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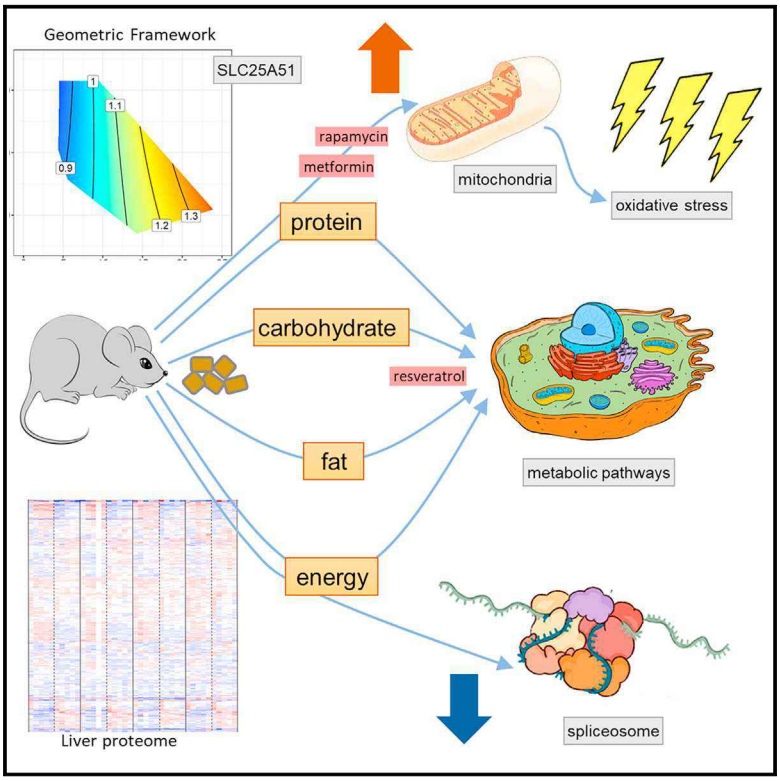
Article

Nutritional reprogramming of mouse liver proteome is dampened by metformin, resveratrol, and rapamycin

Graphical abstract



Highlights

- Dietary energy intake has a negative correlation with abundance of spliceosome proteins
- Protein intake correlates with abundance of mitochondrial proteins and oxidative stress
- Metformin, rapamycin, and resveratrol dampen responses to nutrients
- Abundance of SLC25A51, the mitochondrial NAD transporter increased with protein intake

Authors

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In brief

In a study of the hepatic proteome, Le Couteur et al. show that dietary energy and macronutrients influence fundamental cellular machinery, including the spliceosome and mitochondria. Metformin, rapamycin, and resveratrol broadly dampened the proteomic responses to diet rather than acting on specific nutrient sensing pathways. The impact of diet was substantially more significant than that of drugs.

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Nutritional reprogramming of mouse liver proteome is dampened by metformin, resveratrol, and rapamycin

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SUMMARY

Nutrient sensing pathways influence metabolic health and aging, offering the possibility that diet might be used therapeutically, alone or with drugs targeting these pathways. We used the Geometric Framework for Nutrition to study interactive and comparative effects of diet and drugs on the hepatic proteome in mice across 40 dietary treatments differing in macronutrient ratios, energy density, and drug treatment (metformin, rapamycin, resveratrol). There was a strong negative correlation between dietary energy and the spliceosome and a strong positive correlation between dietary protein and mitochondria, generating oxidative stress at high protein intake. Metformin, rapamycin, and resveratrol had lesser effects than and dampened responses to diet. Rapamycin and metformin reduced mitochondrial responses to dietary protein while the effects of carbohydrates and fat were downregulated by resveratrol. Dietary composition has a powerful impact on the hepatic proteome, not just on metabolic pathways but fundamental processes such as mitochondrial function and RNA splicing.

INTRODUCTION

Aging and age-related health are strongly influenced by nutrition over the lifespan (Fontana and Partridge, 2015). The most widely studied nutritional intervention that delays aging and age-related disease is caloric restriction, which increases lifespan in many taxa, from yeast to monkeys (Ingram and de Cabo, 2017). The cellular pathways that provide mechanistic links between caloric restriction and aging have been studied intensively, motivated by the possibility that drugs acting on these pathways might replicate the benefits of caloric restriction without reduction in food intake (Partridge et al., 2020). Nutrient sensing pathways that are proposed to link caloric restriction with aging include those regulated by mechanistic target of rapamycin (mTOR), sirtuins (SIRT1), 5′ adenosine monophosphate-activated protein kinase (AMPK), and insulin/insulin growth factor-1 (IGF-1) (Madeo et al., 2019). Activation or inhibition of these pathways influence the response to caloric restriction, and in some cases, lifespan. Metformin, rapamycin, and resveratrol—which act on AMPK, MTOR, and SIRT1 pathways—increase lifespan in some species (Baur et al., 2006; Harrison et al., 2009; Madeo et al., 2019; Martin-Montalvo et al., 2013).

More recently, the effects of manipulating dietary macronutrients have been studied. Protein restriction was found to extend lifespan while high fat diets and high protein diets may accelerate aging and reduce lifespan (Austad and Hoffman, 2021; Simpson et al., 2017). The interaction between protein and carbohydrate intake is also important. As dietary protein is replaced by complex carbohydrates rather than fat, lifespan increases in mice and invertebrate animals (Le Couteur et al., 2016; Lee et al., 2008; Solon-Biet et al., 2014). Many of these macronutrient studies used a nutritional geometry approach, where the effects of macronutrients, calories, and food intake on outcomes such as lifespan are partitioned, integrated, and interrogated across a wide topology of diets differing in macronutrient and energy content (Raubenheimer et al., 2016; Simpson and Raubenheimer, 1999, 2014).

Furthermore, there are interactions between drugs that act on these nutrient sensing pathways and the background diets against which they are tested (Madeo et al., 2019). A well-known example is the SIRT1 agonist resveratrol, which is associated with increased lifespan in mice on a high-fat diet (Baur et al., 2006) but not on a standard diet (Pearson et al., 2008). Drugs that act on nutrient sensing pathways would also be expected to influence the normal physiological responses to different types of diet.

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The main organ that responds to changes in nutrition and orchestrates the systemic response to nutrition is the liver. The metabolic pathways in the liver that respond to fluctuations in nutrients include those involved in their synthesis and degradation, storage or utilization for energy, and transformation into other cellular substrates (Rui, 2014). Regulating these pathways allows the liver to maintain a stable systemic metabolic milieu despite changes in dietary composition and intake. The effects on the liver of caloric restriction or drugs acting on the nutrient sensing pathways have been studied individually using transcriptomic and, less commonly, proteomic approaches. Such studies mostly emphasized the impact of caloric restriction on pathways involved in aging such as autophagy, mitochondria, and inflammation (Barger et al., 2015; Derous et al., 2017).

Here we address two key questions: 1) how do diet and drugs compare in the nature and amplitude of their effects on metabolic networks, and 2) how do nutrients and drugs interact in their effects? These are among the major issues to resolve before translating such interventions either widely in human populations or with precision in individuals.

RESULTS

Macronutrients, not drugs, influence phenotype

Mice (male C57BL/6J) were maintained on one of ten diets differing in the ratio of macronutrients and with either standard energy density (14.8 kJ/g) or low energy density (8 kJ/g), which was achieved by diluting the food with non-digestible cellulose (Figure S1A; Table S1; STAR Methods). Each diet contained either metformin (0.1% w/w), rapamycin (14 mg/kg), resveratrol (0.04% w/w), or a control vehicle. Mice had *ad libitum* access to their diets, which were commenced at 12 weeks of age and continued until termination of the study at 40 weeks of age. There were 335 mice (control n = 81, metformin n = 87, rapamycin n = 85, resveratrol n = 82) that completed the study. Metformin, rapamycin, and resveratrol did not influence intake of macronutrients (Figure S1B). Blood levels for metformin, rapamycin, and resveratrol were not influenced by energy density (Figure 1A) and were at or above levels reported in humans administered these drugs (Chimento et al., 2019; Kajbaf et al., 2016; Shao et al., 2020).

The Geometric Framework was used to interpret outcomes (Raubenheimer et al., 2016; Simpson and Raubenheimer, 2014). The clearest finding is that diets that were high in protein and fat were associated with highest levels of body weight, % body fat, insulin resistance (insulin × glucose product), and IGF-1 (corresponding to the red area of each surface). This peak was not influenced by metformin, rapamycin, or resveratrol (Figures 1B–1E), nor did the drugs substantially influence the relationship between the vector through the peak and the trait as food intake increased (Figures 1F–1I). Rapamycin was associated with normal glucose × insulin product but increased glucose levels (Figure 1D; Figures S1C and S1D), as reported elsewhere (Reifsnyder et al., 2020).

Energy density influences many major metabolic pathways in the liver

Proteomic analysis quantified 4,939 hepatic proteins with complete datasets across all the diet and drug groups (two to four mice per group). Principal component analysis (PCA) revealed that the proteome was strongly influenced by energy intake (Figure 2A). Energy intake of mice on low energy diets was 30% less than energy intake of mice on standard energy diets (33.1 ± 3.4 versus 47.9 ± 8.5 kJ/mouse/day).

The abundance of 973 and 996 proteins were positively or negatively correlated with energy intake, respectively in control animals (Figure 2B). Proteins with abundance that was positively correlated with energy intake were involved in the metabolism of nutrients including glycolysis (e.g. PFKL, ALDOB, ENO1, GAPDH), gluconeogenesis (e.g. PGK1, PGAM1), glycogenolysis (e.g. AGL, GYG, PGM1, PYGL), glycogen synthesis (e.g. GBE1, GCK, GYG), tricarboxylic acid cycle (e.g. CS, DLAT), lipogenesis (e.g AGPAT2, FASN, SCD1), lipolysis (e.g. MGLL, ANGPTL3, PLIN4), beta oxidation (e.g. ACAA2, ACADM, HADHB), amino acid metabolism (e.g. BAAT, BCKDK, GLUD1) and ketogenesis (e.g. HMGCS, HMGCL, BDH). Likewise, enrichment analysis of these proteins revealed mostly pathways involved with the metabolism of nutrients (Figure 2C).

Low energy intake upregulates the spliceosome

Proteins with abundance that was negatively correlated with energy intake included many involved with RNA metabolism and splicing (Figure 2D). Enrichment analysis revealed pathways associated with RNA processing, and in particular the spliceosome (Figure 2C). The abundance of about one half of proteins involved with spliceosome was negatively correlated with energy intake. Of all the proteins negatively correlated with energy intake, 15% were spliceosome-related proteins. These included most of the heterogeneous nuclear ribonucleoproteins (HNRNPs L, R, F, M, U, UL, A0, HI, A2, DL, AB, D, UL1, H2, K, AB, A1, C, H3, LL; PTBP1, RALY) and serine/arginine-rich splicing factors (SRSF1, 2, 5, 6, 7, 9, 10, 11). HNRNPs and SRSFs are major regulators of splicing (Wilkinson et al., 2020). These conclusions were consistent with analysis using generalized additive models (GAMs) (Figures S2A and S2B).

An association between the spliceosome and caloric restriction has been reported in *C. elegans* (Heintz et al., 2017), *D. melanogaster* (Gao et al., 2020) mice (Swindell, 2009) and Rhesus monkeys (Rhoads et al., 2018; Rhoads et al., 2020). In monkeys, caloric restriction was achieved by providing the monkeys with 30% less food than *ad libitum*-fed monkeys and this led to increased lifespan (Colman et al., 2009). In our study, a 30% reduction in energy intake was achieved in *ad libitum*-fed mice by dietary dilution. Previously, we found that this did not increase lifespan (Solon-Biet et al., 2014). This implies that upregulation of splicing by a reduction in energy intake from caloric dilution does not necessarily delay aging and increase lifespan, as is seen with the reduction of energy intake from standard caloric restriction where animals are given less food. Reduced energy intake induced by caloric dilution avoids the profound neuroendocrine responses to fasting in standard caloric restriction models which may be necessary for its lifespan effects (Speakman et al., 2016). Regardless, there is a robust response of the spliceosome to reduced energy intake across species.

Rapamycin, resveratrol, and metformin narrow the responses to energy intake

Rapamycin, resveratrol, and metformin reduced the number of proteins with abundance that was either positively or negatively correlated with energy intake (Figure 2B). Rapamycin and

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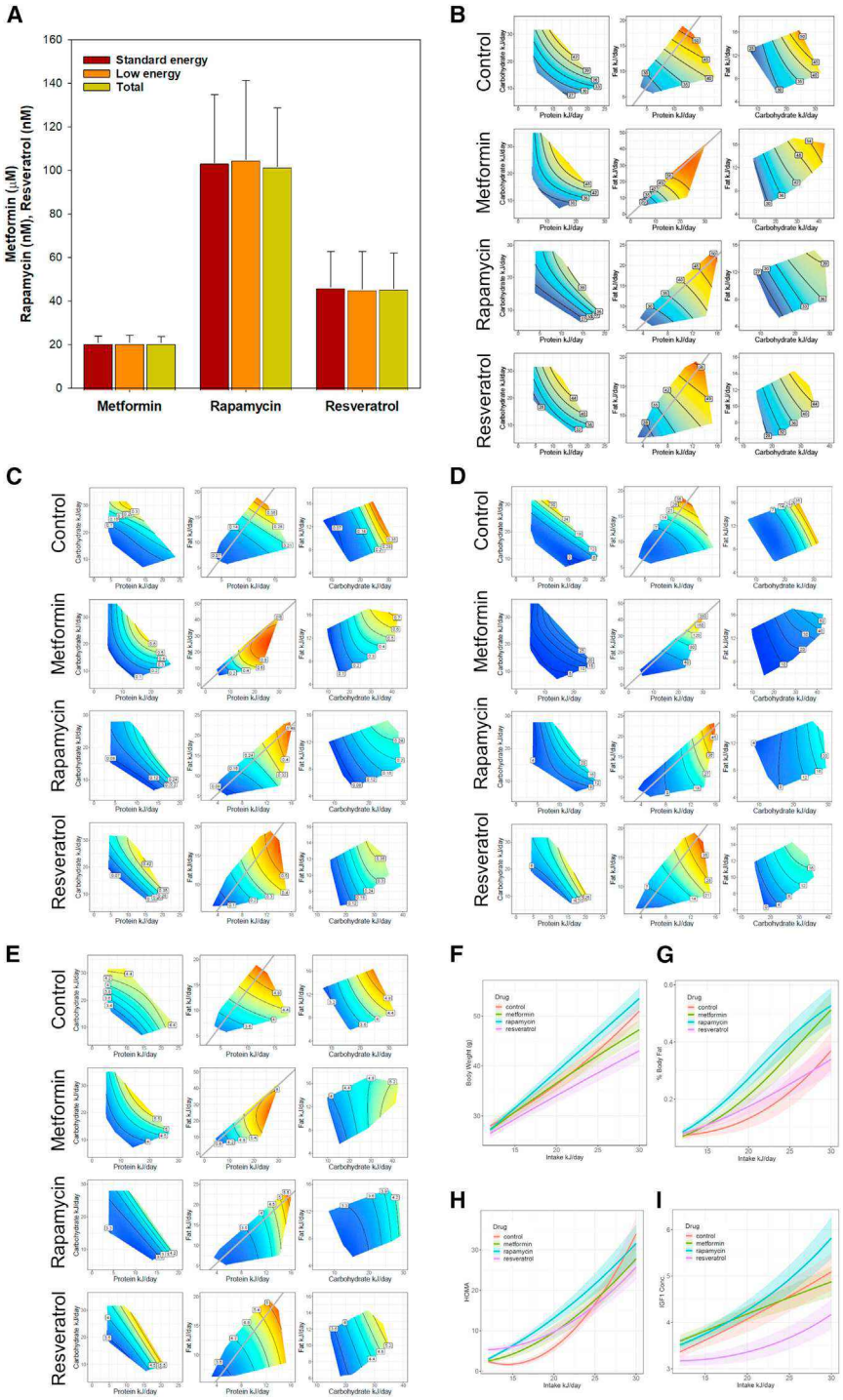

Figure 1. Nutrition and phenotypic outcomes

(A) Blood levels of metformin, rapamycin, and resveratrol in mice receiving these drugs. Results are represented as mean ± SD.

(B–D) Geometric framework surface response curves for (B) body weight (g), (C) percentage body fat, (D) insulin resistance (insulin [ng.mL^{-1}] × glucose [mM.L^{-1}]), and (E) IGF-1 (ng.ml^{-1}). For each group (control, metformin, rapamycin, resveratrol) there are three surfaces that are bounded by the axes: protein intake versus carbohydrate intake, protein versus fat intake, and carbohydrate versus fat intake. The color of the surface varies from red, which is the highest value, to blue, which is the lowest value.

(F–I) The relationship between food intake and phenotype along the vectors demonstrated in (B)–(E).

*Statistical analyses for response surfaces are provided in Table S2

See also Figure S1.

resveratrol reduced the number and/or statistical significance of the pathways associated with energy intake, while metformin had minimal effect (Figures 2E and 2F).

Metformin, rapamycin, and resveratrol also significantly influenced the slope of the relationship between the abundance of some proteins and energy intake. The drugs mostly dampened or reversed the slope of the relationship between energy intake and protein abundance and rarely increased the slope of the relationship (Figure 3A). Rapamycin and resveratrol impacted on more proteins than did metformin. Rapamycin and resveratrol had the largest effect on pathways including monocarboxylic acid metabolism, oxidation-reduction processes, and the peroxisome (Figure 3B).

For those proteins with abundance that was negatively correlated with energy intake, rapamycin and resveratrol reduced splicing and RNA metabolism pathways (Figure 3C). They also reversed the relationship of proteins involved with the lysosome, carbohydrate metabolism, and VEGF pathways (Figures 3B and 3C).

Together, these analyses indicate that low energy intake drives RNA metabolic pathways, particularly the spliceosome, while high energy intake is associated with upregulation of a suite of metabolic pathways involved with the metabolism of nutrients. Overall, metformin, rapamycin, and resveratrol narrowed these responses, either by reducing the number of proteins that were significantly correlated with energy intake or by dampening and even reversing the slope of the relationship between protein abundance and energy intake. Rapamycin and resveratrol had larger impacts than metformin, including downregulation of the spliceosome response to low energy intake.

The effect of these drugs is likely secondary to their role in regulation of protein synthesis. All three drugs inhibit MTORC1, either directly or indirectly, which is the key regulator of translation via downstream effects on 4EBP and S6K1 (Howell et al., 2017; Kennedy and Lamming, 2016; Villa-Cuesta et al., 2011). Here, the abundance of these three proteins (MTOR, S6K1,

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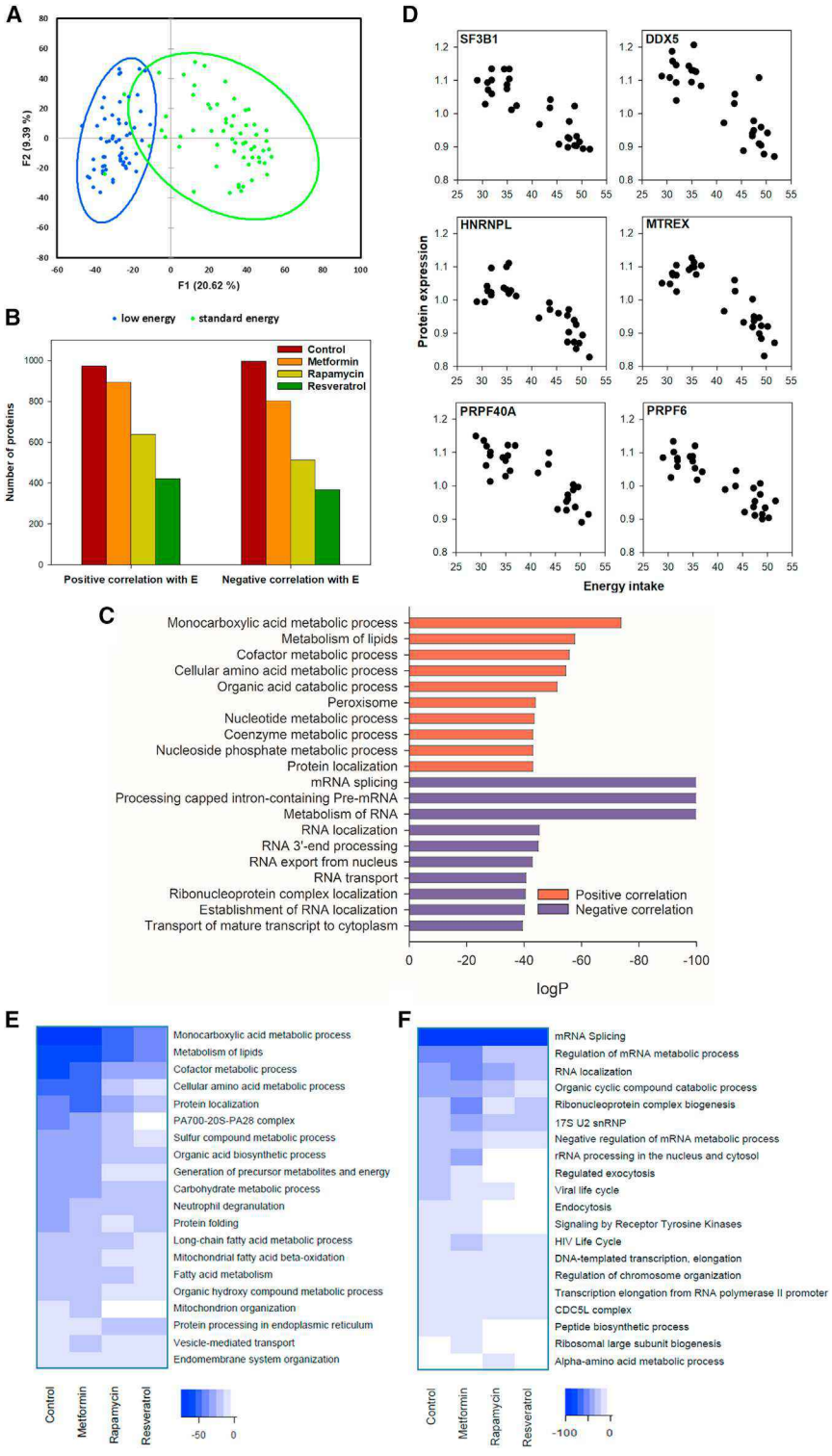

Figure 2. Dietary energy and hepatic proteome

(A) PCA demonstrated separation of proteome on the basis of low dietary energy and standard dietary energy.

(B) Comparison of control, metformin, rapamycin, and resveratrol groups for the number of proteins that were significantly correlated, positively or negatively, with energy intake.

(C) Enrichment analysis showing pathways that were associated with proteins with abundance that was positively (red) or negatively (blue) correlated with dietary energy intake.

(D) The relationship between dietary energy and the abundance of several key proteins associated with spliceosome (Splicing factor 3B subunit 1, SF3B1, $r = -0.87$, $p < 10^{-3}$; DEAD box protein 5 DDX5, $r = -0.84$, $p < 10^{-3}$; Heterogeneous nuclear ribonucleoprotein L, HNRNPL, $r = -0.83$, $p < 10^{-3}$; Exosome RNA helicase MTR4, MTREX, $r = -0.86$, $p < 10^{-3}$; Pre-mRNA-processing factor 40 homolog A, PRPF40A, $r = -0.84$, $p < 10^{-3}$; Pre-mRNA-processing factor 6, PRPF6; $r = -0.87$, $p < 10^{-3}$). Heatmaps comparing the effects (logP) of metformin, rapamycin, and resveratrol on pathways identified by enrichment analysis of proteins where abundance was positively correlated (E) or negatively correlated (F) with energy intake. The most intense blue color identifies pathways that are most strongly associated with the proteins in the control or drug groups. When a drug has influenced a pathway, the blue intensity will be different to the control.

See also Figure S2.

4EBP) was not influenced by energy intake or by rapamycin, metformin, or resveratrol; however, the actions of these proteins are regulated by phosphorylation rather than abundance.

Metformin, rapamycin, and resveratrol have been considered to be caloric restriction-mimetics, meaning that they recapitulate many of the phenotypic features of caloric restriction without reducing energy intake (Madeo et al., 2019). If these drugs are caloric restriction-mimetics, then in our experimental model, they should replace some of proteins that are positively correlated with energy intake with proteins that are negatively correlated with energy intake. To test this, a Venn diagram was created comparing proteins that are negatively correlated with energy intake and proteins that are positively correlated with energy intake in the presence of metformin, rapamycin, or resveratrol. There were no shared proteins apart from two proteins with rapamycin (Figure 3D). Although the drugs, particularly resveratrol, reversed the slope of the positive relationship between energy intake and the abundance of many proteins (Figure 3A), none of these proteins overlapped with those with abundance that was negatively correlated with energy intake (Figure 3E). However, it is important to note that reduction in energy intake by caloric dilution is different to caloric restriction because it avoids neuroendocrine responses to fasting (Speakman et al., 2016).

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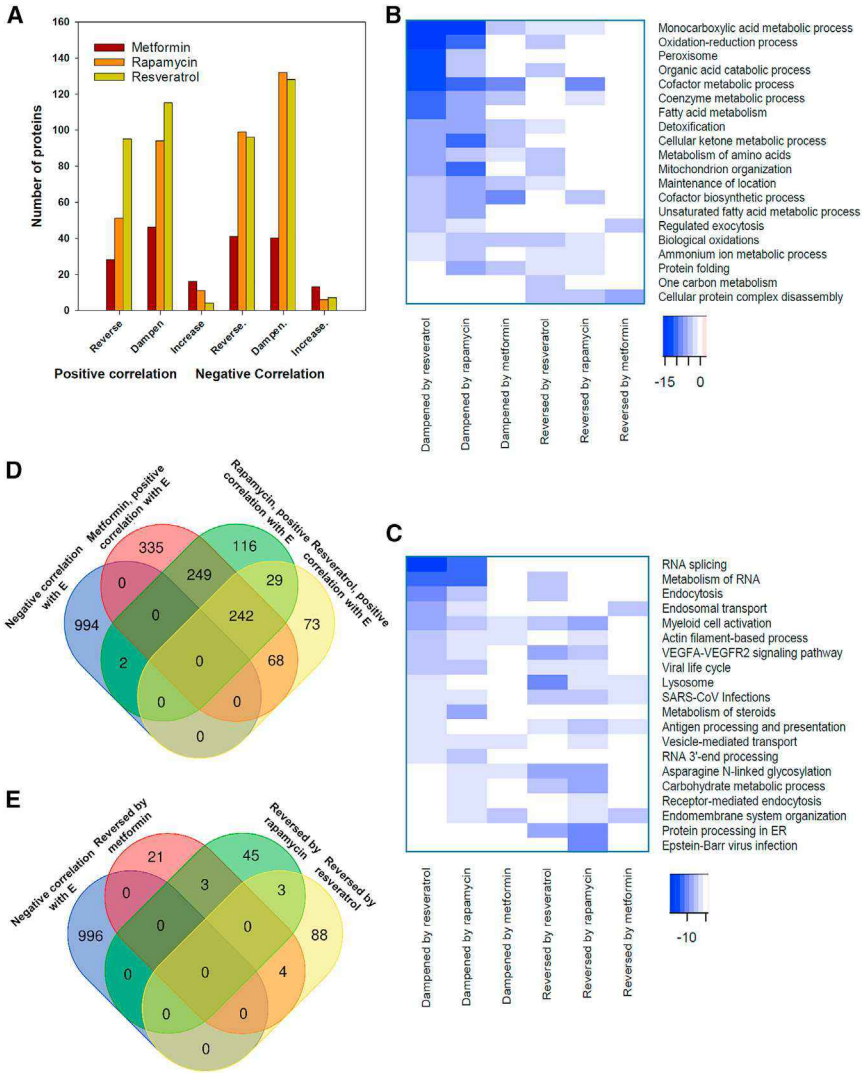

Figure 3. The effects of metformin, rapamycin, and resveratrol on the response of the hepatic proteome to dietary energy

(A) The number of proteins where the correlation between abundance and energy intake was significantly altered by metformin, rapamycin, or resveratrol. The slope of the relationship between abundance and energy intake could either be increased, dampened, or reversed with metformin, rapamycin, or resveratrol compared to the control groups.

(B and C) Heatmaps showing the effects (logP) of metformin, rapamycin, and resveratrol on pathways identified by enrichment analysis of those proteins where the relationship between abundance and energy intake was positive (B) or negative (C) and was significantly altered by these drugs. The most intense blue color corresponds to those pathways that are most dampened or reversed by metformin, rapamycin, or resveratrol.

(D) Venn diagram comparing those proteins where abundance was negatively correlated with energy intake (reflecting caloric restriction) and those proteins that were positively correlated with energy intake and treated with either metformin, rapamycin, or resveratrol.

(E) Venn diagram comparing those proteins where abundance was negatively correlated with energy intake and those proteins where their abundance had a significant positive correlation with energy intake that was reversed with administration of either metformin, rapamycin, or resveratrol.

Metformin, rapamycin, and resveratrol are reported to act on nutrient sensing and aging pathways. We evaluated whether these drugs altered the number of proteins that make up each of the following pathways: insulin/IGF1, MTOR, NFκB, sirtuin, oxidative stress, autophagy, or xenobiotic metabolism (Derous et al., 2017). There was no evidence on PCA that metformin, rapamycin, and resveratrol influenced the abundance of proteins involved in these pathways (Figure S3). Absence of change in MTOR signaling in liver with long-term rapamycin treatment has been reported by others (Fok et al., 2014b).

Protein intake upregulates mitochondrial proteins and pathways, generating oxidative stress

PCA showed that the highest protein diet (protein 55%) was differentiated from the other lower protein diets (protein 10%–33%) (Figure 4A). There were 499, 356, and 262 proteins with abundance that were positively correlated with protein intake, carbohydrate intake, or fat intake, respectively. There were 166, 323, and 213 proteins with abundance that were negatively correlated with protein intake, carbohydrate intake, or fat intake, respectively (Figures 4B and 4C).

There were many mitochondrial proteins with abundance positively correlated with protein intake (Figure 4D) as well as mitochondrial pathways on enrichment analysis (Figure 4E). Protein intake was also associated with the abundance of the gluconeogenic enzymes, aspartate aminotransferases (GOT1, GOT2), and alanine aminotransferase (GPT2). The conclusions from GAMs analysis were similar, confirming the association of protein intake with mitochondrial pathways (Figure S4D).

Transcription factors associated with protein intake identified by enrichment analysis were PPARGCIA (PGC1A), NRF1, SP1, SP3, and PPARD (Figure S4E). PGC1A is a master regulator of mitochondria secondary to its interactions with NRF1 (Miller et al., 2019). SIRT3 is a mitochondrial sirtuin and regulator of mitochondrial function identified as a link between caloric restriction and aging (Barger et al., 2015) The abundance of SIRT3 was positively associated with protein intake ($r = 0.6$, adjusted $p < 0.01$) but not the other macronutrients.

Unlike excess carbohydrates and fat which are stored directly as glycogen and triglycerides, respectively, excess amino acids are not stored in the liver. Protein synthesis is maximal on standard dietary protein and does not increase when dietary protein is increased beyond normal intake (Zaragozá et al., 1987). The only strategy available for the liver to dispose of excess dietary amino acids is to divert them toward energy production by mitochondria. High-protein diets have been linked with increases in mitochondrial number and size (Zaragozá et al., 1987) and increased

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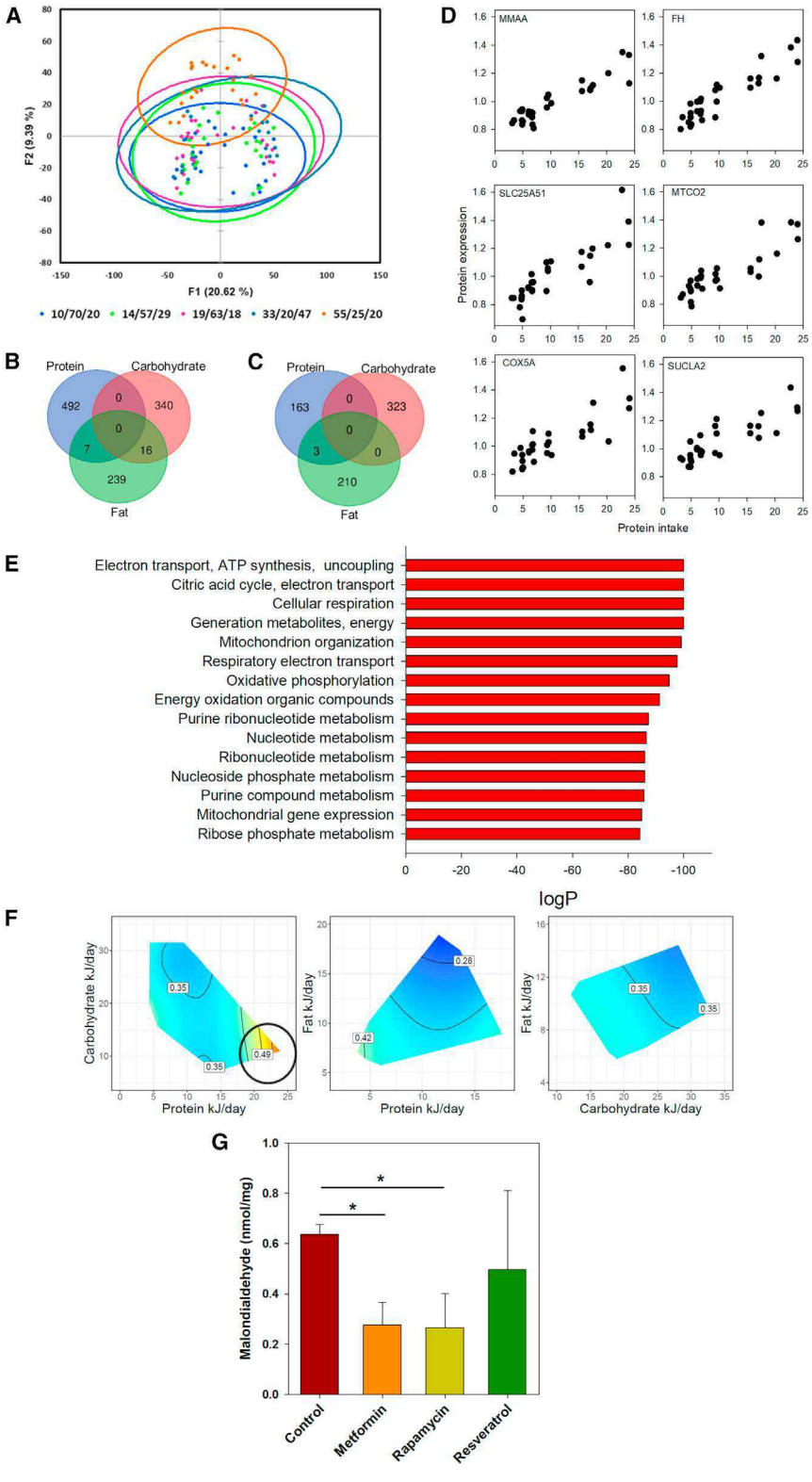

Figure 4. Macronutrients and the hepatic proteome

(A) PCA demonstrated differentiation of the high protein diet from the other diets.

(B and C) Venn diagram of proteins with abundance that was positively (B) correlated and negatively (C) correlated with intake of protein, carbohydrate, or fat.

(D) The relationship between protein intake and the abundance of mitochondrial proteins (Methylmalonic aciduria type A protein, MMAA, $r = 0.92$, $p < 10^{-3}$; Fumarate hydratase, FH, $r = 0.92$, $p < 10^{-3}$; Mitochondrial nicotinamide adenine dinucleotide transporter, SLC25A51, $r = 0.87$, $p < 10^{-3}$; Cytochrome *c* oxidase subunit 2, MTCO2, $r = 0.87$, $p < 10^{-3}$; Cytochrome *c* oxidase subunit 5A, COX5A, $r = 0.84$; Succinate-CoA ligase [ADP-forming] subunit beta, SUCLA2, $r = 0.84$, $p < 10^{-3}$).

(E) Enrichment analysis showing pathways associated with proteins with abundance that were positively correlated with dietary protein intake.

(F) Geometric Framework surface response curves for liver levels of malondialdehyde (nmol/mg) in control mice.

(G) The effect of metformin, rapamycin, and resveratrol on liver levels of malondialdehyde in mice on high-protein diet (55% protein, standard energy). (n = 5–6 per group, Kruskal-Wallis test $p < 0.01$, *$p < 0.05$ Dunns test).

*Statistical analysis for response surface is provided in Table S3

See also Figure S4.

expression of genes influencing mitochondria (Gokarn et al., 2018) as well as abundance of mitochondrial proteins as shown here. In order to determine whether this generated oxidative stress, malondialdehyde concentrations were measured. High protein intake was associated with increased malondialdehyde levels (Figure 4F). This was reduced significantly by metformin and rapamycin, but not resveratrol, in mice on the high protein diets (Figure 4G). In addition, PCA showed that high-protein diets differed from the other lower protein diets in terms of the abundance of proteins involved in oxidative stress pathways (Figure S3H).

Dietary protein upregulates mitochondrial NAD$^+$ transporter, SLC25A51, and downregulates NENF, a potential regulator of appetite

There was a positive correlation of SLC25A51 with protein intake in control animals (Figures 4D and 5A). Rapamycin, metformin, and resveratrol did not reverse the relationship between protein intake and SLC25A51 abundance (Figure 5A). SLC25A51 is the mitochondrial NAD$^+$ transporter (Luongo et al., 2020). We also found that SLC25A51 abundance was negatively correlated with the abundance of NMRK1 and NAMPT and positively correlated with the abundance of NMNAT3 and NNMT (Figure 5B),

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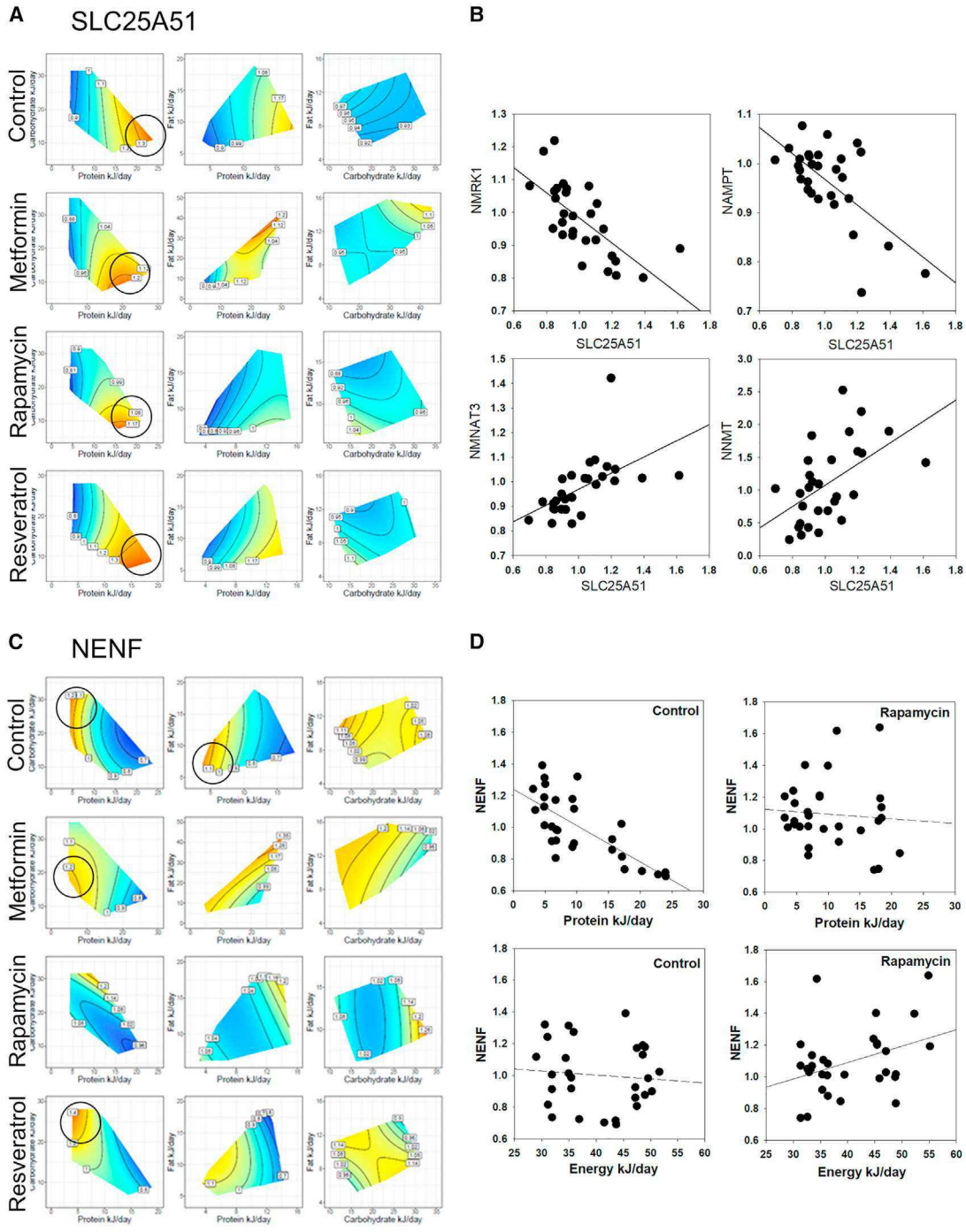

Figure 5. Protein intake and SLC25A15 and NENF

(A) Geometric Framework surface response curves for the abundance of SLC25A51. There is upregulation of SLC25A51 with high protein intake.

(B) The relationship between SLC25A51 abundance and the abundance of key proteins in the mitochondrial NAD^+ pathways: Nicotinamide riboside kinase 1, NMRK1, $r = -0.68$, $p < 10^{-3}$; Nicotinamide phosphoribosyltransferase, NAMPT, $r = -0.65$, $p < 10^{-3}$; Nicotinamide nucleotide adenylyltransferase 3, NMNAT3, $r = 0.56$, $p < 10^{-2}$; Nicotinamide N-methyltransferase, NNMT, $r = 53$, $p < 10^{-2}$).

(C) Geometric Framework surface response curves for the abundance of NENF. There is upregulation of NENF in low protein diets, which is attenuated significantly by rapamycin.

(D) The relationship between NENF abundance and intake of protein and energy, in control mice ($r = -0.73$ $p < 10^{-5}$, $r = -0.1$ ns, respectively) and those on rapamycin ($r = -0.1$ ns; $r = 0.37$ $p < 0.05$, respectively).

*Statistical analyses for response surfaces are provided in Table S5.

See also Figure S5.

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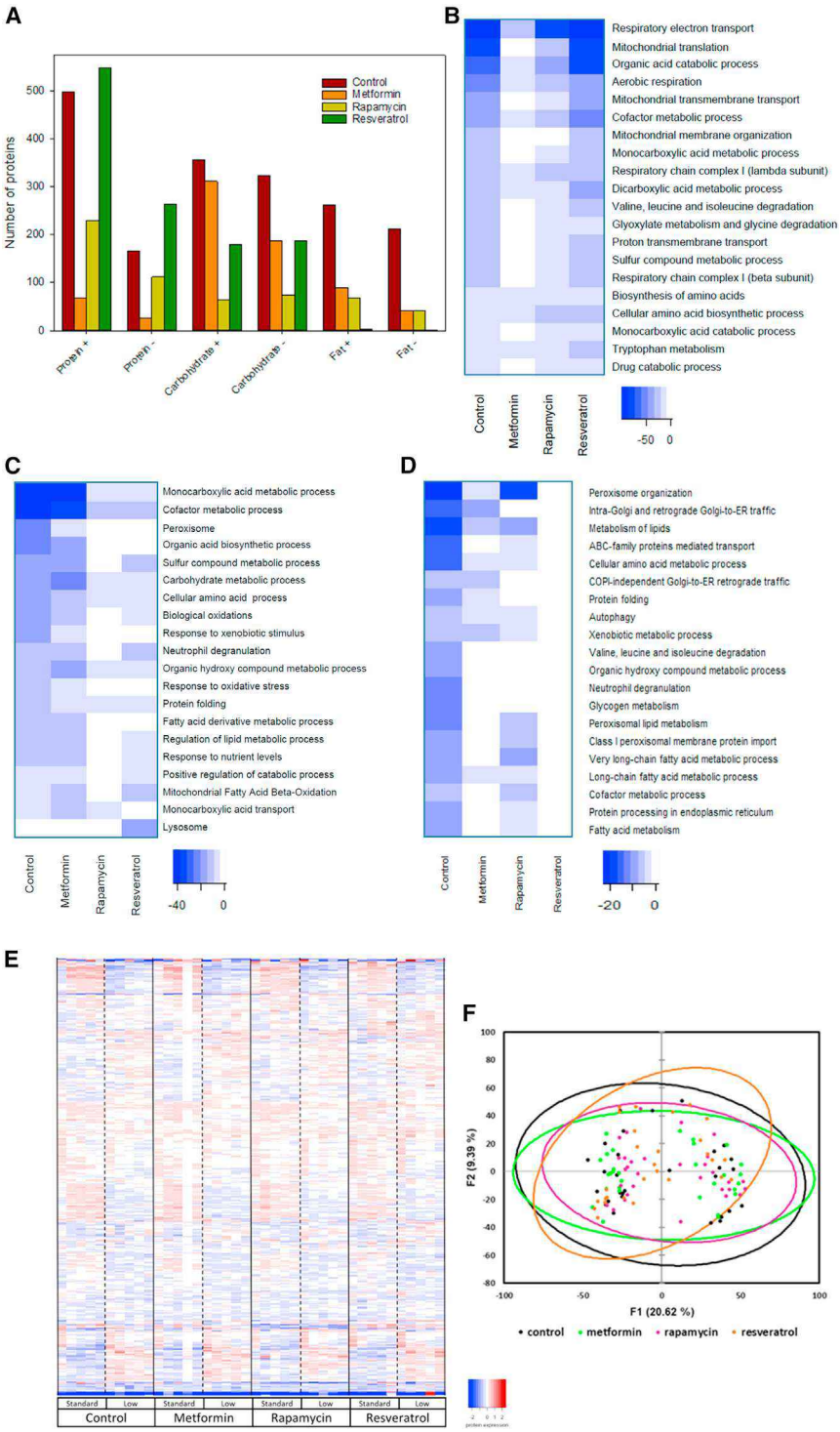

Figure 6. The effects of metformin, rapamycin, and resveratrol on the number of proteins where abundance is influenced by macronutrient intake

(A) Comparison of control, metformin, rapamycin, and resveratrol groups for the number of proteins with abundance that significantly correlates, positively or negatively, with intake of protein, carbohydrates, and fat.

(B–D) Enrichment analysis showing pathways associated with proteins where abundance was positively correlated with protein intake (B), carbohydrate (C), or fat intake (D), comparing control, metformin, rapamycin, and resveratrol groups.

(E) Heatmap showing protein abundance. Each lane is pooled from two to four livers. The columns have been ordered according to drug group, energy density, and then by macronutrient composition.

(F) PCA comparing control versus metformin, rapamycin, and resveratrol.

*Statistical analyses are provided in the supplemental information.

See also Figure S6.

consistent with expected responses to elevated levels of mitochondrial NAD^+.

Given the possibility that liver mitochondrial function regulates responses to dietary protein, we postulated that liver mitochondria might generate a signal to regulate protein intake. NENF (neudesin) was among the top ten proteins that were negatively correlated with protein intake. Rapamycin abolished the effect of any macronutrient on NENF and generally led to high overall levels (Figure 5C). NENF is a neurotrophic factor that has not been widely studied but is expressed in the liver in both mitochondria and endoplasmic reticulum (Ohta et al., 2015a; Ohta et al., 2015b) and influences food intake (Byerly et al., 2013; Ohta et al., 2015b). We found a negative correlation between NENF abundance and dietary protein, which might indicate that NENF stimulates protein intake. Through the protein leverage effect (Simpson and Raubenheimer, 2005) this could influence the intake of other macronutrients and energy. The effects of rapamycin indicate that protein sensing via the MTOR pathway inhibits NENF abundance.

Macronutrients upregulate pathways linked to their metabolism

Apart from the marked relationship between protein intake and mitochondrial proteins, all the macronutrients influenced a wide range of metabolic pathways involved with carbohydrate, fat, and amino acid metabolism (Figures 6A–6D). Protein intake was positively correlated with many amino acid metabolic pathways (valine, leucine and isoleucine degradation, biosynthesis of amino acids, tryptophan metabolism); carbohydrate intake was positively correlated with carbohydrate metabolic pathways (monocarboxylic acid metabolic process, carbohydrate metabolic process); and fat intake was positively correlated with fat metabolic pathways (peroxisome

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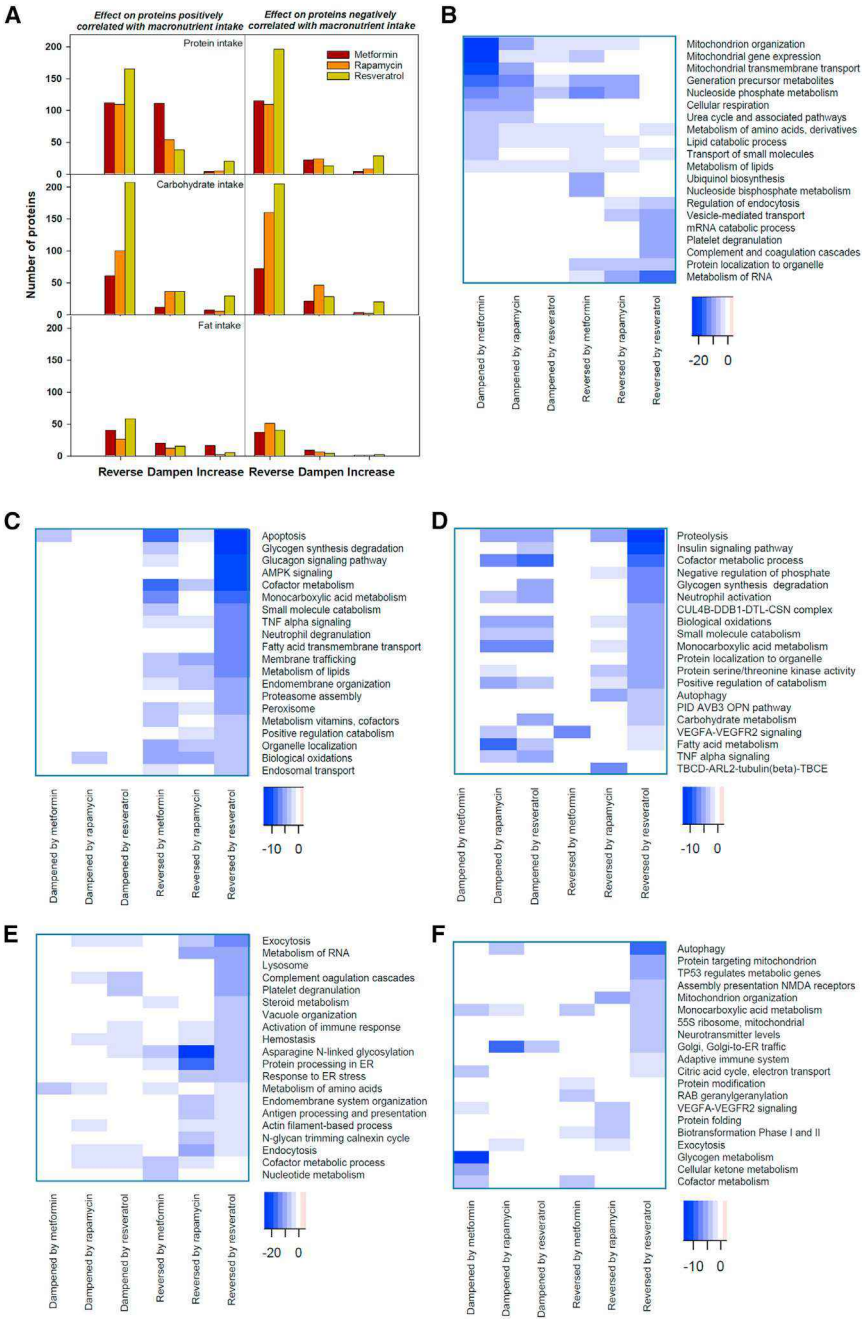

Figure 7. The effects of metformin, rapamycin, and resveratrol on the correlation between protein abundance and macronutrient intake

(A) The number of proteins where the correlation between abundance and intake of protein, carbohydrate, or fat was significantly altered by metformin, rapamycin, or resveratrol.

(B and C) Heatmap generated by enrichment analysis of proteins where there was a positive (B) or negative (C) correlation between abundance and intake of protein and where this correlation was significantly altered by metformin, rapamycin, or resveratrol.

(D and E) Heatmap generated by enrichment analysis of proteins where there was a positive (D) or negative correlation (E) between abundance and intake of carbohydrates and where the slope of this correlation was significantly altered by metformin, rapamycin, or resveratrol.

(F) Heatmap generated by enrichment analysis of proteins where there was a positive correlation between abundance and intake of fat and where the slope of this this correlation was significantly altered by metformin, rapamycin, or resveratrol.

organization, metabolism of lipids, peroxisomal lipid metabolism, very long chain and long chain fatty acid metabolic processes).

Metformin, rapamycin, and resveratrol dampen the responses to dietary macronutrients

Metformin and rapamycin reduced the number of proteins with abundance that was correlated, positively or negatively, with all macronutrients and associated pathways (Figure 6A). Resveratrol had a large effect on proteins and pathways influenced by the intake of carbohydrates and fat but not protein (Figures 6B–6D; Figure S6).

The effects of these drugs on the proteome were not as substantial as those generated by dietary components. A heatmap of protein abundance across all diets and drugs shows that the main effect on liver proteome was energy intake rather than drugs (Figure 6E). PCA was unable to separate the drug groups and controls (Figure 6F). Comparing the PCAs (Figures 2A, 4A, and 6F) indicates that the greatest effects on the proteome were generated by dietary energy, then macronutrients, then drugs.

'Metformin, rapamycin, and resveratrol also significantly altered the slope of the relationship between macronutrient intake and the abundance of proteins. The main effect was either to dampen or reverse the correlations, rather than increase the correlation (Figure 7A). This effect was greatest for resveratrol, reversing the positive or negative correlation between the abundance of many proteins and the intake of either protein or carbohydrates. The drugs had much less effect on proteins with abundance that were correlated with fat intake. Enrichment analysis revealed that the positive impact of protein intake on the abundance of mitochondrial proteins was dampened by all three drugs (Figure 7B). Overall, resveratrol had the greatest effects, reversing the effects of macronutrients on many pathways, not only metabolic pathways but a range of others including autophagy, inflammatory responses, exocytosis, and apoptosis (Figures 7C–7F). Resveratrol also increased the slope of the association between the abundance of many proteins and macronutrients, although

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this was much less frequent with rapamycin and metformin. Resveratrol has a major role in the epigenetic regulation of transcription as well as in influencing translation via MTOR (Fernandes et al., 2017). This provides a mechanism for its greater effects compared to rapamycin and metformin, which only influence translation via MTOR.

Given the association between protein intake and the abundance of mitochondrial proteins and the established effects of metformin, rapamycin, and resveratrol on mitochondrial function, the effects of these drugs on the main mitochondrial pathways were examined. Metformin and rapamycin both substantially reduced the abundance of mitochondrial proteins upregulated by protein intake, while resveratrol had minimal effect. This is consistent with our finding that metformin and rapamycin, but not resveratrol, reduced oxidative stress associated with protein intake. Notably, metformin substantially reduced the number of complex 1 proteins that were upregulated by protein intake, supporting its complex 1 mode of action *in vivo* and at a micromolar concentration (Table S4; Pecinová et al., 2019). Transcription factor enrichment analysis showed that both metformin and rapamycin, but not resveratrol, suppressed the response of PPARGC1A (Figure S4E) to protein intake.

DISCUSSION

The liver is the primary organ that responds to nutrition and regulates systemic homeostasis of carbohydrates, fat, and proteins. Variation in energy intake is dealt with by regulating the mobilization and synthesis of energy stores (glycogen, triglycerides) and the rate of production of energy substrates (ATP, NAD). Caloric restriction, the main nutritional intervention that delays aging, causes an extensive integrated reprogramming of liver metabolism (Rhoads et al., 2018). Similarly, in our study the nutritional factor that influenced the hepatic proteome the most was the energy density of the diet (where low energy density was generated by dilution with non-digestible cellulose). Pathways that were positively correlated with energy intake in the liver included a suite of processes involved in the metabolism of carbohydrates, fat, and amino acids.

Mice on the diluted diets ate more food but consumed about 30% less energy than those mice on the standard energy diet. This is similar in extent to standard caloric restriction experimental models where animals are given less food, rather than diluted food. Therefore, we anticipated some overlap between our results for proteins with abundance that were negatively correlated with energy intake and those reported for caloric restriction studies. Low energy intake was strongly associated with processes involved in RNA metabolism, particularly splicing and the spliceosome. This has been noted recently in standard caloric restriction models in *C. elegans* (Heintz et al., 2017), *D. melanogaster* (Gao et al., 2020), mice (Swindell, 2009), and monkeys (Rhoads et al., 2018; Rhoads et al., 2020). In the monkey study it was concluded that upregulation of splicing indicates that alternate transcripts regulate the broad response to energy depletion (Rhoads et al., 2018). Alternatively, reduction in translation secondary to caloric restriction might be reciprocated with increased translational fidelity, potentially improving cellular resilience and delaying aging (Swindell, 2009). This reciprocal relationship is supported by our observation that metformin, rapamycin, and resveratrol reduced both the abundance of proteins and splicing pathways. Aging is associated with altered splicing and reduced splicing efficiency in animals from *C. elegans* to humans (Lee et al., 2016). Splicing homeostasis predicts life expectancy in *C. elegans*, and age-related changes in splicing are reversed by caloric restriction (Heintz et al., 2017). RNA metabolism and splicing are important components of cellular proteostasis, recognized as one of the "hallmarks of aging" (Lopez-Otín et al., 2013). Our results emphasize that regulation of splicing is highly nutrient dependent. Short-term fasting and refeeding influence splicing of G6PD, a key enzyme in *de novo* lipogenesis, and this is mediated via abundance of SRSFs and HNRNPs. (Cyphert et al., 2013; Walsh et al., 2013). Many SRSFs and most HNRNPs were influenced by energy intake in our study, supporting their role in linking long-term nutrition and splicing.

Among the wide range of effects of macronutrients on the liver proteome, the association between protein intake and mitochondrial proteins and pathways was the most striking. There have been only a few previous studies linking dietary protein and mitochondria in the liver, including an ultrastructural study and two transcriptomic studies (Gokarn et al., 2018; Schwarz et al., 2012; Zaragozá et al., 1987). Excess dietary protein cannot be stored in the liver; therefore the only mechanisms that the liver has to deal with excess intake of dietary protein are the anaplerotic pathways that utilize amino acids for energy production in mitochondria and the conversion of amino acid carbon to glucose and fatty acids. High protein intake was associated with increased oxidative stress, presumably the consequence of excessive mitochondrial function beyond maintenance of normal function that occurs in healthy aging where oxidative stress is minimized (Bornstein et al., 2020). This provides a mechanistic link between high protein diet and accelerated aging. On the other hand, we previously found that low-protein, high-carbohydrate diets were associated with longer lifespan (Solon-Biet et al., 2014), and such diets would be associated with lower abundance of mitochondrial proteins leading to lower levels of oxidative stress.

Dietary protein upregulated the mitochondrial NAD^+ transporter SLC25A51 (Luongo et al., 2020), and this is the first report of external regulation of its abundance. Given the key role of SLC25A51 in mitochondrial function, it may link dietary protein intake with mitochondria. Another potential mechanism to counter excessive protein intake is the reduction in protein appetite. It is intriguing that NENF, a newly described neurotrophic protein that influences food intake (Byerly et al., 2013; Ohta et al., 2015b), was strongly influenced by protein intake. Further studies will determine whether it is a protein-sensing regulator of protein appetite.

The effects of metformin, rapamycin, and resveratrol on the hepatic proteome were not as large as the effects of dietary constituents and mostly involved reduction in the proteomic response to the intake of energy and macronutrients. This is most likely secondary to the inhibitory effects of these drugs on global protein translation, mediated directly or indirectly via MTOR (Howell et al., 2017; Kennedy and Lamming, 2016; Villa-Cuesta et al., 2011). Rapamycin has been shown by others to reduce bulk protein synthesis in the liver (Fok et al., 2014b; Karunadharma et al., 2015); however, there were substantial differences between their effects, related both to the underlying diets and the target pathways. Resveratrol more frequently increased the strength or reversed the slope of the correlations between macronutrient intake and the abundance of proteins and

influenced more proteins than metformin and rapamycin. Resveratrol influences epigenetic regulation of gene transcription (Fernandes et al., 2017) that provides a mechanism for its additional impact on protein abundance compared to metformin and rapamycin, which regulate protein synthesis only at the translational level. Resveratrol mostly acted on proteins that were influenced by intake of fat and carbohydrates, rather than protein. This is consistent with the finding that the anti-aging effects of resveratrol were only seen in mice on high fat diets but not standard diets (Baur et al., 2006; Pearson et al., 2008). Some of the effects of resveratrol are mediated by activation of SIRT1, an enzyme that requires NAD^+ as a cofactor, and therefore will be influenced by intake of fat and carbohydrates that more directly influence cellular bioenergetics than protein. On the other hand, metformin and rapamycin both dampened the upregulation of mitochondrial proteins and pathways driven by higher protein intake. This response matches the differential effects of these drugs on the number of proteins that were significantly influence by protein intake. The acute mitochondrial effects of metformin may include either activation of AMPK leading to increased mitochondrial activity or inhibition of complex 1 leading to reduced mitochondrial function (Pecinová et al., 2019). However, a proteomic study in *ApoE* knockout mice found that four months of metformin downregulated many mitochondrial pathways (Stachowicz et al., 2012), consistent with our findings. Similarly, rapamycin acutely lowered mitochondrial activity (Schieke et al., 2006) and reduced mitochondrial protein abundance (Fok et al., 2014b; Karunadharma et al., 2015).

Although metformin, rapamycin, and resveratrol are considered to be caloric restriction-mimetics, we did not find any evidence that they were influencing the abundance of proteins linked with caloric restriction, aging, or the nutrient sensing pathways. This might be because many of these pathways are regulated by phosphorylation rather than abundance. It also reflects the difference between caloric dilution and caloric restriction. Both are associated with a similar reduction in energy intake; however, *ad libitum* access to diluted food mitigates the profound behavioral and neuroendocrine consequences of fasting (Speakman et al., 2016). The effects of caloric restriction and rapamycin on the hepatic transcriptome and metabolome have been compared, and it was found that there was little overlap between the effects of rapamycin and caloric restriction and that dietary intervention had a greater impact than rapamycin (Fok et al., 2014a; Fok et al., 2014b). A similar conclusion was drawn in a study of global protein synthesis, where the half-lives of proteins were influenced more by caloric restriction than rapamycin (Karunadharma et al., 2015). Even though MTOR is the target for both rapamycin and dietary protein, we did not find any effects of rapamycin on the proteome that were differentially influenced by dietary protein intake.

In conclusion, we utilized the Geometric Framework to interpret the complex relationships between diet and physiological responses of the hepatic proteome over a broad range of macronutrient intakes in the presence or absence of metformin, rapamycin, or resveratrol. Among the very large number of proteins and pathways influenced by diet, the major findings were that low energy intake increased the abundance of spliceosome proteins, while protein intake was positively correlated with the abundance of mitochondrial proteins, leading to oxidative stress. Overall metformin, rapamycin, and resveratrol reduced these proteomic responses to diet indicating their role in suppressing protein synthesis; however, there were important differences between these drugs at the level of the macronutrients and the pathways that were targeted. Dietary energy and macronutrients had much greater impact on protein abundance than the drugs.

Limitations of study

The major issue is that this is a complex dataset where dietary macronutrients, dietary energy, and three different drugs were studied, generating 40 different treatment groups. As we had done previously with a study of the effects of macronutrients on the hepatic transcriptome, we used different analytical methods to interrogate the data based on Pearson's correlation coefficient and GAMs in an attempt to increase the robustness of our conclusions. Only male C57BL/6J mice were studied which limits applicability. Other analyses for post-translational modification of proteins which influence protein function were not performed. There may be circadian rhythm in the abundance of some proteins, which could not be detected since all samples were taken at the same time point. We did not evaluate the microbiome, which is influenced both by diet (Holmes et al., 2017) and drugs such as metformin and rapamycin (Bauer et al., 2018; Bitto et al., 2016) with effects on systemic metabolic responses.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2021.10.016.

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AUTHOR CONTRIBUTIONS

S.J.S., D.L.C., V.C.C., and D.E.J. designed the study. D.L.C., S.M.S., and S.J.S. wrote the first draft. S.M.S. managed the study. T.P., A.B., G.J.C., S.M.S., V.C.C., and J.A.W. performed and managed animal experiments;

B.L.P. performed proteomics; N.J.H. performed drug levels; R.G., A.M.S., and S.M.S. performed modeling.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ketamil (100mg/mL as Ketamine Hydrochloride)	Ilium	APVMA no. 51188c
Xylazil-20 (20mg/mL Xylazine as Hydrochloride)	Ilium	APVMA no. 38653
Metformin	Farmahispania	CAS 1115-70-4
Resveratrol	Sirtris Pharmaceuticals	SRT501
Rapamycin	Rapamycin Holdings	16-0202-027-12
a-D Glucose	Sigma Aldrich	158968
trans-Resveratrol-d4	Cayman Chemicals	13130
Rapamycin-d3	Cayman Chemicals	22093
Metformin-d6 (hydrochloride)	Cayman Chemicals	16921
Metformin (hydrochloride)	Cayman Chemicals	13118
trans-Resveratrol	Cayman Chemicals	70675
Rapamycin	Cayman Chemicals	1568-5
Critical commercial assays		
Ultra Sensitive Mouse Insulin ELISA Kit	CrystalChem	90080: RRID:AB_2783626
Fibroblast Growth Factor 21 Mouse/Rat ELISA	BioVendor	RD291108200R: RRID: AB_2783730
Lipid Peroxidation (MDA) Assay Kit (Colorimetric/Fluorometric)	Abcam	ab118790
GPO-PAP Triglyceride kit	Roche Diagnostics	11730711 216
Deposited data		
Mendeley data: 10.17632/vy22skn8ps.1		
Experimental models: organisms/strains		
Mouse: C57BL/6J	Animal Resources Centre	MGI:3028467
Software and algorithms		
DB EchoMRI software	N/A	N/A
XLstat	XLstat Addinsoft 2020	https://www.xlstat.com
R	R Core Team	N/A
Metascape	N/A	http://metascape.org
Bioinformatics Evolutionary Genomics online program	N/A	http://bioinformatics.psb.ugent.be/webtools/Venn/
Other		
Mouse Diet: 55p 25f 20c 8mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-117
Mouse Diet: SF16-117 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-033
Mouse Diet: SF16-117 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-034
Mouse Diet: SF16-117 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-035
Mouse diet: 33p 20f 47c 8mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-118
Mouse Diet: SF16-118 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-036
Mouse Diet: SF16-118 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-037
Mouse Diet: SF16-118 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-038
Mouse Diet: 19p 63f 17c 8mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-119
Mouse Diet: SF16-119 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-039
Mouse Diet: SF16-119 + resveratrol 0.01%	Specialty Feeds Pty Ltd	SF17-040
Mouse Diet: SF16-119 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-041
Mouse Diet: 14p 57f 29c 8mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-120
Mouse Diet: SF16-120 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-042

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse Diet: SF16-120 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-043
Mouse Diet: SF16-120 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-044
Mouse Diet: 10p 70f 20c 8mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-121
Mouse Diet: SF16-121 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-045
Mouse Diet: SF16-121 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-046
Mouse Diet: SF16-121 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-047
Mouse Diet: 55P 20F 25C 15mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-112
Mouse Diet: SF16-112 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-018
Mouse Diet: SF16-112 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-019
Mouse Diet: SF16-112 + 14mg/kg rapamycin	Specialty Feeds Pty Ltd	SF17-020
Mouse Diet: 33p 20f 47c 15mj AIN93G	Specialty Feeds Pty Ltd	SF16-113
Mouse Diet: SF16-113 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-021
Mouse Diet: SF16-113 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-022
Mouse Diet: SF16-113 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-023
Mouse Diet: 19p 73f 17c 15mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-114
Mouse Diet: SF16-114 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-024
Mouse Diet: SF16-114 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-025
Mouse Diet: SF16-114 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-026
Mouse Diet: 14p 57f 29c 15mj mod AIN93G	Specialty Feeds Pty Ltd	SF16-115
Mouse Diet: SF16-115 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-027
Mouse Diet: SF16-115 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-028
Mouse Diet: SF16-115 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-029
Mouse Diet: 10p 70f 20c 15mj AIN93G	Specialty Feeds Pty Ltd	SF16-116
Mouse Diet: SF16-116 + metformin 0.1%	Specialty Feeds Pty Ltd	SF17-030
Mouse Diet: SF16-116 + resveratrol 0.04%	Specialty Feeds Pty Ltd	SF17-031
Mouse Diet: SF16-116 + rapamycin 14mg/kg	Specialty Feeds Pty Ltd	SF17-032

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the Lead Contact, David Le Couteur (david.lecouteur@sydney.edu.au)

Materials availability

The study did not generate new unique reagents or materials

Data and code availability

1. Data Protein abundance data were deposited on Mendeley and are publicly available as the date of publication:
https://data.mendeley.com/datasets/vy22skn8ps/draft?a=e5083855-6fb2-48af-950c-5eaf79fa9e7f
https://doi.org/10.17632/vy22skn8ps.1
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028928
2. Code. Code for R scripts available at
https://github.com/AlistairMcNairSenior/Pathways_GFN.
3. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

C57BL/6J male mice were obtained from the Animal Resources Centre (WA, Australia) at four weeks of age and housed at the Charles Perkins Centre (25C, 12 h light:dark cycle, AIN93-G chow until commenced on specialized diets). Mice were housed at four per cage and were not exercised. At 12 weeks of age, mice were randomly allocated to one of 40 different experimental groups, and had *ad*

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libitum access to their diet. Body weights and food intakes were measured monthly. Four animals were euthanized due to weight loss greater than 20% as an ethical endpoint. All these mice were on the 55% protein diets and were replaced. At six and 10 months of age, mice underwent glucose tolerance tests and body composition analysis. At 10 months mice were anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg, intraperitoneal) and killed by exsanguination while anesthetized. Tissue samples were collected and snap frozen using freeze clamps and liquid nitrogen. Tissue collection was performed between 10:00 and 12:00, 4–6 h after initiation of the light cycle and when animals had completed their overnight feeding period. The study was approved by the University of Sydney Animal Ethics Committee (Protocol 2016/1026).

Diets and Drugs

Ten diets with five different ratios of protein, carbohydrates and fat (protein:carbohydrate:fat = 10:70:20, 14:57:29, 19:63:18, 33:20:47 or 55:25:20 as % of kJ/g) at either of two different energy contents (standard diet 14.8 kJ/g, low energy 8 kJ/g) (Specialty Feeds, WA, Australia, Catalog numbers are in key resources table) were used. The low energy diets were formulated with 50% non-digestible cellulose fiber (Solon-Biet et al., 2014). Food intake was corrected for orts in cage bedding and used to calculate intake of protein, carbohydrates, fat and energy.

Drugs were added to the chow at the following concentrations: metformin 0.1% (Martin-Montalvo et al., 2013), rapamycin 14 mg/kg (Miller et al., 2011) and resveratrol 0.04% (Baur et al., 2006) while control diets contained no drug. Mice (n = 8 per group) were commenced on one of the 40 diets (10 diets, three drugs or control, Table S1) at 12 weeks of age and maintained on their diet until 10 months of age they were euthanized as the experimental endpoint.

METHOD DETAILS

Body Composition

Body composition was assessed in conscious mice using an EchoMRI 900 (EchoMRI, TX, USA) at six and 10 months of age.

Glucose Tolerance Tests

Glucose tolerance tests were performed at six and 10 months of age. Mice were fasted for four h. Basal blood samples were obtained by tail tipping and blood glucose measured using a clinical glucometer (Accu-Chek Performa, Roche Diagnostics Australia Pty Ltd). Glucose (2 g/kg lean mass) was administered via oral gavage. Blood was collected at baseline, 15, 30, 45, 60 and 90 min. The incremental area under the curve (AUC) was calculated.

Insulin resistance was calculated from insulin x glucose product.

Metabolic Hormones

Mouse IGF-1 and insulin were measured by ELISA, following the manufacturer's instructions (Crystal Chem, IL).

Liver malondialdehyde

Oxidative stress was measured using a Lipid Peroxidation Assay Kit (Malondialdehyde (MDA assay) (Abcam, catalog no. ab118790). 10 μg of frozen liver was homogenized in lysis buffer. 600 μl of thiobarbituric acid was added to 200ul of supernatant from each homogenized sample. Samples and standards were incubated at 95°C for 60 min. Samples were cooled on ice for 10 min and centrifuged at 13,000 g for 10 min at 4°C. 200 μl of sample and standard were pipetted into a 96-well plate for analysis. Fluorescence was measured at 532/553 (Ex/Em) using an Infinite M1000 PRO plate reader (Tecan).

Blood drug levels

Blood levels of metformin and trans-resveratrol were measured using an Agilent 6460A triple quadrupole mass spectrometers (Agilent Technologies, Santa Clara, CA, USA). Rapamycin levels were measured using a Shimadzu triple quadrupole LCMS system (LCMS-8050, Nexera LC-30AD, ShimadzuCorp., Kyoto, Japan).

Proteomics

Liver was lysed in 6 M guanidine in 100 mM Tris pH 8.5 containing 10 mM tris(2-carboxyethyl)phosphine and 40 mM 2-Chloroacetamide by tip-probe sonication. The samples were heated at 95°C for 5 min and centrifuged at 20,000 x g for 5 min at 4°C. The supernatant was precipitated with 4 volumes of acetone overnight and protein centrifuged at 5,000 x g for 5 min at 4°C. The protein pellet was washed with 80% acetone and resuspended in 10% trifluoroethanol in 100 mM HEPEs, pH 7.9. Protein determination was performed with BCA, normalized to 20 μg/20 μl and digested with 0.4 μg of sequencing grade trypsin and 0.4 μg of LysC (Wako, Japan). Peptides were labeled with 40 μg of 10-plex Tandem Mass Tags (TMT) in a final concentration of 50% acetonitrile at room temperature for 1.5 h followed by de-acylation with a final concentration of 0.3% hydroxylamine and quenching with a final concentration of 1% trifluoroacetic acid. Isotopically labeled peptides for each individual TMT 10-plex experiment were pooled and dried by vacuum centrifugation to ~50 μl. A total of fifteen TMT 10-plex experiments were performed containing nine channels of individual livers and the 10th channel (label 131) a pooled internal control that was identical across all experiments. The dried peptides were desalted by SDB-RPS microcolumns and dried by vacuum centrifugation. Thirty micrograms of peptide were fractionated on an in-house fabricated 25 cm x 320 μm column packed with C18BEH particles (3 μm, Waters). Peptides were separated on a gradient of

0 – 30% acetonitrile containing 10 mM ammonium formate (pH 7.9) over 60 min at 6 μl/min using an Agilent 1260 HPLC and detection at 210 nm with a total of 48 fractions collected and concatenated down to 12.

Peptides were analyzed on a Dionex ultra-high pressure liquid chromatography system coupled to an Orbitrap Lumos mass spectrometer. Briefly, peptides were separated on 40 cm x 75 μm column containing 1.9 um C18AQ Reprosil particles on a linear gradient of 2- 30% acetonitrile over 2 h. Electrospray ionisation was performed at 2.3 kV and positively charged peptides detected via a full scan MS (350-1550 m/z, 1e6 AGC, 60K resolution, 50 ms injection time) followed data-dependent MS/MS analysis performed with CID of 35% normalized collision energy (NCE) (rapid scan rate, 2e4 AGC, 50 ms injection time, 10 ms activation time, 0.7 m/z isolation) of the top 10 most abundant peptides. Synchronous-precursor selection with MS3 (SPS-MS3) analysis was enabled with HCD of 60 NCE (50K resolution, 1e5 AGC, 105 ms injection time) (McAlister et al., 2014). Data were processed with Proteome Discoverer v2.3 and searched against the Mouse UniProt database (November 2018) using SEQUEST. The precursor MS tolerance were set to 20 ppm and the MS/MS tolerance was set to 0.8 Da with a maximum of 2 miss-cleavage. The peptides were searched with oxidation of methionine set as variable modification, and TMT tags on peptide N terminus / lysine and carbamidomethylation of cysteine set as a fixed modification. All data was searched as a single batch and the peptide spectral matches (PSMs) of each database search filtered to 1% FDR using a target/decoy approach with Percolator (Kall et al., 2007). The filtered PSMs from each database search were grouped and q-values generated at the peptide level with the Qvality algorithm. Finally, the grouped peptide data was further filtered to 1% protein FDR using Protein Validator. Quantification was performed with the reporter ion quantification node for TMT quantification in Proteome Discoverer. TMT precision was set to 20 ppm and corrected for isotopic impurities. Only spectra with < 50% co-isolation interference were used for quantification with an average signal-to-noise filter of > 10.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results are presented as mean ± SD. Principal component analyses, parametric and non-parametric t tests and ANOVA were performed using XLstat (XLstat, Addinsoft (2020) XLStat statistical and data analysis solution. NY, USA. https://www.xlstat.com).

To identify proteins with abundance that was significantly influenced by macronutrients and energy, the correlations between the abundance of each protein and intakes of protein, carbohydrates, fat and energy were determined using both Pearson's coefficient and Generalized Additive Models (GAMs) as described previously (Gokarn et al., 2018) (Data S2). The P values generated by both Pearson's coefficient and GAMS were corrected using Benjamini Hochberg correction with FDR = 0.05. GAMs allow the effects of the intakes of protein, carbohydrates, fat and energy to be calculated together, but does not provide a statistical value to determine whether the relationship is positive or negative. Generalized Additive Models (GAMs) were used to generate response surfaces and to assess the effects of macronutrient intake on outcomes. GAMs were implemented in R (R Core Team, 2013) using the mgcv package. A separate GAM was implemented for each outcome. In each GAM the daily intakes of the three macronutrients were fitted as three-dimensional smooth term (s(protein, carbohydrate, fat)) with smoothing by drug group (4-level categorical variable); and drug group was also fitted as a parametric additive effect (allowing separate intercepts). These three-dimensional terms incorporate the main effects of each macronutrient and their interactions. To determine whether metformin, rapamycin and resveratrol significantly altered the relationship between the intake of protein, carbohydrates, fat or energy with the abundance of proteins, the correlation coefficients were compared from the differences in the Z statistic between the control group and each of the drug groups. Enrichment analyses were performed using Metascape (http://metascape.org) (Zhou et al., 2019). Venn analyses were undertaken using the Bioinformatics Evolutionary Genomics online program (http://bioinformatics.psb.ugent.be/webtools/Venn/).