⌘ *Author's Choice*

Physical Interaction between MYCN Oncogene and Polycomb Repressive Complex 2 (PRC2) in Neuroblastoma

FUNCTIONAL AND THERAPEUTIC IMPLICATIONS[*][S]

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Background: The neuroblastoma oncogene MYCN and the PRC2 members EZH2 and SUZ12 are regulators of gene transcription.
Results: MYCN and PRC2 form a repressive complex on the promoter of the tumor suppressor gene *CLU*.
Conclusion: PRC2 members are recruited by MYCN to repress gene expression and induce tumorigenesis.
Significance: Reactivation of MYCN-PRC2-repressed genes by epigenetic drugs could be of clinical value in neuroblastoma.

CLU (clusterin) is a tumor suppressor gene that we have previously shown to be negatively modulated by the MYCN proto-oncogene, but the mechanism of repression was unclear. Here, we show that MYCN inhibits the expression of *CLU* by direct interaction with the non-canonical E box sequence CACGCG in the 5′-flanking region. Binding of MYCN to the *CLU* gene induces bivalent epigenetic marks and recruitment of repressive proteins such as histone deacetylases and Polycomb members. MYCN physically binds *in vitro* and *in vivo* to EZH2, a component of the Polycomb repressive complex 2, required to repress *CLU*. Notably, EZH2 interacts with the Myc box domain 3, a segment of MYC known to be essential for its transforming effects. The expression of *CLU* can be restored in MYCN-amplified cells by epigenetic drugs with therapeutic results. Importantly, the anticancer effects of the drugs are ablated if *CLU* expression is blunted by RNA interference. Our study implies that MYC tumorigenesis can be effectively antagonized by epigenetic drugs that interfere with the recruitment of chromatin modifiers at repressive E boxes of tumor suppressor genes such as *CLU*.

Epigenetic modification of the DNA or associated proteins is thought to be involved in the control of gene expression in normal and pathological contexts. In cancer, many genes appear to be deregulated by aberrant rearrangements of the chromatin, which frequently occurs during neoplastic transformation. For example, methylation of the DNA is generally increased in cancer cells, leading to the silencing of tumor suppressor genes (1). Deacetylation of the histones, proteins tightly associated to the DNA, is also an important mechanism by which cancer cells reduce or silence the expression of genes that restrict cell proliferation or increase cell death (2, 3)

MYCN is a member of the MYC family of transcription factors frequently amplified in neuroblastoma, a childhood cancer originating from the peripheral nervous system (4). Amplification of *MYCN* is a poor prognostic factor in cancer patients, and transgenic expression of *MYCN* in the neuroectoderm causes neuroblastoma with high penetrance in mice (4, 5). Conversely, ablating the expression of MYCN in human neuroblastoma cell lines causes inhibition of their proliferation and stimulates apoptosis (6–8). Thus, in neuroblastoma patients carrying amplification of the gene, *MYCN* is likely to be required and sufficient to cause a fatal form of the disease.

It has been hypothesized that one of the mechanisms by which increased expression of *MYCN* drives tumorigenesis is by increasing the expression of cell cycle-related genes such as ornitine decarboxylase via direct promoter interaction and transactivation (9). More recently, it was observed that MYC proteins can also suppress gene expression indirectly, by interacting with sequence-specific transcription factors such as SP1 and MIZ1 and bringing transcriptional co-repressors near the transcription initiation site of the growth suppressor gene p21 (10–12). Using this mechanism, MYCN could induce transcriptional silencing of genes involved in negative regulation of cell proliferation and transformation. A further mechanism by which MYCN could mediate its oncogenic effects is by modulating microRNAs. We and others have shown that MYCN can induce the expression of transcripts of the 17–92 cluster of microRNAs. Among the targets of the 17–92 microRNA cluster, p21, *BIM*, and *CLU* appear to be critically involved in MYCN tumorigenesis (13–15). A sensible hypothesis is that the aberrant expression of MYCN could modify gene expression both via direct and indirect mechanisms.

In this study, we focus our attention on one of the MYCN-regulated genes, the putative tumor suppressor gene *CLU*. In a previous work, we showed that *CLU* is a suppressor of MYCN tumorigenesis *in vivo*, and silencing of *CLU* is required by MYCN to exert its malignant behavior (13). We noticed the presence of potential MYC-binding sites (also known as E box) in the human *CLU* promoter, so we wondered whether MYCN could modulate the expression of *CLU* via a direct mechanism.

EXPERIMENTAL PROCEDURES

Cell Culture—The human neuroblastoma cell lines SH-SY5Y, IMR-32, and HEK-293 cells were obtained from the American Type Culture Collection (ATCC). The human neuroblastoma cell lines LAN-1, Kelly, and Tet-Off 21N (stably transfected with a tetracycline-controlled transactivator protein and an expression vector encoding *MYCN* cDNA, which is switched off in the presence of Tet)[4] were described previously (16–18). Primary neuroblastoma cells were obtained by mechanically disaggregating a tumor resection from a patient with a MYCN-amplified, relapsing tumor. Written consent for the utilization of the tumor material was obtained from the family. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 50 μg/ml gentamicin, with the exception of Kelly, which were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin-streptomycin. For growth assays, cells were plated at a density of 1.5×10^5 cells/well in six-well plates. Cells were counted with a hemocytometer, and cell death was scored by trypan blue dye staining. For epigenetic drug treatments, cells were plated at a density of 5×10^4 cells/well in six-well plates. After 24 h, cells were exposed to trichostatin A (1 mM), valproic acid (1.5 mM), or 5-aza-2′-deoxycytidine (10 mM) for 24 or 48 h.

Plasmid Construction and Transfections—To generate the pGL2 clusterin WT reporter vector, the *CLU* human promoter region containing the putative MYCN binding site (E box) was first amplified by PCR from genomic DNA (the primers used were as follows: 5′-cgactggtaccctgtgtgtgctctcttctccagca-3′ (forward) and 5′-ttcgatcgaattggggctggctgcaaacctg-3′ (reverse)) and ligated in the TOPO vector using the TOPO TA cloning kit (Invitrogen). The *CLU* promoter segment was cut with KpnI and HindIII and subcloned into the luciferase pGL2 promoter vector (Promega Biosciences, Promega Corp., San Luis Oispo, CA). The pGL2 clusterin MUT reporter vector was obtained by mutation of the E box sequence located at −482 from the transcription start site (wt, cacgcg; mutant, TCTGCT) by site-directed mutagenesis (QuikChange multisite-directed mutagenesis kit, Stratagene). The MYCN expression vector lacking the MYC box domain 3 (MB3) domain (amino acids 187–241) was obtained by amplifying the plasmid pCMV14-MYCN-3Xflag (19) by PCR using the following primers: forward, ACCAGCGG-CGGCGACCACAA; reverse, GCACTCGGCGGCCGGGT-GGG. The PCR product was ligated and verified by sequencing. The pGL2 clusterin WT or the pGL2 clusterin MUT reporter vectors were transiently transfected in the presence or absence of

CMV-MYCN into SH-SY5Y cells using Lipofectamine 2000 reagent (Invitrogen). Luciferease assays were carried out 36 h post transfections. PcDNA 3.1(+)/His- MYCN, pSUPER.gfp+neo.*CLU* and pSUPER.gfp+neo.Scr have been described previously (13). SH-SY5Y cells were transfected with pcDNA 3.1(+)/His-MYCN, and a mix population was obtained after selection in medium containing 0.8 mg/ml G418 (Invitrogen) for 3 weeks. IMR-32 and LAN-1 cells were transfected with pSUPER.gfp+neo.*CLU* or pSUPER.gfp+neo.Scr plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were maintained in selection medium containing 1.0 mg/ml G418 for 3 weeks to select individual clones with or without expression of *CLU*.

In Vitro Invasion and Cell Viability Assays—*In vitro* invasion assay was carried out as reported previously (13). Briefly 2.5 × 10^4 neuroblastoma cells were resuspended in serum-free medium and seeded on the top of the invasion assay chamber following manufacturer's instructions (BD Biosciences). Medium containing 10% fetal calf serum served as chemo-attractant in the bottom chamber.

Electrophoretic Mobility Shift Assay—Nuclei were prepared from SHSY-5Y cells stably transfected with empty or MYCN expressing CMV vector. Cells were harvested in hypotonic buffer (10 nM Tris-HCl, pH 7.8, 5 mM KCl, 2 mM MgCl$_2$, 1 mM DTT, protease inhibitors) and subsequently lysed in the hypotonic buffer supplemented with 0.25% Nonidet P-40 for 5 min on ice. Nuclei were pelleted, washed twice with hypotonic buffer, and suspended in 2 volumes of hypotonic buffer containing 0.3 M NaCl. The suspension was kept on ice for 30 min and centrifuged, and the supernatant was used directly for EMSA. Double-stranded DNA oligomers containing the wild type (5′-GGGGCTCCCAGATGGGCACGCGAGTTCAGG-CTCTTCC-3′) or mutant (5′-GGGGCTCCCAGATGGGCT-TAGGAGTTCAGGCTCTTCC-3′) putative MYCN-binding site (underlined) were labeled with [γ-^{32}P]dATP and used as probes for gel shift analysis. Nuclear cell extract (5 μg) and 1 ng of probes were mixed for 30 min at room temperature in 25 μl of binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 4% Ficoll 400 (v/v), 1 mM DTT, 1.5 μg poly(dI-dC)). Shifted protein complexes were resolved by electrophoresis in a 5% native polyacrylamide gel.

Standard ChIP and Dual Chromatin Immunoprecipitation Assays—Standard ChIP and dual ChIP assays were performed as described (19). Antibodies used in this study were as follows: IgG (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA), GAL4 (IgG2A negative control) (Santa Cruz Biotechnology), MYCN (sc53993, Santa Cruz Biotechnology), Max (sc197, Santa Cruz Biotechnology), HDAC1 (Ab7028, Abcam), HDAC2 (05-814, Upstate), Bmi1 (Ab 14389), EZH2 (5246, Cell Signaling), SUZ12 (Ab12073, Abcam), AcH3 (07-599, Upstate, H3K4-2Me (07-030, Upstate), H3K9-3Me (07-442, Upstate), H3K27-3Me (07-449, Upstate), H4K20-3Me (07-463, Upstate). The primers used were as follows: BS2 (E box), 5′-ttgtgtcttggactg-ggaca-3′ (forward) and 5′-gaaagcaagggggagctttct-3′ (reverse); −1000, 5′-tccatagtcctgatcctgaactg-3′ (forward) and 5′-tttggagc-cagggatgtttaag-3 (reverse); +1000, 5′-gtggagcattgggcacaactg-3′ (forward) and 5′-ccagaggcaaaggttagcactg-3′ (reverse); +3000, 5′-

ccttgttaatgtgctacttgagtgtc-3′ (forward) and 5′-tcacagaaccaacag-gacgatg-3′ (reverse).

GST Pulldown Assay—The different MYCN segments were cloned into the pGEX-3X plasmid, in frame with GST. Recombinant 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 EZH2 protein (TNT® Quick Coupled Transcription/Translation System, Promega, Madison, WI) pretreated with DNase (GE Healthcare, Waukesha, WI). Purified complexes were separated on SDS-PAGE and analyzed by Western blotting using anti-EZH2 antibodies (5246, Cell Signaling).

Co-immunoprecipitations—For co-immunoprecipitation experiments, we used IMR32 because this cell line showed the strongest expression of Polycomb repressive complex 2 members compared with other MYCN-amplified neuroblastoma cell lines. Cells were washed twice in PBS and lysed in 3.2 ml of IP buffer (20 ml of HEPES, 175 mM NaCl, 10% glycerol, 0,5% Nonidet P-40, 1 mM EDTA, 1 mM DTT, protease, and phosphatase inhibitors) and kept on ice for 30 min. Cell debris were eliminated by centrifugation, and the supernatant was collected and incubated with 10 mg of the following antibodies: SUZ 12 (ab 12073, Abcam), EZH2 (clone AC22, Upstate, Millipore), MYCN (sc-53993, Santa Cruz Biotechnology). As control antibodies, we used Fyn (sc-434, Santa Cruz Biotechnology) and TRAF2 (N-19, Santa Cruz Biotechnology). After six washes in 1× PBS, immunoprecipitated samples were run onto a SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was blocked in PBS containing 5% dry milk and 0.1% Nonidet P-40 and incubated with the different antibodies overnight at 4 °C. The filters were then incubated with the appropriate secondary antibody labeled with horseradish peroxidase, followed by incubation with a chemiluminescent substrate (ECL, Amersham Biosciences). Proteins were detected by exposing the nitrocellulose filter to an autoradiographic film. All experiments were repeated for at least three times in duplicates. All data for statistical analysis were calculated as means ± S.E. Differences among groups were determined with an unpaired *t* test. A probability value of $p = 0.05$ or less was considered significant.

Analysis of Acetylated Histones—LAN-1 cells were treated with 300 nM TSA, 3 mM valproic acid or a combination of both drugs for 24 and 48 h. After treatment, nuclei were obtained by resuspending cells in low salt buffer (10 mM HEPES, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM sodium flourophosphate, 1 mM PMSF, and protease inhibitor mixtures). After centrifugation, nuclei were lysed in high salt buffer (10 mM HEPES, pH 8, 450 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM sodium fluorophosphate, 1 mM PMSF, and protease inhibitor mixture). 20 μg of nuclear extracts were loaded onto a SDS-PAGE and analyzed by Western blot analysis. AcH3 (07-599) and H1 (05-547) antibodies were from Millipore.

RESULTS

MYCN Binds to the 5′-Flanking Sequence of the CLU Gene in Vitro and in Vivo—Scanning of the human *CLU* gene revealed two potential E boxes (named BS1 and BS2) in close proximity of the transcription start site (Fig. 1*A*). We firstly investigated whether MYCN could interact with the putative E boxes *in vitro*. We quickly ruled out BS1 as a genuine E box sequence (data not shown), but a probe containing BS2 could be shifted by cell lysates containing exogenously expressed MYCN protein. The interaction was specific because it was ablated by point mutations in BS2 (Fig. 1*B*). We next assessed whether MYCN was bound to the *CLU* promoter in neuroblastoma cells *in vivo*. Chromatin IP analysis was carried out using the MYCN amplified LAN-1 and Kelly neuroblastoma cell lines or non-amplified SH-SY5Y cells. This assay demonstrated that endogenously expressed MYCN binds to the *CLU* promoter in neuroblastoma cells *in vivo* (Fig. 1*C*). As a control, we used primers that amplify a region of the *CLU* promoter that does not contain the E box. As expected, no amplification was observed using these primers after immunoprecipitation with the MYCN antibody (supplemental Fig. S1).

Bioinformatic Assessment of the BS2 E Box Sequence in Human Neuroblastomas—To verify whether BS2 could be found in other MYCN-regulated genes, we carried out a bioinformatic analysis asking the following question: is the sequence CACGCG enriched in the flanking region of genes whose expression is regulated with, and possibly by, MYCN in neuroblastoma tumors? We observed a significant and robust enrichment of the non-canonical E box BS2 in MYCN co-regulated genes in a large neuroblastoma data set (neuroblastoma prognosis database -Oberthuer Lab-) in the Oncogenomics repository (http://pob.abcc.ncifcrf.gov/cgi-bin/JK). The enrichment was equal or superior to that observed with the canonical E box sequence CACGTG (Table 1). Interestingly, only the non-canonical E box showed a slight but statistically significant enrichment in the promoter of genes whose expression is anti-regulated with that of MYCN (*i.e.* genes that have an expression pattern similar to *CLU*) (Table 2). Overall, the bioinformatic analysis indicates that the non-canonical E box BS2 is, similar to the canonical, involved in MYCN transcriptional activation of target genes. However, BS2 but not the canonical E box motif could also mediate MYCN repression of a set of genes in human neuroblastomas (the complete list of genes correlated and anti-correlated with BS2 is shown in supplemental Table S1).

The Non-canonical BS2 E Box Sequence Mediates Negative Regulation of the CLU Promoter by MYCN—To understand whether binding of MYCN to the *CLU* promoter was functionally relevant, we subcloned a section of the *CLU* promoter, containing BS2, in front of the luciferase gene. We also generated a luciferase vector with the *CLU* promoter containing point mutations that ablate the binding of MYCN (as shown in the gel shift assay in Fig. 1*B*). We then transfected the different constructs into SH-SY5Y neuroblastoma cells with or without a plasmid expressing MYCN. We observed that luciferase activity decreased in a statistically significant manner when the *CLU* promoter was co-transfected with the *MYCN* vector compared with the control vector. Importantly, mutation of the E box completely abrogated the repressive effect of MYCN, suggesting that binding to the E box is essential for negative regulation of the *CLU* promoter (Fig. 1*D*).

MYCN Causes a Bivalent Modification of the Chromatin Surrounding the E Box Binding Site in the CLU Promoter and Recruitment of Histone Deacetylases (HDACs) and Polycomb Proteins—To understand how mechanistically MYCN could cause transcriptional repression of the *CLU* promoter, we per-
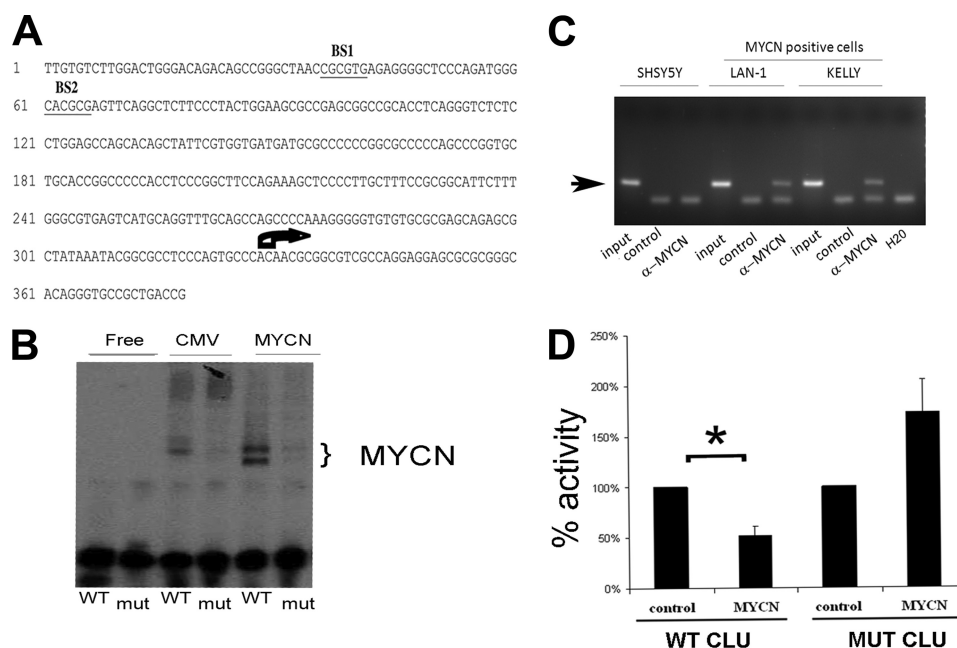
A

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                                          BS1
1    TTGTGTCTTGGACTGGGACAGACAGCCGGGCTAACCGCGTGAGAGGGGCTCCCAGATGGG
BS2
61   CACGCGAGTTCAGGCTCTTCCCTACTGGAAGCGCCGAGCGGCCGCACCTCAGGGTCTCTC
121  CTGGAGCCAGCACAGCTATTCGTGGTGATGATGCGCCCCCCGGCGCCCCCAGCCCGGTGC
181  TGCACCGGCCCCCACCTCCCGGCTTCCAGAAAGCTCCCCTTGCTTTCCGCGGCATTCTTT
241  GGGCGTGAGTCATGCAGGTTTGCAGCCAGCCCCAAAGGGGGTGTGTGCGCGAGCAGAGCG
301  CTATAAATACGGCGCCTCCCAGTGCCCACAACGCGGCGTCGCCAGGAGGAGCGCGCGGGC
361  ACAGGGTGCCGCTGACCG
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C

B

D

FIGURE 1. **MYCN binds to a non-canonical, repressive E box in the 5′-flanking region of *CLU*.** *A*, the promoter region of the human *CLU* gene proximal to the transcription start site (indicated by the *arrow*) is shown. Two E box-like sequences and putative binding **s**equences for MYCN are indicated by BS1 and BS2. *B*, gel shift assay. Cell lysates from SH-SY5Y cells stably transfected with empty CMV or MYCN vectors were mixed with radioactive probes encompassing the wild type (WT) or mutated (*Mut*) binding sequence BS2 as indicated. A specific complex containing MYCN is indicated by the *bracket*, and *free* indicates the probe without lysates. Note that naïve SH-SY5Y cells do express a small amount of endogenous MYCN, explaining the faint band shift observed in empty vector (CMV)-transfected cells. *C*, chromatin immunoprecipitation assay. The SH-SY5Y, LAN-1 and Kelly neuroblastoma cell lines were subjected to chromatin IP assays with MYCN or GAL4 (control) antibodies. A specific product (indicated by the *arrow*) is amplified with 10% of the input chromatin after immunoprecipitation with the MYCN, but not GAL4, antibody. *D*, a segment of the *CLU* promoter containing the putative BS2 binding site characterized in the gel shift analysis was subcloned upstream of the luciferase gene, in the wild type or mutant conformations, and co-transfected into non-MYCN amplified SH-SY5Y cells with or without the CMV-MYCN expression vector, as indicated. The activity of the promoters in the absence of MYCN was set to 100% and is indicated in the Y axis. S.D. are indicated by the *error bars*. Statistical significance (indicated by the *asterisk*) was assessed using the Student's *t* test ($p = 0.0413$; $n = 4$).

TABLE 1

E box motif enrichment in the MYCN co-regulated set of the Obertheuer data set

The canonical and BS2 sequence were scored in at least one (>0), 10% (>0.1), or 50% (>0.5) of the transcripts originating from MYCN co-regulated genes. The noncanonical sequence was scored in both orientations (indicated by $+/-$), whereas the canonical sequence was only scored in one orientation because it is a palindrome. Significant *p* values are shown in boldface type.

	p value	Enrichment
CACGTG		
>0	**8.55×10^{-3}**	1.24
>0.1	**1.31×10^{-3}**	1.32
>0.5	**1.13×10^{-4}**	1.65
CACGCG		
(+/		
−)		
>0	**6.98×10^{-5}**	1.35
>0.1	**1.55×10^{-5}**	1.40
>0.5	**1.07×10^{-7}**	1.74

TABLE 2

E box motif enrichment in the MYCN anti-correlated set of the Oberthuer data set

Significant *p* values are shown in boldface type.

	p value	Enrichment
CACGTG		
>0	0.210	1.15
>0.1	0.854	0.96
>0.5	0.629	1.09
CACGCG		
(+/		
−)		
>0	**0.001**	1.38
>0.1	**0.003**	1.36
>0.5	0.070	1.32

formed chromatin IP assays and quantitative PCR to quantitatively assess the binding of chromatin proteins, scanning ~5 kb of the *CLU* promoter surrounding the BS2 MYCN-binding site. For these experiments, we used a neuroblastoma cell line, SHEP 21N, which contains an expression vector (MYCN Tet-Off) that allows the conditional expression of MYCN. As expected, the MYCN signal was enriched in correspondence of the E box BS2 in the absence of tetracycline. MAX was also found bound to the E box, but its presence was not dependent on MYCN expression. Notably, although binding of MYCN was associated with active chromatin marks, such as acetylated histone H3 and dimethylated lysine 4 on histone H3, negative marks such as

trimethylated lysine 9 and 27 of histone H3 were observed immediately downstream of the E box. In agreement with this observation, several chromatin remodeling factors associated with transcriptional repression such as HDAC 1/2 and Polycomb complex factors (BMI1, EZH2, and SUZ12) were detected around the BS2 E box or 1000 bp downstream of the E box sequence in the presence of MYCN, respectively (Fig. 2). This "bivalent" configuration is typical of repressed, developmentally regulated genes, which are poised to be activated by physiological stimuli. Interestingly, in the absence of MYCN we observed a very strong increase of histone H3 pan-acetylation and dimethylated lysine 4, suggestive of a rearrangement of the chromatin compatible with transcriptional activation. Indeed, repressive proteins and negative epigenetic marks, such as the
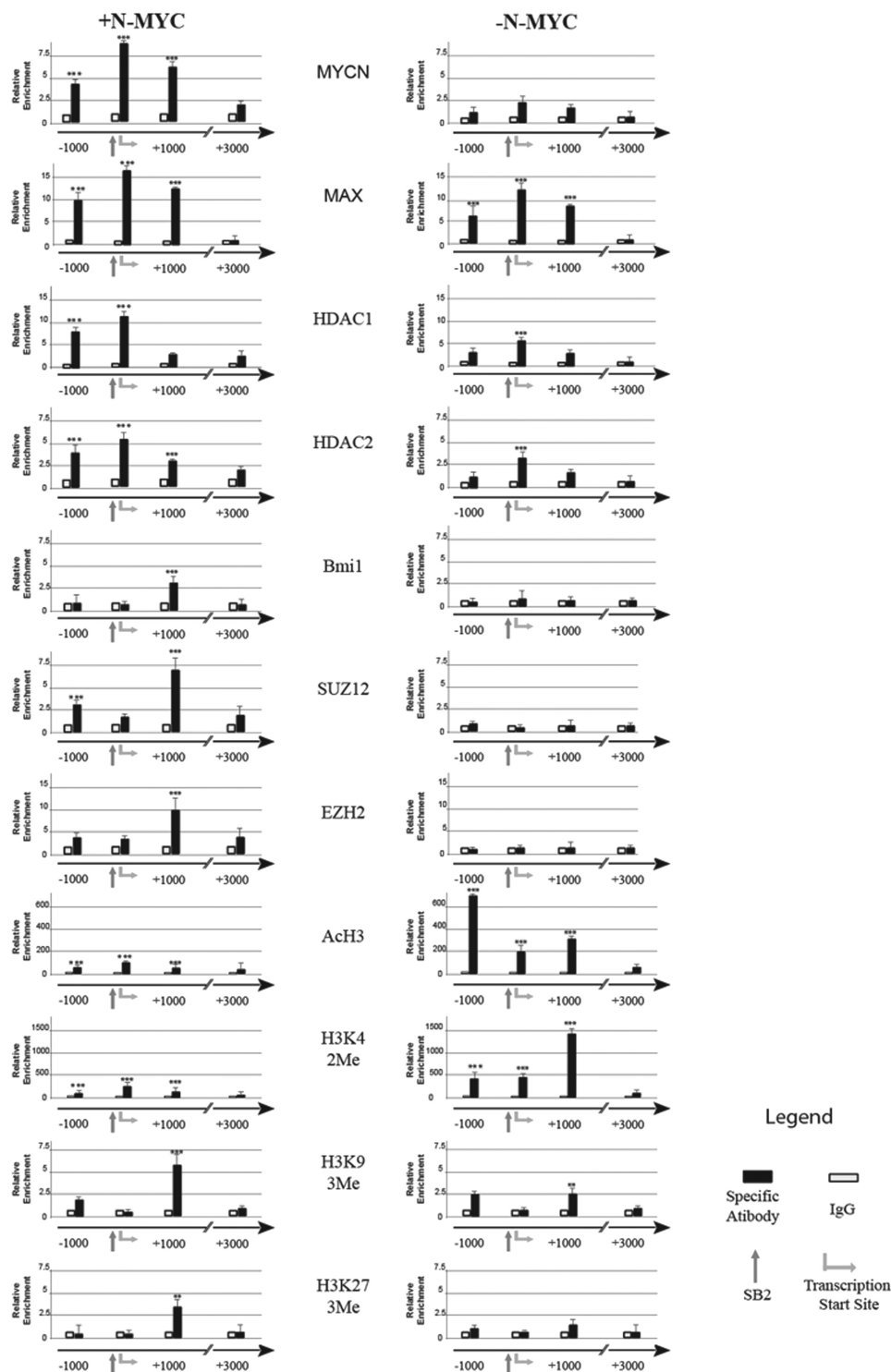
FIGURE 2. **Binding of MYCN on the *CLU* promoter is associated with recruitment of chromatin remodeling proteins and bivalent epigenetic marks.** Chromatin immunoprecipitation analysis of the *CLU* promoter region, surrounding the BS2 E box, was carried out in the presence or absence of MYCN, as indicated, using a Tet-Off system. Chromatin was immunoprecipitated using antibodies against the indicated proteins. The number of base pairs upstream ($-$) or downstream ($+$) the E box are indicated in the X axis. In the Y axis is indicated the fold of enrichment over the control IgG antibody. *Error bars* indicate S.D. ***, $p < 0.001$; **, $p < 0.01$ ($n = 3$).

Polycomb factors and trimethylated lysine 9 and 27 on histone H3, were reduced or absent (Fig. 2).

MYCN Interacts with the PRC2 Complex via EZH2—Enrichment of polycomb members in the proximity of the BS2 binding site prompted us to investigate whether MYCN could physi-

cally recruit inhibitory Polycomb proteins at the *CLU* promoter. Co-immunoprecipitation experiments indicated that endogenous EZH2 is physically associated with MYCN in *MYCN*-amplified IMR32 cells (Fig. 3*A* and supplemental Fig. S2). The histone methyltransferase EZH2 and SUZ12 are part
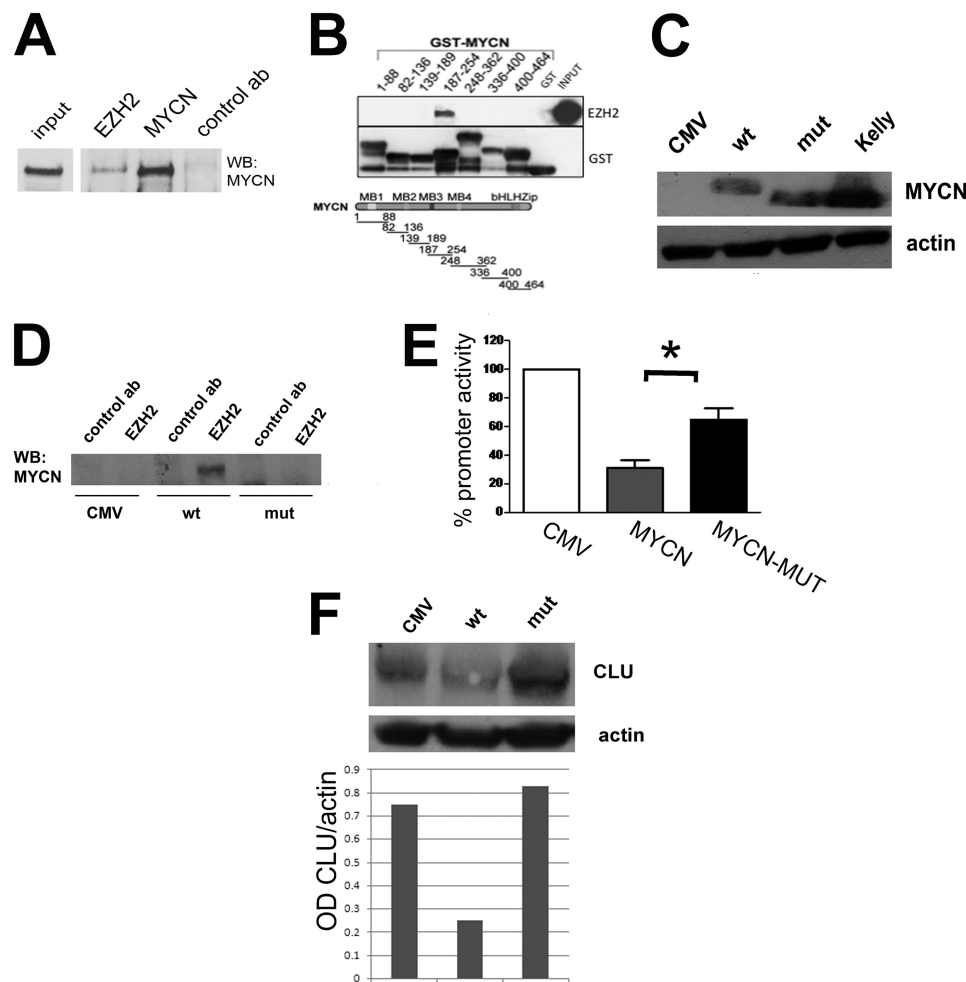
FIGURE 3. **MYCN physically interacts with Polycomb repressive complex 2 via EZH2.** *A*, co-immunoprecipitation assay showing the interaction of endogenous EZH2 and MYCN in IMR-32 cells. Cell lysates were immunoprecipitated with the antibodies indicated on the *top* of the gel and subjected to Western blot analysis (*WB*) with the MYCN antibody. *B*, immobilized GST-MYCN polypeptides were incubated with equal amounts of the *in vitro* translated EZH2 protein, separated by SDS-PAGE, and probed with an anti-EZH2 antibody. The diagram in the *bottom* shows full-length MYCN protein in which the functional domains are indicated by different shades of *gray*. The MYCN segments cloned in the GST expression vector are in *black*, and *numbers* indicate amino acid positions. *C*, Western blot analysis showing equal expression of the wild type and mutant (*mut*; lacking amino acids 187–241) MYCN proteins in SH-SH5Y cells stably transfected with the different MYCN constructs. The *MYCN*-amplified cell line Kelly is used as a positive control for MYCN expression. *D*, co-immunoprecipitation analysis in SH-SY5Y cells transfected with the wild type or mutant MYCN constructs showing interaction of EZH2 wild type, but not mutant, MYCN protein. *E*, luciferase assay of SH-SY5Y cells expressing the wild type or mutant MYCN constructs, transiently transfected with the *CLU* promoter. *Error bars* indicate S.D., and the *asterisk* indicates statistical significance (Student's *t* test; $p = 0.01$, $n = 4$). *F*, Western blot analysis showing expression of *CLU* in SH-SY5Y cells stably transfected with the wild type or mutant MYCN plasmids. The *bottom panel* depicts the densitometric analysis of MYCN expression relative to actin.

of a complex called Polycomb repressive complex 2 that methylates lysine 27 on histone H3 (21). As expected, EZH2 and SUZ12 are tightly associated in IMR32 cells (supplemental Fig. S2), suggesting that MYCN could bind to the EZH2-SUZ12 complex. MYCN interacts with EZH2 via a short segment in the central part of the protein containing the MB3 (Fig. 3*B*). Notably, a mutant version of MYCN lacking the MB3 domain and incapable to bind to EZH2 *in vitro* and *in vivo* (Fig. 3, *C* and *D*, and supplemental Fig. S3) was significantly less efficient as a transcriptional repressor than the wild type MYCN protein in luciferase assays, although it bound to the non-canonical E box CACGCG *in vitro* with an efficiency similar to that of the wild type protein (Fig. 3*E* and supplemental Fig. S4). Furthermore, the MYCN mutant was unable to inhibit endogenous *CLU* expression in SH-SY5Y neuroblastoma cells (Fig. 3*F* and supplemental Fig. S5). The direct role of EZH2 in repressing *CLU* transcription in neuroblastoma cells has been recently demon-

strated in a study from Thiele and co-workers (22). Overall, these results indicate that MYCN recruits EZH2 to the *CLU* promoter to induce transcriptional silencing.

Therapeutic Potential of CLU Reactivation in MYCN-amplified Neuroblastomas—In the next set of experiments, we assessed whether *CLU* reactivation could have clinical relevance in the context of *MYCN*-amplified tumors. HDAC inhibitors are compounds that inhibit, in addition to their specific HDAC targets, Polycomb group proteins (23). Exposure of the *MYCN*-amplified cell lines LAN-1 and Kelly to valproic acid and trichostatin A but not the demethylating drug azacytidine strongly induced *CLU* expression after 24 h (Fig. 4*A*). Notably, treatment of neuroblastoma cells with trichostatin A or valproic acid alone or in combinations caused a marked increase of pan-acetylated histone H3 of LAN-1 cells, strengthening the hypothesis that the reversal of epigenetic silencing at the *CLU* locus is responsible for the reactivation of gene expression (sup-
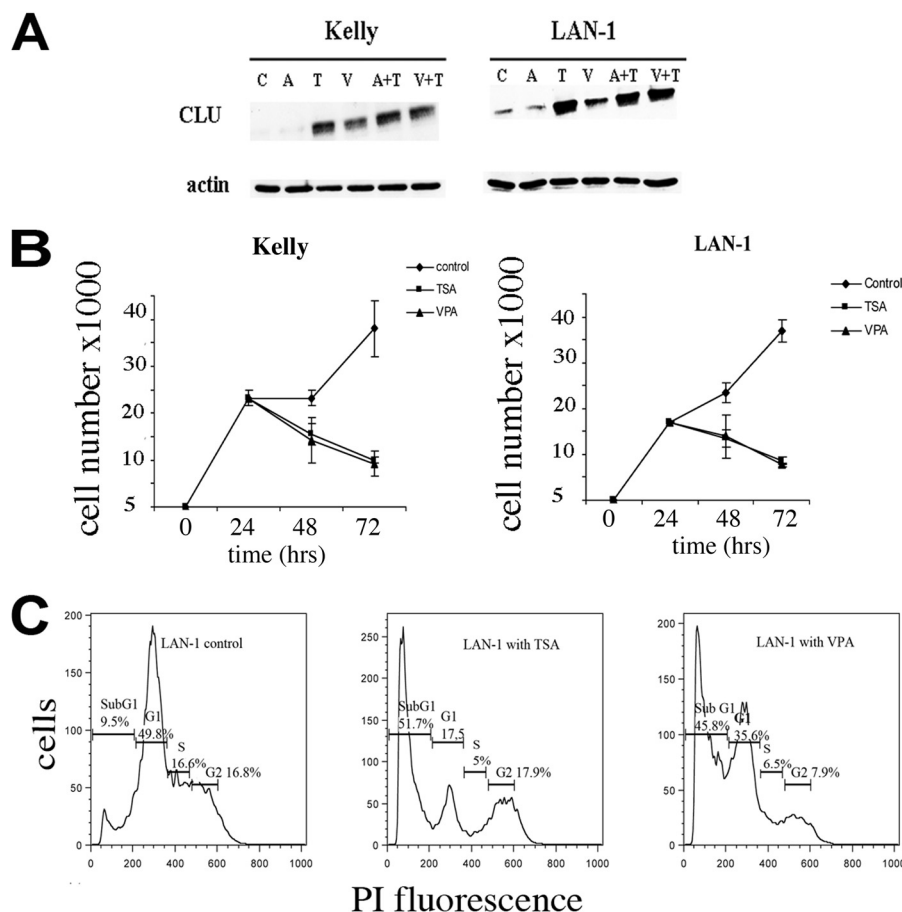
FIGURE 4. **HDAC inhibitors reactivate *CLU* expression in MYCN amplified cell lines.** *A*, MYCN amplified cell lines Kelly and LAN-1 were exposed to azacytidine (*A*), trichostatin A (*T*), valproic acid (*V*), or their combinations as indicated. *C* indicates control untreated cells. Expression of *CLU* or actin used as a reference control was assessed by Western blot analysis. *B*, proliferation curves of Kelly and LAN-1 cells in the presence or absence of TSA or valproic acid (*VPA*). The *error bars* indicate S.D. of values from triplicate wells. The experiment was repeated twice with similar results; a representative experiment is shown. *C*, cell cycle profiles of LAN-1 cells were evaluated after 48 h in the presence or absence of trichostatin A or valproic acid. The experiment was repeated twice with similar results; a representative experiment is shown. *PI*, phosphatidylinositol.

plemental Fig. S6). Next, we investigated whether the epigenetic drugs caused modifications of the biological behavior of the neuroblastoma cell lines. We first evaluated the proliferation rates of LAN-1 and Kelly in the presence or absence of drugs. Trichostatin A and valproic acid strongly inhibited the proliferation of neuroblastoma cells (Fig. 4*B*). The inhibitory effect was caused by apoptosis, indicated by the presence of a large increase of cells with fragmented sub-G_1 DNA, and reduced cell cycle activity, demonstrated by decreased number of cells in the S phase (Fig. 4*C*).

Short term cultures of cells dissected from fresh tumors deriving from a *MYCN* transgenic mouse (TH-*MYCN*) or a child with metastatic neuroblastoma with amplification of MYCN were cultured in the presence or absence of TSA. We observed that, in the presence of the drug, *CLU* expression was reactivated in parallel with changes in cell cycle profiles and induction of sub-G_1 fragmented DNA, diagnostic of apoptosis, validating the effect of HDAC inhibitors in primary tumor cells (Fig. 5). The induction of apoptosis in human neuroblastoma cells was confirmed by annexin V staining (supplemental Fig. S7).

Valproic acid is an orally available drug currently used in the clinic as an antiepileptic; thus, it was of interest to investigate whether it showed anticancer activity in xenotransplantation experiments. We injected the flanks of immunodeficient mice with LAN-1 cells and randomize the mice in valproic acid and control groups. There was a significant suppression of tumor growth in mice consuming valproic acid, suggesting that the drug could potentially be useful for the treatment of this childhood neoplasia (supplemental Fig. S8).

CLU Reactivation Is Key for the Anti-invasive effects of Epigenetic Drugs on MYCN-expressing Tumor Cells—To understand whether reactivation of *CLU* is required to mediate the biological effects of trichostatin A and valproic acid, we transfected the LAN-1 (*MYCN*-amplified) neuroblastoma cell line with a *CLU*-specific shRNA construct validated in a previous study (13). After selection with G418, we chose two clones that showed a reduced expression of *CLU* in the presence or absence of epigenetic drugs (Fig. 6*A*). We showed previously that *CLU* inhibits the metastatic potential of neuroblastoma cells (13, 24), we thus investigated whether the epigenetic drugs acted as inhibitors of neuroblastoma metastasis using an *in vitro* invasion assay. Invasion of LAN-1 cells was significantly inhibited in the presence of the epigenetic drugs. This effect was not a consequence of cell death because neuroblastoma cells were exposed to the drugs for 24 h, and only viable cells were seeded
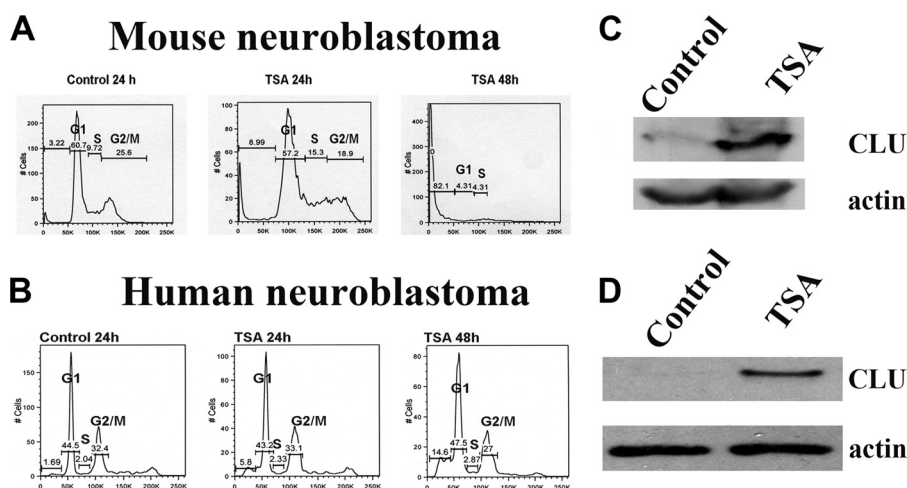
FIGURE 5. **Trichostatin A induces apoptosis and *CLU* reactivation in primary neuroblastoma cells.** Mouse neuroblastoma cells from MYCN transgenic mice (*A*) or a human neuroblastoma metastasis (*B*) were cultured *in vitro* in the presence or absence of TSA for 24 h. Cells were stained with propidium iodide, and cell cycle profiles were assessed by FACS analysis. Western blot analysis with a *CLU* antibody confirmed that *CLU* is reactivated after incubation of mouse (*C*) and human (*D*) neuroblastoma cells with TSA.

for the assay, which was carried out in the absence of the drugs. Importantly, ablation of *CLU* by the shRNA construct completely abolished the antimetastatic effects of the epigenetic drugs (Fig. 6*B* and supplemental Fig. S9).

DISCUSSION

CLU is a multifunctional protein that we have recently shown to act as a haploinsufficient tumor suppressor gene in neuroblastoma. MYCN inhibits the expression of *CLU* mRNA and protein in neuroblastoma cell lines and primary tumors in part by inducing oncogenic microRNAs belonging to the miR17–92 cluster (13). However, the miR17–92 cluster inhibits *CLU* indirectly, through the TGF-β pathway (25). The presence of MYC binding motifs in the *CLU* promoter prompted us to ask whether MYCN could regulate *CLU* expression directly.

We first observed that MYCN binds to and transcriptionally regulates the *CLU* gene in a negative manner, through a non-canonical E box element in the 5′-flanking sequence. MYC proteins have been shown to bind to different versions of the classical E box motif "CACGTG," but the E box characterized in this study "CACGCG" is peculiar because it mediates negative instead of positive regulation. In previous studies, it was reported that MYCN represses other tumor suppressor genes indirectly, by interacting with the transcription factors SP1 and MIZ1 (12, 19, 26). To our knowledge, our study is the first to report that a MYC member causes transcriptional repression by recruiting histone-modifying enzymes such as Polycomb proteins via an E box. MYCN interacts with the Polycomb member EZH2 through the MYC box domain 3, MB3. This is a notable finding because it was shown that this element is crucially required for MYC transcriptional repression, and deletion of MB3 impairs the transforming activity of MYC (27). An intriguing hypothesis stemming from these observations is that the interaction between the MB3 element and EZH2 critically links MYC transforming activity to the recruitment of Polycomb transcriptional repressors.

Another notable finding of our study is that the binding of MYCN to the *CLU* promoter causes a chromatin rearrange-

ment that is compatible with the bivalent state, typical of developmentally regulated genes in stem cells or genes silenced in cancer (28–30). Thus, MYC oncoproteins could confer stem-like features and aggressive phenotype to cancer cells by physically recruiting Polycomb group proteins and HDACs at tumor suppressor genes such as *CLU* and inducing the bivalent epigenetic state. This notion is corroborated by a recent study from the Einsenman group in which c-MYC was shown to impose widespread changes in histone methylation patterns in ES cells. Furthermore, it was noted that a subset of Polycomb-bound genes with bivalent histone methylation patterns are bound and regulated in response to altered c-MYC levels (31). It will be of interest to assess whether the ability of c-MYC to induce cell reprogramming and/or transformation also involves the formation of bivalent epigenetic marks around E boxes at the promoters of other tumor suppressor genes.

Infants and children with cancer are particularly sensitive to the toxic effects of chemotherapeutic drugs, which cause a number of adverse sequelae, including cognitive impairments, retarded development, and secondary neoplasms (4, 32). In neuroblastoma, amplification of *MYCN* marks a class of patients with metastatic disease whom need to be treated aggressively. Despite therapy, many children die with relapsing disease. It is therefore essential to explore other therapeutic avenues to find a treatment approach that is more effective and safer for children with aggressive forms of neuroblastoma.

There is a growing interest in histone deacetylase inhibitors for cancer therapy because they are relatively safe and have shown promising results in clinical trials (33, 34). An important advance in the understanding of the mechanisms of action and the clinical potential of these compounds would be to determine the critical biomarkers that predict a positive response to treatment. For example, in a cohort of patients with cutaneous T cell lymphoma, oral administration of the HDAC inhibitor lbh589 caused the up-regulation of 23 genes in biopsies taken after the treatments (20). We have shown here that MYC-mediated repression of *CLU* can be reversed by HDAC inhibitors,
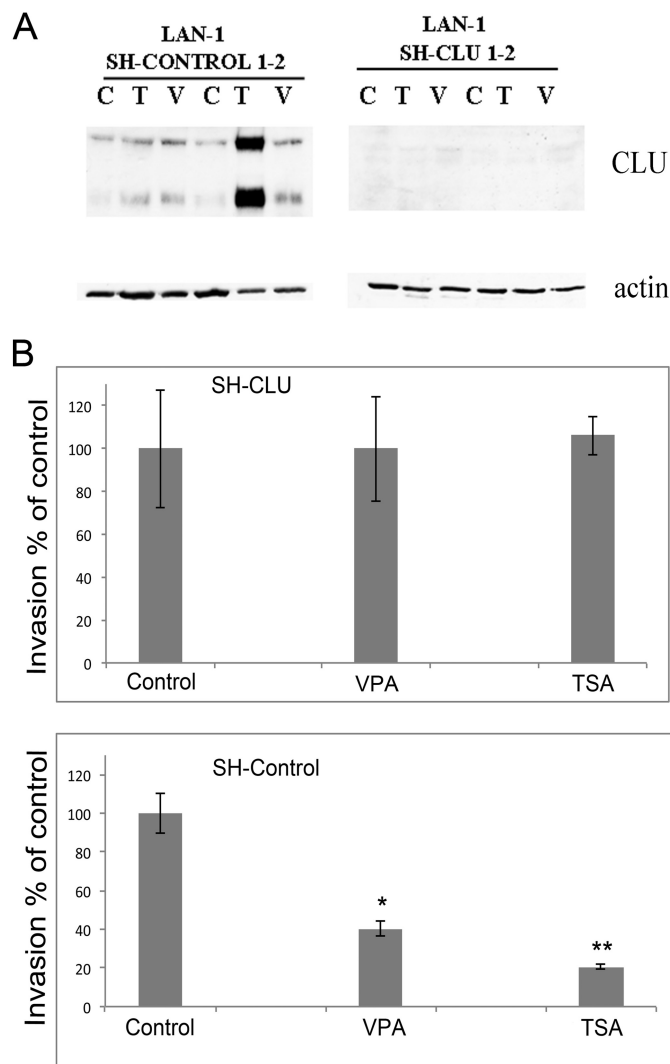
FIGURE 6. ***CLU* mediates the antimetastatic activity of HDAC inhibitors in *in vitro* invasion assays.** *A*, Western blot assays showing the expression of *CLU* or actin used as a loading control in LAN-1 cell lines transfected with a shRNA control (the shRNA control 1 cell line is shown in *lanes 1–3*, and the shRNA control 2 in *lanes 4–6*) or an shRNA vector targeting *CLU* (the shRNA *CLU* 1 and 2 cell lines are shown in *lanes 1–3* and *4–6*, respectively). *T*, trichostatin A; *V*, valproic acid; *C*, vehicle control. *B*, LAN-1 cells with or without *CLU* expression (clone 1) were plated in the top wells of *in vitro* invasion chambers after 24 h of exposure to the HDAC inhibitors TSA and valproic acid (*VPA*). Only live cells (as assessed by trypan blue dye exclusion assay) were plated in the invasion chamber. The number of vehicle-treated cells seeded in the top well divided by the number of cells passing the Matrigel membrane and recovered in the bottom chamber was set as 100% invasion index. The *error bars* indicate S.D. from triplicate wells. *, $p = 0.001$; **, $p = 0.0006$ ($n = 3$).

resulting in anticancer activity. In the clinic, the presence of CLU, a secreted protein, in the sera of cancer patients could serve as a biomarker to predict a favorable response after treatments with epigenetic drugs.

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