Restoration of the molecular clock is tumor suppressive in neuroblastoma

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MYCN activation is a hallmark of advanced neuroblastoma (NB) and a known master regulator of metabolic reprogramming, favoring NB adaptation to its microenvironment. We found that the expression of the main regulators of the molecular clock loops is profoundly disrupted in MYCN-amplified NB patients, and this disruption independently predicts poor clinical outcome. MYCN induces the expression of clock repressors and downregulates the one of clock activators by directly binding to their promoters. Ultimately, MYCN attenuates the molecular clock by suppressing BMAL1 expression and oscillation, thereby promoting cell survival. Reestablishment of the activity of the clock activator RORα via its genetic overexpression and its stimulation through the agonist SR1078, restores BMAL1 expression and oscillation, effectively blocks MYCN-mediated tumor growth and de novo lipogenesis, and sensitizes NB tumors to conventional chemotherapy. In conclusion, reactivation of RORα could serve as a therapeutic strategy for MYCN-amplified NBs by blocking the dysregulation of molecular clock and cell metabolism mediated by MYCN.
ncogenic expression of the MYC family members (e.g., MYC and MYCN) drives many human cancers; however, strategies aiming at chemically disrupting their functions have proven difficult to develop. The MYCN oncogene is amplified in almost half of all high-risk neuroblastomas (NBs) and is the primary oncogene driving this malignancy. Overexpressed MYCN causes spontaneous, high-penetration NB in mice, originating from neuroblast precursor cells. In tumors with activated MYC or MYCN, the high demand for growth and biomass accumulation required for tumor progression is achieved by metabolic reprogramming. MYC drives several metabolic pathways associated with cell growth, including increased glucose and glutamine uptake, stimulation of mitochondrial biogenesis and nucleotide synthesis, and enhanced de novo lipid synthesis. In particular, MYC directly activates the expression of lipogenic enzymes (e.g., ACACA, FASN, and SCD1), promoting de novo fatty acid (FA) synthesis from citrate. In addition, MYC interacts with the related transcriptional regulator MondoA to control SREBP1-dependent lipid biosynthesis. However, far less is known about MYCN regulation of cell metabolism. MYCN loss of function suppresses FA β-oxidation and leads to lipid droplet accumulation in NB cells, suggesting that aggressive NBs require FA as an energy source.

Cellular metabolism and circadian rhythm intimately cross-talk, and MYC regulates rhythmic cell metabolism. Moreover, genetic ablation of clock genes enhances glucose and glutamine consumption in vivo. The core molecular clock is composed of the heterodimer CLOCK/BMAL1, which activates transcription of the PER and CRY genes via binding at specific E-box sequences. In turn, PER1/2 and CRY1/2 suppress CLOCK/BMAL1. This transcriptional feedback loop ensures a 24-h transcription of the PER and CRY genes via binding at specific E-box sequences. Notably, the expression of both RORα and BMAL1 is strongly repressed in MYCN-amplified (MNA) NB (cohort 1, Fig. 1; cohort 2, Supplementary Fig. 1a), and this correlates with poor overall (OS) and progression-free survival (PFS) in all patient cohorts (Fig. 1 and Supplementary Fig. 1). This association is retained when the analysis is restricted to stage 3 and 4 patients (Supplementary Fig. 1b, c). Conversely, REV-ERBα expression is significantly higher in MNA NB (cohort 1, Fig. 1; cohort 2, Supplementary Fig. 1a). Notably, we found that RORα expression predicts outcome independently of current prognostic factors, including age at diagnosis, International Neuroblastoma Staging System (INSS) stage, and MYCN status (Supplementary Table 1). Collectively, our data define the low expression of the positive arm of the clock (RORA-BMAL1) as an unfavorable prognostic marker in NB.

MYCN disrupts the molecular clock by direct binding to clock gene promoters. Due to the significant association between circadian genes and MYCN expression in patients, we next examined whether MYCN could alter clock gene expression. To test this hypothesis, we conditionally overexpressed MYCN in MYCN3 (Tet-ON) cells and conditionally silenced MYCN in LAN5 MNA cells (LAN5 ShMYCN). Ectopic MYCN expression resulted in significant upregulation of the circadian repressor REV-ERBα, and downregulation of the circadian activators RORA and BMAL1. However, depletion of MYCN completely restored their expression levels (Fig. 2a). We then used a human NB line SK-N-AS that lacks amplification of MYCN and stably expresses their expression levels (Fig. 2a). We then used a human NB line SK-N-AS that lacks amplification of MYCN and stably expresses inducible wild-type MYCN-ERTM (estrogen receptor tamoxifen mutant). When stimulated with 4-hydroxytamoxifen (4OHT), MYCN-ER translocates into the nucleus and upregulates MYCN targets. MYCN transcriptional activation led to significant upregulation of REV-ERBα and downregulation of RORα and BMAL1 both at mRNA and protein levels (Fig. 2b). As a control, we demonstrated that both doxycycline (DOX) and 4OHT do not alter clock gene expression (DBP, BMAL1, and PER2) in parental
LAN5 and SK-N-AS cells (Supplementary Fig. 2). Collectively, these data support a role for MYCN in altering circadian gene expression, and specifically suppressing the positive arm of the clock.

We then asked whether MYCN could also disrupt circadian rhythmicity. We subjected DOX-induced MYCN3 and LAN5 ShMYCN cells to real-time luminescence analysis using a previously established Per2-luc adenovirus reporter32 and found that MYCN induction also impairs the circadian oscillation of PER2 by both lengthening the circadian period and reducing the circadian amplitude (Supplementary Fig. 3). These results are consistent with previously reported impaired BMAL1 oscillation in MYC overexpressing cell lines14.

To address the mechanism of MYCN regulation of the clock, we performed MYCN chromatin immunoprecipitation (ChIP)-qPCR analysis in both MNA cells and MYCN Tet-OFF (Tet-21/N) cells, in which MYCN is turned off upon DOX treatment (Fig. 2c and Supplementary Fig. 4). We found significant enrichment of MYCN binding to the promoter regions of REV-ERBa, RORα (transcript variants 1 and 4), and BMAL1 in both MNA cell lines. Moreover, turning off MYCN fully abrogates MYCN binding (Fig. 2c). These findings suggest that MYCN directly attenuates the clock by repressing the clock activators RORα and BMAL1, and inducing the clock repressor REV-ERBa. Because MYCN induces transcriptional repression by indirectly binding to DNA in part through interactions with MIZ133, we performed MYCN ChIP-qPCR analysis in LAN5 cells upon genetic depletion of MIZ1 (LAN5 ShMIZ1) to determine whether loss of MIZ1 altered MYCN occupancy at clock activators genes. Effective knockdown of MIZ1 (Supplementary Fig. 5a, b) significantly reduces MYCN binding at the promoter regions of BMAL1 and RORα (Supplementary Fig. 5c, d), suggesting that MYCN repression of the clock requires MIZ1.

RORα activity rescues MYCN-mediated repression of BMAL1 expression and oscillation. Synthetic ligands for RORs have been recently characterized, including the RORα/γ agonist SR107834. Unlike most family members, the RORs recognize and bind as monomers to specific sequences of DNA (ROR response elements, RORE) in the regulatory region of the target genes. When bound to these elements, they constitutively recruit co-activators resulting in continual transcriptional activation35. To test whether RORα signaling is active in NB cells, we used two approaches:

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Fig. 1 Clock gene expression is altered in primary NB tumor samples and correlates with poor clinical outcomes. Kaplan-Meier analysis of overall survival (OS) in patient cohort 1 (Kocak n = 476, GEO: GSE45547). Graphs depict p-values corrected for multiple testing (Bonferroni correction) of cutoff levels for RORα, BMAL1, and REV-ERBa. Correlations between clock gene expression, INSS stages, and MYCN amplification status are shown. MNA = MYCN-amplified; S1–4S = stage1-4S; 476 of the 649 samples were annotated with survival data. The box plot is defined by two lines at the 25th and 75th percentile. A line is drawn inside the box at the 50th percentile.
RORα pharmacological activation (via the agonist SR1078) and RORα genetic overexpression. Two MNA cell lines (LAN5 and SK-N-BE(2)-C) were treated with SR1078 and the mRNA expression of RORα target genes containing a RORE in their promoters (G6Pase, FGF-21, and BMAL1) was determined. SR1078 effectively activates target gene transcription in both cell lines (Fig. 3a). We next examined whether genetic overexpression of RORα could activate downstream signaling as well. To ectopically express RORα, we inserted a lentiviral DOX-inducible vector containing the sequence of RORα (isoform 1) into MNA LAN5 cells because these cells showed relatively low RORα expression compared to other cell lines tested. Conditional RORα overexpression resulted in robust transcriptional activation of its target genes (Fig. 3a). Furthermore, SR1078 and the genetic
overexpression of RORα efficiently induced BMAL1 protein expression in both systems (Fig. 3b).

We then asked whether SR1078 and RORα overexpression could rescue MYCN-mediated repression of BMAL1 expression and oscillation. For this purpose, we employed two inducible MYCN cell systems: MYCN3 (Tet-ON) and SK-N-AS MYCN-ER cells. As expected, all known and identified MYCN-target genes (ODC1, REV-ERBa, DKK1, RORA, and BMAL1) were significantly induced or repressed upon turning on MYCN. Notably, BMAL1 mRNA and protein levels were repressed in both systems upon MYCN activation. However, SR1078 treatment and RORα genetic overexpression were both able to completely rescue BMAL1 mRNA and protein levels (Fig. 3c). Moreover, active MYCN-ER (MYCN-ON) greatly impaired circadian oscillation of BMAL1 in SK-N-AS MYCN-ER cells. However, overexpression of RORα was capable of both restoring BMAL1 expression, and also fully rescuing its circadian oscillation (Fig. 3d). Similarly, turning ON RORα highly enhanced BMAL1 oscillation in MNA cells (Supplementary Fig. 6). Collectively, these data indicate that RORα activity effectively counteracts MYCN-mediated repression of BMAL1 expression and oscillation.

MYC and other cell cycle regulators have been shown to be clock-regulated35, suggesting that multiple feedback loops exist between MYC and the components of the molecular clock. Thus, we asked whether restoration of the clock could alter c-MYC and MYCN expression. MYCN protein stabilization was notably decreased by ROR agonist SR1078 in MNA cells (LAN5 and SK-N-AS BE2[C]); however, no changes in c-MYC protein levels were detected in non-MNA c-MYC overexpressing cells (SH-SY5Y) (Supplementary Fig. 7), suggesting that this clock-mediated regulation is MYCN-dependent.

Restoration of BMAL1 via RORα activation inhibits cell growth and lipogenic gene expression. Because MYCN repression of the clock correlates with poor clinical outcomes, we then asked whether restoration of the clock via activation of RORα could have a functional significance and alter NB cell phenotype. SR1078 inhibits cell growth in both MNA and non-MNA cell lines. However, this effect is greater in MNA compared to non-MNA cells. Similarly, MNA cells show higher caspase-mediated apoptosis following SR1078 treatment (Fig. 4a). Genetic overexpression of RORα (RORα Tet-ON) also profoundly restricts cell growth and induces cell cycle arrest (Fig. 4b and Supplementary Fig. 8). However, depletion of BMAL1 (Supplementary Fig. 9) promotes cell growth and almost completely rescues the block in cell viability and the apoptosis induced both by SR1078 and RORα overexpression (Fig. 4c), suggesting that the anti-tumor activity of RORα is dependent on BMAL1. Because cell viability assays such as MTT and CCK-8 sense metabolic processes, we used cell counting as an alternative method and confirmed the same changes in cell proliferation (Supplementary Fig. 10). In addition, because SR1078 can stabilize p5334, we tested whether this also occurs in NB. We detected no changes in p53 protein levels upon SR1078 treatment in multiple MNA lines. Moreover, genetic depletion of p53 did not affect the response to SR1078 (Supplementary Fig. 11), suggesting that SR1078 alters cell viability in a p53-independent manner.

To further elucidate the molecular mechanisms by which SR1078 impairs NB cell survival, we performed RNA-sequencing analysis in LAN5 cells with and without SR1078 treatment for 8 h. A total of 712 genes were differentially regulated (p < 0.05): 459 downregulated and 253 upregulated (Fig. 4d). Reactome pathway enrichment analysis showed that the most downregulated biological processes were cholesterol biosynthesis and regulation by SREBP (adjusted p = 2.44E−09), metabolism of lipids and lipoproteins (adjusted p = 1.01E−03), and tubulin folding (adjusted p = 1.39E−03), while amino acid transporters (adjusted p = 9.13E−04) were the most upregulated (Fig. 4d and Supplementary Data 1), suggesting that restoration of the clock alters cell metabolism. Overall, 34 out of the 459 SR1078-downregulated genes belonged to the top enriched lipid pathways (cholesterol biosynthesis and regulation by SREBP, and metabolism of lipids and lipoproteins, in blue, Supplementary Fig. 12a). We further selected ten genes specifically involved in cholesterol synthesis (IDL-1, HMGR, HMGCS1, and MVK2) and FA metabolism (FABP3, FABP5, ELOVL2, ELOVL6, ACSL3, and SCD1) and validated their expression after SR1078 treatment by q-PCR in MNA cells. Both these gene sets were significantly downregulated by SR1078 in LAN5 cells (Supplementary Fig. 12b). Cell metabolism is tightly controlled by the molecular clock36 as both metabolites and metabolic gene expression are subject to circadian oscillations39. Altogether, these data indicate that activation of RORα inhibits NB cell survival by restoring BMAL1 expression and constraining lipogenic gene expression.

Activation of RORα restores BMAL1 and blocks NB tumor growth by inhibiting lipid metabolism. Our patient and in vitro data both suggest that clock activators RORα and BMAL1 are downregulated in MNA NB. These data also suggest that activation of RORα restricts cell survival. We, therefore, examined the in vivo anti-tumor activity of restoring RORα function via SR1078 and genetic overexpression. For this purpose, we used an orthotopic NB mouse model and generated MNA xenografts by implanting MNA cells under the renal capsule of nude mice. This model faithfully recapitulates the aggressive and highly vascular phenotype of primary NB40. LAN5 cells were engrafted and SR1078 treatment was initiated 2 weeks after implantation. Vehicle control and SR1078 (15 mg/kg) were given i.p. daily for 14 days, and the effect on tumor growth was compared between groups at the end of the treatment. SR1078 treatment resulted in significant inhibition of tumor growth (p = 0.02). The ability of SR1078 to block tumor growth was confirmed in a second MNA xenograft model, derived from MNA IMR32 cells (p = 0.0004). Notably, SR1078 anti-tumor activity was associated with the...
restoration of BMAL1 protein levels in the xenograft tumors (Fig. 5a). However, SR1078 was not able to block tumor growth in a non-MNA xenograft model of SK-N-AS cells ($p = 0.5116$) (Fig. 5b), suggesting that the tumor-suppressive function of ROR$\alpha$ activation is MYCN-dependent. The general health conditions of the mice and their body weights were recorded weekly throughout the studies and no apparent signs of toxicity related to SR1078 treatment were noted (Supplementary Fig. 13). We then engrafted LANS ROR$\alpha$ Tet-ON cells and evaluated their ability to grow in nude mice with and without DOX treatment to induce ROR$\alpha$ overexpression. Overexpression of ROR$\alpha$ significantly blocked tumor growth ($p = 0.013$), and DOX-induced tumors also showed higher BMAL1 protein levels (Fig. 5c). In addition, compared to control tumors, tumor cell proliferation was

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**Figures**: The figures illustrate the expression levels of BMAL1, ROR$\alpha$, G6Pase, and FGF-21 in different conditions. The mRNA levels are quantified using qPCR, and the results are presented as fold changes (FC) compared to control conditions. The graphs show the expression patterns under various treatments, including DOX, 4OHT, and SR1078. The results are consistent with the text description, indicating the regulatory effects of these treatments on the expression of genes involved in the circadian rhythm and tumor suppression.
Fig. 3 RORα activity rescues MYCN-mediated repression of BMAL1 expression and oscillation. **a** mRNA expression of RORα target genes (BMAL1, G6Pase, and FGF-21) in MNA LANS and SK-N-NE(2)-C cells treated with SR1078 (10 µM for 24 h), and LANS RORα Tet-ON cells cultured with (RORα-ON) and without (RORα-OFF) DOX (2 µg/ml) for 48 h. Data are mean ± SD (n = 3; ***p < 0.0001; two-tailed unpaired t-test). **b** Upper panel: BMAL1 protein expression in LANS and SK-N-NE(2)-C cells treated with SR1078. Lower panel: RORα and BMAL1 protein expression in MNA LANS RORα Tet-ON cells cultured with (RORα-ON) and without (RORα-OFF) DOX (2 µg/ml) for 24 h. Data were analyzed by densitometry (Image J v1.42). CyPb served as a loading control and BMAL1/RORα/CyPb ratios were determined (n = 2). **c** Upper panel: mRNA expression of MYCN target genes in MYCN3 cells treated with (MYCN-ON) and without (MYCN-OFF) DOX (1 µg/ml) for 48 h, and in SK-N-AS MYCN-ER cells treated with and without 4OHT (1 µM) for 48 h. Data are mean ± SD (n = 3; **p < 0.001; two-tailed unpaired t-test). Lower panel: BMAL1 mRNA and protein expression in MYCN3 cells under MYCN-OFF, MYCN-ON, and MYCN-ON + SR1078 (5 µM for 24 h) conditions. BMAL1 mRNA and protein expression in SK-N-AS MYCN-ER RORα Tet-ON cells upon MYCN activation (4OHT 1 µM for 24 h) with and without RORα overexpression (DOX 2 µg/ml for 24 h). Protein expression data were analyzed by densitometry (Image J v1.42a) and BMAL1/CyPb ratios determined (n = 2). **d** SK-N-AS MYCN-ER RORα cells were cultured with and without 4OHT (MYCN-ON/OFF), DOX (RORα-ON/OFF), and 4OHT + DOX for 24 h and then synchronized with 50% horse serum for 2 h. Cells were collected every 6 h from 16 to 64 h after synchronization. 4-OHT and DOX treatments were applied during and after cell synchronization. BMAL1 mRNA expression was determined by q-PCR and normalized to 18S expression. Data are mean ± SEM (n = 2; p < 0.0001 at all conditions and time points by two-way ANOVA with Tukey’s multiple comparisons test). BMAL1 oscillates at all conditions (p < 0.05) according to the JTK-rhythmicity test. FC fold change.

significantly lower in tumors that were both treated with SR1078 and overexpressing RORα (p < 0.0001 and p = 0.0012, respectively). Although SR1078 induced a robust apoptotic response in the treated tumors compared to controls (p = 0.0358), only a mild effect on cell apoptosis was observed in RORα-overexpressing tumors (Supplementary Fig. 14). Collectively, our data suggest that restoration of BMAL1 is tumor suppressive in NB.

The RNA-seq data indicated that SR1078 inhibits NB cell survival in part by suppressing genes involved in lipid metabolism (Fig. 4d). To determine whether SR1078 suppresses tumor growth by perturbing lipid metabolism, we performed lipidomics analysis in control (n = 10) and SR1078-treated (n = 8) LAN5 tumors. Supporting our RNA-seq data, we found that intratumoral levels of glycerolipids, such as triglycerides (TGs) and their precursors diglycerides (DGs) were significantly reduced by SR1078 (FDR < 0.25; Fig. 5d and Supplementary Data 2), suggesting that activation of RORα effectively inhibits in vivo lipogenesis. In contrast, the intratumoral cholesterol esters were increased by SR1078 (Supplementary Fig. 15). This finding challenges our RNA-seq data in which cholesterol biosynthesis genes were suppressed by SR1078, suggesting a potential compensatory upregulation of cholesterol uptake in these tumors. Because FAs are required for DG and TG synthesis, we further determined what changes in FA composition were induced by SR1078 using liquid chromatography-mass spectrometry (LC-MS)-based FA profiling. Notably, SR1078 significantly reduces the levels of intratumoral FAs including C12:0, C16:0, C18:1, and C20:3, all of which serve as side chains of the reduced DG and TG groups (Fig. 5e and Supplementary Table 2). Altogether, these data suggest that SR1078 blocks tumor growth by inhibiting FA metabolism and glycerolipid synthesis.

Activation of RORα opposes MYCN-driven lipogenesis. Recent reports have emphasized the role of MYC in reprogramming lipid metabolism to enhance tumorigenesis41. MYC induces SREBP1c-dependent de novo FA synthesis and directly activates the transcription of key lipogenic enzymes to support cancer growth42. On the other hand, RORs are also key regulators of lipid metabolism with tissue-dependent effects35. Nobiletin (a natural flavonoid) mediated activation of RORα blocks tumor growth by inhibiting FA synthesis and desaturation activity, stable isotope labeling and gas chromatography-mass spectrometry (GC-MS) were employed for FA determination. Turning on MYCN expression significantly increases the concentration of de novo synthesized C14:0 (myristic acid) and C16:0 (palmitic acid), and enhances SCD1 activity as indicated by the 13C16-labeled C16:1 (palmitoleic acid) to 13C16-labeled C16:0 ratio. However, SR1078 restores these FA levels, suggesting that activation of RORα efficiently blocks MYCN-mediated de novo FA synthesis and desaturation (Fig. 6e). Because the most profound effect of SR1078 was on SCD1 expression and activity, we next examined how SCD1 expression relates to NB clinical outcome. SCD1 expression is strongly associated with poor clinical outcomes and MYCN amplification (Fig. 6f). Moreover, ChIP-qPCR analyses in MYCN Tet-OFF cells confirmed a significant enrichment of MYCN binding at the SCD1 promoter, which is fully abrogated upon turning off MYCN (Fig. 6g), suggesting that MYCN directly transcriptionally induces SCD1 to promote lipogenesis. Selected FAs (e.g., oleic acid), the main product of SCD1, have been shown to rescue cell viability in cancer cells treated with FA synthesis inhibitors (e.g., TOFA)36. We found that culture media supplemented with oleic acid partially rescues the viability of MNA cells treated with SR1078 (Fig. 6h), suggesting that SR1078 targets MYCN-mediated SCD1 activity.

Activation of RORα sensitizes NB tumors to conventional chemotherapy. We hypothesized that activation of RORα, by restoring the molecular clock and cell metabolism, could sensitize NB to conventional therapy. To investigate whether SR1078 alters the sensitivity of NB cells to conventional therapies such as 

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etoposide (VP16), MNA cells were exposed to different concentrations of SR1078 and VP16 as single agents and in combination (IC50 doses). VP16 IC50 values were significantly reduced (10.3, 4.2, and 10.3 fold for LAN5, IMR32, and NGP cells, respectively) when cells were exposed to the combination therapy (Fig. 7a). SR1078 IC50 values were also robustly reduced (13.6, 29, and 40 fold, respectively) when cells were treated with the combination therapy (Supplementary Fig. 17). Moreover, the addition of low-dose SR1078 strongly enhanced the cell apoptosis induced by VP16 in all three cell lines (Fig. 7a). We then tested the anti-tumor activity of our combination therapy in vivo, using orthotopic xenografts generated from MNA NGP cells. As expected, single agents SR1078 and VP16 significantly blocked tumor growth (p < 0.05), and SR1078 strongly sensitized NB
Fig. 4 RORα activation inhibits cell survival via BMAL1 and constrains lipogenic gene expression. a Cell viability (MTT assay) and apoptosis (caspase 3/7 assay) in MNA (LAN5, IMR32) and non-MNA (SH-SYSY, SK-N-AS) cells following treatment with SR1078 for 24 h (apoptosis) and 72 h (cell viability). IC50 values are shown in the lower panel. Cell viability data are mean ± SD and are representative from n = 2 biological replicates (n = 4 technical replicates). MNA cell lines in red; non-MNA cell lines in black. Apoptosis data are mean ± SD (n = 3; two-way ANOVA with Tukey’s multiple comparisons test). b Cell proliferation of LAN5 RORα Tet-ON cells cultured with (RORα-ON) and without (RORα-OFF) DOX over 8 days. Data are mean ± SD (n = 3; ***p < 0.0001; two-tailed unpaired t-test). c Cell cycle analysis of LAN5 RORα-ON and LAN5 RORα-ON cells following 10 days of DOX (2 μg/ml) induction. Data are mean ± SD (n = 2). c Left panel: cell growth of LAN5 SiCTRL and LAN5 SiBMAL1 cells in the presence and absence of SR1078 (5 μM) for 4 days. Data are mean ± SD (n = 3; two-tailed unpaired t-test). Middle panel: Caspase 3/7 activation in LAN5 SiCTRL and LAN5 SiBMAL1 cells following SR1078 treatment (10 μM) for 24 h. Data are mean ± SD (n = 3; two-tailed unpaired t-test). Right panel: cell growth of LAN5 RORα SiCTRL and LAN5 RORα SiBMAL1 cells treated with (+Tet, RORα-ON) and without (−Tet, RORα-OFF) DOX for 4 days. Data are mean ± SD (n = 3, ***p < 0.0001; two-tailed unpaired t-test). d Left panel: Heat map of differentially expressed genes in LAN5 cells following SR1078 treatment (10 μM for 8 h). Right panel: Most enriched up- and downregulated pathways by SR1078 in LAN5 cells (p < 0.05). Blue = downregulated genes, red = upregulated genes.

Discussion

We have shown here that MYCN suppresses the molecular clock in NB. In turn, restoration of the clock effectively counteracts MYCN-mediated cell growth and metabolism. MYCN-amplification, which occurs in 50% of high-risk NB, distinguishes tumors with a high relapse rate and poor survival. We found that MYCN directly activates the clock repressor REV-ERBα and suppresses the activators RORα and BMAL1. This dysregulation is a powerful NB prognostic marker: low RORα and BMAL1 expression and high REV-ERBα expression strongly associated with poor clinical outcomes, independently of other prognostic factors.

The downregulation of the clock by MYCN is consistent with previous studies showing that ectopic MYC expression activates REV-ERBα to dampen BMAL1 expression and oscillation and downregulates core clock gene expression via MIZ1. Indeed, in the context of transcriptional repression MYC does not directly contact DNA but associates with DNA-bound transcription factors, such as MIZ1 and/or Sp1. Our data show that depletion of MIZ1 reduces the MYCN binding at the promoter regions of BMAL1 and RORα, suggesting that MIZ1 mediates MYCN repression of the clock in NB cells. This is further supported by the observation that MYCN physically binds the BMAL1 promoter despite the absence of a functional E-box and in the region where MIZ1 binds. Importantly, we show that MYCN suppresses the clock activators RORα and BMAL1 to sustain cell proliferation and rewire cell metabolism, suggesting that this dysregulation has a functional significance and may contribute to NB oncogenesis. Depending on the context, BMAL1 can function as a tumor suppressor or oncogene. Interestingly, MYC and BMAL1 are basic helix-loop-helix DNA-binding transcription factors, which recognize the same E-box sequences in the regulatory regions of target genes. Our ChIP-qPCR data revealed reduced BMAL1 occupancy of target E-box sites in lipogenic genes, which is rescued by ectopic RORα. Thus, we can speculate that oncogenic MYCN plays a role in disrupting BMAL1-controlled transcription and metabolism. RORα and BMAL1 gene silencing is found in several other cancers, including hematological malignancies. RORα also play important roles in tumor metabolism and inflammation. Genetic studies have shown that attenuated RORα leads to various metabolic abnormalities in mice, including hyperglycemia and glucose intolerance. Moreover, RORα inhibits inflammation by interfering with NF-kB signaling.

These observations prompted us to investigate whether restoring the clock could counteract the effects of MYCN on cell growth and metabolism. To restore the core clock component BMAL1 and clock function, we pharmacologically activated the circadian activator RORα via SR1078, a synthetic RORα/γ ligand agonist that selectively binds to the ligand-binding domain of the receptors promoting the transcription of target genes, such as BMAL1. Our data indicate that restoration of the molecular clock is tumor suppressive in NB. Both SR1078 and genetic overexpression of RORα effectively restore BMAL1 expression and oscillation, and block tumor growth in MNA, but not in non-MNA orthotopic xenografts, suggesting that the anti-tumor effect of SR1078 is MYCN-dependent. Importantly, the anti-tumor activity of SR1078 and RORαs is abolished following depletion of BMAL1, further supporting that the core clock component BMAL1 functions as a tumor suppressor in NB. These emerging connections between clock disruption and oncogenesis suggest that circadian interventions could be employed for anti-cancer treatment. Supporting this concept, recent work suggests that pharmacological agonists of REV-ERBs are selectively lethal to cancer cells and block glioblastoma (GBM) and GBM stem cell growth. We further hypothesized that SR1078, by restoring the molecular clock and cell metabolism, could be used in combination with conventional therapies to reduce toxicity and enhance chemotherapeutic viability. We find that SR1078 significantly improves the anti-tumor activity of the standard-of-care drug etoposide in MNA xenografts, suggesting that pharmacological modulation of the clock could be explored in future combination studies.

Metabolic rewiring is a key function of MYC oncogenes. Although several c-MYC metabolic functions are well characterized, the role of MYCN in metabolic reprogramming is less defined. MYC, in coordination with SREBP1, controls lipogenesis, which is required for both initiation and maintenance of tumorigenic growth. Moreover, inhibition of FA oxidation dampens tumor growth of triple-negative breast cancers overexpressing MYC. Interestingly, the activity of some of these lipogenic enzymes has been previously reported to be clock-controlled and linked to oncogenesis, suggesting that changes in their circadian activity may affect tumor growth. We demonstrate here that MYCN directly induces lipogenic gene expression and lipid intermediates, which are constrained by SR1078 in a BMAL1-dependent fashion. Moreover, SR1078 profoundly inhibits lipid metabolism in vivo by reducing glycerolipids and their FA components. Thus, we postulate that MYCN-mediated repression of the clock could induce the unconstrained expression of lipogenic genes and activate lipid metabolism to support tumor growth. However, restoration of the clock effectively constrains MYCN-mediated lipogenesis. We show that MYCN directly binds and upregulates the stearoyl-CoA desaturase-1 (SCD1), which catalyzes the synthesis of mono-unsaturated from saturated FA. In turn, activation of RORα blocks MYCN-induced SCD1 expression and activity. Moreover, supplementation with oleic acid, the major product of SCD1, rescues the viability of NB cells, confirming that SR1078 functions by blocking
MYCN-mediated lipogenesis. SCD1 expression is frequently elevated in cancer and its inhibition has shown some anti-tumor activity. We find that high SCD1 expression predicts poor clinical outcomes in NB and its expression directly correlates with MYCN amplification. This could represent a potentially targetable metabolic vulnerability.

Overall, our results demonstrated that restoration of the molecular clock via activation of RORα blocks MYCN-driven tumor growth and lipid metabolism (Supplementary Fig. 18). These novel findings implicate RORα activation as a potential therapeutic strategy for blocking MYCN-mediated dysregulation of the molecular clock and metabolism in high-risk NB.
Fig. 5 SR1078 and RO Rx genetic overexpression block tumor growth and inhibit lipid metabolism. a Tumor weights (mean ± SD) from MNA LANS- and IMR32-derived xenografts treated with vehicle control or SR1078 (15 mg/kg i.p. for 14 days) (LANS: p = 0.02, Mann–Whitney test, control group n = 10 and SR1078 group n = 8; IMR32: p = 0.0004, Mann–Whitney test, control group n = 11 and SR1078 group n = 10). BMAL1 protein expression in representative LANS-5 xenografted tumor lysates from SR1078 treated mice and controls. b Tumor weights (mean ± SD) from non-MNA SK-N-AS derived xenografts treated with vehicle control or SR1078 (15 mg/kg i.p. for 14 days) (p = 0.5116, Mann–Whitney test, control group n = 10 and SR1078 group n = 10). c Tumor weights (mean ± SD) from DOX-induced (RO Rx-ON) and controls (RO Rx-OFF) mice (p = 0.013, Mann–Whitney test, RO Rx-Off group n = 6 and RO Rx-ON group n = 8). BMAL1 protein expression in tumor lysates from representative RO Rx-ON and RO Rx-OFF mice. d Heat map and relative levels of altered DG and TG in LANS-derived xenografts treated with vehicle control (n = 10) or SR1078 (n = 8; 15 mg/kg i.p. for 14 days). Groups were compared by a two-tailed unpaired t-test. P-values were adjusted by the Benjamini–Hochberg method to obtain FDR. Changes with FDR < 0.25 were selected for heat map presentation; *FDR < 0.25. Yellow = upregulated lipids; blue = downregulated lipids. e Heat map and relative levels of altered intratumoral FAs (FDR < 0.25) in LANS-derived xenografts treated with vehicle control (n = 8) or SR1078 (n = 7; 15 mg/kg i.p. for 14 days). Groups were compared by a two-tailed unpaired t-test. P-values were adjusted by the Benjamini–Hochberg procedure to obtain FDR. Changes with FDR < 0.25 were selected for heat map presentation; *FDR < 0.25. AHA aminohexanotanico acid, DDA dodecanolico acid, DGD digliceridi, DHA docosahexanoidico acid, DTA docosatetraenoico acid, EPA eicosapentanoico acid, ETR etoricoxibico acid, HDA hexadecanico acid, ODA octadecanico acid (stearico acid), ODDA octadecanico acid (oleico acid); TG trigliceridi. For panels (d) and (e), the box plot is defined by two lines at the 25th and 75th percentiles. A line is drawn inside the box at the 50th percentile.

Methods

Cell lines and culture conditions. SH-SY5Y, IMR32, SK-N-BE(2)-C, SK-N-AS, NGP (ATCC), LANS, and CHLA225 (Metelitsa, BCM, Houston, TX), and MYCN3 (Shoeth, BCM, Houston, TX) NB cell lines were maintained in RPMI 1640 medium. Tet-211N (Perini, Bologna, Italy) cells were grown in DMEM high glucose medium and kept in selection with neomycin (4418, Santa Cruz Biotechnology# sc-29065A, 0.2 mg/ml) and hygromycin (Sigma Aldrich® H3274, 0.150 mg/ml). All cell lines were validated by genotyping and regularly tested for mycoplasma. To turn off MYCN, doxycycline (DOX; Santa Cruz Biotechnology# sc-204734A) was added at a final concentration of 2 µg/ml. SK-N-AS MYCN-ER (Altman, Roche–Sta., USA) cells were maintained in DMEM high glucose and sodium pyruvate. To activate MYCN transcription, cells were treated with 1 µM 4-hydroxytamoxifen (Sigma Aldrich® T716) for 48 h. Cell lines were validated by genotyping and confirmed by expression of CD56, Nestin, MYCN, and tyrosine hydroxylase within the past 12 months. SR1078 (Calbiochem® 557352) was used at a concentration of 5–15 µM for in vitro studies. BSA-conjugated oleic acid (Sigma Aldrich® O3008) was supplemented in the culture media (10–500 µM).

Primary NBs survival data. mRNA gene expression analyses were performed within R2: a genomics analysis and visualization platform (http://r2.amhc.illinois.edu), using datasets: NB Kocak (n = 649, GEO: GSE45457, patient cohort 1), NB Versteege (n = 88, GEO: GSE16476 88/122, patient cohort 2), and NB Research Consortium (NRC, n = 283, GEO: GSE99647, patient cohort 3). In the Kocak dataset, 476 of the 649 samples were annotated with survival data. To test the association of gene expression with survival, individual gene expression profiles were dichotomized by a median split into “high” or “low” groups, and Kaplan–Meier survival curves were plotted for each group. A Cox regression model was used to test for the independence of each MYCN-related gene expression from the other significant factors: MYCN amplification, age, and INSS stages. The Mann–Whitney U test was used to evaluate the significant differences among quantitative variables. The significance of correlation analyses between MYCN and ROR α was evaluated by the Spearman test.

Plasmid constructs. To generate MYCN-inducible MYCN3 cells, MYCN cDNA was cloned into a pTR2-Hygro vector (BD Biosciences) containing a tetracycline-responsive promoter. The construct was then transfected into a SHEP subclone stably expressing the tetracycline response element and selected with hygromycin. For LANS-ShMYCN cells, GIPZ human MYCN lentiviral clones (V21HS.36755 and V3LHS.322662) were obtained from the Cell-Based Screening Service core at Baylor College of Medicine (BCM). MYCN–shRNA sequences were inserted in the lentiviral gene silencing vector pLduc1, which has a dual fluorescent system consisting of a constitutive cassette (rtTA3 and eGFP) to detect infection efficiency and a turbroRFP (trRFP)-shRNA cassette activated upon DOX treatment. To generate LANS and SK-N-AS RO Rx conditional overexpressing cells, RO RxT (Origene RC209296) was cloned into the pENTR/D-TOPO vector (Invitrogen) and then gateway cloned into a pLduc1 lentiviral vector with RO Rx-HA under the control of the tetracycline-inducible promoter. LANS cells were transduced with lentiviral vectors at a multiplicity of infection sufficient to achieve greater than 90% infection as determined by the presence of GFP fluorescence. BMAL1 siRNAs (scrambled and BMAL1 targetable) were obtained from Thermo Scientific (Dharmacon SMARTpool siRNAs, which consists of a pool of four siRNA sequences, SO-2564383G). To generate LANS Shp53 cells, second-generation lentiviruses expressing Shp53 and ShLuc were used as previously described. Briefly, 293 T cells were transfected with pSLPW construct along with pVSVG and pLV-CMV-delta 8.2 by using lipofectamine. Virus-containing supernatants were collected at 48 h and 72 h and NB cells transduced in the presence of 8 mg/ml polybrene (Sigma).

In vitro functional assays. To test the effect of SR1078 on cell viability, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out as previously described. Cell proliferation was assessed using the CCK-8 Kit (Dojindo, Kumamoto, Japan) according to the manufacturer’s instructions. Briefly, 3 × 10³ cells/well were seeded in 96-well plates. At determined times, CCK-8 (10 µl) was added to the wells and incubated at 37 °C for 4 h, and absorbance (450 nm) was measured. Caspase 3/7-mediated apoptosis was determined using the Caspase-Glo assay kit (Promega). Briefly, 5 × 10³ cells/well were seeded in 96-well plates and subjected to different treatments for 24–48 h. Equal volumes of customer supplied caspase substrate and 1x caspase reagent were added to the wells, and luminescence was measured on a plate-reading luminometer after 3 h incubation. Cell cycle distribution was measured by PI Flow Cytometry kit (abcam® 139418). LANS RO Rx cells were harvested and fixed (70% ethanol for 2 h). Cells were stained with 50 µg/ml PI for 30 min before FACS analysis (BD LSR II with BD FACS DIVA v6.1.3). Cell population (%) in G0-G1-S, G2 and S phases was analyzed by FlowJo v7.6.1.

Real-time q-PCR and western blotting. Total RNA was extracted and purified with RNaseasy kit (QIAGEN). Real-time PCR was performed with SYBR green using the LightCycler 480 II system. AHA aminohexanotanico acid, DDA dodecanolico acid, DGD digliceridi, DHA docosahexanoidico acid, DTA docosatetraenoico acid, EPA eicosapentanoico acid, ETR etoricoxibico acid, HDA hexadecanico acid, ODA octadecanico acid (stearico acid), ODDA octadecanico acid (oleico acid); TG trigliceridi. For panels (d) and (e), the box plot is defined by two lines at the 25th and 75th percentiles. A line is drawn inside the box at the 50th percentile.

RNA-seq analysis. We generated libraries with the Illumina TruSeq Stranded mRNA (p/n 20020594) kit starting with 15 ng of RNA, and we sequenced with the NextSeq 500, PE75, v2.5 reagent kit. Differential expression programs from RNA-seq data of LANS cells treated with SR1078 (10 µM) for 8 h were performed by the Ingenuity Systems. Fastq files were aligned to the reference genome (hg38.p12) using STAR (v2.4.2) with default settings to produce transcripts per million (tpm) estimates for each ensemble gene. When identifying up- and downregulated genes, we required genes to significantly be downregulated by t-test and with a consistent fold change above 1.5 or below 0.67 compared to controls. Expression values were offset by adding a pseudo count of 1 tpm for each gene to exclude genes with low expression estimates. Gene set enrichment for Reactome pathways based on dysregulated genes was performed in Enrichr. Pathways in the same category were grouped. Significantly genes were sorted by p-value and associated with pathway terms.

Chromatin immunoprecipitation (ChIP). The MYCN ChIP assays were performed as follows. Briefly, 1 × 10⁶ cells were cross-linked using 1% formaldehyde, and the cross-linked cell was resuspended in cell lysis buffer following 3500g centrifugation, RIPA sonication buffer was added to complete nuclei lysis. DNA shearing was performed by...
sonication using the Bioruptor PLUS (Diagenode). A small aliquot of sonicated material was put aside, and the remaining sample was immunoprecipitated using 5 μg of ChIP-grade antibodies (B8.4.B, Santa Cruz Biotechnology# sc-53993). Rec-sepharose Protein A or G beads (Invitrogen) were used to immobilize immunocomplexes and RNAse-A treatment (37 °C 1 h) and reverse crosslinking was performed using Proteinase K (Roche) for 6 h at 65 °C. Immunoprecipitated DNA was purified using phenol/chloroform and ethanol precipitation techniques. DNA was analyzed by q-PCR and normalized by the fold enrichment method (2\(^{-\Delta\Delta CT}\)) using the primers listed in Supplementary Table 3. The ABCA10 transcription start site was used as a negative control DNA region for MYCN. The BMAL1-ChIP assays were performed using the ChIP-IT® Express Enzymatic (Active Motif# 53009) following the manufacturer’s protocol. Approximately, 1.5 × 10^7 cells were fixed using 1% formaldehyde. The fixation reactions were stopped by adding Glycine Fix-Stop solution. After washing with ice-cold PBS, cells were collected in a solution containing PMSF and centrifuged at 4 °C. The cell pellet was disrupted with a Dounce homogenizer in 1 ml ice-cold lysis buffer containing protease inhibitors.
inhibitor and PMSF. After centrifugation, nuclei were resuspended in digestion buffer containing protease inhibitor and PMSF, then incubated at 37 °C for 5 min. Chromatin was then sheared to 200–300 bp by adding an enzyme shearing cocktail for 10 min at 37°C. Samples were centrifuged at 21,000xg for 10 min at 4°C. One percent aliquot of this material was retained as “input” DNA. The remaining chromatin sample was divided, one-half was immunoprecipitated with the BMAL1 antibody (Abcam# 93806), and the second half was used for a mock immunoprecipitation with a control IgG. Real-time PCR amplification was carried out using 2.5 μL of DNA sample, using primers listed in Supplemental Table 3.

Circadian oscillations. Real-time bioluminescence monitoring. MYCN3 and LAN5 cells were treated with or without 1 μM DOX for 48 h before infection with Per2-dluc adenovirus as previously described64 for another 24 h. Cells were then serum-starved for 16 h and plated on glass bottoms. Per2-dluc activity was measured after cell synchronization.

FA measurement. FA tracing was performed using perfluorobenzyl bromide (PFBBr) derivatization and GC-MS negative chemical ionization as previously described66. SP-2380 columns (Supelco, Inc.) were used to allow better peak resolution of newly synthesized FAs. Separation was performed on a Luna 3μm phenyl-hexyl column (150 × 2 mm) using 10 mM ammonium acetate buffer containing 50% acetonitrile and 0.1% acetic acid at a flow rate of 0.2 ml/min with a gradient starting at 40% B at 0 min, 50% B at 10 min, 75% B at 15 min, and 100% B at 16 min to 64 h after synchronization. 4-OHT and DOX treatments were applied during and after cell synchronization. BMAL1 mRNA expression was determined by q-PCR and normalized to 18S expression. To test for rhythmicity, JTK_Cycle65 was used to determine the concentration of each FA. To determine de novo lipogenesis, deuterated water (D2O) (atom 99%, Cambridge Isotope Laboratory) was added to the media to reach an enrichment of 2.5%. The percentage contribution of newly made FA was calculated as total [H]-labeled FA/(H-labeled body content × 100) (n = number of exchangeable hydrogens). The ratio was determined by mass spectrometry to quantify the relative incorporation of deuterium into a given lipid class.

Lipidomics. Sample Preparation was as follows: Tumor samples were homogenized with an equal ratio of 0.15 M KCl and methanol followed by 400 μL of dichloromethane and 2 μL of acetic acid. Internal standards and quality control samples were prepared as previously described67. Brieﬂy, in equimolar concentration of 10 μM each sample batch buying Biotector (Cell Signaling Technology). After centrifugation (5 min 6500 g), the lower organic layer was collected and dried under nitrogen. Before mass spectrometric (MS) analysis, the dried extract was suspended in 100 μL of solution (10:85 acetonitrile/water/isopropanol alcohol containing 10 mM ammonium acetate) and subjected to liquid chromatography (LC) separation and coupling to tandem mass spectrometry (TOF) analysis (Shimadzu, CTO-20A Nexera X2 41 HPLC system). MS analysis was carried out on a Triple TOF 5600 equipped with Turbo VTM ion source (AB Sciex). Data were acquired with Analyst TF software v1.8 (AB Sciex). Lipids were separated on the agilent HSS UPLC T3 column (Waters) at 55°C. The initial mobile phase consists of acetonitrile/water (40:60 v/v) to 100% acetonitrile at 1.4 mL/min. The lower organic layer was collected and converted to acetonitrile/water/isopropanol (10:85:5 v/v) with 10 mM ammonium acetate with gradient elution at a flow rate of 0.4 mL/min and an injection volume of 5 μL. Operating source conditions for Triple TOF scan for positive ionization: source voltage 5500 V, declustering potential (DP) 60 V, source T 450°C, ion source gas 1 (GS1) 40 psi, ion source gas 2 (GS2) 35 psi, curtain gas (CUR) high, and collision energy 10 V. Conditions for negative ionization: source voltage −4500 V, DP 60 V, GS1 40 psi, GS2 45 psi, CUR 30 psi, and collision −10 V. The MS/MS spectra were controlled by data-dependent
acquisition with dynamic background subtraction, charge monitoring, and dynamic exclusion of former target ions for 9 s. Rolling collision energy spread was set to generate a collision energy ramp around a collision energy center point with the goal of providing a richer MS/MS spectrum of lipids. Mass accuracy was maintained by the use of an automated calibrant delivery system interfaced to the second inlet of the DuoSpray source. Data processing and statistical analysis were performed as previously described.\(^6\)\(^7\) Briefly, data were normalized by median inter-quantile range normalization and were log2 transformed. The two groups were compared by Student’s t-test. \(p\)-values were adjusted by the Benjamini–Hochberg procedure to obtain FDR. Changes with FDR < 0.25 were selected for heat map presentation.

### Statistical analysis
Data were collected in Microsoft Excel 2013 and analyzed in GraphPad Prism v7. All in vitro assays are expressed as mean ± standard deviation (SD) and performed in triplicate. Data are compared using a two-sided unpaired t-test, one or two-way ANOVA with Tukey’s, Sidak’s, and Dunnett’s multiple comparisons test. Tumor weights are expressed as mean ± SD and compared using Mann-Whitney tests; \(p\)-values < 0.05 were considered statistically significant. Rhythmicity was tested using the non-parametric test, JTK_Cycle, R version 4.0.3, package MetaCycle.

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

### Data availability
Publicly available clinical datasets GSE45547, GSE16476, and GSE85047 were analyzed with R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). RNA-
sequencing data generated in this study have been deposited in the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/), accession number: PRJEB6158. All Figures and Supplementary Figures have associated raw data. Raw data of Figs. 1–7 and Supplementary Fig. 1–18, and uncropped blots are provided as one Source Data file. All the other data are available within the article and its Supplementary Information. Source data are provided with this paper.

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References

1. Eilers, M. & Eisenman, R. N. Myc’s broad reach. Genes Dev. 22, 2755–2766 (2008).
2. Mari, J. M. Recent advances in neuroblastoma. N. Engl. J. Med. 362, 2202–2211 (2010).
3. Weiss, W. A., Aldeape, K., Mohapatra, G., Feinstein, B. G. & Bishop, J. M. Targeted expression of MYCN causes neuroblastoma in transgenic mice. EMBO J. 16, 2985–2995 (1997).
4. Hsieh, A. L., Walton, Z. E., Altman, B. J., Stine, Z. E. & Dang, C. V. MYC and metabolism on the path to cancer. Semin. Cell Dev. Biol. 43, 11–21 (2015).
5. Cantor, J. R. & Sabatini, D. M. Cancer cell metabolism: one hallmark, many mechanisms. Cell 150, 1011–1026 (2013).
6. DeBerardinis, R. J. & Cheng, T. Q.’s next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 29, 313–324 (2010).
7. Li, F. et al. Myc stimulates nucleated mitochondrial genes and mitochondrial biogenesis. Mol. Cell. Biol. 25, 6235–6244 (2005).
8. Liu, Y. C. et al. Global regulation of nucleotide biosynthetic genes by c-Myc. PLoS ONE 3, e2722 (2008).
9. Morrish, F. et al. Myc-dependent mitochondrial generation of acetyl-CoA contributes to fatty acid biosynthesis and histone acetylation during cell cycle entry. J. Biol. Chem. 285, 36267–36274 (2010).
10. Loven, J. et al. Reviving global gene expression analysis. Cell 151, 476–482 (2012).
11. Carroll, P. A. et al. Deregulated Myc requires MondoA/Mlx for metabolic reprogramming and tumorigenesis. Cancer Cell 27, 271–285 (2015).
12. Zirath, H. et al. MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells. Proc. Natl. Acad. Sci. USA 110, 10528–10532 (2013).
13. Mohawk, J. A., Green, C. B. & Takahashi, J. S. Central and peripheral circadian clocks in mammals. Annu. Rev. Neurosci. 35, 445–462 (2012).
14. Altman, B. J. et al. MYC disrupts the circadian clock and metabolism in cancer cells. Cell Metab. 22, 1099–1109 (2015).
15. Papagiannakopoulos, T. et al. Circadian rhythm disruption promotes lung tumorigenesis. Cell Metab. 24, 324–331 (2016).
16. Sato, T. K. et al. Feedback repression is required for mammalian circadian clock function. Nat. Genet. 38, 312–319 (2006).
17. Cho, H. et al. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485, 123–127 (2012).
18. Kojetin, D. J. & Burris, T. P. REV-ERB and ROR nuclear receptors as drug targets. Nat. Rev. Drug Discov. 13, 197–216 (2014).
19. Shostak, A. et al. MYC/MIZ1-dependent gene repression inversely coordinates circadian clocks in mammals. Annu. Rev. Neurosci. 35, 445–462 (2012).
20. Altman, B. J. et al. MYC disrupts the circadian clock and metabolism in cancer cells. Cell Metab. 22, 1099–1109 (2015).
21. Papagiannakopoulos, T. et al. Circadian rhythm disruption promotes lung tumorigenesis. Cell Metab. 24, 324–331 (2016).
22. Sato, T. K. et al. Feedback repression is required for mammalian circadian clock function. Nat. Genet. 38, 312–319 (2006).
23. Cho, H. et al. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485, 123–127 (2012).
24. Kojetin, D. J. & Burris, T. P. REV-ERB and ROR nuclear receptors as drug targets. Nat. Rev. Drug Discov. 13, 197–216 (2014).
25. Shostak, A. et al. MYC/MIZ1-dependent gene repression inversely coordinates the circadian clock with cell cycle and proliferation. Nat. Commun. 7, 11807 (2016).
26. Yang, G., Wang, C., Evers, B. M., Zhou, B. P. & Xu, R. ROR alpha suppresses breast tumor invasion by inducing SEMA3F expression. Cancer Res. 72, 1728–1739 (2012).
27. Kottorou, A. E. et al. Altered expression of NF-Y and RORA in colorectal adenocarcinomas. Acta Histochem. 114, 535–561 (2012).
28. Moretti, R. M., Montagnani Marelli, M., Sala, A., Motta, M. & Limonta, P. Activation of the orphan nuclear receptor RORalpha counters the proliferative effect of fatty acids on prostate cancer cells: crucial role of 5-lipoxygenase. Int. J. Cancer. 112, 87–93 (2004).
29. Fu, R. D., Qiu, C. H., Chen, H. A., Zhang, G. Z. & Lu, M. Q. Retinoic acid receptor-related receptor alpha (RORalpha) is a prognostic marker for hepatocellular carcinoma. Tumour Biol. 35, 763–7610 (2014).
30. Wang, Y., Solt, L. A., Kojetin, D. J. & Burris, T. P. Regulation of p53 stability and apoptosis by a ROR agonist. PLoS ONE 7, e34921 (2012).
31. Lee, J. M. et al. RORalpha attenuates Wnt/beta-catenin signaling by PKCalpha-dependent phosphorylation in colon cancer. Mol. Cell 37, 183–195 (2010).
32. Taniguchi, H. et al. Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies. Cancer Res. 69, 8447–8454 (2009).
33. Zeng, Z. L. et al. Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer. Clin. Cancer Res. 20, 1042–1052 (2014).
34. Wang, Y. et al. MYC disrupts the circadian clock and metabolism in cancer cells. Cell Metab. 22, 1099–1109 (2015).
35. Papagiannakopoulos, T. et al. Circadian rhythm disruption promotes lung tumorigenesis. Cell Metab. 24, 324–331 (2016).
36. Sato, T. K. et al. Feedback repression is required for mammalian circadian clock function. Nat. Genet. 38, 312–319 (2006).
37. Cho, H. et al. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485, 123–127 (2012).
38. Kojetin, D. J. & Burris, T. P. REV-ERB and ROR nuclear receptors as drug targets. Nat. Rev. Drug Discov. 13, 197–216 (2014).
39. Shostak, A. et al. MYC/MIZ1-dependent gene repression inversely coordinates the circadian clock with cell cycle and proliferation. Nat. Commun. 7, 11807 (2016).
40. Yang, G., Wang, C., Evers, B. M., Zhou, B. P. & Xu, R. RORalpha suppresses breast tumor invasion by inducing SEMA3F expression. Cancer Res. 72, 1728–1739 (2012).
41. Kottorou, A. E. et al. Altered expression of NF-Y and RORA in colorectal adenocarcinomas. Acta Histochem. 114, 535–561 (2012).
42. Moretti, R. M., Montagnani Marelli, M., Sala, A., Motta, M. & Limonta, P. Activation of the orphan nuclear receptor RORalpha counters the proliferative effect of fatty acids on prostate cancer cells: crucial role of 5-lipoxygenase. Int. J. Cancer. 112, 87–93 (2004).
43. Fu, R. D., Qiu, C. H., Chen, H. A., Zhang, G. Z. & Lu, M. Q. Retinoic acid receptor-related receptor alpha (RORalpha) is a prognostic marker for hepatocellular carcinoma. Tumour Biol. 35, 763–7610 (2014).
44. Wang, Y., Solt, L. A., Kojetin, D. J. & Burris, T. P. Regulation of p53 stability and apoptosis by a ROR agonist. PLoS ONE 7, e34921 (2012).
45. Lee, J. M. et al. RORalpha attenuates Wnt/beta-catenin signaling by PKCalpha-dependent phosphorylation in colon cancer. Mol. Cell 37, 183–195 (2010).
46. Taniguchi, H. et al. Epigenetic inactivation of the circadian clock gene BMAL1 in hematologic malignancies. Cancer Res. 69, 8447–8454 (2009).
47. Zeng, Z. L. et al. Overexpression of the circadian clock gene Bmal1 increases sensitivity to oxaliplatin in colorectal cancer. Clin. Cancer Res. 20, 1042–1052 (2014).
56. Comerford, S. A. et al. Acetate dependence of tumors. Cell 159, 1591–1602 (2014).
57. Li, J. et al. Partial characterization of a cDNA for human stearoyl-CoA desaturase and changes in its mRNA expression in some normal and malignant tissues. Int. J. Cancer 57, 348–352 (1994).
58. Roongta, U. V. et al. Cancer cell dependence on unsaturated fatty acids implicates stearoyl-CoA desaturase as a target for cancer therapy. Mol. Cancer Res. 9, 1551–1561 (2011).
59. Slack, A. et al. The p53 regulatory gene MDM2 is a direct transcriptional target of MYC in neuroblastoma. Proc. Natl. Acad. Sci. USA 102, 731–736 (2005).
60. Barbieri, E. et al. A p53 drug response signature identifies prognostic genes in high-risk neuroblastoma. PloS ONE 8, e79843 (2013).
61. Barbieri, E. et al. MDM2 inhibition sensitizes neuroblastoma to chemotherapy-induced apoptotic cell death. Mol. Cancer Ther. 5, 2358–2365 (2006).
62. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2013).
63. Kuleshov, M. V. et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 44, W90–W97 (2016).
64. Stashi, E. et al. SRC-2 is an essential coactivator for orchestrating metabolism and circadian rhythm. Cell Rep. 6, 635–645 (2014).
65. Hughes, M. E., Hogenesch, J. B. & Kornacker, K. JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J. Biol. Rhythm. 25, 372–380 (2010).
66. Mohammad, M. A. & Haymond, M. W. Regulation of lipid synthesis genes and milk fat production in human mammary epithelial cells during secretory activation. Am. J. Physiol. Endocrinol. Metab. 305, E700–E716 (2013).
67. Vantaku, V. et al. Multi-omics integration analysis robustly predicts high-grade patient survival and identifies CPT1B effect on fatty acid metabolism in bladder cancer. Clin. Cancer Res. 25, 3689–3701 (2019).
68. White, J. J. & Sillito, R. V. Genetic silencing of olvilocerebellar synapses causes dystonia-like behaviour in mice. Nat. Commun. 8, 14912 (2017).
69. Piyaratnha, D. W. B. et al. Distinct lipidomic landscapes associated with clinical stages of urothelial cancer of the bladder. Eur. Urol. Focus 4, 907–915 (2018).

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Competing interests
The authors declare no competing interests.

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