## SUPPLEMENTARY DATA

# MYCN and HDAC2 cooperate to repress miR-183 signaling in neuroblastoma

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## SUPPLEMENTARY MATERIALS AND METHODS

### microRNA profiling.

Total RNA from BE(2)-C cells was isolated using miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in water. The quality of total RNA was checked by gel analysis using the total RNA Nanochip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Waldbronn, Germany). Only samples with RNA index values greater than 7 were selected for microRNA profiling. RNA concentrations were determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Probe labeling and Illumina BeadArray Hybridization is a modification of an assay that was developed for high-throughput gene expression profiling, the DASL® Assay (cDNA-mediated annealing, selection, extension and ligation, (59)). The miRNA method similarly targets specific sequences with sets of oligonucleotides that are extended, and then labeled during PCR amplification. If not stated otherwise, 200 ng of total RNA were first polyadenylated using Poly-A Polymerase, incubated at 37°C for 60 min, then heat inactivated at 70°C for 10 min. The introduced poly A tail was then used as a priming site for cDNA synthesis, incubated at 42°C for 60 min, then heat inactivated at 70°C for 10 min. The primer used for cDNA synthesis was biotinylated and contained a universal PCR primer sequence that was used later in the assay. After cDNA synthesis, miRNAs were individually interrogated using specific oligonucleotides. A single miRNA-specific oligo (MSO) is designed against each mature miRNA sequence, which consists of three parts: at the 5′-end is another universal PCR priming site; in the middle is an address sequence used for capturing the product on the array; and at the 3′-end is a miRNA-specific sequence. The second universal PCR priming site is shared among all MSO's, and each address sequence is associated uniquely with each of the 1146 miRNA targets. As controls, central mismatch probes for miRNAs hsa-let-7a, let-7c, let-7f, miR-152 and miR-182, and 3′-end mismatch probes for small nuclear RNAs RNU24 and RNU66 were used. The subsequent assay process and array hybridization were performed as described previously (59). Briefly, 15 µl of the cDNA synthesis reaction was added to 5 µl of the multiplexed MSO pool and 30 µl of a reagent containing streptavidin

paramagnetic particles, heated to 70°C, and allowed to anneal to 40°C. All 1146 human miRNAs were assayed simultaneously. After binding and washing, the annealed MSOs were extended through the cDNA primer, forming an amplifiable product. The extended oligos were eluted from the streptavidin beads and added to a PCR reaction, in which one of the universal primers was Cy3 labeled and the other universal primer was biotinylated. The PCR products were captured on streptavidin paramagnetic beads, washed and denatured to yield single-stranded fluorescent molecules to hybridize to the arrays. The universal arrays used for fluorescent reporting consist of capture oligos immobilized on beads and randomly assembled onto the ends of fiber optic bundles, which are arranged in a matrix to match a 96-well plate (Sentrix® Array Matrix, Illumina, (60)). The identity of each bead is determined before hybridization to the miRNA assay product. Arrays were scanned on the BeadArray Reader, and automatic image registration and intensity extraction software was used to derive intensity data per bead type corresponding to each miRNA (61). Data were analyzed using Chipster software, version 1.4.7 (http://chipster.csc.fi/) (62).

**Cloning of inducible miR-183 and miR negative ctrl expression plasmids and generation of inducible cell lines.**

The human miR-183 sequence together with flanking DNA sequences was amplified by PCR using the following primers: forward: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCAGTCTGGGTGATGTGG-3', reverse: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGCGGGCTCTCTGGGGACAC-3'. The sequence of the miR-neg ctrl miRNA negative control was amplified from the pcDNA 6.2-GW/EmGFP-miR-neg control plasmid (Invitrogen) with the following primers: forward: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGGAGGCTTGCTGAAGGCT-3', reverse: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGCCATTTGTTCCATGTGAG-3'. For the generation of tetracycline/doxycycline-inducible miR-183 or miR-neg ctrl expression plasmids, the amplicons were cloned using the Gateway Technology with Clonase II protocol (Invitrogen) into the pT-REx-DEST30 plasmid according to the manufacturer's instructions.

Plasmids were stably transfected into BE(2)-C cells together with the plasmid pcDNA 6/TR (Invitrogen), encoding the Tet repressor. After selection with G418 (1.5 mg/ml) and blasticidin (7.5 µg/ml), individual clones were tested for miR-183 or miR-neg ctrl expression upon tetracycline/doxycycline treatment by quantitative RT-PCR.

**Inducible *MYCN* knockdown cell line.**

Stable transfected IMR-32 cell line expressing small hairpin RNA (shRNA) against MYCN under control of the Tet-repressor (IMR32-6TR-MYCNsh) was generated according to the protocol described in Muth et al., 2010 (63).

**Trypan blue cell viability assay.**

Cells were seeded, cultured and treated as indicated. Viability and cell number were evaluated by automated trypan blue staining using the VI-CELL Cell Viability Analyzer (Beckman Coulter, Krefeld, Germany).

**Caspase-3-like activity assay.**

Cells were seeded, treated as indicated, collected with supernatant and lysed in cell lysis buffer (Biovision, Mountain View, CA, USA) for 10 min on ice. Thereafter, the reaction buffer containing the AFC-labeled caspase-3-specific peptide, DEVD (Biovision), was added. Caspase-3-like activity was measured at 37°C in black 96-well plates using a fluorescence plate reader with a 380 nm excitation filter and a 530 nm emission filter (64).

**Flow cytometry.**

For apoptosis measurements, cells were stained with propidium iodide and analyzed in Fl-2 in logarithmic mode with a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) using CellQuest Pro software (BD Biosciences) (64).

**Soft agar assay**.

Anchorage-independent colony formation was tested with Chemicon's Cell Transformation Assay Kit according to the manufacturer's instructions. For induction of miR expression, cells were treated with doxycycline (100 ng/ml). Colonies were stained with crystal violet and automatically counted with Image J 1.44p software.

**Table S1. miR-183 induction by HDACi compared to solvent control treatment.**

| | significance of miR-183 induction in different cell lines after treatment for 24 h (p-value) | | | |
|---|---|---|---|---|
| **HDACi** | **BE(2)-C** | **Kelly** | **SH-SY5Y** | **SH-EP** |
| Panobinostat | 0.0043 | 0.0017 | 0.0073 | 0.0297 |
| PCI-24781 | < 0.0001 | 0.0022 | 0.0140 | 0.0357 |
| Vorinostat | 0.0003 | 0.0003 | 0.0126 | 0.0182 |
| Entinostat | 0.0018 | 0.0016 | 0.0165 | 0.0499 |
| Tubacin | 0.0989 | 0.4781 | 0.2929 | 0.0815 |
| Compound 2 | 0.3670 | 0.8226 | 0.9477 | 0.0038 |
| Trichostatin A | 0.0003 | 0.0032 | 0.0011 | 0.0007 |

**Table S2. Sequences of primers for qRT-PCR.**

| gene | forward primer (5'-3') | reverse primer (5'-3') | reference |
|---|---|---|---|
| *HDAC1* | TGA CGA GTC CTA TGA GGC CAT T | CCG CAC TAG GCT GG AAC ATC | - |
| *HDAC2* | TGT GAG ATT CCC AAT GAG TTG C | GGT AAC ATG CGC AAA TTT TCA A | (55) |
| *HDAC3* | CCT CAC TGA CCG GGT CAT | ACC TGT GCC AGG GAA GAA GTA A | - |
| *HDAC4* | GAG GTT GAG CGT GAG CAA GAT | TAG CGG TGG AGG GAC ATG TAC | - |
| *HDAC5* | GTC TCG GCT CTG CTC AGT GTA GA | GGC CAC TGC GTT GAT GTT G | - |
| *HDAC6* | CAA GGA ACA CAG TTC ACC TTC G | GTT CCA AGG CAC ATT GAT GGT A | - |
| *HDAC7* | AGG ACA AGA GCA AGC GAA GTG | TTC AGA ATC ACC TCC GCT AGC T | - |
| *HDAC8* | CCA AGA GGG CGA TGA TGA TC | GTG GCT GGG CAG TCA TAA CC | (55) |
| *HDAC9* | AGT GTG AGA CGC AGA CGC TTA G | TTT GCT GTC GCA TTT GTT CTT T | - |
| *HDAC10* | ATC TCT TTG AGG ATG ACC CCA G | ACT GCG TCT GCA TCT GAC TCT C | - |
| *HDAC11* | CAA TGG GCA TGA GCG AGA C | TGT GGC GGT TGT AGA CAT CC | (65) |
| *HPRT* | TGA CAC TGG CAA AAC AAT GCA | GGT CCT TTT CAC CAG CAA GCT | (22) |
| *SDHA* | TGG GAA CAA GAG GGC ATC TG | CCA CCA CTG CAT CAA ATT CAT G | (22) |

**Table S3. Knockdown efficiency of *HDAC1-11* targeting RNAi.** BE(2)-C cells were transiently transfected with 2 siRNAs (siRNA#1, #2) to specifically silence each HDAC or negative control siRNAs (siNC#1, siNC#2), and knockdown efficiency was determined using qRT-PCR 96 h after transfection. Relative expression to mock-transfected cells is presented as mean ± SD.

| | **relative mRNA expression level compared with mock** | | | | |
|---|---|---|---|---|---|
| **gene** | **untreated** | **siNC#1** | **siNC#2** | **siRNA#1** | **siRNA#2** |
| *HDAC1* | 1.18 ± 0.27 | 1.41 ± 0.59 | 0.91 ± 0.02 | 0.11 ± 0.03 | 0.18 ± 0.18 |
| *HDAC2* | 0.98 ± 0.01 | 1.13 ± 0.24 | 1.01 ± 0.11 | 0.13 ± 0.00 | 0.06 ± 0.02 |
| *HDAC3* | 1.24 ± 0.22 | 1.05 ± 0.18 | 1.03 ± 0.30 | 0.30 ± 0.03 | 0.19 ± 0.02 |
| *HDAC4* | 0.91 ± 0.11 | 0.78 ± 0.04 | 1.08 ± 0.34 | 0.14 ± 0.05 | 0.51 ± 0.05 |
| *HDAC5* | 0.98 ± 0.06 | 0.84 ± 0.12 | 0.86 ± 0.08 | 0.40 ± 0.23 | 0.17 ± 0.02 |
| *HDAC6* | 0.95 ± 0.15 | 1.01 ± 0.31 | 0.95 ± 0.19 | 0.25 ± 0.07 | 0.19 ± 0.06 |
| *HDAC7* | 1.12 ± 0.21 | 1.00 ± 0.03 | 0.99 ± 0.12 | 0.19 ± 0.02 | 0.40 ± 0.23 |
| *HDAC8* | 1.06 ± 0.27 | 0.85 ± 0.11 | 0.99 ± 0.16 | 0.06 ± 0.04 | 0.27 ± 0.12 |
| *HDAC9* | 0.98 ± 0.05 | 1.01 ± 0.05 | 0.71 ± 0.18 | 0.19 ± 0.10 | 0.25 ± 0.03 |
| *HDAC10* | 1.46 ± 0.63 | 1.34 ± 0.35 | 0.89 ± 0.15 | 0.33 ± 0.12 | 0.26 ± 0.23 |
| *HDAC11* | 0.83 ± 0.06 | 1.34 ± 0.31 | 1.02 ± 0.13 | 0.12 ± 0.00 | 0.22 ± 0.02 |

**Table S4. Order information of siRNAs.**

| gene | siRNA# | distributor | cat. # |
|---|---|---|---|
| *HDAC1* | 1 | Qiagen | SI00070609 |
| | 2 | Qiagen | SI00070623 |
| *HDAC2* | 1 | Ambion | 120209 |
| | 2 | Qiagen | SI00434959 |
| *HDAC3* | 1 | Qiagen | SI00057337 |
| | 2 | Qiagen | SI03057901 |
| *HDAC4* | 1 | Qiagen | SI03082282 |
| | 2 | Ambion | 107926 |
| *HDAC5* | 1 | Qiagen | SI00077735 |
| | 2 | Qiagen | SI03066007 |
| *HDAC6* | 1 | Qiagen | SI02663808 |
| | 2 | Qiagen | SI04438490 |
| *HDAC7* | 1 | Qiagen | SI02777726 |
| | 2 | Qiagen | SI04439715 |
| *HDAC8* | 1 | Qiagen | SI00122080 |
| | 2 | Qiagen | SI03099593 |
| *HDAC9* | 1 | Qiagen | SI00148372 |
| | 2 | Qiagen | SI00148393 |
| *HDAC10* | 1 | Qiagen | SI00141736 |
| | 2 | Qiagen | SI00141757 |
| *HDAC11* | 1 | Qiagen | SI03039085 |
| | 2 | Qiagen | SI03084158 |

## Figure S1

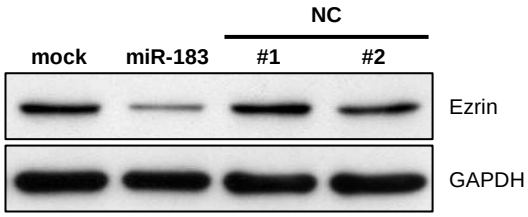


**Figure S1. Transfection efficacy of enforced miR-183 expression was monitored by down-regulation of the known miR-183 target Ezrin.**
BE(2)-C cells were transiently transfected with miR-183 or negative controls (NC#1 and #2). Ezrin expression was analyzed by western blot 96 h post-transfection. GAPDH served as a loading control.

## Figure S2

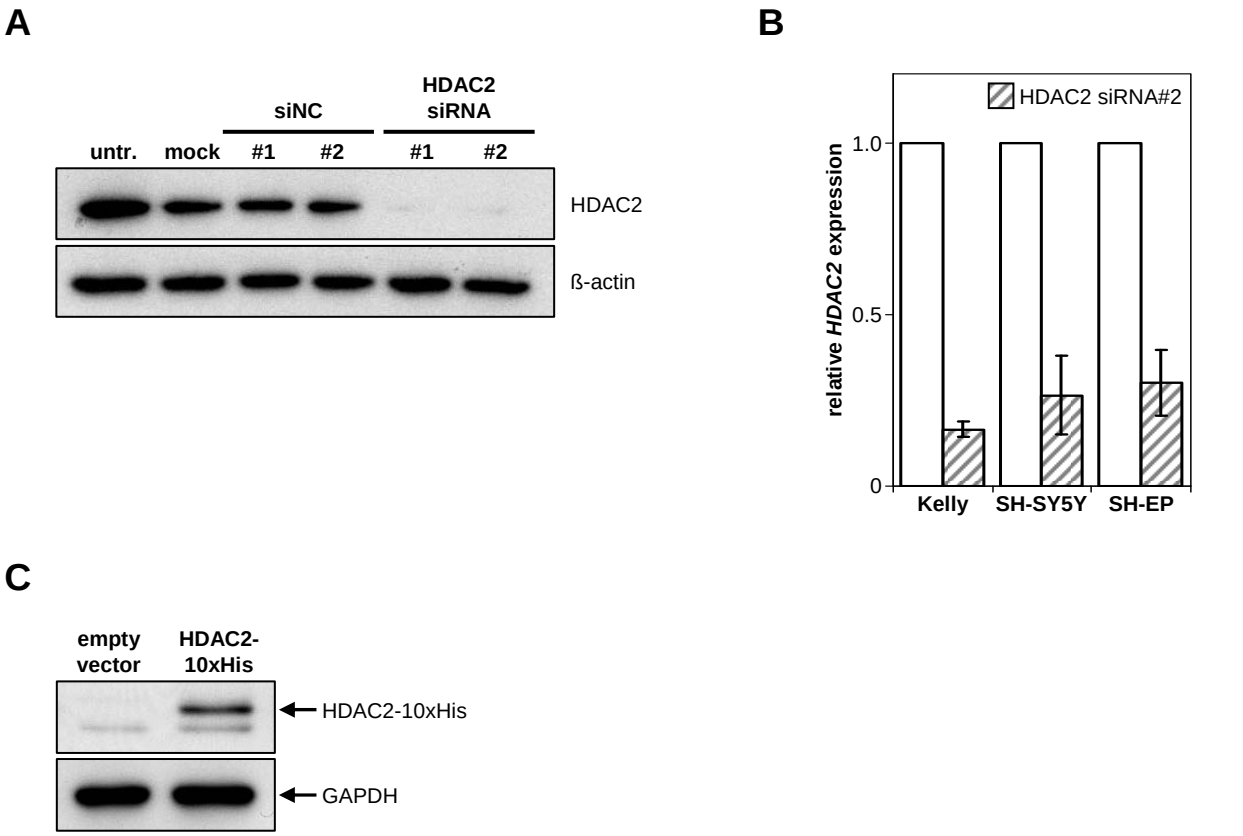


**Figure S2. *HDAC2* knockdown and enforced expression of HDAC2 in neuroblastoma cell lines.**
**(A)** Depletion of HDAC2 on protein level in BE(2)-C cells after knockdown. Cells were transiently transfected with two different siRNAs targeting *HDAC2* (siRNA#1 or #2) or negative controls (siNC#1 or #2). HDAC2 protein level was analyzed 96 h after transfection. by western blot. ß-actin served as a loading control. **(B)** Neuroblastoma cell lines were transiently transfected with siRNA#2 targeting HDAC2. *HDAC2* expression was analyzed via qRT-PCR 96 h after transfection. Mean expression relative to mock-transfected cells is presented (± SD). **(C)** Enforced expression of His-tagged HDAC2. BE(2)-C cells were transiently transfected with a HDAC2-His-tagged encoding plasmid. Enforced expression of His-tagged HDAC2 was analyzed by western blot with a His-tag-specific antibody 72 h after transfection. GAPDH served as a loading control.

## SUPPLEMENTARY REFERENCES


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