Supporting Information

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SI Materials and Methods

Cell lines. Human NB cell lines SK-N-AS, SK-N-SH, SIMA (N3), SH-SY5Y, LAN-5, SK-N-BE(2), HTLA-230, IMR-32, BE2 (M17), and lung carcinoma H1299 cells were obtained from ATCC (Manassas, VA, USA) and cultured in their suggested medium at 37 °C in 5 % CO_2. Mouse NB N1E-115 cells were kept in culture as described (1). NB cells were treated with all-*trans*-Retinoic acid (ATRA) (Sigma, Saint Louis, MO, USA) for the indicated times and concentrations. Control cells were treated in the presence of DMSO (Sigma). Cell lines utilized were tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza) every 3 months.

Protein analysis and antibodies. Western blot (WB) analysis was carried out as already described (2). Antibodies were ZNF281: ab101318 (Abcam, Cambridge, UK); Synapsin II (Syn II): ab76494 [EPR3277] (Abcam); GAPDH: G8795 (Sigma, St. Louis, Missouri, USA); c-Myc: 9E10 (sc-40 - Santa Cruz Biotechnology); MYCN: B8.4.B (sc-53993 - Santa Cruz Biotechnology); β–actin: AC-15 (a5441 - Sigma, Saint Louis, MO, USA); NEFM: ab1987 (Millipore, Billerica, MA, USA); HA: antiHA.11 clone 16B12 (ab130275 Abcam), anti-mouse-HRP-conjugated (Bio-Rad, Hercules, CA, USA; cat. no.: 170-5047) and anti-rabbit-HRP-conjugated (Bio-Rad; cat. no.: 170-6515)

RNA extraction, cell transfection, siRNA silencing and real-time qPCR analyses. RNA was extracted with the RNeasy Mini Kit 50 (Qiagen) according to standard procedures. We utilized a previously described expression vector encoding for TAp73β–HA (3) for transfection of NB cell lines and H1299 cells. Lipofectamine 2000 Transfection Reagent (Invitrogen) was used for transfections according to the manufacturer's suggestions. The following siRNAs were utilized for silencing: siRNA against ZNF281 (siZNF281) and with scrambled siRNAs (scr) (L-006958-00 for siZNF281 and D-001810-10 for scr - Dharmacon, Lafayette, CO, USA); anti-MYCN siRNA (L-003913-01 - Dharmacon); anti-MYC siRNA (L-003282-02 - Dharmacon) and scrambled siRNAs control (src) (D-001810-10 - Dharmacon). Pre-miR34a (PM11030 - ThermoFisher Scientific) and nc control (AM17110 - ThermoFisher Scientific) were used for transfection of NB cells. Backbone modified anti-miR were: anti-hsa-miR34a-5p mircury LNA Power Inhibitor (41100982 - Exiqon, Denmark) and mircury LNA Power Inhibitor Negative Control (199006 - Exiqon) were used for transfection of NB cells and H1299 cells according to the manufacturer's instructions using Lipofectamine RNAiMAX transfection Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and qPCR were carried out as previously described (4).

Functional Assays. Cells were co-transfected with *wild-type* and mutant reporters containing the *wt* and the mutated 3'UTR of human ZNF281 and the pre-miR34a and nc control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions. Luciferase assays were carried out using the Dual Luciferase Reporter assay (Promega). Each experimental point was analyzed in triplicate in three independent experiments. Statistical significance was calculated by unpaired two-tailed Student's t-test.

Lentiviral infections. LAN-5 cells were infected with the lentiviral vector pLVTHM-sh-scrambled, pLVTHM-sh281-I and pLVTHM-sh281-II as described (2). Two independent clones infected with sh-ZNF281 (sh-281-I and sh-281-II) and a mixed population infected with sh-scrambled (sh-ctrl) were analyzed.

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Cell proliferation. EdU incorporation and FACS analysis were performed using the Click-IT EdU Alexa Fluor 488 Flow Cytofluorimetry kit (C10425, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Array analysis. BE2-M17 cells were silenced for 72h with siRNA against ZNF281 or with a scrambled control. RNA was extracted with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Agilent 8x60k Human Gene Expression arrays (Agilent Technologies LDA UK Limited, Cheadle, Cheshire) were used to evaluate changes in gene expression. For each sample, 100 ng RNA was fluorescently labelled with Cy3, using the Agilent Low Input Quick Amp labelling kit (one-colour), according to the manufacturer's instructions. 600 ng of each labelled sample was hybridised to arrays at 65° C for 17 h with rotation at 10rpm. Following hybridisation, the arrays were scanned using an Agilent SureScan High Resolution scanner, and Agilent Feature Extraction software applied to the resulting images. Data of the array analysis are accessible (GEO accession number: GSE112029).

Chromatin crossliking immuno-precipitation. ChIP analyses were carried out using a commercial Kit MAGnify Chromatin Immunoprecipitation System (Invitrogen) and ChIP-grade antibody anti-Zfp281 ab101318 (Abcam). Primers used for amplification of the regions including the potential ZNF281 binding sites in the promoter regions of GDNF, NRP2, Axin2 (positive control) and 16q22 (negative control) are listed in the Supplementary Table S1. PCR was carried out using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer's instructions.

NB datasets analysis. Bioinformatic analysis was carried out utilizing the Normal Brain Development – BrainSpan (524 samples); GSE 45547 Tumor Neuroblastoma - Kocak - dataset (649 patients) in R2 genomics analysis and visualization platform (http://r2.amc.nl) or the GSE49710 neuroblastoma dataset (260 patients) (5). Mean values were analyzed for statistical differences with the two-tailed unpaired t-test.

Immunofluorescence analysis. Immuno-fluorescence analyses were carried out on NB cells seeded onto glass coverslips at $5x10^4$ cells/cm^2 and treated with ATRA (Sigma) for the indicated times and concentrations. Control cells were treated with DMSO (Sigma). After treatment cells were rinsed in PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were washed with PBS and permeabilized with 0,2% Triton X-100 in PBS for 10 minutes at room temperature. Cells were incubated with Anti-Neurofilament 160 antibody (N5264 – Sigma) diluted 1:40 in PBS/1% BSA for 1h at room temperature. Coverslips were then washed three times with PBS for 5 minutes at room temperature and incubated with Alexa Fluor 594 anti-mouse secondary antibody (1:1000 – ThermoFisher Scientific) and DAPI (1:1000) diluted in PBS/1% BSA for 1h at room temperature. Coverslips were washed with PBS three times for 5 minutes at room temperature and mounted with ProLong™ Gold antifade reagent (Invitrogen). Cells were examined under a Nikon laser scanning fluorescence confocal microscope (Nikon Eclipse Ti). Micrographs were analyzed with a specific software package (NIS element AR4.00.04).

1. De Laurenzi V, *et al*. (2000) Induction of neuronal differentiation by p73 in a neuroblastoma cell line. *J Biol Chem* 275(20):15226-15231.
2. Pieraccioli M, Imbastari F, Antonov A, Melino G, & Raschella G (2013) Activation of miR200 by c-Myb depends on ZEB1 expression and miR200 promoter methylation. *Cell Cycle* 12(14):2309-2320.
3. Agostini M, *et al*. (2011) Neuronal differentiation by TAp73 is mediated by microRNA-34a regulation of synaptic protein targets. *Proc Natl Acad Sci U S A* 108(52):21093-21098.
4. Pieraccioli M, *et al*. (2016) ZNF281 contributes to the DNA damage response by controlling the expression of XRCC2 and XRCC4. *Oncogene* 35(20):2592-2601.
5. Antonov AV, *et al*. (2014) PPISURV: a novel bioinformatics tool for uncovering the hidden role of specific genes in cancer survival outcome. *Oncogene* 33(13):1621-1628.

Table S1.

Oligonucleotides used for cloning sh-ctrl, shZNF281 and human ZNF281 3'UTR.

Name	Sequence (5' to 3')
sh-ctrl F	AGTGTACGCGTGAATCTCATTCGATGCATATTCAAGAGATA TGCATCGAATGAGATTCTTTTATCGATCGGTA
sh-ctrl R	TACCGATCGATAAAAGAATCTCATTCGATGCATATCTCTTG AATATGCATCGAATGAGATTCACGCGTACACT
sh281-I F	AGTGTACGCGTCCAGAATCTCAGGGAATCAAATTCAAGAGA TTTGATTCCCTGAGATTCTGGTTTTTATCGATCGGTA
sh281-I R	TACCGATCGATAAAAACCAGAATCTCAGGGAATCAAATCTC TTGAATTTGATTCCCTGAGATTCTGGACGCGTACACT
sh281-II F	AGTGTACGCGTGCTCTAAATGCTGAAATTAAATTCAAGAGA TTTAATTTCAGCATTTAGAGCTTTTTATCGATCGGTA
sh281-II R	TACCGATCGATAAAAAGCTCTAAATGCTGAAATTAAATCTC TTGAATTTAATTTCAGCATTTAGAGCACGCGTACACT
hZNF281 3'UTR F	AGCATCTAGAGGTCCCAAAAGTGGCCAGGCTGGAGGTCTTC
hZNF281 3'UTR R	CAGGTCTAGACAGTGGTAAAGGTCCTCTCCATCCTTTG
hZNF281 3'UTR mut F	CTAATGTAATTTTGTTTTATTTTGGGAATGTTTCTACACGATC
hZNF281 3'UTR mut R	GATCGTGTAGAAACATTCCCAAAATAAAACAAAATTACATT AG

Oligonucleotides used for qPCR

Name	Sequence (5' to 3')
human β-actin F	GTTGCTATCCAGGCTGTGCTA
human β-actin R	AATGTCACGCACGATTTCCCG
human ZNF281 F	GAGGACACATAGTGGAGAAAAGCC
human ZNF281 R	TGAGACAACACAGCCAGATTACCC
human MYCN F	CCTCCAACACCAAGGCTGTC
human MYCN R	TCTTGGGACGCACAGTGATG
Human GDNF F	AGTGCTTCCTAGAAGAGAGCG
Human GDNF R	GAGCCGCTGCAGTACCTAAA
Human NRP2 F	GTCTCCTACAGCCTAAACGGCA
Human NRP2 R	GGGTCAAACCTTCGGATGTCA

Oligonucleotides used for ChIP analysis

Name	Sequence (5' to 3')
Human Axin2 F	CCAACTCACTCAGGGGAGAC
Human Axin2 R	GATTCTTGGCACAGGCAGTAG
Human Chr. 16q22 F	CTACTCACTTATCCATCCAGGCTAC
Human Chr. 16q22 R	ATTTCACACACTCAGACATCACAG
Human GDNF F	AACTTTAAGAGGTGGGAGGAGTAT
Human GDNF R	CCGCCCACACCTCATTCTTC
Human NRP2 F	TAATCAGGAACACAGAAGGGGC
Human NRP2 R	CTCGCCGAGTGTCTGTGC

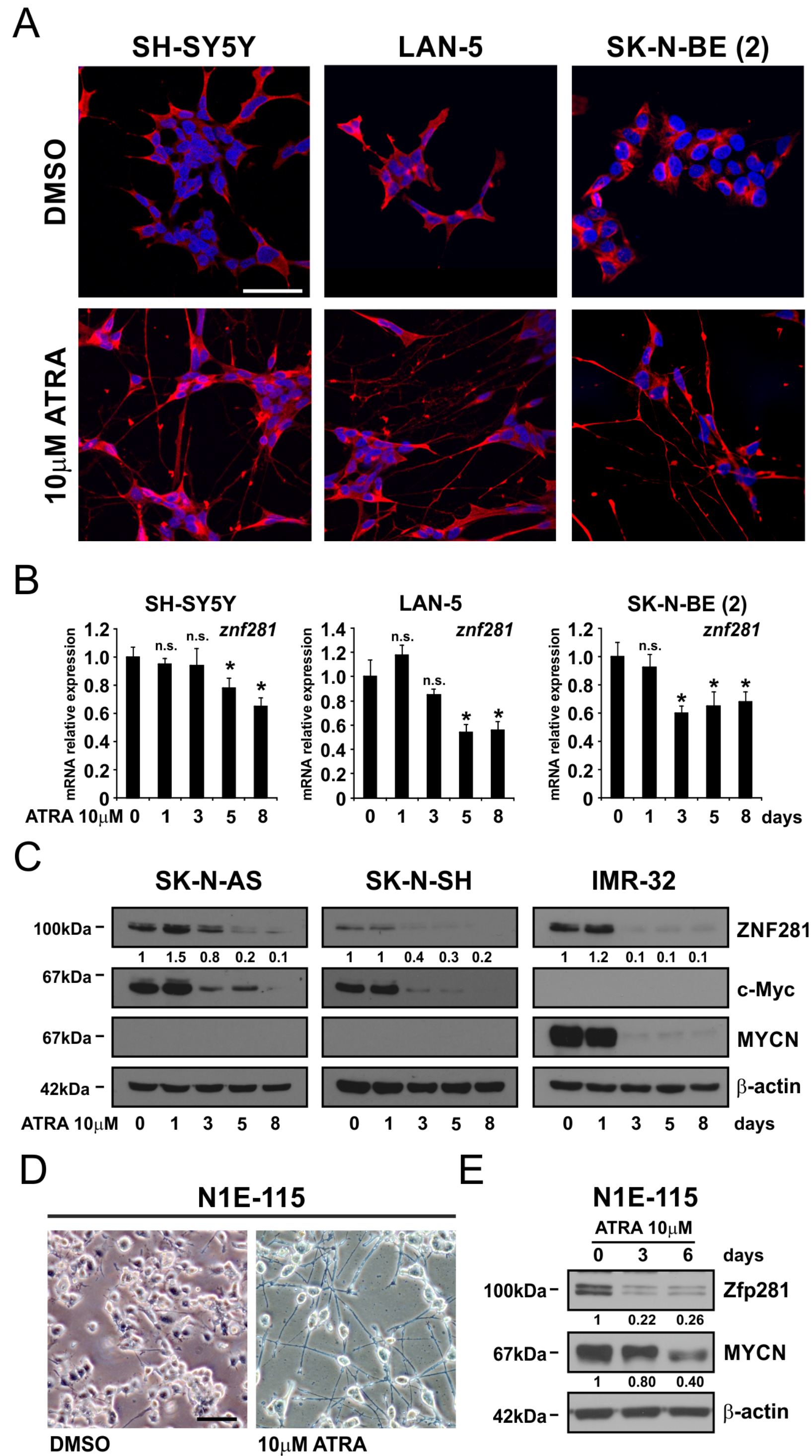

Fig. S1. A: ATRA-induced differentiation of NB cell lines. NEFM was detected by immunofluorescence (red) in ATRA-treated cells (10 μM for 8 days). Nuclei were counterstained with DAPI (blue). Scale bar is 50 μm. **B**: qPCR to measure the expression of ZNF281. Values are reported ± standard deviation (SD) of triplicate biological replicates. **C**: WB analyses to detect ZNF281, c-Myc and MYCN in the indicated human NB cell lines treated with 10 μM ATRA. Antibodies used were as in Figure 1. (**D**) Morphology and (**E**) WB analysis of murine NB cell line N1E-115 cultured in 10% FBS and treated with 10 μM ATRA for the indicated time. Scale bar is 100 μm. Numbers below the corresponding blot represent densitometric analysis normalized to the housekeeping gene. All blots in the Figure were repeated at least two times with similar results.

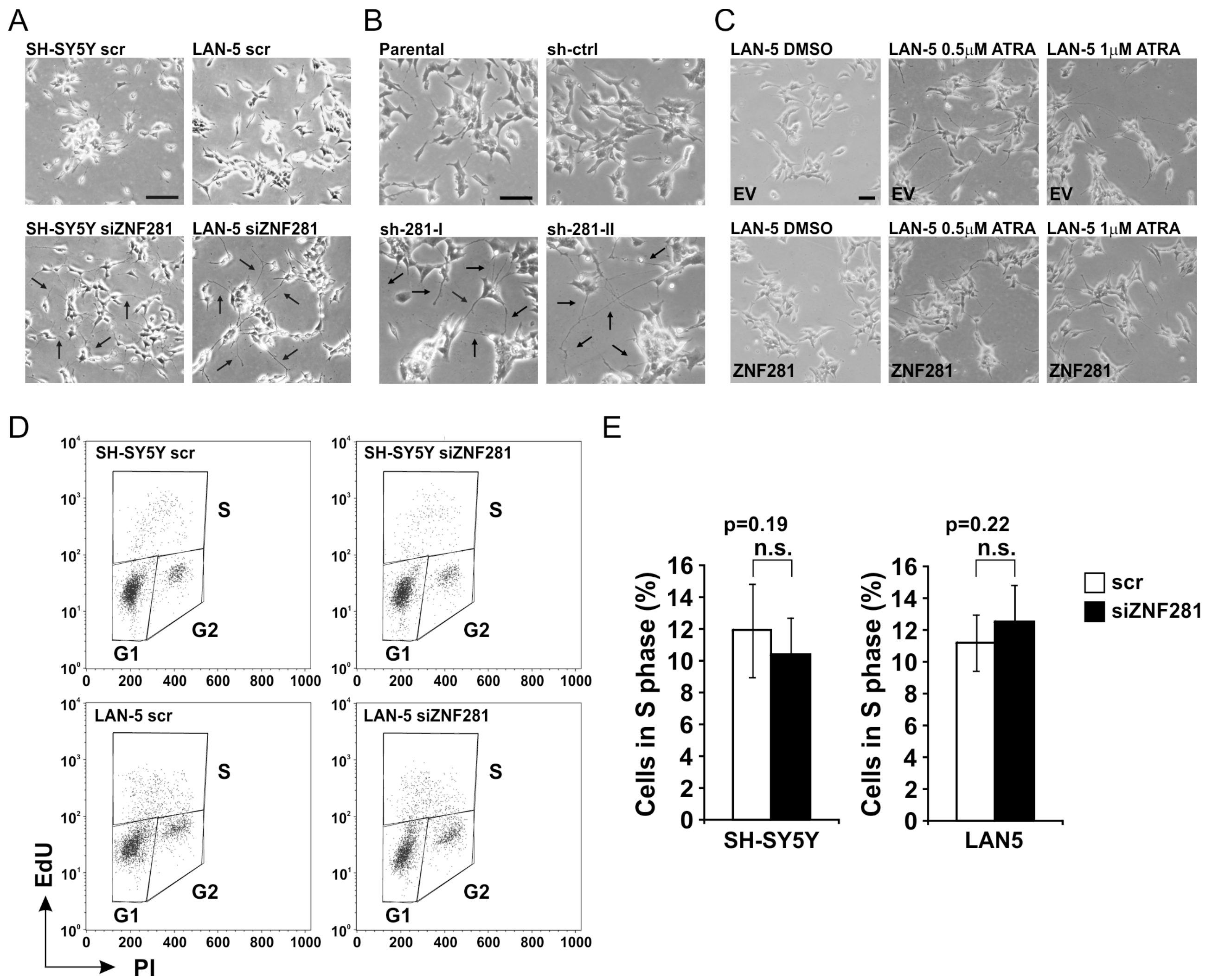

Fig. S2. A: Morphology of SH-SY5Y and LAN-5 human NB cell lines transfected with a pool of siRNA against ZNF281 (siZNF281) or with scrambled siRNAs (scr) (see Fig. 1 for details). Arrows indicate neurites. Scale bar is 100 μm. **B**: the human NB cell line LAN-5 infected with the lentiviral vector pLVTHM-sh-scrambled, pLVTHM-sh281-I and pLVTHM-sh281-II: two independent clones infected with sh-ZNF281 (sh-281-I and sh-281-II) and a mixed population infected with sh-scrambled (sh-ctrl) were analyzed. Arrows indicate neurites. Scale bar is 100 μm. **C**: the human NB cell line LAN-5 transfected with an expression vector for human ZNF281 and treated with ATRA at the indicated concentrations for 3 days. Scale bar is 50 μm. **D**: Cell cycle distribution and S-phase EdU incorporation (**E**) of SH-SY5Y and LAN-5 cells transfected with a pool of siRNAs against ZNF281 (siZNF281) or with scrambled siRNAs (scr). Ten thousand gated events were analysed. n.s.: not significant. Error bars are S.D. of triplicate biological replicates.

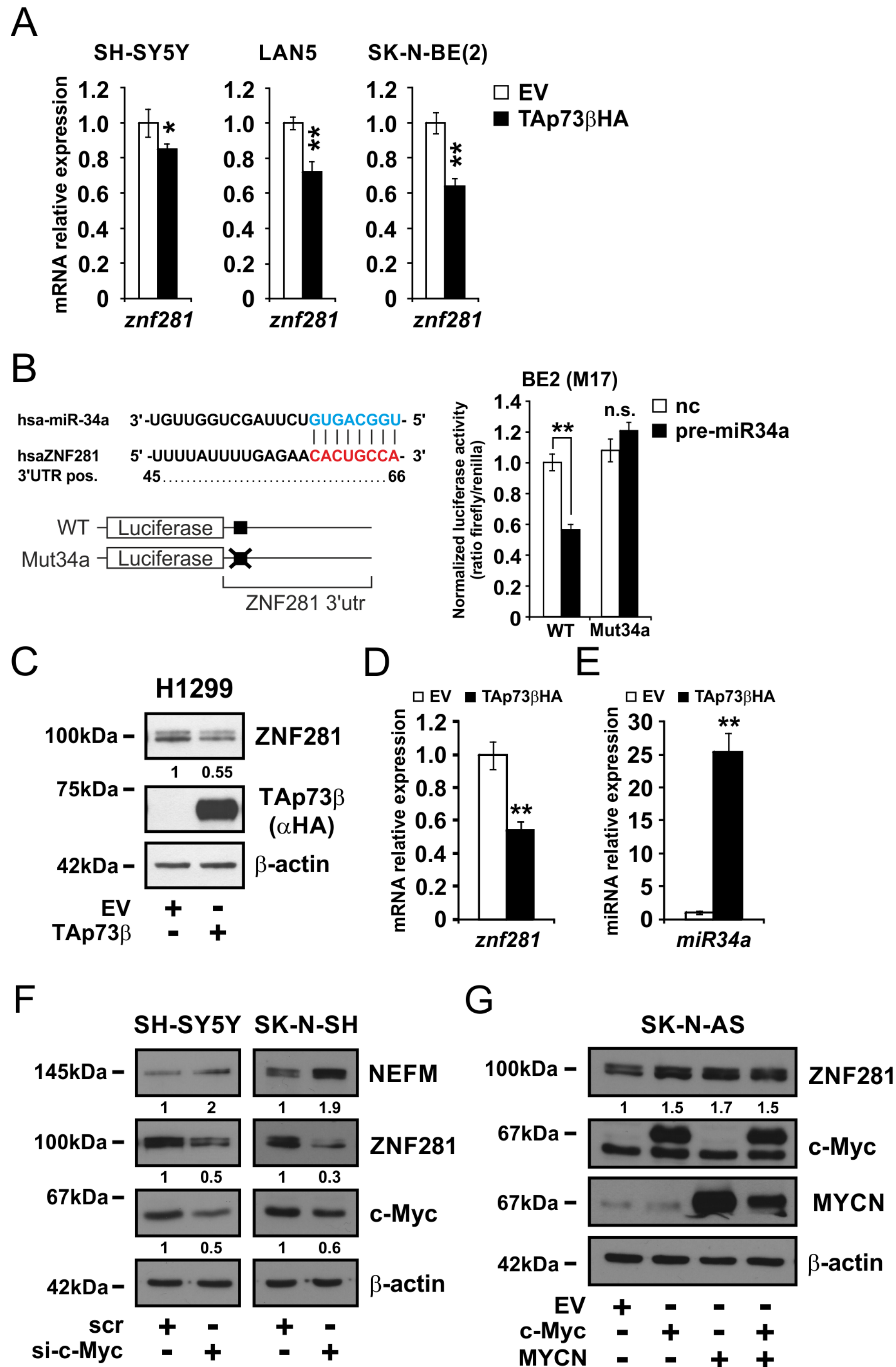

Fig. S3. **A**: qPCR to measure the expression of ZNF281 in the indicated human NB cell lines transfected with an expression vector for TAp73β-HA or with the empty vector (EV). Asterisks indicate statistical significance (*p< 0.05, ** p< 0.01) of the difference between EV and TAp73β-HA-transfected cells calculated with unpaired two-tailed Student's t test. **B**: Luciferase assays were carried out with the *wt* and mutated 3'UTR region of the human ZNF281 gene that contains a miR34a binding site (scheme on the left). Asterisks in the graph indicate statistical significance (** p< 0.01, unpaired two-tailed Student's t test). Assays were repeated three times in triplicate. **C:** WB analysis of H1299 cells transfected with an expression vector for TAp73β-HA or with the empty vector (EV). Numbers below the corresponding blot represent densitometric analysis normalized to the housekeeping gene. **D**: qPCR to measure the expression of ZNF281 and of miR34a (**E**) in H1299 cells transfected with TAp73β-HA or EV. Asterisks indicate statistical significance (** p< 0.01) of the difference between EV and TAp73βHA-transfected cells calculated with unpaired two-tailed Student's t test. **F:** WB analysis of SH-SY5Y and SK-N-SH cells transfected with a pool of anti-MYC siRNA or with scrambled siRNAs control (src) for 96 hrs. **G:** WB analysis of SK-N-AS cells transfected with an expression vector for c-Myc or MYCN or with the empty vector (EV). Numbers below the corresponding blot represent densitometric analysis normalized to the housekeeping gene. Blots are representative of 2 to 4 biological replicates.

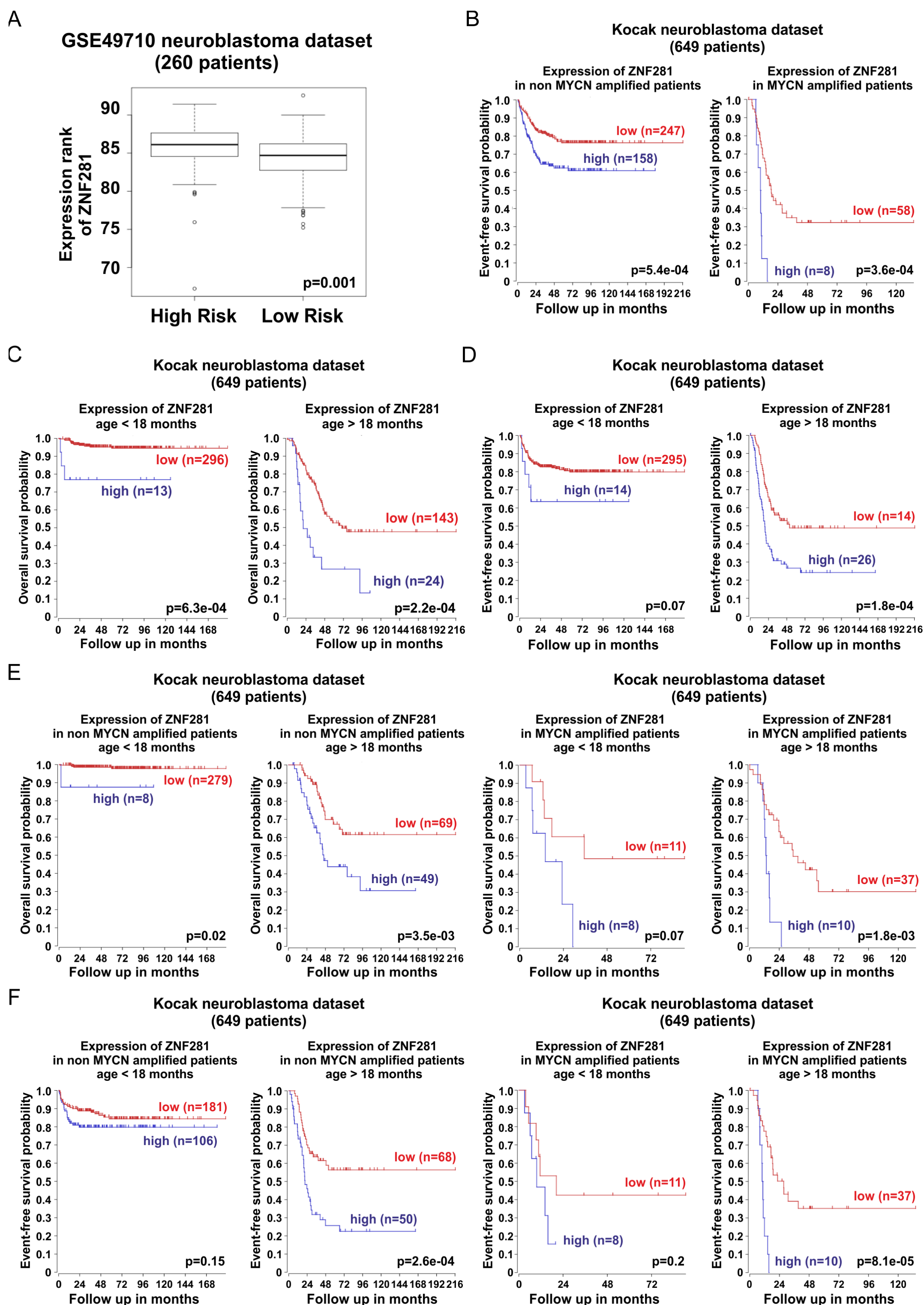

Fig. S4. A: expression of ZNF281 in high risk NB patients (stage 4) versus low-intermediate-risk patients (stages 1, 2, 3, 4S). All data used for the analysis are from the GSE49710 neuroblastoma dataset (260 patients). Boxes indicate the median (horizontal line); whiskers, distances from the highest and lowest value to each end of the box that are within 1.5 X box length; outliers are indicated as dots. Mean values were compared with the two-tailed unpaired t-test. **B-E**: Kaplan Meier (KM) event-free and overall survival probability (as indicated in the Figure) analyses in the MYCN non-amp and in the MYCN-amp subcohorts (**B**), in the <18 month and >18 month subcohorts (**C and D**), in the in the MYCN non-amp<18 month and in the MYCN non-amp >18 month subcohorts, in the MYCN-amp<18 month and in the MYCN-amp>18 month subcohorts (**E**), in the MYCN non-amp <18 month and in the MYCN non-amp>18 month, in the MYCN-amp<18 month and in the MYCN-amp >18 month subcohorts (**F**) according to ZNF281 expression. All data used for the KM analyses are from the GSE 45547 Tumor Neuroblastoma - Kocak - dataset (649 patients) in R2 genomics analysis and visualization platform (http://r2.amc.nl). The R2 system gives the cut-off value of ZNF281 expression levels. The difference between the curves for ZNF281 high and ZNF281 low groups are compared by chi-squared test.

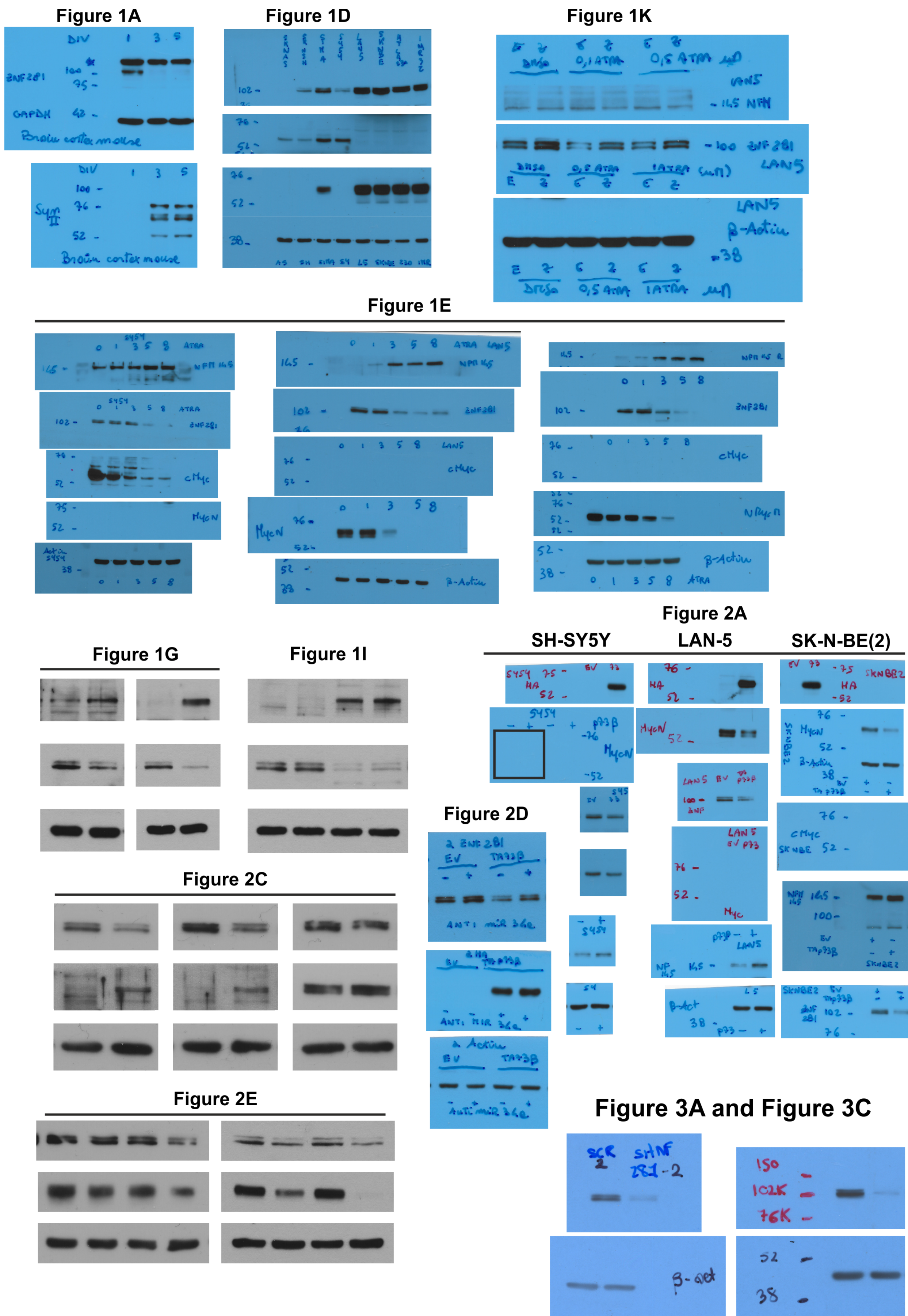

Fig. S5. Uncropped images from which WB analyses in Fig. 1-2 -3 were derived.

Figure 3F

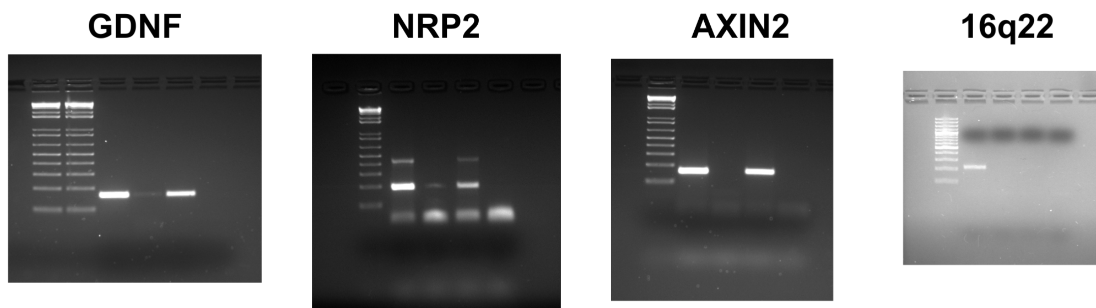

Figure S1C

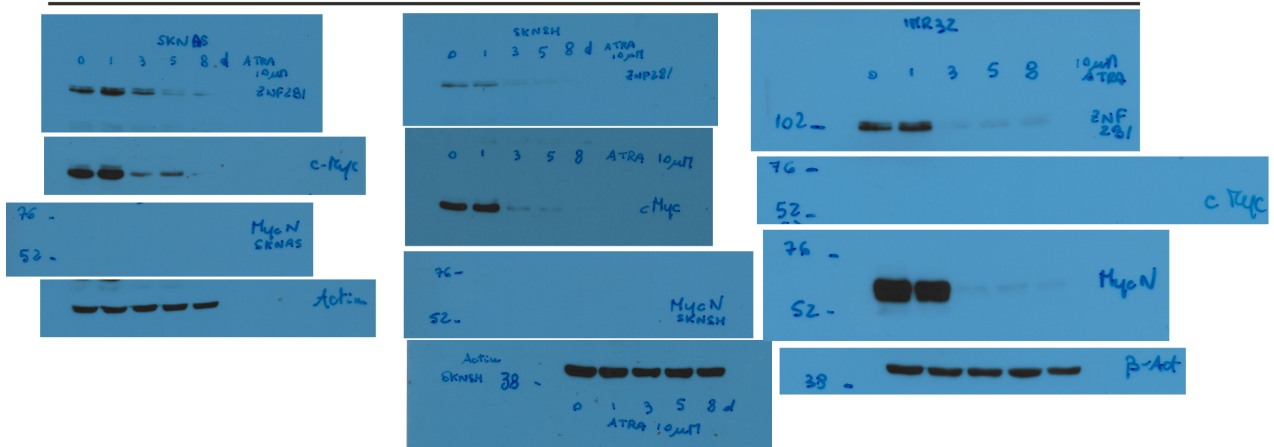

Figure S1E

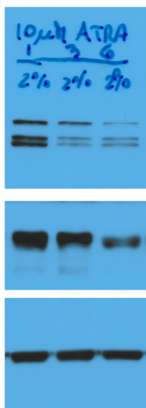

Figure S3B

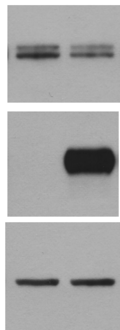

Figure S3E

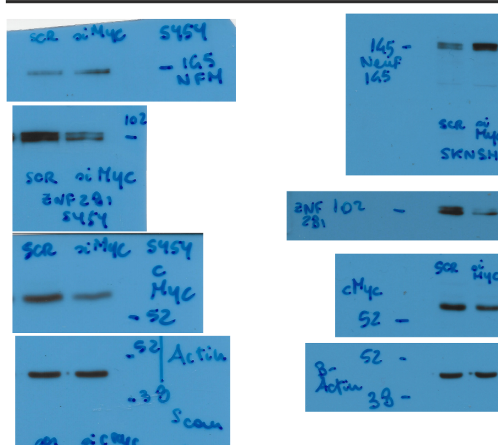

Figure S3F

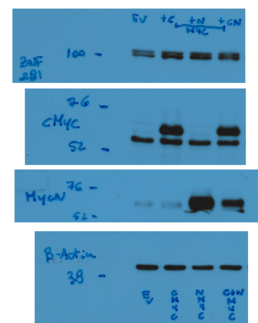

Fig. S6. Uncropped images from which ChIP analysis in Fig. 3F and WB analyses in Supplementary Fig. S1 and S3 were derived.