Trans-ancestry Fine Mapping and Molecular Assays Identify Regulatory Variants at the ANGPTL8 HDL-C GWAS Locus

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ABSTRACT Recent genome-wide association studies (GWAS) have identified variants associated with high-density lipoprotein cholesterol (HDL-C) located in or near the ANGPTL8 gene. Given the extensive sharing of GWAS loci across populations, we hypothesized that at least one shared variant at this locus affects HDL-C. The HDL-C-associated variants are coincident with expression quantitative trait loci for ANGPTL8 and DOCK6 in subcutaneous adipose tissue; however, only ANGPTL8 expression levels are associated with HDL-C levels. We identified a 400-bp promoter region of ANGPTL8 and enhancer regions within 5 kb that contribute to regulating expression in liver and adipose. To identify variants functionally responsible for the HDL-C association, we performed fine-mapping analyses and selected 13 candidate variants that overlap putative regulatory regions to test for allelic differences in regulatory function. Of these variants, rs12463177-G increased transcriptional activity (1.5-fold, \( P = 0.004 \)) and showed differential protein binding. Six additional variants (rs17699089, rs200788077, rs56322906, rs3760782, rs737337, and rs3745683) showed evidence of allelic differences in transcriptional activity and/or protein binding. Taken together, these data suggest a regulatory mechanism at the ANGPTL8 HDL-C GWAS locus involving tissue-selective expression and at least one functional variant.

To date, at least 157 loci have been associated with high-density lipoprotein cholesterol (HDL-C) in genome-wide association studies (GWAS) (Willer et al. 2013). Analyses of European, African American, Mexican, Pima Indian, and East Asian participants have identified four different lead variants associated with HDL-C located in or near angiopeptin-like protein 8 (ANGPTL8) (Coram et al. 2013; Weissglas-Volkov et al. 2013; Willer et al. 2013; Nair et al. 2016; Spracklen et al. 2017). ANGPTL8 is a small protein of 198 amino acids; the gene is located on chromosome 19 within intron 14 of dedicator of cytokinesis 6 (DOCK6), and is transcribed in the opposite direction of DOCK6. European GWAS meta-analyses identified rs737337 (\( P = 4.6 \times 10^{-17}, N = 185,000 \)) (Teslovich et al. 2010; Willer et al. 2013) as the lead variant associated with HDL-C; this variant was also associated with total cholesterol (\( P = 4.1 \times 10^{-5} \)) but not triglycerides (\( P = 0.12 \)) or low-density lipoprotein cholesterol (LDL-C, \( P = 0.26 \)). rs737337 is located 2.8 kb upstream of the ANGPTL8 transcription start site (RefSeq NM_018687) and is a synonymous variant in exon 19 of DOCK6 (Thr723, RefSeq NM_020812). In an African American population, the lead variant rs12979813 was only associated with HDL-C (\( P = 1.9 \times 10^{-8}, N = 12,157 \)) (Coram et al. 2013), which is located 7.5 kb upstream of ANGPTL8 and in intron 22 of DOCK6. In a joint analysis of Mexican and Pima Indian samples, the lead
variant rs2278426 was associated with HDL-C \((P = 3.4 \times 10^{-9}, N = 4361)\) and total cholesterol \((P = 5.0 \times 10^{-6})\) (Weissglas-Volkov et al. 2013; Hanson et al. 2016), which is a nonsynonymous variant in exon 2 of \textit{ANGPTL8} (Arg59Trp) that exhibits relatively high linkage disequilibrium (LD) with the European lead rs737337 \([r^2 = 0.74, 1000G\] Admixed American (AMR)] and moderate LD with the African American lead rs12979813 \([r^2 = 0.52\) AMR]. Finally, in recent lipid associations in East Asian samples, lead variant rs3760782 was the strongest HDL-C–associated variant \((P = 8.8 \times 10^{-11})\) and another variant in strong LD \((r^2 = 0.95)\) is the lead variant for total cholesterol \((P = 1.5 \times 10^{-15})\) and LDL-C \((P = 1.8 \times 10^{-9})\) (Spracklen et al. 2017). rs3760782 is located 3.5 kb upstream of \textit{ANGPTL8} and in intron 20 of \textit{DOCK6}. Given the extensive sharing of GWAS loci across populations (Visscher et al., 2012), we hypothesized that at least one shared variant at the \textit{ANGPTL8} locus affects HDL-C in all of these populations.

\textit{ANGPTL8} is a recently defined gene also called C19orf80, LOC55908, refeeding-induced fat and liver (RIFL), TD26, hepatocellular carcinoma–associated gene, lipasin, and betatrophin (Quagliarini et al. 2012; Ren et al. 2012; Zhang 2012). Serum \textit{ANGPTL8} protein levels have been associated with many metabolic phenotypes including type 1 and type 2 diabetes (Espes et al. 2014a; Fu et al. 2014b; Hu et al. 2014; Yamada et al., 2015), obesity (Fu et al. 2014b; Lee et al. 2016), and nonalcoholic fatty liver disease (Lee et al. 2016). The gene is mainly expressed in liver and adipose tissues, despite being entirely contained within one intron of the ubiquitously expressed \textit{DOCK6} (Quagliarini et al. 2012; GTEx Consortium 2013). The precise mechanisms of action of \textit{ANGPTL8} remain unclear. \textit{ANGPTL8} is secreted into the plasma and is involved in triglyceride storage in adipose tissue; knockout mice gained less fat than wild-type (Wang et al. 2013) mice and \textit{Angptl8} knockout in 3T3L1 mouse adipocytes led to decreased triglyceride content (Ren et al. 2012). \textit{ANGPTL8} expression is increased in response to stress, it upregulates early growth response transcription factor and downregulates adipose triglyceride lipase, suggesting a role in lipid homeostasis (Zhang et al. 2015). \textit{ANGPTL8} contains a lipoprotein lipase inhibitory motif and inhibits LPL function when coexpressed with \textit{ANGPTL3} (Haller et al. 2017). Additionally, serum \textit{ANGPTL8} protein is inversely associated with HDL-C levels (Yi et al. 2015) supporting a role of \textit{ANGPTL8} in HDL-C metabolism.

\textit{DOCK6} is a member of the dedicator of cytokinesis family of atypical guanine nucleotide exchange factors. \textit{DOCK6} is expressed in many tissues, with highest levels of expression in the lungs, thyroid, and adipose (GTEx Consortium 2013). \textit{DOCK6} functions as a guanine nucleotide exchange factor for RAC1 and CDC42 and has roles in cytoskeletal remodeling and neurite outgrowth (Miyamoto et al. 2007). Mutations in \textit{DOCK6} can cause Adams-Oliver syndrome, an actin cytoskeletonopathy characterized by limb and skin defects (Shaheen et al. 2011). \textit{DOCK6} does not have functions that obviously relate to HDL-C metabolism.

Two coding variants in \textit{ANGPTL8} have been proposed to affect HDL-C levels. A rare \textit{ANGPTL8} nonsense variant, rs145464906 (MAF = 0.001 in >42,000 individuals of European ancestry) encoding Gln121Ter, is associated with increased HDL-C \((P = 5.1 \times 10^{-13})\) and increased triglycerides \((P = 0.003)\) in an exome-based association analysis (Peloso et al. 2014). Based on conditional analysis (Peloso et al. 2014) and LD \((r^2 < 0.01)\), rs145464906 is independent of the reported \textit{ANGPTL8} GWAS variants. The common Mexican and Pima Indian lead variant, rs2278426 encoding Arg59Trp, has been proposed to increase cleavage of \textit{ANGPTL3}, leading to decreased LPL activity and lower HDL-C (Hanson et al. 2016). Although coding variants have been identified, the variants responsible for the common HDL-C association signal at this locus remain unclear, and may include regulatory variants. While a simple mechanism involving a single variant that alters transcription of a single gene is straightforward, multiple variants and/or multiple genes may contribute to the functional consequences of a GWAS signal (Corradin et al. 2014; He et al. 2015; Roman et al. 2015).

In this study, we describe an association between the HDL-C locus variants and subcutaneous adipose level of \textit{ANGPTL8} RNA. We show that a subset of HDL-C–associated variants overlap regions with strong evidence of regulatory activity (Bernstein et al. 2010; Encode Project Consortium 2012), and we use trans-ancestry fine-mapping and functional assays to identify variants exhibiting allelic differences in regulatory activity at the \textit{ANGPTL8} HDL-C GWAS locus.

**MATERIALS AND METHODS**

**Study population and phenotypes**

The METabolic Syndrome In Men (METSIM) study includes 10,197 men, aged from 45 to 73 yr, randomly selected from Kuopio, Eastern Finland, and examined in 2005–2010 (Stancakova et al. 2009; Laakso et al. 2017). The Ethics Committee of the University of Eastern Finland in Kuopio and the Kuopio University Hospital approved the METSIM study, and it was carried out in accordance with the Helsinki Declaration. Triglyceride and lipoprotein characteristics were measured via proton nuclear magnetic-resonance (NMR) or enzymatic assays in 10,079 METSIM participants (Inouye et al. 2010). DNA samples were genotyped on the Illumina OmniExpress and HumanCoreExome arrays, and additional genotypes were imputed using the Go2D integrative haplotypes reference panel as previously described (Fuchsberger et al. 2016).

We also analyzed a set of 8421 self-identified African American participants from the Women’s Health Initiative SNP Health Association Resource (WHI-SHARe) study. Details of the study design, cohort characteristics, written informed consent, and study approval by local Human Subjects Committees have been described previously (Hays et al. 2003). Participants who had consented to genetic research were genotyped on the Affymetrix 6.0 array and additional genotypes were imputed using the 1000 Genomes Project data as a reference panel, as previously described (Reiner et al. 2012).

**Association, conditional, and haplotype analysis**

For METSIM, we performed a preliminary test for association between ~19 million genetic variants and 72 lipid and lipoprotein subclasses (Davis J.P., Huyghe J.R., Jackson A.U., Stringham H.M., Teslovich, T.M., Welch R.P., Fuchsberger C., Locke A.E., Narisu N., Chines P.S., Kangas A.J., Soininen P., Ala-Korpela M., Kuusisto J., Collins F.S., Laakso M., Mohlke K.L., Boehnke M., unpublished data). To better observe the relative differences between variants, we use the association of variants with the concentration of phospholipids in medium HDL because the association with this trait is stronger \((P = 1.9 \times 10^{-7})\) than with HDL-C \((P = 7.7 \times 10^{-4})\). We assumed an additive mode of
inheritance and accounted for cryptic relatedness between individuals using the EMMAX mixed model (gemma) test) implemented in EPACTS (Kang et al. 2010). After accounting for age, BMI, smoking status, and lipid-lowering medication status as covariates, trait value residuals were inverse normal transformed. We carried out reciprocal conditional analyses with candidate variants within the associated signal to evaluate the potential presence of a second association signal by adjusting for specific genetic variants, including METSIM lead variant rs737337 and Women’s Health Initiative (WHI) lead variant rs4804154. LD in locus plots was calculated from the METSIM imputed genotypes, and all chromosome coordinates correspond to hg19.

For WHI, association analysis was performed under an additive genetic model using linear regression adjusted for covariates. The imputed allelic dosage at each variant was tested via MACH2QTL (Li et al. 2010). Genome-wide African ancestry proportion, age, BMI, and smoking history were included as covariates. Genome-wide African ancestry proportion was derived from locus-specific smoking history were included as covariates. Genome-wide African ancestry proportion was derived from locus-specific ancestry, which has a correlation of 0.99 with the first principal component of population structure. Ancestry estimation has been described previously (Coram et al. 2013). To assess the potential presence of multiple, independent variants at the same locus influencing HDL-C trait, we repeated regression analyses, conditioning on rs4804154. LD in locus plots was calculated from WHI-imputed genotypes, and all chromosome coordinates correspond to hg19.

We constructed haplotypes based on five variants at ANGPTL8 that were previously reported as the GWAS index variants (rs4804154, rs737337, and rs2278426) in studies of different ancestry groups (Teslovich et al. 2010; Coram et al. 2013; Weissglas-Volkov et al. 2013; Willer et al. 2013; Hanson et al. 2016) or are proxies (rs7345683 and rs760782) in high LD with both the European and African American lead variants. We performed haplotype analyses using the “haplo.stat” R package (Schaid et al. 2002), estimated haplotypes and haplotype frequencies using the haplo.em function, and tested for association between haplotypes and the concentration of phospholipids in medium HDL (METSIM) or HDL-C (WHI) level using the haplo.glm function. We assumed an additive model in which the regression coefficient represented the expected change in inverse normalized HDL-C level with each additional copy of the specific haplotype compared with the reference haplotype. The same covariates used for single variant analysis were also used in the haplotype analysis model.

Expression quantitative trait association
RNA from subcutaneous adipose tissue was extracted and the expression levels of probesets were measured using Affymetrix Human Genome U219 Array in 770 METSIM participants (Civelek et al. 2016). The expression quantitative trait locus (eQTL) for ANGPTL8 was not previously reported because of a gene name difference with RefSeq and Ensembl. Expression data were normalized using Robust Multi-Array Average (RMA) analysis (Irizarry et al. 2003). eQTL associations were performed as previously described (Civelek et al. 2016). Briefly, we applied PEER analysis (Stegle et al. 2010) to account for complex non-genetic factors in the RMA-normalized gene expression levels. We adjusted for 35 inferred confounding factors and inverse normal transformed the PEER-processed residuals. In eQTL analysis, we used genotype dosages imputed using Haplotype Reference Consortium data (McCarthy et al. 2016) to test for the variant association with expression levels of all genes within 1 Mb of rs737337. We assumed an additive mode of inheritance and accounted for cryptic relatedness between individuals using the EMMAX mixed model implemented in EPACTS (Kang et al. 2010). Additionally, we performed conditional analysis on the expression level of ANGPTL8 by adjusting for rs4804155 and on the expression level of DOCK6 by adjusting for rs17699089 to assess the presence of multiple, independent associations. We also performed conditional analysis using candidate functional variant rs12463177 to assess the coincidence of GWAS and eQTL signals. To examine the relationship between RMA-normalized expression levels and HDL-C, we adjusted both traits for age and BMI, inverse normal transformed the residuals, and then tested for association in regression analyses.

MANTRA
To fine-map the ANGPTL8 locus, we performed trans-ancestry meta-analysis using MANTRA (Morris 2011). MANTRA accounts for the shared similarity in closely related populations using Bayesian partition model assuming the same underlying allelic effect. It models the effect heterogeneity among distant populations by clustering according to the shared ancestry and allelic effects. We conducted the analysis based on the association summary statistics from GWAS in METSIM and WHI and included variants present in both METSIM and WHI. We constructed a 99% credible set of variants by ranking all variants according to their Bayes factors.

CAVIAR and PAINTOR
To prioritize functional variants, we analyzed variants at the ANGPTL8 locus using both CAVIAR (Hormozdarian et al. 2014) and PAINTOR (Kichaev et al. 2014). CAVIAR estimates the posterior probability of a variant being functional by jointly modeling the P-values and Β association statistics, and PAINTOR leverages functional genomic annotation data, in addition to association strength, to prioritize functional variants. Both methods allow for multiple functional variants at the risk locus. Using consistent alleles for METSIM and WHI African Americans, we calculated Z scores based on P-values and the sign of Β from GWAS in these studies. For METSIM, the LD matrix was calculated based on European data from the 1000 Genomes Project Phase 1. For WHI African Americans, LD was calculated based on imputed data as previously described (Reiner et al. 2012). In CAVIAR, we returned the credible set that contains all of the true functional variants with 95% confidence level. The annotation matrix used in PAINTOR contained data from the ENCODE project accessed through the UCSC Genome Browser using the Table Browser tool. The matrix included HepG2 H3K4me1, H3K4me2, H3K4me3, and H3K27ac histone marks, DNase hypersensitivity clusters (ENCODE v3) and transcription factor binding (ENCODE v2) from the Broad Institute. Presence or absence of overlap was determined by the UCSC Table Browser intersection with the signal tracks. In PAINTOR, analysis was performed for METSIM alone (Finns), WHI alone (African Americans), and the two data sets together. We set the number of functional variants as 2, 3, 4, or 5 based on feasible running time. PAINTOR predicted variants to be functional based on a posterior probability >0.1, a threshold suggested previously (Kichaev et al. 2014).

Functional annotation
To identify regulatory overlap of HDL-C-associated variants with histone marks, transcription factor binding, and DNase hypersensitivity sites, we used data from the ENCODE and Roadmap Epigenome projects accessed through the UCSC genome browser (Bernstein et al. 2010; Encode Project Consortium 2012). RNA-seq data were obtained from the Roadmap Epigenome Project (Bernstein et al. 2010; adult liver and adipose tissue) or a previous publication (Parker et al. 2013; HepG2). We evaluated previously published positive correlations of DNase hypersensitivity sites with gene expression in liver cell types (Sheffield et al. 2013). LD calculations for selecting candidate variants were based
on 1000 Genomes Phase 1 data. The AFR dataset was used for African American LD and EUR for European LD.

**Cell culture**

HepG2, SW872, SGBS, 293T, 3T3L1, Huh-7, and MIN6 cell lines were maintained at 37°C with 5% CO₂. HepG2 cells (ATCC, HB-8065) were cultured in MEM-alpha (Gibco) supplemented with 10% FBS and 1 mM sodium pyruvate. SW872 cells (ATCC, HTB92) were cultured in DMEM:F12 (Sigma) supplemented with 10% FBS. SGBS cells (Wabitsch et al. 2001) were generously provided by Dr. Martin Wabitsch (University of Ulm) and cultured in DMEM:F12 (Sigma) supplemented with 10% FBS and 5% 3.3 mM biotin/1.7 mM panthotenate solution. SW872 and SGBS cells were transduced in the undifferentiated, preadipocyte state. 293T cells (ATCC, CRL-3216) were cultured in DMEM (Sigma) supplemented with 10% FBS and 200 mM L-glutamine. 3T3-L1 cells (ATCC, CL-173) were cultured and differentiated as described in the ATCC protocol. Huh-7 cells (ICR0403, Japanese Collection of Research Bioresources Cell Bank, National Institute of Biomedical Innovation), were cultured in DMEM with high glucose (Gibco) with 10% FBS, 1 mM sodium pyruvate, and 1 mM nonessential amino acids (Sigma), and 1 mM L-glutamine. MIN6 cells (Miyazaki et al. 1990) were cultured in DMEM (Sigma), supplemented with 10% FBS, 1 mM sodium pyruvate, and 0.1 mM β-mercaptoethanol.

**Dual luciferase transcriptional reporter assays**

We PCR-amplified fragments surrounding each regulatory region or variant with five PRIME Mastermix (five PRIME) or Phusion High-Fidelity Polymerase (New England Biosystems) with the primers listed in Supplemental Material, Table S1 in File S1 from DNA of individuals homozygous for alleles associated with increased or decreased HDL-C. Gateway attB sites were included in primers and Gateway cloning was used to insert fragments into a Gateway-compatible pGL4.23 (minimal promoter) or pGL4.10 (promoterless) firefly luciferase reporter vector (Promega). Fragments containing HDL-C-associated variants are designated as forward or reverse based on their orientation with respect to the genome and the ANGPTL8 gene. The 5-kb and promoter regions were only cloned in the forward orientation (pGL4.10) because they include the ANGPTL8 promoter. Regions were isolated based on epigenetic marks of promoter and/or enhancer regions surrounding the HDL-C-associated variants. We isolated three to five independent clones (biological replicates) for each allele for each orientation and verified by sequencing. Each clone was cotransfected with Renilla luciferase vector in duplicate (HepG2, 293T, Huh-7, SW872) or triplicate (SGBS, 3T3L1) wells (technical replicates) using FUGENE 6 (HepG2, 293T, Huh-7, SW872, 3T3L1; Promega). Lipofectamine 2000 (Invitrogen; Life Technologies), or Lipofectamine 3000 (SBS; Life Technologies). Twenty-four (SGBS) or 48 h (all other cell types) after transfection, we collected cell lysates and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity of the clones containing the PCR fragments was normalized to Renilla luciferase readings to control for differences in transfection efficiency. We repeated all luciferase transcriptional reporter experiments on independent days and obtained consistent results. Data are reported as fold change in activity relative to an empty pGL4.23 or pGL4.10 vector. We used two-sided Student’s t-tests to compare luciferase activity between alleles or haplotypes.

**Electrophoretic mobility shift assays**

For electrophoretic mobility shift assays (EMSAs), we prepared nuclear cell extracts from HepG2, Huh-7, SGBS, 3T3L1, SW872, and MIN6 cells using the NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific). Protein concentration was measured with a BCA assay (Thermo Scientific), and lysates were stored at −80°C until use. We designed 17–19 bp biotin- or IR-Dye 700-labeled oligos around the HDL-C-associated variants (Table S1 in File S1) for both alleles. We annealed double-stranded oligos and performed binding reactions as previously described (Kulzer et al. 2014). We used 4–6 μg of antibody in supershift assays and 100–200 ng CEBPP-purified protein for select EMSAs. Binding reactions were run on nondenaturing PAGE DNA retardation gels in 0.5× TBE (Lonza). For biotin-labeled oligos, we transferred the reactions to Biodyne nylon membranes (Thermo Scientific), cross-linked them on a UV cross-linker (Stratagene), and detected DNA–protein complexes by chemiluminescence. For IR-DYE 700-labeled oligos, we imaged gels on a LiCor Odyssey CLx Imaging System. We repeated all EMSA experiments on another day with consistent results.

**Data availability**

Plasmids are available upon request. Supplemental figures and tables contain all relevant data.

**RESULTS**

**Regulation of tissue-selective expression of ANGPTL8**

ANGPTL8 expression is largely restricted to liver and adipose tissues (Quaglia et al. 2012; GTEx Consortium 2013). To determine drivers of ANGPTL8 tissue specificity, we tested candidate regulatory regions in human hepatocyte (HepG2, Huh-7), human preadipocyte (SGBS), human adipocyte (SW872), mouse adipocyte (3T3-L1), human embryonic kidney (293T), and mouse islet β (MIN6) cell lines. We tested a region extending 400 bp upstream of the transcription start site that spans epigenetic marks characteristic of promoters, and a regulatory region that extends 5 kb upstream of the transcription start site (regions shown in Figure 1). The 400 bp promoter contains no HDL-C–associated variants; the 5-kb region contains seven HDL-C–associated variants (rs200788077, rs56322906, rs6511729, rs3760782, rs737337, rs737338, and rs3745683). We tested these candidate regions in transcriptional reporter luciferase assays. In comparison to empty vector, the promoter increased transcriptional activity fivefold in HepG2 (P < 0.0001) and 1.6-fold in Huh-7 (P = 0.04), but not in any other cell type (P > 0.5, Figure 2A). The 5-kb region increased transcriptional activity in HepG2 by eightfold in HepG2 (P = 0.0007) and sixfold in Huh-7 (P = 0.002) compared with empty vector and increased transcriptional activity twofold in preadipocyte (SW872, P = 0.0007) and adipocyte (3T3-L1, P = 0.023) cells compared with empty vector (Figure 2B). Neither region showed transcriptional activity in MIN6 or 293T cells. These data suggest that the 400-bp region contains promoter regulatory elements contributing to tissue specificity in liver, but may be mediated by additional enhancer elements, especially in adipocytes.

**Characterization of the ANGPTL8 HDL-C GWAS locus**

To characterize and determine shared variants of the ANGPTL8 association signal across populations, we analyzed the METSIM study of Finns (N = 8380) and the African Americans subset (N = 8244) from the WHI (Coram et al. 2013). In a preliminary METSIM analysis, the variants most strongly associated with HDL-C at the ANGPTL8 locus were rs737337 (P = 7.7 × 10⁻⁴, β = −0.09) and its LD proxies; these variants were more strongly associated with the concentration of phospholipids in medium HDL particles (P = 1.9 × 10⁻⁷, β = −0.14; Figure 3A and Table S2 in File S1) than HDL-C (P = 7.7 × 10⁻⁴, β = −0.14) (Davis J. P., Huyghe J.R., Jackson A.U., Stringham H.M., Teslovich, 2013). In a preliminary METSIM analysis, the variants most strongly associated with HDL-C at the ANGPTL8 locus were rs737337 (P = 7.7 × 10⁻⁴, β = −0.09) and its LD proxies; these variants were more strongly associated with the concentration of phospholipids in medium HDL particles (P = 1.9 × 10⁻⁷, β = −0.14; Figure 3A and Table S2 in File S1) than HDL-C (P = 7.7 × 10⁻⁴, β = −0.14) (Davis J. P., Huyghe J.R., Jackson A.U., Stringham H.M., Teslovich, 2013). In a preliminary METSIM analysis, the variants most strongly associated with HDL-C at the ANGPTL8 locus were rs737337 (P = 7.7 × 10⁻⁴, β = −0.09) and its LD proxies; these variants were more strongly associated with the concentration of phospholipids in medium HDL particles (P = 1.9 × 10⁻⁷, β = −0.14; Figure 3A and Table S2 in File S1) than HDL-C (P = 7.7 × 10⁻⁴, β = −0.14) (Davis J. P., Huyghe J.R., Jackson A.U., Stringham H.M., Teslovich, 2013). In a preliminary METSIM analysis, the variants most strongly associated with HDL-C at the ANGPTL8 locus were rs737337 (P = 7.7 × 10⁻⁴, β = −0.09) and its LD proxies; these variants were more strongly associated with the concentration of phospholipids in medium HDL particles (P = 1.9 × 10⁻⁷, β = −0.14; Figure 3A and Table S2 in File S1) than HDL-C (P = 7.7 × 10⁻⁴, β = −0.14) (Davis J. P., Huyghe J.R., Jackson A.U., Stringham H.M., Teslovich, 2013).
T.M., Welch R.P., Fuchsberger C., Locke A.E., Narisu N., Chines P.S., Kangas A.J., Soininen P., Ala-Korpela M., Kuusisto J., Collins F.S., Laakso M., Mohlke K.L., Boehnke M., unpublished data). Association analyses conditioned on rs737337 showed no evidence for any additional signals (all $P = 8.0 \times 10^{-4}$, Figure S1B and Table S2 in File S1). In WHI, the lead variant associated with HDL-C is rs4804154 ($P = 8.4 \times 10^{-17}$), located 13 kb from rs737337, and we did not observe evidence of any additional signals ($P = 4.8 \times 10^{-5}$, Figure S1D and Table S3 in File S1). rs4804154 is in low LD with the reported African American lead variant rs12979813 ($r^2 = 0.18$ AFR). rs12979813 is the most strongly associated genotyped variant in the previously reported African American sample (Coram et al. 2013), which includes the WHI study, but rs4804154 is the strongest variant after imputation in WHI. These two lead variants, rs737337 and rs4804154, are in moderate pairwise LD in Europeans ($r^2 = 0.67$ EUR) and in low pairwise LD in Africans ($r^2 = 0.26$ AFR). rs737337 was among the most strongly associated variants in WHI ($P = 9.1 \times 10^{-10}$) and the same was true of rs4804154 in METSIM ($P = 5.7 \times 10^{-5}$). The rare coding variant rs154564906 in ANGPTL8 (Peloso et al. 2014) is not present in METSIM and was not significantly associated with HDL-C in WHI ($P = 0.30$). Conditioning on the common coding variant rs2278426 attenuates, but does not abolish, the signal in both populations (METSIM $P = 0.03$; WHI $P = 1.82 \times 10^{-4}$). Conditioning on the lead variant abolished the association with rs2278426 (WHI $P = 0.78$; METSIM, $P = 0.86$), suggesting that the regulatory variants capture more of the association signal than rs2278426 alone. Because many GWAS loci are shared across populations, the presence of an association signal in both WHI and METSIM supports the hypothesis of at least one shared functional variant across populations.

To further characterize the locus across populations, we conducted haplotype association analyses in METSIM and WHI (Table 1). By comparing variants across haplotypes and populations, we can hypothesize which variants represent the inheritance pattern of variants that have a functional effect. We included five variants in the analyses: the lead variants from European (rs737337), African American (rs4804154), Mexican/Pima Indian (rs2278426), and East Asian (rs3760782) studies and the one variant (rs3745683) in high LD ($r^2 = 0.8$) with the leads in all four studies. The most common haplotype in both METSIM and WHI contained alleles individually associated with increased HDL-C (haplotype 1, Table 1). In both studies, the haplotype containing all the alleles individually associated with decreased HDL-C (haplotype 3) showed the strongest association with HDL-C ($\beta = -0.044, P < 1.0 \times 10^{-22}$ in WHI and $\beta = -0.146, P = 5.3 \times 10^{-7}$ in METSIM). Haplotypes 1 and 2 differed only for rs737337 alleles. Haplotype 2 is common in African Americans (21% frequency) and nearly absent in Finns (0.02% frequency), which explains the different extent of $r^2$-based LD in these populations.
small effect sizes of haplotype 2 ($\beta = 0.01$ in WHI), 3 ($\beta = 0.044$ in WHI, $\beta = 0.146$ in METSIM), and 5 ($\beta = 0.009$ in WHI, $\beta = 0.049$ in METSIM) suggest that rs737337, rs2278426, and rs4804154 may contribute to, but are not alone responsible for, the association signal. These data are consistent with a signal shared across populations driven by one or more functional variants represented by rs3745683 and rs3760782, with potential additional contributions from rs737337, rs2278426, and/or other proxies.

eQTL associations with HDL-C–associated variants and nearby genes

To determine which gene(s) the HDL-C–associated variants may affect, we investigated eQTL associations. We observed an eQTL association in subcutaneous adipose tissue from 770 METSIM study participants for both ANGPTL8 (rs4804154, $P = 1.0 \times 10^{-9}$; Figure S2 in File S1, and Table 2) and DOCK6 (rs17699089, $P = 7.2 \times 10^{-7}$; Figure S2 in File S1, Table 2, and Table S4 in File S1), but not any other gene within 1 Mb of rs737337 (Table S4 in File S1). The METSIM and WHI HDL-C–associated variants were among the variants most strongly associated with both ANGPTL8 and DOCK6 mRNA levels (Figure 3 and Table S4 in File S1), suggesting that the same variants associated with HDL-C may act by affecting expression level of ANGPTL8 and/or DOCK6. Conditional analyses were performed to confirm the coincidence of the GWAS and eQTL signals. For both genes, alleles associated with lower HDL-C levels were associated with lower mRNA levels. In addition, ANGPTL8 mRNA level was associated with HDL-C level in METSIM samples ($P = 0.017$, Figure S3 in File S1), whereas DOCK6 was not ($P = 0.42$, Figure S3 in File S1).

Evidence that the variants most strongly associated with HDL-C are also most strongly associated with ANGPTL8 mRNA levels suggests that a regulatory mechanism acts at this GWAS locus.

Selection of variants to test for allelic differences in regulatory activity

Prioritizing variants at GWAS loci for functional study can be challenging, especially at loci with regulatory mechanisms. To narrow
Table 1 Haplotype association analyses in the WHI and METSIM studies

| Haplotype | Reference | Effect | SE | P Value | Effect | SE | P Value | Effect | SE | P Value |
|-----------|-----------|--------|----|---------|--------|----|---------|--------|----|---------|
| WHI African American Participants | | | | | | | | | | |
| 1 | C C T G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 2 | C C G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 3 | T T C A | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 4 | T C T G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| METSIM Finnish participants | | | | | | | | | | |
| 1 | C C T G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 2 | C C G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 3 | T T C A | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |
| 4 | T C T G C | REF | | 0.042 | 0.026 | 0.019 | 0.006 | 8.8E-16 | 0.016 | 0.016 | 0.017 |

Table 2 Functional characterization of candidate variants

We examined the candidate variants for allelic differences in assays of regulatory function in human liver-derived (HepG2, Huh-7), preadipocyte (SGBS), and adipocyte (SW872) cells. We chose these cell types because ANGPTL8 is expressed in liver and adipose, they had the highest transcriptional activity in our cell type-specificity assays, and they have roles in HDL-C metabolism. We tested variants individually or as a haplotype in luciferase transcriptional reporter assays and/or in EMSA (Figure S6 and Figure S7 in File S1). Among the variants analyzed, rs12463177, which was identified in all three fine-mapping analyses and is a candidate at $r^2 > 0.5$ in both populations ($r^2 = 0.74$ with rs737337 EUR, $r^2 = 0.93$ with rs4804154 AFR), and is 8 kb upstream from the ANGPTL8 transcription start site showed significant ($P < 0.05$) allelic differences in two assays of regulatory function (Figure 4). In luciferase transcriptional reporter assays in HepG2, a 697-bp region containing rs12463177-G showed 1.2 to 1.4-fold increased enhancer activity ($P = 0.02$ forward orientation; $P = 0.004$ reverse) compared with rs12463177-C. While modest, this allelic difference was replicated in three independent experiments. The significant effect on transcriptional activity was not replicated in HuH-7, SGBS, and SW872 cells, in which this region showed repressor activity; however, the trend between the alleles is consistent with HepG2, at least in the reverse orientation (Figure S6 in File S1). In EMSAs using HepG2 and SGBS nuclear extract, the rs12463177-G allele showed increased binding (Figure 4, and Figure S7 and Figure S8 in File S1). The direction of effect of rs12463177 transcriptional activity is consistent with the eQTL direction. A functional role for rs12463177 is consistent with additional evidence that rs12463177 overlaps a DNase hypersensitivity site that
was previously correlated to ANGPTL8 expression (Sheffield et al. 2013; green rectangle in Figure 1) and that the eQTL association signal is attenuated when conditioned on rs12463177 (Figure S2 in File S1 and Table 2). Rs17699089 was not tested in transcriptional reporter assays as the most strong enhancer activity in transcriptional activity and/or protein binding, rs12463177 showed by far the strongest enhancer activity in transcriptional activity between the two haplotypes (Figure S11 in File S1). Rs737337 showed by far the strongest enhancer activity in transcriptional activity between the two haplotypes (Figure S11 in File S1). Rs737337 showed by far the strongest enhancer activity in transcriptional activity between the two haplotypes (Figure S11 in File S1). Rs737337 showed by far the strongest enhancer activity in transcriptional activity between the two haplotypes (Figure S11 in File S1).

To examine the effect on transcriptional activity of multiple variants together with the ANGPTL8 promoter, we tested 5-kb haplotypes located immediately upstream of the ANGPTL8 transcription start site. This region includes five of the seven variants showing allelic differences in protein binding and/or transcriptional activity when examined separately (rs200788077, rs56322906, rs3760782, rs737337, and rs3745683; Figure S6 in File S1) and two variants that did not show evidence of allelic differences (rs6511729 and rs737338; Figure S9 in File S1). These results suggest a complex regulatory mechanism involving enhancer and repressor regions that work in concert to regulate expression.}

**DISCUSSION**

In this study, we examined the tissue specificity of ANGPTL8, reported the first eQTL for variants at the ANGPTL8 HDL-C GWAS locus in adipose tissue, and identified variants at the GWAS locus that exhibit allelic differences in assays of regulatory function. We found that a 400-bp promoter is the main driver of tissue specificity in liver, and that expression may be mediated by additional enhancer elements within 5 kb upstream of ANGPTL8, especially in adipocytes. Of seven candidate regulatory variants that showed allelic differences in transcriptional activity and/or protein binding, rs12463177 showed clearest allelic differences consistent with the direction of the ANGPTL8 eQTL. These data suggest that multiple regions and potentially multiple variants regulate ANGPTL8 expression in liver and adipose tissue.

**Figure 4** Allelic differences in regulatory assays of rs12463177 (A) A 698-bp region containing either allele of rs12463177 was cloned into the pGL4.23 vector and transfected in HepG2 cells. Data are represented as the mean ± SD of 3–5 biological replicates. Luciferase activity was normalized to empty vector (EV) and P-values were determined by t-tests. * P < 0.05, ** P < 0.01. (B) Differential protein binding was evaluated in vitro using EMSA. IR-labeled probes containing either allele of rs12463177 were incubated with 10 µg HepG2 nuclear protein. The arrow shows stronger binding for rs12463177-G. Consistent binding was observed with SGBS nuclear protein (Figure S7 in File S1). HDL-C—Increasing alleles are presented first.
associated with HDL-C (Yi et al. 2015). This direction is opposite of the association we observed with ANGPTL8 expression and HDL-C; however, others have suggested that ELISA methods may not consistently quantify serum ANGPTL8 levels (Fu et al. 2014a). Furthermore, ANGPTL3 inhibits lipoprotein lipase when coexpressed with ANGPTL8, giving a direct connection to lipid metabolism (Haller et al. 2017). While we cannot rule out a role for DOCK6, these lines of evidence and a rare coding variant in ANGPTL3, giving a direct connection to lipid metabolism (Haller et al. 2014) suggest ANGPTL8 as the most likely target gene at this HDL-C GWAS locus.

The ANGPTL8 HDL-C GWAS locus exhibited unusual characteristics in fine-mapping because the associated variants defined by LD $r^2$, with the lead GWAS variants span a larger chromosomal region in African Americans than in Europeans, opposite of most loci in the genome (International HapMap Consortium 2003). Our haplotype association analyses showed that the LD differences are due to a haplotype with 20% frequency in African Americans from WHI that is essentially not observed in Finns from METSIM (0.02% frequency). At this locus, low-frequency haplotypes (frequency < 0.03; Table 1) distinguish $r^2$ thresholds of 0.8 and 0.5 in Finns, and the $r^2$ dependence on allele frequency suggests that a LD threshold of $r^2 > 0.8$ for selecting candidate variants may be too restrictive and miss potentially functional variants. Here, we considered an initial set of candidate variants based on a more liberal threshold of $r^2 > 0.5$, resulting in 42 total variants. We then tested nine variants predicted by MANTRA, CAVIAR, and PAINTOR and that overlapped regulatory datasets in functional assays. Seven of nine variants showed evidence of allelic differences (Figure 5), but only rs12463177 and rs17699089 showed differences consistent with the direction of the eQTL association. Of four additional variants that overlapped regulatory regions but were not predicted to be functional in fine-mapping assays, none showed evidence of regulatory activity. Taken together, these data suggest that the joint use of fine-mapping and regulatory overlap can successfully identify variants exhibiting allelic differences in functional assays.

The mechanism by which the variants that showed allelic differences in functional assays may work in concert remains unclear. The effect on transcriptional activity of rs12463177 was modest and only significant in one cell type (Figure 4 and Figure S6 in File S1). This marginal effect observed in cell lines may not represent the physiological effect in vivo. The magnitude of effect of rs737337 was much greater (50-fold increased transcriptional activity), but did not show significant allelic differences, in contrast to a previous report (Schmidt et al. 2015). rs737337 exhibited strong liver-specific, allele-specific protein binding, but the specific transcription factor(s) remain unknown. One possible mechanism is that the regulatory region containing rs737337 is a strong enhancer that drives expression of ANGPTL8, but that the region containing rs12463177 is important for regulating allele-specific expression. Transcriptional regulators bound at the multiple variants may act together via chromosomal looping with the ANGPTL8 promoter. Further experiments, especially in vivo, are needed to elucidate the precise roles and interactions of the seven variants that showed allelic differences in transcriptional activity and/or protein binding.

In this study, we identified ANGPTL8 as the target gene at this HDL-C GWAS locus, determined regulatory drivers of tissue specificity, and combined fine-mapping approaches and regulatory overlap with experimental assays to identify variants that may contribute to the HDL-C GWAS signal at ANGPTL8. Identifying variants underlying GWAS loci contributes to a growing understanding of target genes, their direction of effect, and metabolic phenotypes. Our results are consistent with previously described results at other GWAS loci where multiple common regulatory variants act together (Corradin et al. 2014; He et al. 2015; Roman et al. 2015), and continued work on the ANGPTL8 locus will further clarify the complex mechanism of these variants.

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