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Supplemental Material to:

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Glutathione participates in the modulation of starvation-induced autophagy in carcinoma cells

Autophagy 2012; 8(12)
http://dx.doi.org/10.4161/auto.22037

www.landesbioscience.com/journals/autophagy/article/22037

Figure S1

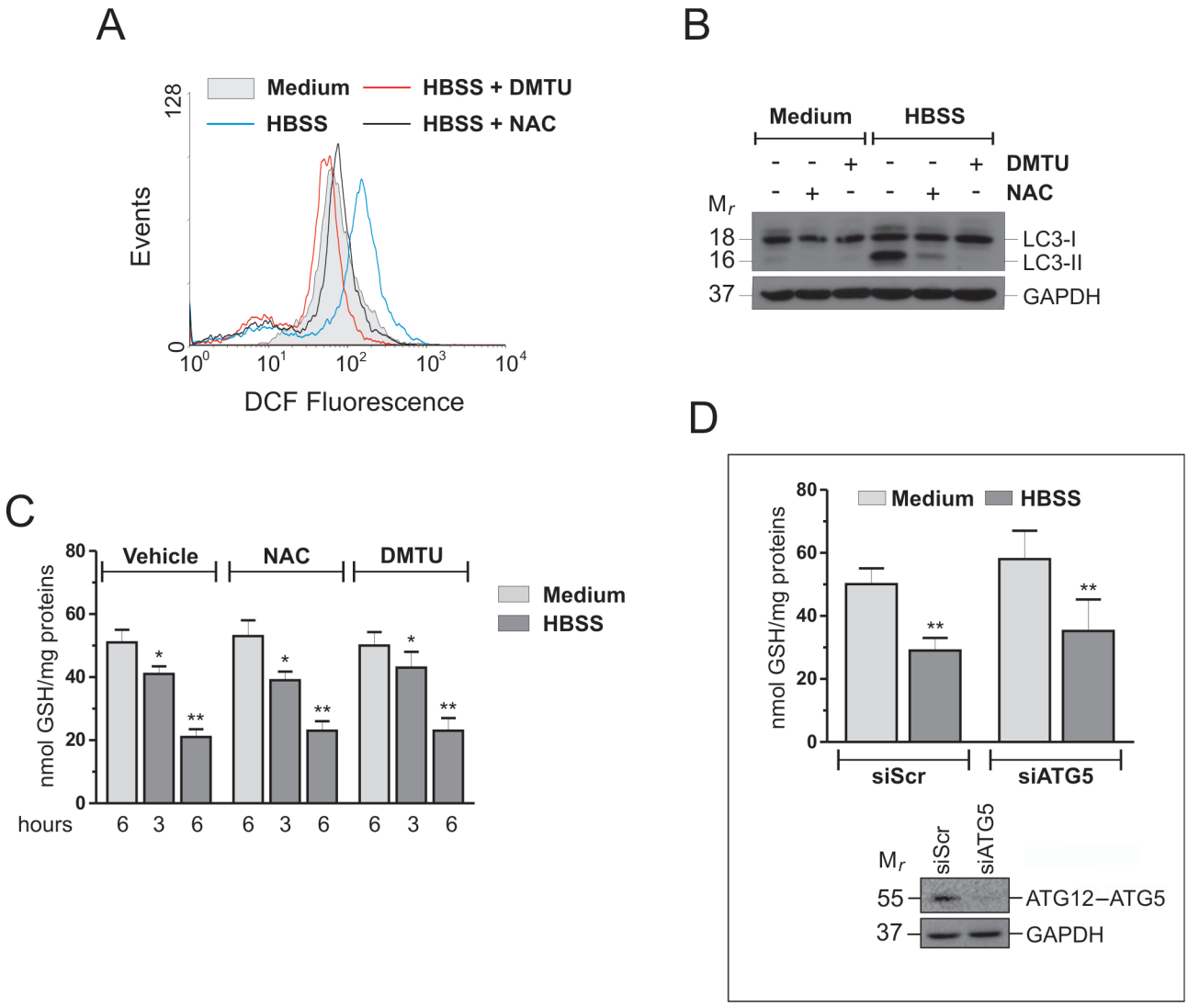
A
Medium
HBSS
HBSS + DMTU
HBSS + NAC
Events
128
0
10^0
10^1
10^2
10^3
10^4
DCF Fluorescence
B
Medium
HBSS
DMTU
NAC
M_r
18
16
37
LC3-I
LC3-II
GAPDH
C
Vehicle
NAC
DMTU
nmol GSH/mg proteins
80
60
40
20
0
hours
6 3 6 6 3 6 6 3 6
Medium
HBSS
D
Medium
HBSS
nmol GSH/mg proteins
80
60
40
20
0
siScr
siATG5
siScr
siATG5
M_r
55
37
ATG12–ATG5
GAPDH

Figure S2

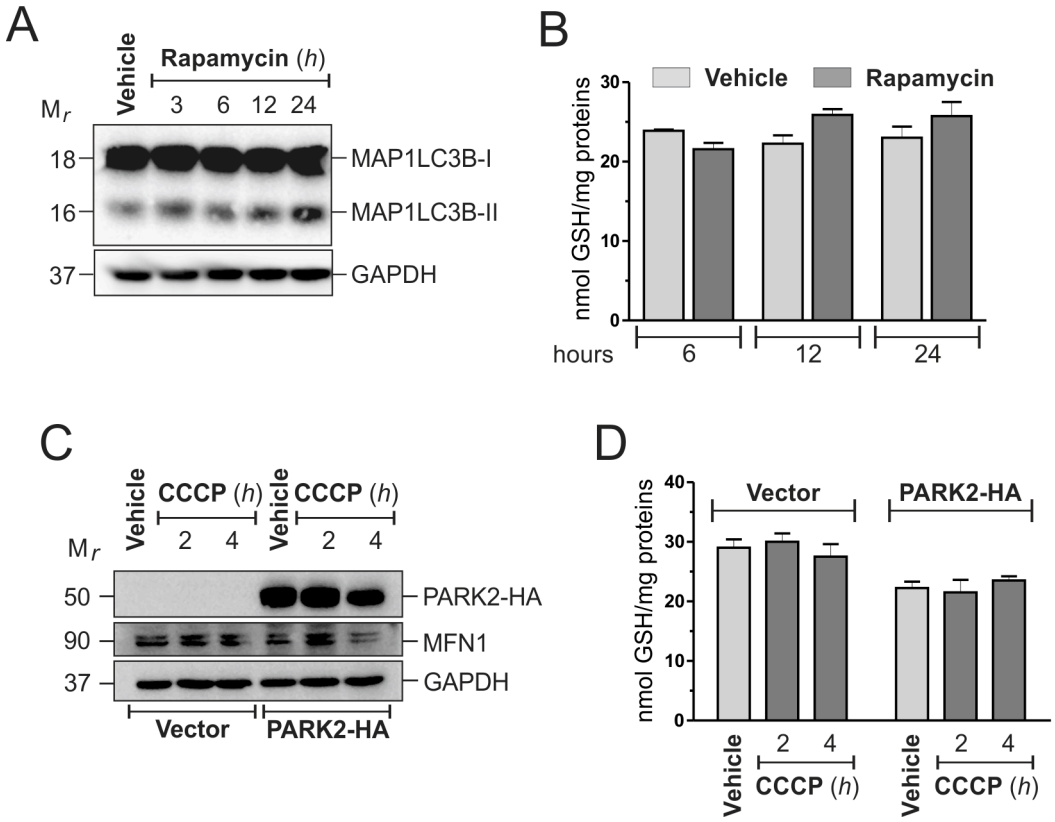
A
Vehicle
Rapamycin (h)
Mr
3 6 12 24
18
16
37
MAP1LC3B-I
MAP1LC3B-II
GAPDH
B
Vehicle
Rapamycin
nmol GSH/mg proteins
30
20
10
0
hours
6
12
24
C
Vehicle
CCCP (h)
Vehicle
CCCP (h)
Mr
2 4
2 4
50
90
37
PARK2-HA
MFN1
GAPDH
Vector
PARK2-HA
D
nmol GSH/mg proteins
40
30
20
10
0
Vector
PARK2-HA
Vehicle
2 4
CCCP (h)
Vehicle
2 4
CCCP (h)

Figure S3

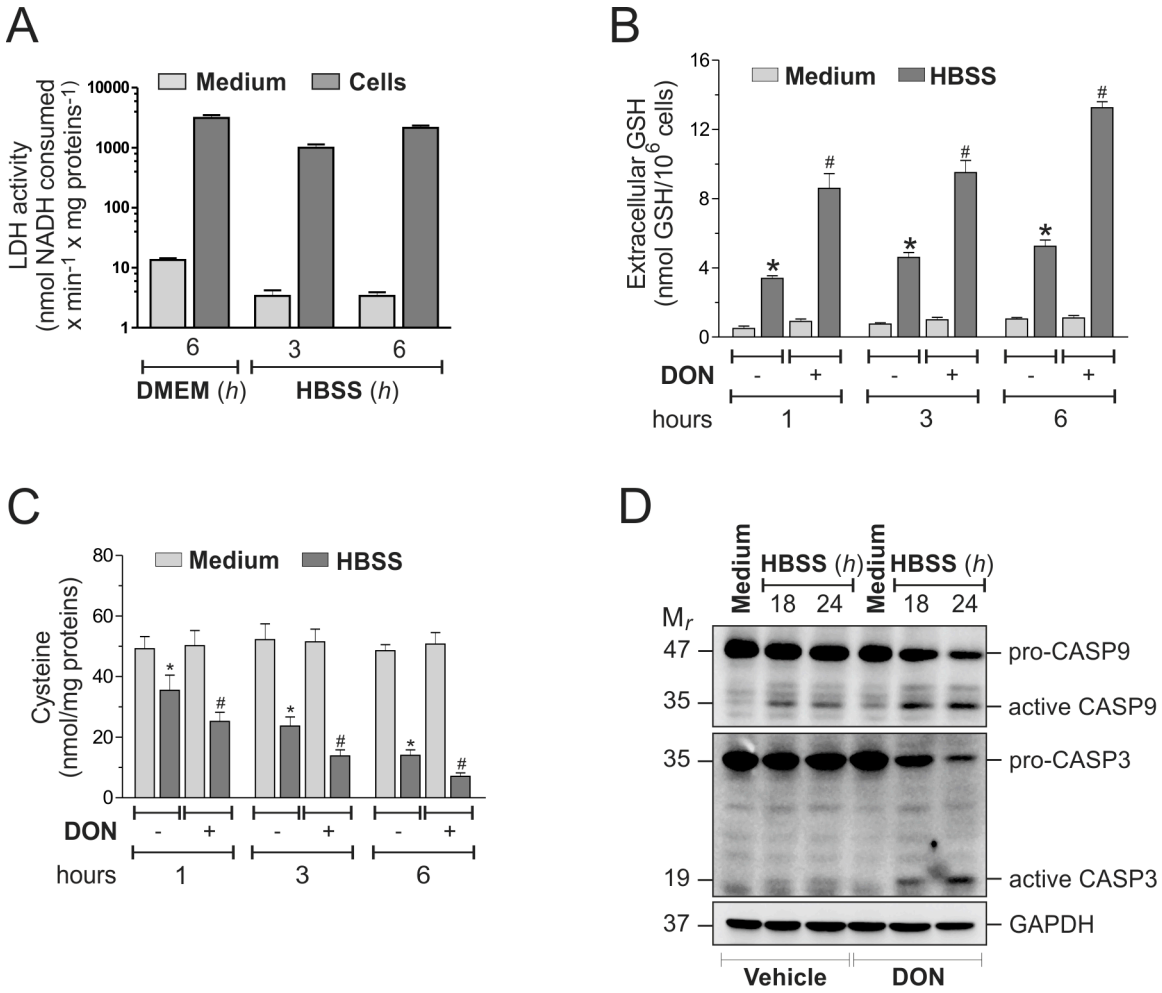
A
Medium
Cells
LDH activity
(nmol NADH consumed
x min-1 x mg proteins-1)
10000
1000
100
10
1
6
3
6
DMEM (h)
HBSS (h)
B
Medium
HBSS
Extracellular GSH
(nmol GSH/10^6 cells)
16
12
8
4
0
DON
-
+
hours
1
3
6
C
Medium
HBSS
Cysteine
(nmol/mg proteins)
80
60
40
20
0
DON
hours
1
3
6
D
Medium
HBSS (h)
18
24
Mr
47
35
pro-CASP9
active CASP9
pro-CASP3
19
active CASP3
37
GAPDH
Vehicle
DON

Figure S4

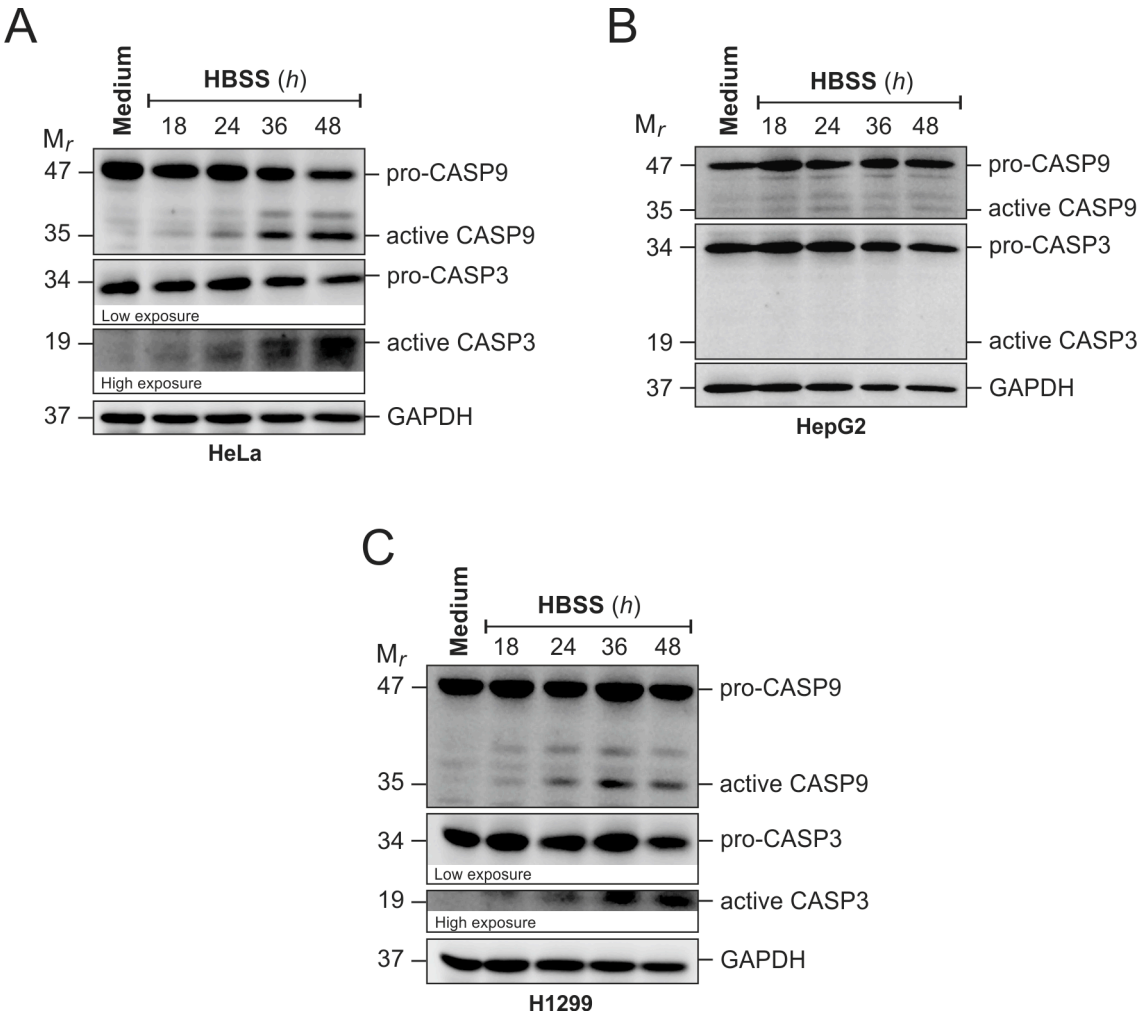
A
Medium
HBSS (h)
18 24 36 48
Mr
47
35
34
19
37
pro-CASP9
active CASP9
pro-CASP3
Low exposure
active CASP3
High exposure
GAPDH
HeLa
B
Medium
HBSS (h)
18 24 36 48
Mr
47
35
34
19
37
pro-CASP9
active CASP9
pro-CASP3
active CASP3
GAPDH
HepG2
C
Medium
HBSS (h)
18 24 36 48
Mr
47
35
34
19
37
pro-CASP9
active CASP9
pro-CASP3
Low exposure
active CASP3
High exposure
GAPDH
H1299

Figure S5

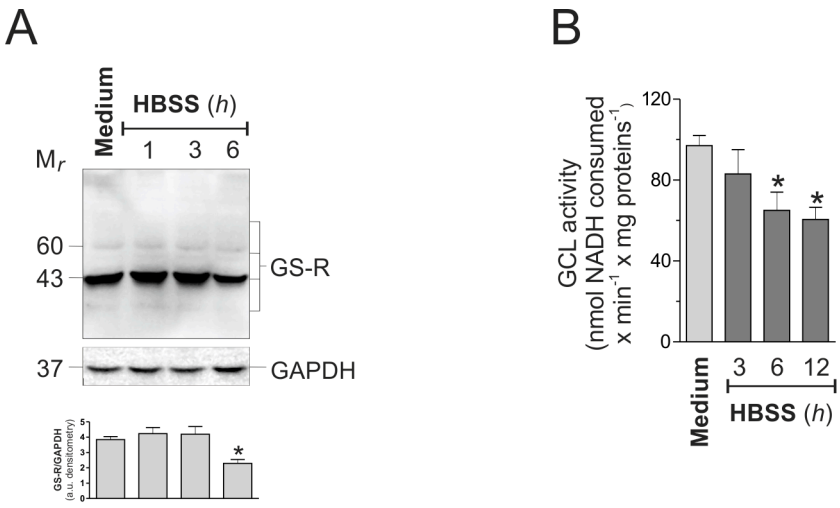
A
Medium
HBSS (h)
1
3
6
Mr
60
43
GS-R
37
GAPDH
GS-R/GAPDH
(a.u. densitometry)
*
B
GCL activity
(nmol NADH consumed
x min-1 x mg proteins-1)
120
80
40
0
*
*
Medium
3
6
12
HBSS (h)

Figure S6

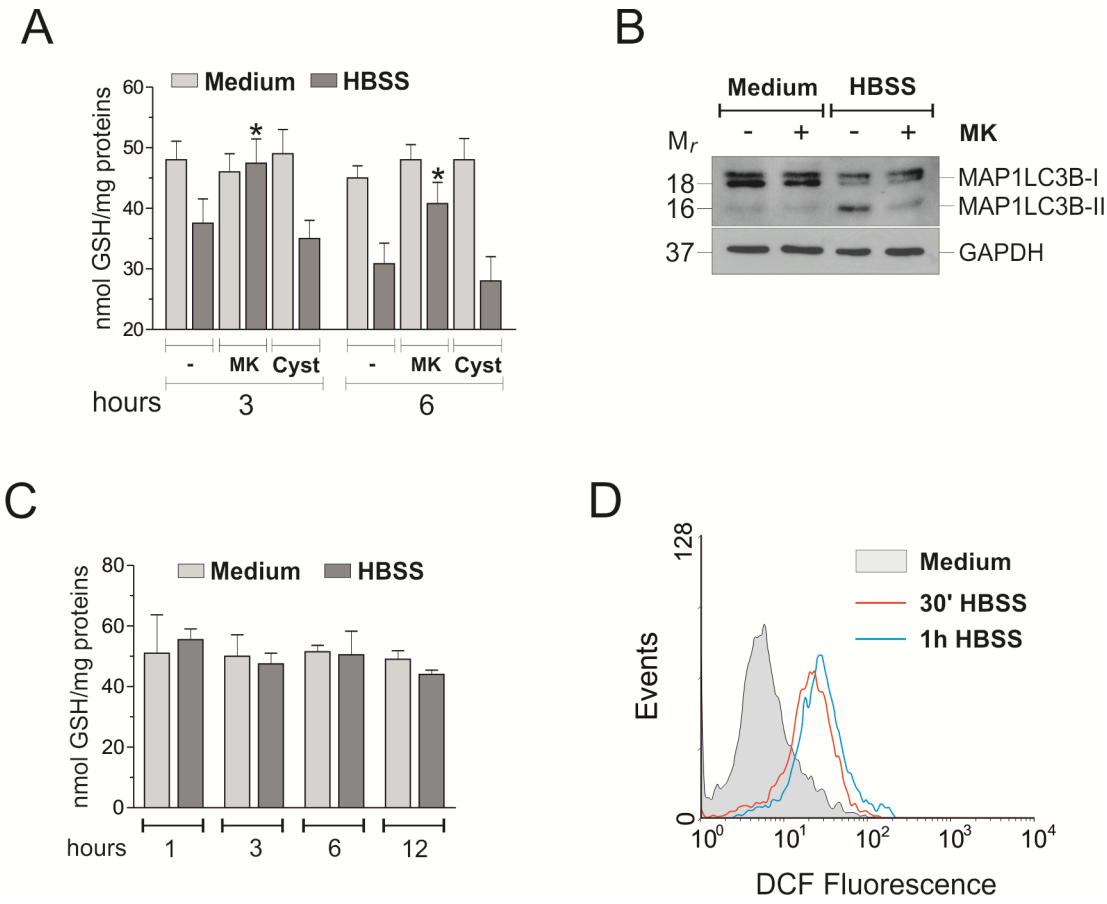
A
Medium
HBSS
nmol GSH/mg proteins
60
50
40
30
20
*
*
-
MK
Cyst
-
MK
Cyst
hours
3
6
B
Medium
HBSS
Mr
-
+
-
+
MK
18
16
37
MAP1LC3B-I
MAP1LC3B-II
GAPDH
C
Medium
HBSS
nmol GSH/mg proteins
80
60
40
20
0
hours
1
3
6
12
D
128
0
Events
Medium
30' HBSS
1h HBSS
10^0
10^1
10^2
10^3
10^4
DCF Fluorescence

Figure S7

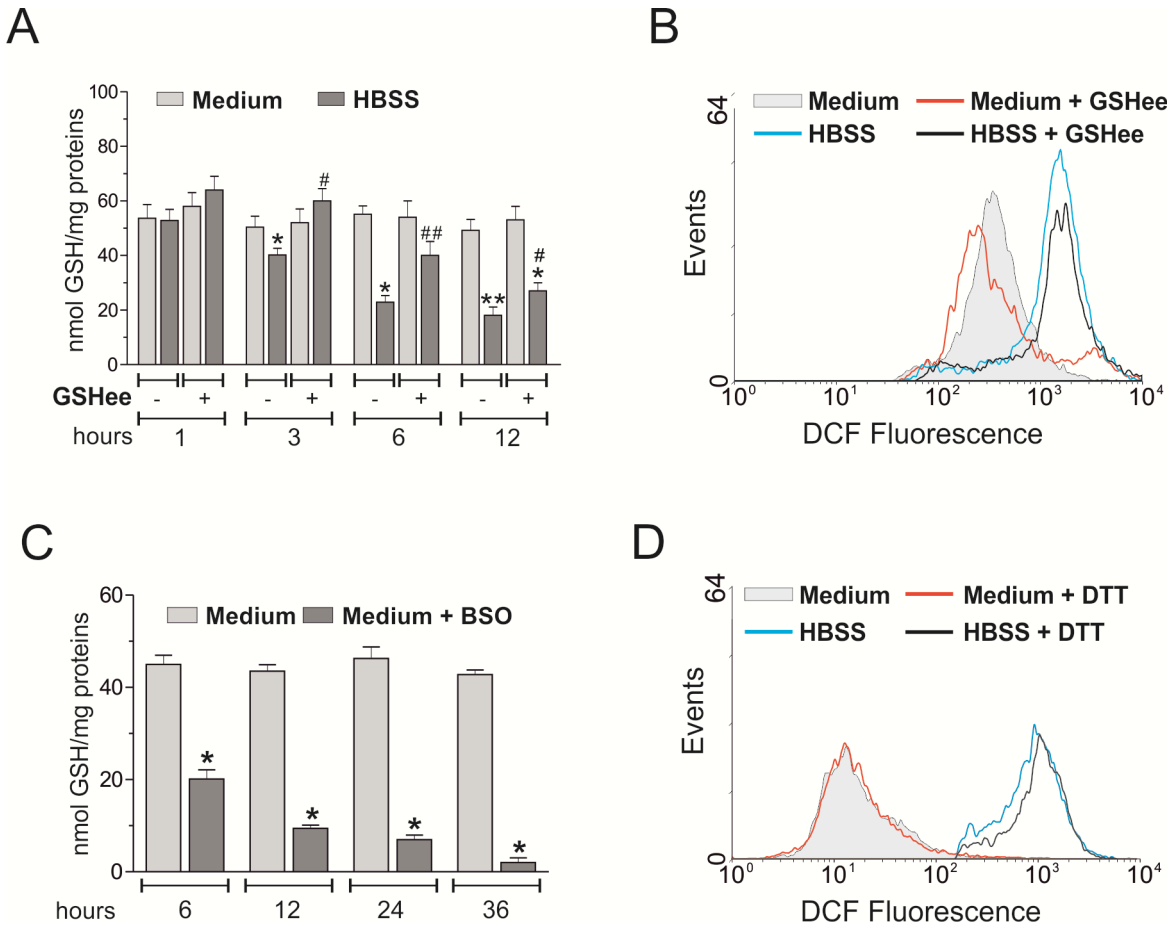
A
Medium
HBSS
nmol GSH/mg proteins
100
80
60
40
20
0
GSHee
-
+
hours
1
3
6
12
B
Medium
Medium + GSHee
HBSS
HBSS + GSHee
Events
64
0
10^0
10^1
10^2
10^3
10^4
DCF Fluorescence
C
Medium
Medium + BSO
nmol GSH/mg proteins
60
40
20
0
hours
6
12
24
36
D
Medium
Medium + DTT
HBSS
HBSS + DTT
Events
64
0
10^0
10^1
10^2
10^3
10^4
DCF Fluorescence

Figure S1.

(A) HeLa cells were starved for 1 h with HBSS, HBSS supplemented with 5 mM NAC or HBSS supplemented with 20 mM DMTU. Thirty minutes before the end of the experimental time cells were incubated with 50 μM H2-DCFDA and cytofluorometrically analyzed for intracellular ROS content. Cytofluorometric histograms are from one experiment out of five that gave similar results. (B) HeLa cells were starved as in (A). After 3 h, 50 μg of total protein extract were loaded onto each lane for detection of MAP1LC3B. GAPDH was used as loading control. (C) HeLa cells were starved as in (A). At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 5 independent experiments. *p <0.05 against medium. **p<0.01 against medium. (D) HeLa cells were transfected with either a nontargeting siRNA (siScr) or an siRNA targeting ATG5 (si*ATG5*), starved for 6 h and intracellular GSH levels determined as in (C). *p <0.05 against medium. #p<0.05 against siScr HBSS (upper panel). Concomitantly, siScr and si*ATG5* cells were harvested and lysed. Twenty-five μg of total protein extract were loaded onto each lane for detection of ATG5. GAPDH was used as loading control (lower panel). Western blots are from one experiment representative of three that gave similar results.

Figure S2.

(A) HeLa cells were treated with 500 nM rapamycin for the indicated times. Fifty μg of total protein extract were loaded onto each lane for detection of MAP1LC3B. GAPDH was used as loading control. Western blots are from one experiment representative of three that gave similar results. (B) HeLa cells were treated with 500 nM rapamycin. At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 3 independent experiments. (C) HeLa cells were transfected with either a PARK2-HA-containing plasmid or an empty vector. Twelve hours after transfection, cells were treated with 20 μM CCCP for the indicated times. Thirty μg of total protein extract were loaded onto each lane for detection of MFN1. GAPDH was used as loading

control and HA was used as transfection control. Western blots are from one experiment representative of three that gave similar results. Alternatively (D), cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 3 independent experiments.

Figure S3.

(A) HeLa cells were starved with HBSS. At indicated times, cells and 500 µl of medium were collected and LDH activity determined spectrophotometrically following the oxidation of NADH at 340 nm. Data are expressed as nmoles of NADH consumed x min-1 x mg proteins-1 and represent the mean ± SD of n = 3 independent experiments. (B) HeLa cells were starved with HBSS or HBSS supplemented with 1 mM DON. At indicated times, 500 µl of cell medium was collected and GSH determined by HPLC. Data are expressed as nmoles of GSH/10^6 cells and represent the mean ± SD of n = 5 independent experiments. *p <0.05 against medium. #p<0.05 against HBSS. (C) HeLa cells treated as in (B). At indicated times, cells were harvested and intracellular cysteine was determined by HPLC analysis. Data are expressed as nmoles of cysteine/mg of proteins and represent the mean ± SD of n = 3 independent experiments. *p <0.05 against control. #p<0.05 against HBSS. (D) HeLa cells were starved with HBSS or HBSS supplemented with 1 mM DON for the indicated times. Twenty-five µg of total protein extract were loaded onto each lane for detection of pro and active forms of CASP3 and CASP9. GAPDH was used as loading control. Western blots are from one experiment representative of three that gave similar results.

Figure S4.

HeLa (A), HepG2 (B) and H1299 (C) cells were starved with HBSS for the indicated times. Twenty-five µg of total protein extract were loaded onto each lane for detection of pro and active forms of CASP3 and CASP9. GAPDH was used as loading control. Western blots are from one experiment representative of three that gave similar results.

Figure S5.

(A) HeLa cells were starved with HBSS. At indicated times, 30 μg of total protein extract were loaded onto each lane for detection of glutathionylated proteins (GS-R). GAPDH was used as loading control (upper panel). Densitometric analysis of GS-R levels normalized to GAPDH. *p <0.05 against medium (bottom panel). (B) HeLa cells were starved with HBSS. At indicated times, cells were collected and GCL activity determined spectrophotometrically following the oxidation of NADH at 340 nm. Data are expressed as nmoles of NADH consumed x min-1 x mg proteins-1 and represent the mean ± SD of n = 3 independent experiments. *p <0.05 against medium.

Figure S6.

(A) HeLa cells were starved with HBSS, HBSS supplemented with 5 μM MK571 (MK) or HBSS supplemented with 1 mM cystathionine (Cyst). At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 5 independent experiments. *p <0.05 against HBSS. (B) HeLa cells were starved with HBSS or HBSS supplemented with 5 mM MK. After 3 h of starvation, 50 μg of total protein extract were loaded onto each lane for detection of MAP1LC3B. GAPDH was used as loading control. Western blots are from one experiment representative of three that gave similar results. (C) MCF-7 cells were starved with HBSS. At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 5 independent experiments. (D) MCF-7 cells were starved with HBSS for the indicated times. Thirty minutes before the end of the experimental time cells were incubated with 50 μM H2-DCFDA and cytofluorometrically analyzed for intracellular ROS content. Cytofluorometric histograms are from one experiment out of five that gave similar results.

Figure S7.

(A) HeLa cells were starved with HBSS or HBSS supplemented with 10 mM GSHee. At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± SD of n = 5 independent experiments. *p <0.05 against medium. **p<0.01 against medium. #p<0.05 against HBSS. ##p<0.01 against HBSS. (B) HeLa cells were starved for 1 h with HBSS or HBSS supplemented with 10 mM GSHee. Thirty minutes before the end of the experimental time, cells were incubated with 50 μM H2-DCFDA, and analyzed for intracellular ROS content. Cytofluorometric histograms are from one experiment out of five that gave similar results. (C) HeLa cells were treated with 1 mM BSO. At indicated times, cells were harvested and intracellular GSH was determined by HPLC analysis. Data are expressed as nmoles of GSH/mg of proteins and represent the mean ± S.D. of three different experiments. *p <0.05 against medium. (D) HeLa cells were starved with HBSS or HBSS supplemented with 1 mM DTT for 1 h and analyzed as in (B). Cytofluorometric histograms are from one experiment out of five that gave similar results.