



LKB1 and AMPK α 1 are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia.

Gao Sun, Gabriela da Silva Xavier, Tracy Gorman, Claire Priest, Antonia Solomou, David J Hodson, Marc Foretz, Benoit Viollet, Pedro-Luis Herrera, Helen Parker, et al.

► To cite this version:

Gao Sun, Gabriela da Silva Xavier, Tracy Gorman, Claire Priest, Antonia Solomou, et al.. LKB1 and AMPK α 1 are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia.. *Molecular metabolism*, 2015, 4 (4), pp.277-86. 10.1016/j.molmet.2015.01.006 . inserm-01159152

HAL Id: inserm-01159152

<https://inserm.hal.science/inserm-01159152>

Submitted on 2 Jun 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



LKB1 and AMPK α 1 are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia

Gao Sun¹, Gabriela da Silva Xavier¹, Tracy Gorman², Claire Priest², Antonia Solomou¹, David J. Hodson¹, Marc Foretz^{3,4,5}, Benoit Viollet^{3,4,5}, Pedro-Luis Herrera⁶, Helen Parker⁷, Frank Reimann⁷, Fiona M. Gribble⁷, Stephanie Migrenne⁸, Christophe Magnan⁸, Anna Marley², Guy A. Rutter^{1,*}

ABSTRACT

Aims/Hypothesis: Glucagon release from pancreatic alpha cells is required for normal glucose homeostasis 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 (α LKB1KO), or the catalytic alpha subunits of AMPK (α AMPKdKO, $-\alpha$ 1KO, $-\alpha$ 2KO), selectively in \sim 45% of alpha cells in mice by deleting the corresponding *loxP* alleles with a preproglucagon promoter (PPG) *Cre*.

Results: Blood glucose levels in male α LKB1KO mice were lower during intraperitoneal glucose, aminoimidazole carboxamide ribonucleotide (AICAR) or arginine tolerance tests, and glucose infusion rates were increased in hypoglycemic clamps ($p < 0.01$). α LKB1KO mice also displayed impaired hypoglycemia-induced glucagon release. Glucose infusion rates were also elevated ($p < 0.001$) in α AMPK α 1 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 α LKB1KO, α AMPKdKO, and $-\alpha$ 1KO, but not $-\alpha$ 2KO islets.

Conclusions/Interpretation: An LKB1-dependent signalling cassette, involving but not restricted to AMPK α 1, is required in pancreatic alpha cells for the control of glucagon release by glucose.

© 2015 Published by Elsevier GmbH. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/3.0/>).

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-Jeghers 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

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

Received January 6, 2015 • Revision received January 14, 2015 • Accepted January 17, 2015 • Available online 31 January 2015

<http://dx.doi.org/10.1016/j.molmet.2015.01.006>

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 ($\alpha 1$ and $\alpha 2$) 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 α TC1-9 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 (α LKB1KO), and for one (AMPK $\alpha 1$ KO, $\alpha 2$ KO) or both (α AMPKdKO) AMPK α catalytic subunits selectively in pancreatic alpha cells using preproglucagon (*PPG*)-*Cre* driven recombination. We show that LKB1 signalling, at least partly mediated via AMPK $\alpha 1$, 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 *lox'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 α LKB1KO mice (*Lkb1*^{fl/fl}, *Cre* positive). α LKB1KO 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 homeostasis [34] or to lead to significant recombination in extra-pancreatic tissues [11]. α LKB1KO mice and littermate controls were born at the expected Mendelian ratios.

2.2. Generation of mutant mice selectively lacking AMPK $\alpha 1$ and $\alpha 2$ in pancreatic alpha cells

Mice homozygous for *Ampk* $\alpha 1$ ^{fl/fl} were crossed with mice heterozygous for *Ampk* $\alpha 2$ ^{fl/+}. The resulting double heterozygotes (*Ampk* $\alpha 1$ ^{fl/+}, $\alpha 2$ ^{fl/+}) were crossed with *PPG-Cre*-expressing animals [33] to generate triple heterozygous mice (*Ampk* $\alpha 1$ ^{fl/+}, $\alpha 2$ ^{fl/+}, *Cre* positive). The latter were then bred with mice homozygous for both *lox'd* *Ampk* $\alpha 1$ and $\alpha 2$ alleles (*Ampk* $\alpha 1$ ^{fl/fl}, $\alpha 2$ ^{fl/fl}) to produce α AMPKdKO mice (*Ampk* $\alpha 1$ ^{fl/fl}, $\alpha 2$ ^{fl/fl}, *Cre* positive). α AMPKdKO mice were further

crossed with *Ampk* $\alpha 1$ ^{fl/fl}, $\alpha 2$ ^{fl/fl} mice to generate littermate controls (*Ampk* $\alpha 1$ ^{fl/fl}, $\alpha 2$ ^{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 μ l) 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 (Accucheck; 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 μ l) from the tail vein was collected at 0, 10 and 30 min after injection into EDTA coated tubes containing 1 μ l DPP IV inhibitor (final concentration: 100 μ mol/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 ¹²⁵I 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 μ l/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 \pm SEM.

3. RESULTS

3.1. Generation of mice deleted selectively in the pancreatic alpha cell for LKB1 or AMPK α -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-*LoxP*-STOP control cassette (Rosa26tdRFP) [35] against animals bearing *Cre* recombinase under the control of a 0.6 kb fragment of the rat preproglucagon 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

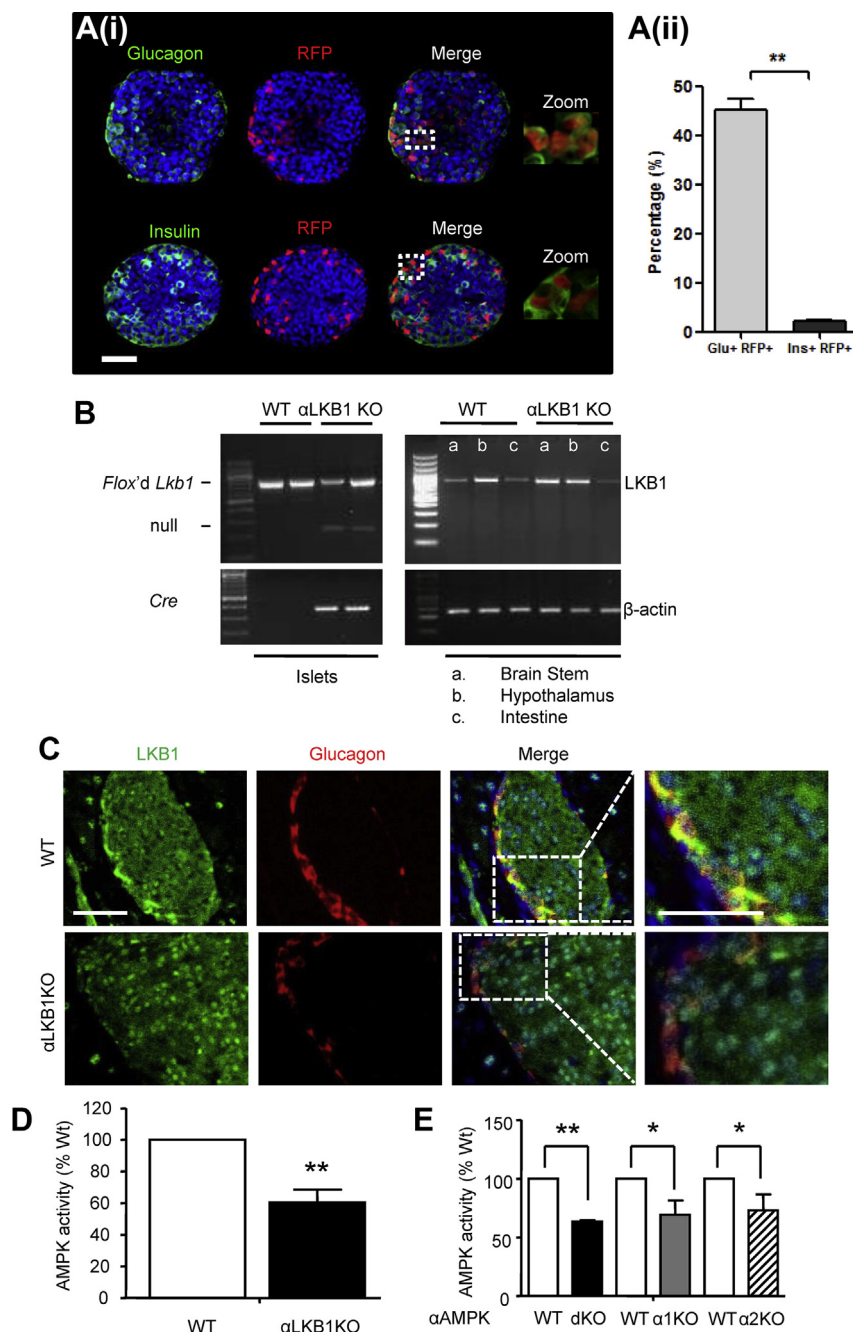


Figure 1: Generation of alpha cell specific LKB1 and AMPK α subunit(s) KO mice (A) (i) Representative immunostaining of pancreatic islets isolated from *PPG-Cre:RFP* mice using antibodies against RFP (1:100) and either glucagon (1:2000; green) or insulin (1:200; green). Nuclei are shown by DAPI (blue) 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 μ m. (D,E) Total AMPK activity in islets of α LKB1KO (D), α AMPK KO (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 \pm 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 (α LKB1KO) by crossing animals in which exons 3–6 of the *Lkb1* gene were flanked with *LoxP* sites [32] with *PPG-Cre* mice [33]. The excision of exons 3–6 mediated by *Cre* was observed in pancreatic islets of α LKB1KO 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 α LKB1KO mice (Figure 1C). Moreover, total AMPK activity was reduced by almost 40% in whole islets from these mice ($60.5 \pm 8.1\%$ 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 (α AMPKdKO) or single (α AMPK α 1KO and α 2KO) catalytic α -subunits selectively in pancreatic alpha cells by crossing either double AMPK α 1 and α 2 or single α -isoform *floxed* mice with *PPG-Cre* deleter mice as above. *Cre*-mediated recombination led to decreases in AMPK activity of $\sim 37\%$, $\sim 32\%$ and $\sim 27\%$ in islets from α AMPKdKO, α 1KO and α 2KO mice, respectively (Figure 1E), the more modest change in islets from

α AMPKdKO 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 α LKB1KO 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 α AMPKdKO 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 α LKB1KO or α AMPK KOs compared to wild-type littermates (Figure S2a–c).

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

Examined at eight weeks of age, male α LKB1KO mice maintained lower overnight fasting blood glucose levels (Figure 2A), while the levels in α AMPKKOs 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 α AMPK α 1KO and α AMPKdKO mice versus controls (Figure S2g–i). Plasma insulin levels were not different between

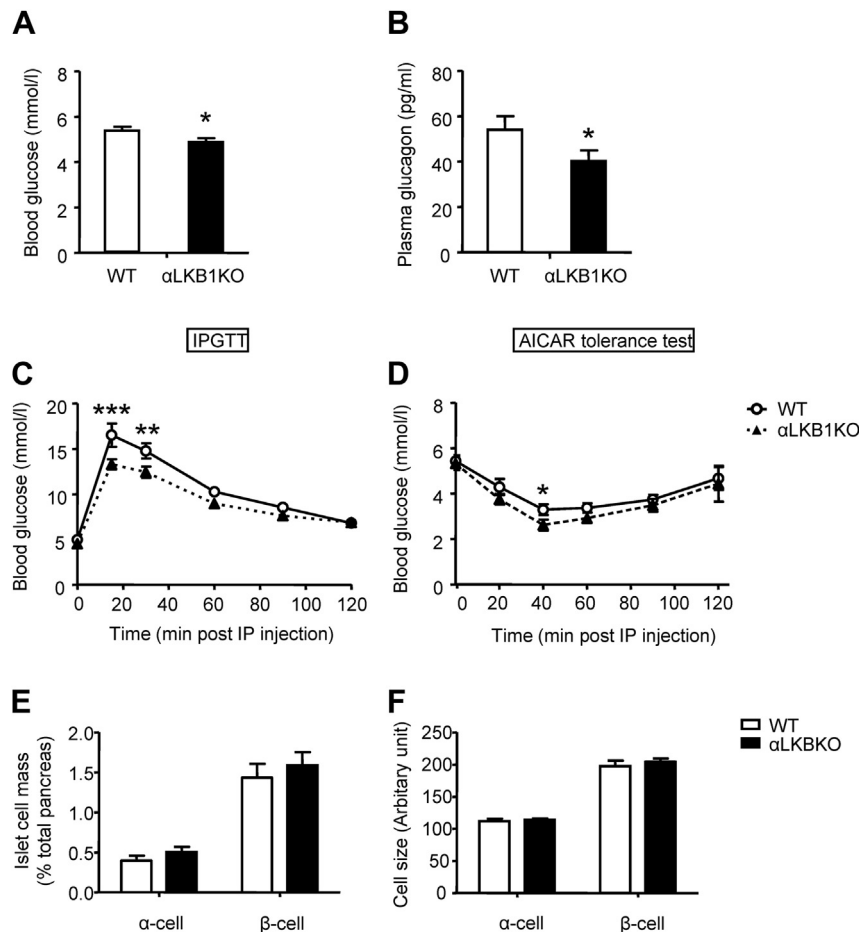


Figure 2: Male α LKB1KO mice display lowered fasting glucose and glucagon levels, and improved glucose and AICAR tolerance. Fasting blood glucose (A) and plasma glucagon levels (B), and intraperitoneal glucose (C) and AICAR (D) tolerance of 8 week-old male mice. Data are expressed as means \pm SEM; $n = 12-22$ (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 α LKB1KO, $n = 5$ vs. 0.078 ± 0.019 nmol/l in WT, $n = 5$). However, eight week-old male α LKB1KO 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: $62.8 \pm 3.8\%$ of initial value in α LKB1KO vs. $70.9 \pm 4.3\%$ in wild type; Figure S3a). None of the α AMPK 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 α LKB1KO mice (51 ± 3 vs. $40\% \pm 3\%$ 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 α LKB1KO 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 α AMPK KO mice (Figure S4b, d, f).

We next tested whether lower fasting glucagon levels, as observed in α LKB1KO mice, may affect the expression of gluconeogenic genes in the liver [41]. Of three genes (PEPCK, G6Pase and PGC-1 α) examined in 24 week-old male α LKB1KO 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 α LKB1KO 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 α LKB1KO (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 α LKB1KO and α AMPK α 1KO 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 α LKB1KO mice. We therefore further explored the impact of deleting LKB1 or AMPK α isoforms in the alpha cell using Hyperinsulinemic-hypoglycemic clamps. When α LKB1KO (Figure 3A, B) or α AMPK α 1KO (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), α LKB1KO animals showed impaired glucagon secretion under these conditions (Figure 3F). Likewise, α AMPK α 1KO mice showed a strong tendency towards lowered glucagon secretion ($p = 0.06$ at 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 α AMPKdKO mice vs controls (Figure S6).

3.4. α LKB1KO and α AMPK KO mice display unaltered alpha cell morphology, total mass and single cell size

The above results together suggested that deletion of LKB1 or AMPK α 1 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, α LKB1KO, α AMPK α 1KO 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 α LKB1KO versus control alpha cells (Figure S7).

3.5. Glucagon secretion is sensitized to inhibition by glucose in islets isolated from α LKB1KO or α AMPK α 1KO mice

The above studies suggested that glucagon secretion from alpha cells is likely to be impaired *in vivo* after the deletion of LKB1 or AMPK α 1 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 α LKB1 KO or α AMPK KO ($-\alpha$ 1, $-\alpha$ 2, 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, α LKB1KO or α AMPK α 1KO 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 α LKB1KO or α AMPK α 1KO 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 μ mol/l epinephrine (at 17 mmol/l glucose) were preserved in α LKB1KO (Figure 5A) and in α AMPK α KO (not shown) mouse islets.

Ablation of AMPK α 2 (Figure 5C) or of both AMPK α 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 AMPK α 2 (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

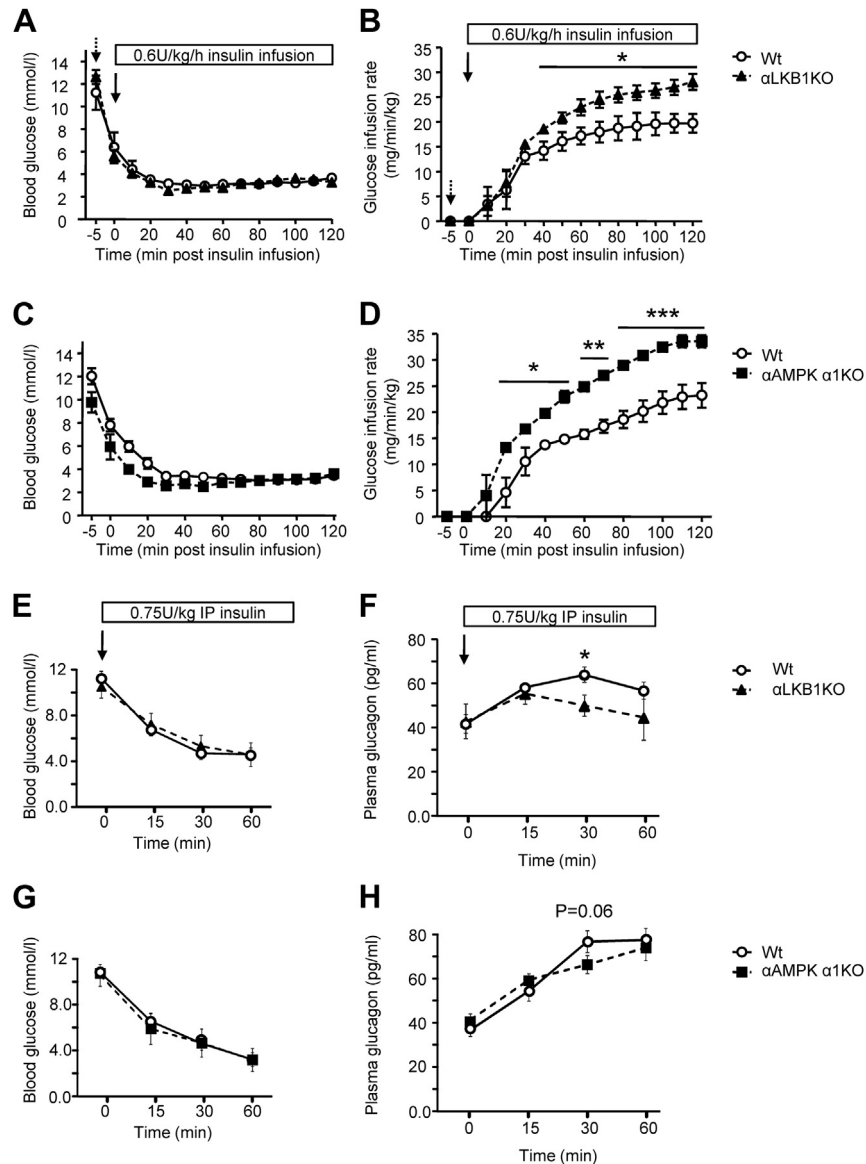


Figure 3: Both α LKB1KO and α AMPK α 1KO mice exhibit enhanced glucose sensitivity during hyperinsulinemic-hypoglycemic clamps and impaired glucagon release during insulin tolerance tests. Blood glucose levels (A, C) and glucose infusion rates (B, D) of α LKB1KO (A, B) or α AMPK α 1KO mice (C, D) and 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; 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 α LKB1KO mice. (G, H) as (E, F), using AMPK α 1KO mice and controls. Data are expressed as means \pm 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 homeostasis 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 AMPK α 1 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

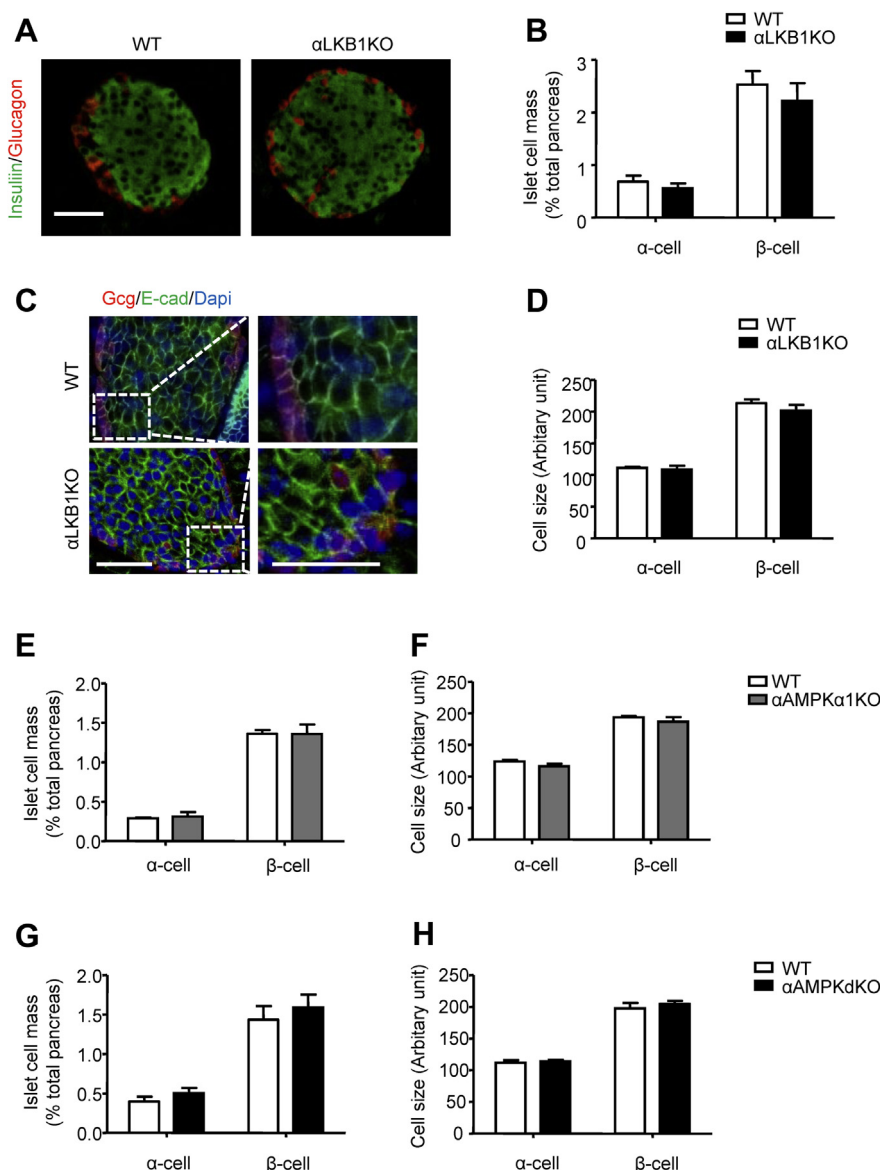


Figure 4: α LKB1KO and α AMPK α 1KO, -dKO mouse islets show normal alpha cell morphology, size and mass. (A) Representative immunostaining of pancreatic sections using guinea pig anti-insulin (green, 1:200) and mouse anti-glucagon antibodies (Red, 1:2000). (B, E, G) Quantitation of relative α - and β -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 \pm SEM. $n = 5$ mice per genotype. Scale bar, 40 μ m. 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 AMPK α 1 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 ($\sim 7\%$ 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–

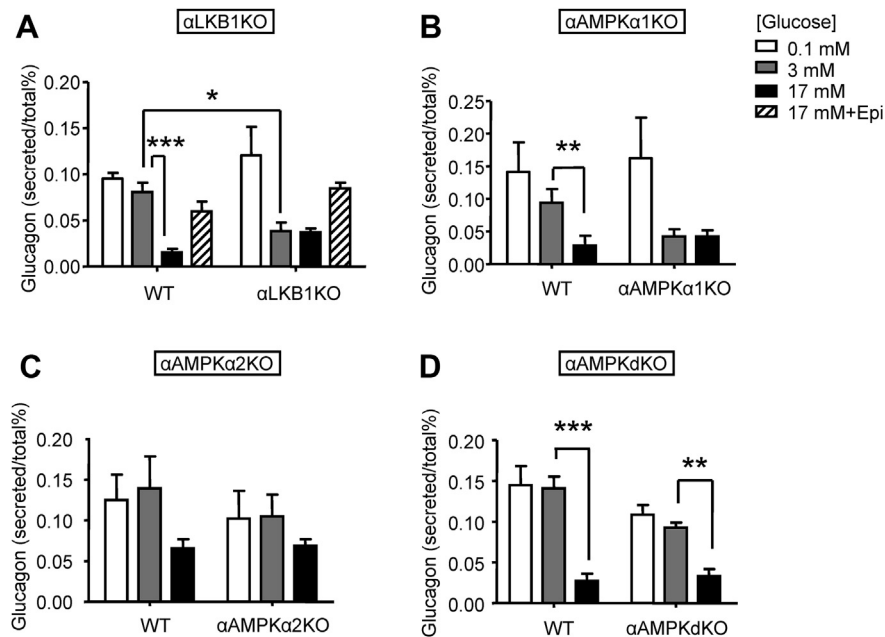


Figure 5: αLKB1 KO and αAMPKα1, -dKO mouse islets secrete less glucagon in response to low (3 mmol/l) glucose. Glucagon secretion from 12 size-matched islets isolated from αLKB1KO (A), αAMPKα1KO (B), α2KO and (C), dKO mice (D) and their respective wild type littermate controls, statically incubated for 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 α AMPKdKO islets could conceivably contribute to the normalization of responses in the latter model. Future studies will be needed to test these possibilities.

ACKNOWLEDGEMENT

This study was funded by grants to GAR from the Wellcome Trust (Programme 081958/Z/07/Z; Senior Investigator Award WT098424AIA), the MRC (UK; Project GO401641; Programme MR/J0003042/1), and Diabetes UK (BDA 11/0004210). DJH thanks Diabetes UK for an RD Lawrence Fellowship (BDA 12/0004431). The work leading to this publication also received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 155005 (IMIDIA), resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in kind contribution. We thank Prof. Nabeel Bardeesy for the kind gift of rabbit anti-LKB1 antibody. GLP-1 assays were performed with the support of the MRC Centre for Obesity and Related Metabolic Diseases (MRC CORD), Cambridge.

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>

REFERENCES

- Gromada, J., Franklin, I., Wollheim, C.B., 2007. Alpha-cells of the endocrine pancreas: 35 years of research but the enigma remains. *Endocrine Reviews* 28:84–116.
- Cryer, P.E., 2002. The pathophysiology of hypoglycaemia in diabetes. *Diabetes, Nutrition & Metabolism* 15:330–333.
- Kaneto, A., Miki, E., Kosaka, K., 1974. Effects of vagal stimulation on glucagon and insulin secretion. *Endocrinology* 95:1005–1010.
- Ahren, B., Veith, R.C., Taborsky Jr., G.J., 1987. Sympathetic nerve stimulation versus pancreatic norepinephrine infusion in the dog: 1). Effects on basal release of insulin and glucagon. *Endocrinology* 121:323–331.
- Taborsky Jr., G.J., Ahren, B., Havel, P.J., 1998. Autonomic mediation of glucagon secretion during hypoglycemia: implications for impaired alpha-cell responses in type 1 diabetes. *Diabetes* 47:995–1005.
- Rutter, G.A., 2009. Regulating glucagon secretion: somatostatin in the spotlight. *Diabetes* 58:299–301.
- Ravier, M.A., Rutter, G.A., 2005. Glucose or insulin, but not zinc ions, inhibit glucagon secretion from mouse pancreatic alpha-cells. *Diabetes* 54:1789–1797.
- Vieira, E., Salehi, A., Gylfe, E., 2007. Glucose inhibits glucagon secretion by a direct effect on mouse pancreatic alpha cells. *Diabetologia* 50:370–379.
- Gilon, P., Bertrand, G., Loubatieres-Mariani, M.M., Remacle, C., Henquin, J.C., 1991. The influence of gamma-aminobutyric acid on hormone release by the mouse and rat endocrine pancreas. *Endocrinology* 129:2521–2529.
- Bailey, S.J., Ravier, M.A., Rutter, G.A., 2007. Glucose-dependent regulation of gamma-aminobutyric acid (GABA A) receptor expression in mouse pancreatic islet alpha-cells. *Diabetes* 56:320–327.
- Kawamori, D., Kurpad, A.J., Hu, J., Liew, C.W., Shih, J.L., Ford, E.L., et al., 2009. Insulin signaling in alpha cells modulates glucagon secretion in vivo. *Cell Metabolism* 9:350–361.
- Ishihara, H., Maechler, P., Gjinovci, A., Herrera, P.L., Wollheim, C.B., 2003. Islet beta-cell secretion determines glucagon release from neighbouring alpha-cells. *Nature Cell Biology* 5:330–335.
- Franklin, I., Gromada, J., Gjinovci, A., Theander, S., Wollheim, C.B., 2005. Beta-cell secretory products activate alpha-cell ATP-dependent potassium channels to inhibit glucagon release. *Diabetes* 54:1808–1815.
- Ramracheya, R., Ward, C., Shigeto, M., Walker, J.N., Amisten, S., Zhang, Q., et al., 2010. Membrane potential-dependent inactivation of voltage-gated ion channels in alpha-cells inhibits glucagon secretion from human islets. *Diabetes* 59:2198–2208.
- Hardie, D.G., 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature Reviews Molecular Cell Biology* 8:774–785.
- Xiao, B., Sanders, M.J., Underwood, E., Heath, R., Mayer, F.V., Carmena, D., et al., 2011. Structure of mammalian AMPK and its regulation by ADP. *Nature* 472:230–233.
- Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., et al., 1998. A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* 391:184–187.
- Hawley, S.A., Pan, D.A., Mustard, K.J., Ross, L., Bain, J., Edelman, A.M., et al., 2005. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metabolism* 2:9–19.
- Woods, A., Dickerson, K., Heath, R., Hong, S.P., Momcilovic, M., Johnstone, S.R., et al., 2005. Ca^{2+} /calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metabolism* 2:21–33.
- Momcilovic, M., Hong, S.P., Carlson, M., 2006. Mammalian TAK1 activates Snf1 protein kinase in yeast and phosphorylates AMP-activated protein kinase in vitro. *The Journal of Biological Chemistry* 281:25336–25343.
- Garcia-Haro, L., Garcia-Gimeno, M.A., Neumann, D., Beullens, M., Bollen, M., Sanz, P., 2010. The PP1-R6 protein phosphatase holoenzyme is involved in the glucose-induced dephosphorylation and inactivation of AMP-activated protein kinase, a key regulator of insulin secretion, in MIN6 beta cells. *The FASEB Journal* 24:5080–5091.
- Leclerc, I., Sun, G., Morris, C., Fernandez-Millan, E., Nyirenda, M., Rutter, G.A., 2011. AMP-activated protein kinase regulates glucagon secretion from mouse pancreatic alpha cells. *Diabetologia* 54:125–134.
- da Silva Xavier, G., Leclerc, I., Salt, I.P., Doiron, B., Hardie, D.G., Kahn, A., et al., 2000. Role of AMP-activated protein kinase in the regulation by glucose of islet beta cell gene expression. *The Proceedings of the National Academy of Sciences* 97:4023–4028.
- da Silva Xavier, G., Leclerc, I., Varadi, A., Tsuboi, T., Moule, S.K., Rutter, G.A., 2003. Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression. *Biochemical Journal* 371:761–774.
- Kefas, B.A., Cai, Y., Kerckhofs, K., Ling, Z., Martens, G., Heimberg, H., et al., 2004. Metformin-induced stimulation of AMP-activated protein kinase in beta-cells impairs their glucose responsiveness and can lead to apoptosis. *Biochemical Pharmacology* 68:409–416.
- Fu, A., Ng, A.C., Depatie, C., Wijesekara, N., He, Y., Wang, G.S., et al., 2009. Loss of Lkb1 in adult beta cells increases beta cell mass and enhances glucose tolerance in mice. *Cell Metabolism* 10:285–295.
- Granot, Z., Swisa, A., Magenheimer, J., Stolovich-Rain, M., Fujimoto, W., Manduchi, E., et al., 2009. LKB1 regulates pancreatic beta cell size, polarity, and function. *Cell Metabolism* 10:296–308.
- Sun, G., Tarasov, A.I., McGinty, J.A., French, P.M., McDonald, A., Leclerc, I., et al., 2010. LKB1 deletion with the R1P2.Cre transgene modifies pancreatic beta-cell morphology and enhances insulin secretion in vivo. *The American Journal of Physiology — Endocrinology and Metabolism* 298:E1261–E1273.

- [29] Beall, C., Piipari, K., Al-Qassab, H., Smith, M.A., Parker, N., Carling, D., et al., 2010. Loss of AMP-activated protein kinase alpha2 subunit in mouse beta-cells impairs glucose-stimulated insulin secretion and inhibits their sensitivity to hypoglycaemia. *Biochemical Journal* 429:323–333.
- [30] Sun, G., Tarasov, A.I., McGinty, J., McDonald, A., da Silva Xavier, G., Gorman, T., et al., 2010. Ablation of AMP-activated protein kinase alpha1 and alpha2 from mouse pancreatic beta cells and RIP2.Cre neurons suppresses insulin release in vivo. *Diabetologia* 53:924–936.
- [31] Wicksteed, B., Brissova, M., Yan, W., Opland, D.M., Plank, J.L., Reinert, R.B., et al., 2010. Conditional gene targeting in mouse pancreatic ss-Cells: analysis of ectopic Cre transgene expression in the brain. *Diabetes* 59:3090–3098.
- [32] Bardeesy, N., Sinha, M., Hezel, A.F., Signoretti, S., Hathaway, N.A., Sharpless, N.E., et al., 2002. Loss of the Lkb1 tumour suppressor provokes intestinal polyposis but resistance to transformation. *Nature* 419:162–167.
- [33] Herrera, P.L., 2000. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* 127:2317–2322.
- [34] Quoix, N., Cheng-Xue, R., Guiot, Y., Herrera, P.L., Henquin, J.C., Gilon, P., 2007. The GluCre-ROSA26EYFP mouse: a new model for easy identification of living pancreatic alpha-cells. *FEBS Letter* 581:4235–4240.
- [35] Parker, H.E., Adriaenssens, A., Rogers, G., Richards, P., Koepsell, H., Reimann, F., et al., 2012. Predominant role of active versus facilitative glucose transport for glucagon-like peptide-1 secretion. *Diabetologia* 55:2445–2455.
- [36] Viollet, B., Andreelli, F., Jorgensen, S.B., Perrin, C., Geloën, A., Flamez, D., et al., 2003. The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity. *The Journal of Clinical Investigation* 111: 91–98.
- [37] Varadi, A., Tsuboi, T., Rutter, G.A., 2005. Myosin Va transports dense core secretory vesicles in pancreatic MIN6 beta-cells. *Molecular Biology of the Cell* 16:2670–2680.
- [38] Thorel, F., Damond, N., Chera, S., Wiederkehr, A., Thorens, B., Meda, P., et al., 2011. Normal glucagon signaling and {beta}-Cell function after Near-total {alpha}-Cell ablation in adult mice. *Diabetes* 60:2872–2882.
- [39] Lizcano, J.M., Goransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., et al., 2004. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO Journal* 23:833–843.
- [40] Foretz, M., Ancellin, N., Andreelli, F., Saintillan, Y., Grondin, P., Kahn, A., et al., 2005. Short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. *Diabetes* 54:1331–1339.
- [41] Jiang, G., Zhang, B.B., 2003. Glucagon and regulation of glucose metabolism. *The American Journal of Physiology — Endocrinology and Metabolism* 284: E671–E678.
- [42] Unger, R.H., Aguilar-Parada, E., Muller, W.A., Eisentraut, A.M., 1970. Studies of pancreatic alpha cell function in normal and diabetic subjects. *The Journal of Clinical Investigation* 49:837–848.
- [43] Dann, S.G., Selvaraj, A., Thomas, G., 2007. mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends in Molecular Medicine* 13:252–259.
- [44] Shah, P., Basu, A., Basu, R., Rizza, R., 1999. Impact of lack of suppression of glucagon on glucose tolerance in humans. *American Journal of Physiology* 277:E283–E290.
- [45] Conarello, S.L., Jiang, G., Mu, J., Li, Z., Woods, J., Zycband, E., et al., 2007. Glucagon receptor knockout mice are resistant to diet-induced obesity and streptozotocin-mediated beta cell loss and hyperglycaemia. *Diabetologia* 50: 142–150.
- [46] Lee, Y., Wang, M.Y., Du, X.Q., Charron, M.J., Unger, R.H., 2011. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* 60:391–397.
- [47] Rutter, G.A., Hodson, D.J., 2014. Beta cell connectivity in pancreatic islets: a type 2 diabetes target? *Cellular and Molecular Life Sciences*. Oct 17. [Epub ahead of print].
- [48] Zac-Varghese, S., Trapp, S., Richards, P., Sayers, S., Sun, G., Bloom, S.R., et al., 2014. The Peutz-Jeghers kinase LKB1 suppresses polyp growth from intestinal cells of a proglucagon-expressing lineage in mice. *Disease Models & Mechanisms* 7:1275–1286.
- [49] Drewnowski, A., Popkin, B.M., 1997. The nutrition transition: new trends in the global diet. *Nutrition Reviews* 55:31–43.
- [50] Gopel, S.O., Kanno, T., Barg, S., Weng, X.G., Gromada, J., Rorsman, P., 2000. Regulation of glucagon release in mouse -cells by KATP channels and inactivation of TTX-sensitive Na⁺ channels. *The Journal of Physiology* 528:509–520.
- [51] Liu, Y.J., Vieira, E., Gylfe, E., 2004. A store-operated mechanism determines the activity of the electrically excitable glucagon-secreting pancreatic alpha-cell. *Cell Calcium* 35:357–365.
- [52] Pagliassotti, M.J., Cherrington, A.D., 1992. Regulation of net hepatic glucose uptake in vivo. *The Annual Review of Physiology* 54:847–860.
- [53] Ayala, J.E., Bracy, D.P., McGuinness, O.P., Wasserman, D.H., 2006. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes* 55:390–397.
- [54] Long, Y.C., Zierath, J.R., 2006. AMP-activated protein kinase signaling in metabolic regulation. *The Journal of Clinical Investigation* 116:1776–1783.
- [55] Sakamaki, J.I., Fu, A., Reeks, C., Baird, S., Depatie, C., Al, A.M., et al., 2014. Role of the SIK2-p35-PJA2 complex in pancreatic beta-cell functional compensation. *Nature Cell Biology* 16:234–244.