Dietary fat, but not dietary protein or carbohydrate (sucrose), regulates energy intake and causes adiposity in mice

Authors:

Sumei Hu[1#], Lu Wang[1,2,3#], Dengbao Yang[1#], Li Li[1,2#], Jacques Togo[1,2#], Yingga Wu[1,2#], Quansheng Liu[4], Baoguo Li[1,2], Min Li[1,2], Guanlin Wang[1,2,3], Xueying Zhang[1,2,3], Jianbo Li[5], Yanchao Xu[1], Elspeth Couper[3], Andrew Whittington-Davies[3], Mohsen Mazidi[1,2], Lijuan Luo[4], Shengnan Wang[4], Alex Douglas[3], John R. Speakman[1,3,6*]

Affiliations:

[1]State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, 100101, PRC

[2]University of Chinese Academy of Sciences, Shijingshan District, Beijing, 100049, PRC

[3]Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen AB24 2TZ, Scotland, UK

[4]Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou, 510260, PRC

[5]University of Dali, Dali, Yunnan Province, 671000, PRC

[6]Lead Contact, [#]equal contribution

[*]corresponding author Contact information: John R. Speakman,

E: j.speakman@abdn.ac.uk

T: +44 7771808275 F: +44 1224 272396

Summary: The impacts of different macronutrients on body weight regulation remain unresolved, with different studies suggesting increased dietary fat, increased dietary carbohydrates (particularly sugars) or reduced dietary protein may all stimulate over consumption and drive obesity. We exposed C57BL/6 mice to 29 different diets varying from 8.3 to 80% fat, 10 to 80% carbohydrate, 5 to 30% protein and 5 to 30% sucrose. Only increased dietary fat content was associated with elevated energy intake and adiposity. This was associated with increased gene expression in the 5-HT receptors, and the dopamine and opioid signaling pathways in the hypothalamus. We replicated the core findings in four other mouse strains (DBA/2, BALB/c, FVB and C3H). Mice regulate their food consumption primarily to meet an energy rather than a protein target, but this system can be over-ridden by hedonic factors linked to fat, but not sucrose, consumption.

Keywords: fat intake, sucrose intake, protein leverage hypothesis, protein target, energy regulation, homeostatic control, hedonic overdrive, mice, adiposity, hypothalamic hunger pathway, obesity, FGF signaling, mTOR signaling

Introduction

Obesity is a global health issue. According to the WHO in 2014, there were 1.9 billion overweight adults in the world, of which 600 million had obesity (WHO, 2016). This is a major health problem because obesity is a risk factor for numerous chronic diseases (Haslam and James, 2005). It is widely agreed that obesity results from prolonged positive energy balance (Hall et al., 2012). The relative roles of reduced energy expenditure and elevated intake in the etiology of such imbalance has been disputed (Prentice and Jebb, 1995; Swinburn et al., 2011; Westerterp and Speakman, 2008). However, data suggest that obesity is driven at least in part, if not completely, by overconsumption of energy (Swinburn et al., 2011; Westerterp and Speakman, 2008). The reasons for such overconsumption have been strongly debated (van Dam and Seidell, 2007; Willett, 1998). Changing macronutrient composition of the food may be a contributory factor, yet despite decades of study, there is still little consensus over whether high fat, or high sugar, or both are responsible for the elevated intake (van Dam and Seidell, 2007; Willett, 1998). Some much needed clarity has been brought to the field recently by the 'nutritional geometry' approach (Simpson and Raubenheimer, 2012). This aims to set individual dietary selection and its consequences into an n-dimensional framework, where nutritional behavior can be understood as animals attempting to reach nutritional targets that have been molded by evolution to optimize, for example, reproduction or survival (Simpson and Raubenheimer, 2012).

Applications of this approach to many species suggests that animals may eat food primarily to achieve a target intake of protein (Felton et al., 2009; Hawley et al., 2016; Raubenheimer and Simpson, 1997), which may be driven in part by FGF signaling (Gosby et al., 2016). This contrasts

the classical interpretation that food intake serves primarily to match energy demands. Attempting to ingest a target protein intake may lead to overconsumption of energy when the protein content of the diet declines. The protein leverage hypothesis, therefore, posits that calorie consumption is driven largely by declining dietary protein content (relative to energy), and individuals overconsume energy when attempting to meet their protein target (Simpson and Raubenheimer, 2005), thereby maintaining protein intake relatively constant (Fig. 1 A). In contrast, the energy regulation model suggests animals eat food primarily to match their energy demands, and hence faced with lowered protein content they are not stimulated to overconsume, and may avoid elevated adiposity, but at the potential threat of protein malnutrition (Fig. 1 B). The hedonic over-ride hypothesis posits that normally individuals homeostatically regulate their energy intake in relation to energy demands (Fig. 1D), and hence are normally in energy balance. However, this homeostatic control may be overridden by hedonic factors linked to consumption of various macronutrients in particular sugars (e.g. sucrose) and fat (Berridge et al., 2010; Berthoud, 2011; Berthoud and Morrison, 2008; Berthoud et al., 2017). This hedonic effect drives individuals into positive energy balance, resulting in weight gain, and has been exceptionally characterized as food addiction (Avena et al., 2008; Gearhardt et al., 2011). These models assume that energy expenditure reflects energy requirements and that energy expenditure is therefore independent of dietary macronutrient composition.

These models (Fig. 1) illustrate a method to quantify the extent of protein leverage. If there is perfect leverage, then the protein intake plotted against protein content of the diet will extrapolate to the intercept at a value equal to the intake for the highest protein content diet (Fig. 1 A). This pattern might be termed 100% protein leverage. In contrast, if the animals regulate their intake of energy, then the plot of realized protein intake against dietary protein content would extrapolate to zero. This

could be termed 0% protein leverage. If the animals pursue a mixed strategy (Fig. 1 C), then the extrapolation of the protein intake to protein content line to the intercept provides a measure of the % effect of protein leverage in the diet choice. For example, if the gradient connecting protein intake to protein content of the diet intercepted at a point half way between 0 and the intake at the highest protein intake, then the % effect of protein leverage would be 50%. These models also assume that the responses are linear across the range of protein contents in the diet. Other patterns are possible and would require more complex fitting procedures. The leveraging of total intake by protein has been widely supported by empirical observations in a wide range of animals from insects to non-human primates (Felton et al., 2009; Gosby et al., 2016; Hawley et al., 2016; Raubenheimer and Simpson, 1997) and humans (Gosby et al., 2011; Martens et al., 2013; Martinez-Cordero et al., 2012; Simpson et al., 2003), although the percentage contribution of protein leverage to total intake has generally not been quantified as explicitly as indicated by the above approach. Nevertheless, protein leverage is increasingly being used to understand aspects of comparative feeding biology and the human obesity epidemic (Bekelman et al., 2017).

Since the mouse is a widespread model used to understand human obesity, we aimed to explore how changes in macronutrient composition of the diet impacts food intake. In particular how changes in protein, carbohydrate (sugar) and fat contents of the diet leverage intake and cause adiposity. In total, we used 30 different diets varying orthogonally in their macronutrient composition to allow separation of the different macronutrient effects. We combined these observations with gene expression profiling of the hypothalamus and adipose tissue to assess if FGF pathways were stimulated, and measurements of aminopeptidase activity in the alimentary tract, to explore the underlying mechanisms by which protein, carbohydrate or fat may exert its effects on appetite. The

most extensive work was performed in C57BL/6 mice, and then the core observations were repeated in 4 other strains (BALB/c, C3H, DBA/2 and FVB) which include strains classically regarded as 'resistant' to obesity.

Results

The overall experimental designs and the outcomes are summarized in Table 1.

Protein leverage hypothesis is not supported

C57BL/6 mice were fed for 3 months on 2 series of six diets with variable protein (5-30% by energy) and constant high (60%) or low fat (20% by energy) (12 diets in total with 20 mice per diet: diet details in Table S1). The energy intakes of the mice fed on the 5% protein diet with either 60% fat or 20% fat were slightly higher than the mice fed on diets with higher protein contents, while there was no difference between the other protein levels (Figs. 2 A, B). At both levels of fat in the diet, total energy intake over the final week of measurement did not differ significantly in relation to protein content from 10% to 30%, with only a significant difference between 5% protein group and other protein groups ($p = 1.40 \times 10^{-6}$ for 60% fat and $p = 2.52 \times 10^{-5}$ for 20% fat: Figs. 2 A, B). The consequence was that protein intake was strongly and linearly, directly related to the protein content in the diet ($p = 2.80 \times 10^{-28}$ for 60% fat and $p = 5.43 \times 10^{-62}$ for 20% fat) (Fig. 2 A, B). These relationships had intercepts that did not differ significantly (at $p < 0.01$) from zero ($p = 0.103$ for 60% fat and $p = 0.045$ for 20% fat). The percentage protein leverage was at most 3.5 to 4% (nsd to zero). The same patterns were observed if we used the average food intake over the entire 12 weeks rather than intake over the final ten days (Figs. S1 A, B).

Body adiposity increased with increasing dietary protein content

On the 60% fat diets with variable protein contents, body mass, lean mass and adiposity all *increased* with increasing protein content in the diets at the end of the experimental period ($p = 1.92 \times 10^{-5}$ for body weight, $p = 2.30 \times 10^{-4}$ for fat mass and $p = 2.10 \times 10^{-4}$ for lean mass) (Fig. 2 C), opposite the predictions of the protein leverage hypothesis. On the 20% fat diets, body mass, body adiposity and lean mass also increased as protein content increased from 5% to 20% protein, and then decreased when protein increased from 20 to 30% ($p = 0.024$ for body weight, $p = 0.005$ for fat mass and $p = 0.002$ for lean mass) (Fig. 2 D). Changes in the overall adiposity (% fatness) showed the same trends as fat mass (Figs. S2 A, B). The patterns of overall adiposity were also reflected in the changes in size of the individual fat depots (Figs. S2 E, F).

Mice may theoretically burn off excess calorie intake by becoming more physically active. However, physical activity of the mice did not follow any consistent trend in the mice fed on diets with different protein contents, and there were no significant differences between the different protein groups ($p = 0.712$ for 60% fat and $p = 0.452$ for 20% fat). DEE ($p = 0.603$ for 60% fat and $p = 0.128$ for 20% fat) and REE ($p = 0.874$ for 60% fat and $p = 0.214$ for 20% fat) also followed the same pattern under either high fat or low fat conditions, with no significant changes between groups fed on diets with different protein levels (Figs. 2 E, F). RER was independent of protein content of the diets, except a significant difference between 5% and 30% protein at 60% fat ($p = 0.016$ and $p = 0.108$ for 20% fat). The estimated energy expenditure of mice over the entire duration of the manipulation calculated using the software of Guo and Hall (2009) (Table 2) showed the same trends as the point estimates of metabolism.

The activity of the aminopeptidase-N in the gut increased with the increasing protein content at 20% fat (Fig. 2 G-H) ($p = 0.002$ for 20% fat and a trend $p = 0.071$ for 60% fat). Small leveraging of total energy intake by protein may still in theory cause adiposity if maintained over protracted periods. There were significant differences in body mass and fat mass between the 5% protein group and other protein groups with either 60% fat ($p = 4.35 \times 10^{-31}$ for body weight and $p = 2.30 \times 10^{-4}$

for fat mass) or 20% fat ($p = 9.29 \times 10^{-31}$ for body weight and $p = 0.005$ for fat mass) during the last week of measurement (Figs. 2 C-D).

Responses of four other mouse strains on diets with variable protein content were consistent with C57BL/6 mice

Mice from four other strains (BALB/c, C3H, DBA/2 and FVB) were used to validate the core results with respect to protein on C57BL/6 mice, by feeding them on diets containing variable protein and constant high fat (60% fat). In all four strains, we repeated the observation in the C57BL/6 strain that there was a linear decrease in absolute protein intake with declining protein content in the diet ($p = 5.67 \times 10^{-7}$ for BALB/c, $p = 9.00 \times 10^{-20}$ for C3H, $p = 7.12 \times 10^{-34}$ for DBA/2, $p = 2.74 \times 10^{-21}$ for FVB, respectively) (Fig. 3). Energy intake of BALB/c mice and FVB mice showed a slight but significant increase as protein content decreased, with no differences when protein content was over 10% ($p = 2.08 \times 10^{-5}$ for BALB/c and $p = 0.045$ for FVB) (Figs. 3 A, G). Total energy intake was independent of the protein content in C3H mice ($p = 0.517$) and DBA/2 ($p = 0.132$) (Figs. 3 E, G). The same patterns were observed if we used the average food intake over the entire experiment period rather than intake over the final ten days (Figs. S3 A, C, E, G). The calculated percentage protein leverage was 12.2% in BALB/c mice ($p = 0.047$), 6.1% in C3H mice ($p = 0.019$), 1.3% in DBA/2 mice ($p = 0.479$) and 4.65% in FVB mice ($p = 0.089$). In spite of these slight trends for increased energy intake at lower protein levels in two of the strains, body mass, fat and lean mass of BALB/c and C3H mice were both *increased* when the protein content in the diets increased from 5% to 20%. Fat content then decreased when the protein content was increased from 20% to 30% (BALB/c: $p = 4.73 \times 10^{-8}$ for fat mass and $p = 0.008$ for lean mass; C3H: $p = 1.17 \times 10^{-5}$ for fat mass and $p = 0.009$ for lean mass) (Figs. 3 B, D). In the other 2 strains there were no significant body composition differences between different protein groups (Figs. 3 F, H) (DBA/2: $p = 0.669$ for fat mass and $p = 0.301$ for lean mass; FVB: $p = 0.843$ for fat mass and $p = 0.505$ for lean mass). Changes in the overall adiposity (% fatness)

showed the same trends as fat mass (Figs. S4 A, C, E, G). Overall, the responses of the 5 different strains were consistent that altering the protein content of the diet across a large range, produced only minimal impacts on energy intake, but enormous differences in protein intake. These changes had no significant impact on adiposity.

Dietary fat content drives energy intake and body adiposity of mice

Another 2 series of six diets with fixed protein content (10% or 25%) and variable fat content were designed and fed to C57BL/6 mice to investigate the effect of dietary fat content on body adiposity. Fat intake (during the last 10 days) increased linearly and significantly with increasing fat content in the diet either with 10% protein ($p = 2.18 \times 10^{-45}$) or 25% protein ($p = 4.27 \times 10^{-68}$) (Figs. 4 A, B). Increased fat intake was related to higher energy intake of the mice fed on diets with either 10% protein ($p = 3.24 \times 10^{-12}$) or 25% protein ($p = 6.26 \times 10^{-12}$), even though the mice reduced their food intake when the fat content was increased in the diet ($p = 8.71 \times 10^{-17}$ for 10% protein, $p = 5.36 \times 10^{-8}$ for 25% protein) (Figs. 4 A, B). Consequently, the increased energy intake caused the increase in body weight ($p = 3.45 \times 10^{-7}$ for 10% protein, $p = 1.68 \times 10^{-28}$ for 25% protein) and body fat mass ($p = 1.51 \times 10^{-9}$ for 10% protein, $p = 3.02 \times 10^{-24}$ for 25% protein), when dietary fat content was lower than 60% (Figs. 4 C, D). The same patterns were observed if we used the average intake over the 12 weeks of study rather than intake in the last 10 days (Figs. S1 C, D). Body weight and fat mass of the mice were slightly decreased because of significantly reduced food intake, when fat content in the diet was higher than 60% (Figs. 4 C, D)**.** No significant changes were observed in the lean mass of mice fed on diets with variable fat content either with 10% protein ($p = 1.66 \times 10^{-4}$) or 25% protein ($p = 0.003$). Trends in adiposity (%fatness) and the sizes of individual fat depots mirrored the pattern of change in fat mass (Figs. S2 C, D, G, H). Therefore, increasing dietary fat content up to 60% fat leads to increased energy intake and causes adiposity in mice; however, further increase in the fat content led to a slight decrease in the energy intake via reduction in the absolute weight of food intake, and, as a consequence, body

weight and fat mass decreased.

The activity of aminopeptidase-N showed a trend similar to body weight and body fatness in either 10% protein or 25% protein groups, however, there was no significant differences ($p = 0.079$ for 10% protein, $p = 0.057$ for 25% protein) (Figs. 4 G, H). There was no significant trend in the change of physical activity. Significantly higher physical activity was observed in the mice fed on diet with 10% fat, in comparison with mice fed on diets with higher fat content when protein was fixed at 10% ($p = 2.71 \times 10^{-5}$), with no differences between other diet groups (Fig. 4 E). When protein was fixed at 25%, there were no significant differences in physical activity between mice fed on diets with variable fat content, except significantly higher physical activity in mice fed with diets with 25% fat (Fig. 4 F). No significant differences were observed in DEE ($p = 0.060$ for 10% protein and $p = 0.737$ for 25% protein) and REE ($p = 0.017$ for 10% protein and $p = 0.421$ for 25% protein) either with 10% or 25% protein (Figs. 4 E, F). Significantly higher RER were observed in the mice fed on diets with fat content lower than 30% either with 10% or 25% protein, with no differences between mice fed on diets with fat content over 30% regardless of protein content ($p = 1.78 \times 10^{-8}$ for 10% protein, $p = 5.23 \times 10^{-9}$ for 25% protein) (Figs. 4 E, F).

The responses of four other strains on diets with variable fat content confirmed that dietary fat regulates energy intake and adiposity even in strains regarded as 'obesity resistant'

The effect of dietary fat content was also replicated in the other 4 mouse strains (Fig. 5)**.** Energy intake of BALB/c and C3H mice increased when fat content in the diet increased to 50% fat, which was then decreased when fat content increased further to 80% ($p = 3.33 \times 10^{-7}$ for BALB/c mice, $p = 3.92 \times 10^{-4}$ for C3H mice) (Figs 5 A, C). The weight of food ingested by these two mouse strains decreased gradually when fat content increased from 10% to 80% ($p = 8.06 \times 10^{-12}$ for BALB/c mice, $p = 2.06 \times 10^{-7}$ for C3H mice) (Figs 5 A, C). Energy intake of DBA/2 and FVB mice increased gradually with increasing fat content in the diet ($p = 2.56 \times 10^{-4}$ for DBA/2 mice, $p = 0.019$ for FVB

mice), even though the mice decreased the weight of food intake ($p = 8.10 \times 10^{-7}$ for DBA/2 mice, $p = 0.002$ for FVB mice) (Figs 5 E, G). Body weight ($p = 2.08 \times 10^{-4}$ for BALB/c, $p = 4.63 \times 10^{-7}$ for C3H, $p = 1.48 \times 10^{-8}$ for DBA/2 and $p = 3.004 \times 10^{-6}$ for FVB) and body fatness ($p = 2.05 \times 10^{-4}$ for BALB/c, $p = 3.88 \times 10^{-8}$ for C3H, $p = 1.67 \times 10^{-8}$ for DBA/2 and $p = 4.17 \times 10^{-7}$ for FVB) were increased significantly in all 4 stains with similar trends, while there were no significant differences in body lean mass in all 4 strains ($p = 0.093$ for BALB/c, $p = 0.001$ for C3H, $p = 0.020$ for DBA/2 and $p = 0.023$ for FVB) (Figs 5 B, D, F, H).

Sucrose content in the diet does not drive energy intake and affect adiposity in mice

In all the above diets dietary sucrose was fixed at 5% by energy. The effect of sucrose on body adiposity was investigated by fixing protein content at 25% and fat content at 41.7%, while varying the contribution of the sucrose in the carbohydrate fraction from 5 to 30 % of total energy (see Table S1 for diet details). Sucrose intake also increased linearly in relation to sucrose content in the diet ($p = 8.0 \times 10^{-39}$) (Fig. S5 A). Energy intake over the last 10 days, however, remained constant in the mice fed on diets with variable sucrose content, with no significant differences between different sucrose groups ($p = 0.320$). Energy intake over the entire experimental period also showed the same trend (fig. S5 B). Body weight ($p = 0.855$), fat mass ($p = 0.620$) and lean mass ($p = 0.902$) also did not differ significantly in relation to the sucrose content (Fig. S5 C). No significant differences were observed in DEE ($p = 0.435$), REE ($p = 0.350$), RER ($p = 0.473$) and physical activity ($p = 0.994$) (Fig. S5 D).

Hypothalamic hunger signaling pathways and adipose tissue browning related signaling pathways of mice fed on diets with variable protein and fat content

To investigate if protein and fat contents of the diets had effects on energy balance via the canonical hunger signaling pathways in the hypothalamus, RNAseq was performed on RNA

extracted from the hypothalami of mice exposed to the different diets, followed by alignment using standard tools, and pathway analysis using the IPA software. We then explored the impacts of fat and protein on gene expression profiles across all diets using general linear modelling (GLM), with gene expression as the dependent variables and dietary levels and interactions of the two macronutrients as the independent variables. There were no significant associations (GLM: $p \geq 0.05$) between the four primary hypothalamic genes that drive hunger (*Pomc*, *Cart*, *Agrp*, and *Npy*) and the level of protein in the diet (Fig. 6; table S2). There were also no significant associations between protein contents of the diets and gene expression of components of the melanocortin signaling, dopamine or opioid receptor systems (Fig. 6; table S2). However, dietary protein content was associated significantly with elevated gene expression levels of three serotonin (5-HT) receptors, *Htr1a*, *Htr4* and *Htr5a* (Figs. 6; table S2). Consistent with the small non-significant calculated impacts of protein contents on overall intake, these data indicate that enormous 6 fold changes in dietary protein (from 5 to 30%) did not have any significant impacts on hunger signaling pathways in the hypothalamus. There were only seven significant changes in hypothalamic gene expression in the FGF signaling pathway (7/76 genes), including a positive correlation between the expression of *Fgf2* and protein content (Figs. S6B; table S3). Changes in the expression levels of 16/152 genes in the mTOR signaling pathway were significantly associated with dietary protein levels, most (12/16) of which were negatively related to elevated protein content of the diet (figs. S6A; table S4).

A suggested mechanism by which animals may avoid obesity is by burning off excess energy via upregulation of white adipose tissue (WAT) browning. To investigate if protein content of the diet had effects on energy balance via the browning related signaling pathways in the WAT, RNAseq was performed on RNA extracted from the sWAT and eWAT of mice exposed to the different diets, followed by alignment using standard tools, and pathway analysis using the IPA software. *Ucp1*, *Cpt1b*, *Acaca*, *Acacb*, *Pnpla2*, *Fabp4*, *Acsl1*, *Adrb1*, *Adrb2*, *Adrb3*, *Ppargc1a*, *Pparg*, *Fgf21*, *Fgfr1*, *Fgfr2*, *Fgfr3*, *Fgfr4*, *Fgfrl1*, *Cidea* are general thermogenic related genes. There were significant negative

associations between the protein contents in the diet and the expression of *Ucp1, Acaca, Fgf21 and Cidea* (fig. S7B; table S5) in WAT. *Prdm16, Tgfb1, Bmp7, Ebf2, Tbx1, Tnfrsf9, Tmem26, Slc27a1, Hoxc9, Mtus1, Kcnk3* are genes specifically related to WAT browning. *Bmp7* and *Tnfrsf9* showed significant negative associations to the dietary protein contents but the other 'browning genes' were unrelated (figs. S7B; table S5). *Sirt1, Mtor, Cyp26b1, Eya2, Hspb7, Pdk4, Rnf34, Fam63b, Egln3, Stac2, Tns2, Fgf1, Fgf10* are also involved in the browning signaling pathways. There were also no significant regressions between protein content of the diet and these genes (figs. S7B; table S5). In WAT, only 5/76 genes involved in FGF signaling showed changes in expression, including a negative correlation between protein content of the diet and *Fgf21* gene expression itself, (figs. S7C; table S6). In WAT, only 4/152 genes in the mTOR signaling pathway were correlated with dietary protein levels, and expression of Mtor itself was not significantly changed (Fig. S7A; table S7).

Significantly positive associations were evident between the fat levels of the diet and the main hedonic signaling systems linked to food intake, ie., dopamine (*Drd1* and *Drd6*) and opioid receptor (*Oprk1*, *Oprd1* and *Oprm1*) systems (Fig. 7; table S2). There was no significant association between fat contents and components of the melanocortin signaling pathway (Fig. 7; table S2). However, surprisingly there were strong significant negative associations between fat content and the two primary hypothalamic genes that drive hunger (*Npy* and *Agrp*: Fig. 7). This was not paralleled by associations between dietary fat content and the primary hunger-suppressing genes (*Pomc* and *Cart*) (Fig. 7; table S2). Nevertheless positive associations between fat content and elements of the serotonin (5-HT) receptor (*Htr2a, Htr2c, Htr1a, Htr1b, Htr5a* and *Htr4*) signaling, upregulation of which is also generally considered inhibitory of intake, were significant. These changes were consistent with the system attempting to compensate the enhanced hedonic signals, and were mirrored by the reduced weight of food ingested as the fat content increased. In addition, significantly positive associations were observed between the dietary fat levels and insulin signaling components (*Insr*, *Stat3* and *Jak2*), IGF signaling (*Igf1* and *Igf1r*), and the growth hormone receptor

(GR) (Fig. 7; table S2). In hypothalamic FGF signaling, 46/76 genes showed significant changes in gene expression in relation to dietary fat changes, 35 (including changes in *Fgf1, 2, 9, 10, 12, 13, 14* and *18*) of which were positively associated to fat content of the diet, and the other 11 (including change in *Fgfrl1*) were negative (Fig. S6 D; table S3). In hypothalamic mTOR signaling, 110/152 genes showed significant changes in gene expression, 42 of which were positively correlated to dietary fat while the other 68 were negatively correlated (Fig. S6 C; table S4).

We also investigated the impact of dietary fat levels on browning related signaling pathways in the WAT. There were significant negative relationships between the levels of fat in the diet and gene expressions of *Ucp1, Acaca, Acacb Fgfr2, Fgfr3, Fgfr4* and *Cidea* (figs. S7E; table S5). *Bmp7, Egln3* and *Tns2* showed significant negative associations to the dietary fat content while *Tgfb1, Pdk4* and *Fgf1* were positive (Fig. S7E; table S5). With respect to WAT FGF signaling, there were 30/76 significant correlations to dietary fat content in gene expression in components of the FGF pathway, 18 of which were positive while the others were negative (Fig. S7F; table S6). Expressions of *Fgf1, 13* and *18* were positively correlated with dietary fat, while gene expression of *Fgf2, 3, 4, 11* and *12*, were negatively correlated to dietary fat contents (Fig. S7F; table S6). In the WAT 46/152 genes in the mTOR signaling pathway correlated significantly to dietary fat content, 29 of which were positive while the others were negative, and there was no significant change in expression of *Mtor* itself (Fig. S7D; table S7).

Discussion

Alternative ideas about food intake regulation include the protein leverage hypothesis, the homeostatic energy regulation model and the hedonic overdrive model (Fig 1). It is clear from the responses to the diets where protein varied, but fat contents were kept constant, that the responses of these mice conformed most closely to the pattern illustrated in Fig. 1B: regulating energy rather than protein intake. The percentage level of protein leverage was not significantly different to zero in all

five strains (using p = 0.05 and Bonferroni correction for multiple testing). We therefore found little evidence to support the notion that as protein content declined the mice were stimulated to consume more food to compensate. In 4/5 strains there was a non-significant trend in the direction of elevated intake, but in BALB/c mice it reached significance. This absence of a strong effect was mirrored by the lack of stimulation of the main hunger pathway in the hypothalami of C57BL/6 mice in response to declining dietary protein, and there was no impact on FGF signaling. Nevertheless, small increases in energy intake over protracted durations such as used in the current experiment could in theory lead to elevated fat deposition. However, the trends we observed were insufficient to generate elevated adiposity in any of the strains. The pattern of change in the aminopeptidase enzyme levels mirrored the protein levels in the diet. Mice did not upregulate this enzyme to facilitate greater uptake of protein as its levels in the diet declined, but rather its expression mirrored the amount of protein that had to be handled as a by-product of taking in a fixed quantity of food energy that varied in its protein content. These data suggest that in mice food intake is primarily regulated by energy requirements (energy homeostatic model), and that protein does not leverage intake to cause adiposity.

Our conclusions contrast several previous studies of the protein leverage hypothesis across a wide range of species from insects to humans. These also include two studies of mice (Huang et al., 2013; Sorensen et al., 2008). Several differences between our study and these previous studies may explain the different conclusions drawn. First, the mice in the previous studies were younger when the experimental manipulations started. In one case (Sorensen et al., 2008), the mice were only 5 weeks old and hence still growing. For growing animals, protein intake may be more important to facilitate growth and hence may play a greater role in driving intake. Second, the diets covered a different range of fat and protein contents. In particular, their diets had lower fat content (c. 15% by energy) compared with 20 and 60% by energy used here. Moreover, the range of protein contents was also different: from 10 to 50% by energy compared with 5 to 30% by energy used here. The biggest difference, however, is how the effects of protein on intake were quantified and described. Hence, while energy intake

increased as the protein content declined, and hence the data were claimed to support the protein leverage hypothesis, the effect was modest (from 1780 kJ to 2100 kJ). Using the same approach for quantification used here, the extent of protein leverage was only 4.6% in NMRI mice (Sorensen et al., 2008) and 4.9% in C57BL/6 mice (Huang et al., 2013), similar to the observations here. In our case, this was not significantly different to zero and insufficient to drive elevated adiposity.

When we allowed the fat content of the diets to vary, but held the protein content constant there was a clear stimulatory effect of the fat content on total energy intake. At low fat contents (< 40% fat) the mice ate very similar weights of food, but because the energy density was greater as the fat content increased, their energy intakes increased. Above 50% fat in the diet the mice reduced the weight they consumed, but still continued to eat more energy than when feeding on the low fat diets. This elevated consumption of energy led to increased adiposity which was highest at dietary fat levels of 50-60%. The normal homeostatic regulation of energy intake revealed when we varied protein content was therefore perturbed by elevated levels of fat and hence more similar to the hedonic overdrive model (Fig 1D). This elevated energy intake was associated with hypothalamic stimulation of the dopamine, opioid and 5-HT receptor systems, all pointing to increased reward when ingesting higher levels of fat. These data indicate that overconsumption of energy occurs in mice primarily because dietary fat stimulates hypothalamic hedonic systems that override the homeostatic control (Fig 1D see also Berthoud 2008). These stimulatory changes in the hypothalamic hedonic system appeared to be counter-regulated by reductions in the primary hypothalamic drivers of hunger (*Npy* and *Agrp*) and increases in elements of the 5-HT signaling system. This pattern was associated with reductions in the actual weight of food that was ingested as fat content increased, but until fat contents were elevated above 60% this effect was insufficient to blunt energy intake. The responses of the mice to fat intake therefore departed from the simple linear models introduced above (Fig 1). Since we only measured gene expression in the hypothalamus we can only make inferences with respect to the hedonic system located in this brain region. Clearly other brain other regions mediate reward and may be involved in

the impact of fat on dietary intake. We found very limited evidence of stimulated white adipose tissue browning as dietary fat content increased, not changes in physical activity levels, and these were insufficient to have any impact on whole body metabolic rates. When dietary fat levels are fixed, variation in other macronutrients appears largely irrelevant for weight and adiposity regulation. Interestingly, given the strong focus in public health messages about refined sugar intake (e.g. the WHO 2015 guidelines that sugar intake should be limited to 10% of energy) we found no stimulation of energy intake or elevated adiposity by varying levels of sucrose in the diet (up to 30% by calories) independent of other macronutrient changes.

These data have one of two important implications. First, if humans respond in the same way as mice to dietary macronutrient manipulations, with adiposity due only to the hedonic qualities of fat, then a declining protein content of diets over the time course of the obesity epidemic is unlikely to have been a causal factor driving elevated consumption of energy, and hence obesity (Simpson and Raubenheimer, 2005). Hence, increasing protein in the diet will likely not be an effective counter-measure. Alternatively, if humans do not respond in the same way as mice, and are susceptible to protein leverage (Gosby et al., 2011; Martens et al., 2013; Martinez-Cordero et al., 2012; Simpson et al., 2003) and to dietary sucrose (Fortino et al., 2007; Kuhnle et al., 2015), then this calls into question the validity of using mice as a model to study energy regulation and obesity in humans.

Author Contributions

S.H. analyzed the data and co-wrote the manuscript. D.Y. and L.L. managed the overall day to day experimental process and contributed to the data collection. L.W. and A.D. performed the RNA extractions and the RNASeq and IPA related analysis. Q.L., L.J. and W.N. performed the analysis of the activity of aminopeptidase-N in the jejunum. Y.W., Y.X., B.L., M.L., G.W., X.Z., J.L., E.C., A.W.D., M.M. & J.T. were involved in the initial experimental design and performed body weight, food intake

and body composition measurements. J.R.S. directed the project, conceived and designed the experiments, contributed to the analysis and co-wrote the paper. All authors approved the final version prior to submission for publication.

Acknowledgements

Full details of methods and materials are provided in the supplementary materials and the project has been registered at the Open Science Framework (doi: 10.17605/OSF.IO/YH9GZ). The study was funded by Chinese Academy of Sciences Strategic program (XDB13030100), the 1000 Talents program and a Wolfson merit award to J.R.S, and by the National Natural Science Foundation of China (31570409) and funds from Guangdong Academy of Sciences (2016GDASPT-0215; 2017GDASCX-0107) to Q.L. The authors declare no conflicting financial interests.

References

Anders, S., McCarthy, D.J., Chen, Y., Okoniewski, M., Smyth, G.K., Huber, W., and Robinson, M.D. (2013). Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nature protocols *8*, 1765-1786.

Arch, J.R., Hislop, D., Wang, S.J., and Speakman, J.R. (2006). Some mathematical and technical issues in the measurement and interpretation of open-circuit indirect calorimetry in small animals. International journal of obesity (2005) *30*, 1322-1331.

Avena, N.M., Rada, P., and Hoebel, B.G. (2008). Evidence for sugar addiction: behavioral and neurochemical effects of intermittent, excessive sugar intake. Neuroscience and biobehavioral reviews *32*, 20-39.

Bekelman, T.A., Santamaría-Ulloa, C., Dufour, D.L., Marín-Arias, L., and Dengo, A.L. (2017). Using the protein leverage hypothesis to understand socioeconomic variation in obesity. American Journal of Human Biology *29*, n/a-n/a.

Berridge, K.C., Ho, C.Y., Richard, J.M., and DiFeliceantonio, A.G. (2010). The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. Brain research *1350*, 43-64.

Berthoud, H.R. (2011). Metabolic and hedonic drives in the neural control of appetite: who is the boss? Current opinion in neurobiology *21*, 888-896.

Berthoud, H.R., and Morrison, C. (2008). The brain, appetite, and obesity. Annual review of psychology *59*, 55-92.

Berthoud, H.R., Munzberg, H., and Morrison, C.D. (2017). Blaming the Brain for Obesity: Integration of Hedonic and Homeostatic Mechanisms. Gastroenterology *152*, 1728-1738.

Bullard, J.H., Purdom, E., Hansen, K.D., and Dudoit, S. (2010). Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC bioinformatics *11*, 94.

Chen, Y., Lun, A.T.L., and Smyth, G.K. (2014). Differential expression analysis of complex RNA -seq experiments using edgeR. In: Statistical Analysis of Next Generation Sequence Data, Somnath Datta and Daniel S Nettleton (eds), Springer, New York.

Derous, D., Mitchell, S.E., Green, C.L., Chen, L., Han, J.D., Wang, Y., Promislow, D.E., Lusseau, D., Speakman, J.R., and Douglas, A. (2016). The effects of graded levels of calorie restriction: VI. Impact of short-term graded calorie restriction on transcriptomic responses of the hypothalamic hunger and circadian signaling pathways. Aging *8*, 642-663.

Felton, A.M., Felton, A., Raubenheimer, D., Simpson, S.J., Foley, W.J., Wood, J.T., Wallis, I.R., and Lindenmayer, D.B. (2009). Protein content of diets dictates the daily energy intake of a free-ranging

primate. Behavioral Ecology *20*, 685-690.

Fortino, M.A., Lombardo, Y.B., and Chicco, A. (2007). The reduction of dietary sucrose improves dyslipidemia, adiposity, and insulin secretion in an insulin-resistant rat model. Nutrition *23*, 489-497.

Gearhardt, A.N., Yokum, S., Orr, P.T., Stice, E., Corbin, W.R., and Brownell, K.D. (2011). Neural correlates of food addiction. Archives of general psychiatry *68*, 808-816.

Gosby, A.K., Conigrave Ad Fau - Lau, N.S., Lau Ns Fau - Iglesias, M.A., Iglesias Ma Fau - Hall, R.M., Hall Rm Fau - Jebb, S.A., Jebb Sa Fau - Brand-Miller, J., Brand-Miller J Fau - Caterson, I.D., Caterson Id Fau - Raubenheimer, D., Raubenheimer D Fau - Simpson, S.J., and Simpson, S.J. (2011). Testing protein leverage in lean humans: a randomised controlled experimental study.

Gosby, A.K., Lau, N.S., Tam, C.S., Iglesias, M.A., Morrison, C.D., Caterson, I.D., Brand-Miller, J., Conigrave, A.D., Raubenheimer, D., and Simpson, S.J. (2016). Raised FGF-21 and Triglycerides Accompany Increased Energy Intake Driven by Protein Leverage in Lean, Healthy Individuals: A Randomised Trial. PloS one *11*, e0161003.

Guo, J., and Hall, K.D. (2009). Estimating the Continuous-Time Dynamics of Energy and Fat Metabolism in Mice. PLoS computational biology *5*, e1000511.

Guo, J., Jou, W., Gavrilova, O., and Hall, K.D. (2009). Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets. PloS one *4*, e5370.

Hall, K.D., Heymsfield, S.B., Kemnitz, J.W., Klein, S., Schoeller, D.A., and Speakman, J.R. (2012). Energy balance and its components: implications for body weight regulation. The American Journal of Clinical Nutrition *95*, 989-994.

Haslam, D.W., and James, W.P.T. (2005). Obesity. The Lancet *366*, 1197-1209.

Hawley, J., Simpson, S.J., and Wilder, S.M. (2016). Flesh flies regulate the consumption of 3

macronutrients to maximize lifespan and egg production. Behavioral Ecology *27*, 245-251.

Huang, X., Hancock, D.P., Gosby, A.K., McMahon, A.C., Solon, S.M.C., Le Couteur, D.G., Conigrave, A.D., Raubenheimer, D., and Simpson, S.J. (2013). Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. Obesity *21*, 85-92.

Kuhnle, G.G., Tasevska, N., Lentjes, M.A., Griffin, J.L., Sims, M.A., Richardson, L., Aspinall, S.M., Mulligan, A.A., Luben, R.N., and Khaw, K.T. (2015). Association between sucrose intake and risk of overweight and obesity in a prospective sub-cohort of the European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk). Public health nutrition *18*, 2815-2824.

Liu, Q.S., and Wang, D.H. (2007). Effects of diet quality on phenotypic flexibility of organ size and digestive function in Mongolian gerbils (Meriones unguiculatus). Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology *177*, 509-518.

Liu, Q.S., Zhang, Z.Q., Caviedes-Vidal, E., and Wang, D.H. (2013). Seasonal plasticity of gut morphology and small intestinal enzymes in free-living Mongolian gerbils. Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology *183*, 511-523.

Lund, S.P., Nettleton, D., McCarthy, D.J., and Smyth, G.K. (2012). Detecting differential expression in RNA-sequence data using quasi-likelihood with shrunken dispersion estimates. Statistical Applications in Genetics and Molecular Biology Volume 11, Issue 5, Article 8.

Lu, J., Tomfohr, J.K., and Kepler, T.B. (2005). Identifying differential expression in multiple SAGE libraries: an overdispersed log-linear model approach. BMC bioinformatics *6*, 165.

Maroux, S., Louvard, D., and Baratti, J. (1973). The aminopeptidase from hog intestinal brush border. Biochimica et biophysica acta *321*, 282-295.

Martens, E.A., Lemmens S.G., and Westerterp-Plantenga, M.S. (2013). Protein leverage affects energy

intake of high-protein diets in humans.

Martinez-Cordero, C., Kuzawa, C.W., Sloboda, D.M., Stewart, J., Simpson, S.J., and Raubenheimer, D. (2012). Testing the Protein Leverage Hypothesis in a free-living human population. Appetite *59*, 312-315.

McCarthy, D.J., Chen, Y., and Smyth, G.K. (2012). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Research *40*, 42884297.

Nixon, J.P., Zhang, M., Wang, C., Kuskowski, M.A., Novak, C.M., Levine, J.A., Billington, C.J., and Kotz, C.M. (2010). Evaluation of a quantitative magnetic resonance imaging system for whole body composition analysis in rodents. Obesity (Silver Spring, Md.) *18*, 1652-1659.

Prentice, A.M., and Jebb, S.A. (1995). Obesity in Britain: gluttony or sloth? BMJ (Clinical research ed.) *311*, 437-439.

R Core Team (2015). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. (https://www.R-project.org/).

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139–140.

Robinson, M.D., and Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biology *11*, R25.

Robinson, M.D., and Smyth, G.K. (2007). Moderated statistical tests for assessing differences in tag abundance. Bioinformatics *23*, 2881–2887.

Robinson, M.D., and Smyth, G.K. (2008). Small sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics *9*, 321–332.

Simpson, S.J., Batley, R., and Raubenheimer, D. (2003). Geometric analysis of macronutrient intake

in humans: the power of protein? Appetite *41*, 123-140.

Simpson, S.J., and Raubenheimer, D. (2005). Obesity: the protein leverage hypothesis. Obesity reviews : an official journal of the International Association for the Study of Obesity *6*, 133-142.

Simpson, S.J., and Raubenheimer, D. (2012). The Nature of Nutrition: A Unifying Framework from Animal Adaptation to Human Obesity. (Princeton Unverisity Press).

Sorensen, A., Mayntz, D., Raubenheimer, D., and Simpson, S.J. (2008). Protein-leverage in mice: the geometry of macronutrient balancing and consequences for fat deposition. Obesity (Silver Spring, Md.) *16*, 566-571.

Speakman, J.R. (2013). Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. Front Physiol *4*, 34.

Speakman, J.R., and Keijer, J. (2012). Not so hot: Optimal housing temperatures for mice to mimic the thermal environment of humans. Molecular metabolism *2*, 5-9.

Swinburn, B.A., Sacks, G., Hall, K.D., McPherson, K., Finegood, D.T., Moodie, M.L., and Gortmaker, S.L. (2011). The global obesity pandemic: shaped by global drivers and local environments. The Lancet *378*, 804-814.

Tschop, M.H., Speakman, J.R., Arch, J.R., Auwerx, J., Bruning, J.C., Chan, L., Eckel, R.H., Farese, R.V., Jr., Galgani, J.E., Hambly, C., et al. (2011). A guide to analysis of mouse energy metabolism. Nature methods *9*, 57-63.

van Dam, R.M., and Seidell, J.C. (2007). Carbohydrate intake and obesity. Eur J Clin Nutr *61*, S75-S99.

Weir, J.B. (1949). New methods for calculating metabolic rate with special reference to protein metabolism. The Journal of physiology *109*, 1-9.

Westerterp, K.R., and Speakman, J.R. (2008). Physical activity energy expenditure has not declined since the 1980s and matches energy expenditures of wild mammals. International journal of obesity (2005) *32*, 1256-1263.

World Health Organization (WHO). (2016). *Obesity and overweight*. (*http://www.who.int/mediacentre/factsheets/fs311/en/*).

Willett, W.C. (1998). Dietary fat and obesity: an unconvincing relation. The American Journal of Clinical Nutrition *68*, 1149-1150.

Zhang, L.N., Morgan, D.G., Clapham, J.C., and Speakman, J.R. (2012). Factors predicting nongenetic variability in body weight gain induced by a high-fat diet in inbred C57BL/6J mice. Obesity (Silver Spring, Md.) *20*, 1179-1188.

Materials and Methods

Ethical statement. All procedures were reviewed and approved by the Institutional Review Board, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences: approval numbers AP2014011 and AP2015004, pertaining to the study of C57BL/6 mice and the 4 additional strains study, respectively.

Registration. This project has been registered at the open science framework (doi: 10.17605/OSF.IO/YH9GZ).

Mice. C57BL/6, DBA/2, BALB/c, FVB and C3H mouse strains were used. All the mice were male and purchased at age 8 weeks from Charles River and acclimated to the animal house for 2 weeks. We exposed these mice to a panel of 24 different diets that varied in their fat, carbohydrate and protein contents (details below). The primary outcomes were food intake and body weight/adiposity. Based on the previously reported variation in the response of C57BL/6 mice to high fat diet (Zhang et al., 2012), a power analysis indicated that to detect an effect size of 0.3 g/day in mean food intake between groups with 80% power at alpha = 0.05 in a one-way ANOVA with 6 levels, a sample of 20 per group was necessary. A total of 480 male C57BL/6 mice were therefore used to investigate the effect of protein and fat content on adiposity, at 20 mice per diet. For the validation work on the other strains, we reduced the sample size to 8 per group. This increased the detectable effect size at 80% power to 0.48 g per day. Finally, we fixed the protein content at 25% and fat content at 41.7%, with only changing sucrose content (5% to 30%) in the diet, to investigate if changing sucrose content affect body fatness in mice. C57BL/6 mice were used at 10 mice per diet. In total, the number of mice used across all experiments was 924.

All mice were housed individually (in a specific pathogen free facility) and maintained in

environmentally controlled conditions (temperature 22-24°C, 12:12 LD cycle lights on at 0730h). Appropriate housing temperature for studies of mice and how best to mimic the situation in humans is disputed. Here we followed the suggestion in Speakman and Keiger (2012) that when provided with nesting and bedding materials standard room temperature (22-24 ^{o}C) may not be too far from representative. They were provided *ad libitum* access to food and water and were monitored for health status daily. All mice were fed a standard diet with 10% fat and 20% protein (D12450B, Research Diets Ltd) for 2 weeks as the baseline period. Following 2 weeks of baseline monitoring (at age 12 weeks), all mice were randomly allocated to different groups and switched to the experimental diets for 10-12 weeks (12 weeks for C57Bl/6 and 10 weeks for the other 4 strains). After 10-12 weeks all mice were sacrificed and dissected.

Experimental diets. In total, mice were fed on 5 diet series, each series consisting of 6 different diets (total = 30 diets), full details of which are in Supplementary materials Table S1. In the first two series (Series 1: D14071601 – D14071606 and series 2: D14071607 – D14071612) we fixed the level of fat at 20% (series 1) or 60% (series 2) by energy, and varied the protein content from 5% to 30% by energy. The protein source was casein. The balance was made up by carbohydrate (roughly equal mix of corn starch and maltodextrose). The source of fat was a mix of cocoa butter, coconut oil, menhaden oil, palm oil and sunflower oil. This mix was designed to match the balance of saturated, mono-unsaturated and polyunsaturated fats (ratio 47.5: 36.8: 15.8) and the n-6: n-3 ratio (14.7: 1) in the typical western diet. The proportions of the different fat constituents and hence fatty acid distributions did not change as the total fat content changed. Sucrose and cellulose were both fixed 5% by energy and weight respectively, and all diets were supplemented with a standard vitamin and mineral mix. In the second two series of diets (series 3: D14071613 – D14071618 and series 4: D14071619 – D14071624) we

fixed the level of protein at 10% (series 3) or 25% (series 4) by energy and then allowed the fat content to vary. When the protein was at 10% the six fat contents were 10, 30, 40 50 70 and 80%. When the protein was 25% the six fat contents were 8.3, 25, 33.3, 41.7, 58.3 and 66.6%. Fat, protein and carbohydrate composition were the same as those in the first 2 series. In these diets the sucrose, cellulose and vitamin and mineral contents were the same as the diets in series 1 and 2. In a fifth series of diets we fixed the fat at 41.7%, and the protein at 25% and then allowed the sucrose to vary between 5% and 30% in 5% steps (diet codes D16053101 to D16053105). All these diets can be ordered direct from research diets (www.researchdiets.com) using the diet codes provided.

Body weight, food intake and body composition measurements. Over the 2 week baseline period, body weight (BW) and food intake (FI) were measured and recorded daily. Following this, all mice were exposed to the experimental diets for 12 weeks and BW and FI were also measured daily. Body composition including fat mass and lean mass of all mice were measured using an EchoMRI^{TM} Body Composition Analyzer (Nixon et al., 2010) weekly over the 12 week experimental period. Canola oil was used as the standard for the measurements. Food intake was measured form the weight of food that went missing from the food hopper each day. Mice occasionally pulled pellets of food through the hopper bars and so a thorough search of the cage was made to return any uneaten food to the hopper before weighing. Very occasionally mice ground their food into small pieces and the large number of small fragments in the bedding could not be measured. We eliminated occasional days when the lab notes indicated this had been a problem. For the individuals that consistently ground their food we eliminated the entire track of food intake data. The numbers of animals that ground food and were eliminated were as follows: n = 11 for C57BL/6 mice, n = 0 for BALB/c and DBA/2 mice, n = 7 for C3H mice, and n = 2 for FVB mice.

Energy expenditure and physical activity measurements. Mice were moved to a TSE PhenoMaster/LabMaster system for 2 days after 10 weeks on the experimental diets. This duration is sufficient to obtain an accurate measure of energy metabolism (Speakman, 2013). After calibrating the system with the reference gases (20.950%/0.05% for O_2/CO_2), the oxygen (O_2) consumption (mL/min), carbon dioxide (CO_2) production (mL/min), respiratory exchange rate (RER = VCO_2/VO_2) and locomotor activity (Counts), as well as food and water intake (g), were recorded. Measurements were taken at 1-min intervals for the whole period. Energy expenditure (EE) was calculated from O_2 consumption and CO_2 production according to the Weir Equation: EE (kJ/day) = ((3.9 x VO_2 (mL/min) + 1.1 x VCO_2 (mL/min)) x 1440 (min)/1000 x 4.184 (Weir, 1949). In addition to point estimates of energy expenditure we also used the software provided by Guo and Hall (2009)/Guo et al. (2009) to calculate the energy expenditure over the entire 12 week manipulation period from the data on body weight, body fat mass and food intake changes.

The activity of aminopeptidase-N assay. Aminopeptidase-N assays were performed according to a previously reported method (Liu and Wang, 2007; Liu et al., 2013). Immediately after sacrifice, the small intestines were opened and rinsed with cold 0.9% NaCl solution. Briefly, 0.8~1.0 cm pieces of jejunum (in mid-region of small intestines) were snap frozen in liquid nitrogen and then kept at -80°C for enzyme assays. Aminopeptidase-N assays were carried out using L-alanine-p-nitroanilide as a substrate (Maroux et al., 1973). Prior to enzyme assays, intestinal samples were thawed at 4°C and homogenized (30 s, using a homogenizer setting in 10 000 rpm) in 0.9% NaCl (1:10, w/v) in an ice-water bath. Ten μL tissue homogenate was mixed with 1 mL assay solution (2.04 mM L-alanine-p-nitroanilide in 0.2 M phosphate buffer (NaH_2PO_4/Na_2HPO_4, pH 7.0)) and incubated at 37 °C for 10 min. The reaction was then terminated with 3 mL chilled 2 N acetic acid. The absorbance was measured

at 384 nm. The activity was determined using a p-nitroanilide standard curve, and presented as activity per unit intestinal wet mass (μmol /min·g wet tissue).

RNA isolation and transcriptome analysis (hypothalamus and white adipose tissue). From each diet group, the hypothalami of 8/20 individuals were collected. The left halves of two, and the right halves of another two, were pooled together as one sample, and the same was performed with the other 4 hypothalami, resulting in each diet group having 2 pooled samples of 4 hypothalami (n = 48 samples in total across 24 diets). From each diet group, the sWAT and eWAT of 12/20 individuals were also collected. A small piece from each of six sWAT collections were pooled together as one sample, and the same was performed with the other six eWAT collections. In this way each diet group had one pooled sWAT sample and one pooled eWAT sample (also n = 48 across 24 diets). The total RNA of the hypothalamus and white adipose tissues was isolated using the RNeasy Mini Kit (QIAGEN, 74104) according to manufacturer's protocol. All sequencing was carried out using the Illumina NextSeq 500 sequencer. RNA fragments were sequenced by 75 bp long reads from paired ends (PE 2x75 bp, 150 bp per fragment). FASTQ data files were analyzed using FASTQC (a quality control tool for high throughput sequence data; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Paired-end reads were mapped to the *Mus musculus* genome (GRCm38) using Bowtie 2-2.1.0, Samtools-0.1.19, and TopHat-2.0.10; uniquely mapped reads for each gene were counted against the GTF file of GRCm38 provided by Ensembl (release 81) using HTSeq-0.6.1p1 using the strand = reverse; after obtaining the count data from the TopHat-HTSeq pipeline, genes with the counts per million (CPM) value ≥ 1 in at least one of the 24 diets group were retained (Anders et al., 2013).

Statistical analysis. Statistical analysis were performed using the R platform (R Core Team, 2015), IBM SPSS 20, GraphPad Prism 6.0 and Microsoft Excel. All values are expressed as mean ± SD.

Whole animal oxygen consumption and energy expenditures were evaluated using ANCOVA (Arch et al., 2006; Tschop et al., 2012). One-way ANOVA with Bonferroni post-testing were calculated using IBM SPSS 20. Differences were considered significant if $p < 0.05$. The transcriptome data counts were analyzed by using the R package 'edgeR' (version 3.12.0, R version 3.2.2) (Robinson and Smyth, 2007; Robinson and Smyth, 2008; Robinson et al., 2010; Robinson and Oshlack, 2010; Lund et al.,2012; McCarthy et al., 2012; Chen et al., 2014) to apply General Linear Modelling (GLM) to analyse the simultaneous effects of both protein and fat content of diets. The protein or fat level changes were treated as continuous numeric covariates; Let π_{gi} be the fraction of all mapped reads in the i_{th} sample that originate from gene g. The total number of mapped reads in library i was denoted by Ni and the number that map to the gene g by y_{gi}. Then:

$E(y_{gi}) = \mu_{gi} = N_i\pi_{gi}$

$\log(\mu_{gi}) = x_i\beta_g + \alpha_g + \log(N_i)$ (Bullard et al., 2010; Lu et al., 2005)

Here x_i is a vector of covariates that specifies the treatment conditions applied to RNA sample i, β_g is a vector of regression coefficients by which the covariate effects are mediated for gene g, and α_g is a vector of intercepts. The use of the negative binomial distribution is equivalent to treating the π_{gi} as gamma distributed. The GLM model used here was: ˜p+f+p:f, which regresses gene expression against the protein (p) and fat contents (f) of diets, as well as their interaction (p:f). However, when the effect of the interaction was not significant (p value ≥ 0.05), the interaction term was dropped and a revised model (~p+f) was utilised. To make the comparisons we used the specific energy balance data that pertained to the individuals that had been selected for the tissue collections to perform RNAseq. In all cases these subsets of individuals did not differ from the entire group of individuals on each diet. The statistical significance was set at p value < 0.05. The p values and beta coefficients were loaded into

the Ingenuity Pathway Analysis (IPA) program (Ingenuity Systems; http://www.ingenuity.com/) for core analysis. We used a custom built pathway for hunger signaling in the hypothalamus (Derous et al., 2016) to visualize the dietary impacts on hunger signaling, and the canonical pathways for FGF and mTOR signaling available in IPA.

Figures

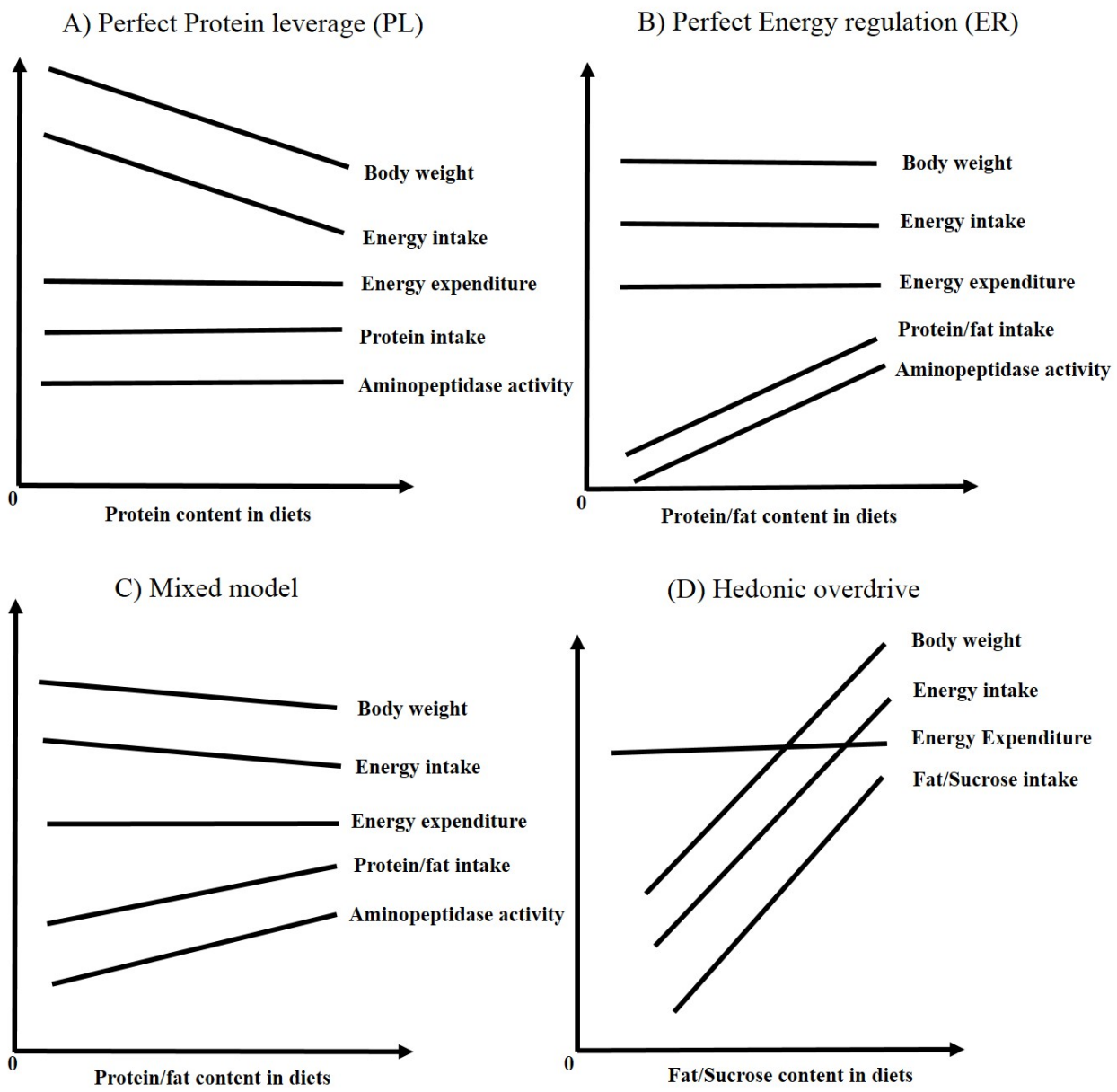


Fig. 1 Different models of energy intake, body weight and energy expenditure in mice. Trends of body weight, energy intake, energy expenditure, absolute protein intake and the activity of aminopeptidase in (A) the perfect protein leverage model. (B) The perfect energy regulation model and (C) in a mixed model of protein leverage and energy regulation. (D) Hedonic overdrive model.

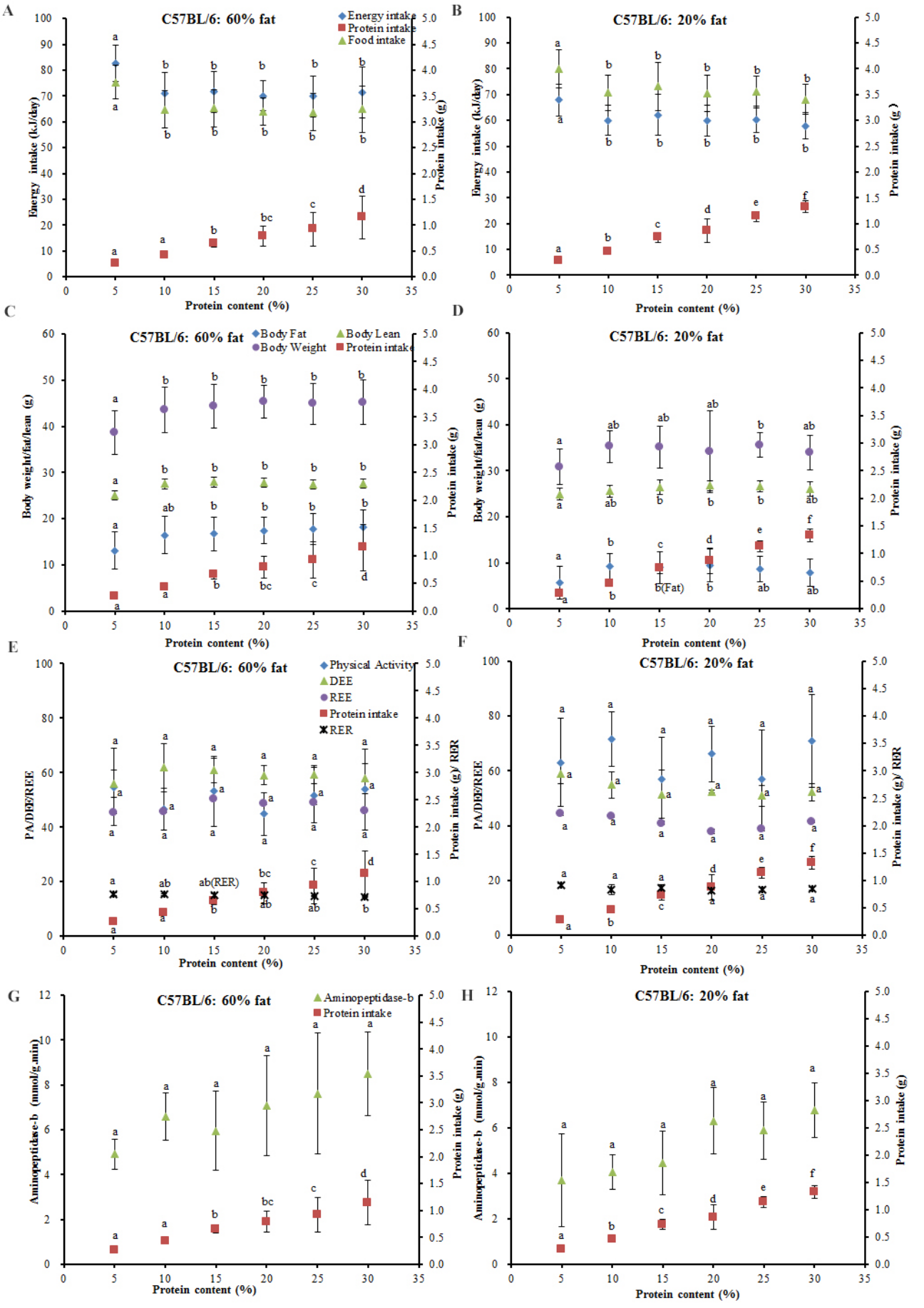

A
C57BL/6: 60% fat
Energy intake
Protein intake
Food intake
Energy intake (kJ/day)
Protein intake (g)
Protein content (%)
B
C57BL/6: 20% fat
Energy intake (kJ/day)
Protein intake (g)
Protein content (%)
C
C57BL/6: 60% fat
Body Fat
Body Lean
Body Weight
Protein intake
Body weight/fat/lean (g)
Protein intake (g)
Protein content (%)
D
C57BL/6: 20% fat
Body weight/fat/lean (g)
Protein intake (g)
b(Fat)
Protein content (%)
E
C57BL/6: 60% fat
Physical Activity
DEE
REE
Protein intake
RER
PA/DEE/REE
Protein intake (g)/ RER
ab(RER)
Protein content (%)
F
C57BL/6: 20% fat
PA/DEE/REE
Protein intake (g)/ RER
Protein content (%)
G
C57BL/6: 60% fat
Aminopeptidase-b
Protein intake
Aminopeptidase-b (nmol/g.min)
Protein intake (g)
Protein content (%)
H
C57BL/6: 20% fat
Aminopeptidase-b (nmol/g.min)
Protein intake (g)
Protein content (%)

Fig. 2 Changes of energy intake, body composition, energy expenditure and activity of aminopeptidase-N of mice following exposure to diets with variable protein content and fixed fat (60% and 20%). Values are represented as mean ± SD. (A-B) Energy intake averaged over the last 10 days of measurement. (C-D) Body composition. (E-F) Energy expenditure and physical activity. (G-H) Activity of aminopeptidase-N. Groups with a same letter were not significantly different ($p > .05$). A total of 120 mice were used with 20 mice per diet.

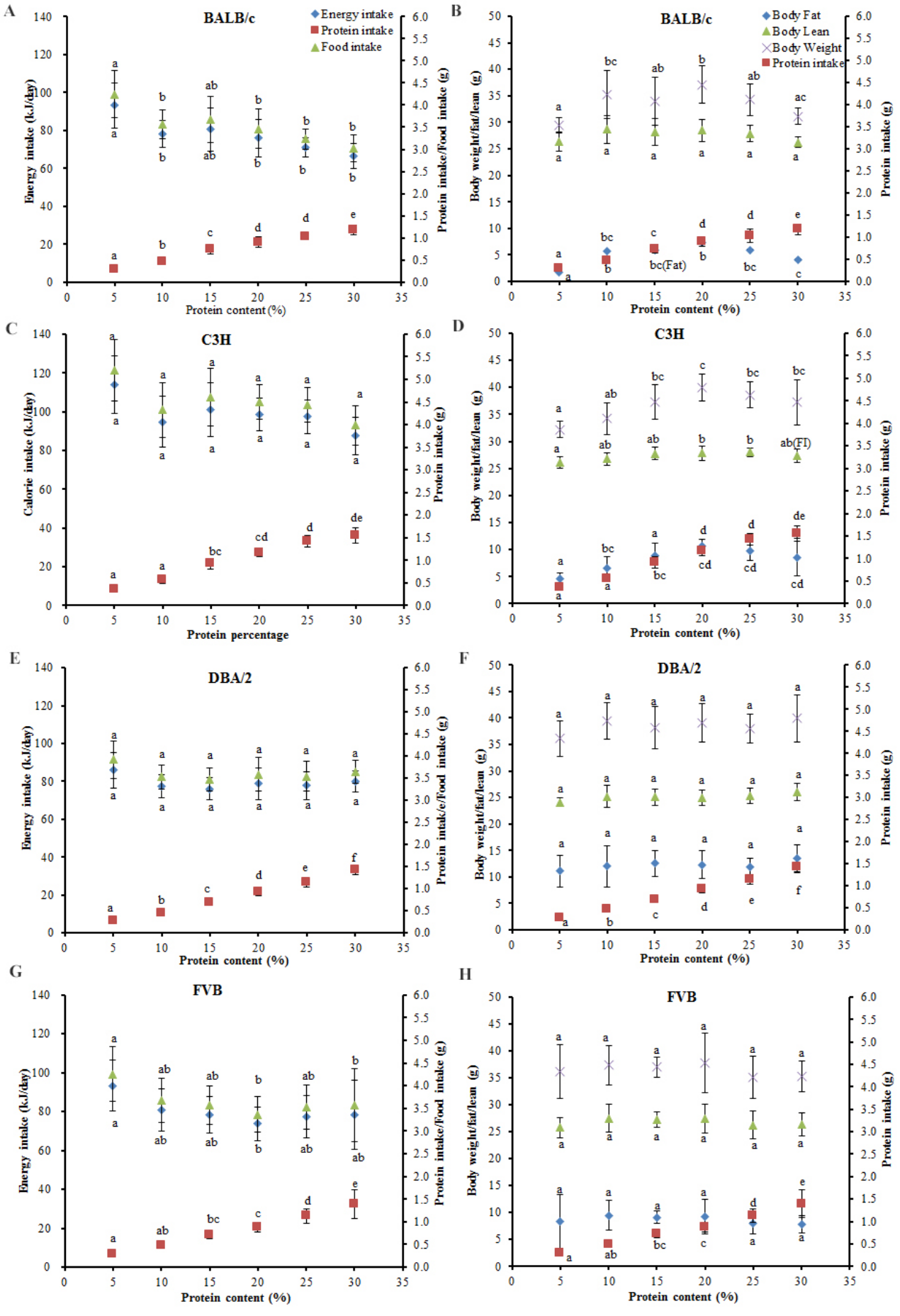

A
BALB/c
Energy intake
Protein intake
Food intake
Energy intake (kJ/day)
Protein intake/Food intake (g)
Protein content (%)
B
BALB/c
Body Fat
Body Lean
Body Weight
Protein intake
Body weight/fat/lean (g)
Protein intake (g)
Protein content (%)
C
C3H
Calorie intake (kJ/day)
Protein intake (g)
Protein percentage
D
C3H
Body weight/fat/lean (g)
Protein intake (g)
Protein content (%)
E
DBA/2
Energy intake (kJ/day)
Protein intake/e/Food intake (g)
Protein content (%)
F
DBA/2
Body weight/fat/lean (g)
Protein intake (g)
Protein content (%)
G
FVB
Energy intake (kJ/day)
Protein intake/Food intake (g)
Protein content (%)
H
FVB
Body weight/fat/lean (g)
Protein intake (g)
Protein content (%)

Fig. 3 Energy intake, body weight and body composition changes in four mouse strains after feeding on diets with variable protein contents and 60% fat for 12 weeks. Values are represented as mean ± SD. (A-B) DBA/2 mice. (C-D) BALB/c mice. (E-F) FVB mice. (G-H) C3H mice. Groups with a same letter were not significantly different ($p > .05$) Energy intake was averaged over the last 10 days of dietary manipulation. A total of 60 mice of each strain was used (10 per diet).

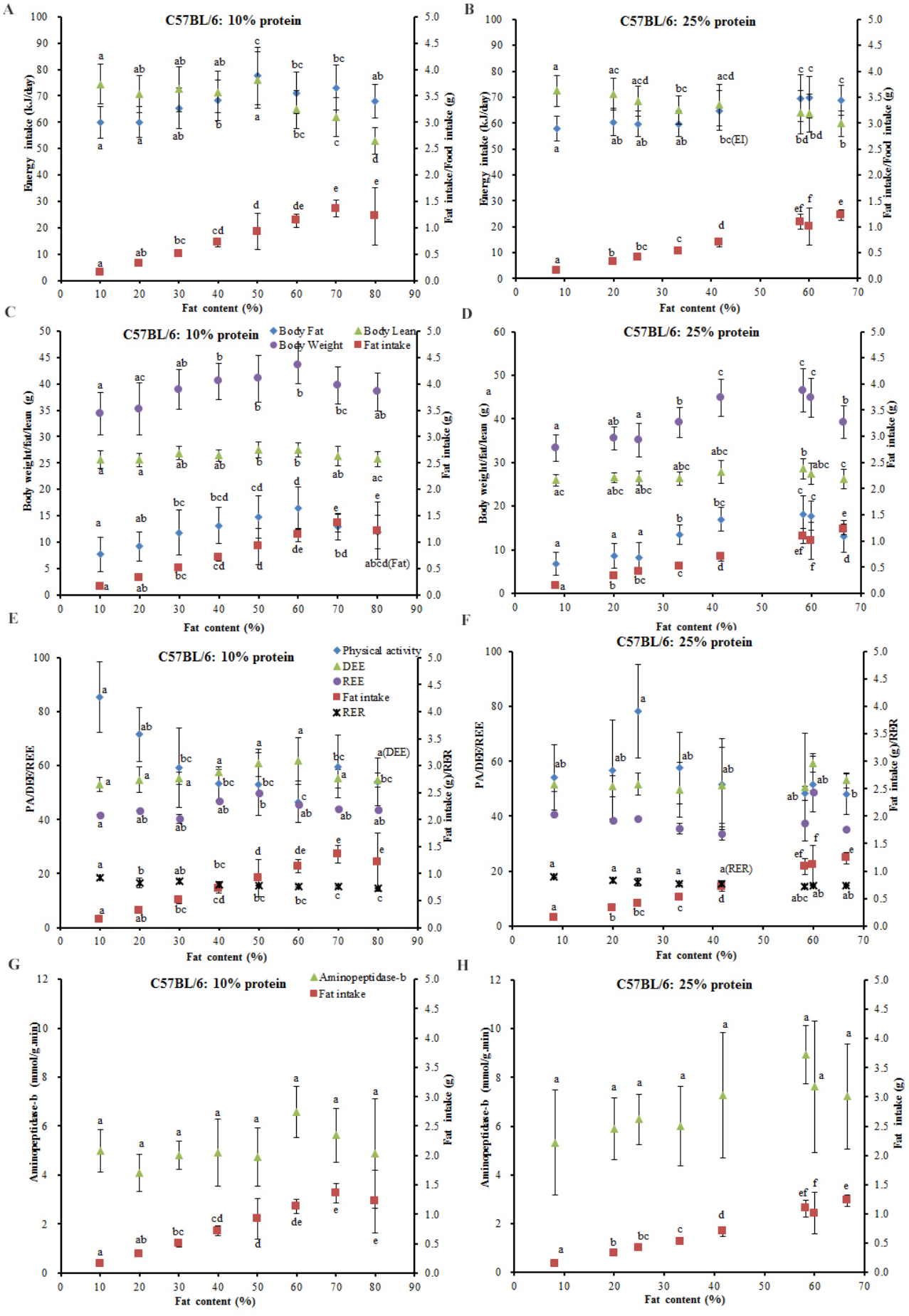

A
C57BL/6: 10% protein
Energy intake (kJ/day)
Fat intake/Food intake (g)
Fat content (%)
B
C57BL/6: 25% protein
Energy intake (kJ/day)
Fat intake/Food intake (g)
Fat content (%)
C
C57BL/6: 10% protein
Body Fat
Body Lean
Body Weight
Fat intake
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)
D
C57BL/6: 25% protein
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)
E
C57BL/6: 10% protein
Physical activity
DEE
REE
Fat intake
RER
PA/DEE/REE
Fat intake (g)/RER
Fat content (%)
F
C57BL/6: 25% protein
PA/DEE/REE
Fat intake (g)/RER
Fat content (%)
G
C57BL/6: 10% protein
Aminopeptidase-b
Fat intake
Aminopeptidase-b (nmol/g.min)
Fat intake (g)
Fat content (%)
H
C57BL/6: 25% protein
Aminopeptidase-b (nmol/g.min)
Fat intake (g)
Fat content (%)

Fig. 4 Changes of energy intake, body composition, energy expenditure. and activity of aminopeptidase-N of C57BL/6 mice following exposure to diets with variable fat content and fixed protein (10% and 25%). Values are represented as mean ± SD. (A-B) Energy intake averaged over the last 10 days of measurement. (C-D) Body composition. (E-F) Energy expenditure and physical activity. (G-H) Activity of aminopeptidase-N. Groups with a same letter were not significantly different ($P > .05$). A total of 120 mice were used with 20 mice per diet.

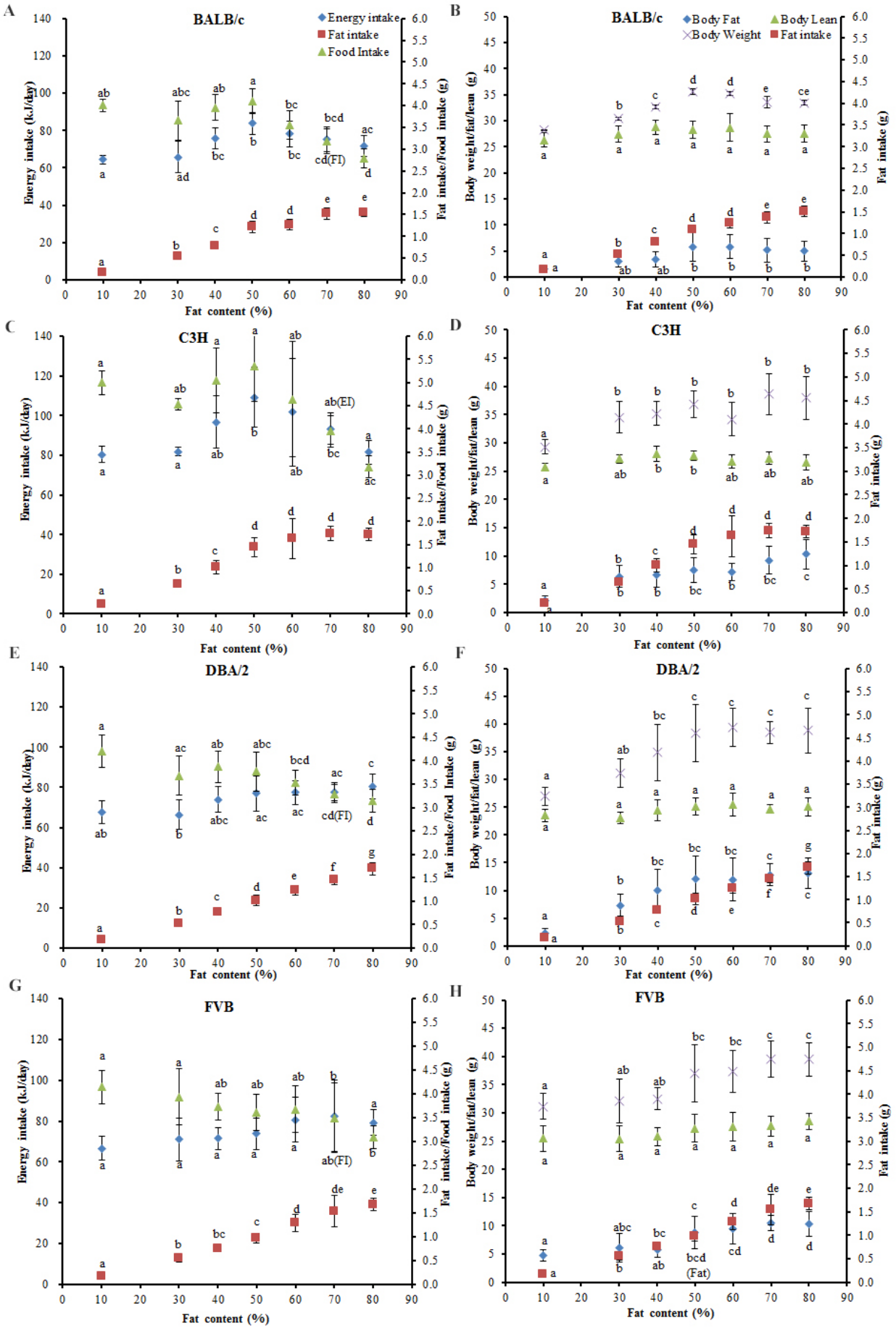

A
BALB/c
Energy intake
Fat intake
Food Intake
Energy intake (kJ/day)
Fat intake/Food intake (g)
Fat content (%)
B
BALB/c
Body Fat
Body Lean
Body Weight
Fat intake
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)
C
C3H
Energy intake (kJ/day)
Fat intake/Food intake (g)
Fat content (%)
D
C3H
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)
E
DBA/2
Energy intake (kJ/day)
Fat intake/Food Intake (g)
Fat content (%)
F
DBA/2
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)
G
FVB
Energy intake (kJ/day)
Fat intake/Food intake (g)
Fat content (%)
H
FVB
Body weight/fat/lean (g)
Fat intake (g)
Fat content (%)

Fig. 5 Energy intake, body weight and body composition changes in four mouse strains after feeding on diets with variable fat contents and 10% protein for 12 weeks. Values are represented as mean ± SD. (A-B) DBA/2 mice. (C-D) BALB/c mice. (E-F) FVB mice. (G-H) C3H mice. Groups with a same letter were not significantly different ($p < .05$). Energy intake was averaged over the last 10 days of measurement. A total of 60 mice per strain was used at 10 mice per diet.

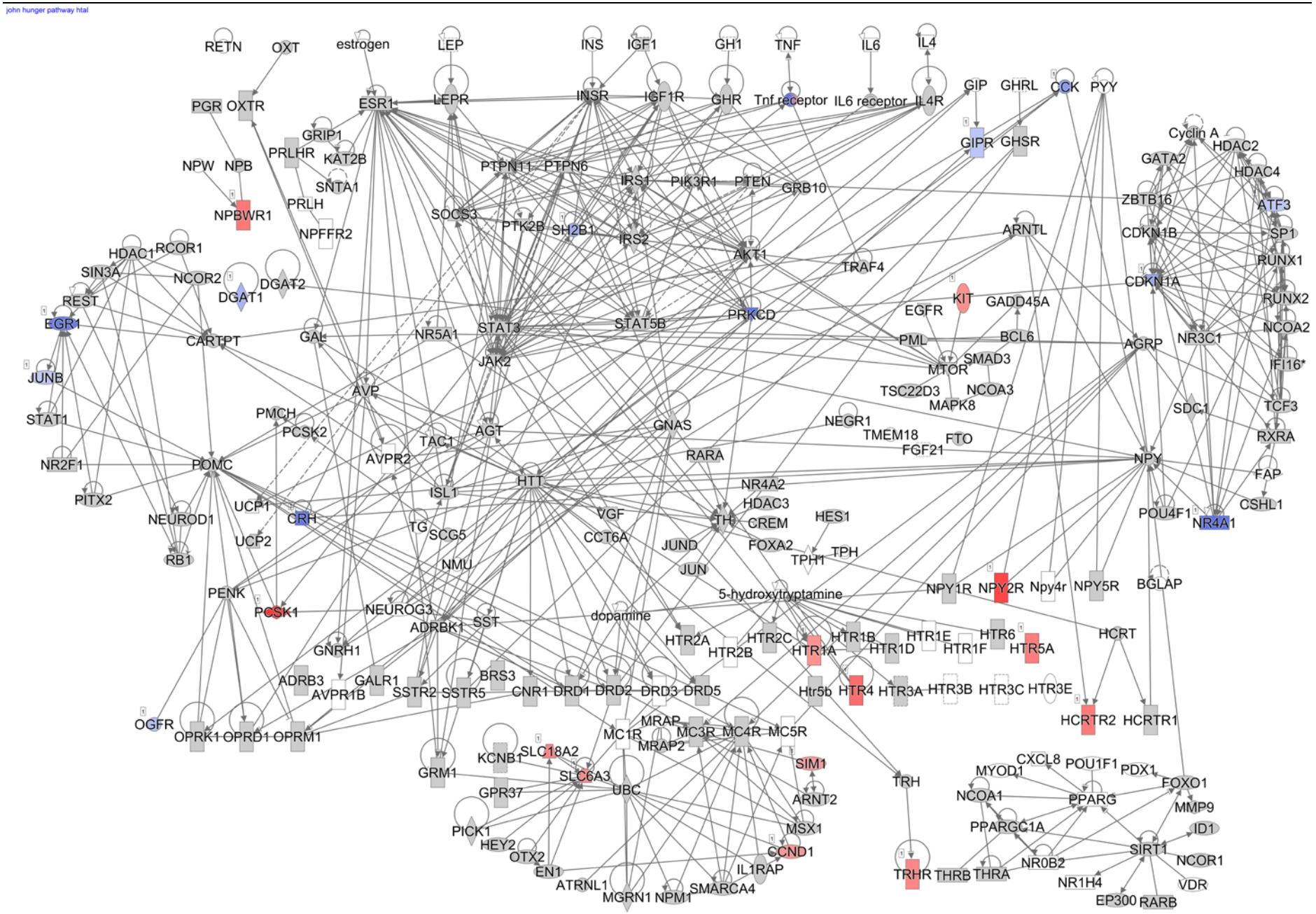

john hunger pathway htal
RETN
OXT
estrogen
LEP
INS
IGF1
GH1
TNF
IL6
IL4
PGR
OXTR
ESR1
LEPR
INSR
IGF1R
GHR
Tnf receptor
IL6 receptor
IL4R
GIP
GHRL
CCK
PYY
GRIP1
PRLHR
KAT2B
GIPR
GHSR
NPW
NPB
SNTA1
PTPN11
PTPN6
IRS1
PIK3R1
PTEN
GRB10
PRLH
NPBWR1
NPFFR2
SOCS3
PTK2B
SH2B1
IRS2
AKT1
TRAF4
ARNTL
HDAC1
RCOR1
SIN3A
NCOR2
DGAT2
DGAT1
REST
EGR1
CARTPT
GAL
NR5A1
STAT3
STAT5B
PRKCD
KIT
EGFR
GADD45A
PML
BCL6
AGRP
JAK2
MTOR
SMAD3
JUNB
AVP
TSC22D3
NCOA3
MAPK8
PMCH
PCSK2
STAT1
TAC1
AGT
GNAS
NEGR1
TMEM18
FGF21
FTO
AVPR2
RARA
NR2F1
POMC
NPY
HTT
NR4A2
PITX2
ISL1
HDAC3
UCP1
CRH
VGF
TH
CREM
HES1
NEUROD1
TG
SCG5
CCT6A
JUND
FOXA2
TPH1
TPH
UCP2
RB1
NMU
JUN
PENK
PCSK1
NEUROG3
5-hydroxytryptamine
NPY1R
NPY2R
Npy4r
NPY5R
BGLAP
ADRBK1
SST
dopamine
GNRH1
HTR2A
HTR2C
HTR1B
HTR1E
HTR6
HCRT
HTR2B
HTR1A
HTR1D
HTR1F
HTR5A
ADRB3
GALR1
BRS3
AVPR1B
SSTR2
SSTR5
CNR1
DRD1
DRD2
DRD3
DRD5
Htr5b
HTR4
HTR3A
HTR3B
HTR3C
HTR3E
HCRTR2
HCRTR1
OGFR
MRAP
MC1R
MC3R
MC4R
MC5R
MRAP2
OPRK1
OPRD1
OPRM1
KCNB1
SLC18A2
SIM1
GRM1
SLC6A3
UBC
TRH
GPR37
ARNT2
MSX1
PICK1
HEY2
CCND1
OTX2
EN1
IL1RAP
ATRNL1
SMARCA4
MGRN1
NPM1
TRHR
THRB
THRA
Cyclin A
HDAC2
GATA2
HDAC4
ZBTB16
ATF3
CDKN1B
SP1
RUNX1
CDKN1A
RUNX2
NR3C1
NCOA2
IFI16*
SDC1
TCF3
RXRA
NPY
FAP
CSHL1
POU4F1
NR4A1
CXCL8
POU1F1
PDX1
FOXO1
MYOD1
NCOA1
PPARG
MMP9
PPARGC1A
ID1
SIRT1
NCOR1
NR0B2
NR1H4
VDR
EP300
RARB
© 2000-2017 QIAGEN. All rights reserved.

Fig. 6 Hunger pathway diagram showing GLM regression of gene expression against dietary protein contents in key hunger and feeding behavior related genes in the hypothalamus of C57BL/6 mice. Red indicates the positive and blue indicates the negative regressions with the protein or fat levels in the diets ($p < 0.05$). Intensity of the color is related to the absolute values of log10 (p value). Grey indicates no significance. A total of 48 pooled samples were used in the analysis across the different protein levels, each sample being pooled from 4 animals.

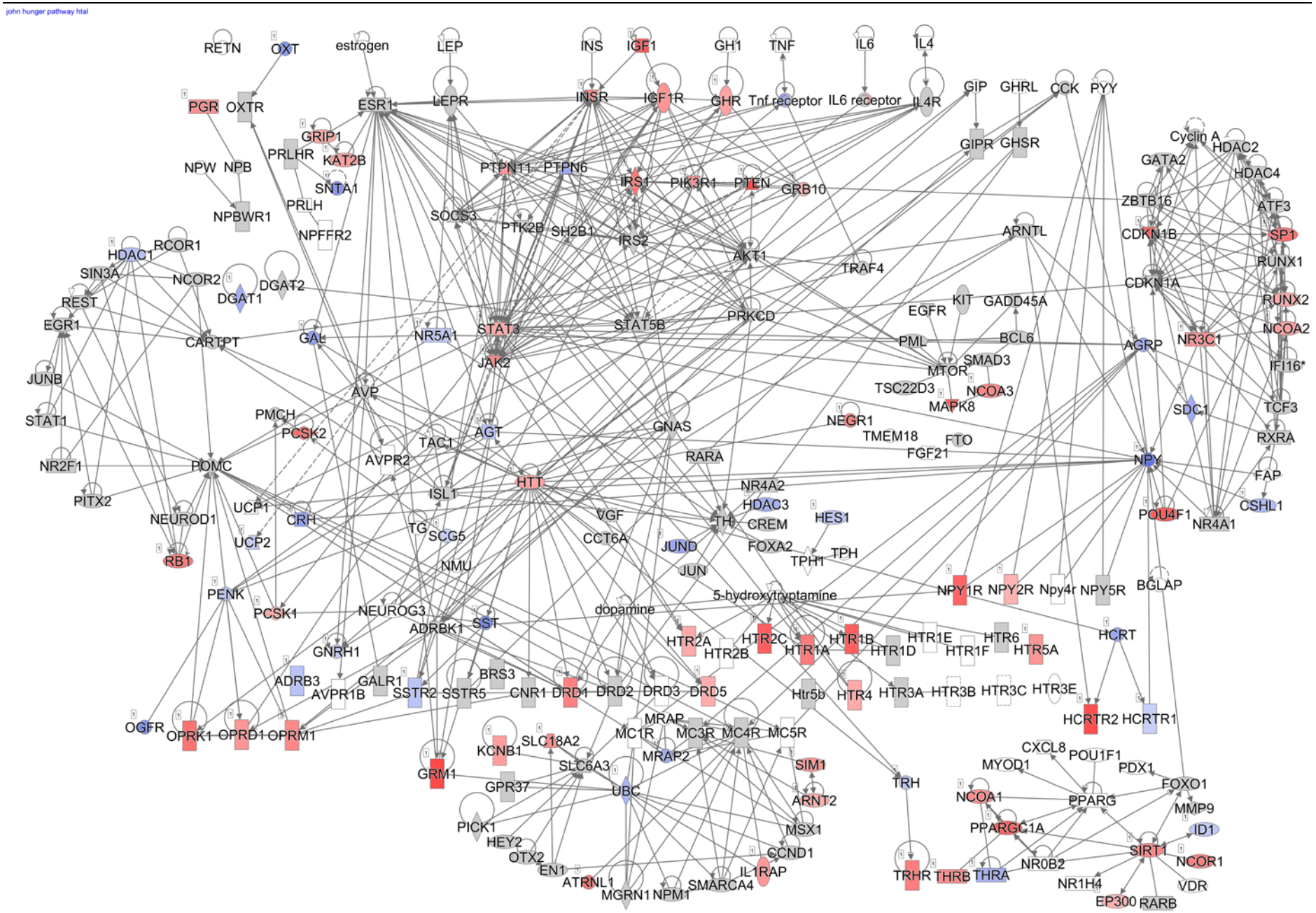


Fig. 7 Hunger pathway diagram showing GLM regression of gene expression against dietary fat contents in key hunger and feeding behavior related genes in the hypothalamus of C57BL/6 mice. Red indicates the positive and blue indicates the negative regressions with the protein or fat levels in the diets ($p < 0.05$). Intensity of the color is related to the absolute values of log10 (p value). Grey indicates no significance. A total of 48 pooled samples were used in the analysis across the different fat levels, each sample being pooled from 4 animals.

Table 1 Summary of experiments performed and main findings.

Experiments	Design	Main Findings
Experiment 1: Manipulation of dietary protein levels at fixed fat contents	• Two series of 6 diets with six fold variation in protein content by energy (5 to 30%) • Series 1 had 20% fat and series 2 60% fat (by energy) • C57BL/6 mice exposed to all 12 diets • BALB/c, C3H, DBA and FVB strains exposed to 6 diets with high fat only (series 2) • Main outcomes, food intake, adiposity, energy expenditure. • RNAseq performed on brain and adipose tissues	• Protein levels were unrelated to energy intake in C57BL/6 mice • Adiposity increased as protein level increased on 20% fat (series 1) • No major changes in hypothalamic gene expression levels • No evidence for White adipose tissue browning or changes in energy expenditure • Same patterns were observed in other 4 strains • Protein leverage was less than 5% in all strains except BALB/c (12%)
Experiment 2: Manipulation of dietary fat levels at fixed protein contents	• Two series of 6 diets with 8 fold variation in fat content (10-80% and 8.3 to 66.6%) • Series 3 had 10% protein and series 4 had 25% protein (by energy) • C57BL/6 mice exposed to all 12 diets • BALB/c, C3H, DBA and FVB strains exposed to 6 diets with high protein only (series 4) • Main outcomes, food intake, adiposity, energy expenditure.	• Weight of food ingested was stable at fat contents up to 50% and thereafter declined • Food energy ingested progressively increased to 50% fat content and then stabilised • Adiposity reached a peak on diets with 50-60% fat content (by energy) • Hypothalamic gene expression shows increase in expression of genes in reward pathways in relation to dietary fat • AgRP and NPY both downregulated in relation to dietary fat levels • No evidence for white adipose tissue browning or effects on energy expenditure • Same patterns were observed in the other 4 strains • Dietary fat is a key driver of energy intake and adiposity
Experiment 3: Manipulation of dietary sucrose levels at fixed fat and protein contents	• C57BL/6 mice exposed to 6 diets with six fold variation in sucrose levels (5 to 30%) at fixed protein (25%) and fat (41.7%) [all % values by energy] (series 5) • Main outcomes, food intake, adiposity, energy expenditure.	• Food intake and adiposity unrelated to sucrose levels in diet

Table 2 Averaged energy expenditure calculated using the software of Guo and Hall (2009) and averaged energy intake of 5 mouse strains over 12 weeks of experimental period.

			Protein content %								
			5	10	15	20	25	30			p value
	60%	**EE(kJ/day)**	77.78±5.53^{a}	70.17±9.98^{b}	67.38±6.57^{b}	68.66±6.85^{b}	66.21±8.04^{b}	65.09±8.30^{b}			4.32 x 10^{-6}
	Fat	**EI(kJ/day)**	81.87±5.92^{a}	75.04±9.17^{ab}	72.86±6.32^{b}	72.21±6.69^{b}	73.48±12.10^{b}	70.24±6.88^{b}			2.49 x 10^{-4}
	20%	**EE(kJ/day)**	67.85±4.87^{a}	58.96±4.39^{b}	59.20±5.95^{b}	57.25±4.28^{b}	58.97±4.04^{b}	56.37±4.74^{b}			1.56 x 10^{-11}
	Fat	**EI(kJ/day)**	67.90±5.27^{a}	60.52±4.15^{b}	60.58±6.21^{b}	58.84±3.99^{b}	60.55±4.12^{b}	57.62±4.75^{b}			7.62 x 10^{-9}
			Fat content %								
C57BL/6			10	20	30	40	50	60	70	80	p value
	10%	**EE(kJ/day)**	58.32±4.33^{a}	58.96±4.39^{a}	63.41±6.44^{ac}	65.95±8.90^{ab}	72.32±11.58^{b}	70.17±9.98b^{c}	65.92±9.41^{ab}	64.37±8.21^{ab}	2.60 x 10^{-7}
	Protein	**EI(kJ/day)**	59.26±4.45^{a}	60.52±4.15^{a}	66.31±5.79^{ab}	70.7±8.50^{b}	78.87±13.04^{b}	75.04±9.17^{b}	71.86±11.44^{b}	72.06±13.01^{b}	5.06 x 10^{-11}
			8.3	25	25	33.3	41.7	58.3	60	66.6	
	25%	**EE(kJ/day)**	55.72±3.89^{a}	58.95±4.14^{ac}	57.65±3.84^{ac}	57.81±3.8^{ac}	65.50±6.51^{b}	65.92±7.00^{b}	66.27±8.23^{b}	63.10±5.57^{bc}	7.36 x 10^{-12}
	Protein	**EI(kJ/day)**	56.55±3.68^{a}	60.55±4.12^{a}	59.14±3.92^{a}	61.03±3.87^{a}	69.60±6.46^{b}	71.26±6.86^{b}	73.48±12.10^{b}	67.85±5.82^{b}	2.31x 10^{-18}
			Protein content %								
			5	10	15	20	25	30			p value
	60%	**EE(kJ/day)**	92.56±3.36^{a}	76.44±7.07^{bc}	84.97±9.04^{ab}	74.24±7.59^{c}	70.15±4.82^{cd}	63.72±2.11^{d}			6.31 x 10^{-11}
	Fat	**EI(kJ/day)**	95.37±3.94^{a}	79.48±7.66^{bc}	84.92±11.55^{ab}	77.53±8.36^{bc}	72.36±6.35^{cd}	65.27±2.52^{d}			8.75 x 10^{-9}
BALB/c			**Fat content %**								
			10	30	40	50	60	70	80		p value
	10%	**EE(kJ/day)**	62.66±2.75^{a}	66.93±2.95^{ab}	73.71±3.89^{bd}	86.83±8.44^{c}	76.44±7.07^{d}	79.47±7.00^{cd}	71.66±3.48^{bd}		2.03 x 10^{-10}
	Protein	**EI(kJ/day)**	63.26±2.67^{a}	67.67±3.28^{ac}	75.21±3.47^{bc}	92.09±10.15^{d}	79.48±7.66^{b}	81.36±6.92^{b}	73.24±3.66^{d}		1.28 x 10^{-11}
C3H			**Protein content %**								

			5	10	15	20	25	30		p value
	60% Fat	**EE(kJ/day)**	114.51±19.78[a]	99.76±21.20[ab]	97.83±14.17[ab]	94.24±8.33[ab]	93.86±8.67[ab]	83.86±9.99[b]		0.007
		EI(kJ/day)	114.03±14.95[a]	94.83±13.24[ab]	100.91±13.92[ab]	98.69±8.49[ab]	97.35±8.49[ab]	87.43±9.60[b]		0.006
			Fat content %							
			10	30	40	50	60	70	80	p value
	10% Protein	**EE(kJ/day)**	74.81±3.01[a]	76.56±2.66[ac]	90.83±12.70[ace]	112.01±16.70[b]	99.76±21.20[be]	94.15±8.55[bcd]	78.95±3.07[ad]	1.47 x 10^{-7}
		EI(kJ/day)	73.15±3.76[a]	78.8±3.49[ab]	92.51±12.71[b]	112.72±14.81[c]	94.83±13.24[a]	98.14±9.93[bc]	82.46±3.42[ab]	1.89 x 10^{-9}
DBA/2			**Protein content %**							
			5	10	15	20	25	30		p value
	60% Fat	**EE(kJ/day)**	83.35±4.65[a]	75.67±7.82[ab]	73.48±5.21[b]	79.91±7.83[ab]	76.15±5.55[ab]	74.27±4.80[ab]		0.02
		EI(kJ/day)	86.89±4.39[a]	79.47±9.11[b]	78.39±6.47[b]	84.71±8.91[b]	80.16±5.97[b]	78.29±6.38[b]		0.084
			Fat content %							
			10	30	40	50	60	70	80	p value
	10% Protein	**EE(kJ/day)**	65.15±3.53[a]	66.84±4.23[ac]	73.46±4.41[ab]	77.97±8.21[b]	75.67±7.82[bc]	78.62±6.51[b]	76.93±2.52[b]	1.39 x 10^{-5}
		EI(kJ/day)	64.80±3.85[a]	68.36±4.03[ab]	76.39±4.25[bc]	82.35±8.89[c]	79.47±9.11[c]	80.82±4.35[bc]	81.61±3.49[c]	6.33 x 10^{-8}
			Protein content %							
			5	10	15	20	25	30		p value
	60% Fat	**EE(kJ/day)**	94.89±6.21[a]	75.52±6.79[b]	76.06±4.82[b]	75.32±9.48[b]	73.99±3.70[b]	71.42±3.78[b]		1.39 x 10^{-8}
FVB		**EI(kJ/day)**	98.02±7.89[a]	78.55±7.06[b]	77.92±3.97[b]	77.69±9.70[b]	78.06±6.06[b]	76.86±9.16[b]		3.75 x 10^{-6}
			Fat content %							
			10	30	40	50	60	70	80	p value
		EE(kJ/day)	66.26±4.35[a]	68.81±4.80[ac]	77.02±8.16[ab]	77.29±8.19[bc]	75.52±6.79[ab]	80.22±10.03[b]	77.92±2.97[bc]	0.001

	10% Protein	**EI(kJ/day)**	68.26±4.9[a]	70.08±5.27[abd]	71.94±4.32[abd]	80.9±9.75[bc]	78.55±7.06[abc]	83.64±10.53[bc]	79.75±4.85[c]	1.44 x 10^{-4}

Footnote: Groups with a same letter were not significantly different (P > .05).

Supplemental Information:

Figures:

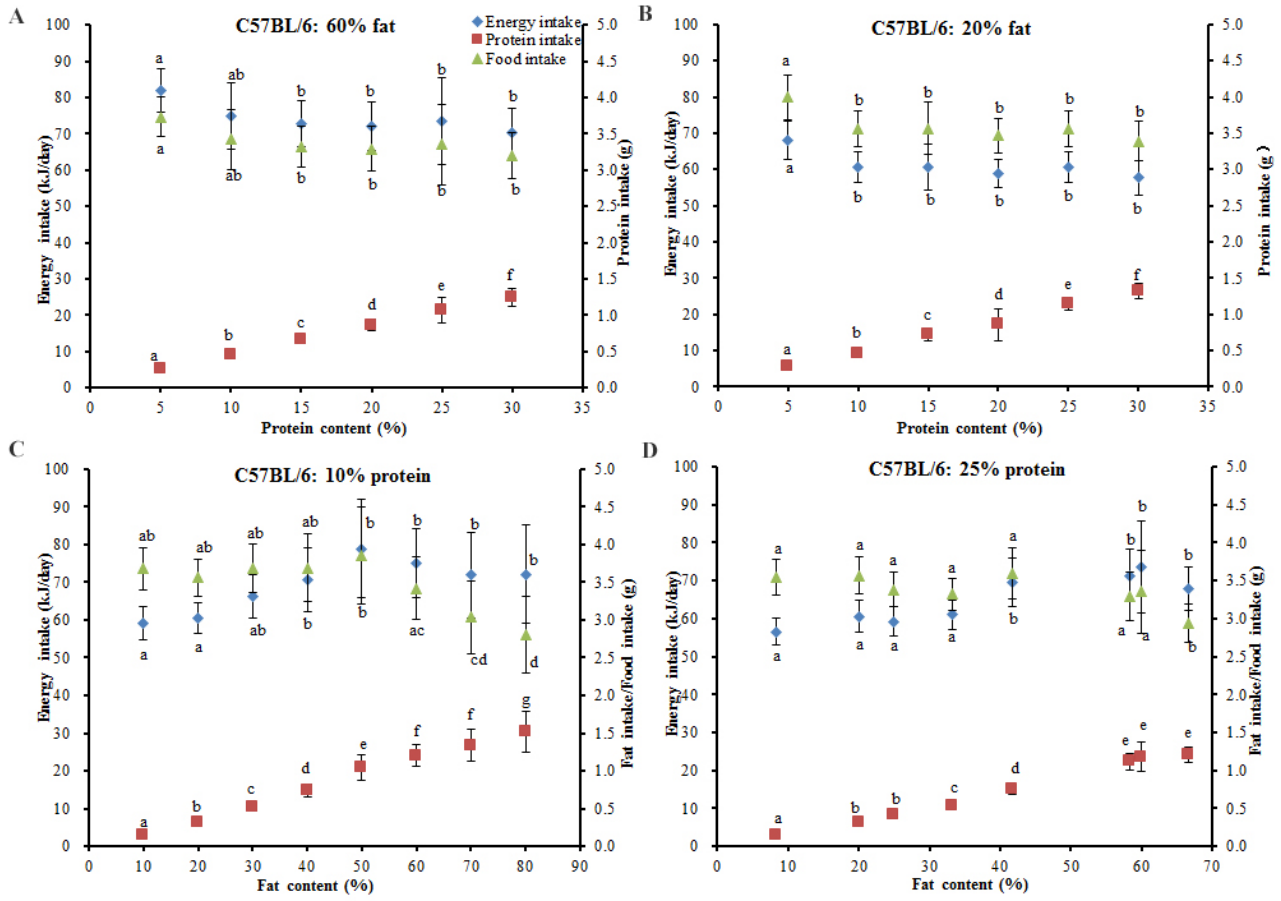


Fig. S1 Energy intake of C57BL/6 mice over the entire 12 week period on the experimental diets.

Panels A and B are variable protein contents at fixed fat. Panels C and D are variable fat content at fixed protein. Values are represented as mean ± SD. Groups with a same letter were not significantly different ($P > .05$).

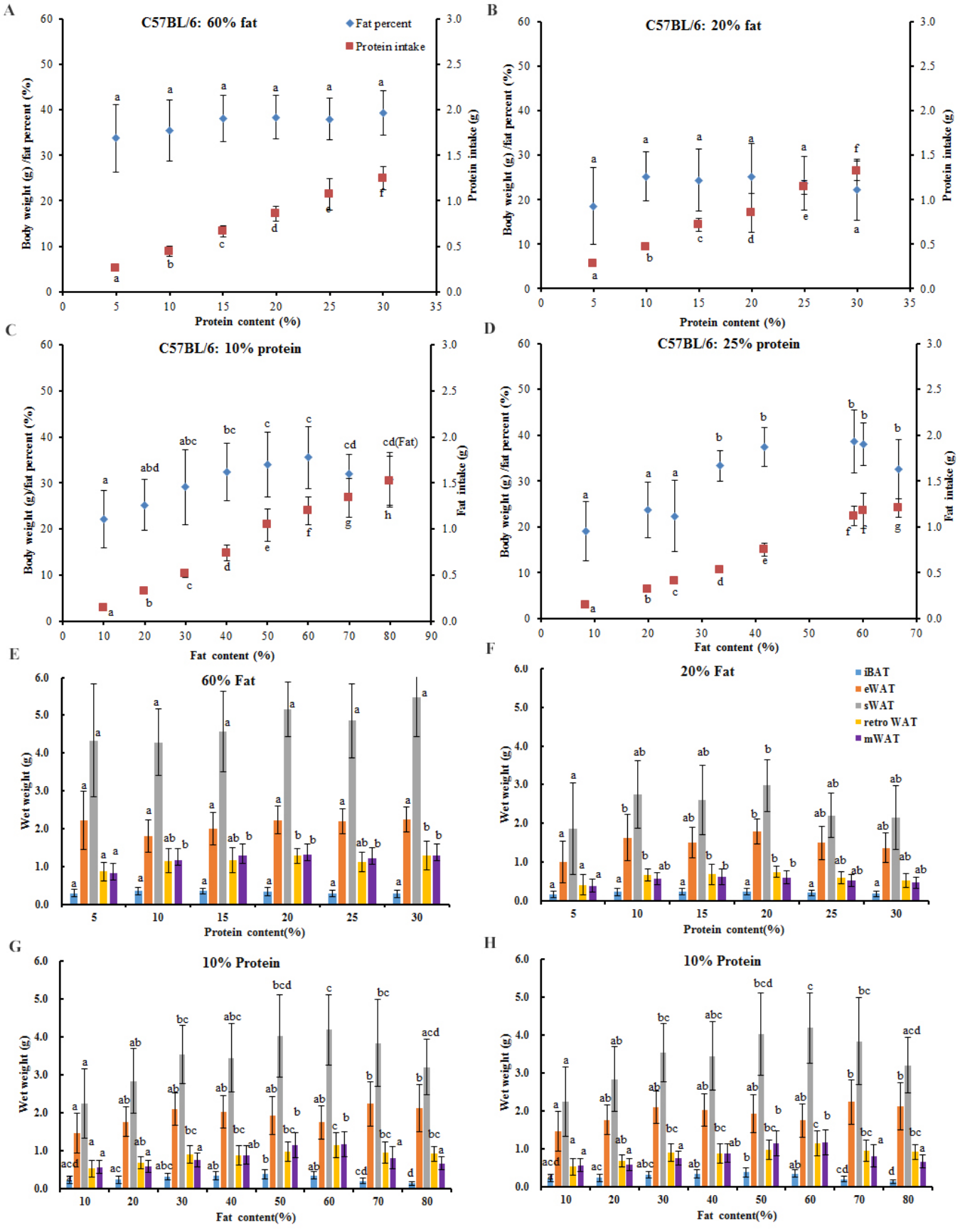


Fig. S2 Adiposity and individual fat depots of C57BL/6 mice following 12 weeks exposure to

experimental diets. A, B, C, D, fat percentage of C57BL/6 mice; E, F, G, H, individual fat depots of C57BL/6 mice. Values are represented as mean ± SD. Groups with a same letter were not significantly different (P > .05).

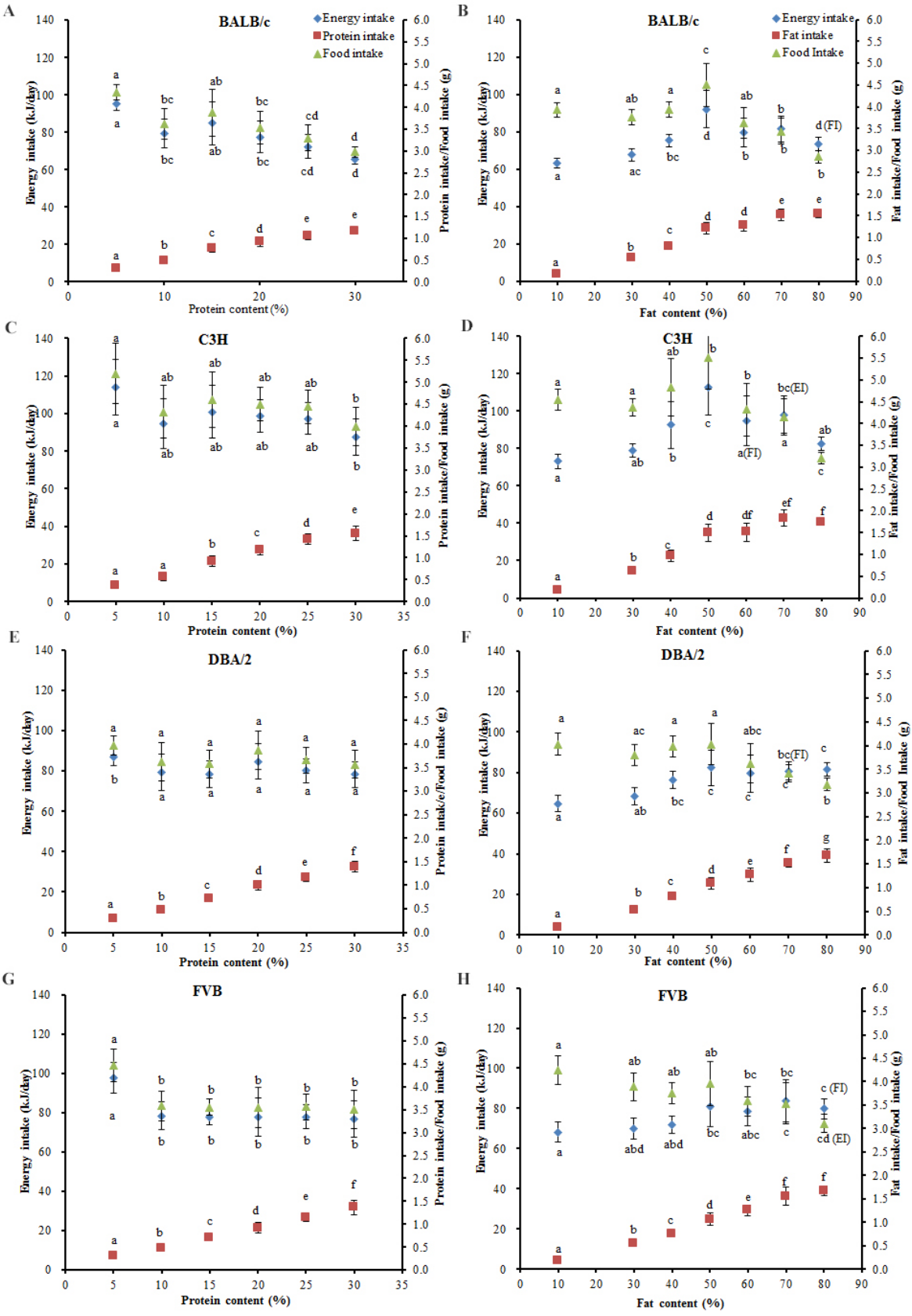


Fig. S3 Energy intake of 4 mouse strains averaged over the entire 12 week experimental period on experimental diets. Left hand panels are diets with fixed fat (60%) and variable protein. Right

hand panels are diets with fixed protein and variable fat. Strains are indicated at the top of each panel. Values are represented as mean ± SD. Groups with a same letter were not significantly different ($P > .05$).

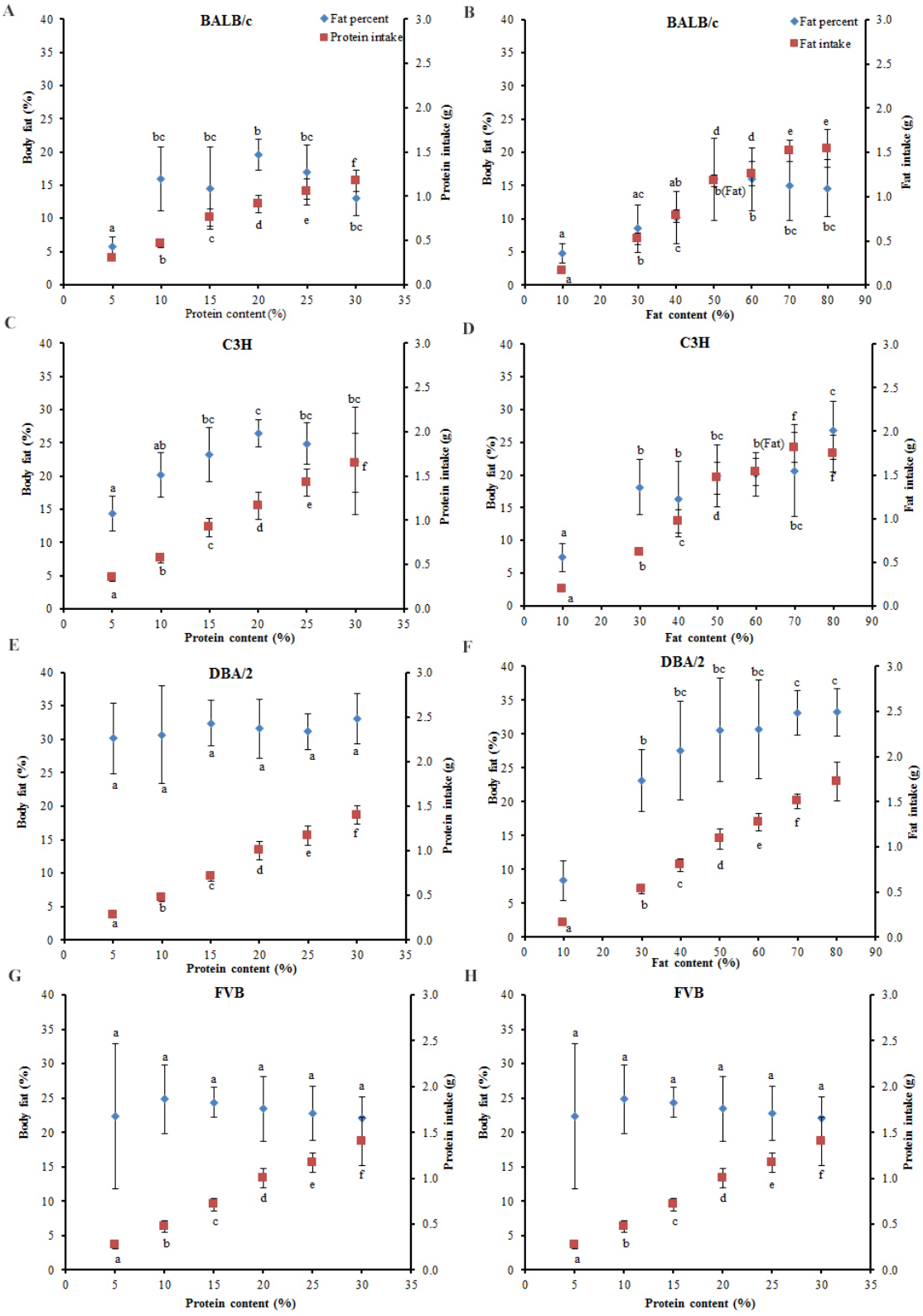


Fig. S4 Adiposity of 4 mouse strains after exposure to experimental diets. Left hand panels are diets with fixed fat (60%) and variable protein. Right hand panels are diets with fixed protein and variable fat. Stains are indicated at the top of each panel. Values are represented as mean ± SD. Groups

with a same letter were not significantly different ($p > 0.05$).

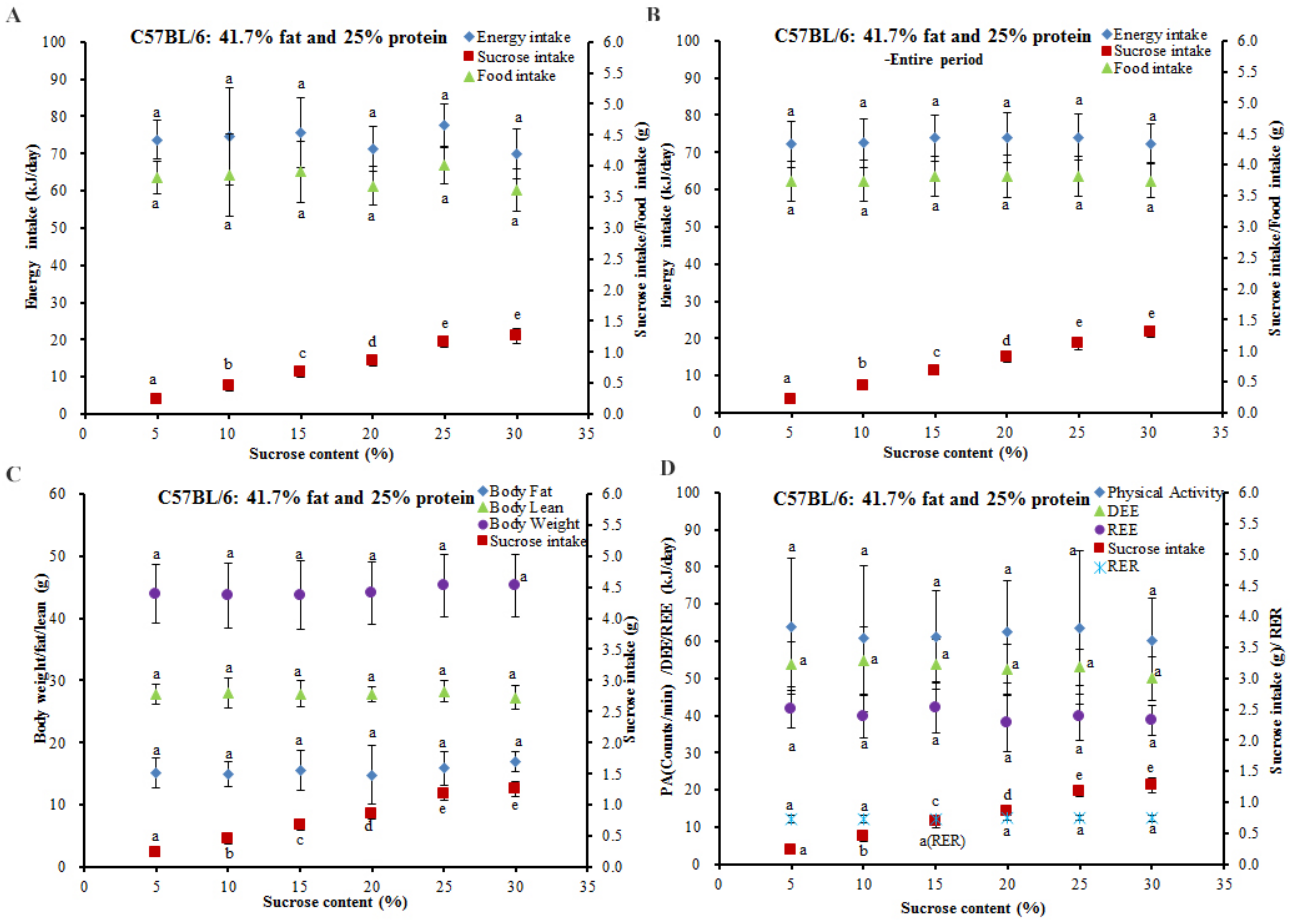
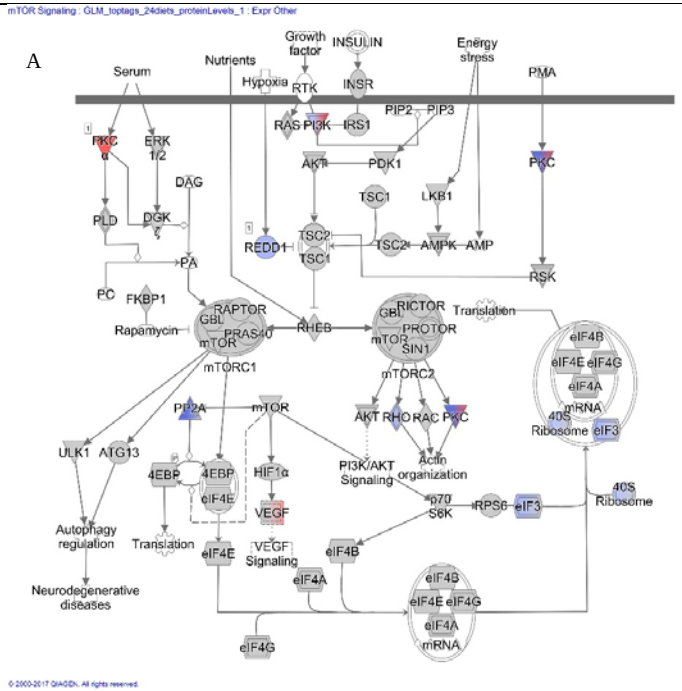
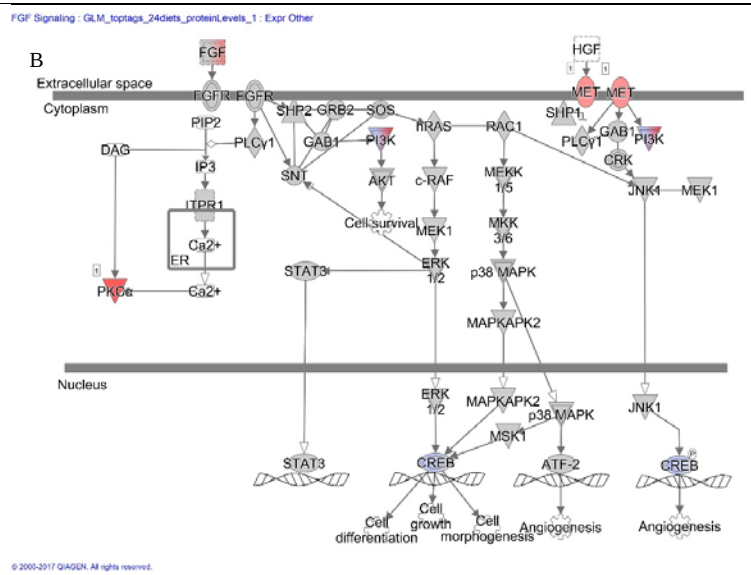
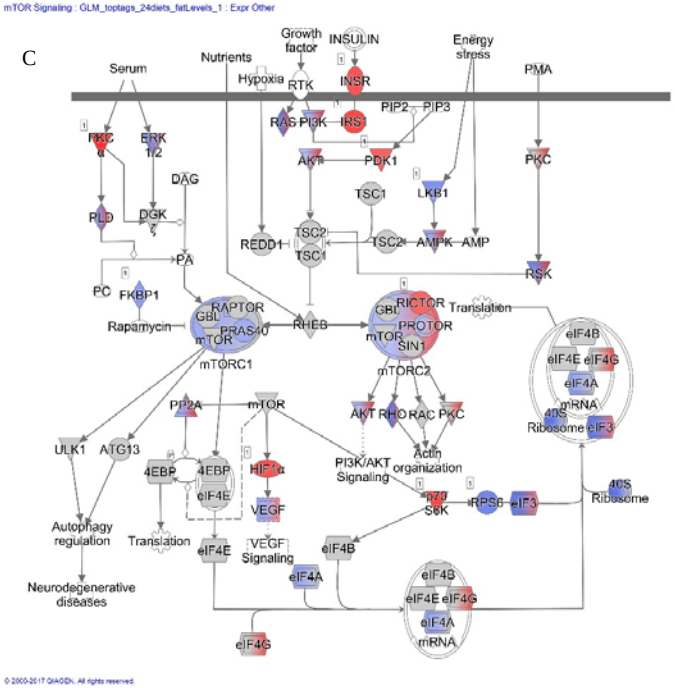


Fig. S5 Responses of C57BL/6 mice following exposure to diets with variable sucrose content and fixed 25% protein and 41.7% fat. (A) energy intake over the last 10 days of the experiment, (B) energy intake over the entire 12 week experimental period, (C) body composition and (D) energy expenditure of mice. Values are represented as mean ± SD. Groups with a same letter were not significantly different (P > .05). A total of 60 mice per strain was used at 10 mice per diet.


mTOR Signaling : GLM_toptags_24diets_proteinLevels_1 : Expr Other
A
Growth factor
INSULIN
Energy stress
Serum
Nutrients
Hypoxia
RTK
INSR
PMA
PIP2
PIP3
RAS
PI3K
IRS1
PKC α
ERK 1/2
AKT
PDK1
PKC
DAG
TSC1
LKB1
PLD
DGK ζ
REDD1
TSC2
AMPK
AMP
PA
RSK
PC
FKBP1
RAPTOR
GBL
PRAS40
RHEB
RICTOR
PROTOR
SIN1
Translation
Rapamycin
mTOR
mTORC1
mTORC2
eIF4B
eIF4E
eIF4G
eIF4A
mRNA
40S Ribosome
eIF3
PP2A
AKT
RHO
RAC
PKC
ULK1
ATG13
4EBP
HIF1α
PI3K/AKT Signaling
Actin organization
p70 S6K
RPS6
VEGF
Autophagy regulation
Translation
VEGF Signaling
Neurodegenerative diseases
© 2000-2017 QIAGEN. All rights reserved.


FGF Signaling : GLM_toptags_24diets_proteinLevels_1 : Expr Other
B
Extracellular space
Cytoplasm
FGF
HGF
FGFR
MET
SHP2
GRB2
SOS
SHIP1
GAB1
PI3K
PLCγ1
CRK
PIP2
DAG
IP3
SNT
AKT
c-RAF
MEKK 1/5
JNK1
MEK1
ITPR1
Ca2+
ER
Cell survival
MKK 3/6
STAT3
ERK 1/2
p38 MAPK
PKCα
MAPKAPK2
Nucleus
MSK1
ATF-2
CREB
Cell differentiation
Cell growth
Cell morphogenesis
Angiogenesis
© 2000-2017 QIAGEN. All rights reserved.


mTOR Signaling : GLM_toptags_24diets_fatLevels_1 : Expr Other
C
Growth factor
INSULIN
Energy stress
Serum
Nutrients
Hypoxia
RTK
INSR
PMA
PIP2
PIP3
RAS
PI3K
IRS1
PKC α
ERK 1/2
AKT
PDK1
PKC
DAG
TSC1
LKB1
PLD
DGK ζ
REDD1
TSC2
AMPK
AMP
PA
RSK
PC
FKBP1
RAPTOR
GBL
PRAS40
RHEB
RICTOR
PROTOR
SIN1
Translation
Rapamycin
mTOR
mTORC1
mTORC2
eIF4B
eIF4E
eIF4G
eIF4A
mRNA
40S Ribosome
eIF3
PP2A
AKT
RHO
RAC
PKC
ULK1
ATG13
4EBP
HIF1α
PI3K/AKT Signaling
Actin organization
p70 S6K
RPS6
VEGF
Autophagy regulation
Translation
VEGF Signaling
Neurodegenerative diseases
© 2000-2017 QIAGEN. All rights reserved.

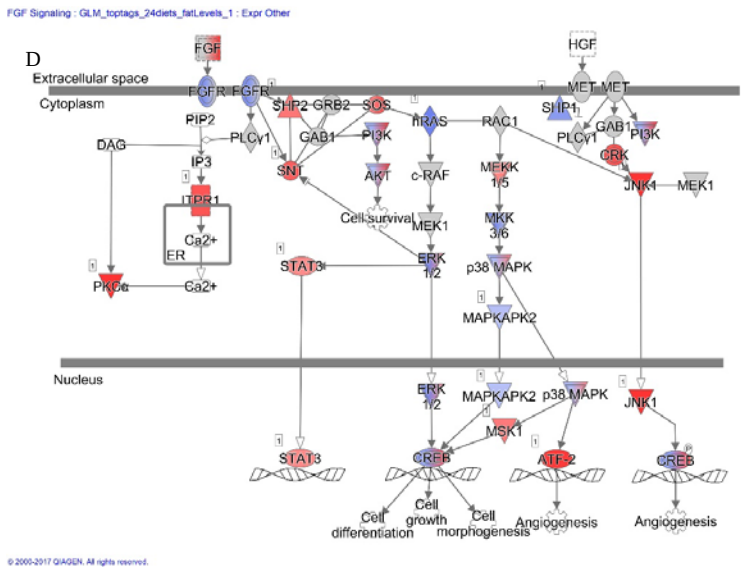
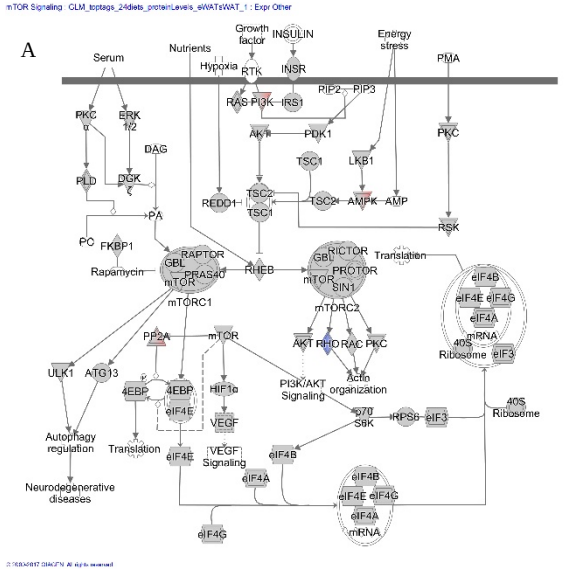
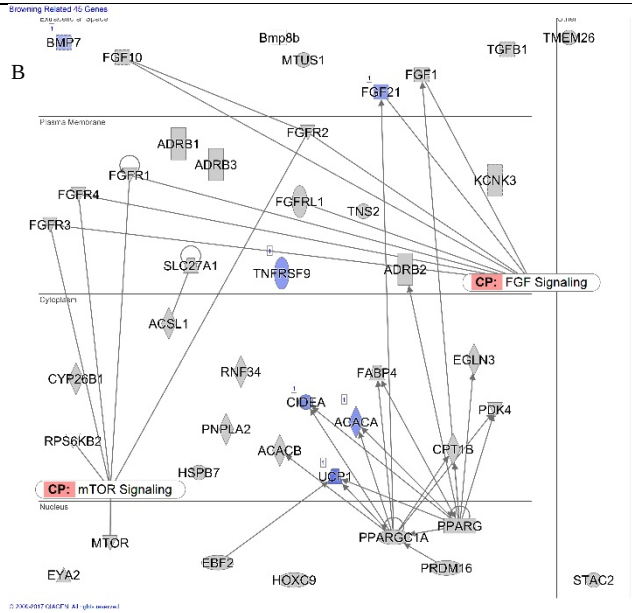
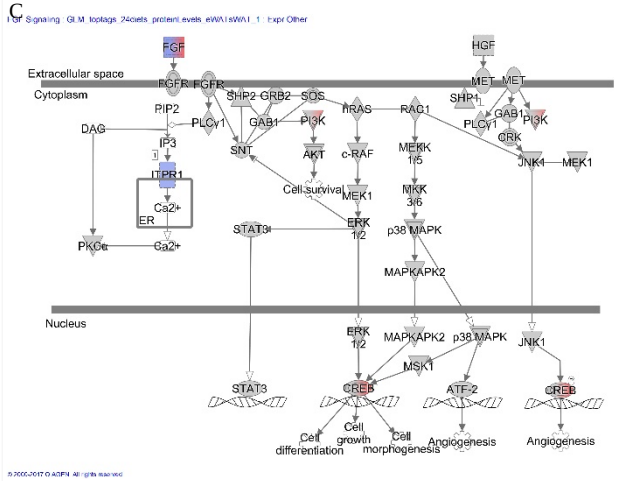
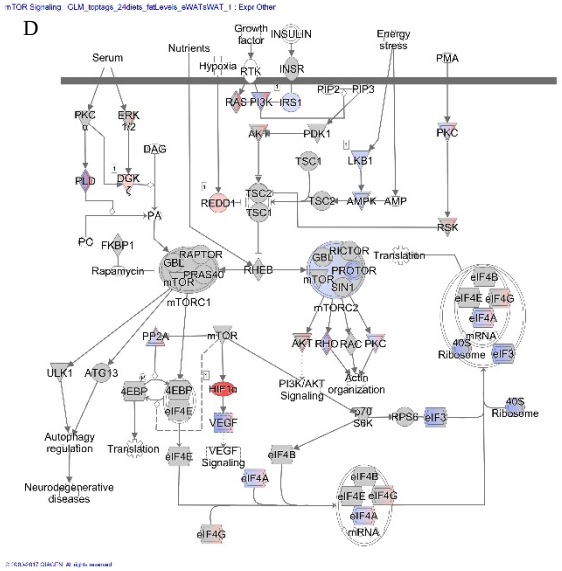

FGF Signaling : GLM_toptags_24diets_fatLevels_1 : Expr Other
D
Extracellular space
Cytoplasm
FGF
HGF
FGFR
MET
SHP2
GRB2
SOS
SHIP1
GAB1
PI3K
PLCγ1
CRK
PIP2
DAG
IP3
SNT
AKT
c-RAF
MEKK 1/5
JNK1
MEK1
ITPR1
Ca2+
ER
Cell survival
MKK 3/6
STAT3
ERK 1/2
p38 MAPK
PKCα
MAPKAPK2
Nucleus
MSK1
ATF-2
CREB
Cell differentiation
Cell growth
Cell morphogenesis
Angiogenesis
© 2000-2017 QIAGEN. All rights reserved.

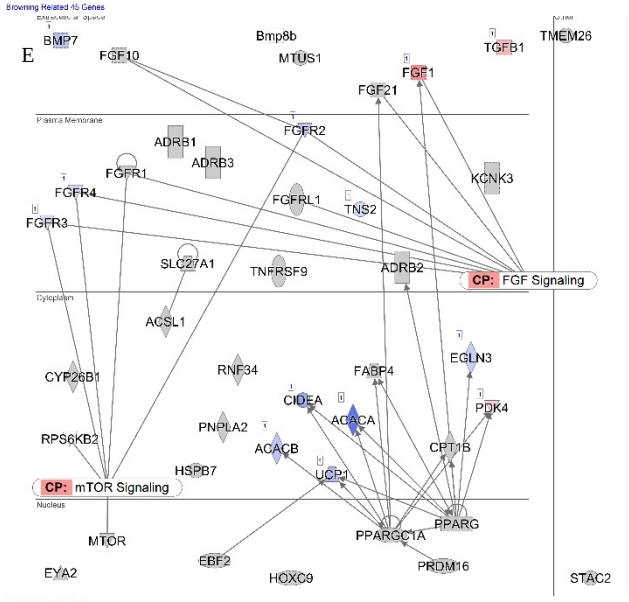
Fig. S6 GLM regressions of gene expression to dietary protein or fat contents in mTOR Signaling and FGF Signaling in the hypothalamus of C57BL/6 mouse. (A) mTOR Signaling and (B) FGF Signaling (at variant protein levels). (C) mTOR Signaling and (D) FGF Signaling (at variant fat levels). Red indicates the positive and blue indicates the negative regressions with the protein or fat levels in the diets ($p < 0.05$). Intensity of the color is related to the absolute values of log10 (p value). Grey indicates no significance. A total of 24 pooled samples across the different protein levels were used in the analysis, each sample being pooled form 4 individuals.


A
mTOR Signaling
Growth factor
INSULIN
Energy stress
Serum
Nutrients
Hypoxia
RTK
INSR
PMA
RAS
PI3K
IRS1
PIP2
PIP3
PKC
ERK 1/2
AKT
PDK1
DAG
TSC1
LKB1
PLD
DGK
TSC2
REDD1
AMPK
AMP
RSK
PA
PC
FKBP1
Rapamycin
RAPTOR
GBL
PRAS40
mTOR
RHEB
RICTOR
PROTOR
SIN1
Translation
mTORC1
mTORC2
eIF4B
eIF4E
eIF4G
eIF4A
mRNA
40S Ribosome
eIF3
PP2A
AKT
RHO
RAC
PKC
ULK1
ATG13
4EBP
HIF1α
PI3K/AKT Signaling
Actin organization
p70 S6K
RPS6
Autophagy regulation
Translation
VEGF
VEGF Signaling
Neurodegenerative diseases


Browning Related 45 Genes
B
BMP7
Bmp8b
TGFB1
TMEM26
FGF10
MTUS1
FGF1
FGF21
Plasma Membrane
FGFR2
ADRB1
ADRB3
FGFR1
KCNK3
FGFR4
FGFRL1
TNS2
FGFR3
SLC27A1
TNFRSF9
ADRB2
CP: FGF Signaling
Cytoplasm
ACSL1
CYP26B1
RNF34
FABP4
EGLN3
CIDEA
ACACA
PDK4
RPS6KB2
PNPLA2
ACACB
CPT1B
HSPB7
UCP1
CP: mTOR Signaling
Nucleus
PPARGC1A
PPARG
MTOR
EBF2
PRDM16
EYA2
HOXC9
STAC2


C
FGF Signaling
Extracellular space
Cytoplasm
FGF
FGFR
HGF
MET
SHP2
GRB2
SOS
SHP1
GAB1
PI3K
PLCγ1
DAG
PIP2
IP3
ITPR1
Ca2+
ER
PKCα
SNT
iRAS
RAC1
AKT
c-RAF
MEKK 1/5
MKK 3/6
JNK1
MEK1
Cell survival
ERK 1/2
STAT3
p38 MAPK
MAPKAPK2
Nucleus
MSK1
CREB
ATF-2
Cell differentiation
Cell growth
Cell morphogenesis
Angiogenesis


D
mTOR Signaling
Growth factor
INSULIN
Energy stress
Serum
Nutrients
Hypoxia
RTK
INSR
PMA
RAS
PI3K
IRS1
PIP2
PIP3
PKC
ERK 1/2
AKT
PDK1
DAG
TSC1
LKB1
PLD
DGK
TSC2
REDD1
AMPK
AMP
RSK
PA
PC
FKBP1
Rapamycin
RAPTOR
GBL
PRAS40
mTOR
RHEB
RICTOR
PROTOR
SIN1
Translation
mTORC1
mTORC2
eIF4B
eIF4E
eIF4G
eIF4A
mRNA
40S Ribosome
eIF3
PP2A
AKT
RHO
RAC
PKC
ULK1
ATG13
4EBP
HIF1α
PI3K/AKT Signaling
Actin organization
p70 S6K
RPS6
Autophagy regulation
Translation
VEGF
VEGF Signaling
Neurodegenerative diseases


Browning Related 45 Genes
E
BMP7
Bmp8b
TGFB1
TMEM26
FGF10
MTUS1
FGF1
FGF21
Plasma Membrane
FGFR2
ADRB1
ADRB3
FGFR1
KCNK3
FGFR4
FGFRL1
TNS2
FGFR3
SLC27A1
TNFRSF9
ADRB2
CP: FGF Signaling
Cytoplasm
ACSL1
CYP26B1
RNF34
FABP4
EGLN3
CIDEA
ACACA
PDK4
RPS6KB2
PNPLA2
ACACB
CPT1B
HSPB7
UCP1
CP: mTOR Signaling
Nucleus
PPARGC1A
PPARG
MTOR
EBF2
PRDM16
EYA2
HOXC9
STAC2

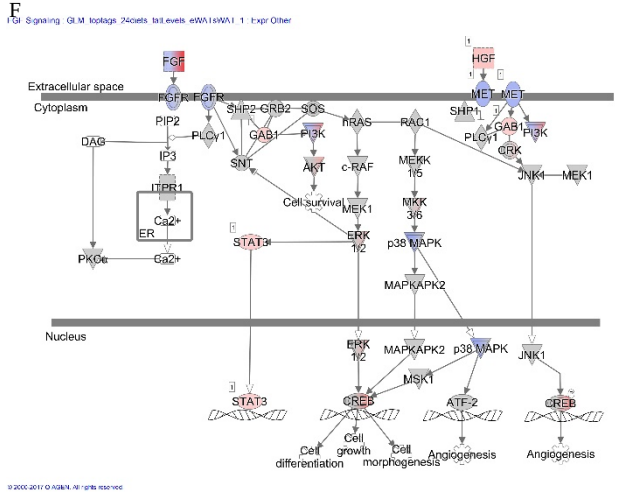

F
FGF Signaling
Extracellular space
Cytoplasm
FGF
FGFR
HGF
MET
SHP2
GRB2
SOS
SHP1
GAB1
PI3K
PLCγ1
DAG
PIP2
IP3
ITPR1
Ca2+
ER
PKCα
SNT
iRAS
RAC1
AKT
c-RAF
MEKK 1/5
MKK 3/6
JNK1
MEK1
Cell survival
ERK 1/2
STAT3
p38 MAPK
MAPKAPK2
Nucleus
MSK1
CREB
ATF-2
Cell differentiation
Cell growth
Cell morphogenesis
Angiogenesis

Fig. S7 GLM regressions of gene expression to dietary protein or fat contents in browning related pathways, mTOR Signaling and FGF Signaling of the WAT of C57BL/6 mouse. (A) mTOR Signaling, (B) browning related genes, and (C) FGF Signaling (at variant protein levels). (D) mTOR Signaling, (E) browning related genes, and (F) FGF Signaling (at variant fat levels). Red indicates the positive and blue indicates the negative regressions with the protein or fat levels in the diets ($p < 0.05$). Intensity of the color is related to the absolute values of log10 (p value). Grey indicates no significance. A total of 24 pooled samples across the different protein levels were used in the anlaysis, each sample being pooled from 4 individuals.

Supplemental Table Legends (Separate)

Table S1 Composition of the 29 experimental diets.

Table S2 Hunger pathway of hypothalamus under variable dietary protein or fat contents. Related to Fig.6 and 7.

Table S3 FGF signaling of hypothalamus under variable dietary protein or fat contents. Related to Fig. S2 and main text.

Table S4 mTOR signaling of hypothalamus under variable dietary protein or fat contents. Related to Fig. S2 and main text.

Table S5 Browning related genes of WAT under variable dietary protein or fat contents. Related to Fig. S3 and main text.

Table S6 FGF signaling of WAT under variable dietary protein or fat contents. Related to Fig. S3 and main text.

Table S7 mTOR signaling of WAT under variable dietary protein or fat contents. Related to Fig. S3 and main text.