Supplementary Information:

Supplemental Methods:

Animals:

Male wild type C57BL/6 mice and LDL-receptor (Ldlr) deficient mice on the C57BL/6 background (B6.129S7-*Ldlr*^{tm1Her}/J, stock no. 002207, hence called Ldlr-/-), 8-10 weeks of age, were obtained from Jackson Laboratory.

Apobec1–/–; *APOB*-Tg; *Ldlr*+/– (LAhB-H) mice were generated by crossing *Apobec*-/-;*Ldlr*-/- and *Apobec*-/-;*Ldlr*+/+ mice (*1, 2*) with human *APOB* transgenic mice. LAhB-H mice have elevated plasma LDL cholesterol, apoB100 and triglyceride levels. By introducing the human *APOB* gene, plasma lipid profile more closely resembling human can be achieved, making this model a highly "humanized" mouse model. Mice used for this study were between 12-22 weeks of age. Blood samples were collected after 4 hours fasting.

The generation of *Trib1* deficient mice on a mixed 129/SV x C57BL/6 was previously described elsewhere (*3*). Mice used in this study were backcrossed onto the C57BL/6 background for at least 4 generations using marker-assisted breeding (*4*). Sex matched wild-type littermates were used as controls throughout the study. Mice used for this study were between 8-10 weeks of age. Blood samples were collected after 7 hours fasting.

The animals were housed in colony cages in specific pathogen-free facilities with a 12hr light/ dark cycle at the University of Pennsylvania and The Rockefeller University. Mice had free access to water and were fed a standard rodent chow diet containing 4.5% fat (No. 5001: Lab Diets, St. Louis, MO) ad libitum. All studies were performed in male mice and littermates were used as controls. Animal experiments were reviewed and approved by the Institutional Animal Care and Use Committees of the Rockefeller University and University of Pennsylvania.

Trib1 Gene overexpression studies in mouse liver:

Recombinant Adeno-Associated Viruses (AAV) were engineered to over-express murine Trib1 (Genbank ID BC006800), as previously described (*5*). The murine Trib1 cDNA (Open Biosystems, catalogue number MMM1013-64257) was subcloned into a specialized vector for use by the University of Pennsylvania's Penn Vector Core for production of AAV8 viral particles expressing Trib1. Primer sequences used for cloning are available upon request. All clones were verified by sequencing at the Department of Medical Genetics DNA sequencing facility, University of Pennsylvania. AAV8 was generated using a chimeric packaging construct in which the Rep gene from AAV2 has been fused with the Cap gene from AAV8. Empty AAV8 viral particles were also obtained from the Penn Vector Core and used as control.

Unless otherwise stated, mice received either 1 x 10^{12} viral particles of null AAV (AAV_null) or 1 x 10^{12} viral particles of AAV encoding Trib1 (AAV_trib1) in PBS (total volume of 300 µl per mouse), via intraperitoneal injection. Blood samples were collected after a four hour fast, from retro-orbital plexus using heparinized capillary tubes at baseline and at various times following after AAV injection.
After sacrifice of the mice, terminal bleeds were collected and livers were harvested and analyzed for gene expression as described below.

Blood parameters
All measurements were done in plasma obtained from fasted mice (at least 4h fasting). Blood samples were collected from anesthetized mice via puncture of the retro-orbital venous plexus or cardiac exsanguination. Plasma was separated by centrifugation at 8000x g for 10 min. Plasma cholesterol and triglycerides were determined enzymatically using coloremetric assay kits (Roche Diagnostics, Thermo Scientific). Alanine aminotransferase (ALT) levels were measured on a Cobas MIRA autoanalyzer (Roche Diagnostic Systems).
To analyze the distribution of lipoproteins, pooled plasma (160µl) from each group (n=5;), was separated by fast performance liquid chromatography (FPLC) gel filtration (Amersham Pharmacia Biotech) as previously described (*6*). Cholesterol and triglyceride content of the fractions were measured enzymatically using Infinity reagents (Thermo Scientific).
Plasma lipoproteins were isolated by sequential ultracentrifugation from 60 µl of plasma at densities (d) of <1.006 g/ml (very-low-density lipoprotein), 1.006 ≤ d ≤ 1.063 g/ml (intermediate-density lipoprotein and low-density lipoprotein), and d >1.063 g/ml (high-density lipoprotein) using a 42.2Ti rotor (Beckman Coulter), as previously described (*7*). Cholesterol and triglycerides were determined enzymatically.
NMR lipoprotein analysis of pooled plasma samples was performed by LipoScience Inc.

Measurements of hepatic lipids:
Frozen mouse liver samples were homogenized in PBS, followed by lipid extraction with methyl-tert-butyl ether as described by Matyash et al (*8*). Lipid extracts were dried down in 1%Titon-X in chloroform under nitrogen and redissolved in H_2O. Hepatic concentrations of cholesterol and triglycerides were measured using commercial kits (Roche Diagnostics) and normalized per gram liver weight.

Real time PCR analysis
TRIB1 mRNA expression in 16 human tissues was analyzed by quantitative RT-PCR in Human MTC multiple tissue cDNA Panels I and II (Clontech).
In mouse studies, RNA was extracted from snap frozen liver samples using TriZol Reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) according to the manufacturers' recommendations. cDNA was generated from 2µg of RNA using Superscript III (Invitrogen) enzyme. Quantitative RT-PCR was carried out with SYBR Green PCR core reagents or TaqMan® Gene Expression Assays (Applied Biosystems) on an ABI PRISM 7900 Sequence Detector (PE Applied Biosystems). The relative expression levels of

mRNA were quantified with the comparative Ct (ΔΔCt) method using beta-actin as housekeeping gene.
All primer sequences for the real time PCR analysis are available from the authors' upon request.

In vivo VLDL secretion assay
VLDL-TG secretion rates were determined as previously described (*6*). In brief, mice were fasted for 4h and intraperitoneally injected with 400µl of Pluronic-407 (1mg/g body weight) resuspended in PBS. Blood was collected via retro-orbital bleeds prior to injection and at indicated timepoints (1h, 2h, 4h after injection). Plasma triglyceride levels were measured enzymatically as described above. The TG production rate (µmol/kg/h) was calculated from the difference in plasma TG levels over a given interval following detergent injection. TG production rates were then expressed as relative changes compared to respective controls.

Generation of HepG2 cells with stable Trib1 overexpression
HepG2 cells with stable overexpression of human TRIB1 (HepG2_trib1) or cell-surface marker ΔLNGFR (HepG2_control) were generated using recombinant retroviruses as previously described (*9-11*). Briefly, the full-length human trib1 cDNA with a C-terminal FLAG-protein tag was subcloned into the Moloney murine leukemia virus-derived SFG vector containing the encephalomyocarditis virus internal ribosomal entry site (IRES) and the eGFP gene (Clontech Laboratories, Palo, Alto, CA). 293GPG-packaging cells were transfected with each plasmid using calcium chloride. HepG2 cells were transduced daily for 3 d by spinoculation at 80g at 25 °C for 1 h with cell-free retroviral supernatant in 6-well plates coated with 15 µg/ml retronectin (Takara Biomedicals, Otsu, Japan) in the presence of polybrene (8 µg/ml). Four days post-transduction, fluorescence-activated cell sort selection based on eGFP was used to select positive HepG2 cells. Subsequently, expression of Trib1 was verified by quantitative RT-PCR and western blotting (using an antibody against the FLAG tag). HepG2 cells were maintained in DMEM containing 10% FBS and 1% antibiotic/antimycotic solution (Invitrogen) unless otherwise described.

Preparation and culture of ex vivo mouse hepatocytes
Mice of the Apobec–/–; APOB Tg; Ldlr+/– background that had been administered AAV vectors were used as the source of primary hepatocytes. Hepatocytes were isolated by liver perfusion from Trib1 and control mice 21 days following AAV injection, as previously described (*12*). In brief, mice were anesthetized with ketamine and xylazine and then dissected to expose the liver, portal vein, and inferior vena cava. A catheter was inserted into the portal vein and sutured in place. The livers were perfused with buffer for five minutes to remove all red blood cells, followed by digestion in situ by running digestion media through the catheter for 15 minutes. The livers were transferred to 10 mm dishes with 15 ml of hepatocyte wash media and run through a mesh into 50 mL conical tubes to separate the cells. The cells were centifuged at 50 g at 4°C to remove non-parenchymal cells (including Kupffer cells and endothelial cells). The hepatocyte pellets were washed twice with hepatocyte wash media and resuspended in 25 ml PBS + 25 ml of Percoll solution (45 ml Percoll + 5 mL 10x PBS + 100µl of 1M

HEPES). The cells were then centrifuged at 115 g for five minutes at 4°C to pellet the viable hepatocytes. The hepatocytes were resuspended in Hepatozyme media (Invitrogen) + 10% FBS + 1% amino acids and seeded at subconfluency ($3x10^5$ cells/well) on 12 well type I collagen-coated plates. After four hours attachment at 37°C in a 5% CO2 incubation, the media was replaced with Hepatozyme media (Invitrogen) supplemented with 1% antibiotic/antimycotic and cells were cultured overnight. Cell viability was analyzed before plating by Trypan blue exclusion.

Ex vivo triglyceride synthesis in mouse primary hepatocytes and HepG2 cells

To determine the rate of triglyceride synthesis ex vivo, primary hepatocytes of AAV_trib1 and AAV_null treated mice and HepG2 cells with TRIB1 overexpression (HepG2_trib1) or expression of a control vector (HepG2_control) were cultured in DMEM in the presence of 0.4 mM oleic acid conjugated with BSA (Sigma) or BSA only (HepG2 cells) and 5 µCi [3H]-1,2,3 glycerol (American Radiolabeled Biochemicals) for up to 4h. The cells were then washed three times with ice-cold PBS and treated twice with 1mL of hexane:isopropanol (3:2, vol/vol) to extract cellular lipids. The collected media was subjected to Bligh-Dyer lipid extraction (*13*) and the hexane:isopropanol fractions from cells were dried in a rotary evaporator. Lipid fractions were solubilized in chloroform and separated by thin layer chromatography (TLC) in a hexane-diethyl ether-acetic acid solvent system (170:30:1. v/v/v). The triglyceride band was identified by comparison to a purified triolein standard, excised and radioactivity measured by scintillation counting. In the experiments using HepG2 cells, TLC plates were exposed on tritium sensitive phosphor storage screens and read out on a Storm Phosphorimager (GE Healthcare). Densitometric analysis of the triglyceride bands was performed using the Image Quant software (Molecular Dynamics) and values were expressed as arbitrary units (AU). All data was normalized to total cellular protein measured with the DC Rrotein Kit (BioRad).

Metabolic Labeling and Immunoprecipitation of apoB in HepG2 cells

HepG2 cells with TRIB1 overexpression (HepG2_trib1) or expression of a control vector (HepG2_control) were seeded in triplicates on 6-well plates and grown to 80% confluency. For labeling experiments, cells were switched to methionine/cysteine-free DMEM containing 1% FBS and 400 µM oleic acid complexed to BSA. After 1h pre-incubation, 200µCi per well of ^{35}S-methionine/cysteine (Perkin Elmer) was added and cells were labeled for 3h before harvesting media. ApoB was immunoprecipitated from the conditioned media with a polyclonal goat antibody against human apoB (Calbiochem) and samples were resolved by SDS-PAGE. The gel was fixed (50% Methanol/10% acetic acid/40% H_2O) and after drying, exposed on a phosphor storage screen. Densitometric analysis was performed using the Image Quant software (Molecular Dynamics). Data was normalized to total secreted protein levels determined by tricholoroacetic acid (TCA) precipitation from conditioned media, followed by scintillation counting.

Immunofluorescence and Microscopy

Immunofluorescence staining of HepG2_Trib1 and HepG2_control cells was performed using a standard protocol. Briefly, cells were grown on glass coverslips coated with collagen (BD Bioscience), washed with PBS, and fixed with 3.7% paraformaldehyde in

PBST (PBS, 0.1% Triton-X-100). Following three washing steps with PBST, cells were blocked at RT for at least 30 min in PBST containing 2% BSA. The cells were subsequently incubated with primary mouse-anti FLAG M2 antibody (Sigma-Aldrich, 1: 1000) diluted in PBST containing 2% BSA and 3% normal goat serum overnight at 4°C. The primary antibody was detected with an anti mouse Rhodamine Red-conjugated secondary antibody (1:1500, Jackson Immuno Research Lab Inc.). Slides were counterstained with TO-PRO-3 iodide (Invitrogen) for 5 min and mounted with ProLong Gold antifade reagent (Invitrogen). Stainings were analyzed using an upright Zeiss LSM 510 confocal laser scanning microscope. Images were obtained using the Zeiss LSM software, and Photoshop CS4 was used to prepare figures.

Statistical Analyses:

Data are presented as the mean ± standard error of the mean (SEM). Results were analyzed by Student's t-test (two-tailed) or ANOVA with Dunnett's post-test, when multiple comparisons to control group were made. Statistical significance was defined as $p < 0.05$.

References:

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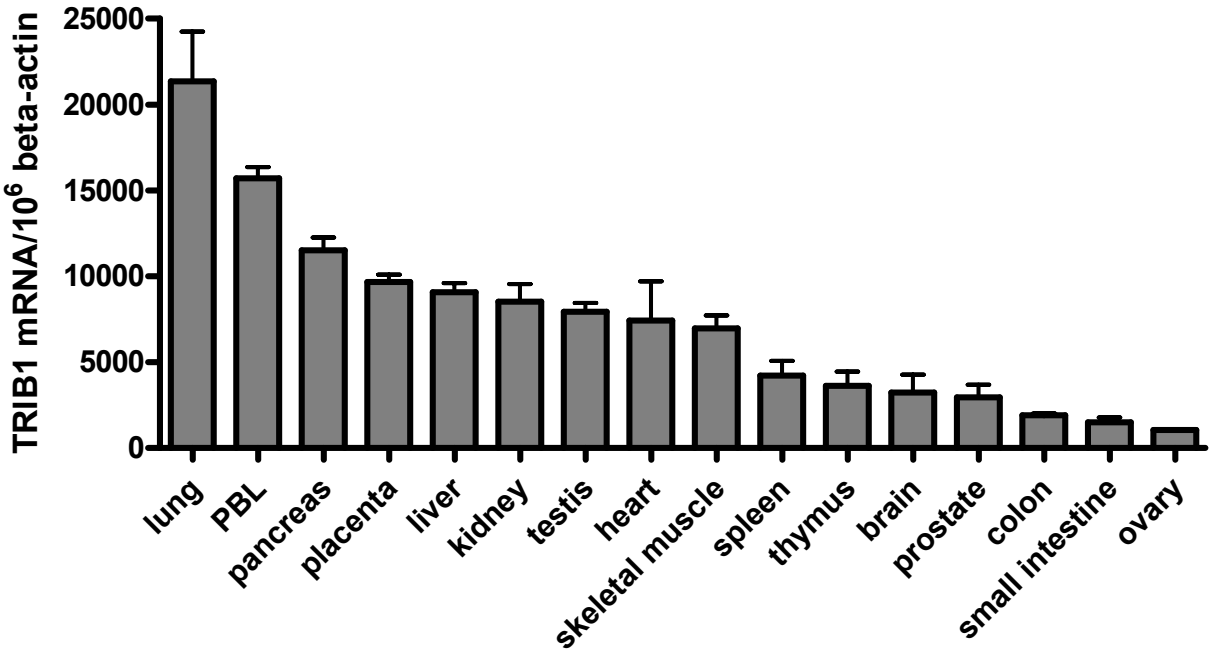
	Plasma cholesterol (mg/dL)	Plasma TG (mg/dL)	Human ApoB (mg/dL)
Wild type	**109± 20**	**52 ± 5**	**N/A**
LDLR-/-	**220 ± 15**	**69± 9**	**N/A**
LDLR+/-/Apobec-/-hApoBtsg (LAhB-H)	**242± 23**	**102 ± 10**	**132 ± 17**

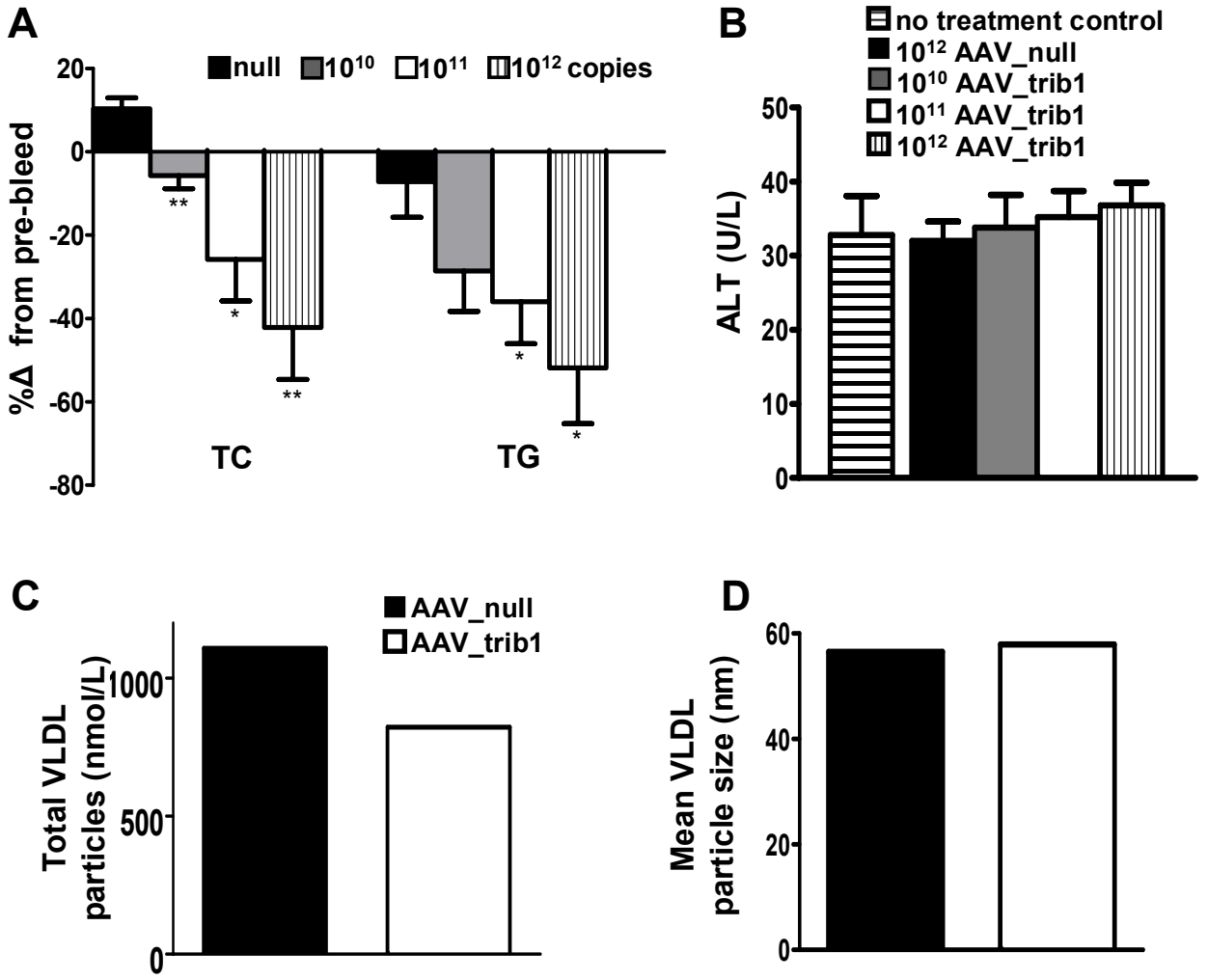
N>10 per genotype

Supplemental Table 1: Plasma lipids (measured after 4h fast) in LDLR/Apobec-/-hApoBtsg (LAhB-H) mice, in comparison to LDLR-/- and wild-type C57BL6J mice. LAhB-H mice have elevated plasma cholesterol, apoB100 and triglyceride levels. By introducing the human APOB gene, plasma lipid profile more closely resembling human is achieved, making this model a highly "humanized" mouse model. Mice used for this study were between 12-22 weeks of age and were fed standard chow diet. Values represent mean ± SD.

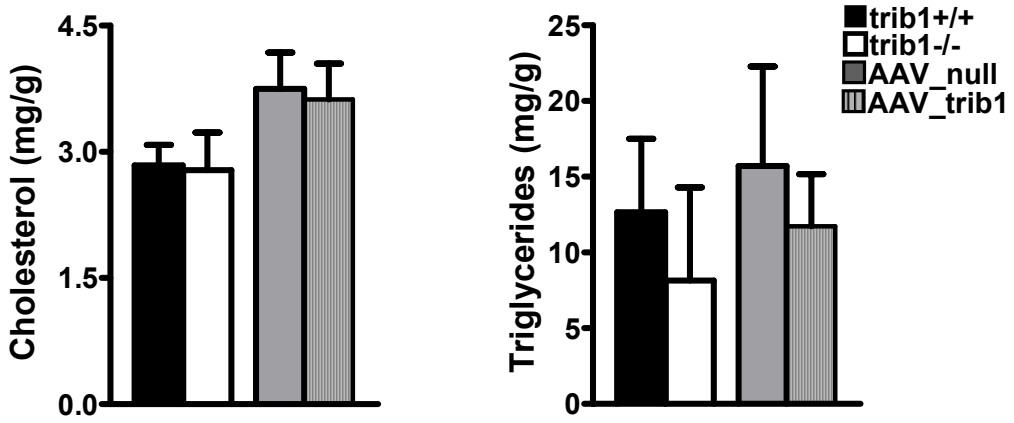
Supplemental Figure 1: Linkage disequilibrium plot of human chromosome 8q24. Trib1 is the only annotated gene in the haplotype block containing the associated SNP (rs17321515). The location of the associated SNP is marked in red. Linkage disequilibrium is displayed as r^2 values. Data was generated using Haploview (HapMap Data Release 27, NCBI B36 assembly).



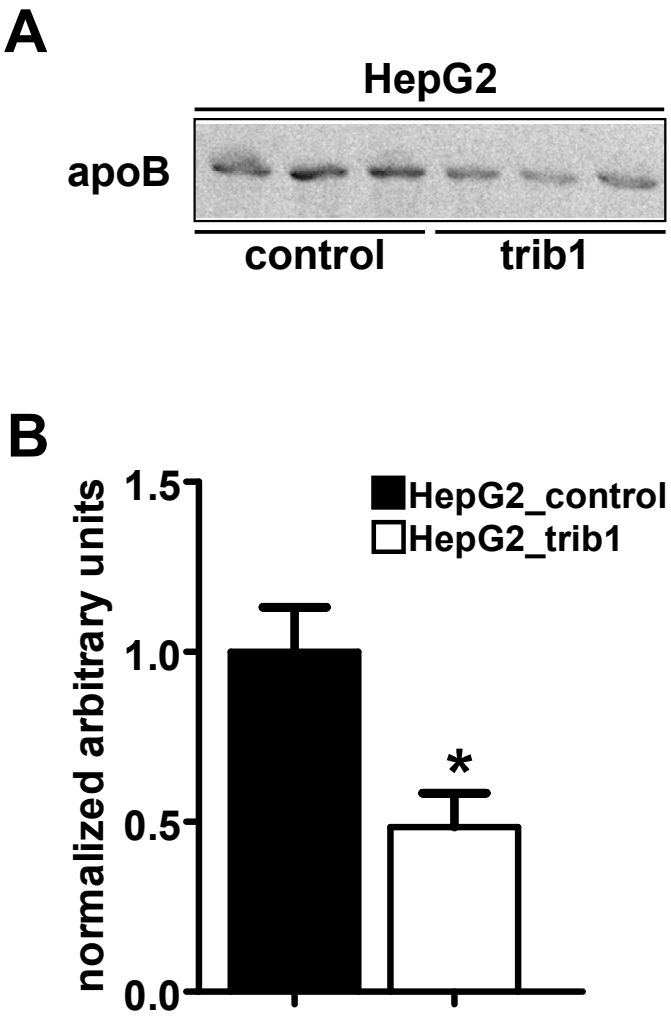
Supplemental Figure 2: TRIB1 is ubiquitously expressed across 16 human tissues. mRNA expression levels of TRIB1 were determined by quantitative RT-PCR using cDNAs from MTC Multiple Human Tissue cDNA Panels I and II (Clontech). PBL= peripheral blood leukocytes



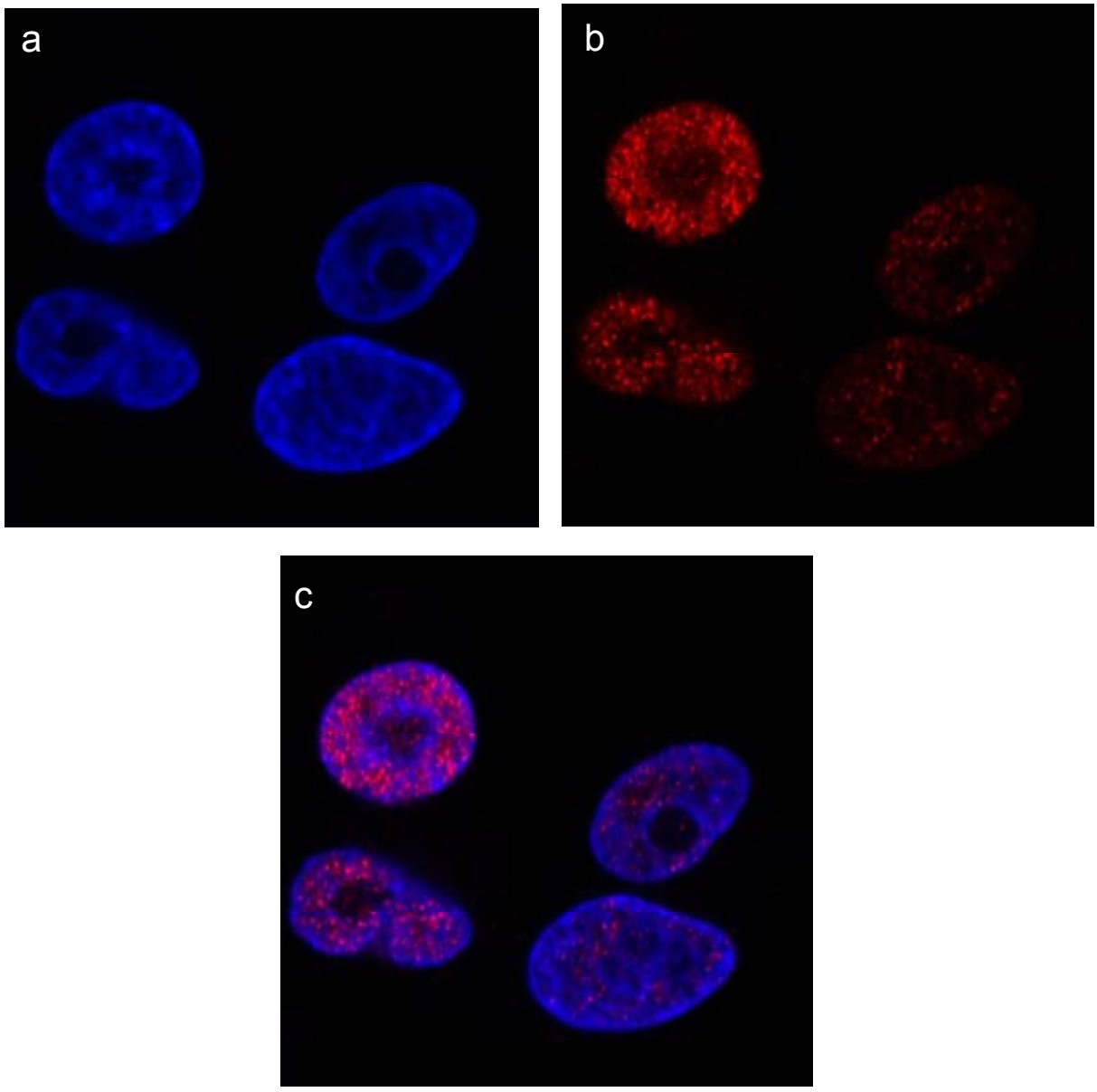
Supplemental Figure 3: **(A)** Dose dependant reduction of plasma total cholesterol (TC) and TG in male C57/BL6 mice (n=5) receiving either 10^{12} genome copies control (AAV_null), 10^{10},10^{11} or 10^{12} genome copies AAV_trib1. Post-injection measurements were performed two weeks after virus injection and are presented as % change compared to pre-injection levels. *p<0.05, **p<0.005; AAV_null versus AAV_trib1 groups **(B)** AAV8 injection does not increase plasma ALT levels in mice. ALT levels were measured in plasma of mice before AAV8 injection (no treatment control) and 2 weeks after injection of 10^{12} copies control AAV_null or various doses of AAV_trib1 (10^{10}, 10^{11}, 10^{12} copies). **(C)–(D)** NMR analysis of pooled plasma samples from the VLDL-TG secretion study with LAhB-H mice at 4h after P-407 injection. **(C)** Total plasma VLDL particle concentration and **(D)** mean VLDL particle size in AAV_trib1 or AAV_null treated LAhB-H mice.



Supplemental Figure 4: Hepatic cholesterol and triglyceride levels (n=6 per group) are not significantly different between trib1-/-, trib1_AAV and respective control groups.



Supplemental Figure 5: Effect of *TRIB1* overexpression on apoB secretion in HepG2 cells. **(A)** HepG2 cells overexpressing *TRIB1* (HepG2_trib1) or control vector (HepG2_control) were radiolabeled with ^{35}S-methionine/cysteine for 3h, followed by immunoprecipitation of apoB from conditioned media, SDS-polyacrylamide gel electrophoresis and autoradiography. **(B)** Densitometric analysis of labeled apoB secreted from HepG2 cells. ApoB measurements were normalized to radioactive counts in trichloroacetic acid precipitated proteins from conditioned media, representing total secreted proteins. *$p<0.05$



Supplemental Figure 6: TRIB1 localizes to the nucleus of hepatocytes. Immunofluorescence imaging in HepG2 cells expressing TRIB1 with a C-terminal FLAG tag. (a) TO-PRO-3 iodide stain (nuclear staining), (b) mouse anti-FLAG M2 antibody, detected with anti mouse Rhodamine Red-conjugated secondary antibody, (c) merged image.