Figure S1, related to Figure 1. 0.5 mM 2-DG does not inhibit the increase induced by staurosporine (STS) (A) and higher concentrations of 2-DG induce autophagy in the presence of glucose (B).
(A) NRVMs were cultured in the presence or absence of 1 μM staurosporine (STS) for 20 hrs. Some cells were treated with 0.5 mM 2-DG. n=4, ***P<0.001.
(B) NRVMs were cultured in with glucose in the presence of 2-DG at various concentrations for 16 hrs and subjected to Western blotting. n=5. ***P<0.001 vs no 2-DG. ns; not significant.

Figure S2, related to Figure 4. Phosphorylation of AMPK (A), ACC (B), Raptor (C) or Akt (D) is not altered by knockdown or overexpression of HK-II, nor 2-DG, in NRVMs. Rapamycin induced autophagy is not inhibited by 2-DG nor HK-II knockdown (E). Cells were cultured in DMEM or no-glucose (NG) DMEM for 16 hrs, harvested, and subjected to Western blotting. Some cells were transfected with control siRNA (si-Ctrl) or HK-II siRNA (si-HK-II), or infected with GFP (AdGFP) or HK-II adenovirus (AdHK-II). Phosphorylation at Thr172 of AMPK (n=8), Ser79 of ACC (n=7), Ser792 of Raptor (n=7) and Ser473 of Akt (n=9) were used to assess activity. * *P*<0.05, ***P*<0.01, ***P<0.001 vs control. ns; not significant.

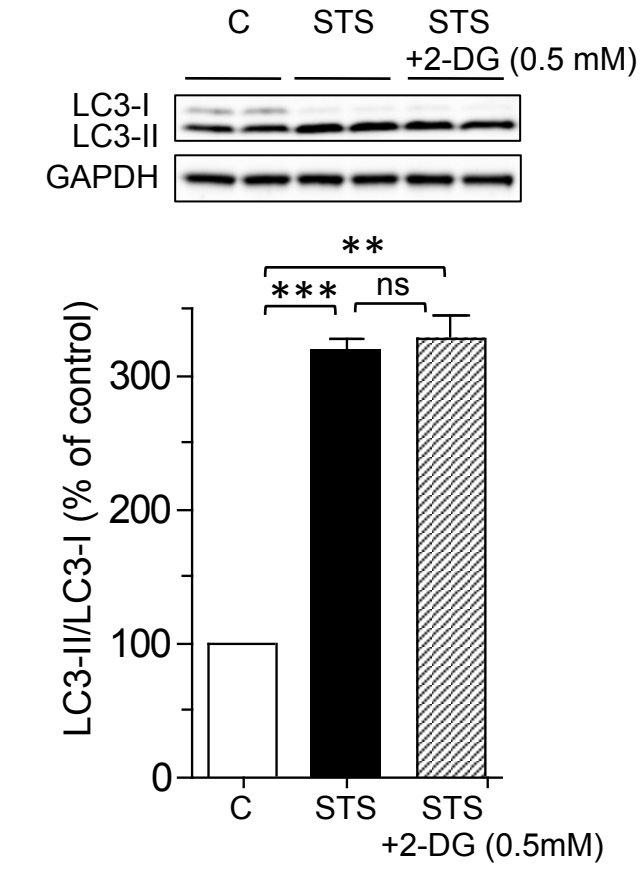
Figure S3, related to Figure 4. Hexokinase-I (HK-I) knockdown does not affect the decrease in P-p70S6K induced by glucose deprivation, nor 2-DG inhibition. Cells were transfected with control siRNA (si-Ctrl), HK-I siRNA and subjected to glucose deprivation for 16 hrs with or without 2-DG (0.5 mM), harvested, and subjected to Western blotting. ** P< 0.01, *** P<0.001. n=5-6.

Figure S4, related to Figure 5. Higher concentrations of 5-thio-glucose (5-TG) up to 5 mM do not affect the LC3-II/LC3-I ratio (A) and mitochondrial association of HK-II is not required for the HK-II mediated autophagic effect in the absence of glucose (B).
(A) NRVMs were cultured in DMEM or no-glucose (NG) DMEM for 16 hrs in the presence of 2-DG or 5-TG, and subjected to Western blotting.
(B) NRVMs expressing GFP (control), WT HK-II or N-terminus deletion mutant (ΔN) HK-II were subjected to glucose deprivation for 16 hrs. n=7. *** P<0.001.

Figure S5 related to Figure 6. Co-localization of HK-II and mTOR is increased by glucose deprivation and decreased by addition of 2-DG. NRVMs were subjected to glucose deprivation with or without 2-DG for 16 hrs and immunofluorescence using confocal microscopy was obtained.

Figure S6, related to Figure 7. Phosphorylation of ULK-1 at Ser555 is increased by glucose deprivation and this response is enhanced by WT HK-II, but not by F199A mutant (A). Add-back of WT HK-II, but not F199A mutant, recovers autophagy in HK-II knocked-down NRVMs (B). Model of the pathway by which HK-II regulates autophagy in response to glucose deprivation (C).
(A) NRVMs expressing GFP (control), WT HK-II or F199A mutant (FA) HK-II were subjected to glucose deprivation for 16 hrs * P<0.05, n=8.
(B) NRVMs were transfected with control siRNA (si-Ctrl) or HK-II siRNA (si-HK-II #1). After 48 hrs transfection, cells were infected with adenovirus encoding WT or F199A (FA) HK-II and cultured for a further 24 hrs. Glucose deprivation (16 hrs) induced increase in the ratio of LC3-II/LC3-I was assessed by Western blotting (n=8).

A


C
STS
STS
+2-DG (0.5 mM)
LC3-I
LC3-II
GAPDH
**
***
ns
LC3-II/LC3-I (% of control)
300
200
100
0
C
STS
STS
+2-DG (0.5mM)

B

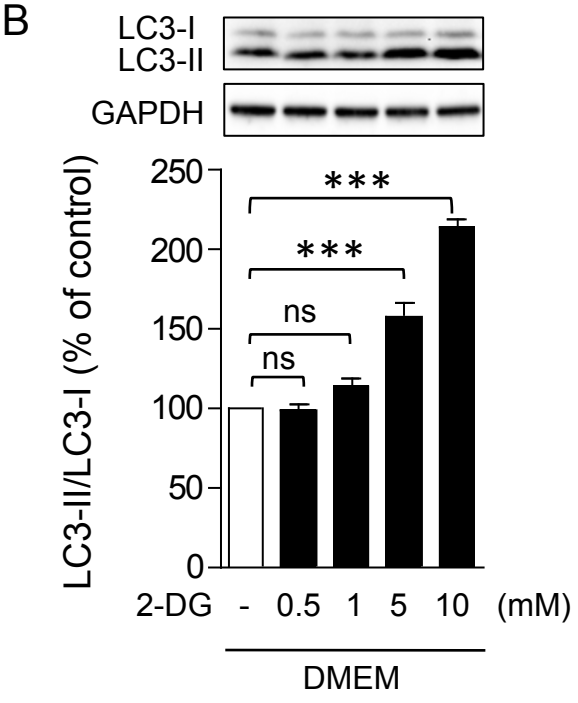
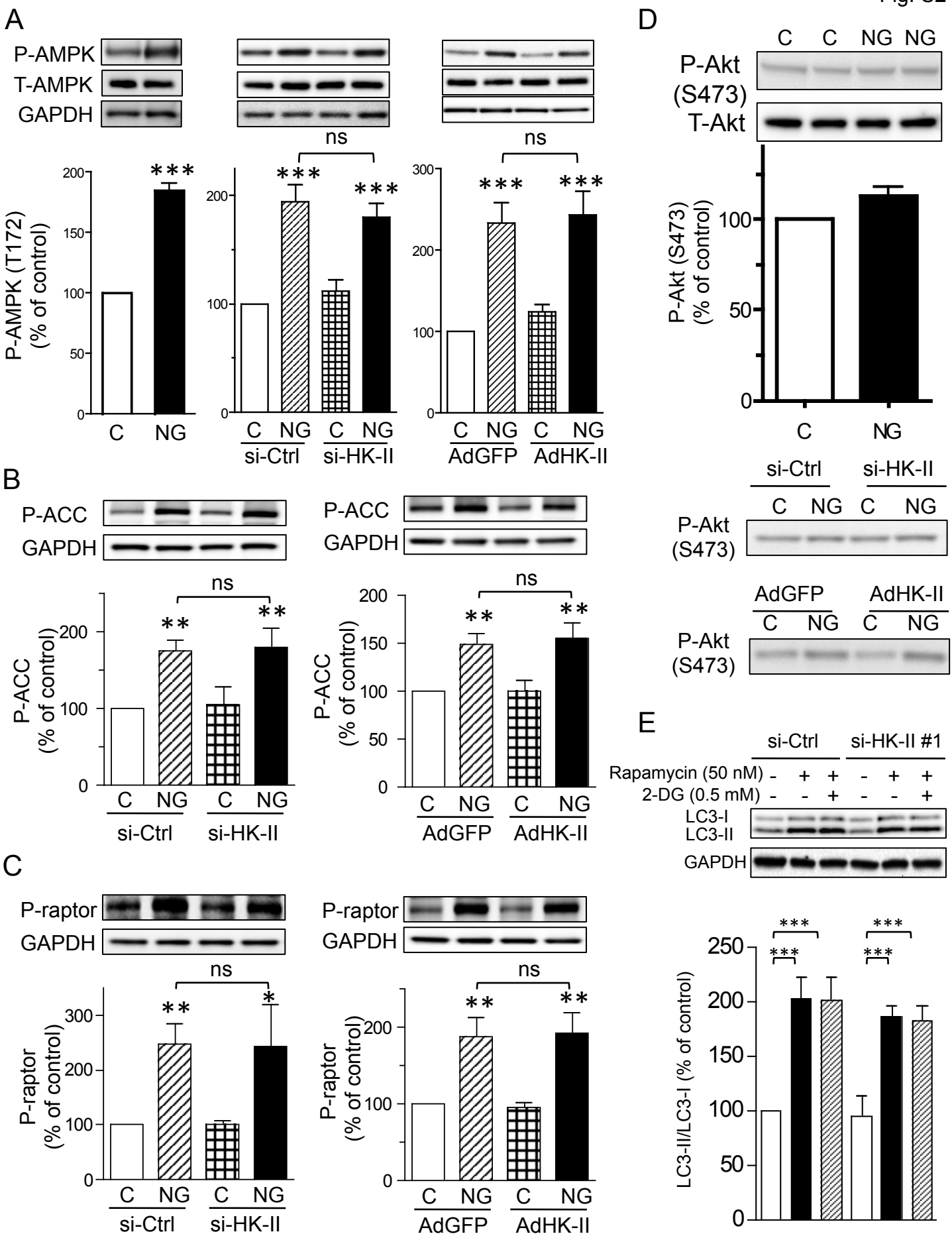
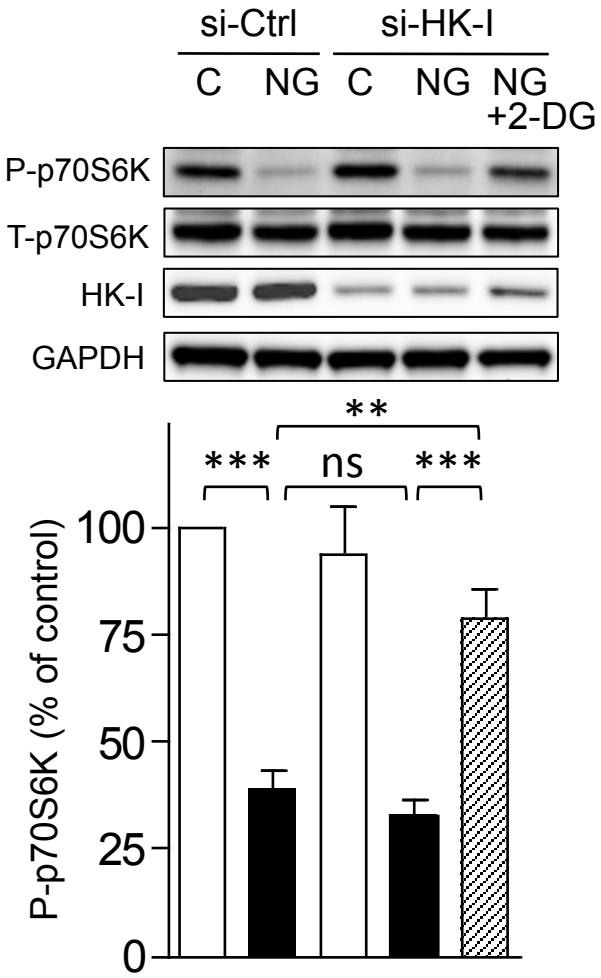
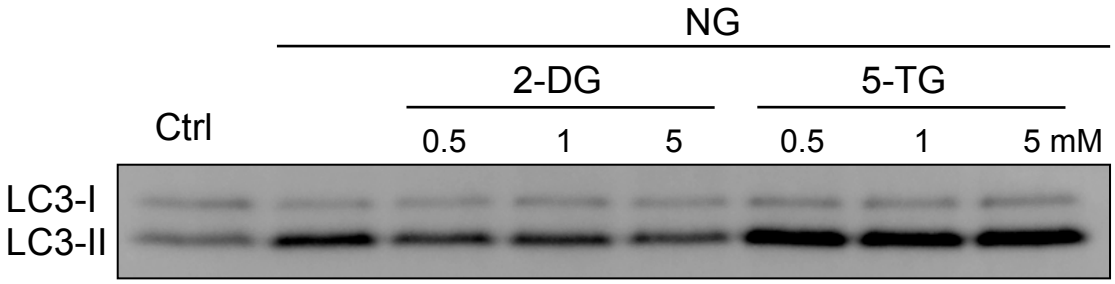

LC3-I
LC3-II
GAPDH
250
200
150
100
50
0
***
***
ns
ns
LC3-II/LC3-I (% of control)
2-DG - 0.5 1 5 10 (mM)
DMEM

Fig. S2

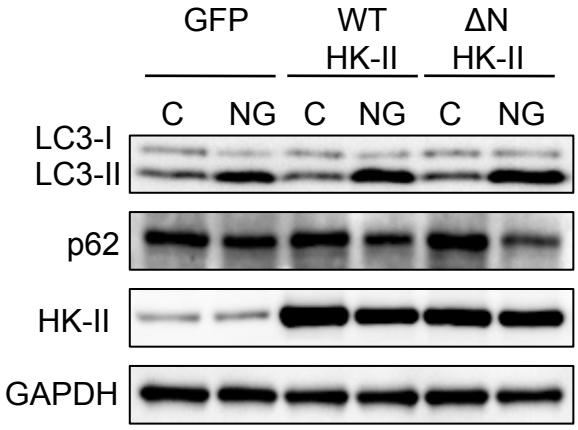

A
P-AMPK
T-AMPK
GAPDH
P-AMPK (T172) (% of control)
ns
***
C NG
si-Ctrl si-HK-II
AdGFP AdHK-II
B
P-ACC
GAPDH
P-ACC (% of control)
**
C NG C NG
si-Ctrl si-HK-II
AdGFP AdHK-II
C
P-raptor
GAPDH
P-raptor (% of control)
*
D
P-Akt (S473)
T-Akt
P-Akt (S473) (% of control)
E
si-Ctrl
si-HK-II #1
Rapamycin (50 nM)
2-DG (0.5 mM)
LC3-I
LC3-II
GAPDH
LC3-II/LC3-I (% of control)


si-Ctrl
si-HK-I
C
NG
C
NG
NG
+2-DG
P-p70S6K
T-p70S6K
HK-I
GAPDH
**
***
ns
***
P-p70S6K (% of control)
100
75
50
25
0

A


NG
2-DG
5-TG
Ctrl
0.5
1
5
0.5
1
5 mM
LC3-I
LC3-II

B


GFP
WT HK-II
ΔN HK-II
C
NG
C
NG
C
NG
LC3-I
LC3-II
p62
HK-II
GAPDH

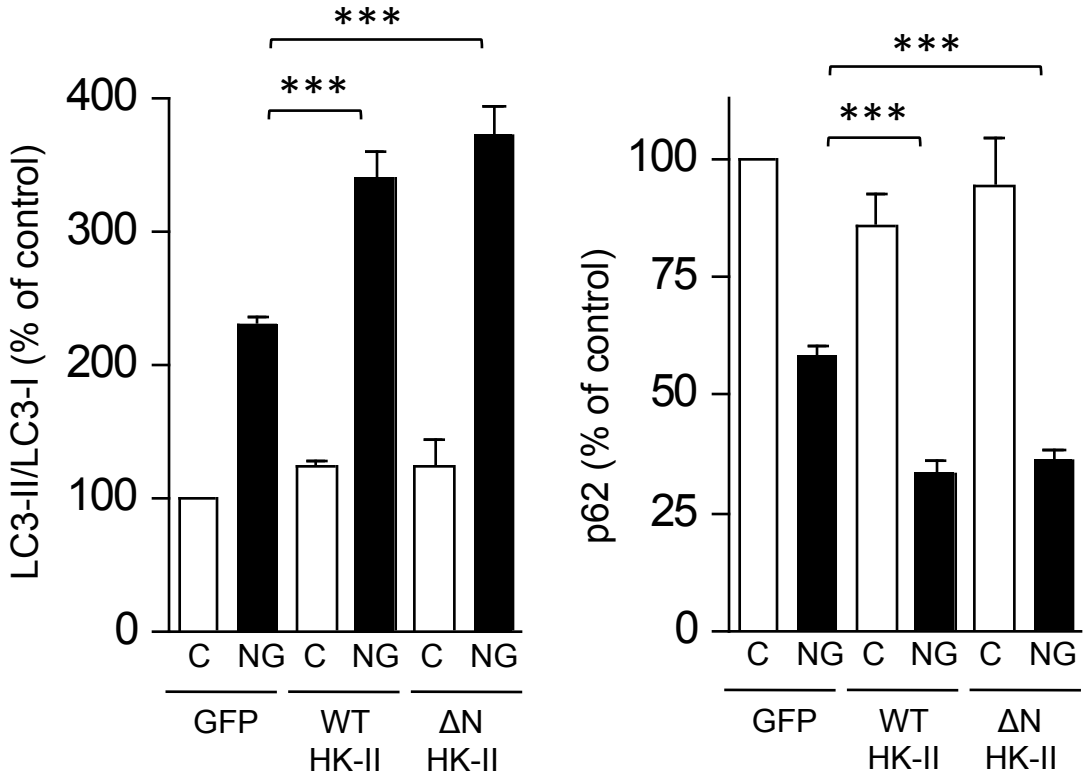
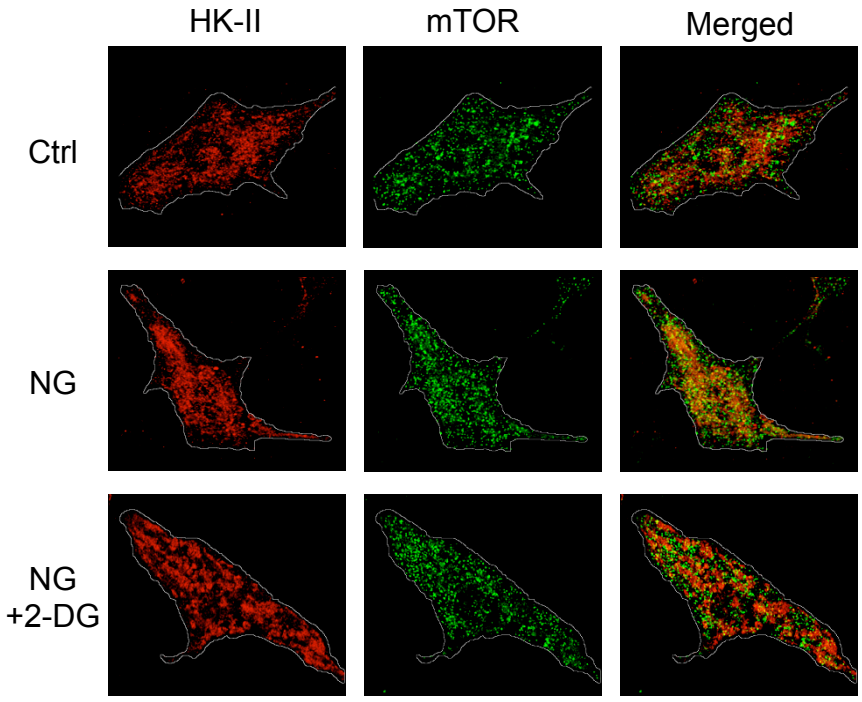
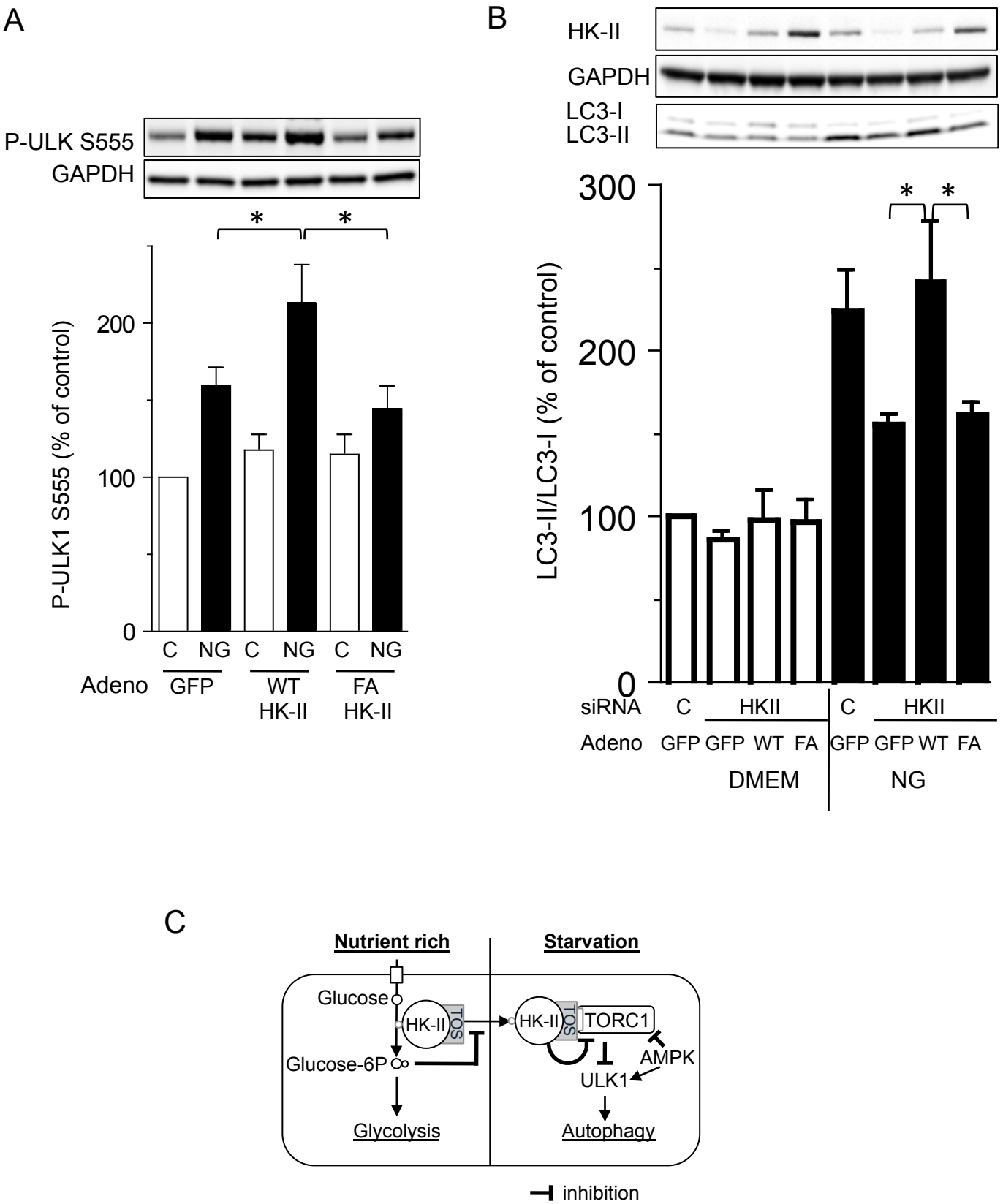

***
***
LC3-II/LC3-I (% of control)
400
300
200
100
0
C
NG
C
NG
C
NG
GFP
WT HK-II
ΔN HK-II
***
***
p62 (% of control)
100
75
50
25
0
C
NG
C
NG
C
NG
GFP
WT HK-II
ΔN HK-II

Fig. S5




A
P-ULK S555
GAPDH
P-ULK1 S555 (% of control)
200
100
0
C NG C NG C NG
Adeno GFP WT HK-II FA HK-II
B
HK-II
GAPDH
LC3-I
LC3-II
LC3-II/LC3-I (% of control)
300
200
100
0
siRNA C HKII C HKII
Adeno GFP GFP WT FA GFP GFP WT FA
DMEM
NG
C
Nutrient rich
Starvation
Glucose
HK-II
TOS
Glucose-6P
Glycolysis
HK-II
TOS
TORC1
AMPK
ULK1
Autophagy
inhibition

Supplemental Materials and Methods

Cell Culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-2 day-old Sprague-Dawley rat pups using a kit (Worthington). Myocytes were plated at a density of $3.5x10^4/cm^2$ and maintained overnight in Dulbecco's modified Eagle's medium (DMEM high glucose; Invitrogen), supplemented with 15% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). Cells were serum starved for 24 hrs prior to glucose deprivation. Adenoviral infection was carried out as described previously (Miyamoto et al., 2005); after serum starvation, cells were infected with wild-type HK-II, F199A HK-II, kinase-dead HK-II (KD HK-II), N-terminus deletion mutant (ΔN), GFP (control) or LC3-GFP adenovirus for 16 hrs, washed and cultured for a further 8 hrs prior to glucose deprivation.

Transfection of cardiomyocytes with siRNA

Pre-designed HK-II ON-TARGET*plus* siRNA (siHK-II #1) for rat and control siRNA were purchased from Thermo Scientific. Pre-designed HK-II siRNA (siHK-II #2) was also purchased from Qiagen. NRVMs were transfected with siRNA using DharmaFECT-I transfection reagent (Thermo Scientific). 2 µmol siRNA were transfected into $1x10^6$ cells (6 cm dish). siRNA and DharmaFECT-1 were individually incubated in conical tubes containing 0.5 ml OPTI-MEM media (GIBCO) at room temperature for 5 min, mixed and incubated at room temperature for 20 min. Media in culture dishes were replaced with fresh 10% FBS/high-glucose DMEM (3 ml for 6 cm dish) and siRNA/DharmaFECT-I mixtures (1 ml/dish) were added. After overnight incubation, cells were washed and cultured for another 72 hrs in serum free media (DMEM high glucose).

Western blotting and Immunoprecipitation

Cardiomyocytes were washed three times with ice-cold PBS, harvested in RIPA buffer; composed of 150 mM NaCl, 50 mM Tris (pH7.4), 1% NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 0.2 mM EDTA, supplemented with 200 µmol Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/L PNPP and 1 mmol PMSF. Samples were nutated at 4˚C for 10 min, spun down at 20,000 x *g* for 7 min and supernatants were saved as whole cell lysates. Cell lysates were mixed with LDS sample buffer and reducing agent (Invitrogen), heated at 75˚C for 10 min and equal amounts of protein (10-30 µg) were loaded onto SDS-PAGE (Invitrogen NuPage system). Gels were run on ice and proteins were transferred to PVDF membranes (Millipore). Membranes were blocked using 5% milk TBS-Tween for 1 hour at room temperature and probed at 4°C overnight using the following antibodies at 1:1000 dilution in 5% BSA/TBS-tween. The phospho-acetyl-coA carboxylase, hexokinase-II (rabbit monoclonal), hexokinase-I (rabbit polyclonal), LC-3 (rabbit polyclonal), total- and phosphorylated AMPK (rabbit monoclonal), phosphorylated Akt (Ser473), GAPDH, total- and phosphorylated-p70S6K (Thr389), total and phosphorylated-4E-BP1 (Thr37/46), raptor (rabbit monoclonal), mTOR (rabbit monoclonal), GβL and phosphorylated ULK-1 (Ser757 or S555) (rabbit polyclonal) were from Cell Signaling technology. p62 antibody was purchased from BD biosciences. Blots were washed with TBS-tween (5 min x 5 times) and incubated with secondary antibodies (1:2000~1:5000 dilution) in 5% milk/TBS-Tween for 1 hour.

For immunoprecipitation studies, cells were washed with ice-cold PBS twice and lysed in hypotonic/digitonin buffer (20 mM PIPES [pH7.2], 5 mM EDTA, 3 mM $MgCl_2$, 10 mM glycerophosphate, 10 mM pyrophosphate, 0.02% digitonin plus protease and phosphatase inhibitors). In some experiments, 0.5% NP-40 alternative or 0.3% CHAPS were used instead of digitonin. After 40 min nutation at 4°C, samples were spun down at 20,000 x *g* for 7 min and supernatants were saved. HK-II or mTOR was immunoprecipitated using antibody to HK-II (Santa Cruz, C-14 goat polyclonal) or mTOR (EMD, mouse monoclonal), respectively. Cell

lysates were precleaned with Protein A/G PLUS-agarose beads for 30 min at 4°C and 150-300 μg of total protein was then incubated with HK-II or mTOR antibody (4 μg) and protein A/G PLUS agarose (Santa Cruz Biotechnology; 30 μl of 50% slurry) at 4˚C overnight. Immunocomplexes were washed with ice-cold lysis buffer 4 times, and beads were boiled in 2x LDS buffer to elute captured protein and subjected to Western blotting. Band intensities were analyzed by AlphaView SA (Cell Biosciences Inc.), background was subtracted followed by normalization to that of loading control obtained from the same gel (GAPDH for whole cell lysate and target molecule of immunoprecipitation for the study of HK-II-TORC1 interaction) and percentage of control was obtained.

Immunofluorescence study

NRVMs were grown on gelatin coated glass coverslips in 12 well plates. The cells were serum starved >24 hours before washing and replacing the medium with either glucose containing, glucose-free or glucose-free supplemented with 300 μM 2-DG. After 17 hours, the cells were fixed for 5 minutes using 4% paraformaldehyde in PBS and then washed 3x with PBS before storing at 4^{o}C. When required, cells were permeabilized using 0.1% Triton X-100 for 5 minutes, washed 3x with PBS before addition of 5% BSA (fraction V) for 30 mins. Antibodies (mTOR: 2983 (CST); HKII: sc-6521 (SCBT)) were diluted in 0.1% BSA in PBS and incubated overnight at 4˚C. The cells were then washed 3x 5 minutes in PBS before addition of Alexa 488 donkey anti-rabbit and Alexa 568 donkey anti-goat (Invitrogen) secondaries. Cells were again washed 3x 5 minutes in PBS and mounted on glass slides using Vectorshield with DAPI (Vector Labs). Pictures were aquired using an Olympus Fluoview 1000 confocal microscope.

Cell Death ELISA Assay

DNA fragmentation, indicative of apoptosis, was detected using the cell death detection ELISA^{PLUS} (Roche Applied Science). Cells were washed with ice-cold PBS twice and harvested in cytosolic extraction buffer (20 mmol Tris pH 7.6, 3 mmol EDTA, 3 mmol EGTA, 125 mmol NaCl, 20 mmol β-glycerophosphate and 0.4% Nonidet P-40 alternative plus protease and phosphatase inhibitors). Cell lysates (5 μl) were incubated with anti-histone-biotin antibody and anti-DNA-peroxidase antibody in a streptavidin-coated 96 well plate on an orbital shaker (60 rpm) for 2 hours at room temperature. Subsequently, wells were washed with incubation buffer and, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid substrate (100 μl per well) was added. Absorbance was measured at 405 nm using an Infinite M200pro plate reader (Tecan) (Del Re et al., 2007, 2008; Miyamoto et al., 2008).

Statistical Analysis

Results are reported as averages ± SEM. Comparisons between two groups were accomplished using unpaired Student's *t* test. Experiments with more than two groups were compared by ANOVA followed by the Tukey post-hoc test. A P-value of <0.05 was considered statistically significant.

Supplemental References

Del Re, D.P., Miyamoto, S., and Brown, J.H. (2007). RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. J Biol Chem *282*, 8069-8078.

Del Re, D.P., Miyamoto, S., and Brown, J.H. (2008). Focal adhesion kinase as a RhoA-activable signaling scaffold mediating Akt activation and cardiomyocyte protection. J Biol Chem *283*, 35622-35629.

Miyamoto, S., Howes, A.L., Adams, J.W., Dorn, G.W., 2nd, and Brown, J.H. (2005). Ca2+ dysregulation induces mitochondrial depolarization and apoptosis: role of Na+/Ca2+ exchanger and AKT. J Biol Chem *280*, 38505-38512.

Miyamoto, S., Murphy, A.N., and Brown, J.H. (2008). Akt mediates mitochondrial protection in cardiomyocytes through phosphorylation of mitochondrial hexokinase-II. Cell Death Differ *15*, 521-529.