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1 **Role of liver AMPK and GCN2 kinases in the control of protein metabolism in response**
2 **to postprandial and/or long-term effects of dietary protein intake**

3

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23

24

25 AMPK: AMP-activated kinase
26 ASR: absolute synthesis rate
27 Atf4: activating transcription factor 4
28 Ddit3: DNA damage-inducible transcript 3
29 eIF2 α : eukaryotic initiation factor 2 α
30 FSR: fractional protein synthesis rate
31 GCN2: general control non-derepressible 2
32 HP: high-protein
33 KO: knock-out
34 LP: low-protein
35 mTOR: mammalian target of rapamycin
36 NP: normo-protein
37 P: protein content
38 P20: 20% protein diet
39 Trib3: tribbles homolog 3
40 TSC1: tuberous sclerosis complex 1
41 TSC2: tuberous sclerosis complex 2
42 ULK1: Unc-51 like autophagy activating kinase
43 WT: wild-type
44

45 **Abstract**

46 *Purpose*

47 Protein synthesis and proteolysis are known to be controlled through mTOR (mammalian target of
48 rapamycin), AMPK (AMP-activated kinase) and GCN2 (general control non-derepressible 2) pathways,
49 depending on the nutritional condition. This study aimed at investigating the contribution of liver AMPK
50 and GCN2 on the adaptation to high variations in protein intake.

51 *Methods*

52 To evaluate the answer of protein pathways to high or low protein diet, male wild-type mice and
53 genetically modified mice from C57BL/6 background with liver-specific AMPK- or GCN2-knockout
54 were fed from day 25 diets differing in their protein level as energy: LP (5%), NP (14%) and HP (54%).
55 Two hours after a 1 g test meal, protein synthesis rate was measured after a ¹³C valine flooding dose.
56 The gene expression of key enzymes involved in proteolysis and GNC2 signaling pathway were
57 quantified.

58 *Results*

59 The HP diet but not the LP diet was associated with a decrease in fractional synthesis rate (FSR) by 29%
60 in the liver compared to NP diet. The expression of mRNA encoding ubiquitin and Cathepsin D was not
61 sensitive to the protein content. The deletion of AMPK or GCN2 in the liver did not affect nor protein
62 synthesis rates and neither proteolysis markers in the liver or in the muscle, whatever the protein intake.
63 In the postprandial state, protein level alters protein synthesis in the liver but not in the muscle.

64 *Conclusions*

65 Taken together, these results suggest that liver AMPK and GCN2 are not involved in this adaptation to
66 high and low protein diet observed in the postprandial period.

67

68 **Keywords :** protein synthesis; proteolysis; knock-out mice; high-protein diet; low protein diet; liver

69

70

71 **Introduction**

72 The question of the adequate protein content in the diet to satisfy metabolic functions and sustain protein
73 turnover has been extensively debated regarding the metabolic adaptation to low or high protein diets
74 [1, 2]. Among them, protein fluxes are of interest as key determinants of organ protein pools.

75 Protein synthesis and proteolysis are two highly controlled processes influenced by amino acid
76 availability. Studies have reported that feeding a low-protein diet for 15 days decreased protein synthesis
77 in the muscle of male rats in the fed state [3, 4]. Similarly, a protein deficient diet is associated with
78 decreased protein synthesis in the fed state in the liver, the intestine and the skin of pigs of both sex [5].
79 Yoshizawa *et al.* have shown that compared to a 20% protein diet, refeeding male Sprague-Dawley rats
80 with a protein-free diet is associated with a 40% decrease in protein synthesis rate in the muscle and in
81 the liver [6]. Inversely, increasing protein content to a certain extent can stimulate protein synthesis, but
82 this process rapidly reaches maximum levels and paradoxical effects were observed with high protein
83 diets. Indeed, increasing the amount of protein in the diet from 12.7 to 20.7% for 14 days increased
84 protein synthesis rate in the liver, kidney, pancreas and muscle in male and female piglets during the
85 postprandial period [7]. However, the effects of very high-protein diets on protein synthesis are more
86 variable. In the muscle, some studies have shown that high protein diets had no effect on muscle protein
87 synthesis in the fasted or fed states in rats [8] or could decrease it after adaptation periods of 21 or 30
88 days [9, 10]. In visceral organs, especially in the liver, high protein diet was reported to lower protein
89 synthesis rate in fasted and fed states in rats adapted for 14 days to the diet [11, 12] but the mechanisms
90 are not elucidated. Concerning proteolysis, feeding rats for two weeks low [3, 4] or high protein diets
91 [11, 13] decreased the activity of ubiquitin-proteasome, cathepsin and caspase systems in rodents in the
92 fed state. In these adaptation processes, the liver plays a central role in the channeling of amino acids in
93 energy and protein pathways.

94 The availability of AA in tissues is known to interfere with several kinases that upregulate or
95 downregulate protein synthesis and proteolysis. The two kinases AMPK (AMP-activated kinase) and
96 GCN2 (general control non-derepressible 2) are involved in the control of protein synthesis and
97 proteolysis. GCN2 was first identified as a stress kinase involved in amino acid deficiency signaling.
98 When the availability of one or several amino acid decreases, GCN2, in turn, phosphorylates the

99 eukaryotic initiation factor 2 α (eIF2 α) which leads to the blockade of translation initiation and protein
100 synthesis [14].

101 Whereas AA deficiency activates GCN2, it was shown to downregulate mTOR, probably through
102 AMPK pathway [15]. In addition, AMPK integrates signals related to energy availability in the cell like
103 glucose and glycogen concentrations leading to its inhibition [16, 17].

104 While the role of mTOR has been extensively studied, the role of AMPK and GCN2 in the control of
105 protein synthesis and proteolysis in response to the variation of dietary protein level is less documented.

106 Especially, the role of the hepatic sensing remains unknown. In order to evaluate the contribution of
107 liver AMPK and GCN2 on the adaptation of protein synthesis to protein intake modulation, wild-type
108 mice and genetically modified mice with liver-specific AMPK- or GCN2-knockout, were fed diets
109 differing in their protein content and different parameters related to protein synthesis and metabolism
110 were examined.

111

112 **Methods**

113 The study was approved by the French National Animal Care Committee (number 14-15) and conformed
114 to the European legislation on the use of laboratory animals.

115 **Animals**

116 In total, 102 males, 27 wild-type AMPK mice (WT-AMPK) and 23 AMPK-KO liver specific mice (KO-
117 AMPK) and 27 wild-type GCN2 mice (WT-GCN2) and 25 GCN2-KO liver specific mice (KO-GCN2),
118 were generated in the light and temperature-controlled animal facility of AgroParisTech (12:12 h
119 reversed light/dark cycle, lights on from 9:00 pm to 9:00 am, 24 °C, 55% humidity). To obtain AMPK-
120 KO liver specific mice, C57BL/6 albumin-Cre mice were crossed with mice bearing floxed AMPK α 2.
121 Then, offspring were crossed with mice bearing floxed AMPK α 1. To obtain GCN2-KO liver specific
122 mice, C57BL/6 albumin-Cre mice were crossed with mice bearing floxed GCN2. Specific genomic
123 deletions were confirmed using genotyping and Western-blots protein analysis.

124 Breeder mice were conventionally housed, were fed *ad libitum* a diet containing 20% of protein
125 throughout the test and had freely access to tap water. Pups were weaned from dams at the age of 25
126 days and placed in single cage at the start of the experiment. Cages were covered with woodchips and
127 enriched with a cardboard tube and red plastic housing.

128 **Study Design**

129 Two independent studies related to the liver specific gene deletion of AMPK or GCN2 were conducted.
130 Mice were fed for three weeks on a normo-protein diet (NP, 14 % as energy) as a run-in prior to the test.
131 Mice were then randomly allocated to the same NP diet or switched on low-protein (LP, 5% as energy)
132 or high-protein (HP, 54 % as energy) diet during another three-week period constituting the test-
133 period. **Diets were isoenergetic by increasing the protein content at the expense of carbohydrate (Table**
134 **1).** The protein content of the HP diet was chosen accordingly to that used in studies from our group
135 [18, 19] and others [9, 20] allowing inter-studies comparisons. The LP content in protein of 5 %
136 corresponds to a sufficient but not too severe deficiency at which adaptations of protein metabolism
137 were observed [3, 21]. A period of three weeks is considered sufficient for protein metabolism to adapt
138 to the protein level in the diet [18, 22, 23]. During this period, food intake and body weight were

139 followed on a daily basis. At the end of the test-period, after a twelve hour fast starting from 7:00 pm,
140 mice were fed a calibrated meal of 1g of their test-diet at 7:00 am and returned to their home cage. One
141 hour and forty minutes later after making sure meal was consumed totally, mice were injected i.p. with
142 ¹³C-valine (150 μmol/100 g body weight), as already implemented in mice [24] and in pigs [25]. Twenty
143 minutes later, they were killed with an overdose of pentobarbital (50mg/kg). Sampling were therefore
144 performed two hours after meal onset around 9:00 am.
145 Samples of liver and quadriceps muscle were rapidly frozen in liquid nitrogen and then stored at -80°C
146 for further protein synthesis rate measurement. Other samples of liver and quadriceps muscle were
147 placed in TRIzol (Invitrogen) and frozen at -80°C for PCR analysis.

148

149 **Measurements of *in vivo* protein synthesis**

150 Protein content (P_{content}) was estimated from total nitrogen content on lyophilized samples of liver and
151 quadriceps muscle using an elemental analyzer (Vario Micro Cube, Elementar, Lyon, France) and
152 atropine as a standard. The ^{13}C -valine enrichments in free and protein-bound amino acids in liver and
153 quadriceps muscle were determined as previously described [19]. Briefly, after homogenization in liquid
154 nitrogen, the tissue was deproteinized with 10 % 5-sulfosalicylic acid solution. The supernatant was
155 collected for the determination of the ^{13}C -valine enrichment in free amino acids and the protein pellet
156 was hydrolyzed (6M HCl at 110 °C for 48h) to obtain individual amino acids from protein. Free and
157 protein-bound amino acids were purified through cation-exchange resin (AG 50X8, 100-200 mesh,
158 BioRad) using 4M NH_4OH as elution solution. Free amino acids were analyzed as tert-
159 butyldimethylsilyl derivatives by GC/MS using a GC 6890N gas chromatograph coupled to a MS 5973N
160 (Agilent Technologies) operated in selective ion monitoring mode (ions at m/z 288 and 289) after
161 electron impact ionization. Protein-bound amino acids were analyzed after N-acetylpropyl derivatization
162 [26] by gas chromatography–combustion-isotope ratio mass spectrometry (GC-c-IRMS) using a GC
163 7890B (Agilent Technologies) coupled *via* GC5 interface with an IRMS (Isoprime, Manchester, UK)
164 The fractional protein synthesis rate (FSR, %/day) in tissues was calculated according to the following
165 formula:

$$166 \quad \text{FSR} = \frac{E_{\text{protein-bound valine}} - E_{\text{basal}}}{E_{\text{free valine}} \times t_{\text{inc}}} \times 100$$

167 where $E_{\text{protein-bound valine}}$ and $E_{\text{free valine}}$ are the protein-bound and free ^{13}C valine enrichments in the tissues
168 and t_{inc} is the incorporation time of ^{13}C -valine.

169 The absolute synthesis rate (ASR, g/d) was determined as $\text{ASR} = \text{FSR} \times P$, where P is tissue total
170 protein content.

171 **RNA preparation and gene expression and biochemical measurement**

172 RNA preparation and gene expression measurement in the liver and in muscle followed the same
173 protocol described previously in [27]. We measured the expression of genes involved in proteolysis
174 (*Cathepsin D*, *Ulk1* and *Ubiquitin*) and in GCN2 signaling pathway (*Gcn2*, *Ddit3*, *Trib3* and *Atf4*). The

175 primers used for qPCR are detailed in Supplemental Table 1. Data are expressed relatively to the values
176 of the WT mice fed the NP diet.

177 **Statistical analysis**

178 Data are presented as means \pm SEM. The effects of the diets and genotype were tested by two-way
179 ANOVA with interaction using R[®] software. Pairwise comparisons were performed with Post-hoc
180 Bonferroni tests for multiple comparisons, p-values were multiplied by the number of comparisons
181 performed. Differences were considered significant at $P < 0.05$.

182

183 **Results**

184 **Food intake, body weight, tissue mass and composition**

185 In the AMPK study, final body weight was affected by the protein content in the diet ($P<0.05$) but it
186 was not correlated to food intake. Indeed, mice fed the NP diet weighed more than HP and LP-diets fed
187 mice (Table 2). No effect of neither the genotype nor the protein content was observed in the GCN2
188 study ($P>0.05$). Interestingly, final body weight and liver mass were lower in KO-AMPK mice
189 compared to WT-AMPK mice ($P<0.05$).

190 Cumulative food intake increased with the decrease in protein content of the diet in the two experiments
191 so that food intake of LP-diet fed mice was 1.8-fold the one of HP-diet fed mice ($P<0.0001$, Table 2).

192 There was an effect of the AMPK deletion on feed intake as shown by the increased food intake in mice
193 fed the NP diet. GCN2 deletion had no effect on food intake ($P<0.05$, Table 2).

194 In the two experiments, weight and protein concentration in liver increased in parallel with the increase
195 in protein content of the diet ($P<0.05$) whereas muscle weight and protein content were affected neither
196 by the protein intake nor by the genotype ($P>0.05$, Table 2).

197 **Post-prandial tissue protein synthesis rates**

198 Before euthanasia, mice were all fed a calibrated meal of 1g, in order to overcome the differences on
199 energy intake that could have impacted protein synthesis rates. We also made sure that the pellet was
200 ingested within 30 minutes after meal onset.

201 First, our results showed that the fractional synthesis rate (FSR) was more than ten times higher in the
202 liver than in the muscle (Figure 1). There was an effect of the protein content of the diet that decreased
203 the protein anabolism in the liver for low and high protein content, compared to NP diet ($P<0.05$). Liver
204 FSR decreased by 29% in HP fed mice compared to NP fed mice ($P<0.05$, Figure 1A). Additionally, in
205 the GCN2 study, liver FSR tended to be lower ($P<0.06$) in LP fed mice compared to NP fed mice.
206 Similarly, there was a trend ($P=0.07$, AMPK study) or an effect ($P<0.05$, GCN2 study) of the protein
207 diet on the ASR that was numerically the lowest in the HP and LP groups (Figure 1C). The effect was
208 especially marked in the GCN2 experiment where LP fed mice, because of their smaller liver weight
209 and protein concentration, exhibited a significant decrease in ASR compared to NP fed mice ($P<0.05$).

210 Protein synthesis rate in the muscle was insensitive to dietary protein level ($P>0.05$, Figure 1B-1D). No
211 effect of liver GCN2 or AMPK deletion was noticed on both FSR and ASR in the liver or in the muscle
212 (Figure 1).

213 **Postprandial gene expression involved in proteolysis**

214 We have investigated the effect of protein intake on two markers of autophagy in the postprandial state,
215 Cathepsin D and ULK1 and one marker of ubiquitin proteasome pathway, Ubiquitin.

216 On the basis on mRNA results, gene expression in the liver remained insensitive to genotype and protein
217 level ($P>0.05$) except in the GCN2 experiment where liver Cathepsin D and ULK1 expressions were
218 inversely related to the protein content of the diet ($P<0.001$ and $P<0.05$, respectively, Table 3). In
219 particular, Cathepsin D mRNA abundance was higher in the LP group compared to the two other groups
220 ($P<0.001$, Table 3). Gene expression in the muscle was neither affected by the deletion of hepatic AMPK
221 or GCN2 nor by the protein content of the diet ($P>0.05$, Table 3). Interestingly, in the liver the expression
222 levels of *Atf4* (encoding activating transcription factor 4 – ATF4), *Ddit3* (encoding DNA damage-
223 inducible transcript 3 - CHOP) and *Trib3* (encoding tribbles homolog – TRB3), were lower in GCN2
224 KO mice under the LP diet compared with WT mice (Table 4). In the AMPK experiment, the level of
225 expression of these genes responded mainly to the difference in protein level and exhibited an increased
226 expression in response to LP diet. Interestingly, the deletion of AMPK tended to increase *GCN2*
227 expression ($P=0.06$) and increased significantly *Atf4* expression in the liver ($P<0.01$, Table 4).

228 **Postprandial plasma urea and albumin:**

229 In the two experiments, plasma urea was higher in HP fed mice in the postprandial state ($P<0.01$)
230 whereas no genotype effect was observed ($P>0.05$), Table 5). No effect of the deletion or protein level
231 of the diet was observed for plasma albumin ($P>0.05$, Table 5).

232 Discussion

233 The purpose of this study was to explore the mechanisms of adaptation to low and high protein diet
234 regarding liver protein metabolism. For this purpose, WT mice and mice in which AMPK or GCN2 was
235 deleted in the liver were fed during three weeks with low, normo- and high protein diets. We observed
236 that, in contrast to muscle, the protein content of the diet affected liver mass and composition as well as
237 protein synthesis rate. Indeed, the consumption of a HP diet was associated with a marked decrease in
238 FSR by 29% compared to NP diet. Surprisingly, the deletion of AMPK or GCN2 in the liver did not
239 affect postprandial protein metabolism markers (lean mass, FSR, gene encoding for proteolysis) in the
240 liver or in the muscle, whatever the protein content in the diet.

241 Our results on the effect of a HP diet are in line with our previous works in rats that reported that the
242 consumption of HP diet lowered protein synthesis rates in the liver but did not affect muscle protein
243 turnover [11, 12]. Moreover, we have reported that the main metabolic response to HP diet concerned
244 the stimulation of AA oxidation, leading to a large rise in urea production [18]. Using ¹⁵N-¹³C dietary
245 amino acids, the dietary AA cumulative deamination 4h after meal was reported to be about 8 times
246 higher in rats adapted to HP than in those adapted to NP diet and the dietary AA cumulative oxidation
247 was doubled in HP rats in comparison with NP rats [28]. Accordingly, in the present study we have also
248 observed that plasma urea was increased in HP conditions. Thus, because of the rapid saturation of
249 protein anabolic process [18], the excess of dietary amino acids provided by HP diet were deaminated
250 and further oxidized.

251 In the present study as in previous ones, we observed both a decrease in protein anabolic rate in the liver
252 and an increase in protein content in the liver. We previously explained this paradox by a higher decrease
253 of proteolysis fluxes in the postprandial state after a HP diet compared to NP in rats. **This was associated**
254 **with a decrease in Cathepsin D and Ubiquitin** expression in the liver [29]. In other studies, a LP diet
255 was associated with a decrease in caspase and proteasome systems in the muscle [3, 4] and an increase
256 in autophagy in the liver [30]. In our work, the proteolysis indicators that we studied were mostly
257 insensitive to dietary protein content and genotype with the exception of Cathepsin D in LP-fed mice
258 and ULK1 in HP-fed mice in the GCN2 experiment. This observation together with the downward trend

259 of protein synthesis in LP diet could explain why these mice exhibited an important decrease in their
260 liver protein content.

261 LP diet intake is known to decrease tissue protein synthesis [3–5]. Consistently, we observed such an
262 effect in the GCN2 study, where LP diet intake tended to decrease FSR compare to NP and had a
263 negative effect on ASR in the liver, without any effect on muscle protein synthesis rate. This effect was
264 less marked in the AMPK study. We have no logic explanation for the differences between the WT mice
265 of both studies, except the lab origin of the mice that differs. In the present experiment, no effect of the
266 protein level of the diet was observed for plasma albumin, suggesting that the protein deficiency was
267 not too severe. Perhaps a longer dietary intervention would have resulted in more severe effects as LP
268 diets are usually associated with decrease in plasma protein content [5].

269 We did not observe any effect of the deletion of AMPK or GCN2 on protein synthesis rate. Although
270 surprising, these results can be explained by the experimental conditions as animals were studies in the
271 fed state. First, we can hypothesize that in the postprandial state, because of the load of amino acids and
272 energy provided by the meal, both kinases are not highly phosphorylated and activated in the liver of
273 WT mice. In this context, it is not possible to observe any difference in protein synthesis rate between
274 WT and genetically modified mice. Moreover, it has been reported that GCN2 is activated when the diet
275 is deprived in indispensable amino acids (-leucine or – tryptophan) [31]. Although we explored a low
276 protein level, the amino acid concentration after a 1g meal may be not low enough to induce the
277 activation state of these stress kinase. Second, we can also hypothesize that adaptation mechanisms
278 compensate for the KO of GCN2 and AMPK. Indeed, GCN2 is known to mediate the different effects
279 of a LP diet [32]. It was reported that KO-GCN2 mice do not exhibit any increase in food intake and
280 energy expenditure in the first days after the introduction of a LP diet compared to WT mice [32]. The
281 same team reported that in KO-GCN2 mice, two weeks after being on a LP diet, an adaptation
282 mechanism compensated for the absence of GCN2 and restored the effects of a low protein diet on food
283 intake and energy expenditure [33]. In our study, KO-GCN2 mice were fed during three weeks on a LP
284 diet and this adaptation mechanism could have taken place. This might explain for the absence of any
285 difference on protein synthesis rates and proteolysis between WT-GCN2 and KO-GCN2 mice despite
286 the fact that KO-GCN2 failed to induce the expression of *Atf4*, *Ddit3* and *Trib3* in response to LP diet.

287 However, in our study, the difference in food intake between the groups was visible from the start of the
288 experiment suggesting that food intake regulation is not controlled by GCN2 at the liver level.

289

290 We acknowledge that our study has some limitations. First, we failed to report the level of
291 phosphorylation and activation of the different pathways including the mTOR pathway due to the too
292 long time of sample storing before performing the analysis. Second, AMPK and GCN2 are sensitive to
293 deficiency in energy and amino acids, respectively. These situations are most likely to occur when
294 animals are in the fasted state. As our measurements were performed in the postprandial period, it is
295 possible that this could not allow to reveal the involvement of these pathways in long-term regulations
296 to the modulation of diet composition. Additional measurements performed during the fasted state could
297 have overcome this issue.

298

299 In conclusion, our study could not evidence any effect of GCN2 or AMPK deletion on protein
300 metabolism in mice fed different protein levels. The modulation of protein content in diet showed
301 consistent effects with those already reported on LP and HP diet, especially on the protein FSR in the
302 liver, whereas the expression of genes involved in proteolysis were not altered. During the postprandial
303 period, liver AMPK and GCN2, did not appear to play a major role on the control of the rate of protein
304 synthesis in the liver and in the muscle, suggesting that in the fed state, these two kinase do not play an
305 important role in the control protein metabolism in response to amino acid excess or deprivation.

306 **Authors' contributions:**

307 B.V., A-C.M., P.F., M.F supplied the mice models. T-C.D., D.A.M., D.T., P.C.E, C.G. designed the
308 study. T.C.D., P.C.E., D.A.M., J.P., J.M., N.K. conducted research. T.C.D. D.A.M., D.T., P.F, M.F.
309 interpreted the data. T.C.D., D.A.M. wrote the manuscript. T-C.D. and D.A.M. had primary
310 responsibility for final content. All authors have read and approved the manuscript submission.

311

312

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314 This work was supported by UMR PNCA

315 **Conflict of interest**

316 All authors have no conflict of interest.

317 **Ethics approval**

318 This study was conducted in accordance to the European directive for the use and care of laboratory
319 animals (2010/63/UE), and received the agreement of French National Animal Care Committee (number
320 14-15).

321

322 **Table 1** Macronutrient composition of the diets. Diets were prepared by the “atelier de préparation des
 323 aliments”, UPAE, INRA, Jouy en Josas, France. Energy density is computed assuming a
 324 metabolizable energy of 16.7 kJ/g for carbohydrates and proteins and 37.7 kJ/g for fat.
 325

	P20	NP	LP	HP
Weight content (g/kg)				
Milk proteins	200	140	50	530
Starch	570	622	700	287
Sucrose	93	100	113	46
Soy Oil	40	40	40	40
Minerals	35	35	35	35
Vitamins	10	10	10	10
cellulose	50	50	50	50
choline	2	2	2	2
Energy content (%)				
Protein	20.5	14.5	5.2	54.6
Carbohydrate	68.5	75.0	84.0	34.9
Fat	10.5	10.5	10.5	10.5
Energy density (kcal/g)	3.48	3.48	3.47	3.49

326
 327 Diets were prepared by the “atelier de préparation des aliments,” UPAE, INRA, Jouy en Josas, France.
 328 Energy density is computed assuming a metabolizable energy of 4.00 kcal/g for carbohydrates and
 329 proteins and 9.00 kcal/g for fat. HP, high-protein diet; LP, low-protein diet; NP, normo-protein diet; P20
 330 : 20% protein diet.

Table 2 Initial and final body weight, cumulative food intake, tissue mass and protein content of WT-AMPK, KO-AMPK, WT-GCN2 and KO-GCN2 mice fed NP, LP or HP diets for three weeks.

Diet	NP		LP		HP		Genotype	Test diet	Interaction	
Genotype	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK				
	Initial body weight (g)	13.87 ± 0.62	14.43 ± 0.46	14.28 ± 0.41	13.61 ± 0.47	14.20 ± 0.40	14.15 ± 0.34	NS	NS	NS
	Final body weight (g)	24.74 ± 0.41	23.7 ± 0.66 ^a	22.93 ± 0.32	22.29 ± 0.34 ^b	23.79 ± 0.71	23.14 ± 0.73 ^{ab}	<0.05	<0.05	NS
	Cumulative food intake (g)	71.8 ± 1.3	79.1 ± 1.9* ^a	93.4 ± 1.20	94.4 ± 2.1 ^b	54.1 ± 1.4	53.6 ± 1.6 ^c	<0.05	<0.0001	NS
Liver	Weight (g)	1.00 ± 0.02	0.88 ± 0.07 ^{ab}	0.92 ± 0.02	0.86 ± 0.02 ^a	1.07 ± 0.05	0.97 ± 0.04 ^b	<0.05	<0.05	NS
	mg of protein/100 mg of tissue	17.58 ± 0.76	17.39 ± 0.45 ^a	16.29 ± 0.58	15.99 ± 0.65 ^a	19.67 ± 0.68	18.48 ± 0.6 ^b	NS	<0.001	NS
Muscle	Weight (g)	0.36 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	NS	NS	NS
	mg of protein/100 mg of tissue	19.74 ± 0.59	20.71 ± 0.41	19.66 ± 0.4	20.75 ± 0.53	20.17 ± 0.52	19.83 ± 0.72	NS	NS	NS
Genotype	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2				
	Initial body weight (g)	13.99 ± 0.38	14.58 ± 0.38	14.79 ± 0.33	13.95 ± 0.29	14.68 ± 0.58	14.05 ± 0.46	NS	NS	NS
	Final body weight (g)	23.17 ± 0.48	23.54 ± 0.55	22.07 ± 0.44	21.60 ± 0.58	22.67 ± 0.78	22.56 ± 0.79	NS	NS	NS
	Cumulative food intake (g)	68.26 ± 3.16	70.93 ± 2.83 ^a	93.64 ± 2.74	88.45 ± 3.16 ^b	54.26 ± 2.58	52.37 ± 3.80 ^c	NS	<0.0001	NS
Liver	Weight (g)	0.87 ± 0.04	0.87 ± 0.04 ^a	0.75 ± 0.05	0.72 ± 0.06 ^b	1.00 ± 0.06	1.00 ± 0.06 ^a	NS	<0.0001	NS
	mg of protein/100 mg of tissue	20.3 ± 0.88	18.8 ± 0.64 ^a	16.58 ± 0.69	16.46 ± 0.53 ^b	19.85 ± 0.45	19.31 ± 0.94	NS	<0.0001	NS
Muscle	Weight (g)	0.39 ± 0.01	0.4 ± 0.01	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.39 ± 0.01	NS	NS	NS
	mg of protein/100 mg of tissue	22.35 ± 0.72	22.25 ± 0.45	20.52 ± 0.84	20.46 ± 0.65	22.17 ± 0.84	20.22 ± 0.03	NS	NS	NS

Data are mean ± SEM (n =7-9).

^{a,b,c} Different letters within a line mean statistically different values between test diets (post hoc Bonferroni tests for multiple comparisons, P<0.05).

* Significant difference between WT and KO within the same diet (post hoc Bonferroni tests for multiple comparisons, P<0.05).

Table 3 mRNA abundance of Cathepsin D, ULK1 and Ubiquitin in liver and muscle of WT-AMPK, KO-AMPK, WT-GCN2 and KO-GCN2 mice fed NP, LP or HP diets two hours after meal onset in the postprandial state.

Diet		NP		LP		HP		Genotype	Test diet	Interaction
Genotype		WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK			
Liver	Cathepsin D	1 ± 0.08	1.14 ± 0.08	0.98 ± 0.06	0.96 ± 0.08	1.15 ± 0.11	1.04 ± 0.04	NS	NS	NS
	ULK1	1 ± 0.09	1.11 ± 0.11	1.16 ± 0.11	1.17 ± 0.13	1.01 ± 0.09	1.03 ± 0.10	NS	NS	NS
	Ubiquitin	1 ± 0.09	1.22 ± 0.10	1.06 ± 0.09	0.94 ± 0.13	1.04 ± 0.09	0.99 ± 0.07	NS	NS	NS
Muscle	Cathepsin D	1 ± 0.05	0.8 ± 0.06	0.85 ± 0.04	0.91 ± 0.07	0.98 ± 0.06	0.95 ± 0.05	NS	NS	NS
	Ubiquitin	1 ± 0.17	1.05 ± 0.08	1.14 ± 0.04	1.27 ± 0.08	1.17 ± 0.10	1.28 ± 0.07	NS	NS	NS
Genotype		WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2	Genotype	Test diet	Interaction
Liver	Cathepsin D	1 ± 0.10	0.97 ± 0.07 ^a	1.54 ± 0.28	2.08 ± 0.31 ^b	0.86 ± 0.13	1.22 ± 0.21 ^a			
	ULK1	1 ± 0.13	1.02 ± 0.12	1.05 ± 0.11	1.32 ± 0.16	0.88 ± 0.15	0.79 ± 0.15	NS	<0.05	NS
	Ubiquitin	1 ± 0.34	0.79 ± 0.06	0.93 ± 0.14	1.17 ± 0.14	0.97 ± 0.22	1.29 ± 0.19	NS	NS	NS
Muscle	Cathepsin D	1 ± 0.12	0.81 ± 0.02	0.73 ± 0.04	1.20 ± 0.24	1.10 ± 0.37	0.90 ± 0.06	NS	NS	NS
	Ubiquitin	1 ± 0.11	0.83 ± 0.05	0.81 ± 0.03	0.91 ± 0.06	0.96 ± 0.15	0.73 ± 0.05	NS	NS	NS

Data are mean ± SEM (n=7-9).

^{a,b,c} Different letters within a line mean statistically different values between test diets (post hoc Bonferroni tests for multiple comparisons, P<0.05).

Table 4 mRNA abundance in liver of WT-AMPK, KO-AMPK, WT-GCN2 and KO-GCN2 mice fed NP, LP or HP diets two hours after meal onset in the postprandial state.

Diet	NP		LP		HP		Genotype	Test diet	Interaction
Genotype	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK			
GCN2	1 ± 0.14	1.32 ± 0.09	1.28 ± 0.10	1.63 ± 0.14	1.41 ± 0.13	1.28 ± 0.13	0.06	0.06	NS
CHOP	1 ± 0.25	1.40 ± 0.23 ^a	1.70 ± 0.25	1.83 ± 0.17 ^b	1.21 ± 0.29	0.93 ± 0.15 ^a	NS	<0.05	NS
TRB3	1 ± 0.10	2.18 ± 0.27 ^a	11.9 ± 3.93	11.1 ± 9.66 ^b	1.34 ± 0.19	0.98 ± 0.19 ^a	NS	<0.0001	NS
ATF4	1 ± 0.11	1.76 ± 0.15 ^{ab}	1.51 ± 0.12	1.62 ± 0.16 ^a	1.18 ± 0.14	1.19 ± 0.09 ^b	<0.01	<0.05	<0.05
Genotype	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2			
GCN2	1 ± 0.23	0.16 ± 0.02	0.84 ± 0.09	0.25 ± 0.03	0.92 ± 0.14	0.20 ± 0.04	<0.0001	NS	NS
CHOP	1 ± 0.16	0.90 ± 0.08 ^a	7.28 ± 2.11	1.33 ± 0.29* ^b	1.27 ± 0.21	1.69 ± 0.28 ^a	<0.01	<0.01	<0.01
TRB3	1 ± 0.10	0.96 ± 0.17 ^a	35.1 ± 12.4	2.23 ± 0.88* ^b	1.20 ± 0.42	2.80 ± 1.19 ^a	<0.01	<0.01	<0.01
ATF4	1 ± 0.18	0.98 ± 0.08	2.15 ± 0.43	1.09 ± 0.15* ^b	1.25 ± 0.46	1.17 ± 0.14	0.08	0.07	NS

Data are mean ± SEM (n =7-9).

^{A,B,C} Different letters within a line mean statistically different values between test diets (post hoc Bonferroni tests for multiple comparisons, P<0.05).

* Significant difference between WT and KO within the same diet (post hoc Bonferroni tests for multiple comparisons, P<0.05).

Table 5 Plasma urea and albumin of WT-AMPK, KO-AMPK, WT-GCN2 and KO-GCN2 mice fed NP, LP or HP diets two hours after meal onset in the postprandial state.

Diet	NP		LP		HP		Genotype	Test diet	Interaction
Genotype	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK	WT-AMPK	KO-AMPK			
Plasma Urea (mmol/l)	14.56 ± 0.8	12.92 ± 0.67 ^a	9.34 ± 0.63	9.51 ± 0.62 ^b	22.19 ± 0.91	25.16 ± 2.43 ^c	NS	<0.0001	NS
Plasma Albumine (g/l)	27.54 ± 0.83	25.3 ± 1.37	25.42 ± 0.8	26.35 ± 0.79	26 ± 1.33	26.12 ± 1.35	NS	NS	NS
Genotype	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2	WT-GCN2	KO-GCN2			
Plasma Urea (mmol/l)	15 ± 1.88	17.09 ± 1.94 ^a	15.66 ± 1.76	11.04 ± 1.77 ^b	27.46 ± 3.07	29.4 ± 4.77 ^b	NS	<0.001	NS
Plasma Albumine (g/l)	27.99 ± 4.9	31.66 ± 4.23	27.65 ± 2.54	24.72 ± 4	28.53 ± 3.65	29.1 ± 4.37	NS	NS	NS

Data are mean ± SEM (n =7-9).

^{a,b,c} Different letters within a line mean statistically different values between test diets (post hoc Bonferroni tests for multiple comparisons, P<0.05).

References

1. Morrison CD, Laeger T (2015) Protein-dependent regulation of feeding and metabolism. *Trends Endocrinol Metab* 26:256–262. <https://doi.org/10.1016/j.tem.2015.02.008>
2. Hursel R, Martens EAP, Gonnissen HKJ, et al (2015) Prolonged Adaptation to a Low or High Protein Diet Does Not Modulate Basal Muscle Protein Synthesis Rates - A Substudy. *PloS One* 10:e0137183. <https://doi.org/10.1371/journal.pone.0137183>
3. Batistela E, Pereira MP, Siqueira JT, et al (2014) Decreased rate of protein synthesis, caspase-3 activity, and ubiquitin–proteasome proteolysis in soleus muscles from growing rats fed a low-protein, high-carbohydrate diet. *Can J Physiol Pharmacol* 92:445–454. <https://doi.org/10.1139/cjpp-2013-0290>
4. dos Santos MP, Batistela E, Pereira MP, et al (2016) Higher insulin sensitivity in EDL muscle of rats fed a low-protein, high-carbohydrate diet inhibits the caspase-3 and ubiquitin-proteasome proteolytic systems but does not increase protein synthesis. *J Nutr Biochem* 34:89–98. <https://doi.org/10.1016/j.jnutbio.2016.04.008>
5. Wykes LJ, Fiorotto M, Burrin DG, et al (1996) Chronic low protein intake reduces tissue protein synthesis in a pig model of protein malnutrition. *J Nutr* 126:1481
6. Yoshizawa F, Kimball SR, Vary TC, Jefferson LS (1998) Effect of dietary protein on translation initiation in rat skeletal muscle and liver. *Am J Physiol-Endocrinol Metab* 275:E814–E820
7. Deng D, Yao K, Chu W, et al (2009) Impaired translation initiation activation and reduced protein synthesis in weaned piglets fed a low-protein diet. *J Nutr Biochem* 20:544–552. <https://doi.org/10.1016/j.jnutbio.2008.05.014>
8. Chevalier L, Bos C, Gryson C, et al (2009) High-protein diets differentially modulate protein content and protein synthesis in visceral and peripheral tissues in rats. *Nutr Burbank Los Angel Cty Calif* 25:932–939. <https://doi.org/10.1016/j.nut.2009.01.013>
9. Masanes R, Fernandez-Lopez JA, Alemany M, et al (1999) Effect of dietary protein content on tissue protein synthesis rates in Zucker lean rats. *Nutr Res* 19:1017–1026

10. Taillandier D, Guezennec C-Y, Patureau-Mirand P, et al (1996) A high protein diet does not improve protein synthesis in the nonweight-bearing rat tibialis anterior muscle. *J Nutr* 126:266–272
11. Chevalier L, Bos C, Gryson C, et al (2009) High-protein diets differentially modulate protein content and protein synthesis in visceral and peripheral tissues in rats. *Nutr* Burbank Los Angel Cty Calif 25:932–939. <https://doi.org/10.1016/j.nut.2009.01.013>
12. Chevalier L, Bos C, Azzout-Marniche D, et al (2010) Dietary protein regulates hepatic constitutive protein anabolism in rats in a dose-dependent manner and independently of energy nutrient composition. *Am J Physiol Regul Integr Comp Physiol* 299:R1720-1730. <https://doi.org/10.1152/ajpregu.00497.2010>
13. Chotechuan N, Azzout-Marniche D, Bos C, et al (2011) Down-regulation of the ubiquitin-proteasome proteolysis system by amino acids and insulin involves the adenosine monophosphate-activated protein kinase and mammalian target of rapamycin pathways in rat hepatocytes. *Amino Acids* 41:457–468. <https://doi.org/10.1007/s00726-010-0765-2>
14. Dever TE, Hinnebusch AG (2005) GCN2 whets the appetite for amino acids. *Mol Cell* 18:141–142. <https://doi.org/10.1016/j.molcel.2005.03.023>
15. Thomson A, Smart K, Somerville MS, et al (2019) The Ussing chamber system for measuring intestinal permeability in health and disease. *BMC Gastroenterol* 19:98. <https://doi.org/10.1186/s12876-019-1002-4>
16. Janzen NR, Whitfield J, Hoffman NJ (2018) Interactive Roles for AMPK and Glycogen from Cellular Energy Sensing to Exercise Metabolism. *Int J Mol Sci* 19:3344. <https://doi.org/10.3390/ijms19113344>
17. Rothschild JA, Islam H, Bishop DJ, et al (2021) Factors Influencing AMPK Activation During Cycling Exercise: A Pooled Analysis and Meta-Regression. *Sports Med Auckl NZ*. <https://doi.org/10.1007/s40279-021-01610-x>
18. Morens C, Gaudichon C, Metges CC, et al (2000) A High-Protein Meal Exceeds Anabolic and Catabolic Capacities in Rats Adapted to a Normal Protein Diet. *J Nutr* 130:2312–2321. <https://doi.org/10.1093/jn/130.9.2312>

19. Vidal-Lletjós S, Khodorova NV, Piscuc M, et al (2021) Tissue-specific effect of colitis on protein synthesis in mice: impact of the dietary protein content. *Eur J Nutr* 60:1669–1677. <https://doi.org/10.1007/s00394-020-02365-3>
20. Petzke KJ, Elsner A, Proll J, et al (2000) Long-term high protein intake does not increase oxidative stress in rats. *J Nutr* 130:2889–2896. <https://doi.org/10.1093/jn/130.12.2889>
21. Schutt AK, Blesson CS, Hsu JW, et al (2019) Preovulatory exposure to a protein-restricted diet disrupts amino acid kinetics and alters mitochondrial structure and function in the rat oocyte and is partially rescued by folic acid. *Reprod Biol Endocrinol RBE* 17:12. <https://doi.org/10.1186/s12958-019-0458-y>
22. Chevalier L, Bos C, Gryson C, et al (2009) High-protein diets differentially modulate protein content and protein synthesis in visceral and peripheral tissues in rats. *Nutr Burbank Los Angel Cty Calif* 25:932–939. <https://doi.org/10.1016/j.nut.2009.01.013>
23. Stepien M, Azzout-Marniche D, Even PC, et al (2016) Adaptation to a high-protein diet progressively increases the postprandial accumulation of carbon skeletons from dietary amino acids in rats. *Am J Physiol-Regul Integr Comp Physiol* 311:R771–R778. <https://doi.org/10.1152/ajpregu.00040.2016>
24. Welle S, Bhatt K, Pinkert CA (2006) Myofibrillar protein synthesis in myostatin-deficient mice. *Am J Physiol Endocrinol Metab* 290:E409–415. <https://doi.org/10.1152/ajpendo.00433.2005>
25. Bregendahl K, Yang X, Liu L, et al (2008) Fractional protein synthesis rates are similar when measured by intraperitoneal or intravenous flooding doses of L-[ring-2H5]phenylalanine in combination with a rapid regimen of sampling in piglets. *J Nutr* 138:1976–1981. <https://doi.org/10.1093/jn/138.10.1976>
26. Corr LT, Berstan R, Evershed RP (2007) Optimisation of derivatisation procedures for the determination of delta13C values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry. *21:3759–3771*
27. Chalvon-Demersay T, Even PC, Tomé D, et al (2016) Low-protein diet induces, whereas high-protein diet reduces hepatic FGF21 production in mice, but glucose and not amino acids up-regulate FGF21 in cultured hepatocytes. *J Nutr Biochem* 36:60–67. <https://doi.org/10.1016/j.jnutbio.2016.07.002>

28. Fromentin C, Azzout-Marniche D, Tomé D, et al (2011) The postprandial use of dietary amino acids as an energy substrate is delayed after the deamination process in rats adapted for 2 weeks to a high protein diet. *Amino Acids* 40:1461–1472. <https://doi.org/10.1007/s00726-010-0756-3>
29. Chotechuan N, Azzout-Marniche D, Bos C, et al (2011) Down-regulation of the ubiquitin-proteasome proteolysis system by amino acids and insulin involves the adenosine monophosphate-activated protein kinase and mammalian target of rapamycin pathways in rat hepatocytes. *Amino Acids* 41:457–468. <https://doi.org/10.1007/s00726-010-0765-2>
30. Henagan TM, Laeger T, Navard AM, et al (2016) Hepatic autophagy contributes to the metabolic response to dietary protein restriction. *Metabolism* 65:805–815. <https://doi.org/10.1016/j.metabol.2016.02.015>
31. Guo F, Cavener DR (2007) The GCN2 eIF2 α kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab* 5:103–114. <https://doi.org/10.1016/j.cmet.2007.01.001>
32. Laeger T, Henagan TM, Albarado DC, et al (2014) FGF21 is an endocrine signal of protein restriction. *J Clin Invest* 124:3913–3922. <https://doi.org/10.1172/JCI74915>
33. Laeger T, Albarado DC, Burke SJ, et al (2016) Metabolic Responses to Dietary Protein Restriction Require an Increase in FGF21 that Is Delayed by the Absence of GCN2. *Cell Rep* 16:707–716. <https://doi.org/10.1016/j.celrep.2016.06.044>

Fig. 1 Fractional synthesis rate (FSR) and absolute synthesis rate (ASR) in liver (**A** and **C**) and muscle (**B** and **D**) in WT-AMPK, KO-AMPK, WT-GCN2 and KO-GCN2 mice fed NP, LP or HP diets. ^{a,b,c} Different letters within a line mean statistically different values between test diets (post hoc Bonferonni tests for multiple comparisons, $P < 0.05$). Data are mean \pm SEM (n =7-9).