1 **Fine mapping of genes determining extrafusal fiber properties in murine soleus**
2 **muscle**

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Abstract

Introduction. Muscle fiber cross-sectional area (CSA) and proportion of different fiber types are important determinants of muscle function and overall metabolism. Genetic variation plays a substantial role in phenotypic variation of these traits, however, the underlying genes remain poorly understood.

Aims. This study aimed to map quantitative trait loci (QTL) affecting differences in soleus muscle fiber traits between the LG/J and SM/J mouse strains.

Methods. Fiber number, CSA, and proportion of oxidative type I fibers were assessed in the soleus of 334 genotyped female and male mice of the F_{34} generation of advanced intercross lines (AIL) derived from the LG/J and SM/J strains. To increase the QTL detection power, these data were combined with 94 soleus samples from the F_2 intercross of the same strains. Transcriptome of the soleus muscle of LG/J and SM/J females was analysed using microarray.

Results. Genome-wide association analysis mapped 4 QTL (genome-wide $p<0.05$) affecting the properties of muscle fibers to Chromosome 2, 3, 4 and 11. A 1.5-LOD QTL support interval ranged between 2.36 Mb and 4.67 Mb. Based on the genomic sequence information, functional and transcriptome data, candidate genes were identified for each of these QTL.

Conclusion. Combination of analyses in F_2 and F_{34} AIL populations with transcriptome and genomic sequence data in the parental strains is an effective strategy for refining QTL and nomination of the candidate genes.

Key words: skeletal muscle, muscle fiber types, genetic variation

51 **Introduction**

52 Skeletal muscle plays a broad range of biological functions including locomotion,
53 thermoregulation, respiration, postural support, protection of bones and viscera; as well as
54 serving as a source of amino acids in times of starvation or disease. Muscle tissue in
55 livestock also provides an essential source of dietary proteins. In humans, there is more
56 than a 2-fold difference in muscle mass between individuals of similar age and same sex (3,
57 33). This is the outcome of variability in the number of muscle fibers and their size (51).
58 These differences are of clinical relevance. Variability in muscle mass significantly impacts
59 energy expenditure (58), influencing preponderance to obesity. In addition, individuals with
60 lower muscle mass may be more vulnerable to impairment of these vital functions due to
61 aging and/or disease related muscle loss. It has recently been reported that there is a
62 positive association between muscle mass and longevity in older adults (66).

63

64 Human skeletal muscles are mainly comprised of a mixture of type I, IIA and IIX muscle
65 fibers (62). The number of fibers, their size and varying proportions of the fiber types affect
66 morphological and functional properties of the muscle (6). A larger diameter of the fibers
67 and higher number of fibers typically leads to augmented muscular strength and power (25,
68 28). The proportion of type I muscle fibers is a factor determining success in endurance
69 sporting events (15, 18) and overall metabolism in humans (24, 29, 44, 74). In livestock,
70 proportion of oxidative type I fibers is associated with meat quality (65).

71

72 In humans, genetic factors account for around half of the variation in strength (19, 24, 74)
73 and the upper limit heritability is even greater (over 0.9) for muscle mass (26). Heritability
74 estimates of proportion of type I fibers is also high, ranging between 0.4 and 0.9, indicating
75 that genetic factors play an important role in determining muscle fiber properties (37, 63).
76 Effects of genetic factors on muscle fibers have also been demonstrated in mouse (20, 22,

77 59), cattle (68), sheep (10, 38) and pig (71). However, the specific genes underlying these
78 effects remain largely elusive.

79

80 Attempts at mapping the polygenic architecture of muscle fiber properties in mouse (11), pig
81 (17, 43, 52, 55, 77), cattle (1) and carp (80) have been made. A number of QTL have been
82 identified in these studies. However, the resolution achieved in the F_2 population is not
83 adequate for reliable nomination of the candidate genes in the majority of the QTLs of
84 polygenic traits. The mouse soleus muscle (primarily consists of type I and IIA fiber types),
85 closely resembles the fiber type composition of human skeletal muscles (primarily comprised
86 of type I, IIA and IIX fiber types), and is therefore a particularly interesting experimental
87 model. In our previous study, we mapped soleus muscle fiber traits in an F_2 intercross
88 between the LG/J and SM/J laboratory mouse strains (11). These strains differ in a number
89 of muscular phenotypes, with the LG/J strain displaying a greater proportion of type I fibers,
90 and a greater cross-sectional area (CSA) of type I and IIA muscle fibers. We identified in
91 that study three significant QTLs contributing to the difference in the CSA of muscle fibers
92 between LG/J and SM/J strains (11). Regions of conserved synteny from the identified loci
93 were also implicated in fiber phenotypes in pig supporting the importance of these genomic
94 regions in determining muscle fiber properties. However, the exact genes underlying their
95 effects remain to be determined.

96

97 Integration of advanced study populations, high throughput gene expression technology and
98 increasing availability of knockout models aid identification of the causative genes.
99 Nomination of the genes underlying QTL effects can be facilitated by improving the mapping
100 resolution, and by utilising genomic sequence and transcriptome information. Advanced
101 intercross lines (AIL) have been proposed as a powerful population for mapping QTLs (16).
102 It has been demonstrated recently that a joint F_2 and AIL analysis can combine the

103 advantages of both mapping populations by increasing the power to detect QTLs and
104 achieving a higher mapping resolution of various traits in mice (13, 47). Additionally, testing
105 for differences in specific gene expression has led to several nominations of quantitative trait
106 genes (30, 35). For validation of such candidate genes, phenotypic effects of relevant
107 alleles can be examined in experimental populations where these alleles segregate albeit on
108 a different genetic background. In addition, available knockout models offers particularly
109 attractive option for validation experiments.

110 In the present study we aimed to fine-map QTL and nominate candidate genes affecting the
111 CSA and proportion of oxidative type I fibers in the soleus muscle in a combined analysis of
112 F_2 and F_{34} AIL mice, and by cross referencing QTL data with soleus transcriptome profiles in
113 the parental strains. Further filtering of the emerged candidates was carried out in an
114 independent AIL and a knockout model.

115

116 **Methods**

117 Muscle Samples

118 This study was carried out on soleus muscles dissected from females and males of the F_{34}
119 advanced intercross lines (AIL) of the LG/J and SM/J inbred strains. Animals were
120 maintained as previously described (13) and sacrificed at 94 ± 4 days. All procedures were
121 approved by the Institutional Animal Care and Use Committee of the University of Chicago.
122 Soleus muscle samples from F_{34} AIL mice described in our previous study (47) were
123 subjected to histological analyses. The final sample size used in the present study was 334
124 F_{34} mice, 142 females and 192 males, after discarding samples of poor tissue quality. A set
125 of 94 F2 samples (38 females and 56 males) described in our previous study (11) was also
126 used in order to increase the QTL detection power.

127 In addition, we also analysed soleus muscle samples for two hypothesis driven studies
128 aimed at testing the effects of identified candidate genes on percentage of oxidative, type I
129 fibers. First, we examined solei samples from the *Chd6* ATPase knockout (n=6),
130 heterozygous (n=4) and wild type (n=4) females. The generation of the *Chd6* mutant mice
131 has been previously reported (40). Briefly, the genetic manipulation generated an allele with
132 the ATPase domain of *Chd6* (exon 12) flanked by loxP sites so that the action of Cre
133 recombinase would delete this domain. The mice were mated to a germline Cre-expressing
134 strain (Jackson lab strain 003465) to delete both exon 12 and the neomycin resistance
135 marker used for the targeting. Subsequently breeding generated the *Chd6* ATPase
136 knockout mice utilized in the present study. Second, solei of the advanced intercross mice
137 (generations F_9-F_{12}), all homozygous carries of the C57BL/6J (n=22) or DBA/2J (n=23)
138 alleles at the region harbouring the *Alad* gene were selected from the tissue bank of our
139 previous study (9).

140 Phenotype assessment

141 The soleus muscles were frozen in isopentane cooled in liquid nitrogen. Transverse
142 sections from the belly of the muscle were cut at a thickness of 10 μm with a cryotome
143 (Leica CM1850UV) at -20˚C. The muscle sections were subjected to ATPase staining (acid
144 pre-incubation, pH 4.47) to distinguish between fiber types (8). Microscopic images of
145 stained sections were taken at x5 and x20 magnification.

146 The following phenotypes were assessed: muscle fiber number (type I and IIA) and percent
147 of type I muscle fibers, cross-sectional area (CSA) of type I and type IIA fibers (**Figure 1**).
148 Muscle fiber traits were manually analysed using ImageJ software (NIH-version 1.43). 25
149 measurements of each fiber type were taken using the freehand selection tool at x20
150 magnification to obtain a value representing the mean CSA of type I or type IIA fibers for that
151 muscle. This was deemed as a representative sample by empirical testing as described
152 previously (11). Total number of type I and type IIA muscle fibers were counted using the
153 ImageJ cell counter plugin on x5 magnification images. As all fibers in mouse soleus pass
154 through the belly of the muscle (69), this method provides an accurate estimate of the
155 number of fibers constituting the muscle. Total number of type I fibers and total number of
156 type IIA fibers were counted, permitting derivation of percentage of type I fibers. Over the
157 course of the study ~200,000 muscle fibers were counted and ~6,700 fibers measured for
158 CSA.

159 <u>Statistical analyses</u>

160 The GraphPad Prism version 5.0 statistical package was used (GraphPad software, La Jolla,
161 CA). Data are presented as mean ± SD, unless otherwise stated. The CSA of type I and
162 type IIA fibers were analysed using a two-way (sex and fiber type) paired-measures (type I
163 and type IIA fibers) ANOVA.

164 <u>Genotyping and QTL mapping</u>

165 Mice were genotyped using a custom designed SNP array that included 4,610 polymorphic
166 SNPs that were approximately evenly distributed across the genome, as described

167 previously (13). The genome-wide association analysis was performed in the combined
168 population of the F_{34} and recently published F_2 intercrosses (11) using the R package
169 QTLRel (12). This software accounted for the complex relationships (e.g., sibling, half-
170 sibling, cousins) among the F_{34} mice by using a mixed model, as previously described (12,
171 13). Due to the sex differences in muscle mass in these mice (47), and the discovery of sex
172 specific QTL in other studies (45, 46), we included sex as an additive and interacting
173 covariate. Threshold of significance was estimated by 1000 permutations (14). We defined
174 the support interval for each QTL as the 1.5-LOD drop off on either side of the peak marker.
175 This interval was expressed in physical map position (Mb) by using the nearest genotyped
176 SNP that flanked the support interval, based on the mouse genome build GRCm38.p3.

177 Transcriptome analysis

178 Soleus muscle tissues from 92-day old LG/J and SM/J females (n=3 of each strain) were
179 used. RNA was isolated using TRIzol (Invitrogen Life Technologies, Carlsbad, CA) followed
180 by purification and DNase digestion using RNeasy minikits (Qiagen, Venlo, Netherlands)
181 according to the manufacturer's instructions. Quantification of total RNA was performed on
182 a NanoDrop spectrophotometer (Thermo Scientific) and quality tested on an Agilent
183 Tapestation with R6K Screentapes (RIN ≥7.3). Generation of sense strand cDNA from
184 purified total RNA (Ambion® WT expression kit, Ambion, Austin, Texas) followed by
185 fragmentation and labelling (GeneChip WT labelling kit, Affymetrix, Santa Clara, CA) were
186 performed according to the manufacturer's instructions. Hybridisation, washing, staining and
187 scanning of microarrays were carried out on Affymetrix Mouse Gene 2.0 ST microarrays
188 according to the manufacturer's standard protocols using a GeneChip Fluidics station 450
189 and GCS3000 scanner (Affymetrix®, Santa Clara, CA). Microarray data are available in the
190 ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-
191 5290.

192

Data pre-processing and quality control analysis was performed using Affymetrix® Genechip® Expression Console v1.2. Probe cell intensity data on the Mouse Gene 2.0 ST array (CEL files) were processed using the RMA16 algorithm (Affymetrix, Santa Clara, CA, USA) which fits a robust linear model at probe level by employing background correction, quantile normalisation of log2 transformed data and summarisation to probe level data (CHP files, 41,345 probe sets).

Data was analysed for differentially expressed genes in Partek® Genomics Suite® version 6.6, build 6.15.0730 (Partek Inc., St Louis, MO) using a Mus musculus build mm10 annotation file for Mouse Gene 2.0 ST microarrays (MoGene-2_0-st-v1.na35.mm10). CEL files (Expression Console v 1.2, Affymetrix, Santa Clara, CA) were imported to Partek Genomics Suite v 6.6 and processed using RMA normalisation with background correction of log2 transformed data and probe set summarisation by median polish. Differential expression analysis between the LG/J and SM/J strains of all genes (n=41,345 transcript clusters) was determined by 1-way ANOVA with Storey's FDR, and q-value ≤ 0.05 considered significant (n=819 genes differentially expressed $\geq$ 1.2 fold; see **Supplementary Table 1**).

To assess transcription of positional candidate genes in each strain, a hypothesis driven analysis of differential gene expression was performed between the LG/J and SM/J strains on all genes mapping to the support interval defined for each QTL in the GWAS described above. Using Partek Genomics Suite v.6.6, a total of 159 genes that were represented on the mouse Gene 2.0ST microarray, were identified in Mus musculus genome build GRCm38, mm10 within mapping co-ordinates Chr2:158908559–162608559 (26 genes), Chr3:33308451–35708451 (15 genes), Chr4:57605946–62913639 (77 genes) or Chr 11:27900000–31500000 (41 genes). 1-way ANOVA identified differentially expressed genes between the LG/J and SM/J strains ($P<0.05$). Fold change was calculated using the geometric mean of samples in each group.

Candidate genes

Nomination of the candidate genes was based on the following three criteria. First, we scrutinized polymorphisms in positional candidates between the LG/J and SM/J strains. The emphasis was on the indels and SNPs that would affect the coding sequence and lead to changes in amino acids. To assess whether amino acid substitution would influence the function of a protein, evolutionary conservation at the site of substitution and properties of substituted amino acids were considered using three different bioinformatics tools as described by Nikolskiy and colleagues (56). Second, we examined expression of positional candidates across a panel of over ninety mouse tissues and cell types available in BioGPS GeneAtlas MOE430, gcrma dataset (79). This analysis permits a quantitative comparison of transcript abundance of a gene between tissues. We considered that an abundant expression in skeletal muscle lineage, i.e. muscle tissue and/or C2C12 myogenic cell line, implies functional and/or structural relevance of a gene in this tissue. Third, we compared gene expression levels in the soleus muscle between the two strains as described in the previous section. Expression difference in this analysis might point at the strain-specific, genotype-dependent mechanism underlying the phenotypic difference.

Results

Phenotypic analyses

CSA. Cross section analysis of soleus muscle fibers were done on mice of both sexes from the F_{34} cohort. For muscle fiber cross-sectional area, we observed a statistically significant sex by fiber type interaction (P<0.0001). In the female F_{34} mice there was no significant difference between type I and type IIA muscle fiber areas (913 ± 229 μm^2, n=140; and 952 ± 242 μm^2, n=140 respectively; P=0.2). However, there was a significant difference within the males, with the type I muscle fiber area being smaller than IIA fiber area (1084 ± 238 μm^2,

245 n=187; and 1215 ± 294 μm^2, n=187 respectively; P< 0.0001). Muscle fiber area was lower
246 in females than males for type I CSA, (P < 0.0001) and type IIA CSA (P < 0.0001).

247 **Percentage of type I fibers.** The number of type I fibers as a percentage of total fibers
248 varied substantially between individuals, ranging from 30% to 67% in females, and from 26%
249 to 59% in males (**Figure 1**) and was greater in females than males (46 ± 8%, n=142; and 39
250 ± 6%, n=189; respectively; P < 0.0001).

251 **Total fiber number**. No difference was observed in the total soleus fiber number between
252 females and males (646 ± 102, n=120, and 667 ± 105, n=177, respectively; P= 0.0979).

253

254 QTL analyses

255 Muscle fiber traits approximated the normal distribution in both the F_2 and F_{34} population
256 (**Supplementary Figure 1**). We identified significant QTL (at the 1% or 5% level of genome-
257 wide statistical significance) (39) for CSA of type I and type IIA fibers and the percentage of
258 type I fibers. We also identified chromosome-wide significant QTL for CSA of type I and type
259 IIA fibers, the percentage of type I fibers and total fiber number (**Table 1**). The size of the
260 support interval of these QTL ranged from 0.4-40.7 Mb, with a median of 4.6 Mb.

261 The QTL at the genome-wide level of significance for CSA of type I and type IIA fibers on
262 chromosome 3 was named *Mfq5*. The QTL at the genome-wide level of significance for the
263 percentage of type I fibers on chromosome 2 and 4 were named *Mfq4* and *Mfq6*,
264 respectively. The SM/J allele conferred a greater percentage of type I fibers at *Mfq4*, and a
265 greater CSA at *Mfq5*. The LG/J allele conferred a greater percentage of type I fibers at *Mfq6*
266 locus.

267 A significant QTL affecting CSA of type I and type IIA fibers was also detected on
268 chromosome 11 (**Figure 2**) within the same region as locus *Mfq3*, previously identified in the
269 F_2 intercross of the same parental strains (11). The QTL exhibited male-specificity in both

270 type I and IIA fibers of the F_{34} mice (**Figure 3**). Because this QTL recapitulated properties of
271 the *Mfq3* locus, which we also found to be male specific in the F_2 population, we concluded
272 that the same locus has been refined in F_{34} and did not assign a new name for this QTL.
273 Earlier reported *Mfq2* locus has been refined in a similar manner; a QTL on chromosome 6
274 affecting CSA of type I and type IIA fibers (at 1% chromosome specific threshold) was
275 engulfed by the support interval of *Mfq2* and also replicated its increasing allele, LG/J, in
276 both females and males (not shown).

277 Gene expression analyses

278 We hypothesized that each identified QTL harbours one or more genetic variants that drive
279 phenotypic differences by means of differential gene expression. Hypothesis driven analysis
280 of differential expression in soleus muscle was performed between LG/J and SM/J strains for
281 the genes in the most robust QTLs affecting fiber CSA or % Type I fibers (*Mfq3*, *Mfq4*, *Mfq5*
282 and *Mfq6*). The Mouse Gene 2.0 ST expression array contains 159 genes residing within
283 the support intervals of these QTLs (**Supplementary Table 2**). Twenty genes (**Table 2**)
284 showed evidence of differential expression (ANOVA, p≤0.05), 2 of which, *Alad* and *Hdhd3*,
285 were significant after correction for the multiple testing problem (Storey's FDR q≤0.05).
286 Compared to other tissues and cell types, expression of differentially expressed genes *Mafb,*
287 *Acyp2* and *Mtif2* (**Table 2**), is particularly enriched in skeletal muscle (BioGPS, Mouse
288 MOE430 gene expression data).

289 Genomic analyses

290 Positional candidates with non-synonymous polymorphisms provide a plausible genetic
291 cause for the phenotypic differences. Based on the genomic sequence of the LG/J and
292 SM/J strains (56), we identified 21 genes in the QTL regions with non-synonymous
293 polymorphisms predicted to affect protein function by at least one out of three algorithms
294 used in the analysis (**Supplementary Table 3**). Four of those genes (*Mfq3*: *Mtif2*, *Rtn4*,
295 *Psme4*; *Mfq5*: *Dnajc19*) are prioritized further because of their preferential expression in

296 muscle lineage (differentiated muscle and/or C2C12 myoblasts) compared to other tissues
297 and cell types. Among those, the *Mtif2* gene differs by 3 (rs26871496, rs26871494,
298 rs29436813) and *Rtn4* by 9 (rs29473364, rs29469198, rs13463765, rs29465940,
299 rs26857726, rs26857725, rs29474377, rs26857722, rs26857721) amino acids between the
300 two strains. At all SNPs the SM/J strains carries reference while the LG/J strain the
301 alternative allele.

302 Candidate gene analyses

303 The *Chd6* gene emerged as a differentially expressed positional candidate for the *Mfq4*
304 locus affecting percentage of type I fibers (**Table 2**). To test its effect we examined soleus
305 muscles of *Chd6* knockout, heterozygous and wild type littermates. This analysis however
306 revealed that the genotype of the animals did not have a significant effect (P=0.30) on the
307 percentage of type I fibers (**Figure 4**).

308 The *Alad* gene emerged as a candidate for another locus affecting proportion of type I fibers,
309 *Mfq6*. In the animals of an advanced intercross between the C57BL/6J and DBA/2J strains
310 (these strains carry one or three copies of *Alad*, respectively (3)), we examined if percentage
311 of type I fibers was genotype-dependent. The analysis revealed no difference in the
312 percentage of type I fibres between the carriers of the C57BL/6J and the DBA/2J alleles, 42
313 ± 7% and 42 ± 8%, respectively.

314 **Discussion**

315 A previous study on muscle weight in LG/J and SM/J strains identified a two-fold difference
316 in soleus muscle size (47). We then explored the cellular and genetic mechanisms
317 contributing to this phenomenon, finding that the difference was largely due to the CSA of
318 muscle fibers and we mapped QTL affecting muscle fiber traits in an F_2 intercross between
319 the LG/J and SM/J strains (11). The present study, which utilizes the F_{34} advanced
320 intercross, verified, refined and expanded our earlier findings.

321 A number of studies have previously reported the effects of *Stat5a* and *Stat5b* (36), *Pgc-1α*
322 (42), *Ky* (4), myostatin (54), leptin (61), calcineurin (76), *Sod1* (5), alpha-actinin-3 (50),
323 dystrophin (7), *Tbx15* (41) and IIB myosin heavy chains (2) genes on muscle fiber area in
324 knockout or mutant models. In addition, *Pgc-1α* (75), calcineurin (76), *Foxo1* (34) and
325 myostatin (20) are reported to affect the proportion of muscle fiber types. However, the
326 genomic positions of these genes have not been linked to muscle fiber differences between
327 the LG/J and SM/J strains, implicating involvement of novel genes.

328 **Muscle fiber number**. The number of fibers is an important determinant of muscle size and
329 functional properties. It is set during embryogenesis and the first post-natal week in mice
330 (78). The number of muscle fibers in males (667 ± 105) and females (646 ± 102) of the F_{34}
331 population was comparable to that observed in the soleus of the F_2 population (645 ± 102
332 and 595 ± 107, respectively), and within the range of the fiber count observed in solei of a
333 variety of different strains of mice ~250-~900 fibers (32, 49, 57, 70, 72).

334 From these data it emerged that males and females are born with a similar number of fibers
335 in soleus muscle, and that the sex difference in muscle weight (males have approximately
336 30% larger soleus than females) is due to the difference in fiber size. Comparison of the
337 parental strains also revealed a similar number of fibers (11), despite the 2-fold difference in
338 soleus weight (47), demonstrating that size rather than number of fibers determines variation
339 in muscle weight between the LG/J and SM/J strains.

340 **Fiber area.** The CSA of muscle fibers in the LG/J strain is 49% to 90% greater than the
341 corresponding fibers in the SM/J strain, indicating that this variable accounts for a large
342 portion of the muscle mass difference between the strains (47).

343 The area of type I (1084 ± 238 μm^2 and 913 ± 229 μm^2 for males and females, respectively)
344 and type IIA (1215 ± 294 μm^2 and 952 ± 242 μm^2, respectively) of the F_{34} mice was
345 comparable to the corresponding fiber area of the F_2 mice of the same lineage (11) and it is
346 within the range reported for the type I, between 920 μm^2 and 1780 μm^2 (32, 57, 70), and
347 type IIA fiber area, between 700 μm^2 and 1400 μm^2 (32, 70), in various inbred mouse
348 strains.

349 **Percentage of type I fibers.** The percentage of type I fibers in male (39 ± 6%) and female
350 (47 ± 8%) F_{34} mice were also within the range of previous studies, which showed the
351 percentage of type I fibers in the soleus muscle fluctuates between ~25 and ~66% (32, 57,
352 70).

353 In the F_{34} mice we replicated our observation in the F_2 population that the percentage of type
354 I fibers was significantly greater in females than males. This sex difference is also observed
355 in various human muscles where, in general, women have a higher percentage of type I
356 muscle fibers than males (27, 53, 60, 64, 67). The phenomenon is likely to be explained, at
357 least partly, by the effect of androgens; castration leads to a higher percentage of type I
358 fibers in the soleus of male mice (73).

359 **Validation and refinement of genetic architecture.** In the present study, we validated and
360 refined the genetic architecture of muscle fibers identified in an F_2 intercross between the
361 same parental strains (11). In order to increase QTL detection power, we increased sample
362 size by combining the F_{34} and F_2 data. The median mapping resolution of 4.6 Mb for muscle
363 fiber QTLs was comparable with 3.7 Mb of muscle weight QTLs obtained in the same
364 population albeit using ~1,600 fewer genetic markers than in the present analysis (47). A
365 genome-wide significant QTL identified in the present study between 27.9 Mb and 31.4 Mb

366 on chromosome 11 (**Table 1**) overlapped with a significant QTL, *Mfq3*, mapped in the F_2
367 population (11). In addition to the chromosomal location, the increasing allele of this locus
368 (LG/J) and its male-specific effect (**Figure 3**) were also replicated in F_{34}, suggesting that the
369 same gene(s) were involved in two different populations and permitting us to refine the *Mfq3*
370 locus from 51.6 Mb to 3.57 Mb. The presence of two satellite QTL proximal of the refined
371 *Mfq3* (**Table 1**) suggests that the QTL observed in the F_2 population (11) might have been
372 an outcome of up to three linked loci.

373 The recently reported "mini-muscle" locus, mapped to 67.1–70.2 Mb on chromosome 11,
374 affects muscle fiber area and proportion of fiber types (21-23). However, the mutation
375 responsible for the "mini-muscle" phenotype maps to an intron of *Myh4* gene located at 67.2
376 Mb (31), between the support intervals of two adjacent QTLs affecting fiber type between the
377 LG/J and SM/J strains (**Table 1**). Together, these data suggest that a number of genes
378 residing on chromosome 11 might be involved in the regulation of muscle fiber phenotypes.

379 The QTL affecting the CSA of type I and type IIA fibers on chromosome 6, albeit at 1%
380 chromosome-wide threshold of significance (**Table 1**), overlapped with the *Mfq2* locus found
381 in the F_2 population, characterized by the same increasing allele, LG/J. Thus, the support
382 interval of *Mfq2* could be considered to be 5.18 Mb rather than the previously reported 56.5
383 Mb. Importantly, the immediate proximity of the refined region (Chr 6: 110.8-116.0 Mb) to
384 the syntenic region (Chr 6:116.0-118.0 Mb) implicated in the QTL affecting the diameter of
385 pig IIA fibers (17) suggest that the same genes could be underlying the effects of these
386 QTLs in mice.

387 A QTL affecting percentage of type I fibers (at 10% chromosome-wide threshold) on
388 chromosome 1 (67.6to 70.8 Mb) overlapped with *Mfq1* locus which influenced the CSA of
389 type I and type IIA fiber area in the F_2 population (11). However, because the CSA and
390 percentage of type I fibers are poorly correlated traits both in the F_{34} (**Supplementary Table**

391 **4**) and the F_2 mice (11), it is likely that different genes are underlying the *Mfq1* locus and the
392 QTL identified in the F_{34} population. Further studies are required to clarify this observation.

393 **Transcriptome analysis**

394 In the present study, the expressed transcriptome in soleus muscle of the parental strains
395 was examined in order to facilitate nomination of the candidate genes within the refined QTL.
396 We hypothesized that if the phenotypic effect of the QTL was brought about by the allele
397 specific abundance of transcripts encoded by genes within the QTL, such genes would be
398 differentially expressed in the transcriptome between the parental strains. Comparison of
399 expression of the genes within the four most robust QTLs identified *Alad* and *Hdhd3* genes
400 as potential candidates for the *Mfq6* locus, which affects the proportion of type I fibers.
401 Transcripts of both genes are more abundant in the LG/J compared to the SM/J strain. This
402 is consistent with our findings in the TA muscle of the same strains (48). Of these two
403 identified candidate genes, transcripts of *Alad* are ~20 times more abundant in the mouse
404 muscle than *Hdhd3,* regardless of strain. In addition, *Alad* may play a role during
405 myogenesis as its expression in C2C12 myogenic cells is 5-fold higher compared to
406 differentiated muscle (79).

407 **Candidate genes.**

408 The support intervals of four most robust QTLs harbor 159 genes (**Supplementary Table 2**).
409 These regions were scrutinized further for the genes fulfilling one of the following criteria:
410 presence of the functional variants (i.e. non-synonymous SNPs predicted to alter function of
411 encoded protein); abundance of transcript in muscle lineage, particularly in comparison to
412 other tissues and cell types; differential expression in the soleus of the two strains; and by
413 comparing genomic sequence between the LG/J and SM/J strains a list of 21 genes was
414 highlighted (**Supplementary Table 3**) with the strain-specific functional variants. Using
415 bioinformatics, 4 genes abundantly and/or preferentially expressed in skeletal muscle
416 compared to other types of tissues and cells were identified. Our own analysis of gene

417 expression in soleus muscle highlighted a set of 20 genes differentially expressed between
418 the two strains (**Table 2**). Intersection of all these lists permitted us to prioritise nine
419 candidate genes which appeared on more than one of these lists and/or for which
420 independent and accessible validation models were available (i.e. *Chd6* and *Alad*). Because
421 neither the *Chd6* (**Figure 4**) nor *Alad* genes were found to affect proportion of type I fibres in
422 the way predicted by the QTL analyses, the list of prioritised candidates was reduced to 7
423 genes annotated in **Supplementary Table 5**. Three out of four QTLs contain one (*Mfq6*) or
424 more candidate genes. All candidates are abundantly transcribed in muscle lineage with
425 *Psme4*, *Acyp2* and *Mafb* showing the highest level of expression in skeletal muscle
426 compared to other tissues and cells. None of the seven candidates have been previously
427 implicated to affect properties of skeletal muscle fibres although some of them have been
428 implicated in cardiomyopathy or function as transcription factors (**Supplementary Table 5**).
429 Thus, genomic and gene expression analyses permitted focusing on a limited number of
430 positional candidates in the future validation studies for establishing the causative genes.

431 **Conclusion**

432 In conclusion, we have refined the genetic architecture affecting cross sectional area of
433 soleus muscle fibers and proportion of type I fibers in the LG/J and SM/J derived lineage.
434 Integrating QTL mapping, genomic and transcriptome data from homologous muscle
435 highlighted several candidate genes that may underpin muscle phenotypes critical to health
436 and disease and worthy of follow up analyses.

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439

440

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444

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450

451 Author contributions

452 A.L. conceived and supervised the study, A.M.C. phenotyped muscle samples, A.A.P.
453 provided genotypes and oversaw the QTL analyses, R.C. designed the QTLRel software
454 used in the QTL analysis, A.M.C. carried out QTL mapping, M.E.S, C.M. and E.C.D. did
455 transcriptome analysis, S.N.F. and J.L.F. generated and provided *Chd6* knockout samples,
456 A.L. and A.M.C. wrote the manuscript with input from all co-authors.

457

458

459 **Figure legends**

460 **Figure 1. Individual variability in proportion of oxidative fibers.** Representative images
461 of F_{34} female soleus cross-sections following myosin ATPase staining (acid pre-incubation).
462 Dark fibers type I, pale fibers type IIA.

463

464 **Figure 2. Type I fiber cross-sectional area QTL on chromosome 11.** Analyses were
465 carried out in the F_2intercross and in the combined F_2 and F_{34}populations. *X*-axis indicates
466 the relative position in the linkage map in centimorgan (cM). The thresholds are at the level
467 of 0.05 genome wise significance for the F_2 output (dotted line) and combined output (solid
468 line).

469

470 **Figure 3. Sex specificity of *Mfq3* locus on cross-sectional area (CSA) of soleus type I**
471 **and IIA fibers in the F_{34} intercross.** Mean and SEM. Genotype at the peak marker: LG,
472 homozygous for LG/J allele; H, heterozygous; SM, homozygous for SM/J allele.

473

474

475 **Figure4. Percentage of type I fibers in the soleus muscle of 4 month old *Chd6***
476 **knockout (KO), heterozygous (HET) and wild-type (WT) females.** There is no difference
477 in percentage of type I muscle fibers in the soleus muscle between knockout, heterozygotes
478 and wild-type groups (P=0.3041). Each data point is from a single mouse, horizontal lines
479 represent group mean.

480

481 **Table 1.** Characteristics of muscle fiber QTL in combined analyses of the F_2 and F_{34}
482 intercrosses derived from the LG/J and SM/J strains.

Chr	Thr**	Level***	Start Mb†	End Mb	Size Mb	Trait	Locus¥
1	C	0.1	67.6	70.7	3.1	% Type I	
1	C	0.1	193.9	194.3	0.4	% Type I & CSA2A	
2	C	0.1	92.4	104.8	12.4	% Type I	
2	C	0.05	139.6	145.6	6.0	% Type I	
2	**G**	**0.01**	158.8	162.5	3.7	**% Type I**	***Mfq4 (SM)***
3	**G**	**0.05**	**33.6**	**40.0**	**6.4**	**CSA1 & CSA2A**	***Mfq5 (SM)***
4	**G**	**0.05**	**57.7**	**62.7**	**5.0**	**% Type I**	***Mfq6 (LG)***
4	C	0.05	103.9	106.1	2.2	% Type I	
6	C	0.05	81.9	84.1	2.2	CSAIIA	
6	C	0.01	110.8	116.0	5.2	CSA1 & CSA2A	*Mfq2* (LG)*
7	C	0.05	138.4	140.0	1.6	% Type I	
8	C	0.1	7.4	12.4	5.0	% Type I	
8	C	0.05	89.0	92.4	3.4	TOTAL	
8	C	0.01	121.9	128.6	6.7	TOTAL	
10	G	0.1	120.7	121.3	0.6	% Type I	
11	C	0.1	12.4	17.2	4.8	CSAIIA	
11	C	0.1	19.1	23.1	4.0	CSAIIA	
11	**G**	**0.01**	**28.0**	**31.5**	**3.5**	**CSA1 & CSA2A**	***Mfq3* (LG)***
11	C	0.1	62.5	64.2	1.7	% Type I	
11	C	0.1	70.6	76.2	5.6	% Type I	
12	C	0.1	27.6	29.3	1.7	CSA1	
13	C	0.01	5.3	9.9	4.6	% Type I	
13	C	0.05	71.5	74.0	2.5	CSAIIA	
14	C	0.05	93.6	102.3	8.7	CSAIIA	
15	C	0.1	12.1	20.3	8.2	TOTAL	
16	C	0.05	68.9	75.1	6.2	CSA1 & CSA2A	
X	C	0.01	11.8	52.5	40.7	TOTAL	

483 * refined previously identified QTL in the LG/J and SM/J F_2 intercross (47).

484 ** C – chromosome-wide threshold, G- genome-wide threshold

485 *** Level of significance

486 ¥ LG –increasing allele is LG/J, SM- increasing allele is SM/J

487 † Genomic positions based on GRCm38.p3.

488

489 **Table 2.** Positional candidate genes differentially expressed between LG/J and SM/J soleus muscles.

Chr	QTL	Probe set ID	*Gene*	p-value*	Fold-Change**	Gene name***
2	*Mfq4*	17393868	***Mafb***	0.033	-1.77	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (avian)
		17393910	*Chd6*	0.042724	-1.13	chromodomain helicase DNA binding protein 6
3	*Mfq5*	17404652	*Gm24780*	0.032914	-1.98	Predicted gene Gm24780, predicted protein is B4HDV3.
		17396801	*Ttc14*	0.033387	-1.14	tetratricopeptide repeat domain 14
		17396876	----	0.0219376	-1.80	There are no assigned mRNA sequences for this probe set. The probe set lies within lncRNA Sox2ot (Sox2 overlapping transcript, non-protein coding)
4	*Mfq6*	17425606	*Gm12526*	0.046791	-1.17	predicted gene 12526
		17414380	*Gm24277*	0.00525644	-2.07	Gm24277 a known snRNA. The probeset also lies within an intronic region of RefSeq gene Pakap (PALM2-AKAP2),a read through transcript on chromosome 4
		17425701	*Mir3095*	0.0298235	-1.78	Mir3095 (Entrez ID 100526502; EST ENSMUST00000175552).
		17426097	*Mup3*	0.00304	-1.49	major urinary protein 3
		17426126	*Fkbp15*	0.018038	-1.10	FK506 binding protein 15
		17414545	*Slc31a1*	0.049799	1.12	solute carrier family 31, member 1
		17426166	*Cdc26*	0.021407	-1.29	cell division cycle 26
		17426198	*Hdhd3*	0.000869	1.86	haloacid dehalogenase-like hydrolase domain containing 3
		17426206	*Alad*	9.75E-05	1.91	aminolevulinate, delta-, dehydratase
		17414600	*Rgs3*	0.023096	1.08	regulator of G-protein signaling 3
11	*Mfq3*	17248064	***Mtif2***	0.015517	-1.16	mitochondrial translational initiation factor 2
		17261285	*LOC102637613*	0.00432682	1.68	linc RNA [AK084560 (EST)/ Gm12092 (predicted gene)].
		17248127	**----**	0.00187422	-1.46	There are no assigned mRNA sequences for this transcript. The probe set lies within an intron of *Sptbn1*.
		17261393	***Acyp2***	0.011777	1.26	acylphosphatase 2, muscle type
		17248196	*Asb3*	0.014221	-1.15	ankyrin repeat and SOCS box-containing 3

490

491 * ANOVA p-value for strain effect; ** Fold change uses SM/J as baseline (negative values indicate LG/J expression is down compared to SM/J,
492 positive values LG/J expression up compared to SM/J); bold indicates that gene is predominantly and/ or strongly expressed in skeletal muscle
493 tissue (79). *** For probe sets not designed against an annotated gene, genes at the genomic loci of the Affymetrix probeset were identified in
494 UCSC genome browser using mouse genome build GRCm38.

495

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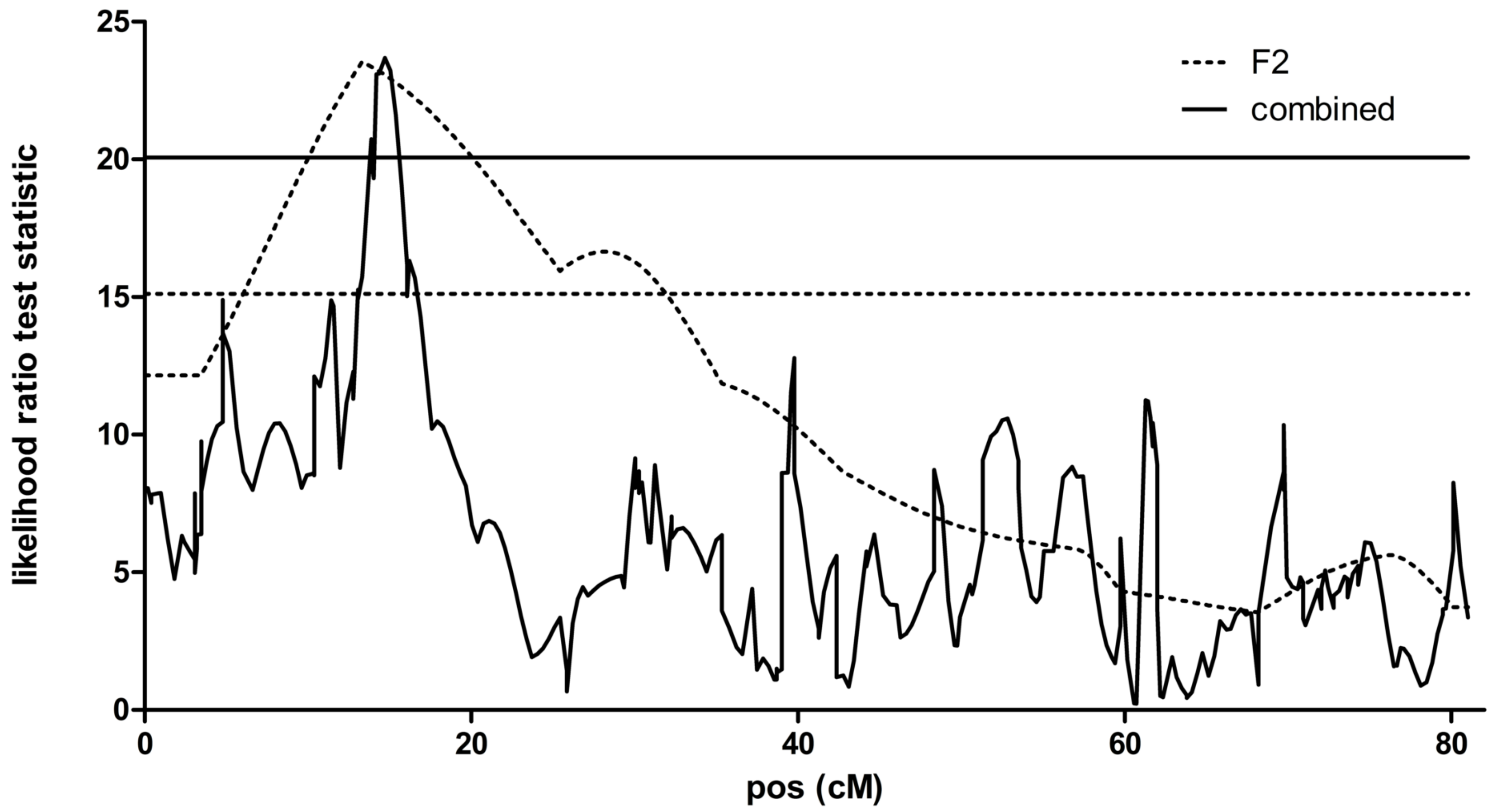
737

A

B

500 µm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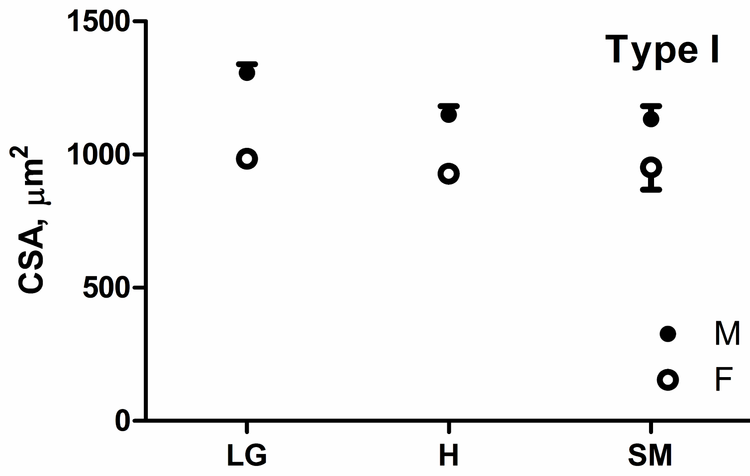
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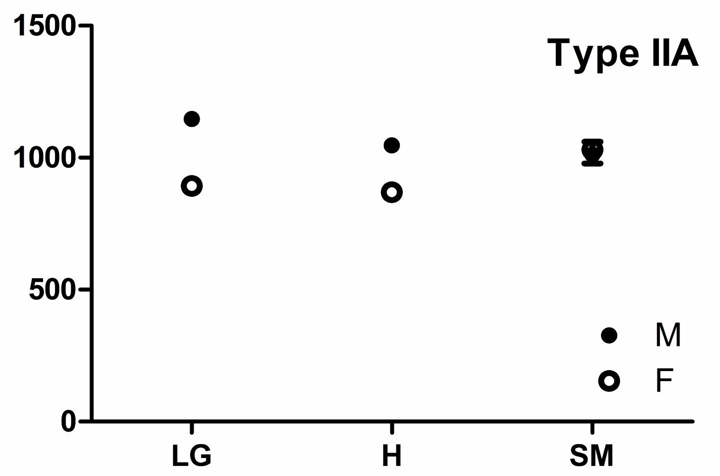
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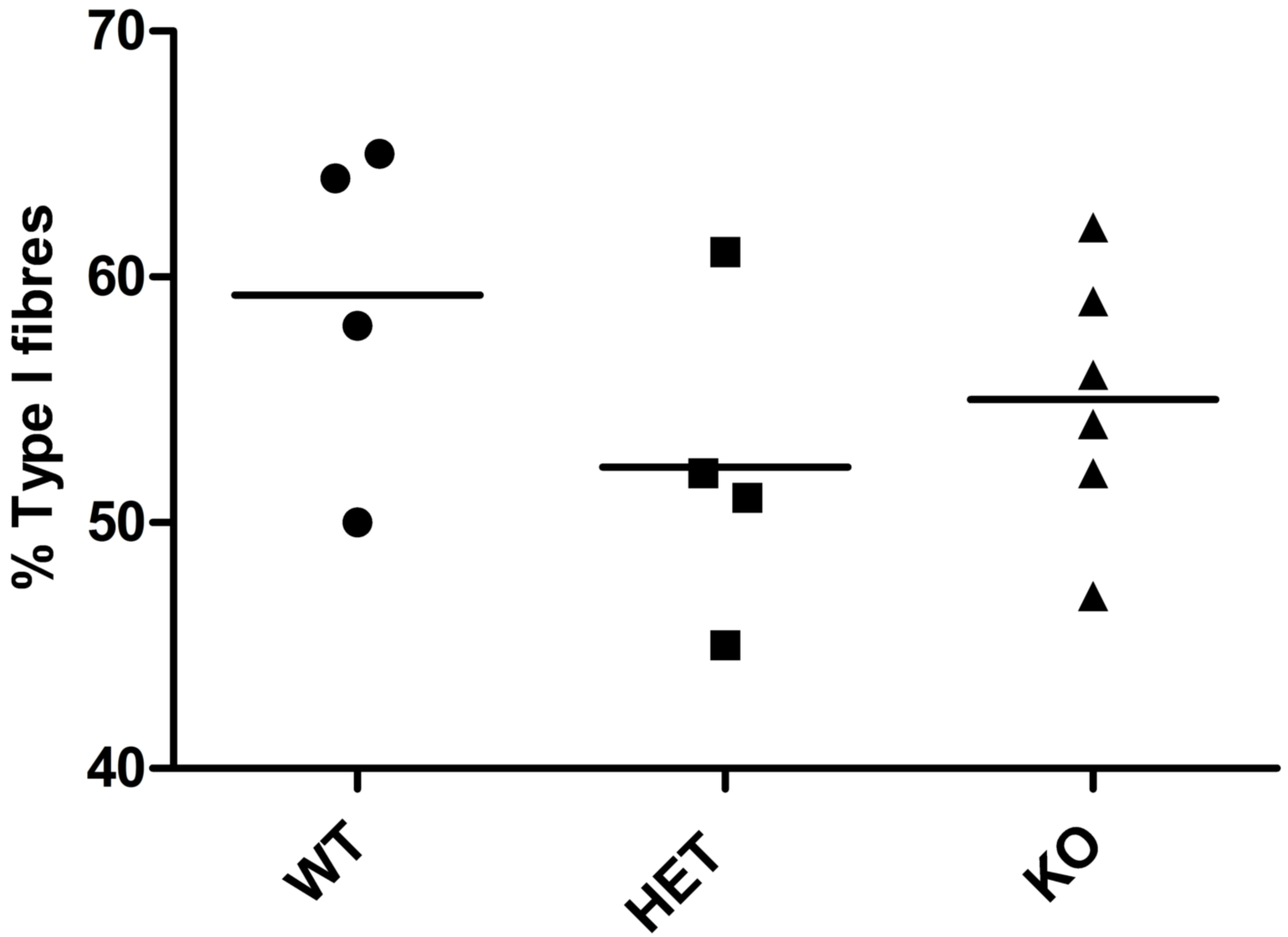

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