

Local myostatin inhibition improves skeletal muscle glucose uptake in insulin resistant high fat diet-fed mice

Article

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1 **Local myostatin inhibition improves skeletal muscle glucose**
2 **uptake in insulin resistant high fat diet-fed mice**

3

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5

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10

11 **Running head**

12 Myostatin inhibition improves muscle glucose uptake

13

14

15 **Author contributions**

16 WE, MC & KF designed experiments, WE, MC & DC performed experiments, WE,

17 MC & KF analysed data and WE wrote the paper with input from DC, MC and KF

18

19

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30 **Abstract**

31 Myostatin inhibition is thought to improve whole body insulin sensitivity and mitigate
32 the development of insulin resistance in models of obesity. However, although
33 myostatin is known to be a major regulator of skeletal muscle mass, the direct effects
34 of myostatin inhibition in muscle on glucose uptake and the mechanisms which may
35 underlie this are still unclear. We investigated the effect of local myostatin inhibition
36 by adeno-associated virus-mediated overexpression of the myostatin pro-peptide on
37 insulin-stimulated skeletal muscle glucose disposal in chow-fed or high fat diet-fed
38 mice and evaluated the molecular pathways that might mediate this. We found that
39 myostatin inhibition improved glucose disposal in obese high fat diet-fed mice
40 alongside the induction of muscle hypertrophy, but did not have an impact in chow-
41 fed mice. This improvement was not associated with greater glucose transporter or
42 peroxisome proliferator-activated receptor gamma coactivator-1 α expression or 5'
43 AMP-activated protein kinase activation as previously suggested. Instead,
44 transcriptomic analysis suggested that the improvement in glucose disposal was
45 associated with significant enrichment in genes involved in fatty acid metabolism and
46 translation of mitochondrial genes. Thus, myostatin inhibition improves muscle
47 insulin-stimulated glucose disposal in obese high fat diet-fed mice independent of
48 muscle hypertrophy, potentially involving previously unidentified pathways.

49

50 **Keywords**

51 Myostatin – Skeletal muscle – Glucose uptake – Insulin resistance - Diabetes

52

53 **Introduction**

54 The development of skeletal muscle insulin resistance is an important feature of type
55 2 diabetes because skeletal muscle is a major site of post-prandial glucose uptake.
56 In addition, the development of insulin resistance is thought to be a feature of the
57 loss of muscle mass during aging, known as sarcopenia. Inhibition of myostatin, a
58 negative regulator of muscle size, has received significant attention as a potential
59 therapeutic strategy for the improvement of both muscle strength (32) and insulin
60 sensitivity (5), and the mitigation of the pathological features of the metabolic
61 syndrome. Myostatin is a member of the transforming growth factor- β family of
62 proteins that is secreted and activates Smad2/3 signalling in cells in an
63 autocrine/paracrine fashion by binding the activin type 2A and 2B receptors (20). It is
64 expressed predominantly in muscle and (at the mRNA level) at much lower levels in
65 adipose tissue (1).

66

67 Knockout of the myostatin gene causes significant enlargement of skeletal muscles
68 through both hyperplasia and hypertrophy (24), but this increase in muscle mass is
69 not mirrored by an increase in muscle strength (2). In contrast, post-natal inhibition of
70 myostatin causes muscle hypertrophy, but not hyperplasia, (3, 19), and results in a
71 concomitant increase in muscle strength (12, 23). Much less is known about the
72 effect of myostatin inhibition on muscle glucose uptake and insulin sensitivity.
73 Myostatin gene knockout prevents fat mass gain during the lifespan of chow-fed
74 mice (25) and most evidence indicates that the genetic loss of myostatin improves
75 glucose tolerance and/or insulin sensitivity in mouse models of (extreme) obesity
76 (13, 14, 40). Post-natal systemic myostatin antibody treatment increases skeletal
77 muscle mass (4, 5, 18, 34, 39) and increases whole body insulin sensitivity in aged
78 chow-fed mice, but not young mice being fed either regular chow or a high fat diet
79 (5). Thus, myostatin inhibition-induced muscle hypertrophy is not always
80 accompanied by an increase in insulin sensitivity, suggesting that hypertrophy is not
81 sufficient and another, muscle mass-independent effect is required or that a potential
82 threshold in the increase in muscle mass exists for an effect on insulin sensitivity to
83 occur.

84 In addition, the mechanism through which myostatin acts to improve insulin
85 sensitivity in skeletal muscle remains unclear. Systemic administration or
86 overexpression of myostatin inhibitors affects the action of myostatin originating

87 from, and acting on, other tissues important for controlling whole body insulin
88 sensitivity, such as white and brown adipose tissue (33). Thus, it remains unclear
89 whether myostatin inhibition improves muscle insulin sensitivity in models of insulin
90 resistance through a local effect or whether this effect is mediated by alterations in
91 systemic factors. Myostatin inhibition has been proposed to stimulate signalling
92 through the Akt pathway (36) and to increase 5' AMP-activated protein kinase
93 (AMPK) activity (11, 43) in skeletal muscle. These pathways control the translocation
94 of glucose transporters to the plasma membrane as part of insulin-dependent and
95 insulin-independent signalling mechanisms, respectively (16, 17). In addition, we
96 have previously shown greater expression of the GLUT1 and GLUT4 glucose
97 transporters after local myostatin inhibition, which was associated with enhanced
98 muscle glucose disposal in rat muscle (8). Finally, myostatin inhibition-dependent
99 activation of an AMPK-peroxisome proliferator-activated receptor gamma coactivator
100 (PGC)-1 α pathway has been suggested to stimulate the formation of brown fat by
101 increasing the secretion of the hormone irisin from skeletal muscle (30).

102

103 Generation of active myostatin requires cleavage and subsequent dimerization of a
104 precursor protein. The NH₂-terminal latency-associated peptide (ProMyo) sequesters
105 the myostatin dimer and prevents it from binding to the activin type 2A and 2B
106 receptors (20). Here, we show that local skeletal muscle myostatin inhibition using
107 an adeno-associated virus (AAV) expressing the ProMyo peptide increases insulin-
108 stimulated glucose uptake in high-fat diet-fed mice, but not in chow-fed mice despite
109 the presence of significant muscle hypertrophy. In contrast to previous work, this was
110 not associated with increased PGC-1 α or glucose transporter expression.

111

112

113 **Methods**

114 *Preparation of adeno-associated virus*

115 The adeno-associated virus (AAV) construct containing a modified myostatin
116 propeptide sequence fused to a mouse immunoglobulin G2a (IgG2a) moiety under
117 control of a CAGG promoter was as previously described (12, 41). AAV2/8 ProMyo
118 viral particles were produced and titered by Vector Core (Nantes, France).

119 *Animals*

120 Male C57BL/6 mice (Harlan Laboratories) and myostatin knockout mice on a
121 C57BL/6 background (24) were housed in animal facilities at the Royal Veterinary
122 College or the University of Reading under a 12:12 hour day-night cycle with
123 standard chow or a high fat diet and water available *ad libitum*. The high fat diet was
124 obtained from Research Diets (New Brunswick, USA; #D12451), and contained 45%
125 of calories derived from fat (lard and soy bean oil), 35% from carbohydrates and
126 20% from protein. All experimental procedures were carried out under a United
127 Kingdom Home Office licence in compliance with the Animals (Scientific Procedures)
128 Act 1986.

129

130 For intramuscular administration of AAV8 ProMyo, mice were anaesthetized with
131 isoflurane (4% induction, 2% maintenance) and the anterior aspect of the lower limbs
132 was shaved. AAV ProMyo (5×10^{10} virus particles in 50 μ l PBS-MK) was injected into
133 the cranial compartment of the left lower leg with a 29-gauge insulin syringe, while
134 the right leg was injected with 50 μ l PBS-MK as a paired control.

135

136 *Intraperitoneal insulin and glucose tolerance tests*

137 For intraperitoneal insulin tolerance tests (IPITT), mice were fasted for 3–4 hours
138 before administering insulin. Insulin was prepared at 100 iu/ml in normal saline and
139 used to resuspend nitrogen-dried 2-[1,2-³H(N)]-deoxy-D-glucose (0.37 MBq). Basal
140 blood glucose was measured in tail blood with an Accu-Check Advantage meter
141 (Roche Diagnostics, Burgess Hill, West Sussex, UK). A further 10 μ l of blood was
142 collected in microfuge tube containing 1 iu heparin in saline, mixed and placed on
143 ice. Immediately afterwards, insulin and deoxyglucose tracer was administered
144 intraperitoneally at a dose of 0.75 iu/kg. At 15, 30, 60 and 90 minutes after insulin

145 administration, blood glucose was measured as described above. After taking the
146 final blood sample, the mice were euthanized by cervical dislocation and tibialis
147 cranialis (TC), extensor digitorum longus, soleus muscles and epididymal fat pads
148 were collected, weighed and frozen in liquid nitrogen-cooled isopentane.
149 Intraperitoneal glucose tolerance test (IPGTT) was conducted and glucose clearance
150 into TC muscle was determined as described previously (7).

151

152 *In vivo study design*

153 For the time course analysis of the effect of myostatin inhibition, 3-month-old
154 C57BL/6 males were given an intramuscular injection of AAV ProMyo as described
155 above, and were kept for 1, 2, 4 or 10 weeks before being subjected to an IPITT or
156 an IPGTT, after which they were euthanized and their muscles harvested and snap-
157 frozen (n=10 per time point).

158

159 To determine the effect of myostatin inhibition in HFD-fed mice, C57BL/6 males were
160 switched from regular chow to high fat diet (HFD) at 8 weeks of age, while
161 contemporaneous controls were kept on a normal chow diet. Four weeks later, mice
162 were given intramuscular injections of AAV8 ProMyo into one TC muscle, while the
163 contra-lateral limb was injected with saline. Mice were kept for 2 or 10 weeks post-
164 injection, after which mice (n=10 per group) underwent an IPITT, before euthanasia
165 and muscle collection. The remaining mice were euthanized and their muscles were
166 harvested without undergoing an IPITT (n=8 per group).

167

168 *RNA analysis*

169 TC muscles from mice harvested without IPITT 10 weeks after AAV or saline
170 injection were homogenized in Tri-reagent (Sigma-Aldrich) and RNA was extracted
171 according to the manufacturer's instructions. One microgram of RNA was reverse
172 transcribed using a qScript cDNA synthesis kit (Quanta Biosciences). Transcript
173 levels were quantified in duplicate by real-time PCR using PerfeCta SYBR Green
174 FastMix (Quanta Biosciences). A serial dilution of a mixture of cDNA from all
175 samples was prepared and used to construct a standard curve for relative
176 quantification of target transcripts, expression of which were normalized to that of

177 CNSK2A2, a reference gene which was defined after geNorm analysis
178 (<https://genorm.cmgg.be/>). The primer sequences used are listed in Table 1.

179 For microarray analysis, independent pooled RNA samples were prepared by mixing
180 500 ng total RNA from four independent samples to create two RNA pools from both
181 ProMyo-overexpressing and saline-injected muscle samples. Total RNA integrity for
182 each replicate was determined using by Bioanalyzer on a RNA Pico Chip (Agilent
183 Technologies, as per manufacturer's instructions). For microarray analysis, labelled
184 extracts were prepared from total RNA samples using the Nugen Ovation V2 system
185 followed by Nugen Encore Biotin Labelling Kit (Nugen Technologies Inc, as per
186 manufacturer's instructions). Subsequently, samples were hybridized to Affymetrix
187 Mouse 430_2 GeneChips as per manufacturer's guidelines (Nugen Technologies Inc
188 and ThermoFisher Scientific)

189

190 Raw intensity data were processed with the RMA algorithm with quantile
191 normalisation using the Affymetrix Expression Console software. The resulting
192 expression data were subjected to gene set enrichment analysis (GSEA) (26, 35)
193 using GSEA software v_3.0. Input data were the bi-weight average signal (log2).
194 Genes without a gene symbol were excluded and data were collapsed into single
195 gene symbols before the analysis, using the median expression value for each gene
196 symbol (20630 genes remained). All genes were ranked on the basis of differential
197 expression between ProMyo and saline-injected muscles, defined as the real values
198 of Diff_of_Classes. Three separate GSEA runs were performed; one with the gene
199 sets KEGG_FATTY_ACID_METABOLISM,
200 KEGG_OXIDATIVE_PHOSPHORYLATION and
201 KEGG_INSULIN_SIGNALING_PATHWAY, one with ten gene sets related to
202 inflammation (see table 2) and one with collection c5.all.v6.1, a collection of 5,917
203 gene sets based on gene ontologies. All gene sets were obtained from Molecular
204 Signature Database v6.1. GSEAs were run with permutation of gene sets (n=1,000),
205 using the weighted enrichment statistic, and default parameters for gene set size
206 (minimum size 15, maximum size 500). A false discovery rate of <0.05 was accepted
207 as being significant. Subsequent Leading Edge Analysis was performed to determine
208 overlap between significantly enriched gene sets in genes mostly responsible for the
209 enrichment score.

210 The microarray data have been uploaded to Gene Expression Omnibus (accession
211 GSE130622). Output enrichment plots for gene sets listed in table 2 are available
212 upon request.

213

214

215 *Western blotting*

216 Liquid nitrogen-powdered muscle was lysed in cold RIPA buffer (50 mM TRIS-HCl
217 (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% v/v Nonidet P40 substitute, 0.25% sodium
218 deoxycholate, plus freshly added protease inhibitor cocktail (Sigma), phosphatase
219 inhibitor cocktail (Sigma) and 20 mM beta-glycerophosphate). Protein concentration
220 was determined using the Bio-rad DC protein assay. Samples were adjusted to the
221 same concentration with RIPA buffer and SDS-PAGE loading buffer containing beta-
222 mercaptoethanol (2% final concentration) was added. Samples containing 40 µg
223 protein were separated on pre-cast 4–12% Bis-Tris gels (Life Technologies) and
224 blotted onto PVDF membranes. Membranes were blocked in 5% non-fat dried milk in
225 Tris-buffered saline with 0.5% tween-20 (TBS-T) and incubated overnight at 4°C with
226 primary antibody diluted in TBS-T, 5% BSA. Membranes were subsequently
227 incubated with horseradish-conjugated species-specific secondary antibodies
228 (Millipore) in TBS-T containing 5% non-fat milk powder. Membranes were washed 3
229 × 5 min in TBS-T after each step. Antibodies were then detected with ECL Plus (Bio-
230 rad) and an ImageQuant LAS4000 mini (GE Healthcare). The following primary
231 antibodies were used: anti-Akt #9272, anti-pSer473-Akt #9271, anti-AMPKα #2532,
232 anti-pThr172-AMPKα, anti-ACC #3662, anti-pSer79-ACC #3661, anti-α-actinin
233 #6487, anti-GAPDH #5174 and anti-Cblb #9498 from Cell Signaling Technology;
234 anti-PGC-1α #54481 and OXPHOS antibody cocktail #110413 from Abcam; and
235 anti-GLUT4 #sc-53566 from Santa Cruz Biotechnology.

236

237 *Statistical analysis*

238 Statistical analyses were carried out using SigmaPlot v12.3 or GraphPad Prism v6.
239 Data from time course experiments, and experiments involving different diets and
240 myostatin inhibition were analysed with Two-Way Repeated Measures ANOVA, with
241 time or myostatin inhibition as the paired factor. The Sidak-Holm method was used
242 for *post-hoc* testing. Two-group comparisons were performed using *t*-tests, after
243 verifying equality of variance and normality. The threshold for statistical significance

244 was set at $p < 0.05$. Data are displayed as mean \pm standard error of the mean
245 (S.E.M), or as mean plus individual data points.
246

247 **Results**

248

249 *Myostatin inhibition increases muscle size, but not insulin-stimulated glucose*
250 *disposal in chow-fed mice*

251 Overexpression of ProMyo in TC muscles of chow-fed mice for 1, 2, 4 or 10 weeks
252 resulted in significant muscle hypertrophy in wild-type mice (Figure 1A) but not in
253 myostatin knockout mice (Figure 1B). As expected, muscle mass was substantially
254 higher in the myostatin knockout mice compared to the wild type mice (Figure 1A
255 &1B). However, inhibition of the activin type 2B receptor has been shown to lead to
256 additional hypertrophy in myostatin knockout mice (21) and the absence of such an
257 effect of AAV ProMyo on muscle mass in the knockout mice implies that the
258 construct is myostatin-specific. Insulin-stimulated glucose disposal, as measured by
259 IPITT, was not different between saline- and AAV ProMyo-injected muscles at any of
260 the time points (Figure 1C). Total glucose uptake into muscle was higher after 4 and
261 10 weeks ProMyo overexpression by virtue of the significant increases in muscle
262 mass (Figure 1D). As we have previously demonstrated higher muscle glucose
263 uptake per unit muscle mass in ProMyo overexpressing rat muscle during a glucose
264 tolerance test (IPGTT) (8), we measured glucose uptake during an IPGTT in mice
265 after two weeks of ProMyo overexpression. However, we found no difference in
266 glucose uptake between saline and AAV-ProMyo-injected muscles (Figure 1E).
267 These data suggest that although myostatin inhibition-induced muscle hypertrophy
268 increases total glucose disposal into muscle under conditions of hyperinsulinaemia,
269 muscle glucose uptake per unit muscle mass is not increased by myostatin inhibition
270 in chow-fed mice.

271

272 *Myostatin inhibition increases insulin-stimulated glucose disposal in muscle of HFD-*
273 *fed mice*

274 We next investigated whether local myostatin inhibition would improve muscle
275 glucose uptake in HFD-fed mice. Mice were given an intramuscular injection of AAV8
276 ProMyo or saline after 4 weeks of HFD-feeding. One group of mice was analysed
277 after 2 weeks of myostatin inhibition (6-week total duration of HFD). At this time
278 point, total body mass was not significantly greater in the HFD-fed mice (Figure 2A),
279 but epididymal fat pad mass was significantly increased (Figure 2B), indicating
280 visceral fat accumulation. We did not detect a significant difference in fasting or

281 IPITT blood glucose levels between diet groups (Figure 2C), but HFD-fed mice
282 displayed a delayed reduction in blood glucose levels in response to insulin (Figure
283 2D), which is indicative of whole body insulin resistance. AAV8 ProMyo-injected TC
284 muscles from both chow- and HFD-fed mice showed significant hypertrophy (+9.7%
285 and +8.8% vs. saline, respectively; Figure 2E). However, neither chow-fed nor HFD-
286 fed mice showed an increase in glucose uptake into ProMyo-overexpressing TC
287 muscles (Figure 2F). These data suggest that 2 weeks of myostatin inhibition in
288 muscle is not sufficient to significantly increase insulin-stimulated glucose disposal in
289 muscles of HFD-fed mice.

290

291 After 10 weeks of myostatin inhibition (14 weeks of HFD-feeding), we observed a
292 more severe metabolic phenotype. A significant increase in total body mass was
293 detected (Figure 3A) and fat pad mass had increased to a greater extent in HFD-fed
294 mice (Figure 3B). In addition, HFD-fed mice showed significantly higher fasting
295 glucose levels than chow-fed controls and had a clearly delayed response to insulin
296 during the IPITT (Figure 3C and 3D). Muscle mass was substantially greater in
297 ProMyo overexpressing muscles than in saline-treated controls in both chow
298 (+22.8%) and HFD (+23.2%) groups (Figure 3E). At this time point the ProMyo
299 overexpressing muscles of the insulin-resistant HFD-fed mice showed significantly
300 higher glucose uptake per unit mass during the IPITT than saline-injected controls
301 (by ~78%) (Figure 3F). On this basis, total muscle glucose disposal can be estimated
302 to be increased by 115–121% of that of control muscles. Taken together, these data
303 suggest that 10 weeks of myostatin inhibition in mouse muscle specifically increases
304 insulin-stimulated muscle glucose disposal in HFD-fed, but not chow-fed mice,
305 despite the presence of significant muscle hypertrophy in both groups of mice.

306

307 *Effect of myostatin inhibition on potential regulators of insulin sensitivity*

308 We next determined whether myostatin inhibition selectively induced changes in
309 pathways controlling GLUT translocation and/or expression in HFD-fed mice.
310 Muscles from mice not subjected to IPITT were used for the analysis. Measurement
311 of pro-myostatin mRNA, which includes the sequence for the inhibitory ProMyo
312 peptide, showed robust overexpression of the ProMyo construct in muscles of chow-
313 and HFD-fed mice after 10 weeks of myostatin inhibition (87 ± 16 -fold & 57 ± 9 -fold,
314 respectively, Figure 4A). Slc2a1/GLUT1 mRNA levels were lower in ProMyo-

315 overexpressing muscles (Figure 4B). Slc2a4/GLUT4 mRNA levels were significantly
316 lower in ProMyo-overexpressing muscles of HFD-fed mice compared to ProMyo-
317 overexpressing muscles of chow-fed mice (Figure 4C). GLUT1 and GLUT4 protein
318 levels did not differ among any of the groups (Figure 4D-4F). These data suggest
319 that, unexpectedly, GLUT expression was unaltered or reduced at the transcript level
320 by myostatin inhibition. Basal Ser473 phosphorylation of Akt and Thr172
321 phosphorylation of AMPK α were similar between diet groups or in ProMyo-
322 overexpressing muscles (Figure 5A & 5C). However, Akt protein levels were
323 increased in ProMyo-overexpressing muscles of chow-fed mice, but not of HFD-fed
324 mice (Figure 5B). AMPK α protein levels did not differ among the groups (Figure 5D).
325 Likewise, basal Ser79 phosphorylation of acetyl-CoA carboxylase (ACC), which is
326 indicative of AMPK activity, was similar among the groups (Figure 5E). ACC
327 expression was increased in ProMyo-overexpressing muscles, although there was
328 no significant difference within individual diet groups (Figure 5F). PGC-1 α protein
329 levels were significantly higher in HFD-fed mice but there was no effect of ProMyo
330 overexpression (Figure 5G). These data suggest that the basal activities of pathways
331 controlling GLUT expression and translocation were not specifically upregulated in
332 HFD-fed mice by ProMyo overexpression.

333

334 We explored the possibility that myostatin inhibition affects the expression of two
335 more recently identified regulators of both myogenesis and muscle insulin sensitivity.
336 Mitsugumin 53 (MG53) and Casitas B-cell lymphoma-b (Cbl-b) are E3 ligases that
337 target the insulin receptor substrate 1 (IRS-1) for proteasomal degradation and their
338 expression is thought to inhibit myoblast differentiation (42) and induce muscle
339 atrophy during muscle unloading (29). Furthermore, MG53 is thought to induce
340 muscle insulin resistance in response to high fat diet-feeding (31). Unexpectedly,
341 transcript levels of MG53 were unaffected by HFD-feeding and were higher in
342 ProMyo overexpressing muscles (Figure 6A). Cbl-b mRNA expression was not
343 affected by ProMyo overexpression (Figure 6B), but its protein level was lower in
344 HFD-fed mice (Figure 6C & 6D). Together, these data suggest unexpected changes
345 in the expression of MG53 and Cbl-b in response to both high fat-diet feeding and
346 myostatin inhibition that are unlikely to explain the observed effects on insulin-
347 stimulated muscle glucose disposal.

348

349 *Mitochondrial ribosomal protein transcripts are enriched after myostatin inhibition*

350 To obtain insight into possible alternative mechanisms for the effect of myostatin
351 inhibition on skeletal muscle glucose uptake, we performed GSEA on transcriptomic
352 data obtained from muscles from HFD-fed mice subjected to 10 weeks of myostatin
353 inhibition and their paired saline-injected controls. We detected a 600-fold increase
354 in IgG2A expression, which was due to the presence of IgG2A sequence in the AAV
355 ProMyo-Fc construct. Therefore, this transcript was removed from the GSEA. To
356 explain the observed increase in glucose uptake, we hypothesized that genes
357 related to insulin signaling, fatty acid metabolism and oxidative phosphorylation may
358 be enriched in the ProMyo overexpressing muscles. We detected significant
359 enrichment of the KEGG_FATTY_ACID_METABOLISM gene set, but not of the
360 KEGG_INSULIN_SIGNALING_PATHWAY or
361 KEGG_OXIDATIVE_PHOSPHORYLATION gene sets (Table 2 & Table 3). It has
362 been suggested that the effect of myostatin inhibition on glucose uptake in HFD-fed
363 mice can be explained by reduced muscle inflammation (11). Therefore, we
364 determined the enrichment of gene sets related to inflammation in our muscle
365 samples. However, none of these gene sets showed significant enrichment in either
366 saline-treated or ProMyo-overexpressing muscles (Table 2).

367 To explore possible novel pathways involved in mediating the effect of myostatin
368 inhibition on muscle glucose uptake, we repeated the GSEA with a large collection of
369 gene sets based on gene ontologies. We found four gene sets that were enriched in
370 ProMyo overexpressing muscles at a FDR of <5% (Table 2). These gene sets show
371 a significant amount of overlap in that all contain mitochondrial ribosomal proteins.
372 Subsequent analysis of the overlap in the genes responsible for the significant
373 enrichment scores indeed mostly identified genes encoding mitochondrial ribosomal
374 proteins and other genes involved in mitochondrial translation (Table 4), suggesting
375 that myostatin inhibition increases the expression of these genes in the muscle of
376 HFD-fed mice. Because the transcriptomic data suggest that the translation of
377 mitochondrial DNA-encoded genes might be specifically enhanced, we analysed the
378 protein levels of the nuclear DNA-encoded ubiquinol-cytochrome c reductase core
379 protein 2 (UQCRC2) and succinate dehydrogenase complex iron sulfur subunit B
380 (SDHB), and the mitochondrial DNA-encoded cytochrome c oxidase I (MTCO1).
381 However, we found no differences in either the nuclear or mitochondrially encoded
382 oxidative phosphorylation complex subunits among the groups (Figure 7).

384 **Discussion**

385

386 Myostatin inhibition is thought to improve muscle glucose disposal but the
387 mechanisms whereby this is achieved and their quantitative importance are unclear.
388 We show here that myostatin inhibition increases insulin-stimulated glucose disposal
389 in skeletal muscle of HFD-fed mice, but not chow-fed mice, at a time point at which
390 substantial muscle hypertrophy had occurred. Unexpectedly, this was not associated
391 with higher expression of GLUT1/GLUT4 or PGC-1 α , or basal phosphorylation of Akt
392 or AMPK, which have been proposed to be regulators of the positive effects of
393 myostatin inhibition on insulin sensitivity. However, we observed significant
394 enrichment of genes involved in fatty acid metabolism and mitochondrial translation
395 following myostatin inhibition.

396

397 The lack of an effect of local myostatin inhibition on muscle insulin-stimulated
398 glucose disposal in chow-fed mice was unexpected. Beneficial effects on glucose
399 uptake and insulin sensitivity have been observed in multiple models of genetically
400 induced muscle hypertrophy (6, 9, 10), implying the possibility of a common
401 mechanism that leads to greater muscle glucose uptake. Furthermore, our previous
402 experiments in rats demonstrated greater glucose disposal during an IPGTT after 17
403 days of overexpression of the same ProMyo-Fc construct, which was associated with
404 increased expression of GLUT1 and GLUT4 (8). However, muscle glucose uptake
405 per unit muscle mass during an IPITT was unchanged throughout the full time
406 course of the development of muscle hypertrophy (Figure 1C). This demonstrates
407 that increases in muscle glucose disposal rate are not the inevitable result of
408 myostatin inhibition-induced muscle hypertrophy.

409 We observed significantly higher insulin-stimulated glucose disposal in ProMyo
410 overexpressing muscles after 10 weeks, but not 2 weeks, of local myostatin inhibition
411 in mice on an HFD (Figure 2F & 3F). The mice from the 10 week time point showed
412 a more severe metabolic phenotype as a result of a longer duration of HFD feeding,
413 with further increases in visceral fat accumulation, high fasting blood glucose and
414 clear insulin resistance compared to the chow-fed mice and the two week time point
415 (Figure 2 & 3). Despite this, glucose uptake was not lower in the muscles of the
416 HFD-fed mice compared to those of the chow-fed mice (Figure 2F & 3F). Other

417 groups have shown that feeding C57Bl/6 mice a similar HFD (i.e. 45% of calories
418 from fat) for a similar or shorter amount of time leads to insulin resistance in TC
419 muscle (37). The IPITT method we used to measure muscle glucose uptake involves
420 a significant amount of variation between animals which can make it difficult to detect
421 between-animal effects such as those of the diet.

422 Nevertheless, our data suggest that the beneficial effect of myostatin inhibition on
423 muscle glucose disposal requires a muscle hypertrophy-independent factor, which
424 may be associated with the development of a severe metabolic phenotype. We have
425 previously shown that local myostatin inhibition increases muscle glucose disposal to
426 a much greater extent than muscle size in chow-fed rats (8), suggesting this factor
427 may not be exclusively related to metabolic disease. Myostatin antibody treatment
428 resulted in muscle hypertrophy in chow- and HFD-fed young mice and chow-fed old
429 mice, but only increased whole body insulin sensitivity in the old mice (5). The
430 absence of improvements in insulin sensitivity in young mice in the experiments by
431 Camporez *et al.* might be explained by a requirement for a minimum level of
432 hypertrophy in combination with the presence of a significant metabolic phenotype,
433 as the mice from the 2 week myostatin inhibition time point in our study displayed a
434 similar degree of hypertrophy, had been fed a HFD for a similar duration, and
435 displayed no increase in glucose disposal with myostatin inhibition (Figure 2).
436 However, it is unclear why an improvement was observed in old mice despite the
437 presence of a similar degree of muscle hypertrophy in young mice.

438

439 Together these data suggest that an increase in insulin-stimulated glucose disposal
440 into muscle by postnatal myostatin inhibition in young mice requires a significant
441 metabolic phenotype combined with a long duration of inhibition, although rats
442 appear to be more sensitive to the effects of myostatin inhibition (8). The reason for
443 the difference in the effect of ProMyo overexpression in chow-fed mice and rats is
444 unclear. Our chow-fed mouse data set (Figure 1) includes a time point that is similar
445 to the time point used in our previous rat study, at which the degree of hypertrophy
446 was similar and at which we assessed glucose uptake during both an IPITT and an
447 IPGTT, which was the test performed in the rats. It is possible that the larger content
448 of the more insulin-sensitive type 1/2A muscle fibres in the rat TC muscle compared
449 to that of the mouse TC muscle (~5/25% vs ~0/5%, respectively) played a role in the
450 different response to myostatin inhibition, and that local myostatin inhibition in a

451 mouse muscle with a fibre type composition more similar to that of the rat TC would
452 have shown a similar increase in glucose uptake.

453

454 The data have potential implications for the clinical translation of myostatin inhibition
455 for the treatment of insulin resistance, as it may be that more obese individuals are
456 more likely to benefit from myostatin inhibitors. Obesity does not appear to affect the
457 degree of muscle hypertrophy resulting from myostatin inhibition (Figure 3E), and
458 indeed the magnitude of the increase in insulin-stimulated glucose disposal exceeds
459 that of the increase in muscle mass (Figure 3F). Thus, myostatin inhibition leads to
460 substantial improvements in total insulin-stimulated muscle glucose disposal, which
461 implies a treatment strategy utilising this approach would have positive effects in
462 obese insulin resistant patients.

463

464 Among the potential mechanisms which have been suggested to explain the positive
465 effects of myostatin inhibition on muscle or whole body glucose uptake are higher
466 expression of glucose transporters GLUT1 and GLUT4 (8), activation of Akt (27, 36),
467 and stimulation of brown fat formation by increasing the secretion of the hormone
468 irisin from skeletal muscle through an AMPK-PGC-1 α -dependent mechanism (30).
469 We did not demonstrate higher expression of any of these molecules in the ProMyo-
470 overexpressing TC muscles from HFD-fed mice (Figure 4 & 5), and we found no
471 evidence of increased basal AMPK activity (Figure 5). However, we cannot exclude
472 the possibility that GLUT translocation from the cytosol to the plasma membrane was
473 increased separately of any effect on GLUT expression. There was an increase in
474 PGC-1 α protein levels in HFD mice, which is consistent with the existing literature
475 (15), but there was no effect of ProMyo overexpression (Figure 5G). This brings into
476 question whether myostatin inhibition in muscle is sufficient to increase PGC-1 α
477 expression. In support of this, lower PGC-1 α protein levels have been detected in
478 myostatin knockout mice (22) and lower PGC-1 α transcript levels were found in mice
479 treated with AAV ProMyo (28). In addition, we detected no increase in expression of
480 the insulin receptor-targeting E3 ligases MG53 and Cbl-b in HFD-fed mice, while
481 myostatin inhibition unexpectedly increased the expression of MG53, which has
482 previously been suggested to be responsible for muscle insulin resistance in
483 response to HFD feeding (31) (Figure 6). Together, these data suggest that whole
484 body insulin resistance was not associated with higher expression of these IRS-1-

485 targeting E3 ligases in muscle and that the improvement of muscle glucose disposal
486 resulting from myostatin inhibition was not associated with a decrease in their
487 expression.

488

489 The results of our experiments are contrasting with existing hypotheses regarding
490 the mechanism through which myostatin might increase skeletal muscle glucose
491 disposal, and therefore suggest that other mechanisms exist. In accordance with
492 previous observations (11) we found enrichment of gene sets associated with fatty
493 acid (FA) oxidation including acyl-CoA synthases and acyl-CoA dehydrogenases
494 (Table 2 & 3), which catalyse the initial steps in the beta-oxidation of FAs. We
495 speculate that higher expression of these sets of genes could lead to improved
496 metabolism of fatty acids, and thus greater insulin sensitivity. For example, an
497 increase in carnitine palmitoyl transferase 1 (CPT1) activity (one of the genes which
498 showed increased expression in ProMyo-overexpressing muscles) in skeletal muscle
499 improves insulin sensitivity in HFD-fed mice (38). In addition, we observed
500 enrichment of genes involved in mitochondrial translation, including those expressing
501 mitochondrial ribosomal proteins, following myostatin inhibition in muscles of HFD-
502 fed mice (Table 2 & 4). Reductions in OXPHOS gene expression in human muscle
503 have been associated with type 2 diabetes (26), but we found no significant
504 enrichment of a gene set related to oxidative phosphorylation with myostatin
505 inhibition (Table 2). In accordance with this, we found no difference in the protein
506 levels of two nuclear-encoded subunits of oxidative phosphorylation complexes
507 (Figure 7A & 7B). The protein levels of a mitochondrially encoded protein also did
508 not differ between saline-treated and ProMyo-overexpressing muscles (Figure 7C),
509 which argues against a general increase in translation of mitochondrial proteins.
510 Although any causal relationship between myostatin inhibition, changes in
511 mitochondrial gene expression and improvements in muscle glucose uptake requires
512 further investigation, it is possible that an enhancement in mitochondrial translation
513 could explain why myostatin inhibition potentiates the effects of exercise on whole-
514 body insulin sensitivity and running distance in aged mice (18).

515

516 We conclude that local post-natal myostatin inhibition improves insulin-stimulated
517 muscle glucose disposal in obese HFD-fed mice. This is not secondary to muscle
518 hypertrophy and is not observed in young insulin-sensitive mice. This effect does not

519 appear to rely on the upregulation of glucose transporter and PGC-1 α expression but
520 was associated with expression changes in previously unidentified pathways related
521 to mitochondrial function. These findings point towards further areas for future
522 investigations into the mechanism of the effects of myostatin inhibition, and suggest
523 that therapeutic myostatin inhibition may be effective in improving muscle glucose
524 uptake primarily in obese insulin-resistant individuals.

525

526

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530

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534

535 **Disclosures**

536 The authors have no conflict of interest to declare.

537

538

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716 **Figure 1: AAV8 ProMyo increases muscle mass but not insulin-stimulated**
717 **glucose disposal in chow-fed mice**

718 A: Mass of saline- or AAV8 ProMyo-injected tibialis cranialis (TC) muscle at 1, 2, 4 &
719 10 weeks post-injection (n=10 per group). B: TC muscle masses of 2-month-old
720 myostatin null mice 4 weeks after a single intramuscular injection of AAV8 ProMyo
721 (n=3 per group). C: Glucose uptake per unit muscle mass during an intraperitoneal
722 (i/p) insulin tolerance test (IPITT) at the indicated times after saline or AAV8 ProMyo
723 injection. D: Total muscle glucose uptake at the indicated times after saline or AAV8
724 ProMyo injection. E: Glucose uptake per unit muscle mass during an i/p glucose
725 tolerance test (IPGTT) 2 weeks after saline or AAV8 ProMyo injection. Data are
726 shown as mean + S.E.M. ** $p < 0.01$ vs. Saline at the same time point. *** $p < 0.001$ vs.
727 Saline at the same time point. Two-way Repeated Measures ANOVA with Sidak-
728 Holm posthoc test (A, C, D); Paired t-test (B, E).

729

730 **Figure 2: Muscle insulin-stimulated glucose disposal is not affected by 2**
731 **weeks of myostatin inhibition in mice on a high fat diet**

732 Body mass (A) and epididymal fat pad mass (B) of chow-fed and high fat diet (HFD)-
733 fed mice after 2 weeks of intramuscular ProMyo overexpression (n=10 per group). C:
734 Blood glucose concentration during IPITT. D: Normalized blood glucose
735 concentration during IPITT. Data in A-D are shown as mean +/- S.E.M. E: Muscle
736 mass in chow-fed and HFD-fed mice 2 weeks after saline or AAV8 ProMyo injection.
737 F: Glucose uptake into TC muscle during the IPITT. Bars in E & F show mean values
738 and data points connected by a line represent contralateral muscle pairs from the
739 same animal. ** Indicates $p < 0.01$ vs. control. *** Indicates $p < 0.001$ vs. control.
740 Unpaired t-test (A, B); Two-way Repeated Measures ANOVA with Sidak-Holm
741 posthoc test (C, D, E, F)

742 **Figure 3: Higher skeletal muscle insulin-stimulated glucose disposal after 10**
743 **weeks of myostatin inhibition in mice on a high fat diet**

744 Body mass (A) and epididymal fat pad mass (B) of chow-fed and high fat diet (HFD)-
745 fed mice after 10 weeks of intramuscular ProMyo overexpression (n=10 per group).
746 C: Blood glucose concentration during IPITT. D: Normalized blood glucose
747 concentration during IPITT. Data in A-D are shown as mean +/- S.E.M. E: Muscle
748 mass in chow-fed and HFD-fed mice 10 weeks after saline or AAV8 ProMyo
749 injection. F: Glucose uptake into TC muscle during the IPITT. Bars in E & F show
750 mean values and data points connected by a line represent contralateral muscle
751 pairs from the same animal. * Indicates $p < 0.05$ vs. control. ** Indicates $p < 0.01$ vs.
752 control. *** Indicates $p < 0.001$ vs. control. Unpaired t-test (A, B); Two-way Repeated
753 Measures ANOVA with Sidak-Holm posthoc test (C, D, E, F).

754

755

756 **Figure 4: Effect of 10 weeks of myostatin inhibition in HFD-fed mice on**
757 **glucose transporter expression**

758 A-C: Real-time PCR analysis of ProMyo (A), Slc2a1/GLUT1 (B) and Slc2a4/GLUT4
759 (C) transcript levels in chow- and HFD-fed mice (n=8 per group). A significant
760 ANOVA main effect of ProMyo on GLUT1 transcript levels (B) is indicated. D & E:
761 Western blot quantification of protein levels of GLUT1 (D, n=8 per group) and GLUT4
762 (E, n=7-8 per group). F: Example western blot images. Samples from intra-animal
763 muscle pairs are indicated by lines underneath the blot images. ProMyo (+) or saline
764 (-) treatment is indicated. Data are shown as mean + S.E.M. * $p < 0.05$. ** $p < 0.01$. ***
765 $p < 0.001$. Two-way Repeated Measures ANOVA with Sidak-Holm posthoc test (A-E).

766

767 **Figure 5: Effect of 10 weeks of myostatin inhibition in HFD-fed mice on**
768 **signalling pathways controlling glucose transporters**

769 A-G: Western blot quantification of phospho- and total levels of Akt (A & B), AMPK
770 (C & D), ACC (E & F), and PGC-1 α protein (G) (n=6-8 per group). Significant
771 ANOVA main effects of ProMyo on ACC levels (F) and of diet on PGC-1 α levels (G)
772 are indicated. H: Example western blot images. Samples from intra-animal muscle
773 pairs are indicated by lines underneath the blot images. ProMyo (+) or saline (-)
774 treatment is indicated. The vertical spaces between blot images indicate lanes that
775 were on the same blot but from which other lanes have been cropped out. Data are
776 shown as mean + S.E.M. * $p < 0.05$. ** $p < 0.01$. Two-way Repeated Measures ANOVA
777 with Sidak-Holm posthoc test (A-G).

778

779

780 **Figure 6: Effect of 10 weeks of myostatin inhibition in HFD-fed mice on insulin**
781 **signalling-controlling E3 ligases**

782 A-B: Real-time PCR analysis of MG53 (A) and Cblb (B) transcript levels in chow and
783 HFD-fed mice (n=8 per group). C: Western blot quantification of Cbl-b protein levels
784 (n=8 per group). Significant ANOVA main effects of ProMyo on MG53 transcript
785 levels (A) and of diet on Cbl-b protein levels (C) are indicated. D: Example western
786 blot images. Samples from intra-animal muscle pairs are indicated by lines
787 underneath the blot images. ProMyo (+) or saline (-) treatment is indicated. Data are
788 shown as mean + S.E.M. * $p < 0.05$. Two-way Repeated Measures ANOVA with
789 Sidak-Holm posthoc test (A, B, C).

790

791

792 **Figure 7: Effect of 10 weeks of myostatin inhibition in HFD-fed mice on**
793 **mitochondrial protein levels**

794 A-C: Western blot quantification of nuclear-encoded complex III subunit UQCRC2
795 (A) and complex II subunit SDHB (B), and mitochondrially-encoded complex IV
796 subunit MTCO1 (C) protein levels (n=8 per group). D: Example western blot images.
797 Samples from intra-animal muscle pairs are indicated by lines underneath the blot
798 images. ProMyo (+) or saline (-) treatment is indicated. Data are shown as mean +
799 S.E.M. No significant differences were detected. Two-way Repeated Measures
800 ANOVA with Sidak-Holm posthoc test (A, B, C).

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Figure 1

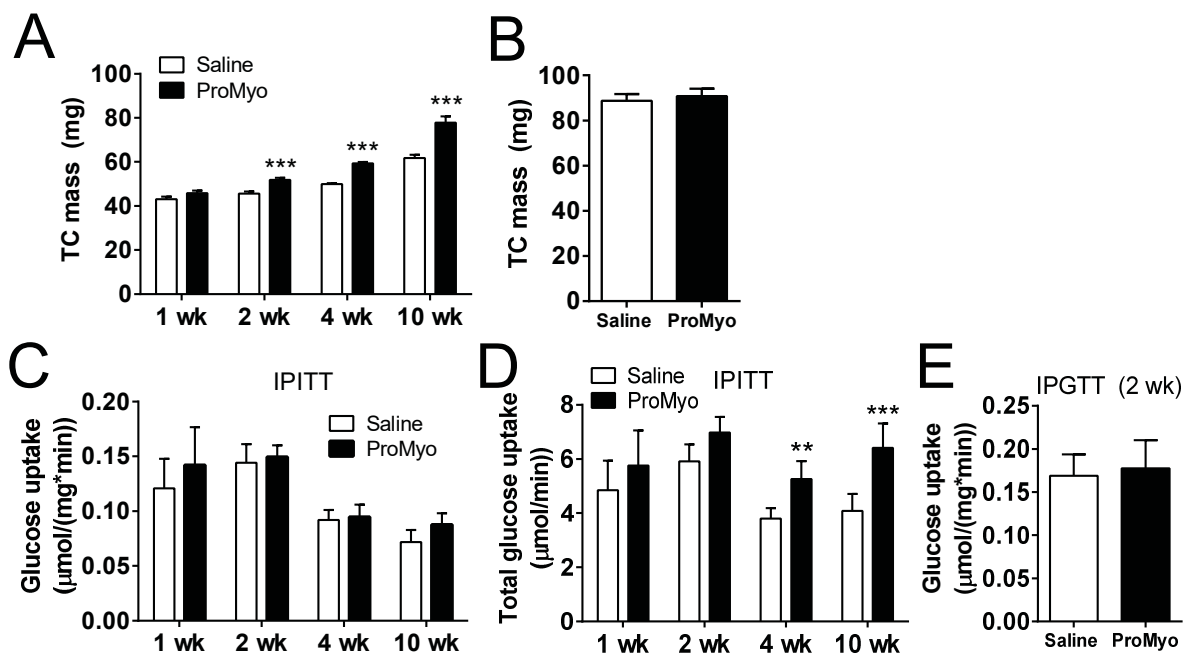


Figure 2

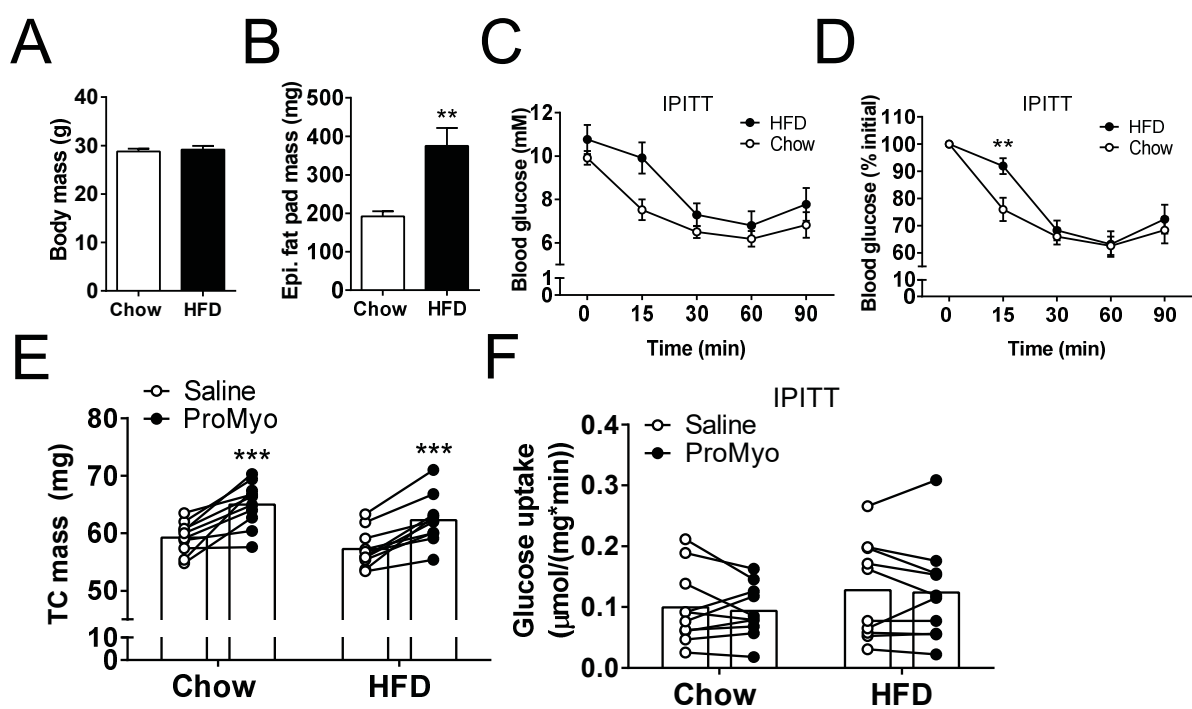


Figure 3

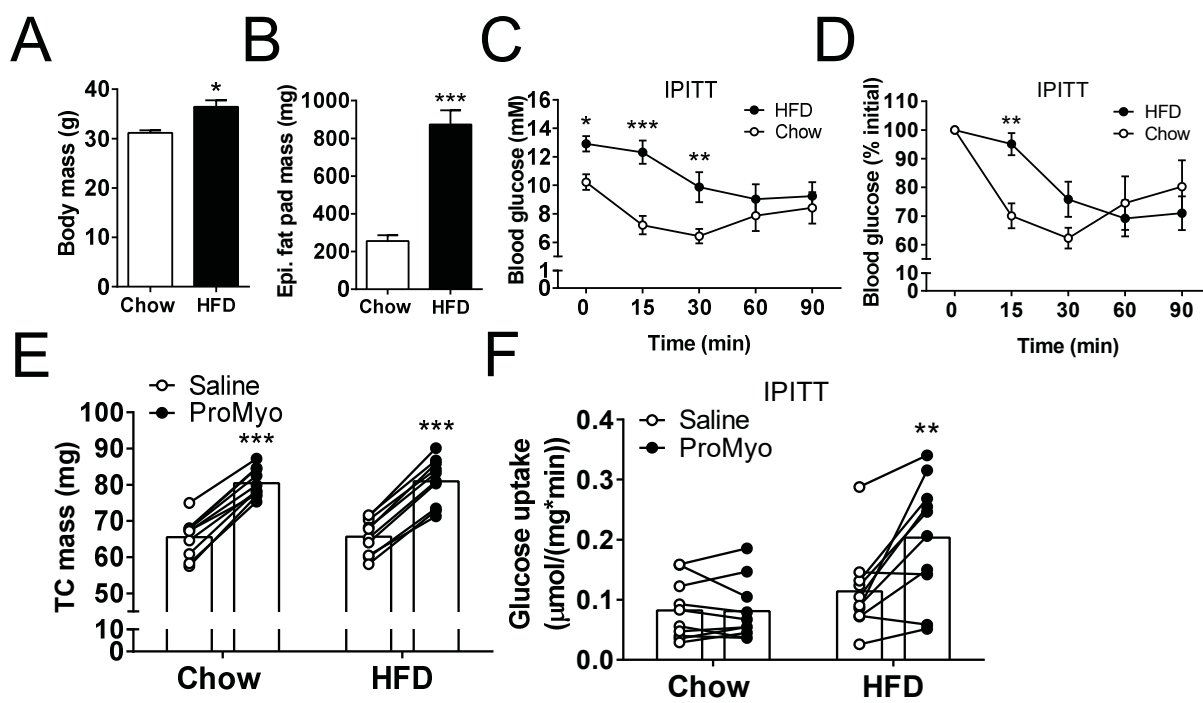


Figure 4

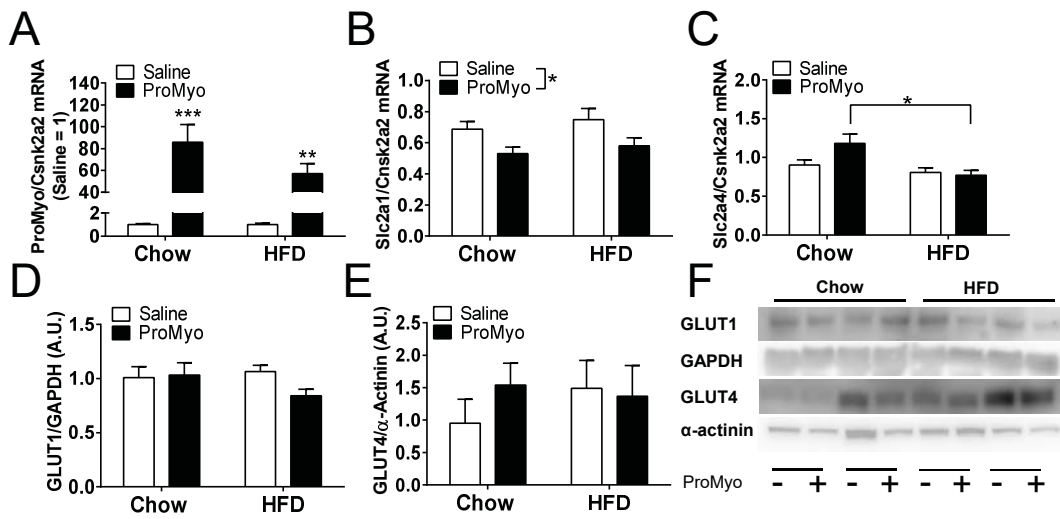


Figure 5

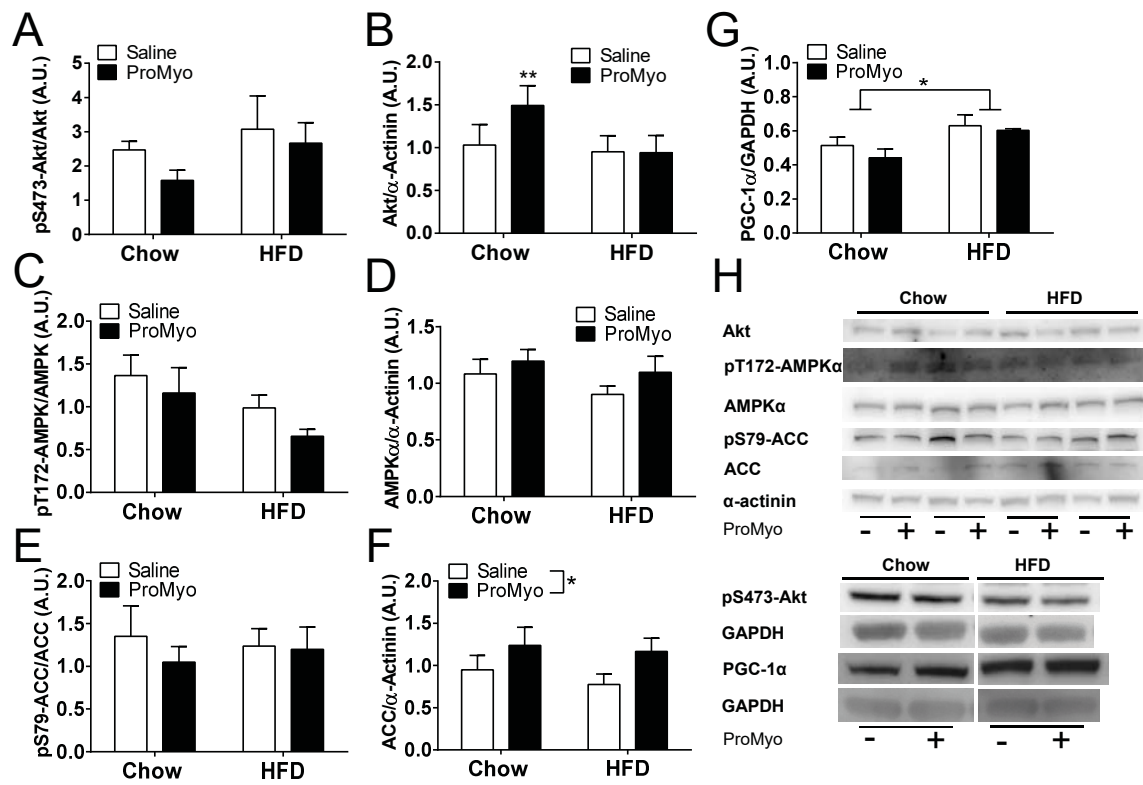


Figure 6

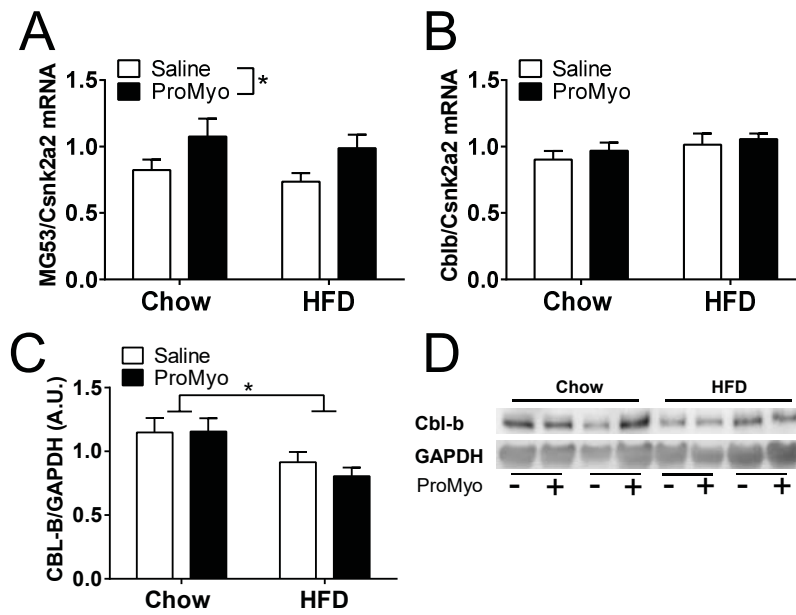
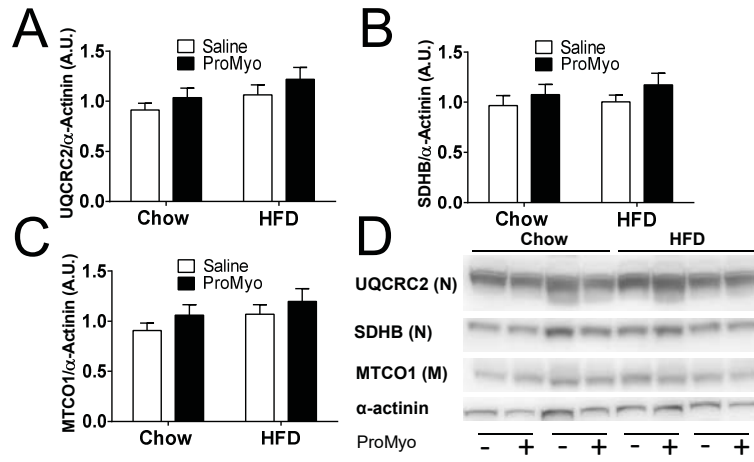


Figure 7



Transcript	Primers	Reference
ProMyo	Fw: 5'-GGCACTGGTATTTGGCAGAG-3' Rv: 5'-GTCCTGGGAAGGTTACAGCA-3'	
Mstn exon 1	Fw: 5'-TGTTTATATTTACCTGTTTCATGCTGAT-3' Rv: 5'-GCCCTCTTTTTCCACATTTTC-3'	
Slc2a4/GLUT4	Fw: 5'-ACACTGGTCCTAGCTGTATTCT-3' Rv: 5'-CCAGCCACGTTGCATTGTA-3'	
Slc2a1/GLUT1	Fw: 5'-CGGGGTCTTAAGTGCATCAG-3' Rv: 5'-CTCCCACAGCCAACATGAGG-3'	
MG53	Fw: 5'-TGTGTGCCTCGCTCGGTTC-3' Rv: 5'-TCTGCTTCACGGTCCAGAGAA-3'	(31)
Cblb	Fw: 5'-GAGCCTCGCAGGACTATGAC-3' Rv: 5'-CTGGCCACTTCCACGTTATT-3'	(29)

Table 1: Sequences of primers used for real-time RT-PCR analysis

Gene set	Size	ES	NES	FDR q value
KEGG_FATTY_ACID_METABOLISM	34	0.57	1.93	0.003
KEGG_OXIDATIVE_PHOSPHORYLATION	105	0.17	0.71	1.000
KEGG_INSULIN_SIGNALING_PATHWAY	123	0.16	0.68	0.970
GO_INFLAMMATORY_RESPONSE	454	-0.28	-1.39	0.145
GO_MACROPHAGE_ACTIVATION	31	-0.44	-1.43	0.172
GO_REGULATION_OF_MACROPHAGE_ACTIVATION	26	-0.35	-1.05	0.484
GO_MACROPHAGE_DIFFERENTIATION	19	-0.54	-1.56	0.128
GO_REGULATION_OF_MACROPHAGE_DIFFERENTIATION	20	-0.19	-0.57	0.986
GO_LEUKOCYTE_ACTIVATION	414	-0.22	-1.13	0.357
GO_T_CELL_MEDIATED_IMMUNITY	28	0.37	1.07	0.628
GO_REGULATION_OF_T_CELL_PROLIFERATION	147	0.23	0.98	0.504
GO_CYTOKINE_PRODUCTION	120	-0.22	-0.90	0.788
GO_CYTOKINE_MEDIATED_SIGNALING_PATHWAY	452	-0.24	-1.18	0.359
GO_TRANSLATIONAL_TERMINATION	86	0.53	2.14	0.024
GO_ORGANELLAR_RIBOSOME	68	0.55	2.12	0.020
GO_TRANSLATIONAL_ELONGATION	102	0.50	2.05	0.047
GO_MITOCHONDRIAL_TRANSLATION	95	0.50	2.05	0.036

Table 2: Gene set enrichment analysis of transcriptomics data from ProMyo overexpressing TA muscles of HFD-fed mice

Size: Number of genes in gene set; ES: Enrichment score; NES: Normalized enrichment score; FDR: False discovery rate (Significance threshold: $q < 0.05$). Positive ES indicates enrichment of gene set in ProMyo-overexpressing muscles, negative ES indicates enrichment in saline-treated muscles.

Gene symbol	Gene title	Rank
ACSL3	acyl-CoA synthetase long-chain family member 3	7
ACSL4	acyl-CoA synthetase long-chain family member 4	251
ALDH9A1	aldehyde dehydrogenase 9 family, member A1	913
ECHS1	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	1,412
ACSL6	acyl-CoA synthetase long-chain family member 6	1,568
CPT1A	carnitine palmitoyltransferase 1A (liver)	1,781
ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	1,973
ACADS	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	1,980
GCDH	glutaryl-Coenzyme A dehydrogenase	2,575
ACSL1	acyl-CoA synthetase long-chain family member 1	2,974
ACAA2	acetyl-Coenzyme A acyltransferase 2	3,095
HADHA	hydroxyacyl-Coenzyme A dehydrogenase, alpha subunit	3,181
ACADVL	acyl-Coenzyme A dehydrogenase, very long chain	3,187
HADH	hydroxyacyl-Coenzyme A dehydrogenase	3,398
ADH4	alcohol dehydrogenase 4 (class II), pi polypeptide	4,035
ACADL	acyl-Coenzyme A dehydrogenase, long chain	4,169

Table 3: Leading edge genes of KEGG_FATTY_ACID_METABOLISM gene set enriched in ProMyo-overexpressing vs. saline-treated muscles

Rank indicates the ranking of the gene in the list of all genes (total: 20,630) ranked on the basis of differential expression between ProMyo-overexpressing and saline-treated muscles.

Gene symbol	Gene title	Rank
MTG1	mitochondrial GTPase 1 homolog (<i>S. cerevisiae</i>)	202
GFM2	G elongation factor, mitochondrial 2	257
MRPS22	mitochondrial ribosomal protein S22	381
MRPS33	mitochondrial ribosomal protein S33	476
MRPL22	mitochondrial ribosomal protein L22	609
MTERFD2	MTERF domain containing 2	738
MRPS35	mitochondrial ribosomal protein S35	752
MRPS12	mitochondrial ribosomal protein S12	874
MRPL21	mitochondrial ribosomal protein L21	964
MRPS5	mitochondrial ribosomal protein S5	1,159
MRPL19	mitochondrial ribosomal protein L19	1,230
ABTB1	ankyrin repeat and BTB (POZ) domain containing 1	1,280
MRPS31	mitochondrial ribosomal protein S31	1,539
MRPL44	mitochondrial ribosomal protein L44	1,556
EIF5A2	eukaryotic translation initiation factor 5A2	1,745
MRPL3	mitochondrial ribosomal protein L3	1,759
TUFM	Tu translation elongation factor, mitochondrial	1,771
HARS	histidyl-tRNA synthetase	1,963
MRP63	mitochondrial ribosomal protein 63	2,008
MRPL18	mitochondrial ribosomal protein L18	2,017
MTRF1L	mitochondrial translational release factor 1-like	2,018
MRPS27	mitochondrial ribosomal protein S27	2,102
MRPL2	mitochondrial ribosomal protein L2	2,188
MRPL32	mitochondrial ribosomal protein L32	2,215
MRPL47	mitochondrial ribosomal protein L47	2,325
MRPS30	mitochondrial ribosomal protein S30	2,500
EEFSEC	eukaryotic elongation factor, selenocysteine-tRNA-specific	2,759
MRPL55	mitochondrial ribosomal protein L55	2,762
MRPL24	mitochondrial ribosomal protein L24	2,865
MRPS18A	mitochondrial ribosomal protein S18A	3,106
MRPL46	mitochondrial ribosomal protein L46	3,217

GFM1	G elongation factor, mitochondrial 1	3,349
MTIF2	mitochondrial translational initiation factor 2	3,428
EEF1D	eukaryotic translation elongation factor 1 delta	3,519
MRPL40	mitochondrial ribosomal protein L40	3,558
MRPS18C	mitochondrial ribosomal protein S18C	3,569
MRPS34	mitochondrial ribosomal protein S34	3,696
MRPS18B	mitochondrial ribosomal protein S18B	3,709
MRPS28	mitochondrial ribosomal protein S28	3,786
MRPS25	mitochondrial ribosomal protein S25	3,787
GSPT1	G1 to S phase transition 1	3,828
MRPS2	mitochondrial ribosomal protein S2	3,956
MRPL14	mitochondrial ribosomal protein L14	3,988
MRPS6	mitochondrial ribosomal protein S6	4,178
MRPL51	mitochondrial ribosomal protein L51	4,218
MRPS16	mitochondrial ribosomal protein S16	4,225
MRRF	mitochondrial ribosome recycling factor	4,419
MRPL54	mitochondrial ribosomal protein L54	4,603
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	4,640
NSUN4	NOL1/NOP2/Sun domain family, member 4	4,659
MRPL20	mitochondrial ribosomal protein L20	4,671

Table 4: Leading edge genes of gene ontology-based gene sets enriched in ProMyo-overexpressing vs. saline-treated muscles

Rank indicates the ranking of the gene in the list of all genes (total: 20,630) ranked on the basis of differential expression between ProMyo-overexpressing and saline-treated muscles.