.J., et al., *Myc downregulation by transforming growth factor beta required for activation of the p15(Ink4b) G(1) arrest pathway.* Mol Cell Biol, 1999. **19**(9): p. 5913-22.
164. Dauphinot, L., et al., *Analysis of the expression of cell cycle regulators in Ewing cell lines: EWS-FLI-1 modulates p57KIP2and c-Myc expression.* Oncogene, 2001. **20**(25): p. 3258-65.

165. Yang, B.S., J.D. Gilbert, and S.O. Freytag, *Overexpression of Myc suppresses CCAAT transcription factor/nuclear factor 1-dependent promoters in vivo.* Mol Cell Biol, 1993. **13**(5): p. 3093-102.
166. Freytag, S.O. and T.J. Geddes, *Reciprocal regulation of adipogenesis by Myc and C/EBP alpha.* Science, 1992. **256**(5055): p. 379-82.
167. Amundson, S.A., et al., *Myc suppresses induction of the growth arrest genes gadd34, gadd45, and gadd153 by DNA-damaging agents.* Oncogene, 1998. **17**(17): p. 2149-54.
168. Barsyte-Lovejoy, D., D.Y. Mao, and L.Z. Penn, *c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism.* Oncogene, 2004. **23**(19): p. 3481-6.
169. Kime, L. and S.C. Wright, *Mad4 is regulated by a transcriptional repressor complex that contains Miz-1 and c-Myc.* Biochem J, 2003. **370**(Pt 1): p. 291-8.
170. Versteeg, R., et al., *c-myc down-regulates class I HLA expression in human melanomas.* Embo J, 1988. **7**(4): p. 1023-9.
171. Inghirami, G., et al., *Down-regulation of LFA-1 adhesion receptors by C-myc oncogene in human B lymphoblastoid cells.* Science, 1990. **250**(4981): p. 682-6.
172. Coller, H.A., et al., *Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3260-5.
173. Tikhonenko, A.T., D.J. Black, and M.L. Linial, *Viral Myc oncoproteins in infected fibroblasts down-modulate thrombospondin-1, a possible tumor suppressor gene.* J Biol Chem, 1996. **271**(48): p. 30741-7.
174. Wu, K.J., A. Polack, and R. Dalla-Favera, *Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC.* Science, 1999. **283**(5402): p. 676-9.
175. Peukert, K., et al., *An alternative pathway for gene regulation by Myc.* Embo J, 1997. **16**(18): p. 5672-86.
176. Feng, X.H., et al., *Direct interaction of c-Myc with Smad2 and Smad3 to inhibit TGF-beta-mediated induction of the CDK inhibitor p15(Ink4B).* Mol Cell, 2002. **9**(1): p. 133-43.
177. Izumi, H., et al., *Mechanism for the transcriptional repression by c-Myc on PDGF beta-receptor.* J Cell Sci, 2001. **114**(Pt 8): p. 1533-44.
178. Mao, D.Y., et al., *Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression.* Curr Biol, 2003. **13**(10): p. 882-6.
179. Kleine-Kohlbrecher, D., S. Adhikary, and M. Eilers, *Mechanisms of transcriptional repression by Myc.* Curr Top Microbiol Immunol, 2006. **302**: p. 51-62.

180. Adhikary, S., et al., *The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation.* Cell, 2005. **123**(3): p. 409-21.
181. Brenner, C., et al., *Myc represses transcription through recruitment of DNA methyltransferase corepressor.* Embo J, 2005. **24**(2): p. 336-46.
182. Fuks, F., et al., *Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription.* Embo J, 2001. **20**(10): p. 2536-44.
183. Orian, A., et al., *A Myc-Groucho complex integrates EGF and Notch signaling to regulate neural development.* Proc Natl Acad Sci U S A, 2007. **104**(40): p. 15771-6.
184. Zhang, J., et al., *The repression of human differentiation-related gene NDRG2 expression by Myc via Miz-1-dependent interaction with the NDRG2 core promoter.* J Biol Chem, 2006. **281**(51): p. 39159-68.
185. Zhang, J., et al., *Human differentiation-related gene NDRG1 is a Myc downstream-regulated gene that is repressed by Myc on the core promoter region.* Gene, 2008. **417**(1-2): p. 5-12.
186. Brodeur, G.M., et al., *Biology and genetics of human neuroblastomas.* J Pediatr Hematol Oncol, 1997. **19**(2): p. 93-101.
187. Jinbo, T., et al., *Coamplification of the L-myc and N-myc oncogenes in a neuroblastoma cell line.* Jpn J Cancer Res, 1989. **80**(4): p. 299-301.
188. Corvi, R., et al., *Non-syntenic amplification of MDM2 and MYCN in human neuroblastoma.* Oncogene, 1995. **10**(6): p. 1081-6.
189. Roy, N.V., et al., *Identification of two distinct chromosome 12-derived amplification units in neuroblastoma cell line NGP.* Cancer Genetics and Cytogenetics, 1995. **82**(2): p. 151-154.
190. Brinkschmidt, C., et al., *Comparative genomic hybridization (CGH) analysis of neuroblastomas--an important methodological approach in paediatric tumour pathology.* J Pathol, 1997. **181**(4): p. 394-400.
191. Lastowska, M., et al., *Comparative genomic hybridization study of primary neuroblastoma tumors. United Kingdom Children's Cancer Study Group.* Genes Chromosomes Cancer, 1997. **18**(3): p. 162-9.
192. Vandesompele, J., et al., *Genetic heterogeneity of neuroblastoma studied by comparative genomic hybridization.* Genes Chromosomes Cancer, 1998. **23**(2): p. 141-52.
193. Ireland, C.M., *Activated N-ras oncogenes in human neuroblastoma.* Cancer Res, 1989. **49**(20): p. 5530-3.
194. Moley, J.F., et al., *Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers.* Cancer Res, 1991. **51**(6): p. 1596-9.

195. Tanaka, T., et al., *Expression of Ha-ras oncogene products in human neuroblastomas and the significant correlation with a patient's prognosis.* Cancer Res, 1988. **48**(4): p. 1030-4.

196. Vogan, K., et al., *Absence of p53 gene mutations in primary neuroblastomas.* Cancer Res, 1993. **53**(21): p. 5269-73.

197. Hosoi, G., et al., *Low frequency of the p53 gene mutations in neuroblastoma.* Cancer, 1994. **73**(12): p. 3087-93.

198. Keshelava, N., et al., *Loss of p53 function confers high-level multidrug resistance in neuroblastoma cell lines.* Cancer Res, 2001. **61**(16): p. 6185-93.

199. Tweddle, D.A., et al., *Evidence for the development of p53 mutations after cytotoxic therapy in a neuroblastoma cell line.* Cancer Res, 2001. **61**(1): p. 8-13.

200. Moll, U.M., et al., *Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors*

Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(10): p. 4407-4411.

201. Goldman, S.C., et al., *The p53 signal transduction pathway is intact in human neuroblastoma despite cytoplasmic localization.* American Journal of Pathology, 1996. **148**(5): p. 1381-1385.

202. Slack, A., G. Lozano, and J.M. Shohet, *MDM2 as MYCN transcriptional target: Implications for neuroblastoma pathogenesis.* Cancer Letters

Advances in basic and translational neuroblastoma research, 2005. **228**(1-2): p. 21-27.

203. Puisieux, A., S. Valsesia-Wittmann, and S. Ansieau, *A twist for survival and cancer progression.* 2005. **94**(1): p. 13-17.

204. Beltinger, C.P., et al., *No CDKN2 Mutations in Neuroblastomas.* Cancer Res, 1995. **55**(10): p. 2053-2055.

205. The, I., et al., *Neurofibromatosis type 1 gene mutations in neuroblastoma.* Nat Genet, 1993. **3**(1): p. 62-6.

206. Brodeur, G.M., et al., *Cytogenetic features of human neuroblastomas and cell lines.* Cancer Res, 1981. **41**(11 Pt 1): p. 4678-86.

207. White, P.S., et al., *A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3*

Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(12): p. 5520-5524.

208. Martinsson, T., et al., *Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms.* Cancer Res, 1995. **55**(23): p. 5681-6.

209. White, P.S., et al., *Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma.* 2004. **24**(16): p. 2684-2694.

210. Ejeskär, K., et al., *Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours.* Medical and Pediatric Oncology, 2001. **36**(1): p. 61-66.

211. Caron, H., et al., *Chromosome bands 1p35-36 contain two distinct neuroblastoma tumor suppressor loci, one of which is imprinted.* Genes, Chromosomes and Cancer, 2001. **30**(2): p. 168-174.

212. Bauer, A., et al., *Smallest region of overlapping deletion in 1p36 in human neuroblastoma: A 1 Mbp cosmid and PAC contig.* Genes, Chromosomes and Cancer, 2001. **31**(3): p. 228-239.

213. Maris, J.M., et al., *Comprehensive analysis of chromosome 1p deletions in neuroblastoma.* Medical and Pediatric Oncology, 2001. **36**(1): p. 32-36.

214. Plantaz, D., et al., *Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification.* Int J Cancer, 2001. **91**(5): p. 680-6.

215. Guo, C., et al., *Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas.* Oncogene, 1999. **18**(35): p. 4948-57.

216. Attiyeh, E.F., et al., *Chromosome 1p and 11q deletions and outcome in neuroblastoma.* N Engl J Med, 2005. **353**(21): p. 2243-53.

217. Srivatsan, E.S., K.L. Ying, and R.C. Seeger, *Deletion of chromosome 11 and of 14q sequences in neuroblastoma.* Genes Chromosomes Cancer, 1993. **7**(1): p. 32-7.

218. Suzuki, T., et al., *Frequent loss of heterozygosity on chromosome 14q in neuroblastoma.* Cancer Res, 1989. **49**(5): p. 1095-8.

219. Hoshi, M., et al., *Detailed deletion mapping of chromosome band 14q32 in human neuroblastoma defines a 1.1-Mb region of common allelic loss.* Br J Cancer, 2000. **82**(11): p. 1801-7.

220. Thompson, P.M., et al., *Loss of heterozygosity for chromosome 14q in neuroblastoma.* Med Pediatr Oncol, 2001. **36**(1): p. 28-31.

221. Caron, H., *Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma.* Medical and Pediatric Oncology, 1995. **24**(4): p. 215-221.

222. Bown, N., et al., *Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma.* N Engl J Med, 1999. **340**(25): p. 1954-61.

223. Van Roy, N., et al., *Analysis of 1;17 translocation breakpoints in neuroblastoma: implications for mapping of neuroblastoma genes.* European Journal of Cancer, 1997. **33**(12): p. 1974-1978.

224. Schleiermacher, G., et al., *Variety and complexity of chromosome 17 translocations in neuroblastoma.* Genes Chromosomes Cancer, 2004. **39**(2): p. 143-50.

225. Lastowska, M., et al., *Breakpoint position on 17q identifies the most aggressive neuroblastoma tumors.* Genes, Chromosomes and Cancer, 2002. **34**(4): p. 428-436.

226. Islam, A., et al., *High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma.* Oncogene, 2000. **19**(5): p. 617-23.

227. Godfried, M.B., et al., *The N-myc and c-myc downstream pathways include the chromosome 17q genes nm23-H1 and nm23-H2.* Oncogene, 2002. **21**(13): p. 2097-101.

228. Saito-Ohara, F., et al., *PPM1D is a potential target for 17q gain in neuroblastoma.* Cancer Res, 2003. **63**(8): p. 1876-83.

229. Look, A., et al., *Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study.* J Clin Oncol, 1991. **9**(4): p. 581-591.

230. Bowman, L., et al., *Genetic staging of unresectable or metastatic neuroblastoma in infants: a Pediatric Oncology Group study*

10.1093/jnci/89.5.373. J. Natl. Cancer Inst., 1997. **89**(5): p. 373-380.

231. Bagatell, R., et al., *Outcomes of Children With Intermediate-Risk Neuroblastoma After Treatment Stratified by MYCN Status and Tumor Cell Ploidy*

10.1200/JCO.2004.00.2931. J Clin Oncol, 2005. **23**(34): p. 8819-8827.

232. Kaneko, Y., et al., *Different Karyotypic Patterns in Early and Advanced Stage Neuroblastomas.* Cancer Res, 1987. **47**(1): p. 311-318.

233. Nakagawara, A., *Trk receptor tyrosine kinases: a bridge between cancer and neural development.* Cancer Lett, 2001. **169**(2): p. 107-14.

234. Barbacid, M., et al., *The trk family of tyrosine protein kinase receptors.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 1991. **1072**(2-3): p. 115-127.

235. Huang, E.J. and L.F. Reichardt, *Trk receptors: roles in neuronal signal transduction.* Annu Rev Biochem, 2003. **72**: p. 609-42.

236. Birren, S.J., L. Lo, and D.J. Anderson, *Sympathetic neuroblasts undergo a developmental switch in trophic dependence.* Development, 1993. **119**(3): p. 597-610.

237. Nakagawara, A., et al., *Inverse Relationship between trk Expression and N-myc Amplification in Human Neuroblastomas.* Cancer Res, 1992. **52**(5): p. 1364-1368.

238. Tanaka, T., et al., *trk A gene expression in neuroblastoma. The clinical significance of an immunohistochemical study.* Cancer, 1995. **76**(6): p. 1086-95.

239. Nakagawara, A., *The NGF story and neuroblastoma.* Med Pediatr Oncol, 1998. **31**(2): p. 113-5.

240. Nakagawara, A., et al., *Expression and function of TRK-B and BDNF in human neuroblastomas.* Mol Cell Biol, 1994. **14**(1): p. 759-67.

241. Acheson, A., et al., *A BDNF autocrine loop in adult sensory neurons prevents cell death.* Nature, 1995. **374**(6521): p. 450-3.

242. Matsumoto, K., et al., *Expression of Brain-derived Neurotrophic Factor and p145TrkB Affects Survival, Differentiation, and Invasiveness of Human Neuroblastoma Cells.* Cancer Res, 1995. **55**(8): p. 1798-1806.

243. Douma, S., et al., *Suppression of anoikis and induction of metastasis by the neurotrophic receptor TrkB.* Nature, 2004. **430**(7003): p. 1034-9.

244. Ho, R., et al., *Resistance to chemotherapy mediated by TrkB in neuroblastomas.* Cancer Res, 2002. **62**(22): p. 6462-6.

245. Eggert, A., et al., *Expression of neurotrophin receptor TrkA inhibits angiogenesis in neuroblastoma.* Med Pediatr Oncol, 2000. **35**(6): p. 569-72.

246. Yamashiro, D.J., et al., *Expression of TrkC in favorable human neuroblastomas.* Oncogene, 1996. **12**(1): p. 37-41.

247. Ryden, M., et al., *Expression of mRNA for the neurotrophin receptor trkC in neuroblastomas with favourable tumour stage and good prognosis.* Br J Cancer, 1996. **74**(5): p. 773-9.

248. Mahadeo, D., et al., *High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors.* J Biol Chem, 1994. **269**(9): p. 6884-91.

249. Lee, K.F., A.M. Davies, and R. Jaenisch, *p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF.* Development, 1994. **120**(4): p. 1027-33.

250. Bibel, M., E. Hoppe, and Y.A. Barde, *Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR.* Embo J, 1999. **18**(3): p. 616-22.
251. Benedetti, M., A. Levi, and M.V. Chao, *Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness.* Proc Natl Acad Sci U S A, 1993. **90**(16): p. 7859-63.
252. Casaccia-Bonnefil, P., C. Gu, and M.V. Chao, *Neurotrophins in cell survival/death decisions.* Adv Exp Med Biol, 1999. **468**: p. 275-82.
253. Hempstead, B.L., *The many faces of p75NTR.* Curr Opin Neurobiol, 2002. **12**(3): p. 260-7.
254. Wiese, S., et al., *The role of p75NTR in modulating neurotrophin survival effects in developing motoneurons.* Eur J Neurosci, 1999. **11**(5): p. 1668-76.
255. MacPhee, I.J. and P.A. Barker, *Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduces TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain.* J Biol Chem, 1997. **272**(38): p. 23547-51.
256. Hansford, L.M., et al., *Mechanisms of embryonal tumor initiation: distinct roles for MycN expression and MYCN amplification.* Proc Natl Acad Sci U S A, 2004. **101**(34): p. 12664-9.
257. Dean, M., A. Rzhetsky, and R. Allikmets, *The human ATP-binding cassette (ABC) transporter superfamily.* Genome Res, 2001. **11**(7): p. 1156-66.
258. Allikmets, R., et al., *Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database.* Hum Mol Genet, 1996. **5**(10): p. 1649-55.
259. Decottignies, A. and A. Goffeau, *Complete inventory of the yeast ABC proteins.* Nat Genet, 1997. **15**(2): p. 137-45.
260. Higgins, C.F., *ABC transporters: from microorganisms to man.* Annu Rev Cell Biol, 1992. **8**: p. 67-113.
261. Michaelis, S. and C. Berkower, *Sequence comparison of yeast ATP-binding cassette proteins.* Cold Spring Harb Symp Quant Biol, 1995. **60**: p. 291-307.
262. Goldstein, L.J., et al., *Expression of the multidrug resistance, MDR1, gene in neuroblastomas.* J Clin Oncol, 1990. **8**(1): p. 128-36.
263. Chan, H.S., et al., *P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma.* N Engl J Med, 1991. **325**(23): p. 1608-14.
264. Juliano, R.L. and V. Ling, *A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants.* Biochim Biophys Acta, 1976. **455**(1): p. 152-62.
265. Cole, S.P., et al., *Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.* Science, 1992. **258**(5088): p. 1650-4.

266. Deeley, R.G., C. Westlake, and S.P. Cole, *Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins.* Physiol Rev, 2006. **86**(3): p. 849-99.
267. Borst, P., et al., *A family of drug transporters: the multidrug resistance-associated proteins.* J Natl Cancer Inst, 2000. **92**(16): p. 1295-302.
268. Paffhausen, T., M. Schwab, and F. Westermann, *Targeted MYCN expression affects cytotoxic potential of chemotherapeutic drugs in neuroblastoma cells.* Cancer Lett, 2006.
269. Gottesman, M.M., T. Fojo, and S.E. Bates, *Multidrug resistance in cancer: role of ATP-dependent transporters.* Nat Rev Cancer, 2002. **2**(1): p. 48-58.
270. Manohar, C.F., et al., *MYCN-mediated regulation of the MRP1 promoter in human neuroblastoma.* Oncogene, 2004. **23**(3): p. 753-62.
271. Norris, M.D., et al., *Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan in vitro.* Mol Cancer Ther, 2005. **4**(4): p. 547-53.
272. Lorand, L. and R.M. Graham, *Transglutaminases: crosslinking enzymes with pleiotropic functions.* Nat Rev Mol Cell Biol, 2003. **4**(2): p. 140-56.
273. Rodolfo, C., et al., *Tissue transglutaminase is a multifunctional BH3-only protein.* J Biol Chem, 2004. **279**(52): p. 54783-92.
274. Piacentini, M., et al., *Transglutaminase overexpression sensitizes neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress.* J Neurochem, 2002. **81**(5): p. 1061-72.
275. Pelengaris, S., et al., *Reversible activation of c-Myc in skin: induction of a complex neoplastic phenotype by a single oncogenic lesion.* Mol Cell, 1999. **3**(5): p. 565-77.
276. Felsher, D.W. and J.M. Bishop, *Reversible tumorigenesis by MYC in hematopoietic lineages.* Mol Cell, 1999. **4**(2): p. 199-207.
277. Kang, J.H., et al., *MYCN silencing induces differentiation and apoptosis in human neuroblastoma cells.* Biochem Biophys Res Commun, 2006. **351**(1): p. 192-7.
278. Nara, K., et al., *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, 2007. **30**(5): p. 1189-96.
279. Santini, V., A. Gozzini, and G. Ferrari, *Histone deacetylase inhibitors: molecular and biological activity as a premise to clinical application.* Curr Drug Metab, 2007. **8**(4): p. 383-93.

280. Rasheed, W.K., R.W. Johnstone, and H.M. Prince, *Histone deacetylase inhibitors in cancer therapy.* Expert Opin Investig Drugs, 2007. **16**(5): p. 659-78.

281. Keshelava, N., et al., *Histone deacetylase 1 gene expression and sensitization of multidrug-resistant neuroblastoma cell lines to cytotoxic agents by depsipeptide.* J Natl Cancer Inst, 2007. **99**(14): p. 1107-19.

282. Furchert, S.E., et al., *Inhibitors of histone deacetylases as potential therapeutic tools for high-risk embryonal tumors of the nervous system of childhood.* Int J Cancer, 2007. **120**(8): p. 1787-94.

283. Lutz, W., et al., *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, 1996. **13**(4): p. 803-12.

284. Porro, A. and G. Perini, *The Epigenome-Network of Excellence.* Epigenetics protocol database. http://www.epigenome-noe.net, 2007.

285. Chiba, T., et al., *Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells.* J Hepatol, 2004. **41**(3): p. 436-45.

286. He, L.Z., et al., *Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia.* J Clin Invest, 2001. **108**(9): p. 1321-30.

287. Gray, S.G., et al., *Microarray profiling of the effects of histone deacetylase inhibitors on gene expression in cancer cell lines.* Int J Oncol, 2004. **24**(4): p. 773-95.

288. Zhang, X.D., et al., *The histone deacetylase inhibitor suberic bishydroxamate regulates the expression of multiple apoptotic mediators and induces mitochondria-dependent apoptosis of melanoma cells.* Mol Cancer Ther, 2004. **3**(4): p. 425-35.

289. Grimmer, M.R. and W.A. Weiss, *Childhood tumors of the nervous system as disorders of normal development.* Curr Opin Pediatr, 2006. **18**(6): p. 634-8.

290. Gartel, A.L. and K. Shchors, *Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes.* Exp Cell Res, 2003. **283**(1): p. 17-21.

291. Wanzel, M., S. Herold, and M. Eilers, *Transcriptional repression by Myc.* Trends Cell Biol, 2003. **13**(3): p. 146-50.

292. Ikegaki, N., et al., *De novo identification of MIZ-1 (ZBTB17) encoding a MYC-interacting zinc-finger protein as a new favorable neuroblastoma gene.* Clin Cancer Res, 2007. **13**(20): p. 6001-9.

293. Miller, F.D. and D.R. Kaplan, *Neurotrophin signalling pathways regulating neuronal apoptosis.* Cell Mol Life Sci, 2001. **58**(8): p. 1045-53.

294. Sofroniew, M.V., C.L. Howe, and W.C. Mobley, *Nerve growth factor signaling, neuroprotection, and neural repair.* Annu Rev Neurosci, 2001. **24**: p. 1217-81.

295. Bibel, M. and Y.A. Barde, *Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system.* Genes Dev, 2000. **14**(23): p. 2919-37.

296. Dechant, G., *Molecular interactions between neurotrophin receptors.* Cell Tissue Res, 2001. **305**(2): p. 229-38.

297. Lavoie, J.F., et al., *TrkA induces apoptosis of neuroblastoma cells and does so via a p53-dependent mechanism.* J Biol Chem, 2005. **280**(32): p. 29199-207.

298. Ozben, T., *Mechanisms and strategies to overcome multiple drug resistance in cancer.* FEBS Lett, 2006. **580**(12): p. 2903-9.

299. Borst, P. and R.O. Elferink, *Mammalian ABC transporters in health and disease.* Annu Rev Biochem, 2002. **71**: p. 537-92.

300. Dean, M. and T. Annilo, *Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates.* Annu Rev Genomics Hum Genet, 2005. **6**: p. 123-42.

301. Bordow, S.B., et al., *Prognostic significance of MYCN oncogene expression in childhood neuroblastoma.* J Clin Oncol, 1998. **16**(10): p. 3286-94.

302. Scotto, K.W., *Transcriptional regulation of ABC drug transporters.* Oncogene, 2003. **22**(47): p. 7496-511.

303. Tatebe, S., F.A. Sinicrope, and M.T. Kuo, *Induction of multidrug resistance proteins MRP1 and MRP3 and gamma-glutamylcysteine synthetase gene expression by nonsteroidal anti-inflammatory drugs in human colon cancer cells.* Biochem Biophys Res Commun, 2002. **290**(5): p. 1427-33.

304. Tucholski, J., M. Lesort, and G.V. Johnson, *Tissue transglutaminase is essential for neurite outgrowth in human neuroblastoma SH-SY5Y cells.* Neuroscience, 2001. **102**(2): p. 481-91.

305. Balajthy, Z., et al., *Tissue-transglutaminase contributes to neutrophil granulocyte differentiation and functions.* Blood, 2006. **108**(6): p. 2045-54.

306. Singh, U.S., et al., *Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells.* J Biol Chem, 2003. **278**(1): p. 391-9.

307. Jiang, G., et al., *c-Myc and Sp1 contribute to proviral latency by recruiting histone deacetylase 1 to the human immunodeficiency virus type 1 promoter.* J Virol, 2007. **81**(20): p. 10914-23.

308. Stern, D.F., et al., *Differential responsiveness of myc- and ras-transfected cells to growth factors: selective stimulation of myc-transfected cells by epidermal growth factor.* Mol Cell Biol, 1986. **6**(3): p. 870-7.