Supplemental figure

Fig. S1. Cysteine is capable to inhibit ferroptosis independent of GSH synthesis.

(A) Analysis of lipid ROS level upon cystine (CC) deprivation in MEFs. MEFs were treated in the absence or presence of CC (400 μM). After incubated for 8 h, cells were stained with BODIPY 581/591 C11 and subsequently analyzed by flow cytometry. Cells that are positive for BODIPY 581/591 C11 are defined as accumulation of lipid ROS. (B) Detection of cell death in the context of CC in MEFs. MEFs were cultured for 12 h as described in Fig. S1 (A) and followed by staining with propidium iodide (PI). Cell death was measured by flow cytometry analysis. Cells that are positive for PI were considered as dead ones. (C and D) Analysis of lipid ROS level and cell death upon cystine (CC) deprivation in A549 cells. Cells were cultured in the absence or presence of CC (400 μM) for 8 h for evaluation of lipid ROS (C) as described in Fig. S1 (A), or for 12 h for cell death detection (D) as described in Fig. S1 (B). Ferrastain-1 (Fer-1, 10 μM) and deferoxamine (DFO, 80 μM), two classical inhibitors of ferroptosis, were included as negative control. (E-H) Assay of lipid ROS level and cell death upon impairment of GSH synthesis with L-buthionine sulfoximine (BSO). HT1080 cells and MEFs were cultured in the absence or presence of indicated amount BSO for 24 h. Cells were collected for assessment of lipid ROS level (E and G) and cell death (F and H) as described in Fig. S1 (A and B), respectively. (I) Measurement of glutathione (GSH) and oxidized glutathione (GSSG) level upon impairment of GSH synthesis with BSO in A549 cells. A549 cells were cultured in the absence or presence of indicated concentrations of BSO for 24 h and subjected to assay of GSH and GSSG level. (J and K) Assay of lipid ROS level and cell death induced by BSO in A549 cells. Cells acquired from Fig. S1 (I) were subjected to detection of lipid ROS (J) as described in Fig. S1 (A) and cell death (K) as described in Fig. S1 (B). (L) Knockdown of subunit of glutamate-cysteine ligase (GCLC) in HT1080 cells with siRNAs. Cells were transfected with siRNAs against GCLC. Following 60 h incubation, total cell extracts were analyzed for GCLC expression with Western blot using antibody against GCLC. (M) Evaluation of GSH and GSSG level for catalytic subunit of glutamate-cysteine ligase (GCLC)-depleted HT1080 cells. GCLC-depleted HT1080 cells acquired from Fig. S1 (L) were subjected to assessment of GSH and GSSG level. (N and O) Assessment of lipid ROS and cell death for GCLC-depleted HT1080 cells. GCLC-depleted HT1080 cells acquired from Fig. S1 (L) were subjected to measurement of lipid ROS (N) as described in Fig. S1 (A), and detection of cell death (O) as described in Fig. S1 (B). Data represent as mean ± SD of three independent experiments, with significance determined by one-way ANOVA test. $^{**}P < 0.01$; $^{****}P<0.0001$; ns, nonsignificant.

Fig. S2. Homocysteine is capable to inhibit cystine deprivation-induced ferroptosis.

(A and B) Assay for lipid ROS and cell death in response to homocysteine (Hcy) treatment in MEFs. MEFs were cultured with indicated concentration of Hcy in the absence of Cystine (CC). After 8 h for culture, cells were collected and subjected to lipid ROS analysis (A) as described in Fig. S1 (A). After 10 h of incubation, cells were subjected to evaluation of cell death (B) as described in Fig. S1 (B). (C and D) Measurement of the impact of Hcy on lipid ROS and cell death in HT1080 cells. In the absence or presence of cysteine (Cys, 400 μM), cystine (CC, 400 μM), homocysteine (Hcy, 400 μM), L-homocystine (L-hcy2, 400 μM), or D-homocystine (D-hcy2, 400 μM), cells were incubated for 10 h for lipid ROS assessment (C) as described in Fig. S1 (A), or for 14 h for evaluation of cell death (D) as described in Fig. S1 (B). The presence of CC (400 μM) in Fig. S2 (A and B), Fer-1 (10 μM) and DFO (80 μM) in Fig. S2 (A-C) were the negative control. One representative experiment out of three independent biological replicas was shown.

Fig. S3. The role of homocysteine in ferroptosis inhibition is independent of transsulfuration pathway.

(A) Knockdown of cystathionine β-synthase (CBS) in HT1080 cells with siRNAs. HT1080 cells were transfected with siRNAs against CBS or scramble. Following 48 h incubation, total cell extracts were analyzed for CBS expression with Western blot using antibody against CBS. (B and C) Evaluation of lipid ROS and cell death for CBS-depleted HT1080 cells. Cells acquired from Fig. S3 (A) were incubated with or without cystine (CC, 400 μM) in the absence or presence of homocysteine (Hcy, 400 μM) as indicated. Cells were incubated for 10 h for detection of lipid ROS (B) as described in Fig. S1 (A), or for 14 h for cell death measurement (C) as described in Fig. S1 (B). (D) Assay of MAT2A, SAHH, CBS, and CTH expression. HT1080 cells and MEFs were cultured and subjected to analyzed for MAT2A, SAHH, CBS, and CTH expression with Western blot using corresponding antibodies. (E and F) Evaluation of lipid ROS and cell death for cystathionine γ-lyase (CTH) inhibition in MEFs. MEFs were treated with homocysteine (Hcy, 400 μM) or/and DL-propargylglycine (PAG, 100 μM), a chemical CTH inhibitor, in the absence of CC as indicated. Cells were treated for 8 h for analysis of lipid ROS (E) as described in Fig. S1 (A) or for 12 h for evaluation of cell death (F) as described in Fig. S1 (B). (G and H) Assessment of the effect of cystathionine (Cta) on lipid ROS and cell death in CTH-inhibited MEFs. MEFs were treated with Cta (400 μM) or/and PAG (100 μM) in the absence of CC as indicated. After treatment for 8 h or 12 h respectively, lipid ROS (G) or cell death (H) were evaluated as described in Fig. S1 (A and B). (I and J) Measurement of lipid ROS and cell death induced by Hcy (400 μM), Cta (400 μM) or PAG (400 μM) in the absence of CC. In the context of different reagent combinations as indicated, HT1080 cells were cultured for 8 h for lipid ROS determination (I) as described in Fig. S1 (A) or for 12 h for evaluation of cell death (J) as described in Fig. S1 (B). One representative experiment out of three independent biological replicas was shown.

Fig. S4. The effect of cysteine on anti-ferroptosis relies on GPX4.

(A and B) Impact of GPX4 inhibition on lipid ROS level and cell death in the presence of cysteine (Cys) in A549 cells. Cells were cultured with or without RSL3 (2 μM) in the presence of Cys (400 μM). Following 4 h or 8 h incubation, cells were subjected to evaluation of lipid ROS (A) as described in Fig. S1 (A) or cell death detection (B) as described in Fig. S1 (B). (C) Overexpression of Flag-xCT in HT1080 cells. HT1080 cells were transfected with pcDNA3.1-3×Flag containing *xCT* gene and cultured for 24 h. After incubated with RSL3 (2 μM) for 4 h, total cell lysates were analyzed for Flag-xCT expression with Western blot using antibody against Flag. One representative image of three independent biological replicas was shown. (D and E) Evaluation of lipid ROS and cell death for xCT overexpression in HT1080 cells. Cells acquired from Fig. S4 (C) were incubated for 4 h for lipid ROS assessment (D) as described in Fig. S1 (A), or for 8 h for evaluation of cell death (E) as described in Fig. S1 (B). Ferrastain-1 (Fer-1, 10 μM) were included as negative control. (F and G) Analysis of lipid ROS and cell death upon GSH synthesis inhibition and GPX4 inactivation in HT1080 cells. Following 20 h of treatment with BSO (0.2 mM), cells were incubated in the absence or presence of RSL3 (2 μM) as indicated for 4 h for lipid ROS assessment (F) as described in Fig. S1 (A), or for 8 h for evaluation of cell death (G) as described in Fig. S1 (B). (H) Detection of intracellular H_2S. HT1080 cells were cultured with NaHS (100 μM) in the absence of Cys for 10 h. After treated with RSL3 (2 μM) for 4 h, cells were collected and subjected to assessment of H_2S. (I and J) Analysis of lipid ROS and cell death in the context of elevated intracellular H_2S. Cells acquired from Fig. S4 (H) were cultured for 4 h for lipid ROS detection (I) as described in Fig. S1 (A), or for 8 h for analysis of cell death (J) as described in Fig. S1 (B). Data represent as mean ± SD of three independent experiments, with significance determined by one-way ANOVA test or t text. $^{**}P<0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, nonsignificant.

Fig. S1

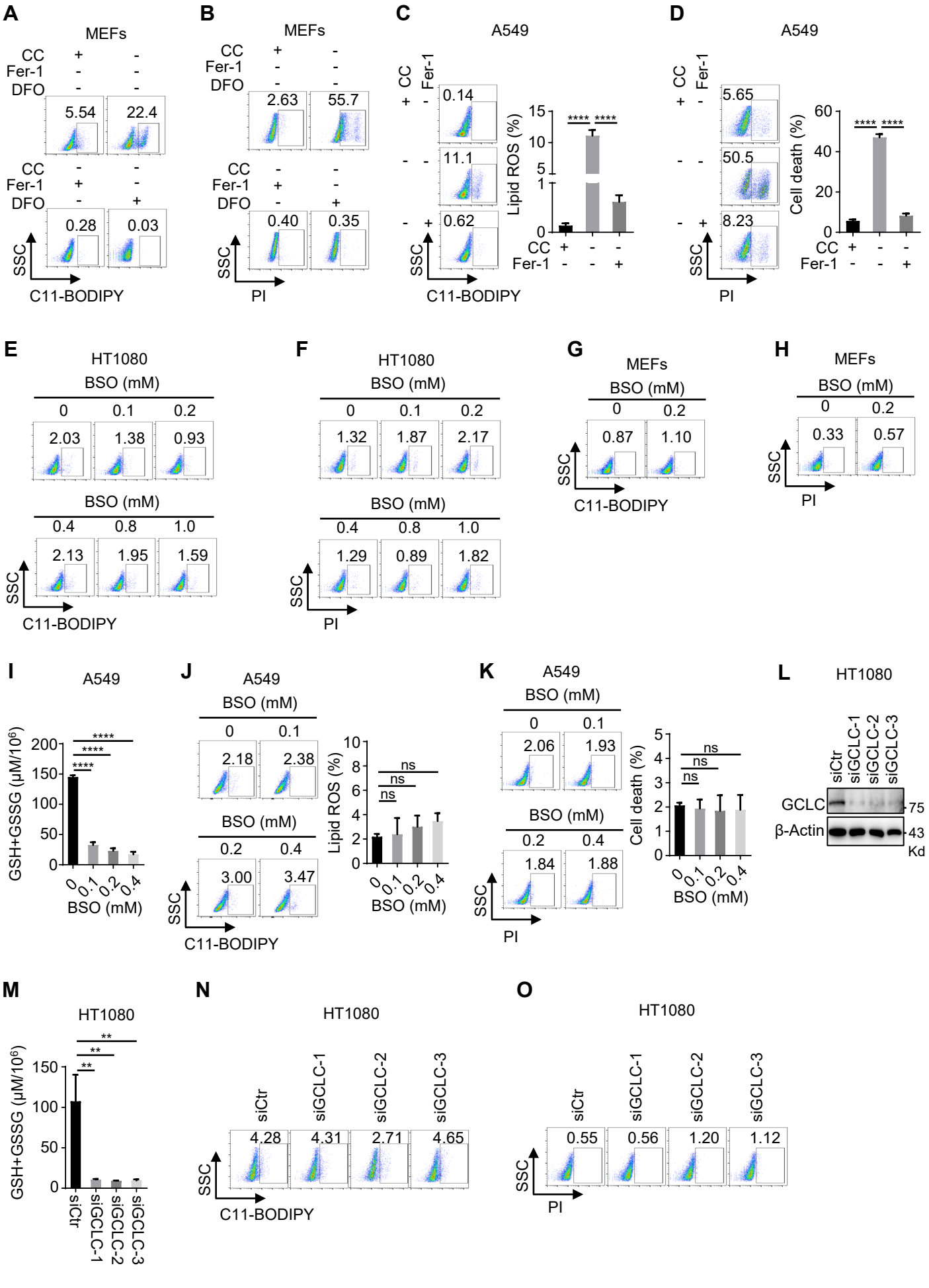

A
MEFs
CC + -
Fer-1 - -
DFO - -
5.54
22.4
CC - -
Fer-1 + -
DFO - +
0.28
0.03
SSC
C11-BODIPY
B
MEFs
CC + -
Fer-1 - -
DFO - -
2.63
55.7
CC - -
Fer-1 + -
DFO - +
0.40
0.35
SSC
PI
C
A549
+ CC - Fer-1
0.14
- - 11.1
- + 0.62
SSC
C11-BODIPY
Lipid ROS (%)
15
10
5
1
0
**** ****
CC + - -
Fer-1 - - +
D
A549
+ CC - Fer-1
5.65
- - 50.5
- + 8.23
SSC
PI
Cell death (%)
60
40
20
0
**** ****
CC + - -
Fer-1 - - +
E
HT1080
BSO (mM)
0 0.1 0.2
2.03
1.38
0.93
BSO (mM)
0.4 0.8 1.0
2.13
1.95
1.59
SSC
C11-BODIPY
F
HT1080
BSO (mM)
0 0.1 0.2
1.32
1.87
2.17
BSO (mM)
0.4 0.8 1.0
1.29
0.89
1.82
SSC
PI
G
MEFs
BSO (mM)
0 0.2
0.87
1.10
SSC
C11-BODIPY
H
MEFs
BSO (mM)
0 0.2
0.33
0.57
SSC
PI
I
A549
GSH+GSSG (μM/10^6)
200
150
100
50
0
**** **** ****
0 0.1 0.2 0.4
BSO (mM)
J
A549
BSO (mM)
0 0.1
2.18
2.38
BSO (mM)
0.2 0.4
3.00
3.47
SSC
C11-BODIPY
Lipid ROS (%)
10
8
6
4
2
0
ns ns ns
0 0.1 0.2 0.4
BSO (mM)
K
A549
BSO (mM)
0 0.1
2.06
1.93
BSO (mM)
0.2 0.4
1.84
1.88
SSC
PI
Cell death (%)
5
4
3
2
1
0
ns ns ns
0 0.1 0.2 0.4
BSO (mM)
L
HT1080
siCtr
siGCLC-1
siGCLC-2
siGCLC-3
GCLC
β-Actin
75
43
Kd
M
HT1080
GSH+GSSG (μM/10^6)
150
100
50
0
** ** **
siCtr
siGCLC-1
siGCLC-2
siGCLC-3
N
HT1080
siCtr
siGCLC-1
siGCLC-2
siGCLC-3
4.28
4.31
2.71
4.65
SSC
C11-BODIPY
O
HT1080
siCtr
siGCLC-1
siGCLC-2
siGCLC-3
0.55
0.56
1.20
1.12
SSC
PI

Fig. S2

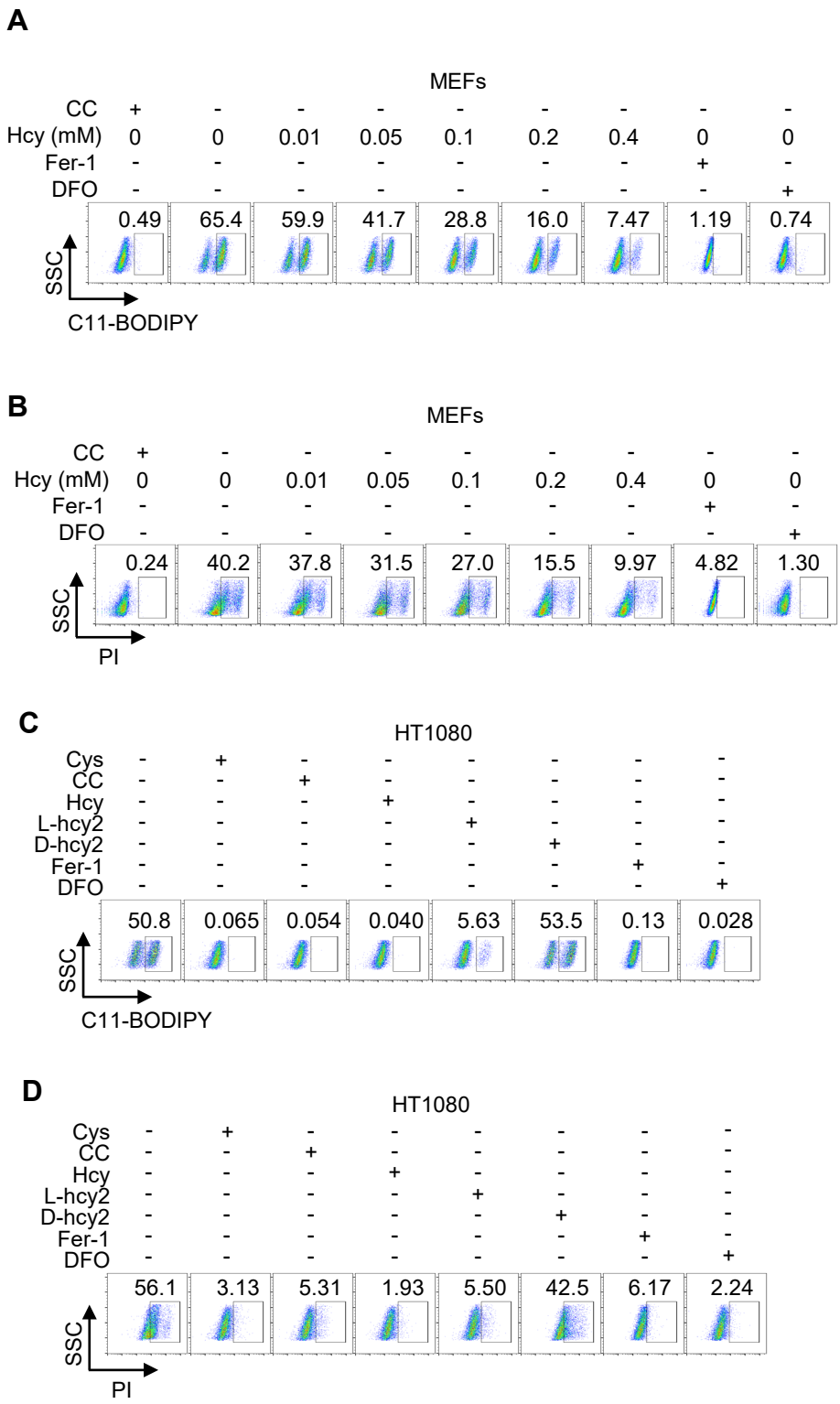

A
MEFs
CC + - - - - - - - -
Hcy (mM) 0 0 0.01 0.05 0.1 0.2 0.4 0 0
Fer-1 - - - - - - - + -
DFO - - - - - - - - +
0.49 65.4 59.9 41.7 28.8 16.0 7.47 1.19 0.74
SSC
C11-BODIPY
B
MEFs
CC + - - - - - - - -
Hcy (mM) 0 0 0.01 0.05 0.1 0.2 0.4 0 0
Fer-1 - - - - - - - + -
DFO - - - - - - - - +
0.24 40.2 37.8 31.5 27.0 15.5 9.97 4.82 1.30
SSC
PI
C
HT1080
Cys - + - - - - - -
CC - - + - - - - -
Hcy - - - + - - - -
L-hcy2 - - - - + - - -
D-hcy2 - - - - - + - -
Fer-1 - - - - - - + -
DFO - - - - - - - +
50.8 0.065 0.054 0.040 5.63 53.5 0.13 0.028
SSC
C11-BODIPY
D
HT1080
Cys - + - - - - - -
CC - - + - - - - -
Hcy - - - + - - - -
L-hcy2 - - - - + - - -
D-hcy2 - - - - - + - -
Fer-1 - - - - - - + -
DFO - - - - - - - +
56.1 3.13 5.31 1.93 5.50 42.5 6.17 2.24
SSC
PI

Fig. S3

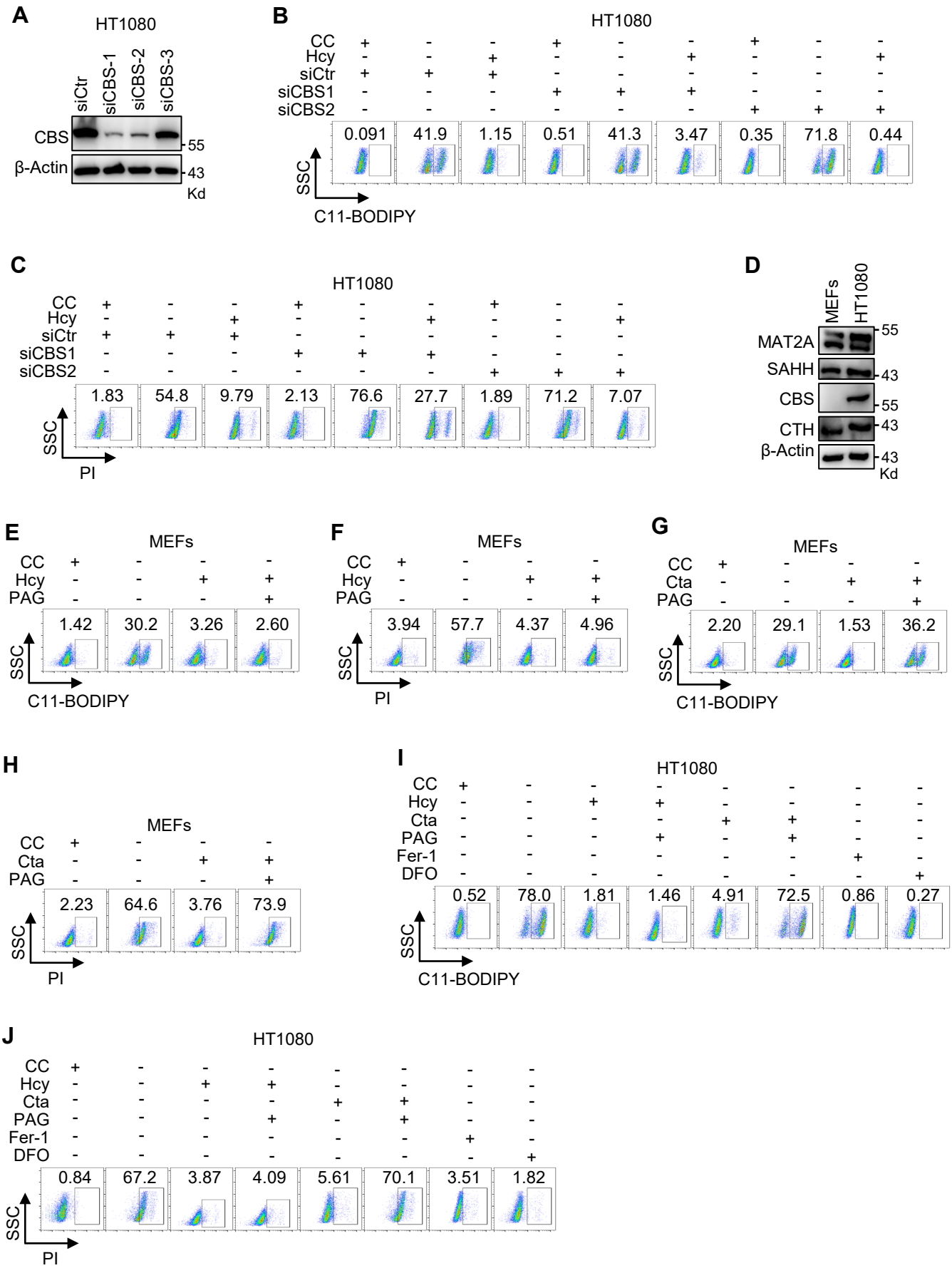
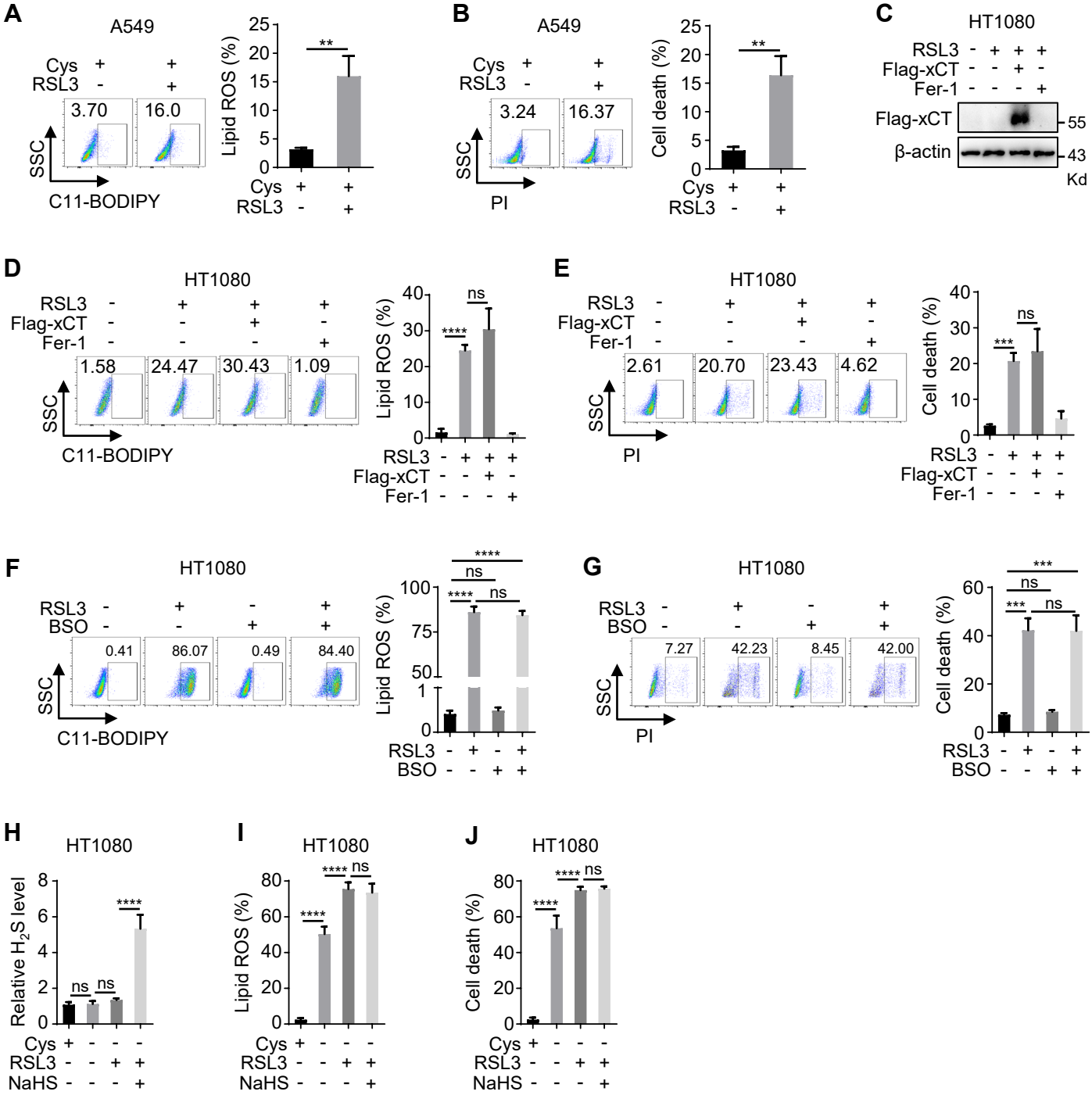

A
HT1080
siCtr
siCBS-1
siCBS-2
siCBS-3
CBS
β-Actin
55
43
Kd
B
HT1080
CC
Hcy
siCtr
siCBS1
siCBS2
0.091
41.9
1.15
0.51
41.3
3.47
0.35
71.8
0.44
SSC
C11-BODIPY
C
HT1080
CC
Hcy
siCtr
siCBS1
siCBS2
1.83
54.8
9.79
2.13
76.6
27.7
1.89
71.2
7.07
SSC
PI
D
MEFs
HT1080
MAT2A
SAHH
CBS
CTH
β-Actin
55
43
55
43
43
Kd
E
MEFs
CC
Hcy
PAG
1.42
30.2
3.26
2.60
SSC
C11-BODIPY
F
MEFs
CC
Hcy
PAG
3.94
57.7
4.37
4.96
SSC
PI
G
MEFs
CC
Cta
PAG
2.20
29.1
1.53
36.2
SSC
C11-BODIPY
H
MEFs
CC
Cta
PAG
2.23
64.6
3.76
73.9
SSC
PI
I
HT1080
CC
Hcy
Cta
PAG
Fer-1
DFO
0.52
78.0
1.81
1.46
4.91
72.5
0.86
0.27
SSC
C11-BODIPY
J
HT1080
CC
Hcy
Cta
PAG
Fer-1
DFO
0.84
67.2
3.87
4.09
5.61
70.1
3.51
1.82
SSC
PI

Fig. S4


A
A549
Cys + +
RSL3 - +
3.70
16.0
SSC
C11-BODIPY
Lipid ROS (%)
**
B
A549
3.24
16.37
PI
Cell death (%)
C
HT1080
RSL3 - + + +
Flag-xCT - - + -
Fer-1 - - - +
Flag-xCT
β-actin
55
43
Kd
D
HT1080
1.58
24.47
30.43
1.09
****
ns
E
HT1080
2.61
20.70
23.43
4.62
***
F
HT1080
BSO - - + +
0.41
86.07
0.49
84.40
G
HT1080
7.27
42.23
8.45
42.00
H
HT1080
Relative H2S level
NaHS - - - +
I
HT1080
J
HT1080