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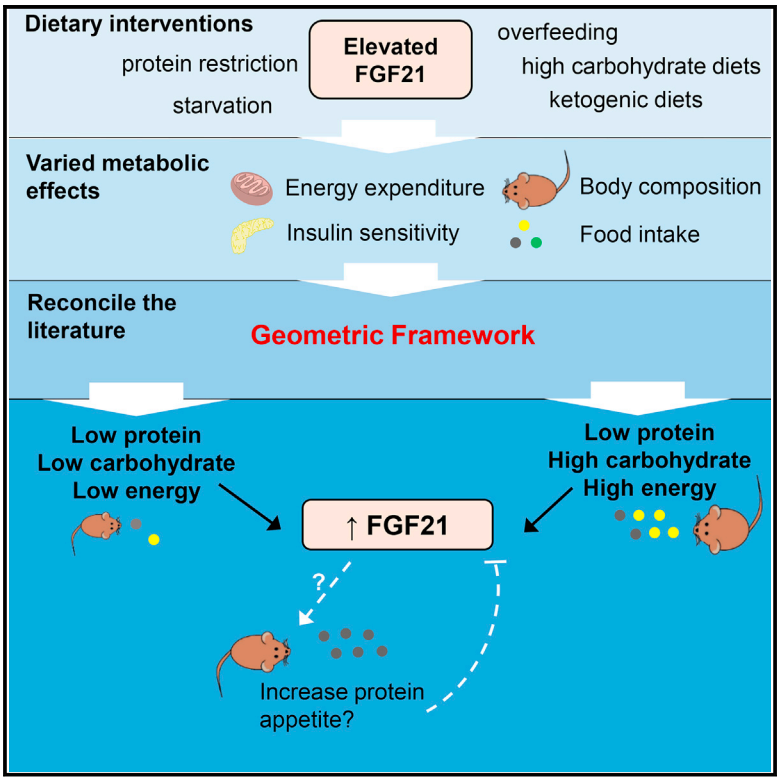
Cell Metabolism

Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework

Graphical Abstract



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In Brief

FGF21 is elevated in seemingly opposite nutrient contexts such as starvation, overfeeding, protein restriction, and ketogenic and high-carbohydrate diets. Using the Geometric Framework and 25 different types of diets, Solon-Biet et al. reconcile these findings and demonstrate that maximal FGF21 elevation occurs on low protein, high carbohydrate intakes.

Highlights

- FGF21 is maximally elevated under low protein, high carbohydrate intakes
- The Geometric Framework reconciles conflicting findings on FGF21 elevation
- Metabolic effects of FGF21 are dependent on nutrient context

Accession Numbers

GSE85998

Solon-Biet et al., 2016, Cell Metabolism *24*, 1–11
October 11, 2016
http://dx.doi.org/10.1016/j.cmet.2016.09.001

CellPress

Please cite this article in press as: Solon-Biet et al., Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework, Cell Metabolism (2016), http://dx.doi.org/10.1016/j.cmet.2016.09.001

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Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework

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http://dx.doi.org/10.1016/j.cmet.2016.09.001

SUMMARY

Fibroblast growth factor 21 (FGF21) is the first known endocrine signal activated by protein restriction. Although FGF21 is robustly elevated in low-protein environments, increased FGF21 is also seen in various other contexts such as fasting, overfeeding, ketogenic diets, and high-carbohydrate diets, leaving its nutritional context and physiological role unresolved and controversial. Here, we use the Geometric Framework, a nutritional modeling platform, to help reconcile these apparently conflicting findings in mice confined to one of 25 diets that varied in protein, carbohydrate, and fat content. We show that FGF21 was elevated under low protein intakes and maximally when low protein was coupled with high carbohydrate intakes. Our results explain how elevation of FGF21 occurs both under starvation and hyperphagia, and show that the metabolic outcomes associated with elevated FGF21 depend on the nutritional context, differing according to whether the animal is in a state of under- or overfeeding.

INTRODUCTION

Many organisms from slime molds to mammals have a powerful appetite for protein and a specific capacity to regulate its intake (Dussutour et al., 2010; Mayntz et al., 2005, 2009; Raubenheimer et al., 2015; Simpson and Raubenheimer, 2005, 2012; Sørensen et al., 2008). In mammals, behavioral mechanisms to achieve a specific protein "intake target" result in compensatory feeding on diets low in protein and high in carbohydrates or fat, with important implications for excess energy intake, obesity, and metabolic dysfunction (Gosby et al., 2014, 2016; Raubenheimer et al., 2015; Simpson and Raubenheimer, 2005; Sørensen et al., 2008). Recently, the ratio of dietary protein to the other macronutrients has been shown to influence immunity, growth, reproduction, metabolism, and aging (Le Couteur et al., 2015; Lee et al., 2008; Ponton et al., 2011; Solon-Biet et al., 2014, 2015a; Sørensen et al., 2008). However, despite its wide-ranging effects, the mechanisms that control protein intake are still unclear (Fromentin et al., 2012; Morrison and Laeger, 2015; Morrison et al., 2012).

Recent evidence has identified fibroblast growth factor 21 (FGF21) as the first known endocrine signal activated by protein restriction (De Sousa-Coelho et al., 2012; Laeger et al., 2014; Morrison and Laeger, 2015). Although circulating FGF21 is primarily derived from the liver, it is also expressed in several key metabolic tissues, including the gut, brain, adipose tissue, muscle, and pancreas (Morrison and Laeger, 2015; Potthoff and Finck, 2014), and regulates several critical metabolic functions, such as gluconeogenesis, mitochondrial function, ketogenesis, and lipid metabolism, via actions in the brain (Chau et al., 2010). The fact that FGF21 knockout mice do not increase food intake or show changes in energy expenditure similar to wild-type mice when challenged with low-protein diets identifies FGF21 as a key regulator that coordinates the metabolic response to protein deprivation (Laeger et al., 2014). Increases in FGF21 occur with protein restriction independent of total energy intake in mice and humans. Putative mechanisms include amino acid sensors such as general control non-derepressible 2 (GCN2) and, potentially, signals arising from protein catabolism that promote *FGF21* expression and increase circulating levels (De Sousa-Coelho et al., 2012; Laeger et al., 2014, 2016). Similar effects have been seen in response to dietary methionine restriction (Lees et al., 2014; Stone et al., 2014), suggesting that

deficiencies of specific amino acids may underlie the effect of protein deprivation on FGF21.

FGF21 interacts with other key nutrient-sensing pathways such as 5′ AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) (Chau et al., 2010), suggesting a key role for FGF21 in linking nutrition, health, and aging. Increased expression and/or activation of these pathways has been shown to improve metabolic profiles and lifespan in various organisms (Cantó and Auwerx, 2011; McCarty, 2004; Mercken et al., 2014). AMPK and SIRT1 interact closely with other nutrient sensors such as the amino acid-sensitive mechanistic target of rapamycin (mTOR) and insulin/insulin-like growth factor 1 (insulin/IGF-1) signaling pathways. We recently showed that circulating branched-chain amino acid levels in mice (BCAA; isoleucine, leucine, and valine) were strongly positively associated with increased protein intake and hepatic mTOR activation (Solon-Biet et al., 2014), an adverse outcome for age-related metabolic health (Laplante and Sabatini, 2012; Solon-Biet et al., 2015b). Together, these pathways share many downstream targets that regulate critical cell processes including mitochondrial function, cellular metabolism, autophagy, and protein synthesis. These findings indicate that FGF21 may be a key regulator linking nutrient sensing to metabolic and health outcomes.

Although FGF21 is robustly elevated in protein/amino acid restriction, its physiological role remains uncertain (Laeger et al., 2014; Maida et al., 2016). FGF21 is increased in the starved condition and on exposure to low-protein ketogenic diets (Fazeli et al., 2015; Kharitonenkov and Larsen, 2011; Markan et al., 2014; Potthoff and Finck, 2014). Based on these findings, one would assume that overfeeding would reduce FGF21, but this is not the case. In fact, FGF21 is elevated in overfeeding, obesity, and insulin resistance (Kharitonenkov and Larsen, 2011; Markan et al., 2014). FGF21 resistance is unlikely to underlie this effect as injection of FGF21 to obese and insulin-resistant rodents and humans enhances insulin sensitivity, suppresses gluconeogenesis (Xu et al., 2009), and promotes rapid weight loss by increasing energy expenditure (Emanuelli et al., 2014; Sarruf et al., 2010), while having no effect when given to lean, healthy mice.

The first step in resolving the FGF21 paradox will be to determine how the interaction of specific macronutrients and energy influence FGF21 expression and secretion and relate these back to metabolic outcomes. Experimental dietary designs employed to date have not permitted analysis of this type and have resulted in conflicting interpretations of the roles of FGF21 in the context of low energy intakes (Inagaki et al., 2007; Markan et al., 2014; Potthoff et al., 2009), high energy intakes (Dushay et al., 2010; Zhang et al., 2008), low protein intakes (De Sousa-Coelho et al., 2012; Laeger et al., 2014; Lees et al., 2014; Stone et al., 2014), and high carbohydrate intakes (Dushay et al., 2014; Iizuka et al., 2009; Sánchez et al., 2009; Uebanso et al., 2011). Here, we use the Geometric Framework (GF) to distinguish between the role of macronutrients and energy intake on FGF21 expression and circulation. The GF is a multidimensional framework that has successfully modeled and quantified simultaneous impacts of macronutrients and energy on food intake, body composition, lifespan, reproductive function, cardiometabolic health, immune status, mitochondrial function, and nutrient signaling pathways (Le Couteur et al., 2015; Simpson and Raubenheimer, 2012; Solon-Biet et al., 2014, 2015c). We use the GF to show that FGF21 responds both to low protein intake and, most strongly, to a combination of low protein coupled with high carbohydrate. Hence, elevation of FGF21 can occur under conditions of starvation or hyperphagia. We propose that its different metabolic outcomes depend on the nutritional context, differing according to whether the animal is in a state of under- or overfeeding.

RESULTS

We used the GF to investigate the effect of macronutrient balance on FGF21 and its relationship with metabolic health in 15-month-old mice. Data shown are derived from a previously reported study on the effects of macronutrients and energy on late-life cardiometabolic health and lifespan (Solon-Biet et al., 2014). A total of 858 mice were chronically fed one of 25 diets varying in protein, carbohydrate, fat, and total energy density from weaning. Energy density was manipulated by the addition of cellulose, yielding three energy groups: 8, 13, and 17 kJ/g (low, medium, and high energy). A subset of mice was culled at 15 months and various metabolic measures quantified (Solon-Biet et al., 2014). Studies were also conducted on HepG2 cells to investigate whether findings in vivo could be recapitulated in vitro. Data were modeled as response surfaces, fitted using thin-plate spline procedures in R and analyzed using generalized additive modeling (GAM), as previously described in detail in Solon-Biet et al. (2014). Here, response surfaces are presented as 3D and 2D heatmaps and show different phenotypic outcomes. For each response surface, areas in red indicate highest values for a given response, and decrease in elevation to areas in dark blue, which indicate the lowest values. Complete statistical analyses are provided for each surface in the Supplemental Information, available online.

FGF21 Expression and Circulation Were Elevated with Low Protein Intakes and Maximal When Low Protein Was Coupled with High Carbohydrate Intakes

Using data from chronically fed 15-month-old mice, we first plotted hepatic *FGF21* mRNA expression and circulating FGF21 levels as response surfaces mapped onto arrays of macronutrient and energy intakes. Response surfaces were plotted as two-nutrient slices from the full three-nutrient surface, sliced at the median of the third nutrient dimension (shown in parentheses). Reduced protein intake robustly increased FGF21. Mice consuming less than 5 kJ/day of protein had consistently higher hepatic *FGF21* mRNA expression (Figure 1A; $p < 0.001$; Table S4) and circulating FGF21 levels (Figure 1B; $p < 0.001$; Table S4). Importantly, these surfaces show that FGF21 was not driven by energy intake alone. Although protein had overwhelmingly the strongest effect, with no other term in the three-nutrient GAM reaching statistical significance (Table S4), it was apparent from the response surface visualization that the highest levels of circulating FGF21 were associated with a combination of low protein and high carbohydrate intakes (Figure 1B). When individual macronutrient intakes were plotted, plasma FGF21 levels correlated negatively with protein intake ($R = -0.580$; $p < 0.01$) but were positively correlated with carbohydrate intake ($R = 0.488$; $p < 0.001$), with no detectable effect of fat intake or total energy intake ($R = 0.082$, $p = 0.402$ and $R = 0.177$, $p = 0.068$, respectively) (Figures 1C–1F; Table S5). These data suggest a role for the

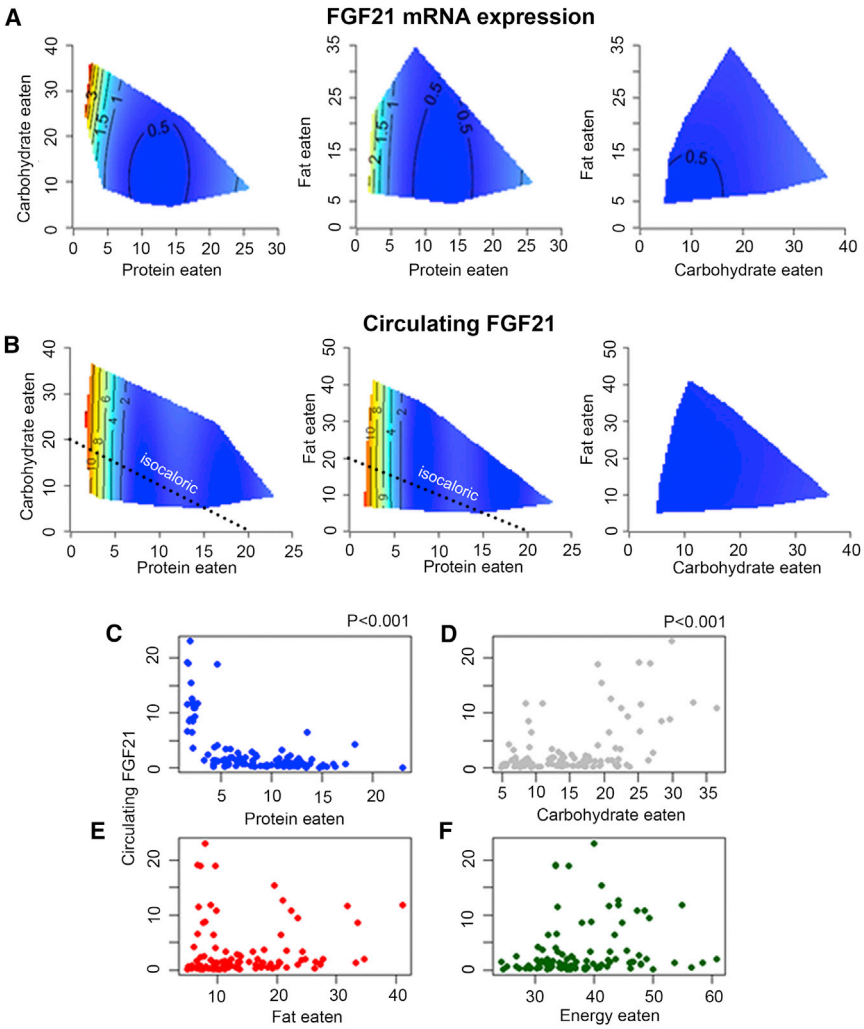

Figure 1. FGF21 Is a Signal of Protein Deprivation, Not Energy Deprivation

(A and B) Three-dimensional response surfaces showing macronutrient intakes (kJ/day) versus (A) hepatic FGF21 mRNA expression and (B) circulating plasma FGF21 levels (ng/mL).

(C–F) Relationship between intakes (kJ/day) of macronutrients, energy, and plasma FGF21 levels. There were no significant associations between FGF21 and fat or total energy intakes. Correlation coefficients for protein, carbohydrate, fat, and energy are −0.580, 0.488, 0.082, and 0.177, respectively.

See also Tables S4 and S5.

balance of dietary protein to carbohydrate on FGF21. To determine whether the increase of FGF21 levels seen chronically is similar to those secondary to acute changes in diet, FGF21 levels were measured in mice commenced on a 5% protein diet over 24 hr. Plasma FGF21 levels reached levels seen at 15 months within 24 hr (Figure S1) and were similar to acute changes reported by Laeger et al. (2014) and von Holstein-Rathlou et al. (2016).

Given the absence of an effect of fat consumed, and to focus in more detail on the interaction between protein and carbohydrate intakes, we next reduced the three-nutrient statistical model by removing terms associated with fat intake. While protein intake consistently appeared as the main driver of both mRNA expression and circulating levels of FGF21 in vivo, GAM and associated response surfaces confirmed that maximal levels occurred when protein intake was low and carbohydrate intake was high (a ratio of protein to carbohydrate intakes [P:C] of 1:12; Figures 2A and 2B) (see Table S5). Even when non-protein intake (i.e., the sum of carbohydrate and fat) was combined and plotted against protein intake, no significant effect of non-protein intake was observed, confirming that the main influence on FGF21 is derived from carbohydrates rather than fat (Figure S2; Table S5).

Two recent studies have reported that FGF21 selectively affects appetite for sweet mono- and disaccharides (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). In our study, mice were fed carbohydrates primarily as starch with additional sucrose; therefore, the results were analyzed to determine whether the carbohydrate effect on FGF21 was mediated by sucrose or starch. Geometric analysis showed that dietary sucrose had no independent effect on FGF21; however, there was an association between FGF21 and the interaction between dietary protein and sucrose, with FGF21 elevated in high-sucrose conditions only when protein intake was low. Starch showed similar but stronger effects on circulating levels of FGF21, which were only elevated under the combination of low protein and high starch intakes (Figure S2; Table S5). These data suggest that the nutritional context at which FGF21 is most elevated is dependent on the balance of protein to carbohydrate and that this balance may be important in FGF21-mediated suppression of sugar appetite.

To explore possible nutrient signals associated with protein and carbohydrate intakes, we next analyzed FGF21 as a function of circulating amino acids and glucose and found that the interaction between them had a statistically significant effect on FGF21 (Figure 2C; $p < 0.001$; Table S5). Among circulating amino acids, the BCAAs (isoleucine, leucine, and valine) were the only amino acids that correlated positively with protein intake (as reported in Solon-Biet et al., 2014), and in turn BCAAs were the most strongly correlated to FGF21 (Figure 2D; R = −0.373; Figure S3).

To see whether these effects of BCAAs and glucose seen in vivo could be recapitulated in vitro, we incubated human HepG2 liver cells in media containing one of 25 concentrations of BCAAs and glucose, mimicking physiological concentrations found in the mice (Table S2). Although glucose significantly influenced FGF21 protein expression, maximal levels were only found when there was a combination of low BCAAs and high glucose levels (Figure 2E). As we had reported previously in vivo

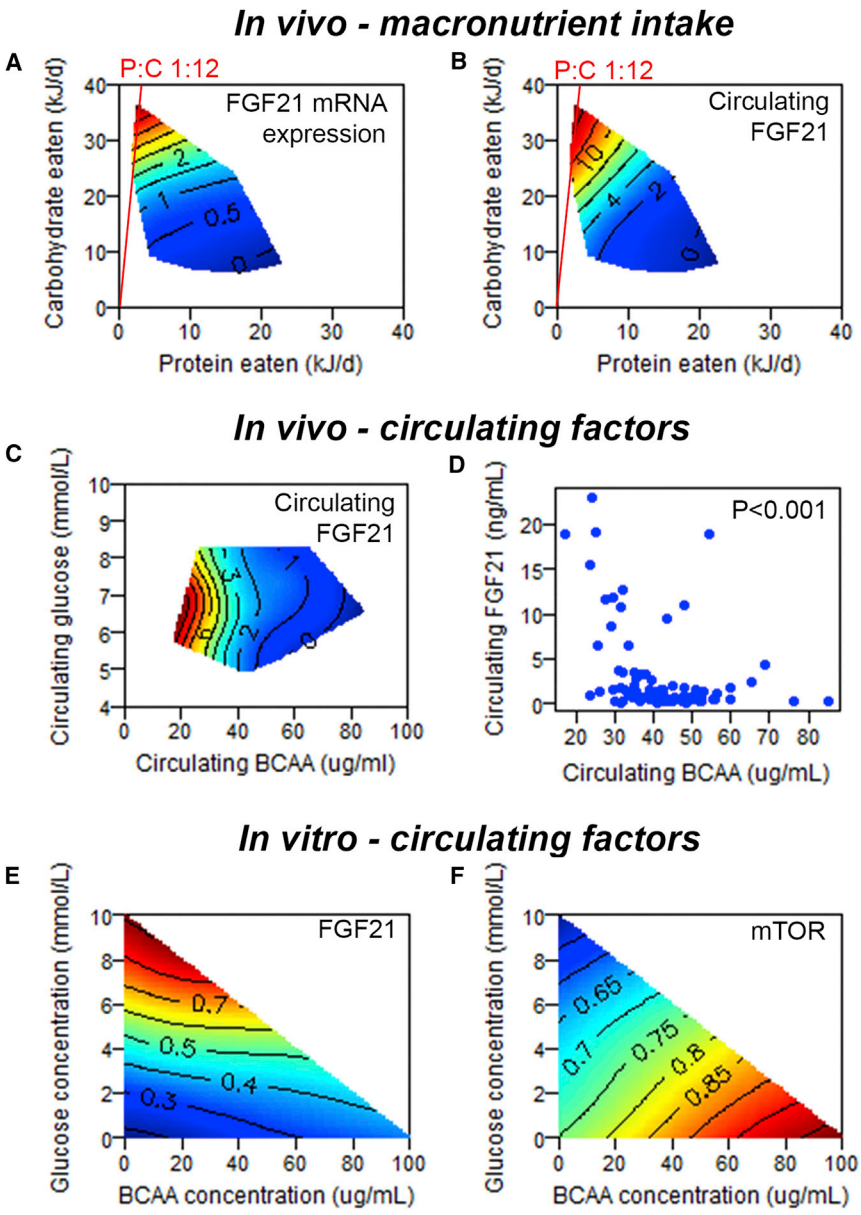

Figure 2. Hepatic Expression and Plasma FGF21 Levels Are Maximal in Low-Protein, High-Carbohydrate Conditions

(A and B) Two-dimensional response surfaces showing the effect of protein and carbohydrate intakes on (A) FGF21 hepatic mRNA expression and (B) circulating FGF21 levels (ng/mL).

(C) BCAA (μg/mL) versus glucose (mmol/L) in mice.

(D) Relationship between BCAA and plasma FGF21 in vivo (R = 0.0373).

(E and F) In vitro studies using HepG2 cells show the effect of BCAAs and glucose on (E) FGF21 and (F) mTOR activation (intensity units via western blot).

See also Figures S1 and S2 and Table S5.

(Solon-Biet et al., 2014), mTOR activation was greatest with the opposite nutrient ratio, i.e., high BCAA with low glucose (Figure 2F).

FGF21 and Metabolic Phenotype

We next explored the metabolic phenotypes associated with elevated FGF21, using data derived from our earlier GF analysis of this same cohort of mice (Solon-Biet et al., 2014). As an illustration, Figure 3A indicates two contrasting phenotypes (1 and 2) that occur within the region of elevated FGF21 in protein-carbohydrate intake space. Phenotype 1 is hyperphagic, driven by compensatory feeding for protein on low-protein diets, resulting in increased body fat but remaining insulin sensitive. Phenotype 2, on the other hand, is energy restricted (achieved by the addition of non-digestible fiber to the diet), lean, and insulin sensitive. Corresponding response surfaces to reflect this metabolic phenotype are adapted from work published by Solon-Biet et al. (2014) and are shown in Figures 3B–3I. Here, body mass was reduced with an overall reduction in protein intake; however, this reduction was associated with an increase in percent body fat, but only when energy intake was high, as seen in phenotype 1 (and also where mice were confined to low-protein, high-fat diets). Blood pressure, glucose tolerance, high-density lipoprotein (HDL), and bone mineral density were all improved in phenotype 1 in comparison to phenotype 2. Clearly, elevated FGF21 was not associated with a single metabolic profile; rather, metabolic outcomes reflect the ratio of protein to carbohydrate ingested and total energy intake.

Protein Intake Influenced UCP1, Glucose Metabolism, and IGF-1 Levels

The effect of diet on uncoupling protein 1 (UCP1) as a reflection of energy expenditure was measured by protein expression of UCP1 from intrascapular brown adipose tissue (BAT; Figure 4A). Increased UCP1 protein expression occurred on low protein intakes, consistent with the same nutrient context that elevated FGF21 (Figures 1A and 1B). As protein intake increased, UCP1 trended downward; however, these data were not statistically significant. These metabolic associations were further explored by measuring markers of gluconeogenesis, namely hepatic mRNA expression levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) (Figure 4B; Table S4). We found that *PEPCK*, a rate-controlling step in gluconeogenesis, was most highly overexpressed when protein intake was maximal and when combined with a low carbohydrate or fat intake ($p = 0.002$). Response surfaces showed opposing patterns to *FGF21* expression (Figure 1A), linking high expression of *FGF21* to suppression of gluconeogenesis. Just as increased protein intake increased *PEPCK* expression, the probability of elevated glycogen accumulation in the liver was also linked to

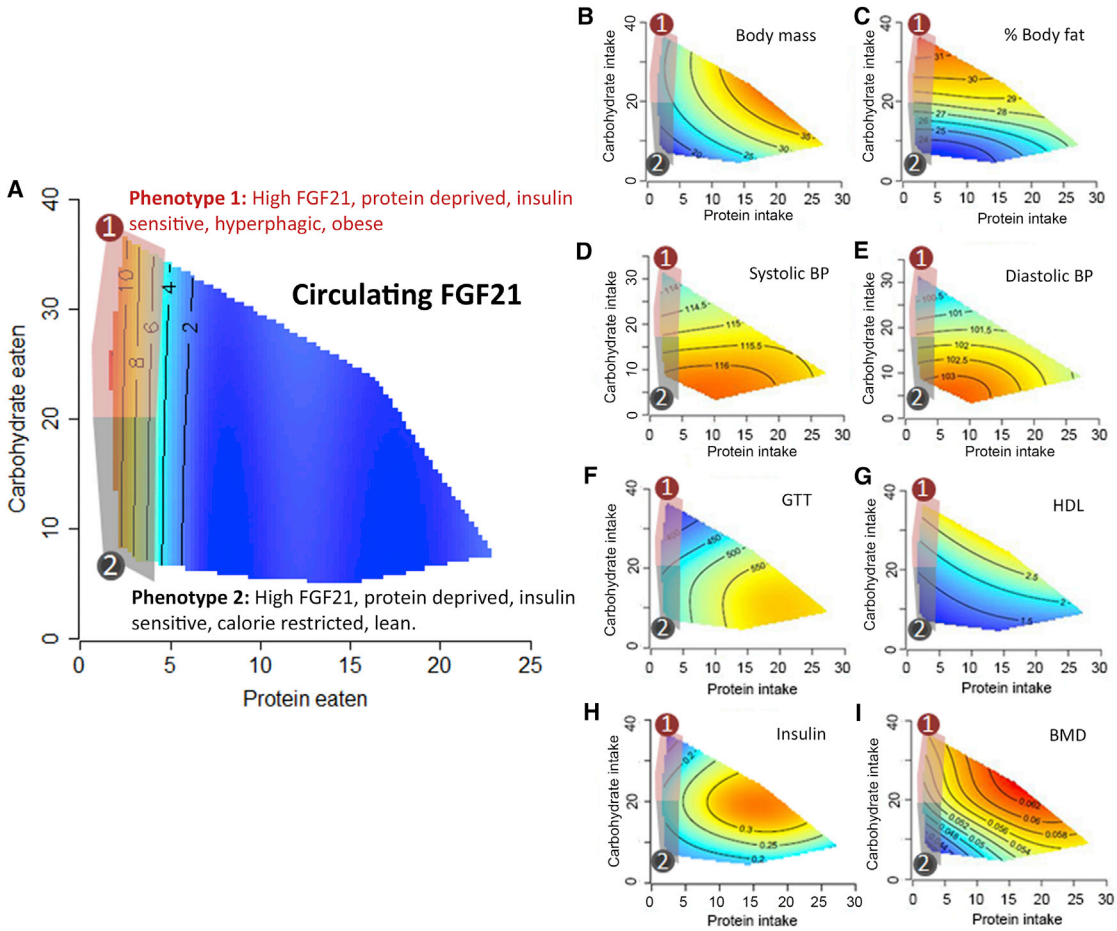

Figure 3. Elevated Circulating FGF21 Levels Are Associated with Various Metabolic Phenotypes

FGF21 levels were robustly elevated by low-protein diets, but the nutritional context in which this occurred resulted in different metabolic phenotypes.

(A) Two contrasting phenotypes are superimposed onto a response surface for circulating FGF21 levels. These metabolic outcomes were previously published in Solon-Biet et al. (2014). Phenotypes 1 and 2 both have high FGF21 levels, are protein deprived, and are insulin sensitive. However, phenotype 1 is hyperphagic due to compensatory for protein, resulting in obesity. Phenotype 2 is calorie restricted and lean.

(B–I) The effects of protein and carbohydrate intakes on (B) body mass (g), (C) percent body fat, (D) systolic blood pressure (mm/Hg), (E) diastolic blood pressure (mm/Hg), (F) area under the curve from glucose tolerance test, (G) HDL (mmol/L), (H) insulin (ng/mL), and (I) bone mineral density (g/cm^2). It is clear that the two high-FGF21 phenotypic conditions are different in their metabolic profiles. Adapted from Solon-Biet et al. (2014).

increased protein intake, reflecting increased gluconeogenesis on these diets (Figures 4C and 4E). The response surface for IGF-1 showed that protein intake also had the most dominant effect, followed by carbohydrate intake ($p < 0.001$ and $p = 0.039$, respectively), with no statistically significant effect of fat intake or total energy intake (Figure 4D). Hence, in these 15-month-old mice, the low-protein, high-carbohydrate nutritional context at which FGF21 was elevated (Figure 3) corresponded strikingly to the nutritional context where UCP1 was maximal but where *PEPCK* expression and circulating IGF-1 levels were at their lowest. This antagonistic effect of FGF21 and IGF-1 is consistent with findings that show reduced circulating IGF-1 in transgenic mice overexpressing FGF21 (Zhang et al., 2012).

ATF5 Gene Expression Correlates Closely with Dietary Protein and FGF21 Expression and Levels

To investigate cellular pathways that link dietary macronutrients with *FGF21* expression and production by the liver, microarray gene expression studies were performed on 48 livers across all diets. The gene whose expression most closely correlated with *FGF21* was *ATF5* ($p = 1.28 \times 10^{-13}$, $R = 0.8974$) (Table S6; Figure S4). Increased *ATF5* expression was confirmed by qPCR (Figure S4; Tables S4 and S6). By contrast, *ATF4*, which is considered to be a key mechanism linking diet (amino acid deprivation) to FGF21 (De Sousa-Coelho et al., 2013), was not statistically significant. *ATF5* expression by qPCR was also negatively correlated with circulating BCAA levels ($p = 0.004$, $R = -0.322$; Figure S4), suggesting a potential role in linking diet and FGF21.

DISCUSSION

FGF21 is an emerging endocrine regulator that influences several metabolic functions critical for health. As such, it is an important potential drug target for the treatment of diabetes and other metabolic disorders (Potthoff and Finck, 2014). There are paradoxical results in the literature regarding triggers for its

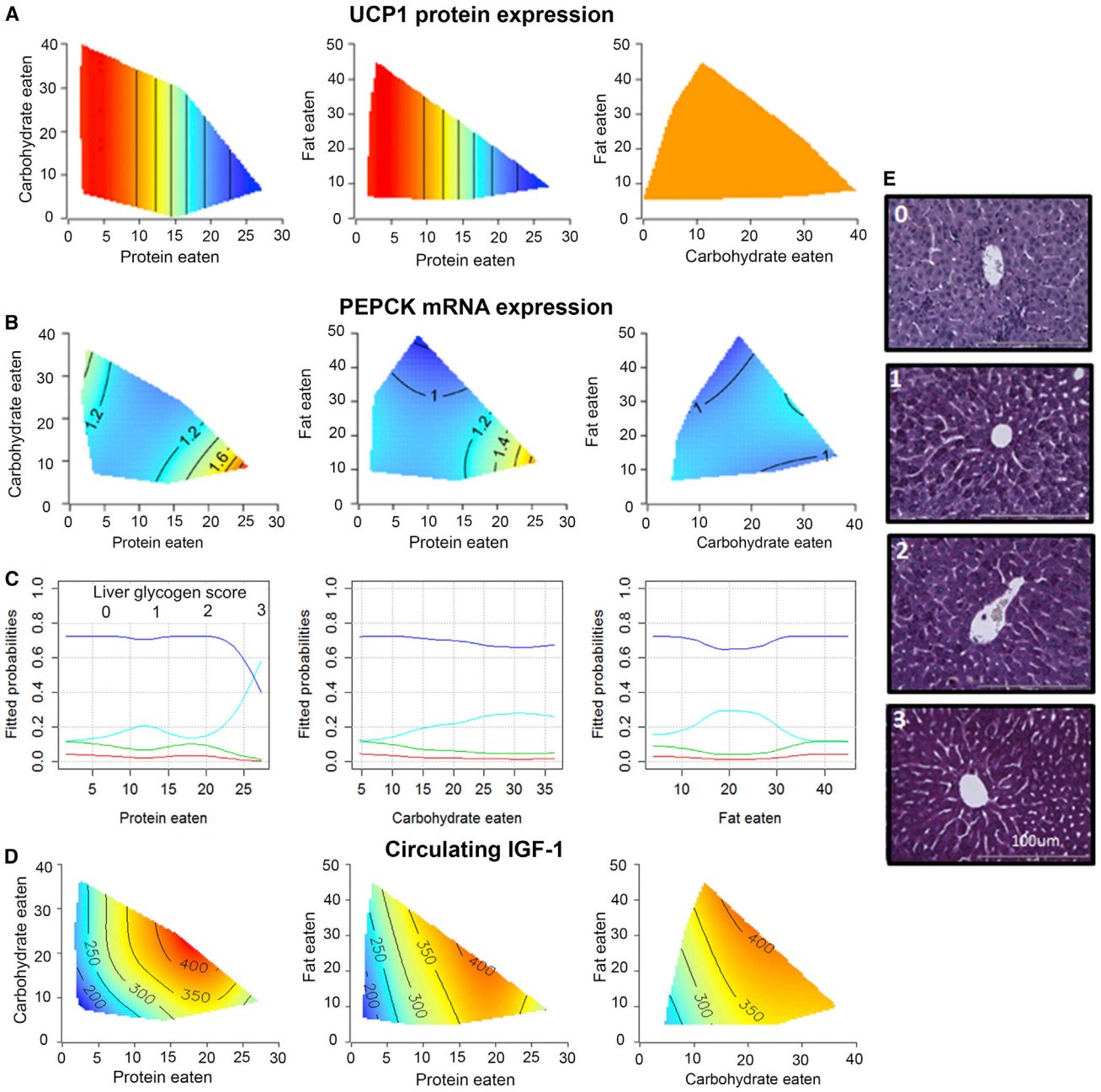

Figure 4. The Impacts of Protein Intake on UCP1, Glucose Metabolism, and IGF-1 Levels

(A) UCP1 from BAT was maximal on low protein intakes, reflecting elevated energy expenditure, and is shown as intensity units via western blot.

(B) PEPCK mRNA expression was decreased with low protein intakes and elevated with higher protein intakes, consistent with findings that excess protein intake results in impaired gluconeogenesis.

(C) Relationship between macronutrient intakes and hepatic glycogen storage. High protein intake increased the probability of glycogen accumulation in the liver.

(D) IGF-1 was increased with high protein intakes and responded oppositely to FGF21.

(E) Glycogen storage was graded from 0 to 3 and stained with PAS.

See also Figure S3 and Table S4.

secretion and its physiological role, with currently no consistent metabolic signature of elevated FGF21 expression or circulation. FGF21 is increased in various conditions such as overfeeding, obesity, insulin resistance, starvation, protein/amino acid deprivation, low-protein ketogenic diets, and high carbohydrate feeding (Badman et al., 2007; Fazeli et al., 2015; Kharitonenkov and Larsen, 2011; Laeger et al., 2014; Lees et al., 2014; Markan et al., 2014; Stemmer et al., 2015), yet the metabolic effects are very different in each nutritional context. The current work suggests that these findings can be reconciled using the GF.

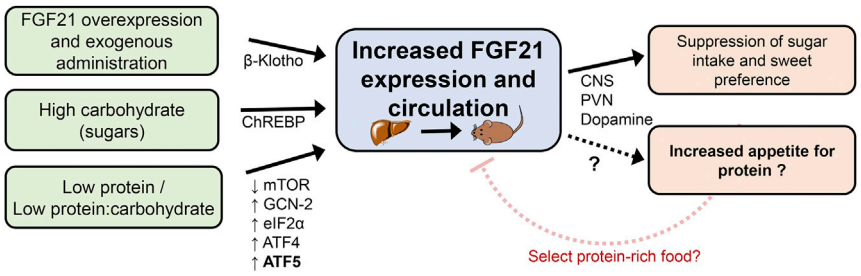

Figure 5. Model of FGF21-Mediated Effects on Appetite

FGF21 liver expression and circulating levels are influenced by genetic, pharmaceutical, and nutritional interventions and have been shown to suppress the intake of sugar and influence sweet preference. It remains to be determined whether increased FGF21 results in an increase in protein appetite, but this, in combination with depressed carbohydrate appetite, would be consistent with FGF21 being highest under the combination of simultaneously elevated carbohydrate intake and low protein intake. Increased protein appetite would then lead to the selection of protein-rich foods, which inhibits FGF21 levels. Restriction of amino acids, such as that seen in low protein or low protein to carbohydrate intakes, decreases mTOR activation, activates cellular pathways such as GCN2, and increases phosphorylation of eIF2α. Subsequent activation of ATF4 contributes to increased FGF21 expression and circulation and is a key mechanism linking diet to FGF21. Our findings show that ATF5 was robustly increased with low protein intakes; however, its exact role in mediating the actions of FGF21 remains unclear.

Our data support reports that low protein intake plays the major role in driving FGF21 expression and secretion (Laeger et al., 2014), with maximal elevation of FGF21 occurring when low protein intakes were coupled with high carbohydrate intakes. There were two contrasting circumstances in which protein intake was low: (1) low protein, high energy intakes or (2) low protein, low energy intakes. In the first condition, low-protein diets fed ad libitum drive compensatory feeding for protein (termed "protein leverage"; Simpson and Raubenheimer, 2005, 2012; Sørensen et al., 2008), resulting in hyperphagia in an attempt to reach a target protein intake and promoting adipogenesis and increased fat mass while leaving the animal still somewhat protein deprived (e.g., phenotype 1 in Figure 3 and low-protein, high-fat diets; Solon-Biet et al., 2014). The increased food intake on lower percent protein diets is reported to be accompanied by increased energy expenditure, presumably in part through diet-induced thermogenesis (Huang et al., 2013; Solon-Biet et al., 2015a). In the short term (up to 8 weeks), increased energy expenditure protects mice against gain in fat mass (Solon-Biet et al., 2015a), but it is unable to prevent increased adiposity in the long term (Huang et al., 2013; Solon-Biet et al., 2014; Sørensen et al., 2008). Consistent with these results, recent evidence has shown that FGF21 increases expression of *UCP*1 in adipose tissue to induce thermogenesis (Fisher et al., 2012) and can explain the suppression of hepatic gluconeogenesis (Sarruf et al., 2010; Xu et al., 2009) in this condition. However, the role of diet-induced thermogenesis on *UCP1* expression in BAT over long-term feeding conditions remains unclear. Exposure to 16 weeks of low-protein diets resulted in BAT hyperplasia but reduced *UCP1* expression (Huang et al., 2013), suggesting that other mechanisms, such as the beiging of white adipose depots, may be involved (Kazak et al., 2015).

Earlier, we reported that the low protein, high carbohydrate condition (phenotype 1 in Figure 3) is associated with favorable late-life health outcomes, such as increased lifespan, improved insulin sensitivity and glucose metabolism, increased mitochondrial function, and better blood pressure and lipid profiles, despite increased adiposity (Solon-Biet et al., 2014). Under such circumstances, circulating FGF21 levels were elevated, as they were on low protein, high fat intakes—a phenotype with less favorable metabolic outcomes than low protein, high carbohydrate intakes (Solon-Biet et al., 2014). These results explain reports that circulating FGF21 is elevated under obesity. By contrast, in the low protein, low energy condition (phenotype 2 in Figure 3), circulating FGF21 was also elevated, but mice were both protein and energy restricted, equivalent to the starved condition. This nutritional context is associated with leanness, insulin sensitivity, a net catabolic state, reduced reproduction (Owen et al., 2013; Solon-Biet et al., 2015c), and decreased energy expenditure, consistent with findings that FGF21 acts via the brain under such circumstances to reduce energy expenditure and stimulate fatty acid oxidation and ketogenesis (Inagaki et al., 2007).

Hence, our data show that FGF21 acts in a context-dependent manner and is an indicator of low protein intake and, to a lesser degree, a low-protein, high-carbohydrate ratio. Such a protein imbalance could help explain why, when administered to obese or insulin-resistant rodents or humans, FGF21 enhanced insulin sensitivity and suppressed hepatic gluconeogenesis (Xu et al., 2009), whereas when administered to a lean, metabolically healthy animal in protein and energy balance, it had no detectable effects on metabolism (Kharitonenkov and Larsen, 2011; Markan et al., 2014).

Mice, like other animals, have been shown to regulate consumption of both protein and carbohydrate independently to an "intake target" (Simpson and Raubenheimer, 2012; Sørensen et al., 2008). Regulating intake to meet this target requires that mice, under conditions of a low-protein, high-carbohydrate diet, have an elevated appetite for protein and a depressed appetite for carbohydrate in order to invoke compensatory food selection to rebalance the diet (Sørensen et al., 2008). These are the circumstances under which FGF21 is most elevated. Elevated FGF21 has been reported to depress sugar appetite (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016), and it is predicted, but as yet unproven, that FGF21 will stimulate protein appetite, thereby leading mice to reject high-carbohydrate, low-protein foods in favor of foods with the opposite macronutrient balance (i.e., low carbohydrate, high protein; Figure 5). This prediction is consistent with the negative-feedback signal proposed by von Holstein-Rathlou et al. (2016), where elevation of FGF21 by sugar ingestion limits further sugar intake.

In low amino acid environments, signaling to FGF21 has been shown to occur via phosphorylation of the protein kinase known as GCN2, subsequent phosphorylation of eukaryotic initiation factor 2α (eIF2α), and increased activation and synthesis of activating transcription factor 4 (ATF4). Together, this pathway upregulates hepatic *FGF21* expression and secretion in response to

protein restriction (Chotechuang et al., 2009; De Sousa-Coelho et al., 2012; Laeger et al., 2014; Soultoukis and Partridge, 2016). However, recent work has shown that GCN2-dependent activation of FGF21 occurs only in acute feeding conditions and suggests that other mechanisms in this pathway act to induce FGF21 under chronic exposure to low-protein environments (Laeger et al., 2016).

eIF2α-dependent activation of *ATF4* is well established as playing a key role in the adaptation to dietary stress and is a key link between amino acid deprivation and *FGF21* induction (De Sousa-Coelho et al., 2013; Morrison and Laeger, 2015). However, the role of other transcription factors subject to eIF2α phosphorylation is unknown. Like *ATF4*, ATF5 is highly expressed in the liver (Hansen et al., 2002), but its role in mediating the response to amino acid deprivation/imbalance is unclear. *ATF5* is also induced via phosphorylation of eIF2α, and knockout of *ATF4* in mouse embryonic fibroblast (MEF) cells significantly reduces *ATF5* mRNA levels, indicating that these transcription factors are closely linked (Zhou et al., 2008). Our data suggest that *ATF5* may also play an important role in eIF2α-dependent activation of the FGF21 response. We found that *ATF5* was most highly expressed with low protein intakes (Figure S4; Table S6), coinciding with the dietary conditions that induced the highest circulating FGF21 levels, suggesting that *ATF5* may be an important mechanism in FGF21 induction during amino acid restriction. We also found that low levels of BCAAs were strongly associated with increased *FGF21* and *ATF5* liver expression, linking this pathway to protein intake and potentially other nutrient-sensing pathways that respond to protein intake.

Currently, four pathways are strongly implicated in nutrient sensing and the dependent control of metabolism: mTOR, insulin/IGF-1, AMPK, and the SIRT1 pathways, which all interact and share targets that regulate metabolism and aging. mTOR is involved in regulating cell growth, proliferation, and protein synthesis, integrating input from the insulin/IGF-1 pathway, dietary protein intake, and, in particular, BCAAs (Chotechuang et al., 2009; Solon-Biet et al., 2014). We have previously reported that decreased hepatic mTOR activation is associated with improved markers of cardiometabolic health and extended longevity in the same cohort of mice. Here, we have shown both in vivo and in vitro that mTOR was activated in nutrient conditions (high P:C) that were the opposite of those that activated FGF21 (low P:C), indicating that these two pathways may be linked. Cornu et al. (2014) showed that amino acids can influence hepatic *FGF21* expression via mTOR and downstream effects on peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). We found that the low P:C environment that supports highest FGF21 expression and circulation was also the same nutrient condition that is associated with the lowest IGF-1 levels. These findings are consistent with findings that FGF21 upregulation causes a corresponding reduction in IGF-1 (Inagaki et al., 2008) (Figure 4). The insulin/IGF-1 signaling (IIS) pathway responds to changes in insulin levels and is sensitive to energy, protein, and BCAA content. Reducing the action of this pathway (Fontana et al., 2010) improves metabolic health in a range of animals. AMPK and SIRT1 act largely antagonistically to mTOR and insulin/IGF-1. They are activated in response to cellular energy levels, and increased activation of these pathways is associated with improved metabolic outcomes such as insulin sensitization and increased lifespan. FGF21 activates the AMPK and SIRT1 pathways, regulating energy homeostasis and increasing mitochondrial oxidative function (Chau et al., 2010), providing a key link between nutritional state, nutrient signaling, and metabolic health outcomes.

In conclusion, we have shown that the GF can help to reconcile apparently contradictory reports regarding the significance of an elevation of FGF21 and its metabolic effects, which are highly dependent on nutritional context. An important next step will be to determine whether elevation of FGF21 is coupled to the specific control of protein appetite, and how it modulates metabolic outcomes in a manner that reflects the nutritional context of the animal.

EXPERIMENTAL PROCEDURES

We have previously published detailed methods for this study regarding diet, lifespan, late-life metabolic health, and reproduction (Le Couteur et al., 2015; Solon-Biet et al., 2014, 2015c). Here, additional studies were conducted to explore the role of FGF21 in macronutrient balance and metabolic health. Detailed methods can be found in the Supplemental Experimental Procedures.

Animals and Husbandry

A total of 858 3-week-old male and female C57Bl6/J mice (n = 858) from the Animal Resources Centre were housed in the ANZAC Research Institute. Mice were given ad libitum access to one of 25 experimental diets and water (Table S1). Diets varied in protein (P), carbohydrate (C), fat (F), and energy (E) content. Energy manipulations were done through the addition of cellulose, allowing for low, medium, and high energy density diets (8, 13, and 17 kJ/g). Food intake was measured weekly for 6 months and monthly thereafter. At 15 months of age, 183 mice spanning across diets were sacrificed and tissues collected for various analyses. All protocols were performed in accordance with the Sydney Local Health District Animal Welfare Committee (protocol no. 2009/003).

For acute experiments, 8-week-old male C57Bl6/J mice (n = 8) from the Animal Resources Centre were used and protocols completed in accordance with the University of Sydney Animal Ethics Committee (AEC 2015/881). Mice were given ad libitum access to a 5% protein, 75% carbohydrate, and 20% fat diet and tail vein blood collected at 0 and 24 hr for analysis of circulating FGF21 levels.

Measurement of Circulating Factors

Plasma FGF21 was measured using the FGF21 mouse/rat ELISA kit (Biovendor). Plasma IGF-1 levels were measured using the mouse IGF-1 ELISA kit (Crystal Chem) and the plasma insulin was quantified using the Mouse Ultrasensitive Insulin ELISA kit (ALPCO Diagnostics). Circulating free amino acids were analyzed at the Australian Proteome Analysis Facility of Macquarie University using the Waters AccQ-Tag Ultra Chemistry Kit (Water Corporation), as has been previously reported (Solon-Biet et al., 2014).

Cell Culture

To determine the effect of BCAA and glucose balance on nutrient signaling in vitro, we chose 25 diet treatments spanning across the response surfaces seen in our previous work (Solon-Biet et al., 2014). Glucose concentrations ranged from 0 to 10 mM and BCAA concentrations ranged from 0 to 100 μg/mL (Table S2). HepG2 cells (human hepatocellular carcinoma cell line) were grown in DMEM supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin and incubated in standard humidified conditions (37°C with 5% CO_2).

Western Blot Analysis

Cell pellets, frozen liver tissue, and frozen intrascapular BAT (~30 mg) were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors

(Roche Mini cOmplete Protease Inhibitor Cocktail Tablets and Roche PhosSTOP Phosphotase Inhibitor Cocktail Tablets). For tissues, samples were lysed using the TissueLyser (QIAGEN) and then sonicated. After lysing, all samples were centrifuged (4°C, 10 min, 16,000 g), the supernatant was collected, and the protein content was quantified using bicinchoninic acid (BCA) assay (Sigma).

Gene Expression

Total RNA from liver and BAT was extracted using the Trizol method (Sigma) and quantified spectrophotometrically using a NanoDrop (Thermo Scientific), and RNA integrity was quantified using the Bioanalyzer (Agilent). Samples with an RNA integrity number (RIN) <6 were excluded from further downstream applications. For microarray, RNA from liver was extracted using the QIAGEN Allprep Mini Kit (QIAGEN) and quantified spectrophotometrically using a NanoDrop (Thermo Scientific), and RNA integrity was quantified using the Bioanalyzer (Agilent). Samples with an RIN >7 were analyzed by Affymetrix Mouse Gene ST array at the Ramaciotti Centre for Genomics (University of New South Wales; GEO: GSE85998).

Histology

Paraffin-embedded liver tissue was sectioned at 4 μm and stained with periodic acid-Schiff (PAS) to assess hepatic glycogen storage. The extent of storage was assessed and scored (0, 1, 2, or 3) by three independent observers blinded to tissue category.

Statistics

All data were analyzed in R v.3.1.3. Data involving response surfaces were analyzed using GAM and previously explained in detail in Solon-Biet et al. (2014). Correlations were analyzed using Pearson's correlation. Hepatic glycogen storage was assessed as a score ranging from 0 to 3 by four independent scorers. The scores were then modeled with an ordinal regression (proportional odds) and fitted with the help of the vector generalized additive model (VGAM) package in R, which allows the incorporation of spline terms into the model.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE85998.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.09.001.

AUTHOR CONTRIBUTIONS

S.M.S.-B. performed experiments, analyzed the data, and wrote the manuscript. V.C.C., M.H., T.P., D.W., A.C.M., A.W., J.D.-W., K.A.W., J.R.K., F.P., R.G., and J.W. performed experiments. K.R. analyzed the data. A.D.C., D.E.J., D.R., and C.D.M. assisted in preparing the manuscript. S.J.S. and D.G.L.C. conceived and supervised the project and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia (project grant 571328, program grant 571408), the Ageing and Alzheimers Institute (AAAI) of Concord Hospital, and the Australian Research Council (Discovery Project grant 160101119). S.M.S.-B. was supported by a National Medical Health and Research Council of Australia Peter Doherty Early Career Fellowship (grant 1110098).

Received: December 10, 2015
Revised: June 30, 2016
Accepted: August 31, 2016
Published: September 29, 2016

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