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Heterogeneity of neuroblastoma cell identity revealed by transcriptional circuitries

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Neuroblastoma is a tumor of the peripheral sympathetic nervous system¹, derived from multipotent neural crest cells (NCCs). To define Core Regulatory Circuitries (CRCs) controlling the gene expression program of neuroblastoma, we established and analyzed the neuroblastoma super-enhancer landscape. We discovered three types of identity in neuroblastoma cell lines: a sympathetic noradrenergic identity defined by a CRC module including the PHOX2B, HAND2 and GATA3 transcription factors (TFs); an NCC-like identity, driven by a CRC module containing AP-1 family TFs; a mixed type further deconvoluted at the single cell level. Treatment of the mixed type with chemotherapeutic agents resulted in enrichment of NCC-like cells. The noradrenergic module was validated by ChIP-seq. Functional studies demonstrated dependency of neuroblastoma with noradrenergic identity on PHOX2B, evocative of lineage addiction. Most neuroblastoma primary tumors express TFs from the noradrenergic and NCC-like modules. Our data demonstrate a novel aspect of tumor heterogeneity relevant for neuroblastoma treatment strategies.

Keywords: neuroblastoma, neural crest cells, ChIP-seq, super-enhancers, core regulatory circuitries, transcription factors, cell proliferation

Nearly one in six patients who die of a childhood cancer had a neuroblastoma, a tumor of the peripheral sympathetic nervous system¹. Several genes including *MYCN*², *ALK*³⁻⁶ and *TERT*^{7,8} have been shown to act as major drivers of neuroblastoma oncogenesis. In this work, we have determined the core transcriptional regulatory circuitries (CRCs)⁹ that govern the gene expression program of neuroblastoma. CRCs, which can be defined by super-enhancer (SE) mapping of H3K27 acetylation mark (H3K27ac) and further sequence motif analysis, provide integrative information about cell identity^{9,10}.

We examined a panel of twenty-five neuroblastoma cell lines (Table S1) and two primary human neural crest cell (hNCC) lines¹¹. SEs were defined by the ROSE algorithm¹² modified to account for copy number changes. Principal Component Analysis (PCA), based on scores of SEs identified in at least two neuroblastoma cell lines or in both hNCC lines (n=5975) revealed two distinct groups (Figure 1a): group I with 18 neuroblastoma cell lines and group II comprising the GIMEN, SH-EP and GICAN neuroblastoma cell lines. Group II closely resembled the hNCC lines in this analysis. Four neuroblastoma cell lines occupied an intermediate position between groups I and II. These included the phenotypically heterogeneous SK-N-SH cell line whereas its sub-clones, SH-SY5Y and SH-EP, were included in groups I and II, respectively. This result is consistent with SH-SY5Y cells displaying neurite-like processes and expressing noradrenergic biosynthetic enzymes TH and DBH (“N” phenotype), and SH-EP cells exhibiting a substrate-adherent “S” phenotype without expression of TH and DBH¹³. We also profiled the SE landscape of six patient-derived xenografts (PDXs), five of them with *MYCN* amplification (Table S2). All PDXs clustered with group I when included in the PCA (Figure 1a).

SEs were then sorted according to the median H3K27ac signal for each group (Figure 1b and 1c, respectively; Table S3). In group I, the strongest SEs comprise a set of transcription factor (TF) loci including *HAND2*, *PHOX2A/PHOX2B* and *GATA2/GATA3* and the *ALK* oncogene locus (Figure 1b, 1d, 1e, Figures S1 and S2). These findings are consistent with previous SE data on a few neuroblastoma cell lines^{14,15}. *PHOX2B*, *HAND2*, and *GATA3* are known to participate in a complex TF network controlling normal sympathetic neuron specification and differentiation^{16,17}. Recurrent SEs in these TFs therefore appear to be a hallmark of sympathetic cell identity. Most SEs of group II overlapped with SEs of hNCC lines (Figure 1c), consistent with the results of the PCA analysis.

Our analysis found *MYCN* SEs for 10 out of 18 cell lines of group I, with or without

MYCN amplification, and 3 PDX (Figure S3). No groups linked to the *MYCN* or *ALK* status were revealed in the PCA. Furthermore, supervised analysis of SE scores did not indicate SEs associated with *MYCN* amplification or *ALK* mutations (Tables S4 and S5). Cell lines with a *PHOX2B* mutation were observed in group I (SH-SY5Y), group II (SH-EP) or in the intermediate group (SK-N-SH).

To detect driver TFs for groups I and II, we used i-cisTarget to find DNA sequence motifs enriched in the SEs with the highest score. For group I, this analysis identified a TAATYYAATTA binding motif common to several homeobox proteins, including *PHOX2B* and *PHOX2A* (Figure S4). During sympathetic nervous system development *PHOX2B* regulates *PHOX2A* expression¹⁸. Both TFs are highly expressed in most neuroblastoma cell lines and primary tumors (Figure S5). *PHOX2B* was undetectable at the transcript and protein level solely in the group II cell lines as well as in the hNCC lines, but was expressed in all other neuroblastoma cell lines (Figure 1f; Figure S6). There was a corresponding lack of SE at *PHOX2B* and no TH and DBH transcription in GIMEN, SH-EP and GICAN (Figure S7). I-cisTarget analysis showed enrichment in AP-1 motif in group II and in the hNCC lines (Figures S8 and S9). AP-1 is a heterodimer composed of FOS and JUN family members both of which are expressed in immature hNCC¹¹. These results suggest that *PHOX2B* participates in the activity of neuroblastoma group I SEs while the AP-1 complex TFs influence the SE landscape of group II.

A CRC calling algorithm^{9,19} identified *PHOX2B* as group I-specific CRC TF and FOSL1, FOSL2 and JUN for group II (Figure S10), consistent with our i-cisTarget results. We therefore searched for TFs predicted to be in a CRC either with *PHOX2B* or a FOS/JUN family member (Figure 1g). Cell lines showing an intermediate position in the PCA had a CRC that included several TFs of both *PHOX2B*-associated and FOS/JUN-associated sets. Most of TFs of the latter set are expressed in neural crest cells and/or mesenchymal neural crest derivatives. The CRCs of the six PDXs were highly similar to those of the group I cell lines (Figure 1g). As we documented that SE strength linearly correlated with gene expression (Figure S11), we used the latter to further define fully connected TF modules. This analysis revealed two main TF modules distinguishing groups I and II (Figure 1h). *PHOX2B*, GATA3 and HAND2 were present in module 1 whereas module 2 included FOSL1 and FOSL2. These modules were anti-correlated at the gene expression level. Western blot analysis confirmed the co-expression of several TFs in

group I or group II (Figure S12). Furthermore, single cell analysis showed that the SK-N-AS and SK-N-SH cell lines are heterogeneous and comprise cells expressing TFs of either module 1 or module 2 within the same population (Figure 1i, Figure S13, Table S6).

Taken together, these data demonstrate a novel type of heterogeneity in neuroblastoma cell lines and suggest that individual cells assume either a sympathetic noradrenergic identity, characterized by a CRC module including PHOX2B, HAND2 and GATA3, and subsequent expression of the enzymes TH and/or DBH; or an NCC-like identity, characterized by expression of a distinct module including FOS and JUN family members but lacking PHOX2B and noradrenergic marker expression. Both types of identity are observed in several heterogeneous cell lines. All cell lines with MYCN amplification except one (CHP-212) had a noradrenergic identity whereas cell lines without MYCN amplification displayed any of the three identities (Figure 1g).

To explore whether the different identity classes seen in neuroblastoma cell lines are also characteristic for neuroblastoma primary tumors, expression data from a large set of primary tumors (n=498; dataset GSE49711)²⁰ were studied. Correlations between expression of the TFs of each module identified in cell line CRCs were calculated for these primary tumors. We found strong positive correlations between PHOX2B, HAND2, and GATA3 of module 1 as well as between the TFs of the NCC-like module (Figure 2a). We also observed anti-correlations between PHOX2B, HAND2, and GATA3 on the one hand, and TFs of the NCC-like module on the other hand. These results therefore confirm the data obtained with cell lines and further define a PHOX2B/HAND2/GATA3 noradrenergic CRC module in primary neuroblastomas. Next, we used the average expression of the two modules to explore primary tumor identity. All but two tumors showed high expression of the noradrenergic module (Figure 2b). A continuum was observed between low to high values of the NCC-like module, suggesting heterogeneity of cell identity in primary tumors. The remaining two cases with low noradrenergic and high NCC-like module expression may correspond to rare cases with full NCC-like identity, as described for group II cell lines. Similar to the cell lines (Figure S14), lower expression of the NCC-like module was observed in the majority of MYCN-amplified tumors (two-sided Wilcoxon signed-rank test p-value 1.01×10^{-10}). A role for MYCN in downregulation of genes from this module is consistent with its promotion of peripheral neuron differentiation from multipotent avian NCC²¹.

Next, expression of the NCC-like and noradrenergic modules was evaluated in a series of

10 diagnosis/relapse sample pairs²². Different patterns were observed between the two disease stages (Figure 2c). To address a possible link between heterogeneity of cell identity and treatment response, we investigated the effect of chemotherapy on the NCC-like SH-EP and noradrenergic SH-SY5Y cell lines. SH-EP cells were more resistant to the three agents used (Figure S15). Treatment of the parental SK-N-SH cell line with doxorubicin or cisplatin resulted in the respective decreased or increased expression of module 1 and 2 (Figure 2d). Enrichment of cells with an NCC-like identity thus correlates with better drug resistance. However, we cannot exclude that treatment may also induce transdifferentiation from noradrenergic to NCC-like identity. The observation that tumors at relapse are not systematically enriched in NCC-like cells supports the concept of plasticity in the reversion of cell identity. This may rely on a switch from adrenergic to NCC-like identity under chemotherapy and from NCC-like to noradrenergic after treatment. Altogether, these data underline the importance of targeting both types of cells during treatment.

Strong correlations between PHOX2B, HAND2 and GATA3 expression were observed both in cell lines and tumors. Phox2b directly binds Hand2 protein²³, and Phox2b, Hand2 and Gata3 cross-regulate during sympathetic nervous system development¹⁶. We therefore performed ChIP-seq analysis for these TFs in the CLB-GA neuroblastoma cell line and identified binding motifs for PHOX2B, HAND2 (zinc finger TF) and GATA3 (bHLH leucine zipper TF) (Figure 3a). Binding regions for all three TFs corresponded to the H3K27ac peaks in the *PHOX2B*, *GATA3*, *HAND2* and *ALK* SEs and also in the *MYCN* SE (Figure 3b and 3c, Figure S16). These results therefore confirm the biological existence of the noradrenergic module, showing that PHOX2B, HAND2 and GATA3 are SE-regulated and bind to the SEs of each other (Figure 3d). We next investigated the occupancy by these TFs of 4,336 SE regions predicted in at least two neuroblastoma cell lines. SE regions were ranked according to average SE score and intersection with TF binding sites was evaluated. Over 90% of the strong and recurrent neuroblastoma SEs were co-occupied by PHOX2B, HAND2 and GATA3 (Figure 3e). Additionally, positional binding analysis showed that HAND2, PHOX2B and GATA3 bind the same ~400 bp-long regions within active regulatory regions (Figure 3f). Altogether, our results demonstrate that PHOX2B, HAND2 and GATA3 are master TFs defining the SE landscape of neuroblastoma cell lines with a noradrenergic identity.

It has been demonstrated that cancer dependencies can be found among SE-marked

genes^{19,24}. Although missense and frameshift *PHOX2B* mutations predispose to neuroblastoma^{25,26}, its role in sporadic neuroblastoma remains poorly understood. *Phox2b* knock-out mice completely lack autonomic structures¹⁷ whereas conditional knock-out leads to decreased neuroblast proliferation²⁷. An effect of *PHOX2B* knockdown on neuroblastoma cell proliferation has been previously suggested²⁸. To further document the consequence of *PHOX2B* knockdown on neuroblastoma growth, we generated a doxycycline-inducible anti-*PHOX2B* short-hairpin RNA (shRNA) expression system in noradrenergic CLB-GA and SH-SY5Y cells. Inducible decrease of *PHOX2B* protein (Figure 4a, 4c) resulted in significant inhibition of neuroblastoma cell growth (Figure 4b, 4d, Figure S17). Decreased expression of *PHOX2B* in CLB-GA cells also impaired tumor growth *in vivo* (Figure 4e and 4f, Figure S18). We then evaluated whether *PHOX2B* decrease was sufficient to change the noradrenergic identity of the CLB-GA and SH-SY5Y cell lines to an NCC-like identity. However, data obtained by RNA-seq and RT-q-PCR suggested that the residual level of *PHOX2B* was sufficient to maintain a noradrenergic identity (Figure S19). This observation is consistent with the noradrenergic identity of the CLB-PE cell line in which *PHOX2B* expression is low but detected at the protein and RNA levels (Figures 1f and S6).

We observed a reduction of proliferation upon *HAND2* and *GATA3* knockdown in several cell lines, consistently with previous data on *GATA3* knockdown¹⁵ (Figure S20). These results are in line with *Hand2* and *Gata3* controlling sympathetic neuroblast proliferation¹⁶. Neuroblastoma cells of noradrenergic identity therefore appear to be addicted to these key lineage TFs as well as to *PHOX2B*²⁹.

In conclusion, our work provides fundamental insights into the transcriptomic and epigenomic landscape of neuroblastoma. Distinct TF networks predicate different tumor identities, corresponding to sympathetic noradrenergic or NCC-like identity. Most primary tumors comprise cells of both identities, revealing a novel aspect of tumor heterogeneity. Neuroblastoma treatment should benefit from specifically targeting both identities.

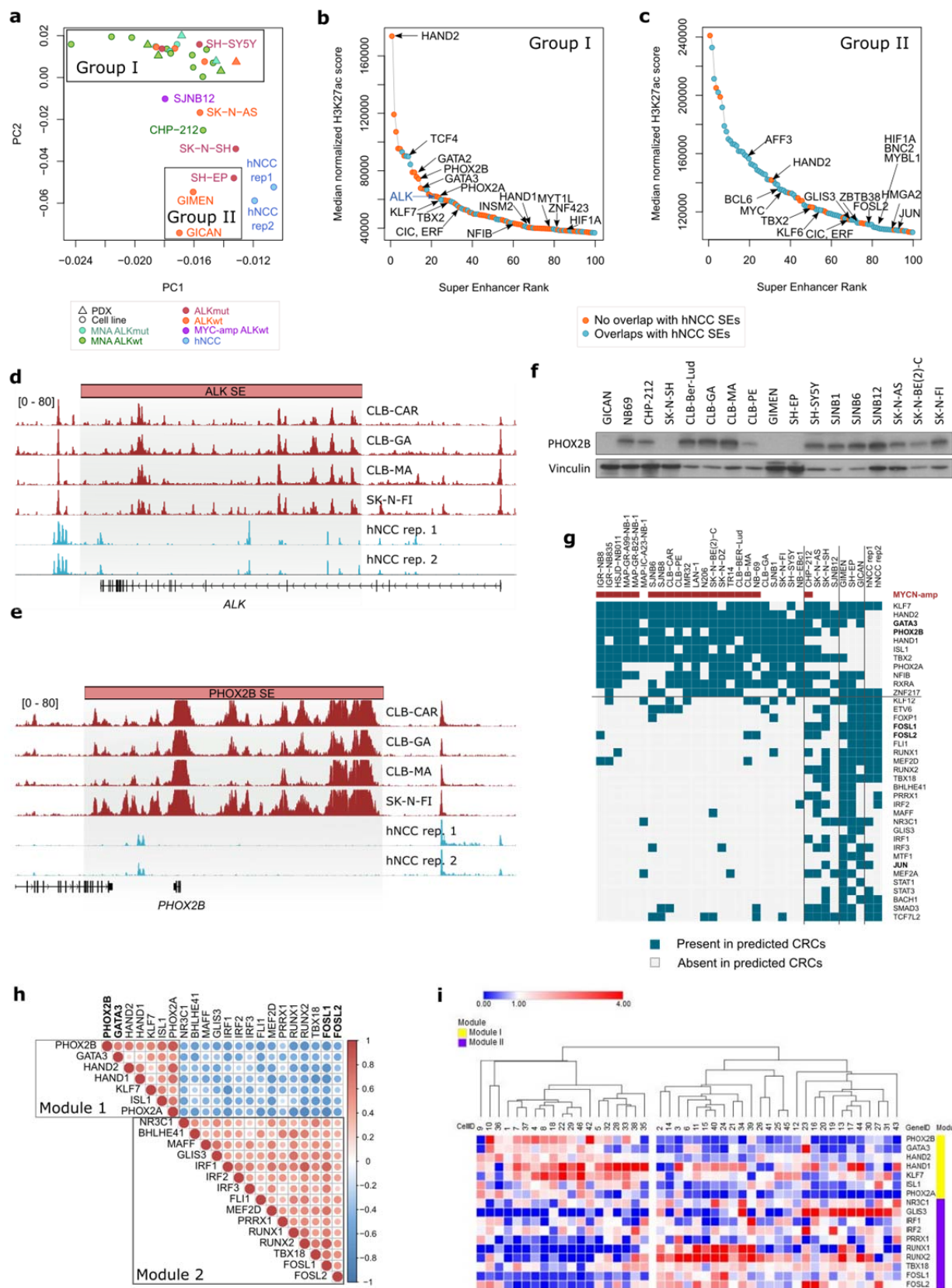


Figure 1. SE landscape reveals various CRCs and identities in neuroblastoma cell lines. **a**, Principal Component Analysis (PCA) based on neuroblastoma and hNCC SE log scores. MNA: MYCN amplification. **b, c**, Ranked plot for the 100 SEs with the highest median H3K27ac score in neuroblastoma cell line groups I and II, respectively. TFs are indicated in black with arrows. **d, e**, Tracks for ChIP-seq profiles for H3K27ac binding at *ALK* and *PHOX2B* SEs, respectively. **f**, Western blot analysis of PHOX2B and vinculin as a loading control in a panel of neuroblastoma cell lines. *SK-N-SH cells correspond to batch 1*. **g**, TFs predicted to participate in a CRC with PHOX2B (upper part) or with a FOS/JUN family member (lower part) in neuroblastoma cell lines. TFs whose binding motifs are enriched in SEs of group I and II are shown in bold. **h**, Pearson correlation matrix for the *expression values of 22 TFs identified in CRCs of cell lines* shows strong positive correlations within module 1 and module 2; *correlation is calculated for RNA-seq data in neuroblastoma cell lines and PDX (n=31)*. **i**, Single cell analysis reveals heterogeneity of cell identity in the SK-N-AS cell line. Expression of TF of modules 1 and 2 was evaluated by RT-q-PCR and data were normalized to the SK-N-AS cell population overall.

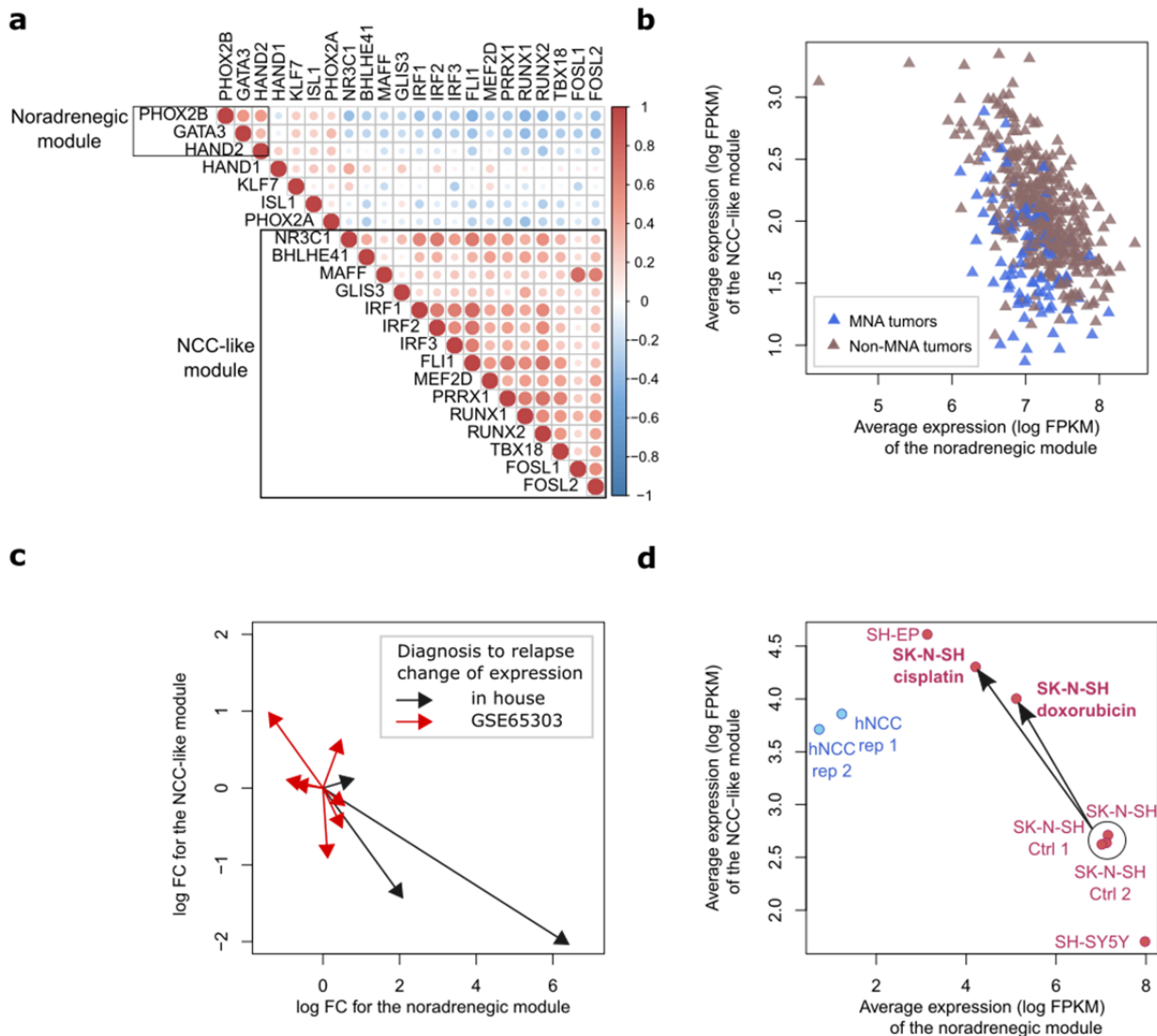


Figure 2. Different identity of neuroblastoma primary tumors and impact of chemotherapy on cell identity. **a**, Pearson correlation matrix for the 22 TFs identified in CRCs of cell lines in a set of 498 neuroblastoma primary tumors. **b**, Mean expressions of the noradrenergic and NCC-like modules negatively correlate in the whole set of tumors (Pearson $R = -0.49$, one-sided permutation test $p\text{-value} < 10^{-10}$) and define a continuum between full noradrenergic and NCC-like cases. Blue: tumors with *MYCN* amplification. **c**, Identity of tumor pairs at diagnosis and relapse revealed by expression profiling. The series includes 7 pairs from the GSE65303 dataset²² (red) and 3 in-house pairs (black). **d**, Treatment of SK-N-SH cells with chemotherapy favors cells with an NCC-like identity. Cells used in this experiment (batch 2) were more noradrenergic compared to the ones used in the ChIP-seq experiment (batch 1).

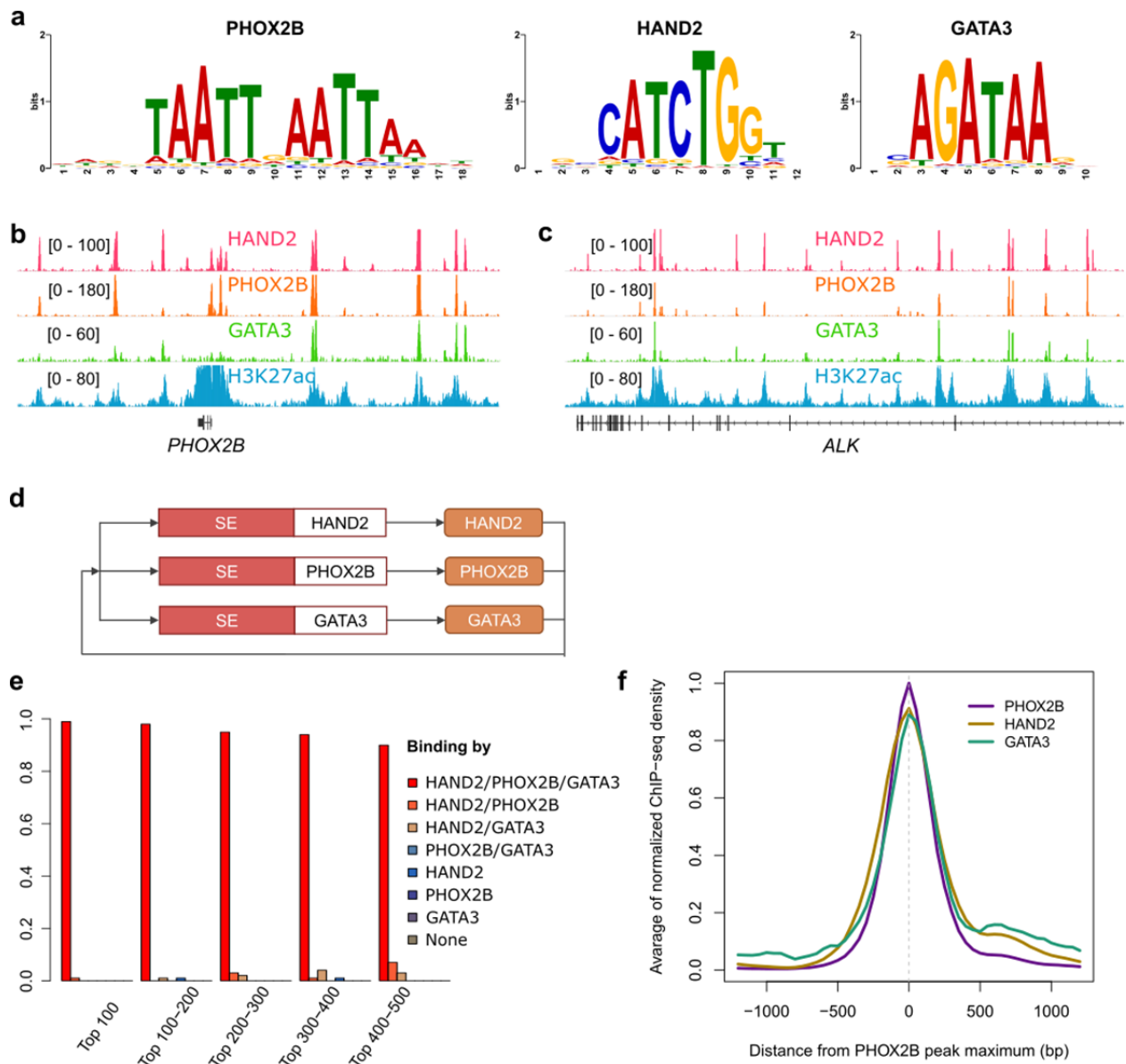


Figure 3. PHOX2B, HAND2 and GATA3 are master transcription factors defining the SE landscape of noradrenergic neuroblastoma. **a**, *De novo* identification of PHOX2B, HAND2 and GATA3 TF binding motifs. **b** and **c**, Tracks for ChIP-seq profiles for PHOX2B, HAND2, GATA3 and H3K27ac binding at *PHOX2B* and *ALK* SEs, respectively. **d**, CRC of activating TFs that define a noradrenergic module. **e**, Neuroblastoma SEs defined by H3K27ac peaks are simultaneously occupied by PHOX2B, HAND2 and GATA3. **f**, HAND2, PHOX2B and GATA3 bind closely located regions within neuroblastoma SEs (summary of densities of 2,078 binding sites corresponding to 500 top neuroblastoma SEs).

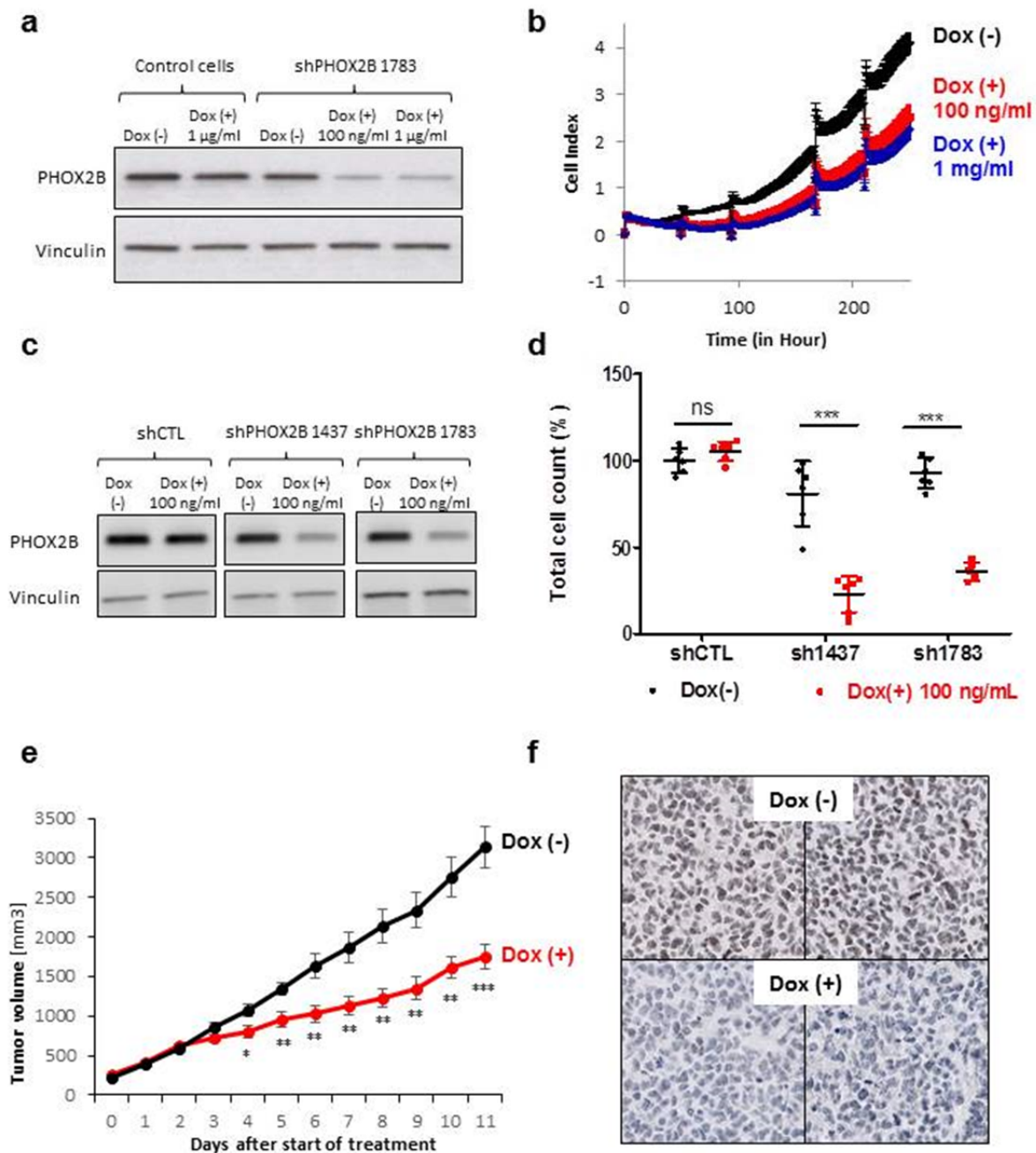


Figure 4. PHOX2B is critical for the growth of noradrenergic neuroblastoma cells. **a**, PHOX2B knockdown following doxycycline treatment was confirmed at 72 h by immunoblot (loading control: vinculin) in CLB-GA neuroblastoma cells infected with a shRNA targeting *PHOX2B* vector. **b**, xCELLigenceTM proliferation kinetics of infected CLB-GA cells in absence

or presence of doxycycline at 100 ng/ml or 1 μ g/ml. Data shown are the mean $\pm$ s.d. of results obtained in the different conditions (n=5 technical replicates). **c**, PHOX2B immunoblot of SH-SY5Y neuroblastoma cells infected with 2 different shRNA vectors targeting *PHOX2B*, at 72 h. **d**, Cell counts for the SH-SY5Y cell line infected with sh1437 or sh1783 vectors targeting *PHOX2B* or with the control shCTL vector. 10^5 cells were plated in 24-well plates at day 0 in the absence or presence of doxycycline at 100 ng/ml. The number of living cells was counted at day 8 (Mean $\pm$ s.d.; n=6 replicates). **e**, Growth curves for subcutaneously xenografted sh1783 transduced CLB-GA cells. When tumors reached a volume of around 170 mm³, doxycycline and sucrose (Dox +) or sucrose alone (Dox -) was added to the drinking water (Mean $\pm$ s.e.m.; n =8 mice per group). P values were determined via two-tailed unpaired Welch's t-test (***: p<0.001). **f**, PHOX2B immunohistochemistry (brown) combined with Hematoxylin staining in two xenografts treated with doxycycline and two control xenografts.

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Online Methods

Neuroblastoma and hNCC cell lines

Neuroblastoma cell lines used in this study have been previously described³⁰. [CHP-212](#), [IMR-32](#), [SH-SY5Y](#), [SK-N-AS](#), [SK-N-BE\(2\)C](#), [SK-N-DZ](#), [SK-NF-I](#) and [SK-N-SH](#) were obtained from the American Type Culture Collection (ATCC). CLB cell lines were derived by V. Combaret (Lyon, France). The SH-EP and LAN-1 cell lines have been kindly provided by M. Schwab (Heidelberg, Germany) and J. Couturier (Paris, France). Lines GIMEN, N206, SJNB1, SJNB6, SJNB8, SJNB12 and TR-14 were obtained from R. Versteeg (Amsterdam, The Netherlands) and line GICAN was a kind gift from M. Ponzoni (Genova, Italy). The NB69 and NB-EBc1 cell lines were obtained from the European Collection of Authenticated Cell Cultures and from the Children's Oncology Group, respectively. A first batch of SK-N-SH cells (batch 1) was used for the ChIP-seq and single cell analysis. A second batch (batch 2) was used for the evaluation of the chemotherapeutic agents. Batch 2 was enriched in adrenergic cells. Cell line authentication was performed by comparison of the genomic copy number profile calculated from the input ChIP-seq data obtained using Control-FREEC³¹ (see below) with SNP array profile and STR profiling for ATCC cell lines. Cells were checked routinely by PCR for the absence of mycoplasma. Neuroblastoma cell lines were cultured at 37°C with 5% CO₂ in a humidified atmosphere in RPMI (GE Healthcare, for CLB cell lines, [SH-EP](#), [GICAN](#) and [NB69](#)), in IMDM (Gibco) for [NB-EBc1](#) (according to the provided conditions) or DMEM (GE Healthcare, for other cell lines), with 10%, 15% or 20% FCS (Eurobio) and 100 µg/ml penicillin/streptomycin (Gibco). Primary hNCC lines were grown as previously described³² under bioethical approval PFS14-011 from the French Biomedical Agency for the use of human embryonic material to S. Zaffran. Briefly, cells were grown in Glutamax DMEM:F12 [Gibco] supplemented with 12% FCS (Eurobio), 100 µg/ml penicillin/streptomycin, 10 mM HEPES, 100 ng/ml hydrocortisone, 10 µg/ml transferrin, 400 pg/ml 3,3,5-thio-iodo-thyronine, 10 pg/ml glucagon, 100 pg/ml epidermal growth factor, 1 ng/ml insulin and 200 pg/ml fibroblast growth factor 2 (all products supplied by Sigma-Aldrich except EGF and FGF2 from Gibco).

PDX models

Neuroblastoma PDXs were obtained from stage L2 (MAP-IC-A23-NB-1), stage 3 (IGR-NB8) or stage 4 (IGR-N835, MAP-GR-A99-NB-1, MAP-GR-B25-NB-1 and HSJD-NB-011³³). None of them was related to the used cell lines. All PDXs but MAP-IC-A23-NB-1 had *MYCN* amplification. PDXs IGR-NB8, IGR-N835^{34,35} were obtained using female Swiss nude mice of 6-8 weeks at engraftment whereas female NSG mice were used for MAP-GR-A99-NB-1 and MAP-GR-B25 PDXs. These PDX models are developed and maintained within the project "Development of Pediatric PDX models" approved by the experimental ethic committee 26 (CEEA26 – Gustave Roussy) under the number 2015032614359689v7. The MAP-IC-A23-NB-1 (IC-pPDX-17) and HSJD-NB-011 models were obtained using female SCID mice of 10-11 weeks or female Swiss nude mice of 3-6 weeks at engraftment. Animal studies at SJD were approved by the local animal care and use committee (Comite Etico de Experimentacion Animal at Universidad de Barcelona, protocol 135/11). All experiments were performed in accordance with European legislation. MAP-IC-A23-NB-1, MAP-GR-A99-NB-1 and MAP-GR-B25-NB-1 PDXs were obtained through the Mappyacts protocol (clinicaltrial.gov: NCT02613962).

Patient samples

Three diagnosis/relapse pairs of tumors (Pair1/2/3-Diagnosis and Relapse; all stage 4; Table S2) were studied in this work. The relapse samples were obtained through the Mappyacts protocol. The MAP-GR-B25-NB-1 PDX was derived from the relapse of pair 1. Analysis of biological material from patients, including study of expression profiles of neuroblastoma samples was approved by the Institut Curie's Institutional Review Board. This study was authorized by the decision of the ethics committees « Comité de Protection des Personnes Sud-Est IV », references L07–95 and L12–171, “Comité de Protection des Personnes Ile de France 1”, reference 0811728 and “Comité de Protection des Personnes Ile de France 3” reference 3272. Written informed consent was obtained from parents or guardians according to national law.

ChIP-sequencing

H3K27ac, PHOX2B, HAND2 and GATA3 chromatin immunoprecipitation (ChIP) was performed using the iDeal ChIP-seq kit for Histones or iDeal ChIP-seq kit for Transcription Factors (Diagenode) using the following antibodies: ab4729 (rabbit polyclonal, Abcam) for H3K27ac, sc-376997X (mouse monoclonal), sc-9409 and sc-22206X (goat polyclonal) from Santa Cruz Biotechnology for PHOX2B, HAND2 and GATA3, respectively. Ten million cells were cross-linked with 1% formaldehyde for 10 min followed by quenching with 125 mM glycine final concentration for 5 min at room temperature. Chromatin was isolated by the addition of lysis buffer, and lysates sonicated to obtain sheared chromatin to an average length of ~ 300 bp. ChIP was performed with chromatin of 1 million cells for H3K27ac and 3.75 million cells for transcription factors. The equivalent of 1% of chromatin used for TFs was kept to quantify input and reverse cross-linked 4h at 65°C with proteinase K. ChIP was performed overnight at 4°C on a rotating wheel with 1 µg of antibody for H3K27ac, 2 µg for HAND2 and 5 µg for PHOX2B and GATA3. Protein A-coated magnetic beads were precleared with antibodies 3h at 4°C only for transcription factors. After ChIP, chromatin was eluted 30 min at room temperature and reverse cross-linked 4h at 65°C with proteinase K. DNA was precipitated and purified with magnetic beads with the Ipure kit (Diagenode). Before sequencing, ChIP efficiency was validated by quantitative PCR for each antibody on specific genomic regions using powerSYBR® Green Master mix (Applied Biosystems) and compared for each primer pair to the input DNA. Primers are available upon request.

For PDX samples, frozen tumors were reduced to powder with a pestle and then resuspended in PBS. Crosslinking of chromatin was performed by adding 1% formaldehyde for 8 min with agitation on a rotating wheel. Lysis of cells, fragmentation of chromatin and ChIP were performed as described above for cell lines using the iDeal ChIP-seq kit for Histones.

Illumina sequencing libraries were prepared from ChIP and input DNA using the TruSeq ChIP library preparation kit according to the manufacturer's protocol. Briefly, DNA were subjected to consecutive steps of end-repair, dA-tailing and ligation to TruSeq indexed Illumina adapters. Size-selection was performed only for the H3K27ac ChIP (100 – 600 bp). After a final amplification step of 14 cycles, the resulting DNA libraries were quantified using a qPCR method (KAPA library quantification kit) and sequenced on the Illumina HiSeq2500 instrument

(rapid run mode; single reads 100 nts).

ChIP-seq analysis

ChIP-seq reads were mapped to the human reference genome hg19/GRCh37 using Bowtie2 v2.1.0³⁶. Low mapping quality reads ($Q < 20$) were discarded; duplicate reads were kept in order to detect signal in genomic amplification regions. Enriched regions (peaks) were called using HMCAN v1.30³⁷ with the following parameters: min fragment length 100 bp, median fragment length 250 bp, maximal fragment length 400 bp, small bin length 50 bp, large bin length 25 kb, p-value threshold 0.05, merging distance 200 bp, number of iterations 20, final threshold 0.1, removing duplicates: False. Regions from the hg19 ENCODE blacklist³⁸ were excluded from the analysis. HMCAN output included ChIP density profiles corrected for the GC-content and copy number bias (*.wig) and narrow and large enrichment regions further called peaks (*.bed). Density profiles were then normalized between samples with an in-house R script based on the median density values in the 5,000 highest peaks discounting the first 100 peaks as they may correspond to amplification regions. Peaks with low signal (i.e., low HMCAN score values) were discarded (in-house script correlating peak length and peak signal, <https://github.com/BoevaLab/LILY/>).

The Control-FREEC³¹ algorithm was applied to input samples (default parameters; input: *.bam files) to obtain copy number profiles of each cell line. These profiles matched known copy number profiles for these neuroblastoma cell lines.

To call enhancers and super-enhancers, a modified version of ROSE^{12,39} dubbed LILY was used (<http://BoevaLab.com/LILY/>). First, large H3K27ac peaks were stitched together, using a default distance of 12.5 kb, while promoter regions (± 2.5 Kb from the transcription start site) were excluded. Then each region received a SE score corresponding to the sum of normalized H3K27ac density values (already corrected for copy number and GC-content bias by HMCAN³⁷). The regions were sorted according to the SE score. The threshold of the score distinguishing typical enhancers from SEs was determined by ROSE. For twenty-five neuroblastoma cell lines, the average number of SEs identified per cell line was 1,252 (standard deviation 385). The highest number of SEs was detected in GIMEN and SH-EP (1,901 and 1,819 regions respectively).

ChIP-seq experiments for H3K27ac were performed once for every sample except for the CLB-GA cell line for which the experiment was performed in two biological replicates. We used these replicate samples to document the reproducibility of the SE calling and SE score calculation (Figure S21). Among the top 500 SEs of replicate 1, 93% were annotated as active SEs in replicate 2.

To generate a list of neuroblastoma SEs, we superimposed the SE regions predicted in the twenty-five cell lines and excluded regions shorter than 12 Kb. In order to avoid stitching of several neighboring SE regions into one, long regions with several sub-peaks were separated in sub-regions using as a threshold one half of the median number of SEs. Overall, 4,336 regions with overlapping SEs detected in more than one sample were annotated as putative neuroblastoma SEs (Table S3). SEs were assigned to the RefSeq genes (hg19, version Sep 16, 2016) using the information about locations of topologically associating domains (TADs) in eight human cell lines⁴⁰. Among all genes located in the same TAD with a SE and therefore possibly regulated by a SE, we selected these with the highest correlation between the gene expression and the SE score in the 33 samples of these study (threshold 0.361 corresponding to the adjusted p-value ('FDR') of 0.05, Figure S11). Of note, each gene can have several SE regions and each SE can be assigned to a number of genes (Table S3). In total, neuroblastoma SEs were assigned to 4,791 genes. Similarly, we detected and assigned to genes 1,639 SEs active in both hNCC samples.

For further analysis, we kept only SE regions active in at least two neuroblastoma cell lines or hNCC samples (5,975 regions). This was done to remove cell-line specific events and false positive predictions of SE regions.

Principal Component Analysis (PCA) for 33 samples (25 neuroblastoma cell lines, 6 neuroblastoma PDXs and 2 hNCC lines) was performed on log2 values of SE scores of 5,975 SEs. Table S3 shows contributions of the SE regions to the first two principal components. Analysis of samples in the first principal components suggested their separation into group I (CLB-GA, CLB-MA, CLB-CAR, CLB-BER-Lud, CLB-PE, NB69, NB-EBc1, SJNB1, SJNB6, SJNB8, IMR-32, LAN-1, N206, SK-N-BE(2)C, SK-N-DZ, SK-N-FI, TR14, SH-SY5Y), group II (GICAN, SH-EP, GIMEN) and the intermediate group (SK-N-SH, SK-N-AS, SJNB12, CHP-212). Table S3 includes information about fold changes and p-values for the two-sided Wilcoxon test for differential analysis of SE scores between group I and II.

To detect known transcription factor binding motifs enriched in neuroblastoma SEs (cell lines of group I and II) and SEs of hNCC, we applied the i-cisTarget⁴¹ method to the list of 2,227, 1,850 and 1,640 valley regions in H3K27ac peaks overlapping the 100 top SEs of group I and II and hNCC, respectively.

CRC in the neuroblastoma cell lines, PDX samples and hNCC lines were detected using COLTRON¹⁹ based on the list of samples' SEs with the following properties: (i) SE score correlated with gene expression in our set of 31 NB samples and (2) SE region was detected in more than 2 cell lines in our study. We then parsed the files with ranked cliques to see whether a given TF was predicted to be involved in a CRC of a given sample. We kept TFs present in over 50% of cell lines from group I (n=18) or group II (n=3). This resulted in 69 TFs. From the COLTRON predictions, we excluded 17 transcription factors that were not associated with a SE in our analysis (Figure S10). As motif enrichment analysis discovered a significant enrichment in homeobox and AP-1 motifs of neuroblastoma SEs (Figures S4 and S8), among these 52 TFs, we selected those that were predicted by COLTRON to occur in the same CRC as the homeobox TF PHOX2B or AP-1 TFs (JUN, JUNB, FOSL1 or FOSL2) in more than 50% of cell lines of group I or II. This resulted in 37 TFs (Figure 1g). Clustering of the 37 genes (hclust, McQuitty method) based on the correlation of their expression defined two modules (module 1, n = 7, includes PHOX2B; module 2, n=15, includes FOSL1 and FOSL2) (Figure S22).

Motif discovery in ChIP-seq peaks of GATA3, HAND2 and PHOX2B was performed using the Position Analysis tool of the RSAT package⁴² (Oligonucleotide size: HAND2: 5; GATA3: 5; PHOX2B: 8).

To calculate average ChIP-seq density profiles around the PHOX2B peak maximum positions, we first extracted all 2,400 bp regions centered on PHOX2B ChIP-seq binding sites and kept those that overlapped peaks of all three TFs. We obtained 14,693 such regions throughout the whole human genome for the CLB-GA cell line. 2,078 out of them were located within the 500 strongest neuroblastoma SEs. ChIP-seq density for each TF for each region was rescaled to have a maximum value of 1 corresponding to the peak maximum. We then plotted the average rescaled density for the 2,078 regions.

RNA-sequencing and transcriptome read alignment

Total RNA was extracted from fresh cells or frozen tumors using TRIzol® Reagent (Invitrogen),

or AllPrep DNA/RNA Mini Kit (Qiagen) or NucleoSpin RNA kit (Macherey-Nagel; for the SK-N-SH cell line treated with chemotherapy). All samples were subjected to quality control on a Bioanalyzer instrument and only RNA with RIN (RNA Integrity Number) > 6 were used for sequencing. RNA sequencing libraries were prepared from 1 µg of total RNA using the Illumina TruSeq Stranded mRNA Library preparation kit which allows performing a strand-specific sequencing. A first step of polyA selection using magnetic beads is done to focus sequencing on polyadenylated transcripts. After fragmentation, cDNA synthesis was performed and resulting fragments were used for dA-tailing and then ligated to the TruSeq indexed adapters. PCR amplification is finally achieved to create the final cDNA library. After qPCR quantification, sequencing was carried out using 2 x 50 cycles (paired-end reads 50 nts) for all samples (except SH-EP, 2 x 100; Pair1-Relapse and Pair3-Relapse, 2 x 75; Pair2-Relapse, 2 x 150). Sequencing was performed with the Illumina HiSeq2500 instrument (high output mode) except for cases Pair1-Relapse and Pair2-Relapse analyzed with the NextSeq500 instrument and Pair3-Relapse3 analyzed on a HiSeq4000 instrument. Reads were aligned to the human reference genome hg19/GRCh37 using TopHat2 v2.0.6⁴³ with the following parameters: global alignment, no mismatch in the 22 bp seed, up to three mismatches in the read, library type fr-firststrand. Gene expression values (FPKM=fragments per kilobase per million reads) were computed by Cufflinks v2.2.1⁴⁴ and further normalization between samples was done using quantile normalization (R/Bioconductor package limma)⁴⁵.

Western blots

Western blots were carried out using standard protocols with the following antibodies: PHOX2B (sc-376997 from Santa Cruz Biotechnology at 1:500) and anti-vinculin (ab18058 from Abcam at 1:1,000). Membranes were then incubated with an anti-mouse immunoglobulin G (IgG) horseradish peroxidase-coupled secondary antibody (1:3,000, NA931V) from GE Healthcare. Proteins were detected by enhanced chemiluminescence (PerkinElmer).

Single cell gene expression analysis

Single cells loading, capture and mRNA pre-amplification were performed following the Fluidigm user manual “Using C1 to Capture Cells from Cell Culture and Perform

Preamplification Using Delta Gene Assays". Briefly, cells were dissociated using TrypLE Express reagent (Gibco), washed 2 times in PBS and 2,000-4,000 cells were loaded onto a medium size (10-17µm) C1 single-cell auto prep IFC (Fluidigm). The capture efficiency was assessed by imaging capture sites under the microscope and cell viability was investigated with ethidium homodimer-1 and calcein AM stains (LIVE/DEAD kit, Thermo Fisher Scientific). Capture sites containing more than one cell or a dead cell were later excluded. Lysis, reverse transcription, and specific target preamplification steps were done on the C1 machine according to the Fluidigm user manual. Preamplification was done with inventoried pairs of unlabeled primers coupled with a Taqman probe FAM-MGB (Applied Biosystems TaqMan Gene Expression Assays, Thermo Fischer Scientific) for each of the module 1 and 2 genes and 4 housekeeping genes (GAPDH, ACTG1, ACTB and RPL15). Preamplification products were harvested and high throughput real-time PCR was performed using the Fluidigm Biomark HD system with 48.48 gene expression Dynamic Arrays. For each cell line, a bulk control representative of 400 cells was processed the same way as the single cells. The raw data were first analyzed with the Fluidigm Real-Time PCR Analysis Software and exported to csv files for further analysis.

Gene expression value was normalized using the geometric mean of all 4 housekeeping genes Ct values of a given cell, cells were excluded if this geometric mean was >16.5. The Livak method ($2^{-\Delta\Delta C_t}$) was applied using the gene expression values of the SK-N-SH cell line population as reference for the relative expression. Hierarchical clustering was performed using one minus Pearson correlation with an average linkage method including heatmap using Morpheus platform (<https://software.broadinstitute.org/morpheus>).

Treatment of cell lines with chemotherapy

SH-EP and SH-SY5Y cell lines were plated in 96-well plates two days before the addition of cisplatin, etoposide or doxorubicin. Seeding densities for each cell lines were optimized to reach 80% of confluency in the untreated cells. Cells were treated with chemotherapeutic agents for 48 h. Cell viability was then measured using the *in vitro* Toxicology Assay Kit, Resazurin-based, following manufacturer's instructions (Sigma-Aldrich).

SK-N-SH cells were plated in 6-well plates and then treated with cisplatin (7.5 µM) or

doxorubicin (100 nM) for 7 days, medium and drugs were changed every 2 days. RNAs were extracted using NucleoSpin RNA kit (Macherey-Nagel).

Doxycycline-inducible shRNA systems

PHOX2B-specific short hairpin RNAs sh1783 (5'-CCGGTGGGAAGGCAGAAACCATTAATCTCGAGTTTAATGGTTTCTGCCTTCCATTTTGTG-3') and sh1437 (5'-CCGGAGTAATCGCGCTAAGAATAAACTCGAGTTTATTCTTAGCGCGATTACTTTTTTGTG-3') were selected from Sigma Mission shRNA library and cloned into the pLKO-Tet-On all-in-one system⁴⁶ (Addgene). Lentiviral particles were produced in HEK293T cells and CLB-GA cells were infected as previously described⁴. SH-SY5Y cells were incubated with viral particles for 48 hours without polybrene. Selection with puromycin (Invitrogen) at 400 ng/ml or 1 µg/ml, respectively, was performed 24 h after infection and maintained during all culture experiments, for CLB-GA and SH-SY5Y cells, respectively. PHOX2B knockdown efficacy was assessed by Western blot 24 h/48 h/96 h after the addition of doxycycline (100 ng/ml or 1 µg/ml). For colony formation assays, 6×10^4 transduced cells were plated at day 0 in 6-well dishes and stained with crystal violet at day 11⁴⁷.

Proliferation assays

Cells were counted in real-time with an xCELLigenceTM instrument (ACEA Biosciences) monitoring impedance across gold microelectrodes. 10^4 infected CLB-GA or SH-SY5Y cells were seeded per well of a 96-well plate in 200 µl medium containing doxycycline at 100 ng/ml or 1 µg/ml (quintuplicates per group) or no doxycycline. Medium was refreshed after 48 h. For cell counting, 2×10^4 infected CLB-GA or 10^5 infected SH-SY5Y were plated in 24-well plates in the presence or absence of doxycycline at 100 ng/ml or 1 µg/ml. The number of living cells was counted at day 4, 7, 10 and 14 (triplicates per group) for CLB-GA and at day 8 (n=5/6 technical replicates) for SH-SY5Y cells using a Vi-cell XR Cell Viability Analyzer (Beckman Coulter)

Statistical analysis

To calculate p-values for Pearson correlation (null hypothesis consisted in zero Pearson correlation), we implemented a one-sided permutation test. Number of permutations was 10^4

when calculating p-values for correlation between SE score and gene expression (Figure S11) and 10^6 in the test for correlation between gene expression of the noradrenergic and NCC-like modules in the set of tumors (Figure 2). This test does not need the data to follow the normal distribution and does not require equal variation between the groups that are statistically compared.

Xenotransplantation experiments and mice

10×10^6 CLB-GA cells transduced with the shRNA against PHOX2B (sh1783) were injected subcutaneously in the flanks of 6-week-old NSG mice (Charles Rivers Laboratories) in an equal mix of PBS and Matrigel (BD Biosciences). When tumors reached a volume of around 170 mm^3 , mice were randomly assigned to the control (5% sucrose in drinking water) or the treatment (doxycycline (2 mg/l) and 5% sucrose in drinking water) groups. Tumor growth was monitored with a caliper every day. Mice were killed once tumors reached a volume of around $3,000 \text{ mm}^3$ calculated as $V = a/2 \times b \times ((a+b)/2)$ with a being the largest diameter and b the smallest. Experiments were conducted in accordance with the recommendations of the European Community (86/609/EEC), the French Competent Authority, and UKCCCR (guidelines for the welfare and use of animals in cancer research). Approval for this study was received from Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (authorization number 5524-20 160531 1607151 v5).

PHOX2B immunohistochemistry

Mice tumors were fixed with acidified formal alcohol (AFA) for 24 h and paraffin-embedded. Labelling was performed on $4 \mu\text{m}$ sections with the BOND-III instrument (Leica Microsystems) using the Bond Polymer Refine Detection™ (Leica) kit. Briefly, sections were deparaffinized, antigen retrieval was performed with an EDTA-based solution (Leica) for 20 minutes at pH 9, and sections were stained with a rabbit polyclonal anti-PHOX2B antibody (Abcam, EPR14423, 1/1000).

siRNA and growth assays

HAND2 and GATA3 knockdown was performed with 20 nM siRNA (Hs_HAND2_3 #SI00131915, Hs_HAND2_6 #SI03046736, Hs_GATA3_7 #SI04202681 and Hs_GATA3_8

#SI04212446; Control siRNA #1027281; Qiagen) using RNAimax transfection reagent (Thermo Fisher Scientific). The number of living cells was counted using a Vi-cell XR Cell Viability Analyzer (Beckman Coulter) (n=5 or 6 technical replicates).

Data Availability

Raw data for cell line ChIP-seq and RNA-seq, and processed data for the cell lines, tumors and PDXs are available in Gene Expression Omnibus (GEO) under accession number GSE90683. Raw data for PDX ChIP-seq and RNA-seq will be available through EGA, as well as RNA-seq data for patient samples.

Reviewers can access to the GEO submission using this link: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=khcvkaailpulxqt&acc=GSE90683>.

Code availability

The code of the pipeline for the SE detection from cancer ChIP-seq data is available at <http://boevalab.com/LILY/>.

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Author information

Contributions

V.B. and I.J.-L. conceived the study, analyzed the data and wrote the manuscript. V.B. coordinated bioinformatics analysis and I.J.-L. coordinated the whole study. C.L. performed *in vitro* experiments and all ChIP experiments and participated in the study design. A.P. generated and analyzed the doxycycline-inducible anti-*PHOX2B* shRNA cell lines. S.D. performed the single cell analysis and study of chemotherapeutic agents. C.P.-E. performed the *in vivo* experiments and contributed *in vitro* experiments. V.R. performed all sequencing experiments. H.E. and S.T. provided hNCC cell lines and V.C. provided neuroblastoma cell lines. A.L. performed alignment of RNA-seq and ChIP-seq data. E.D.-D., B.G., D.S. and A.M.C. provided neuroblastoma PDXs. I.M. performed the reproducibility analysis. E.D. and B.D. generated the Biomark data. M.F.O. and T.G.P.G. generated lentiviral particles and provided help with lentiviral infections. S.B. coordinated and supervised sequencing experiments. G.S. participated in the study design and provided the in-house pairs of diagnosis/relapse samples with the help of E.L., G.P. and B.G. S.G.-L. participated in RNA-seq analysis. E.B. provided computational infrastructure and data storage. H.R. and T.D. provided expertise in sympathetic nervous development and transcription factors. I.J.-L and O.D. provided laboratory infrastructure. I.J.-L, V.B. and O.D. provided financial support. All authors read and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Extended data figures and tables

Supplementary Table 1: *MYCN*, *ALK* and *PHOX2B* status of neuroblastoma cell lines and PDXs. Amp., amplification; Non amp., non amplified; WT, wild-type. NA: not available.

* As determined by Sanger sequencing of exons 23 and 25.

□ *MYCN* locus: 5 copies; *MYC* locus: 7 copies; *ALK* locus: 7 copies (from Control-FREEC analysis)

In frame deletion in the second PolyAlanine track; functional change unknown.

Supplementary Table 2: Patient clinical data.

Supplementary Table 3: Characteristics of neuroblastoma and hNCC super-enhancers. Group I: all neuroblastoma cell lines with the exception of SH-EP, GIMEN, *GICAN*, SK-N-AS, SJNB12, SK-N-SH and CHP-212; Group II: SH-EP, GIMEN and *GICAN*.

Supplementary Table 4: Supervised analysis of SE scores according to *MYCN* status.

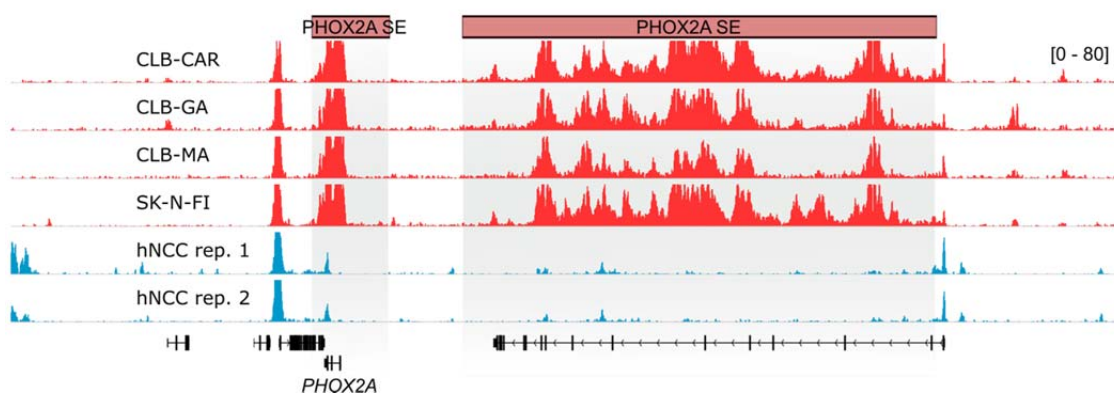
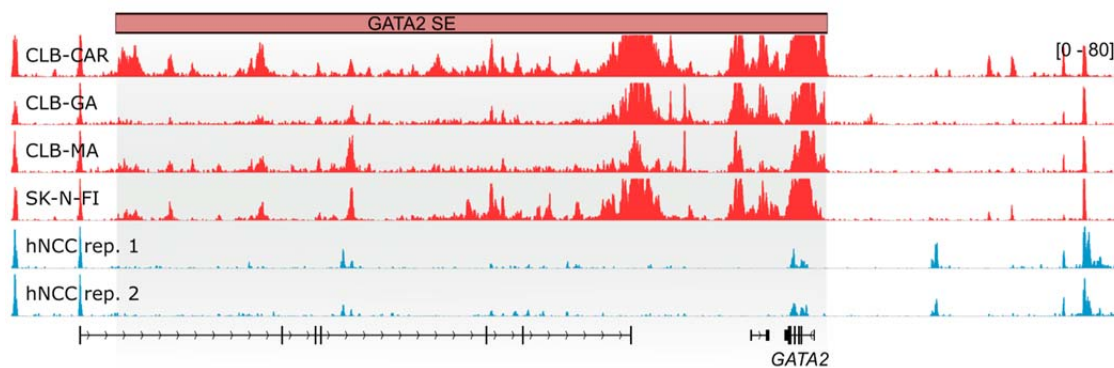
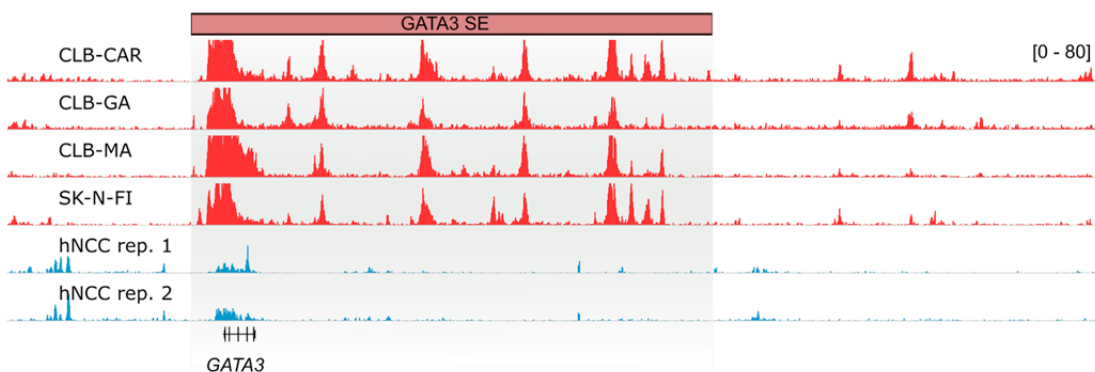
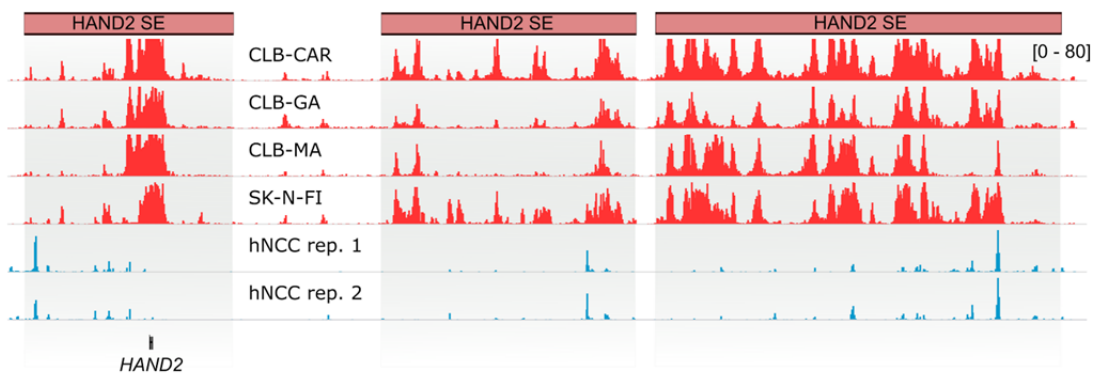
809 **Supplementary Table 5:** Supervised analysis of SE scores according to ALK status.

810

811 **Supplementary Table 6:** Raw Ct values measured for housekeeping genes (GAPDH, ACTG1,
812 ACTB, RPL15) and TFs of modules 1 and 2 for single cells of the SK-N-AS, SH-EP, SH-SY5Y
813 and SK-N-SH cell lines using the Fluidigm Biomark HD.

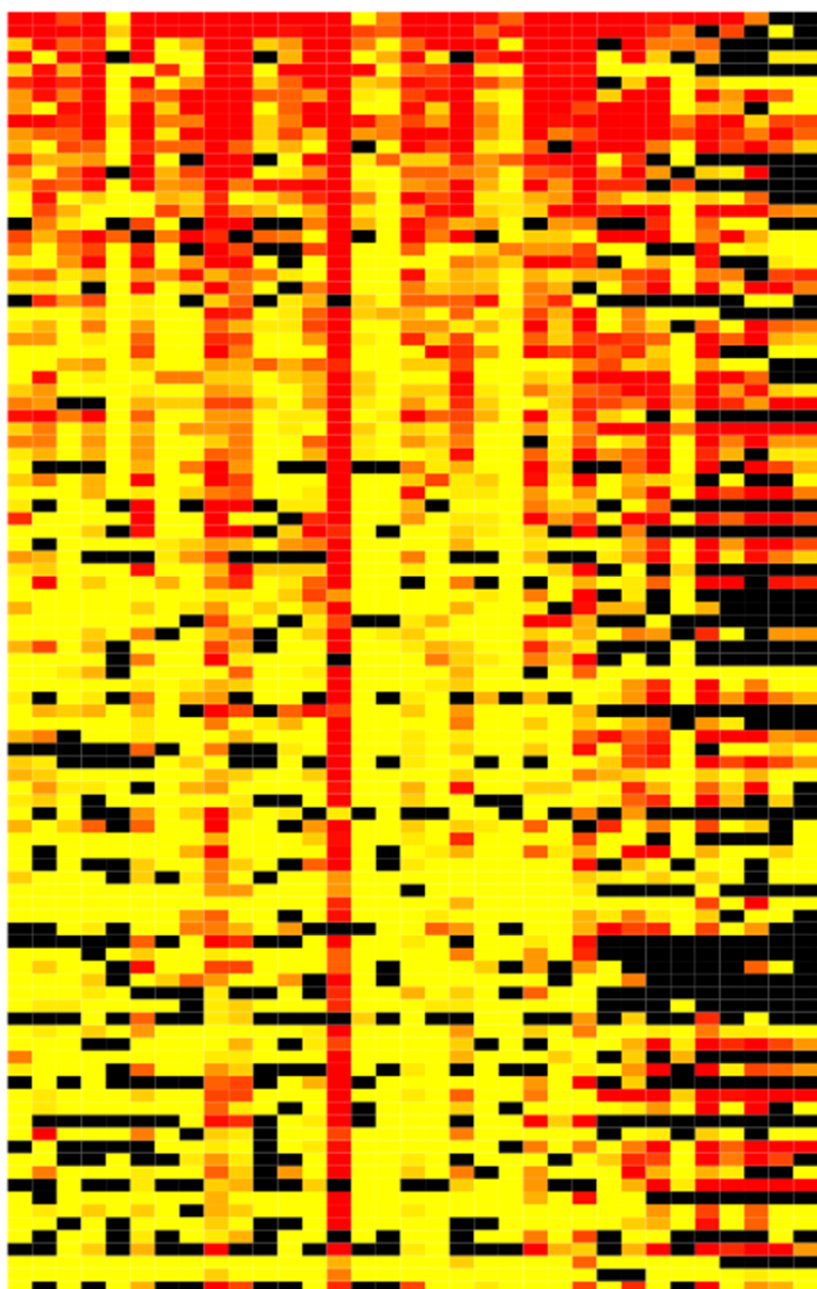
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Supplementary Figure 1: Tracks for ChIP-seq profiles of H3K27ac at *HAND2*, *GATA3*,
GATA2, and *PHOX2A* SEs.

a

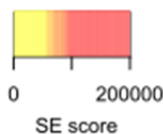


IGR-NB8
IGR-NB35
HSJD-NB011
MAP-GR-A99-NB-1
MAP-GR-B25-NB-1
MAP-IC-A23-NB-1
SUNB6
SUNB8
CLB-CAR
CLB-PE
IMR32
LAN-1
N206
SK-N-BE(2)-C
SK-N-DZ
TR14
CLB-BER-Lud
CLB-MA
NB-69
CLB-GA
SUNB1
SK-N-FI
SH-SY5Y
NB-EBc1
CHP-212
SK-N-AS
SK-N-SH
SUNB12
GIMEN
SH-EP
GICAN
hNCC rep1
hNCC rep2

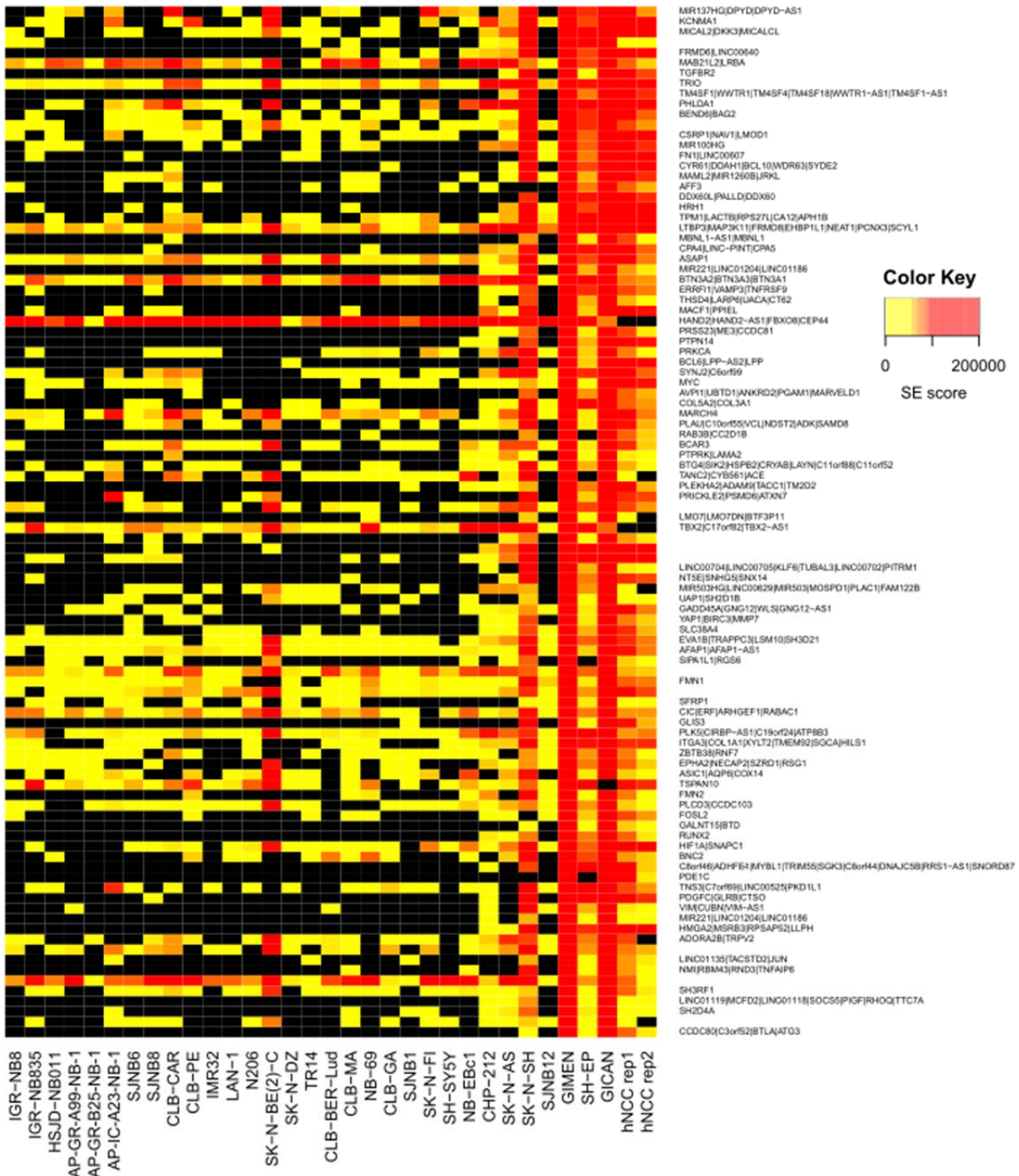
Associated genes

HAND2|HAND2-AS1|FBXO8|CEP44
GSE1|MR5093|GINS2
SLIT3|FBLL1|MR218-2
DLK1|BEGAIN|RTL1|MEG8|MEG9|MEG3|INO0052|SLC25A47
EML5|TTC8|CENK10
MAML3
TCF4
MA521L2|LRSB
GATA2|GATA2-AS1|RLVBL1|DNAJB8|DNAJB8-AS1|EEFSEC
UNC5C|BMPR1B
PHOX2B|LMCH1|LINC00582
GATA3|GATA3-AS1|TAP3
BTN3A2|BTN3A3|BTN3A1
KIF26B|SMYD3
PRR34-AS1|MRLET7BHC|PRR34
PER3
MARCH4
ALK
PHOX2A|CLPB|NUMA1|LRTOMT|PDE2A
KLF7
TENM4
HAND2|HAND2-AS1|HMOB2|SAP30
TBX2|C17orf82|TBX2-AS1
IGFBP4|TNF4
KIF26A|TMEM179
LTBP3|MAP3K11|FRMD8|EBHP1L1|NEAT1|PCNO3|SCYL1
CIC|ERF|ARHGAP1|RABAC1
JRAJ1C-AS1
STRAB1|CCDC33|SLR2|SLR
ASAP1
FAM155A
MCF2|C2orf61|TTC7A|EPCAM
ZNF608
TP53|11|TP53N18
TP53N10
CHRNA3|CHRNA4|CHRNA5|CRABP1|CIB2|REB2|ADAMT57|SH2D7|WDR61
ARHGAP4|AVPR2
EXOC4
VEGFA
RBM22|GLT1D1|ADGRD1|TMEM132C
BEND5|ELAVL4|AGBL4
RBM20|GRAM
ICA1|GLOC1|NDPH1|PRA3|MO5|UMAD1
MA521L1|NBEA|RFC3
PLK5|CRBP-AS1|C19orf24|ATP6B3
LINC00578
NOL4L
NFIB
FAM102A|FAM125B
ZFP6L2
BCOR
SEC24D|SYNPO2
INSM2
HAND1
SSH2
COL3A1|GULP1
ASTN2
BCOR
CADM1
SYT1
PKR1
MYT1L|LINC01250
SESN3
IER5L
ZNF423
DENND3
HAND2|HAND2-AS1
TRIO
HIF1A|SNAPC1
ADORA2B|TRPV2
NCOA7
TPM1|ACTB|IRPS27|UCA12|APH1B
NHSL2|CITED1|RGAD4|ERCC6L
LGALS3BP
ATF7|TNF2|IGFBP6|NFFR|RARG
CPEB4
MR13THG|DPYD|DPYD-AS1
CBX1|SP2
DCP2|MCC
POLIN5|BMPR1B

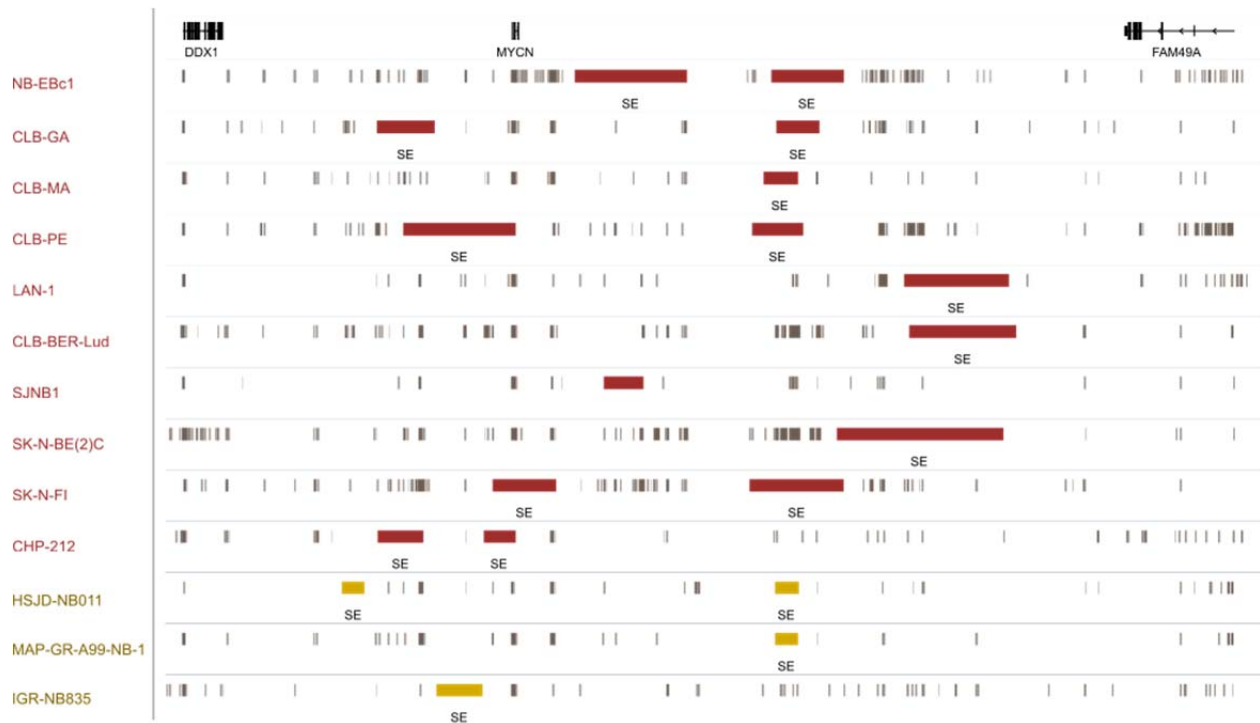
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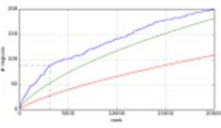
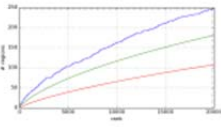
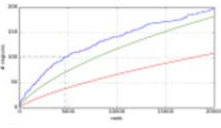
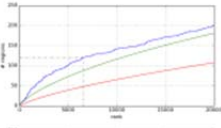
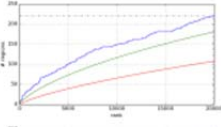
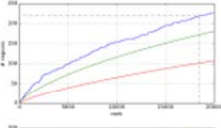
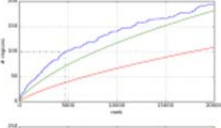
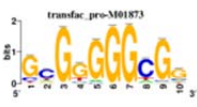
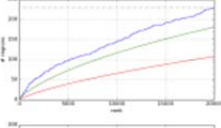
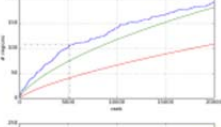
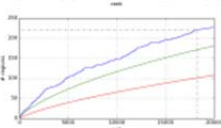
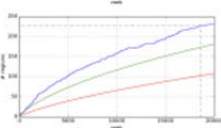
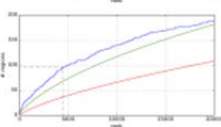
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Supplementary Figure 2: Heatmap of the SE scores for the 6 PDX, 25 NB cell lines and hNCC cells: top SEs of group I (a); top SEs of group II (b); SE is not detected (black).



Supplementary Figure 3: Locations of SE regions predicted for the *MYCN* locus; SEs in NB cell lines (red); SEs in PDX (yellow); typical enhancers and active promoters (grey).

#	Feature	NES	Logo	Recovery Curve
1.	TAAYYNAATTA-PROP1-full	4.21		
2.	PGAM2	3.98		
3.	TAATYYAATTA-PHOX2B-DBD	3.98		
4.	GATA1, GATA2, GATA3, GATA4, GATA5, GATA6	3.94		
5.	ZCCHC14	3.85		
6.	WT1	3.84		
7.	TAATYYAATTA-PHOX2A-DBD	3.76		
8.	EGR1	3.75		
9.	TAATYTAATTA-PRRX1-full	3.74		
10.	SP1:SP3	3.72		
11.	SP4	3.72		
12.	ALX4	3.71		

Supplementary Figure 4: i-cis Target summary (database v3.0) on H3K27ac peak valleys of the top 100 strongest SEs identified in group I. NES, Normalized enrichment score. NES threshold 3.7. NES and recovery curves are explained at the i-cisTarget website:

849 <https://gbiomed.kuleuven.be/apps/lcb/i-cisTarget/> .

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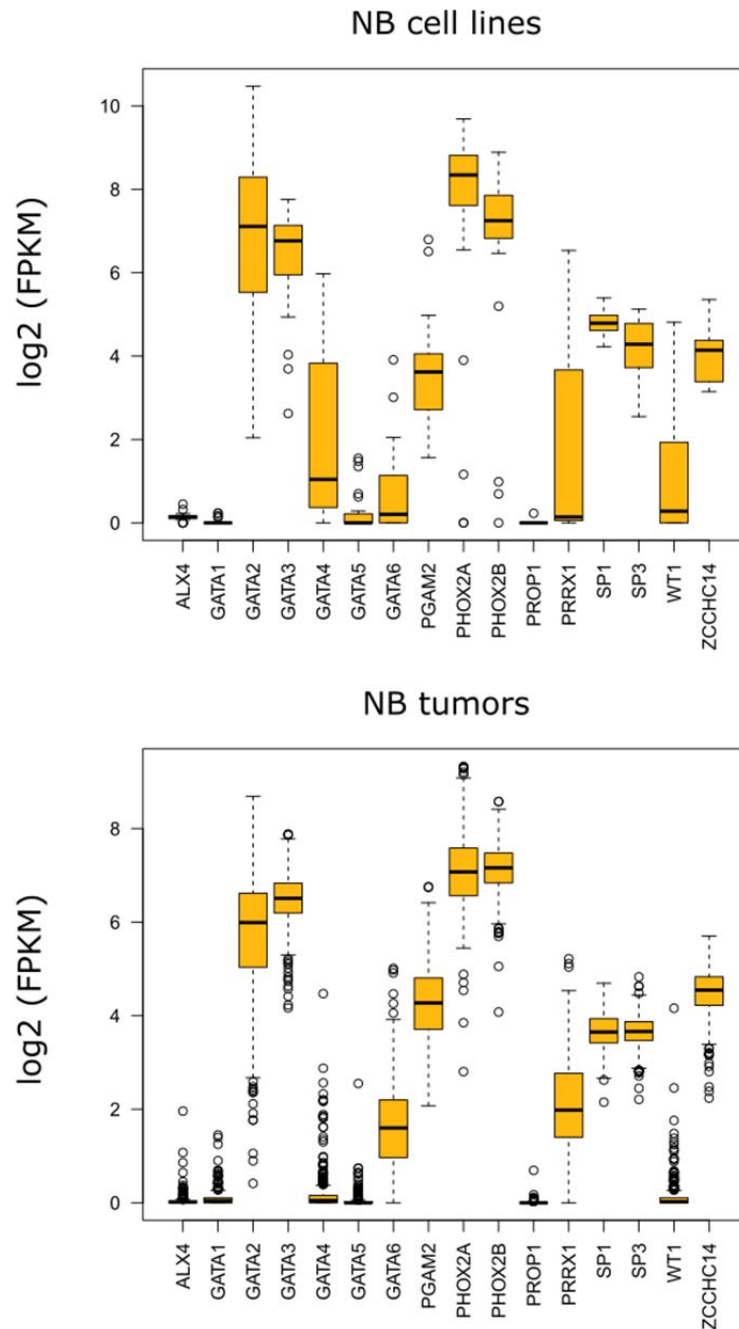
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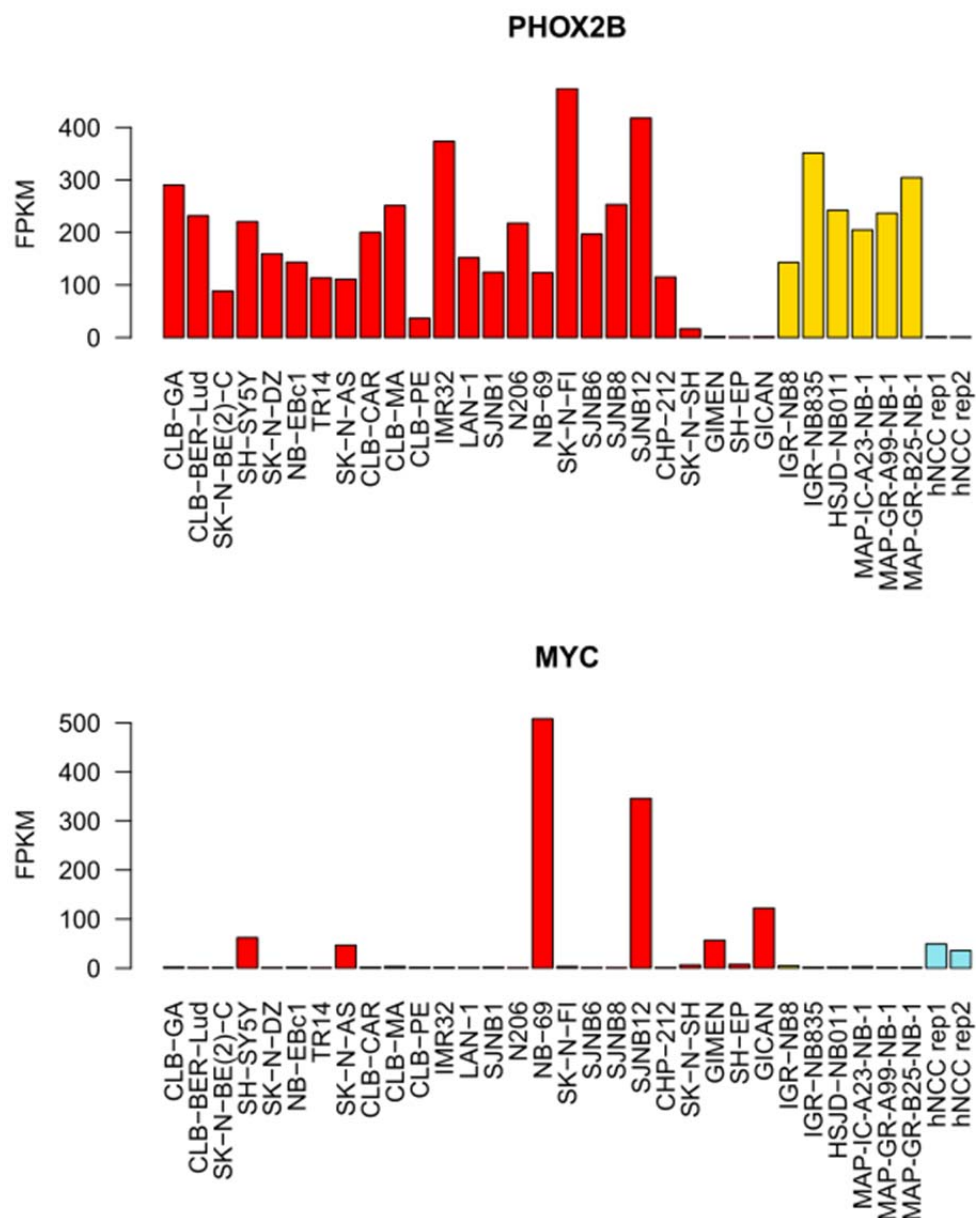
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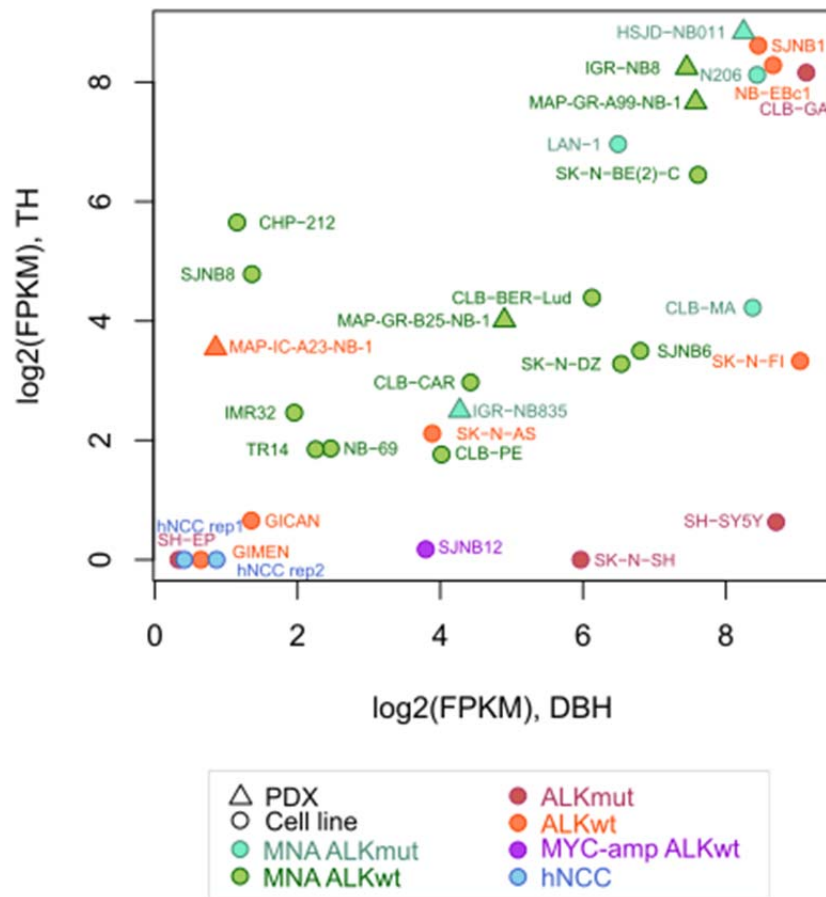
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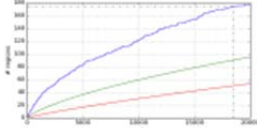
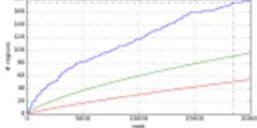
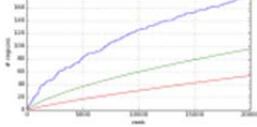
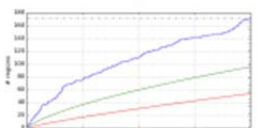
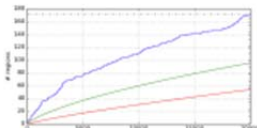
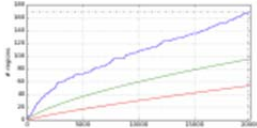
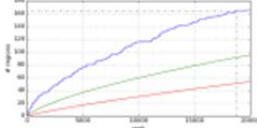
Supplementary Figure 5: Log2 FPKM expression values for genes coding for TFs whose binding motifs are enriched in valleys of H3K27ac peaks of the top 100 strongest SEs identified in group I, in neuroblastoma (NB) cell lines (our data) and NB primary tumors (498 tumors, dataset GSE49711). The box represents the middle 50% of values; the black line inside the box indicates the median.



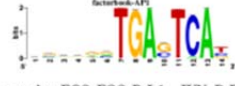
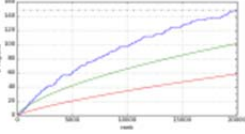
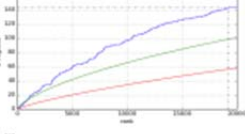
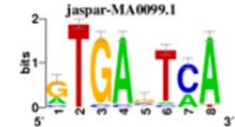
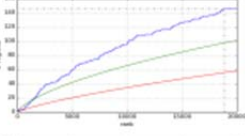
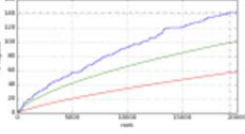
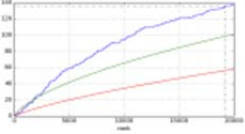
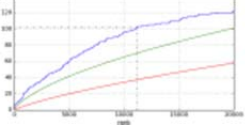
Supplementary Figure 6: *PHOX2B* and *MYC* expression levels measured by RNA-seq in neuroblastoma cell lines (red), PDX (yellow) and hNCC lines (blue).



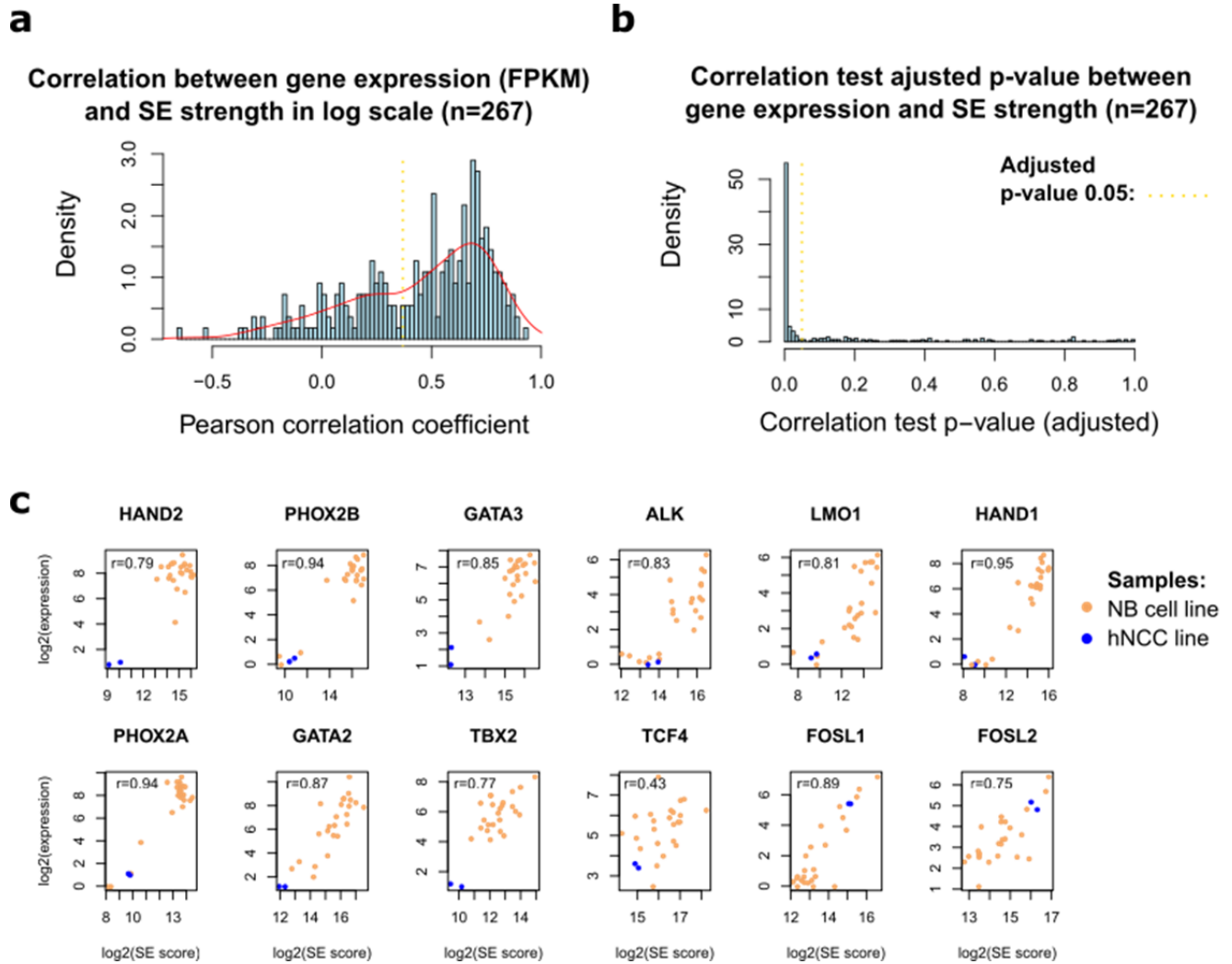
Supplementary Figure 7: Log2 FPKM expression values of *DBH* and *TH* in hNCC and neuroblastoma cell lines and PDX measured by RNA-seq.

#	Feature	NES	Logo	Recovery Curve
1.	JUN	7.12		
2.	AP-1: BATF, SPI1, FOS, JUNB, JUN, JUND, FOSL1, FOSL2	6.95		
3.	ARNT	6.88		
4.	FOS	6.40		
5.	JUNB	6.38		
6.	FOSL1	6.29		
7.	FOSB, FOS, FOSL1, FOSL2	6.23		

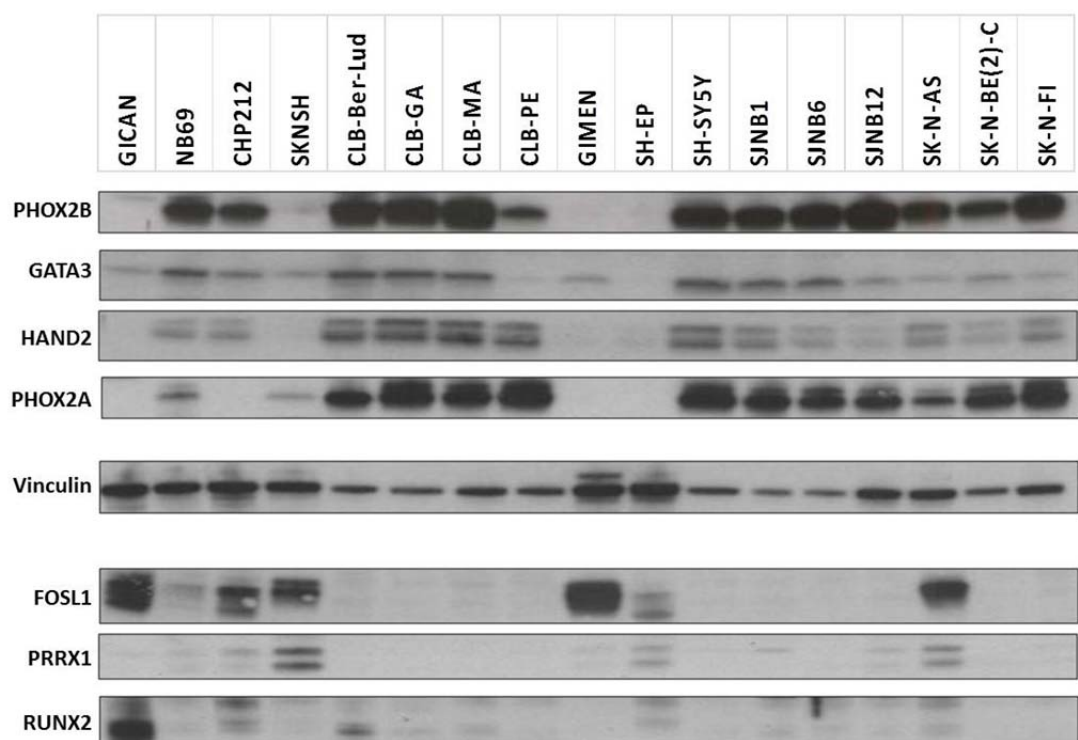
Supplementary Figure 8: i-cis Target summary (database v3.0) on H3K27ac peak valleys of the top 100 strongest SEs identified in group II. NES, Normalized enrichment score. NES threshold 6.2. NES and recovery curves are explained at the i-cisTarget website: <https://gbiomed.kuleuven.be/apps/lcb/i-cisTarget/>.

#	Feature	NES	Logo	Recovery Curve
1.	BATF, FOS, JUNB, JUN, JUND, FOSL1, FOSL2	4.11		
2.	FOS, JUNB, FOSL1, JUND, FOSB	4.00		
3.	FOS	3.99		
4.	FOSB, FOS, FOSL1, FOSL2	3.80		
5.	BACH2	3.88		
6.	EZR	3.71		

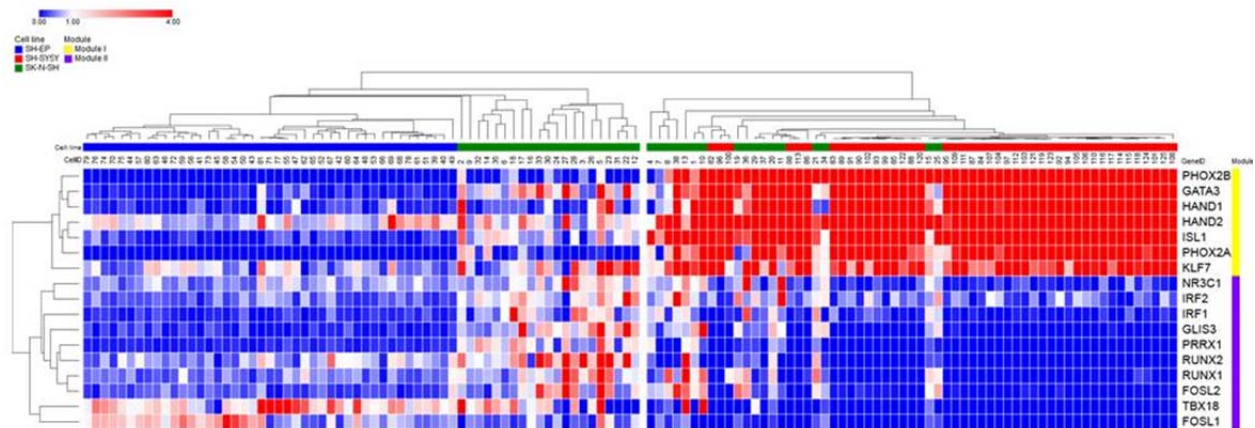
Supplementary Figure 9: i-cis Target summary (database v3.0) on H3K27ac peak valleys of the top 100 strongest SEs identified in hNCC. NES, Normalized enrichment score. NES threshold 3.7. AUC threshold 0.015. NES and recovery curves are explained at the i-cisTarget website: <https://gbiomed.kuleuven.be/apps/lcb/i-cisTarget/>.



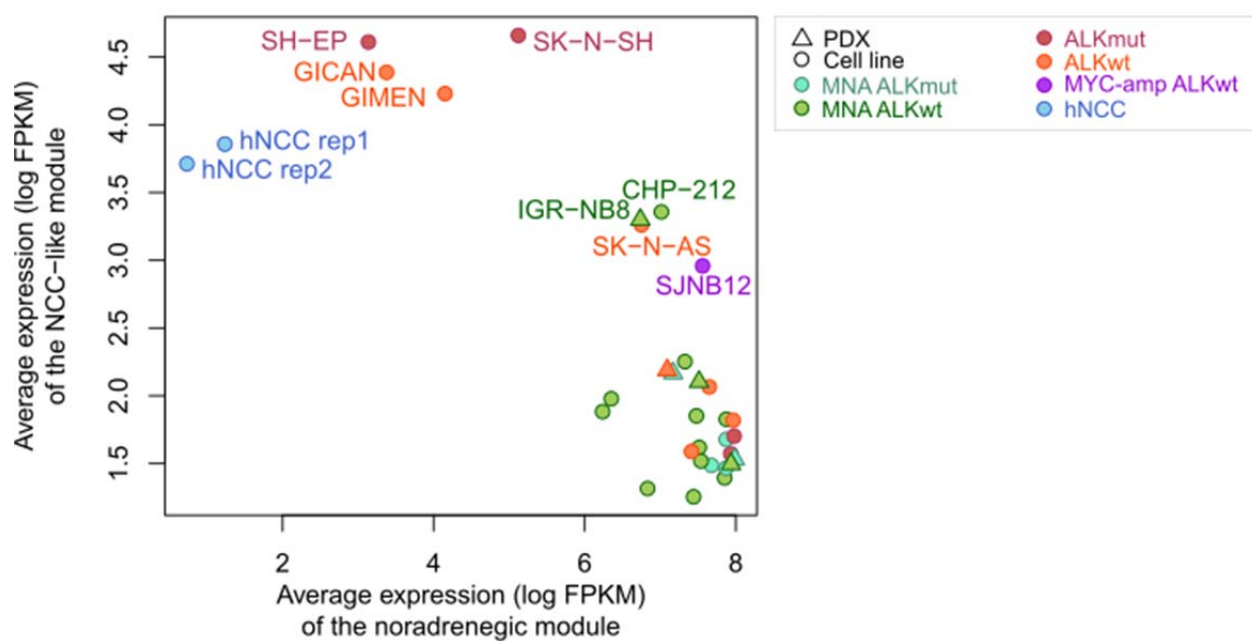
Supplementary Figure 11: Gene expression linearly correlates with SE score (in log scale). **a**, Pearson correlation coefficients were calculated for 267 SE regions corresponding to only one gene and detected in at least 2 neuroblastoma samples. Pearson correlation one-sided permutation tests were performed on the set of 25 neuroblastoma cell lines and 2 hNCC samples; **p-values adjusted with the FDR method**. **b**, Distribution of corresponding adjusted p-values for 267 SE regions. **c**, Examples of correlation between SE score and expression of particular genes. Orange: neuroblastoma cell lines; blue: hNCC samples.



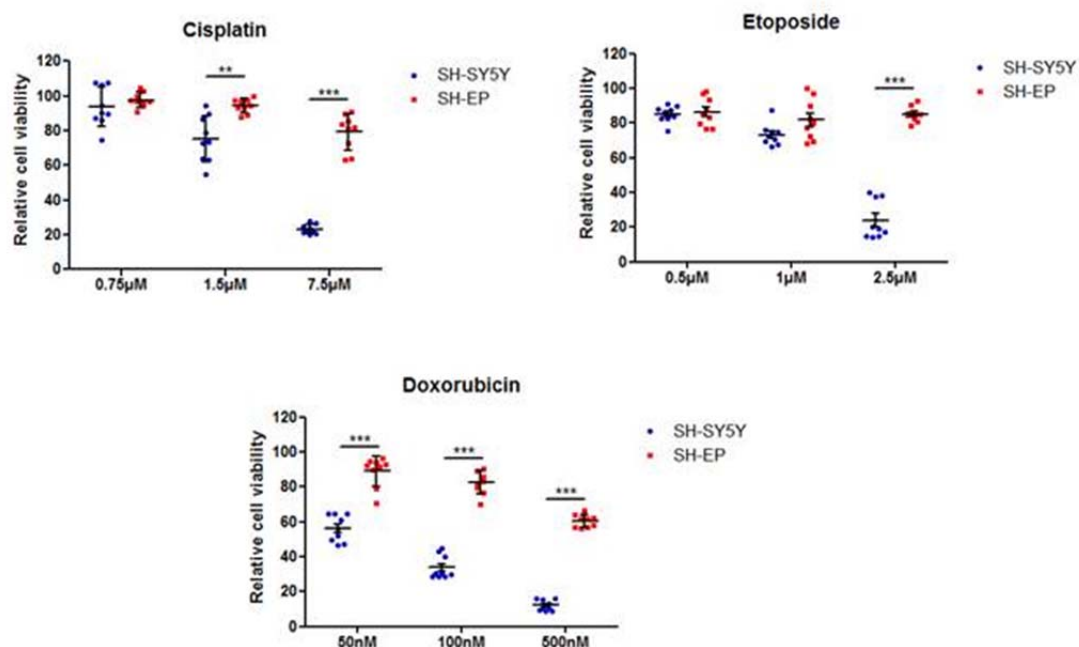
Supplementary Figure 12: Western blot analysis of PHOX2B, GATA3 (CST #5852, D13C9), HAND2 (sc-9409), PHOX2A (sc-8978), FOSL1 (sc-28310), PRRX1 (Sigma HPA051084), RUNX2 (sc-101145) and vinculin in a panel of neuroblastoma cell lines. All antibodies were used at 1:500 except the GATA3 antibody used at 1:1000.



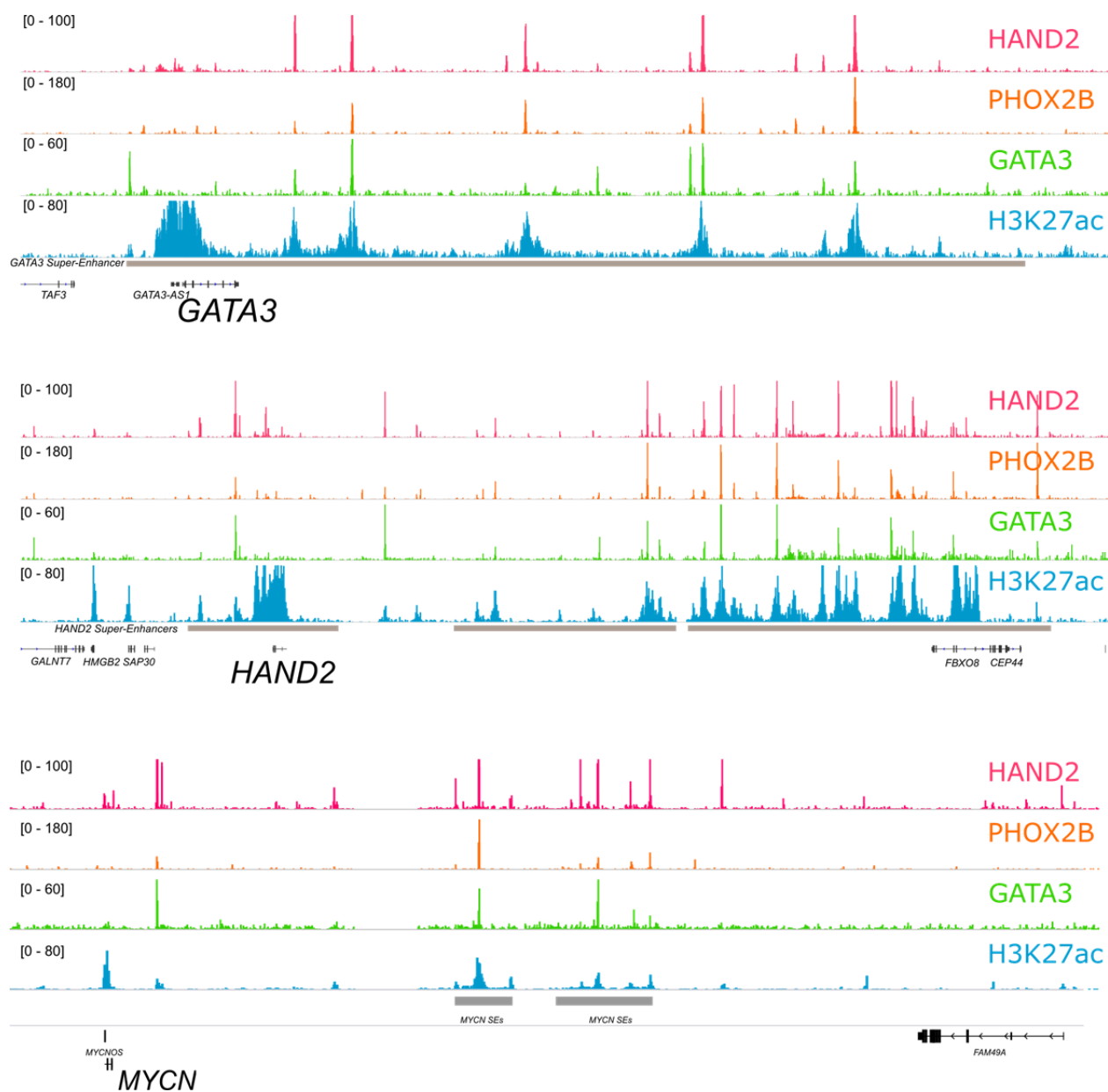
Supplementary Figure 13: Clustering of SK-N-SH, SH-EP and SH-SY-5Y single cells analyzed by RT-q-PCR for the expression of TFs of modules 1 and 2. The first group of cells includes all SH-EP cells as well as some cells of the SK-N-SH cell line; a second group includes all SH-SY5Y cells as well as cells of the SK-N-SH cell line. RT-q-PCR data were normalized to the SK-N-SH cell line population for the three cell lines using the geometric mean of the four housekeeping genes.



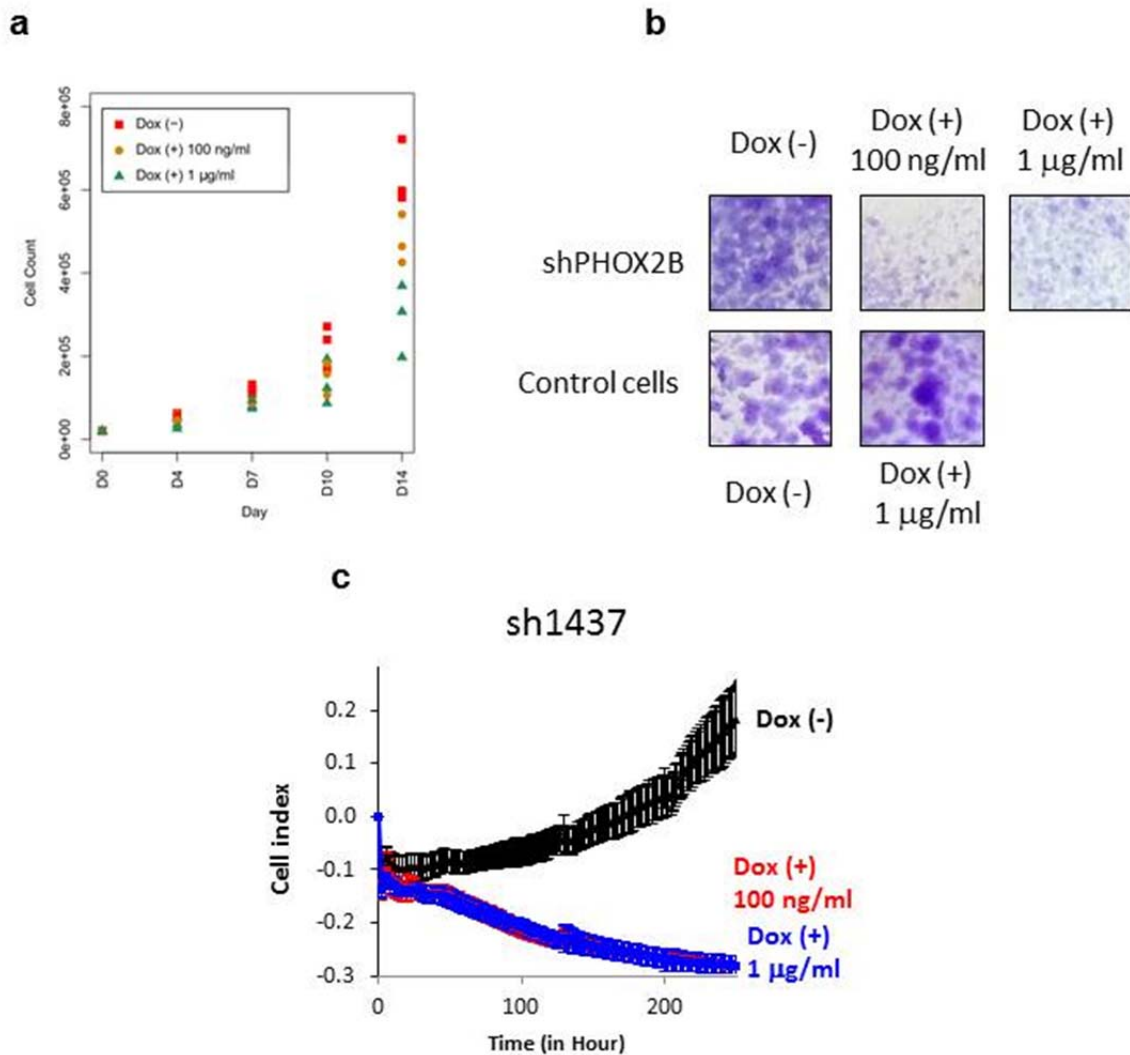
Supplementary Figure 14: Expression of modules 1 and 2 in neuroblastoma cell lines and PDXs. Average is calculated for log₂ FPKM values.



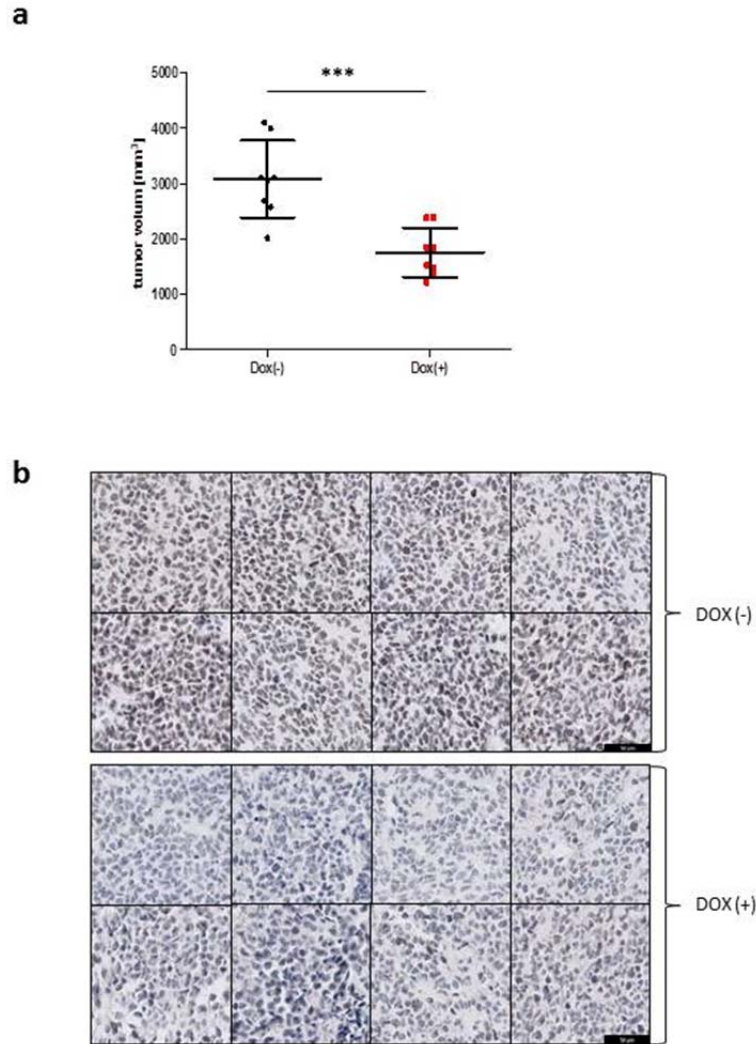
Supplementary Figure 15: NCC-like SH-EP cells are more resistant to chemotherapy than noradrenergic SH-SY5Y cells (n=9 technical replicates per condition; P values were determined via two-tailed unpaired Welch's t-test (***: p<0.001)).



Supplementary Figure 16: Tracks for ChIP-seq profiles for PHOX2B, HAND2, GATA3 and H3K27ac binding at the *GATA3* (top), *HAND2* (middle) and *MYCN* (bottom) SEs in the CLB-GA cell line.

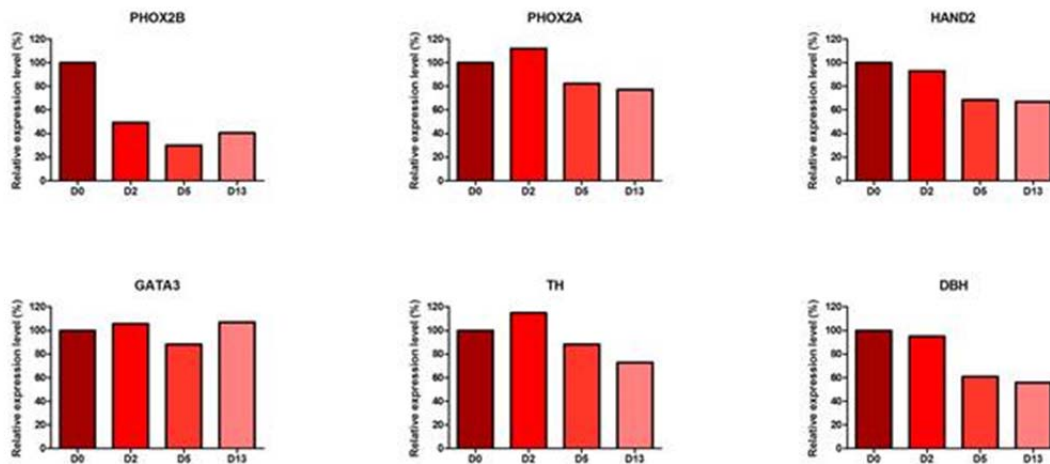


Supplementary Figure 17: a, Validation of xCELLigence™ results by cell counting for the CLB-GA cell line infected with the sh1783 vector targeting PHOX2B. 2×10^4 cells were plated in 24-well plates at day 0 in the absence or presence of doxycycline at 100 ng/ml or 1 µg/ml. The number of living cells was counted at day 4, 7, 10 and 14. **b**, Decreased foci formation of CLB-GA cells upon doxycycline-induced PHOX2B knockdown. Doxycycline at 1 µg/ml did not affect growth of CLB-GA non-infected control cells. **c**, xCELLigence™ proliferation kinetics for the SH-SY5Y cell line infected with the sh1437 vector targeting PHOX2B, respectively. Data shown are the mean $\pm$ s.d. of results obtained in the different conditions (n=5 technical replicates)

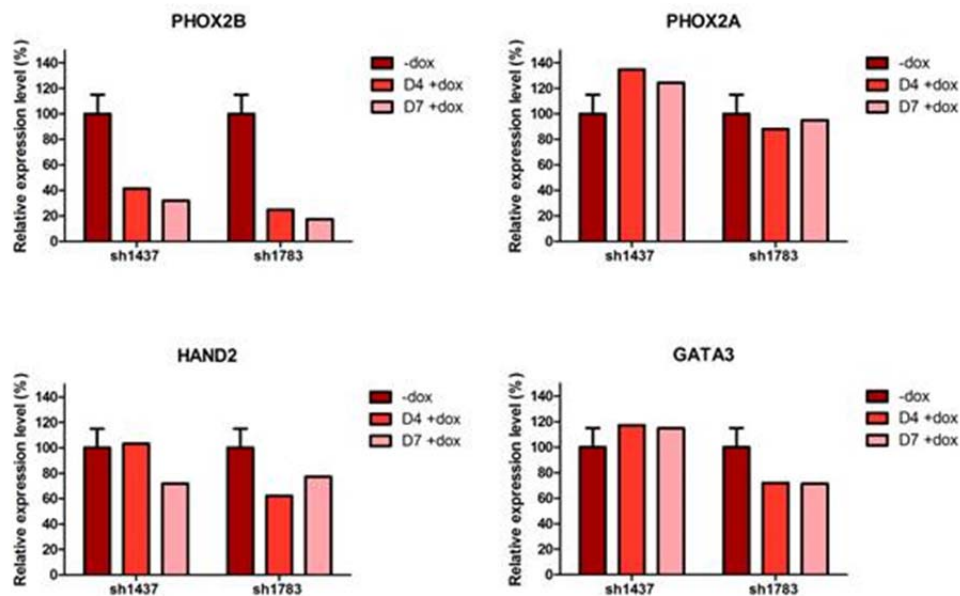


Supplementary Figure 18: a, Tumor volume of mouse xenografts of CLB-GA cells transduced with sh1783 targeting PHOX2B after 11 days of treatment with sucrose alone (Dox(-)) or doxycycline and sucrose (Dox(+)). **b**, PHOX2B expression analyzed by immunohistochemistry (EPR14423-Abcam) in mouse xenografts of CLB-GA cells transduced with sh1783 targeting PHOX2B treated or not with doxycycline (DOX). Each panel corresponds to a different tumor (n=8 tumors in each group).

a



b



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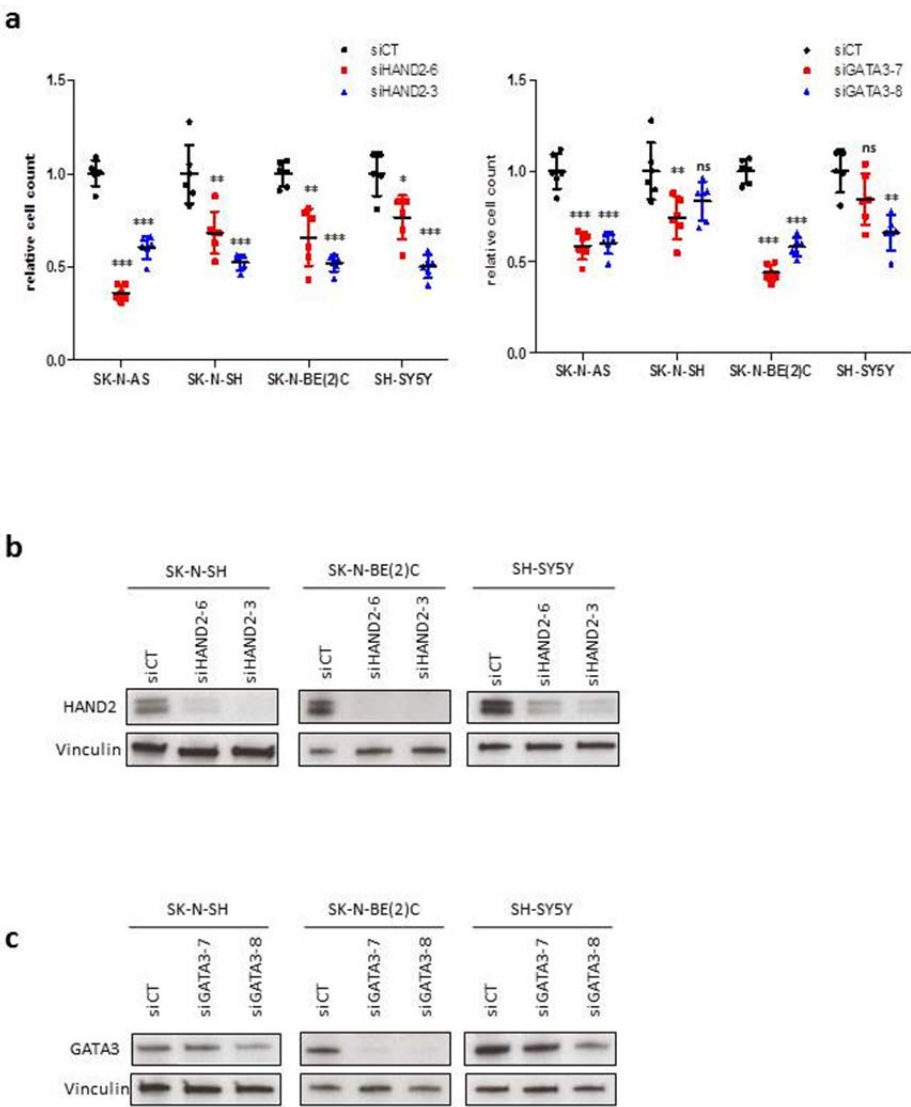
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Supplementary Figure 19: Impact of PHOX2B decrease on the expression profiles of CLB-GA and SH-SY5Y cells. a, RNA-seq was performed on CLB-GA cells transduced with sh1783 targeting PHOX2B after 2, 5 and 13 days of doxycycline treatment and on untreated cells (D0). Expression levels (FPKM values) for each day are compared to the untreated cells (100%). PHOX2B knockdown resulted in a modest decrease of PHOX2A, HAND2, TH and DBH. No expression of the genes of the NCC-like module was detected in any of the conditions. **b**, Expression of genes of modules 1 and 2 was evaluated by RT-q-PCR on SH-SY5Y cells

transduced with sh1437 or sh1783 targeting PHOX2B after 4 and 7 days of doxycycline treatment and compared to untreated cells (100%). GAPDH was used as a reference gene. No strong changes were observed following PHOX2B decrease. FOSL1, RUNX2 and PRRX1 were not detected neither in the untreated condition nor after PHOX2B knockdown. TaqMan(r) Gene Expression Assays (Thermo Fischer Scientific) used in this assays: GAPDH (4326317E), PHOX2B (Hs00243679_m1), HAND2 (Hs00232769_m1), GATA3 (Hs00231122_m1), PHOX2A (Hs00605931_mH), FOSL1 (Hs04187685_m1), RUNX2 (Hs01047973_m1), PRRX1 (Hs00246567_m1).



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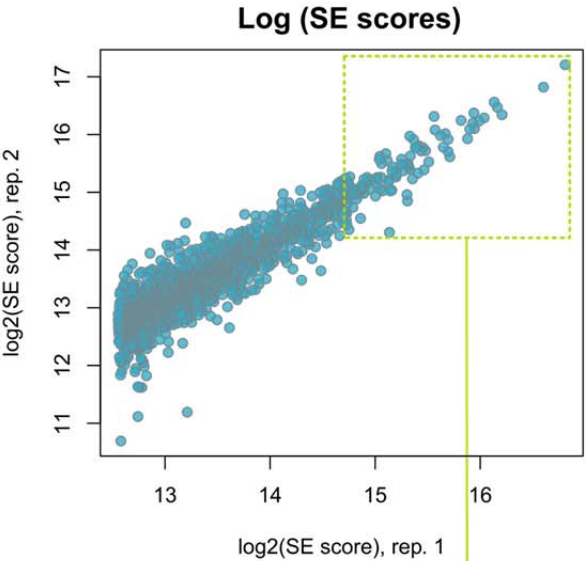
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Supplementary Figure 20: HAND2 and GATA3 knockdown impairs proliferation of SK-N-AS, SK-N-SH, SK-N-BE(2)C and SH-SY5Y cell lines. **a**, Cell counting of cells treated with siRNA targeting HAND2, GATA3 or with a control siRNA (at 3 days post-treatment for SK-N-BE(2)C, 5 days for SK-N-SH and 6 days for SK-N-AS and SH-SY5Y). (n=5 or 6 technical replicates, mean +/- s.d.). P values were determined via two-tailed unpaired Welch's t-test (***: p<0.001). **b and c**, Western blots for HAND2, GATA3, or vinculin.

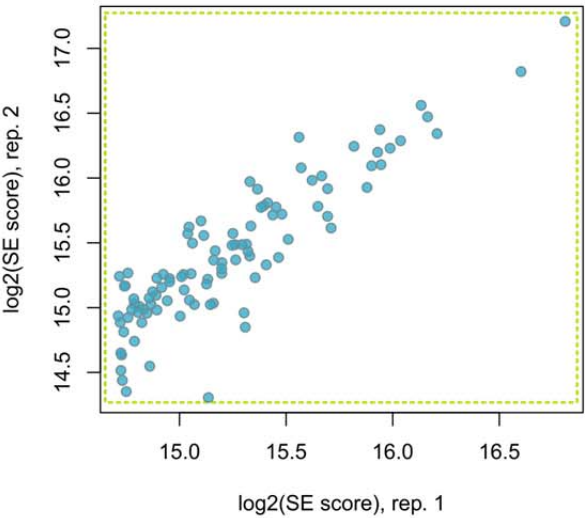
a

	Proportion detected as SEs in rep. 2	Proportion detected as Enhancer in rep. 2
Top 100 SEs, rep. 1	100%	0%
Top 500 SEs, rep. 1	93%	6.6%
All SEs, rep. 1	75.6%	23.3%

b



c



d

	Pearson correlation coef. of SE log scores in rep. 1 and rep. 2	Spearman correlation coef. of SE log scores in rep. 1 and rep. 2
Top 100 SEs, rep. 1	0.90	0.86
Top 500 SEs, rep. 1	0.92	0.88
All SEs, rep. 1	0.93	0.90

Supplementary Figure 21: Reproducibility analysis of SE calling and score assessment. ChIP-seq experiment for the H3K27ac mark was performed in duplicate for the CLB-GA cell line. **a**, Proportion of active SEs from replicate 1 detected as SEs in replicate 2. **b**, **c**, Correlation

1058 between normalized values of SE scores in CLB-GA replicate 1 and 2, shown for all the SEs of
1059 replicate 1 **(b)** and the top 100 SEs of replicate 1 **(c)**. **d**, Correlation coefficient for SEs scores
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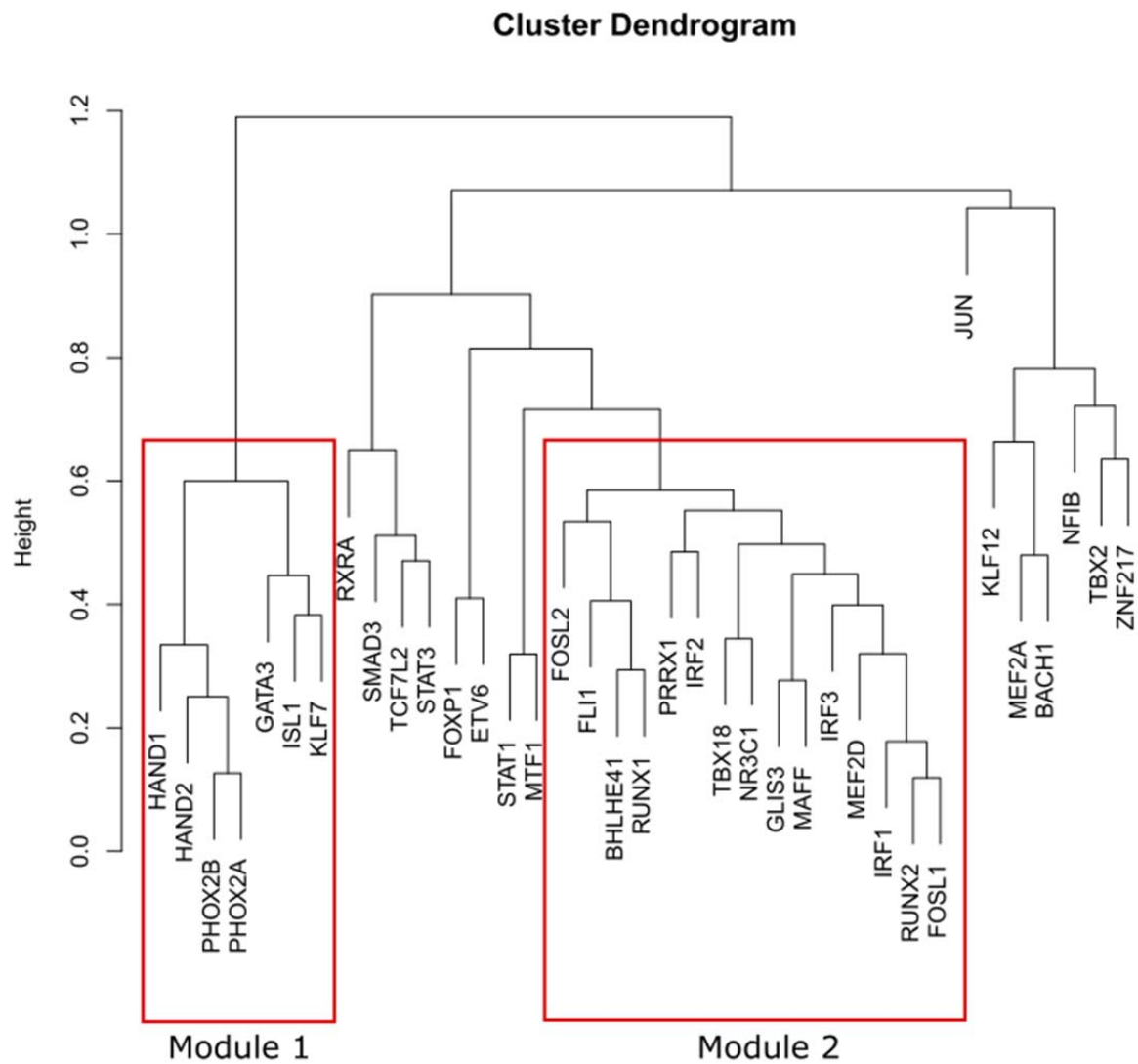
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Supplementary Figure 22: Clustering of 37 genes from CRCs of neuroblastoma group I and II based on their expression correlation in NB cell lines and PDX (R package ‘hclust’ with the McQuitty method). Two modules were defined.

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