ORIGINAL RESEARCH

Functional Analysis of the *TRIB1* Associated Locus Linked to Plasma Triglycerides and Coronary Artery Disease

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***Background*—**The *TRIB1* locus has been linked to hepatic triglyceride metabolism in mice and to plasma triglycerides and coronary artery disease in humans. The lipid-associated single nucleotide polymorphisms (SNPs), identified by genome-wide association studies, are located ≈30 kb downstream from *TRIB1,* suggesting complex regulatory effects on genes or pathways relevant to hepatic triglyceride metabolism. The goal of this study was to investigate the functional relationship between common SNPs at the *TRIB1* locus and plasma lipid traits.

***Methods and Results*—**Characterization of the risk locus reveals that it encompasses a gene, *TRIB1*-associated locus (*TRIBAL*), composed of a well-conserved promoter region and an alternatively spliced transcript. Bioinformatic analysis and resequencing identified a single SNP, rs2001844, within the promoter region that associates with increased plasma triglycerides and reduced high-density lipoprotein cholesterol and coronary artery disease risk. Further, correction for triglycerides as a covariate indicated that the genome-wide association studies association is largely dependent on triglycerides. In addition, we show that rs2001844 is an expression trait locus (eQTL) for *TRIB1* expression in blood and alters *TRIBAL* promoter activity in a reporter assay model. The *TRIBAL* transcript has features typical of long noncoding RNAs, including poor sequence conservation. Modulation of *TRIBAL* expression had limited impact on either *TRIB1* or lipid regulatory genes mRNA levels in human hepatocyte models. In contrast, *TRIB1* knockdown markedly increased *TRIBAL* expression in HepG2 cells and primary human hepatocytes.

***Conclusions*—**These studies demonstrate an interplay between a novel locus, *TRIBAL,* and *TRIB1*. *TRIBAL* is located in the genome-wide association studies identified risk locus, responds to altered expression of *TRIB1,* harbors a risk SNP that is an eQTL for *TRIB1* expression, and associates with plasma triglyceride concentrations. **(*J Am Heart Assoc*. 2014;3:e000884 doi: 10.1161/JAHA.114.000884)**

Key Words: cardiovascular diseases • lipids • lipoproteins • liver

Several genome-wide association studies (GWAS) have identified a region in the vicinity (≈30 kb) of the *TRIB1*coding region associated with plasma levels of triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), and coronary artery disease (CAD).[1–6] The proximity of the associated region to *TRIB1* suggested that the Trib1 protein might constitute the bona fide effecter of the GWAS-identified risk alleles. However, the *TRIB1* coding region and risk SNPs are not in linkage disequilibrium (LD), consistent with possible independent functions. Although not previously implicated in lipoprotein metabolism, in a mouse model, hepatic overexpression of *Trib1* lowered plasma TGs by reducing VLDL production, an effect attributed to downregulation of lipogenic genes (*Acc1, Fas, Scd1*) and upregulation of genes involved in fatty acid oxidation (*Cpt1a, Cpt2, Acox1*). Liver-specific *Trib1* knockout mice exhibited opposite changes in lipogenic gene expression and plasma TGs.[7] Implicit in this model, higher Trib1 levels would be expected to protect against atherosclerosis by reducing hepatic TG synthesis and increasing fatty acid oxidation.

Recent studies by Satoh et al demonstrate the challenge of defining a simple function for *TRIB1*.[8] They elegantly demonstrated that *Trib1* deficiency limited to the hematopoietic compartment (M2-like macrophages) is associated with increased lipolysis in adipose tissue. Thus, *TRIB1* is likely to play multiple, tissue-specific roles with complex systemic implications. Equally at odds with the simple antiatherogenic

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model, Ostertag et al demonstrated that *Trib1* haploinsufficiency in the mouse protects against obesity without altering serum TG.[9] The latter study revealed inflammation regulation as an important facet of *Trib1* function. In view of the accepted role of inflammation in atherosclerosis progression, this may explain a part of the association of the *TRIB1* locus with CAD. Importantly, the GWAS identified the *8q24.13* locus for TG and CAD has not yet been identified as an expression trait locus (eQTL) for *TRIB1*, suggesting that the genomic effects of this locus may not be limited to alterations in expression of the Trib1 protein.[3]

At the molecular level, the mechanism by which *TRIB1* regulates TG metabolism is poorly understood.[10] Clearly, *TRIB1* could regulate transcription by operating as a coactivator and more broadly by regulating transcription factor function.[10] Our understanding of Trib1 function stems largely from tumorigenic models where *TRIB1* has been implicated as an oncogene in leukemia.[11] Key to the oncogenic properties of Trib1 is its ability to promote degradation of CCAAT/Enhancer-Binding Protein Alpha via its stimulatory interaction with MAP2K1 and RFWD2. Interestingly, *Trib2*, which shares *Trib1*'s oncogenic properties, has been implicated in murine adipocyte differentiation via CCAAT/Enhancer-Binding Protein Beta regulation.[12] In addition and consistent with a role in the human vascular disease, a Trib2 epitope has been identified in atheroma supporting a possible role in plaque development.[13] Finally, Trib3, the last member of the family, also plays a key regulatory role in lipid metabolism in adipose tissue via regulation of *Acc1* and inhibition of fatty acid synthesis.[14] Despite evidence for a role of all 3 members of the Tribbles family in some facet of lipid metabolism, only the *TRIB1* locus has been associated with plasma TG concentrations in humans.

Here, we identify a novel long noncoding RNA (lncRNA) in the *TRIB1*-associated locus (*TRIBAL)* that encompasses risk SNPs for TG and HDL-C. lncRNA, broadly defined as RNAs longer than 200 bp, with no protein coding potential, have attracted considerable interest as important transcriptional regulators.[15,16] The shear abundance of these transcripts vis-à-vis the total transcriptome hints to important functions that are just beginning to be elucidated. Nevertheless, the relatively poor sequence conservation of lncRNA vs mRNA as a group has cast doubt on their relevance to biology, and many lncRNA have been proposed to constitute transcriptional noise.[17] Alternatively, it has been proposed that RNA structure and function might be preserved despite a lack of sequence conservation. Nevertheless, there is general consensus that some lncRNA are indeed biologically important, consistent with significant genome-wide expression changes observed on knock-down of certain lncRNA.[18] In recent years, their roles have expanded considerably as potentially new classes of lncRNA have been described including circular RNAs (ciRNA) that are proposed to function as molecular sponges for miRNA and enhancer RNAs (eRNA) that redefine the enhancer element paradigm.[19,20]

Efforts to systematically categorize lncRNAs are under way.[21] On the one hand, lncRNA can resemble mRNA transcripts: they tend to be polyadenylated, can be found in the cytosol, and typically consist of multiple exons. On the other hand, some lncRNA compartmentalize in the nucleus, are expressed at a lower level, and tend to be poorly conserved.[17,21] To date, most functional nuclear lncRNA have been associated with gene expression regulation in *cis*, usually defined as acting on genes located 3′ or 5′ of the lncRNA gene.[15,17] According to 1 prevailing model, nuclear lncRNA regulate the expression of specific neighboring protein coding genes by regulating their epigenetic status via the recruitment of specific remodeling complexes. However, analyses of whole genome transcription appear equally, if not more, consistent with longer distance interactions by lncRNA.[17]

Here, we demonstrate a functional interplay between a novel lncRNA termed *TRIBAL* and *TRIB1*. *TRIBAL* responds to altered expression of *TRIB1* and harbors a risk SNP that is an eQTL for *TRIB1* expression in blood and associates with plasma TG concentrations.

Methods

Research Cohort

Patients were all part of the Ottawa Heart Study and recruited as described previously. Subjects for CAD cases included white men and women from the Ottawa Heart Study with severe premature CAD that resulted in coronary revascularization. The study was approved by the Human Research Ethics Committee of the Ottawa Heart Institute; written inform consent was obtained from all participants. In all CAD cases, the documented onset was under 60 years of age. Diabetics and individuals with plasma cholesterol levels indicative of monogenic hypercholesterolemia (>280 mg/dL) were excluded from the study. Written and informed consent was obtained from all participants. For SNP genotyping at the *TRIB1/TRIBAL* locus, controls included healthy white men (>65 years) and women (>70 years) with no symptoms and no previous personal or family history of CAD. For TRIB1 mRNA expression analysis in whole blood, the chosen control subjects were not on lipid-altering medication and were matched for age, sex, and body mass index. Written and informed consent was obtained from all participants.

Cell Culture

Primary human hepatocytes were obtained from Corning Life Sciences from multiple donors, 1 donor per replicate. HepG2,

Huh7, and COS-7 were from ATCC. Transfections were performed with Fugene HD (Promega) or Lipofectamine RNAimax (Life Technologies) for plasmids or oligonucleotides, respectively. HepG2 and Huh7 cells were maintained in low-glucose DMEM (Life Technologies) supplemented with 10% FBS and penicillin/streptomycin (P-S, Life Technologies). COS-7 cells were maintained in high-glucose DMEM (Life Technologies) containing 10% FBS and P-S. Human hepatocytes were obtained from Corning Life Sciences, preattached to collagen-coated plates. On arrival, medium was changed to low-glucose DMEM containing a 1:500 dilution of gentamicin/amphoterin-B (Lonza) for 20 hours before the experiment. After a fresh media change, knock-downs were performed for 48 hours in 12-well plates by using 60 nmol/L (hepatocytes) or 20 nmol/L (HepG2/Huh7) of 2′-*O*-(2-methoxy)ethyl–modified antisense oligonucleotides (sequences in supplemental sequences) with 4.8 mL of Lipofectamine RNAimax in 1 mL final volume (0.2 mL OptiMEM+0.8 mL of DMEM). Overexpression was performed by using lentiviral constructs (PLVX-derived) coding for *TRIBAL* form 1 (*TRIBAL1*). Infections were performed in the presence of 4 μg/mL polybrene with concentrated viral supernatants (Lenti-X, Clontech). Viral supernatants were obtained from 293FT cells (Life Technologies) cultured for 3 days after being cotransfected with psPAX2 (4.5 μg), pMD2.G (1.5 μg) and PLVX-derived (6 μg) constructs per 10-cm culture dish. Stable cell lines (Huh7 and HepG2) were selected with puromycin (2 μg/mL) for 3 days, after which cells were passaged in the absence of puromycin. Cells were maintained for 5 to 10 additional passages before analysis. Expression levels of *TRIBAL* increased ≈150 and 600-fold, respectively, in Huh7 and HepG2.

Quantitative Real-Time PCR

For routine quantitative reverse transcription PCR (qRT-PCR), RNA was isolated by using the Roche High Pure RNA isolation kit. Total RNA (0.5 to 1 μg) was reverse transcribed with the Transcriptor First Strand cDNA synthesis kit from Roche Diagnostics. A combination of oligo(dT) and random hexamer primers was used, as indicated by the supplier. Quantitative real time PCR was performed by using the Roche LightCycler 480 SybrGreen I Master mix, and reactions were run on a LightCycler480 (Roche). *TRIBAL* signal typically yielded crossing point (Cp) values around 32. Unless mentioned otherwise, Cp values were normalized to peptidylprolyl isomerase A (PPIA).

Rapid Amplification of DNA Ends

Total RNA was obtained from HepG2 by using Trizol (Life Technologies). Rapid amplification of DNA ends (RACE) was performed by using the 5′/3′ RACE kit, 2nd Generation (Roche Diagnostics) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA for 60 minutes at 55°C followed by 5 minutes at 85°C and purified with the High Pure PCR Purification Kit (Roche Diagnostics) according to the modifications described in the RACE kit instructions. All PCRs were run on the ABI GenAmp PCR system 9700. *TRIBAL* amplification required 2 rounds of PCR with the Roche FastStart Taq DNA polymerase, dNTPack (Roche Diagnostics). For 3′ RACE, in the first round of PCR, each reaction contained a gene-specific forward primer targeting exon 1 or 2 and 1× GC-rich solution. The first-round PCR products were diluted 1:20 for the second round by using a nesting primer, under the following conditions: (1) an initial denaturation for 2 minutes at 95°C; (2) 10 cycles of 95°C for 15 seconds, 57°C for 30 seconds, 72°C for 40 seconds; (3) 25 cycles of 95°C for 15 seconds, 57°C for 30 seconds, 72°C for 40 seconds (+20 s/cycle for the 25 cycles); and (4) a final elongation at 72°C for 7 minutes. For 5′ RACE, the purified cDNA was used for the 2-step poly(A)-tailing reaction with terminal transferase at 37°C for 30 minutes followed by a 10-minute incubation at 70°C to inactivate the enzyme. PCR conditions were as follows: (1) an initial denaturation for 2 minutes at 95°C and (2) 45 cycles of 95°C for 15 seconds, 57°C for 1 minutes, 72°C for 90 seconds. In the first round, a 3:1 ratio of oligo(dT)-anchor primer and a nested primer was used, while a ratio of 1:1 was used in the second round. For the second round, a shorter anchor primer was used to allow for more compatible melting temperatures. Finally, several primers were designed upstream from one another to "chase" the 5′ end of the transcripts. Chasing required 1 round of PCR and was performed by using the conditions just described.

Plasmid Constructs

Reporter constructs were derived from pGL3.0 (Promega). The lentiviral vector (PLVX) was from Clontech. All constructs were generated by conventional cloning methodologies by using PCR and sequencing to ascertain identity. Primers used for cloning are described in the supplemental sequence information file.

Reporter Assays and Cignal Finder Reporter Arrays

Inserts were generated by PCR by using the PfuUltraII system from Stratagene (Agilent Technologies) and subcloned in pGL3.0 basic. All plasmids were affinity-purified (Qiagen) and transfected with Fugene HD (Promega) in 80% confluent COS-7 and HepG2 in 12-well plates. Unless otherwise indicated, 1 μg of total plasmid was transfected per well containing 2%

pRL-SV40 renilla (Promega). For Cignal Finder, HepG2 or Huh7 cells seeded (80 000 cells per well) in Cignal Finder Signal Transduction 45-Pathway Reporter Arrays were reverse-transfected with Attractene (0.75 μL in 50 μL Opti-MEM). Cells were lysed in 0.1 mL of passive lysis buffer, and 20 μL was used per luciferase/renilla assay. All assays were performed 24 hours posttransfection in triplicate in a GLOMAX apparatus.

Cellular Fractionation and Nuclear Enrichment

Nuclear fractionation was obtained as described by Rinn et al.[22] Relative abundance of *TRIBAL*, U1, and PPIA in comparison with total signal obtained from whole cell lysates was measured by using qRT-PCR. All primer sequences used are described in the supplemental sequence information section. *TRIBAL* amount was quantified by using a pair of primers targeting exons 1 and 2. The fraction of nuclear to total signal was determined for each transcript. *TRIBAL* values were normalized to the relative nuclear signal of PPIA and U1.

Chromatin RNA Precipitation

The procedure was performed as described in detail by Chu et al.[23] Briefly, 3×10-cm dishes of near-confluent HepG2 cells were used for each pulldown (1 dish per pulldown condition). Cells were rinsed briefly in PBS and fixed in 1% formaldehyde in PBS for 10 minutes followed by a 10-minute glycine block. Following 1 additional rinse in PBS, cells were lysed in 50 mmol/L Tris-HCl, 10 mmol/L EDTA, and 1% SDS containing RNase inhibitor (1:400 dilution), PMSF, and a Roche protease inhibitor mix. Cells were then frozen in liquid N_2 and stored at −80°C. Thawed lysates were sonicated in a Bioruptor (Diagenode) by using 1.5 mL of cell suspension in 15-mL Falcon tubes for 30 minutes with 30-seconds-on/60-seconds-off cycles. Following a brief centrifugation (5 minutes, 13 000*g*) the supernatant were diluted 1:2 in hybridization buffer (50 mmol/L Tris-HCl, 750 mmol/L NaCl, 1 mmol/L EDTA, and 1% SDS containing RNase inhibitor [1:400 dilution]), PMSF, and a Roche protease inhibitor cocktail and precleared for 30 minutes with streptavidin-coupled magnetic beads (PureProteome, Millipore). The supernatant was recovered and incubated in the absence of oligonucleotides or in the presence of 2 sets of 5 biotinylated nucleotides (see supplemental information) with nonoverlapping sequences. Incubation was performed for 90 minutes at 37°C followed by 10 washes (4 minutes per wash) in 2× SSC and 0.5% SDS at 37°C. Aliquots were then processed separately for RNA and DNA analyses. RNA was recovered by successive proteinase K treatment, TriPure extraction, and purification with the RNAeasy Kit (Qiagen). Recovered RNAs were quantified by qRT-PCR. Bead-associated DNA was first released by 2 rounds of 2 μL each of RNase H (Epicenter, 10 U/μL) and RNase A (Roche, 10 mg/mL) digests, followed by proteinase K treatment. DNA was further purified by a combination of phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation with 2.5 volumes of ethanol and GenElute LPA (Sigma-Aldrich) for 20 minutes on ice. The recovered material was resuspended in 30 μL EB buffer (Qiagen) and quantified by qPCR. All the oligonucleotides are described in the supplemental sequence information section.

Transcription Array Analyses

Stable pools of HepG2 expressing *TRIBAL1* were generated by lentiviral infection with PLVX*TRIBAL1* (or PLVX control) followed by puromycin selection (2 μg/mL). For Huh7, *TRIBAL* was suppressed for 48 hours with the *TRIBAL*-specific antisense oligonucleotides or a nontarget control before RNA isolation. Samples from 3 independent experiments were processed for RNA. For Huh7, total RNA (200 ng) from each of the experimental and control group samples and universal reference total RNA (Agilent) were used to synthesize double-stranded cDNA and cyanine-labeled cRNA according to the manufacturer's instruction. Experimental and/or control cRNAs were labeled with cyanine 5-CTP, and reference RNAs were labeled with cyanine 3-CTP. Cyanine-labeled cRNAs were synthesized by using T7 RNA polymerase in vitro transcription kit (Agilent Technologies) and later purified by using RNeasy Mini Kit (Qiagen). Each experimental or control Cy-5 labeled cRNA sample was mixed with equimolar amount of Cy-3 labeled reference cRNA and were hybridized to Agilent SurePrint G3 Hmn GE 8×60K oligonucleotide microarrays (Agilent Technologies Inc, Mississauga, ON, Canada). Arrays were scanned on an Agilent G2505B scanner. Data from the scanned images were extracted by using Agilent Feature Extraction software version 11. A reference randomized block design was used to analyze gene expression microarray data. Data were normalized by using the locally weighted scatterplot smoothing regression modeling method, and statistical significance of the differentially expressed genes was analyzed by using microarray ANOVA with R statistical software (http://www.r-project.org). The F statistic was used to test the treatment effects, and *P* values were estimated by using the permutation method with residual shuffling. The false discovery rate multiple testing correction[24] was applied to minimize any false-positive results. Fold change calculations were based on the least-square means. For overexpression experiments (HepG2), isolated RNAs were processed at the Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON, Canada). Labeled cRNAs were hybridized on GeneChip Human Transcriptome Arrays 2.0,

and results were analyzed by using Expression Console and the Transcriptome Analysis Console from Affymetrix. The analyses are available at the Gene Expression Omnibus (accession number GSE54957).

Statistical Analyses

For cell biology experiments, 3 independent replicates, each quantified by using 2 or 3 technical replicates, were analyzed. Samples were normalized to their matched control first (nontarget oligonucleotide control, empty vector, etc) to obtain relative changes for each experiment; relative changes were then averaged across 3 or 4 experiments. Statistical analyses for biologic experiments were performed by using 2-tailed unpaired Student's *t* tests, and differences were deemed significant if $P<0.05$. Nonparametric tests (Mann–Whitney U test) were then performed, which corroborated the *t* test results ($P<0.05$). Error bars correspond to 95% CIs ($\alpha=0.05$).

Allelic frequencies were analyzed by using Fisher's exact test to generate the indicated *P* values. Odds ratios (ORs) for CAD were determined with 95% CIs by using a logistic regression model fit. LD between SNPs was determined by using R Program 2.10.1. For CAD/TG/SNP association analyses in larger cohorts, association of candidate SNPs with CAD and TG phenotypes was measured by using linear and logistic models implemented in PLINK v1.07[25]; both methods allow for inclusion of covariates in the analysis. Linear regression was conducted to analyze the association of TGs with SNPs. The first 2 principal components (pc1 and pc2) of ancestry as determined by EIGENSTRAT[26] were included as covariates in the analysis. Logistic regression was conducted to quantify the association of the CAD with SNPs, after adjustment for pc1 and pc2. To determine if the association of CAD with SNPs is dependent on TGs, we also conducted a logistic regression analysis by including TGs, pc1, and pc2 as covariates in the analysis.

Results

Identification of 2 Common *TRIB1*-Associated SNPs That Correlate With CAD and Changes in Lipoprotein Profile

To address the relationship of risk SNPs for TG/CAD, we carried out phylogenetic analysis and identified a ≈300-bp conserved region (CNS) proximal to most of the GWAS identified risk SNPs (Figure 1A and 1B). This region was resequenced by using DNA from a sample of CAD cases and controls, matched for age and sex, who had participated in the Ottawa Heart Study. We identified 2 common SNPs (rs2001844 and rs2001845), neither of which is in the same LD block as the *TRIB1* coding region (Figure 1C), that displayed a strong association with CAD with allele-specific OR ≈1.5 (Table 1). These SNPs are in LD with the TG-associated SNP rs2001945[27] and, of these, rs2001844 displayed a stronger association with the TG and CAD risk SNP, rs17321515 ($D'=1$, $r^2=0.98$).[3] As expected from their unequal LD with rs17321515, rs2001844 and rs2001845 were moderately linked ($D'=1$, $r^2=0.38$). Of note, subsequent to our analysis, a recent gene-centric meta-analysis of plasma lipid traits identified rs2001844 to have the largest effect size of the 40 SNPs investigated in this region.[28]

Having established its relationship to CAD, the association of rs2001844 with lipid traits was investigated in healthy control subjects who were not receiving lipid-modifying therapy; CAD patients were excluded from this first analysis due to confounding effects of medication regimens. The rs2001844 CAD risk allele was associated with increased TGs and decreased HDL-C (Figure 2A). These data appear most consistent with an additive model for TGs and a nonadditive model for HDL-C.

While these data suggest that changes in lipid profiles underlie the CAD association with rs2001844, this remained to be investigated. To this end, the association was examined in a larger cohort consisting of both healthy and CAD patients, focusing on the contribution of TGs. As shown in Table 2, when TGs were excluded from the logistic regression analysis, rs2001844 was significantly associated with CAD, consistent with our earlier findings. When the analysis was adjusted for TGs, however, the association with CAD was considerably attenuated, indicating that the association of rs2001844 with CAD can be explained largely by the plasma TGs effect. Similar findings were obtained for rs2001945 and rs2001845, implying that the whole locus operates similarly.

rs2001844 Is an eQTL for *TRIB1*

Previous GWAS that used lymphoblastoid cells did not identify an eQTL for *TRIB1* expression in the TG and CAD risk locus. To investigate a potential relationship in untransformed cells, Paxgene-derived RNA was isolated from whole blood of 160 genotyped patients, and *TRIB1* mRNA was quantified by qRT-PCR (Figure 2B). These data indicate that rs2001844 is associated with *TRIB1* mRNA expression as assessed by using a 1-way ANOVA ($P=0.035$), with the risk genotype being associated with 20% lower expression of *TRIB1* (AA versus BB, $P=0.008$, unpaired *t* test).

Identification of *TRIBAL* Transcripts 3′ of the CNS Region

Expression data from the ENSEMBL and UCSC databases revealed 2 distinct transcripts of unknown function (ie, ENST00000522815 and TCONS_00014852 [UCSC]) that

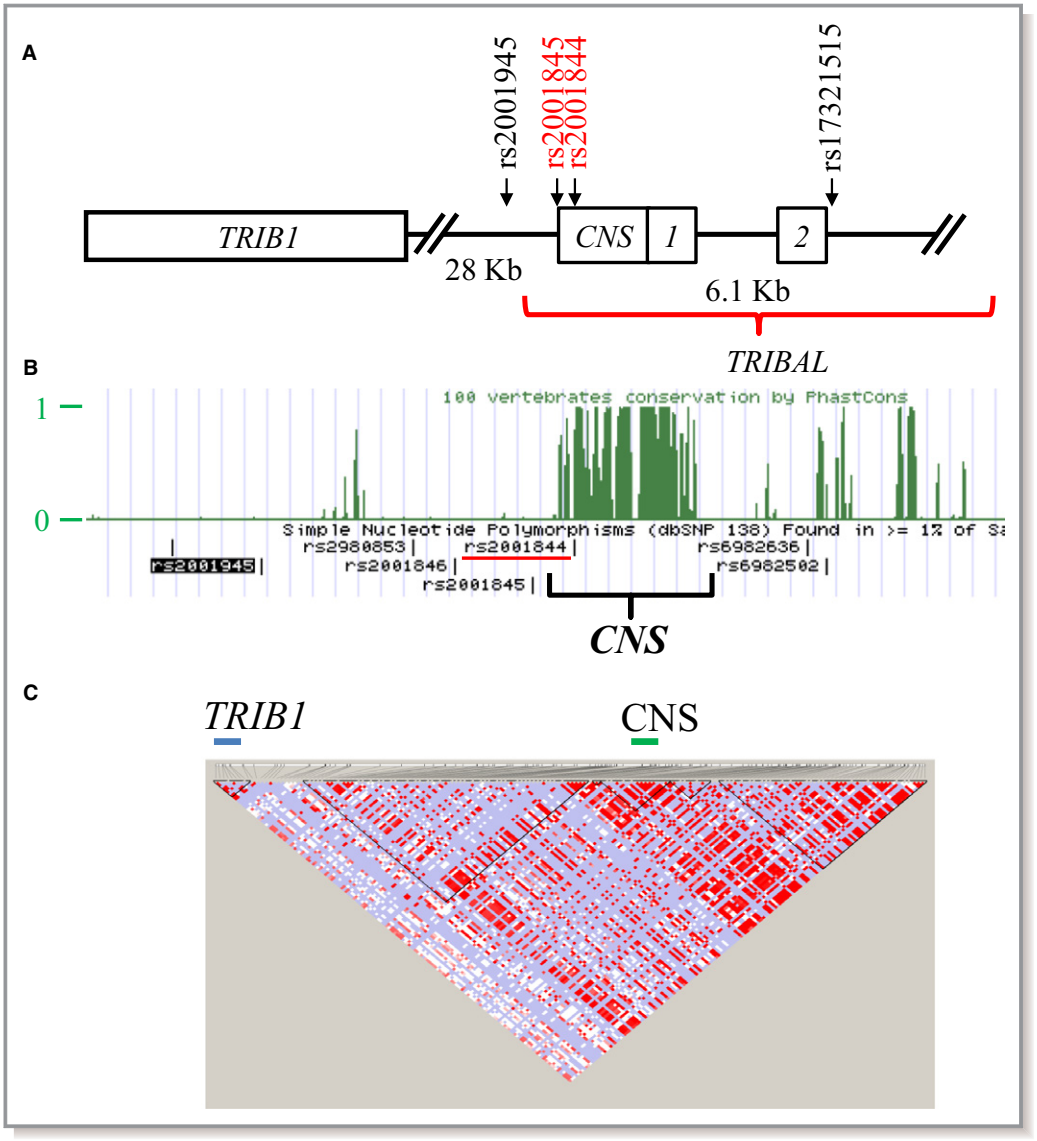

Figure 1. Conserved region (CNS) and its relationship with TG and CAD-associated SNP identified by GWAS. A, The position of the SNP identified in this study are shown in red relative to the CAD/TG-associated SNP (black). *TRIBAL* exons are numbered. B, CNS region and its conservation. PhastCons (UCSC Genome Browser) analysis of CNS and surrounding region, focusing on vertebrates. SNPs are indicated at bottom. Of common SNPs, only rs2001844 (underlined in red) is located within the ≈500-bp CNS region. The TG associated SNP rs2001945 is boxed in black. C, *TRIB1* and the CAD-associated SNPs occupy distinct LD blocks. LD plot generated by HaploView (version 4.2) highlighting the lack of linkage between SNPs associated with the *TRIB1* coding region and the SNPs associated with CAD. Analysis was performed using the 1000 Genome indexed SNPs spanning chromosome 8: 126447308 to 126486409. Blocks shown (black triangles) were defined by CIs. CAD indicates coronary artery disease; CNS, conserved region; GWAS, genome-wide association studies; LD, linkage disequilibrium; SNP, single nucleotide polymorphism; TG, triglycerides.

were either juxtaposed or overlapping the CAD-associated TG SNPs (Figure 3). Further, the corresponding putative gene ENSG00000253111 appeared to encode a third distinct transcript (ENST00000521991). All 3 transcripts consisted of multiple exons and appeared to lack protein coding potential, consistent with their classification as lncRNAs. In view of the close linear proximity of this locus to *TRIB1*, the functional association between both regions as assessed by previous GWAS, and the convolution of the existing nomenclature, we refer to the ENSG00000253111 locus as *TRIBAL* (TRIB1 associated locus) throughout this work. Reverse transcription PCR analysis detected a transcript encompassing 2 exons (designated exons 1 and 2) in HepG2 cells and, to a lesser extent, in peripheral blood mononuclear cells obtained from

Table 1. Allele Frequencies, CAD Association, and LD Data for SNPs Within Evolutionarily Conserved Regions Genotyped in CAD Cases and Controls From the Ottawa Heart Study

SNP	Chromosome 8 Location	Phenotype	AA	AB	BB	MAF	Allelic *P* Value	Odds Ratio (95% CI)	LD With rs2001945
rs2001845	126478644	Cases	106	122	38	0.37	0.0062	1.46 (1.19 to 1.87)	D′=1
		Controls	145	98	28	0.28			r^2=0.52
rs2001844	126478745	Cases	47	125	94	0.59	0.004	1.50 (1.18 to 1.90)	D′=1
		Controls	74	131	66	0.48			r^2=0.81

Minor allele frequencies (MAF) were compared between CAD cases and controls for each SNP. Statistical analysis was based on a univariate logistic regression model. rs2001945 is a previously identified TG-associated SNP. Both SNPs are in partial LD with one another (D′=1, r^2=0.38), according to Haploreg (CEU population); A=major allele, B=minor allele. CAD indicates coronary artery disease; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

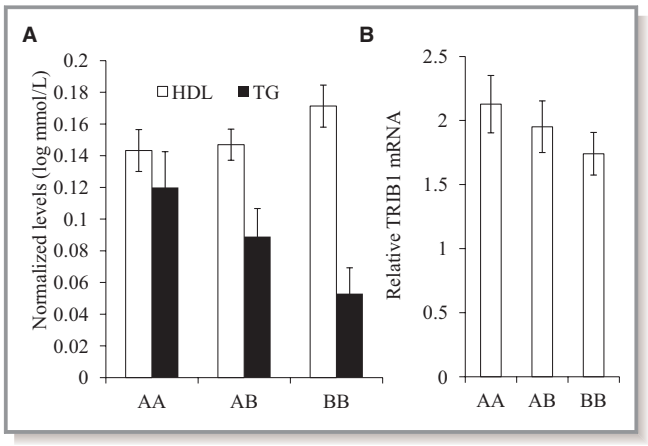

Figure 2. Risk SNP at rs2001844 is associated with higher TG and lower HDL levels. A, Fasting levels of HDL and TG (normalized) in non-CAD patients with homozygous risk (AA, n=385), heterozygous (AB, n=666) and homozygous risk (BB, n=346) genotypes. Error bars represent 95% CIs. For HDL, statistically significant differences were observed for AA vs BB (*P*=0.0036) and AB vs BB (*P*=0.0044) only. For TG, all groups were statistically different (*P*<0.05). B, Levels of *TRIB1* transcript were measured in a total of 160 patients (50 AA, 56 AB, 54 BB) well matched for sex, age, and BMI. Data are normalized to *PPIA* for each patient, and bars represent the means±95% CIs *TRIB1* mRNA levels were statistically significant between AA and BB only (*P*=0.008). BMI indicates body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; SNP, single nucleotide polymorphism; TG, triglycerides.

whole blood (Figure 4). Examination of the Illumina RNAseq data (Human Body Map) via the UCSC Genome Browser revealed expression of low abundance transcription over the 2 exons as determined by RNAseq.

Implication of *TRIBAL* Transcription in *TRIB1* Regulation

Due to the close proximity of this transcript to risk SNPs and the effect of rs2001844 on *TRIB1* mRNA, we first investigated the potential role of *TRIBAL* in the regulation of *TRIB1* transcription. We hypothesized that rs2001844 or a linked SNP could regulate *TRIBAL* expression level, which in turn might alter *TRIB1* expression. Unfortunately, due to the very low expression level of *TRIBAL* in blood, we were not able to accurately quantify *TRIBAL* transcripts by using qRT-PCR. Reporter constructs were thus used to determine the effects of rs2001844 on *TRIBAL1* expression. Of note, the region encompassing rs2001844 displays epigenetic marks consistent with a regulatory function, including several acetylation and methylation marks, DNase I accessibility, and potential conserved transcription factor binding sites (UCSC browser and RegulomeDB). We reasoned that the risk SNP may alter promoter activity of the *TRIBAL* transcript. Transfection in COS-7 and HepG2 cells indicated that the CNS region harbors weak promoter-like activity, increasing basal transcription by a factor of 3- to 4-fold over the promoter-free constructs (Figure 5A). Further deleting the construct to the core CNS1 enhanced the activity somewhat, but further reduction gradually decreased its promoter activity (Figure 5B). The influence of the risk allele was then evaluated by comparing sequences derived from control subjects differing only in the rs2001844 allele (A or G). Of note, constructs containing the risk allele (A) displayed significantly less activity than the reference allele, suggesting that *TRIBAL* expression may be altered by rs2001844, an eQTL for *TRIB1* expression as demonstrated earlier (Figure 5C).

Identification of Multiple *TRIBAL* Transcripts in HepG2 Cells

Expression data indicated the presence of transcripts containing both exons 1 and 2 but fell short of addressing whether the transcript(s) extended further, as hinted by the several forms reported in publicly available data. To obtain a complete *TRIBAL* sequence, we performed 5′ and 3′ RACE to extend in both directions from exon 1 in HepG2 cells. The 5′ RACE extended exon 1 somewhat over the available sequences but identified no additional exons. The 3′ RACE, on the other hand, identified multiple novel exons and corresponding transcripts, covering a genomic span of

Table 2. Association of Risk SNPs With CAD Depends on TGs

SNP	Chr8 Location	Allele	Cases/Controls	Adjusted for pc1+pc2		Adjusted for pc1+pc2+TGs	
				Odds Ratio	*P* Value	Odds Ratio	*P* Value
rs2001945	126477978	G	4628/3920	1.1504	9.35E–06	1.088	1.79E–02
rs2001845	126478644	A	6243/4284	1.145	2.31E–05	1.0993	8.08E–03
rs2001844	126478745	G	6243/4284	0.8736	5.00E–06	0.9133	5.98E–03

After removing SNPs with missingness per SNP >5%, Hardy-Weinberg equilibrium <0.0001, mean allele frequency <0.01, info <0.4, and subjects with >10% genotype missingness, we measured the association of each candidate SNP with CAD and its dependence on TGs as explained under Statistical Analysis in the Methods section. CAD indicates coronary artery disease; SNP, single nucleotide polymorphism; TGs, triglycerides.

≈60 kbp (Figure 3 and supplemental sequence information). Conservation analysis revealed *TRIBAL* exons to be poorly conserved (Figure 6). Of the transcripts recovered, those consisting of exons 1, 2, and 7 were the most frequent. Cellular fractionation of HepG2 cells demonstrated a clear nuclear enrichment of *TRIBAL* relative to PPIA, on par with that of the snRNA U1, as well as a relatively short half-life of ≈2 hours (Figure 7).

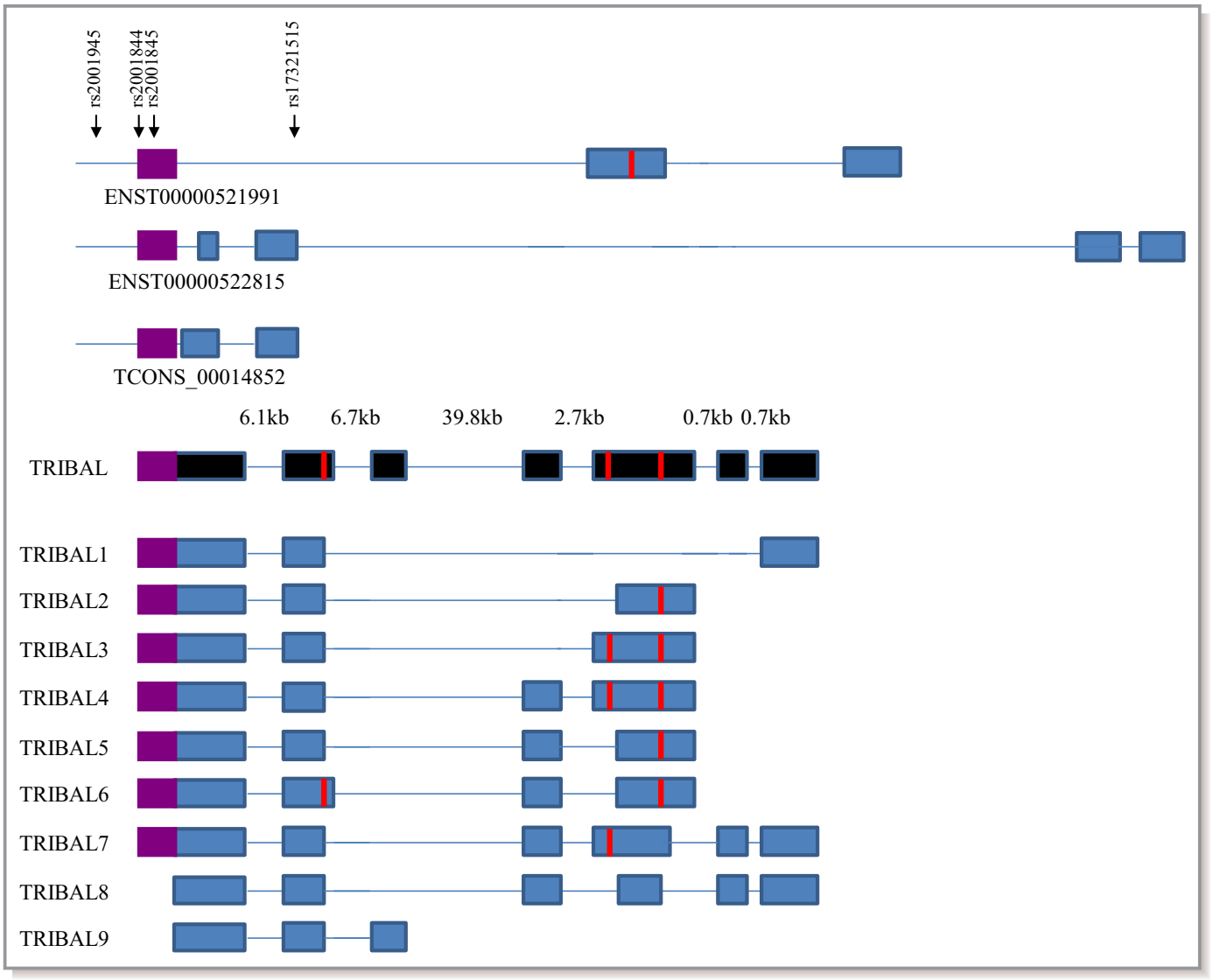

Figure 3. *TRIBAL* spliced variants. Top, exon distribution of the reported forms of *TRIBAL* observed in HepG2. Relative positions of coronary artery disease/triglycerides relevant single nucleotide polymorphisms are indicated with arrows. Conserved (CNS) region is in magenta. Bottom, *TRIBAL* forms identified by RACE in HepG2 cells. *TRIBAL1* was the most consistently recovered version.

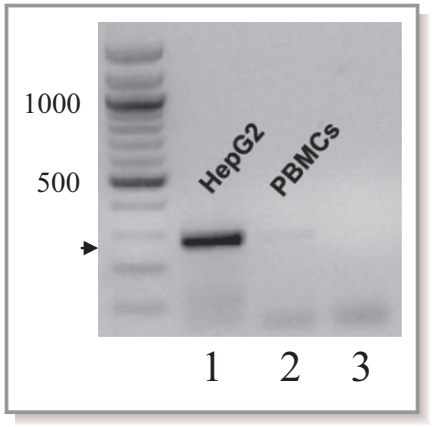

Figure 4. Detection of a transcript containing *TRIBAL* exons 1 and 2 in HepG2 and peripheral blood mononuclear cells (PBMCs). *TRIBAL* transcripts were amplified from HepG2 (lane 1) and PBMCs (lane 2) RNA by reverse transcription PCR analysis using a pair of oligonucleotides matching exons 1 and 2 as described in the Methods section. The amplified products were visualized following agarose gel eletrophoresis and ethidium bromide detection. Lane 3 represents a non–reverse-transcribed peripheral blood mononuclear cell control. Arrowhead indicates migration position of *TRIBAL* amplicons.

Effect of Expression of *TRIBAL* Transcripts on *TRIB1* Expression

Localization of *TRIBAL* to the nucleus hinted at a gene regulatory function. While these results suggested that *TRIBAL* transcription might regulate *TRIB1* levels, they did not address whether the transcripts themselves played any role in the process. To test the contribution of the *TRIBAL* transcripts to *TRIB1* expression, *TRIBAL* expression was attenuated by using an antisense oligonucleotide targeting exon 1 (Figure 8A). A ≈60% reduction in *TRIBAL* abundance resulted in a ≈10% decrease in *TRIB1* in Huh7 cells and primary human hepatocytes, reaching statistical significance only in Huh7 cells; HepG2 cells, in contrast, showed no significant change. In view of the incomplete downregulation leading to possible threshold effects, the impact of *TRIBAL* overexpression was investigated. Among the various *TRIBAL* forms identified earlier, *TRIBAL1* was selected since it represented the most frequently isolated transcript by RACE, presumably reflecting higher endogenous levels and greater biologic significance. Introduction of high levels of *TRIBAL1* had no significant impact on *TRIB1* mRNA levels in any of the human hepatic cell types studied (Figure 8B). Thus, the overexpression data are inconsistent with a direct role of *TRIBAL* in *TRIB1* regulation. To further probe the interplay between the 2 genes, we next investigated the impact of

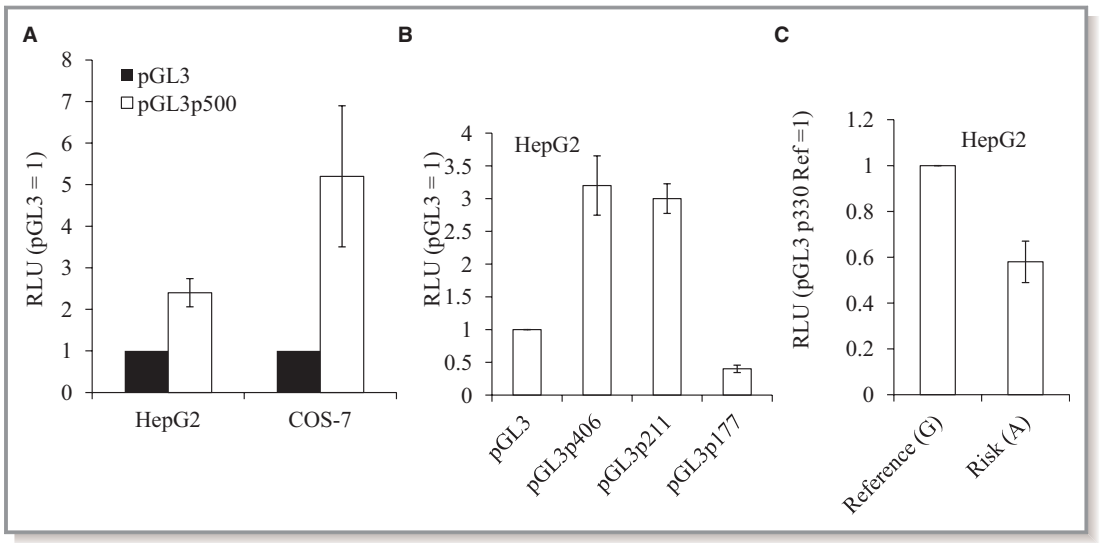

Figure 5. Characterization of the conserved region proximal to the *TRIBAL1* transcript. A, HepG2 and COS-7 cells were transfected with empty pGL3 basic or pGL3 basic harboring the ≈500-bp region located 28 kb 3′ of *TRIB1*. Plasmids were cotransfected with pRL-SV40. Luciferase (firefly and renilla) activity was measured 24 hours posttransfection. B, Same as in A except 3′ deletions of the 406-bp region were tested. C, Substitution of the risk allele in pGL3p211 blunts promoter activity. Oligonucleotides used to generate the truncations are described in the supplemental sequence information. Values were first normalized to pGL3 (set to 1) and then averaged across 3 biologic replicates. Differences with the control (pGL3 or Reference [G], as appropriate) were all statistically significant. Error bars represent 95% CIs. RLU indicates relative light units.

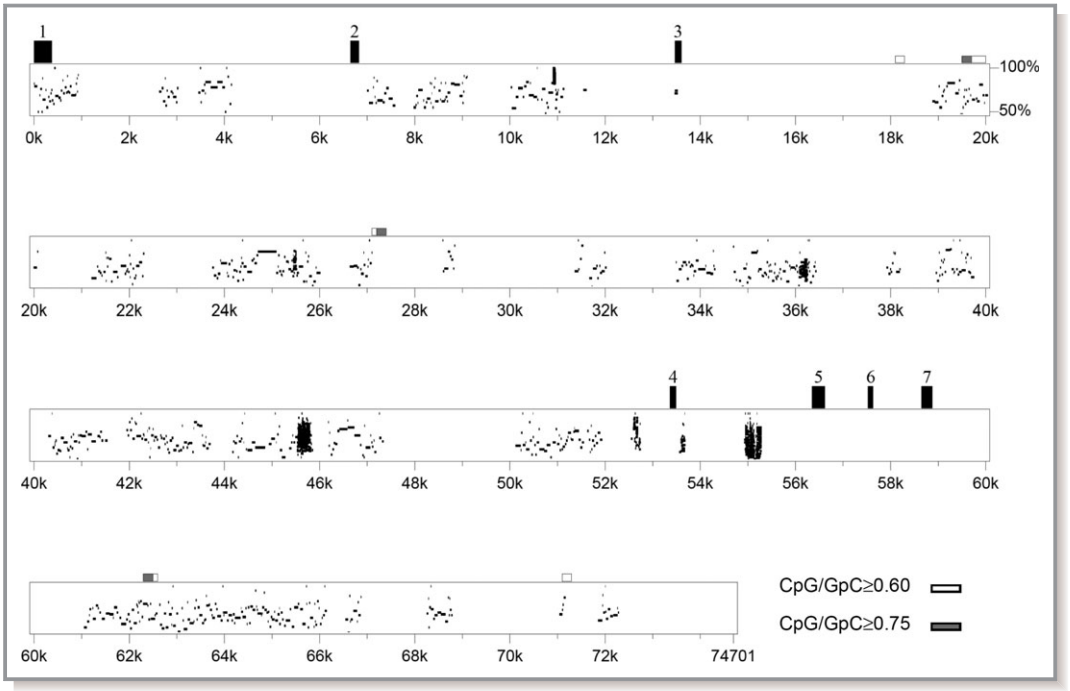

Figure 6. Mouse and human sequences were aligned using PIPmaker (http://www.bx.psu.edu/miller_lab/). Position of the human exons are shown on top, numbered 1 to 7. Note that the exons identified in human correspond to poorly conserved regions.

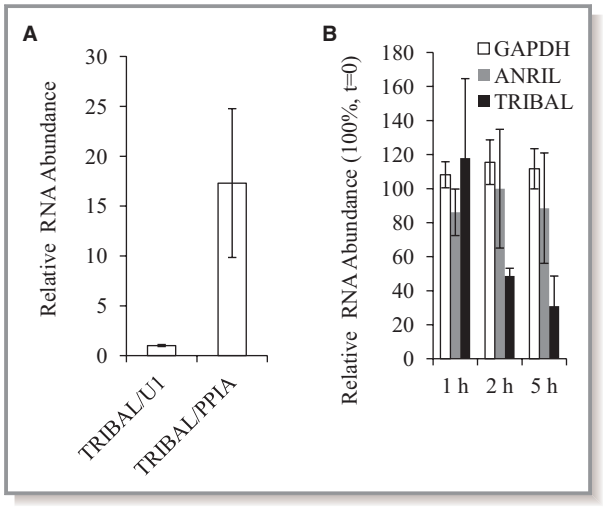

Figure 7. *TRIBAL* transcripts reside in the nucleus and display a moderately short half-life. A, Localization of *TRIBAL* transcripts. *TRIBAL*, *PPIA*, and *U1* RNAs were quantified by qRT-PCR in total HepG2 lysates and isolated nuclei. Relative nuclear abundance (defined as nuclear over total signal) was measured for all 3 transcripts by qRT-PCR and nuclear enrichment is expressed relative to U1 and PPIA nuclear signals. B, Stability of *TRIBAL* transcripts. Abundance of *GAPDH*, *ANRIL* (another long noncoding RNA), and *TRIBAL* transcripts in HepG2 cells treated with actinomycin D (5 μg/mL) for the indicated time, as assessed by qRT-PCR. Data are the average of 3 experiments. Error bars represent 95% CIs. GAPDH indicates glyceraldehyde-3-phosphate dehydrogenase; PPIA, Peptidylprolyl Isomerase A; qRT-PCR, quantitative reverse transcription polymerase chain reaction; *TRIBAL*, *TRIB1*-associated locus.

TRIB1 knock-down on *TRIBAL*. We reasoned that *TRIBAL* might regulate *TRIB1* chiefly under conditions where the latter is perturbed. Notably, *TRIB1* knock-down strongly stimulated *TRIBAL* expression in HepG2 and primary human hepatocytes (Figure 8C).

Absence of Specific *TRIBAL* Transcript Recruitment to *TRIB1*

Given the nonreciprocal effects of *TRIBAL* and *TRIB1* expression, we next determined the occupancy of *TRIBAL* on *TRIB1* by using chromatin RNA immunoprecipitation (ChIRP). Presence of *TRIBAL* on the *TRIB1* regulatory sequences might offer a mechanism of transcriptional regulation that could be turned on or off in response to appropriate cellular cues. We focused our efforts on *TRIBAL1*-overexpressing cells as detection of the endogenous transcript was difficult due to the combination of low expression levels and cross-linking conditions. To ensure specificity, ChIRP uses 2 distinct pools of oligonucleotides, and a pull-down qualifies as genuine if, and only if, both sets of target-specific oligonucleotides lead to similar enrichment. While *TRIBAL1* was indeed isolated and markedly enriched relative to *PPIA*, we found no evidence of preferential association of *TRIBAL1* in or adjacent to the *TRIB1* promoter region (Figure 9). Consistent with the lack of preferential recruitment, there was no evidence of *TRIB1* promoter regulation by *TRIBAL1* in reporter assay experiments (Figure 10).

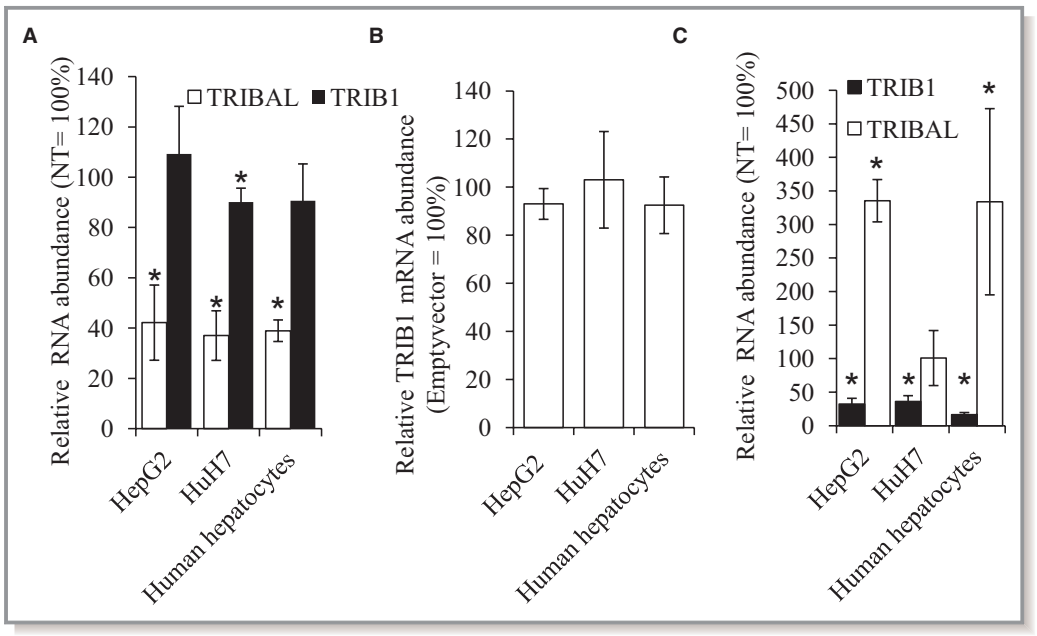

Figure 8. Modulation of *TRIBAL* transcript levels fails to impact *TRIB1* mRNA abundance. A, Knock-down of *TRIBAL* lncRNA. Cells were incubated for 48 hours prior harvest with a *TRIBAL*-targeting oligonucleotide or a control oligonucleotide (NT). B, Overexpression of *TRIBAL1* in primary human hepatocytes and HepG2. HepG2 cells and primary human hepatocytes were infected with *TRIBAL1*-expressing (or control) lentivirus. HepG2 and Huh7 clones were stably selected with puromycin while hepatocytes were harvested 48 hours post infection. C, Knock-down of *TRIB1* increases *TRIBAL* transcript levels; the same ss in A except that *TRIB1* was targeted with an antisense nucleotide against *TRIB1*. Values were normalized to the NT (A and C) or empty vector (B). Numbers shown represent the average of 3 or 4 experiments. *Statistically significant change from the respective nontarget (NT: A and C) or empty vector (B) control values. *TRIBAL* indicates *TRIB1*-associated locus.

Key Lipid Metabolism Regulatory Genes Are Unaffected by Perturbations of *TRIBAL* in Huh7 Cells

While these results indicated a subtle interaction between *TRIBAL* transcripts and *TRIB1*, they did not address possible *TRIB1*-independent contributions of *TRIBAL* to lipid metabolism. To address this point, the expression levels of several key genes, including several previously demonstrated to be affected by *TRIB1* downregulation,[7] were tested in response to *TRIBAL* downregulation and overexpression. Reducing *TRIBAL* expression by ≈60% in Huh7 or increasing expression by >100-fold had no significant impact on any lipid regulatory genes tested (Figure 11). Thus, despite the link between rs2001844 and plasma TGs, the *TRIBAL* transcript does not appear to directly affect the expression of key hepatic lipid regulatory genes.

Implication of *TRIBAL* in Small GTPase and G Protein–Coupled Signaling

With the limited impact of *TRIBAL* level manipulation on key lipid genes and *TRIB1* expression, the search for putative targets was broadened. First, interrogations of public databases (CSG, University of Michigan; ScanDB) with rs2001844 or rs2001845 failed to reveal any genome-wide significant ($P<5\times10^{-8}$) genetic associations. In view of the paucity of information obtained from these early efforts, microarray analyses of transcriptional responses to *TRIBAL* perturbation were performed. Changes in response to *TRIBAL* downregulation were first assessed; Huh7 were used rather than HepG2 because the former exhibited more extensive and consistent downregulation of *TRIBAL*. In addition to *TRIBAL* itself (not shown), only 2 targets with large (>1.5) absolute changes were observed, both lncRNA of unknown function (Table 3). Next, the transcriptional impact of *TRIBAL1* overexpression was examined by using stable HepG2-derived cell lines. Again, no protein coding genes exhibited a >1.5-fold change (Table 3). However, several piwi RNAs (piRNA) were identified, as well as *SNORD109B*, the only indexed protein coding gene to exhibit a >1.5-fold change. Of interest, *SNORD109B*, although poorly characterized, has been implicated in Prader–Willi disease, a syndromic obesity phenotype.

Limiting the analysis to >1.5-fold changes might miss small yet biologically relevant differences. To obtain a global picture of the changes entailed, DAVID analyses (http://david.abcc.

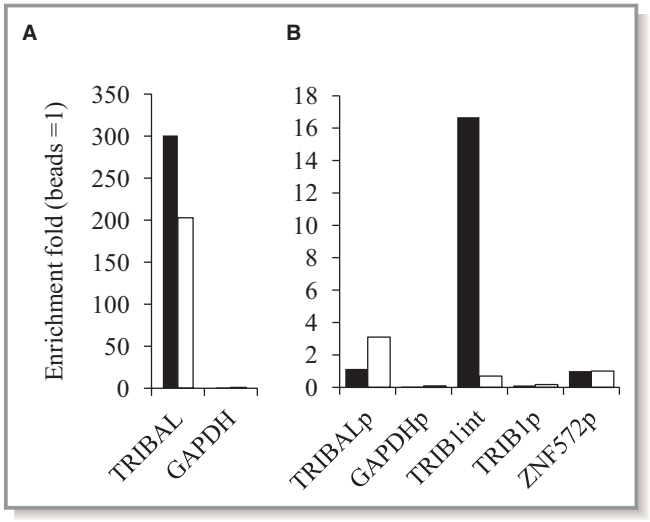

Figure 9. No preferential *TRIBAL1* association with *TRIB1* genomic regions. ChIRP analysis was performed on HepG2 cells overexpressing *TRIBAL1* using 2 sets of nonoverlapping biotinylated oligonucleotides reverse complement to the *TRIBAL1* transcript. A, qRT-PCR analysis. *TRIBAL1* enrichment achieved with both sets of oligonucleotides (solid, set 1 and open bars, set 2) over beads alone. As a control, *GAPDH* shows no enrichment. B, qPCR amplification of the DNA fraction associated with the *TRIBAL1* pull-down. Two unrelated promoter regions (GAPDH and ZNF572) serve as non-specific controls. Data shown are representative of 4 independent experiments. *TRIBAL* indicates *TRIB1*-associated locus.

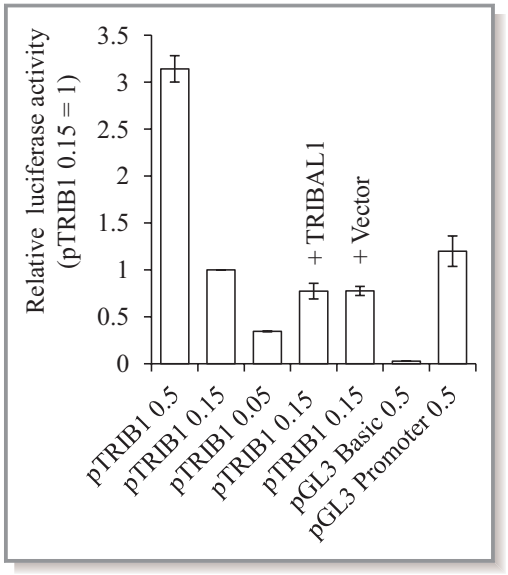

Figure 10. *TRIBAL1* overexpression fails to affect *TRIB1* promoter activity. Indicated pGL3-derived constructs were transfected in HepG2 cells (12-well plate), and luciferase and renilla luminescence were measured 24 hours later. pTRIB1 contains a 1.9-kb fragment of the Trib1 promoter region (see supplemental sequence information for the PCR primers used). PLVX*TRIBAL1* (*TRIBAL1*) or PLVX (Vector) (0.35 μg) was included where indicated. Data are normalized to pRL-SV40 included as internal transfection control, and data are expressed relative to pTRIB1 (0.15 μg). pGL3 basic and pGL3 promoter (SV40 promoter) are shown for comparison. PLVX*TRIBAL1* transfection resulted in >1000 fold-increase over endogenous levels. PCR indicates polymerase chain reaction; *TRIBAL*, *TRIB1*-associated locus.

ncifcrf.gov/) were performed on the top statistically significant changes (adjusted $P<0.05$) from both analyses. Down-regulation of *TRIBAL* resulted (false discovery rate <0.05) in perturbation of genes involved in small GTPase regulation, while *TRIBAL1* overexpression was associated with G protein–coupled receptor (*GPCR*) signaling (Table 4), suggesting that *TRIBAL* may impinge on GTP metabolism.

Multiple Pathways Affected by *TRIBAL* Overexpression Revealed by Promoter Arrays

These experiments focused on transcriptional regulation, which constitutes one of several layers of biologic homeostasis. To obtain a more complete picture of the potential role of *TRIBAL*, we explored the impact of *TRIBAL* on posttranscriptional regulation by using Cignal Reporter Arrays (SABiosciences); these are designed to probe the status of several pathways simultaneously by measuring the activity of promoter elements functionally validated for each pathway. Of the 45 pathways tested, *TRIBAL1* overexpression in HepG2 and Huh7 hepatoma cells impinged on several distinct pathways ($P<0.05$) (Figure 12). In addition, using less stringent criteria ($P<0.1$ for at least 1 cell type), shared pathway trends across the 2 cell types (KLF4, JNK, Oct4) emerged.

Discussion

In an investigation of the functional relationship of a noncoding region at 8q24.13, downstream from the *TRIB1* gene, to plasma TG and CAD risk, we have identified and characterized a novel gene locus (*TRIBAL*) that codes for multiple lncRNA splice variants. In addition, we demonstrate that *TRIBAL* contains a conserved promoter region in tight LD with risk SNPs previously identified in multiple GWAS. Furthermore, we identify an SNP, rs2001844, within the 5′ region of *TRIBAL* that alters promoter activity and is associated with: plasma TG concentrations, *TRIB1* expression in blood cells of healthy subjects, and the risk for CAD. These findings provide a partial mechanistic link between the *TRIB1* locus and CAD risk. Before this work, a direct association of *TRIB1* with plasma TG and CAD risk was inferred from mouse models of *Trib1* knock-down and overexpression under the assumption that the risk locus exerted its effects via *TRIB1*. While the relative linear proximity of *TRIB1* and *TRIBAL* makes

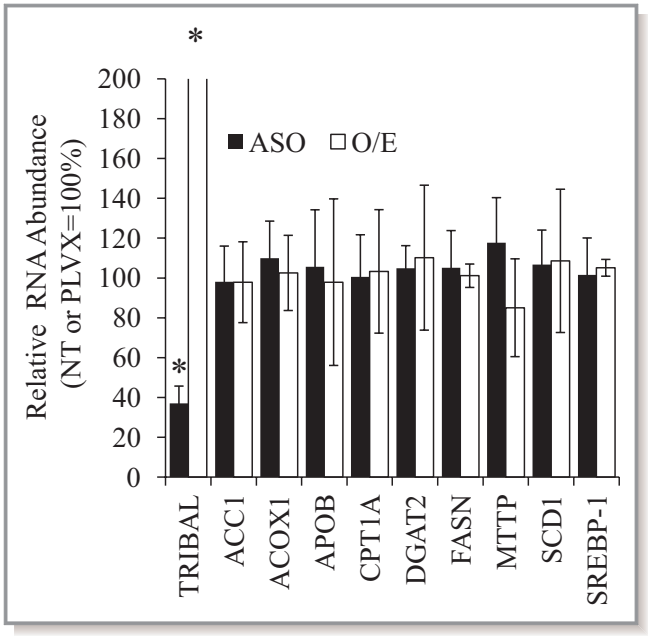

Figure 11. Expression levels of lipid metabolism genes are not affected by changes in *TRIBAL* message level. Transcription of the indicated genes was assessed in Huh7 by qRT-PCR following targeting of *TRIBAL* lncRNA with antisense oligonucleotides (ASO) or stable overexpression of *TRIBAL1* (O/E) generated by lentiviral infection. Incubation with the ASO was performed for 48 hours before cell harvest. Data are normalized to corresponding nontarget (ASO vs NT) or empty vector (O/E vs PLVX). Data represent the average of 3 biologic replicates ±95% CIs. *Statistically significant change. *TRIBAL1* expression was increased 151-fold (±48, 95% CI). ACC1 indicates Acetyl-CoA Carboxylase Alpha; ACOX1, Acyl-CoA Oxidase 1, Palmitoyl; APOB, apolipoprotein B; CPT1A, Carnitine Palmitoyltransferase 1A; DGAT2, Diacylglycerol O-Acyltransferase 2; FASN, fatty acid synthase; MTTP, Microsomal Triglyceride Transfer Protein; SCD1, Stearoyl-CoA Desaturase (Delta-9-Desaturase); SREBP-1, Sterol Regulatory Element Binding Transcription Factor 1; *TRIBAL*, *TRIB1*-associated locus.

Table 3. Changes in Response to *TRIBAL* Levels Assessed by Transcription Array Analyses

Transcript Cluster ID	Fold Change (Linear)	Gene Symbol
TRIBAL1 O/E		
DQ599087,uc001nas.1	−2.62	—
Small nucleolar RNA, C/D box 109B	−1.90	SNORD109B
DQ590525,uc001jym.1	−1.81	—
DQ597713,uc004alm.2	−1.72	—
DQ577812,uc021qhx.1	−1.68	—
DQ589460,uc010rgf.2	−1.62	—
JA202363,uc022bun.1	1.50	—
OTTHUMT00000006709/lnc-OR4F29-3	1.51	—
DQ577137,uc001xwa.3	1.74	—
DQ594093,uc021vmi.1	1.84	—
TRIBAL knock-down		
lincRNA:chr21:44907688-44909690 forward strand	−2.32	—
lincRNA:chr7:29652000-29661000 forward strand	2.36	—

Transcripts displaying greater than 1.5× linear changes in response to *TRIBAL1* overexpression (*TRIBAL1 O/E*) or *TRIBAL* knock-down are listed.

a *cis* regulation model appealing, the complex architecture of the genome permits long-range interactions that could equally account for the relationship of the *TRIBAL* region to plasma lipids and CAD.[29]

While not identified in the original GWAS for TG or CAD, subsequent to our analysis, a gene-centric meta-analysis of plasma lipid traits identified rs2001844 as having the largest effect size of the 40 SNPs investigated in this region.[28] In addition, analysis of rs2001844 predicted function by RegulomeDB yields a relatively high score (2b) consistent with a functional role and, notably, rs2001844 is the only reported common SNP within the CNS region that has the potential to alter cognate regulatory motifs (HaploReg). More specifically, a G (reference)-to-A (risk) substitution, associated with a decrease in *TRIBAL* promoter activity in our assays, is predicted to significantly decrease association of the p300 coactivator and promote heat shock gactor binding. Interestingly, p300 was found to interact with a DNA fragment matching exon 1 of *TRIBAL* by ChIP-seq in both HepG2 and HeLa cells (ENCODE).

Our data appear globally consistent with a previous report,[7] demonstrating that *Trib1* deficiency in mice results in increased plasma TG. More specifically, the current study demonstrates that the CAD risk SNPs are associated with increased plasma TGs and decreased *TRIB1* in the blood of normolipemic subjects, consistent with, but by no means proof of, an atheroprotective role for *TRIB1* in humans. Burkhardt et al[7] demonstrated a profound influence of hepatocyte *Trib1* on murine TG metabolism. The specific contributions of hepatic *TRIB1* expression to these processes in human hepatocytes remain to be addressed, but it is important to note that the TG risk SNPs at the human *TRIB1* locus are also linked to hepatic steatosis, implicating effects mediated by altered hepatic lipogenesis, fatty acid oxidation, or VLDL assembly and secretion.[30] Extrahepatic roles are also plausible. Satoh et al[9] uncovered novel metabolic roles for *Trib1* that extend into the macrophage compartment, in turn affecting adipocyte metabolism. Given the association of risk SNPs with *TRIB1* expression in PBMCs observed here, the role of *Trib1* in macrophages is of interest to our understanding of this locus.

The data presented here demonstrate that *TRIBAL* responds to altered expression of *TRIB1* but are not support-

Table 4. Analyses of Statistically Significant Changes According to the Database for Annotation, Visualization and Integrated Discovery (DAVID)

Category	Term	*P* Value	Fold	FDR
TRIBAL1 O/E				
GOTERM_BP_FAT	GO:0007186~G protein–coupled receptor protein	4E–07	2.8	6.42E–04
REACTOME	REACT_14797:Signaling by GPCR	1.7E–06	3.2	1.06E–03
GOTERM_BP_FAT	GO:0007166~cell surface receptor linked signal transduction	1.1E–05	2.1	1.81E–02
TRIBAL knock-down				
GOTERM_BP_FAT	GO:0032318~regulation of Ras GTPase activity	1.4E–05	3.5	2.45E–02
GOTERM_BP_FAT	GO:0051056~regulation of small GTPase mediated signal	2.3E–05	2.4	4.05E–02

Genes with significantly affected changes (without correction for multiple testing) following *TRIBAL1* overexpression (O/E) or knock-down were analyzed for pathway enrichment. Pathways that are significantly enriched (FDR <0.05) are shown. FDR indicates false discovery rate; fold, fold enrichment.

ive of a role for *TRIBAL* transcripts in the regulation of hepatocyte *TRIB1*. Manipulation of *TRIBAL1* transcript abundance by antisense or overexpression approaches failed to alter *TRIB1* in a consistent manner, although a small effect was observed in the Huh7 hepatocyte cell line. Results from overexpression experiments are largely consistent with the knock-down data and are indicative of functional independence of the 2 transcripts. In addition, we did not detect specific recruitment of *TRIBAL1* to the *TRIB1* promoter in HepG2 cells. By analogy with transcription factors where occupancy is not tantamount to gene regulation, *TRIBAL* transcripts could be recruited to chromatin in a primed but inactive state. The data presented here are inconsistent with this model, although there remains the possibility that *TRIBAL1* may interact with *TRIB1* via a regulatory region that we have not examined. ChIRP experiments were designed to interrogate regions proximal to the *TRIB1* promoter and did not provide evidence of binding at or upstream of the 5′ sequence of *TRIB1*. To extend these findings, in future experiments we will use a ChIRPseq system[31] to identify genome-wide *TRIBAL1* chromatin binding sites. One additional caveat to the overexpression approach is that we selected one of several identified *TRIBAL* transcript variants. Analogous to several other lncRNAs, *TRIBAL* transcripts are subject to alternative splicing and it is unclear whether the "major" form identified so far is the most biologically relevant in human hepatocytes. Clearly, a full understanding of the relationship among *TRIBAL, TRIB1,* and CAD/TG will necessitate further characterization of the various *TRIBAL* transcripts, in hepatocytes as well as in other CAD-relevant tissues.

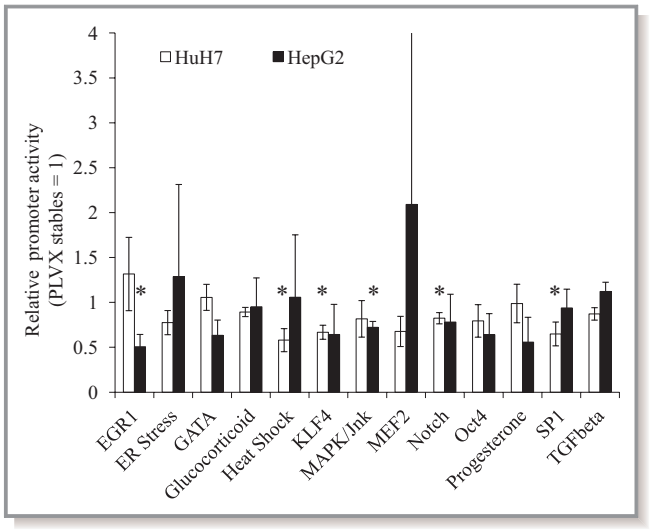

Figure 12. Long-term impact of *TRIBAL1* overexpression. HepG2 (black bars) and Huh7 (white bars) cells, stably expressing *TRIBAL1* or vector (PLVX) alone were assessed for pathway activation status using Cignal Reporter arrays. Relative reporter activity (firefly/renilla) was divided by the PLVX infected population values. Values represent the mean of 3 biologic replicates ±95% CI. Overexpression of *TRIBAL1* increased *TRIBAL* signal ≈200 for Huh7 and 800× for HepG2. Data shown focus on pathways trending toward significance (of 45 total pathways tested) in either cell type ($P<0.1$, 2-tailed Student's *t* test). *Statistically significant changes ($P<0.05$). ER indicates Endoplasmic reticulum; EGR1, Early growth response protein 1; KLF4, Kruppel-Like Factor 4; MAPK, mitogen-activated protein kinase; Jnk, c-Jun N-terminal kinase; MEF2, myocyte enhancer factor-2; TGFbeta, Transforming Growth Factor Beta; *TRIBAL*, *TRIB1*-associated locus.

As proposed previously for other poorly conserved lncRNA, *TRIBAL* expression may simply arise from transcriptional noise secondary to local and transient chromatin opening,[32] and the GWAS locus regulation may not necessitate specific, or even any, *TRIBAL* transcripts. Rather, the unwinding itself, with no obligate transcription, could act as a *TRIB1* locus regulatory switch via an enhancer-like looping mechanism for instance.[33] It is possible that the increased *TRIBAL* abundance observed following *TRIB1* knock-down is a byproduct of a local chromatin rearrangement aimed at restoring *TRIB1* mRNA levels. According to some estimates, up to 40% of the genome

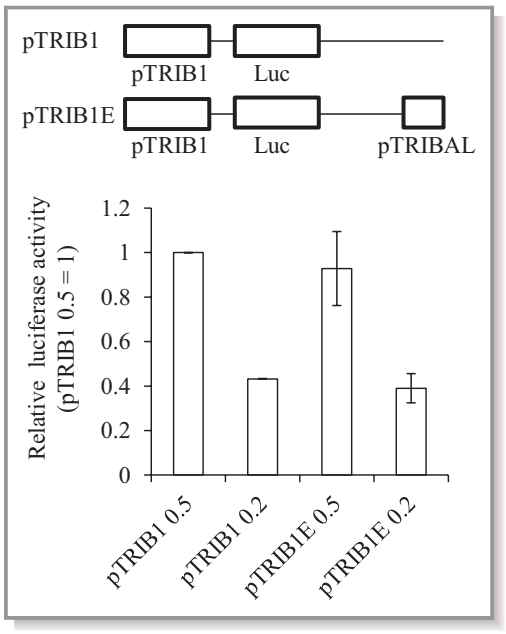

Figure 13. No measurable cis regulation by *TRIBAL* conserved region on *TRIB1* promoter region. Indicated pGL3-derived constructs were transfected in HepG2 cells, and levels of luciferase and renilla were measured 24 hours later. pTRIB1 contains a 1.9-kb fragment of *TRIB1* promoter region. pTRIBAL corresponds to a 497-bp fragment encompassing the conserved *TRIBAL* promoter region. Amounts transfected are indicated (12-well plates; μg). Data are normalized to pRL-SV40 and expressed relative to the "pTRIB1 0.5" values. Data represent the mean of 2 experiments (±95% CIs) and are representative of 3. *TRIBAL* indicates *TRIB1*-associated locus.

may harbor enhancer-like functions.[29] Interestingly, the CNS region shares chromatin features found, albeit not exclusively, in weak enhancers according to publicly available ENCODE data. Early studies in our laboratories using *TRIB1* promoter reporter constructs have thus far identified no such activity (Figure 13). However, while this manuscript was in revision, Ishizuka et al[34] reported that the poorly conserved region immediately downstream of the CNS region, roughly spanning exon 1 and intron 1 of *TRIBAL*, harbors enhancer-like activity in transient reporter assays. Certainly, these experiments point to a possible functional link between *TRIBAL* and *TRIB1* but fall short of addressing whether the region identified affects endogenous *TRIB1* (or *TRIBAL*) expression. Testing this model adequately requires in-depth analyses probing the topology of the genome around the *TRIB1* locus, in the presence of either *TRIBAL* control or risk alleles.

Further arguing against transcriptional noise, our analyses reveal that perturbation of expression by *TRIBAL* knock-down or *TRIBAL1* overexpression resulted into systematic expression changes in transcripts associated with small monomeric Ras GTPases and GPCR signaling, respectively. Interestingly, Ras is a well-characterized regulator of mitogen-activated protein kinase signaling, which we have recently shown to be implicated in *TRIB1* mRNA stability[35] hinting that *TRIBAL* could affect *TRIB1* levels in *trans*, via putative alterations in mitogen-activated protein kinase kinase/extracellular signal-regulated kinases signaling. The implication of mitogen-activated protein kinases is consistent with the overexpression results as elements from both GPCR and Ras signaling can converge on mitogen-activated protein kinase.[36] In view of the lack of consistent effect of altered *TRIBAL* expression, *TRIB1* mRNA levels in primary human hepatocytes or hepatocyte cell lines, any impact of *TRIBAL* in liver is likely to be modest. Nevertheless, these results point to potential regulatory nodes that may link *TRIBAL* to *TRIB1* in extrahepatic settings.

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Disclosures

None.

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