The de novo synthesis of the nonessential amino acid serine is often upregulated in cancer. In this study, we demonstrate that the serine catabolic enzyme, mitochondrial serine hydroxymethyltransferase (SHMT2), is induced when MYC-transformed cells are subjected to hypoxia. In mitochondria, SHMT2 can initiate the degradation of serine to CO₂ and NH₄⁺, resulting in net production of NADPH from NADP⁺. Knockdown of SHMT2 in MYC-dependent cells reduced cellular NADPH:NADP⁺ ratio, increased cellular reactive oxygen species, and triggered hypoxia-induced cell death. In vivo, SHMT2 suppression led to impaired tumor growth. In MYC-amplified neuroblastoma patient samples, there was a significant correlation between SHMT2 and hypoxia-inducible factor-1 α (HIF1α), and SHMT2 expression correlated with unfavorable patient prognosis. Together, these data demonstrate that mitochondrial serine catabolism supports tumor growth by maintaining mitochondrial redox balance and cell survival.

**SIGNIFICANCE:** In this study, we demonstrate that the mitochondrial enzyme SHMT2 is induced upon hypoxic stress and is critical for maintaining NADPH production and redox balance to support tumor cell survival and growth. *Cancer Discov;* 4(12): 1406–17. © 2014 AACR.

Serine Catabolism Regulates Mitochondrial Redox Control during Hypoxia

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INTRODUCTION

Since Otto H. Warburg discovered in the 1920s that tumor cells show high glucose consumption (1), oncologists have been interested in finding out how tumor cells adapt metabolic pathways to obtain advantages in cell proliferation and tumor growth. Previously, we and others demonstrated that serine is an activator of pyruvate kinase M2 (PKM2), the pyruvate kinase isoform ubiquitously found in cancers that catalyzes the final reaction in glycolysis (2, 3). Intracellular serine depletion reduces PKM2 enzymatic activity, leading to the accumulation of upstream glycolytic intermediates, including 3-phosphoglycerate, which provides the precursor for de novo serine synthesis. Two recent reports suggest that the genomic regions encoding phosphoglycerate dehydrogenase (PHGDH), the first enzyme in the serine synthetic pathway, are amplified in breast cancer and melanomas (4, 5), leading to diversion of the glycolytic intermediate 3-phosphoglycerate to serine synthesis. The cytosolic synthesis of serine in many cancer cells appears to be in excess of that needed to support macromolecular synthesis (4). These observations led us to consider whether serine catabolism also contributes to tumor cell survival and proliferation.

Serine catabolism is initiated by serine hydroxymethyltransferase (SHMT) activity, catalyzed in the cytosol by SHMT1 and in the mitochondrion by SHMT2. SHMTs catalyze a reversible reaction converting serine to glycine, with concurrent methylene-tetrahydrofolate (THF) generation. Increased SHMT enzyme activity has been detected in human colon cancer and rat sarcoma (6). Although it is possible that serine catabolism contributes to the anabolic needs of a growing cell for glycine, whether serine catabolism contributes to antioxidative defense for cell survival has not been investigated.

One common problem faced by solid tumors is hypoxia, or oxygen deficiency. The hypoxia-inducible factors (HIF) are the major transcriptional regulators of hypoxic adaptation of tumor cells. HIFs are heterodimeric transcription factors composed of an oxygen-regulated α subunit and a constitutively expressed β subunit. Under normoxia, the α subunits are hydroxylated on proline residues, enabling recognition by the von Hippel–Lindau (VHL) tumor suppressor followed by proteosomal degradation. As hydroxylation is inhibited under hypoxia, the α subunits accumulate and form heterodimers with the β subunit to regulate the expression of hundreds of genes (7, 8). As oxygen is the terminal electron acceptor of the mitochondrial electron transport chain (ETC), under hypoxia reduced oxygen levels lead to electrons leaking out from ETC, forming reactive oxygen species (ROS; refs. 9, 10). This creates a redox stress in tumor mitochondria. Pyruvate dehydrogenase kinase 1 (PDK1), a HIF1 target, has been shown to suppress pyruvate entry into the tricarboxylic acid (TCA) cycle, thus reducing ROS generation and cell death (11). However, it is unclear whether there are other metabolic pathways regulated by HIF that influence redox and cell viability in mitochondria.

Here, we present evidence for a critical role of mitochondrial serine catabolism in NADPH production and redox regulation under hypoxia. Specifically, we show that the mitochondrial isoform of SHMT, SHMT2, is induced by...
levels, consequently leading to cell death. Depletion of SHMT2 in hypoxic cells increases ROS in cytosol, whereas SHMT2 is in the mitochondrion (Fig. 1A). Two SHMT isoforms have been identified in mammals (12, 13): SHMT1 is localized to the cytosol, whereas SHMT2 is in the mitochondrion (Fig. 1A). Using the Oncomine database (14), we found that SHMT2, but not SHMT1, is overexpressed in a variety of human cancers (Fig. 1B). Because PHGDH, the first enzyme in the de novo serine synthetic pathway, has been shown to be upregulated in cancers (4, 5), we next determined whether either SHMT isoform’s expression was correlated with PHGDH in cancer. We examined the relationship between the two SHMT isoforms with PHGDH in human neuroblastoma samples. SHMT2 showed a stronger correlation with PHGDH expression (r = 0.67) than that with SHMT1 (r = 0.34; Fig. 1C). In addition, the correlation of SHMT2 and PHGDH expression is remarkably more prominent in samples from patients who died from their disease (r = 0.9) compared with the correlation found in samples from patients where the neuroblastoma regressed (alive; r = 0.42; Supplementary Fig. S1A). A similar analysis was performed using RNA sequencing data from human breast cancer samples. Consistently, the correlation

**RESULTS**

**The Mitochondrial Isoform SHMT2 Is Upregulated in Cancers and Coexpressed with PHGDH**

Increased PHGDH enzyme activity is associated with upregulation of SHMT enzyme activity in tumors (6), suggesting that SHMT may be critical for downstream serine catabolism that promotes tumor development. Two SHMT isoforms have been identified in mammals (12, 13): SHMT1 is localized in cytosol, whereas SHMT2 is in the mitochondrion (Fig. 1A).
Hypoxia-Induced SHMT2 Promotes Cell Survival

To identify whether changes in other metabolic enzymes were associated with SHMT2 overexpression, we analyzed gene expression data from other cancer samples (15). We found that the expression of phosphoglycerate kinase isoenzyme 1 (PGK1) and lactate dehydrogenase A (LDHA) significantly correlates with SHMT2 expression ($r^2_{PGK1 \text{ vs } SHMT2} = 0.87$, $P < 0.001$; $r^2_{LDHA \text{ vs } SHMT2} = 0.84$, $P < 0.001$). Because PGK1 and LDHA are both HIF1α targets that are induced upon hypoxia (16, 17), we asked whether SHMT2 is also regulated under hypoxia through HIF. A promoter analysis for SHMT2 identified two hypoxia response elements (HRE), which are the binding sites for HIFs (Fig. 2A), suggesting that HIF may be responsible for the upregulation of SHMT2. To test whether SHMT2 is induced upon hypoxia, Kelly cells (a neuroblastoma cell line) were exposed to 0.5% O2 for 6 or 16 hours. Hypoxia significantly induced SHMT2 mRNA expression, whereas the expression of SHMT1 was not increased (Fig. 2B). In addition, treating cells with CoCl2, a HIF stabilizer, also induced SHMT2 expression (Fig. 2C). Because HIF2α expression is tissue-specific whereas HIF1α is ubiquitously expressed (8), we asked whether SHMT2 is also regulated by HIF1α. Kelly cells were transfected with 30 nmol/L control siRNA or siRNA targeting HIF1A or HIF2A. After 48 hours, cells were exposed to 0.5% O2 for 8 (for qPCR) or 16 hours (for immunoblot). mRNA levels were measured using qPCR and normalized to normoxia control (left). HIF1α and HIF2α knockdown were confirmed by immunoblot (right). Nor, normoxia; hyp, hypoxia. B to D, data represent mean ± SD of triplicate PCR reactions, and representative of three independent experiments is shown. *, $P < 0.05$; **, $P < 0.01$, determined by the Student two-tailed t test.

**Figure 2.** The hypoxic induction of SHMT2 depends on HIF1α. A, the promoter sequence of human (h) SHMT2. Red, E-boxes (CACGTG); green, hypoxia response elements (HRE; NCGTG). B, hypoxia induces SHMT2 expression. Kelly cells were exposed to normoxia or 0.5% O2 for 6 or 16 hours. mRNA levels were measured using qPCR and normalized to normoxia control. C, CoCl2 treatment induces SHMT2 expression. Kelly cells were treated with 50 μmol/L CoCl2 for 5 or 16 hours. mRNA levels were measured using qPCR and normalized to normoxia control. D, the hypoxic induction of SHMT2 depends on HIF1α. Kelly cells were transfected with 30 nmol/L control siRNA or siRNA targeting HIF1A or HIF2A. After 48 hours, cells were exposed to 0.5% O2 for 8 (for qPCR) or 16 hours (for immunoblot). mRNA levels were measured using qPCR and normalized to normoxia control (left). HIF1α and HIF2α knockdown were confirmed by immunoblot (right). Nor, normoxia; hyp, hypoxia. B to D, data represent mean ± SD of triplicate PCR reactions, and representative of three independent experiments is shown. *, $P < 0.05$; **, $P < 0.01$, determined by the Student two-tailed t test.

of SHMT2 and PHGDH expression ($r = 0.45$) is stronger than the correlation of SHMT1 and PHGDH expression ($r = 0.17$; Fig. 1D). In addition, Kaplan–Meier survival analysis indicated that patients with breast cancer with low SHMT2 expression survive better than the patients with high SHMT2 expression (Supplementary Fig. S1B). Together, these data suggest that the mitochondrial isoform SHMT2 is an important utilizer of serine produced through enhanced PHGDH activity in tumors.

**SHMT2 Is Induced upon Hypoxia in a HIF1α-Dependent Manner**

To identify whether changes in other metabolic enzymes were associated with SHMT2 overexpression, we analyzed gene expression data from other cancer samples (15). We found that the expression of phosphoglycerate kinase isoenzyme 1 (PGK1) and lactate dehydrogenase A (LDHA) significantly correlates with SHMT2 expression ($r^2_{PGK1 \text{ vs } SHMT2} = 0.87$, $P < 0.001$; $r^2_{LDHA \text{ vs } SHMT2} = 0.84$, $P < 0.001$). Because PGK1 and LDHA are both HIF1α targets that are induced upon hypoxia (16, 17), we asked whether SHMT2 is also regulated under hypoxia through HIF. A promoter analysis for SHMT2 identified two hypoxia response elements (HRE), which are the binding sites for HIFs (Fig. 2A), suggesting that HIF may be responsible for the upregulation of SHMT2. To test whether SHMT2 is induced upon hypoxia, Kelly cells (a neuroblastoma cell line) were exposed to 0.5% O2 for 6 or 16 hours. Hypoxia significantly induced SHMT2 mRNA expression, whereas the expression of SHMT1 was not increased (Fig. 2B). In addition, treating cells with CoCl2, a HIF stabilizer, also induced SHMT2 expression (Fig. 2C). Because HIF2α expression is tissue-specific whereas HIF1α is ubiquitously expressed (8),
we next determined which HIF isoform is responsible for the hypoxic induction of SHMT2. The hypoxic induction of SHMT2 was reduced in HIF1α-knockdown cells, whereas knocking down HIF2α did not affect SHMT2 expression under hypoxia (Fig. 2D and Supplementary Fig. S2), suggesting that the hypoxic induction of SHMT2 depends on HIF1.

The Hypoxic Induction of SHMT2 Requires MYC Amplification

Intriguingly, the hypoxic induction of SHMT2 was not observed in every cell line we tested. Two neuroblastoma cell lines, Kelly and SK-N-BE(2), and one glioblastoma cell line, SF188, showed significant SHMT2 induction under hypoxia, but others (SH-SY5Y, H293T, H1299, and HT1080) did not upregulate SHMT2 upon hypoxia (Fig. 3A and Supplementary Fig. S3). Because SHMT2 is a reported MYC target gene (18, 19), and two MYC binding sites (E-Box) were localized adjacent to the two HREs in the SHMT2 promoter (Fig. 2A), we hypothesized that the hypoxic induction of SHMT2 also requires MYC. Indeed, the three cell lines that demonstrated SHMT2 induction under hypoxia all expressed high levels of N-MYC or c-MYC, whereas the other cell lines had low or undetectable MYC expression (Fig. 3A and Supplementary Fig. S3). To determine whether MYC is necessary for the hypoxic induction of SHMT2, we repressed MYC activity in SF188 cells using siRNA targeting MYC. Downregulation of MYC expression not only reduced basal SHMT2 expression, but also eliminated the hypoxic induction of SHMT2 (Fig. 3B). Moreover, overexpressing MYC in SH-SY5Y cells conferred the ability to upregulate SHMT2 under hypoxia (Fig. 3C). The fact that cells expressing c-MYC also possess the ability to upregulate SHMT2 indicates that the hypoxic induction of SHMT2 is not limited to MYCN-amplified neuroblastoma. These data are consistent with the recent demonstration that elevated MYC induces transcriptional amplification of adaptive gene expression programs (20).
Hypoxia-Induced SHMT2 Promotes Cell Survival

SHMT2 Is Necessary for Maintaining Redox Homeostasis and Cell Survival under Hypoxia

Because transcriptional upregulation of HIF targets contributes to hypoxic adaptation, whether SHMT2 contributes to metabolic adaptation to such conditions was investigated.

Stable cell lines were generated through infection with lentiviruses carrying pLKO–shSHMT2 and pLKO–shNT (nontargeting control) vectors. The knockdown efficiency was confirmed by immunoblotting under normoxia and hypoxia (Fig. 4A). To measure the metabolic flux from serine to glycine, the shNT

Figure 4. SHMT2 is necessary for maintaining redox balance and cell survival under hypoxia. A, Kelly cells were infected by lentivirus expressing shRNA-targeting SHMT2 (two independent vectors #1 and #2) or a nontargeting control (shNT). After puromycin selection, stable pool of cells was exposed to 0.5% O2 for 24 and 48 hours. SHMT2 knockdown was confirmed by immunoblot. B, Kelly cells were exposed to 0.5% O2 for 16 hours, and then labeled with 0.4 mmol/L U-13C-serine for 2 hours in DMEM without serine or glycine. The relative amounts of U-13C-serine and U-13C-glycine were measured using GC-MS. All values are normalized to normoxic shNT control cells (data represent mean ± S.D of three biologic repeats, and representative of two independent experiments is shown). C, Kelly cells were exposed to normoxia (Nor) or 0.5% O2 (Hyp) for 6 hours. ROS levels were measured using a flow cytometry-based DCFH assay. All values are normalized to normoxic shNT control cells (data represent mean ± SE of three independent experiments). D, Kelly cells were exposed to normoxia (Nor) or 0.5% O2 (Hyp) for 24 hours. Cellular NADPH and NADP+ were measured using LC/MS (data represent mean ± SE of three biologic repeats, and representative of three independent experiments is shown). E, cellular GSH/GSSG ratios were measured using a glutathione colorimetric assay kit. F, Kelly cells were exposed to normoxia (Nor) or 0.5% O2 (Hyp) for 48 hours with or without 3 mmol/L NAC, and percentage cell death was determined using Trypan Blue staining. G, SH-SY5Y cells were infected by lentivirus expressing pLenti-GIII-CMV-SHMT2 or vector control (VC). After puromycin selection, cells were exposed to normoxia (Nor) or 0.5% O2 (Hyp) for 48 hours. Percentage cell death was determined using Trypan Blue staining. F and G, data represent mean ± SD of three biologic repeats, and representative of three independent experiments is shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001, determined by the Student two-tailed t test.
and shSHMT2 cells were exposed to normoxia or hypoxia for 16 hours, and then U-13C-serine was added to their medium. The amounts of U-13C-glycine generated were determined using gas chromatography–mass spectroscopy (GC-MS). The metabolic fluxes from serine to glycine had a more than 50% reduction in shSHMT2 cells under both normoxia and hypoxia, indicating that there is significant flux from serine to glycine catalyzed by SHMT2 in these cells (Fig. 4B).

Methylene–THF produced by SHMT2 can potentially contribute to de novo thymidylate synthesis in the mitochondria. However, the mitochondrial DNA:nuclear DNA ratio of stable shSHMT2 cells was not reduced under either normoxia or hypoxia (Supplementary Fig. S4A). Methylene–THF can also be oxidized through the folate metabolism to generate NADPH, which is important for maintaining cellular redox balance (21–24). Consistent with these findings, SHMT2-knockdown cells (shSHMT2) showed a more profound increase of ROS under hypoxia than control cells (shNT; Fig. 4C). Staining with a mitochondrion-specific probe, MitoTracker Red CM-H2Ros (25), confirmed that SHMT2 repression caused elevated ROS in the mitochondria (Supplementary Fig. S4B). We also measured the NADPH:NADP+ ratio after hypoxic treatment using LC/MS. SHMT2 suppression caused a significant reduction of the NADPH:NADP+ ratio under hypoxia (Fig. 4D). This decrease was associated with a lower glutathione/glutathione disulfide (GSH/GSSG) ratio (Fig. 4E). In addition, using electron microscopy, swollen mitochondria were observed in shSHMT2 cells under hypoxia, indicating that these mitochondria are under stress (Supplementary Fig. S4C).

Because increased ROS in hypoxic mitochondria leads to cell death, we tested whether repressing SHMT2 expression compromises cell viability under hypoxia. shSHMT2 cells had significantly more cell death than control cells upon hypoxia. The antioxidant N-acetylcysteine (NAC) rescued the death of shSHMT2 cells under hypoxia, indicating that the increased cell death caused by SHMT2 repression was due to elevated ROS (Fig. 4F). To determine whether SHMT2 induction is sufficient to protect cells from hypoxic stress, we overexpressed SHMT2 in SH-SY5Y cells, which do not have N-MYC amplification and do not induce SHMT2 under hypoxia (Fig. 3A). Forced SHMT2 overexpression significantly promoted the survival of SH-SY5Y cells under hypoxia, suggesting that SHMT2 is both necessary and sufficient for protecting cells from hypoxia-induced cell death (Fig. 4G). SHMT2 also protects cells from death when exposed to limitations in both oxygen and glucose (Supplementary Fig. S5).

SHMT2 Is Needed for Tumor Growth In Vivo and Is Upregulated in MYCN-Amplified and Aggressive Neuroblastoma Samples

To test whether SHMT2 contributes to tumor growth in vivo, we performed a xenograft experiment by injecting shNT or shSHMT2 Kelly cells in nude mice. SHMT2 knockdown significantly inhibited xenograft tumor growth (Fig. 5A–C; Supplementary Table S1). To further examine whether HIF1α–dependent SHMT2 induction is relevant to human tumors, expression of SHMT2 and HIF1α was studied using immunohistochemistry in a well-characterized human neuroblastoma tissue microarray (26). Consistent with the observation that MYC is required for the hypoxic induction of SHMT2, SHMT2 and HIF1α show a significant correlation in the MYCN-amplified patient group but not in tumor samples without MYCN amplification (Fig. 6A and B). Furthermore, SHMT2 is highly expressed in poorly differentiated neuroblastoma and in patient samples with unfavorable prognosis (Fig. 6C–E).

DISCUSSION

SHMT2 transfers a methyl group from serine to THF, producing glycine and methylene–THF. Glycine and one-carbon units contribute to purine and thymidine synthesis, which are necessary for nucleic acid synthesis and cell proliferation (27–29). Here, we demonstrate that methylene–THF is critical for producing NADPH and repressing ROS generation in mitochondria. Cellular NADPH is essential for redox defense and reductive biosynthesis of amino acids, deoxynucleotides, and lipids. Previous studies concluded that major NADPH sources are the oxidative pentose phosphate pathway (oxPPP), malic enzyme (ME), and isocitrate dehydrogenase (IDH; ref. 30). In contrast, the folate pathway has been regarded primarily as a one-carbon unit source for de novo nucleotide synthesis, not typically considered as an important NADPH source. However, the present data suggest that SHMT2-dependent production of methylene–THF in mitochondria contributes to mitochondrial NADPH generation and redox balance during hypoxia (Fig. 7).

During tumor progression, hypoxia develops when tumor growth exceeds the ability of available vasculature to supply tumor cells with oxygen and nutrients. Clinically, tumor...
Hypoxia-Induced SHMT2 Promotes Cell Survival

Hypoxia is a significant obstacle to treatment because hypoxic tumor cells are more resistant to radiotherapy (31) and chemotherapy (32, 33). Hypoxia also contributes to metastasis (34, 35) and angiogenesis. Therefore, it is important to elucidate the mechanisms by which hypoxia affects tumor physiology at the cellular and molecular levels, which can lead to the development of novel therapeutic methods that effectively target hypoxic tumors and ultimately improve patient outcome. HIF1α and MYC play critical roles in cancer cell metabolism. In fact, both of them can upregulate glycolysis-related enzymes, including HK2, LDHA, and PDK1 (36, 37). The upregulation of these enzymes not only accelerates glucose uptake and glycolysis but also promotes the Warburg effect in two ways: On the one hand, LDHA channels pyruvate toward lactate production; on the other hand, pyruvate entry into the TCA cycle is blocked by PDK1, which phosphorylates and inhibits pyruvate dehydrogenase (PDH) activity (11, 38). HIF1α is able to reprogram cancer cells to anaerobic glycolysis and reduce mitochondrial oxidative phosphorylation under low-oxygen conditions, thus protecting cells from ROS accumulation. Here, we demonstrate that HIF1α and MYC cooperatively upregulate SHMT2, resulting in improved cell survival.

Figure 6. Expression of SHMT2 and HIF1α correlate in aggressive neuroblastomas. Human neuroblastoma microarray with 123 tumor samples in duplicates were stained for SHMT2 and HIF1α. Staining was quantified using the Matlab software. A, representative images of HIF1α and SHMT2 [×40] from MYCN-amplified or MYCN-nonamplified neuroblastoma samples. B, SHMT2 and HIF1α staining correlate in MYCN-amplified tumor samples, but not in tumors without MYCN amplification. C to E, SHMT2 expression significantly correlates with tumor dedifferentiation and poor prognosis. C, ANOVA, ***, P < 0.001. D, Student test, **, P < 0.01. E, ANOVA, ***, P < 0.001.
in increased NADPH generation and enhanced redox balance during hypoxia. The induction of SHMT2 under conditions of hypoxia likely contributes to maintaining mitochondrial catalabolism of serine even when the rate of consumption of one-carbon units for nucleotide metabolism declines. It is likely that SHMT2 is the key enzyme that connects serine metabolism to mitochondrial redox control in cancer. The fact that hypoxic induction of SHMT2 happens only in MYC-amplified cancer cells makes it an attractive target for cancer therapy. The successful usage of antifolate drugs in cancer treatment suggests that the discovery of potent SHMT2 inhibitors may also provide significant benefits for the treatment of cancer.

**METHODS**

**Bioinformatic Analysis**

Expression data of human neuroblastoma tissue samples (GSE16237; Affymetrix Human Genome U133 Plus 2.0 Array) were obtained from the NCBI Gene Expression Omnibus database (39). Expression used in the analysis is the average expression of the multiple probes for the gene. Fifty-one neuroblastoma samples total, including patients who died of their tumors (n = 12) and patients in whom tumors regressed spontaneously (alive; n = 39) were analyzed. The RNA sequencing data of 919 breast cancer samples were obtained from The Cancer Genome Atlas (TCGA) database. The renal cancer dataset was obtained from the Oncomine database.

**Cell Lines and RNAi**

Kelly (36), SK-N-BE(2) (36), SF188 (40), H1299(2), H293T, and HT1080 (ATCC) cells were cultured in DMEM. SH-SY5Y (41) cells were cultured in DMEM. SH-SY5Y cells overexpressing SHMT2 were generated using the same packaging system and pLenti-GIII-CMV-SHMT2 plasmid (ABMgood). For HIF1α, HIF2α, and c-MYC knockdown, siRNA targeting each gene (Ambion; 30 nmol/L) were transfected into Kelly or SF188 cells using Lipofectamine RNAiMAX (Invitrogen). After 48 hours, cells were subjected to hypoxia treatment.

**Glutathione Assay**

Glutathione assay was performed using a Glutathione colorimetric assay kit (BioVision) following the manufacturer’s protocol.

**Mass Spectrometry Analysis**

For the measurement of NADPH/NADP⁺ levels, Kelly cells were exposed to 0.5% oxygen for 24 hours, metabolism was quenched, and metabolites extracted by aspirating media and immediately adding 80:20 methanol:water at −80°C. Supernatants from two rounds of methanol:water extraction were combined, dried under nitrogen, and resuspended in high-performance liquid chromatography (HPLC) water. The LC/MS method involved reversed-phase ion-pairing chromatography coupled by negative mode electrospray ionization to a stand-alone orbitrap mass spectrometer (Thermo Scientific) scanning from m/z 85–1,000 at 1 Hz at 100,000 resolution (42–44), with LC separation on a Synergy Hydro-RP column (100 mm × 2 mm, 2.5 μm particle size; Phenomenex) using a gradient of solvent A (97:3 H₂O/MeOH) with 10 mmol/L tributylamine and 15 mmol/L acetic acid) and solvent B (100% MeOH). The gradient was 0 minutes, 0% B; 2.5 minutes, 0% B; 5 minutes, 20% B; 7.5 minutes, 20% B; 13 minutes, 55% B; 15.5 minutes, 95% B; 18.5 minutes, 95% B; 19 minutes, 0% B; 25 minutes, 0% B. Injection volume was 10 μL, flow rate 200 μL/min, and column temperature 25°C. Data were analyzed using the MAVEN software suite (45).
For isotope labeling and tracing experiments, Kelly cells were exposed to 21% or 0.5% oxygen for 16 hours, and then labeled with 0.4 mmol/L U-13C-serine and 2 hours in DMEM without serum or glycine. After being washed twice with ice-cold PBS, metabolites were extracted using 80:20 methanol:water at -80°C containing 20 μmol/L D-2HG (D-2-hydroxyglyutaric-2,3,4,4-t, acid) as an internal standard. Supernatants were dried in an evaporator (Genevac EZ-2) under nitrogen, dissolved in methoxamine/pyridine (40 mg/mL), derivatized using acetonitrile:N-methyl-N-tertbutyldimethylsilyl trifluoroacetamide (MTBSTFA, Regis), and heated at 37°C for 30 minutes to derivatize the metabolites. GC-MS was conducted. The signals of U-13C-serine and U-13C-glucose were normalized to internal standard and packed cell volume. Natural isotope abundance was corrected using IsoCor (46).

Immunoblot

The following antibodies were used: SHMT1, SHMT2 (Sigma), HIF1α, HIF2α (Novus), c-MYC, N-MYC (Santa Cruz), and COX IV (Abcam).

Reverse Transcription and Real-Time PCR

Total RNA was extracted following the TRizol Reagent (Invitrogen) protocol. Three micrograms of total RNA was used in reverse transcription following the SuperScript III (Invitrogen) protocol. Quantitative PCR was performed on a 7900HT Sequence Detection System (Applied Biosystems) using Taqman Gene Expression Assays (Applied Biosystems). Gene expression data were normalized to 18S rRNA.

ROS Measurement and Cell Death Assay

Cellular ROS was measured according to published protocols (47). Briefly, Kelly cells were exposed to hypoxia for 6 hours and then incubated with 5 μmol/L CM-H2DCFDA (Invitrogen) for 30 minutes. Cells were trypsinized, and mean FL1 fluorescence was measured by flow cytometry. Mitochondrial ROS were measured using MitoTracker Red CM-H2Ros dye (Invitrogen; ref. 25). Kelly cells were exposed to hypoxia for 6 hours and then incubated with 0.5 μmol/L MitoTracker Red CM-H2Ros dye for 30 minutes. To measure cell death, cells were stained with Trypan Blue. Stained/unstained cells were counted and cell death percentages were calculated.

Immunohistochemistry and Automated Scoring

Neuroblastoma cases were obtained from the University of Pennsylvania following approval from the Institutional Review Board. All cases were deidentified before analysis and were contained in a previously well-characterized tissue microarray (26). Tumor samples consisted of 123 neuroblastomas (34 differentiated, 89 poorly differentiated), 33 ganglioneuroblastomas (17 intermixed and 16 nodular), and 11 ganglioneuromas. Control tissues included brain, adrenal, placenta, and tonsil. All samples were documented with the age at diagnosis, disease stage, MYCN gene amplification status, mitosiskaryorrhexis index, prognosis, and histopathologic classification according to the International Neuroblastoma Pathologic Classification, Children’s Oncology Group (INPC, COG). An independent pathologist evaluated all cases before analyses.

Immunohistochemical studies and quantification were performed as previously described (48). In brief, immunostaining was performed using the Discovery XT processor (Ventana Medical Systems). Tissue sections were blocked for 30 minutes in 10% normal goat serum in 2% BSA in PBS. Sections were incubated for 5 hours with the rabbit anti-SHMT2 (Sigma; concentration, 0.3 μg/mL) or mouse anti-HIF1α (concentration, 10 μg/mL) antibodies. Tissue sections were then incubated for 60 minutes with biotinylated goat anti-rabbit or anti-mouse IgG (Vector Labs) at a 1:200 dilution. Blocker D, Streptavidin–HRP, and DAB Detection Kit (Ventana Medical Systems) were used according to the manufacturer’s instructions.

Slides were scanned using an Aperio ScanScope Scanner (Aperio) and viewed through the Aperio ImageScope software program. For automated scoring, an individual blinded to the experimental design captured JPEG images from each tissue core (circular area of 315 cm2 corresponding to the entire core) at 10x magnification on the Aperio ImageScope viewing program. Automated quantification of immunostaining (described as pixel units) on each JPEG was conducted using an automated analysis program with Matlab’s image processing toolbox based on previously described methodology. The algorithm was based on color segmentation with RGB color differentiation, K-Means Clustering, and background–foreground separation with Otsu’s thresholding. To arrive at a score for each tissue core, the number of extracted pixels was multiplied by their average intensity for each core (depicted as pixel units). The final score for SHMT2 or HIF1α for a given case was calculated by averaging the score of two cores (for each case).

Disclosure of Potential Conflicts of Interest

J.D. Rabinowitz has ownership interest (including patents) in Agios Pharmaceuticals and is a consultant/advisory board member for Atlas Ventures. C.B. Thompson is a member of the board of directors of Merck and Charles River Laboratories; has ownership interest (including patents) in Merck, Agios Pharmaceuticals, Charles River Laboratories, University of Michigan, University of Pennsylvania, and University of Chicago; and is a consultant/advisory board member for Agios Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J. Ye, J. Fan, J.R. Cross, C.B. Thompson Development of methodology: J. Ye, S. Venneti, L.W.S. Finley, C. Lu, J.R. Cross Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Ye, J. Fan, S. Venneti, Y.-W. Wan, B.R. Pawel, G. Qing, J.D. Rabinowitz Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): J. Ye, J. Fan, S. Venneti, Y.-W. Wan, L.W.S. Finley, J.R. Cross, Z. Liu, M. Celeste Simon Writing, review, and/or revision of the manuscript: J. Ye, S. Venneti, C. Lu, T. Lindsten, M. Celeste Simon, J.D. Rabinowitz, C.B. Thompson Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Venneti, J. Zhang, T. Lindsten Study supervision: C.B. Thompson Other (provided cell lines and plasmids): M. Celeste Simon

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