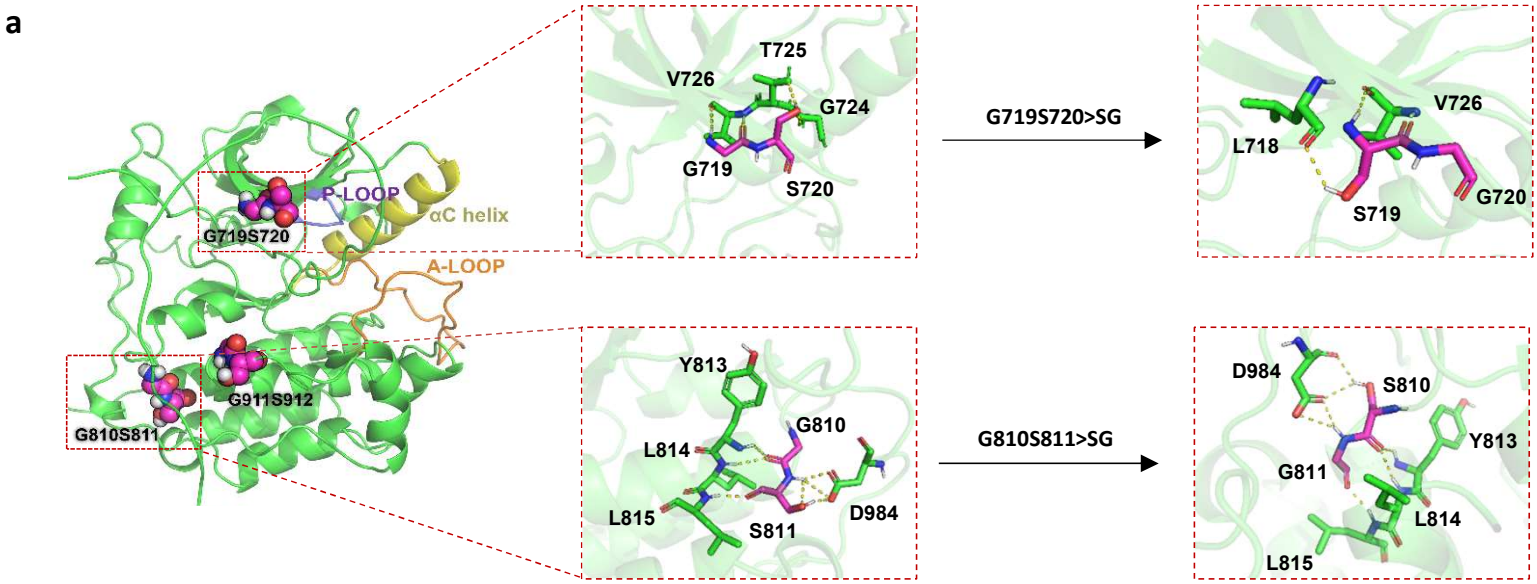
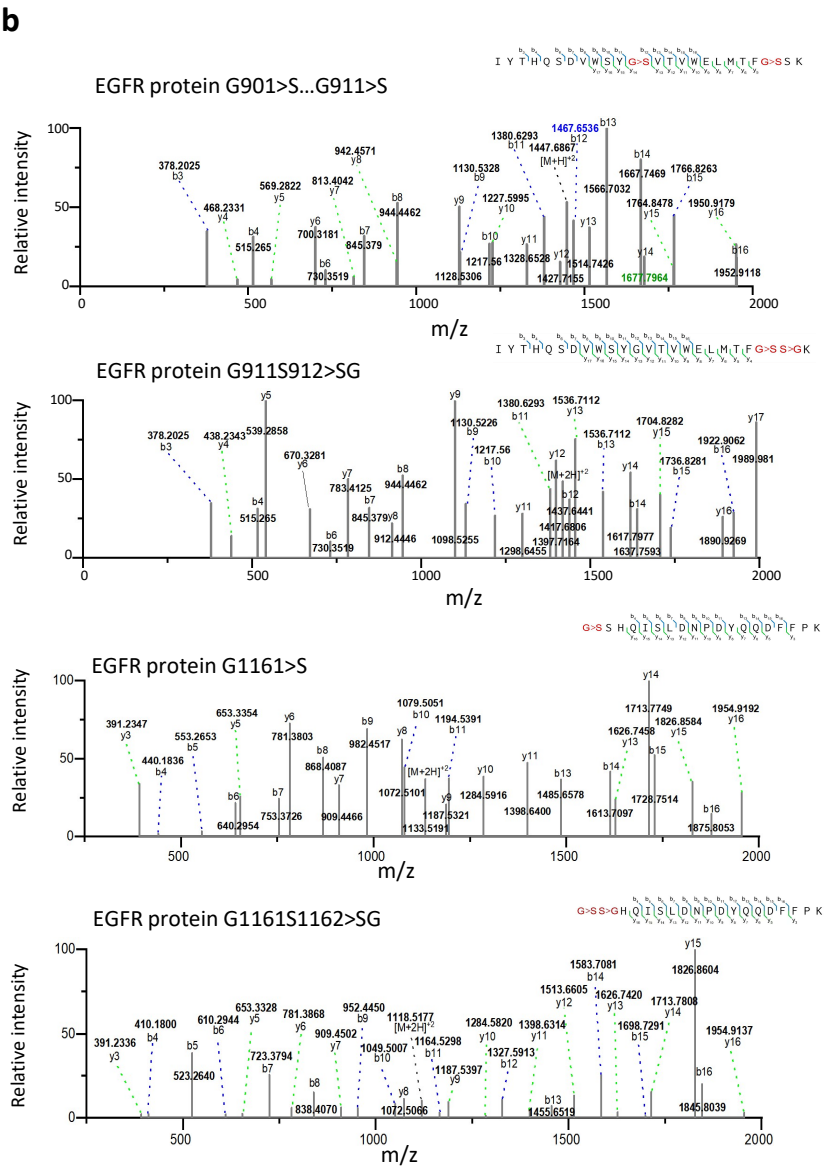
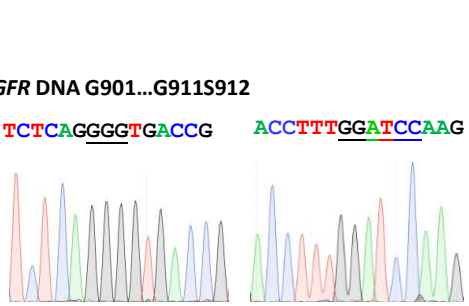






Extended Data Fig.1| The GS island editing alters the EGFR protein conformation.

Extended Data Fig.1| The GS island editing alters EGFR protein conformation.

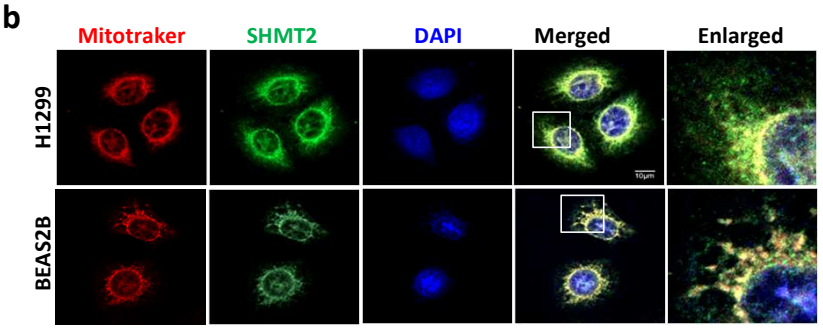
a. Seven GS islands are mapped along the cytoplasmic region of the EGFR protein. While G719, G810, and G911 GS islands are located within the TK domain, G1103, G1161, G1054, and G1189 are located within the N-terminal regulatory domain. The local residues for G719 and S720 residues of G719 GS island contacting and for G810 and S811 residues of G810 island contacting are shown to have been altered due to GS>SG editing or gene mutation corresponding to the specific GS islands.

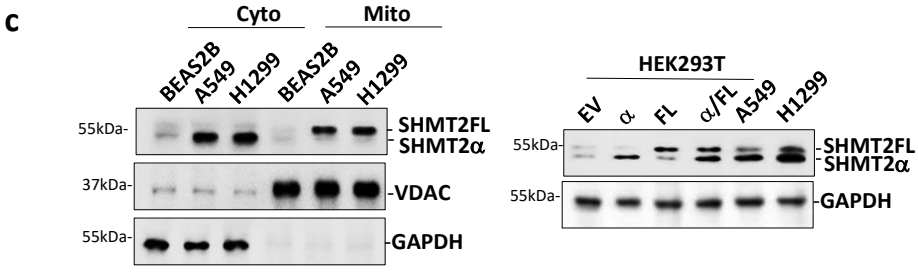
b. It presents mass-mass spectra of peptides with G>S editing recovered from EGFR proteins prepared from a lung cancer adenocarcinoma sample. A peptide with G901>S and G911>S editing sites was recovered (upper), while a G911S912>SG switch editing peptide was recovered with an untypical trypsinization pattern (middle). A G1161S1162>SG switch editing peptide was also recovered from EGFR protein with an untypical trypsinization pattern.

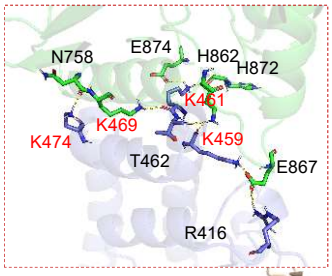
c. The DNA sequence analysis of the local G901 motif of *EGFR* gene in the above sample used in panel ***b***. It revealed no mutations on G901 and G911S912 motif of *EGFR* gene from the patient sample for mass spectrometry analysis.

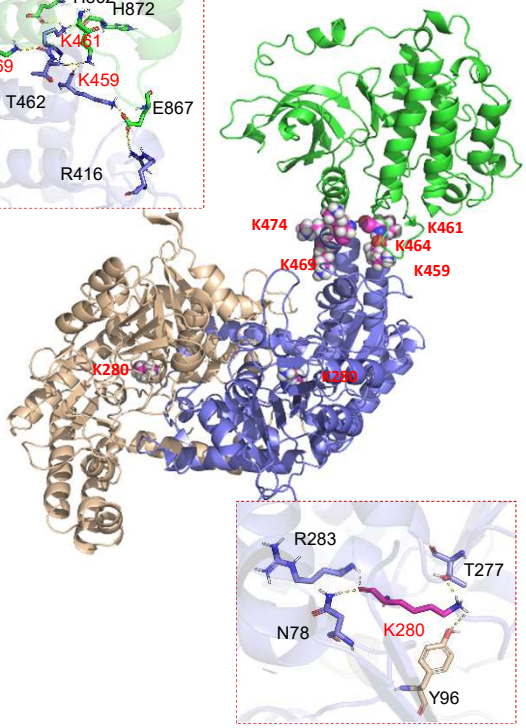
a

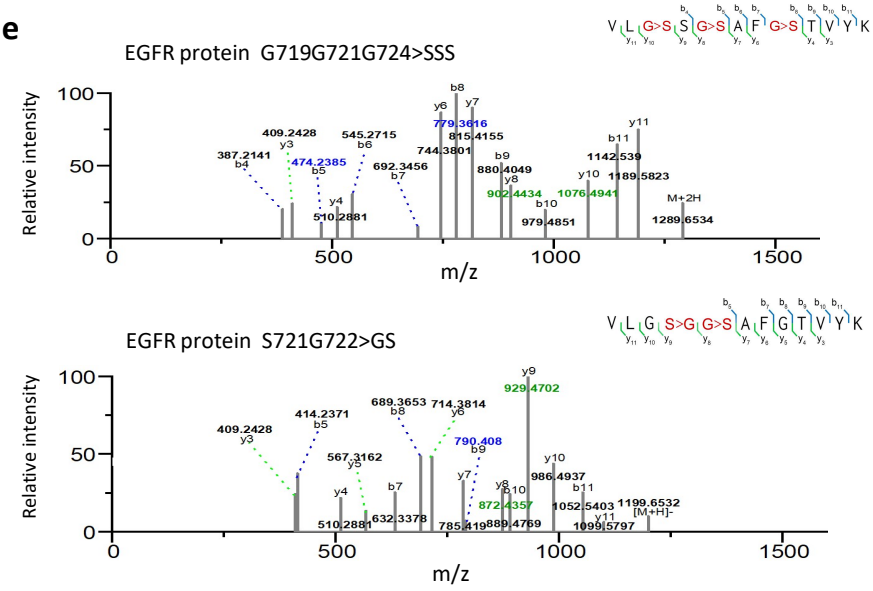
Cell line	Protein	Coverage	Peptide	PSM	Unique Peptide
A549	EGFR	63%	79	157	76
	SHMT2	12%	4	4	4
H1299	EGFR	66%	89	232	87
	SHMT2	25%	7	7	7
H460	EGFR	55%	58	111	57
	SHMT2	42%	15	28	15

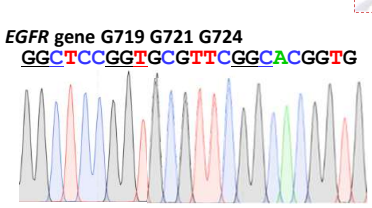


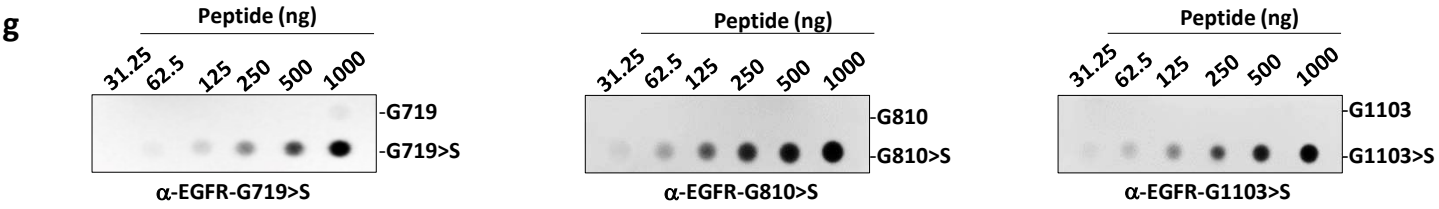


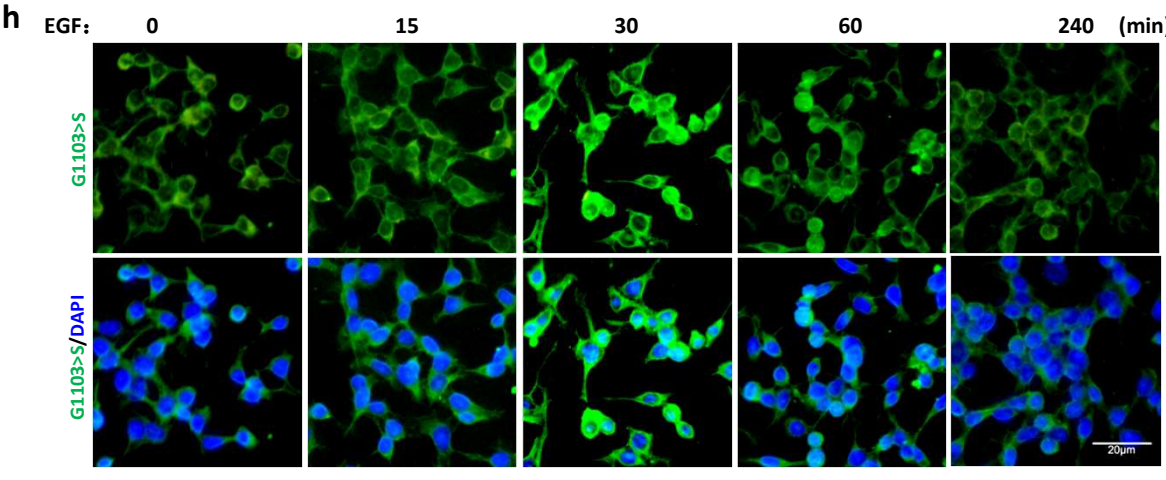












Extended Data Fig. 2| Cytosolic SHMT2 associates with EGFR via its N-terminal 5K cluster for hydroxymethylation initiation.

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a. Immunoprecipitated EGFR and its associated proteins were subjected to mass spectrometry for identification, and SHMT2 protein was identified as binding with EGFR protein within H1299, A549, and H460 cell lines.

b. H1299 and BEAS2B cells were fluorescently stained with anti-SHMT2 as well as with Mitotracker and DAPI.

c. In BEAS2B, A549, and H1299 cells, cytosolic and mitochondrial fractions were analyzed in Western blot for SHMT2 localization. GAPDH and VDAC were included as the markers of cytosolic and mitochondrial fractions respectively (left panel). *SHMT2* in full length and *SHMT2*α (1-21Δ) were transiently overexpressed in HEK293T cells. WCL were prepared from these HEK293T transfectants as well as A549 and H1299 cells were compared for SHMT2 full length and SHMT2α expression (right panel).

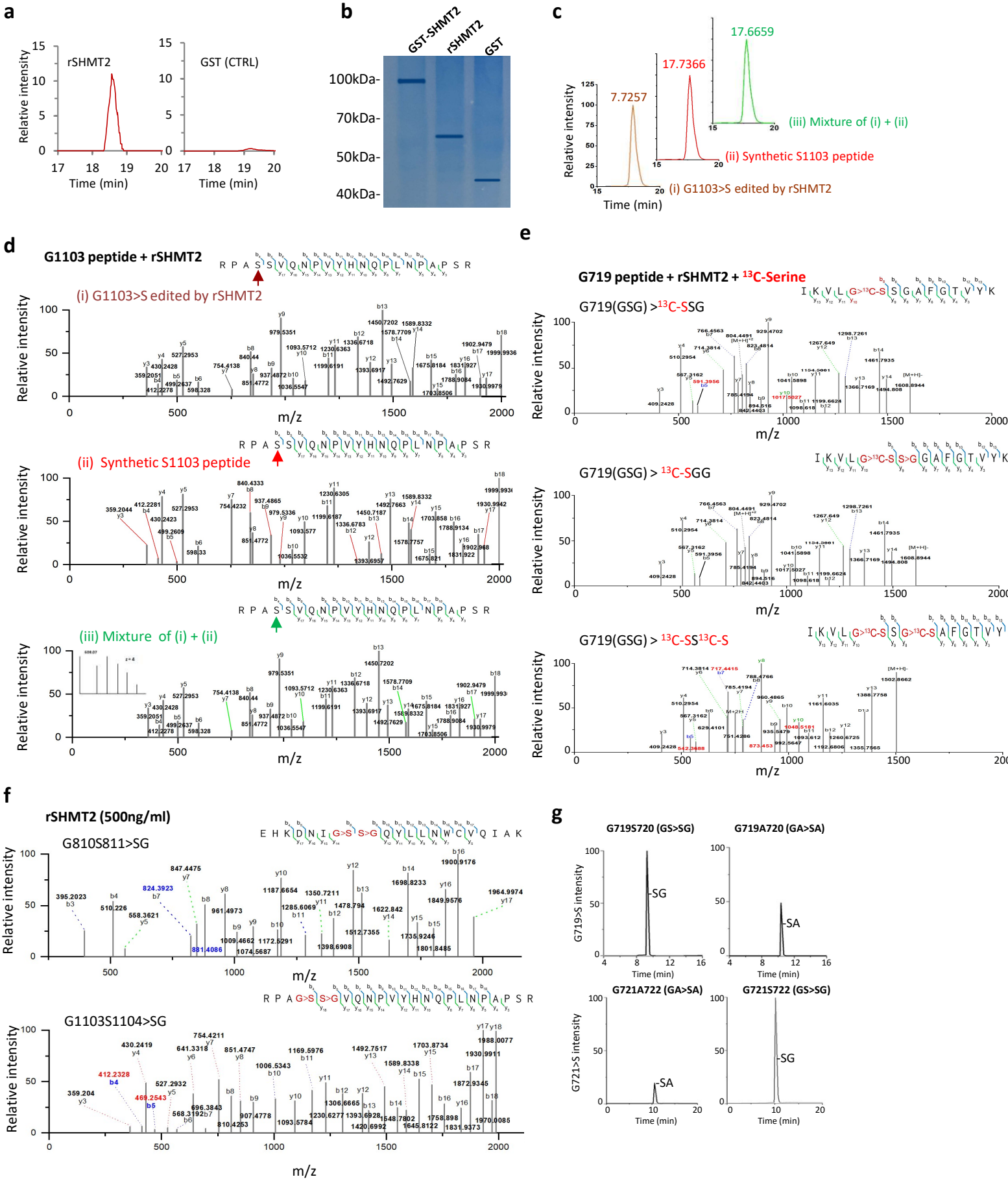
d. Structural analysis revealed that the N-terminal 5K cluster of SHMT2 protein is involved in the interaction with EGFR cytoplasmic domain. The N-terminal 5Ks of SHMT2 protein form hydrophilic bonds with the charged residues of the EGFR protein. Meanwhile, K280 of SHMT2 resides within the center for dimer or tetramer of SHMT2 proteins, and K280 forms bonds with other residues locally.

e. Mass-mass spectra obtained for the peptides of EGFR protein bearing triple G>S mutations (G719>S, G721>S, G724>S) and G720S721>SG mutation. EGFR proteins were purified from HEK293T cells transfected with EGFR along with SHMT2.

f. DNA motif covered the cluster of glycine and serine residues (G719, G721, G724) was sequenced.

g. The dot blot results of polyclonal antibodies prepared for G719>S, G810>S, and G1103>S, respectively. Control and G>S peptides with the sequence of G719 (IKVLG$_{719}$-SGAFGTVYK), G810 (REHKDNIS$_{810}$SQYL), and G1103 (SVPKRPAS$_{1103}$SVQNP) were also spotted on the nitrocellulose membrane with indicated concentrations. Antibody dilution with 1:1000 fold was applied.

h. The time course up to 240 minutes, depicting EGF treatment of H1299 cells were treated with EGF (100 ng/ml) up to 240 minutes. EGFR protein G>S hydroxymethylation induction was immunostained with anti-EGFR G1103>S along with DAPI staining.



Extended Data Fig. 3| SHMT2 catalyzes hydroxymethylation in MFTH dependent and independent manners.

Extended Data Fig. 3| SHMT2 catalyzes hydroxymethylation in MFTH-dependent and independent manners.

a. LC-mass spectra of *in vitro*-derived G1103>S peptide. SHMT2 and GST control proteins were used for the *in vitro* assay with G1103-peptide ($RPAG_{1103}SVQNPVYH$).

b. Purified GST-SHMT2, rSHMT2 and GST were separated in 10% SDS-PAGE gel and stained with Coomassie Brilliant blue.

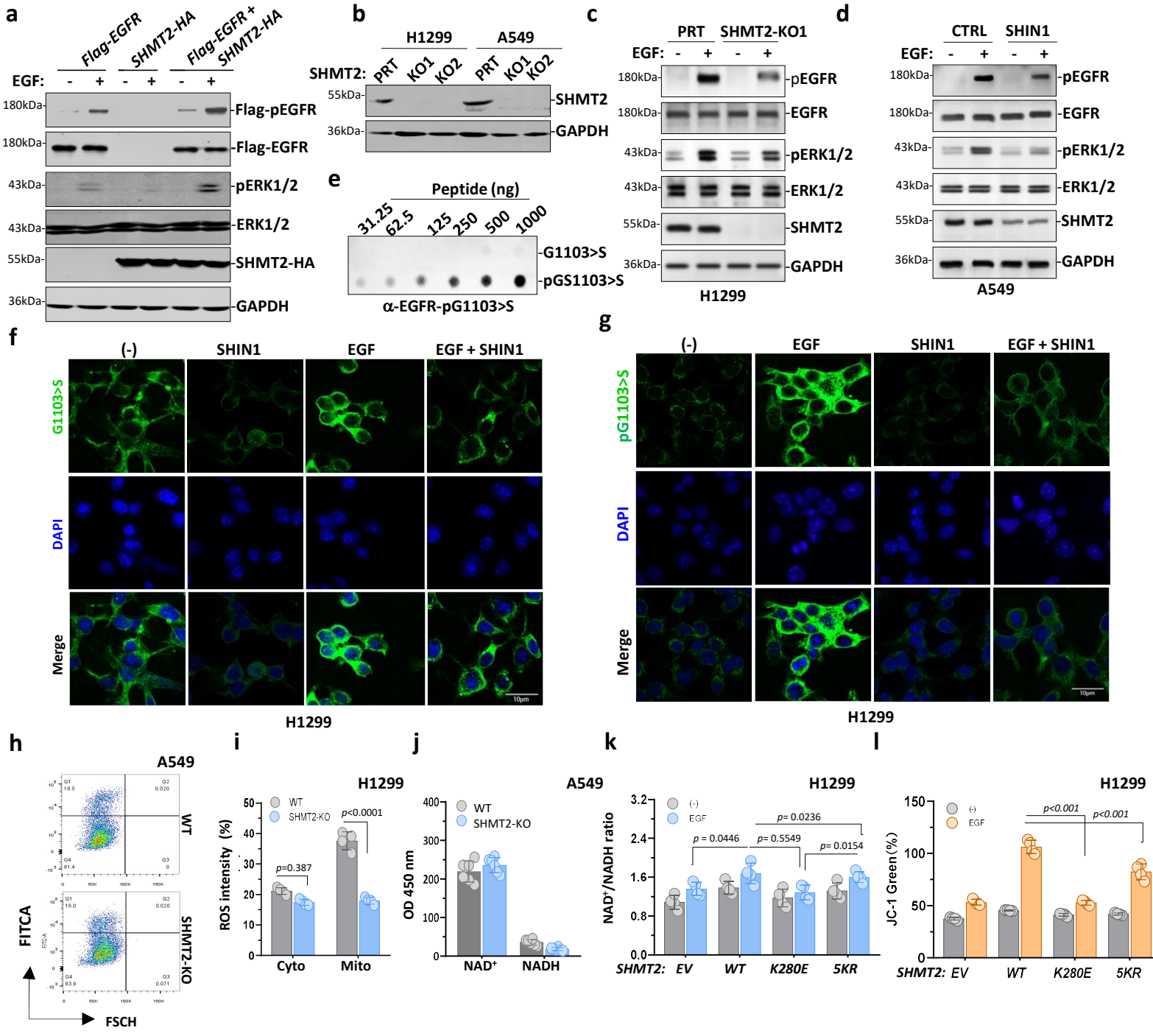
c. LC-Mass spectra obtained from (i) SHMT2-catalyzed G1103-peptide hydroxymethylation reaction *in vitro*, (ii) synthetic S1103 peptide spectrum, and (iii) mixture of (i) and (ii) overlapping spectrum.

d. The mass-mass spectra of G1103>S peptide were obtained from the *in vitro* reaction (i), synthesized S1103 peptide (ii), and the mixture of the two (iii).

e. A synthesized G719-peptide ($IKVLG_{719}SGAFGTVYK$) was incubated with purified rSHMT2 in an *in vitro* assay condition, which included free isotope labeled amino acid ^{13}C-serine, PLP, THF, and ATP. The mass-mass spectra (AB6600 mass spectrometry) were obtained to show G719 peptides with G719>^{13}C-S, G719>^{13}C-SS720>G, and G719>^{13}C-SG721>^{13}C-S.

f. Synthesized G810-peptide or G1103 peptide was incubated with a high dose (500 ng/ml) of purified rSHMT2 (GST-SHMT2) in an *in vitro* assay condition, which included free amino acid ^{13}C-serine, PLP, and MTHF. The reaction was analyzed with AB6600 mass spectrometry for GS>SG SENA editing mutation induction.

g. Synthesized wild type G719 peptide ($IKVL\underline{G_{719}S}GAFGTVYK$) and S720>A G719 peptide ($IKVL\underline{G_{719}A}GAFGTVYK$) were compared for their response to SHMT2 in G719>S editing mutation induction under *in vitro* reaction condition (upper panel). The above synthesized wild type G719-peptide and A722>S mutated G719 peptide ($IKVLGS\underline{G_{721}S}FGTVYK$) were compared for their response to SHMT2 in G721>S editing induction under *in vitro* reaction condition (low panel).



Extended Data Fig.4|Cytosolic SHMT2 affects EGFR protein signaling with a moderate effect on cytosolic metabolism.

Extended Data Fig.4| Cytosolic SHMT2 affects EGFR protein signaling with a moderate effect on metabolism.

a. WCL prepared from HEK293T cells transfected with EV, Flag-EGFR, SHMT2-HA or Flag-EGFR/SHMT2-HA were analyzed in Western blot. EGFR auto-phosphorylation and ERK activation were detected using anti-EGFR-pY1068 and anti-ERK1/2-pT202/pY204 respectively. The protein expression levels of Flag-EGFR (anti-Flag), SHMT2-HA (anti-HA), ERK, and GAPDH were included.

b. Parental (PRT) and SHMT2 knocked out (KO1 and KO2) of H1299 and A549 cells were analyzed in Western blot for SHMT2 expression.

c. PRT and SHMT2 KO cells were treated with EGF (100 ng/ml) or left no treatment for 30 minutes, EGFR-pY1068 and ERK1/2-pT202/pY204 were analyzed in Western blot.

d. A549 cells were treated with DMSO (CTRL) or 5 mM SHIN1 in DMSO pretreatment for 24 hours followed by EGF (100 ng/ml) treatment or no treatment for an additional 30 minutes; EGFR phosphorylation and ERK1/2 activation were analyzed in Western blot as above.

e. Dot blot to show poly-clonal antibody against pG1103>S. G1103>S control peptide and pG1103>S peptide were spotted on the nitrocellulose membrane with indicated amounts.

f. In H1299 cells were incubated with or without 5 mM SHIN1 for 24 hours followed by EGF treatment, glycine hydroxymethylation mediated G>S was detected via fluorescent immunostaining with specific antibody against EGFR-G1103>S. Nuclear DAPI staining was included.

g. In H1299 cells that received 5 mM SHIN1 incubation followed by EGF treatment, EGFR phosphorylation on S1103 (G1103>pS) was detected with specific antibody recognizing EGFR-pS1103. Nuclear staining with DAPI was included.

h. The cytoplasmic ROS level was analyzed in WT or SHMT2-KO A549 cells; WT and SHMT2-KO A549 cells were incubated with 10μM 2',7'-DCFH-DA, and intracellular ROS production was determined using flow cytometry (Ex 488 nm, Em 525 nm). Each ROS analysis was performed in triplicates, and a representative result was shown here.

i. The ROS levels in cytoplasm and mitochondria were analyzed in indicated H1299 cells.

j. Cytosolic NAD+ and NADH levels were examined in H1299 cells with OD450nm.

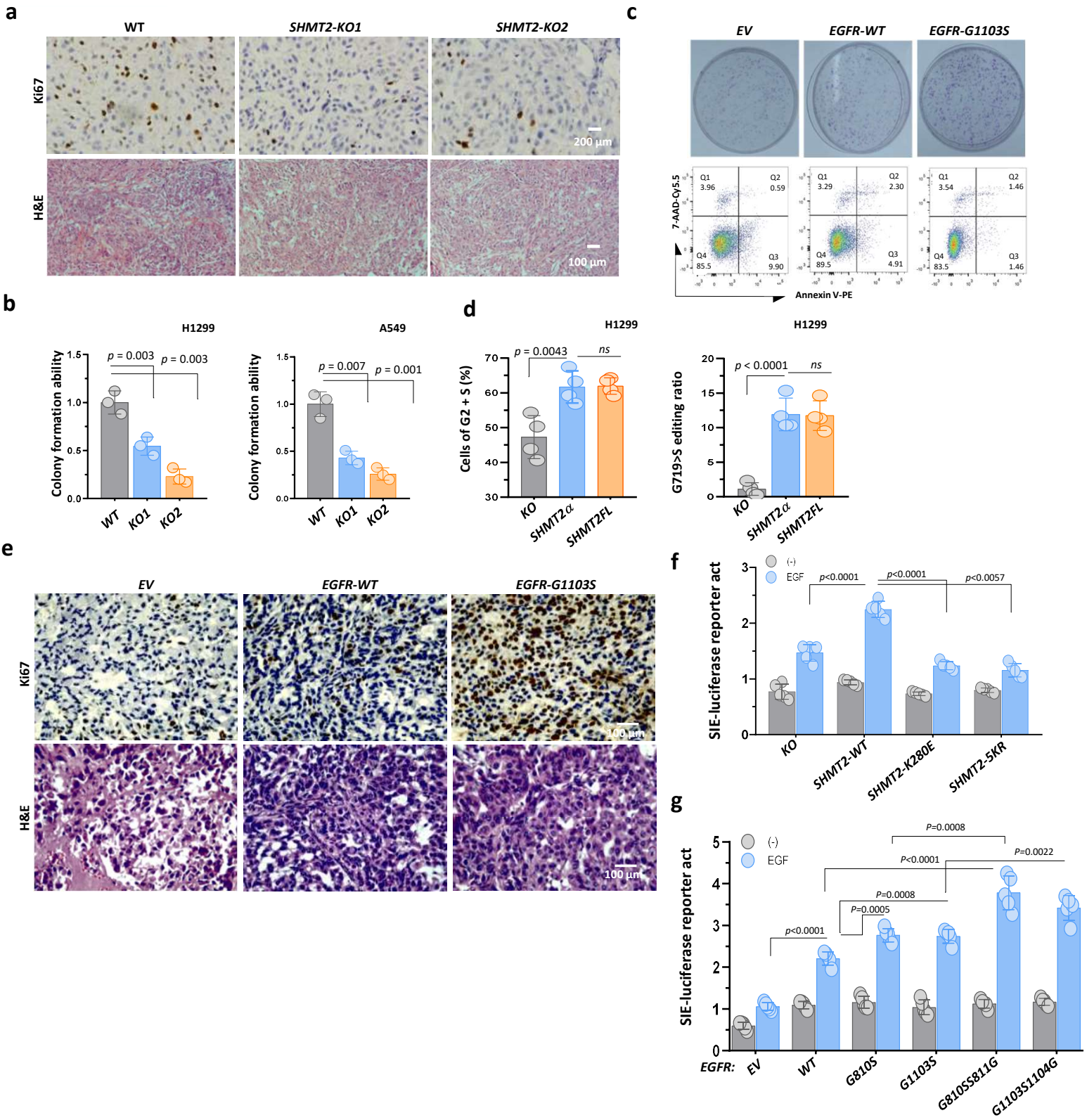
k. Cytosolic NAD+/NADH ratio was analyzed in SHMT2-KO H1299 cells expressing SHMT2 variants (EV, SHMT2-WT, K280E and 5KR) and treated with EGF (100 ng/ml, 30 minutes).

l. The JC-1 probe was applied to analyze mitochondrial *membrane* potential by flow cytometry in parental H1299 cells as well as in H1299 cells with SHMT2-KO background expressing SHMT2-WT, SHMT2-K280E, and SHMT2-5KR.

m. Free amino acid glycine was converted into serine by rSHMT2 *in vitro*. Purified rSHMT2 proteins including WT, K280E, and 5KR forms as well as the control protein GST were incubated with glycine and 5N,10N-MTHF in the cell free reaction buffer. DL-β-phenylserine content was then detected and quantified.

n. Free amino acid serine was converted into glycine in by rSHMT2 *in vitro*. Purified rSHMT2 proteins the control protein GST as above in ***m*** were incubated with serine and THF in the cell free reaction buffer. Glycine content was then detected and quantified.

o. Absolute quantitative CE–MS measurement of intracellular amino acids to compare levels between WT and SHMT2-KO H1299 cells that were not subjected to media change for 96 hours. Amino acids were in units of pg per ml from $1x10^6$ cells.



Extended Data Fig.5|G>S obtained from hydroxymethylation editing or *EGFR* gene mutation effects EGFR signaling similarly.

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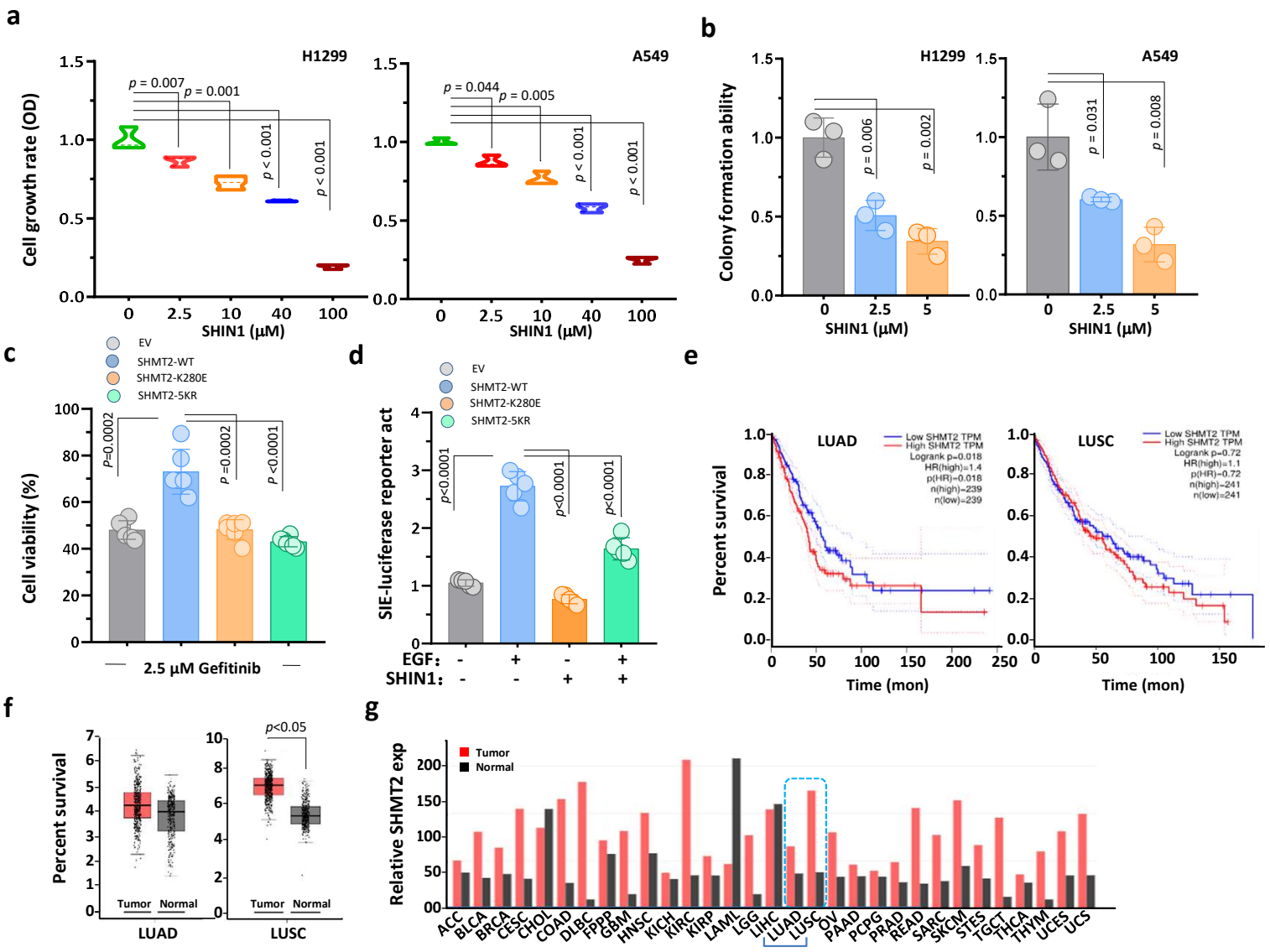
a. Xenograft tumors of *SHMT2-KO (KO1, KO2)* A549 cells and parental A549 cells (WT) implanted subcutaneously in mice and stained them with Ki67 and H&E.
b. Colony formation abilities in mice by comparing *SHMT2-KO1* and *SHMT2-KO2* H1299 cells to parental H1299 cells (WT), mean ± SD, n = 3 (left panel). Colony formation abilities in mice by comparing SHMT2-KO1 and -KO2 A549 cells to parental A549 cells (WT), mean ± SD, n = 3 (right panel).
c. Colony formation ability was applied to test the cell viability of A549 cells expressing EV, *EGFR-WT* or *EGFR-G1103S* cDNA (upper panel). Flow cytometry analysis of apoptotic cells in A549 cells using Annexin-V and 7-AAD staining (bottom panel).
d. Flow cytometry was performed using PI staining to analyze SHMT2 KO H1299 cells and SHMT2 KO H1299 cells with SHMT2α or SHMT2 full length reintroduction. Cells in G2 and S phases were quantitated (left panel). EGFR proteins immunoprecipited from the above H1299 cells were analyzed with mass spectrometry for G719>S editing analysis and peptides with G719>S were quantitated (right panel). The peptide ionic peaks obtained from mass spectrometry were analyzed using the MS software analysis. The G719>S and G719 signal intensity (peak area) were statistically analyzed using Prism 8.0.2 software for the ratio calculation.
e. A549 xenograft tumors expressing EV, EGFR-WT, or EGFR-G1103S were implanted subcutaneously in nude mice and analyzed for H&E staining and IHC with anti-Ki67.
f. In HEK293T cells, the SIE-luciferase reporter was transfected along with EV or SHMT2 variants followed by EGF treatment or no treatment for an additional 6 hours. STAT3 transcription activity based on SIE-luciferase reporter activation was then analyzed.
g. In HEK293T cells, the SIE-luciferase reporter was transfected along with EV or EGFR variants for EGF-activated STAT3 transcription activity analysis as above.



Extended Data Fig.6|SHIN1 inhibits lung cancer cell growth.

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a. To assess the inhibitory effect of SHIN1 on lung cancer cell growth, we treated H1299 and A549 cells with various doses of SHIN1 and analyzed cell growth rates with the CCK8 assay, mean ± SD, n = 3.
b. Colony formation analysis on H1299 cells (left) and A549 cells (right) treated with 2.5 mM or 5 mM SHIN1 for 24 hours, mean ± SD, n = 3.
c. In SHMT2-KO H1299 cells reintroduced with SHMT2, cell viability was analyzed in response to 2.5 mM Gefitinib treatment for 24 hours.
d. In HEK293T cells, EGFR and SIE-luciferase reporter were co-trransfected for 36 hours, followed by treatment with EGF, no EGF, or EGF plus SHIN1 for an additional 6 hours. These transfectants were subjected to STAT3 SIE-luciferase reporter activation analysis.
e. Overall survival in lung adenocarcinoma (LUAD) patients with low SHMT2 expression levels (n=239) was compared to those with high SHMT2 expression levels (n=239) (left), and in lung squamous cell carcinoma (LUSC) patients with low SHMT2 expression levels (n=241) compared to those with high SHMT2 expression levels (n=241). We obtained these results from the TCGA database analysis.
f. Utilizing the TCGA database analysis to create a box plot of SHMT2 expression in patients with LUAD (T) (n=483) and unaffected controls (N) (n=347), as well as patients with LUSC (T) (n=486) and unaffected controls (N) (n=338).
g. Through a database analysis of TCGA (The Cancer Genome Atlas http://cancergenome.nih.gov/), SHMT2 gene expression levels in tumor tissues were compared with normal tissues from patients with various types of cancers.