Fumarate Hydratase Deletion in Pancreatic β Cells Leads to Progressive Diabetes

Highlights
- Fh1 loss in β cells causes progressive Hif1α-independent diabetes
- Fh1 loss in β cells impairs ATP generation, electrical activity, and GSIS
- Elevated fumarate is a feature of diabetic murine and human islets
- “Normoglycemia” restores GSIS in Fh1βKO islets

In Brief
Adam et al. have shown that progressive diabetes develops if fumarate hydratase is deleted in mouse pancreatic β cells. Such β cells exhibit elevated fumarate and protein succination and show progressively reduced ATP production and insulin secretion. The depleted insulin response to glucose recovers when diabetic islets are cultured in reduced glucose.

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Fumarate Hydratase Deletion in Pancreatic β Cells Leads to Progressive Diabetes

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SUMMARY
We explored the role of the Krebs cycle enzyme fumarate hydratase (FH) in glucose-stimulated insulin secretion (GSIS). Mice lacking Fh1 in pancreatic β cells (Fh1 KO mice) appear normal for 6–8 weeks but then develop progressive glucose intolerance and diabetes. Glucose tolerance is rescued by expression of mitochondrial or cytosolic FH but not by deletion of Hif1α or Nrf2. Progressive hyperglycemia in Fh1 KO mice led to dysregulated metabolism in β cells, a decrease in glucose-induced ATP production, electrical activity, cytoplasmic [Ca2+]i elevation, and GSIS. FH loss resulted in elevated intracellular fumarate, promoting succination of critical cysteines in GAPDH, GMPR, and PARK 7/DJ-1 and cytoplasmic acidification. Intracellular fumarate levels were increased in islets exposed to high glucose and in islets from human donors with type 2 diabetes (T2D). The impaired GSIS in islets from diabetic Fh1 KO mice was ameliorated after culture under normoglycemic conditions. These studies highlight the role of FH and dysregulated mitochondrial metabolism in T2D.

INTRODUCTION
Diabetes is an increasing and serious global health and financial problem (Ashcroft and Rorsman, 2012), characterized by defective insulin secretion from the β cells of the pancreatic islets, which causes elevated blood glucose. Mitochondrial production of ATP plays a key role in glucose-stimulated insulin secretion (GSIS) (Maechler and Wollheim, 1999); an increase in intracellular ATP closes ATP-sensitive K+ channels (KATP channels) in the β cell plasma membrane, triggering depolarization and Ca2+-dependent electrical activity. The resulting rise in cytoplasmic Ca2+ initiates exocytosis of insulin granules. In addition to this “triggering” effect, glucose amplifies insulin secretion at a stage subsequent to Ca2+ influx. Several intracellular factors might mediate this amplifying effect, including ATP, NADPH, and glutamate (Henquin, 2011).

The Krebs-cycle enzyme fumarate hydratase (FH) catalyzes the hydration of fumarate to malate. FH is also a tumor suppressor, mutated in hereditary leiomyomatosis and renal cell cancer (HLRCC) (Launonen et al., 2001). Loss of FH activity results in the intracellular accumulation of fumarate, the stabilization of hypoxia-inducible factor 1α (HIF1α), and activation of HIF-dependent pathways, including glucose metabolism (Adam et al., 2014).

The high levels of fumarate that accumulate in FH-deficient cells cause post-translational modification of cysteine residues in proteins to form S-(2-succino) cysteine (2SC), a process...
known as succination (Alderson et al., 2006). This induces loss of activity of the mitochondrial Krebs-cycle enzyme aconitase (Ternette et al., 2013), activation of the antioxidant response sensor nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Adam et al., 2011), and elevation of reactive oxygen species (ROS) signaling (Sullivan et al., 2013; Zheng et al., 2015). Succination has been described in fat and skeletal muscle cells of some diabetic animal models (Nagai et al., 2007; Thomas et al., 2012). Its functional consequences include inactivation of the glycolytic enzyme. However, Fh1 was deleted specifically in pancreatic β cells (Fh1KO mice). These mice had normal glucose tolerance for the first 6–8 weeks of life, and their β cells had essentially normal properties, including GSIS, despite the lack of a key Krebs-cycle enzyme. However, Fh1KO mice subsequently developed rapidly progressing diabetes, culminating in severe glucose intolerance, reduced islet insulin content, and almost complete loss of GSIS.

RESULTS

Mice Lacking Fh1 in Pancreatic β Cells Exhibit Hif1α-Independent Glucose Intolerance

We generated animals in which Fh1 was deleted specifically in pancreatic β cells (Fh1KO mice) by intercrossing an Fh1 conditional knockout mouse (Pollard et al., 2007) with mice expressing Cre recombinase driven by the rat insulin promoter (Rip2-Cre mice; Herrera, 2000). Control (CTL) mice were either Fh1<sup>fl/fl</sup>Rip2-Cre<sup>−/−</sup> or Fh1<sup>fl/fl</sup>Rip2-Cre<sup>+/−</sup> littermates. Deletion of Fh1 in β cells was confirmed at the protein level in islets of 9- to 12-week-old mice (Figure 1). No marked differences were seen in gross histology between CTL and Fh1KO mice in islets (Figures 1A and 1F). Immunohistochemistry (IHC) showed that all cells in CTL islets exhibited uniform expression of FH, which was lost within the core of Fh1KO islets. However, some islet cells, most likely α and β cells, retained FH (Figures 1B, 1G, and 1H). Fh1 loss and elevated furamidate lead to stabilization of HIF1α and subsequent nuclear localization (Isaacs et al., 2005; Pollard et al., 2005). Figures 1C and 1H show nuclear localization of HIF1α in most cells of Fh1KO islets, but not in CTL islets or the exocrine pancreas. No marked differences were observed in insulin or glucagon IHC between CTL and Fh1KO islets (Figures 1D, 1E, 1I, and 1J).

Deletion of Fh1 was confirmed in Fh1KO islets from 9- to 12-week-old mice at the mRNA level. A small amount of Fh1 mRNA remained in Fh1KO islets, likely reflecting its presence in non-β cells (Figure 1K). Expression of the Hif1α target genes pyruvate dehydrogenase kinase 1 (Pdk1) and prolyl hydroxylase dehydrogenase 3 (Phd3/Egln3) was increased in Fh1KO islets (Figure 1K).

Blood glucose levels were measured in free-fed CTL and Fh1KO littermates from 6 to 20 weeks. While young (6–8 weeks of age) Fh1KO mice were nearly normoglycemic, they subsequently (after ≥9 weeks of age) developed severe hyperglycemia (>20 mM) (Figure 1L). Plasma glucose in CTL mice was stable at all ages (<10 mM). For simplicity, here we refer to CTL and Fh1KO littermates at 6–9, 9–12, and >15 weeks of age as stages I–III, respectively, to match the progressive diabetes in Fh1KO mice. Stages I (non-diabetic [ND]), II (diabetic), and III (severely diabetic) have free-fed blood glucose levels of 12.8 mM ± 0.6 mM, 22.6 mM ± 1.2 mM, and 30.4 mM ± 0.9 mM (Figure 1L). The progression of diabetes was confirmed in glucose tolerance tests, which revealed mild intolerance between 9 and 12 weeks of age (stage II) and severe intolerance by 15 weeks (stage III) (Figures 1M and 1N). No age-dependent deterioration of glucose tolerance was observed in CTL mice.

To assess whether the glucose intolerance of Fh1KO mice is mediated by HIF1α stabilization, we crossed Fh1KO and Hif1α<sup>−/−</sup> mice (Cramer et al., 2003) to produce β-cell-specific deletion of both Fh1 and Hif1α (Fh1Hif1αKO mice). Deletion of Hif1α, confirmed by loss of HIF1α staining in all nuclei of Fh1Hif1αKO islets (Figure 1O), did not ameliorate the glucose intolerance of Fh1KO mice (Figure 1P). Loss of Hif1α also leads to stabilization of NRF2 and activation of downstream pathways, typified by increased expression of Hmox1 (confirmed in Figure S1D) (Adam et al., 2011). The role of Nrf2 in β cell function is unclear, proposed to protect from oxidative damage and blunt GSIS (Urano et al., 2013). To determine the contribution of Nrf2 to glucose homeostasis, we crossed Fh1KO with a constitutive knockout of Nrf2 (Ittoh et al., 1997) to delete both Fh1 and Nrf2 in the β cell (Fh1Nrf2<sup>−/−</sup>KO littermates). The glucose intolerance of Fh1Nrf2<sup>−/−</sup>KO mice was unaltered in the Fh1Nrf2<sup>−/−</sup>DKO mice (Figure S1A). In contrast, crossing Fh1KO mice with mice stably expressing either full-length human FH (Fh1KO+FH) or cytoplasmic FH (FH<sup>CP</sup>) (Fh1KO+FH<sup>CP</sup>) (Adam et al., 2013) fully reversed the glucose intolerance tested in stage III mice and restored the mRNA expression of Egln3 and Hmox1 (Figures 1Q and S1B–S1D), and these mice were normoglycemic for >1 year.

Deletion of Fh1 in β Cells Results in Progressive Loss of GSIS

To explore the cause of glucose intolerance in Fh1KO mice, we examined insulin secretion from the perfused pancreas. Glucose elevation from 1 mM to 6 mM transiently stimulated insulin release ~10-fold in stage II CTL mice but not in Fh1KO littermates (Figure 2A). Nevertheless, the response to 20 mM glucose was almost superimposable in both genotypes. In stage III Fh1KO mice, the response to both 6 mM and 20 mM glucose was 85% less than in CTL (Figure 2B). Similar effects on GSIS were obtained in static incubations (Figure S2). The mitochondrial substrate α-ketoisocaproic acid (α-KIC) stimulated insulin secretion ~5-fold in CTL islets but had no effect in Fh1KO islets (Figure S2).
These differences in insulin release correlated with a reduction in insulin content. In islets from stage II mice, insulin content and insulin granule density were reduced by ~50% in Fh1KO islets (Figures 2C–2E). Pancreatic insulin content was 97% less in stage III Fh1KO mice, compared to CTL littermates (Figure 2F), and there was a marked decrease in insulin-positive cells (Figure 2G). When insulin secretion data are normalized to basal values, secretion from stage II Fh1KO islets remains strongly reduced, suggesting that there is a functional defect (Figure S2).

**Fh1KO Islets Exhibit Dysregulated Metabolism**

As mitochondrial metabolism plays a key role in GSIS (Ashcroft and Rorsman, 2012) and Fh1 deletion disrupts the Krebs cycle, we analyzed glucose utilization ([^1]H_2O production, reflecting combined flux through the glycolytic and pentose phosphate pathways) and oxidation (CO_2 production, reflecting mitochondrial metabolism) (Hellman et al., 1971). In both CTL and Fh1KO stage II islets, glucose oxidation increased 4-fold when glucose was increased from 1 to 20 mM (Figure 3A). Glucose utilization also increased 4-fold, but the effect was 35%–40% greater in Fh1KO than CTL islets (Figure 3B), suggestive of increased aerobic glycolysis (O’Flaherty et al., 2010).

Metabolite analysis in islets from stage II mice confirmed an overall pattern of changes similar to that observed in other FH-deficient cells (Adam et al., 2011; Frezza et al., 2011). In particular, levels of fumarate, argininosuccinate, and adenylosuccinate were increased, and aspartate was reduced (Figure 3C; Table S1), indicating that FH loss leads to stimulation of the urea cycle/arginine biosynthesis pathway (Adam et al., 2013; Zheng et al., 2013). Levels of AMP, uridine monophosphate (UMP), and guanosine monophosphate (GMP) were decreased, suggesting both purine and pyrimidine metabolism are compromised as a consequence of aspartate depletion, but likely reflect altered adenylosuccinate metabolism due to elevated fumarate (Bulusu et al., 2011). Importantly, no difference was observed in oxidized or reduced glutathione between CTL and Fh1KO islets, suggesting no major change in ROS or ROS signaling (Sullivan et al., 2013). Islet fumarate and argininosuccinate content...
were normalized following re-expression of either FH or FH\textsuperscript{cyt} in mice (Figures S3A and S3B).

The lack of FH means there is a loss of Krebs-cycle intermediates for every glucose molecule entering the mitochondria. Since metabolism might be maintained by enhanced utilization of amino acids (anaplerosis), we measured 16 key amino acids in islets at both 1 mM and 20 mM glucose. Glucose elevation increased levels of 15 amino acids in CTL islets but had little effect in stage II Fh1\textsuperscript{b} KO islets (Figures 3D, S3C, and S3D).

Glutamate may play a key role in both glucose- and incretin-induced insulin secretion, via the amplifying (non-K\textsubscript{ATP}-dependent) pathway (Maechler and Wollheim, 1999). Because the glucose-induced increase in glutamate was lower in Fh1\textsuperscript{b} KO islets than in CTL islets (Figure 3D), we investigated the amplifying pathway of insulin secretion using islets from stage II animals depolarized with 70 mM extracellular K\textsuperscript{+} (to test the amplifying effect of glucose). Increasing glucose from 2 mM to 20 mM amplified insulin secretion by 600% in CTL islets but only by 150% in Fh1\textsuperscript{b} KO islets (Figure 3E). Addition of exogenous membrane-permeable dimethyl glutamate (DMG; 5 mM) had no effect in CTL islets at either 2 mM or 20 mM glucose but potentiated GSIS in Fh1\textsuperscript{b} KO islets in response to 20 mM glucose (Figure 3E). This suggests that the lack of intermediates, including glutamate, may underlie the impaired insulin release of Fh1\textsuperscript{b} KO islets. Exogenous membrane-permeable dimethyl fumarate (DMF; 5 mM) did not inhibit insulin secretion in CTL islets (Figure 3F), indicating that the acute increase of intracellular fumarate in Fh1\textsuperscript{b} KO islets is not the cause of the impaired GSIS.

Some aspects of altered metabolism in Fh1\textsuperscript{b} KO islets were also investigated by comparing glutamine (m\textsubscript{0} to m\textsubscript{+5}) and glucose (m\textsubscript{0} to m\textsubscript{+6}) isotopomers in islets from stage II Fh1\textsuperscript{b} KO and CTL mice incubated in 1 mM or 20 mM glucose with either [U-\textsuperscript{13}C\textsubscript{5}]-glutamine or \textsuperscript{13}C\textsubscript{6}-glucose (Figure S4). Increased levels of fumarate and argininosuccinate, derived from glutamine, were observed in islets subsequently exposed to 20 mM glucose (Figures S4H and S4M). Substitution of the isotopomers m\textsubscript{+2}, m\textsubscript{+3}, m\textsubscript{+4}, and m\textsubscript{+5} for the naturally occurring m\textsubscript{+0} and m\textsubscript{+1} forms was increased significantly in both glutamate and aspartate in both CTL and Fh1\textsuperscript{b} KO islets subsequently exposed to 20 mM glucose (Figures S4C and S4D). Islets cultured at 20 mM glucose utilized glutamine to generate uridine 5'-diphosphate (UDP)-N-acetylglucosamine (Figures S4J and S4K), a precursor for synthesis of glycosaminoglycans, proteoglycans, and glycolipids (Bond and Hanover, 2013). Substitution of the isotopomers m+2, m+3, m+4, and m+5 for the naturally occurring m+0 and m+1 forms was increased significantly in both glutamate and aspartate in both CTL and Fh1\textsuperscript{b} KO islets incubated with 20 mM glucose (Figures S4J, S4L, S4N, and S4O).
Figure 3. Ablation of Fh1 Causes Dysregulated Metabolism

(A) Glucose oxidation in stage II Fh1ΔKO and CTL islets (n = 7 CTL and n = 16 Fh1ΔKO mice in 3 experiments) at 1 mM and 20 mM glucose. **p < 0.01 versus 1 mM glucose; not significant between CTL and Fh1ΔKO islets.

(B) Glucose utilization in CTL and Fh1ΔKO islets from stage II mice (n = 7 mice of each genotype in 3 experiments) at 1 mM and 20 mM glucose. ***p < 0.001 versus 1 mM glucose; **p < 0.01 between CTL and Fh1ΔKO at 20 mM glucose.

(C) Heatmap of metabolites (measured by CE-TOFMS) that show significant concentration differences between islets from stage II CTL (n = 8 mice) and Fh1ΔKO (n = 7 mice) mice incubated at 5 mM glucose for 1 hr. Red and green for Fh1ΔKO islets indicate metabolites that are significantly increased or decreased, respectively, versus CTL islets (p < 0.05); Student’s t test. Each column represents values for islets from one animal. Absolute values are given in Table S1. Branch points indicate metabolites linked in common pathways.

(D) Amino acid content in islets of stage II Fh1ΔKO (gray; n = 12) and CTL (black; n = 8) mice at 20 mM glucose, expressed relative to that at 1 mM glucose (20G/1G) (n = 30 islets per group analyzed in triplicate in 5 experiments). Abbreviations: Asp, aspartate; Glu, glutamic acid; Ser, serine; Gln, glutamine; His, histidine; Gly,

(legend continued on next page)
Progressive Loss of GSIS in Fh1+jKO Mice Correlates with Reduced ATP Production and β Cell Electrical Activity

Next, we compared the effects of glucose on ATP production and electrical activity in CTL and Fh1+jKO mice. The glucose-induced increase in the ATP/ADP ratio was identical in stage I Fh1+jKO and CTL islets (Figure 4A). However, β cells in islets from stage III Fh1+jKO mice exhibited far smaller glucose-induced increases in the ATP/ADP ratio than age-matched CTL β cells (Figures 4B and 4C).

Glucose-induced electrical activity in β cells from stage II Fh1+jKO mice was similar to that of CTL islets (Figure 4D). However, clear differences were observed in stage III mice. Whereas CTL β cells responded to 20 mM glucose with membrane depolarization and action potential firing, β cells in stage III Fh1+jKO islets were refractory to glucose stimulation (Figure 4E). In such glucose-unresponsive Fh1+jKO β cells, electrical activity was elicited by the KATP-channel blocker tolbutamidine. Together, these data suggest that glucose is unable to inhibit KATP channels in stage III Fh1-deficient β cells, because ATP generation is reduced.

Fh1 Deletion Causes Disruption of β Cell Mitochondrial Ultrastructure

The morphology, size, and distribution of mitochondria in β cells of stage II mice were analyzed by electron microscopy. Whereas mitochondria in CTL β cells had normal morphology with clear cristae, many mitochondria in Fh1+jKO β cells were swollen without clear cristae, and some were very large (>1.25 μm in diameter). There was larger range in the mitochondrial area in Fh1+jKO β cells, suggesting an imbalance between mitochondrial fission and fusion (Figures S5A and S5B). Mitochondria in islets from Fh1+jKO+FH (full-length FH rescue) or Fh1+jKO+FHcyt (cytoplasmic FH rescue) mice were similar to those of CTL mice (Figure S5B).

Protein Succination in Diabetic Islets

Fumarate reacts with cysteine residues in proteins in a non-enzymatic process known as succination. Using an antibody (2SC) that labels succininated proteins specifically (Blatnik et al., 2008), we confirmed that succination was detected in Fh1+jKO β cells and not in CTL β cells (Figures 5A and 5B).

Culture of adipocytes in high glucose elevates fumarate and results in protein succination (Nagai et al., 2007). Therefore, we determined quantitatively whether hyperglycemia would increase fumarate in islets. Fumarate levels were ~100-fold higher in stage II Fh1+jKO islets, even after 1 hr incubation at 1 mM glucose, yet they were further increased at 20 mM glucose (Figure 5C). Glucose also increased fumarate levels in CTL islets; 1 hr incubation at 20 mM glucose increased fumarate levels 12-fold, compared to islets incubated at 1 mM glucose. This was not due to a reduction in FH (Figure 5D) and, thus, is likely a consequence of the enhanced glucose metabolism. Similar acute (1 hr) effects of high glucose on fumarate content were observed in ND human islets (Figures 5E and 5F). Interestingly, the fumarate content of islets from donors with type 2 diabetes (T2D) was higher than that in ND islets, and high glucose produced no further increase (Figure 5F).

Tandem mass spectrometry (MS/MS) analysis of islets from stage II Fh1+jKO mice islets detected succination of key cysteine residues in glyceraldehyde 3-phosphate dehydrogenase (GAPDH; residue C150) (Figures 5G and 5H), guanosine monophosphate reductase (GMPR; C186) (Figure S6A; Table S2), and Parkinson’s disease (autosomal recessive, early onset) 7 (PARK 7/DJ-1; C106). Succination of PARK7/DJ-1 (C106) was also observed in islets from a human T2D donor (Figure S6B; Table S2).

Succination of residue C150 of GAPDH was increased by 270% in Fh1+jKO islets (Figures 5G and 5H). Succination of GAPDH, detected in the gastrocnemius muscle of diabetic rats, has been shown to reduce enzyme activity (Blatnik et al., 2008). This might limit glucose flux through glycolysis, leading to accumulation of upstream glycolytic 3- and 6-carbon intermediates. We tested the possible functional consequences by culturing CTL islets for 24 hr in the presence of 10 mM of the triose D-glyceraldehyde. This resulted in the complete loss of GSIS and correlated with a slight increase in islet insulin content (Figures S7A and S7B).

Impact of Fh1 Deletion on Cytosolic Calcium and pH

Links have been proposed between glucose and pH (Shepherd and Henquin, 1995). The loss of glucose- and Ca2+-dependent electrical activity in stage III Fh1+jKO β cells is predicted to cause an alteration in Ca2+ handling. Intracellular accumulation of fumarate, the anion of fumaric acid, may lower cytoplasmic pH (pHi) and thereby compromise GSIS, effects compounded by the inhibition of GAPDH and accumulation of acidic trioses. Therefore, we performed parallel measurements of [Ca2+]i and pH; in stages I and III Fh1+jKO β cells and CTL cells. Changes in [Ca2+]i were similar in stage I CTL and Fh1+jKO β cells (Figures 6A and 6B). Thus, glucose, glyceroldehyde, and high K+-induced membrane depolarization increased [Ca2+]i. Tolbutamide had little additional stimulatory effect on [Ca2+]i, when tested in the presence of 20 mM glucose (cf. Figure 4E). Although basal pHi was slightly lower in β cells from stage I Fh1+jKO mice than in CTL cells (as expected from the elevated fumarate), the responses to glucose and glyceroldehyde were similar (Figures 6A, 6B, and 6E): glucose and glyceroldehyde lowered pHi.

glycine; Thr, threonine; Arg, arginine; Tau, tauroine; Ala, alanine; Tyr, tyrosine; GABA, gamma aminobutyric acid; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine.

(B) Insulin secretion from islets from stage II Fh1+jKO (gray) and CTL (black) islets (n = 4 experiments; n = 3 mice per genotype) in the presence of 70 mM KCl and 2 mM or 20 mM glucose and 5 mM dimethylglutamate (DMG), as indicated. *p < 0.01 versus 2 mM glucose in Fh1+jKO; **p < 0.0001 versus 2 mM glucose (CTL).

(F) Insulin secretion in wild-type islets (n = 13 experiments; n = 3 mice) at 1 mM and 20 mM glucose with the addition of dimethyl fumarate (5 mM DMF). **p < 0.001 or better versus 1 mM glucose.

Error bars represent ± SEM. See also Figures S3 and S4 and Table S1.
Glucose-induced electrical activity in b cells. Traces are representative of 5 (CTL) or 4 (Fh1 KO) mice. Each trace is the average of >200 cells.

Reduced ATP Production with Age
Basal pHi was lower in stage III Fh1 KO mice. Responses are normalized to the ratio at 1 mM glucose. *p < 0.05 versus CTL; #p < 0.05 versus basal (3 mM glucose).

Impaired GSIS in Fh1 KO Islets Is a Consequence of Hyperglycemia
We reasoned that the deterioration of GSIS in Fh1 KO islets over time might be a consequence of the progressive hyperglycemia (Brereton et al., 2014). We tested whether the impairment of GSIS could be reversed. Freshly isolated islets from stage III Fh1 KO mice were refractory to 6 mM glucose, and the response to 20 mM glucose was only 30% of that seen in CTL islets (Figure 7A), echoing the data from the perfusion experiments (Figure 2B).

After culture at 12 mM glucose for ~65 hr, secretory responses in CTL islets were essentially the same as in freshly isolated islets. However, GSIS at 20 mM glucose was dramatically improved in Fh1 KO islets and approached that of the CTL islets (Figure 7B). Insulin content was 35% lower in Fh1 KO than in CTL islets (Figure 7C).

Finally, we tested whether the loss of GSIS in stage III Fh1 KO islets reflects a direct "glucotoxic" effect or is related to accelerated glucose metabolism. Distinct from what was seen following culture at 12 mM glucose (Figures 7A and 7B), islets from stage III Fh1 KO mice cultured for 3 days at 20 mM glucose showed no recovery of GSIS. By contrast, islets cultured in the presence of the glucokinase inhibitor mannoheptulose (Zelent et al., 2005; Coore and Randle, 1964) exhibited some (limited) glucose responsiveness (Figure 7D).

DISCUSSION
We show here that lack of the Krebs-cycle enzyme FH causes a progressive deterioration of \( b \) cell function, resulting in severe diabetes associated with impaired oxidative metabolism, ATP production, intracellular calcium handling, and cytosolic acidification.

Distinct from the mouse model in which \( Vhl \) is deleted in \( b \) cells (Zehetner et al., 2008; Cantley et al., 2009), we found that the diabetes associated with \( Fh1 \) loss is \( Hif1 \alpha \) and \( Nrf2 \) independent. Our data are consistent with earlier observations that, while \( Fh1 \) deletion leads to the accumulation of fumarate, and \( Hif1 \alpha \) is stabilized, many associated functional changes are independent of \( HIF1 \alpha \) (O’Flaherty et al., 2010; Adam et al., 2013; Ternette et al., 2013). Similar to previous studies, we were unable to detect \( HIF1 \alpha \) in normal \( b \) cells, and deletion of \( HIF1 \alpha \) alone did not disrupt glucose homeostasis (Zehetner et al., 2008; Cantley et al., 2009). However, it remains unclear whether \( b \) cell dysfunction and diabetes are caused by \( HIF1 \alpha \) stabilization or vice versa (Cantley et al., 2010; Girgis et al., 2012). In the Fh1 KO mouse, loss of glucose regulation appears entirely attributable to FH. The hyperglycemia that develops may drive \( HIF1 \alpha \) stabilization, perhaps compounding GSIS impairment further in a feedback loop.

Why Do Young (Stage 1) Fh1 KO Mice Have Normal Glucose Tolerance?
Given the importance of mitochondrial metabolism for insulin secretion, the mild phenotype of stage I Fh1 KO mice was...
surprising, not least as Fh1 deletion led to >100-fold accumulation of fumarate, consistent with arrest of the Krebs cycle at FH. Because insulin secretion was unimpaired, this suggests that refilling of Krebs-cycle intermediates (anaplerosis) must occur distal to FH. Labeling experiments in mouse embryonic fibroblasts (MEFs) lacking Fh1 show that fumarate exits the mitochondria to the cytoplasm but is then metabolized to aspartate via the urea cycle, which re-enters the Krebs cycle (Adam et al., 2013). Studying metabolism in murine pancreatic islets is difficult because of the limited amounts of material available, especially from the diabetic islets. Our analyses of Fh1 KO islets by capillary electrophoresis time of flight MS (CE-TOFMS) indicate similar disruption of the urea cycle and purine metabolism and explain, in part, why the diabetic phenotype of Fh1 KO mice can be rescued by re-expression of cytosolic FH. Moreover, Fh1 KO islets exhibit increased utilization and oxidation of glucose, consistent with aerobic glycolysis, recapitulating previous observations made when Fh1 was deleted in MEFs and mouse kidney (O’Flaherty et al., 2010; Adam et al., 2013).

Normally, excess Krebs-cycle reactants are used to produce amino acids via cataplerosis (Choi et al., 2011; MacDonald et al., 2005). However, in Fh1 KO islets, amino acid content tended to be reduced, and glucose did not increase the content of several amino acids; for example, glutamate. This is of interest because glutamate has been implicated in the modulation of GSIS (Maechler and Wollheim, 2000). Exogenous dimethyl glutamate restored GSIS in Fh1 KO islets but had no effect in CTL islets. These data support the idea that glutamate functions as an “amplification signal.” Another possibility is that exogenous glutamate may restore GSIS by feeding into the Krebs cycle and partially restoring mitochondrial metabolism (Frezza et al., 2011).

Despite the ∼50% reduction in insulin content, the response to 20 mM glucose was maintained in stage II Fh1 KO mice. We speculate that Fh1 KO β cells may compensate for the reduction in insulin content by stimulation of insulin exocytosis. Fh1 KO islets contain significantly elevated levels of adenylsuccinate, which has been proposed to stimulate exocytosis by inhibition of the sentrin/SUMO-specific protease 1 (Gooding et al., 2015).

**Rapid Progression to Diabetes in Stage II Fh1 KO Mice**

Once slight hyperglycemia developed, rapid deterioration of glycemic control was observed (up to 10 mM/week). We speculate that a small elevation of plasma glucose induces a vicious cycle of impaired insulin secretion and hyperglycemia. In part, this arises because, as blood glucose increases, more glucose enters metabolism, causing even greater stress on the β cell with increased fumarate levels. It is possible that metabolism of cytosolic fumarate and anaplerosis eventually become unable to maintain the Krebs cycle and that, once this occurs, rapid deterioration of both GSIS and glucose tolerance occurs.

**Why Do Fh1 KO Mice Become Glucose Intolerant?**

Our results indicate that Fh1 KO mice eventually develop diabetes because glucose is no longer able to stimulate insulin secretion. This is because of a failure of mitochondrial metabolism that culminates in impaired ATP production, defective KATP-channel closure, and suppression of electrical activity and [Ca2+].

Why does ATP production fail? Pumping H+ across the inner mitochondrial membrane, the electron transport chain produces the alkalization of the mitochondrial matrix (Wiederkehr et al., 2009) and membrane hyperpolarization, which are needed to drive ATP synthesis in β cells. Fumaric acid (C4H4O4) is an acid with two hydroxyl groups with pKa of 3.0 and 4.4; thus, loss of FH activity can accordingly be expected to result in acidification not just of the cytoplasm (experimentally measured) but also of the mitochondrial matrix.

There is a sigmoidal relationship between glucose concentration and mitochondrial metabolism (Ashcroft et al., 1970; Helman et al., 1971). At low glucose concentrations, the Krebs cycle runs at a fairly low rate, and the amount of fumarate generated and deposited in the cytosol will be low. Thus, if Fh1 KO mice remain normoglycemic, cytoplasmic acidification will be modest. In agreement with this idea, glucose-responsive β-cells...
from stage I Fh1KO mice had a higher pH than β cells from stage III mice. At higher plasma glucose levels, mitochondrial glucose metabolism will accelerate due to the increased substrate, leading to greater fumarate generation, acidification of both cytoplasmic and mitochondrial matrices, and a progressive impairment of ATP production. Also, acidification may influence the activity of other enzymes involved in β cell metabolism with optimal activity at alkaline pH (e.g., Bernstein and Everse, 1978; Lai and Cooper, 1986; Willson and Tipton, 1980). These effects may be aggravated by downregulation of key genes involved in mitochondrial metabolism, as seen in a mouse model of diabetes caused by a gain-of-function mutation in the KATP channel (βV59M mice; Brereton et al., 2016). These scenarios are not mutually exclusive, and it is possible that they operate in parallel and that both contribute to the loss of ATP production and GSIS. Both these hypotheses predict that inhibition of glucose metabolism should exert a protective effect on β cell metabolism. This proved to be the case, as GSIS was restored in Fh1KO islets following “normoglycemic” culture or culture in high glucose with the glucokinase inhibitor mannoheptulose.

**Fumarate Accumulation Leads to Protein Succination**

Our data demonstrate that elevation of intracellular fumarate is associated with “hyperglycemia” in both mouse and human islets. We detected succination of critical cysteines in GAPDH, GMPR, and PARK7/DJ-1 proteins in Fh1KO islets. Succination of GAPDH has been reported previously in adipocytes in diabetic db/db and ob/ob mice. It is a marker of impaired mitochondrial metabolism and has functional effects (Blatnik et al., 2008; Frizzell et al., 2011). Reduced PARK7/DJ1 activity is compatible with the small mitochondria (suggestive of mitochondrial fragmentation and impaired function) seen in Fh1KO islets. Interestingly, we found that fumarate levels are elevated in islets from T2D donors and may, via succination, explain why expression of PARK7 is...
reduced in T2D islets (Jain et al., 2012). Importantly, it also suggests that mitochondrial metabolism is impaired in islets, leading to elevated fumarate and succination.

Conclusions

Our studies suggest a cycle in which progressive hyperglycemia in Fh1jKO mice leads to the deterioration of metabolism, culminating in the loss of GSIS and frank diabetes. Although β cells from young Fh1jKO mice are glucose responsive, subtle differences exist between Fh1jKO and CTL islets. Thus, GSIS is impaired at 6 mM glucose in Fh1jKO mice, compared to CTL mice. This defect may underlie the slight elevation in plasma glucose that precedes the more rapid deterioration in glucose tolerance. Our hypothesis is consistent with the proposal that glucose that precedes the more rapid deterioration in glucose tolerance.

Conclusions

Our studies suggest a cycle in which progressive hyperglycemia and impaired GSIS (Weir and Bonner-Weir, 2004).

In severely diabetic mice expressing the gain-of-function KATP channel mutation V59M (Brereton et al., 2014), the adverse effects on β cell function and insulin content were reversed following restoration of normoglycemia. Similarly, the severe impairment of GSIS seen in diabetic (stage III) Fh1jKO islets was almost fully corrected simply by culturing the islets under “normoglycemic” conditions. This agrees with the report that GSIS is restored in long-term T2D patients following normalization of plasma glucose levels and suppression of hepatic glucose production induced by a low-calorie diet (Lim et al., 2011).

We propose that the Fh1jKO mouse provides a valuable new model for T2D. In particular, it is not obese, and the glucose intolerance develops in an age-dependent fashion. Thus, it provides a useful tool for studying the progression observed in T2D and to interrogate the systemic and cellular consequences of metabolic dysfunction in the pancreatic β cell without the complications of altered diet and/or obesity.

EXPERIMENTAL PROCEDURES

See also Supplemental Experimental Procedures.

Mice

Animal experiments were conducted in accordance with the UK Animals Scientific Procedures Act (1986) and University of Oxford local ethical guidelines. We used male and female adult mice of the following strains: Fh1tm1Pjpfl/flRip2-Cre recombinase, Fh1tm1Pjpfl/flRip2-Cre+/−, (Fh1tm1Pjpfl/flRip2-Cre+/− crossed with Tg(Ins2-Cre)2Wd/Cre recombinase, Rip2-Cre+/−), Fh1tm1Pjpfl/flRip2-Cre+/− Gt(DOsa); Rtf161-cre; Tg(Cdh16-cre)91Igr, Fh1tm1Pjpfl/flRip2-Cre+/− Gt(Rosa)26So1m1(CAG-Fry)Fypp; Tg(Cdh16-cre)91Igr, and Fh1tm1Pjpfl/flRip2-Cre−/− Nrf2−/− mice (designated Fh1jKO, Fh1jKO+FH, Fh1jKOFh, Fh1jKO+NRF2, respectively) and littermate controls (designated CTL). The constitutive Nrf2 KO mouse was generated from an embryonic stem cell (ESC) clone obtained from Riken, Japan. Genotyping was performed by Transnetyx, but primer details and PCR conditions can be obtained from J.A. NMRI and C57BL/6J mice (designated wild-type) purchased commercially were used in a few cases.

IHC

Mouse tissues were fixed in 10% neutral-buffered formalin, dehydrated, and processed for paraffin wax embedding and sectioning (3 μm). H&E sections were generated for all samples. IHC was carried out using the EnVision Kit (Dako) as per the manufacturer’s protocol, with the following antibodies: FH (Autogen Bioclear), HIF1α (Cayman), insulin (MP Biomedicals), glucagon (MP Biomedicals and Sigma), and 2SC (Nagai et al., 2007).
Intraperitoneal Glucose Tolerance Test

Blood glucose levels were determined with an Accuchek Aviva meter after 16 hr of fasting and at time points following intraperitoneal injection of 2 g glucose per kilogram of body weight. Animals were culled at the end of the test, and pancreata were processed as described earlier.

Hormone Secretion and Content Measurements

Insulin secretion was measured by in situ pancreas perfusion or in static incubations of isolated islets (Zhang et al., 2013). Insulin content was determined in parallel from isolated islets or from mouse pancreata harvested separately.

Quantitative Imaging of ATP, Ca²⁺, and pH

Time-lapse imaging of the ATP/ADP ratio in mouse islets was performed using 10×14× magnification on a Zeiss AxioZoom.V16 microscope. Islets were infected with an adenovirus (3 × 10⁵ plaque-forming units [PFUs] per islet) delivering Perceval, a recombinant sensor of ATP/ADP (Berg et al., 2009). Groups of islets isolated from CTL and Fh1/IKO animals were imaged simultaneously 24 hr post-infection at glucose concentrations as indicated, with single-cell resolution. Time-lapse images were collected every 30 s, and the bath solution was perfused at 60 μL/min at 34°C.

Simultaneous time-lapse imaging of [Ca²⁺], and pH in mouse islets was performed on the inverted Zeiss Axiovert 200 microscope, equipped with the Zeiss LSM 510-META laser confocal scanning system, using a 40×1.3 NA objective. Mouse islets were loaded with 6 μM of the Ca²⁺-sensitive dye Fluo-4 for 90 min before being transferred to a separate solution containing 6 μM of the pH-sensitive dye SNARF-5F (both dyes from Molecular Probes) for a further 50 min at room temperature and imaged using an open chamber at 34°C. The bath solution containing various stimuli was perfused continuously at the rate of 200 μL/min. The ratiometric dye SNARF-5F was excited at 543 nm, and emission was collected at 650 nm and 600 nm. Fluo-4 was excited at 488 nm and imaged at 530 nm. Images were acquired at the frequency of 0.03 Hz. The pH calibration of each trace was performed using high K⁺-nigericin technique. Valinomycin (5 μM) was added to the extracellular solution to abolish the K⁺ gradient (Tarasov et al., 2012, 2013).

Statistical Analysis

Image sequences were analyzed (registration, background subtraction, ROI intensity versus time analysis) using open-source Fiji software (http://fiji.sc/Fiji). The numerical time series data were analyzed using the IgorPro package (WaveMetrics). Statistical significance of the differences between paired or unpaired samples was tested using Friedman or Kruskall-Wallis tests, with single-cell resolution. Time-lapse images were collected every 30 s, and the bath solution was perfused at 60 μL/min at 34°C.
Hellman, B., Sehlin, J., and Taljedal, I.B. (1971). Effects of glucose and other Metab. between HIFs, type 2 diabetes, and metabolic syndrome. Trends Endocrinol. cell Reports, 3, 372–380.

Alderson, N.L., Wang, Y., Blatnik, M., Frizzell, N., Walla, M.D., Lyons, T.J., Alt, N., Carson, J.A., Nagai, R., Thorpe, S.R., and Baynes, J.W. (2006). S-2-Succinyl)cysteine: a novel chemical modification of tissue proteins by a Krebs cycle intermediate. Arch. Biochem. Biophys. 450, 1–8.

Ashcroft, F.M., and Rorsman, P. (2012). Diabetes mellitus and the β cell: the last ten years. Cell 148, 1160–1171.

Ashcroft, S.J., Hedgeson, C.J., and Randle, P.J. (1970). Glucose metabolism in mouse pancreatic islets. Biochem. J. 118, 143–154.

Berg, J., Hung, Y.P., and Yellen, G. (2009). A genetically encoded fluorescent reporter of ATP:ADP ratio. Nat. Methods 6, 161–166.

Bernstein, L.H., and Everse, J. (1978). Studies on the mechanism of the malate dehydrogenase reaction. J. Biol. Chem. 253, 8702–8707.

Blatnik, M., Frizzell, N., Thorpe, S.R., and Baynes, J.W. (2008). Inactivation of glyceraldehyde-3-phosphate dehydrogenase by fumarate in diabetic formation of S-2-succinyl)cysteine, a novel chemical modification of protein and possible biomarker of mitochondrial stress. Diabetes 57, 41–49.

Bond, M.R., and Hanover, J.A. (2013). O-GlcNAc cycling: a link between metabolism and chronic disease. Annu. Rev. Nutr. 33, 205–229.

Brereton, M.F., Iberl, M., Shimomura, K., Zhang, Q., Adriaenssens, A.E., Proks, P., Sipliota, I., Dace, W., Mattis, K.K., Ramacheya, R., et al. (2014). Reversible changes in pancreatic islet structure and function produced by elevated blood glucose. Nat. Commun. 5, 4639.

Brereton, M.F., Rohm, M., Shimomura, K., Holland, C., Tornovsky-Babey, S., Dadon, D., Iberl, M., Chibalina, M.V., Lee, S., Glaser, B., et al. (2016). Hyperglycemia induces metabolic dysfunction and glycoprotein accumulation in pancreatic β-cells. Nat. Commun. 7, 13496.

Bulusu, V., Jayaraman, V., and Balaram, H. (2011). Metabolic fate of fumarate, a side product of the purine salvage pathway in the intraerythrocytic stages of Plasmodium falciparum. J. Biol. Chem. 286, 9236–9245.

Cantley, J., Selman, C., Shukla, D., Abramov, A.Y., Forstreuter, F., Esteban, M.A., Clar et, M., Lingard, S.J., Cle lments, M., Harten, S.K., et al. (2009). Deletion of the von Hippel-Lindau gene in pancreatic β-cells impairs glucose homeostasis in mice. J. Clin. Invest. 119, 125–135.

Cantley, J., Grey, S.T., Maxwell, P.H., and Withers, D.J. (2010). The hypoxia response pathway and β-cell function. Diabetes Obes. Metab. 12 (Suppl. 2), 159–167.

Choi, S.E., Lee, Y.J., Hwang, G.S., Chung, J.H., Lee, S.J., Han, S.J., Kim, H.J., Lee, K.W., Kim, Y., et al. (2011). Supplement of TCA cycle intermediates protects against high glucose/palmitate-induced INS-1 beta cell death. Arch. Biochem. Biophys. 505, 231–241.

Coore, H.G., and Randle, P.J. (1964). Inhibition of glucose phosphorylation by mannnoheptulose. Bioch. J. 91, 56–69.

Cramer, T., Yamashita, Y., Clausen, B.E., Förster, I., Pavlinski, R., Mackman, N., Haase, V.H., Jaenisch, R., Cott, J., O’Flaherty, L., and Newsholme, P., Herva, R., and Aaltonen, L.A. (2001). Inherited susceptibility to uterine leiomyomas and renal cell cancer. Proc. Natl. Acad. Sci. USA 98, 3387–3392.

Lim, E.L., Hollingsworth, K.G., Arbisala, B.S., Chen, M.J., Mathers, J.C., and Taylor, R. (2011). Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver tracyglycerol. Diabetologia 54, 2506–2514.

Lai, J.C., and Cooper, A.J. (1986). Brain alpha-ketoglutarate dehydrogenase complex: kinetic properties, regional distribution, and effects of inhibitors. J. Neurochem. 47, 1376–1386.

Maehlc, P., and Wollheim, C.B. (1999). Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature 402, 685–689.

Maechel, P., and Wollheim, C.B. (2000). Mitochondrial signals in glucose-stimulated insulin secretion in the beta cell. J. Physiol. 529, 49–56.

Merker, E.D., Metz, T.O., Smith, R.D., Baynes, J.W., and Frizzell, N. (2014). The succinate proteme. Mass Spectrom. Rev. 33, 98–109.

Nagai, R., Brock, J.W., Blatnik, M., Baatz, J.E., Baud, J.E., Baud, J., Walla, M.D., Thorpe, S.R., Baynes, J.W., and Frizzell, N. (2007). Succination of protein thiols during adipocyte maturation: a biomarker of mitochondrial stress. J. Biol. Chem. 282, 34219–34228.

O’Flaherty, L., Adam, J., Heath, L.C., Zhdanov, A.V., Chung, Y.L., Miranda, M.X., Croft, J., Olpin, S., Clarke, K., Pugh, C.W., et al. (2010). Dysregulation of hypoxia pathways in fumarate hydratase-deficient cells is independent of defective mitochondrial metabolism. Hum. Mol. Genet. 19, 3844–3851.

Pollard, P.J., Brière, J.J., Alum, N.A., Barwell, J., Barclay, E., Wortham, N.C., Hunt, T., Mitchell, M., Olpin, S., Moat, S.J., et al. (2005). Accumulation of Krebs cycle intermediates and over-expression of Hif1alpha in tumours which result from germline FH and SDH mutations. Hum. Mol. Genet. 14, 2231–2239.

Pollard, P.J., Spencer-Dene, B., Shukla, D., Howarth, K., Nye, E., El-Bahrawy, M., Deheragoda, M., Joannou, M., McDonald, S., Martin, A., et al. (2007). Targeted inactivation of fh1 causes proliferative renal cyst development and activation of the hypoxia pathway. Cancer Cell 11, 311–319.

R Development Core Team (2016). R: A language and environment for statistical computing (R Foundation for Statistical Computing). http://www.R-project.org/.

Shepherd, R.M., and Henquin, J.C. (1995). The role of metabolism, cytoplasmic Ca2+ and pH-regulating exchangers in glucose-induced rise of cytoplasmic pH in normal mouse pancreatic islets. J. Biol. Chem. 270, 7915–7921.

Spéigel, P., Malmgren, S., Sharoyko, V.V., Newsholme, P., Koeck, T., and Mulder, H. (2011). Metabolomic analyses reveal profound differences in
glycolytic and tricarboxylic acid cycle metabolism in glucose-responsive and -unresponsive clonal β-cell lines. Biochem. J. 435, 277–284.
Sullivan, L.B., Martinez-Garcia, E., Nguyen, H., Mullen, A.R., Dufour, E., Sudarshan, S., Licht, J.D., Deberardinis, R.J., and Chandel, N.S. (2013). The proto-oncometabolite fumarate binds glutathione to amplify ROS-dependent signaling. Mol. Cell 51, 236–248.
Tarasov, A.I., Semplici, F., Ravier, M.A., Bellomo, E.A., Pullen, T.J., Gilon, P., Sekler, I., Rizzuto, R., and Rutter, G.A. (2012). The mitochondrial Ca2+ uniporter MCU is essential for glucose-induced ATP increases in pancreatic β-cells. PLoS ONE 7, e39722.
Tarasov, A.I., Semplici, F., Li, D., Rizzuto, R., Ravier, M.A., Gilon, P., and Rutter, G.A. (2013). Frequency-dependent mitochondrial Ca2+ accumulation regulates ATP synthesis in pancreatic β cells. Pflugers Arch. 465, 543–554.
Ternette, N., Yang, M., Larroya, M., Kitagawa, M., O’Flaherty, L., Wolhutter, K., Igarashi, K., Saito, K., Kato, K., Fischer, R., et al. (2013). Inhibition of mitochondrial aconitase by succination in fumarate hydratase deficiency. Cell Rep. 3, 689–700.
Thomas, S.A., Storey, K.B., Baynes, J.W., and Frizzell, N. (2012). Tissue distribution of S-(2-succino)cysteine (2SC), a biomarker of mitochondrial stress in obesity and diabetes. Obesity (Silver Spring) 20, 263–269.
Uruno, A., Furusawa, Y., Yagishita, Y., Futakomi, T., Muramatsu, H., Negishi, T., Sugawara, A., Kessler, T.W., and Yamamoto, M. (2013). The Keap1-Nrf2 system prevents onset of diabetes mellitus. Mol. Cell. Biol. 33, 2996–3010.
Weir, G.C., and Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. Diabetes 53 (Suppl. 3), S16–S21.
Wiederkehr, A., Park, K.-S., Dupont, O., Demaurex, N., Pozzan, T., Cline, G.W., and Wolheim, C.B. (2009). Matrix alkalization: a novel mitochondrial signal for sustained pancreatic β-cell activation. EMBO J. 28, 417–428.
Willson, V.J.C., and Tipton, K.F. (1980). The effect of pH on the allosteric behaviour of ox-brain NAD+-dependent isocitrate dehydrogenase. Eur. J. Biochem. 109, 411–416.
Zehetner, J., Danzer, C., Collins, S., Eckhardt, K., Gerber, P.A., Balbighi, P., Galvanovskis, J., Shimomura, K., Ashcroft, F.M., Thorens, B., et al. (2008). PVHL is a regulator of glucose metabolism and insulin secretion in pancreatic β cells. Genes Dev. 22, 3135–3146.
Zelent, D., Najafi, H., Odili, S., Buettiger, C., Weik-Collins, H., Li, C., Doliba, N., Grimsby, J., and Matschinsky, F.M. (2005). Glucokinase and glucose homeostasis: proven concepts and new ideas. Biochem. Soc. Trans. 33, 306–310.
Zhang, Q., Ramracheya, R., Lohmann, C., Tarasov, A., Bengtsson, M., Braha, O., Braun, M., Breton, M., Collins, S., Galvanovskis, J., et al. (2013). Role of KATP channels in glucose-regulated glucagon secretion and impaired counterregulation in type 2 diabetes. Cell Metab. 18, 871–882.
Zheng, L., MacKenzie, E.D., Karim, S.A., Hedley, A., Blyth, K., Kalna, G., Watson, D.G., Szlosarek, P., Frezza, C., and Gottlieb, E. (2013). Reversed argininosuccinate lyase activity in fumarate hydratase-deficient cancer cells. Cancer Metab. 1, 12.
Zheng, L., Cardaci, S., Jerby, L., MacKenzie, E.D., Sciacovelli, M., Johnson, T.I., Gaude, E., King, A., Leach, J.D., Edrada-Ebel, R., et al. (2015). Fumarate induces redox-dependent senescence by modifying glutathione metabolism. Nat. Commun. 6, 6001.
Supplemental Information

Fumarate Hydratase Deletion in Pancreatic β Cells Leads to Progressive Diabetes

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Supplemental Figure S1. Introduction of full length (FH) and cytoplasmic (FH\textsuperscript{cyt}) human fumarate hydratase rescues dysregulated metabolism in Fh1\textsuperscript{β}KO islets.

Supplemental Figure S2. Glucose stimulated insulin secretion is blunted in Fh1\textsuperscript{β}KO islets

Supplemental Figure S3. Glucose stimulated amino acid response in CTL and Fh1\textsuperscript{β}KO islets

Supplemental Figure S4. Metabolite analysis with stable isotope tracer [U-\textsuperscript{13}C\textsubscript{5}]-glutamine or [\textsuperscript{13}C\textsubscript{6}]-glucose of murine Fh1\textsuperscript{β}KO islets and CTL islets.

Supplemental Figure S5. Introduction of full length (FH) or cytoplasmic (FH\textsuperscript{cyt}) human fumarate hydratase restores mitochondrial morphology in β-cells lacking Fh1.

Supplemental Figure S6. Evidence of elevated protein succination in pancreatic islets from Fh1\textsuperscript{β}KO mice and human diabetic donors.

Supplemental Figure S7. Glyceraldehyde blunts GSIS in CTL islets.

Supplemental Table 1. Metabolite analysis of murine Fh1\textsuperscript{β}KO islets compared to CTL islets confirms Fh1 loss leads to dysregulated metabolism.

Supplemental Table 2. Screening protein succination in Fh1\textsuperscript{β}KO murine islets, non-diabetic and T2D human islets by mass spectrometry.
Supplemental Experimental Procedures

RNA isolation, reverse transcription and quantitative PCR (qPCR)
Total RNA was extracted from freshly-picked size-matched islets using Tri-reagent (Sigma) according to the manufacturer’s protocol. Single-strand cDNA was synthesized from total RNA (2 µg) using a high-capacity cDNA kit (Applied Biosystems) and analyzed by multiplex qPCR using Taqman gene expression assays on a StepOne thermocycler (Applied Biosystems). Normalization was to β-actin mRNA and relative gene expression was calculated using the ΔΔCT method. DNA corresponding to 50 ng of RNA template was used and three biological replicates, each in duplicate, were performed for each experiment. Statistical comparisons were performed using an unpaired Student’s t test.

Mouse Islet Isolation, culture and hormone secretion
Islets were isolated by collagenase (Sigma) or liberase (Roche) digestion. Collagenase or liberase was injected into the pancreas, which was then excised and incubated for 10–14 min at 37°C. Digestion was stopped by the addition of 10 ml of ice-cold Hank’s Balanced Salt (HBSS; Sigma UK) supplemented with 0.1% bovine serum albumin (BSA) (Invitrogen, UK). The islets were left to sediment by gravity and washed further in HBSS with BSA to remove the remaining exocrine tissue. Mouse islets were transferred to RPMI-1640 (Sigma, UK) supplemented with 5 mM glucose, 100 U/ml penicillin, 10 µg/ml streptomycin and 10% fetal calf serum. Isolated mouse islets were cultured in this medium at 37 °C in a humidified atmosphere (5% CO2/95% air) for 2 hr prior to experiments.

Hormone secretion was measured from batches of 10-12 islets and was counted as an experiment (unless otherwise stated). Size-matched islets were hand-picked and washed twice in glucose-free RPMI-1640 (Sigma; supplemented with 100 U/ml penicillin, 10 µg/ml streptomycin and 10% fetal calf serum). The islets were pre-incubated for 1 hr in a humidified chamber at 37 °C (5% CO2/95% air) in 300 µl of Krebs-Ringer buffer (KRB) which contained the following (mM) 140 NaCl, 3.6 KCl, 2.6 CaCl2, 0.5 MgSO4·7H2O, 0.5 NaH2PO4, 2 NaHCO3, 5 HEPES and 2 mg/ml BSA (pH adjusted to 7.4) and 1 mM glucose. The pre-incubation buffer was discarded and the islets were incubated for a further 1 hr, as indicated. In one set of experiments (Figure 3E), hormone secretion was measured in the presence of 70 mM KCl (indicated in the text) to bypass electrical activity and investigate the amplifying effect of glucose.

An aliquot of the supernatant was collected and stored at -20°C for quantification of insulin or glucagon secretion by radioimmunoassay. The remaining supernatant was discarded and the islets were lysed in 100 µl of ice-cold acid ethanol solution (containing ethanol, H2O and HCl in a ratio of 52:17:1) to release their hormone content. The lysates were immediately frozen at -20 °C. Radio-immunoassays for insulin and glucagon were performed using kits (Millipore and Eurodiagnostica respectively) following the manufacturer’s protocols.

Insulin and glucagon content of whole pancreas was determined by freezing and then grinding the pancreas, after weighing. This ground tissue was then transferred to ice-cold acid ethanol (as per above) and sonicated prior to determination of hormone content.

Whole-pancreas perfusion
Dynamic measurements of insulin secretion were performed using in situ pancreatic perfusion. Briefly, the aorta was cannulated by ligating above the coeliac artery and below the superior mesenteric artery, and the pancreas was perfused with KRB solution at a rate of ~0.45 ml/min using an Ismatec Reglo Digital MS2/12 peristaltic pump. The perfusate was maintained at 37 °C with a Warner Instruments temperature control unit TC-32 4B in conjunction with a tube heater (Warner Instruments P/N 64-0102) and a Harvard Apparatus heated rodent operating table. The effluent was collected, using a Teledyne ISCO Foxy R1 fraction collector. The pancreas was first perfused for 20 min with 1 mM glucose before commencing the experiment to establish the basal rate of secretion.

Membrane potential recordings
Freshly isolated mouse islets were immobilized by using a large bore glass electrode and electrical activity was measured from β cells on the periphery of the islets using an EPC-10 amplifier (HEKA Electronics) and Pulse software (version 8.81, HEKA Electronics). β-cells were identified by Na+ current inactivation pattern and electrical activity in response to different glucose concentrations. In order to maintain metabolically intact cells, perforated patch whole-cell configuration was used. The extracellular solution is composed of (mM) 140 NaCl, 3.6 KCl, 0.5 MgSO4, 0.5 NaH2PO4, 1.5 CaCl2, 5 NaHCO3 and 10 HEPES (pH7.4 with NaOH). The intracellular solution contained (mM) 76 K2SO4, 10 NaCl, 10 KCl, 1 MgCl2 and 5 HEPES (pH 7.35 with KOH). Perforation
was achieved by the pore-forming antibiotic amphotericin B (at the final concentration of 40 μg/ml) added into the intracellular solution.

**Measurement of [5-3H]-glucose utilization and [U-14C]-glucose oxidation**

Groups of islets were incubated in KRB containing BSA (Sigma, 0.2%) and 1 or 20 mM glucose at 37 °C for 90 min in vials. Metabolism was stopped by the addition of HCl. The CO₂ produced during oxidation was captured in 400 μl phenylethylamine (Sigma), diluted 1:1 v/v in methanol, during a 90 min incubation period. The 3H₂O produced was left to equilibrate to 2 ml water added to the vials, for a further 24 hr. Biodegradable scintillation liquid (10 ml) was then added to either the 400 μl phenylethylamine or to the 2 ml H₂O, in a scintillation vial. The ³⁷CO₂ and the ³²H₂O were measured in a scintillation counter. Radiolabeled glucose and scintillation liquid were obtained from Perkin Elmer.

**HPLC quantification of amino acids**

Islet amino acids were separated by reverse-phase HPLC after pre-column derivatization with o-phthalaldehyde and quantified by fluorescence detection. Groups of 30 islets were analyzed in triplicate from Stage II Fh1βKO and control (CTL) littermates. The content and release of the measured islet amino acids were studied following previously published methods (Hernandez-Fisac et al, 2006). The residual incubation medium was aspirated, the islets were washed twice with 100 μl PBS and their amino acids extracted with 30 μl of 10% (w/v) 5-sulfosalicylic acid.

**Metabolite analysis (CE-TOFMS) of islets**

The levels and pattern of incorporation of the respective labels in metabolites (fmol/cell) were determined by capillary electrophoresis time of flight mass spectrometry (CE-TOFMS) analysis. Isolated islets from individual mice of the same genotype were pooled and then separated into experimental groups. These islets were then washed and incubated at 37 °C in replicates under appropriate experimental conditions. Labelling studies were performed on islets isolated from Stage II Fh1βKO and age-matched control mice and cultured in RPMI-1640 (Sigma, UK) medium supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin and 10% fetal calf serum containing either the stable isotope tracer [U-¹³C₆]-glutamine for 3 hr or ¹⁵N-glucose for 1 hr at 1 or 20 mM glucose. At the end of the incubation, the islets were washed twice in an excess of 5% mannitol in water (Wako) and 200 μl of methanol was added containing 3 standards (methionine sulfone, 2-morpholinooethanesulfonate and D-Camphor-10-sulfonic acid, each at 25 μM). The islets were then left to rest for 10 min prior to freezing and storage at -80 °C. Frozen islet samples were prepared and analyzed by CE-TOFMS as described previously (Soga et al, 2006; Soga et al, 2009).

**Electron microscopy and comparative quantitation of mitochondria**

Isolated islets from Stage II CTL and Fh1βKO mice were fixed in 2.5% glutaraldehyde (Sigma, UK) in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide, block stained in 2% uranyl acetate and embedded in Spurr’s resin or London Resin Gold resin (LRG) (Agar Scientific, UK). Ultrathin sections were cut onto nickel grids, contrast was enhanced with 2% uranyl acetate and lead citrate and sections examined in a Jeol 1010 microscope. For morphometry, mitochondrial cytoplasmic density and sectional area (size) were quantified from micrographs (x800 magnification) taken of 25-30 β cells in 3-5 islets from each experimental group.

**Culture of human islets and CE-TOFMS**

Human pancreata (Figure 5E and Table S2) were obtained with ethical approval and clinical consent from non-diabetic donors. Islets were isolated in the Diabetes Research & Wellness Foundation Human Islet Isolation unit (Oxford, UK) using modified versions of published procedures (Lake et al., 1989). Following isolation, islets were cultured in CMRL medium containing 5.5 mM glucose and 2 mM L-glutamine. Hand-picked islets were size-matched and cultured for 1 or 24 hr in RPMI containing glucose as indicated. Islets were pre-incubated in Krebs-Ringer buffer (KRB) containing 2 mg/ml BSA and 3 mM glucose for 1 hr at 37 °C, followed by a 1 hr test incubation in KRB supplemented with glucose as indicated. At the end of the incubation time the islets were processed for CE-TOFMS as described above for mouse islets.

**Culture of human islets and GC/MS analysis**

For these experiments (Figure 5F), human islets were obtained from the Nordic Center For Clinical Islet Transplantation (Uppsala, Sweden). Experimental procedures were approved by the Lund University Ethical Board. These were in compliance with the Declaration of Helsinki (2000) and the World Medical Association. Islets were processed according as previously described (Fadista et al., 2014). Islets were incubated 30 min at 2.8 mM glucose and then stimulated with either 2.8 or 16.7 mM glucose for 1 hr. The islets were transferred into 300 μl ice-cold extraction solvent. Metabolite extracts were analyzed on an Agilent 6890N gas chromatograph (Agilent Technologies, Atlanta, GA) equipped with an Agilent 7683B auto-sampler (Agilent Technologies) and
coupled to a LECO Pegasus III TOFMS electron impact time-of-flight mass spectrometer (LECO Corp., St. Joseph, MI).

**Immunofluorescence and immunoblotting**

Immunofluorescence was performed using the same antibodies as for IHC with Alexa Fluor® secondary antibodies (Molecular Probes, Life technologies) and an FITC-conjugated anti-V5 antibody (Life technologies) using a Zeiss LSM510 META confocal imaging system.

Immunoblotting was performed as described previously (O’Flaherty et al., 2010) using primary antibodies against FH (Autogen Bioclear) and calnexin (Calbiochem).

**Mass spectrometry (MS) and Proteome analysis**

Isolated pancreatic islets were lysed in 7 M Urea /SDS buffer and either run on an SDS-PAGE or processed in solution. For in solution samples, depletion of serum albumin was achieved by addition of ice-cold ethanol to a final concentration of 42% to the lysates and reconstitution of protein pellets in a buffer containing 6 M urea and 100 mM Tris (pH 7.8). Unmodified cysteine residues were alkylated in reducing conditions (10 mM DTT) using 30 mM 4-vinylpyridine (resulting in pyridylethylolation, PE) or 50 mM iodoacetamide (carbamidomethylation, CA). Proteins were then subjected to trypsin digestion. In solution samples were further purified on Sep-Pak C18 columns (Waters).

Analysis of resulting peptides was performed on either an LTQ Orbitrap Velos or a Q-Exactive (Thermo Fisher Scientific).

**LTQ Orbitrap Velos**

Peptides were separated on an Acquity nano UPLC system (Waters) supplemented with a 25 cm C18 column, 1.7 µm particle size (Waters). They were eluted by applying a 60-180 min linear gradient from 1% buffer A (0.1% formic acid in water) to 40% buffer B (0.1% formic acid in acetonitrile) at a flow rate of 250 nl/min. Collision-induced dissociation (CID) was induced on the twenty most abundant ions per full MS scan using an isolation width of 1.5 Da. All fragmented precursor ions were actively excluded from repeated MS/MS analysis for 15 s.

**Q-Exactive**

Peptides were separated on a Ultimate 3000 RSLCnano System utilizing a PepMap C18 column, 2 µm particle size, 75 µm x 50 cm (Thermo Scientific). A 60 min linear gradient was applied from 1% buffer A (0.1% formic acid, 5% DMSO in water) to 40% buffer B (0.1% formic acid, 5% DMSO in acetonitrile) at a flow rate of 250 nl/min. Collision-induced dissociation (CID) was induced on the fifteen most abundant ions per full MS scan using an isolation width of 1.5 Da. All fragmented precursor ions were actively excluded from repeated MS/MS analysis for 15 s.

All samples were analyzed in triplicate. Feature identification was performed by generation of Mascot generic files (mgf) using Proteowizard and analysis with either Mascot v2.3.01 or Peaks 7 searching the human or mouse SwissProt database.

Normalisation of MS runs and label-free quantitation of detected features was performed using Peaks 7 (Bioinformatics solutions). All features with a significant change between both conditions (ANOVA p-value < 0.05) were included in the analysis and imported to Ingenuity.
**Supplemental References**

Fadista, J., Vikman, P., Laakso, E.O., Mollet, I.G., Esguerra, J.L., Taneera, J., Storm, P., Osmark, P., Ladenvall, C., Prasad, R.B., et al. (2014). Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. *PNAS* **111**, 13924-13929.

Hernandez-Fisac, I., Fernandez-Pascual, S., Ortsater, H., Pizarro-Delgado, J., Martin del Rio, R., Bergsten, P., Tamarit-Rodriguez, J. (2006). Oxo-4-methylpentanoic acid directs the metabolism of GABA into the Krebs cycle in rat pancreatic islets. *Biochem J* **400**, 81-89

Lake, S.P., Bassett, P.D., Larkins, A., Revell, J., Walczak, K., Chamberlain, J., Rumford, G.M., London, N.J., Veitch, P.S., Bell, P.R. et al. (1989). Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator. *Diabetes* **38** Suppl 1, 143-145.

O’Flaherty, L., Adam, J., Heather, L.C., Zhdanov, A.V., Chung, Y.L., Miranda, M.X., Croft, J., Olpin, S., Clarke, K., Pugh, C.W. et al. (2010). Dysregulation of hypoxia pathways in fumarate hydratase-deficient cells is independent of defective mitochondrial metabolism. *Hum Mol Genet* **19**, 3844-3851.

Soga, T., Baran, R., Suematsu, M., Ueno, Y., Ikeda, S., Sakurakawa, T., Kakazu, Y., Ishikawa, T., Robert, M., Nishioka, T. et al. (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. *J Biol Chem* **281**, 16768-16776.

Soga, T., Igarashi, K., Ito, C., Mizobuchi, K., Zimmermann, H.P., Tomita, M. (2009). Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry. *Anal Chem* **81**, 6165-6174.
Figure S1. Introduction of full length (FH) and cytoplasmic (FHcyt) human fumarate hydratase rescues dysregulated metabolism in Fh1βKO islets. Related to Figure 1.

(A) Deletion of Nrf2 did not alter the glucose tolerance in Stage II Fh1βKO compared to littermate CTL mice. IPGTT was performed in Fh1βKONrf2CTL, Fh1βKONrf2Het, Fh1βNrf2DKO and age matched CTL, Fh1CTLNrf2KO and Fh1CTLNrf2Het mice (n= 3 experiments using a total of at least 8 mice per genotype).

(B-D) Analysis of gene expression by qPCR in isolated islets from Stage II CTL (blue), Fh1βKO (red), Fh1βKO+FH (green) and Fh1βKO+FHcyt (brown) mice for murine fumarate hydratase, Fh1 (B), Egl-9 family hypoxia-inducible factor 3, Egln3 (C), and heme oxygenase-1, Hmox1 (D) (n=3 experiments using islets from at least 9 mice per genotype). These data confirm that although murine Fhl remains deleted in the β-cells of Fh1βKO, Fh1βKO+FH and Fh1βKO+FHcyt mice, the Hif1α target gene Egln3 and the Nrf2 target gene Hmox1 both show mRNA expression in islets from Fh1βKO+FH and Fh1βKO+FHcyt mice comparable to that of CTL islets by virtue of expression of both full length and cytoplasmic-specific human FH. In contrast, the mRNA levels of Egln3 and Hmox1 are elevated in the Fh1βKO islets because of elevated fumarate leading to the subsequent stabilization of both HIF1α and NRF2. mRNA levels were normalized to those of Actb. #p<0.0001 versus CTL. Error bars indicate ± SEM.
Figure S2. Glucose stimulated insulin secretion is blunted in Fh1βKO islets. Related to Figure 2.
Insulin secretion in age-matched CTL and Stage II Fh1βKO islets following 1 hr stimulation with glucose or α-ketoisocaproic acid (α-KIC; 10 mM). Results are displayed with insulin secretion expressed normalized to a percentage of the basal value (2 mM glucose). Approximate basal secretion is indicated (dashed line), (n=islets from at least 3 mice per genotype). ***p<0.001 versus 2 mM glucose CTL, §§p<0.01, §§§p<0.001 for comparison of the same condition between FH1βKO and CTL. Error bars indicate ± SEM.
Figures in the main text display insulin secretion data as absolute values.
Data have been normalized to the basal insulin release to compensate for any decrease in secretion due to lowered insulin content. The fact that stimulated insulin secretion remains strongly reduced in Fh1βKO islets argues that there is a functional defect.
Figure S3. Glucose stimulated amino acid response in CTL and Fh1βKO islets. Related to Figure 3.

(A-B) Analysis by CE-TOFMS of islet content of significant metabolites from Stage II CTL (blue), Fh1βKO (red), Fh1βKO+FH (green) and Fh1βKO+FHcyt (brown) mice demonstrated that β cell fumarate (A) and argininosuccinate (B) contents were normalized to levels comparable to CTL mice following re-expression of full-length or cytoplasmic-specific human FH. #p<0.0001 versus CTL; ND not detected. (n=3 samples from 12-15 mice per experimental group).

(C-D) Amino acid content measured by HPLC in Stage II CTL (n=8 mice) and Fh1βKO (n=12 mice) islets after culture in 1 mM glucose (C) or 20 mM glucose (D) for 1 hr. (n=30 islets per genotype were analyzed in triplicate in 5 experiments). Abbreviations: Asp, aspartate; Glu, glutamic acid; Ser, serine; Gln, glutamine; His, histidine; Gly, glycine; Thr, threonine; Arg, arginine; Tau, taurine; Ala, alanine; Tyr, tyrosine; GABA, gamma aminobutyric acid; Val, valine; Phe, phenylalanine; Ile, isoleucine and Leu, leucine. Error bars represent ± SEM.
Figure S4. Metabolite analysis with stable isotope tracer $[^{13}\text{C}_5]$-[glutamine or $^{13}\text{C}_6$]-[glucose of murine Fh1βKO islets and CTL islets. Related to Figure 3.

(A-O) Analysis by CE-TOFMS of metabolite levels (relative area) and the pattern of $^{13}\text{C}$ incorporation in islets isolated from age-matched CTL (black) and Stage II Fh1βKO (grey) mice following culture in medium containing either $[^{13}\text{C}_5]$-glutamine for 3 hr (A-H and M) or $^{13}\text{C}_6$-glucose for 1 hr (I-L and N-O) in cells and stimulation at 1 or 20 mM glucose. The samples are underivatized and uncorrected for natural abundance of $^{13}\text{C}$.

(M) Changes in the percentage incorporation by glucose stimulation (1 and 20 mM glucose; 1G and 20G) of glutamine isotopomers (m0 to m+5) into UDP-N-acetylglucosamine following culture for 3 hr of isolated CTL and Fh1βKO islets in medium containing the stable isotope $[^{13}\text{C}_5]$-glutamine.

(N) Changes in the percentage incorporation by glucose stimulation (1 and 20 mM glucose) of glucose isotopomers (m0-m+6) into glutamate following culture for 1 hr of isolated CTL and Fh1βKO islets in medium containing the stable isotope $^{13}\text{C}_6$-glucose.

(O) Changes in the percentage incorporation by glucose stimulation (1 and 20 mM glucose) of glucose isotopomers (m0-m+6) into aspartate following culture for 1 hr of isolated CTL and Fh1βKO islets in medium containing the stable isotope $^{13}\text{C}_6$-glucose.

(n=3 experimental groups of pooled islets for each experimental condition from at least 3 mice per genotype).

These data indicate that at 1 mM glucose none of the intracellular glutamate is derived from glutamine in CTL islets compared to ~50% in Fh1βKO islets. At 20 mM glucose, both the CTL and Fh1βKO islets use glutamine to produce glutamate. The small amount of islet tissue that could be isolated precluded a broad-spectrum analysis. However, at 1 mM glucose (1G) essentially none of the glutamate in the CTL islets came from labelled glutamine. By contrast, ~50% of the glutamate is derived from glutamine in Fh1βKO islets as label travels via the oxidative route of the Krebs cycle into fumarate and argininosuccinate. Most of this is from the direct conversion of glutamine to glutamate (m+5), but detection of m+3 may result from entry of fumarate into the urea cycle, allowing Asp m+4 to be produced. At 20 mM glucose, both the CTL and Fh1βKO islets use glutamine to produce glutamate, and both m+5 and m+3 are observed.
Figure S5. Introduction of full length (FH) or cytoplasmic (FHcyt) human fumarate hydratase restores mitochondrial morphology in β cells lacking Fh1. Related to Figure 4.

(A) Electron micrographs of β cells in Stage II CTL and Fh1βKO islets highlighting some very large mitochondria (red arrow) and smaller mitochondria (blue arrow) in the Fh1βKO β cells compared to CTL islets. Abbreviations: g, secretory granule; m, mitochondrion; n, nucleus. Scale bar: 500 nm.

(B) Relative mitochondrial area (mitochondrial area divided by cytoplasmic area; maximum to minimum range) compared in β cells in Stage II CTL (blue), Fh1βKO (red), Fh1βKO+FH (green) and Fh1βKO+FHcyt (brown) mice showing the larger range of mitochondrial areas in Fh1βKO islets and normalization following reintroduction of FH. (n=25-30 β cells in 3-5 islets per genotype). Error bars represent ± SEM.
Figure S6. Evidence of elevated protein succination in pancreatic islets from Fh1βKO mice and human diabetic donors. Related to Figure 5.

(A) MS/MS spectra for peptide 178VGVPGSVCTTR189 showing increased succination in guanosine 5'-monophosphate oxidoreductase (GMP reductase 1) at cysteine 186 (C186) in Stage II Fh1βKO islets. Both theoretical mass and detected mass (in brackets) are given for each assigned fragment ion. Peptide fragments of different mass that contain the modified residue are highlighted in red. PE indicates the modification of cysteine residues at C186 to pyridylethyl-cysteine.

(B) MS/MS spectra showing either succination (2S-C) or carbamidoethylation (C-C) at cysteine 106 (C106) in the 100GLIAICAGPTALLAHEIGFGSK122 peptide of human DJ-1 derived from islets of a T2D donor. Matching fragment ion peaks between the two peptide species that do not contain the modified residue are highlighted in green, whereas peptide fragments of different mass that contain the modified residue are highlighted in red. The calculated peptide mass based on the detected m/z (m: mass, z: charge) value of the doubly charged precursor peptide ion ([M+2H]⁺) and the calculated ([M] calc.) and theoretical peptide mass ([M] theor.) are stated for both peptide species. Detected N- and C-terminal fragment ions are indicated in the peptide sequence, assigned in the spectrum and depicted as follows: b: N-terminal fragment ion; y: C-terminal fragment ion; *: fragment ion minus NH₃; and ′: doubly charged fragment ion. Both theoretical mass (in brackets) and detected mass are given for each assigned fragment ion.
Figure S7. Glyceraldehyde blunts GSIS in CTL islets. Related to Figures 5 and 6.

(A) Insulin secretion measured during 1 hr incubations at 1, 6 and 20 mM glucose following 24 hr culture of wild-type islets from C57BL/6J mice with 10 mM glyceraldehyde. (n=6 experimental groups of islets for each condition assayed in 2 experiments; islets harvested from 6 mice in total). Data are mean values ± SEM of secretion expressed as per cent of hormone content. ****p<0.0001 versus 1 mM glucose; ¶¶¶¶p<0.0001 versus 6 mM glucose; §§§§p<0.0001 comparing same condition between groups.

(B) Insulin content of the islets in A.
| Metabolite                | WT   | WT   | WT   | WT   | WT   | WT   | WT   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | KO   | T-test |
|--------------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Argininosuccinate        | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 9.19E-01 | 8.75E-01 | 9.39E-01 | 9.58E-01 | 9.13E-01 | 9.57E-01 | 8.28E-01 | 8.72E-17 |
| Fumarate                 | 6.53E-02 | 7.15E-02 | 1.22E-01 | 6.28E-02 | 6.81E-02 | 1.34E-01 | 7.82E-02 | 5.63E-02 | 1.20E+00 | 1.05E+00 | 1.06E+00 | 1.15E+00 | 1.21E+00 | 1.25E+00 | 1.16E+00 | 1.45E-14 |
| Glucose-6-phosphate      | 2.46E-02 | 5.92E-02 | 4.63E-02 | 3.91E-02 | 3.26E-02 | 3.90E-02 | 3.86E-02 | 3.21E-02 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 3.39E-02 | 7.42E-05 |
| Aspartate                | 1.26E+00 | 1.41E+00 | 1.54E+00 | 1.31E+00 | 1.19E+00 | 1.41E+00 | 1.33E+00 | 1.17E+00 | 1.06E+00 | 8.90E-01 | 8.96E-01 | 1.09E+00 | 1.09E+00 | 1.02E+00 | 1.08E+00 | 1.09E-04 |
| Adenylosuccinate         | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 7.07E-02 | 6.39E-02 | 5.89E-02 | 1.99E-01 | 7.08E-02 | 8.88E-02 | 1.44E-01 | 1.27E-04 |
| Cysteine-glutathione     | 8.49E-02 | 1.02E-01 | 1.09E-01 | 6.87E-02 | 3.75E-02 | 9.96E-02 | 6.01E-02 | 9.32E-02 | 6.97E-02 | 5.69E-02 | 0.00E+00 | 1.80E-02 | 4.40E-02 | 2.47E-02 | 1.84E-02 | 2.07E-03 |
| disulphide - Divalent    | 3.85E-01 | 5.45E-01 | 5.70E-01 | 2.40E-01 | 3.68E-01 | 6.06E-01 | 4.10E-01 | 2.74E-01 | 1.13E-01 | 1.80E-01 | 1.26E-01 | 3.26E-01 | 2.23E-01 | 9.76E-02 | 2.97E-01 | 2.29E-03 |
| Adenosine                | 2.02E-01 | 2.18E-01 | 2.79E-01 | 1.91E-01 | 1.85E-01 | 2.90E-01 | 1.83E-01 | 3.62E-01 | 3.95E-01 | 3.60E-01 | 3.85E-01 | 3.51E-01 | 3.18E-01 | 2.71E-01 | 3.19E-01 | 3.37E-03 |
| Cystathionine            | 9.10E-02 | 1.35E-01 | 1.31E-01 | 6.77E-02 | 1.15E-01 | 1.75E-01 | 1.24E-01 | 8.39E-02 | 0.00E+00 | 0.00E+00 | 0.00E+00 | 1.06E-01 | 7.35E-02 | 3.50E-02 | 1.05E-01 | 6.30E-03 |
| Uridine                  | 5.13E-02 | 1.23E-01 | 1.22E-01 | 6.01E-02 | 5.36E-02 | 9.18E-02 | 1.02E-01 | 9.68E-02 | 5.64E-02 | 4.67E-02 | 0.00E+00 | 6.31E-02 | 5.47E-02 | 5.22E-02 | 5.48E-02 | 9.36E-03 |
| monophosphate            | 7.11E-01 | 8.16E-01 | 7.00E-01 | 5.59E-01 | 7.92E-01 | 8.52E-01 | 6.80E-01 | 5.38E-01 | 4.27E-01 | 5.24E-01 | 5.85E-01 | 6.51E-01 | 5.04E-01 | 6.46E-01 | 1.21E-02 |
| Guanidinoacetate         | 5.33E-01 | 7.11E-01 | 8.16E-01 | 7.00E-01 | 5.59E-01 | 7.92E-01 | 8.52E-01 | 6.80E-01 | 5.38E-01 | 4.27E-01 | 5.24E-01 | 5.85E-01 | 6.51E-01 | 5.04E-01 | 6.46E-01 | 1.21E-02 |
| Gamma                    | 4.20E-01 | 4.94E-01 | 6.34E-01 | 5.39E-01 | 3.69E-01 | 5.79E-01 | 5.25E-01 | 5.01E-01 | 5.25E-01 | 3.40E-01 | 3.57E-01 | 3.85E-01 | 4.12E-01 | 3.78E-01 | 3.84E-01 | 1.32E-02 |
Table S1. Metabolite analysis of murine Fh1βKO islets compared to CTL islets confirms Fh1 loss leads to dysregulated metabolism. Related to Figure 3.

Analysis by CE-TOFMS of islet cellular concentrations (fmol/cell) for metabolites. Islets were isolated from individual Stage II Fh1βKO (n=7) and age-matched CTL (n=8) mice and incubated for 1 hr in 5 mM glucose then prepared for CE-TOFMS as described above. Metabolites listed show significance of p<0.05 or better between CTL and Fh1βKO islets (Student’s t-test).

| Metabolite               | WT    | WT    | WT    | WT    | WT    | WT    | KO    | KO    | KO    | KO    | KO    | KO    | KO    | KO    | T-test |
|--------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| cis-Aconitate            | 3.12E-01 | 2.00E-01 | 8.11E-02 | 1.50E-01 | 1.14E-01 | 1.74E-01 | 2.73E-01 | 2.48E-01 | 4.36E-01 | 6.40E-01 | 2.32E-01 | 3.24E-01 | 2.47E-01 | 2.88E-01 | 1.61E-02 |
| Guanosine monophosphate  | 1.63E-01 | 2.64E-01 | 2.72E-01 | 1.30E-01 | 1.47E-01 | 3.75E-01 | 1.99E-01 | 1.62E-01 | 0.00E+00 | 1.29E-01 | 0.00E+00 | 2.25E-01 | 1.01E-01 | 0.00E+00 | 1.77E-01 | 1.78E-02 |
| Glycine                  | 7.74E-01 | 8.17E-01 | 1.13E+00 | 9.82E-01 | 7.38E-01 | 9.04E-01 | 9.38E-01 | 7.81E-01 | 1.11E+00 | 9.61E-01 | 1.05E+00 | 9.93E-01 | 1.08E+00 | 9.74E-01 | 9.92E-01 | 2.21E-02 |
| Glucose 1-phosphate      | 9.78E-02 | 1.16E-01 | 1.47E-01 | 1.05E-01 | 5.91E-02 | 9.12E-02 | 9.92E-02 | 9.52E-02 | 8.20E-02 | 7.47E-02 | 6.40E-02 | 4.43E-02 | 1.01E-01 | 9.05E-02 | 5.39E-02 | 3.03E-02 |
| Isocitrate               | 6.27E-01 | 4.60E-01 | 1.57E-01 | 4.03E-01 | 2.00E-01 | 1.47E-01 | 3.55E-01 | 4.76E-01 | 4.75E-01 | 7.13E-01 | 1.09E+00 | 4.79E-01 | 5.49E-01 | 4.15E-01 | 5.01E-01 | 3.35E-02 |
| Glycero-phosphorylcholine| 4.44E-01 | 5.25E-01 | 6.75E-01 | 6.77E-01 | 3.99E-01 | 6.25E-01 | 7.73E-01 | 6.96E-01 | 5.94E-01 | 5.88E-01 | 7.59E-01 | 8.09E-01 | 7.48E-01 | 9.04E-01 | 9.57E-01 | 3.70E-02 |
Table S2. Screening protein succination in Fh1βKO murine islets, non-diabetic and T2D human islets by mass spectrometry. Related to Figure 5.
This list of proteins was established using a false discovery rate of 1%. Protein sequence was confirmed on http://www.uniprot.org/. Quantitative analysis of protein changes was determined using Peaks (Bioinformatics solutions) and the proteins that were detected with significant change of abundance between both conditions are listed. P is the probability that the match between the MS/MS query and the peptide sequence is random. (n=150 islets isolated from 3 Stage II Fh1βKO mice; approximately 150 islets from each of 2 diabetic and 2 nondiabetic adult donors).