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GENETIC AND PHARMACOLOGICAL MODULATION OF THE MYCN/MAX/MXD NETWORK

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ABSTRACT

Amplification of the MYCN gene is common to many types of infancy cancer of mostly neuroendocrine origin, including neuroblastoma (NB). Since identification of the correlation between MYCN status and poor NB prognosis, many efforts have been made to develop efficient MYCN targeting drugs. The rationale for choosing MYCN as a therapeutic target for NB treatment lies in its restricted spatial and temporal expression related to the early stages of embryonic development and its undetectable levels in adult tissue. Moreover, it is found deregulated in highly malignant cancers.

As the other MYC oncoproteins, MYCN forms a functional transcription factor when associated with another basic helix-loop-helix-leucine-zipper BHLHz protein named MAX and carries out its functions as a positive modulator of gene involved in proliferation and self-renewal. Together with MYCN, MAX belongs to an extended network of transcription factors which interact with each other binding DNA and regulating transcription of target genes. Thus, MYCN transcriptional activation is dependent on MAX availability. On the other hand, repressive side of the network is represented by MNT-MAX dimers which repress transcription of MYCN target genes.

In this thesis, two approaches have been adopted to antagonize the oncogenic features of MYCN-overexpressed NB cells.

A first genetic approach is based on the modulation of the expression levels of MAX and MNT network proteins. Published gene arrays on Kocak dataset cohort of 643 patients highlight that low levels of MYCN antagonists MAX and MNT in presence of MYCN amplification, are related to good clinical outcome. Using RNA interference in MYCN-amplified cells, we have demonstrated that downregulation of MAX and MNT affects in first instance cell proliferation. Moreover, using retinoic acid treatment, it has been demonstrated that knockdown of MAX and MNT promotes neuronal differentiation and induces both expression of positive prognostic markers and downregulation of negative ones. Thus, suppression of the malignant phenotype after MAX and MNT silencing has been confirmed.

On the other hand, same Kocak dataset pointed out the correlation between high levels of MAX and MNT and short term survival probability in MYCN amplified NB patients. Using transient transfection of MYCN-amplified cell lines, the correlation between high

MYCN antagonist levels and the enhancement of the malignant phenotype has been confirmed. In this first part of the work, it has been established that it is not the unique MYCN amplification to exactly reflect the biology of NB, but MYCN functions are strongly related to the expression of MYC/MAX/MXD network proteins.

To date, despite the attractiveness of MYCN as a therapeutic target and the discovery of new MYCN targeting compounds, most of these drugs have not passed the pre-clinical stage because of their non-specificity or selection of resistant clones. A second approach to counteract MYCN oncogenicity has been proposed and a novel iron chelator M606 derivative of hydroxyquinolones was identified for its ability to downregulate MYCN only in actively proliferating cells. Moreover, characterizing M606 action on MYCN promoter, deregulation in E2F/RB pathway has been found.

Through chromatin immunoprecipitation, it was highlighted the role of deacetylation and not methylation in mediating MYCN downregulation. E2F1 and E2F2 consensus have been found necessary to induce M606-mediated downregulation of a reporter gene under control of MYCN promoter.

Activator E2F family are proteins involved in cell cycle regulation and their activity is strictly related to RB phosphorylation state. We further pointed out the role of M606 in inducing hypophosphorylated state of RB supporting the hypothesis of RB sequestration of E2F transactivation domain, thus resulting in downregulation of E2F target genes such as MYCN. Same results were obtained using Exjade, an iron chelator used in NBs clinical trial provide evidence for a general mechanism related to iron chelator compound family.

Studies have revealed E2F/RB pathway involvement in the negative regulation of MYCN induced by TGFβ and via RT-qPCR it has been suggest a putative role of TGF cytokine family in mediate M606 downregulation. Thus, in this part of the work M606 action has been characterized and through comparison with Exjade, lines of evidence of deregulated E2F/RB pathways in Neuroblastoma have been further provided.

Overall, the present results support the possibility to offset MYCN driven oncogenesis through genetic or pharmacological approaches.

INTRODUCTION

1 Neuroblastoma

1.1 Clinical and Biological Characteristics of Neuroblastoma

Neuroblastoma (NB) is an embryonal malignant solid tumor of the sympathetic nervous system. It is the most frequently diagnosed neoplasm during infancy, characterized by a median age at diagnosis of 19 months but more than 90% of the diagnosed cases are children aged less than 5 years [1].

The tumor often grows rapidly and there is a direct correlation between age and extent of disease. In almost half of all patients at the time of diagnosis, the disease has already metastatized to the bone marrow, liver, cortical bone, skin and the lymph tissue and it is usually refractory to chemotherapy [2].

It is the second most frequent extracranial malignant cancer occurring in childhood after leukemia and, among the various solid tumors of infancy, after those affecting the central nervous system, it is the most common (8-10%), with an incidence of 10.5 cases per million per year [3].

Neural crest-related precursor cells are the presumptive source of this neoplasm: this transient population of cells produces multipotent progenitor cells that migrate and give rise to the enteric nervous system, the peripheral nervous system, pigment cells, Schwann cells, and cells of the craniofacial skeleton and adrenal medulla [4].

Given the wide lineage these cells can give rise to, the tumor can arise anywhere along the sympathetic chain concurring to the heterogeneous histology and pathology of NB.

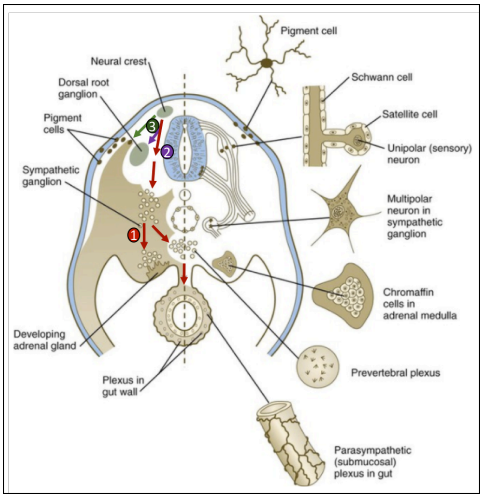


Fig.1. Neural crest migratory pathways.

Left, Pathways in the early embryo. Red arrows indicate the first emigrating cells which follow the ventral sympathoadrenal line. Purple arrow highlights the second wave of emigrating cells (pathway 2) that follows the ventrolateral pathway. Pathway 3 indicated by the green arrow underlines the last cells that leave the neural tube and through the dorsolateral pathway, go on to differentiate into pigment cells.

Right, Cells originate after migration of neural crest cells [4].

Most primary tumors (65%) appear to arise in the abdomen especially in the medullary region of the adrenal glands and in the extra-adrenal paraspinal ganglia followed by the mediastinum [3]. Thus, NB is thought to be preferentially a sympathoadrenal lineage

neural crest-derived tumor. Less common sites are the sympathetic ganglia in the neck, head, chest and pelvis [5].

Given its origin, the disease has overall a broad spectrum of clinical features directly related to the site, extent and biological features of the primary tumor and the presence of metastasis [6]. Cancer grade is directly correlated to degree of cellular and extra-cellular maturation: in children with a median age of less than 2 years the most undifferentiated and aggressive NB form is commonly found, while ganglioneuroma, the more mature tumor type, affects older age groups [6]. During the past years, a significant enhancement in survival probability of low risk NB patients has been achieved, but for what concerns high-risk clinical phenotype patients, long term survival is still less than 40% [7].

However, because of unexpected clinical behaviors of this tumor, such as maturation or spontaneous regression in some patients and lethality prior to reproductive age in others, it is possible to observe different clinical pictures at the onset of NB:

Localized tumors: about 40% of patients has a localized accidentally discovered disease that usually respond to chemotherapy and/or surgical removal.

Metastatic disease: about half of the patients have hematogenous metastasis, the spread of the primary tumor into adjacent lymph nodes is less common [3].

4S disease: in 1971 D'Angio and colleagues first described this distinctive condition with a case study on 5% of patients. The primary tumor has reduced dimensions with various metastasis that spontaneously regress. Interestingly, among all tumors, NB is characterized by the highest percentage (5-10%) of spontaneous regression or differentiation into ganglioneuroma without pharmacological treatment [8]. Given these clinical data, comprehension of molecular mechanisms underlying spontaneous regression/differentiation may advantage therapeutic approaches to drive these phenomena.

1.2 Clinical Prognostic Indicators: Grading and Staging

Clinical staging, essential to help predict outcome and select suitable therapy, classifies patients into high, intermediate and low risk groups based on:

Age: the main prognostic factor, inversely related to outcome and independent of tumor stage [9].

International Neuroblastoma Pathology Classification (INPC): after revision of the original Shimada system, it describes four categories of NB patients divided for their

degree of neuroblastic maturation toward ganglion cells, schwannian stromal development and mitosis-karyorrhexis index (MKI):

• Neuroblastoma Schwannian stroma-poor with variable histology, consisting of a large population of small poorly differentiated or differentiating neuroblasts

• Ganglioneuroblastoma intermixed Schwannian stroma-rich with a favorable histology, consisting of low-malignancy ganglion cells capable to metastasize

• Nodular ganglioneuroblastoma, both Schwannian stroma-rich or poor characterized by favorable or unfavorable histology

• Ganglioneuroma, benign form characterized by presence of fully mature ganglion cells and predominance of dense stroma of Schwann cells [10].

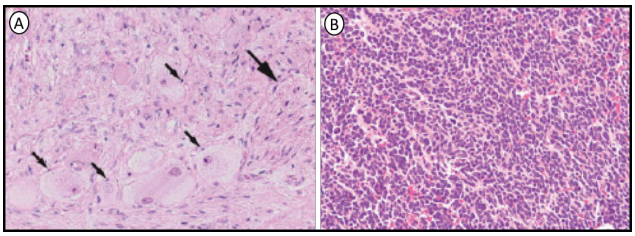


Fig.2. Differentiation grade in NBs.
A, Black arrows indicate ganglion and Schwann cells, peculiarity of stroma-rich NBs.
B, Stroma-poor NB consists of densely packed small round blue cells with small cytoplasm [3].

Tumor stage International Neuroblastoma Staging System: the currently used system based on degree of surgical excision of primary tumor and involvement of lymph nodes (Tab.1) [8].

STAGE	SURGICAL RESECTION	LYMPH NODES INVOLVMENT
1	Complete gross excision of localized tumor, with or without positive microscopic margins	Negative ipsilateral non-adherent lymph nodes Nodes attached to and removed with the primary tumor may be positive
2A	Incomplete gross excision of localized tumor	Negative ipsilateral non-adherent lymph nodes Nodes attached to and removed with the primary tumor may be positive
2B	Complete or incomplete gross excision of localized tumor	Positive ipsilateral non-adherent lymph nodes Controlateral lymph nodes negative
3	• Unresectable unilateral tumor inflitrating across the midline • Localized unilateral tumor • Unresectable midline tumor with bilateral extension	• Positive or negative regional lymph nodes • Controlateral positive regional lymph nodes • Unresectable due to positive bilateral lymph nodes
4	Any primary tumor with involvement of ski, bone, bone marrow, liver and/or other organs	Dissemination of distant lymph nodes
4S	Localized primary tumor with involvement of skin, liver and/or less than 10% of bone marrow cellularity **(only applies to children less than 1 year of age)**	

Table 1. International Neuroblastoma Staging System [8].

Biological manifestations (i.e. MYCN amplification and DNA ploidy): there is a reproducible correlation between a high MKI and presence of adverse clinical and biological manifestations [11].

1.3 Genetic Abnormalities in NB and Biological Prognostic Indicators

Neuroblastoma displays profound genetic heterogeneity that reflects the possibility to run into different tumor subtypes that range from spontaneous regression to rapid progression and death.

While its anatomic pattern of origin is well defined, the etiology of NB remains still unclear and even though the majority of cases appear to be sporadic, there are lines of evidence of hereditary NB.

Familial genetic lesions: Hereditary NB is both rare and heterogeneous and occurs in less than 5% of all NBs. This minor subset of patients inherits a genetic predisposition to disease that follows an autosomal dominant inheritance pattern with incomplete penetrance and is related to germ-line mutations in the short arm of chromosome 16 (16p12-13), though, to date, no causal genes have been characterized [12,13].

Germline mutations in paired-like homeobox 2B (PHOX2B) on chromosome 4p13, are the first specific predisposition mutations identified in NB; this gene is a regulator of autonomic nervous system development and it is expressed early in the developing sympathoadrenal progenitors promoting neuron formation and differentiation. Mutations in PHOX2B were found in about 7% of familial NB cases and only 2% of total cases suggesting that these mutations could give selective advantages to tumor cells but they are likely not sufficient to drive NB pathogenesis [14].

Other germline mutation commonly found in familial NB cause activation of ALK signaling. Anaplastic lymphoma kinase ALK (Ch. 2p23) is a member of receptor tyrosine kinases and is thought to have a role in the normal development of the central and peripheral nervous system. De Brouwer et al demonstrated that these mutations are found in similar frequencies in favorable and unfavorable outcome cases and occur in about 50% of familial NB cases but also 12% of sporadic NB cases [15]. Concordance in mutation patterns for NB in twins during childhood indicates the predominance of hereditary factors in tumorigenesis, whereas discordance in older twins underline the crucial role of random mutations and/or other factors [16-17].

Sporadic NB may also show a germline contribution, with greater effect sizes for rare pathogenic variants. Genome-wide association studies have allowed the identification of risk polymorphisms in several large independent studies. Maris and colleagues characterized three common SNPs within two overlapping genes, FLJ22536 and FLJ44180

at 6p22 locus: high-risk NB and low patient survival was significantly associated with homozygosity for any of these risk alleles. Association with high-risk NB was found also for the low frequency germline variants of BRCA1-associated RING domain1 (BARD1) gene [14]. Heterogeneity of the disease suggests that pattern of acquired genetic alterations defines the tumor phenotype: it has been found that somatic change such as change in tumor-cell ploidy or gain/loss of alleles and oncogene activation are the main mutations associated with sporadic NB development [18].

DNA plody: Low-stage tumors are hyperdiploid or near-triploid, usually associated with good prognosis and commonly found in patients of less than 1 years of age. Despite the clinical predictability of ploidy in infants, this prognostic significance is lost for patients older than 1-2 years. This is reasonably due to the presence of several structural rearrangements in hyperdiploid/near-triploid tumors of older patients that are absent in infants with whole chromosome gains [19].

Chromosome gain: Trisomy of chromosome 17q occurs in about half of the NB primary tumors. Gain of 17q is associated with tumor malignancy, poor prognosis and MYCN amplification but its definition as independent prognostic factor is still controversial [12]. Although the breakpoint of 17q varies, the aberration is often due to unbalanced translocations of segment 17q21-qter to the distal part of chromosomes 1p or 11q. Translocated genetic region could reach 20Mb of 17q counting for more than 200 genes and at least 30 translocation sites on 20 different chromosomes have been identified [5,3].

Chromosome loss and tumor suppressor genes: Loss of heterozygosity LOH of the short arm of chromosome 1p has been found in about 25-35% of all NBs and in 70% of the advanced stages [20]. It correlates both with gain of 17q and MYCN amplification and it is independently associated with poor clinical outcome [21]. Although the responsible mechanism for this aberration is still unknown, relevance of 1p LOH is underlined by studies indicating that transfer chromosome 1p material into human NB cells *in vitro* induce suppression of tumorigenicity and differentiation [22]. Potential candidate tumor suppressor genes (TSGs) in the 1p LOH span a region of about 260kb at 1p36.3 and to date, three putative TSGs have been identified: microRNA-34a (mir-34a), the chromodomain helicase DNA-binding domain 5 (CHD5) and the kinesin superfamily protein 1B beta (KIF1B) all three of these proteins with a role in cell growth [23-25].

LOH of 11q has been identified in approximately 40-45% of primary tumors without MYCN amplification. Although it is inversely correlated with MYCN amplification, this aberration is related to adverse clinical features and histology resulting in a useful marker in determining the prognosis for MYCN-not amplified NBs [26]. Putative TSGs identified are H2AFAX with a role in genomic stability and CADM1 which encodes for an adhesion protein involved in neural cell development [27].

About 16-27% of NB patients show LOH of 14q without showing association with a precise clinical stage, suggesting that this aberration may be a universal early event during tumor development [28]. However, it is not the single genetic marker, but the overall segmental genomic profile of tumors that adds information to patient prognosis [18].

Abnormal expression of the neurotrophin receptor: The Trk family of neutrophin signaling receptors (TRKA, TRKB and TRKC) are strongly correlated with the biologic and clinical features of NB tumors mediating proliferation, differentiation and death via the binding of nerve growth factor ligands [29]. Favorable NBs show expression of both TRKA and TRKC. High levels of the nerve growth factor receptor TRKA in association with NGFR and very low/no MYCN expression, were found in favorable NBs while a novel TRKA splice variant promoting cell survival and angiogenesis has been found in advanced-stage tumors. In contrast, unfavorable MYCN amplified tumors often co-expresses the full length TRKB neurotrophin receptor and its ligand brain-derived neurotrophic factor BDNF. Interestigly, the truncated form of TRKB is expressed in some favorable NB tumors. These tumors also express low level of TRKC ligand neurotrophn-3 and high levels of TRKA [30-31].

Amplification of loci: Amplification with the strongest prognostic value in NB involves MYCN gene. It is present in approximately 20% of all cases and 40% of advanced stages of disease and even in patients with lower stages of disease and it is related to highly malignant phenotype and poor prognosis. Schwab and colleagues identified the amplified genetic region as a large region ranging from 100kb to 1Mb from locus 2p24 copied to form double-minute chromatin bodies (DMs) that are extrachromosomal circular elements that probably accumulate by uneven segregation during mitosis. The amplified DNA region can also integrate into chromosomal locus to form homogeneously staining regions or HSRs, typical of the cultured NB cells [5,32].

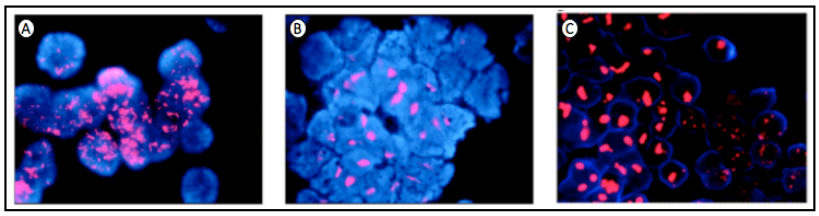


Fig.3. Image of fluorescence *in situ* hybridization image of neuroblastomas depicting *MYCN* amplification manifested as DMs (*A*), HSRs (*B*), or dmins + HSRs (*C*) [33].

The significance and the relevance of *MYCN* amplification in NB pathogenesis was first established in the early 1980's from Brodeur and Seeger that pointed out the association with advanced stages of disease and poor prognosis [34].

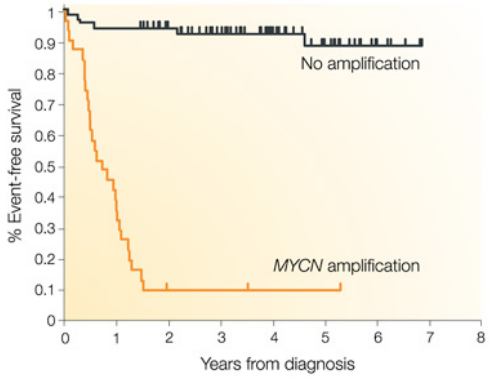


Fig.4. Kaplan–Meier survival curve of infants less than 1 year of age with metastatic NB. The 3-year event-free survival probability (EFS) of MYCN-not amplified NB patients was 93%, whereas those with MYCN amplified tumor had only a 10% EFS [5].

Years later, Weiss et all, revealed the overall impact of MYCN confirming that overexpression of MYCN alone was sufficient to initiate NB formation in mice [35]. Even if there is a corresponding overexpression of MYCN protein in MYCN-amplified NBs, it is still controversial whether MYCN protein or mRNA has prognostic significance in tumor lacking MYCN amplification [5].

However, since MYCN oncogene amplification occurs in about 20% of NBs, other factors may probably be involved in the early stages of tumor initiation and formation. For instance, ALK activation results in increased expression of MYCN transcription and increased stabilization of MYCN protein likely via activation of AKT and ERK. Furthermore, genomic localization of ALK and MYCN on chromosome 2p may explain their co-amplification in NBs. However, no NB has been shown to amplify another gene that did not also amplify MYCN [36]. Several other amplifications have been also identified in NB cases and include amplification of DDXI gene at 2p24, MDM2 gene at 12q13, the MYCL gene at 1p32 and unidentified DNA region from chromosome loci 2p22 and 2p13 [37,38].

2 Genetics of Neuroblastoma

2.1 MYC Oncogenes and Oncoproteins

The Myc proto-oncogene family is composed by three well-defined members, MYC, MYCN and L-MYC. From Bishop et al. pioneering work arose MYC discovery as the cellular homolog to the transforming gene v-myc of the avian MC29 myelocytomatosis transforming retrovirus [39,40].

On the heels of this revelation were the discoveries of other two genes paralog to MYC and related in gene structure and nucleotide sequence that were shown by cytogenetic analyses to be amplified in various human cancers. In 1983 MYCN was identified as an amplified gene found in NBs, while later small-cell lung cancers were found to carry amplified L-MYC [41,42]. Then, Schwab and colleagues point out the involvement of Myc oncogene family amplification in a variety of cancers, including retinoblastoma, small cell lung cancer cells, glioblastoma, medulloblastoma and astrocytoma [43,44].

Although a good degree of homology, Myc family members are characterized by a slightly different expression pattern that is tissue- and stage-specific: MYC is specifically expressed in proliferating tissues in the adult, whereas MYCN is present in many proliferating neonatal tissues and at highest levels in pre-B cells, forebrain, kidney, intestine and hindbrain. During gastrulation, MYCN levels are high in the embryonic mesoderm whereas MYC expression is mostly restricted to extraembryonic tissues. During differentiation, MYCN expression has been shown to persist in some tissues such as the retina, telencephalon, and intestine, where MYC is downregulated [45,46]. Both proliferative and differentiative compartments of the neural tube and brain expressed L-MYC, together with the developing kidney, as well as the newborn lung [47,48].

Structurally, genes have a three-exon organization with long 5′ and 3′ untranslated regions (UTRs). Exon 2 and 3 contain the highly homologous major coding domain, whereas first exon is not conserved among family members but rather has regulatory functions [49]. They encode similarly sized nuclear phosphoproteins (~50–55 kDa for MYC and MYCN while ~40 kDa for L-MYC) which contain highly conserved clusters of amino acids that are likely to be important for nuclear targeting, nucleic acid binding and *in vitro* transforming activities [50]. Organization of Myc transcription factors is similar among paralogs and, to a lesser extent, its orthologs throughout evolution especially for certain

domains, whereas many of the sequence outside these well-defined domains are divergent among paralogs. In Figure 5, the functionally important conserved regions are diagrammed.

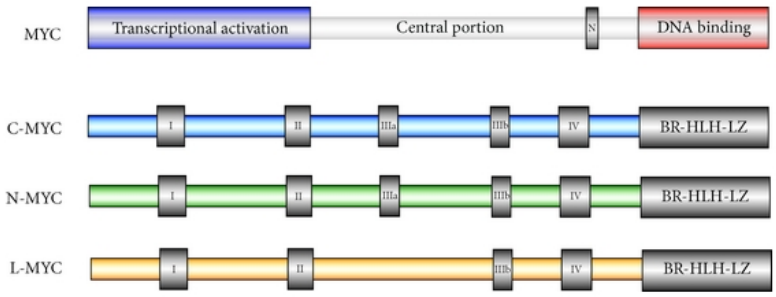


Fig.5. Architecture of the MYC oncoprotein family. On the top, it is shown a generic representation of MYC protein. Below is a representation of conserved sequences present in the other family members. c-MYC is drawn to scale at 439 amino acids while N- and L-MYC proteins are shown not in scale. They are respectively 464 and 364 amino acids in length, due to differences in the length of nonconserved sequences. MYC proteins possess: 1) a large unstructured amino-terminal region named TAD involved in transcriptional activation and containing the conserved region named MYC boxes MBI mainly involved in the regulation of MYC protein stability, and MBII that modulates the interaction with proteins such as GCN5, TRRAP, TIP48 and TIP60; 2) a middle segment rich in proline, glutamic acid, threonine and proline residues PEST as well as two conserved MYC boxes MBIII and MBIV and a nuclear localization sequence; 3) a C-terminal region with the basic helix-loop-helix leucine zipper bHLHz domain, necessary for the nucleic acid binding and for interaction with MAX [51].

Compared to other oncoproteins such as SRC or RAS, MYC is unique because its coding region is rarely mutated. Instead, MYC's oncogenic properties are unleashed by regulatory mutations leading to unconstrained high levels of expression [51].

2.1.1 MYCN Functional Activity as Activator and Repressor

As for all proto-oncogenes, distinction must be drawn between their "normal" functions in untransformed cells and their "pathological" functions in tumor cells: even if the two are related, carcinogenic functions may represent only a subset of the physiological. MYC acts as a sensor integrating cellular signals and mediating transcriptional response that drives various cellular mechanisms. By activation (cyclin D1, D2, E, cdk4, cdc25a, id2, cul1, cks2) and repression (p15, p21, p27) of its target genes, MYC activates cyclin D1(D2, D3)/Cdk4(6) as well as cyclin E/Cdk2 and inactivates retinoblastoma protein RB driving cells through G_1-phase and resulting in S-phase entry. Thus, the Myc protein family potently stimulates proliferation and inhibits differentiation [52]. Given the involvement of this transcription factors also in cell growth, immortality and genomic instability, in reducing cell adhesion and stimulating angiogenesis and metastasis, it is well established that deregulated proteins possess high transformation potential and are associated with poor prognosis in various types of tumors [53]. However, in the absence of sufficient

amounts of survival factors, MYC can induce apoptosis. This represents a security mechanism against hyperproliferative signaling and limits its proliferation-stimulating effect to situations where proliferation is appropriate [54].

The MYC family of proteins are an atypical family of transcription factors in that they can regulate approximately 10-15% of the whole genome [55]. As for the other members of the family, MYCN can exerts its functions as transcription factor only when associated with a small protein named MYC Interacting Protein X or MAX. As with all transcription factors, MYCN directly or indirectly recruits different sets of interacting proteins or cofactors to engage the basal transcriptional machinery. However, MYC–MAX interacts with a multiplicity of factors such as chromatin remodelers or antipausing factors, as well as other transcription factors such as MIZ-1 and the estrogen receptor. Thus, MYCN's precise function in transcriptional activation and repression reflects the specific factors recruited, the chromatin context of the target gene and the presence of other transcription factors proximal to the binding site [53]. Furthermore, MYCN was shown to binds to histone modifiers and remodel domain of euchromatin that contain active gene highlighting a function that is independent from its activity as transcription factor and leading to hypothesized that MYCN can act as an enhancer and regulate genes at distance [56].

MYCN Canonical transactivation: The most relevant model of MYCN-mediated transcription activation postulates that MYCN increases local histone acetylation to keep chromatin in an active state in the promoter regions of target genes through interaction with TRRAP (TRansactivation/tRansformation Associated Protein). TRRAP acts as a scaffold for assembling multiprotein complexes to chromatin involving histone acetylation HAT complexes such as the SAGA complex SPT/ADA/GCN5/Acetyltransferase or the TRRAP-TIP60 complex, formed by TIP60 an H2A/H4 acetylase and the ATPase/helicase motif-containing cofactors TIP48/49 involved in chromatin remodeling that moves or displaces nucleosomes [57-59]. Factors such as positive transcription elongation factor b P-TEFb and TFII-H that stimulate transcriptional elongation through phosphorylation of Ser2 of RNA polymerase C-terminal domain, can also be recruited to the MYC/MAX dimer. Lastly, MYC has also a function in controlling RNA pol II promoter clearance through regulation of RNA pol II kinases expression via mRNA cap methylation, polysome loading and rate of translation [60].

MYCN Repression: MYC-MAX heterodimers can also interact with other transcription factors and bind to Inr initiator element consensus to repress transcription. There are evidences that MYCN can directly be recruited to non-E-box promoter of target genes through association with other transcription factors, including SP1/SP3, MIZ-1, and NF-YB/NF-YC. Myc-interacting zinc finger protein Miz-1 transactivates tumor suppressor genes as well as genes involved in cell cycle regulation through recruitment of the histone acetyltransferase p300. MYC interaction with Miz-1 and other transcription factors causes repression by recruiting HDACs to MYC-Miz complex and moreover through disruption of Miz-p300 interaction. Moreover, MYC-Miz complex inhibits MYC ubiquitination and subsequent degradation promoting MYC stabilization. [61] MYC is also involved in repression of target gene promoters via methylation, indeed MYC-MIZ-1 complex can also recruit the DNA methyltransferase DNMT3a. MYC represses many genes through induction of the tumor suppressor PTEN, which activates the histone-lysine *N*-methyltransferase EZH2 via AKT phosphorylation inducing genome trimethylation of H3K27. Moreover, this is also the autoregolatory mechanisms used by MYC to limits its own expression [58,61]. Repression of genes that do not contain Inr sequences occurs through specific GC-rich regions recognized by MYC-formed complexes with factor such as the zinc-finger SP1 and subsequent recruitment of chromatin modifiers such as HDAC1 [62].

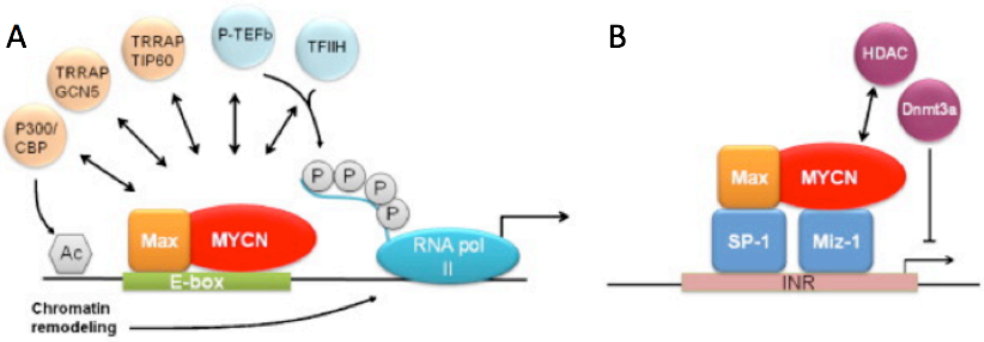


Fig.6. Mechanisms of MYCN activation and repression of target gene transcription. A) MYCN-MAX transactivation occurs via E-box binding. Recruitment of histone acetyl transferases (HATs) including p300/CBP, GCN5- and Tip60-containing TRRAP complexes result in an open chromatin state, and MYCN-promoted phosphorylation of the C-terminal domain of RNA polymerase II. B) Repression mediated by MYCN of Miz-1/Sp-1 induced transcription. MYCN-MAX dimer recruits various factors including DNA methylase 3a (Dnmt3a) and histone deacetylases (HDACs) that induce a repressed chromatin state [62].

2.1.2 Regulating MYCN Expression

The involvement of the Myc protein family in so many cellular mechanisms necessitates to finely control its expression and protein fate at almost every level known to molecular biology: transcription initiation and elongation, translation, stability of mRNA and protein and furthermore, protein activity is regulated by posttranslational modifications and interacting proteins [55]. Regulation occurs also through short half-lives (~30 minutes) of MYC mRNA transcripts and proteins and through their fine expression as cells enter the cell cycle. Indeed, MYCN has a strictly spatial and temporal expression related to embryonal development of the peripheral nervous system [4]. Neural crest-related precursor cells are multipotential and migrating cells that give rise to the enteric nervous system, the peripheral nervous system, pigment cells, Schwann cells and cells of the adrenal medulla and craniofacial skeleton. MYCN expression is high when these migrating cells are in highly proliferative state, while MYCN expression decreases when cells undergo differentiation. Any dysregulation of MYCN expression impairs the ability of progenitor cells to undergo differentiation [45]. Furthermore, tissue specific expression of MYCN is due to the presence of TSE sequence (tissue-specific element) within the first intron that acts at posttranscriptional level [63].

Regulation of MYCN protein level: Several proteins have been described that alter the stability of MYCN mRNA and its translation efficiency [64]. Interestingly, some of the well characterized proteins mediating MYCN mRNA stability binds to AU-reach elements ARE at 3'UTR, some of them with a positive action such as Mdm2, Hud and Wig-1, and others with a negative action on MYCN stability, i.e. TAp73. Furthermore, MYCN seems to auto-regulate itself through modulation of target genes encoding for proteins involved in its stability. Indeed, Mdm2 is a direct positive target of MYCN, while MYCN can repress transcription of TAp43 [65-68].

Several studies identified individual miRNAs targeting 3'UTR of MYCN as involved in negative regulation of its stability or translation (e.g. miR-30e, miR-29a/b/c, miR-34a, miR-19a/b, miR-101, miR-181a, miR-202, miR-449a/b and let-7c/e). The best characterized *MYCN*-targeting miRNA is miR-34a located at ch.1p36, a frequently deleted region in *MYCN*-amplified NBs. Overexpression of mir-34a in MYCN-amplified NB cell lines, as well as let-7 and miR-202, inhibits MYCN mRNA translation [25,69]. However, miRNAs-MYCN interactions are mutual, as MYCN itself targets miRNAs.

Indeed Speleman et al. demonstrate that MYCN-targeting miRNAs are downregulated during MYCN-driven tumor formation [70].

Another level of MYCN protein regulation is interdependent posttranslational modifications, that affect the activity, stability and degradation of MYCN. As for other MYC family members, MYCN is phosphorylated at multiple sites located over the entire protein. Luscher et al. identified a region that lies within a PEST sequence involved in MYCN degradation as substrate of kinase CK2, an enzyme which shows enhanced expression in many tumors [71-73]. Also the dimerization partner of MYC, MAX is a CK2 substrate and phosphorylation of MAX results in change in DNA binding properties of MYC/MAX complexes. Together these findings suggest a role of CK2 in stabilizing MYC protein and in regulate DNA binding properties [74].

A further phosphorylation area located within the TAD of MYCN, involves Thr-58 and Ser-62 amino acid residues whose phosphorylation is tightly interdependent. In response to activation of the MAPK signaling, Cdk1 phosphorylates Ser-62 providing a priming for GSK3β binding, a second kinase which subsequently phosphorylates Thr-58. Dephosphorylation of Ser-62 through PP2A protein phosphatase 2A sensitizes MYC proteins to bind the tumor suppressors Fbxw7 or Huwe1 and recruit a ubiquitination complex to MYCN protein. Aurora A kinase has been recently identified as overexpressed at both mRNA and protein level in primary MYCN amplified NBs and its high expression levels correlates with a high-risk group and poor prognosis. It has been shown that AurKA inhibit degradation of ubiquitinated MYCN via inhibition of Fbxw7 pathway [75,76].

Another post-translational modification to which MYC is subject is lysine acetylation. Indeed, it has been established that stimulation of acetylation decreases ubiquitination of MYC and enhances its stability, given also that some of the identified acetylation sites overlap with the ubiquitination sites [75]. In absence of growth factor-dependent signals, proteosomal degradation of MYCN allows cells to exit the cell cycle and undergo differentiation [76].

Transcriptional control of MYCN: First, MYCN gene expression is regulated at transcriptional level. MYCN promoter region has been shown to have consensus for transcription factors that positively (e.g. Shh, E2F, Sp1-3) or negatively (e.g. ROR) regulates its expression in response to different mitogenic signals [64].

During the last years, it was shown that MYCN is an essential downstream effector of Sonic hedgehog (Shh) signaling pathway and this transcriptional activation is required for both normal and neoplastic proliferation of neuronal precursors in the cerebellum. Importantly, mouse model with constitutively activated Shh signaling results in medulloblastoma formation but no evidence of hyperproliferation or tumor formation was detected in mice nullizygous for MYCN [77]. MYCN is also a pivotal target for Wnt signaling in promoting proliferation and differentiation of neocortical neurons via β-catenin/Tcf3 transcription complex pathway [78].

Regulatory sequences are also located outside the promoter region: the previously mentioned TSE located within intron 1 lies near a consensus binding site for RORα1 and RVR designated as RORE (ROR response element). Negative regulation of ROR was demonstrated through mutation of RORE sequence and subsequent increase of the oncogenic potential of MYCN gene in rat embryonic fibroblast [79]. The multiple signal transduction cascade that target MYCN promoter are often deregulated in cancer cells and contribute to enhanced its expression but amplification of MYCN in the form of DMs or HSRs and the resulting overexpression seems to be of greater impact for the malignancy of the NBs [75,11].

Strieder et al. identified three transcription factors involved in MYCN expression in MYCN amplified NB cells. His work demonstrates the binding of E2F-1, 2 and 3 to the proximal MYCN promoter *in vivo* and that inhibition of E2F activity in through overexpression of $p16^{INK4A}$ induced a reduction MYCN expression. There is also lines of evidence that E2F proteins are involved in the negative regulation of *MYCN* by TGF-β and retinoic acid. These data underline the role of E2F transcription factors in both *MYCN* activation and repression in neuroblastoma [80]. E2F/Rb pathway is essential for modulation of cell cycle, differentiation and survival of various cell types in the developing and adult CNS and, although neither RB gene mutations nor any other protein mutations known to inactivate the pRb pathway in other types of tumor have been detected in NBs, recent evidence suggests that E2F/Rb activity in NBs may be deregulated [81].

2.2 The E2F/Rb Pathway in Disease Development

Proper development of CNS requires coordination between proliferation, differentiation and survival input signals from the environment that activate transduction pathways and converge on the E2F/Rb molecular switch [82]. One of the hallmarks of cancer cells is their insensitivity to antiproliferative signals, such as antigrowth factors. From novel studies, the central role of retinoblastoma protein RB and its relatives p107 and p130 have emerged, in mediating these antiproliferative signals. Indeed, pRb pathway is found deregulated in a large number human cancers. This pathway involves also the negative regulators of cell proliferation INK4 family cyclin-dependent kinase inhibitors, and the positively acting Cyclin D/CDK4-CDK-6. The downstream effectors in the pathway are the E2F transcription factors [83].

2.2.1 RB Proteins

RB1 was the first identified tumor suppressor gene whose sequential loss or inactivation of both alleles was claimed to be responsible for the retinoblastoma. Gene encodes for pRB, a member of the pocket protein family RB together with its relatives p107 and p130 encoded respectively by RBL1 and RBL2 genes. The central core of RB-family proteins is the 'small pocket' domain defined as the minimal domain necessary to bind to viral oncoproteins such as simian virus TAg through their peptide LXCXE motif, essential for stable interaction and present in many chromatin regulating proteins with which RB proteins interact [84]. (Fig. 6)

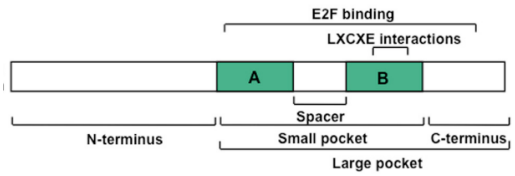


Fig.7. Architecture of the RB protein family. The main homology sequence lies in the well-conserved *small pocket* region, which consists of A and B domains (*green*) that represent a single cyclin fold domain together with a flexible interdomain linkers. The 'large pocket' is the minimal growth suppressing domain of RB-family proteins and it is capable of binding E2F transcription factors as well as viral proteins. p130 and p107 have also insertion subject to regulatory phosphorylation to maintain protein stability and a CDKs interacting regions. Revisited from [84].

The large pocket (C-terminal domain and small pocket) is sufficient to interact with E2F family of transcription factors to control proliferation, apoptosis, differentiation and development. RB but not its relatives, has a docking site used only by E2F1, and a short peptide region in the C-terminus that is competitively occupied by cyclin/cyclin dependent kinases (CDKs) or protein phosphatase 1 (PP1) [84].

2.2.2 E2F Proteins

E2F transcription factor family positively (E2F-1 to E2F-3) and negatively (E2F-3b to E2F-8) regulates expression of genes involved in cell cycle regulation and synthesis of DNA through binding to the canonical consensus TTTCCCGC and its slight variations in the promoter of target genes [85].

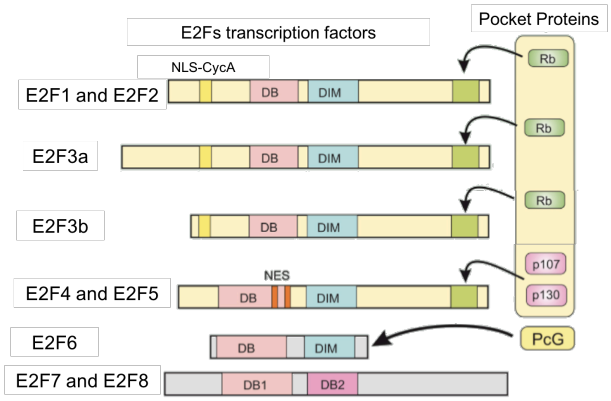


Fig.8. Domain structure of E2F family and their Rb family-binding partners. All E2F proteins have a DNA-binding domain (DB *pink*), and most of them also have a dimerization domain (DIM *blue*) which allows interactions with an obligate dimerization partner (DP). The Rb family-binding domain (*green*) located in the C-terminal region of the activators E2F1–E2F3 is responsible for binding retinoblastoma protein (Rb), whereas p107 and p130 bind to a similar domain on the repressors E2F4 and E2F5. E2F6 and E2F7 do not bind pocket proteins, but E2F6 binds to the Polycomb group of proteins (*PcG*). Thus, repressor E2Fs form two subgroups; E2F3b-E2F5, which bind RBs to form a functional repressive complex, and E2F6-E2F8, which act independently of Rb binding. The E2F1–E2F3 isoforms have a Cyclin A domain (*CycA yellow*) and a nuclear localization signal (*NLS*), whereas E2F4 and E2F5 have two nuclear export signals (*NESs*). Revisited from [85].

De Gregori et al. point out the overlapping functions of the E2Fs among the members of activators and repressors in the cell cycle control, but also unique functions during tissue homeostasis, development and tumor formation. Loss of all three activator E2Fs and/or overexpression of E2Fs 4 and 5 results in cell cycle arrest but mutant mouse models provide evidence for E2F-1 specific role also in apoptosis [86].

The regulatory essence of E2F complexes is based on the ability to interact with pocket proteins but also to bind DNA with high affinity, moreover, classical E2Fs (E2F1-E2F6) need to form heterodimers with DP proteins. Hypophosphorylated pocket proteins can bind E2F sequestering their transactivation domain and recruiting chromatin modifiers but in contrast, hyperphophorylated RB are unable to interact with thereby liberating their transactivation domain and allowing transcription of E2F target genes. Association of Rb to activator E2Fs masks their transactivation domain responsible for transcription of target genes and furthermore induces recruitment of chromatin remodeling complexes (e.g. HDAC); whereas binding of RB proteins to repressor E2Fs (E2F4-5) mediates recruitment of repression complexes to repress transcription [81,87].

2.2.3 Overview on E2F/RB Pathway and Cancer

Main hallmark of cancer cells is insensitivity to antiproliferative stimuli and during the last decade there has been a growing interest for the central role of RB proteins in mediating these antiproliferative signals for various reasons. Firstly, RB pathway is found deregulated in a large number human cancers and pathology. Alzheimer, Parkinson's disease and Amyotrophic lateral sclerosis show active E2F and aberrant or hyperphosphorylated Rb expression resulting in increased expression of E2F target genes, thus leading to the notion that neurodegeneration could be consequent to activation of apoptosis after abortive cell-cycle re-entry [88-90]. Indeed, the previously mentioned role of E2F1 in apoptosis was also confirmed in cortical neurons: its depletion conferred protection to cerebellar granular neurons from dopamine-induced cell death and to cortical neurons from ☙-amyloid induced death [81,91].

Moreover, this pathway involves the main regulator of cell proliferation [83].

Involvement in Cell cycle: E2F/RB pathway is a master cell cycle regulator, and the impairment of the pathway appears to be a necessary step in human oncogenesis. Phosphorylation of RB family proteins is sequentially mediated by different cyclin-CDK complexes in a temporal specific manner. In response to mitogens, cyclin D expression is upregulated and it forms complexes with CDK4 or CDK6 and phosphorylates Rb proteins preventing RB from binding to E2Fs. Thus, activator E2Fs, whose expression is maximum during S phase transition, are able to induce transcription when free from RB. On the other hand, repressor E2Fs are expressed all through the cell cycle, especially since their typical expression in quiescent cells, and play main roles in transcriptional repression in G0/G1 bound by Rb proteins [83]. The fine balance between repressor and activator E2Fs regulate cell cycle progression [92,93]. Disruption of the RB pathway induces cell cycle arrest through upregulation of CDK inhibitor $p21^{Cip1}$ or $p27^{Kip1}$ or if cells fail inducing cell cycle arrest, cells undergo apoptosis via activation of p53 pathway or induction of TAp73, a p53 family member, which activates various pro-apoptotic genes [87].

Evidence of the role of activator and repressor E2Fs in proliferation come from knockout or deregulation study: loss of all three activating E2Fs and/or overexpression of E2Fs 4 and 5 results in cell cycle arrest. Moreover, ectopic expression of activators E2Fs in quiescent immortalized rodent fibroblasts is sufficient to drive them into S phase. Mutant mouse models provide evidence of E2F1 specific role also in apoptosis and in proliferation

of progenitors of the central nervous system; knockdown of E2F3 points out its role in the proliferation of adult neural precursor. [84,85].

Effect of RB mutations in mouse models are indicated to be embryonic lethal and RB null mice shown increased apoptosis in the CNS especially in spinal cord, spinal ganglia and hindbrain.

Interestingly, despite RB expression in both intermediate and ventricular zone of the neural tube during development, the apoptotic cells distribution in the $Rb^{-/-}$ mice were confined to the intermediate zone, containing progenitors committed to the neuronal lineage suggesting a role for RB in the proliferation of adult neural precursor [94-96].

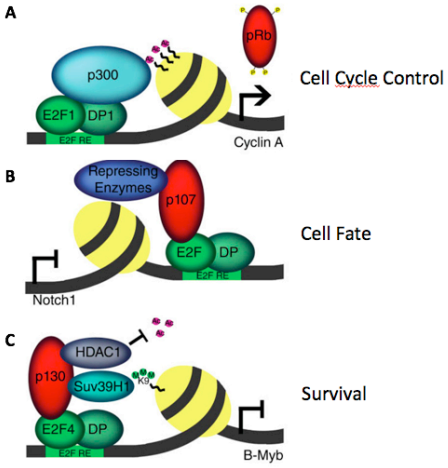


Fig.9. Examples of the known mechanisms of transcriptional regulation with which E2Fs coordinate cell-cycle progression, survival, cell fate, and proper cell development. A) acetyltransferase p300 is recruited by E2F1 to chromatin to promote the expression of cell-cycle related genes. B) An example of E2F-mediated repression during neuronal development: repressor E2F is necessary for the p107 recruitment to the Notch1 promoter. C) E2F4 is involved in adult neuronal survival by recruiting a complex formed by p130, HDAC1, and the methyltransferase Suv39H1 to the promoter of the proapoptotic gene B-Myb. Revisited from [81]

In the E2F/RB-mediated onset of pathology there could be distinct transcriptional mechanisms mediated by deregulated E2F or physiologically activated E2F. Although the released from RB protein, physiologically activated and deregulated E2F are functionally different: the first one activates only typical E2F targets, whereas deregulated E2F induced transcription of both typical and atypical E2F targets. This is due to the temporarily release from RB of physiologically activated E2F that is predicted, however, to be 'under control' of RB. On the other hand, deregulated E2F is totally out of RB control because of dysfunction on the related pathway. Atypical E2F target genes include the first identified ARF gene, the major activator of p53 pathways, the CDK inhibitor $p27^{Kip1}$ and the tumor suppressor Tap73 [87].

2.2.3 E2F/RB Pathway and Neuroblastoma

During past years, efforts have been made to clarify the genetic programmes at the base of generation of cell diversity in the nervous system. Recent finding points out that the diversity in the nervous system is due to extensive interactions and synergies between transcription factors of the homodomain HD and bHLHz families. Indeed, HD proteins such as Pax6 and Otx2 were shown to pattern to the neural primordium and the expression in mice of proneural bHLHz proteins such as Mash1, Math1 and Ngn1-3 were shown to induce generation of differentiated neurons [97]. Cooperation between proneural and inhibitory bHLHz and HD proteins was further demonstrated by Sugimori and colleagues' work, underlining a molecular code that results in cell cycle exit and differentiation and thus in spatial and temporal patterns of neurogenesis and gliogenesis [98].

During embryogenesis, RB proteins control proliferation and activation of a neurogenic gene expression program of specific populations of neuroblasts. Various neurogenic transcription factors are involved in neuroblasts differentiation and their transcriptional activity is finely controlled by ID (inhibitor of DNA binding) protein family through a sequestration mechanism.

ID protein family comprises four conserved transcriptional regulators firstly recognized for their ability to repress bHLHz protein activities and then also activities of ETS, paired box PAX and RB proteins. ID proteins have a role in the timing control of stem cell differentiation and cell fate during normal development [99].

Because the ID proteins are known to sequester RB, it is convincing that the amount of RB levels during early development could be crucial for the appropriate activation of the commitment program. Although the deeper understanding of ID functions in cancers maintenance and progression, the transforming role of ID proteins is postulated on the basis of their capacity to inhibit the massive and multimodal tumor-suppressive RB pathways through direct binding ID2/ID4-RB or indirectly through the blocking of ETS-mediated expression of INK4A by ID1 [100].

ID overexpression was also proposed as prognostic indicators in Neuroblastoma and not surprisingly, NB cell lines carrying MYCN amplification, usually overexpress ID2 to constitutively bypass the cell cycle checkpoint imposed by RB, providing another missing piece of the puzzle for determining the onset of NBs [101]. Although to date, we still lack a comprehensive picture of the downstream molecular events that are controlled by the reciprocal regulation of RB and ID proteins, evidences suggest a role for aberrant

modulation or impairment of ID/RB/E2F regulatory pathway during embryogenesis resulting in the formation of NB [99,101].

2.3 MYCN/MAX/MXD Network

Within two years of MYCN's discovery in NBs, amplification of MYCN was shown to correlate with poor prognosis in patients, a biomarker that is still used today to stratify risk. MYCN transcription factor belongs to an extended network defined by the presence of a basic helix-loop-helix leucine zipper (bHLHz) motif that is known to mediate protein-protein interactions among members of the network and DNA binding. Indeed, MYC is known to heterodimerize with MAX at consensus Enhancer-box (E-box) sequences located into or in the immediate proximity of promoters or enhancers of the regulated genes to activate transcription. Both canonical (CACGTG) or not (CANNTG) E-boxes are the sequence recognized by all the MYCN/MAX/MXD network proteins which MYCN belongs to [51].

2.3.1 Main members of the Network

Myc Interacting Factor X, MAX: MYC family functions are mediated by MAX, a stable bHLHz protein that, in contrast with MYC family members, is expressed in both resting and proliferating cells, regardless of MYC levels. [54] The heterodimer "signature" comprises groups of target genes involved in pluripotency and growth, but also proteins with a role in the normal development of sympathoadrenal lineage cells, such as the multidrug resistance protein 1 (MRP1), α-prothymosin, MASH1, Id2, telomerase, MCM7, Pax-3, leukemia inhibitory factor, activin A and MDM2. [50] [12] As previously mentioned, MYCN-mediated transcriptional activation occurs through interaction with MAX and subsequent recruitment of TRRAP complexes.

MAX network transcriptional repressor MNT and Max Interacting Proteins, MXD: From the presence of MAX in cell lines with no MYC expression and from its weakly homodimerization capability, arises the evidence that MAX can form heterodimers with other related proteins of the network, known as MXD1-4 and the more distantly related MNT. These proteins, that compose the MAD protein family readily heterodimerize with MAX and bind DNA at the same E-box sequence as MYCN, but function as transcriptional repressor acting as MYCN main antagonists [102]. The capability of

MXD1-4 and MNT proteins to act as transcriptional repressor, further strengthen the model in which MXD proteins antagonize MYC in regulating many aspects of cell biology [103]. In contrast to MYC, proteins of the MXD family possess an mSin3 interaction domain SID (Fig.8) that has a critical role in ensuring MXD/MNT functions. While MXD with an impaired SID has little biological activity, MNTΔSID gain transforming activity suggesting other relevant MNT functions [104].

MGA: the bHLHz MAX protein binds also MGA. It differs from other members of the network which in general are rather small molecules and secondly, because it has additional functional domains that allow it to act both as an activator or repressor. Indeed, MGA has two distinct DNA binding domains, a T domain and bHLHz DNA-binding motifs, suggesting it can interact with DNA not only at E-boxes level but also through sequences recognized by its T-box DNA binding motif. MGA is known to take part to a repressive complex that binds MYC and E2F target genes [104].

MondoA, ChREBP and Max like Protein, MLX: the bHLHz MAX like protein MLX heterodimerizes also with ChREBP/MondoB and MondoA as well as with a part of MXD family proteins. While MYC, MAX and MXD proteins localize exclusively in the nucleus, MLX and MONDO proteins have mainly cytoplasm localization but, in response to metabolic stimuli, can shuttle between the two compartments. MXD–MLX dimers can bind both non canonical or canonical E-box sequences, and repress transcription, acting similarly to MXD–MAX heterodimers [105,106]. Target genes of MondoA/ChREBP-MLX dimers are involved in glutamine and glucose metabolism that are essential for cells both in physiological and pathological conditions [54].

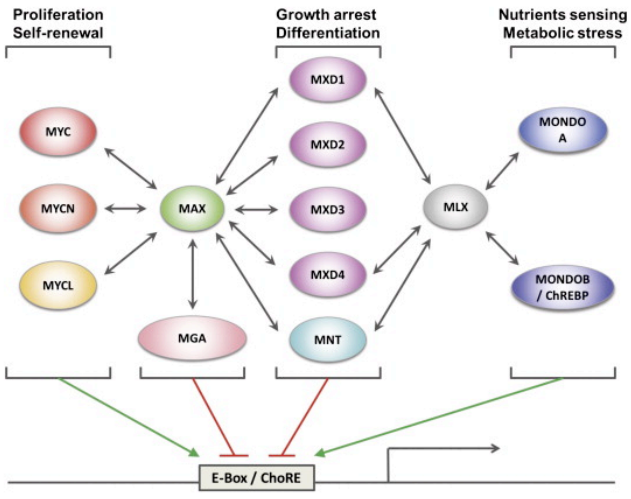


Fig.7. Diagram of the MYCN/MAX/MXD network. All members are represented and two-headed arrows underline their interactions. Green arrows indicate transcriptional activation whereas red lines, repression functions. E-Box, Enhancer-box; ChoRE, Carbohydrate response element. [102]

Diagrammed in Figure 7 the representation of the enlarged network through which Mondo, MXD and MYC gene families mediate a wide transcriptional response to growth arrest, mitogenic and metabolic signals. Structural differences between the network members directly reflect the deep functional activities of these proteins (Figure 8).

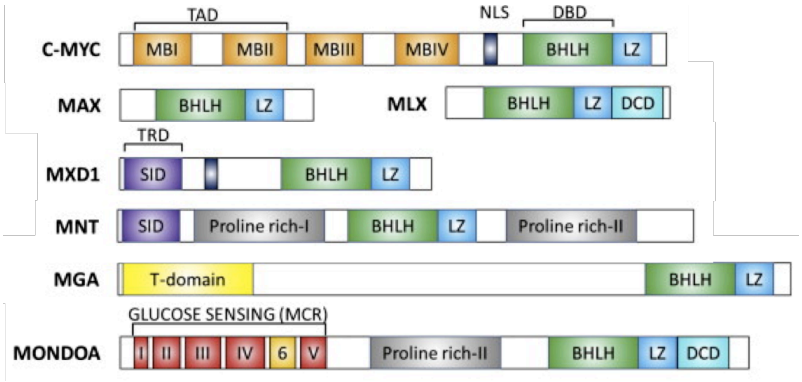


Fig.8. Representation of the main domains of the members of the network (representative member of each subfamily is depicted).
BHLH, Basic helix loop helix; LZ, leucine zipper; MBI-IV, MYC box domains; TAD, transcriptional activation domain; DCD, dimerization and cytoplasmic localization domain; NLS, nuclear localization signal; SID, SID3-interacting domain; TRD, transcriptional repression domain. The Glucose sensing domain is composed by six conserved regions named MONDO Conserved Region (MCR). Revisited from [102].

Given the intricate organization of the network, it is justifiable to consider that imbalance in the expression profile of the network members may have a significant biological impact by changing the complex physiological interactions between the members themselves. This notion is based first, on the consideration that MYC, MXD, or MONDO family proteins compete for binding to MAX or MLX limiting their free quantity required for other connections. A second consideration is that the same e-box could be target of both transcriptional activation and repression induced by different heterodimers imposing various consequences in terms of gene target expression [106].

2.3.2 MAX role in the balance of MYCN activities

To further investigate MYCN functions as activator and repressor, it is also necessary to understand the importance of MAX in preserving the equilibrium among activated and repressed transcription of MYCN target genes. Evidence for MAX germline loss-of-function mutations in patients suffering from pheochromocytoma has highlighted the role of MAX as tumor suppressor gene. All mutations are located within the bHLHz domain, necessary for the stability and specificity of dimer formation and for DNA recognition and the major part of these affected the casein kinase II phosphorylation sites involved in

MAX-mediated DNA binding [51,107,108]. Further evidence for the role of MAX in balancing MYCN target genes comes from the reintroduction of MAX in PC12 cells that induces transcriptional repression and a decrease in growth rate. PC12 is a pheochromocytoma cell line derived from the rat adrenal medulla that does not express MAX protein. Additional experiments on the same cell line highlights MYC independent function as a transcriptional regulator despite the lack of MAX interactor. Therefore, MYC-MAX complexes may not be the only condition for MYC-dependent gene expression. As result of MAX mutations, it is an imbalance in the network that is also responsible for pheochromocytomas and this could also be due to increased MLX recruitment [109]. All these findings confirm the role of imbalance of the MYC/MAX/MXD axis in the growth of aggressive neural tumors, and argue that the genetic hallmark of malign neuroblastoma, i.e. MYCN amplification could lead to an active MAX-indipendent MYCN function.

Interestingly, degree of NBs aggressiveness depends directly on the MYCN copy number, therefore ablation of MAX-MNT or MAX-MYC dimers in pheochromocytoma could contribute to the same oncogenic MYC deregulation that occurs in NB [5,109].

Although MYC independent activities, it has been supposed that MAX may also be required for the correct folding of MYC [110]. Moreover, MAX is the only member of the network that homodimerizes both *in vitro* and *in vivo*, even though MAX homodimers are less stable than MYC-MAX heterodimers or other heterodimers of the Myc network [111]. Because MAX lacks a transactivation domain, MAX homodimers fail to regulate transcription, but it was demonstrated that, while MYC overexpression activates, MAX over-expression has been reported to repress the transcription of genes bearing c-MYC binding sites. Furthermore, MAX-mediated repression is relieved by overexpression of c-MYC [112].

Recent studies suggest that the amount of MAX is limited and MYC and MNT really compete for binding to. To date, there is no further information on the different binding capacity of MYC or MXD proteins [113, 114].

Alternate mRNA splicing produces two major highly conserved MAX isoforms, the longer of which MAXL differs from the shorter form MAXS for a 9-amino acid insert. Both proteins are expressed at approximately equal levels in most cell types and, unlike MYC, are highly stable and unresponsive to the proliferative state of the cell. *In vitro* shift assay

demonstrated that MAXL can bind DNA more strongly than MAXS and *in vivo* analysis on rat fibroblast pointed out that MAXL is able to repress a MYC-responsive reporter gene whereas MAXS had little effect on its expression. In comparison to MAX(S)-overexpressing cells, ones overexpressing MAXL showed reduced expression of exogenous or endogenous MYC responsive genes, grew more slowly, showed accelerated apoptosis after growth factor depletion. These differential effects of two MAX isoform can at least be explained partially by the differences in their DNA binding abilities and their effects on target gene expression [115].

2.3.3 Evidence for MNT as a MYCN antagonist

Because of its chromosomal location (ch.17p) frequently deleted in cancer, MNT is the mainly studied transcriptional factor of the network and, it is ubiquitously expressed as MAX, with no fluctuations among cell cycle [108]. The conventional model in which MNT and MXD proteins act as MYCN antagonists, has been supported by studies demonstrating that overexpression of these factors led to growth arrest and suppressed MYC/RAS induced transformation of fibroblasts. Furthermore, conditional MNT deletions in T cells or in mammary epithelium of mice display hyperproliferation and bring to development of respectively T-cell lymphomas and mammary adenocarcinomas [116]. Moreover, murine embryonic fibroblasts (MEFs) derived from these mice, exhibit many of the hallmark characteristics (i.e. low growth rate, prone to apoptosis, efficiently avoid senescence) of cells subjected to forced MYC expression suggesting that an analogous mechanism of tumorigenesis is operating [117].

In addition, MYCN and MNT are known to target the same genes. Evidences for MNT antagonisms of MYC also come from murine lymphoid cells. In primary T cells, MYC is required for the proliferation and growth of immature thymocytes but its growth can be inhibited by ectopic expression of MXD proteins [54]. Furthermore, MNT deficient cells like cells that overexpress MYC, have increased proliferation and sensitivity to conditions that induce apoptosis [118].

Functional antagonisms between MYC and MNT/MXD: While MYC proteins act as transcriptional activator through their TAD domain, MXD or MNT–MAX heterodimers result in transcriptional repression through their SIN3 interaction domain (SID) with which can bind one of four paired amphipathic α-helical (PAH2) motifs within the large

corepressor complex known as mSIN3 and exerts its main function as repressor through HDAC1- and 2-mediated histone deacetylation [116]. Thus, a second level of biological antagonism, in addition to competition for MAX and DNA binding, occurs via histone acetylation and deacetylation. As for MNT-MAX heterodimers, also MNT-MLX repress transcription through interaction with mSIN3 recruitment of HDACs [4].

Another level of MYC/MXD competition is histone methylation. MXD recruit histone demethylase inducing H3K4me3 demethylation at the TERT promoter thus inducing decreased expression of TERT in human promyelocytic leukemia cells HL-60 during DMSO-induced differentiation [119,120]. Taken together, these data lead to hypothesize that the MXD proteins can antagonize MYC function through inhibition and promotion of both histone acetylation and methylation. However, further binding analysis of MYC and MXD family proteins will be required to fully understand the functional relationships among network factors [54].

2.3.4 Role of the Network in cancer

Expression and function of network members, which both collaborate or antagonize with MYC, can affect biological outcome of MYC-deregulated tumor. Altered expression of MYC is necessary to establishment and maintenance of many tumor and it was proposed that MXD family members could antagonize MYC function acting as tumor suppressors. Schreiber et al. first proposed MXD2 as tumor suppressor gene as its constitutive deletion in mice resulted in a tumor-prone phenotype and hyperplasia but to date, there is no further evidence supporting the idea of MXD family as tumor suppressors [121]. Suppression of MXD1 in mice results in a mild phenotype with just slight maturation impairment of granulocyte progenitors [122]. Mice lacking MXD2 displayed a hyperplastic phenotype in multiple tissues while suppression of MXD3 seems to sharpen sensitivity to apoptotic stimuli. The bland phenotypes of the single-gene MXD1–4 knockouts could be due to redundancy among paralogs or, moreover to their irrelevant expression in the context of MYC-driven oncogenesis given their limited expression at arrest and differentiation time points [123].

This may not be the case for MGA and MNT, which appear to act as tumor suppressors [54].

Loss-of-function mutations in MGA have been identified in chronic lymphocytic leukemia and recent studies on MNT deletion show that it acts as antagonist but also allowing MYC's oncogenic activities through balancing MYC induced apoptosis or proliferation [124,125].

It's interesting to highlight that the lethal effect of combined MNT loss and MYC overexpression in T cells prevents the formation of highly penetrant thymomas that form in MNT replete T cells overexpressing MYC. In the same cell lines, but also in MEFs, MNT deficiency and MYC overexpression is coupled with high levels of mitochondria-generated superoxide/ROS that, when accumulated beyond thresholds could be toxic and induce apoptosis. Moreover, these cells exhibit low oxygen consumption, thus leading to hypothesize that defective oxidative phosphorylation may contribute to excessive ROS production. Less is still known about loss of MNT in high MYC context but the existing data underline the role for MNT in supporting the hypermetabolic state induced by mitogens and high MYC activity [117].

It is important to note that MXD/MNT proteins maintain their repressive activity also when bound to MLX thus, the phenotypes resulting from altered expression of MXD/MNT may be due to by heterodimerization with MLX. While MXD-MLX dimers mediate repression function, MONDO-MLX represent the positive transcriptional arm of the network which control the lipidic and glucidic cellular metabolism. Although MONDO-MLX pathway has not been well investigated, there is evidence that silencing of MONDOA in NB cells induces apoptosis underlining a cooperation between MONDOA and MYC in inducing tumorigenesis [126].

It is well known the MYC capability to negatively autoregulate its own expression through the involvement of the Polycomb complex, usually found impaired in cancers. Due to the findings that Miz-MYC complex repress MXD4 in erythroleukemia cells, and following the discovery of MYC-mediated upregulation of Mondo proteins, it is possible to extend the regulatory cross talk among all network members [127-129].

Differentiation: Grade of differentiation is used as a measure of cancer progression and aggressive MYCN-amplified NBs cells are characterized by a low grade of differentiation. MYCN acts as regulator of a set of genes involved both directly and indirectly in neuronal differentiation processes [8].

In NBs context, the Trk family of neurotrophin signaling receptors are strongly correlated to the biological and clinical features such as differentiation grade of NB tumors. Trk receptors have critical role in the development and maintenance of the central and peripheral nervous systems and their expression is differential and stage disease specific (see par.1.3). MYC regulation of TRK expression was investigated and it was pointed out that NGFR expression is repressed through binding of MYCN to the NGFR promoter. Furthermore, siRNA against MYCN in NB cells, could re-induce NGFR expression and sensitize neuroblastoma cells to NGF-mediated apoptosis [30,31].

During the progression of the differentiation process, MXD1–MAX complexes displaced MYC–MAX heterodimers resulting in repression of MYC target genes. The involvement of the MYC/MAX/MXD network results evident also from the temporal tight regulation of member expression: while MNT and MAX appear to be expressed independently from cell cycle, MXD expression is confined to differentiated cells. [125]. On the other hand, minor effects in mice with MXD depletion has led to the idea that MXD proteins may not directly offset MYC activity but somehow they act together with other differentiation factors to repress proliferation and growth genes induced by MYC before differentiation [54].

Proliferation: Tumorigenesis is the result of cell cycle disorganization and thus, uncontrolled cellular proliferation. These events involved conservatory molecules such as cell cycle genes and their products: cyclins, cyclin dependent kinases (Cdks), Cdk inhibitors (CKI) and extra cellular factors (i.e. growth factors). MYC is known to be associated with proliferation, whereas MXD proteins expression is linked to non-proliferating cells. MXD3 and MNT are exceptions to this inverse MXD expression pattern: MXD3 expression is restricted to cells in S phase, whereas MNT is present regardless of cell cycle and differentiation [118,122].

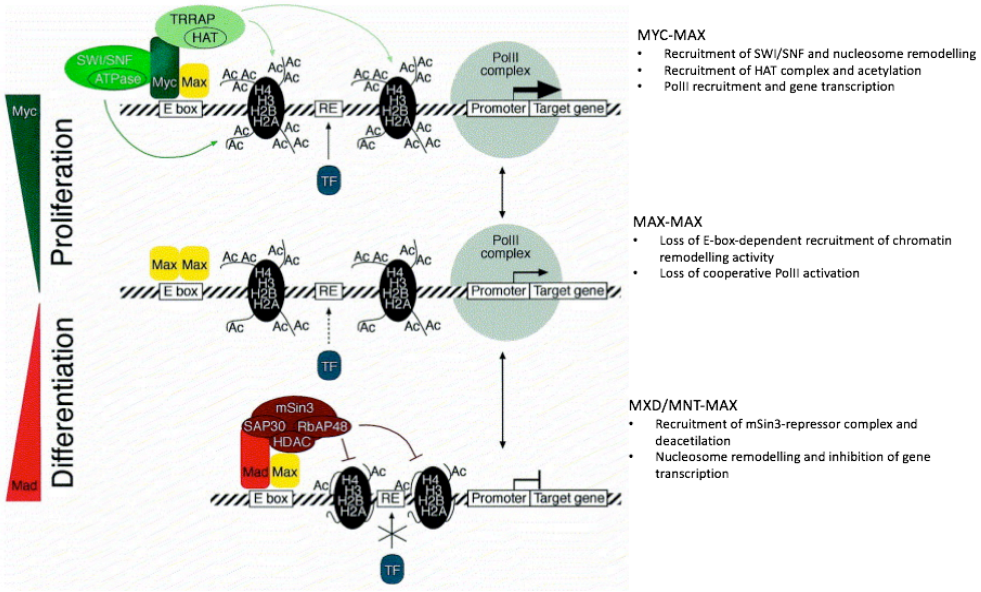


Fig.8. Representation of the control of promoter activity by MYC/MAX/MXD network members during proliferation or differentiation and proposed mechanisms. *Upper panel:* MYC binds to SWI/SNF complex involved in nucleosome remodeling; interacts with TRRAP complexes necessary for change in the acetylation status of core histones. Other components are involved including TFs and subunits of the PolII complex. *Middle panel:* MAX has not been reported to bind TFs and dimers do not influence directly promoter activity but compete for DNA binding. *Lower panel:* mSin3-dependent repressor complex is recruited through MXD proteins and induce deacetylation of core histones and increased chromatin compaction. E box: refers to a DNA element that is recognized by MYC/MAX/MXD network members; RE: response element for transcriptional regulators; Ac: acetylation of histones; TF: transcription factor; HAT: histone acetyl transferase; HDAC; histone deacetylase; revisited from [104]

In the context of the neuroblastoma, MYCN is able to affect the balance between proliferation and differentiation by promoting transcriptional activation of several genes involved in cell proliferation. When ectopically induced, it stimulates the re-entry of quiescent cells into cell cycle and shortens the progression through the cell cycle, specifically reducing the G1 phase and decreasing cell attachment to the extracellular matrix. Nevertheless, reduction of MYCN expression level, using MYCN anti-sense, promotes cell cycle arrest, differentiation, and apoptosis [62].

3 Current treatment strategies for Neuroblastoma

To date, current treatment for NB consists of surgery, chemotherapy, radiation, and biotherapy but the main treatment for NB is based on chemotherapy, which reduces the size of primary tumor facilitating surgery and eradicates distant metastasis. Surgical intervention is recommended in all cases in which tumor location permits; local radiotherapy is helpful in completing therapy. High dose chemotherapy is based on the combined use of cytotoxic drugs belonging to different molecular class types, however these treatments could lead to possible selection of a drug-resistant cellular clones and moreover, delivery of non-specific cytotoxic drugs leads to high incidence of side effects due to high grade of non-specificity, meaning a significant obstacle for NB treatment.

To date, main drugs targeting MYCN that passed the pre-clinical trial belong to iron chelator class. Epidemiologic studies pointed out higher cancer incidence in iron overload individuals compared to iron deplete individuals which shown low incidence of tumor formation [130,131]. Iron is an essential element for metabolism and cell cycle progression and thus it is particularly required to cancer cells and various studies highlighted that association between high iron load and cancer may be due to free radical production and reduction in ROS protection mechanisms, or moreover to inhibition to nutrient sensing or impairment of immune systems. Gold standard iron chelator was DFO desferoxamine and Exjade, used *in* vitro but also *in vivo* to treat NB patients in many clinical trials and many studies are going on about mechanisms interesting growth inhibition by iron depletion. These studies could lead to help in development of new more potent iron chelators with low lipophilicity, a characteristic that may interacts with different biochemical processes *in vitro* lead often to side effects [132,133].

Due to side effects of non-specific cytotoxic drugs and due to possible selection of chemo-resistant cellular clones, there is the need to develop novel tumor specific therapies that could be less toxic than current approaches and to date, molecular target therapy has received much attention. Targets of the molecular therapy are specific molecules responsible for cancer progression and thus involved in proliferation, differentiation, genetic instability and metastasis [134]. Methods mainly investigated involved peptide nucleic acids, which specifically hybridize to DNA or RNA inhibiting transcription or translation of a target gene; antisense oligonucleotides that hybridize to and inhibit the mRNA of a specific gene and small interfering RNA siRNA, which induces silencing of

target genes by inducing degradation of complementary mRNA. Another method consists in developing synthetic small molecule inhibitors which potentially show the ability to interfere with a molecular target at multiple levels [135]. Synthetic small molecules are favored by the pharmaceutical industry because of their attractive pharmacokinetic properties, indeed they can easily diffuse into tumor cells inhibit expression of a target gene or function of protein. Inhibition is usually due to direct interaction with the specific protein preventing its interaction with other factors or inducing conformational changes [136].

3.1 MYCN as a Therapeutic Target

Distinct molecular targets have been identified as possible targets for NB treatment and great interest was elicited by MYCN oncoproteins. Given its restricted spatial and temporal expression limited to the early stages of embryonic development and given its virtually undetectable levels in normal post-natal tissues, MYCN could be an elite target for novel therapies [5]. Furthermore, MYCN offers multiple levels of targeted inhibition. Different research groups have studied downregulation of MYCN in MYCN-amplified NB cell lines using different approaches such as PNA or RNAi with variable results depending on experimental conditions and cell lines but collectively these studies suggest that target MYCN results in growth arrest, apoptosis and/or morphological differentiation [53]. For instance, MYCN inhibition through retinoic acid or antisense oligonucleotides has already demonstrated to induce neuronal differentiation in NB cells and to reduce tumor growth in Neuroblastoma mouse model. [7]. It has been extensively reported that single oncogene inactivation within a primary tumor is sometimes sufficient to induce tumor regression, thus it is possible to speculate that direct targeting of MYCN could be promising for neuroblastoma therapy.

3.1.1 Target MYCN Transcription

Recently studied showed MYC suppression after inhibition of the BET bromodomain family of chromatin adaptors. Transcription of c-Myc is related to an increased histone acetylation, a covalent chromatin modification associated with transcriptional activation. BET proteins (bromodomain and extra-terminal family proteins) associate with acetylated chromatin facilitating transcriptional activation by increasing the binding of transcriptional

activators. A molecule named JQ1 able to target BET proteins was identified. Action of JQ1 on c-MYC expression results in preventing the recruitment of coactivator proteins necessary for transcriptional initiation and mRNA elongation thus leading to disruption of mRNA synthesis. JQ1 treatment in mouse model of multiple myeloma shows its effects on tumor burden but treatment on MYCN-amplified NB cell lines and on NB mouse model results in MYCN expression decrease, although this effect was far less dramatic than that observed in a c-MYC driven cell line [137,138].

Thus, another level of MYCN inhibitions could be epigenome regulation. Balance between DNA methylation and histone acetylation can be pathologically altered especially in favor of DNA hypermethylation and histone deacetylation. Hence, there is a growing interest for HDAC inhibitors and many of these are on preclinical trial. For instance, tumorigenesis in NB mouse model was decreased after Cambinol treatment, a SIRT1 inhibitor; treatment with class I and II HDAC inhibitor, Trichostatin A results in reduced tumor weight and volume in the same mouse model [139-1341]. Therefore, HDAC inhibitors may be a viable route to target *MYCN*-amplified NBs.

3.1.1 Target MYCN Proteins

Galderisi et al demonstrated a 3-fold decrease in MYCN mRNA levels after antisense oligonucleotides treatment in NB cells but phenotypic effects depended on cell lines used. After MYCN antisense oligonucleotide treatment, N-types neuronal cells underwent differentiation while treatment of Schwannian S-type cells results in both differentiation and apoptosis [142].

MYCN mRNA inhibition was shown also through treatment with retinoic acid, a vitamin A metabolite. Treatment has been shown to induce neuronal differentiation in NB cells in a mechanism that involves $p27^{Kip1}$, a key mediator of growth arrest [143].

As described in paragraph 2.1.2 MYC protein proteolysis is a result of subsequential step of phosphorylation at multiple sites that involved GSK-3β kinase and PP2A phosphatase [75,76]. Inhibition of PI3K can decrease MYCN protein levels through promoting MYCN phosphorylation by blocking a PI3K-driven inhibitory GSK-3β phosphorylation [36]. Various cancers frequently exhibit alteration in PI3K/mTOR pathway: activation of this pathway is related to resistance to apoptosis, increased growth rate and deregulated energy supply mechanisms. mTOR inhibitors are known to downregulate MYCN protein and

inhibit both *in vitro* and *in vivo* NB growth. Moreover, NB cell lines lacking GSK-3β phosphorylation site were resistant to treatment with PI3K/mTOR inhibitors [144].

Another level of MYCN downregulation can be by inhibition of proteins that stabilize MYCN proteins. Proteins such as AURKA that prevent dephosphorylation at T58, result in stabilizing MYCN through direct protein-protein interaction and making MYCN less degradable by proteasome. Interestingly, high levels of AURKA are associated to MYCN-amplified NBs, mediated potentially by MYCN itself. Thus, AURKA could represent a strategy to treat MYCN-amplified NBs, given also its possible ligand binding site. Different compounds targeting AURKA have been tested both *in vivo* and *in vitro* [144,146]. It is also possible that targeting proteins downstream MYCN pathways might be an alternative strategy to indirect inhibit MYCN. Indeed, targeting MDM2, for instance with Nutlin-3 or MI-63, also results in downregulation of MYCN mRNA blocking its expression through inhibitions of MDM2 binding to the 3'UTR of MYCN [147].

Despite promising evidence for MYCN as a therapeutic target, no MYC or MYCN inhibitors have yet passed the preclinical trial, and further studies are needed to develop efficient MYCN inhibitors.

3.2 Targeting the MYC/MAX/MXD Pathway

Site-directed mutagenesis experiments have underlined the critical role of various amino acids located within the bHLHz domain in the interactions among MYC/MAX/MXD network members. Therefore, it was suggested that the bHLHz domain could be the target for therapeutic strategies or for developing inhibitors and several compounds with the ability to interfere with MYC-MAX have already been tried. Inhibitors of protein-protein interactions are usually designed on the sequence of dimerization domain and often target the small discrete binding sites. Impairment of MYC-MAX dimerization through use of small-molecule results in decreased c-MYC-induced oncogenic transformation of chicken embryo fibroblasts *in vitro*. The discovery of low molecular weight inhibitors for MYC-MAX complex has shown that binding between large protein interfaces may also be disrupted by small molecules [148]. On the other hand, there is evidence for MYC to retains substantial MAX-independent activities, thus this may not be the best strategy for all tumor types [109].

Endogenous inhibition of MYCN activity was identified after characterization of the network which MYC proteins belong to: antagonists such as MAX-MAX dimers have

been found to compete for binding to E-box sequences and to indirectly inhibit transcription while, antagonists such as MXD/MNT-MAX directly repress transcription of MYCN target genes through repression complex recruitment [111]. Strategies to restore protein functions are now considered viable anticancer therapeutic approaches and this may be an option for MAX-null pheochromocytoma patients or for antagonizing MYCN activity in MYCN-amplified NBs [107]. Inhibition of MYC function by exogenous antisense approaches, or overexpression of dominant-negative alleles of MNT or MXD proteins, severely impairs the growth-factor-induced proliferation of cells in culture [125]. Evidences reported in paragraph 2.3 highlight the hypothesis that through modulation of MYCN/MAX/MXD network it is possible to antagonize MYCN activities.

AIM OF THE PROJECT

Neuroblastoma (NB) is the most frequently diagnosed malignant solid tumor during infancy and affects the developing sympathetic nervous system. While its anatomic pattern of origin is well defined, the etiology of NB remains still unclear. Neuroblastoma displays profound clinical heterogeneity that reflects the wide differentiation spectrum of the neuroblast cells from which it originates. Moreover, NB has also deep genetic heterogeneity that is linked to possibility to run into different tumor subtypes that range from spontaneous regression to rapid progression and death. The presence of different hallmarks allows to divide patients into risk categories. Almost half of all patients at the time of diagnosis have stage 4 disease, which represent the more malignant stages with presence of hematogenous metastasis.

About half of high risk NB patients shows MYCN amplification, which is, to date, the strongest prognostic indicator of the NB disease. This genetic abnormality is frequently found in many types of infancy cancers of mostly neuroendocrine origin, including medulloblastoma, astrocytoma, Wilms' tumor, small cell lung cancer, osteosarcoma and rhabdomyosarcoma. Moreover, it has been demonstrated that the sole MYCN amplification is sufficient to initiate NB formation in mice.

Given the strong prognostic value of MYCN amplification and its role in driving tumor formation, MYCN provides an attractive therapeutic target for treatment of NB. To date, many efforts have been made to develop strategies to inhibit MYCN expression and/or MYCN-mediated transcription pathways but unsuccessful. This is probably due to the presence of MYCN within an intricate network of various proteins named MYCN/MAX/MXD protein network whose proteins can bind the same DNA consensus, however resulting in both activation or repression.

As the other MYC oncoproteins, MYCN forms a functional transcription factor when associated with another basic helix-loop-helix-leucine-zipper BHLHz protein named MAX and carries out its functions as positive modulator of gene involved in proliferation and self-renewal. Together with MYCN, MAX belongs to the MYCN/MAX/MXD network of transcription factors which interact with each other binding DNA and regulating transcription of target genes. Thus, MYCN transcriptional activation is dependent on MAX availability. Moreover, MAX can both *in* vivo and *in vitro* homodimerize and weakly inhibits MYCN target genes. On the other hand, the repressive side of the network is represented by MNT-MAX dimers which actively repress transcription of MYCN target

genes through its inhibition domain. Thus, the sole MYCN amplification may not exactly reflect the biological activity of this factor but MYCN functions are strongly related to the expression of MYC/MAX/MXD network proteins.

Aim of my thesis is to provide a better comprehension of molecular mechanisms underlying MYCN oncogenicity to advantage therapeutic approaches to contrast these phenomena. Thus, two approaches through which antagonize oncogenic characteristics in MYCN overexpressed NB cells have been evaluated.

A first genetic approach is based on the modulation of the expression levels of MAX and MNT network proteins. Published gene arrays on Kocak dataset cohort of 643 patients highlight that low levels of MYCN antagonists MAX and MNT in presence of MYCN amplification, are related to good clinical outcome. On the other hand, same Kocak dataset pointed out correlation between high levels of MAX and MNT and short term survival probability in MYCN amplified NB patients.
Using RNA interference and overexpression in MYCN-amplified cells, we want to confirm that downregulation of MAX and MNT suppresses the malignant phenotype of NB cell lines, whereas high MYCN antagonist levels correlate with enhancement of the malignant phenotype.

To date, despite the attractiveness of MYCN as a therapeutic target and the discovery of new MYCN targeting compounds, most of them have not passed the pre-clinical stage because of their non-specificity or selection of resistant clones. A second approach through elucidate mechanisms underlying the MYCN-driven oncogenicity is based on the study of a new compound isolated for its ability to downregulate MYCN only in actively proliferating cells. To better characterize action of M606, the novel iron chelator derivative of hydroxyquinolones isolated, reporter gene assay was used to test compound action on MYCN promoter.
Through chromatin immunoprecipitation, chromatin status has been investigated and moreover, through comparison with Exjade, another iron chelator used to treat NBs, it was investigated whether this may be or not a generalized mechanism related to iron chelator class of compounds.

RESULTS

1 Genetic Modulation of the MYCN/MAX/MXD Network

1.1 Expression of MAX and MNT in MYCN-amplified Neuroblastoma is related to short event free survival probability

MYCN amplification is predictive for clinical outcome in NBs but MYCN can form a functional transcription factor only when associated with MAX, Moreover, MAX availability firmly depends on the expression levels of other network members, such as MNT. Thus, the sole MYCN amplification can not exactly reflect the biological activity of this factor but MYCN functions are strongly related to the expression of MYC/MAX/MXD network proteins.

To understand how MAX and MNT expression impact on Neuroblastoma, experimental data present in published gene expression arrays were evaluated. Genomics analysis and visualization R2 platform (http://r2.amc.nl) were used to evaluate a possible correlation between MYCN amplification and MNT or MAX expression in Neuroblastoma. Thus, meta-analysis was conducted using publically available microarray based on Kocak gene expression datasets which profiles a cohort of 649 NB tumors stratified for age and sex, presence of MYCN amplification and stage of disease. Data were graphed as survival probability without relapse events (event free survival EFS) [149].

As shown in figure 1.C, high levels of MAX and MNT in MYCN amplified NB patients are strongly related to short term event free probability. When data are plotted without taking in account MYCN stratification, MAX and MNT high expression loses its correlation with poor clinical outcome, and in contrast seems that their expression has a favorable prognostic impact both in mixed NBs and in MYCN-not amplified NBs (Fig.1 A shows MYCN not amplified NBs while Fig.1B shows mixed cohort of NBs).

Based on these intriguing findings, it was hypothesized that it is not the exclusive MYCN expression to determine poor prognosis, but a displacement in the members of the network in such a way that prognosis may depend on the fine balance between MYCN and its counterparts.

Thus, it has been decided to elucidate how modulation of expression levels of MAX and MNT may define cellular behaviors in MYCN-mediated tumorigenesis.

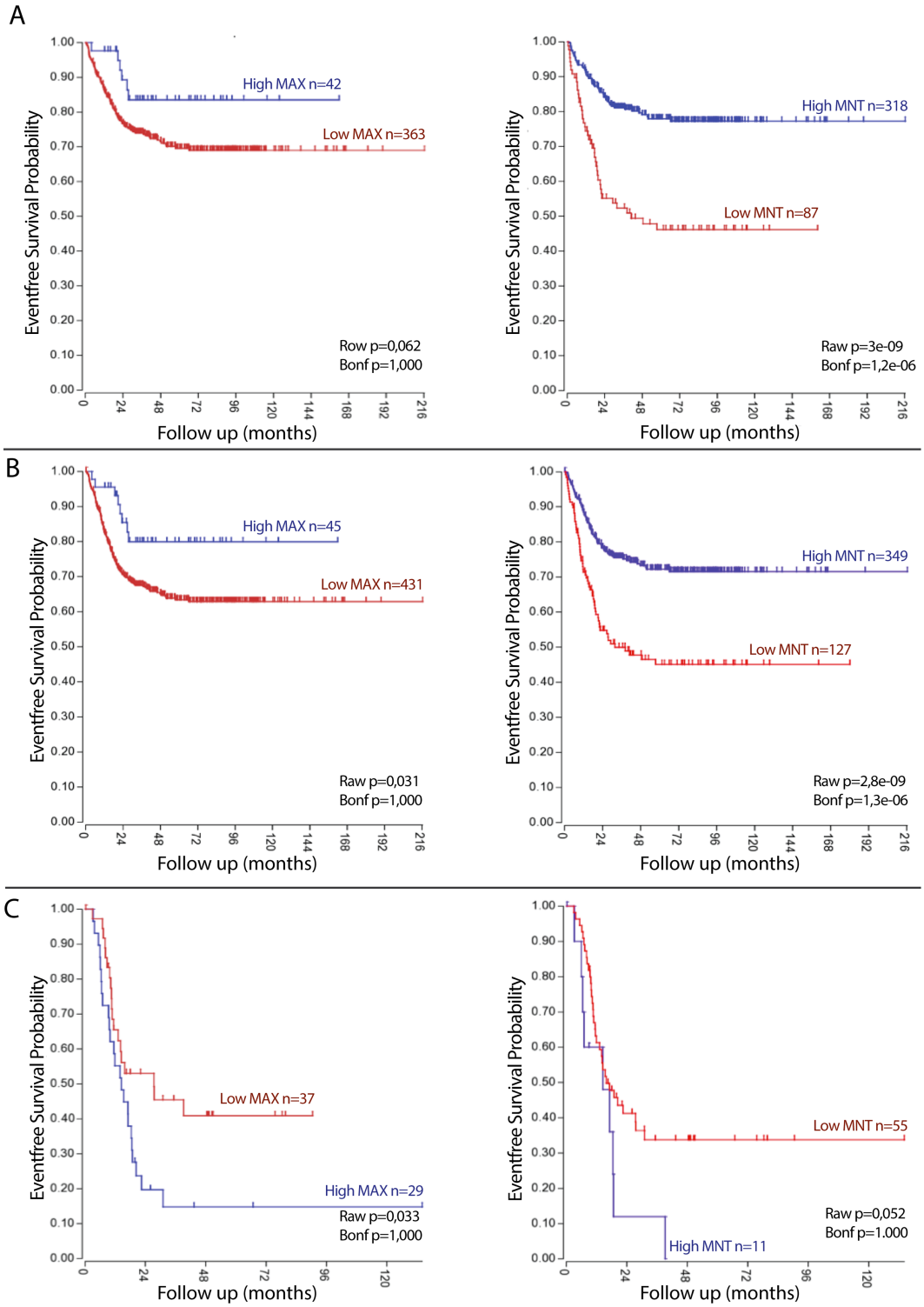


Fig.1. Kaplan–Meier curve estimates for EFS according to classification by the SVM_th10 predictor. Red curves represent low mRNA expression, while blue curves represent high mRNA expression. Characteristic of the involved patients are defined in ref [149]. Published data derived from R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) database.

A. The MAX *(left)* and MNT (*right)* relative Kaplan–Meier survival EFS for the sub-cohort of Neuroblastoma patients stratified for the absence of MYCN amplification (*n* = 476);

B. The MAX *(left)* and MNT *(right)* relative Kaplan–Meier survival EFS for the complete validation cohort of patients with neuroblastoma (*n* = 405);

C. The MAX *(left)* and MNT relative Kaplan–Meier survival EFS *(right)* for NBs patient sub-cohort showing MYCN amplification (n=66).

1.2 Suppression of the malignant phenotype in MYCN-amplified NB cell line by knockdown of MAX and MNT

1.2.1 Silencing of MAX and MNT in BE(2)-C induces cell growth rate decrease through upregulation of p21 ^{Cip1}

Tumorigenesis is the result of cell cycle deregulation, leading to an uncontrolled cellular proliferation. MYCN, together with its essential dimerization partner MAX, has an important role in transcriptional regulation of gene whose products are involved in proliferation. Moreover, MNT protein expression is coincident with that of MYC, regardless of cell cycle and differentiation [118,122].

To further examine the hypothesis that the modulation of the network members could change NB biological features in response to MYCN amplification status, SK-N-BE(2)-C NB cell line (here named BE(2)-C) were transducted with lentivirus expressing shRNA against MAX and MNT and a control shRNA and growth rate was evaluated. Specifically, 5 shRNAs against each factor were tested in MYCN-amplified BE(2)-C cells and validated through qRT-PCR and western blot and the most efficient was chosen (data not shown). After transduction with the most active shRNA, knockdown was validated through Western blot and qRT-PCR (Figure 2.A and 2.B).

Proliferation was evaluated through automated cell counter via stains propidium iodide stains of nucleic acid and 24 hours were chosen as time points. Plotted in figure 2.C growth curve indicating that knockdown of MAX and MNT strongly reduce cell number through days.

$p21^{Cip1}$ is a potent cyclin-dependent kinase inhibitor 1 that inhibits CDK2 and CDK1 complexes and thus, mediates cell cycle G_1 phase arrest. It is also known to be down-modulated in the majority of NBs and $p21^{Cip1}$ expression induced significant neurite extension in neuroblastoma cells. Figure 2.D shows a strong upregulation of $p21^{Cip1}$ mRNA levels after MAX knockdown. MNT downregulation can also induce increase in $p21^{Cip1}$ mRNA in BE(2)-C although less dramatically.

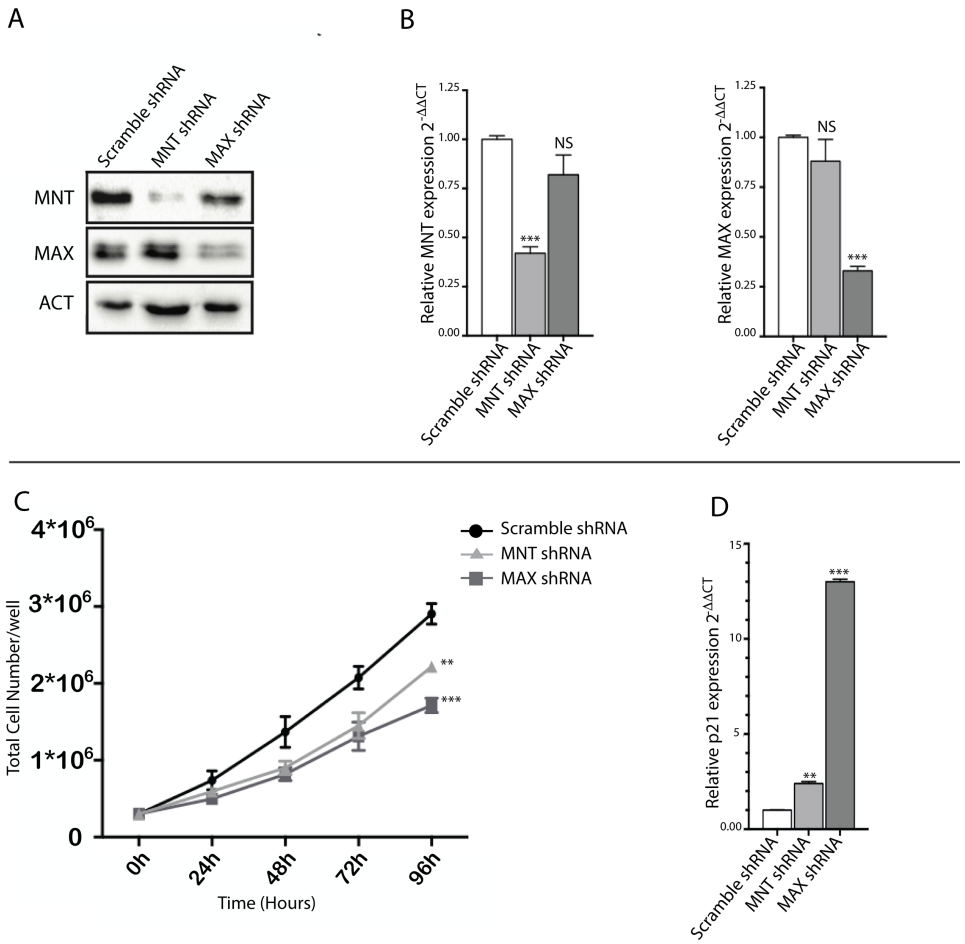


Fig.2.Genetic silencing of MAX and MNT reduces malignant phenotype in NB MYCN-amplified cell line impairing cell proliferation. BE(2)-C were transducted with scramble shRNA and shRNA against MAX and MNT, followed by selection with 1ugr/mL with puromycine. All data shown as mean of at least 3 independent experiments ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *, ** and *** indicate P<.05, .01 and .001 respectively. NS not statistically significant.
A. Western blot on MAX and MNT knockdown BE(2)-C.
B. qRT-PCR on MAX and MNT mRNA. White bar indicates control shRNA sample, bright and dark gray are respectively MNT and MAX shRNA samples. Internal reference gene was GlUcuronidaSeBeta, GUSB;

C. Silencing of MAX and MNT impairs cell proliferation. Cell growth rate of each groups were assessed by cell counting every 24 hours and expressed as number of cells per well;

D. qRT-PCR showing p21 increased value after shRNA transduction. White bar indicates control shRNA sample, bright and dark gray are respectively MNT and MAX shRNA samples. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

1.2.2 RA promotes differentiation in MAX- and MNT KD BE(2)-C

Grade of differentiation is a measure of cancer progression and MYCN is known to be a regulator of a set of genes involved both directly and indirectly in neuronal differentiation processes [8]. NB cells retain some features of neural crest progenitors, such as the ability to undergo neuronal differentiation in the presence of appropriate signals such as Retinoic Acid.

To further understand how MAX and MNT knockdown can affect cellular behaviors of MYCN-amplified NB cells, the capability of cells to undergo differentiation was evaluated after stimulation with 10μM of Retinoic Acid RA, a derivative of vitamin A.

Expression levels of the neuronal marker genes neuromodulin (GAP43) and neurofilament middle chain (NEFM) and morphological signs of differentiation in response to RA treatment were evaluated. Neuronal differentiation includes the elaboration of a large network of axons and dendrites, which are referred non-specifically as neurites when grown in culture. Thus, after seeding cells at proper density, medium was replaced every day with fresh medium with addition of RA. Morphological changes were evaluated every 24 hours. Average length of the neurites was obtained by manually tracing the length of all neurite outgrowths from neuron's cell body using NIH-ImageJ software. Total number of neurites were divided by the total number of cells present.

Results demonstrate that BE(2)-C knockdown of MAX induces the most typical morphological changes observed during neuronal differentiation. Figure 3.A shows a strong increase in neurite extension already after 3 days of RA treatment and moreover these cells shows decreased neurite branching (Figure 3.B) and decreased cell numbers (Figure 3.C). Left panel of Figure 3.D underline the typical neurite alignment shown by MAX-KD cells.

On the other hand, downregulation of MNT in BE(2)-C seems not to induce morphological changes associated with neuronal differentiation.

Through analysis of the two neuronal markers GAP43 and NEFM, we investigated whether RA treatment could lead cells to undergo differentiation into neuronal like cells. NEFM is middle-molecular-weight neurofilament protein that comprises the axoskeleton and is usually associated with axonal neurite caliber and commonly used as a biomarker of neuronal damage. GAP43 or neuromodulin is a marker of an effective regenerative response in the nervous system associated with nerve growth and filopodia induction. Results indicated in figure 3.E indicate that RA induction of differentiation leads MAX- and MNT-KD cells to express high levels of GAP43 but relatively low levels of NEFM.

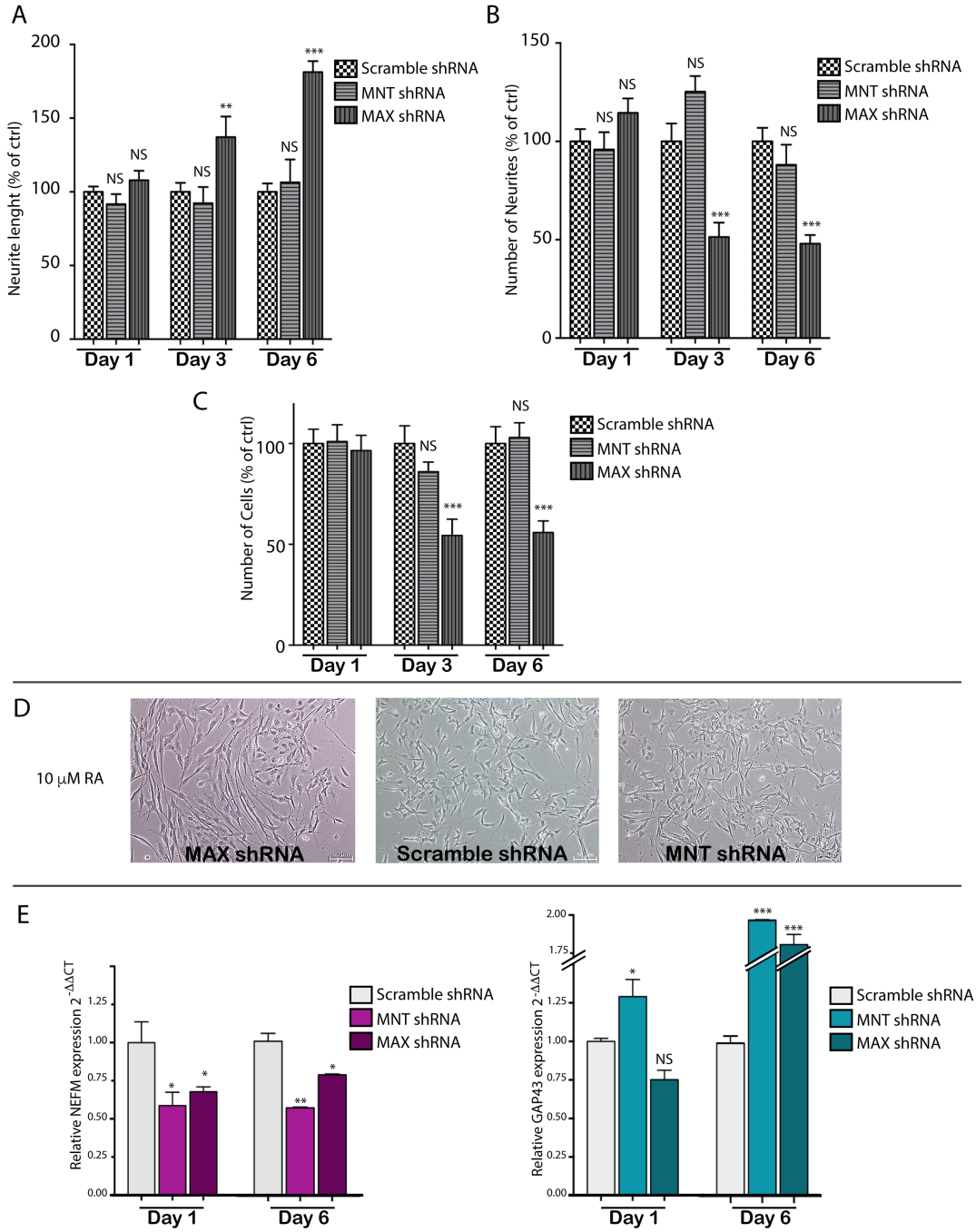


Fig.3.Genetic silencing of MAX leads to retinoic acid-induced neurite formation in BE(2)-C transducted with scramble shRNA and shRNA against MAX and MNT. All data shown as mean of at least 3 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *, ** and *** indicate P<.05, .01 and .001 respectively. NS not statistically significant.

A. Average neurite length after 3 and 6 days of 10μM RA treatment plotted as percentage of the control;

B. Average number of neurites after 3 and 6 days 10μM of RA treatment plotted as percentage of the control and normalized on cell numbers;

C. Average number of cells following 3 and 6 days of 10μM of RA treatment plotted as percentage of the control and normalized on cell numbers;

D. Morphological features of shRNA cells after 6 days of 10μM RA. Panel on the left shows shMAX cells with an increased neurite alignment. Panel in the middle represents control and panel in the right indicates shMNT cells;

E. qRT-PCR on transducted cells. Purple panel in the left indicates NEFM mRNA level while green panel on the right represents GAP43 mRNA expression levels. Light gray bar indicates control shRNA sample. X axis indicates days of RA induction. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

1.2.3 Silencing of MAX and MNT induces differential expression of the main prognostic markers

The Trk family of neurotrophin signaling receptors are strongly related to differentiation grade of NB tumors and *in vitro* are strong signals promoting neurite outgrowth. Expression of neurotrophin receptor TRKA and NGFR is commonly found in favorable NBs and are considered as positive prognostic marker of the disease. MYCN/SP1/Miz1 complex is known to target core promoter of TRKA and NGFR genes to induce repressed chromatin status and thus, downregulation of the two receptors. Moreover, NGFR and TRKA are considered a marker of early and immature neuronal differentiation [150,151].

To further characterize MAX and MNT knockdown, neurotrophin signaling receptors have been evaluated. Figure 4.A indicates a strong upregulation of the two neurotrophin receptors TRKA and NGFR in presence of a weaker reduction of MYCN mRNA level when both MAX and MNT were downregulated in BE(2)-C. All these findings strongly correlate with the gene expression arrays on figure 1.C based on clinical data which show correlation between high survival probability and MAX or MNT low levels in MYCN-amplified NBs.

Although direct correlation of TRKA/NGFR with positive clinical outcome, the neurotrophin signaling receptors TRKB is preferentially expressed together with its ligand BDNF in NBs with poor prognosis. Their expression provides invasive and metastatic capability to tumor cells and moreover, there are lines of evidence that BDNF/TRKB signaling in NB cells is known to enhance therapy resistance. Results shown in figure 4.B indicates upregulation of TRKB mRNA in MNT-KD cells but in absence of any BDNF increase thus, no autocrine pathway of BDNF/TRKB signaling is activated.

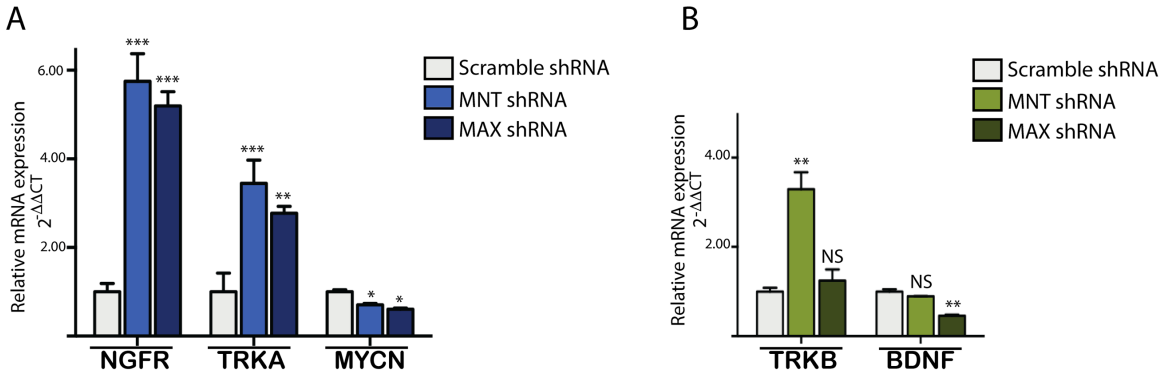


Fig.4. MAX and MNT knockdown induces high expression of TRKA and NGFR neurotrophin receptors in BE(2)-C. All data shown as mean of at least 3 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *, ** and *** indicate P<.05, .01 and .001 respectively. NS not statistically significant. Internal reference gene used was GlUcuronidaSeBeta, GUSB.
A. qRT-PCR of positive prognostic markers compared to MYCN expression level on transducted cells. Light gray bar indicates control shRNA sample. X axis indicates mRNA analyzed. Samples are indicated on legend;

B. qRT-PCR of negative prognostic markers on transducted cells. Light gray bar indicates control shRNA sample. X axis indicates mRNA analyzed. Samples are indicated on legend.

Among other prognostic markers, metalloproteinases and their inhibitors were analyzed. Extracellular matrix proteolysis and degradation depend on the net ratio between matrix metalloproteinases and their tissue inhibitor metalloproteinases TIMP. Increased expression in NBs of MMP-2 in association with low levels of TIMP-2 is associated with poor clinical outcome and advanced clinical stages [152].

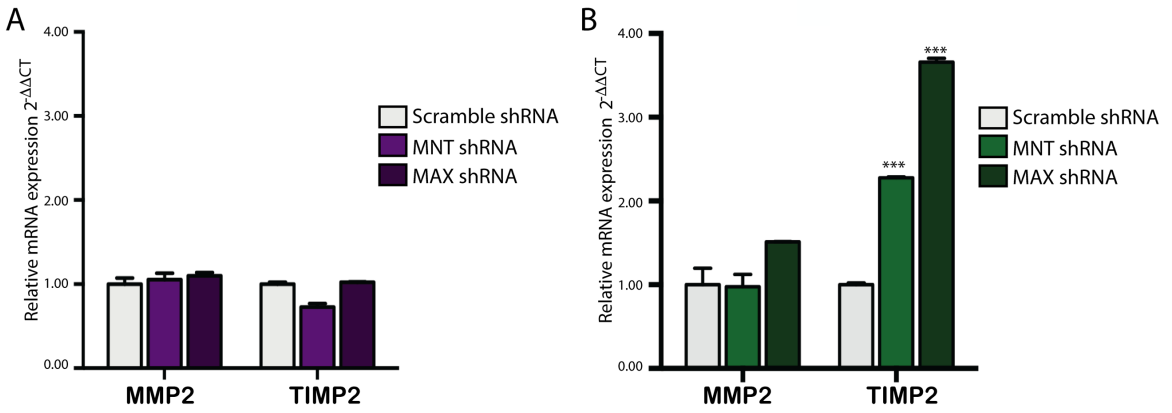


Fig.5. MAX and MNT silencing in BE(2)-C induces no change in MMP-2/TIMP-2 ratio before RA treatment and enhanced TIMP2 expression after RA treatment. All data shown as mean of at least 3 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *** indicates P<.001. Internal reference gene used was GlUcuronidaSeBeta, GUSB.
A. qRT-PCR on transducted cells before retinoic acid treatment. Light gray bar indicates control shRNA sample. X axis indicates MMP and TIMP mRNA analyzed. Samples are indicated on legend. Data are not statistically significant compared to control shRNA;

B. qRT-PCR on transducted cells after 6 days of retinoic acid treatment shows enhanced TIMP2 expression. Light gray bar indicates control shRNA sample. X axis indicates MMP and TIMP mRNA analyzed.

Results indicated in figure 5.A shows that MNT- and MAX-KD cells do not induce change in MMP-2/TIMP-2 ratio, but after induction of differentiation with retinoic acid, balance is

strongly shifted towards TIMP-2 expression with the strongest effect for MAX-silenced NB cells compared to control.

On the other hand, MMP-9 seems not to be associated with NB clinical stage and prognosis and it is usually not expressed in neuroblastoma cells but rather from stromal cells. It is known that MMP-9 promotes differentiation of adult neural progenitor cells and in mouse model of MYC-induced pancreatic neuroendocrine tumors, MMP-9 knockdown promotes tumor invasion [152-154]. Moreover, RA treatment is known to promote MMP-9 in SK-N-BE neuroblastoma cell line in association with phenotypic neuronal-like differentiation BE neuroblastoma cell line in association with phenotypic neuronal-like differentiation [155]. Figure 6 indicates that knockdown of MAX and MNT leads to expression of MMP-9 and this expression is enhanced after RA treatment compared to control.

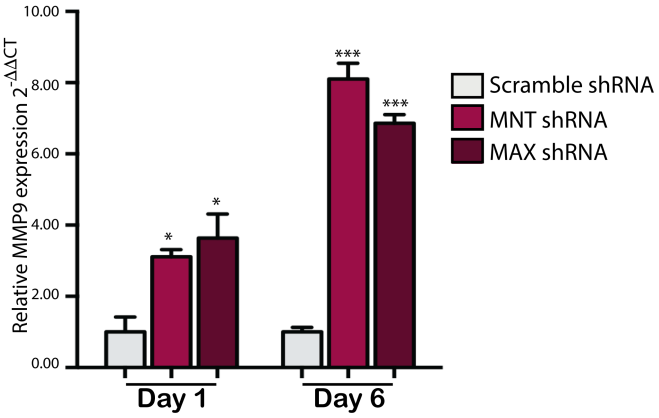


Fig.6. qRT-PCR on transducted BE(2)-C cells shows that MAX and MNT Knockdown induces expression of MMP-9 before RA treatment (DAY 1) and RA treatment further enhanced its expression. All data shown as mean of at least 3 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; **, *** indicate P<.01 and .001. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

1.2.4 Knockdown of MAX in BE(2)-C impairs BE(2)-C motility

To strengthen the evidence that modulation of MAX and MNT protein levels may affect cellular behaviors, motility activity was evaluated through wound closure assay. Results in in figure 7.A and B pointed out that downregulation of MAX strongly impairs migratory activity of BE(2)-C, whereas MNT knockdown do not impair motility.

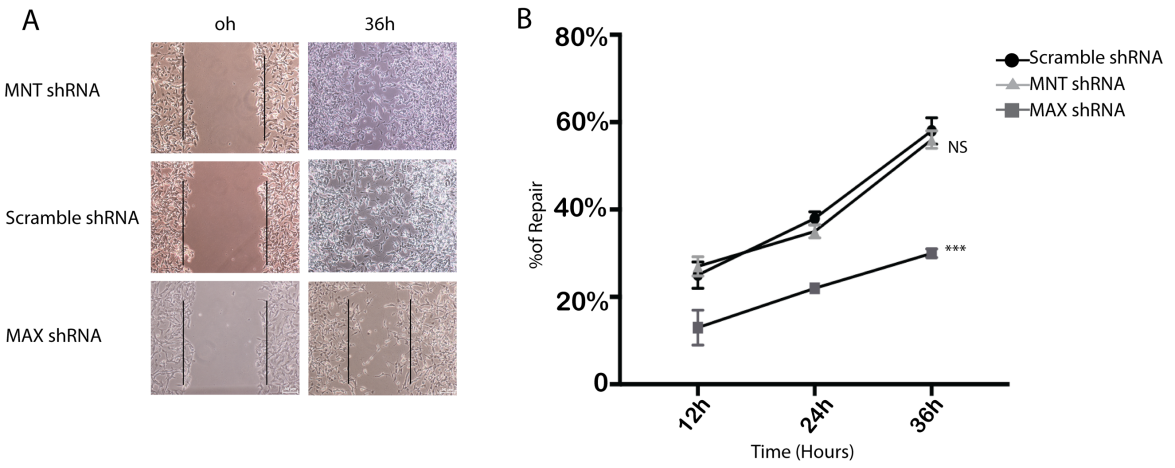


Fig.7. Knockdown of MAX impair motility of BE(2)-C transduced cells as measured by wound closure assay for 36 hours, compared with scramble shRNA cells. All data shown as mean of at least 3 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *** indicated P<.001. NS not statistically significant.
A. Representative images of wound closure assay;

B. Wound closure assay on BE(2)-C transducted with shRNA against MNT *(light gray)* and MAX *(dark gray)* expresses as percentage of repair. Samples are listed in legend.

1.3 Upregulation of MAX and MNT protein levels enhances the malignant phenotype of BE(2)-C

As previously mentioned and indicated in figure 1.A and 1.B, high levels of MAX and MNT in MYCN amplified NB patients are strongly related to bad prognosis. Thus, it was pointed out that it is not the exclusive MYCN expression to determine poor prognosis, but a displacement between MYCN and its network counterparts. To strengthen the hypothesis that through the modulation of the network members it is possible to counteract MYCN oncogenicity, MAX and MNT were overexpressed in MYCN amplified NB cell line.

1.3.1 Overexpression of MAX and MNT in BE(2)-C leads to increased proliferation rate

Specifically, BE(2)-C were transfected with constructs bearing MNT and long and short isoforms of MAX, here named respectively MAXL and MAXS. To distinguish ectopic from endogenous expression, all proteins were conjugated with a flag.

Effective overexpression was validated through Western blot analysis (figure 8.A). Proliferation rate was evaluated as previously mentioned for MNT and MAX knockdown.

Analysis conducted reveal that expression of MAXL and MNT in BE(2)-C leads to a strongly increase in growth rate. On the other hand, overexpression of MAXS induces no change in proliferation rate of BE(2)-C (figure 8.B)

Through qRT-PCR, p21 ^{Cip1} mRNA levels were evaluated and results indicate a strong downregulation in both MNT and MAXL overexpressing cells, whereas overexpression of MAXS results in no change in p21 ^{Cip1} mRNA levels (figure 8.C).

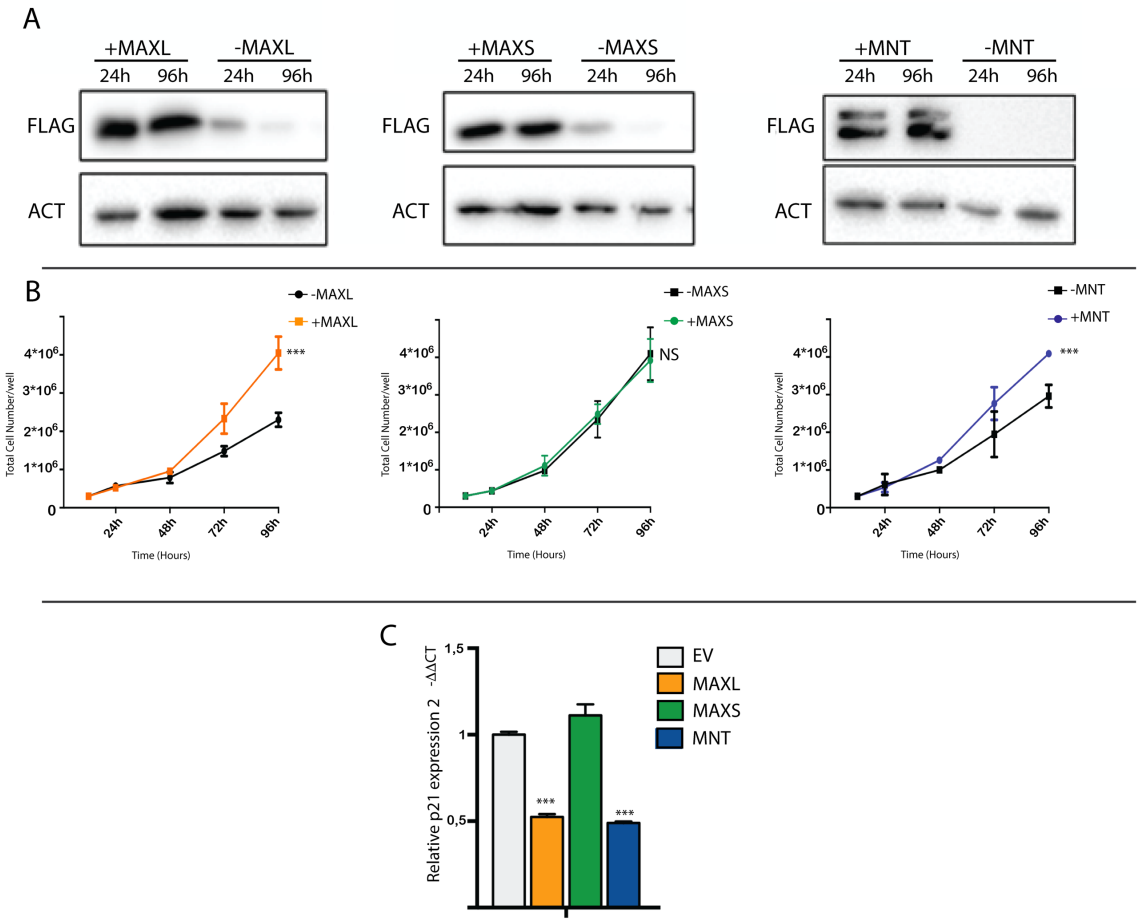


Fig.8. Transient overexpression of MAXL and MNT in BE(2)-C induces change in proliferation rate. All data shown as mean of at least 2 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; **, *** indicate P<.01 and .001 NS not statistically significant.

A. Western blot analysis validates MAXL, MAXS and MNT overexpression and their inducible decreased level after addition of tetracycline;

B. Overexpression of MAXL and MNT induce high proliferation rate compared to tetracycline treated cells. Cell growth rate of each group were assessed by cell counting every 24 hours and expressed as number of cells per well;

C. qRT-PCR showing p21 decreased value after 48 hours of MAXL and MNT overexpression. Light gray bar indicates control empty vector sample, other samples are listed in legend. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

1.3.2 Effect of RA treatment in MAXL, MAXS and MNT overexpressing cells

To better characterize the role of MAX and MNT overexpression in determining cellular behaviors of MYCN-amplified NB cells, the capability of transfected cells to undergo differentiation was evaluated. After stimulation with 10μM of Retinoic Acid, morphological signs of differentiation and expression levels of the neuronal marker genes GAP43 and NEFM were evaluated. Average length and total number of neurites per number of cells were obtained as previously described.

Results indicate that when MAXS and MNT were overexpressed no significant morphological change was appreciated, whereas overexpression of MAXL results in a slight decrease in neurites formation. MYCN-amplified NB tumors and cell lines present up to 100 fold increase of MYCN expression levels and in particular, BE(2)-C are 4-stage tumor cells deriving from bone marrow metastasis with MYCN-HSRs and thus considered highly malignant cell type [5]. Worsening its cancerous phenotype grade seems to be a hard effort, especially using a transient system.

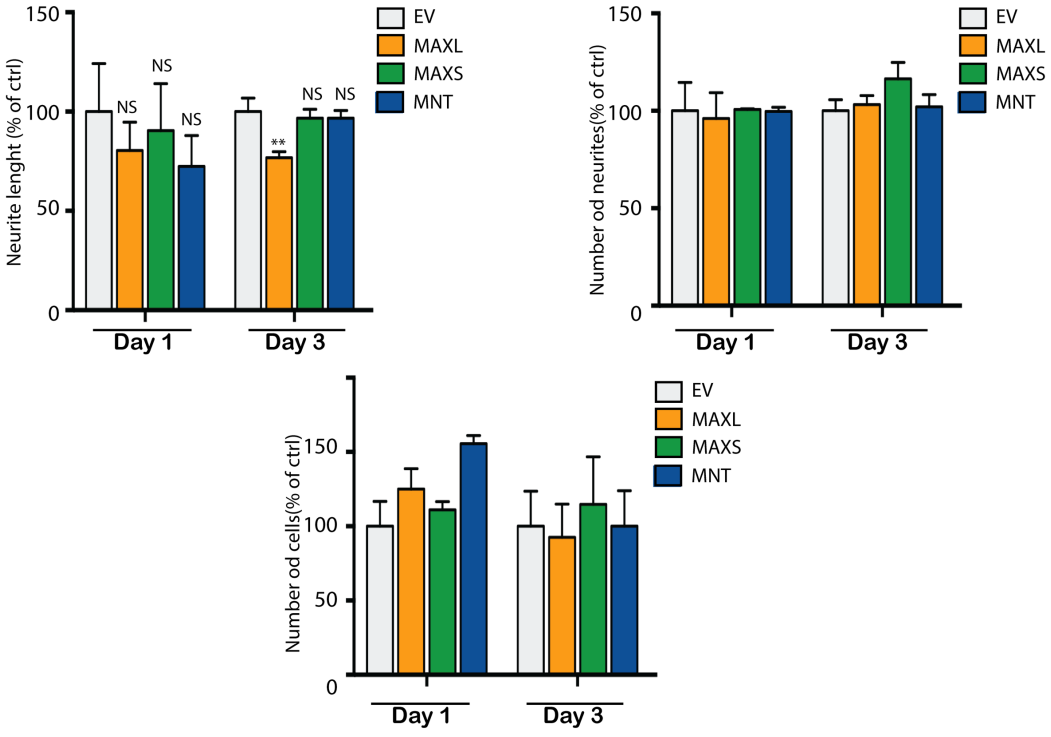


Fig.9. Transient overexpression of MAXL and MNT in BE(2)-C do not induce change in neurite length. All data shown as mean of at least 2 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; NS not statistically significant.
Effect of 10μM of RA treatment on MAXL, MAXS and MNT overexpressing cells. Upper left panel shows average of neurite length plotted as percentage of the control. Upper right panel shows number of neurites plotted as percentage of control and normalized on cell numbers. All data are NS. Lower panel shows number of cells after RA treatment. All data are normalized on tetracycline treated samples. All data are NS.

From analysis of neuronal differentiation markers GAP43 and NEFM emerges the different role of MAX long and short isoforms. When BE(2)-C are transfected with MAXS, all two neuronal markers are strongly expressed thus suggesting a protective and pro-differentiative role. On the other hand, MAXL overexpression do not to impair neuronal marker expression. In MNT overexpressing cells, all two neuronal marker mRNA levels were found significantly decreased suggesting an opposite role for MAXS and MNT.

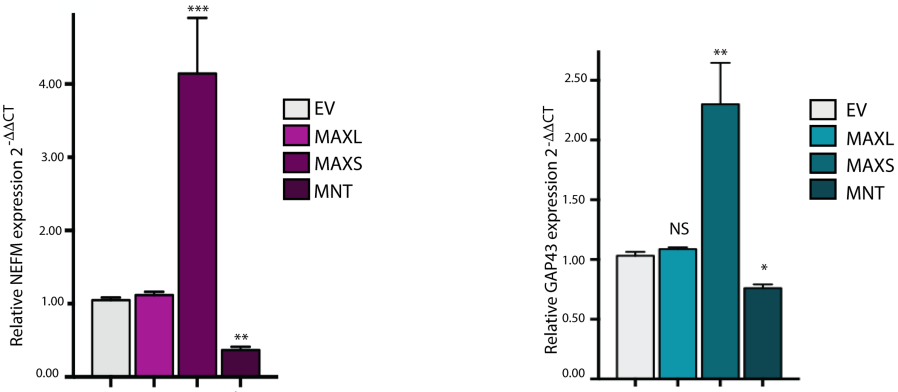


Fig.10. Transient overexpression of MAXL and MNT in BE(2)-C induces change in neurite length. All data shown as mean of at least 2 independent experiments ± SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *, **, *** indicate P<.05, .01 and .001. NS not statistically significant.
qRT-PCR on transducted cells. Purple panel on the left indicates NEFM mRNA level while green panel on the right represents GAP43 mRNA expression levels. Light gray bar indicates control EV sample. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

1.3.3 Overexpression of network members affects prognostic markers inducing gain in migratory capability

NGFR and TRKA are considered markers of early and immature neuronal differentiation [150,151]. Given the role of TRKA and NGFR in promoting *in vitro* neurite outgrowth and given their expression in favorable NBs, to better characterize MAX and MNT overexpressing cells TRKA and NGFR mRNA levels were evaluated.

Results indicate that both TRKA and NGFR mRNA expression was found significantly high after transient expression of MAX short isoform, thus strengthening the hypothesis of the protective MAXS role compared to MAXL. To reinforce this evidence, expression of TRKB, commonly found in poor prognosis NBs, is significantly high in MAXL and MNT while not in MAX short isoform.

Another intriguing finding that emerges from analysis of neuronal and positive prognostic markers is the opposite role of MAXS and MNT: as shown for GAP43 and NEFM, also TRKA and NGFR were found significantly decreased after MNT overexpression and not after MAXS overexpression. Modulation of neurotrophin signaling receptors occurs without any change in MYCN levels.

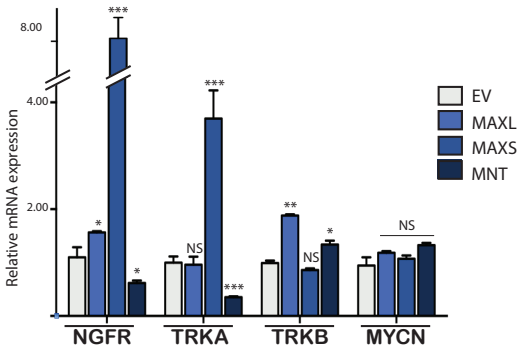


Fig.11. qRT-PCR of positive prognostic markers compared to MYCN expression level on transducted cells. Light gray bar indicates control sample. X axis indicates mRNA analyzed. Samples are indicated on legend; All data shown as mean of at least 2 independent experiments ±SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; *, ** and *** indicate P<.05, .01 and .001 respectively. NS not statistically significant. Internal reference gene used was GlUcuronidaSeBeta, GUSB.

Migratory activity was then investigated through wound closure assay.

Results underline that overexpression of MAXL and MNT induce a slightly trend in increase motility capability of BE(2)-C, whereas overexpression of MAXS do not impair motility.

As previously point out, BE(2)-C are highly malignant tumor cells with a replicative cell cycle of 18 hours (data from ATCC Standards Development Organization) that makes it difficult to analyze a predicted more malignant phenotype.

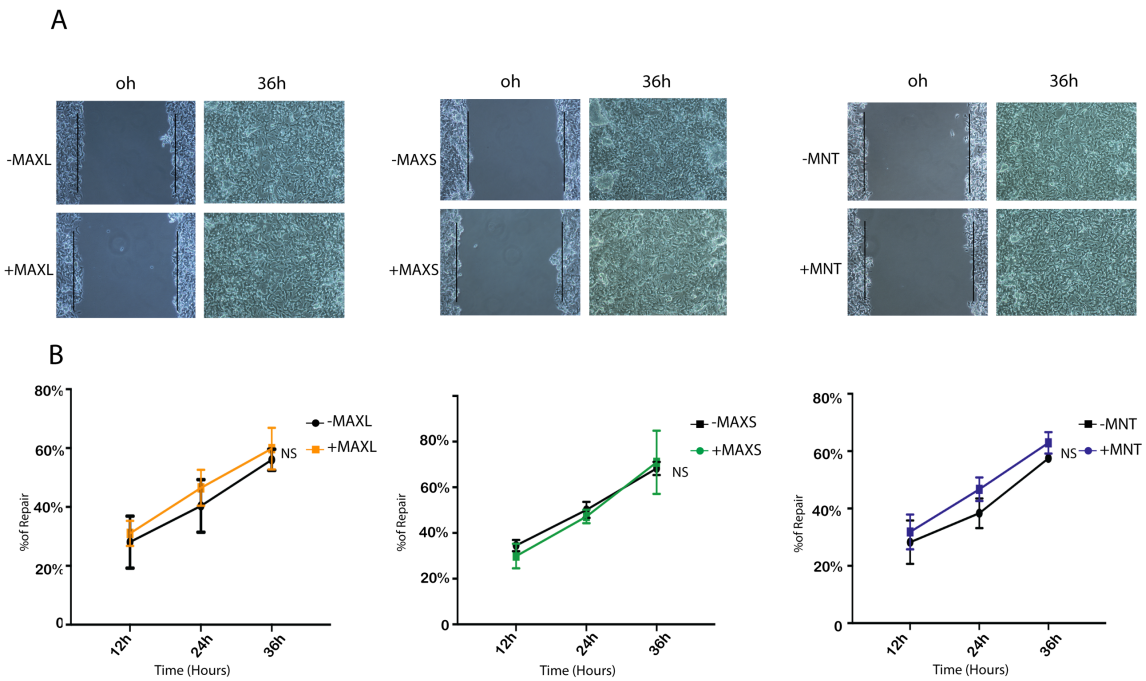


Fig.12. Wound closure assay for 36 hours, compared with tetracycline-treated cells. All data shown as mean of at least 2 independent experiments ±SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; NS not statistically significant.
A. Representative images of wound closure assay;

B. Wound closure assay on BE(2)-C transfected with MAXL*(left orange panel)*, MAXS *(middle green panel)* and MNT *(right blue panel)* and expresses as percentage of repair.

2 Pharmacological Modulation of the MYCN/MAX/MXD Network

Conventional approaches to treat NB involved surgery, radiotherapy and especially administration of non-specific cytotoxic drugs that lead to high incidence of side effects due to high grade of aspecificity. Here resides the need to develop novel less toxic molecular-target therapy. There are many clinical trial testing drugs that target various molecules but the most attractive molecule to target remains MYCN. The rationale for choosing MYCN as a therapeutic target for treatment of neuroblastoma lies firstly in its deregulated expression in highly malignant tumors, and moreover, MYCN amplification is associated with patient poor clinical outcome. Additionally, its expression in normal tissue is restricted to the early stages of embryonic development, and is virtually undetectable in normal adult tissues.
To date, despite the attractiveness of MYCN as a therapeutic target and the discovery of new

MYCN targeting compounds, most of them have not passed the pre-clinical stage because of their non-specificity or selection of resistant clones.

2.1 Chemical library screening identifies M606 as MYCN inhibitors

From the collaboration with the research group of Dr. Michelle Haber (Children's Cancer Institute, Australia, Sidney) a chemical library comprising 34.000 molecules were screened and a derivative of hydroxyquinolones named M606 was isolated for its strong ability to downregulate MYCN both at protein and mRNA level as shown in Figure 12.C and for its ability to reduce oncogenic behaviors of NB MYCN amplified cell lines (data not shown). Then, it was questioned if M606 may act by inducing change in MYCN mRNA or protein stability. For this purpose, BE(2)-C were treated with cycloheximide, inhibitor of proteic synthesis and with Actinomycin D, an antibiotic inhibitor of DNA synthesis. At regular time points, respectively total protein samples and mRNA samples were evaluated through Western blot and qRT-PCR analysis. Panel D of Figure 12 illustrates that M606 does not induce change in protein or mRNA decay time and since there is no change in stability, it was therefore hypothesized that it can act at transcriptional level.

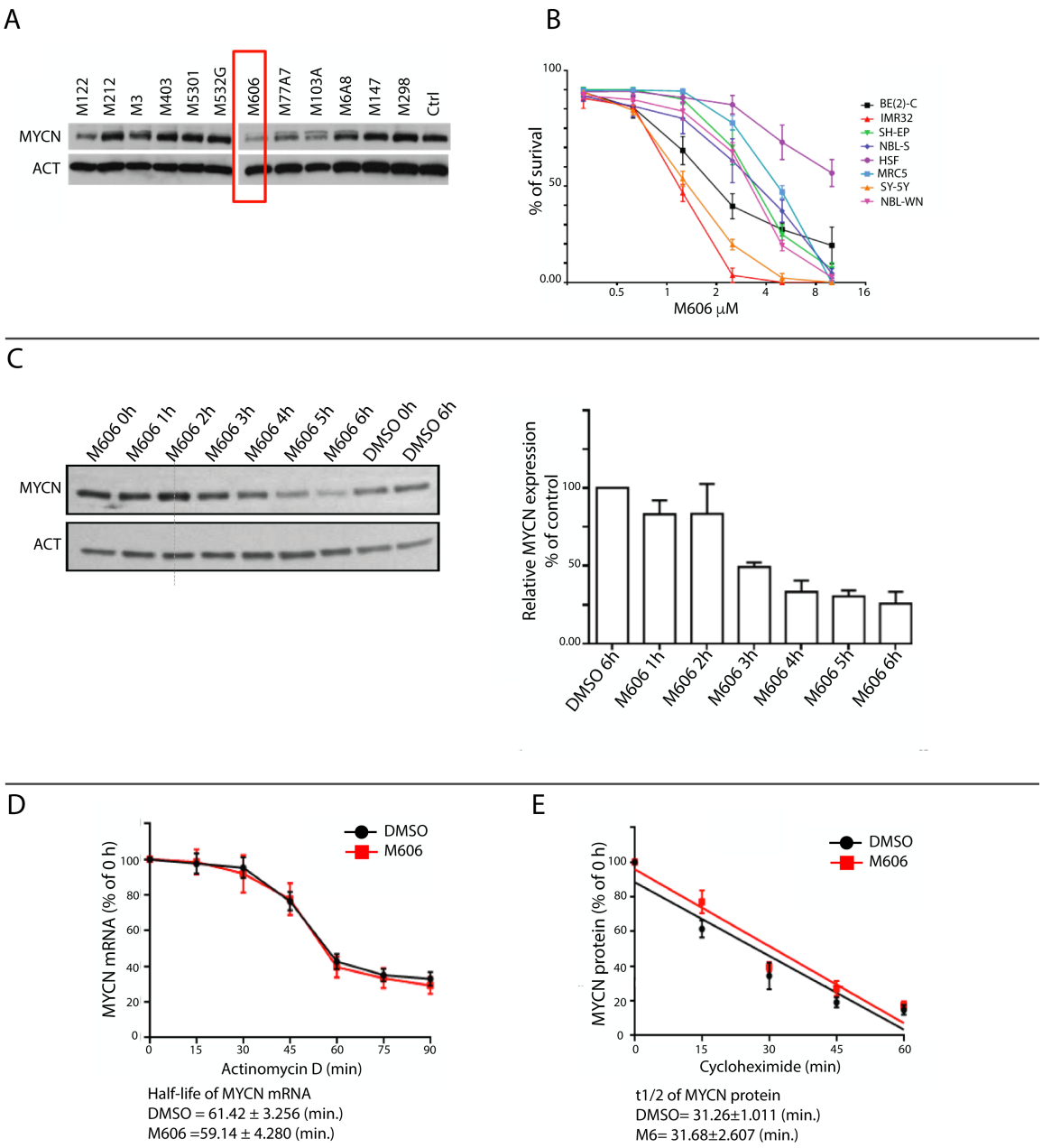


Fig.13. Screening on a large chemical library of unique compounds in a cell-based readout system leads to identification of M606.

A. Identification of M606 compound for its ability to decrease MYCN protein level compared to other drugs tested. Here represented just few compounds tested on BE(2)-C;

B. Cytotoxicity assay on different NB MYCN-amplified cell lines with increasing concentration of M606;

C. M606 can strongly decrease MYCN levels. BE(2)-C treated with 15μM of M606 for the listed time points. *Left panel:* Western blot analysis; *right panel:* qRT-PCR expressing MYCN levels as percentage of control;

D. MYCN mRNA levels plotted as a function of time after M6 treatment and after the addition of actinomycin D. In red are shown treated sample decay while in black mRNA levels of cells treated with DMSO as control;

E. MYCN protein levels plotted as a function of time after M6 treatment and after the addition of cycloheximide. In red are shown treated sample decay while in black protein levels of cells treated with DMSO as control.

2.2 M606-mediated MYCN downregulation depends on cell density

Sensitivity to pharmacological treatments could be dramatically different depending on cell confluency. Many cancer chemotherapy agents selectively kill actively dividing cells therefore sub-confluent cell cultures while sparing cells that are not dividing. Since the gene expression patterns of growing and cell cycle arrested cells are different, this can also result into selective expression of membrane transporters, metabolizing enzymes, or binding proteins that could affect pharmacological treatment. On the other hand, many drugs have similar effect in confluent as well as in sub-confluent conditions. M606 action on MYCN protein is effective only in actively proliferating cells.

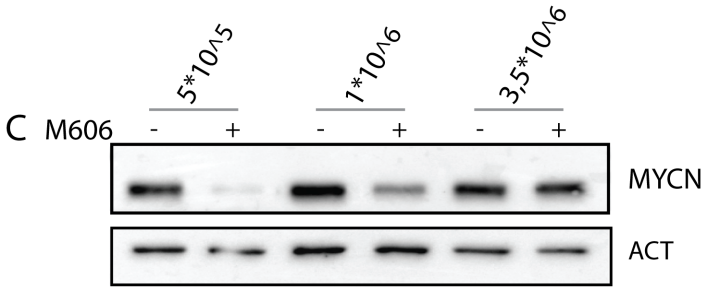


Fig.13. Western blot analysis on BE(2)-C to test action of M606 on cell confluence; actin was used as internal control.

2.3 M606-mediated MYCN downregulation can be reverted by iron addition

M606 is a derivative of hydroxyquinolones which shows ability to chelate iron. To date, main drugs targeting MYCN that passed the pre-clinical trial belong to iron chelator class. Epidemiologic studies pointed out higher cancer incidence in iron overload individuals compared to iron deplete individuals which shown low incidence of tumor formation [130,131]. Iron is an essential element for metabolism and cell cycle progression and thus it is particularly required to cancer cells.

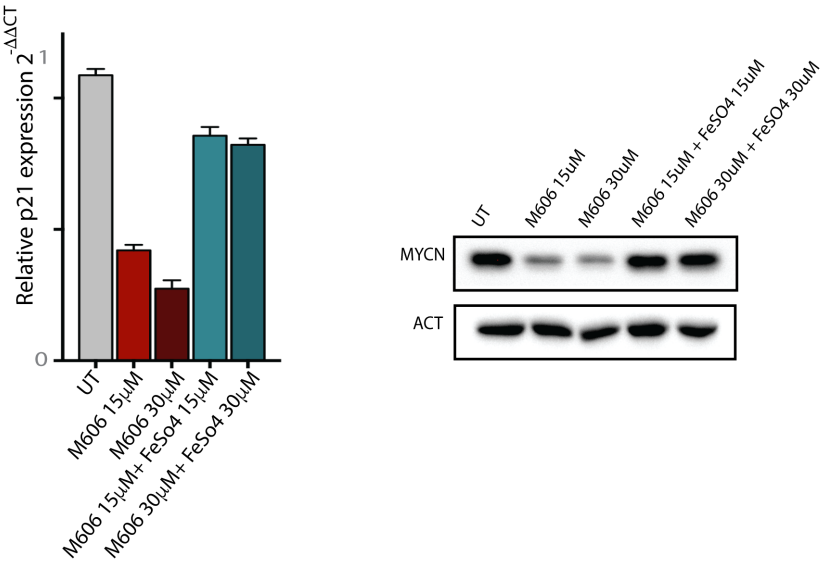


Fig.14. Right panel shows qRT-PCR on BE(2)-C treated with 15μM of M606. Western blot analysis on treated cells is shown on left panel; actin was used as internal control.

2.4 M606-induced MYCN downregulation occurs through decrease in chromatin acetylation pattern and iron addition restores acetylation status of chromatin

To better understand the mechanisms of M606 action on MYCN expression, acetylation status of MYCN promoter was analyzed. Histone acetyltransferases HATs act antagonistically to HDACs to control chromatin accessibility for gene transcription. Also, DNA methylation works in gene silencing allowing densely packing of DNA and thus, lowering gene expression. Moreover, recent findings have shown that various tumors as NBs, show recurrent genetic mutations converging on epigenetic mechanisms. To define the epigenetic mechanisms that drive the M606-mediated MYCN downregulation, chromatin immunoprecipitation (ChIP) was performed using H3 pan-acetylated antibody on BE(2)-C treated for 12 hours with 15μM of M606. Results highlight role of acetylation and not in methylation in MYCN downregulation induced by compounds. ChIP shows a decrease in acetylation that can be blocked by addition of equimolar of iron (figure 14.A and B).

DNA methylation pattern was further investigated performing a methylated DNA immunoprecipitation using an antibody raised against 5-methylcytosine (5mC). Results show no change in methylation after M606 treatment (figure 14.C and D).

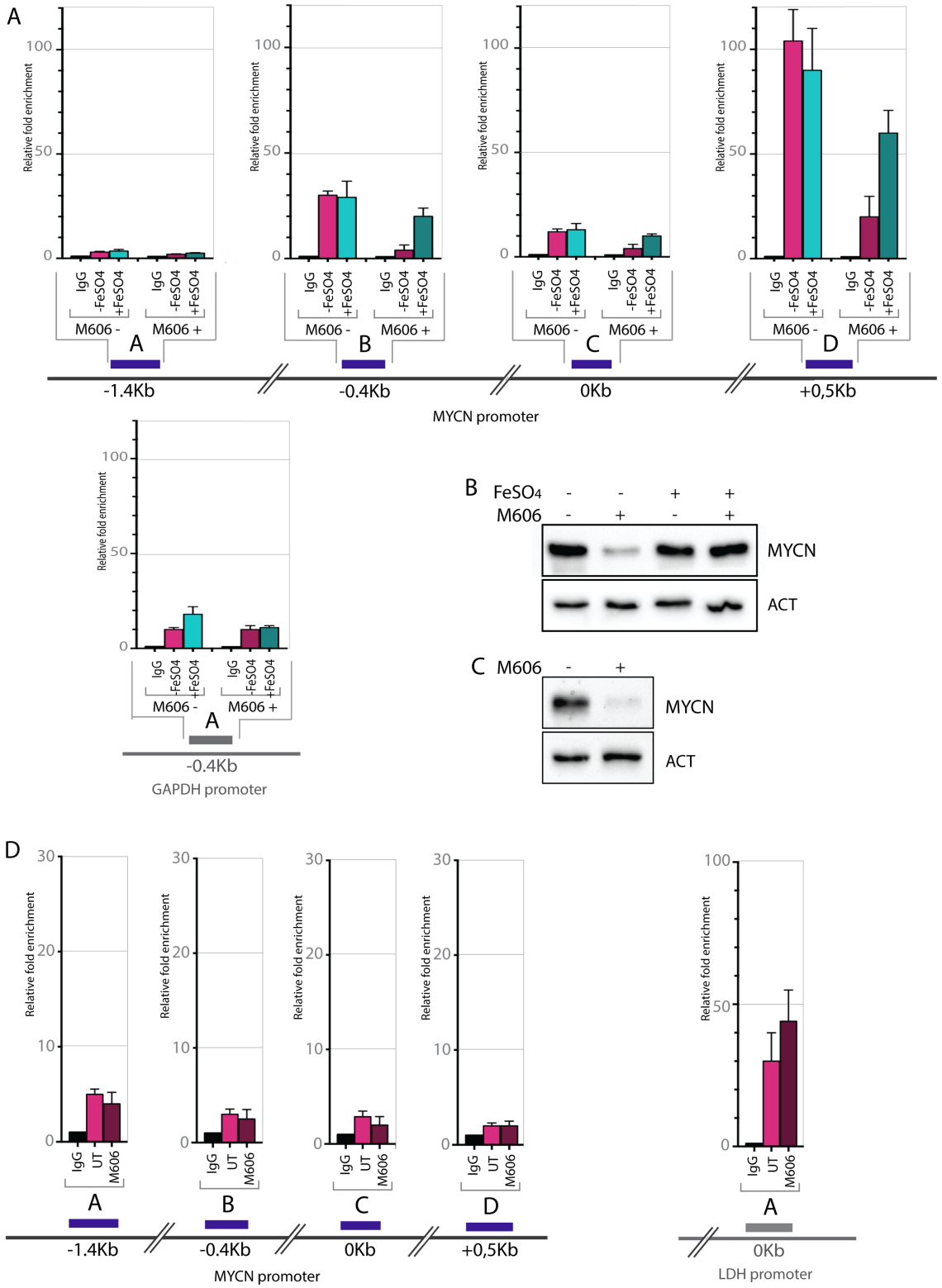


Fig.15. Acetylation and methylation pattern of MYCN promoter after 12h of M606 treatment. Mean of two independent experiments ±SEM standard error of the mean. Data are presented as fold enrichment normalized on enrichment obtained using pre-immune serum (IgG).

A. ChIP on MYCN promoter of BE(2)-C treated with 15µM M606 and with equimolar of $FeSO_4$. GAPDH promoter is used as positive experimental control.

B-C. Western blot analysis on ChIP sample (B) and MeDIP sample (D). Actin was used as internal control.

D. MeDIP on MYCN promoter of BE(2)-C treated with 15µM M606. LDH is used as positive experimental control.

2.5 Identification of the minimum M606 responsive region

Since M606 induces no change in MYCN protein and mRNA stability, but rather its inhibition leads to a decreased acetylation pattern, it was hypothesized that it can act at transcriptional level. To support this hypothesis, a reporter gene assay was used to evaluate M606 action on MYCN promoter.

2.5.1 Identification of the minimum M606 responsive region in the MYCN promoter

MYCN promoter was cloned upstream luciferase firefly coding sequence and the activity of MYCN_LUC construct after 12 hours of M606 treatment was tested. BE(2)-C were cotransfected with MYCN promoter_LUC construct and a construct carrying another luciferase that acts as internal calibrator of the system. In figure 15, it is shown the workflow of the analysis and the related graphs. Given the existence of two MYCN mRNAs from RefSeq, 2.9Kb comprising all two putative MYCN promoters were analyzed. Figure 15.A shows the three constructs used comprising the two MYCN promoter and the whole 2.9kb construct. Through a reporter gene assay all constructs were tested for their ability to drive LUC transcription. Data are reported as ratio between treated on not treated, normalized on values of exon 62 of dystrophin and expressed in RLU or relative light units.

Results indicate that the action of M606 on the construct -1385;-2 of MYCN promoter leads to a strongly decrease in luciferase activity. As consequence, also the whole MYCN promoter respond to M606 action (figure 15.B)

Consequently, to verify the possible existence of one or more M6 responsive regions, 9 deletion constructs were generated and tested. These constructs were designed to have 200 bp of serial deletions with 50 bp overlapped (Figure 15.C) . After BE(2)-C transfection and M606 treatment, it was identified construct with deletion 8 as responsible of M606 action on MYCN promoter. Absence of this region in the MYCN promoter lead to M606 insensitivity and thus result in no RLU variation (Figure 15.D). Also construct with deletion 9 shows a partially insensitivity to M606 action therefore it was decided to test all 200bp that were deleted in constructs 8 and 9 and moreover to test a negative random region of the MYCN promoter (Figure 15.E right panel).

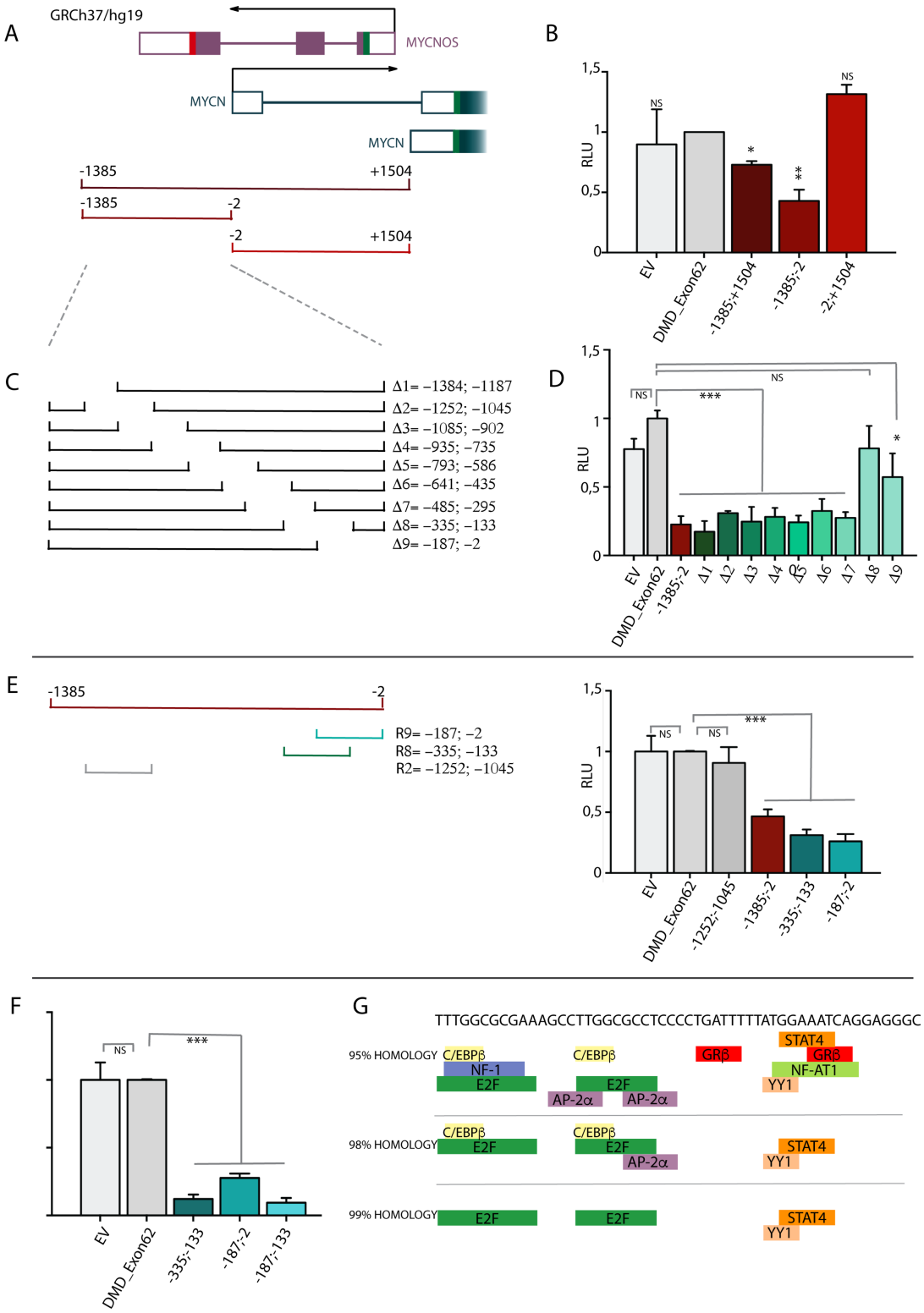


Fig.16. Workflow and dual luciferase assay. Luciferase activity is expressed in RLU. Data are represented as mean of at least 3 independent experiments ±SEM standard error of the mean. Data were normalized on exon 62 of dystrophin DMD. All grey bar represents negative controls.

A. Schematic representation of the three MYCN promoter tested;

B. Dual luciferase reporter assay on BE(2)-C after 12 hours of 15µM M606 action. Constructs tested are represented in red;

C. Schematic representation of the 9 deletion constructs tested;

D. Dual luciferase reporter assay on BE(2)-C after 12 hours of 15μM M606 action. Whole MYCN promoter in red is used as positive control. In green are represented all deletion constructs;

E. Schematic representation of the three regions of 200pb of MYCN promoter tested on the left panel. Right panel shows dual luciferase assay of the three construct. Whole MYCN promoter in red is used as positive control. Dark green bars indicate construct carrying 200pb deleted in construct 8 and 9;

F. Dual luciferase reporter assay on BE(2)-C after 12 hours of 15μM M606 action. Constructs carrying region deleted in construct 8 and 9 were used as positive control *(dark green)*. Constructs carrying the 50 overlapped bp are represented in turquoise;

G. *In silico* analyses of putative transcription factors performed with different homology degree.

The presence of random region upstream the luciferase gene does not yield any M606 response, whereas the presence of the 200bp deleted in constructs 8 and 9 lead to a strongly decrease in luciferase activity thus suggesting their role in M606-mediated MYCN downregulation (Figure 15.E left panel). As previously mentioned, two constructs have 50bp in common and this suggest that the putative involved region may span this region. Therefore, 50pb were cloned and tested through reporter assay and result evident in Figure 15.F its involvement in mediate M606 action on MYCN promoter. To identify putative factors involved whose consensus span this 50bp, *in silico* analyses were performed using **P**redicted **P**rokaryotic **R**egulatory **P**roteins P2RP software (figure 16.G) [157]. Analysis performed filtered for transcriptional factors with 99% of homology revealed 2 E2F consensus and stat4 and YY1 consensus sequences.

2.5.2 M606 action on MYC protein and identification of homology sequence between MYC and MYCN

Interestingly, results in figure 16.A indicate that compound acts also on cMYC inducing a decrease in its expression in different NB cell lines but also non-neuronal cell line as HepG2 (data not shown). Given this evidence, a further *in silico* analysis was performed to identify a common region between MYCN and cMYC promoter and a region of 24bp comprising three E2F sites, two of which are inversely oriented and overlapped and one TIE TGFβ inhibitory element was identified (figure 14.B). This region spans 24 bp upstream of the multiple transcription start site, which is highly conserved in the human and mouse MYC genes and mediate major part of promoter activity and responsiveness to TGFβ [80]. Thus, to better define the role of this sequence in mediate M606 action, 3 further constructs were created and tested. The first construct has whole MYCN promoter sequence except the putative 24bp, while second and third constructs comprise whole MYCN promoter except a

deletion of respectively the two inversely oriented and overlapped E2F sites and E2F/TIE sites.

Results underline that deletion of the whole E2Fs/TIE sites and deletion of the E2F1-2 consensus from the whole 1383bp of MYCN promoter disrupt the action of M606 on MYCN thus strongly supporting the hypothesis of E2F involvement in MYCN downregulation (figure 16.C). Given the derivation of BE(2)-C from stage 4 treated patients, it was questioned if this could be a generalizing mechanism of action or it could be BE(2)-C specific.

To strengthen the hypothesis of generalizing mechanism involving E2Fs in M606 downregulation of MYCN, the same assay was performed also in another neuroblastoma cell line LAN1 obtaining same result (Figure 16.D).

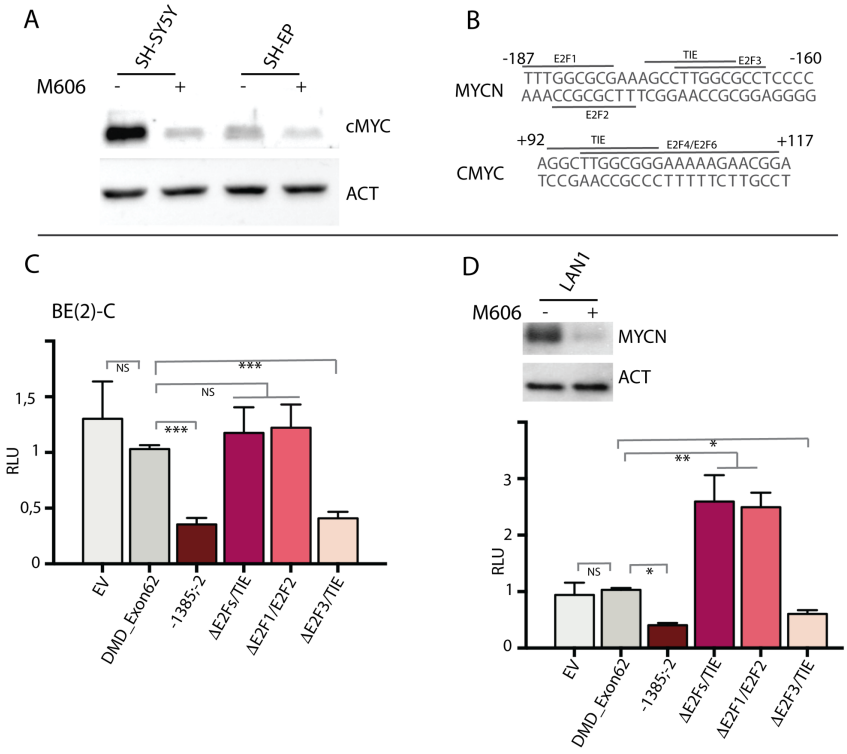


Fig.17. Identification of homology sequence between MYC and MYCN. Data are represented as mean of at least 3 independent experiments ±SEM standard error of the mean. Data were normalized on exon 62 of dystrophin DMD. All grey bar represents negative controls.

A. Western blot analysis performed on SH-SY5Y and SH-EP after 12 hours of 15μM M606 treatment;

B. Schematic representation of the homology sequence between MYCN and cMYC promoter region;

C. Dual luciferase assay performed on BE(2)-C transfected with deletion variants of the whole MYCN promoter. MYCN whole promoter was used as positive control *(dark red)* data are normalized on exon 62 of DMD. In purple is represented construct with E2Fs/TIE deletion. In pink construct with E2F1/E2F-2 site deletion and in light pink construct with E2F3/TIE deletion;

D. Upper panel shows 15μM M606 action on MYCN in LAN1 after 12 hours of treatment. Lower panel shows dual luciferase assay performed on LAN1 transfected with deletion variants of the whole MYCN promoter. MYCN whole promoter was used as positive control *(dark red)* data are normalized on exon 62 of DMD. In purple is represented construct with E2Fs/TIE deletion. In pink construct with E2F1/E2F-2 site deletion and in light pink construct with E2F3/TIE deletion.

2.6 Downregulation of MYCN involved dephosphorylation of RB protein and change in RB pathway related genes

E2F proteins regulate various target genes involved in various mechanisms such as DNA repair, cell cycle progression or apoptosis that finely regulate the balance between growth promotion and suppression. The 8 E2F members can be classified into different subgroups based on their structure, affinity for different members of pRB family and putative function. Members of the activator subgroup which consists of E2F1 2 e 3, act as transcriptional activators when unbound to RB, one of the pocket proteins with which activator E2Fs associate. It is well established role of impairment of E2F/RB pathways in tumor (here reported in paragraph 2.2.3). During quiescent phase activator E2Fs are sequestered by hypophosphorylated RB resulting in suppression of E2Fs target genes and suppression is further strengthen by recruitment of chromatin-modifying factors. On the other hand, growth stimuli result in expression of CDKs which phosphorylate RB thus, leading E2Fs in active status.

Given the involvement of activator E2F in mediate M606 downregulation of MYCN, it was hypothesized a change in phosphorylation pattern of RB protein. Results show decreased in hyperphosphorylated state of RB in two neuroblastoma cell lines BE(2)-C and LAN1 (Figure 17.A and C). To better understand whether this could be a generalized mechanism common to iron chelator drug class, another iron chelator Exjade or EXJ was tested. Results indicates that also Exjade induces a decrease in MYCN protein level and favors hypophosphorylated RB state. Action of Exj on MYCN promoter both in LAN and in BE(2)-C involves E2Fs sites (Figure 17.B and D).

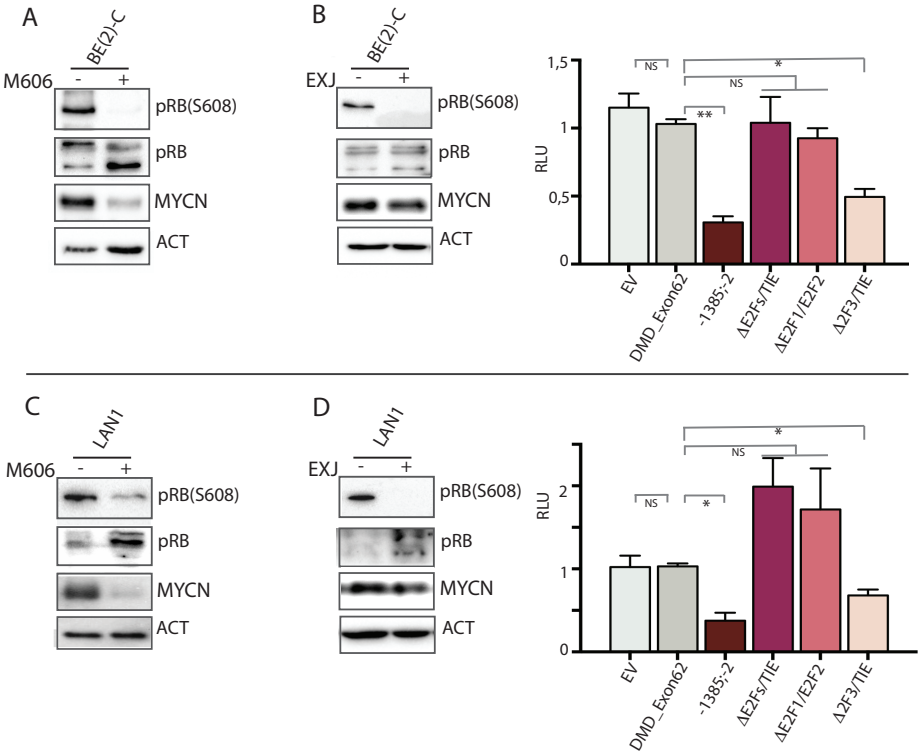


Fig.18. Downregulation of MYCN involved dephosphorylation of RB protein. Data are represented as mean of at least 3 independent experiments ±SEM standard error of the mean. Data were normalized on exon 62 of dystrophin DMD. All grey bars represent negative controls.
A. Western blot analysis on BE(2)-C after 12 hours of 15μM M606 treatment;

B. Western blot analysis on BE(2)-C after 12 hours of 100μM Exjade treatment on right panel and dual luciferase assay on left panel.

C. Western blot analysis on LAN1 after 12 hours of 15μM M606 treatment;

D. Western blot analysis on LAN1 after 12 hours of 100μM Exjade treatment on right panel and dual luciferase assay on left panel.

2.7 M606 induces change in expression of RB pathway related genes and MYCN/MAX/MXD Network members

Region of 24 bp upstream of the multiple transcription start site of MYCN promoter identified as involved in M606 action is known to mediate responsiveness to TGFβ [80]. Transforming growth factor β represents a cytokine that causes growth inhibition in many lymphoid and epithelial cell types by inducing hypophosphorylation of RB protein. Thus, it was hypothesized the involvement of TGFβ in mediate RB dephosphorilation. Results indicates a strongly increased in p21 (CDKN1A gene) emerged, accordingly with cell cycle exit. Moreover, analysis reveals a drug-related alteration of E2F members. Exjade treatment in both BE(2)-C and LAN1 results in increased levels of activator E2Fs and E2F7, a repressive protein which regulates transcription without association to RB. Same Exjade

treatment induce upregulation in all TGF–related mRNA in BE(2)-C including TGFα, TGFβ1-2-3 and TGF receptors and in LAN1 results in TGFβ1-3 and TGF receptors increase. M606 action on both BE(2)-C and LAN1 results in TGFβ1-2-3 increase and in LAN1 induces also upregulation of TGF receptors.

It is known that in the E2F/RB-mediated onset of pathology there could be distinct transcriptional mechanisms mediated by deregulated E2F or physiologically activated E2F and peculiarity of deregulated E2Fs is the ability to induce transcription of both typical and atypical E2F target genes due to dysfunction on the related pathway. Data reveals no variation of atypical E2F target gene CDKN1B ($p27^{Kip1}$) both in LAN1 and BE(2)-C.

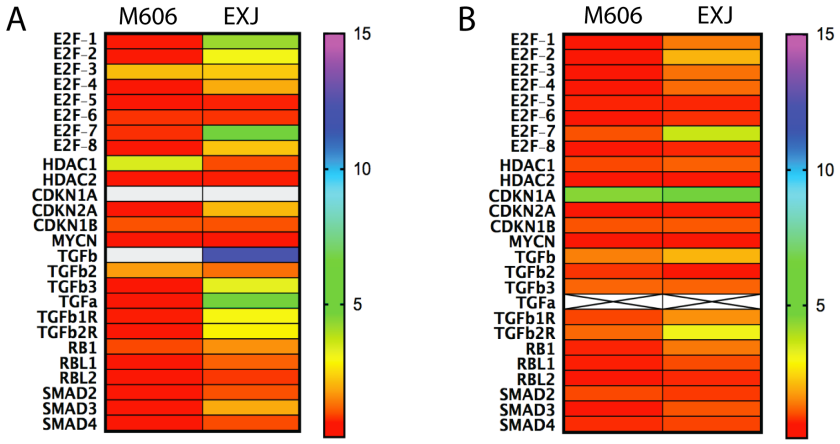


Fig.19. qRT-PCR of RB pathway related genes on BE(2)C (**panel A**) and LAN1 (**panel B**) after 15μM and 100μM of respectively M606 and Exjade treatment for 12 hours. Data are expressed normalized on untreated samples. White colored panels indicate out of range values while cross indicate data excluded from analysis. All data shown as mean of at least 2 independent experiments ±SEM standard error of the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test; Internal reference gene used was GlUcuronidaSeBeta, GUSB.

MXD proteins are members of the bHLHz motif network which MYCN belongs to together with MAX and MNT. While MNT and MAX appear to be expressed independently from cell cycle, MXD expression is confined to differentiated quiescent cells, thus limited to arrest and differentiation time points.

Results on BE(2)-C shows induction of MAX and MXD proteins and in parallel a decrease in MYCN protein levels.

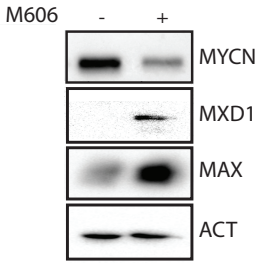


Fig.20. Western blot analysis on BE(2)-C treated for 12 hours with 15μM of M606. Actin was used as internal control.

DISCUSSION

Amplification of MYCN gene is common to many types of infancy cancers of mostly neuroendocrine origin, including medulloblastoma, astrocytoma, Wilms' tumor, small cell lung cancer, osteosarcoma, rhabdomyosarcoma and about 25% of neuroblastoma (NB) [43,44]. Neuroblastoma is an embryonal malignant tumor characterized by deep genetic heterogeneity that results in different NB subtypes. A subset of malignant NBs is characterized by MYCN oncogene amplification and overexpression that strongly correlate with poor prognosis [5]. Weiss et all, revealed that overexpression of MYCN alone was sufficient to initiate NB formation in mice [35]. Moreover, no NB has been shown to amplify another gene that did not also amplify MYCN and, its protein coding domain is rarely mutated making reside its oncogenic properties on regulatory mutations that lead to uncontrolled high expression [36,51]. Given these observations, it was suggested that inhibition of MYCN expression and/or MYCN-mediated transcription pathways provides an attractive therapeutic target for treatment of NB.

1 Genetic modulation of the MYCN/MAX/MXD network

MYCN is a member of the MYC gene family and encodes for a transcription factor which can regulate approximately 10-15% of the whole genome both directly associated to DNA and indirectly via recruitment of other transcription factors already associated to regulatory DNA regions [55]. Direct MYCN-related activation of transcription requires heterodimerization with its obligatory partner MAX that results in a functional transcription factors which can bind E-box sequences located near promoters or enhancers of target genes. MAX, together with MYCN, belongs to an extended network of transcription factors defined by the presence of a BHLHz motif known to mediate protein-protein interactions among members of the network and DNA binding. Role of MAX in modulate MYCN activity resides in its ability to both *in vitro* and *in vivo* homodimerize and bind E-boxes leading to transcriptional repression of MYCN target genes.

Another level of MAX-mediated MYCN inhibition is MAX availability: among all members of the network, MNT is known as the main antagonists of MYCN at first instance, for the capability to bind MAX limiting its amount for MYCN binding, but moreover for the presence of SIN3 interaction domain (SID) with which can exert its main function as repressor of MYCN target genes [116,119,120]. Impairment of the network in cancers are

well reported in literature and the role of MAX and MNT in cancer has been widely discussed in chapter 2.3 of the introduction.

All these findings lead to the observation that the sole MYCN amplification can not exactly reflect the biological activity of MYCN but its functions are strongly related to the expression of MYC/MAX/MXD Network proteins. In the present work, it has been highlighted the role of the imbalance in the network in determine the aggravation or the improvement of oncogenic cell behaviors.

Meta-analysis conducts using published gene arrays on Kocak dataset of 649 NB patients highlighted that high levels of MAX and MNT in MYCN amplified NB patients are strongly related to short term event free probability. On the other hand, emerged that low levels of MYCN antagonists in presence of MYCN amplification, are related to good clinical outcome [149].

In the present work, using RNA interference in MYCN-amplified cells we have demonstrated that downregulation of MAX and MNT leads to suppression of the malignant phenotype affecting cell proliferation and promoting neuronal differentiation and positive prognostic markers and moreover, through transient overexpression, it has been confirmed that high levels of MYCN antagonists correlate with improvement of malignant cellular phenotype in presence of MYCN amplification.

Modulation of MAX: downregulation of MAX leads to impairment in cell cycle progression. MYCN-MAX heterodimer is known to have an important role in transcriptional regulation of genes whose products are involved in proliferation [52]. Recent studies have shown that upregulation in MAX-MYCN dimer globally elevate the transcription rate of almost all genes normally expressed in that specific cellular system [157]. Thus, it is likely that limited amount of MAX may be the cause of reduction in proliferation.

$p21^{Cip1}$ is a potent cyclin-dependent kinase inhibitor 1 that inhibits CDK2 and CDK1 complexes mediating cell cycle G_1 phase arrest. Many primary neuroblastoma shows $p21^{Cip1}$ downmodulation, which is particularly severe in patients with MYCN amplification [158]. Moreover, it is known that MYCN-MAX represses the expression of $p21^{CIP1}$, by forming a complex with transcriptional regulators, such as the MIZ-1 and SP1 thereby promoting cell growth and cancer onset [62]. Moreover, overexpression of $p21^{Cip1}$ is related to significant neurite extension in neuroblastoma cells [159]. Accordingly with these findings, in the present work it has been demonstrated that knockdown of MAX leads to

upregulation of $p21^{Cip1}$ supporting the hypothesis that limited amount of MAX may be the cause of modulation of MYCN target genes involved in proliferation and differentiation.

Neuroblastoma cells retain some features of neural crest progenitors, such as the ability to undergo neuronal differentiation in the presence of appropriate signals. When MAX is downregulated, BE(2)-C rapidly undergo neuronal differentiation after RA stimuli showing most typical morphological changes observed during neuronal differentiation: extensive neurite extension, decreased neurite branching, typical neurite alignment and upregulation of neuronal marker GAP43.

The differentiation ability of PC12 cells emphasizes that MAX is not necessary for the differentiation process. PC12 are pheochromocytoma cells lacking MAX expression which are known to undergo differentiation after stimuli and to induce *in vitro* transcription of a reporter gene linked to the MYC-MAX DNA binding site [107]. The existence of this cell line underlines that MYC-MAX complexes may not be absolute requirement for the whole MYC function and disruption of MYCN-MAX dimer mitigate malignant phenotype. To strengthen this hypothesis, it has been demonstrated that chemical inhibition of MYCN-MAX dimerization induces cell cycle arrest, differentiation and apoptosis *in vitro* and increased survival in MYCN transgenic mice model [160]. On the other hand, these cells fail to upregulate NEFM, the middle-molecular-weight neurofilament protein used as a biomarker of neuronal damage and whose down-regulation is a commonly found in neurodegenerative diseases as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease, thus suggesting a neuronal damage despite tendency to differentiate into neuronal phenotype [161].

We have shown that MAX downregulation in MYCN-amplified cell line results in expression of TRKA and NGFR with no change in TRKB/BDNF expression. High levels of the nerve growth factor receptor TRKA in association with NGFR are considered a positive prognostic markers commonly found in favorable NBs, whereas expression of TRKB in association with its ligand brain-derived neurotrophic factor BDNF is restricted to malignant NBs [29-31]. Moreover, TRKA and NGFR are MYCN negative targets and their expression induces differentiation and neurite outgrowth in PC12 pheochromocytoma cell line lacking MAX expression [162]. These lines of evidence suggest that limited availability of MAX may be the cause of MYCN target genes modulation.

Prognostic value of MMP/TIMP ratio in NB help in prediction of clinical outcome and define clinical stages. We have demonstrated that MAX knockdown in SK-N-BE(2)-C is associated with a shift in balance towards TIMP-2 expression after RA-treatment. Ratio between metalloproteinase MMP-2 and its inhibitor TIMP-2 is responsible for extracellular matrix proteolysis and degradation. Increased expression in NBs of MMP-2 in association with low levels of TIMP-2 is associated with poor clinical outcome and advanced clinical stages [152].

MMP-9 seems not to be associated with NB clinical stage and prognosis although it is well established its role in differentiation of adult neural progenitor cells. Experimental data reinforcing this evidence highlights association between MMP-9 expression in SK-N-BE after RA treatment and phenotypic neuronal-like differentiation. Moreover, MMP-9 knockdown promotes tumor invasion in mouse model of MYC-induced pancreatic neuroendocrine tumors [152-155]. In line with these findings, it has been here reported that knockdown of MAX induces increased expression of MMP-9 and RA induction of differentiation can enhance this expression.

It is well known the role of MYCN expression in promoting cellular migration of neuroblastoma cells and moreover it has been highlighted that enhanced migration is proportional to MYCN levels [163,164]. We have demonstrated that MAX knockdown results in impairment of migratory capability. According to clinical data, here it has been demonstrated that silencing of MAX in presence of MYCN amplification results in reduced aggressive phenotype and lines of evidence suggest that main effects are related to limitation in the availability of MAX.

On the other hand, accordingly to clinical Kocak dataset, MAX overexpression enhances cancerous features of BE(2)-C. As previously mentioned, BE(2)-C are highly malignant MYCN amplified cell type and worsening its cancerous phenotype grade is a hard effort. Alternate mRNA splicing produces two major highly conserved MAX isoforms, the longer of which MAXL differs from the shorter form MAXS for an insertion of 9-amino acid at codon11. Both proteins are expressed at approximately equal levels in most cell types but evidence suggests that MAXL can bind DNA more strongly than MAXS and that MAXL is able to repress a MYC-responsive reporter gene whereas MAXS had little effect on its expression but rather stimulated its expression. Furthermore, cells overexpressing MAXS

and not ones overexpressing MAXL do not shows variation in growth rate and accelerated apoptosis after growth factor depletion, suggesting its protective role [115].

In the present work we confirm that MAXL and not MAXS overexpression in BE(2)-C results in increased proliferation rate through downregulation of $p21^{Cip1}$. Moreover, MAXL overexpression leads to a slightly decrease in neurite formation after RA treatment without affecting expression of positive prognostic markers but rather inducing TRKB, a negative prognostic marker found in unfavorable NBs [165,166].
MAXL overexpressing cells shows also a slightly increase in motility. It is known that coexpression of MAXL with cMYC has a stimulatory effect due to its predilection to heterodimerize [115]. Our evidences confirm the stimulatory action of MAXL and not MAXS in worsening of the malignant phenotype of neuroblastoma cells.

Moreover, here we shown that overexpression of MAXS results in no modulation of neither proliferation nor migration but rather upregulation of positive prognostic markers TRKA and NGFR and upregulation of neuronal differentiation markers GAP43 and NEFM, strengthening the hypothesis of the protective MAXS role compared to MAXL [115,167]. MAXL and MAXS are the major isoforms of this protein and how they differ functionally from one another has not been determined. Accordingly, to literature, we have shown that two isoforms regulate gene expression, cell cycle progression, motility and differentiation in different ways. This could be probably due to a different pattern of CKII phosphorylation, which moreover, is known to affect MAX binding to DNA and regulate association with MXD proteins, although further experiments need to be done to fully explain their different biological behaviors [167].

Modulation of MNT: downregulation of MNT leads to impairment in growth rate not strictly dependent to upregulation of $p21^{Cip1}$. Whereas MYCN-MAX are known to actively promotes transcription through recruitment of coactivators thus inhibition leads to absence of dimer formation, MAX-MNT repress transcription through recruitment of mSin3 corepressor and involvement of deacetylases [116-120]. As a consequence, it is expected that MNT loss results in worsening of cancerous phenotype but it was not.

Role of MNT in apoptosis emerges from experiments highlighting the lethal effect of combined MNT loss and MYC overexpression in T cells and MEFs also associated with high levels of ROS production that, when accumulated beyond thresholds could be toxic and induce apoptosis. [117,169]. Thus, inhibition of malignant phenotype mediated by MAX-

downregulation may be due to lack in MYCN-MAX dimer formation, whereas loss of MNT may probably activate apoptotic pathways.

MNT knock down cells exhibit expression of TRKB in absence of BDNF. Coexpression of bone derived neurotrophic factor receptor and its ligand are negative prognostic markers in NBs associated with poor prognosis, and providing invasive and metastatic capability and enhancing therapy resistance [29-31] On the other hand, many studies underline the role of the sole TRKB expression in neurite formation thus suggesting its putative role in neuronal differentiation [165-166]. Accordingly, MNT-KD cells show strong upregulation of neuronal marker GAP43 and furthermore, RA induction results in increased levels of MMP-9 that is known to be associated with phenotypic neuronal-like differentiation, accordingly to what found for MAX-KD cells [152-153]. However, these cells do not develop any neurtic process, suggesting the requirement of MNT protein to complete differentiation process.

MNT knock down cells exhibit expression of positive prognostic markers TRKA and NGFR and a balance in MMP/TIMP that favors TIMP-2 expression after RA treatment.
The importance of MNT also emerges from its constant expression regardless of the cell cycle or the proliferative status. Indeed, these cells fail to upregulate NEFM, leading to suggest a neuronal damage in agreement with activation of apoptotic pathways [150]. Seems likely that MAX-MNT or MLX-MNT affinity and DNA binding could not be identical, thus phenotype observed after partially depletion of MNT could be a result in unbalance between formation of these dimers [168].
We have demonstrated correlation between good clinical prognosis in presence of MNT loss and cellular model of MNT knockdown.

On the other hand, overexpression of MNT results in enhanced malignant phenotype of BE(2)-C with strongly effect on proliferation rate through downregulation of $p21^{Cip}$, low levels of differentiation and positive prognostic markers TRKA and NGFR and moreover upregulation of TRKB expression, commonly found in unfavorable NBs. A slight enhancement in migratory capability is also observed.
MNT is known to mediate the repressive side also of the MONDO-MLX pathway, thus it is possible to ascribe the resulting phenotype even to an impairment in this side of the network. Through MNT ChIP-Seq data from ENCODE it was highlighted the presence of a subsets of MNT target genes that may not overlap with MYC-MAX target genes and whose action is still to investigate [168]. MNT is known to interact with Sin3 to induce transcriptional

repression and deletion of SID domain converts MNT from repressor to activator [171]. Given repressive effect of MNT mediated by mSin3 and given also mSin3 limited amount within cells, it is likely that effects of MNT upregulation may be probably linked to the presence of these subsets of genes.

Interestingly, MYC and MNT levels appear to autoregulate through a feedback system in which ectopic MYC expression induces endogenous expression of MNT and absence of MNT decrease MYC levels as a needed to counteract MYC excessive apoptosis both normally and in the context of tumorigenesis [169,170]. OX40 receptor activated T cells have a very robust proliferative response associated with both induction of MYC, MNT and MXD4 that appear to have main function in limiting apoptosis sustaining T cell proliferation [170]. Moreover, MNT and MXD4 are known to decreased during nerula stage in Xenopus, immediately before neural tube development, stages in which MYCN expression together with other necessary factors, is essential in migrating neural crest cells for the rapid expansion of progenitor population and then decrease during neural differentiation [76,172].

Fully explanation of the mechanisms underlying worsening of cell phenotype after MNT upregulation in MYCN amplified condition need further elucidation that, to date, are still lacking. Whereas a huge amount of literature exists on the role of imbalance in network members in physiological conditions, but less is known about impairment of the network in MYC or MYCN amplified conditions. Canonical scheme of MNT as a main MYCN antagonist should be probably revaluated in light of this unexpected role of MNT. Different independent studies underline that loss of MNT both via deletion or inactivation is not selective for oncogenesis as expected but may even be detrimental and selected against [117,118,168].

2 Pharmacological modulation of the MYCN/MAX/MXD network

To date, current treatment for NB consists of surgery, chemotherapy, radiation, and biotherapy but the main treatment for NB is based on chemotherapy combining different cytotoxic drugs that often has MYCN as molecular target [132,133]. The rationale for choose MYCN as a therapeutic target for treatment of NBs lies firstly in its deregulated expression commonly found in malignant tumors and associated with poor patient outcome. Furthermore, it has a restricted expression related to the early stages of embryonic

development and virtually undetectable in normal post-natal tissues. Despite the attractiveness of MYCN as a therapeutic target and the discovery of new MYCN targeting compounds, most of them have not passed the pre-clinical stage because of their non-specificity or selection of resistant clones. The main molecular class of cytotoxic compound used affecting MYCN expression is iron chelator [132,133] Epidemiologic studies on higher cancer incidence in iron overload individuals have pointed out the role of iron in cancer and many clinical trial are going on testing new iron chelator compounds to treat high risk NBs [130,131].

In the present work, it has been identified the molecular pathways of MYCN downregulation induced by M606, a novel iron chelator compound derivative of hydroxyquinolones. M606 can strongly induces MYCN protein and mRNA downregulation without affecting stability but rather acting at transcriptional level.

Combined IFN-γ and retinoic acid therapy correlates also with decrease in acetylation, indicative of a chromatin in a transcriptionally repressed state [173]. Here we demonstrate that M606-induced MYCN downregulation occurs through decrease in chromatin acetylation and not methylation pattern.

Moreover, M606 action on MYCN protein is related only to actively proliferating cells. Many cancer chemotherapy agents are known to selectively kill actively dividing cells (sub-confluent cell cultures) rather than sparing cells that are not dividing [174].

In the present work, it has been shown that E2F1 and E2F2 consensus have been found necessary to induce M606 impairment of MYCN promoter driven transcription of a reporter gene. E2Fs sites on MYCN promoter are upstream of the multiple transcription start site, a region highly conserved in the human and mouse MYC genes and mediate major part of promoter activity and responsiveness to TGFβ and comprises three E2F sites, two of which are inversely oriented and overlapped and one TIE TGFβ inhibitory element was identified [80].

Strieder et al. demonstrates the binding of E2F1, 2 and E2F3 to the proximal MYCN promoter *in vivo* and that inhibition of E2F activity in through overexpression of $p16^{INK4A}$ induced a reduction MYCN expression [80].

E2F/Rb pathway is essential for modulation of cell cycle, differentiation and survival of various cell types in the developing and adult CNS [94-96]. Although neither RB gene

mutations nor any other protein mutations known to inactivate the pRb pathway in other types of tumors have been detected in NBs, recent evidence suggests that E2F/Rb activity in NBs may be deregulated [81]. pRb pathway is found deregulated in many human cancers and neurodegenerative diseases such as Alzheimer, Parkinson's disease and Amyotrophic lateral sclerosis show active E2F and aberrant or hyperphosphorylated RB expression resulting in increased expression of E2F target genes [88-90]. The regulatory essence of E2F activator complexes is based on the ability to interact with pocket proteins but also to bind DNA with high affinity. Association of hypophosphorylated Rb to activator E2Fs sequesters their transactivation domain responsible for transcription of target genes and furthermore induces recruitment of chromatin remodeling complexes (e.g. HDAC), whereas hyperphophorylated RB are unable to interact with liberating their transactivation domain and allowing transcription of E2F target genes [81].

In the present work, it was pointed out that M606 can induce hypophosphorylated status of RB supporting the hypothesis of RB sequestration of E2F transactivation domain, thus resulting in E2F target gene downregulation. Here, it has been demonstrated that also Exjade, another iron chelator can act through the same mechanisms leading to hypothesize the presence of a generalized iron chelator mechanism that act via RB dephosphorylation.

It is known that in the E2F/RB-mediated onset of pathology there could be distinct transcriptional mechanisms mediated by deregulated E2F or physiologically activated E2F and peculiarity of deregulated E2Fs is the ability to induce transcription of both typical and atypical E2F target genes due to dysfunction on the related pathway [87]. In the present work, it has been demonstrated that both Exjade and M606 fail to modulate the atypical E2F target gene $p27^{Kip1}$ thus suggesting a physiologically activation of E2F released from RB.

The cyclin-dependent kinase inhibitor $p21^{Cip1}$ mediates cell cycle G_1 phase arrest and is related to significant neurite extension in neuroblastoma cells [159]. Moreover, it is known that MYCN-MAX represses the expression of $p21^{CIP1}$, by forming a complex with transcriptional regulators, such as the MIZ-1 and SP1 thereby promoting cell growth and cancer onset [62]. $p21^{Cip1}$ have been shown to be upregulated after M606 treatment.

There is also evidence that E2F proteins are involved in the negative regulation of MYCN induced by TGFβ and the 24bp identified region is known to mediate responsiveness to TGFβ [80]. Via qRT-PCR it has been shown upregulation of TGFβ and TGF receptor in both BE(2)-C and LAN1 suggesting an autocrine pathway of autoinhibition

that need further to be fully clarified. Exjade treatment also induces modulation of E2F protein family, contrary to M606. This could be probably due to their different iron capability or molecular structure.

Combined treatment with IFN-γ and retinoic acid is known to induce MYCN downregulation but further upregulation of MXD protein that is known to be associated with differentiation cellular phenotype [173]. MXD proteins are members of the bHLHz motif network which MYCN belongs to together with MAX and MNT. While MNT and MAX appear to be expressed independently from cell cycle, MXD expression is confined to differentiated quiescent cells, thus limited to arrest and differentiation time points. Accordingly with these findings, we demonstrate that M606 induce expression of MXD1 and MAX in SK-N-BE cells in presence of low levels of MYCN protein.

3 Final Remarks

Overall these studies provide evidence supporting the hypothesis that it is not the unique MYCN amplification that reflect the biological activity of MYCN but rather this is strongly related to the expression of MYCN/MAX/MAD network proteins. Overall, our results fully mimic the clinical condition in which a direct correlation between downregulation of MAX and MNT and good prognosis in MYCN amplified NB patients is observed. We have demonstrated that:

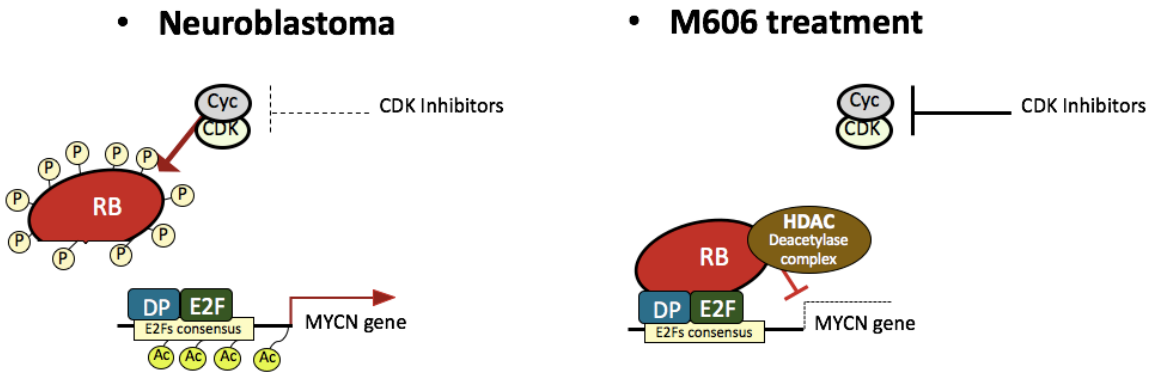
- knockdown of MAX leads to suppression of the malignant phenotype affecting cell proliferation, promoting neuronal differentiation and enhanced expression of differentiation and positive prognostic markers in MYCN amplified cell line;
- knockdown and MNT leads to attenuation of the malignant phenotype inducing decrease in cell proliferation and enhanced expression of differentiation and positive prognostic markers in MYCN amplified cell line.

We further demonstrate correlation between high levels of MAX and MNT protein in MYCN amplified context and poor prognosis accordingly to clinical data in which expression of MYCN counterparts is related to short therm survival:

- high levels of MYCN antagonists correlate with improvement of malignant cellular phenotype in presence of MYCN amplification affecting proliferation, migration and inducing expression of negative prognostic markers and repression of positive ones

Our study reveal also a dualism between two MAX long and short isoforms highlighting that they differentially regulate gene expression, cell cycle progression, motility and differentiation in different way. Our evidences confirm the stimulatory action of MAXL and not MAXS in worsening of the malignant phenotype of neuroblastoma cells and point out basis for further study on two MAX isoforms and their expression pattern in low and high risk NBs.

In the second part of the present work, a novel compound named M606 was characterized for its ability to repress MYCN at transcriptional level. Its action on MYCN promoter involved 24 bp upstream of the multiple cloning site of MYCN promoter and activator E2F consensus seems to be necessary.



Schematic representation of the hypothesized mechanism of M606 action proposed for the downregulation of MYCN.

Here it is hypothesized the mechanism of action involved in MYCN downregulation mediated by two iron chelators: iron chelation through both Exjade and M606 treatment induces upregulation of CDK inhibitors and results in hypophosphorylated status of RB protein that can bind activator E2Fs and sequester their transactivation domain. Repression of MYCN promoter is strengthen to the further recruitment of chromatin remodeling complexes.

Our findings provide evidence for deregulated E2F/RB pathways in NBs supporting the hypothesis of presence of physiologically activated E2Fs and not deregulated ones.
In conclusion, we have partially elucidated the molecular pathways through which a novel iron chelator mediates MYCN downregulation.

MATERIALS AND METHODS

NEUROBLASTOMA CELL CULTURES

Human neuroblastoma SK-N-BE(2)-C and LAN1 cell lines were maintained and cultured in high glucose DMEM containing 10% of fetal bovine serum, L-glutamine 2mM, Penicillin–Streptomycin solution with penicillin 100 U/mL of penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO2 at 37 °C.

DRUGS AND REAGENTS

When indicated, cells were treated with 15µM of M606, 100µM of Exjade or in combination with $FeSO_4$ 15µM for 12hours, or in combination with 15µM of Panobinostat (*Aurogene*). Drugs were resuspendend in DMSO and stored at -80°C, whereas Iron was freshly prepared for each experiment. M606 was kindly provide by research group of Dr. Michelle Haber from Children's Cancer Institute, Australia, Sidney. All trans-Retinoic acid *(Sigma-Aldrich)* is resuspended in DMSO and used at 10µM of final concentration.

RNA EXTRACTION

The procedure is described for two 100mm dishes of cultured cells at 70% of confluence. Same procedure is adopted for M606 experiments with cultured cells grown in five 100mm dishes at 20-30% of density. *Sigma-Aldrich TriReagent* protocol is adopted. Remove medium and rinse dishes with 1mL of PBS1X. Add 1mL of trypsin-EDTA 0,05% to plate to detach cells and neutralize trypsin action with 5mL of DMEM and transfer the suspension in a 15mL tube. Centrifuge 5m 1000rpm and remove supernatant. Add 1mL of TriRiagent to the pellet, gently mix and incubate for 5min at RT. Add 200 µL of chloroform and vortex. Incubate for 5min at RT. Centrifuge 5m 12000rpm at 4°C and transfer aqueous phase into a new tube containing 500 µL of isopropyl alcohol and vortex. Incubate for 5min at RT. Centrifuge 15min 12000rpm at 4°C and remove supernatant. Wash twice pellet with 1mL of EtOH 75% diluted in DEPC treated water. Centrifuge 10min 12000rpm at 4°C and remove supernatant and let the pellet dry. Resuspend it in 50 µL of water treated with DEPC and heat sample 10min at 55°C.

DNase I TREATMENT

After quantification of RNA by spectrophotometric analysis to verify quality (ratio 260/280 > 1.8, ration 260/230 > 1.7), DNase treatment is necessary to digest the contaminant genomic DNA. DNase treatment of 1,5 µgr of RNA is performed using the

DNA-free kit (*Ambion*, *Life Technologies*), with 0,75 U of rDNase I in 15 μL of final volume. Mix is incubated for 30min at 37°C and that DNase is inactivated using 0.1 volume of Inactivation Reagent. Incubate 2min RT, mixing occasionally. Centrifuge 2min 10000rpm RT and transfer RNA in a fresh tube.

REVERSE TRANSCRIPTASE REACTION

The RT-PCR was designed for the reproducible and sensitive detection and analysis of RNA molecules in a two-step process. Avian reverse transcriptase RT (*Bio-Rad*) with reduced RNase H activity, was engineered to have higher thermal stability and to produce higher yields of full-length cDNA. Reverse transcription is performed using iScript Reverse Transcription Supermix (*Bio-Rad*). 5x ready to use cocktail contains MMLV RT(RNAseH+), RNAse inhibitor, random hexamers and oligo-dTs, buffer, MgCl2, dNTPs, buffer and stabilizers. Reverse transcription is performed on 1 μgr of RNA in 20 μL of final volume. Thermal cycler setting provided by BioRad is as follow: priming for 5min at 25°C, Reverse Transcription for 30min at 42°C and inactivation at 85°C for 5 min. Add the appropriate water volume up to 100 μL and store at -20°C or use for qPCR immediately.

REAL TIME QUANTITATIVE PCR

SsoAdvanced Universal SYBR Green (*BioRad*) for ICycler CFX96 is used in the quantitative real time PCR. SYBR Green I dye is an asymmetrical cyanine dye for dsDNA stain resulting in DNA-dye complexes that absorb light at 497 nm, and emit fluorescence at 520 nm recorded, in our condition, after annealing and elongation steps.

It is a 2X ready to use mix with all components, except template and primers: 1μM of Fluorescein Reference Dye, enhancers, dNTPs, MgCl2, polymerase, SYBR Green I dye and stabilizers. For multiple reactions, master mix is prepared and after adding the appropriate master mix volume to each tube, the unique reaction components are added. Forward and reverse primers are used at final concentration of 200nM. 20ngr of template are used. The TaqDNA polymerase provided in the SuperMix has been chemically modified to block polymerase activity at RT, allowing room-temperature reaction setting up and long term storage at 4°C. Activity is restored after a 10-minutes incubation in PCR cycling. Protocol used in ICycler CFX96 for qPCR is as follows: polymerase activation and DNA denaturation for 30sec 95°C, 40 cycles of denaturation for 15sec at 95°C and elongation for 30sec at 60°C. Melting curve analysis is the final step characterized by an

increment of 0.5°C 2-5 sec/step of the temperature from 65°C to 95°C. Primer used in qPCR are listed below:

NAME	SEQUENCE FOREWARD	SEQUENCE REVERSE
MYCN_qRT-PCR	GATGCACCCCCACAGAAGAA	CTCCGAGTCAGAGTTTCGGG
GUSB_qRT-PCR	AGCCTGGAGCAAGACAGTGG	ATACAGATAGGCAGGGCGTTCG
CDKN1A(p21) _qRT-PCR	ATTAGCAGCGGAACAAGGA	CAACGTTAGTGCCAGGAA
MAX_qRT-PCR	GAAGGGGAGGGGGAAGTC	CCGTGTTGTGTGTGTGTGTG
MNT_qRT-PCR	CAGGAGGGCCCATCTGAAAG	CCTTCCTCTTCAGGGACTGGA
TRKA_qRT-PCR	TCAACAACGGCAACTACACG	TGAACTCGAAAGGGTTGTCC
TRKB_qRT-PCR	GGGACACCACGAACAGAAGT	CACCACAGCATAGACCGAGA
NEFM_qRT-PCR	TCAGCGGCTCCCCGTCCAGT	TCTCGGCGGAGCTGAGCATGG
GAP43_qRT-PCR	AGGGAACCTGGTCTCTGGGTTGT	TCGTCACCCAGGTGATGCTGTGA
TIMP2_qRT-PCR	GTTCAAAGGGCCTGAGAAGGA	CGGCCTTTCCTGCAATGAGA
MMP2_qRT-PCR	TGATGGCATCGCTCAGATCC	GGCCTCGTATACCGCATCAA
MMP9_qRT-PCR	CTTTGAGTCCGGTGGACGAT	TCGCCAGTACTTCCCATCCT
BDNF_qRT-PCR	TCAAGCCTCTTGAACCTGCC	TGCCCCCATGAAAGAAGCAA
E2F1_qRT-PCR	TCGTAGCATTGCAGACCCTG	TGAAAGTTCTCCGAAGAGTCCA
E2F2_qRT-PCR	CAACATCCAGTGGGTAGGCA	TGCTCCGTGTTCATCAGCTC
E2F3_qRT-PCR	GTTGTGAAAGCCCCTCCAGA	AATGGGCCCTTGGGTACTTG
E2F4_qRT-PCR	ACCCCACAGGTGTTTTGGAA	GGGGCAAACACTTCTGAGGA
E2F5_qRT-PCR	TCTTCAGCAGGATCTATTAGTGG	TGTAGTCATCTGCCGGGGTA
E2F6_qRT-PCR	ATAAGGAGCACCAACGGACC	TCCCGACACCTTCAGACCTT
E2F7_qRT-PCR	CAGGCAGCCCAGACTAGATT	TCTTCGGGGCCATCCTTGAT
E2F8_qRT-PCR	ATCCCAACCCTGTGTGAAT	CGAAATGCGTCGACGTTCA
TGFa_qRT-PCR	GCCCAGATTCCCACACTCAG	ACGTACCCAGAATGGCAGAC
TGFb_qRT-PCR	GGAAATTGAGGGCTTTCGCC	AGTGAACCCGTTGATGTCCA
TGFb2_qRT-PCR	AAGAAGCGTGCTTTGGATGC	AAAGTGGACGTAGGCAGCAA
TGFb3_qRT-PCR	CCCAGCTCTAAGCGGAATGA	TAGCGCTGTTTGGCAATGTG
RB1_qRT-PCR	GCCTCTCGTCAGGCTTGAGT	CCAAGCTCTCTCTCTGACATGA
RBL1_qRT-PCR	TCTAACAATGGCCACAGCCC	GCATCATTTGCGACACCATGT
RBL2_qRT-PCR	AGAGGAGAGGGGAGACCTCA	GGGAGAGAGTGGAGGAGCAT
TGFb1R_qRT-PCR	GGTTCCGTGAGGCAGAGATT	CACCAACCAGAGCTGAGTCC
TGFB2R_qRT-PCR	GCAGCATCACCTCCATCTGT	TTGGGGTCATGGCAAACTGT
SMAD2_qRT-PCR	TGGGGACTGAGTACACCAAA	ACGACCATCAAGAGACCTGG
SMAD3_qRT-PCR	TGCAAGATCCCACCAGGATG	GGGTCAACTGGTAGACAGCC
SMAD4_qRT-PCR	CCAATCATCCTGCTCCTGAGT	TAGGGCAGCTTGAAGGAACC
CDKN2A(p14) _qRT-PCR	GTTTTCGTGGTTCACATCCCG	CATCATGACCTGGTCTTCTAGG
CDKN1B(p27) _qRT-PCR	TAATTGGGGCTCCGGCTAAC	GAAGAATCGTCGGTTGCAGGT

TOTAL AND NUCLEAR PROTEIN ISOLATION

First step is common to both protocols: remove medium and rinse dishes with 1mL of PBS1X. Detach cells from the plate adding 1mL of trypsin-EDTA 0,05% to plate and neutralize trypsin action with 5mL of DMEM and transfer the suspension in a 15mL tube. Centrifuge 5m 1000rpm and remove supernatant.

The procedure is for the total protein extraction is described for 1100mm dishes of cultured cells at 70% of confluence. Same procedure is adopted for M606 experiments with cultured cells grown in five 100mm dishes at 20-30% of density. Resuspend pellet into about 100 µL of ice-cold RIPA buffer. Vortex and incubate in ice for 15min. Sonicate for 10min at maximum intensity. Centrifuge 20min 13000rpm at 4°C and transfer the supernatant to a new tube. Store at -80°C.

The procedure for the nucleic protein extraction is described for two 100mm dishes of cultured cells at 70% of confluence. Same procedure is adopted for M606 experiments with cultured cells grown in five 100mm dishes at 20-30% of density. Resuspend pellet into 500 µL of hypotonic solution. Centrifuge 5min 1500rpm at 4°C and again resuspend pellet in 500 µL of hypotonic solution let tube incubate for 15min in ice. Add 500 µL of hypotonic solution + NP40 0,4% (final concentration of 0,2%) and let tube incubate for 15min in ice (10min for LAN1). Centrifuge 10min 6000rpm and resuspend nuclei in high salt solution. Rotate the sample 1hour in cold room vortexing every 10min. Centrifuge 15min 13000rpm at 4°C. transfer supernatant in a fresh tube and quantify it.

Note: M606 experiment were performed using variation of high salt solution listed below.

RIPA BUFFER	HYPOTONIC SOLUTION	HIGH SALT SOLUTION	HIGH SALT SOLUTION M606
TrisHCl pH 7.5 50mM	Hepes 10mM	Hepes 10mM	Hepes 10mM
NaCl 150mM	NaCl 50mM	NaCl 420mM	NaCl 420mM
Na Doc 0.5%	EDTA 1mM	EDTA 1mM	$MgCl_2$ 1mM
Np40 1%	DTT 1mM	glycerol 10%	glycerol 10%
SDS 0.1%	$Na_4P_2O_7$ 1mM	$Na_4P_2O_7$ 1mM	$Na_4P_2O_7$ 1mM
PMSF 1mM	Na_3VO_4 1mM	Na_3VO_4 1mM	Na_3VO_4 1mM
Complete 1X	Na_2PO_3F 1mM	Na_2PO_3F 1mM	Na_2PO_3F 1mM
	PMSF 1mM	PMSF 1mM	PMSF 1mM
	Complete 1X	Complete 1X	Complete 1X

Quantification is performed using BCA methods (*ThermoScientific*) with standard protocol supplied by company.

WESTERN BLOT ANALYSIS AND ANTIBODIES

Protein mixes obtained by nuclear or total extraction were separated by SDS-PAGE in 10% or 12% poly-acrylamide gels.

	10%	12%	15%
40% poly acryl-amide	2.5 ml	3 ml	3.75 ml
TrisHCl 1.5M pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 µl	100 µl	100 µl
10% APS	100 µl	100 µl	100 µl
TEMED	5 µl	5 µl	5 µl
mqH_2O	To 10 ml	To 10 ml	To 10 ml

50-100 µgr of nuclear extracts or 30-50 µgr of total extracts were loaded.

Running buffer: tris 25mM, glycine 190mM, SDS 0,1% pH 8.3

Transfer buffer: tris 25mM, glycine 190mM, Methanol 20% pH 8.3

Blocking solution: NaCl 150mM, TrisHCl 20mM pH 8, Dried Milk 4%

All antibodies were purchased from Santa-Cruz: MNT (sc-769); Actin (sc-1616); MAX (sc-197); MYCN (sc-53993); RB (sc-50); pRBSer608 (sc-56174). Antibody FLAG M2 (F3165) was purchased from Sigma.

ShRNA PRODUCTION AND SILENCING ASSAYS

Briefly, packaging cells chosen for viral production were HEK 293T. cells were transfected using Effectene kit (QUIAGEN) with packaging vectors from Addgene: pMD2.G (#12259), psPAX2 (#12260) and pLKO.1 TRC ShRNA backbone plasmids. pLKO.1 Lentiviral Scramble shRNA (#RHS6848) and pLKO.1 Lentiviral MAX (#RHS3986) and MNT (#RHS2039) were purchased at Open Biosystems-GE Dharmacon.

Optimization experiment was carried out using puromycin dose response or kill curve and testing various Multiplicity of infection (MOI). For shRNA experiments, SK-N-BE(2)-C cells were transduced for 12 hours with MOI 10 and polybrene concentration at 10µg/ml, following selection with 50 mg/ml puromycin.

DAY 1: plate 6x10^6 HEK 293T

DAY 2: Prepare the following mix x each sample: 100 µL Buffer EC, 0.5 µgr psPAX2, 1 µgr pMD2.G. Vortex and add the specific pLKO.1 plus 4 µL enhancer solution. Vortex and incubate RT 15min. Add Effectene 16 µL and incubate for 30 min RT. During the incubation, change medium to the 293T cells plated the day before with 1 mL of fresh medium and then add to the cells. Virus production will start after about 12 hours.

DAY 3: After 12 hours from transfection, change medium to 293T to collect virus in a fresh medium. Prepare the cells will be infected (BE(2)-C). Plate 3x10^6 cells in a 6 well dished.

DAY 4: filter the supernatant using a 0.45 µm filter, use it or store at - 80°C until use.
INFECTION: Some cells as primary cells are sensitive to Polybrene, thus determine the suitable concentration of Polybrene is recommended. Aspirate the medium and infect cells with 250 to 500 µl viral supernatant. Add fresh medium to a final volume of 500 µL adding 5 µL of 1 µg/µl Polybrene.
DAY 5: change medium with fresh DMEM and puromycin 1µgr/mL. It is recommended to perform a puromycin kill dose before experiments.

TRANSIENT TRANSFECTIONS

BE Tet-Off cells were obtained after transfection of SK-N-BE(2)-C with Tet-Off Advanced transactivator plasmid that is a modified transactivator protein optimized for expression in mammalian cells. The inducible promoter, PTight, provides low basal expression and is tightly controlled by induction with tetracycline. After transfection of BE(2)-C using Lipofectamine® 2000 (*Life Technologies*), single clone were selected adding G418 to 0.5 mg/ml. single clones were tested for their ability to induce tetracycline response using Dual Luciferase assay and transfecting selected clones with a construct carrying Luciferase construct under a tetracycline responsive promoter (pTRE-Tight-Luc). Selected clone was transfected with pTRE3G_MAXL3xFLAG, pTRE3G_MAXS3xFLAG and pTRE3G_MNT3xFLAG. Experiments were conduct also on empty vector alone. To insert 3xFlag in frame with CDS, selected genes were firstly cloned into pCMV14 and then into pTRE3G. MNT(**NM_020310.2**), MAXL(**NM_002382.4**) and MAXS(**NM_145112.2**) were cloned.
Listed below, primers used:

NAME	SEQUENCE FOREWARD	SEQUENCE REVERSE
MNT_pTRE3G	TTTAGATCTATGAGCATAGAGACGCTACTGG	TTTCATATGCTTGTCATCGTCATCCTTGTAG
MAX_pTRE3G	TTTAGATCTATGCAGCGATAACGATGACATC	TTTGCTAGCGGATCACTACTTGTCAT
MNT_pCMV14	AAAAGCTTATGAGCATAGAGACGCTACTGGAGG	AAGGATCCAGCCAGCTTGAGTGTGCTGAC
MAXL_pCMV14	TTTAAGCTTATGAGCGATAACGATGACATCGAG	TTTGGATCCGCTGGCCTCCATCCG
MAXS_pCMV14	TTTAAGCTTATGAGCGATAACGATGACATCGAG	TTTGGATCCGCTGGCCTCCATCCG

CELLULAR ASSAY

Wound closure assay was performed to evaluate cells migration. Cells were plated and when 70% of confluence were reached, a tip was used to remove a part of cell layer from

different separate areas of the growth substrate. To allow stabilization of cells, let them stay for 15min and then replace medium to remove debris and start wound closure assay photographing areas at regular time points. Final wound size was the result of average of 3 independent experiments. Analysis were performed using ImageJ.

To evaluate cell proliferation, 300.000 cells were plated in 6-well plate and every day cells were counted using trypan blue exclusion method and Nucleocassette from *Chemometec*. The growth curve was results of average of at least three independent experiments.

To evaluate neurite outgrowth, 3 × 10^4 cells were plated onto a 100mm dish in DMEM with 0,1% FBS for 48h. Medium was then replaced with complete DMEM with 10% FBS and differentiation was induced adding every day retinoic acid ATRA 10 μM for 6 days in half fresh medium and half conditioned medium. Photographs of different areas of the cultures were taken every day with a phase-contrast microscope. Neurite outgrowth, number of cells, and number of neurites was measured using the image analysis software ImageJ. Were scored as positive cells with neuritic extensions at least twice the cell body.

LUCIFERASE ASSAY

The DLR Dual-Luciferase® Reporter Assay System from Promega, gives an efficient means of performing dual-reporter assays. The term "dual" refers to the sequential detection of the activities of two luciferases: firefly from *Photinus pyralis* and Renilla from *Renilla reniformis*. Renilla luciferase-expressing vector is used as internal calibrator of the system due to its constitutively basal expression. Firefly vector carries promoter sequence of interest. Firefly activity is misured adding LAR II reagent and after quantification, the addition of Stop & Glo® reagent to the same tube allow quenching of firefly signal and detection of Renilla activity.

Briefly, 1x10^6 neuroblastoma cells were plated in 24 well plate. The day after, cells were transfected using LTX (*Life Technologies*), and protocol provided by companies.

NOTE: for BE(2)-C 1.2 μL of LTX was used; for LAN1 1.8 μL of LTX was used in the same final volume.

For M606 experiments on MYCN promoter, different time points were tested and 12 hours after transfection were chosen. At the selected time point, add 100 μL of passive lysis buffer and mix for 20min at RT. Samples are ready to be measured or be stored at -

80°C. Dispense 30 µL of LAR II into the luminometer tubes then add 20 µL of sample and read the luminescence. Then add 30 µL of Stop & Glo Reagent and mix briefly by pipetting and read the renilla signal. Data are expressed in RLU or Relative Light Units, an arbitrary unit to define firefly activity on renilla activity.

NAME	SEQUENCE FOREWARD	SEQUENCE REVERSE
MYCNp_-1385;1504	AAAAAGCTTTCACAGCCTGCGCTTTGAAG	AAACTCGAGGCTGTTCCTGGCTGCAGAAT
MYCNp_-1385;-2	AAAAAGCTTTCACAGCCTGCGCTTTGAAG	TTTGCTAGCGGATCACTACTTGTCAT
MYCNp_-2;1504	AAACTCGAGAAAGGACAGGATGCTAGGCG	AAAAAGCTTGCTGTTCCTGGCTGCAGAAT
Δ1	AGATCTCGAGCCCGGGCTAG	CGTGGTCATCATCATAATAATAGCTGAC
Δ2	CGCCACCATCAAATCCTGGCTTTAC	CAGCAGCTTTCCTTTTTCCCTTCAAC
Δ3	GAAATGGAGTTCGTAGTGCCTCCTA	TTAACTTGGGAGCCCTGGGG
Δ4	CAGGCGAATCTGATTTACCGAGATGG	GCAGCTCCGCTTTCTGCTCA
Δ5	GACTGTCCACGCGTCCTCAC	CCAGAGGTCTTGTTCCTAAGGGGG
Δ6	AAATAAATAAGTGCGAGCTACGAGGGT	GGGGGCTGGGTTAGAAGCAT
Δ7	CTTGCAGGGAGGTTGCTCCT	TTCTCAGAGTGCAGCCGGTG
Δ8	GAGCCTGGCAATTGCTTGTCATT	GGGGTAAAGCCGCTTTCCTCT
Δ9	GCCACTTGCTTTTCTTTGCAGAGA	AAGCTTGGCATTCCGGTACTGT
MYCNp_-1252;-1045	TTTGGTACCGGAAGGGAAGGGGCCAAT	TTTGAGCTCTCACTACTTCGTTTCTTTGTGCC
MYCNp_-335;-133	TTTGGTACCGCAGGGTGGGTGCTGCATT	TTTGAGCTCGCCCTCCTGATTTCCATAAAAATCAGGGG
MYCNp_-187;-2	TTTGGTACCTTTTGGCGCGAAAGCCTTGG	TTTGCTAGCGGATCACTACTTGTCAT
MYCNp_E2F1/E2F2	AAGCCACTTGCTTTTCTTTGCAGAGAGAAG	GCCTTGGCGCCTCCCCTGATTTTT
MYCNp_E2F3/TIE	TTTCGCGCCAAAAGCCACTTGCTTT	CCCCTGATTTTTATGGAAATCAGGAGG
MYCNp_E2Fs/TIE	AAGCCACTTGCTTTTCTTTGCAGAGAGAAG	CCCCTGATTTTTATGGAAATCAGGAGG

CH-IP CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation were performed on M606 treated neuroblastoma cells and thus the specific protocol is intended for treated human neuroblastoma cells growing adhesively. One of the critical steps in ChIP regards the chromatin fragmentation conditions, which need to be experimentally determine for each cell types used.

Count and centrifuge 1x10^7 cells for each IP points and resuspend in 10mL of DMEM (if M606 was added to cells prior experiment, add DMEM with M606 addition again). Add 270 µL of formaldehyde from a 37% and incubate RT rotating at 13rpm for 10min. Add 0,5 ml of glycine from a 2,5 M stock solution mix rapidly and incubate RT rotating at 13rpm for 10min. centrifuge 5min 1500rpm at 4°C then keep sample on ice. Remove supernatant and add 10mL of PBS1X washing cells for 3 times centrifuging for 5min 1500rpm at 4°C. Add 0,5mL of ice-cold Cell Lysis Buffer to the pellet and gently mix tube. Centrifuge 10min 3000rpm at 4°C and remove supernatant. Add to pellet 200 µL ice-

cold RIPA buffer with SDS 0,7% and mix. Incubate 20min on ice. Sonicate 2 times for 15sec at low power and 5 times for 15sec at high power mixing samples after each sonicate cycle. Sonicate preclearing: centrifuge samples 15min at 14000 rpm at 4°C and transfer supernatant in a new tube containing 30 µL of beads coated with Immobilized Protein A. Incubate 30min 13rpm in rotation at 4°C. Spin down 5min 3000 rpm at 4°C. Transfer the supernatant in a new fresh tube end, after putting aside 30 µL for INPUT DNA preparation, add 5 µgr of specific antibody. Incubate O/N 13rpm in rotation at 4°C. For input DNA preparation, dilute to 100 µL with RIPA wash and store at -20°C. The day after, add to sample 30 µL of coated beads and incubate 30min 13rpm in rotation RT then centrifuge 3min 3000rpm 15°C and proceed with the following washing steps: twice with 1mL RIPA wash; 3 times with 1mL Washing buffer; twice with 1mL TE buffer always rotating 3min RT and spin down 3min 3000rpm 15°C. Recover the supernatant and add 70 µL of TE buffer to the beads and also to the input and add RNAse A 10 µgr and incubate 1hour 1000rpm at 37°C. Add 20 µl Proteinase 74 K Buffer 5X and 6 µl Proteinase K (19 mg/mL) and incubate 6hours 950 rpm at 65°C. Centrifuge 10min at 6000 rpm at 4°C and transfer the supernatant to a Phase Lock gel tube. Add same volume of phenol/chlorophorm/isoamylalcohol mix and centrifuge 3min 13000rpm RT. Transfer aqueous phase into a new tube and add 1/10 of Na acetate 3M pH 5.2, 1 µL glycogen from a 20 mg/mL stock solution and 2,5v of EtOH then vortex and let DNA precipitate O/N at -2’°C. The day after centrifuge 30min 13000rpm at 4°C then wash pellet twice with 1mM of EtOH 70%. Resuspend pellet of IP-DNA and input sample in 50uL of water and quantify input to create proper dilution at 1 µgr/µL. Use 2-4 µl of IP-DNA for Real Time PCR analysis.

Buffers used are listed below:

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

METHYLATED DNA IMMUNOPRECIPITATION

Methylated DNA immunoprecipitation were performed on M606 treated neuroblastoma cells and thus the specific protocol is intended for treated human neuroblastoma cells growing adhesively. One of the critical steps in ChIP regards the chromatin fragmentation conditions, which need to be experimentally determine for each cell types used.

Genomic extraction was performed using 5prime kit. 20 µgr of genomic DNA were used for each IP point in a final volume of 300 µL of FB buffer 1X. Perform 3 sonication at high power for 10min and then denature DNA at 98°C for 15min and put on ice. Add 50 µgL of pre-cleared beads (pre-incubated with salmon sperm for 4 hours, washed 3 times with 1mL of FB and after centrifuge 2min 3000rpm RT resuspended in half of initial volume) and incubate on wheel 15min 4°C. Spin down 1min 14000rpm for 4°C and transfer supernatant into a new tube. Split sample in input 20 µL and MeC: 270 µL and 330 µL of FB buffer 1X. Add 10 µgr of antibody anti-MeC and incubate O/N on wheel at 4°C. The day after, add 50 µL of coated beads to sample and incubate rotating 30min RT. Immunoprecipitate sample spinning 4000rpm 5min and discard supernatant. Wash pellet 4 times with FB1X and twice with TE buffer spinning each time 2min 4000 RT after 2min of incubation on wheel. Resuspend pellet in 70 µL of TE and proceed with RNAse and Proteinase K treatment as for ChIP. Precipitation with phenol/chlorophorm/isoamylalcohol was performed as for ChIP. Primers used for ChIP and MeDIP:

TSS FW MYCNp	GCCTTCTCTCTGCAAAGAAAAGC
TSS RV MYCNp	AAGGAGAGGAAAGCGGCTTTAC
-400 FW MYCNp	GCGCCCCTCTTCTTTCAATTTG
-400 RV MYCNp	ACGAAAGAAGGGTAGTCCGAAG
-1400 FW MYCNp	ATCCAATGGTGAGGTGAAGAGG
-1400 RV MYCNp	TCCTTAGGCTTGTTTGGAGAGAG
500 FW MYCNp	GGACTGTTTCTGCTTCCGAAAC
500 RV MYCNp	GTAAGGGCTGCAAAAGGATTAGG
1500 FW MYCNp	TGTCTGTCGGTTGCAGTGTTG
1500 RV MYCNp	TCCGCCCCGTTCGTTTTAATAC
TSS FW GAPDH	GGCTACTAGCGGTTTTACGGG
TSS RV GAPDH	GCTGCGGGCTCAATTTATAGAAACC
-400 FW GAPDH	CTGAGCAGTCCGGTGTCACTAC
-400 REV GAPDH	CCTCCCCTTTCTTTCTTTCAA
1500 FW GAPDH	TCCTGATTTCTGGAAAAGAGCTA
1500 REV GAPDH	GGAAGAGGGGAAGCTGTATTTTA
LDH TSS FW	GCGTTTCATTACCACCCTCT
LDH TSS REV	GTCCAGATCACCGAATGCT

STATISTICAL ANALYSIS

All experiments are performed at least 3 times. Data were analyzed with Graphpad Prism 6 software and expressed as mean ± standard deviation (SD). Analysis of Variance (ANOVA) among groups was used to analyze difference among groups. two-sided unpaired t test was used for two groups. Survival analyses and two genes correlation were performed according to the method of Kaplan and Meier and two-sided log-rank tests (193). A probability value of 0.05 or less was considered statistically significant.

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