The role of MYCN-mediated transcriptional repression in neuronal physiopathology

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Abstract

MYC is a transcription factor that can activate transcription of several targets by direct binding to their promoters at specific DNA sequences (E-box).

However, recent findings have also shown that it can exert its biological role by repressing transcription of other set of genes. C-MYC can mediate repression on its target genes through interaction with factors bound to promoter regions but not through direct recognition of typical E-Boxes.

In this thesis, we investigated whether MYCN can also repress gene transcription and how this is mechanistically achieved.

Moreover, expression of TRKA, P75NTR and ABCC3 is attenuated in aggressive MYCN-amplified tumors, suggesting a causal link between elevated MYCN activity and transcriptional repression of these three genes.

We found that MYCN is physically associated with gene promoters in vivo in proximity of the transcriptional start sites and this association requires interactions with SP1 and/or MIZ-1 (i.e. TRKA, P75NTR and ABCC3).

Furthermore, we show that this interaction could interfere with SP1 and MIZ-1 activation functions by recruiting co-repressors such as DNMT3a or HDACs (i.e. TRKA and P75NTR).

Studies in vitro suggest that MYCN interacts through distinct domains with SP1, MIZ-1 and HDAC1 supporting the idea that MYCN may form different complexes by interacting with different proteins.

Forced re-expression of endogenous TRKA and P75NTR with exposure to the HDAC inhibitor TSA sensitizes neuroblastoma to NGF-mediated apoptosis, whereas ectopic expression of ABCC3 induces decrease in cell motility without interfering with growth.

Finally, using shRNA whole genome library, we dissected the P75NTR repression trying to identify novel factors inside and/or outside MYCN complex for future therapeutic approaches.

Overall, our results support a model in which MYCN, like c-MYC, can repress gene transcription by direct interaction with SP1 and/or MIZ-1, and provide further lines of evidence on the importance of transcriptional repression induced by Myc in tumor biology.
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Introduction
1. The Nervous System (a quick overview)

The nervous system is known as the more elaborate and fascinating organ system in human. It is composed of a network of very specialized cells that coordinate every action and signals transmission between different parts of an organism. In most of the subjects belonging to the animals kingdom it is divided in two different parts related to anatomical features:

a) **Central nervous system (CNS):** that integrates the information received from all parts of the body and coordinates their activity. It consists in spinal cord, brain and retina.

b) **Peripheral nervous system (PNS):** that is made up of the nerves and ganglia outside of the brain and the spinal cord. Its main function is to connect the CNS to the muscles and organs in the body in both directions. A further division can be done in:

1. **Sensory Nervous System:** that sends information to the CNS collected from internal organs or from external stimuli.

2. **Motor Nervous System:** that carries information from CNS to organs, muscles and glands and can be further subdivided in:

   I. **Somatic Nervous System:** that controls skeletal muscles and sensory organs.

   II. **Autonomic Nervous System:** controls involuntary muscles, such as smooth and cardiac.

![Fig.1. The Human Nervous System](image-url)
1.1 Neurons

The more representative cell types forming the nervous system are the neurons (Fig 2).

A typical neuron possess a cell body (soma), dendrites and axon, moreover it has a particular membrane morphology that reflects its cell-to-cell communication function.

The soma can give rise to numerous dendrites but never to more the one axon. There are different kinds of neuronal classification and all of these are based on different cellular features.

Based on **structure**: 

i) **Unipolar**: dendrite and axon emerging from the same process;  
ii) **Bipolar**: axon and dendrite are located on the opposite side of the soma;  
iii) **Multipolar**: they have more than one dendrites;

Based on **function**: 

i) **Afferent neurons**: also known as sensory neurons that convey the information from tissue and organs to the CNS; 
ii) **Efferent neurons**: involved in the signal transmission process from CNS to periphery;  
iii) **Interneurons**: connect neurons with a specific region of CNS.
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Each neuron can communicate with another through a contact in a process known as synapse in which the synaptic signal (of electrical or chemical nature) usually runs from the axon of one neuron to the soma and/or dendrites of another one.

1.2 Glia Cells

Other types of cells that contribute to form the nervous system are glia cells. These cell have a non-neuronal origin and provide support, nutrition and other several function to nervous system [1]. In the human brain, it is estimated that the total number of glia roughly equals the number of neurons, although the proportions can vary in different brain areas. The most important functions of glial cells consists in supporting neurons and holding them in place; supplying nutrients to neurons; insulating neurons electrically; destroying pathogens and remove dead neurons; and providing guidance cues directing the axons of neurons to their targets. A very important type of glial cell (oligodendrocytes in the central nervous system, and Schwann cells in the peripheral nervous system) generates layers of a fatty substance called myelin that wrap around axons and provide electrical insulation allowing neurons to transmit action potentials much more rapidly and efficiently.

Astrocytes represents the majority of the CNS glia cells, they are the most abundant glia cells in brain and their main function is to bloody supply neurons. Moreover they regulate the external chemical environment of neurons by removing excess ions, and recycling neurotransmitters released during synaptic transmission. The current theory suggests that astrocytes may be the predominant "building blocks" of the blood-brain barrier.

![Glia and neuronal cells](image)
2. Nervous System Cancers

The term nervous system cancer includes a wide range of cancers occurred both in central nervous system and in peripheral nervous system. Nowadays cancers of the brain and nervous system are the second most common type of childhood cancer, after leukemia. It has been estimated that around 23,000 new cases of primary malignant brain and central nervous system (CNS) tumors were diagnosed in the United States in 2011; of those, approximately 3,000 were new cases of childhood primary brain and CNS tumors. (http://www.cbtrus.org/).

Based on these evidences, the malignant tumors can be divided in two main groups:

- **CNS’s Cancers**: There are more than 100 types of primary brain tumors, and about 6% of all brain tumors cannot be assigned an exact type. These types of cancers are considered to be among the most devastating of all cancers due to the brain and spinal cord complexity. The effects can be devastating for the patient when cancer attacks the CNS. It has been found that 20%–40% of all cancers metastasize to the brain [2, 3]. Among the most famous cancers we have:
  - *Tumours of Neuroepithelial Tissue*
    - Astrocytic Tumours
    - Oligodendroglial Tumours
    - Oligoastrocytic Tumours
    - Embryonal Tumours
  - *Tumours of the Meninges*
  - **Metastatic Tumours**: they rise in another place but they can metastasize to the brain.

- **PNS’s Cancers**: Peripheral Neuroblastic tumors, also known as PNT, are so unique that those factors useful in the routine practice of surgical pathology, such as positive surgical margins, tumor necrosis, vascular invasion, and even hematogenous/lymphatic spread, are not necessarily significant indicators of aggressive tumor progression and a poor clinical outcome of the patients. Biologic properties are often more important and critical than those conventional prognostic factors for predicting clinical behavior of the tumor in individual cases [4].
  - *Tumours of Cranial and Paraspinal Nerves*
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- Neurilemmoma/Schwannoma
- Ganglioneuroma, Ganglioneuroblastoma and Neuroblastoma

3. Neuroblastoma (an overview)

Neuroblastoma is a malignant disease described for the first time in 1864 from a German pathologist R.L.K Virchow. He noted nodular distension of the suprarenal gland with more than one swelling that arose from the adrenal medulla. But only in 1910 J.H Wright uses for the first time the term Neuroblastoma while he was studying the migration of primitive nerve cells during the embryogenesis and he observed a development of tumors of similar appearance in different sites within the body [5]. Nowadays neuroblastoma (NB) is the most common extra cranial solid tumor 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 pediatric 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 and about 1300 in Europe. 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 [6]. Neuroblastoma originates from neural crest that is an embryonic structure formed between the third and the fourth week of human embryonic development. During the development the neural crest cells migrate to many specific regions to form a variety of structure including the sympathetic nervous system (SNS). In vitro studies have shown that growth factors play a crucial role in lineage determination in neural crest stem cells, in fact, a way to obtaining primary culture from of immature neurons and neuroendocrine cells from rat is to grow the cells in presence of Nerve Growth Factor (NGF) and low levels environment of glucocorticoids [7]. By analyzing the transcriptional profiles of neuroblastoma cells it becomes evident that they share the expression of a set of genes within the SNS cells and as for many tumors neuroblastoma has low expression of some specific lineage markers. One also frequently finds expression of genes or antigens normally linked to migrating neural crest cells as MYCN, progenitor cells of other lineage as C-KIT and NEUROD, or early sympatho-adrenal progenitor cells as MYCN; HASH-1;
dHAND [8-10]. It is still an open question whether the expression of these genes reflect crest cell characteristics or instead features of an early progenitor stage. Most primary tumors (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla gland. Other common sites include the neck, chest, and pelvis. The disease is remarkable for its broad spectrum of clinical behavior where signs and symptoms are highly variable and dependent on site of primary tumor as well as the presence or absence of metastatic disease [6, 11]. Even if a substantial improvement in outcome of certain well-defined subsets of patients has been obtained during the past few years, the outcome for children with a high-risk clinical phenotype has improved only modestly and the long-term survival is still less than 40% [12, 13].

![Image](image.png)

**Fig.5.** Onset sites of Neuroblastoma.

Based on a histological point of view neuroblastoma can be classified in:

- **Immature:** they are the larger population of small neuroblasts, characterized from a high rate of undifferentiating form and little cytoplasm (neuroblastoma, malignant).
- **Partially mature:** consisting of ganglion cells capable of to metastasize.
- **Mature:** ganglion cells organized in cluster and surrounded by a stroma of Schwann cells (ganglioneuroma, benign).
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Fig. 6. 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 [11].

Nowadays the general accepted method in neuroblastoma staging is *International Neuroblastoma Staging System* (shown in Table 1) that divide the pathology in several stage 1, 2A, 2B, 3, 4 and 4s each one reflecting a different dissemination status of the tumors [14]. However, these clinical features are imperfect predictors of tumor behavior, so further prognostic markers are needed. Advances in understanding of neuroblastoma came from cytogenetic 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 [11, 15].

A peculiarity of neuroblastoma tumors is that in some cases they spontaneously regress. This phenomena was described for the first time by D’Angio and colleagues in 1971 [16]. They described the uncommon behavior of 4s stage in which infants although showed a small localized primary tumors with metastasis in several organs like liver, skin or bone marrow they always completely regressed. 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%. This is the reason why
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probably the number of spontaneous regression rate, due to asymptomatic neuroblastomas, could be probably much higher [15].

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

Table 1. International Neuroblastoma Staging System

3.1 Genetics abnormalities in Neuroblastoma

Subsets of patients show a genetic predisposition to develop neuroblastoma, and this predisposition follows an AUTOSOMAL-DOMINANT pattern of inheritance. Literature data report that roughly 22% of all neuroblastoma could be the result of a germinal mutation [17]. This hypothesis is reinforced by studies showing that the median age at diagnosis of patients with familial neuroblastoma is reduced from 18 months to 9 months [15]. Although some patients have a predisposition
to the disease, most neuroblastomas occur sporadically. It has been found that gain of alleles, activation of oncogene, loss of alleles and/or change in cell ploidy are important steps in neuroblastoma development.

- **DNA content:** In spite of the fact that most tumors have karyotypes in the diploid range, tumors from patients with low stages are often hyperploid [18, 19]. Unfortunately, this aspect is not easy to assess since cells karyotyping assays are most of the time unsuccessful.

- **Amplification of loci:** the most important and significant amplification that occur in neuroblastoma involves MYCN gene's locus at 2p24 but I'll discuss later about that and its relevance in this type of cancer. Other amplifications include 2p22, 2p13, 12q13 (MDM2 gene), and 1p32 (MYCL gene) [20-23]. However, no neuroblastoma has been shown to amplify another gene that did not also amplify MYCN. Other abnormalities can occur at 4q, 6p, 7q, 11q and 18q [24-26].

- **Trisomy of 17q:** it is one of the most common abnormalities in neuroblastomas. It has been registered in 50% of cases [27, 28]. The gene (or genes) mapping in this chromosome regions responsible for the selective advantage is (are) unknown, though they may most likely have been proposed genes with anti-apoptotic function with consequences on surviving rate [29].

- **HRAS oncogene activation:** Activating mutations of RAS proto-oncogene are rare in neuroblastoma [30, 31]. However, there are some lines of evidence that high expression levels of HRAS in neuroblastoma can correlate with a lower stage disease and good prognosis [32]. Activation of RAS proteins may result from activation of tyrosine kinase receptor (TRK receptors such as TRKA).

- **Chromosomal deletions or allelic low:** Deletions of some chromosomes are common in neuroblastoma disease and generally correlate with different stages of aggressivity and prognosis. For instance, deletion of 1p occurs in 35% of cases and is associated with MYCN amplification [33-35]. It is a good marker for the cancer progression but not for a valuation of survival. Three regions of 1p have been found involved by a different research groups (see Fig 7).

On the other hand, deletions in 11q and 14q counter correlate with 1p and MYCN status [36]. Notably 11q LOH was associated with event-free survival but only in patients that lack MYCN amplification. Presumably the cause of this is that a very few amount of tumors have 11q loss and MYCN amplification and when the two abnormalities are concomitant the prognostic impact of MYCN is dominant.
Fig. 7. Loss of heterozigosity of chromosome 1p. This genetic map shows the different regions identified by Brodeur [37], Schawb [38], Hyashi [39], Nakagawara [40], Martinsson [35] [41] and Versteeg [40].

- **Specific tumor-suppressor genes:** the TP53 gene, which encodes the P53, is one of the most commonly mutated genes in human tumors. P53 is a key regulator of cell cycle and its inactivation can contribute to tumor progression. The role of this gene in neuroblastoma is still controversial. In fact TP53 is rarely mutated in primary neuroblastoma [42, 43]. Recent evidences show that TP53 gene might be more often mutated in cell lines that are derived from patients relapse that in primary tumors [44, 45]. CDKN2A (also known as INK4A or p16) has been found deleted or mutated in several neuroblastomas. Is well known that CDKN2A plays an important role in cell cycle control. As well as CDKN2A also NF1 (a negative regulator of Ras signal pathway) has been found altered but both these gene alterations seem to be uncommon in primary tumors [46, 47].

- **ABCC subfamily:** Although the prognostic value of the ATP-binding cassette, subfamily C (ABCC) transporters in childhood neuroblastoma is usually attributed to their role in cytotoxic drug efflux, certain observations have suggested that these multidrug transporters might contribute to the malignant phenotype independent of cytotoxic drug efflux (see below).

### 3.2 MYC oncoproteins and neuroblastoma

The role of MYC oncogene family in the biology of normal and cancer cells has been intensively studied since 1980s. This family (comprising c-MYC; MYCN and
MYCL) is one of the most studied groups of proteins in biology. The deregulation on Myc genes is involved in a wide range of cancer types. They normally respond to different kind of signals driving cells proliferation, growth, apoptosis, metabolism, cell-size control, genome integrity and differentiation. The Myc family members share a good degree of homology (see Fig 8) but they are characterized from a slightly different expression pattern.

![Fig.8](image_url) The three MYC proteins (c-MYC, MYCN and MYCL). The N terminus of Myc contains the transactivation domain and the C terminus contains the DNA-binding domain. The MYC boxes I, II, III and IV are indicated in red. The basic helix-loop-helix/Leucine zipper (b/HLH/LZ) domain is indicated in green. MYC box II (MBII) has been shown to have a crucial role in most of the biological activities of Myc.

The expression of c-MYC gene is quite constant during embryonic development and is detected in adult tissues with a high rate of proliferation. MYCN is subjected to a strict temporal and spatial expression pattern as shown by comparison of fetal and adult brain cells [48] and by analyses of fetal mouse tissues during the development [49, 50]. For instance, MYCN expression starts to be detected in murine development at day 7.5 [51] reaches its maximal level at days 9.5-11.5 and then decreases after day 12.5 [50]. MYCN in expressed in several tissues such as heart, limb buds and neural tube [49] and during the organogenesis in lung, liver and stomach [52]. Moreover, MYCN expression is highly dynamic in time as well as in space. At birth time it is expressed in brain, kidney, intestine, lung and heart but it becomes downregulated after several days or weeks, depending on the specific tissue taken in consideration. Studies in 2002 have been demonstrated that its expression is essential for a normal development since its inactivation leads to a large set of defects [53]. This intricate expression pattern reflects a severe control mechanism that is achieved by converge of several tissue-specific, stage-specific and signals on MYCN promoter elements most likely different from those found in the c-MYC promoter.
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elements. Indeed, several data shown that MYCN is an exclusive and essential downstream effector of Shh signaling during cerebellar growth whereas c-MYC is required for Wnt/β-catenin pathways [54, 55]. Generally increase of Myc levels occurs through both transcriptional and post-transcriptional mechanisms and appears to be an immediate early response (about 2 hours) to most mitogenic factors [56]. On the contrary, anti-proliferative signals trigger rapid downregulation of Myc expression [57].

3.2.1 Myc/Max/Mad transcription factor network
As mentioned before, Myc oncoproteins contain both leucine zipper and helix-loop-helix motifs [58, 59]. These proteins motifs are documented in sequence-specific DNA binding protein, thus Myc can bind the DNA. However this evidence was nothing until the identification of Max in 1991 [60]. Max is a small protein that can homodimerize and binds the DNA and the binding function of the Max-Max dimer is inhibited by phosphorylation [61]. Max can also form heterodimer with Myc family proteins and this heterodimers are not sensitive to any phosphorylation as seen previously for Max-Max dimer [61]. On the other hand, no stable Myc homodimers have been found present in vivo. Max can also dimerize with an other set of protein such as Mad1, Mad2 (Mx1), Mad3, Mad4 and Mnt or better know as Mad family members. In fact, has been shown that they behave more or less like Myc [62].

Fig.9. Max-interacting proteins. Max forms heterodimers with members of the Myc and Mad protein families as well as with the Mnt and Mga proteins. Each of these proteins interacts with Max through its BR/HLH/LZ domain.
Myc/Max dimer can bind the DNA on specific sequences also known as E-Box (CANNTG, the most popular is CACGTG) with a weak transcriptional activity (from 2 to 4 fold) [60, 63, 64]. In contrast, the Mad/Max heterodimer acts a transcriptional repressor at the same binding sites [65-67].

The ability in transcriptional modulation of the dimers derives from specific domains that they can interact with a set of co-activators or co-repressors in order to form different complexes. For instance, the transcriptional activation of Myc is mediated by the recruitment of histone acetyltransferases (HATs) through the interaction between the Myc MBII motif and TRRAP (transactivation/transformation associated protein), whereas Mad/Max acts as transcriptional repressor by recruiting HDACs (histone deacetylase proteins) through the adaptor protein SIN-3 [68, 69].
3.2.2 MYCN and Neuroblastoma

In 1983 Schwab and colleagues identified a Myc-related oncogene (MYCN) located on the distal arm of chromosome 2 encoding a phosphoprotein (MYCN) of molecular weight of 65/67 KDa localized in the nucleus and capable to bind the DNA on a hexameric sequence (see above) [70, 71]. Surprisingly a large region from this site becomes amplified in some neuroblastoma cases and the MYCN locus is copied to form an extrachromosomal circular element or DMs (double-minute chromatin bodies) or a homogenously staining region (HSR) with retention of wild type copy [72].

Evidence suggests that all the copies derived from amplification are transcriptionally active [73, 74] and this leads to an increase on MYCN mRNA levels form 50 to 100 fold thus generating high endogenous levels of MYCN protein. Amplification and overexpression of MYCN is generally associated with advanced stages in neuroblastoma and poor prognosis, in fact cells with high levels of MYCN are characterized by a rapid tumor progression and low level of differentiation even in infants and patients with low stage of disease [75, 76]. Furthermore, studies show a strong correlation between MYCN amplification, and deletions on chromosome 1 and 17q gain.

It has been demonstrated that MYCN is amplified in 30% of advanced neuroblastoma cases and 22% of total cases display a number of copies form 50 to 100 times. Overall, these results make MYCN amplification as one of the most significant prognostic factors for neuroblastoma outcome (see Table.2 and Fig 13).
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| Table 2: Correlation of MYCN amplification and stage in neuroblastomas |
|-----------------------------|---------------------|-------------------|
| Stage at diagnosis          | MYCN amplification  | 3-year survival   |
| Benign ganglioneuromas      | 0/64 (0%)           | 100%              |
| Low stages (1,2)            | 31/772 (4%)         | 90%               |
| Stage 4S                    | 15/190 (8%)         | 80%               |
| Advanced stages (3,4)       | 612/1,974 (31%)     | 30%               |
| TOTAL                       | 658/3,000 (22%)     | 50%               |

Table 2. Analysis on 3000 neuroblastoma patients.

Fig.13. Survival of infants with metastatic neuroblastoma based on MYCN status. A Kaplan–Meier survival curve of infants less than 1 year of age with metastatic neuroblastoma.

3.2.3 MYCN as an activator

In yeast and mammalian MYCN transfected cells the exogenous MYCN overexpression is sufficient to activate several synthetic reporters containing proximal E-Box [65, 77]. Furthermore, MYCN regulates natural E-box containing promoters or sequences derived from a putative Myc target genes [64, 78-80]. The Myc/Max heterodimer has been seen to have a weak transcription activity (from 2 to 10 fold) both endogenously and in transient assay [77] and these evidences has been confirmed by different microarray experiments in 2004 [81]. These results corroborate the transcriptional role of Myc even if, as mentioned before, its activity is weaker when compared to other transactivators.

Generally, MYCN through its transactivation domains (TAD) can recruit the basal transcription machinery either directly or indirectly. In almost every case, TAD function implicates interactions with specific set of proteins. The dominant model suggests that Myc, when bound to the DNA, increases local histone acetylation.
Indeed MYCN has been found to interact with histone acetyltransferase complexes including TRRAP and either GCN5 (general control of amino-acid synthesis protein 5) or TIP60 which preferentially acetylate histones H3 and H4 respectively [69, 82] (fig 13a). Myc can also bind the p300/CBP (CREB-Binding Protein) acetyltransferases [83], but the effect of this binding is still controversial. In fact a 2003 study showed that CBP could acetylate Myc itself leading to a change in Myc protein ubiquitination and activity [83]. An acetylated chromatin state results in an open DNA that provides more accessible docking sites for acetyl histone binding proteins such as GCN5 and SWI/SNF chromatin remodeling complex with consequential induction of transcription [84, 85]. Nowadays is certain that recruitment of acetyltransferase proteins is the major mechanism of transactivation and is utilized from other transcription factor s as TCF (T-cell factor), E2F, p53 and Gal4 [86]. Most of the Myc target genes are transcribed by RNA polymerase II. Other target genes are CDKA (cyclin dependent kinase 4) [87], CDC25A [88], cyclin D2 [89, 90] and other members of E2F family [91]. Furthermore, Myc has been found to stimulate expression of several genes that are directly involved in cell size and growth or that encode for ribosomal proteins [92], translation factors and metabolic enzymes [93]. This is consistent with the evidence that Myc, through the binding with TRRAP and subsequent acetylation, is present in vivo at both RNA polymerase III and RNA polymerase I dependent genes [92, 94-96].

Myc can also regulate the transcription at the level of transcriptional elongation, in fact is well know that RNA pol II is recruited in proximity of the start sites with its C-terminus tail (CTD) in hypophosphorylated state. Phosphorylation of the CTD occurs during transcription and elongation steps, whereas the CTD must be dephosphorylated to allow RNA pol II to be recycled. Has been shown that Myc, by its TADs domains, can directly interact with the CTD tail of RNA pol II and increase the phosphorylation (see Fig 14b) [97, 98].
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.

Moreover Myc can promote the methylation of 5’mRNA guanidine (cap) that is a general essential step for gene expression. Recent studies show that Myc has an important role also in DNA replication. During the cell cycle, the whole genome needs to be correctly replicated and segregated to the “daughter cells”. Any kind of disruption in this pathway results in cell cycle arrest or at worst in mutation and/or genomic instability [99]. Myc was found to bind to numerous components of the pre-replicative complex and localize to early sites of DNA replication [100, 101]. The last recent studies demonstrated that Myc could also be involved in a polycistronic microRNA regulation making its role in the tumorigenesis even more intriguing [102, 103].

Taken together, these findings reveal an apparent discrepancy between Myc’s dramatic effects on cellular function and its weak transcriptional activation. Recent experiments have been shown that hypothetically, Myc could bind thousands of sites present in the genome (about 15% of the genes) as well as intergenic regions [81, 104-106] thus Myc could regulate a significant portion of all genes in an organism. Certainly, the potentially Myc’s binding sites in vivo are more than the number of Myc molecules in proliferating cells, suggesting that each site is bound only temporarily by Myc (hit and run theory) [57]. In the end, there are several evidences that MYCN may play an important role in the human
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genome organization regulating global cellular euchromatin. MYCN maintains 90-95% of euchromatic histone marks: H3K9 acetylation and methylation and H3K4 modifications in human neuroblastoma with the enhancer like function [107]. The absence of Myc in neuronal stem cells causes nuclear condensation and a spread of heterochromatic portion. Nonetheless, it is intriguing that it can bind intergenic regions even if they are not enriched for the E-Box specific sequence. In these cases it is supposed that Myc can bind the DNA through an indirect association with chromatin. Furthermore, Myc has been shown to possess another interesting feature completely independent from E-Box context; indeed Myc can act as well as transcriptional repressor on a specific set of genes [108].

3.2.4 MYCN as a repressor

For several years it has been observed that high levels of Myc expression in transformed cell lines correlate with down-regulation of specific mRNAs. Indeed, in 1980 a number of studies have been demonstrated the Myc participates in a negative feedback loop [108, 109]. Other indications that Myc might also function as a transcriptional repressor came from a genome-wide analyses where has been shown that Myc can repress at least as many genes as it activates [57]. While, the mechanism by which Myc can promote the transcription of its targets is well understood and established, very little is known about its role in transcription repression. The use of a serial deletion mutants have focused the attention on the importance on Myc Box II (MBII) and BR/HLH/LZ region in both activation and repression activity.
Originally, no DNA consensus sequence for transcriptional repression mediated by Myc has been identified. This reinforced the possibility that this mechanism is simply an indirect consequence of the altered physiological state of a cell that is induced by Myc. Indeed, there are data supporting an indirect mechanism of gene repression by Myc [110, 111]. Furthermore, DNA elements required for the repression mediated by Myc have been demonstrated to be within the promoters of repressed targets genes, thus indicating that Myc-repression occurs at a transcriptional level [108].

The repressed genes belonging to different classes: the first class consists in genes that encode for proteins selectively expressed in quiescent cells or involved in cell proliferation. Among these we have $P21^{\text{Cip1}}$ [112-116],
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$P27^{kip1}$ [117], $P15^{ink4b}$ [115, 118, 119], $P16^{ink4c}$ [93], $P57^{kip2}$ [120], and further the differentiation-inducing protein C/EBP-α [121, 122], the growth-arrest proteins GAS1 and GAS2 [123], the growth-arrest and DNA damage proteins GADD34, GADD45, GADD153 [124] and the Myc-antagonist MAD4 [125]. All the genes in the list point to a role for Myc-mediated gene repression in the control of cellular growth, differentiation and response to DNA damage. It appears clear that the repression of each individual gene could contribute to the phenotype of Myc-transformed cells.

The second class encompasses genes that are involved in cell adhesion and cell surface markers for instance class I HLA molecules in melanoma cells, the $\alpha3\beta1$ integrin in neuroblastoma and the $LFA-1$ cell adhesion protein in the transformed B-cell [62, 126, 127]. In fact, altered cell adhesion is a hallmark of many tumors as well as in the Myc-transformed cells [128].

Finally, genes involved in metabolic pathways such as $H$-ferritin and thrombospondin [129, 130]. Alteration in these gene expressions correlates with angiogenesis.

These results indicate that Myc has a massive combination of functions that, when altered, could increase the replicative potential of the cells and causing tumors.

The Myc-repression mechanism has been better elucidate with the identification of both DNA sequences and specific Myc-binding proteins that are involved in the repression. Recent studies show that not all genes are repressed by Myc through the same mechanism.

Some Myc repressed targets contain a subclass of initiator elements (INRs consensus, YYCAYYYYY, where Y represents a pyrimidine base T/C), which are usually but not exclusively, on TATA less promoters. INRs elements are recognized by TFII-D as well as a number of regulatory proteins like TFII-I, YY1 and the Myc-interacting zinc finger protein1 (MIZ-1). Has been demonstrated that all these three proteins interact with BR/HLH/LZ region of Myc [62]. While there has been a little follow-up on the initial evidences on TFII-I and YY1, the association Miz-1/Myc has been confirmed and shown to promote stabilization of Myc by inhibiting its ubiquitination and degradation [131].

$MIZ-1$ (also known as $ZBTB17$) gene encodes for a protein of 721 aa characterized from a 13 zinc-finger domains (N-terminus) and a BTB/POZ
domain which is a protein/protein interaction domain found in a multiple zinc-finger proteins. Thus, Miz-1 interacts with Myc “outside” the HLH domain, but do not interact with Mad, Max and Mnt [131, 132]. Someone else Myc repressed genes, such as GADD45, do not contain INR sequences and the repression appears to be mediated by a GC-rich regions that are recognized by other factors like WT1 or P53 [124]. Another important GC binding protein that seems to be involved in this repression mechanism is the basal transcription factor 1 (specific protein 1 or SP1). SP1 is a zinc-finger protein of 785 aa involved in many cellular processes including differentiation, growth, apoptosis, responses to DNA damage and chromatin remodeling. It has 2 TADs domains and normally recruits TBP. Furthermore, results show that repression by Myc could occur through the SMAD and NF-Y binding sites due to a direct protein/protein interaction between Myc and these factors [133, 134]. It is clear that several pathways of repression exist (Fig 15).

Finally, the mechanistic model is that Myc/Max heterodimers interacts with transcriptional activators that are bound directly to DNA through enhancer or INR elements and these multi-protein complexes could displace co-activator and recruit co-repressor [135-137]. However, as mentioned before, some genes are repressed by Myc through a mechanism that does not involve Max [114, 138]. In these cases, Myc recruits DNMT3A (DNA methyltransferase) to the Myc/Miz-1 complex on the promoter as show for P21 repression, indicating that the repression could at least partially be mediated by methylation of its target regions [139]. Since DNMT3A is complexed

Fig.15. Myc mediated repression (A) INR dependent (B) INR independent.
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with histone deacetylase proteins, its recruitment might lead to a local histone deacetylation and inhibition of transcription [140].

To date still less is known about MYCN mediated repression. Only few genes have been found repressed by MYCN:

- **NDRG1**: This gene encodes for a cytoplasmic protein involved in stress responses, hormone responses, cell growth, and differentiation [141].
- **NDRG2**: This is a cytoplasmic protein that may play a role in neurite outgrowth [142].
- **TG2**: a protein involved in apoptosis [55].
- **CCNG2**: a cell cycle regulator [143].
- **MKP3**: (also known as DUSP6) is a dual specificity protein phosphatase [144].

This is the reason why this thesis will be focused to the study of the mechanism(s) underlying the pivotal role played by MYCN.

Furthermore, recent findings show that Myc is one of the fundamental factors in the “magic quartet” that can reprogram somatic cells to induced pluripotent stem cells (iPS). Ectopic expression of Myc increases the ability of OCT-4, SOX2 and KLF4 to induce iPS formation form mouse and human cells of 10 fold [145-148].

Taken together these results show that both activation and repression are maybe required for Myc biological functions and reveal another level of complexity hidden behind “the Myc family”.

3.3 Expression and function of Trk and p75\(^\text{NTR}\) in Neuroblastoma

The factors that are responsible for regulating the malignant transformation of sympathetic neuroblasts to neuroblastoma cells are not completely well

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Fig.16. Schematic models of MYCN-mediated repression on TG2 (A), CCNG2 (B) and MKP3 (C) genes.
understood, but they probably involve one or more neurotrophin-receptor pathways that signal the cell to differentiate or undergo to apoptosis.

The TRK (NTRK) family of neurotrophin receptors plays an important role in development and maintenance of the central and peripheral nervous system. This family consists of TRKA (NTRK1), TRKB (NTRK2) and TRKC (NTRK3).

The principal ligands for these receptors are: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4-5). All the TRK receptors do not show the same affinity for all the ligands mentioned before (Fig 17). TRKA is high-affinity receptors for NGF, TRKB for BDNF, NT3 and NT4-5 while TRKC has a good binding affinity with NT3. All the neurotrophins bind also with lower affinity to another receptor known as P75NTR (NGFR).

In normal sympathetic ganglia, most of the mature neurons at the perinatal stages express TRKA at high levels as a result of a “switching” of expression from TRKB and TRKC [149].

A massive physiological apoptosis occurs after the expression of TRKA and entering in G1 cell cycle arrest. Knockout mice for TRKA, TRKB or TRKC display an overlapping spectrum of abnormalities in central and in the peripheral nervous system [150-152].

TRK was discovered as an oncogene fused with tropomyosin gene in the extracellular domain [153] from which its name is tropomyosin related kinase (TRK).
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Comparison of the sequences of TRK family members to those other transmembrane tyrosine kinase indicated that they are a completely novel family of cellular surface proteins.

They are characterized from:

- An extracellular domain of 50-80 KDa with the neurotrophin binding site
- A transmembrane domain of 25 a.a
- A cytoplasmic domain of 130-150 KDa recognized by ATP and with tyrosine-kinase activity
- A portion with numerous tyrosines that are phosphorylated for the activation of the receptors.

The bond of the ligand to the receptors induces a structural change in the extracellular portion, causing the dimerization and activating the cytoplasmic kinase function for the cross-phosphorylation.

TRKA is a transmembrane receptor that acts as a homodimer (Fig 18). Explanted neuroblastoma cells with high level of TRKA differentiate when expose to NGF or undergo apoptosis in absence of NGF [154]. The NGF/TRKA network could provoke differentiation or regression in good prognosis neuroblastomas depending on the particular microenvironment.
Fig.19. Signal-transduction pathway of the TRKA tyrosine kinase receptor. Binding of nerve growth factor (NGF) leads to TRKA auto-phosphorylation and activation of various signalling cascades. Proteins interact directly with the TRK intracellular domain are SHC, PLCγ1, SH2B and IAPs. 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 phosphatidylinositol 3-kinase (PI3K).

In neuroblastoma TRKA is expressed in tumors with favorable outcome that often showed spontaneous regression. Such tumors usually affect patients under one year of age, with low stage and their DNA is aneuploid. Furthermore, TRKA expression is strongly downregulated in aggressive neuroblastomas that usually have MYCN oncogene over-expression and loss of the region 1p36. The combination of TRKA expression and MYCN amplification provide even greater prognostic power [15, 154-158] (Fig 20).

Fig.20. 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 MYCN (B).
In contrast to TRKA, TRKB is preferentially expressed in aggressive neuroblastoma, especially those with MYCN amplified [159]. It is physiologically expressed in normal sympathetic neurons at an early stage compared to TRKA. In the MYCN amplified tumors may suggest that the arrest of differentiation has occurred before TRKA expression. Oddly, has been found a truncated form of TRKB lacking the catalytic tyrosine kinase domain in favorable tumors.

TRKC was found expressed in low stage neuroblastoma, and like TRKA, it is not expressed in tumors carrying MYCN amplification [160, 161].

P75NTR, also known as NGFR, was cloned for the first time in 1986. It is able to activate a distinct set of signaling pathways within cells that can be synergistic or antagonistic to those activated by TRK receptors. Most of this pathways are pro-apoptotic indeed it belongs to the tumor necrosis factor receptor (TNFR)/ Fas death-receptor super-family, but are suppressed by TRK receptor-initiated signaling.

P75NTR may form a homodimer or a heterodimer with the TRKA receptor. In this second case its presence increases the rate of NGF association with TRKA [162-165].

The biological role of P75NTR in neuroblastoma is still unclear. However, recent evidence suggests that the intracellular region of P75NTR has a death domain which might send signals to induce neuronal cell death [166].

Theoretically, the P75NTR expression could lead to either cell death or differentiation in response to ligand, depending from the presence or the absence of TRKA [167, 168]. As for TRKA, the expression of P75NTR is strongly downregulated in aggressive neuroblastoma having MYCN amplification.

It appears that TRKs and P75NTR are key molecules in the understanding of neuroblastoma biology. However, much still remains unknown, including what regulates expression and function of neurotrophin receptors in neuroblastoma. Taken together, the evidences suggest that there may be a direct involvement of MYCN in the repression of TRKA and P75NTR and this mechanism may play a pivotal role in malignancy of neuroblastoma.

3.4 Chemioresistance in neuroblastoma

It is well known that amplification of MYCN oncogene occurs in 30% of primary untreated neuroblastomas and it is associated with advanced stage disease, rapid progression and unfavorable prognosis [72].
Moreover, this patient subgroup often shows a multiple drug resistance phenotype (MDR) that develops from the treatment of the tumors with chemotherapeutic drugs and increase with the intensity of the therapy accommodation.

There are two general models of resistance to anticancer drugs: those that impair delivery of anticancer drugs to tumor cells and those that arise in the cancer cells themself due to genetic and epigenetic alterations that affect drug sensitivity. In this second case cancer cells start to pump the drugs out by increasing the activity of efflux pumps, such as ATP-dependent transporters [169].

The ATP-binding cassette genes (ABC) represent the largest family of transporter genes and many of those are implicated in disease process and/or drugs resistance [170-173]. ABC genes are widely conserved between species; there are 25 ABC in E.Coli, 29 in S. cervisae, 56 in C. elegans, 56 in Drosophila, 51 in Mouse and 48 in homo sapiens. Human ABCs are localized on 16 different autosomes and only 2 of them reside on the X chromosome.

The prototype ABC protein binds ATP and uses this energy to transport molecules of different nature across the cell membranes (see Fig 21).

Analysis of amino acid sequences alignments of the ATP-binding domains has allowed the ABC genes to be classified into subfamilies. There are seven ABC

![Diagram of ABC transporters](image)
gene subfamilies in the human genome and for the most part they contain genes that display high identity in the trans-membrane domains (TM) and have identical gene organization. However, the function of ABC genes poorly corresponds to subfamily organization.

Two of the best-characterized ABC transporters in neuroblastoma are P-glycoprotein (encoded by MRD gene) and the multi resistant-associated protein MRP.

While the role of MRP is still controversial and fails to predict the outcome (see Fig 22), a significant improvement has been done by studying MRP genes. MRP encodes a novel membrane transport protein whose over-expression confers a resistance profile similar to that mediated form MRD [174]. The discovered of ABCC1 (MRP1) stimulated a genomic search of homologous leading to the discovery of 12 additional members of the ABCC subfamily transporters [175]. Such as ABCC1 many ABCC family members have the potential to confer drug resistance, according with the theory that cancer cells may combine several different types of transporters to gain drug resistance [176].

Treatment of neuroblastoma includes cytotoxic agents (as Topoisomerase I inhibitors and taxanes), multi-drug resistance modulators, apoptosis modulators, gene therapy and inhibitors of angiogenesis. In particular, in neuroblastoma, the chemotherapeutic protocols combine alkylating agents, topoisomerase inhibitors and antibiotics.

Following initial treatment with cytotoxic drugs, tumors appear highly responsive and display a good percentage or complete remission in about 80% of cases, even those classified with unfavorable prognostic outcome [177]. Although many high-risk neuroblastomas initially respond to the first cycle of intensive chemotherapy, they frequently become refractory to treatment as the disease progress. Member of alkylating agents or platinum compounds, like cisplatin and cycle-phosphoamide respectively, are normally used in the chemotherapeutic treatment in neuroblastoma. These drugs are not substrate of ABCC1 [178] maybe this is the explanation why even the tumors with high levels of ABCC1 initially respond to chemotherapy and additional drug resistance lately appears [179].
ABCC1 expression is considered a powerful marker for neuroblastoma prognosis. In fact, high levels of ABCC1 expression are strongly correlated with reduction in survival [180].

![Fig. 22. Expression of the MDR1 (ABCB1) and MRP1 (ABCC1) genes in 60 neuroblastoma patients. The survival of patients whose tumours expressed high levels of ABCC1 is significantly worse than those whose tumours expressed low levels [180].](image)

Also ABCC4 (encoded by MRP4 gene) is expressed in primary neuroblastoma and its over-expression is significantly associated with MYCN amplification and ABCC1 expression. The drug resistance phenotype of MRP4 has been to date thought to encompass primarily nucleoside analogues (including anti-retroviral agents) and methatrexate. ABCC4 is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38. Thus, like MRP1 also high expression of MRP4 gene correlates with poor clinical outcome and in neuroblastoma [181] (see Fig 23)

![Fig. 23. Survival in 52 neuroblastoma patients according to expression of the MRP4 (ABCC4) gene [181].](image)

Our group in collaboration with Dr. Michelle Haber (Children’s Cancer Institute Australia, Sidney) was able to demonstrate that MYCN and c-MYC can transcriptionally regulate set of ATP-binding cassette transporter genes.
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Specifically, they found that MYCN drives the expression of ABCC1 and ABCC4 in neuroblastoma through a direct binding in their promoter sequences. These evidences support that the levels of Myc directly affect the malignant behavior of neuroblastoma cells in vitro and tumor aggressive in vivo.

We also found that in primary neuroblastoma low levels of ABCC3 expression (MRP3) are predictive for clinical outcome and counter-correlate with MYCN levels. The substrates of ABCC3 transporter gene are still unknown. So, as in the case of TRKA and P75NTR the relationship between ABCC3 and MYCN remains to be defined.

4. Genome scale loss-of-function: RNA interference

Genome sequencing efforts have transformed the nature of biological inquiry and have led to an increased need for tools that enable functional studies on the genome scale. In model organisms, genome scale loss-of-function genetic approaches have revealed rich, often unappreciated insights into many biological processes. Sequencing the Saccharomyces cerevisiae genome deeply altered experimental approach and led to the creation and large use of a yeast gene-deletion collection that has facilitated studies of gene function [182]. Similarly, in model organism such as Caenorhabditis elegans and Drosophila melanogaster the discover of RNA interference (RNAi) can be exploited to suppress gene expression [183] [184] has lead to a rapid identification of the genes involved in many biological pathways through powerful loss-of-function screens [185-190]. Although powerful genetic tools already exist for both D. melanogaster and C. elegans, the availability of genome-scale libraries of RNAi reagent has facilitated comprehensive and, at the same time, increasingly complex loss-of-function screens.

RNAi regulates gene expression through sequence-specific targeting of mRNA making possible the production of large-scale libraries direct toward each gene in the genome. RNAi also suppresses gene expression in mammalian cells [191] and chemically synthesized siRNA have become essential tools for biological studies. Indeed, screens in human cell using commercially available libraries of synthetic siRNA have identified modulator of apoptosis [192, 193], cell survival [194] and kinase required for endocytosis processes [195].

In 1998, Fire and Mello discover a mechanism of gene silencing based on the presence of small RNA in C. elegans. These gene silencing is on evolutionarily
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A conserved process and is highly dependent on gene sequence. It includes endogenous micro RNA (miRNA) and exogenous siRNA or shRNA. During the years the mechanism of RNAi has been well investigated and now appears clear that this process is initiated in the nucleus and terminated in the cytoplasm. In the nucleus, RNA polymerase II binds to promoter and transcribe a long RNA precursor as part of one arm of an ~80 nucleotide RNA stem-loop that in turn forms part of a several hundred nucleotides long miRNA precursor (primary miRNA or pri-miRNA) [196, 197]. When a stem-loop precursor is found in the 3’ UTR, a transcript may be used as a pri-miRNA and as mRNA. Recent studies show that even the RNA polymerase III is involved in the transcription of particular set of miRNAs but the mechanism is still under investigation [198].

Subsequently, the double strand RNA structure of hairpins in a pri-miRNA is recognized from nuclear proteins such as DGCR and Drosha forming the “microprocessor” complex. In this complex, DGCR8 orients the catalytic RNase III domain of Drosha (a ribonuclease protein) that cuts RNA about eleven nucleotides from the hairpins liberating them. The product results in a pre-miRNA (precursor-miRNA). Pre-miRNA hairpins are exported from the nucleus to the cytoplasm by Exportin-5 where they are recognized and cleaved by Dicer in 20-25 nucleotide fragments with 2 nt of overhang at 3’. One of the two strand will be incorporated in the RISC (RNA induced Silencing complex) and will guide the entire complex to the target mRNA (Fig 24).

Fig.24. A schematic representation of siRNA, shRNA and miRNA pathways[199].
Introduction

Unfortunately, many mammalian cells appear to be resistant to the transfection methods usually used to introduce synthetic siRNAs into cells. An alternative approach to the transfection is to transduce (infect) mammalian cells with viruses carrying the expression sequence of a short hairpin RNA (shRNA) that encodes a specific siRNA within the cells; this approach can achieve stable, long time and highly effective gene suppression in a variety of cells types [200-203].

For instance, TRC library has been created in 2006 from the RNAi Consortium with the goal of enabling large-scale loss-of-function screens through the development of RNAi libraries and methodologies for their use. This library is designed on the lentiviral vector PLKO.1 (Fig 25), to maximize viral titers and resistance to plasmid recombination. The PLKO.1 lentiviral vector is a derivative of PRRRLSIN.cPPT.PGK/GFP/WRE and is a third-generation self-inactivating lentiviral vector that can be used also as a second-generation [204-206]. Human U6 promoter drives the expression of shRNAs and the vector contains a PGK promoter that regulates the expression of the puromycin resistance gene to allow the selection of transduced cells (fig 24). Viruses are generated using a three plasmid packaging system (two for the second generation) that separates the gag, pol from rev and from the gene encoding the VSV-G coat onto separate vector to minimize the potential for recombination to create replication competent viruses (low than 0.05%).

At July 2006 the shRNA library of TRC contained over 135.000 clones targeting 14.300 human genes. Each gene is target by an average of five distinct constructs and at least four shRNA are available for over 96% of targeted genes. The hairpin sequences contain stems of 21 nucleotides that exactly match the
target transcript and are selected using an algorithm designed to maximize the efficiency of knockdown, to avoid off-targets effects and to cover all the transcripts. All the genome cover has been reached on spring 2007 and the library is still under upgrading processes.

Other famous Lentiviral library are pGIPZ library that integrate the GFP protein to monitor the efficiency of transduction and the DECIPHER Pooled Lentiviral shRNA Libraries.

Furthermore, library based on Lentivirus can be used to transduce both dividing and non-dividing cells.

Numerous are the libraries based on retroviral vector such as pSM2C and pRS libraries. On contrary, retroviral libraries are used to infect only dividing cells. Due to their robustness and specificity siRNA and shRNA are also significantly used to silence cancer-related targets. A large number of preclinical studies have offered good outcome by silencing genes critical for tumor processes. For instance, metastatic pancreatic cancer is one of the most aggressive and deadly tumors with a life expectancy of 4-6 months. Pancreatic duodenal homebox-1 oncogene (PDX-1) has been found overexpressed in pancreatic adenocarcinoma associated to poor prognosis. Silencing of PDX-1 expression through the use of a specific shRNA represents an attractive approach to inhibit tumor growth and immunohistochemistry examination showed a good reduction of PDX-1 expression compared with the patients control group [207, 208]. RNAi technology has been used to inhibit tumor metastasis potential. For example in advanced prostatic cancer where the silencing of PIN1 and VEGFR1 gene expression significantly inhibits tumor growth metastasis and angiogenesis processes respectively [209-211].

Moreover, RNAi can be used to inhibit tumor growth in combination with chemotherapy or radiation therapy [212].
Results

1. **MYCN expression is inversely correlated with that of TRKA, P75\textsuperscript{NTR} and ABCC3.**

As mentioned before, high expression of TRKA, P75\textsuperscript{NTR} and ABCC3 is associated with good prognosis and lower-stage neuroblastomas and is strongly downregulated when MYCN is overexpressed. Their expression is considered a powerful prognostic factor for favorable outcome.

To determine whether MYCN can regulate at transcriptional level the expression of our genes we analyzed their expression profiles in different neuroblastoma cells, clones and systems.

To this purpose, we used TET-21/N inducible cells, a human neuroblastoma cell line in which MYCN ectopic expression is transcriptional downregulated by adding tetracycline to the culture medium [213]. Transcription levels of TRKA, P75\textsuperscript{NTR} and ABCC3 were measured by qRT-PCR as a function of tetracycline treat and correlate with that of MYCN. As shown in Fig 1A-B all the analyzed gene expression increased significantly upon a repression of MYCN expression.

Second, we generated several stable cell clones by stable transforming SH-SY 5Y, a human neuroblastoma cell line in which the expression of MYCN is low, with an expression vector carrying the MYCN coding sequence. The Fig 1C-D shows that each cellular clone that overexpresses MYCN can repress the transcription of TRKA, P75\textsuperscript{NTR} and ABCC3 as compared to parental cell SH-SY 5Y and to the clone carrying the empty vector.

Finally, we treated SK-N-BE and L-AN-1, two human neuroblastoma cell lines with high level of MYCN expression due to an amplification, with a specific siRNA targeting the MYCN mRNA. Results of Fig 1E-F show that strong silencing of MYCN upregulates TRKA, P75\textsuperscript{NTR} and ABCC3 as demonstrated by Western blotting and for qRT-PCR.

APEX1, is a gene positively regulated by MYCN and was used as a positive control in every system tested. As expected, its repression occurs when MYCN is downregulated.

All these findings support the initial hypothesis pointing to a direct role of MYCN in repressing this specific set of genes.
Fig. 1. The expression of MYCN counter-correlates with the expression of TRKA, P75NTR and ABCC3 in neuroblastoma systems. A. Quantification of genes mRNA by qRT-PCR as a function of MYCN expression in TET-21/N system. B. Western blot assay. C. Expression of TRKA, P75NTR and ABCC3 in neuroblastoma clones select for MYCN ectopic expression. D. Western blotting. E. Western blot F. effect of MYCN knockout on the TRKA, P75NTR and ABCC3 expression by qRT-PCR.
Results

2. MYCN mediates repression of TRKA, P75\textsuperscript{NTR} and ABCC3 acting on the core promoter regions.

To formally confirm the direct role of MYCN in the repression of TRKA, P75\textsuperscript{NTR} and ABCC3 we generated different luciferase constructs obtained by cloning the promoter regions of these genes into pGL3-basic (see Fig 2). The luciferase activity (RLU) was measured for each of these constructs in different cellular systems as a function of MYCN expression:

- **TET-21/N** system in presence or absence of tetracycline induction.
- **SH-SY 5Y** cell clones as a function of the level of MYCN overexpression.
- **HeLa cells** as a function of the amount of the pCMV-MYCN expression vector co-transfected with the luc-reporters mentioned before. Furthermore, the use of HeLa cells extends our analysis in a different model from neuroblastoma.

As shown in Fig 2, MYCN overexpression negatively influences the luciferase activity of TRKA, P75NTR and MRP3 full-length promoters while on the contrary we did not measure any change on the same promoters carrying deletions of the putative core binding regions. Furthermore, expression of MYCN does not affect the activity of a viral promoter such as Cytomegalovirus (CMV) that becomes responsive when one of the three core promoters is cloned downstream. APEX1 -1900/+443 construct represents our positive control and negative control respectively.

Overall, through deletions we identified the minimal responsive regions of each promoter to MYCN thus demonstrating that the core promoter of TRKA, P75\textsuperscript{NTR} and ABCC3 are required for MYCN-mediated repression phenomena. These findings encouraged us to further investigate the mechanism by which MYCN can promote transcription repression.
Results

Fig. 2. Whole promoters and respectively deletion mutants of the tested genes were cloned into Firefly Luciferase reporter (pGL3-basic). Luciferase activity was tested in presence/absence of MYCN in different conditions. Luciferase activity was normalized to that of Renilla luciferase. APEX1 and CMV are the controls.

3. Repression of the neurotrophin receptor genes TRKA and $p75_{NTR}$ in neuroblastoma.

3.1. Bioinformatics analysis reveals SP1 and MIZ-1 binding sequences.

Through luciferase assays we identified the minimal responsive region for TRKA and $p75_{NTR}$ to MYCN. To better address this point, we bioinformatically analyzed both core promoter regions and we found several consensus sequences for SP1 and MIZ-1 transcription factors (see fig 3). As mentioned in the introduction, scientific literature supports the idea that MYC can be recruited in an indirect manner on the target genes through its interactions with SP1 or MIZ-1. Indeed, SP1 and MIZ-1 binding sequence are also present in the cell cycle regulator gene $P21$ that results repress by MYC in different contexts. Moreover, Brenner and colleagues demonstrated that c-MYC could recruit DNMT3a on some genes thus promoting their repression. A couple of years ago, we have established that TRKA and $p75_{NTR}$ promoter are not methylated so we hypothesized that a
different mechanism from DNA methylation may determine the repression of neurotrophin receptor genes and it is may be similar to that described for P21.

Fig. 3. Cartoon representation of TRKA, P75NTR and P21 promoters. The distribution of SP1 (yellow rectangle) and MIZ-1 (red rectangle) sites is very similar among the three promoters.

3.2 MYCN is physically associated on the core promoter of TRKA and p75NTR in vivo.

According to the model generally accepted for Myc-mediated repression was reasonable to think that also MYCN does not bind directly DNA (in fact there are no putative E-box within the core promoter of both receptors), but through interactions with other proteins that can bind the DNA directly. Based on this, we performed dual-crosslinking chromatin immune precipitation assays (dual ChIP), a variant of standard ChIP in which two different crosslinking agents are used: Di (N-succynidimil) glutarate (DSG) that promotes the fixation of protein-protein bonds and the formaldehyde that causes links between proteins and DNA. In this way, we assured that even the proteins that not directly bind the DNA can be recovered [214].

Results in Fig 4 show that when we performed a dual ChIP on SK-N-BE neuroblastoma cell line we found that MYCN together with its partner MAX, SP1 and MIZ-1 can specifically co-occupy the core promoter region both of TRKA and p75NTR. To establish the presence of MIZ-1 we performed dual ChIP assay in an inducible clone derived from SK-N-BE, which expresses MIZ-1-HA protein after ponasterone treatment. This allowed us to bypass the problem derived from absence of high quality commercial antibodies against MIZ-1 using an antibody direct against the HA tag.
Fig. 4. Dual ChIP reveals that MYCN is physically associated with the core promoter regions of repressed genes in SK-N-BE. Results are expressed as a relative enrichment calculated as the ratio between the enrichment obtained with the specific antibody and the one obtained with the pre-immune serum (IgG). Amplicon A has been used as a negative control. Results represent the average of three independent experiments of qRT-PCR. Standard error is indicated.
3.3 MYCN, SP1 and MIZ-1 interact one with each other forming a complex through distinct domains.

Based on the previous results we hypothesized that MYC, SP1 and MIZ-1 could interact forming a “ternary complex” capable to bind the DNA and to induce repression on its target genes. In order to confirm this idea, we performed a co-immunoprecipitation assay. We co-transfected HEK 293 cells with expression vector for MYCN with FLAG and another one for SP1-HA or for MIZ-1-HA tag proteins. Fig 5 shows that when MYCN-FLAG was immunoprecipitated using an anti-FLAG we specifically recovered both SP1-HA and MIZ-1-HA (Fig 5A). We obtained the same results when SP1-HA or MIZ-1-HA was immunoprecipitated with the HA antibody. Moreover, to confirm the specificity of these interactions, we showed that MAD-FLAG (another member of Myc/Mad/Max network) cannot recover neither SP1-HA that MIZ-1-HA (Fig 5A).

Furthermore, we performed the same assay using the endogenous expression of each of our interested proteins in SK-N-BE cells and we obtained the same results (Fig 5B).

Finally, to map the domains involved in the interactions observed above, we generated several MYCN deleted proteins fused with the GST. We tested each constructs in GST-pull down assay incubating each fragments with in vitro translated SP1-HA or MIZ-1-HA proteins (Fig 5C).

We found that MYCN interacts with SP1 and MIZ-1 through two distinct domains, one located on N-terminus (MBII) and the other one placed at C-terminus in proximity of the br/HLH/LZ domain respectively.
Results

Fig. 5. Co-immunoprecipitation and GST pull down assays. A. Tagged proteins were co-expressed in HEK293 cells. Immunoprecipitation was performed using an anti-HA antibody and the western blot was developed using anti-FLAG antibody. Mad-FLAG is the negative control. B. Co-immunoprecipitation using endogenous proteins in SK-N-BE. Antibodies SP1, MIZ1 and MYCN were used for immunoprecipitation and western blot detection. C. GST pull down schematic representation of MYCN. GST assays were performed incubating MYCN fragments with SP1-HA or MIZ-1-HA in vitro translated proteins. Anti-HA antibody was used for immunodetection.
Results

3.4 MYCN, SP1 and MIZ-1 are required for TRKA and P75NTR repression and their silencing sensitize neuroblastoma cells to NGF mediated apoptosis.

To better characterize the critical role played by MYCN, SP1 and MIZ-1 in neurotrophin receptors repression we decided to silence each component of this putative complex (with the specific siRNAs).

The silencing of the expression of our genes is sufficient to re-activate the expression of TRKA and P75NTR in SK-N-BE neuroblastoma cells (Fig 6A-B).

Fig.6. Functional role of MYCN, SP1 and MIZ-1 in TRKA and P75NTR repression. A. Quantification of the tested genes transcripts by qRT-PCR. Fold differences are expressed as a function of RNAi knockout condition of MYCN, SP1 or MIZ-1.

In order to investigate the biological importance of and P75NTR re-expression we silenced MYCN, SP1 or MIZ-1 in SK-N-BE neuroblastoma cells. We registered a significance increase in the percentage of the cells undergoing apoptosis only when cells treated with siRNA were also treated with NGF (Fig 7). The cell cycle was analyzed by BrdU incorporation assay and the cells in sub-G1 peak were considered as apoptotic cells (Fig 7A).

Furthermore we tested the apoptosis using an anti-PARP antibody on whole protein extract from SK-N-BE (Fig 7B). PARP (Poly-ADP-Rybose-Polymerase) is a protein of 113 KDa recognized from certain caspases activated during early stages apoptosis. As expected, the fragment 89 KDa of the PARP is clearly detectable only in the presence of RNAi treatment assist with treatment with NGF.
Results

Fig. 7: TRKA and P75NTR re-expression increases the sensitivity of neuroblastoma cells to apoptosis after a treatment with NGF 50 ng/ml. A. BrdU incorporation assay for cell cycle analysis. B. Western blot assay with anti-PARP. Staurosporine has been used as a positive apoptotic inducer.

3.5 HDAC1 is an important co-factor in MYCN-mediated repression on TRKA and P75NTR promoter genes.

As for TG2 gene (data not shown), we wondered if some chromatin modifier could be involved in the repression process mediated by the oncogene MYCN [55]. Based on this, we investigated whether HDAC1 was also present at the repressed TRKA and P75NTR promoters in TET21/N, SK-N-BE, LAN-1 and SH-SY5Y neuroblastoma cells. ChIP assays in figure 8 show that HDAC1 is bound to
the core promoter regions only when MYCN is co-expressed and that histone deacetylation is strongly dependent on MYCN expression.

**Fig. 8:** Chromatin immunoprecipitation assays were performed in different neuroblastoma cell lines and clones using anti-HDAC1, anti-MYCN, anti-PanH3 antibodies. Relative enrichments are expressed as the ratio between the enrichment obtained with the specific antibody and the one obtained with the pre-immune serum (IgG). **A.** SK-N-BE and LA-N-1 neuroblastoma cell lines are characterized by a high level of endogenous MYCN. **B.** SH-SY 5Y cell clones **C.** TET-21/N neuroblastoma cell system. Results represent the average of 3 independent experiments.
Results

3.6 TSA (trichostatin A) treatment induces reactivation of TRKA and $P75^{NTR}$ and sensitizes neuroblastoma cells to NGF-mediated apoptosis.

To provide evidence that the repression of TRKA and $P75^{NTR}$ is induced, at least in part, through histone deacetylation we attempt to inhibit histone deacetylase using trichostatin A (TSA). We treated SK-N-BE and LA-N-1 with 250nM of TSA for 48 hrs and as shown in Fig 9A we obtained an increase in the expression of both TRKA and $P75^{NTR}$ from 10 to 15 fold.

Furthermore, we analyzed the same phenomena in TET-21/N system as a function of the presence (-TET) or the absence (+TET) of MYCN. Results in figure 10B show the synergic effect of the MYCN downregulation with the TSA treatment.

![Graphs showing qRT-PCR analyses](image)

**Fig.9.** qRT-PCR analyses. Fold enrichment is represented as a function of the condition without TSA (A) and without TSA and tetracycline (B). Results represent the average of three independent experiments.

Finally, we tested the biological effect of the TSA inducted re-expression of TRKA and $P75^{NTR}$ treating the cells with TSA for 48 hours and adding NGF (50 ng/ml).

We performed WB assay using PARP cleaved form as a biochemical marker of apoptosis. Fig 10 shows that NGF can induce massive apoptosis in cells re-expressing TRKA and $p75^{NTR}$ after TSA treatment.
Results

Fig.10. Western blot assay on SK-N-BE after TSA treatment and NGF addition using the anti-PARP antibody. Cleaved form of PARP (89 KDa) is a marker of apoptosis. Staurosporine (St.) is a positive control of apoptosis.

3.7 Final remarks
Based on our findings we can suggest a schematic model in which the complex formed by MYCN, SP1 and MIZ-1 is bound to the core promoter regions of TRKA and P75NTR and recruits HDAC1 inducing a deacetylated status of the chromatin and a consequence repression of the transcription (Fig 11).

Fig.11. Schematic representation of MYCN-mediated repression mechanism in neuroblastoma cells.

4. Repression of ABCC3 (ATP-binding cassette transporter)

4.1 Bioinformatics analysis reveals SP1 binding sites.
As mentioned in the introduction, ABCC3 gene encodes for ABCC3 ATP-binding cassette protein, a protein characterized by a high prognostic potential and involved in the phenomena of the chemoresistance in cancer. Moreover, the ABCC3 expression profile counter-correlates with MYCN expression in neuroblastoma.

Similarly to the rational applied for TRKA and P75NTR we analyzed ABCC3 gene promoter for the presence of MYCN, SP1 and MIZ-1 binding sites. Like neurotrophin receptors, ABCC3 promoter does not contain E-Boxes in the
Results

proximity of its transcriptional start sites, whereas it contains SP1 binding site (GC region). Surprisingly, no MIZ-1 binding sites have been found in close proximity of the start (Fig 12).

![Schematic representation of ABCC3 promoter. A. Cartoon model with SP1 sites (yellow rectangles). B. CpG islands analyses of the ABCC3 promoter using ebi.ac.uk tool for CpG islands finding.](image)

**Fig.12.** Schematic representation of ABCC3 promoter. A. Cartoon model with SP1 sites (yellow rectangles). B. CpG islands analyses of the ABCC3 promoter using ebi.ac.uk tool for CpG islands finding.

### 4.2 MYCN represses ABCC3 promoter through the interaction with SP1.

We performed a dual-ChIP on ABCC3 promoter checking if SP1 and MYCN can co-occupy the same core promoter region thus regulating, as for neurotrophin receptor genes, its expression (Fig 13).

![MYCN is physically associated with the core promoter of ABCC3. Dual-ChIP and quantitative PCR were applied to SK-N-BE cell line. Fold enrichment is relative to the pre-immune serum (IgG). Results represent the mean ± SE of three independent ChIP experiments.](image)

**Fig.13.** MYCN is physically associated with the core promoter of ABCC3. Dual-ChIP and quantitative PCR were applied to SK-N-BE cell line. Fold enrichment is relative to the pre-immune serum (IgG). Results represent the mean ± SE of three independent ChIP experiments.

Results show that both SP1 and MYCN (and its partner MAX) bind the ABCC3 promoter on a specific region containing SP1 binding sites.

Taken together these results support the hypothesis that MYCN can repress some specific target genes even in absence of MIZ-1.
4.3 ABCC3 expression levels affect multiple neuroblastoma cell behaviors.

To investigate the biological significance of suppressed ABCC3 expression in neuroblastoma we worked together with the group of Dr. Michelle Haber (Children's Cancer Institute, Australia, Sidney). In the last few years, we have already demonstrated that high levels of ABCC1 and ABCC4 are driven by MYCN overexpression that binds directly (with its partner MAX) to their promoter sequences thus influencing neuroblastoma behavior and prognosis. As a model for ABCC3 studies we choose SK-N-BE, which display low endogenous levels of ABCC3. We generated stable clones in which the expression of ABCC3 was vehicled from CMV promoter (Fig 15A). Moreover, we generated clones carrying a mutant form in the ATP binding domain of ABCC3 coding sequence. Cell clones were investigated for different parameters and we found that expression of ABCC3 reduces migratory activity (Fig 15B-C) without significantly interfering with cell proliferation (Fig 15D-E).

Furthermore, mutations that affect the ATP binding site (V1322F) restores a wild type phenotype without interfering with ABCC3 expression and its membrane localization (Fig 15A and fig 14).

We can conclude that ABCC3 plays a critical role in multiple aspects of tumor cell phenotype independent from any role in cytotoxic drug efflux and drug resistance.

**Fig.14.** Expression of FLAG-ABCC3. Immunofluorescent detection of FLAG-tagged ABCC3 in SK-N-BE neuroblastoma cell clones expressing either ABCC3-wt (clones A12, B12) or ABCC3 V1322F mutant (clones C4, D1). Cells were stained with both monoclonal antibody M2 anti-FLAG (FLAG), and DAPI to visualize nuclei.
Results

Fig. 15. Impact of ABCC3 gene expression on human SK-N-BE neuroblastoma cell characteristics. A Western blot analysis of ABCC3 protein expression either ABCC3-wt (clones A12, B12) or ABCC3 V1322F mutant (clones C4, D1) constructs. B Representative images of wound closure assay; Scale bar, 125 µm. C Expression of ABCC3-wt and impaired motility of SK-N-BE clones, as measured by wound closure assay for 24 hours, compared with empty vector clone. D Colony-forming assay and E Bromodeoxyuridine (BrdU) incorporation upon expression of ABCC3-wt or its catalytically inactive mutant in SK-N-BE cells. One-way analysis of variance followed by two-sided t tests vs control were used to generate P values. Means are derived from three replicate experiments and error bars represent 95% confidence intervals.

5. Whole genome shRNA screening identifies novel factors involved in P75NTR gene transcriptional control.

Even if we elucidated the mechanism by which MYCN can repress P75NTR neurotrophin receptor gene appears clear that we did not identify all the factors involved in this complicated pathways.

Based on these evidences, we performed a whole genome shRNA screening in collaboration with Dr. Michael Green (UMASS, Worcester, USA) to identify those
Factors whose silencing could promote the re-expression of $P75^{NTR}$ using the Openbiosystem Lentiviral library created on PLKO.1 vector.

5.1 Set-up of the parameters for the infection.

The first step for a successful screening consisted in optimizing each parameter as a function of our cell model. From the previous results emerged that $P75^{NTR}$ is repressed by MYCN whose silencing drives a re-expression of the neurotrophin gene. As shown in Fig 16, we recapitulated the results described before.

**Fig.16.** Re-expression of $P75^{NTR}$ as a function of MYCN silencing. A. qRT-PCR B. FACS; the numbers indicate the percentage of the cell re-expressing $P75^{NTR}$ as a function of the non silencing control infection (TRC_NS) C. Western blot.

Another important step in screening assays based on fluorescence-activated cell sorting is the quality and the specificity of the antibody used. To assess the efficiency of the antibody we generated a pCDNA4 plasmid driving the expression of $P75^{NTR}$ coding-sequence. HEK-293T were transiently transfected and $P75^{NTR}$ expression was tested for qRT-PCR and FACS (see Fig 17A-B).
Fig.17. P75NTR antibody results specific for P75NTR detection and responds specifically to the minimum variation in the levels of the neutrophin gene expression. A. qRT-PCR on HEK-293T transiently transfected with either pCDNA4-P75NTR or pCDNA4. B. FACS analysis C. Silencing of P75NTR endogenous in SK-N-BE is efficiently recognized from our antibody even for FACS. D. qRT-PCR on SK-N-BE to assess the efficiency of the silencing. Furthermore we silenced P75NTR expression using a set of 5 shRNAs in SK-N-BE and we assessed the efficiency of the antibody to recognize a minimum variation on P75NTR expression even at low levels (SK-N-BE cell line expresses low levels of endogenous P75NTR see above).
Results

As shown in Fig 17C-D our antibody results capable to detect the minimum variation of cellular P75\textsuperscript{NTR}.

5.2 Infection of SK-N-BE using PLKO.1 whole library.
We performed a whole genome shRNA infection in SK-N-BE neuroblastoma cells, which display low levels of endogenous P75\textsuperscript{NTR}.

Viruses were packaged using HEK-293T cells and the viral titer was determined using the targeting cells (SK-N-BE). To obtain high percentage of cells with a single insertion in the genome we transduced the SK-N-BE with a M.O.I of 0.3 (see Fig 18).

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**Fig.18.** Poisson distribution of viral insertions into the genome depending from M.O.I.

Cells re-expressing \textit{P75\textsuperscript{NTR}} were sorted using a fluorescence-activated cell sorting (FACS) and collected separately (see Fig 19). To minimize the false positive rate, only the single cells and not apoptotic or apoptotic-like were included in the analyses. Experimental procedures are described accurately in Material and Methods.
5.3 Identification of the potential candidates

We extracted the DNA from the sorted population and we amplified by PCR a specific region representing the potential shRNA directs against the putative “candidate gene”. The first round of sequences identified 8 genes that, after shRNA-mediated knockdown, resulted in significant $P75^{NTR}$ re-expression. These genes are listed in Table 1.

<table>
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<td>tumor necrosis factor, alpha-induced protein 3</td>
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<tr>
<td>SPHK2</td>
<td>sphingosine kinase 2</td>
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<tr>
<td>GPR152</td>
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<td>heterogeneous nuclear ribonucleoprotein C</td>
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<td>GSK3β</td>
<td>glycogen synthase kinase 3 beta</td>
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Table 1. Genes required for $P75^{NTR}$ silencing.
Results

5.4. shRNA-mediated knockdown of the candidate genes induces $P75^{NTR}$ re-expression.

The data collected so far have to be considered preliminary. Further experiments are required to consider these candidates significant.

To validate the candidate genes obtained from the screen, we performed qRT-PCR and, where has been possible, western blot and FACS assays.

qRT-PCR results in Fig 20 demonstrated that our putative candidate genes can induce a re-expression of $P75^{NTR}$ from 3 to 30 fold and most of them seem to have a more powerful effect than MYCN itself. Moreover, using at least 2 distinct shRNAs we excluded the off-target effects.

![Fig.20](image)

**Fig.20.** Specific knockdown induce $P75^{NTR}$ re-expression. Fold enrichment is calculated as a function of the SK-N-BE infected cells using TRC_NS virus.

Additionally, we assessed (up now only for two of them) the re-expression of $P75^{NTR}$ through a western blot. Fig 21 confirms their potential involvement in the neurotrophin receptor gene silencing and demonstrates a good rate of correlation between signal obtained in qRT-PCR and effective protein re-expression.

![Fig.21](image)

**Fig.21.** Western blot analysis on SK-N-BE after infection with either specific shRNAs or non-silencing shRNA (TRC_NS). Cells were infected for 24 hrs and selected with puromycin ($3\mu$g/ml) for 5 days.
Results

Based on the previous results, we selected the best shRNA for each of our candidates and we tested the re-expression of $P75^{NTR}$ by FACS.

Results in Fig 22 demonstrate that the selective knockdown of our candidates promotes the re-expression of the neurotrophin receptor gene, and most of them have a greater effect if compared to MYCN silencing.

![Graphs showing FACS results for different shRNAs](image)

**Fig. 22.** Fluorescence-activated cell sorting assays on SK-N-BE. Cells were infected with either the specific shRNAs or non-silencing (TRC_NS) for 24 hours and selected with puromycin (3 µg/ml) for 5 days.

Cell population was stained with primary anti-P75NTR antibody (BD 557194) dil 1:1000 and secondary antibody APC conjugated (Jackson 115-136-146). Positive cells (APC+) have been identified using BD Scaler Flow cytometer.

### 5.5 Incoming experiments.

Results collected so far indicate that further validation experiments have to be done to ensure the potential of our candidates in $P75^{NTR}$ regulation. First of all, we have to complete western blot analyses checking the formal re-expression of $P75^{NTR}$ and the efficiency of the knockdown of our candidates. Second, we have to test the biological significant of this re-expression. Furthermore, as seen for MYCN mediated repression, to better address the last point we will investigate the response of $P75^{NTR}$ re-expressing cells to NGF and pro-NGF treatments measuring the apoptosis induction.
Discussion
MYCN is a member of the MYC proto-oncogene family that also comprises c-MYC and MYCL. The gene was first discovered in neuroblastoma cell lines as amplified DNA with homology to viral myc.

The Myc’s family onco-proteins are characterized from a highly conserved br/HLH/LZ DNA binding motif by which they can dimerize with proteins of MAX subfamily and act as a transcription factor through a direct binding with a specific DNA sequence called E-box (CACGTG) [62, 64]. Nonetheless, it has been shown that the dimeric complex (Myc-Max) can also well recognize degenerate variants of classical E-box known as “non-canonical E-box” (CATGTG and CAGCGC).

Like other Myc members, MYCN controls the expression of many target genes involved in several critical cellular processes such as proliferation, growth, protein synthesis, metabolism, cell size, genome integrity, apoptosis and differentiation [215].

They were commonly known as a transcriptional activator in fact the dimer can recruit transcriptional co-factors such as TRRA-P or p300/CBP to promote transcriptional events (see introduction).

Lately, genome-wide analyses demonstrated that Myc represses at least as many targets as it activates thus focusing the attention on a novel function of these amazing proteins. In the repression events, Myc binds to other transcription factors and inhibits transcription of their downstream targets. In this way, cell cycle regulators, pro-apoptotic and cell adhesion genes can be repressed thus promoting rapid growth and aggressive phenotype.

Deregulated expression of MYCN is often found in neuroblastoma and in several other cancers, frequently of embryonic and/or neuroendocrine origin. These tumors originate from tissues where MYCN is normally expressed and include retinoblastoma [216], Wilm’s tumor [217], rhabdomyosarcoma [218], medulloblastoma [219], glioblastoma [220], and small cell lung cancer [221].

Neuroblastoma is the most common extracranial solid tumor of the childhood and is responsible of higher number of cancer-related deaths in infants [15]. So far, MYCN amplification status remains one of the most critical predictor of neuroblastoma prognosis and outcome [15, 74, 76] although other important factors have been identified as important for prognosis prediction as ploidy status,
loss of chromosome 1p, expression of ABCCs (ABBC1 and ABCC4) and expression of TRKA and P75\textsuperscript{NTR} neurotrophin receptor genes (see introduction). Our results show that MYCN amplification and overexpression induces repression of a subset of genes targeting their core promoter region:

- TRKA and P75\textsuperscript{NTR} neurotrophin receptor genes
- ATP binding cassette transporter gene 3 (ABCC3)
- Cell cycle inhibitor P21 (already demonstrated for c-MYC but never for MYCN)

1. Neurotrophin receptor genes repression

We have analyzed the role of MYCN in the transcriptional repression of TRKA and P75\textsuperscript{NTR} in neuroblastoma. Our results show that MYCN is physically engaged with the core promoter region of both genes through interaction with 2 other transcription factors, SP1 and MIZ-1.

SP1 and MIZ-1 are normally transcriptional activators promoting TRKA and P75\textsuperscript{NTR} expression. In our case, it seems that they can work together forming a platform cable of recruiting MYCN. In support of our idea we performed Co-IP assays and we demonstrated that MYCN, SP1 and MIZ-1 can interact with one other. Moreover, we mapped the specific regions of MYCN directly involved in this interaction and we finding that the MBII domain of MYCN interacts with SP1 whereas the BR/HLH/LZ domain interacts with MIZ-1 respectively.

We also demonstrated MYCN, SP1 and MIZ-1 are all necessary to mediate repression and that the silencing of one members of this “ternary complex” is sufficient to promote a disruption of the complex and a consequential TRKA and P75\textsuperscript{NTR} re-expression.

How does MYCN exert his repression activity? There are two possible and different answers to this question. One possibility is that MYCN could interfere, after its recruitments in the complex, with the transactivation functions of SP1 and MIZ-1 blocking their activity. Another possibility is that the ternary complex, formed in presence of high levels of free MYCN, can recruit other additional factors such as chromatin modifier agents thus promoting changes in chromatin structure and accessibility. This second hypothesis is without any doubt more intriguing and it is supported by several lines of evidence deriving from c-MYC studies, in which c-MYC promotes P21 repression by recruiting DNMT3a (see introduction) Our results show that MYCN recruits HDAC1 on TRKA and P75\textsuperscript{NTR}
core promoter regions and its recruitment can induce a strong decrease in their acetylation levels, thereby minimizing their accessibility to polymerase and other transcription factors. Indeed, ChIP assays and treatment with HDACs inhibitors such as TSA demonstrate that: 1) HDAC1 can co-occupy the same region of MYCN, SP1 and MIZ-1 on the neurotrophin core promoter genes 2) Inhibition of HDAC1 can reactivate transcription of the two genes thus relieving the repression effects of MYCN.

What is the biological relevance of this mechanism for neuroblastoma? Surprisingly, even if neuroblastoma is one of the most deadly cancers in childhood, it is also characterized by high degree of spontaneous regression. This peculiarity seems to be directly correlated with differentiation or massive cell death events that occur in concomitance with high expression levels of TRKA expression, considered a predictor of positive outcome. Advanced stages neuroblastomas normally have a fast progression and lack both neurotrophin receptor genes expression. This is confirmed also in transgenic mice where over expression of MYCN promotes a progressive downregulation of TRKA and $P75^{NTR}$ increases the rate of growth.

Interestingly, our findings also demonstrate that resumed expression of TRKA and P75NTR, obtained through alternative and distinct approaches appears to, sensitize neuroblastoma cells to NGF-mediated apoptosis whereas it has not effect on differentiation.

Why apoptosis and not differentiation? In fact, several evidences described by the scientific literature link the co-expression of TRKA and $P75^{NTR}$ to cell differentiation or growth inhibition. We have two explanations supporting our results. First of all, recent studies have shown that under particular circumstance, TRKA re-expression, in addition to that of $P75^{NTR}$, could promote apoptotic pathways transduction [222-225]. Second, in our experiments we always obtained a quite larger $P75^{NTR}$ re-expression compared to TRKA, suggesting that $P75^{NTR}$ signaling may prevail on that established through TRKA.

However, still many aspect remain to be elucidated on the complexity of $P75^{NTR}$ and TRKA signaling pathways.

Collected together our findings provide a mechanistic rational between MYCN overexpression and neurotrophin receptor genes repression in neuroblastoma, highlighting the anti-oncogenic role of NGF/$P75^{NTR}$/TRKA pathways and lay
Discussion

foundation to searching and developing novel therapeutic approaches to treat neuroblastoma.

2. Repression of ABCC3.
Chemoresistance of cancer cells is in part the consequence of a misregulation of the activity of membrane protein, called ATP-binding cassette transporters, responsible for the efflux of chemotherapeutic drugs [172] [226].
In neuroblastoma cells ABCC1, ABCC3 and ABCC4 are the most strongly regulated genes belonging to ABCC subfamily. In fact, we discovered that ABCC1, ABCC3 and ABCC4 expression can stratify neuroblastoma patients into groups having excellent, intermediate and poor outcome and this combination of factors is considered one of the most powerful prognostic marker.
Furthermore, these genes are the only ones in the entire ABCC subfamily found to be directly regulated from MYCN specifically ABCC1 and ABCC4 are positively regulated whereas ABCC3 results to be inhibited.
In this thesis, I focused my work investigating the molecular mechanism by which MYCN can negatively regulate ABCC3 expression trying to verify if MYCN mediated repression process can involve a different complex in function of the target gene that has to be repressed. Moreover, providing a mechanistic a rationale of ABCC3 repression means found different putative therapeutic targets for a possible drugs developing.
First of all, we found that specific overexpression of ABCC3 as well as silencing of ABCC1 or ABCC4 genes cause reduction in cell motility without interfere with cellular growth. In particular we provided the first evidence that ABCC3 gene is a downstream target of MYCN. As shown in the results, ABCC3 gene promoter contains several SP1 binding sites located around the TSS whereas no E-Box has been found. Using dual-ChIP we demonstrated that SP1 and MYCN can occupy the same region of DNA on the ABCC3 promoters and that also MAX is involved in this type of repressing complex. Recent evidences support ta tumor suppressor role of ABCC3 in the oncogenesis, thus explaining the reason why it became transcriptionally silent during the tumors development [227]
3. shRNA whole genome screening

Immunohistochemical analyses of $P75^{NTR}$ expression on a tissue microarray of 93 primary neuroblastic tumors revealed that it is expressed in neuroblastic cells of ganglioneuromas/ganglioneuroblastomas as well as differentiating neuroblastomas, but not in poorly differentiated neuroblastomas. Furthermore in an unrelated cohort of 110 neuroblastic tumors, $P75^{NTR}$ mRNA expression levels correlated with differentiation, and patients with tumors that expressed $P75^{NTR}$ at high levels had an increased survival. Ectopic $P75^{NTR}$ expression in the neuroblastoma cell line significantly reduced proliferation, increased the fraction of apoptotic cells in vitro and resulted in a loss of tumorigenicity in nude mice.

As mentioned in the introduction, $P75^{NTR}$ binds NGF and related neurotrophins with low affinity, but its effect is still unclear and controversial. It has been shown that cells overexpressing $P75^{NTR}$ increase the number of high and low-affinity NGF binding sites in TRKA [228]. This is probably due to a direct interaction with TRK receptors that induces a conformational change or $P75^{NTR}$ may also play a role in ligand presentation [229]. $P75^{NTR}$ expression may increase the sensitivity of TRKA to low concentrations of NGF and at the same time it can induce apoptosis in the presence of NGF [230-233]. Nevertheless, the effect of P75NTR on the cellular response to neurotrophins is complex and may depend on the concentration of ligand, the ratio of receptors, the cell type in which it is expressed, and its stage of differentiation [229, 234, 235].

To identify new regulators of $P75^{NTR}$ and well elucidate its function and its regulation in the peripheral nervous system development and diseases, we performed a whole genome shRNA screening and identified several potential candidates that can directly or indirectly control the transcription of $P75^{NTR}$.

Surprisingly most of the candidates are receptors or proteins with cytosolic localization involved in different processes and apparently not associated with transcription factors.

Why we found more receptors than transcription factors?

One possible explanation is that we used strict parameters for our screening thus selecting only those genes whose silencing induces a strong re-expression of $P75^{NTR}$ (from 4 to 50 fold). This hypothesis is supported by what we showed in
the results chapter where at the “ternary complex” disruption corresponds a $P75^{NTR}$ reactivation of a maximum of 4 fold. Furthermore, receptors are usually on top of a signaling pathway and when their expression is misregulated the effect spreads on all the components belonging at that specific way resulting in consequential signal amplification.

Moreover, several evidences suggest that some of our potential candidates are not exhaustively studied in particular in the neuroblastoma context. $TNFAIP3$ encodes for a zinc-finger protein that inhibits nuclear factor-$\kappa$B and avoids TNF induced apoptosis. $SPHK2$ encodes for a kinase involved in the sphingolipids metabolism. Its product is sphingosine 1-P a bioactive lipid mediator upregulated in different kind of cancers. It should be also mentioned that $SPHK2$ was found as part of a repressive complex capable of blocking transcription of the $P21$ gene. In that case $SPHK2$ can interact with HDAC1/2 to keep histone acetylation at low levels. However when $SPHK2$ is phosphorylated through the PI3-K signal pathways it becomes active and starts producing sphingosine 1-P which is a selective inhibitor of HDAC activity. Interestingly, sphingosine 1-P is a specific substrate of ABCC1 efflux activity thus suggesting that $SPHK2$ may affect neuroblastoma biology through different pathways: one involved in the control of $P75^{NTR}$ expression and a second one involving a functional axis with ABCC1.

In contrast to $SPHK2$, almost nothing is known about $GPR152$, $MAGEA12$ and $TRDN$ function and their role in nervous system diseases and cancers. $GPR152$ belongs to G-protein coupled receptor family. This family is wide and its members are capable to interact with a wide variety of molecules on the outer surface of cells. Usually each receptor binds to one or a few specific molecules that activate the receptor, which changes its shape. The receptor can then activate proteins called G proteins within cells. In a process called signal transduction, active G proteins trigger a complex network of signaling pathways that ultimately influence many cell functions.

Researchers have identified more than a thousand G protein-coupled receptors in humans and other organisms. Many of these receptors are predicted to be olfactory receptors, which allow organisms to recognize different smells. Other G protein-coupled receptors are involved in vision, the immune system, and the autonomic nervous system. Additionally, several major brain chemicals
(neurotransmitters) relay signals between nerve cells using G protein-coupled receptors. These neurotransmitters are critical for regulating behavior and mood. G protein-coupled receptors are involved in many human diseases, including various forms of cancer. Researchers estimate that about half of all currently available drugs have been designed to target these receptors.

On contrary, EPHA2 is tyrosine kinase receptor and has been demonstrated that it acts during the nervous system developing but is not yet correlated with neuroblastoma or apoptosis.

Finally, GSK3β is a serine/threonine kinase involved in more than forty different pathways. In mammalian it is recently subject of several studies because implicated in numerous disease and cancers. For instance in 2008 Kotliarova and colleagues demonstrated that in c-MYC-dependent glioma inhibition of GSK3-beta or alpha using different agents result in an increased sensitivity to apoptosis and in a block in intracellular nuclear factor-κB activity [236]. Moreover, will be very fascinating also elucidate the correlation between MYCN and GSK3 wondering if it will act as c-MYC or it has a totally different role.

Luckily, is easier to find commercial drugs targeting receptors than transcription factors and this will help me in further validation assay.

4. Final remarks

Overall our findings provide further clarifications on mechanism of MYCN transcriptional repression in Neuroblastoma and how these may contribute to tumor progression, chemoresistance and lack to apoptosis sensitivity particularly in those cancer where MYCN expression is deregulated.

Curiously, although my thesis has been focused on Neuroblastoma and MYCN, there are numerous evidences demonstrating that c-MYC and MYCN generally share several common targets and characteristics in cancer (as ABCC1, ABCC3 and ABCC4 regulation). In normal conditions, the heterodimer MYC/MAX promotes cell cycle progression: thus physiologically MYC expression is tightly regulated and restricted during G1/S transition. Furthermore, MYC proteins have short half-lives (in order of 20-30 minutes) whereas MAX is more stable and constitutively expressed (fig 1).
Fig.1. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in physiological conditions.

We believed that when MYC expression is deregulated during the whole cell cycle the MYC/MAX heterodimer can become overabundant and can interact with novel partners such as SP1 or MIZ-1 outside the usual E-box context thus promoting neoplastic phenotype.

Moreover, when significantly overexpressed (like in advanced stage neuroblastoma) Myc may establish a set of novel interactions not strictly connected to Max such with DNMT3a, SIRT1 and HDACs (fig 2 and fig 3).

Fig.2. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in physiological conditions.
Several years and resources have been spent looking for an efficient therapy focused on blocking MYC onco-proteins, but they have been a big failure. Our studies, in particular the shRNA screening against P75NTR, are a new hope to understand and discover who are the factors that act directly with MYCN by themselves which contribute in neuroblastoma tumorigenesis and how we can interfere with their function avoiding all the side effects as chemoresistance and apoptosis bypassing.

**Fig.3.** Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in physiological conditions.
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CELL CULTURES

Human neuroblastoma SK-N-BE(2)C, TET-21/N, SHEP and IMR-32 cells were cultured in DMEM containing 10% heat-inactivated FBS and 50 mg/ml gentamycin. Human neuroblastoma, SH-SY5Y and L-AN-1 cells were cultured in RPMI medium 1640 containing 10% FBS and 50 mg/ml gentamycin. TET-21/N 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 suspension 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 for 5 minutes at 12000rpm at 4°C. Transfer aqueous 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.
RT-PCR SYSTEM

The RT-PCR was designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step process. 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)), 2 µg 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 RT (15 units/ 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 20 min. 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 TaqDNA 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 TaqDNA polymerase, SYBR GreenER fluorescent dye, 1 µM Fluorescein.
Reference Dye, MgCl₂, 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 TaqDNA 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 adjust 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 [106] 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
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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 for 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 ethanol 100% 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.

<table>
<thead>
<tr>
<th>Cell Lysis Buffer</th>
<th>RIPA Buffer</th>
<th>Washing buffer</th>
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</thead>
<tbody>
<tr>
<td>5 mM PIPES pH 8</td>
<td>150mM NaCl</td>
<td>100mM TrisHCl pH 8</td>
</tr>
<tr>
<td>85 mM KCl</td>
<td>1% NP40</td>
<td>500mM LiCl</td>
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<tr>
<td>0.5% NP40</td>
<td>0.5% NaDox</td>
<td>1% NP40</td>
</tr>
<tr>
<td>1 mM PMSF</td>
<td>0.1% SDS</td>
<td>1% NaDox</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>50 mM TrisHCl pH 8</td>
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<tr>
<td></td>
<td>1 mM PMSF</td>
<td>Protase inhibitor cocktail</td>
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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 10^7 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
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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 [106] 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 [106] 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
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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 (disuccinimydyl 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.

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
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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-MYCN 1-88, GST-MYCN 82-254, GST-MYCN 249-361 and GST-MYCN 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.
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.
PRODUCTION OF TRC VIRAL SUPERNATANT

We routinely use QIAGEN Effectene Transfection Reagent, which works very well for us. Detailed protocols are provided with the kit. The protocol below has been slightly modified from the QIAGEN kit protocol, in that it uses slightly more DNA.

**Reagents**

293T cells (ATCC)

293T is a highly transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted.

Cell culture medium

Effectene reagent (Qiagen)

EC buffer (comes with the QIAGEN Effectene kit)

Enhancer (comes with the QIAGEN Effectene kit)

TRC plasmid DNA (purchased from the RNAi Core Facility)

psPAX2 (Addgene) → This is the packaging vector

pMD2.G (Addgene) → This is the envelope vector

0.45 µm filter (Millipore)

**Method**

Day 1: Plate 1.0x10^6 to 1.2x10^6 293T cells in a 6-well plate.

Day 2: a. In a sterile microfuge tube, combine 1 µg of TRC or pGIPZ plasmid DNA with 1 µg psPAX2 and 0.5 µg pMD2.G (2:2:1 ratio) in 100 µl EC buffer.
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Add 3.2 µl Enhancer. Mixed by brief vortexing and then spin down to collect the contents of the tube. Incubate at room temperature for 5 minutes. Add 10 µl Effectene reagent, mix by brief vortexing and incubate for another 20-30 minutes at room temperature.

b. During the incubation, re-feed the 293T cells (that have been plated out the day before) with 1.6 ml of fresh medium. 293T cells peel off easily, use extreme care to re-feed the cells.

c. After the 20-30 minute incubation, add 0.6 ml medium to the DNA-Effectene mixture.

Mix well and drop carefully onto the cells.

Day 3: Re-feed the transfected cells with 2.5 ml fresh medium. 293T cells peel off easily, use extreme care to re-feed the cells.

Day 4: 48 hours after infection, filter the supernatant through a 0.45 µ filter, aliquot and store at -80°C until ready for use.

INFECTION USING TRC VIRAL SUPERNATANT

The following protocol works well with most commonly used cancer cell lines. However, be aware that some cells, particularly primary cells, are extremely sensitive to Polybrene. It is therefore a good idea to pre-determine the most suitable concentration of Polybrene to be used in the infection.

**Reagents**

Cells of interest to be infected

Cell culture medium

Viral supernatant (purchased from the RNAi Core Facility, or produced from TRC plasmid DNA [see accompanying protocol])

Polybrene, 1 µg/µl (Sigma)

Puromycin (various sources such as Sigma and Clontech)

The ideal concentration of puromycin should be pre-determined based on the cell line.

**Method**

Day 1: Plate 1x10^5 to 1.25x10^5 cells per well in a 6-well plate.
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Day 2: Aspirate the medium and infect cells with 250 to 500 µl viral supernatant. Add fresh medium to a final volume of 1 ml. Add 10 µl (or pre-determined optimized amount) of 1 µg/µl Polybrene.

Day 3: Re-feed the cells with fresh medium.

Day 4: Start puromycin selection or check the cells under a fluorescence microscope for GFP expression (if using the pGIPZ system).

After 5 to 7 days of puromycin selection, the cells are ready to use in assays.

FACSCalibur ANALZYER

Reagents:
PBS1x cold; Tripine 1x; RPMI or DMEM 0,1% FBS without phenol red; plate 96 well,

If necessary treat the cells with:
• Wash cells with PBS1X
• PFA 4% in PBS1x 15’
• Wash twice with RPMI+FBS 0,2%
• Resuspend the cell in RMPI+FBS 0,2% + Saponin 0,5% 15’
• All the incubation step should be done with saponin

Or
• Dump the media from the cell culture
• Wash 1 time with PBS1x (not necessary cold)
• Tripinize the cell with the opportune volume of Tripine 1x
• Add media for neutralize tripine and put the cells in a new tube
• Centrifuge at 250 rcf at RT for 5’
• Resuspend the cell in media and plate in 96 well in 100ul for well. (vortex gently or pipetting)
• Centrifuge the cells and resuspend them in 40 µl of media+ Abl°1:1000-1:250
• Incubate for 20’ at on Ice then add 160 µl of media
• Wash 3 times with media resuspending the pellet
• Resuspend the cells in 50 µl media AbII° 1:200 for 20’ on Ice (cover) then add 150 µl of media
Materials and Methods

- Wash 2 times with media and resuspend in the appropriate volume (depends on cell density)
- Transfer the resuspended cells in the tube.

<table>
<thead>
<tr>
<th>Laser</th>
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<tr>
<td><strong>Blue 488 nm</strong></td>
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<td>670 LP</td>
<td>PerCP, PerCP-Cy5.5, PE-Cy5, PE-Cy5.5, PE-Cy7, 7AAD, PerCP-eFluor 710, PI*</td>
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<tr>
<td></td>
<td>B</td>
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<td>C</td>
<td>530/30</td>
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<td><strong>Red 635 nm</strong></td>
<td>A</td>
<td>661/16</td>
<td>APC, Cy5, Alexa 633, Alexa 647</td>
</tr>
</tbody>
</table>

* The emission wavelength of PI is ~575 nm so be aware that compensation issues may arise when using this dye in combination with other fluorochromes.

Not all dyes listed can be used in combination, particularly tandem dyes which are excitable by the red laser.
Bibliography


Bibliography


Bibliography

Bibliography


213. Lutz, W., et al., Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and


N-Myc Regulates Expression of the Detoxifying Enzyme Glutathione Transferase GSTP1, a Marker of Poor Outcome in Neuroblastoma.


Author’s Affiliations: Children’s Cancer Institute Australia for Medical Research, Lowy Cancer Research Centre, Randwick, Sydney, New South Wales, Australia; Department of Biology, University of Bonn, Bonn, Germany; Children’s Medical Research Institute and Children’s Oncology Group Registry and Data Center, Dana-Farber Harvard Cancer Care and Children’s Hospital Boston, Massachusetts.

Abstract

Amplification of the transcription factor MYCN is associated with poor outcome and a multidrug-resistant phenotype in neuroblastoma. n-Myc regulates the expression of several ATP-binding cassette (ABC) transporter genes, thus affecting global drug efflux. Because these transporters do not confer resistance to several important cytotoxic agents used to treat neuroblastoma, we explored the prognostic significance and transcriptional regulation of the phase II detoxifying enzyme, glutathione S-transferase P1 (GSTP1). Using quantitative real-time PCR, GSTP1 gene expression was assayed in a retrospective cohort of 51 patients and subsequently in a cohort of 207 prospectively accrued primary neuroblastomas. These data along with GSTP1 expression data from an independent microarray study of 291 neuroblastoma samples were correlated with established prognostic indicators and disease outcomes. High levels of GSTP1 were associated with decreased event-free and overall survival in all three cohorts. Multivariable analyses, including age at diagnosis, tumor stage, and MYCN amplification status, were conducted on the two larger cohorts, indicating the prognostic significance of GSTP1 expression levels in this setting. Mechanistic investigations revealed that GSTP1 is a direct transcriptional target of n-Myc in neuroblastoma cells. Together, our findings reveal that n-Myc regulates GSTP1 along with ABC transporters that act to control drug metabolism and efflux. Furthermore, they imply that strategies to jointly alter these key multidrug resistance mechanisms may have therapeutic implications to manage neuroblastomas and other malignancies driven by amplified MYC family genes. Cancer Res; 72(4): 845-53. 62011 AACR.


ABCC multidrug transporters in childhood neuroblastoma: clinical and biological effects independent of cytotoxic drug efflux.


Experimental Therapeutics and Molecular Diagnostics Program, Children’s Cancer Institute Australia for Medical Research, Lowy Cancer Research Centre, University of New South Wales, P.O. Box 81, Randwick, NSW 2031, Australia.

Abstract

BACKGROUND: Although the prognostic value of the ATP-binding cassette, subfamily C (ABCC) transporters in childhood neuroblastoma is usually attributed to their role in cytotoxic drug efflux, certain observations have suggested that these multidrug transporters may also contribute to the malignant phenotype independent of cytotoxic drug efflux.

METHODS: A n-myc myelocytomatosis virus related oncogene, neuroblastoma derived (MYCN)-driven transgenic mouse neuroblastoma model was employed with an ABCG2 deficient mouse strain (858 MYCN(+/−)), 205 MYCN(+1+) mice) or, alternatively, treated with the ABCG1 inhibitor, Verapamil (n = 20). ABCG genes were suppressed using short interfering RNA or overexpressed by stable transfection in neuroblastoma cell lines BE(2)-C, SH-EP, and SH-SY5Y, which were then assessed for wound closure ability, clonogenic capacity, morphological differentiation, and cell growth. Real-time quantitative polymerase chain reaction was used to examine the clinical significance of ABCF family gene expression in a large prospectively accrued cohort of patients (n = 209) with primary neuroblastomas. Kaplan-Meier survival analysis and Cox regression were used to test for associations with event-free and overall survival. Except where noted, all statistical tests were two-sided.

RESULTS: Inhibition of ABCG2 clinically significantly inhibited neuroblastoma development in MYCN transgenic mice (mean age for palpable tumor-bearing mice, 47.2 days; control mice, 41.9 days; hazard ratio [HR] = 0.3, 95% confidence interval [CI] = 2.85 to 9.32; P < .001). Suppression of ABCG1 in vitro inhibited wound closures (P < .001) and clonogenicity (P = .006). Suppression of ABCG4 enhanced morphological differentiation (P < .001) and inhibited cell growth (P < .001). Analysis of 209 neuroblastoma patient tumors revealed that, in contrast to ABCG1 and ABCG4, low rather than high ABCG3 expression was associated with reduced event-free survival (HR of recurrence or death = 2.4, 95% CI = 1.4 to 4.2; P = .001), with 23 of 53 patients with low ABCG3 expression experiencing recurrence or death compared with 31 of 155 patients with high ABCG3. Moreover, overexpression of ABCG2 in vitro inhibited neuroblastoma cell migration (P < .001) and clonogenicity (P = .03). The combined expression of ABCG1, ABCG3, and ABCG4 was associated with patients having an adverse event, such that of the 12 patients with the "poor prognosis" expression pattern, 10 experienced recurrence or death (HR of recurrence or death = 12.3, 95% CI = 6.2 to 27; P < .001).

CONCLUSION: ABCG transporters can affect neuroblastoma biology independently of their role in chemotherapeutic drug efflux, enhancing their potential as targets for therapeutic intervention.

Comment in


SIRT1 promotes N-Myc onco genesis through a positive feedback loop involving the effects of MKP3 and ERK on N-Myc protein stability.


Children’s Cancer Institute Australia for Medical Research, Randwick, Australia.

Abstract

The N-Myc oncoprotein is a critical factor in neuroblastoma tumorigenesis which requires additional mechanisms converting a low-level to a high-level N-Myc expression. N-Myc protein is stabilized when phosphorylated at Serine 62 by phosphorylated ERK protein. Here we describe a novel positive feedback loop whereby N-Myc directly induced the transcription of the class III histone deacetylase SIRT1, which in turn increased N-Myc protein stability. SIRT1 binds to Myc Box I domain of N-Myc protein to form a novel transcriptional repressor complex at gene promoter of mitogen-activated protein kinase phosphatase 3 (MKP3), leading to transcriptional repression of MKP3, ERK protein phosphorylation, N-Myc protein phosphorylation at Serine 62, and N-Myc protein stabilization. Importantly, SIRT1 was up-regulated, MKP3 down-regulated, in pre-cancerous cells, and preventative treatment with the SIRT1 inhibitor Camitin reduced tumors in TH-MYCN transgenic mice. Our data demonstrate the important roles of SIRT1 in N-Myc oncosilting and SIRT1 inhibitors in the prevention and therapy of N-Myc-induced neuroblastoma.
c-MYC oncoprotein dictates transcriptional profiles of ATP-binding cassette transporter genes in chronic myelogenous leukemia CD34+ hematopoietic progenitor cells.


Department of Biology, University of Bologna, Bologna, Italy.

Abstract
Resistance to chemotherapeutic agents remains one of the major impediments to a successful treatment of chronic myeloid leukemia (CML).

Misregulation of the activity of a specific group of ABC-binding cassette transporters (ABCs) is responsible for reducing the intracellular concentration of drugs in leukemia cells. Moreover, a consistent body of evidence also suggests that ABC transporters play a role in cancer progression beyond the efflux of cytotoxic drugs. Despite a large number of studies that investigated the function of the ABC transporters, little is known about the transcriptional regulation of the ABC genes. Here, we present data showing that the oncoprotein c-MYC is a direct transcriptional regulator of a large set of ABC transporters in CML. Furthermore, molecular analysis carried out in CD34+ hematopoietic cell precursors of 21 CML patients reveals that the overexpression of ABC transporters driven by c-MYC is a peculiar characteristic of the CD34+ population in CML and was not found either in the population of mononuclear cells from which they had been purified nor in CD34+ cells isolated from healthy donors. Finally, we describe how the methylation state of Cpg islands may regulate the access of c-MYC to ABC genes, a well-studied gene associated with multidrug resistance in CML, hence, affecting its expression. Taken together, our findings support a model in which c-MYC-driven transcriptional events, combined with epigenetic mechanisms, direct and regulate the expression of ABC genes with possible implications in tumor malignancy and drug efflux in CML.


APP-dependent up-regulation of Ptc1 underlies proliferation impairment of neural precursors in Down syndrome.

Nazzari S, Mitraga VM, Valleri E, Puchta C, Rizzi S, Quisti S, Perni G, Barbareschi R, Cioni E.

Department of Human and General Physiology, University of Bologna, Italy.

Abstract
Mental retardation in Down syndrome (DS) appears to be related to severe neuropathies impairment during critical phases of brain development. Recent lines of evidence in the cerebellum of a mouse model for DS (the Ts65Dn mouse) have shown a defensive responsiveness to Sonic Hedgehog (Shh), a potent mitogen that controls cell division during brain development, suggesting involvement of the Shh pathway in the neuropathological defects of DS. Based on these premises, we sought to identify the molecular mechanisms underlying the derangement of the Shh pathway in neural precursor cells (NPCs) from Ts65Dn mice. By using an in vitro model of NPCs obtained from the subventricular zone and hippocampus, we found that trismic NPCs had an increased expression of the Shh receptor Patched 1 (Ptc1), a membrane protein that suppresses the action of a second receptor, Smoothened (Smoo). Therefore maintaining the pathway in a repressed state. Partial silencing of Ptc1 expression in trismic NPCs restored cell proliferation, indicating that proliferation impairment was due to Ptc1 overexpression. The overexpression of Ptc1 in trismic NPCs resulted from increased levels of AICD, a transcription-promoting fragment of amyloid precursor protein (APP), and increased AICD binding to the Ptc1 promoter. Our data provide novel evidence that Ptc1 overexpression underlies the derangement of the Shh pathway in trismic NPCs with consequent proliferation impairment. The demonstration that Ptc1 overexpression in trismic NPCs is due to an APP fragment provides a link between this trismic gene and the defective neuronal production that characterizes the DS brain.


A SP1/MIZI/MYC repression complex recruits HDAC1 at the TRKA and p75NTR promoters and affects neuroblastic malignancy by inhibiting the cell response to NGF.


University of Bologna, Department of Biology, Bologna, Italy.

Abstract
Neuroblastoma is the most common extracranial solid tumor of childhood. One important factor that predicts a favorable prognosis is the robust expression of the TRKA and p75NTR neurotrophin receptor genes. Interestingly, TRKA and p75NTR expression is often attenuated in aggressive MYCN-amplified tumors, suggesting a causal link between elevated MYCN activity and the transcriptional repression of TRKA and p75NTR, but the precise mechanisms involved remain elusive. We have shown that MYCN also acts directly to repress TRKA and p75NTR gene transcription. Specifically, we found that MYCN levels were critical for repression and that MYCN targeted proximal core promoter regions by forming a repression complex with transcription factors SP1 and MIZ1. When bound to the TRKA and p75NTR promoters, MYCN recruited the histone deacetylase HDAC1 to induce a repressed chromatin state. Forced re-expression of exogenous TRKA and p75NTR with exposure to the HDAC inhibitor TSA sensitized neuroblastoma cells to NGF-mediated apoptosis. By directly connecting MYCN to the repression of TRKA and p75NTR, our findings establish a key pathway of clinical pathogenicity and aggressiveness in neuroblastoma.

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Direct and coordinate regulation of ATP-binding cassette transporter genes by Myc factors generates specific transcription signatures that significantly affect the chemoresistance phenotype of cancer cells.


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Abstract
Increased expression of specific ATP-binding cassette (ABC) transporters is known to mediate the efflux of chemotherapeutic agents from cancer cells. Therefore, establishing how ABC transporter genes are controlled at their transcriptional level may help provide insight into the role of these multifaceted transporters in the malignant phenotype. We have investigated ABC transporter gene expression in a large neuroblastoma data set of 251 tumor samples. Clustering analysis demonstrated a strong association between differential ABC gene expression patterns in tumor samples and amplification of the MYCN oncogene, suggesting a correlation with MYCN function. Using expression profiling and chromatin immunoprecipitation studies, we show that MYCN oncoprotein coordinately regulates transcription of specific ABC transporter genes, by acting as either an activator or a repressor. Finally, we extend these notions to c-MYC showing that it can also regulate the same set of ABC transporter genes in other tumor cells through similar dynamics. Overall, our findings provide insight into MYC-driven molecular mechanisms that contribute to coordinate transcriptional regulation of a large set of ABC transporter genes, thus affecting global drug efflux.