Figure S1

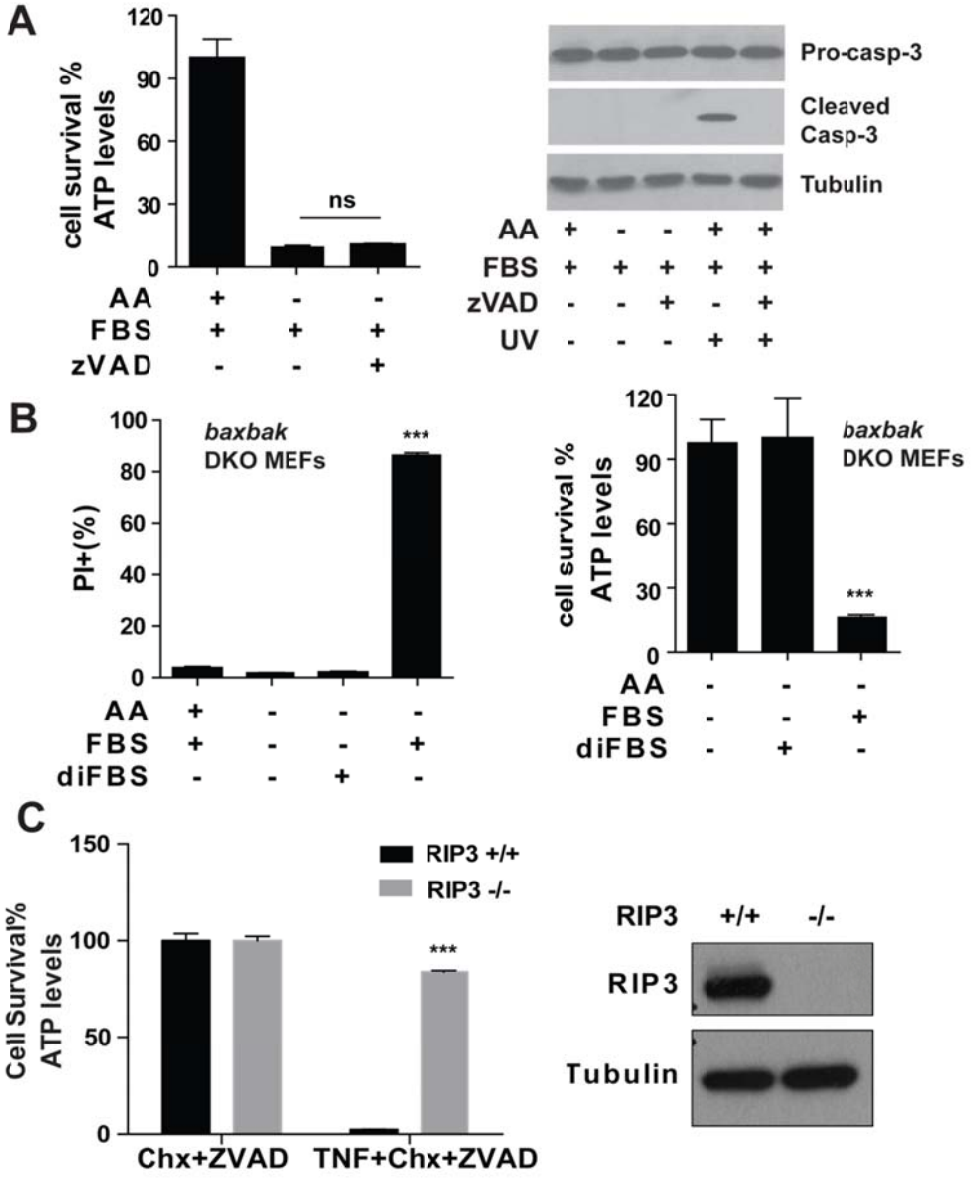

A
cell survival %
ATP levels
120
90
60
30
0
ns
AA + - -
FBS + + +
zVAD - - +
Pro-casp-3
Cleaved
Casp-3
Tubulin
AA + - - + +
FBS + + + + +
zVAD - - + - +
UV - - - + +
B
PI+(%)
100
80
60
40
20
0
baxbak
DKO MEFs
***
AA + - - -
FBS + - - +
diFBS - - + -
cell survival %
ATP levels
120
90
60
30
0
baxbak
DKO MEFs
***
AA - - -
FBS - - +
diFBS - + -
C
Cell Survival%
ATP levels
150
100
50
0
RIP3 +/+
RIP3 -/-
***
Chx+ZVAD
TNF+Chx+ZVAD
RIP3 +/+ -/-
RIP3
Tubulin

Figure S2

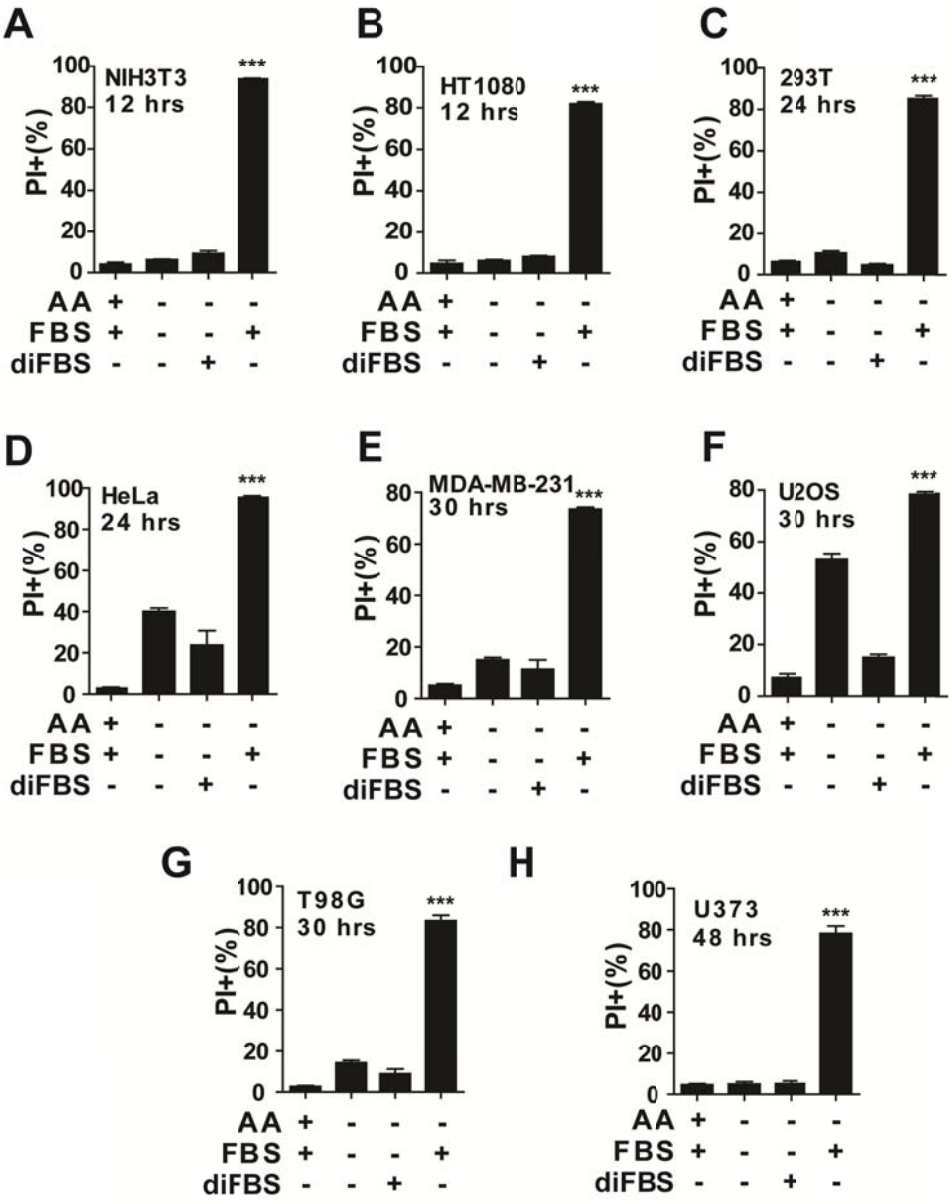

A
NIH3T3
12 hrs
B
HT1080
12 hrs
C
293T
24 hrs
D
HeLa
24 hrs
E
MDA-MB-231
30 hrs
F
U2OS
30 hrs
G
T98G
30 hrs
H
U373
48 hrs
PI+(%)
AA
FBS
diFBS
***

Figure S3

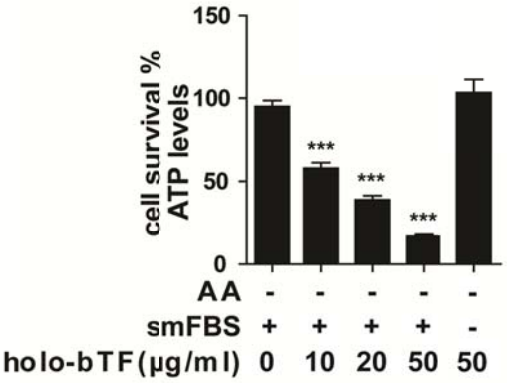

150
100
50
0
cell survival %
ATP levels
***
***
***
AA - - - - -
smFBS + + + + -
holo-bTF(µg/ml) 0 10 20 50 50

Figure S4

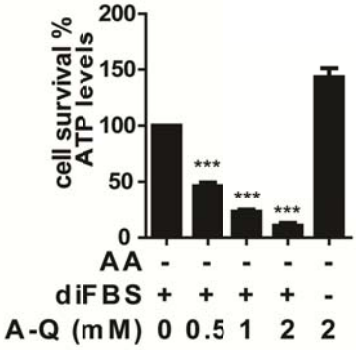

cell survival %
ATP levels
200
150
100
50
0
***
***
***
AA - - - - -
diFBS + + + + -
A-Q (mM) 0 0.5 1 2 2

Figure S5

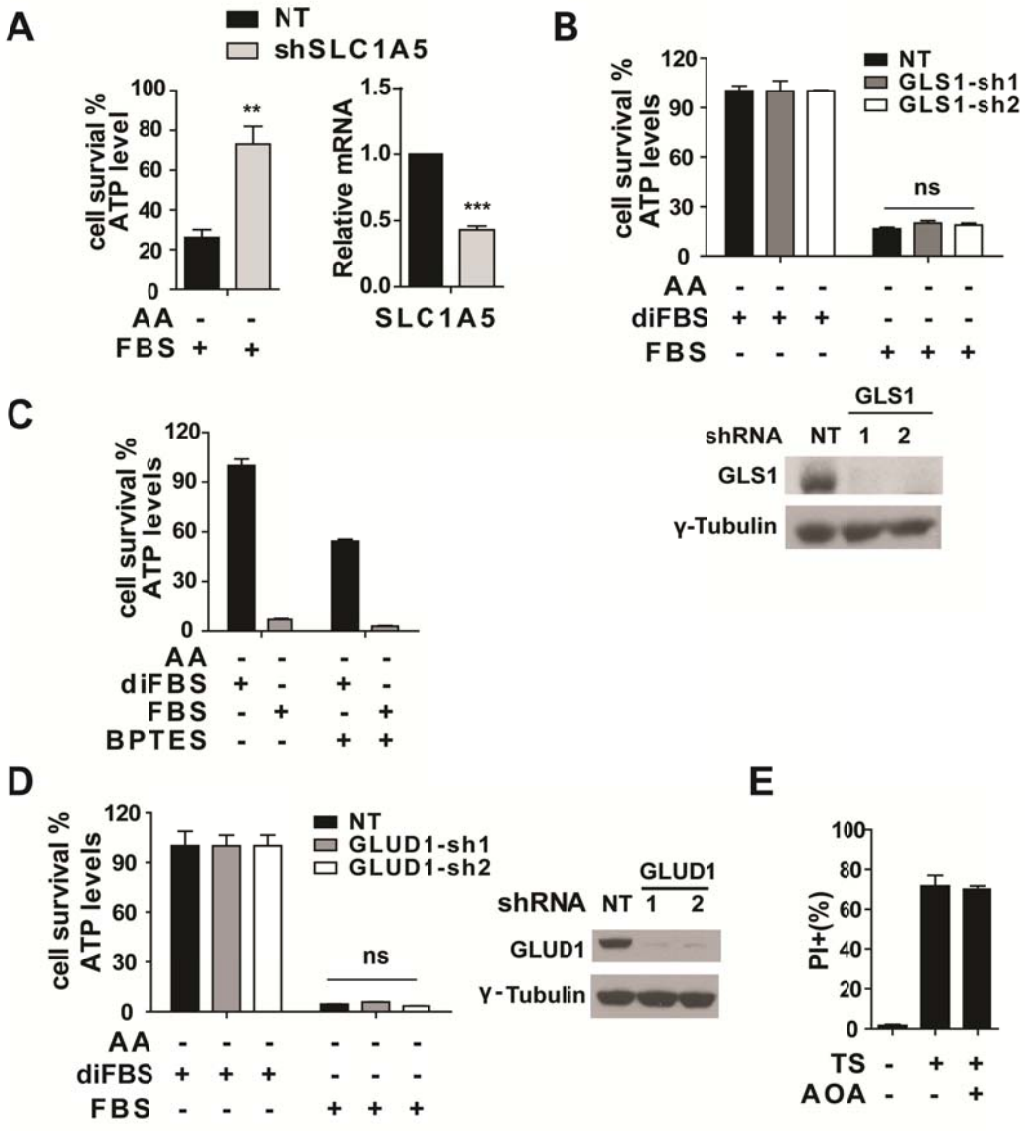

A
NT
shSLC1A5
cell survial % ATP level
100
80
60
40
20
0
**
AA - -
FBS + +
Relative mRNA
1.5
1.0
0.5
0.0
***
SLC1A5
B
NT
GLS1-sh1
GLS1-sh2
cell survival % ATP levels
120
90
60
30
0
ns
AA - - - - - -
diFBS + + + - - -
FBS - - - + + +
GLS1
shRNA NT 1 2
GLS1
γ-Tubulin
C
cell survival % ATP levels
120
90
60
30
0
AA - - - -
diFBS + - + -
FBS - + - +
BPTES - - + +
D
NT
GLUD1-sh1
GLUD1-sh2
cell survival % ATP levels
120
90
60
30
0
ns
AA - - - - - -
diFBS + + + - - -
FBS - - - + + +
GLUD1
shRNA NT 1 2
GLUD1
γ-Tubulin
E
PI+(%)
100
80
60
40
20
0
TS - + +
AOA - - +

Figure S6

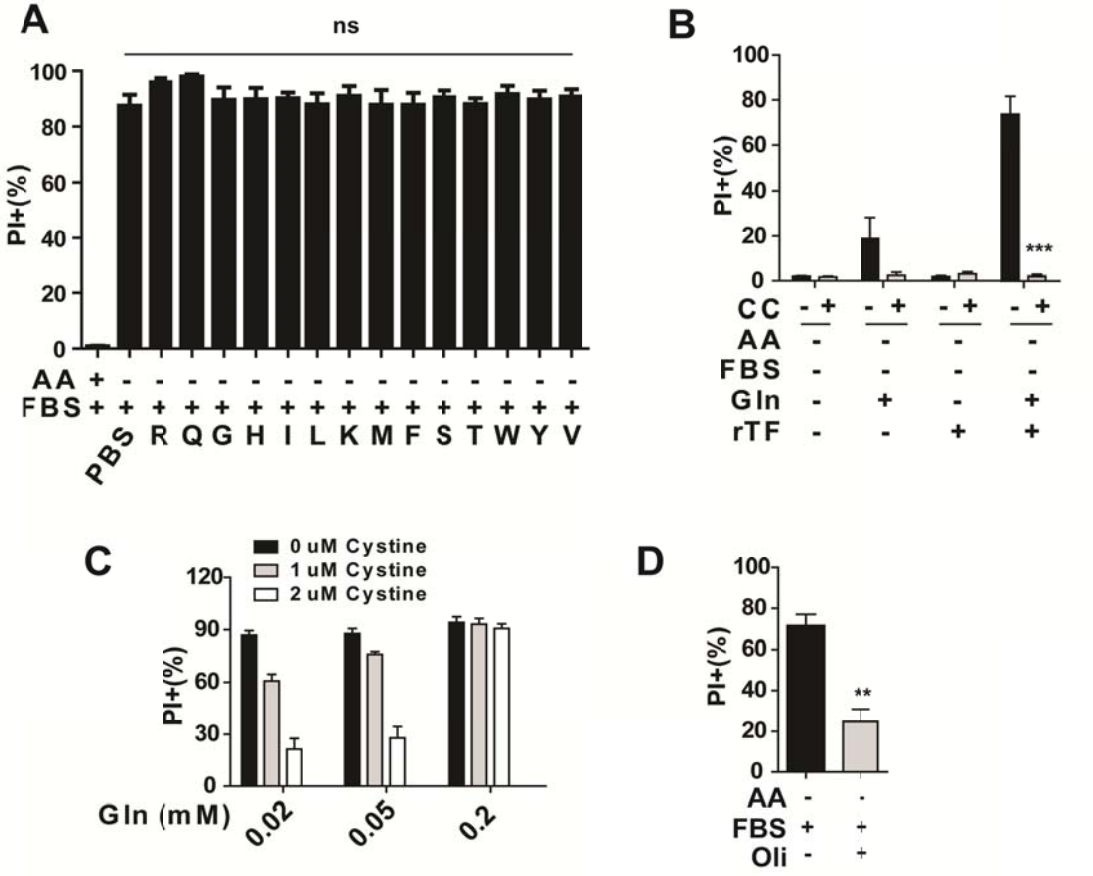
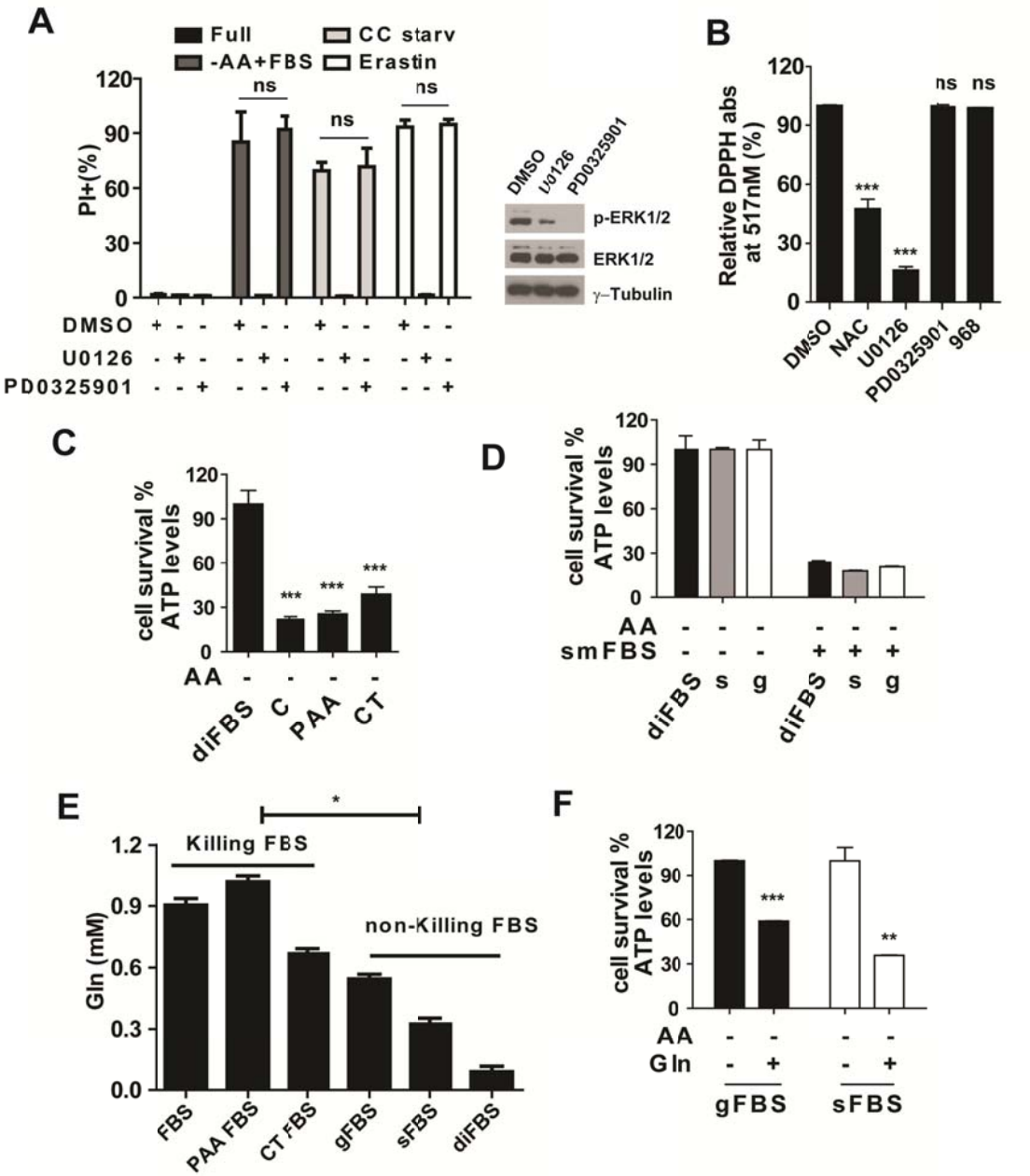

A
ns
PI+(%)
100
80
60
40
20
0
AA + - - - - - - - - - - - - - - -
FBS + + + + + + + + + + + + + + + +
PBS R Q G H I L K M F S T W Y V
B
PI+(%)
100
80
60
40
20
0
***
CC - + - + - + - +
AA - - - -
FBS - - - -
Gln - + - +
rTF - - + +
C
0 uM Cystine
1 uM Cystine
2 uM Cystine
PI+(%)
120
90
60
30
0
Gln (mM)
0.02
0.05
0.2
D
PI+(%)
100
80
60
40
20
0
**
AA - -
FBS + +
Oli - +

Figure S7


A
Full
CC starv
-AA+FBS
Erastin
PI+(%)
ns
DMSO
U0126
PD0325901
p-ERK1/2
ERK1/2
γ–Tubulin
B
Relative DPPH abs at 517nM (%)
DMSO
NAC
U0126
PD0325901
968
C
cell survival % ATP levels
AA
diFBS
C
PAA
CT
D
smFBS
s
g
E
Killing FBS
non-Killing FBS
Gln (mM)
FBS
PAA FBS
CT FBS
gFBS
sFBS
diFBS
F
Gln
gFBS
sFBS

Supplemental Figure Legends

Figure S1 related to Figure 1. Serum induces potent non-apoptotic, RIP3-indepednent cell death upon amino acid starvation.

(A) Caspase inhibitor zVAD cannot block serum-induced cell death. MEFs were treated with or without zVAD-FMK as indicated for 12 hrs. Cell viability was determined by measuring cellular ATP levels. Western blot showing that zVAD can block caspase-3 activation induced by UV light.

(**B**) Bax and Bak are not required for serum-induced cell death. *bax/bak*-DKO MEFs were treated as indicated for 12 hrs. Left: Cell death was quantitated by PI staining followed by flow cytometry. Right: Cell viability was determined by measuring cellular ATP levels. diFBS: dialyzed FBS, which did not induce cell death as full serum (FBS) did.

(**C**) RIP3 is required for TNFα-induced necrosis. RIP3+/+ or RIP3-/- MEFs were treated as indicated and cell viability was determined by measuring cellular ATP levels. Cycloheximide (Chx): 1μg/ml; zVAD: 20 μM, TNFα: 100 ng/ml. Western blot confirmed the knockout of RIP3. Data are presented as mean ± SEM, n=3 (***P < 0.001 by unpaired Student t-test).

Figure S2 related to Figure 2. Multiple types of cancerous and noncancerous cells can undergo serum-dependent necrosis.

The results of 8 different cell lines are presented. Cell death was measured by propidium iodide (PI) stained coupled with flow cytometry. diFBS: dialyzed FBS, which did not induce cell death as full serum (FBS) did.

Data are presented as mean ± SEM, n=3 (***P < 0.001 by unpaired Student t-test).

Figure S3 related to Figure 3. Iron-bound bovine holo-transferrin can induce cell death under amino acid starvation conditions in a smFBS-dependent manner.

bak/bax-DKO MEFs were treated for 12 hrs as indicated. Cell viability was determined by measuring cellular ATP levels. holo-bTF: bovine holo-transferrin.

Data are presented as mean ± SEM, n=3 (***P < 0.001 by unpaired Student t-test).

Figure S4 related to Figure 4. L-alanine-L-glutamine (A-Q) mimics the killing activity of L-glutamine.

MEFs were treated as indicated for 12 hrs and cell viability was determined by measuring cellular ATP levels.

Data are presented as mean ± SEM, n=3 (***P < 0.001 by unpaired Student t-test).

Figure S5 related to Figure 5. Glutaminolysis mediates serum-dependent necrosis.

(**A**) RNAi knockdown of SLC1A5 inhibited serum-dependent necrosis. Left: MEFs expressing non-targeting (NT) shRNA or shRNA targeting SLC1A5 were treated as indicated for 12 hrs and cell viability was subsequently measured. Right: qPCR measurement of SLC1A5 mRNA levels in MEFs infected with NT shRNA or shRNA targeting SLC1A5.

(**B**) GLS1 knockdown cannot block serum-dependent necrosis in MEFs. MEFs infected with NT shRNA or two independent shRNAs against GLS1 were treated as indicated for 12 hrs and cell viability was subsequently measured. Western blot (lower panel) confirmed the knockdown of GLS1 expression.

(**C**) GLS1 inhibitor BPTES failed to block serum-dependent necrosis in MEFs. MEFs were treated as indicated for 12 hrs, in the presence or absence of BPTES (Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide; 10 μM).

(**D**) GLUD1 is dispensable for serum-dependent necrosis in MEFs. MEFs infected with Non-Targeting (NT) shRNA or two independent shRNAs against GLUD1 were treated as indicated for 12 hrs, and cell viability was subsequently measured.

(**E**) AOA cannot inhibit TNF-α induced apoptosis. TS: TNF-α (50 ng/ml) + SMAC mimetic (0.5 μM); AOA: 0.5 mM.

Data are presented as mean ± SEM, n=3 (**P < 0.01, ***P < 0.001 by unpaired Student t-test).

Figure S6 related to Figure 6. Cystine but no other amino acid can inhibit serum-induced necrosis.

(**A**) MEFs were cultured for 12 hrs as indicated with addition of different amino acids. Cell death was determined by PI staining coupled with flow cytometry**.**

(**B**) Cystine can inhibit transferrin/glutamine-induced necrosis in *baxbak* DKO MEFs. DKO MEFs were treated for 12 hrs as indicated. Cell death was determined by PI staining coupled with flow cytometry**.**

(**C**) Partial cystine starvation is sufficient to induce necrosis in a glutamine dose-independent manner. Cell death was determined by PI staining coupled with flow cytometry**.**

(D) Inhibition of mitochondrial oxidative phosphorylation activity dramatically inhibited serum induced cell death. Cell death was determined by PI staining coupled with flow cytometry**.** Oli: Oligomycin (5 μM).

Data are presented as mean ± SEM, n=3 (**P < 0.01, ***P < 0.001 by unpaired Student t-test).

Figure S7 related to Figure 7. MEK inhibition is not sufficient to block ferroptosis, and the ferroptotic cell death-inducing activity of FBS from different sources correlates with seral L-glutamine concentrations

(A) MEFs were treated as indicated for 12 hrs and cell viability was determined by measuring cellular ATP levels. MEFs were treated for 12 hrs as indicated. Cell death was determined by PI staining coupled with flow cytometry**.** PD0325901, 10 μM; U0126, 10 μM. Western blotting confirmed the inhibition of MEK1/2 kinase activity by PD0325901 and U0126.

(**B**) The antioxidant activity of different compounds. The in vitro antioxidant activity was analyzed by a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay measuring reduction of DPPH.

(**C**) Three independent sources of FBS (C, PAA, and CT) from different companies are competent to induce cell death of MEFs under amino acid-starvation conditions. MEFs were treated for 12 hrs as indicated. Cell viability was determined by measuring ATP level**.**

(**D**) Two sources of FBS (s and g) are incompetent to induce cell death of MEFs under amino acid-starvation conditions. As diFBS, the killing activity of incompetent FBS can be restored by adding smFBS generated from competent FBS. MEFs were treated for 12 hrs as indicated. Cell viability was determined by measuring ATP level**.**

(**E)** The concentration of L-Glutamine measured in both killing and non-killing FBS.

(**F**) The killing activity of incompetent FBS can be restored by supplementation of L-Gln (4 mM). MEFs were treated for 12 hrs as indicated. Cell viability was determined by measuring ATP level**.**

Data are presented as mean ± SEM, n=3 (*P < 0.05, **P < 0.01, ***P < 0.001 by unpaired Student t-test).

Movie S1 related to Figure 1. Cell Death induced by amino acid/serum-double starvation in MEFs.

Movie S2 related to Figure 1. Serum-dependent necrosis in MEFs upon amino acid starvation (confocal movie, corresponding to the still images in Fig. 1e).

Movie S3 related to Figure 1. Serum-dependent necrosis in MEFs upon amino acid starvation.

Movie S4 (related to Figure 2). MEFs grown in amino acid-free medium in the presence of dialyzed serum (diFBS).

Supplemental Experimental Procedures

Cell culture

Unless specified otherwise, all mammalian cells are maintained in DMEM medium with high-glucose, sodium pyruvate (1 mM), glutamine (2 mM), penicillin (U/ml), streptomycin (0.1mg/ml) and 10% (v/v) FBS at 37°C and 5% CO_2.

Induction and measurement of cell death

To induce cell death, 80%-confluent cells were washed with PBS twice, and then cultured in amino acid-free medium, with specific factors added as indicated in individual experiments. Cell

death was analyzed by propidium iodide (PI) staining coupled with microscopy or flow cytometry. Alternatively, cell viability was determined using the CellTiter-Glo luminescent Cell Viability Assay (Promega). In assays using WT MEFs, viability was calculated by normalizing ATP levels to cells treated with amino acid-starvation in the presence of 10% (v/v) diFBS, while in assays using *bax/bak*-DKO MEFs, ATP levels were normalized to cells treated with amino acid and FBS double starvation.

Antibodies

Primary antibodies used were anti-bovine transferrin (BETHYL, Cat #A10-122A), anti-TfR (Life Science, Cat #136800), anti-Caspase3 (Cell Signaling, Cat #9665), anti-γ-Tubulin (Sigma, Cat #T6557), anti-GLS1 (Proteintech, Cat #12855-1), anti-GLS2 (Prosci, Cat #6217), anti-RIP3 (Prosci, Cat #2283), anti-GLUD1 (Cell signaling, Cat #9828), sheep IgG (BETHYL, Cat #P130-100), anti-pERK1/2 (Cell Signaling, Cat #4370S), and anti-ERK1/2 (Cell Signaling, Cat #9107).

Reagents

Compound 968 was purchased from Millipore (Cat #352010). MEK1/2 inhibitor (PD0325901) was purchased from Millipore (Cat #444966). U0126 was purchased from Cell Signaling Technology （Cat#9903）. zVAD was purchased from ENZO (Cat #ALX-260-020). Erastin was purchased from Millipore (Cat#329600). Ferrostatin-1 (Fer-1) was purchased from XcessBio (Cat# M60042). SMAC mimetic (Birinapant) was purchased from Selleck Chemicals (Cat # S7015). The source of commercial transferrins is as follows: bovine holo-transferrin (Sigma Cat # T1283), bovine apo-transferrin (Sigma Cat # T1428), and recombinant human holo-transferrin (Sigma Cat # T3705). All other chemicals were purchased from Sigma-Aldrich. Different Fetal Bovine Serum preparations used in this study are as follows: FBS (GEMINI, Cat #100-125; Lot #A51C05A), PAA FBS (PAA, Cat #A15-201; Lot #A20111-7008), CT FBS

(Clontech, Cat #631106; Lot #A301097018), sFBS (sigma, Cat #F2442; Lot #12H045) and gFBS (Gibco, Cat #10437-028; Lot #1036512).

Purification and identification of transferrin from FBS

All purification steps were carried out at 4^{o}C, and chromatography was performed with an Amersham FPLC system. For the purification, 20 ml FBS (664 mg protein) was applied to 50-70 % (saturation) ammonium sulfate precipitation. The protein pellet (242 mg) that contained the activity was dissolved in 4 ml Buffer A (20 mM Hepes, PH 7.5 10 mM NaCl) and dialyzed overnight. The activity was applied to HiTrap SP Sepharose (GE Healthcare). The flow-through containing the activity was subjected to HiTrap Q Sepharose (GE Healthcare). After washing the column with Buffer A, the fractions was eluted by a gradient of 10-300 mM NaCl in Buffer A. Activity-containing fractions were further fractionated with HiTrap Heparin Sepharose (GE Healthcare) by using a gradient 10-300 mM NaCl in Buffer A. Fractions of 1 ml was collected. After dialysis, filtered with 0.2 mM filter, the fractions was assayed for activity. SDS-PAGE and Coomassie staining (Bio-Rad) was preformed, and a single band correlated with the killing activity was subject to protein identity determination by mass spectrometry analysis (MALDI-TOF-MS/MS). The activity was identified as bovine transferrin.

Immuno-depletion of transferrin

To deplete transferrin from serum, amino acid- free DMEM medium containing 10% FBS was incubated with control IgG or anti-bovine transferrin antibody bound to Protein G Agarose (GE Healthcare) overnight at 4^{o}C. Protein G Agarose was removed by centrifugation and the supernatant was assayed for killing activity.

Purification and identification of L-glutamine from FBS

FBS was filtered through Centrifugal Filter Units (MWCO 10 KDa) (Millipore) to obtain small molecule fraction (smFBS). One ml of smFBS was dried under vacuum and dissolved in 1 ml methanol and insoluble material was removed by centrifugation. The supernatant was dried and dissolved in 750-µl methanol first and then mixed with 614-µl acetonitrile (final ratio of methanol: acetonitrile is 55:45). After incubating the mix for 30 min at 4^{o}C, precipitated material was removed by centrifugation, and the supernatant was dried and dissolved in 750-µl methanol. Aliquot of 150-µl was mixed with 1350-µl acetonitrile (final ratio of methanol: acetonitrile is 10:90) and incubated for 30 min at 4^{o}C. The insoluble material was obtained by centrifugation and then dissolved in 75-µl ddH_2O. An aliquot of 50-µl as input was applied to a reversed phase XDB-C18 (4.6 x 250 mm) HPLC analytical column (Agilent). Separation was achieved by use of step elution consisting of A (ddH_2O) and B (methanol) as following: 0.00-11.00 min: 100% A, flow rate 1 ml/min; 11.00-21.00 min: 100% B, flow rate 1 ml/min. All fractions were dried and dissolved in 100-µl ddH_2O, and 25-µl of each fraction was subjected to activity assay. The fraction with the highest killing activity was subjected to mass spectrometry (PE SCIEX API-100 LC/MS system, mass range: 30.0 to 500.0 by amu).

Quantitative RT-PCR

Total RNA was extracted using Aurum Total RNA mini kit (Bio-Rad) and reverse transcription was preformed from 400 ng of total RNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative RT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad) using a CFX connect Real-time System (Bio-Rad). The relative level of mRNA was calculated by comparative Ct method using Actin as control. The primers for SLC38A1 are SLC38A1-F: TGGTGACCATCATACTCTTG and SLC38a1-R: TCAGTGGCCTTCGTCGGTGC; the primers for Actin are: ACTIN-F: GGCACCACACCTTCTACAATG and ACTIN-R:

GGGGTGTTGAAGGTCTCAAAC; the primers for GCLC are GCLC-F: TGAGCATAGACACCATCATC and GCLC-R: GGTAGTTCAGAATACTGCATC; the primers for SLC1A5 are SLC1A5-F: TCTCCTTGATCTTGGCCGTG and SLC1A5-R: TCGAACGTGGCACTGGAGTC; the primers for GOT1 are GOT1-F: GAACACACCAATCTACGTATC and GOT1-R: GCTTCCACTGCTCTCTGGAGTC.

Lentiviral-Mediated shRNA interference

MISSION lentiviral shRNA clones targeting mouse TfR, SLC38A1, GLUD1, GLS1, GLS2 and non-targeting control construct were purchased from Sigma-Aldrich. Lentivirus was packaged in 293T cells, and used to infect target cells which were then selected with puromycin for at least 3 days prior to use in experiments. The clones ID for the shRNA are TfR-sh1: TRCN0000375693, TfR-sh2 TRCN0000375695; SLC38A1 KD: TRCN0000069231; GLS1-sh1: TRCN0000253163, GLS-sh2: TRCN0000253167; GLS2-sh1: TRCN0000177027, GLS2-sh2: TRCN0000198217; GLUD1-sh1: TRCN0000041506, GLUD1-sh2: TRCN0000041507, GCLC KD: TRCN0000311454; SLC1A5 KD: TRCN0000340676; GOT1 KD: TRCN0000119795.

Time-lapse microscopy

Live cell imaging of H2b-mcherry expressing MEFs was performed on glass-bottom 6-well plates (MatTek, Ashland, MA) using a Nikon Ti-E inverted microscope attached to a CoolSNAP CCD camera (Photometrics). Fluorescence and differential interference contrast (DIC) images were acquired every 7 minutes, and images were analyzed using NIS elements software (Nikon) and ImageJ software (NIH). For confocal imaging, MEFs were grown on 35mm glass bottom plates (MatTek, Ashland, MA) and DIC images were acquired every 5 minutes with the Ultraview Vox spinning disc confocal system (Perkin Elmer) equipped with a Yokogawa CSU-X1 spinning disc head, and EMCCD camera (Hamamatsu C9100-13), and coupled with a Nikon

Ti-E microscope. Image analysis was performed with Volocity software (Perkin Elemer). All imaging was carried out in incubation chambers at 5% CO_2 and 37°C.

Glutamine concentration measurement

The concentrations of glutamine in different FBS were measured by an YSI 7000 enzymatic analyzer according to the manual.

GSH measurement

$2x10^5$ MEFs were seeded in 6-well plates. One day later, cells were treated as indicated for 6 hours. Cells were harvested and cell numbers were determined. Total glutathione was measured as described previously(Rahman et al., 2006).

Measurement of reactive oxygen species (ROS)

MEFs were treated as indicated, and then 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Life Technologies Cat# D-399) was added and incubated for 1 hour. Excess H_2DCFDA was removed by washing the cells twice with PBS. Labeled cells were trypsinized and resuspended in PBS plus 5% FBS. Oxidation of H_2DCFDA to the highly fluorescent 2',7'-dichlorofluorescein (DCF) is proportional to ROS generation and was analyzed using a flow cytometer (Fortessa, BD Biosciences). A minimum of 10,000 cells was analyzed per condition.

2,2-Diphenyl-1-picrylhydrazyl Assay for antioxidant activity

The experiment was performed as described previously (Blois, 1958; Dixon et al., 2012). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma Cat#D9132) was dissolved in methanol to a final concentration of 50 μM. The tested compounds were added to 1 ml of DPPH solution with a final concentration of 50 μM. Samples were mixed well and incubated at room temperature for 1 hr. The absorbance at 517 nm (indicating the concentration of non-reduced DPPH) was measured

using methanol as control. Results were normalized to DMSO (which has no antioxidant activity; set as 100%).

Ischemia-reperfusion (I/R) analysis using isolated hearts

Male C57BL/6J mice weighing 25–30 g at age 12–14 weeks were used in all experiments and maintained in a temperature-controlled room with alternating 12:12-h light-dark cycles. Experiments were performed using an isovolumic isolated heart preparation as published and modified for the use in mice hearts (Ananthakrishnan et al., 2009; Hwang et al., 2004). Hearts from 12–14 weeks aged wild-type mice were isolated, and retrograde perfused at 37°C in a non-recirculating mode through the aorta at a rate of 2.5 ml/min. Hearts were perfused with modified Krebs-Henseleit (KH) buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 5 mM glucose, 0.4 mM palmitate, 0.2 mM glutamine, 10 μg/ml human recombinant transferrin, 0.4 mM BSA, and 70 mU/l insulin). Left ventricular developed pressure (LVDP) was measured using a latex balloon in the left ventricle. LVDP and coronary perfusion pressure were monitored continuously on a four-channel Gould recorder. Hearts were perfused either with KH buffer containing vehicle (DMSO) or the Compounds throughout the I/R protocol. After an equilibration period of 30 min, global ischemia was performed for 30 min followed by 60 minutes of reperfusion. Cardiac injury due to I/R stress was assessed by measuring LDH release in the perfusates that were collected during 60 min of reperfusion. Infarct area was measured using 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. After 60 min of reperfusion, the heart is perfused with Evans blue in-situ and then removed. Hearts were sliced into cross-sections at approximately 1-mm intervals. The sections were embedded in the TTC solution at 37°C for 10 min, and area of infarct as a percent of the whole heart was quantified as described. Functional recovery of LVDP was expressed by comparing to the initial LVDP before ischemia.

All animal experiments were approved by the Institutional Animal Care and Use Committees of New York University School of Medicine and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub. No. 85–23, 1996).

Statistical analyses

All statistical analyses were performed using Prism 5.0c GraphPad Software. P values were calculated with unpaired Student's t test. Data are presented as mean ± SEM from 3 independent experiments (*P < 0.05, **P < 0.01 ***P < 0.001 by unpaired Student t-test).

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