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*Cancer Res* 2011;71:404-412. Published OnlineFirst December 1, 2010.

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# A SP1/MIZ1/MYCN Repression Complex Recruits HDAC1 at the *TRKA* and *p75NTR* Promoters and Affects Neuroblastoma Malignancy by Inhibiting the Cell Response to NGF

Nunzio Iraci<sup>1</sup>, Daniel Diolaiti<sup>1,2</sup>, Antonella Papa<sup>1,3</sup>, Antonio Porro<sup>1,4</sup>, Emanuele Valli<sup>1</sup>, Samuele Gherardi<sup>1</sup>, Steffi Herold<sup>5</sup>, Martin Eilers<sup>5</sup>, Roberto Bernardoni<sup>1</sup>, Giuliano Della Valle<sup>1</sup>, and Giovanni Perini<sup>1</sup>

## Abstract

Neuroblastoma is the most common extracranial solid tumor of childhood. One important factor that predicts a favorable prognosis is the robust expression of the *TRKA* and *p75NTR* neurotrophin receptor genes. Interestingly, *TRKA* and *p75NTR* expression is often attenuated in aggressive *MYCN*-amplified tumors, suggesting a causal link between elevated *MYCN* activity and the transcriptional repression of *TRKA* and *p75NTR*, but the precise mechanisms involved are unclear. Here, we show that *MYCN* acts directly to repress *TRKA* and *p75NTR* gene transcription. Specifically, we found that *MYCN* levels were critical for repression and that *MYCN* targeted proximal/core promoter regions by forming a repression complex with transcription factors SP1 and MIZ1. When bound to the *TRKA* and *p75NTR* promoters, *MYCN* recruited the histone deacetylase HDAC1 to induce a repressed chromatin state. Forced re-expression of endogenous *TRKA* and *p75NTR* with exposure to the HDAC inhibitor TSA sensitized neuroblastoma cells to NGF-mediated apoptosis. By directly connecting *MYCN* to the repression of *TRKA* and *p75NTR*, our findings establish a key pathway of clinical pathogenicity and aggressiveness in neuroblastoma. *Cancer Res*; 71(2); 404–12. ©2010 AACR.

## Introduction

Neuroblastoma is the most common solid extracranial childhood tumor that originates from the sympathetic nervous systems (1, 2). *MYCN* amplification has been long proposed as a critical predictor of neuroblastoma outcome (3, 4), although a few other genetic markers have been recently described as important for prognosis, such as ploidy status, loss of chromosome 1p (5), expression of ABC drug transporters (6, 7), and expression of neurotrophin receptors (8–10).

It has been shown that *TRKA* is expressed at high levels in neuroblastomas with a biologically favorable outcome. Low-risk neuroblastomas usually express high *TRKA* and respond

to NGF, and thus, affect cell growth and differentiation (8, 9). Similarly, *p75NTR* has also been considered a favorable prognostic indicator, though its role in neuroblastoma development is less clear (10).

*TRKA* is a transmembrane receptor that functions as a homodimer. The binding of NGF to a homodimer of *TRKA* activates the autophosphorylation of the receptor, the docking of signaling proteins, signal transduction, and the induction of gene transcription. The activation of specific signaling pathways has been linked to the survival, differentiation, and development of sympathetic neurons, whereas the inhibition of *TRKA* activation can lead to altered cell growth, depending in part on the state of differentiation of the cell (11, 12). *p75NTR* is a member of the tumor necrosis factor receptor (TNFR) superfamily and binds all members of the NGF family of neurotrophins with low affinity (13). The biological consequences of *p75NTR* expression are controversial in that, sometimes, it can mediate apoptosis in developing neurons even in the absence of NGF or the *TRKA* receptor. The effect of *p75NTR* on the cellular response to neurotrophins is complex and may depend on the concentration of the ligand, the cell type, and also the stage of cell differentiation (14).

The 2 receptors are, however, often coexpressed in the same cellular background, and depending on their relative ratio, they appear to control the overall effect of NGF on neuronal biology (14, 15).

Many studies have shown that *TRKA* mRNA levels counter-correlated with *MYCN* amplification. Importantly, the combination of *TRKA* expression and *MYCN* amplification provided

**Authors' Affiliations:** <sup>1</sup>University of Bologna, Department of Biology, Bologna, Italy; <sup>2</sup>Fred Hutchinson Cancer Research Center, Division of Basic Sciences, Seattle, Washington; <sup>3</sup>Cancer Genetics Program, Beth Israel Deaconess Cancer Center, Harvard Medical School, Boston, Massachusetts; <sup>4</sup>Ecole Polytechnique Federale de Lausanne, ISREC-Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland; and <sup>5</sup>Theodor-Boveri-Institute, Physiological Chemistry Biocenter Am Hubland, Würzburg, Germany

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

N. Iraci and D. Diolaiti contributed equally to this study.

**Corresponding Author:** Giovanni Perini, University of Bologna, Department of Biology, via F. Selmi, 3; 40126 Bologna, Italy. Phone: 39-0512094167; Fax: 39-0512094286. E-mail: [giovanni.perini@unibo.it](mailto:giovanni.perini@unibo.it)

doi: 10.1158/0008-5472.CAN-10-2627

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even greater prognostic power (9, 16–18). MYCN is a member of the MYC family of transcription factors, whose activity is mostly restricted to the developing nervous system (19–21). As is the case for other MYC proteins, MYCN induces tumor formation and is an important marker of poor prognosis in tumors of neuronal origins (22, 23). The controlled expression of MYCN in a transgenic animal model is sufficient to induce neuroblastoma formation (24). Recently, Marshall and colleagues have shown that transgenic mice overexpressing MYCN in perinatal root ganglia develop neuroblastoma at approximately 6 weeks of age and that such tumor cells displayed a reduced expression of *TRKA* and *p75NTR* and progressed to a more malignant phenotype (25).

Although these data clearly suggest a functional link between MYCN expression and the NGF/*TRKA*/*p75NTR* pathways, how MYCN contributes to the silencing of the expression of the receptors has yet to be defined.

In this study, we investigated how MYCN affects the transcription of *TRKA* and *p75NTR* genes in neuroblastoma cells. Here, we show that MYCN is physically associated with the proximal/core promoters of NGF receptors and can negatively affect their transcription by interacting with the SP1 and MIZ1 transcription factors. In this context, MYCN can recruit HDAC1 to silence neurotrophin-receptor expression. Finally, we show that drugs targeting epigenetic pathways can relieve the transcriptional repression of NGF receptors and can induce massive apoptosis by sensitizing neuroblastoma cells to NGF.

## Materials and Methods

### Cell culture

TET-21/N, SK-N-BE, SH-SY5Y, LA-N-1, HeLa, and HEK-293 cell lines were cultured as previously described (26). Cell lines were obtained from Manfred Schwab (TET-21/N), German Collection of Microorganisms and Cell Cultures (SK-N-BE, SH-SY5Y), Arturo Sala (LA-N-1), and Michael Green (HeLa, HEK-293) between 1997 and 2005 and were systematically validated for *MYCN* copy number and the expression of specific markers, such as MYCN and tyrosine hydroxylase, upon receipt and during usage by Dr. Giuliano Della Valle who runs the cytogenetic facility, Department of Biology, University of Bologna, Italy.

The human TET-21/N cell line was derived from SHEP neuroblastoma cells transfected with 2 plasmids: pUHD15-1, which expresses the tTA transactivator gene, and the plasmid pHUD10-3/MYCN, which expresses the human MYCN. To switch off MYCN expression, TET-21/N cells were cultured in the presence of 2  $\mu$ g/ml of tetracycline (Sigma-Aldrich) for 48 hours. Stable clones expressing MYCN were generated by the transfection of SH-SY5Y or SK-N-BE cells with the p3XFLAG-CMV-14-MYCN construct (Sigma-Aldrich). Inducible clones expressing c-terminal tagged HA-SP1 or HA-MIZ1 were generated via the pVgRXXR/pIND system (Invitrogen).

### Gene expression analyses

RNA from cell lines were prepared with the RNeasy Mini kit (Qiagen) and reverse-transcribed with SuperScript III (Invitrogen).

Real-time quantitative PCR (RQ-PCR) was done with an iQ SYBR Green Supermix and the iQ5 Cycler thermocycler (Bio-Rad). RNA expression values were normalized to GUSB. Primers for RQ-PCR are listed in Supplementary Table S1.

### RNAi gene silencing

Cells were transfected with scrambled or gene-specific siRNA with a Lipofectamine RNAiMAX reagent (Invitrogen). siRNAs targeting MYCN and MIZ1 were purchased from Dharmacon, and those targeting SP1 were purchased from Invitrogen.

### Immunoblotting analysis

Western blots were done as previously described (26). The antibodies used were MYCN (sc-53993, Santa Cruz), *TRKA* (2505, Cell Signaling), *p75NTR* (9PIG323, Promega), APEX1 (NB100-504, Novus Biologicals), SP1 (07-645, Upstate), MIZ1 (sc-22837, Santa Cruz), p21 (05-345, Upstate), FLAG M2 (F1804, Sigma-Aldrich), HA-probe (sc-805, Santa Cruz), PARP (sc-7150, Santa Cruz), and  $\beta$ -ACTIN (A2066, Sigma Aldrich).

### Luciferase assay

The *TRKA*, *p75NTR*, *APEX1*, and *p21* promoters were amplified by PCR and cloned into the pGL3-basic vector (Promega). The Renilla-TK vector was used as an internal control. The activity of firefly or *Renilla* luciferase was measured with the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions.

### Dual chromatin immunoprecipitation assays

Dual ChIP cross-linking was done as previously described (27, 28). Antibodies used in this study were IgG (sc-2027, Santa Cruz), SP1 (07-645, Upstate), MYCN (sc-53993, Santa Cruz), MAX (sc-197, Santa Cruz), HA-probe (sc-805, Santa Cruz), HDAC1 (05-614, Upstate), and anti-Acetyl-Histone H3 (06-599, Upstate).

Specific pairs of primers used for quantitative ChIP are listed in Supplementary Table S2.

### Protein coimmunoprecipitation assay

Nuclear protein lysates extracted from transiently transfected HEK-293 cells (100 mg) or from SK-N-BE cells (1 mg) were immunoprecipitated with 1 mg of specific antibodies. Eluted proteins were separated on SDS/PAGE and analyzed by Western blot analysis.

### GST pull-down assay

GST-MYCN proteins were expressed in BL21 *Escherichia coli* cells, purified, and immobilized onto glutathione agarose beads (Sigma-Aldrich). GST beads were then incubated with *in vitro*-translated SP1-HA and MIZ1-HA proteins (TNT Quick Coupled Transcription/Translation System, Promega) pre-treated with DNase (GE Healthcare). Purified complexes were separated on SDS/PAGE and analyzed by Western blotting.

### Fluorescence activated cell sorter analysis

SK-N-BE cells treated with specific siRNA were cultured for 48 hours in the presence of NGF (10 nmol/L) before the

addition of BrdU 20 mmol/L for 1 hour. Cells were then incubated with an anti-BrdU antibody (Becton Dickinson). Indirect staining was achieved through the use of a secondary goat anti-mouse antibody coupled with Alexa Fluor 448 (Molecular Probe).

### Statistical analysis

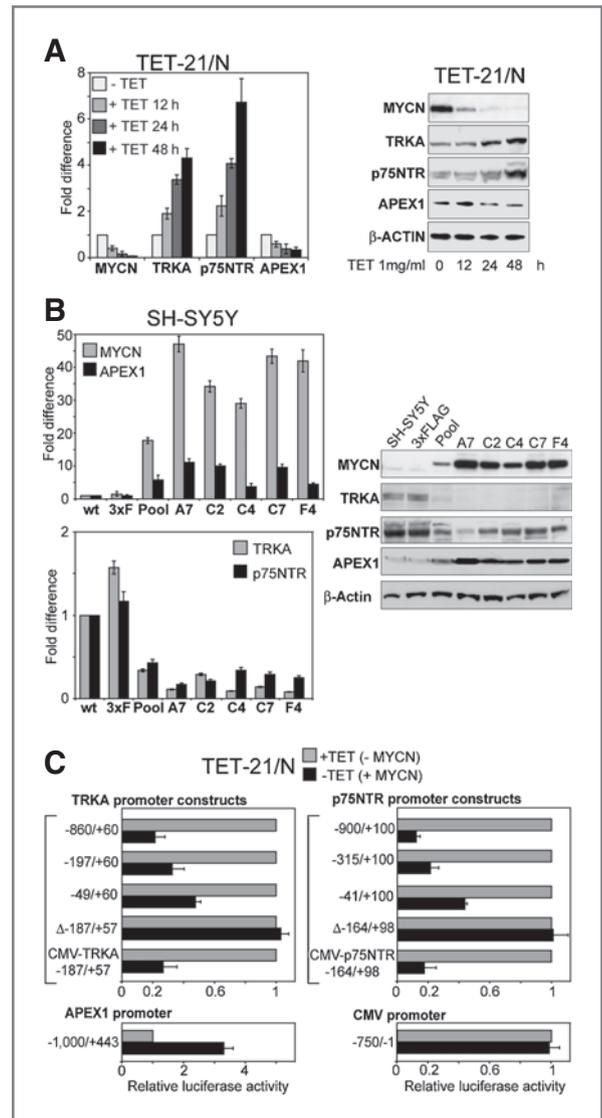
All experiments were repeated for at least 3 times in duplicates. All data for statistical analysis were calculated as mean  $\pm$  standard error. Differences among groups were determined for significance via ANOVA, whereas differences for 2 groups were determined with an unpaired *t*-test. A probability value of  $p = 0.05$  or less was considered significant.

## Results

### MYCN represses *TRKA* and *p75NTR* through their core promoter

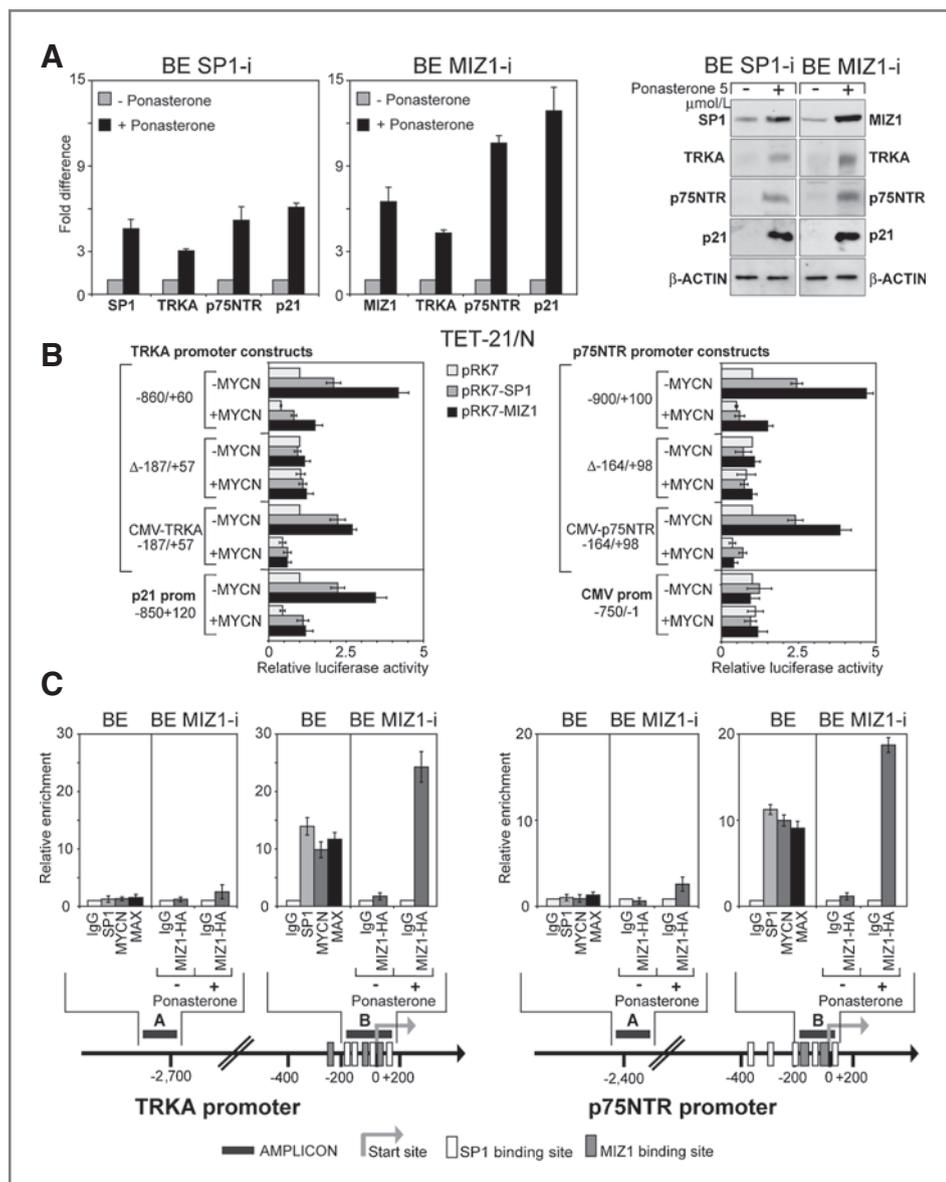
To determine whether MYCN can affect the expression of *TRKA* and *p75NTR*, the expression of the 2 receptors was analyzed in several distinct cell systems and under different conditions. First, the expression of *TRKA* and *p75NTR* was analyzed in the human TET-21/N neuroblastoma cell line conditionally expressing MYCN under the control of the tet-off (tetracycline) promoter (29). The expression of MYCN, *TRKA*, and *p75NTR* was determined in these cells as a function of tetracycline treatment. The results of Figure 1A show that both the mRNA and the protein levels of *TRKA* and *p75NTR* substantially increase upon the silencing of MYCN expression by the addition of tetracycline to the medium. As a control, we checked the expression of *APEX1*, a gene positively regulated by MYCN, and as expected, it decreased when MYCN was downregulated. Second, human neuroblastoma SH-SY5Y cells, which carry a single copy of MYCN and express detectable levels of *TRKA* and *p75NTR* (Fig. 1B), were stably transformed with a vector overexpressing a FLAG-MYCN derivative protein. Several cell clones were selected and screened for high MYCN expression. One clone transfected with the empty vector was also selected and used as a negative control. A comparison of *TRKA* and *p75NTR* expression between parental SH-SY5Y cells and derived clones showed a dramatic reduction in *TRKA* and *p75NTR* expression in cells with high MYCN (Fig. 1B). Finally, the expression of *TRKA* and *p75NTR* was monitored in human neuroblastoma cell lines in which MYCN had been silenced through RNAi. As shown in Supplementary Figure S1, RNAi-mediated silencing of MYCN promotes the upregulation of *TRKA* and *p75NTR* expression. Collectively, these data confirm a primary role of MYCN on *TRKA* and *p75NTR* transcription regulation.

To investigate how MYCN regulates *TRKA* and *p75NTR* transcription, the promoter regions of the 2 genes were cloned into the basic luciferase reporter vector pGL3b. The recombinant reporter vectors [*TRKA*(-860/+60); *p75NTR*(-900/+100)] were transfected in TET-21/N cells, and luciferase activity was determined as a function of MYCN expression. Results show that promoter activity correlated negatively with MYCN expression, indicating that MYCN represses these



**Figure 1.** MYCN affects *TRKA* and *p75NTR* expression in neuroblastoma cells by targeting their core promoter regions. **A**, mRNA and protein levels of *TRKA*, *p75NTR*, and *MYCN* were determined by qRT-PCR and WB, respectively. *APEX1*, a positive control for the qRT-PCR;  $\beta$ -ACTIN loading control of the WB. **B**, expression of *TRKA*, *p75NTR*, and *MYCN* in several SH-SY5Y cell clones engineered to express high levels of MYCN-FLAG. Levels of expression were compared with that of the parental cell line (5Y wt) and with that of a cell clone transfected with the empty vector (3XFLAG). **C**, *TRKA* and *p75NTR* promoters were cloned upstream of the pGL3basic-Luciferase vector. The minimal region responding to MYCN was mapped by deletion mutagenesis. As a control, the MYCN-responsive region of the *TRKA* and *p75NTR* promoters was separately cloned downstream of the CMV promoter of a CMV-luc reporter. Luciferase activity was monitored in TET-21/N cells as a function of MYCN. Luc activity in the (+)MYCN condition was determined as a percentage of the activity of the same reporter in the (-)MYCN condition. *APEX1* promoter was used as a control. Results are the average  $\pm$  SE of 5 independent experiments.

genes at the level of transcription (Fig. 1C). Similar results were also obtained when reporters were transfected into the SH-SY5Y cell clones or cotransfected along with an MYCN expression vector in HeLa cells (Supplementary Fig. S2).



**Figure 2.** MYCN requires SP1 and MIZ1 transcription factors to repress transcription *in vivo*. **A**, induction of either SP1 or MIZ1 promotes the expression of endogenous *TRKA* and *p75NTR* genes. Two SK-N-BE cell clones were obtained that expressed SP1 or MIZ1 in a ponasterone-dependent fashion. mRNA (left) and protein (right) levels are shown as a function of SP1- or MIZ1-induced expression. **B**, TET-21/N cells were cotransfected with the *TRKA*- or *p75NTR*-luc reporters along with a vector overexpressing either SP1 or MIZ1. Luciferase assay was done as a function of MYCN expression. **C**, dual ChIP was employed to determine the *in vivo* binding of MYCN to *TRKA* and *p75NTR* promoters. Dual ChIP was done on SK-N-BE cells to analyze SP1 distribution, and it was done on SK-N-BE MIZ1-i +/- ponasterone to determine the association of MIZ1 to tested promoters. **A** and **B** represent 1 region, distal from the transcription start site, and the region surrounding the TSS, respectively. Relative enrichment of a given promoter region obtained with a specific antibody was compared with that obtained with pre-serum (IgG), which was set to 1 in the graph. Results are the average  $\pm$  SE of 4 independent experiments.

Furthermore, mapping of the promoters revealed that the core promoters of *TRKA* (-187/+57) and *p75NTR* (-164/+98) were responsive to MYCN. This point was also confirmed by showing that when the proximal/core promoter regions were cloned immediately downstream of the CMV minimal promoter, which is typically not responsive to MYCN (see diagram of Supplementary Fig. S2), the derived constructs (*CMV-TRKA* -187/+57; *CMV-p75NTR* -164/+98) were downregulated in a MYCN-dependent fashion. These findings suggest that MYCN targets the proximal/core promoter regions of *TRKA* and *p75NTR*.

#### MYCN collaborates with SP1 and MIZ1 to repress transcription *in vivo*

Given that c-MYC has been shown to repress the transcription of a few genes, such as *p15INK4b* and *p21CIP/WAF*, by interacting with either SP1 or MIZ1 transcription factors (30–

33), the *TRKA* and *p75NTR* promoter regions were analyzed for the presence of consensus DNA binding sites for SP1 and MIZ1. Bioinformatic analysis predicted putative SP1 or MIZ1 binding sites around the start site of transcription for the *TRKA* and *p75NTR* genes (see diagrams in Fig. 2C). To investigate the role of MIZ1 and SP1 in the transcription regulation of *TRKA* and *p75NTR*, we generated 2 SK-N-BE stable cell clones that expressed SP1 or MIZ1 from a ponasterone-inducible promoter. Ponasterone-induced expression of SP1 and MIZ1 was robust and resulted in a significant increase in the endogenous mRNA and protein levels of *TRKA* and *p75NTR* receptors (Fig. 2A). Importantly, inducing SP1 or MIZ1 expression also increased the expression of Luc-reporter vectors (see above) containing the core promoters of *TRKA* and *p75NTR* but not those lacking the core promoter sequence (Supplementary Fig. S3A). Next, to determine whether the expression status of MYCN affects the ability of SP1 or MIZ1 to

regulate the transcription of *TRKA* and *p75NTR*, the promoter reporters were cotransfected along with either an SP1 or an MIZ1 expression vector into TET21/N cells and in SH-SY5Y cell clones, and luciferase activity was found by the presence or absence of MYCN expression. The results showed that MYCN expression muted the response of the *TRKA*(-860/+60) and *p75NTR*(-900/+100) luc-reporters to SP1 or MIZ1. This suggests MYCN is regulating SP1 and MIZ1 activity at the proximal/core promoter elements (Fig. 2B and Supplementary Fig. S3B).

Furthermore, we analyzed whether MYCN can directly target the core promoter regions of *TRKA* and *p75NTR* *in vivo*. For this purpose, we used dual cross-linking chromatin immunoprecipitation (dual ChIP) assay.(27, 28). Dual ChIP was done on SK-N-BE cells, and MYCN cross-linking was examined at 2 DNA regions of each promoter analyzed. The results show that MYCN can specifically associate with the proximal/core promoter regions of *TRKA* and *p75NTR* *in vivo* (Fig. 2C). We also found that SP1 and MIZ1 robustly cross-linked to the same region occupied by MYCN (Fig. 2C). In the

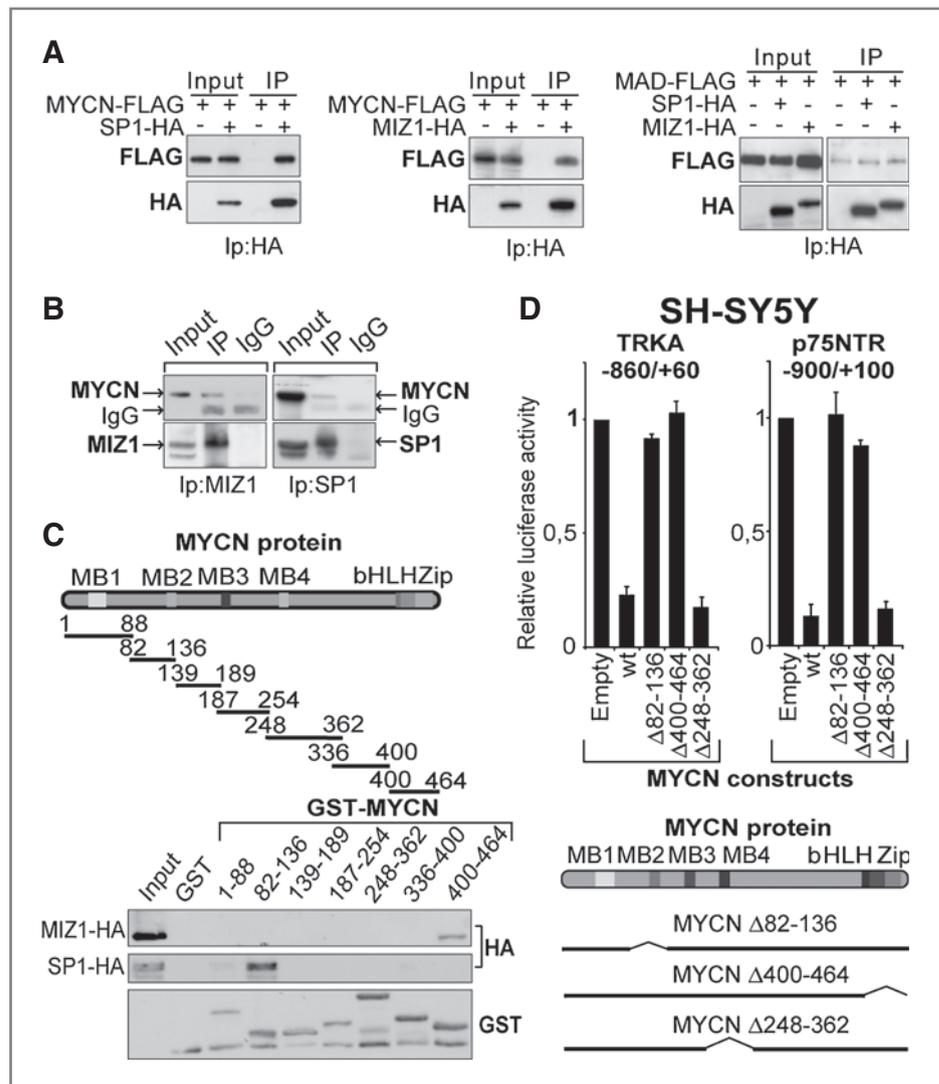
case of MIZ1, dual ChIP was done on cells expressing the MIZ1-HA protein, and an anti-HA antibody was used for immunoprecipitation due to the lack of commercial anti-MIZ1 antibodies that work in our experimental conditions. The same set of experiments was done on the *p21CIP/WAF* proximal/core promoter, which served as a positive control (Supplementary Fig. S4).

These data suggest that MYCN associates with the same promoter regions of *TRKA* and *p75NTR* recognized by SP1 or MIZ1.

### MYCN binds SP1 and MIZ1 through distinct domains

To establish whether MYCN can interact with SP1 or MIZ1, we did coimmunoprecipitation assays in cell extracts. Specifically, HEK-293 cells were cotransfected with vectors expressing MYCN-FLAG along with a HA-tagged version of either SP1 (SP1-HA) or MIZ1 (MIZ1-HA). SP1 or MIZ1 was immunoprecipitated with HA antibodies, and the presence of MYCN was determined by Western blotting (WB). We found that MYCN can interact with both proteins (Fig. 3A). Furthermore,

**Figure 3.** MYCN can interact with both SP1 and MIZ1 through distinct domains. **A**, a FLAG-MYCN protein was expressed in HEK-293 cells along with either SP1-HA or MIZ1-HA. Coimmunoprecipitation was done with an anti-HA monoclonal antibody (IP-HA), and co-IP products were probed with an anti-FLAG antibody in WB. **B**, coimmunoprecipitation of endogenous proteins. Protein complexes immunoprecipitated with either an anti-SP1 or an anti-MIZ1 antibody were probed with an anti-MYCN in WB. **C**, GST-MYCN proteins were used in a GST pull-down assay. Immobilized GST-MYCN proteins were loaded with *in vitro*-translated SP1-HA or MIZ1-HA proteins. GST-MYCN complexes were analyzed by WB. **D**, MYCN proteins lacking the MYCN domains interacting with SP1 or MIZ1 were tested on the *TRKA*-luc and the *p75NTR*-luc reporters in SH-SY5Y cells. The relative luciferase activity determined by a full-length MYCN and derived mutants was compared with that obtained with the empty vector, which was set to 1. Results represent the average  $\pm$  S.E. of 3 independent transfections.



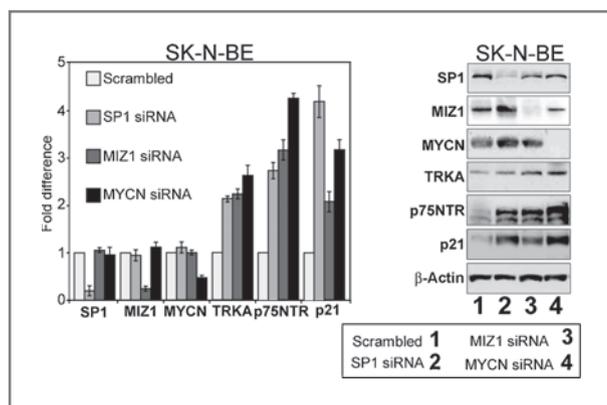
these interactions were confirmed by showing that MYCN coimmunoprecipitated with endogenous SP1 and MIZ1 from SK-N-BE cells (Fig 3B). The interaction was judged to be specific because, used in the same conditions, MAD, a bZIP protein of the MYC-MAX family, did not coimmunoprecipitate (Fig. 3A, right).

Next, to map the regions of MYCN involved in its interaction with SP1 and/or MIZ1, several GST-MYCN mutants were constructed and used in a GST pull-down assay with *in vitro*-translated SP1 and MIZ1 proteins (Fig. 3C). The results show that the region between aa 82 and 136 of MYCN, carrying the MBII domain, could specifically pull down SP1, whereas the region between aa 400 and 464, carrying the bHLHZip domain, could pull down MIZ1. Thus, SP1 and MIZ1 interact with distinct regions of MYCN.

To demonstrate a functional relevance of the regions of MYCN binding SP1 and MIZ1, a plasmid expressing MYCN mutants with either one of the 2 domains having been deleted, were cotransfected along with the *TRKA* or the *p75NTR* luciferase reporter (Fig. 3D). Results show that MYCN proteins missing either the MBDII or the bHLHZip domain could not repress the transcription of the luc reporters (Fig. 3D). On the other hand, a MYCN protein carrying a deletion between aa 248 and 362 but leaving the MBDII and bHLHZip domains intact still retained the ability to repress transcription efficiently; thus, suggesting that both domains are critical for MYCN-mediated repression.

### SP1 and MIZ1 are both required for MYCN-mediated repression *in vivo*

To support this model, we have systematically silenced the expression of MYCN, SP1, or MIZ1 in SK-N-BE cells by using specific siRNAs and analyzed the resulting expression of endogenous *TRKA* and *p75NTR* (Fig. 4). Results show that the silencing of any of the 3 transcription factors individually



**Figure 4.** Cellular depletion of SP1, MIZ1, or MYCN induces the expression of *TRKA* and *p75NTR*. SK-N-BE cells were treated with siRNAs against SP1, MIZ1, or MYCN respectively. A scrambled siRNA was used as a negative control. siRNAs employed include (1) scrambled, (2) SP1 siRNA, (3) MIZ1 siRNA, and (4) MYCN siRNA. The expression of SP1, MIZ1, MYCN, *TRKA*, and *p75NTR* mRNAs was determined by RT-PCR (left), whereas proteins were analyzed by WB (right). p21/CIP/WAF and  $\beta$ -ACTIN served as a positive and a negative control, respectively. Results are the average  $\pm$  SE of 4 independent experiments.

was sufficient to induce the re-expression of both *TRKA* and *p75NTR* mRNA and protein. Again, these findings are consistent with a model in which MYCN requires both SP1 and MIZ1 to exert a repression function.

To further corroborate this model, we turned to bioinformatic analysis. We searched for genes known to be regulated by SP1 and containing SP1 binding sites in close proximity to the transcription start site but lacking MIZ1 binding sites. One prediction is that this class of genes will not be repressed by MYCN even when MYCN is highly expressed. Then, we checked whether these genes were included in the list of MYC-responsive genes on the MYC Cancer Gene web site (<http://www.myc-cancer-gene.org>; ref. 34). If not, it was reasonable to suppose that such genes were responsive only to SP1 but not to MYC. Finally, genes that fit this criterion were further analyzed for the presence of MIZ1 binding sites in their core promoter regions.

Through this approach, we could identify Matrix Metalloproteinase 2 (*MMP2*) as one gene that carries SP1 binding sites but not MIZ1 binding sites in its core promoter.

Consistently, with these observations, we could show that SP1 stimulates the transcription of *MMP2* in SK-N-BE/SP1-i cells when induced by ponasterone, whereas MIZ1 could not (Supplementary Fig. S5A). *MMP2* did not change its transcription level as a function of MYCN expression in TET21/N, SK-N-BE, and LA-N-1 neuroblastoma cells, indicating that MYCN does not regulate this gene (Supplementary Fig. S5B). Furthermore, dual ChIP assay showed that SP1 can bind the *MMP2* promoter very efficiently, whereas MIZ1 and MYCN cannot (Supplementary Fig. S5C).

Taken together, the data suggest that, even in the presence of high cellular levels of MYCN, the binding of SP1 alone is not sufficient to recruit MYCN at gene promoters and that other factors, particularly MIZ1, appear to be important for the association of MYCN with repressed target genes.

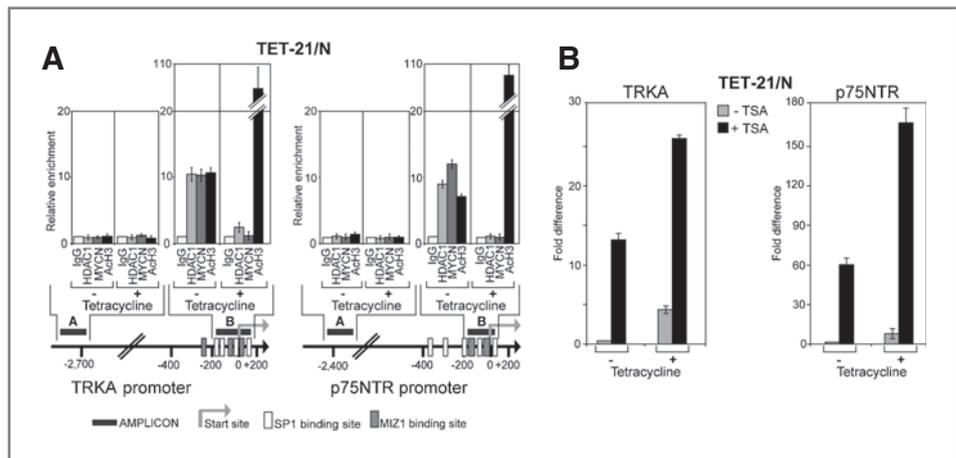
### SP1/MIZ1/MYCN repression complex recruit HDAC1 at the *TRKA* and *p75NTR* proximal/core promoter regions

Since we previously showed that MYCN can bind the histone deacetylase HDAC1 protein (35), we investigated whether HDAC1 was also present at the repressed (high MYCN) *TRKA* and *p75NTR* promoters in TET21/N, SK-N-BE, LAN-1 and SH-SY5Y cells. Indeed, ChIP assays show that HDAC1 is bound to the core promoter regions only when MYCN is coexpressed and that histone deacetylation is strongly dependent on MYCN expression (Figs. 5A and Supplementary Fig. S6A and B). To provide evidence that the repression is mediated, at least in part, through histone deacetylation, we attempted to reactivate the expression pharmacologically. The treatment of TET21/N cells with TSA for 48 hours led to a significant re-expression of both *TRKA* and *p75NTR* (Fig. 5B). These results suggest that MYCN represses gene transcription by recruiting HDAC1 and deacetylating chromatin.

### Re-expression of *TRKA* and *p75NTR* sensitizes neuroblastoma cells to NGF-mediated apoptosis

To examine the consequences of the reactivation of *TRKA* and *p75NTR* expression in neuroblastoma biology, we

**Figure 5.** MYCN mediates transcription repression by recruiting HDAC1 at *TRKA* and *p75NTR* promoters *in vivo*. A, dual ChIP was done as described in Figure 2C. The analysis was done with anti-HDAC1 and Pan-AcH3 antibodies. B, TET21/N cells were treated with TSA (250 nmol/L) for 48 hours. *TRKA* and *p75NTR* mRNA reactivation was monitored as a function of both TSA and MYCN expression. Results represent the average  $\pm$  SE of 3 independent experiments.



analyzed the effect of nerve growth factor (NGF) on SK-N-BE neuroblastoma cells that were induced to express good levels of *TRKA* and *p75NTR* by RNAi silencing of any of the SP1/MIZ1/MYCN transcription factors. Fluorescence activated cell sorting (FACS) assays show that NGF can induce massive apoptosis in cells re-expressing *TRKA* and *p75NTR* (Fig. 6A). Furthermore, the cleavage of PARP, determined by WB, was used as a biochemical marker of apoptosis (Fig. 6B). More importantly, similar results were obtained when the expression of *TRKA* and *p75NTR* was reactivated by treating cells with TSA (Fig. 6C).

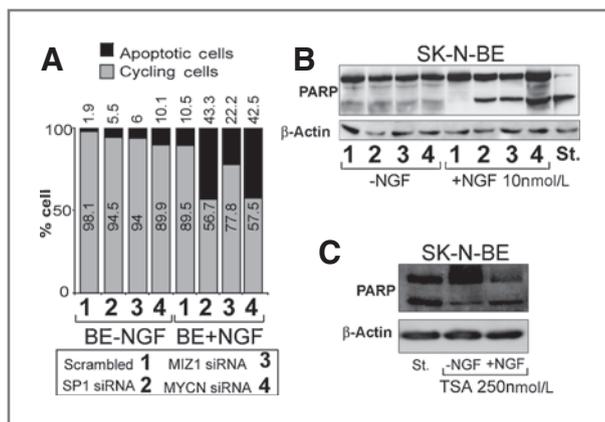
## Discussion

In this manuscript, we have analyzed the role of MYCN in the transcriptional repression of *TRKA* and *p75NTR*, 2 neurotrophin receptors, in neuroblastoma cells. Our results show

that MYCN exerts its function as a repressor by targeting the proximal/core promoter regions of the *TRKA* and *p75NTR*. MYCN appears to be physically engaged with the core promoter regions through interaction with 2 other transcription factors, SP1 and MIZ1. In part, this mechanism is similar to how *c-MYC* represses the *p21CIP/WAF* gene (33). However, we are the first to demonstrate that MYCN directly interacts with both SP1 and MIZ1 to downregulate transcription. More specifically, our data suggest that MYCN targets the *TRKA* and *p75NTR* core promoters by associating with a protein platform requiring the presence of both SP1 and MIZ1. Previous studies showed that *c-MYC*-mediated repression was linked to either SP1 or MIZ1 without clearly explaining whether the 2 factors acted independently or in a concerted fashion (30, 32, 33). Based on our findings, we propose that, although necessary, SP1 is not sufficient to mediate MYCN repression and that at least one other player, MIZ1, is needed to bring MYCN to repressed promoters.

How does MYCN specify transcription repression? One possibility is that it may interfere with the transactivation functions of SP1 and MIZ1, 2 positive transcription regulators. Another intriguing possibility is that MYCN, by its association with SP1 and MIZ1, may subsequently recruit specific chromatin enzymes that modify DNA or promote chromatin condensation. Although it has been shown that *c-MYC* can recruit DNMT3a at the *p21CIP/WAF* gene promoter (36), this is unlikely to occur at *TRKA* and *p75NTR* promoters because the CpG island regions within these promoters are totally unmethylated in several neuroblastoma cell lines and do not change with MYCN expression levels (Supplementary Fig. S7). On the other hand, we showed that MYCN can recruit HDAC1, and the overall histone acetylation of the core promoters of *TRKA* and *p75NTR* is low. Thus, we favor a model whereby MYCN, SP1, and MIZ1 form a platform to recruit HDAC1 to silence gene expression by deacetylating chromatin.

What is the biological relevance of this mechanism for neuroblastoma? It has been previously proposed that the expression of *TRKA* and/or *p75NTR* may be a favorable predictor of clinical outcome. Indeed, neuroblastomas at an advanced stage of progression lack *TRKA* and *p75NTR* expression, and transgenic mice overexpressing MYCN give rise to



**Figure 6.** Forced re-expression of *TRKA* and *p75NTR* sensitizes neuroblastoma cells to NGF-mediated cell death. A, SK-N-BE cells were incubated with scrambled siRNA (1), SP1 siRNA (2), MIZ1 siRNA (3), or MYCN siRNA (4) along with 10 nmol/L NGF for 48 hours. Cell death was determined via FACS, evaluating the extent of the sub-G<sub>1</sub> cell population peak. B, cell death was also monitored by a WB assay with an anti-PARP antibody: full-length (p113) and cleaved forms (p89) of PARP are shown. Staurosporine was used as a control. C, WB of PARP obtained from cells treated with TSA (250 nmol/L) with or without NGF (10 nmol/L) for 48 hours.

neuroblastomas that progressively lose the expression of *TRKA* and *p75NTR* (8–10). Surprisingly, our findings show that resumed expression of *TRKA* and *p75NTR* sensitizes neuroblastoma cells to NGF-mediated apoptosis. Indeed, the effect of NGF on cells coexpressing *TRKA* and *p75NTR* has often been linked to cell differentiation or growth inhibition. However, very recent studies have shown that, in addition to *p75NTR* and under certain circumstances, *TRKA* may trigger an apoptotic signaling (37–40). Therefore, it may be possible that, in our conditions and particularly when *TRKA* and *p75NTR* expression is forced, NGF can convey an intracellular cell death message through both receptors.

Thereby, 1 possible contribution of MYCN to the progression of neuroblastoma may be to desensitize them to NGF signaling by the repression of NGF receptors. This would allow neuroblastoma cells to escape cell death mediated by NGF and progress toward a more malignant phenotype.

In conclusion, our findings have provided a mechanistic rationale to the long-known correlation between MYCN amplification and overexpression, and by directly connecting MYCN to the repression of *TRKA* and *p75NTR*, we have established a key pathway of clinical pathogenicity and aggressiveness in neuroblastoma.

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## Disclosure of Potential Conflicts of Interest

All authors declare no competing financial interest in relation to the work described.

## Acknowledgments

We thank Manfred Schwab for the MYCN monoclonal antibody, Donatella Manzoni for excellent technical assistance with cell cultures, Robert N. Eisenman for critical reading, and Joseph C. Reese for critical reading and text revision.

## Grant Support

N. Iraci, A. Papa, A. Porro, E. Valli were supported by the PhD Program in Molecular and Cellular Physiology of the University of Bologna; S. Gherardi was supported by a senior fellowship from Programma Regionale per la Ricerca industriale, l'Innovazione e il Trasferimento Tecnologico (PRITT). This work was supported by grants from the Italian Ministry of Research and Education-PRIN (G. Della Valle, G. Perini), AIRC (G. Della Valle, G. Perini), PRITT (G. Della Valle), University of Bologna (G. Della Valle, G. Perini).

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Received July 19, 2010; revised September 29, 2010; accepted November 10, 2010; published OnlineFirst December 21, 2010.

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