

# FGF21 does not require adipocyte AMP-activated protein kinase (AMPK) or the phosphorylation of acetyl-CoA carboxylase (ACC) to mediate improvements in whole-body glucose homeostasis



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## ABSTRACT

**Objective:** Fibroblast growth factor 21 (FGF21) shows great potential for the treatment of obesity and type 2 diabetes, as its long-acting analogue reduces body weight and improves lipid profiles of participants in clinical studies; however, the intracellular mechanisms mediating these effects are poorly understood. AMP-activated protein kinase (AMPK) is an important energy sensor of the cell and a molecular target for anti-diabetic medications. This work examined the role of AMPK in mediating the glucose and lipid-lowering effects of FGF21.

**Methods:** Inducible adipocyte AMPK  $\beta 1\beta 2$  knockout mice ( $i\beta 1\beta 2AKO$ ) and littermate controls were fed a high fat diet (HFD) and treated with native FGF21 or saline for two weeks. Additionally, HFD-fed mice with knock-in mutations on the AMPK phosphorylation sites of acetyl-CoA carboxylase (ACC)1 and ACC2 (DKI mice) along with wild-type (WT) controls received long-acting FGF21 for two weeks.

**Results:** Consistent with previous studies, FGF21 treatment significantly reduced body weight, adiposity, and liver lipids in HFD fed mice. To add, FGF21 improved circulating lipids, glycemic control, and insulin sensitivity. These effects were independent of adipocyte AMPK and were not associated with changes in browning of white (WAT) and brown adipose tissue (BAT). Lastly, we assessed whether FGF21 exerted its effects through the AMPK/ACC axis, which is critical in the therapeutic benefits of the anti-diabetic medication metformin. ACC DKI mice had improved glucose and insulin tolerance and a reduction in body weight, body fat and hepatic steatosis similar to WT mice in response to FGF21 administration.

**Conclusions:** These data illustrate that the metabolic improvements upon FGF21 administration are independent of adipocyte AMPK, and do not require the inhibitory action of AMPK on ACC. This is in contrast to the anti-diabetic medication metformin and suggests that the treatment of obesity and diabetes with the combination of FGF21 and AMPK activators merits consideration.

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**Keywords** FGF21; AMPK; ACC; Adipocyte; Brown fat; Obesity; Diabetes

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**Abbreviations:** ACC, acetyl-CoA carboxylase; ACC DKI, ACC1-S79A and ACC2-S212A double knock-in; AKT, protein kinase B; AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; COX, cytochrome *c* oxidase; CreER<sup>T2</sup>, Cre recombinase — estrogen receptor T2; CNS, central nervous system; DAG, diacylglycerol; FFA, free fatty acid; FGF21, fibroblast growth factor 21; FGF1c, fibroblast growth factor receptor 1c; GTT, glucose tolerance test; gWAT, gonadal white adipose tissue; H&E, hematoxylin and eosin; HFD, high fat diet;  $i\beta 1\beta 2AKO$ , inducible AMPK  $\beta 1\beta 2$  adipocyte knockout; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; KLB, beta klotho; RER, respiratory exchange ratio; mTORC1, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; TAG, triacylglycerol; UCP1, uncoupling protein 1; WAT, white adipose tissue; WT, wildtype

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## 1. INTRODUCTION

Initially identified as a hormone secreted by the liver that could potentially stimulate glucose uptake in adipocytes [1], fibroblast growth factor 21 (FGF21) is an endocrine factor that exerts potent anti-obesity and anti-diabetic effects in rodents and non-human primates [2–8]. Furthermore, long-acting FGF21 analogues in phase 1 clinical trials decrease body weight and improve the lipid profile of patients with type 2 diabetes [6,9]. In pre-clinical rodent models, FGF21 administration increases energy expenditure and improves glucose and lipid homeostasis [2]. These effects have been related to increases in glucose uptake and triglyceride clearance in white (WAT) and brown adipose tissue (BAT) [10] and require the expression of the FGF21 receptor, fibroblast growth factor receptor 1c (FGFR1c), and cofactor beta klotho (KLB) [11,12]. Findings from several FGF21 studies have also shown increases in the browning of WAT; a process whereby WAT acquires characteristics of brown fat such as increases in mitochondrial number and the expression of uncoupling protein 1 (UCP1). While recent studies have suggested that metabolic improvements mediated by FGF21 do not require UCP1 [13,14] nor intrascapular BAT [2], other groups have shown that UCP1 is required for FGF21-mediated glucose disposal [15] (see Straub and Wolfrum for a minireview [16]). Although it has recently been recognized that FGF21 may exert many of its pharmacological effects through the central nervous system (CNS) [17], the intracellular mechanisms mediating the beneficial metabolic effects of FGF21 on adipose tissue metabolism *in vivo* are currently not fully elucidated.

Previous studies have shown that FGF21 administration activates the energy sensor AMP-activated protein kinase (AMPK) to promote mitochondrial biogenesis and greater mitochondrial oxidative function in cultured rodent and human adipocytes [18]. AMPK is a central regulator of the energy status of the cell, regulating a myriad of metabolic pathways [19–22]. AMPK in WAT and BAT is activated by sympathetic drive through mechanisms that are not fully understood but are thought to occur, at least in part, by increased lipolysis [23–25]. Our previous work demonstrated that adipocyte AMPK is required for acute BAT-mediated thermogenesis, the browning of WAT in response to  $\beta$ -adrenergic stimuli, and protection against the deleterious effects of a high fat diet (HFD) via the regulation of the mitochondrial quality control pathway (mitophagy) [23]. Furthermore, the inducible deletion of adipocyte AMPK in adult mice did not alter adipocyte lipolysis [23]. However, whether adipocyte AMPK is important for the glucose and lipid-lowering effects of FGF21 remains to be determined.

Chronic FGF21 treatment reduces hepatic triglyceride levels by promoting fat oxidation [26,27] and suppressing *de novo* lipogenesis in the liver of rodents, effects associated with an increase in AMPK activation [2]. In addition, discrete effects of FGF21, such as suppression of hepatic glucose output [28], are thought to involve action on the liver [2,29,30]. Interestingly, the therapeutic effects of metformin, a first line therapy for the control of blood glucose levels, requires AMPK regulation of acetyl-CoA carboxylase (ACC) to suppress *de novo* lipogenesis and promote hepatic fat oxidation [31]. Together, these data suggest that FGF21 might also mediate improvements in blood glucose levels through the AMPK/ACC axis in the liver.

Considering that FGF21 is in clinical trials for the treatment of obesity and diabetes, a deeper understanding of the responsible *in vivo* signaling pathways has obvious medical benefits. In particular, both clinical studies published to date do not demonstrate robust glycemic control, despite weight loss [6,9]. Since adipose tissue is a key organ that directly mediates the effects of FGF21 [6,17,32,33], a better understanding of adipocyte-specific signaling events is required in the

context of improvements in whole-body metabolism. Moreover, since metformin is a first line therapy for glycemic control in diabetic patients, it would be of great benefit to understand if FGF21 also requires the AMPK/ACC axis to improve blood glucose levels. To this end, we utilized an inducible model for the deletion of AMPK in adipocytes of adult mice [23] to examine whether the metabolic improvements of FGF21 therapy require adipocyte AMPK *in vivo*. To circumvent the deleterious effects of AMPK removal prior to HFD challenge [23], we performed deletion of adipocyte AMPK following HFD treatment. We also tested whether FGF21 requires AMPK-mediated phosphorylation of ACC to improve blood glucose and hepatic lipid levels by utilizing a knock-in model in which AMPK lacks the ability to inactivate ACC. Overall, we demonstrated that the beneficial metabolic effects of pharmacological administration of FGF21 on energy metabolism, fuel selection, body weight, insulin tolerance, and liver lipids are not mediated through adipocyte AMPK or the upregulation of a thermogenic program in BAT or inguinal WAT (iWAT). Additionally, FGF21 administration does not require the AMPK/ACC pathway to reduce hepatic lipid content or improve glucose homeostasis and insulin sensitivity. Taken together, these findings offer new insights into the mechanism of FGF21 action and open up the possibility of understanding the disconnect between rodent and clinical data on glucose endpoints.

## 2. MATERIALS AND METHODS

### 2.1. Animals

All experiments were approved by the McMaster University Animals Ethics Committee and conducted under appropriate Canadian guidelines for animal research. Mice lacking adipocyte AMPK  $\beta$ 1 $\beta$ 2 ( $\beta$ 1 $\beta$ 2AKO) along with littermate controls were generated as previously described [23]. Tamoxifen (Cayman Chemical; MI, USA), dissolved in sunflower oil, was given via oral gavage for five consecutive days in adult males to induce the deletion of adipocyte AMPK  $\beta$ 1 $\beta$ 2 after 10 weeks of a high fat diet (HFD, 45% kcal fat) (D12451, Research Diets; New Brunswick, New Jersey). At week 14 of HFD, control and  $\beta$ 1 $\beta$ 2AKO mice were anesthetized with Avertin (2,2,2-tribromoethanol dissolved in methyl-2-butanol, Sigma-Aldrich; ON, Canada; 0.5 mg/g body) and implanted dorsally with two week mini-osmotic pumps (1002, Alzet; CA, USA) delivering 0.35 mg/kg/day of native FGF21 or 0.9% sodium chloride (saline) and incisions closed with perma-hand silk suture (Ethicon; ON, Canada). Food and water were provided ad libitum and mice were housed at 23 °C with a 12-hour light/dark cycle.

Male ACC1-S79A and ACC2-S212A knock-in mutation mice (ACC DKI) were as previously described and were put on a 45% HFD at 3–4 months of age [31]. At week 10 of HFD, wildtype and ACC DKI mice received subcutaneous injections of long-acting FGF21 (PF-05231023) at 10 mg/kg twice a week for 2 weeks.

### 2.2. Metabolic parameters and blood measurements

Metabolic monitoring was performed using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments; OH, USA). Body fat was determined using a time-domain NMR whole-body composition analyzer (minispec LF90II, Bruker; MA, USA) and normalized to body weight. A glucose tolerance test (GTT) or insulin tolerance test (ITT) was performed with 6-hour fasted mice on day 13 of treatment, after intraperitoneal injection of 1.0 g/kg of glucose or 1 U/kg insulin, respectively, for HFD-fed mice [34]. Blood glucose levels were determined using a One Touch Ultra Glucometer (LifeScan, Canada). FFA were measured by isolating plasma via centrifugation at 4 °C, diluting in PBS at a 1:5 ratio, and using a non-esterified fatty acid

colorimetric assay kit (NEFA-HR 2, Wako Diagnostics; VA, USA). Plasma insulin and adiponectin were quantified by ELISA (Millipore).

### 2.3. In-vivo glucose clearance

Tissue-specific glucose uptake, stimulated by 1.0 U/kg of insulin via intraperitoneal injection, was performed by tail vein injection of 2  $\mu$ Ci of 3H-2-deoxy-D-glucose (2-DG) 15 min prior to sacrifice, as previously described [35]. Rates of tissue-specific glucose uptake were measured by analyzing radioactivity from tissues in both the deproteinized and proteinized state using 0.3 mM BaOH and 0.3 mM Zn(SO<sub>4</sub>) (Sigma-Aldrich; ON, Canada).

### 2.4. Immunoblotting and cytochrome c oxidase (COX) activity

Tissue lysates were prepared in lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 100 mM NaF, 10 Na-pyrophosphate, 5 EDTA mM, 250 mM sucrose, 1 mM DTT, and 1 mM Na-orthovanadate, 1% Triton X, and Complete protease inhibitor cocktail (Roche)). Western samples were prepared using 4x SDS sample buffer (40% glycerol, 240 mM, Tris-HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5%  $\beta$ -mercaptoethanol, with a 1:50 dilution of 1 M DTT) and boiled at 95 °C for 5 min. 20  $\mu$ g of protein was loaded per well for iWAT and 5  $\mu$ g of protein for BAT, as this amount avoids oversaturation of the UCP1 signal. Immunoblotting was performed as previously described [23]. Using antibodies purchased from Cell Signaling Technology (AKT, #9272; pAKT Ser473, #4058; anti-Rabbit IgG horseradish peroxidase (HRP)-linked, #7074; and anti-Mouse IgG HRP-linked; #7076), Alpha Diagnostic International (UCP1, #UCP11-A), and Invitrogen ( $\beta$ -tubulin, #32-2600), blocked membranes were incubated in primary antibody overnight at 4 °C (5% BSA in TBST).

In order to determine phospho/total protein ratios, membranes were first probed for the phospho protein, stripped for 30 min at 37 °C using Restore Plus (Thermo Scientific; ON, Canada) to ensure removal of phospho signal, and re-probed overnight using antibody against the total protein. UCP1 protein levels were normalized to the loading control  $\beta$ -tubulin. Densitometrical analysis was performed using Image J Software (NIH; MD, USA).

COX activity was measured in tissue lysates as prepared above and as previously described [23].

### 2.5. Histology

Samples harvested from mice were initially stored and fixed in 10% formalin (24–48 h) and subsequently processed for paraffin embedding and stained with H&E by the Department of Pathology and Molecular Medicine at McMaster University Children's Hospital. Images of the liver (20x) and BAT (10x) were taken using a Nikon 90i Eclipse (Nikon Inc.; NY, USA).

### 2.6. Lipid analysis

Plasma and lipid analysis was performed as previously described [6]. Briefly, liver tissue samples were homogenized in Methanol:Water (1:1 v/v) and then lipids were extracted with Dichloromethane: Isopropanol: Methanol (25:10:65, v/v/v) and analyzed by mass spec against internal standards. Liver triglyceride levels in ACC-DKI mice were measured using a Triglyceride Assay Kit (Cayman Chemical) as previously described [23].

### 2.7. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed to determine mRNA expression of browning and mitochondrial markers as previously described [36]. Briefly, RNA was isolated using TRIzol reagent (Invitrogen; CA, USA) and applied to columns (RNeasy kit, Qiagen; CA, USA) for purification. All Taqman primers were purchased from Invitrogen [23] (Hadh, Mm00492535\_m1;

Cpt1b, Mm00487191\_g1; Ppargc1a, Mm01208835\_m1; Ppara, Mm00440939\_m1; Ucp1, Mm01244861\_m1; Ppia, Mm02342430\_g1; Mt-co2, Mm03294838\_g1; Cox8b, Mm00432648\_m1; Cidea, Mm00432554\_m1), and relative gene expression was calculated using the comparative Ct (2- $\Delta$ CT) method. Values were normalized to the housekeeping gene Ppia and subsequently expressed as relative to control saline.

### 2.8. Statistical analyses

Results were analyzed by two-way ANOVA using GraphPad Prism software. Repeated measures two-way ANOVA was used for GTT and ITT data. All values are presented as mean  $\pm$  SEM and significance was accepted at  $p \leq 0.05$ .

## 3. RESULTS

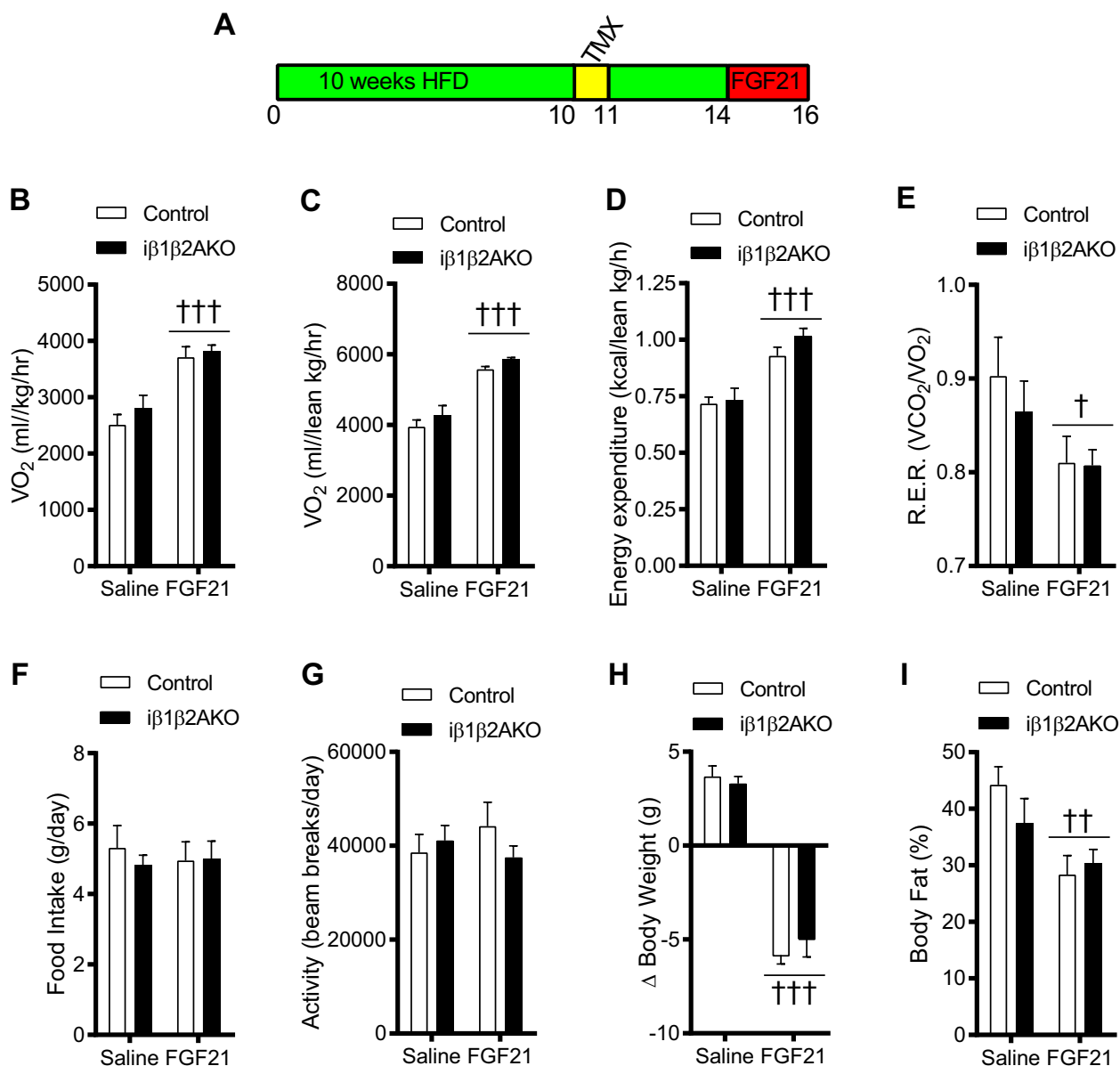
### 3.1. FGF21 increases energy expenditure, reduces adiposity, and improves blood parameters independent of adipocyte AMPK

To examine the role of adipocyte AMPK in mediating the metabolic effects of FGF21, double floxed AMPK  $\beta$ 1 and  $\beta$ 2 mice with or without the Adiponectin Cre recombinase — estrogen receptor T2 (CreER<sup>T2</sup>) transgene were subjected to HFD treatment for 10 weeks followed by tamoxifen-induced deletion of AMPK (Figure 1A). We evaluated the efficiency of deletion by determining phosphorylation of ACC, the most sensitive method for detecting cellular AMPK activity, and found no detectable phosphorylation of ACC in BAT and iWAT of mice deficient for AMPK (Supplemental Figure 1). Following three weeks to allow for the appropriate genetic recombination and recovery, mice treated with native FGF21 for two weeks showed increased oxygen consumption and energy expenditure (Figure 1B–D) and lowered respiratory exchange ratio (R.E.R.) (Figure 1E) in both control and mice deficient for AMPK in adipocytes. The increase in metabolic activity by FGF21 was similar when the data were normalized to lean body mass (Figure 1B vs. C) and was not due to differences in food intake and physical activity, as these parameters were unchanged upon FGF21 administration (Figure 1F and G). Concomitant with greater metabolic activity, FGF21 treatment reduced body weight (Figure 1H) and adiposity (Figure 1I) in both genotypes. Overall, these data indicate that FGF21 does not require adipocyte AMPK to mediate an increase in energy expenditure and a reduction in body fat.

Adiponectin has been suggested to be an important mediator for the insulin sensitizing effects of FGF21 [3,37] and is an activator of AMPK [38]. Indeed, FGF21 administration increases circulating adiponectin in humans [6,9]. FGF21 administration caused a modest trend of increased circulating adiponectin levels in control ( $p = 0.068$ ) (Figure 2A) but not in  $\beta$ 1 $\beta$ 2AKO mice; however, serum insulin levels were dramatically reduced in both genotypes (Figure 2B). Despite the much lower insulin levels, WT and  $\beta$ 1 $\beta$ 2AKO mice treated with FGF21 had lower fed (Figure 2C) and fasted blood glucose levels (Figure 2D). FGF21 treatment also lowered plasma free fatty acid (FFA) and triacylglycerol (TAG) levels in both genotypes to a similar extent (Figure 2E and F). Overall, these data indicate that FGF21 lowers serum insulin, blood glucose, and plasma TAGs and FFAs independently of adipocyte AMPK.

### 3.2. FGF21 improves insulin sensitivity and promotes glucose uptake in BAT independently of adipocyte AMPK

Consistent with lower blood glucose and plasma insulin, FGF21 improved insulin-mediated glucose disposal in both control and  $\beta$ 1 $\beta$ 2AKO mice (Figure 3A and B). To determine which tissues were mediating the improvements in insulin-mediated glucose uptake, we assessed 2-deoxyglucose uptake following an intraperitoneal injection

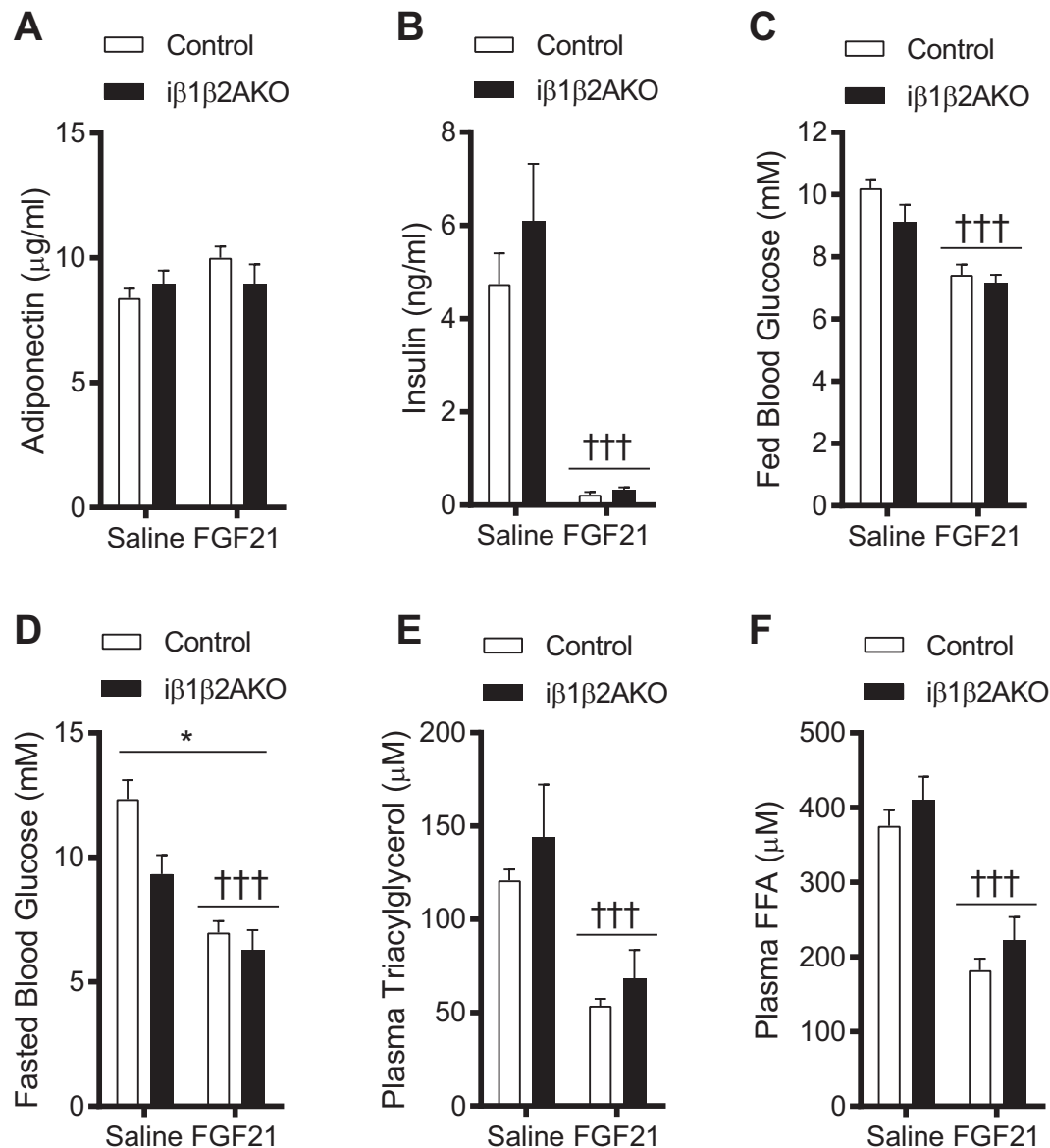


**Figure 1: Native FGF21 increases energy metabolism, fuel selection, and body weight regulation in both control and  $i\beta 1\beta 2$ AKO mice.** (A) timeline of experiment with 45% high fat diet (HFD), in weeks, consisting of a week of tamoxifen (TMX) treatment to induce the deletion of both AMPK beta subunits ( $\beta 1$  and  $\beta 2$ ) only in adipocytes, and two weeks of native FGF21 administration using implanted osmotic mini-pumps. Mice were maintained on a HFD throughout the illustrated timeline and FGF21 treatment was administered for two weeks. (B–G) oxygen consumption ( $VO_2$ ) normalized to whole body mass (B) and lean body mass (C), energy expenditure normalized to lean body mass (D) respiratory exchange ratio (R.E.R.) (E), food intake (F), and activity levels (G) of control and  $i\beta 1\beta 2$ AKO mice treated with saline or native FGF21 for 14 days ( $n = 4$  per group). Change in body weights (H) and fat percentage (I) of control and  $i\beta 1\beta 2$ AKO mice treated with saline or native FGF21 for 14 days ( $n = 4$ –7 per group). Data are means  $\pm$  SEM with  $+++ p < 0.001$ ,  $++ p < 0.01$ ,  $+ p < 0.05$  denoting a general treatment effect as determined by a two-way ANOVA.

of insulin. FGF21 increased glucose uptake in BAT (Figure 3C), but this effect was not significant in iWAT (Figure 3D). Interestingly, FGF21 had an overall effect of lowering glucose uptake in gonadal WAT (gWAT), liver and quadriceps muscle (Figure 3E–G). To determine if FGF21 treatment was associated with improvements in adipose tissue insulin signaling, and whether there were differences in  $i\beta 1\beta 2$ AKO mice, we examined AKT phosphorylation status in BAT and iWAT. FGF21 treatment increased phosphorylation of AKT in both BAT (Figure 3H and I) and iWAT (Figure 3H and J) following an acute injection of insulin. These data indicate that FGF21 treatment improves BAT insulin action and that these effects do not require AMPK.

### 3.3. FGF21 reduces hepatic steatosis without increasing WAT browning

FGF21 reduces non-alcoholic fatty liver disease (NAFLD) [2,5] while inducible deletion of adipocyte AMPK prior to HFD treatment promotes NAFLD [23]. Consistent with previous reports [2,29], FGF21 treatment resulted in lower hepatic lipid content, as indicated by lower lipid-laden white area present within cells in hematoxylin and eosin (H&E) stains (Figure 4A), which was further confirmed by a reduction in liver diacylglycerol (DAG) (Figure 4B) and TAG (Figure 4C) levels. In contrast to DAG and TAG levels, liver ceramide levels were increased with FGF21 treatment (Figure 4D).



**Figure 2: The effects of native FGF21 on specific blood parameters in control and  $i\beta 1\beta 2$ AKO mice.** Circulating adiponectin (A) and insulin (B) levels of control and  $i\beta 1\beta 2$ AKO mice treated with saline or native FGF21 for 14 days ( $n = 4-7$  per group). Blood glucose levels in the fed (C) and 12 h fasted (D) state ( $n = 4-7$  per group). E and F: end-point plasma triacylglycerol (E) and free fatty acids (FFA) (F) of Control and  $i\beta 1\beta 2$ AKO mice treated with saline or native FGF21 ( $n = 4-7$  per group). Data are means  $\pm$  SEM with †††  $p < 0.001$  denoting a general treatment effect and \*  $p < 0.05$  denoting a general genotype effect as determined by a two-way ANOVA.

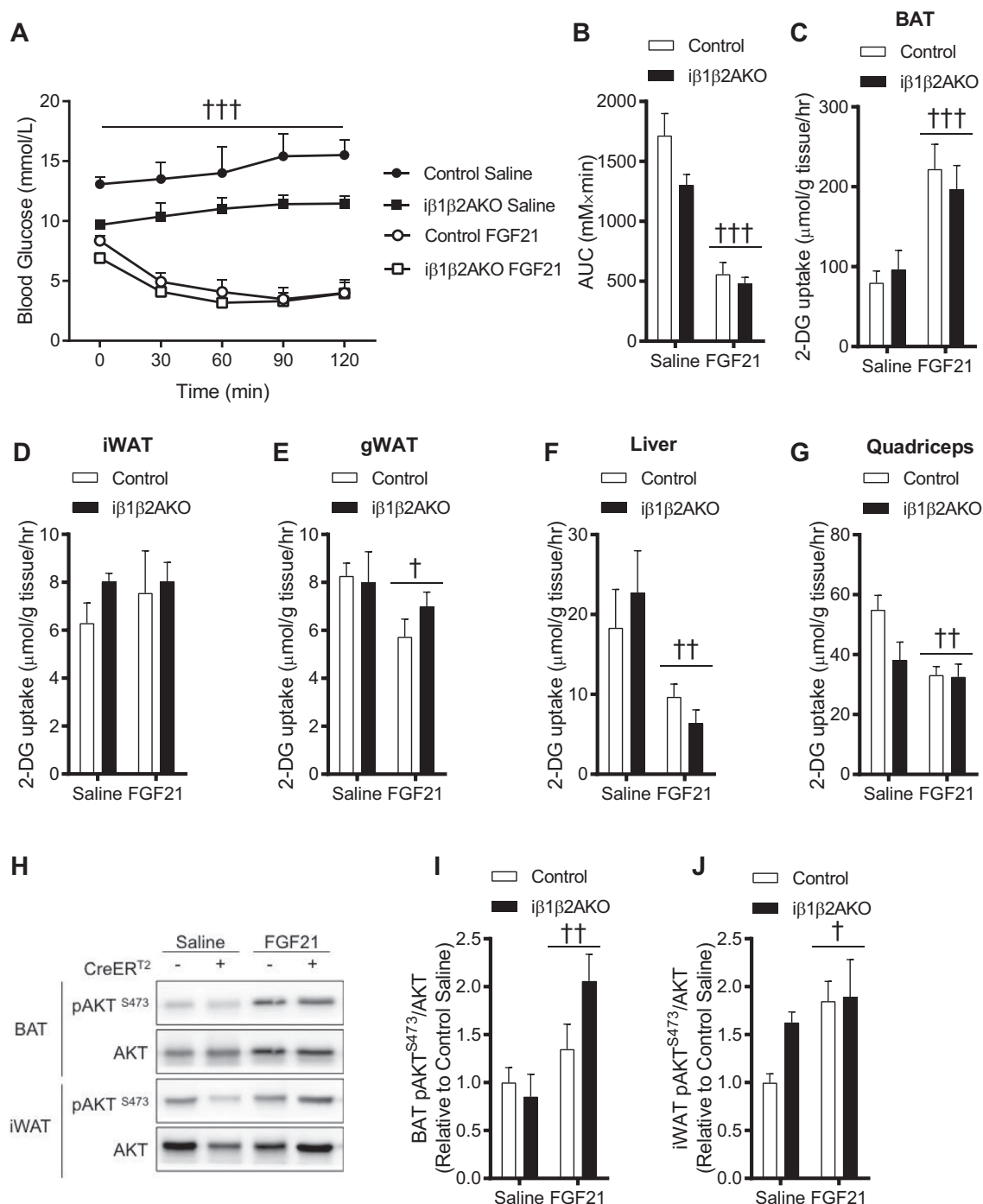
FGF21 has been associated with improvements in BAT function and increased WAT browning; however, the role of UCP1 and WAT browning in mediating the metabolic improvements induced by pharmacological administration of FGF21 are somewhat dichotomous [13,15,16]. FGF21 treatment reduced both BAT and iWAT mass (Figure 5A and B, respectively) and reduced the presence of lipid in BAT, as indicated by H&E staining (Figure 5C). FGF21 treatment had a modest overall effect on increasing UCP1 and MT-CO2 mRNA levels in BAT of control and  $i\beta 1\beta 2$ AKO mice (Figure 5D), but not in iWAT (Figure 5F). Consistent with an increase in MT-CO2, FGF21 increased mitochondrial cytochrome *c* oxidase (COX) activity in the BAT of both control and  $i\beta 1\beta 2$ AKO mice (Figure 5E). FGF21 increased Ppargc1a mRNA levels in iWAT, but the expression of other browning markers was unaffected (Figure 5F). FGF21 treatment did not affect UCP1 protein levels in BAT, while there was an overall effect for lower UCP1

levels in the BAT of  $i\beta 1\beta 2$ AKO mice (Figure 5G and H). The expression of UCP1 protein in iWAT, while highly variable, was unaffected by FGF21 treatment in either Control or  $i\beta 1\beta 2$ AKO mice (Figure 5G and I).

#### 3.4. FGF21-mediated improvements in glucose homeostasis, insulin sensitivity and hepatic lipid content are independent of phosphorylation of ACC by AMPK

The accumulation of hepatic lipids is thought to be critical in liver insulin resistance and causative in the dysregulation of whole-body glucose homeostasis [39]. One of the molecular mechanisms for hepatic lipid accumulation is the process of *de novo* lipogenesis. FGF21 has been shown to suppress hepatic glucose output [28] and *de novo* lipogenesis, effects that were associated with the activation of AMPK [2]. To examine whether the metabolic improvements resulting from FGF21 administration are due to AMPK's inhibitory

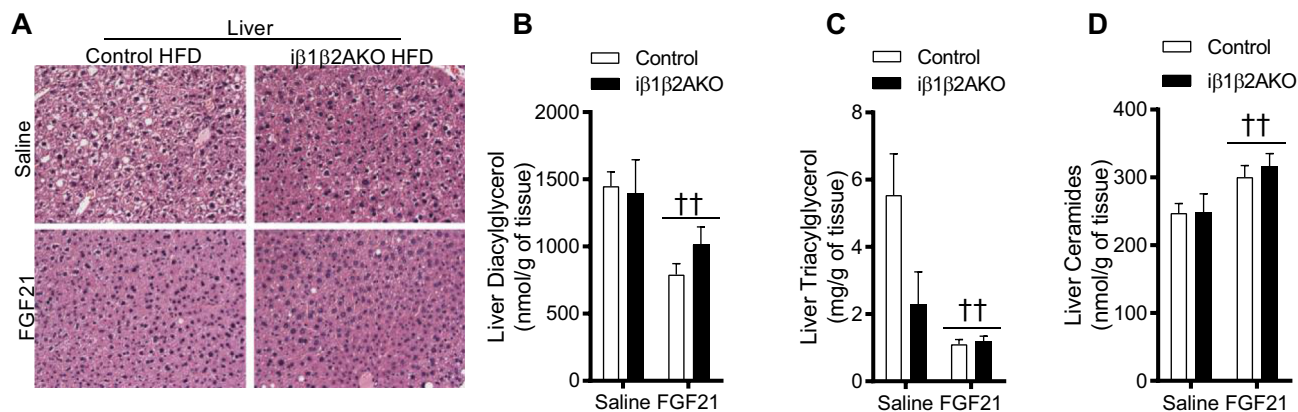




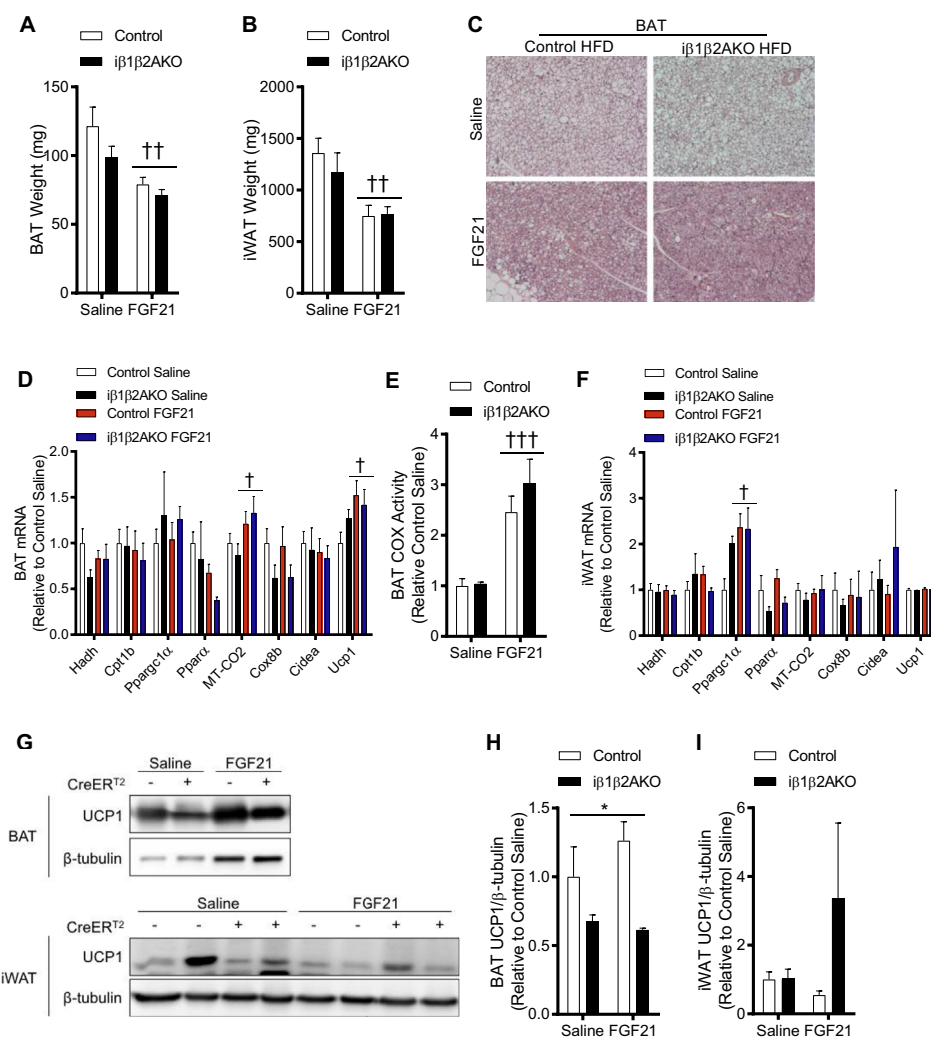
**Figure 3: Control and  $i\beta 1\beta 2$ AKO mice treated with native FGF21 for two weeks have improved insulin sensitivity.** A and B: insulin tolerance test (ITT: 1 U/kg) (A) and area under the curve (AUC) (B) of control and  $i\beta 1\beta 2$ AKO mice treated with native FGF21 on indicated diet ( $n = 4-7$  per group). C–G: tissue glucose uptake measured using 2-DG of BAT (C), iWAT (D), gWAT (E), liver (F), and quadriceps muscle (G) in control and  $i\beta 1\beta 2$ AKO mice treated with saline or native FGF21 ( $n = 4-7$  per group). H–J: representative total and phospho (S473) AKT immunoblotting (H) with quantification in BAT (I:  $n = 4-7$  per group) and iWAT (J:  $n = 3-7$  per group) of 10-week HFD and 2-week treated (saline or native FGF21) control and  $i\beta 1\beta 2$ AKO mice. Data are means  $\pm$  SEM with  $+++ p < 0.001$ ,  $++ p < 0.01$ ,  $\dagger p < 0.05$  denoting a general treatment effect as determined by a two-way ANOVA (A: two-way repeated measures ANOVA).

action on ACC — a key rate-limiting enzyme in the lipogenesis pathway and regulator of hepatic fat oxidation — we utilized a previously described knock-in mutation mouse model for ACC where AMPK lacks the ability to phosphorylate and inactivate ACC [31]. In addition, we utilized a long-acting form of FGF21 (PF-05231023) that demonstrated efficacy in humans [6]. Consistent with previous

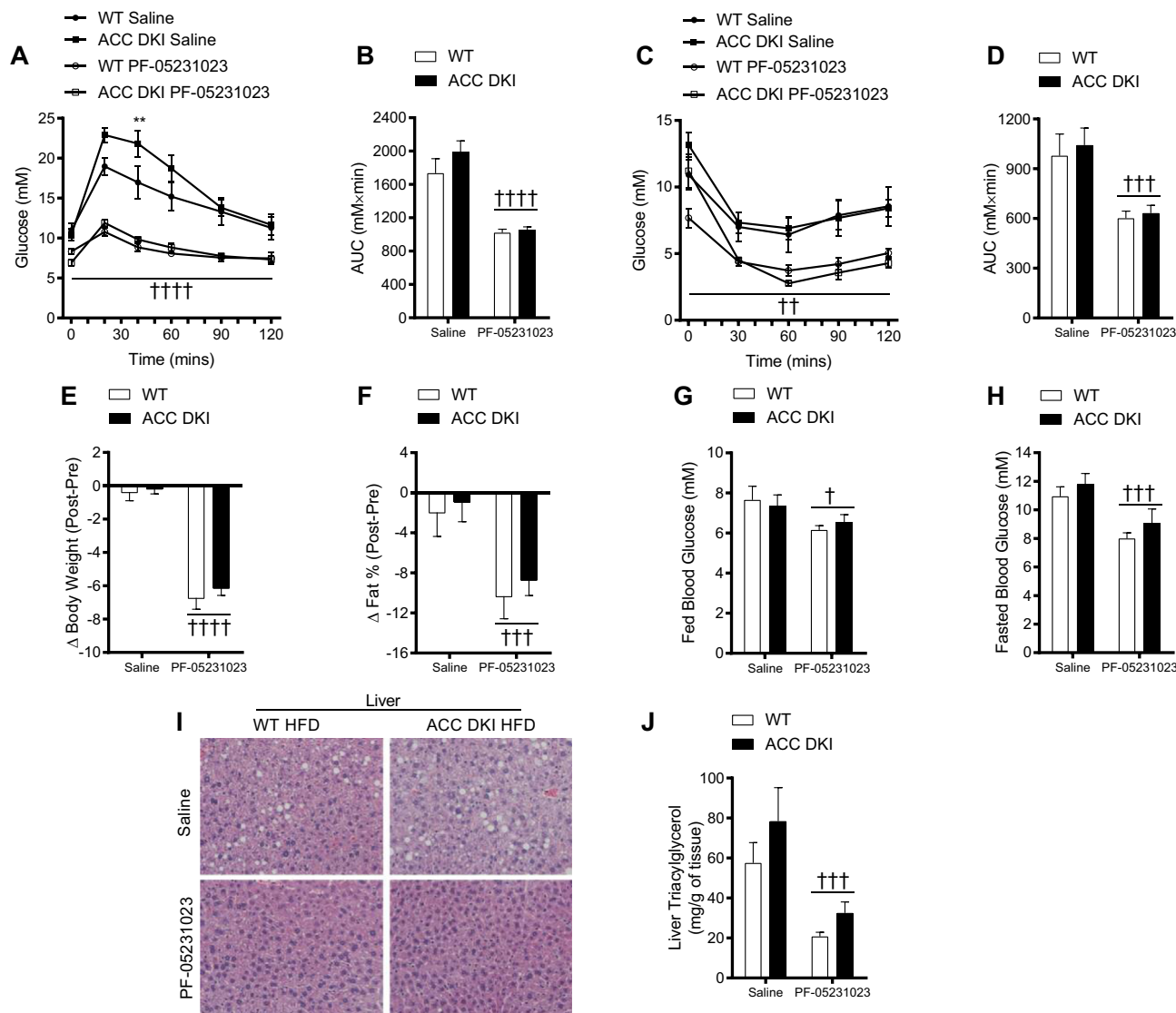
studies [2,5] and the current data in  $i\beta 1\beta 2$ AKO mice, FGF21 significantly improved glucose tolerance (Figure 6A and B) and insulin sensitivity (Figure 6C and D). However, these effects were independent of the ability of AMPK to phosphorylate and inactivate ACC. Additionally, FGF21 administration led to reduced body weight (Figure 6E), adiposity (Figure 6F), and lowered fed (Figure 6G) and



**Figure 4: Treatment with native FGF21 for two weeks reduces liver lipids in mice fed a high fat diet (45%) for 10 weeks.** (A) representative H&E liver stains (20x) of control and iβ1β2AKO mice treated with saline or native FGF21. B and C: Liver levels of diacylglycerol (B) and triacylglycerol (C) as measured by mass spec (n = 4–7 per group). (D) Liver ceramides as measured by mass spec (n = 4–7 per group). Data are means ± SEM with †† p < 0.01 denoting a general treatment effect as determined by a two-way ANOVA.



**Figure 5: The beneficial effects of native FGF21 in control and iβ1β2AKO mice are not driven by the upregulation of a thermogenic program *in vivo*.** A and B: tissue weights of BAT (A) and iWAT (B) of control and iβ1β2AKO mice treated with saline or native FGF21 for 14 days (n = 4–7 per group). (C) representative H&E stains (10x) of BAT from control and iβ1β2AKO mice fed a high fat diet (45%) for 10 weeks and treated with saline or native FGF21 for 14 days. D and E: mRNA expression of mitochondrial and browning markers (Hadh, MT-CO2, Cox8b, Cpt1b, Ppargc1α, Pparα, Cidea, Ucp1) in BAT (D) and iWAT (E) (n = 4–7 per group). (F) mitochondrial COX activity in whole tissue BAT lysates from control and iβ1β2AKO mice treated with FGF21 (n = 4–7 per group). G–H: representative UCP1 and β-tubulin immunoblotting (G) with quantification in BAT (H) and iWAT (I) of high fat diet-fed and two week treated (saline or native FGF21) control and iβ1β2AKO mice (n = 4–7 per group). Data are means ± SEM with ††† p < 0.001, †† p < 0.01, † p < 0.05 denoting a general treatment effect and \* p < 0.05 denoting a general genotype effect as determined by a two-way ANOVA.



**Figure 6: Long-acting (PF-05231023) FGF21-mediated improvements in insulin sensitivity and hepatic lipid content are not due to AMPK's suppression of ACC.** A–D: glucose tolerance test (GTT; 1 g/kg) (A) with area under the curve (AUC) (B) and insulin tolerance test (ITT; 1 U/kg) (C) with area under the curve (D) of wildtype and ACC DKI fed a 45% high fat diet for 10 weeks and treated with saline or long-acting FGF21 for two weeks ( $n = 4$  per group). Change in body weights (E) and in fat percent (F) in wildtype and ACC DKI mice treated with saline or PF-05231023 ( $n = 8$  per group). Blood glucose levels in the fed (G) and 12-hr fasted (H) state of wildtype and ACC DKI mice treated with saline or long-acting FGF21 for two weeks ( $n = 8$  per group). I and J: representative H&E liver stains (20x) (I) and levels of triacylglycerol (J) in the liver of wildtype and ACC DKI mice treated with saline or long-acting FGF21 for two weeks ( $n = 8$  per group). Data are means  $\pm$  SEM with  $++++ p < 0.0001$ ,  $+++ p < 0.001$ ,  $++ p < 0.01$ ,  $+ p < 0.05$  denoting a general treatment effect and  $** p < 0.01$  denoting a general genotype effect as determined by a two-way ANOVA (A and C: two-way repeated measures ANOVA).

fasted-state (Figure 6H) blood glucose levels, irrespective of genotype. Lastly, the administration of long-acting FGF21 substantially lowered hepatic lipid content, marked by the reduced lipid containing area within H&E stained liver sections (Figure 6I) and reduced liver triacylglycerol levels (Figure 6J).

#### 4. DISCUSSION

In the current study, we examined whether pharmacological administration of FGF21 requires adipocyte AMPK to mediate its beneficial effects on metabolic homeostasis. Our previous work demonstrated that adipocyte AMPK was necessary to maintain BAT function, mediate increases in WAT browning and protect against the development of hepatic steatosis in rodents on a HFD [23]. Our current results demonstrate that FGF21 administration does not require adipocyte

AMPK to mediate improvements in glucose homeostasis, lipid homeostasis, insulin sensitivity, and reductions in body weight *in vivo*. Furthermore, we tested whether AMPK's inhibitory phosphorylation of ACC mediated the metabolic benefits of FGF21 administration and showed no genotype differences in the treated groups.

In mice, FGF21-mediated improvements in metabolic homeostasis have also been associated with the browning of WAT [2,14]. We found that FGF21 treatment increased energy expenditure, reduced body fat mass, and improved insulin sensitivity without increases in BAT or iWAT UCP1 protein levels. Indeed, as previously reported [23], we found that BAT UCP1 levels were lower in mice deficient for adipocyte AMPK, yet FGF21 improved metabolic homeostasis in these mice. FGF21 increased mitochondrial COX activity in both genotypes, which may explain how FGF21 might be mediating its effects independently of adipocyte AMPK, possibly via UCP1-independent mechanisms (see



below). We did not observe changes in overall WAT browning, as measured by gene expression analyses and UCP1 protein levels, although FGF21 treatment did increase *Ppargc1 $\alpha$*  mRNA levels, which could still be involved in mediating the effects of FGF21 [18,40] in this model.

We found that FGF21 increased glucose uptake in BAT and lowered uptake in the liver and quadriceps muscle, effects which were independent of adipocyte AMPK. Since we did not perform the hyperinsulinemic–euglycemic clamp technique, we cannot rule out the potential effects of FGF21 on suppressing hepatic glucose output [2,28]. FGF21 also increased tissue insulin action in BAT and iWAT independently of adipocyte AMPK; however, FGF21 did not significantly increase tissue glucose uptake in iWAT. These data suggest that the target tissue for the glucose lowering effects of FGF21 is likely BAT, although this does not require increased BAT UCP1 protein levels or AMPK. These data could explain the lack of glucose lowering in humans since the subjects recruited for the clinical studies were not measured for the presence of functional BAT. An alternate pathway is that FGF21 might promote UCP1-independent thermogenic mechanisms to mediate some of its beneficial metabolic effects. Recently, a phospho-proteomic approach to identify the signaling pathways activated downstream of FGF21 in adipocytes uncovered the metabolic sensor mammalian target of rapamycin (mTORC1) as an important mediator of the effects of FGF21 *in vitro* [41]. mTORC1 was required for FGF21-mediated increase in UCP1 mRNA, adiponectin secretion, and glucose uptake [41]. Future work should determine whether FGF21 requires adipocyte mTORC1 to mediate improvements in whole-body metabolism.

FGF21 likely improves hepatic steatosis and liver insulin action by reducing lipogenesis and enhancing lipid oxidation [2,27,29,30]. As metabolic improvements upon FGF21 administration are associated with reduced expression of ACC1 and ACC2 in liver [27], we used previously described knock-in mutation mice (ACC DK1) to test whether AMPK's inhibitory phosphorylation of ACC (ACC1-S79 and ACC2-S212) mediated the metabolic benefits of FGF21 administration. When ACC is phosphorylated by AMPK, as occurs with metformin treatment, malonyl-CoA levels are reduced, leading to the suppression of *de novo* lipogenesis and the reduction of hepatic lipid content and insulin resistance [31,42]. However, we observed no differences between wildtype and ACC DK1 mice in response to FGF21 administration over two weeks. These data indicate that FGF21 does not share a similar mechanism to metformin for the amelioration of lipid content and insulin sensitivity [31].

In conclusion, we report that FGF21 improves metabolic homeostasis in HFD-fed mice, effects that are independent of adipocyte AMPK or its downstream substrate ACC, and which were not associated with increases in BAT or WAT browning. These data suggest that combinatorial treatment of obesity and diabetes with FGF21 and AMPK activators [31,43] may have enhanced efficacy since they function through different signaling pathways. Future work is required to determine whether FGF21 mediates its effects via UCP1-independent futile cycling pathways, such as greater lipid turnover [44] or a creatine substrate cycle [45].

#### AUTHOR CONTRIBUTIONS

E.P.M., E.M.D., A.M.F., S.T., and G.R.S. designed the experiments. E.P.M., E.M.D., A.M.F., V.Z.Z., J.D.C., J.M.Y., D.M.E., and A.L. performed the experiments and testing. E.P.M., E.M.D., A.M.F., V.Z.Z., J.D.C., B.K., J.G.G., D.M.E., A.L., and S.T. provided technical expertise and analyzed data. E.P.M., E.M.D., and G.R.S. wrote the manuscript. All authors edited the manuscript and provided comments.

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#### CONFLICT OF INTEREST

The authors declare no competing financial interests.

#### APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.molmet.2017.04.001>.

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