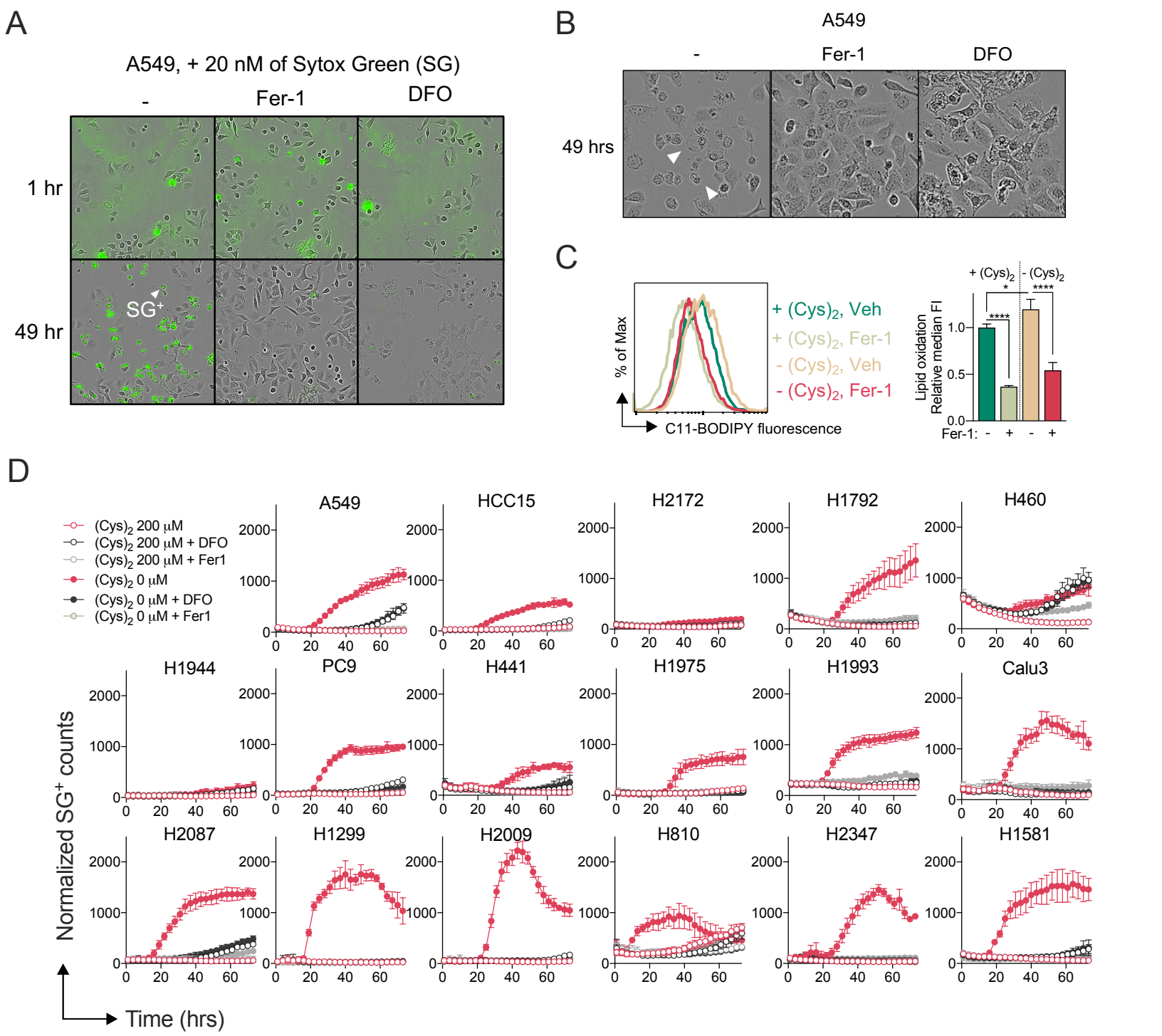

A
A549, + 20 nM of Sytox Green (SG)
-
Fer-1
DFO
1 hr
49 hr
SG+
B
A549
-
Fer-1
DFO
49 hrs
C
% of Max
C11-BODIPY fluorescence
+ (Cys)2, Veh
+ (Cys)2, Fer-1
- (Cys)2, Veh
- (Cys)2, Fer-1
Lipid oxidation
Relative median FI
+ (Cys)2
- (Cys)2
Fer-1: - + - +
D
(Cys)2 200 μM
(Cys)2 200 μM + DFO
(Cys)2 200 μM + Fer1
(Cys)2 0 μM
(Cys)2 0 μM + DFO
(Cys)2 0 μM + Fer1
A549
HCC15
H2172
H1792
H460
H1944
PC9
H441
H1975
H1993
Calu3
H2087
H1299
H2009
H810
H2347
H1581
Normalized SG+ counts
Time (hrs)

Figure S1. Transsulfuration cannot support NSCLC cysteine pools (related to Figure 1). (A) A representative image of Sytox Green (SG) stained A549 cells following cystine starvation for 1 or 49 hours in the presence of Vehicle (0.1% DMSO), Fer-1 (10 μM), or DFO (100 μM). Images show the same well position at the two different time points (1 or 49 hrs). A representative Sytox Green positive (SG^+) cell is indicated with a white arrow. (B) A representative image of non-stained A549 cells following 49 hours of cystine starvation treated with Vehicle (0.1% DMSO), Fer-1 (10 μM), or DFO (100 μM). Representative ferroptotic cells are indicated with white arrows. (C) Measurement of A549 cell lipid peroxidation under cystine starved (0 μM) or replete (200 μM) conditions treated with Vehicle (0.1% DMSO) or Fer-1 (10 μM) for 10 hrs (N=3). The median fluorescence intensity (median FI) of oxidized BODIPY-C11 signal was normalized by mean value of vehicle treated cystine replete conditions. (D) Incucyte time course measurement of NSCLC cell death under cystine starved (0 μM) or replete (200 μM) conditions treated with Vehicle (0.1% DMSO), Ferrostatin-1 (Fer-1, 10 μM) or DFO (100 μM) (N=4). Cell death was determined by Sytox Green every 3 hours over 73 hours and normalized to cell density. For C and D, data are shown as mean ± SD. N is the number of biological replicates. *P<0.05 and ****P<0.0001. For C, a one-way ANOVA with Bonferroni's multiple comparison test was used for statistical analyses.

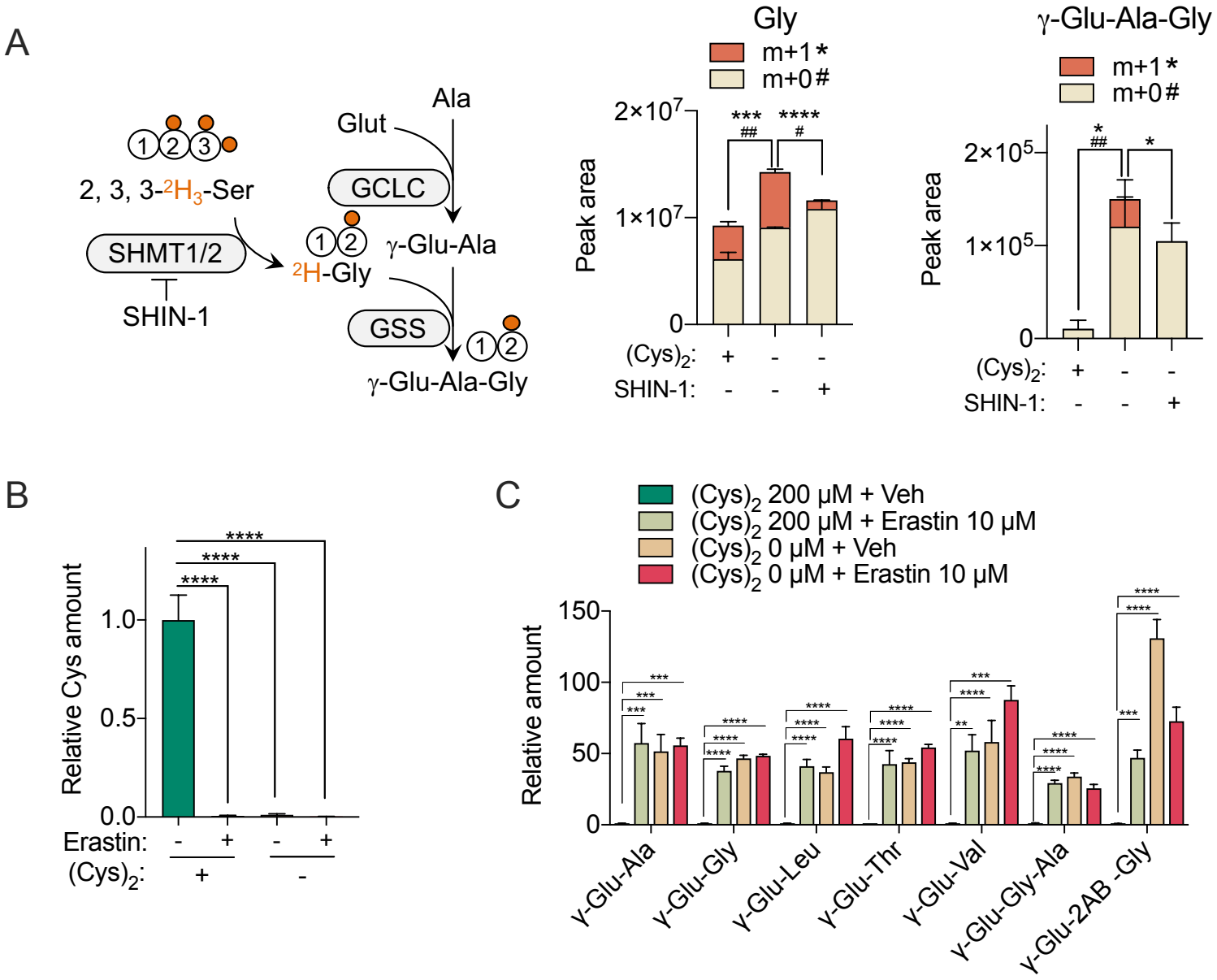

A
2, 3, 3-²H₃-Ser
SHMT1/2
SHIN-1
²H-Gly
Glut
Ala
GCLC
γ-Glu-Ala
GSS
γ-Glu-Ala-Gly
Gly
m+1*
m+0#
Peak area
2×10⁷
1×10⁷
0
(Cys)₂:
SHIN-1:
γ-Glu-Ala-Gly
2×10⁵
1×10⁵
B
Relative Cys amount
1.0
0.5
0.0
Erastin:
(Cys)₂:
C
(Cys)₂ 200 μM + Veh
(Cys)₂ 200 μM + Erastin 10 μM
(Cys)₂ 0 μM + Veh
(Cys)₂ 0 μM + Erastin 10 μM
Relative amount
150
100
50
0
γ-Glu-Ala
γ-Glu-Gly
γ-Glu-Leu
γ-Glu-Thr
γ-Glu-Val
γ-Glu-Gly-Ala
γ-Glu-2AB -Gly

Figure S2. Cystine starvation induces glutamate-derived γ-glutamyl-peptide accumulation (related to Figure 2). (A) A549 cell 2, 3, 3-$^{2}H_{3}$-Ser tracing under cystine replete or starved conditions in the presence and absence of SHIN-1 (0.5 μM) for 12 hrs (N=3). (B-C) Intracellular (B) cysteine and (C) γ-Glu-peptides levels in A549 cells under cystine replete or starved conditions in the presence and absence of erastin (10 μM) for 12 hrs (N=3). For A-C, data are presented as mean ± SD. N is number of biological replicates. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001; $^{\#}$P<0.05, $^{\#\#}$P<0.01. For A-C, a one-way ANOVA with Bonferroni's multiple comparison test was used for statistical analyses.

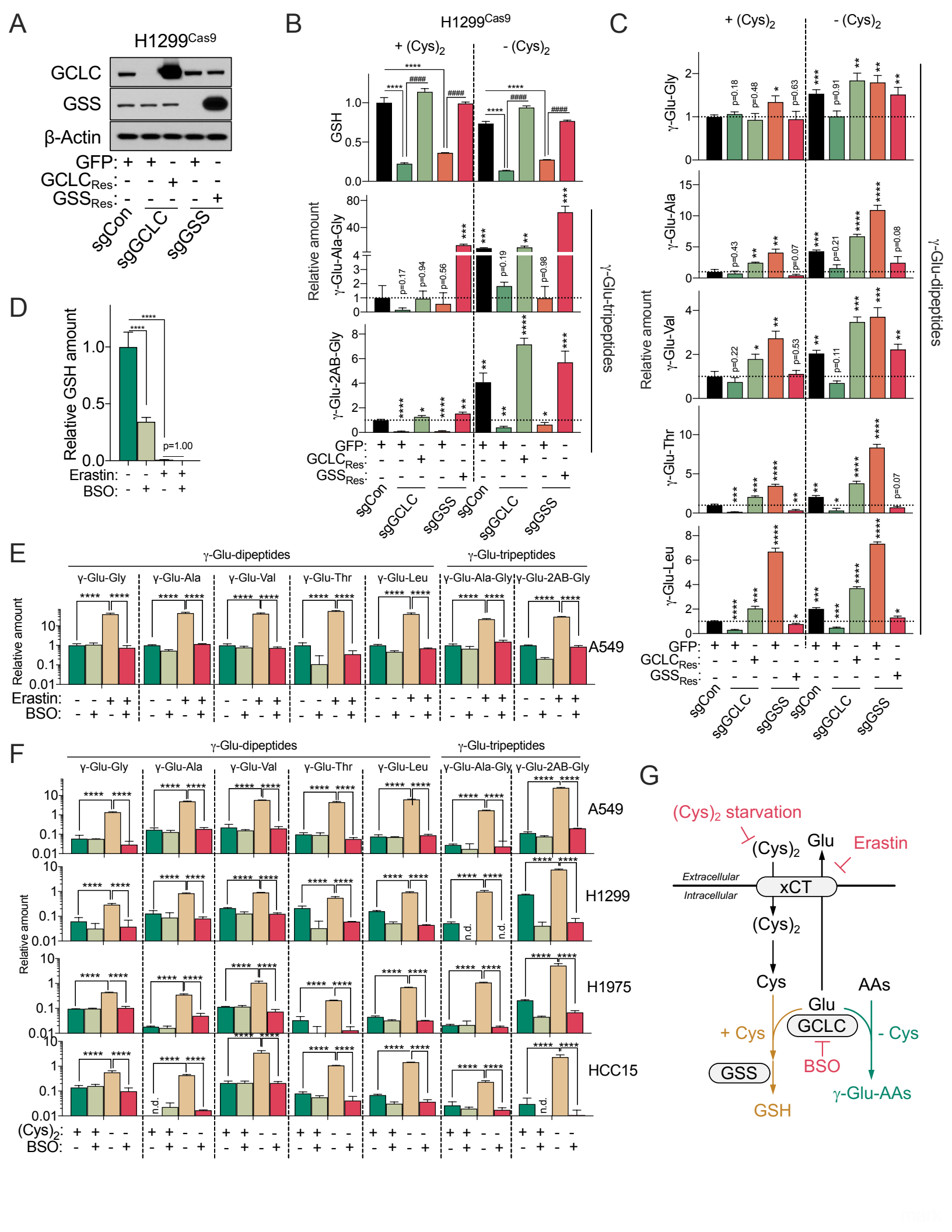

A
H1299Cas9
GCLC
GSS
β-Actin
GFP:
GCLCRes:
GSSRes:
sgCon
sgGCLC
sgGSS
B
H1299Cas9
+ (Cys)2
- (Cys)2
GSH
Relative amount
γ-Glu-Ala-Gly
γ-Glu-2AB-Gly
γ-Glu-tripeptides
C
γ-Glu-Gly
γ-Glu-Ala
γ-Glu-Val
γ-Glu-Thr
γ-Glu-Leu
γ-Glu-dipeptides
D
Relative GSH amount
p=1.00
Erastin:
BSO:
E
γ-Glu-dipeptides
γ-Glu-tripeptides
γ-Glu-Ala-Gly
γ-Glu-2AB-Gly
A549
F
H1299
H1975
HCC15
n.d.
(Cys)2:
G
(Cys)2 starvation
Erastin
Extracellular
Intracellular
xCT
Glu
Cys
AAs
GCLC
+ Cys
- Cys
BSO
GSS
GSH
γ-Glu-AAs

Figure S3. GCLC mediates γ-glutamyl-peptide synthesis in cell culture (related to Figure 3). (A) Representative immunoblots of GCLC and GSS expression from $H1299^{Cas9}$ cells transduced with sgRNAs (sgCon, sgGCLC or sgGSS), followed by reconstitution with GFP (+GFP), sgRNA-resistant GCLC (+$GCLC_{Res}$), or sgRNA-resistant GSS (+GSS_{Res}). β-actin was used as a loading control. (B-C) Intracellular γ-Glu-tripeptides (B) and γ-Glu-dipeptides (C) in the cells from (A) under cystine replete or starved conditions for 3 hrs (N=3). For B-C, data were normalized to the mean value of sgCon + GFP cells under cystine replete conditions. (D-E) Intracellular GSH (D) and γ-Glu-peptide levels (E) in A549 cells treated with erastin (10 μM) and/or BSO (100 μM) for 12 hrs (N=3). (F) Intracellular γ-Glu-peptides levels in four NSCLC cell lines under cystine replete or starved conditions in the presence and absence of BSO (100 μM) for 12 hrs (N=3). (G) Schematics depicting the effect of cystine availability and GCLC activity on γ-Glu-peptide production. For B - F, data are presented as mean ± SD. N is number of biological replicates. n.d. not detected. **P<0.01, ***P<0.001, and ****P<0.0001; ####P<0.0001. For GSH in B, one-way ANOVA with Bonferroni's multiple comparison test was used for the statistical comparison between sgCon + GFP, sgGCLC + GFP, and sgGSS + GFP. For the statistical comparison between sgGCLC + GFP and sgGCLC + $GCLC_{Res}$ or sgGSS + GFP and sgGSS + GSS_{Res}, an unpaired two-tailed t test was used. For γ-Glu-Ala-Gly and γ-Glu-2AB-Gly in B and C, an unpaired two-tailed t test was used for the comparison with parental cells under cystine replete conditions [+$(Cys)_2$]. For D-F, a one-way ANOVA with Bonferroni's multiple comparison test was used for statistical analyses.

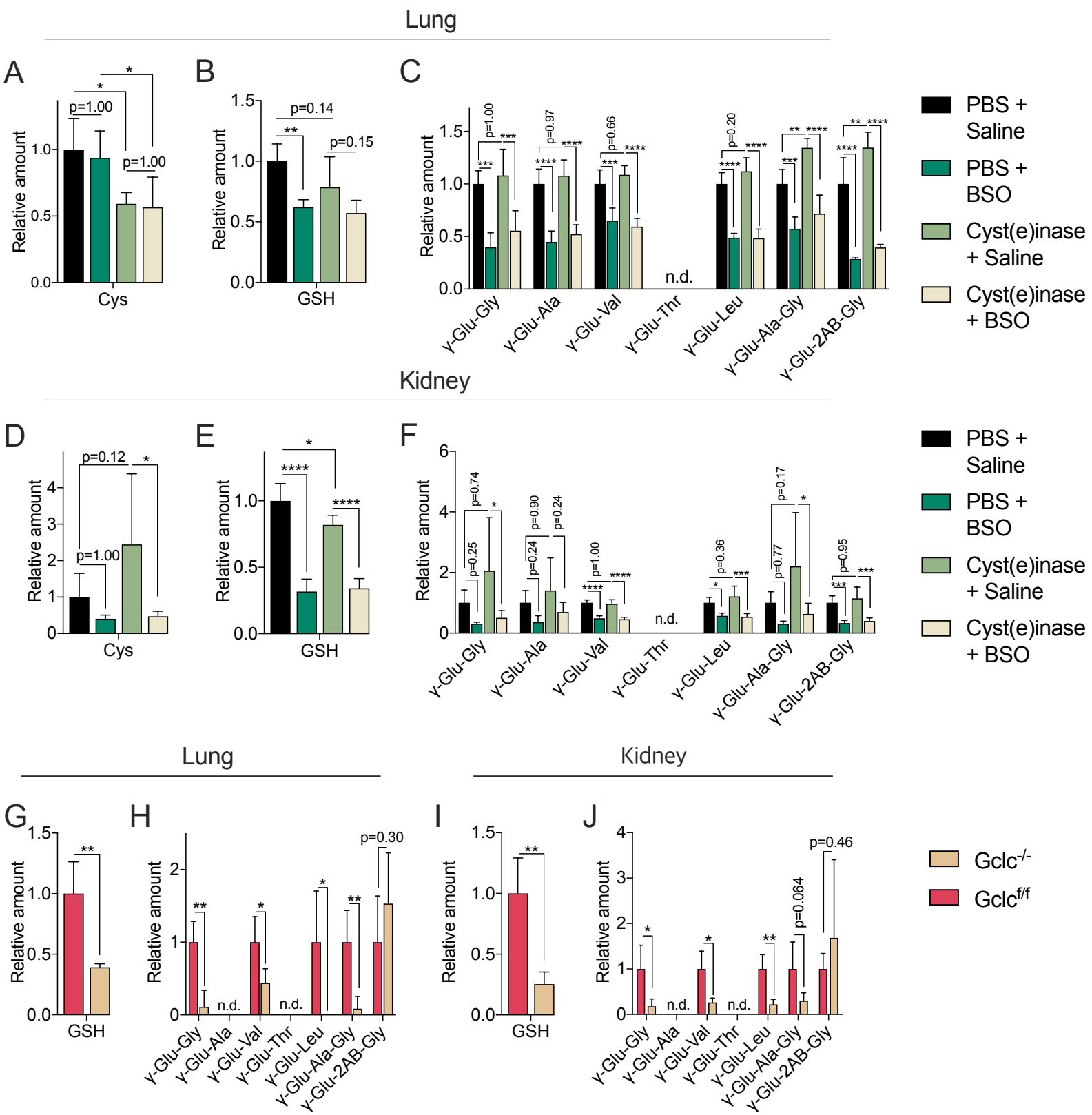
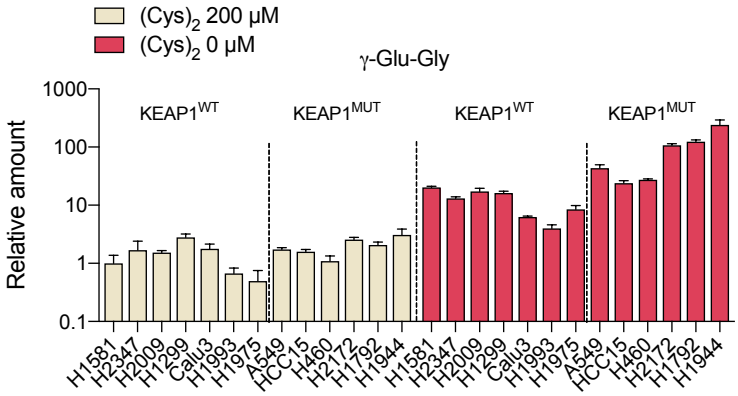
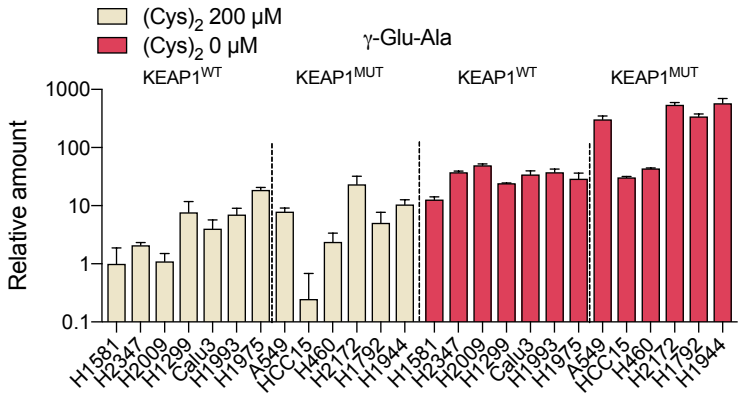
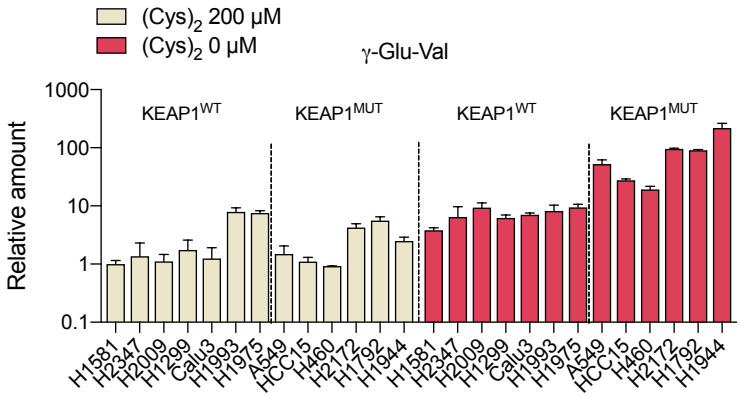

Lung
A
B
C
Relative amount
Cys
GSH
p=1.00
p=0.14
p=0.15
n.d.
γ-Glu-Gly
γ-Glu-Ala
γ-Glu-Val
γ-Glu-Thr
γ-Glu-Leu
γ-Glu-Ala-Gly
γ-Glu-2AB-Gly
PBS + Saline
PBS + BSO
Cyst(e)inase + Saline
Cyst(e)inase + BSO
Kidney
D
E
F
p=0.12
p=0.74
p=0.25
p=0.90
p=0.24
p=0.36
p=0.77
p=0.17
p=0.95
Lung
Kidney
G
H
I
J
Gclc-/-
Gclc f/f
p=0.30
p=0.46
p=0.064

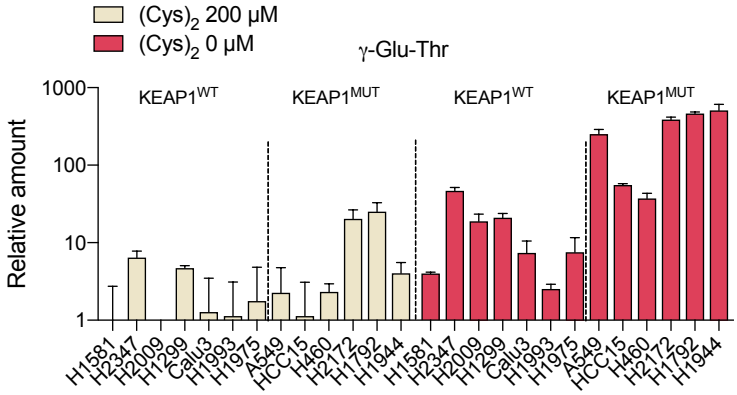
Figure S4. GCLC mediates γ-glutamyl-peptide synthesis in vivo (related to Figure 4). (A-C) Analysis of lung cysteine (A), GSH (B), and γ-Glu-peptides levels (C) in mice treated with Cyst(e)inase or PBS, together with BSO or saline. (D-F) Analysis of kidney cysteine (D), GSH (E), and γ-Glu-peptides levels (F) in the mice from (A-C). The metabolite levels were normalized to the mean value of PBS/saline treated mice (N=5). (G-H) Analysis of lung GSH (G) and γ-Glu-peptides levels (H) in $Gclc^{f/f}$ and $Gclc^{-/-}$ mice. (I-J) Analysis of kidney GSH (I) and γ-Glu-peptides levels (J) in the mice from (G-H). The metabolite levels are normalized to the mean value of $Gclc^{f/f}$ mouse tissues (N=4). For A-J, data are presented as mean ± SD. N is number of biological replicates. n.d., not detected; *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. For A-F, a one-way ANOVA with Bonferroni's multiple comparison test was used for statistical analyses. For G-J, an unpaired two-tailed t test was used for the statistical comparisons.

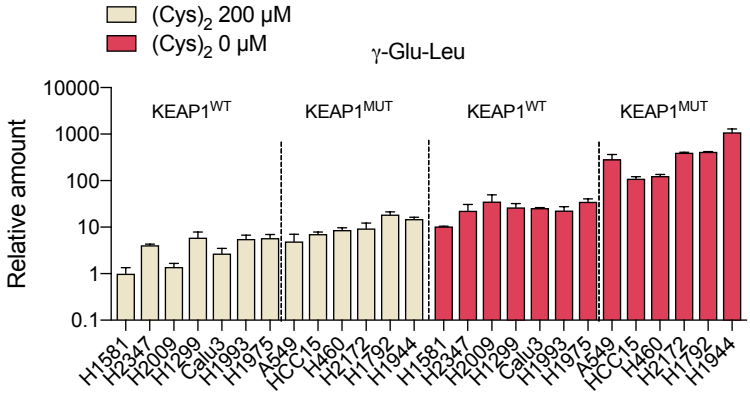
A

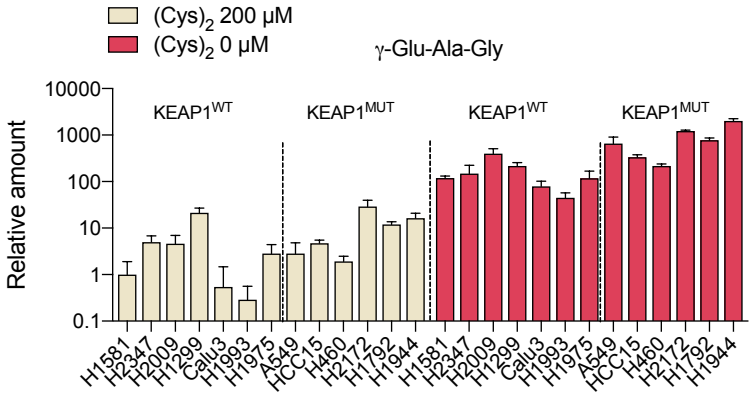

(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Gly
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944


(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Ala
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944


(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Val
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944


(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Thr
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
1000
100
10
1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944


(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Leu
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
10000
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944


(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-Ala-Gly
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
10000
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944

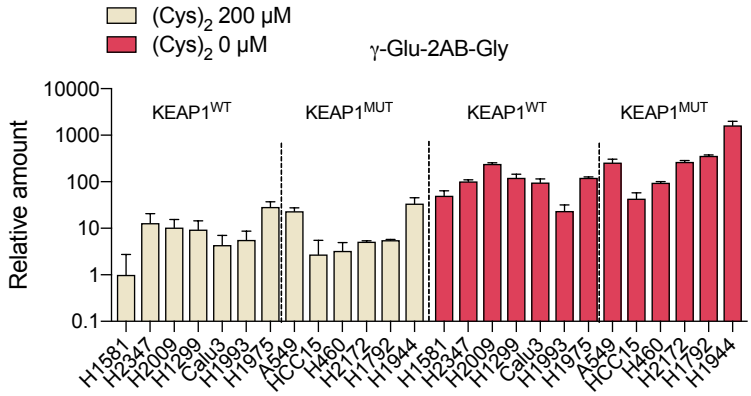

(Cys)2 200 μM
(Cys)2 0 μM
γ-Glu-2AB-Gly
Relative amount
KEAP1WT
KEAP1MUT
KEAP1WT
KEAP1MUT
10000
1000
100
10
1
0.1
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944

Figure S5. NRF2 promotes γ-glutamyl-peptide synthesis via GCLC (related to Figure 5). (A) Analysis of relative γ-Glu-dipeptide levels in KEAP1^{WT} (N=7) and KEAP^{MUT} (N=6) NSCLC cell lines. The γ-Glu-peptides were analyzed following culture in cystine replete or starved conditions for 12 hrs. The γ-Glu-peptide amount of each NSCLC cell line was normalized to the mean value of H1581 cells under cystine replete conditions.

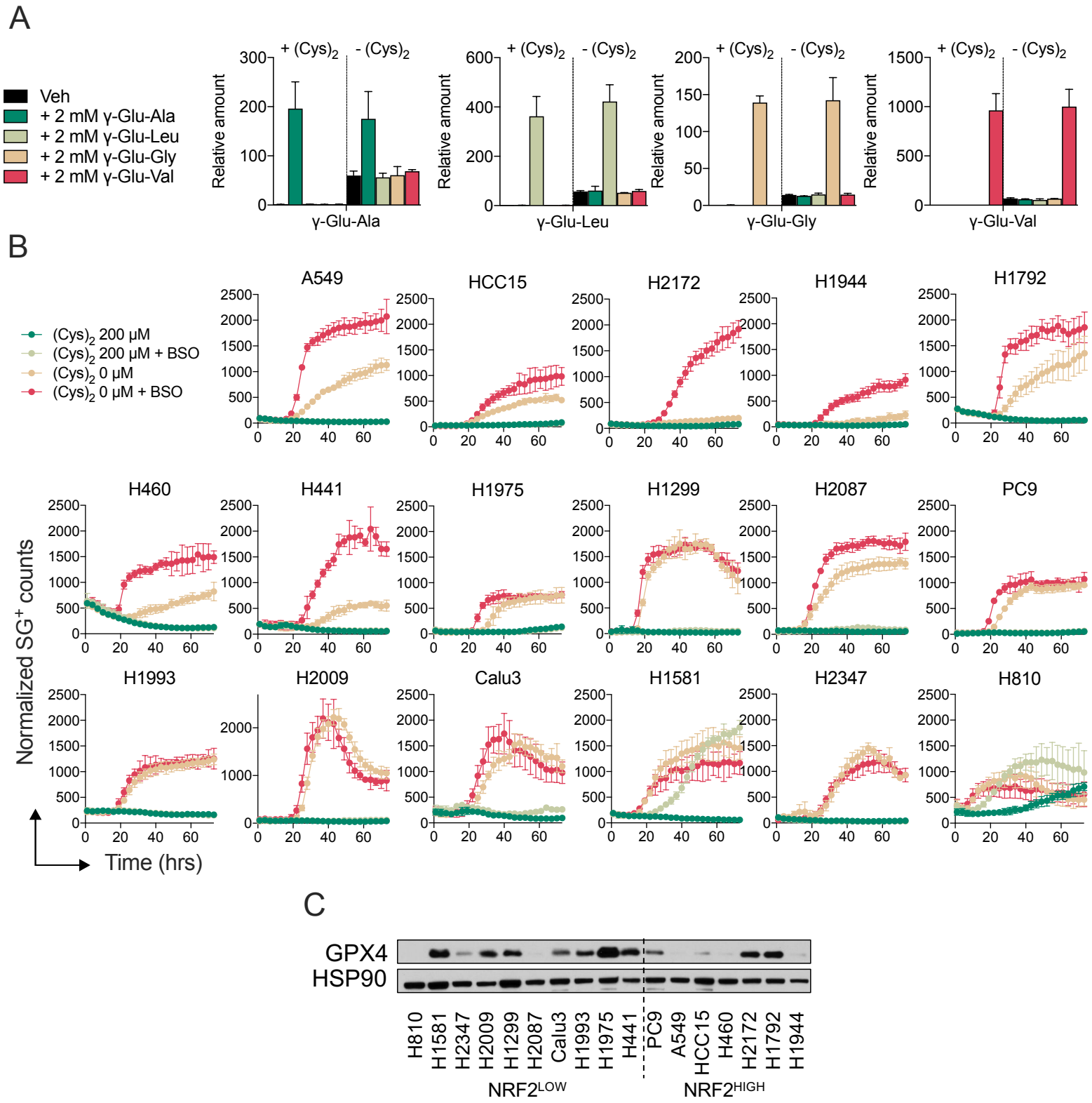

A
Veh
+ 2 mM γ-Glu-Ala
+ 2 mM γ-Glu-Leu
+ 2 mM γ-Glu-Gly
+ 2 mM γ-Glu-Val
+ (Cys)2
- (Cys)2
Relative amount
γ-Glu-Ala
γ-Glu-Leu
γ-Glu-Gly
γ-Glu-Val
B
(Cys)2 200 μM
(Cys)2 200 μM + BSO
(Cys)2 0 μM
(Cys)2 0 μM + BSO
A549
HCC15
H2172
H1944
H1792
H460
H441
H1975
H1299
H2087
PC9
H1993
H2009
Calu3
H1581
H2347
H810
Normalized SG+ counts
Time (hrs)
C
GPX4
HSP90
H810
H1581
H2347
H2009
H1299
H2087
Calu3
H1993
H1975
H441
PC9
A549
HCC15
H460
H2172
H1792
H1944
NRF2LOW
NRF2HIGH

Figure S6. Dipeptide synthesis protects KEAP1 mutant cells from ferroptosis (related to Figure 6). (A) Determination of the ability of exogenous γ-Glu-dipeptides to elevate intracellular γ-Glu-dipeptide levels. A549 cells were treated with the indicated concentration of γ-Glu-dipeptides for 12 hours under cystine replete and starved conditions and intracellular levels were assayed. γ-Glu-peptide amounts were normalized by the mean value of cystine replete conditions (N=3). (B) NSCLC cell death was monitored using Sytox Green (SG) every 3 hours with the Incucyte system. Cells were cultured under cystine starved or replete conditions and treated with vehicle (0.1% DMSO) or BSO (100 μM) for 73 hours (N=4). Vehicle-treated cystine replete and starved data are from Figure S1D. (C) Representative immunoblot of GPX4 expression in $NRF2^{LOW}$ and $NRF2^{HIGH}$ NSCLC cell lines. This blot is a reprobing of the GCLM blot shown in Figure 5A and shares the loading control (HSP90). For A and B, data are presented as mean ± SD. N is number of biological replicates.

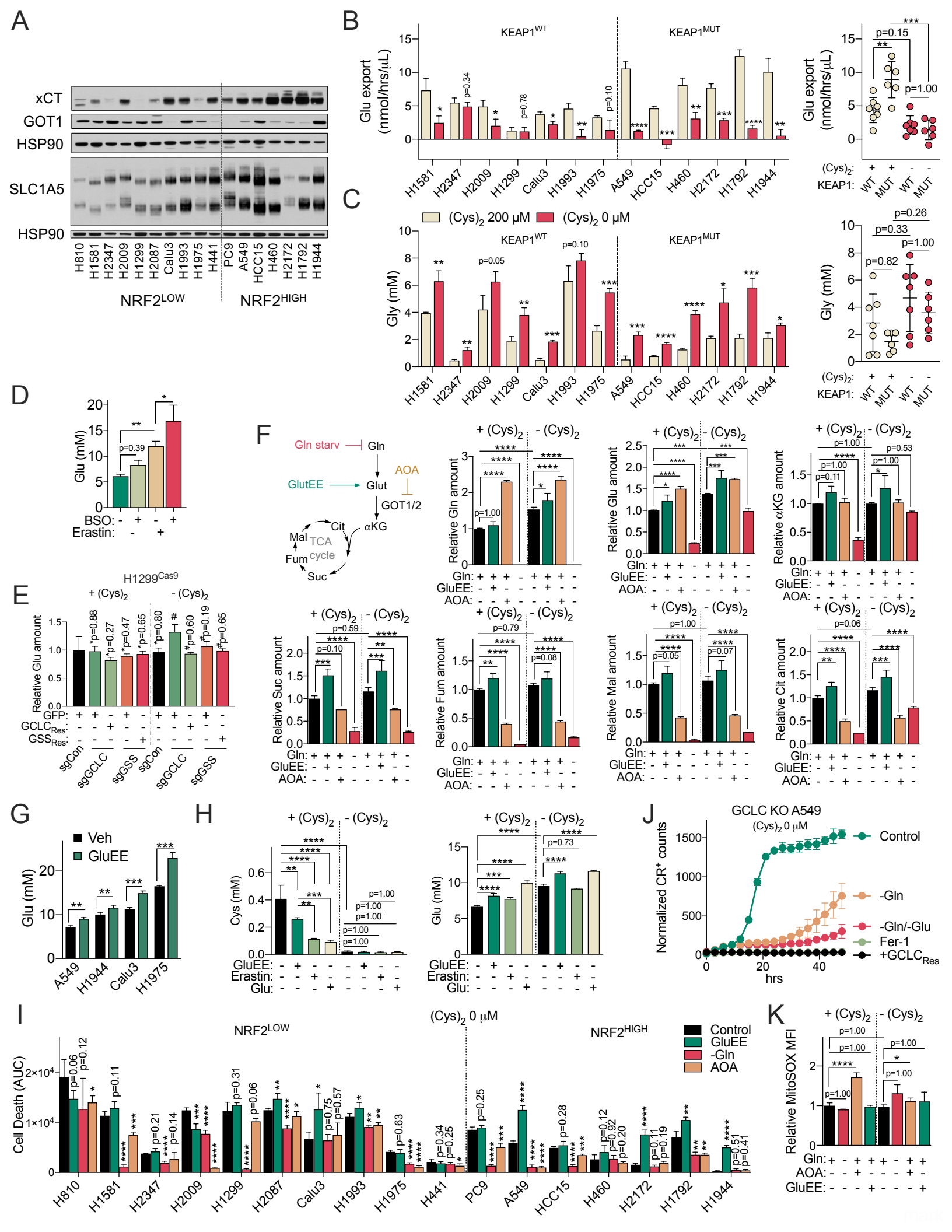

A
xCT
GOT1
HSP90
SLC1A5
HSP90
H810 H1581 H2347 H2009 H1299 H2087 Calu3 H1993 H1975 H441 PC9 A549 HCC15 H460 H2172 H1792 H1944
NRF2LOW
NRF2HIGH
B
KEAP1WT
KEAP1MUT
Glu export (nmol/hrs/μL)
H1581 H2347 H2009 H1299 Calu3 H1993 H1975 A549 HCC15 H460 H2172 H1792 H1944
p=0.15
p=1.00
(Cys)2: + + - -
KEAP1: WT MUT WT MUT
C
(Cys)2 200 μM
(Cys)2 0 μM
KEAP1WT
KEAP1MUT
Gly (mM)
p=0.26
p=0.33
p=1.00
p=0.82
D
Glu (mM)
p=0.39
BSO: - + - +
Erastin: - +
E
H1299Cas9
+ (Cys)2
- (Cys)2
Relative Glu amount
GFP:
GCLCRes:
GSSRes:
sgCon sgGCLC sgGSS sgCon sgGCLC sgGSS
F
Gln starv
Gln
GlutEE
Glut
AOA
GOT1/2
Cit
αKG
Mal
TCA cycle
Fum
Suc
Relative Gln amount
Relative Glu amount
Relative αKG amount
Relative Suc amount
Relative Fum amount
Relative Mal amount
Relative Cit amount
Gln:
GluEE:
AOA:
G
Veh
GluEE
Glu (mM)
A549 H1944 Calu3 H1975
H
Cys (mM)
Glu (mM)
GluEE:
Erastin:
Glu:
J
GCLC KO A549
(Cys)2 0 μM
Normalized CR+ counts
Control
-Gln
-Gln/-Glu
Fer-1
+GCLCRes
hrs
I
NRF2LOW
(Cys)2 0 μM
NRF2HIGH
Control
GluEE
-Gln
AOA
Cell Death (AUC)
H810 H1581 H2347 H2009 H1299 H2087 Calu3 H1993 H1975 H441 PC9 A549 HCC15 H460 H2172 H1792 H1944
K
+ (Cys)2
- (Cys)2
Relative MitoSOX MFI
Gln:
AOA:
GluEE:

Figure S7. Dipeptide synthesis scavenges glutamate (related to Figure 7). (A) Representative immunoblot of xCT, GOT1, and SLC1A5 in $NRF2^{LOW}$ and $NRF2^{HIGH}$ NSCLC cell lines. The xCT and GOT1 blots share a membrane with the NRF2 blot shown in Figure 5A and share the same loading control (HSP90). The SLC1A5 blot is a reprobing of the same membrane as the GCLC and GSS blots shown in Figure 5A and shares the loading control (HSP90). (B) (Left) Determination of the Glu export rate in NSCLC cell lines under cystine replete and starved conditions for 12 hrs (N=3). (Right) Comparison of the Glu export rate between KEAP1 ^{WT} (N=7) and KEAP1 ^{MUT} NSCLC (N=6) cells. (C) (Left) Analysis of intracellular Gly levels in NSCLC cell lines under cystine replete and starved conditions for 12 hrs (N=3). (Right) Comparison of Gly levels between KEAP1 ^{WT} (N=7) and KEAP1 ^{MUT} NSCLC (N=6) cells. (D) Analysis of intracellular Glu concentrations in A549 cells treated with erastin (10 μM) in the presence and absence of 100 μM BSO for 12 hrs (N=3). (E) Analysis of relative intracellular Glu levels in the $H1299^{Cas9}$ transduced with sgRNAs (sgCon, sgGCLC or sgGSS), followed by reconstitution with GFP (+GFP), sgRNA-resistant GCLC ($+GCLC_{Res}$), or sgRNA-resistant GSS ($+GSS_{Res}$) under cystine replete or starved conditions for 3 hrs (N=3). (F) Analysis of intracellular Gln, Glu, α-ketoglutarate (αKG), succinic acid (Suc), Fumaric acid (Fum), Malic acid (Mal), and Citric acid (Cit) under cystine replete and starved conditions for 12 hrs in the presence and absence of GluEE (5 mM), AOA (0.5 mM), or Gln starvation. (N=3). (G) Analysis of intracellular A549, H1944, Calu3, and H1975 cell Glu levels in the presence of GluEE (5 mM) for 12 hrs (N=3). (H) Analysis of intracellular A549 cell Cys (left) and Glu (right) levels following culture in cystine replete and starved conditions in the presence of GluEE (5 mM), Erastin (0.5 μM), or Glu (5 mM) for 12 hours (N=3). (I) Analysis of the death of NSCLC cell lines treated with GluEE (5 mM), AOA (0.5 mM) or starved of Gln. Cells were starved of cystine and cell death was monitored by Sytox Green every 3 hours for 49 hours, followed by AUC calculation. (N=4 except H1299, cystine 0 μM + GluEE: N=3). (J) Analysis of the death of GCLC KO A549 cells reconstituted with GCLC ($+GCLC_{Res}$), treated with Fer-1 (10 μM), or starved of Gln with or without Glu. Cells were starved of cystine for the indicated time points and death was monitored by Cytotox Red (CR) every 3 hours, and counts were normalized to density (N=3). (K) Analysis of A549 cell mitochondrial superoxide levels with MitoSOX under cystine starved and replete

conditions in the presence of AOA (0.5 mM), GluEE (5 mM), or Gln starvation for 8.5 hrs (N=3). MFI = mean fluorescence intensity. For B-K, data is presented as mean ± SD. N is number of biological replicates. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. For B (left), C (left), E, and G, unpaired two-tailed t tests were used for the statistical comparisons. For E, * is for the comparison with sgCon + GFP under cystine replete conditions [+$(Cys)_2$] and # is for the comparison between sg Con + GFP under cystine starved conditions [-$(Cys)_2$]. For B (right), C (right), D, F, H, I, and K, one-way ANOVA with Bonferroni's multiple comparison tests were used for statistical analyses.