A murine model of the human CREBRF[R457Q] obesity-risk variant does not influence energy or glucose homeostasis in response to nutritional stress

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Abstract

Obesity and diabetes have strong heritable components, yet the genetic contributions to these diseases remain largely unexplained. In humans, a missense variant in Creb3 regulatory factor (CREBRF) [rs373863828 (p.Arg457Gln); CREBRF^{R457Q}] is strongly associated with increased odds of obesity but decreased odds of diabetes. Although virtually nothing is known about CREBRF's mechanism of action, emerging evidence implicates it in the adaptive transcriptional response to nutritional stress downstream of TORC1. The objectives of this study were to generate a murine model with knockin of the orthologous variant in mice (CREBRF^{R458Q}) and to test the hypothesis that this CREBRF variant promotes obesity and protects against diabetes by regulating energy and glucose homeostasis downstream of TORC1. To test this hypothesis, we performed extensive phenotypic analysis of CREBRF^{R458Q} knockin mice at baseline and in response to acute (fasting/refeeding), chronic (low- and high-fat diet feeding), and extreme (prolonged fasting) nutritional stress as well as with pharmacological TORC1 inhibition. The results demonstrate that the murine CREBRF^{R458Q} model of the human CREBRF^{R457Q} variant does not influence energy/glucose homeostasis in response to these interventions. Alternative preclinical models and/or studies in humans will be required to decipher the mechanisms linking this variant to human health and disease.

Introduction

Obesity is a global public health threat that is associated with additional metabolic abnormalities (i.e., insulin resistance, dyslipidemia) that increase the risk of common diseases such as diabetes and cardiovascular disease. Although obesity clearly has a complex and multifactorial etiology, it has a strong heritable component (>80% in some studies). Over the past several decades, large genome-wide association studies (GWAS) have sought to identify the genetic contributions to this heritability. These studies have been instrumental in linking numerous genes/loci to anthropometric and/or metabolic traits, including body mass index (BMI)/obesity (1, 2), regional adiposity/insulin biology (2, 3), and glycemia/diabetes (4). These studies have revealed important insights into the underlying physiology/pathophysiology of these traits such as the critical role of central neuroendocrine regulation of energy homeostasis in obesity (1, 2), adipose tissue biology in insulin resistance (2, 3), and beta cell biology in diabetes (4). Despite these insights, the majority of heritability of these metabolic traits remains largely unexplained. For example, genes/loci identified by GWAS account for less than 3% of the variance in BMI (1). Furthermore, these GWAS are historically enriched in populations with European ancestry and have not adequately assessed genetic contributions from underrepresented groups (5), many of which are disproportionately affected by obesity, diabetes, and cardiometabolic diseases. Identifying novel genes/pathways underlying this "missing heritability" across diverse populations could improve prevention and/or treatment of obesity and its complications.

To bridge this knowledge gap, our research group has established an ongoing collaboration with the Samoan Ministry of Health to understand and address cardiometabolic disease risk in Samoans, a geographically isolated population with a particularly high prevalence of obesity. This collaborative effort resulted in the identification of a missense variant in Creb3 regulatory factor (CREBRF) [rs373863828 (p.Arg457Gln); CREBRF^{R457Q}] by GWAS in Samoans that was strongly associated with BMI (6). This obesity-risk variant (odds ratio for obesity of 1.305 per copy) is unique in that, even though it has a BMI-increasing effect size greater than any other known common obesity-risk variant (1.36–1.45 kg/m^2 CREBRF^{R457Q} versus only 0.37 kg/m^2 for the FTO locus), it is paradoxically associated with relative

83 protection from diabetes (odds ratio for diabetes of 0.586 after controlling for BMI) and other metabolic

84 complications of obesity (7). This variant is also positively related to linear height in humans (8-10).

85 Although this variant is extremely rare in populations most commonly studied in GWAS (i.e., Europeans),

86 it is very common in Samoans (~46% carry at least one copy of the risk allele). The relationship between

87 CREBRF[R457Q] and obesity/diabetes risk has now been replicated by numerous other groups in Oceanic

88 populations (9, 11-15). However, despite its large effect size and high prevalence in this population, the

89 mechanisms by which the CREBRF[R457Q] variant contributes to obesity and obesity-associated traits

90 remain unknown.

91   CREBRF (also known as Luman/CREB3 Recruitment Factor, LRF) was initially identified in 2008

92 as a novel protein that binds to CREB3 and inhibits CREB3-mediated activation of the unfolded protein

93 response (UPR) in mice (16). Global targeted deletion of CREBRF in mice results in lower total body

94 mass (17), suggesting that the human CREBRF[R457Q] variant may be a gain-of-function variant with

95 respect to body mass / energy homeostasis. This hypothesis is further supported by our data

96 demonstrating that ectopic overexpression of the human CREBRF[R457Q] variant in a murine 3T3-L1

97 adipocyte model promotes adipogenesis, decreases mitochondrial respiration, and increases fat storage

98 (6). While these data implicate adipocytes in the pathophysiology of the CREBRF[R457Q] variant, the relative

99 contributions of other tissues and cell types where CREBRF is also expressed remain unknown (6).

100 CREBRF is highly regulated by nutritional status and TORC1 action (increased by fasting/starvation and

101 TORC1 inhibition; decreased by refeeding and TORC1 activation) (6, 18). Overexpression of CREBRF

102 in 3T3-L1 adipocytes protects against starvation (6), whereas deletion of CREBRF in Drosophila S6 cells,

103 larvae, and adults increases susceptibility to starvation (18). These data suggest that CREBRF plays a

104 critical role in the cellular and physiological response to nutritional stress. In addition, mechanistic studies

105 in Drosophila demonstrate that CREBRF mediates the majority of the downstream effects of TORC1

106 inhibition by rapamycin (18). Although similar findings have yet to be confirmed in mammals, these data

107 nonetheless implicate CREBRF in one of the most well-known energy sensing pathways in biology.

108   Despite the potential importance of these findings, virtually nothing is known about the function

109 or physiological relevance of CREBRF or the CREBRF[R457Q] variant. Our overall goal is to understand

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110   how the CREBRF^{R457Q} variant influences energy and metabolic homeostasis. Based on the above data,

111   we hypothesized that the CREBRF^{R457Q} variant promotes obesity and protects against diabetes by

112   regulating gene pathways involved in energy and glucose homeostasis downstream of TORC1. To test

113   this hypothesis, we generated an animal model of the human CREBRF^{R457Q} variant by using CRISPR-

114   Cas9 gene-editing technology to knockin the orthologous mutation in mice (CREBRF^{R458Q}). We then

115   phenotyped these mice at baseline and in response to acute (fasting/refeeding), chronic (low- and high-

116   fat diet feeding), and extreme (prolonged fasting) nutritional stress as well as with pharmacological

117   TORC1 inhibition.

Materials and Methods

Generation and validation of *Crebrf* knockin (*Crebrf*KI) mice.

CREBRF[R458Q] knockin (*Crebrf*KI) mice were generated using CRISPR-Cas9 gene-editing technology in a congenic C57BL/6J mouse background strain with the assistance of the University of Pittsburgh Transgenic and Gene Targeting (TGT) Core (19-21). Briefly, a double-strand DNA break was introduced in proximity of the murine R458 codon by targeting the Cas9 nuclease with a small guide RNA (sgRNA) (murine *Crebrf* target sequence: 5'-CTGGTACATATTACTTGGCA-3', chr17:26,757,961-26,757,980 (mm10)). The desired genomic DNA substitutions were introduced by co-injection of a PAGE-purified single stranded oligodeoxynucleotide (ssODN) ultramer to serve as template for homology-directed repair (HDR). Two substitutions (lower cap bold in the sequence below; see also **Fig 1A**) were introduced: i) a silent substitution (TTC to TcC) to introduce a BamH1 site (underlined) approximately 20 bp away from the R458 codon, to serve as a proxy for the mutation for genotyping purposes; and ii) a coding substitution (CGA to CaA) to introduce the desired R458Q knockin variant. A PCR-generated sgRNA template was used for sgRNA synthesis using the MEGAshortscript T7 Kit (ThermoFisher Scientific), and Cas9 mRNA was produced using a linearized plasmid as template for the in vitro transcription mMESSAGE mMACHINE T7 Ultra Kit (ThermoFisher Scientific). Both sgRNA and Cas9 mRNA were purified using the MEGAclear Kit (ThermoFisher Scientific). C57BL/6J pronuclear-stage zygotes, obtained by natural mating of superovulated females, were microinjected with sgRNA (10 ng/µL, each) and Cas9 mRNA (20 ng/µL), and the ssODN (1µM). Injected zygotes were cultured overnight and 2-cell embryos were transferred to pseudopregnant CD1 mice to obtain potential founder mice.

The presence or absence of the desired knockin allele was confirmed in several ways. First, purified tail DNA from potential founders were screened using a two-step nested PCR-RFLP assay. The first amplification (with genomic DNA as template) used forward primer 5' TTTAATGCCTGGCACCATTT and reverse primer 5' GAACGAGGCAGAGGATTCAA to generate a 700 bp product (PCR product 1,

7

143 PCR1). The second amplification (with PCR product 1 as template) used forward primer 5'

144 TGACAATTGTGGGACCATGT and reverse primer 5' GAACGAGGCAGAGGATTCAA to generate a 370

145 bp product (PCR product 2, PCR2). Both steps used PCR conditions of 95°C x 30s / 55°C x 30s / 72°C

146 x 30s for 35 cycles. The second PCR product was then digested with BamH1 and resolved on agarose,

147 resulting in 370 bp (plus 700 bp) bands for the wild-type (WT) allele and a 221 bp and 149 bp (plus 479

148 bp) bands for the knockin (KI) allele (**Fig 1B**). Second, for founders positive for the KI allele by the PCR-

149 RFLP assay, the 700 bp PCR products were subcloned into the pCR™4-TOPO® TA vector and

150 transfected into *Escherichia coli* DH5α cells (TOPO 4 Kit, ThermoFisher Scientific) followed by plasmid

151 preparation and direct Sanger sequencing. In this way, a founder carrying the desired KI allele was

152 confirmed and then mated to WT C57BL/6J mice to expand the colony. Subsequently, mice were

153 genotyped using our previously published locked nucleic acid assays (21). All oligonucleotide sequences

154 used in this study are listed in **S1 Table**.

155

156 **Animals.**

157 Mice were housed under standard conditions (25°C, 14:10 h light:dark cycle) with *ad libitum*

158 access to a control low-fat diet (LFD: Research Diets D12450Hi; 10/70/20 kcal% fat/carbohydrate/protein;

159 3.82 kcal/g) or high-fat diet (HFD: Research Diets D12451i; 45/35/20 kcal% fat/carbohydrate/protein;

160 4.73 kcal/g) from weaning until sacrifice, except as otherwise noted (i.e., during fasting). The LFD was

161 specifically formulated as the control for the HFD. For the longitudinal comprehensive phenotyping

162 experiment, male and female WT and KI mice were fed LFD and HFD until 22 weeks of age and then

163 sacrificed in fasted (16h) or refed (24h of fasting followed by 16h of refeeding) states. For the prolonged

164 fasting experiment, a separate cohort of female WT and KI mice were ad lib fed HFD as above until 36

165 weeks of age. After acclimation to single housing in metabolic cages (Sable Promethion system), they

166 were then fasted until they lost 25% of their initial body weight and then refed until they re-established

167 their initial body weight. For in vivo mTORC1 inhibition experiments, a separate cohort of male WT and

168 KI mice were ad lib fed LFD or HFD as above, but were treated with either vehicle (Veh: sterile 10% PEG

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169 400/8% ethanol plus an equal volume of 10% Tween 80) or rapamycin (Rapa: rapamycin 4 mg/kg body

170 weight dissolved in the above vehicle; rapamycin was obtained LC Laboratories) via an intraperitoneal

171 (ip) injection every other day for three weeks prior to sacrifice in the ad lib fed state at 22 weeks of age

172 based on a previously reported protocol (22). Animal experiments were approved by the University of

173 Pittsburgh IACUC (Protocol #20107971) and conducted in conformity with PHS Policy for Care and Use

174 of Laboratory Animals (PHS Assurance #D16-00118).

175

Metabolic and biochemical analyses.

177 Body composition, energy expenditure, and metabolic parameters were performed as described

178 (23-25). Body composition was determined by echo magnetic resonance imaging (Echo Medical

179 Systems). Energy intake and expenditure, energy substrate utilization, and activity were determined by

180 indirect calorimetry (Promethion Metabolic Phenotyping Cages from Sable Systems International).

181 Plasma glucose was measured using a One-Touch FastTake glucometer (Lifescan). For glucose

182 tolerance tests (GTTs), mice were injected ip with 1.5 g/kg glucose following a 6-h fast. Other serum

183 parameters were determined using the following kits: insulin (Ultra Sensitive Mouse Insulin ELISA kit;

184 Crystal Chem), triacylglycerol or TAG (Pointe Scientific Triglycerides Liquid Reagents; Fisher Scientific);

185 non-esterified fatty acids or NEFAs (HR series NEFA-HR[2] Reagents; Wako Diagnostics), leptin (mouse

186 leptin ELISA, Crystal Chem); and adiponectin (mouse adiponectin ELISA, Crystal Chem). Liver TAGs

187 (Infinity Triglycerides Liquid Stable Reagent; Thermo Scientific) were determined following tissue lipid

188 extraction in chloroform:methanol (2:1), followed by acidification with H_2SO_4 and phase separation by

189 centrifugation, and reconstitution in 60 µl of tert-butanol plus 40 µl of a 2:1 mixture of Triton X-114 (23).

190

Gene expression analysis.

192 RNA was isolated using RNeasy Lipid Tissue Mini Kit with on-column DNase treatment (Qiagen)

193 and then reverse transcribed into cDNA using qScript Supermix (Quanta Biosciences). Relative gene

194 expression was then determined by quantitative PCR (qPCR; ABI QuantStudio3 System) using the

9

195 standard curve method after amplification of cDNA with gene-specific primer-probe sets using PerfeCTa

196 Fastmix II Fastmix (Quantabio). Taqman Gene Expression Assays were Mm01299053_m1 for *Crebrf*

197 and Mm02342430_g1 for reference gene *Ppia* (ThermoFisher). qPCR was conducted in accordance with

198 MIQE guidelines (26).

199

Protein analysis by immunoblotting.

201 All antibodies used in this study are listed in **S2 Table**. Immunoblotting was performed as

202 described (27). Briefly, tissues were homogenized in ice-cold RIPA lysis buffer (250mM Tris-HCl, pH 7.4,

203 750 mM NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulphate (SDS), 100

204 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl (PMSF) and 1% cocktail protein protease and

205 phosphatase inhibitors (Sigma), pH 8.0). Lysates were centrifuged at 14,000x *g* for 30 min at 4 °C, and

206 supernatants were stored at -80 °C until analysis. Lysate protein was resolved by SDS-PAGE (Novex

207 NuPAGE 4-12% Bis-Tris gels, Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membranes

208 (Millipore sigma). Phosphorylated and total proteins were identified by immunoblotting using the following

209 primary antibodies: Phospho-S6 Ribosomal Protein (Ser240/244) Antibody #2215 and α-Tubulin (DM1A)

210 Mouse mAb #3873 (Cell Signaling Technologies) at 1:1000 dilution. Immunoblots were evaluated using

211 IRDye® 680RD donkey anti-mouse IgG and 800CW donkey anti-rabbit IgG (LI-COR) at 1:15,000 and

212 scanned on an Odyssey Clx Imaging System (LI-COR). Fluorescent signals were quantified using Image

213 Studio Software (LI-COR).

214

Statistical analysis.

216 Results are expressed as mean (SD). Comparisons were made using general linear models

217 followed by determination of simple effects for pair-wise comparisons if relevant (SPSS Software v27).

218 For repeated measurements, comparisons were made by generalized linear model with time as a within

219 subject variable. For indirect calorimetry data, comparisons were made using generalized estimated

220 equations with time as a within subject variable. Dependent variables and fixed effects for each analysis

10

221   are indicated in the text and figure legends. Males and females were analyzed separately. For all

222   analyses, p values of <0.05 were considered statistically significant.

223

Data and resources availability.

225   1) Data sharing. Data supporting the findings, methods, and conclusions in this manuscript are

226   available within the manuscript in the supplemental online material, or from the corresponding author.

227   2) Resource sharing. Information related to antibodies, primers, and key reagents are summarized in

228   supplemental online material. Unique resources used in this manuscript are available from

229   corresponding author. The *Crebrf*KI mouse model or its derivatives (embryos, sperm) will be available

230   for distribution from the corresponding author or deposited with a commercial vendor.

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Results

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Generation and validation of *Crebrf* knockin (*Crebrf*KI) mice.

234   Our previous study identified a nonsynonymous variant in CREBRF (rs373863828) to be strongly

235   associated with obesity and diabetes risk in humans (6). The variant is a guanine (G) to adenine (A)

236   substitution that changes the amino acid at position 457 from an arginine [Arg, R] to a glutamine (Gln,

237   Q), designated CREBRF^{R457Q} in humans (**S1A-B Fig**). CREBRF DNA and protein sequences are highly

238   conserved in vertebrates. Comparison of human to mouse protein sequences revealed that the

239   homologous amino acid in mice was at position 458 (**S1B Fig**). To generate *Crebrf*KI mice, CRISPR-

240   Cas9 gene-editing technology was used to replace the guanine (G) to adenine (A) in genomic DNA of

241   congenic C57BL/6J mice (comparable to the rs373863828[A] minor allele in humans), to generate the

242   homologous mutant protein, designated CREBRF^{R458Q} in mice. The annotated murine wild-type (WT) and

243   genome-edited knockin (KI) alleles (**Fig 1A**) were confirmed by direct sequencing and restriction fragment

244   length polymorphisms (RFLP) (**Fig 1B**). Resulting mice homozygous for the KI allele are subsequently

245   designated *Crebrf*KI or KI mice. Gene expression analysis confirmed *Crebrf* mRNA expression across

246   multiple tissues in WT and KI mice, and this expression did not differ between genotypes (**Fig 1C**).

247   Endogenous CREBRF protein expression is known to be low and transiently expressed at baseline (16).

248   Consistently, CREBRF protein was below the limit of detection in peripheral tissues of both WT and KI

249   mice. Experimental WT and KI mice, generated from heterozygous x heterozygous crosses, were viable

250   and fertile with the expected Mendelian ratios of genotype and sex in resulting pups.

251

252   **Fig 1. Generation and validation of *Crebrf* knockin (*Crebrf*KI) mice.   (A)** The CREBRF^{R458Q} knockin

253   allele was generate using CRISPR-Cas9 gene-editing technology. Genomic (top) and protein (bottom)

254   sequence of the wild-type (WT) and gene-edited knockin (KI) alleles are shown. To generate the KI allele,

255   two substitutions were introduced: i) a silent substitution (TTC to TcC) to introduce a BamH1 site

256   (underlined with restriction cut site shown with red arrowhead) about 20 bp away from R458 codon, to

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257   serve as a proxy for the mutation for genotyping purpose; and ii) a coding substitution (CGA to CaA) to

258   introduce the desired R458Q knockin variant (surrounded by the black box); **(B)** The WT and KI alleles

259   were confirmed by direct sequencing and restriction length polymorphism (RFLP) analysis. The

260   schematic for the two-step nested PCR RFLP assay is shown on the left, and the resulting representative

261   gels, confirming homozygous WT, heterozygous (Het), and homozygous KI mice, are shown on the right.

262   **(C)** *Crebrf* mRNA expression by qPCR in multiple tissues of WT and KI mice (M and F, 6-week-old, ad

263   lib fed LFD, n=6/group except testes and ovaries n=3/group). Data are murine *Crebrf* relative to reference

264   gene *Ppib* and expressed relative to WT testes. Testes, ovaries, hypothalamus (hypothal), cardiac

265   muscle (Card Musc), gastrocnemius-plantaris-soleus skeletal muscle (Sk Musc), spleen, brown adipose

266   tissue (brown AT), perigonadal white adipose tissue (white AT), liver, and pancreas show no genotype

267   effects on Crebrf mRNA expression. Unpaired two-tailed t-tests were used to compare gene expression

268   (dependent variable) between genotype (fixed factor). Data are mean (SD). $p < 0.05$ was considered

269   significant.

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271

272   **The CREBRF^{R458Q} variant does not affect body weight, body composition, or**

273   **linear growth at baseline (low-fat diet) or in response to chronic nutritional**

274   **overload (high-fat diet) in mice.**

275   The human CREBRF^{R457Q} variant is associated with greater body mass (6, 11-13) and linear

276   height (8-10) in humans. To determine the effect of the murine CREBRF^{R458Q} variant on these

277   parameters, we evaluated energy homeostasis, body composition, and linear growth in male and female

278   WT and KI mice under control nutritional conditions (low-fat diet, LFD; males only) and in response to

279   chronic nutritional overload (high-fat diet, HFD; males and females) from weaning until sacrifice at 22

280   weeks of age. No differences were identified between genotypes for longitudinal total body mass (**Fig**

281   **2A**), fat mass (**Fig 2B**), or lean mass (**Fig 2C**) in male (**Fig 2, blue, top row**) or female (**Fig 2, red,**

282   **bottom row**) mice on either diet. In addition, at the time of sacrifice, no differences were identified

13

283   between genotypes for two different measures of linear growth: tibia length and nose-rump length (**Fig**

284   **2D**) or for individual tissue weights (**S2A-B Fig**). Notably, as positive controls, the expected effects of diet

285   on these parameters were present, i.e., higher total body mass and fat mass with HFD-feeding (**Fig 2A-**

286   **B top row**; **S2A Fig**). Thus, the murine CREBRF^{R458Q} variant does not affect body weight, body

287   composition, or linear growth at baseline or in response to chronic nutritional overload in mice.

288

289   **Fig 2. Effect of the CREBRF^{R458Q} variant on body weight, body composition, and linear growth on**

290   **control diet (low-fat diet) and in response to chronic nutritional overload (high-fat diet) in mice.**

291   Male (M, top row, n=12-14/group for LFD and 30-31/group for HFD) and female (F, bottom row, n=27-

292   38/group) wild-type (WT) and knockin (KI) mice were fed low-fat diet (LFD) or high-fat diet (HFD) weaning

293   until sacrifice at 22 weeks of age (or 36 weeks of age in Fig 5). Total body mass (**A**), fat mass (**B**), and

294   lean mass (**C**) were determined at the ages indicated. Tibia and Nose-rump length (**D**) were determined

295   at 22 weeks of age. General linear models with (**A-C**) or without (**D**) time as a within subject variable were

296   used to compare the above dependent variables between D=diet (LFD, HFD) and G=genotype (WT, KI)

297   as fixed factors. Data are mean (SD). Main effects with a significance level of $p<0.05$ are indicated. Main

298   effects with a significance level of ≥0.05 are labeled as non-significant (ns).

299

300

301   **The CREBRF^{R458Q} variant does not affect energy homeostasis in response to**

302   **acute (<24h fasting/refeeding) or chronic (high-fat diet) nutritional stress in**

303   **mice.**

304   CREBRF has been shown to be highly regulated by nutritional stress in mammalian cells (6) and

305   Drosophila (18). Since genotype could lead to equal or conditional changes in energy intake and

306   expenditure that do not induce a net change in body mass or composition, we additionally evaluated

307   these parameters in WT and KI mice in response to both acute nutritional stress (<24h fasting and

308   refeeding) and chronic nutritional stress (LFD and HFD feeding). As an initial step, we performed a

309   detailed analysis of phenotypic parameters in association with indirect calorimetry and activity monitoring

310   using a comprehensive laboratory animal metabolic measurement system in 12-week-old male mice fed

311   LFD and HFD. The overall effect for each parameter is expressed as both cumulative values over time

312   (**Fig 3, top row**) and rates per interval (light-dark intervals within fed-fasted-refed intervals) (**Fig 3,**

313   **bottom row**). No differences were identified between genotypes for total body mass (**Fig 3A**), energy

314   intake per total body mass (**Fig 3B**), energy expenditure per total body mass (**Fig 3C**), or activity per

315   mouse (**Fig 3A**), regardless of diet, nutritional status, or light cycle. As positive controls, the expected

316   significant effects of diet, nutritional status, and light cycle on these parameters were present, i.e., higher

317   total body mass, energy intake per total body mass, and activity per mouse with HFD compared to LFD,

318   fed/refed compared to fasted states, and dark compared to light cycle; as well as higher energy

319   expenditure per total body mass with fed/refed compared to fasted states, and dark compared to light

320   cycle. Thus, the murine CREBRF^{R458Q} variant does not affect total body mass, energy intake, energy

321   expenditure, or activity in response to either acute nutritional stress (fasting and refeeding) or chronic

322   nutritional stress (LFD and HFD feeding).

323

324   **Fig 3. Effect of the CREBRF^{R458Q} variant on energy homeostasis in response to acute (<24h**

325   **fasting/refeeding) or chronic (high-fat diet) nutritional stress in mice**. Male (M, 12-week-old, n=7-

326   14/group) wild-type (WT) and knockin (KI) mice fed control low-fat diet (LFD) or high-fat diet (HFD) were

327   evaluated for changes in total body mass **(A)**, energy intake **(B)**, energy expenditure **(C)**, and activity **(D)**

328   in response to changes in nutritional status (fed, fasted, refed) over time using indirect calorimetry and

329   activity monitors (Sable System). The protocol was as follows: 24-36 hours of acclimation (data not

330   shown), 24 hours of ad lib feeding (6PM-6PM), 24 hours of fasting (6PM-6PM), 15 hours of refeeding

331   (6PM-9AM). Feeding status and lighting status (dark=black bar; light=white bar) are shown on the top of

332   each graph. Data are presented in two ways: Top row = mean (without SD for figure clarity) for each

333   variable over the course of the entire study (**A**=absolute value, **B-D**=cumulative values); or Bottom row =

334   mean (SD) for each variable during each 12-hour light-dark interval (**A**=absolute value; **B-D**=average per

335   hour for that interval). Generalized estimated equations with time as a within subject variable were used

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336 to compare the above dependent variables between D=diet (LFD, HFD), N=nutritional status (fed, fasted,

337 refed), L=lighting status (dark, light), and G=genotype (WT, KI) as fixed factors. Main effects with a

338 significance level of p<0.05 are indicated. Main effects with a significance level of ≥0.05 are labeled as

339 non-significant (ns).

340

341

The CREBRF^{R458Q} variant does not affect energy substrate utilization, glucose homeostasis, or lipid homeostasis in response to acute (<24h fasting/refeeding) or chronic (high-fat diet) nutritional stress in mice.

345 The human CREBRF^{R457Q} variant is associated with relative protection from metabolic

346 complications of obesity, most notably lower fasting glucose and lower diabetes risk (6, 11-13) as well as

347 absence of expected obesity-associated increase in serum lipids in humans (6). To determine the effect

348 of the murine CREBRF^{R458Q} variant on these parameters, we evaluated energy substrate utilization,

349 glucose homeostasis, and lipid homeostasis in response to acute nutritional stress (<24h fasting and

350 refeeding) and chronic nutritional stress (LFD and HFD feeding) in WT and KI mice. We first determined

351 the respiratory exchange ratio (RER) using the above indirect calorimetry experiment in 12-week-old

352 male mice fed LFD and HFD as above (**Fig 4A**). No differences were identified between genotypes for

353 RER, regardless of diet, nutritional status, or light cycle. As positive controls, the expected significant

354 effects of diet, nutritional status, and light cycle on these parameters were present, i.e., higher RER with

355 LFD- compared to HFD, fed/refed compared to fasted states, and dark compare to light cycle (reflecting

356 greater utilization of carbohydrate over lipid substrates under these conditions). Thus, the murine

357 CREBRF^{R458Q} variant does not affect the ability to switch between glucose and lipid as energy substrates

358 in response to acute or chronic nutritional stress. We next determined blood glucose at 4-week intervals

359 from weaning until sacrifice in HFD-fed male (**Fig 4B-C, blue top**) and female (**Fig 4B-C, red, bottom)**

360 mice in response to fasting (**Fig 4B**) and refeeding (**Fig 4C**). No differences were identified between

361 genotypes for blood glucose at any time point, regardless of nutritional status. As a positive control, the

362 expected significant effects of nutritional status on blood glucose were present, i.e., higher blood glucose

363 in the refed compared to fasted states. We next determined the blood glucose response to an

364 intraperitoneal glucose load using glucose tolerance tests in male (**Fig 4D, blue, top**) and female (**Fig**

365 **4D, red, bottom**) mice. No differences were identified between genotypes for glucose tolerance,

366 regardless of diet. As a positive control, the expected significant effects of diet on glucose tolerance were

367 present, i.e., worse glucose tolerance and greater area under the curve for glucose (AUCg) with HFD

368 compared to LFD.

369 We additionally determined serum parameters for glucose and lipid homeostasis in LFD- and

370 HFD-fed male (**Fig 4E-J, blue, top**) and HFD-fed female (**Fig 4E-J, red, bottom**) mice following sacrifice

371 in the fasted and refed states. No differences were identified between genotypes for glucose (**Fig 4E**),

372 insulin (**Fig 4F**), triacylglycerols (**Fig 4G**), non-esterified fatty acids (**Fig 4H**), adiponectin (**Fig 4I**), or leptin

373 (**Fig 4J**), regardless of diet or nutritional status. Likewise, there were no differences between genotypes

374 for hepatic triacylglycerol content under these conditions (data not shown). As a positive control, the

375 expected effects of diet and nutritional status on these parameters were observed, i.e., higher glucose,

376 adiponectin, and leptin with HFD; higher glucose and insulin, lower NEFA and TAG with refeeding.

377 Finally, we measured *Crebrf* mRNA expression and regulation by nutritional status and diet in

378 metabolically-relevant tissues (**Fig 4K-M**), including adipose tissue (**Fig 4K**), skeletal muscle (**Fig 4L**),

379 and liver (**Fig 4M**). *Crebrf* mRNA was regulated by nutritional status (higher with fasting compared to

380 refeeding) in all three tissues and by diet (higher in LFD compared to HFD) in muscle. In contrast, *Crebrf*

381 mRNA expression did not differ by genotype in any of the three tissues. Thus, the CREBRF^{R458Q} variant

382 does not affect energy substrate utilization, glucose homeostasis, or lipid homeostasis in response to

383 acute (<24h fasting/refeeding) or chronic (HFD) nutritional stress in mice, despite regulation of *Crebrf*

384 mRNA expression in response to these interventions.

385

386 **Fig 4. Effect of the CREBRF^{R458Q} variant on energy substrate utilization, glucose homeostasis, and**

387 **lipid homeostasis in response to acute (<24h fasting/refeeding) or chronic (high-fat diet)**

388 **nutritional stress in mice**. Respiratory exchange ratio (RER) in male (M, 12-week-old, n=7-14/group)

389   wild-type (WT) and knockin (KI) mice fed low-fat diet (LFD) or high-fat diet (HFD) during changes in

390   nutritional status (fed, fasted, refed) over time using indirect calorimetry (Sable System). The protocol,

391   parameters, and analyses are described in the legend for **Fig 3**. **(B-C)** Blood glucose (dependent

392   variable) at 4-week intervals from weaning until sacrifice in HFD-fed male (M, top, n=18/group) and

393   female (F, bottom, n=14-18/group) wild-type (WT) and knockin (KI) mice in response to fasting (**B**, 16h)

394   and refeeding (**C**, 16h fast followed by 12h refeeding). For clarity, B and C are shown as separate graphs

395   but data are analyzed together. **(D)** Glucose tolerance tests (GTTs, left) and area under the curve for

396   glucose (AUCg, right) in male (M, top, 19-week-old, n=12-18/group) and female (F, bottom, 19 -week-

397   old, n=10-18/group) wild-type (WT) and knockin (KI) mice. **(E-J)** Serum parameters in LFD- and HFD-fed

398   male (E-J, top) and female (E-J, bottom) WT and KI mice sacrificed at 22-weeks-old in the fasted (16h)

399   or refed (24h of fasting followed by 16h of refeeding) states (4-9/group): **(E)** glucose, **(F)** insulin, **(G)**

400   triacylglycerol (TAG), **(H)** non-esterified fatty acids (NEFA), **(I)** adiponectin, and **(J)** leptin. **(K-M)**

401   Expression of *Crebrf* relative to *Ppia* mRNA in adipose tissue **(K)**, gastrocnemius skeletal muscle **(L)**,

402   and liver **(M)** in the same mice as (E-J). General linear models with **(B-D)** or without **(E-M)** time as a

403   within subject variable were used to compare the above dependent variables between D=diet (LFD,

404   HFD), N=nutritional status (fasted, refed), and G=genotype (WT, KI) as fixed factors. Data are mean

405   (SD). Main effects with a significance level of $p < 0.05$ are indicated. Main effects with a significance level

406   of ≥0.05 are labeled as non-significant (ns).

407

408

409   **The CREBRF[R458Q] variant does not affect energy or metabolic homeostasis**

410   **in response to prolonged nutritional stress (>24h fasting) in mice.**

411   Evidence from Drosophila (18) and murine 3T3-L1 adipocytes (6) implicates CREBRF in the

412   cellular and physiological adaptation to starvation. We, therefore, hypothesized that physiological

413   manifestations of the murine CREBRF[R458Q] variant might require the more extreme physiological

414   challenge of prolonged fasting. To test this hypothesis, female WT and KI mice (F, 36-week-old,

18

415   n=8/group) were fasted until they achieved 25% weight loss, followed by refeeding ad libitum for the

416   same duration, while closely monitoring body mass, fat mass, lean mass, blood glucose, and indirect

417   calorimetry (**Fig 5**) (28). At baseline, littermate weight-matched mice had comparable body mass (**Fig**

418   **5A**), lean mass (**Fig 5B**), and fat mass (**Fig 5C**). Mice responded remarkably well to prolonged starvation,

419   exhibiting normal physical and behavioral characteristics. In response to prolonged to fasting, mice lost

420   ~25% of their initial body mass (**Fig 5A, right**), and the rate of loss with fasting as well as rate of gain

421   with refeeding were similar for percent total body mass (**Fig 5A**), lean mass (**Fig 5B**), and fat mass (**Fig**

422   **5C**). Thus, the CREBRF^{R458Q} variant does not differentially affect body mass or composition during

423   prolonged fasting. We next looked at the effect of prolonged fasting on energy substrate homeostasis

424   and utilization. In this smaller sub-cohort, there was a slightly lower baseline blood glucose (p<0.05) and

425   RER (p=0.09) in *Crebrf*KI mice that was inconsistent with result in the larger group (**Fig 4**), likely reflecting

426   a cohort effect in timing since last feeding in the ad lib fed state rather than true difference between

427   groups. Notably, during prolonged fasting, blood glucose (**Fig 5D**) and RER (**Fig 5E**) converged to similar

428   minimums and rebounded identically after refeeding in both genotypes. Thus, the CREBRF^{R458Q} variant

429   does not differentially affect glucose homeostasis or energy substrate utilization during prolonged fasting.

430   Thus, overall, the CREBRF^{R458Q} variant does not affect energy or metabolic homeostasis in response to

431   prolonged nutritional stress (>24h fasting) in mice.

432

433   **Fig 5. Effect of the CREBRF^{R458Q} variant energy or metabolic homeostasis in response to**

434   **prolonged nutritional stress (>24h fasting) in mice.** Female (F, 36-week-old, HFD-fed, n=8/group)

435   wild-type (WT) and knockin (KI) mice were subjected to a monitored fast until they achieved 25% loss of

436   their initial body mass, followed by refeeding ad libitum. Body mass, lean mass, fat mass, blood glucose,

437   and indirect calorimetry were determined during fasting and refeeding. (**A**) Baseline total body mass after

438   ad lib feeding (**A, left**), and during prolonged fasting and refeeding (**A, right**). (**B**) Baseline lean mass

439   after ad lib feeding (**B, left**), and during prolonged fasting and refeeding (**B, right**). (**C**) Baseline fat mass

440   after ad lib feeding (**C, left**), and during prolonged fasting and refeeding (**C, right**). (**D**) Baseline blood

441   glucose after ad lib feeding (**D, left**), and during prolonged fasting and refeeding (**D**, **right**). (**E**) Baseline

442 respiratory exchange ratio (RER) after ad lib feeding (**E, left**), and during prolonged fasting and refeeding

443 (**E, right**). Baseline data (**A-E, left**) were analyzed by unpaired two-tailed t-test. Indirect calorimetry data

444 was analyzed as described in Fig 3. Other data were analyzed using general linear models with time as

445 a within subject variable and G=genotype (WT, KI) as the fixed factor. Data are mean (SD). Main effects

446 with a significance level of p<0.05 are indicated.

447

448

449 **The CREBRF^R458Q variant does not affect energy or metabolic homeostasis**

450 **in response to mTORC1 inhibition in mice.**

451   The Drosophila homolog of CREBRF, REPTOR, is induced by the TORC1 inhibitor, rapamycin,

452 and mediates the majority of the downstream transcriptional response to rapamycin-mediated TORC1

453 inhibition, primarily via its (de)phosphorylation-dependent translocation to the nucleus (18). Although

454 there is no evidence of the latter in mammalian cells, we have confirmed that rapamycin induces CREBRF

455 mRNA expression in murine 3T3-L1 cells (6). We, therefore, hypothesized that the physiological

456 manifestations of the murine CREBRF^R458Q variant might be TORC1-specific, and therefore may be

457 unmasked by rapamycin treatment. To test this hypothesis, we treated LFD- and HFD-fed male mice with

458 vehicle or rapamycin 4 mg/kg body weight via intraperitoneal (ip) injection every other day for three weeks

459 prior to sacrifice at ~22 weeks of age in the ad lib-fed state (**Fig 6**). We first confirmed the effectiveness

460 of our pharmacological intervention. As expected, rapamycin treatment decreased phosphorylated

461 ribosomal S6 (pS6), a known downstream target of TORC1, in all tissues examined (adipose tissue,

462 skeletal muscle, and liver) (**Fig 6A**). We next confirmed the expression and regulation of *Crebrf* mRNA

463 in metabolically-relevant tissues of rapamycin-treated mice (**Fig 6B-D**). Rapamycin increased *Crebrf*

464 mRNA expression in adipose tissue and skeletal muscle but not liver, whereas genotype did not influence

465 *Crebrf* mRNA expression, regardless of treatment. Consistent with previous reports (22), rapamycin

466 treatment resulted in a clear and significant impairment of glucose tolerance and increased area under

467 the curve for glucose (AUCg) in both HFD-fed (**Fig 6E**) and LFD-fed (**Fig 6F**) mice. However, there was

20

468   no effect of genotype on the glycemic response to rapamycin treatment in mice on either diet. Similar

469   results were obtained for other relevant outcome measures noted in (22) (data not shown). Thus, the

470   CREBRF^{R458Q} variant does not affect energy or metabolic homeostasis in response to mTORC1 inhibition

471   in mice.

472

473   **Fig 6. Effect of CREBRF^{R458Q} on energy or metabolic homeostasis in response to mTORC1**

474   **inhibition in mice.** Male (M, n=6-7/group) wild-type (WT) and knockin (KI) mice were fed low-fat diet

475   (LFD) or high-fat diet (HFD) from weaning until sacrifice at 22 weeks of age were treated with vehicle

476   (Veh) or rapamycin (Rapa) 4 mg/kg body weight via intraperitoneal (ip) injection every other day for three

477   weeks prior to sacrifice at ~22 weeks of age in the ad lib fed state. **(A)** Protein expression of Ser240/244

478   phosphorylated ribosomal protein S6 (pS6) relative to tubulin control by Western blotting in adipose tissue

479   (top), gastrocnemius skeletal muscle (middle), and liver (bottom) [left, quantification; right, actual blots).

480   **(B-D)** *Crebrf* mRNA relative to *Ppia* control in adipose tissue **(B)**, gastrocnemius skeletal muscle **(C)**, and

481   liver **(D)** of mice treated with rapamycin. **(E-F)** Glucose tolerance tests (GTTs, left) and area under the

482   curve for glucose (AUCg, right) in rapamycin-treated HFD-fed **(E)** and LFD-fed **(F)** mice. General linear

483   models with **(E-F,** GTTs**)** or without **(A-D)** time as a within subject variable were used to compare the

484   above dependent variables between R=rapamycin treatment (rapamycin, vehicle) and G=genotype (WT,

485   KI) as fixed factors. Data are mean (SD). Main effects with a significance level of p<0.05 are indicated.

486   Main effects with a significance level of ≥0.05 are labeled as non-significant (ns).

Discussion

The goal of this investigation was to determine the function and physiological relevance of the human CREBRF^{R457Q} obesity/diabetes risk variant by generating and evaluating a murine "knockin" model with the orthologous CREBRF^{R458Q} variant. However, despite strong evidence linking the CREBRF^{R457Q} variant to obesity and diabetes risk in humans, we were not able to detect any genotype effects of the CREBRF^{R458Q} variant on energy, glucose, or metabolic phenotypes in mice. Furthermore, we examined these phenotypes at baseline as well as under conditions of acute (<24h fasting/refeeding), chronic (low- and high-fat diet feeding up to 20w), and extreme (5d starvation) nutritional stress, as well as in response to pharmacological stress (inhibition of TORC1 by rapamycin). Although we did not observe an effect of genotype, we did demonstrate that *Crebrf* mRNA expression is induced by fasting (adipose tissue, skeletal muscle, and liver) and rapamycin-mediated inhibition of TORC1 (adipose tissue, skeletal muscle), which has not been previously reported. The data presented suggest that the CREBRF^{R458Q} knockin mouse model is not likely to be an effective model for understanding the function and physiological relevance of this gene/variant.

There are several possible explanations for why the CREBRF^{R458Q} knockin mouse model did not recapitulate the phenotype of human CREBRF^{R457Q} carriers. Notably, we conducted several quality control experiments to confirm that we indeed successfully engineered the desired orthologous CREBRF^{R458Q} knockin allele in mice. We confirmed this by directly sequencing the allele, as well as by developing three separate genotyping methods and by genotyping mice with at least two methods at weaning and again after sacrifice. Thus, we are confident that we evaluated a valid mouse model. In addition, a separate group independently created a CREBRF^{R458Q} knockin mouse model and likewise found no effect of genotype on body mass, fasting blood glucose, or fasting plasma insulin. Unlike us, they reported an effect on body length in male mice only (10). Importantly, our phenotypic evaluation of the CREBRF^{R458Q} knockin mouse model presented is much more extensive and includes acute, chronic, and extreme nutritional and pharmacological stresses.

22

513   It is possible that the CREBRF^{R457Q} variant is causal/relevant in humans, but the orthologous

514   CREBRF^{R458Q} variant in mice is not. A corollary to this possibility is that perhaps the murine CREBRF^{R458Q}

515   variant influences cellular biology in mice that remain undetectable at the physiological level due to

516   compensatory mechanisms. However, we observed no evidence of this. Two other possibilities are that

517   the effect size of CREBRF^{R458Q} variant in mice was too small to detect or was not detectable under the

518   specific conditions tested in mice. To address this issue, we assessed body mass (the primary effect

519   observed in human carriers of this variant) over 22 weeks on all mice generated for this study to maximize

520   power to detect differences. We also focused on the main effects of genotypes for numerous dependent

521   variables at baseline and under nutritional and pharmacological stress. However, we cannot exclude the

522   possibility that an effect of genotype might be detectable under other conditions.

523   It is also possible that the human CREBRF^{R457Q} variant is not the causative variant contributing to

524   obesity and diabetes risk in humans. However, we do not believe this to be the case for two reasons: 1)

525   the human CREBRF^{R457Q} variant is strongly and repeatedly linked to metabolic traits in humans

526   (especially BMI, glucose, and height), and 2) CREBRF is linked to multiple biological processes known

527   to contribute to these metabolic traits. Regarding the former, in our original report, we confirmed the

528   relationship between the CREBRF^{R457Q} variant and BMI in an initial cohort of 3,072 Samoans and in a

529   separate replication cohort of 2,102 additional Samoans. This relationship between the CREBRF^{R457Q}

530   variant and BMI was highly significant (β 1.356, p 1.12 x 10^{-13}), as were the associations with obesity risk

531   (OR 1.305, p 1.12 x 10^{-5}) and diabetes risk (OR 0.586, p 6.68 x 10^{-9} after adjusting for BMI) (6). The

532   relationship between CREBRF^{R457Q} and obesity/diabetes risk has now been replicated by numerous

533   groups in other populations across Oceania (9, 11-15). These data provide strong support for the

534   observed associations, but are not sufficient to prove causality.

535   Regarding the biological evidence linking CREBRF and/or the CREBRF^{R457Q} variant to

536   metabolism, we have previously demonstrated that ectopic overexpression of human CREBRF^{R457Q} in a

537   murine 3T3-L1 adipocyte model promotes adipogenesis, decreases mitochondrial respiration, and

538   increases fat storage (6), consistent with the observed whole-body phenotypes in human carriers of the

539   CREBRF^{R457Q} allele, and providing functional support for a causal role of this variant. Although additional

23

540 mechanistic studies of CREBRF^{R457Q} variant are still underway, multiple studies have emerged linking

541 (wild-type) CREBRF to multiple proteins/pathways known to be critically involved in energy and metabolic

542 homeostasis including CREB-family proteins (29, 30), TORC1 (18, 31), Akt (32), and glucocorticoid

543 receptor (17, 33) across different species (i.e., human, mouse, goat, Drosophila) and cell types (i.e.,

544 adipocyte, neurons, gastric cells, endometrial cells, etc.). In vivo, genetic manipulation of endogenous

545 CREBRF in mice and Drosophila results in dramatic phenotypes related to energy and metabolic

546 homeostasis (17, 18). Specifically, global deletion of CREBRF in mice or Drosophila decreases body

547 mass, whereas the opposite phenotype is observed in human carriers of the CREBRF^{R457Q} allele. We

548 have separately confirmed that global deletion of murine CREBRF in mice decreases body mass and

549 protects against diet-induced obesity, whereas global overexpression of murine CREBRF in mice has

550 the opposite effect (Kershaw, publication in progress). These data suggest that CREBRF does indeed

551 contribute to energy and metabolic homeostasis, and that global and/or tissue-specific targeted deletion

552 and/or overexpression of endogenous murine CREBRF may be more useful mouse models to

553 understand the complexities of this gene. Such studies could lead to more targeted mechanistic

554 hypotheses related to the CREBRF^{R457Q} variant that could be tested using cellular models, especially

555 "knockin" (rather than ectopic expression) models of CREBRF^{R457Q} in human cell lines.

556   Alternatively, it is possible that one or more variants in linkage disequilibrium with the

557 CREBRF^{R457Q} variant, rather than (or in combination with) the CREBRF^{R457Q} variant itself may be the

558 primary driver(s) of obesity/diabetes risk in humans. In our original report, we actually identified several

559 variants in proximity to CREBRF that were in high linkage disequilibrium (6). We concentrated on three

560 variants: 1) rs12513649 - located 35,727 bp upstream of the CREBRF start site (between the ATP6V0E1

561 and CREBRF genes), 2) rs150207780 – located in the intronic region between exon1 and exon 2 of

562 CREBRF, 3) rs373863828 (i.e., CREBRF^{R457Q}) – located in a highly conserved region (GERP score 5.49)

563 in exon 5 of CREBRF. Since CREBRF^{R457Q} was a missense variant that was predicted to be highly

564 damaging (SIFT, 0.03; PolyPhen-2, 0.996), it was the leading candidate. In addition, a potential causal

565 role is supported by the aforementioned data in murine 3T3-L1 adipocytes. In considering the other

566 variants noted above, both rs12513649 and rs150207780 are both present at minor allele frequencies of

24

567   6.8% and 7.7% in East Asian individuals without rs373863828 (5-173045049-C-G, 5-173079668-C-T and

568   5-173108771-G-A, respectively, in gnomAD 3.1 (34), but no association between them and BMI (35, 36)

569   or T2D (37) has been reported. Though variants far upstream and/or in introns are less likely to contribute

570   to phenotypes, additional studies of these and other variants in linkage disequilibrium with the

571   CREBRF^{R457Q} variant, alone and in combination, may be required to clarify their individual and combined

572   effects in human and mouse cells.

573   Despite these findings, CREBRF remains a potentially important yet poorly understood

574   gene/protein linked to human health and disease. Thus far, the CREBRF^{R457Q} variant is associated with

575   increased obesity risk and decreased diabetes risk in humans (6, 9, 11-14) as well as height (8, 10) and

576   body composition/lean mass (38). However, the endogenous wild-type (non-variant) CREBRF

577   gene/protein has been linked, either directly or indirectly, to many other clinically-relevant physiological

578   and/or pathological processes in mammals, including susceptibility to viral infection (29), endometrial

579   function during pregnancy (31), angiogenesis (39), neuroendocrine function and behavior (17),

580   Alzheimer's disease (40), and cancer (32, 41-45). Thus, there continues to be an urgent need to

581   understand the mechanism by which this gene/protein contributes to normal biology and disease, and in

582   particular, the specific effect of the CREBRF^{R457Q} variant, which is highly prevalent in Oceanic

583   populations.

584   In summary, the CREBRF^{R458Q} knockin mouse model of the human CREBRF^{R457Q} obesity-risk

585   variant does not influence energy or glucose homeostasis in response to nutritional stress. To better

586   understand the physiological relevance of CREBRF, future studies could focus on murine models with

587   global or tissues-specific deletion or overexpression of endogenous CREBRF. Such studies are likely to

588   reveal novel insights into "normal" CREBRF function that would permit more targeted hypothesis testing

589   related to the CREBRF variant in the future. In addition, studies examining the human CREBRF^{R457Q}

590   variant (or variants) in human cells will be instrumental in confirming causality and well as dissecting the

591   underlying mechanisms. Finally, additional studies in human carriers of the CREBRF^{R457Q} variant would

592   enhance the deeper and broader understanding of how this variant might impact human health in the

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593 quest to identify tangible strategies for prevention and treatment of obesity and associated metabolic

594 abnormalities in humans.

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747

Supporting information

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750 **S1-S3 Tables. Oligonucleotide (S1 Table), antibody (S2 Table), and key reagent (S3 Table)**

751 **information.**

752

753 **S1 Fig. Comparison of human and mouse CREBRF protein sequence.**

754 **(A)** Illustration of the amino acid change from an arginine (Arg, R) to a glutamine (Gln, Q) at position

755 457 in humans (Rs373863828) or 458 in mouse. **(B)** The human and mouse protein sequences are on the

756 top and bottom line, respectively, with the comparison between the two sequences in the middle. Overall,

757 the human and mouse CREBRF protein sequence are highly homologous (~94%). The human R457

758 corresponds to mouse R458 (shown in RED).

759

760 **S2 Fig. Effect of the CREBRF^{R458Q} variant on tissue weights at the time of sacrifice in response to**

761 **nutritional and pharmacological interventions.**

762 **(A-B)** Tissue weights following acute and chronic nutritional interventions. Male **(A)** and female **(B)**

763 wildtype (WT) and Crebrf^{R458Q} knockin (KI) mice were fed low-fat diet (LFD) or high-fat diet (LFD) from

764 weaning until 22 weeks of age and then sacrificed in the fasted state (16h fast) or refed state (24h fast

765 followed by 16h refeeding) (M, n=7-9 per group; F 7-11 per group). General linear models were used to

766 compare each tissue (dependent variables) between D=diet (LFD, HFD), N=nutritional status (fasted,

767 refed), and G=genotype (WT, KI) as fixed factors. Data are mean (SD). Main effects with a significance
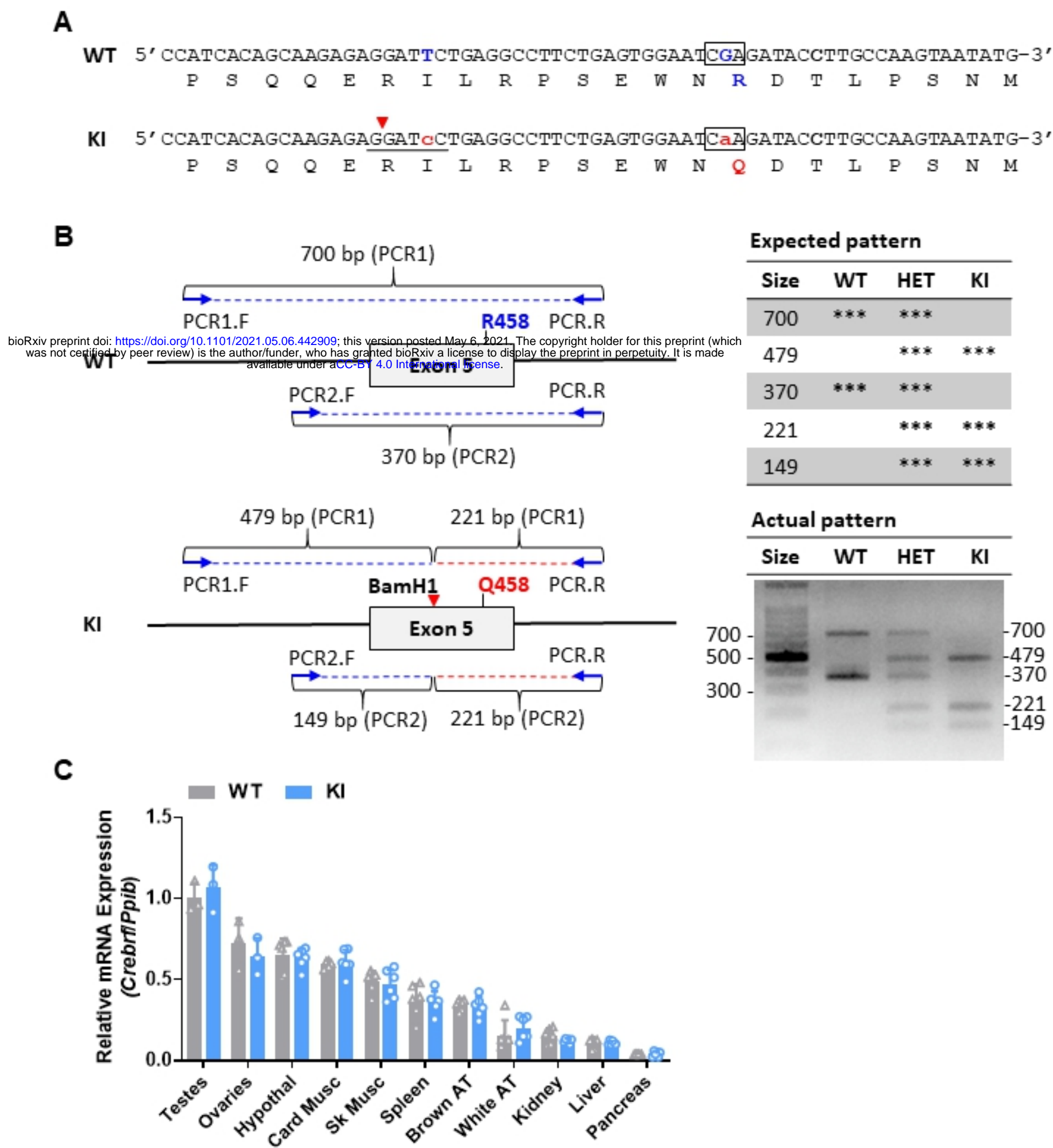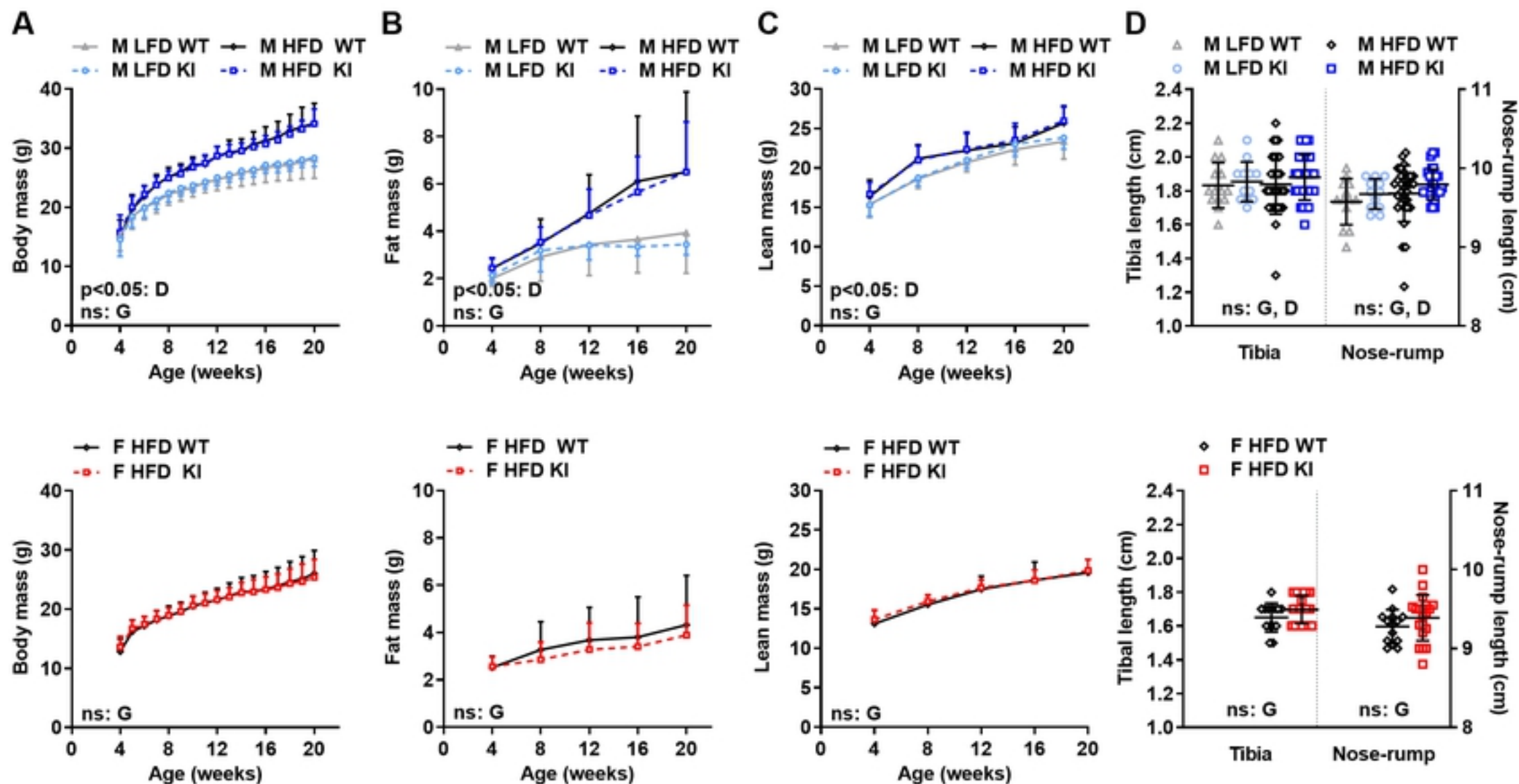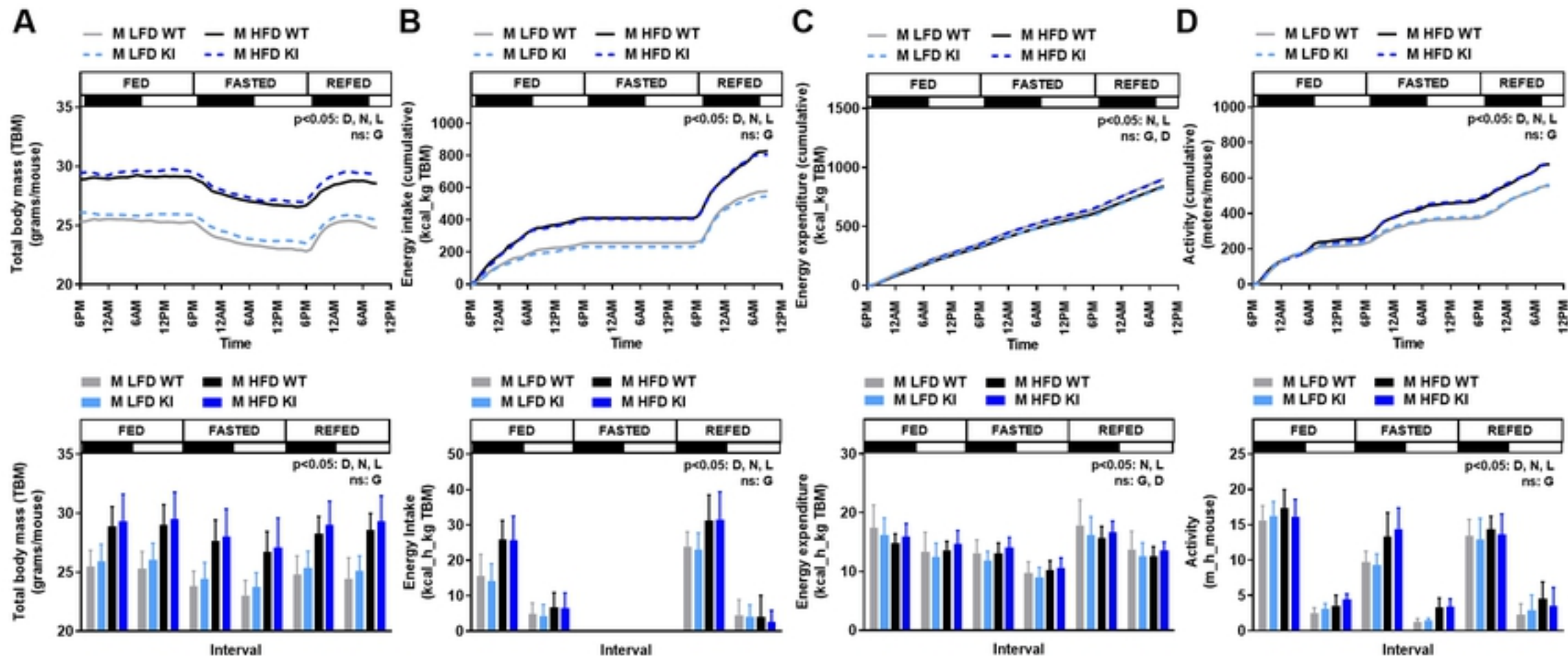
768 level of $p<0.05$ are indicated.

769

770 **S3 Fig. Original Gels.**

A

WT 5'-CCATCACAGCAAGAGAGGATTCTGAGGCCTTCTGAGTGGAATCGAGATACCTTGCCAAGTAATATG-3'
      P   S   Q   Q   E   R   I   L   R   P   S   E   W   N   R   D   T   L   P   S   N   M

KI 5'-CCATCACAGCAAGAGAGGATcCTGAGGCCTTCTGAGTGGAATCaAGATACCTTGCCAAGTAATATG-3'
      P   S   Q   Q   E   R   I   L   R   P   S   E   W   N   Q   D   T   L   P   S   N   M

B

Expected pattern

Size	WT	HET	KI
700	***	***	
479		***	***
370	***	***	
221		***	***
149		***	***

Actual pattern

C

Fig1

Fig2

Fig3

Fig4

Fig5

Fig6