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Article

Sort1, Encoded by the Cardiovascular Risk Locus 1p13.3, Is a Regulator of Hepatic Lipoprotein Export

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SUMMARY

Recent genome-wide association studies (GWAS) have revealed strong association of hypercholesterolemia and myocardial infarction with SNPs on human chromosome 1p13.3. This locus covers three genes: *SORT1*, *CELSR2*, and *PSRC1*. We demonstrate that sortilin, encoded by *SORT1*, is an intracellular sorting receptor for apolipoprotein (apo) B100. It interacts with apoB100 in the Golgi and facilitates the formation and hepatic export of apoB100-containing lipoproteins, thereby regulating plasma low-density lipoprotein (LDL) cholesterol. Absence of sortilin in gene-targeted mice reduces secretion of lipoproteins from the liver and ameliorates hypercholesterolemia and atherosclerotic lesion formation in LDL receptor-deficient animals. In contrast, sortilin overexpression stimulates hepatic release of lipoproteins and increases plasma LDL levels. Our data have uncovered a regulatory pathway in hepatic lipoprotein export and suggest a molecular explanation for the cardiovascular risk being associated with 1p13.3.

INTRODUCTION

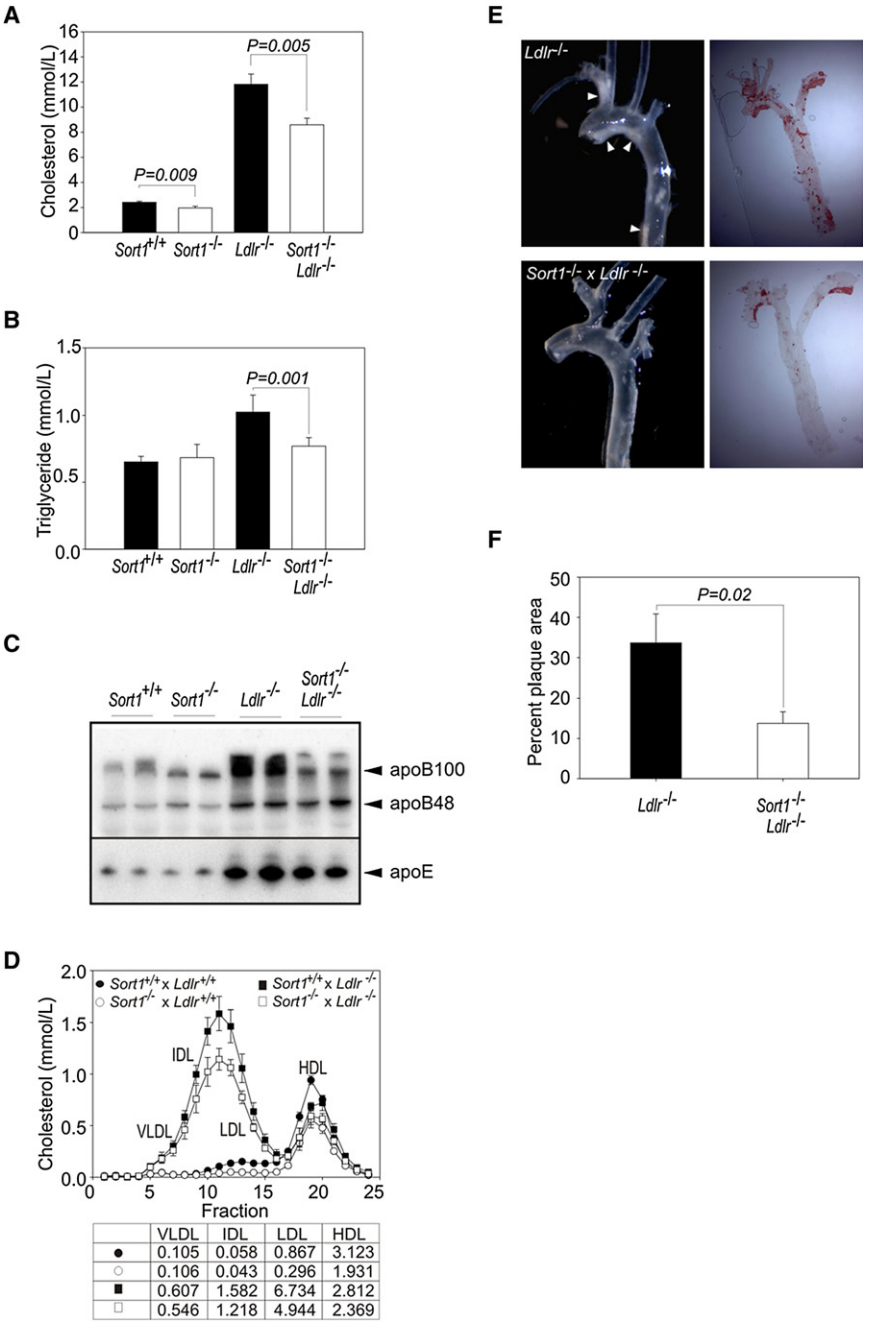
Elevated levels of LDL are key contributors to atherosclerosis and ischemic heart disease, the leading cause of morbidity and mortality worldwide. The high heritability of circulating LDL levels is well established, and earlier studies of individuals with extreme lipid values or families with Mendelian forms of dyslipidemias have identified key genes involved in control of LDL metabolism (Havel and Kane, 2001).

LDL is mainly generated by lipolysis in the circulation from liver-derived very low-density lipoproteins (VLDLs) that contain apoB100 as a main structural component. Genetic variants of apoB100, causing low VLDL secretion, are associated with reduced plasma LDL (Hooper et al., 2005). Similarly, mutations in apoB or the microsomal triglyceride carrier protein (MTP) essential for lipidation of apoB in hepatocytes result in impaired release of VLDL particles from the liver and in reduced plasma LDL cholesterol levels (Sharp et al., 1993; Young et al., 1990). In contrast to defects in hepatic release of lipoproteins, monogenic diseases that affect clearance of circulating LDL particles as in familial hypercholesterolemia, an inheritable defect in the LDL receptor gene, result in massive increase in circulating LDL levels and in elevated risk of cardiovascular disease (Goldstein et al., 2010).

While rare monogenic diseases have been instrumental in establishing main concepts of hepatic lipoprotein metabolism, many more genes may be involved in modulation of lipid homeostasis and in conferring risk for CAD in the general population. While the contribution of individual risk genes may be modest compared with major familial disease genes, the compound action of these modifiers is likely to play a decisive role in the complex genetic of CAD. To identify novel modifiers and risk factors in lipid metabolism, a number of genome-wide association studies (GWAS) have been carried out aiming to identify new gene loci associated with plasma LDL and cholesterol levels or with myocardial infarction.

Remarkably, GWAS replicated in several populations have identified three common single-nucleotide polymorphisms (SNPs) on 1p13.3 strongly associated with reduced plasma LDL and coronary heart disease (Linsel-Nitschke et al., 2009; Kathiresan et al., 2008, 2009; Willer et al., 2008; Samani et al., 2007, 2008, 2009; Sandhu et al., 2008; Karvanen et al., 2009; Muendlein et al., 2009; Schadt et al., 2008). These SNPs lie in a noncoding region in the vicinity of *CELSR2*, *PSRC1*, and *SORT1* that encodes cadherin EGF LAG seven-pass G-type receptor 2, proline/serine-rich coiled-coil 1, and sortilin, respectively. Which of the three genes may represent the cardiovascular risk gene is unclear.

Sortilin is one of five members of the Vps10p domain receptor family, a group of multifunctional proteins typically found in intracellular compartments of the *trans*-Golgi network (TGN) and early endosomes (Willnow et al., 2008). The unifying structural motif in this gene family is the Vps10p domain, a ten-bladed β-propeller fold that composes part of the extracellular domain of all



	VLDL	IDL	LDL	HDL
●	0.105	0.058	0.867	3.123
○	0.106	0.043	0.296	1.931
■	0.607	1.582	6.734	2.812
□	0.546	1.218	4.944	2.369

Figure 1. Lipoprotein Metabolism in Wild-Type and Sortilin-Deficient Mice

(A and B) (A) Plasma cholesterol and (B) triglyceride levels in mice fed a Western-type diet for 6 weeks (*Sort1*$^{+/+}$, n = 46; *Sort1*$^{-/-}$, n = 15; *Ldlr*$^{-/-}$, n = 26; *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ mice, n = 18).

(C) Western blot analysis of apoB100, apoB48, and apoE in plasma samples from mice of the indicated genotypes.

(D) FPLC profiles of plasma samples from *Sort1*$^{+/+}$ (n = 3), *Sort1*$^{-/-}$ (n = 6), *Ldlr*$^{-/-}$ (n = 4), and *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ (n = 5) mice. Cholesterol concentrations (mM) in VLDL, IDL, LDL, and HDL fractions, respectively, are shown below.

(E) Atherosclerotic lesions (arrowheads) in the aorta of *Ldlr*$^{-/-}$ but not of *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ mice fed a Western-type diet for 8 months as shown by bright-field microscopy (left panel) or oil red O (right pane). Representative examples from a total of six animals in each group are shown.

(F) Percent plaque area in the aortas of the mice of the indicated genotypes (n = 6 per group). All values are depicted as mean ± SEM.

receptors (Quistgaard et al., 2009). In cells, sortilin is synthesized as inactive precursor that is incapable of ligand binding due to a 40 amino acid propeptide that precludes ligands from entering the binding site inside a tunnel formed by the tertiary structure of the β-propeller (Munck et al., 1999; Quistgaard et al., 2009). In the TGN, proconvertases liberate the propeptide conferring activity to the receptor (Munck et al., 1999).

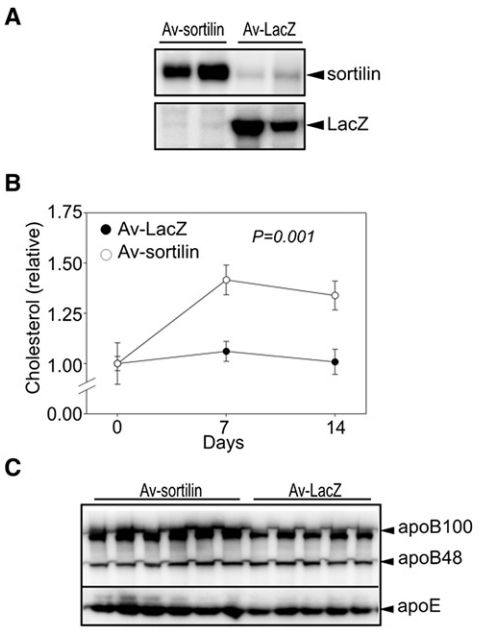
Among other functions, Vps10p domain receptors are believed to assist in sorting of target proteins in the secretory and/or the endosomal pathways. Yet sortilin has also been recognized as a crucial component of the signaling complex whereby proneurotrophins control survival of neurons (Jansen et al., 2007; Nykjaer et al., 2004). Although sortilin has been shown to bind apoA-V (Nilsson et al., 2008) and lipoprotein lipase (Nielsen et al., 1999), a potential contribution of this receptor to lipoprotein metabolism remains enigmatic.

The present study aimed at exploring the relevance of sortilin as a cardiovascular risk factor.

RESULTS

Loss of Sortilin Expression Protects from Hypercholesterolemia

Mice deficient in sortilin (*Sort1*$^{-/-}$) fed a Western-type diet exhibit a significant 20% reduction in plasma cholesterol (2.5 ± 0.3 versus 2.0 ± 0.2 mmol/L) but not triglyceride levels compared with wild-type controls (Figures 1A and 1B). When crossed with the LDL receptor-deficient line (*Ldlr*$^{-/-}$) to increase plasma LDL levels, absence of sortilin diminished plasma cholesterol from 11.9 ± 0.8 in *Ldlr*$^{-/-}$ to 8.4 ± 0.5 mmol/L in (*Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$) double knockout (DKO) mice, corresponding to an ~30% decline. Triglycerides were reduced from 1.3 ± 0.1 to 0.8 ± 0.1 mmol/L (Figures 1A and 1B). Using western blot analysis, we noticed a decrease in plasma levels of apoB100, but not of apoB48, in DKO animals (Figure 1C). Densitometric scanning of replicate western blot experiments revealed a 48.2% ± 3.19% decrease in apoB100 in the DKOs (n = 7) compared with *Ldlr*$^{-/-}$ mice (n = 6) (p = 0.001). This observation was substantiated by FPLC analysis, which revealed that



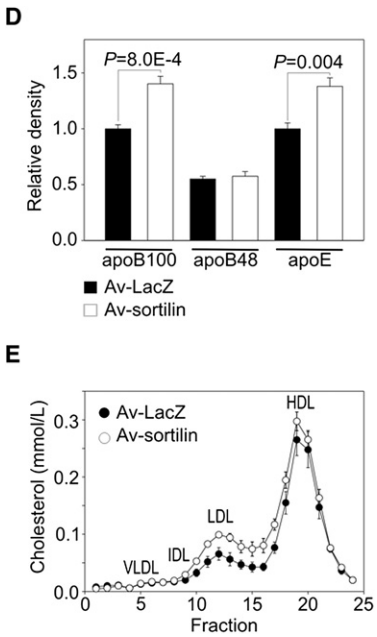


Figure 2. Lipoprotein Metabolism in Mice following Adenovirus-Mediated Hepatic Overexpression of Sortilin

(A) Western blot analysis of sortilin and lacZ expression in liver samples of mice treated with adenoviruses encoding sortilin (Av-sortilin) or β-galactosidase (Av-lacZ).

(B) Plasma cholesterol levels in Av-sortilin (n = 6)- and Av-lacZ (n = 5)-treated mice fed a Western-type diet.

(C) Immunoblot analysis of apoB100, apoB48, and apoE in plasma samples from virus-infected animals as indicated.

(D) Quantification of intensities of the immunoreactive bands in the western blot in (C). Data are normalized to the intensity of apoB100 in Av-lacZ-treated mice (set at 1).

(E) Mean FPLC profiles of three samples from both virus-treated groups at day 14 postinfection. All values are depicted as mean ± SEM.

plasma cholesterol contained in the IDL and LDL fractions was significantly reduced in DKO mice (Figure 1D). In line with reduced plasma cholesterol, we also observed a considerable reduction in the extent of atherosclerotic lesions in the aortas of *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ compared with *Ldlr*$^{-/-}$ mice as shown by bright-field microscopy of whole-mount aortas and by Oil Red O staining (Figure 1E). Quantification of six animals per group demonstrated a reduction in the percentage of plaque area from 33.68% ± 7.18% in the LDLR-deficient mice to 13.77% ± 2.86% in the *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ animals, corresponding to an overall ~59% (p = 0.018) decrease in atherosclerotic lesion formation (Figure 1F).

Overexpression of Sortilin Increases Plasma Cholesterol

Outside the nervous system, sortilin is expressed in adipose tissue and in liver, tissues that both contribute to systemic lipoprotein metabolism (see Figure S1A available online). In both human and mouse livers, sortilin expression was confined to hepatocytes (Figures S1B and S1C). To test whether hepatic dysfunction may account for the LDL phenotype seen in sortilin-deficient animals, we overexpressed the receptor selectively in hepatocytes in vivo by adenoviral gene transfer. We reasoned that enhanced expression, as opposed to loss of activity in sortilin knockout mice, should increase plasma LDL.

To this end, recombinant adenoviruses encoding sortilin (Av-sortilin) or β-galactosidase (Av-lacZ) were injected into the tail veins of wild-type mice. Seven days later, robust overexpression of the transgenes in the livers of mice was seen (Figure 2A). When comparing plasma cholesterol levels between the groups, Av-sortilin-injected animals showed an ~42% increase in total cholesterol (p = 0.001; Figure 2B). Hypercholesterolemia sustained for the time period observed here (14 days). In contrast, plasma cholesterol levels in Av-lacZ-treated mice remained unchanged compared to that of noninjected controls (Figure 2B). Likewise, Av-sortilin application in DKO animals reverted the LDL cholesterol-lowering effect of the sortilin gene defect and resulted in a hypercholesterolemic phenotype in DKO (from 8.70 ± 0.48 mM to 11.56 ± 0.11 mM; p = 0.04) comparable to that of the *Ldlr*$^{-/-}$ line (11.87 ± 0.73 mM) (Figure S2A). The increase in plasma cholesterol in Av-sortilin-injected animals was accompanied by a rise in plasma apoB100 (~44%), but not apoB48 content, suggesting that sortilin selectively impacts on apoB100 bioavailability (Figures 2C and 2D). FPLC analysis confirmed accumulation of LDL/IDL particles in the circulation of Av-sortilin- compared with Av-lacZ-treated mice (Figure 2E). HDL was only marginally affected. Plasma apoE levels were also increased in mice overexpressing sortilin (Figure 2D). This finding is in line with the fact that hepatic apoE secretion is positively correlated with VLDL production, as the apolipoprotein is associated with these liver-derived triglyceride-rich particles.

To demonstrate that the reduced hepatic lipoprotein export in *Sort*$^{-/-}$ mice was not accounted for by secondary changes in gene expression or hepatic morphology, we analyzed liver samples by gene arrays and by histology. In the transcriptome analysis, of 84 key genes in hepatic lipoprotein and cholesterol metabolism tested here (Figures S2B and S2C), only two transcripts were changed by more than 3-fold in sortilin-deficient mice: transcriptional regulating factor 1 (Trerf1/TReP-132), a regulator of the steroidogenic pathway but with unknown functions in the liver (Guo et al., 2007), was reduced 6.48-fold. The second gene encoded cholesterol 7α-hydroxylase (CYP7A1), an enzyme that catalyzes the first reaction in the cholesterol catabolic pathway in the liver converting cholesterol into bile acids (Norlin and Wikvall, 2007). A 3.43-fold increase in CYP7A1 expression was confirmed by quantitative RT-PCR (Figure S2D) and western blotting (inset to Figure S2D), suggesting that hepatic lipid accumulation might be affected by sortilin deficiency. To exclude a confounding influence from LDL

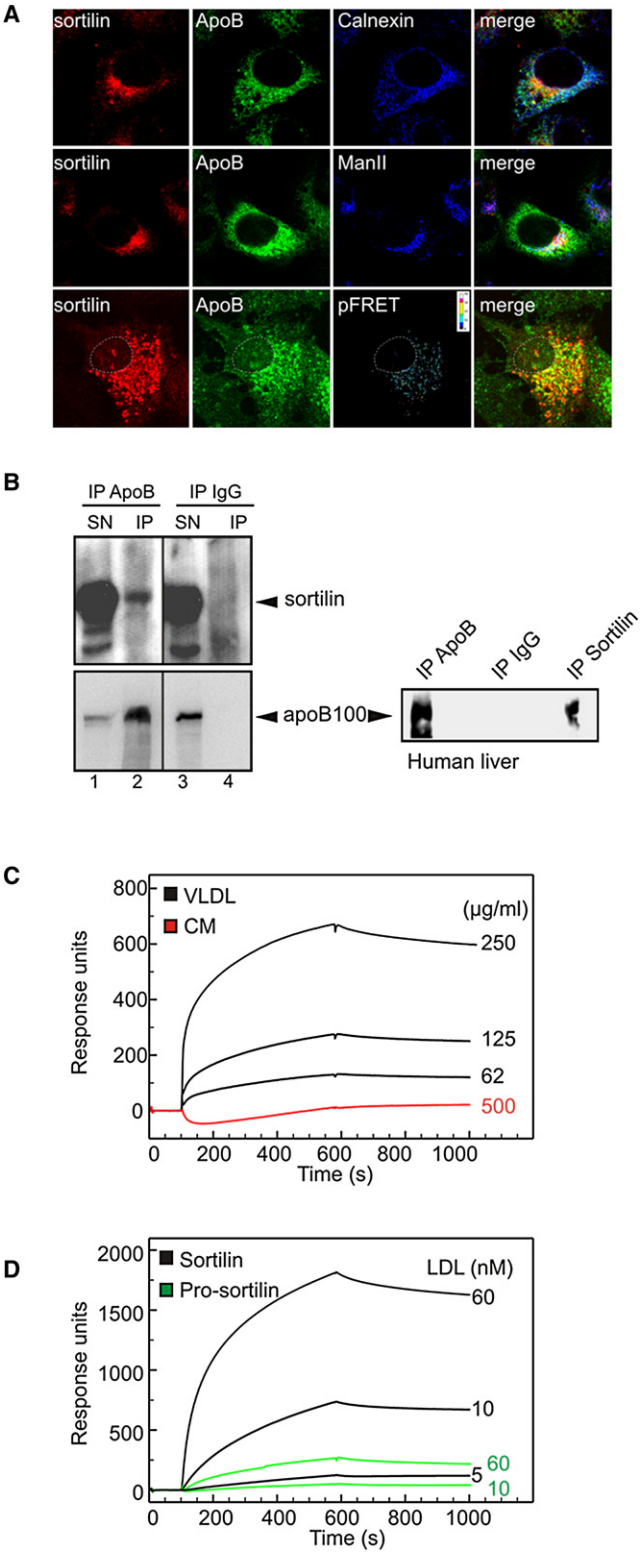


Figure 3. Binding of apoB100 to Sortilin

(A) Immunofluorescence detection of endogenous sortilin and apoB100 in HepG2 cells. Sortilin (red) and apoB100 (green) colocalize in a mannosidase II-positive (blue, middle panel), but not in a calnexin-positive (blue, upper panel), cellular compartment. Sortilin (red) interacts with apoB100 (green) in the perinuclear region as demonstrated by pFRET efficiency (lower panel). Nuclei are indicated by dotted circles.

(B) (Left) Coimmunoprecipitation of endogenous sortilin with anti-apoB IgG (IP ApoB, lane 2), but not with control IgG (IP IgG, lane 4) from HepG2 cells. IP designates the immunoprecipitate (lanes 2 and 4) and SN the corresponding supernatant of the IP reaction (lanes 1 and 3). Samples were probed for sortilin (upper panel) and apoB100 (lower panel) (B, right). Coimmunoprecipitation of endogenous apoB from human liver with anti-sortilin IgG (lane 3) but not with nonimmune control IgG (lane 2). As a positive control, immunoprecipitation of apoB100 with anti-apoB antisera is shown in lane 1.

(C) SPR analysis showing binding of the indicated concentrations of mouse VLDL but not of human chylomicrons (CM) to the immobilized sortilin ectodomain (0.108 fmol/mm^2).

(D) BIAcore analysis of a concentration series of human LDL showing binding to the immobilized ectodomain of mature sortilin but not to cleavage-resistant prosortilin (0.096 fmol/mm^2). The calculated affinity (Kd) for sortilin is 1–2 nM.

endocytosis, we compared the lipid content in livers from *Ldlr*$^{-/-}$ and *Sort1*$^{-/-}$ × *Ldlr*$^{-/-}$ mice. Despite indistinguishable morphology of livers from the two genotypes, Sudan staining uncovered a reduction in hepatic lipid accumulation in the DKOs (Figure S2E). In accordance, biochemical quantification revealed a decrease in hepatic triglycerides from 159.09 ± 29.16 to 83.22 ± 10.80 μg/mg tissue, corresponding to an ~48% reduction in the DKO animals ($p = 0.04$). Cholesterol levels were unchanged in these mice (3.84 ± 0.22 versus 3.81 ± 0.18 μg/mg). This observation suggests that increased Cyp7a1 expression may enhance biliary excretion shunt from the liver of receptor-deficient mice.

Expression of the remaining 82 genes was unaffected. Most importantly, *Apob* transcript levels were unaltered (Figure S2B). Also, transcript levels for *Psrc1* and *Celsr2*, the other two candidate genes at 1p13.3, were unchanged in sortilin-deficient mice as shown by quantitative RT-PCR (Figure S2F).

Sortilin Functionally Interacts with apoB100 but Not apoB48

Based on a function for Vps10p domain receptors as intracellular sorting proteins, we hypothesized that sortilin may interact with apoB100 and facilitate hepatic VLDL biosynthesis and release. To explore this possibility, we first studied if endogenous sortilin and apoB100 colocalize in the human hepatoma cell line HepG2 using immunofluorescence microscopy (Figure 3A and Figures S3A and S3B). We found that apoB100 is predominantly present in the endoplasmic reticulum (ER) (calnexin-positive), in line with previous reports (Boren et al., 1990; Sakata et al., 2001), but also in *medial*- to *trans*-Golgi compartments (mannosidase II positive). Interestingly, sortilin colocalized with apoB100 selectively in the Golgi. By fluorescence resonance energy transfer (FRET) and after subtraction of the signals from donor only and acceptor only to correct for spectral bleach through, we calculated an average pFRET of ~10% ± 4% (Figure 3A and Figure S3C), indicating a high degree of apoB100 binding to sortilin in this secretory compartment. To further substantiate the ability of apoB and sortilin to physically interact, we performed coimmunoprecipitation of endogenous sortilin with anti-apoB antisera from hepatoma cells. In these studies, a distinct fraction of sortilin coprecipitated with apoB, in line with a transient interaction of the two proteins in the TGN (Figure 3B). Coimmunoprecipitation of

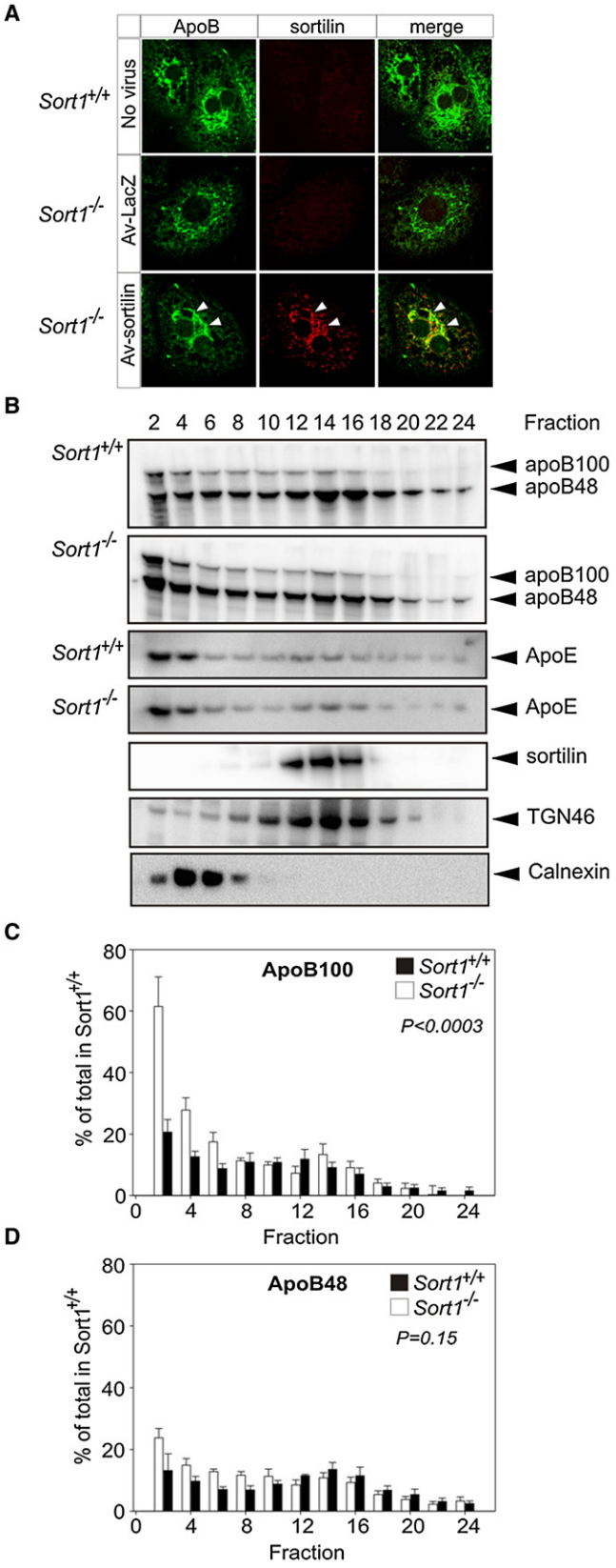


Figure 4. Sortilin Expression Modulates Subcellular Distribution of apoB100 in Mouse Hepatocytes

(A) Immunofluorescence detection of apoB (green) and sortilin (red) in primary hepatocytes from *Sort*$^{+/+}$ wild-type mice or from *Sort*$^{-/-}$ animals treated with lacZ (Av-lacZ) or sortilin-expressing (Av-sortilin) adenoviruses. Sortilin overexpression relocates apoB from an ER-like to a Golgi-like compartment in *Sort*$^{-/-}$ cells (arrowheads), similar to the pattern seen for apoB100 in *Sort*$^{+/+}$ cells. Of note, the anti-sortilin antisera applied does recognize the human receptor expressed from the viral gene construct, but not the endogenous murine protein in noninfected wild-type cells (upper panel).

(B) Subcellular fractionation of hepatic microsomal membranes. In wild-type mice, apoB100 and apoB48 colocalize with sortilin in Golgi-enriched fractions positive for TGN46 (fractions 12–16). In sortilin-deficient mice, apoB100 is shifted to lighter fractions corresponding to the calnexin-positive ER (fractions 2–8).

(C and D) (C) Relative distribution of apoB100 and (D) apoB48 in subcellular fractions from four independent experiments as determined by densitometric scanning of western blots exemplified in (B). Values (mean ± SEM) are normalized to the total expression of apoB100 and apoB48 in wild-type mice, respectively.

both proteins was also documented from human liver specimens (Figure 3B).

Next, we tested which apoB isoform, apoB100 or apoB48, was capable of sortilin binding. To do so, the extracellular domain of sortilin was immobilized on a sensor chip and VLDL binding to the protein domain was characterized by surface plasmon resonance (SPR) analysis. To exclude potential binding through apoE, we used VLDL particles purified from apoE-deficient mice. As shown in Figure 3C, purified VLDL particles exhibited a robust binding to sortilin. Because VLDL contains both apoB100 and apoB48, we also tested binding of human chylomicrons (CMs) that contain apoB48 and apoE as well as that of LDL particles that carry apoB100 only. While we observed strong interaction of the sortilin ectodomain with LDL (Figure 3D; Kd ~1–2 nM), CM were completely inactive (Figure 3C). The specificity of VLDL and LDL interaction with sortilin was further evidenced using competition experiments with neurotensin and the receptor-associated protein (RAP), potent inhibitors of ligand binding to sortilin (Nielsen et al., 1999). Neurotensin (20 μM) inhibited VLDL (125 μg/ml) and LDL (60 nM) binding by ~33% and 42%, respectively. RAP (5 μM) completely abolished interaction between LDL and sortilin ~98%. We concluded that apoB100, but not apoB48, is capable of binding to sortilin with high affinity, an interaction which likely proceeds in late Golgi compartments.

Sortilin Modulates Intracellular Trafficking and Release of apoB100

To gain further insights into the molecular mechanism by which sortilin affects plasma LDL, we overexpressed lacZ or sortilin by adenoviral gene transfer in *Sort1*$^{-/-}$ hepatocytes (Figure 4A). While in wild-type cells apoB was mainly present in a perinuclear Golgi-like compartment, hepatocytes lacking sortilin and transfected with Av-lacZ showed a dispersed apoB staining throughout the cell, consistent with a predominant ER localization. In contrast, re-expression of sortilin (Av-sortilin) in *Sort1*$^{-/-}$ hepatocytes resulted in a robust accumulation of apoB100 in a Golgi-like perinuclear compartment that also contained sortilin (Figure 4A and Figure S4). This observation

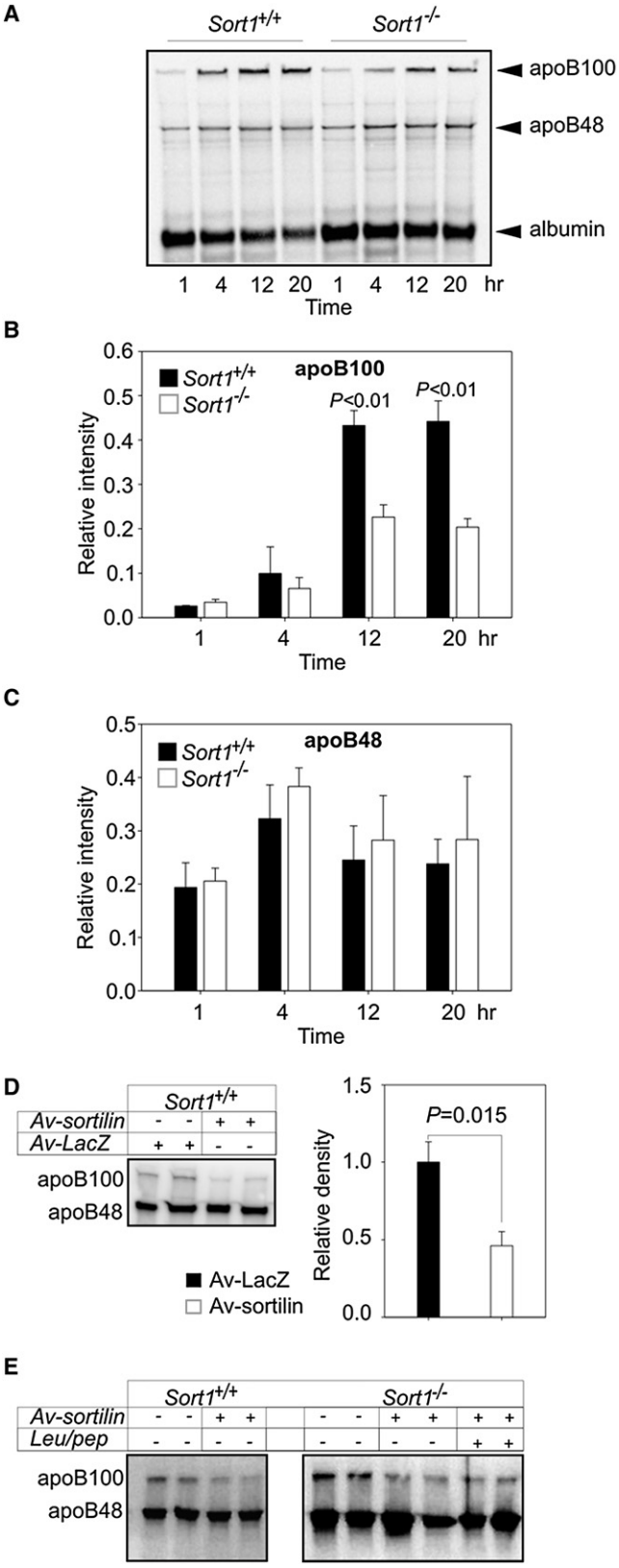


Figure 5. Sortilin Regulates apoB100 Secretion, but Not Degradation, in Primary Hepatocytes

(A) Immunoprecipitation of apoB100 and apoB48 from the medium of metabolically labeled primary hepatocytes of the indicated genotypes. Albumin was immunodetected as internal reference for metabolic labeling and secretion efficiency.

(B and C) (B) Quantification of data sets for apoB100 and (C) apoB48 as exemplified in (A) (n = 4 for each genotype). Intensities (mean ± SEM) are given relative to the total amount of apoB100 and apoB48, respectively, in wild-type samples (set at 1).

(D) Immunoblots for apoB100 and apoB48 in wild-type hepatocytes infected with Av-sortilin or Av-lacZ (left) and quantification thereof (right). Values are mean ± SEM.

(E) ApoB100 and apoB48 in wild-type and *Sort1*$^{-/-}$ mouse hepatocytes. Where indicated, replicate cell cultures were incubated with sortilin-expressing adenovirus (Av-sortilin) and with the lysosomal inhibitors leupeptin and pepstatin A.

corroborated our model that sortilin is capable of binding to apoB100, thereby affecting its subcellular localization.

As well as in cultured cells, we also characterized the impact of sortilin deficiency on intracellular trafficking of apoB100 in vivo. We subjected microsomal membranes from livers of wild-type and sortilin-deficient mice to subcellular fractionation and determined the distribution of apoB100, apoB48, apoE, and sortilin by immunoblotting. In wild-type mice, apoB100 and apoB48 were present in ER but also in Golgi-enriched fractions where they colocalized with sortilin (fractions 12–16; Figure 4B). Disruption of *Sort1* shifted the distribution of apoB100 toward ER fractions (fractions 2–6), suggesting that sortilin is required for efficient transit of apoB100 through the biosynthetic pathway (Figures 4B and 4C; $p < 0.0003$). In the absence of sortilin, there was also a tendency for buildup of apoB48 in the ER, but this effect was not significant (Figures 4B and 4D; $p = 0.15$). Sortilin deficiency did not affect subcellular localization of apoE (Figure 4B).

Calculating the total amount of apoB100 present in the subcellular fractions revealed a 57% increase in apolipoprotein content in the livers of *sortilin*$^{-/-}$ compared with *sortilin*$^{+/+}$ mice, albeit at normal *Apob* transcript levels (Figure S2B). Metabolic labeling of primary hepatocytes substantiated faulty release of apoB100 from sortilin-deficient cells. Thus, after an extended chase period, ~54% less apoB100 molecules were secreted into the medium of *Sort1*$^{-/-}$ hepatocytes compared with control cells (Figures 5A and 5B). Excretion of apoB48, on the other hand, was not affected (Figures 5A and 5C). In support of sortilin-mediated apoB100 secretion, adenoviral-mediated overexpression of sortilin, but not of lacZ, in wild-type hepatocytes was accompanied by an ~54% reduction in cellular apoB100 (but not apoB48) as shown by western blotting (Figure 5D). Likewise, apoB100 accumulation was notably diminished following sortilin overexpression in both wild-type and in *Sort1*$^{-/-}$ hepatocytes (Figure 5E).

Sortilin has been proposed to mediate TGN to endosome/lysosome sorting of target proteins. To exclude any confounding effects from lysosomal sorting on the cellular apoB100 concentration, we also treated sortilin overexpressing hepatocytes with lysosomal enzyme inhibitors leupeptin and pepstatin A. When comparing the cellular apoB100 content with and without treatment, we failed to see an obvious accumulation of the apoprotein in the presence of lysosomal inhibitors, suggesting that sortilin is

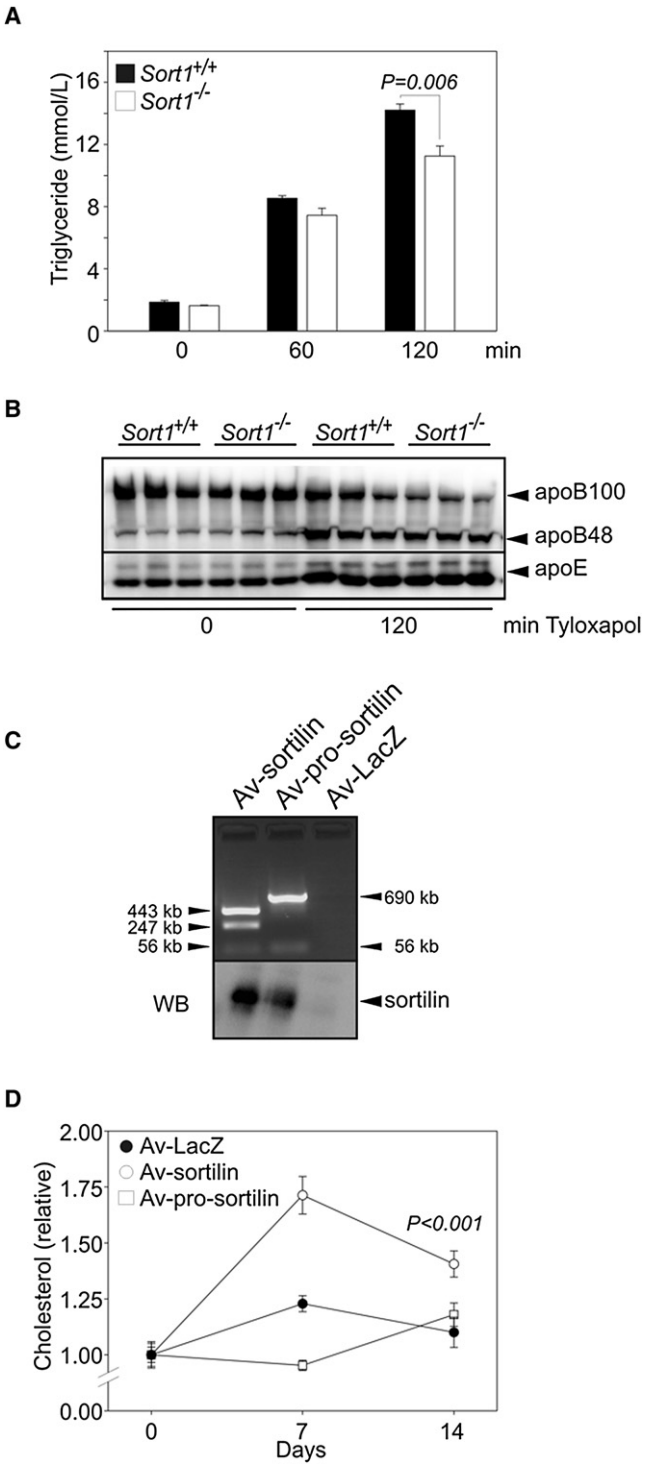


Figure 6. Sortilin Facilitates Hepatic Lipoprotein Export via Binding of apoB100 in the *trans*-Golgi Network

(A) Mice of the indicated genotypes were treated with tyloxapol, and plasma samples were collected at the indicated time points and analyzed for triglyceride concentrations (n = 5 animals per group). Triglycerides values are mean ± SEM.

(B) Western blot analysis of apoB100, apoB48, and apoE in representative plasma samples from the experiment shown in (A).

(C) Genotyping of livers from mice treated with Av-sortilin and Av-prosortilin. Introduction of a point mutation in prosortilin disrupts a BsaHI restriction site in a RT-PCR product (upper panel). Virus expression is confirmed by western blotting (lower panel).

(D) Plasma cholesterol levels in mice fed a Western-type diet and infected with Av-sortilin (n = 6), Av-prosortilin (n = 6), or Av-lacZ (n = 6) for the indicated periods of time. Cholesterol values are mean ± SEM relative to plasma levels prior to virus treatment.

unlikely to mediate endosome/lysosome sorting of apoB100 (Figure 5E).

Sortilin Promotes Hepatic Export of apoB100-Containing Lipoproteins In Vivo

In a recent study, recombinant overexpression of sortilin in human embryonic kidney 293 cells resulted in a modest increase in cellular uptake of LDL (Linsel-Nitschke et al., 2009). Thus, a potential role for this receptor in promoting clearance of LDL was proposed. This hypothesis contrasts with our data from sortilin-deficient mice that exhibit reduced levels of LDL compared to mice expressing the receptor (Figure 1D). To test an effect of endogenous sortilin on hepatic LDL uptake, we measured LDL receptor expression and endocytic uptake of purified LDL in primary hepatocytes from wild-type and sortilin-deficient animals. No difference in LDL receptor expression (Figure S2B) or LDL uptake (Figure S5) was seen comparing genotypes.

Taken together, our cellular studies suggested that sortilin binds apoB100 in the distal secretory pathway and regulates its sorting and/or incorporation into nascent lipoprotein particles, thereby facilitating their release from cells. To confirm that this model also applies to the in vivo situation, we injected wild-type and *Sort1*$^{-/-}$ mice on a Western-type diet intravenously with tyloxapol (Triton WR-1339), an inhibitor of lipolysis that prevents breakdown of VLDL to LDL. Two hours after tyloxapol application, triglyceride levels (indicative of newly secreted VLDL particles) were ~22% lower in *Sort1*$^{-/-}$ compared with wild-type animals (Figure 6A, p = 0.006). Total cholesterol levels were unchanged, in accordance with fatty acids being a major component of VLDL particles (Figure S6A). In wild-type animals, the levels of apoB100 remained fairly constant following tyloxapol treatment, likely reflecting that VLDL production is balanced by a simultaneous clearance of pre-existing LDL particles during the 2 hr time course of the experiment (Figure 6B). In contrast, there was noticeably less apoB100 in the plasma of sortilin-deficient mice. ApoB48 and apoE accumulated in the plasma of tyloxapol-treated animals regardless of the genotype (Figure 6B).

Lastly, we further elucidated the cellular mechanism whereby sortilin may control biogenesis and hepatic release of VLDL. We took advantage of a sortilin mutant, denoted prosortilin, that harbors mutations in the amino acid sequence required for proteolytic cleavage of the receptor propeptide. Because the propeptide blocks entry of ligands to the binding pocket, the activity of sortilin is conditioned on the release of the propeptide from the receptor by proconvertases in the TGN (Munck et al., 1999). Using SPR analysis we showed that LDL was not able to bind to prosortilin (Figure 3D). Moreover, addition of 5 mM recombinant propeptide inhibited binding of VLDL (125 μg/ml) to mature sortilin by ~85%. These findings suggested that the interaction between apoB100 and sortilin likely occurs in a distal

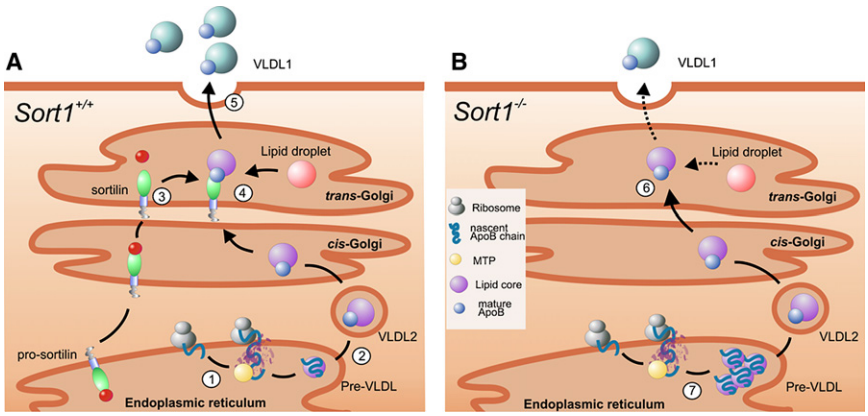


Figure 7. Model of Sortilin Action in Hepatic Lipoprotein Export

(A) Model of VLDL production in the secretory pathway of hepatocytes involving the activity of MTP in the ER and sorting receptor sortilin in the TGN.

(B) Impaired formation and release of VLDL1 in sortilin-deficient hepatocytes result in blockade of apoB100 export form the ER.

Golgi compartment where activation of the receptor takes place. To substantiate the physiological relevance of this finding, we overexpressed prosortilin by adenoviral-mediated gene transfer (Av-prosortilin) in mice and compared the effects to those elicited by overexpression of the wild-type receptor (Av-sortilin). Infection of mice in both groups (Figure 6C, upper panel) resulted in similar levels of hepatic overexpression of the two receptor variants (Figure 6C, lower panel). Yet prosortilin was unable to increase cholesterol levels in the animals as opposed to the mature wild-type receptor, which went up by ~70% at day 7 (Figure 6D). Similarly to Av-prosortilin, treatment with Av-lacZ control virus preparations did not affect systemic cholesterol levels (Figure 6D). In agreement with increased sortilin-mediated VLDL release, total triglycerides were increased by ~138% (Figure S6B). Thus, we concluded that sortilin mediates its effect on lipoprotein export in the TGN by direct interaction with apoB100.

DISCUSSION

Previous studies identified SNPs at 1p13.3 strongly associated with plasma LDL and risk of CAD. This locus covers the genes *SORT1*, *PSRC1*, and *CELSR2*. Using transgenic mouse models that either overexpress or lack sortilin, we identified sortilin as a regulator of hepatic lipoprotein production and plasma LDL cholesterol. According to our model, the receptor facilitates trafficking and assembly of VLDL in the distal Golgi compartment of liver cells where formation of triglyceride-rich particles from lipidated apoB100 proceeds (reviewed in Olofsson et al., 2007).

In wild-type hepatocytes, formation of VLDL particles starts with lipidation of nascent apoB100 polypeptide chains that are being translocated into the lumen of the ER (Figure 7A). Lipidation of the apolipoprotein involves the activity of MTP (Figure 7A, step 1). Primordial particles (pre-VLDL) are further lipidated to form a triglyceride-poor form of VLDL (VLDL2; Figure 7A, step 2) that reaches the Golgi, where it is converted into triglyceride-rich VLDL1 by addition of triglycerides from lipid droplets (Figure 7A, step 4). We propose that assembly and release of VLDL1 (steps 4 and 5) may require interaction of apoB100 with mature sortilin that becomes proteolytically activated in the distal Golgi (step 3). The presence of the propeptide in prosortilin prevents premature association of the receptor with nascent apoB100 chains in the ER. Based on our findings, loss of sortilin activity likely impairs formation and release of VLDL1 in the Golgi (Figure 7B, step 6), and thereby impedes vesicular transport of apoB100 through the secretory pathway. This block results in backlog of apoB100 trafficking and impaired release of pre-VLDL from the ER (Figure 7B, step 7).

Many phenotypes seen in sortilin-deficient mice are also observed in conditions of hypo- and abetalipoproteinemia, supporting a role for the receptor in promotion of VLDL production. For example, genetic ablation or pharmacological blockade of MTP reduces hepatic secretion of apoB100, but only modestly apoB48, and, consequently, impairs VLDL export from the liver (Chang et al., 1999; Liao et al., 2003; Raabe et al., 1999). The same phenotype is seen when inactivating *Sort1*. Enhanced production and release of apoB100-containing lipoproteins are also seen when overexpressing wild-type sortilin (Figures 2 and 6D). As opposed to models of hypobetalipoproteinemia, sortilin-deficient hepatocytes do not accumulate cholesterol in the liver. One possible explanation is the apparent increase in expression of CYP7A1, presumably resulting in enhanced biliary excretion of the sterol. The effect of sortilin on hepatic lipoprotein metabolism in vivo is critically dependent on the ability of the receptor to interact with ligands, as prosortilin that is devoid in apoB100 binding fails to modulate plasma cholesterol levels (Figure 6D).

Mechanistically, hypo- and abetalipoproteinemia caused by apoB and MTP gene defects, respectively, are associated with a dysfunction in the ER (Chang et al., 1999; Du et al., 1994; Raabe et al., 1999). In contrast, sortilin deficiency seems to impair lipoprotein secretion at a later stage along the secretory pathway, likely in transit through the Golgi. A general function for sortilin and related Vps10p domain receptors in protein sorting at the TGN has previously been proposed based on their structural homology to the vacuolar protein sorting 10 protein (VPS10P), a sorting receptor in *Yeast* that directs carboxypeptidase Y to the vacuole (Marcusson et al., 1994). This hypothesis was supported by findings in mammalian cell lines that the cytoplasmic tail of sortilin was able to direct chimeric mannose 6-phosphate receptors from the TGN into endosomal/lysosomal pathways (Nielsen et al., 2001).

A proposed role for Vps10p domain receptors in sorting processes at TGN was confirmed using gene-targeting or knockdown approaches as discussed below. However, these studies failed to support a function for the receptors in targeting proteins into lysosomal degradation. Rather, the mammalian

receptors proved to be involved in trafficking of target proteins through the late secretory pathway to the cell surface. For example, inactivation of SORLA, a sorting receptor for the amyloid precursor protein (APP), resulted in enhanced shunt of APP to the cell surface and in aggravated proteolytic processing into amyloid β-peptides (Andersen et al., 2005; Schmidt et al., 2007). Knockdown of sortilin expression in primary neurons impaired regulated release of its ligand brain-derived neurotrophic factor (Chen et al., 2005). Also, new studies presented here did not reveal a distinct effect of sortilin on targeting of apoB100 to lysosomal pathways (Figures 5D and 5E). This observation is in line with the fact that posttranscriptional regulation of apoB100 biogenesis by proteolytic degradation mainly involves autophagosomes and, to some extent, also proteasomes and the ER (Pan et al., 2008; Hooper et al., 2005).

Levels of sortilin activity are directly correlated with plasma LDL cholesterol concentrations in mice. Thus, loss of receptor activity reduces plasma cholesterol levels by 20% on the wild-type LDL receptor background and by 30% in *Ldlr*$^{-/-}$ animals (Figure 1A). As a result, development of atherosclerotic plaques in the aorta of *Ldlr*$^{-/-}$ mice is considerably impaired on the sortilin knockout background (Figure 1E). When the activity of the receptor is increased (as in Av-sortilin treated mice), plasma cholesterol levels rise by up to 75% (Figures 2B and 6D). Similar changes in plasma LDL and cholesterol have been identified in individuals with the minor 1p13.3 allele variant. Based on epidemiological studies, each minor allele translates into an ~4%–9% reduction in plasma cholesterol, depending on the ethnic group studied (Linsel-Nitschke et al., 2009; Willer et al., 2008; Keebler et al., 2009; Nakayama et al., 2009). As a consequence, carriers of the minor allele are subject to 13%–29% lower risk of CAD (Kathiresan et al., 2009; Samani et al., 2007; Linsel-Nitschke et al., 2009). Thus, even modest changes in LDL cholesterol associated with altered expression of sortilin may translate into a substantial risk burden. Of course, experiments presented in this study do not exclude a contribution from *PSRC1* and *CELSR2*, also covered by the 1p13.3 locus to cardiovascular phenotypes associated with 1p13.3. Obviously, lack of information concerning the functional roles of these two genes in context of the cardiovascular system impairs formulation of such hypotheses. However, it is of note that levels of expression of these two genes were unchanged in *Sort1*$^{-/-}$ mice (Figure S2F).

A positive correlation of plasma cholesterol with sortilin activity documented in mice is intriguing given the fact that a recent study demonstrated modestly increased levels of *SORT1* transcripts in carriers of the minor allele variant despite reduced plasma LDL levels (Linsel-Nitschke et al., 2009). Based on their results, the authors argued that sortilin may act as a clearance pathway for LDL promoting hepatic catabolism of the lipoprotein when expression of the receptor is increased. Obviously, this hypothesis contrasts with reduced levels of plasma LDL and cholesterol in sortilin-deficient mice shown here. When considering this controversy, one has to keep in mind that the exact mechanism of how SNPs associated with *SORT1* affect receptor activity is unclear at present. Any interpretation is complicated by the fact that no functional SNPs have been identified in the *SORT1* coding region and that no information on the amount of receptor protein or activity in the human liver is available for SNPs identified so far. Although it is intuitive that protein concentration may correlate with mRNA level, this statement is only true globally. For individual genes, the correlation of transcript and protein level is weak, as shown in multiple genome studies (Greenbaum et al., 2001; Ideker et al., 2001). Furthermore, *SORT1*, *PSRC1*, and *CELSR2* are all in the same linkage disequilibrium block, and the minor allele variant also regulates expression of *PSRC1* and *CELSR2*, genes that could exhibit opposing functions to that of sortilin. Indeed, using quantitative trait mapping in mouse intercrosses, Schadt et al. identified that low expression of *PSRC1* correlates with low LDL cholesterol (Schadt et al., 2008). Thus, *SORT1* mRNA levels alone may be a poor predictor of hepatic protein activity.

Lowering LDL cholesterol is considered one of the most efficient strategies to reduce the risk of CAD (Visser et al., 2008). Identification of regulators in lipoprotein metabolism, such as sortilin, will help to develop therapeutic strategies aimed at reducing plasma LDL cholesterol, the single most predictive cardiovascular risk factor.

EXPERIMENTAL PROCEDURES

Animal Experimentation

Congenic *Sort1*$^{-/-}$ mice on C57BL/6J background (>98.5%) have been described previously (Jansen et al., 2007). *Ldlr*$^{-/-}$ line (C57BL/6J) was obtained from Charles River Laboratories, Wilmington, MA. Unless stated otherwise, experiments were carried out on mice fed a Western-type diet (Altromin Western Diet, Brogaarden, Denmark) for 2 weeks. Permission for animal experimentation was obtained (J. 2006/561-1206).

Lipoprotein FPLC, Cholesterol, and Triglyceride

Plasma cholesterol and triglycerides were determined in fasted animals using the "Cholesterol CHOD-PAP" and "Triglycerides GPO-PAP" kits (Roche, Basel, Switzerland). Measurement of lipoprotein profiles was conducted by TNO Biosciences, Leiden, The Netherlands.

Preparation of Adenoviruses

Adenoviruses encoding human sortilin (amino acids 1–833), human prosortilin lacking a functional furin site (RWRR to RWGG), or lacZ were propagated by ViraQuest Inc., North Liberty, IA. Mice were injected with 2E9 pfus intravenously. Hepatocytes were infected with 20 pfu/cell.

Coimmunoprecipitation

Lysed HepG2 cells (1% Nonidet P-40, 0.25% deoxycholate), incubated with anti-apoB (ab20737, Abcam, England) or nonimmune IgG, were precipitated using protein G agarose (Roche Applied Science, Germany).

Immunofluorescence Analysis

HepG2 cells or primary hepatocytes were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked in 10% FCS, and incubated with antibodies against sortilin, apoB100, mannosidase II, and/or calnexin for 24 hr at 4°C. Secondary fluorescent-labeled antibodies were used for visualization.

Pulse-Chase Experiments

Primary hepatocytes were metabolically labeled with L-[^{35}S]cysteine and L-[^{35}S]methionine in the presence of Brefeldin A, washed and incubated in complete medium, and chased for the times indicated (Munck et al., 1999). Medium was collected, immunoprecipitated using gamma-bind Sepharose coated with anti-albumin (A0433, Sigma Aldrich) or anti-apoB (ab20737, Abcam), and analyzed by SDS-PAGE and fluorography.

Surface Plasmon Resonance Analysis

Sortilin and prosortilin were immobilized on a CM5 chip at 0.108 and 0.096 fmol/mm^2, respectively. Sample and running buffer was 10 mM HEPES, 150 mM (NH4)2SO4, 1.5 mM CaCl2, 1 mM EGTA, 0.005% Tween-20 (pH 7.4). The SPR signal was expressed in relative response units (RUs) after

subtraction of the RU in a control flow channel. Kinetic parameters were determined using BIAevaluation 3.1 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2010.08.006.

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