



# Roux-en-Y gastric bypass surgery is effective in fibroblast growth factor-21 deficient mice

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## ABSTRACT

**Objective:** The mechanisms by which bariatric surgeries so effectively and lastingly reduce body weight and normalize metabolic dysfunction are not well understood. Fibroblast growth factor-21 (FGF21) is a key regulator of metabolism and is currently considered for treatment of obesity. Although elevated by acute food deprivation, it is downregulated after weight loss induced by chronic calorie restriction but not after Roux-en-Y gastric bypass surgery. Therefore, the goal of the present study was to assess the role of FGF21-signaling in the beneficial effects of Roux-en-Y gastric bypass surgery (RYGB).

**Methods:** High-fat diet-induced obese FGF21-deficient (FGF21<sup>-/-</sup>) and wildtype (WT) mice were subjected to RYGB, sham surgery, or caloric restriction to match body weight of RYGB mice. Body weight, body composition, food intake, energy expenditure, glucose tolerance, and insulin sensitivity, as well as plasma levels and hepatic mRNA expression of FGF21 were measured.

**Results:** Hepatic expression and plasma levels of FGF21 are higher after RYGB compared with similar weight loss induced by caloric restriction, suggesting that elevated FGF21 might play a role in preventing increased hunger and weight regain after RYGB. However, although the body weight differential between RYGB and sham surgery was significantly reduced in FGF21<sup>-/-</sup> mice, RYGB induced similarly sustained body weight and fat mass loss, initial reduction of food intake, increased energy expenditure, and improvements in glycemic control in FGF21<sup>-/-</sup> and WT mice.

**Conclusions:** FGF21 signaling is not a critical single factor for the beneficial metabolic effects of RYGB. This may open up the possibility to use FGF21 as adjuvant therapy in patients with ineffective bariatric surgeries.

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**Keywords** Obesity; Diabetes; Body weight; Food intake; Energy expenditure; Glucose tolerance; Insulin; Leptin

## 1. INTRODUCTION

There are currently few options for effective and lasting treatment of obesity and its associated comorbidities. Even though bariatric surgery produces substantial and sustained weight loss in a majority of patients [1], it is unable to completely reverse morbid obesity and does not prevent weight regain and diabetes relapse in a significant portion of patients [2]. Considerable advances have also recently been made with pharmacological therapies, either alone or as combination therapies targeting more than one physiological pathway. In animal studies, some of these new pharmacological agents are able to produce body weight loss that starts to rival bariatric surgeries [3–5], but long-term efficacy and safety in humans remain to be confirmed for most of these candidates [6–8].

Fibroblast growth factor 21 (FGF21) is one of these agents that has profound beneficial effects on body weight and metabolism in pre-clinical obesity models [9–15]. FGF21 is produced mainly by the liver and acts both peripherally and in the brain [16]. It has been linked to the regulation of carbohydrate and lipid metabolism in response to starvation and dietary protein restriction [17–22]. Given the semi-

starvation state early after bariatric surgery, it is plausible that FGF21-signaling is recruited to orchestrate an adaptive response resulting in a new body weight set point.

Interestingly, FGF21 serum levels and hepatic expression are increased in patients with obesity, type 2 diabetes, and nonalcoholic fatty liver disease [23–26], suggesting that the metabolic syndrome is associated with FGF21-resistance, similar to leptin-resistance. In morbidly obese females with type 2 diabetes, weight loss induced by a very low calorie diet or by gastric banding lowered FGF21 levels, but RYGB, in contrast, elevated FGF21 levels [27]. Similarly, while FGF21 levels were reduced after weight loss induced by dieting and vertical sleeve gastrectomy (VSG), they remained elevated after RYGB [26]. On the basis of these findings, it has been suggested that the relative elevation of FGF21 could contribute to the beneficial effects of RYGB [27]. FGF21 signaling could be part of the mechanism by which bariatric surgery physiologically lowers the defended level of body weight and improves glycemic control [28].

The mechanisms leading to sustained weight loss after gastric bypass surgery are not well understood. Weight loss induced by calorie restriction triggers strong counter-regulatory responses such as

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heightened hunger and lower metabolism, eventually leading to abandonment of dieting and weight regain [29]. In contrast, weight loss induced by gastric bypass surgery does not appear to trigger such responses in a majority of patients [30]. Identification of the molecular mechanisms underlying this resistance to counter-regulatory responses after gastric bypass surgery could lead to powerful new 'knife-less' approaches in the prevention and treatment of obesity. Alternatively, it is important to demonstrate which metabolically beneficial molecular mechanisms act independent of, or in parallel to, the mechanisms utilized by bariatric surgery. Such mechanisms could potentially be exploited as adjuvant therapies in bariatric surgery patients who do not respond satisfactorily to the surgery. To distinguish these two possibilities, we carried out Roux-en-Y gastric bypass surgery (RYGB) in FGF-deficient (FGF21<sup>-/-</sup>) and wildtype (WT) mice and compared the effects on body weight, body composition, food intake and choice, energy expenditure, and glycemic control.

## 2. MATERIALS AND METHODS

### 2.1. Animals and diets

Male *Fgf21*<sup>-/-</sup> and wildtype mice on a C57BL/6 background were obtained from a breeding colony at the Pennington Biomedical Research Center that was originally established by a gift from Dr. Steven Kliewer (University of Texas Southwestern, Dallas, Texas, USA) [18]. At the age of 8 weeks, all mice were provided a two-choice diet, consisting of high-fat diet (Kcal%: Carb, 20 [sucrose 6.8, maltodextrin 12.3]; Fat, 60; Prot, 20, Diet D12492, Research Diets, New Brunswick, NJ) and regular mouse chow (Kcal%: Carb, 58 [sucrose, 5.26; glucose, 0.26; fructose 0.37; lactose, 2.7; starch, 44]; Fat, 13; Prot, 28.5, # 5001, Purina LabDiet, Richmond, IN) for 10 weeks before surgery. Mice were initially housed in group shoe box cages with corn cob bedding and transferred to individual cages with wire mesh floors for the measurement of food intake. Animals were kept in climate controlled rooms at 22 ± 2 °C with a 12/12 h light/dark cycle (lights on from 0700 to 1900 h) except for the metabolic chambers, in which temperature was raised to 29 °C for 5 days.

All procedures were approved by the Institutional Animal Care and Use Committee of the Pennington Biomedical Research Center and strictly adhered to the standards of the National Institutes of Health.

### 2.2. RYGB and sham surgery

RYGB was carried out according to a protocol described in detail earlier [31]. Briefly, in a jejuno-gastric anastomosis, the cut end of the mid-jejunum was connected with a very small gastric pouch and the other end of the cut jejunum was anastomosed to the lower jejunum, resulting in a 5–6 cm long Roux limb, a 6–7 cm long biliopancreatic limb, and a 15–18 cm long common limb. Sham surgery consisted of laparotomy only, without transection of jejunum and stomach.

To match body weight of mice after RYGB surgery, access to the two-choice diet was restricted to about 50–70% of pre-surgical ad libitum intake in an additional control group. Pre-weighed amounts of food were given at 1000–1200 h during the light period.

### 2.3. Measurement of body weight, body composition, and food intake

Body weight and food intake were measured daily except for weekends. Total food intake in kcal was derived from intake of high-fat (5.24 kcal/g) and regular chow diet (3.02 kcal/g) and by taking spillage into account. Body composition was measured before surgery and every 4 weeks after surgery using a Minispec LF 90 NMR Analyzer (Bruker Corporation, The Woodlands, TX).

### 2.4. Measurement of energy expenditure, RER, and locomotor activity

8 weeks after surgery, all mice were adapted to eating food from hanging baskets with training lids on their home cages for 3 days. They were then transferred to individual metabolic chambers (Phenomaster/Labmaster, TSE Systems, Germany) for continuous monitoring of O<sub>2</sub> and CO<sub>2</sub> consumption, food and water intake, as well as locomotor activity (beam breaks). Animals were in the metabolic chambers for 4 days at 23 °C and 3 days at 29 °C.

### 2.5. Measurement of IP glucose tolerance, HOMA insulin resistance, and leptin

Six weeks after surgery, intraperitoneal glucose tolerance was tested in overnight (15–17 h) food deprived mice by administering 2 g/kg of  $\alpha$ -D-glucose (10% in sterile water, i.p.). Tail blood was analyzed by a glucometer (Onetouch Ultra Glucometer, LifeScan INC, Milpitas, CA; Onetouch Ultra Strips, LifeScan INC, Milpitas, CA). At 5 min before the injection, larger samples of 100  $\mu$ l of whole blood were collected using heparinized capillary tubes (Fisherbrand Microhematocrit Capillary Tubes, Thermo Fisher Scientific, Waltham, MA) into centrifuge tubes containing 4.5  $\mu$ l of a protease inhibitor cocktail (1.5  $\mu$ l of each of the following: Protease inhibitor, Sigma, St. Louis, MO; DDP-IV inhibitor, EMD Millipore, St. Charles, MO; Pefabloc SC, Roche, Indianapolis, IN) and immediately centrifuged at 4 °C and 3000 RPM for 10 min to separate the plasma from the whole blood. Plasma aliquots were frozen in liquid nitrogen and stored at –80 °C prior to processing. Plasma was subjected to ELISA for measurement of insulin and leptin concentrations (MMHMAG-44K Milliplex map mouse metabolic hormone magnetic bead panel – metabolism multiplex assay, EMD Millipore, St. Charles, MO).

### 2.6. Measurement of hepatic expression and plasma FGF21 levels

At the end of the experiment, mice were killed by decapitation between 09:00 and 13:00 h after 3–4 h of food deprivation. Trunk blood was collected and treated as above and plasma FGF21 was measured using ELISA (Mouse and Rat FGF-21 ELISA; BioVendor, No. RD291108200R). The liver was harvested and immediately frozen at –80 °C. Total RNA was extracted from liver using TRIzol reagent following the manufacturer's protocol (15596018, Invitrogen). RNA quality and quantity were determined by spectrophotometry using a NanoDrop (Thermo Scientific). cDNA synthesis was performed with M-MLV reverse transcriptase (M1701, Promega), and mRNA was quantified on the ABI 7900 platform using the SYBR green methodology in optical 384-well plates (Applied Biosystems). Primer pairs were designed using NCBI Primer-BLAST with at least one primer spanning an exon–exon boundary. Target gene expression was normalized with cyclophilin B as the endogenous control.

### 2.7. Statistical analysis

Body weight, body composition, food intake, chow preference, energy expenditure, locomotor activity, RER, blood glucose, and blood insulin data were all analyzed with two-way ANOVA with surgery and genotype as between-subjects variables. Bonferroni corrected multiple comparisons were used for comparisons of specific data points. Energy expenditure was additionally analyzed by ANCOVA using a tool provided by the Mouse Metabolic Phenotyping Core, Vanderbilt University (MMPC, Nashville, TN) (<https://www.mmpc.org/shared/regression.aspx>; December 2015). This tool is used to analyze changes in energy expenditure between groups while adjusting for total body mass. All data are reported as mean ± SEM.

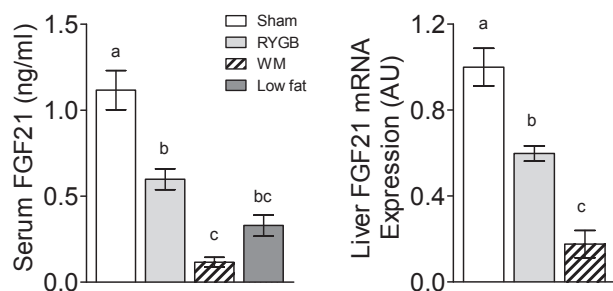
### 3. RESULTS

#### 3.1. Hepatic expression and plasma levels of FGF21 are higher after RYGB compared with similar weight loss induced by caloric restriction

Consistent with the human literature, FGF21 plasma and hepatic FGF21 gene expression levels were highest in sham-operated obese and lowest in calorie-restricted WT mice (Figure 1). Although lower than in sham mice, FGF21 levels were higher after RYGB compared with calorie-restricted mice with similar body weight (WM). FGF21 levels after RYGB were also slightly, but not significantly, higher compared with never obese, lean mice fed a low fat diet from one of our earlier studies [21].

#### 3.2. Body weight and composition are similarly affected by RYGB in FGF21<sup>-/-</sup> and WT mice

Confirming our earlier findings [31], RYGB and sham surgery were without complications and mortality. All RYGB mice were able to ingest some solid food on the day after surgery. As shown in Figure 2A and B, FGF21<sup>-/-</sup> and WT mice gained body weight similarly on the high-fat diet and responded similarly to RYGB, with a rapid weight loss phase for the first 2 weeks followed by a plateau phase with relative weight stability lasting to the end of the observation period at 11 weeks after surgery. FGF21<sup>-/-</sup> mice responded to sham surgery with a slower recovery from the initial body weight loss, resulting in slightly but significantly less weight gain at the end of the study. When adjusted for initial body weight, there was a significant effect of genotype on percent body weight change in the sham group (2-way ANOVA, main effect of genotype:  $F[1,10] = 9.7$ ,  $p < 0.05$ ). The RYGB-induced changes in body weight were largely accounted for by changes in fat mass, with relatively modest changes in lean mass (Figure 3A–C). To mechanistically better understand RYGB-induced metabolic changes, we matched non-surgical mice to the body weight after RYGB by caloric restriction (WM). Even though weight loss was the same for RYGB and WM animals, body composition was different. While the weight loss in RYGB mice was almost exclusively fat, WM mice of both genotypes lost significantly less fat mass (Figure 3A) but more lean mass (Figure 3B). Therefore, the adiposity index was significantly higher in WM mice of both genotypes as compared to RYGB (Figure 3C). The weights of inguinal, epididymal,



**Figure 1: FGF21 plasma levels and hepatic mRNA expression in obese mice and after weight loss induced by RYGB or caloric restriction.** Fasting plasma concentrations and hepatic mRNA expression of FGF21 were measured in high-fat diet-induced obese mice subjected to sham surgery (Sham,  $n = 4$ ), RYGB ( $n = 4$ ), or caloric restriction to match body weight of RYGB mice (WM,  $n = 4$ ). For comparison, fasting plasma levels of FGF21 are also shown for low-fat fed mice ( $n = 18$ ). Bars that do not share the same letters are significantly different from each other ( $p < 0.05$ , based on ANOVA, followed by Bonferroni-corrected multiple comparison tests).

and retroperitoneal fat pads, measured at the end of the observation period, were similarly affected by genotype and surgery (Figure 3D). Finally, interscapular brown fat weight was also similarly decreased by RYGB and WM in both genotypes (Figure 3E).

In summary, RYGB-induced changes in body weight and composition were very similar in FGF21<sup>-/-</sup> and WT mice, strongly suggesting that FGF21-signaling alone is not critical for the body weight-lowering effects of RYGB.

#### 3.3. RYGB reduces early food intake and feed efficiency similarly in FGF21<sup>-/-</sup> and WT mice

Body weight and composition are determined by contributions from both energy intake and expenditure, and we have previously shown in our mouse model that food intake is mainly suppressed early after surgery while increased energy expenditure likely contributes to the sustained weight loss [31]. We confirmed these dynamics in the WT mice of the present study and found no significant differences to this pattern in the FGF21<sup>-/-</sup> mouse. Total food intake of RYGB mice of both genotypes was significantly lower compared with sham-operated mice during the first 10 days after surgery (WT:  $t = 3.348$ ,  $p < 0.01$ ; KO:  $t = 4.133$ ,  $p < 0.001$ ) (Figure 4A). For the rest of the observation period (days 11–76), food intake returned to pre-surgical levels in both surgical groups and genotypes, although there was a general, non-significant trend for RYGB mice to eat slightly more food than sham-operated mice. The amount of food required for weight-matching was approximately 40% lower than intake of RYGB mice for both genotypes for the period of days 11–76 (Figure 4A and B), clearly indicating changes in energy efficiency and expenditure (see below). Finally, the strong RYGB-induced decrease in feed efficiency was similar for both genotypes (Figure 4C).

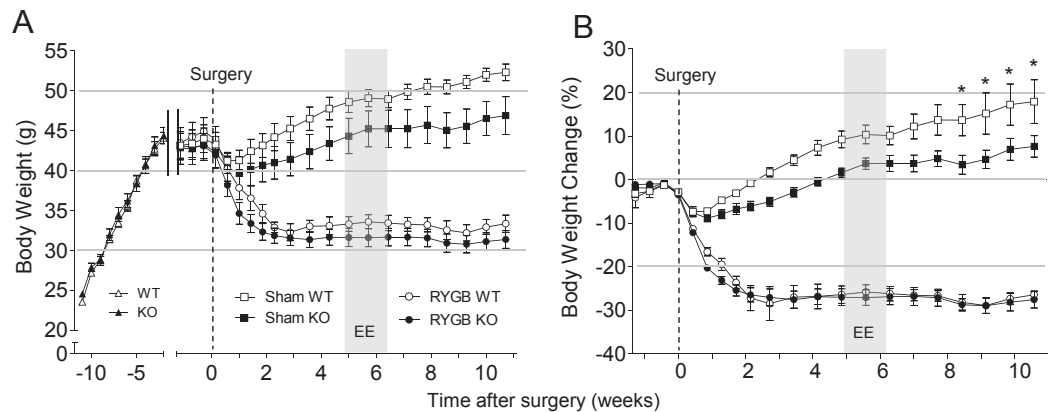
#### 3.4. FGF21<sup>-/-</sup> mice do not show RYGB-induced decrease in high-fat preference

Continuous exposure to a two-choice diet allowed an estimate of changes in food choice, since a decreased preference for energy-dense sweet and fatty foods has been demonstrated in both bariatric surgery patients and rodents [30,32,33]. Given that FGF21 treatment reduces sugar appetite [34,35], differential effects on food choice after RYGB in FGF21<sup>-/-</sup> and WT mice could have been expected. Although, preference for the high-fat diet was initially similarly decreased by RYGB in both genotypes (Figure 4D), FGF21<sup>-/-</sup> mice exhibited higher fat preference during the last 10 days of the observation period, (Figure 4E; main effect of genotype:  $F[1,23] = 34.11$ ,  $p < 0.001$ ; main effect of surgery:  $F[1,23] = 47.87$ ,  $p < 0.001$ ; interaction:  $F[1,23] = 6.414$ ,  $p < 0.05$ ), consistent with the idea that FGF21-signaling is required for RYGB-induced reduction of preference for high-fat.

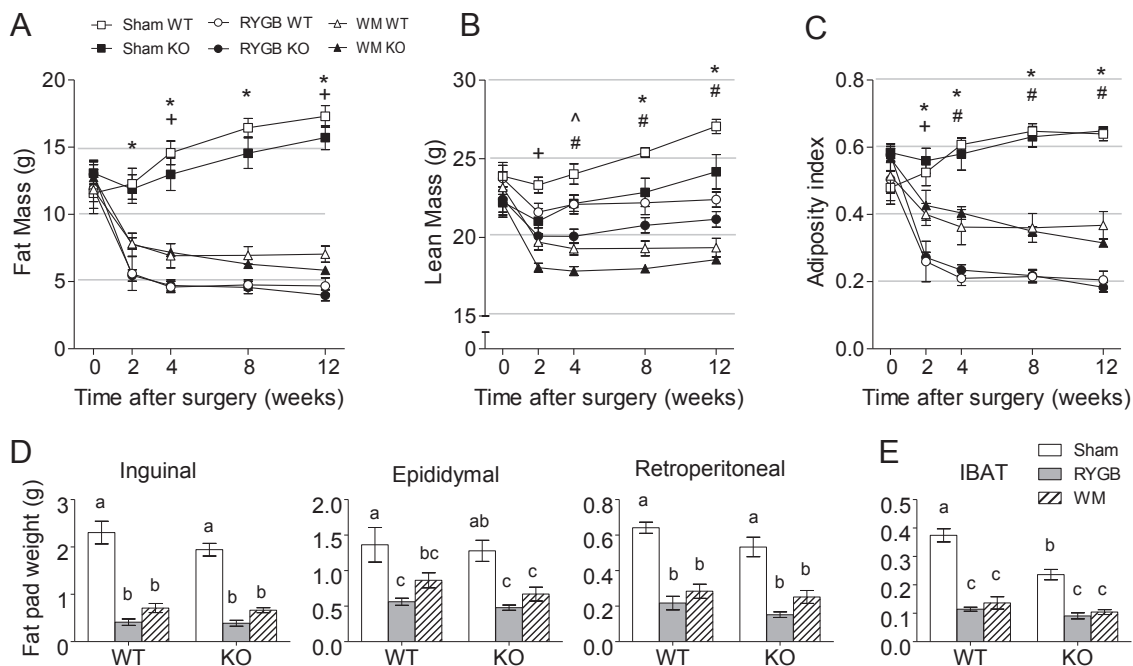
#### 3.5. Energy expenditure, but not locomotor activity, is similarly increased after RYGB in FGF21<sup>-/-</sup> and WT mice

Overall, the effects of RYGB on energy expenditure 8 weeks post-surgery were similar in FGF21<sup>-/-</sup> and WT mice (Figure 5). Because the considerable controversy regarding correct normalization of energy expenditure data and ideal environmental temperature, we expressed energy expenditure in several different ways and measured it at both room temperature and near thermoneutrality.

Total EE not adjusted for body weight was highest in sham, intermediate in RYGB, and lowest in WM mice at both temperatures (Figure 5A and D) and thus tracked body weights across the groups. Adjusting EE for total body mass using ANCOVA resulted in similarly increased EE after RYGB compared to WM in both genotypes (Figure 5A and D).



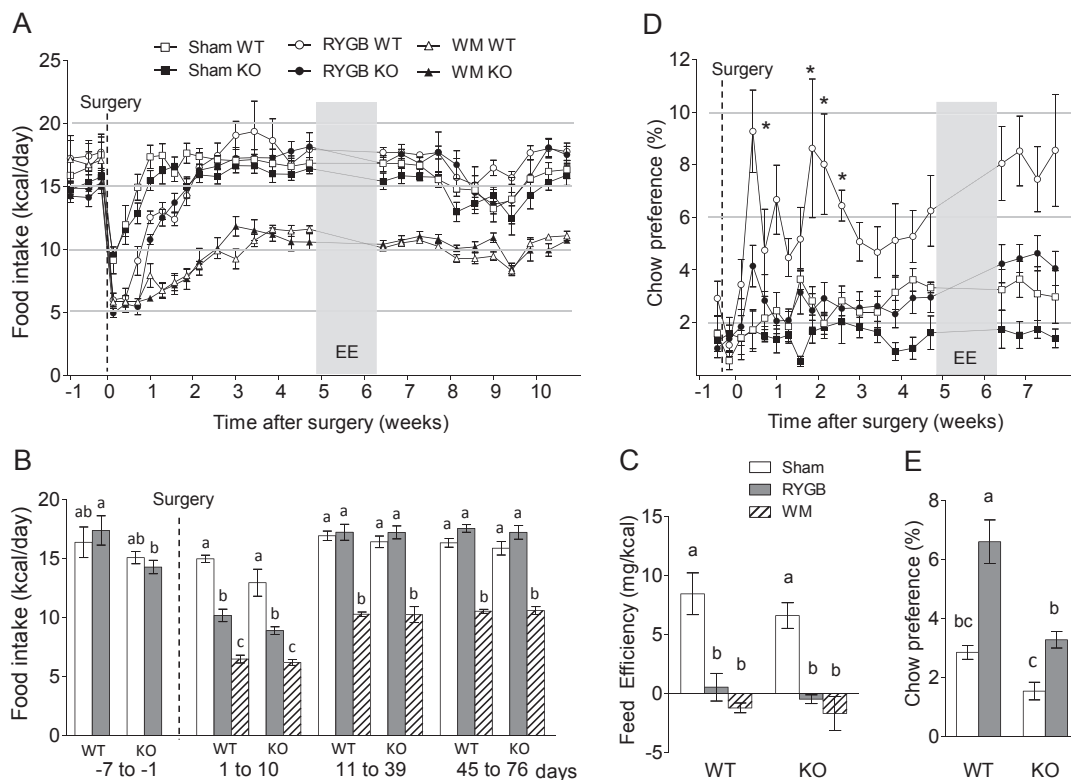
**Figure 2: The effects of RYGB on body weight are similar in  $FGF21^{-/-}$  and WT mice.** A, B: Absolute and percent change of body weight in wildtype and  $FGF21^{-/-}$  mice. WT (open triangle,  $n = 12$ ) and KO (closed triangle,  $n = 23$ ) mice were exposed to a high-fat diet for 12 weeks and then subjected to either RYGB (RYGB WT, open circle,  $n = 4$ ; RYGB KO, closed circle,  $n = 9$ ) or sham surgery (Sham WT, open square,  $n = 4$ ; Sham KO, closed square,  $n = 7$ ). The surgery effect (Sham vs. RYGB) was highly significant for both genotypes after the first post-surgical week. \* $p < 0.05$ , Sham KO vs. Sham WT mice (based on Bonferroni-corrected multiple comparisons after one-way repeated measures ANOVA).



**Figure 3: The effects of RYGB on body composition are similar in  $FGF21^{-/-}$  and WT mice.** A–C: Fat mass, lean mass, and adiposity index (fat mass/lean mass) in WT (open symbols) and  $FGF21^{-/-}$  mice (closed symbols) before and after RYGB (circles), sham surgery (squares), or weight-matching (triangles). \* $p < 0.05$ , RYGB vs. Sham, both genotypes; † $p < 0.05$ , RYGB vs. Sham, KO only; # $p < 0.05$ , RYGB vs. WM, both genotypes; + $p < 0.05$ , RYGB vs. WM, KO only (based on separate 2-way ANOVA at each time point followed by Bonferroni-corrected multiple comparison tests). Means  $\pm$  SEM,  $n = 4$ –12 mice per group. D, E: Inguinal, epididymal, retroperitoneal, and BAT fat pad weights 10 weeks after RYGB (gray bars), sham surgery (white bars), and weight-matching (shaded bars). Means  $\pm$  SEM,  $n = 4$ –12 mice per group. Bars that do not share the same letters are significantly different from each other ( $p < 0.05$ , based on ANOVA, followed by Bonferroni-corrected multiple comparison tests).

Specifically, at 23 °C, EE of both WT and KO mice was higher in RYGB compared with WM mice (WT: +11.3%,  $p < 0.05$ ; KO: +10.5%,  $p < 0.05$ ). At 29 °C, EE of both WT and KO mice was higher in RYGB compared with WM mice (WT: +33.1%,  $p < 0.005$ ; KO: +31.5%,  $p < 0.0001$ ). However, ANCOVA-adjusted EE was not significantly different in RYGB compared with Sham for both genotypes and at both temperatures (Figure 5A and D). While this RYGB-induced increase in the total energy expenditure of WT mice at 29 °C was accompanied by

higher locomotor activity (+31.5  $\pm$  19.1%,  $p < 0.05$ ), it occurred in the face of lower locomotor activity in KO mice (–24.7  $\pm$  7.3%,  $p < 0.05$ ; Figure 5C and F). This could indicate an important role for  $FGF21$  in the coupling between body weight loss and physical activity. This deficit, however, was obviously not important for the overall regulation of body weight after RYGB. The respiratory exchange ratio (RER) was significantly higher in RYGB mice of both genotypes and at both temperatures compared with WM mice (Figure 5B and E).



**Figure 4:** Effect of RYGB on food intake, feed efficiency and food choice in wildtype (WT) and  $FGF21^{-/-}$  (KO) mice. All mice were on a two choice diet consisting of high-fat and regular (low-fat) chow. **A:** Daily total food intake before and for 12 weeks after surgery for WT (open symbols) and  $FGF21^{-/-}$  mice (closed symbols) after RYGB (circles), sham surgery (squares), or weight-matching (triangles, see panel b for significant differences). **B:** Mean daily total food intake during 4 different periods before and after surgery. **C:** Feed efficiency calculated on the basis of body weight gained per kcal food ingested during the period from 15 to 76 days after surgery. **D, E:** Preference ratio for chow over high-fat diet before and after surgery (**D**) and mean preference ratio for post-surgical weeks 7–8 (**E**). \* $p < 0.05$  RYGB/WT vs both Sham/WT and RYGB/KO. Means  $\pm$  SEM,  $n = 4$ –12 mice. Bars that do not share the same letters are significantly different from each other ( $p < 0.05$ , based on ANOVA, followed by Bonferroni-corrected multiple comparison tests).

### 3.6. Improved glucose tolerance and insulin sensitivity after RYGB are largely accounted for by weight loss and are similarly affected in $FGF21^{-/-}$ and WT mice

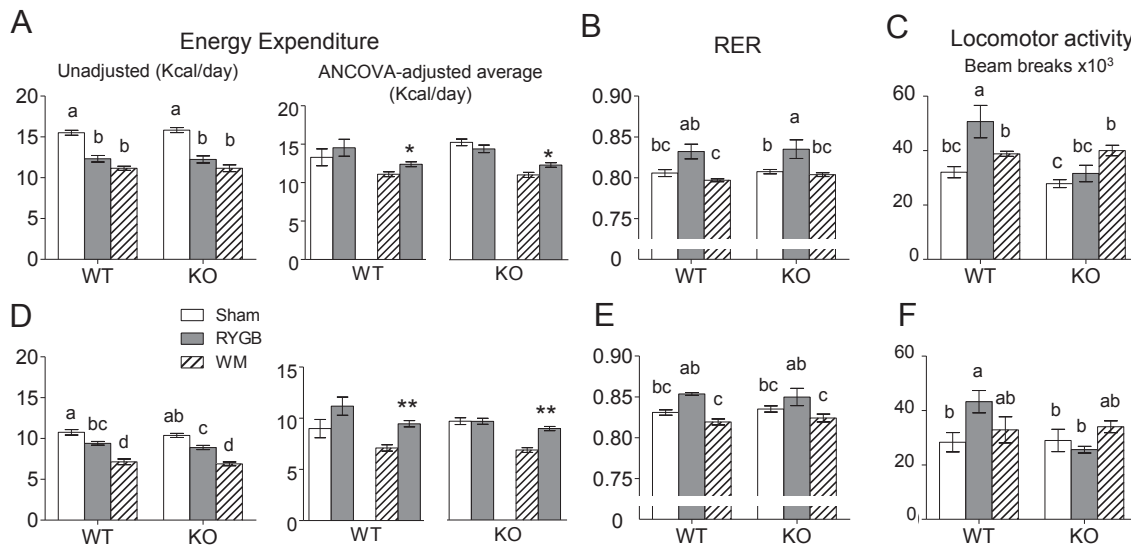
Given the controversial views regarding improvements of glycemic control after RYGB [36–39], it is important to have a weight-matched control group. At 6 weeks after surgery, fasting blood glucose was not significantly different between RYGB and sham mice of both genotypes (Figure 6A), although it was significantly reduced in WM mice. Intraperitoneal glucose tolerance curves showed similar peak values at 30 min, but recovery at 60 and 120 min was faster in RYGB compared with sham mice for both genotypes. However, the 0–120 min AUC did not show significant main effects of either surgery or genotype (Figure 6B).

There was a significant main effect of surgical condition ( $F[2,32] = 26.25$ ,  $p < 0.0001$ ) but not genotype ( $F[1,32] = 0.08$ , n.s.) on fasting insulin, with a 3-fold increase in the sham group compared to both RYGB and WM mice in both genotypes ( $p < 0.001$ ) (Figure 6C). Similarly, there was a significant main effect of surgical condition ( $F[2,32] = 33.94$ ,  $p < 0.0001$ ) but not genotype ( $F[1,32] = 0.6$ , n.s.) on HOMA-IR, with about 6-fold increases in sham vs. RYGB and sham vs. WM in WT mice, and about 3-fold increases in  $FGF21^{-/-}$  mice (all groups:  $p < 0.001$ ) (Figure 6D).

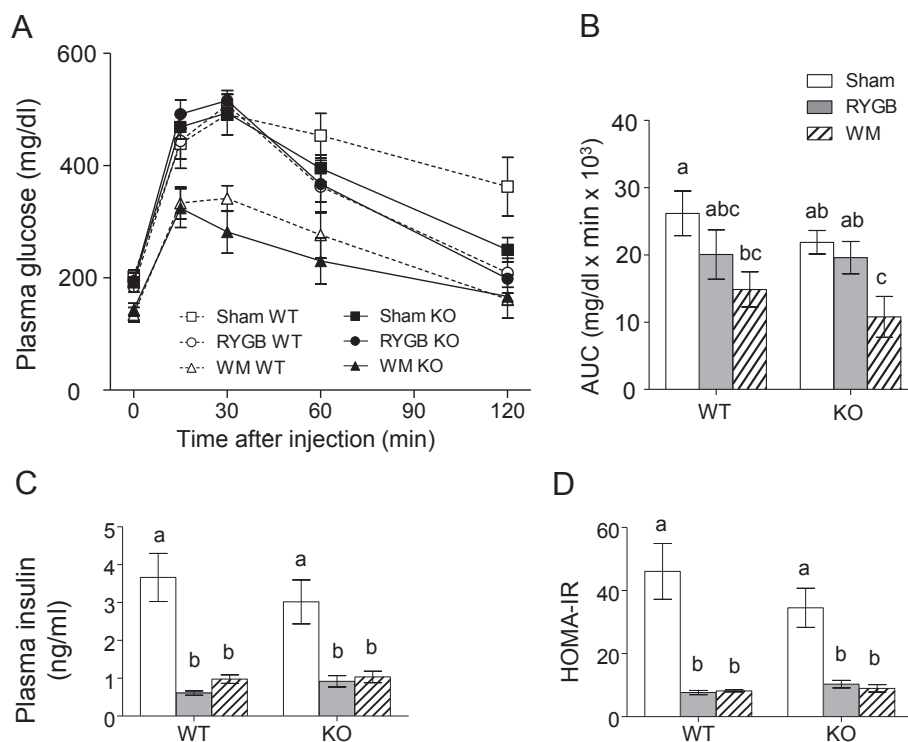
## 4. DISCUSSION

The role of  $FGF21$  in metabolic homeostasis is still incompletely understood. Clearly, acute nutrient restriction, particularly protein

restriction, increases hepatic mRNA expression and circulating levels of  $FGF21$  [17,18,21]. However,  $FGF21$  levels are high under conditions of obesity, type 2 diabetes, and nonalcoholic fatty liver disease [23–26], and, as demonstrated here, in obese mice. Given the similarities to another starvation hormone, leptin, it had been suggested that  $FGF21$  levels rise in obesity in response to relative  $FGF21$ -resistance [23]. Furthermore, the response to chronic calorie restriction induced by different manipulations indicates a special mechanism engaged selectively by RYGB. While chronic calorie restriction induced by low calorie diets, gastric banding, and VSG all decrease  $FGF21$ , RYGB increases  $FGF21$  levels [26,27]. Somewhat consistent with these findings, we show here that  $FGF21$  plasma levels and hepatic expression are higher after RYGB compared with similar weight loss induced with caloric restriction. However, in contrast to the findings in humans [27],  $FGF21$  levels were significantly lower in RYGB compared with sham-operated, obese mice. Therefore, although RYGB did not increase  $FGF21$  levels above those of sham,  $FGF21$  levels also did not fall as far as would be expected based on the dietary restriction and weight reduction. This outcome suggests that RYGB does indeed act to increase  $FGF21$ , but that this RYGB-induced increase is counterbalanced by the intervening weight loss and concomitant reduction in  $FGF21$ . This ability of RYGB to attenuate weight loss-induced reductions in  $FGF21$  is consistent with a more recent study assessing the effect of RYGB in humans [26] and also highlights the need to include weight matched controls for proper interpretation.



**Figure 5: The effects of RYGB on energy expenditure, but not locomotor activity, are similar in wildtype and  $FGF21^{-/-}$  mice.** Energy expenditure, RER, and locomotor activity as measured in metabolic chambers at 23 °C (A–C) and 29 °C (D–F) at 8–10 weeks after surgery. Means  $\pm$  SEM,  $n = 4–12$  mice per group. Bars that do not share the same letters are significantly different from each other;  $p < 0.05$ , based on ANOVA, followed by Bonferroni-corrected multiple comparison tests. Comparisons of ANCOVA-adjusted average EE were made pairwise for each genotype and temperature; \* $p < 0.05$ ; \*\* $p < 0.0001$ .



**Figure 6: Glucose tolerance, fasting plasma insulin, and insulin resistance (HOMA-IR) are similarly affected at 6 weeks after surgery in  $FGF21^{-/-}$  and WT mice.** A: Intraperitoneal glucose tolerance test in WT (open symbols) and  $FGF21^{-/-}$  mice (closed symbols) after RYGB (circles), sham surgery (squares), and weight-matching (triangles). B: Areas under the curve for plasma glucose, C: Fasting plasma insulin and D: HOMA-IR insulin resistance based on fasting plasma glucose/fasting plasma insulin. Means  $\pm$  SEM,  $n = 4–12$  mice per group. Bars that do not share the same letters are significantly different from each other ( $p < 0.05$ , based on ANOVA, followed by Bonferroni-corrected multiple comparison tests).

The mechanisms responsible for RYGB's effects on hepatic FGF21 expression and plasma levels are not clear and have not been addressed in the present study. RYGB is well known to alter nutrient absorption and gastrointestinal tract physiology, produce unique

changes in gut hormone levels [40,41], and alter hepatic metabolism, and it seems possible that any one of these effects could contribute to alterations in hepatic FGF21 expression. We also can not exclude a role for the changed diet preference after RYGB, although it seems unlikely

that the very small increase in chow preference after RYGB is a major mechanism. Considering that our understanding of the cellular mechanisms governing FGF21 expression are also limited, significant effort will be required to fully delineate the mechanisms underlying FGF21 regulation in response to RYGB.

The observation that FGF21 levels do not fall as precipitously as expected following RYGB suggests that FGF21 signaling may be involved in a mechanism that suppresses the powerful adaptive biological responses typically seen after calorie restriction-induced weight loss. Without FGF21 signaling, less weight loss after RYGB could be expected. However, contrary to these expectations, RYGB was just as effective in reducing body weight, fat mass, and food intake as well as in increasing energy expenditure (relative to calorie-restricted, weight-matched controls) in FGF21<sup>-/-</sup> compared with WT mice. There was, however, a significantly smaller difference in final body weight achieved between sham surgery and RYGB in FGF21-deficient mice, but this attenuated effect of RYGB was mainly due to reduced body weight gain in sham operated FGF21-deficient mice. Unlike in a previous report [42], susceptibility to high-fat diet-induced obesity was not increased in our FGF21<sup>-/-</sup> mice. The reasons for this difference are not clear, as both knockout strains were on C57BL6J background. However, we cannot rule out a somewhat different outcome of RYGB in mice with higher susceptibility to high-fat diet-induced obesity.

There were also some subtle differences in RYGB-induced effects on food choice and locomotor activity. Specifically, preference for chow was significantly higher after RYGB compared with sham surgery in WT mice, an observation we have made before in mice and rats [31,43]. This increased chow preference after RYGB was much attenuated and not statistically significant in FGF21<sup>-/-</sup> mice. A role for FGF21-signaling in macronutrient preference was demonstrated in two recent reports [34,35]. FGF21 administration decreases [35] and FGF21-deficiency increases [34] simple sugar preference in mice by a feedback mechanism involving  $\beta$ -Klotho-dependent FGF21 signaling in the hypothalamus. Therefore, the lower preference for chow (and higher preference for the high-fat diet) in FGF21-deficient mice observed in our study could be explained by the higher content of sweet-tasting sugars (glucose, sucrose, fructose, and polycose) in the high-fat (~19%) compared with chow (~6%).

Overall, our findings demonstrate that while FGF21-signaling is not a critical single mechanism mediating RYGB's weight-lowering effects, it may nevertheless contribute to the overall beneficial effects of RYGB. Confirming earlier reports [31,44], our mouse model identified relative hyper-metabolism as an important factor in RYGB's effect on energy balance and body weight. While FGF21 has clearly been linked to increases in EE in rodents, the results also indicate that this hyper-metabolism does not require FGF21. The WM group was instrumental for demonstrating this hyper-metabolism. First, changes in energy expenditure are strongly invoked by the fact that WM mice had to be fed significantly less energy to maintain the same body weight as RYGB mice, as previously demonstrated by Liou et al. [45]. Second, comparing EE of RYGB and WM mice at thermoneutrality in metabolic chambers revealed a substantial increase in EE (15–17%) in RYGB mice of both genotypes, enough to account for the sustained reduction in body weight.

There was an interesting genotype/surgery interaction for locomotor activity measured in the metabolic chambers. While activity was almost the same for sham and WM mice of both genotypes, activity at 8 weeks after RYGB was significantly lower in KO vs. WT mice (–41% at 29 °C and by –38% at 23 °C). In WT mice, activity after RYGB was higher compared with WM and could have accounted for much of their increased energy expenditure. In stark contrast, in FGF21<sup>-/-</sup> mice,

activity after RYGB was lower compared with WM, in the face of increased energy expenditure, suggesting that FGF21-signaling contributes to RYGB's stimulatory effect on locomotor activity and thus indirectly enhances non-activity related thermogenesis.

RYGB induced significant changes in glycemic control, most notably a marked reduction of plasma insulin levels and improved HOMA-IR. This effect was also consistent across genotypes, suggesting that the glycemic effects of RYGB also do not require FGF21. This insulin lowering effect in our RYGB model seems to be strictly secondary to weight loss, as WM mice showed the same improvement in glucose tolerance and insulin sensitivity. These results are consistent with previous work in humans indicating that much of the metabolic effect of RYGB is secondary to the hypocaloric state and early weight loss [36–39].

There are some limitations of our study. First, considering the knockout strategy used, we cannot rule out developmental adaptations potentially masking a contribution of FGF21-signaling in RYGB's beneficial effects. Future studies with tissue-specific and inducible knockout strategies will be necessary. Second, evidence for increased FGF21-signaling after RYGB, as demonstrated here and by others [27,46], is not as overwhelming as, for example, for hypersecretion of GLP-1 (see [47] for review). However, even with this vast evidence for a role of GLP-1, none of the studies using various models of GLP-1 signaling-deficiency found this to affect the outcome of different bariatric surgeries [47–49]. Thus, involvement of a particular signaling pathway does not necessarily depend on demonstrations of surgery-induced changes in that pathway. Finally, our assessment of FGF21 signaling pathway activity is incomplete, as we only measured FGF21 mRNA expression in the liver. A more comprehensive assessment of both FGF21 mRNA and the FGF21 receptor complex in the liver and other tissues such as adipose tissue will be necessary to fully understand changes in FGF21 signaling after RYGB.

## 5. CONCLUSIONS

FGF21-signaling is not a critical single factor required for RYGB to lower body weight and improve glycemic control but may play a minor role in food choice and locomotor activity. However, the findings in this study do not rule out the possibility that FGF21 acts as an important co-factor with other putative mechanisms. If FGF21 is not directly involved in RYGB's effects on energy balance, it might be useful as an adjuvant future therapy in patients with failed or sub-optimal bariatric surgery outcomes.

## AUTHOR CONTRIBUTIONS

CDM, HM, and HRB conceived the study and designed the experiments. HRB wrote the original draft of the manuscript, supervised, and administered the project. HRB and CDM provided resources and acquired funding for the project. CDM, HM, and JY reviewed and edited the manuscript. HZ and RLT conducted the research. MBM and HRB formally analyzed, curated, and visualized the data.

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## CONFLICT OF INTEREST

None of the authors declare any conflict of interest.

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