Transcription factor-7–like 2 (TCF7L2) gene acts downstream of the Lkb1/Stk11 kinase to control mTOR signaling, β cell growth, and insulin secretion

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Type 2 diabetes currently affects 415 million individuals worldwide, and this number is expected to rise to >600 million by 2040 (www.diabetesatlas.org).2 Pancreatic β cell failure is an essential, if still poorly understood, component of disease development and progression (1).

Genome-wide association studies have identified more than 100 loci associated with disease risk (2) with the majority affecting insulin secretion rather than the action of the hormone. Although in a few cases the likely effector transcript has been identified (3, 4), for most loci neither the causal gene nor its mechanism of action at the cellular level has been defined. Of the commonly inherited risk variants, those in the transcription factor-7–like 2 (TCF7L2/TCF4) gene, including rs7903146, display among the highest odds ratio for exaggerated type 2 diabetes risk (~1.2/allele) (5). The identified single-nucleotide polymorphism (SNP) rs7903146 is located in the third intron of TCF7L2 and has been estimated to contribute to 10–25% of all cases of diabetes lean patients (6) TCF7L2 lies at the foot of the wingless (Wnt) signaling pathway activated both by Wnt ligands and by certain growth factors (e.g. insulin and IGF-1), which act through receptor tyrosine kinases (7). In the presence of Wnt ligands, a signaling cascade results in stabilization and nuclear localization of β-catenin, which interacts with T cell–specific factor/lymphoid enhancer–binding factor to control transcription of target genes. In the absence of Wnt ligands, β-catenin is degraded by protein complexes, including axin-2 and glycogen synthase kinase 3β (GSK3β) (8).

Several studies have explored the role of TCF7L2 in insulin secretion in model systems. Thus, inhibition of TCF7L2 activity in a human or in rat insulinoma cell line (9, 10) inhibited insulin secretion in response to glucose. Likewise, deletion of the TCF7L2 gene selectively in the β cell in mice (11, 12) reduced insulin production in older animals and impaired the expansion of β cell mass in response to a high-fat diet (11, 12). Finally, in a separate study (13), re-expression of TCF7L2 on a null background improved glucose tolerance. Importantly, the degree to which the action of disease-risk variants on the β cell may be

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This article contains Figs. S1 and S2 and Tables S1 and S2.

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3 The abbreviations used are: TCF7L2, transcription factor-7–like 2; AMPK, AMP-activated protein kinase; GSK3, glycogen synthase kinase 3; LKB1, liver kinase B1; mTOR, mammalian target of rapamycin; TSC, tuberous sclerosis complex; qPCR, quantitative PCR; KO, knockout; dKO, double knockout; DAPI, 4’,6-diamidino-2-phenylindole; rpS6, ribosomal protein subunit S6; HTFR, homogenous time-resolved fluorescence; KHB, Krebs-HEPES-bicarbonate; ANOVA, analysis of variance.
context-dependent is unclear. Thus, TCF7L2 variants could have different pathophysiological effects among the five different subpopulations of diabetic patients identified in a recent study (14). The mechanisms, including the genetic drivers, behind these differences remain obscure.

Here, we have explored the impact of Tcf7l2 deletion in a model of β cell expansion driven by artificially enhanced growth factor signaling. Several earlier observations have suggested that a reciprocal relationship may exist between the tumor suppressor liver kinase B1 (LKB1/STK11) and TCF7L2 signaling in other systems. First, the LKB1/STK11 homologue XEEK1 is required for Wnt signaling in Xenopus laevis and acts by phosphorylating and inactivating GSK3 (15). Moreover, in Peutz-Jeghers syndrome, Wnt signaling activation is correlated to LKB1 expression (16). Similarly, in esophageal carcinoma patients, LKB1 is down-regulated and Wnt target genes are up-regulated through inhibition of GSK3β activity (17). We (18, 19) and others (20, 21) have shown previously that inactivation of LKB1 in the β cell leads to a substantial increase in insulin production and improved glucose tolerance. LKB1 is a tumor suppressor mutated in Peutz-Jeghers syndrome, a premalignant condition characterized by hamartomatous polyps and an increased risk of all cancers (22, 23). Although the mechanisms involved remain to be fully elucidated, increases in β cell mass (18), changes in the signaling pathways activated by glucose (19, 24), and alterations in cellular morphology and polarity (18, 20, 21) all appear to play a role in enhancing insulin secretion in the Lkb1-null β cell. Acting via the fuel-sensitive enzyme AMP-activated protein kinase (AMPK), and the tuberous sclerosis complex TSC1–TSC2, LKB1 also inhibits mammalian target of rapamycin (mTOR) signaling to restrict protein synthesis and cell division (25). This pathway may oppose β cell expansion in the adult because AMPK is likely to be active in these cells in the fasting state (26, 27).

To explore the above possibilities, we used an epistasis approach to examine the impact on the pancreatic β cell of deleting Tcf7l2 in the absence of Lkb1 alleles. We show that, in contrast to the action of Tcf7l2 ablation to impair insulin secretion in WT mice, loss of this transcription factor on an Lkb1-null background further increases insulin secretion, β cell size, and β cell mass and augments mTOR activity, consistent with a role for TCF7L2 as an inhibitor of mTOR signaling.

Because a strategy generating all possible genotypes would have produced mice homozygous for deletion of both alleles at a frequency of 1 per 64 pups, we designed instead two separate breeding colonies to reduce animal numbers in accordance with the 3Rs. The following offspring were produced and named as follows (group 1): control (Ins1Cre+/−:Lkb1f+:Tcf7l2f+), βLkb1-KO (Ins1Cre+/−:Lkb1f+:Tcf7l2f+), and βLkb1-KO-Tcf7l2-het (Ins1Cre+/−:Lkb1f+:Tcf7l2f+/+; Fig. 1A, group 1). The second breeding strategy (group 2) generated littermates βLkb1-KO-Tcf7l2-het (Ins1Cre+/−:Lkb1f+:Tcf7l2f+) and βLkb1-KO-Tcf7l2-dKO (Ins1Cre+/−:Lkb1f+:Tcf7l2f−/−; Fig. 1B, group 2).

We first measured Lkb1 and Tcf7l2 gene expression in isolated islets using RT-qPCR analysis. The level of endogenous Lkb1 mRNA was strongly decreased in the presence of Cre transgene when one single or both Tcf7l2 alleles were floxed as expected. Likewise, the level of Tcf7l2 mRNA was decreased when both alleles were floxed (βLkb1−/−Tcf7l2−/−dKO) compared with control. Importantly, we observed no significant differences in the level of Lkb1 and Tcf7l2 mRNAs between βLkb1-KO-Tcf7l2-het mice from either group 1 or 2. Of note, deletion of Lkb1 significantly increased Tcf7l2 expression when both Tcf7l2 alleles were present in βLkb1-KO mice (Fig. 1, C and D). Likewise, we observed decreased LKB1 and TCF7L2 protein expression in isolated islets from βLkb1-KO, βLkb1-KO-Tcf7l2-het, and βLkb1−/−Tcf7l2−/−dKO compared with control islets (Fig. 1E).

Deletion in the β cell of two Tcf7l2 alleles in an Lkb1-null background improves oral glucose tolerance and insulin secretion

Consistent with previous findings (18–21, 28), deletion of both Lkb1 alleles in the β cell improved glucose tolerance in mice aged 8 weeks (Fig. 2, A and B). These changes were not associated with any alteration in body weight (Fig. S1A). Glucose tolerance was not further affected by the additional deletion of a single Tcf7l2 allele (Fig. 2, A and B). Deletion of Lkb1 alone also lowered fed glycemia, and this action was attenuated by the additional deletion of Tcf7l2 (Fig. S1B). Insulin sensitivity was unchanged by deletion of a single Tcf7l2 allele (Fig. S1D).

As described previously (18–21, 28), Lkb1 deletion substantially increased insulin release in response to glucose in vivo (Fig. 2C). Interestingly, glucose-stimulated insulin secretion in vitro only tended to increase on an Lkb1-null background compared with control (Fig. 2D). Monoallelic Tcf7l2 deletion had little further impact on these changes such that the glycemic phenotype of βLkb1−/−Tcf7l2−/−het did not differ from βLkb1−/−KO mice in vivo, but insulin release was enhanced in vitro (Fig. 2, C and D). In contrast, when βLkb1−/−KO-Tcf7l2−/−het mice were compared with homozygous βLkb1−/−Tcf7l2−/−dKO animals deleted for both Tcf7l2 alleles, we observed a further improvement in glucose tolerance (Fig. 3, A and B) but unchanged, body weight, fed glycemia, and insulin sensitivity (Fig. S2, A, C, and E). A substantial (∼2-fold) increase in acute insulin release in response to glucose injection was also observed in vivo (Fig. 3, C and D) when comparing βLkb1−/−Tcf7l2−/−dKO with βLkb1−/−KO-Tcf7l2−/−het littermates. Likewise, comparing islets isolated from mice deleted for both versus a single Tcf7l2 allele, insulin secretion was significantly increased

Results
Generation of β cell–specific Lkb1/Tcf7l2 double-knockout mice

To study the impact of the deletion of Tcf7l2 and Lkb1 in the pancreatic β cell, we established breeding pairs on a mixed background (C57BL/6J, FVB/NJ, and 129S1/SvlmJ) to produce offspring deleted for Lkb1 and/or Tcf7l2 selectively in the β cell using the highly selective Cre deleter strain Ins1Cre in which Cre recombinase is inserted into the Ins1 locus (28, 29) (Fig. 1, A and B). Deletion at other sites, including the brain, is minimal in this model, and, importantly, the transgene does not carry the human growth hormone minigene present in alternative Cre strains (e.g. RIP2.Cre) (30). Consequently, effects of Ins1Cre expression alone on glucose homeostasis are not observed.

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in response to elevated glucose but not to depolarization with KCl (Fig. 3E). Thus, deletion of Tcf7l2 on an Lkb1-null background exerts an effect whose direction is opposite to that seen in control islets (11, 12). In females, deletion of one or two Tcf7l2 alleles on an Lkb1-null background did not affect oral glucose tolerance, insulin sensitivity, fed glycemia, and body weight compared with Lkb1 deletion only (BLkb1-KO; Fig. S2).

Next, we sought to explore intracellular free calcium (Ca²⁺) dynamics to elucidate whether these may be altered and contribute to the enhanced insulin secretion. Islets derived from
βLkb1-KO mice displayed a delayed and decreased response to high glucose in free cytosolic Ca^{2+} increases compared with control animals (Fig. 4, A and B). A similar degree of impairment was observed after the additional deletion of a single Tcf7l2 allele. Interestingly, islets from βLkb1-KO mice showed a decreased response to depolarization with KCl compared with control mice, whereas βLkb1-KO-Tcf7l2-het islets displayed a similar response to KCl compared with control islets (Fig. 4, A and C). In group 2, no difference in response to high glucose or KCl was noted between islets from βLkb1-KO-Tcf7l2-het and βLkb1-Tcf7l2-DKO mice (Fig. 4, D, E, and F).

**Impact of Lkb1 and Tcf7l2 deletion on islet morphology**

We next examined β cell size and the distribution of β cells within the islet in pancreatic slices (Fig. 5, A and B). Cellular proliferation was also assessed through Ki-67 staining (Fig. 5C). As reported previously (18–21), deletion of Lkb1 increased the number of “rosette-like” structures within each islet, as identified using the adherens junction marker E-cadherin, likely reflecting a change in cellular polarity (Fig. 6, A and B) (see (18, 21). The number of rosette structures was not significantly affected by deletion of a single Tcf7l2 allele, whereas the deletion of both alleles tended (p = 0.055) to increase this number, a change that may also contribute to the enhanced secretion observed (18, 21).

β cell mass did not show any significant differences after Lkb1 deletion, and deletion of a single Tcf7l2 allele had no further effect (Fig. 6C). In contrast, deletion of both Tcf7l2 alleles caused a substantial (>30%) and significant increase in β cell mass as examined in βLkb1-Tcf7l2-dKO versus βLkb1-KO-Tcf7l2-het littermates. Correspondingly, β cell proliferation, examined by Ki-67 staining, was not affected by Lkb1 deletion alone or the loss of a single Tcf7l2 allele but significantly increased when two Tcf7l2 alleles were deleted (Figs. 5C and 6, E and F). β cell size, as assessed by comparing islet volume with the number of DAPI-labeled nuclei/islet, was significantly increased by Lkb1 deletion but not further affected by either mono- or biallelic deletion of Tcf7l2 (Fig. 6, G and H).

**Impact of Tcf7l2 deletion on mTOR signaling**

As described previously (18, 20), mTOR signaling is implicated in β cell hypertrophy when Lkb1 is deleted. We therefore examined whether Tcf7l2 deletion may impact mTOR signaling. Whereas deletion of Lkb1 alone had no effect on the levels of phospho-ribosomal protein subunit S6 (rpS6) (Fig. 7, A, B, and D), a significant increase was observed in βLkb1-Tcf7l2-dKO versus βLkb1-Tcf7l2-het islets by immunostaining of pancreatic slices (Fig. 7, A and C) and confirmed by Western (immuno)blotting (Fig. 7E).

**Regulation of Wnt signaling**

Finally, we explored the effects of LKB1 and TCF7L2 deletion on genes in the Wnt/β-catenin pathway. We found that β-catenin, the transcriptional activator for the T cell–specific
factor family of transcription factors, and axin-2, a negative loop regulator of Wnt signaling, tend to be down-regulated in the absence of LKB1 (Fig. 8A). However, axin-2 was up-regulated by LKB1 and TCF7L2 deletion (Fig. 8B). Therefore, it is possible that a cross-talk exists between LKB1 and Wnt/TCF7L2 signaling in pancreatic islets and that this could be involved in controlling β cell proliferation. Furthermore, it is possible that Lkb1 could be a regulator of Wnt signaling and that TCF7L2 could contribute to loop regulation of Wnt signaling involved in proliferative signaling induced by Wnt ligands.

Discussion

The overall aim of the present study was to determine whether, under conditions of exaggerated β cell proliferation, the role of Tcf7l2 may differ from that previously described in animals placed under metabolic stress imposed by aging or by a high-fat diet (11, 12). To this end, we used a mouse model in which Lkb1 was deleted selectively in β cells, mimicking, at least in part, changes during early development (31, 32), pregnancy (33, 34), and insulin resistance.
prior to the onset of type 2 diabetes (35, 36). This seemed an important question given that apparent differences in action have previously been described for other genome-wide association study–identified type 2 diabetes genes, such as \( SLC30A8 \) (37–39), when modeled in mice.

Strikingly, we demonstrated that the direction of the effect of \( Tcf7l2 \) deletion is reversed under these conditions (11, 12) versus those seen under metabolic stress (11, 12). Given that \( TCF7L2 \) is normally considered to be a positive regulator of the cell cycle and thus proliferative (40, 41), this result was unexpected. We therefore considered carefully the possibility that this might be due to alterations elsewhere in the genome given that the \( Lkb1 \) alleles (FVB/N/129S1) were carried by animals with a slightly different genetic background from the floxed \( Tcf7l2 \) strain (C57BL/6J) used (see Table S1). Although this possibility cannot be excluded absolutely, we believe it is unlikely given that both FVB/N (42) and 129S1 (43) animals display similar glucose tolerance on a regular chow diet as C57BL/6 mice.

As an alternative explanation, we speculated that \( Tcf7l2 \) acts as a negative regulator of mTOR signaling. This view was supported by the data shown in Fig. 7, which demonstrated increased mTOR signaling after deletion of both, but not a single, \( Tcf7l2 \) allele. Interestingly, in the present study, we saw relatively little effect of \( Lkb1 \) deletion on mTOR signaling in the presence of \( Tcf7l2 \) despite the predicted activation of the downstream TSC1–TSC2 complex in the absence of AMPK activity (25). Nevertheless and interestingly, loss of \( TCF7L2 \) impacted the alterations in beta cell apical–basolateral polarity observed

Figure 4. Deletion of one or two \( Tcf7l2 \) alleles did not further alter \( \text{Ca}^{2+} \) dynamics in response to glucose but restored responses to KCl in males. A, changes in free cytosolic \( \text{Ca}^{2+} \) in response to 3 mmol/liter glucose (3G), 17 mmol/liter glucose (17G), and 20 mmol/liter KCl in group 1. B, quantification of area under the curve (AUC) for glucose responses. C, quantification of area under the curve for KCl responses. D, free cytosolic \( \text{Ca}^{2+} \) changes in response to 3 mmol/liter glucose (3G), 17 mmol/liter glucose (17G), and 20 mmol/liter KCl in group 2. E, quantification of area under the curve for glucose responses. F, quantification of area under the curve for KCl responses. Each plot represents the average of 16–29 islets (\( n = 3 \) per genotype; *, \( p < 0.05 \); **, \( p < 0.01 \); ****, \( p < 0.0001 \)). Error bars represent the mean ± S.E.; ns, not significant. a.u., arbitrary unit.
after Lkb1 ablation (18–21, 28), which led to alterations in the number of “rosette” structures. The latter changes have previously been ascribed to alterations in signaling by the AMPK-related kinase MARK2/Par1b (21).

Interestingly, increased Tcf7l2 and decreased β-catenin mRNA levels were also observed after Lkb1 deletion in the present study, providing evidence for an interaction between these genes in the β cell wherein LKB1 represses Tcf7l2 expression (Fig. 8). Moreover, we found that axin-2 was regulated positively and negatively, respectively, by Lkb1 and Tcf7l2.

We also noted that deletion of Tcf7l2 on an Lkb1-null background resulted in changes in β cell growth (i.e. hypertrophy and hyperplasia) but also increased β cell function (secretion of insulin as normalized to total insulin content). Although increased mTOR signaling provides a likely mechanism for the former, the mechanisms driving increased insulin secretion remain unclear. In recent studies, we (19) and others (24) demonstrated that loss of LKB1 signaling resulted in marked alterations in glucose signaling to ATP generation and calcium dynamics such that the so-called “amplifying” pathway of insu-
secretion, chiefly reliant on the closure of ATP-dependent K⁺ channels (46) and calcium influx. Instead, the new findings point toward a further enhancement of the amplifying pathways for insulin secretion in β-cells lacking both Tcf7l2 alleles in the absence of Lkb1.

The present data may also provide a mechanistic underpinning for other findings in the literature that have pointed to a possible interaction between nutrient levels and TCF7L2 action. For example, the action of TCF7L2 risk (T) allele rs7903146 depended on plasma glucose levels during oral glucose tolerance tests (47) with deleterious actions being most apparent at high glucose and a tendency to be protective at low glucose. We would note that, although the direction of this effect might appear to be the reverse of that reported here in mice, the above study chiefly interrogated the actions of incretins on insulin secretion; incretins were not examined here. Nevertheless, glucose-dependent suppression of AMPK activity, likely to mimic the effect of Lkb1 deletion on mTOR activity, may provide a means through which changes in glycinemia modulate the direction of the effect of TCF7L2 variants on type 2 diabetes risk.

In summary, we demonstrate here that Tcf7l2 acts as a modifier gene for Lkb1 in the β cell, affecting islet polarity, cellular proliferation, and mass via mTOR signaling. These findings may be relevant for our understanding of the actions of human TCF7L2 variants on type 2 diabetes risk in different individuals and settings (14).

### Experimental procedures

#### Generation of mutant mice lacking LKB1 and TCF7L2 selectively in pancreatic β cells

Mice homozygous for the floxed Lkb1/Stk11 gene (mixed FVB/129S1 and C57BL/6 background) (18) were crossed to mice homozygous for floxed (f/f) Tcf7l2 alleles (C57BL/6 background) (12). The resulting double heterozygotes (Lkb1f/f:Tcf7l2f/f) were crossed with double heterozygous mice, and the latter were then bred with mice expressing Cre recombinase at the insulin 1 locus (Ins1Cre) (28, 29). Subsequently, two separate breeding colonies were established to produce the following offspring and named as follows (group 1): control (Ins1Cre−/−:Lkb1f/f:Tcf7l2f/f), βLkb1-KO (Ins1Cre+−/−:Lkb1f/f:Tcf7l2f/f), and βLkb1-KO-Tcf7l2-het (Ins1Cre+−/−:Lkb1f/f:Tcf7l2f/f; Fig. 1A). The second breeding strategy generated littermates βLkb1-KO-Tcf7l2-het (Ins1Cre+−/−:Lkb1f/f:Tcf7l2f/f) and βLkb1-KO-Tcf7l2-dKO (Ins1Cre+−/−:Lkb1f/f:Tcf7l2f/f; Fig. 1B, group 2). The genetic background of the resulting crosses was quantified by SNP genome scanning analysis (The Jackson Laboratory; Table S1).

#### 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. Unless otherwise stated, data presented are those obtained using male mice. All in vivo procedures described were performed at the Imperial College Central Biomedical Service and approved by the UK Home Office Animals Scientific Procedures Act, 1986 (HO License PPL PA0377F07 to I. L.).

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**Figure 6. Effects of Lkb1 or Tcf7l2 deletion on β cell size and mass in males.** A and B, rosette-like structure count per islet (n = 3 mice/genotype) in group 1 (A) and in group 2 (B). C and D, β cell mass is the ratio of insulin-positive staining to the total pancreatic surface and pancreas weight (n = 6–7 mice/genotype). E and F, quantification of Ki-67–positive and insulin-positive cells based on 10–15 islets per pancreas (n = 3–4 mice/genotype). G and H, mean β cell size measured as the ratio of the insulin-positive staining surface area to the number of β cells (n = 3–4 mice/genotype) in group 1 (G) and in group 2 (H), n = 0.05; **, p < 0.01. White bars, control; black bars, βLkb1-KO; gray bars, βLkb1-KO-Tcf7l2-het (group 1); gray hatched bars, βLkb1-KO-Tcf7l2-het (group 2); black dotted bars, βLkb1-KO-Tcf7l2-dKO. Error bars represent the mean ± S.E.; ns, not significant.
Measurement of metabolic parameters in vivo

Glucose tolerance was performed on 15-h–fasted mice after an oral gavage of glucose (2 g/kg of body weight). Tail venous blood glucose was monitored at 0, 15, 30, 60, 90, and 120 min after glucose administration. Insulin tolerance was performed on 5-h–fasted mice after an intraperitoneal injection of insulin (0.75 unit/kg of body weight; Humulin® S; Lilly). Tail venous blood glucose was monitored at 0, 15, 30, and 60 min. In vivo glucose-stimulated insulin secretion was assessed after intraperitoneal injection of glucose (3 g/kg), and blood was collected at 0, 2.5, 5, and 15 postinjection. Plasma insulin levels were measured using a homogenous time-resolved fluorescence (HTRF) mouse insulin kit (Cisbio, France).

Isolation of mouse islets

Islets were isolated by digestion with collagenase as described (48). In brief, pancreata were inflated with a collagenase solution (1 mg/ml) and placed in a water bath at 37 °C for 12 min. After several washes, the islets were purified on a Histopaque gradient (Sigma-Aldrich), and isolated islets were cul-

Figure 7. Deletion of Tcf7l2 increases mTOR activity in Lkb1-null islets from males. A, representative immunofluorescence staining of pancreatic sections from random-fed 10-week-old males using rabbit anti-phospho-ribosomal protein S6 (Ser-235/236) (p-rpS6; 1:100; red) and guinea pig anti-insulin antibodies (1:200; red). White arrows represent phospho-rpS6 and insulin colocalization. Scale bars, 100 μm. B and C, quantification of phospho-rpS6–positive staining of total β cells per islet based on 15–20 islets per pancreas from n = 4–5 mice/genotype. White bars, control; black bars, βLkb1-KO; gray bars, βLkb1-KO-Tcf7l2-het (B), gray hatched bars, βLkb1-KO-Tcf7l2-het (C); and black dotted bars, βLkb1-Tcf7l2-dKO. D and E, mouse pancreatic islets were isolated from animals of group 1 (D) and group 2 (E). Error bars represent the mean ± S.E.; *, p < 0.05; ns, not significant.

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bicarbonate (KHB) solution (130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl\textsubscript{2}, 0.5 mM MgSO\textsubscript{4}, 0.5 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM NaHCO\textsubscript{3}, 10 mM HEPES, and 0.1% (w/v) BSA, pH 7.4) containing 3 mM glucose. To stimulate ex vivo glucose-stimulated insulin secretion and allowed to recover overnight.

**Ex vivo glucose-stimulated insulin secretion**

Insulin secretion assays on isolated mouse islets were performed as described previously (18). In brief, 10 size-matched islets per condition were incubated for 1 h in Krebs-HEPES-bicarbonate (KHB) solution (130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl\textsubscript{2}, 0.5 mM MgSO\textsubscript{4}, 0.5 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM NaHCO\textsubscript{3}, 10 mM HEPES, and 0.1% (w/v) BSA, pH 7.4) containing 3 mM glucose. Subsequently, islets were incubated for 30 min in KHB solution with either 3 mM glucose, 17 mM glucose, or 30 mM KCl. Secreted insulin and total insulin were quantified using an HTRF insulin kit (Cisbio) in a PHERAstar reader (BMG Labtech, UK) following the manufacturer’s guidelines.

**RNA extraction and quantitative real-time PCR analysis**

RNA was isolated and purified from fresh isolated islets (50–200) with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA purity and concentration were measured by spectrophotometry (Nanodrop, Thermo Fisher), and only RNA samples with an absorption ratio between 1.8 and 2.0 for 260/280 nm were used. cDNA was synthesized using 200 ng of RNA using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems) including random primers. For quantitative real-time PCR, we used SYBR Green PCR Master Mix (Life Technologies) and the primers sequences in Table S2.

**Immunohistochemistry and islet morphology**

Isolated pancreata were removed from euthanized mice, fixed overnight in 10% (v/v) formalin, and subsequently embedded in paraffin wax. Sections (5 μm) were cut and fixed in Superfrost slides. Slides were prepared as detailed previously (28). For antigen retrieval before specific antigen detection, sections were treated with Tris-EDTA buffer, pH 9.0, at 95 °C for 20 min. Primary antibodies used were anti-guinea pig insulin (1:200; Dako), anti-mouse glucagon (1:1000; Sigma-Aldrich), anti-E-cadherin (1:100; Cell Signaling Technology), and anti-Ki-67 (1:200; Abcam, UK). Slides were visualized using an Axiovert 200 M microscope (Zeiss, Germany) with Alexa Fluor 488 goat anti-guinea pig IgG, Alexa Fluor 568 donkey anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG, or Alexa Fluor 568 goat anti-guinea pig IgG (Invitrogen). ImageJ software (Wayne Rasband, National Institute of Mental Health) was used to calculate the β cell mass and size. We determined the percentage of pancreatic surface that was insulin- or glucagon-positive as measured in four sections separated by 75 μm in the z axis from six to seven mice of each genotype. For Ki-67 and E-cadherin detection, pancreata from three 10-week-old mice in each genotype were examined. At least three 5-μm sections per mouse at least 150 μm apart were analyzed. To quantify the number of rosette-like structures (i.e. 8–10 cells arranged concentrically around an identifiable central “core”) in islets (18), we used E-cadherin and DAPI staining of pancreatic sections. Structures were included where the void at the center was negative for DAPI. Ten islets per mouse and three mice per genotype were assessed.

**Western (immuno)blotting**

After isolation, islets were collected and lysed in ice-cold buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% deoxycholate, 5 mM EDTA, and 1% Triton X-100) containing protease inhibitor mixture (Roche) and phosphatase inhibitors (Sigma-Aldrich). Lysates from 125 islets were denatured for 5 min at 95 °C in Laemmli buffer, resolved by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes before immunoblotting. Intensities were quantified using ImageJ.

**Antibodies**

The following antibodies were used in Western (immuno)blot analysis and immunohistochemistry: rabbit anti-phospho-S6 ribosomal protein (Ser-235/236) (Cell Signaling Technology), mouse anti-α-tubulin (Sigma-Aldrich), rabbit anti-E-cadherin (Cell Signaling Technology), guinea pig anti-insulin (Dako), mouse anti-glucagon (Sigma-Aldrich), and rabbit anti-Ki-67 (Abcam).

**Measurement of intracellular free calcium**

Whole isolated islets were incubated with Fura-8AM (Invitrogen) for 45 min at 37 °C in KHB containing 3 mmol/liter glucose. Fluorescence imaging was performed using a Nipkow spinning disk head, allowing rapid scanning of islet areas for prolonged periods of time with minimal phototoxicity. Velocity
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software (PerkinElmer Life Sciences) provided the interface while islets were kept at 37 °C and constantly perfused with KHB containing 3 or 17 mmol/liter glucose or 20 mmol/liter KCl. For each experiment, we used 16–29 islets. Imaging data were analyzed with ImageJ software using an in-house macro (49).

Statistical analysis

GraphPad Prism 7.0 was used for statistical analysis. Statistical significance was evaluated by two-tailed paired Student’s t test and one- or two-way ANOVA with a Bonferroni or Tukey post hoc test when appropriate. All data are shown as means ± S.E. p values of <0.05 were considered statistically significant.

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References

1. Kahn, S. E., Zraika, S., Utzschneider, K. M., and Hull, R. L. (2009) The β cell lesion in type 2 diabetes: there has to be a primary functional abnormality. *Diabetologia* 52, 1003–1012 CrossRef Medline

2. Fuchsberger, C., Flannick, J., Teslovich, T. M., Mahajan, A., Agarwala, V., Gaulton, K. J., Ma, C., Fontanillas, P., Moutsianas, L., McCarthy, D. I., Rivas, M. A., Perry, J. R. B., Sim, X., Blackwell, T. W., Robertson, N. R., et al. (2016) The genetic architecture of type 2 diabetes. *Nature* 536, 41–47 CrossRef Medline

3. van de Bunt, M., Manning Fox, J. E., Dai, X., Barrett, A., Grey, C., Li, L., Bennett, A. J., Johnson, P. R., Rajorje, R. V., Gaulton, K. J., Dermitzakis, E. T., MacDonald, P. E., McCarthy, M. I., and Glyn, A. L. (2015) Transcript expression data from human islets links regulatory signals from genome-wide association studies for type 2 diabetes and glimic signals to their downstream effectors. *PloS Genet.* 11, e1005694 CrossRef Medline

4. Mehta, Z. B., Fine, N., Pullen, T. J., Cane, M. C., Hu, M., Chabosseau, P., Meur, G., Velayas-Baexa, A., Monaco, A. P., Marselli, L., Marchetti, P., and Rutter, G. A. (2016) Changes in the expression of the type 2 diabetes-associated gene VPS13C in the β cell are associated with glucose intolerance in humans and mice. *Am. J. Physiol. Endocrinol. Metab.* 311, E488–E507 CrossRef Medline

5. Grant, S. F., Thorleifsson, G., Reynolds, I., Benediktsson, R., Manolescu, A., Sainz, J., Helgason, A., Stefansson, H., Emilsson, V., Helgadottir, A., Styrkarsdottir, U., Magnusson, K. P., Walters, G. B., Palsdottir, E., Jonsdottir, T., et al. (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nature* 438, 320–323 CrossRef Medline

6. Zeggini, E., and McCarthy, M. I. (2007) TCF7L2: the biggest story in diabetes genetics since HLA? *Diabetologia* 50, 1–4 CrossRef Medline

7. Brantjes, H., Barker, N., van Es, J., and Clevers, H. (2002) TCF: Lady justice casting the final verdict on the outcome of Wnt signalling. *Bioch. Biophys. Acta* 1583, 255–261 CrossRef Medline

8. Habener, J. F., and Liu, Z. (2014) Wnt signalling in pancreatic islets, in *Islets of Langerhans* (Islam, M., ed) pp. 391–419, Springer, Dordrecht, Netherlands

9. da Silva Xavier, G., Golder, M. K., McDonagh, A., Tarasov, A. I., Carzanga, R., Kronenberg, B., Bag, S., and Rutter, G. A. (2009) TCF7L2 regulates late events in insulin secretion from pancreatic islet β-cells. *Diabetes* 58, 894–905 CrossRef Medline

10. Zhou, Y., Park, S. Y., Su, J., Bailey, K., Ottosson-Laasko, E., Shcherbina, L., Oskolkov, N., Zhang, E., Thevenin, T., Fadista, J., Bennett, H., Vikman, P., Wierup, N., Fex, M., Rung, J., et al. (2014) TCF7L2 is a master regulator of insulin production and processing. *Hum. Mol. Genet.* 23, 6419–6431 CrossRef Medline

11. da Silva Xavier, G., Mondragon, A., Sun, G., Chen, L., McGinty, J. A., French, P. M., and Rutter, G. A. (2012) Abnormal glucose tolerance and insulin secretion in pancreas-specific Tcf7l2 null mice. *Diabetologia* 55, 2667–2676 CrossRef Medline

12. Mitchell, R. K., Mondragón, A., Chen, L., McGinty, J. A., French, P. M., Ferrer, J., Thorens, B., Hodson, D. I., Rutter, G. A., and Da Silva Xavier, G. (2015) Selective disruption of Tcf7l2 in the pancreatic β cell impairs secretory function and lowers β cell mass. *Hum. Mol. Genet.* 24, 1390–1399 CrossRef Medline

13. Savic, D., Ye, H., Aneas, I., Park, S. Y., Bell, G. I., and Noreaga, M. A. (2011) Alterations in TCF7L2 expression define its role as a key regulator of glucose metabolism. *Genome Res.* 21, 1417–1425 CrossRef Medline

14. Ahlgvist, E., Storm, P., Kärjämäki, A., Marttinen, M., Dorkhan, M., Carlsson, A., Vikman, P., Prasad, R. B., Aby, D. M., Almgren, P., Wessman, Y., Shaat, N., Spégel, P., Mulder, H., Lindholm, E., et al. (2018) Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. *Lancet Diabetes Endocrinol.* 6, 361–369 CrossRef Medline

15. Ossipova, O., Bardeesy, N., DePinho, R. A., and Green, J. B. (2003) LKB1 regulates Wnt signalling in vertebrate development. *Nat. Cell Biol.* 5, 889–894 CrossRef Medline

16. Ma, Y., Zhang, G., Fu, X., Xia, O., Zhan, C., Li, L., Wang, Z., and Wu, B. (2010) Wnt signaling may be activated in a subset of Peutz-Jeghers syndrome polyps closely correlating to LKB1 expression. *Oncol. Rep.* 23, 1569–1576 Medline

17. Liu, K., Luo, Y., Tian, H., Yu, K. Z., He, J. X., and Shen, W. Y. (2014) The tumor suppressor LKB1 antagonizes WNT signaling pathway through modulating GSK3β activity in cell growth of esophageal carcinoma. *Tumour Biol.* 35, 995–1002 CrossRef Medline

18. Sun, G., Tarasov, A. I., McGinty, J. A., French, P. M., McDonald, A., Leclerc, I., and Rutter, G. A. (2010) LKB1 deletion with the RIP. CRE transgene modifies pancreatic β-cell morphology and enhances insulin secretion in vivo. *Am. J. Physiol. Endocrinol. Metab.* 298, E1261–E1273 CrossRef Medline

19. Swisa, A., Granot, Z., Tamara, N., Sayers, S., Bardeesy, N., Phillipson, L., Hodson, D. J., Wickstrom, J. D., Rutter, G. A., Leibowitz, G., Glaser, B., and Dor, Y. (2015) Loss of liver kinase B1 (LKB1) in β cells enhances glucose-stimulated insulin secretion despite profound mitochondrial defects. *J. Biol. Chem.* 290, 20934–20946 CrossRef Medline

20. Fu, A., Ng, A. C., Depatic, C., Wijesekara, N., He, Y., Wang, G., Bardeesy, N., Scott, F. W., Touyz, R. M., Wheeler, M. B., and Scroeton, R. A. (2009) Loss of Lkb1 in adult β cells increases β cell mass and enhances glucose tolerance in mice. *Cell Metab.* 10, 285–295 CrossRef Medline

21. Granot, Z., Swisa, A., Magenheim, I., Stolovich-Rain, M., Fujimoto, W., Manduchi, E., Miki, T., Lennerz, J. K., Stoeckert, C. J., Jr., Meyuhas, O., Seino, S., Permutt, M. A., Pwica-Woms, H., Bardeesy, N., and Dor, Y. (2009) LKB1 regulates pancreatic β-cell size, polarity, and function. *Cell Metab.* 10, 296–308 CrossRef Medline

22. Boardman, L. A., Thibodeau, S. N., Schaid, D. J., Lindor, N. M., McDonnell, S. K., Burgart, L. J., Ahlquist, D. A., Podratz, K. C., Pittelkow, M., and Hartmann, L. C. (1998) Increased risk for cancer in patients with the Peutz-Jeghers syndrome. *Ann. Intern. Med.* 128, 896–899 CrossRef Medline

23. Jenne, D. E., Reimann, H., Neuz, J., Friedel, W., Loff, S., Jeschke, R., Müller, O., Karcher, T., and Schiemann, P. W. (1991) Peutz-Jeghers syndrome is caused by an oncogenic mutation in the cloned human gene encoding T-KC. *Cell* 66, 290–308 CrossRef Medline
from pancreatic β cells, and may regulate insulin release. Biochem. J. 335, 533–539 CrossRef Medline

27. da Silva Xavier, G., Leclerc, I., Varadi, A., Tsuboi, T., Moule, S. K., and Rutter, G. A. (2003) Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and proproinsulin gene expression. Biochem. J. 371, 761–774 CrossRef Medline

28. Kone, M., Pullen, T. J., Sun, G., Ibberson, M., Martinez-Sanchez, A., Sayers, S., Nguyen-Tu, M. S., Kantor, C., Swisa, A., Dor, Y., Gorman, T., Ferrer, J., Thorens, B., Reimann, F., Gribble, F., et al. (2014) LKB1 and AMPK differentially regulate pancreatic β-cell identity. FASEB J. 28, 4972–4985 CrossRef Medline

29. Thorens, B., Tarussio, D., Maestro, M. A., Rovira, M., Heikkilä, E., and Pound, L. D., Sarkar, S. A., Benninger, R. K., Wang, Y., Suwanichkul, A., Shadoan, M. K., Printz, R. L., Oeser, J. K., Lee, C. E., Piston, D. W., Mcguinness, O. P., Hutton, J. C., Powell, D. R., and O’Brien, R. M. (2009) Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion. Biochem. J. 421, 371–376 CrossRef Medline

30. Brouwers, B., de Faudeur, G., Oeser, J. K., Lee, C. E., Piston, D. W., and Rutter, G. A. (2013) Role for LKB1 in adult β cell mass dynamics in adult mice. J. Biol. Chem. 288, 34171–34180 CrossRef Medline

31. Bouwens, L., and Rooman, I. (2005) Regulation of pancreatic β-cell mass. Physiol. Rev. 85, 1255–1270 CrossRef Medline

32. Ackermann, A. M., and Gannon, M. (2007) Molecular regulation of pancreatic β-cell mass development, maintenance, and expansion. J. Mol. Endocrinol. 38, 193–206 CrossRef Medline

33. Rieck, S., and Kaestner, K. H. (2010) Expansion of β-cell mass in response to pregnancy. Trends Endocrinol. Metab. 21, 151–158 CrossRef Medline

34. Cruciani-Guglielmacci, C., Bellini, L., Denom, J., Oshima, M., Fernandez, N., Normandie-Levi, P., Berney, X. P., Kulkarni, R. N., and Ashcroft, F. M., and Rorsman, P. (2013) KATP channels and islet hormone secretion. Mol. Metab. 2, 4–12 CrossRef Medline

35. Sachdeva, M. M., and Stoffers, D. A. (2009) Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal β-cell mass expansion. Mol. Endocrinol. 23, 747–758 CrossRef Medline

36. Mezza, T., Muscogiuri, G., Sorice, G. P., Clemente, G., Hu, J., Pontecorvi, A., Holst, J. J., Giaccari, A., and Kulikarni, R. N. (2014) Insulin resistance alters islet morphology in nondiabetic humans. Diabetes 63, 994–1007 CrossRef Medline

37. Pound, L. D., Sarkar, S. A., Benninger, R. K., Wang, Y., Suwanichkul, A., Shadoan, M. K., Printz, R. L., Oeser, J. K., Lee, C. E., Piston, D. W., McGuinness, O. P., Hutton, J. C., Powell, D. R., and O’Brien, R. M. (2009) Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion. Biochem. J. 421, 371–376 CrossRef Medline

38. Nicolson, T. J., Bellomo, E. A., Wijesekara, N., Loder, M. K., Baldwin, J. M., Gyuulkhandanyan, A. V., Koshkin, V., Tarasov, A. I., Carzaniga, R., Kronenberg, K., Taneja, T. K., da Silva Xavier, Libert, S., Fрогул, P., Scharffm, R., et al. (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. Diabetes 58, 2070–2083 CrossRef Medline

39. Rutter, G. A., and Chimienti, F. (2015) SLC30A8 mutations in type 2 diabetes. Diabetesologia 58, 31–36 CrossRef Medline

40. Muncan, V., Faro, A., Haraeis, A. P., Hurlstone, A. F., Wiendlone, M., van Es, J., Borling, J., Bethel, H., Zivkovic, D., and Clevors, H. (2007) T-cell factor 4 (Tcf7l2) maintains proliferative compartments in zebrafish intestine. EMBO Rep. 8, 966–973 CrossRef Medline

41. Clevors, H. (2006) Wntβ-catenin signaling in development and disease. Cell 127, 469–480 CrossRef Medline

42. Montgomery, M. K., Hallahan, N. L., Brown, S. H., Liu, M., Mitchell, T. W., Cooney, G. J., and Turner, N. (2013) Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. Diabetologia 56, 1129–1139 CrossRef Medline

43. Sachdeva, M. M., and Stoffers, D. A. (2009) Minireview: meeting the demand for insulin: molecular mechanisms of adaptive postnatal β-cell mass expansion. Mol. Endocrinol. 23, 747–758 CrossRef Medline

44. Henquin, J. C. (2000) Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes 49, 1751–1760 CrossRef Medline

45. Rutter, G. A., Pullen, T. J., Hodson, D. J., and Martinez-Sanchez, A. (2015) Pancreatic β-cell identity, glucose sensing and the control of insulin secretion. Biochem. J. 466, 203–218 CrossRef Medline

46. Ashcroft, F. M., and Rorsman, P. (2013) KATP channels and islet hormone secretion: new insights and controversies. Nat. Rev. Endocrinol. 9, 660–669 CrossRef Medline

47. Heni, M., Ketterer, C., Thamer, C., Herzberg-Schäfer, S. A., Gouthoff, M., Stefan, N., Machicaco, F., Staiger, H., Fritsche, A., and Haring, H. U. (2010) Glycemia determines the effect of type 2 diabetes risk genes on insulin secretion. Diabetes 59, 3247–3252 CrossRef Medline

48. Ravier, M. A., and Rutter, G. A. (2010) Isolation and culture of mouse pancreatic islets for ex vivo imaging studies with trappable or recombinant fluorescent probes. Methods Mol. Biol. 633, 171–184 CrossRef Medline

49. Mitchell, R. K., Nguyen-Tu, M. S., Chabosseau, P., Callingham, R. M., Pullen, T. J., Cheung, R., Leclerc, I., Hodson, D. J., and Rutter, G. A. (2017) The transcription factor Pax6 is required for pancreatic β cell identity, glucose-regulated ATP synthesis, and Ca2+ dynamics in adult mice. J. Biol. Chem. 292, 8892–8906 CrossRef Medline