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Supporting Online Material for

Kinase LKB1 Mediates Glucose Homeostasis in Liver, and Therapeutic Effects of Metformin

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Materials & Methods

Mice colony monitoring and tissue isolation. Lkb1 $^{+/+}$, $^{L/+}$, or $^{L/L}$ mice were maintained on an FVB/N genetic background as previously described (Bardeesy et al., 2002). 8-week old male ob/ob mice were purchased from Jackson labs (Bar Harbor, ME). Experimental mice were cervically dislocated and liver and muscle were harvested immediately and either processed for histological analysis (4% paraformaldehyde) or frozen in liquid nitrogen for molecular studies. These samples were then placed frozen into Nunc tubes, pulverized in liquid nitrogen, and homogenized in lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 50 mM NaF, 5 mM β-glycero-phosphate, 50 nM calyculin A, 1 mM $Na3VO_4$, 10 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 4 μg/ml aprotinin) on ice for 30s using a tissue homogenizer. Adenovirus CMV-Cre was purchased from the University of Iowa Gene Transfer Vector Core (Iowa City, IA). In brief, 8-10 week old male $LKB1^{+/+}$ and $LKB1^{L/L}$ littermates were tail vein injected with 75ul of adenoviral-Cre (titer between 1-4 x 10^{10} pfu/ml). For blood glucose measurement, mice were fasted 18h o/n and then glucose was analyzed using a glucometer (Bayer). Insulin levels in plasma were measured using UltraSensitive Rat Insulin ELISA Kit (Crystal Chem, Inc, Downers' Grove, IL). Glucose/ insulin tolerance tests were administered as described previously (*S1*). For metformin experiments, LKB1+/+ or L/L littermates were places on high fat diet (Harlan Tecklan, Madison, WI; 55/Fat) for 8 weeks. For metformin experiments, mice were injected intraperitoneally with 250mg/kg metformin in 0.9% sterile saline or just 0.9% sterile saline similar to Zou et al, 2004 (*S2*). Mice were first fasted 18h o/n and blood glucose was measured at 12:00 PM (day0). Food was re-administered and mice were injected with metformin or saline at 10:00AM for the next two days (day1, day 2). Mice were then fasted at 6:00PM day 2 and injected with metformin at 10:00AM and blood glucose was measured at 12:00PM day 3.

Reagents and Cell Lines

Anti-phospho-AMPK (T172), total AMPK alpha, anti-ACC, anti-phospho-S6K1(T389), anti-phospho ribosomal protein S6 (S235/236), anti-ribosomal protein S6, anti-phospho-4EBP1(Ser65), anti-eIF4E antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-LKB1, anti-PGC1α, anti-TORC2 antiserum was previously described (*S3, S4*). Anti-Flag antibodies (M2 monoclonal and Flag polyclonal) were from Sigma. Metformin was obtained from Sigma. Constructs used: Flag-wild-type LKB1. Flag-kinase dead (K78I) LKB1, Flag-wild-type TORC2, FLAG-TORC2 S171A, were described previously (*S5, S6*). Adenovirus bearing shRNA for TORC2 or control scrambled sequence (US ctl) were prepared as previously described (*S2*). HeLa cells were obtained from ATCC. HeLa cells were transiently transfected using Fugene transfection reagent (Roche) according to manufacturer's suggestions.

Biochemistry

HeLa cells or tissue extracts were lysed in 20mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 50 mM NaF, 5 mM β-glycero-phosphate, 50 nM calyculin A, 1 mM Na3VO4, 10 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 4 μg/ml aprotinin as described above. After centrifugation

for 15min at 15G to remove insoluble material, lysates were equilibrated for protein levels using the BCA method (Pierce) and resolved on 6 to 12% SDS-PAGE gels, depending on the experiment. Gels were transferred to PVDF and western blotted. All primary antibodies were diluted in TBS-T/5%BSA and incubated overnight at 4°, except the TORC2 and PGC1a antibodies which were diluted in TBS-T/5%milk. Immunoprecipitations were performed as previously described (*S7*).

Immunofluorescence:

Tissues were fixed in ice-cold 4% paraformaldehyde overnight and embedded in paraffin. Slides were rehydrated by washing three times in xylene for 5 minutes, twice in 100% alcohol for ten minutes, twice in 95% alcohol for ten minutes, and twice in distilled water for five minutes. Antigen unmasking was done by placing slides into a closed chamber with 1mM EDTA, pH 8.0, and microwaving on high power until solution boils (1 minute), then incubating the chamber at 94°C for 15 minutes. Slides were then rinsed three times in distilled water for five minutes and once in TBS-T (Tris-buffered saline + 0.1% Tween) for five minutes. Sections were blocked in TBS-T + 5% normal goat serum (NGS) for one hour at room temperature and incubated overnight at 4°C in TBS-T + 5%NGS + rabbit anti-TORC2 at a dilution of 1:1600. The following day, the slides were washed three times in TBS-T for five minutes and then incubated for 45 minutes in the dark at room temperature in TBS-T + 5% NGS + goat anti-rabbit-AF594 (Molecular Probes) at a dilution of 1:200. The slides were then washed three times in the dark in TBS-T for five minutes. Slides were mounted with PBS + 50% glycerol + 0.5% n-propyl gallate and pictures were taken on a Zeiss microscope with the appropriate filter sets.

Quantitative Real Time PCR:

RNA samples were diluted to a concentration of 0.14 mg/ml and 1 μg (7 μl) of each sample was reverse transcribed with the SuperScriptIII RT kit from Invitrogen according to the manufacturer's instructions. The resulting cDNAs were diluted four-fold and 2 μl (1/40 of the total cDNA product) was used in each PCR reaction. Quantitative real time PCR was performed using Bio-Rad Sybr Green supermix and an MJ Research thermocycler driven by Opticon Monitor 2.0 software.

Fig.S1

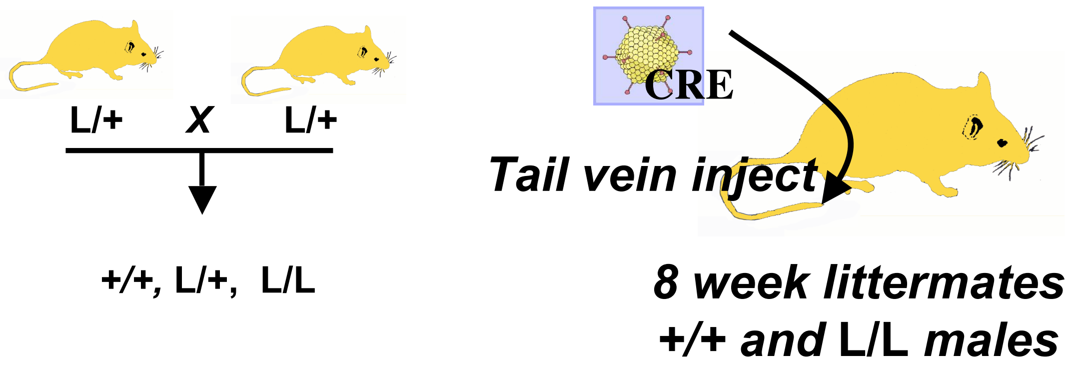


Fig.S2

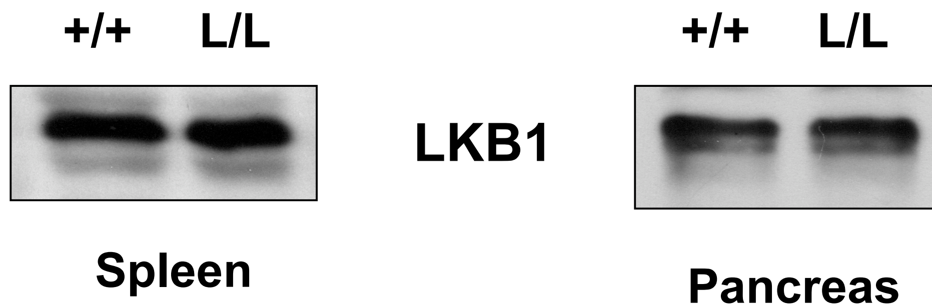


Fig.S3

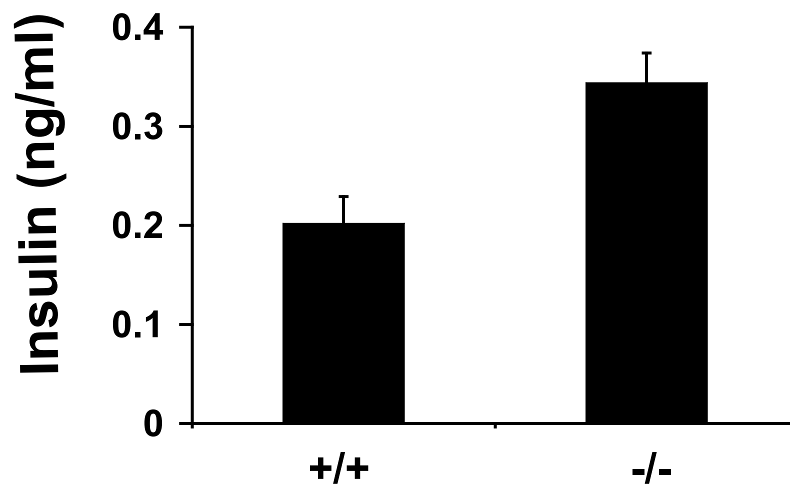


Fig. S4

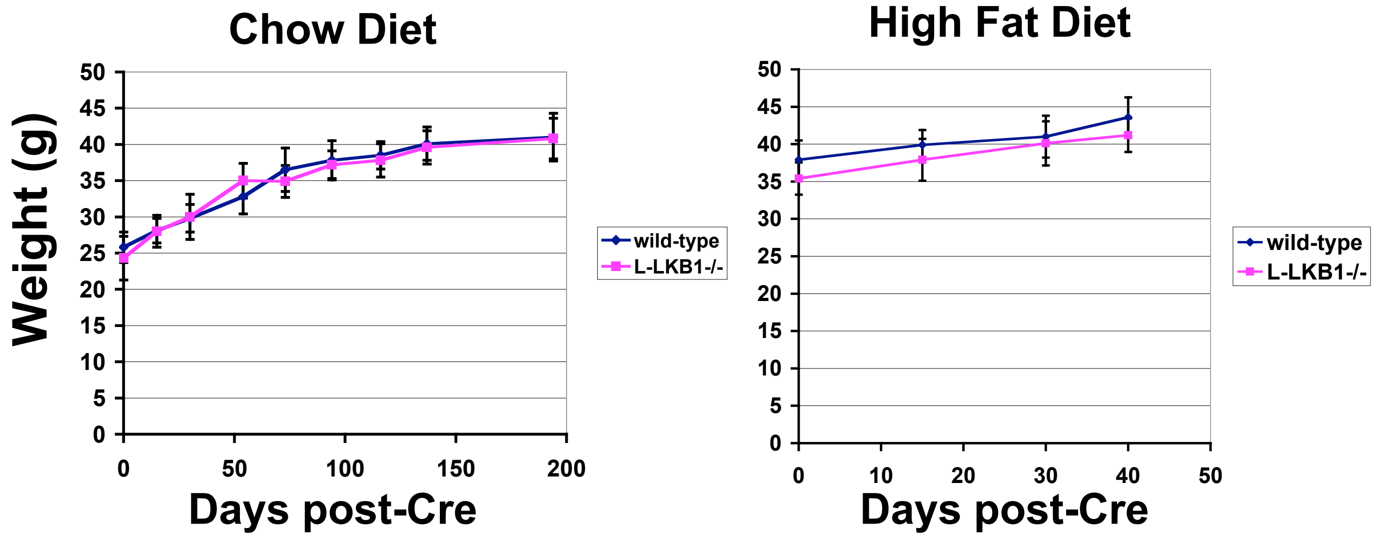


Fig. S5

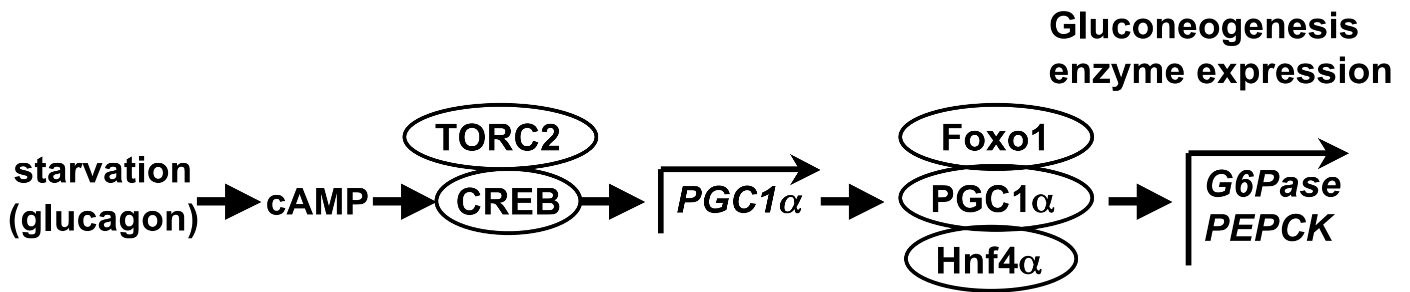
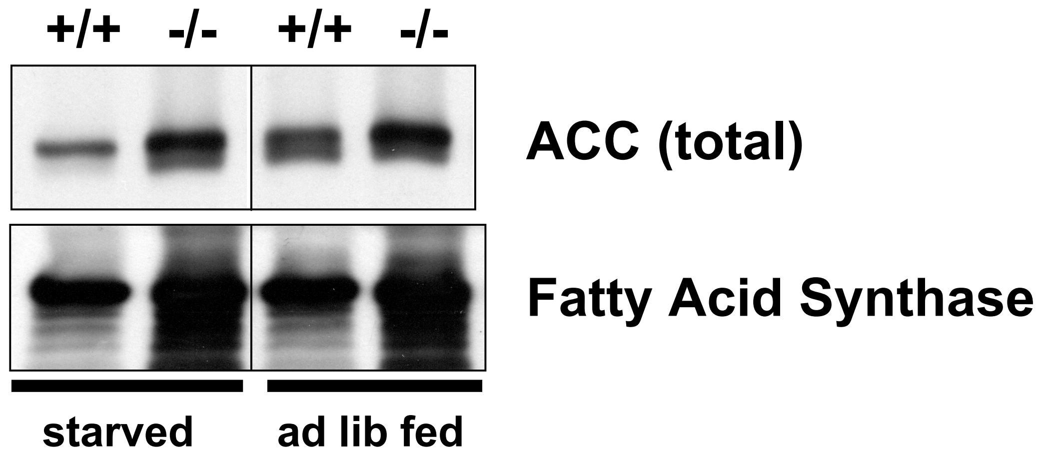


Fig. S6



Supplemental Figure Legends:

Figure S1. Strategy for deletion of LKB1 in adult liver. Male and female L/+ mice were intercrossed to generate cohorts of +/+ and L/L littermates. 8-10 week old males of each genotype were then injected with adenovirus bearing CMV-Cre which homes to hepatocytes when introduced to the bloodstream via tail vein injection.

Figure S2. Adenoviral Cre only affects LKB1 protein levels in liver. Immunoblots with anti-LKB1 antibody examining LKB1 protein levels in total cell extracts from spleen and pancreas from wild-type and L/L mice 4 weeks after adenoviral Cre injection.

Figure S3. LKB1 loss in liver leads to increased blood insulin levels. Mice of the indicated genotypes were fasted for 18h at 4 weeks after adenoviral Cre injection and then blood insulin levels were monitored using an insulin ELISA kit.

Figure S4. LKB1+/+ and L/L mice show similar patterns of weight gain. 8-week old +/+ or LKB1 L/L littermate male mice were tail-vein injected with adenoviral Cre and their weight was measured at various timepoints post-adenoviral Cre injection on either chow or high fat diets.

Figure S5. **Current model for transcriptional control of gluconeogenesis.** Fasting induces hormones such as glucagons, which in turn results in the production of cAMP in hepatocytes. The CREB transcription factor binds to co-activators such as TORC2 and becomes transcriptionally active, driving the expression of the PGC1α co-activator. PGC1α then binds to the nuclear hormone receptors HNF4α and the forkhead family member Foxo1 and drives the transcription of key gluconeogenic enzymes such as G6Pase and PEPCK. CREB and TORC2 are also known to directly contribute to the transcription of G6Pase and PEPCK.

Figure S6. Loss of LKB1 in liver leads to increased protein expression of ACC1 and FAS, two critical targets of SREBP-1. Two weeks post-adenoviral Cre injection, mice of the indicated genotypes were fasted for 18h overnight or fed ad libitium and then sacrificed. Total cell extracts were made from liver or muscle and immunoblotted for total ACC1 levels or Fatty Acid Synthase levels.

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