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Supplemental Information

The Histone H3 Methyltransferase G9A Epigenetically Activates the Serine-Glycine Synthesis Pathway to Sustain Cancer Cell Survival and Proliferation

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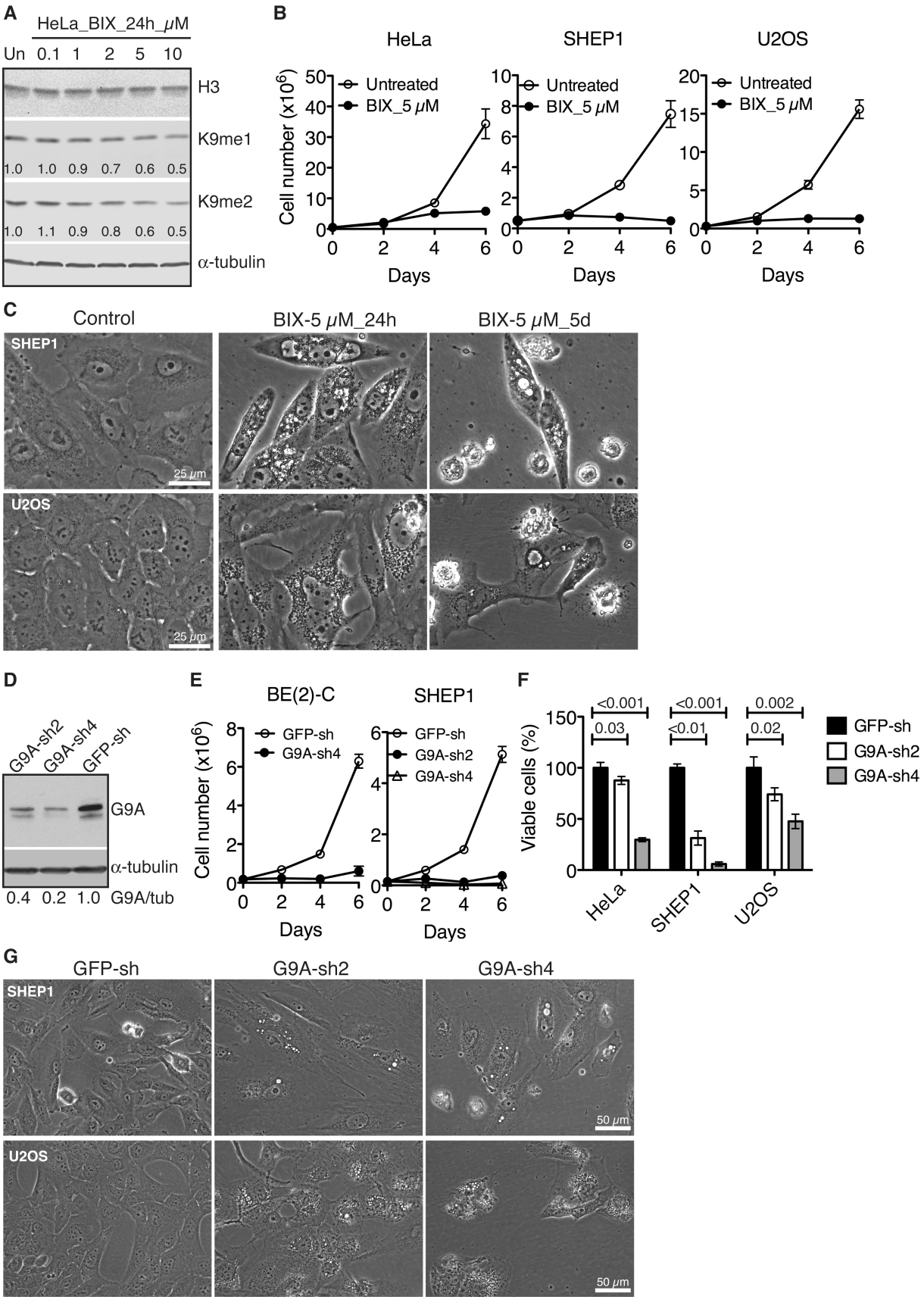
A
HeLa_BIX_24h_μM
Un 0.1 1 2 5 10
H3
K9me1
1.0 1.0 0.9 0.7 0.6 0.5
K9me2
1.0 1.1 0.9 0.8 0.6 0.5
α-tubulin
B
HeLa
SHEP1
U2OS
Untreated
BIX_5 μM
Cell number (x10^6)
Days
C
Control
BIX-5 μM_24h
BIX-5 μM_5d
SHEP1
U2OS
25 μm
D
G9A-sh2
G9A-sh4
GFP-sh
G9A
α-tubulin
0.4 0.2 1.0 G9A/tub
E
BE(2)-C
SHEP1
GFP-sh
G9A-sh4
G9A-sh2
Cell number (x10^6)
Days
F
Viable cells (%)
<0.001
0.03
<0.001
<0.01
0.002
0.02
GFP-sh
G9A-sh2
G9A-sh4
HeLa
SHEP1
U2OS
G
GFP-sh
G9A-sh2
G9A-sh4
SHEP1
U2OS
50 μm

Figure S1. G9A inhibition or silencing suppresses cell proliferation and survival, Related to Figure 1.

(A) Immunoblotting and quantification of H3K9me1 and H3K9me2 (against histone H3) in HeLa cells with or without BIX treatment.

(B) Growth assay of the indicated cell lines with or without BIX treatment. Error bars represent SD of three independent experiments.

(C) Micrographs of the indicated cancer cell lines with or without BIX treatment.

(D) Immunoblotting of G9A in HeLa cells expressing GFP shRNA (GFP-sh) or G9A-sh sequences. G9A levels were quantified against α-tubulin.

(E-F), Growth (E) and survival (F) assays of the indicated cell lines expressing GFP-sh, G9A-sh2 or G9A-sh4. Error bars represent SD of three independent experiments.

(G) Micrographs of U2OS and SHEP1 cells expressing GFP-sh, G9A-sh2 or G9A-sh4.

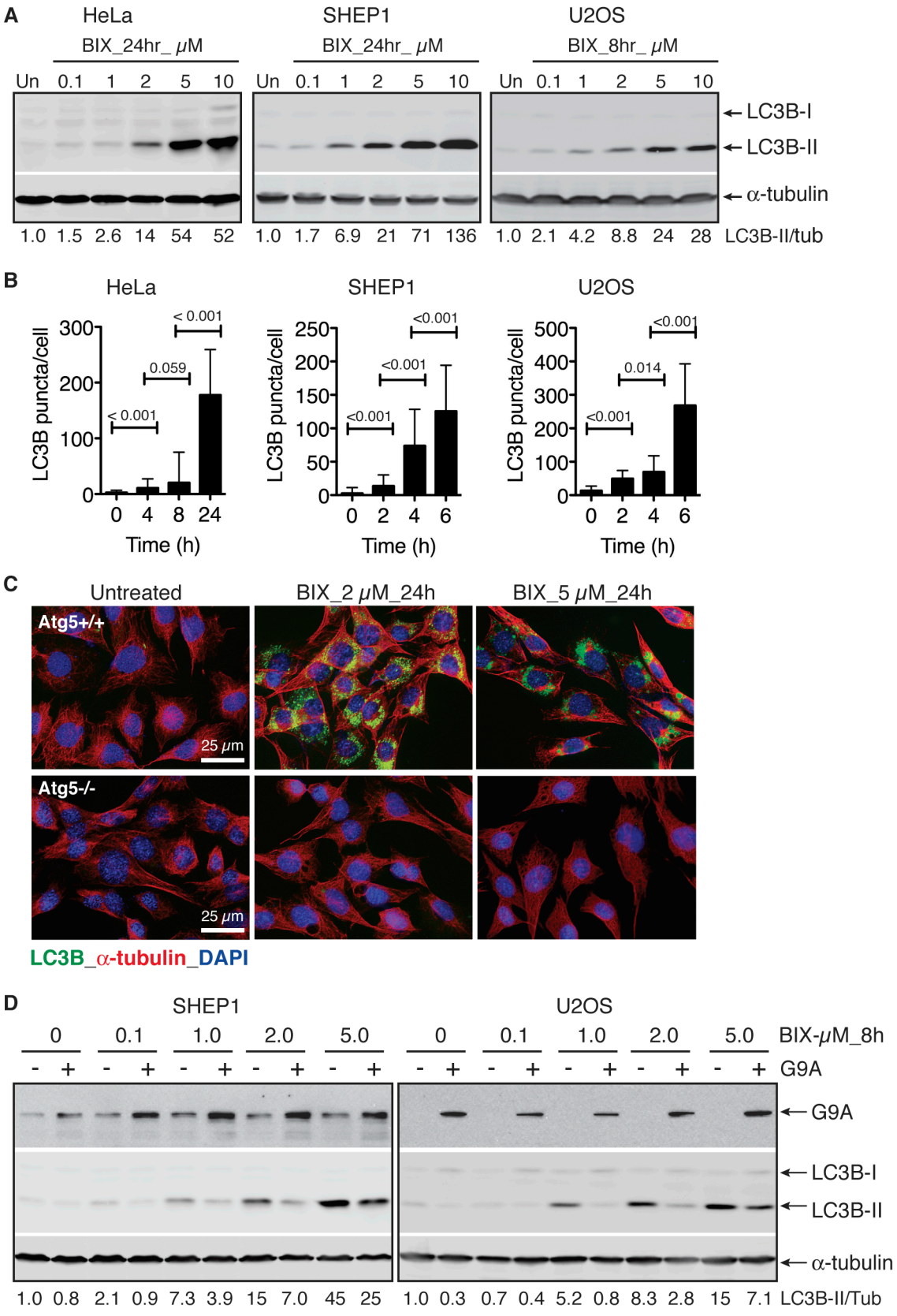
A
HeLa
BIX_24hr_ μM
Un 0.1 1 2 5 10
SHEP1
BIX_24hr_ μM
Un 0.1 1 2 5 10
U2OS
BIX_8hr_ μM
Un 0.1 1 2 5 10
LC3B-I
LC3B-II
α-tubulin
1.0 1.5 2.6 14 54 52
1.0 1.7 6.9 21 71 136
1.0 2.1 4.2 8.8 24 28
LC3B-II/tub
B
HeLa
SHEP1
U2OS
LC3B puncta/cell
< 0.001
0.059
< 0.001
<0.001
<0.001
<0.001
0.014
<0.001
0 4 8 24
0 2 4 6
0 2 4 6
Time (h)
Time (h)
Time (h)
C
Untreated
BIX_2 μM_24h
BIX_5 μM_24h
Atg5+/+
Atg5-/-
25 μm
25 μm
LC3B_α-tubulin_DAPI
D
SHEP1
U2OS
0 0.1 1.0 2.0 5.0 0 0.1 1.0 2.0 5.0 BIX-μM_8h
- + - + - + - + - + - + - + - + - + - + G9A
G9A
LC3B-I
LC3B-II
α-tubulin
1.0 0.8 2.1 0.9 7.3 3.9 15 7.0 45 25
1.0 0.3 0.7 0.4 5.2 0.8 8.3 2.8 15 7.1
LC3B-II/Tub

Figure S2. G9A inhibition induces autophagy, Related to Figure 1

(A) Immunoblotting of LC3B in the indicated cell lines either untreated or treated with increasing concentrations of BIX. LC3B-II levels were quantified against α-tubulin.

(B) Time-course study and quantification of LC3B-positive autophagosomes in the indicated cell lines with or without 5 μM BIX treatment. Error bars represent SD (n = 100 cells).

(C) Immunofluorescence staining of LC3B-positive autophagosomes in $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs with or without BIX treatment.

(D) Immunoblotting of G9A and LC3B in untreated or BIX-treated U2OS and SHEP1 cells with or without G9A overexpression. LC3B-II levels were quantified against α-tubulin.

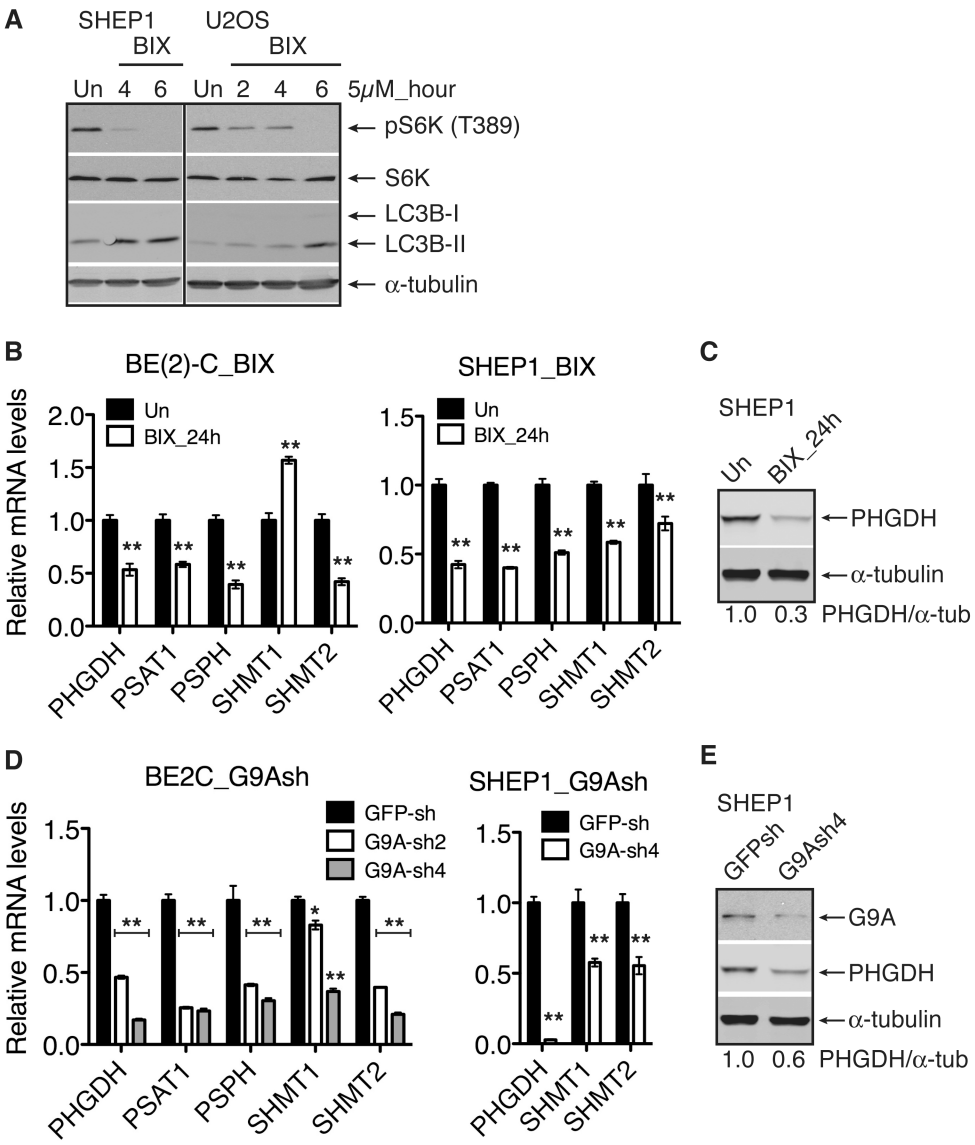

Figure S3. G9A inhibition or silencing represses serine-glycine biosynthesis, Related to Figure 2.

(A) Immunoblotting of S6K, pS6K (T389), and LC3B in U2OS and SHEP1 cells either untreated or treated with 5 μM BIX for the indicated times. α-tubulin levels are shown as loading control. (B) qRT-PCR analysis of mRNA expression of serine-glycine biosynthesis enzyme genes in BE(2)-C and SHEP1 cells with or without 5 μM BIX treatment. Error bars represent SD (n = 3). (C) Immunoblotting of PHGDH in SHEP1 cells with or without 5 μM BIX treatment. α-tubulin levels are shown as loading control. (D) qRT-PCR analysis of mRNA expression of serine-glycine biosynthesis enzyme genes in BE(2)-C and SHEP1 cells with or without G9A silencing. Error bars represent SD (n = 3). (E) Immunoblotting of PHGDH in SHEP1 cells with or without G9A silencing. α-tubulin levels are shown as loading control. *p < 0.01; **p < 0.001.

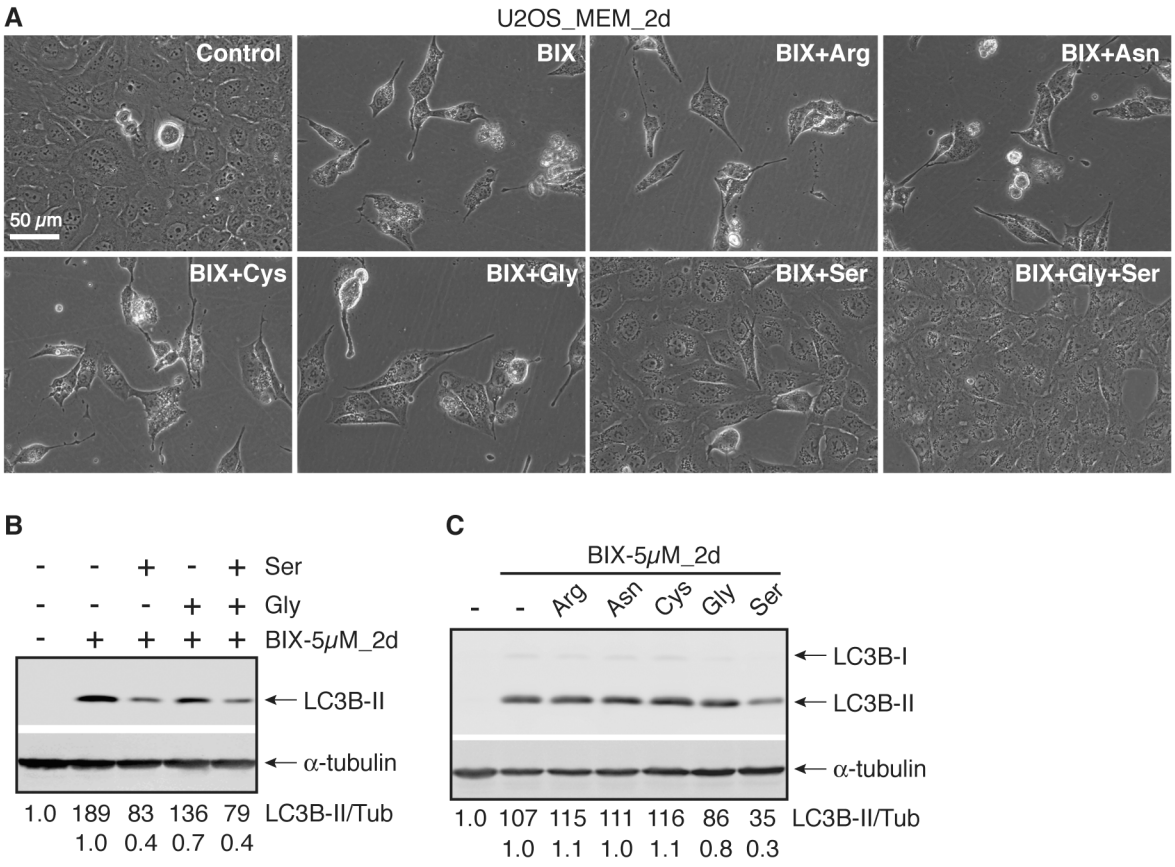

Figure S4. Supplemental serine rescues the phenotype of G9A inhibition, Related to Figure 3

(A) Micrographs of U2OS cells cultured in MEM for 2 days with or without 5 μM BIX in the presence or absence of the indicated supplemental amino acids.

(B-C) Immunoblotting of LC3B in cell samples from (A). LC3B-II levels were quantified against α-tubulin.

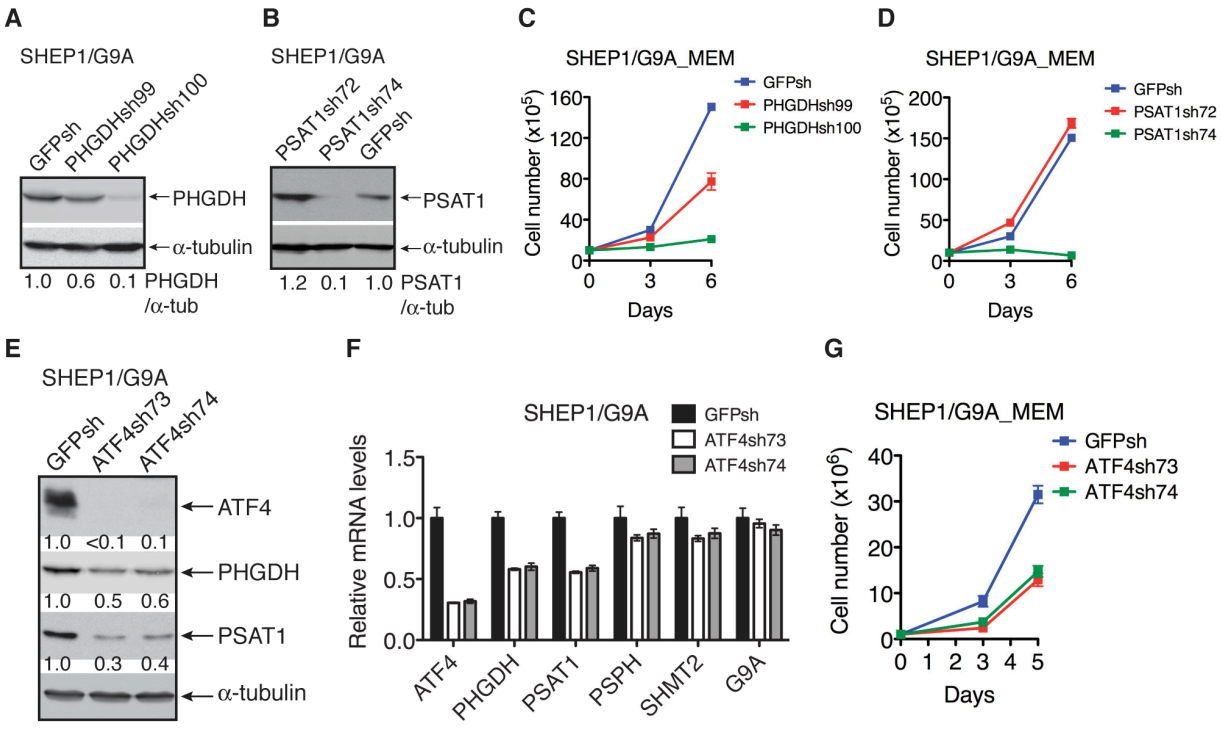

Figure S5. Activation of serine-glycine biosynthesis is required for G9A to promote cell proliferation, related to Figure 7.

(A-B) Immunoblotting of PHGDH and PSAT1 in G9A-overexpressing SHEP1 cells with shRNA-mediated knockdown of PHGDH (A) or PSAT1 (B).

(C-D) Growth assay of G9A-overexpressing SHEP1 cells with shRNA-mediated knockdown of PHGDH (C) or PSAT1 (D). Error bars represent SD (n =3).

(E-F) Immunoblot (E) and RT-PCR (F) analysis of the expression of serine-glycine biosynthesis enzyme genes in G9A-overexpressing SHEP1 cells with shRNA-mediated knockdown of ATF4.

(G) Growth assay of G9A-overexpressing SHEP1 cells with shRNA-mediated knockdown of ATF4. Error bars represent SD (n =3).

Table S1. BIX-responsive genes (±1.5 fold, $p<0.01$), Related to Figure 2.

Table S2. GO analysis of downregulated BIX-responsive genes identified by RNA-seq (top 10 GO biological process terms based on FDR), Related to Figure 4.

Table S3. RT-PCR primers, Related to Figures 2, 4, 6 and 7

Primer set	Forward	Reverse
ATF4	TTCTCCAGCGACAAGGCTAAGG	CTCCAACATCCAATCTGTCCCG
CCNA2	CTCTACACAGTCACGGGACAAAG	CTGTGGTGCTTTGAGGTAGGTC
CCNB1	GACCTGTGTCAGGCTTTCTCTG	GGTATTTTGGTCTGACTGCTTGC
CCNB2	CAACCAGAGCAGCACAAGTAGC	GGAGCCAACTTTTCCATCTGTAC
CDC25C	AGAAGCCCATCGTCCCTTTGGA	GCAGGATACTGGTTCAGAGACC
PHGDH	CTTACCAGTGCCTTCTCTCCAC	GCTTAGGCAGTTCCCAGCATTC
PSAT1	ACTTCCTGTCCAAGCCAGTGGA	CTGCACCTTGTATTCCAGGACC
PSPH	GACAGCACGGTCATCAGAGAAG	CGCTCTGTGAGAGCAGCTTTGA
SHMT1	TGAACACTGCCATGTGGTGACC	CTCTTTGCCAGTCTTGGGATCC
SHMT2	GCCTCATTGACTACAACCAGCTG	ATGTCTGCCAGCAGGTGTGCTT
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

Supplemental Experimental Procedures

Overexpression and RNA interference

Cell lines overexpressing G9A, SHMT1 or SHMT2 were generated by lentiviral infection with pLenti6-MK1-EHMT2/G9A-V5 (Addgene), pCDH-pur-SHMT1 or pCDH-pur-SHMT2 (both from Thermo Scientific). Cell lines with knockdown of G9A, PHGDH, PSAT1 or ATF4 were established by infection with lentiviruses expressing shRNA against G9A (RHS4533-EG10919, Thermo Scientific), PHGDH (RHS4533-EG26227, Thermo Scientific), PSAT1 (RHS4533-EG29968, Thermo Scientific) or ATF4 (TRCN0000013573 and TRCN0000013574, Sigma-Aldrich). U2OS cells expressing mCherry-GFP-LC3 were established by infection with pBabe-puro-mCherry-GFP-LC3 retroviruses (Addgene).

Electron microscopy

Approximately 1.5 x 10^7 U2OS cells with or without 5 μM BIX for 24 hr were harvested and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer (pH 7.4). The samples were post-fixed in 2% osmium tetroxide in NaCac, stained with 2% uranyl acetate, dehydrated with a graded ethanol series and embedded in Epon-Araldite resin. Thin sections were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems), collected on copper grids and stained with uranyl acetate and lead citrate. Cells were observed in a JEM 1230 transmission electron microscope (JEOL) and imaged with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan).

Autophagosomes were identified by morphology as described (Periyasamy-Thandavan et al., 2008).

Soft agar and xenograft assays

Cells (2000/well) were mixed with 0.3% Noble agar in DMEM growth medium and plated onto six-well plates containing a solidified bottom layer (0.6% Noble agar in DMEM growth medium). After 14 days, colonies were stained with 5 mg/ml MTT (Sigma-Aldrich) and photographed. For xenograft assay, 6-week-old female NOD.SCID/NCr mice (NCI-Frederick) were injected subcutaneously at both flanks with 3 x 10^6 SHEP1 or U2OS cells overexpressing GFP or G9A in 200 µl serum-free DMEM. Six weeks after injection, tumors were removed and weighed. All animal experiments were pre-approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Primary antibodies

The following primary antibodies were used in immunoblotting: rabbit anti-ATF4 (sc-200, Santa Cruz Biotechnology, 1:1000), rabbit anti-cyclin A (sc-751, Santa Cruz Biotechnology, 1:200), rabbit anti-cyclin B1 (sc-752, Santa Cruz Biotechnology, 1:200), mouse anti-histone H3 (05-499, Millipore, 1:1000), mouse anti-H3K9me1 (17-680, Millipore, 1:2000), rabbit anti-H3K9me2 (17-648, Millipore, 1:500), mouse anti-G9A (B.133.9, Pierce, 1:1000), rabbit anti-LC3B (3868, Cell Signaling, 1:1000), rabbit anti-PHGDH (HPA021241, Sigma-Aldrich, 1:300), rabbit anti-PSAT1 (201020002, Novus, 1:3000), mouse anti-S6K (sc-8418, Santa Cruz Biotechnology, 1:400), rabbit anti-pS6K

(T389) (9205, Cell Signaling, 1:1000), rabbit anti-β-actin (600-401-886, Rockland Immunochemicals, 1:2000), and mouse anti-α-tubulin (B-5-1-2, Sigma-Aldrich, 1:4000). For immunofluorescence:

ChIP-qPCR primers

The primer pair PHGDH_50 covers the *PHGDH* promoter region from -50 to +57 (+1 for TSS): *PHGDH_50* forward: GAGCTTTGGCTGAGATGGAGA, *PHGDH_50* reverse: CTCAAACTCTCCGCGACTCC. The primer pairs PSAT1_106 and PSAT1_165 cover the *PSAT1* promoter regions from -106 to +101 and -165 to -87, respectively: *PSAT1_106* forward: CGCGAACCAATTAGCGCAG, *PSAT1_106* reverse: CCCAAAGTTGACCACCTGCC; *PSAT1_165* forward: AGGAGCAACTGCTTCGACTC, *PSAT1_165* reverse: CCTGCGCTAATTGGTTCGC. The primer set for the *GAPDH* coding region was provided by Millipore as positive control for anti-H3K9me1 and anti-H3K9me2 ChIP (forward: GGCTCCCACCTTTCTCATCC, reverse: GGCCATCCACAGTCTTCTGG).

Supplemental Reference

Periyasamy-Thandavan, S., Jiang, M., Wei, Q., Smith, R., Yin, X.-M., and Dong, Z. (2008). Autophagy is cytoprotective during cisplatin injury of renal proximal tubular cells. Kidney Int *74*, 631-640.