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

Document Version:
Peer reviewed version

1 **<u>Fine mapping of genes determining extrafusal fiber properties in murine soleus</u>**

2 **<u>muscle</u>**

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27 **Abstract**

28 **Introduction.** Muscle fiber cross-sectional area (CSA) and proportion of different fiber types

29 are important determinants of muscle function and overall metabolism. Genetic variation

30 plays a substantial role in phenotypic variation of these traits, however, the underlying genes

31 remain poorly understood.

32 **Aims.** This study aimed to map quantitative trait loci (QTL) affecting differences in soleus

33 muscle fiber traits between the LG/J and SM/J mouse strains.

34 **Methods.** Fiber number, CSA, and proportion of oxidative type I fibers were assessed in the

35 soleus of 334 genotyped female and male mice of the F_{34} generation of advanced intercross

36 lines (AIL) derived from the LG/J and SM/J strains. To increase the QTL detection power,

37 these data were combined with 94 soleus samples from the F_2 intercross of the same

38 strains. Transcriptome of the soleus muscle of LG/J and SM/J females was analysed using

39 microarray.

40 **Results.** Genome-wide association analysis mapped 4 QTL (genome-wide p<0.05)

41 affecting the properties of muscle fibers to Chromosome 2, 3, 4 and 11. A 1.5-LOD QTL

42 support interval ranged between 2.36 Mb and 4.67 Mb. Based on the genomic sequence

43 information, functional and transcriptome data, candidate genes were identified for each of

44 these QTL.

45 **Conclusion.** Combination of analyses in F_2 and F_{34} AIL populations with transcriptome and

46 genomic sequence data in the parental strains is an effective strategy for refining QTL and

47 nomination of the candidate genes.

48

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

50

51 **<u>Introduction</u>**

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   <u>Transcriptome analysis</u>

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

193　Data pre-processing and quality control analysis was performed using Affymetrix®

194　Genechip® Expression Console v1.2. Probe cell intensity data on the Mouse Gene 2.0 ST

195　array (CEL files) were processed using the RMA16 algorithm (Affymetrix, Santa Clara, CA,

196　USA) which fits a robust linear model at probe level by employing background correction,

197　quantile normalisation of log2 transformed data and summarisation to probe level data (CHP

198　files, 41,345 probe sets).

199

200　Data was analysed for differentially expressed genes in Partek® Genomics Suite® version

201　6.6, build 6.15.0730 (Partek Inc., St Louis, MO) using a Mus musculus build mm10

202　annotation file for Mouse Gene 2.0 ST microarrays (MoGene-2_0-st-v1.na35.mm10). CEL

203　files (Expression Console v 1.2, Affymetrix, Santa Clara, CA) were imported to Partek

204　Genomics Suite v 6.6 and processed using RMA normalisation with background correction

205　of log2 transformed data and probe set summarisation by median polish. Differential

206　expression analysis between the LG/J and SM/J strains of all genes (n=41,345 transcript

207　clusters) was determined by 1-way ANOVA with Storey's FDR, and q-value ≤0.05

208　considered significant (n=819 genes differentially expressed ≥ 1.2 fold; see **Supplementary**

209　**Table 1**).

210　To assess transcription of positional candidate genes in each strain, a hypothesis driven

211　analysis of differential gene expression was performed between the LG/J and SM/J strains

212　on all genes mapping to the support interval defined for each QTL in the GWAS described

213　above. Using Partek Genomics Suite v.6.6, a total of 159 genes that were represented on

214　the mouse Gene 2.0ST microarray, were identified in Mus musculus genome build

215　GRCm38, mm10 within mapping co-ordinates Chr2:158908559–162608559 (26 genes),

216　Chr3:33308451–35708451 (15 genes), Chr4:57605946–62913639 (77 genes) or Chr

217　11:27900000–31500000 (41 genes). 1-way ANOVA identified differentially expressed genes

218　between the LG/J and SM/J strains (P<0.05). Fold change was calculated using the

219　geometric mean of samples in each group.

220   <u>Candidate genes</u>

221   Nomination of the candidate genes was based on the following three criteria. First, we

222   scrutinized polymorphisms in positional candidates between the LG/J and SM/J strains. The

223   emphasis was on the indels and SNPs that would affect the coding sequence and lead to

224   changes in amino acids. To assess whether amino acid substitution would influence the

225   function of a protein, evolutionary conservation at the site of substitution and properties of

226   substituted amino acids were considered using three different bioinformatics tools as

227   described by Nikolskiy and colleagues (56). Second, we examined expression of positional

228   candidates across a panel of over ninety mouse tissues and cell types available in BioGPS

229   GeneAtlas MOE430, gcrma dataset (79). This analysis permits a quantitative comparison of

230   transcript abundance of a gene between tissues. We considered that an abundant

231   expression in skeletal muscle lineage, i.e. muscle tissue and/or C2C12 myogenic cell line,

232   implies functional and/or structural relevance of a gene in this tissue. Third, we compared

233   gene expression levels in the soleus muscle between the two strains as described in the

234   previous section. Expression difference in this analysis might point at the strain-specific,

235   genotype-dependent mechanism underlying the phenotypic difference.

236

237   **<u>Results</u>**

238   <u>Phenotypic analyses</u>

239   **CSA**. Cross section analysis of soleus muscle fibers were done on mice of both sexes from

240   the F_{34} cohort. For muscle fiber cross-sectional area, we observed a statistically significant

241   sex by fiber type interaction (P<0.0001). In the female F_{34} mice there was no significant

242   difference between type I and type IIA muscle fiber areas (913 ± 229 μm^2, n=140; and 952 ±

243   242 μm^2, n=140 respectively; P=0.2). However, there was a significant difference within the

244   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   <u>QTL analyses</u>

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   <u>Gene expression analyses</u>

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, $p \leq 0.05$), 2 of which, *Alad* and *Hdhd3*,

285   were significant after correction for the multiple testing problem (Storey's FDR $q \leq 0.05$).

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   <u>Genomic analyses</u>

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   <u>Candidate gene analyses</u>

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.

Discussion

314

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 ($39 \pm 6\%$) and female

350   ($47 \pm 8\%$) 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.

437

438

439

440

441   Grants

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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

Chromosome 11

likelihood ratio test statistic

pos (cM)

F2
combined