INVENTORY OF SUPPLEMENTAL INFORMATION

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse Studies

C57BL/6J and FVB mice were obtained from Jackson labs. *Tsc1*$^{fl/fl}$ and L*Tsc1*KO mice on a C57BL/6J background were described previously (Yecies et al., 2011), with L*Tsc1*KO mice generated from crosses between *Tsc1*$^{fl/fl}$ and *Alb-Cre Tsc1*$^{fl/fl}$ mice, through the albumin-Cre transgene (Postic and Magnuson, 2000). PCR genotyping for *Tsc1* and Cre alleles was performed as described (Kwiatkowski et al., 2002). Homozygous *AMPK*α*1*$^{fl/fl}$ and *AMPK*α*2*$^{fl/fl}$ mice previously described (Hasenour et al., 2014) on an FVB genetic background were bred with or without albumin-CreER^{T2} expression (Imai et al., 2000) to generate wild type controls (*AMPK*α*1*$^{fl/fl}$/α*2*$^{fl/fl}$) or inducible liver-specific deletion of both catalytic subunits of AMPK (*L-AMPK*α*1*/α*2-DKO*). The final *AMPK*α*1*$^{fl/fl}$/α*2*$^{fl/fl}$ and L-*AMPK*α*1*/α*2*-DKO mice used were generated through tamoxifen treatment every other day for a total of 3 i.p. injections (1 mg tamoxifen/mouse per injection), thereby inducing deletion of AMPK in the albumin-creER^{T2} expressing L-*AMPK*α*1*/α*2*-DKO mice only. The experiments described, or primary hepatocyte isolation, were carried out approximately 2 weeks post-tamoxifen injections. Mice were fed a normal chow diet (9% kcal from fat) prior to the experiments described. For fasting-refeeding

studies, mice were fasted overnight and either euthanized or refed normal chow for 2 h or 6 h. Vehicle (0.9% saline) or metformin (Sigma, PHR1084) reconstituted in 0.9% saline was administered via intraperitoneal injection 1 h prior to euthanization. Liver tissue was harvested immediately and flash frozen in liquid nitrogen. All animal procedures were approved by either the Harvard Medical Area's or Salk Institute's Institutional Animal Care and Use Committee.

Primary Hepatocytes

Primary mouse hepatocytes were isolated as previously described (Yecies et al., 2011) from 10- to 12-week-old mice by portal vein perfusion with Liberase TM (Roche, 5401119001) and Percoll (Sigma, P4937) gradient purification. Hepatocytes were plated at $2x10^5$ cells per well of a 12-well or $4.5x10^5$ cells per well of a 6-well plate in DMEM (Corning, 10-017CV) containing 5% FBS, and allowed to adhere for 5h on plates pre-coated with collagen (Sigma, C3867). Following adhesion, cells were washed twice in DMEM and incubated overnight in DMEM containing 5% FBS, or serum-free DMEM prior to the indicated treatments. For adenovirus experiments, cells were washed 5 h after plating, infected with virus (University of Iowa Viral Vector Core Facility) at a 10-fold multiplicity of infection and incubated overnight. Cells were then washed twice in DMEM containing 5% FBS and incubated for 24 h prior to metformin treatment for 5 h. Cryopreserved human hepatocytes were obtained from ThermoFisher (Lot # Hu8150) and cultured according to their protocol. Briefly, cells were thawed in CHRM (ThermoFisher CM7000), centrifuged at 100xg for 10 min, resuspended in Williams E medium without phenol red, containing thawing/plating supplements (ThermoFisher CM3000: 5% Fetal Bovine Serum, 1uM Dexamethasone, 1% Pen/Strep, 4ug/mL Human recombinant insulin, 2mM GlutaMAX, 15 mM HEPES, pH 7.4) and plated onto collagen-coated plates at 600K/ 6-well for protein synthesis, or 300K/well of 12-well for parallel protein lysates. 4 h later cells were washed twice to remove floating cells, and media was replaced for incubation overnight. The

following day media was refreshed 1 h prior to addition of metformin at the indicated concentrations.

Protein Extraction and Immunoblotting

Liver tissue lysates were generated from 50-100 mg pieces of frozen liver tissue dounce-homogenized in NP40 lysis buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 1 mM EDTA, 1% NP-40 [Igepal CA-630], 5 % glycerol, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate, and protease inhibitor cocktail [Sigma, P8340]). For experiments with the *AMPK$\alpha 1^{fl/fl}/\alpha 2^{fl/fl}$* and L-*AMPK$\alpha 1/\alpha 2$*-DKO mice or hepatocytes, protein extracts were prepared in a Triton X100-based lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50mM NaF, 2.5 mM sodium pyrophosphate, 2 mM b-glycero-phosphate, 1 mM sodium orthovanadate, 50 nM calyculin A, and protease inhibitor cocktail (Roche)). Primary hepatocytes or liver tissue homegenates were lysed on ice for 10 min with shaking in lysis buffer. Lysates were centrifuged at 16,000 x g for 10 min at 4°C. Protein extracts were normalized by Bradford assay (BioRad, 500-0006), separated by SDS-PAGE, and transferred onto PVDF membranes for immunoblotting (EMD Millipore, IPVH00010). Where indicated, band intensities were quantified using ImageJ (NIH).

The following primary antibodies obtained from Cell Signaling Technology, unless otherwise noted, were used for immunoblotting: phospho-AMPK-T172 (#4188, #2535), AMPK (#2603, #2532), TSC1 (#6935), S6K1 (#2708, #9202), phospho-S6K1-T389 (#9234, Millipore, 07-018), Raptor (#2280), phospho-Raptor-S792 (#2083) or -S722 (Millipore, 09-104), phospho-4EBP1-S65 (#13443, #9451), 4EBP1 (#9644, #9452), S6 (#2217), phospho-S6-235/236 (#2211, #4858), phospho-S6-S240/S244 (#2215), and β-actin (Sigma, A5316 or A5441). Horseradish

peroxidase-conjugated secondary antibodies were anti-rabbit IgG (#7074) and anti-mouse IgG (#7076).

Oxygen Consumption Rates

Oxygen consumption rates (OCR) were measured on an XF24 Seahorse extracellular flux analyzer. Primary hepatocytes were seeded in XF 24-well cell culture microplates (Seahorse Bioscience, 100867-100) at 2×10^4 cells/well, with at least four samples per condition. 5 h after plating, cells were washed twice in DMEM, and incubated in DMEM overnight. Assays were initiated the following day after washing cells twice in unbuffered Seahorse assay media (Seahorse Bioscience, 102365-100) supplemented with 25 mM glucose and 2 mM sodium pyruvate, pH 7.4, pre-warmed to 37°C. Cells were pre-equilibrated in the assay medium for 60 min at 37ºC without CO_2, while assay cartridges were prepared and calibrated. After the equilibration period, cells were subjected to three baseline measurements before injection of metformin to a final concentration of 1 mM. Oxygen consumption is reported as the average OCR at 60 minutes of metformin treatment relative to untreated on the same plate (at least 4 samples per condition), averaged from four independent experiments.

Cap Binding and Protein Synthesis

For m^7GTP affinity purification, primary hepatocytes were plated at $1.2x10^6$ cells per 6 cm dish. Cells were serum-starved overnight, pre-treated with 100 nM insulin for 20 min, followed by addition of vehicle or 1 mM metformin for 5 h. Cells were lysed on ice for 10 min in pull-down buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP40, 2 mM DTT, protease inhibitor), scraped, vortexed and centrifuged at 16,000 x g for 10 min. γ-Aminophenyl-m^7GTP agarose beads (Jena Bioscience, AC-155) were washed 3X in wash buffer (lysis buffer with 1

mM $MgCl_2$ instead of 5 mM), blocked in 1% BSA in wash buffer for 30 min, washed 2X in wash buffer, and resuspended in an equal volume. 30 µL of bead slurry was added to 1 mg of protein lysate per sample and rotated for 1h at 4ºC. Beads were washed 3X in wash buffer, resuspended in 1.5X Laemli buffer, boiled and separated by SDS-PAGE.

For measurements of relative rates of protein synthesis, primary hepatocytes were cultured in 6-well plates in serum-free DMEM for 16 h, followed by stimulation with 100 nM insulin (Alpha-Diagnostic, INSL16-N-5) for 20 min prior to treatment with or without 1 mM metformin for 5 h, with duplicate samples per condition. Cells were washed twice and changed to methionine-free DMEM (Cystine/methionine/glutamine-free DMEM [Invitrogen, 21013-024] with L-cystine and L-glutamine added back) for 30 min, then changed to the same medium containing 50 µCi/mL ^{35}S-methionine (PerkinElmer, NEG009L005MC) for 20 min. Cells were washed twice in ice cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 % sodium deoxycholate, 0.1% SDS, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM glycerol 2-phosphate, 50 mM NaF, 0.5 mM sodium orthovanadate, and protease inhibitor). Samples were normalized by protein concentration, separated by SDS-PAGE, transferred to PVDF and analyzed by autoradiogram and subsequent immunoblot. Relative rates of protein synthesis were quantified using ImageJ (NIH) analysis of the entire lane for each sample on scanned autoradiograms and graphed as percent of untreated cells, averaged from duplicate samples over 5 independent experiments.

Plasma Metformin measurement

Male FVB mice (age 14 weeks) were fasted overnight, refed for 2h with saline, 200mg/kg or 250mg/kg metformin for the last hour. Mice were anesthetized prior to blood collection. Retro-

orbital blood was collected into EDTA microvette tubes (Sarstedt 16444100), and plasma samples were obtained by centrifugation. Metformin concentrations were measured using a mass spectrometry based approach described previously (Chandel et al., 2016). 40 μL of plasma was mixed with 40 μL ACN, and 20 uL 50:50 ACN/H_2O spiked with 0.5 nmol metformin-d6 (Cayman Chemicals) as an internal standard. Samples were then vortexed for 30 s followed by centrifugation at 15000g for 10 min at 4 °C. A portion of supernatant (5 μL) was subjected to LC/MS analysis using a Thermo TSQ Quantiva instrument fitted with an Acquity UPLC BEH C18 column (1.7 μM; 2.1 mm x 100 mm; Waters). The LC conditions were the same as previously described (Chandel et al., 2016). MS analyses were performed using electrospray ionization (ESI) in positive ion mode with the following source parameters: spray voltage of 3.5 kV, ion transfer tube temperature of 325 °C, and vaporizer temperature of 275 °C . MRM [collision energy (CE) of 22 V, RF lens set at 77 V] was used to detect metformin (m/z 130.1 → 71.1) and metformin-d_6 (m/z 136.2 → 77.1).

Statistical Analyses

Data are presented as mean ± SEM and unless otherwise noted, statistical significance was determined using a Student's two-tailed t-test, with significance being considered at P-values < 0.05. For S1B significance was determined by two-way ANOVA and Tukey's post hoc test.

SUPPLEMENTAL REFERENCES

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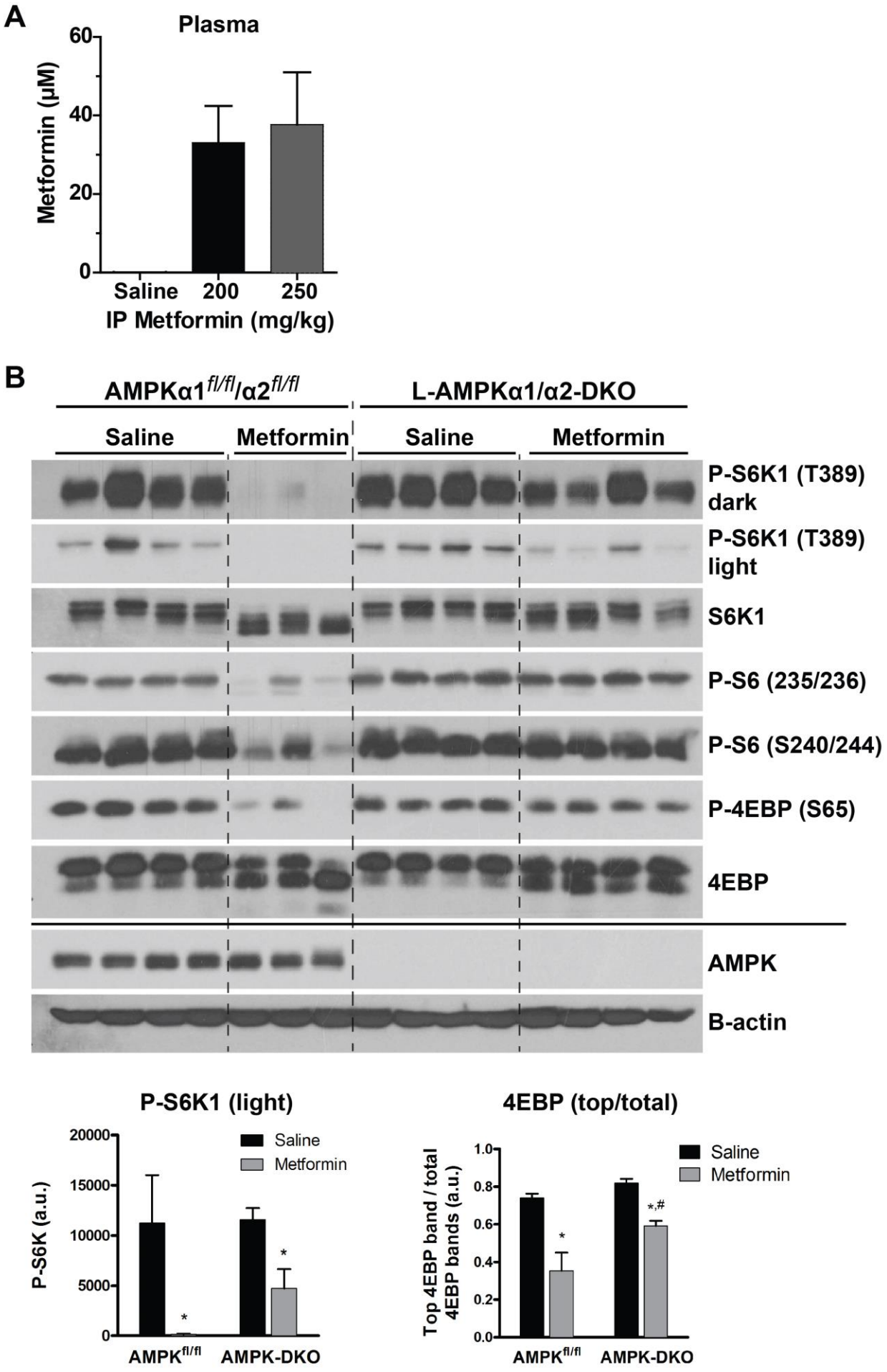
SUPPLEMENTAL FIGURE LEGEND

Supplementary Figure 1. Data in support of Figure 1.

(A) Mass spectrometric analysis of metformin concentrations in plasma from male mice (age 14 weeks) fasted overnight, then refed for 2h and treated with saline, 200mg/kg or 250mg/kg metformin via i.p. injection 1 h prior to sac. Data are mean ± SEM (n = 5 biological replicates per group).

(B) Male *AMPKα1$^{fl/fl}$/α2$^{fl/fl}$* or *L-AMPKα1/α2-DKO* mice (age 12 weeks) were fasted overnight, refed for 2 h and treated with saline or 250 mg/kg metformin for the last 1 h (n=4 per treatment). For the P-S6K1 (T389) blot, light and dark exposures are provided to better assess differences. Quantitation of P-S6K (light exposure) and phosphorylated 4EBP (top band of 4EBP/total 4EBP) is shown in graphical format below as mean +/- SEM (n=3-4). * indicates significant difference between saline and metformin treatment within the same genotype, # indicates significant difference between metformin treated *AMPKα1$^{fl/fl}$/α2$^{fl/fl}$* (AMPK$^{fl/fl}$) and metformin treated *L-AMPKα1/α2-DKO* (AMPK-DKO) as calculated by two-way ANOVA and Tukey's post hoc test.


A
Plasma
Metformin (μM)
60
40
20
0
Saline
200
250
IP Metformin (mg/kg)
B
AMPKα1fl/fl/α2fl/fl
L-AMPKα1/α2-DKO
Saline
Metformin
Saline
Metformin
P-S6K1 (T389) dark
P-S6K1 (T389) light
S6K1
P-S6 (235/236)
P-S6 (S240/244)
P-4EBP (S65)
4EBP
AMPK
B-actin
P-S6K1 (light)
P-S6K (a.u.)
20000
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Saline
Metformin
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AMPKfl/fl
AMPK-DKO
4EBP (top/total)
Top 4EBP band / total 4EBP bands (a.u.)
1.0
0.8
0.6
0.4
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Saline
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AMPKfl/fl
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