MYCN amplification and ATRX mutations are incompatible in neuroblastoma

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Aggressive cancers often have activating mutations in growth-controlling oncogenes and inactivating mutations in tumor-suppressor genes. In neuroblastoma, amplification of the MYCN oncogene and inactivation of the ATRX tumor-suppressor gene correlate with high-risk disease and poor prognosis. Here we show that ATRX mutations and MYCN amplification are mutually exclusive across all ages and stages in neuroblastoma. Using human cell lines and mouse models, we found that elevated MYCN expression and ATRX mutations are incompatible. Elevated MYCN levels promote metabolic reprogramming, mitochondrial dysfunction, reactive-oxygen species generation, and DNA-replicative stress. The combination of replicative stress caused by defects in the ATRX-histone chaperone complex, and that induced by MYCN-mediated metabolic reprogramming, leads to synthetic lethality. Therefore, ATRX and MYCN represent an unusual example, where inactivation of a tumor-suppressor gene and activation of an oncogene are incompatible. This synthetic lethality may eventually be exploited to improve outcomes for patients with high-risk neuroblastoma.

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Neuroblastoma is the most common extracranial solid tumor of childhood. MYCN amplification and age at diagnosis are the two most powerful predictors of outcome, with survival rates 5–10 times higher in infants than in adolescents or young adults. Previous genomic analyses of stage 4 pediatric neuroblastoma samples identified the ATRX mutations in patients that were typically older than 5 yr, had an indolent disease course, and poor overall survival (OS)1,13. One important function of ATRX is recognition of guanine (G)-rich stretches of DNA and deposition of the H3.3 histone variant to prevent the formation of G-quadruplex (G4) structures, which can block DNA replication or transcription. G-rich repeats are also found at telomeres and centromeres; ATRX forms a complex with DAXX to deposit H3.3 in those regions to maintain their integrity. In cells lacking ATRX, H3.3 is not efficiently deposited at the telomeric G-rich regions, G4 structures form, and replication forks stall. Consequently, telomeres undergo homologous recombination leading to alternative lengthening of telomeres (ALT). The formation of G4 structures in other G-rich repetitive regions of the genome can cause replicative stress or block transcription. Indeed, H3.3 is deposited at actively transcribed regions of the genome. Particular regions of the genome may also affect transcription by targeting the PRC2 complex to transcriptional silencing associated with tumorigenesis in a variety of cancers. One important function of ATRX is recognition of guanine (G)-rich stretches of DNA and deposition of the H3.3 histone variant to prevent the formation of G-quadruplex (G4) structures, which can block DNA replication or transcription. MYCN regulates diverse cellular processes during development and in cancer. For example, elevated MYCN leads to increased glycolytic flux and glutaminolysis to promote metabolic reprogramming associated with tumorigenesis in a variety of cancers including neuroblastomas. MYCN-induced glutaminolysis in neuroblastoma elevates reactive-oxygen species (ROS) and DNA-replicative stress. Indeed, one of the hallmarks of neuroblastoma is the DNA mutation signature associated with ROS induction. Consequently, neuroblastoma cells exhibit increased sensitivity to pharmacological agents that induce oxidative stress. Here we demonstrate that the DNA-replicative stress induced by ATRX mutations and MYCN amplification cause synthetic lethality in neuroblastoma. This is unusual because oncogene activation and tumor-suppressor inactivation often work in concert to promote tumorigenesis not cancer cell death.

Results
ATRX and MYCN mutations in neuroblastoma. To complement previous neuroblastoma studies from the Therapeutically Applicable Research to Generate Effective Treatment (TARGET) initiative and the Pediatric Cancer Genome Project (PCGP), we obtained neuroblastoma samples from 473 patients (122 unpaired and 351 paired tumor/germline) from the Children’s Oncology Group (COG) (Table 1). We identified single-nucleotide variations, small indels, and other somatic mutations in the coding region of ATRX via custom capture and Illumina sequencing using probes spanning the entire ATRX locus and whole-exome sequencing of 828 germline and tumor samples. We also included MYCN in the capture probe set to determine its copy number. We identified 19 somatic ATRX mutations, 15 of those were internal deletions predicted to encode truncated proteins (Fig. 1a).

Significantly higher ATRX-mutation frequencies are detected in patients with International Neuroblastoma Staging System (INSS) stage 4 disease (8.6%), high-risk subgroup (14.6%), 11q loss of heterozygosity (LOH), or unfavorable histology and in those who are 18 mo or older at diagnosis (Table 1, Fig. 1b). ATRX-mutant neuroblastomas were significantly more likely to have ALT than ATRX wild-type tumors (89.5% (17/19) vs. 22.2% (4/18); p < 0.0001, two-tailed Fisher’s exact test). In addition, black patients had a significantly higher frequency of ATRX mutation than did those in other racial categories (9.1% vs. 3.3%; p = 0.0348, two-tailed Fisher’s exact test) (Table 1). ATRX mutation frequency did not differ based on sex, ALK status, tumor grade, mitosis–karyorrhexis index, or ploidy (Table 1).

ATRX mutations were associated with significantly lower 4-year event-free survival (EFS) rates among the groups with non-INSS stage 4 disease (p = 0.0007, two-tailed Fisher’s exact test), INSS stage 4 disease (p = 0.0128, two-tailed Fisher’s exact test), ages 5–12 y (p = 0.0006, two-tailed Fisher’s exact test) and older than 12 y (p = 0.0038, two-tailed Fisher’s exact test) at diagnosis in the COG cohort (Supplementary Data 1 and Fig. 1c, d). Compared with those without a mutation, patients harboring an ATRX mutation were 5.0 times more likely to experience a adverse event (p < 0.0001) and 3.4 times more likely to die of their disease (p = 0.0046, two-tailed Fisher’s exact test) (Supplementary Data 1).

In the cohort of 819 tumors, ATRX mutations (n = 64) and MYCN amplification (n = 140) showed statistically significant mutual exclusivity (p = 0.0037, Cochrane-Mantel-Haenszel test) (Fig. 1e and Supplementary Note 1). Only 1 of 819 (0.1%) tumors had both lesions. However, this was an unusual discordant sample because it was scored as MYCN-amplified by fluorescence in situ hybridization (FISH) in COG’s neuroblastoma reference lab but non-amplified based on normalized read depth from our custom capture Illumina next-generation sequencing data (Supplementary Fig. 1A). To resolve this discrepancy, we repeated

### Table 1 Distribution of ATRX mutations in neuroblastoma.

| Classificationa | Grouping | N(%)b | ATRX mutation (%)c | p-value |
|-----------------|----------|-------|-------------------|--------|
| INSS stage      | Non-stage 4 | 315 (65.8) | 5 (1.6) | 0.0002 |
| Stage 4         | Stage 4 | 142 (34.2) | 13 (9.2) | 0.0002 |
| Risk Group      | Low/intermediate | 375 (79.6) | 5 (1.3) | 4.4e-7 |
| High            | High | 96 (20.4) | 14 (14.6) | 0.0002 |
| Sex             | Female | 253 (53.4) | 12 (4.7) | 0.3830 |
| Male            | Male | 221 (46.6) | 7 (3.2) | 0.3830 |
| MYCN (FISH)     | Not amplified | 431 (91.7) | 18 (4.2) | 0.00001 |
| Amplified       | Amplified | 39 (8.3) | 22 (56.4) | 0.00001 |
| MYCN (NGS)      | Not amplified | 447 (94.1) | 18 (4.3) | 0.0680 |
| Amplified       | Amplified | 28 (5.9) | 0 (0) | 0.0680 |
| ALK             | No mutation | 454 (95.6) | 17 (3.7) | 0.2026 |
| Mutation        | Mutation | 21 (4.4) | 2 (9.5) | 4.9e-4 |
| Ploidy          | Hyperdiploid | 277 (60.7) | 10 (3.6) | 0.6454 |
| Diploid         | Diploid | 179 (39.3) | 8 (4.5) | 0.6454 |
| 11q LOH          | No | 301 (91.8) | 5 (1.7) | 4.9e-4 |
| Yes             | Yes | 27 (8.2) | 5 (18.5) | 0.00001 |
| Ip LOH          | No | 290 (88.4) | 9 (3.1) | 1.0000 |
| Yes             | Yes | 38 (11.6) | 1 (2.6) | 1.0000 |
| Histology       | Favorable | 314 (69.8) | 1 (0.3) | 9.3e-9 |
| Unfavorable     | Unfavorable | 136 (30.2) | 17 (12.5) | 9.3e-9 |
| Grade           | Differentiating | 43 (10.9) | 3 (7.0) | 0.7168 |
| Poorly/undifferentiated | Poorly/undifferentiated | 283 (68.6) | 15 (5.3) | 0.7168 |
| MKI             | Low/intermediate | 298 (69.3) | 16 (6.0) | 0.2356 |
| High            | High | 32 (8.7) | 0 (0) | 0.2356 |
| Race            | Black | 55 (12.7) | 5 (9.1) | 0.0348 |
| other           | other | 378 (87.3) | 12 (3.2) | 0.0348 |

aINSS international neuroblastoma staging system, MYCN amplification was assessed by FISH at each COG participating center and by next-generation sequencing (NGS) in our sequence analysis, mkii, MKI-mitosis–karyorrhexis index.
bNumber of patients with percentage in parentheses.
cThere were 19 ATRX mutations identified but for some tumors, the classifier was not applied so the total number of samples and the total number of ATRX-mutant samples is lower.
the FISH analysis and found regional heterogeneity of MYCN amplification (Fig. 1f–h). Specifically, the region with MYCN amplification had a high mitosis–karyorrhexis index and was less likely to have ultrabright telomere foci, characteristic of ALT, in ATRX-mutant neuroblastoma cells (Fig. 1f–h and Supplementary Fig. 1B–E).

To determine whether ATRX mutations and MYCN amplification are incompatible in vivo, we conditionally inactivated ATRX...
in two genetically engineered neuroblastoma mouse models (Fig. 1i–l)\(^{17,18}\). One model (LSL-MYCN;Dbh-iCre) is a transgenic line with conditional expression of MYCN from Rosa26a locus by Cre expressed in the dopaminergic cells of the sympathoadrenal lineage\(^{19}\). The other model includes the Th-ALK\(^{21,22}\) transgene that potentiates MYCN-mediated tumorigenesis\(^{20}\). For each of these models, tumor formation was reduced, and survival was significantly increased when Atrx was simultaneously inactivated with elevated MYCN expression (Fig. 1k–l). The few tumors that did form in the Atrx\(^{lox/lox}\);LSL-MYCN;Dbh-iCre;ALK\(^{Fl/+}\) mice all had evidence of an intact Atrx allele, suggesting that rare cells that did not undergo Atrx inactivation contributed to tumor formation. It is also possible that ALC signaling partially rescued the synthetic lethality. Atrx inactivation has been shown to reduce cell survival in brain, muscles and testes\(^{21,22}\). Histopathological review of adrenal gland and paravertebral sympathetic ganglia from LSL-MYCN;Dbh-iCre and Atrx\(^{lox/lox}\);LSL-MYCN;Dbh-iCre mice at 3 weeks and 1 year of age showed no differences (Supplementary Fig. 2, Supplementary Note 2), suggesting that the lack of tumor formation was not due to death of the NB cell of origin.

**ATRX mutations are incompatible with elevated MYCN.** To extend our data to humans, we characterized the expression of MYCN, ATRX, and DAXX proteins across 12 human cancer cell lines (Fig. 2a). We used eight neuroblastoma cell lines, including three MYCN-amplified lines (IMR32, SKNB2E, NB-5) or moderately elevated levels of MYCN (NBL-S) (Supplementary Data 2), two lines (SKNMM and CHLA90) produce ATRX with an in-frame deletion (ATRX\(^{IFD}\)) and are diploid for MYCN (Fig. 2a), one osteosarcoma cell line with no detectable expression of ATRX (U2OS) and three lines as controls.

To knockdown ATRX, we used two different lentiviral vectors expressing shRNAs targeting ATRX (#9 and #20) (Fig. 2b). The optimal knockdown was ~86% in HeLa cells after 48 h with ATRX shRNA–20. Two weeks after introducing shRNAs, temolome content, ALT status and the proportions of cells in different phases of the cell cycle did not differ in any cell line tested (Supplementary Fig. 3A–I). Knocking down ATRX decreased colony formation in MYCN-amplified neuroblastoma cells (IMR32 and NB-5) but not cell lines expressing wild-type MYCN (Fig. 2c, d).

To inactivate ATRX in the MYCN-amplified neuroblastoma cell lines, we designed and validated two guide RNAs (gRNA-2 and gRNA-5) to exon 6 of ATRX (Fig. 2e, f). We transfected 293T, SKNB2E, and IMR32 cells with each gRNA and a plasmid expressing Cas-9 and purified the transfected cells by flow cytometry. At 2, 10, and 14 days after plating the purified cells, we harvested DNA, performed PCR using primers spanning both of the two gRNA-target sequences, and performed Illumina MiSeq analysis to calculate the proportion of mutated alleles in each sample. Although the ATRX-mutant alleles were abundant in 293T and SKNB2E cells 2 days after plating, those cells harboring ATRX-mutant alleles were lost by 10 days in culture (Fig. 2g and Supplementary Fig. 3i). In MYCN-amplified IMR32 cells, no mutant alleles were detected at any timepoint, though a control gRNA to the AAAS1 locus led to efficient mutagenesis. A similar study performed with a gRNA to DAXX had no effect on the viability of 293T, SKNB2E, or IMR32 cells (Supplementary Fig. 3K-M). Taken together, our data suggest that ATRX inactivation is incompatible with MYCN amplification in neuroblastoma, which is consistent with data from the Project Achilles (https://depmap.org/portal/achilles/).

Next, we induced the expression of MYCN in ATRX-mutant neuroblastoma cells by using a doxycycline-inducible vector (Fig. 2h). Initially, we used two ATRX-mutant cell lines (SKNMM and U2OS), a MYCN-amplified cell line (SKNB2E), and a cell line that is wild-type for both genes (SKNF1) (Fig. 2a). The levels of MYCN achieved with doxycycline induction in both ATRX-mutant lines were similar to those in MYCN-amplified cell lines (Fig. 2h).

The growth rates of SKNF1MYCN and SKNB2E MYCN cells, after 10 days in culture ± doxycycline, showed no difference with induction of MYCN expression, but by 6 days in culture, the two ATRX-mutant lines showed a marked loss of cells (Fig. 2i, j). A parallel experiment performed with stable cell lines that had only the doxycycline-inducible vector lacking MYCN showed no effect on cell growth. More than 95% of the cells in the SKNMM MYCN cultures were lost by 8–10 days in the presence of doxycycline (Fig. 2k). Live imaging of cells ± doxycycline on Days 6–8, the peak period of cell loss (Fig. 2I), enabled us to determine the timing of cell death in 44 cells from 13 different movies (Supplementary Data 3). In total, 54% of the cells died after initiating cytokinesis; the remaining cells died 20.2 ± 12.8 h (mean ± standard deviation) after cytokinesis. These data were extended to five other ATRX-mutant cell lines including two glioma cell lines that had isogenic ATRX-wild-type controls (Supplementary Fig. 4)\(^{23}\). A marker of DNA-replicative stress (pRPA32) was upregulated in the absence of ATRX and in the presence of doxycycline. There was no effect of ectopic MYCN expression on a DAXX-mutant cell line (Supplementary Fig. 4A, B). A small number of SKNMM MYCN cells escaped cell death and continued to grow for 45 days in the presence of doxycycline. The ATRX\(^{IFD}\) was still expressed and the MYCN transgene was still present, but MYCN protein was lost (Fig. 2m).

To determine whether an elevated level of MYCN is incompatible with ATRX mutation in human neuroblastoma cells in vivo, we performed an in vivo growth competition assay. In one cohort of mice, SKNMM MYCN cells labeled with luciferase and YFP were mixed in a 1:1 ratio with unlabeled control SKNMM CONT cells,
which contained the doxycycline-inducible vector lacking MYCN. In a second cohort, the SKNMMCONT cells were labeled with luciferase and YFP and the SKNMMMYCN cells were unlabeled. Cells were injected into the para-adrenal space of 25 immunocompromised mice for each cohort. The luciferase was monitored every week, and ultrasound was performed every 2 weeks. Animals were randomized to +doxycycline and –doxycycline groups once the tumor was larger than 14 mm³ by three-dimensional ultrasound and/or 1.4 × 10⁷ photons/s/cm²/sr by xenogen imaging (Supplementary Data 4). The SKNMMMYCN cells were outcompeted by the
SKNMMCONT cells in vivo, as determined by xenogen and ultrasound (Fig. 2n–q).

The epigenomic landscape of MYCN-amplified and ATRX-mutant NB. MYCN is a global transcriptional regulator that activates and represses genes controlling cell division, cell size, and cell differentiation during development. MYCN, the MYCN gene is highly expressed in neuroblastoma cells, compared with that in wild-type ATRX neuroblastoma, we selected eight cell lines that encompass the ATRX status, including those with MYCN amplification or ATRX mutations (Fig. 3a).

We also included eight orthotopic patient-derived xenografts (O-PDXs) that were derived by par-adorenal injection of patient tumors into immunocompromised mice as described previously (Fig. 3a). One of those O-PDXs (SJNBL047443_X1) has an ATRX in-frame deletion (Fig. 3b, c and Supplementary Data 2). Autopsy tumor material from a patient with ATRX-mutant neuroblastoma (SJNBL030014_D) was also included (Fig. 3a and Supplementary Data 2). We performed whole-genome bisulfite sequencing (WGS; Supplementary Fig. 5 and Data 5), RNA-sequencing, and ChIP-seq of eight histone marks (H3K4me1, H3k4me2, H3K4me3, H3K27me3, H3K27Ac, H3K36me3, H3K9/14Ac, and H3K9me3), CTCF, BRD4, and RNA polymerase II (PolII) across all eight cell lines, the eight O-PDX tumors, the autopsy sample, and normal fetal adrenal medulla. We also performed MYCN ChIP-seq on three O-PDXs with MYCN amplification (SJNBL046_X, SJNBL012407-X1, and SJNBL013762_X1) and the SKNFMYCEN, SKNBE2MYCN, and SKNNMMMYCN cell lines with doxycycline on Day 4 in culture as well as matched controls. To relate gene expression changes to epigenetic changes induced by MYCN, we performed ChIP-seq for the eight histone marks, CTCF, Brd4, and RNA PolII in the SKNNMMMYCN cells ± doxycycline on Day 4 in culture.

To define the chromatin states and analyze the transitions thereof across the genome, we performed chromatin Hidden Markov Modeling (ChromHMM) using all 393 ChIP-seq data sets (Fig. 3d, e and Supplementary Data 6). To share these data, we have developed the SKNNMMMYCN or U2OSMYCN cell lines (Supplementary Data 7). This is consistent with previous data showing that MYC regulates RNA PolII pause release not transcriptional initiation. These upregulated target genes are enriched for the pathways involved in metabolism (e.g., SLC3A2, SLC7A5) and mitochondrial gene expression (e.g., PNPT1) (Fig. 3f, g, Supplementary Fig. 6B-E, and Data 7), consistent with previous studies, demonstrating that elevated MYC/MYCN induces metabolic reprogramming in cancer cells.

Metabolic reprogramming induced by MYCN. One form of metabolic reprogramming induced by MYCN/MYC is glutamine addiction. The glutamine transporter (SLC1A5) involved in glutamine uptake and the bidirectional transporter (SLC7A5–SLC3A2) involved in glutamine efflux coupled with essential amino acid import are direct targets of MYCN and are upregulated in SKNNMMMYCN cells in the presence of doxycycline (Fig. 3g, Supplementary Data 7). Following MYCN induction, we quantified glutamine metabolism in SKNNMMMYCN cells ± doxycycline on Day 4 in culture and measured eight tricarboxylic acid (TCA) cycle cellular metabolites. The proportions of five carbon-labeled glutamate and α-ketoglutarate increased, consistent with glutamine utilization for the TCA cycle as well as reductive carboxylation to produce five carbon-labeled citrate (Fig. 4a, b).

Inefficient use of glutamine and/or glucose by cancer cells can lead to the export of glycolytic intermediates such as lactate. The pH of the medium was quickly acidified in subconfluent cultures of SKNNMMMYCN or U2OSMYCN cells in the presence of doxycycline; however, SKNBE2MYCN and SKNFMYCEN cells showed no difference in the pH of their media (Fig. 4c, d). The reduced pH correlated with an increase in lactate in the culture medium and dependence on glutamine for survival (Fig. 4e and Supplementary Fig. 7A). To provide additional data on the metabolic reprogramming induced by MYCN in ATRX-deficient cells, we performed a more-comprehensive metabolic profiling of 54 metabolites using 13C-labeled glucose and 13C-labeled glutamine (Fig. 4f, g and Supplementary Data 8). Glucose was the major source of lactate; relatively few of the carbons from glucose were used for the TCA cycle (Fig. 4f, g and Supplementary Data 8). In contrast, glutamine was converted to α-ketoglutarate for the TCA cycle and reductive carboxylation.

Glutamine is also an important mitochondrial substrate; cells must precisely balance the expression and activity of proteins...
encoded by the nucleus with those encoded by the mitochondria to maintain homeostasis. Cells also upregulate pathways required to mitigate the ROS that are a natural byproduct of mitochondrial metabolism to prevent excessive protein or DNA damage. For example, glutamine uptake in cancer cells can elevate glutathione levels because glutamine is converted to the glutathione precursor, glutamate. Cell lines that were sensitive to glutamine depletion in the medium also had significantly elevated levels of glutathione (Supplementary Fig. 7B). ROS and DNA damage were increased in the absence of glutamine (Supplementary Fig. C, D).

Pathways involved in mitochondrial homeostasis were upregulated in SKNMM\textsuperscript{MYCN} or U2OS\textsuperscript{MYCN} cells in the presence of doxycycline (Supplementary Data 7). Little change in mitochondrial mass was measured using MitoTracker Green in SKNMM\textsuperscript{MYCN} or U2OS\textsuperscript{MYCN} cells ± doxycycline, but the mitochondrial membrane potential (Δψ\textsubscript{m})...
measured with tetrathiomolybdate-ethyl ester (TMRE) was significantly lower in the presence of doxycycline (Fig. 4h, i). To assess mitochondrial function in MYCN-overexpressing SKNMM cells, we measured the oxygen consumption rate. Induction of MYCN in SKNMM cells decreased basal mitochondrial respiration and respiratory reserve capacity, relative to that seen in un-induced SKNMM cells (Fig. 4j, k and Supplementary Fig. 8). Transmission electron micrographs showed that SKNMM MYCN cells in the presence of doxycycline had more disrupted mitochondria on Day 4 and subsequent timepoints than did SKNMM MYCN cells maintained in culture without doxycycline or the other cell lines (Fig. 4l–o, Supplementary Fig. 9). Similar results were obtained for the ATRX-deficient U2OS MYCN cells but not ATRX-wild-type SKNBE2-MYCN cells (Supplementary Fig. 9).

Replicative stress in ATRX-mutant neuroblastomas expressing MYCN. The mitochondrial dysfunction described above may lead to the accumulation of ROS and in turn contribute to replicative stress through DNA damage. The ROS increase began at Day 4 and persisted until Day 6 in SKNMM MYCN cells in the presence of doxycycline (Fig. 5a). This was accompanied by increased expression of γH2AX protein, a biomarker for DNA double-strand breaks (Fig. 5b, c). Spectral karyotyping showed a significant increase in cells with DNA fragmentation and translocation in the SKNMM MYCN cells in the presence of doxycycline (Fig. 5d). We also detected increased DNA fragmentation in SKNMM MYCN and U2OS MYCN cells in the presence of doxycycline by using a single-cell gel electrophoresis assay (Fig. 5e–g).

Cells with DNA-replicative stress are sensitive to hydroxyurea31. Thus, we maintained SKNMM MYCN cells cultured ± doxycycline with different concentrations of hydroxyurea (12.5, 25, 50, or 100 μM on Days 1–4) and measured cell viability by CellTiter-Glo on Day 4. The SKNMM cells expressing MYCN were significantly more sensitive to hydroxyurea at 50 or 100 μM concentrations (Supplementary Fig. 10A). The DNA content in each cell line, ± doxycycline at Day 4 in culture, showed an increase in S and G2/M phases in the SKNMM MYCN cells in the presence of doxycycline, which was consistent with cell cycle arrest as a result of DNA damage (Supplementary Fig. 10B). The ROS scavenger N-acetyl cysteine had a small but reproducible effect on reducing cell death induced by ectopic MYCN expression in SKNMM cells (Supplementary Fig. 10C, D).

To identify other pathways that may modulate to MYCN-induced cell death in ATRX-deficient cells, we performed a dose–response screen using two drug libraries. The first was a collection of 177 oncology drugs and compounds in late clinical development (Phase II or later). The second was a customized library of 34 drugs and compounds that target proteins or pathways that have been reported to reduce MYC/MYCN oncogenic activity (Data S9). Within the oncology drug set, 37 compounds selectively potentiated killing of SKNMM MYCN cells in the presence of doxycycline (Supplementary Data 9); the majority (59.4%, 22/37) were drugs that induce DNA damage, inhibit DNA repair or replication, or inhibit cell cycle checkpoints (Supplementary Fig. 10E–G and Data 9). The only two drugs that partially rescued the cell death were a third-generation retinoid derivative (bexarotene) and an mTORC1/2 inhibitor (INK128) (Supplementary Fig. 10H and Data 9). The bexarotene result was particularly interesting because retinoids are an effective treatment for neuroblastoma (i.e., they reduce MYCN expression and induce differentiation). These results were independently validated using retinoic acid (Fig. 5h, i).

Together, these data suggest that MYCN induction causes metabolic reprogramming and mitochondrial dysfunction that contribute to increased ROS and DNA-replicative stress. It may be synthetically lethal in ATRX-mutant cells because they already have DNA-replicative stress in part owing to reduced ability to resolve G4 DNA structures. A subset of genes that are normally induced in MYCN-amplified neuroblastomas also may have failed to be activated in the ATRX-mutant neuroblastomas owing to the DNA and/or histone modifications. We identified one gene that met those criteria. CUX2 encodes a homeodomain protein with three CUT repeats that is expressed in the developing nervous system and important for the repair of oxidative DNA damage32. CUX2 is a direct MYCN target and is upregulated in MYCN-amplified neuroblastomas but not ATRX-mutant neuroblastomas (Fig. 5j). The CUX2 promoter is epigenetically repressed by H3K27me3 in ATRX-mutant neuroblastomas and not induced in SKNMM cells in the presence of doxycycline (Fig. 5j). There was a partial rescue of cell death in SKNMM MYCN cells in the presence of doxycycline when CUX2 was ectopically expressed (Fig. 5k), suggesting that CUX2 is important for oxidative stress in MYCN-amplified neuroblastomas. CUX2 is not induced by retinoic acid in 11 different NB cell lines (Supplementary Fig. 11 and Data 10) so the mechanism of partial rescue by retinoic acid is distinct from that of CUX2. Combining NAC with ectopic expression of CUX2 did not significantly increase survival over NAC or CUX2 alone (Supplementary Fig. 11B).

Heterochromatic H3.3 deposition is altered in ATRX-mutant NB. ATRX has an important role in H3.3 deposition in chromatin33. In addition to telomeres and pericentric heterochromatin, ATRX helps to resolve G-quadruplex (G4) structures in the DNA by deposition of H3.3 at those sites across the genome33,34. This is important because G4 structures can inhibit DNA replication and transcription33,34. G4 structures often overlap with noncanonical DNA:RNA hybrids called R-loops because both structures are important for DNA replication, or inhibit cell cycle checkpoints. It may contribute to increased ROS and DNA-replicative stress. It may resolve G4 DNA structures. A subset of genes that are normally induced in MYCN-amplified neuroblastomas also may have failed to be activated in the ATRX-mutant neuroblastomas owing to the DNA and/or histone modifications. We identified one gene that met those criteria. CUX2 encodes a homeodomain protein with three CUT repeats that is expressed in the developing nervous system and important for the repair of oxidative DNA damage32. CUX2 is a direct MYCN target and is upregulated in MYCN-amplified neuroblastomas but not ATRX-mutant neuroblastomas (Fig. 5j). The CUX2 promoter is epigenetically repressed by H3K27me3 in ATRX-mutant neuroblastomas and not induced in SKNMM cells in the presence of doxycycline (Fig. 5j). There was a partial rescue of cell death in SKNMM MYCN cells in the presence of doxycycline when CUX2 was ectopically expressed (Fig. 5k), suggesting that CUX2 is important for oxidative stress in MYCN-amplified neuroblastomas. CUX2 is not induced by retinoic acid in 11 different NB cell lines (Supplementary Fig. 11 and Data 10) so the mechanism of partial rescue by retinoic acid is distinct from that of CUX2. Combining NAC with ectopic expression of CUX2 did not significantly increase survival over NAC or CUX2 alone (Supplementary Fig. 11B).
IMR32), ATRX-mutant cells (SKNMM and CHLA90) and MYCN non-amplified/ATRX wild-type cells (LAN6 and SKNFI). We identified the reproducible peaks in each group (ATRX, MYCN, and WT) and created a reference list of 51,919 H3.3 peaks across our data set. Among those peaks, 18,436 (35%) overlapped with DNA sequences that are predicted to form G4 structures and 70% (12,932) of those are predicted to form R-loops42. As expected, there were fewer H3.3 peaks in the ATRX group and nearly a twofold reduction in H3.3 peaks overlapping G4 sequences (Fig. 6a). For every H3.3 peak in the data set, we assigned it to a
Fig. 4 Expression of MYCN in ATRX-mutant cells leads to metabolic reprogramming and mitochondrial dysfunction. a Simplified drawing of the TCA cycle and reductive carboxylation, blue circles: 13C derived from 13C5-glutamine. b Bar plot (mean of two technical replicates) of 13C-labeled isopomers in SKNMMMYCN cells on day 4 ± doxycycline (M = 5) of labeling with 13C5-glutamine. The arrows indicate an increase in M = + 5 glutamate and M = + 5 α-ketoglutarate (the most abundant and direct derivatives of 13C5-glutamine) in the presence of doxycycline. c Photograph of culture media on day 3 ± doxycycline. d Line plots of media pH ± doxycycline for each day. The culture media were changed on Day 3. e Histogram (mean of two biological replicates) of the levels of lactate at Day 4 in SKNMMMYCN cells ± doxycycline. f Simplified drawing of the glycolysis highlighting the production of lactate from 13C5-glucose. g Bar plots of 13C-labeled isopomers of lactate and α-ketoglutarate in the medium or cells after 18 h of labeling with 13C5-glucose or 13C5-glutamine, respectively, at Day 4 in SKNMMMYCN cells ± doxycycline, with concentrations indicated on the bars. h, i Box and Whiskers plots of MitoTracker Green (H) and TMRE (I) staining ± doxycycline on Day 4. Each Box shows the 10th to 90th percentiles range of data (at least 15,000 cells), the line represents the median and the Whiskers show the minimum and maximum data range. P values were calculated using two-tailed Mann–Whitney non-parametric test. j, k Representative results of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) (mean and SEM of three technical replicates) for 2.5 × 10^4 SKNMMMYCN cells grown under the Mito-Stress assay conditions for the Seahorse. The experiment was done three times with similar results. l–o Representative electron micrograph of mitochondria from SKNMMMYCN cells after 4 days ± doxycycline. l, n showing disruption in the mitochondrial cristae (arrows). Bar plot of scoring of mitochondrial morphology for the SKNMMMYCN cells ± doxycycline, numbers of scored mitochondria are indicated m, o. P value was calculated using Chi-square test. DOX doxycycline, FCCP carbonyl cyanide-p-trifluoromethoxyphenylhydrazine, Olig oligomycin, Rot/AA rotenone/antimycin A, TCA tricarboxylic acid, TMRE tetramethylrhodamine ethyl ester.

Discussion

Mutually exclusive mutation profiles are not uncommon in cancer cells; however, many are thought to result from targeting the same oncogenic pathway. For example, inactivation of the RB1 tumor-suppressor gene is often exclusive of amplification of genes encoding cyclins (e.g., CCNE1) because they target the same pathway. Examples of synthetic lethality caused by oncogene activation and tumor-suppressor mutation are much less common, and few (if any) have been validated in vivo.

Here, we show that amplification of the MYCN oncogene and inactivation of the ATRX tumor-suppressor gene are mutually exclusive in neuroblastomas from patients of all ages and stages of disease. One discrepant tumor sample may have contained two separate clones, but more-detailed analysis was not possible owing to limited tissue. In mouse models and cell lines, the combination of elevated MYCN expression and ATRX loss led to synthetic lethality. Ectopic MYCN caused metabolic reprogramming, mitochondrial dysfunction, ROS production, and DNA damage in ATRX-mutant neuroblastoma cells. We propose that MYCN amplification and ATRX mutations are incompatible in neuroblastoma, because both lead to DNA-replicative stress. Consistent with this model, the synthetic lethality was partially rescued by genes that reduce oxidative stress (CUX2) and pharmacological agents that induce differentiation (retinoic acid) or reduce ROS levels (N-acetyl cysteine). Similarly, pharmacological agents that induced replicative stress through DNA damage exacerbated the synthetic lethality.

ATRX-mutant neuroblastomas have several unique features, relative to other ATRX-mutant cancers. First, DAXX was not mutated in our cohort, whereas in pancreatic neuroendocrine tumors (PanNETs), ATRX, and DAXX mutations have approximately the same frequency and are mutually exclusive. Second, ATRX-mutant neuroblastomas have worse outcome, but patients with ATRX/DAXX-mutant PanNETs tumors have prolonged survival. Third, ATRX mutations in neuroblastomas are often in-frame deletions that remove approximately half of the amino terminus of the protein. In other cancers, the mutations are indels or nonsense mutations. In the original study describing ATRX mutations in neuroblastoma, there was no difference in outcome or clinical presentation for patients with in-frame deletions versus missense or nonsense mutations. However, that cohort was relatively small and a much larger study of ATRX-mutant neuroblastomas would be required to determine if there is any genotype–phenotype correlation for the type of ATRX mutation.

Previous studies suggest that amino acids 1–841 of ATRX are sufficient for localization to heterochromatin. This region is deleted in the ATRXΔC-terminal in most ATRX-mutant neuroblastomas. The ADD domain (amino acids 168–293) binds to histone H3 tails, with preferential binding to H3K9me3 domains that lack H3K4 methylation. HP1 can bind H3K9me3 and recruit ATRX through the HP1-interacting domain (amino acids 586–590), even in the absence of the ADD domain in ATRX. Therefore, in neuroblastomas with in-frame deletions, we propose that they lack the
heterochromatin-binding domain and have defects in H3.3-chaperone function that is required for genome maintenance. Our data also suggest that there are defects in H3.3 deposition at promoters and enhancers for genes involved in neuronal differentiation, particularly for those with G4 structures predicted to form R-loops. Indeed, one of the major pathways that was deregulated is involved in retinoic acid response. This is important because retinoic acid induces differentiation of NB cells and is part of the treatment...
regimen for NB patients. It is possible that there may be a block in retinoic acid response in ATRX-mutant NB cells and this may contribute to the poor outcome for those patients.

Based on our data, we propose that ATRX mutation contributes to tumorigenesis in two ways. First, defects in H3.3 deposition at telomeres leads to telomere maintenance through ALT. Second, ATRX mutations lead to defects in H3.3 deposition at G4 structures in promoters and enhancers of genes involved in neuronal differentiation including retinoic acid-responsive genes. As a result, gene expression is attenuated and ATRX-mutant neuroblastoma cells continue to proliferate. Although DAXX mutations can also contribute to ALT, they do not alter H3.3 deposition at G4 structures. We propose that both mechanisms (ALT and G4 resolution) are essential for tumorigenesis and that is why we did not detect any DAXX mutations in our cohort.

Although the inability of ATRX-mutant neuroblastoma cells to resolve G4 structures promotes tumorigenesis by preventing differentiation, it also causes DNA-replicative stress and slows tumor growth. Indeed, it was recently shown that ATRX can play a role in suppressing R-loop (RNA-DNA hybrid) formation. R-loops can lead to collapse of the DNA replication fork and replicative stress. This may be why ATRX-mutant neuroblastomas are slow growing and indolent. DNA-replicative stress leads to synthetic lethality with ectopic MYCN expression. This genetic synthetic lethality may eventually be exploited in the clinic by reducing the function of ATRX in patients with MYCN-amplified tumors or inducing metabolic (or other changes) caused by MYCN expression in ATRX-mutant tumors. To achieve this ambitious goal in high-risk neuroblastoma, much will need to be learned about the downstream targets of ATRX and MYCN that contribute to this phenotype.

Methods
Uncropped blots and flowcytometric analysis gating. All uncropped gels and blots are shown in Supplementary Fig. 12. Flow cytometric analysis gating information is shown in Supplementary Fig. 13.

Patient samples. Patients were eligible for inclusion in the analytic cohort if they enrolled in the COG Neuroblastoma Biology study ANBL00B1 before treatment, had a confirmed diagnosis of neuroblastoma; and had reported outcome data. Informed consent of the patients and/or parents/legal guardians was obtained at the time of enrollment to ANBL00B1. Detailed description of the statistical analysis is provided in the supplemental information.

Statistical analysis of ATRX mutations and clinical features. The cohort used in this analysis consists of 477 neuroblastoma patients (of which 476 had clinical data and 475 had outcome data available) representing a mixture of risk levels, disease stages, and ages at diagnosis. All are enrolled on COG protocol ANBL1288 (“Analysis of ATRX Mutations in Neuroblastoma”) and were assayed for ATRX mutations. Roughly 80% of the patients were diagnosed between 2010 and 2012, with the remaining 20% diagnosed between 2001 and 2009.

The frequency of ATRX mutations, along with Coppers’ exact 95% confidence intervals, were calculated across the entire cohort and in subgroups formed by 12-year age categories (12 yrs at diagnosis) and INSS stage categories (1, 2 A/B or 3, 4, 5, and 4). Chi-square tests or Fisher’s exact tests, depending on sample size, were used to test for associations between ATRX mutations and clinical factors. EFS and OS were compared between patients with and without ATRX mutations, across the entire cohort and in subgroups based on stage, age, and risk. EFS and OS Cox models were used to assess the prognostic strength of ATRX mutations in the presence of standard clinical risk factors.

A forward-selection process was used to construct parsimonious Cox models. At the beginning of the forward-selection process, the following candidate variables were considered to be on equal footing: age at diagnosis (< 18 months vs. > 18 months), INSS stage (non-stage 4 vs. stage 4), sex, MYCN status (non-amplified vs. amplified), ALK mutation, ploidy (hyperdiploid vs. diploid), 1p (no LOH vs. LOH), INPC histology (favorable vs. unfavorable), ATRX mutation, mitosis–karyorhexis index (MKI, low/intermediate vs. high), and grade (totally undifferentiated/poorly differentiated vs. differentiating). Variables were entered into the model one at a time, with the variable chosen for entry being the one that is most significant at each step, per a Wald test. If at any difference in the process history was chosen to enter the model, then MKI, grade, and age at diagnosis were no longer considered for entry, as histology is confounded with these variables. Conversely, if at any point MKI, grade, or age at diagnosis entered the model, then histology was no longer considered for entry. The selection process ended when all remaining candidate variables failed to reach significance at the 0.05 level.

EFS time was measured as the number of days from diagnosis to date of relapse, disease progression, secondary malignancy, death, or, if no event occurred, date of last contact. OS time was measured as the number of days from diagnosis to date of death, or, if the patient did not die, date of last contact. OS time was assessed both by the neuroblastoma reference lab as part of ANBL00B1 enrollment and by St. Jude Children’s Research Hospital. There were some discrepancies between the two, particularly in one patient with an ATRX mutation (an aberration which previous research suggests is mutually exclusive from MYCN amplification), whom the six neuroblastoma reference lab determined to have MYCN amplification, whereas St. Jude detected no amplification. Owing to these discrepancies, both sets of MYCN amplification data were considered individually in this analysis.

Outcome was not compared in the <18 months old at diagnosis subgroup, as there were no patients with ATRX mutations in that subgroup. Nor was outcome compared in the 18 mo–5yrs old at diagnosis subgroup, as only one patient in that subgroup had an ATRX mutation.

The median follow-up time for patients who did not have an event was 3.9 years. The median follow-up time for patients who did not die was also 3.9 years. The forward-selection process substituting MYCN amplification data from Dr. Federico’s lab for those from the NBL reference lab yielded the same final EFS and OS Cox models. The effect of ATRX mutations on outcome was also tested among patients with INSS stage 4 disease who were at or above 5 years of age at the time of diagnosis. This subgroup was of interest because of its association with a chronic/indolent course of disease. A notable difference between this subgroup and the subgroups in which cases of concurrent ATRX and MYCN amplification were observed, was the number of patients with high-risk disease; the subgroup of older INSS stage 4 patients was entirely high-risk, whereas the other subgroups included a mixture of risk levels. This prompted us to perform a subgroup analysis based on risk group (high vs. low/intermediate). There were five low/intermediate risk patients with ATRX mutations, two of which experienced an event and one subsequently died. The difference in effect of ATRX mutations between high- and non-high-risk disease groups was also detected in the Cox model of EFS that included an interaction term for mutation and risk level.

Mutual exclusivity between ATRX mutations and MYCN amplification in neuroblastoma was done by merging the COG, TARGET, and PCGP data.
Custom capture for ATRX, MYCN and ARID1A/B. Targeted enrichment was performed using the Seqcap EZchoice Kit (Roche) according to vendor instructions for the Kapa workflow with 500 ng of genomic DNA as the starting input for library construction.

Exome sequencing. Whole-exome sequencing was conducted using the SeqCap EZ HGSC VCRome (Roche) according to manufacturers’ instructions.

MYCN FISH. Purified human NMYC BAC DNA (RP11-1183P10) was labeled with a red-dUTP (AF594, Molecular Probes) and human chromosome 2 control (2q11.2) BAC DNA (RP11-527J8) was labeled with a green-dUTP (AF488, Molecular Probes) both by nick translation. The paraffin slides were deparaffinized with xylene 2 × 10 min each at room temperature (RT), placed in ethyl alcohol 3 × 5 min each at RT, air-dried, placed in 10% buffered formalin for 1 h at RT. The slides were then placed in pepsin (8 mg/ml) in 0.1N HCL for 3 min, rinsed in dH2O 2 min each at RT, air-dried, placed in 10% buffered formalin for 1 h at RT. The slides in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. The probe and slide were co-denatured at 90 °C for 12 min and incubated overnight at 37 °C on a Thermobrite. In brief, washed in PN and then stained with 4,6-diamidino-2-phenylindole (DAPI) (1 µg/ml). Images were captured using a
Fig. 6 H3.3 deposition is altered at G4 sequences in ATRX-mutant neuroblastomas. a Stack bar plot of H3.3 peaks in the ATRX-deficient (CHLA90, SKNMM), MYCN amplified (SKNBE2, IMR32), and ATRX wild type, MYCN non-amplified (LAN6, SKNF1) cell lines. The H3.3 peaks that overlap with G4 sequences are shown in gray. b Stack bar plot of H3.3 peaks that are constitutive (C) across all three groups (ATRX, MYCN, WT), enriched (E) in ATRX mutant, depleted (D) in ATRX mutant, or overlap (O) between ATRX mutant and MYCN or WT. The H3.3 peaks that overlap with G4 sequences are shown in gray and the number of peaks for the D group are shown. c Piechart of the location of D group H3.3 peaks that have a correlation between the presence or absence of an H3.3 peak and ChromHMM state. Separate pie charts are shown for those that overlap with G4 sequences and those that lack G4 sequences. d Heatmap of the ChromHMM states used in this study with color coding. e Stack bar plot of the distribution of ChromHMM states for each cell line in the non-genic regions that have D group H3.3 peaks that correlate with ChromHMM state and overlap with G4 sequences or lack G4 sequences. g ChromHMM, WGBS, and ChiP-seq tracks for the DUSP26 gene in SKNMM (ATRX mutant) and SKNBE2 (MYCN amplified) cells. Gene expression is indicated (FPKM) and G4 motif sequences are shown below the ChromHMM tracks. h ChromHMM, WGBS, and ChiP-seq tracks for the SLCA2 gene in SKNMM:MYCN cells with and without doxycycline. Gene expression is indicated (FPKM) and G4 motif sequences are shown below the ChromHMM tracks. DOX doxycycline, ChromHMM chromatin hidden markov modeling, G4 guanine quadruplex structure.

| Table 2 Numbers of enrolled mice for neuroblastoma genetic mouse model survival study. |
|---------------------------------|----------------|----------------|----------------|----------------|
| Strain                          | Enrolled       | Did not reach the survival age | Censored       | Reported in the survival analysis |
| MYCN                           | 45             | 8                           | 1#             | 36                          |
| MYCN-ATRX                      | 59             | 2##                         | 15##           | 44                          |
| MYCN-ALK                       | 18             | 1                           | 0              | 17                          |
| MYCN-ALK-ATRX                  | 47             | 12                          | 8*             | 27                          |

*Found dead no tumors. **Hunched and lethargic no tumors, large spleen. *Malocclusion, four mice with fight wounds, paralysed hind limbs, found dead no tumors, large thymus (dysplasia).

Plat-Apochromat × 63 objective on a Zeiss Axios Imager.Z2 microscope and GenASIs scanner (ScanView software version 7.2.7).

ATRX IHC. ATRX immunohistochemistry was done on formalin-fixed 4-μm-thick paraffin sections using Leica Polymer Reine Detection kit (Leica Microsystems, Wetzlar, Germany) on a Leica Bond system. Epitope retrieval was done by heating ChromHMM tracks.

Genetic mouse models. To test the incompatibility between ATRX mutations and MYCN overexpression in LSL-MYC:Dbh-Cre mice, the mice express MYCN from the Rosa26 locus when a stop sequence is floxed by Cre recombinase under the control of Dbh promoter in sympathetic ganglion cells. We breed LSL-MYCN:Dbh-Cre mice to ATRXlox mice to inactivate AtrX in the same tissues. We also tested the consequences of AtrX inactivation when Th-ALK1174Ala is added18 (LSL-MYC: Dbh-Cre:Th-ALK1174D, Th-ALK1174Ala mice constitutively express ALK1174D, the most common activating ALK mutation found in human neuroblastoma, under the control of Th promoter).

Whole-genome sequencing. DNA from the tumors and matching germline samples was sequenced and analyzed as described previously54. In brief, 250–500 ng of genomic DNA was input for library construction using Illumina-compatible adapters, and four to six cycles of amplification were performed with KAPA HiFi Hotstart ReadyMix (KAPA Biosystems). The reference human genome assembly NCBII Build 37 was used for mapping samples. WGS’s mapping, coverage, and quality assessment, single-nucleotide variation (SNV)/indel detection, tier annotation for sequence mutations, and identification of LOH have been described previously55. Structural variations were analyzed and annotated using CREST18. Copy number variations were identified by evaluating the difference of read depth for each tumor and its matching normal using CONSENSING17. WGS data were uploaded at EMBL with accession number EGA00001002528.

CUT&RUN and analysis. We did automated CUT&RUN (AutoCUT&RUN) for H3.3 as described in detail https://www.protocols.io/view/autocut-run-genome-wide-profiling-of-chromatin-pro-ufetje. In brief, cells were bound to concanavalin A-coated magnetic beads (Bangs Laboratories), permethylated with digoxigenin and then incubated with primary antibody against H3.3 (Abnova, clone 2D7-H1), followed by incubation with rabbit anti-mouse IgG (Abcam, ab64580). Cells were arrayed on a 96-well plate and the rest of the reaction was carried out on a Beckman Biomek FX, including digestion with proteinA-MNase, ligation of adapters and library preparation. Size distributions of prepared libraries were assessed using an Agilent 4200 TapeStation. Libraries were pooled and at equimolar concentrations and 25 × 25 bp paired-end sequencing on an Illumina HiSeq 2500 was performed.

To find differential binding sites for H3.3, for each CUT&RUN sample, we called peaks twice by MACS2 (paired-end mode) that one using default FDR value cutoff 0.05 as high confidence peak set and the other using 0.5 as low confidence peak set. Then for each of the three groups, we compiled the reproducible peak set that required a high confidence peak also overlap a low confidence peak from the other samples within the sample group. Then we merged the reproducible peaks from three group to create a reference peak set and counted fragments (fragment size <2000 bp) overlap the reference peaks for each sample. At last, we performed statically tests using Voom58. To check their overlapping with G4 motifs and R-Loops, we downloaded G4 Motifs from supplementary data of ref. 59 and lifted over them from hg18 to hg19, we got 100,780 regions. We

Trypsin (10 mg/ml, Sigma Cat # T9935) filtered and then suspended in a Matrigel basement membrane matrix (BD Bioscience catalog number 354234) at a concentration of 2 × 105 cells per 10-μl and placed on ice for injection. The cells were injected in the para-adenal region of the mice with ultrasound guidance under anesthesia.

Histopathologic review of genetically engineered mouse models. Adrenals and paraverterbal sympathetic ganglia from Atrxlox:LSL-MYCN:Dbh-Cre mice at the age of three weeks and 1 year by hematoxylin and eosin (HE) staining and quality assessment, single-nucleotide variation (SNV)/indel detection, tier annotation for sequence mutations, and identification of LOH have been described previously55. Structural variations were analyzed and annotated using CREST18. Copy number variations were identified by evaluating the difference of read depth for each tumor and its matching normal using CONSENSING17. WGS data were uploaded at EMBL with accession number EGA00001002528.

Orthotopic patient-derived xenografts. Mouse studies were performed in a strict accordance with the recommendations in the Guide to Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at St. Jude Children’s Research Hospital. All efforts were made to minimize suffering. All mice were housed in accordance with approved IACUC protocols. Animals were housed on a 12–12 light cycle (light on 6:00, off 18:00) and provided food and water ad libitum.

Patient-derived xenograft cells were generated and maintained orthotopically in athymic nude (Jackson Laboratories, strain code 007850), NSG (Jackson Laboratories, strain code 005557) or C57BL/6 scid mice (Jackson Laboratories, strain code 001913) as described previously44. Tumor cells were dissociated using Trypsin (10 mg/ml, Sigma Cat # T9935) filtered and then suspended in a Matrigel basement membrane matrix (BD Bioscience catalog number 354234) at a concentration of 2 × 105 cells per 10-μl and placed on ice for injection. The cells were injected in the para-adenal region of the mice with ultrasound guidance under anesthesia.
downloaded 169,222 “RLFS in genic and proximal regions” from http://rloop.bii.a-star.edu.sg/ for hg19 as predicted R-Loops regions66.

Whole-genome bisulfite sequencing and analysis. Genomic DNA was extracted using the DNeasy Kit (QIAGEN #69504) according to the manufacturer’s protocol and quantified with a Nanodrop (ThermoScientific). Whole-genomic bisulfite conversion and sequencing were done by Hudson Alpha (Huntsville, AL).

Sequencing data were aligned to the hg19 human genome using BSMAP2.74. We first performed statistical test of differentially methylated locus (DML) between MYCN-amplified and non-MYCN-amplified samples using DMLTest function (smoothing = TRUE) in DSS (Wu et al. 2015), the results were then used to detect differentially methylated regions (DMRs) using CalDMR function in DSS, the p value threshold for calling DMR is 0.01. The minimum length for DMR is 50 bps and the minimum number of CpG sites for DMR is 3. The minimum methylation difference is 0.2. The DMRs that overlap with the promoters (1 kb flanking the TSS) of differentially expressed genes (at least twofold changes and adjusted p value ≤ 0.1) were deemed to be associated with gene expressions.

RNA-seq and analysis. RNA was extracted from cultured cells and tissues using RNeasy Kit (QIAGEN #74104) or TRizol (Life Technologies) preparations via a phenol–chloroform extraction or Direct-zol RNA MiniPrep (Zymo Research # R2052) following manufacturers’ protocols. RNA concentration was measured using a NanoDrop (Thermo Fisher scientific, Waltham, MA) and the quality of RNA was determined with a bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were prepared using the TrueSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA) from 500–ng total RNA. Pair-end deep sequencing was done with Illumina HiSeq 2500 sequencers (Illumina, San Diego, CA). FASTQ sequences were mapped to the human genome hg19 (GRCh37-lite) and Gene-based FPKM quantification was calculated using DESeq2 and adjusted p-value ≤ 0.05 (35).

For MYCN ChIP-Seq, we used rabbit polyclonal antibody (Active Motif Cat# 61185). This antibody was validated by small-scale and large-scale sequencing by active motif as well as by us in St. Jude Childhood Solid Tumor Network website (https://www.stjude.org/CSTN/)(30)

We applied the Hidden Markov Model trained for 18 states from previously published data to the data from this manuscript. Expanded chromHMM state 13 were defined as proportion of promoter downstream (TSS ~ +2 kb) in SKNNM54 - 50% to SKNNM57 in DOX condition (TSS + >20000 bp). The intervals were used for MYCN-binding site. Enrichr66 were used for pathway analysis.

ChIP-Seq analysis. ChIP-Seq analysis was done as described previously64. In brief, ChIP-Seq reads were aligned to human genome hg19 (GRCh37-lite) using BWA software (version 0.7.12-r1039, default parameter) and the duplicated reads were marked using Picard software. We kept only non-duplicated reads for the analysis using samtools (parameter “-f 1 -f 1024” version 1.2). The coverage of the pre-processed reads by downstream analysis were filtered using ENCODE data criteria. We calculated relative strand correlation value (RSC) and estimated the fragment size under support of R (version 2.14.0) with packages (tools’ version 1.17.7) and bitops (version 1.0-6). We required at least 10 million unique reads for peak source loci H3K4me2/3, H3K9ac14ac, H3K27Ac, Ctcf, RNA PolIII, 3′/5′ for RSC > 1, 20 million unique reads for broad markers (H3K4me1, H3K9me3, H3K27me3, H3K36me3) and 10 million unique mapped reads for inputs with RSC < 1. All samples were manually inspected and the SPP (version 1.1) was used to generate the cross-correlation plot. Then we generated a GO term list and three GO term lists for each condition were generated for the peak regions. GO term lists were generated using GOstat for pathway analysis.

Super-enhancer and core regulator circuit analysis. Super-enhancers and core regulator circuit analysis have been described as described previously64. We called auto-regulators by CRCMapper67 based on super-enhancers called by ROSE68. We provided two sets of results that one only used H3K27ac data, while the other one excluded H3K27ac peaks overlapping promoter (defined by H3K4me3 peaks) before calling super-enhancer by ROSE.

ChromHMM. ChromHMM models were generated for y as described before34. In brief, Non-duplicated aligned reads were extended by fragment size, and ChIP-Seq (version 1.10) with “-c colfids 0.1,2,5 -center” for BinarizeBed was used for chromatin state modeling. To choose the state number, we first modeled all samples together from seven states to 33 states and selected the model with 18 states upon manual inspection. For better visualization of the dynamics of HMM state across stages, we normalized color intensity by the maximum total percentage of state covering a gene and flanking region. We reduced the interval for an HMM state to half bar and the intensity to half the normalized amount if it did not rank in the top two HMM state for a gene. As HMM states could be assigned by multiple genes, the maximum total percentage across genes was used for normalization.

Immuno-blotting. The medium was removed and the cells were washed twice with PBS then the cells were scrapped in 1 ml PBS and centrifuged at 2000 × g for 5 min at 4 °C to pellet the cells. PBS was removed and the cells were flash-frozen in dry ice and stored at −80 °C until use. Cell pellets were 120 μg each and used for 15 min in radioimmunoprecipitation assay buffer (Sigma Cat. #R0278) supplemented with 1 × Halt protease inhibitor (Thermo Fisher, Cat. #7834) and 5 mM ethylene diaminetetraacetic acid (EDTA). Lysate were then centrifuged for 15 min at 10,000 rpm at 4 °C. Protein concentration in the cell lysates was determined with a bioanalyzer (Agilent Technologies, Santa Clara, CA). Membranes were washed three times in PBS with 0.1% Tween-20 at room temperature and one time in PBS. The membranes were then scanned by Odyssey ( LI-COR Biosciences, Lincoln, NE). Band intensities were quantified using ImageStudio Lite software (LI-COR Biosciences, Lincoln, NE).

Telemore FISH. After a 5-hour colcemid incubation, cells from both samples were harvested using routine cytogenetic methods. A commercially prepared directly fluorescent isothiocyanate-labeled PNA telome probe (Dako, Cat. # 5327) was used for this analysis. Dako protocols were followed for the pretreatment of the data before calling super-enhancer by ROSE.
Telomeres/cell, fluorescence/cell, and telomere/cell. A fixed exposure time is used for all cells.

**Telomere qPCR.** Telomere qPCR was done as described previously (Cheung et al.) DNA was extracted using DNAEasy Kit (Qiagen, Cat. #69504). qPCR was done on 10-ng DNA template using SYBR green Select master mix (Thermo Fisher, Cat. #4472908). Two sets of primer pairs were used in separate reactions were used to amplify the telomeric sequence and a common locus RPLP0, which was used as an internal control. The reactions were done in duplicates and the average delta C (t) was calculated for each sample by subtracting the average Ct (t) values of the telomeric reaction from those of the internal control reactions. The primers used were

Telomere F: 5'-CGGTTTGTGGTTGGTTGTTGTTGTTGTTGGTGTTGG-3'
Telomere R: 5'-GGCAGGCTACCTACCTACCTACCTACCTACCAA-3'
Control, locus: RPLP0 F: 5'-CAGCAAGTTGGAGGATGTAACCC-3'
RPLP0 R: 5'-CCATTCTCATCAACCGGTTCACA-3'

**Atrx shRNAs.** Eight ATRX-specific shRNA lentiviral vectors were purchased from Dhamacon as bacteriophage glycol vaccines (Cat. # V2LHS-092920 (#9), V2LHS-202920 (#20), V2LHS-22887 (#22), V2LHS-375686 (#37), V2THS-22887 (#87), V2THS-213229 (#30), V2THS-255090 (#13), and V2THS-2081895 (#95)). Lentiviruses were made in HEK293T cells by cotransfecting the viral vectors with three packaging plasmids. The supernatants containing the viral particles after 48 and 72 h were collected, filtered, concentrated using ultracentrifugation, and titrated in HeLa cells using the GFP or RFP reporters in the vector. In addition, two non-silencing RNA plasmid particles were also isolated (Cat. # 88434 and RHS3436). Cells were transduced with equal number of viral particles and transduced cells were selected with flow cytometry or puromycin after 48 h post transduction.

**Cell cycle analysis.** Cells were trypsinized and harvested, pelleted and suspended in 1 ml propidium iodide (0.05 mg/ml propidium iodide (Sigma, Cat. # P4864), 0.1% sodium citrate, 0.1% Triton X-100). The cells were then treated with 10-ml 0.2 mg/ml of ribonuclease A (Colbiochem, Cat. #556746) for 30 min at room temperature, filtered through 40-μm nylon mesh, and analyzed by flow cytometry for the DNA content.

**Colony assays.** Cells were transduced with a lentivirus expressing ATRX shRNA #9, ATRX shRNA #20, or scrambled control shRNA at multiplicity of infection 5 for 24 h. Then the cells were harvested, suspended into single cells in a pre-warmed medium, counted and seeded at 300 cells/well in six-well plates. The plates were shaken gently every 1–2 min for 10 min. Fresh medium was added every 4 days. After 2–3 weeks when colonies were visible, cells were washed, fixed in 1% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet.

**CRISPR/Cas-9 targeting ATRX and DAXX.** Guide RNAs (gRNAs) were designed targeting an early and conserved exon between isoforms of the hATRX and hDAXX genes. Spectral imaging was performed to identify gRNAs within at least 3 bp of mismatch to other sites in the human genome. gRNAs were further validated in human K562 cells for cutting efficiency using targeted deep sequencing. Two validated hATRX (g2 and g5) and one hDAXX (g11) gRNA were used in this study. The sequences of gRNAs are:
hATRX g2: 5’-CTGCACTGCTTTGGACAGACNGG-3’
hATRX g5: 5’-CAGCTAGGGCTCTAATGANGG-3’

Cells were transiently transfected with PC326-Cas-9 plasmid (to express Cas-9; Addgene # 43945), either Z/E-hATRX.g2 or Z/E-hATRX.g5 (to express hATRX gRNA; the backbone plasmid Addgene # 43860) and pUB-GFP (to express GFP; Addgene # 11155) in a ratio of 7:7:1, respectively using Mirus 2020 (Mirus Biotechnology Cat. # 2020). After 48 h, the cells were sorted for GFP-positive cells. Half of the sorted cells were pelleted immediately and stored at ~80°C and the remaining cells were seeded in six-well plates and harvested at different time points. For targeting efficiency after CRISPR-particle targeting in IMR92 cells, we transplanted the cells with the same mix of plasmids but added a control plasmid expressing gRNA targeting the hAAV5 locus (Addgene # 41818) in a ratio of 4:4:6:1 for hAAV5-gRNA: ATRX gRNA: Cas-9: gRNA expressing plasmids, respectively. DNA was extracted from the cell pellets using DNAeasy kit (Qiagen, Cat. # 69504) and PCR was done to amplify the targeted region using primers to which MiSeq adapters were annexed and the gel-purified PCR products were subjected to deep sequencing.

The primers used are (adapter sequences are underlines)

hATRX F: TGGTGGAGCTCGATGAAAGGCAGGTGAAATTT
hATRX R: GTTCGTCCTGGGCTTTGATGAAGTGAAGGGTTTCT

hAAV5 F: TCATGGACGTCATGGAAGAGAGCCACCTGATGATGTGTTGCTC
hAAV5 R: GTTCGTCCTGGGCTTTGATGAAGTGAAGGGTTTCT

The percentage of indels was determined using CRISPResso software as described before.

**Comet assay.** Alkaline comet assay was done using CometAssay Reagent Kit (Trevigen, Cat. # 4252-40-K) following the manufacturer’s protocol. In brief, cells were harvested and suspended in PBS buffer at a concentration of 1 x 10⁵ cells/ml. Cells were combined with LMAgarose in a ratio 1:10 at 37 °C and 50-μl of the mix was spread over the CometSlide. The doxycycline-treated and untreated samples were loaded onto two different wells on the same slide. Slides were left at 4 °C for 30 min in the dark to allow the agarose to polymerize. The slides were then incubated in a freshly made Alkaline Unwinding Solution (200 mM NaOH, 1 mM EDTA, pH > 13) overnight at 4 °C in the dark. Electrophoresis was done for 4 °C at 30 V for 1 h in the Alkaline Electrophoresis Solution (200 mM NaOH, 1 mM EDTA, pH > 13). The slides were washed twice in deionized H₂O, dried at 37 °C and stored in the dark. To analyze the samples, nuclei suspended in the agarose gel were stained by 100-μl of SYBR Gold (Thermo Fisher, Cat. # 11494) diluted 1:30,000 in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Then the slides were rinsed in water and dried completely at 37 °C. Slides were examined by a fluorescence microscope for excitation/ emission: 490/522 and 10-15 images were taken per sample. Percentage of DNA in the tail was quantified in the images using ImageJ software by measuring fluorescent intensity in the tail/totall fluorescence × 100. Mann–Whitney non-parametric statistical test was used to compare the scores in the samples with or without doxycycline.

**Spectral karyotyping (SKY) analysis.** Cells were treated with colcemid for 5 h at 37 °C then the cells were harvested for cytogenetic analysis. SKY analysis was done using a commercial spectrally calibrated probe from spectral imaging (Carlbad, CA) following the manufacturer’s protocol. Fifty metaphase cells from every sample were scored for the presence of DNA fragmentations. Statistical analysis was done using Fisher exact test.

**Hydroxyurea sensitivity.** The cells were seeded in 96-well plates at a density of 2500 cells per well overnight. Next day (day 0), the medium was changed with a fresh medium with or without 2 μg/ml doxycycline. After 24 h of doxycycline treatment (day 1), the medium was changed again with fresh medium containing serially diluted hydroxyurea with or without doxycycline. At day 4, the cell viability was assessed using the CellTitre-Glo cell viability assay kit (Promega, Cat. G7572) using PHERAstar FSX (BMG Labtech, San Diego, California).

**Inducible MYCN.** To make the doxycycline-inducible cell lines, we used plenti4/TO/V5 DEST system (Invitrogen, Cat. # V480-20) following the manufacturer’s protocol. In brief, the cells were transfected with the plenti4/TO lentivirus to constitutively express the Tet repressor and the cells were selected with 10-μg/ml blasticidin-HCL (Thermo Fisher, R21001) in the medium. The cells were then transduced with the V5 DEST-MYC or V5 DEST-control lentivirus and were selected with 10-μg/ml zeocin (Thermo Fisher, R25001) in the medium. The V5 DEST-MYC virus was a gift from the Freeman lab at St. Jude Children’s Research Hospital. The cells were maintained in a selective medium containing 5-μg/ml blasticidin-HCL and 5-μg/ml zeocin. We also cloned MYCN into another dox-inducible system using Lent-X Tet-One (Takara Cat# 631847) following manufacturer protocol. We confirmed the sequence of the construct by sanger sequencing. Cells were transduced with Lenti-X Tet-One lentiviruses containing MYCN or with the empty vector. Selection for the cells containing the construct was done using puromycin and the cells were maintained in puromycin-containing media. Induction of MYCN expression was done by adding doxycycline to the medium to a final concentration of 1–2 μg/ml.

**Growth curve.** The cells were plated in six-well plates at a density of 50,000 cells per well over night. Then the medium over the cells was changed with a fresh medium with or without 1–2 μg/ml doxycycline. Three wells in each line were harvested every day and counted for the total number of cells per well using a hemocytometer.

**Live imaging.** Live cell time-lapse imaging experiments for SKNNM-MYCN in the presence or absence of doxycycline were performed using a Nikon C2 confocal configured on a TE2000E2 microscope with a 20 x 0.8 NA Plan Apo lens (Nikon Instruments Inc., Melville, NY). A 640 nm DPSS lasers was used to simultaneously excite Annexin V-Alexa Fluor 647 and generate the DIC images. During imaging experiments, the cells were maintained at 37 °C and 5% CO₂, and images were collected every 30 min for 67 h. Images were acquired and processed using NIS Elements software.

**In vivo mixing experiment.** SKNNM-MYCN and SKNNM-CONT cells were labeled with luciferase-YFP (yellow fluorescent protein) using a lentivirus. Two mixed cell
pools were prepared in a 1:1 ratio: labeled SKNMMMYCN cells/unlabeled SKNMMCONT and labeled SKNMMCONT /unlabeled SKNMMMYCN. Cell mixes were injected in the flank of each region of the mice (four million cells/mouse) with ultrasound guidance under anesthesia as explained before. In this experiment, we used NOD.CB17-Prkdcscid/J female mice (Jackson laboratories, Stock # 001303). The mice were followed up for the tumor formation using ultrasound scanning every 2 weeks, xenogen signal every week and palpation every week. Mice were enrolled in the study when the tumor was at least 1 mm² in size using the ultrasound scanning. Enrolled mice were randomly assigned to receive either doxycycline (2 mg/ml) in the drinking water or continue on regular water.

The labeled/unlabeled cell mixes were also maintained in culture and the ratio of YFP-expressing cells in each cell mix was determined weekly using flow cytometric analysis.

**Lactate measurements.** Lactic acid was measured using Lactate Assay Kit (Sigma Cat. # MAK064) following the manufacturer’s protocol. The absorbance at 570 nm (A570) was measured for a colorimetric detection of the lactate using the microplate reader SpectraMax-M5 (Molecular Devices, Sunnyvale, California).

**GC-MS-mediated analysis of 13C-labeled TCA metabolites.** The analytical procedure applied was based on the one previously published28. In brief, cells were cultured in glutamine-free medium (10% fetal bovine serum (FBS), penicillin/streptomycin) supplemented with 4 mM of standard glutamine or [U-13C]glutamine (682-H-CLM-1822-H; Cambridge Isotope Laboratories, Inc. for 5 h. Next, cells were trypsinized, washed with ice-cold saline solution, snap-frozen, and stored in liquid nitrogen for further processing. Extraction of hydrophilic metabolites was performed using two volumes of methanol/water/solution (2:1 v/v, high-performance liquid chromatography (HPLC)-grade, spiked with 5 nM of norvaline as an internal standard), homogenization and mixing with one volume of chloroform (HPLC-grade). Derivationization with 2% methoxylamine-HCl solution and GC-MS-grade MTBSTFA + 1% t-BDMCS reagent was performed as previously described. Samples were analyzed in split-less mode using Agilent 7890B GC system equipped with a 5977B MSD, Agilent 5975ms, + 1 mm EZ, 60 m, 0.25, 0.25 μm column, helium carrier gas and Mass Hunter software package. Chromatographic gradient conditions: separation time 36 min; start at 80 °C, 1 min; ramp to 250 °C at 70°C/min; ramp to 300 °C at 50 °C/min and hold for 9 min. MSD settings: source 150 °C; quadrupole 150 °C; interface 300 °C; injector 250 °C, El source 70 eV, El pressure 1.8 x 10⁻⁵ torr. Signals were acquired using SIM mode conditions as described before (full scan range 50 m/z at ~ 2 scans/1000 m/z; MTBSTFA/s/for< s (N = 3)). Enrichment of 13C-labeled isotopomers of analyzed compounds was corrected by natural abundance using “standard glutamine” medium-incubated sample as reference.

**Detecting metabolites using CE-TOFMS.** MYCN-induced and un-induced SKNMMTYC cells were grown on either glutamine-free or glucose free completed medium-incubated sample as reference.

**TEM analysis of mitochondria.** The samples were fixed with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4, and post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer with 0.3% potassium ferricyanide at 29 K with some portion of the nucleus visible, printed and all mitochondrion profiles were scored blindly. Mitochondria were fixed in 2% formaldehyde in 0.1 M sodium cacodylate buffer pH 7.4, and post fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer with 0.3% potassium ferricyanide at 29 K with some portion of the nucleus visible, printed and all mitochondrion profiles were scored blindly. Mitochondria were scored 0 = normal (aligned cristri and normal outer membrane), 1 = abnormal (swollen and disorganized cristri or disrupted outer membrane) or 2 = very abnormal (both swollen and disorganized cristri and severe outer membrane disruption).

**Gamm H2AX immunostaining.** Cells were harvested and incubated on poly-L-lysine-treated (Sigma, Cat. # R2920) slides for 30 min at 37 °C. The cells were fixed in 4% paraformaldehyde in FBS at 4 °C overnight, blocked by normal donkey serum for 1 h at room temperature and incubated with mouse anti Phospho Ser139-histone H2AX antibody (Millipore, Cat. # 5–636) 1:10,000 overnight at 4 °C. The slides were then washed three times in PBS and incubated with donkey anti-mouse Cy3 IgG antibodies (Millipore, Cat. # AP192C) 1:500 for 1 h at room temperature and incubated with mouse anti Phospho Ser139-histone H2AX antibody (Millipore, Cat. # 5–636) 1:10,000 overnight at 4 °C. The slides were washed three times in PBS, counter-stained with DAPI for 10 min, washed three times in PBS and mounted with Prolong Gold Antifade Mountant (Thermo Fischer, Cat. # P36930).

**Measuring ROS.** ROS was assayed using 2′,7′-dichlorofluorescin diacetate (DCFDA) cellular ROS detection assay kit (Abcam # ab138531) or CellRox Green (ThermoFischer # C10444) following manufacturers’ protocol. For DCFDA cellular ROS assay, cells were plated overnight in 96-well plates (25,000 cells per well) in their complete medium with or without doxycycline. The fluorescence excitation/emission 485/535 was measured using the microplate reader.
SpectraMax-M5 (Molecular Devices, Sunnyvale, California). For CellRox Green, cells grow in 10-cm plates. CellRox Green was added to the medium 1:500 and incubated for 30 min then cells were harvested and analyzed using flow cytometry.

**Measuring cellular glutathione.** The cells were plated in 96-well plates overnight at a density of 6000 cells per well in their complete medium with or without doxycycline. Glutathione concentration per well was measured using GSH-Glo Glutathione Assay Kit (Promega # V9611) according to the manufacturer’s protocol. The luminescence was measured using PHERAstar FSX (BMG Labtech, San Diego, California).

**Drug screening.** Cell screening was done on MYCN-induced and un-induced SKMMyCN cells as previously described with the following modifications: to determine optimal cell plating density, SKMMyCN cells were plated on a flat-bottomed, white 96-well plate (Corning Cat#3917), at eight different cell densities ranging from 10 cells/well to 10,000 cells/well; 12 wells per cell intensity per plate. Twenty-four hours after plating, medium was changed with or without doxycycline. Medium was changed after that with or without doxycycline every 2 or 4 days. One plate from every testing condition was read every day for 11 days using CellTiter-Glo (CTG, Promega Cat#G7573) on PHERAstar FSX (BMG Labtech, San Diego, California). Cell density of 1250 cells per well and medium change once every 4 days was determined to have good signal to noise separation while maintaining logarithmic growth in both induced and un-induced cells. We tested different dimethyl sulfoxide (DMSO) concentrations for the selected cell density and confirmed minimal cell death with up to 0.197% DMSO. Positive control compound selection was performed with eight positive control compounds and candidates (doxorubicin HCl, staurosporine, etoposide, SN-38, bortezomib, cyclohexamide, panobinostat, and TAKA123) arrayed in single-point concentration and 1:3 dilution series. Based on successful cell killing Staurosporine was chosen as positive control for cell screenings. Assay validation in 384-well plates was performed with staurosporine.

**Dose-response curve experiment.** A schematic representation of the dose–response curve experiment is illustrated (Supplementary Data 14). Cells were plated in 96-well plates (Corning Cat#3917) at 1250 cells/100 μL; 12 cells per compound plate. Approximately 24 h following plating, medium was changed with fresh medium with or without doxycycline; six-cell plates per a compound plate each. Two days after MYCN induction, medium was changed again with fresh medium with or without doxycycline and cells were drugged with the compound plates and a positive control plates using a Biomek FX (Beckman Coulter) liquid handler equipped with a pin tool. The pin tool transferred 112 nL compound stock, resulting in 890-fold compound dilution. After 3 days of drugging, half of the plates were read. On day 4 post drugging, medium was changed with fresh medium again with or without doxycycline and fresh drugs were added to the medium using the Biomek FX. After 4 days of the second drugging, the other half of the plates were read. The compound plate contained compounds dissolved in DMSO arrayed in a 1:3-dilution series in columns 1–10 with eight compounds per plate (target concentration 10 mM or 1 mM). The positive control plate was empty from column 1–10; in columns 11–12, it contained DMSO and the positive control. Two biological replicates were performed for each 8 days experiment, with three technical replicates per biological replicate.

**Drug screening analysis.** Normalization, fitting, and visualization of dose–response experiments were performed using custom code written in the R programming language (version 3.3.2; R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/). Dose–response curve data analysis for cytotoxical compounds was done as previously described before. Raw CellTiter-Glo (CTG) luminescence signals (RLU) were first log2 transformed, then normalized to the mean of the positive and negative controls on each plate.

In order to detect drugs that reduced MYCN-induced cell death, traditional dose–response analysis was not appropriate because protective drugs would often protect at low concentrations, but eventually induce cytotoxicity at high concentrations. To detect a protective effect, we needed to quantify the increase in CTG signal at low concentration before any decrease in signal at high concentration. To do so, the CTG RLU from all wells were first log2 transformed for each plate, the value for each well was then normalized by subtracting out the mean log2 RLU for negative control wells. The normalized activity of each compound was then fit using the function of the log10 drug concentration using a smooth spline (smooth.spline function in R with default parameters). For each fit, the area under the curve (AUC) for the portion of the spline with RLU above the RLU of the lowest drug concentration tested was calculated. Higher values for this AUC metric indicate greater protection against loss of cell viability as measured by the CTG assay.

**CX2 expression in SKMMyCN cells.** SKMMyCN cells were transduced by a lentiviral vector (pLenti-C-mGF-P2A-Puro) from Origene expressing either CX2 (Cat# RC22263614V) or GFP control construct (Cat# PS100093V). GFP-positive cells were sorted after 48 h and maintained in a selective media containing 1.5-μg puromycin per mL.

**Statistics and reproducibility.** We have made every effort to ensure reproducibility of our data by, when possible, repeating the experiments using independent samples, including positive and negative controls and using multiple approaches to confirm our observations. We stated the sample number for each experiment and how many times each experiment was repeated in the figure legends. However, the following experiments were done once:

Fig. 1f–i This was a unique case in which there was a discrepancy in COG FISH report and next-generation sequencing report. This sample was carefully studied as described in the results section.

Fig. 3b This is the first and only O-PDX developed from ATRX-mutant neuroblastoma, as explained in the text.

Fig. 5h This experiment was done to confirm the high-throughput screening and was done in a dose response.

Fig. 5i This experiment was done once. However, the ability of RA to reduce MYCN level is well-established in literature.

Supplementary Fig. 1B–1SE: Was done to all the clinical samples as mentioned in the material and methods.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Whole-genome bisulfite sequencing data and ChIP-seq data have been deposited in the EMBL-EBI database under the accession code EGAS00001002575. Whole-exome sequencing data have been deposited in the EMBL-EBI database under the accession code EGAS00001002528. All of our extensive epigenetic data and analysis are freely available in a cloud-based viewer (https://pecan.st Jude.cloud/proteinpaint/study/mycn_nbl_2018). All O-PDX tumors described here are freely available with no obligation to collaborate through the Childhood Solid Tumor Network (http://www.stjudetumorstudy.org). We downloaded G4 Motifs from supplementary data of Du et al. 2009. We downloaded 169,222 R-Loop domains in genomic and proximal regions from http://loop.bi.a-star.edu.sg. All the other data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author upon reasonable request.

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Author contributions
M.Z., S.F., X.C., and M.A.D. conceived and designed the experiments and analysis. M.Z., E.S., J.J., L.G., R.N., J.N., J.E., H.M., D.Y., Y.L., M.M.K., M.Y., S.M.P.-M., M.R.C., A.R., P.V., S.L., A.S., J.F.S., and M.P.M. collected the data. M.Z., Y.F., B.X., X.C., S.F., J.W., C.V.R., A.N., and M.A.D. performed analysis. M.D.H., R.E.G., and S.M.P.-M. provided samples and reagents. M.Z., L.G., R.N., J.N., J.E., H.M., D.Y., Y.L., M.M.K., M.Y., S.M.P.-M., M.R.C., A.R., P.V., S.L., A.S., J.F.S., and M.P.M. collected the data. M.Z., Y.F., B.X., X.C., S.F., J.W., C.V.R., A.N., and M.A.D. performed analysis. M.D.H., R.E.G., and S.M.P.-M. provided samples and reagents. S.F., A.P., J.Z., E.R.M., R.K.W., S.H., J.R.D., and M.A.D. provided project leadership and guidance. X.Z. developed the visualization tools and web portal. M.Z., S.F., X.C., A.P., and M.A.D. wrote the paper.

Competing interests
The authors declare no competing interests.

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