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Transgenic expression and genetic variation of LMF1 affect LPL activity in mice and humans

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Abstract

Objective—Lipoprotein lipase (LPL) is a principal enzyme in lipoprotein metabolism, tissue lipid utilization and energy metabolism. LPL is synthesized by parenchymal cells in adipose, heart and muscle tissues followed by secretion to extracellular sites, where lipolyic function is exerted. The catalytic activity of LPL is attained during post-translational maturation, which involves glycosylation, folding and subunit assembly within the endoplasmic reticulum (ER). A lipase-chaperone, lipase maturation factor 1 (Lmf1), has recently emerged as a critical factor in this process. Previous studies demonstrated that loss-of-function mutations of Lmf1 result in diminished lipase activity and severe hypertriglyceridemia in mice and human subjects. The objective of this study is to investigate whether, beyond its role as a required factor in lipase maturation, variation in Lmf1 expression is sufficient to modulate LPL activity *in vivo*.

Methods and Results—To assess the effects of Lmf1 overexpression in adipose and muscle tissues, we generated aP2-Lmf1 and Mck-Lmf1 transgenic mice. Characterization of relevant tissues revealed increased LPL activity in both mouse strains. In the omental and subcutaneous adipose depots, Lmf1 overexpression was associated with increased LPL specific activity without changes in LPL mass. In contrast, increased LPL activity was due to elevated LPL protein level in heart and gonadal adipose tissue. To extend these studies to humans, we detected association between *LMF1* gene variants and post-heparin LPL activity in a dyslipidemic cohort.

Conclusions—Our results suggest that variation in Lmf1 expression is a post-translational determinant of LPL activity.

Lipoprotein lipase (LPL) is a principal enzyme in plasma lipid metabolism.[1] Through the hydrolysis of triglycerides (TG) associated with lipoprotein particles such as chylomicrons and very low-density lipoproteins, LPL releases fatty acids for utilization and storage in various tissues. LPL is the rate-limiting enzyme in tissue absorption of dietary and endogenously produced TG, hence its activity is a key determinant of tissue lipid partitioning as well as plasma TG levels. Indeed, LPL-deficiency results in elevated plasma

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DISCLOSURE
None.

TG,[2] whereas LPL-transgenic mice exhibit increased tissue lipid content and insulin resistance.[3] Consistent with mouse models, genetic variation in the LPL gene is associated with insulin resistance,[4] plasma TG concentration and coronary artery disease[5] in human populations. Thus, tissue LPL activity is a critical determinant of metabolic traits in health and disease.

LPL is synthesized in the endoplasmic reticulum (ER) of parenchymal cells in muscle and adipose tissue followed by secretion and transport to the vascular endothelium, the functional site of LPL action. LPL attains catalytic activity within the ER in a multistep process, herein referred to as lipase maturation, which involves glycosylation, glycan processing, folding and the assembly of homodimers.[6] In adipocytes, only about 70-80% of newly synthesized LPL is converted into active enzyme, whereas the rest remains permanently inactive and undergoes degradation.[7-9] Thus, post-translational maturation represents a bottleneck in the generation of active LPL in adipocytes.[10]

Several factors have been implicated in lipase maturation including components of the calnexin/calreticulin and BiP/Grp94 chaperone systems.[11] In addition to general chaperones, which are involved in the post-translational processing of most proteins traveling through the ER, a lipase-specific chaperone, lipase maturation factor 1 (Lmf1), has also been described.[12] Lmf1 was identified as the gene affected in a mutant mouse strain (cld, combined lipase deficient), which exhibits severe hypertriglyceridemia owing to a lack of LPL activity. Although LPL protein is normally expressed in *cld* mutant mice, it fails to attain the catalytically active homodimer conformation and is subject to retention in the ER and degradation.[13] Lmf1 is a polytopic protein of the ER membrane and it physically interacts with LPL through a soluble region exposed to the ER lumen.[14] Interestingly, LPL is not the only lipase affected by Lmf1, as hepatic lipase (HL) and endothelial lipase (EL) activities are also diminished in Lmf1 deficiency in both mouse and human subjects.[12, 15, 16] Similarly to LPL, HL and EL also require homodimerization for catalytic activity.[17, 18] In contrast, the enzymatic activity of pancreatic lipase, a related enzyme that is functional as a monomer,[19] is not dependent on Lmf1 function.[20] Based on these observations, Lmf1 has been proposed to play a role in the assembly of folded lipase subunits into active dimers, or stabilization of the latter.[21]

To date, the identity of factors limiting the efficiency of LPL maturation *in vivo* has remained unknown. Based on *in vitro* studies implicating dimerization as a major bottleneck in lipase maturation[22] and the suspected role of Lmf1 in this process, we hypothesized that Lmf1 may be a rate-limiting factor in the generation of active LPL.[23] To test this hypothesis, we overexpressed Lmf1 in adipose, heart and muscle tissues of transgenic mice. Our results demonstrate that elevated cellular levels of Lmf1 result in increased LPL activity in all tissues tested. While Lmf1 overexpression increased LPL specific activity, but not LPL mass, in omental and subcutaneous adipose tissues, the converse (i.e. higher LPL mass, but no change in specific activity) was observed in heart and the gonadal adipose depot. We also extended these studies to humans by investigating the relationship between LMF1 and LPL using association analysis and detected significant association between genetic variants in *LMF1* and post-heparin LPL activity. Taken together, our results indicate that Lmf1 is not only required for lipase maturation, but experimental and natural variation in Lmf1 expression can also modulate LPL activity *in vivo*.

METHODS

A detailed description of methods is provided in the Supplemental Materials section available online at http://atvb.ahajournals.org. aP2-Lmf1 and Mck-Lmf1 transgenic mice have been generated on the FVB/J genetic background, maintained in a specific pathogen-

free facility under 14:10 hour light cycle and were fed a chow diet. All animal studies were approved by the Institutional Animal Care and Use Committee at the Cedars-Sinai Medical Center. Human study participants were recruited in the Helsinki and Turku University Central Hospitals and gave informed consent. The study design was approved by the ethics committees of the participating centers.

RESULTS

Elevated LPL activity in aP2-Lmf1 transgenic mice

To investigate the role of Lmf1 in adipose tissue, we generated transgenic (Tg) mice with elevated Lmf1 expression using the 5.4-kb enhancer/promoter region of the aP2 gene (Fig. 1A), which directs expression in terminally differentiated adipocytes.[24, 25] To facilitate the detection of exogenous Lmf1 protein in tissues, we included an N-terminal myc epitope tag, which does not interfere with the lipase maturation function of Lmf1.[26] As determined by immunoblotting with an anti-myc antibody, the transgene-derived myc-Lmf1 protein was specifically expressed in subcutaneous, omental and, at a lower level, gonadal adipose tissue in aP2-Lmf1 mice (Fig. 1B and 1C). To assess the relative expression of endogenous vs exogenous Lmf1, we performed immunoblotting with an antibody recognizing the C-terminus of the protein.[12] Myc-Lmf1 expression in gonadal adipose was comparable (0.75-fold higher) to that of the endogenous protein, whereas omental (2.1-fold) and subcutaneous (2.9-fold) adipose tissues exhibited higher levels (Fig. 1C).

We previously demonstrated that loss-of-function mutations in *Lmf1* result in diminished LPL activities.[12] To test if LPL is also affected by elevated Lmf1 expression, we determined lipase activities in tissues of aP2-Lmf1 mice. LPL activities were significantly higher in all Tg adipose depots in both fasted (Fig. 2A) and fed (Supplementary Fig. S1) conditions. Furthermore, similar results were obtained in a second, independent line of aP2-Lmf1 mice (Supplementary Fig. S2). These results suggest that endogenous Lmf1 levels limit the expression of LPL activity in adipose tissue.

Total tissue lipase activities measured in our experiments include both intracellular (i.e. before secretion from adipocytes) and extracellular LPL, the latter representing functionally relevant enzyme. To determine if LPL activity is increased in the functional compartment of Tg adipose tissue, fat pads were incubated with heparin *ex vivo* and lipase activity released into the medium was measured. Heparin-released LPL activity from Tg adipose was 3-fold elevated compared to wild-type tissue (Supplementary Fig. S3A) indicating that Lmf1 overexpression results in increased secreted enzyme activity.

Next, we investigated potential molecular mechanisms responsible for increased LPL activity in transgenic tissues. Consistent with the post-translational role of Lmf1 in LPL maturation, LPL mRNA expression was unchanged in tissues of aP2-Lmf1 mice (Supplementary Fig. S4A). However, the analysis of LPL protein expression by ELISA revealed distinct differences among adipose depots. In gonadal adipose, LPL mass was significantly increased in female aP2-Lmf1 mice and showed a trend toward that effect in males, which explains the higher LPL activities detected in this tissue (Fig. 2B). In contrast, LPL mass was largely unaffected in omental and subcutaneous depots, with the exception of omental adipose in male mice (Fig. 2B). These data suggest that in omental and subcutaneous adipose, a larger proportion of total LPL mass was present in a catalytically active form in aP2-Lmf1 mice. Indeed, calculation of LPL specific activity (i.e. LPL activity per LPL mass) demonstrated that increased LPL activities were due to higher specific activity of the enzyme in these tissues (Fig. 2C).

To assess the potential metabolic consequences of elevated adipose tissue LPL activity, we characterized metabolic parameters in aP2-Lmf1 mice. No changes were detected in body weight or composition, plasma parameters including lipid, glucose and insulin levels or post-heparin LPL activity (Supplementary Table 1).

Elevated LPL activity in Mck-Lmf1 transgenic mice

The results obtained in aP2-Lmf1 mice prompted us to investigate whether the effects of Lmf1 overexpression on LPL extend to other major LPL-expressing tissues, such as heart and skeletal muscle. We used a 6.5-kb genomic DNA fragment encompassing the promoter, enhancer 1, exon 1 and intron 2 of the Mck gene to drive myc-Lmf1 expression in skeletal muscle and heart (Fig. 3A).[27] As expected, myc-Lmf1 was specifically expressed in these tissues (Fig. 3B). Comparison of exogenous and endogenous Lmf1 levels demonstrated several fold overexpression of myc-Lmf1 protein in Tg muscle (12-fold) and heart (21-fold) tissues (Fig. 3C).

Consistent with aP2-Lmf1 mice, Lmf1 overexpression also increased tissue LPL activities in heart and muscle of Mck-Lmf1 mice under both fasted (Fig. 4A) and fed (Supplementary Fig. S5) conditions. These results were confirmed in a second Mck-Lmf1 mouse line representing an independent transgene integration event (Supplementary Fig. S6). Similar to aP2-Lmf1 adipose tissue, *ex vivo* heparin-release experiments demonstrated increased secreted LPL activity in the extracellular compartment in Mck-Lmf1 muscle (Supplementary Fig. S3B).

Consistent with a post-translational mechanism, elevated LPL activities in Mck-Lmf1 tissues were not associated with increased LPL mRNA expression (Supplementary Fig. S4B). However, in contrast to aP2-Lmf1 adipose tissue, increased LPL activity was primarily due to higher LPL protein levels (Fig. 4B). The only exception to this is female muscle tissue, which exhibited elevated LPL specific activity in Tg animals (Fig. 4C).

Similar to aP2-Lmf1 mice, post-heparin LPL activity and metabolic parameters were indistinguishable between Mck-Lmf1 and wild-type littermates with the exception of slightly lower HDL-cholesterol levels in Tg males (Supplementary Table 2).

LMF1 is associated with post-heparin LPL activities in humans

Although tissue-specific overexpression of Lmf1 did not result in elevated plasma LPL activities in our transgenic mouse models, we hypothesized that genetic variation resulting in altered LMF1 expression in all tissues may affect post-heparin plasma LPL activity in human subjects. To investigate whether common genetic variation influences LPL activity in human samples, we performed a tag-SNP approach in the *LMF1* gene region (130kb). We genotyped 20 SNPs capturing >90% of the SNPs with minor allele frequency (MAF) ≥10% in *LMF1* in 1,100 individuals from 92 Finnish dyslipidemic families. We did not attempt to capture genetic variation with MAF <10%, as our study sample is not sufficiently powered to detect the effects of such variants. The SNPs were tested with continuous post-heparin LPL activity levels in family-based association analyses using quantitative transmission disequilibrium test (QTDT).[28, 29] In these extended families, the heritability estimate for LPL was 0.36 (SE = 0.08; $p = 8 \times 10^{-7}$) indicating that LPL activity has a clear genetic component comparable to the heritability estimates of serum lipid levels.[30]

We identified a common SNP (MAF = 0.4), rs3751666, in intron 1 of *LMF1* to be associated with LPL activity ($p = 3 \times 10^{-4}$) (Fig. 5 and Supplementary Table 3). This result is gene-wide significant, as it surpasses the Bonferroni correction for 20 SNPs tested (Bonferroni-adjusted p = 0.006). There was one other SNP (rs3829491) surpassing the Bonferroni correction (Bonferroni-adjusted p = 0.038), however, this SNP does not represent an

independent association signal as it is in high linkage disequilibrium (LD) with rs3751666 ($r^2 = 0.96$) (Supplementary Table 3). The association of rs3751666 was only nominally significant with plasma TGs and HDL-cholesterol ($p = 0.03$ and 0.01, respectively).

Next, we used an imputation method to extend our association analysis to tagged and non-tagged SNPs in the genomic region of *LMF1* (57 additional SNPs). We imputed genotype dosage (expected allele counts 0-2) using the MACH program[31] and analysis of dosage data was performed with QTDT. Overall, we obtained the strongest evidence of association for the genotyped rs3751666 SNP even though we tested for association with many more common SNPs (MAF >5%) in the region (Supplementary Fig. S7). Furthermore, the strength of the association signal of the regional SNPs was highly correlated with the strength of their pairwise LD with rs3751666 ($r = -0.7$ and $p = 1 \times 10^{-12}$) (Supplementary Fig. S7) suggesting that rs3751666 is the only independent common association signal at this locus. The genotypic means (± SEM) of LPL activity adjusted for age, sex and kinship were 225 ± 1.07 μmol FA/h/ml for the A/A common homozygotes, 217 ± 1.06 μmol FA/h/ml for the A/G heterozygotes, and 210 ± 1.1 μmol FA/h/ml for the G/G rare homozygotes. In agreement with Lmf1 transgenic mouse data, these results suggest that variation in LMF1 expression and/or activity may modulate LPL activity in humans. We did not observe significant associations between the 20 SNPs and post-heparin HL activity, consistent with this lipase being less dependent on LMF1 for maturation than LPL.[12]

DISCUSSION

Previous characterization of loss-of-function mutations identified Lmf1 as a critical factor in the post-translational maturation of lipases in mice and human subjects.[12, 15] The goal of the present study was to investigate whether altered Lmf1 expression also affects this process. We pursued two approaches to address this issue. First, we generated transgenic mouse lines overexpressing Lmf1 in various tissues. Characterization of these mice demonstrated that LPL activity was elevated in all transgenic tissues tested. Second, we sought genetic evidence for an effect of variable LMF1 expression on LPL in human subjects. Consistent with the mouse studies, we detected significant association between *LMF1* SNPs and LPL activity in a dyslipidemic cohort. Taken together, our results extend the previously established role of Lmf1 as a required factor for lipase maturation and suggest that the expression level of this chaperone is a determinant of LPL activity.

Although LPL activity was uniformly increased in all Lmf1-transgenic tissues, initially distinct mechanisms seemed to be operating in different tissues. In some (gonadal adipose, heart and muscle), elevated LPL mass was clearly responsible for increased LPL activity. In others (omental and subcutaneous adipose), higher specific activity of LPL without changes in LPL mass explained elevated tissue LPL activity. Despite the apparent differences, the direct effects of Lmf1 overexpression are likely to be the same in all tissues, namely enhanced LPL dimer assembly in the ER and increased rate of secretion of the active enzyme, as demonstrated in our study. However, the fate of extracellular LPL is tissue context-dependent and determined in large part by the expression of angiopoietin-like protein 4 (Angptl4), which dissociates and inactivates the LPL dimer,[32] and the turnover rate of inactive LPL as a function of tissue perfusion and hepatic degradation.[33] Consistent with such tissue-specific differences, LPL specific activity was found to be highly variable between human adipose depots and adipose vs heart tissue in the mouse.[34, 35] In conclusion, LPL mass and specific activity in a given tissue are subject to the effects of intra- and extracellular biosynthetic and degradation pathways and the apparent variability in our transgenic models is a likely reflection of this complexity.

The aP2 promoter-driven transgenic construct resulted in variable expression in different adipose depots allowing us to assess the relationship between Lmf1 levels and LPL activity. Consistent with previous reports, subcutaneous and gonadal depots exhibited the highest and lowest level of Lmf1 transgene expression, respectively.[36] Accordingly, LPL activities showed a similar overall pattern across adipose depots and in general seemed to be commensurate with the degree of Lmf1 overexpression. An important caveat to the study on Mck-Lmf1 mice is that the transgene was expressed at supraphysiological levels, which limits the interpretation of this model. Nonetheless, in general agreement with the aP2-Lmf1 transgenics, LPL activity was elevated in the heart and muscle of Mck-Lmf1 mice, although several-fold overexpression of Lmf1 resulted in only 30-50% increases in LPL activity. It is conceivable that the relatively modest impact on LPL is due to a saturation effect. Alternatively, the differences between aP2- and Mck-Lmf1 transgenics may reflect distinct mechanisms involved in the regulation of LPL activity in adipose and muscle tissues.[37, 38]

The metabolic effects of elevated tissue LPL activities remained undetectable in our transgenic mouse models. This is not unexpected and is consistent with previous studies on LPL-transgenic mice. For example, LPL overexpression in aP2-LPL mice, which exhibit similarly elevated LPL activities in adipose tissue as aP2-Lmf1 mice, had no effect on plasma lipids, post-heparin LPL activity or other metabolic traits.[39] Likewise, Mck-LPL transgenic mice with muscle-specific LPL overexpression exhibited wild-type levels of plasma lipids, glucose and insulin.[3] However, in contrast to tissue-specific models, transgenic mice overexpressing LPL in all tissues showed elevated plasma LPL activity and altered lipid traits.[40-42] Taken together, these results suggest that the metabolic effects of increased LPL activity limited to individual tissues may be too small to be detected or masked by compensatory changes. Potential compensatory mechanisms may involve reduced LPL expression in non-transgenic tissues or changes in the activities of post-translational regulators of LPL, such as apolipoproteins C2, C3, A5, and angiopoietin-like proteins 3 and 4.[1]

Our transgenic mouse studies suggested that Lmf1 expression level is a determinant of LPL activity. To extend this conclusion to humans, we tested the effects of *LMF1* variation on LPL by performing association analyses between common genetic variants in the *LMF1* region and post-heparin plasma LPL activity in a dyslipidemic cohort. To the best of our knowledge, this is the first study to investigate the effects of common variants on LPL activity in human dyslipidemia. We detected gene-wide significant association between LPL activity and a tag-SNP (rs3751666) within the first intron of *LMF1* suggesting that variation in LMF1 also influences LPL activity in humans. The association signal may be due to LD with functional polymorphism(s) affecting the expression or function of LMF1. Alternatively, as rs3751666 occurs within a short distance (28 bp) from exon 2, it may directly affect splicing of the *LMF1* message. Additional larger cohorts characterized for the lipase phenotype will be required to replicate the association with rs3751666 and fine-map the functional variant.

The apparent lack of associations between *LMF1* and plasma lipid levels in our study and a previous GWAS[5] is likely due to insufficient power to detect small effects. Consistent with the generally small effects of common variants, the impact of rs3751666 on LPL activity is relatively modest. Furthermore, LPL is only one of many factors in the determination of plasma lipid levels. Indeed, LPL is responsible for only 8.8% and 5.6% of the variation in plasma TG and HDL-C levels, respectively, in our cohort. In summary, we provided genetic evidence indicating that common variants of *LMF1* affect plasma LPL activity in humans, which is consistent with the quantitative effects of Lmf1 expression on tissue LPL in mouse models. Although association with lipids could not be detected in the present study, the role of LMF1 in plasma lipid metabolism has been previously established. In particular, rare

LMF1 variants, which were not interrogated in the current analysis or other GWAS, have been demonstrated to exert major effects on plasma TG levels.[12, 15, 43]

The main conclusion of the present study is that variation in Lmf1 expression is associated with changes in LPL activities. Nonetheless, important questions remain to be addressed in future studies. For example, the molecular mechanisms responsible for increased LPL specific activity in some transgenic tissues, but elevated LPL mass in others is not well understood. It is also unclear whether HL and EL are similarly affected by Lmf1 overexpression. Finally, our results raise the interesting possibility of lipase regulation through the modulation of Lmf1 expression and/or activity. Further studies are warranted to investigate the physiological contexts and molecular mechanisms affecting Lmf1 expression and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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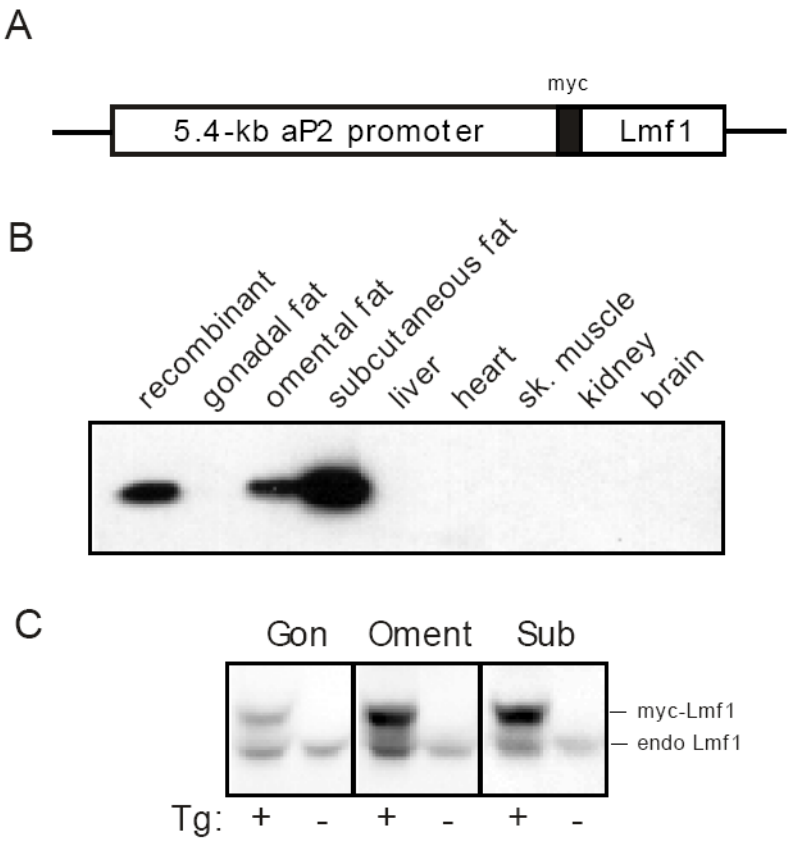

Figure 1.

Lmf1 expression in aP2-Lmf1 transgenic mice. **A**, Schematic representation of the transgene construct containing a 5.4-kb fragment from the aP2 promoter, myc epitope tag and Lmf1 cDNA. **B**, Western blot analysis of aP2-Lmf1 tissues using anti-myc antibody for detection. Equal amounts of total tissue protein were loaded in each lane. The first lane ('recombinant') represents cell lysate from transfected HEK293 cells to indicate the size of recombinant myc-Lmf1 protein. **C**, Western blot analysis of aP2-Lmf1 adipose depots using an Lmf1-specific antibody detecting both endogenous and transgene-expressed proteins.

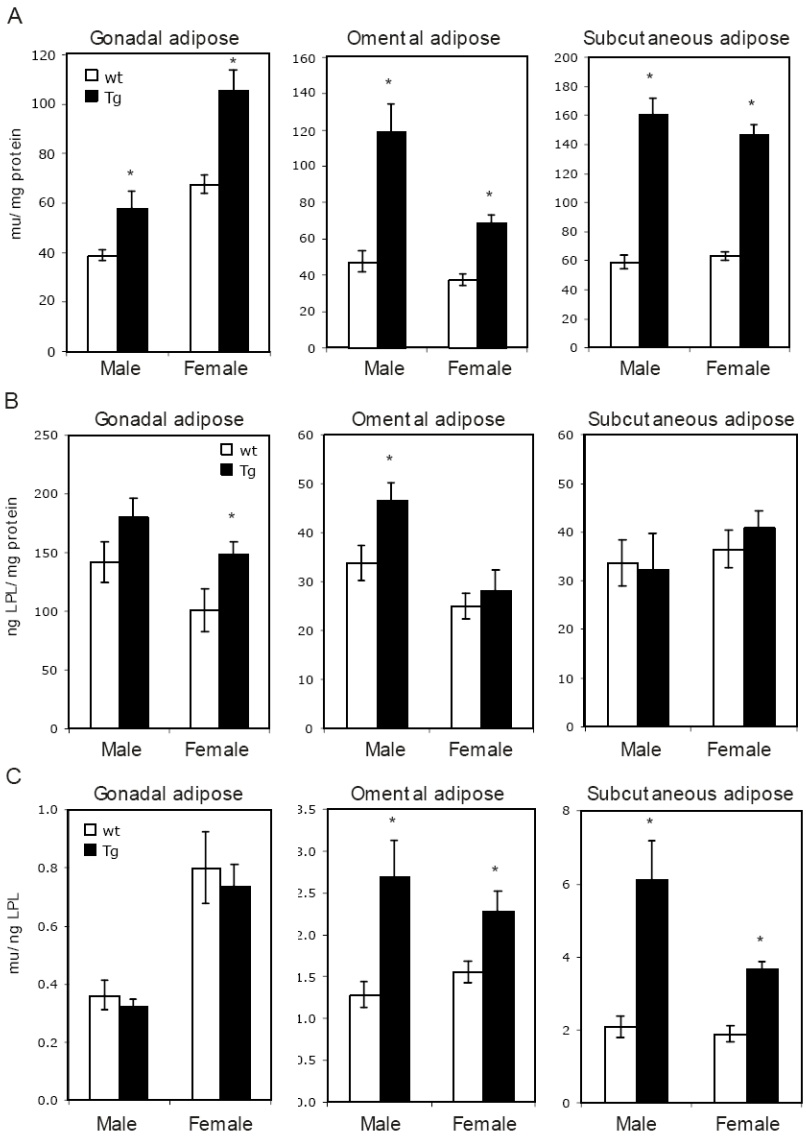

Figure 2.

LPL activity, protein expression and specific activity in fasting aP2-Lmf1 mice. **A,** Tissue LPL activity in wild-type (open bars, n = 8-10) and Tg (filled bars, n = 6-7) adipose depots. **B,** LPL protein mass in the same tissue samples shown in panel A. **C,** LPL specific activity calculated as the ratio of tissue LPL activity and LPL mass. *p<0.05

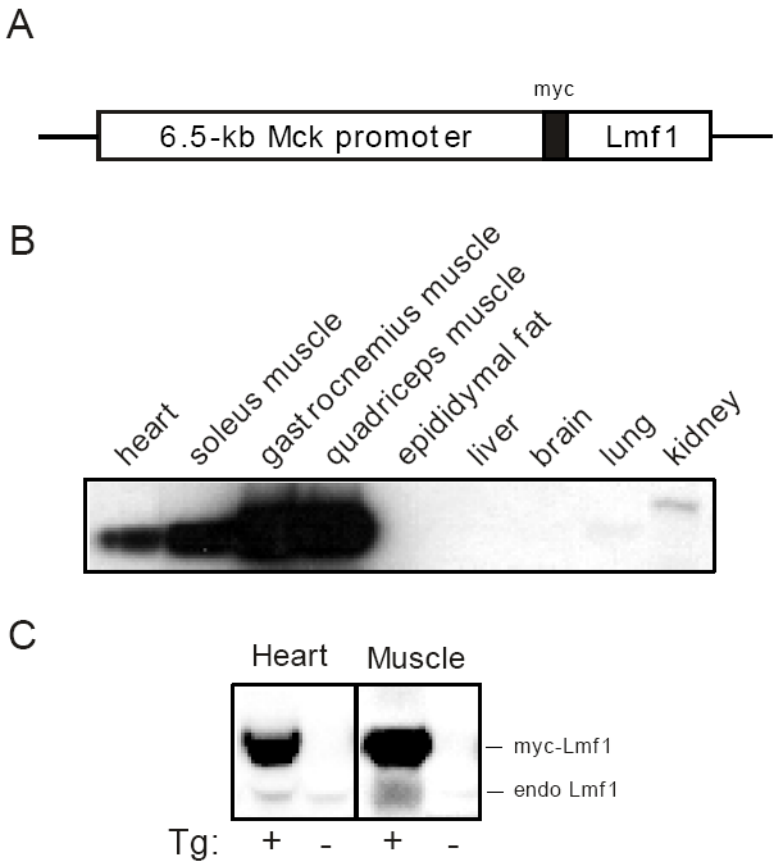

Figure 3.

Lmf1 expression in Mck-Lmf1 transgenic mice. **A**, Schematic representation of the transgene construct containing a 6.5-kb fragment from the Mck promoter, myc epitope tag and Lmf1 cDNA. **B**, Western blot analysis of Mck-Lmf1 tissues using anti-myc antibody for detection. Equal amounts of total protein were loaded in each lane. **C**, Western blot analysis of Mck-Lmf1 heart and muscle using an Lmf1-specific antibody detecting both endogenous and transgene-derived proteins.

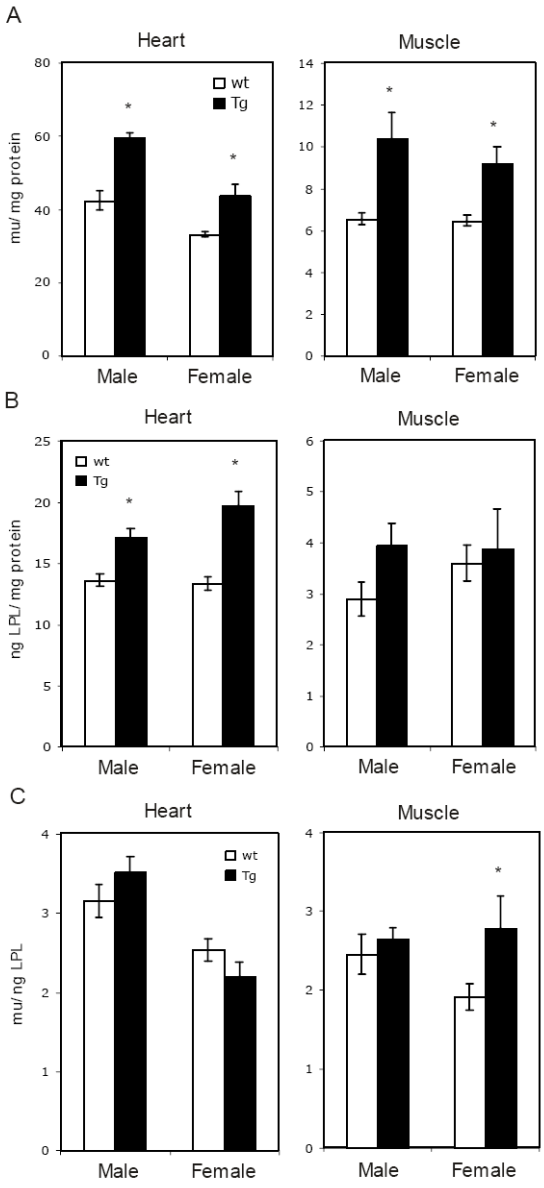

Figure 4.

LPL activity, protein expression and specific activity in fasting Mck-Lmf1 mice. **A,** Total tissue LPL activity in wild-type (open bars, n = 8) and Tg (filled bars, n = 6-8) heart and quadriceps muscle. **B,** LPL protein mass in the same tissue samples shown in panel A. **C,** LPL specific activity calculated as the ratio of tissue LPL activity and LPL mass. *p<0.05

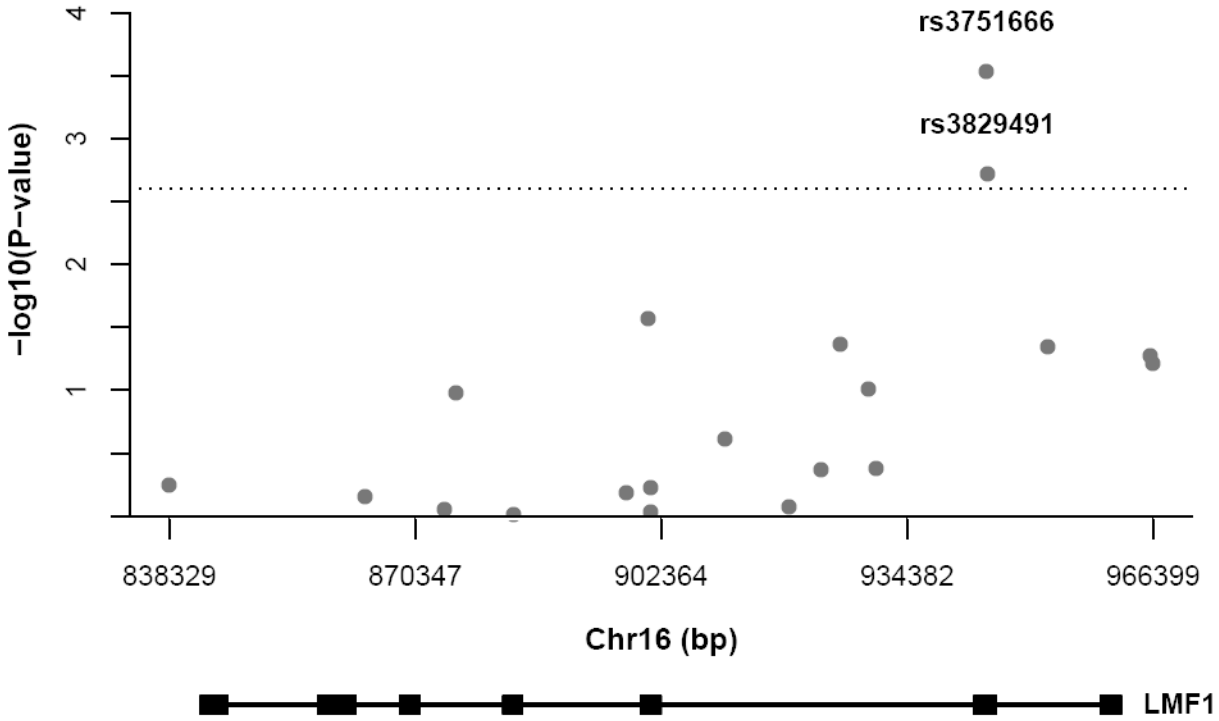

Figure 5.
Association results across the LMF1 gene. The –log10 of the p-values obtained from the QTDT analyses for quantitative LPL activity (range 77-487 μmol/ml) are shown. The dashed line indicates the Bonferroni-corrected significance threshold [$\log_{10}(2.50\times10^{-3})$]. The location of the SNPs is shown in relation to the gene structure of LMF1.