




## RESEARCH ARTICLE

**REVISED** Hepatic deletion of p110 $\alpha$  and p85 $\alpha$  results in insulin resistance despite sustained IRS1-associated phosphatidylinositol kinase activity [version 2; referees: 2 approved, 1 approved with reservations]

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**Abstract**

**Background:** Class IA phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) is an integral mediator of insulin signaling. The p110 catalytic and p85 regulatory subunits of PI3K are the products of separate genes, and while they come together to make the active heterodimer, they have opposing roles in insulin signaling and action. Deletion of hepatic p110 $\alpha$  results in an impaired insulin signal and severe insulin resistance, whereas deletion of hepatic p85 $\alpha$  results in improved insulin sensitivity due to sustained levels of phosphatidylinositol (3,4,5)-trisphosphate. Here, we created mice with combined hepatic deletion of p110 $\alpha$  and p85 $\alpha$  (L-DKO) to study the impact on insulin signaling and whole body glucose homeostasis.


**Methods:** Six-week old male flox control and L-DKO mice were studied over a period of 18 weeks, during which weight and glucose levels were monitored, and glucose tolerance tests, insulin tolerance test and pyruvate tolerance test were performed. Fasting insulin, insulin signaling mediators, PI3K activity and insulin receptor substrate (IRS)1-associated phosphatidylinositol kinase activity were examined at 10 weeks. Liver, muscle and white adipose tissue weight was recorded at 10 weeks and 25 weeks.


**Results:** The L-DKO mice showed a blunted insulin signal downstream of PI3K, developed markedly impaired glucose tolerance, hyperinsulinemia and had decreased liver and adipose tissue weights. Surprisingly, however, these mice displayed normal hepatic glucose production, normal insulin tolerance, and intact IRS1-associated phosphatidylinositol kinase activity without compensatory upregulated signaling of other classes of PI3K.

**Conclusions:** The data demonstrate an unexpectedly overall mild metabolic phenotype of the L-DKO mice, suggesting that lipid kinases other than PI3Ks might partially compensate for the loss of p110 $\alpha$ /p85 $\alpha$  by signaling through other nodes than Akt/Protein Kinase B.

**Open Peer Review**

Referee Status:   

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	1	2	3
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<b>version 2</b>	report	report	report
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**Keywords**

phosphatidylinositol-4, 5-bisphosphate 3-kinase, p110, p85, insulin receptor substrate, insulin resistance, glucose intolerance

Comments (0)

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**REVISED** Amendments from Version 1

We have made the changes required by the referees for Figure 1 and also added glucagon measurements to Figure 4. Essentially, this means that more samples have been added and western blots quantified to make the results clearer and more convincing; Dataset 1 and Dataset 4 have been updated.

**See referee reports**

## Introduction

Class IA phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) is a central mediator of a number of membrane receptor signaling pathways, including the insulin signaling pathway<sup>1,2</sup>. Following receptor activation by insulin, PI3K binds to tyrosine-phosphorylated amino acids of the insulin receptor substrates (IRS), resulting in PI3K activation and the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>). PIP<sub>3</sub> has high affinity for the pleckstrin homology (PH) domain of the downstream target Akt/Protein Kinase B (PKB). The interaction of PIP<sub>3</sub> with the PH domain enables phosphorylation of Akt/PKB by phosphoinositide dependent kinase (PDK) 1 and PDK 2, ultimately triggering a number of metabolic actions, such as lipogenesis, glycogen synthesis, inhibition of hepatic glucose output and increased glucose uptake in muscle and adipose tissue.

Class IA PI3Ks consist of two subunits. The catalytic subunit, p110, contains the kinase domain responsible for the formation of PIP<sub>3</sub>. The regulatory subunit, the most common of which is p85 $\alpha$ , binds to phosphorylated tyrosine residues in tyrosine kinases and their substrate proteins via its SH2 domain, leading to activation of PI3K activity. Both the regulatory and catalytic subunits exist as several different isoforms. In humans, there are four known catalytic subunit isoforms: p110 $\alpha$ , p110 $\beta$ , p110 $\delta$  and p37 $\delta$ . p110 $\alpha$ , p110 $\beta$  and p110 $\delta$  are encoded by three different genes, *PIK3CA*, *PIK3CB* and *PIK3CD*, respectively (reviewed in 1), whereas p37 $\delta$  (*PIK3CD\_v2*) is a splice variant of p110 $\delta$ <sup>3,4</sup>. We and others have shown that of these catalytic subunits, p110 $\alpha$  is the major contributor for transmitting the insulin signal<sup>5-7</sup>, whereas p110 $\beta$  becomes active primarily in response to G protein-coupled receptor signaling and plays a role in proliferation<sup>8,9</sup>. p110 $\delta$  is more cell specific than p110 $\alpha$  and p110 $\beta$ , and plays an important role in immune cells and the embryonic nervous system<sup>10-12</sup>.

The regulatory subunits are also encoded by three genes, *PIK3R1*, *PIK3R2* and *PIK3R3*. Their primary gene products are p85 $\alpha$ , p85 $\beta$  and p55 $\gamma$ , respectively (reviewed in 1). *PIK3R1* also encodes two splice variants of p85 $\alpha$ , p55 $\alpha$  and p50 $\alpha$ , which have more limited tissue distribution. p85 $\alpha$  is the major regulatory subunit isoform, constituting 65%–75% of the intracellular pool of regulatory subunits in most cells<sup>13</sup>. Despite the crucial role of the regulatory subunits of Class IA PI3K in mediating insulin-dependent PI3K signaling<sup>14,15</sup>, mice with a knockout (KO) of the p85 $\alpha$  regulatory subunit display increased insulin sensitivity, increased levels of PIP<sub>3</sub> lipids, elevated Akt/PKB activity and improved glucose tolerance<sup>13,16-19</sup>. The molecular

mechanisms that underlie this negative regulation by p85 appear to be complex and include unbalanced stoichiometry between subunits<sup>20,21</sup>; effects of p85 on both protecting p110 from degradation while partially inhibiting its kinase activity<sup>16,19,21,22</sup>; retention of PI3K in an inactive vesicle compartment<sup>23</sup>; links between p85 $\alpha$  and PTEN activity<sup>24</sup>; links between p85 $\alpha$  and JNK activity leading to IRS1 serine phosphorylation and inhibition of IRS1-mediated effects, and links between p85 $\alpha$  and XBP-1 in modifying the unfolded protein response<sup>25</sup>.

To dissect the intricate equilibrium between the catalytic and regulatory subunits of PI3K, as well as the opposing and complex roles of p110 $\alpha$  and p85 $\alpha$  in insulin signaling and action, in the present study, we have investigated the impact of a combined hepatic deletion of p110 $\alpha$  and p85 $\alpha$  on insulin signaling and whole body glucose homeostasis.

## Methods

### Animals

All mice in this study were on a 129Sv-C57Bl/6 mixed genetic background. To create the liver double knock-out mice, p110 $\alpha$  lox-lox mice<sup>7</sup> were crossed with p85 $\alpha$  lox-lox mice, hemizygous for the Albumin-Cre recombinase transgene<sup>14</sup>. Mice were housed on a 12-hour light cycle and fed a standard rodent chow and water *ad libitum*. All protocols for animal use and euthanasia were approved by the Gothenburg Ethical Committee on Animal Experiments, in accordance with Swedish guidelines and Directive 2010/63/EU for animal experiments, and by the Animal Care Use Committee of the Joslin Diabetes Center and Harvard Medical School in accordance with National Institutes of Health guidelines. All efforts were made to ameliorate any suffering of the mice by reducing stress, housing mice in small groups with items that stimulate their natural activity, and allowing the mice to recover 1–2 weeks after each procedure (such as measuring blood glucose, glucose tolerance test etc). During the insulin tolerance test, the mice were monitored closely to not fall too low in blood glucose levels.

For each experiment, a group of 5–12 male mice per genotype were used. The mice were studied from 6 weeks of age to 25 weeks of age. During this time weight and fasting blood glucose levels were measured every two weeks. Glucose tolerance test was performed at 8 weeks, 16 weeks and 24 weeks. Pyruvate tolerance test was performed at 15 weeks and insulin tolerance test was performed at 19 weeks.

### Metabolic and physiological procedures

Animals were fasted overnight and anesthetized with 2-2-2 tribromoethanol (Sigma-Aldrich, St Louis MO), followed by injection of 5 U of insulin (Actrapid, Novo Nordisk Inc., Plainsboro Township, NJ) or saline via the inferior vena cava. Five minutes after the injection, the liver, muscle and white adipose tissue (WAT) were excised, weighed, and snap-frozen in liquid nitrogen.

Glucose tolerance test was performed by intraperitoneal (i.p.) injection of 2 g glucose/kg BW after an overnight fast. Insulin tolerance test was performed by i.p. injection of 1.25 U insulin/kg BW. Pyruvate tolerance test was performed by i.p.

injection of 2 g of pyruvate/kg BW after an over-night fast. Insulin and glucagon was measured with ELISA (Crystal Chem Inc., Downer Grover, IL).

### RNA extraction and gene expression analysis

RNA was extracted by homogenization of liver tissue in RLT buffer (Qiagen, Valencia, CA) followed by extraction using the RNeasy kit (Qiagen, Valencia, CA). For gene analysis, cDNA was prepared using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA) with random hexamer primers. Gene expression was analyzed by real-time reverse transcription-PCR (RT-PCR) on an ABI Prism sequence detection system (Applied Biosystems, Foster City, CA). The cycling conditions used were an initial 95°C 10-minute step followed by 40 cycles of 95°C for 15s and 60°C for 60s. Samples were normalized to the 18S rRNA gene. Primer sequences are available in [Table 1](#).

### Protein extraction and analysis

Liver tissue was homogenized in lysis buffer containing 25 mM Tris-HCl, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM EGTA, 1 mM EDTA, 1% NP-40 and protease inhibitors (Sigma-Aldrich, St Louis MO), then allowed to incubate at 4°C for one hour. Extracts were centrifuged at 55,000 rpm (Beckman 70.1 Ti rotor) for one hour, and the supernatant was stored at -80°C. WAT was homogenized in lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 25 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM orthovanadate, and protease inhibitors (Sigma-Aldrich, St Louis MO) followed by incubation for 2 h at 4°C. The samples were then centrifuged at 12,000 rpm for 15 min, and the supernatant was collected and stored at -80°C. Protein analysis was made by SDS-PAGE and subsequent western blot. Briefly, protein samples were loaded onto 4–12% Bis-Tris protein gels (Thermo-Fisher Scientific, Waltham, MA) and subjected to gel electrophoresis using 25mM Tris, 192 mM glycine and 0.1% SDS as running buffer. Samples were transferred onto a

nitrocellulose membrane and the membranes were incubated in 5% skim milk solution for 1 h followed by primary antibody incubation according to the manufacturer's protocol for each antibody. Membranes were washed 2×5 min and 1×15 min in PBS with 0.1% Tween and then incubated with the secondary antibody for 1h followed by another washing procedure. Immunoprecipitation was performed using magnetic beads coated with protein G (Pierce Biotechnology Inc, Rockford, IL). All western blots and immunoprecipitation experiments were performed with a minimum of four replicates (four separate samples).

### Antibodies

IRS1 (RRID:AB\_2127860, rabbit monoclonal, 1:50, Cell Signaling Technology Inc, cat# 3407 for western blot), IRS1 (RRID:AB\_631842, rabbit polyclonal, 10µl per reaction, Santa Cruz Biotechnology Inc, cat# sc-559 for immunoprecipitation), p110α (rabbit monoclonal, 1:1000 for western blot and 1:50 for immunoprecipitation, Cell Signaling Technology Inc, cat# 4249), p110β (RRID:AB\_2165246, rabbit monoclonal, 1:500 for western blot and 1:50 for immunoprecipitation, Cell Signaling Technology Inc, cat# 3011), p110γ (RRID:AB\_10828316, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 5405), p110δ (mouse monoclonal, 1:500, Becton, Dickinson and Company, cat# 611015), p101 (RRID:AB\_10829448, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 5569), Vps34 (RRID:AB\_2299765, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 4263), p150 (rabbit polyclonal, 1:1000, Cell Signaling Technology Inc, cat# 14580), Akt/PKB (RRID:AB\_329827, rabbit polyclonal, 1:1000, Cell Signaling Technology Inc, cat# 9272), pS-Akt/PKB (RRID:AB\_329825, rabbit polyclonal, 1:1000, Cell Signaling Technology Inc, cat# 9271), pT-Akt/PKB (RRID:AB\_2255933, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 2965), pT-p70S6K (RRID:AB\_330944, rabbit polyclonal, 1:1000, Cell Signaling

**Table 1. Primer used for gene expression analysis.**

Gene	Type	Sequence/Assay ID#	
<i>Pik3ca</i>	Gene expression assay	Mm00435673_m1	Thermo Fisher Scientific
<i>Pik3cb</i>	Gene expression assay	Mm00659576_m1	Thermo Fisher Scientific
<i>Pik3r1</i>	Gene expression assay	Mm01282781_m1	Thermo Fisher Scientific
<i>Pik3r2</i>	Gene expression assay	Mm00435694_m1	Thermo Fisher Scientific
<i>G6pc</i>	Forward Primer	5'-ACTGTGGGCATCAATCTCCT	
<i>G6pc</i>	Reverse Primer	5'-ACAGGTGACAGGGAAGTCTGCT	
<i>G6pc</i>	Probe	5'-[6FAM]TGGGTGGCAGTGGTCGG	
<i>Pck1</i>	Forward Primer	5'-TGGATGTCGGAAGAGGACTT	
<i>Pck1</i>	Reverse Primer	5'-AGTGGCCCCATGCTGAAT	
<i>Pck1</i>	Probe	5'-[6FAM]GTGCATGAAAGGCCGCA	
<i>Fbp1</i>	Forward Primer	5'-AGCTGCTGAATCCGCTCTG	
<i>Fbp1</i>	Reverse Primer	5'-TTGATCACCAGGTCATTGGA	
<i>Fbp1</i>	Probe	5'-[6FAM]GCGGGCATCGCACAGCT	
<i>18S</i>	Gene expression assay	4310893E	Thermo Fisher Scientific

Technology Inc, cat# 9205), p70S6K (rabbit polyclonal, 1:500, Cell Signaling Technology Inc, cat# 9202), p85 $\alpha$  (RRID: AB\_2268174, rabbit monoclonal, 1:1000, Abcam, cat# 22653), p85-pan (RRID: AB\_10831521, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 4257), pT202/Y204-ERK (RRID: AB\_2315112, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 4370), ERK (RRID: AB\_390779, rabbit monoclonal, 1:1000, Cell Signaling Technology Inc, cat# 4695), p55 $\gamma$  (mouse monoclonal, 1:2000, Abcam, cat# ab186612), PIK3C2 $\alpha$  (rabbit polyclonal, 1:1000, MyBiosource, cat# MBS9202698), PIK3C2 $\gamma$  (rabbit polyclonal, 1:1000, MyBiosource, cat# MBS820611). Rabbit secondary antibody (RRID: AB\_772206, HRP-linked from donkey, 1:1000, GE Healthcare Life Sciences, cat# NA934), mouse secondary antibody (RRID: AB\_772210, HRP-linked from sheep, 1:1000, GE Healthcare Life Sciences, cat# NA931).

### **In vitro kinase assays**

Immunoprecipitates, using protein G Dynabeads (Life Technologies), of liver protein lysates were prepared with p110 $\alpha$  and p110 $\beta$  antibodies or IRS1 antibody. The PI3K assay was performed as previously described<sup>13</sup>. Briefly, immunoprecipitates were incubated with 5  $\mu$ g PI substrate (phosphatidylinositol from bovine liver), 20 mM MgCl<sub>2</sub>, 8  $\mu$ M cold ATP and 0.5  $\mu$ l radio-labeled [ $\gamma$ -<sup>32</sup>P]-ATP (1.11\*10<sup>14</sup> bq/mmol) in PI3K reaction buffer (20 mM Tris-HCl, 100 mM NaCl and 0.5 mM EGTA) for 25 min at room temperature. The resulting radioactively labeled PIP was analyzed with thin layer chromatography and phosphorimaging (FLA-3000, Fujifilm). Prior to these experiments, titration experiments of bead concentration and PI substrate concentration were performed to ensure precipitation of equal amounts of protein, as well as optimal PI concentration to obtain maximal enzyme activity.

### **Statistics**

All data are presented as mean  $\pm$  standard error of the mean (SEM). Student's t-test was used for statistical analysis between two unpaired groups. A p-value of <0.05 was considered statistically significant. The statistical software used was GraphPad Prism 7.00.

## **Results**

### **Deletion of hepatic p110 $\alpha$ and p85 $\alpha$ results in impaired insulin signaling downstream of PI3K**

Mice with a liver-specific deletion of p110 $\alpha$  and p85 $\alpha$ , termed hereafter liver double knockout (L-DKO) mice, were created by breeding mice carrying homozygous floxed *Pik3ca* and *Pik3r1* alleles<sup>14,26</sup> with transgenic mice carrying the Cre recombinase driven by the albumin promoter (albumin-Cre). Deletion of *Pik3ca* and *Pik3r1* in the liver resulted in markedly reduced gene and protein expression of p110 $\alpha$  and p85 $\alpha$  (Figures 1A, 1C, 1E), as well as impaired activation of the downstream targets Akt/PKB, with decreased phosphorylation of serine 473 and threonine 308, and p70S6 kinase (Figure 1F-I). p110 $\beta$  gene expression was not affected by the deletion of p110 $\alpha$  and p85 $\alpha$  (Figure 1B). p85 $\beta$  gene expression was slightly, but significantly, decreased in the L-DKO livers (Figure 1D). As expected, in the floxed control mice, there was an increase in the amount of p110 $\alpha$  associated with IRS1 in response to insulin compared to basal conditions, whereas no p110 $\alpha$  was associated with IRS1 in

the L-DKO mice (Figure 1K and 1L). MAPK signaling, as shown by ERK phosphorylation, was unchanged in the L-DKO mice compared to controls (Figure 1F and 1J).

Previous studies have shown that the interaction between the regulatory and catalytic subunits of PI3K to form dimers has a mutual stabilizing effect on both subunits, whereas the monomeric forms are more readily subjected to degradation<sup>19,21,22</sup>. We hypothesized that more p85 $\beta$  would bind to IRS1 when p85 $\alpha$  was absent, thereby maintaining p110 $\beta$  stabilization. However, only very low levels of p85 $\beta$  protein were detected in the liver of the L-DKO mice, as shown both in assessment of total p85 protein (Figure 1E) and in p85 immunoprecipitates with IRS1 (Figure 1K and 1O), similar to what we have reported earlier in liver-specific p85 $\alpha$  knock-out mice<sup>14</sup>. The expression of p55 $\gamma$  regulatory isoform protein was not affected by deletion of p110 $\alpha$  and p85 $\alpha$  (Figure 1E), nor was the amount bound to IRS1 (Figure 1K and 1P). In contrast, total p110 $\beta$  protein expression was decreased in the L-DKO mice compared to controls (Figure 1E), likely due to destabilization of this catalytic isoform in the absence of p85 $\alpha$ , supporting an insufficiency for p85 $\beta$  to compensate for the loss of p85 $\alpha$ . Interestingly, despite overall decreased protein expression of p110 $\beta$ , similar amounts of p110 $\beta$  were associated with IRS1 in controls and L-DKO mice (Figure 1K and 1M). The third catalytic isoform of class IA PI3Ks, p110 $\delta$ , has been shown to have a major role in immune cells and the embryonic nervous system, but not in other tissues<sup>10-12</sup>. Consistent with this, we found only very small amounts of full length p110 $\delta$  in whole liver lysates or bound to IRS1 (Figure 1K). However, the antibody picked up a band of about 70 kDa in whole lysates and in IRS1 immunoprecipitates (Figure 1K). The amount of this protein did not appear to be consistently different between controls and L-DKO mice or between basal and insulin-stimulated samples, so is likely non-specific.

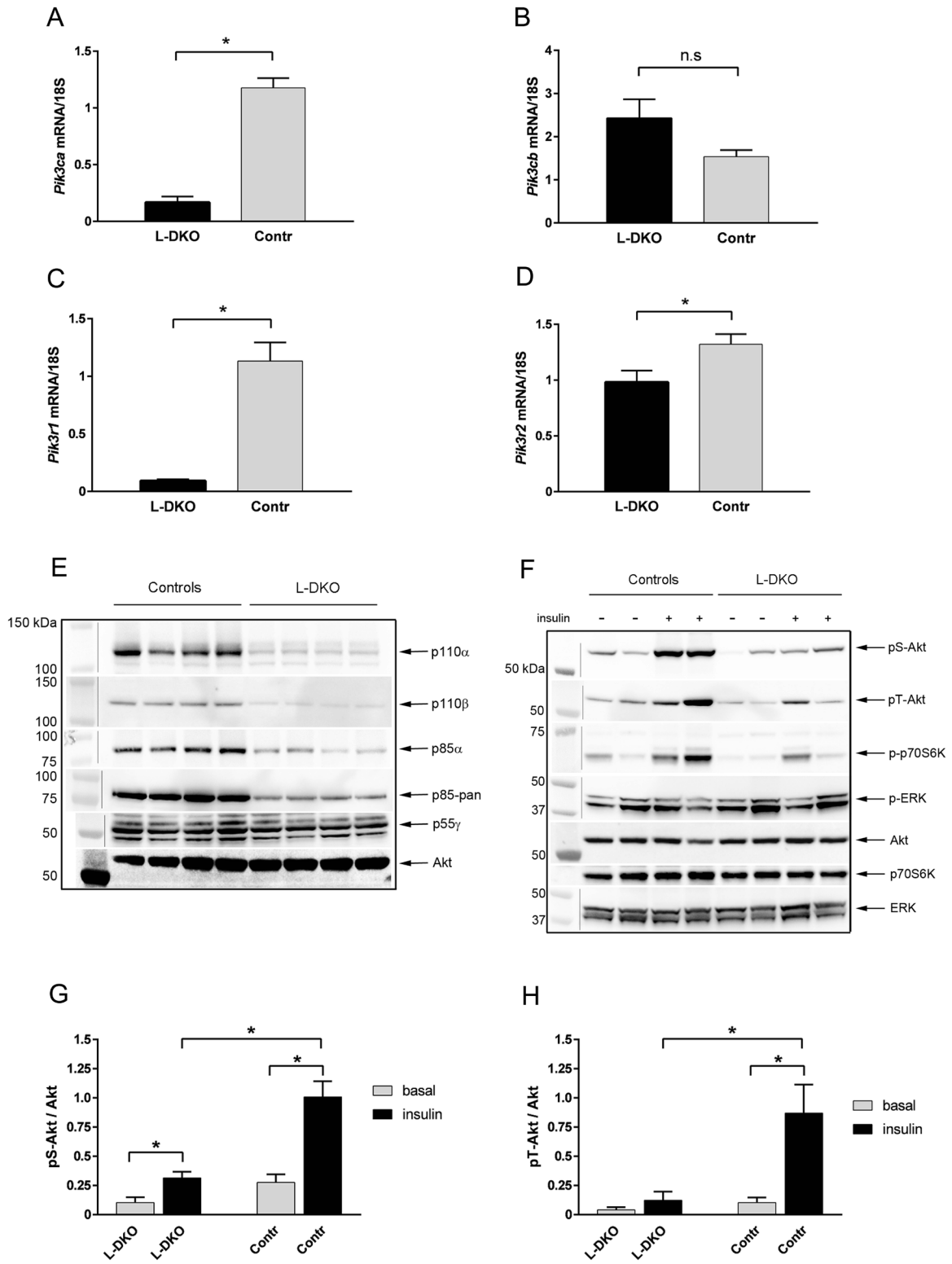
### **IRS1-associated phosphatidylinositol kinase activity is intact in the L-DKO mice**

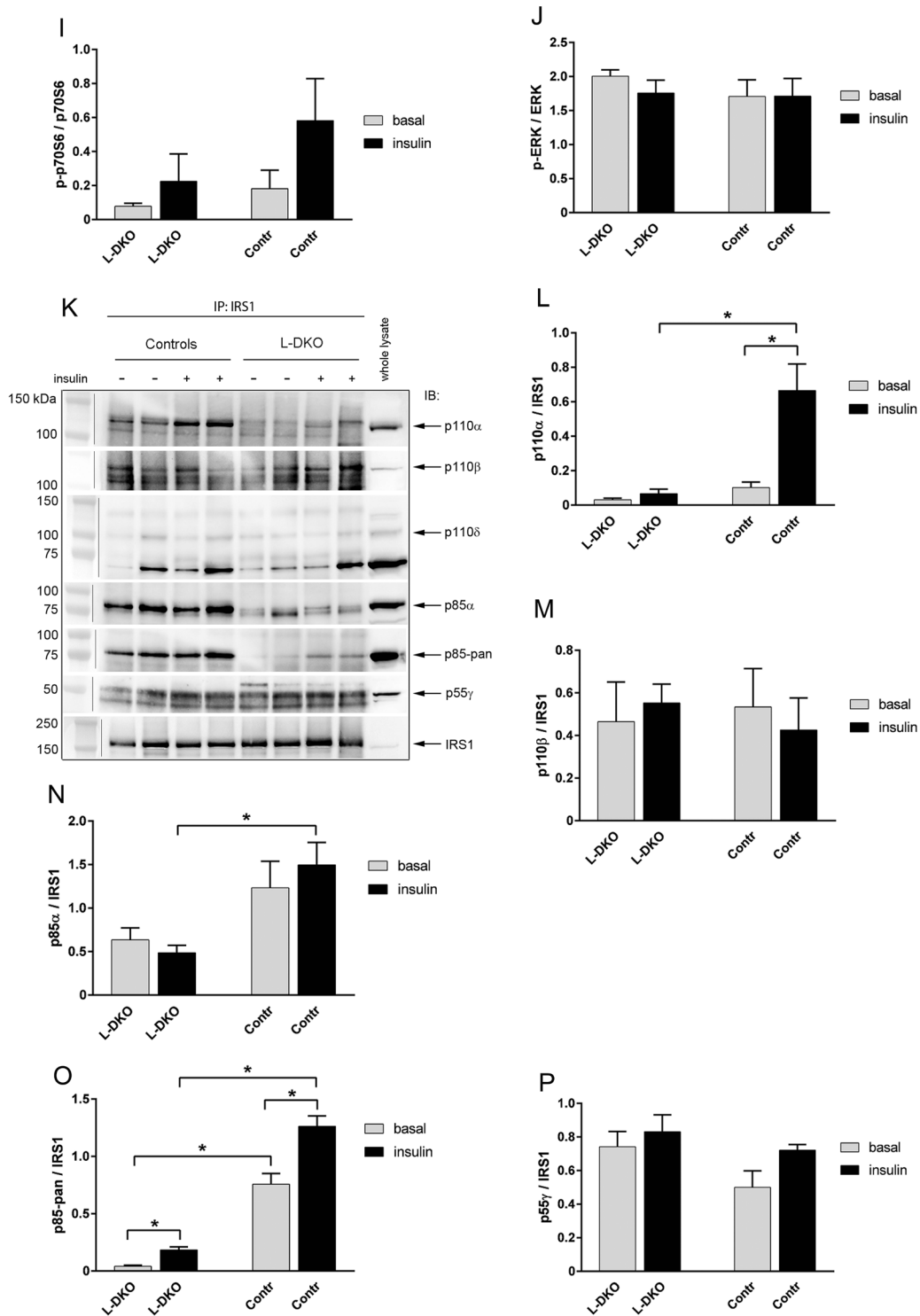
As expected, p110 $\alpha$  kinase activity, as assessed by the ability to add the 3'-phosphate group to phosphoinositides in p110 $\alpha$  immunoprecipitates, was markedly decreased both in the basal and insulin-stimulated states of the L-DKO mice compared to controls (Figure 2A). Overall p110 $\beta$  kinase activity was much lower than p110 $\alpha$  kinase activity in control mice, consistent with previous studies<sup>7,27</sup>, and was not changed in the basal state between controls and L-DKO mice (Figure 2B). However, p110 $\beta$  kinase activity was significantly decreased in the insulin-stimulated state of the L-DKO mice (Figure 2B), even though similar amounts of p110 $\beta$  protein were associated with IRS1 in controls and L-DKO mice (Figure 1K and 1M). Surprisingly, IRS1-associated phosphatidylinositol kinase activity in response to insulin was intact in the L-DKO, despite lack of p110 $\alpha$  kinase activity and decreased p110 $\beta$  activity (Figure 2C).

### **The intact IRS1-associated kinase activity in the L-DKO mice cannot be explained by compensatory upregulation of other classes of PI3K**

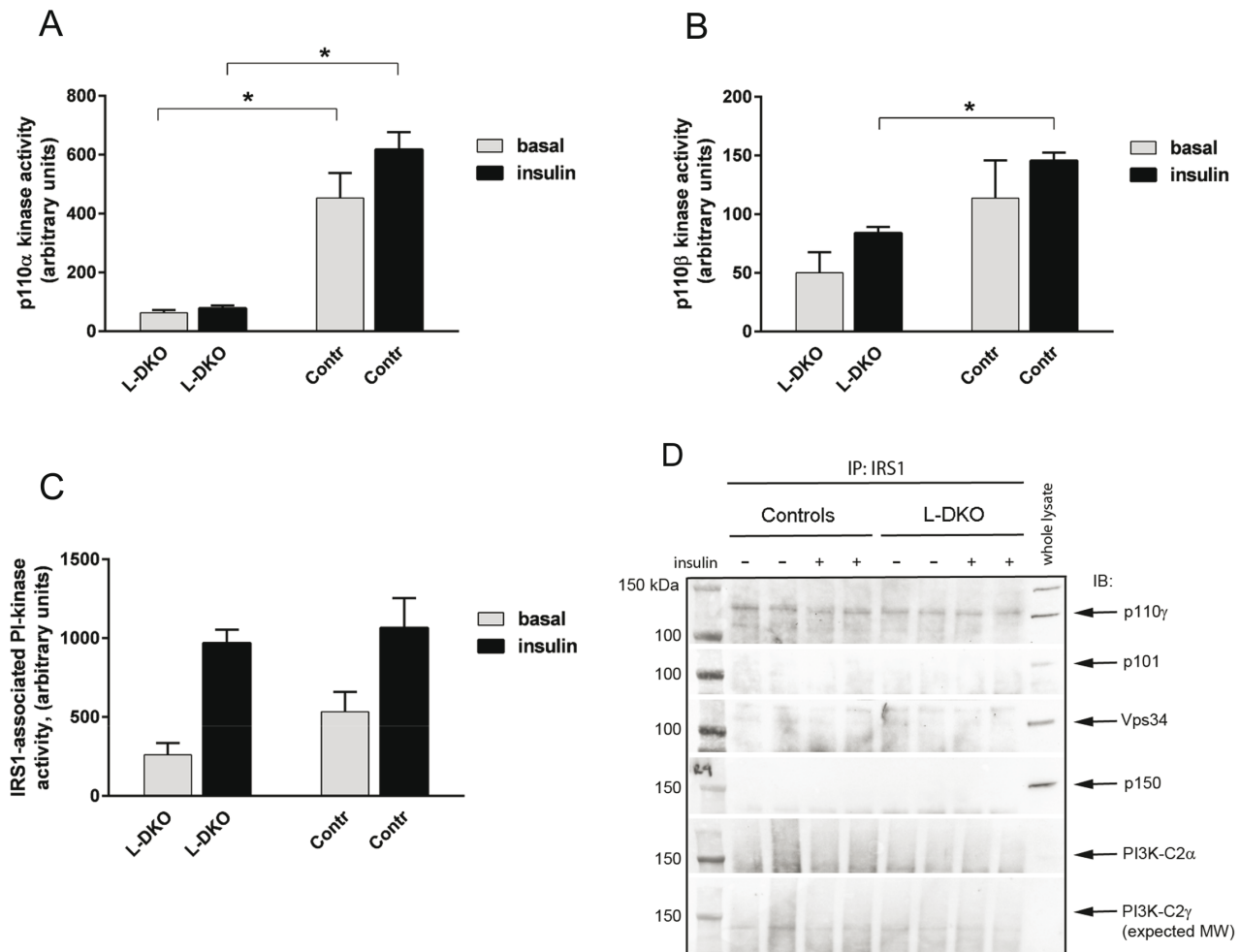
To explore if other classes of phosphoinositide 3-kinases were responsible for the intact IRS1-associated activity, we investigated the association of IRS1 with class IB-, class II and class III members of phosphoinositide 3-kinases in liver lysates







**Figure 1. Gene- and protein-expression of regulatory and catalytic subunits of PI3K and associated insulin signaling mediators.** mRNA expression of (A) *Pik3ca*, (B) *Pik3cb*, (C) *Pik3r1* and (D) *Pik3r2* in livers of flox controls and L-DKO mice. (E) Representative western blot of protein expression of p110 $\alpha$ , p110 $\beta$ , p85 $\alpha$ , p85-pan (detects both p85 isoforms) and p55 $\gamma$ . Total Akt was used as a loading control. (F, phosphorylated Akt/PKB (Ser 473 and Thr 308), phosphorylated p70S6 kinase, and phosphorylated ERK in livers of flox controls and L-DKO mice. Total Akt, p70S6K and ERK was used as loading controls. (G–J) Quantification measurements of the western blots shown in (F) of pS-Akt, pT-Akt, p-p70S6K and p-ERK respectively. (K) Representative western blot of immunoprecipitation experiments with antibodies for IRS1 and subsequent immunoblotting with antibodies for p110 $\alpha$ , p110 $\beta$ , p110 $\delta$ , p85 $\alpha$ , p85-pan (detects both p85 isoforms) and p55 $\gamma$ . The whole-lysate reference sample was from an insulin-treated flox control mouse. (L–P) Quantification measurements of the western blots shown in (K) of p110 $\alpha$ , p110 $\beta$ , p85 $\alpha$ , p85-pan and p55 $\gamma$ . IP = immunoprecipitation, IB = immunoblot. Basal condition is indicated with a minus (-) sign, insulin-treated condition is indicated with a plus (+) sign. Basal conditions refer to fasting overnight and injection of saline through the vena cava. Insulin treatment refers to injection of 5 U of insulin through the vena cava 5 min prior to euthanization. Error bars indicate SEM (n = 5–8). \*, p < 0.05 compared to controls. n.s. = non significant. Images containing a vertical line are composites taken from a single original image.



**Figure 2. PI3K activity and assessment of the association of IRS1 with class IB-, class II- and class III-members of phosphoinositide 3-kinases.** (A) p110 $\alpha$  lipid kinase activity, (B) p110 $\beta$  lipid kinase activity, and (C) IRS1-associated phosphatidylinositol (PI) kinase activity in L-DKO mice and controls were assessed by immunoprecipitation. (D) Representative western blot of immunoprecipitation experiments with antibodies for IRS1 and subsequent immunoblotting with antibodies for p110 $\gamma$ , p101, Vps34, p150, PI3K-C2 $\alpha$  and PI3K-C2 $\gamma$ . The whole-lysate reference sample was from an insulin-treated flox control mouse. IP = immunoprecipitation, IB = immunoblot. Basal condition is indicated with a minus (-) sign, insulin-treated condition is indicated with a plus (+) sign. Basal conditions refer to fasting of mice overnight and injection of saline through the vena cava. Insulin treatment refers to injection of 5 U of insulin through the vena cava 5 min prior to euthanization. Error bars indicate SEM (n = 4). \*, p < 0.05 compared to controls.

from controls and L-DKO mice. Class IB PI3K consists of the p110 $\gamma$  catalytic subunit and the p101 regulatory subunit. The amount of p101 was low in whole lysates and no p101 was associated with IRS1 in either control mice or L-DKO mice (Figure 2D). p110 $\gamma$  was associated with IRS1, but the amounts were similar between controls and L-DKO mice (Figure 2D). Class III PI3K catalytic subunit Vps34 and regulatory subunit p150 were present in whole lysates, but not associated with IRS1 in either control mice or L-DKO mice (Figure 2D). Class II PI3K consists of only one subunit, but exists in several isoforms. The PI3K-C2 $\alpha$  isoform is ubiquitously expressed, whereas PI3K-C2 $\gamma$  has been reported to have a more limited tissue distribution, including hepatocytes<sup>28,29</sup>. However, we detected only little or no PI3K-C2 $\alpha$  or PI3K-C2 $\gamma$  in whole liver lysates or associated with IRS1 in controls or L-DKO mice (Figure 2D). Thus, other classes of phosphoinositide 3-kinases did not compensate

for the loss of p110 $\alpha$  and p85 $\alpha$  in the L-DKO mice and could not explain the intact IRS1-associated phosphatidylinositol kinase activity in the absence of p110 $\alpha$  and p85 $\alpha$ .

#### L-DKO mice have decreased liver weight and WAT weight compared to controls

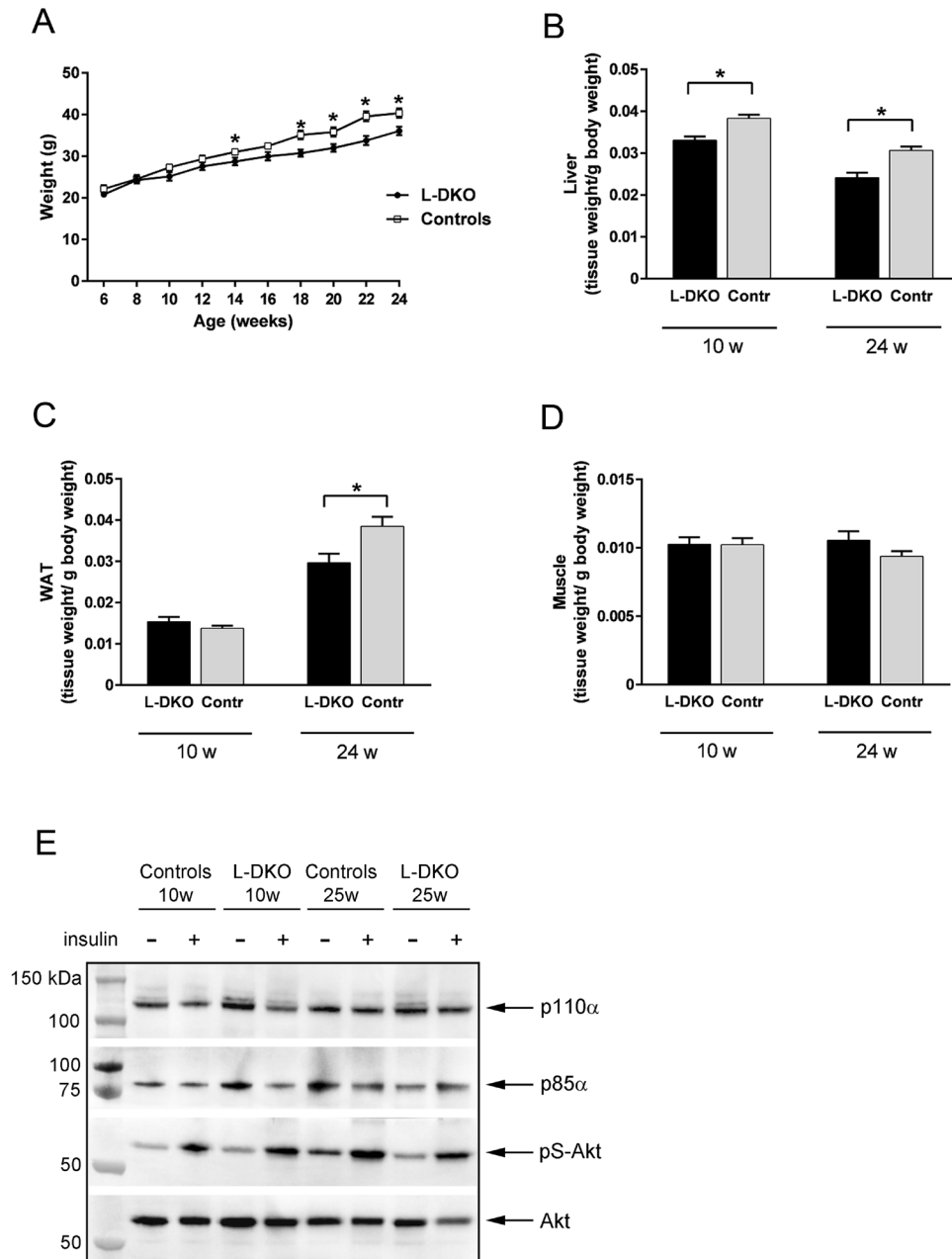
On standard chow (4% fat content by weight, 12% by calories), L-DKO mice had similar body weights to the flox control mice until 10–12 weeks of age, after which the L-DKO mice showed slower weight gain compared to controls (Figure 3A). At least part of this decrease was due to a decrease in liver weight. Thus, by 10 weeks of age, the ratio of liver weight to body weight in the L-DKO mice was decreased by 13% compared to control mice (Figure 3B), whereas there was no difference in WAT weight or muscle weight (Figures 3C and 3D). At 24 weeks of age, liver weight remained decreased (18%), and there



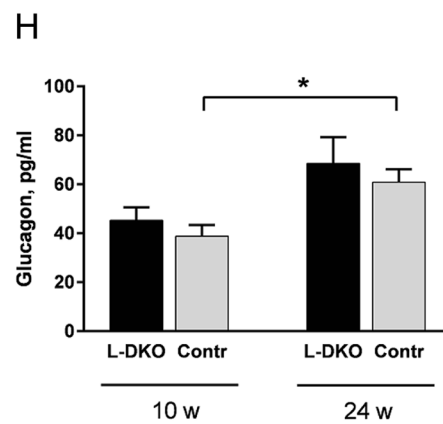
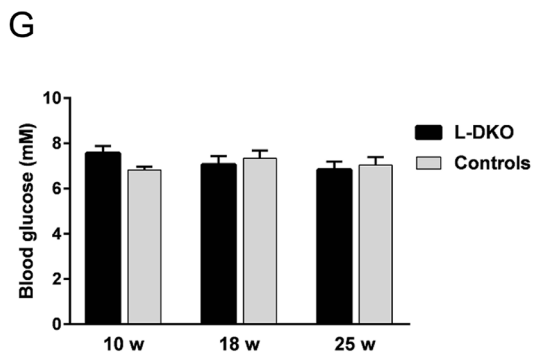
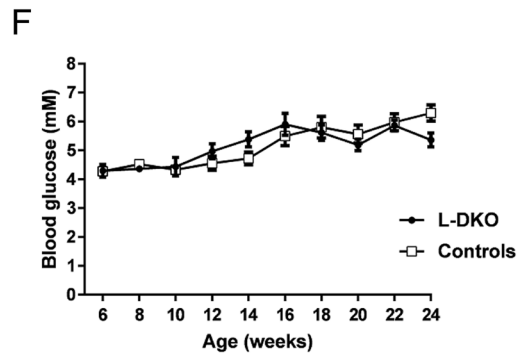
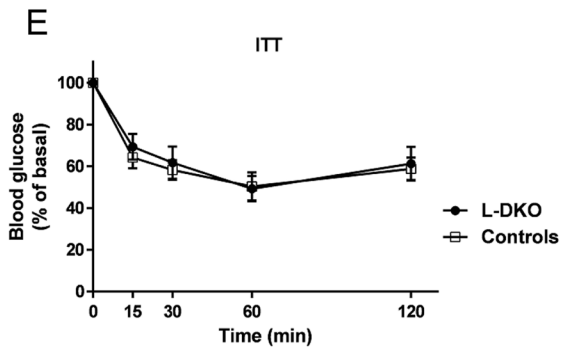
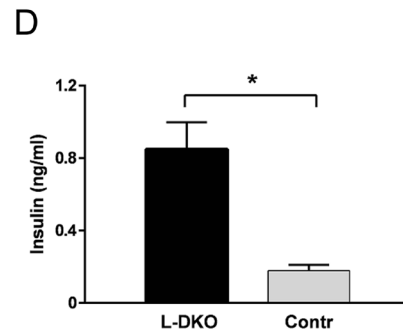
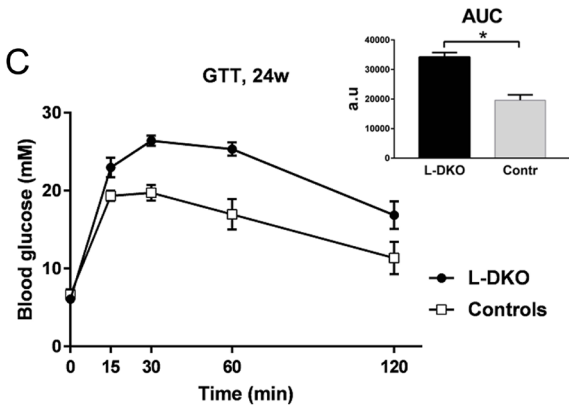
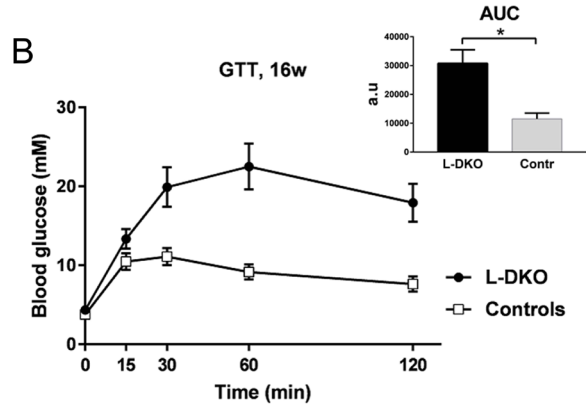
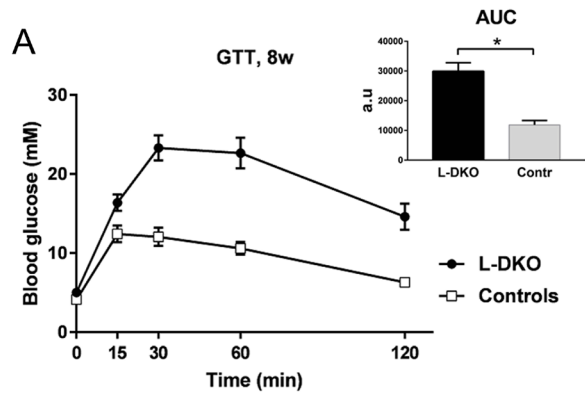
was also a significant decreased WAT weight (22%) for the L-DKO mice compared to controls (Figures 3B and 3C). To assess a possible change in insulin sensitivity in WAT, associated with the decreased WAT mass, we investigated insulin signaling mediators in this tissue. WAT p110 $\alpha$ - and p85 $\alpha$  expression was similar in controls and L-DKO mice as was Akt/PKB phosphorylation in response to insulin (Figure 3E). There was no difference in insulin-stimulated Akt/PKB-activation at 10w compared to 25w in either controls or L-DKO mice (Figure 3E).

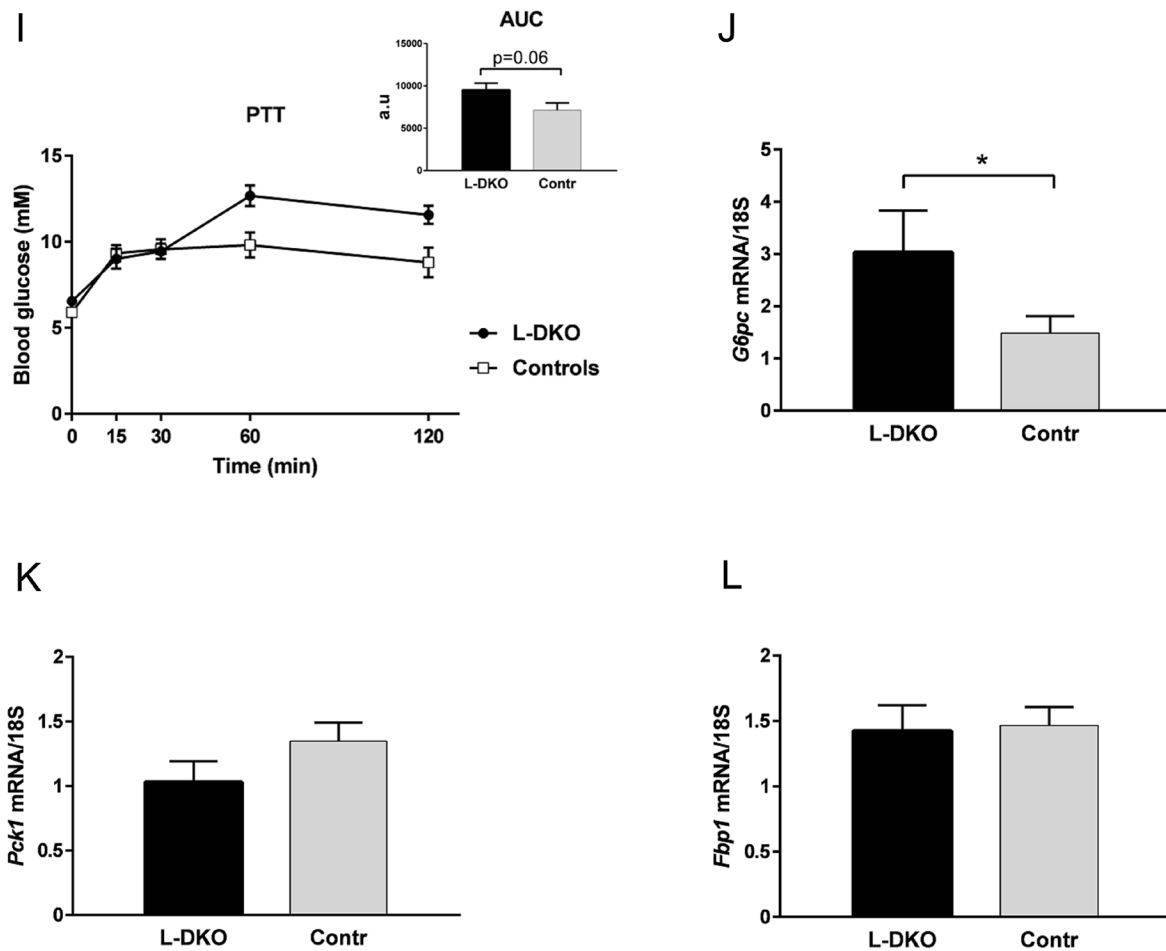
**L-DKO mice have impaired glucose tolerance, but normal insulin tolerance and normal blood glucose levels**

Despite similar body weight (Figure 3A), L-DKO mice were severely glucose intolerant as early as at 8 weeks of age (Figure 4A), and remained similarly glucose intolerant throughout the 24 week study (Figures 4B and 4C). This was associated with markedly increased fasting insulin levels (Figure 4D). However, somewhat surprisingly, the L-DKO mice showed a normal response to exogenous insulin during an intraperitoneal



**Figure 3. Body weight, tissue weights and adipose insulin signaling.** (A) whole body weight, (B) liver weight, (C) white adipose tissue (WAT) weight and (D) muscle weight of 10 and 24 week old male L-DKO mice and controls. (E) Representative western blot of p110 $\alpha$ , p85 $\alpha$  and phosphorylated Akt/PKB (Ser 473) in WAT of 10 and 25 week old male flox controls and L-DKO mice. Total Akt/PKB was used as a loading control. Basal condition is indicated with a minus (-) sign, insulin-treated condition is indicated with a plus (+) sign. Basal conditions refer to fasting of mice overnight and injection of saline through the vena cava. Insulin treatment refers to injection of 5 U of insulin through the vena cava 5 min prior to euthanization. Error bars indicate SEM (n = 8-17). \*, p < 0.05 compared to controls. w = weeks.





**Figure 4. Glucose homeostasis.** Glucose tolerance test at (A) 8 weeks, (B) 16 weeks and (C) 24 weeks of age; (D) fasting insulin levels at 10 weeks of age; (E) insulin tolerance test at 19 weeks of age; (F) fasting glucose levels; (G) random fed glucose levels; (H) fasting glucagon levels; (I) pyruvate tolerance test at 15 weeks of age; (J–L) hepatic mRNA expression of the gluconeogenic markers glucose 6-phosphatase (*G6pc*), phosphoenolpyruvate carboxykinase (*Pck1*) or fructose 1,6-bisphosphatase (*Fbp1*). L-DKO mice and controls were given 2 g glucose/kg body weight intraperitoneally (i.p.) for the glucose tolerance test or 1.25 U insulin/kg body weight i.p. for the insulin tolerance test or 2 g pyruvate/kg body weight i.p. for the pyruvate tolerance test. Blood glucose was measured at 0, 15, 30, 60, and 120 min. Error bars indicate SEM (n = 6–12). \*, p < 0.05. The insets in the glucose tolerance test graphs and the pyruvate tolerance test graph show the area under the curve (AUC) with subtracted basal glucose values.

insulin tolerance test (Figure 4E). Despite the markedly impaired glucose tolerance and marked hyperinsulinemia, fasting and random fed glucose levels in the L-DKO mice remained similar between controls and L-DKO mice (Figures 4F and 4G). Circulating glucagon levels were also similar between L-DKO mice and controls both at 10 weeks of age and 24 weeks of age (Figure 4H).

#### L-DKO mice display normal hepatic glucose production

Increased gluconeogenesis is one of the hallmarks of hepatic insulin resistance in type 2 diabetes. To determine whether the impaired glucose tolerance in L-DKO mice was due to increased hepatic glucose output, we subjected these animals to a challenge with pyruvate, the major gluconeogenic substrate. Over the 120 min period following administration of pyruvate,

there was a trend toward increased glucose levels in L-DKO mice compared to controls, but this was not statistically significant (Figure 4I). This was associated with a significant change in hepatic gene expression of the gluconeogenic enzyme glucose 6-phosphatase (*G6pc*) (Figure 4J), whereas gene expression of the other key mediators of gluconeogenesis, phosphoenolpyruvate carboxykinase (*Pck1*) and fructose 1,6-bisphosphatase (*Fbp1*), remained similar between control mice and L-DKO mice (Figures 4K and 4L).

Dataset 1. Raw data for gene and protein expression shown in Figure 1

<http://dx.doi.org/10.5256/f1000research.12418.d205909>

**Dataset 2. Raw data for PI3K activity measurements and protein expression shown in Figure 2**

<http://dx.doi.org/10.5256/f1000research.12418.d175494>

**Dataset 3. Raw data for body and tissue weights and protein expression shown in Figure 3**

<http://dx.doi.org/10.5256/f1000research.12418.d175495>

**Dataset 4. Raw data for metabolic procedures and measurements and gene expression shown in Figure 4**

<http://dx.doi.org/10.5256/f1000research.12418.d205910>

## Discussion

In this study, we investigated the impact of a combined deletion of p110 $\alpha$  and p85 $\alpha$  on insulin signaling and glucose homeostasis. For this purpose, we created mice with a liver-specific deletion of the major catalytic and major regulatory subunits of PI3K: *Pik3ca* and *Pik3r1*. We have previously shown that hepatic deletion of only p110 $\alpha$  results in severe insulin resistance and impaired glucose tolerance, signifying that p110 $\alpha$  is crucial for mediating insulin signaling<sup>7</sup>. Moreover, mice deficient in all p85 isoforms in either muscle or liver exhibit severely impaired insulin signaling in these tissues<sup>14,15</sup>. The liver plays a crucial role in maintaining glucose homeostasis; we therefore hypothesized that deleting both these isoforms would result in severe and overt diabetes.

As expected, in the L-DKO mice, p110 $\alpha$  catalytic activity was blunted and, as a result, the activation of the signal downstream of PI3K was markedly decreased. However, the L-DKO mice showed normal fasting and fed blood glucose levels throughout the study (24 weeks) and normal insulin tolerance. Glucose tolerance was impaired in the L-DKO mice and circulating insulin levels were markedly elevated, but to a degree similar to mice with only p110 $\alpha$  deleted in the liver (L-p110 $\alpha$  KO)<sup>7</sup>. Surprisingly, despite abolished p110 $\alpha$  activity, we observed an intact total IRS1-associated phosphatidylinositol (PI) kinase activity in the L-DKO mice. This finding was very different from what we previously observed in L-p110 $\alpha$  KO mice, which only have p110 $\alpha$  deleted in the liver<sup>7</sup>. In the L-p110 $\alpha$  KO mice, insulin-stimulated IRS1-associated PI kinase activity was markedly blunted<sup>7</sup>. This suggests that it is the loss of the regulatory subunit that accounted for the preserved IRS1-associated activation, possibly by enabling other phosphatidylinositol kinases to bind to IRS1. However, there did not appear to be any compensatory effects of other known catalytic and regulatory class IA subunit isoforms for the action of insulin. Thus, we did not detect any differences in the IRS1-associated amounts of p110 $\beta$ , p110 $\delta$  or p55 $\gamma$  in controls and L-DKO mice in response to insulin and only very small amounts of p85 $\beta$  associated with IRS1 in the L-DKO mice. Similarly, no evidence of compensatory effects by class IB, class II or class III members of phosphoinositide 3-kinases were found.

Previous studies by us and others, including our study of the L-p110 $\alpha$  KO mice, have shown that p110 $\beta$  is unable to compensate for the loss of p110 $\alpha$ <sup>7,26,30</sup>. However, we observed similar amounts of IRS1-associated p110 $\beta$  in the L-DKO mice and controls despite overall total decreased levels of p110 $\beta$  in the liver. We therefore speculated that perhaps p110 $\beta$  activity was increased in response to insulin in the L-DKO mice compared to controls, which would explain the sustained IRS1-associated PI kinase activity. We found no difference in the p110 $\beta$  activity between the controls and the L-DKO mice in the basal state. In addition, the p110 $\beta$  kinase activity was significantly decreased, rather than increased, in the insulin-stimulated state of L-DKO mice. We thus conclude that the sustained IRS1-associated PI kinase activity in the L-DKO mice is not due to increased activity of p110 $\beta$ .

Although the amount of IRS1-associated p110 $\beta$  was similar in controls and L-DKO, the total amount of p110 $\beta$  was decreased in the L-DKO mice. We, and others, have previously reported that the dimeric interaction between the regulatory and the catalytic subunits results in stabilization of the subunits, whereas the monomeric forms are more readily subjected to degradation<sup>18,20-21</sup>. Therefore, the absence of the major regulatory subunit p85 $\alpha$  in the L-DKO mice probably subjects p110 $\beta$  to more rapid degradation. In this context, observing similar amounts of p110 $\beta$  associated with IRS1 in controls and L-DKO mice is somewhat surprising, but is likely due to stabilization of a fraction of p110 $\beta$  subunits by interaction with IRS1-associated p85 $\beta$  or p55 $\gamma$ .

Lack of the major regulatory subunit in the L-DKO mice, accompanied by absence of increased activation of p110 $\beta$  or compensatory increased expression of other phosphoinositide 3-kinases, suggests that presence of other classes of phosphatidylinositol kinases, perhaps PI4K and PI5K, account for the intact IRS1-associated kinase activity by directly binding to IRS1. PI4Ks have been described as mediators of endosomal trafficking from the Golgi and to be involved in EGF-stimulated phosphoinositide signaling<sup>31</sup>. Type II PI4Ks interact with the EGF receptor, but they are not known to interact with IRS1. Type III PI4Ks are structurally related to PI3Ks, with a high degree of conservation between their catalytic domains and sensitivity to wortmannin<sup>31</sup>. The isoform PI4KIII $\alpha$  has been reported to be functionally connected to PI3K in FGF signaling during pectoral fin development in the zebra fish<sup>32</sup>.

PI5K exists as two separate classes, PI(3)P5K and PI(4)P5K, phosphorylating the D5 position of the inositol ring of phosphatidylinositol 3-phosphate and phosphatidylinositol 4-phosphate, respectively. Of the PI(4)P5Ks, the isozyme PIP5Kc has been shown to respond to, and become phosphorylated by, various hormones and growth factors, such as EGF, and play a role in actin cytoskeletal reorganization, clathrin-dependent endocytosis, membrane ruffle formation, etc.<sup>33</sup>. However, a direct effect on insulin signaling and interaction with IRSs by PIP5Kc has not been reported. PI(3)P5K, also called PIKfyve, and has been quite extensively studied and reported to be involved in membrane trafficking, stress- or hormone-induced

signaling, ion channel activity, cytoskeletal dynamics, nuclear transport, gene transcription and cell cycle progression<sup>34</sup>. Interestingly, PIKfyve is regulated by insulin, recruiting PIKfyve to inner membranes, where insulin receptor and IRSs are also found<sup>35</sup>, and co-precipitates with p110 and p85 subunits in 3T3-L1 adipocytes<sup>36</sup>. Thus, PIKfyve appears to be a possible contributor to the sustained IRS1-associated kinase activity in the L-DKO mice. However, PIKfyve expression has been reported to be rather tissue specific, mainly expressed in adipose tissue, muscle and brain<sup>37,38</sup> and expression in the liver appears low<sup>37,38</sup>. A more extensive follow-up investigation of the various phosphoinositides in the L-DKO livers compared to controls may help elucidating the phosphatidylinositol kinase responsible for the sustained IRS1-associated kinase activity in the L-DKO mice.

The absence of both p85 $\alpha$  and p110 $\alpha$ , as seen in the L-DKO mice, would logically result in a very severely impaired metabolic phenotype. The L-DKO mice had markedly elevated circulating insulin levels, impaired glucose tolerance and showed a trend toward increased rates of hepatic glucose output when given pyruvate. However, fasted and fed glucose levels were not different between controls and L-DKO mice, i.e., randomly fed L-DKO mice were not diabetic and insulin tolerance tests were normal. Part of this protection might be the fact that L-DKO mice had less accumulation of WAT with age. In addition, it is possible that the high circulating insulin levels reflect an impaired hepatic insulin clearance rather than insulin resistance in the muscle, which would explain the paradoxical normal insulin tolerance. Interestingly, body weight, WAT weight and hepatic glucose output were significantly increased and insulin tolerance severely impaired in L-p110 $\alpha$  KO mice<sup>7</sup>, which lack only p110 $\alpha$  in liver, demonstrating that L-DKO mice showed an overall less severe metabolic phenotype compared to L-p110 $\alpha$  KO mice.

In summary, deletion of hepatic p110 $\alpha$  and p85 $\alpha$  results in an impaired insulin signal and impaired glucose homeostasis, but shows an overall less severe metabolic phenotype compared to mice with only p110 $\alpha$  deleted in the liver. Although other PI3Ks were unable to compensate for the loss of p110 $\alpha$

and p85 $\alpha$ , IRS1-associated phosphatidylinositol kinase activity was surprisingly still intact, possibly due to interaction of IRS1 with other classes of phosphatidylinositol kinases.

**Abbreviations:** Fbp, fructose-1,6-bisphosphatase; G6pc, glucose-6-phosphatase; GTT, glucose tolerance test; ITT, insulin tolerance test; L-DKO, liver double knockout; Pck, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKB, protein kinase B; PTT, pyruvate tolerance test; WAT, white adipose tissue

### Data availability

**Dataset 1:** Raw data for gene and protein expression shown in [Figure 1](#). DOI, [10.5256/f1000research.12418.d205909](https://doi.org/10.5256/f1000research.12418.d205909)<sup>39</sup>

**Dataset 2:** Raw data for PI3K activity measurements and protein expression shown in [Figure 2](#). DOI, [10.5256/f1000research.12418.d175494](https://doi.org/10.5256/f1000research.12418.d175494)<sup>40</sup>

**Dataset 3:** Raw data for body and tissue weights and protein expression shown in [Figure 3](#). DOI, [10.5256/f1000research.12418.d175495](https://doi.org/10.5256/f1000research.12418.d175495)<sup>41</sup>

**Dataset 4:** Raw data for metabolic procedures and measurements and gene expression shown in [Figure 4](#). DOI, [10.5256/f1000research.12418.d205910](https://doi.org/10.5256/f1000research.12418.d205910)<sup>42</sup>

### Competing interests

No competing interests were disclosed.

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[Data Source](#)
  41. Chaudhari A, Ejeskär K, Wettergren Y, *et al.*: Dataset 3 in: Hepatic deletion of p110 $\alpha$  and p85 $\alpha$  results in insulin resistance despite sustained IRS1-associated phosphatidylinositol kinase activity. *F1000Research*. 2017.  
[Data Source](#)
  42. Chaudhari A, Ejeskär K, Wettergren Y, *et al.*: Dataset 4 in: Hepatic deletion of p110 $\alpha$  and p85 $\alpha$  results in insulin resistance despite sustained IRS1-associated phosphatidylinositol kinase activity. *F1000Research*. 2018.  
[Data Source](#)

# Open Peer Review

Current Referee Status:



## Version 2

Referee Report 05 July 2018

doi:10.5256/f1000research.16404.r34701



**Lily Q. Dong**

Department of Cell Systems & Anatomy, University of Texas Health at San Antonio, San Antonio, TX, USA

The revised version is acceptable.

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Referee Report 28 June 2018

doi:10.5256/f1000research.16404.r34702



**Richard Z. Lin , Lisa Ballou**

Department of Physiology and Biophysics, Stony Brook School of Medicine, Stony Brook, NY, USA

The manuscript is still not fully acceptable. Please see our comments below:

Fig. 2.: The conclusion that p110 $\beta$  is not responsible for at least some of the the IRS1-associated kinase activity in L-DKO mice is still not well supported by the evidence. The western blots in Fig. 1K are not convincing, and the quantified western blots in Fig. 1M have large error bars. In Fig. 2B, the p110beta antibody used for IP might be much less efficient than the IRS1 antibody in pulling down the enzyme. The IRS1 IPs should be assayed in the presence and absence of TGX-221, which is highly selective for p110beta in vitro. This assay is not more complicated than the ones already shown in the figure.

Fig. 3: Please indicate in the legend which muscles and WAT depots were analyzed.

**Competing Interests:** No competing interests were disclosed.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Referee Report 25 June 2018

doi:10.5256/f1000research.16404.r34700



**James R. Woodgett** , **Prital Patel**

Lunenfeld-Tanenbaum Research Institute, Sinai Health System & University of Toronto, Toronto, ON, Canada

The revised version fully satisfies my comments previously made with respect to the original submission.

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Signal transduction

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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### Version 1

Referee Report 15 November 2017

doi:10.5256/f1000research.13447.r27377



**Richard Z. Lin**, **Lisa Ballou**

Department of Physiology and Biophysics, Stony Brook School of Medicine, Stony Brook, NY, USA

#### General Comments:

The authors made a very interesting observation that L-DKO mice, lacking hepatic p110 $\alpha$  and p85 $\alpha$ , have a milder metabolic phenotype than mice lacking only hepatic p110 $\alpha$ . These mice have normal fasting blood glucose levels but not surprisingly they are glucose intolerant. Interestingly, the liver and WAT weights were reduced. Liver triglyceride levels were not measured to determine if the fat content of the liver was also reduced. Metabolic cage measurements would be revealing to rule in or out changes in energy expenditure of the animals. It would also be informative to know if a high-fat diet causes L-DKO animals to develop fasting hyperglycemia and/or if the fatty liver associated with this type of diet is accentuated or attenuated.

In general, the results from the insulin signaling experiments are not convincing. It is unclear why the authors concluded that the residual signaling to Akt is not due to Class I PI3Ks. IRS-1 pull-downs from L-DKO liver have substantial PI kinase activity. Better experiments are needed to resolve this research question (see comment below).

#### Specific Comments:

Figures 1F & 1G, Figure 2 and Figure 3E. (a) The dose of insulin used to study insulin signaling was extremely high. 5 U injected into a 30 g mouse is 167 U/kg body weight. Compare that to the 1.25 U/kg body weight used for the insulin tolerance test. Some of the signaling changes could be due to activation of an unusually high percentage of insulin receptors or insulin activation of IGF1 receptors that would not occur under physiological conditions. Attempts to explain the pathophysiological phenotypes of the L-DKO mouse based on the signaling results is therefore problematic. (b) The legends say that "Basal

conditions refer to fasting of mice overnight.” Were the control mice anesthetized and injected ivc with saline, as stated under Methods? If so, the legends could be more specific.

Figure 1F. Total Akt and total p70S6K should be shown.

Figure 1G. (a) Why did insulin treatment of control mice not increase the amount of p85 $\alpha$  (or other regulatory subunit) associated with IRS-1? (b) Use of a pan p85 antibody to estimate changes in p85 $\beta$  expression is not convincing. Lack of a good mouse p85 $\beta$  antibody is a limitation to the study. (c) The conclusion that similar amounts of IRS-1-bound p110 $\beta$  were pulled down in control and L-DKO mice is not convincing based on the blot.

Figure 2. The conclusion that p110 $\beta$  and the other PI3Ks examined by western blotting are not responsible for the IRS-1-associated kinase activity is premature. The IPs should be assayed in the presence and absence of selective inhibitors to determine the contribution of p110 $\beta$  and the other enzymes to the PI kinase activity. Considering that hepatocytes make up 75-80% of the cell types in the liver, it is also possible that some of the kinase activity in the IRS-1 IP comes from p110 $\alpha$  in the 20-25% of cells that are not targeted by albumin-Cre.

Figure 3. Histological examination of the livers should be included. Liver triglyceride levels should be measured.

Figures 3C & 3D. The specific WAT depots and muscles examined should be identified.

Figure 4A-D. Fasting insulin is higher in the L-DKO mice at 10 weeks of age. Presumably, this is why these mice are euglycemic. Insulin levels should be measured before and after the glucose challenge with mice in one of the GTT age groups. This experiment will assess if the glucose intolerance is due to inability of the L-DKO mice to mount a further insulin response.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Author Response 21 May 2018

**Victoria Rotter Sopasakis**, University of Gothenburg, Sweden

Response to general comments:

We agree with the reviewers that all these studies would be very interesting, but would take several years to complete and is outside the scope of this particular study.

Response to specific comments:

1a. The dose of insulin used is what is normally used for these types of experiments. If the reviewers would be right, i.e unusually high activation of insulin would occur, this would be the case in all our other similar studies (and all other scientists' studies who are doing similar experiments), for example in the study where we knocked out p110a in the liver. Since this is not the case, we don't believe that this a major problem for the interpretation of our data.

1b. Yes, the control mice were anesthetized and injected ivc with saline (basal conditions) or insulin. This has been added to the figure legends as required.

2. Total Akt and total p70S6K blots have been added to Figure 1F.

3a. We have added more experiments to this figure (n=4) and made quantitative graphs of the results. The amount of p85a associated with IRS1 still remains similar between basal and insulin-stimulated conditions, whereas the p85-pan antibody shows increased amounts of total p85 in response to insulin (figures 1N and 1O).

3b. We have previously (before submitting the manuscript) tried various different p85b antibodies without any success. Some work well for human tumor tissue for example, but give no band at all for our mouse liver lysates. Other antibodies we tried fail to deliver a band for both human and mouse tissues. This is the reason we chose to use the p85-pan antibody to indirectly show p85b protein expression. Since both Dr Woodgett and Drs Lin and Ballou requested a p85b antibody we have tried again to make various p85b antibodies on the market to work for our liver lysates and have spent months now trying to optimize the western blot conditions for these antibodies. We are not satisfied with the results. The only conclusion we can draw is that either 1) the p85b antibodies available on the market simply does not work well for mouse tissue or 2) there is not much p85b expressed in mouse liver. Based on the p85-pan westerns that we performed as well as previous studies including Taniguchi et al, we believe that there simply is not that much p85b expressed in mouse liver.

3c. More experiments have been added (n=4) and quantifying graphs are now included in the figure (figure 1M).

4. These types of experiments are all very interesting. However, they are complicated, particularly considering that inhibitors are never 100% selective and specific. We therefore question the gain of these experiments in relation to the work and resources that have to be invested to complete them.

5. Histological experiments and liver triglyceride level measurements are outside the scope of this



study.

6. These types of experiments would not lead to increased understanding of the underlying mechanisms of our data. We therefore question the gain of these experiments in relation to the work and resources that have to be invested to complete them.

7. It is quite possible that the L-DKO mice are not able to further increase the insulin secretion in response to the glucose load in the GTT considering the very high insulin levels these mice have normally. We have added a glucagon ELISA (new figure 4H) that shows normal glucagon levels for DKO mice both at 10 w of age and 24 w of age. The normal glucagon levels and the very high insulin levels in the DKO mice indicates that the pancreas is working normally in these mice and the underlying mechanism for the glucose intolerance is therefore not likely at the level of the pancreas.

**Competing Interests:** No competing interests were disclosed.

Referee Report 08 November 2017

doi:10.5256/f1000research.13447.r27376



**Lily Q. Dong**

Department of Cell Systems & Anatomy, University of Texas Health at San Antonio, San Antonio, TX, USA

Chaudhari et al. reported the physiologic roles of hepatic p110a and p85a in regulating insulin signaling and glucose homeostasis. They concluded that hepatic deletion of p110a and p85a (L-DKO) led to mild metabolic phenotype of the L-DKO mice and proposed that lipid kinases other than PI3Ks might compensate for the loss of p110a/p85a by signaling through unknown signaling pathway other than Akt signaling. Since deletion of p85a leads to improved insulin sensitivity and deletion of p110a leads to impaired insulin signaling and insulin resistance, it is interesting to learn that L-DKO mice showed an overall less severe metabolic phenotype compared to the L-p110a KO mice.

1. Although the expression levels of p110b were decreased in the liver of L-DKO mice (Fig. 1E), IRS1-associated p110b levels were comparable between the control and KO mice (Fig. 1G). In addition, notable levels of p110b activity were maintained in the L-DKO mice and insulin treatment can stimulate the activity although it is 50% compared to the control (Fig. 2B). It is unclear why the authors concluded that the intact IRS1-associated kinase activity in the KO mice are not from the remaining p110b.
2. The KO mice showed significantly impaired glucose tolerance and markedly increased fasting insulin levels (Fig. 4). What were the pancreatic phenotype of the KO mice? What were the levels of glucagon in the KO mice?

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 21 May 2018

**Victoria Rotter Sopasakis**, University of Gothenburg, Sweden

1. We don't believe that the intact IRS1-associated kinase activity in the DKO mice are from the remaining p110b for the following reasons:

The total degree of kinase activity is very much smaller for p110b compared to p110a. This is an observation we have made over and over again for liver lysates in response to insulin in several different studies (the a.u numbers of the y-axis in the graphs in figure 2 demonstrates this to some extent). However, we still wanted to be sure that we were correct to rule out a role of p110b since we observed similar amounts of p110b bound to IRS1 in the DKO and controls. It was therefore important to look at the kinase activity associated with IRS1. The p110b-associated kinase activity in response to insulin was significantly decreased in the DKO mice compared to controls (figure 2b). These two observations ( 1) very much smaller degree of p110b activity over all compared to p110a kinase activity and 2) decreased IRS1-associated p110b activity in the DKO in response to insulin) makes it highly unlikely that the sustained IRS1-associated kinase activity is due to compensatory levels of p110b kinase activity).

2. We have performed a glucagon ELISA assay for the mice (new figure 4H). There were no differences in glucagon serum levels between DKO mice and controls at either 10 weeks of age or 24 weeks of age. The high fasting insulin levels in the DKO mice suggest that insulin production and secretion by the pancreas is intact and since no differences in glucagon secretion was observed even at older age we conclude that the pancreatic phenotype is likely very similar between DKO mice and controls.

**Competing Interests:** No competing interests were disclosed.

Referee Report 14 September 2017

doi:10.5256/f1000research.13447.r25513

**James R. Woodgett** , **Prital Patel**

Lunenfeld-Tanenbaum Research Institute, Sinai Health System & University of Toronto, Toronto, ON, Canada

In this study, the authors generated a liver-specific double knockout of the regulatory and catalytic subunit of Class 1A PI3K. Previously, single inactivation of either the catalytic or regulatory subunits had been shown to result in opposite effects on insulin resistance vs sensitivity. The authors demonstrated through *in vivo* studies in male mice (129Sv-C57Bl/6) that defects in glucose tolerance as well as changes in liver, fat and body weight occur upon deletion of both the regulatory PIK3R1 (p85 $\alpha$ ) and catalytic subunit PIK3CA (p110 $\alpha$ ) but retained normal production of glucoses from the liver.

**Specific Comments:**

1. Figure 1E (page 6): the authors state (on page 5) that low levels of p85 $\beta$  were detected in the L-DKO mice compared to controls. This was indirectly demonstrated through the use of a pan-p85 antibody that detects both p85 $\alpha$  and p85 $\beta$ . Mice with liver-specific deletion of p85 $\alpha$  on a p85 $\beta$  null background have been previously characterized (Taniguchi *et al.*). There, the overall levels of p85 $\beta$  detected utilizing a pan p85 $\beta$  antibody in a lysate derived from a liver that was wild type for p85 $\beta$  and null for p85 $\alpha$  was minimal. Therefore, it is possible that the overall protein content reduction observed using the pan p85 antibody in Figure 1E may be explained by the deletion of p85 $\alpha$  from the liver, and may not be due to any change in p85 $\beta$  levels. To directly compare wild type and L-DKO samples, a specific p85 $\beta$  antibody should be used. Loading controls should also be provided for some panels in Figure 1.
2. Figure 1F to show impaired activation, total AKT blots should be provided (similar to Figure 3E). Total levels of p70S6K also need to be measured. In Figure 1G, the authors state that although p110 $\beta$  levels are reduced, the amount associated with IRS-1 is unchanged. However, due to the variability across samples in both control and L-DKO, and the overall poor quality of the blot, these conclusions don't inspire confidence. These data should be quantitated. Additionally, the p110 $\beta$  band on the last lane appears to run slightly higher compared to the rest of the bands and the control raising possible questions over the specificity of the p110 $\beta$  antibody used for IP. Are similar results observed using a distinct antibody? In line with the comments in point 1, the authors state that the overall amount of p85 $\beta$  associated IRS-1 does not change. This conclusion requires use of a p85 $\beta$  selective antibody.
3. As noted by the authors, there is known complexity between the structural roles of the various PI3K subunits and isoforms, with effects on protein stability and access to IRS-1 binding sites. There appears, as noted above, to be significant variation in levels of the untargetted subunits across the 2 typically shown animals (e.g. Figure 1G). If other animals were measured, a composite of the levels (e.g. n=4) would provide much better insight into the levels (if any) of compensation, allowing a clearer picture of what may be underlying the glucose metabolic effects. It is also possible there was downstream adaptation given the IRS-1 is negatively regulated by PI3K signaling (and appears somewhat higher in the DKOs (Figure 1G).

4. For the PI3K lipid kinase activity measurements (chromatographic separation, Figure 2 shows the quantitation), how were the separate plates normalized?

Minor grammatical error:

Page 11, second paragraph line 10- "and" is added in error.

\*Taniguchi, C. M., T. Kondo, M. Sajan, J. Luo, R. Bronson, T. Asano, R. Farese, L. C. Cantley and C. R. Kahn (2006). "Divergent regulation of hepatic glucose and lipid metabolism by phosphoinositide 3-kinase via Akt and PKC $\lambda$ /zeta." *Cell Metab* 3(5): 343-353.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Signal transduction

**We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Author Response 21 May 2018

**Victoria Rotter Sopasakis**, University of Gothenburg, Sweden

1. We totally agree with the Dr Woodgett's point here and it is essentially what we tried to convey in the manuscript, but to make it clearer we have added an extra sentence including the reference to Taniguchi et al (page 9, row 7).

We have previously (before submitting the manuscript) tried various different p85b antibodies without any success. Some work well for human tumor tissue for example, but give no band at all for our mouse liver lysates. Other antibodies we tried fail to deliver a band for both human and mouse tissues. This is the reason we chose to use the p85-pan antibody to indirectly show p85b protein expression. Since both Dr Woodgett and Drs Lin and Ballou requested a p85b antibody we have tried again to make various p85b antibodies on the market to work for our liver lysates and

have spent months now trying to optimize the western blot conditions for these antibodies. We are not satisfied with the results. The only conclusion we can draw is that either 1) the p85b antibodies available on the market simply does not work well for mouse tissue or 2) there is not much p85b expressed in mouse liver (just as Dr Woodgett points out above). Based on the p85-pan westerns that we performed as well as previous studies including Taniguchi et al, to which Dr Woodgett referred, we believe that there simply is not that much p85b expressed in mouse liver. Loading controls have been added to figure 1 E and 1F, including Akt and p70S6K.

2.

- Total Akt blot has been added to figure 1F
- Total p70S6K has been added to figure 1F
- Blots in figure 1G (=new figure 1K) have been quantitated, except for p110d, which is so weak there is no point to quantify it. Graphs of the quantitation have been added to figure 1 (new figures 1L-P). n=4 in these quantification graphs, i.e we have added more samples for each condition to cover small variations in the immunoprecipitation and western blot experiments.
- p85b has not been added for the same reason as in figure 1E, see above.

3.

- In the quantification graphs added to figure 1F (new figure 1G-J) n=4, ie we have added new experiments with different samples. The WB pictures become very large when adding so many panels, so we decided including all four replicates in the quantification graphs. However, all blots are provided in the raw data sets for the referee to see.
- Same for figure 1G (new figure 1K), i.e n=4. Quantification graphs have been added (new figures 1L-P).

4. The plates were normalized to a reference sample = active p110a/p85 complex (see original plate pictures in the raw data sets).

5. Can not find this error. Please specify.

**Competing Interests:** No competing interests were disclosed.

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