TM6SF2 is a regulator of liver fat metabolism influencing triglyceride secretion and hepatic lipid droplet content

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Genome-wide association studies have identified a locus on chromosome 19 associated with plasma triglyceride (TG) concentration and nonalcoholic fatty liver disease. However, the identity and functional role of the gene(s) responsible for these associations remain unknown. Of 19 expressed genes contained in this locus, none has previously been implicated in lipid metabolism. We performed gene expression studies and expression quantitative trait locus analysis in 206 human liver samples to identify the putative causal gene. Transmembrane 6 superfamily member 2 (*TM6SF2*), a gene with hitherto unknown function, expressed predominantly in liver and intestine, was identified as the putative causal gene. *TM6SF2* encodes a protein of 351 amino acids with 7–10 predicted transmembrane domains. Otherwise, no other protein features were identified which could help to elucidate the function of TM6SF2. Protein subcellular localization studies with confocal microscopy demonstrated that TM6SF2 is localized in the endoplasmic reticulum and the ER-Golgi intermediate compartment of human liver cells. Functional studies for secretion of TG-rich lipoproteins (TRLs) and lipid droplet content were performed in human hepatoma Huh7 and HepG2 cells using confocal microscopy and siRNA inhibition and overexpression techniques. In agreement with the genome-wide association data, it was found that *TM6SF2* siRNA inhibition was associated with reduced secretion of TRLs and increased cellular TG concentration and lipid droplet content, whereas *TM6SF2* overexpression reduced liver cell steatosis. We conclude that TM6SF2 is a regulator of liver fat metabolism with opposing effects on the secretion of TRLs and hepatic lipid droplet content.

TM6SF2 expression | TM6SF2 function | TM6SF2 identification | 19p12 locus

Genome-wide association (GWA) studies have been used as an unbiased tool to identify common single nucleotide polymorphisms (SNPs) associated with plasma lipoprotein traits in population studies (1–6). Some of these lead SNPs are found in or near genes known to cause dyslipidemias, thus facilitating the identification of the functional process that governs their relationship with the lipoprotein trait. However, many of the newly discovered lead SNPs are either found in genomic regions devoid of expressed genes or are present in genomic regions harboring a large number of genes previously not implicated in lipoprotein metabolism. An example of the later condition is the locus in chromosome 19, designated *CILP2*, *NCAN*, or *PBX4* locus and hereafter termed 19p12 locus, which has been identified in several GWA studies for relationships with plasma triglyceride (TG) and cholesterol concentrations (1–6). The lead SNP (rs10401969) in the 19p12 locus is part of a large linkage block containing 19 expressed genes, none of which has previously been implicated in lipid metabolism. In addition, GWA studies have also found evidence for an association between the 19p12 locus and nonalcoholic fatty liver disease (7, 8), coronary heart disease (5, 9), and diabetes mellitus (10, 11). Obviously, these conditions pose a considerable challenge for the identification of the functional gene(s) in the 19p12 locus responsible for the observed relationships with different phenotypes.

Recent studies have demonstrated that expression quantitative trait locus (eQTL) analysis provides a powerful tool for the identification of the functional gene in a locus identified by GWA analysis. For example, eQTL analysis was used successfully for the identification of *SORT1* as the functional gene responsible for the relationship between a locus on chromosome 1p13 and plasma low-density lipoprotein (LDL) cholesterol concentration (12). Here we have used gene expression and eQTL analysis of human liver samples to uncover the functional gene in the 19p12 locus responsible for the relationship between the lead SNP rs10401969 and plasma lipid levels. Presence of the minor allele of rs10401969 was found to be associated with reduced transcript levels of transmembrane 6 superfamily member 2 (*TM6SF2*). Moreover, a significant, positive relationship was observed between hepatic expression of *TM6SF2* and plasma TG concentration. These observations suggested that *TM6SF2* is the putative functional gene in the 19p12 locus responsible for the observed relationships with plasma TG levels. Subsequent functional studies in human hepatoma cells corroborated that TM6SF2 is involved in liver fat metabolism by influencing secretion of TG-rich lipoproteins (TRLs) and hepatic lipid droplet content.

Significance

Genome-wide association studies have uncovered a genetic locus in chromosome 19 associated with the plasma triglyceride (TG) concentration, a risk factor for coronary heart disease. The identity and functional role of the gene responsible for this association is unknown. Gene expression analysis of 206 human liver samples led to the identification of transmembrane 6 superfamily member 2 (*TM6SF2*), a gene with hitherto unknown function, as the putative causal gene. Functional studies in human liver cells demonstrated that inhibition of *TM6SF2* was associated with reduced secretion of TG-rich lipoproteins (TRLs) and increased cellular TG concentration, while *TM6SF2* overexpression reduced cellular TG concentration. We conclude that TM6SF2 is a novel regulator of liver fat metabolism with opposing effects on the secretion of TRLs and hepatic TG content.

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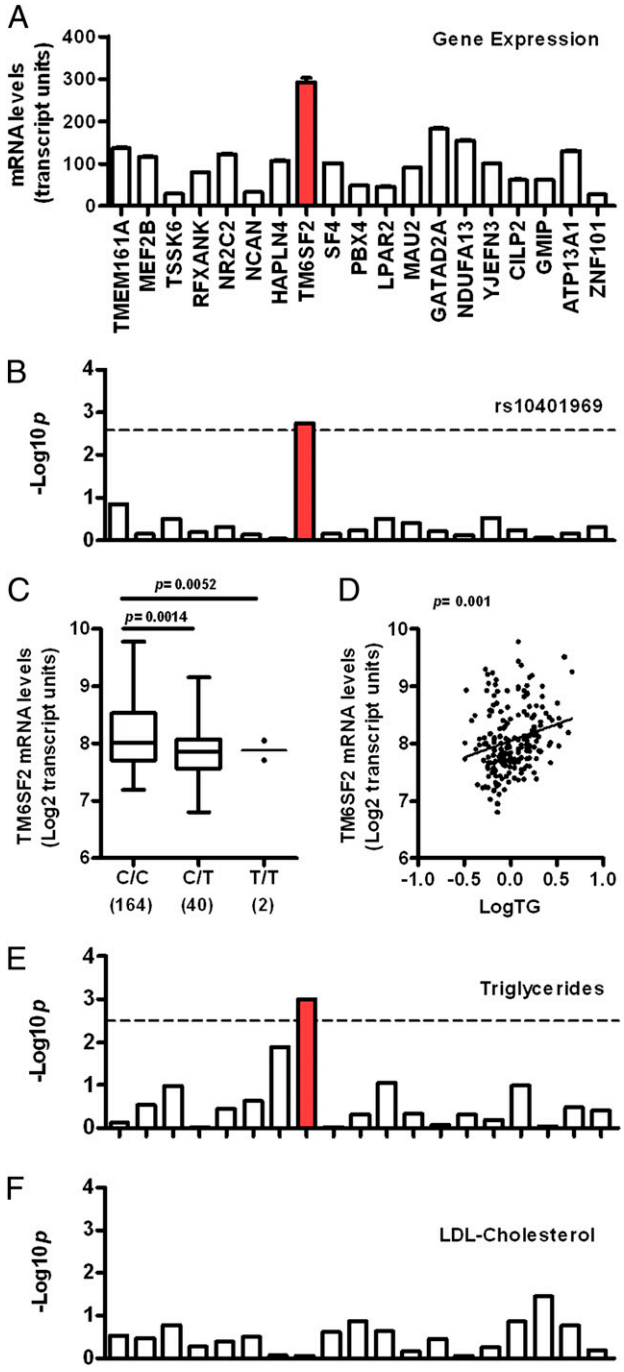

Fig. 1. Identification of TM6SF2 as putative functional gene/protein in the chromosome 19p12 locus associated with plasma TG concentration. (*A*) Comparison of the hepatic mRNA levels for all 19 genes in the 19p12 locus. mRNA levels are expressed in arbitrary units (designated transcript units) and represent mean ± SD of 206 human liver samples. (*B*) eQTL analysis of the GWA lead SNP rs10401969 for all 19 genes in the 19p12 locus, as analyzed in 206 human liver samples using multiple linear regression analysis. (*C*) Relationship between rs10401969 genotype and *TM6SF2* mRNA levels in 206 human liver samples. The data were analyzed by ANOVA (upper horizontal bar) and by Student's *t* test (lower horizontal bar). (*D*) Relationship between *TM6SF2* mRNA levels and plasma TG concentration in 206 subjects using multiple linear regression analysis. (*E* and *F*) Comparison of the $-\log^{10} P$ values for the relationships between the mRNA levels of all 19 genes in the 19p12 locus and the plasma TG (*E*) and LDL cholesterol (*F*) concentrations analyzed in 206 individuals. The dotted line in *B* and *E* represents $P = 0.05$ corrected for 19 tests ($P = 0.0026$).

Results

Relationships Between rs10401969 Genotype, Hepatic Expression of TM6SF2, and Plasma TG Concentration. The mRNA levels of all 19 genes in the 19p12 locus were analyzed in 206 human liver biopsies using Affymetrix GeneChip Human exon 1.0 ST arrays. As shown in Fig. 1*A*, all 19 genes were expressed in human liver, with the highest mRNA levels for *TM6SF2*. This gene was originally described in conjunction with the identification of *TM6SF1* (13), but no detailed analysis of *TM6SF2* has been reported. eQTL analysis was performed between the lead SNP rs10401969 and the hepatic expression of all 19 genes in the 19p12 locus (Fig. 1*B*). Multiple linear regression analysis demonstrated a significant relationship ($P = 0.0018$) between *TM6SF2* mRNA expression and rs10401969, coded by the number of major alleles of the SNP. This relationship remained significant following correction for multiple testing ($P = 0.05$ corrected for 19 tests is 0.0026, indicated by the dotted line in Fig. 1*B*). No relationships were observed between rs10401969 and expression of other genes in the 19p12 locus (Fig. 1*B*). As shown in Fig. 1*C*, the minor T-allele of rs10401969 was associated with reduced expression of *TM6SF2* ($P = 0.0052$, analyzed by ANOVA). Note that only two subjects homozygous for the minor T-allele were identified in this cohort. Nevertheless, a significant difference in *TM6SF2* mRNA level was observed for subjects with the major C/C genotype compared with subjects with the C/T genotype ($P = 0.0014$, analyzed by Student *t* test).

Because recent GWA studies reported associations between the minor allele of rs10401969 and reduced plasma triglyceride levels (1–6), we analyzed the relationship between hepatic *TM6SF2* mRNA level and plasma lipoprotein concentrations. As shown in Fig. 1 *D* and *E*, a positive relationship was observed between hepatic *TM6SF2* mRNA levels and plasma TG concentration ($P = 0.0010$, as analyzed by multiple linear regression) whereas no relationships were found with plasma LDL cholesterol (Fig. 1*F*) and HDL cholesterol (Fig. S1) levels. Overall, these observations provide evidence for a link between rs10401969 genotype, hepatic expression of *TM6SF2*, and plasma TG concentration, suggesting that *TM6SF2* is the functional gene in the 19p12 locus involved in TG metabolism.

Characterization of TM6SF2. Human *TM6SF1* and *TM6SF2* are located on chromosomes 15 and 19, respectively. *TM6SF2* encodes a protein of 351 amino acids with 68% similarity and 52% identity to TM6SF1 (13). Protein pattern and domain prediction softwares predicted 7–10 transmembrane domains for TM6SF2. Considerable conservation was observed for the predicted protein sequence of TM6SF2 in the cow, dog, guinea pig, mouse, rat, and human.

In agreement with a report by Morris et al. (10), we identified a nonsynonymous variant (rs58542926) in exon 6 of *TM6SF2* exhibiting near-complete allelic association ($r^2 > 0.99$) in our cohorts with rs10401969, but this change is predicted by SIFT (14) to have no appreciable effect on protein function. Otherwise, no other protein features were identified which could help to elucidate the function of TM6SF2.

We evaluated the expression of *TM6SF2* in human tissues and found, in agreement with Dezso et al. (15), substantial *TM6SF2* mRNA levels in liver and intestine, whereas all other tissues analyzed showed low *TM6SF2* mRNA levels (Fig. S2). The tissue expression of *TM6SF2* resembles the expression pattern of apolipoprotein B (*APOB*), coding for the essential structural protein for the synthesis and secretion of TRLs from liver and intestine (Fig. S2). In agreement with the *TM6SF1* Northern blot analysis reported by Carim-Todd et al. (13), it was found that the *TM6SF1* mRNA levels were low in human intestine tissue, whereas *TM6SF1* mRNA levels were below the detection threshold in the expression analysis of the 206 human liver samples.

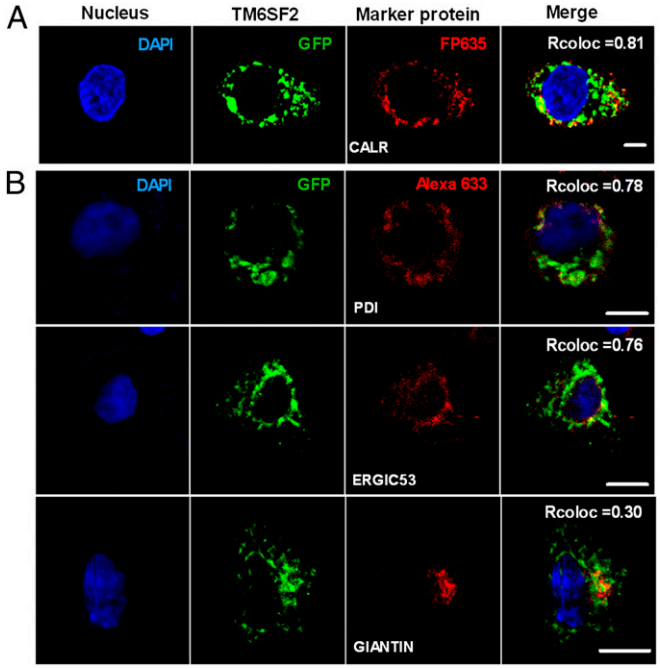

Fig. 2. Subcellular localization of TM6SF2. (*A*) Human hepatoma Huh7 cells were transfected with GFP-tagged TM6SF2 and FP635-tagged CALR, a marker for ER. Following 48-h incubation, cells were fixed and subjected to fluorescence analysis. (*B*) Huh7 cells were transfected with GFP-tagged TM6SF2. Following 48-h incubation, cells were stained for PDI, ERGIC53, or GIANTIN, markers for ER, ERGIC, and Golgi, respectively. Colocalization was quantified using Pearson correlation (Rcoloc) and represents mean values of one to three cells evaluated in four to six independent experiments. (Scale bar, 10 μm.)

Confocal microscopy was used to determine the subcellular localization of TM6SF2 in human hepatoma Huh7 and HepG2 cells. Expression of full-length TM6SF2 containing a C-terminal GFP tag in human hepatoma Huh7 cells revealed a perinuclear lattice-like staining, indicating localization to the endoplasmic reticulum (ER) (Fig. 2*A*). A similar pattern was seen in HepG2 cells. Considerable overlap was observed between TM6SF2 and the ER marker calreticulin (CALR) containing a C-terminal FP635 tag (Fig. 2*A*). Colocalization of these proteins was quantified using Pearson correlation analysis, where values exceeding 0.5 indicate appreciable overlap and 1.0 indicates complete colocalization. The Pearson correlation (Rcoloc) value for TM6SF2 and CALR was 0.83 ± 0.04 (mean ± SD, $n = 5$), compatible with a predominant localization of TM6SF2 in the ER in human hepatoma cells. No evidence was found for localization of TM6SF2 in other membranous structures, like cell membrane, mitochondria, and lipid droplets.

The possible involvement of TM6SF2 in ER–Golgi transport was subsequently evaluated by immunofluorescence staining of TM6SF2 transfected cells with antibodies to the ER marker protein disulphide isomerase (PDI), the ER-Golgi intermediate compartment (ERGIC) marker protein ERGIC53, and the Golgi marker protein GIANTIN. As shown in Fig. 2*B*, considerable overlap between TM6SF2 and both PDI and ERGIC53 was observed, whereas only limited overlap was seen for TM6SF2 and GIANTIN. The Rcoloc values (mean ± SD) for PDI, ERGIC53, and GIANTIN in relation to TM6SF2 were 0.78 ± 0.05 ($n = 10$), 0.80 ± 0.03 ($n = 10$), and 0.30 ± 0.08 ($n = 10$), respectively. As control, we analyzed in separate experiments the colocalization of CALR transfected cells with immunofluorescence staining for PDI, ERIGIC53, and GIANTIN (Fig. S3). As expected, considerable overlap was observed between CALR and PDI, whereas only limited overlap was seen between CALR and both ERGIC53 and GIANTIN. Taken together, these data indicate that TM6SF2 is mainly localized to the ER and ERGIC, and not to the Golgi.

TM6SF2 Influences Secretion of TRLs. Functional analysis of TM6SF2 was conducted in the human hepatoma Huh7 and HepG2 cell lines using transient transfection techniques with specific *TM6SF2* siRNA probes. In preliminary experiments, both cell lines were found to have *TM6SF2* mRNA levels comparable to human liver, while both cell lines exhibited borderline detectable *TM6SF1* mRNA levels, in agreement with the *TM6SF1* expression data from the human liver samples. Two different *TM6SF2* siRNA probes were used throughout these studies, and comparable results were obtained for both probes. *TM6SF2* siRNA inhibition reduced the *TM6SF2* mRNA levels in Huh7 and HepG2 cells to 27 ± 7% and 24 ± 5% of control values, respectively (mean ± SD of $n > 15$). No compensatory increases in the expression of *TM6SF1* were observed. Western blot analysis demonstrated that *TM6SF2* siRNA inhibition was associated with substantial reduction in TM6SF2 protein in Huh7 cells (Fig. 3 *A* and *B*) and HepG2 cells (Fig. 3 *C* and *D*). *TM6SF2* inhibition did not influence cell viability and had no effect on cell proliferation, as assessed by flow cytometry and quantification of mRNA and protein concentrations.

For an initial evaluation of the effect of *TM6SF2* inhibition on cell function, we analyzed in both Huh7 and HepG2 cells the effects of *TM6SF2* siRNA inhibition on the mRNA levels of 10 genes with prominent roles in hepatic lipid metabolism. As shown in Fig. 3*E*, no significant changes were observed in the expression of genes involved in secretion and metabolism of TRLs (*APOB*, *MTTP*, *LDLR*) and lipid droplet proteins (*PLIN2*, *PLIN3*). However, significant reductions (defined by a decrease with $P < 0.01$ in both Huh7 and HepG2 cells) were seen in the expression of several genes involved in triglyceride synthesis (*ACSS2*, *DGAT1*, *DGAT2*, *PNPLA3*), whereas no change was observed in the expression of *FASN*.

The effect of TM6SF2 inhibition on the secretion of TRLs was subsequently analyzed in Huh7 and HepG2 cells using a methodology that measures the secretion of the protein moiety of TRLs using an ELISA for APOB and the lipid moiety of TRLs by quantification of the secretion of C^{14} triglycerides following incubation with C^{14}-labeled glycerol (16). In agreement with our earlier study (16), the rates of APOB and TG secretion from these cells were found to be linear over time and recovered in the VLDL–LDL size range, as analyzed by molecular sieve chromatography, whereas no evidence was found of extracellular hydrolysis or reuptake of the secreted lipoproteins. As shown in Fig. 3*F*, *TM6SF2* siRNA inhibition leads to a markedly reduced secretion of TG by Huh7 and HepG2 cells, whereas more modest reductions in the secretion of APOB by the hepatoma cells was observed.

TM6SF2 Influences Hepatic Lipid Droplet Metabolism. A recent GWA study identified an association between rs2228603 in the 19p12 locus and nonalcoholic fatty liver disease (7). Although rs2228603 is in strong allelic association with rs10401969 ($r^2 > 0.96$), we hypothesized that TM6SF2, in addition to its role in hepatic TRL secretion, is also involved in hepatic lipid droplet metabolism. We addressed this question with the same *TM6SF2* inhibition procedures as were used for TRL secretion analysis. As shown in Fig. 4 *A* and *E*, *TM6SF2* inhibition increased the cellular TG concentrations in Huh7 and HepG2 cells, respectively. This observation is in line with the GWA data that demonstrated that the minor allele of rs2228603 is associated with increased liver fat (7).

The effect of *TM6SF2* inhibition on lipid droplet area and size was subsequently studied using confocal microscopy. As shown in Fig. 4*B* and quantified in Fig. 4*C*, *TM6SF2* inhibition leads

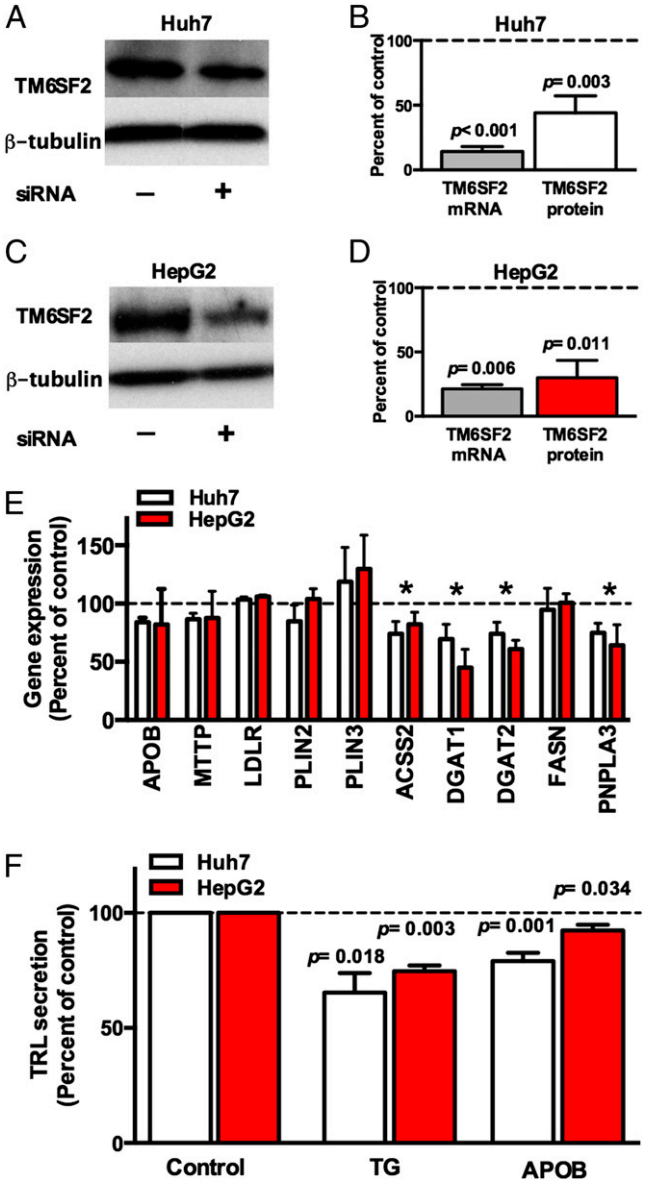

Fig. 3. *TM6SF2* siRNA inhibition decreases secretion of TRLs by human hepatoma Huh7 and HepG2 cells. (*A* and *C*) Western blot analysis of human hepatoma Huh7 (*A*) and HepG2 (*C*) cells treated for 48 h with *TM6SF2*-specific or control siRNA probes. (*B* and *D*) Quantitation of *TM6SF2* mRNA levels and TM6SF2 protein concentration estimated from the Western blot experiments shown in *A* and *C*. *TM6SF2* mRNA levels were quantified and standardized with RPLP0. TM6SF2 protein concentrations were quantified in the same experiments and standardized for the β-tubulin concentration. The values are expressed as percent of control experiments (indicated with a dotted line) and represent mean ± SD of four independent experiments. (*E*) Effect of *TM6SF2* siRNA silencing on the expression of genes involved in hepatic lipid metabolism in human hepatoma Huh7 and HepG2 cells. The values are expressed as percent of control experiments (indicated with a dotted line) and represent mean ± SD of six to eight independent experiments. **P* < 0.01 in both Huh7 and HepG2 cells. (*F*) Effect of *TM6SF2* inhibition on triglyceride (TG) and APOB secretion by human hepatoma Huh7 and HepG2 cells. The values are expressed as percent of control experiments (indicated with a dotted line) and represent mean ± SD of six to eight independent experiments.

to ∼twofold increase in lipid droplet area in Huh7 cells, and a similar phenomenon was observed in HepG2 cells (Fig. 4 *F* and *G*). The marked increase in lipid droplet area is the consequence of an increase in the number of lipid droplets as well as a shift in the overall lipid droplet size distribution toward larger lipid droplets, as illustrated for Huh7 cells in Fig. 4*B* and quantified in Fig. 4*D*, and for HepG2 cells in Fig. 4*F* and quantified in Fig. 4*H*.

We used transient overexpression with full-length *TM6SF2* plasmids as an alternative tool to evaluate the effect of TM6SF2 on lipid droplet metabolism. We observed that 10–25% of the Huh7 cells were overexpressing FP635-tagged TM6SF2 without detrimental effects on cell viability, which makes it possible to compare directly hepatoma cells expressing FP635-tagged TM6SF2 with cells not expressing FP635-tagged TM6SF2. As shown in Fig. 5 (*Upper*), cells expressing FP635-tagged TM6SF2 are characterized by a decrease in the number and size of the lipid droplets compared with cells that do not express FP635-tagged TM6SF2. As control, we analyzed the transient expression of FP635-tagged *CALR* and found no difference in the lipid droplet area of *CALR* transfected cells compared with non-transfected cells (Fig. 5, *Lower*). Cell pairs (composed of one cell expressing and the other cell not expressing the FP635-tagged protein) were identified and subjected to qualitative and quantitative analysis. As shown in Fig. S4, Huh7 cells overexpressing FP635-tagged TM6SF2 showed a significant reduction in lipid droplet area/cell area, whereas no effects on lipid droplet area/cell area were observed in cells overexpression FP635-tagged CALR. A similar study was performed using GFP-tagged *TM6SF2* transfected cells stained with Nile Red for quantification of lipid droplets, and essentially the same results were obtained (Fig. S5). Overall, these studies indicate that *TM6SF2* expression influences hepatic steatosis in Huh7 cells.

Discussion

The primary aim of this study was to uncover the gene(s) responsible for the relationship between the 19p12 locus, identified in several GWA studies (1–6), and the plasma TG concentration. Using a combination of gene expression and eQTL analysis of human liver samples, we identified *TM6SF2* as the putative functional gene in this locus. Subsequent in vitro studies in human hepatoma Huh7 and HepG2 cell lines demonstrated that *TM6SF2* siRNA inhibition reduces the secretion of TRLs. These observations are in line with the eQTL and GWA data that demonstrated that the minor allele of the lead SNP in the GWA studies is associated with reduced *TM6SF2* mRNA levels and reduced plasma TG concentration, respectively. The possible involvement of TM6SF2 in secretion of TRLs is supported by the prominent tissue expression of *TM6SF2* in liver and intestine, two tissues with critical roles in the secretion of TRLs. Moreover, subcellular localization studies found an overlapping localization pattern of TM6SF2 with ER and ERGIC, cellular compartments with a major function in hepatic TG secretion. Overall, these studies indicate that TM6SF2 is the functional protein responsible for the relationship between the 19p12 locus and the plasma TG concentration.

A recent GWA study identified an association between non-alcoholic fatty liver disease (NAFLD) and the 19p12 locus (7). Although the lead SNP of this GWA study is in strong allelic association with rs10401969, we hypothesized that TM6SF2 could be involved in hepatic steatosis. Unfortunately, in the absence of quantitative data on the lipid content of the human liver samples, we were unable to test this hypothesis directly. However, in vitro studies in human hepatoma Huh7 and HepG2 cells demonstrated that *TM6SF2* siRNA inhibition increased lipid droplet content, whereas overexpression of TM6SF2 was associated with reduced lipid droplet content. These observations are in line with the eQTL data reported here and the results from population studies (7, 8) demonstrating that the minor allele of the lead SNP is associated with reduced *TM6SF2* mRNA levels and increased hepatic lipid content, suggesting that TM6SF2 plays a role in the development of NAFLD.

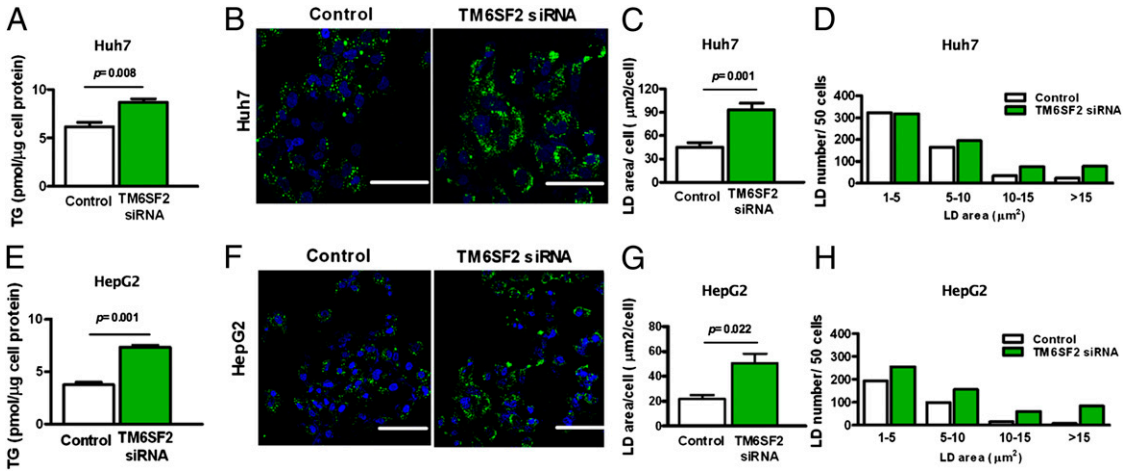

Fig. 4. *TM6SF2* siRNA inhibition increases lipid droplet content of human hepatoma Huh7 and HepG2 cells. Human hepatoma Huh7 (*A*–*D*) and HepG2 (*E*–*H*) cells were analyzed 48 h after siRNA inhibition with *TM6SF2*-specific or control siRNA probes. (*A* and *E*) Effect of *TM6SF2* siRNA inhibition on cellular TG concentration of Huh7 (*A*) or HepG2 (*E*) cells. (*B* and *F*) Representative fields of 20–30 Huh7 (*B*) or HepG2 (*F*) cells stained with DAPI and BODIPY493/503 and analyzed by confocal microscopy. (*C* and *G*) Effect of *TM6SF2* siRNA inhibition on lipid droplet (LD) area per cell area of Huh7 (*C*) or HepG2 (*G*) cells. (*D* and *H*) Effect of *TM6SF2* siRNA inhibition on size distribution of lipid droplets in Huh7 (*D*) and HepG2 (*H*) cells. Values in *A*, *C*, *E*, and *G* represent mean ± SD of six to eight independent experiments. (Scale bar, 75 µm.)

While this study was under review, it was reported by two different research groups (17, 18) that knockdown of *Tm6sf2* or transient *TM6SF2* overexpression in mice alters serum lipid profiles and influences hepatic TG content. The current study extends these reports and demonstrates in human liver cells that TM6SF2 plays a role in both secretion of TRLs and hepatic lipid droplet content. Indeed, it is remarkable that the results from the GWA studies (1–8), the knockdown and overexpression studies in mice (17, 18), and the functional analysis of TM6SF2 in human hepatoma cell lines (this study) provide consistent evidence for opposing effects of TM6SF2 on TRL secretion and hepatic lipid droplet content: Reduced expression of *TM6SF2* was associated with reduced secretion of TRLs and increased hepatic lipid droplet content, whereas increased expression of *TM6SF2* was related to decreased hepatic lipid droplet content. Needless to say, none of these reports resolve the precise functional role of TM6SF2 in these processes. However, the current study generated a number of observations that may help to address this question. First, we confirmed that TM6SF2 was prominently expressed in tissues involved in secretion of TRLs. Moreover, we found that *TM6SF2* siRNA inhibition was associated with a greater reduction of TG secretion compared with APOB secretion in both Huh7 and HepG2 cells, suggesting a role of TM6SF2 in the supply of lipids for TRL synthesis. In addition, we observed that TM6SF2 is found in ER and ERGIC of liver cells, indicating the involvement of TM6SF2 in the early stages of TRL synthesis. Finally, we noted that *TM6SF2* siRNA inhibition was associated with marked reductions in the expression of several genes involved in TG synthesis, underlining the key role of TM6SF2 in lipid metabolism. Overall, these observations

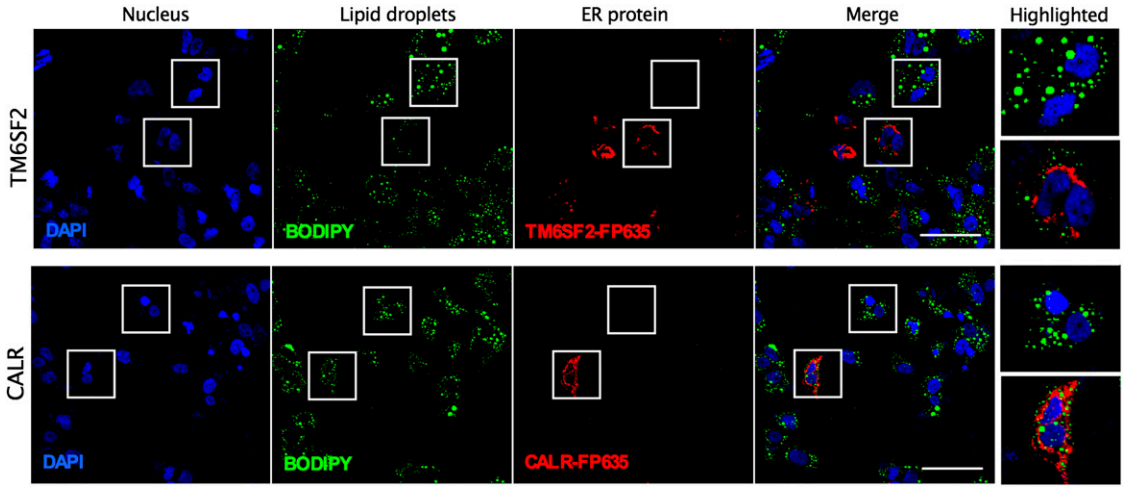

Fig. 5. TM6SF2 overexpression reduces lipid droplet content of human hepatoma Huh7 cells. Human hepatoma Huh7 cells were transfected with FP635-tagged TM6SF2 plasmids or FP635-tagged CALR (control) plasmids. Following 48-h incubation, cells were stained with BODIPY493/503 and analyzed by confocal microscopy. Representative fields of 20–30 cells transfected with FP635-tagged TM6SF2 plasmids (*Upper*) and FP635-tagged CALR plasmids (*Lower*) are shown. Note that in *Upper*, the lipid droplet content (green color) is considerably lower in TM6SF2 transfected cells (red color) compared with the nontransfected cell (absence of red color). In contrast, no difference in lipid droplet content is observed for the CALR transfected cells, as shown in *Lower*. (Scale bar, 50 µm.)

suggest that TM6SF2 is primarily involved in the regulation of TRL synthesis and secretion, with secondary effects on lipid droplet content.

In summary, through a series of studies involving gene expression and eQTL analysis of human liver samples and functional studies in the human hepatoma Huh7 and HepG2 cell lines, we identified TM6SF2 as a regulator of liver fat metabolism influencing the secretion of TRLs and hepatic lipid droplet content. The observed dual role of TM6SF2 in TRL secretion and hepatic TG content is in line with data from population studies demonstrating associations between the 19p12 locus and plasma TG concentration (1–6) and hepatic steatosis (7, 8). The clinical importance of this locus is underlined by its relationships to coronary artery disease (5, 9, 18, 19) and type 2 diabetes (10, 11). It is expected that further analysis of TM6SF2 will uncover the biological function of TM6SF2 in hepatic lipid metabolism, thereby providing new insights into the complex relationship between liver TG metabolism, coronary artery disease, and type 2 diabetes mellitus.

Materials and Methods

Gene Expression and eQTL Analysis. Liver biopsies were obtained from patients undergoing aortic valve surgery as part of the Advanced Study of Aortic Pathology (20). All protocols were approved by the ethics committee of the Karolinska Institutet, and informed consent was obtained from all participants according to the Helsinki Declaration. Gene expression was analyzed in all liver samples using the Affymetrix GeneChip Human exon 1.0 ST array (Affymetrix). Genotyping was performed using Taqman assays (Applied Biosystems, Life Technologies). Standard plasma lipid analysis was performed with automated commercial assays.

Cell Culture Conditions and Transfection Studies. Human hepatoma HepG2 and Huh7 cells were obtained from the American Type Culture Collection (HB-8065) and the Health Science Research Resources Bank (cell no. JCRB0403; Osaka, Japan), respectively. Cells were grown in Dulbecco's Modified Eagle's Medium (Gibco, Life Technologies) supplemented with 10% FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin. Lipofectamine 2000 (Invitrogen, Life Technologies) was used as transfection reagent. siRNA oligonucleotides specific for *TM6SF2* (catalog nos. s28703 and 279297) were purchased from Ambion (Life Technologies). *Trans*IT-2020 Transfection Reagent (Mirus Bio LLC) was used as transfection reagent for the overexpression studies. C-terminal GFP-tagged or FP635-tagged full-length human TM6SF2 and CALR probes were obtained from OriGene.

Confocal Microscopy. Hepatoma cells were cultured in four-well chamber slides, fixed for 20 min with 4% paraformaldehyde, washed with PBS and permeabilized for 45 min with saponin in block buffer (1.5g glycine, 3g BSA, 100 mL PBS, 2 mL 0.5% saponin). Cells were stained for 1 h with PDI, ERGIC53, or GIANTIN mouse monoclonal antibodies (Enzo Life Sciences), washed three time with PBS, and incubated for 1 h with goat antimouse Alexa Fluor 488 secondary antibody (Invitrogen, Life Technologies). Cells were washed three times with PBS, and coverslips were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories). For the lipid droplet analysis, cells were stained for 10 min with 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (BODIPY493/503, Molecular Probes, Life Technologies) and mounted with ProLong Gold Antifade (Invitrogen, Life Technologies). Images were obtained using a Leica SP5 confocal microscope, equipped with a 63 × 1.4 lens and diode and argon lasers. All images were generated using ImageJ software (http://rsb.info.nih.gov/ij/). Lipid droplet area was quantified in each chamber slide well in 12–15 randomly chosen fields of 30–50 cells using the Particle analysis Plugin of the ImageJ software. The number and area of the cells in each field were determined using the ImageJ cell counter and particle analysis Plugin. The average lipid droplet area/cell was calculated by dividing the overall lipid droplet area by the number of cells in the same field. The average lipid droplet area/cell area was calculated by dividing the total lipid droplet area by the cell area.

Assays. The TM6SF2 antibody used in the Western blot experiments was purchased from Abnova. Gene expression in the cell culture experiments was quantified by real-time quantitative PCR using Taqman assays (Applied Biosystems, Life Technologies); RPLP0 was used as standard. TG secretion was quantified following 24 h incubation of cells with ^{14}C glycerol (PerkinElmer) at a final concentration of 2.85 mCi/mL (16). The lipids were extracted from the cell culture medium, separated by TLC, and the radioactivity associated with the TGs was measured. The APOB in the cell culture medium was quantified by ELISA (ALerCHEK). Cellular TG and protein concentrations were measured with assays supplied by Biovision and Thermo Fisher Scientific.

Statistical Analysis. The distribution of continuous variables in groups was expressed as mean ± SD. Differences in continuous variables between groups were tested by Student´s *t* test. mRNA values are reported in arbitrary units (designated transcript units) when the hepatic expression of different genes were compared. The transcript units and the plasma triglyceride levels were logarithmically transformed for all other statistical analysis. Multiple linear regression was used to test the association between mRNA expression and the SNP coded by the number of major alleles, and ANOVA analysis was used to test the association between mRNA expression and the SNP genotypes. All statistical analysis was performed with GraphPad Prism software. The Rcoloc values in the colocalization studies were calculated using the colocalization Plugin of the ImageJ software.

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1. Willer CJ, et al. (2008) Newly identified loci that influence lipid concentrations and risk of coronary artery disease. *Nat Genet* 40(2):161–169.
2. Kathiresan S, et al. (2008) Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nat Genet* 40(2):189–197.
3. Aulchenko YS, et al.; ENGAGE Consortium (2009) Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. *Nat Genet* 41(1):47–55.
4. Kathiresan S, et al. (2009) Common variants at 30 loci contribute to polygenic dyslipidemia. *Nat Genet* 41(1):56–65.
5. Teslovich TM, et al. (2010) Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 466(7307):707–713.
6. Willer CJ, et al.; Global Lipids Genetics Consortium (2013) Discovery and refinement of loci associated with lipid levels. *Nat Genet* 45(11):1274–1283.
7. Speliotes EK, et al.; NASH CRN; GIANT Consortium; MAGIC Investigators; GOLD Consortium (2011) Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. *PLoS Genet* 7(3):e1001324.
8. Gorden A, et al.; GOLD Consortium (2013) Genetic variation at NCAN locus is associated with inflammation and fibrosis in non-alcoholic fatty liver disease in morbid obesity. *Hum Hered* 75(1):34–43.
9. Do R, et al. (2013) Common variants associated with plasma triglycerides and risk for coronary artery disease. *Nat Genet* 45(11):1345–1352.
10. Morris AP, et al.; Wellcome Trust Case Control Consortium; Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) Investigators; Genetic Investigation of ANthropometric Traits (GIANT) Consortium; Asian Genetic Epidemiology Network–Type 2 Diabetes (AGEN-T2D) Consortium; South Asian Type 2 Diabetes (SAT2D) Consortium; DIAbetes Genetics Replication And Meta-analysis (DIAGRAM) Consortium (2012) Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. *Nat Genet* 44(9):981–990.
11. Saxena R, et al.; Look AHEAD Research Group; DIAGRAM consortium (2012) Large-scale gene-centric meta-analysis across 39 studies identifies type 2 diabetes loci. *Am J Hum Genet* 90(3):410–425.
12. Musunuru K, et al. (2010) From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. *Nature* 466(7307):714–719.
13. Carim-Todd L, Escarceller M, Estivill X, Sumoy L (2000) Cloning of the novel gene TM6SF1 reveals conservation of clusters of paralogous genes between human chromosomes 15q24→q26 and 19p13.3→p12. *Cytogenet Cell Genet* 90(3-4):255–260.
14. Ng PC, Henikoff S (2003) SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 31(13):3812–3814.
15. Dezso Z, et al. (2008) A comprehensive functional analysis of tissue specificity of human gene expression. *BMC Biol* 6:49.
16. Krapivner S, et al. (2010) DGAT1 participates in the effect of HNF4A on hepatic secretion of triglyceride-rich lipoproteins. *Arterioscler Thromb Vasc Biol* 30(5):962–967.
17. Kozlitina J, et al. (2014) Exome-wide association study identifies a *TM6SF2* variant that confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 46(4):352–356.
18. Holmen OL, et al. (2014) Systematic evaluation of coding variation identifies a candidate causal variant in *TM6SF2* influencing total cholesterol and myocardial infarction risk. *Nat Genet* 46(4):345–351.
19. Zhou L, et al. (2011) Genetic variants at newly identified lipid loci are associated with coronary heart disease in a Chinese Han population. *PLoS ONE* 6(11):e27481.
20. Folkersen L, et al.; BiKE and ASAP study groups (2010) Association of genetic risk variants with expression of proximal genes identifies novel susceptibility genes for cardiovascular disease. *Circ Cardiovasc Genet* 3(4):365–373.