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Research Paper

FGF21 Has a Sex-Specific Role in Calorie-Restriction-Induced Beiging of White Adipose Tissue in Mice

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Calorie restriction (CR) promotes healthspan and extends the lifespan of diverse organisms, including mice, and there is intense interest in understanding the molecular mechanisms by which CR functions. Some studies have demonstrated that CR induces fibroblast growth factor 21 (FGF21), a hormone that regulates energy balance and that when overexpressed, promotes metabolic health and longevity in mice, but the role of FGF21 in the response to CR has not been fully investigated. We directly examined the role of FGF21 in the physiological and metabolic response to a CR diet by feeding *Fgf21*$^{-/-}$ and wild-type control mice either an *ad libitum* diet or a 30% CR diet for 15 weeks. Here, we find that FGF21 is largely dispensable for CR-induced improvements in body composition and energy balance, but that the lack of *Fgf21* blunts CR-induced changes in glucose regulation and insulin sensitivity in females. Surprisingly, despite not affecting CR-induced changes in energy expenditure, loss of *Fgf21* significantly blunts CR-induced beiging of white adipose tissue (WAT) in male but not female mice. Our results shed new light on the molecular mechanisms involved in the beneficial effects of a CR diet, clarify that FGF21 is largely dispensable for the metabolic effects of a CR diet, and highlight a sex-dependent role for FGF21 in the molecular adaptation of WAT to CR.

Introduction

Calorie restriction (CR), defined as a dietary regimen in which calories are reduced without malnutrition, promotes healthspan and increases lifespan in diverse organisms ranging from yeast to mice and nonhuman primates[1–4]. CR promotes metabolic health in mammals, reducing adiposity and improving blood sugar control[3,5–7]. Despite almost a century of effort, the physiological and molecular mechanisms by which CR functions are still not totally understood, stymieing efforts to develop CR mimetics which could promote health in the rapidly aging global populace.

As CR works to slow aging in all tissues, it has been suggested that CR may work in part through endocrine factors. The roles of hormones, including growth hormone, insulin, insulin-like growth factor 1 (IGF-1), and adiponectin, have been explored by numerous groups, but evidence that one of these hormones is responsible for the beneficial effects of CR remains elusive[7–9]. One endocrine factor that could play a role in CR that has not yet been fully explored is fibroblast growth factor 21 (FGF21).

FGF21 is a hormone produced by multiple tissues including the liver and white adipose tissue (WAT) in response to a diverse array of nutrient stresses, including fasting, protein restriction (PR), and restriction of specific dietary amino acids[10–13]. FGF21 promotes insulin sensitivity and regulates energy balance by promoting food consumption, activating brown adipose tissue (BAT) and stimulating the beiging of inguinal white adipose tissue (iWAT). FGF21 mediates the adaptive starvation response to induce ketogenesis, gluconeogenesis, lipolysis, and lipid β-oxidation[14–17]. In addition to mimicking many of the beneficial metabolic effects of a CR diet, overexpression of FGF21 significantly extends lifespan[18]. Intriguingly, several studies have found that FGF21 is induced by CR in mice and rats[19,20], though to different extents; we also have observed an effect of CR on blood levels of FGF21.

Here, we directly examine the role of FGF21 in the metabolic response to CR by examining the effects of CR in wild-type (WT) and *Fgf21*$^{-/-}$ (KO) mice of both sexes. We find that FGF21 is largely dispensable for the metabolic benefits of a CR diet in both males and females. Both WT and KO mice placed on CR become lean, glucose tolerant, and insulin sensitive, with a minor effect of *Fgf21* deletion on fasting blood glucose (FBG) levels in female mice. Similarly, we find that FGF21 is not required for the effects of CR on energy balance. Surprisingly,

we find that loss of *Fgf21* blunts the CR-induced reprogramming of WAT metabolism in male mice. We conclude that despite the similarity in FGF21-induced and CR-induced phenotypes, and our discovery of a role for FGF21 in the CR-induced reprogramming of WAT, FGF21 is largely dispensable for the effects of CR on metabolic health.

Materials and Methods

Animal care, housing, and diet

All procedures were performed in conformance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee of the William S. Middleton Memorial Veterans Hospital. Male and female WT and KO mice were generated by crossing CMV (cytomegalovirus)-Cre mice[21] from The Jackson Laboratory (006054) with mice expressing a floxed allele of *Fgf21*[22] from The Jackson Laboratory (022361), and then crossed with C57BL/6J mice to remove CMV-Cre. All mice were acclimated to the animal research facility for at least 1 week before entering studies. All animals were housed in static microisolator cages in a specific pathogen-free mouse facility with a 12:12-hour light–dark cycle, maintained at approximately 22°C.

At approximately 9 weeks of age, all animals were singly housed and placed on 2018 Teklad Global 18% Protein Rodent Diet for 1 week before randomization. At 10 weeks of age, mice were randomized to either an *ad libitum* (AL) diet or a 30% CR diet, in which animals were fed once per day during the beginning of the light period, at approximately 7 a.m. A stepwise reduction in food intake starting at 20% was carried out for mice in the CR group in week 1 before maintenance of a 30% restriction. The caloric intake of the mice in the AL group was calculated weekly to determine the appropriate number of calories to feed the mice in the CR groups. This feeding schedule has been widely used and extends the lifespan of mice[5,7,23].

Metabolic phenotyping

Glucose, insulin, and pyruvate tolerance tests (PTTs) were performed by fasting all mice for 7 hours or overnight (~21–22 hours) and then injecting either glucose (1 g/kg), insulin (0.5 U/kg), or pyruvate (2 g/kg) intraperitoneally[7,24]. Glucose measurements were taken using a Bayer Contour blood glucose meter and test strips. Mouse body composition was determined using an EchoMRI Body Composition Analyzer. For assay of multiple metabolic parameters (O_2, CO_2, food consumption, and activity tracking), mice were acclimatized to housing in a Columbus Instruments Oxymax/CLAMS-HC metabolic chamber system for approximately 24 hours, and data from a continuous 24-hour period were then recorded and analyzed. AL-fed animals had AL access to diet; CR groups were fed once per day at the beginning of the light cycle.

Collection of tissues for molecular and histological analysis

For analysis of plasma FGF21 levels in **Figure 1A**, mice were sacrificed at 7 a.m. prior to the regularly scheduled morning feeding time of the CR-fed animals. For all other experiments, mice were euthanized in the fed state after approximately 15 weeks. Mice euthanized in the fed state had all food removed starting at 3 p.m. the day prior to sacrifice, fed at 7 a.m. the day of sacrifice, and then euthanized 3 hours later. Following blood collection via submandibular bleeding, mice were euthanized by cervical dislocation, and most tissues were rapidly collected, weighed, and then snap frozen in liquid nitrogen. A portion of iWAT was fixed in 10% formalin for 48 hours before being transferred to 70% ethanol, sectioned, and hematoxylin and eosin stained by the University of Wisconsin Carbone Cancer Center Experimental Pathology Laboratory. Images of the iWAT were taken using an EVOS microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a magnification of 40× as previously described[13,25]. Scale bars were inserted automatically or manually by the investigator. For quantification of adipocyte size, six independent fields were obtained for each tissue from each mouse and quantified using ImageJ (NIH, Bethesda, MD, USA). For quantification of multilocular cells, a blinded participant counted the number of multilocular cells per image for each mouse using ImageJ (NIH, Bethesda, MD, USA).

Quantitative real-time Polymerase Chain Reaction (PCR)

RNA was extracted from iWAT using TRIzol Reagent according to the manufacturer's protocol (Thermo Fisher Scientific). The concentration and purity of RNA were determined by absorbance at 260/280 nm using Nanodrop (Thermo Fisher Scientific). 1 μg of RNA was used to generate cDNA (Superscript III; Invitrogen, Carlsbad, CA, USA). Oligo dT primers and primers for real-time PCR were obtained from Integrated DNA Technologies (Coralville, IA, USA); sequences are in Table S1. Reactions were run on a StepOne Plus machine (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Master Mix (Invitrogen). Actin was used to normalize the results from gene-specific reactions.

Enzyme-linked immunosorbent assay (ELISA) assays and kits

Blood plasma for insulin was taken the morning of euthanasia from fasted mice prior to refeeding as well as from the terminal submandibular bleeding. Blood plasma for FGF21 was obtained from the terminal submandibular bleeding. Plasma insulin was quantified using an ultrasensitive mouse insulin ELISA kit (90080), from Crystal Chem (Elk Grove Village, IL, USA). Blood FGF21 levels were assayed by a mouse/rat FGF21 quantikine ELISA kit (MF2100) from R&D Systems (Minneapolis, MN, USA).

Statistics

Data are presented as the mean ± standard error of the mean (SEM) unless otherwise specified. Statistical analyses were performed using one-way or two-way analysis of variance (ANOVA) followed by Sidak or Tukey–Kramer post hoc tests, as specified in the figure legends. Other statistical details are available in the figure legends. Energy expenditure differences were detected using analysis of covariance (ANCOVA). ANCOVA assumes a linear relationship between the variables and their covariates. If the slope is equal between groups, then the regression lines are parallel, and elevation is then tested to determine any differences (i.e., if slopes are statistically significantly different, elevation will not be determined). In all figures, n represents the number of biologically independent animals. Sample sizes were chosen based on our previously published experimental results with the effects of dietary interventions[7,13,25–27]. Data distribution was assumed to be normal, but this was not formally

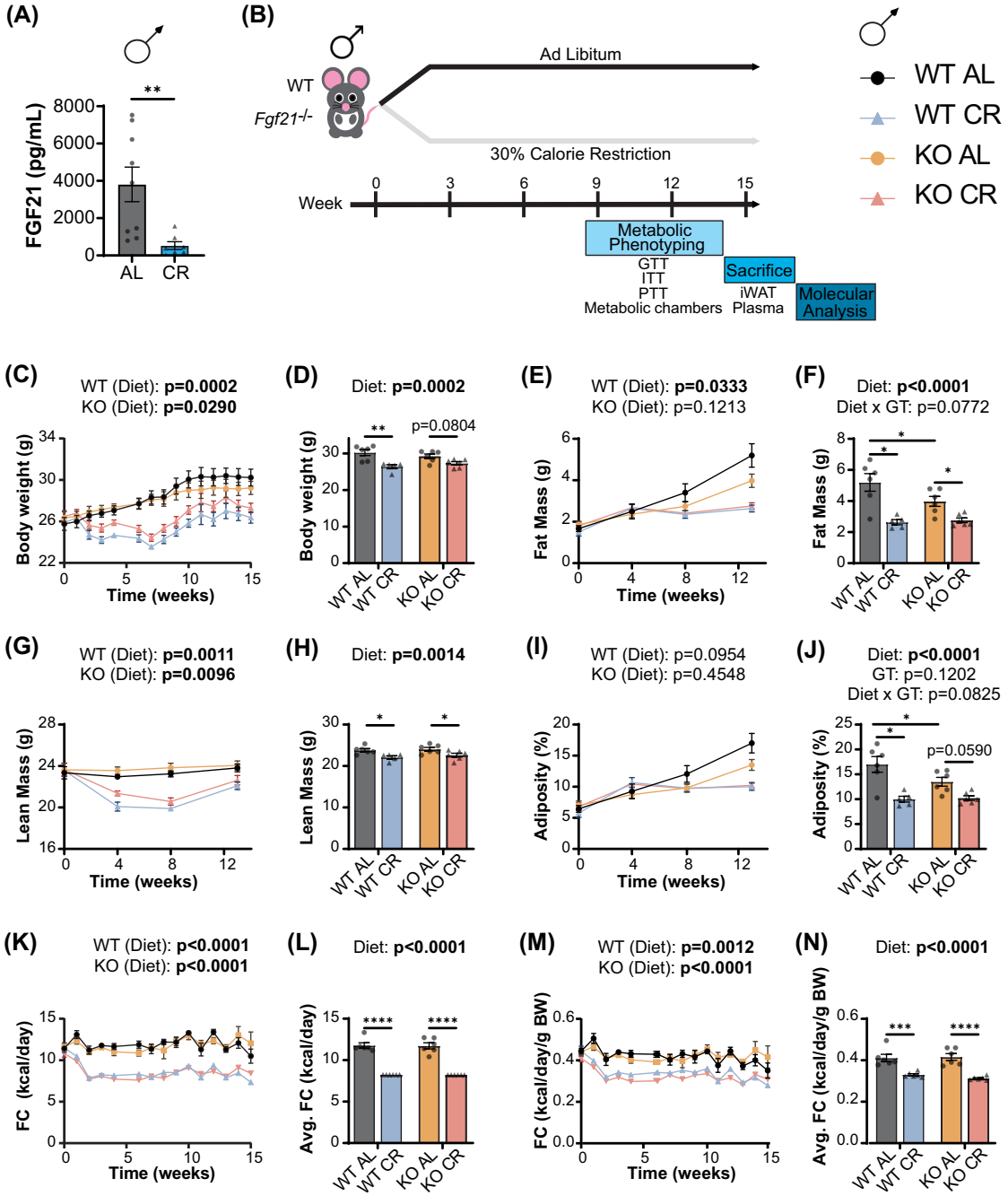


Figure 1. Loss of *Fgf21* does not impact the effect of calorie restriction (CR) on body weight, body composition, and food consumption in male mice. (A) Plasma fibroblast growth factor 21 (FGF21) levels from male mice on either an *ad libitum* (AL) or CR diet for 20 weeks (B) Experimental design. Body weight measurement of male mice on (C) the indicated diets with (D) final body weight at 15 weeks. Fat mass measurement of male mice on (E) the indicated diets with (F) final fat mass at 13 weeks. Lean mass measurement of male mice on (G) the indicated diets with (H) final lean mass at 13 weeks. Adiposity of male mice on the (I) indicated diets with (J) final adiposity at 13 weeks. Food consumption (FC; kcal/day) of male mice on (K) the indicated diets with (L) average food consumed. Food consumption per gram of body weight (kcal/day/g BW) of male mice on (M) the indicated diets with (N) average food consumed per gram of body weight. In (A), n = 9 males/group, **p = 0.0027, unpaired two-tailed t test; (C–N) n = 5–6 mice/group. For longitudinal studies, statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way repeated measures (RM) analysis of variance (ANOVA) or residual maximum-likelihood (REML) analysis conducted individually for each genotype. For analyses of weight or body composition as a single time point, or analysis of average food consumption, statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 Sidak's test post two-way ANOVA. Data represented as mean ± SEM.

tested. Phenotypic data (i.e., body weight, body composition, metabolic health) analyses for principal component analysis (PCA) plots were performed in R (v. 4.0.3) using gplots. PCA plots were produced by imputing missing data and scaling the data using the R package "missMDA" using the PCA analysis, and plots were generated using the R packages "factoextra" and "factoMine"[28,29]. For the heatmap, gene expression data were logged to the base 2 before the plot was generated[30].

Randomization

All studies were performed on animals or on tissues collected from animals. Animals of each sex and strain were randomized into groups of equivalent weight before the beginning of the *in vivo* studies.

Results

Loss of FGF21 does not affect the response of body weight and composition to a CR diet

The effect of CR on FGF21 levels, as well as the role of FGF21 in the response to CR, is unclear[19,20]. We determined FGF21 levels in the blood of both male and female AL-fed mice and mice in which calories were restricted by 30% using Envigo Global 2018 chow, a regimen which we and others have shown extends the lifespan of C57BL/6J mice[5,31]. We observed a significant increase of FGF21 level in CR-fed female mice relative to AL-fed controls, but not males, which actually had decreased levels of circulating FGF21 (**Figs. 1A**, **2A**).

We recently showed that *Fgf21* has sex-dependent roles in the response of C57BL/6J mice to PR, with *Fgf21* being vital to many of the metabolic responses to PR in male but not in female mice[32]. We therefore hypothesized that loss of *Fgf21* might have sex-dependent impacts on the response to CR, particularly with respect to metabolic health improvements. To examine this possibility, we placed WT and KO male and female mice on either AL or CR diets for 15 weeks (**Figs. 1B**, **2B**). Deletion of *Fgf21* was confirmed by PCR genotyping, ELISA, and examining *Fgf21* gene expression in the iWAT (**Fig. S1A–D**).

CR is well known to reduce weight gain and adiposity, and we therefore followed the weight and body composition of the mice during the course of the study. Both male WT and male KO mice had reduced weight gain on a CR diet, with a reduction in both lean mass and fat mass gain; the overall effect, as we expected, was one of reduced adiposity (**Fig. 1C–J**). While the reduction in weight and lean mass by CR was very similar in both WT and KO mice, there was a greater reduction of fat mass and adiposity in WT mice due to a reduced level of fat and reduced adiposity in AL-fed KO mice. In contrast, in female mice a CR diet reduced weight gain and lean mass gain in both WT and KO mice, but did not have decrease fat mass gain or adiposity in either genotype (**Fig. 2C–J**). In accordance with the similar response of both WT and KO mice, food intake was similar between WT and KO mice in both sexes (**Figs. 1K–N**, **2K–N**).

Loss of *Fgf21* has sex-specific impacts on aspects of the response of glucose homeostasis to CR

In mammals fed a CR diet, one of the most striking and broadly conserved effects is improved glucose homeostasis, which manifests in improved glucose tolerance and improved sensitivity to insulin. As administration of FGF21 promotes insulin sensitivity, we hypothesized that mice lacking *Fgf21* would have a reduced improvement in glucose homeostasis when fed a CR diet. After 8 weeks, which we and others have found is sufficient for CR to improve glucose homeostasis[5,33], we performed glucose, insulin, and PTTs. We performed glucose tolerance tests and insulin tolerance tests after both a 7- and 21-hour fasting period to better understand their postprandial glucose regulation (7 hours) and glucose regulation after a fasting period in which CR animals are accustomed to (21 hours). Although we understand that typically animals fast for 3–5 hours to prevent drastic changes in liver function, performing these tests at these times may provide better insight into the regulation of glucose homeostasis in a CR-fed mouse.

We observed that both male WT and KO mice had robust improvements in glucose tolerance when fed a CR diet, whether fasted for 7 or 21 hours (**Fig. 3A**, **B**). In agreement with other recent work from our laboratory, while CR-fed mice of both genotypes were extremely insulin sensitive following a prolonged fast, both WT and KO CR-fed mice had a postprandial increase in insulin resistance, which is suggestive of having a tighter regulation of their blood glucose levels (**Fig. 3C**). After a longer fast, CR-fed mice regardless of genotype were more insulin sensitive (**Fig. 3D**). Fasting glucose and insulin levels were similar in all groups of male mice (**Fig. 3E–H**). However, KO CR-fed males had increased FBG compared to WT CR-fed males after a 21-hour fast (**Fig. 3F**). In addition, after refeeding, KO AL-fed males had higher insulin levels than mice in any other group (**Fig. 3H**).

Investigating the mechanisms by which CR improves glucose tolerance, we found that CR-fed mice of both genotypes had significantly improved tolerance to pyruvate, indicating improved suppression of hepatic gluconeogenesis (**Fig. 3I**). However, CR-fed WT male mice were more pyruvate tolerant than CR-fed KO mice (**Fig. 3I**).

In agreement with what we observed in male mice, we observed improved glucose tolerance with CR in both WT and KO females after 7 hours of fasting (**Fig. 4A**). However, the effect of CR after 21 hours of fasting was significantly different only in WT mice; CR did not significantly improve glucose tolerance in KO females at this time point (**Fig. 4B**). Similar to males, CR-fed females of both genotypes were extremely insulin sensitive following a prolonged fast; however, unlike in males, KO CR-fed mice did not have a postprandial increase in insulin resistance (**Fig. 4C**, **D**). Fasting insulin and glucose levels in KO female mice responded to CR differently than KO male mice (**Fig. 4E–H**). KO CR-fed females had increased FBG compared to WT CR-fed females after 16 or 21 hours of fasting, whereas males only showed this effect after 21 hours of fasting (**Fig. 4E**, **F**). With regards to fasting insulin levels, female mice did not show a statistically significant effect of diet, and KO CR-fed females had significantly higher insulin levels following refeeding than their WT CR-fed counterparts (**Fig. 4G**, **H**). As with males, CR-fed females of both genotypes show improved suppression of hepatic gluconeogenesis during a PTT (**Fig. 4I**). We conclude that *Fgf21* plays a role in the regulation of glucose metabolism in CR-fed female mice, but not in male mice.

Loss of *Fgf21* does not affect CR-induced changes in components of energy balance and fuel utilization

We next observed respiration, energy expenditure, and spontaneous activity using metabolic CLAMS-HC cages. CR has been previously shown to have a very distinct respiratory exchange ratio (RER) curve and reduced energy expenditure[5]. As *Fgf21* is a critical regulator of energy balance through modulation of food intake and energy expenditure, we hypothesized that loss of *Fgf21* will affect energy balance in CR-fed mice. We, therefore, placed the mice in metabolic chambers, allowing us to determine energy expenditure via indirect calorimetry while also assessing food consumption, activity, and fuel source utilization.

We examined the contribution of diet and genotype to energy expenditure in male mice over a 24-hour period, correcting for differences in body weight (**Fig. 5A**) and lean mass (**Fig. 5B**) using ANCOVA. Male mice on CR diets had decreased energy

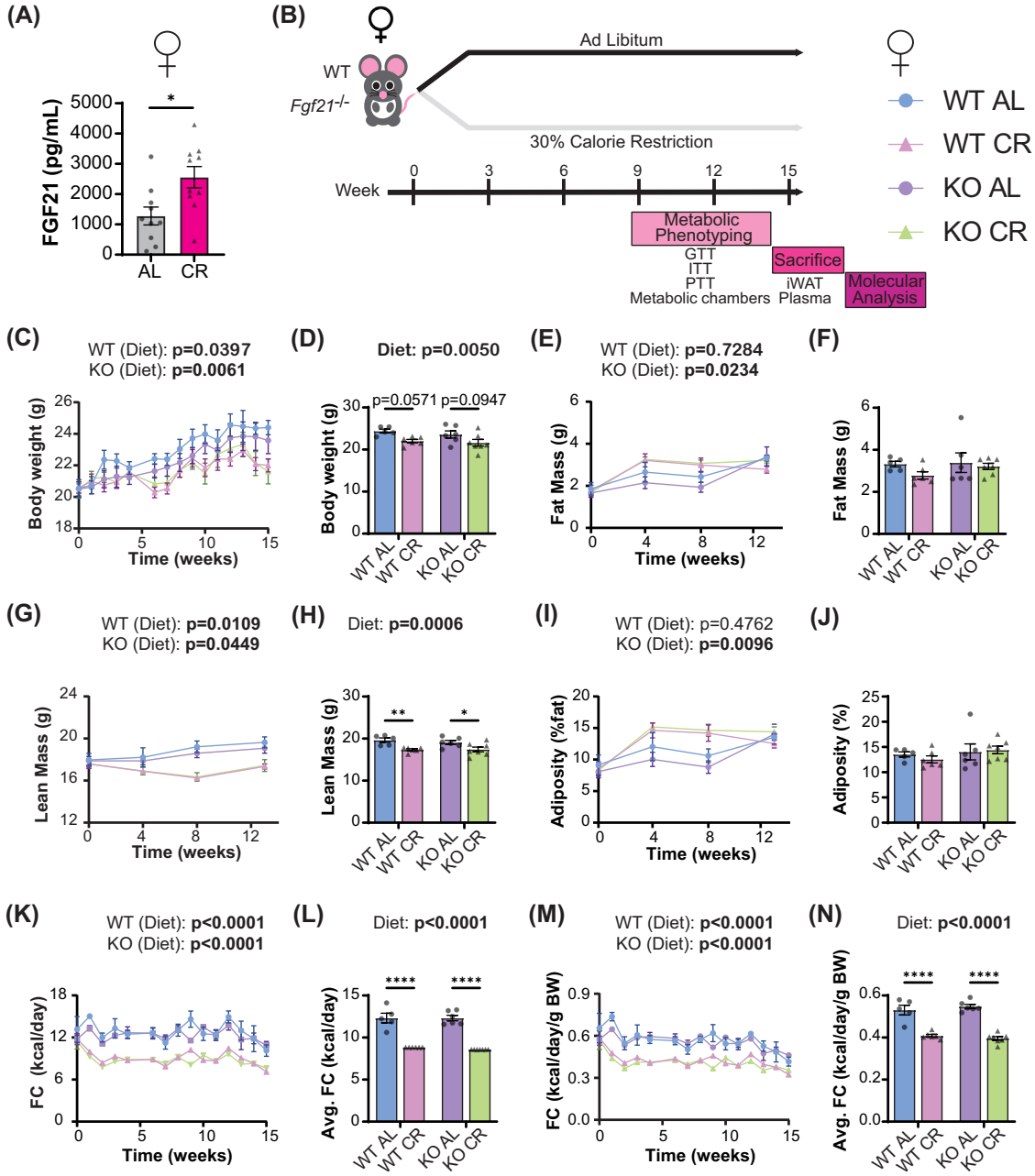


Figure 2. Loss of *Fgf21* does not impact the effect of CR on body weight, body composition, and food consumption in female mice. (A) Plasma FGF21 levels from female mice on either an AL or CR diet for 20 weeks (B) Experimental design. Body weight measurement of female mice on (C) the indicated diets with (D) final body weight at 15 weeks. Fat mass measurement of female mice on (E) the indicated diets with (F) final fat mass at 13 weeks. Lean mass measurement of female mice on (G) the indicated diets with (H) final lean mass at 13 weeks. Adiposity of female mice on (I) the indicated diets with (J) final adiposity at 13 weeks. Food consumption (FC; kcal/day) of female mice on (K) the indicated diets with (L) average food consumed. Food consumption per gram of body weight (kcal/day/g BW) of female mice on (M) the indicated diets with (N) average food consumed per gram of body weight. In (A), n = 10 females/group, *p = 0.0122, unpaired two-tailed t test; (C–N) n = 5–7 mice/group. For longitudinal studies, statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way RM ANOVA or REML analysis conducted individually for each genotype. For analyses of weight or body composition as a single time point, or analysis of average food consumption, statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA; *p < 0.05, **p < 0.01, ****p < 0.0001 Sidak's test post two-way ANOVA. Data represented as mean ± SEM.

expenditure relative to AL-fed male mice, regardless of genotype (Fig. 5A, B). CR mice undergo rapid lipogenesis following refeeding, then sustain themselves via the utilization of these stored lipids[7,34]. We determined substrate utilization by examining the RER, the ratio of O_2 consumed and CO_2 produced; a value close to 1.0 indicates that carbohydrates are primarily utilized for energy production and for active *de novo* lipogenesis, whereas a value approaching 0.7 indicates that lipids are the predominant energy source[35]. Both WT and KO CR-fed male mice displayed a distinct pattern in RER, with a rapid increase in RER indicating lipogenesis following once-per-day feeding (Fig. 5C–D). We also observed no effect of genotype on spontaneous activity, although

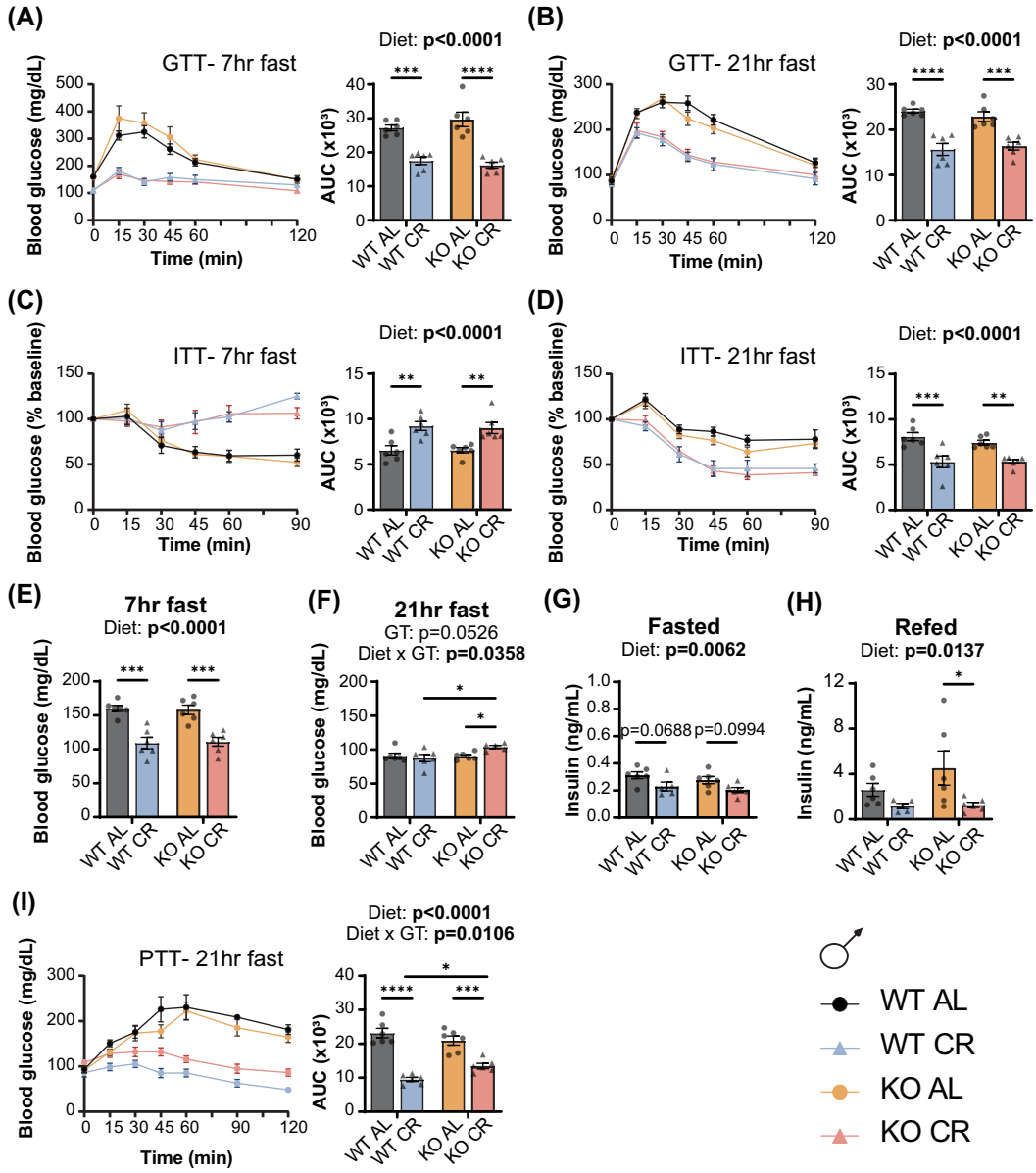


Figure 3. FGF21 is largely dispensable for the effects of CR on glucose homeostasis in male mice. A glucose tolerance test (GTT) was conducted after a (A) 7- and (B) 21-hour fast. An insulin tolerance test (ITT) was conducted after a (C) 7- and (D) 21-hour fast. Fasting blood glucose (FBG) was measured after a (E) 7- and (F) 21-hour fast prior to the start of the ITT in panels (C and D). Plasma insulin was determined (G) after a 16-hour fast and (H) after a 3-hour refeeding after 15 weeks on diet. (I) A pyruvate tolerance test (PTT) was conducted after a 21-hour fast. In panels (A–F, and I), GTTs, ITTs, and PTT were performed between 8 and 13 weeks on diet regimens. In panels (A–I), n = 5–6 mice/group; statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 from a Sidak's post test examining the effect of parameters identified as significant in the two-way ANOVA; AUC, area under the curve. Data represented as mean ± SEM.

there was an overall trend of CR-fed males toward increased activity (p = 0.06) (Fig. 5E). Female mice behaved similarly to male mice, with strong effects of diet but not genotype on energy expenditure and RER (Fig. 5F–I). While female CR-fed mice also had an overall trend toward increased activity (p = 0.0589), we observed a trend toward decreased activity of KO females (p = 0.0719) (Fig. 5J).

Loss of *Fgf21* blunts CR-induced reprogramming of WATs in males but not females

Adipose tissue plays a central role in the response to CR;[36] in mice, CR promotes the beiging of iWAT, increasing the expression of thermogenic, lipogenic, and lipolytic genes[7,37]. FGF21 plays a key role in adipose tissue, promoting energy expenditure in response to various stimuli, in part through promoting the beiging of iWAT[1,38–42]. We therefore, examined the role of FGF21 in CR-induced beiging of iWAT. We examined the expression of thermogenic, lipogenic, and lipolytic genes, increases in which are characteristic of beiging iWAT, and we examined iWAT histology.

As expected, in male CR-fed mice, expression of thermogenic genes *Ucp1*, *Cidea*, and *Elovl3* was increased (Fig. 6A–C). For all three genes, there was a diminished effect of CR in the KO mice lacking *Fgf21*. We also looked at the lipogenic genes *Dgat1*, *Fasn*, and *Acc1*. We found that a CR-diet-induced *Acc1* and *Dgat1* in

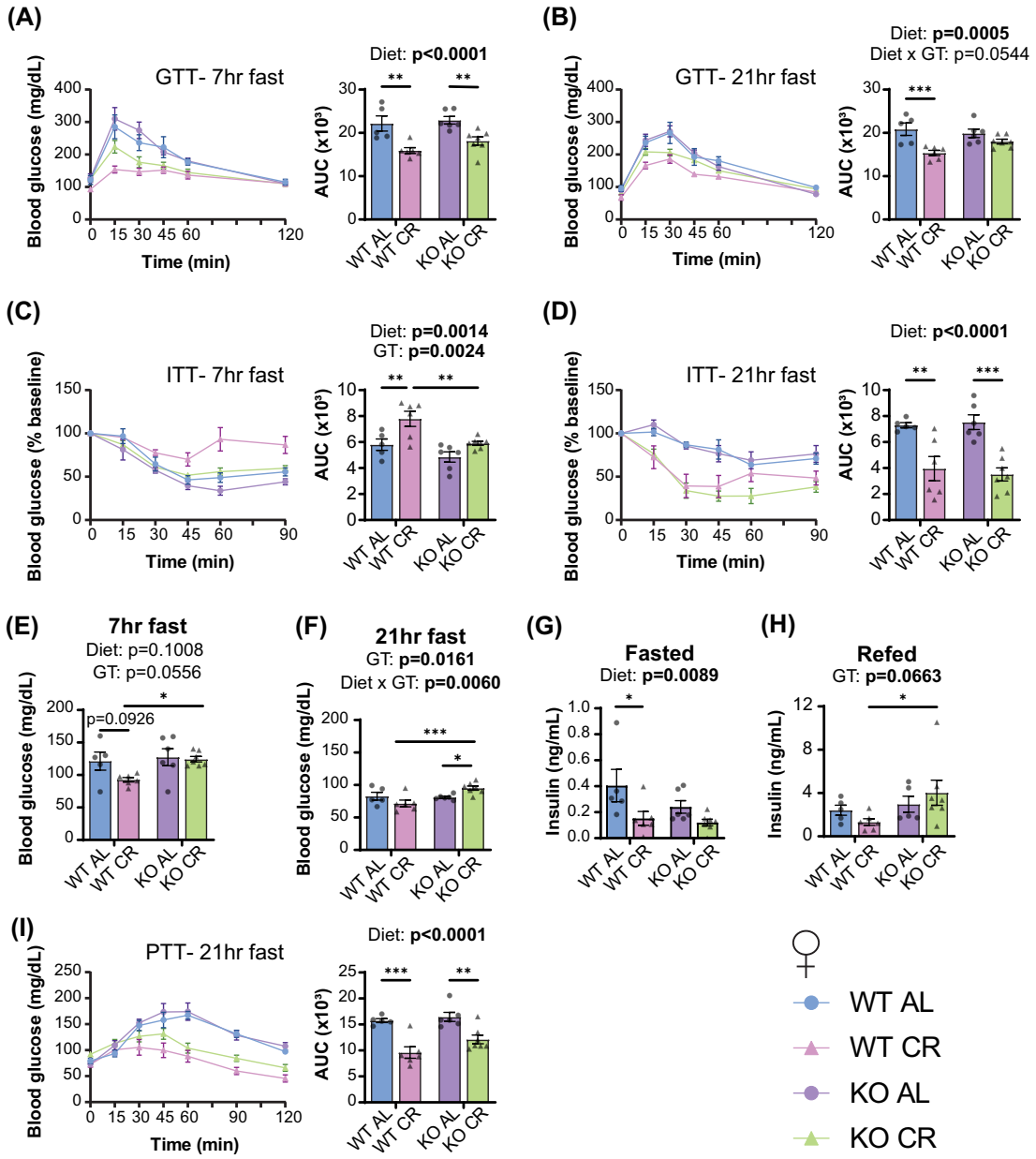
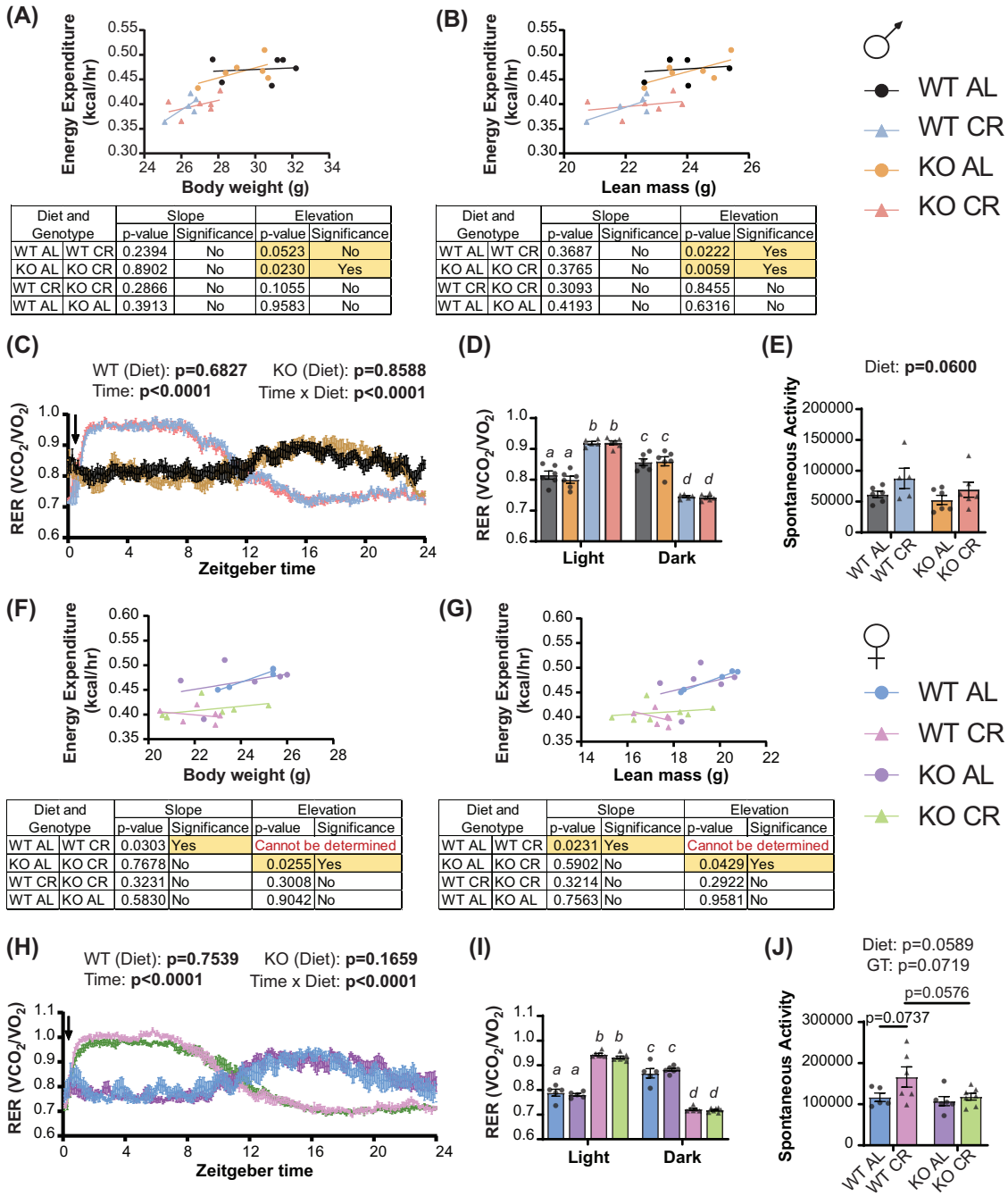


Figure 4. Loss of *Fgf21* impacts glucose homeostasis in female mice under specific feeding conditions. A GTT was conducted after a (A) 7- and (B) 21-hour fast. An ITT was conducted after a (C) 7- and (D) 21-hour fast. FBG was measured after a (E) 7- and (F) 21-hour fast prior to the start of the ITT in panels (C and D). Plasma insulin was determined (G) after a 16-hour fast and (H) after a 3-hour refeeding after 15 weeks on diet. (I) A PTT was conducted after a 21-hour fast. In panels (A–F, and I), GTTs, ITTs, and PTT were performed between 8 and 13 weeks on diet regimens. In panels (A–I), n = 5–7 mice/group; statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ from a Sidak's post test examining the effect of parameters identified as significant in the two-way ANOVA. Data represented as mean ± SEM.

both WT males and KO males; however, there was a significant effect of genotype and a significant diet × genotype interaction on the induction of *Fasn* by CR, with significantly less induction of *Fasn* in KO than in WT males (Fig. 6D–F). Finally, we examined the expression of two lipolytic genes, *Atgl* and *Lipe*. Similar to *Fasn*, we found a significant induction of both genes by CR in WT males, but a significant effect of genotype and a significant diet × genotype interaction resulting from a blunting of the induction of these genes in CR-fed KO males (Fig. 6G–H). Finally, we characterized iWAT histology. We observed a strong visual effect of CR on multilocularity in WT males, indicative of adipose tissue beiging, but a reduced effect in KO mice, which is supported by the quantification of multilocular cells per animal (Fig. 6I–K). Therefore, loss of *Fgf21* seems to slightly blunt CR-induced beiging of male mice.

In contrast, in female mice we observed a substantially decreased effect of CR on gene expression as compared to males, even in WT mice (Fig. 7A–H). There was not a significant effect of CR on the expression of either *Ucp1* or *Lipe*. There was a significant overall effect of CR on the expression of the thermogenic genes *Cidea* and *Elovl3*, but in contrast to males, a similar effect was seen in both WT and KO females



Diet and Genotype		Slope		Elevation	
		p-value	Significance	p-value	Significance
WT AL	WT CR	0.2394	No	0.0523	No
KO AL	KO CR	0.8902	No	0.0230	Yes
WT CR	KO CR	0.2866	No	0.1055	No
WT AL	KO AL	0.3913	No	0.9583	No

Diet and Genotype		Slope		Elevation	
		p-value	Significance	p-value	Significance
WT AL	WT CR	0.3687	No	0.0222	Yes
KO AL	KO CR	0.3765	No	0.0059	Yes
WT CR	KO CR	0.3093	No	0.8455	No
WT AL	KO AL	0.4193	No	0.6316	No

Diet and Genotype		Slope		Elevation	
		p-value	Significance	p-value	Significance
WT AL	WT CR	0.0303	Yes	Cannot be determined	
KO AL	KO CR	0.7678	No	0.0255	Yes
WT CR	KO CR	0.3231	No	0.3008	No
WT AL	KO AL	0.5830	No	0.9042	No

Diet and Genotype		Slope		Elevation	
		p-value	Significance	p-value	Significance
WT AL	WT CR	0.0231	Yes	Cannot be determined	
KO AL	KO CR	0.5902	No	0.0429	Yes
WT CR	KO CR	0.3214	No	0.2922	No
WT AL	KO AL	0.7563	No	0.9581	No

Figure 5. Loss of *Fgf21* does not affect heat, respiratory exchange ratio (RER), or spontaneous activity in CR-fed male and female mice. Energy expenditure of male mice as a function of (A) body weight and (B) lean mass over a 24-hour period. RER of male mice over the course of (C) a 24-hour period and (D) averaged during the light and dark cycles. Arrow indicates feeding time of CR mice. (E) Spontaneous activity of male mice. Energy expenditure of female mice as a function of (F) body weight and (G) lean mass over a 24-hour period. RER of female mice over the course of (H) a 24-hour period and (I) averaged during the light and dark cycles. Arrow indicates feeding time of CR mice. (J) Spontaneous activity of female mice. In panels (A–J), energy expenditure, RER, and spontaneous activity were determined after mice were on the dietary regimens for 13–15 weeks; n = 5–6 (males); n = 5–7 (females). In panels (A,B, and F–G), data for each individual mouse are plotted; simple linear regression (analysis of covariance) was calculated to determine if the slopes or elevations are equal; if the slopes are significantly different, differences in elevation cannot be determined. In panels (C and H), statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way RM ANOVA or REML analysis conducted individually for each genotype. In panels (D and I), statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA conducted separately for the light and dark cycles; means with the same letter are not significantly different from each other, Tukey test post ANOVA, $p < 0.05$. In panels (E and J), statistics for the overall effects of genotype, diet, and the interaction represent the p-value from a two-way ANOVA; *$p < 0.05$, Sidak's test post two-way ANOVA. Data represented as mean ± SEM.

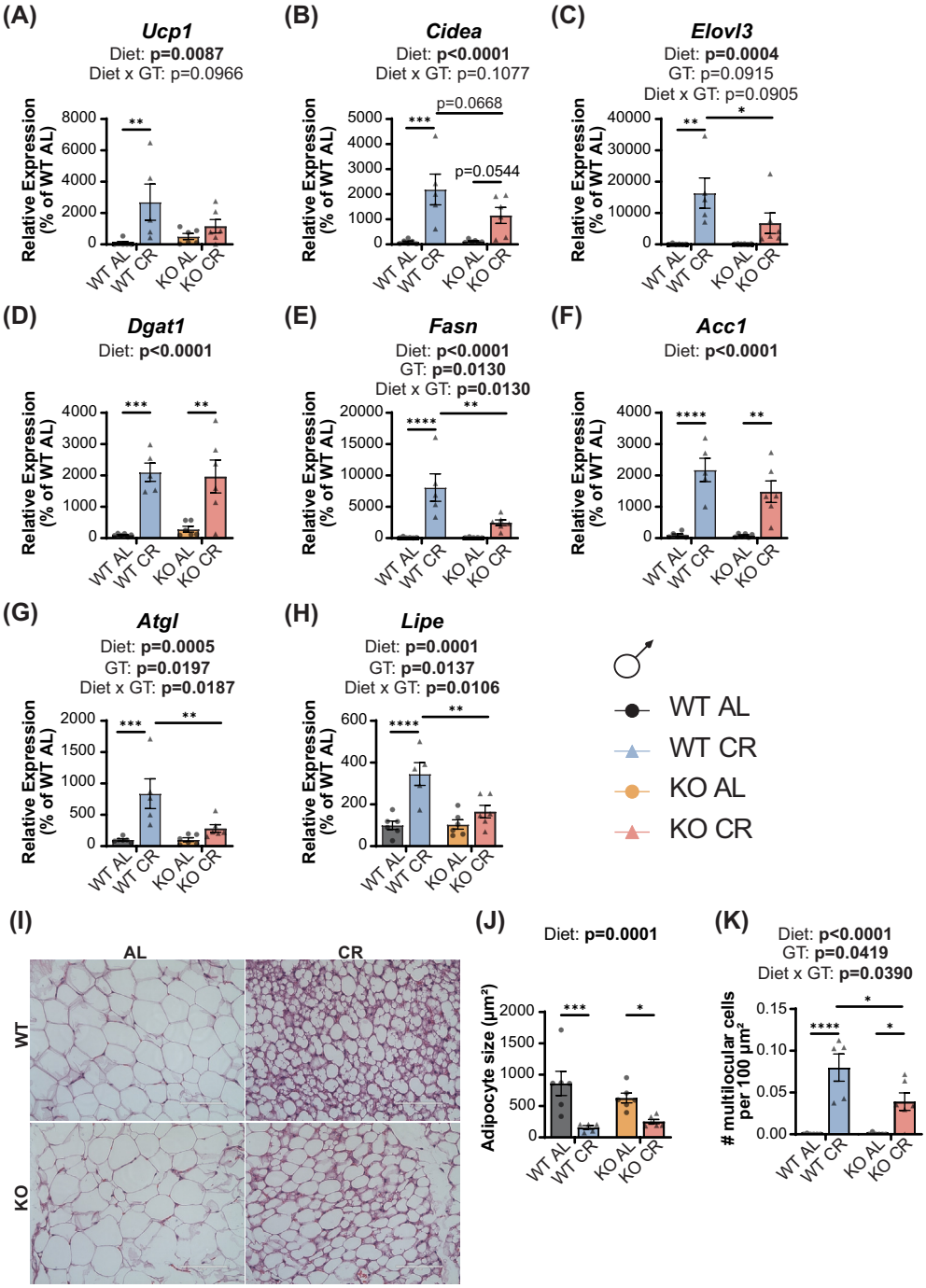


Figure 6. Loss of *Fgf21* blunts CR-induced beiging in the inguinal white adipose tissue (iWAT) of male mice. The expression of three thermogenic genes, (A) *Ucp1*, (B) *Cidea*, and (C) *Elovl3*, was quantified in the iWAT of male mice. The expression of three lipogenic genes, (D) *Dgat1*, (E) *Fasn*, and (F) *Acc1*, was quantified in the iWAT of male mice. The expression of the lipolytic genes (G) *Atgl* and (H) *Lipe* was quantified in the iWAT of male mice. (I) Hematoxylin and eosin (HE) staining (representative images; scale bar = 100 μm, 40× magnification) from iWAT of male mice. (J) Quantified adipocyte size (μm^2) from HE-stained iWAT images from male mice. (K) The number of multilocular cells per 100 μm^2 of HE-stained iWAT images of male mice. In panels (A–H, and J–K), n = 5–6 mice/group; statistics for the overall effects of genotype, diet, and the interaction represents the p-value from a two-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 from a Sidak's post test examining the effect of parameters identified as significant in the two-way ANOVA; AUC, area under the curve. Data represented as mean ± SEM.

(Fig. 7B, C). Also, in contrast to males, deletion of *Fgf21* did not impact lipogenic or lipolytic gene expression in CR-fed females (Fig. 7D–H). Histological examination of iWAT suggests that CR increases beiging equally well in both WT and KO female mice (Fig. 7I–K).

Discussion

CR improves metabolic health and longevity across diverse species[3,5,43–45]. Many of the metabolic effects of CR are at first glance similar to those induced by the energy balance hormone

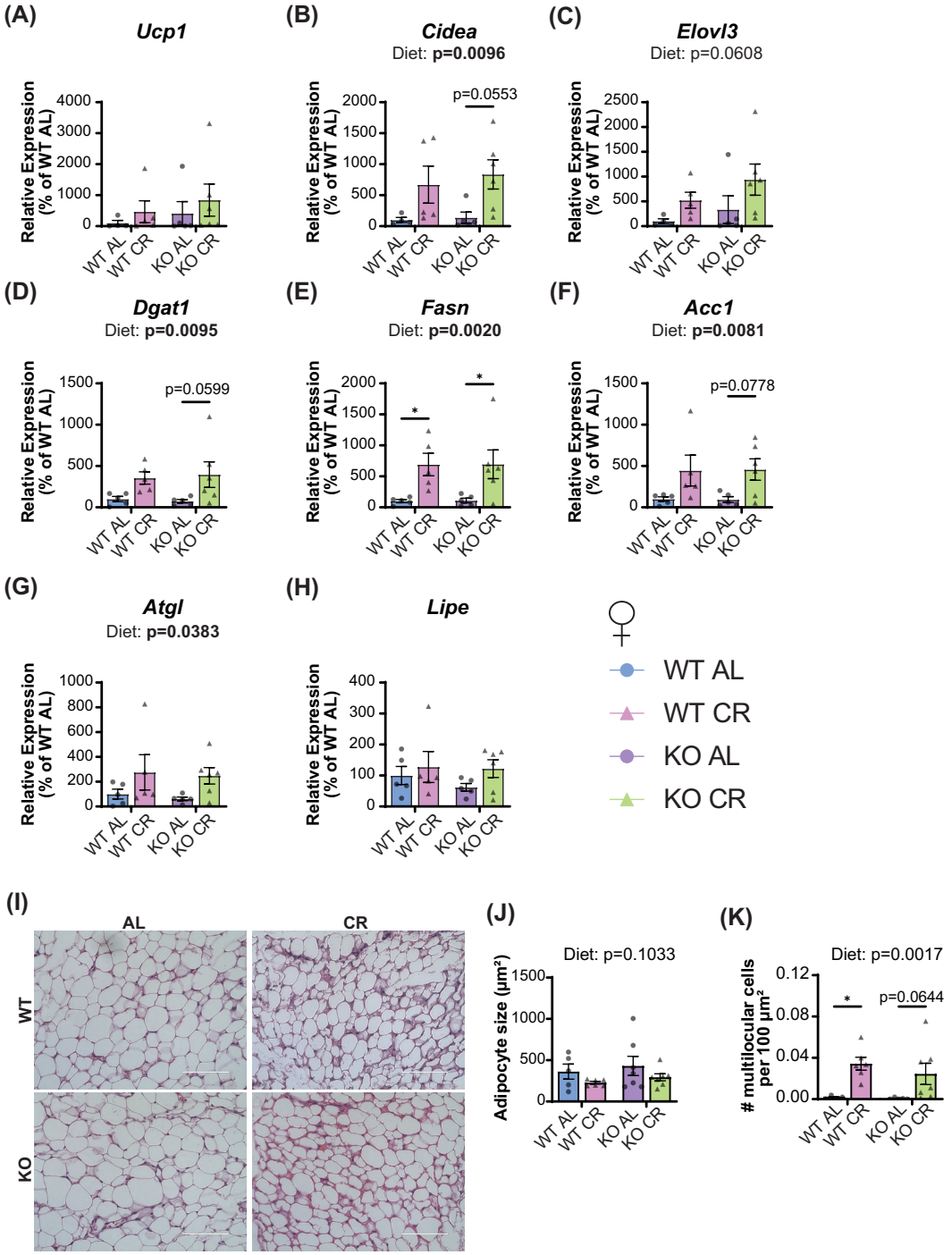


Figure 7. Loss of *Fgf21* does not impact CR-induced beiging in the iWAT of female mice. The expression of three thermogenic genes, (A) *Ucp1*, (B) *Cidea*, and (C) *Elovl3*, was quantified in the iWAT of female mice. The expression of three lipogenic genes, (D) *Dgat1*, (E) *Fasn*, and (F) *Acc1*, was quantified in the iWAT of female mice. The expression of the lipolytic genes (G) *Atgl* and (H) *Lipe* was quantified in the iWAT of female mice. (I) HE staining (representative images; scale bar = 100 μm, 40× magnification) from iWAT of female mice. (J) Quantified adipocyte size (μm^2) from HE-stained iWAT images from female mice. (K) The number of multilocular cells per 100 μm^2 of HE-stained iWAT images of female mice. In panels (A–H, and J–K), n = 4–7 mice/group; statistics for the overall effects of genotype, diet, and the interaction represent the p-value from a two-way ANOVA; *p < 0.05 from a Sidak's post test examining the effect of parameters identified as significant in the two-way ANOVA. Data represented as mean ± SEM.

FGF21. FGF21 promotes the browning/beiging of iWAT as well as activating BAT, thereby leading to increased energy expenditure and reduced body weight, several dietary interventions that increase FGF21 extend lifespan, and overexpression of FGF21 is sufficient to extend mouse lifespan[1,18,38,39,42,46]. Some work has identified a role for FGF21 in specific phenotypes of CR in male mice and rats[19,20], and we found that morning-fed CR mice display a sex-specific induction of FGF21 in their natural feeding state (Figs. 1A, 2A). While it is plausible that CR promotes metabolic health in part via FGF21, the requirement for FGF21 in the

metabolic response to CR has not been comprehensively investigated.

Here, we examined the role of FGF21 in the metabolic response to 30% CR in male and female mice fed once per day in the morning, a paradigm widely used in previous studies. We find that deletion of *Fgf21* does not substantially alter the effect of CR on body weight, composition, or food consumption in either males or females (**Fig. 8A**). However, when we examined the regulation of glucose homeostasis, we identified several roles for FGF21 in the response to CR. In males, WT mice fed a CR diet had better suppression of glucose production from pyruvate and higher FBG than CR-fed KO males. We observed a similar trend in females, with KO females also showing a blunted improvement in glucose tolerance on a CR diet as compared to WT mice when examined after a prolonged fast, and KO CR-fed females also had increased FBG at two time points. WT female mice, but not KO females, showed a postprandial increase in insulin resistance. Our results are consistent with a minor role for *Fgf21* in the suppression of hepatic glucose output during CR in both sexes, as well as literature showing that administration of FGF21 promotes hepatic insulin sensitivity[47].

Loss of *Fgf21* also does not substantially alter the effects of CR on energy balance in either male or female mice. This was rather surprising, as the role of FGF21 in these phenotypes in response to restriction of protein or dietary amino acids has been well established[10,13,48,49]. A limitation of the literature is that much of this work has been performed in C57BL/6J male mice, and we have found that many effects of PR depend on sex and strain. This includes the role of FGF21, which we have found plays an important role in the response to PR in C57BL/6J males, but not C57BL/6J females[32]. Our results are consistent with a model in which FGF21 is not essential for the effects of CR on energy balance.

In light of this conclusion, it is somewhat surprising that loss of *Fgf21* blunts the beiging of WAT of male mice placed on a CR diet (**Fig. 8B**). We find that CR-fed KO male mice do not have increased expression of the thermogenic gene *Ucp1*, typically thought of as a main driver for thermogenesis during beiging. However, the expression of two other thermogenic genes, *Cidea* and *Elovl3*, are also elevated by CR in males, although the induction of these genes by CR is blunted in the absence of FGF21. *Elovl3*, which leads to an increase in very long-chain fatty acids, is an adaptive thermogenesis marker found to be increased in BAT in response to cold exposure[50]. Futile cycling between lipogenic and lipolytic gene expression has also been shown to be a characteristic of beiging WAT[13,51]. We also found blunted expression of lipogenic and lipolytic genes in KO CR-fed male mice relative to WT CR-fed males. We also found a reduced effect of multilocular adipocytes at the histological level, suggesting that loss of *Fgf21* is required for the full induction of beiging

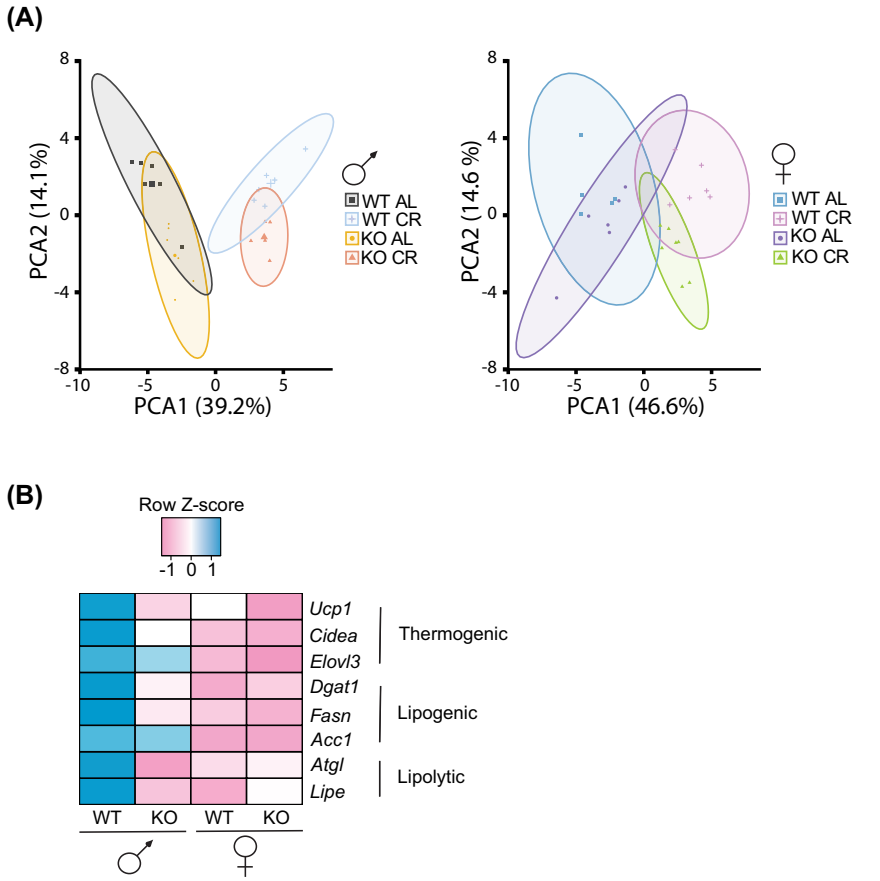


Figure 8. Loss of *Fgf21* does not affect the overall phenotypic response to CR, but blunts the induction of iWAT genes in a sex-specific manner. (A) Phenotypic measurements of individual mice visualized by genotype and diet groups and split by sex indicating separation along PC1 and PC2; 95% confidence ellipses are indicated. (B) Log_2 fold changes in gene expression induced by CR in the iWAT of each genotype and sex of mice were z-scaled normalized.

by CR in male mice. Intriguingly, these effects are sex-specific, as in CR-fed female mice, beiging is not induced to the same extent as in the males, loss of *Fgf21* does not impact thermogenic, lipogenic, or lipolytic gene induction in response to CR in females, and KO female mice on a CR diet have histologically similar iWAT to WT CR-fed female mice. We speculate that these sex differences may be due to the CR regimen itself, such that females may need a different CR regimen to experience the same metabolic benefit as males, as indicated by previous research showing that different sexes maximally benefited from different degrees of CR[31].

Limitations of this study include that our study lasted less than 4 months; there may be more prominent differences in loss of *Fgf21* under a longer CR duration that we did not see in this short time frame, and we did not examine the contribution of FGF21 to the effects of CR on frailty, cognition, or lifespan. We examined the role of FGF21 on the response of iWAT to CR, but we did not examine the molecular response of other adipose depots or other tissues. We collected tissues for molecular examination in the refed state, but due to the distinct eating patterns of CR-fed mice, examining tissues in other feeding states might prove insightful. We fed CR mice in the morning shortly after lights on; while this intervention extends lifespan[5,7], recently it has been suggested that there may be distinct effects of feeding mice in the morning or evening[52], and examining CR mice fed in the evening might find different results. Finally, Green and colleagues showed that only C57BL/6J males had a robust induction of FGF21 in response to PR, whereas females had a reduced response ($p = 0.056$), and FGF21 did not increase in response to PR in either sex of DBA/2J or UM-HET3 mice[32]. Examining the response to CR in multiple strains might reveal more details about the role of FGF21 as well as FGF21-independent mechanisms in the metabolic response to CR.

In summary, here we have tested the hypothesis that the nutrient-responsive hormone FGF21 is a key mediator of the CR-induced improvements in body composition, glucose homeostasis, and energy balance. We have determined that deletion of *Fgf21* from the whole body does not impair the ability of CR to improve body composition or energy balance and has relatively minor effects on CR-induced improvements in glucose regulation. In agreement with the literature linking FGF21 to the beiging of iWAT, we find that FGF21 plays a key role in CR-induced beiging of iWAT, but only in males. Here, we disprove the hypothesis that FGF21 mediates the metabolic benefits of CR, bringing us one step closer to identifying the true molecular mediators of the beneficial effects of CR. Notably, we have not excluded a role for FGF21 in the effects of CR on frailty or lifespan. In addition, we have not excluded the role of factors upstream of FGF21, including the general control nonderepressible 2 kinase, activating transcription factor 4, nor factors downstream of FGF21, such as IGF-1 and mechanistic target of rapamycin, in the response to CR.

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Author Contributions

M.F.C., H.H.P., and D.W.L. conceived of and designed the experiments. M.F.C., I.A., C.Y.Y., R.B., A.M.B., I.K., and M.M.S. performed the experiments. M.F.C., I.K., C.L.G., and D.W.L. analyzed the data and wrote this article.

Declaration of Interests

D.W.L. has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mechanistic target of rapamycin (mTOR) inhibitors for the treatment of various diseases. The remaining authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplemental Information

Supplemental information can be found online at https://doi.org/10.59368/agingbio.20230002.

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