Metformin reduces liver glucose production by inhibition of fructose-1,6-bisphosphatase

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Metformin is a first-line drug for the treatment of individuals with type 2 diabetes, yet its precise mechanism of action remains unclear. Metformin exerts its antihyperglycemic action primarily through lowering hepatic glucose production (HGP). This suppression is thought to be mediated through inhibition of mitochondrial respiratory complex I, and thus elevation of 5′-adenosine monophosphate (AMP) levels and the activation of AMP-activated protein kinase (AMPK), though this proposition has been challenged given results in mice lacking hepatic AMPK. Here we report that the AMP-inhibited enzyme fructose-1,6-bisphosphatase-1 (FBP1), a rate-controlling enzyme in gluconeogenesis, functions as a major contributor to the therapeutic action of metformin. We observe this during a metformin tolerance test and in a metformin-euglycemic clamp that we have developed. The antihyperglycemic effect of metformin in high-fat diet–fed diabetic FBP1-KI mice was also significantly blunted compared to wild-type controls. Collectively, we show a new mechanism of action for metformin and provide further evidence that molecular targeting of FBP1 can have antihyperglycemic effects.

Diabetes is characterized by impaired glucose homeostasis partly due to abnormally elevated HGP. The biguanide drug metformin (N,N-dimethylbiguanide) works principally through inhibition of HGP, although enhanced glucose disposal has also been reported in some studies. It is widely accepted that metformin inhibits mitochondrial respiration through complex I, reducing hepatocellular energy charge. A previous study examined whether AMPK activation is responsible for the therapeutic effects of metformin; the reported data support a mechanism involving AMPK-dependent inhibition of HGP and lipogenesis, although an inhibitor of questionable selectivity was used in the study.

Indeed, a recent study has demonstrated that inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) by AMPK plays an important role in metformin-induced improvements in insulin action by maintaining hepatic lipid homeostasis. However, the importance of AMPK in metformin action on HGP has been challenged in experiments using mice lacking hepatic AMPK. Recent studies report that metformin inhibits HGP through hepatic AMPK-independent mechanisms, either by attenuating the ability of glucagon to increase 3′,5′-cyclic adenosine monophosphate (cAMP) levels and promote HGP or through direct inhibition of mitochondrial glyceraldehyde-3-phosphate dehydrogenase and the subsequent increase in cytosolic free [NADH]/[NAD+], leading to impaired utilization of lactate for gluconeogenesis. These findings suggest that the underlying mechanisms responsible for the HGP- and glucose-lowering effects of metformin in diabetes may not be explained by any single target or pathway. Interestingly, a widely used pharmacological AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP mimic, profoundly suppressed glucose output in hepatocytes lacking AMPK, indicating AMP per se, but not AMPK, plays a vital role in suppressing HGP. Similarly, a tight correlation between the magnitude of increase in [AMP]/[ATP] and inhibition of glucose output in hepatocytes has been noted.

Given that the anabolic process of gluconeogenesis is energetically costly, hepatocytes must balance this energy demand with production, thereby maintaining energy homeostasis. Hepatocytes are equipped with a mechanism to control the rate of hepatic gluconeogenesis in response to energy status, and FBP1 (Braunschweig Enzyme Database (BRENDA) entry: EC 3.1.3.11) has long been recognized as a key component. FBP1 catalyzes the irreversible hydrolysis of fructose-1,6-bisphosphate (F-1,6-P2) to fructose-6-phosphate (F6P) and inorganic phosphate (P) in the presence of divalent cations. FBP1 is a key rate-controlling enzyme in the gluconeogenic pathway, and individuals with FBP1 deficiency present with hypoglycemia and metabolic acidosis due to impaired gluconeogenesis. FBP1 activity is regulated synergistically by the allosteric inhibitors AMP and F-2,6-P2. AMP noncompetitively inhibits FBP1 by binding to a unique allosteric site, whereas F-2,6-P2 binds to the active site in competition with F-1,6-P2. Although levels of F-2,6-P2 are largely under hormonal control, AMP concentration is thought to be mediated through inhibition of mitochondrial respiratory complex I, and thus elevation of 5′-adenosine monophosphate (AMP) levels and the activation of AMP-activated protein kinase (AMPK), though this proposition has been challenged given results in mice lacking hepatic AMPK. Here we report that the AMP-inhibited enzyme fructose-1,6-bisphosphatase-1 (FBP1), a rate-controlling enzyme in gluconeogenesis, functions as a major contributor to the therapeutic action of metformin. We observe this during a metformin tolerance test and in a metformin-euglycemic clamp that we have developed. The antihyperglycemic effect of metformin in high-fat diet–fed diabetic FBP1-KI mice was also significantly blunted compared to wild-type controls. Collectively, we show a new mechanism of action for metformin and provide further evidence that molecular targeting of FBP1 can have antihyperglycemic effects.

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Results
Identification of an AMP-insensitive FBP1 mutant. Demonstrating the importance of allosteric regulation of a rate-controlling enzyme in metabolic flux control in vivo is difficult partly because of the lack of an established experimental strategy. The only definitive approach is to generate a KI animal model that specifically renders the target enzyme insensitive to the ligand of interest while leaving all other modes of regulation intact\(^1\). Such a mutant cannot be designed from basic principles and can only be chosen on the basis of the available or predicted structure combined with detailed in vitro enzyme kinetic analysis. Before designing such a point mutant of FBP1, we first established that metformin does not have a direct inhibitory effect on FBP1 up to 10 mM (Supplementary Fig. 1a). Neither does it appear to inhibit the reported target AMP deaminase 1 (AMPD1)\(^2\) (Supplementary Fig. 1a).

Next, to identify an AMP-resistant FBP1 mutant, we performed structure-guided mutagenesis based on the reported structure of the human FBP1–AMP complex\(^3\) (protein data bank (PDB) ID: 1PTA) (Fig. 1a) and evolutionary conservation of key AMP-contacting residues (Supplementary Fig. 1b). Several point mutants designed to disrupt AMP binding to mouse FBP1 were prepared using an Fbp1 null Escherichia coli strain. Native FBP1 from mouse liver was purified and used as a reference material to validate recombinant mouse FBP1 with a polyhistidine tag at the N-terminus (6HIS–FBP1). We obtained high-purity recombinant and native FBP1 as judged by Coomassie-stained SDS-PAGE gels (Fig. 1b). For inhibition of recombinant wild-type (WT) and native mouse liver FBP1 activity, half-maximal inhibitory concentration (IC\(_{50}\)) values of AMP were comparable at ~14 and ~20 μM, respectively (Fig. 1c). Among other ligands (F-1,6-P\(_2\), Mg\(^2+\)) and inhibitor (5-inosine monophosphate (IMP)), inhibition was not observed for FBP1 with a polyhistidine tag at the N-terminus (6HIS–FBP1) (Supplementary Fig. 1a). We went on to determine the IC\(_{50}\) for FBP1-G27P inhibition compared to WT protein and activity of FBP1 with a polyhistidine tag at the N-terminus (6HIS–FBP1). For inhibition of FBP1, we first established that metformin does not have a direct inhibitory effect on FBP1 up to 10 mM (Supplementary Fig. 1a).

FBP1-G27P-KI mouse model. To establish whether AMP-mediated inhibition of FBP1 activity contributes to the antihyperglycemic action of metformin in vivo, we generated an FBP1-G27P-KI mouse model in which the codon for glycine 27 of Fbp1 was modified to encode proline (Fig. 1d). FBP1 is predominantly expressed in liver and kidney and to a much lesser extent in testes and small intestine (Supplementary Fig. 2a). Although expression of FBP1 in islets has been reported\(^3\), in our study it was undetectable using a highly specific antibody (Supplementary Fig. 2a). We confirmed that expression and activity of FBP1 were comparable between homozygous FBP1-G27P-KI (KI mice) and control WT mice in liver and kidney, although FBP1 expression in KI mice was modestly higher in small intestine and lower in testes compared to WT mice (Fig. 1e and Supplementary Fig. 2b,c). Assayed in crude liver extracts, AMP exhibited an IC\(_{50}\) for FBP1-G27P inhibition that was >400-fold higher than that for WT FBP1 (Fig. 1e), which is far beyond the physiological range of cellular AMP concentrations\(^4\).

KI mice were born at the expected Mendelian frequency and displayed similar body weight and growth curves (data not shown), food intake and respiratory exchange ratio as well as locomotor activity to that of WT mice (Supplementary Fig. 3a–f). Relative to WT, KI mice exhibited similar blood glucose, plasma insulin, glucagon and leptin levels as well as hepatic glycogen content under fasted and refeed (4 h ad libitum food access following overnight fast) conditions (Fig. 2a–c). KI mice also displayed normal blood glucose tolerance (Fig. 2f) and gluconeogenic capacity, assessed by the pyruvate-tolerance test (Fig. 2g). In accordance with these observations, immunoblot analysis revealed comparable expression of major metabolic proteins involved in hepatic glucose metabolism (for example, GLUT2, glucokinase (GCK) and its regulator protein GCKR, hexokinase 1 (HXK1), phosphofructokinase (PFK1), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB1), FBP1 and glucose-6-phosphate dehydrogenase (G6PD)), glycogen metabolism (for example, glycogen synthase (GYS2) and glycogen phosphorylase (PYGL)) and gluconeogenesis (for example, catalytic and transporter subunit of glucose-6-phosphate phosphatase (G6PC/T), cytosolic/mitochondrial isoforms of phosphoenolpyruvate carboxykinase (PEPCK-C/M), pyruvate carboxylase (PC) and pyruvate kinase (PKLR)) as well as the major metformin transporter organic cation transporter 1 (OCT1) between WT and KI mice under fasted and refeed conditions (Fig. 2h and Supplementary Fig. 4). Moreover, activities of the major gluconeogenic and glycolytic enzymes were similar between WT and KI mice under both fasted and refeed conditions (Supplementary Fig. 5a–i). At the transcript level, mRNA expression levels of genes involved in gluconeogenesis (Pck1, Pparg1c and Foxo1a) and lipogenesis (Fasn) were similar between WT and KI mice (Supplementary Fig. 5j–m). Conversely, the G6pc mRNA level was significantly lower in KI mice under both fasted and refeed conditions (Supplementary Fig. 5n), although this did not translate into a difference at the protein level (Fig. 2h and Supplementary Fig. 4b) or in activity (Supplementary Fig. 5b).

Generation and characterization of an AMP-insensitive FBP1-G27P-KI mouse model. To establish whether AMP-mediated
Fig. 1 | Generation of the AMP-insensitive FBP1-G27P-KI mouse model. a, The structure of human FBP1 is represented as ribbons. AMP and interacting residues (numbered from the initiator methionine) are shown as sticks. Dashed lines represent hydrogen-bonding interactions, whereas residues making hydrophobic contacts are illustrated as sticks and transparent surfaces. Red and blue spheres represent backbone oxygen and nitrogen atoms, respectively. b, Coomassie-stained SDS-PAGE of mouse liver FBPase and recombinant mouse 6HIS–FBP1 preparations, with single point mutations designed to disrupt AMP binding. c, AMP inhibition curves of mouse 6HIS–FBP1 mutants. FBPase activity is expressed as a ratio of the maximum activity in the absence of AMP (V/Vo). IC50 values represent the mean ± s.d. of three independent measurements on two enzyme preparations. The line graph is representative of the results from a single preparation. d, Schematic illustrating the targeting strategy used to generate C57BL/6NT ac FBP1-G27P-KI mice. Exons and FRT recombination sites are represented by dark gray boxes and triangles, respectively. The KI allele containing the mutation encoding p.G27P in exon 1 is shaded pale gray. Correct recombination was confirmed by Southern blotting of PsiI and KpnI digests of genomic DNA isolated from targeted embryonic stem cells with the corresponding 5′ and 3′ probes (black boxes). Genotyping of the constitutive KI allele was performed by PCR of genomic DNA using primers P1 and P2. e, Top, Liver biopsies from overnight-fasted (16 h) or refed (4 h) WT and KI mice were assayed for Fbp1 mRNA expression by qPCR (left) or FBPase activity (right) by spectrophotometric assay. Bottom, The line graph shows AMP inhibition curves of FBPase activity in liver homogenates expressed as a ratio of the activity in the absence of AMP (V/Vo). Results represent mean ± s.d.; n = 5–7 mice per group.
Prior to testing the effect of AMP mimetic compounds, FBP1-G27P-KI mice are resistant to the hypoglycemic action of normal hepatic energy and metabolic homeostasis as well as whole-body glucose homeostasis. Taken together, these results demonstrated that KI mice possess normal hepatic energy and metabolic homeostasis as well as whole-body glucose homeostasis.

**FBP1-G27P-KI mice are resistant to the hypoglycemic action of AMP mimetic compounds.** Prior to testing the effect of metformin, we wanted to confirm whether KI mice are resistant to AMP-mediated lowering of blood glucose in vivo. For this purpose, we initially used the AMP mimetic FBPase inhibitor MB06322 (ref. 19), the prodrug of MB05032 (Fig. 3a and Supplementary Table 1). We observed that recombinant mouse FBP1 was approximately twofold less sensitive to MB05032 compared to rat FBP1 in vitro, whereas sensitivity to AMP was comparable between the two species (Fig. 3b). As all available preclinical data were only obtained from rats, we took the species difference in drug response into account, and 75 mg of MB06322 per kg body weight (mg kg\(^{-1}\)) was administered intraperitoneally (i.p.) to WT and KI mice. In WT mice, MB06322 treatment resulted in a robust and sustained decrease (~40% at 2–3 h post injection) in blood glucose levels (Fig. 3c), which was accompanied by an increase in blood lactate levels (Fig. 3d). In contrast, even though plasma concentration of the drug was comparable between genotypes (Fig. 3e), MB06322 had no significant effect on both blood glucose and lactate concentration in KI mice (Fig. 3f,g). We next sought to determine whether the well-documented hypoglycemic effect of AICAR\(^{20}\), which is converted intracellularly to the AMP-mimetic ZMP (Fig. 4a), is mediated through ZMP-dependent inhibition of FBP1 in vivo. Administration of AICAR (250 mg kg\(^{-1}\), i.p.) resulted in a profound (up to ~60%) decrease in blood glucose levels in WT (Fig. 4b) but not KI (Fig. 4c) mice, whereas the plasma concentration of AICAR was similar between the two groups (Fig. 4d). Consistent with the results observed with MB06322, AICAR induced a marked increase in blood lactate levels in WT mice (Fig. 4e), but the increase was only modest in KI mice (Fig. 4f). Plasma glucagon levels were increased in AICAR-treated WT (Fig. 4g) but not KI (Fig. 4h) mice, most likely to counteract the rapid induction of hypoglycemia (Fig. 4b). There was no significant change in plasma insulin levels (Fig. 4i). As anticipated, AICAR robustly stimulated phosphorylation of liver AMPK and its bona fide substrate ACC in both WT and KI mice (Fig. 4k,l). This was accompanied by a
A profound increase in liver ZMP and ZTP concentrations in both groups of mice (Supplementary Table 4). As previously reported in both intact animals\(^1,29\) and isolated hepatocytes\(^12,21\), AICAR administration in WT mice resulted in a substantial decrease in the total adenine nucleotide pool (Supplementary Table 4). The conversion of AICAR to ZMP by adenosine kinase consumes ATP and acts as a trap for phosphate, similar to the metabolic consequences of a large fructose bolus. This effect is further enhanced by substantial substrate cycling between AICAR and ZMP due to dephosphorylation by 5′-nucleotidase\(^22\). This leads to depletion of Pi, resulting in deinhibition of AMP deaminase and the loss of adenine nucleotides. However, this mechanism cannot be fully responsible, as AICAR had minimal effect on adenine nucleotides in KI mice despite similar accumulation of ZMP (Supplementary Table 4). Furthermore, AICAR was previously observed to have no significant effect on ATP levels in fed mice\(^22\). The common factor here is likely the absence of an acute glucose-lowering effect and inhibition of a high gluconeogenic flux, which leads to the accumulation of additional phosphorylated species (for example, F-1,6-P₂ and the triose phosphates) as additional sinks for Pi (data not shown). Interestingly, AICAR treatment lead to a similar increase in hepatic NAD⁺ in both WT and KI mice (Supplementary Table 4), which was likely due to AMPK-dependent inhibition of fatty acid synthesis and the subsequent increase in β-oxidation.

In skeletal muscle, AICAR failed to increase AMPKα T172 phosphorylation and activity in both WT and KI mice (Supplementary Fig. 6a–c), although it enhanced phosphorylation of ACC and TBC1 domain family member 1 (TBC1D1), most likely via ZMP-dependent allosteric activation of AMPK (Supplementary Fig. 6d,e). Notably, AICAR was unable to stimulate phosphorylation of RAPTOR, which is a marker of more robust AMPK activation\(^23\) (Supplementary Fig. 6f). Indeed, ZMP concentration and [ZMP]:[ATP] ratio in skeletal muscle were > 50-fold and > 150-fold less, respectively, than that detected in liver following AICAR treatment, a consequence of the considerable first-pass metabolism of AICAR and low plasma concentration (Supplementary Table 4 and Supplementary Fig. 6g). Thus, it is presumed that the magnitude of AMPK activation following AICAR treatment in skeletal muscle was below the threshold to cause lowering of blood glucose in KI mice through promoting glucose uptake in this tissue. Collectively,
using two AMP mimetic drugs, we have confirmed that the FBP1-G27P-KI model is suitable to investigate the effect of an AMP-elevating agent (i.e., metformin) on blood glucose in vivo.

FBP1-G27P-KI mice are metformin intolerant. To determine whether KI mice exhibited altered responses to an acute dose of metformin, we performed a metformin-tolerance test. As metformin affects intestinal glucose absorption, we intraperitoneally injected glucose (2 g kg⁻¹) following an oral administration of 250 mg kg⁻¹ metformin (Supplementary Fig. 7a), a dose commonly used in rodents to elicit an acute glucose-lowering effect. In WT mice, prior administration of metformin promoted significantly faster disappearance of blood glucose compared to vehicle-treated control (Fig. 5a). In contrast, the glucose-lowering effect of metformin was...
Fig. 5 | FBP1-G27P-KI mice exhibit resistance to the acute glucose-lowering effect of metformin. a, b, Vehicle (water) or metformin (250 mg kg⁻¹ p.o.) was administered to fasted (16h) WT (a) and KI (b) mice and after 45 min, glucose tolerance (2 g kg⁻¹ i.p.) was assessed by monitoring blood glucose over a period of 2h. Results represent mean ± s.e.m.; n = 18 (WT-vehicle), 17 (WT-metformin), 15 (KI-vehicle) and 15 (KI-metformin) mice. *P < 0.05 (vehicle versus metformin). c–e, Mice were fasted for 16h and dosed with vehicle (water) or metformin (250 mg kg⁻¹ p.o.). After 1h exposure, blood and liver biopsies were taken and assayed for metformin (c). Western blotting of ACC and AMPKα phosphorylation in livers from vehicle and metformin-treated mice. Representative results from three mice per group are shown (d). Quantitative analysis of pT172-AMPKα. Results are expressed as the pT172-AMPKα/AMPKα ratio normalized to the WT-vehicle group (e). n = 5 mice per group. f, g, Arterial blood glucose (f) and GIR (g) during metformin-euglycemic clamp in WT and KI mice. Mice were fasted for 5h and infused intravenously with metformin (rate of 3.75 mg kg⁻¹ min⁻¹) and a variable infusion of 50% glucose to maintain euglycemia at 120 mg dl⁻¹ over a period of 120 min. *P < 0.05 (WT versus KI). h, Plasma and liver metformin concentrations at the end of the clamp period. i–l, Rates of endogenous glucose production (EndoRa) (i), gluconeogenesis (GNG) (j), glycogenolysis (GYG) (k) and glucose disappearance (Rd) (l) during the resting period (5h fasted) and steady state of the metformin clamp (average from 100–120 min). Results represent mean ± s.e.m.; n = 8 (WT-resting), 8 or 9 (WT-clamp), 10 (KI-resting) and 9–11 (KI-clamp) mice. *P < 0.05. Statistical significance was determined using an unpaired, two-tailed Student’s t-test and an alpha level of 0.05.
markedly lower in KI mice (Fig. 5b), even though plasma and liver metformin levels (~125–150 μM and ~0.7 μmol g⁻¹, respectively) were comparable between the groups of mice (Fig. 5c). Metformin caused a comparable increase in hepatic [AMP]/[ATP] (approximately twofold) and decreased energy charge (~10%) in both groups of mice (Supplementary Table 5). This change in energy status was associated with a robust increase in phosphorylation of liver AMPK and ACC (Fig. 5d,e). To further investigate whether KI mice were resistant to the glucose-lowering effect of metformin in vivo, we developed and optimized a ‘metformin-euglycemic clamp’ protocol in conscious, unrestrained mice (Supplementary Fig. 7b). A similar technique has been used previously to assess the effect of metformin on HGP in mice26. Two different doses of metformin (1.875 mg kg⁻¹ min⁻¹ and 3.75 mg kg⁻¹ min⁻¹) were tested. Metformin was infused intravenously at a constant rate while the glucose infusion rate (GIR) was adjusted to maintain euglycemia (Fig. 5f). We observed that the lower dose (1.875 mg kg⁻¹ min⁻¹) failed to significantly increase GIR from baseline in WT (and also KI) mice under the euglycemic condition (Supplementary Fig. 7c,d), even though metformin had reached ~170 μM and ~0.8 μmol g⁻¹ in plasma and liver, respectively (Supplementary Fig. 7e). These concentrations were comparable to those observed following an acute oral administration of 250 mg kg⁻¹ (Fig. 5c). This is likely due to differences in the route of administration. The first pass of metformin from the gastrointestinal tract, via the portal vein and liver, into systemic circulation is crucial for the glucose-lowering effect. First, administration of metformin via the portal–hepatic pathway produces more profound glucose lowering than direct systemic infusion27. Second, it has been proposed that there is also a direct effect of metformin on the gut itself. For example, it has been recently suggested that metformin reduces HGP through a gut–brain–liver neuronal network via activation of AMPK in the duodenum, resulting in release of GLP-1 (ref. 28). Another study has shown that metformin reshapes the gut microbiota through interacting with different bacteria, possibly via metal homeostasis29.
Infusion of a higher dose of metformin (3.75 mg kg\(^{-1}\) min\(^{-1}\)) resulted in a substantial increase in GIR, leading to steady state by the end of the 120-min clamp in WT mice (Fig. 5g). In contrast, KI mice displayed only a modest increase in GIR (Fig. 5g), even though plasma, liver and muscle metformin as well as plasma insulin concentrations were similar between the groups of mice (Fig. 5h and Supplementary Fig. 7f). Combining the metformin-euglycemic clamp with administration of \(^{2}\)H stable isotope tracers enabled the quantification of endogenous glucose production (EndoRa), including the relative contribution of glycogenolysis and gluconeogenesis. EndoRa was significantly suppressed (\(-20\%) in WT mice during the clamp (Fig. 5i). This was due to reduction of both gluconeogenesis and glycogenolysis (Fig. 5j,k). In contrast, metformin-induced suppression of EndoRa, gluconeogenesis and glycogenolysis was ablated in KI mice (Fig. 5i–k). Overall, the clamp study revealed that KI mice were largely insensitive to the glucose-lowering effect of metformin because of ablation of metformin-induced suppression of HGP. However, it should be noted that owing to the systemic route of delivery (as mentioned above), much higher and suprapharmacological doses were needed to elicit a robust glucose-lowering effect, resulting in an artificially higher glucose disposal rate (\(-40–50\%) increase in both WT and KI) than is seen at therapeutic doses (Fig. 5l). It has been shown that metformin can stimulate glucose uptake in isolated rat skeletal muscle at suprapharmacological doses (but not at clinical doses due to the absence of OCT1 (ref.\(^{30}\)) by activating AMPK\(^{5}\), which could sensitize insulin action and further promote glucose uptake in muscle\(^{7}\). In support of this premise, metformin concentration in skeletal muscle was increased twofold when infused at the higher rate (3.75 mg kg\(^{-1}\) min\(^{-1}\)) compared to the lower rate (1.875 mg kg\(^{-1}\) min\(^{-1}\)) (Supplementary Fig. 7g). The molecular basis underlying reduced glycogenolysis in WT mice during the clamp is unknown, as we observed no significant difference in the levels of phosphorylation or activity of glycogen synthase (GS) and glycogen phosphorylase (GPα) as well as hepatic glycogen content between groups of mice at the end of the clamp (Supplementary Fig. 7h–m). We monitored tissue distribution and pharmacokinetics of metformin delivered via the systemic route and performed positron emission tomography (PET) analysis following an acute intravenous infusion of \(^{13}C\)metformin. We found that the kinetics and total metformin uptake in liver was comparable between WT and KI mice (Supplementary Fig. 8), consistent with the snapshot measurements of hepatic metformin concentration as shown in Fig. 5c and Supplementary Fig. 7c. In addition, PET analysis highlighted a rapid and marked accumulation of metformin in the bladder, corroborating the need for a much higher dose to achieve a glucose-lowering effect via the systemic route than the gastrointestinal route.

**FBP1-G27P-KI mice are resistant to the acute hypoglycemic action of metformin in an obesity-induced model of diabetes**

We next assessed whether KI mice were resistant to the glucose-lowering effect of metformin under the hyperglycemic (diabetic) condition. WT and KI mice were fed a high-fat diet (HFD) for 10 weeks, and both groups of mice had similar profiles of weight gain and food intake over the period of dietary intervention (Fig. 6a,b). HFD-fed WT and KI mice showed hallmark features of type 2 diabetes, including glucose intolerance, hyperglycemia, hyperinsulinemia as well as hypertriglyceridemia (Fig. 6c–f). At the end of HFD intervention, we orally treated WT and KI mice with metformin (250 mg kg\(^{-1}\) or vehicle (water) and monitored blood glucose levels 2h following metformin treatment. We found that metformin, but not vehicle, produced a significant reduction of blood glucose levels in WT mice (\(-30–40\%)\), but the effect was significantly blunted in KI mice (P = 0.047; Fig. 6g,h). We verified that hepatic metformin levels (Fig. 6i) and the magnitude of changes in [AMP]:[ATP] and energy charge (Supplementary Table 6) as well as associated increases in AMPK phosphorylation (Fig. 6j) were comparable between genotypes. Inhibition of gluconeogenesis by metformin in WT mice could also be supported by a modest fall in hepatic glucose and G6P levels, which was blunted in KI mice. Similarly, inhibition of the step catalyzed by FBP1 was suggested by a decrease in F6P (P = 0.053) and a concomitant increase in F-1,6-P\(_2\), in livers from metformin-treated mice (P = 0.091) that was lower in KI mice (Supplementary Table 7). However, given that there was a modest (\(-10\%) but significant effect of decreased blood glucose following metformin treatment in KI mice (Fig. 6h), there must be additional mechanisms, independent of AMP-mediated FBP1 inhibition, to lower blood glucose. This is unsurprising given that metformin has multiple proposed primary and secondary targets\(^{3}\), including mitochondrial complex I\(^{\alpha3}\) and glycerol-3-phosphate dehydrogenase\(^{35}\) in the liver, as well as duodenal AMPK\(^{6}\). Although it has been proposed that one of the mechanisms of action of metformin involves a reduction in hepatic CAMP (a key mediator of glucagon signaling), there was no significant effect (Fig. 6k) and an actual increase in downstream phosphorylation of PKA substrates (i.e., pS33-PFKFB1 and pS133-cAMP response element–binding protein (CREB)) (Supplementary Fig. 9a,b) under the conditions of our model system (it has been reported that the reduction in cAMP (ref.\(^{30}\)) by activating AMPK\(^{5}\), which could sensitize insulin action and further promote glucose uptake in muscle\(^{7}\). In support of this premise, metformin concentration in skeletal muscle was increased twofold when infused at the higher rate (3.75 mg kg\(^{-1}\) min\(^{-1}\)) compared to the lower rate (1.875 mg kg\(^{-1}\) min\(^{-1}\)) (Supplementary Fig. 7g). The molecular basis underlying reduced glycogenolysis in WT mice during the clamp is unknown, as we observed no significant difference in the levels of phosphorylation or activity of glycogen synthase (GS) and glycogen phosphorylase (GPα) as well as hepatic glycogen content between groups of mice at the end of the clamp (Supplementary Fig. 7h–m). We monitored tissue distribution and pharmacokinetics of metformin delivered via the systemic route and performed positron emission tomography (PET) analysis following an acute intravenous infusion of \(^{13}C\)metformin. We found that the kinetics and total metformin uptake in liver was comparable between WT and KI mice (Supplementary Fig. 8), consistent with the snapshot measurements of hepatic metformin concentration as shown in Fig. 5c and Supplementary Fig. 7c. In addition, PET analysis highlighted a rapid and marked accumulation of metformin in the bladder, corroborating the need for a much higher dose to achieve a glucose-lowering effect via the systemic route than the gastrointestinal route.

**Discussion**

Metformin has been in use for more than 50 years as an antihyperglycemic agent for the treatment of diabetes. Despite the clinical success of metformin, there is no clear consensus regarding its mode of action, and multiple seemingly contradictory mechanisms have been proposed. However, a common narrative emerges when a clear distinction is drawn between acute versus chronic effects of metformin that can be mediated by either direct or indirect effects on HGP by metabolic or genic means. We have focused specifically on the acute effect of metformin on HGP, where it is clear that AMPK is dispensable. Hepatic AMPK-null mice do not exhibit a defect in either steady-state glycerol, pyruvate tolerance or the acute glucose-lowering effect of metformin\(^{3}\). Furthermore, treatment of cultured hepatocytes\(^{3}\) or intravenous infusion of the specific AMPK activator A769662 has no effect on glucose production\(^{40}\). Although hepatic LKB1-null mice present with severe hyperglycemia and hyperlipidemia due to upregulation of CREB and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α) transcriptional targets (Pck1 and G6pc)\(^{40}\), recent work suggests that this is due to impaired activity of the AMPK-related kinase, salt-inducible kinase (SIK)\(^{41}\), and not AMPK. Much is often made of the short-term changes in Pck1 and G6pc mRNA in response to pharmacological activation of AMPK, but when protein levels are assessed, they are invariably unaltered. Indeed, a poor correlation has been noted between the expression of gluconeogenic genes and HGP\(^{42}\). In short, although biguanides can clearly activate AMPK, it is neither sufficient nor necessary for acute inhibition of HGP.

Obesity-induced diabetes is a standard model in rodent studies, and it is generally accepted that the associated hyperglycemia is a consequence of hepatic insulin resistance in fatty livers. It is well established that AMPK is a critical regulator of lipogenesis, and chronic treatment of diabetic mice with metformin profoundly improves glucose tolerance by reducing hepatic steatosis and improving insulin resistance. Of note, mice expressing nonphosphorylatable mutants of ACC1 and ACC2 (ACC1/2-KI), were resistant to the lipid- and glucose-lowering effect of chronic metformin treatment. Notably, the acute hypoglycemic effect of a single dose of metformin was unaffected\(^{7}\). The same mechanism likely underpins the glucose-lowering effect of chronic A769662
treatment in obese ob/ob mice, in which dramatic reductions in hepatic and plasma lipid were observed. Consequently, AMPK plays a role in the chronic, indirect inhibition of HGP by alleviating hepatic insulin resistance.

Plasma metformin concentration in humans is markedly variable, due in part to the complex pharmacokinetics of the drug and profound intersubject variations in absorption and elimination. It is difficult to quote a meaningful elimination half-life (t½) because the time course of plasma concentrations of metformin follows a multiphasic pattern—however, values in the range from 1.7 to 4.5 h have been reported. Plasma metformin concentration in patients treated with a normally prescribed dose of metformin (1 g, twice per day) has been reported within the range of 0.4–32 μM (plasma levels obtained 14 h after last drug administration) in 159 subjects with well-regulated type 2 diabetes under controlled conditions. In contrast, plasma metformin levels between 0 and 113 mg·l–1 (868 μM) have been measured in random blood samples from diabetic subjects. Both studies have provided useful information, as the former gives an estimate of nadir during multiple dosing, whereas the latter provides a valuable estimate of the maximum concentration of metformin during clinical use. However, overall it has to-date been a challenge to establish the therapeutic range of metformin concentrations in plasma.

The current debate on metformin has turned toward the issue of the validity of the concentrations and doses used in rodent models. It has long been recognized that the effective hypoglycemic dose exhibits a marked species dependence, and researchers rightly use doses that produce robust, reproducible effects. In this regard, it is difficult to compare studies that have not measured plasma and/or liver accumulation as the drug formulation, route of administration and degree of fasting will impact the pharmacokinetics. Species differences in OCT1 expression have also been identified; although both rodent and human liver express OCT1, its expression in human intestines appears much lower than in mice. As metformin is administered orally, differences in intestinal OCT1 expression may affect portal vein levels of metformin and thereby hepatic uptake without detectable effects on metformin levels in peripheral veins. In addition, hepatic exposure to the drug depends not only on OCT1, but also on multidrug and toxin extrusion (MATE) isoforms acting as influx and efflux transporters, respectively. Differential hepatic expression of MATE1 between humans and rodents has been reported, which may also affect metformin kinetics. It has been observed that a glucose-lowering effect occurs in response to a single dose of 50 mg·kg–1 in rats. However, it should be noted that metformin (50 mg·kg–1) was given intravenously, which led to plasma metformin concentration of ~74 μM 30 min after administration, whereas 100 mg·kg–1 and 250 mg·kg–1 doses increased plasma metformin concentration of 345 μM and 1,300 μM, respectively. Further, the study exploring the effect of metformin in ACC1/2-KI mice used a chronic dose of 50 mg·kg–1 (i.p.) for 12 weeks; however, a dose of 200 mg·kg–1 was needed (50 mg·kg–1 had no effect) to observe a glucose-lowering effect in single-dose experiments. Taken together, the dose range of 200–350 mg·kg–1 has consistently been used in many contemporary studies have resulted in misleading values reported for hepatic adenine nucleotides ([ATP]:[AMP] ≤ 10, often approaching 1). Hepatic AMP concentration is a very sensitive indicator of stress and increases by ~10-fold within just 30 seconds of hypoxia. Consequently, tissues must be freeze-clamped in situ to ensure accurate measurements and reveal subtle changes in energy status. Even then, AMPK can easily be activated by increases in free AMP concentrations that are below the limit of quantification. Consequently, AMPK is a sensitive reporter of changes in [AMP]:[ATP], and pT172-AMPKα is a more reliable indicator of changes in the AMP concentration than technically demanding direct measurements. Hence, it can be argued that a consensus emerges that metformin induces changes in hepatic energy status which is sufficient to modify the activity of sensors such as AMPK and FBP1. Nonetheless, we observed here a modest but significant reduction in energy charge in response to metformin treatment in the liver, which may have partly contributed to suppression of energy-demanding gluconeogenic flux.

Understanding the mechanism through which metformin reduces HGP and normalizes blood glucose levels in hyperglycemic type 2 diabetes is of considerable importance. Our results show that metformin induces a mild energy stress in liver, leading to an increase in AMP concentration that allosterically inhibits FBP1 to lower HGP. This is potentially a powerful mechanism, as the subsequent increase in F-1,6-P2 will activate PK and increase glycolytic flux. Our study further supports the advancement of FBP1 as a key target for the treatment of type 2 diabetes, either directly using targeted inhibition or indirectly as a consequence of inducing energy stress. The later mechanism may contribute substantially to the apparent glucose-lowering effect of many biologically active secondary metabolites and to new antidiabetic drugs exploiting the emerging concept of mild mitochondrial uncoupling.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41591-018-0159-7.

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Author contributions
R.W.H. and K.S. designed the study. R.W.H. performed all biochemical assays and the majority of in vivo experiments, assisted by K.S. Analysis of FBP1 structure and design of the mutants were performed by E.Z. and F.S. M.P. performed molecular cloning and

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mutagenesis of FBP1. N.J. and E.I.S. performed the [11 C]metformin-uptake kinetics study and analyzed the data. C.C.H. and L.L. performed the metformin euglycemic clamp and analyzed the data. D.H.W. supervised C.C.H. and L.L. and contributed to interpretation of data from the clamp study. R.W.H. and K.S. wrote the manuscript. All authors reviewed, edited and approved the manuscript.

Competing interests
K.S. is a full-time employee of the Nestlé Institute of Health Sciences S.A., Switzerland.

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Methods

Materials. E. coli strain DF657 was sourced from the Coli Genetic Stock Centre (CGSC, Yale University), and BL21-CodonPlus(DE3)-RIL was from Agilent.

Caspases were from BD Biosciences. Talon cobalt IMAC resin was from Clonetech. pET28a–FBP1 was purchased from Novagen.

Cloning and mutagenesis. Mouse Fbp1 (NCBI reference: AJ132693.1) was amplified from IMAGE EST 5054854 using KOD Hot Start DNA Polymerase (Merck Millipore) and cloned into the BamHI NotI sites to produce PET28a-6HIS–FBP1 and pET15 6HIS–FBP1. Mutagenesis of the G6pc was performed using the QuickChange method (Agilent) but using KOD Hot Start DNA Polymerase. The phosphatase domain of mouse 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (NCBI reference: NM_008824.3) covering amino acids 251–440 was amplified from mouse liver RNA (Agilent no. 736009-41) using GoQaq1 step RT–iQR kit (Promega). The resulting PCR product was ligated into pCSEX-4P-1 vector (GE Healthcare) as a BamHI–NotI fragment. Spinach chloroplast fructose-1,6-biphosphatase 58–415 was cloned from a synthetic fragment (GeneArt Strings, based on Uniprot P224118) and ligated into a modified PET-15 plasmid as a BamHl–NotI fragment. The sequence of all constructs was verified by in vitro sequencing using the BigDye Terminator 3.1 kit on a 3500xl Genetic analyzer (ABI-Invitrogen).

Preparation of rFBP1. 6HIS–FBP1 was expressed in the Fbp–null E. coli strain, DF657(DE3), as described by Giroux et al.51. DF657 was sourced from the CGSC (Yale University, CT, USA), and the DE3 lysogen was prepared using the JDE3 lysogenization kit (Novagen no. 69734). Cells were transformed with pET28a–FBP1 and cultured in minimal medium (M9 salts, 2 mM MgSO4, 0.4% (wt/vol) glycerol, 5 μM thiamine–HCl, 0.5% (wt/vol) casamino acids and 50 μg ml−1 kanamycin) overnight at 37 °C. Minimal medium (0.5–11 containing 25 μg ml−1 kanamycin) was inoculated 1:10 with the starter culture and induced with 0.4 mM IPTG at an OD600 of 0.4–0.6 for 16 h at 37 °C. The resulting cell lysis was performed in 5 ml 50 mM phosphate pH 7, 150 mM NaCl, 0.5 mM tris(2-carboxyethyl) phenolphosphate (TECP), 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg ml−1 leupeptin by sonication (1 min, 40% amplitude) and were clarified at 20,000 g for 20 min at 4 °C. 6HIS–FBP1 was batched to Co2+–charged IMAC resin (Talon) for 30 min at 4 °C, washed with 10 volumes of lysis buffer, 10 volumes of 5 M imidazole and eluted with 5 volumes of 1 M imidazole in 50 mM imidazole pH 7.4, 0.3 M KCl, 0.2 mM EDTA and 0.5 M TCEP over Sephadex G-25, concentrated using 10-kD MWCO centrifugal devices (Sartorius Visipass) and stored at −20 °C in 50% (vol/vol) glycerol. The presence of imidazole in concentrated solutions of 6HIS–FBP1 was essential to prevent aggregation. Untagged FBP1 was prepared by on-column cleavage of 6HIS–LELVIGQGPSS–FBP1 (constructed in PET15 and prepared as described above) with 50 μg HRV 3C protease per mg 6HIS–FBP1 in 50 mM TES pH 7.4, 150 mM KCl, 0.5 mM TCEP. Preparations were polished over a Superdex 200 10/300GL column equilibrated with 50 mM TES pH 7.4, 0.3 M KCl and 1 mM dithiothreitol (DTT) and stored at −20 °C in 50% (vol/vol) glycerol. Preparations were stable for at least 6 months. As a reference, FBPase was purified from mouse liver essentially as described by Tashima et al.52. Briefly, 20 g liver from C57BL/6 N mice was homogenized in four volumes of 20 mM phosphate pH 7, 150 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 2 μg ml−1 leupeptin and was clarified at 10,000 g for 20 min at 4 °C. The supernatant was subjected to heat denaturation at 60 °C for 1 min and centrifuged at 20,000 g for 20 min at 4 °C. The supernatant was subjected to a 55–75% ammonium sulfate cut, and the resulting pellet was dissolved in 1 mM EDTA and dialyzed overnight against 10 mM sodium malonate pH 6.2, 1 mM EDTA (Buffer A). FBPase was further batched to batch 30 g Pi P11 phosphocellulose, washed with 300 ml Buffer A using suction and transferred on XK26/20 column. XK26/20 column was preconditioned with buffer A containing 50 mM NaCl until absorbance at a wavelength of 280 nm (A280) decreased to 0.05 and FBPase was eluted with 2 mM F1,6-P2 and 20 μM 5′-AMP. Positive fractions were dialyzed against Buffer A and added to pH was adjusted to 5.8 with malonic acid; then, these were applied to a 1.6 × 10 cm column of Blue Sepharose FF (C16/20). The column was washed with 10 ml sodium malonate pH 5.8 and 1 mM EDTA, and elution was performed with 1 mM F1,6-P2 and 1 mM AMP. The preparation was polished over a Superdex 200 10/300GL column equilibrated with 20 mM phosphate pH 7, 150 mM KCl and 1 mM EDTA and stored in 50% (vol/vol) glycerol at −20 °C.

FBPase assay. FBPase activity was determined by monitoring the formation of F6P using a coupled spectrophotometric assay. The specific activity of FBP1 was determined in 1 ml reactions containing 50 mM TES pH 7.4, 0.2 mM NADPH, 0.1 M KCl, 0.05 mM EDTA, 2 mM (NH4)2SO4, 2 mM MgCl2, 0.05% (wt/vol) BSA, 2 mM 2-mercaptoethanol, 0.8 μM phenylphosphoglucomerase isozyme and 0.5 μM 6GPD. Reactions were started by the addition of 35 μM F1,6-P2 and the absorbance was monitored at 30 °C. Reaction rates were calculated from the linear phase assuming ε(NADPH)=6.22 mM−1 cm−1. 1 U is defined as 1 μmol F6P formed per min at 30 °C. Where appropriate, 1 μM AMP deaminase (purified from chicken muscle using P11 phosphocellulose, essentially as described by Smiley et al.53) was added to remove contaminating AMP from NADPH, as determined by Han et al.41. The activity ratio at pH 7.2 and pH 9.4 was determined under similar conditions in reactions buffered with 50 mM bis-tris propane at the appropriate pH. Other kinetic properties were determined in a 96-well format in nonbinding black microplates (Greiner no. 659900), in which the quantity of NADP+ was reduced to 0.15 mM, and reactions were monitored by the increase in absorbance.
in fluorescence ($\lambda_{ex} = 345$ nm, $\lambda_{em} = 465$ nm) calibrated by the addition of 5 nmol F6P. $K_0$ (F-1,6-P$_2$) was determined at 2 mM Mg$^{2+}$ and fitted to equation 1:

$$V = \frac{V_0}{1 + \frac{[S]}{K_m}}$$

Where: $V_0$, initial velocity; $V_0$, maximum velocity; $[S]$, F-1,6-P$_2$ concentration; $K_m$, Michaelis constant for S. $K_0$, apparent substrate inhibition constant; and b, factor determining maximum activity at high [S]. $K_0$(Mg$^{2+}$) was determined at 35 μM F-1,6-P$_2$ and fitted to equation 2:

$$V = \frac{V_0}{1 + \frac{[S]}{K_m + [S]}}$$

Where: $V_0$, initial velocity; $V_0$, maximum velocity; [S], Mg$^{2+}$ concentration; $K_m$, Michaelis constant for S and h, coefficient for inhibitory compounds was determined at 2 mM Mg$^{2+}$ and 35 μM F-1,6-P$_2$ and fitted to equation 3:

$$V = \frac{V_0}{1 + \frac{[S]}{K_m + [S]}}$$

Where: $V_0$, initial velocity; $V_0$, maximum velocity in the absence of inhibitor; I, concentration of inhibitor; $I_50$, concentration of inhibitor that gives 50% inhibition; and h, coefficient.

Stock solutions of F-1,6-P$_2$ were standardized by enzymatic assay in reactions containing 50 mM imidazole pH 7.1, 0.15 mM NAD$^+$, 0.02 U/ml aldolase, 1.2 U/ml triosephosphate isomerase and 0.16 U/ml g-glucose-3-phosphate dehydrogenase. Stock solutions of AMP, 5'-inosine monophosphate (IMP) and 5'-AICAR monophosphate (ZMP) were prepared in 20 mM TES pH 7.4, neutralized with NaOH and standardized by ultraviolet (UV) absorbance in 0.1 M phosphate pH 7 at $A_{260}$ (ε$_{AMP}$ = 15.4 μM$^{-1}$ cm$^{-1}$), $A_{260}$ (ε$_{IMP}$ = 12.2 μM$^{-1}$ cm$^{-1}$) and $A_{260}$ (ε$_{F}^{26}$, 26.5 μM$^{-1}$ cm$^{-1}$).

The thermal stability assay was performed on untagged FBP1 preparations using a Roche LightCycler 480 II. FBP1 was diluted to 0.1 U/ml G6PD, 0.2 U/ml phosphoglucose isomerase and 0.25 U/ml GST-FBPase-2 F6P. Km(F-1,6-P$_2$) was determined at 2 mM Mg$^{2+}$, 0.1 mM EDTA and 0.15 mM NADP$^+$.

The melting temperature (Tm) was determined from the maximum of the first derivative of the fluorescence at 345 nm, A249 ($\lambda_{em}$, 580 nm; melt temperature and incubation in primary antibodies prepared in TBS-T containing 5% (wt/vol) BSA overnight at 4 °C. Membranes were developed using HRP-conjugated antibodies (AF680 and AF790) on nitrocellulose membranes using an Odyssey CLx imaging system (Li-COR). Tissue homogenization.

Liver biopsies were powdered in a liquid nitrogen-cooled mortar and pestle and homogenized in 10 volumes of extraction buffer using a rotor-stator homogenizer (Polytron, Kinematica AG). For western blotting and assay of G6PC, PK and FBP1, tissues were homogenized in 50 mM tris-HCl pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1% (wt/vol) Triton X-100, 20 mM glycerol-2-phosphate buffer. Samples were denatured at 95 °C for 2 min, fractionated by SDS-PAGE and stained with colloidal Coomassie G-250.

Immobilized ligand affinity binding. FBP1 (2 μg) in 20 mM TES pH 7.4, 100 mM KC1, 1 mM MgCl$_2$, 0.1 mM F-1,6-P$_2$ and 0.01% (wt/vol) Brij-35 was mixed with 5 μl 2'3'-O-(2-aminomethyl-carbamoyl)-adenosine 5'-monophosphate (2'3'-EDA-AMP)-agarose or unconjugated agarose for 30 min at 4 °C. Excess free ligand (0.5 mM AMP) was included as a negative control. Resin was pelleted at full-speed for 5 s, washed in 3 x 0.5 ml binding buffer and eluted with 20 μl Laemmli sample buffer. Samples were denatured at 95 °C for 2 min, fractionated by SD-SAGE and stained with colloidal Coomassie G-250.

Animals. Animal studies were approved by the local ethics committee, and all protocols were approved by the Service Vétérinaire Cantonale (Lausanne, Switzerland) under license V2841. C57BL6/N Tac FBP1-G27P mice were generated by Taconic Biosciences GmbH as described in Fig. 1d. Animals were kept in a standard temperature- and humidity-controlled environment on a 12-h light and 12-h dark cycle and had free access to water and standard chow or a HFD that was 60% kcal fat (Research Diets Inc. D12492), as described. [125] Metformin PET was performed in accordance with the Danish Animal Experimentation Act and the European convention for the protection of vertebrate animals used for experimental and other purposes and was approved by the Animal Experiments Inspectorate, Denmark. Metformin-euglycemic clamps were completed with the approval of the Vanderbilt Animal Care and Use Committee; for these studies, animals were housed on a 12-h light and 12-h dark cycle in a temperature (23°C) and humidity-stable environment. Mice were maintained on a standard chow diet (5L0D LabDiet, St. Louis, MO). Male mice between the ages of 14 and 22 weeks were used for all procedures. Basic phenotyping was performed by PhenoPro (Illkirch, France) in a licensed animal facility (agreement no. A67-218-40). All experiments were approved by the local ethical committee, and investigators were not blinded.

Tissue homogenization. Liver biopsies were powdered in a liquid nitrogen-cooled mortar and pestle and homogenized in 10 volumes of extraction buffer using a rotor-stator homogenizer (Polytron, Kinematica AG). For western blotting and assay of G6PC, PK and FBP1, tissues were homogenized in 50 mM tris-HCl pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1% (wt/vol) Triton X-100, 20 mM glycerol-2-phosphate buffer. Samples were denatured at 95 °C for 2 min, fractionated by SDS-PAGE and stained with colloidal Coomassie G-250.

Metabolic phenotyping. Mice were weighed and food consumption was monitored on the day so that all procedures commenced at 09:00 the following morning. Blood glucose was monitored using a glucometer (AlphaTRAK 2, Abbott Logistics B.V.) on venous blood drawn from the tail. Blood lactate was assayed using lactate dehydrogenase (Coomassie Pro 2, Arkay Inc.). The genotype of experimental animals was confirmed by PCR on 1 μl blood lysed in 20 μl 20 mM NaOH, 60% (wt/vol) PEG-200 (ref. 58) using KAPA2G polymerase. Glucose tolerance was determined by administration of 2g kg$^{-1}$.
d-glucose p.o. or i.p. from a 20% (wt/vol) glucose solution after 16 h fast. Pyruvate
tolerance was determined by administration of 1 g/kg pyruvate (free acid) i.p. from a
12.7% (wt/vol) sodium pyruvate solution (pH 6) after a 16-h fast. The pyruvate
stock solution was standardised by assay in 50 mM phosphate pH 7.0 (15.0 μM NADH
and 0.1 U ml−1 lactate dehydrogenase). AICAR tolerance was determined by administration
of 250 mg/kg i.p. from a 12.5 mg ml−1 solution in 0.9% (wt/vol) saline after a 16-h fast. The acute glucose lowering effect of MB06332 was assessed by administration of 75 mg/kg i.p. from a 7.5 mg ml−1 solution in a vehicle composed
of 10:16 Soluene HS 15PEG 400water (comprising was dissolved (PEG 400, gentle heat/sonication, combined with warm liquid solutol HS 15 and dispersed in water).
Energy expenditure, food intake and spontaneous activity (beam-break) were determined by indirect calorimetry (Labmaster, TSE Systems GmbH, Germany).
Following a 3-h acclimatization period, mice were monitored for a 21-h period from
14:00 on day 1 to 23:00 on day 2 (12-h light and 12-h dark cycle at 21 ± 2 °C).

Plasma metabolites. Blood was drawn by tail bleeding into lithium heparin–
coated capillaries (Sarstedt Micromed CB-200), and plasma was prepared by
centrifugation at 3,000 g for 5 min at 4 °C. Plasma was stored at −80 °C before
analysis. Insulin and glucagon were determined by sandwich ELISA using kits from
Mercodia (no. 10-1290-01 and no. 10-1281-01). Leptin was measured using an
ELISA from Merck Millipore (no. EZML-82K). Triglyceride was determined using an
enzymatic assay from Sigma (TRIO100).

Metformin-tolerance test. Animals were starved for 16h, and 250 mg kg−1
metformin–HCl was administered p.o. by gavage. After 45 min, resting blood
glucose was monitored at t = 0. Metformin and 5,5-diphenylhydantoin were resolved isocratically with
reverse-phase chromatography on a Dionex Ultimate-3000 RS HPLC, essentially as
Metformin was assayed in plasma and tissues by ion-pair
reversed-phase chromatography on a Dionex Ultimate-3000 RS HPLC, essentially as
described by Zarghi et al.15. Plasma was deproteinised with three volumes 80%
(vol/vol) acetonitrile containing 5 μg ml−1 5,5-diphenylhydantoin as internal standard.
Protein was pelleted at 10,000 g for 5 min, and the supernatant was
used directly. Standards were prepared by spiking metformin (1 mg/ml−1  standard
prepared in methanol and stored at −20 °C) into drug-free heparinized plasma
(Innovative Research Inc. no. ICM-5S78-S) so that the final concentration after solvent extraction ranged from 0.3 to 5 μg ml−1. Tissue samples were powdered in a
liquid nitrogen–cooled mortar and pestle and were homogenized with 10 volumes
of ice-cold 40 mM potassium phosphate pH 8.1, and
were taken
for determining plasma glucose concentration after 120 min. The end of the procedure,
−30 μl of blood was drawn into heparinised capillaries for determining plasma metformin concentration.

Metformin assay. Metformin was assayed in plasma and tissue by ion-pair
reversed-phase high-performance liquid chromatography on a Dionex Ultimate-3000 RS HPLC, essentially as
described by Zarghi et al.15. Plasma was deproteinised with three volumes 80%
(vol/vol) acetonitrile containing 5 μg ml−1 5,5-diphenylhydantoin as internal standard.
Protein was pelleted at 10,000 g for 5 min, and the supernatant was
used directly. Standards were prepared by spiking metformin (1 mg/ml−1  standard
prepared in methanol and stored at −20 °C) into drug-free heparinized plasma
(Innovative Research Inc. no. ICM-5S78-S) so that the final concentration after solvent extraction ranged from 0.3 to 5 μg ml−1. Tissue samples were powdered in a
liquid nitrogen–cooled mortar and pestle and were homogenized with 10 volumes
of ice-cold 40 mM potassium phosphate pH 8.1, and
were taken
for determining plasma glucose concentration after 120 min. The end of the procedure,
−30 μl of blood was drawn into heparinised capillaries for determining plasma metformin concentration.

Prior to the onset of the study an estimation
of the required sample size was determined according to:
\[ n = \frac{\left( z_{1-\alpha/2} + z_{1-\beta} \right)^2 \times \left( \sigma_2 + \sigma_1 \right)}{\left( \sigma_2 - \sigma_1 \right)^2} \]
where the level of significance was α = 0.05 and desired power was 1 − β = 0.8. The quantities \( z_{1-\alpha/2} \) and \( z_{1-\beta} \) are critical values from the normal distribution being 1.96 and 0.8416, respectively. The sample size, n, has a value of 2 mg kg−1
min−1. This value was derived from initial studies in our laboratory using stable isotopes to quantify in vivo EndoRa in fasted mice. 2 (2 mg kg−1 min−1), which represents the difference we aimed to identify for EndoRa. As such, the resulting sample size rounded off at n = 8. Experimenters were blinded to the genotype until the conclusion of the study. Values were excluded from the reported means on the basis of the following predetermined exclusion criteria:

1. Following completion of metformin clamps for all mice designated for study, the GIRs were assessed. Any mouse that displayed a GIR outlier during the steady-state–sampling period (100–120 min) was removed from the study after gas chromatography–mass spectrometry (GC–MS) analysis. Outliers were designated as those with values ± 1.5 s.d. from the
group mean of a specific time point.
2. Estimated glucose fluxes (EndoRa, Rd, gluconeogenesis and glycogenolysis) were excluded from reported means if the value was ≥ 2 s.d. from the

group mean.
3. Mice were chronically catheterized approximately 7 d before study, as described previously. Briefly, catheters were implanted in the carotid artery and jugular vein for sampling and infusion, respectively. Animals were housed individually
post-surgery and were monitored for distress. Prior to study, mice were within 10% of presurgery weight. On the day of study, mice were placed in bedded containers with
food or water at 07:00 (t = 0), 5 h before initiation of the clamp.

An arterial blood sample (80 μl) was drawn for evaluating the unlabeled, natural isotope abundance of glucose after 3 h of fasting (t = 120 min). Subsequently, a bolus of H2O (99.9%) was delivered over 25 min to enrich total body water to 4.5% A. [6,6-2H2]glucose prime (80 mg kg−1) was dissolved in the bolus. Following the prime, [6,6-2H2]glucose was continuously infused (0.8 mg kg−1 min−1) for the
remainder of the fasting period. An arterial blood sample (110 μl) was taken to
correlate basal glucose and insulin levels to the injection kinetics of the [6,6-2H2]glucose prime. Metformin was delivered as a continuous infusion (1.875 or 3.75 mg kg−1
min−1), followed by a variable infusion of 50% dextrose (8% [6,6-2H2]glucose) to clamp blood glucose levels at 120 mg d−1. All infused solutions were prepared in a
4.5% H2O2 saline solution. Blood glucose was monitored (AccuCheck; Roche
Analytical, Indianapolis, IN) every 10 min, and donor erythrocytes were infused to
maintain hematocrit levels during the study. Three arterial blood samples
(100 μl each) were obtained during the clamp steady-state period 90 min after
metformin infusion was initiated for determination of glucose fluxes as well as arterial glucose, insulin and/or metformin levels. Plasma was stored at −20 °C after
analysis. Mice were rapidly euthanised through cervical dislocation immediately after the injection of the last sample. Tissues were rapidly dissected (within 30 min), freeze-clamped in liquid nitrogen and stored at −80 °C until further analysis.

Preparation of glucose derivatives for GC–MS analysis. Plasma samples were separated into three aliquots. Each aliquot was derivatized separately to obtain di-0-isopropylidene propionate, aldinolide pentaprionate and methoxylol pentaprionate derivatives of glucose, as previously described3–11.

GC–MS analysis. For gas chromatography–mass spectrometry (GC–MS) analysis, we employed an Agilent 7890A gas chromatography system with an HP-5ms
capillary column (Agilent J&W Scientific) interfaced with an Agilent 5975C mass spectrometer, and analysis was executed as previously described3 with minor modifications. Injection volumes were 1 μl with purge flow times between 20 and 120 s. A custom MATLAB function was used to integrate each derivative peak in order to obtain mass isotope distribution (MIDs) for the following ion ranges: aldinolide, m/z 173–178, 259–264, 284–289, and 370–375; methoxylol, m/z 145–149; di-0-isopropylidene, m/z 301–308. MIDs of each fragment were
calculated as the averages of two injections per sample. Root mean square error
was determined to provide uncertainty and was calculated by comparing the MIDs of unlabeled glucose samples to the theoretical MIDs obtained from the known abundances of naturally occurring isotopes.
Glucose positional deuterium enrichment analysis. The positional deuterium enrichment at each carbon of glucose was determined by least-squares regression as previously described with the use of six glucose fragments, all glucose isopyamic up to M+2, and INCA software (available at https://mfa.vueinversion.com/mfa). Goodness of fit was assessed by a chi-square test, and confidence intervals of 95% were determined as previously described. Fits were accepted according to a chi-square test (P = 0.05) with nine degrees of freedom.

Glucose kinetics. The infusion rate of [6,6-2H2]glucose and model-derived plasma [6,6-2H2]glucose enrichment were used to determine glucose turnover (Rt). Assuming steady-state conditions, glucose disappearance (Rd; mg kg−1 min−1) is equivalent to Rt. Endogenous glucose production (EndoRa; mg kg−1 min−1) was calculated by subtracting the GIR from total Rt. The model-derived positional deuterium enrichment at carbon 5 (D5) and carbon 2 (D2) of plasma glucose allowed for the fractional contribution of gluconeogenesis and glycogenolysis to be determined as previously outlined. Briefly, GNG was obtained by calculating the ratio between D5 and D2 (GNG = D5 / D2). GNG to EndoRa was determined using the equation GNG = 1 - GNG. Multiplying by EndoRa allowed for absolute rates of glycogenolysis and gluconeogenesis to be calculated. Glucose flux rates for the three clamp steady-state samples were averaged to obtain representative values for each mouse.

[11C]Metformin microPET. [11C]Metformin was synthesized by methylation of 1-methylbiguanide with [11 C]methyl triflate and prepared as a solution to a chi-square test (P = 0.05) with nine degrees of freedom.

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containing 50 mM HEPES pH 7.4, 0.1 M KCl, 0.1 mM EDTA, 7.5 mM KCl, 0.1 mM MgCl₂, 0.5 mM MgSO₄, 2.5 mM ADP, 0.2 mM NADH, 10 mM NAD⁺, 15 mM 2-mercaptetoethanol, 0.1 mM microcinycin-LR, 0.05% (wt/vol) BSA, 3.2 µM lactate dehydrogenase and 1.3 or 6.6 mM phosphoenolpyruvate.

6-phosphofructo-1-kinase. 6-phosphofructo-1-kinase (BRENDA: EC 2.7.1.11) activity was determined using the method of Castano et al. 46. Extracts (40µg) were incubated in reactions containing 50 mM HEPES pH 7.1, 0.1 M KCl, 0.5 mM MgCl₂, 1.5 mM ATP, 0.25 mM fructose-6-phosphate, 0.75 mM glucose-6-phosphate, 0.1 mM AMP, 0.5 mM Pi, 1 mM NH₄Cl, 0.2 mM NADH, 0.05% (wt/vol) BSA, 2 mM 2-mercaptoethanol, 10 µM rotenone, 1 µM aldolase, 10 µM triosephosphate isomerase, 2 µM glyceraldehyde-3-phosphate dehydrogenase and 1 µM phosphoglucoisomerase at 30 °C. Coupling enzymes were buffer-exchanged over sephadex G-25 equilibrated with 10 mM tris-HCl pH 7.1 to remove sulfite.

PK. Total PK (BRENDA: EC 2.7.1.40) activity and ratio at 1.3 or 6.6 mM phosphoenolpyruvate at 66 mM KCl was determined as described by Blair et al. 50. Lysates (10µg) were incubated in reactions containing 100 mM tris-HCl pH 7.5, 66 mM KCl, 10 mM MgCl₂, 2.5 mM ADP, 0.2 mM NADH, 10 µM rotenone, 100 µM microcinycin-LR, 0.05% (wt/vol) BSA, 2 mM lactate dehydrogenase and 1.3 or 6.6 mM phosphoenolpyruvate.

PEPCK-C. PEPCK-C (BRENDA: EC 4.1.1.32) was assayed using the method of Peters et al. 47. Lysates (100µg) were incubated in reactions containing 50 mM tris-HCl pH 7.4, 1 mM MnCl₂, 0.1 mM EDTA, 0.05% (wt/vol) BSA, 0.5 mM PEP, 0.2 mM NADH, 0.1 µM rotenone, 0.2 mM 2-deoxy-GDP, 2 µM UTP, malate dehydrogenase and either 20 mM NaCl or 20 mM NaHCO₃ (saturated with CO₂) at 30 °C.

FBP1. FBP1 (BRENDA: EC 3.1.3.11) activity was assayed as described above.

AMPK. AMPK (BRENDA: EC 3.5.4.6) was assayed in reactions containing 50 mM MOPS pH 7.2, 100 mM KCl, 1 mM DTT, 0.05% (wt/vol) BSA, 1 mM ATP; 0.2 mM AMP; 7.5 mM 2-oxoglutarate, 0.15 mM NADH and 5 U ml⁻¹ glutamate dehydrogenase. All assays were performed in a final volume of 200 µl and monitored by changes in A₄₁₂. Initial rates were determined from the linear phase of the reaction.

AMPK phosphotransferase (BRENDA: EC 2.7.1.11) activity was assayed using immunoprecipitates. Briefly, lysates (50µg) were incubated with 2 µg anti-AMPKα1 or anti-AMPKα2 and 5 µl protein G-Sepharose for 2 h at 4 °C. Immune complexes were pelleted at 500 × g for 1 min and washed with 3 x 1 ml lysis buffer and 2 x 1 ml 50 mM tris-HCl pH 8, 0.1 mM EDTA. Phosphotransferase activity was determined in reactions containing 30 mM HEPES pH 7.5, 10 mM MgCl₂, 0.1 mM EGTA, 0.1 mM [γ³²P]ATP (250 CPM pmol⁻¹), 0.1 mM AMARAASAAALARRR-C(32P)₅ and 100 µM Grb2 AAALARRR-C(32P)₅. Reactions were quenched by spotting onto P81 filters and immersed in 75 mM phosphoric acid. Filters were washed 3 x 10 min with 75 mM phosphoric acid and rinsed with acetone. [³²P] incorporation was determined by Chenery counting. With the exception of AMPK, 1 U = 1 µmol product formed per min at 30 °C. For AMPK, 1 U = 1 nmol phosphor磷酸ate incorporated per min at 30 °C.

Data analysis. For models was performed by least-squares nonlinear regression using Levenberg–Marquardt minimization using GraphPad Prism v5.0. AUC was calculated using the trapezoidal rule with subtraction of the area under the baseline (AUC₀₋₅) being the net area under the curve. Statistical significance was determined using the unpaired, two-tailed Student’s t-test and an alpha level of 0.05. All data were normally distributed based D’Agostino–Pearson omnibus tests, and sample variance was similarity between groups was compared.

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

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| Item                                                                 | Confirmed |
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| The statistical test(s) used AND whether they are one- or two-sided | N/A       |
| Only common tests should be described solely by name; describe more complex techniques in the Methods section. | N/A       |
| A description of all covariates tested | N/A       |
| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | N/A       |
| A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | N/A       |
| For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted | N/A       |
| Give P values as exact values whenever suitable. | N/A       |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | N/A       |
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| Clearly defined error bars | N/A       |
| State explicitly what error bars represent (e.g. SD, SE, CI) | N/A       |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | N/A |
| Data analysis  | N/A |

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
For mouse studies, sample size was based on power calculations informed by accumulated knowledge from similar studies in our lab and reported in the literature.

Data exclusions
Although rarely happened (5-10%), mice used for GTT and MTT experiments were excluded from analysis if peak blood glucose level was <20 mM (attributable to variation inherent in IP administrations). Mice were excluded from the metformin euglycaemic clamp if values were greater than 1.5 standard deviations from the group mean (as described in detail under “Metformin-euglycemic clamp” section in online methods section).

Replication
In vitro studies on recombinant FBP1 are based on three independent experiments from two preparations of enzyme. In vivo studies, AICAR treatment, MB06322 treatment, HFD/metformin treatment, and metformin clamp (for comparison between WT vs. KI) were performed once. Other in vivo experiments were replicated 2-4 times.

Randomization
Age- and sex-matched mice were arbitrarily assigned to experiment groups.

Blinding
Investigators were unblinded.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study
☒ Unique biological materials
☒ Antibodies
☒ Eukaryotic cell lines
☒ Palaeontology
☒ Animals and other organisms
☒ Human research participants

Methods

n/a Involved in the study
☒ ChIP-seq
☒ Flow cytometry
☒ MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Unique materials (e.g. FBP1 mutant cDNA constructs) are available.

Antibodies

Antibodies used

- PEPCK-M (6924), pS641 GYS (3891), GYS (3886), pT389 p70S6K (#9234), p70S6K (#2708), pS240/244 S6 (#2215), S6 (#2217), pS79/p212 ACC1/2 (#3661), ACC1/2 (#3676), pT172 AMPKa1/2 (#2535), AMPKa1/2 (#2532), AMPKa1/2 mAb (#2793), pT792 RAPTOR (#2083), RAPTOR (#2280), HXK1 (#2024), G6PD (#12263), TBC1D1 (#4629), pS133 CREB (#9198) and streptavidin-HRP (Abcam). G6PT (sc-135479) and FBPI (sc-32435) were from Sigma. SLC22A1/OCT1 (#ACT-011) antibody was from Alomone Labs. pS237 TBC1D1 was from Merck Millipore (07-2268). PYGL (15851-1-AP) was from Proteintech. pS15 PYGL (S961A) was from DSTT. AMPKa1 and AMPKa2 antibodies used for immunoprecipitation were raised in sheep against C-355TSPPDSFLDDHHLTR369 and C-352MDDSAMHIPPGLKPH366 (human sequences) respectively. GLUT2 antibody was provided by Bernard Thorens (University of Lausanne, Switzerland). GCK/HXK4 antibody was provided by Mark Magnuson (Vanderbilt University, TN). GCKR antibody was from Masakazu Shioya (Vanderbilt University, TN). G6PC antibody was provided by Giles Mithieux (University of Lyon, France). pS33 PFKFB1 antibody was provided by Jianxin Xie (Cell Signaling Technology). PFKFB1 antibody was provided by Simone Baltrusch (University of Rostock, Germany).
pS8 GYS2 antibody was provided by Joan Guinovart (University of Barcelona, Spain).

Validation

All antibodies sourced from Cell Signaling Technology are well-validated by the manufacturer and are widely used in the scientific community for Western blotting and detected single bands of the correct MW and exhibited the expected response to physiological stimuli. Anti-G6PT (sc-135479), FBP1 (sc-32435), PFKL (ab181064), PKLR (ab171744) and PYGL (15851-1-AP) were validated for Western blotting using a panel of mouse tissue homogenates and were accepted for use based on the strong detection of single bands of the correct MW and appropriate tissue distribution. AMPKα1 and AMPKα2 antibodies (for immunoprecipitation) have been validated using AMPKα1/AMPKα2 KO or LKB1 KO tissue/cell lysates (reported in PMID: 17855357/PMID: 16332922). pS237 TBC1D1 was validated in muscle cell/tissue lysates treated with AMPK activators (PMID: 27826658). PEPCK-C antibody (ab28455) was validated using PEPCK-C KO tissue lysates (unreported results from KS). SLC22A1/OCT1 (#ACT-011) was validated for immunoprecipitation/Western blotting using liver biopsies from WT and OCT1 KO mice (unreported results from KS and RWH using material supplied by NJ). G6PC antibody was validated using G6PC KO tissue lysates (reported in PMID: 21109326). GCK and GCKR antibodies were validated using respective KO tissue lysates (reported in PMID: 14985368 and PMID: 10713097). PFKFB1 antibody was validated in rat/mouse liver lysates (reported in PMID: 15047617). GLUT2 antibody was validated using GLUT2 KO tissue lysates (reported in PMID: 10823833). pS33 PFKFB1 antibody was validated in glucagon-treated mouse hepatocytes (reported in PMID: 25088745), pS15 PYGL (S961A) was validated for Western blotting using cultured cells overexpressing WT and S15A PYGL (unreported results from KS and RWH). pS8 GYS2 antibody was validated using cultured cells overexpressing WT and S8A GYS2 (reported in PMID: 19124463).

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Animals and other organisms          |
|--------------------------------------|
| Laboratory animals                   |
| C57BL/6NTac FBP1 G27P Knockin mice were generated by Taconic Biosciences GmbH and male mice between the ages of 14-22 weeks were used for all experiments. |
| Wild animals                         |
| The study did not involve wild animals. |
| Field-collected samples              |
| The study did not involve field-collected samples. |