Selective gene dependencies in MYCN-amplified neuroblastoma include the core transcriptional regulatory circuitry

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Childhood high-risk neuroblastomas with MYCN gene amplification are difficult to treat effectively. This has focused attention on tumor-specific gene dependencies that underlie tumorigenesis and thus provide valuable targets for the development of novel therapeutics. Using unbiased genome-scale CRISPR–Cas9 approaches to detect genes involved in tumor cell growth and survival, we identified 147 candidate gene dependencies selective for MYCN-amplified neuroblastoma cell lines, compared to over 300 other human cancer cell lines. We then used genome-wide chromatin-immunoprecipitation coupled to high-throughput sequencing analysis to demonstrate that a small number of essential transcription factors—MYCN, HAND2, ISL1, PHOX2B, GATA3, and TBX2—are members of the transcriptional core regulatory circuitry (CRC) that maintains cell state in MYCN-amplified neuroblastoma. To disable the CRC, we tested a combination of BRD4 and CDK7 inhibitors, which act synergistically, in vitro and in vivo, with rapid downregulation of CRC transcription factor gene expression. This study defines a set of critical dependency genes in MYCN-amplified neuroblastoma that are essential for cell state and survival in this tumor.

We conducted systematic genome-scale CRISPR–Cas9 screens in 341 human cancer cell lines, including 9 neuroblastoma lines with MYCN-amplification. Our cell line panel also included an additional two neuroblastoma cell lines, SKNFI and SKNAS, which do not harbor MYCN-amplification but do express high levels of MYCN or c-MYC, respectively. We identified 147 candidate genes that were selectively essential to the growth and survival of MYCN-amplified neuroblastoma cell lines (Supplementary Table 1). The guide RNAs targeting these genes showed greater depletion in the 9 MYCN-amplified neuroblastoma cell lines than in the other human cancer cell lines representing 26 tumor types. Classification of the 147 dependencies by gene ontology molecular function identified a diverse set of genes encoding proteins in numerous functional categories (Fig. 1a)⁹,¹⁰, with overrepresentation of ontologies for nucleic acid binding (12%, $P=8.48\times10^{-3}$) and transcription factor activity or binding (20%, $P=6.78\times10^{-3}$). Many dependency genes have not been previously associated with the sympathoadrenal cell lineage. Others have been reported to be involved in neural crest cell specification and neuroblastoma pathogenesis¹¹-²⁰. This set of transcriptional regulatory genes is highly connected by putative protein–protein interactions in the STRING database²¹ (Fig. 1b). Representative dependency scores in MYCN-amplified neuroblastoma compared to other tumor cells are shown in Fig. 1c,d for the genes HAND2 and ISL1, and for a subset of other genes with nucleic acid binding and/or transcription factor activities in Supplementary Fig. 1. Our results show that many transcription factor genes with selective dependency in MYCN-amplified neuroblastoma cells also displayed dependency in the two MYCN non-amplified cell lines studied so far, although additional MYCN non-amplified lines will need to be studied before firm conclusions can be drawn. We next performed transient small-interfering RNA (siRNA) knockdown targeting messenger RNAs that encode five transcription factor dependencies (HAND2, ISL1, PHOX2B, GATA3, and TBX2) to independently verify that these genes represent dependencies in MYCN-amplified neuroblastoma cell lines. Depletion of expression of these genes resulted in suppression of colony formation and induction of apoptosis (Fig. 1e,f and Supplementary Figs. 2 and 3). Thus, MYCN-amplified neuroblastoma cells rely on a discrete set of genes that are either unique to, or only minimally shared with, other tumor types.

The gene dependencies we identified suggest that diverse sets of complex pathways are activated to support the viability of MYCN-amplified neuroblastoma. Given the overrepresentation of transcription factor and nucleic acid binding activities among the 147 gene dependencies (Fig. 1a), we hypothesized that these genes were specifically required for transcriptional regulation in neuroblastoma cells. Previous studies have demonstrated that lineage-specific differences in cell state during development are determined by tissue-specific CRC that may be subverted to drive malignant transformation²²,²³. CRCs are formed by transcription factors assembled into positive, feed-forward autoregulatory loops that establish and maintain cell lineage and identity through their extended regulatory networks²⁴,²⁵. CRC transcription factor genes are typically marked by extensive stretches of acetylation at histone H3-Lysine 27 (H3K27ac), a genomic feature characteristic of high levels of gene
expression, termed super-enhancers\textsuperscript{26,27} (Fig. 2a). To investigate the relevance of a CRC framework to the dependency genes identified earlier, we performed chromatin-immunoprecipitation coupled to high-throughput sequencing (ChIP-seq) for H3K27ac in 5 MYCN-amplified neuroblastoma cell lines, identifying a shared set of 77 genes associated with super-enhancers across all 5 cell lines (Fig. 2b and Supplementary Table 2). As observed in the selective dependencies, the super-enhancer-associated genes in MYCN-amplified neuroblastoma cells were overrepresented among ontologies\textsuperscript{9,10} including nucleic acid binding (21%, $P = 3.93 \times 10^{-3}$) and transcription factor activity or binding (22%, $P = 4.29 \times 10^{-3}$). Examples of the H3K27ac signals of highly ranked transcription factor genes are shown in Fig. 2c and Supplementary Fig. 4.

When these 77 super-enhancer-associated genes were compared with the 147 selective dependencies in MYCN-amplified neuroblastoma identified by the CRISPR–Cas9 screen, 11 of the super-enhancer-associated genes were selective dependencies and 10 of 11 (91%) of these genes encoded transcription factors (Fig. 2d). This represents 11 of 69 super-enhancer-associated genes targeted by specific single guide RNAs (15.9%, $P = 1.11 \times 10^{-6}$ for enrichment), since 8 of the super-enhancer-associated genes were non-coding and were not targeted in the screen. To verify that these genes are
expressed in primary human neuroblastomas, we examined the expression level of each super-enhancer-associated, CRISPR–Cas9 dependency nucleic acid binding or transcription factor gene in four primary neuroblastoma tumor expression datasets. The majority of the super-enhancer-associated CRISPR–Cas9 dependency genes exhibited high levels of expression in primary neuroblastomas as compared to multiple other types of primary tumors (Supplementary Fig. 5) with LDB1 as an exception.

Recent evidence has suggested the presence of two super-enhancer-associated transcriptional networks controlling neuroblastoma cell state, one representing a multipotent neural crest cell-like or mesenchymal cell state (NCC/MES) and a second representing sympathoadrenal cells, referred to as a noradrenergic (NOR/ADR) cell state28,29. The majority of cell lines and low passage primary tumors, regardless of MYCN-amplification, displayed super-enhancers associated with this core group of transcription factors (Supplementary Fig. 6a,b). Furthermore, analysis of neuroblastoma cell lines subdivided by transcriptional network subtype demonstrated that these CRC transcription factors were also marked by super-enhancers, although to a lesser degree in the NCC/MES cell state. Analysis of all neuroblastoma cell lines in our genome-scale CRISPR–Cas9 screen demonstrated gene expression signatures consistent with either NOR/ADRn or NCC/MES cell states (Supplementary Fig. 6c). Expression of CRC transcription factor genes was elevated in both neuroblastoma subtypes compared to cell lines derived from other tumor types (Supplementary Fig. 6d).

Given these findings, we hypothesized that the CRC responsible for specifying neuroblastoma cell fate is formed by these super-enhancer-marked transcription factor dependencies. To test this hypothesis, we performed ChIP-seq in BE2C and Kelly cells with antibodies recognizing MYCN and five super-enhancer-regulated transcription factor dependencies. These data demonstrated that HAND2, ISL1, PHOX2B, GATA3, TBX2, and MYCN bind consensus DNA sequences adjacent to each other to form dense clusters of transcription factors comprising distinct ‘epicentres’ within their own enhancer regions (Fig. 3a and Supplementary Fig. 7). These epicentres occupied discrete regions of open chromatin delineated by assay for transposable-accessible chromatin using sequencing (ATAC-seq). Binding of each transcription factor was preferentially found in gene enhancers containing enriched consensus binding motifs (Supplementary Table 3 and Supplementary Fig. 8), suggesting that these transcription factors collectively regulate the expression of their own genes, and each of the other five transcription factors evaluated by ChIP, and form a feed-forward autoregulatory CRC. When overexpressed, MYCN is thought to bind active
promoter and enhancer elements throughout the genome, similar to the activities of MYC, resulting in amplified transcription that overcomes rate-limiting constraints on cell proliferation and survival. Genome-wide ChIP-seq analysis demonstrated that MYCN binds coordinately with other members of the CRC to regions of open chromatin marked by enhancers across the genome (Fig. 3b), indicating that it may function as a CRC member or, alternatively, as a general transcriptional amplifier as has recently been reported. Amplification of the MYCN gene raised the background signal of input DNA across the amplified region, precluding formal evaluation of CRC transcription factor binding to any potential MYCN enhancer (Supplementary Fig. 9). Analysis demonstrated a nearly twofold statistical enrichment of binding of all five CRC transcription factors, with or without MYCN, to dependency genes ($P < 0.01$ for binding to dependency genes versus remainder of expressed genes in the CRISPR–Cas9 screen by two-sided Fisher's exact test). However, MYCN and these transcription factors are also present at genes that are not selective neuroblastoma dependencies.

To establish co-regulation of CRC component genes, we determined the gene expression levels by reverse transcription...
qPCR (RT–qPCR) after transient siRNA-mediated knockdown of each gene (Fig. 3c and Supplementary Fig. 10). We observed decreased expression levels of all CRC transcription factors after knocking down each individual CRC gene. The impact of CRC transcription factor depletion on the super-enhancer landscape of the cell remains to be studied; however, these data implicate these genes and their encoded transcription factors in a feed-forward autoregulatory mechanism that drives MYCN-amplified neuroblastoma (Fig. 3d).

Efforts to target aberrant transcriptional activity in tumors such as MYCN-amplified neuroblastoma have centered on agents that inhibit cyclin-dependent kinases and BET-bromodomain-dependent pathways that act as mediators of MYCN-driven initiation and elongation of transcription.35,36. Based on the feed-forward, autoregulatory CRC we have identified, we postulated that MYCN-amplified neuroblastoma cells could be targeted synergistically by combining JQ1 (BRD4 inhibitor) and THZ1 (CDK7 inhibitor). While treatment with JQ1 or THZ1 alone reduced the growth of Kelly and BE2C cells (Fig. 4a), combination treatment dramatically reduced cell numbers and induced apoptosis (Fig. 4a and Supplementary Fig. 11). Comparison of results after treatment with multiple concentrations of JQ1 and THZ1 alone and together demonstrated synergistic inhibition of viability of a panel of both MYCN-amplified and non-amplified neuroblastoma cell lines by the Chou–Talalay
combination index (Fig. 4b and Supplementary Fig. 12) [47–49]. Next, we subcutaneously xenografted BE2C cells in nude mice and treated tumor-bearing mice with vehicle control, JQ1, THZ1, or the combination. In contrast to control and single agent-treated mice, combination inhibition with JQ1 and THZ1 led to reduced tumor progression and increased survival compared to either single agent alone without a substantial effect on animal weight (Fig. 4c and Supplementary Fig. 13). These data indicate that compounds disrupting transcriptional initiation and elongation can serve as a synergeticly potent and effective method of tumor inhibition in MYCN-amplified neuroblastoma in vitro and in vivo.

To evaluate the effects of combination treatment on both CRC and global gene expression, we treated BE2C cells with a synergistic combination of JQ1 for 0, 1, 4, and 12h, and performed gene expression analysis with spike-in controls. In comparison to the top 1% of highest expressed transcripts, CRC mRNA levels rapidly decreased in expression after 1h of treatment (Fig. 4d,e). We observed that the majority of downregulated genes at 4h of combined JQ1 and THZ1 treatment were bound by all CRC members, suggesting that they represent a network of genes directly regulated by the CRC (Supplementary Fig. 14). By 12h of treatment, the majority of all mRNA transcripts were downregulated, indicative of general transcriptional collapse. These results were confirmed by RT–qPCR analysis of expression levels of CRC and non-CRC member genes (ACTB, HPRT) in BE2C and Kelly cells (Supplementary Fig. 15). The expression level of each of the six transcription factor genes was dramatically reduced by the combination of JQ1 and THZ1, with more limited effects noted with either drug alone. These results underscore the effects of JQ1 and THZ1 combination treatment in MYCN-amplified neuroblastoma, although the complete effects of combination transcriptional disruption remain to be elucidated.

Our findings identify a collection of genes representing diverse vulnerabilities in MYCN-amplified neuroblastoma, including the transcription factors that form an autoregulatory CRC specific for these cells. At least six of the transcription factors encoded by members of the dependency group belong to this CRC, each of which directly regulates the expression of its own gene as well as those encoding the other CRC transcription factors. This CRC also controls an extended regulatory network of genes, which we suggest contributes to initiation and maintenance of the transformed phenotype in MYCN-amplified neuroblastoma. We hypothesize that a similar set of transcription factors form a CRC in MYCN non-amplified neuroblastoma, due to reliance on high-level expression of MYC or MYCN in these tumors. Our results underscore the interconnected autoregulatory mechanisms underlying the expression and activity of CRC transcription factors. This dependence on transcriptional activity leads to the enhanced vulnerability of these cells to combinatorial targeting of key nuclear proteins that mediate transcriptional initiation and elongation. A subset of the selective dependencies that we have identified in this tumor probably reflect the consequences of transformation by the CRC, and it will be important to investigate these genes and the pathways they control as a source of additional ‘druggable’ targets in this tumor.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-018-0191-z.

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Author contributions
A.D.D., M.W.Z., N.V.D., K.S., B.J.A., R.A.Y. and A.T.L. conceived the study and designed the experiments. A.D.D. and M.W.Z. performed genomic meta-analyses, ChIP-seq, ATAC-seq, siRNA, low-throughput CRISPR-Cas9, pharmacologic, and gene expression assays. N.W. and S.H. performed ChIP-seq and ATAC-seq experiments. A.B.I., A.D.D., N.V.D., and K.S. performed and interpreted the drug activity and synergy studies. N.V.D., K.S., J.M.K., F.V., D.E.R., A.T., W.C.H., and T.R.G. performed and analyzed the genome-scale CRISPR-Cas9 screening experiments. N.V.D., B.J.A., and R.A.Y. performed computational analysis. A.D.D., M.W.Z., N.V.D., K.S., and A.T.L. wrote the manuscript with input from all authors.

Competing interests
R.A.Y. is a founder and shareholder of Syros Pharmaceuticals, which is discovering and developing therapeutics directed at transcriptional pathways in cancer. K.S. and W.C.H. consulted for Novartis Pharmaceuticals as part of the Dana–Farber Cancer Institute/Novartis Drug Discovery Program. B.J.A. is a shareholder of Syros Pharmaceuticals. No other potential conflicts of interest are declared.

Additional information
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**Methods**

**Cell lines.** Kelly and BE2C neuroblastoma cell lines were obtained from the American Type Culture Collection (BE2C) and DSMZ (Kelly). All cell lines used for the genome-scale CRISPR-Cas9 screen have been previously described. All cell lines were short tandem repeat (STR) profiled to ensure identity. Neuroblastoma cell lines were cultured in RPMI media containing 10% fetal bovine serum and 1% penicillin-streptomycin and validated to be free of *Mycoplasma* species.

**In vivo tumor models.** We adhered to animal protocols approved by the Dana–Farber Cancer Institute Animal Care and Use Committee. Animals were maintained according to institutional guidelines. Eight-week-old female nude mice were subcutaneously implanted with 1 × 10⁶ BE2C cells in 30% matrigel/PBS. Mice were assigned to four groups: vehicle, JQ1, THZ1, or the combination of JQ1 and THZ1. Treatment with small-molecule inhibitors was initiated once tumors engrafted and reached 100–150 mm³. Compounds were solubilized in 10% DMSO and 90% 5% dextrse in water, JQ1 was delivered at 50 mg kg⁻¹ daily by intraperitoneal injection, and THZ1 was delivered at 10 mg kg⁻¹ twice daily by intraperitoneal injection. Mice were treated for 14 d and followed for survival. Tumors were measured by calipers twice weekly. Mice were weighed twice a week. Animals were euthanized according to institutional guidelines when tumors reached 2,000 mm³ in length or width, or if animals became moribund.

**CRISPR–Cas9 screening and analysis.** The CRISPR–Cas9 screen was performed with the Avana library containing approximately 70,000 guides and an average of 4 guides per gene. Details of library construction, cell transduction, and analysis are found in the Supporting Note. This version of the Avana dataset contains 341 cell lines, including 9 MYCN-amplified neuroblastoma lines (CHP212, IMR52, Kelly, KPNYN, H137N, SKNB2, SKNDZ, and SIMA). This dataset includes 2 MYCN non-amplified neuroblastoma cell lines, SKNAS and SKNFL, which were excluded from the enrichment analysis for a total of 339 cell lines analyzed. Other cell lines in the screen represent 26 different types of tumors (breast 26, chondrosarcoma 1, colorectal 25, endometrial 12, esophageal 10, head and neck 8, leukemia 9, liver 13, lung non-small cell 45, lymphoma 4, medulloblastoma 4, melanoma 2, osteosarcoma 4, ovarian 31, prostate 22, renal 16, rhabdoid 1, soft tissue sarcoma 5, thyroid 1, urinary tract 19) and were combined to create the comparison group, as detailed previously.

Dependency scores were generated for each gene in each cell line using the CERES algorithm. Briefly, CERES is a computational method to estimate gene dependency levels from CRISPR-Cas9 essentiality screens while accounting for the copy-number-specific effect, as well as variable single guide RNA activity. The gene dependency scores generated by CERES are absolute dependency scores with each guide RNA measured using the Kolmogorov–Smirnov test with a false discovery rate less than 0.05 using the Benjamini–Hochberg multiple testing correction.

Candidate dependencies were filtered to only include those that also had an absolute dependency effect before the decision-making described above. Genes that had overall negative scores across all cancer lines in the screen were considered to be consistent with pan-essentiality and were excluded. Candidate dependency genes were further filtered to include those with expression in MYCN-amplified neuroblastoma in the Cancer Cell Line Encyclopedia. Thus, final scores reflect selective dependencies relative to findings with all other tumor types in the screen.

**Gene ontology classification.** Gene ontologies were assigned using the Gene Ontology subset (GO-slim) molecular function ontologies. These 42 GO-slim molecular functions were combined to create broader classifications of genes such as transcription factor activity or binding (Supplementary Table 4). In each class molecular functions were combined to create broader classifications of genes such as dependencies if they had more than 2 core essential genes.

Guide RNAs and an average dependency score of −1 for a set of positive control non-amplified neuroblastoma cell lines, SKNAS and SKNFL, which were added to the screen to confirm functional knockdown. Jenflux was delivered at 100 ng μl⁻¹ daily with the Nextera protocol. Transposed DNA was then purified using the MinElute kit (Qiagen). Library amplification was performed using NEBNext High-Fidelity 2X PCR Master Mix (NEB), with SYBR Green I dye (Invitrogen) and Nextera primers Ad1 and Ad2 (x) at 25 μM. Amplified libraries were purified with the PCR purification kit (Qiagen), and quality control was performed using the 2200 Tapestation (Agilent).

**Synergy studies and pharmacologic inhibitors.** Synergy screening was performed with THZ1 synthesized by the Gray Laboratory (Dana–Farber Cancer Institute) and THZ1 synthesized by the Bradner Laboratory (Dana–Farber Cancer Institute). Experiments were validated with compounds from commercially available sources: JQ1 (Sigma–Aldrich) and THZ1 (EMD Millipore). For assessment of synergy, neuroblastoma cell lines were plated in 384-well tissue culture–treated plates at a density of 25,000 cells ml⁻¹. Cells were then treated with single or combinations of compounds and subsequently analyzed for cell viability on days 0, 1, and 3 using the Cell-Titer Glo luminescent assay kit (Promega) per the manufacturer’s instructions. Luminescence was read on a Fluostar Omega Reader (BMG Labtech). For cell growth assays and gene expression studies, JQ1 was used at 3 μM and THZ1 at 78 nM (Kelly) or 125 nM (BE2C), with DMSO as a vehicle control. Synergy was assessed by the Chou–Talalay Combination index, isobologram, and excess over Bliss methods.

**Statistical analysis.** Data from the ChiP-seq and CRISPR–Cas9 screens were analyzed as described above. Animal experiments were analyzed by two-sided analysis of variance (ANOVA) and post hoc Turkey test, t-tests, or one-sided Fisher exact tests as appropriate for multiple or pair wise comparisons. Statistical significance was defined as a P < 0.05. Data were analyzed with GraphPad Prism 7.01, and all error bars represent standard deviation unless otherwise noted.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Code availability. Custom code written in R/python to perform analyses of CRISPR–Cas9 and ChIP-seq data is available upon reasonable request.

Data availability. Gene dependency scores from the CRISPR–Cas9 screen are publicly available at https://doi.org/10.6084/m9.figshare.5520160.v1,44. ChIP-seq (GSE94822), ATAC-seq (GSE94823), and spike-in microarray (GSE108914) data are available through GEO; for further details, see Supplementary Table 6.

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Experimental design

1. Sample size

Describe how sample size was determined.

For cellular and molecular assays, sample sizes were chosen based on expected phenotypes and previous experience with assay variability. Results from genome-scale CRISPR assays inferred strong phenotypic effects resulting from the disruption of essential genes and thus 3-6 technical replicates were chosen for most experiments. For colony formation, immunoblotting, qRT-PCR and drug treatment assays, experiments were completed at least in triplicate biological replicates. Technical replicates representing the same cells assayed at the same time were limited and not included in the total n value. N values denoted in the figure legends represent independent biological replicates. ChIP-seq experimental design is detailed in ChIP-seq reporting summary. CRISPR-Cas9 experiments for genome-scale analysis were completed using 70,000 guides and an average of 4 guides per gene. The CRISPR sgRNA library contains 1000 non-targeting guides as negative controls. Animal experiments were completed with a total of 8 animals per treatment group.

2. Data exclusions

Describe any data exclusions.

Outlier analysis for qRT-PCR studies were performed and values representing greater than or less than 3 standard deviations from the mean were excluded. No data exclusions were performed for other assays. For CRISPR-Cas9 experiments, a pool of guides was transduced into a population of cells, and cells were cultured for approximately 21 days in vitro. At the end of the assay, barcodes for each guide were sequenced for each cell line in replicate. Reads per kilobase were calculated for each replicate and then the log2 fold change compared to the initial plasmid pool was calculated for each guide. Samples with poor replicate reproducibility, as well as guides that have low representation in the initial plasmid pool, were removed from analysis. In total, each gene was assayed with, on average, 4 guides per gene.

3. Replication

Describe whether the experimental findings were reliably reproduced.

Experimental findings were reliably reproduced by multiple independent individuals. For example, combination JQ1+THZ1 experiments for cDNA expression analysis as well as effects on cell growth were replicated independently by three individuals at distinct timepoints. CRISPR-Cas9 screening was validated by low throughput siRNA analysis (Figure 1E,F).

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Experimental groups were defined based on appropriate biological and technical controls. Groups of animals were subcutaneously implanted with 1x10^6 BE2C cells the right flank of 7-8 week old nude female mice. Treatment was started when tumors reached 100-150mm3 with rolling admission into all 4 groups.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Whole genome ChIP-seq analysis was confirmed by two individuals blinded to the identity of the proteins involved. CRISPR-cas9 screening was
Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
|     | ☒ | The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
|     | ☒ | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly. |
|     | ☒ | A statement indicating how many times each experiment was replicated |
|     | ☐ | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
|     | ☒ | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
|     | ☒ | The test results (e.g. \( p \) values) given as exact values whenever possible and with confidence intervals noted |
|     | ☒ | A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
|     | ☒ | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study.

CERES (Meyers RM et al. Nature Genetics 2017)
GraphPad Prism 7.01
Microsoft Excel 2016
Microsoft Word 2016
Python
RStudio Version 1.1.383
R version 3.3.2 with standard packages
R packages dplyr 0.7.4; tidyr 0.8.0; matrixStats 0.53.1; dslice 1.1.5; biomaRt 2.30.0; GSEABase 1.36.0; ggplot2 2.2.1; ggrepel 0.7.0; extrafont 0.17; affy 1.56.0
FlowJo v.10.4.2
Bowtie 1.2
MACS - 1.4.2
UCSC Genome Browser - 21 Feb 2016
ROSE
bamToGFF 0.1
bedtools v2.26.0
FIMO 4.11.4
AME 4.11.4
JASPAR - JASPAR_CORE_2014 and JASPAR_CORE_2016
HOCOMOCO - HOCOMOCOv10_HUMAN_mono CIS-BP 1.02
Adobe Illustrator v.CC
R2 database (2017)

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.
8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials used in experiments are commercially available from various sources.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Immunoblotting was carried out with the following antibodies:

- PHOX2B-B11X (Santa-Cruz Biotechnology, catalog number: sc376997X, lot number: H0712, dilution: 1:500)
- ISL1 clone 39.4D5 (Developmental Studies Hybridoma Bank, catalog number: 39.4D5, lot number: 8/4/16, dilution: 1:250)
- HAND2 A12X (Santa-Cruz Biotechnology, catalog number: sc398167X, lot number: K1716, dilution: 1:500)
- TBX2 (Abcam, catalog number: ab33298, lot number: GR296812-2, dilution: 1:1000)
- GATA3 (Pierce Biotechnology, catalog number: MA-1028, lot number: PE203382, dilution: 1:1000).

- Betactin (Cell Signaling Technology, catalog number: 4967, lot number: 9, dilution: 1:1000)
- cleaved caspase-3 (Cell Signaling Technology, catalog number: 9665, lot number: 7, dilution: 1:500)
- PARP-1 (Cell Signaling Technology, catalog number: 9542, lot number: 15, dilution: 1:1000).

Secondary antibodies were:

- Anti-mouse IgG HRP-Linked (Cell Signaling Technology, catalog number: 7076, lot number: 31, dilution: 1:5000).
- Anti-rabbit IgG HRP-Linked (Cell Signaling Technology, catalog number: 7074, lot number: 26, dilution: 1:5000).

Antibodies for western blotting against CRC members were confirmed specific by transient siRNA against the target protein, followed by western blotting. Antibodies against beta-actin, cleaved caspase-3 and cleaved PARP-1 were confirmed specific by the manufacturer.

Antibodies for ChIP-Seq were confirmed specific by immunoprecipitation-western blotting with control and siRNA-treated cell lysates, per the recommendations of ENCODE.

The following antibodies were used for ChIP-Seq:

- GATA3 (Pierce Biotechnology, catalog number: MA-1028, lot number: PE203382).
- HAND2 M19X (Santa-Cruz Biotechnology, catalog number: sc9409X, lot number: C230)
- ISL1 (Developmental Studies Hybridoma Bank, catalog number:40.3A4, lot number: 8/22/13)
- MYCN (Invitrogen Biotechnology, catalog number: MA-1-16638, lot number: RK2291965)
- PHOX2B (Santa-Cruz Biotechnology, catalog number: sc376997X, lot number: H0712)
- TBX2 (Abcam, catalog number: ab33298, lot number: GR296812-2)
- H3K27ac (Abcam Biotechnology, catalog number: ab4729, lot number: GR312651-1)
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. Kelly and BE2C neuroblastoma cell lines were obtained from ATCC (BE2C) or DSMZ (Kelly). All cell lines used for the genome-scale CRISPR-Cas9 screen are detailed in Meyers RM et al. (Nature Genetics 2017).
   b. Describe the method of cell line authentication used. All cell lines used were STR tested for identity at either the Dana-Farber Cancer Institute molecular diagnostics core facility or The Broad Institute of MIT and Harvard.
   c. Report whether the cell lines were tested for mycoplasma contamination. All cells were routinely tested for mycoplasma and confirmed negative.
   d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified lines listed in the ICLAC database were used in this study.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
    Provide details on animals and/or animal-derived materials used in the study.
    Eight-week old female nude mice were obtained from Charles River Laboratories and subcutaneously xengrafted with 1x10^6 BE2C cells in 30% matrigel/PBS. Animals were treated with IP injection of compound as detailed in the manuscript once tumors engrafted and reached 100-150mm^3. Mice were sacrificed when tumors reached a maximum of 2000 mm in length or width or if they became moribund per institutional policies.

Policy information about studies involving human research participants

12. Description of human research participants
    Describe the covariate-relevant population characteristics of the human research participants.
    This study did not involve human research participants.
ChIP-seq Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data deposition

1. For all ChIP-seq data:
   - a. Confirm that both raw and final processed data have been deposited in a public database such as GEO.
   - b. Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

2. Provide all necessary reviewer access links. (The entry may remain private before publication.)

   Link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94822
   Accession key: gtohowcphgwmv

3. Provide a list of all files available in the database submission.

   PROCESSED DATA FILES:
   - GSM2486153_20161217_5291.7830.rpm.WIG.gz
   - GSM2486155_20161217_5285.7822.rpm.WIG.gz
   - GSM2486156_20161217_5286.7824.rpm.WIG.gz
   - GSM2486158_20161217_5293.7826.rpm.WIG.gz
   - GSM2486159_20161217_5288.7816.rpm.WIG.gz
   - GSM2486161_20161217_5292.7832.rpm.WIG.gz
   - GSM2486170_20161217_5283.7818.rpm.WIG.gz
   - GSM2486157_20161215_5261.7792.rpm.WIG.gz
   - GSM2486162_20161215_5265.7794.rpm.WIG.gz
   - GSM2486165_20161215_5263.7802.rpm.WIG.gz
   - GSM2486166_20161215_5281.7810.rpm.WIG.gz
   - GSM2915909_20170616_5888.8198.rpm.WIG.gz
   - GSM2915910_20170616_5890.8210.rpm.WIG.gz
   - GSM2915911_20170616_5889.8216.rpm.WIG.gz

   RAW FILES:
   - 20161217_5291.20161217_BE2C_PHOX2B_MWZ3782_S6_R1_001.fastq.gz
   - 20161217_5285.20161217_BE2C_HAND2_M19X_MWZ3782_S4_R1_001.fastq.gz
   - 20161217_5286.20161217_BE2C_Input_MWZ3782_S11_R1_001.fastq.gz
   - 20161217_5293.20161222_BE2C_MYCN_MWZ3782_S1_R1_001.fastq.gz
   - 20161217_5288.20161217_BE2C_ISL1_MWZ3782_S1_R1_001.fastq.gz
   - 20161217_5292.20161217_BE2C_TBX2_MWZ3782_S7_R1_001.fastq.gz
   - 20161217_5283.20161217_BE2C_GATA3_MWZ3782_S8_R1_001.fastq.gz
   - 20161215_5261.20161215_Kelly_GATA3_MWZ3781_S8_R1_001.fastq.gz
   - 20161215_5265.20161215_Kelly_ISL1_MWZ3781_S1_R1_001.fastq.gz
4. If available, provide a link to an anonymized genome browser session (e.g. UCSC).

N/A

### Methodological details

5. Describe the experimental replicates.

For each experiment, four individual P150 plates of BE2C or Kelly cells were fixed and pooled to achieve a total of 1x10E8 cells per pellet. 10 pellets were sonicated per experiment and sonicated nuclear extracts were pooled, prior to division such that 10% of the sonicated nuclear extract was used for each ChIP.

6. Describe the sequencing depth for each experiment.

ChIP-sequencing was performed using Illumina NextSeq 500 single-end 75bp reads. Read counts for ChIP targets were as follows:

| Sample       | Total Reads | Mapped Reads |
|--------------|-------------|--------------|
| BE2C_Input   | 45170421    | 36931058     |
| Kelly_PHOX2B | 58964693    | 45237905     |
| Kelly_GATA3  | 37758294    | 30181530     |
| Kelly_MYCN   | 53494531    | 40042506     |
| BE2C_GATA3   | 41674459    | 35660539     |
| Kelly_Input  | 76794317    | 54614212     |
| Kelly_ISL1   | 51315749    | 37962697     |
| BE2C_HAND2   | 51031589    | 43855881     |
| BE2C_JSL1    | 38836431    | 33091551     |
| BE2C_PHOX2B  | 32878170    | 28794150     |
| Kelly_TBX2   | 70568114    | 54000987     |
| BE2C_TBX2    | 31741229    | 27241668     |

7. Describe the antibodies used for the ChIP-seq experiments.

Antibodies were first validated by IP with total protein lysates collected from BE2C and Kelly cells. The antibodies used for ChIP are as follows:

- GATA3 (Thermo Fisher MA1028)
- HAND2 (Santa Cruz sc9409X)
- ISL1 (Developmental Studies Hybridoma Bank 40.3A4)
- MYCN (Invitrogen Biotechnology, MA-1-16638)
- PHOX2B (Santa Cruz sc376997X)
- TBX2 (Abcam ab33298)
- H3K27ac (Abcam ab4729)

8. Describe the peak calling parameters.

Regions enriched in ChIP-seq signal were identified using MACS with corresponding control and parameters –keep-dup=auto and –p 1e-9. Regions displayed in Figure 3B were created from the
9. Describe the methods used to ensure data quality. Data quality was assessed using MACS by comparing peak enrichment over input controls with a p cutoff value of 1e-9.

10. Describe the software used to collect and analyze the ChIP-seq data. Reads were aligned to the human genome (hg19) using bowtie with parameters –k 2 –m 2 –e 70 –best and –l set to the read length.

For visualization, WIG files were created from aligned ChIP-Seq read positions using MACS with parameters –w –S –space=50 –nomodel –shiftsize=200 to artificially extend reads to be 200bp and to calculate their density in 50bp bins.

Heatmaps of ChIP-Seq signal in these regions were made by counting reads in bins dividing each site using bamToGFF (https://github.com/bradnerComputation). The display heatmap was created using heatmap.2 in R.

Super-enhancers in Kelly and BE2C were identified using ROSE (https://bitbucket.org/young_computation/rose), as described in Mansour et al. with some modifications. Briefly, two sets of peaks of H3K27ac were identified using MACS with parameter sets –keep-dup=auto –p 1e-9 and –keep-dup=all –p 1e-9. Peaks identified that contact the region chr2:14817188-17228298 were discarded, because they fall within the genomically amplified regions around MYCN. It can be assumed that MYCN is associated with a super-enhancer due to the exceptionally high signal in this region. The collapsed union of regions called using both MACS parameter sets that do not contact the discarded MYCN-proximal region were used as input for ROSE.