# Epigenetic silencing of the DNA mismatch repair gene, *MLH1*, induced by hypoxic stress in a pathway dependent on the histone demethylase, LSD1

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## Supplemental Information

## Supplemental Experimental Procedures

**ChIP assay antibodies, relating to the ChIP assay in the main text.** Antibodies specific for the following histone marks were used in the ChIP assays: acetyl-K9 H3 (07-352, Millipore), trimethyl-K9 H3 (17-625, Millipore), mono/di/trimethyl-K4 H3 (05-791, Millipore), monomethyl-K4 H3 (07-463, Millipore), dimethyl-K4 H3 (07-030, Millipore), trimethyl-K4 H3 (ab8580, Abcam), LSD1 (39186, Active Motif).

**ChIP assay primers, relating to the ChIP assay in the main text.** The *MLH1p*F1/R1 primer set has been described previously (Bindra and Glazer, 2007) . The *ACTB-2p*F1/R1 primers were purchased from Active Motif (71005).

**Construct used in Fig. S8, a supplemental data for Fig.1 in the the main text**. Lentivirus shRNA vectors for Max knockdown were obtained from Sigma-Aldrich (Max: TRCN0000231550, and TRCN0000231551 ).

**Antibodies used for Western blotting in the main text.** The primary antibodies used for western blotting were as follows: β-actin (C4; Santa Cruz Biotechnology), anti-JARID1B/PLU-1 (ab50958, Abcam), anti-CoREST (07-455, Millipore), anti-JARID1A/RBP2 (#3867, Cell Signaling), and anti-LSD1 (#4218s, Cell Signaling), anti-MLH1 (#3515, Cell Signaling), mono/di/trimethyl-K4 H3 (05-791, Millipore), dimethyl-K4 H3 (07-030, Millipore), Max (SC-197; Santa Cruz Biotechnology).

**Quantitative real-time PCR analysis, a method relating to main text Fig. 1 and Fig. 2.**

For quantitative real-time PCR analysis of *MLH1* mRNA expression, total RNA was prepared using RNAeasy Miniprep Kit (Qiagen). 2 μg RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was used in PCR reactions containing Taqman Universal PCR Master Mix (Applied Biosystems) with premixed Taqman probes and primers for *MLH1* and 18S (Applied Biosystems). The Mx3000p real time PCR system (Stratagene) was used to monitor fluorescence intensity in real-time to allow quantitative comparisons.

**β-Galactosidase mutation assay, used in Figure S3B, a supplemental data for main text Fig. 1.** The pCAR-OF plasmid, in which the gene contains a 58-bp out-of-frame $(CA)_{29}$ insertion at the 5' end of its coding region, was transfected by using cationic lipids into HeLa cells, along with the gWIZ luciferase vector (0.5g) as a normalization control. Three hours later, medium containing the transfection mixture was removed. Cells were replenished with fresh medium and were cultured under either normoxic or hypoxic conditions. After 48 h, cells were lysed, and the β-galactosidase and luciferase activities were measured under each condition. The value for β-galactosidase expression were normalized to the luciferase control and averaged over replicate samples.

**PMR value determination, relating to Methylight assay in main text Fig.6**: PMR (Percentage of fully methylated reference) was used to define the amount of cytosine methylated DNA at the *MLH1* promoter. The percentage of fully methylated molecules at *MLH1* promoter was calculated by dividing the *MLH1*: *COL2A1* ratio of a sample by the *MLH1*: *COL2A1* ratio in fully methylated DNA and multiplying by 100. Given the high efficiency of Q-MSP amplification for both *MLH1* and *COL2A1* genes in this study, PMR values were detected with the comparative $C_T$ method. The relation between the percentages of methylated DNA molecules

and $C_T$ was described as PMR = $2^{-\Delta\Delta CT} \times 100\%$ where $\Delta\Delta C_T = \Delta C_{T(MLH1)} - \Delta C_{T(COL2A1)} = [C_{T(MLH1)} - C_{T(COL2A1)}]_{(Sample)} - [C_{T(MLH1)} - C_{T(COL2A1)}]_{(Full\text{-}methylated)}$.

**Supplemental Figure Legends**

**Fig. S1. Hypoxia-induced histone modifications at the *MLH1* promoter in MCF-7 cells and SW480 cells, supplemental data for main text Fig.1.** MCF-7 cells or SW480 cells were exposed to normoxia (N) or hypoxia (H) for 48 h. Cells were collected for quantitative ChIP analyses using specific antibodies to determine H3K4 methylation or H3K9 acetylation and methylation levels at the *MLH1* promoter. In SW480 cells, western blot and RT-PCR were also performed to analyze MLH1 protein and mRNA expression, respectively. (A) ChIP analysis in MCF-7 cells. Representative agarose gels containing *MLH1* promoter region PCR amplification products are shown. The corresponding quantitative data are shown in Fig. 1. (B). ChIP analysis in SW480 cells. Representative agarose gels containing *MLH1* promoter region PCR amplification products are shown. (C). Quantification of H3K4 methylation levels by real-time PCR at the *MLH1* promoter in the same conditions as in (B). Promoter occupancy levels are expressed as the fold change relative to normoxia, based on three independent ChIP assays, with error bars based on SEs. (D). Western blot analysis of MLH1 protein levels in SW480 after exposure to normoxia (N) or hypoxia (H) for 48 h. (E). Quantitative real-time PCR analysis of *MLH1* mRNA expression levels in normoxic or hypoxic SW480 cells. mRNA levels are expressed as the fold change relative to normoxic control.

**Fig. S2. Western blot analysis of global changes in H3K4 methylation, supplemental data for main text Fig.1.** (A). MCF-7 cells were exposed to normoxia (N) or hypoxia (H) for indicated times, and cells were collected for western blot analysis to determine total H3K4 me1,2,3 and H3K4 me2 protein levels. (B). SW480 cells were exposed to normoxia (N) or

hypoxia (H) for 48 h, and the cells were collected for western blot analysis to determine total H3K4 me1,2,3 and H3K4 me2 protein levels.

**Fig. S3. MLH1 protein levels inversely correlate with levels of CAIX in human SW480 xenograft tumor, and induction of mutagenesis by hypoxia and suppression by forced MLH1 expression, supplemental data for main text Fig.1. (A)** Triple immune fluorescence in SW480 xenograft tumor shows that MLH1 expression inversely correlates with the hypoxia marker, carbonic anhydrase IX (CAIX). (I). Nuclei were stained by DAPI (Blue). (II) MLH1 protein is stained as green. (III) CAIX protein is stained as red. (IV). Merged images. (B). Restoration of β-galactosidase (beta-gal) activity via frameshift mutagenesis in a reporter gene construct in HeLa cells. The cells were transfected with an episomal, replicative vector, pCAR-OF, containing the β-galactosidase gene interrupted by a 58-bp out-of-frame $(CA)_{29}$ insertion tract at the 5' end of its coding region. Restoration of the proper reading frame to generate a functional enzyme occurs when replication slippage errors in the repeated sequence tract are not corrected by MMR. The HeLa cells with pCAR-OF vector were transfected with or without MLH1 expression vector or treated with TSA (300 nM), and then the cells were exposed to normoxia or hypoxia. The experiment was performed three times, and the relative β-galactosidase values were normalized to a value of 1 for the normoxic cells. Standard errors are indicated.

**Fig. S4. Hypoxia induces LSD1 binding to *MLH1* promoter, supplemental data for main text Fig.2.** Chromatin immuneprecipitation (ChIP) assays were performed on SW480 cells following 48 h exposure to normoxia (N) or hypoxia (H; 0.01% $O_2$) using and LSD1 specific antibody to interrogate LSD1 occupancy at the *MLH1* promoter, as indicated. Representative agarose gels containing *MLH1* promoter region PCR amplification products are shown. *ACTB-2* promoter primers were used as positive control for the LSD1 ChIP assay.

**Fig. S5. Hypoxia induces silencing of the *MLH1* promoter in SW480 cel1s, supplemental data for main text Fig.3.** (A) Image of representative cell culture wells showing differential ganciclovir (GCV)-resistant colony formation following growth in normoxia or hypoxia and subsequent GCV selection. (B) Frequency of GCV-resistant clones (indicative of silencing of *MLH1p*-TK-Blast$^R$ expression) following exposure of SW480 cells to normoxia or hypoxia (0.5% $O_2$) for the 35 days. Selection in the presence of GCV was performed under normoxic conditions for 10 additional days. Error bars represent SEs from three replicates. The frequency of the resulting GCV resistant clones indicative of *MLH1* promoter silencing is shown.

**Fig. S6. Knockdown of the H3K4 demethylases, LSD1, PLU-1, and RBP2, does not reactivate the silenced *MLH1* promoter in RKO cells, supplemental data for main text Fig.4.** RKO cells were stably transduced with lentiviral expression constructs for a GFP shRNA or for shRNAs targeting LSD1, PLU-1, or RBP2 to generate RKO GFPsh, RKO LSD1sh, RKO RBP2sh and RKO PLU-1sh cell lines. Each cell line was either untreated or treated with 5-aza-dC at 5 μm or with 5-aza-dC plus trichostatin A (TSA) for 72 h. Western blot analyses were performed determine MLH1 expression levels. The last lane in each panel represents an MCF-7 cell lysate as a positive control for abundant MLH1 expression.

**Fig. S7. LSD1 is required for *MLH1* silencing in RKO cells following reactivation by transient 5-aza-dC exposure under both normoxic and hypoxic conditions, supplemental data for main text Fig.4.** RKO GFPsh cells, RKO LSD1sh cells, and RKO PLU-1sh cells were treated with 5-aza-dC at 5 μm for 8 days. The cells were then placed in standard conditions either in normoxia or in hypoxia (1% $O_2$) for 43 additional days. MLH1 expression was analyzed at the indicated times. (A) Western blot analyses to determine MLH1 expression levels in RKO GFPsh

cells, RKO LSD1sh cells, and RKO PLU-1sh cells immediately after 8 days of 5-aza-dC treatment (indicated as D-8). (B) Western blot analyses to determine MLH1 expression levels in RKO GFPsh cells, RKO LSD1sh cells, and RKO PLU-1sh cells after replacement in standard medium without 5-aza-dC either in normoxia (N) or in hypoxia (H; 1% $O_2$) for additional days as indicated (R-15 for 15 days; R-26 for 26 days; R-36 for 36 days and R-43 for 43 days ).

**Fig. S8. Hypoxia-invoked Max/Mad1 and Max/Mnt pathways are not required for hypoxia-induced histone modifications at the *MLH1* promoter, supplemental data for main text Fig.1.** SW480 cells with Max knockdown were established using lentiviral shRNAs constructs targeting Max. SW480 GFPsh was used as a control cell line. (A) Western blot analyses to determine Max expression levels in SW480 GFPsh and SW480 Maxsh cells. SW480 Maxsh-551 cells were chosen because there was greater Max knockdown in this cell line compared to SW480 Maxsh-550. (B) ChIP analyses for H3K4 methylation levels at the *MLH1* promoter were performed in SW480 GFPsh control cells and SW480 Maxsh-551cells following a 48 h exposure to normoxia (N) or hypoxia (H). ChIP analyses of levels of H3K4 methylation are shown for the *MLH1* promoter as quantified by real-time PCR. Promoter occupancy levels are expressed as the fold change relative to the normoxic SW480 GFPsh cells, based on three independent ChIP assays with error bars based on SEs.

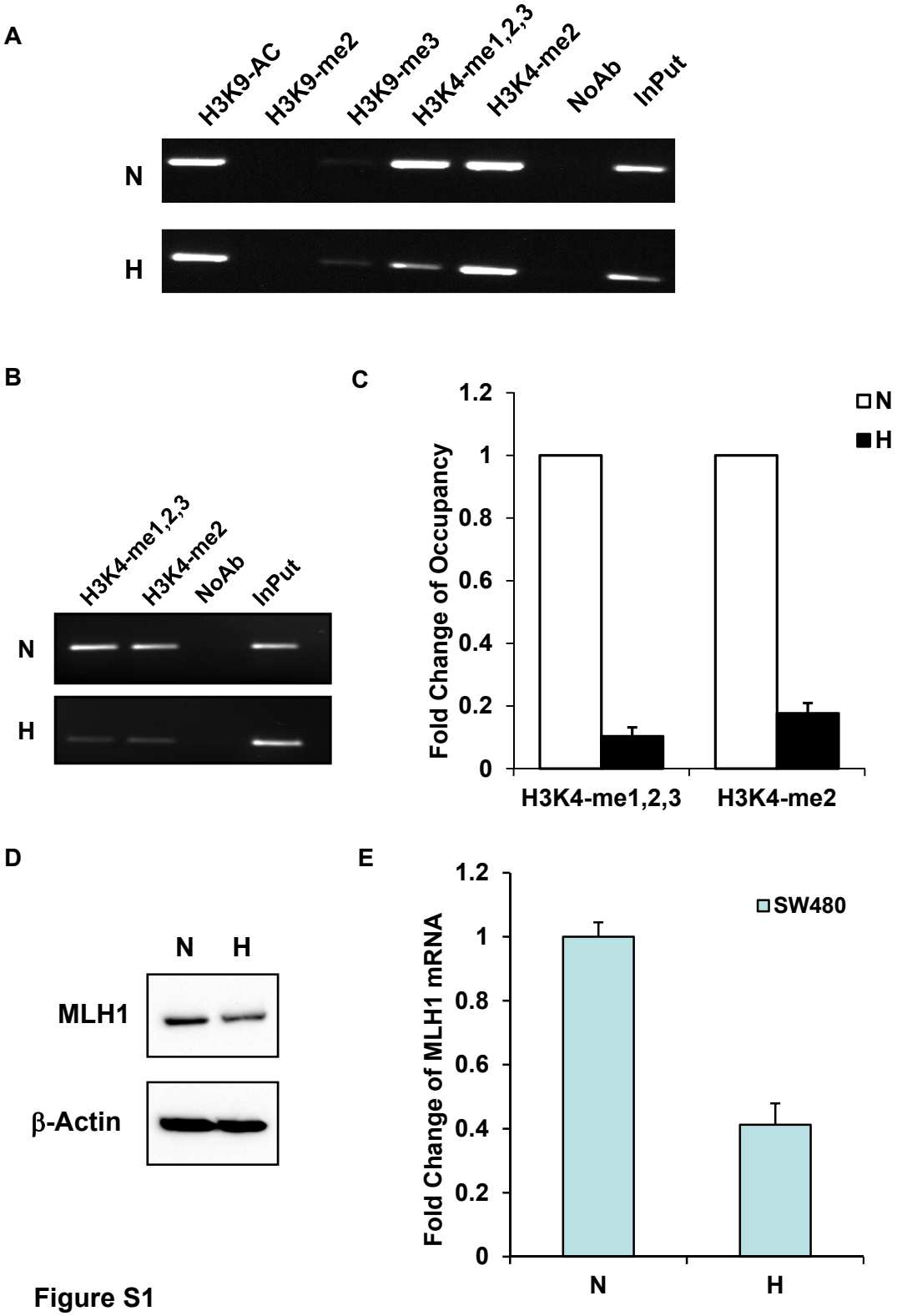
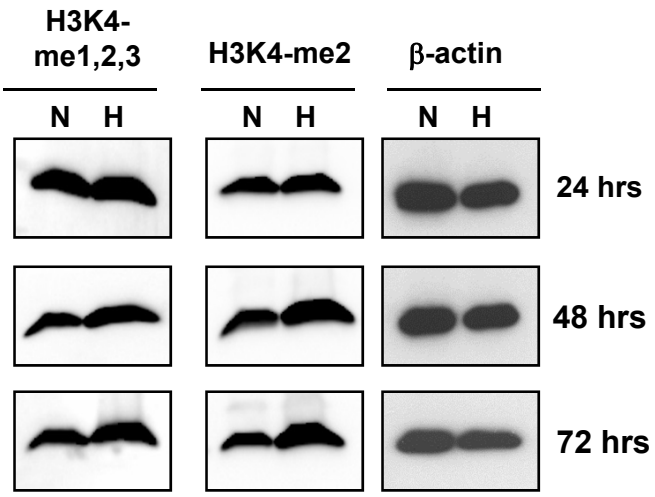

A
H3K9-AC
H3K9-me2
H3K9-me3
H3K4-me1,2,3
H3K4-me2
NoAb
InPut
N
H
B
H3K4-me1,2,3
H3K4-me2
NoAb
InPut
N
H
C
Fold Change of Occupancy
1.2
1
0.8
0.6
0.4
0.2
0
N
H
H3K4-me1,2,3
H3K4-me2
D
N
H
MLH1
β-Actin
E
Fold Change of MLH1 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
SW480
N
H

Figure S1

A


H3K4-me1,2,3
H3K4-me2
β-actin
N H
N H
N H
24 hrs
48 hrs
72 hrs

B

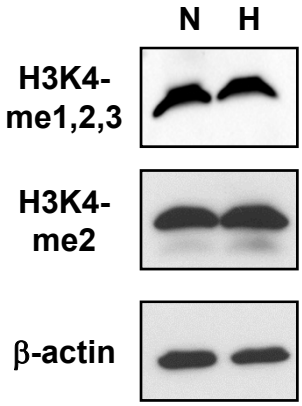
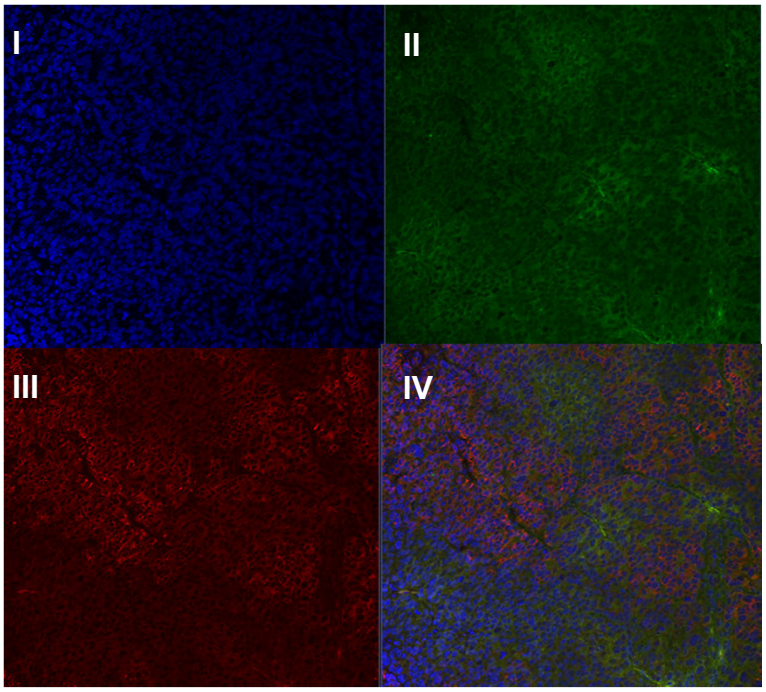

N H
H3K4-me1,2,3
H3K4-me2
β-actin

**Figure S2**

A


I
II
III
IV

B

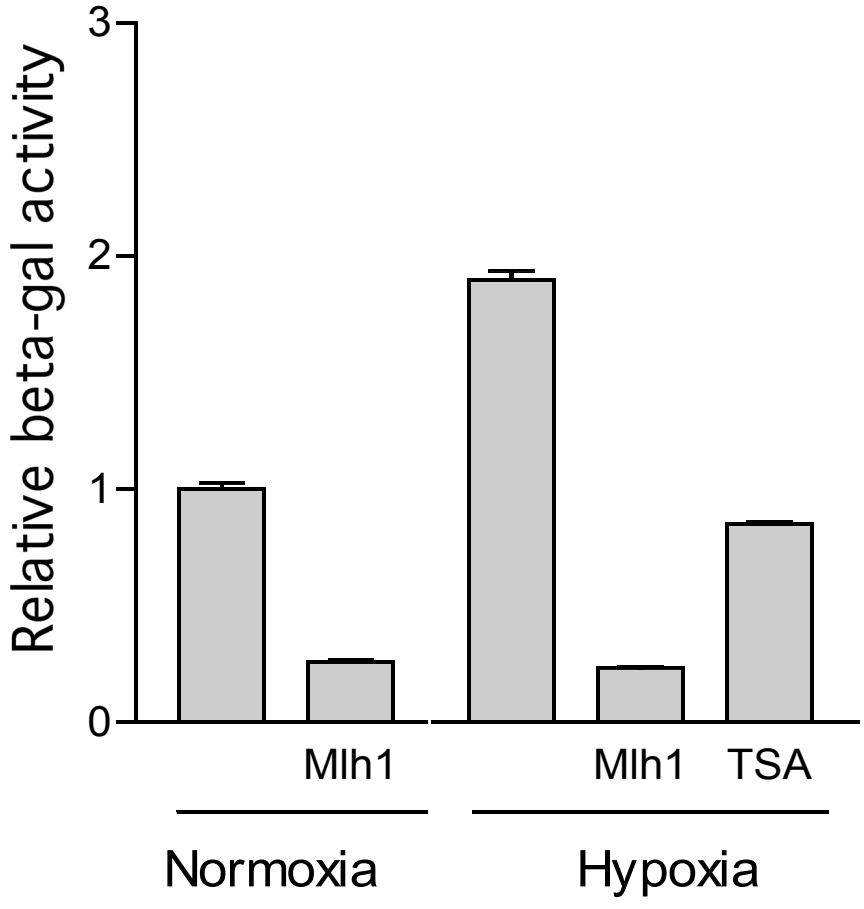

Relative beta-gal activity
3
2
1
0
Mlh1
Mlh1
TSA
Normoxia
Hypoxia

**Figure S3**

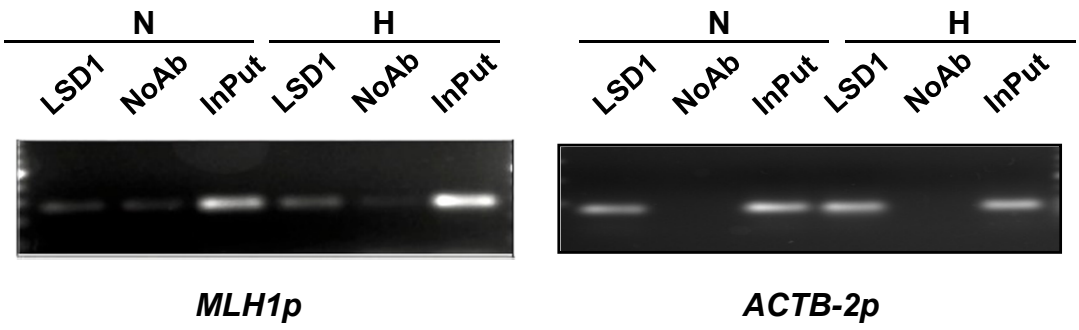

N
H
LSD1
NoAb
InPut
LSD1
NoAb
InPut
N
H
LSD1
NoAb
InPut
LSD1
NoAb
InPut
MLH1p
ACTB-2p

**Figure S4**

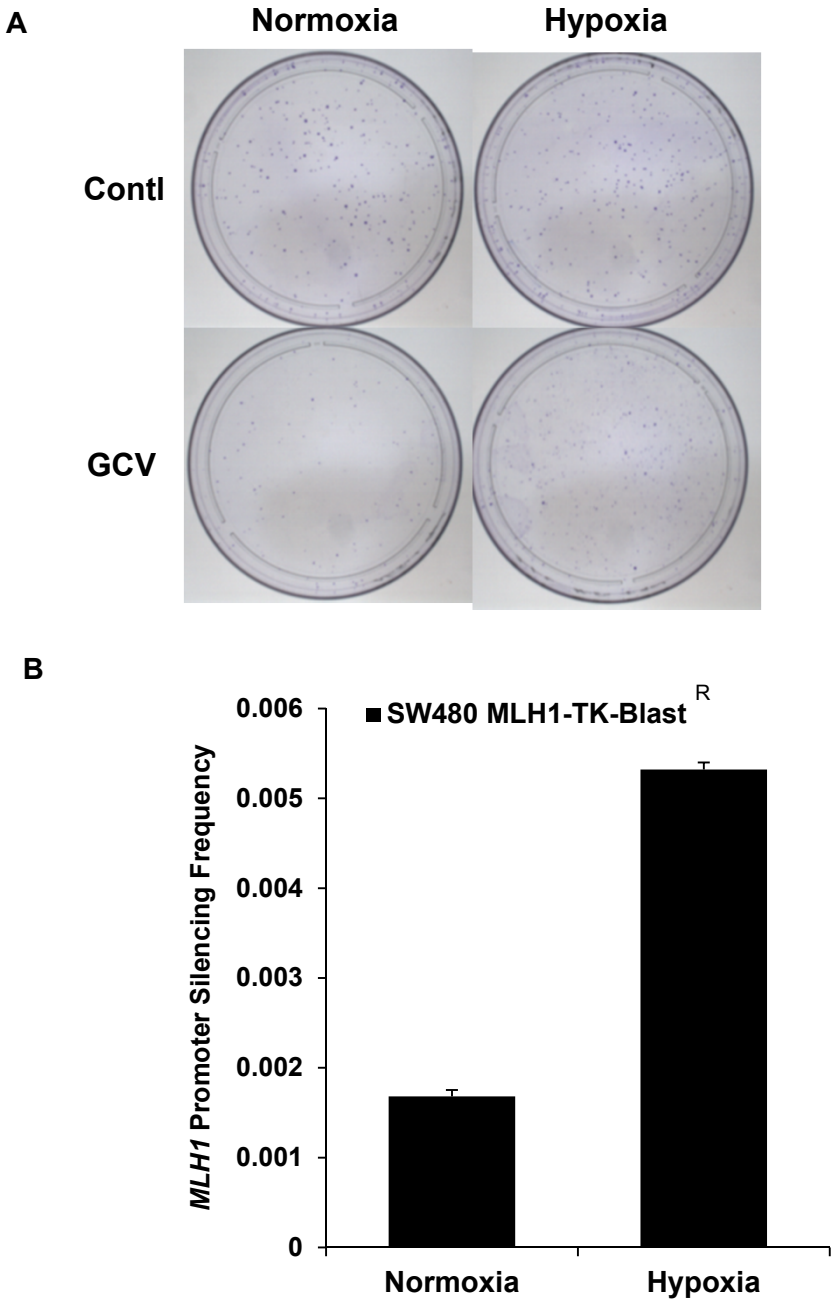

A
Normoxia
Hypoxia
Contl
GCV
B
SW480 MLH1-TK-Blast R
MLH1 Promoter Silencing Frequency
0.006
0.005
0.004
0.003
0.002
0.001
0
Normoxia
Hypoxia

**Figure S5**

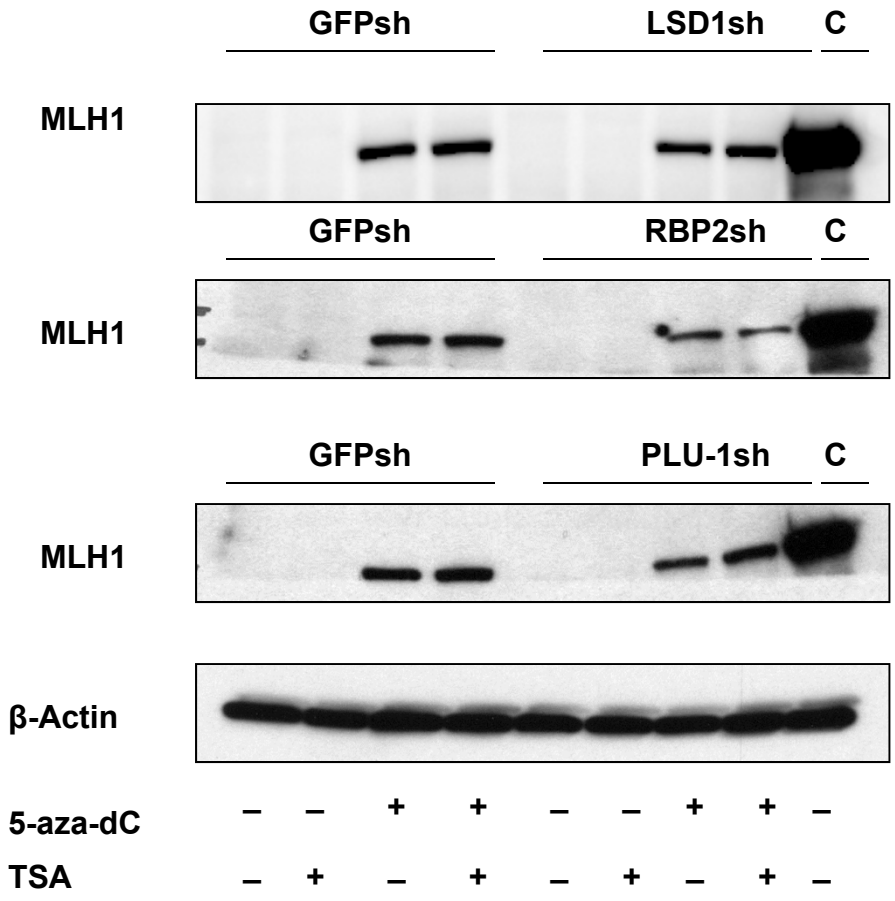

GFPsh
LSD1sh
C
MLH1
GFPsh
RBP2sh
C
MLH1
GFPsh
PLU-1sh
C
MLH1
β-Actin
5-aza-dC – – + + – – + + –
TSA – + – + – + – + –

**Figure S6**

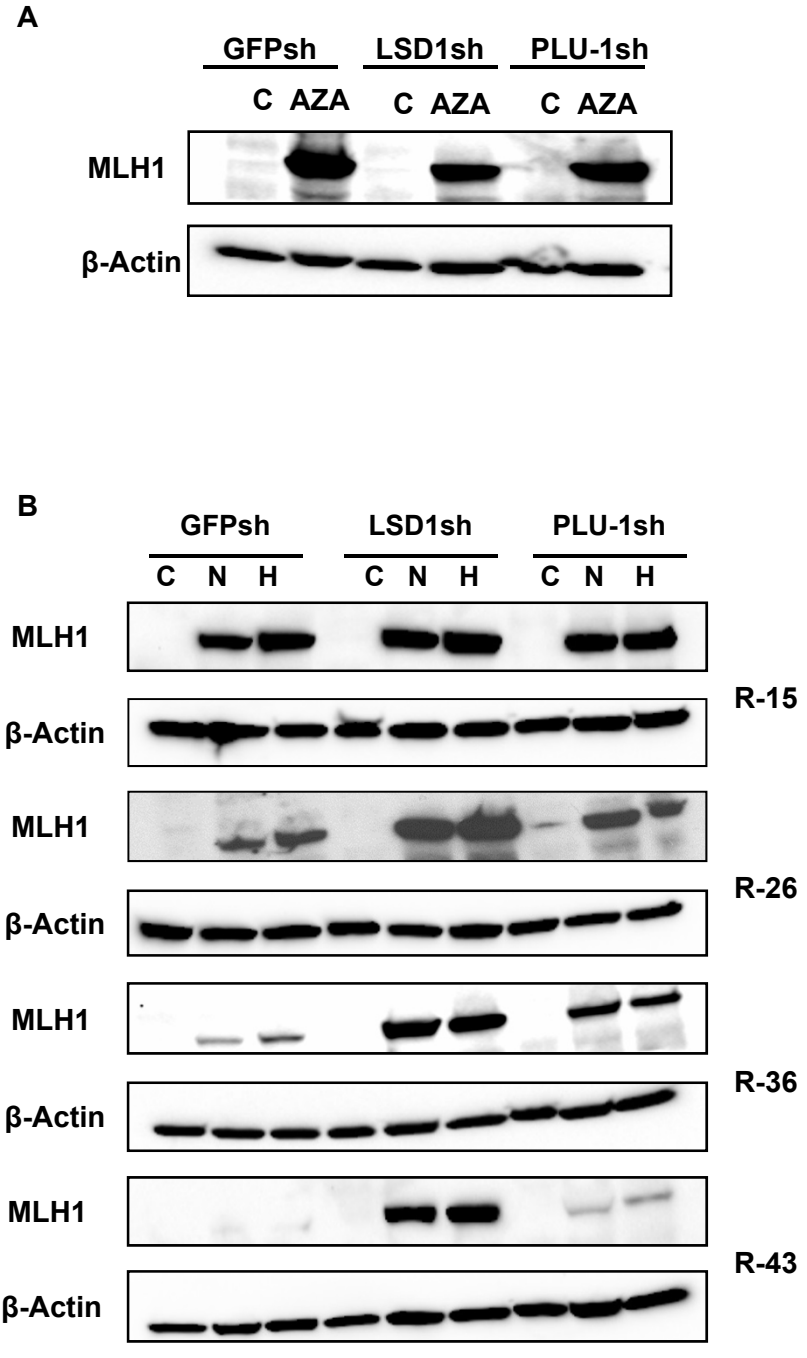
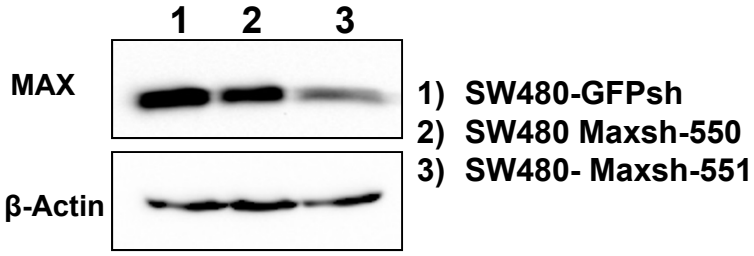

A
GFPsh
LSD1sh
PLU-1sh
C AZA
C AZA
C AZA
MLH1
β-Actin
B
GFPsh
LSD1sh
PLU-1sh
C N H
C N H
C N H
MLH1
β-Actin
R-15
MLH1
β-Actin
R-26
MLH1
β-Actin
R-36
MLH1
β-Actin
R-43

Figure S7

A


1 2 3
MAX
β-Actin
1) SW480-GFPsh
2) SW480 Maxsh-550
3) SW480- Maxsh-551

B

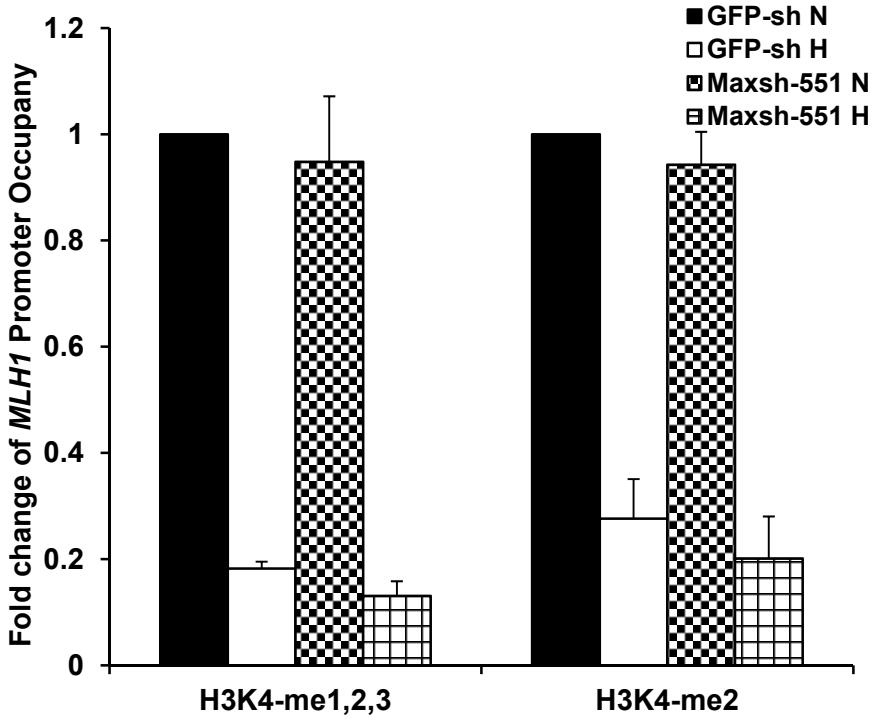

GFP-sh N
GFP-sh H
Maxsh-551 N
Maxsh-551 H
Fold change of MLH1 Promoter Occupany
1.2
1
0.8
0.6
0.4
0.2
0
H3K4-me1,2,3
H3K4-me2

Figure S8