

Metabolic Reprogramming by MYCN Confers Dependence on the Serine-Glycine-One-Carbon Biosynthetic Pathway



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Abstract

MYCN amplification drives the development of neuronal cancers in children and adults. Given the challenge in therapeutically targeting MYCN directly, we searched for MYCN-activated metabolic pathways as potential drug targets. Here we report that neuroblastoma cells with MYCN amplification show increased transcriptional activation of the serine-glycine-one-carbon (SGOC) biosynthetic pathway and an increased dependence on this pathway for supplying glucose-derived carbon for serine and glycine synthesis. Small molecule inhibitors that block this metabolic pathway exhibit selective cytotoxicity to MYCN-amplified cell lines and xenografts by inducing metabolic stress and autophagy. Transcriptional activation of the SGOC pathway in MYCN-amplified cells requires both MYCN and ATF4, which form a positive feedback loop,

with MYCN activation of ATF4 mRNA expression and ATF4 stabilization of MYCN protein by antagonizing FBXW7-mediated MYCN ubiquitination. Collectively, these findings suggest a coupled relationship between metabolic reprogramming and increased sensitivity to metabolic stress, which could be exploited as a strategy for selective cancer therapy.

Significance: This study identifies a MYCN-dependent metabolic vulnerability and suggests a coupled relationship between metabolic reprogramming and increased sensitivity to metabolic stress, which could be exploited for cancer therapy.

See related commentary by Rodriguez Garcia and Arsenian-Henriksson, p. 3818

Introduction

MYCN is a member of the MYC family of oncogenic transcription factors that also include MYC and MYCL. A primary function of MYCN, as well as other MYC family proteins, is to promote cell growth and proliferation through transcriptional regulation (1, 2). Aberrant MYCN activation, mainly via genomic amplification, is

commonly observed in neuronal and neuroendocrine cancers, including neuroblastoma (3), medulloblastoma (4, 5), Wilms tumor (6), retinoblastoma (7), neuroendocrine prostate cancer (8–10), glioblastoma (11, 12), and small-cell lung cancer (13–15). However, MYCN is a poor drug target (16). We reasoned that the cell proliferation program driven by MYCN activation might create a selective pressure on metabolic pathways to meet the biosynthetic demand of growth, thereby imposing metabolic dependences that can be exploited therapeutically. Our investigation reveals that MYCN and ATF4, a master regulator of amino acid metabolism and stress responses (17–19), form a positive feedback loop for transcriptional activation of the serine-glycine-one-carbon (SGOC) metabolic pathway. This metabolic reprogramming generates a SGOC pathway-dependent synthetic lethal effect in MYCN-amplified neuroblastoma cells. Thus, targeting the SGOC metabolic pathway may provide selective therapeutic benefits for patients with MYCN-amplified cancers.

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Materials and Methods

Cell lines and cell culture

Neuroblastoma cell lines BE(2)-C (CRL-2268), IMR32 (CCL-127), SH-SY5Y (CRL2266), SK-N-AS (CRL-2137), SK-N-DZ (CRL-2149), SK-N-FI (CRL-2141), and SK-N-SH (HTB-11) were obtained from ATCC, LA1-55n (06041203) from Sigma-Aldrich, and CHLA-90, LA-N-5, LA-N-6, SMS-KANR, and SMS-KCNR from Children's Oncology Group Cell Culture and Xenograft Repository. NBL-S was a gift from S.L. Cohn (University of Chicago,

Chicago, IL), NLF from M.C. Simon (University of Pennsylvania, Philadelphia, PA), and SHEP1 from V.P. Opipari (University of Michigan, Ann Arbor, MI). All cell lines had been authenticated using short tandem repeat profiling (ATCC) and after authentication, large frozen stocks were made to ensure against contaminations by other cell lines. All cell lines were used within 10 passages after reviving from the frozen stocks and were free of *Mycoplasma* contamination as determined by a LookOut Mycoplasma PCR Kit (Sigma-Aldrich) and staining cells with DAPI every 3 months. IMR32, SHEP1, SH-SY5Y, SK-N-AS, and SK-N-SH were cultured in DMEM (HyClone; SH30022), BE(2)-C in DMEM/Ham's F-12 1:1 (HyClone; SH30023), and all others in RPMI1640 (HyClone; SH30027). All culture media were supplemented with 10% FBS (Atlanta Biologicals; S11050).

Patient data analyses

The Sequencing Quality Control (SEQC) dataset of MAQC-III was downloaded from the NCBI Gene Expression Omnibus (accession number GSE62564; ref. 20). Kaplan–Meier survival analysis based on the SGOC 6-gene signature (*PHGDH*, *PSAT1*, *SHMT2*, *MTHFD1*, *MTHFD1L*, and *MTHFD2*) was performed using the software R 3.3.2. Gene expression correlation analyses were conducted online using the R2 Platform (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>), and the resulting figures and *P* values were downloaded. Gene Ontology (GO) analysis of the *MYCN*-amplification gene signature (21) was performed using the software DAVID (22).

Quantitative reverse-transcription PCR

Total RNA was isolated using TRIzol (Invitrogen). Reverse transcription was performed using an iScript Advanced cDNA Synthesis Kit (Bio-Rad). Quantitative reverse-transcription PCR (qRT-PCR) was performed using a 2× SYBR green qPCR master mix (Bimake) on an iQ5 real-time PCR system (Bio-Rad) with gene-specific primers (Supplementary Information). All samples were normalized to β2 microglobulin (B2M) mRNA levels.

Overexpression and RNA interference

The Retro-X Tet-Off Advanced Inducible System (Clontech) was used to generate neuroblastoma cell lines with inducible *ATF4*, *FBXW7α*, or *MYCN* expression in the absence of doxycycline (Doxy). Human myc-tagged *ATF4* and myc-tagged *FBXW7α* were generated by PCR using pRK-*ATF4* (Addgene; #26114) and pCR4-TOPO-*FBXW7α* (Open Biosystems; MHS4426-99239216) as templates, respectively. The sequences were verified by sequencing and subcloned into pRetroX-Tight-pur (Clontech) and pCDH-CMV-MCS-EF1-puro (SBI System Biosciences). Lentiviral shRNA constructs shATF4-73 (TRCN0000013573), shATF4-74 (TRCN0000013574), shMYCN-94 (TRCN0000020694), and shMYCN-95 (TRCN0000020695) were obtained from Sigma-Aldrich, shPHGDH (RHS3979-9595900) from Thermo Fisher Scientific, and shFBXW7 (RHS4533-NM_001013435) from Open Biosystems. Retroviruses for the expression of *ATF4*, *FBXW7α*, or *MYCN* were produced in 293FT cells using the packaging plasmids pHDM-G and pMD.MLVogp (gifts from R. Mulligan Massachusetts Institute of Technology, Cambridge, MA), and lentiviruses for the expression of shRNA, *ATF4*, or *MYCN* were produced in 293FT cells using the packaging plasmids pLP1, pLP2, and pLP/VSVG (Thermo Fisher Scientific). Retroviral and lentiviral infections of cells were conducted according to standard procedures.

Immunoblotting

Proteins (20–50 μg) were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies (Supplementary Information). Proteins were visualized and quantified using the Odyssey system (LI-COR Biosciences) or a Clarity Western ECL Kit (Bio-Rad) and ImageJ (version 1.50e). Films were exposed for various times for quantification of target proteins within their linear range of detection.

Chromatin immunoprecipitation and quantitative PCR

Chromatin immunoprecipitation (ChIP) was performed as described (23) using the cell lines BE(2)-C, SMS-KCNR, SHEP1_pCDH-MYCN, BE(2)-C_shATF4-73, and BE(2)-C_tetoff-ATF4 (± 2 μg/mL Doxy for 6 days). For each antibody, ~4 × 10⁷ cells were used. Cross-linked chromatin was sheared through sonication (Fisher Scientific Model 150E) and immunoprecipitated using ChIP grade rabbit anti-ATF4 (sc-200), mouse anti-MYCN (B8.4.B, sc-53993), control mouse IgG (sc-2025) or rabbit IgG (sc-2027) from Santa Cruz Biotechnology. For qPCR, 2 independent ChIP samples were analyzed, and each sample was assayed in triplicate using ChIP and quantitative PCR (ChIP-qPCR) primers (Supplementary Information). The number associated with each primer set indicates the position of the forward primer relative to its target gene transcription start site (TSS, +1).

MYCN half-life assay

BE(2)-C_tetoff-ATF4 and SK-N-DZ_tetoff-ATF4 cells were cultured with or without 2 μg/mL Doxy for 5 days, followed by addition of cycloheximide to a final concentration of 10 μg/mL. Samples were then collected at various time points for immunoblot analysis of MYCN protein levels, which were quantified against β-actin using ImageJ.

In vivo ubiquitination assay

The assay was performed as described (24). Briefly, BE(2)-C cells were cotransfected with pFlag-CMV-2-ubiquitin and pCDNA3.1-FBXW7α, along with or without pCDH-ATF4, and BE(2)-C cells expressing shGFP, shATF4-73, shFBXW7-55, or both shATF4-73 and shFBXW7-55 were transfected with pFlag-CMV-2-ubiquitin. Approximately 44 hours after transfection, cells were treated with 10 μmol/L MG-132 for 4 hours, washed with PBS, and lysed with a lysis buffer containing 2% SDS, 150 mmol/L NaCl, 10 mmol/L Tris-HCl, (pH 8.0), 2 mmol/L sodium orthovanadate, and 50 mmol/L sodium fluoride. The lysates were boiled for 10 minutes, sonicated (Fisher Scientific Model 150E, amplitude 40 and time 10 seconds), and diluted 1:10 with a dilution buffer (10 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl, and 1% Triton-X 100), followed by MYCN immunoprecipitation with mouse anti-MYCN (B8.4.B) and Protein G Dynabeads (Thermo Fisher Scientific; 10003D) and immunoblotting using rabbit anti-Flag for Flag-ubiquitin (Sigma-Aldrich; F-7425) and rabbit anti-MYCN (Cell Signaling Technology; #9405).

Inhibitor assays

Small molecule inhibitors NCT-503 (Sigma-Aldrich; SML1659), NCT-503 inactive control (Sigma-Aldrich; SML1671), and CBR-5884 (Sigma-Aldrich; SML1656) were dissolved in DMSO, and stock solutions were aliquoted and stored at –80°C until use. Cells were treated with DMSO, NCT-503, or CBR-5884 for 2 to 3 days, and the number of viable cells was determined by trypan blue exclusion assay. To assess the effect of supplemental

serine on NCT-503 action, cells were cultured in MEM (Gibco; 11095-080) supplemented with 10% dialyzed FBS (Gibco; 26400-044), 4.5 g/L D-(+)-glucose (Sigma-Aldrich; G8769), 1× MEN vitamins (Gibco; 11120-052), 1× sodium pyruvate (Gibco; 11360-070), and nonessential amino acids alanine, asparagine, glutamic acid, glycine, proline with or without 0.4 mmol/L serine.

Amino acid uptake assay

[³H]-Glycine (60 Ci/mmol) and [³H]-Serine (11 Ci/mmol) were purchased from Moravek. Cells in 24-well plates were washed twice with the transport buffer (25 mmol/L HEPES/Tris, pH 7.5, 140 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 0.8 mmol/L MgSO₄, and 5 mmol/L glucose). Transport of 5 μmol/L glycine or serine (0.05 μmol/L [³H]-glycine or [³H]-serine plus 4.95 μmol/L unlabeled glycine or serine) was measured at 37°C for 2.5, 5.0, 10, and 15 minutes. Transport was terminated by aspiration of the transport buffer followed by 3 washes with 2 mL of ice-cold transport buffer. The cells were lysed with 0.5 mL of 1% SDS in 0.2 N NaOH and the radioactivity was determined by scintillation counting.

Metabolomics analysis

SHEP1-pCDH and SHEP1-pCDH-MYCN cells were washed with 5% mannitol and extracted with HPLC grade methanol (Thermo Fisher Scientific; A452-4) containing internal standards according to the instructions provided by Human Metabolome Technologies (HMT). Extracted metabolites were analyzed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) at HMT. Peaks were annotated with putative metabolites from the HMT metabolite database based on their MTs/RTs and *m/z* values determined by TOFMS. The tolerance range for the peak annotation was configured at ±0.5 minutes for MT and ±10 ppm for *m/z*. Peak areas were normalized against the internal standards and then the cell number. Three biological replicate samples (~10⁶ cells/sample) were analyzed for each cell type and *P* values were calculated by Welch *t* test.

Stable isotope flux analysis

BE(2)-C and SK-N-AS cells were cultured in glucose-deficient DMEM (11966-025; Invitrogen) supplemented with 10% dialyzed FBS and 20 mmol/L [U-¹³C]glucose (CLM-1396; Cambridge Isotope Laboratories) in the presence of DMSO or 10 μmol/L NCT-503 for 24 hours. Cells were collected by scraping and centrifugation, and cell pellets were washed once with ice-cold PBS, snap frozen in liquid nitrogen, and stored at -80°C until analysis. Four to six biological replicate samples (~5 × 10⁶ cells/sample) were analyzed for each condition and cell type. Metabolite extraction and deprivation, instrumentation, and data processing are described in Supplementary Information.

Microarray

Total RNA was isolated using TRIzol from 3 biological replicates of BE(2)-C cells treated with DMSO or 10 μmol/L NCT-503 for 48 hours. Affymetrix microarray was performed using the Human Gene 2.0 ST microarray chip. Data were normalized, significance determined by ANOVA, and fold change calculated with the Partek Genomics Suite. GO analysis was performed with DAVID (22) for all differentially expressed genes (≥±1.5 fold, *P* < 0.05), and GSEA was performed as described (25). The NCBI Gene Expression Omnibus (GEO) accession number for the microarray data is GSE120347.

Xenograft assay

BE(2)-C and SK-N-AS cells in 200 μL serum-free DMEM were injected subcutaneously into both flanks of 6-week-old male and female NOD/SCID mice at 2.5 × 10⁶ cells per injection site. Tumor volume was measured every other day using a digital caliper and estimated using the equation $V = (L \times W^2)/2$. Mice bearing tumors of ~100 mm³ were randomly divided into 2 groups (5 per group), stratified by gender and tumor volume, and treated with vehicle (40 μLDMSO) or 64 mg/kg (body weight) NCT-503 delivered daily by intraperitoneal injection for 10 days. Animals were euthanized when their tumors reach ~1.0 cm in diameter. The animal experiments were approved by the Institutional Animal Care and Use Committee of Medical College of Georgia, Augusta University.

Statistical analysis

Quantitative data are presented as mean ± SD and were analyzed for statistical significance by unpaired, 2-tailed Student *t* test or 2-way ANOVA. PHGDH inhibitor dose-response curves were fitted with the 4-parameter equation "log (inhibitor) vs. response - variable slope" and box plots were generated using the box and whiskers (Turkey) plot. For xenograft study, log-rank test was used to account for mouse survival. Unless otherwise stated, all statistical analyses were conducted using GraphPad Prism 7.0d for Mac.

Additional methods

Additional methods are provided in Supplementary Information.

Results

High expression of the SGOC gene signature is associated with neuroblastoma with MYCN amplification

High-risk neuroblastoma is a leading cause of pediatric cancer deaths, and MYCN amplification has a major role in its development (2, 26–28). To search for potential MYCN-dependent metabolic pathways, we performed GO analysis of the MYCN-amplification gene signature consisting of 143 genes upregulated in neuroblastoma tumors with MYCN amplification relative to those without (21). This analysis identified serine-glycine synthesis, one-carbon metabolism, and purine nucleotide biosynthesis as the most significantly upregulated metabolic processes in MYCN-amplified neuroblastoma (Fig. 1A). These metabolic processes are connected by the SGOC pathway (Fig. 1B; refs. 29, 30). Indeed, the MYCN-amplification gene signature contains several genes encoding enzymes within the SGOC pathway (21), including phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), mitochondrial serine hydroxymethyltransferase 2 (SHMT2), cytosolic methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 (MTHFD1), and mitochondrial methylenetetrahydrofolate dehydrogenase 2 (MTHFD2). These enzymes link the glycolytic intermediate 3-phosphoglycerate (3PG) to the production of serine, glycine, and the one-carbon carriers 5,10-methylene-tetrahydrofolate (5,10-MTHF) and 10-formyl-THF (Fig. 1B). Both glycine and 10-formyl-THF donate carbon, and glycine also contributes nitrogen, moieties for purine nucleotide synthesis; 5,10-MTHF is essential for *de novo* thymidylate production as a coenzyme of thymidylate synthase.

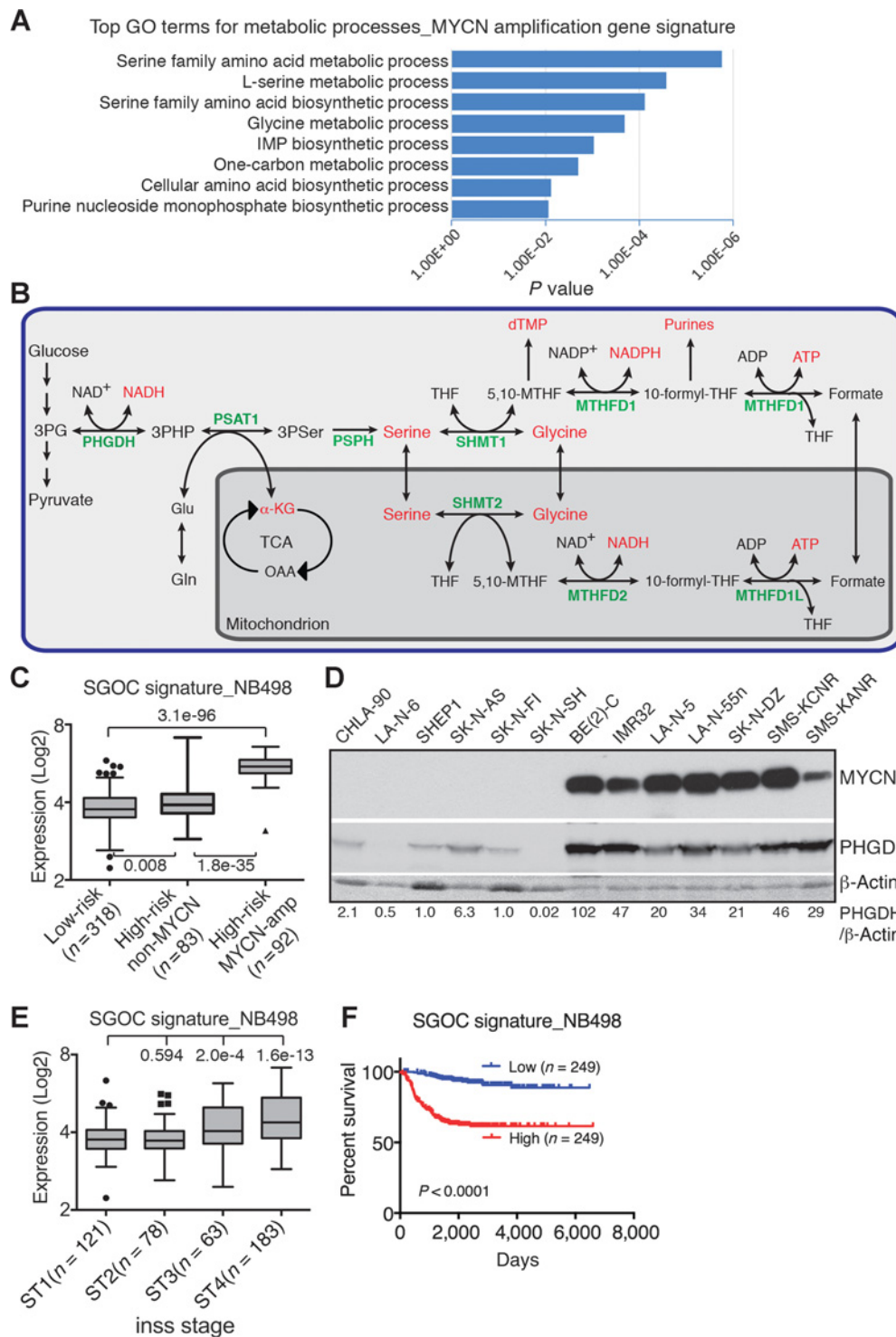


Figure 1. Expression of the SGOC genes is upregulated in *MYCN*-amplified high-risk neuroblastoma. **A**, Top GO terms related to metabolic processes for the *MYCN*-amplification gene signature ($n = 143$). **B**, SGOC metabolic pathway with enzymes indicated in green and key products in red. **C**, Box plot of average mRNA expression of the SGOC 6-gene signature in relation to neuroblastoma risk groups and *MYCN*-amplification status using the SEQC dataset. **D**, Immunoblotting of *MYCN* and *PHGDH* in neuroblastoma cell lines with or without *MYCN* amplification. *PHGDH* levels were quantified against β -actin, with the *PHGDH* level in SHEP1 cells being designated as 1.0. **E**, Box plot of average mRNA expression of the SGOC 6-gene signature in relation to neuroblastoma stages using the SEQC dataset. **F**, Kaplan-Meier survival curves for the SEQC cohort of 498 neuroblastoma patients based on the average mRNA expression of the SGOC 6-gene signature, with log-rank test P value indicated. Unless otherwise indicated, data were analyzed with 2-tailed Student t test, with P values indicated.

We further assessed the correlation between *MYCN* amplification and transcriptional reprogramming of SGOC metabolism in neuroblastoma by analyzing the gene expression profiling data from a cohort of patients with neuroblastoma ($n = 498$, the SEQC dataset; ref. 20). The average expression of the 6 SGOC genes (*PHGDH*, *PSAT1*, *SHMT2*, *MTHFD1*, *MTHFD1L*, and *MTHFD2*) was significantly higher in high-risk tumors with *MYCN* amplification compared with low-risk tumors and to high-risk tumors without *MYCN* amplification (Fig. 1C). We confirmed the observation by analyzing the expression of individual SGOC genes in neuroblastoma tumors with or without *MYCN* amplification (Supplementary Fig. S1A). In agreement with the findings from tumors, *MYCN*-amplified neuroblastoma cell lines expressed markedly higher levels of PHGDH protein in comparison with non-*MYCN*-amplified cell lines (Fig. 1D). This PHGDH expression pattern is specifically associated with *MYCN* amplification but is not a general feature of rapidly proliferating neuroblastoma cell lines as the non-*MYCN*-amplified cell lines SK-N-AS, SK-N-FI, and SHEP1, despite their low PHGDH expression (Fig. 1D), proliferated at similar or faster rates in comparison with the *MYCN*-amplified cell lines BE(2)-C, SK-N-DZ, and SMS-KCNR as determined by cell population doubling time (Supplementary Fig. S1B). Moreover, inhibition of the proliferation of neuroblastoma cell lines by serum deprivation did not reduce PHGDH protein expression (Supplementary Fig. S1C and S1D). Overexpression of PHGDH in non-*MYCN*-amplified cells had no significant impact on cell proliferation (Supplementary Fig. S1E and S1F).

Further analysis of the SEQC dataset revealed that higher expression of the SGOC gene signature is significantly associated with advanced stages of neuroblastoma and poor prognosis in patients with neuroblastoma (Fig. 1E and F). Together, these findings provide genetic evidence for transcriptional activation of the SGOC metabolic pathway in high-risk neuroblastoma with *MYCN* amplification.

MYCN transcriptionally activates SGOC metabolism

We next investigated whether *MYCN* is required for transcriptional activation of the SGOC genes. *MYCN* knockdown by shRNA in *MYCN*-amplified neuroblastoma cell lines reduced mRNA and protein expression of the SGOC genes (Fig. 2A and B; Supplementary S2A). Thus, *MYCN* has a major role in maintaining steady-state expression of the SGOC genes in these cell lines. In addition, *MYCN* depletion inhibited cell proliferation and induced differentiation characterized morphologically by neurite outgrowth (Supplementary Fig. S2B and S2C).

We next investigated whether an increase in *MYCN* level is sufficient to activate SGOC gene transcription. *MYCN* overexpression in non-*MYCN*-amplified neuroblastoma cell lines increased expression of the SGOC genes at the mRNA and protein levels (Fig. 2C–F) and enhanced cell proliferation (Supplementary Fig. S2D). Metabolomic profiling of non-*MYCN*-amplified SHEP1 cells with or without *MYCN* overexpression revealed that *MYCN* overexpression resulted in a significant increase in the intracellular levels of serine and glycine (Supplementary Fig. S2E) but had no effect on serine and glycine transport (Supplementary Fig. S2F), suggesting that *MYCN* overexpression enhanced biosynthetic activity of the SGOC metabolic pathway.

To determine whether *MYCN* directly targets the SGOC genes for transcriptional activation, we performed ChIP-qPCR assays. *MYCN* and other members of the MYC family bind to the

consensus E-box sequence CANNTG. We focused on *PHGDH*, *PSAT1*, *MTHFD1L*, and *MTHFD2*, which contain the canonical E-box sequence CACGTG in their promoters or first introns (Fig. 2G). ChIP-qPCR analysis of the *MYCN*-amplified BE(2)-C cells showed significant levels of endogenous *MYCN* associated with the E-box sequences (Fig. 2H). We also detected significant levels of *MYCN* in the proximal promoter regions of *PHGDH*, *PSAT1*, *MTHFD1L*, and *MTHFD2* (Fig. 2I). We obtained essentially the same results with the *MYCN*-amplified SMS-KCNR cells (Supplementary Fig. S2G and S2H) and the non-*MYCN*-amplified SHEP1 cells with *MYCN* overexpression (Supplementary Fig. S2I). Collectively, these findings suggest that the SGOC genes are direct transcriptional targets of *MYCN*.

ATF4 is a transcriptional activator of the SGOC genes in *MYCN*-amplified cells

ATF4 has a major role in transcriptional activation of enzymes within the serine-glycine synthesis pathway (31–35). Also, it has been shown recently that ATF4 acts downstream of mTORC1 and NRF2 in transcriptional activation of one-carbon flux and nucleotide biosynthesis (36, 37). Given the above findings on *MYCN*, we wondered if ATF4 has a significant role in transcriptional regulation of the SGOC genes in *MYCN*-amplified cells. Analysis of the SEQC dataset revealed a strong positive correlation in mRNA expression levels between *ATF4* and the SGOC genes, except for *PSPH* and *SHMT1* (Supplementary Fig. S3A). Overexpression of ATF4 in both *MYCN*-amplified and non-*MYCN*-amplified cell lines increased SGOC gene expression at the mRNA and protein levels (Fig. 3A and B; Supplementary Fig. S3B and S3C). Conversely, ATF4 depletion by shRNA in *MYCN*-amplified cell lines markedly decreased mRNA and protein expression of the SGOC genes (Fig. 3C and D) and inhibited cell proliferation (Supplementary Fig. S3D). Interestingly, ATF4 overexpression had no significant effect on the proliferation of non-*MYCN*-amplified cell lines (Supplementary Fig. S3E) but significantly enhanced the proliferation of *MYCN*-amplified cell lines (Supplementary Fig. S3F), suggesting that ATF4 might cooperate with *MYCN* in driving cell proliferation. Collectively, these findings indicate an essential role of ATF4 in maintaining SGOC gene expression and sustaining the proliferation of *MYCN*-amplified neuroblastoma cells.

To determine whether ATF4 directly targets the SGOC genes in *MYCN*-amplified cells, we performed ChIP-qPCR analysis of BE(2)-C_tetoff-ATF4 cells. In the absence of induction of exogenous ATF4, significant levels of endogenous ATF4 were found at the promoters of *PHGDH*, *PSAT1*, *SHMT2*, *MTHFD1L*, and *MTHFD2*, as well as those of *ASNS* and *DDIT3*, 2 classic ATF4 target genes (Fig. 3E; refs. 38, 39). Induction of ATF4 further increased the levels of ATF4 at these sites, except for the *MTHFD2* promoter (Fig. 3E). In addition, ATF4 knockdown completely abolished the binding of endogenous ATF4 to the promoters of *PSAT1*, *MTHFD2*, and *DDIT3* (Supplementary Fig. S3G). Thus, the SGOC genes are direct transcriptional targets of ATF4.

ATF4 is a direct target gene of *MYCN* and is required for *MYCN* induction of SGOC genes

Because both *MYCN* and ATF4 are required for transcriptional activation of the SGOC genes in *MYCN*-amplified cells, we investigated how they coordinate their activities in this process. Analysis of the SEQC dataset revealed significantly higher *ATF4*

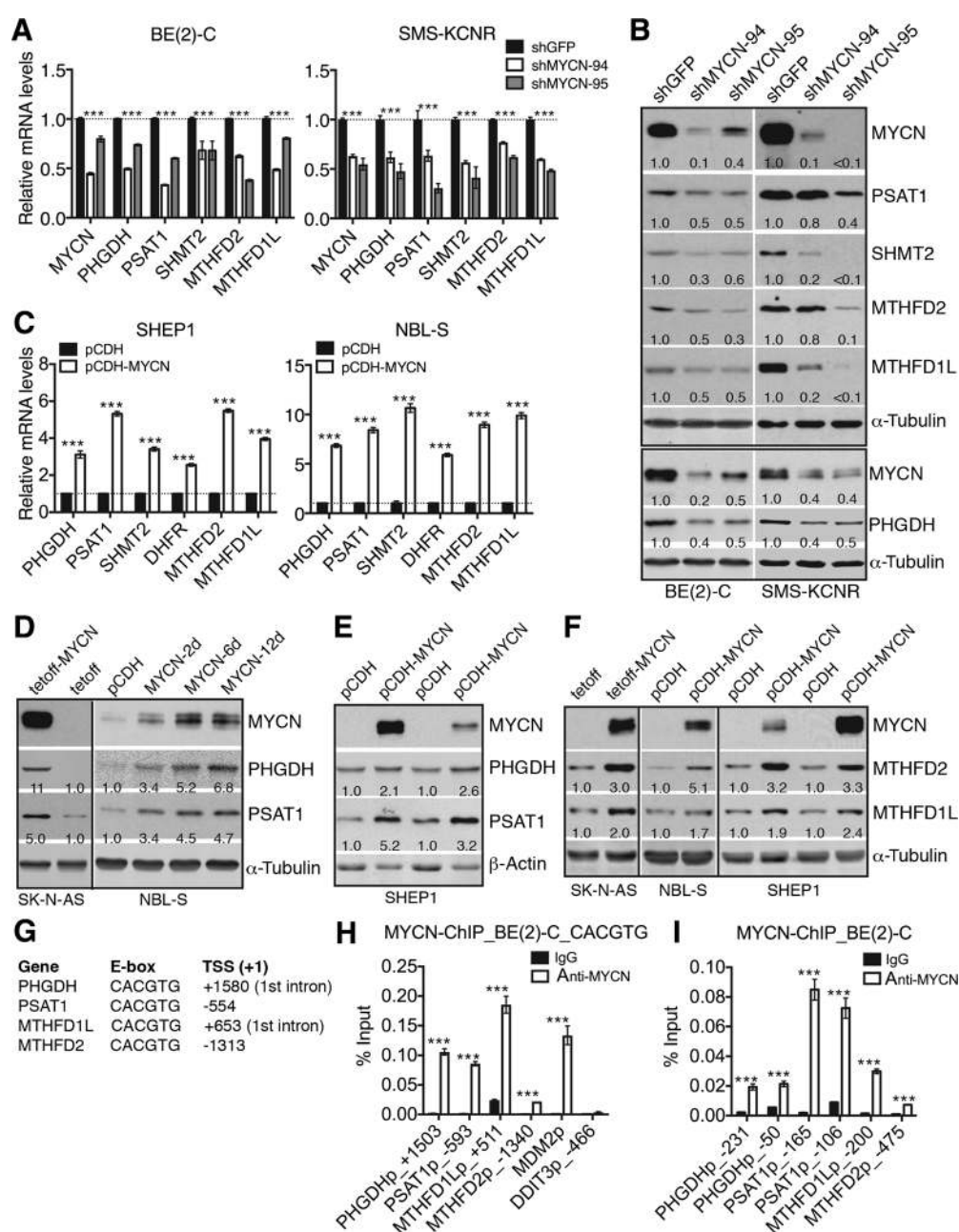


Figure 2.

MYCN transcriptionally activates the SGOC genes. **A** and **B**, qRT-PCR (**A**) and immunoblot (**B**) analyses of SGOC gene expression in *MYCN*-amplified cell lines expressing shRNA to GFP or MYCN. **C**, qRT-PCR analysis of SGOC gene mRNA expression in non-*MYCN*-amplified cell lines without (pCDH) or with (pCDH-MYCN) MYCN overexpression. **D-F**, Immunoblotting of SGOC enzymes in non-*MYCN*-amplified cell lines with constitutive (pCDH-MYCN) or inducible (tetoff-MYCN) MYCN expression. **G**, E-box sequences within the indicated SGOC genes relative to their TSS. **H** and **I**, ChIP-qPCR showing endogenous MYCN binding to the E-box sequences (**H**) and 5' regions (**I**) of the indicated SGOC gene promoters in BE(2)-C cells. The *MDM2* and *DDIT3* promoters were used as positive and negative control, respectively. Error bars represent SD ($n = 3$), and data were analyzed with 2-tailed Student *t* test. Protein levels were quantified against α -tubulin or β -actin. ***, $P < 0.001$.

mRNA levels in tumors with *MYCN* amplification than those without (Supplementary Fig. S4A) and a positive correlation in mRNA expression levels between *ATF4* and *MYCN* (Supplementary Fig. S4B), suggesting that one might regulate the expression of the other or that both are transcriptionally regulated by a common mechanism. To test these models, we examined the abilities of

ATF4 and *MYCN* to regulate the expression of each other. *ATF4* overexpression had no significant effect on *MYCN* mRNA expression (Fig. 4A), whereas *MYCN* overexpression significantly upregulated mRNA and protein expression of *ATF4* (Fig. 4B and C). In addition, *MYCN* knockdown led to a significant reduction in *ATF4* mRNA and protein levels (Fig. 4D and E). Thus, *MYCN* is a

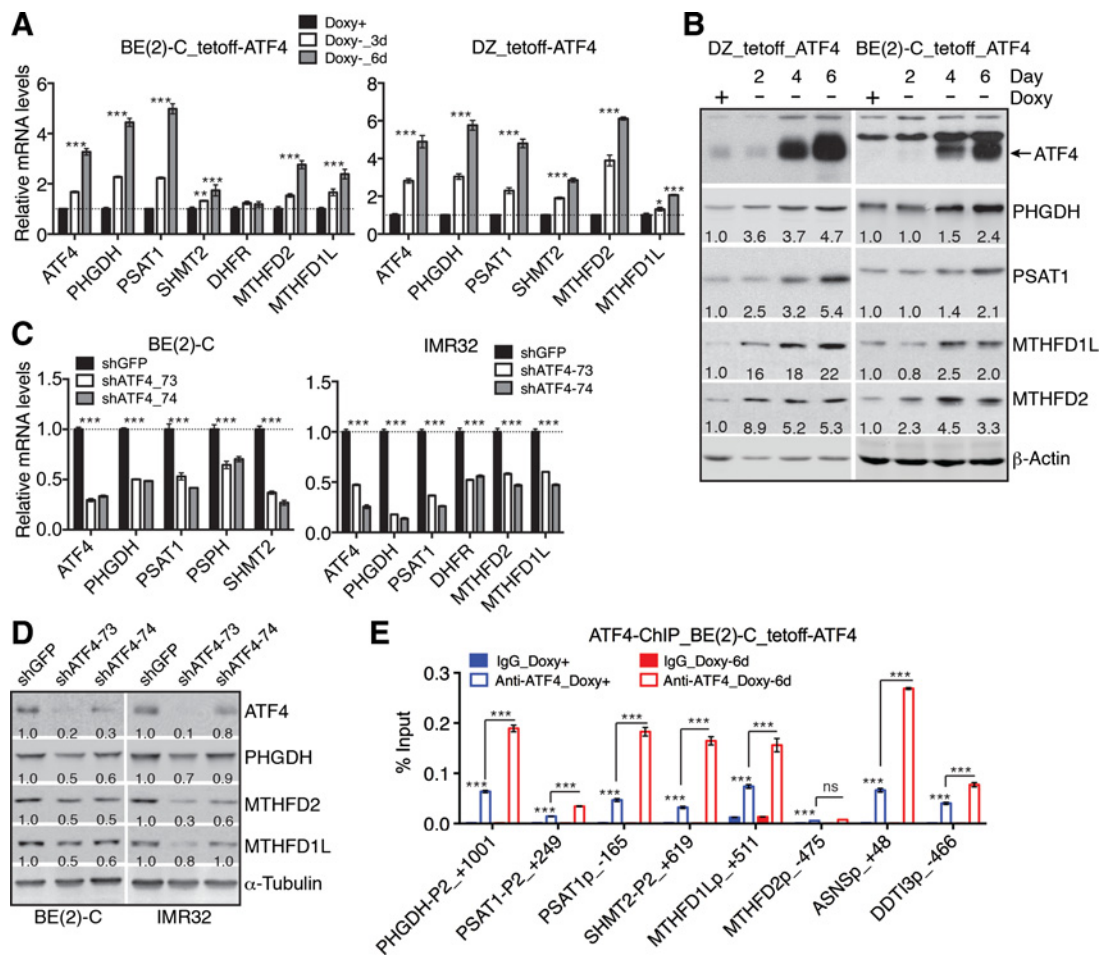


Figure 3.

ATF4 is a key transcriptional activator of SGOC gene expression in MYCN-amplified neuroblastoma cells. **A** and **B**, qRT-PCR (**A**) and immunoblot (**B**) analyses of SGOC gene expression in MYCN-amplified cell lines without (Doxy+) or with (Doxy-) ATF4 induction. **C** and **D**, qRT-PCR (**C**) and immunoblot (**D**) analyses of SGOC gene expression in MYCN-amplified cell lines expressing shRNA to GFP or ATF4. **E**, ChIP-qPCR showing ATF4 binding to the indicated SGOC gene promoters in BE(2)-C cells without (Doxy+) or with (Doxy-6d) ATF4 induction. The *ASNS* and *DDT13* promoters were used as positive control. Error bars represent SD ($n = 3$), and data were analyzed with 2-tailed Student *t* test. Protein levels were quantified against α -tubulin or β -actin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

key transcriptional activator of ATF4 expression in neuroblastoma cells.

The ATF4 gene contains 2 canonical E-box sequences (CACGTG), one in the first intron (+767) and the other in the 3' region (+3915). ChIP-qPCR analysis demonstrated significant levels of endogenous MYCN binding to these regions (Fig. 4F, ATF4p₋+687 and ATF4p₋+3818). In addition, we detected significant levels of MYCN at the ATF4 promoter 5' region (Fig. 4F). Thus, ATF4 is a direct target gene of MYCN.

Collectively, these observations suggest that ATF4 functions downstream of MYCN in transcriptional activation of the SGOC genes. In support of this model, ATF4 knockdown significantly abrogated the ability of MYCN to induce the SGOC genes (Fig. 4G), indicating an essential role of ATF4 in mediating transcriptional activation of the SGOC genes by MYCN.

ATF4 stabilizes MYCN protein

During our ChIP-qPCR studies with BE(2)-C_tetoff-ATF4 cells (Fig. 3E), we unexpectedly observed that following ATF4

induction, there was a significant increase in MYCN levels at the promoters of the SGOC genes (Fig. 5A) and ATF4 (Supplementary Fig. S4C). It has been shown previously that elevated levels of MYC lead to increased MYC occupancy at the promoters of active genes (40, 41). Because ATF4 had no effect MYCN mRNA expression (Fig. 4A), we asked whether it affects MYCN protein expression. Immunoblotting revealed that ATF4 overexpression increased MYCN protein levels (Fig. 5B), whereas ATF4 knockdown reduced MYCN protein expression (Fig. 5C) and increased MYCN protein ubiquitination (Fig. 5D), leading to a decrease in MYCN occupancy at the SGOC gene promoters (Supplementary Fig. S4D). Moreover, ATF4 overexpression resulted in an average of 2.5-fold increase in MYCN protein half-life (Fig. 5E and F). Collectively, these data suggest that ATF4 increases MYCN protein levels by blocking its degradation.

FBXW7 is a component of the SKP1/CUL1/FBXW7 E3 ubiquitin ligase complex that targets MYCN for proteasomal degradation (42, 43). As expected, we found that FBXW7 overexpression

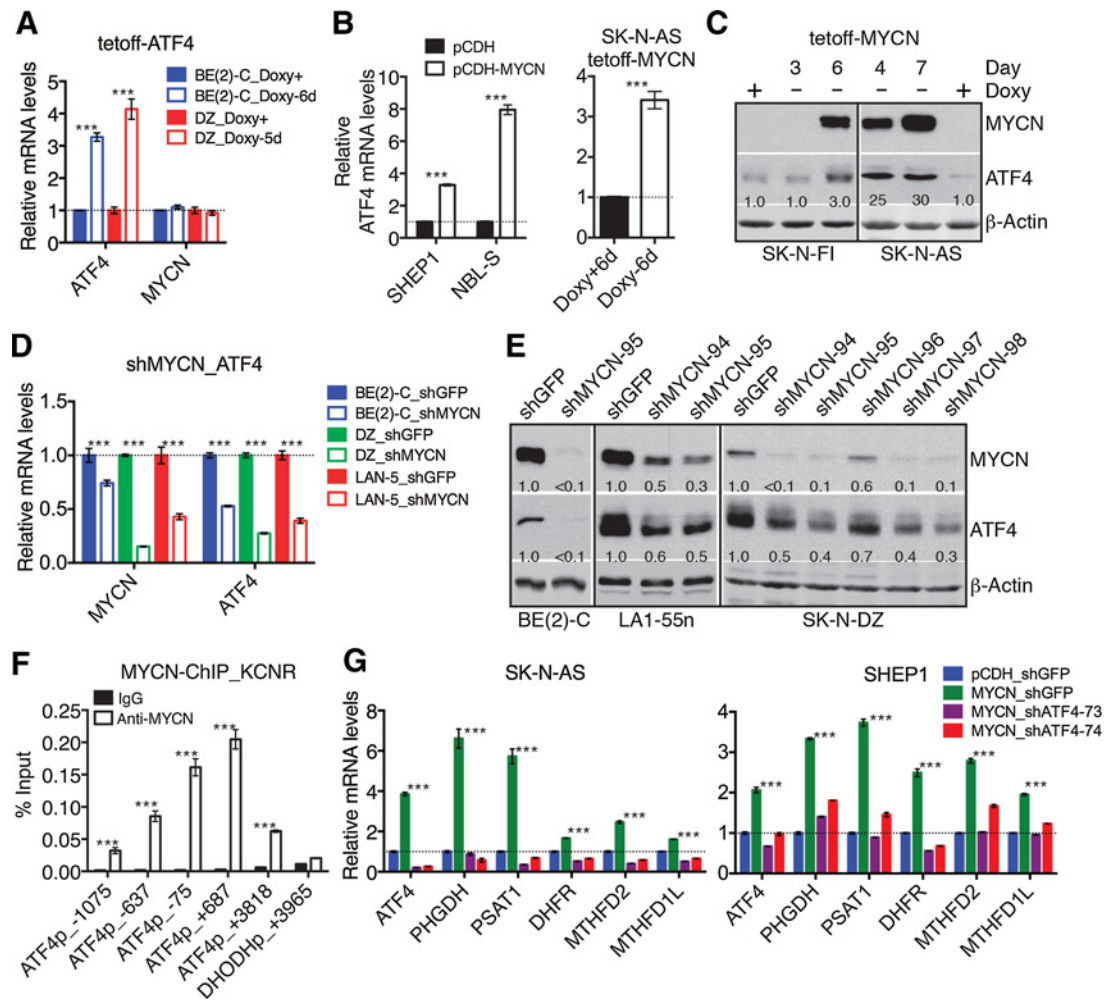


Figure 4. ATF4 is a direct target gene of MYCN and is required for MYCN induction of the SGOC genes. **A**, qRT-PCR analysis of *ATF4* and *MYCN* mRNA expression in BE(2)-C and SK-N-DZ cells without (Doxy+) or with (Doxy-6d) ATF4 induction. **B** and **C**, qRT-PCR (**B**) and immunoblot (**C**) analyses of ATF4 mRNA and protein levels in non-MYCN-amplified cell lines with constitutive (pCDH-MYCN) or inducible (tetoff-MYCN, Doxy-) MYCN expression. **D** and **E**, qRT-PCR (**D**) and immunoblot (**E**) analyses of ATF4 mRNA and protein levels in MYCN-amplified cell lines expressing shRNA to GFP or MYCN. **F**, ChIP-qPCR showing endogenous MYCN binding to multiple sites in the *ATF4* gene in MYCN-amplified SMS-KCNR cells. The *DHODH* 3' region was used as negative control. **G**, qRT-PCR analysis of mRNA levels of *ATF4* and SGOC genes in vector (pCDH) or MYCN-overexpressing (MYCN) cell lines expressing shRNA to GFP or ATF4. Error bars represent SD ($n = 3$), and data were analyzed with 2-tailed Student *t* test. ATF4 and MYCN protein levels were quantified against β -actin. ***, $P < 0.001$.

markedly reduced MYCN protein levels in MYCN-amplified cells (Fig. 5G; Supplementary Fig. S4E), leading to decreased protein levels of SGOC enzymes (Supplementary Fig. S4E). ATF4 overexpression was sufficient to neutralize the effect of FBXW7, leading to increased MYCN protein expression (Fig. 5G) and reduced MYCN protein ubiquitination (Fig. 5H). Conversely, FBXW7 depletion, which had no effect on MYCN mRNA expression (Supplementary Fig. S4F), significantly increased MYCN protein expression, leading to higher PHGDH expression (Fig. 5I). Moreover, when FBXW7 expression was silenced, ATF4 knockdown could no longer increase MYCN protein ubiquitination (Fig. 5I). Thus, both overexpression and knockdown studies suggest that ATF4 stabilizes MYCN protein by antagonizing FBXW7-mediated MYCN ubiquitination and subsequent degradation. These findings, along with those showing that MYCN transcriptionally activates ATF4 expression (Fig. 4B

and C), provide evidence for a MYCN-ATF4 positive feedback loop for transcriptional activation of the SGOC metabolic pathway in MYCN-amplified cells.

MYCN sensitizes neuroblastoma cells to PHGDH inhibition

The observation that the SGOC genes are transcriptionally activated in MYCN-amplified relative to non-MYCN-amplified neuroblastoma cells led us to hypothesize that inhibition of this metabolic pathway might be synthetically lethal with MYCN amplification. We tested this hypothesis in a panel of neuroblastoma cell lines with MYCN amplification [BE(2)-C, LA1-55n, SK-N-DZ, and SMS-KCNR] or without (NBL-S, SHEP1, SH-SY5Y, SK-N-AS, and SK-N-FI). Of note, BE(2)-C and SMS-KCNR cells were derived from relapsed tumors after chemotherapy (44) and, as expected, both cell lines were highly resistant to the chemotherapy drug doxorubicin in comparison with the non-MYCN-amplified

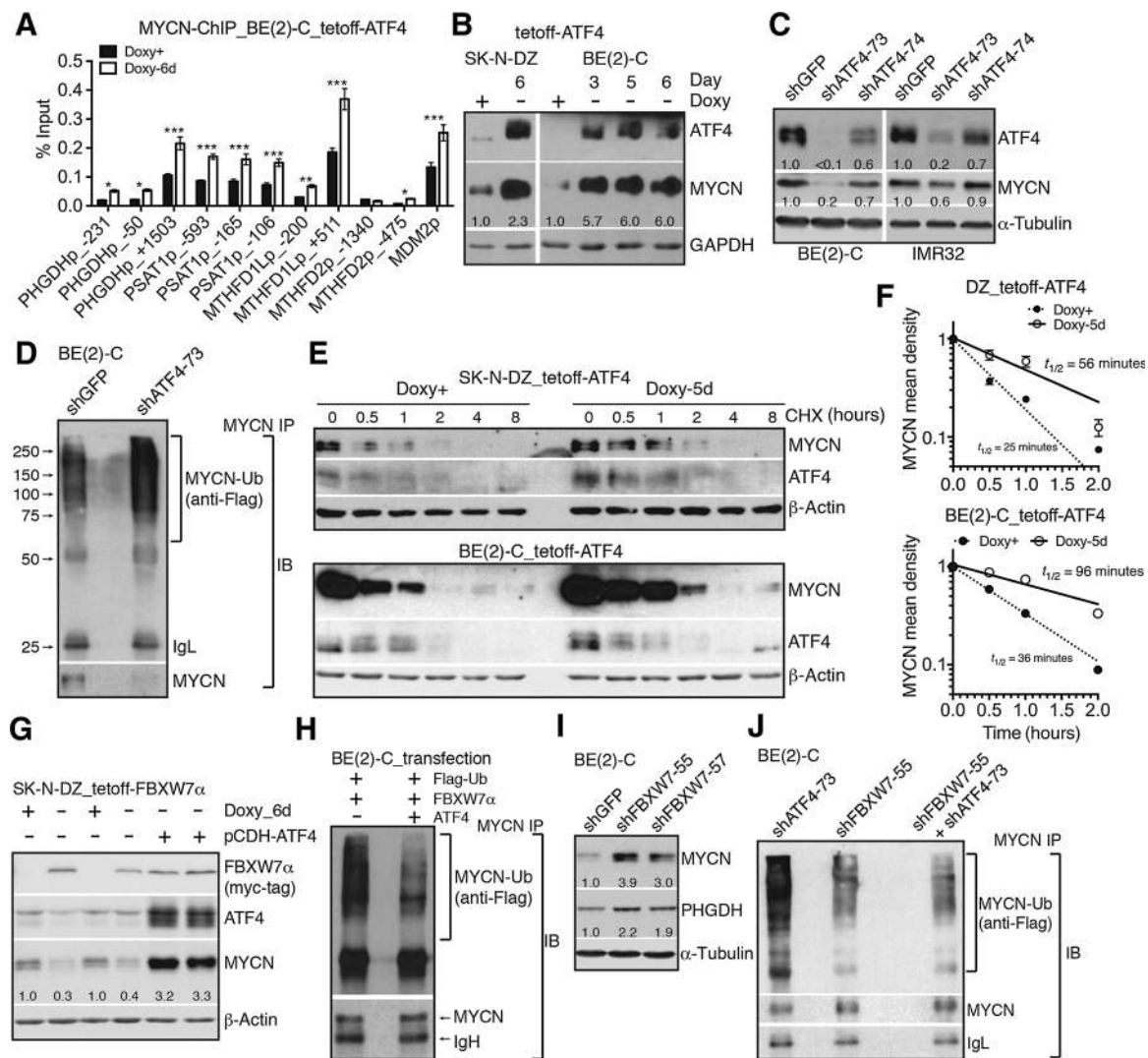


Figure 5.

ATF4 stabilizes MYCN protein. **A**, ChIP-qPCR showing increased MYCN occupancy at the SGOC gene promoters following ATF4 induction (Doxy-6d vs. Doxy+). **B**, Immunoblotting showing increased MYCN protein levels following ATF4 induction (Doxy- vs. Doxy+). **C** and **D**, Immunoblotting showing decreased MYCN protein levels (**C**) and increased MYCN ubiquitination (**D**) following ATF4 knockdown. **E** and **F**, Immunoblotting (**E**) and quantification (**F**) of MYCN protein half-life in BE(2)-C and SK-N-DZ cells without (Doxy+) or with (Doxy-5d) ATF4 induction. Samples were collected at various time points following addition of cycloheximide (CHX). MYCN protein levels were quantified against β -actin and are presented as the fraction of the initial levels at time zero. **G**, Immunoblotting of the indicated proteins in SK-N-DZ cells without (Doxy+) or with (Doxy-6d) FBXW7 α induction and ATF4 overexpression (pCDH-ATF4). **H**, Immunoblotting of MYCN ubiquitination in BE(2)-C cells transfected with plasmids expressing Flag-ubiquitin, FBXW7 α , and/or ATF4. **I**, Immunoblotting showing increased MYCN and PHGDH protein expression following FBXW7 knockdown. **J**, Immunoblotting of MYCN ubiquitination in BE(2)-C cells infected with lentiviruses expressing shATF4-73, shFBXW7-55, or both. Error bars represent SD ($n = 3$), and data were analyzed with 2-tailed Student t test. Protein levels were quantified against α -tubulin, β -actin, or GAPDH. *, $P < 0.05$; ***, $P < 0.001$. IgH, immunoglobulin heavy chain.

cell lines SK-N-AS, SK-N-FI, and SH-SY5Y and the MYCN-amplified cell lines LA1-55n and SK-N-DZ (Fig. 6A).

We treated these cell lines with NCT-503, a small molecule inhibitor of PHGDH (45). NCT-503 ($EC_{50} = 8\text{--}16 \mu\text{mol/L}$) at 10 to 20 $\mu\text{mol/L}$ markedly inhibited the growth and survival of MYCN-amplified cell lines, including the chemoresistant BE(2)-C and SMS-KCNR cells, but had no significant or only a modest effect on non-MYCN-amplified cell lines (Fig. 6B and C; Supplementary Fig. S5A). This effect of NCT-503 was neither a result of nonspecific inhibition of MYCN expression (Supplementary Fig. S5B) nor dependent on the proliferation state since

serum deprivation, which markedly inhibited the proliferation of both non-MYCN- and MYCN-amplified cell lines (Supplementary Fig. S1C), had no significant effect on the differential sensitivities of non-MYCN- and MYCN-amplified cell lines to NCT-503 (Supplementary Fig. S5C). Treatment with NCT-503 negative control compound showed no significant effect on all the cell lines examined.

Consistent with the previous report (45), we observed the growth-inhibiting effect of NCT-503 in the presence (Fig. 6B) or absence (Fig. 6C) of supplemental serine. In addition, non-MYCN-amplified (resistant) SK-N-AS cells and MYCN-amplified

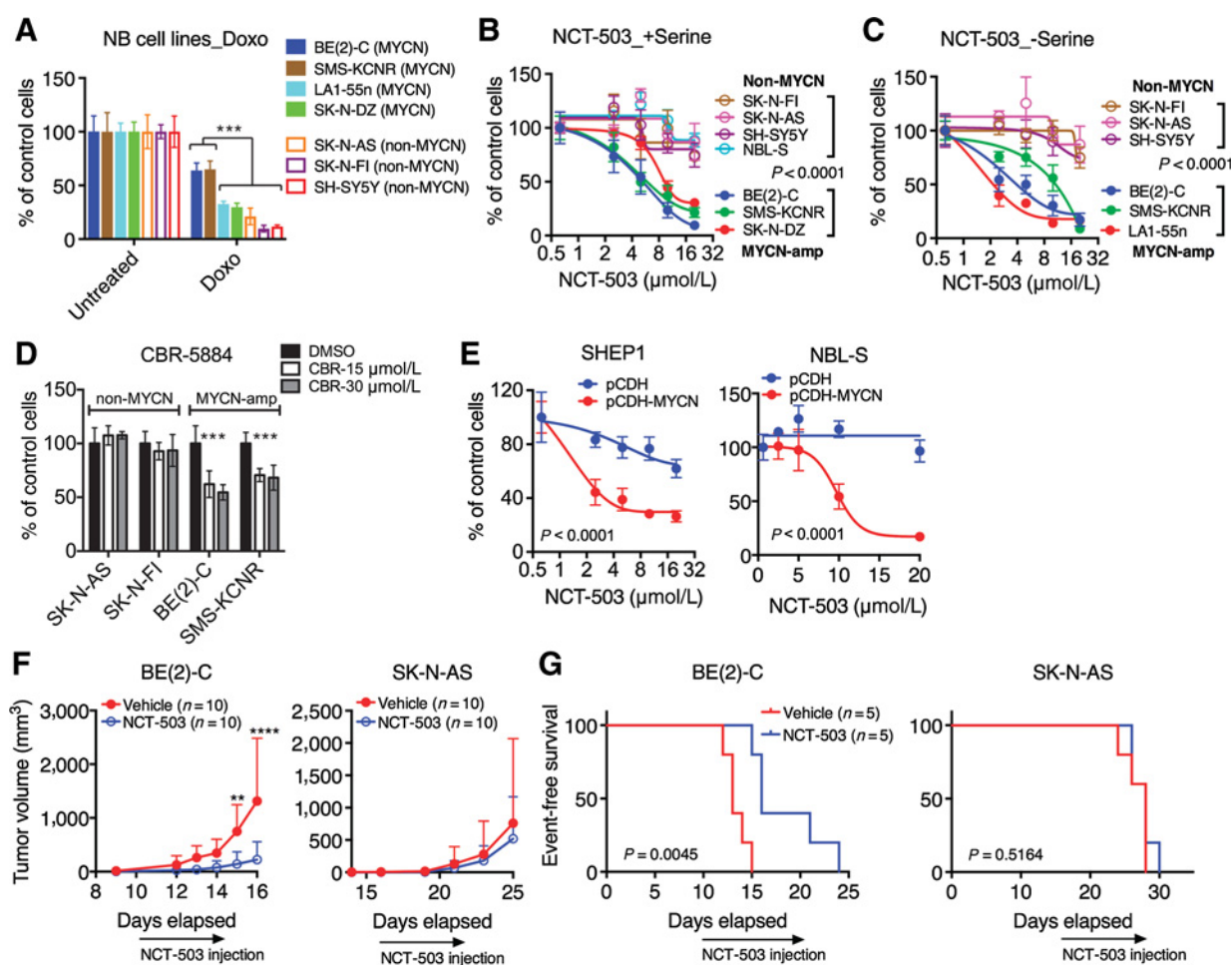


Figure 6. MYCN sensitizes neuroblastoma cells to PHGDH inhibitors. **A**, Survival assays of neuroblastoma cell lines treated with doxorubicin (Doxo) at 0.5 $\mu\text{g}/\text{mL}$ for 2 days. **B–D**, Survival assays of MYCN-amplified vs. non-MYCN-amplified cell lines treated with increasing concentrations of NCT-503 (**B** and **C**) for 2 days or CBR-5884 (**D**) for 3 days in the presence (**B** and **D**) or absence (**C**) of 0.4 mmol/L serine. **E**, Survival assays of SHEP1 and NBL-S cells without (pCDH) or with MYCN (pCDH-MYCN) overexpression treated with increasing concentrations of NCT-503 for 2 days. **F** and **G**, Tumor growth (**F**) and event-free survival (**G**) curves for mice with BE(2)-C or SK-N-AS xenografts treated with vehicle or NCT-503 at 64 mg/kg for 10 days. Treatment started when tumors reached $\sim 100 \text{ mm}^3$. Error bars in **A–E**, SD ($n = 4$). Data were analyzed by 2-tailed Student *t* test (**A** and **D**), 2-way ANOVA (**B**, **C**, **E**, and **F**), or log-rank test (**G**). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

(sensitive) BE(2)-C cells showed no difference in their ability to uptake glycine, whereas BE(2)-C cells displayed a slightly higher activity in transporting serine (Supplementary Fig. S5D). Moreover, NCT-503 treatment modestly inhibited glycine and serine transport of SK-N-AS cells but had no effect on BE(2)-C cells (Supplementary Fig. S5E). Together, these findings suggest that serine and glycine transport is not a major factor in determining the sensitivity of non-MYCN- and MYCN-amplified cell lines to NCT-503.

We confirmed the sensitivity of MYCN-amplified neuroblastoma cells to PHGDH inhibition in parallel studies with another small molecule inhibitor of PHGDH, CBR-5884 (46). Compared with non-MYCN-amplified cell lines, MYCN-amplified cell lines were also significantly more sensitive to 15 to 30 $\mu\text{mol}/\text{L}$ CBR-5884 (Fig. 6D). In addition, MYCN-amplified BE(2)-C and SMS-KCNR cells were significantly more sensitive to PHGDH knockdown than non-MYCN-amplified SHEP1 and SK-N-FI cells

(Supplementary Fig. S5F and S5G). Importantly, MYCN overexpression was sufficient to sensitize non-MYCN-amplified SHEP1 and NBL-S cells to NCT-503 (Fig. 6E; Supplementary Fig. S5H).

We next evaluated NCT-503 antitumor activity in mouse xenograft models established with MYCN-amplified BE(2)-C cells and non-MYCN-amplified SK-N-AS cells. Tumor-bearing mice were treated with vehicle or 64 mg/kg (body weight) of NCT-503 by daily intraperitoneal injection for 10 days or until sacrifice. NCT-503 treatment significantly reduced the growth MYCN-amplified BE(2)-C xenografts [Fig. 6F, BE(2)-C], and prolonged the survival of tumor-bearing mice [Fig. 6G, BE(2)-C]. By contrast, NCT-503 treatment of mice bearing non-MYCN-amplified SK-N-AS xenografts showed no significant effect on tumor growth and mouse survival (Fig. 6F and G, SK-N-AS). In all cases, NCT-503 treatment at the indicated dose and time had no significant effect on mouse body weight (Supplementary Fig. S5I).

Collectively, these findings suggest that increased MYCN expression in neuroblastoma cells imposes a dependence on the SGOC metabolic pathway for survival and proliferation.

PHGDH inhibition triggers metabolic stress in MYCN-amplified cells

The SGOC pathway links glycolysis to the production of serine, glycine, and one-carbon units. To elucidate the metabolic basis for the differential sensitivities of MYCN-amplified and non-MYCN-amplified cells to NCT-503, we performed stable isotope flux analysis of glucose carbon into serine and glycine synthesis (Fig. 7A). NCT-503 treatment significantly reduced the production of m+3 serine and m+2 glycine from [U-¹³C]glucose in MYCN-amplified BE(2)-C cells (Fig. 7B and C, DMSO vs. NCT-503). By contrast, no such effect was observed in non-MYCN-amplified SK-N-AS cells (Fig. 7B and C). Importantly, the proportions of serine and glycine derived from [U-¹³C]glucose (m+3/m+0 serine and m+2/m+0 glycine) were significantly higher in BE(2)-C cells relative to SK-N-AS cells (Fig. 7B and C, BE(2)-C vs. AS). Taken together, these data indicate that MYCN-amplified cells rely more on glucose-derived carbon for serine and glycine synthesis in comparison with non-MYCN-amplified cells, suggesting a metabolic basis for their differential sensitivities to PHGDH inhibition.

To gain a molecular understanding for the selective cytotoxic effect of NCT-503 on MYCN-amplified cells, we performed microarray gene expression profiling of BE(2)-C cells treated with DMSO or 10 μmol/L NCT-503. A total of 3,655 NCT-503-responsive genes ($\geq \pm 1.50$ fold, $P < 0.05$) were identified, with 1,223 genes being upregulated and 2,432 genes downregulated (Supplementary Table S1). GO analysis revealed that genes downregulated by NCT-503 treatment were highly enriched for GO terms associated with DNA replication and G₁- to S-phase transition (Supplementary Fig. S6A; Supplementary Table S2). We obtained essentially the same result with gene set enrichment analysis (GSEA), which showed downregulation of a large number of genes involved in DNA replication and G₁-S cell-cycle transition (Supplementary Fig. S6B). As reported previously (45), NCT-503 treatment induced G₁ arrest (Supplementary Fig. S6C), consistent with a key role of the SGOC pathway in supplying nucleotides for DNA replication.

For genes upregulated by NCT-503 treatment, GO analysis showed a significant enrichment for GO terms associated with various stress responses, including glucose starvation, endoplasmic reticulum stress, and the unfolded protein response, and oxidative stress (Fig. 7D; Supplementary Table S2). Similarly, GSEA revealed that genes involved in the amino acid deprivation response were most significantly enriched and upregulated (Fig. 7E; Supplementary Fig. S6D). These stress-responsive genes, including *ASNS*, *ASS1*, *ATF3*, *CTH*, *DDIT3*, *TRIB3*, and *XBP1* (Supplementary Fig. S6D; Supplementary Tables S1 and S2), are induced by ATF4 in response to various stress signals (19). In addition, several genes within the SGOC pathway were induced by NCT-503, including *PHGDH*, *PSAT1*, *SHMT2*, and *MTHFD2* (Supplementary Table S1). These data suggest that in MYCN-amplified cells, NCT-503 treatment elicited an adaptive response to metabolic stress induced by inhibition of SGOC synthesis.

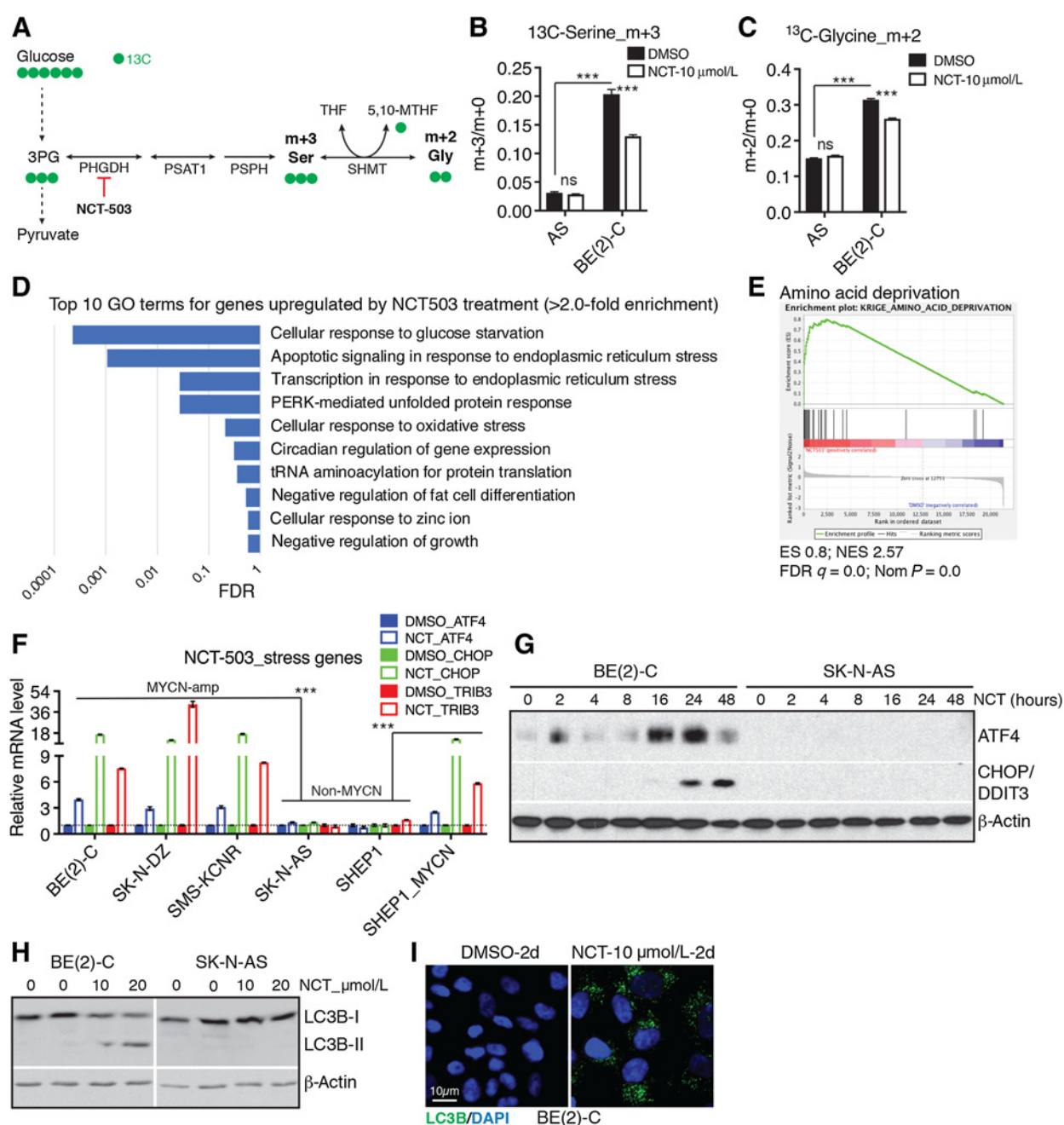
We verified the microarray data by qRT-PCR and immunoblotting, which showed that NCT-503 treatment markedly induced mRNA and protein expression of ATF4, DDIT3 (also known as CHOP) and/or TRIB3 in MYCN-amplified, but not in non-

MYCN-amplified, cell lines (Fig. 7F and G; Supplementary S6E). Overexpression of MYCN, which sensitized non-MYCN-amplified cells to NCT-503 (Fig. 6E) was sufficient to confer NCT-503 induction of *ATF4*, *DDIT3*, and *TRIB3* in non-MYCN-amplified SHEP1 cells (Fig. 7F). Moreover, NCT-503 treatment induced autophagy in MYCN-amplified cells, but not in non-MYCN-amplified cells, as evidenced by increased LC3B-II production and the formation of LC3B-positive puncta (Fig. 7H and I; Supplementary Fig. S6F). Together, these findings suggest that PHGDH inhibition induces metabolic stress in MYCN-amplified neuroblastoma cells, leading to G₁ arrest and autophagy.

Discussion

Our study reveals a MYCN-dependent vulnerability to suppression of the SGOC metabolic pathway. PHGDH inhibition triggered cell-cycle arrest and a metabolic stress response with autophagy in MYCN-amplified neuroblastoma cells, but not in non-MYCN-amplified cells. This increased sensitivity to PHGDH inhibition can be recapitulated by overexpression of MYCN in non-MYCN-amplified cells. Mechanistically, we show that in comparison with non-MYCN-amplified cells, MYCN-amplified cells, likely owing to their higher expression of PHGDH and other SGOC pathway enzymes, have an increased dependence on glucose-derived carbon for the synthesis of serine and glycine (and possibly also one-carbon units as suggested previously; ref. 45). In addition, a recent study showed that PHGDH inhibition in cells with high PHGDH expression could disrupt central carbon metabolism (47), which may help to explain the failure of supplemental serine to rescue the cytotoxic effect of NCT-503. Collectively, these findings suggest a metabolic basis for the differential sensitivities of MYCN-amplified and non-MYCN-amplified neuroblastoma cells to PHGDH inhibitors.

MYCN amplification has a major role in the development of high-risk neuroblastoma, a leading cause of pediatric cancer deaths (2, 26–28). However, targeting MYCN directly for therapy has proved to be challenging (16). An alternative strategy is to target metabolic pathways that are activated by MYCN for sustaining cancer cell growth and proliferation. In support of this idea, we found that blocking SGOC metabolism selectively inhibits the proliferation and survival of MYCN-amplified neuroblastoma cells. Also, a recent study reported that MYCN-amplified neuroblastomas display increased folate dependence and are more sensitive to methotrexate, an inhibitor of dihydrofolate reductase that functions to maintain the folate-one-carbon loop for thymidylate synthesis (48). Together, these findings suggest that targeting MYCN-activated metabolic pathways could be a selective therapeutic strategy against MYCN-amplified tumors. In addition, our findings have implications in treatment of patients with relapsed neuroblastomas carrying MYCN amplification. Although a majority of patients with high-risk neuroblastoma respond to initial therapy, approximately half of them will eventually have a relapse with resistant to further therapies (27). Currently, there are no effective treatment regimens for these patients. We found that PHGDH inhibitors are effective in inhibiting the growth of chemoresistant BE(2)-C and SMS-KCNR cells and of BE(2)-C xenograft tumors. Both BE(2)-C and SMS-KCNR cell lines were derived originally from relapsed tumors after chemotherapy (44). Thus, blocking SGOC metabolism might provide a treatment strategy for relapsed neuroblastomas with MYCN amplification.

**Figure 7.**

PHGDH inhibition reduces glucose flux into the SGOC pathway and induces metabolic stress in *MYCN*-amplified cells. **A**, Diagram for incorporation of ^{13}C from glucose into serine and glycine. **B** and **C**, [^{13}C]glucose flux analysis in non-*MYCN*-amplified SK-N-AS and *MYCN*-amplified BE(2)-C cells treated with DMSO or 10 $\mu\text{mol/L}$ NCT-503 for 24 hours. Shown are the ratio of ^{13}C -labeled m+3 serine or m+2 glycine to unlabeled serine or glycine. Error bars, SD ($n = 4-6$ biological replicates). **D** and **E**, GO analysis of NCT-503-upregulated (**D**) and GSEA of NCT-503-responsive (**E**) genes in BE(2)-C cells treated with 10 $\mu\text{mol/L}$ NCT-503 for 48 hours. **F**, qRT-PCR analysis of stress-responsive genes in *MYCN*-amplified and non-*MYCN*-amplified cell lines treated with DMSO or 10 $\mu\text{mol/L}$ NCT-503 for 48 hours. Error bars, SD ($n = 3$). **G**, Time-course analysis of ATF4 and CHOP (also known as DDIT3) protein expression in BE(2)-C and SK-N-AS cells treated with 10 $\mu\text{mol/L}$ NCT-503. **H**, Immunoblotting of LC3B in BE(2)-C and SK-N-AS cells treated with the indicated concentrations of NCT-503 for 48 hours. **I**, Immunofluorescence of LC3B-positive autophagosomes in BE(2)-C cells treated with 10 $\mu\text{mol/L}$ NCT-503 for 2 days. Data (**B**, **C**, and **F**) were analyzed by 2-tailed Student *t* test. ***, $P < 0.001$.

ATF4 is a master regulator of amino acid metabolism and stress responses (17–19). We found that MYCN binds to the promoter of *ATF4* to increase its mRNA expression. In addition, we show

that MYCN-mediated transcriptional activation of SGOC pathway enzymes requires ATF4. Interestingly, a recent study reported that both MYCN and ATF4 can directly activate the transcription

of *ASCT2* (also known as *SLC1A5*), which encodes a glutamine transporter. It also reported a positive correlation in mRNA expression among *MYCN*, *ATF4*, and *ASCT2* in high-risk neuroblastomas, suggesting a potential coordination between *MYCN* and *ATF4* in regulation of *ASCT2* expression (49). These findings, along with ours, provide evidence for *MYCN* and *ATF4* to regulate a common set of genes in metabolic reprogramming in neuroblastoma. Our study further uncovers a *MYCN*-*ATF4* positive feedback loop: *MYCN* transcriptionally activates *ATF4* expression, and *ATF4* stabilizes *MYCN* protein by antagonizing FBXW7-mediated *MYCN* ubiquitination and degradation. This positive feedback loop may be hardwired into *MYCN*-amplified cancer cells to reinforce the hyperactive state of *SGOC* gene transcription and to boost production outputs of the metabolic pathway required by the *MYCN*-driven growth program. The ability of *ATF4* to stabilize *MYCN* protein also suggests a molecular mechanism for the observation that *ATF4* overexpression significantly enhanced the proliferation of *MYCN*-amplified neuroblastoma cells but had no effect on the proliferation of non-*MYCN*-amplified neuroblastoma cells.

Given the key role of *ATF4* as a master regulator of stress responses, our identification of the *MYCN*-*ATF4* positive feedback loop suggests a molecular mechanism for the stress-sensitive phenotype of *MYCN*-amplified cancer cells. The feedback loop transcriptionally amplifies *SGOC* biosynthesis to sustain cell growth and proliferation, but, at the same time, confers to these cells an increased dependence on the *SGOC* pathway and an intensified stress response to *PHGDH* inhibition. This is reminiscent of the earlier findings that some oncogenes, such as *MYC* and *E2F1*, simultaneously promote cell proliferation and sensitize cells to apoptosis (50). Thus, there might be a coupled relationship between metabolic reprogramming and sensitization to metabolic stress. The notion that cancer cells reprogram their metabolism to meet the biosynthetic challenge of growth and

proliferation but, as a result, are inherently sensitive to metabolic stress may offer numerous opportunities for manipulating cell metabolism in the direction of cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Writing, review, and/or revision of the manuscript: J. Ding, Z. Dong, Y. Zha, H.-F. Ding
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Ye, Z.-C. Ding
Study supervision: Y. Zha, H.-F. Ding

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