1   MAPPING THE EPISTATIC NETWORK UNDERLYING MURINE

2   REPRODUCTIVE FATPAD VARIATION

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24   Running Title: Murine Fatpad Epistasis

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47   ABSTRACT

48   Genome-wide mapping analyses are now commonplace in many species and

49   several networks of interacting loci have been reported. However, relatively few details

50   regarding epistatic interactions and their contribution to complex trait variation in multi-

51   cellular organisms are available and the identification of positional candidate loci for

52   epistatic QTL (epiQTL) is hampered, especially in mammals, by the limited genetic

53   resolution inherent in most study designs. Here we further investigate the genetic

54   architecture of reproductive fatpad weight in mice using the F_{10} generation of the LG,SM

55   Advanced Intercross (AI) line. We apply multiple mapping techniques including a

56   single-locus model, locus-specific composite interval mapping, and tests for multiple

57   QTL per chromosome to the twelve chromosomes known to harbor single locus QTL

58   (slQTL) affecting obesity in this cross. We also perform a genome-wide scan for pair-

59   wise epistasis. Using this combination of approaches we detect 199 peaks spread over all

60   19 autosomes that potentially contribute to trait variation including all eight original F_2

61   loci (*Adip1-8*), novel slQTL peaks on chromosomes 7 and 9, and several novel epistatic

62   loci. Extensive epistasis is confirmed involving both slQTL confidence intervals as well

63   as regions that show no significant additive or dominance effects. These results provide

64   important new insights into mapping complex genetic architectures and the role of

65   epistasis in complex trait variation.

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INTRODUCTION

67   The development and elaboration of techniques such as interval mapping (Lander

68   and Botstein 1989), composite interval mapping (Zeng 1994), and methods based on

69   complex pedigree structures (Jannink et al. 2001) has produced an extensive repertoire

70   for the statistical exploration of genotype-phenotype relationships, especially for single

71   loci. Using these approaches genome-wide analyses have identified single-locus QTL

72   (slQTL) underlying variance in characters as varied as agronomic traits and pest

73   resistance in corn (Papst et al. 2004), life span in fruit flies (Wilson et al. 2006), alkylator

74   induced cancer susceptibility in mice (Fenske et al. 2006), murine skeletal morphology

75   (Kenney-Hunt et al. 2008), and an ever expanding list of human diseases and disorders

76   including Age-Related Macular Degeneration (*e.g.* Klein et al. 2005), Type 2 diabetes

77   (*e.g.* Sladek et al. 2007; Zeggini et al. 2008), and Crohn's disase (*e.g.* Duerr et al. 2006).

78   In addition, several studies have successfully employed epistatic QTL (epiQTL) mapping

79   strategies to describe multi-locus networks (e.g. Cheverud et al. 2001; Stylianou et al.

80   2006; Wentzell et al. 2007; Fawcett et al. 2008, Fawcett et al. 2010).

81   However, most mapping studies in model systems involve either F_2 intercross

82   populations or Recombinant Inbred (RI) strain panels (see also Hanlon et al. 2006).

83   These populations harbor limited recombination and so tend to identify relatively large

84   confidence intervals, complicating the physiological investigation of statistical results.

85   Furthermore, while recombinant Inbred (RI) strain sets represent a four-fold expansion of

86   the F_2 recombination-based map, they require a minimum of 20 generations of brother-

87   sister mating (Silver 1995) and the number of strains per set is usually low, especially in

88   mammals. Conversely, the production of Advanced Intercross (AI) lines involves many

89   generations of outbreeding in a relatively large population. This preserves

90   heterozygosity, retains many more recombinant gametes in the gene pool, decreases the

91   average size of segregating linkage blocks, and increases mapping resolution (Haldane

92   and Waddington 1931; Bartlett and Haldane 1935; Hanson 1959a; Hanson 1959b;

93   Hanson 1959c; Hanson 1959d; Darvasi and Soller 1995; Rockman and Kruglyak 2008).

94   Specifically, the F_{10} generation of a murine AI line represents an approximately five-fold

95   expansion of the F_2 map and thus an improvement in resolution over both F_2 intercross

96   and RI line study designs.

97   Obesity and related phenotypes are among the most intensively studied complex

98   traits in mice and the LG,SM AI has proven particularly useful in the identification of

99   adiposity QTLs. Previous work in this cross has characterized over 70 loci contributing

100 to variance in fatpad weight, body weight and relevant organ weights (Cheverud et al.

101 1999; Cheverud et al. 2001; Cheverud et al. 2004; Fawcett et al. 2008). In addition, a

102 recent study used the combined F_9 and F_{10} generations (Fawcett et al. 2010) to fine-map

103 loci for a suite of obesity related characters and achieved an average confidence interval

104 for fatpad loci of 4.14 Mb. These CI were subsequently tested for epistasis and extensive

105 interaction was confirmed, though several direct effect loci identified in the F_2 and $F_{2/3}$

106 generations failed to replicate and were thus not included. However, in a full genome-

107 wide scan for pair-wise epistasis in the F_2 generation of this cross (Jarvis and Cheverud

108 2009) 38 fatpad loci that were not identified using a single locus mapping model show

109 significant epistatic interactions. Consistent with results from other experimental systems

110 (reviewed in Phillips 2008) this suggests that many biologically relevant loci are invisible

111 to single locus scans. Thus, combining the increased genetic resolution of an F_{10} AI line

112   study, with the full range of single-locus and epistatic mapping strategies promises to

113   produce novel insights into the contribution of epistatic interactions to variation in

114   reproductive fatpad weight. Furthermore, the accumulating data on positional

115   candidate genes (*e.g.* Chehab 2008; Gat-Yablonski and Phillip 2008; Ichihara and

116   Yamada 2008; Cheverud et al. 2009) provides the opportunity to explore functional

117   hypotheses for identified loci and their interactions.

118   Utilizing the F_{10} generation of the LG,SM AI line (Cheverud et al. 2001) we

119   further characterized the complex genetic architecture underlying murine reproductive

120   fatpad weight. We first performed a slQTL scan on the original eight chromosomes

121   harboring direct effect loci in the F_2 generation (1, 6, 7, 8, 9, 12, 13, and 18) as well as

122   the four shown to harbor slQTL in the combined F_9-F_{10} population (3, 4, 10 and 16;

123   Fawcett et al. 2010). Composite interval mapping and two-QTL tests were subsequently

124   performed, the latter when multiple loci on a single chromosome were suspected.

125   Finally, we carried out a full genome-wide scan for pair-wise epistasis. In order to

126   identify the most meaningful set of loci to screen for candidate genes, marker genotypes

127   representing slQTL and epiQTL that exceeded their appropriate thresholds were

128   combined in linear models, first for each chromosome separately and ultimately the entire

129   genetic system. Confidence intervals for peaks that remained significant in the full model

130   were screened for positional candidate loci and potential physiological interactions via

131   both the MGI database (www.informatics.jax.org/) and a literature search.

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133   MATERIALS AND METHODS

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134   **Data:** The population analyzed is the F_{10} generation (N = 1298; 85 full-sib

135   families; average litter size 8.45) of an Advanced Intercross (AI) line generated from an

136   F_2 intercross of the inbred mouse strains SM/J and LG/J (Chai 1956; Chai 1956;

137   Cheverud et al. 1996; Vaughn et al. 1999; Cheverud et al. 2001). The animal facility is

138   maintained at a constant temperature of 21°C with 12-hour light-dark cycles. Animals

139   were fed a standard rodent chow (PicoLab Rodent Chow 20 (#5053) with 12% of its

140   energy from fat, 23% from protein, and 65% from carbohydrate) *ad libitum* and were

141   weaned at 3 weeks of age. After weaning, animals were housed in single sex cages

142   containing no more than 5 individuals.

143   Between the F_2 and F_{10} generations the population was maintained at an effective

144   size of approximately 300 with 75 mating pairs and no variance in family size. Mating

145   between littermates was actively avoided. At greater than 13 weeks of age animals were

146   sacrificed and necropsies performed. The reproductive fatpads of each animal were

147   removed, combined and weighed on a digital scale to the nearest hundredth of a gram.

148   Phenotypes were statistically corrected for age at necropsy, sex, litter size, and parity

149   status (whether or not they were mated to produce the F_{11}) using multiple regression and

150   the residuals used for further analysis. Genotypes for each individual were obtained at

151   1470 polymorphic SNPs across the genome by Illumina (San Diego, USA) GoldenGate

152   Assay using DNA extracted from liver tissue obtained at necropsy. Inter-marker

153   genotypes were imputed at 1 cM intervals using the equations of Haley and Knott (1992).

154   **Mapping Analyses:** A single locus QTL (slQTL) scan at all measured and

155   imputed loci was first conducted on chromosomes 1, 3, 4, 6, 7, 8, 9, 10, 12, 13, 16, and

156   18 using the regression model:

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$$Y_i = \mu + a * X_{ai} + d * X_{di} + \text{error} \qquad (1)$$

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160   where Y_i is the vector of corrected phenotypes, μ is a constant, and X_{ai} and X_{di} are the

161   vectors of genotype scores; a and d are the fitted additive and dominance regression

162   coefficients respectively. The sums of squares for both model terms were pooled for

163   significance testing. The results of the full genome-wide slQTL mapping in the

164   combined F_9-F_{10} generations were previously reported (Fawcett et al. 2010).

165   Composite interval (CI) mapping (Zeng 1994) was applied to the identified,

166   preliminary confidence intervals using the following model:

167

$$Y_{ijk} = \mu + a * X_{ai} + d * X_{di} + \text{error} \,|\, X_{aj}\, X_{dj}\, X_{ak}\, X_{dk} \qquad (2)$$

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170   In this case, X_{aj}, X_{dj}, X_{ak}, and X_{dk} represent vectors of genotype scores at loci greater than

171   20 F_{10} cM up- and down-stream of the confidence interval on whose effects the within-

172   interval regressions were conditioned. This eliminates the effects of proximal and distal

173   QTL on the same chromosome from being confounded with the target QTL. When

174   multiple peaks on the same chromosome were suggested, the fit of all pair-wise two locus

175   models were compared to the appropriate single locus case using a X^2 test with two

176   degrees of freedom ($X^2_{crit} = 2*\text{abs}[\ln(1/p_{one})-\ln(1/p_{two})]$, where p_{one} and p_{two} are p-values

177   from the one and two locus models respectively (Sokal and Rohlf 1995).

178   Finally all genome-wide, between-chromosome, pair-wise combinations of

179   measured and imputed autosomal loci were tested using the following epistatic mapping

180   model:

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182     $Y_{ij} = \mu + aa(X_{ai}*X_{aj}) + ad(X_{ai}*X_{dj}) + da(X_{di}*X_{aj}) + dd(X_{di}*X_{dj}) + error \mid X_{ai} \ X_{di} \ X_{aj} \ X_{dj}$ (3)

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184   where *aa*, *ad*, *da*, and *dd* are the additive-by-additive, additive-by-dominance,

185   dominance-by-additive, and dominance-by-dominance epistasis regression coefficients

186   and $X_{ai} \ X_{di} \ X_{aj} \ X_{dj}$ represent vectors of the corresponding additive and dominance

187   genotypes at the two loci involved. The sums of squares and degrees of freedom for all

188   four epistatic components were pooled for initial significance testing. The raw

189   probability associated with each multiple regression for all mapping analyses above was

190   transformed to a linear scale using the base 10 logarithm of the inverse of the probability

191   of no epistasis (LPR = $\log_{10}(1/p)$) producing values comparable to LOD scores obtained

192   through maximum likelihood analysis (Lander and Botstein 1989).

193   **Thresholds:** Interpretation of these analyses is complicated both by the large

194   number of comparisons involved as well as the family structure present in the population.

195   In order to account for these two issues simultaneously, simulations were performed

196   using the known pedigree of all individuals between the F_2 and F_{10} generations to

197   generate a null distribution of expected effects from which the appropriate single-locus

198   LPR threshold was determined (Fawcett et al. 2008, Norgard et al. 2009). Given a

199   heritability of reproductive fatpad weight in the F_{10} of 0.47 (from sib-correlations)

200   chromosome-specific thresholds for identifying novel slQTL ranged from 6.15

201 (chromosome 8) to 6.6 (chromosome 1). The experiment-wide threshold for novel slQTL

202 detection was 7.34. For the purposes of replication, a corrected point-wise threshold

203 (equivalent to $p = 0.05$) of 3.32 was applied for slQTL peaks within previously identified

204 confidence intervals.

205   Following the method described in Fawcett et al. (2010), the analysis-wide

206 epistasis threshold for the identification of novel interactions was calculated to be 8.33.

207 The threshold for tests between a given slQTL and all other unlinked markers in the

208 analysis was 6.06 and the analogous chromosome-specific thresholds ranged from 4.73

209 (chromosome 8) to 5.25 (chromosome 1). The corrected point-wise threshold for

210 epistatic tests between two slQTL was 3.44. Tests involving slQTL are partially

211 protected from multiple comparisons as they were identified with independent

212 information.

213   **Confidence Intervals:** Due to the complexity of our mapping strategy, the

214 conventional 1 LPR drop criterion was applied to define all reported confidence intervals.

215 When multiple peaks, either slQTL, epiQTL or both, occurred in the same region, the

216 most proximal and most distal 1 LPR drop was used to determine CI endpoints.

217 Confidence intervals (CI) for slQTL peaks were also calculated for each location

218 individually using the standard deviation of the simulated distribution of 1000 mapping

219 iterations involving known effects on simulated chromosomes (Norgard et al. 2009). The

220 two techniques yielded very similar CI for all slQTL though the simulation-based

221 intervals were slightly smaller.

222   **Linear Models:** We constructed and evaluated separate chromosome-specific

223 models using the linear model function in R (R Development Core Team) before

224   combining their results into a full model of the genetic system. This process began with

225   terms representing each significant effect at all slQTL peaks identified by the single locus

226   model (equation 1) and composite interval mapping (equation 2). For example, the

227   chromosome 1 model (see Figure 1A) began with five slQTL terms representing the

228   additive (p = 0.00726) and dominance (p = 0.0007) effects at 20.15 Mb, the additive (p =

229   0.000268) and dominance (p = 0.0383) effects at 70.77 Mb and the dominance effect (p =

230   1.06×10^{-06}) at 134.82 Mb. The additive effect at 134.82 Mb was non-significant in the

231   slQTL mapping model (p = 0.868) and so was not included. Likewise, the chromosome

232   13 model (see Figure 1B) included two terms representing the additive effects at 53.54

233   Mb (p = 3.05×10^{-06}) and 90.61 Mb (p = 4.88×10^{-05}) respectively. In this case, neither

234   dominance effect was significant in the slQTL mapping model (p = 0.798 and p = 0.634)

235   and so both were excluded. When considered jointly, some individual terms (*e.g.* the

236   dominance effect only at 70.77 Mb on chromosome 1) no longer remained significant (p

237   < 0.05) in Type I ANOVA tables (using the "anova" function). Such terms were

238   removed. For those chromosomes not found to harbor slQTL, a similar process was

239   performed beginning with all significant interactions.

240   Next, individual coefficients from the epistatic mapping model (*aa*, *ad, da, dd*;

241   equation 3) at all peaks that exceeded their appropriate thresholds in the epiQTL scan

242   were similarly examined to determine the type or types of interactions occurring. Terms

243   representing all significant interactions were then added step-wise to each appropriate

244   chromosome-specific model. Only epistatic terms that remained significant (p < 0.05) in

245   both Type I and Type II ANOVA tables, using the R functions "anova" and "Anova" (the

246   latter from the package "car") respectively and did not cause any established additive or

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247   dominance effects to become non-significant ($p < 0.05$) were retained to define each final

248   chromosome-specific model. These stringent criteria were established in order to obtain

249   a tractable number of high-confidence CI to screen for positional candidates and

250   physiological interactions.

251   Next, additive and dominance terms from all chromosome-specific models were

252   combined and terms that became non-significant in either Type I or Type II ANOVA

253   tables (or both) were culled to define the "slQTL system." This model included 20 terms

254   at 18 loci (15 additive and 5 dominance; bold in Supplemental Table 1). Epistasis terms

255   significant in the chromosome-specific models were then added stepwise to the slQTL

256   system as above to define the "full model." In addition to the 20 marginal effect terms,

257   this model includes 23 interaction involving 26 different epiQTL confidence intervals.

258   Finally, since many epiQTL peaks occur at locations not represented in the slQTL

259   system, the appropriate additive and dominance terms for each interaction were added to

260   the full model to ensure that the identified epistatic contributions were not unduly biased

261   upward by variance attributable to single locus effects. This had relatively little effect

262   and resulted in the elimination of only 3 interactions, all of which are significant in Type

263   I tests. The results from the full model are reported with these nominally significant

264   terms noted in bold (Table 1, see below).

265   **Candidate Genes:** All CI for peaks identified in the full model were screened for

266   plausible positional candidate genes and known interactions. This involved both queries

267   of the MGI database for functional variants affecting adiposity as well as a broad

268   literature search and was intended to generate meaningful and testable physiological

269   hypotheses regarding the observed statistical associations.

270

271   RESULTS

272   **Replication and Identification:** Significant marginal effects, epistatic effects or

273   both are observed in the F_{10} population on all eight chromosomes harboring the original

274   *Adip* loci and three of the four additional chromosomes implicated in the combined F_9-F_{10}

275   slQTL scan (Supplementary Figure 1). In the F_{10} alone, there were no significant slQTL

276   on chromosome 16. Similar to the results of Fawcett et al. (2010), peak LPR scores from

277   either the single locus scan or composite interval mapping at or near the confidence

278   intervals of five *Adip* loci exceeded the experiment-wide threshold (7.34) even for novel

279   QTL detection (*Adip1*: LPR = 9.2, *Adip2*: LPR = 8.9, *Adip3*: LPR = 8.3, *Adip5*: LPR =

280   9.6, and *Adip8*: LPR = 12.3). All three remaining F_2 loci exceed the point-wise threshold

281   (3.32) required for tests within previously defined confidence intervals (*Adip4*: LPR =

282   5.6, *Adip6*: LPR = 5.24; *Adip7*: LPR = 4.8). Additional slQTL on chromosomes 3, 4, and

283   10 also replicated. Interestingly, the chromosome 4 locus (*Adip24*, Fawcett et al. 2010;

284   LPR = 12.65) roughly corresponds to two loci previously reported in the literature as

285   *Adip11* and *Adip12* in a cross between C57BL/6J and DBA/2J (Keightley et al. 1996;

286   Brockmann et al. 1998; Stylianou et al. 2006). Finally, composite interval mapping

287   revealed novel loci on chromosomes 7 and 9 that both exceed their appropriate

288   chromosome-specific thresholds of 6.36 and 6.38 respectively. A total of 22 potential

289   marginal effect peaks were identified (Supplementary Table 1).

290   **epiQTL Mapping:** In the genome-wide scan for epistasis 177 peaks involving

291   217 interactions exceeded their appropriate significance thresholds and physically cluster

292   into approximately 51 potential epiQTL (Supplementary Table 1). Additive-by-additive

293 interactions were the most common (98), Additive-by-Dominance or Dominance-by-

294 Additive were the next most common (97) and Dominance-by-Dominance interactions

295 were the most rare (22). Consistent with the results of Jarvis and Cheverud (2009) and

296 several other studies (see Phillips 2008), many of these occurred at locations showing no

297 significant marginal effects in this cross, though some occurred at locations significant in

298 slQTL scans in other crosses (Table 1; Figure 1; Supplementary Table 1; Supplementary

299 Figures 2-20).

300   **Linear Models:** In total, we identified 199 slQTL and epiQTL peaks that

301 potentially contribute to trait variation. These cluster into roughly 73 confidence

302 intervals showing a variety of combinations of additive, dominance and epistatic effects

303 (Supplementary Table 1). In order to identify the most robust signals we systematically

304 added vectors of genotype scores representing each into linear models and determined the

305 set that is simultaneously significant in both Type I and Type II tests. We began by

306 establishing a single locus model that contained all slQTL peaks that remain significant

307 together. This slQTL system includes 20 marginal effect terms (15 additive and 5

308 dominance) shows an adjusted R^2 value of 0.2254 (F statistic = 18.64 on 20 and 1281 df).

309 We next added epistatic peaks stepwise to generate a full model of the genetic system.

310 This full model (Table 1) includes 23 additional interaction terms (9 *aa*, 10 *ad/da*, and 4

311 *dd*) involving 26 different epiQTL confidence intervals and shows an adjusted R^2 value

312 of 0.3322 (F statistic = 15.71 on 43 and 1257 df). Using a chi-square goodness of fit test

313 with 23 (43-20) degrees of freedom this represents a highly significant improvement in fit

314 over the base slQTL model ($p < 10^{-25}$). Following the addition of all marginal terms

315 involved in epistasis, three interaction terms become non-significant at the $p < 0.05$ level

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316   in either the Type I or the Type II tables or both (bold terms in Table 1). Removing these

317   interactions from the full model, its adjusted R^2 value is 0.3220 (F statistic = 16.07 on 40

318   and 1260 df), which also represents a highly significant improvement in model fit (p <

319   10^{-20}).

320   **Positional Candidates:** While in-depth functional assays and other detailed

321   molecular studies are required to sort out the biological basis of QTL and their

322   interactions, examination of positional candidate genes in slQTL confidence intervals

323   suggests testable physiological hypotheses for several observed statistical effects. In

324   general, confidence intervals contain a variety of candidate loci including transcription

325   factors, components of various signaling cascades (*e.g.* the *Wnt*, *Insulin,* and *Igf* signaling

326   networks), neuro-endocrine hormones and their receptors, as well as genes directly

327   implicated in glucose processing and metabolism. For example, the CI found at

328   6:133.92-142.67 Mb contains the promising candidate *Lrp6*, a low-density lipoprotein

329   receptor-related protein that is thought to contribute to variation in a variety of metabolic

330   risk factors in humans (Kahn et al. 2007; Mani et al. 2007) and *Cdkn1b*, a cyclin-

331   dependent kinase inhibitor with known effects on pancreatic islet mass in diabetic mice

332   (Uchida et al. 2005). Both *Lrp6* and *Cdkn1b* have differences in expression level in

333   white fat (p = 3.82 x 10^{-12} and 0.013, respectively) and in the liver (p = 1.62 x 10^{-13} and

334   7.48 x 10^{-8}, respectively) between the two parental lines in this cross (Cheverud,

335   unpublished results). The CI 18:58.77-80.76 Mb shows potential functional links to

336   mammalian neurotransmitter signaling via *Htr4* (Gardner et. al. 2008), as do 13:40.74-

337   55.35 Mb via *Cplx2* (Brachya et al. 2006) and *Drd1a* (de Leeuw van Weenen et al. 2009).

338   In addition, the region 6:114.73-121.97 Mb contains neuro-endocrine candidates *Adipor2*

339   (Yamauchi et al. 2007; Ziemke and Mantzoros 2010) and *Ankrd26* (Bera et al. 2008),

340   which also shows a significant difference in expression in liver between LG/J and SM/J

341   (p = 0.0002; Cheverud, unpublished results). Together, these loci suggest a functionally

342   similar genetic architecture to the emerging picture of Type 2 diabetes in humans (Doria

343   et al. 2008).

344   There are also a number of strong candidate loci for observed epistatic

345   interactions. The most striking involves the CIs 13:0-24.24 Mb and 1:118.37-138.01 Mb,

346   which contain *Inhba* and *Inhbb* respectively. The proteins encoded by these loci are

347   components of the Activin and Inhibin complexes which have wide-ranging effects on a

348   variety of physiologic, homeostatic and metabolic processes including mammalian

349   reproduction, inflammation and adipocyte differentiation (Woodruff and Mather 1995;

350   Werner et al. 2006; Hirai et al. 2005). Interestingly, 13:0-24.24 Mb participates in five

351   separate interactions that are significant in the full model (Table 1) and appears to interact

352   with a region (9:68.10-95.10 Mb) containing an important receptor for serotonin (*Htr1b*).

353   Glutamate signaling and metabolism are also likely to underlie a portion of fatpad

354   variation due to epistasis in this cross. The interacting epiQTL CI 1:42.41-52.71 Mb and

355   9:68.10-95.10 Mb contain the enzyme that catalyses the first reaction in the primary

356   pathway for the renal catabolism of glutamine (*Gls*) and the first rate limiting enzyme in

357   glutathione synthesis (*Gclc*) respectively. *Gls* also shows differential expression in white

358   fat cells between the parental lines (p = 0.00097). Ghrelin and its associated pathways

359   also appear as likely candidates. For example, 1:118.37-138.01 Mb contains *Gpr39,* a

360   member of the ghrelin receptor family. This CI interacts with 6:133.92-142.67 Mb which

361   harbors *Pde3a,* a locus known to be downstream of ghrelin signaling in platelets

362   (Elbatarny et al. 2007) and which shows significant differences in gene expression in

363   white fat between SM/J and LG/J (p=0.00018), and 12:73.42-89.12 Mb which contains

364   *Hif1a*, whose protein product increases the expression of *Vegf* (Hoffmann et al. 2008).

365   Interestingly, *Vegfc* shows a significant difference in expression in white fat between the

366   parental lines (p = 0.001) and Vegfb shows differences in liver (p = 0.009). Ghrelin is

367   also known to increase the expression of *Vegf* in human luteal cells (Tropea et al. 2007)

368   and *Vegf* in turn, is thought to be an important regulator of adipogenesis and obesity (Cao

369   2007). A final interesting epiQTL CI is 12:108.99-120.28 Mb. It contains *Dlk1*, *Meg3*,

370   and *Rtl1*, all three of which appear to participate in an interacting (and imprinted)

371   network affecting growth in mice (Gabory et al. 2009).

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373   DISCUSSION

374   While the family structure of an outbred population complicates some aspects of

375   the mapping process, the F_{10} (and later) generations of advanced intercross lines hold an

376   intrinsic advantage in mapping resolution over more conventional study designs. Here

377   this advantage translated into a variety of results with important implications for mapping

378   complex trait variation and new insights into the genetic architecture of murine fatpad

379   weight.

380   The first and most striking result of this analysis from a mapping perspective is

381   the relatively low level of overlap in the physical positions of slQTL and epiQTL peaks

382   despite the analytical bias towards finding epistasis involving slQTLs due to their

383   protected status with respect to multiple comparisons. Though slight discrepancies may

384   be expected due to subtle patterns of linkage, larger map distances between peaks likely

385   indicate that multiple functional variants are present. Indeed, when both types are

386   observed in close proximity, epistatic peaks tend not to line up well with their single-

387   locus counterparts and epiQTL are frequently observed in regions showing no significant

388   marginal effects at all (Figure 1; Table 1; Supplementary Table 1; Supplementary Figures

389   1-20). This supports the notion that a relatively large number of variable, functionally

390   relevant loci exert their influence on complex trait variation primarily via epistatic

391   interactions rather than through conventional additive and dominance effects. It is also

392   interesting to note that some regions interact with multiple locations in the genome. For

393   example, proximal chromosome 13 (13:0-24.24 Mb) shows five significant interactions

394   in the full model including two with separate locations on chromosome 1. Identifying

395   such repeated signals may be useful in developing significance thresholds that help

396   ameliorate the penalties incurred by performing multiple comparisons. Such consistency

397   may also help distinguish epiQTL at the center versus the edges of functional networks.

398   Next, in keeping with observations in congenic lines (*e.g.* Christians et al. 2006)

399   as well as other recent slQTL mapping studies (Fawcett et al. 2010), F_2 confidence

400   intervals were frequently observed to divide into multiple significant slQTL (Figure 1,

401   Supplementary Figure 1). Interestingly, we observe similar splitting of single-locus and

402   epistatic signals. For example, at the proximal end of chromosome 1 (Figure 1A)

403   marginal effect peaks observed in the F_2, combined $F_{2-3,}$ and in an intercross between SM

404   and NZO (*obq7*; Taylor et al. 2001) appear to resolve in our mapping population into

405   three distinct peaks with two marginal effect loci flanking an epiQTL. This suggests that

406   the original F_2 and the subsequent F_{2-3} signals in this cross were composites of both

407   single-locus and epistatic effects and that the boundaries of previously reported CI may

408   have been influenced by epistatic contributions to single-locus values. Thus, current

409   estimates of the number of loci underlying trait variation are likely to be overly

410   conservative and reported effect size estimates are potentially biased by the presence of

411   multiple, closely linked functional elements. Interestingly, it also suggests that

412   confidence intervals identified in other intercross experiments, especially those that share

413   a parental strain, can be productively evaluated under *a priori* epistatic hypotheses, which

414   may also ease issues related to multiple testing. On this account, it is also striking that

415   the epistatic network identified in Stylianou et al. (2006) as Chr4-*Adip11* is centered on a

416   region also identified here as contributing to the epistatic architecture of fatpad weight.

417   The results of composite interval mapping also suggest that adjacent slQTL and

418   epiQTL impact the mapping process. For example, there is a dramatic and unexpected

419   increase in significance (nearly 3 orders of magnitude) for the additive slQTL peak at

420   134.82 Mb on chromosome 1 when composite interval mapping was applied (Figure 1A).

421   While this is the most dramatic example, such effects were repeatedly observed

422   (Supplementary Figure 1) and on chromosomes 7 and 9 this resulted in the identification

423   of two novel loci. Interestingly, this suggests that adjacent functional variants with

424   opposite effects were fixed in the original parental lines during their production. Indeed,

425   inspection of the regression coefficients from the full linear model shows that the

426   epistatic peak closest to the slQTL signal at 134.82 Mb on chromosome 1 (DD with

427   12:73.42-89.12 Mb) and the marginal signal itself share a positive sign. However, the

428   two slightly centromeric interactions involving the additive value on chromosome 1 (AA

429   with 13:0-24.24 Mb and AD with 6:133.92-142.67 Mb) are both negative. Conditioning

430   on these adjacent markers is indeed expected to enhance the signal of the neighboring

431   additive effect, consistent with our observations. Thus, comparing the results of

432   conventional single-locus mapping model and composite interval mapping may be an

433   indirect means of identifying neighboring functional variants. Further mapping in later

434   generations of this Advanced Intercross will provide a great deal of additional

435   information on the sign, magnitude and physiological basis for these observed effects as

436   recombination is expected to further separate their statistical signatures.

437   **Conclusions:** The application of multiple mapping approaches, including an

438   epistatic model, is a vital strategy for characterizing complex genetic architectures.

439   Contrary to suggestions based on human GWAS findings, we found substantial numbers

440   of pair-wise epistatic interactions involving many more loci than show single locus

441   effects that account for an important portion of trait variation. This is likely due to the

442   genetic structure of our experimental population where allele frequencies are

443   intermediate; there are no rare alleles in our mapping system. This is critical since

444   epistasis is known to produce predominantly additive and dominance variance when

445   relatively rare alleles are involved (Cheverud and Routman, 1995; Cheverud, 2000).

446   Here, the use of a combination of techniques was further enhanced by the

447   improved genetic resolution offered by AI lines. While single locus scans remain the

448   most tractable, pair-wise epistatic relationships can now be dissected in great detail as

449   well and the identification of candidate loci for such interactions is possible. This is

450   especially true for characters for which a large body of literature exists describing the

451   mechanistic relationships among candidate genes and related pathologies. In such cases,

452   incorporating *a priori* information regarding functional interactions can be used to help

453   focus epistatic mapping studies and both ease the difficulties associated with multiple

454   comparisons and facilitate the physiological interpretation of statistical results. It is an

455   exciting prospect that even more fine-scale mapping of these loci will be possible in later

456   generations of the LG,SM AI line. Undoubtedly future analyses, coupled with the

457   incorporation of sequence information from the parental lines, will aid in further refining

458   the physiological hypotheses presented here for fatpad variation and greatly contribute to

459   our understanding of the statistical signatures of complex genetic architectures.

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749

749   TABLE 1: Results from the full linear model of the epistatic network underlying murine

750   reproductive fatpad weight in the LG,SM AI line. Chromosome, confidence intervals (Mb), peak

751   locations (Mb), peak LPR scores, nearest SNP to the peak, effect type threshold and threshold

752   value are all given for each term. The appropriate references for any *a priori* hypotheses are

753   listed along with positional candidate loci for both slQTL and epiQTL. Bold terms are nominally

754   significant (p > 0.05) when additive and dominance effects for all interactions are included in the

755   model. References: [1]Cheverud et al. 2001; [2]Fawcett et al. 2008; [3]Taylor and Phillips 1996; [4]Taylor

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760

761

762   FIGURE 1: Mapping results of significant terms from the full model of reproductive fatpad weight

763   in the LG,SM AI line for chromosomes 1 (A) and 13 (B). Results from the single-locus model are

764   given as connected grey dots, composite interval mapping as smooth black lines and epistatic

765   interactions by other connected shapes. Confidence intervals from previous analyses are

766   represented by horizontal bars below the QTL plot.

767

Chr 1	CI 1 Begin (Mb)	CI 1 End (Mb)	Peak 1 (Mb)	Chr 2	CI 2 Begin (Mb)	CI 2 End (Mb)	Peak 2 (Mb)	slQTL LPR	Peak SNP 1	Peak SNP 2	Epistatic LPR	Effect(s)	Threshold Type	Threshold	Reported Adipose QTL in CI(s)	QTL Reference(s)	Candidates (CI 1)	Candidates (CI 2)
1	16.40	21.28	20.15	NA	NA	NA	NA	4.26	rs6334092	NA	NA	A,D	Pointwise	3.32	Adip1; Obq2	1;2;3	Pkhd1	NA
1	65.79	74.08	70.77	NA	NA	NA	NA	4.76	rs6323094	NA	NA	A	Pointwise	6.60	Obq7	4	Vwc2l; Fn1	NA
1	118.37	138.01	134.82	NA	NA	NA	NA	9.17	gnf01.132.831	NA	NA	A	Pointwise	3.32	Obsty1; Gwth1; Obq17	5;6;7	Pik3c2b	NA
3	20.54	27.82	22.51	NA	NA	NA	NA	5.56	rs13477017	NA	NA	A	Pointwise	3.32	None	None	Nlgn1; Ghsr	NA
4	9.71	11.92	10.83	NA	NA	NA	NA	4.78	rs13477558	NA	NA	D	Pointwise	3.32	Unnamed RI QTL	5	Plekhf2	NA
4	78.28	90.30	79.46	NA	NA	NA	NA	11.87	CEL-4-78089985	NA	NA	A	Pointwise	3.32	Adip11; Adip24; Adip11a	2;8;9	Tyrp1	NA
6	114.73	121.97	117.73	NA	NA	NA	NA	5.01	mCV23042866	NA	NA	D	Pointwise	3.32	Adip2; Igf1sl1	1;14	Adipor2; Ankrd26; Pparg	NA
6	133.92	142.67	134.20	NA	NA	NA	NA	8.89	rs13479053	NA	NA	A	Pointwise	3.32	Adip2	1	Lrp6; Grin2b; Cdkn1b	NA
7	30.18	44.44	37.21	NA	NA	NA	NA	4.08	rs6217275	NA	NA	D	Pointwise	3.32	Adip3; Adip3A; Adip3Ab	1;2;8	Tshz3; Plekhf1	NA
7	59.83	77.73	63.51	NA	NA	NA	NA	6.85	rs3717293	NA	NA	A,D	Pointwise	3.32	Tabw; Adip3Ad; Adip25; Obq1	15;8;3	Nipa1; Nipa2; Gabrg3; Gabra5; Gabrb3	NA
7	132.03	143.20	135.24	NA	NA	NA	NA	6.38	CEL-7-116160192	NA	NA	A	New slQTL chr7	6.36	Bsbob2	16	Trim72	NA
8	64.98	90.95	84.79	NA	NA	NA	NA	4.76	rs13479860	NA	NA	A	Pointwise	3.32	Adip4	1;2	Il15	NA
9	61.70	67.72	65.39	NA	NA	NA	NA	6.98	rs13480247	NA	NA	A	New slQTL chr9	6.38	None	None	Mtfmt	NA
9	118.30	125.00	118.88	NA	NA	NA	NA	9.64	rs6316481	NA	NA	A	Pointwise	3.32	Adip5; Adip5a; Adip5b; Adip5c; Obq18	1;2;8;7	Acvr2b	NA
12	60.62	67.43	64.06	NA	NA	NA	NA	5.24	mCV24690992	NA	NA	A	Pointwise	3.32	Adip6; Adip16; Fob2	2;9;19	Lrfn5	NA
13	40.74	55.35	53.54	NA	NA	NA	NA	4.90	rs3699522	NA	NA	A	Pointwise	3.32	Adip7; Adip18; Adip18a; Pfat3	1;2;8;13	Cplx2; Drd1a	NA
18	24.19	56.21	48.82	NA	NA	NA	NA	4.83	rs3684561	NA	NA	A	Pointwise	3.32	Adip8; Adip8a; Adip8b; Kcal1; Mnif2	1;8;20	Sema6a; Hsd17b4	NA
18	58.77	80.76	63.84	NA	NA	NA	NA	12.31	rs13483398	NA	NA	A	Pointwise	3.32	Adip8; Adip8c; Adip8d; Obsty4	1;2;8;5	Adrb2; Htr4	NA
1	42.41	52.71	51.38	9	68.10	95.10	77.25	NA	rs13475863	rs13480288	5.52	DD	QTL x QTL epi	3.44	Adip1; Obq7; Adip5; Mob8	1;4;21	Gls	Gclc
1	118.37	138.01	128.52	6	133.92	142.67	141.48	NA	rs6228473	rs8268650	4.95	AD	QTL x QTL epi	3.44	Obsty1; Gwth1; Obq17; Adip2	1;5;6;7	Gpr39	Pde3a
1	118.37	138.01	128.84	12	73.42	89.12	75.11	NA	rs13476100	rs3687032	4.64	DD	QTL x QTL epi	3.44	Obsty1; Gwth1; Obq17; Adip6	1;5;6;7	Gpr39	Hif1a
1	174.21	189.05	186.63	13	0.00	24.24	23.48	NA	mCV24555989	gnf13.020.621	10.27	AA	QTL x chr1 epi	5.25	Obq9	4	Hlx	Abt1
4	**30.53**	**39.16**	**36.58**	**9**	**118.30**	**125.00**	**123.70**	**NA**	**rs13477649**	**rs8241505**	**6.03**	**DD**	**QTL x QTL epi**	**3.44**	**Unnamed RI QTL; Dob2; Obq18**	**5;7;18**	**Cga**	**Slc6a20a; Slc6a20b**
4	125.68	139.92	130.91	7	132.03	143.20	139.70	NA	rs3673061	rs8236684	4.93	AD	QTL x QTL epi	3.44	Adip12; Qbis1; Afpq2; Adip3	1;9;10;11	Ptpru	Oat
4	143.52	154.77	152.94	7	132.03	143.20	141.88	NA	rs6378384	rs3719258	4.69	AD	QTL x QTL epi	3.44	Adip12; Adip3	1;9	Ajap1	Adam12
6	**33.46**	**46.84**	**37.64**	**9**	**118.30**	**125.00**	**123.70**	**NA**	**rs13478717**	**rs8241505**	**5.11**	**DA**	**QTL x chr6 epi**	**5.09**	**Dob2; Obq18**	**7;18**	**Trim24**	**Ccr9**
6	53.92	71.82	54.18	7	102.32	108.47	105.10	NA	rs13478762	UT-7-90.803899	5.13	AA	QTL x chr7 epi	4.96	Adip2; Obq13	1;4	Crhr2; Ghrhr	Capn5
7	132.03	143.20	137.17	8	42.26	57.10	50.65	NA	rs8236684	rs13479769	5.08	AA	QTL x chr8 epi	4.73	Bsbob2	16	Fgfr2	Ing2
8	124.83	129.12	127.97	9	20.24	39.76	23.57	NA	rs6300613	rs13480112	5.57	AD	QTL x chr9 epi	4.99	Obsty2	5	Disc1	Npsr1
9	20.24	39.76	31.31	12	108.99	120.28	111.04	NA	CEL-9-29909656	CEL-12-104545022	5.36	AA,DD	QTL x chr9 epi	4.99	Carfhg2	17	Kcnj5	Dlk1; Meg3; Rtl1
9	104.05	118.18	109.62	1	191.98	NA	193.61	NA	rs3723953	rs13476308	7.78	AA	QTL x chr1 epi	5.99	Adip5;Dob2	1;18	Fbxw cluster	Nek2
12	108.99	120.28	113.11	1	191.98	NA	195.79	NA	rs13481651	rs13476312	6.06	DA	QTL x chr1 epi	5.25	Adip6; Bsbob4; Mob3	1;2;16;12	Traf3	Hsd11b1
13	0.00	24.24	14.85	1	118.37	138.01	119.02	NA	rs13481702	rs3694226	5.96	AA	QTL x chr1 epi	5.25	Adip7	1	Inhba	Inhbb
13	0.00	24.24	17.38	9	68.10	95.10	82.84	NA	rs3678616	rs13480312	4.54	AA	QTL x QTL epi	3.44	Adip7; Adip5	1	Inhba	Htr1b
13	0.00	24.24	20.21	12	73.42	89.12	82.08	NA	rs6314295	rs3654718	4.78	AA	QTL x QTL epi	3.44	Adip7; Adip6	1	Olfactory receptor cluster	Slc8a3
13	40.74	55.35	43.69	6	80.99	92.88	89.62	NA	rs13481789	rs13479099	4.90	AA	QTL x QTL epi	3.44	Adip7; Adip18; Adip18a; Pfat3; Adip2	1;2;8;13	Ranbp9	Alms1
13	40.74	55.35	45.45	4	143.52	154.77	152.94	NA	rs3688207	rs6378384	4.48	AA	QTL x QTL epi	3.44	Adip7; Adip18; Adip18a; Pfat3; Adip12	1;2;8;9;13	Atxn1	Kcnab2
18	24.19	56.21	37.51	12	60.62	67.43	64.06	NA	gnf18.033.953	mCV24690992	5.88	AD	QTL x QTL epi	3.44	Adip8; Adip8a; Adip8b; Kcal1; Mnif2; Adip6	1;8;20	Pcdhb cluster	Lrfn5
18	**24.19**	**56.21**	**37.93**	**13**	**0.00**	**24.24**	**15.11**	**NA**	**gnf18.033.953**	**rs13481702**	**5.87**	**DA**	**QTL x QTL epi**	**3.44**	**Adip8; Adip8a; Adip8b; Kcal1; Mnif2; Adip7**	**1;8;20**	**Pcdhb cluster**	**Gli3**
18	24.19	56.21	50.47	7	30.18	44.44	30.56	NA	rs13483356	rs13479174	5.76	AD	QTL x QTL epi	3.44	Adip8; Adip8a; Adip8b; Kcal1; Mnif2; Adip3	1;8;20	Hsd17b4	Lrfn3

A)

B)