Supporting Information

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SI Materials and Methods

Datasets. The tumor and normal series used in this study were profiled on Affymetrix U133 arrays and normalized using MAS5.0, with the exception of NB251.

NB88 [R2 annotation: Tumor Neuroblastoma–Versteeg–88–MAS5.0–u133p2; accession no. GSE16476], NB51 [Tumor Neuroblastoma–Hiyama–51–MAS5.0–u133p2; accession no. GSE16237], and NB30 (Tumor Neuroblastoma–Lastowska–30–MAS5.0–u133p2; accession no. GSE13136 (1)] contain neuroblastoma tumors of all stages. NB64 [R2 annotation: Tumor Neuroblastic mixed–Delattre–64–MAS5.0–u133p2; accession no. GSE12460 (2)] consists of 64 neuroblastic tumors, including 53 neuroblastoma tumors of all stages. NB251 [R2 annotation: Tumor Neuroblastoma–Oberthuer–251–amexp255 consists of tumors of all stages and was analyzed on a two-color array (3).

Normal tissue expression profiles [Normal Various–Roth–504–MAS5.0–u133p2; accession no. GSE7307] of 504 samples were divided in nine groups, and a group was added containing all adrenal samples available in the database.

ChIP-on-Chip Analysis. Nine 15-cm dishes of subconfluent IMR32 cells were treated with 1% (vol/vol) formaldehyde for 10 min at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 125 mM. Cells were washed with cold PBS. Nuclei were obtained by harvesting cells in 5 mL swelling buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet-P40, protease inhibitors), incubation on ice for 30 min, homogenation using a syringe and needle (23G), and centrifugation (4500 g) at 4 °C. Nuclear lysates were sonicated five times on ice for 30 s with an Ultrasonic Processor (Sonics) at an amplitude of 30%. The nuclear lysates were divided in 2 × 0.75 mL for MYCN-immunoprecipitation (IP), 2 × 0.75 mL for FLAG-IP (control), and input/chromatin check. The IP samples were six times diluted in ChIP-dilution buffer [1% (vol/vol) TritonX-100, 150 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, protease inhibitors] and precleared (250 μL protein A and 10 μL haring sperm DNA [Roche]) for 30 min at a temperature. ChIP was performed with either 8 μg of monoclonal anti-MYCN antibody (clone B8.4.B, BD Pharmingen) or 8 μg anti-FLAG-antibody (Stratagene) overnight at 4 °C. (The input sample was processed without antibody and protein A beads.) Together with the lysates, 250 μL of protein A beads were incubated for 1 h at 4 °C. The beads were sequentially washed with cold low-salt wash buffer (0.1% SDS, 1% TritonX-100, 150 mM NaCl, 20 mM Tris·HCl, 2 mM EDTA, protease inhibitors), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 500 mM NaCl, 20 mM Tris·HCl, 2 mM EDTA, protease inhibitors), LiCl buffer (1% deoxycholate, 1% Nonidet P-40, 250 mM LiCl, 10 mM Tris·HCl, 1 mM EDTA, protease inhibitors), and finally twice with TE (10 mM Tris·HCl, 1 mM EDTA). DNA was eluted in 250 μL elution buffer (100 mM NaHCO3, 1% SDS, 10 μL), and 20 μL 5 M NaCl was added before de–cross-linking at 65 °C for 4 h. To both ChIP and input, 5 μL 0.5 M EDTA, 10 μL 1M Tris·HCl, pH 6.5, and 5 μL 10 mg/mL Prot K (Roche) was added and incubated at 45 °C for 1 h to degrade protein. RNA was degraded by adding 2.5 μL 10 mg/mL Rnase A (Roche) and incubating for 30 min at 37 °C. DNA was purified using Qiagen PCR purification kit (Qiagen) and quantified with Quant-IT Picogreen (Invitrogen). The specificity of the ChIP was checked by Q-PCR for NME1 (4). The recovered DNA (MYCN-ChIP, FLAG-ChIP and input) was ligation-mediated amplified as described by Ren and Dynlacht (5). Labeling of the material, hybridization to the 2.1 M Deluxe Promoter Array, scanning of the arrays, and peak calling were performed by NimbleGen, Inc.

ChIP-on-Chip Data Analysis. The ChIP-on-chip data, as provided by NimbleGen, were used for subsequent analysis. ^{2}Log signal ratios were used for visualization, and significant peak regions were extracted from the NimbleGen-generated files (*ratio_peaks_mapTo-Features_All_Peaks). Significant peak regions are colored according to the false discovery rate (FDR) score (red, ≤ 0.05; orange, ≤ 0.1; yellow, ≤ 0.2). FDR scores ≤ 0.05 represent the highest confidence of protein binding. Peaks with FDR score between 0.05 and 0.2 also are indicative of a binding site.

For cumulative analysis of binding, the transcription start sites (TSSs) were extracted from the (080814_HG18_Deluxe_Promoter_HX1) file. The TSSs were grouped when more than one TSS was reported for the same gene within 150 bases apart. Next, the data were binned in 50-bp blocks relative to the TSS (corrected for strand orientation), such that only measured regions were included. If a 50-bp block overlapped with a significant region, as described above, then it was marked with 1. If a region was measured, but no significant binding was scored, then it was marked with 0. If the 50-bp bin was not measured on the array, it was marked with "na." Percentage graphs were subsequently made by determining how many of the TSSs contained binding in the 50-bp boxes, excluding those boxes that were not measured. Graphs of the percentage of peaks as a function of the distance to the TSS were generated in R2 (http://r2.amc.nl).

Tissue Microarray. A tissue microarray (TMA) was constructed from formalin-fixed, paraffin-embedded tumor specimens using Manual Tissue Arrayer MTA-1 (Beecher Instruments). Three tissue samples of each tumor were used. TMA blocks were sectioned (4 μm), deparaffinized, immersed in 30% (vol/vol) H_2O_2 in methanol for 20 min, and heat treated at 98 °C (citrate, pH 6) for 20 min. The sections were rinsed with Tris-buffered saline, pH 7.8, and blocked (Ultra V Block, Thermo Scientific) for 10 min. Primary MYCN antibody [#621607, BD Pharmingen; 1:1,000 in Tris-buffered saline with 1% (wt/vol) BSA] was incubated overnight at 4 °C. The Powervision-Ms IgG/HRP (Immunologic) and DAB (Dako) detection system was used to visualize the antibody-binding sites. Sections were counterstained with hematoxylin (Klinipath) and covered with Vectamount. Negative control sections were processed in an identical manner after omitting the primary antibody and showed no staining. The nuclear MYCN expression was scored by two investigators independently.

1. Łastowska M, et al. (2007) Identification of candidate genes involved in neuroblastoma progression by combining genomic and expression microarrays with survival data. *Oncogene* 26(53):7432–7444.
2. Fix A, et al. (2008) Characterization of amplicons in neuroblastoma: High-resolution mapping using DNA microarrays, relationship with outcome, and identification of overexpressed genes. *Genes Chromosomes Cancer* 47(10):819–834.
3. Oberthuer A, et al. (2006) Customized oligonucleotide microarray gene expression-based classification of neuroblastoma patients outperforms current clinical risk stratification. *J Clin Oncol* 24(31):5070–5078.
4. Koppen A, et al. (2007) Direct regulation of the minichromosome maintenance complex by MYCN in neuroblastoma. *Eur J Cancer* 43(16):2413–2422.
5. Ren B, Dynlacht BD (2004) Use of chromatin immunoprecipitation assays in genome-wide location analysis of mammalian transcription factors. *Methods Enzymol* 376: 304–315.

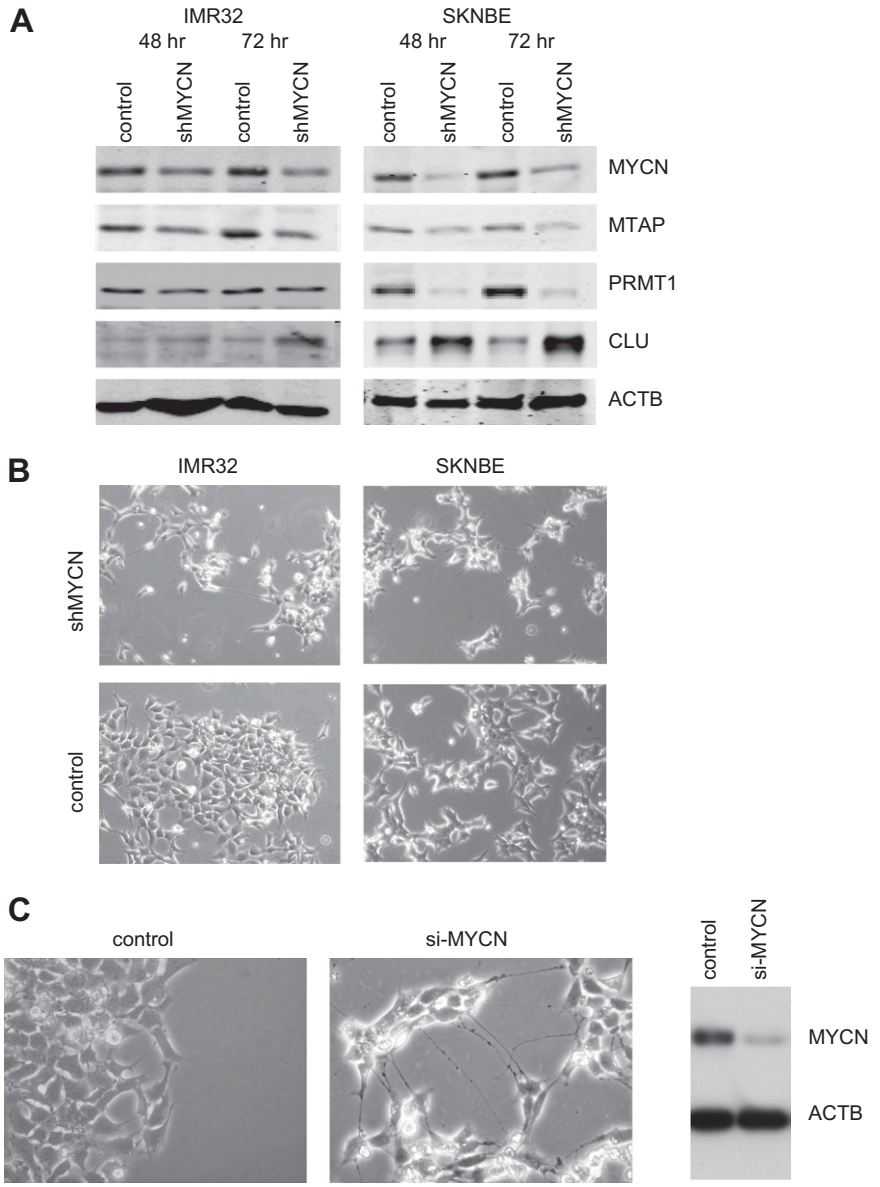

Fig. S1. Protein regulation and phenotype after MYCN silencing. (*A*) IMR32 (*Left*) and SKNBE (*Right*) were transduced with shMYCN or control virus. The time series were analyzed on Western blot with antibodies directed against the indicted proteins. (*B*) Phenotype of IMR32 and SKNBE with shMYCN or control virus. The light microscopy pictures were taken 72 h post transduction. Note the neuronal-like extensions after MYCN silencing. (*C*) Neuronal differentiation after silencing with siRNA directed against MYCN in IMR32. IMR32 was transfected with siRNA directed against MYCN or GFP (control) with a final concentration 200 nM (1). Light microscopy picture (*Left*) and Western blot (*Right*) 48 h post transfection.

1. Valentijn LJ, et al. (2005) Inhibition of a new differentiation pathway in neuroblastoma by copy number defects of N-myc, Cdc42, and nm23 genes. *Cancer Res* 65(8):3136–3145.

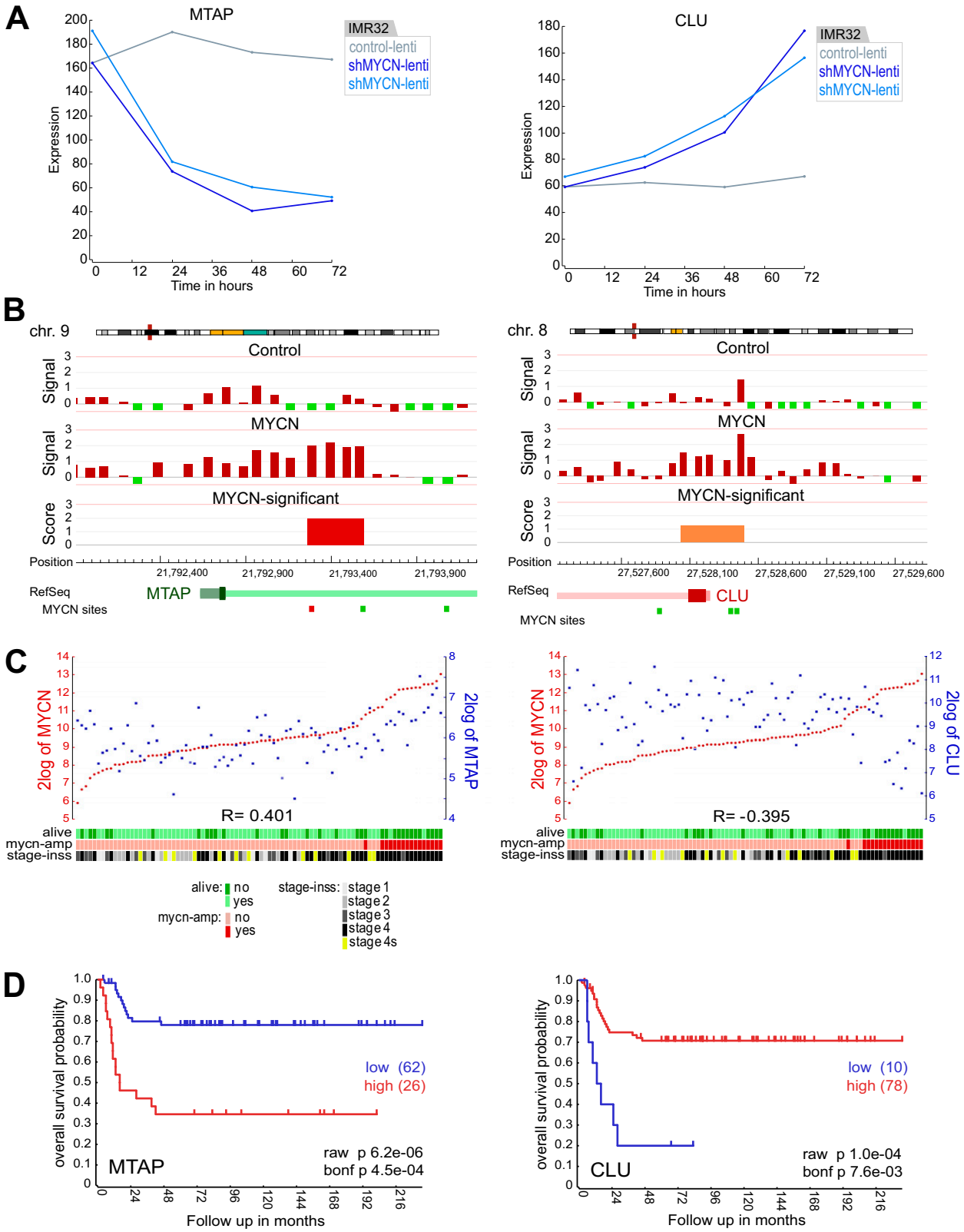

Fig. S2. *MTAP* and *CLU* are regulated by MYCN. *MTAP* (*Left*) is up-regulated and *CLU* (*Right*) down-regulated by MYCN. (*A*) Expression of mRNA according to Affymetrix profiling after silencing of MYCN in IMR32, shMYCN (blue lines), or control virus (gray line). (*B*) ChIP-on-chip analysis in IMR32 with anti-MYCN or control antibody. Signal: raw data of binding ratios (^{2}logfold) in red bars. Score: the significant binding regions (^{2}logfold) colored according to FDR score (red, ≤ 0.05; orange, ≤ 0.1). The reference sequences (green + orientation, red – orientation) are aligned at their chromosomal positions. MYCN-binding sites are indicated in red (CACGTG) and green (alternative, Table S1) boxes. (*C*) YY plot showing the correlation of *MYCN* (red) and *MTAP* or *CLU* (blue) in neuroblastoma tumor series NB88. (*D*) Overall survival analysis in the NB88 set.

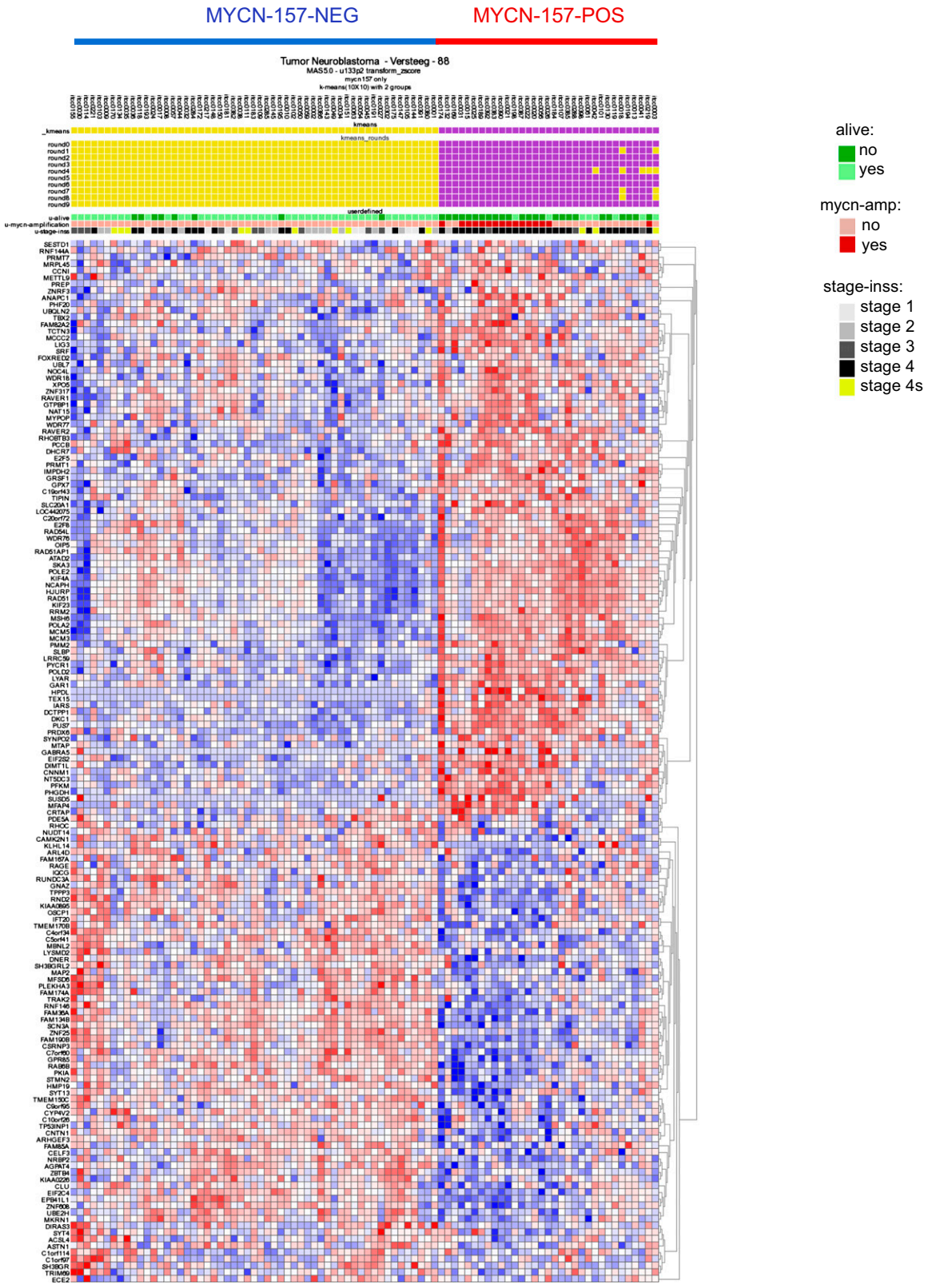

Fig. S3. Clustering according to MYCN-157 expression in NB88 tumor set. Neuroblastoma tumors were clustered in two groups for the expression (z-score) of MYCN-157 with K-means (red, high expression; blue, low expression). The multiple rounds of clustering (10 × 10) are shown in yellow and pink tracks, with the final clusters indicated at the top. Cluster MYCN-157-POS contains *MYCN*-amplified tumors and nonamplified tumors with a similar expression pattern. Cluster MYCN-157-NEG contains only nonamplified tumors. Additional tracks (survival, MYCN-amplification, and International Neuroblastoma Staging System stage) are shown above the clustering and explained in the legend.

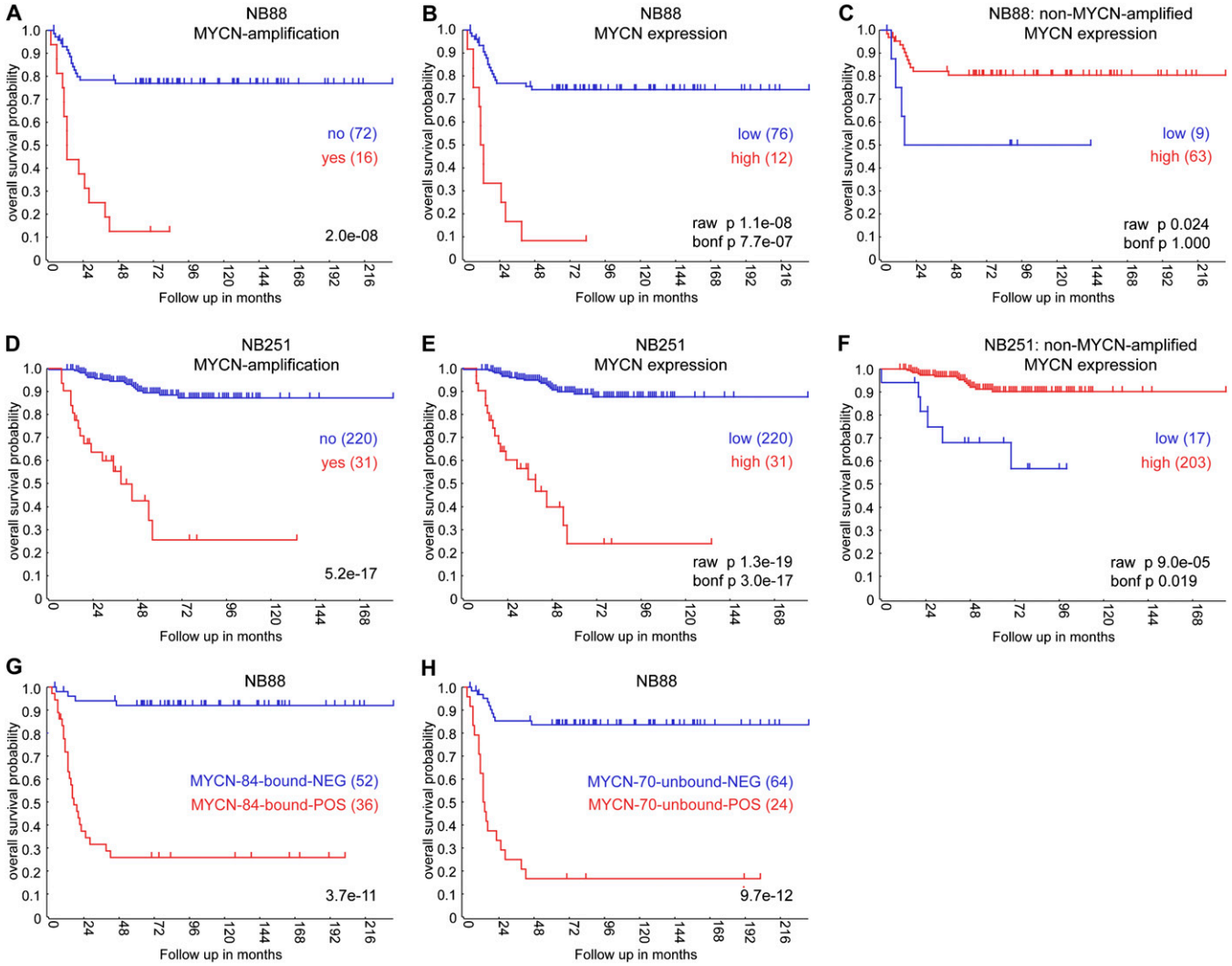

Fig. S4. Overall survival analysis of *MYCN* amplification and *MYCN* expression in NB88 (*A*–*C*) and NB251 (*D*–*F*). (*A*, *D*) Overall survival for *MYCN* amplification. (*B*, *E*) Overall survival for *MYCN* expression. (*C*, *F*) Overall survival for MYCN expression in tumors without *MYCN* amplification. (*G*, *H*) Overall survival of expression of MYCN-bound and -unbound genes predicts the clinical outcome of neuroblastoma in NB88. The genes of the MYCN-157 signature were divided in a group of 84 MYCN-bound (*G*) and 70 MYCN-unbound (*H*) genes. The overall survival of NB88 neuroblastoma tumors clustered according to the expression of these genes is shown.

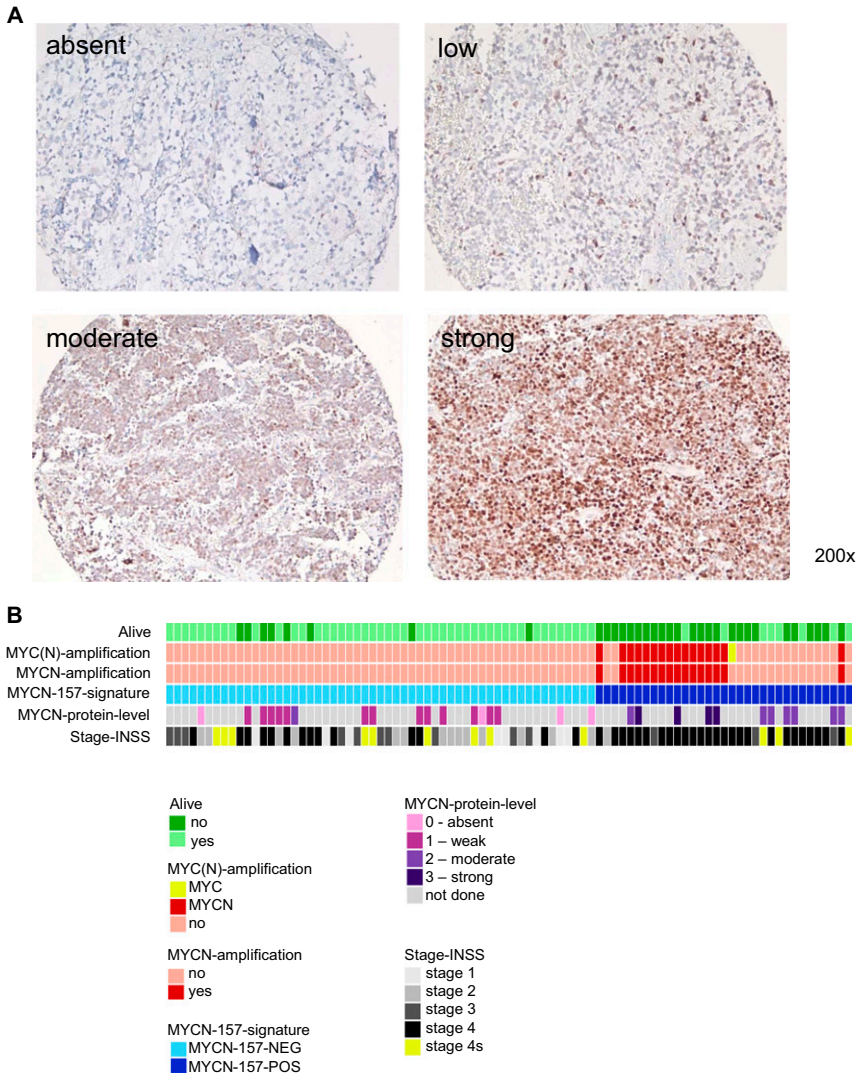

Fig. S5. MYCN protein expression in neuroblastoma tumors. (*A*) Paraffin-embedded neuroblastoma tumors were stained with anti-MYCN and counterstained with hematoxylin. Examples of tumors with different levels of nuclear MYCN expression are shown. (*B*) Overview of properties of the MYCN-157-NEG (light blue) and MYCN-157-POS (dark blue) samples. Different tracks are indicated below.

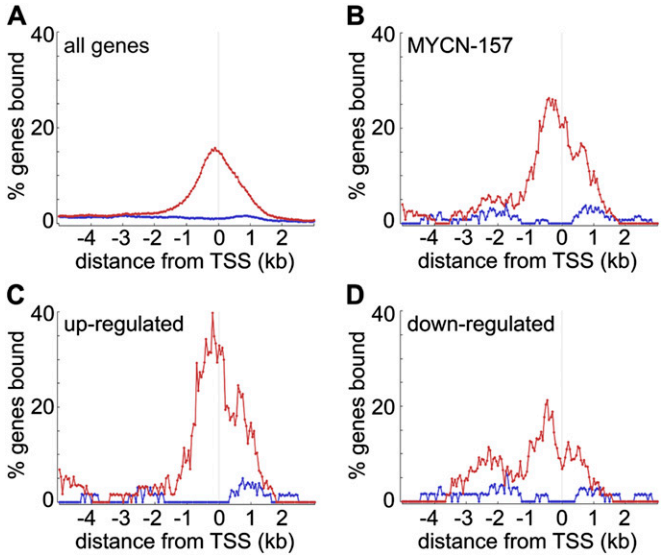

Fig. S6. MYCN binds preferentially to up-regulated genes. The cumulative analysis of ChIP-on-chip in IMR32. The graphs show the percentage of significant (FDR < 0.05) bound promoters of all genes (*A*), the MYCN-157 set (*B*), and the up-regulated (*C*) or down-regulated (*D*) genes of MYCN-157. The percentage of binding is shown relative to the transcription start site (TSS). Red: MYCN; blue: control (anti-flag).

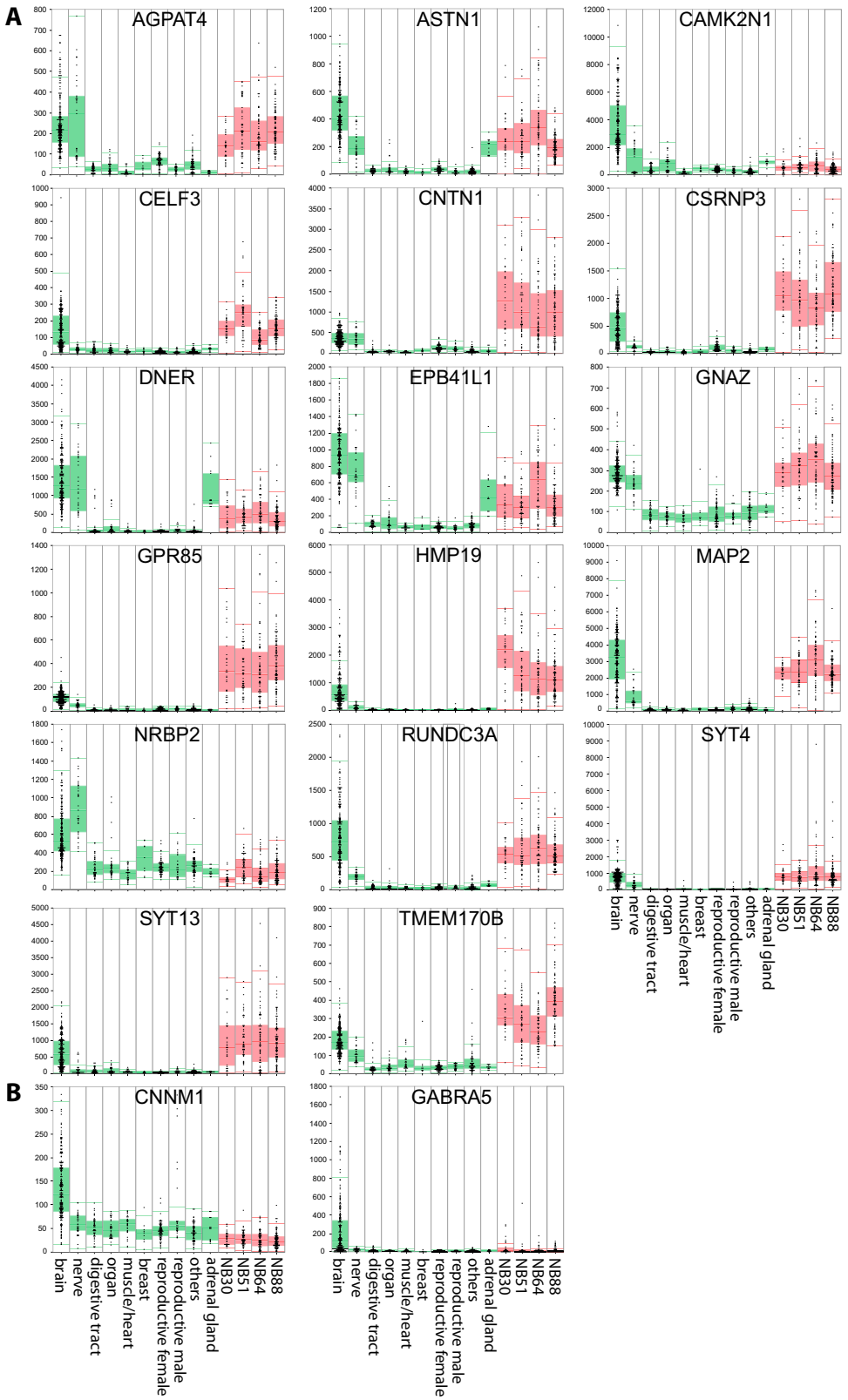

Fig. S7. Neuronal-specific genes are overrepresented in the group of genes down-regulated by MYCN and highly expressed in neuroblastoma. MegaSampler analysis of normal (green) and four independent neuroblastoma tumor series (red). (*A*) Genes down-regulated by MYCN are highly expressed in neuronal tissues (here assigned brain and nerve). Please note the high level of expression in brain, nerve, and all four neuroblastoma tumor series. (*B*) CNMM1 and GABRA5 are up-regulated by MYCN and highly expressed in brain, but are relatively low in neuroblastoma tumors.

Table S1. Cox regression for survival analysis

Variable	Hazard ratio (95% CI)	*P* value
Single		
MYCN-157 (pos vs. neg)	14.87 (5.64–39.22)	<0.0001
MYCN amplification (yes vs. no)	6.25 (3.01–12.97)	<0.0001
Stage (1, 2, 4s vs. 3, 4)	28.5 (3.88–209.7)	0.001
Stage (1, 2, 3, 4s vs. 4)	17.44 (5.25–57.94)	<0.0001
LOH 1p (yes vs. no)	3.56 (0.28–1.69)	<0.0001
Multivariate		
MYCN-157 (pos vs. neg)	12.5 (4.3–36.22)	<0.0001
MYCN amplification (yes vs. no)	1.4 (0.64–3.10)	0.4
MYCN-157 (pos vs. neg)	7.33 (2.69–19.93)	<0.0001*
Stage (1, 2, 4s vs. 3, 4)	10.94 (1.39–86.0)	0.023*
MYCN-157 (pos vs. neg)	5.84 (2.0–17.06)	0.001*
Stage (1, 2, 3, 4s vs. 4)	6.64 (1.75–25.14)	0.005*
MYCN-157 (pos vs. neg)	13.95 (4.84–40.14)	<0.0001
LOH 1p (yes vs. no)	1.06 (0.47–2.37)	0.89
Multivariate MYCN-157 (pos vs. neg)		
Stratus		
MYCN amplification (yes vs. no)	11.72 (4.03–34.09)	<0.0001
Stage (1, 2, 4s vs. 3, 4)	7.3 (2.71–19.69)	<0.0001
Stage (1, 2 ,3, 4s vs. 4)	5.63 (2.0–16.0)	0.001
LOH 1p (yes vs. no)	13.21 (4.60–37.80)	<0.0001

*Independent.

Other Supporting Information Files

Dataset S1 (XLS)