MATERIALS AND METHODS

TMEM55B is a Novel Regulator of Cellular Cholesterol Metabolism

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Expression Array Analyses
Genome-wide gene expression of 480 LCLs established from participants of the Cholesterol and Pharmacogenetics (CAP) clinical trial were incubated with either 2µM activated simvastatin or sham buffer for 24 hours. Simvastatin was obtained as a gift from Merck and activated as previously described. RNA was extracted, converted into the cRNA and quantified using the Illumina HumanRef8v3 expression beadchip and analyzed as previously described. Briefly, expression traits were adjusted for age and gender of the cell donor, exposure batch and cell count on the day of drug exposure, and RNA labeling batch. In addition, data was adjusted for principal components that described greater than 5% of the variation across the dataset and quantile normalized across each gene. This dataset has been deposited into the GEO database under accession number GSE36868. HeLa and fibroblasts cells were sterol depleted by overnight culture in lipoprotein-depleted serum (LPDS) and 1 hr exposure to 2-hydroxy-β-cyclodextrin, and TMEM55B transcript levels were quantified from isolated total cellular RNAs by qRT-PCR as previously described.

RNA-seq Analyses
HepG2 and Hep3B were purchased from ATCC, and Huh7 cells were obtained as a gift from Merck. Cells were maintained in Eagle’s Minimum Essential Media with 10% FBS and 1% penicillin and streptomycin. Cells were treated for 24 hours to conditions of extreme sterol depletion (2µM simvastatin + 10% lipoprotein deficient serum), after which LDL (50µg/ml) or 25-hydroxycholesterol (1µg/ml) was added back and cells were incubated for an additional 24 hours. RNA was isolated and polyA-selected RNA-seq libraries were prepared for Illumina sequencing as previously described. 100 bp paired-end reads were generated and aligned to hg19 using Tophatv1.3.3 and transcripts present in the Ensemblv61 annotation were quantified and tested for differential expression between conditions using Cuffdiffv1.0.3 with the bias correction (-b) and multi-mapping correction (-u) options. Primary hepatocytes from four human donors (Table S1) were purchased from BD Biosciences and maintained in Williams E media without Phenol Red. Cells were treated for 24 hours with 0 nM (n=4), 500 nM (n=4), or 2000 nM (n=3) activated simvastatin in Williams E media supplemented with 10% FBS. Strand-specific RNA-seq libraries were prepared by incorporating dUTP during second-strand cDNA synthesis and degrading UTP-containing strands prior to PCR using UDG. Libraries were sequenced with 100 bp paired end reads on an Illumina HiSeq machine. Fragments were aligned to hg19 using Tophatv2.0.4 and transcripts aligning to known Ensemblv67 genes were counted using HTSeq with the -intersection-strict option, and counts were normalized for library size and variance stabilized using DESeq. Two-sided t-tests were used to test for expression level differences on variance-stabilized data.

Animal studies
Wild caught feral adult male St. Kitts vervets (Cercopithecus aethiops sabaues), also called African green monkeys were studied in two separate experiments as previously described. Briefly, in the first experiment, animals were fed a diet with no added cholesterol (see Rudel et al. 9 for complete diet composition) for 22 weeks, and then fed for 19-weeks with the same diet supplemented with 0.6 mg/kcal cholesterol (n=5). Liver biopsies were collected before and after cholesterol feeding, and tissues were stored at -80°C until analysis. In the second experiment, animals were fed a diet supplemented with 0.002, 0.2 or 0.4 mg/kcal cholesterol (n=5 per diet), and liver biopsies were
collected after 10-weeks. Hepatic cholesterol was quantified as previously described\(^2\). All procedures were approved by the Wake Forest University Animal Care and Use Committee through protocols A04-048 and A10-024. All efforts were made to minimize suffering using the appropriate anesthetic and analgesic agents. Animals were routinely monitored by veterinarians for all medical and dental conditions, with appropriate treatments administered by specialists. Change in LDLR and TMEM55B transcript levels were quantified by qPCR and calculated as fold changes in the first experiment (0.6 mg/kcal cholesterol) from values before and after cholesterol feeding, while those in the second experiments were calculated as the values quantified in the 0.2 mg/kcal or 0.4 mg/kcal cholesterol fed animals divided by the average value of the 0.002 mg/kcal cholesterol fed group. Hepatic cholesterol levels were adjusted for dietary cholesterol. Linear regression was used to assess the relationship between TMEM55B transcript levels and hepatic cholesterol levels using JMP 9.0.

**Knock-down and overexpression studies**

HepG2, Hep3B, and Huh7 cells were reverse transfected with siRNAs (Life Technologies) targeting TMEM55B (TMEM55B-1: S40499, TMEM55B-2: S40498, TMEM55B-3: s40497), SREBF1 (s129), SREBF2 (s27) or a non-targeting control as previously described\(^2\). For overexpression studies, HepG2 cells were transiently transfected with the TMEM55B expression plasmid (SC120728) or matched empty vector (pCMV6-XL4), both purchased from Origene, using the GenJet In Vitro DNA Transfection Reagent Ver. II (SignaGen) and cellular phenotypes quantified 48 hours post-transfection. For determination of TMEM55B-siRNA knockdown efficiency on protein levels, HeLa-Kyoto cells were grown on coverslips, and transfected with either TMEM55B-siRNA or control siRNA Neg9 (Applied Biosystems) as described\(^10\). After 24 hours, cell were transfected again with full-length human TMEM55B linked to GFP (SourceBioscience Cat. No. EX-H3505-M03) using Lipofectamine2000 (Invitrogen) and cultured for 24 hours. Cells were fixed, stained with Hoechst and cell-marker Draq5, and images were acquired on an automated widefield microscope (Olympus) using 20x objectives. Cell outlines were determined in a blinded fashion from the Cy5-channel using ImageJ, and total cell-associated GFP-signal was quantified from a total of 300-400 cells from 10 background-subtracted images per condition from two technical of two biological replicates.

**Transcript quantification**

RNA was extracted and reverse transcribed into cDNA as previously described\(^8\). All assays were performed in triplicate using 50ng cDNA on an ABI PRISM 7900 Sequence Detection System using the qPCR assays shown in Table SIII. Serially diluted standards were created for each qPCR assay, and absolute values (number of molecules/50ng cDNA) were calculated. Grubb’s test for outliers was used to identify statistical outliers from the triplicate values. The median value of replicates remaining was normalized to CLPTM1 as a loading control.

**Cellular phenotyping**

Intracellular cholesterol levels were quantified with the Amplex Red Cholesterol Assay Kit (Life Technologies) following the manufacturer’s protocol. Briefly, cholesterol was extracted from cells with hexane-isopropanol (3:2, v/v), dried under nitrogen and reconstituted with buffer (0.5 M potassium phosphate, pH 7.4, 0.25 M NaCl, 25 mM cholic acid, 0.5% Triton X-100). APOB, APOE and APOCIII were quantified in the cell culture media in triplicate by sandwich-style ELISA. Samples with a coefficient of variation greater than 15% were subject to repeat measurement.

**FACS analyses**

To quantify LDLR cell surface protein, cells were fixed in 1% formaldehyde, incubated with anti-LDLR at 1:100 (Santa Cruz Biotechnology, sc18823) or normal mouse IgG at 1:200 (Santa Cruz Biotechnology, sc2025) for 45 minutes at room temperature, washed and then incubated with goat anti-mouse IgG-FITC at 1:400 (Santa Cruz Biotechnology, sc-2010) for 30 minutes. Fluorescently labeled LDLR was
quantified by fluorescence-activated cell sorting on the on the BD FACS Calibur flow cytometer as the median fluorescence values of 10,000 gated events.

To quantify LDL-C uptake, live cells were incubated with 10 µg/ml Dil-LDL (Biomedical Technologies Inc., bt904) for 3–4 h at 37°C, washed twice with ice-cold PBS, fixed in 1% formaldehyde, and scraped from the wells. Dil was quantified on the BD FACS Calibur as the median fluorescence values of 10,000 gated events.

To determine the effect of TMEM55B knock-down on transferrin receptor levels and activity, HepG2 cells were reverse transfected with one of two different siRNAs from TMEM55B (TMEM-1 or TMEM-2 as described above) or a non-targeting control for 48 hours. Cells were washed with PBS, fixed with 1% formaldehyde, scraped from the plates and resuspended in either 10µL of FITC conjugated anti-Transferrin receptor antibody (Abcam, ab47095) or 20µL of FITC conjugated mouse IgG1 (Abcam, ab91356). Cells were incubated in antibody for 30 minutes at 4°C, washed three times with PBS before quantifying fluorescently-labeled Transferrin receptor by FACS on the BD FACS Calibur flow cytometer as the median fluorescence values of 10,000 gated events.

Multiple replicates of each the NTC, and siRNA treated cells were collected on at least three different days. Fold changes of the siRNA treated cells were calculated as the individual value of the sample divided by the average value of the NTC treated cells on the corresponding day, and individual data points are shown.

**Transferrin-568 uptake analysis**
HeLa-Kyoto cells were transfected with one of six different siRNAs for TMEM55B (A: SI03246733, B: SI00320516, C: SI00320523, D: 131776, E: 131774, F: 37242), after which transferrin-568 intensities in endosome-like particles was quantified by image analysis using an automated screening epifluorescence microscope (ScanR system, Olympus Soft Imaging Solutions). On average 150-200 cells per siRNA spot were imaged using 10x microscope objectives, with all images quality controlled and quantified as previously described.

**Western blot and cycloheximide treatment**
TMEM55B was knocked down in HepG2 cells as described, after which 100 µg/ml cycloheximide (Sigma-Aldrich, C4859) was added and cells collected after 0, 1.5 and 3 hours in RIPA buffer (Sigma-Aldrich, RO278) with protease inhibitor (Thermofisher Scientific, 78430). LDLR and β-actin were quantified by western blot using a mouse anti-LDLR antibody (Abcam, ab14056) and anti-β-actin antibody (Santa Cruz Biotechnology, ACTBD11B7, sc-81178). Band intensities were quantified with Image J.

**REFERENCES**


