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MYC-MEDIATED CONTROL OF GENE TRANSCRIPTION IN CANCER CELLS

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Introduction

MYC oncoproteins

Myc Family

In 1979 a viral oncogene directly involved in transformation induced by Rous Sarcoma Virus (RSV) was discovered, some years later the human homologue was discovered: c-Myc[1].
This new human oncogene was hardly investigated and after few years other two genes homologous to c-Myc was discovered: N-Myc[2] and L-Myc[3]. So three genes characterized by a good degree of homology compose the Myc family.
It has been known for some time that c-Myc, N-Myc and L-Myc are expressed in distinct patterns during embryogenesis [4];[5]. c-Myc tends to be highly expressed in most rapidly proliferating cells and is generally low to absent during quiescence. N-Myc, although present at low levels in many neonatal tissues, is expressed at highest levels in pre-B cells, kidney, forebrain, hindbrain and intestine. In some tissues such as the retina, telencephalon, and intestine, MYCN expression has been shown to persist during differentiation whereas c-Myc is downregulated.[6, 7]
During gastrulation, c-Myc is expressed most abundantly in extraembryonic tissues, whereas MYCN is most abundant in the expanding primitive streak and other regions of the embryonic mesoderm. N-Myc is also down-regulated during the differentiation of this latter tissue to epithelium[8]. L-Myc is expressed in the developing kidney, as well as in the newborn lung and in both proliferative and differentiative compartments of the brain and neural tube [9].
The Myc oncoproteins are transcription factors belonging to a subset of the larger class of proteins containing basic-region/helix–loop–helix/leucin-zipper (BR/HLH/LZ) motifs. BR/HLH/LZ domain is known to mediate protein-protein interactions and DNA binding [10]. They are structurally very similar as shown in Fig.1.

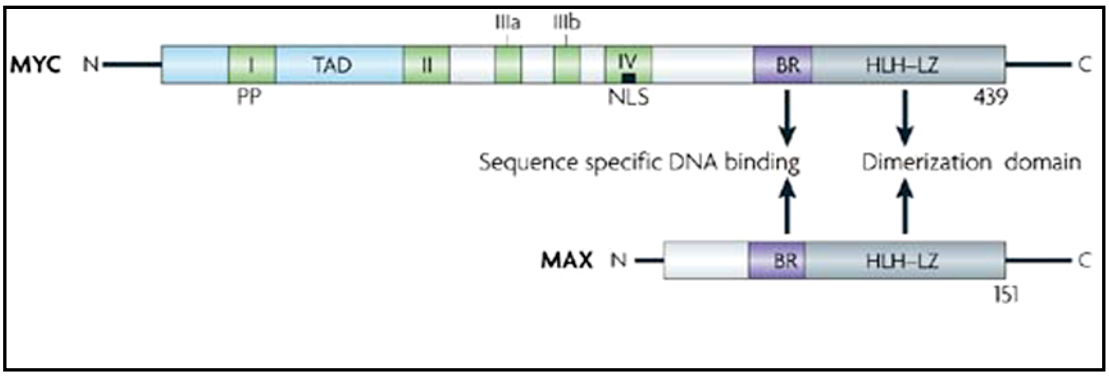

Figure 1. Structural domains of Myc oncoproteins. From N-term to C-term:
Transactivation domain, TAD (amino acids (aa) 1–143): the TAD can confer activation of gene transcription to a heterologous DNA-binding domain.
MYC homology box I, MB I (aa 44–63): MBs are regions highly conserved between MYC, N-MYC and MYCL1; within MB I, stability and activity of MYC are highly regulated through phosphorylation of Thr58 and Ser62.
MB II (aa 128–143): this domain is important for transcriptional repression and activation, region of interaction with TRRAP and other cofactors involved in transformation.
MB IIIa (aa 188–199): this domain is conserved in MYC and N-MYC but not in MYCL1 and shows intermediate transforming potential compared with the activity of the wild type MBII in vivo.
MB IIIb (aa 259–270): this domain is conserved, but no specific function has yet been assigned to it.
MB IV (aa 304–324): this domain is required for full MYC transforming activity and apoptosis.
Primary nuclear localization signal, NLS (aa 320–328): subcellular localization to the nucleus is encoded primarily by this region.
Basic region, BR (aa 355–369): this region is essential for full transformation of primary and immortal cells, and is responsible for specific binding of canonical and non-canonical MYC E-boxes to DNA, with MAX.
Helix–loop–helix–leucine zipper, HLH–LZ (aa 370–439): this domain is essential for full transformation of primary and immortal cells, and is responsible for interaction with MAX [11].

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

The Myc/Mad/Max network comprises a group of nuclear transcription factors whose functions profoundly affect cell behavior [10].

No monomeric Myc proteins have been found in vivo. Instead, Myc is bound to a partner protein, Max (MYC-associated factor-X), itself a small BR/HLH/LZ protein [12]. Max, physiologically, is present in stoichiometric excess to Myc, and can homodimerize and bind DNA, but such Max homodimers appear to be transcriptionally inert [13, 14]. Max can also form heterodimers with several related proteins, known as Mad1, Mxi1 (also known as Mad2), Mad3, Mad4 and Mnt (also known as Rox) (Figure 2). These five proteins, that compose the Mad protein family, behave much like Myc in that they have only weak homodimerization and DNA-binding capacities but readily heterodimerize with Max and bind the DNA [10].

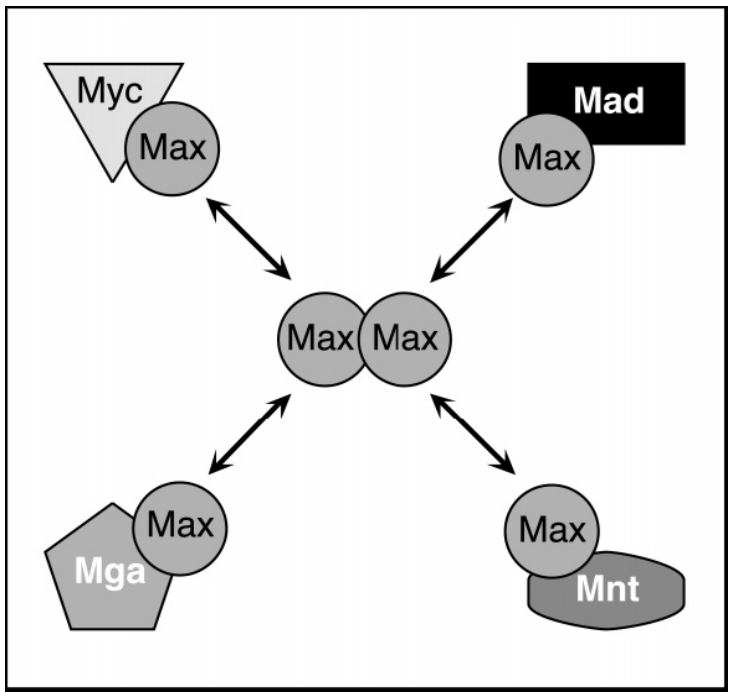

Figure 2. Max-interacting proteins. Max forms heterodimers with members of the Myc and Mad protein families as well as with the Mnt (or Rox) and Mga proteins. Each of these proteins interacts with Max through its BR/HLH/LZ domain [15].

Association of Myc with Max results in the formation of heterocomplexes that recognize the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG) with transcriptional activity (Figure 3) [16-18]. However, in contrast to Myc, which activates transcription at promoters proximal to E-box sites, the Mad-Max heterodimers act as transcriptional repressors at the same binding sites [13] [19] [20].

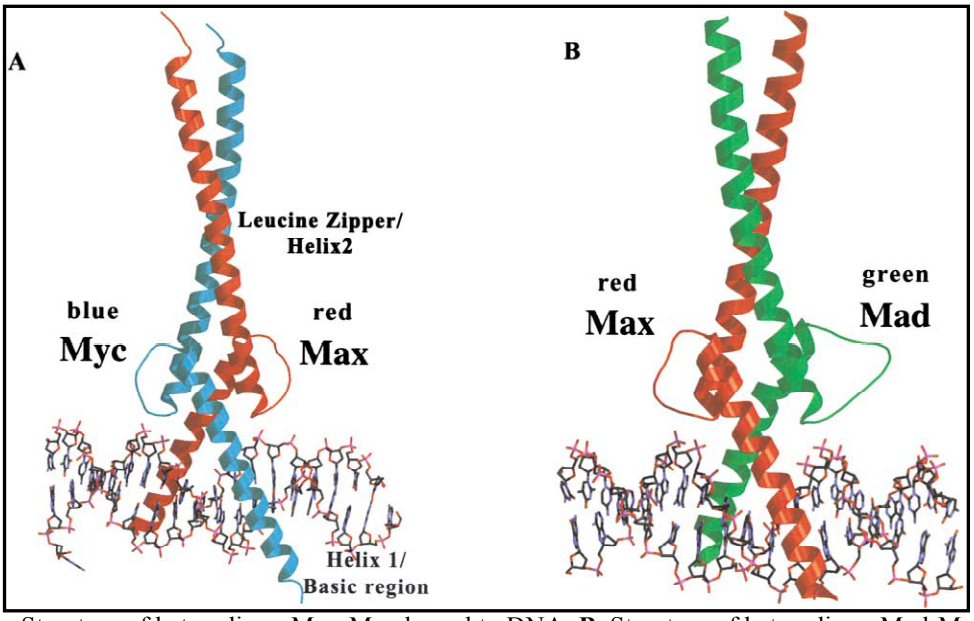

Figure 3. A, Structure of heterodimer Myc-Max bound to DNA; **B,** Structure of heterodimer Mad-Max bound to DNA.

The ability to modulate transcription is derived from specific domains within the Max interacting factors which, in turn, appear to mediate associations with specific coactivators or corepressors, resulting in the formation of higher-order complexes. Furthermore, the different complexes may have antagonistic properties whose functions play out at the level of chromatin structure (see below). The transcription activation function of Myc involves at least in part the recruitment of a histone acetyltransferase (HAT) mediated by TRRAP (transactivation/transformation- associated protein), whereas Mad–Max complexes act as transcriptional repressor by recruiting histone deacetylases (HDACs) through the adaptor protein SIN3 (Figure 4) [21] [22].

In general, Max interacting proteins have short half-lives (on the order of 20–30 min) and their biosynthesis is highly regulated [23]. Max, on the other hand, is stable and constitutively expressed, suggesting that the regulation of the network is largely dependent on the abundance of the Max associated transcription factors that must be tightly regulated [12, 24]. In vivo, Myc–Max complexes are often predominant in proliferating cells, whereas Mad–Max or Mnt–Max complexes are predominant in resting or differentiated cells (see below) [25].

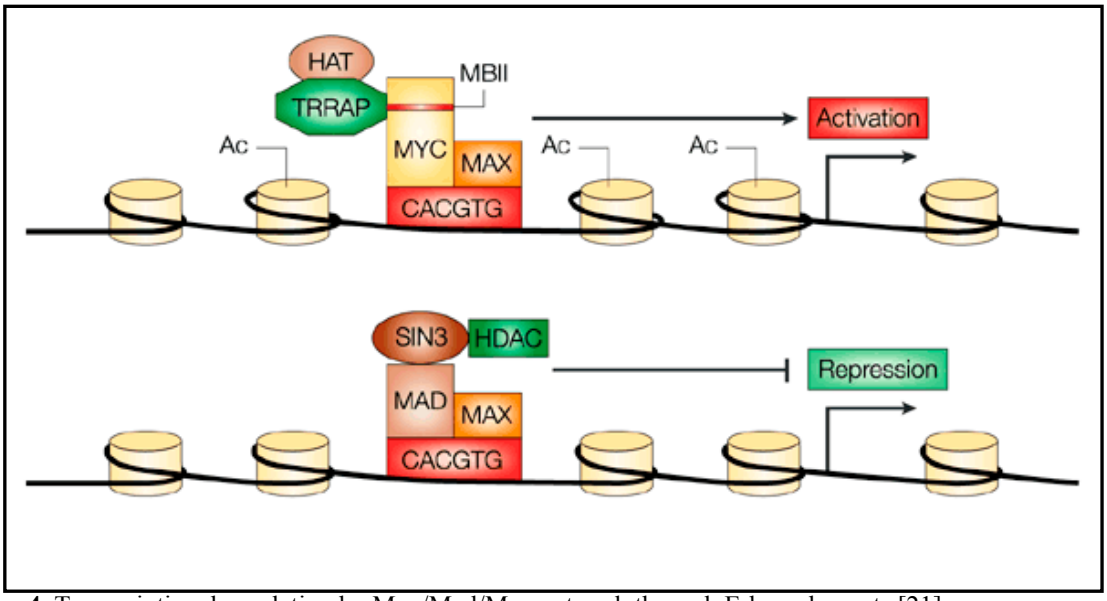

Figure 4. Transcriptional regulation by Myc/Mad/Max network through E-box elements [21].

Our understanding of the Myc/Max/Mad network grew out of research on the MYC oncogene family. The first compelling idea about MYC was that its function was to drive cell growth and proliferation in response to diverse signals. In fact, MYC family genes are broadly expressed during embryogenesis, and targeted deletions of c-MYC or N-MYC genes in mice lead to lethality in mid-gestation embryos [26] [27]. Moreover, there is a strong correlation between MYC expression and proliferation. [9, 28-33]. In cells with activated MYC, G1 phase is often shortened as cells enter the cell cycle, and MYC is essential for G0/G1 to S phase progression [34] [35] [36].

It is now clear that a wide range of growth factors, cytokines, and mitogens induced MYC expression in many cell types by [37-39].The increase in MYC levels occurs through both transcriptional and post-transcriptional mechanisms and appears to occur as an immediate early response (about 2 hours) to most mitogenic factors [40].

On the contrary, anti-proliferative signals trigger rapid downregulation in MYC expression [11]. MYC downregulation is required for cells to exit the cell cycle and undergo differentiation. This important point of regulation is further enforced by the induction and function of the Mad family members in response to differentiation cues [41].

It is important to note, however, that in situations where commitment to a specific lineage is closely linked to an increase in proliferation, Myc can promote differentiation [42].

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

The ability of overexpressed Myc to facilitate proliferation and inhibit terminal differentiation fits well with the fact that tumors of diverse origins contain genetic rearrangements involving MYC family genes, such as genomic amplification in neuroblastoma. Indeed, many of the genomic alterations in MYC result in increased MYC mRNA levels through increased transcription initiation, decreased transcription attenuation, and augmented stability of the MYC messenger RNA [40]. Moreover Myc degradation is carried out through the ubiquitin-mediated proteasome pathway that involves phosphorylation of Thr58 and Ser62 in MB I: many tumor-related mutations in Myc result also in significant stabilization of the protein [43, 44].

One of the most striking findings of the past years has been the discovery that the enhanced expression of Myc proteins contributes to almost every aspect of tumor cell biology [45]. Whereas the ability of Myc to drive unrestricted cell proliferation and to inhibit cell differentiation had long been recognized, more recent work shows that deregulated expression of Myc can drive cell growth and vasculogenesis, reduce cell adhesion, promote metastasis and genomic instability. Conversely, the loss of Myc proteins not only inhibits cell proliferation and cell growth, but can also accelerate differentiation, increase cell adhesion and lead to an excessive response to DNA damage [46].

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

Biological functions of MYC

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

- the high level of relatedness between N-Myc and c-Myc protein structure;
- while c-MYC and MYCN homozygous knockout mice are embryonic lethal, transgenic mice in which MYCN replaced c-MYC showed a gross normal development, indicating that both proteins have largely overlapping functions [26] [27] [47];
- c-Myc and MYCN share >40% of their target genes [48]

Nonetheless, since the late 80s, differences between the two members of the MYC family appeared to be important and seem more pronounced in recent years of research: actually MYCN and c-Myc appear to bind different subsets of genes with different affinities in neuroblastoma tumors and in hematopoietic stem cells [49] [50].

Furthermore, it is well known the existence of direct negative cross-regulation between MYCN and c-Myc [51] [52] [53].

Indeed, by analogy with c-Myc, N-Myc has related functions in regulating cell growth and proliferation, but in a more specialized context. MYCN is subjected to a strict, rather similar temporal and spatial expression pattern, as shown by comparisons of fetal and adult human brain cells [54] and by analysis of tissues from different stages of the fetal and the developing mouse embryo [55] [4]. These results suggest that MYCN may play an important role during mammalian development. The overall expression of the c-MYC gene, in contrast, is virtually constant during embryonic development and occurs in all proliferating cells of an organism.

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

During murine development MYCN mRNA can be detected as early as day 7.5 in the primitive streak [8]. A peak of expression is reached between days 9.5 and 11.5, followed by a sharp decrease after day 12.5 [4]. During this time MYCN mRNA is present in many tissues including heart, limb buds, and neural tube [55]. MYCN mRNA has also been detected during organogenesis in tissues such as hair follicles, lung, liver, and stomach [6]. Within a tissue MYCN expression is not homogeneous. For example, in the heart, expression is restricted to the myocardium; in the liver it occurs mainly in the peripheral layer; in the neural crest it is initially expressed homogeneously, but after colonization of ganglion areas becomes restricted to those cells undergoing neuronal differentiation; in the human fetal kidney it is observed exclusively in the epithelially differentiating mesenchyme; in the somites it is stronger in the posterior than in the anterior half; and in the brain it is observed in the neural precursor cells but becomes more restricted after lineage commitment [56]. Thus MYCN expression is dynamic not only in space but also in time. At birth MYCN is still expressed in the brain, kidney, intestine, lung, and heart but then becomes down-regulated within several days or weeks depending on the tissue. In adults, MYCN expression has mainly been detected at early stages of B-cell development. However, weak expression may be maintained in the adult brain, testis and heart [4]. The lack of MYCN in any tissue where it is normally expressed results in developmental defects [56].

Taken together, these results reveal a complex expression pattern of MYCN which is reflected in a correspondingly complex promoter with multiple tissue-specific, stage-specific, and signal-dependent regulatory elements. As the different expression patterns would lead one to expect, the regulatory regions of c-MYC and MYCN are divergent [56]. Among the candidate pathways involved in differential regulation of Myc proteins are the Sonic hedgehog pathway (Shh) for MYCN activation [57] and the Wnt/beta-catenin pathway for c-MYC activation [58]. Sonic hedgehog (Shh) signaling upregulates expression of the proto-oncogene MYCN in cultured cerebellar granule neuron precursors. The temporal-spatial expression pattern of MYCN, but not other MYC family members, precisely coincides with regions of hedgehog proliferative activity in the developing cerebellum [59].

These findings are confirmed by studies of human tumor cells. MYCN normally inhibits neuronal differentiation by driving germinal cells to maintain their early embryonic short-duration cell cycles and, therefore, favours self-renewal, genetic instability and cancer [56].

Consistent with a critical role of MYCN in the development, overexpression of MYCN within a specific developmental window may cause several pediatric tumors of mostly, but not exclusively, neuroectodermal origin, including neuroblastoma, rhabdomyosarcoma, medulloblastoma, retinoblastoma, astrocytoma, glioblastoma, Wilms' tumors and small cell lung carcinoma [60] [61].

These data are supported by in vivo experiments: transgenic mice overexpressing MYCN in neural crest-derived tissues show frequent development of neuroblastomas [62].

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

It is clear that although neuroblastomas without MYCN amplification show heterogeneity in the level of MYCN expression, they never produce as much MYCN as tumors with amplification do. In fact, several studies failed to find a correlation between the expression of MYCN in non-amplified tumors and an unfavorable outcome, suggesting that a threshold level of MYCN expression has to be exceeded for an unfavorable outcome to occur [63].

MYC as an activator

MYC encodes a phosphoprotein of apparent molecular weight of 65/67 kDa that is localized in the nucleus and binds to DNA [64] [65].

When introduced into cells, MYC can activate transcription of synthetic reporter genes containing promoter proximal E-boxes in both yeast and mammalian cells [13, 14]. In addition, MYCN stimulates natural E-box-containing promoters or sequences derived from putative Myc target genes [18, 66-68].

Myc-Max heterodimers have relatively weak transactivation activity both endogenously and in transient assays [14]. A plethora of microarray studies published recently have shown that Myc proteins activate the majority of target genes by two-fold (generally ranging from 3- to 10-fold transactivation) [69]. Although Myc is now firmly described as a transcription factor, it is certainly feeble compared to other transcription factors.

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

The prevailing model of MYC-mediated transcription postulates, as mentioned above, that MYCN increases local histone acetylation at promoters [46]. MYCN binds to histone acetyltransferase complexes including TRRAP (transformation/transcription-domain-associated protein) and either general control of amino-acid-synthesis protein-5 (GCN5) or TIP60, which preferentially acetylate histones H3 or H4, respectively [70] [22]. Myc also binds to the p300/CBP (CREB-binding protein) acetyltransferases [71].

The resulted histone acetylation then opens the chromatin and provides docking sites for acetyl-histone-binding proteins, including GCN5 and the SWI/SNF chromatin-remodelling complex, both of which correlate with increased transcription (Figure 5a) [72] [73]. Transcription-factor-mediated recruitment of histone acetyltransferases is now recognized to be a major mechanism of transactivation, and many other transcription factors, including TCF (T-cell factor), E2F, the tumor suppressor p53 and Gal4, have been subsequently found to use this mechanism [74]. The region of chromatin opened through Myc would permit subsequent binding and activation by constitutive transcription factors.

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

Among others, target genes include the cyclin-dependent kinase CDK4 [75], the Cdc25A phosphatase that activates CDKs [76], cyclin D2 [77] [78] and the E2F family [79]. In

addition to target genes involved in cell cycle progression, Myc has been found to stimulate expression of multiple genes that control cell size and growth, including those encoding ribosomal proteins, translation factors, and metabolic enzymes [80]. These findings are consistent with the evidence that recruitment of TRRAP and associated acetylation activity is also present in vivo at both RNA polymerase III (RNA pol III)-dependent genes and RNA-pol-I-dependent and ribosomal RNA genes [81] [82] [83] [84].

Recently Myc has been shown to promote oxidative phosphorylation as well as glycolysis through coordinate transcriptional control of the mitochondrial metabolic network [85] [86].

Regulation of transcription also occurs at the level of transcriptional elongation and not just at transcriptional initiation. RNA pol II undergoes a cycle of phosphorylation and dephosphorylation during transcription and, with its C-terminal domain (CTD) in a hypophosphorylated form, RNA pol II is recruited to promoters. Phosphorylation of the CTD occurs during transcription initiation and elongation, whereas the CTD must be dephosphorylated to allow RNA pol II to be recycled for another round of transcription. RNA pol II has been found to pause on most promoters after transcribing approximately 20–40 bases. This model fits well with the finding that Myc stimulates the release of paused RNA pol II from the promoter and stimulates subsequent transcriptional elongation [87]. This correlates with a Myc-dependent increase in RNA pol II phosphorylation: Myc transactivation domain (TAD) binds directly CTD kinases (Figure 5b) [88] [89].Myc induction of RNA pol II phosphorylation occurs globally throughout the nucleus; it can be detected in the total cellular pool of RNA pol II rather than simply at MYC target-gene promoters [90].

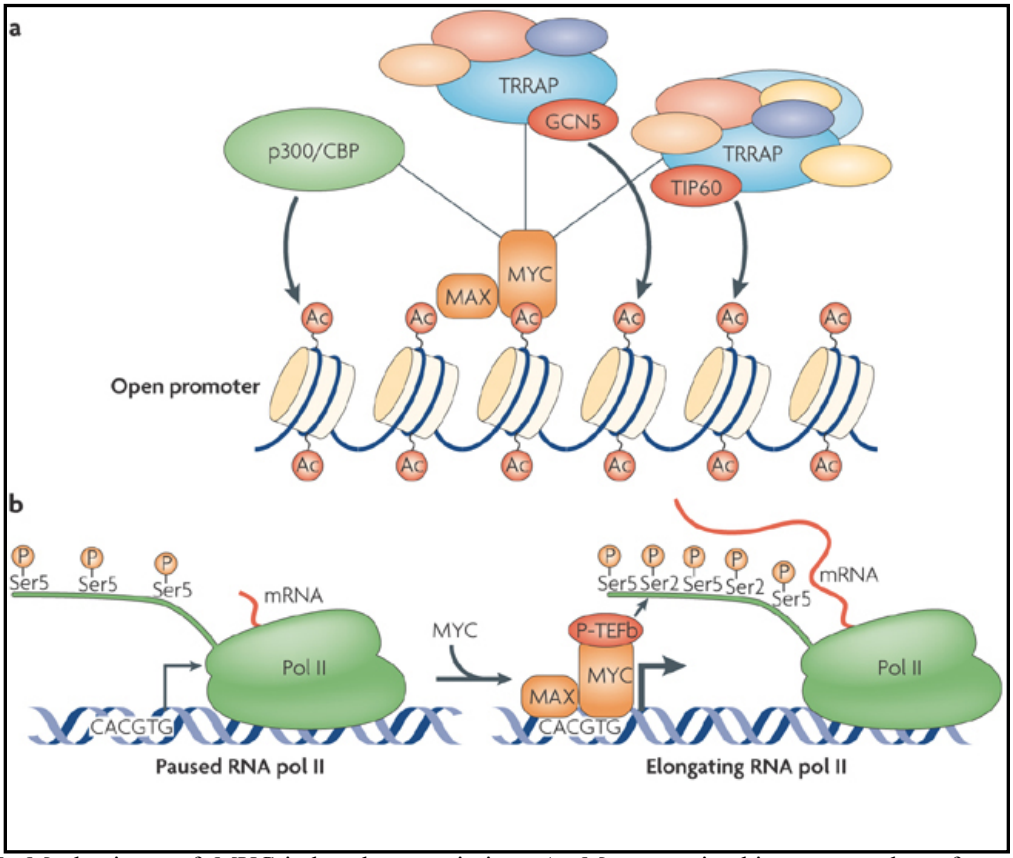

Figure 5. Mechanisms of MYC-induced transcription. **A,** Myc recruits histone acetyltransferases, which promote localized modification of chromatin through acetylation of nucleosomes. **B,** Myc recruits basal transcription factors and promotes the clearance of promoters through RNA polymerase (pol) II. The Myc protein can promote a paused RNA pol to continue transcription of the mRNA by recruiting the P-TEFb (positive transcription-elongation factor-b) complex, which phosphorylates the CTD on Ser2 and promotes transcriptional elongation [91].

Moreover, Myc can control protein expression through mRNA translation by promoting the methylation of the 5' mRNA guanine or 'cap', which is an essential step for protein-coding gene expression. As native levels of Myc regulate the expression of their targets without changes in mRNA abundance, this transcription-independent activity has the potential to influence all aspects of MYC biology in both normal and tumor cells [90].

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

Previous studies have provided a link between MYC and genomic instability, but it was postulated that this was an indirect consequence of transcriptional activity [92]. A recent study describes a direct, non-transcriptional role for MYC in the initiation of DNA

replication. Myc was found to bind numerous components of the pre-replicative complex, and localize to early sites of DNA replication. These observations suggested that MYC might directly control the initiation of S phase and that the MYC effects on genomic instability might not depend on the transcriptional induction of S-phase-promoting genes [93].

Furthermore much excitement has been generated in the past years about the role of non-coding, regulatory RNAs. The first oncogenic microRNA polycistron is shown to be regulated by MYC [94] [95].

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

Indeed, the notion that Myc is a general chromatin regulator is nonetheless consistent with several recent observations concerning MYC function. First, a series of independent expression microarray studies have collectively identified an unexpectedly large group of potential genes that are transcriptionally regulated by Myc [96]. Second, recent experiments directly assessing genomic binding by Myc suggest binding to thousands of sites throughout the genome encompassing approximately 15% of genes as well as intergenic regions [97] [98] [99] [69]. Potentially, therefore, Myc regulates a significant proportion of all genes in an organism. The number of in vivo binding sites exceeds the number of Myc molecules in proliferating cells, which indicates that each site is bound by Myc only temporarily. Most probably, therefore, transcriptional regulation by Myc occurs by a 'hit-and-run' mechanism whereby the relatively brief binding of Myc triggers longer-lasting changes in the chromatin organization at the bound loci [11].

Ultimately there are many evidences that MYCN has a robust role in the human genome in regulating global cellular euchromatin, including that of intergenic regions. Strikingly, MYCN maintains 90% to 95% of total genomic euchromatic marks histone H3 acetylated at lysine 9 and methylated at lysine 4 modifications in human neuroblastoma, with enhancer-like function [100]. In neural stem cells, loss of MYCN is sufficient to cause nuclear condensation, most likely due to a global spread of heterochromatin [100]. Myc's recruitment of histone acetyltransferases such as GCN5 and TIP60, as well as its regulation of histone acetylation at a number of genic loci, suggests that the regulation of euchromatin through histone acetylation is involved. Additional evidence suggests that the Myc-regulated chromatin program involves also methylation of lysine 4 of histone H3, possibly through the demethylase LID [101]. Furthermore Myc may regulate chromatin at a distance such that Myc

binding at one location can influence chromatin at another through higher order chromatin structure.

Intriguingly intergenic binding sites for MYCN are not enriched for E-boxes. Although E-box–independent binding has been reported and may be fairly widespread [102] such binding may be of particular importance for Myc intergenic function.

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

Furthermore, Myc has been shown to possess another feature outside the context of E-boxes: surprisingly Myc can act as well as a transcriptional repressor at certain target promoters (see below) [103].

MYC as a repressor

One of the first indicators that MYC might also function as a transcriptional repressor came from studies published in the 1980s that suggested that MYC participates in a negative feedback loop [104] [105]. After this, genome-wide analyses demonstrate that MYC represses at least as many targets as it activates, further emphasizing the role of repression in MYC function, including transformation [11].

Moreover recent findings show that Myc plays a fundamental role as part of the "magic quartet" of transcription factors that can reprogram somatic cells to induced pluripotent stem (iPS) cells. Ectopic expression of Myc augments the ability of Oct4, Sox2, and Klf4 to induce the formation of pluripotent cells from mouse and human fibroblasts, liver cells, and mature B cells by a factor of twofold to 10-fold, depending on the cell type [106] [107] [108] [109]. Silencing of the somatic cell expression program appears to be an important initial step required for the induction of the ES-like expression program, and it is evident a major contribution of Myc to this first step [110].

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

Initially, no simple consensus sequence for transcriptional repression by Myc had emerged. This opened the possibility that transcriptional repression is simply an indirect consequence of the altered physiological (e.g., transformed) state of a cell that is induced by Myc. Indeed,

there is evidence in the literature for such indirect mechanisms of gene repression by Myc [111] [112].

One argument against the notion that all repression is similarly indirect was the identification of mutants of Myc that distinguish transcriptional activation from repression and the detailed analysis of the resulting phenotypes [113] [114]. Furthermore DNA elements required for Myc-mediated repression has been demonstrated to lie within the promoters of repressed target genes, indicating that Myc repression is likely mediated at the transcriptional level [103].

The repressed genes, like induced genes, fall into multiple functional classes: the first class of genes encodes proteins that are selectively expressed in quiescent cells or that directly or indirectly inhibit cell proliferation. This group encompasses the cell cycle inhibitors p21Cipl [114] [115] [116] [117] [118], p27kipl [119], pl5ink4b [117] [120] [121], pl8ink4c [80], and p57kip2 [122], as well as the differentiation-inducing proteins C/EBP-a [123] [124], the growth-arrest proteins gas1 and gas2 [113], the growth arrest and DNA damage proteins gadd34, gadd45, gadd153 [125] [126], and the Myc-antagonist Mad4 [127]. This long list points to a role for Myc-mediated gene repression in the control of cellular differentiation and in the response to growth arrest signals. It appears clear that the repression of individual genes could significantly contribute to the phenotype of MYC-transformed cells.

A second class of genes that is often repressed by Myc encodes proteins involved in cell adhesion, including a number of integrins: these mRNAs include those encoding cell surface proteins such as the class I HLA molecules in melanoma cells, the $\alpha 3$ $\beta 1$ integrin in neuroblastomas, and the LFA-1 (αL $\beta 2$ integrin) cell adhesion protein in transformed B cells as well as [10, 128] [129]. Altered cell adhesion is a hallmark of many Myc-transformed cells and has been observed in different cell types [10].

Finally, genes involved in metabolic pathway such as thrombospondin and H-ferritin are regulate by Myc [130] [131]. Suppression of thrombospondin plays a causative role in the induction of angiogenesis by Myc.

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

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

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

A number of Myc-repressed targets contain a subclass of initiator elements (INRs; consensus, YYCAYYYYY, where Y is a pyrimidine base), which are usually, but not invariably, present at TATA-less promoters. INR elements are recognized by TFIID as well as a number of regulatory proteins, such as the transcription initiation factor TFII-I, YY-1, and the Myc-interacting zinc-finger protein 1 (Miz-1). Interestingly, the last three proteins have been reported to associate with the BR/HLH/LZ region of Myc [10]. While there has been little follow-up on the initial reports involving Myc interaction with TFII-I or YY-1, the association of Miz-1 with Myc has been recently confirmed and shown to promote stabilization of Myc by inhibiting its ubiquitin-dependent degradation [44]. Miz1 contains 13 zinc fingers and, at its amino-terminus, carries a BTB/POZ-domain, which is a protein/protein interaction domain found in multiple zinc-finger proteins. Miz1 binds to the 'outside' of the helix–loop–helix domain of Myc, but does not interact with Max, Mad or Mnt proteins [114] [132].

Perhaps a stable Myc-Miz1 interaction blocks the ability of Miz1 to initiate transcription at INR-containing and other promoters [132].

Some Myc-repressed genes, such as gadd45, do not contain INR sequences; rather, repression appears to be mediated by a GC-rich region that is potentially recognized by WT1 and p53 [125]. Another GC-rich binding protein that seems to be important for Myc-mediated repression is the basal transcription factor Sp1 (specificity protein 1), a zinc-finger protein involved in gene expression in the early development of an organism. Other results show that repression by Myc happens through the Smad and the NF-Y binding sites due to direct protein/protein interactions between Myc and Smad2 and nuclear factor Y (NFY), respectively [133] [134].

The data clearly support the notion that several pathways of repression exist. Finally the present mechanistic model is that Myc–Max complexes interact with transcriptional activators that are bound directly to DNA through enhancer or initiator elements [135] [136]. These multi-protein complexes are thought to displace co-activators and recruit co-repressors [137].

Indeed some genes are repressed by MYC through a mechanism that does not involve the Max protein [35] [116]. In fact it was shown that Myc recruits a DNA methyltransferase, DNMT3a, to the Myc-Miz1 complex (without Max) on the promoter of p21, indicating that Myc-dependent gene repression could at least partly be mediated by methylation of its target promoters (Figure 6) [138]. Since Dnmt3a is complexed with histone deacetylases, its recruitment by Myc might lead to local histone deacetylation and inhibition of transcription [139]. Recruitment of Dnmt3a by Myc is an attractive mechanism for repression, since it

might provide an explanation of the aberrant DNA methylation of some tumor suppressor genes that is observed in human tumors.

That Myc can form complexes with proteins outside the Max/Mnt context has been recently shown by Orian and colleagues who have found that, in Drosophila, dMyc can directly interact with the co-repressor Groucho without Max to control neuronal development [140].

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

As mentioned above, despite further support for the provocative idea of Myc as a repressor, knowledge of the molecular mechanism lagged behind that of MYC as a transactivator. Nonetheless, it seems likely that both activation and repression are required for Myc biological function. To date still little is known about MYCN-mediated repression. Only few genes have been found to be repressed by MYCN: the differentiation-related NDRG1 and -2 genes (MYCN Downstream-Regulated Genes) via Miz-1-dependent interaction with their core promoter [141] [142] and TG2 via SP1-dependent interaction[143].

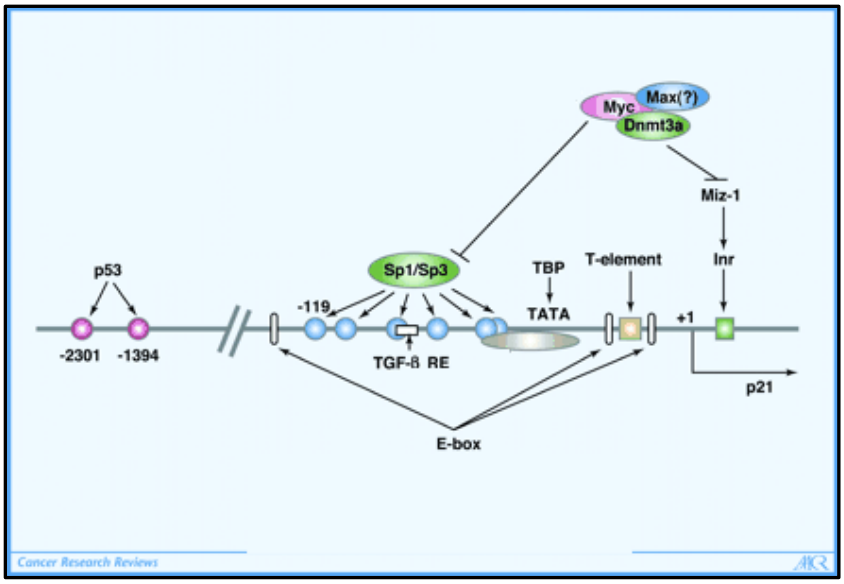

Figure 6. Multiple factors employ distinct mechanisms to repress the p21 promoter. Myc exerts their action through the proximal promoter region. Myc oncoproteins tend to interfere with positive regulators of p21 transcription, such as Sp1 and Miz1 [138].

Myc And Oncogenesis

Deregulated Myc genes are deeply involved in wide range of human cancer: neuroblastoma, osteosarcoma, breast cancer etc. The activation of oncogenic Myc is not due to mutation in

the coding sequence that keeps the protein in an active state (ex. HRAS), but is due principally to three mechanisms of activation:

- ✓ Insertional mutagenesis: transduction of a retrovirus coding for a viral Myc (ex. avian myelocytomatosis retrovirus MC29[1])or insertion upstream of Myc genes of viruses which promoters regulate Myc transcription'[144] (Fig 7a)
- ✓ Chromosomal translocation: after chromosomal translocation Myc became regulated by strongly active promoter[145], this is the case of Burkitt lymphoma in which chromosomes 14,2 or 22, which harbor the Ig heavy and light chain genes, are translocated with chromosome 8, so Myc became regulated by Ig promoter. (Fig 7a)[146, 147].
- ✓ Gene amplification: in cancer cells, Myc gene is present in multiple copies or in the same chromosome or in extrachromosomal DNA named duble-minutes. (Fig 7a)[148-150].

More generally Myc can be deregulated by every kind of mechanisms that converge to Myc overexpression (Fig. 7b) and it exerts his role in oncogenesis through modulation of transcription of several target genes involved in all principal cellular functions.

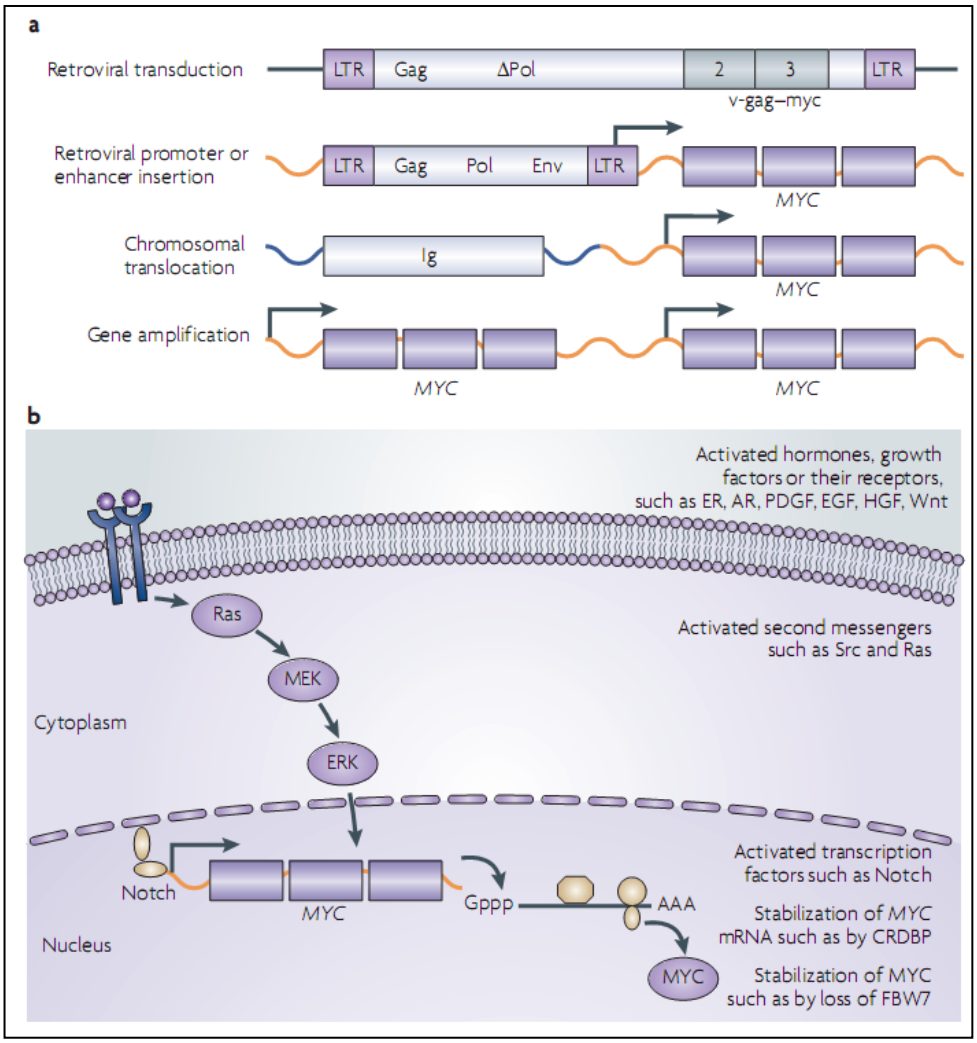

Figure 7. Myc activation mechanisms. **6a** Deregulation could occur as a consequence of gross genetic abnormalities that affect the MYC locus, including retroviral promoter or enhancer insertion, chromosomal translocation and gene amplification. **6b** MYC can be deregulated by many additional mechanisms, including activation of hormones or growth factors, their receptors, second messengers or transcriptional effectors that converge on MYC expression. Alterations in mechanisms that directly or indirectly stabilize MYC mRNA and/or protein can also deregulate expression of this potent oncogene. Adapted from [11]

The main consequence of Myc deregulation is induction of cell proliferation and block of differentiation. In cell whit activated Myc , G1 and G2 are shortened and cells easily enter in S and M phase; this phenomena is manly due to Myc mediated activation of cyclin D1, D2 ,E1, A2, as well as CDK4, CDC25A, E2F1 and E2F2. Myc can also mediate cell cycle progression by directly or indirectly repressing transcription of check point genes like GADD45, GADD153 and CDK inhibitor genes for example P21 and P15 [151]. In conjunction with cell proliferation deregulation of Myc blocks cell differentiation in many different cell types, notably Myc can induce differentiation when commitment to a specific

lineage is linked to proliferation[42]. In conclusion Myc over expression strongly modifies the equilibrium between cell proliferation and differentiation in behalf of proliferation.

The Myc induced cell proliferation is gained by an appropriate increase of cell metabolism and protein synthesis. It's now well know that Myc up-regulates transcription of genes coding for enzymes involved in glucose transport and metabolism such as Glut1, HK2, PKM2, LDHA and PDK1. Furthermore several Myc targets are associated whit mitochondrial biogenesis[152].

The ability of Myc to regulate the transcription of several components of the protein synthesis machinery has been validated in several different cell types. The effect of Myc in stimulating protein synthesis is also supported by a direct role of Myc in promoting ribosome biogenesis and furthermore Myc over expression results in a increased size of nucleoli. Myc also promotes ribosome biogenesis at different levels for example by stimulating rRNA modification and processing through direct control of expression of ribonucleases, rRNA-modifying enzymes and nucleolar proteins such as NMP, Nop52, Nop56 and DKC1. Moreover Myc protein is localized in the nucleolus and directly regulates rRNA synthesis by binding to E-box elements located in the rDNA promoter[153].

Another relevant skill of Myc is its faculty to induce genomic instability. One of the first evidence corroborating this hypothesis was the observation by Mai and co-workers that, after Myc deregulation, DHFR locus such as CyclinD2 and Ribonucleotide Reductase R2 were selectively amplified, and continued Myc expression resulted in rearrangement of the locus, along with additional chromosomal aberrations such as breaks, and the acquisition of extra chromosomal elements. Furthermore Myc induces reactive ROS, which in turn induce DNA breaks. These aberrant phenomena along with Myc induced cell cycling lead to numerical and structural alteration of karyotype and the persistence of these lesions during tumor progression indicates that they are selected for during tumorigenesis[154, 155].

Gene that encodes cytoskeletal and cell adhesion proteins appears to be coordinately downregulated by Myc. The collagen genes and several surface protein genes that mediate adhesion to extracellular matrix (N- and R- cadherins, integrin β_1, fibronectin and fibrillin 1 and 2 are repressed). The repression of these genes implies morphological changes showed by Myc overexpressing cells and the ability to growth in a anchorage-independent way[151].

In the late 1990s the ability of Myc to promote angiogenesis was discovered. Downregulation of thrombospondin is vital to angiogenesis and is potentially achieved through MYC induction of the miR17–92 microRNA cluster, moreover Myc increases expression and release of interleukin 1β that is essential for initiation of angiogenesis.[11]

In my PhD thesis I studied n- and c-Myc mediated transcription respectively in Neuoroblastoma and Osteosarcoma cell lines and theirs involvement in cancer development.

Cellular Models

Osteosarcoma: an overview.

Osteosarcoma (OS) is defined as a malignant mesenchymal tumor in which the cancerous cells produce bone matrix. Osteosarcoma is the most frequent primary bone sarcoma, comprising approximately 15% of all bone tumors and about 0.2% of pediatric tumors overall. The age at diagnosis is on the average 16 years for females and 18 years for males and with low frequency it also occurs in elderly adults. The most common localizations in young adults are areas with rapid bone growth as distal femur, proximal tibia and proximal humerus[156]. OS is associated with several genetic predisposition but most OS tumors are sporadic without familiar patterns. The diagnosis is usually made by radiographic location of tumor lesions and a biopsy for pathologic confirmation. OS has a broad spectrum of histological appearances with common characteristics containing highly proliferative malignant mesenchymal stem cells and the production of osteoid and/or bone by tumor cells. Histologically, OS can be divided into several subtypes. Conventional osteoblastic OS makes up about 70%, whereas chondroblastic and fibroblastic OS tumors are the next most common at 10% each. Other OS types include anaplastic, telangiectatic, giant cell-rich, and small cell OS. Of patients who present no metastatic disease, about 70% will be a long-term survivor. The remaining 30% will develop relapse. Pulmonary metastasis is the most common distant spread and the main cause of death for OS patients.

Unlike others sarcoma, no specific translocation or genetic abnormalities have been identified in OS, but nearly 70% of OS tumors display a multitude of cytogenetic abnormalities. Besides Myc overexpression, another possible cause of genomic instability in OS should be alteration of ALT mechanism. ALT is a recombination-based method that prevents telomeres shortening which induces cellular senescence and apoptosis. ALT mechanism is altered in about 50% of

OS.

Individuals affected by hereditary retinoblastoma (RB) heterozygous for a germline inactivation of RB1 have an approximately 1000 times higher incidence of OS. Genetic alterations of RB1 have been found in up to 70% of sporadic OS cases. Loss of heterozygosis (LOH) of RB1 locus is present in 60% to 70% of OS tumors. The tumor suppressor gene TP53 is located at 17p13, a region frequently identified as abnormal in OS. Alterations in TP53 observed in OS tumors consisted of point mutations (20%–30%, mostly missense mutations), gross gene rearrangements (10%– 20%), and allelic loss (75%–80%)[157].

Among oncogenes the most frequently deregulated are:

- c-Myc . 12 percent of OS have a Myc deregulation comprising amplification and overexpression. Moreover 42 percent of relapsed patients have a c-Myc overexpression.
- FOS and JUN. overexpression of these transcription factor (they act as heterodimers) occurs in 61% of OS tumors.
- MDM2. It is a protein that negatively modulates the activity of TP53. The 12q13 region containing MDM2 gene is amplified in 10% of OS tumors .

Recent findings show that the over expression of c-Myc is correlated to chemoresistance to methotrexate and has a prognostic value in high grade OS even in patients not treated with MTX; this suggests a more wide function of c-Myc in chemoresistance in OS. This hypothesis is corroborated by the work of Hattinger et al [158] where a gain of c-Myc is correlated to doxorubicin resistance and again to MTX resistance.

Actual treatment of OS is composed by multiagents chemotherapy before and after surgical resection of primary tumor, without chemotherapy the long-term survival after resection was less than 20%. The most used chemotherapy combination is cisplatin (*CDDP*), doxorubicin (DX) and high dose of methotrexate (MTX); this therapy improved the long-term survival till to 70% but, still today, about 30% of patients became resistant to chemotherapy agents. Possible mechanisms of drug resistance include alterations in: p-glycoprotein expression, multidrug resistance protein expression, topoisomerase II, glutathione S-transferases, DNA repair, drug metabolism or inactivation, and reduced intracellular influx. Drug resistance can be intrinsic (in the absence of treatment) or acquired (after treatment with chemotherapeutic agents [157].

The most studied gene involved in drugresistance in OS is ABCB1 (P-Glycoprotein/MDR1). Several researchers had correlate ABCB1 expression to poorer overall survivor; moreover recent works have shown a direct correlation between osteosarcoma drug resistance and

ABCB1 overexpression and amplification [158, 159]. ABCB1 is a gene belonging to a wide family of genes named ABC Binding Cassette transporter genes.

Neuroblastoma: an overview

Neuroblastoma is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy. It accounts for more than 7% of malignancies in patients younger than 15 years and around 15% of all pediatric oncology deaths. The overall incidence is about one case in 7,000 live births, and there are about 700 new cases per year in the United States. This incidence is fairly uniform throughout the world, at least for industrialized nations. The median age at diagnosis for neuroblastoma patients is about 18 months; so about 40% are diagnosed by 1 year of age, 75% by 4 years of age and 98% by 10 years of age. [160]

Neuroblastomas are tumors of the sympathetic nervous system. Due to their neural crest cell lineage, neuroblastomas may occur anywhere along the sympathetic ganglia. Most primary tumors (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla. Other common sites of disease include the neck, chest, and pelvis. The disease is remarkable for its broad spectrum of clinical behavior. Although substantial improvement in outcome of certain well-defined subsets of patients has been observed during the past few decades, the outcome for children with a high-risk clinical phenotype has improved only modestly, with long-term survival still less than 40% [161]; [162].

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

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

(Figure 8) [163] [164]

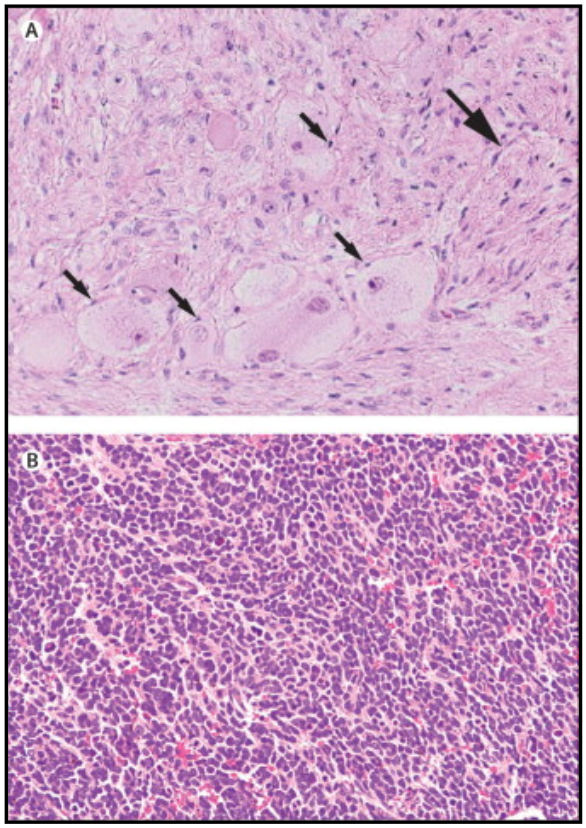

Figure 8. Degree of differentiation in neuroblastoma. **A,** Schwann cells and ganglion cells (indicated by arrows) are prominent in stroma-rich neuroblastoma. **B,** Stroma-poor neuroblastoma consists of densely packed small round blue cells with scant cytoplasm. [165]

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

The generally accepted method is the International Neuroblastoma Staging System (Table 1) [166].

Table 1. International Neuroblastoma Staging System [167]

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

Taken together, the stage of disease, the age of the patient at diagnosis and the site of the primary tumor are the most important clinical variables in predicting patient outcome [168] [160]. The 2-year disease-free survival of patients with stage 1, 2 and 4S is 80–90%, whereas those with stages 3 and 4 have a range of 40–50%. The outcome of infants who are less than 1 year of age is substantially better than older patients with the same stage of disease, particularly those with more advanced stages of disease. Patients with primary tumors in the adrenal gland seem to do worse than patients with tumors originating at other sites.
However, these clinical features are imperfect predictors of tumor behavior.

D'Angio and colleagues [169] first described the striking clinical phenotype of stage 4S (S=special) disease that occurs in about 5% of cases. These infants have small localized primary tumors with metastases in liver, skin, or bone marrow that almost always spontaneously regress. Neuroblastoma has the highest rate of spontaneous regression or differentiation (i.e. into a benign ganglioneuroma) observed in human cancers: the actual frequency of neuroblastomas that are detected clinically and subsequently regress without treatment is 5–10% [170]. However the frequency of true asymptomatic neuroblastomas that regress spontaneously is probably much higher, and might be equal to the number detected clinically. These clinical observations lead to considerable interest in understanding the mechanisms underlying spontaneous regression or differentiation, which in turn may lead to therapeutic approaches to stimulate these phenomena [170].

The etiology of neuroblastoma is still unknown. A subset of patients with neuroblastoma shows a predisposition to develop this disease, and this predisposition follows an autosomal dominant pattern of inheritance with incomplete penetrance. A family history of neuroblastoma is identified in 1–2% of cases [171] [172]. Regression analysis of neuroblastoma data was consistent with Knudson's two-mutation hypothesis for the origin of childhood cancer [173]. The median age at diagnosis of patients with familial neuroblastoma is 9 months, which contrasts with a median age of ~18 months for neuroblastoma in the general population; these patients often have bilateral adrenal or multifocal primary tumors [174] [175]. The concordance for neuroblastoma in twins during infancy indicates that hereditary factors might be predominant, whereas the discordance in older twins indicates that random mutations or other factors might also be important [176].

Traditional genetic analyses have identified the short arm of chromosome 16 (16p12-13) as a likely predisposition locus, though no causal gene has been identified [177].

At the present time, it is unclear if this is the only predisposition locus, or if there are multiple loci, but this locus could account for most high-risk families. Neuroblastoma has also been seen in several patients with constitutional chromosomal rearrangements, including deletions overlapping putative tumor suppressor loci at chromosome bands 1p36 and 11q14-23 [178] [179].

Remarkable disease heterogeneity exists within pedigrees considering affected individuals share the same predisposing genetic lesion, suggesting that the pattern of acquired secondary genetic alterations ultimately defines the tumor phenotype [170].

Schwab and colleagues first identified a novel MYC-related proto-oncogene, MYCN, that was amplified in a panel of neuroblastoma cell lines [2]. MYCN is located on the distal short arm of chromosome 2 (2p24): a large region from this site becomes amplified and the MYCN locus is copied to form an extrachromosomal circular element or DM (double-minute chromatin bodies), with retention of the normal copies of MYCN at 2p24. DMs might accumulate by uneven segregation during mitosis; however, in some cases, the amplified DNA integrates into a chromosomal locus to form an HSR (homogeneously staining regions) (Figure 9) [180] [181] [182] [183]. Other genes might be co-amplified with MYCN in a subset of cases, but MYCN is the only gene that is consistently amplified from this locus [184].

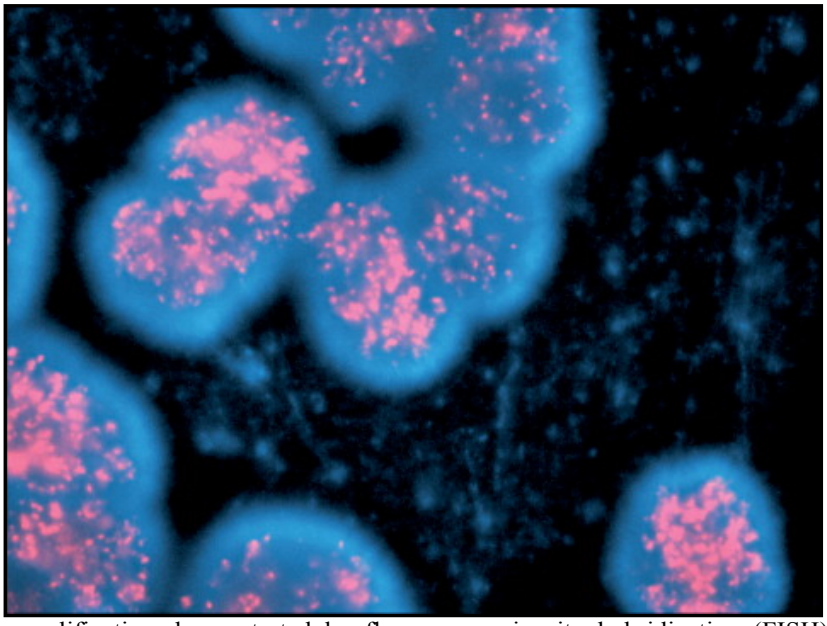

Figure 9. MYCN amplification demonstrated by fluorescence in situ hybridization (FISH) using a labeled MYCN probe. [165]

Currently it is well known that the most consistently genetic aberration associated with poor outcome in neuroblastoma is genomic amplification of MYCN [185] [186]. MYCN amplification occurs in roughly 25% of primary tumors and is strongly correlated with advanced stage disease and treatment failure (Figure 9) [187] [150]. Its association with poor outcome in patients with otherwise favorable disease patterns such as localized tumors or INSS stage 4S disease underscores its biological importance [188] [189] [190].

Thus, amplification of MYCN remains the most important genetically based independent prognostic factor for neuroblastoma and represents an intrinsic biological property of a subset of aggressive neuroblastomas: tumors without amplification at diagnosis rarely, if ever, develop this abnormality [170]. Figure 10

Activation of the MYC genes by amplification is commonly detected in solid human tumors. In neuroblastoma MYCN is amplified from 5- to 300-fold per haploid genome, and all the copies seem to be transcriptionally active [191] [192]. Therefore tumors with MYCN amplification result in an elevated expression of the MYCN protein and this provides selective advantage to the cells through various mechanisms that affect the role of MYCN as a part of the Myc/Mad/Max transcriptional factor network [193] [191].

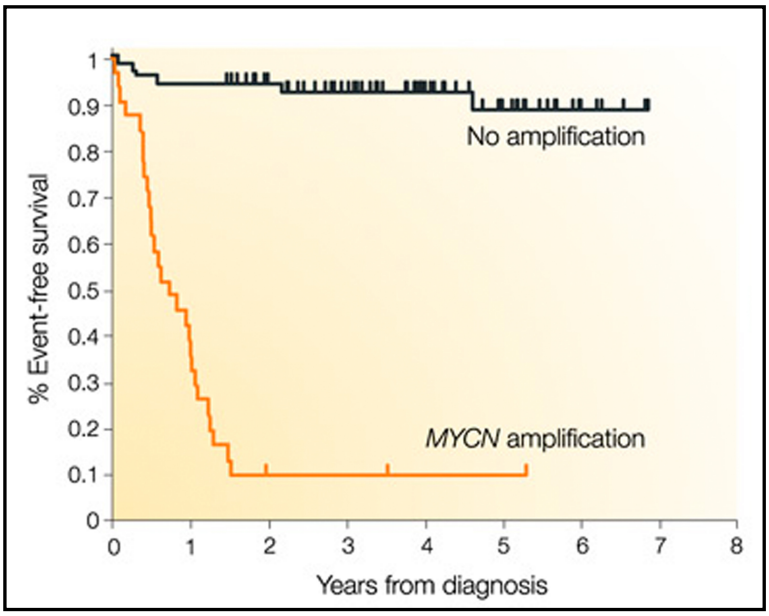

Figure 10. Survival of infants with metastatic neuroblastoma based on MYCN status. A Kaplan–Meier survival curve of infants less than 1 year of age with metastatic neuroblastoma. The 3-year event-free survival (EFS) of infants whose tumors lacked MYCN amplification was 93%, whereas those with tumors that had MYCN amplification had only a 10% EFS [170].

ABC Transporter Genes

The ATP-binding cassette (ABC) transporters are the largest family of transmembrane transporter protein. ABC transporters use the energy from ATP hydrolysis to pump compounds across the membrane or to flip molecules from the inner to the outer leaflet of the membrane [194-196]. While hydrophobic compounds are the most common substrates, ABC transporters are able of transporting metal ions, peptides and sugars.

ABCs are highly conserved between species, indicating that most of these genes have existed since the beginning of eukaryotic evolution. There are 25 ABC genes in E. coli, 29 in S. cerevisiae, 56 in C. Elegans, 56 in Drosophila, 51 in Mouse and 48 in Homo Sapiens [197].

Human ABC genes are localized on 16 different autosomes and 2 genes reside on the X chromosome. Analysis of amino acid sequence alignments of the ATP-binding domains has allowed the ABC genes to be classified into subfamilies. There are seven ABC genes subfamilies in the human genome, from A to G (Table 2). The most part these subfamilies display considerable homology in the trans-membrane (TM) domains and have identical gene organization, and similar intron localization.

Symbol	Alias	Location	Mouse Location	Expression	Function
ABCA1	ABC1	9q31.1	4 23.1	Ubiquitous	Cholesterol efflux onto HDL
ABCA2	ABC2	9q34	2 12.6	Brain	Drug resistance
ABCA3	ABC3, ABCC	16p13.3		Lung	
ABCA4	ABCR	1p22.1–p21	3 61.8	Rod photoreceptors	N-retinylidiene-PE efflux
ABCA5		17q24	11 69	Muscle, heart, testes	
ABCA6		17q24	11 69	Liver	
ABCA7		19p13.3	10 44	Spleen, thymus	
ABCA8		17q24	11 69	Ovary	
ABCA9		17q24	11 69	Heart	
ABCA10		17q24		Muscle, heart	
ABCA12		2q34		Stomach	
ABCA13		7p11–q11		Low in all tissues	
ABCB1	PGY1, MDR	7p21	5 1.0	Adrenal, kidney, brain	Multidrug resistance
ABCB2	TAP1	6p21	17 18.6	All cells	Peptide transport
ABCB3	TAP2	6p21	17 18.6	All cells	Peptide transport
ABCB4	PGY3	7q21.1	5 1.0	Liver	PC transport
ABCB5		7p14		Ubiquitous	
ABCB6	MTABC3	2q36		Mitochondria	Iron transport
ABCB7	ABC7	Xq12–q13	X 39	Mitochondria	Fe/S cluster transport
ABCB8	MABC1	7q36		Mitochondria	
ABCB9		12q24		Heart, brain	
ABCB10	MTABC2	1q42	8 67	Mitochondria	
ABCB11	SPGP	2q24	2 39	Liver	Bile salt transport
ABCC1	MRP1	16p13.1	16	Lung, testes, PBMC	Drug resistance
ABCC2	MRP2	10q24	19 43	Liver	Organic anion efflux
ABCC3	MRP3	17q21.3		Lung, intestine, liver	Drug resistance
ABCC4	MRP4	13q32		Prostate	Nucleoside transport
ABCC5	MRP5	3q27	16 14	Ubiquitous	Nucleoside transport
ABCC6	MRP6	16p13.1		Kidney, liver	
CFTR	ABCC7	7q31.2	6 3.1	Exocrine tissues	Chloride ion channel
ABCC8	SUR	11p15.1	7 41	Pancreas	Sulfonylurea receptor
ABCC9	SUR2	12p12.1	6 70	Heart, muscle	
ABCC10	MRP7	6p21		Low in all tissues	
ABCC11		16q11–q12		Low in all tissues	
ABCC12		16q11–q12		Low in all tissues	
ABCD1	ALD	Xq28	X 29.5	Peroxisomes	VLCFA transport regulation
ABCD2	ALDL1, ALDR	12q11–q12	15 E–F	Peroxisomes	
ABCD3	PXMP1, PMP70	1p22–p21	3 56.6	Peroxisomes	
ABCD4	PMP69, P70R	14q24.3	12 39	Peroxisomes	
ABCE1	OABP, RNS4I	4q31		Ovary, testes, spleen	Oligoadenylate binding protein
ABCF1	ABC50	6p21.33	17 20.5	Ubiquitous	
ABCF2		7q36	13 40	Ubiquitous	
ABCF3		3q25	16 22	Ubiquitous	
ABCG1	ABC8, White	21q22.3	17 A2–B	Ubiquitous	Cholesterol transport?
ABCG2	ABCP, MXR, BCRP	4q22	6 28–29	Placenta, intestine	Toxin efflux, drug resistance
ABCG4	White2	11q23	5 59	Liver	
ABCG5	White3	2p21	17	Liver, intestine	Sterol transport
ABCG8		2p21	17	Liver, intestine	Sterol transport

PBMC, peripheral blood mononuclear cells; VLCFA, very long chain fatty acids.

Table 2. List of human ABC genes, Chromosomal location and Features.[197]

Genes are classified into the ABC superfamily based on the sequence identity of the ATP-binding domain(s), also known as nucleotide-binding folds (NBFs) [195, 197]. These proteins also possess two transmembrane (TM) domains composed of 6–11 membrane-spanning a-helices. The functional transporter can either be a single protein with two NBFs and two TM domains (a full transporter) or be a dimer consisting of two half transporters. Fig. 11.

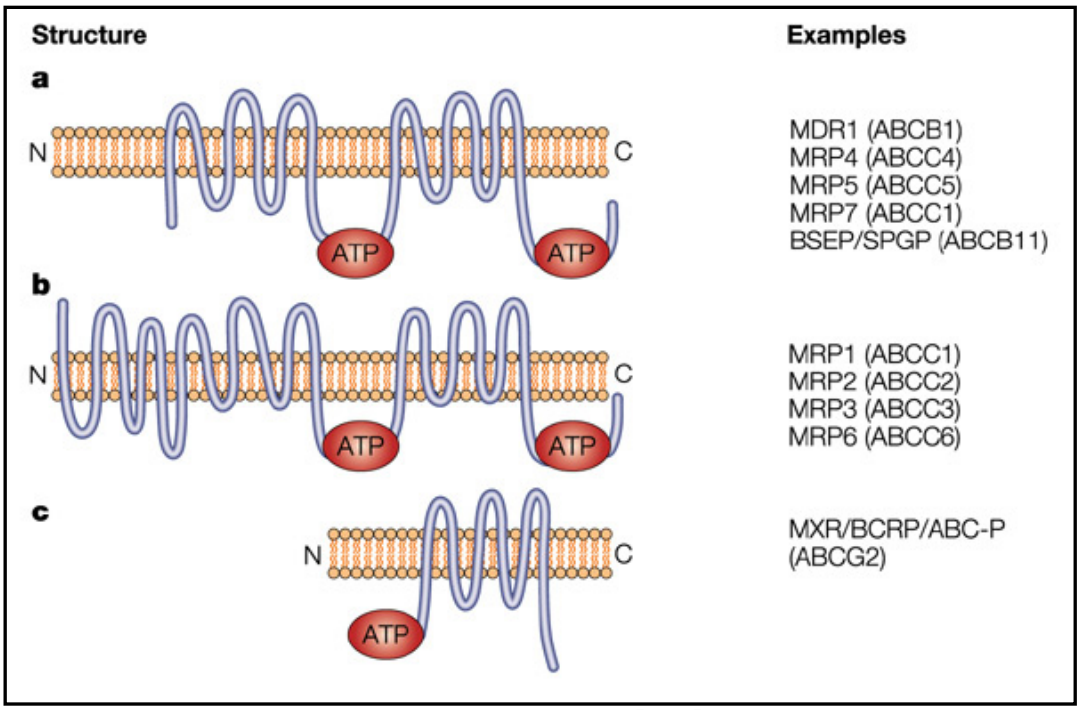

Figure 11. The structure of three categories of ABC transporter [197]

ABCA subfamily is composed of 12 full transporters that are split into two subgroups [197]. The first group (ABCA1–A4, A7, A12, A13) includes seven genes that map to six different chromosomes. The second group of ABCA genes (ABCA5–A6, A8–A10) is organized into a head-to-tail cluster on chromosome 17q24. This gene cluster is also found in the mouse genome.

The expression pattern of the chromosome 17 genes is restricted with ABCA5 and ABCA10 expressed in skeletal muscle, ABCA9 in the heart, ABCA8 in the ovary, and ABCA6 in the liver. No diseases map to the corresponding region of the mouse and human genomes, and the functions are as yet uncharacterized. Probably cluster of ABCA genes in Chr.17 originates by gene duplication. ABCA1 controls the extrusion of membrane phospholipids and cholesterol toward specific plasmatic acceptors, the apolipoproteins. The ABCA1-dependent homeostatic control of the lipid content of the membrane dramatically influences the plasticity and fluidity of the membrane. The ABCA4 gene was found to be highly expressed in rod photoreceptors, and maps to the region of chromosome 1p21 containing the gene for the Stargardt disease, a recessive childhood retinal degeneration syndrome. ABCA4 is believed to mediate transport of Vitamin A derivates [197].

The ABCA2 gene is highly expressed in oligodendrocytes in the brain; the ABCA7 gene highly expressed in the spleen and thymus. The function of these genes, as well as ABCA12

and ACBA13, is not known, although it is tempting to speculate that they similarly participate in cellular lipid homeostasis in specialized environments. This is supported by the recent findings that both ABCA2 and ABCA7 share with ABCA1 a sterol dependent upregulation [197].

The ABCB subfamily is composed of four full transporters and seven half transporters, and this is the only human subfamily to have both types of transporters. The ABCB1 (MDR/PGY1) gene was discovered as a protein overexpressed in certain drug-resistant tumor cell lines. Cells that overexpress this protein display MDR and are resistant to or transport a wide variety of hydrophobic compounds including colchicine, doxorubicin, adriamycin, vinblastine, digoxin, saquinivir, and paclitaxel. ABCB1 is expressed primarily in the liver and blood brain barrier, and is thought to be involved in protecting cells from toxic agents. The gene is duplicated in mice and animals lacking both genes unfortunately display a very limited phenotype and are still viable and fertile. However, they have been very useful models to identify and characterize other drug resistance genes. The ABCB4 and B11 proteins are both located in the liver and participate in the secretion of phophatidylcholine (PC) and bile salts, respectively. Mutations in ABCB4 and ABCB11 are responsible for several forms of progressive familial intrahepatic cholestasis (PFIC). Defects in ABCB4 are responsible for PFIC3, and are associated with intrahepatic cholestasis of pregnancy. Mutations in the ABCB11 gene are found in patients with PFIC2. The ABCB2 and B3 (TAP) genes are half transporters that form a heterodimer to transport digested peptides for MHC1 into the ER. Rare families with defects in these genes display profound immune suppression, as they lack this essential portion of the immune recognition process.

The remaining ABCB subfamily half transporters are expressed in the lysosome (ABCB9) or the mitochondria (ABCB6, B7, B8, and B10). One of the mitochondrial genes (ABCB7) is located on the X-chromosome and mutations in this gene are responsible for X-linked sideroblastic anemia and ataxia (XLSA/A) phenotype. The human ABCB9 plays a role in mitochondrial iron homeostasis and in the biogenesis of cytosolic Fe/S proteins [197].

The ABCC subfamily contains 12 full transporters that perform functions in ion transport, toxin secretion, and signal transduction. The MRPs studied thus far, MRP1–5, are all organic anion pumps, but they differ in substrate specificity, tissue distribution, and intracellular location, MRPs come in two structural types, one characterized by 17 transmembrane segments (MRP1, 2, 3, 6), and one by 12 (MRP4, 5, 7, 8). Cystic fibrosis (CF) is an inherited multisystemic disorder characterized by abnormalities in exocrine gland function consequent

to loss of function of the CFTR transporter (ABCC7). The CFTR protein is unique among ABC proteins in that it is a cAMP-regulated chloride ion channel [198].

The ABCC8 gene was identified as the locus for familial persistent hyperinsulinemic hypoglycemia of infancy, an autosomal recessive disorder characterized by unregulated insulin secretion. The remaining ABCC genes are nine MRP-related genes. ABCC1 (MRP1) was identified as a multidrug resistance gene and demonstrated to transport glutathione conjugates of many toxic compounds. MRP1 is a prototype GS-X pump and a remarkably versatile one. It transports a variety of drugs conjugated to GSH, to sulfate or to glucuronate, as well as anionic drugs and dyes, but also neutral/basic amphipathic drugs and even oxyanions. The oxyanions arsenite and antimonite and the neutral/basic drugs are co-transported with GSH. Notwithstanding this enormous range of substrates transported, MRP1 is not indiscriminate [199]. Similar to ABCB1, ABCC1 transports and confers resistance to a wide variety of toxic substrates, but is not essential for growth or development. ABCC1 can also transport leukotriene C4, a potent chemotactic factor controlling dendritic cell migration from peripheral tissues to lymph nodes [197]. MRP2 and MRP1 have about the same size and putative membrane topology, and they both transport a similar large range of organic anions. However, the tissue distribution of MRP2 is much more restricted than that of MRP1, and MRP2 is located in the apical membrane of epithelial cells, whereas MRP1 is basolateral. MRP2 has an important function in the biliary excretion of endogenous metabolites, such as glucuronosyl-bilirubin, as well as many exogenous compounds. MRP2 is expressed not only in the liver but also in the kidney and the intestine of rats and humans. Mutations in the MRP2 gene cause the Dubin-Johnson syndrome. These patients suffer from an inherited conjugated hyperbilirubinemia, which indicates that bilirubin can enter the hepatocytes and is conjugated with glucuronate, but is not secreted into bile [197].

MRP3 is an organic anion transporter, basolateral like MRP1 [200], and prominently present in liver, gut, and kidney like MRP2. The strong upregulation of MRP3 in the liver under some cholestatic conditions and the ability of MRP3 to transport some bile salts [201] have led to speculations that MRP3 might play a role in the enterohepatic recycling of bile salts and in the removal of toxic organic anions from the hepatocyte under cholestatic conditions [200]. Differently from MRP1 and 2, MRP3 appears unable to transport GSH [200]. This may explain why cells transfected with MRP3 gene constructs are not resistant to most of the anticancer drugs that are probably cotransported with GSH by MRP1/2. Initial studies on MRP3 in a panel of drug-resistant cancer cell lines did not turn up any association between MRP3 levels and resistance [202].

MRP4 and MRP5 are both organic anion pumps, but they have the interesting ability to transport cyclic nucleotides and nucleotide analogs, a class of organic anions apparently not transported by MRP1–3 or 6. The transport of nucleotide analogs by MRP4 and 5 can result in resistance to clinically used base, nucleoside, and nucleotide analogs, at least in transfected cells that highly overproduce MRP4 or 5. The rate of cyclic nucleotide transport by these transporters is low and the physiological role of this transport remains to be defined.

Initial studies on MRP5 showed that this protein is an organic anion pump, able to transport acidic organic dyes, S-(2,4-dinitrophenyl)glutathione, GS-DNP, and GSH, and inhibited by sulfinpyrazone. However, substantial drug resistance in MRP5-transfected cells was found only for 6-mercaptopurine (6MP) and thioguanine (TG), two purine bases that are definitely not acidic [199]. Cells with high expression of MRP4 are highly resistant to PMEA and AZT and much less resistant to other nucleoside analogs used in antiviral therapy, such as lamivudine, ddC, and d4T [203]. MRP4, but not MRP5, confers resistance to short-term incubation with high concentrations of MTX. Like MRP4, MRP5 can transport nucleotide analogs with a normal pyrimidine ring [199]. The list of substrates transported by MRP4 and 5 was substantially broadened by vesicular transport studies. Jedlitschky et al. [204] discovered that MRP5 can transport cyclic GMP and AMP (cGMP and cAMP), and Hopper et al. [205] recently found this for MRP4 as well. The affinity for cGMP is higher than for cAMP. The tissue distribution of MRP4 and MRP5 is still not well known. Recent studies suggest that MRP4 is more widely expressed than initially thought, with the highest levels in kidney and prostate. Analysis of tissue RNA suggests that MRP5 is ubiquitously expressed and the highest levels are found in skeletal muscle and brain. Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and is also able to confer significant resistance in vitro to the topoisomerase I poison irinotecan and its active metabolite SN-38 [206]. No human disease has been associated with alterations in MRP5, and the Mrp5 knock out mouse, has no obvious phenotype. It is possible, however, that the overlapping substrate specificities of MRP5 and MRP4 (and possibly MRP8 and 9) may hide the physiological function of MRP5.

Human MRP6 is mainly expressed in liver and kidney, like Mrp6 (MLP-1), its rat homolog, but low RNA levels have also been detected in other tissues. The substrate specificity of MRP6 is still unknown.

The subfamily D contains 4 genes that encode half transporters expressed exclusively in the peroxisome. ABCD1– 4, which are also called ALDP, ALDR, PMP70, and PMP69, respectively. Interaction between these proteins was demonstrated by co-immunoprecipitation

and yeast two-hybrid assays [199]. One of the genes, ABCD1, is responsible for the X-linked form of ALD, a disorder characterized by neurodegeneration and adrenal deficiency, typically initiating in late childhood. However, there is no correlation between the phenotype of ALD and the genotype at the ABCD1 locus. The functions of the other ABCD family genes have also not been worked out, but the marked sequence similarity (especially for ALDP-ABCD2) suggest that they may exert related functions in fatty acid metabolism. The in vitro demonstration of homo- or heterodimerization of the product of ABCD1 with either ALDRP or PMP70 suggest that different peroxisomal half transporter heterodimer combinations are involved in the import of specific fatty acids or other substrates. ABCD genes are under complex regulation at the transcriptional level, and being very tightly linked to cell lipid metabolism, it is not surprising that they share with the ABCA and ABCG subclasses the sensitivity to the peroxisome proliferator-activated receptor and retinoid X receptor family of nuclear receptors [197].

The ABCE and ABCF subfamilies are composed of genes that have ATP-binding domains that are closely related to those of the other ABC transporters, but these genes do not encode any TM domains. The ABCE subfamily contains a single member, the OABP, ABCE1. This protein recognizes oligoadenylate produced in response to certain viral infections [197]. The ABCE1 gene is the most conserved member of the ABC gene family and is one of the most conserved genes in vertebrate and archaeal genomes [207]. This fact alone suggests that the gene plays an essential role in biology that is common between archae and eukaryotes. In addition, null mutations in the gene are homozygous lethal in every organism that has been examined. The ABCE1 protein was originally identified due to an interaction with and inhibition of RNase L, a nuclease induced by interferon [208]. However, RNase L is not found outside of vertebrates, indicating that ABCE1 has alternate functions. The identification of the role of the ABCE1 protein in ribosome biogenesis and in assembly of the pre-initiation complex of the ribosome in S. cerevisiae provides a function that is both essential and universal to eukaryotes [209]. Recent data show that ABCE1 is essential for *in vitro* and *in vivo* translation of mammalian proteins. Antisera to ABCE1 blocks in vitro translation of mRNA in rabbit reticulocyte lysates but not of poly(U) molecules that can be translated independent of initiation factors. As in yeast, ABCE1 interacts with the eukaryotic initiation factors eIF5 and eIF2 components of the pre-initiation complex. Inhibition of ABCE1 in human cells results in dramatic inhibition of growth, reduction in the amount of large polysomes, and incorporation of labeled amino acids into newly synthesized protein. This is consistent with the results in yeast and supports a critical role for ABCE1 in the initiation of

translation. A recent work of Pisarev et Al. demonstrates that ABCE1 plays a pivotal role in post termination ribosomal recycling by mediating post termination complex dissotiation [210]. The inhibition of ABCE1 could have therapeutic applications. Because the protein is essential to most or all eukaryotes, specific inhibitors could be used in the treatment of pathogens. For example, inhibitors specific to plasmodia, fungi, and/or protozoan parasites could be used to inhibit such organisms as they infect human or other animals. Moreover ABCE1 inhibitors efficiently suppress the growth of human tumor cells. The ABCE1 protein is unusual in containing a Fe-S cluster binding site. It has long been known that Fe-S clusters are assembled in the mitochondria, and this process is essential to the cell. ABCE1 clearly represents one essential Fe-S containing protein [207].

The ABCF proteins each have a pair of NBF, and the best characterized member is the S. cerevisiae GCN20 gene. GCN20 is involved in the activation of the eIF-2 alpha kinase. A human homolog, ABCF1 is part of the ribosome complex and may play a similar role [197].

The human ABCG subfamily contains six half transporters that have an NBF at the N-terminus and a TM domain at the C-terminus: the reverse of the orientation of all other ABC genes. ABCG1 is highly expressed in macrophages and is induced by cholesterol. ABCG4 is highly expressed in the brain. It will be interesting to see if these genes have related functions. The ABCG5 and ABCG8 genes are located head-to-head on the human chromosome 2p15-p16, separated by a region of 200 bp. They are both mutated in families with sitosterolemia, a disorder characterized by defective transport of plant and fish sterols and cholesterol. Sitosterolemia patients display deficient sterol secretion from the intestine and the liver. This genetic evidence indicates that the two half transporters form a functional heterodimer. The analysis of cell lines selected for high level resistance to mitoxantrone led to identification of the ABCG2 (ABCP, MXR1, BCRP) as a multidrug transporter. ABCG2 can use anthracycline anticancer drugs, as well as topotecan, mitoxantrone, or doxorubicin as substrates. The ABCG2 gene is either amplified or rearranged by chromosomal translocations in resistant cell lines. Transfection of ABCG2 into cells confers resistance, consistent with its functioning as a homodimer. The normal function of ABCG2 is not known; however, it is highly expressed in placental trophoblast cells, suggesting that it may pump toxic metabolites from the fetal to the maternal blood supply [197].

Based on the evidences that the transcription factor c-Myc is correlated to chemoresistance in OS and that several ABC transporter are involved in transport of drugs used in OS therapy,

I tested if c-Myc could regulate transcription of ABC genes in OS cell lines.

Cyclin G2

A recent paper of Liu and co-workers[143] shows that MYCN interacts with HDAC1 to repress TGN2 expression, we tested the hypothesis that MYCN could interacts also with HDAC2 and comparing mRNA expression in BE(2)-C transfected with scrambled control siRNA, MYCN siRNA or HDAC2 siRNA, we identified Cyclin G2 (CCNG2) gene as a possible targets of this regulation.

Cyclins represent a group of closely related proteins that regulate cyclin dependent kinases (CDK) activity at specific stage of cell cycle by binding and forming active complexes with specific partner CDKs. Cyclins are thought to confer substrate specificity and regulation (activation, inactivation, localization, binding of subunits and others) to Cdk/cyclin complexes. Moreover the complexes cyclin-CDK are regulated by specific cyclin-kinase inhibitors (CKI). All the 12 different cyclins found in mammalian cells contain a 100 amino-acid homologous region named cyclin box, which is the molecular structure marker of cyclins[211].

Cyclin G is a new member of Cyclin family, which includes Cyclin G1 and Cyclin G2. Although the identity of amino acid sequence and nucleotide sequence of cDNA of Cyclin G1 and Cyclin G2 was 53% and 60% respectively, their biological function and distribution in tissue and organ are very different[212, 213]. The molecular structure of cyclin G differs from other cyclins. N-terminus lacks a “destruction box” sequence controlling the ubiquitin-dependent degradation of mitotic cyclins, while contains an epidermal growth factor receptor (EGF-R/ ErbB) like autophosphorylation motif in its carboxyl terminus. The structure suggested that a role for cyclin G in signal transduction. Cyclin G has not yet been matched with a CDK binding partner, and its biologic function is still elusive. Cyclin G is the only known cyclin that is transcriptionally activated by the p53 tumor suppressor gene. Cloning and sequencing a partial cDNA sequence revealed the cyclin G1 transcript does not encode motifs, which resemble known degradation signals. In contrast, the cyclin G2 protein contains a prototypic protein destabilizing (PEST) rich sequences, which may be responsible for its potential regulated degradation in cell cycle. The cyclin G1 mRNA expression does not fluctuate with cell cycle phase, whereas the expression of cyclin G2 mRNA oscillated with cell cycle and reached peak in the mid-late S phase. In contrast to cyclin G1, cyclin G2 mRNA are weakly expressed in skeletal muscles and heart. It is highly expressed in cerebrum, thymus, spleen, prostate gland and kidney. Notably cyclin G2 transcripts are abundant in

tissue rich in either terminally differentiated cells or cells reacting to growth inhibitory signals and apoptosis. Although Cyclin G1 is a positive regulator and prompts cell proliferation, so far, neither the physiological role nor the biochemical function of cyclin G2 has been defined. The mRNA expression of Cyclin G1 and cyclin G2 can be induced by DNA-damage drug actinomycin-D, which has the p53 dependency for cyclin G1 but the p53 independency for Cyclin G2. Negative growth regulators such as TGF-β1 and dexamethasone can induce cyclin G2. In the growth inhibition state of B cell, the transcription level of cyclin G2 is up-regulated. Ectopic expression of VHL oncosoppressor gene into renal cancer cells with VHL gene defect can induce cyclin G2 expression. Recently it was also showed that lack of cyclin G2 plays an important role in the malignant transformation of papillary carcinoma of the thyroid. Moreover cyclin G2 may play an adjuvant role in the transformation of follicular adenoma to carcinoma [214].

Clusterin

Clusterin (CLU) was described as a glycoprotein present in all body fluids and in most human tissues. In human was first identified by Jenne and Tshopp in 1989 [215]as a component of soluble terminal complement complexes in human serum, then different groups working in different research field's isolated homologues. CLU is expressed at very high levels in many different tissues including dermal fibroblast[216], motorneurons [217, 218]and epithelia[219]. In other tissues, CLU expression is tightly regulated for instance during cell cycle progression[220], atrophy and programmed cell death [221], and in neurodegenerative disorders[222]. Today we know that a nuclear form (nCLU), a cytoplasmic Clusterin and a secreted form of Clusterin (sCLU) exist. Cytoplasmic Clusterin, which originates from retro translocation via the endoplasmic reticulum or Golgi[223], and sCLU are know to cooperate with c-Myc in transformation process [224]and to protect cancer cell from death stimuli[225, 226]. In contrast with this hypothesis recent works show that ectopic expression of Clusterin in prostate cancer cells reduces proliferation [227], moreover overexpression of c-Myc in Rat fibroblast reduces Clusterin expression[228]. An exemplary case is neuroblastoma cells in which Clusterin can mediate resistance to doxorubicin and suppresses in-vitro invasion by inhibiting NF-κB[225, 229]. To reconcile these divergent observations, one possible

explanation is that Clusterin roles in tumorigenesis is context and signal dependent. nCLU, that probably originates from alternative splicing or an alternative translational start site, is a proapoptotic factor at least in breast and prostate cancer cells [230-232].

Clusterin gene is located in chromosome 8p12-21 in close proximity to lipoprotein lipase gene locus. It is organized in 9 exons and codes for three transcript isoforms that have a unique first exon and share the remaining exons from 2 to 9.

The three isoforms are named Isoform1 (NM_001831.2), Isoform2 (NM_203339.1) and Isoform 11032 . Figure 12.

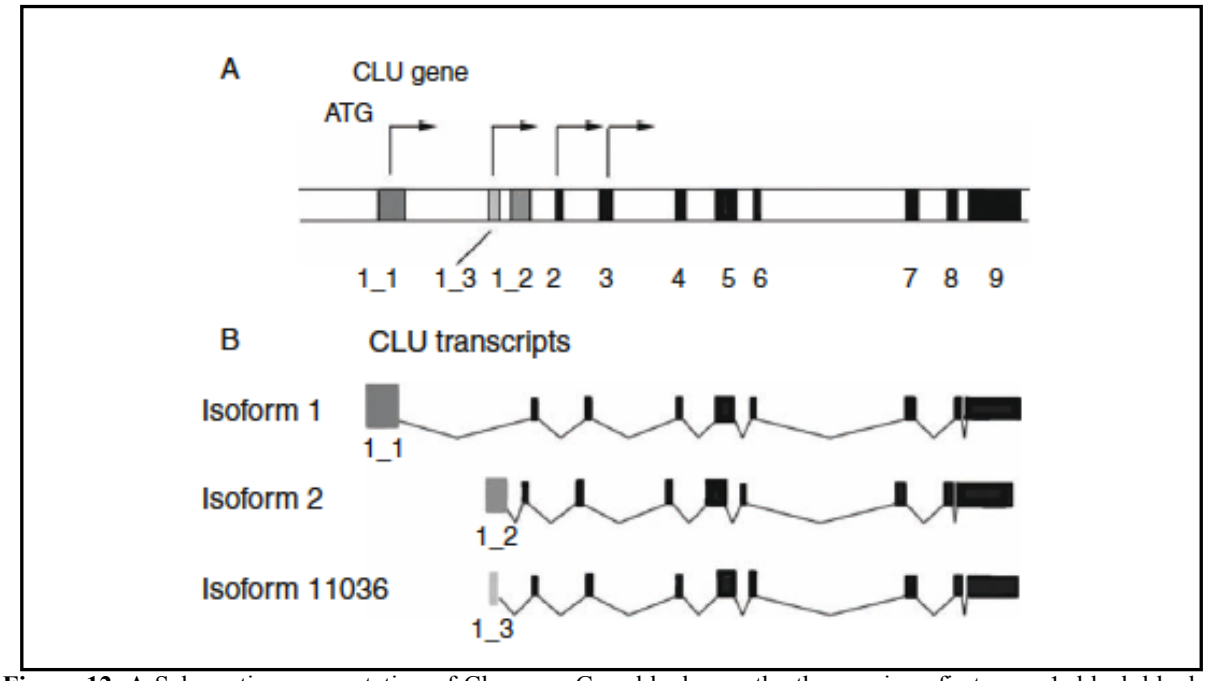

Figure 12. A Schematic representation of Clu gene. Gray blocks are the three unique first exon 1; black blocks represent exons from 2 to 9 and black arrows are the transcriptional start sites in-frame in CLU sequence. B Schematic representation of CLU mRNA variants. Adapted from[233]

Isoform 1 is predicted to produce a protein of 501aa with prevalent citoplasmic/nuclear localization.

Isoform 2 is characterized to have an untranslated exon 1, so the first available ATG is in exon 2, upstream the ER localization leader sequence. This mRNA is predicted to produce a 449aa protein that can be secreted. The 276bp long 5'UTR contains an exstensive secondary structure and an ORF of 57bp coding for a putative regulatory peptide.

In the Isoform11036 the first exon is located between exon 1 of Isoform1 and 2. The first functional in-frame ATG is in the first exon and probably this mRNA code for a 460aa protein with a nuclear localization [234].

All three transcripts hold an additional in-frame ATG in exon 3 and if translation starts from this ATG a shorter form of CLU protein with a supposed nuclear localization is produced [235].

Recent works show that signalling affecting CLU isoforms are different. For example Wnt signalling by TCF1 regulate expression of Isoform1 but not that of Isoform2 [236]; moreover in prostate cancer cell line androgens upregulated transcription of Isoform2 and downregulates that of Isoform1[237] . The explanation of these phenomena could be the existence of different promoters. This was verified only by "in silico" analysis by MatInspector software that highlighted two putative promoters P1 and P2 respectively upstream to Isoform1 and 2 TSS[238]. Figure 13.

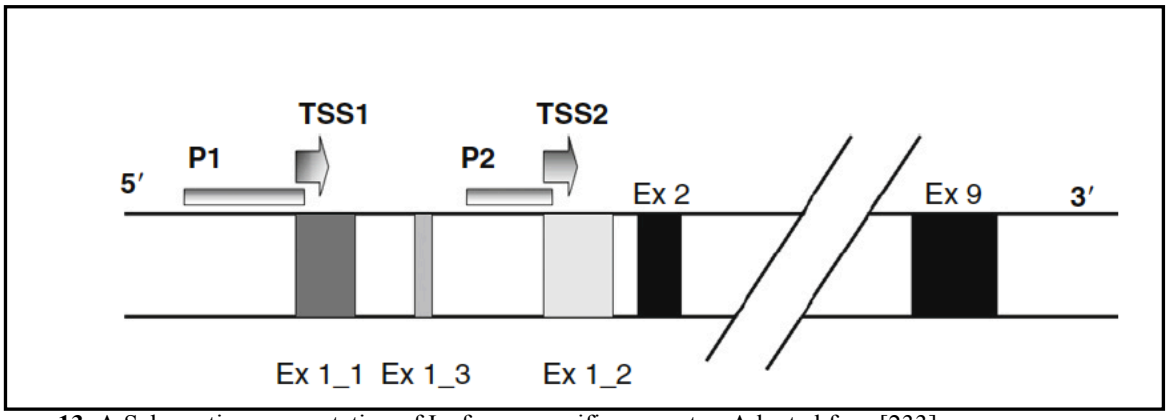

Figure 13. A Schematic representation of Isoforms specific promoter. Adapted from[233]

The P1 promoter is the most studied and it characterized by a conventional TATA box, some potential AP-1 and AP-2 elements[239] and a domain of 14bp conserved among all analysed species named Clusterin Element (CLE). CLE is very similar to Heat-shock Response element, it differs only for a base, and this change is not believed to affect binding of heat-shock factor[240]. A recent study shows that MYCN could indirectly downregulate CLU expression through regulation of miR-17-92 expression[241].

In this PhD thesis we provide the formal demonstration that MYCN could directly regulate Isoform1 expression via direct binding to proximal promoter P1.

Results

Transcriptional Activation

Transcriptional Activation Of ABC genes

Expression profile of c-Myc and 17 ABC transporter genes in 34 biopsy of patients with OS.

In collaboration with Dr. Serra of Istituto Ortopedici Rizzoli (IOR), biopsies of 34 patients and a pool of sane osteoblasts (OBL) were analyzed by Applied Biosystem Custom Micro Fluid Card for c-Myc and 17 ACB transporter genes expression's. In function of c-Myc and ABCs expression's and for each analysed gene, population of 34 patients was divided in two sub-populations High expressing (H) and Low expressing (L) choosing as cut-off value the medians of expression values of each analysed gene in OBL. The analysed ABC are *ABCA2, ABCA6, ABCA7, ABCB1 (MDR1), ABCB9, ABCB10, ABCC1 (MRP1), ABCC2 (MRP2), ABCC4 (MRP4), ABCC5 (MRP5), ABCC11 (MRP8), ABCC12, ABCE1, ABCF1, ABCF2, ABCF3,* and *ABCG2*. Among these genes we founded several which expression correlate to c-Myc expression (Tab. 1).

Correlation		Gene (Expression vs. OBL)	P value (Fisher's exact test)
H/H	L/L	ABCC1	0.04
H/H	L/L	ABCC4	0.05
H/H	L/L	ABCC11	0.005
H/H	L/L	ABCC2	0.003
H/H	L/L	ABCC5	0.04
H/H	L/L	ABCE1	0.005
H/H	L/L	ABCF1	0.0004
H/H	L/L	ABCF2	0.04
H/H	L/L	ABCF3	0.04

Table 1. ABC genes which expression directly correlate to c-Myc one as identified by Affymetrix Custom Gene Card analysis, in 34 biopsies of OS patients.

C-Myc regulates expression of ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 in Osteosarcoma cell line.

To check the hypothesis if c-Myc could directly regulates the transcription of these ABCs, we first performed an *in silico* analysis of gene promoters and we identified several canonical and non canonical E-Boxes in proximity of transcriptional start sites of ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 (Fig.1).

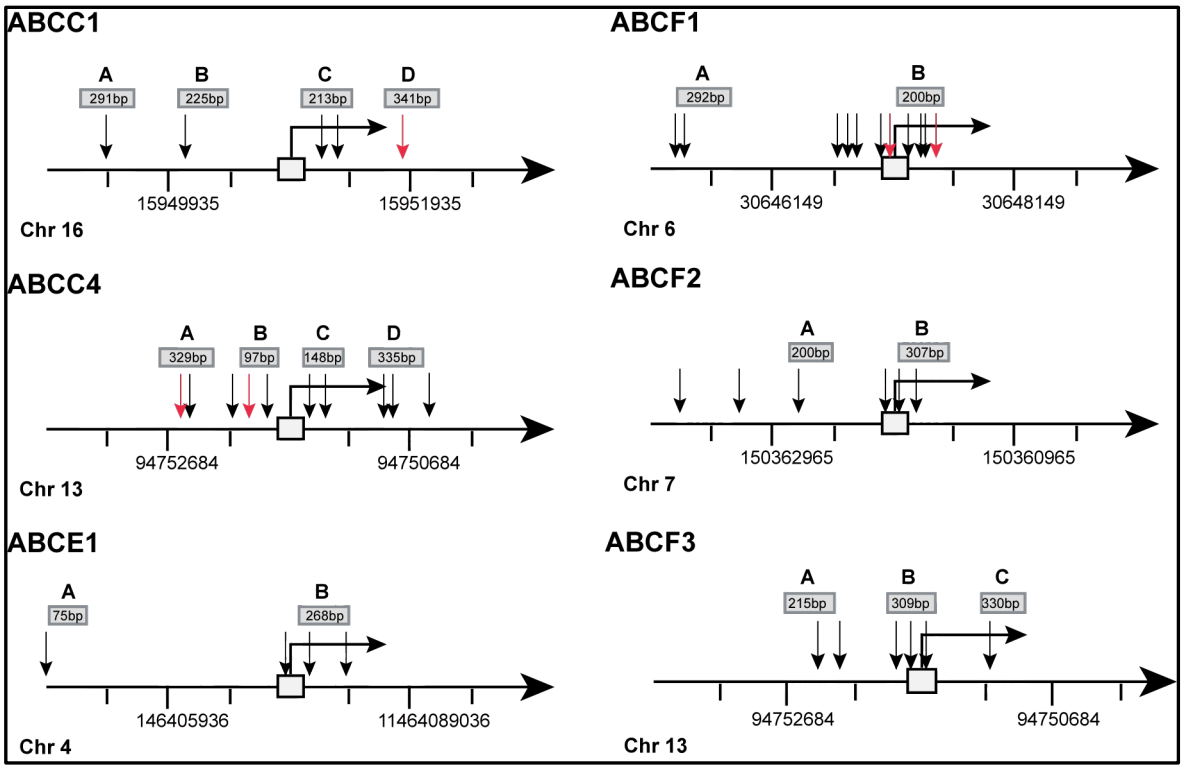

Figure 1. A schematic representation of ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 promoters containing canonical (red arrow) and non-canonical (black arrow) E-boxes. Position and length of ChIP are also showed (letters).

Then we transfected U2OS and SAOS cell line with c-Myc siRNA and we evaluated alteration of the six ABC transporters' expression by Real Time PCR. As shown in fig. 2 reduction of c-Myc expression is correlated to a significant reduction of analysed ABC transporter expression.

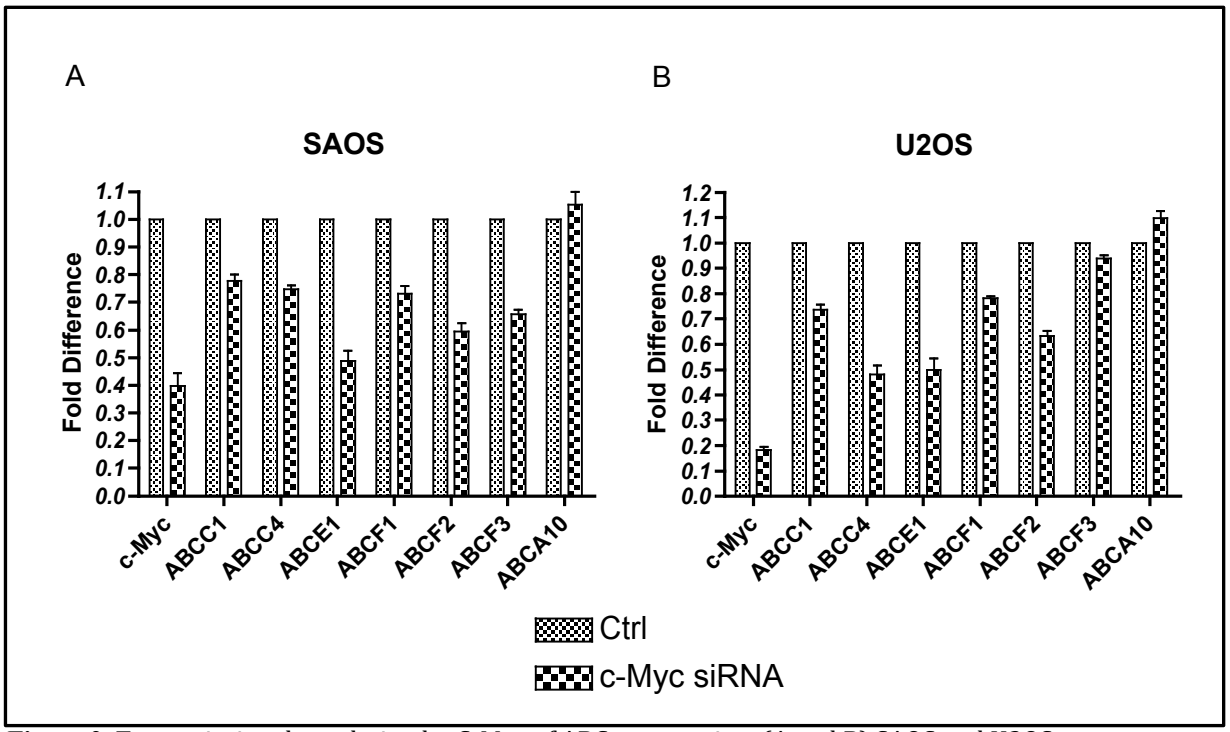

Figure 2. Transcriptional regulation by C-Myc of ABCs expression. (A and B) SAOS and U2OS osteosarcoma cell lines were transfected with scrambled control siRNA, C-Myc siRNA for 24 and 48 hours respectively, followed by RNA extraction and real-time RT-PCR analysis of N-Myc and ABCs expression. N-Myc and ABCs gene expression in control siRNA-transfected cells was artificially set as 1.0.

By Chromatin Immunoprecipitation we confirm that c-Myc directly binds to E-Boxes in gene promoters, the results highlight a strong enrichment of ABC promoters' in c-Myc and Max immunoprecipitation Fig 3.

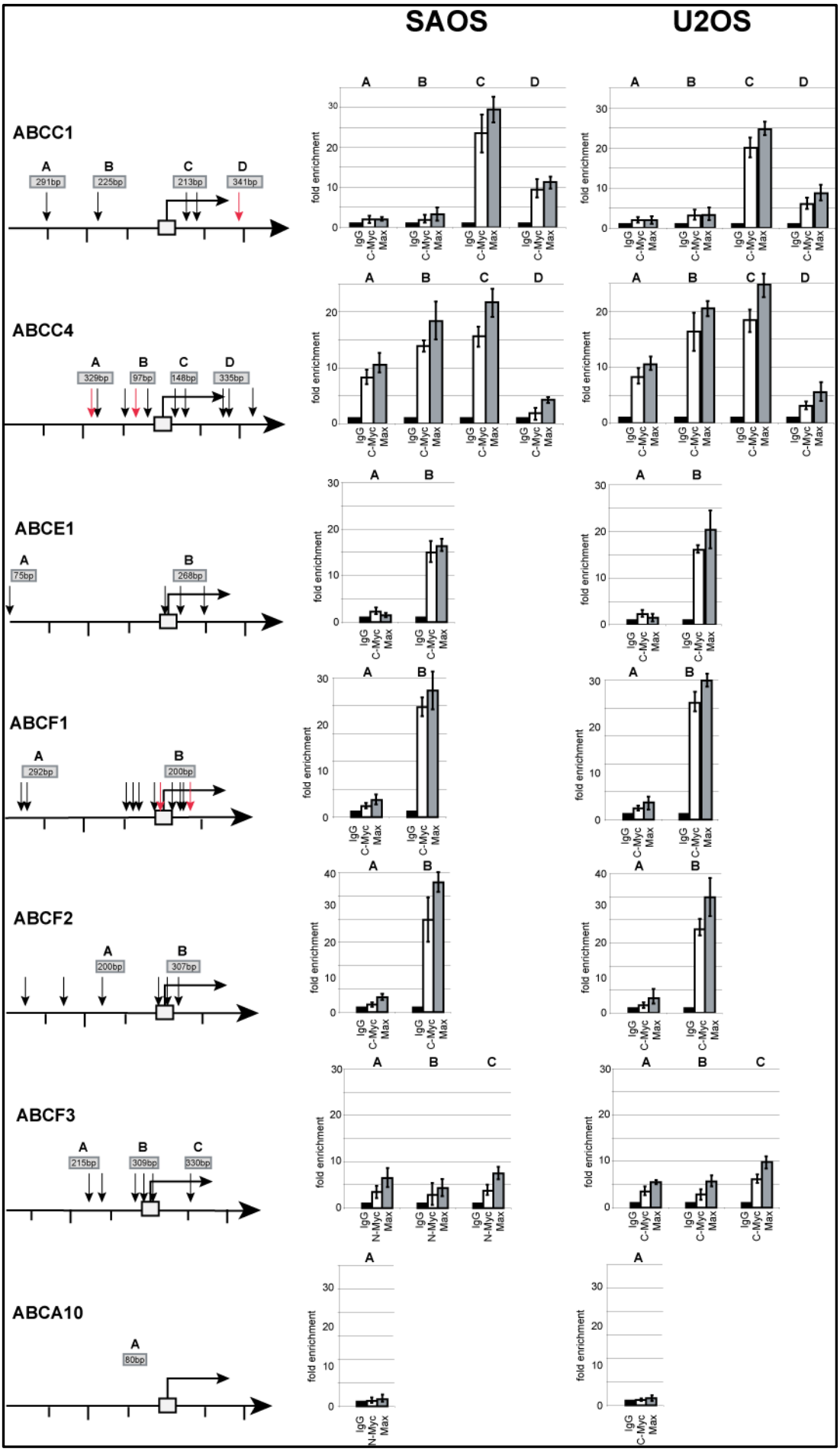
SAOS
U2OS
ABCC1
A 291bp
B 225bp
C 213bp
D 341bp
ABCC4
A 329bp
B 97bp
C 148bp
D 335bp
ABCE1
A 75bp
B 268bp
ABCF1
A 292bp
B 200bp
ABCF2
A 200bp
B 307bp
ABCF3
A 215bp
B 309bp
C 330bp
ABCA10
A 80bp
fold enrichment
IgG
C-Myc
N-Myc
Max

Figure 3. ChIP and quantitative PCR were applied in SAOS and U2OS cell lines. Fold enrichment of ABCs promoter regions immunoprecipitated by C-Myc and Max antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR was performed in triplicate. Results were the average of three independent experiments.

Further we cloned the promoters downstream Luciferase gene in *pGL3-Basic* Vector, then we transiently co-transfected U2OS cell line with Luciferase reporter vector and with or without c-Myc siRNA, as shown in fig. 4 there is a strong reduction in Luciferase activity in presence of c-Myc siRNA.

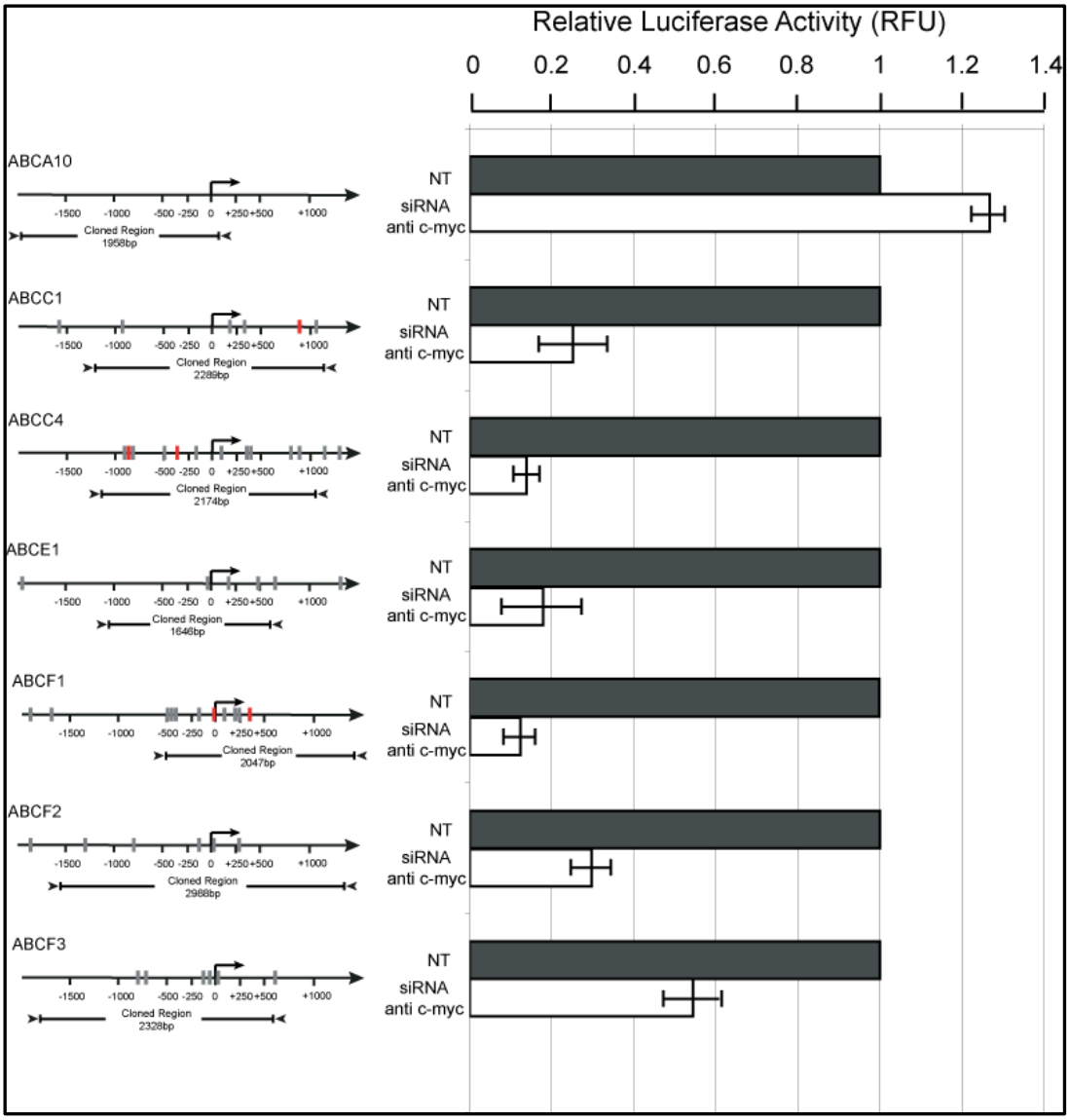

Figure 4. Luciferase activity of the reporter constructs of ABC promoters transfected in U2OS cells was determined in the presence (NT) and absence (siRNA anti c-Myc) of c-Myc expression and normalized to that of Renilla.

C-Myc, ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 expressions are up-regulated in MTX, DX and CDDP resistant Osteosarcoma cell lines

In a recent paper Scionti et al [242] has shown an increase of c-Myc expression in SAOS and U2OS cell line resistant to high dose methotrexate (300ng/ml). New data show that also in DX and CDDP resistant cell lines c-Myc is overexpressed [158]. From these evidences we analysed the expression of the six ABC genes and c-Myc in high dose of MTX, DX and CCDP resistant U2OS and SAOS cell lines. Comparing the expression profile of the analysed genes in U2OS *vs.* relative resistant cell lines, c-Myc and ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 are overexpressed (Fig 5A). Comparison of SAOS *vs.* relative resistant cell lines shows a picture more difficult to interpret, indeed the increase of c-Myc and the

analysed ABC genes is not so clear as in U2OS cell lines and in some cases we observe a sensible reduction in ABC expressions (Fig. 5B).

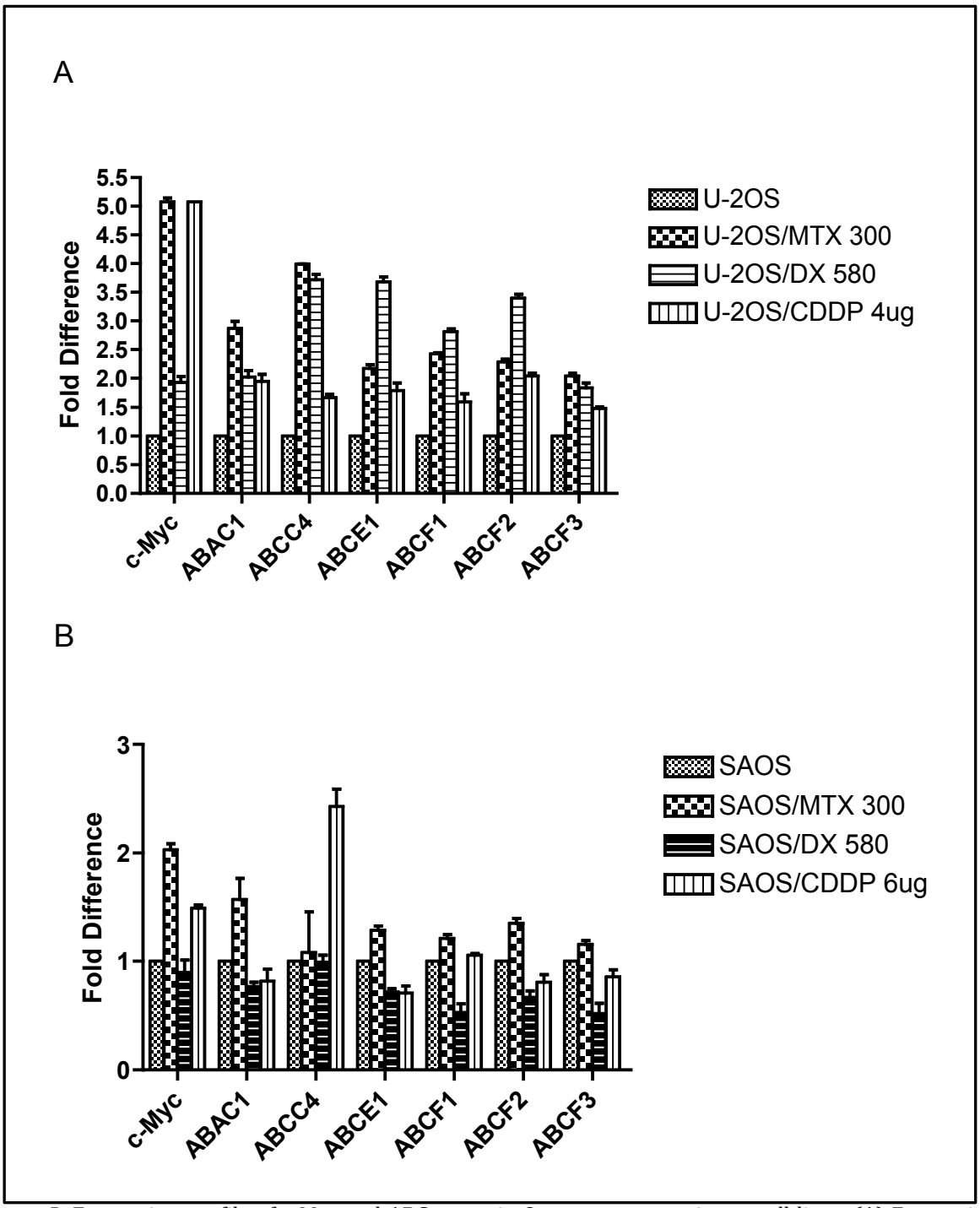

Figure 5. Expression profile of c-Myc and ABC genes in Osteosarcoma resistant cell lines. (A) Expression profile of c-Myc, ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 in U2OS cell line was compared to high dose MTX, DX, CDDP resistant U2OS cell lines. After total RNA extraction, mRNA population was retro transcribed and analysed by Real-Time PCR. (B) Comparison of expression profile among SAOS and SAOS high dose MTX, DX and CDDP resistant cell lines. C-Myc and ABCs gene expression in non-resistant U2OS and SAOS were artificially set as 1.0.

To clarify the gene expression data obtained from SAOS cell lines, we compared expression profile of SAOS and U2OS and we observed that c-Myc and ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 are dramatically overexpressed in SAOS and this could be the cause of a reduced variation of analysed gene expression in SAOS vs. SAOS resistant cell lines (Fig. 6)

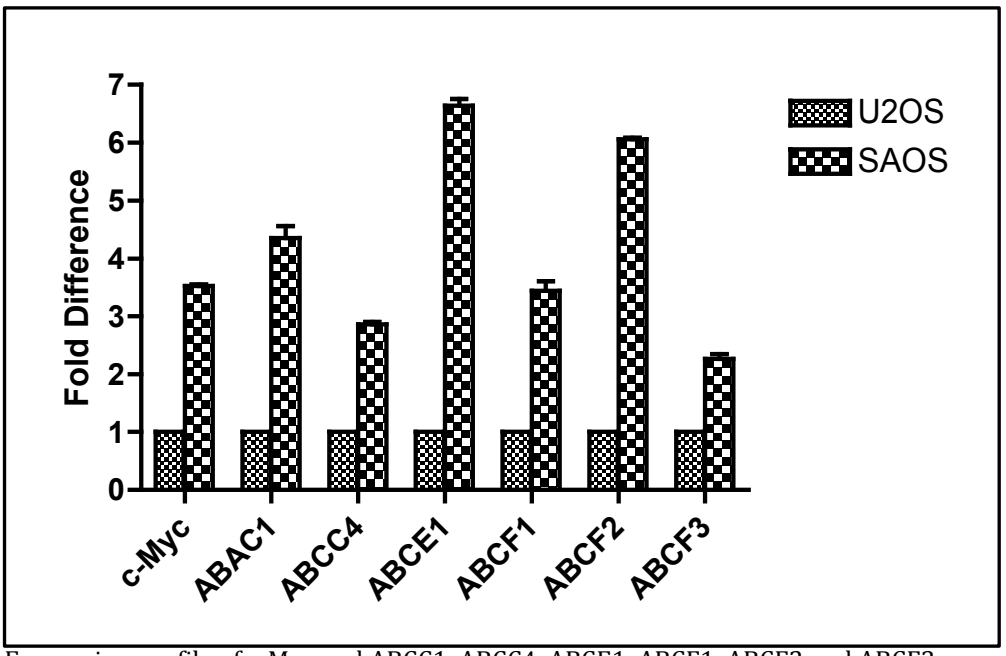

Figure 6. Expression profile of c-Myc and ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 genes in U2OS vs. SAOS cell lines. After total RNA extraction, mRNA population was retro transcribed and analysed by Real-Time PCR. C-Myc and ABCs gene expression in U2OS were artificially set as 1.0.

High expression of c-Myc, ABCC1, ABCC4 and ABCF1 Osteosarcoma cells correlate to more unfavourable outcome.

In collaboration with Dr. Serra of IOR, high and low expression levels of c-Myc, ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 were chosen as criteria to evaluate free event survival at six years of 34 OS patients. For each gene, the High and Low expressing sub-population was analysed by Kaplan-Meier curve. These analyses show that high expression of c-Myc, ABCC1, ABCC4 and ABCF1 correlate to a more unfavourable outcome and could be used as a prognostic factor to predict conventional therapy failure (Fig.7)

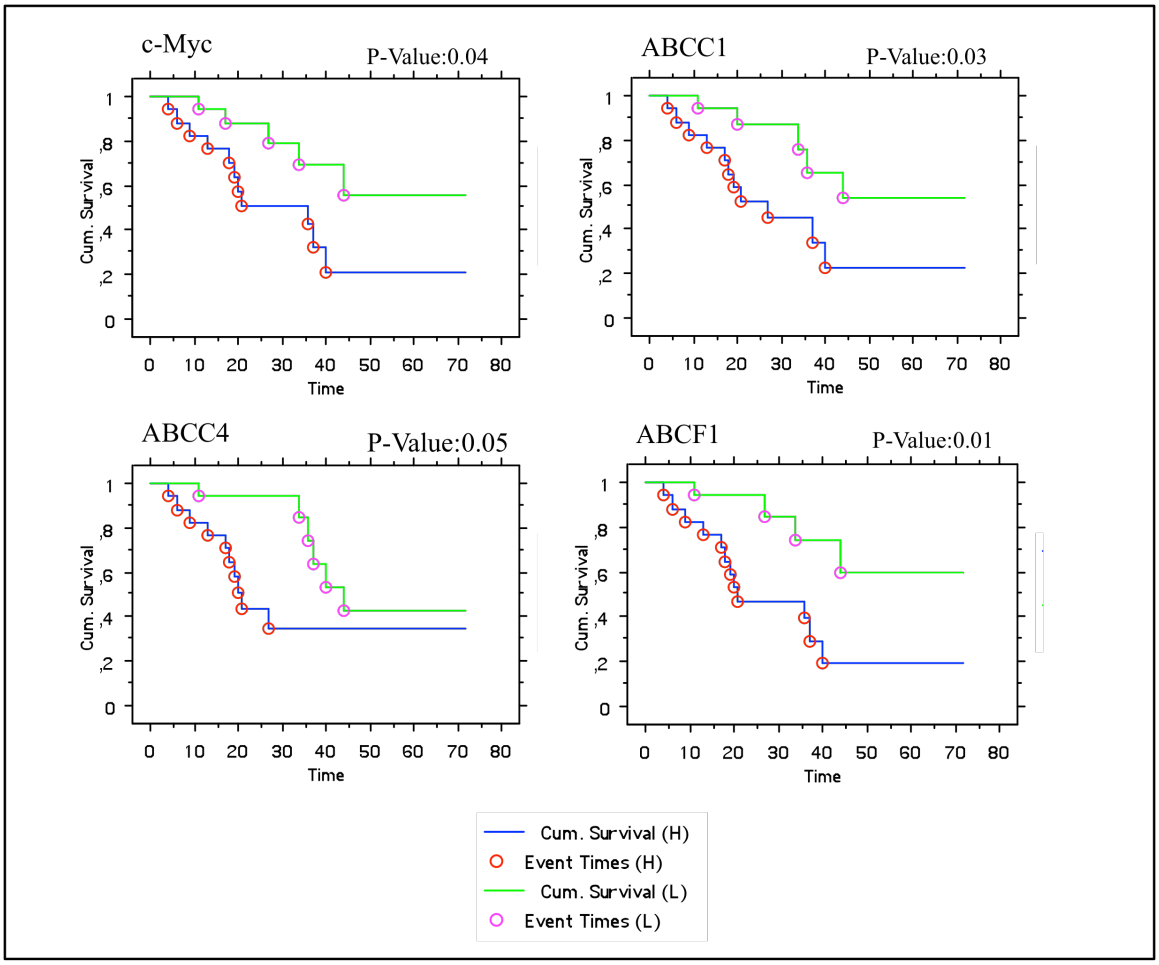

Figure 7. Prognostic impact of c-Myc, ABCC1, ABCC4 and ABCF1 genes that resulted significantly associated with EFS at six years in 34 OS patients, according to Kaplan-Meier curve.

Transcriptional Repression

Transcriptional Repression Of CCNG2

Cyclin G2 is transcriptionally repressed by HDAC2 and MYCN and reactivated by HDAC inhibitors

A recent work of Liu et al[143] showed that MYCN could repress transcription of TG2 by interacting with Sp1 and recruiting HDAC1. In collaboration with group of Dr. Glen Marshall (Children's Cancer Institute Australia, Sidney), we decided to check the hypothesis that MYCN could also interact with HDAC2 to repress gene expression keeping in mind that HDAC1 and HDAC2 have an high grade of homology.

Both MYCN and HDAC2 are well known to exert biological effects through transcriptional regulation. We performed an Affymetrix microarray analysis comparing mRNA expression in BE(2)-C cells transfected with scrambled control siRNA, MYCN siRNA-1 or HDAC2 siRNA-1. In total, 18 genes were commonly upregulated, and 5 genes commonly downregulated, by more than 2 fold by both N-Myc siRNA-1 and HDAC2 siRNA-1 (Table 2). Cyclin G2 (CCNG2) stood out in this microarray analysis as the gene

most dramatically up-regulated by HDAC2 siRNA-1 (9.7 fold), and one of the genes most dramatically activated by MYCN siRNA-1 (4.8 fold).

Gene description	GeneBank accession	N-Myc siRNA	HDAC2 siRNA
ZNF36	AA653300	2.564103	2.864103
HIST1H2BD	BC002842	4.967033	2.747253
SLC35D2	AJ005866	4.638889	2.777778
TP53INP1	AW341649	5.357377	3.278689
MYRL2	BF739795	2.066116	4.859504
KIAA0699	AI934125	2.145923	3.935622
KIAA0265	AF277175	2.320186	3.067285
MIAT	R54042	2.331	4.694639
EST	AA195485	2.380952	3.195
MGC2663	NM_024106	2.487562	3.303
VDUP1	NM_006472	2.638522	3.372
ARHGAP21	AB037845	3.175	2.777778
G3BP2	AB014560	3.011	2.816901
PNRC1	AF279899	3.164557	3.655
ZNF664	BE965646	3.912	3.846154
RBBP2H1A	AF087481	4.291845	8.622
SCG5	NM_003020	4	3.125
CCNG2	**NM_004354**	**4.854369**	**9.700**
EST	AA524005	0.426	0.240
AGPAT5	AU145356	0.411	0.207
UHMK1	AI249980	0.382	0.179
TCF6L1	BE552470	0.347584	0.259645
GNPNAT1	BE789346	0.34638	0.237617

Table 2. Genes commonly regulated by MYCN-siRNA and HDAC2-siRNA as identified by Affymetrix microarray analysis, in Neuroblastoma BE(2)C cells.

To validate the microarray data, we performed real-time RT-PCR analysis of CCNG2 gene expression in BE(2)-C after siRNA transfection. As shown in Figure 8, CCNG2 gene expression is upregulated by MYCN siRNA, HDAC2 siRNA in BE(2)-C.

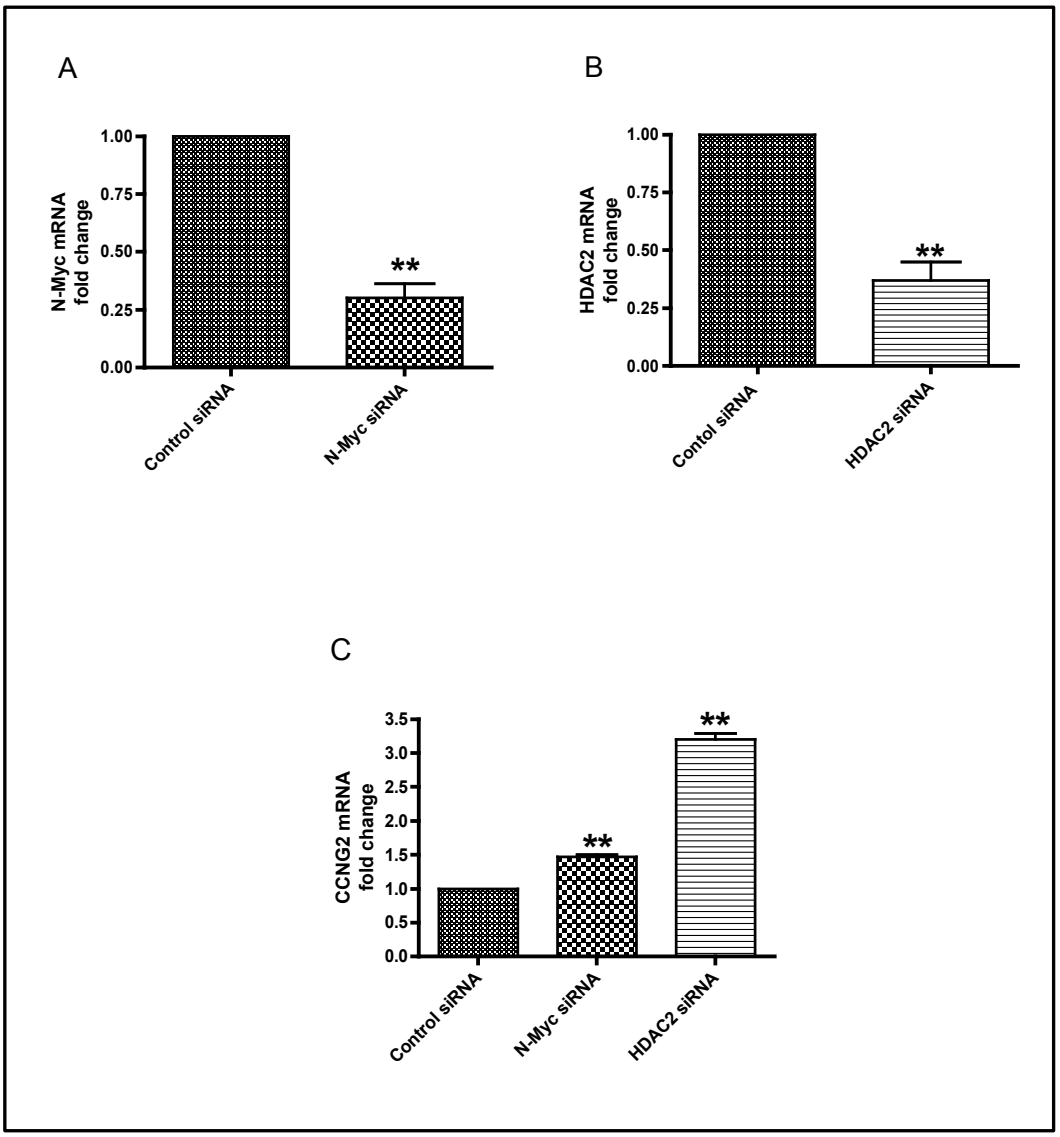

Figure 8. Transcriptional regulation by N-Myc and HDAC2 of CCNG2 expression. (A and B) BE(2)-C neuroblastoma cells was transfected with scrambled control siRNA, N-Myc siRNA or HDAC2 siRNA for 48 hours, followed by RNA extraction and real-time RT-PCR analysis of N-Myc and HDAC2 expression respectively. N-Myc and HDAC2 gene expression in control siRNA-transfected cells was artificially set as 1.0. (C) BE(2)-C neuroblastoma cells was transfected with scrambled control siRNA, N-Myc siRNA or HDAC2 siRNA for 48 hours, followed by RNA extraction and real-time RT-PCR analysis of CCNG2 expression . CCNG2 gene expression in control siRNA-transfected cells was artificially set as 1.0. Symbol ** ($p<0.01$) indicated statistically significant differences. Error bars indicated standard error.

Because pan-HDAC inhibitors repress the deacetylase activity of HDAC2, we examined whether the pan-HDAC inhibitor, trichostatin A (TSA), could reactivate CCNG2 gene expression. As shown in Figure 9, real-time RT-PCR analysis showed that TSA and/or tetracycline reactivated CCNG2 gene expression in TET21/N cell line, a human neuroblastoma cell line carrying a *MYCN* transgene under the control of a TET-OFF promoter. These results suggest that both MYCN and HDAC2 repress CCNG2 gene

expression, and that HDAC inhibitors can be applied to reverse this effect.

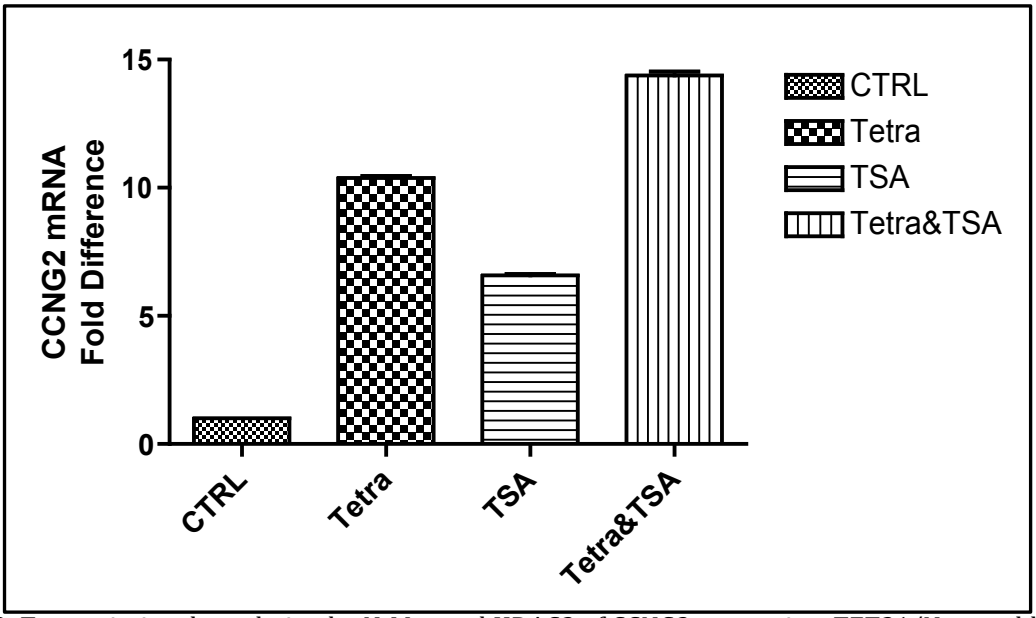

Figure 9. Transcriptional regulation by N-Myc and HDAC2 of CCNG2 expression. TET21/N neuroblastoma cells was treated with tetracycline and/or TSA for 24 hours, followed by RNA extraction and real-time RT-PCR analysis of CCNG2 expression. CCNG2 gene expression in control was artificially set as 1.0.

N-Myc represses CCNG2 gene transcription by directly binding to HDAC2 at the Sp1 consensus site of CCNG2 gene core promoter

Both HDAC2 [243] and N-Myc [143] are known to repress gene transcription by binding to Sp1-binding sites at target gene promoter. As both N-Myc siRNA and HDAC2 siRNA activated CCNG2 gene expression, we tested the hypothesis that N-Myc might repress CCNG2 gene transcription by recruiting HDAC2 to the CCNG2 gene promoter. Bio-informatics analysis of the CCNG2 gene promoter (-3000/+3000 from transcription start site) identified two regions enriched for Sp1 binding sites: one proximal to the transcription start site and a second one located about 1 kb downstream from the transcription start site (Figure 10).

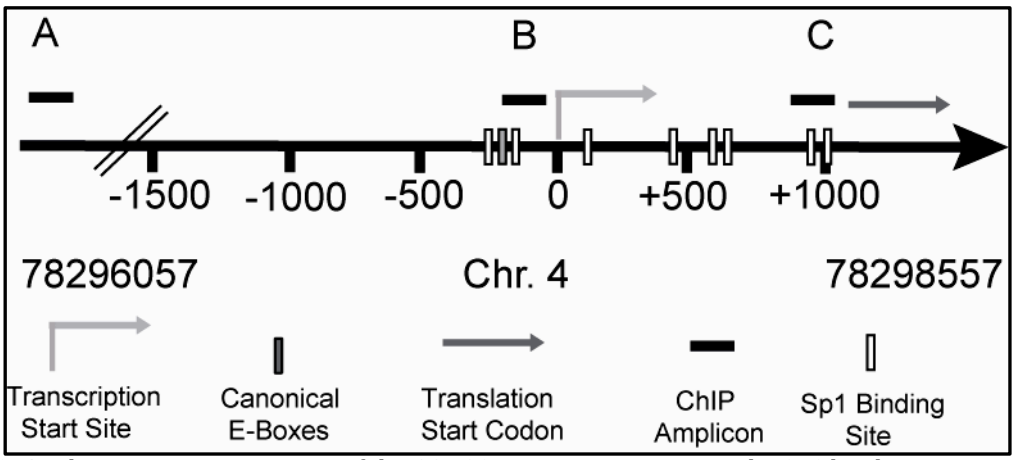

Figure 10. A schematic representation of the CCNG2 promoter containing the Sp1 binding sites

The CCNG2 promoter region was scanned for the presence of N-Myc, Sp1 and HDAC2 binding using the dual cross-linking ChIP assay. A pre-immune serum was used as a negative control to determine the baseline of the non-specific background. As shown in Figure 11, antibodies against N-Myc, HDAC2 and Sp1 all efficiently immunoprecipitated the two regions of the CCNG2 promoter carrying Sp1 binding sites.

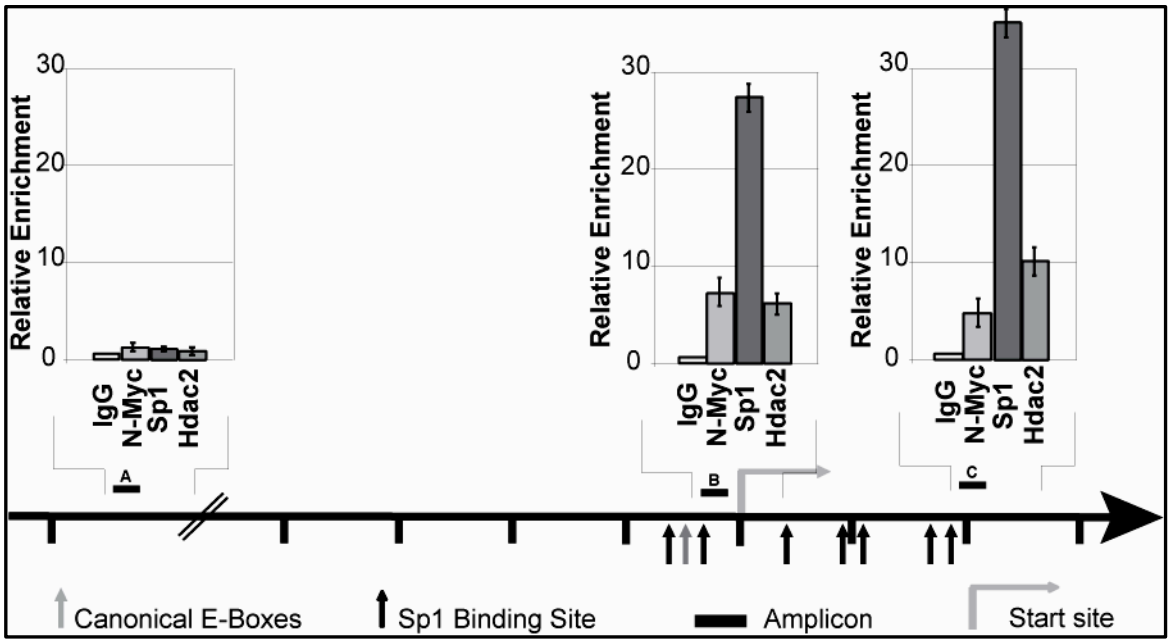

Figure 11. Dual cross-linking ChIP and quantitative PCR were applied in BE(2)-C cells. Fold enrichment of CCNG2 promoter regions immunoprecipitated by anti-Sp1, N-Myc, HDAC2 antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR targeting Amplicon A and Sp1 binding sites (Amplicon B and C) were performed in triplicate. Results were the average of three independent experiments.

To confirm that transcriptional suppression of CCNG2 was mediated by N-Myc, we performed experiments using Luciferase reporter constructs carrying different portions of the CCNG2 promoter: F3(-416/+1188), F2 (+146/+1188), F1(+700/+1188), F2Δ (+146/+825) and F1Δ (+700/+825). Reporters were transiently transfected into the TET21/N cells. Luciferase activity was monitored as a function of N-Myc expression. Results showed that deletion of the Sp1 region downstream of the transcription start site abolished N-Myc-mediated repression of the promoter, suggesting that this region was the target of N-Myc and HDAC2 (Figure 12).

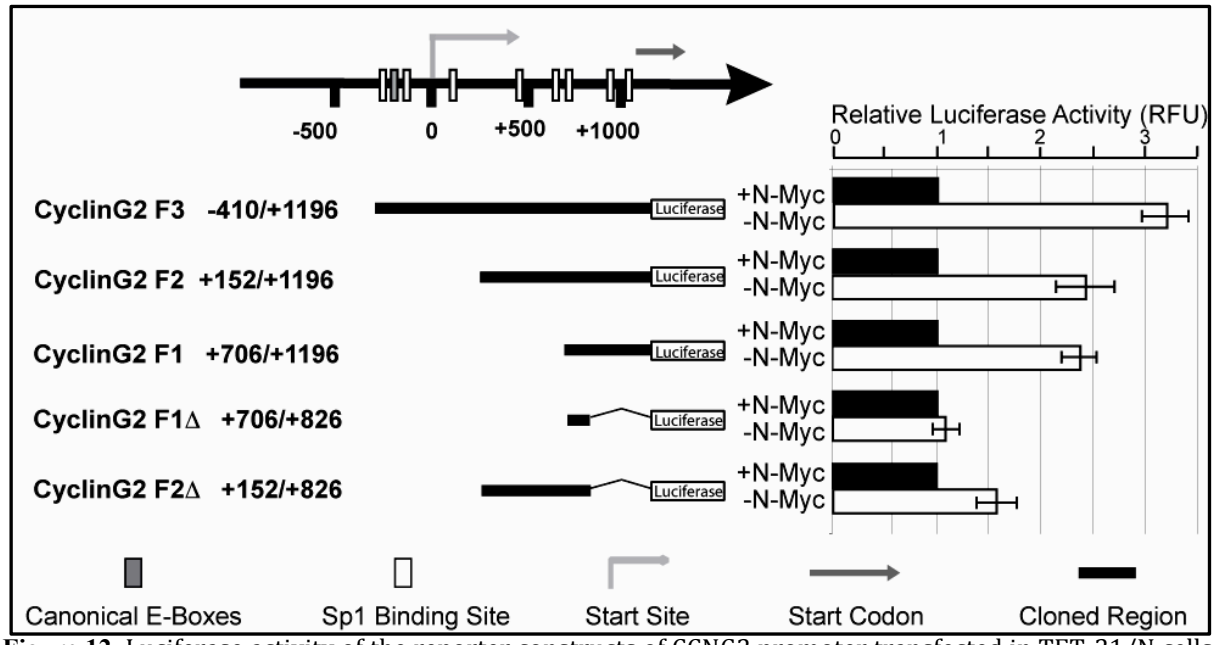

Figure 12. Luciferase activity of the reporter constructs of CCNG2 promoter transfected in TET-21/N cells was determined in the presence (-tetracycline) and absence (+tetracycline) of N-Myc expression and normalized to that of Renilla.

To determine whether N-Myc, HDAC2 and Sp1 are part of the same repressive protein complex, we performed co-immunoprecipitation (IP) assays using BE(2)-C cell nuclear extracts (Figure 13). The extracts were incubated with a specific anti-HDAC2 antibody or with pre-immune IgG as a negative control. The IP-complexes were subsequently analyzed by immunoblot, using antibodies against Sp1, HDAC2 and N-Myc, respectively. Results showed that HDAC2 could co-immunoprecipitate both Sp1 and N-Myc. A reverse experiment in which an anti N-Myc antibody was employed as the immunoprecipitating agent, was also performed. Results showed that N-Myc could efficiently coimmunoprecipitate both HDAC2 and Sp1. Taken together these findings suggest that

N-Myc forms a protein complex along with HDAC2 and Sp1, and that the protein complex represses CCGN2 gene transcription by binding the Sp1 containing region downstream of the transcription start site.

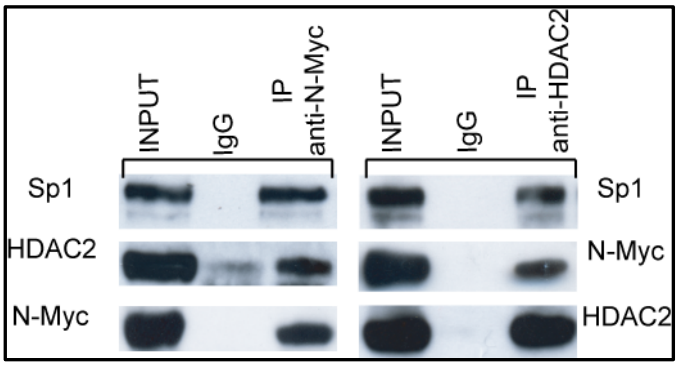

Figure 13. Protein coimmunoprecipitation (IP) of N-Myc (*left*) and HDAC2 (*right*) in BE(2)-C nuclear protein extracts. Products obtained by immunoprecipitating with either anti-N-Myc or anti-HDAC2 antibody were probed with anti-Sp1, anti-HDAC2 and anti-N-Myc antibodies in immunoblot.

Transcriptional repression of CCNG2 contributes to N-Myc and HDAC2-induced cell proliferation.

Unlike most other cyclins, CCNG2 blocks cell cycle progression and induces cell growth arrest [244] [245]. We therefore examined whether transcriptional activation of CCNG2 contributed to cell growth arrest induced by N-Myc siRNA and HDAC2 siRNA. As shown in Figure 14, while repression of CCNG2 gene expression alone did not have an effect on cell proliferation, CCNG2 siRNA partly blocked growth arrest due to N-Myc siRNA and to HDAC2 siRNA in BE(2)-C. These data indicate that transcriptional repression of CCNG2 contributes to N-Myc- and HDAC2-induced cell proliferation in neuroblastoma cells.

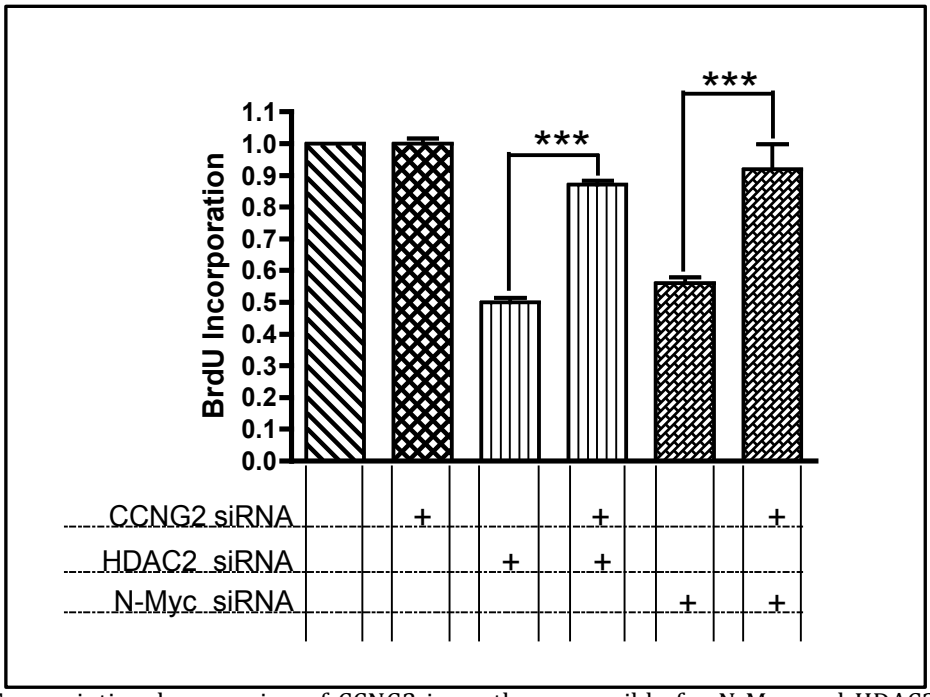

Figure 14. Transcriptional repression of CCNG2 is partly responsible for N-Myc and HDAC2-modulated cell proliferation. BE(2)-C cells was transfected with scrambled control siRNA, N-Myc siRNA, HDAC2 siRNA, CCNG2 siRNA, combination of CCNG2 siRNA and N-Myc siRNA, or combination of CCNG2 siRNA and HDAC2 siRNA. Seventy-two hours after transfection, cell proliferation was examined by BrdU incorporation ELISA and expressed as fold changes. Symbols *** ($p<0.001$) indicated statistically significant differences. Error bars indicated standard error.

Transcriptional Repression Of CLU

MYCN mediates negative regulation of CLU expression in Neuroblastoma cell line.

In a recent work Chayka and colleagues [241] have shown that MYCN induced miR-17-92 microRNA cluster represses Clusterin mRNA translation. In collaboration with Dr. Sala of UCL Institute of Child Health (London, GB) we discovered two non-canonical E-Boxes named BS1 and BS2 by informatical scanning of Clusterin Isoform 1 promoter (Fig 15).

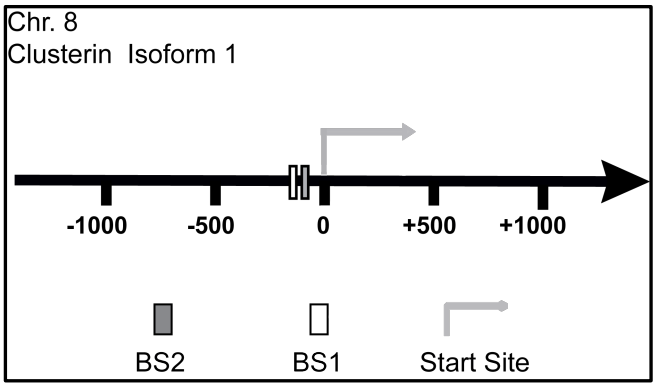

Figure 15. A schematic representation of Clusterin isoform1 promoter containing MYCN binding sites

To check the hypothesis that MYCN could also directly regulate transcription of CLU gene, the proximal promoter of CLU Isoform 1 was cloned upstream Luciferase gene in pGL3-Basic reporter vector. By gel-shift assays, conducted in Sala's Laboratory, BS2 was discovered to be bound by MYCN, so the CLU Isoform1 proximal promoter, carrying a point mutation in BS2 that ablate MYCN binding, was cloned upstream Luciferase gene in pGL3-Basic reporter vector. The two constructs were transfected in SHSY5Y neuroblastoma cell line with or without expression vector of MYCN. Luciferase assay, performed in Sala's Lab, shows a strong reduction of wild type CLU promoter activity in presence of high level of MYCN, this repressive effect was lacking in BS2 mutated CLU promoter. (Fig 16).

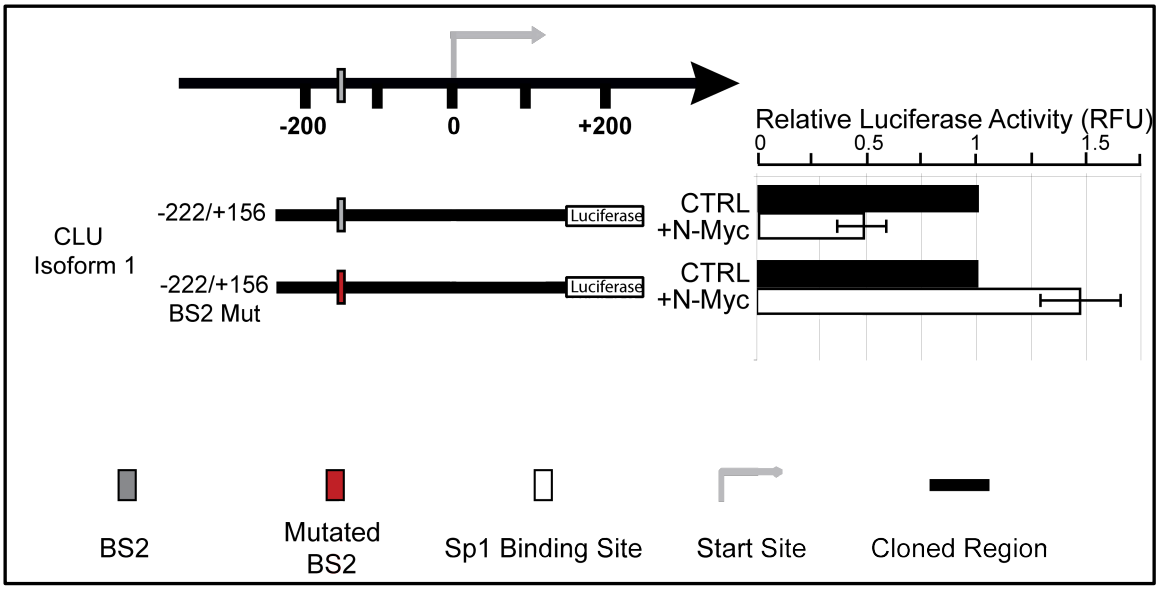

Figure 16. Reporter constructs of Clusterin isoform 1 promoter were co-transfected with or without MYCN expression vector in SHSY5Y neuroblastoma cell line. Luciferase activity was determined after 24h and normalized to that of Renilla.

MYCN directly binds to CLU promoter in TET21/N neuroblastoma cell line.

To verify the binding of MYCN to CLU promoter we performed a ChIP in TET21/N cells using antibodies anti-n-Myc and anti-Max and we scanned by Q-PCR a region of 5Kb surrounding BS2 in CLU promoter. The MYCN and Max signals were enriched in proximity of BS2 (Fig 17).

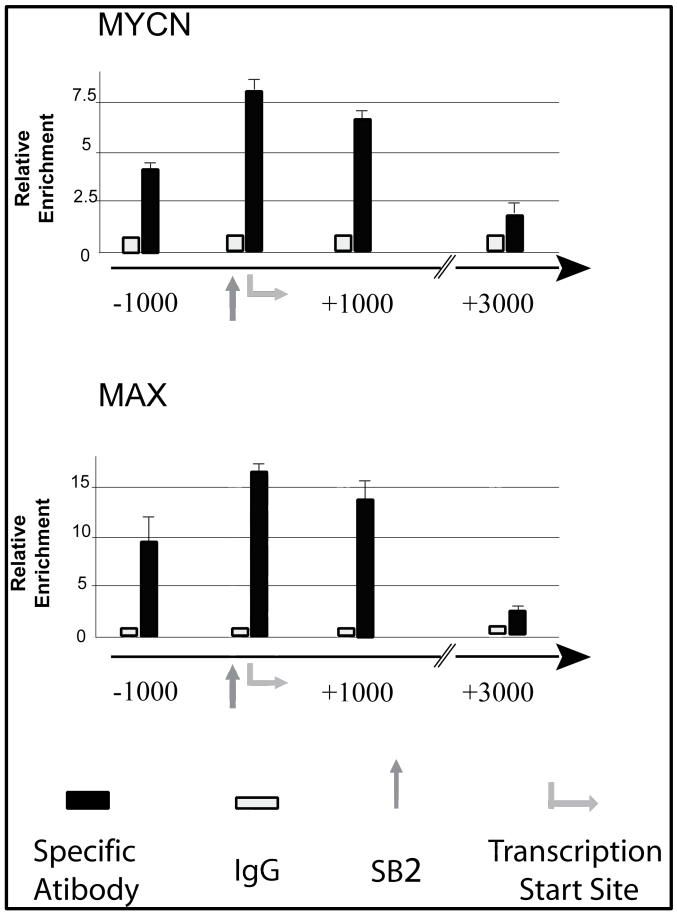

Figure 17. ChIP and quantitative PCR were applied in TET21/N neuroblastoma cell. Fold enrichment of Clusterin promoter regions immunoprecipitated by C-Myc and Max antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR was performed in triplicate. Results were the average of three independent experiments.

Furthermore by Dual-step Chromatin IP we assessed the presence of chromatin remodeling factor associated to transcriptional repression such as HDAC1, HDAC2, BMI1 (component of Polycomb Repressive Complex 1, PRC1) and SUZ12 (component of

Polycomb Repressive Complex 2, PRC2). As shown in fig 18, the same regions enriched in MYCN and Max are enriched for HDAC1 and 2; notably regions, immediately surrounding MYCN/Max and HDACs complexes, are enriched in SUZ12 immunoprecipitate meaning that PRC1 and 2 are involved in CLU repression.

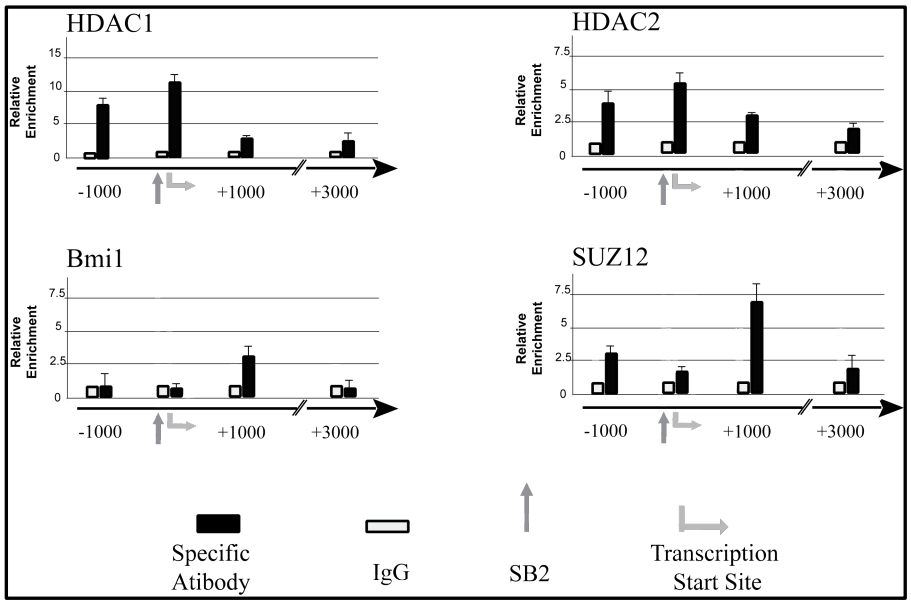

Figure 18. ChIP and quantitative PCR were applied in TET21/N neuroblastoma cell. Fold enrichment of Clusterin promoter regions immunoprecipitated by HDAC1, HDAC2, Bmi1 and SUZ12 antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR was performed in triplicate. Results were the average of three independent experiments.

To verify if these chromatin-remodeling factors should be recruited by MYCN, we performed a Dual-step ChIP in TET21/N treated with tetracycline to switch-off MYCN expression. As shown in figure 19, in absence of MYCN no chromatin-remodeling factor was found associated to CLU promoter, indicating that probably they are recruited by MYCN.

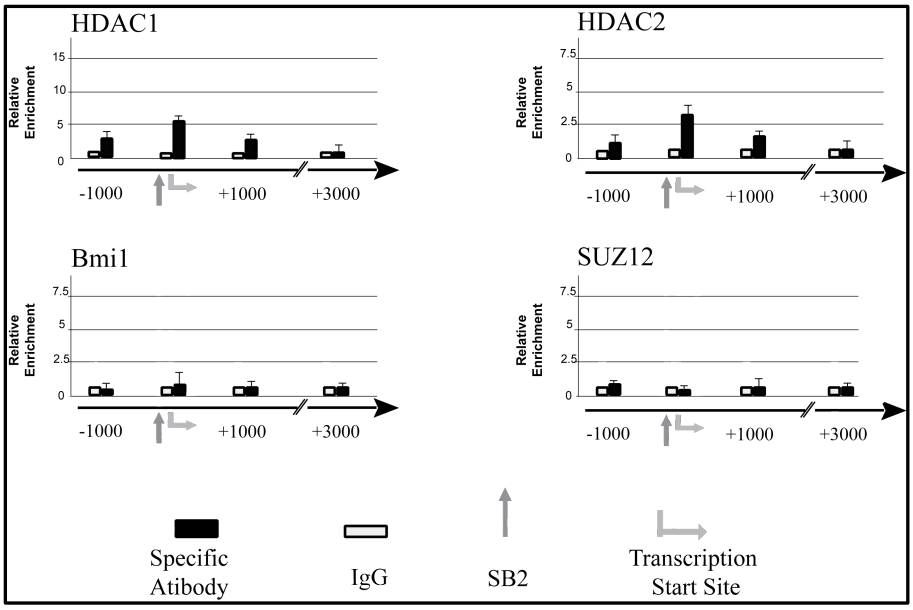

Figure 19. ChIP and quantitative PCR were applied in TET21/N neuroblastoma cell treated with tetracycline. Fold enrichment of Clusterin promoter regions immunoprecipitated by C-Myc, Max, HDAC1, HDAC2, Bmi1 and SUZ12 antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR was performed in triplicate. Results were the average of three independent experiments.

In addition we analyzed histone modification associated to CLU promoter in presence and absence of MYCN in TET21/N. We performed ChIP using antibodies the recognize histone modification involved in transcriptional activation such as acetyl-histone H3 (Ac-H3) and dimethylated lysine 4 on histone H3 (dimethyl-H3K4); and in transcriptional repression such as trimethylated lysine 9 and 27 on histone H3 (trimethyl- H3K9 and H3K27 respectively). In presence of MYCN, we observe good levels of active chromatin markers, as Ac-H3 and dimethyl-H3K4, and very low levels of repressed chromatin markers trimethyl-H3K9 and trimethyl-H3K27. This pattern of histone modification is typical of genes repressed in cancer. In absence of MYCN we found a strong enrichment of CLU promoter in Ac-H3 and dimethyl-H3K4 immunoprecipitate and no enrichment for trimethyl-H3K9 and trimethyl-H3K27. Fig 20

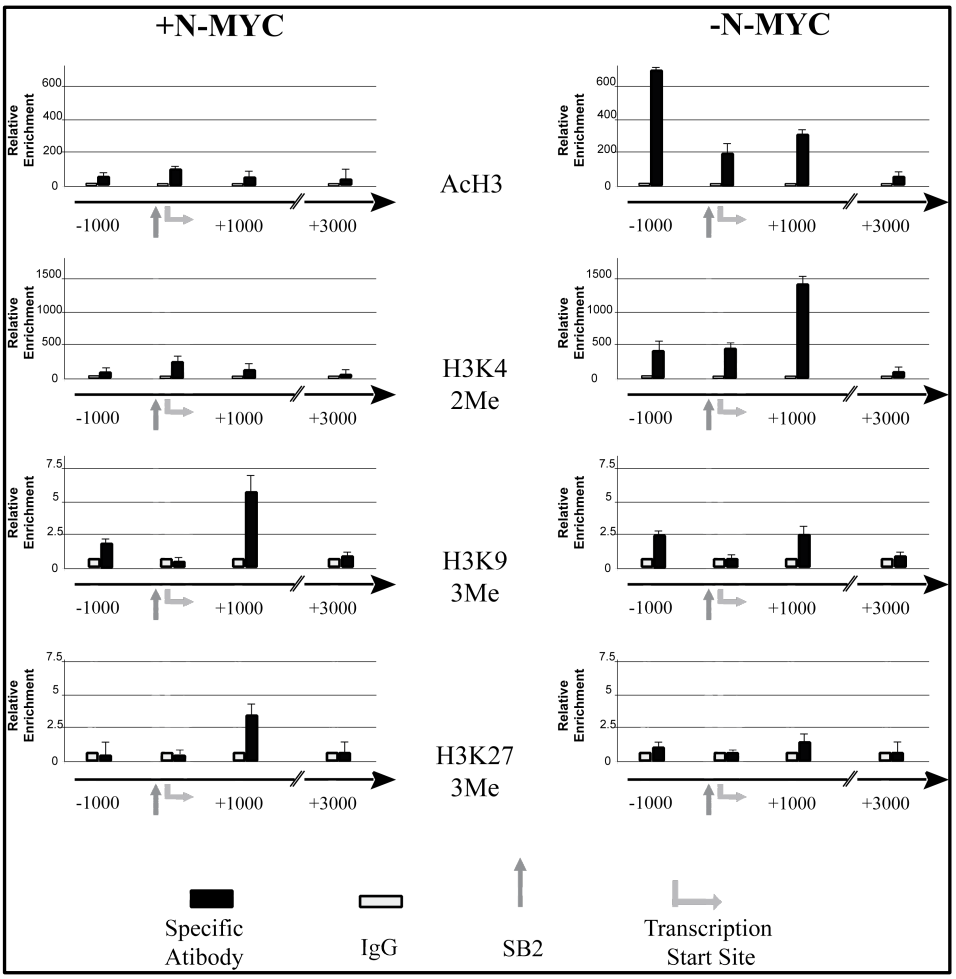

Figure 20. ChIP and quantitative PCR were applied in TET21/N neuroblastoma cell treated or not with tetracycline. Fold enrichment of Clusterin promoter regions immunoprecipitated by Ac-H3, dimethyl-H3K4, trimethyl-H3K9 and trimethyl- H3K27 antibodies and preimmune serum was calculated as the logarithm of the difference between the cycle-threshold obtained with pre-immune serum and the cycle-threshold obtained with the specific antibody. Quantitative PCR was performed in triplicate. Results were the average of three independent experiments.

HDACs inhibitor TSA reactivates CLU isoform 1 expression

The presence of HDACs onto Clusterin promoter suggests that histone deacetylases could play an important role in Clusterin transcriptional repression. To test this hypothesis and to confirm CLU repression by MYCN, we exposed TET21/N cells, a neuroblastoma cell line, to tetracycline or TSA for 24 hours. As shown in Fig.21 both tetracycline and TSA reactivate CLU expression.

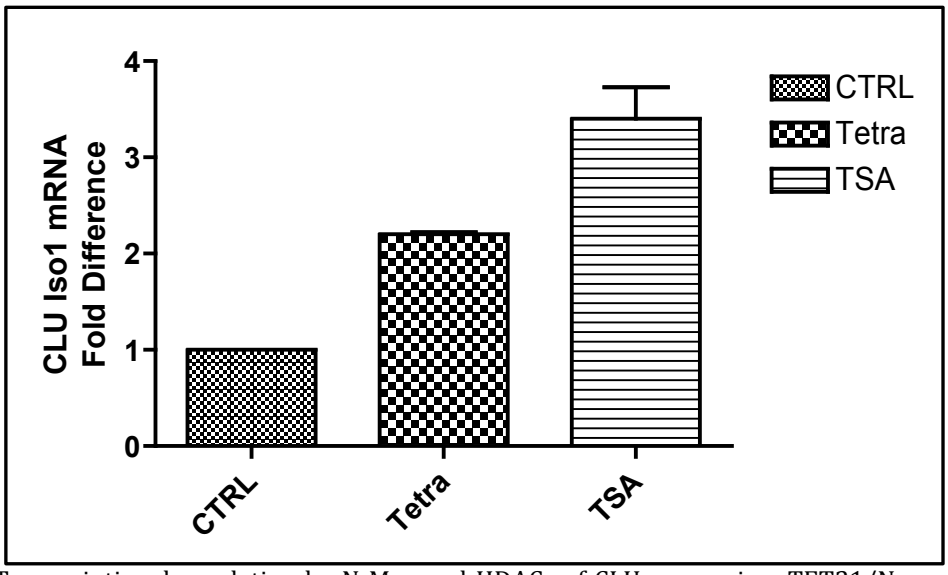

Figure 21. Transcriptional regulation by N-Myc and HDACs of CLU expression. TET21/N neuroblastoma cells was treated with tetracycline or TSA for 24 hours, followed by RNA extraction and real-time RT-PCR analysis of CCNG2 expression. CCNG2 gene expression in control was artificially set as 1.0..

Discussion

The Myc oncoproteins are a well-known family of transcription factors belonging to a subset of the larger class of proteins containing basic-region/helix–loop–helix/leucin-zipper (BR/HLH/LZ) motifs. The Myc family is composed by c-Myc, n-Myc and l-Myc and the most studied components of this family are c-Myc and n-Myc because their expression is deregulated in many kind of cancer (i.e. Osteosarcoma and Neuroblastoma respectively).

Trough BR/HLH/LZ motifs they interact with Max (Myc Associated X-factor), and as heterodimers they act as transcription factor. The better understood mechanism of Myc-mediated transcriptional regulation is activation of gene transcription. The Myc-Max complex recognizes and binds to a specific sequence CACGTG named E-box. Nonetheless it has been shown that beyond "canonical E-boxes" Myc bcan also associate with "non canonical" ones, which are degenerate variants of the consensus CACGTG. The most commonly accepted as non-canonical E-boxes sequences are: CATGTG and CAGCGC.

After binding to target sequences, Myc induces transcriptional activation by recruiting transcriptional co-activator as the Histone acetylase TIP60 and transformation/transcription domain-associated protein TRRAP. Moreover Myc stimulates the release of paused RNA pol II from the promoter [87]and stimulates subsequent transcriptional elongation and increases RNA pol II phosphorylation: Myc transactivation domain (TAD) binds directly CTD kinases [88]. In this thesis we demonstrate that c-Myc/Max complex directly regulates transcription of a panel of ABC genes in OS cell lines. c-Myc amplification or overexpression frequently occurs in Osteosarcoma cell. We first analyzed the correlation between c-Myc and 17 ABC genes in 34 OS biopsies and we discovered nine (ABCC1, ABCC2, ABCC4, ABCC5, ABCC11, ABCE1, ABCF1 ABCF2 and ABCF3) which overexpression correlate to high c-Myc expression. Among these ABC genes we found that ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 are directly and positively regulated by c-Myc in wild type and in high dose (MTX, DX and CCDP) resistant Osteosarcoma cell lines. Furthermore the high expression of c-Myc, ABCC1, ABCC4 and ABCF1 have an high prognostic value in the analyzed osteosarcoma population, suggesting an high probability of conventional therapy failure in patients characterized by this expressional profile. Osteosarcoma is one of most common bone tumors in adolescents. Actual therapy is multi drugs treatment before and after surgical resection and pharmacological cocktail is composed by Methotrexate, Doxorubicin and Cisplatin. Resistance to chemotherapeutic agents is a major obstacle for successful treatment of cancer. The failure of the curative treatment of cancer patients often occurs as a result of intrinsic or acquired drug resistance of the tumors to chemotherapeutic agents. Our results show that Myc directly regulate ABCs transcription also in wild type OS cell lines, suggesting that probably Myc overexpression/amplification could predispose cancer cells to

drug resistance and that these ABCs could play an important role also in tumor biology. The resistance occurs as a cross-resistance to a whole range of drugs with different structures and cellular targets. This phenomenon is called multiple drug resistance (MDR). Multidrug resistance (MDR) severely limits the effectiveness of chemotherapy in a variety of common malignancies and is responsible for the overall poor efficacy of cancer chemotherapy. Often deregulation of ABC genes and c-Myc expression in cancer cells was strongly correlated to MDR. Several ABCs have been correlated to resistance to principal drugs used in OS therapy. ABCC1, ABCC2, ABCC4, ABCC5 and ABCG2 have been related to Methotrexate resistance [246-248]. ABCA3, ABCB1,ABCC1 and ABCG2 are associated to Doxorubicin resistance [249-251], while ABCC2, ABCC4 and ABCF2 were correlated to CCPD resistance [252-254]. As we can infer from scientific literature, ABCC1 and ABCC4 frequently recur to mediate resistance to OS drugs. Intriguingly, a recent review of Fletcher et al [255] suggests a possible role of ABCC1 and ABCC4 not only in MDR but also in cell mobility, making of primary importance the need to use drugs able to inhibit these trans-membrane proteins.

My study has also focused on Myc acting as a repressor. First evidences of the relevance of Myc-mediated transcriptional repression came from genome-wide analyses which demonstrate that Myc represses as many targets as it activates[256]. One of the first mechanism defined for the Myc-mediated transcriptional repression was the interaction of Myc-Max complex with gene promoters by docking the promoter-bound Sp1 and/or Miz1 transcription factors, possibly due to interference with their activation functions or by recruiting other co-repressors like Dnmt3a [115, 137, 257, 258]. Recently Liu and coworkers [143] demonstrated that Myc could repress TG2 transcription by interacting with Sp1 and HDAC1 without Max, this was one of the first evidences in mammals that Myc works outside of the complex with Max. This finding appears consistent with a recent study in which Drosophila dmyc can represses transcription by interacting with Groucho in absence of Max [140].

Inline with these studies we have demonstrated that CCNG2 expression is repressed by an Sp1/MYCN/HDAC2 complex in neuroblastoma cell line. In the model herein proposed, MYCN interacts to Sp1 bound to CCNG2 promoter and recruits HDAC2. A similar model was previously proposed for HDAC1, which shares high homology to HDAC2 [143]. These particular types of complexes may appear unusual considering that Myc is substantially incapable to work as transactivator without Max. However we speculate that an excess of Myc in the cell, due to gene amplification or overexpression, may compete out the constitutive Max allow a “free” Myc to engage with other factor such as HDACs. In the case of CCNG2, transcriptional repression is mediated by the Sp1/MYCN/HDAC2 complex. Interestingly, reactivation of CCNG2 by TSA inhibits

neuroblastoma cell growth thus suggesting that Myc mediated repression represent a necessary step in Myc induced oncogenesis. Cyclin G2 is an atypical member of Cyclin family and its expression is associated with terminal differentiation and apoptosis. Moreover it blocks cell cycle progression and induces cell growth arrest [244, 245, 259]. The presence of HDAC2 in this repressive complex is quite important. HDAC2 promotes cancer cell proliferation [260], and mutation of HDAC2 in mice reduces tumor incidence [261]. These findings suggest that HDACs could be optimal targets for cancer therapy.

Another intriguing hypothesis, which is slowly emerging among Myc experts, is the possibility that the complex Myc-Max could mediate transcriptional repression by direct binding to peculiar E-boxes. This possibility will greatly complicate the complexity level of Myc mediated transcriptional regulation emphasizing the role of context surrounding the E-box. In an elegant work of Palakurthy and coworkers [262] it was shown that Myc could recruit Polycomb Complex 2 after direct binding to DNA and represses transcription of RASSF1A gene. Along this line, my study has shown that MYCN represses CLU expression by a similar transcriptional repression mechanism. CLU is a multifunctional protein involved in many physiological and pathological processes. There are growing evidences that CLU acts as an oncosoppressor in Neuroblastoma [229, 241] and others cancer type [216, 224, 227, 236]. Recently it was shown that MYCN inhibits the expression of CLU in neuroblastoma cell lines and primary tumors in part by inducing oncogenic microRNAs belonging to the miR17-92 cluster [241]. In collaboration with Dr. Sala of UCL Institute of Child Health (London, GB), we firstly observed a non-canonical MYCN binding site in the 5 flanking sequence of Clusterin Isoform1 gene and by reporter assay analyses we measured the repressive effect of MYCN on Clusterin promoter. To confirm the MYCN binding to Clusterin promoter we performed ChIP and Dual-step ChIP in neuroblastoma cell line and we further analyzed the presence of chromatin modifiers (HDACs, PRC1 and PRC2) and histone modifications in presence/absence of MYCN using TET21/N cell lines. Data obtained show that MYCN/Max complex really binds the Clusterin promoter and the same region is immunoprecipitated by HDAC1 and 2 antibodies. Moreover regions immediately flanking Clusterin TSS (~+/-1000 bp) are immunoprecipitated by Suz12 antibody indicating that PRC2 is involved in Clusterin repression. Interestingly in absence of MYCN no chromatin modifier was associated with Clusterin promoter meaning that probably MYCN could have a pivotal role in CLU repression. Another intriguing finding of this study came from analyses of histone modifications of CLU promoter; in absence of MYCN the CLU promoter carries the classic histone modifications typical of a transcriptionally active gene (Ac-H3 and dimethyl-

H3K4), while in presence of MYCN we could observe a strong reduction of activating modifications and a slight increase in repressive modifications (trimethyl-H3K9 and –H3K27). These features resemble the histone modifications found in cancer stem [263] where repressed genes are characterized by a "bivalent" signature of histone modifications. Taken together these findings suggest that the MYCN/Max complex may directly bind to CLU promoter, recruits HDACs thus reducing the level of histone H3 acetylation, and then recruit the PRC1 and PRC2 complexes to modify the histone methylation profile. To our knowledge these are the first evidences for this kind of MYCN-mediated transcriptional repression. Furthermore our results show an increase of Clusterin expression after TSA treatment in neuroblastoma cell line, further reinforcing the idea of epigenetic drugs as novel therapeutic treatments for Neuroblastoma.

It should be noted that HDACs are present in all genes that we found to be repressed by MYCN. Actually more than a dozen HDAC inhibitors are currently in clinical trials for the treatment of malignancies of various organ origins, and the HDAC inhibitor SAHA is already in clinical use for the treatment of cutaneous T cell lymphoma. Our data also highlight the potential application of HDAC inhibitors for the prevention and treatment of MYCN-over-expressing neuroblastoma because HDACs inhibitor TSA significantly reactivates CCNG2 and CLU expression.

Final remarks

Overall these studies provide further clarifications on mechanisms of Myc transcriptional regulation in cancer and on the contribution of Myc to oncogenesis, particularly in those cancer in which Myc expression is deregulated. Overall our results demonstrate that:

- c-Myc directly activates transcription of ABCC1, ABCC4, ABCE1, ABCF1, ABCF2 and ABCF3 in OS cells.
- N-Myc/Hdac2 complex represses CCNG2 transcription in neuroblastoma cells.
- N-Myc/Max complex represses CLU transcription by direct binding to peculiar E-box in neuroblastoma cell line.

Together these findings provide a more general significance to our observations, suggesting that analyzed transcriptional regulations could have a not secondary role in the Myc-mediated oncogenesis.

Taken together our data support the idea that Myc could interact with different partners and it could mediate different transcriptional regulation as a function of its cellular level. In normal condition, one of the principal functions of Myc/Max complex is to promote the cell cycle progression: thus physiologically Myc expression is tightly regulated and restricted during the G1/S transition. Furthermore Myc proteins have short half-lives (on the order of 20–30 min),

whereas Max, on the other hand, is stable and constitutively expressed (Fig. 1).

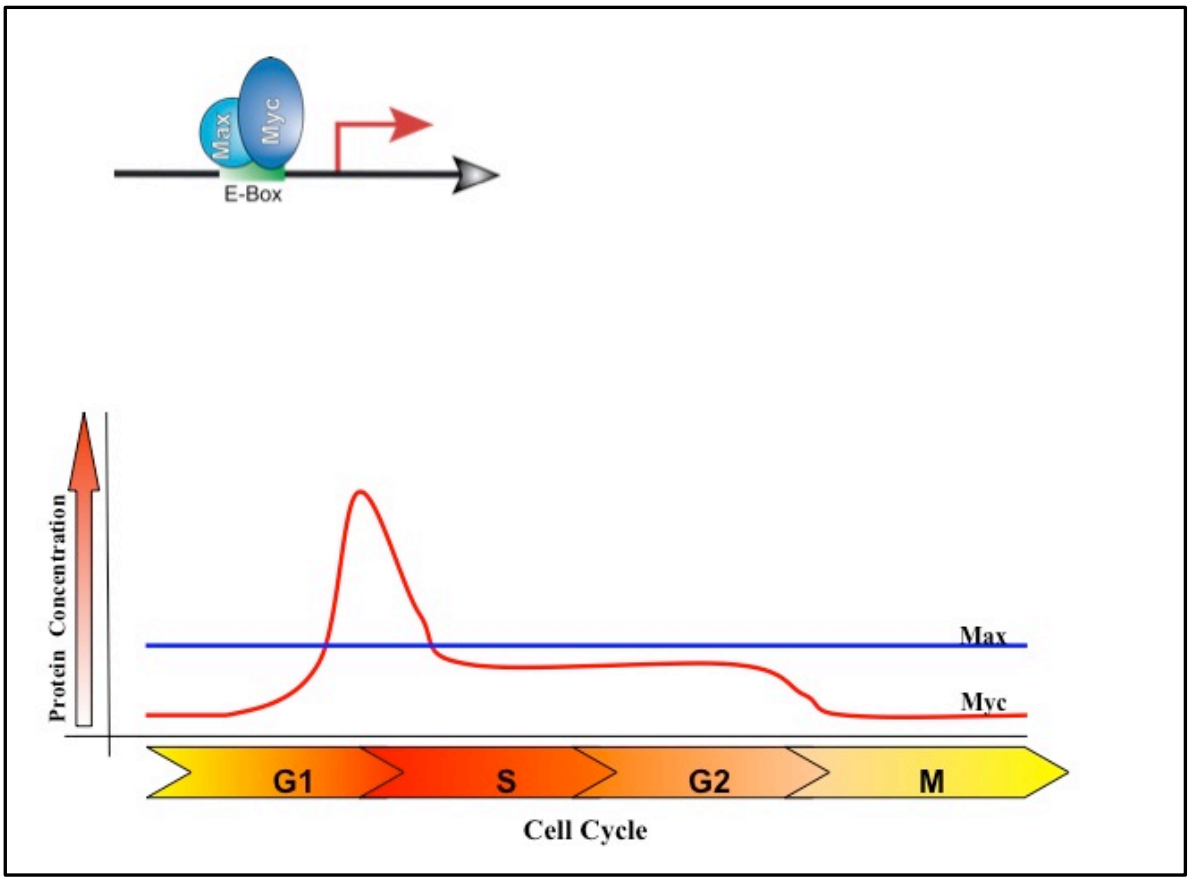

Figure 1. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in physiological conditions

When Myc is deregulated and expressed during the whole cell cycle, the Myc/Max heterodimers may become overabundant and interfere with other transcriptional factors such as Sp1 and Miz1. In this condition the heterodimers could directly bind to lower affinity E-boxes of activated genes and switch off theirs transcriptional activation by recruiting chromatin modifiers (Fig.2)

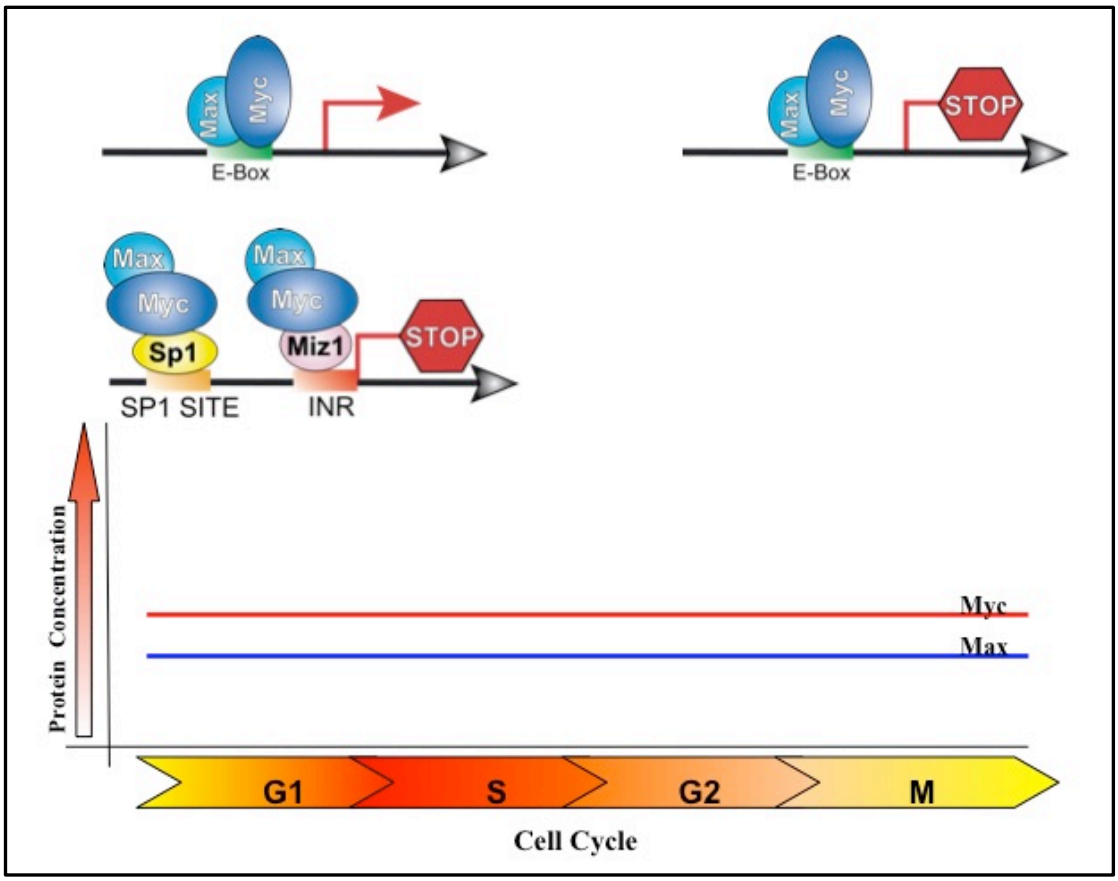

Figure 2. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in pathological conditions

Moreover, when significantly overexpressed, for example as the result of amplification in neuroblastoma or in other tumours, Myc may establish interactions even outside the Max context, such as with Dnmt3a and Hdacs. Fig.3

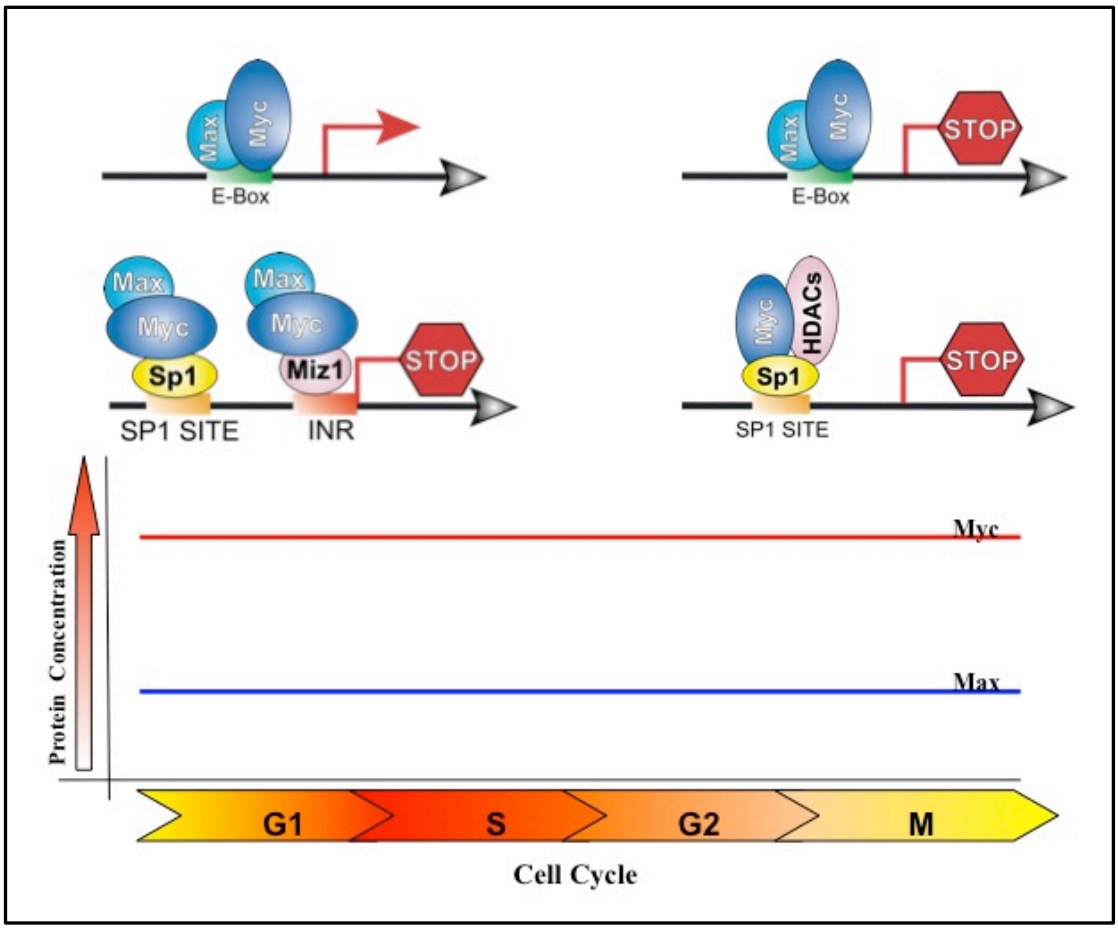

Figure 3. Schematic representation of distinct N-Myc complexes, depending on its cellular levels during cell cycle, in pathological conditions

Materials and Methods

CELL CULTURES

Human neuroblastoma TET-21N and SK-N-BE(2)C cells were cultured in DMEM containing 10% heat-inactivated FBS and 50 mg/ml gentamycin. Tet-21N cells were treated with tetracycline as described (Lutz et al., 1996; Schuhmacher et al., 2001).
Human Osteosarcoma SAOS and U2OS cells were cultured in DMEM containing 10% heat-inactivated FBS and 50 mg/ml gentamycin

RNA EXTRACTION

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

THERMOSCRIPT RT-PCR SYSTEM

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

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

SYBR GREEN qPCR

SYBR GreenER qPCR SuperMix (Invitrogen) for ICycler is a ready to use cocktail containing all components, except primers and template, for real-time quantitative PCR (qPCR) on ICycler BioRad real time instruments that support normalization with Fluoresceina Reference Dye at final concentration of 500nM. It combines a chemically modified "hot-start" version of *Taq*DNA polymerase with integrated uracil DNA glycosilase (UDG) carryover prevention technology and a novel fluorescent dye to deliver excellent sensitivity in the quantification of target sequences, with a linear dose response over a wide range of target concentrations. SYBR GreenER qPCR SuperMix for ICycler was supplied at a 2X concentration and contains hot-start *Taq*DNA polymerase, SYBR GrenER fluorescent dye, 1 μM Fluorescein Reference Dye, $MgCl_2$, dNTPs (with dUTP instead of dTTp), UDG, and stabilizers. The SuperMix formulation can quantify fewer than 10 copies of a target gene, has a broad dynamic range, and is compatible with melting curve analysis. The *Taq*DNA polymerase provided in the SuperMix has been chemically modified to block polymerase activity at ambient temperatures, allowing room-temperature set up and long term storage at 4°C. Activity is restored after a 10-minutes incubation in PCR cycling, providing an automatic hot start for increased sensitivity, specificity and yield. UDG and dUTP in the SuperMix prevent the reamplification of carryover PCR products between reactions. dUTP ensures that any amplified DNA will contain uracil, while UDG removes uracil residues from single or double-stranded DNA. A UDG incubation step before PCR cycling destroys any contaminating dU-containing product from previous reactions. UDG is then inactivated by the high temperatures during normal PCR cycling, thereby allowing the amplification of genuine

target sequences. Fluorescein is included at a final concentration of 500nM to normalize the fluorescent signal on instruments that are compatible with this option. Fluorescein can ajust for non-PCR-related fluctuations in fluorescence between reactions and provides a stable baseline in multiplex reactions. Program real time instrument for PCR reaction as shown following: 50°C for 2 minutes hold (UDG incubation), 95°C for 10 minutes hold (UDG inactivation and DNA polymerase activation), 40 cycles of: 95°C for 15 seconds and 60°C for 60 seconds. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then the unique reaction components (e.g. template, forward and reverse primers at 200nM final concentration). Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate, centrifuge briefly and place reactions in a pre-heated real-time instrument programmed as described above.

ChIP- CHROMATIN IMMUNOPRECIPITATION

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

In each plate add 270 µl of formaldehyde from a 37% stock solution and mix immediately. Incubate samples on a platform shaker for 10 minutes at room temperature. In each plate add 500 ml glycine from a 2,5 M stock solution and mix immediately. Incubate on a platform shaker for 10 minutes at room temperature. Transfer the plates in ice and remove the medium. Harvest the cells with a scraper and then centrifuge at 1500 rpm for 4 minutes in cold centrifuge, then keep samples on ice. Remove the supernatant and wash pellet 3 times with 10 ml ice-cold PBS1X/ 1 mM PMSF. After each washing centrifuge at 1500 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 500 µl ice-cold Cell Lysis Buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Centrifuge at 3000 rpm for 5 minutes at 4°C. Remove supernatant and resuspend pellet in 600 µl ice-cold RIPA buffer. Pipet up and down 10-20 times, then incubate on ice for 10 minutes. Sonication of crosslinked cells is performed in two distinct steps. First, cells are sonicated with a Branson Sonifier 2

times for 15 seconds at 40% setting. Next, cell samples are further sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and pre-clear lysate by incubating it with 50 µl of Immobilized Protein A [99] for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A [99] and incubate by constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer. Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.

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

Cell Lysis Buffer:	**RIPA Buffer**	**Washing buffer**
5 mM PIPES pH 8	150mM NaCl	100mM TrisHCl pH 8
85 mM KCl	1% NP40	500mM LiCl
0,5% NP40	0,5% NaDoc	1% NP40
1 mM PMSF	0,1% SDS	1% NaDoc

Protease inhibitor cocktail [264]	50 mM TrisHCl pH 8
	1 mM PMSF
	Protese inhibitor cocktail [264]

DUAL-STEP CHROMATIN IMMUNOPRECIPITATION

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

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

at 40% setting. Next, cell samples are further sonicated with the Diogene Bioruptor for 20 minutes at high potency in a tank filled with ice/water in order to keep cell samples at low temperature during sonication. (Note 3) Centrifuge samples at 14000 rpm for 15 minutes at 4°C. Transfer supernatant to a new tube and preclear lysate by incubating it with 50 µl of Immobilized Protein A [99] for 15 minutes in the cold room at constant rotation. Centrifuge samples at 3000 rpm for 5 minutes at 4°C. Take the supernatant, after having saved 50 µl aliquot for preparation of INPUT DNA, and add 5 µg of specific antibody. Rotate the sample O/N in the cold room. Add 50 µl of Immobilized Protein A [99] and incubate by constant rotation for 30 minutes at room temperature. Centrifuge the sample at 4000 rpm for 5 minutes at room temperature. Remove the supernatant and proceed to wash the beads. For each wash, incubate the sample by constant rotation fro 3 minutes at room temperature and the centrifuge at 4000 rpm for 2 minutes at room temperature. Wash 4 times with 1 ml Ripa Buffer. Wash 4 times with 1 ml Washing Buffer. Wash 2 times with 1 ml TE buffer. Remove the supernatant and add 200 µl TE buffer to the beads. Add 10 µg RNAse A and incubate at 37°C for 30 minutes. Add 50 µl Proteinase K Buffer 5X and 6 µl Proteinase K (19 mg/ml). Then, incubate at 65°C in a shaker at 950 rpm for 6 hrs. Centrifuge at 14000 rpm for 10 minutes at 4°C, then transfer the supernatant (250 µl) to a new tube.

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

Notes

1).We have tested several crosslinking agents including DSG (disuccinimdyl glutarate), EGS [ethylene glycol bis(succinimidylsuccinate], DMA (dimethyl adipimidate) and DSS (disuccinidimyl suberate). In our conditions, DSG was the one that worked best, although we also obtained good results with EGS.

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

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

Cell Lysis Buffer:	**RIPA Buffer**	**Washing buffer**
5 mM PIPES pH 8	150mM NaCl	100mM TrisHCl pH 8
85 mM KCl	1% NP40	500mM LiCl
0,5% NP40	0,5% NaDoc	1% NP40
1 mM PMSF	0,1% SDS	1% NaDoc
Protease inhibitor cocktail [264]	50 mM TrisHCl pH 8	
	1 mM PMSF	
	Protese inhibitor cocktail [264]	

LUCIFERASE ASSAY

The Dual-Luciferase® Reporter (DLR.) Assay System (Promega) provides an efficient means of performing dual-reporter assays. In the DLR. Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. In the DLR. Assay System, both reporters yield linear assays with subattomole sensitivities and no endogenous activity of either reporter in the experimental host cells. Furthermore, the integrated format of the DLR. Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

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

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

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

CO-IMMUNOPRECIPITATION ASSAYS

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

with immunocomplexes are washed five times with nuclear lysis buffer + NP40 0,25% and boiled in Laemmli sample buffer for 5 min at 100°C. Eluted proteins are separated by SDS-PAGE and analyzed by Western blot.

IMMUNOBLOTTING ANALYSIS

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

GENE SILENCING, TRANSFECTION AND CELLULAR ASSAYS

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

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