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Genetic Variation at PPP1R3B and Changes in Levels of Liver Glycogen and Triglyceride

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Abstract

Genetic variation at rs4240624 on chromosome 8 is associated with an attenuated signal on hepatic computerized tomography (CT), which has been attributed to changes in hepatic fat. The closest coding gene to rs4240624, *PPP1R3B*, encodes a protein that promotes hepatic glycogen synthesis. Here we performed studies to determine if the X-ray attenuation associated with rs4240624 is due to changes in hepatic glycogen or hepatic triglyceride content (HTGC). A sequence variant in complete linkage disequilibrium (LD) with rs4240624, rs4841132, was genotyped in the Dallas Heart Study (DHS), the Dallas Liver Study, and the Copenhagen Cohort (n=112,428) of whom 1,539 had non-viral liver disease. The minor A-allele of rs4841132 was associated with increased hepatic X-ray attenuation (n=1,572, P=4×10⁻⁵), but not with HTGC

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(n=2,674, P=0.58). Rs4841132-A was associated with modest, but significant elevations in serum ALT in the Copenhagen Cohort ($P=3\times 10^{-4}$) and the DHS ($P=0.004$), and with odds ratios for liver disease of 1.13 (95% CI, 0.97-1.31) and 1.23 (1.01-1.51), respectively. Mice lacking PPP1R3B were deficient in hepatic glycogen, whereas HTGC was unchanged. Hepatic overexpression of PPP1R3B caused accumulation of hepatic glycogen and elevated plasma levels of ALT, but did not change HTGC. *Conclusion:* These observations are consistent with the notion that the minor allele of rs4841132 promotes a mild form of hepatic glycogenesis that is associated with hepatic injury.

Keywords

hepatic steatosis; hepatic glycogen; liver injury; metabolism

Fatty liver disease (FLD) has become the most common liver disease in the Western world, affecting approximately 30% of adults in the U.S.^(1,2) The disease is characterized by accumulation of hepatic fat, which over time can cause inflammation (steatohepatitis), and ultimately end-stage liver disease (cirrhosis, liver cancer).⁽¹⁾ Obesity is the major risk factor for FLD, and the increased prevalence of the disorder mirrors the increase in adiposity.⁽¹⁾ Other risk factors for FLD include a high intake of alcohol, a sedentary lifestyle, and a diet rich in refined sugars.^(1, 3, 4)

Apart from these risk factors, genetics plays an important role in FLD development. Several studies report clustering of the disease in families.^(5, 6) The first genome-wide association study (GWAS) for FLD found a common variant in *PNPLA3* (I148M) that is associated with a 2-3 fold increase in HTGC, as measured by proton magnetic resonance spectroscopy (¹H-MRS).⁽⁷⁾ A subsequent GWAS, which used computerized tomography (CT) to assess HTGC, replicated the association with *PNPLA3*, and identified four additional loci (*NCAN*, *LYPLAL1*, *GCKR*, and *PPP1R3B*) that were associated with hepatic steatosis.⁽⁸⁾ Three of these loci (*NCAN*, *LYPLAL1*, and *GCKR*) were also associated with histologic evidence of NAFLD. The fourth locus, *PPP1R3B* (defined by the SNP rs4240624, which is located 175 kb upstream of *PPP1R3B*) was strongly associated with HTGC ($P=10^{-18}$), but not with histologically determined FLD. Thus, *PPP1R3B* may be associated with a benign form of hepatic steatosis that does not progress to steatohepatitis.⁽⁸⁾

Speliotes et. al.⁽⁸⁾ noted that the discrepant effects of variation in *PPP1R3B* on liver fat and histologic FLD may have other explanations. The apparent discrepancy may represent a statistical artifact: either a false positive association with liver fat or a false negative association with histologic indices of disease. Alternatively, genetic variation in *PPP1R3B* locus may be associated with other factors, such as iron or glycogen, that affect hepatic X-ray attenuation independently of HTGC.⁽⁹⁾

The nature of the association between *PPP1R3B* and hepatic steatosis has important implications for FLD. If rs4240624 confers a benign form of hepatic steatosis, then liver TG *per se* is not pathogenic, and other factors must explain the progression of FLD that occurs in a subset of individuals. Subsequent studies replicated the association between rs4240624 and liver fat measured by hepatic X-ray signal attenuation⁽¹⁰⁾ and by ultrasound.⁽¹¹⁾ These findings make false positive association unlikely. They also support the contention that

variation in *PPP1R3B* is associated with HTGC, rather than glycogen, although neither CT nor ultrasound directly measures liver fat content. Gorden *et al.*⁽¹²⁾ failed to find an association between rs4240624 and FLD using histologically determined liver fat content. Therefore, the relationship between the *PPP1R3B* and liver fat content remains equivocal. The notion that rs4240624 is not associated with histologic FLD also requires confirmation. In the study reported by Speliotes *et al.*⁽⁸⁾, the sample that was available for association with NAFLD (n=592) was much smaller than the sample used for analysis of HTGC (n=7176); thus, the possibility of a false negative association cannot be excluded.

The close proximity of rs4240624 to *PPP1R3B* raises the possibility that the association between the SNP and hepatic X-ray attenuation is due to differences in hepatic glycogen rather than TG. *PPP1R3B* encodes a 285-residue protein that plays a key role in hepatic glycogen synthesis^(13–15)(Fig. 1). *PPP1R3B* binds both protein phosphatase 1 (PP1) and glycogen (Fig. 1A). PP1 then dephosphorylates and activates glycogen synthase, which catalyzes the addition of UDP-glucose to glycogen (Fig. 1B). PP1 also dephosphorylates glycogen phosphorylase, inactivating the enzyme that catalyzes release of glucose from glycogen.⁽¹⁶⁾ Thus, the overall effect of *PPP1R3B* is to promote formation of glycogen.⁽¹⁶⁾ Here we tested the hypothesis that the association between rs4841132 (a proxy variant in complete linkage with rs4240624) and hepatic X-ray attenuation is due to changes in hepatic glycogen rather than TG content. Given that excessive glycogen accumulation is known to cause liver damage in patients with glycogen storage diseases⁽¹⁷⁾, we also tested if rs4841132 confers susceptibility to liver disease. Finally, to confirm that the changes observed in hepatic X-ray attenuation are consistent with effects of alteration in hepatic *PPP1R3B* activity, we altered expression of the protein in mice and determined the effects on hepatic glycogen and TG content.

Experimental Procedures

Studies were approved by IRB and ethics committees of the University of Texas Southwestern Medical Center and Copenhagen and were conducted according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Human Subjects

We included participants from the DHS, the Dallas Liver Study, the Copenhagen City Heart Study (CCHS), and the Copenhagen General Population Study (CGPS).

The DHS is a multiethnic, probability-based sample of Dallas County residents that was collected between 2000 and 2002,⁽¹⁸⁾ and 2007 and 2009. Ethnicity was self-reported in accordance with U.S. census categories. For this study we included 2,674 DHS participants in whom HTGC was measured using ¹H-MRS, and 1,275 additional individuals for the analysis of serum ALT levels, and for use as controls for the Dallas Liver Study.

The Dallas Liver Study is a multiethnic sample of patients with liver disease of non-viral etiology. Participants were recruited from liver clinics at UT Southwestern and Parkland Health and Hospital System in Dallas, TX. Participants completed a questionnaire on ethnic/racial background, medical history, lifestyle factors, and family history of liver disease and

other diseases. Additional clinical information was extracted from medical records by a trained technician. We included all African-Americans, European-Americans, and Hispanic-Americans (n=514). As healthy controls we used 514 age-matched and ethnicity-matched participants from the DHS. All individuals with a known etiology to their liver disease were not included.

The CCHS and CGPS are prospective studies of the Danish general population initiated in 1976 and 2003, respectively.⁽¹⁹⁾ All participants from the CCHS and CGPS were white and of Danish descent, as determined by the National Danish Civil Registration System. We combined the CCHS and CGPS into one cohort, totaling 107,447 individuals (Copenhagen Cohort). Individuals with the following ICD codes were included: ICD8: 57109 (alcoholic cirrhosis), 57111 (nonalcoholic steatohepatitis), 57119 (fatty liver), 57192 (unspecified cirrhosis), and 57199 (nonalcoholic cirrhosis), and ICD10: K70.3 (alcoholic cirrhosis), K74.0 (fibrosis or cirrhosis), K74.6 (unspecified cirrhosis), K76.0 (fatty liver), and K76.9 (liver disease, unspecified). These ICD-codes were selected since they are the ones most likely to be used in patients with liver disease of unknown etiology. Data were collected from the National Danish Patient Registry, and the National Danish Causes of Death Registry from January 1, 1977 to November 10, 2014. The National Danish Patient Registry has information on all patient contacts with clinical hospital departments in Denmark, including emergency wards and outpatient clinics (from 1994). The National Danish Causes of Death Registry contains data on the causes of all deaths in Denmark, as reported by hospitals and general practitioners. The diagnoses were combined into a dichotomous liver disease endpoint. In total, 1,025 participants in the Copenhagen Cohort had a diagnosis of liver disease, defined by ICD-codes as described above.

Measurement of HTGC and CT X-ray Attenuation

HTGC was measured in the DHS using ¹H-MRS as previously described.⁽²⁰⁾ Hepatic CT attenuation was measured in 1,572 participants from the CGPS. A random sample of participants from the CGPS was invited to undergo a CT scan of the thorax and upper abdomen (Aquilion One; Toshiba Medical Systems Corporation, Tokyo, Japan). For liver fat assessment, a single, non-contrast, 16 cm volume scan of the upper abdomen was acquired and reconstructed using AIDR3D (Bodyfilter FC12).⁽²¹⁾ CT-X-ray attenuation in liver segments 5-6 was measured using circular ~1.5 cm² regions of interest.

Supplemental Methods

See the Supplemental Methods for details on i) other measurements and genotyping, ii) generation of *Ppp1r3b* KO mice, iii) generation of mice lacking a 10.6 kb region corresponding to *LOC157273*, iv) animal experimental procedures, v) generation of Adeno-PPP1R3B and overexpression of PPP1R3B in mice, vi) transfection of cells with *LOC157273* and PPP1R3B, vii) RT-PCR and RNA-sequencing, and viii) statistical analysis.

Results

Baseline characteristics of the cohorts used in this study are shown (Table 1). The DHS and Dallas Liver Study are multiethnic, and the Copenhagen Cohort is 100% White Europeans.

The frequency of rs4841132-A was 9% in Whites, 12% in African-Americans, and 24% in Hispanic-Americans.

ASSOCIATION BETWEEN RS4841132 AND HEPATIC X-RAY ATTENUATION

Previously, rs4240624 was found to be associated with HTGC.⁽⁸⁾ Analysis of SNPs within the region revealed another variant, rs4841132 that is 635 bp downstream of rs4240624. The two SNPs are in complete LD (Fig. 1C). rs4841132 is located in exon 2 of the major transcript of an intergenic long noncoding RNA (lncRNA), LOC157273, which is located 175 kb upstream of *PPP1R3B* (Fig. 1C). rs4841132-A (minor allele) was associated with increased hepatic X-ray attenuation among 1572 participants from the Copenhagen Cohort (Fig. 2A, left). Mean Hounsfield units (HU) were 58.8, 60.6, and 63.2 for GG-homozygotes, GA-heterozygotes and AA-homozygotes, respectively ($P\text{-trend}=4\times 10^{-5}$). To assess changes in attenuation due to differences in HTGC, we examined the same population after stratifying by the steatogenic PNPLA3(I148M) genotype (Fig. 2A). As expected, 148M was associated with decreased X-ray attenuation ($P\text{-trend}=3\times 10^{-4}$). The absolute per-allele effect size on X-ray attenuation of rs4841132-A was approximately twice that of *PNPLA3*(148M) (+2.4 vs -1.4 HU per allele). Thus, the A-allele would be predicted to be associated with a substantial reduction in HTGC.

ASSOCIATION BETWEEN RS4841132 AND HTGC

Next we examined the relationship between rs4841132-A and HTGC in the DHS where HTGC was measured using ¹H-MRS.⁽²⁰⁾ No association was found between HTGC and rs4841132-A in the entire cohort (Fig. 2B), or after stratifying participants by ethnicity ($P>0.40$, Fig. S1). Using a recessive model (AA vs GG+GA) did not change the null association ($P=0.37$). As shown previously, *PNPLA3*(148M) was strongly associated with HTGC in the DHS ($P=3\times 10^{-16}$) (Fig. 2B, right).⁽⁷⁾ Individuals homozygous for the risk variant (148M) had a 2-fold increase in HTGC compared to individuals homozygous for the non-risk allele (148I). This finding does not support the hypothesis that rs4841132 is associated with HTGC.

ASSOCIATION BETWEEN RS4841132 AND SERUM LIVER ENZYMES

To assess the effect of rs4841132 on hepatic inflammation, we examined the association between the variant and circulating liver enzyme levels. In both the Copenhagen Cohort and the DHS, the A-allele was associated with increased alanine aminotransaminase (ALT) levels (Fig. 3A, $P\text{-trend}=3\times 10^{-4}$ and 0.004, respectively). A similar but weaker association were seen for AST in the larger cohort ($P=0.04$, Table S1), but not in the DHS ($P=0.27$, Table S2). The A-allele was also associated with increased levels of alkaline phosphatase (ALP) in both the DHS ($P=0.001$) and Copenhagen Cohort ($P=9\times 10^{-25}$) (Fig. S2). Consistent with these findings, the minor allele of rs6984305, a sequence variant in LD with rs4841132 ($r^2=0.61$), was associated with increased ALT ($P=0.043$) in a large GWAS of serum liver enzymes.⁽²²⁾ No association was found between rs4841132 and serum levels of bilirubin or gamma-glutamyltransferase in either the Copenhagen Cohort (Table S1) or DHS (Table S2). The association of rs4841132-A with increased plasma ALT levels is not consistent with the hypothesis that the allele is associated with protection from FLD.

ASSOCIATION BETWEEN RS4841132 AND NONVIRAL LIVER DISEASE

Given that the rs4841132-A was associated with elevated liver enzymes, we tested if the variant was associated with increased risk of liver disease. For this analysis we used two cohorts of patients who had liver disease due to obesity (NAFLD) or to alcohol: the Copenhagen Cohort, which included 1,025 patients with liver disease, and 106,422 controls and the Dallas Liver Study, which included 514 patients with liver disease (65% Hispanic-American, 30% European-American, 5% African-American), and 514 age- and ethnicity-matched controls from the DHS (Fig. 3B). Patients with viral hepatitis were excluded. In the Copenhagen Cohort, the odds ratio for liver disease for rs4841132-AA versus -GG homozygotes was 1.91 (95% CI: 1.12-3.26). In both cohorts the rs4841132-A tended towards an association with liver disease, but the association did not reach significance ($P=0.12$) in the Copenhagen Cohort. Excluding individuals with ICD-codes specific for NAFLD ($n=151$) and/or alcoholic cirrhosis ($n=168$) did not change the results (data not shown). The trend was significant in the Dallas Liver Study ($P=0.045$), but the confidence intervals for all genotypes included 1. Meta-analysis across the two studies yielded a summary per-allele odds ratio of 1.16 (1.03-1.31, $P=0.01$). These data are consistent with an association between the rs4841132-A allele and liver disease.

As a positive control for the cohort, we tested the association of PNPLA3 (148M) with liver disease and plasma ALT levels in the Copenhagen Cohort, the DHS, and the Dallas Liver Study (Table S3). As expected, the M-allele was robustly associated with elevated ALT⁽¹⁹⁾, and with increased risk of liver disease.

RS4841132 MAPS TO A LIVER-SPECIFIC NONCODING RNA, LOC157273, THAT IS ASSOCIATED WITH INCREASED EXPRESSION OF PPP1R3B

Fig. 1C shows a LD map of the chromosomal region surrounding rs4841132. No significant LD was observed between rs4841132 and any of the SNPs in either flanking gene: *PPP1R3B* or *TNKS*, which encodes tankyrase, a telomere-associated poly-ADP ribose polymerase. Thus, the association between rs4841132 and hepatic glycogen cannot be attributed to LD with a sequence variation in either of the adjacent coding genes.

As noted above, rs4841132 is located in exon 2 of *LOC157273*, a liver-specific lncRNA expressed in humans and great apes. *LOC157273* resides 175 kb upstream of *PPP1R3B* and 230 kb upstream of *TNKS* (Fig. 1C and 4A). To determine if the variant is associated with differences in levels of *PPP1R3B* or *TNKS* mRNA, we examined an eQTL database ($n=707$ liver samples) collected at Massachusetts General Hospital (Table S4).^(23, 24) In that database, the rs4841132-A was associated with a significantly higher level of *PPP1R3B* mRNA in human livers ($P=9\times 10^{-17}$). This finding supports the hypothesis that the minor allele of rs4841132 increases expression of hepatic *PPP1R3B*, which would be predicted to increase hepatic glycogen synthesis (Fig. 1B). No association was found between rs4841132-A and *TNKS* mRNA levels. In the same database, the A-allele was associated with reduced RNA levels of *LOC157273* in human liver ($P=3\times 10^{-8}$), raising the possibility that effects of rs4841132-A on *PPP1R3B* are mediated by *LOC157273*.

To determine if differences in *LOC157273* expression alter *PPP1R3B* mRNA levels or the glycogen content of cells, we expressed *LOC157273* in three human cell lines that synthesize glycogen: embryonic kidney cells (293 cells),⁽²⁵⁾ and two immortalized human hepatocellular carcinoma cell lines, HepG2 and HuH7-cells. As a positive control, we expressed *PPP1R3B* in 293 cells, which was associated with a 20-fold increase in cellular glycogen content when compared to cells expressing vector alone (Fig. S3A).⁽¹³⁾ In contrast, robust expression of *LOC157273* (Ct-values 14-17 by RT-PCR) in the three cell lines failed to alter cellular levels of *PPP1R3B* mRNA (Ct-values 27-31) or glycogen (Fig. S3B). Thus, *LOC157273* expression appears not to alter expression levels of *PPP1R3B*, or cause changes in glycogen content, at least in these cultured cell lines.

LOC157273 is not conserved in mice (Fig. 4A). To identify transcripts that might serve a function similar to *LOC157273* in mice, we performed whole transcriptome analysis of mouse liver and identified transcripts that mapped to the interval between *Ppp1r3b* and *Tnks*. An unannotated transcript from the locus, which we have called lncRNA1 (mm10, Chr8: 35206516-35207482) was identified (GenBank Accession ID: MF573324). The transcript is predicted to have a single 967-bp exon and is robustly regulated by food intake (Fig. 4B).

To determine if the mouse lncRNA transcribed from the region, or the genomic region *per se*, plays a functional role in regulating *Ppp1r3b*, we used CRISPR to generate mice with a 10.6 kb deletion (mm10, Chr8: 35197190-35207767) that included both the lncRNA1 and the genomic region corresponding to human *LOC157273*. Mice homozygous for the deletion were viable and exhibited no overt phenotype. As anticipated, no lncRNA1 transcript was present in mice homozygous for the deletion (Fig. 4C). Levels of *PPP1R3B* mRNA, glycogen and Tg did not differ in the KO mice (Fig. 4C). Serum glucose levels did not differ between the strains (data not shown). Thus, lncRNA1 and/or the genomic region corresponding to *LOC157273*, appear not to influence expression of *Ppp1r3b* or alter hepatic glycogen or fat content in mice.

HEPATIC GLYCOGEN CONTENT WAS REDUCED AND HTGC WAS UNCHANGED IN *Ppp1r3b*^{-/-} MICE

To test the hypothesis that hepatic X-ray attenuation associated with rs4841132-A is due to an increase in hepatic glycogen content caused by increased expression of *PPP1R3B*, we examined the hepatic glycogen and TG levels in the livers of mice that either lack (KO) or overexpress *PPP1R3B*. As we were performing these studies, Rader and his colleagues developed liver-specific *Ppp1r3b* KO mice.⁽²⁶⁾ These mice had reduced hepatic glycogen due to a reduction in incorporation of glucose into glycogen. The mice also had lower blood glucose levels on both an *ad lib* diet and after a 4 h fast. The effect of *Ppp1r3b* inactivation on liver TG content was not reported.

We used CRISPR to generate mice with a frameshift mutation in *Ppp1r3b* that is predicted to truncate the protein at residue 22 (p.Pro14Leufs*22). Breeding heterozygous carriers of the deletion yielded 14% homozygotes (*Ppp1r3b*^{-/-}) among the offspring (56% *Ppp1r3b*^{+/-} and 30% *Ppp1r3b*^{+/+}). Despite being born in less than the expected Mendelian ratio, *Ppp1r3b*^{-/-} mice were viable, fertile, and had a similar body weight at 8 weeks. In chow-fed male mice (8 weeks), fed for 4 h and then killed, hepatic glycogen content was markedly reduced in

Ppp1r3b^{-/-} mice compared to WT littermates (0.5% vs 4.4% glycogen/liver weight, $P < 0.001$). HTGC was similar in KO and WT mice (Fig. 5A). Plasma levels of cholesterol, TG, glucose and insulin (both fed and fasted for 18 h) were unchanged (Fig. 5A and S4). These findings indicate that variation in *Ppp1r3b* expression has a major impact on liver glycogen levels but is not associated with significant changes in HTGC.

ADENOVIRUS- MEDIATED OVEREXPRESSION OF PPP1R3B IN MOUSE LIVER INCREASES GLYCOGEN CONTENT

Overexpression of PPP1R3B in livers of mice and rats is associated with increased hepatic glycogen content.^(26, 27) Similarly, we found that adenovirus-mediated expression of PPP1R3B at supra-physiological levels in the livers of mice caused a dramatic increase in liver weight and hepatic glycogen content (Fig. 5B). Forty eight hours after injection of recombinant adenoviruses, livers of the mice expressing the mouse PPP1R3B transgene were visibly larger than those injected with the control virus (7.5% vs 6.0% liver/body weight, $P < 0.001$). The hepatic glycogen content was increased in these mice (7.8% vs. 2.4% glycogen/liver weight, $P < 0.001$) (Fig. 5B). The levels of hepatic PPP1R3B mRNA were 4-fold higher in mice expressing the PPP1R3B transgene (Ct=18 vs 20, Fig. S5). No changes in HTGC were seen with PPP1R3B overexpression (Fig. 5B). Plasma levels of ALT, ALP, and cholesterol were increased (Fig. 5B and Fig. S5) whereas TG levels were reduced in the PPP1R3B-treated mice (Fig. 5B); plasma levels of glucose and insulin were unchanged in these animals (Fig. S5).

Discussion

The main finding of this study is that the minor A-allele of rs4841132 is associated with increased hepatic X-ray attenuation on CT scan, but not with hepatic fat content. Our data support a model in which rs4841132-A is associated with increased activity of PPP1R3B, an activator of glycogen synthase and inhibitor of glycogen phosphorylase (Fig. 1). The net effect of the variant is to promote glycogen accumulation in the liver, which increases the attenuation of X-rays by this organ.⁽⁹⁾ The accumulation of glycogen in carriers of rs4841132-A is associated with elevated circulating levels of ALT and also appears to confer increased susceptibility to liver disease. Observations in mice were consistent with this model: genetic manipulation of *Ppp1r3b* activity in mice resulted in large changes in liver glycogen content with no change in HTGC.

In agreement with prior studies, we found that variation at the *PPP1R3B* locus was strongly associated with hepatic X-ray attenuation.⁽⁸⁾ However in our population, the genotype (GG) that was associated with decreased attenuation (consistent with increased fat) was associated with a reduce plasma ALT levels and reduced risk of liver disease. In contrast, the PNPLA3 genotype associated with decreased attenuation and increased HTGC was associated with higher ALT levels and with liver disease. These data are consistent with our observation that rs4841132 was not associated with HTGC (Fig. 2) and with previous studies where histology was used to assay hepatic TG.^(8, 12) A study in the NHANES III cohort reported an association between the rs4240624 variant and HTGC measured using ultrasonography (OR: 1.28; 95% CI, 1.03-1.59; $P = 0.03$).⁽¹¹⁾ However, the lack of association between liver fat

content and PNPLA3(148M) [OR 1.14 (95% CI, 0.97-1.35)] in that study raises concerns about other reported associations. Taken together, the available data support the hypothesis that hepatic glycogen is the factor underlying the robust X-ray attenuation associated with rs4841132-A.

PPP1R3B increases hepatic glycogen accumulation by promoting dephosphorylation and *activation* of glycogen synthase and dephosphorylation and *inactivation* of glycogen phosphorylase, the enzymes governing synthesis and breakdown of hepatic glycogen (Fig. 1B).⁽³¹⁾ The finding that rs4841132-A is associated with higher levels of PPP1R3B mRNA in human livers supports the hypothesis that the allele increases hepatic glycogen content by augmenting PPP1R3B activity.^(23, 24) The mechanism by which rs4841132 influences the transcription of *PPP1R3B* is unknown. Intriguingly, rs4841132 maps to exon 2 of the main transcript of *LOC157273*, a liver-specific lncRNA that is encoded by 3 exons (NR-040039.1) (Fig. 4A). The mature *LOC157273* transcript contains several open reading frames (longest is 119 residues) but these do not appear to be evolutionarily conserved. Could rs4841132 mediate its effect on *PPP1R3B* expression via *LOC157273*, as has been documented for regulation of other protein-coding genes by neighboring lncRNAs? Unfortunately, this hypothesis cannot be tested in a small animal model such as mice because *LOC157273*, like 30% of other lncRNAs, is not present outside of primates.⁽³²⁾

We used whole transcriptome sequencing of mouse liver RNA to identify transcripts from the locus that might perform the same function as *LOC157273*. A single exon-containing lncRNA (lncRNA1 in this paper) sharing no sequence identity with *LOC157273* was regulated in a coordinate fashion with PPP1R3B in response to fasting and refeeding (Fig. 4B). Noncoding RNAs can be expressed from the same location across species, but share little sequence conservation.⁽³³⁾ We hypothesized that the genomic region *per se*, or transcriptional activity at the region, might play a functional role in regulating *Ppp1r3b*. However, genetic deletion of a 10.6 kb genomic region that corresponds to *LOC157273* and includes lncRNA1 did not affect *Ppp1r3b* expression or hepatic glycogen content (Fig. 4C). Thus, we found no evidence that this transcript, or any other sequences syntenic to *LOC157273*, affected expression levels of *Ppp1r3b*.

Rs4841132 is an eQTL for PPP1R3B (minor allele is associated with increased expression) and *LOC157273* (decreased expression) in human liver. Thus, the SNP may affect PPP1R3B by altering expression of *LOC157273*, (ie. *in trans*). We examined the effects of expressing *LOC157273* in immortalized human hepatocyte cell lines (HuH7, HepG2 cells) where endogenous *LOC157273* is expressed at low levels (Ct value>30). Overexpression of the transcript did not influence mRNA levels of *Ppp1r3b* or glycogen content (Fig. S3B). Thus, the mechanism by which sequence variation at the locus affects *PPP1R3B* expression, and the role of the lncRNA *LOC157273* in this interaction, remains unknown. We suspect that replacing the entire ~200 kb genomic region in mice with the corresponding human region might be required to get an experimental handle on this question.⁽³⁴⁾

To determine if the associations observed in human carriers of rs4841132 are consistent with what would be expected from perturbing *PPP1R3B* expression, we generated mice that either lacked or overexpressed the gene and compared their phenotype with the human

association with liver disease will need to be validated in large patient cohorts. Unfortunately, most liver patient cohorts are of European ancestry, and the frequency of rs4841132-A (9% in Whites vs 12% in Blacks and 24% in Hispanics), limits the power to detect associations. In a German/UK GWAS⁽³⁹⁾ of alcoholic liver disease (n=712 alcoholic cirrhotics and 1,426 alcoholic controls), rs4841132-A was not associated with alcoholic cirrhosis (OR: 0.97 [95% CI, 0.76-1.25]). Larger patient cohorts, preferably of Hispanic ancestry, will be required to definitively refute or confirm an association with liver disease.

There were only 1,025 cases with liver disease in the Copenhagen Cohort. Potential explanations for the low prevalence of disease in this population include: 1) the registry-based method of ascertainment selects for symptomatic patients; 2) the median BMI in the Danish cohort was 26 kg/m², lower than in Americans; 3) until recently, NAFLD was under-diagnosed. Nonetheless, the validity of the endpoints used in this paper was supported by the finding that PNPLA3(I148M) was strongly associated with FLD in this study.

Finally, the causal SNP, and the mechanism by which it influences *PPP1R3B* transcription, remains unknown. We studied rs4841132, but there are several other SNPs at the locus that are in high LD, any of which could contribute to the association. We speculated that the lncRNA *LOC157273* could play a role in mediating the effect of rs4841132 on *PPP1R3B* transcription. An alternative possibility is that the causal SNP at the locus acts directly on *PPP1R3B* transcription, and that *LOC157273* is irrelevant.

In conclusion, the minor allele of rs4841132 is associated with increased hepatic X-ray attenuation, and with modestly elevated plasma markers of liver damage. These observations are consistent with the notion that rs4841132 promotes a mild form of hepatic glycogenosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
FLD	fatty liver disease

HTGC	hepatic triglyceride content
1H-MRS	proton magnetic resonance spectroscopy
KO	knockout
NAFLD	nonalcoholic liver disease
lncRNA	long noncoding RNA
PNPLA3	patatin-like phospholipase-domain containing protein 3
PP1	protein phosphatase 1
PPP1R3B	Protein phosphatase 1 regulatory subunit 3B
TG	triglycerides
WT	wildtype
HU	Hounsfield units

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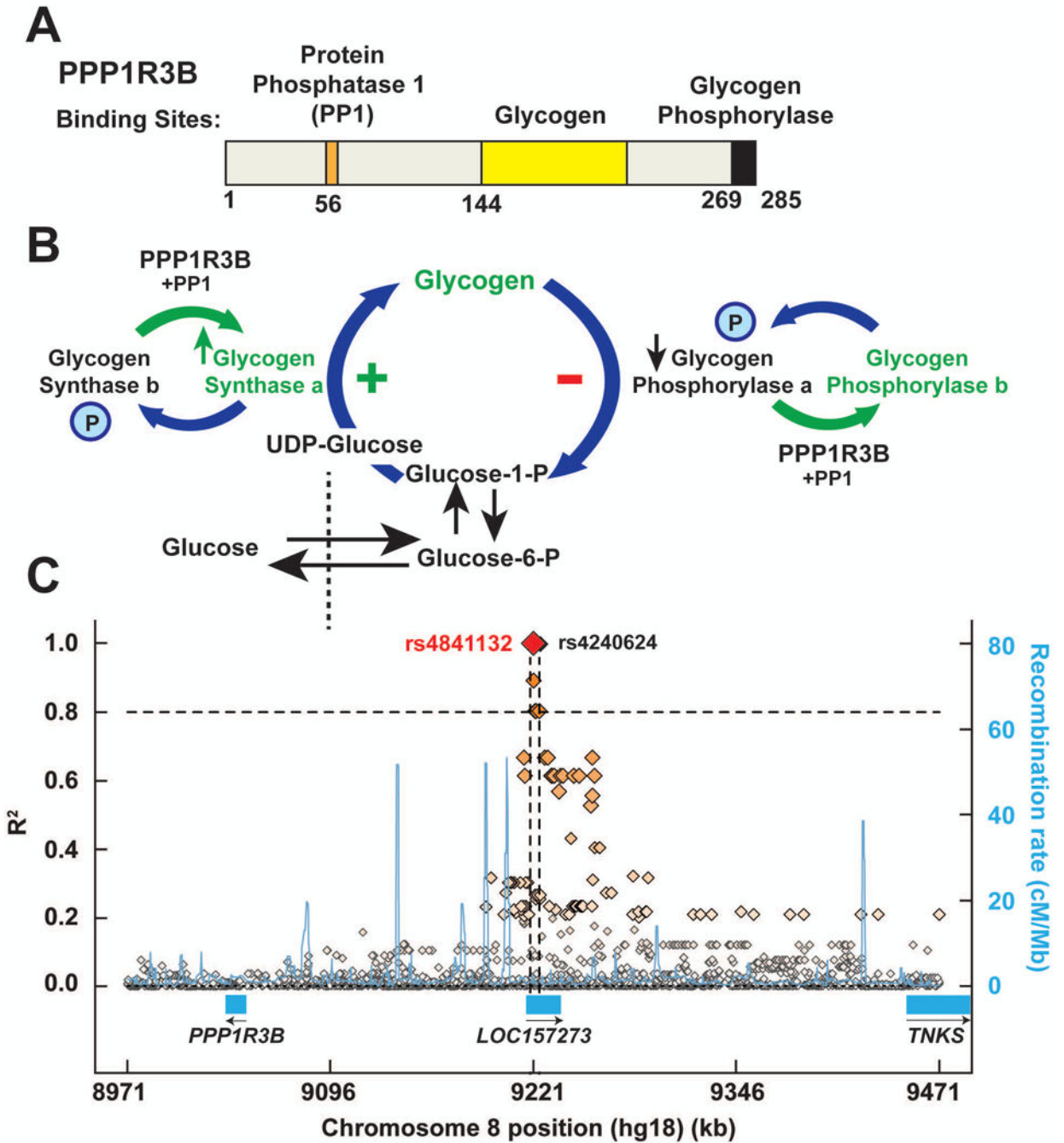


FIG 1. Structure (A), activity (B) and genomic location (C) of PPP1R3B. (A) Functional domains of the PPP1R3B protein showing the regions of the protein that bind protein phosphatase 1 (PP1), glycogen and glycogen phosphorylase. Figure adapted from Doherty *et al.*⁽¹⁵⁾ (B) Schematic of hepatic glycogen metabolism and the function of PPP1R3B. Glucose taken up by the liver is phosphorylated and then converted to UDP-glucose prior to being incorporated into glycogen by glycogen synthase. Glycogen phosphorylase breaks down glycogen, releasing glucose-1-phosphate. PPP1R3B targets protein phosphatase 1 (PP1) to

glycogen synthase and glycogen phosphorylase. PP1 then dephosphorylates these enzymes, leading to activation of glycogen synthase, and de-activation of glycogen phosphorylase. The overall effect of PPP1R3B is to promote glycogen formation. (C) Genomic region flanking rs4841132 (500 kb). The diamonds indicate individual genetic variants. Linkage disequilibrium of the variants with rs4841132 (R^2) is given on Y axis (left) and the recombination hotspots are shown in blue with the recombination rates provided on Y axis (right). Note that both rs4841132 and rs4240624 are located in the liver-specific lncRNA *LOC157273* (exon 2 and intron 2, respectively).

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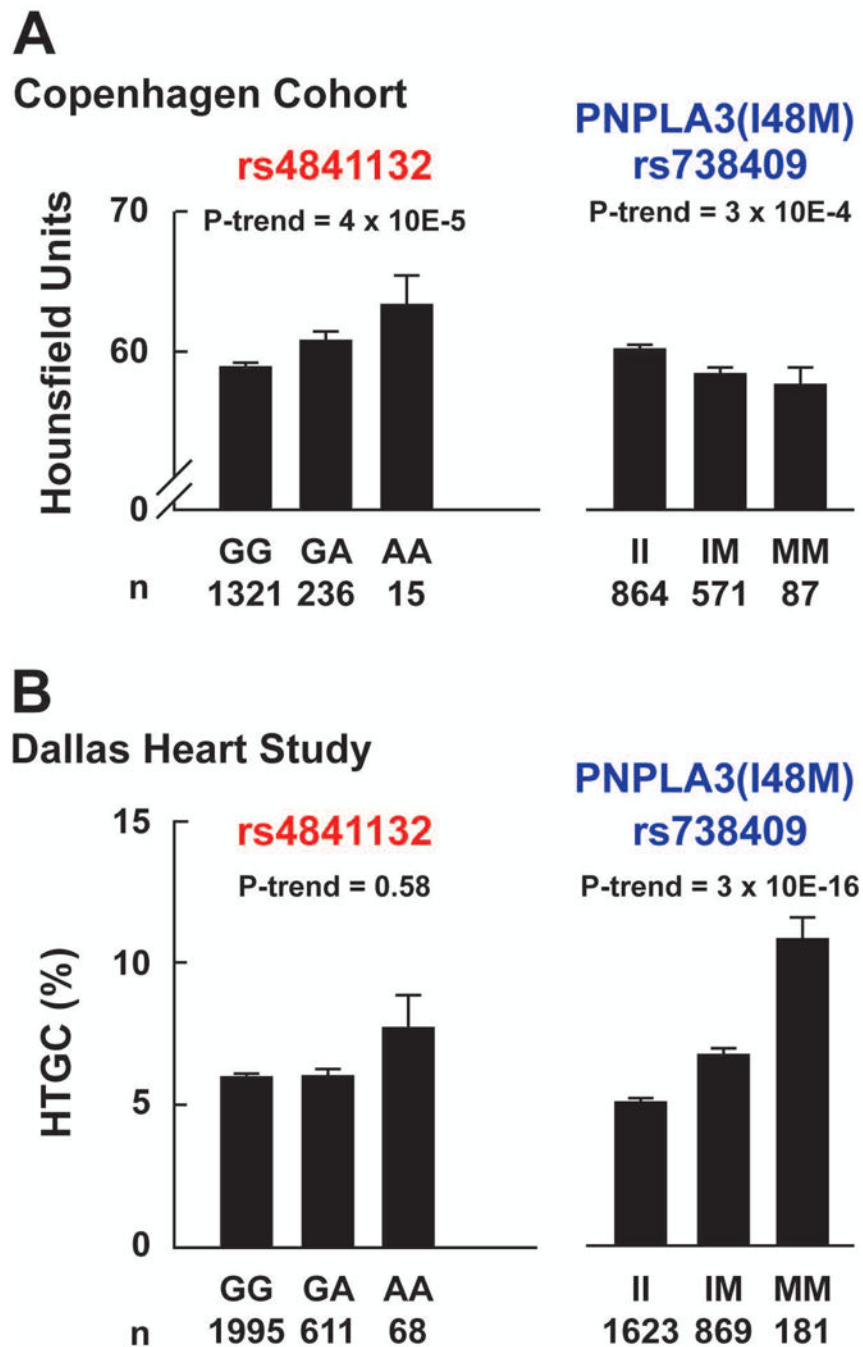


FIG. 2. X-ray attenuation and HTGC as a function of rs4841132 and *PNPLA3*(I48M) (rs738409) genotype. (A) Hepatic CT was measured in 1,572 participants from the Copenhagen Cohort. (B) Magnetic resonance spectroscopy (MRS) was available in 2,674 participants from the Dallas Heart Study. Rs4841132 and rs738409 were genotyped as described in the Methods. P-values are from linear regression, adjusted for age, sex, body mass index and ethnicity. Bars and error bars indicate means and standard errors of the mean. Note: the Y-axes for hepatic CT measurements are cut at 50.

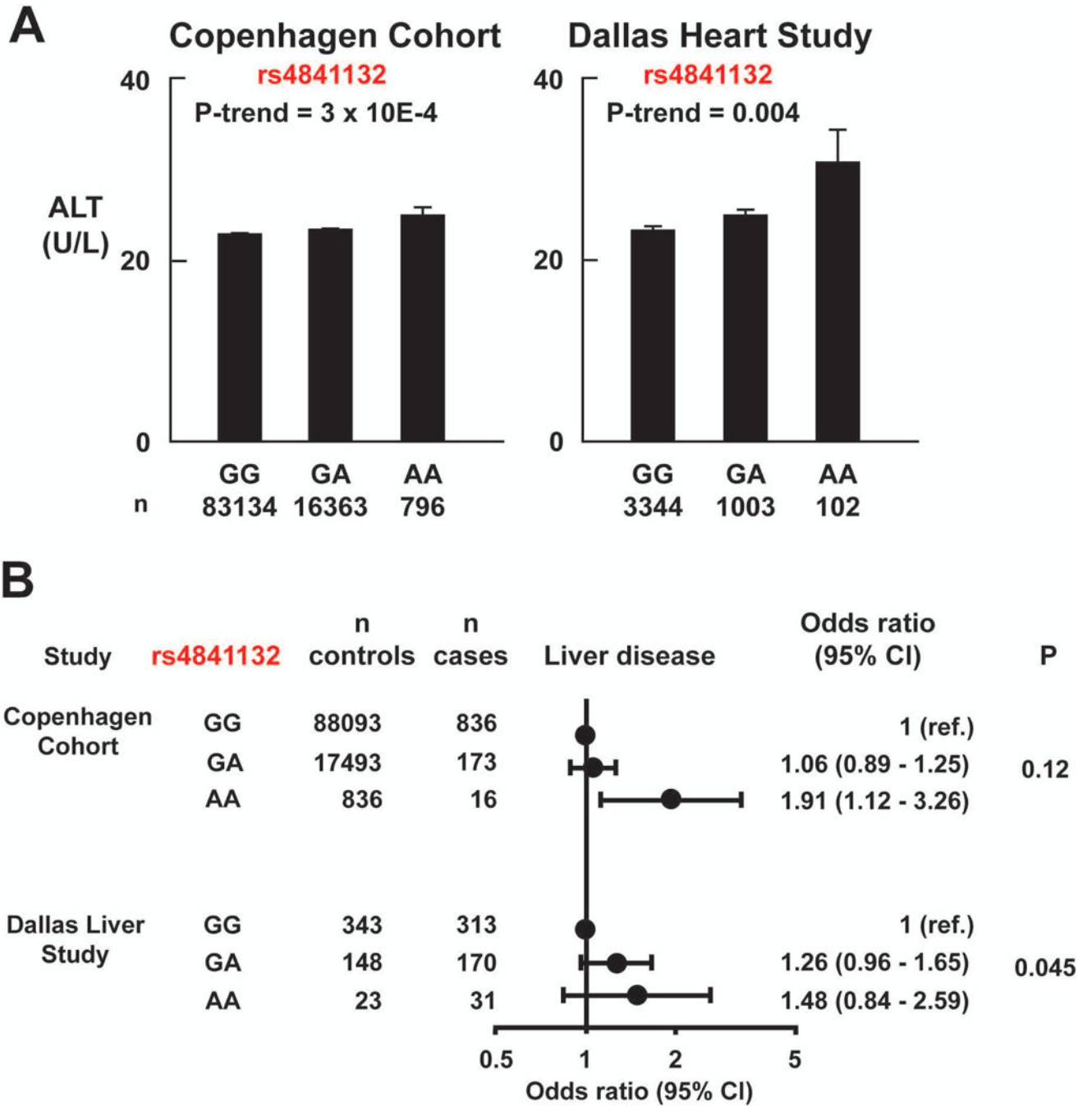


FIG 3. Risk of liver disease as a function of rs4841132 genotype. (A) Association of serum ALT levels with rs4841132 in the Copenhagen Cohort and Dallas Heart Study. Bars and error bars indicate means and standard errors of the mean. P-values are by linear regression, adjusted for age, sex, body mass index and ethnicity. (B) Risk of liver disease in the Copenhagen Cohort and the Dallas Liver Study. Subjects without an ICD-code for liver diseases were used as controls in the Copenhagen Cohort. Healthy subjects from the Dallas Heart Study were used as ethnicity and age-matched controls for the Dallas Liver Study cases.

Homozygotes for the major G-allele acted as the reference group in each study. P-values are tests for trend, adjusted for age, gender and body mass index in the Copenhagen Cohort, and for gender in the Dallas Liver Study.

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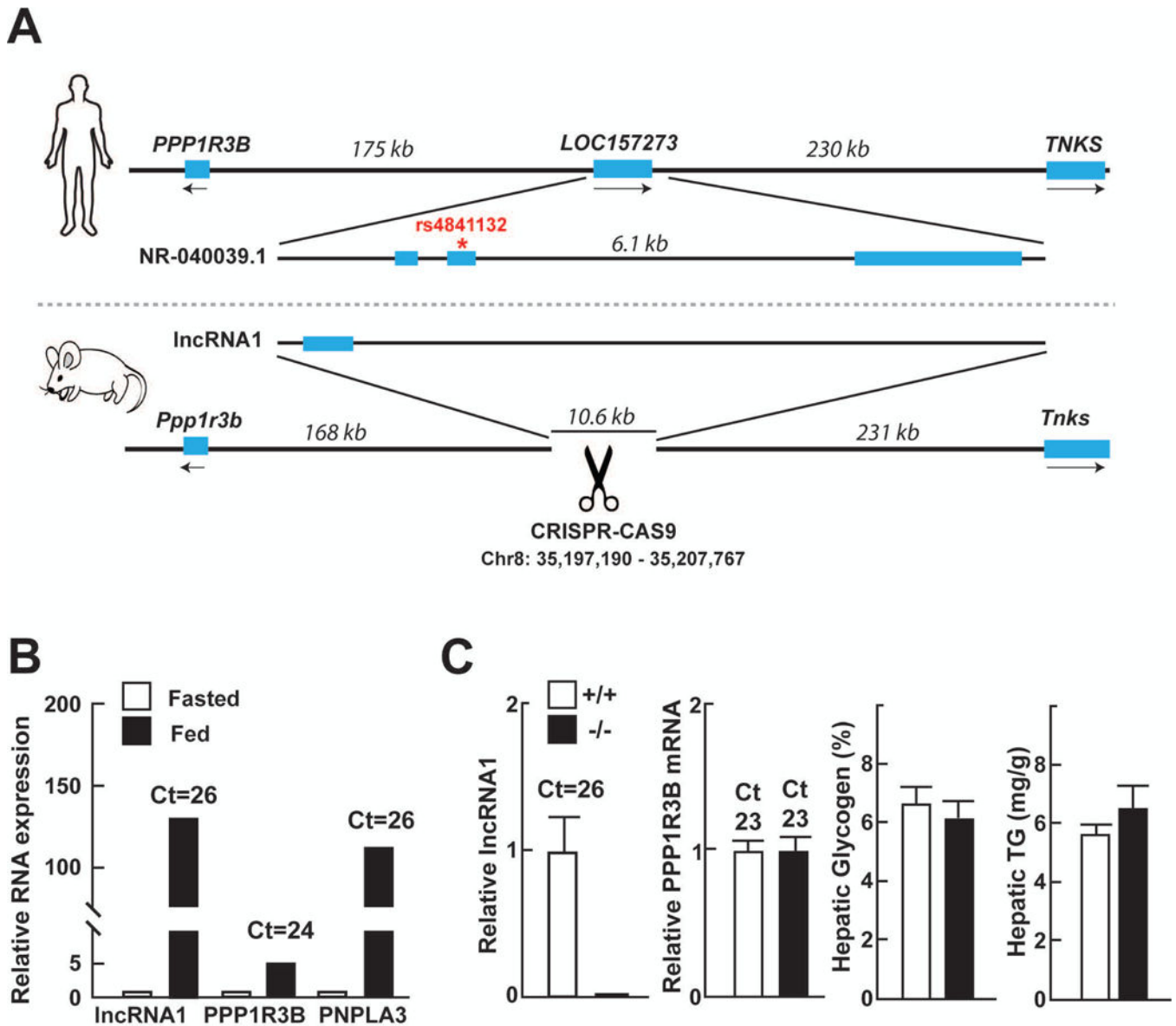


FIG 4. Genomic region of rs4841132 and knock-out of the corresponding region in mice. (A) *LOC157273* is not conserved in mice, but RNA-sequencing of mouse liver identified a lncRNA in the same region. CRISPR/Cas9 was used to generate mice lacking a 10.6 kb region corresponding to *LOC157273*, including the mouse lncRNA. (B) Relative levels of hepatic lnc-RNA1, PPP1R3B, and PNPLA3 mRNA assayed by RT-qPCR in fasted mice and 4 h after refeeding with a chow diet. PNPLA3, which is induced by feeding,⁽⁴⁰⁾ served as a positive control in the experiment. (C) Levels of hepatic PPP1R3B mRNA, glycogen, and TG were not significantly different in mice homozygous for the genomic deletion (-/-) compared to WT controls (+/+).

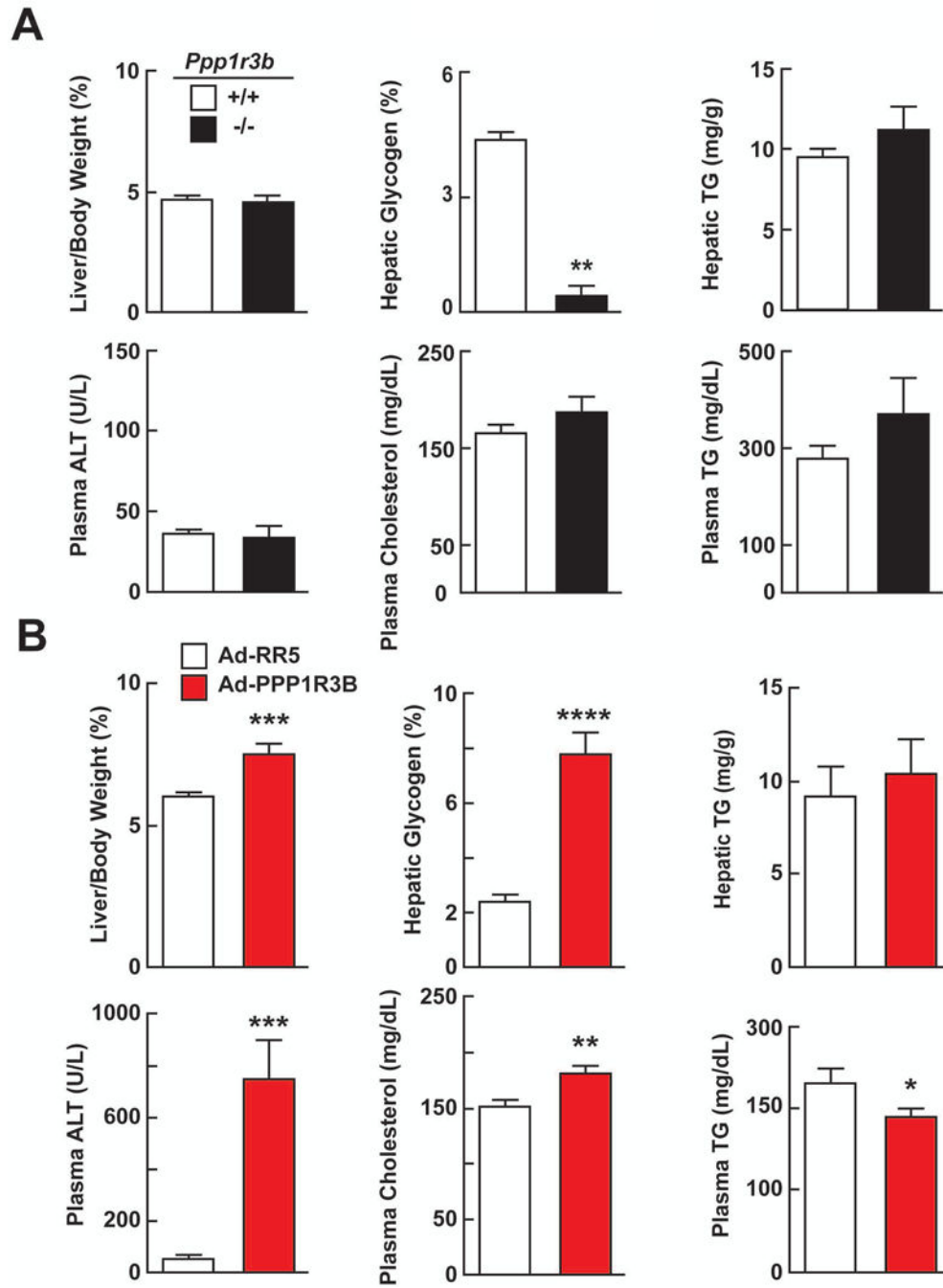


FIG 5. Phenotype of *Ppp1r3b*-KO mice (A) and mice infected with Ad-mouse PPPR1R3B. (A) Food intake was synchronized for 3 days (18 h fasting, 6 h feeding) in male mice (8 wk old, n=3-5/group). At the end of the fasting cycle on the 4th day, the mice were killed 4 h after they were fed. The experiment was repeated and the results were similar. P-values by t-test. * = P<0.05; ** = P<0.001 (B) Phenotype of mice overexpressing mouse PPP1R3B. The tail veins of 8-week old male mice (10/group) were injected with adenovirus expressing PPP1R3B (Ad-PPP1R3B) or an empty control virus (Ad-RR5) (1.25×10^{11} viral particles per

mouse). The chow-fed mice were sacrificed 48 h after the injection. P-values by t-test. * = $P < 0.05$; ** = $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

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Table 1

Baseline characteristics of study participants.

Study	Dallas Heart Study	Copenhagen Cohort	Dallas Liver Study
N	4,467	107,447	514 cases
Age, y	44 (36-53)	58 (48-67)	55 (46-61)
Female, n (%)	2,571 (58)	53,227 (55)	298 (58)
BMI, kg/m ²	29 (26-35)	26 (23-28)	31 (28-35)
European American, %	30	100	26
African American, %	53	0	5
Hispanic American, %	17	0	69

Values are median and interquartile ranges, N or %. Data on age, gender, and BMI were available in ~97,000 from the Copenhagen Cohort.

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