## Supplemental Figure Inventory

**Figure S1: Neither decreasing cell proliferation nor activation of Akt is involved in protecting *TSC2-/-* MEFs from glucose deprivation induced cell death; related to figure 1.**

This figure shows that rapamycin-induced decrease in cell proliferation is not involved in protection from glucose deprivation-induced death. This figure adds to figure 1 by showing that induction of apoptosis is not the cause for glucose addiction in *TSC2-/-* cells. This figure also shows that feedback loop-mediated activation of Akt is not involved in the rapamycin-induced protection.

**Figure S2: Oxidative phosphorylation is necessary for viability and ATP maintenance in the absence of glucose; related to figure 2.**

This figure complements figure 2 by showing that the maintenance of ATP by rapamycin treatment requires OXPHOS.

**Figure S3: Rapamycin-induced autophagy is not required for survival under glucose deprivation; related to figure 3.**

This figure is a prelude to figure 3 by showing that rapamycin-induced autophagy is not the cause for rapamycin-induced ATP maintenance, but rather glutamine (Figure 3).

**Figure S4: Loss of HIF-1a is not sufficient to provide protection under glucose deprivation in *TSC2-/-* MEFs; related to figure 4.**

This figure is a prelude to figure 4 by showing that the inability to produce ATP via the mitochondria via HIF-1$\alpha$ increase is not the reason for requiring mTORC1 inhibition. It leads into figure 4, which shows that mitochondria activity is actually decreased with rapamycin treatment.

**Figure S5: Pyruvate and oxaloacetate can substitute for glutamine to provide protection of *TSC2-/-* cells; related to figure 6.**

This figure shows that TCA cycle intermediates can substitute for glutamine, and complements figure 6, which shows that increasing TCA intermediates does not provide protection.

**Figure S6: EGCG and DON, both inhibitors of glutamine metabolism, induce death of glucose-deprived *TSC2-/-* cells; related to figure 7.**

This figure shows the specificity of glutamine metabolism inhibitors, and complements figure 7, which shows that GDH is necessary for glutamine metabolism following glucose withdrawal.

**Figure S7: The role of eIF4E and S6K1 in mediating rapamycin-induced protection from glucose withdrawal.**

This figure investigates the role of eIF4E and S6K1, two downstream components of mTORC1, in regulating the sensitivity of *TSC-/-* cells to glucose deprivation.

## Supplemental Figure Legends

**Figure S1. Neither decreasing cell proliferation nor activation of Akt is involved in protecting *TSC2-/-* MEFs from glucose deprivation induced cell death.**

A. *TSC2-/- p53-/-* MEFs were grown in the presence of thymidine (3mM) or rapamycin, and were deprived of glucose for 60 hours. Phase images were taken showing viability and are shown in A.

B. Cell proliferation rates with rapamycin or thymidine (3mM) treatment.

C. PI-exclusion assay measured cell viability at 60 hours post glucose starvation. All data is an average (+SEM) of 3 independent experiments.

D. *TSC2-/- p53-/-* MEFs were treated with rapamycin for the indicated times and Akt-P 473 was blotted.

E. Cell viability was observed with phase image at 60 hours post starvation. The *TSC2-/-* MEFs were also treated with rapamycin and DMSO, LY294002 10μM, or Akt IV inhibitor (1μM).

F. PI-exclusion assay measuring viability from B.

G. Western blots measuring the inhibition of Akt-473, S6K1 (Shift), and total Akt.

**Figure S2. Oxidative phosphorylation is necessary for viability and ATP maintenance in the absence of glucose.**

A. *TSC2-/- p53-/-* MEFs that were deprived of glucose and treated with rapamycin were also given oligomycin (Indicated concentrations are in μg/mL). Viability was measured 60 hours post starvation.

B. ATP levels after 24 hours of starvation were measured except oligomycin (0.1 μg/mL) was given 90 minutes prior to lysis. Oligomycin was treated for 24 hours in the sample that contained glucose.

C. The same experiment in A was repeated with antimycin A (2 μg/mL).

**Figure S3. Rapamycin-induced autophagy is not required for survival under glucose deprivation.**

A. LC3-EGFP localization was observed after rapamycin treatment for the indicated times.

B. LC3-EGFP cleavage was measured after the indicated times via western blotting. An antibody against EGFP was used to detect the cleavage.

C. shRNA against Beclin was developed, and the knockdown efficiency is shown. The cleavage of LC3 by rapamycin treatment was measured via western blotting.

D. The cells from C, specifically Beclin shRNA#2,3, were deprived of glucose and treated with rapamycin for 60 hours and viability was measured.

E. 3-methyladenine (3-MA) was used at the indicated concentrations to also block autophagy. Viability, in the absence of glucose with or without rapamycin, was measured after 60 hours.

F. *ATG5+/+* and *ATG5-/-* MEFs were treated with rapamycin for 2 hours, and LC3 cleavage was measured.

G. mTORC1 activity following glucose withdrawal in *ATG5-/-* Rheb S16H expressing cells was measured. Glucose was starved for 12 hours.

H. Cell viability of Rheb S16H expressing *ATG5+/+* and *ATG5-/-* cells was measured following glucose withdrawal for 48 hours.

**Figure S4. Loss of HIF-1a is not sufficient to provide protection under glucose deprivation in *TSC2-/-* MEFs.**

A. Asynchronously growing *TSC2-/-* MEFs were treated with rapamycin for the indicated time points (0, 1, 12, 24 hours). The protein level of HIF-1$\alpha$ was measured via western blotting. * = Nonspecific band. HIF-1$\alpha$ is indicated with an arrow.

B. Knockdown efficiency of shRNAs targeting HIF-1$\alpha$ (#1 and #3). shGFP cells were also treated with rapamycin for 24 hours.

C. *TSC2-/-* MEFs expressing either shGFP, shRNA#1, or shRNA#3 were tested for viability following glucose deprivation. Viability was measured 60 hours post deprivation via PI-exclusion assay.

D. Total ATP levels were determined 24 hours post deprivation in cells with expressing shHIF-1$\alpha$.

**Figure S5. Pyruvate and oxaloacetate can substitute for glutamine to provide protection of *TSC2-/-* cells.**

*TSC2-/-* MEFs were incubated in media containing no glucose with glutamine (4mM), methylpyruvate (10mM), or oxaloacetate (2mM). The cells were also given either rapamycin (A) or cycoheximide (5μM) (B). Viability was measured 48 hours post deprivation. Shown is an average (+SEM) of 3 independent experiments.

**Figure S6. EGCG and DON, both inhibitors of glutamine metabolism, induce death of glucose-deprived *TSC2-/-* cells.**

A. A diagram showing the enzymes involved in glutamine metabolism.

B. EGCG (100μM), a GDH inhibitor, and DON (1mM), a glutaminase inhibitor, were treated to cells grown in glucose containing media or to cells grown in glucose-free media that were protected with rapamycin. Viability was measured 60 hours post deprivation.

C. *TSC2-/- p53-/-* cells were grown in titrating levels of glucose (0, 0.5, 1.0, 2.0, 5.0, 25 mM) and were given vehicle or EGCG (50μM). Cell viability was measured 48 hours post deprivation.

D. EGCG's toxic effects are specific to glutamate metabolism. *TSC2-/-* cells were deprived of both glucose and amino acids. These cells were also give control vehicle or EGCG (50μM) with either glutamine (4mM), glutamate (4mM), OAA (2mM), pyruvate (1mM), or α-ketoglutarate (10mM). Viability was measured 48 hours post treatment/deprivation. (SEM is the average of 3 independent experiments).

E. ROS levels were measured in *TSC2-/-* cells with DFC-DA staining after 12 hours of glucose deprivation.

**Figure S7. The role of eIF4E and S6K1 in mediating rapamycin-induced protection from glucose withdrawal.**

A. *TSC2-/-* cells expressing HA-eIF4E, HA-4E-BP1AA, or pLPCX2 were deprived of glucose for 60 hours with or without rapamycin, and cell viability was measured 60 hours later.

B. shRNA constructs targeting eIF4E were used to generate *TSC2-/-* cells with eIF4E knockdown. Cell viability was measured in these cells after 60 hours of glucose deprivation.

C. Viability of shGFP or shS6K1 expressing cells was measured 60 hours post glucose deprivation. Knockdown efficiency is shown of S6K1 is also shown.

D. Cells from (C) were deprived of glucose and total ATP levels were measured 20 hours post deprivation. ($n = 6$; $p < 0.01$)

## Supplemental References

Christofk, H.R., Vander Heiden, M.G., Harris, M.H., Ramanathan, A., Gerszten, R.E., Wei, R., Fleming, M.D., Schreiber, S.L., and Cantley, L.C. (2008). The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature. *13*, 230-3.

Kimmich, G.A., Randles, J., and Brand, J.S. (1975). Assay of picomole amounts of ATP, ADP, and AMP using the luciferase enzyme system. Analytical Biochemistry. *69*, 187-206.

Watts, R.G. and Howard, T.H. (1992). Evidence for a gelsolin-Rich, labile F-actin pool in human polymorphonuclear leukocytes. Cell Motility and the Cytoskeleton. *21,* 25-37.

Yoon, S.O., Shin, S., Liu, Y., Ballif, B.A., Woo, M.S., Gygi, S.P., and Blenis, J. (2008). Ran-binding protein 3 phosphorylation links the Ras and PI3-kinase pathways to nucleocytoplasmic transport. Molecular Cell. *29*, 362-75.

## Supplemental Experimental Procedures

*Cell Viability Measurements*

All cell viability experiments were conducted with propidium iodide (PI) exclusion assay. In brief, PI was added to culture media (1 μg/mL) for 5 minutes prior to collection. The media from each sample was collected and then the plates were washed briefly with saline. The saline was added to the media collection tube, and then the remaining cells on the plate were detached with trypsin. The cells were then collected and also put into the original media collection tube. The entire tube, which now has the original media with saline wash and trypsinized cells, was centrifuged and the pellet was analyzed for PI-exclusion via FACS (Beckon Dickinson). Additionally, the cells were washed with media lacking glucose and all amino acids (1/2 volume of incubating media) prior to adding the experimental media, which contained dialyzed FBS. For nitrogen mustard experiments, cells were plated overnight (~15-18 hours), and media was changed for new media without pyruvate and with or without glutamine. 8 hours after the change, cells were given different doses of nitrogen mustard. For viability assays with shGDH, we noticed that after 5-6 passages post selection, shGDH#1 and 4 cells were similar to shGFP cells in responding to glucose deprivation. Therefore, we recommend performing experiments relatively quickly post selection as these cells likely compensate to maintain cell growth. For all phase images, the Nikon Eclipse TE300 camera was used, and images were taken at the indicated time points. For GSH depletion experiments, cell viability was also measured 24 hours post nitrogen mustard and BSO treatment. Total GSH levels were measured via GSH-Glo Glutathione Assay (Promega). For ROS measurements, DFC-DA staining (Molecular Probes) was used after 12 hours of deprivation. For both the ROS and GSH assays, the cells were washed with the sample's respective media 2 times prior to incubation.

*Antibodies, Chemicals, shRNAs, Plasmids*

The antibodies used for this study are the following: p53, Raptor, p70, 389(P) p70, S6(P), AMPK$\alpha$1, Thr172 AMPK, ACC(P), Fatty Acid Synthase, cytochrome c, PGC-1$\alpha$, Akt-473, Akt, FOXO1, LC3 and Beclin were all purchased from Cell Signaling Technologies. Antibodies to Actin, BclxL, and EGFP were purchased from Santa Cruz, Inc. Two different antibodies to HIF-1$\alpha$ were used; one was a generous gift from Dr. W. Kaelin (Dana Farber Cancer Institute) and can be purchased from Precision. The other was from Cayman Chemicals. The following chemicals were used in this study: Rapamycin (Calbiochem), 6-Diazo-5-oxo-L-norleucine (Sigma), Thymidine (Sigma), EGCG (Calbiochem), LY294002 (Calbiochem), AKT IV inhibitor (Calbiochem), Cycloheximide (Calbiochem), methylpyruvate (Sigma), oxaloacetic Acid (Sigma), alpha-ketoglutaric acid (Sigma), sodium pyruvate (Sigma), nitrogen mustard (Sigma), succinic acid (Sigma), glutamate (Sigma), aminooxyacetate (Sigma), 3-Methyladenine (Sigma), Oligomycin (Calbiochem), Gramicidin (Sigma), Ouabain Octahydrate (Calbiochem), Antimycin A (Calbiochem), BSO (Sigma). All murine shRNAs, which includes HIF-1$\alpha$, GDH, eIF4E, S6K1, and Beclin, were purchased from open biosystems (pLKO.1 set). pBABE-*TSC1* was a gift from David Kwiatkowski (Brigham and Women's Hospital). *TSC2* was cloned into pLPCX, and the *TSC2* containing viruses were used to reconstitute *TSC2-/- p53-/-* MEFs. pMIG and pMIG-Bcl-xL were purchased from addgene. HA-eIF4E, HA-4E-BP1 37/46 AA, Rheb S16H, and Rheb S20N were all cloned into pLPCX2. The LC3-EGFP construct was a gift from Joan Brugge (Harvard Medical School).

*Fractionation of F- and G- actin*

The *BclxL TSC2-/- p53-/-* MEFs were deprived of glucose and treated with rapamycin or DMSO for 48 hours and lysed in buffer containing 1% triton X (50mM Hepes pH 7.2, 40mM KCl, 10mM EGTA, and appropriate protease inhibitors). The cells were first centrifuged at 15,000 g's for 5 minutes and the supernatant was transferred, and ultracentrifuged at 100,000 g's for 60 minutes. The pellet from the first spin was resuspended in SDS loading buffer and contains the F-actin. The supernatant from the second spin contains the G-actin.

To normalize for loading, protein assay (Bradford) was conducted prior to the first spin and equal protein amounts were spun in the first step. The protocol was originally published by Watts and Howard (Watts and Howard, 1992).

*ATP and ADP measurements*

To measure ATP and ADP amounts, the cells were lysed in somatic releasing buffer (Sigma). Whenever possible, all experiments were conducted in the cold room and all reagents were at ~4° C prior to use. The total ATP amounts were measured via luciferase activity (Sigma) with linear ranges determined for both the samples and standard controls. The data was either graphed as fold increases from glucose deprivation alone or as nmoles of ATP per mg of protein. The ADP levels were measured by adding excess amounts of phosphoenolpyruvate (PEP) and pyruvate kinase (Both from Sigma). This reaction, which converts all the ADP in a sample to ADP, was allowed to progress for 40 minutes in room temperature. The total ATP in this sample was measured via luciferase and the total ADP was determined by subtracting the total nmoles of ATP in the sample ($1^{st}$ reaction) from the second reaction, which contains both ATP and ADP. The ATP/ADP ratio was then determined. A more detailed protocol is described elsewhere (Kimmich et al., 1975).

When plating the cells for both ATP and viability assays, the media was changed when the cell confluency was about 70-80%. This equated to about 650,000 *TSC2-/-* MEFs per 10cm plate. Deprivation was done typically 18-20 hours post plating. It has been our experience that more confluent conditions will take more time to deplete the ATP and induce cell death, while more sparse plating will take less time. To be consistent, we conducted all of our experiments when the cells were at about 70-80% confluency.

*MMP and Total Mitochondria Measurements*

Mitochondria membrane potential (MMP) was measured via FACs with DiOC6 (Invitrogen). Because DiOC6 nonspecifically stains other organelles at high concentrations, we titrated the mean fluorescent intensity (MFI) for with

oligomycin. For total mitochondria staining, we used mitotracker green FM (Invitrogen) as the stain and analyzed the samples via FACS (MFI).

*Oxygen Consumption Assays*

*TSC2-/-* MEFs were plated on 15 cm plates; 24 hours later, the cells were deprived of glucose for 24 hours. Thereafter, the cells were washed in saline and trypsinized. The cells were resuspended in glucose free media and oxygen consumption rates were determined with a polarographic oxygen electrode (Christofk et al., 2008).

*Na+ K+ ATPase Assay*

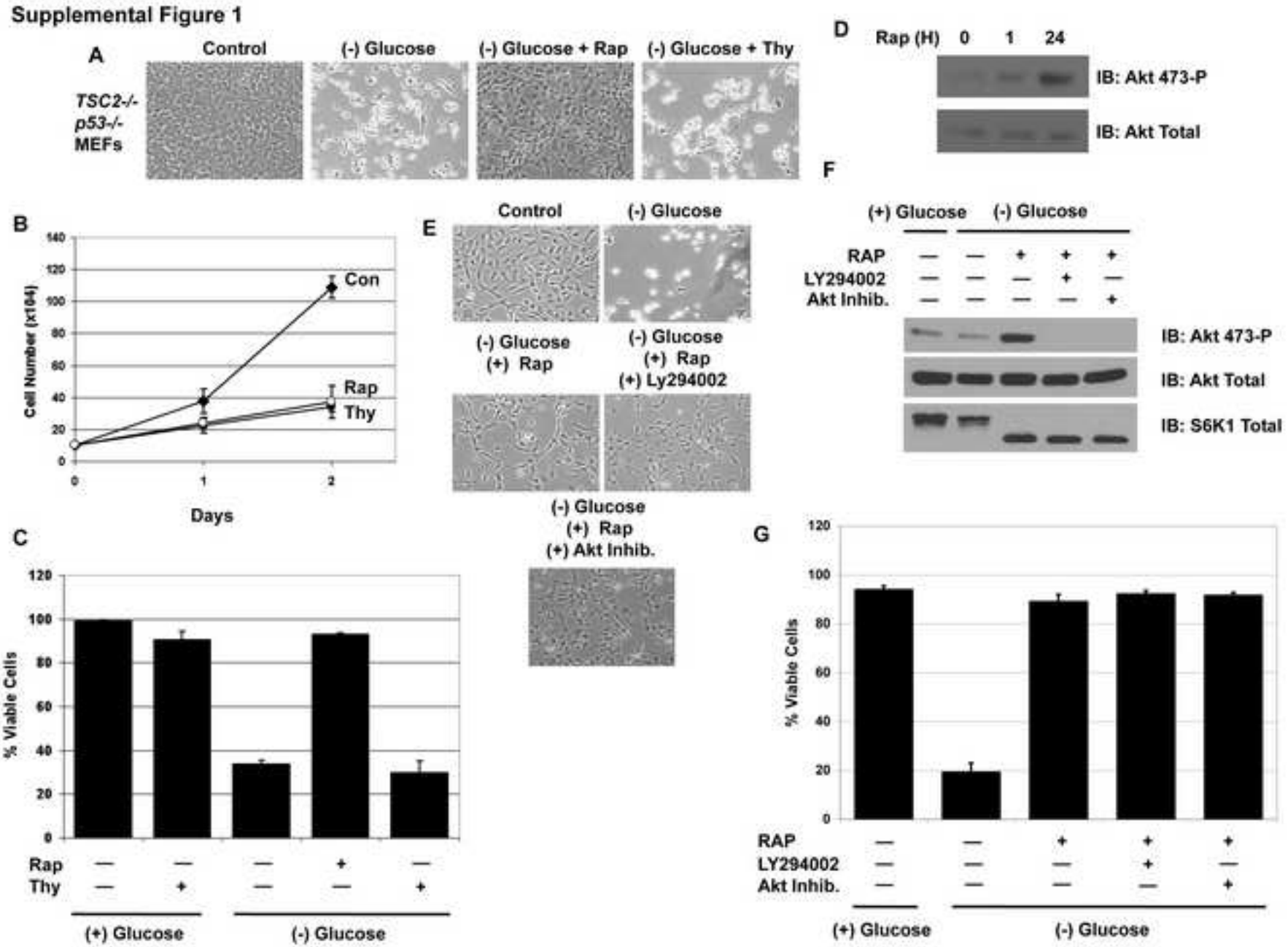
*TSC2-/-* MEFs were treated with rapamycin for 24 hours and incubated with ouabain (2mM) for 15 minutes prior to initiation of assay. The ouabain incubation was in serum-free DMEM, and the assay was also conducted in serum-free DMEM. Thereafter, the cells were given 5 μCi of $^{86}$Rb and incubated for 10 minutes at 37°C. The uptake of $^{86}$Rb was measured via scintillation counter and uptake was normalized to total protein amount. No external sources of ionophores were used to keep the conditions as close as possible to glucose deprivation experiments.

*Autophagy Experiments*

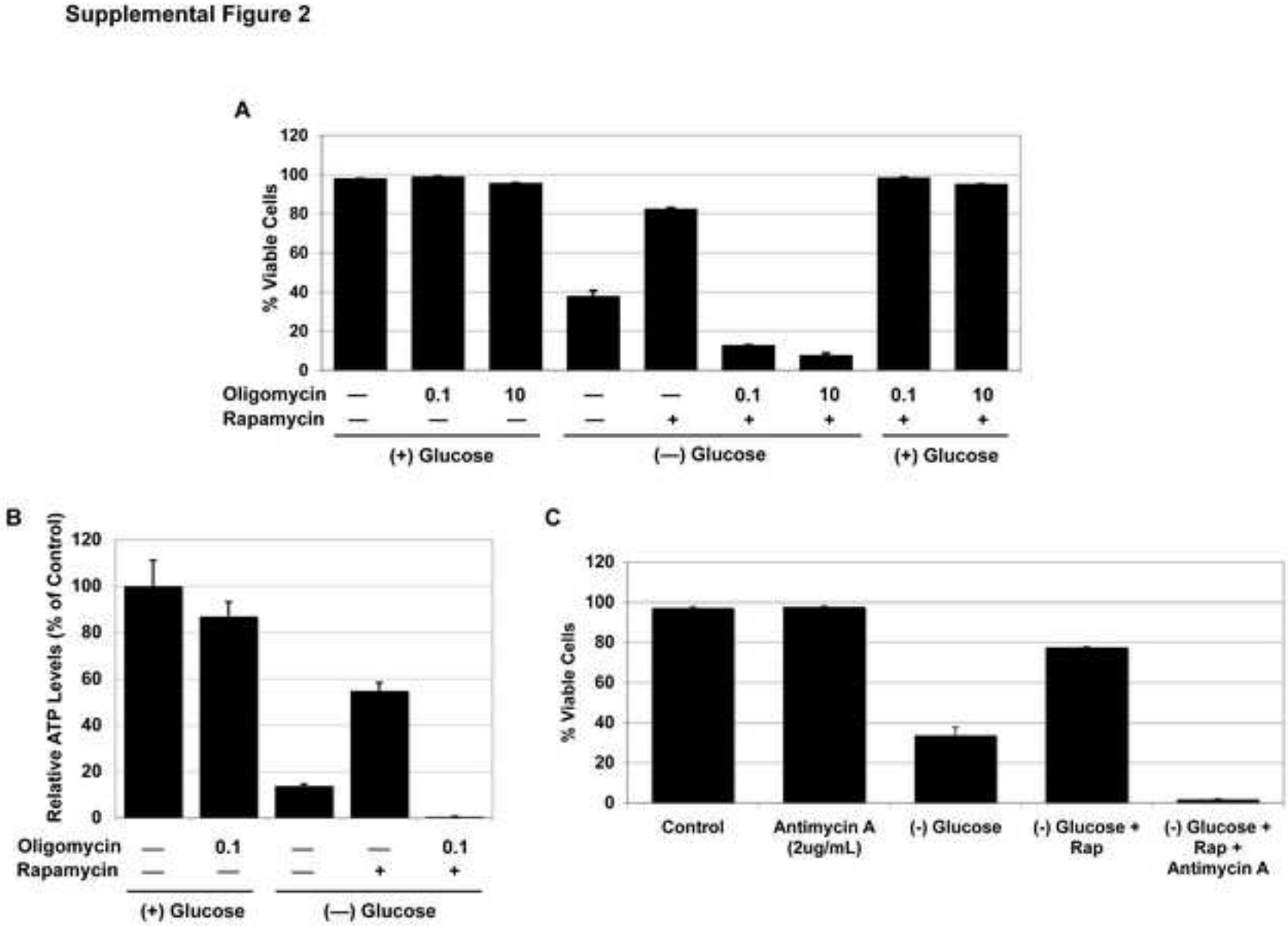
*TSC2-/-* MEFs were infected with viruses that express LC3-EGFP, and the high infection populations were sorted (Dana Farber). Rapamycin was treated and LC3 cleavage was determined by blotting for LC3-EGFP (Anti-GFP). For shBeclin experiments, shRNA was infected first and selected for puromycin survival (3μg/mL). Thereafter, these cells were infected 3 times with the LC3-EGFP virus (every 12 hours), and LC3 cleavage was determined following rapamycin treatment. Because shGFP, which was typically used as a vector control, knocks down LC3-EGFP (Data not shown), we used mock infection as a control for the shBeclin-LC3 EGFP double experiment. For 3-MA experiments, the indicated concentrations (5 and 10 mM) were dissolved directly into the

glucose free media, and viability was determined 60 hours post deprivation. The protocol for fixing and imaging is described in detail elsewhere (Yoon et al., 2008). *ATG5+/+* and *ATG5-/-* MEFs were from Dr. N. Mizushima.

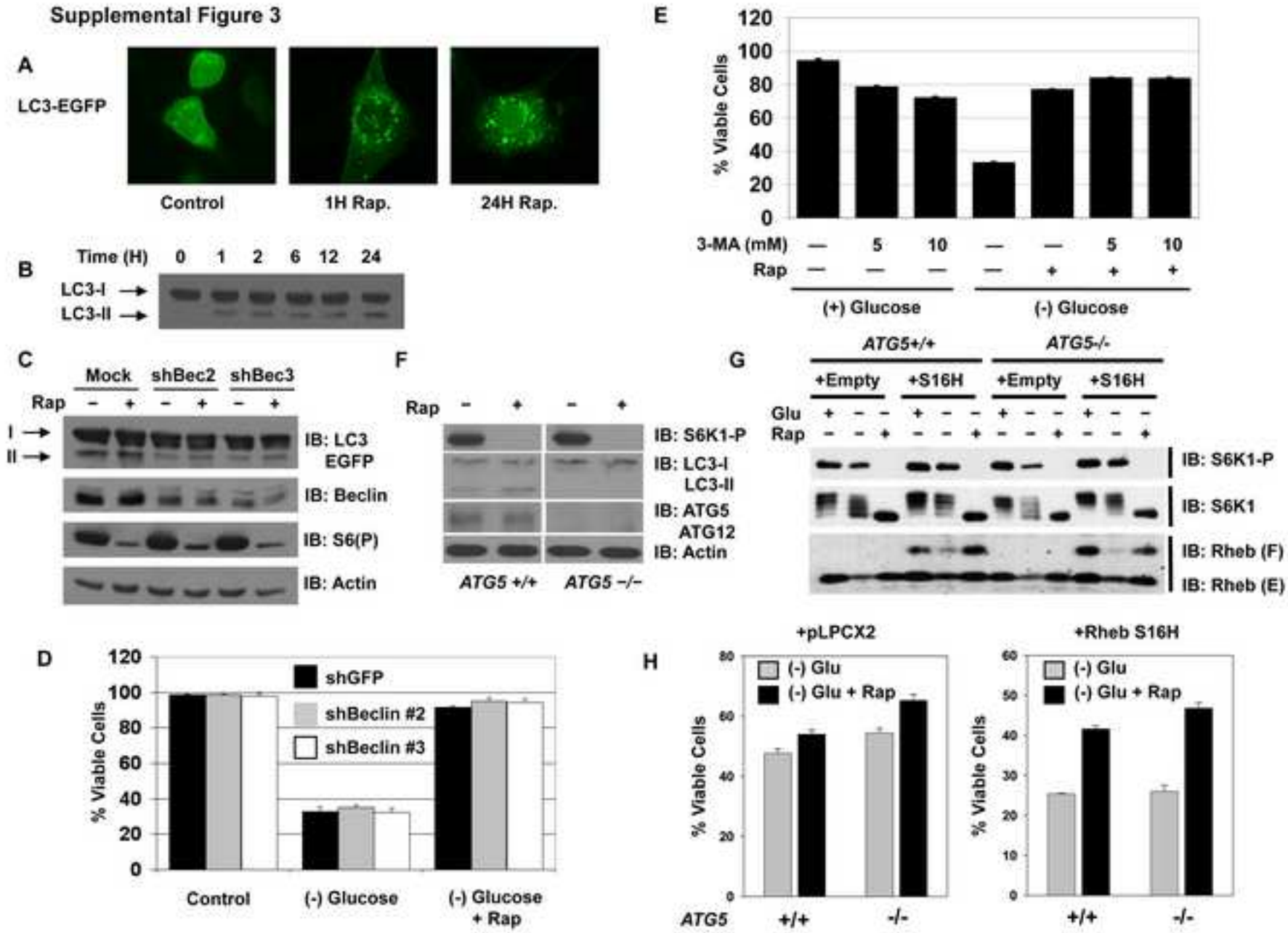
**Supplemental Figure 1**



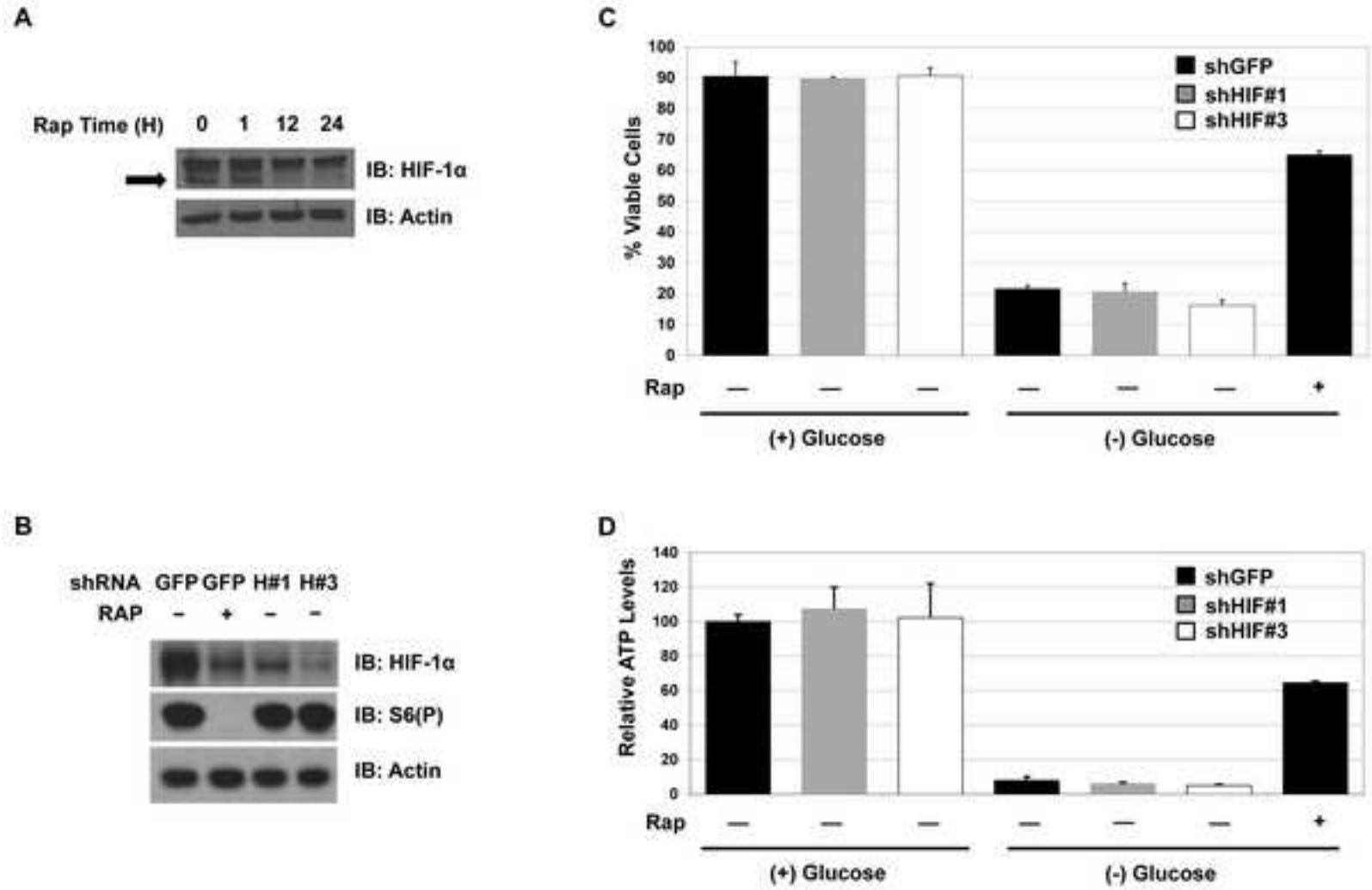
**Supplemental Figure 2**



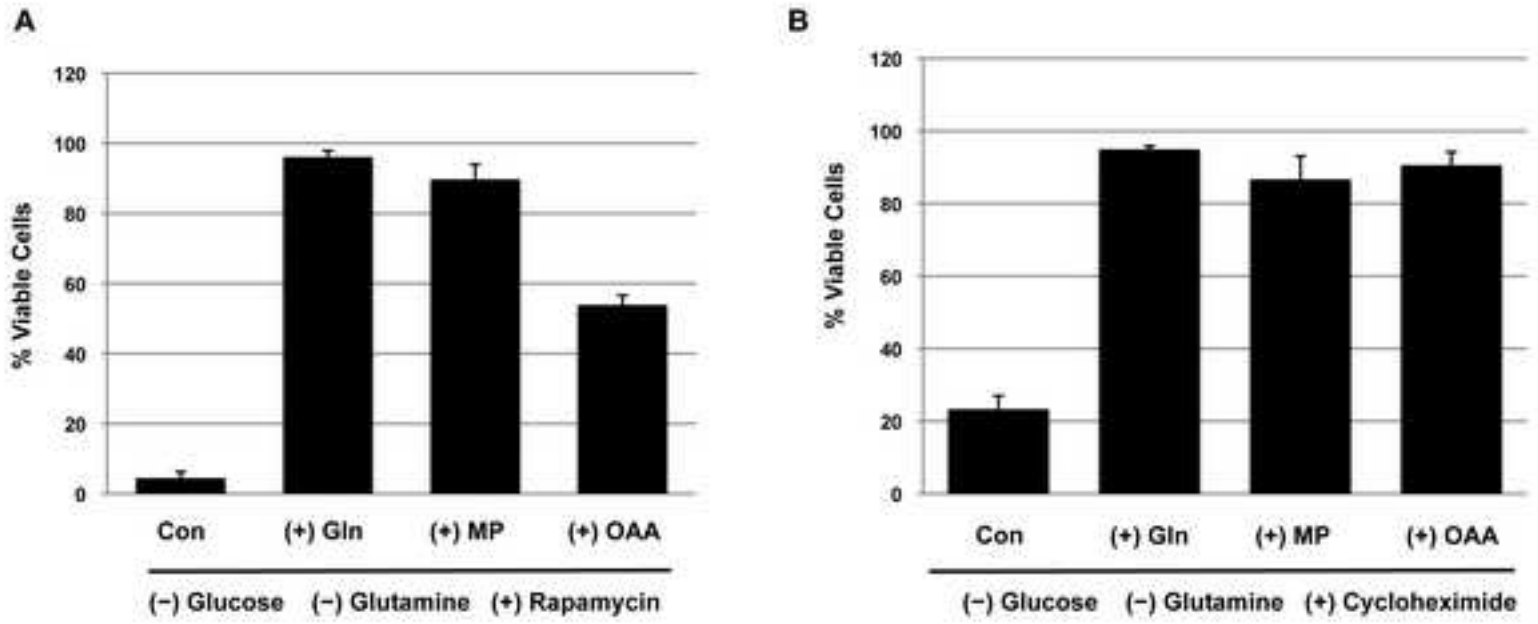
**Supplemental Figure 3**



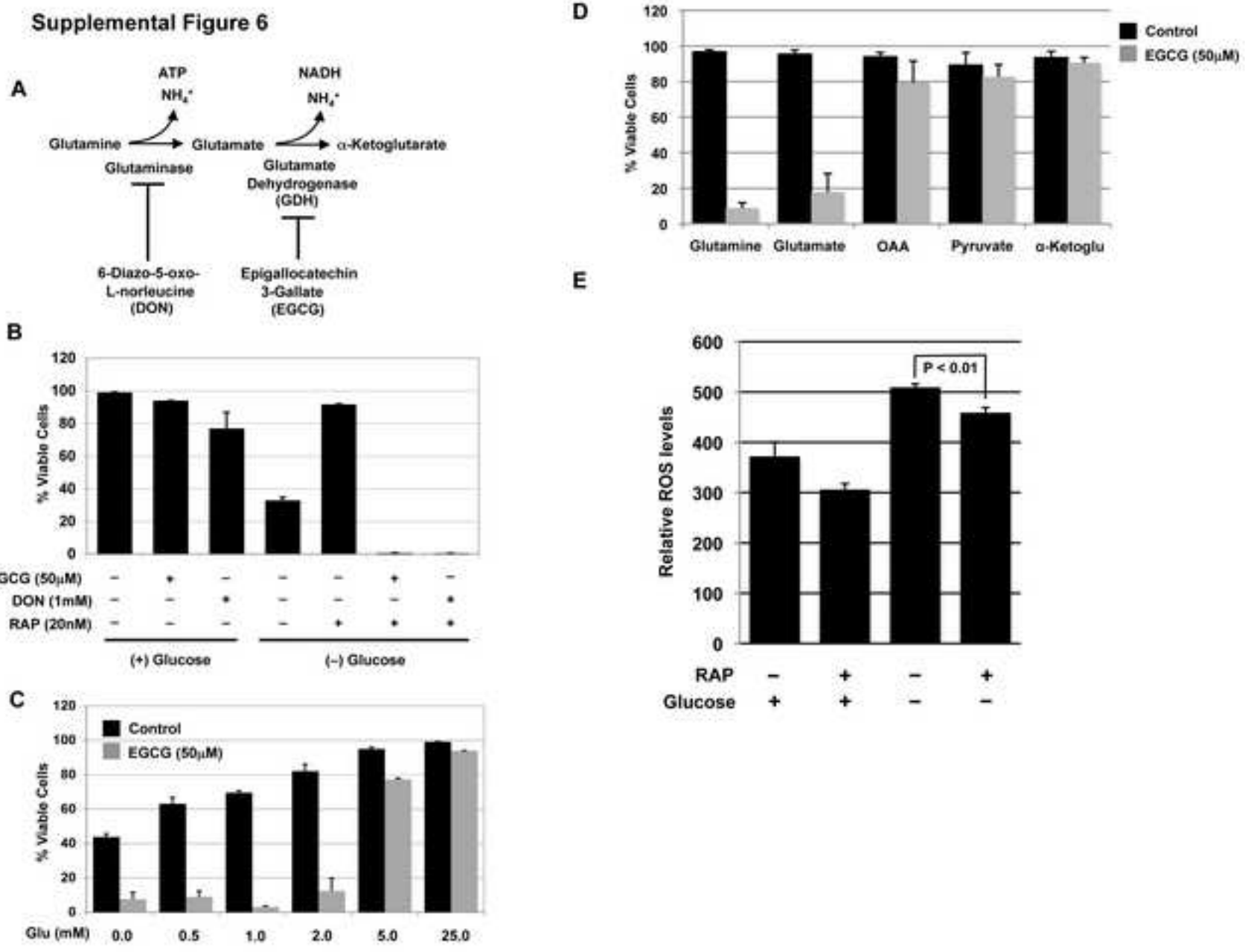
Supplemental Figure 4



Supplemental Figure 5



**Supplemental Figure 6**



**Supplemental Figure 7**