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Original article

MOLECULAR METABOLISM

LKB1 and AMPKα1 are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia

Gao Sun [1], **Gabriela da Silva Xavier** [1], **Tracy Gorman** [2], **Claire Priest** [2], **Antonia Solomou** [1], **David J. Hodson** [1], **Marc Foretz** [3,4,5], **Benoit Viollet** [3,4,5], **Pedro-Luis Herrera** [6], **Helen Parker** [7], **Frank Reimann** [7], **Fiona M. Gribble** [7], **Stephanie Migrenne** [8], **Christophe Magnan** [8], **Anna Marley** [2], **Guy A. Rutter** [1,*]

ABSTRACT

Aims/Hypothesis: Glucagon release from pancreatic alpha cells is required for normal glucose homoeostasis and is dysregulated in both Type 1 and Type 2 diabetes. The tumour suppressor LKB1 (STK11) and the downstream kinase AMP-activated protein kinase (AMPK), modulate cellular metabolism and growth, and AMPK is an important target of the anti-hyperglycaemic agent metformin. While LKB1 and AMPK have emerged recently as regulators of beta cell mass and insulin secretion, the role of these enzymes in the control of glucagon production *in vivo* is unclear.
Methods: Here, we ablated LKB1 (αLKB1KO), or the catalytic alpha subunits of AMPK (αAMPKdKO, -α1KO, -α2KO), selectively in ~45% of alpha cells in mice by deleting the corresponding *flox'*d alleles with a preproglucagon promoter (*PPG*) *Cre*.
Results: Blood glucose levels in male αLKB1KO mice were lower during intraperitoneal glucose, aminoimidazole carboxamide ribonucleotide (AICAR) or arginine tolerance tests, and glucose infusion rates were increased in hypoglycemic clamps ($p < 0.01$). αLKB1KO mice also displayed impaired hypoglycemia-induced glucagon release. Glucose infusion rates were also elevated ($p < 0.001$) in αAMPKα1 null mice, and hypoglycemia-induced plasma glucagon increases tended to be lower ($p = 0.06$). Glucagon secretion from isolated islets was sensitized to the inhibitory action of glucose in αLKB1KO, αAMPKdKO, and -α1KO, but not -α2KO islets.
Conclusions/Interpretation: An LKB1-dependent signalling cassette, involving but not restricted to AMPKα1, is required in pancreatic alpha cells for the control of glucagon release by glucose.

Keywords LKB1; AMPK; Glucagon secretion; PPG; Knockout; Alpha cell

1. INTRODUCTION

Glucagon, secreted by the pancreatic islet alpha cell as blood glucose levels fall, is the key anti-hypoglycemic hormone in mammals, acting as a powerful stimulus for hepatic glucose production [1]. Impaired glucagon release is associated with the episodes of hypoglycemia frequently reported in Type 1 and advanced Type 2 diabetic (T2D) patients treated with insulin or sulphonylureas [2]. On the other hand, elevated circulating glucagon levels contribute to the chronic increase in blood glucose levels characteristic of T2D [1].

Lowered blood glucose levels stimulate glucagon release through multiple mechanisms including changes in parasympathetic [3] and sympathetic tone [4], increases in circulating adrenaline levels [5], as well as through effects of glucose on pancreatic alpha cells [6]. The exact nature of the latter are still debated. Thus, in the mouse, a direct effect of glucose [7,8] and indirect effects of γ-amino butyric acid (GABA) [9,10] and insulin [7,11] released from neighbouring beta cells have all been invoked as regulators of glucagon secretion. Roles for released Zn^{2+} ions have also been proposed [7,12–14].

AMP-activated protein kinase (AMPK) is a heterotrimeric complex comprising α, β and γ subunits that serves as a master energy sensor [15], highly sensitive to intracellular ATP:AMP [15] and ATP:ADP ratios [16]. Liver kinase B1 (LKB1), also called STK11, is a tumour suppressor whose inactivation leads to Peutz-Jegers syndrome [17], characterized by hamartomatous polyps and an increased risk of all cancers. LKB1 phosphorylates at least 13 different protein kinases including AMPK. LKB1 (and alternative protein kinases including calmodulin kinase kinase β [18,19] and transforming growth factor β-activated kinase-1, TAK1) [20], phosphorylate AMPK catalytic α-subunits at Thr172 in the "T-loop". A fall in intracellular energy, leading to

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[1] Section of Cell Biology and Functional Genomics, Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Imperial College London, London, UK [2] AstraZeneca, Alderley Edge, Cheshire, UK [3] Inserm, U1016, Institut Cochin, Paris, France [4] CNRS, UMR8104, Paris, France [5] Université Paris Descartes, Sorbonne Paris cité, Paris, France [6] Department of Genetic Medicine & Development, Faculty of Medicine, University of Geneva, Geneva, Switzerland [7] Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge, UK [8] University Paris Diderot-Paris 7-Unit of Functional and Adaptive Biology (BFA) EAC 7059C NRS, France

*Corresponding author. E-mail: g.rutter@imperial.ac.uk (G.A. Rutter).

Abbreviations: AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; T2D, Type 2 diabetes; PPG, preproglucagon promoter; AICAR, aminoimidazole carboxamide ribonucleotide

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altered occupancy by AMP and/or ADP of sites in the γ-subunit, leads to conformational changes that render T172 in the α-subunit less susceptible to dephosphorylation [16] by protein phosphatases [21]. Activated AMPK then phosphorylates a range of downstream metabolic targets to promote ATP synthesis and inhibit ATP consumption [15].

Through the use of pharmacological AMPK activators and inhibitors [22], we have previously provided evidence that AMPK is a negative regulator of insulin secretion from pancreatic beta cells [23,24]. Furthermore, activation of AMPK by metformin is associated with enhanced beta cell apoptosis [25]. Correspondingly, mice deleted selectively in the beta cell for LKB1 display beta cell hyperplasia and markedly enhanced insulin release [26—28]. By contrast, and unexpectedly, deletion of both AMPK catalytic subunits (α1 and α2) from beta cells causes impaired insulin secretion and glucose intolerance *in vivo* [29,30], possibly as a result of rat insulin promoter-2 (*RIP2*)-*Cre*-mediated recombination in the brain [31].

We have also recently provided evidence from *in vitro* studies that AMPK is involved in the regulation of glucagon release. Thus, activation of AMPK stimulated glucagon release from clonal αTC1-9 cells and mouse pancreatic islets, while a dominant-negative form of the kinase blocked the stimulatory effects of low glucose [22]. However, neither the role of LKB1 nor that of individual AMPK isoforms in controlling glucagon secretion has been examined *in vivo*.

Here, we have generated mice deleted for LKB1 (αLKB1KO), and for one (AMPKα1KO, -α2KO) or both (αAMPKdKO) AMPKα catalytic subunits selectively in pancreatic alpha cells using preproglucagon (*PPG*)-*Cre* driven recombination. We show that LKB1 signalling, at least partly mediated via AMPKα1, is essential for the normal stimulation of glucagon secretion at low glucose levels both *in vitro* and *in vivo*. Moreover, and in marked contrast to its action in the beta cell, we show that LKB1 plays a limited if any role in the control of alpha cell size or total alpha cell mass [26—28].

2. METHODS

2.1. Generation of mutant mice lacking LKB1 selectively in pancreatic alpha cells

Mice heterozygous for *flox*'d alleles of the *Lkb1/Stk11* gene (mixed FVB/129S6 and C57BL/6 background) [32] were obtained from the Mouse Models of Human Cancer Consortium (MMHCC) (www.nih.gov/science/models/mouse/resources/hcc.html) and backcrossed with C57/B6 mice four times. These animals were then bred against *PPG-Cre* expressing mice [33] and the resulting heterozygous offspring were inter-crossed as siblings to generate αLKB1KO mice (*Lkb1*$^{fl/fl}$, *Cre* positive). αLKB1KO mice were further bred with *Lkb1*$^{fl/fl}$ mice to generate littermate controls (*Lkb1*$^{fl/fl}$). It should be noted that the *PPG-Cre* transgene is not reported to exert effects on glucagon secretion or glucose homoeostasis [34] or to lead to significant recombination in extra-pancreatic tissues [11]. αLKB1KO mice and littermate controls were born at the expected Mendelian ratios.

2.2. Generation of mutant mice selectively lacking AMPK α1 and α2 in pancreatic alpha cells

Mice homozygous for *Ampkα1*$^{fl/fl}$ were crossed with mice heterozygous for *Ampkα2*$^{fl/+}$. The resulting double heterozygotes (*Ampkα1*$^{fl/+}$, *α2*$^{fl/+}$) were crossed with *PPG-Cre*-expressing animals [33] to generate triple heterozygous mice (*Ampkα1*$^{fl/+}$, *α2*$^{fl/+}$, *Cre* positive). The latter were then bred with mice homozygous for both *flox*'d *Ampkα1* and *α2* alleles (*Ampkα1*$^{fl/fl}$, *α2*$^{fl/fl}$) to produce αAMPKdKO mice (*Ampkα1*$^{fl/fl}$, *α2*$^{fl/fl}$, *Cre* positive). αAMPKdKO mice were further crossed with *Ampkα1*$^{fl/fl}$, *α2*$^{fl/fl}$ mice to generate littermate controls (*Ampkα1*$^{fl/fl}$, *α2*$^{fl/fl}$). All mice were kept on a C57/B6 background.

2.3. Generation of mice selectively expressing RFP in pancreatic alpha cells

Mice heterozygous for Rosa26tdRFP [35] were crossed with mice heterozygous for *PPG-Cre* to generate double heterozygous mice.

2.4. Mouse maintenance and diet

Animals were housed two to five per individually ventilated cage in a pathogen-free facility with 12 h light/dark cycle and had free access to standard mouse chow diet. All *in vivo* procedures described were performed at the Imperial College Central Biomedical Service and approved by the UK Home Office according to the Animals (Scientific Procedures) Act 1986 of the United Kingdom (PPL 70/7349).

2.5. Glucose, insulin, AICAR and arginine tolerance tests

Intraperitoneal glucose and insulin tolerance tests were performed as previously described [28,30]. For AICAR tolerance, mice fasted for 16 h (water allowed) were intraperitoneally injected with 1.75 g AICAR/kg (Toronto Research Chemicals, North York, Canada). Blood (2—4 μl) from the tail vein was obtained at 0, 20, 40, 60, 90 and 120 min after injection [36]. Blood glucose levels were measured with an automatic glucometer (Accuchek; Roche, Burgess Hill, UK). For arginine tolerance, mice fasted for 16 h (water allowed) were intraperitoneally injected with 3 g/kg L-arginine (pH 7.4; Arginine hydrochloride from Sigma) [11]. Blood (50 μl) from the tail vein was collected at 0, 10 and 30 min after injection into EDTA coated tubes containing 1 μl DPP IV inhibitor (final concentration: 100 μmol/l; Millipore, Watford, UK). Plasma insulin and glucagon levels were measured with an ultrasensitive mouse insulin ELISA kit (Mercodia, Uppsala, Sweden) [37] and a glucagon radioimmunoassay kit with a competitive ^{125}I labelled glucagon (Millipore, Watford, UK) [7] respectively. Experiments were performed on mice aged 8—12 weeks.

2.6. Hyperinsulinemic-hypoglycemic clamp

Nine week-old male mice were implanted with catheters in the right jugular vein under general ketamine (100 mg/kg)/xylazine (10 mg/kg) anaesthesia. Catheters were flushed daily with saline containing heparin (0.4U/100 ml). Mice were housed individually in separate cages for post-surgery recovery. On the day of the clamp study, mice were starved for five hours before catheters were connected onto infusion tubing. Mice were infused with bolus insulin (0.033U) at an infusion rate of 30 μl/min for 5 min. To maintain blood glucose levels close to 2.7—3.3 mmol/l, insulin (0.6 U/kg/h) were infused for 120 min, during which 20% glucose was co-infused with adjustable infusion rates. Blood was collected from tail veins for measurement of glucose levels every 10 or 20 min.

2.7. Other methods

Please see Supplementary Materials and Methods.

2.8. Statistical analysis

Significance was tested using unpaired or paired Student's two-tailed *t*-tests with Bonferroni post-tests for multiple comparisons, or two-way ANOVA with Sidak post-doc tests as required. Analysis was performed using Excel (Microsoft) and Graphpad Prism 4.0 (Graphpad Software). $p < 0.05$ was considered significant and values represent mean ± SEM.

3. RESULTS

3.1. Generation of mice deleted selectively in the pancreatic alpha cell for LKB1 or AMPKα-subunits

To assess the likely efficiency and specificity of recombination in alpha cells, we first crossed mice bearing a tandem dimer red fluorescent protein transgene at the Rosa26 locus distal to a STOP-*Lox*P-STOP control cassette (Rosa26tdRFP) [35] against animals bearing *Cre* recombinase under the control of a 0.6 kb fragment of the rat pre-proglucagon promoter (*PPG*) [38]. This led to almost exclusive expression of *PPG-Cre* in glucagon-positive pancreatic alpha cells, of which ~45% were positive for tdRFP (Figure 1A(i)). Expression in

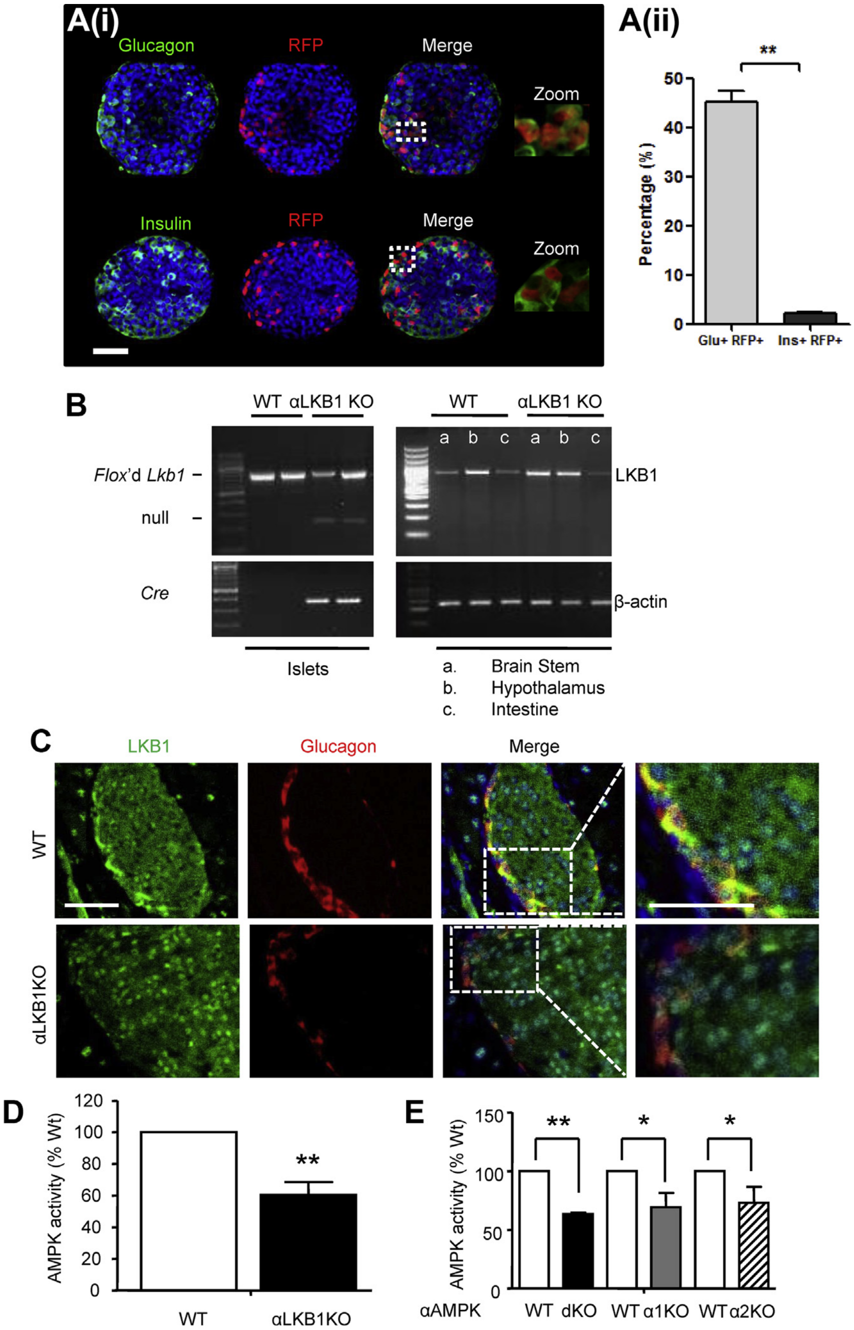

Figure 1: Generation of alpha cell specific LKB1 and AMPK α subunit(s) KO mice (**A**) (i) Representative immunostaining of pancreatic islets isolated from *PPG-Cre:RFP* mice using antibodies against RFP (1:100) and either glucagon (1:2000; green) or insulin (1:200; green). Nuclei are shown by DAPI (blue) staining. (ii) Images of stained islets (5–8) were used to calculate the percentage of cells positive for glucagon or insulin and co-expressing RFP. (**B**) RT-PCR analysis of deletion of exon 3–6 on LKB1 transcript levels in pancreatic islets, brain stems, hypothalamus and intestines. The product sizes were 864 and 300bp for the *flox*'d and null alleles, respectively. (**C**) Representative immunofluorescence staining of pancreatic sections using mouse anti-glucagon (1:2000; red) and rabbit anti-LKB1 (1:100; green) antibodies. Nuclei are shown with DAPI (blue). Scale bar, 75 µm. (**D,E**) Total AMPK activity in islets of αLKB1KO (**D**), αAMPK KOs (**E**) and their wild type littermate control mice (WT). Islets incubated with RPMI media containing 10 mmol/l glucose for 48–72 h, were further incubated with RPMI media containing 3 mmol/l glucose for 1 h before analysis. Data are expressed as means ± SEM; $n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$ between groups using unpaired two-tailed Student's t-tests.

other cell types was below the limit of reliable detection (<3% of insulin-positive cells showed weak staining, likely attributable to background fluorescence; Figure 1A(ii)).

We next generated mice deleted for LKB1 in the alpha cell compartment (αLKB1KO) by crossing animals in which exons 3–6 of the *Lkb1* gene were flanked with *Lox*P sites [32] with *PPG-Cre* mice [33]. The excision of exons 3–6 mediated by *Cre* was observed in pancreatic islets of αLKB1KO mice, but not evident in other preproglucagon-expressing tissues, such as brain stem, hypothalamus, and intestine or in islets from wild type mice (Figure 1B), consistent with previous findings [38]. Correspondingly, LKB1 immunoreactivity was reduced selectively in glucagon-positive cells in pancreatic sections from αLKB1KO mice (Figure 1C). Moreover, total AMPK activity was reduced by almost 40% in whole islets from these mice (60.5 ± 8.1% of wild type, $p < 0.05$; Figure 1D) compatible with a higher expression of LKB1 in alpha than beta cells (ratio of *Lkb1* to *cyclophilin* A mRNA: 0.36 ± 0.09 in FACS-purified alpha *vs.* 0.11 ± 0.03 in beta cells, $p < 0.05$; Figure S1).

Similarly, we created mice deleted for both (αAMPKdKO) or single (αAMPKα1KO and -α2KO) catalytic α-subunits selectively in pancreatic alpha cells by crossing either double AMPKα1 and α2 or single α-isoform *flox*'d mice with *PPG-Cre* deleter mice as above. *Cre*-mediated recombination led to decreases in AMPK activity of ∼37%, ∼32% and ∼27% in islets from αAMPKdKO, -α1KO and -α2KO mice, respectively (Figure 1E), the more modest change in islets from αAMPKdKO mice, possibly reflecting compensatory changes in the expression or activity of the non-deleted isoform or one or more of the 12 other AMPK-related kinase(s) [39].

Consistent with the above demonstration that no or minimal recombination occurred in enteroendocrine L-cells [38], fed plasma GLP-1 levels were identical between genotypes (19.2 ± 4.8 pg/ml in αLKB1KO mice, $n = 3$–5 *vs.* 19.5 ± 1.5 pg/ml in WT, $n = 3$–5; 11.4 ± 0.23 pg/ml, $n = 6$ in αAMPKdKO mice *vs* 13.9 ± 1.97 pg/ml, $n = 4$ in littermate controls). Likewise, supporting the finding that deletion did not occur in ventromedial hypothalamic feeding centres [11], we observed no significant differences of body weight or daily food intake of αLKB1KO or αAMPK KOs compared to wild-type littermates (Figure S2a–c).

3.2. Pancreatic alpha cell-selective deletion of LKB1, but not AMPKα-subunits moderately lowers blood glucose and glucagon levels

Examined at eight weeks of age, male αLKB1KO mice maintained lower overnight fasting blood glucose levels (Figure 2A), while the levels in αAMPKKOs and their littermate controls were comparable (Figure S2d–f). Consistent with these findings, fasting plasma glucagon levels were significantly decreased after LKB1 deletion from alpha cells (Figure 2B), with tendencies towards decreased plasma glucagon in αAMPKα1KO and αAMPKdKO mice *versus* controls (Figure S2g-i). Plasma insulin levels were not different between

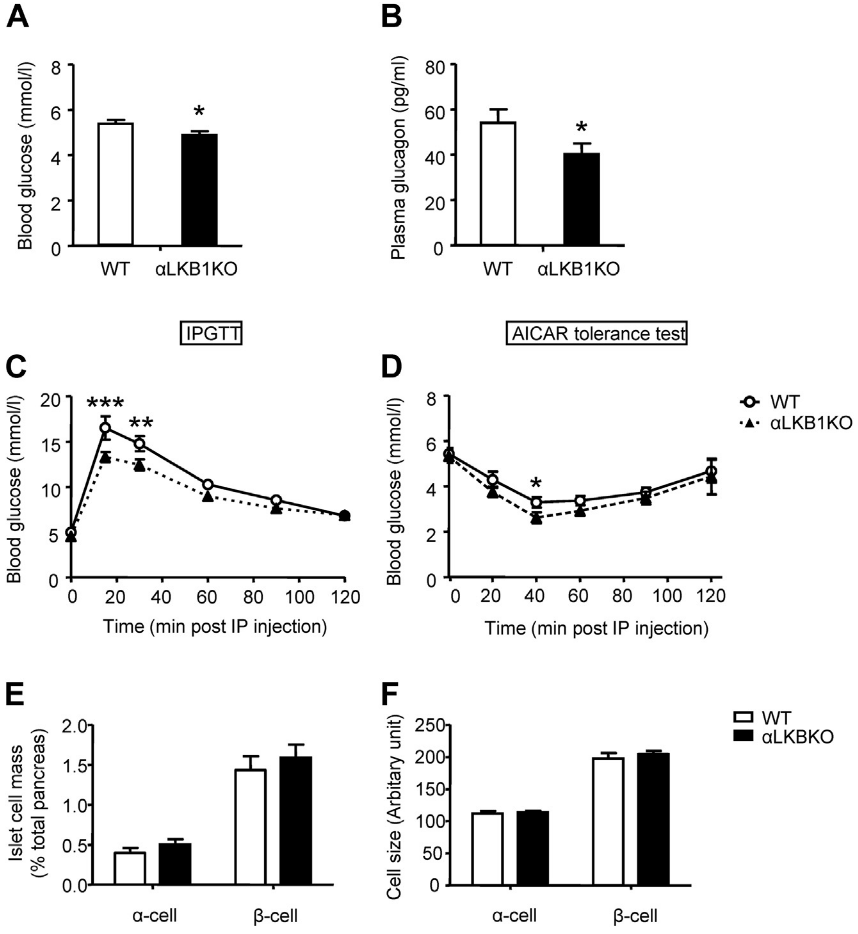

Figure 2: Male αLKB1KO mice display lowered fasting glucose and glucagon levels, and improved glucose and AICAR tolerance. Fasting blood glucose (**A**) and plasma glucagon levels (**B**), and intraperitoneal glucose (**C**) and AICAR (**D**) tolerance of 8 week-old male mice. Data are expressed as means ± SEM; $n = 12$–22 (**A**, **B**) and 7–12 (**C**, **D**) mice per genotype. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between groups using unpaired two-tail t-tests (**A**, **B**, **E**, **F**) or two-way ANOVA with Sidak post hoc comparison (**C**, **D**).

genotypes in the fasted state (0.069 ± 0.022 nmol/l in αLKB1KO, $n = 5$ *vs.* 0.078 ± 0.019 nmol/l in WT, $n = 5$). However, eight week-old male αLKB1KO mice displayed significantly improved tolerance to intraperitoneal (IP) injection of glucose (Figure 2C), with comparable insulin tolerance to wild type littermate controls (15 min post injection: 62.8 ± 3.8% of initial value in αLKB1KO *vs.* 70.9 ± 4.3% in wild type; Figure S3a). None of the αAMPK KO mice displayed any alterations in insulin (Figure S3b-d) or glucose tolerance (Figure S4a, c, e).

To examine the effects of LKB1 deletion in alpha cells in response to hypoglycemia we first challenged mice with the AMP mimetic aminoimidazole carboxamide ribonucleotide (AICAR) which activates AMPK in extrapancreatic tissues (notably liver and muscle) contributing to the effects of this agent to lower blood glucose levels [40]. As shown in Figure 2D, AICAR sharply reduced blood glucose levels in overnight-fasted eight week-old control male mice from 5.72 ± 0.29 mmol/l at 0 min to 3.37 ± 0.21 mmol/l at 40 min post injection ($n = 8$). The same manoeuvre lowered blood glucose even more dramatically, from 5.50 ± 0.30 mmol/l to 2.70 ± 0.22 mmol/l in αLKB1KO mice (51 ± 3 *vs.* 40% ± 3% decrease in KO and wild type respectively, $p = 0.04$). However, plasma glucagon levels at 40 min post AICAR injection were comparable between genotypes (354.0 ± 62.3 pg/ml in KO vs. 501.7 ± 141.9 pg/ml in WT, $n = 5$; data not shown). These results suggest that αLKB1KO mice might develop enhanced peripheral sensitivity to AICAR rather than a blunted glucagon response. None of the above differences were apparent in any of the αAMPK KO mice (Figure S4b, d, f).

We next tested whether lower fasting glucagon levels, as observed in αLKB1KO mice, may affect the expression of gluconeogenic genes in the liver [41]. Of three genes (PEPCK, G6Pase and PGC-1α) examined in 24 week-old male αLKB1KO *versus* control animals, none showed significant differences in expression between genotypes (Figure S4g).

L-arginine stimulates hepatic glucose production, and glucagon and insulin secretion respectively from pancreatic alpha and beta cells [42]. In response to IP injection of L-arginine (3 g/kg) blood glucose levels rose from 5.78 ± 0.27 to 6.95 ± 0.29 mmol/l in wild type mice and from 5.57 ± 0.27 to 5.68 ± 0.41 mmol/l in αLKB1KO mice 10 min after injection (Figure S5a, KO *vs.* wild type, p = 0.007). Correspondingly, at the same time point, plasma glucagon levels tended to be lower in αLKB1KO (290.5 ± 46.8 pg/ml) *vs.* wild type mice (389.9 ± 38.9 pg/ml; p = 0.07; Figure S5b). Plasma insulin levels at this time point were not different between genotypes (Figure S5c).

3.3. Enhanced glucose infusion rates in αLKB1KO and αAMPKα1KO mice *versus* controls under hyperinsulinemic-hypoglycemic clamp and impaired glucagon release during insulin tolerance tests

The above assessments of glucagon release in response to AICAR may conceivably be complicated by impaired AMPK activation in the alpha cell of αLKB1KO mice. We therefore further explored the impact of deleting LKB1 or AMPKα isoforms in the alpha cell using Hyperinsulinemic-hypoglycemic clamps. When αLKB1KO (Figure 3A, B) or αAMPKα1KO (Figure 3C, D) mice were maintained under hypoglycemic conditions by constant intravenous insulin infusion, significantly higher glucose infusion rates were required in each case to maintain ∼3 mmol/l glucose compared to the corresponding littermate controls. Furthermore, and despite showing similar responses in terms of glycaemia during insulin tolerance tests (Figure 3E), αLKB1KO animals showed impaired glucagon secretion under these conditions (Figure 3F). Likewise, αAMPKα1KO mice showed a strong tendency towards lowered glucagon secretion (p = 0.06at 30 min, Figure 3G,H). These data support the view that enhanced glucose disposal observed during clamps in each case was likely due to impaired glucagon release.

No differences were observed in glucose disposal between αAMPKdKO mice *vs* controls (Figure S6).

3.4. αLKB1KO and αAMPK KO mice display unaltered alpha cell morphology, total mass and single cell size

The above results together suggested that deletion of LKB1 or AMPKα1 from the pancreatic alpha cell impairs glucose deprivation-induced glucagon release *in vivo*. To determine whether this may be due to a decrease in alpha cell mass, or in the size of individual alpha cells, we measured these parameters by immunohistochemical analysis of pancreatic sections from control, αLKB1KO, αAMPKα1KO or -dKO mice and corresponding controls (Figure 4). This analysis revealed the expected distribution of cell types within islets in each case, with beta cells concentrated in the core and alpha cells located mainly in the mantle (Figure 4A). Quantification of total alpha cell mass (Figure 4B, E, G) and of single alpha cell size after labelling of the plasma membrane with E-cadherin antibodies (Figure 4C) did not reveal significant genotype-dependent differences (Figure 4D, F, H). Correspondingly, growth factor signalling through the phosphatidylinositol 3′ kinase / Akt / mTOR pathway, which is enhanced after deletion of LKB1 from beta cells [28], was unaffected by the same manipulation in alpha cells (Figure S7). Thus, the degree of phosphorylation of ribosomal protein S6 or of its upstream kinase, S6 kinase (both downstream targets of mTOR signalling) [43], was not different in αLKB1KO *versus* control alpha cells (Figure S7).

3.5. Glucagon secretion is sensitized to inhibition by glucose in islets isolated from αLKB1KO or αAMPKα1KO mice

The above studies suggested that glucagon secretion from alpha cells is likely to be impaired *in vivo* after the deletion of LKB1 or AMPKα1 by mechanisms which do not affect total alpha cell mass. To explore this possibility, we investigated the effects of genomic deletion of these kinases on the regulation of glucagon secretion from pancreatic alpha cells using islets isolated from αLKB1 KO or αAMPK KO (-α1, -α2, dKO) mice. Whereas glucagon release at a low, stimulatory concentration (0.1 mmol/l) of glucose was comparable in islets from mice of all genotypes, αLKB1KO or αAMPKα1KO mice released ∼2.5-fold less glucagon in 30 min at 3 mmol/l glucose than the corresponding wild-type islets (Figure 5A,B). Comparable rates of secretion were observed from islets of all genotypes after incubation at 17 mmol/l glucose (Figure 5). Indeed, glucagon secretion from islets of αLKB1KO or αAMPKα1KO mice was maximally inhibited at 3 mmol/l glucose, indicating enhanced sensitivity to the inhibitory effects of the sugar. By contrast, the dose response to glucose of insulin secretion was not different between wild-type or KO islets in either case (data not shown), arguing against the possibility that the enhanced inhibitory effect of 3 mmol/l glucose on glucagon secretion was due to altered insulin or other beta cell secretion [1]. Furthermore, normal stimulatory effects on glucagon secretion in response to 5 μmol/l epinephrine (at 17 mmol/l glucose) were preserved in αLKB1KO (Figure 5A) and in αAMPKαKO (not shown) mouse islets.

Ablation of AMPKα2 (Figure 5C) or of both AMPKα isoforms (Figure 5D) had no effect on the response of glucagon release to 3 mmol/l (*vs* 0.1 mmol/l) glucose though we noted that the response to 17 mmol/l glucose was impaired after the loss of AMPKα2 (Figure 5C).

4. DISCUSSION

The overall aim of the present study was to explore the cell-autonomous role of LKB1 and of two key downstream kinases in the

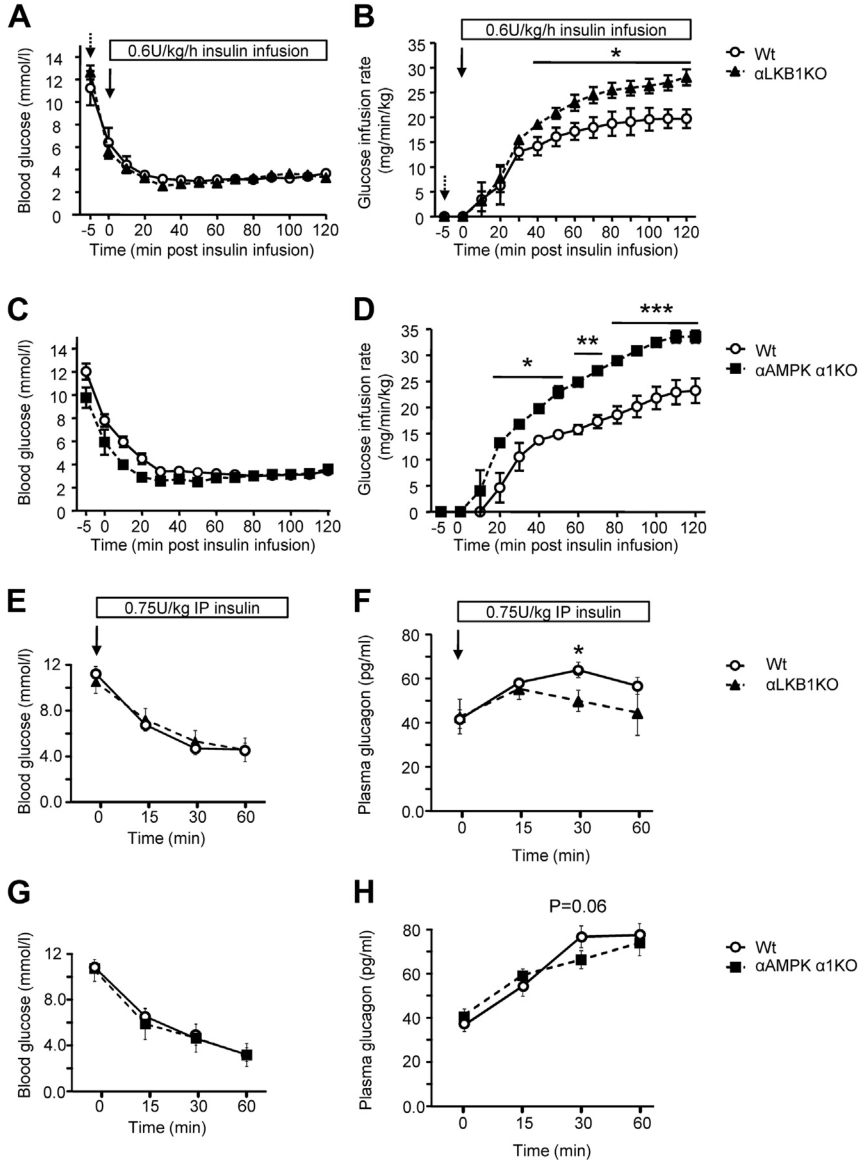

Figure 3: Both αLKB1KO and αAMPKα1KO mice exhibit enhanced glucose sensitivity during hyperinsulinemic-hypoglycemic clamps and impaired glucagon release during insulin tolerance tests. Blood glucose levels (**A**, **C**) and glucose infusion rates (**B**, **D**) of αLKB1KO (**A**, **B**) or αAMPKα1KO mice (**C**, **D**) and their wild type littermate controls during insulin (0.6 U/kg/h) and glucose (20%) co-infusion. Arrow heads with dashed lines, bolus insulin infusion; arrow heads with solid lines, constant insulin (0.6 U/kg/h) infusion. (**E,F**) Changes in plasma glucose (**E**) and glucagon (**F**) after injection of 0.75 U/Kg into wild type or αLKB1KO mice. (**G,H**) as (**E,F**), using AMPKdKO mice and controls. Data are expressed as means ± SEM; n = 4–7 mice per genotype. *p < 0.05, **p < 0.01, ***p < 0.001 between groups by two-way ANOVA with Sidak post hoc comparison (**A**–**D**) or two-tailed Student's t-test with Bonferroni correction (**E**–**H**).

control of glucagon production in the mouse. The concept that modulation of glucagon levels may represent a useful therapeutic approach to some forms of diabetes has existed for many years [44] and was further supported recently by two separate studies in which mice null for glucagon receptors were shown to be resistant to beta cell destruction by streptozotocin induced diabetes [45,46]. On the other hand, substantial (>90%) [38] ablation of alpha cell mass using diphtheria toxin has relatively modest effects on glucose homoeostasis suggesting alpha cell plasticity under some conditions.

4.1. *PPG-Cre* expression is localized in alpha cells

The present (Figure 1A) and previous [33] findings suggest that *PPG-Cre* leads to recombination with high selectivity, but with limited efficiency (∼45%) in alpha cells. Despite this, apparent AMPK activity was markedly (40%) reduced, presumably reflecting the enrichment of LKB1 in alpha and other islet cells [22] and possibly differences between cell types in the expression, stability or regulation of AMPK subunits, or other regulatory enzymes (phosphatases, e.g.). We note that the potential loss of RFP fluorescence during the staining procedure, as well as differences in recombination efficiency at the Rosa26 *versus* the LKB1 *loci*, means that these measurements may in any case under (or over-) estimate of the degree of recombination at the latter.

Nonetheless, we observed changes in glucagon secretion in mice deleted for LKB1 or AMPKα1 with this strategy, suggesting an indispensable role of these kinases in the pancreatic alpha cell. Furthermore, it might be speculated that recombination targets a subset of alpha cells which display a greater degree of control of the whole alpha cell population than others, i.e. that these cells play a "hub" or

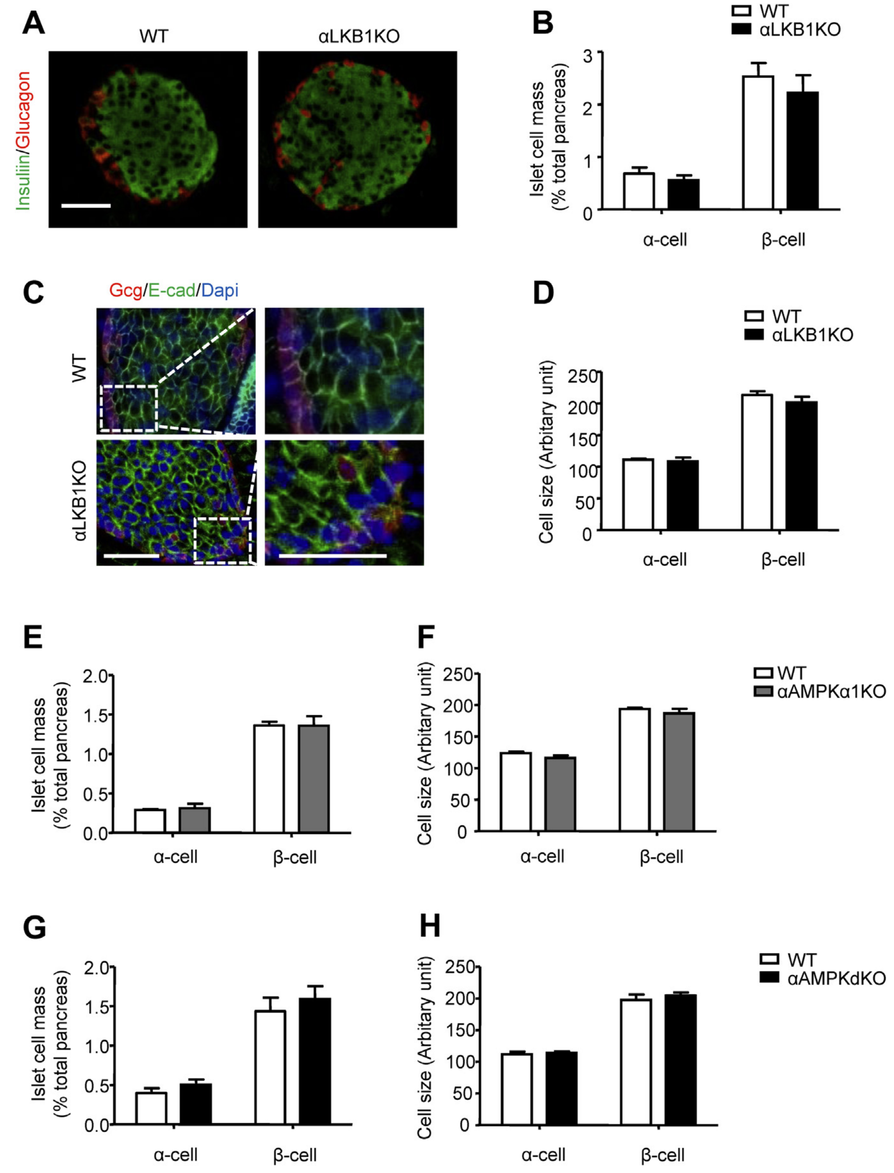

Figure 4: αLKB1KO and αAMPKα1KO, -dKO mouse islets show normal alpha cell morphology, size and mass. (**A**) Representative immunostaining of pancreatic sections using guinea pig anti-insulin (green, 1:200) and mouse anti-glucagon antibodies (Red, 1:2000). (**B**, **E**, **G**) Quantitation of relative α- and β-cell mass based on immunostaining of pancreatic sections. Relative islet cell mass was calculated by expressing total glucagon (α-cell)- or insulin (β-cell)-positive area as a proportion of total autofluorescent pancreatic area. Three to four sections per pancreas were analyzed. (**C**) Representative glucagon (red) and E-cadherin (green) staining of pancreatic sections. Nuclei are shown by DAPI (blue) staining. (**D**, **F**, **H**) Quantification of single alpha and beta cell size. Data are expressed as means ± SEM. $n = 5$ mice per genotype. Scale bar, 40 μm. No statistically significant changes were detected (two-tailed t-test with Bonferroni correction).

"pacemaker" function [47]. Although a comparison with the effects of more complete deletion of LKB1 or AMPKα1 throughout the alpha cell population would test this hypothesis, alternative alpha cell-targeting strategies, e.g. the "iGlu.Cre" mouse [35], can lead to off-target effects [48].

It is relevant to mention that our studies were limited to examination of the phenotype of animals maintained on the low fat (~7% of calories) diet usually used in mouse studies, a diet with a fat value much lower than that of the typical westernized human diet (20–25% fat) [49]. Future studies of the knockout models generated here, both in the context of "humanized" diets and after breeding to backgrounds susceptible to diabetes (*db/db*, e.g.), may also provide interesting information on the role of LKB1 (and AMPK) in controlling glucagon secretion from alpha cells in pathological states in man.

Finally, and although the present findings might seem at odds with the fact that deletion of the majority of the alpha cell complement exerts minor effects on glucose tolerance [38], we note that the effects of hypoglycemia were not examined in this earlier study.

4.2. Deletion of LKB1 in alpha cells does not alter cell size or total pancreatic alpha cell mass

Inactivation of LKB1 selectively in pancreatic beta cells dramatically enhances total insulin output and beta cell mass [26,28]. These changes are accompanied by up-regulation of mTOR signalling [26–

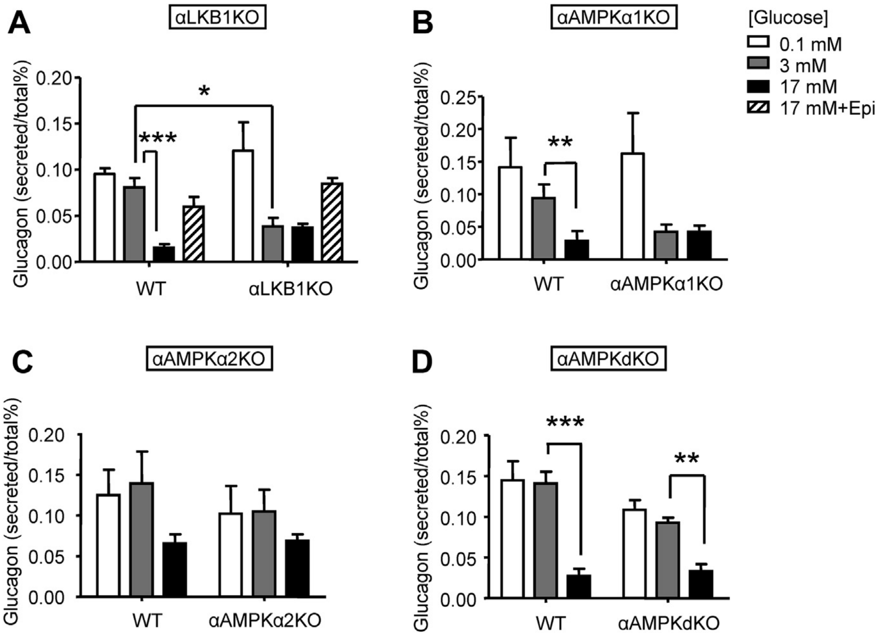

Figure 5: αLKB1 KO and αAMPKα1, -dKO mouse islets secrete less glucagon in response to low (3 mmol/l) glucose. Glucagon secretion from 12 size-matched islets isolated from αLKB1KO (**A**), α AMPKα1KO (**B**), α2KO and (**C**), dKO mice (**D**) and their respective wild type littermate controls, statically incubated for 1 h at 0.1 mmol/l (white bar), 3 mmol/l (grey bars), 17 mmol/l (black bars) glucose, or 17 mmol/l glucose in the presence of 5 μmol/l epinephrine. Eight week-old male mice were used. Data are expressed as means ± SEM; $n = 3$–5. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using two-way ANOVA followed by Sidak post-hoc comparison.

28]. Strikingly, deletion of LKB1 from alpha cells here did not affect overall pancreatic alpha cell mass or individual alpha cell size (Figure 4A–D). Such changes, therefore, cannot explain the observed decreases of glucagon output (Figure 2B) and counter-regulation (Figures 2D and 3) seen *in vivo* in αLKB1KO mice. Analysis of phospho-S6 kinase levels by Western blotting, or staining for phospho-S6 ribosomal protein (rpS6), did not reveal changes in these parameters in alpha cells from αLKB1KO mice compared to controls (Figure S7). Thus, attenuation of mTOR signalling by LKB1 appears to be absent from alpha cells for reasons that are presently unclear.

4.3. LKB1 regulates glucagon secretion from pancreatic alpha cells

The mechanisms through which glucose regulates glucagon release from the pancreatic alpha cell are still incompletely understood. As glucose levels fall, the partial opening of ATP-dependent K^+ channels and of low voltage-gated Ca^{2+} and Na^+ channels has been proposed to activate high voltage-gated Ca^{2+} channels to stimulate glucagon secretion [50]. In an alternative model [51], diminished ATP-dependent Ca^{2+} accumulation by the endoplasmic reticulum may modulate Ca^{2+} influx.

We show here that mice null for LKB1 in pancreatic alpha cells exhibit modestly reduced plasma glucose and glucagon levels *in vivo* (Figure 2A,B) and enhanced insulin sensitivity during hypoglycemic clamps (Figure 3A,B), likely due to reduced glucagon secretion from pancreatic alpha cells. Measurement of portal vein glucagon levels during clamps, which better reflect changes in glucagon secretion from the pancreas than systemic concentrations [52], present a significant technical challenge in the mouse. Moreover, measurement of glucagon from tail vein samples requires the collection of relatively large blood volumes, known to cause substantial increases in catecholamine levels [53]. Consequently, such measurements were not performed here. Nonetheless, examined in isolated islets, inactivation of LKB1 unmasked a clear inhibitory effect of 3 mmol/l glucose on glucagon secretion, a concentration of the sugar at which release was unaffected (versus the rate of release at 0.1 mmol/l glucose) in control islets (Figure 5A). This result is in sharp contrast to the effects of inactivating LKB1 in pancreatic beta cells, where large increases (after one week of deletion in adults) [26,27] or mild decreases (inactivation from E9-11) [28] are seen in glucose-stimulated insulin secretion.

As discussed above, the present results indicate that the absence of LKB1 from alpha cells elicits a marked left-shift in sensitivity to glucose. The mechanism(s) involved will require further investigation but may involve either longer term changes in the expression of key transporters and enzymes involved in glucose sensing (e.g. glucokinase, Ca^{2+} channels) or a more acute role of LKB1 in mediating the metabolic responses to the sugar, as previously proposed [22]. Detailed transcriptomic and proteomic analyses of purified LKB1-null alpha cells will be required to address the former possibility. Of note, since responses to L-arginine were abnormally reduced in αLKB1KO mice compared to controls (Figure S5b) it seems likely that late events in the triggering of glucagon secretion (e.g. secretory granule fusion at the plasma membrane) require LKB1 and/or its downstream targets.

AMPK is the best studied downstream target of LKB1 and plays key roles in the responses to the anti-diabetic drug, metformin [54]. In contrast to the situation in the beta cell [28,30] we reveal here that AMPKα1 is likely, at least in part, to mediate the effects of LKB1 on glucagon secretion in the alpha cell (Figures 3 and 5). Thus, elimination of AMPKα1 partly mimicked the effects of LKB1 deletion of counter-regulatory responses (Figure 3) and on the control of glucagon release (Figure 5), while loss of AMPKα2 exerted more subtle effects. Paradoxically, elimination of both isoforms had no, or minimal, effects on the above, suggesting that compensatory increases in the expression of other genes may normalize glucagon production. Of note, each of the AMPK-related kinases has similar substrate preferences to the canonical AMPK kinases but is regulated by distinct upstream mechanisms. While MARK2 [26] has been implicated in the effects of LKB1 in beta cells, SIK2 elimination impairs insulin release by permitting the accumulation of the CDK5 activator CDK5R1 (p35) and inhibition of Ca^{2+} entry [55]. Loss of SIK2 activity in LKB1-depleted alpha cells may thus lead to defective glucagon release in a similar manner. Furthermore, up-regulation of this enzyme in αAMPKdKO islets could conceivably contribute to the normalization of responses in the latter model. Future studies will be needed to test these possibilities.

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CONFLICT OF INTEREST

The authors declare that there is no duality of interest associated with this manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.01.006

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