Cell Metabolism, *Volume 10*

Supplemental Data

LKB1 Regulates Pancreatic β Cell Size, Polarity, and Function

Zvi Granot, Avital Swisa, Judith Magenheim, Miri Stolovich-Rain, Wakako Fujimoto, Elisabetta Manduchi, Takashi Miki, Jochen K. Lennerz, Christian J. Stoeckert, Jr., Oded Meyuhas, Susumu Seino, M. Alan Permutt, Helen Piwnica-Worms, Nabeel Bardeesy, and Yuval Dor

Supplemental Experimental Procedures

Antibodies

Primary antibodies used for immunostaining were: guinea pig anti insulin (Dako, 1:200), rabbit anti Glut2 (Chemicon, 1:200), mouse anti acetylated alpha tubulin (Sigma, 1:300), mouse anti LKB1 (Santa Cruz, 1:50, requires amplification with TSA kit, NEN), rabbit anti Laminin (Sigma, 1:50), rabbit anti collagen type IV (Chemicon, 1:50), mouse anti E-Cadherin (BD, 1:50), rabbit anti phospho-AMPK alpha (Cell Signaling, 1:100, requires amplification with TSA kit, NEN), rabbit anti phospho-s6 ser235/236 (Cell Signaling, 1:50) and goat anti Pdx1 (a generous gift of Chris Wright, 1:2500). Secondary antibodies were from Jackson immunoresearch. Staining was performed on sections prepared from formalin-fixed, paraffin-embedded pancreata as described before(Nir et al., 2007). Additional antibodies used for western analysis at 1:1000 dilution were: phospho-Mark (rabbit, Cell Signaling #4836, detecting phospho-Mark1 (T215), Mark2 (T208) and Mark3 (T234) but not phospho-Mark4), phospho-ACC, β-actin, MELK, Mark4, Nuak1/Ark5 (rabbit, Cell Signaling); Brsk1, Nuak2, Mark1 (rabbit, Abgent); Mark2 (mouse, Abnova); Brsk2 (goat, Abcam); QSK (rabbit, Upstate); QIK (goat, Santa Cruz), SIK (rabbit, Santa Cruz).

Morphometric Assessment of β Cell Mass

The protocol to assess β cell mass was described in Nir et at, J Clin Invest *117*, 2553-2561 (2007). It consists of the following steps:

- **A.** Take carefully the whole pancreas and weigh
- **B.** Fix, flatten using histological cassette and sponge (thickness about 1mm), prepare paraffin blocks and cut 5 micron sections spanning the whole organ.
- **C.** Take sections in 70 micron intervals for insulin staining (DAB) and hematoxilin counterstain. A single pancreas will normally contain about 12 slides.
- **D.** Acquire 4x images spanning the whole tissue for each slide (30-70 images per slide). Images are taken using an X-Y motorized microscope, and are stitched together automatically, with manual quality control, to generate one large compound image of the whole tissue section. The resolution of compound images is sufficient to

identify single β cells.

E. Calculate for each section the fraction of tissue area covered by insulin immunostaining. This is done automatically with NIS elements, but software selections of areas defined as β cells are examined manually to remove irrelevant brown spots or to add β cells that stained weakly and escaped automatic identification. Importantly, the software calculates the area covered by brown stain with intensity above threshold, without referring to intensity of staining.

F. Calculate the average % coverage by insulin in all sections.

G. Multiply value in F by pancreas weight (in mg) to obtain total β cell mass in mg.

H. Divide value in G by body weight to obtain β cell mass/body weight.

The values in F.G.H for wild type and PLKB mice (n=3 for each genotype) are presented in figure 7sA. Note that in order to calculate β cell mass of a single mouse, we use on average 500 individual non-overlapping 4x images spanning the whole organ. As described in Nir et al 2007, examination of β cell mass in wild type littermates revealed a small inter-individual variation, which is higher in ICR mice compared with c57/bl6 mice.

Supplemental Figure Legends

Figure S1. Examples for Abnormal Localization of Cilia in β Cells of PLKB Mice

Note the homogenous distribution of cilia in wild type islets, and the localization in lateral membranes of β cells. In PLKB islets cilia are often found in clusters (evident in upper panels), and tend to be found in apical membranes, away from the center of rosettes and the clusters of nuclei (evident in bottom panels). All images were taken using a confocal microscope. Red, acetylated alpha tubulin, a cilia marker; blue, Glut2; green, DNA. Arrows point to centers of rosettes, usually containing an autofluorescent erythrocyte. Arrowheads point to cilia. Original magnifications, 600x.

Figure S2. Abnormal Cellular Localization of Glut2 in β Cells of PLKB Mice

A. Staining for Glut2.

B. Co-staining for Glut2 (green), E-Cadherin (red) and DNA (blue).

C. Higher magnification of small groups of β cells stained as in B. Arrows mark centers of rosettes. Arrowheads mark lateral membranes of β cells that have reduced Glut2 staining.

In wild type β cells, Glut2 co-localizes with E-Cadherin (overlap, yellow color). In PLKB β cells, many β cell faces have a greatly reduced Glut2 signal, compared with the intensity of E-Cadherin staining (red color, E-Cadherin+ membranes that stain weakly for Glut2). These faces are frequently lateral, consistent with a loss of Glut2-enriched lateral microvilli. Original magnification, 600x. Analysis was performed on the same mice as in figure 2.

Figure S3. Expression of Direct LKB1 Targets in Islets
Western blot (left) and bioinformatics analysis (right) of expression of all direct phosphorylation targets of LKB1. Is, wild type islets; Mi, Min6; βT, βTctet; In, Ins1; Li, Liver; Br, Brain; He, HeLa cells. P = Present; A = Absent; M = Marginal; N/A = data not available (see methods for details).

Figure S4. LKB1-Dependent Phosphorylation of AMPK in Islets In Vivo Even in the Fed State
A. Staining for p-AMPK (green), pdx1 (red) and DNA (blue). Negative control (no primary antibody) indicates that signal results from binding of secondary antibody to anti p-AMPK antibody rather than a non-specific target. As expected, fasting increases p-AMPK signal intensity. Importantly, islets from fed wild type mice have a stronger signal than islets from fed PLKB mice, indicating that AMPK has LKB1-dependent basal activity even in he absence of energy stress. Confocal immunofluorescence analysis was performed under identical conditions. Mice were 4 months old; in the case of PLKB, tamoxifen was injected at 1 month of age.
B. phosphorylation of AMPK is islets with mosaic deletion of LKB1. PLKB mice received one injection of 125 μg tamoxifen to cause low efficiency deletion of LKB1, and were sacrificed without fasting. In the two islets shown, left panels show staining for LKB1 and right panels show, in serial sections, staining for p-AMPK. Yellow arrows point same LKB1-deficient cells. Cells not expressing LKB1 have undetectable levels of p-AMPK, compared with significant phosphorylation in adjacent wild type cells. This shows AMPK is phosphorylated by LKB1 under non fasting, normoglycemic conditions. The use of mosaic analysis guarantees that wild type and LKB1-deficient cells were exposed to the same non fasting conditions and were processed identically. Staining for LKB1 and p-AMPK cannot be performed on the same section since both antibodies require amplification with TSA.

Figure S5. Increased Phosphorylation of Ribosomal Protein s6 in PLKB Mice and Elimination by Rapamycin
A. wild type mice.
B. wild type mice treated with low dose rapamycin.
C. PLKB mice.
D. PLKB mice treated with rapamycin.
E. negative control – rpS6 phosphorylation deficient knockin mutant ($rpS6^{KI/KI}$).
Blue, phospho-rpS6 (ser 135/136); green, Pdx1. The phospho-rpS6 channel is shown also in monochrome for clarity.

Figure S6. Normal Pattern of rpS6 Phosphorylation in Islets of $Par1b^{-/-}$ Mice
Phospho-rpS6 (ser135/136), Green; Pdx1, Red. Yellow arrows point to p-rpS6+ pdx1+ cells within islets.

Figure S7. β Cell Mass and Insulin Content in PLKB Pancreata

A. β cell mass assessed using morphometry as described before (Nir et al J Clin Invest *117*, 2553-2561 (2007).) and in supplemental procedures. Graphs represent the fraction of pancreas tissue area stained for insulin (left), total beta cell mass in mg, calculated by multiplying the fraction of insulin-stained tissue area by pancreas weight (middle), and β cell mass per body weight (right). N=3 mice per group. NS, non significant ($p>0.05$).

B. representative stitched images of wild type and PLKB mice, stained for insulin. Original resolution of images, before downsampling for presentation online, allows the detection of individual beta cells.

C. insulin content in pancreata from fed and fasted wild type and PLKB mice. PLKB mice do not differ in their pancreatic insulin content, either in absolute values or relative to pancreas weight, in either fed or fasted states. N=4-7 mice per group. Differences between wild type and mutants are non significant.

Figure S8. Characterization of Min6 Cells Infected with Viruses

Retrovirus (pBABE) expressing wild type or dominant negative LKB1. Expression of dominant negative LKB1 reduces significantly the phosphorylation of LKB1 targets. Cells were harvested when cultured in standard Min6 medium containing 25mM glucose and 15% serum. Note LKB1-dependent phosphorylation of AMPK under these conditions.

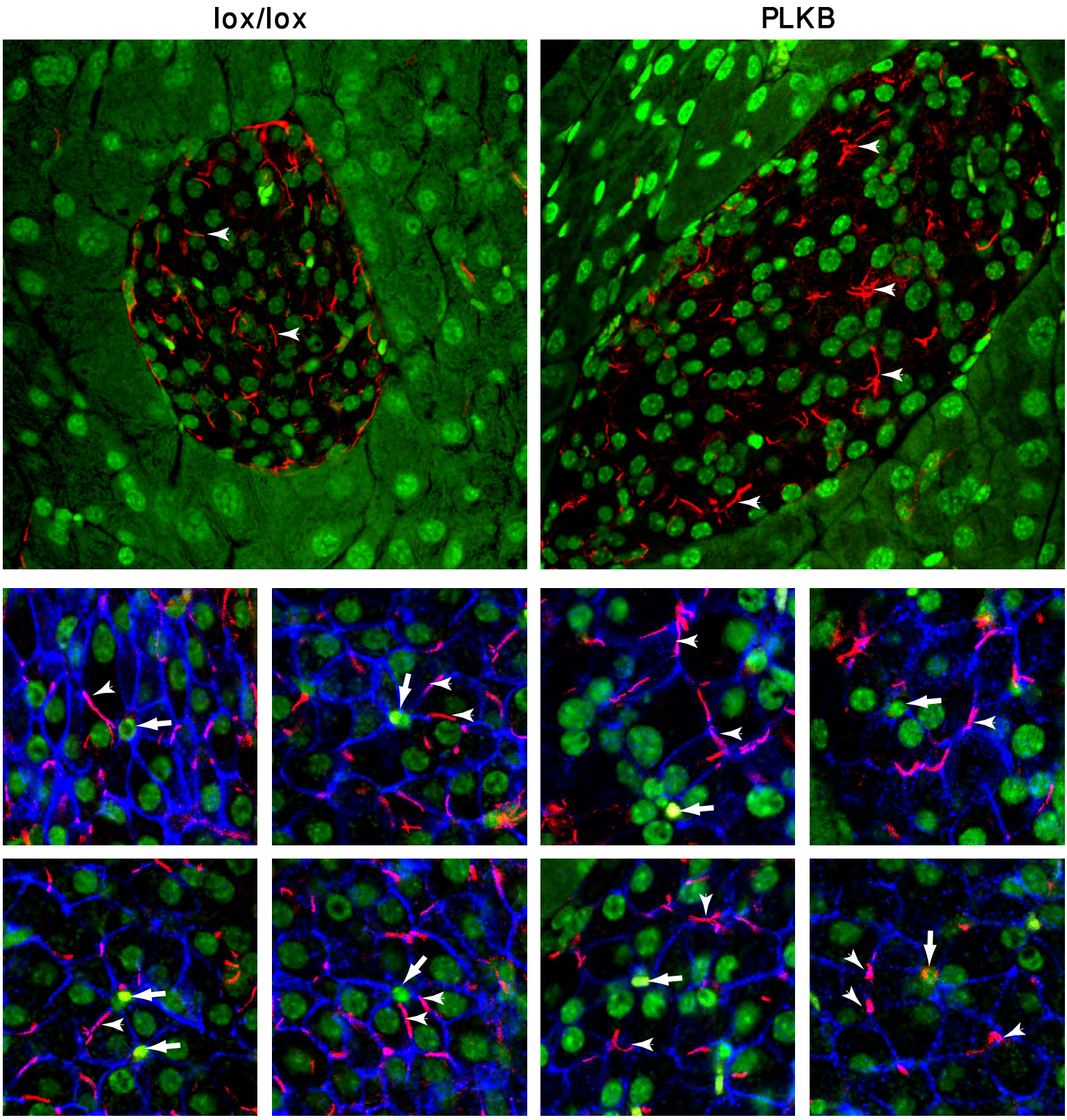


Figure S1

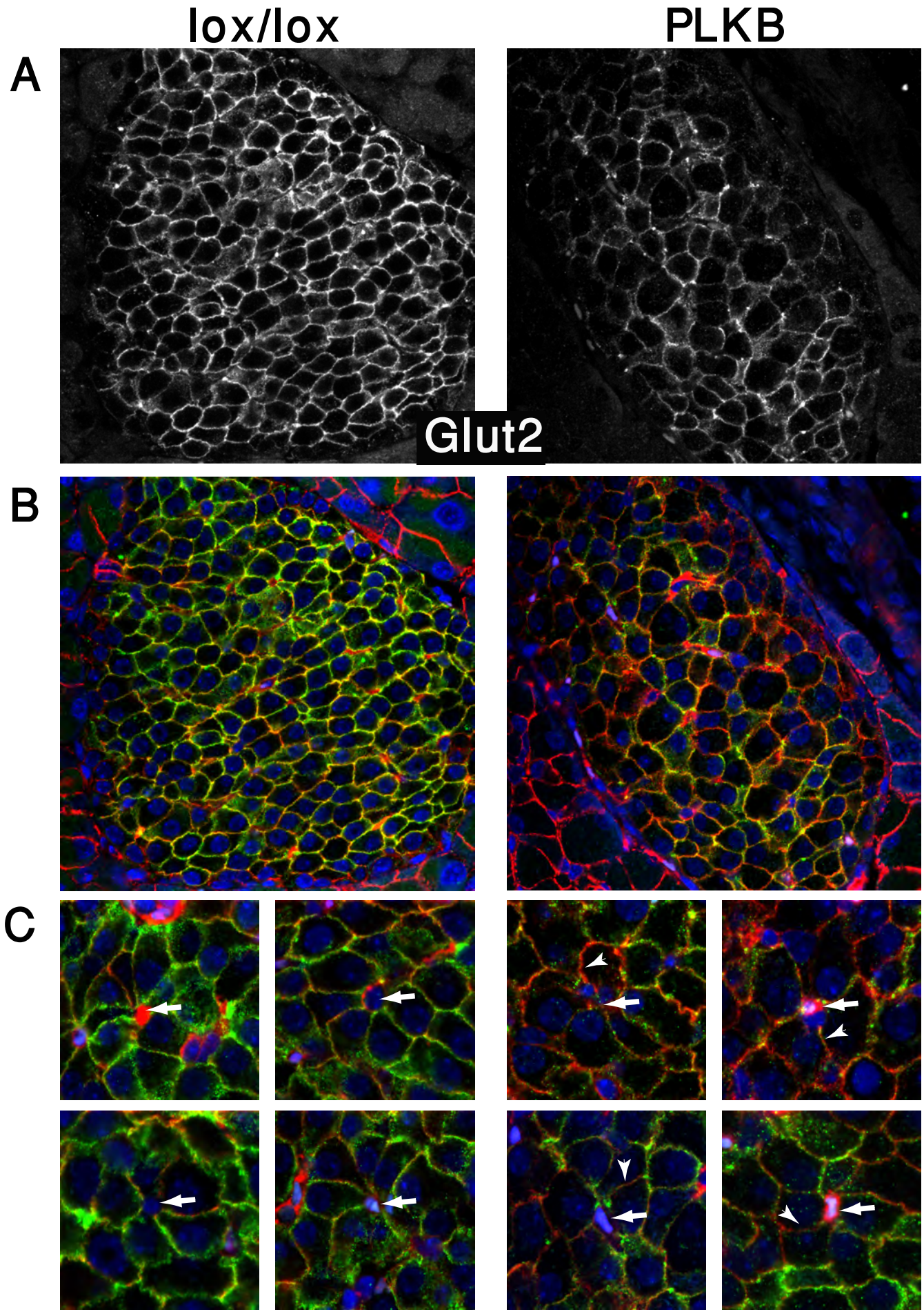
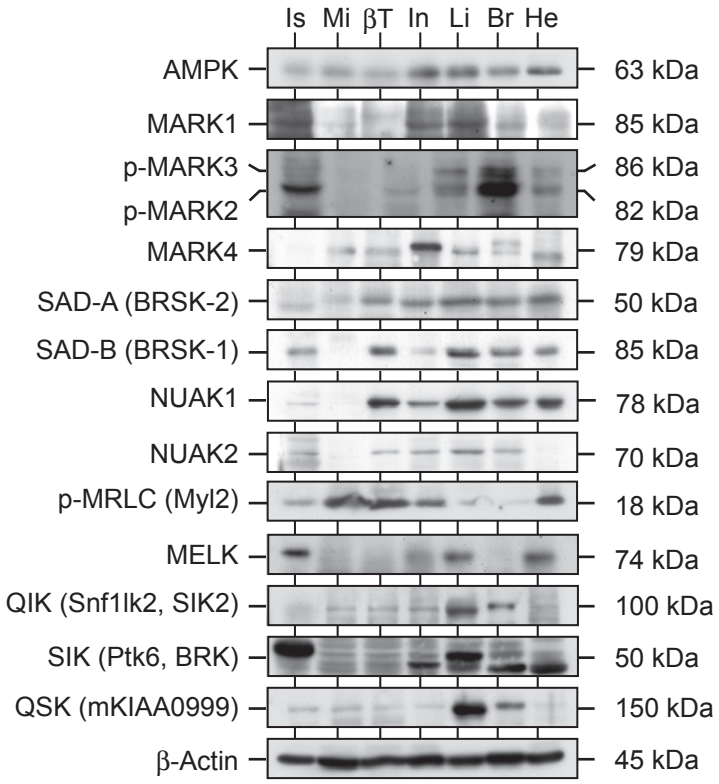


Figure S2

A.



B.

LKB1 target	Transcript
AMPK alpha 1	P
AMPK alpha 2	A
MARK1	P
MARK3	P
MARK2	P
MARK4	M
SAD-A	A
SAD-B	N/A
NUAK1	P
NUAK2	P
MRLC	M
MELK	P
QIK	P
SIK	A
QSK	P

Figure S3

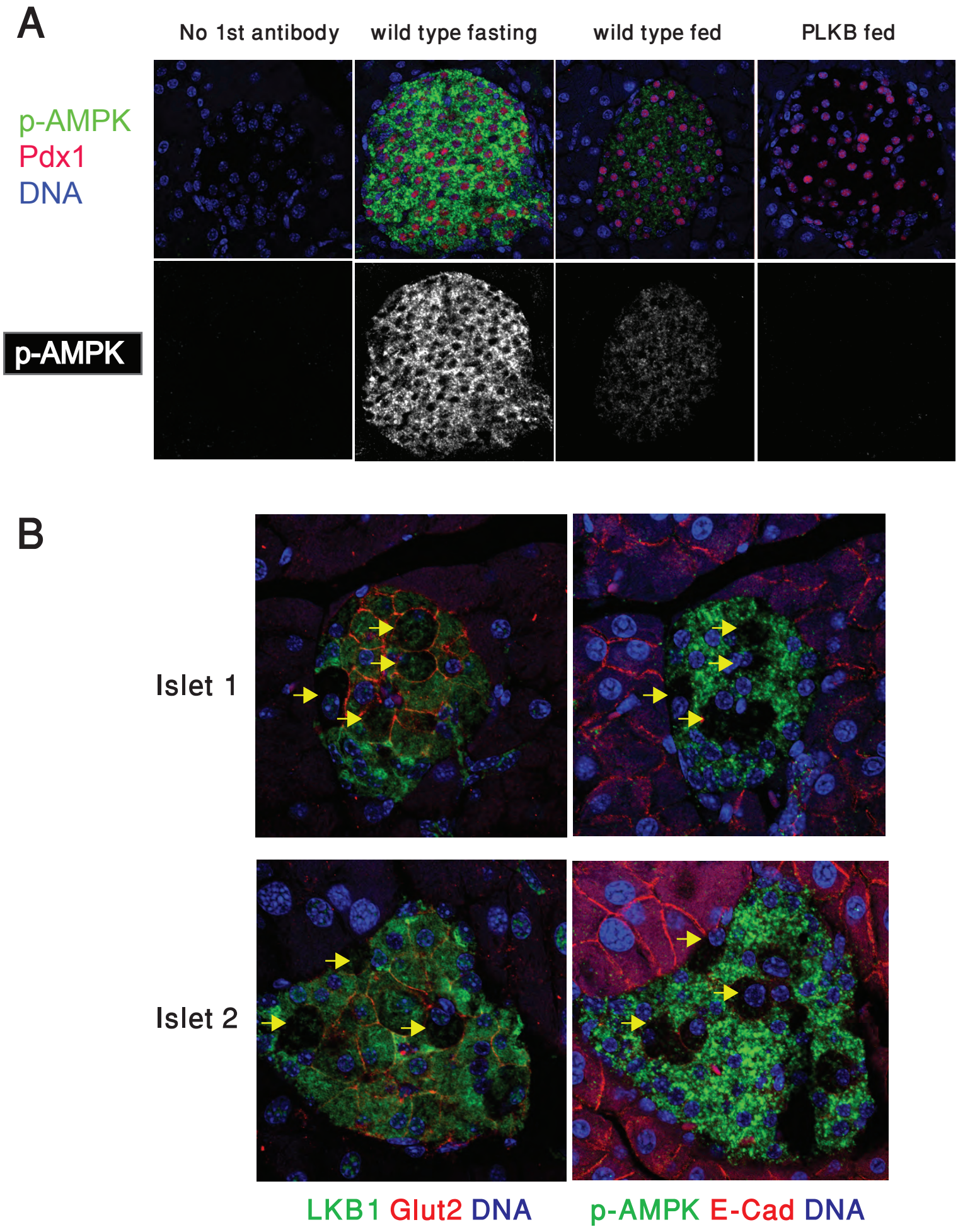

A
No 1st antibody
wild type fasting
wild type fed
PLKB fed
p-AMPK
Pdx1
DNA
p-AMPK
B
Islet 1
Islet 2
LKB1 Glut2 DNA
p-AMPK E-Cad DNA

Figure S4

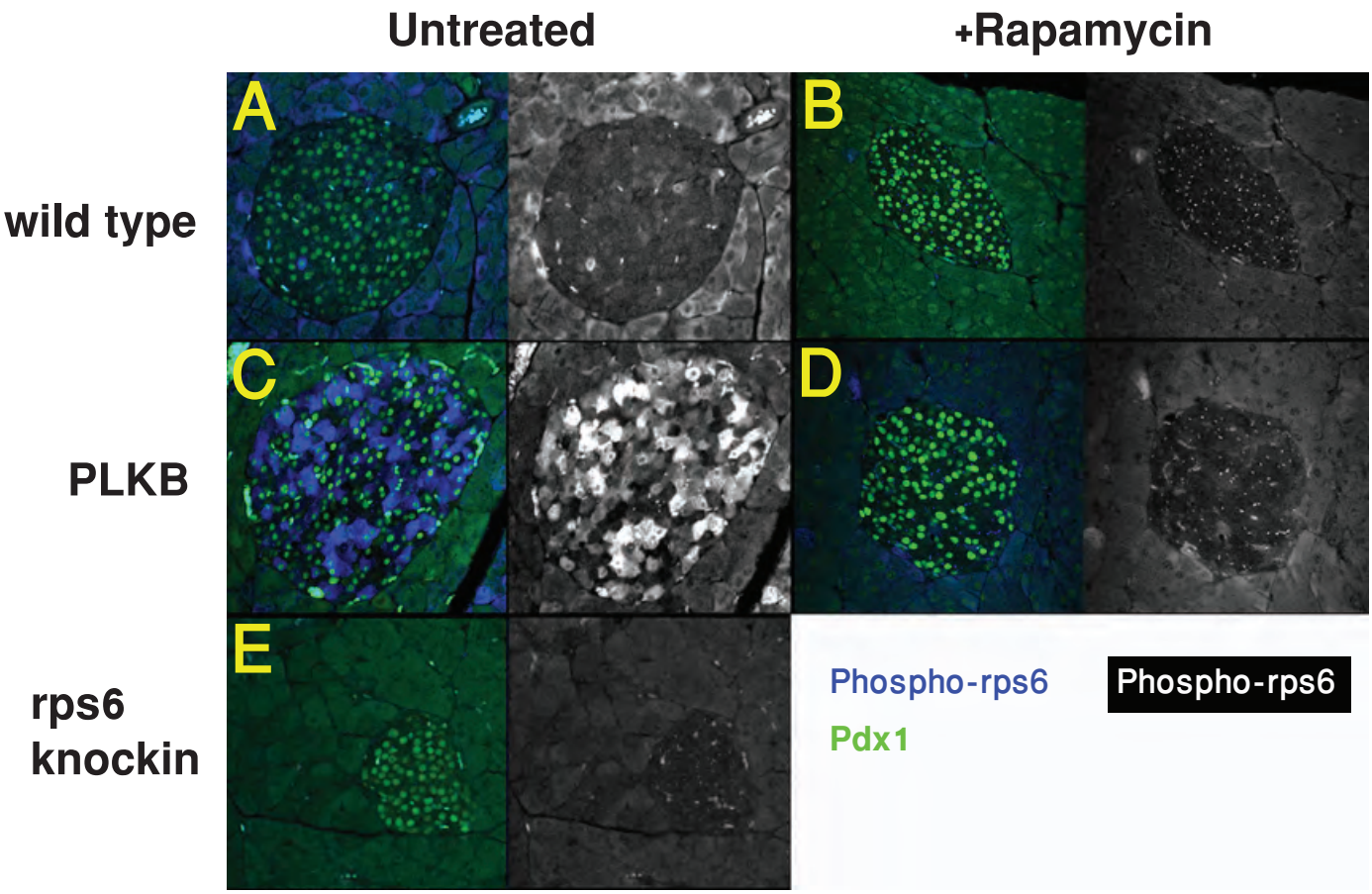

Untreated
+Rapamycin
wild type
PLKB
rps6 knockin
A
B
C
D
E
Phospho-rps6
Pdx1
Phospho-rps6

Figure S5

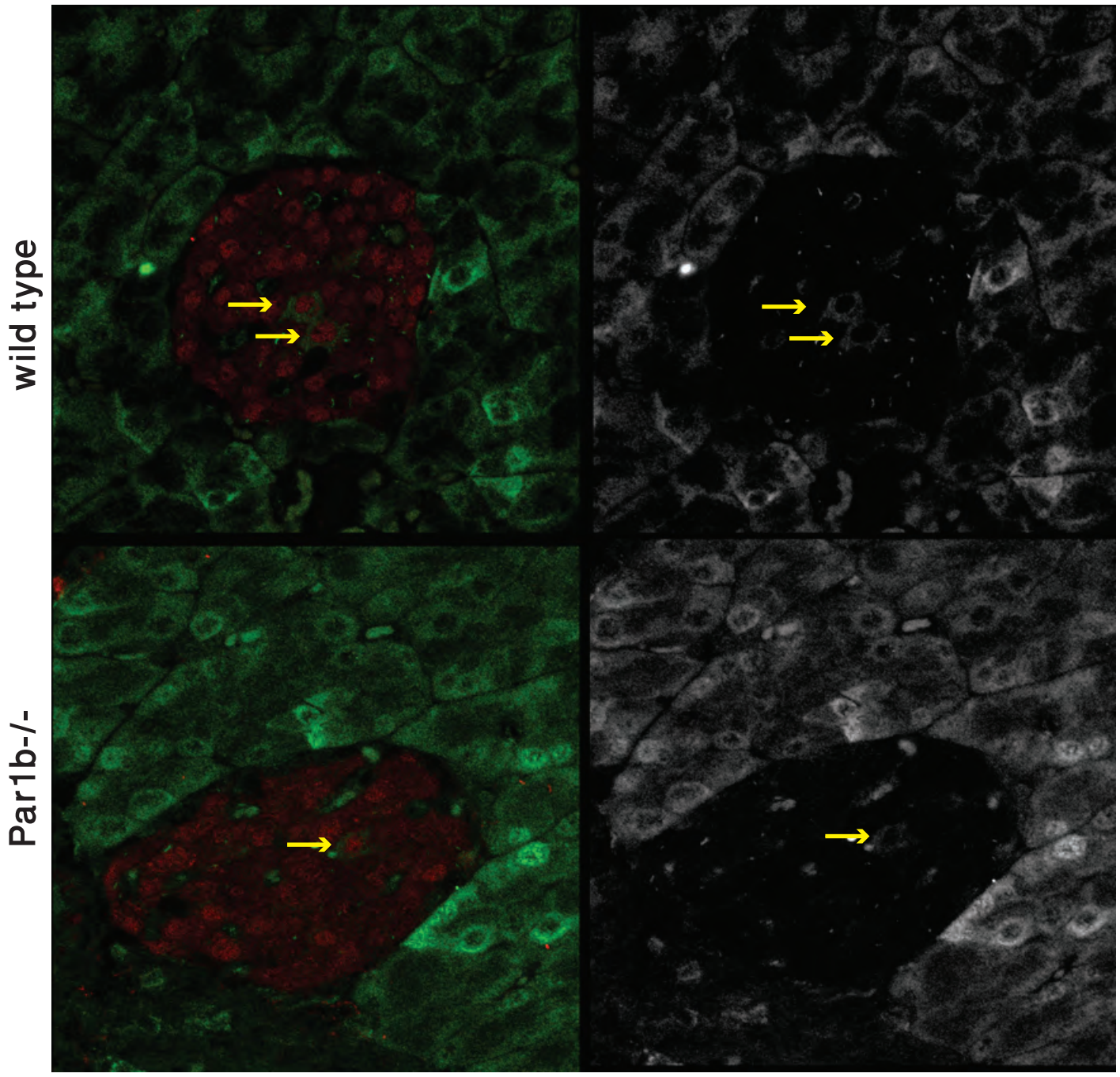
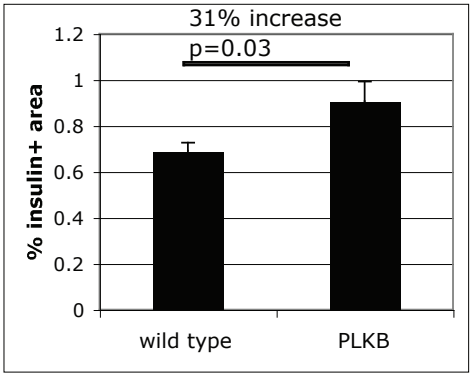
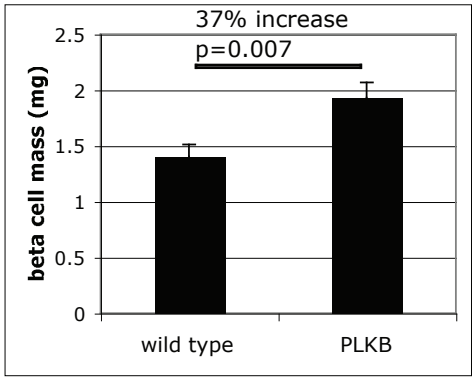
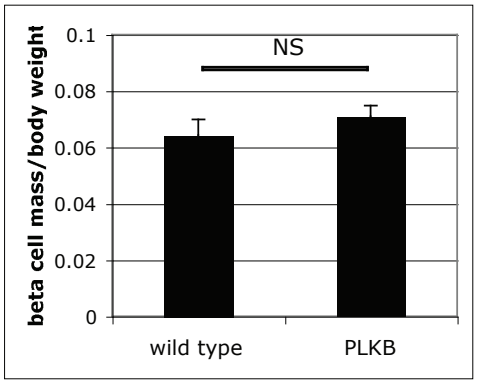


Figure S6

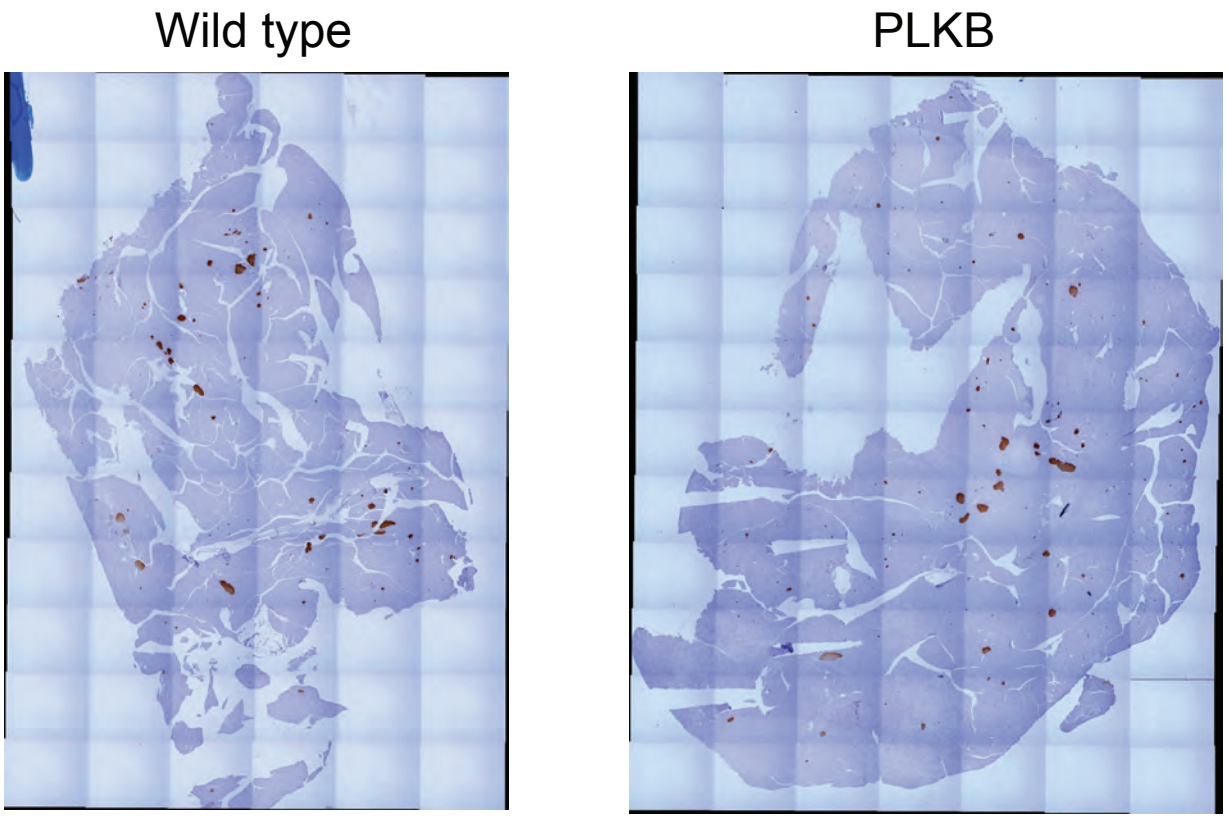
A



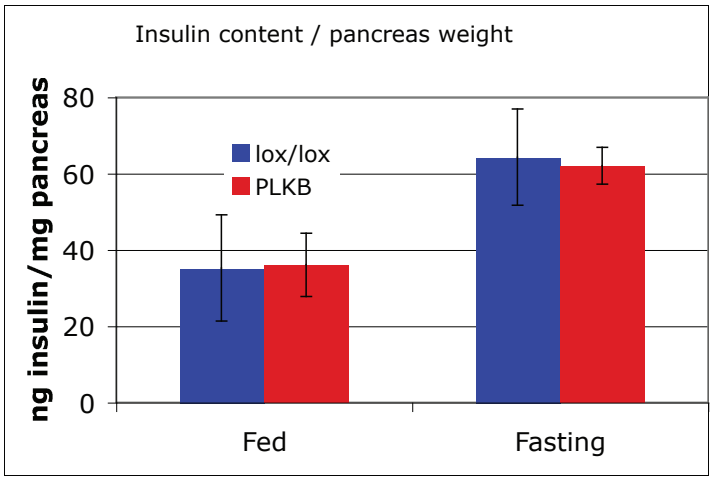




B



C



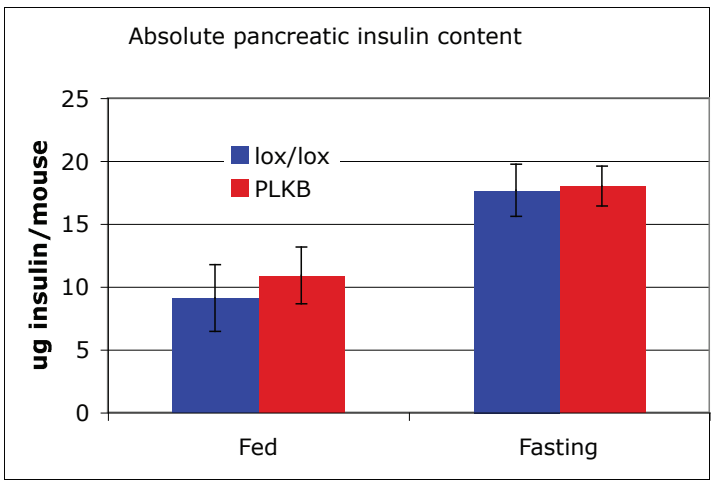


Figure S7

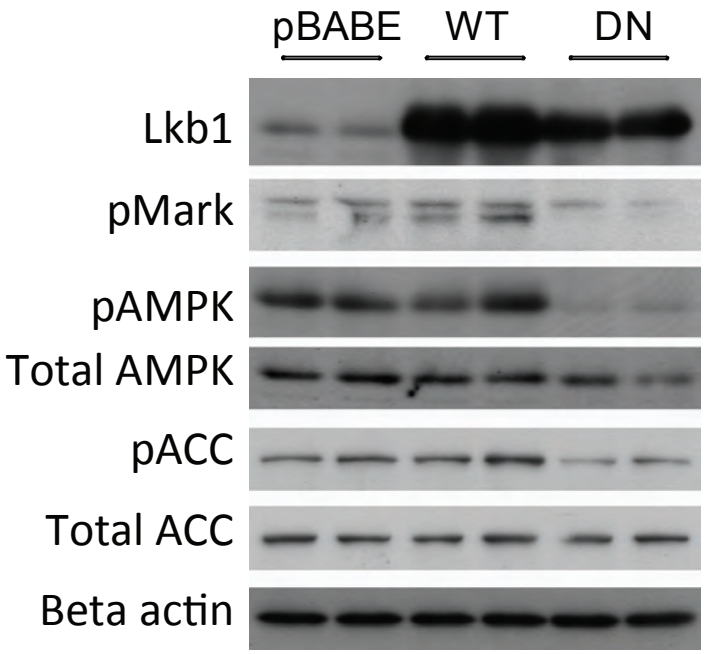

pBABE
WT
DN
Lkb1
pMark
pAMPK
Total AMPK
pACC
Total ACC
Beta actin

Figure S8