1-1-2015

Sortilin: A Protein Involved in Ldl Metabolism and Atherosclerosis

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Abstract
Genome-wide association studies (GWAS) have been used to identify novel genes and loci that contribute to lipid traits and coronary heart disease (CHD) in a causal manner. A locus on chromosome 1p13, which harbors the gene sortilin-1 (SORT1) encoding the protein sortilin is the locus in the human genome with the strongest association with low-density lipoprotein cholesterol (LDL-C) and is also one of the strongest loci associated with CHD. Homozygosity for the minor allele haplotype at 1p13 is associated with a >10 fold increase in hepatic SORT1 expression, a mean 16 mg/dL reduction in plasma LDL-C, and a 40% reduction in CHD risk. Sortilin has been extensively studied in the central nervous system, where it traffics multiple ligands from the Golgi apparatus to the lysosome and also serves as a cell surface endocytosis receptor for a variety of ligands. However, the role of sortilin in other cell types, most notably hepatocytes and macrophages, which are key regulators of lipid metabolism and atherosclerosis development, has not been well studied. Through a series of overexpression and mutagenesis studies in cells and mice, the Rader lab has previously shown that increased sortilin expression in liver reduces plasma LDL-C both by promoting the presecretory lysosomal degradation of the LDL precursor very-low density lipoprotein (VLDL) and by serving as an endocytosis receptor for LDL. The Rader lab has also shown that total body Sort1 deficiency is associated with compromised LDL clearance consistent with overexpression studies; however, it is also associated with a paradoxical reduction in VLDL secretion. Using a variety of liver specific Sort1 deficiency models, as well as reconstitution and mutagenesis studies, I showed that liver specificity is not responsible for the secretion paradox and instead demonstrated that sortilin plays a dual role in VLDL trafficking, serving as a chaperone that facilitates VLDL secretion as well as a transporter that promotes the presecretory degradation of VLDL, depending on the conditions and level of sortilin expression.

Sortilin is strongly associated both with LDL-C levels and with CHD/atherosclerosis. Atherosclerotic cardiovascular disease is driven by elevated LDL-C, thus it is tempting to speculate that the strong association of the SORT1 locus with atherosclerosis is due solely to the LDL-C association. Because sortilin is expressed in macrophages, I hypothesized that macrophage sortilin might influence atherogenesis. Through careful interrogation of the role of sortilin in macrophages, I demonstrated that sortilin plays a role in the development of atherosclerosis independent of plasma LDL-C levels. Specifically, I showed that sortilin serves as an endocytosis receptor for LDL on macrophages, and this represents a physiologically important pathway by which LDL cholesterol enters macrophages and contributes to foam cell formation and atherosclerosis. This work increases our understanding of the role of hepatic and macrophage sortilin in LDL metabolism and atherogenesis, and provides insight into the relationship of the SORT1 locus with LDL-C levels and CHD risk.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Pharmacology

SORTILIN: A PROTEIN INVOLVED IN LDL METABOLISM AND ATHEROSCLEROSIS

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A DISSERTATION

in

Pharmacology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

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DEDICATION:

To my family for their love and support

ACKNOWLEDGMENT

I would like to acknowledge many people who have contributed to my work. I would first like to thank my advisor, Daniel Rader for his support and guidance over the last few years. I would also like to thank Alanna Strong. Her endless help and advice was immeasurable. I would also like to thank members of the Rader lab, Junichiro Tohyama, Dr. Millar, Dr. Billheimer, Dawn Marchadier, Debbie Cromley, Stephanie DerOhannessian, Kevin Trindade, Sumeet Khetarpal, Valeska Redon, Amrith Rodriguez, Mao-sen Sun, Aisha Faruqi, Ed Eduoard, Antonino Picataggi, Donna Conlon, and Linda Carmichael whom made this doable. I would also like to thank my collaborators outside of the Rader lab, Ed Fisher, Howard Kruth, Tina Jin, Muredach Reilly, Hanrui Zhang, Kiran Musunuru, Qiurong Ding, Michael Philips, Sissel Lund-Katz.

I would like to acknowledge my thesis committee: Peter Davies (Chair), Ellen Pure, Ed Fisher, and Rick Assoian for their expertise and insight.

Finally, I would like to thank my family and friends for their support and advice. I would especially like to thank my partner; Roshni Patel, my parents, and the best brother in the universe for all of their support. You have been a source of unbounded joy and happiness. I would like to thank my cousin Hiren Patel and the Pharmacology Graduate Group class: Mike Chiorazzo, Mansi Shinde, Sima Patel, Bridgin Lee, Diana Avery, John O'Donnell, Brian Weiser, and Natalie Daurio. You made my time in graduate school more enjoyable by providing friendship and support. Finally I would like to thank the Pharmacology Graduate Group, especially Sarah Squire, Julie Blendy, and Vladimir Muzykantov for this experience.

ABSTRACT

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Daniel J. Rader

Genome-wide association studies (GWAS) have been used to identify novel genes and loci that contribute to lipid traits and coronary heart disease (CHD) in a causal manner. A locus on chromosome 1p13, which harbors the gene s*ortilin-1* (*SORT1*) encoding the protein sortilin is the locus in the human genome with the strongest association with low-density lipoprotein cholesterol (LDL-C) and is also one of the strongest loci associated with CHD. Homozygosity for the minor allele haplotype at 1p13 is associated with a >10 fold increase in hepatic *SORT1* expression, a mean 16 mg/dL reduction in plasma LDL-C, and a 40% reduction in CHD risk. Sortilin has been extensively studied in the central nervous system, where it traffics multiple ligands from the Golgi apparatus to the lysosome and also serves as a cell surface endocytosis receptor for a variety of ligands. However, the role of sortilin in other cell types, most notably hepatocytes and macrophages, which are key regulators of lipid metabolism and atherosclerosis development, has not been well studied. Through a series of overexpression and mutagenesis studies in cells and mice, the Rader lab has previously shown that increased sortilin expression in liver reduces plasma LDL-C both by promoting the presecretory lysosomal degradation of the LDL precursor very-low density

lipoprotein (VLDL) and by serving as an endocytosis receptor for LDL. The Rader lab has also shown that total body *Sort1* deficiency is associated with compromised LDL clearance consistent with overexpression studies; however, it is also associated with a paradoxical reduction in VLDL secretion. Using a variety of liver specific *Sort1* deficiency models, as well as reconstitution and mutagenesis studies, I showed that liver specificity is not responsible for the secretion paradox and instead demonstrated that sortilin plays a dual role in VLDL trafficking, serving as a chaperone that facilitates VLDL secretion as well as a transporter that promotes the presecretory degradation of VLDL, depending on the conditions and level of sortilin expression.

Sortilin is strongly associated both with LDL-C levels and with CHD/atherosclerosis. Atherosclerotic cardiovascular disease is driven by elevated LDL-C, thus it is tempting to speculate that the strong association of the *SORT1* locus with atherosclerosis is due solely to the LDL-C association. Because sortilin is expressed in macrophages, I hypothesized that macrophage sortilin might influence atherogenesis. Through careful interrogation of the role of sortilin in macrophages, I demonstrated that sortilin plays a role in the development of atherosclerosis independent of plasma LDL-C levels. Specifically, I showed that sortilin serves as an endocytosis receptor for LDL on macrophages, and this represents a physiologically important pathway by which LDL cholesterol enters macrophages and contributes to foam cell formation and atherosclerosis. This work increases our understanding of the role of hepatic and macrophage sortilin in LDL metabolism and atherogenesis, and provides insight into the relationship of the *SORT1* locus with LDL-C levels and CHD risk.

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Chapter One: Introduction

VLDL Production and LDL Catabolism

Low density lipoproteins (LDL) consist of a hydrophilic, polar surface coat composed of phospholipid, free cholesterol, free fatty acids, and apolipoprotein B (apoB) and a hydrophobic, nonpolar core of principally cholesteryl ester with some triglycerides (TG). The principal protein component of all LDL particles is apoB, which is essential to LDL structure and also facilitates its clearance from plasma by binding to the LDL receptor. LDL cholesterol (LDL-C) levels are determined both by the rate of LDL removal from plasma[1] as well as by the rate of production of the LDL precursor very low density lipoprotein (VLDL)[2,3]. VLDL synthesis occurs in the liver, and begins with the synthesis and co-translational lipidation of apoB. ApoB lipidation occurs in the Endoplasmic Reticulum (ER) and is driven by the catalytic activity of microsomal triglyceride transfer protein (MTP) and phospholipid transfer protein (PLTP), which transfers triglycerides and phospholipids to nascent apoB[2]. Lipidated apoB is next transported to the Golgi apparatus for further lipid addition and lipoprotein modification. The fully lipidated apoB is then secreted as VLDL[2].

The VLDL secretion rate is strongly influenced by the pre-secretory degradation of apoB[3]. There are multiple pathways for presecretory apoB degradation, including proteasome and lysosome mediated degradation. When there is insufficient lipid in the ER to lipidate nascent apoB, the apoB particles are retro-translocated out of the ER and targeted for proteasome degradation in a well-characterized pathway called ERAD (ER

associated degradation), which is driven by the ER chaperone Hsp70[4]. ApoB that escapes ER quality control can also be degraded in a post-ER compartment; specifically, Golgi-localized apoB containing particles that are misfolded, oxidized or damaged can be trafficked through autophagy to the lysosome for degradation[4,5]. This pathway, called PERPP (post-ER presecretory proteolysis pathway) is believed to be responsible for the presecretory degradation of VLDL in response to poly-unsaturated fatty acids and insulin[4-6].

LDL levels are also regulated by LDL catabolism[1]. LDL is removed from circulation principally by the hepatic LDL receptor, which binds the apoB in circulating LDL particles and transports it to the endolysosomal system for degradation through clathrin-mediated endocytosis. Other receptors facilitate LDL clearance as well, including low-density lipoprotein related receptor 1 and syndecan-1, although these receptors primarily mediate the uptake of the charged side chains of apoE-containing lipoprotein particles[1,7]. The non-LDL receptor pathways for hepatic clearance of LDL are not fully understood.

Human Genetics of the Chromosome 1p13 SORT1 locus

The most clinically effective drugs for cardiovascular disease currently available target well-known genes and pathways intricately involved in the regulation of cholesterol clearance, biosynthesis and absorption. Interestingly, one of the newest and most promising pharmacotherapies in the treatment of cardiovascular disease is Proprotein convertase subtilisin/kexin type 9 (PCSK9) monoclonal antibodies, which have been recently shown in clinical trials to reduce LDL-C by greater than 60%. PCSK9

has been shown to mediate the turnover of the LDL receptor. PCSK9 was discovered through study of Mendelian disorders of lipid metabolism, highlighting the importance of using genetics as a tool to understand physiology and develop new treatments for disease.

Complex traits such as lipid traits and CHD are influenced by multiple genes of small effect size that contribute to phenotype. Genome-wide association studies (GWAS) have been used to identify such genes and pathways, and this type of unbiased interrogation has been applied to the study of lipid traits and CHD. These studies have identified a number of genes known to be associated with lipid metabolism[8-11] through the study of rare Mendelian disorders of lipid homeostasis, as well as a multitude of novel loci never before associated with heart disease[11-14].

A locus on chromosome 1p13 was identified to have a very strong association with LDL-C. A detailed analysis of this locus was performed to identify the causal variant and causal gene at this locus. Through a series of studies in human cohorts and human derived hepatocytes a common noncoding polymorphism was identified to create a C/EPB (CCAAT/enhancer binding protein) transcription factor-binding site altering the hepatic expression of the *SORT1* gene[15]. Homozygosity for the minor allele haplotype is associated with a >10 fold increase in hepatic *SORT1* expression and a mean 16 mg/dL reduction in plasma LDL-C. Separate studies in sortilin knockout mice also established a role for sortilin in lipoprotein metabolism[16]. Together, these studies established sortilin as the causal gene at the locus underlying the association with LDL-C.

Independently, GWAS for myocardial infarction (MI) and CHD identified the same chromosome 1p13 locus as strongly associated with CHD[11,12,17]. The same variants have also shown to be minor allele haplotype was found to be associated with an approximately 40% reduction in CHD risk[12].

Sortilin Background

The VPS10-domain Family

Sortilin is part of the VPS10 (vacuolar protein sorting 10) receptor family, a family of type-I transmembrane receptors that share an N-terminal propeptide, a VPS10 ligand binding domain, a transmembrane domain and a small cytoplasmic tail that harbors two lysosomal sorting motifs, a tyrosine based sorting motif (YxxL) and a dileucine based sorting motif (DEDLLE). The VPS10 family was originally identified in yeast as a trafficking receptor for carboxypeptidase Y from the Golgi to the vacuole, the yeast lysosome compartment. The VPS10 domain is a 700 amino acid module that folds into a 10-bladed beta propeller structure. There are 5 mammalian VPS10-domain containing proteins: sortilin, the sortilin related receptor (SorLA), and SorCS1-3[18,19].

SorLA is best known for its role as an apolipoprotein E (apoE) receptor, and is believed to serve a neuroprotective role and reduce the risk of Alzheimers Disease and senile plaque formation by promoting the anti-amyloidogenic processing of amyloid precursor protein (APP) by controlling APP as well as BACE (beta secretase 1) trafficking[20,21]. In the vasculature, SorLA increases macrophage scavenger receptor expression, which increases foam cell formation and promotes atherosclerosis[22-25]. The

SorCS1-3 proteins are poorly characterized. They are believed to play a role in Alzheimers Disease[25] development and genome wide association studies suggest a connection to diabetes[26]. Indeed, it has recently been shown that SorCS1 is required for secretory granule replenishment and maximum insulin secretion under conditions of metabolic stress[27].

Sortilin was originally purified by receptor associated protein (RAP) affinity chromatography and neurotensin affinity purification[28]. Sortilin is a 95 kDa, 833 amino acid protein that harbors an N-terminal signal peptide, a 44 amino acid N-terminal propeptide domain, a luminal VPS10 domain, a transmembrane domain and a short cytoplasmic tail homologous to the cation-independent mannose-6 phosphate receptor (CI-M6PR)[28,29]. Sortilin is expressed most strongly in brain, skeletal muscle and adipocytes, with significant expression in liver as well[28,30]. It is a largely intracellular protein with 90% localized to the Golgi and endolysosomal compartments and 10% at the plasma membrane[28,31].

Sortilin trafficking

Sortilin is synthesized in the endoplasmic reticulum (ER) as a proprotein. The propeptide serves as an intrinsic chaperone for sortilin; it facilitates proper folding of the receptor and blocks the VPS10 domain to prevent premature ligand binding[30]. The propeptide domain is cleaved by furin in the trans-Golgi network (TGN) to generate the mature receptor[32]. From the Golgi, sortilin can follow one of three trafficking routes. First, it can be trafficked to the plasma membrane by constitutive secretory vesicles. At the plasma membrane, sortilin can be cleaved by the disintegrin and metalloproteinase

domain-containing protein 10 (ADAM10) to generate a soluble protein comprising the sortilin luminal domain[33]. The majority of sortilin (~95%) is not cleaved by ADAM10 and re-enters the cell through clathrin dependent endocytosis[33]. The sortilin cytoplasmic tail harbors a tyrosine based lysosomal sorting motif (YxxL), which serves as a binding site for adaptor protein 2 (AP2), which recruits clathrin and enables endocytosis[29,34]. Internalized sortilin can then be trafficked back to the Golgi through interaction with the intracellular adaptor proteins retromer, which recognizes the tyrosine based lysosomal sorting motif, and adaptor protein 1 (AP1), which recognizes the dileucine lysosomal sorting motif (DEDLLE).

Second, sortilin can traffic directly from the Golgi to the endolysosomal compartment by binding AP1 and the Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding proteins (GGAs) through its tyrosine and dileucine based sorting motifs, respectively[29,35,36]. Third, in certain cell types, sortilin can bind its ligands intracellularly and be stored in secretory vesicles and undergo regulated secretion[31].

Sortilin undergoes post-translational modifications. It is N-glycosylated in the Endoplasmic Reticulum and also undergoes palmitoylation at cysteine 783, which facilitates retromer binding and lysosome to Golgi recycling[37]. Without palmitoylation, sortilin is retained in the endosomal compartment and degraded[38]. Sortilin localization and degradation is also influenced by interaction with the neurotrophin receptor homolog 2 (NRH2), which interacts with the sortilin cytoplasmic tail and prevents sortilin lysosomal trafficking and instead shunts the receptor to the plasma membrane, thereby

promoting its cell surface localization. This interaction is not dependent on a post-translational modification; instead, it appears to be tightly developmentally regulated[39].

Sortilin at the cell surface

At the cell surface, sortilin serves as both a signaling receptor and as a trafficking receptor. In the central nervous system (CNS) sortilin binds pro-neurotrophins, including pro-nerve growth factor (proNGF) and pro-brain derived neurotrophic factor (proBDNF), which are neuronal growth factors that are incompletely processed and have their pro-proteins still attached. Sortilin and pro-neurotrophins form a complex with the promiscuous p75 neurotrophin receptor ($p75^{NTR}$), and this activates apoptosis in neurons. This signaling pathway is important for proper development of the CNS, but also plays a role in the pathophysiology of aging, neurodegeneration, and spinal cord injury[40-42].

Sortilin also serves as an internalization and degradation receptor for LpL[43], apoAV[44], and progranulin[45]. Interestingly, the same single nucleotide polymorphisms (SNPs) at the 1p13 *SORT1* locus associated with lower LDL-C and reduced risk of CHD are associated with reduced plasma progranulin levels[45]. Functional studies show that increased *SORT1* expression promotes the uptake and degradation of extracellular progranulin, consistent with a role for sortilin as an internalization receptor for this protein.[46].

Sortilin inside the cell

Intracellularly, sortilin shuttles between the Golgi apparatus and endolysosomal compartment, enabling sortilin to serve as a trafficking receptor to target lysosomal

hydrolases to the lysosome for function[29,47]. Indeed, sortilin has been shown to bind a variety of lysosomal hydrolases including sphingomyelinase activator protein, GM2 activating protein, prosaposin, and lysosomal cathepsins, and was found to partially compensate for loss of the mannose-6 phosphate receptor, the primary Golgi to lysosome trafficking receptor for lysosomal hydrolases[48].

Sortilin is also able to traffic proteins to distinct intracellular compartments. For example, sortilin plays an important role in formation and stabilization of the glucose 4 transporter (GLUT4) in skeletal muscle and adipocytes[49]. In these tissues, sortilin binds GLUT4 in the Golgi and traffics it to intracellular vesicles containing insulin regulated aminopeptidase (IRAP), low-density lipoprotein receptor 1 (LRP1), and vesicle associated membrane protein 2 (VAMP2). These vesicles form the insulin responsive compartment (IRC), and translocate to the cell surface upon insulin stimulation. siRNA mediated *SORT1* knockdown depletes GLUT4 and the IRC[49].

Interestingly, it was recently suggested that intracellular sortilin plays a dual role in protein trafficking, and that sortilin can promote the endolysosomal degradation of ligands but also protect them from lysosomal degradation and facilitate their secretion. Specifically, sortilin was shown to bind proBDNF within the secretory pathway[33]. proBDNF bound to full length sortilin was trafficked to the endolysosomal system and degraded; however, if sortilin is cleaved in the Golgi by ADAM10, which separates the ligand binding domain from the lysosomal sorting motifs, the proBDNF:sortilin extracellular domain complex is secreted from cells[33]. The physiological importance of sortilin cleavage is unknown. Sortilin has been extensively studied in the central nervous

and skeletal systems. However, virtually no research on sortilin biology in either the liver or macrophage had been performed prior to the genome wide association studies showing association of the *SORT1* locus with LDL-C and CHD.

Chapter Two: Atherosclerosis

Overview of the Pathophysiology of Atherosclerosis

Atherosclerotic cardiovascular disease is the leading cause of morbidity and mortality in the world, representing an unmet need for the development of pharmacotherapy[50]. Atherosclerosis results from an inflammatory response initiated by the entrapment of lipoproteins in the subendothelial space.[51] Specifically, elevations in plasma LDL-C promote deposition and retention of lipoprotein particles in the subendothelial space, or intima. These retained lipoproteins are modified by proteins secreted by neighboring vascular endothelial cells[52]. The proinflammatory particles induce the activation of the overlying endothelium. Monocytes are recruited into these lesions by chemokines and they differentiate into macrophages, leading to further modification of the retained lipoproteins. Modified lipoproteins are taken up by macrophages, forming cholesterol laden foam cells. These foam cells elicit inflammatory, fibrotic, and thrombotic responses resulting in formation of an atherosclerotic plaque[53]. These plaques can expand slowly into the blood vessel lumen or can acutely rupture inducing a robust inflammatory response. Both mechanisms disrupt blood flow and if they occur within the coronary vasculature cause a myocardial infarction (MI)[54,55].

Haemodynamic and extracellular matrix factors

Haemodynamic forces experienced by arteries are critical in the initiation of atherosclerotic lesions[56,57]. Atherosclerotic lesions generally develop where laminar flow is either disturbed or insufficient to maintain quiescent state endothelium such as sites of curvature and bifurcation. A quiescent state is generally characterized an alignment of endothelial cells in the direction of flow with low rates of proliferation and death. Flow is a survival signal for endothelial cells through effects on multiple signaling pathways, which include phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase 5 (ERK5), and nitric oxide (NO)[58]. Disturbed flow or turbulent blood flow leads to propathologic endothelial cell shear stress, which promotes atherogenic lipoprotein entry and an accumulation of extracellular matrix proteins in the subendothelial space[56,57].

Disturbances in blood flow also activate endothelial mechanotransduction that alters blood vessel morphology and function [56,60]. Mechanical stretch also affects smooth muscle cell function by inducing deformation of the extracellular matrix in which the smooth muscle cells are embedded. Under excessive pressure smooth muscle cells undergo a transformation, in which global gene expression is altered to promote cell proliferation, migration, and extracellular matrix production, processes that promote atherogenesis[59,60]. These changes are driven by many signaling pathways, most notably through Rho kinase, MAPKs, Akt, and the forkhead transcription factors of the FoxO subfamily[59,61-64].

Inflammatory factors

Inflammatory factors are crucial to atherosclerotic lesion initiation and progression[65,66]. Proinflammatory cytokines have been shown to induce expression of endothelial-leukocyte adhesion molecules, which capture monocytes[67]. The first phase known as the capture and rolling phase is mediated by chemokines, CC-chemokine ligand 5 (CCL5) and CXC-chemokine 1 (CXCL1), on endothelial cell glycosaminoglycans and on P-selectin. Next, firm adhesion of monocytes to the endothelium is mediated by Vascular cell adhesion molecule 1 (VCAM1) and intercellular adhesion molecule 1 (ICAM1) which bind to the intergrins very late antigen 4 (VLA4) and lymphocyte function-associated antigen 1 (LFA1), respectively. In fact the adhesion receptor CD44 has been shown to promote atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation[68]. Multiple chemokines interact with cognate receptors (CCR2-CCL2, CX3CR1-CX3CL1, CCR5-CCL5) on various classes of leukocytes and promote direct migration and penetration of leukocytes into the intima[66,67]. It has also been demonstrated in hypercholesterolemic mice that a subset of monocytes marked by high levels of expression of the surface inflammatory marker Ly6c ($Ly6c^{Hi}$) enter plaques early in the development of atherosclerotic lesions[69]. $Ly6c^{Hi}$ monocytes constitute the majority of the monocytes in the plaque, which are thought to be the source of the M1 macrophages (known as the classically activated macrophages). Human plaques have shown M1 macrophages enriched in lipids to be distinct from the less inflammatory M2 macrophages (alternatively activated macrophages)[70]. The factors that promote the polarization to M1 or M2 are not completely known.

Macrophage LDL uptake and foam cell formation

LDL-C is a critical causal factor in atherogenesis and uptake of LDL by macrophages to form 'foam cells' is a key cellular process in atherogenesis. Due to the central role of the foam cell in atherosclerosis development, there is much interest in determining the pathways by which macrophages take up cholesterol[71]. LDL receptor expression in macrophages does not play a role in foam cell formation as evidenced by the abundance of foam cells in humans and mice with homozygous deletions of the LDL receptor gene. Furthermore, the LDL receptor is down regulated by increases in intracellular cholesterol, resulting in a minimal role for the LDL receptor in foam cell formation. Studies by Brown and Goldstein in fibroblasts from individuals lacking the LDL receptor suggest that LDL can enter cells through a receptor independent pathway termed macropinocytosis, in which cells engulf fluid from their surroundings into a macropinosome and all of the contents of the fluid, including any lipoproteins, are brought into the cell[71],[72]. Scavenger receptors are highly expressed on macrophages and participate in the uptake of oxidized and modified lipoproteins[69,73]. Scavenger receptors are not subject to cholesterol down-regulation like the LDL receptor, creating a potentially unsaturatable route of entry for lipoproteins into macrophages. Though scavenger receptors are an important contributor to foam cell formation in vitro[74], genetic deletion of scavenger receptors in mice does not prevent foam cell formation and the degree of lipoprotein oxidation in atherosclerotic lesions is insufficient to activate the scavenger receptor pathway[75]. Macropinocytosis uptake of LDL accounts for seventy percent of macrophage lipoprotein uptake[71]. The pathways that account for the remaining 30% of LDL uptake leading to the formation of foam cells has not yet been identified.

Dissertation Goals

The Rader lab has previously shown that elevated *Sort1* expression reduces LDL by promoting the presecretory lysosomal degradation of VLDL and also by serving as a cell surface receptor for LDL clearance. The lab has also shown that the total body *Sort1*-/- mouse has compromised LDL uptake but a paradoxical reduction in VLDL secretion. The goals of this dissertation were: 1) to better define and explain the relationship of hepatic sortilin deficiency to VLDL secretion; and 2) to characterize the role of macrophage sortilin in LDL uptake, foam cell formation, and atherosclerosis.

Chapter Three: Hepatic sortilin modulates VLDL apoB-100 secretion

Introduction

Genomewide Association Studies (GWAS) were the first to associate the *Sort1* locus with LDL. The Rader lab demonstrated that increased sortilin expression could promote the endolysosomal degradation of precursor of LDL, VLDL. The discovery was achieved through overexpression and mutagenesis studies, where sortilin overexpression was found to reduce VLDL secretion and sortilin mutants defective in their ability to traffic to the endolysosomal system ameliorated the effect of sortilin overexpression on VLDL secretion[76]. Sortilin overexpression reduces VLDL secretion in multiple model systems, a finding which has been widely replicated[77-78]. Interestingly, studies in *Sort1* deficient systems suggested that *Sort1* deficiency is also associated with a paradoxical reduction in VLDL secretion[16,76]. This series of observations in mice—that both hepatic

overexpression and complete genetic deficiency of sortilin reduce VLDL secretion—created uncertainty about the physiology of sortilin and VLDL secretion.

Conversly, the discovery of sortilin as an LDL receptor was accomplished through a series of overexpression, deficiency and mutagenesis studies[47]. Specifically, it was shown that liver sortilin overexpression increased LDL clearance while its deficiency severely compromised LDL clearance. LDL clearance was equally affected by sortilin manipulation in LDL receptor deficient systems, suggesting an LDL receptor independent pathway for sortilin mediated LDL uptake. Through the use of mutants defective in their ability to traffic to the endolysosomal system as well as trafficking mutants that are sequestered at the cell surface the Rader lab was able to demonstrate that sortilin serves as a receptor to facilitate LDL endocytosis and lysosomal degradation.

Though genome wide association studies are valuable tools for identifying genes that contribute to complex traits, they fall short of explaining the mechanistic basis by which the associated genes affect phenotype; specifically, though GWAS pointed to *SORT1* as an important regular of LDL cholesterol, they did not offer any insights on the mechanism by which elevations in *SORT1* expression reduce LDL. One goal of my work was to attempt to reconcile the disparate findings and better understand the relationship of hepatic sortilin expression to VLDL secretion. I entertained a number of hypotheses. The first involved tissue specificity of sortilin expression. The human genetic data as well as the overexpression studies all involved liver specific manipulations, whereas the deficiency studies were all done in mice deficient in sortilin in all tissues. There are numerous examples of genes that have opposing effects in different tissues. My first

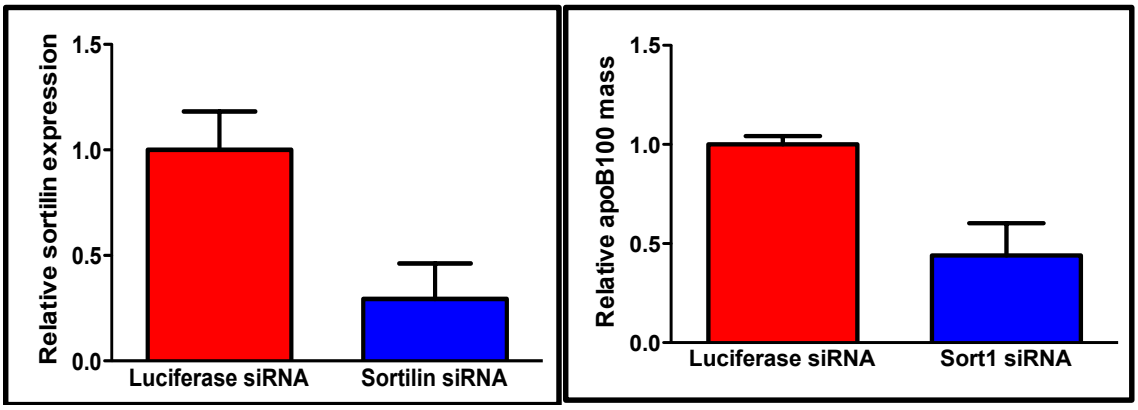
hypothesis is that sortilin may have opposing functions in hepatic and extra-hepatic tissues and that liver-specific deletion of sortilin may have different effects on VLDL secretion than total body sortilin deficiency. The second hypothesis was that at low levels of expression, hepatic sortilin serves as a chaperone for VLDL secretion whereas at higher levels it promotes pre-secretory degradation.

Results:

Sortilin knockdown and deletion in mouse liver decreases VLDL apoB-100 secretion

Our lab has previously shown that sortilin overexpression in mouse liver reduces VLDL secretion[15]. The Rader and Nykjaer labs have also shown that total body *Sort1* deficiency is associated with a paradoxical reduction in VLDL secretion[16,76]. To test whether liver specificity versus total body deficiency is responsible for the paradox, we took a number of approaches to reduce sortilin expression specifically in the liver (the methods are detailed in the Methods section). The first approach I used was siRNAs targeted to murine *Sort1* packaged in a lipidoid particle with well characterized liver specific tropism[80]. Age and sex matched wild-type mice were injected via tail vein with a 2mg/kg mouse specific *Sort1* siRNA or a firefly luciferase siRNA (control) once a week for two weeks (experiment day one and day seven) to achieve maximum *Sort1* knockdown in liver. On day ten, mice were fasted for 4 hours and VLDL secretion was measured by injecting pluronic to inhibit lipolysis (thus all VLDL made and secreted by liver remains in circulation as VLDL) and 35S-methionine/cysteine to label proteins. *Sort1* siRNA reduced hepatic *Sort1* mRNA by 71% ($P = 0.008$, Figure 1A) and decreased

apoB100 secretion by 53% (P value 0.0009, Figure 1B), recapitulating the phenotype of the whole body *Sort1-/-* mouse.



A. B.

Figure 1: siRNA-mediated Sort1 knockdown in liver reduces VLDL ApoB-100 secretion in wildtype mice. A. Quantitative real time PCR for sortilin mRNA was performed on liver normalized to B-actin (71% decrease; n=6 mice per group; P = 0.008). B. Relative VLDL 35S-methionine/cysteine-labeled ApoB-100 normalized to 2 minute plasma counts. (53% decrease; n=6 mice per group; P = 0.0009). This experiment was performed 2 times.

siRNA achieves only transient knockdown of gene expression. siRNA manipulation always carries the risk of off target gene expression changes even after proper screening for off target effects. The risk of off-target effects is compounded by the fact that the siRNAs were packaged in lipid, which has the potential to affect lipid related genes and pathways. We therefore took a second approach to deleting sortilin in the liver; we obtained a *Sort1* conditional knockout mouse in which exon 4 of *Sort1* is flanked by loxP sites, enabling disruption of the endogenous *Sort1* gene upon exposure to cre recombinase (Figure 2).

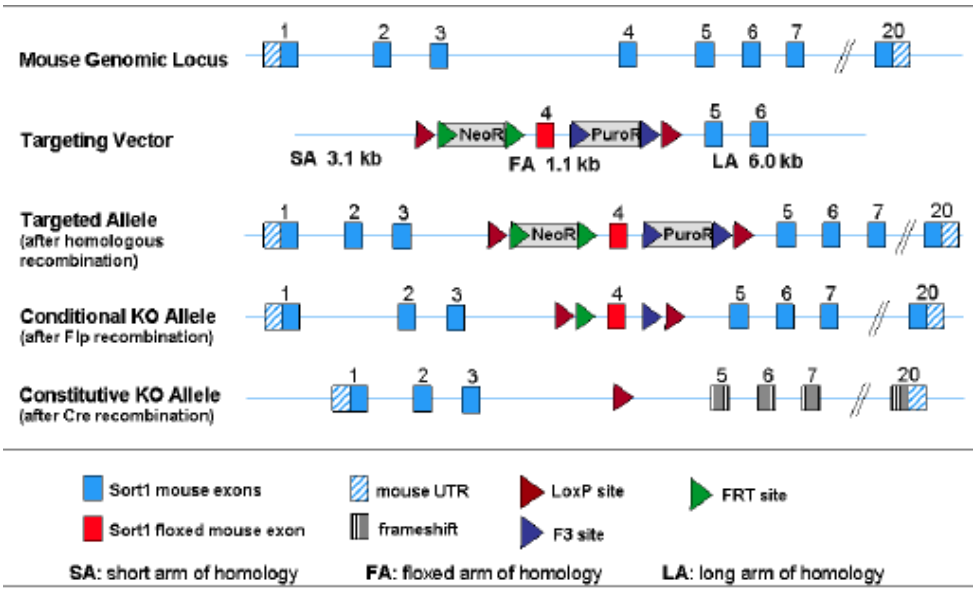


Figure 2: Targeted Allele for *Sort1* fl/fl mouse.

To determine the effect of liver-specific *Sort1* deletion on VLDL secretion, *Sort1 flox/flox* mice (n = 6 mice per group) were injected with 1×10^{12} adeno-associated virus particles expressing Cre recombinase (AAV.Cre) under the control of the liver-specific thyroxine binding globulin (TBG) promoter or null AAV (control). Plasma cholesterol was measured prior to injection, 2, 4, and 6 weeks post injection. Six weeks after AAV injection, *in vivo* apoB-100 secretion studies were performed. AAV.Cre injection reduced liver sortilin protein levels below the threshold of detection (Figure 3a) and no difference in sortilin adipose mRNA expression (Figure 3d), suggesting successful creation of a liver-specific *Sort1* knockout mouse. *Sort1* deficiency in liver did not affect plasma total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides (TG), or non-HDL cholesterol (Figure 3b). Importantly, hepatic-specific deletion of sortilin resulted in a 50% reduction in apoB-100 secretion ($P = 0.005$, Figure 3c). Since our lab has previously shown that sortilin mediates LDL uptake in hepatocytes, we interpret that lack

of change in plasma cholesterol to be a function of a balancing reduction in VLDL production and LDL catabolism.

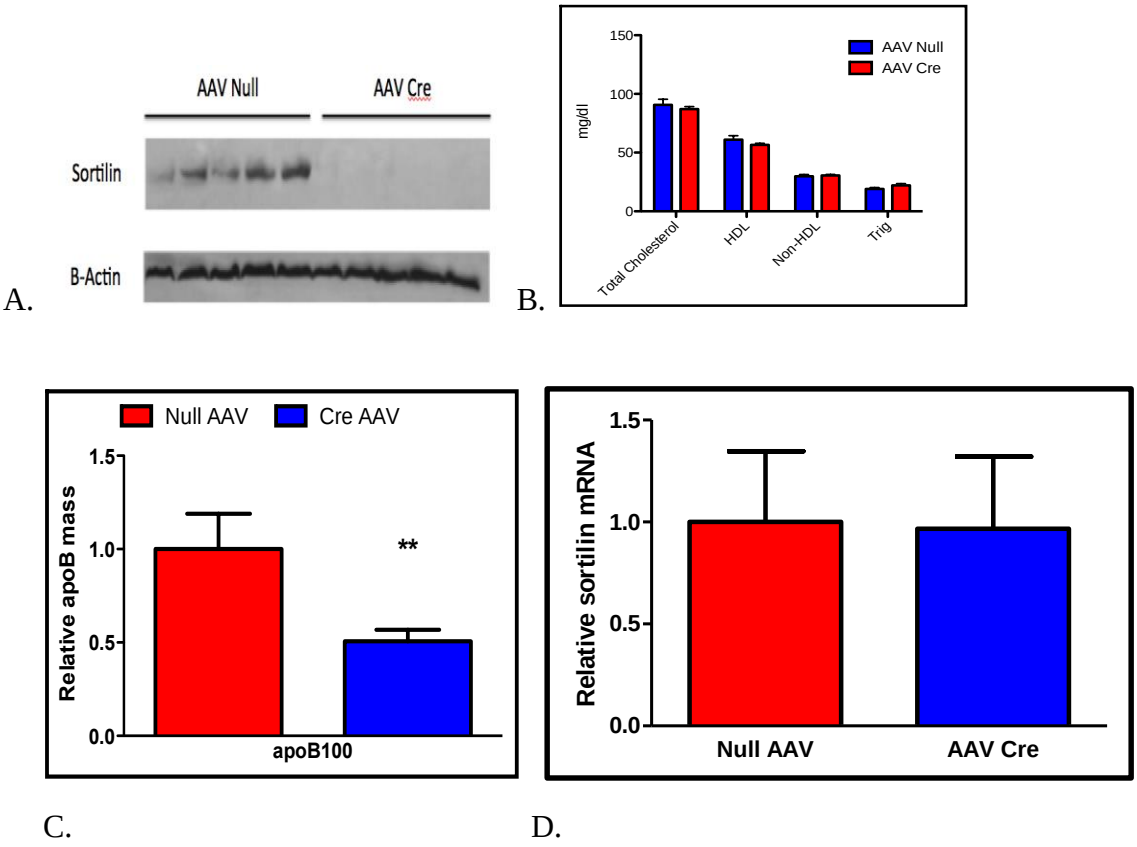
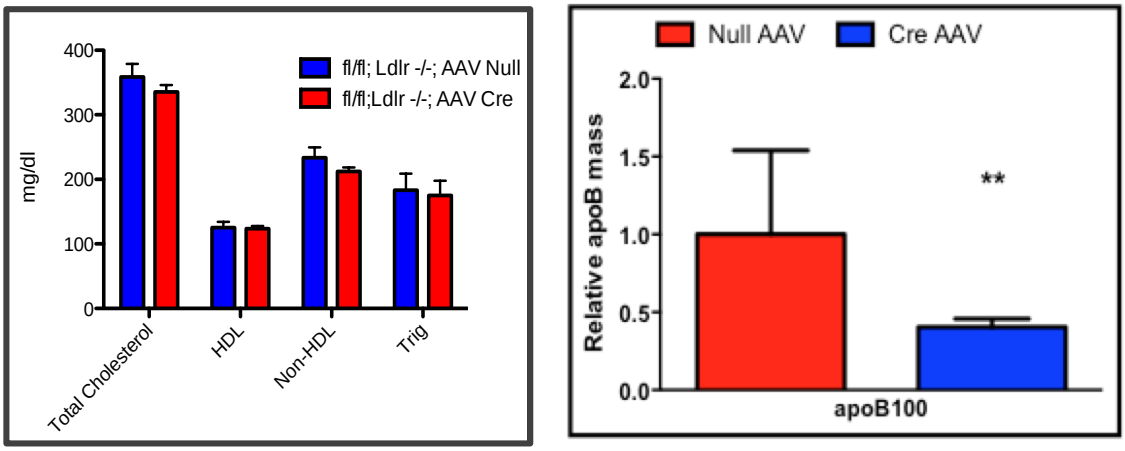


Figure 3: Hepatic-specific deletion of sortilin decreased VLDL apoB-100 secretion. A.Homogenized liver proteins from livers of Sort1 fl/fl mice injected with AAV null or AAV Cre separated by SDS-PAGE and blotted with anti-Sortilin and anti-Bactin antibody. B. Plasma lipids at baseline and after 6 weeks. C. Relative VLDL 35S-methionine/cysteine-labeled ApoB-100 (50% decrease; n=6 mice per group; P = 0.005). D. Relative sortilin adipose RNA expression normalized to Bactin. Experiment was performed 2 times.

The LDL receptor (LDLR) has been reported to influence not only LDL uptake but also VLDL apoB secretion[81]. It is theoretically possible that hepatic sortilin could influence VLDL apoB secretion indirectly by influencing intracellular LDL receptor function. To test the hypothesis that the effect of sortilin deficiency on VLDL apoB-100

secretion is independent of the LDL receptor, *Sort1 fl/fl* mice were bred onto an *Ldlr-/-* background. Sex and age matched *Sort1 fl/fl*; *LDLr-/-* mice were injected with AAV.Cre and null AAV (control) at eight weeks of age (n=6 mice per group) and bled at two, four, and six weeks post injection. VLDL 35S-methionine/cysteine ApoB-100 secretion after pluronic injection was done at six weeks post injection. Liver-specific *Sort1* deficiency on an *LDLr-/-* background did not affect plasma lipids (Figure 4a). However, it was associated with a significant 49% reduction (P value = 0.02) in VLDL apoB-100 secretion (Figure 4b).



A. B.

Figure 4: Hepatic-specific sortilin deficiency on LDLr-/- background reduced VLDL apoB-100 secretion. A. Plasma lipids at baseline and 6 weeks post injection. B. VLDL 35S-methionine/cysteine ApoB-100 secretion where relative apoB 100 mass normalized to 2 minute counts (49% decrease; P = 0.02; n =6 mice per group). Experiment was performed 2 times.

It is possible that there is an extrinsic circulating factor that is affected by hepatic sortilin deficiency and affects VLDL secretion. To ensure that the secretion phenotype is cell-autonomous to hepatocytes, we isolated primary hepatocytes from sortilin deficient

mice and performed metabolic labeling experiments to measure apoB-100 secretion. We used mice on a Apobec-/-; hapoB transgenic background to obtain a more humanized model of apoB secretion; these mice only secrete apoB-100 and not apoB-48 from the liver, similar to humans[82]. We isolated primary hepatocytes from total body *Sort1-/-* mice on a Apobec-/-;hapoB transgenic background and performed an *in vitro* VLDL 35S-methionine/cysteine apoB-100 secretion study. *Sort1* deficiency in primary hepatocytes was associated with a 54% reduction in apoB-100 secretion (Figure 5; P = 0.03). Thus, my data indicate that hepatic *Sort1* deficiency is associated with a significant reduction in VLDL apoB-100 secretion, suggesting sortilin serves a role in VLDL secretion. This suggests that liver specificity may not be responsible for the discrepant findings in *Sort1* overexpression and total body deficiency systems and that the effect of sortilin deficiency in reducing VLDL apoB-100 secretion may be a cell-autonomous effect.

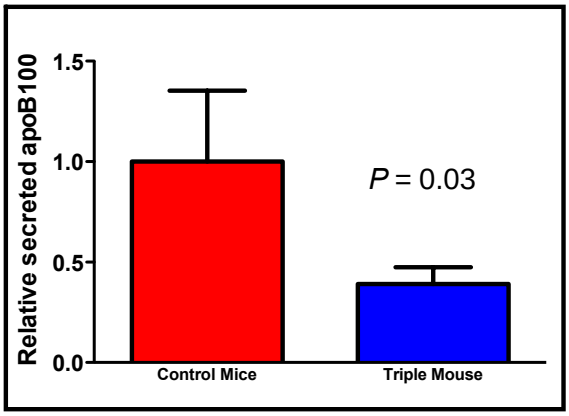
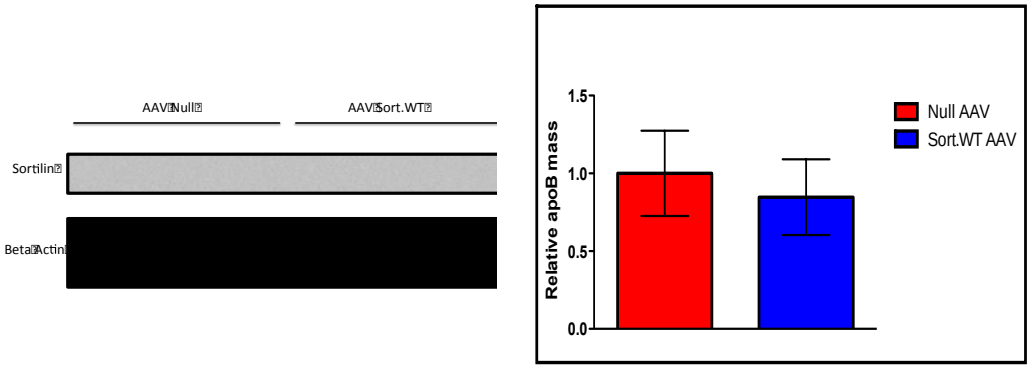


Figure 5: *Sort1* deficiency in primary hepatocytes reduces apoB-100 secretion. Primary hepatocytes from *Apobec1-/-; hAPOB* transgenic (control) and *Sort1-/-; Apobec1-/-; hapoB* transgenic (Triple) mice labeled with 35S-methionine/cysteine, immunuprecipitated apoB 100 normalized to total protein (n =6 wells/ condition; 54% decrease; P = 0.03). Experiment was performed 3 times.

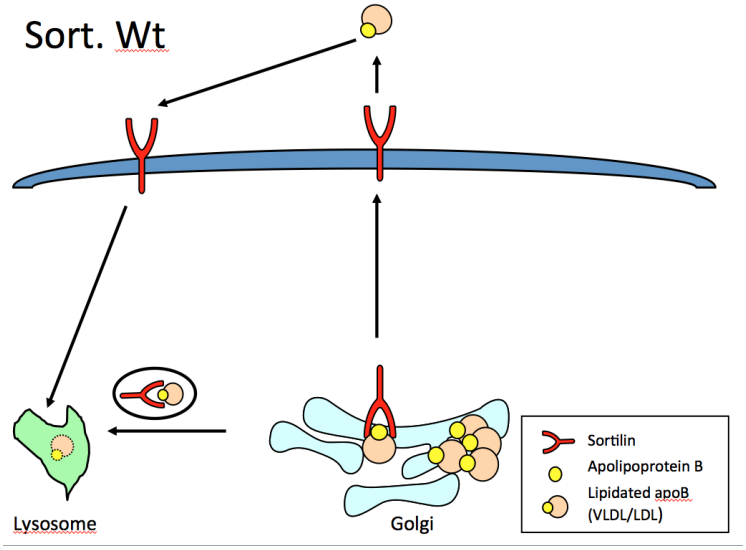
Sort1 serves as a chaperone for VLDL in a cleavage independent manner

The Nykjaer lab previously studied the effect of *Sort1* deficiency on lipid metabolism using an independently generated *Sort1-/-* mouse[16]. They have proposed that sortilin acts as a chaperone for VLDL secretion, namely that sortilin binds presecretory VLDL in the Golgi apparatus and escorts it along the secretory pathway, facilitating its secretion[16]. To test this model, we decided to attempt a reconstitution approach. We injected *Sort1-/-* mice on an *Apobec1-/-; hapoB* transgenic background with 5x10e11 viral particles of wild-type mouse sortilin or null AAV (control, n = 5 mice per group) and determined the effect on VLDL 35S-methionine/cysteine apoB-100 secretion. We successfully achieved hepatic expression—or more likely overexpression—of sortilin in the knockout mice (Figure 6A). Interestingly, hepatic overexpression of *Sort1* in *Sort1-/-* mice had no effect on VLDL apoB-100 secretion (Figure 6B). This result is not consistent with a simple model that sortilin acts as a chaperone to promote VLDL secretion. However, the interpretation is difficult because we are unlikely to have transduced all the hepatocytes in the liver, while at the same time very likely overexpressed sortilin in many hepatocytes. AAV doses low enough to prevent overexpression are not high enough to transduce a majority of hepatocytes. A knockin mouse of sortilin can be made to address this issue. Since sortilin overexpression also reduces VLDL apoB-100 secretion, a net lack of an effect could be the result of reconstitution of expression in some hepatocytes but overexpression in others. The data are broadly consistent with a model in which sortilin is required at some low level to facilitate apoB-100 secretion, but at higher levels of expression it facilitates the endolysosomal degradation of VLDL as seen in individuals

homozygous for the minor allele haplotype at 1p13 and mice overexpressing liver sortilin (Figure 6C).



A. B.



C.

Figure 6: *Sort1* overexpression in *Sort1-/-; Apobec -/-;* human apoB transgenic mice have no net effect on apoB secretion. A. Sortilin immunoblot (n=4 liver lysates/group) B. Pluronic 35S-methionine/cysteine secretion study where relative apoB 100 mass was normalized to 2 minute counts No change seen C. Diagram showing sortilin traffics VLDL from Golgi to lysosome but also LDL from the plasma membrane to the lysosome resulting in no net effect on apoB secretion (n = 5 mice/group.) Experiment was performed 2 times.

Sortilin is known to follow two distinct trafficking routes, from the Golgi apparatus to the endolysosomal system and from the Golgi apparatus to the plasma membrane[28,31]. The Golgi to lysosome trafficking pathway is dominant; with greater than 90% of sortilin shuttled along this pathway[28,31]. The plasma membrane pathway appears to be a less common pathway, with only 10% of sortilin following this route[28,31]. It has been proposed that sortilin can serve as both a chaperone or degrader for its ligands depending on the trafficking route it follows, with the Golgi to plasma membrane pathway promoting ligand secretion and the Golgi to lysosome pathway promoting ligand degradation[33]. It is possible that when AAV is used to express sortilin in the knockout mouse it achieves expression levels so high that it overwhelms the Golgi to plasma membrane chaperone pathway and so most of the overexpressed sortilin follows the pathway to the lysosome and facilitates apoB degradation (Figure 6a). To test this hypothesis, the Golgi to plasma membrane trafficking pathway must be isolated to prevent phenotype masking by sortilin's function in the endolysosomal pathway.

A chaperone function of sortilin for apoB secretion implies that sortilin may directly bind to VLDL. The Rader and Nykjaer labs have each shown that sortilin binds to VLDL by surface plasmon resonance[16,76]. We reasoned that cleaved sortilin might be bound to circulating VLDL and LDL. We performed an immunoblot for sortilin on plasma VLDL and LDL. The experiment indeed showed the presence of sortilin protein on these lipoproteins (Figure 7).

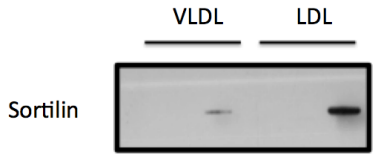
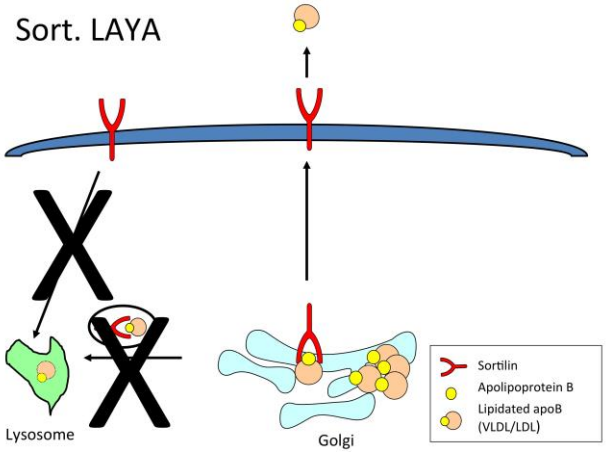


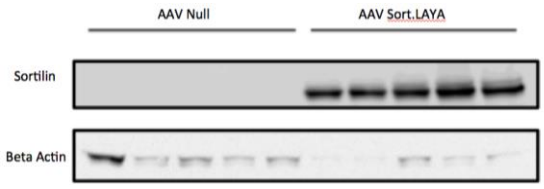
Figure 7: Sortilin is present on VLDL and LDL. Full length sortilin was blotted by western on plasma FPLC fractions of VLDL and LDL pooled from 5 *Sort1-/-;Apobec-/-;*hapoB transgenic mice (lanes 1 and 3) and 5 *Apobec-/-;*hapoB transgenic mice (lanes 2 and 4). Experiment was performed 3 times.

In order to isolate the Golgi to plasma membrane trafficking pathway, we used a sortilin mutant that was incapable of trafficking to the lysosome. Briefly, mutagenesis of the key residues in the dileucine and tyrosine lysosomal sorting motifs to alanine was performed (Y793; L826A/L827A) which we called Sort.LAYA. Previously Sort.LAYA was stably expressed in a HuH7 cell line and shown to localize at the plasma membrane and Golgi (shared with us by cell line creators Quirong Ding and Kiran Musunuru at Harvard). We hypothesized Sort.LAYA overepression would restore the Golgi to cell membrane pathway resulting in an increase in apoB100 secretion (Figure 8a). We injected *Sort1-/-; Apobec1-/-;* hapoB transgenic mice with 5×10^{11} viral particles of null AAV or Sort.LAYA AAV (n=5 per group). After two weeks mice were fasted for four hours for a pluronic 35S-methionine/cysteine apoB-100 secretion study. Mouse livers were harvested for protein analysis. Sort1.LAYA AAV injected mice had sortilin protein present in the liver as seen by immunoblot but no visible sortilin protein was seen in mice injected with null AAV (Figure 8b). Sortilin was present in the plasma of mice expressing Sort1.LAYA (Figure 8c). Importantly, Sort1.LAYA expression was associated with a 135% increase in apoB 100 secretion by P value 0.02 (Figure 8d). Because this mutant is incapable of trafficking to the lysosome, this result suggests that

overexpressed sortilin is capable of facilitating VLDL secretion when it is blocked from trafficking newly synthesized VLDL apoB-100 to the lysosome.



A.



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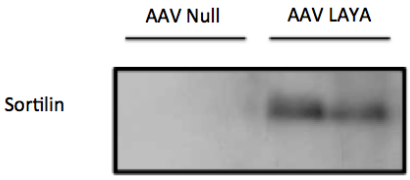

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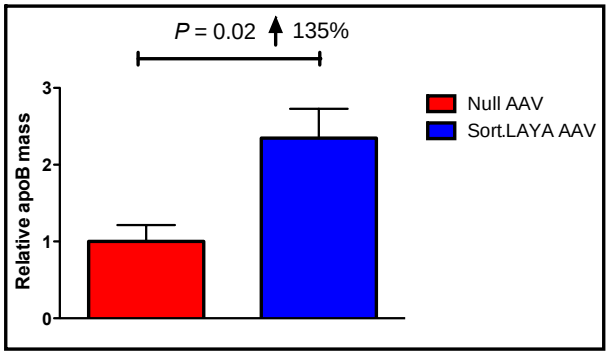


D.

Figure 8: Sort.LAYA increases apoB-100 secretion. A. Diagram showing Sort.LAYA traffics from the Golgi to the plasma membrane; modulating secretion of VLDL. B. Sortilin and Bactin western of livers from mice injected with Null AAV or Sort.LAYA AAV (n=4/group) C. Sortilin western from plasma of mice D. Pluronic 35S-methionine/cysteine secretion study where relative apoB 100 mass was normalized to 2 minute counts (135% increase P value = 0.02 n=6 mice/group). Experiment was performed 2 times

Sort.LAYA restored VLDL secretion in *Sort1-/-* mice; however wild-type sortilin liver specific reconstitution did not, suggesting a complex relationship between sortilin and VLDL secretion. It was recently reported[33] that sortilin serves as both a chaperone and degrader of one of its neuronal ligands proBDNF, depending on its cleavage status. Specifically, the authors show that sortilin is cleaved at the juxtamembrane stalk by the metalloproteinase Adam10 to generate a soluble fragment. Adam10 cleavage occurs intracellularly and at the cell surface. When Adam10 cleaves sortilin, sortilin's ligand binding domain separates from its lysosomal motifs and sortilin and any bound ligands are constitutively secreted. Conversely, when sortilin is not cleaved by Adam10 and remains full length, there is no separation of the ligand binding domain from the lysosomal sorting motifs and sortilin and any bound ligand is trafficked to the endolysosomal system for degradation[33]. One could imagine a similar paradigm for apoB, with full-length sortilin promoting the endolysosomal degradation of VLDL and cleaved sortilin facilitating its secretion.

We tested the hypothesis that sortilin cleavage by Adam10 promotes apoB secretion by using ionomycin, a known activator of Adam10. We first treated McArdle RH-7777 cells stably overexpressing GFP (control) or *Sort1* with vehicle (DMSO) or

ionomycin to determine if it could increase sortilin cleavage into the media. Ionomycin treatment increased sortilin mass in the media (Figure 9a). This suggests that ionomycin directly through Adam10 or indirectly increases sortilin shedding. To determine if the increased sortilin in the media also increases apoB-100 secretion we performed an *in vitro* 35S-methionine/cysteine apoB-100 secretion study. *Sort1* overexpression was associated with a 52% decrease in apoB100 secretion (P = 0.002) (Figure 9b). However, ionomycin treatment to the *Sort1* overexpressing cells was still associated with a 33% decrease in apoB 100 secretion (P = 0.002) (Figure 9c).

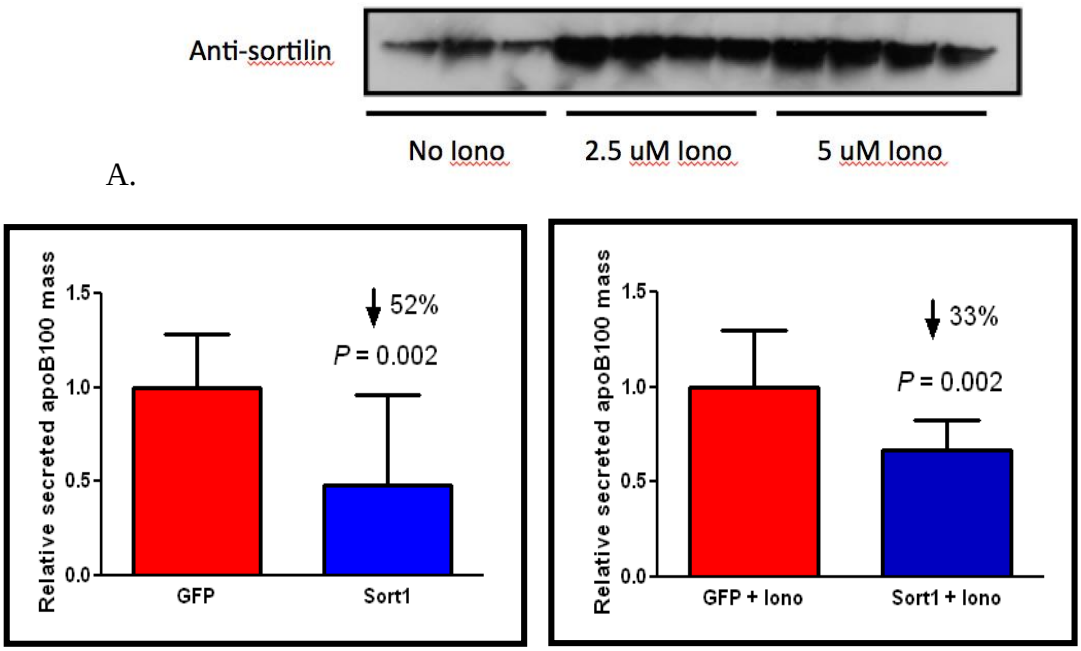
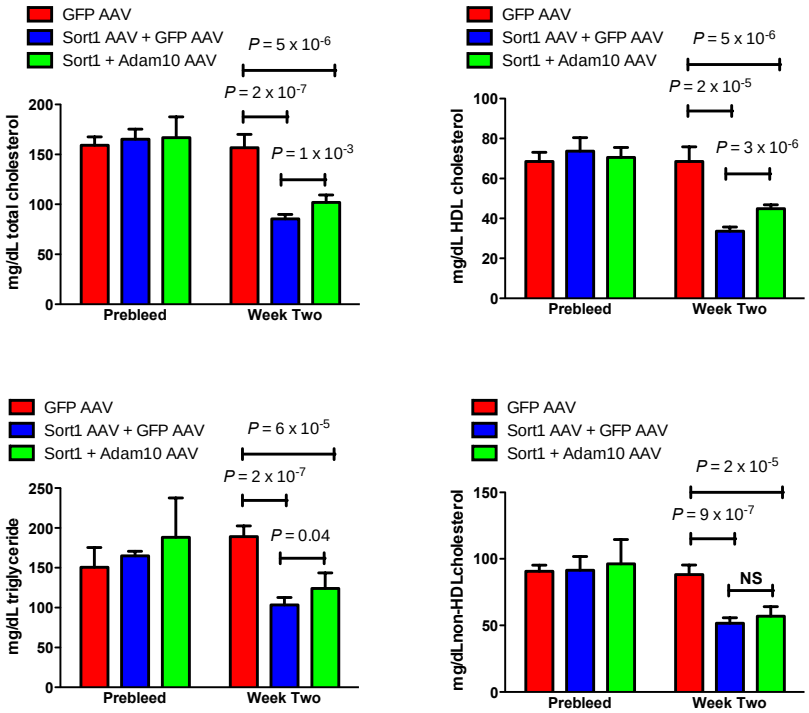
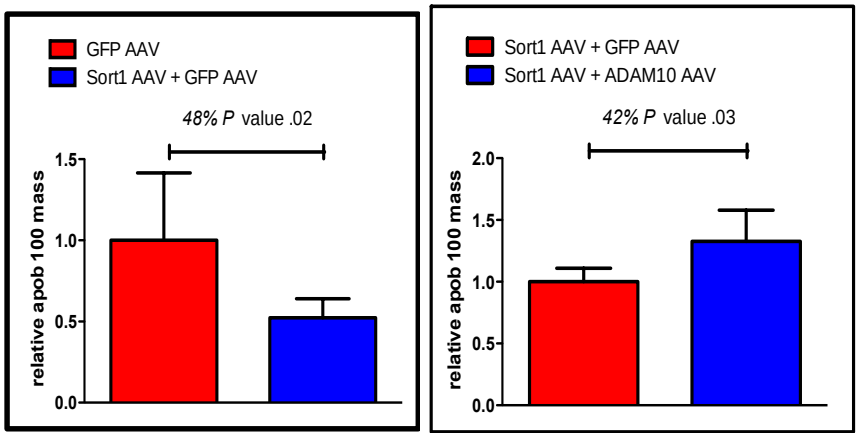


Figure 9A-C: Ionomycin does not increase sortilin mediated apoB 100 secretion. A. Sortilin immunoblot of media from mcArdles cells treated with ionomycin B.-C. Stably overexpressing GFP and *Sort1* mcArdle cells treated with and without ionomycin labeled with 35S-methionine/cysteine, immunuprecipitated apoB 100, and normalized to total protein (n =6 wells/ condition; 52% (P = 0.002) and 33% decrease (P = 0.002) respectively). Experiment was performed 3 times.

Ionomycin may be a nonspecific activator of Adam10. So, we next directly tested the hypothesis that genetic increases in *Adam10* expression will restore apoB100 secretion and therefore cholesterol levels *in vivo*. *Apobec -/-;* hapoB transgenic mice were injected with GFP AAV (1×10^{12} viral particles) or *Sort1* AAV (5×10^{11} viral particles) and GFP AAV (5×10^{11} viral particles) or *Sort1* AAV (5×10^{11} viral particles) and Adam10 AAV (5×10^{11} viral particles). Mice were bled prior to AAV injection and after 2 weeks. *Sort1* overexpression was associated with lower plasma cholesterol levels, but *Adam10* coexpression with *Sort1* did not restore cholesterol levels (Figure 9d). HDL changes because the primary lipid species in mouse is HDL. *Sort1* overexpression reduced apoB 100 secretion 48% P value 0.02, and *Adam10* coexpression with *Sort1* restored apoB 100 secretion 42% P value 0.03 (Figure 9e-f). Importantly, in these experiments we were unable to visualize sortilin in plasma by immunoblot, so it is impossible to determine whether expression of *Adam10* via AAV was sufficient to drive increased sortilin cleavage.



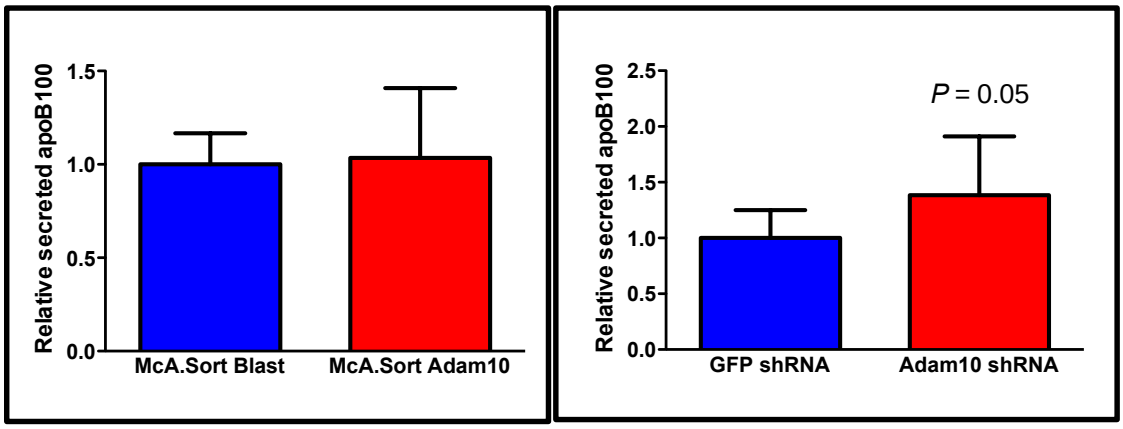
D.



E. F.

Figure 9D-F: Adam10 in vivo overexpression increases VLDL apoB 100 secretion. D. Plasma total cholesterol, HDL, triglycerides, and nonHDL in mice injected with GFP AAV, Sort1 AAV, GFP AAV+ Sort1 AAV, Sort1 AAV +Adam10 AAV 2 weeks post injection E-F. Pluronic 35S-methionine/cysteine secretion study where relative apoB 100 mass was normalized to 2 minute counts

To further test the hypothesis that sortilin cleavage drives VLDL secretion, McArdle cells stably overexpressing *Adam10* were made. *Adam10* overexpression in the context of baseline and increased *Adam10* expression did not increase apoB secretion (Figure 9g). Conversely, we generated *Adam10* knockdown cells using shRNA and also saw no effect on apoB secretion (Figure 9h). Importantly, as was the case for our *in vivo* studies, we were unable to detect sortilin in the media of either cell line making it difficult to conclusively say that there was any effect on sortilin cleavage with manipulation of *Adam10* expression in these cell lines. Given the well-characterized role of Adam10 in receptor cleavage it is likely that cleavage is being affected by *Adam10* expression, which suggests that sortilin cleavage is neither necessary nor sufficient to drive VLDL secretion.



G. H.

Figure 9 G-H: Adam10 overexpression and knockdown has no effect on sortilin mediated apoB 100 secretion. G. Stably overexpressing Blasticidin resistance gene or Adam10 or H. GFP shRNA or ADAM10 shRNA in *Sort1* overexpressing mcArdles labeled with 35S-methionine/cysteine, immunuprecipitated apoB 100, and normalized to

total protein (n =6 wells/ condition no statistically significant difference seen. Experiment was performed 3 times.)

Methods:

In Vivo ApoB 100 Secretion Assay

Mice underwent a four-hour fast to clear chylomicrons from circulation and were then injected intraperitoneally with a 400 uL solution of 1mg/g Pluronic P407-PBS solution to inhibit lipolysis. Fifteen minutes later mice were injected intravenously with 500 uCi of S35-METHIONINE/CYSTEINE to label newly synthesized proteins. Blood was drawn by retro-orbital bleed two minutes after S35-METHIONINE/CYSTEINE injection for normalization and one hour after S35-METHIONINE/CYSTEINE injection for triglyceride measurement and VLDL isolation by ultracentrifugation. Isolated VLDL was run on an SDS-PAGE gel and apoB bands were cut and counted for quantitation of VLDL secretion.

Primary Hepatocyte Isolation

Mice were anesthesized with an intraperitoneal injection of ketamine (70mg/kg) and xylazine (7mg/kg) and then Liver Perfusion Medium (Invitrogen, Carlsbad, CA, USA) followed by Liver Digestion Medium (Invitrogen) were run through the portal vein to remove red blood cells from the liver and to digest extracellular fibrous tissue. Isolated livers were washed with hepatocyte wash medium (Invitrogen), and viable cells were isolated by Percoll gradient centrifugation and plated at a density of 1×10^6 cells per well in 10% FBS and 1% Pen/Strep.

In Vitro apoB 100 secretion assay

Cells were starved for one hour in cysteine and methionine free media and then labeled with S35-METHIONINE/CYSTEINE for three hours. apoB was immunoprecipitated from media and the immunoprecipitate was run on an SDS gel, stained with commassie, and the apoB 100 band was cut and counted. apoB counts were normalized to total cellular protein.

Obtaining DNA for genotyping

Mice were anesthetized with isofluorane and tail tips were cut with razor blades and cauterized. Tails were digested overnight with 180 uL of Buffer ATL and 20uL of Proteinase K (Qiagen, California) at 55 degrees. The Qiagen Symphony (Qiagen, California) was used to extract DNA from the digested tails.

Genotyping whole body Sort1-/- mice

Sort1-/- mice were genotyped using the Expand Long Range, dNTP Pack (Roche, Buckinghamshire, UK). 5 uL of tail DNA was mixed with 5 uL of 5x Long Range Buffer with 12.5 mM $MgCl_2$, 1.5 uL of DMSO, 1.25 uL of PCR nucleotide mix, 9.9 uL of water, 0.35 uL of Expand Long Range enzyme mix and 1 uL of a 10 uM solution of the forward primer (CTCAGGAATGGCATTCTCAG) and 1 uL of a 10 uM solution of the reverse primer (AGCCTTTACCTGGTGTCATC). The PCR cycling conditions were 92 degrees

for 2 minutes, followed by 35 cycles of 92 degrees for 10 seconds, 59 degrees for 15 seconds, and 68 degrees for 6 minutes. There was an additional 7 minute extension cycle at 68 degrees followed by a hold at 4 degrees. PCR products were run on a 1% agarose gel. The wild-type allele produces a 2000 base pair product and the knockout allele produces a 4000 base pair product.

Genotyping Sort1 flox/flox mice

Sort1 fl/fl mice were genotyped using PCR beads (GE Healthcare, Piscataway, New Jersey) and the primers 2899_20 (GAGATGTTAACCTCGCTCAGG) and 2899_30 (CTCTGTCTTAGCTATGTGAACC). We used 1uL of a 10 uM solution of each primer, 18 uL water, and 5 uL of tail DNA. The following PCR cycling conditions were used: 95 degrees for 5 minutes, followed by 35 cycles of 95 degrees for 30 seconds, 60 degrees for 30 seconds, and 72 degrees for 10 minutes. That was followed by a hold at 4 degrees. The PCR reaction for the wild type allele produces a 166-bp band. The PCR for the conditional allele produces a 323 base pair product.

Genotyping LDLr-/- mice

LDLr was genotyped using PCR beads (GE Healthcare, Piscataway, New Jersey) and the primers LDLR1 (AATCCATCTTGTCAATGGCCGATC), LDLR2 (CCATATGCATCCCCAGTCTT) and LDLR3 (GCGATGGATACACTCACTGC). The following PCR cycling conditions were used: 94 degrees for 3 minutes, followed by 35 cycles of 94 degrees for 30 seconds, 65 degrees for 1 minute, and 72 degrees for 1

minute. There was an additional 2 minute extension time followed by a hold at 4 degrees. The PCR reaction for the wild type allele uses primers two and three and produces a 167-bp band. The PCR for the knockout allele uses primers one and two and produces a 350 base pair product.

Adeno-Associated virus injection

Adeno-associated virus (AAV) was prepared by the University of Pennsylvania vector core following standard procedure. AAVs were resuspended in sterile phosphate buffered saline (PBS) to a total volume of 400uL and then injected intraperitoneally into mice.

siRNA preparation

SiRNAs were prepared as a lipidoid formulation designed for liver specific targeting by Alnylam Pharmaceuticals (Cambridge, Massachusetts). The sequences for the *Sort1* duplex were: 5′-uGucAGAAuGGucGAGAcudTsdT-3′ (sense) and 5′-AGUCUCGACcAUUCUGAcAdTsdT-3′ (antisense, 2′-OMe modified nucleotides are in lower case, and phosphorothioate linkages are indicated by "s"). The sequence of the luciferase siRNA duplex is siLuc sense: 5'-CUUACGCUGAGUACUUCGATT-3', antisense: 5'-UCGAAGUACUCAGCGUAAGTT-3'. Formulated siRNAs were diluted in sterile 1x PBS so mice could be injected with 10 uL of siRNA solution per gram of mouse. The total injection volume ranged from 250-270 uL per mouse. SiRNAs were injected via tail vein.

Sacrificing Mice and Harvesting Tissues

Mice were anesthetized by isofluorane inhalation and then a terminal bleed was performed by retro-orbital puncture. Mice were sacrificed by cervical dislocation. Mice were then perfused with 1xPBS to remove red blood cells from the liver and livers were dissected and snap-frozen in liquid nitrogen.

Evaluating Gene Expression

RNA was extracted from mouse liver using Trizol (Invitrogen, Grand Island, NY) and converted to cDNA using the high capacity reverse cDNA kit (Applied Biosystems, Foster City, CA). Murine sortilin was amplified using the Taqman probe Mm_00490905 and murine actin was amplified using the Taqman probe Mm_4352933 (Applied Biosystems, Foster City, California). Real time PCR was performed on an Applied Biosystems 7900HT Real-time PCR system. Sortilin was normalized to actin expression. To evaluate protein expression, livers were homogenized in 1xPBS with protease inhibitor mini tablets (Roche, Indianapolis, Indiana) and 75 ug of protein was loaded onto 10% bis tris gels (Invitrogen, Grand Island, New York) and transferred onto nitrocellulose membranes (Invitrogen, Grand Island, New York). Membranes were probed using rabbit anti sortilin (ab16640) and mouse anti actin (ab8226, Abcam, Cambridge, MA) and mouse anti sortilin (612100, BD Biodesign, Sparks, MD).

Statistics

Statistical analyses were done using 2-tailed paired student's t test for all experiments except one. A 1 way ANOVA followed by a Tukey post-hoc test was used

for the plasma lipids assay of the Adam10 in vivo overexpression experiment. The error bars on graphs are representations of the standard error.

Discussion

Our lab has previously shown that sortilin serves as a bona fide LDL receptor to facilitate the endolysosomal degradation of LDL and also promotes the endolysosomal degradation of the LDL precursor VLDL[76,83]. Interestingly, both *Sort1* overexpression and deficiency are associated with reduced VLDL secretion. Though extensively studied, this paradox remains unexplained[16,76]. *Sort1* is expressed in multiple tissues, and while the overexpression studies involved liver specific manipulations, all deficiency studies were done in mice deficient in sortilin in all tissues, suggesting that tissue specificity could be responsible for the disparate findings in overexpression and deficiency systems. We have shown that tissue specificity may not be responsible for the paradoxical secretion phenotype of the whole body *Sort1-/-* mouse by use of liver specific *Sort1* knockdown using siRNA, liver-specific *Sort1-/-* mice generated through use of the Cre recombinase system, and *Sort1-/-* primary hepatocytes. Liver specific *Sort1* deficiency recapitulates the secretion phenotype of the *Sort1* total body knockout mouse and is also associated with reduced VLDL secretion.

It has been suggested that sortilin serves as a chaperone for VLDL. Liver specific reconstitution of *Sort1-/-* mice with wild-type *Sort1* using AAV does not restore VLDL secretion, suggesting that sortilin does not serve as a pure chaperone for VLDL. AAV is a

powerful research tool to achieve somatic overexpression of genes in mouse liver; however, due to transduction efficiency constraints it cannot be used to effectively reconstitute gene expression, it enables robust overexpression of genes[91]. Sortilin is known to traffic both to the plasma membrane as well as to the endolysosomal system, and the amount of sortilin at the cell surface is regulated with most sortilin remaining intracellular. If sortilin were to serve as a trafficking receptor to facilitate VLDL export it would depend on a dominant Golgi to plasma membrane trafficking pathway. To isolate sortilin's plasma membrane pathway and eliminate confounder effects from sortilin's Golgi to lysosome pathway we used AAV encoding Sort.LAYA, a sortilin mutant defective in its ability to traffic to the endolysosomal system that is instead shuttled to the cell surface. Expression of Sort.LAYA restores VLDL secretion in *Sort1-/-* mice, suggesting that sortilin can serve as a chaperone for VLDL, but the lack of rescue with wild-type *Sort1* suggests a complexity to sortilin's role in VLDL secretion.

Consistent with the hypothesis that sortilin can serve as both a chaperone and degrader of its ligands, Evans et al reported that sortilin facilitates the secretion as well as the lysosomal targeting and degradation of proBDNF [33]. Specifically, Evans et al identified Adam10 as the metalloproteinase that cleaves sortilin at the juxtamembrane stalk both intracellularly and at the plasma membrane, separating sortilin's ligand binding domain from its lysosomal sorting motifs. They further suggested that proBDNF bound to full length sortilin that is not cleaved by Adam10 is trafficked with sortilin to the lysosome for degradation, whereas proBDNF bound to cleaved sortilin is secreted from cells[6]. One can envision a similar paradigm for apoB-containing lipoproteins: under

physiological conditions the majority of sortilin is cleaved by Adam10 and facilitates VLDL secretion, so loss of sortilin reduces VLDL secretion, whereas in the context of increased *Sort1* expression, Adam10 is limiting, and most sortilin remains full length and facilitates the endolysosomal degradation of VLDL. This cleavage pathway may explain the reduction in VLDL secretion seen with both *Sort1* overexpression and deficiency. Our data indicates Adam10 mediated sortilin cleavage is neither necessary nor sufficient for sortilin mediated VLDL chaperoning. This may be due to the different tissues in which the studies were performed (neuronal tissue versus liver) or the ligands intetrogated (proBDNF versus apoB).

Sortilin cleavage therefore cannot explain why reductions in *Sort1* expression in different genetic systems can lead to both increased and decreased VLDL secretion. The answer may lie in recent advances in understanding sortilin trafficking and regulation. It has been shown that 10% of sortilin localizes to the plasma membrane while 90% is intracellular; however, Kim et al found that this ratio can be altered by neurotrophin receptor homolog 2 (Nrh2)[31]. Nrh2 is a molecular switch that promotes the trafficking of sortilin to the cell surface[31]. It is possible that Nrh2 is the factor that drives the secretion phenotype, diverting sortilin from the endolysosomal pathway to the cell surface. Further studies will have to be done to address this hypothesis.

In summary, through liver specific *Sort1* deficiency models as well as reconstitution and mutagenesis studies our findings indicate that liver specificity is not responsible for the secretion paradox and instead demonstrate that sortilin plays a dual role in VLDL trafficking, serving as both a chaperone and degrader of VLDL. Facilitated

secretion is dependent on intact Golgi to cell surface trafficking and cleavage alone is insufficient to restore function.

Chapter Four: Macrophage sortilin promotes LDL uptake, foam cell formation, and atherosclerosis

Patel KM, Strong A, Tohyama J, Jin X, Morales CR, Billheimer J, Millar JS, Kruth H, Rader DJ. Macrophage Sortilin Promotes LDL Uptake, Foam Cell Formation, and Atherosclerosis Circ Res. 2015 Jan 15. pii: CIRCRESAHA.114.305811.

Introduction:

A central hallmark of atherosclerosis is the cholesterol-loaded macrophage or 'foam cell.' Despite decades of research, the molecular mechanisms by which arterial macrophages take up cholesterol-rich lipoproteins, such as low density lipoproteins (LDL), leading to the development of foam cells and atherosclerotic lesions remain to be fully elucidated. Kruth and colleagues have shown that macrophages internalize native LDL through a process of macropinocytosis, although LDL uptake cannot be fully accounted for by this process[71]. Gene deletion of known receptors of modified LDL, such as scavenger receptor A (SRA) and CD36, do not reduce foam cell formation or the development of atherosclerosis in mice[52]. Thus, pathways that mediate macrophage uptake of LDL leading to foam cell formation and atherosclerosis remain of substantial interest.

Unbiased genome-wide association studies (GWAS) of coronary artery disease (CAD) have the potential to identify new pathways involved in atherosclerosis. In one of the first GWAS for CAD, non-coding genetic variants at chromosome 1p13 were reported to be significantly associated with myocardial infarction and CAD[17], a finding that has been widely replicated[11,12]. The same variants have also shown to be significantly associated with plasma levels of LDL cholesterol[8,15,84]. The *SORT1* gene, encoding the protein sortilin, appears to be the causal gene at the locus regulating LDL cholesterol levels[16,76,83]. Sortilin is a type I transmembrane trafficking receptor initially characterized by its ability to serve as a receptor for proneurotrophins[16,76,83] and for its role as a sorting receptor for lysosomal hydrolases[48,85]. Hepatic sortilin expression

modulates VLDL production rates[7, 9 10]; in addition, hepatic sortilin binds LDL and promotes its cellular uptake and lysosomal degradation[76]. Sortilin is also expressed in macrophages, but little is known about its function in this cell type or its relationship to atherosclerosis[86,87]. I hypothesized that macrophage sortilin mediates macrophage LDL uptake. Through a combination of *in vivo* mouse studies and *ex vivo* macrophage studies utilizing *Sort1-/-* macrophages, I show here that macrophage sortilin promotes macrophage LDL uptake, foam cell formation, and atherosclerosis independent of plasma LDL-C levels.

Results

Sortilin deficiency in hematopoetic cells protects against atherosclerosis

Total body *Sort1* deficiency on an *LDLr-/-* background is associated with reduced plasma cholesterol levels, confounding attempts to address its role in atherosclerosis independent of LDL-C levels. We crossed *Sort1-/-* mice onto the background of an atherosclerosis-prone *Apobec1-/-; hAPOB* Tg mouse model, which has a human-like lipoprotein profile, and fed the mice a western type diet for 18 weeks. On this genetic background, total and LDL cholesterol levels were not different in *Sort1-/-* mice compared with *Sort1+/+* mice (Figure 10a,b). This is because liver sortilin serves as a LDL receptor and degrader of VLDL.

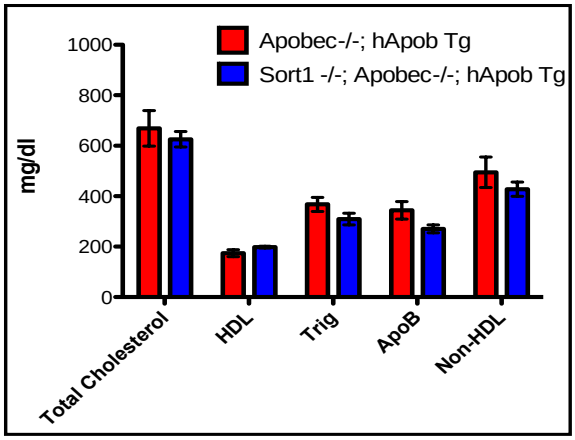


Figure 10A: Biochemical measurements of plasma lipids of Apobec1-/-; hapoB transgenic mice on western diet. Total Cholesterol, HDL, Triglycerides, and human ApoB were measured by MIRA autoanalyzer and Non-HDL was calculated.

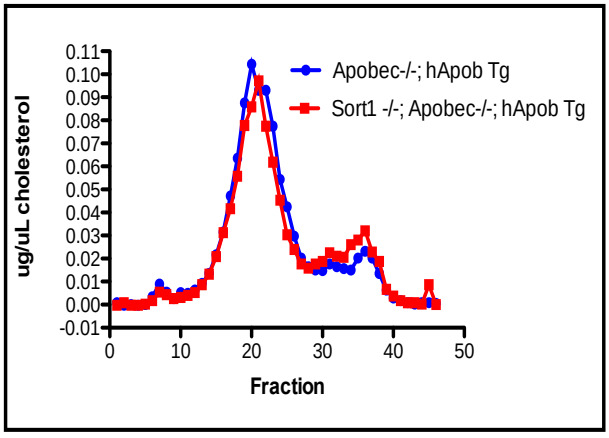


Figure 10B: FPLC profile of pooled plasma. Equal amounts of plasma from each mouse was pooled and run on an FPLC. A cholesterol plate assay was performed on each of the 46 samples from the FPLC and the results are graphed.

After 18 weeks on diet, *Sort1-/-* mice had a 68% reduction in en face aorta lesion area (P <0.0001 Figure 10c,d) and an 87% reduction in aortic root lesion area (P <0.0001 Figure 10e,f) compared with *Sort1+/+* mice, demonstrating a major effect of sortilin deficiency in reducing atherosclerosis despite no effect on plasma cholesterol in this model.

Figure 10C: Oil Red O stained aortas. Mice were placed on western diet for 18 weeks. Aortas were harvested from mice and fixed in 4% paraformaldehyde.

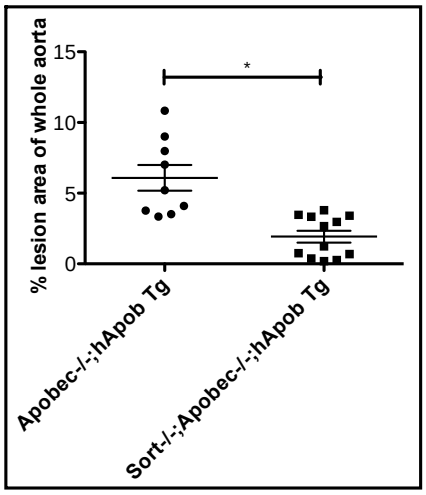


Figure 10D: Quantification of whole aorta. Oil Red O stained area was quantified relative to total surface area.

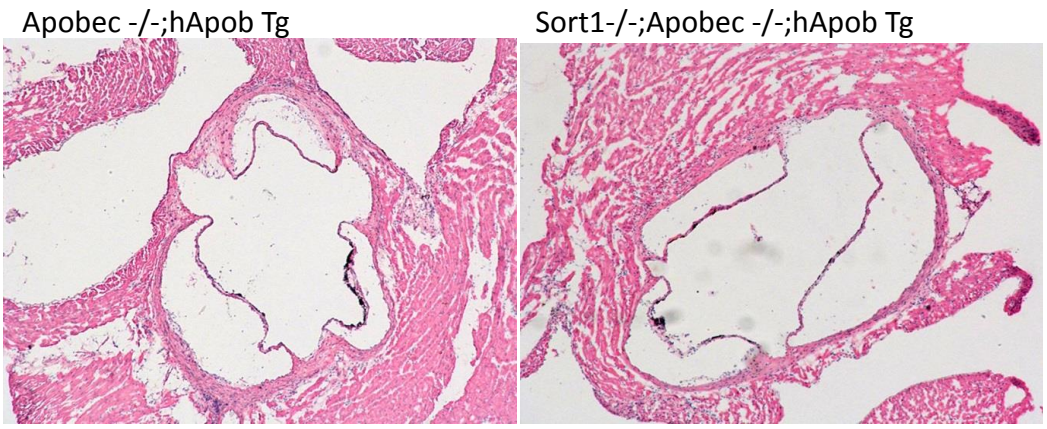


Figure 10E: Aortic roots cross sections. Lesion area was measured over the hematoxylin and eosin stained sections prepared from paraffin embedded hearts.

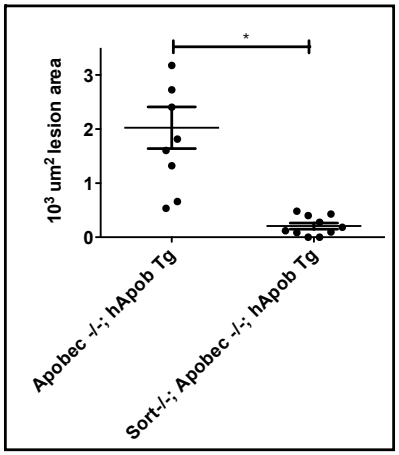


Figure 10F: Quantification of aortic root cross sections. Lesion area was quantified on 3 sections for each mouse.

Macrophages express sortilin and we hypothesized that macrophage sortilin deficiency might account specifically for the reduced atherosclerosis. In order to test this hypothesis, irradiated *LDLr-/-* mice were transplanted with bone marrow from *Sort1-/-;LDLr-/-* mice or *Sort1+/+;LDLr-/-* mice and 6 weeks after transplantation were started on a western type diet and fed for 18 weeks. Bone marrow engraftment was 74% (Figure 11i). Body, liver and spleen weights, plasma cholesterol, peripheral blood counts, and hepatic *Sort1* expression were similar between groups (Figure 11).

Figure 11

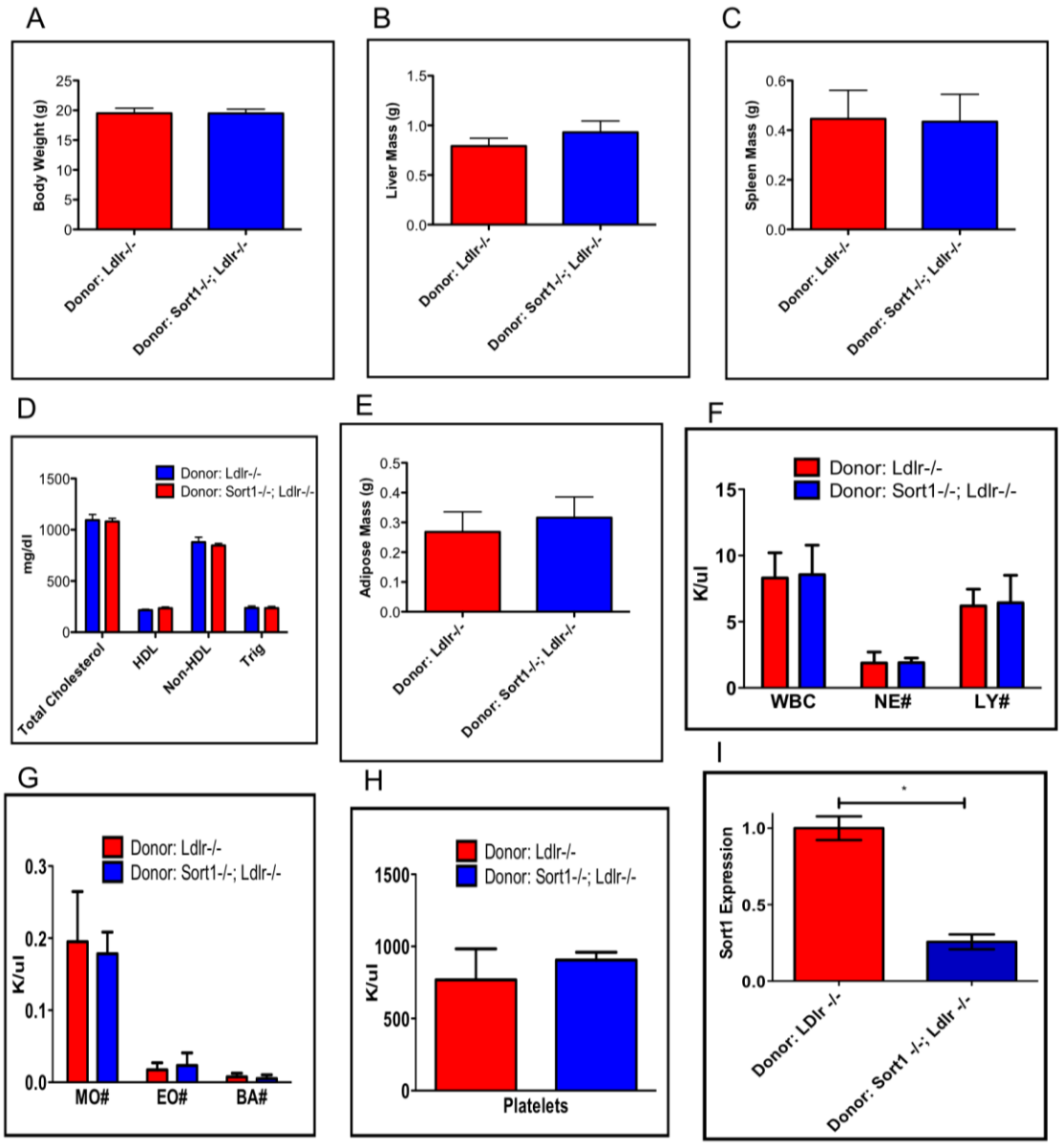


Figure 11: Characterization of recipient LDLr-/- mice carrying donor LDLr-/- or Sort1-/-;LDLr-/- bone marrow. A. *Sort1* deficiency in bone marrow has no effect on body weight. **B.** *Sort1* deficiency in bone marrow has no effect on Liver, **C.** Spleen, or **D.** Adipose Mass. **E.** Sortilin deficiency in bone marrow has no effect on plasma lipids. **F.** *Sort1* deficiency in bone marrow has no effect on white blood cells (WBC), neutrophils (NE#), lymphocytes (LY#), **G.** monocytes **(**MO#), eosinophils (EO#), basophils (BA#). **H.** *Sort1* deficiency in bone marrow has no effect on platelet number. **I.** Bone marrow engraftment was 74% as measured by *Sort1* mRNA expression in spleen normalized to Bactin. N=11 per group P value <0.01

Mice transplanted with *Sort1-/-;LDLr-/-* bone marrow had a 69% reduction in en face aortic lesion area ($P<0.00001$) and a 34% reduction in aortic root lesion area ($P < 0.01$) compared to mice transplanted with *Sort1+/+;LDLr-/-* bone marrow (Figure 12a-d), suggesting that hematopoietic, and potentially macrophage sortilin influences the development of atherosclerotic disease.

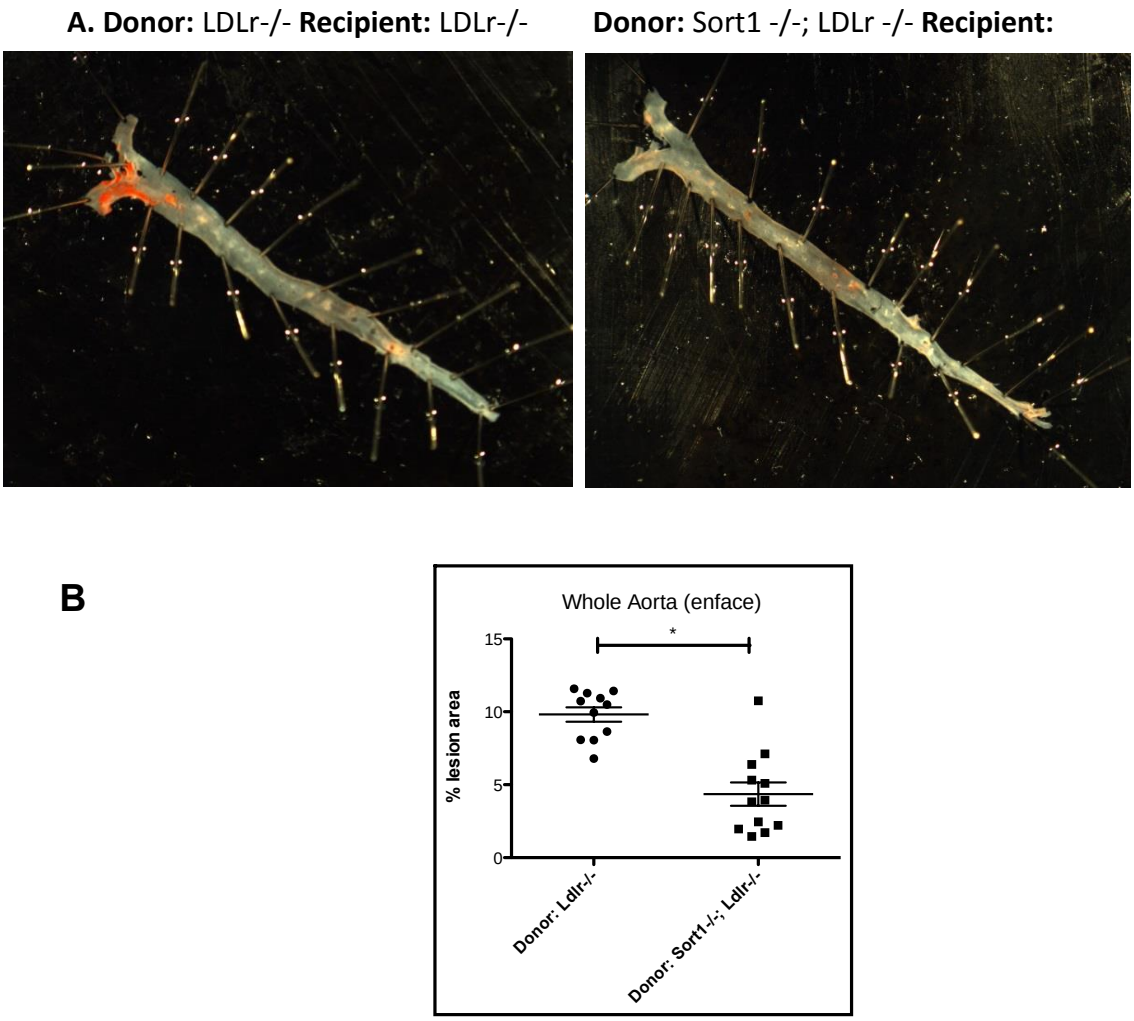


Figure 12 A-B: Whole aorta atherosclerosis of bone marrow transplanted mice. Aortas from n=11 mice per group were isolated, stained, and quantified as previously described.

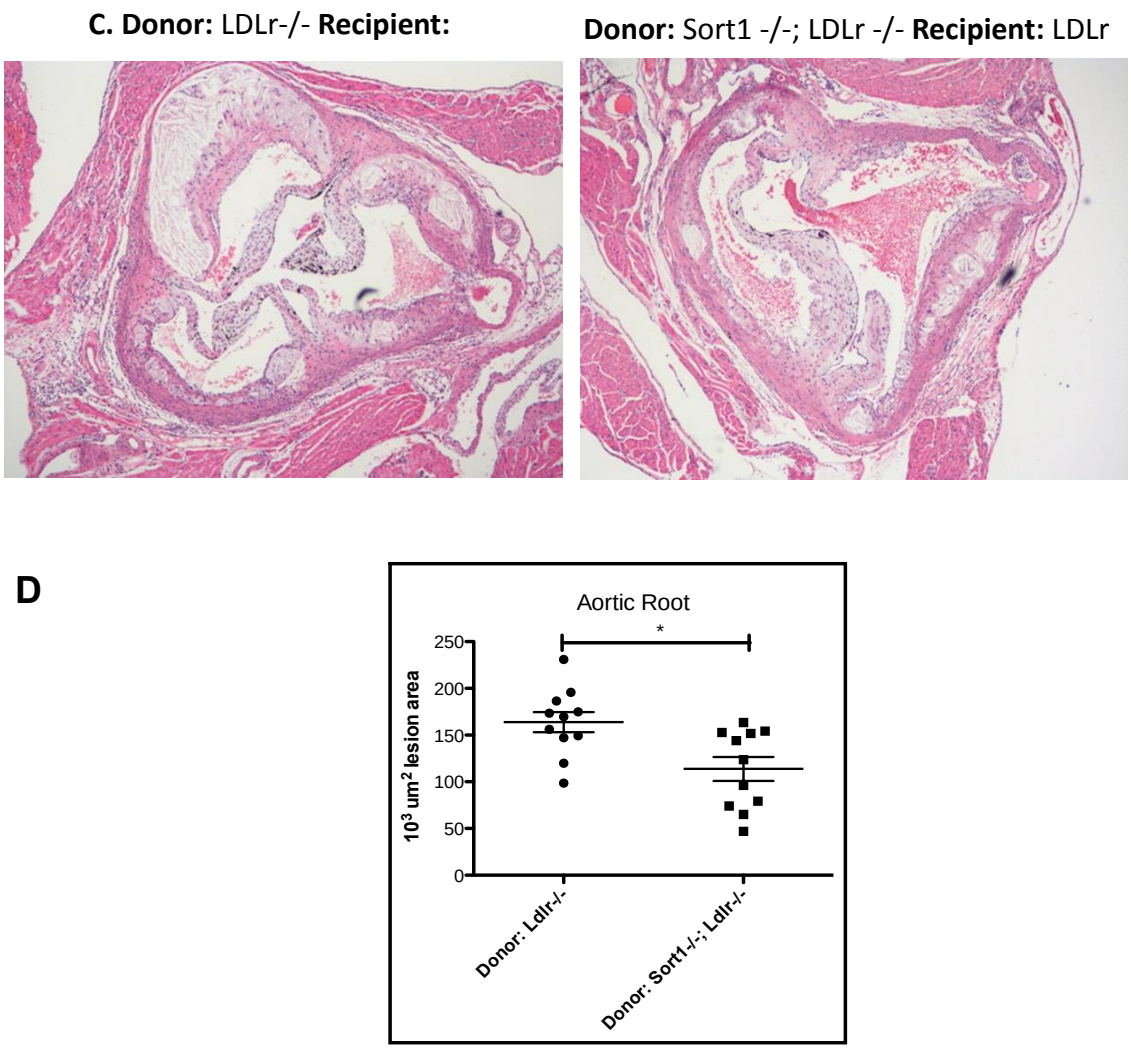


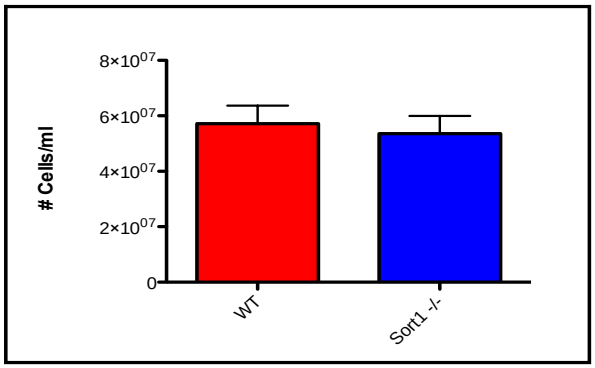
Figure 12 C-D: Aortic root atherosclerosis of bone marrow transplanted mice. Hearts (n=11 per group) were prepped, sectioned, and stained as previously described.

Sortilin deficiency has no effect on thioglycollate-elicited monocyte recruitment or LPS-induced inflammatory response in vivo

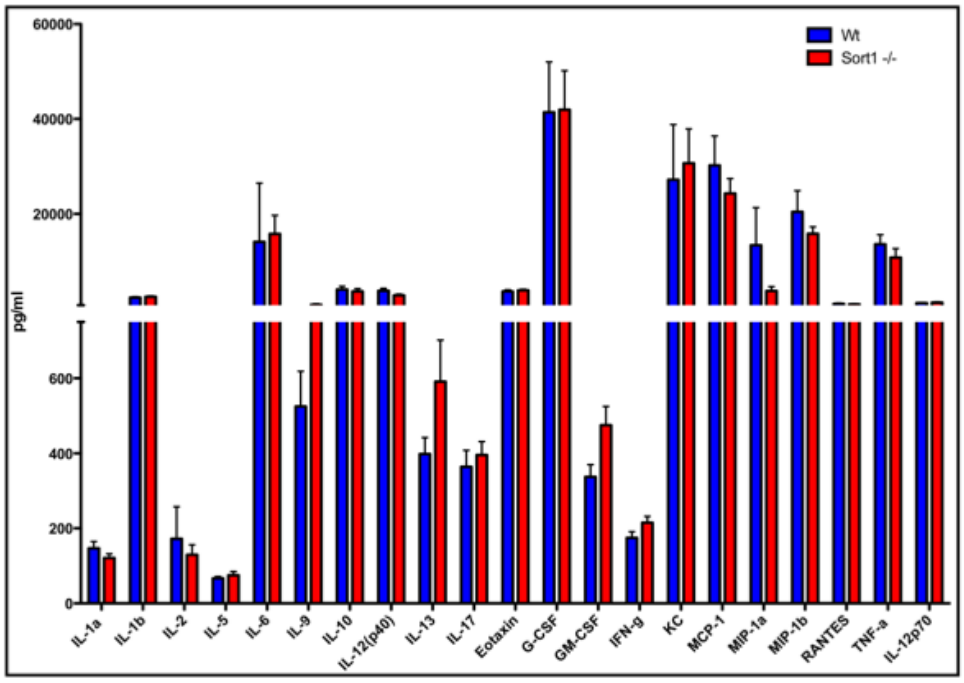
Monocyte recruitment is a key determinant of the macrophage content of atherosclerotic lesions. To determine if *Sort1* deficiency affects macrophage recruitment, *Sort1-/-* and *Sort1*+/+ mice were injected i.p. with thioglycollate to elicit an inflammatory

response. Three days after injection, peritoneal macrophages were harvested and counted. There was no difference in macrophage counts between *Sort1*+/+ and *Sort* -/- mice (Figure 13a). Other methods to assay macrophage recruitment into plaques can be very helpful. Monocyte recruitment and atherosclerosis development is strongly influenced by inflammation and cytokine production. To determine if *Sort1*-/- mice have reduced cytokine levels, cytokine multiplexing assays were performed on *Sort1*-/- and *Sort1*+/+ mice injected with lipopolysaccharide (LPS). Cytokine levels post LPS injection was found to be similar between *Sort1*-/- and *Sort1* +/+ (Figure 13b,c).

A



B



C

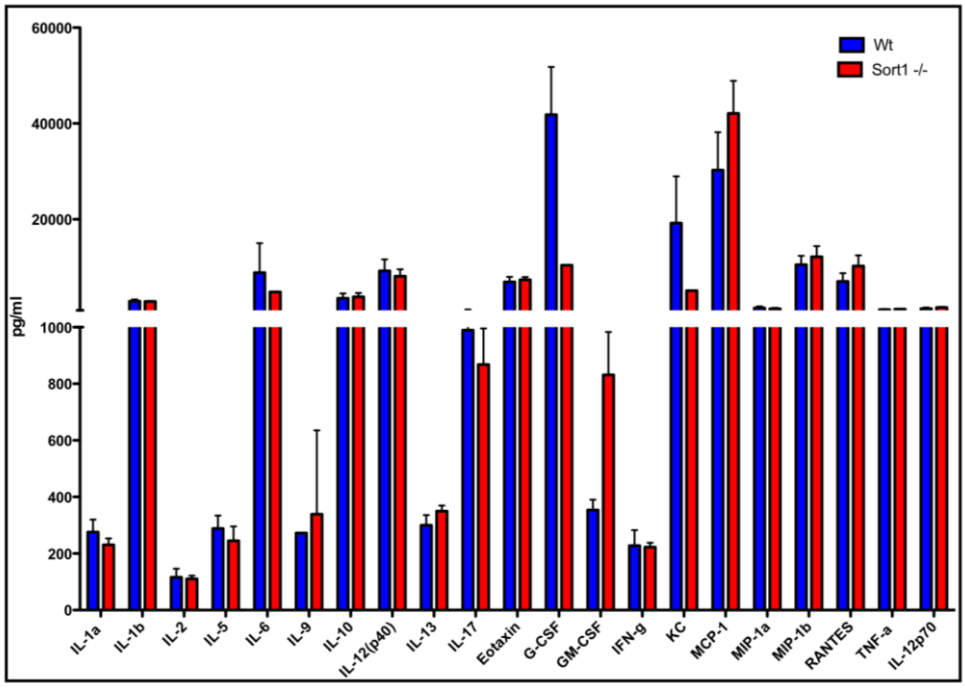


Figure 13: Sortilin deficiency has no effect on macrophage recruitment or modulation of inflammation. A. Thioglycollate (3%w/v) was injected into peritoneal cavity and after 4 days peritoneal macrophages were counted from Sort1 +/+ and Sort -/- mice (n=10) **B.** Serum cytokine levels were measured 2 hours and **C.** 5 hours post lipopolysaccharide injection in Sort1 +/+ and Sort -/- mice (n=10 per group)

Macrophage sortilin deficiency reduces LDL uptake and foam cell formation

To determine if macrophage sortilin deficiency reduces foam cell formation, primary bone marrow macrophages were isolated from *Sort1-/-; LDLr-/-* and control *Sort1+/+; LDLr-/-* mice, cells were differentiated with M-CSF for 7 days, incubated with 1 mg/mL LDL for 5 hours, and Oil Red O staining was performed. *Sort1-/-;LDLr-/-* macrophages had a clear and consistent reduction in Oil Red O staining (Figure 14a).

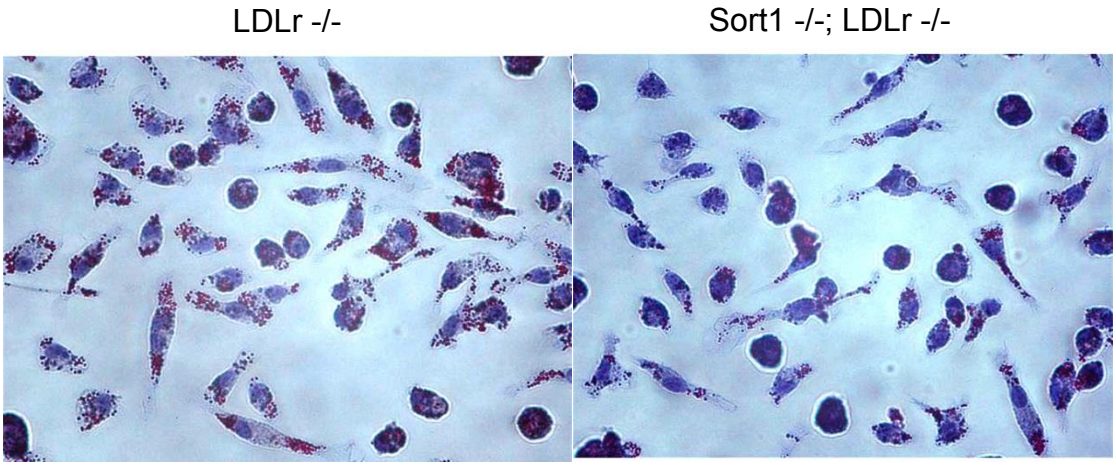


Figure 14A: Oil Red O staining of M-CSF differentiated macrophages. Macrophages were fixed in 4% paraformaldehyde then stained with haematoxylin (blue) and Oil Red O (red). (n=4 wells/group. Experiment was performed 2 times)

Sort1 deficient macrophages had a significant 28% reduction in total cellular cholesterol, a 25% reduction in free cholesterol, and a 32% reduction in cholesteryl ester ($P<0.05$; Figure 14b-d).

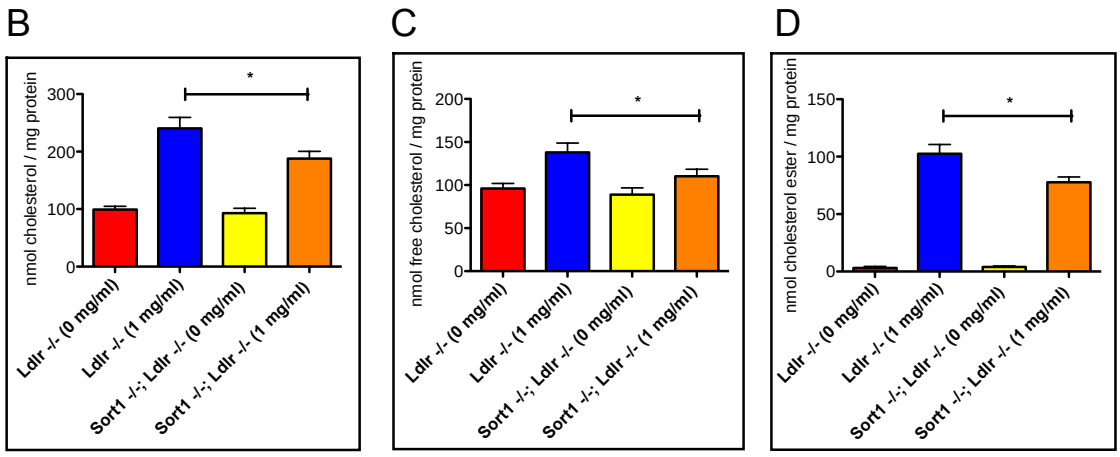


Figure 14 B-D: Cholesterol measurements of M-CSF differentiated macrophages. Macrophages were dissolved in isopropanol and free cholesterol and cholesterol ester were measured by gas chromatography/ mass spectrometry (GC/MS). Total cholesterol was calculated as sum of free cholesterol plus cholesterol ester. (n=3 wells/ group. Experiment was performed 2 times.

In vivo foam cell formation assays were performed by isolating thioglycollate-elicited peritoneal macrophages from *Sort1+/+;Apobec-/-;hApob Tg* and *Sort1-/-;Apobec-/-;hApob Tg* mice fed a western type diet for 18 weeks. Consistent with the *in vitro* loading experiments, macrophages isolated from *Sort1-/-* mice had reduced Oil Red O staining and a significant 33% reduction in cellular cholesterol content compared to macrophages isolated from *Sort1+/+* mice ($P<0.05$; Figure 14e-f). These studies indicated that sortilin-deficient macrophages have reduced capacity to form foam cells when exposed to high levels of LDL.

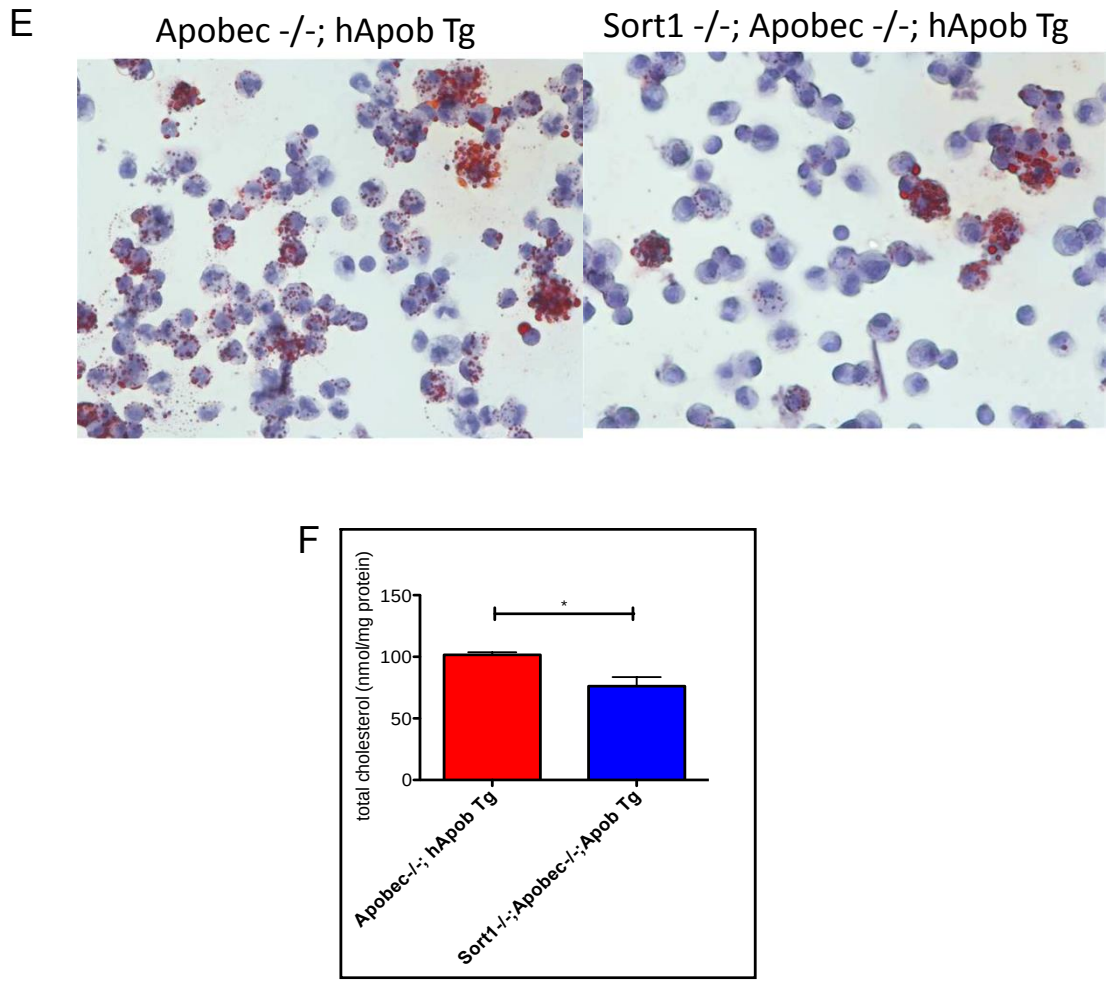


Figure 14 E-F: Oil Red O staining and cholesterol measurements of thioglycollate elicited peritoneal macrophages. Mice were placed on a western diet for 18 weeks. Thioglycollate was injected intraperitoneally and peritoneal macrophages were isolated 4 days thereafter. Oil Red O staining and cholesterol measurements were performed as previously described. (n=3 mice/group. Experiment was performed 2 times.)

As sortilin can act as a receptor for LDL in hepatocytes, we hypothesized that sortilin promotes the internalization of LDL by macrophages. To test the response of sortilin expression to increasing cholesterol concentration in macrophages, thioglycollate-elicited peritoneal macrophages were isolated from wild-type mice and incubated for 24 hours in lipoprotein-deficient serum, lipoprotein-deficient serum supplemented with 25-

hydroxycholesterol to reduce intracellular cholesterol content, or with lipoprotein deficient serum supplemented with high concentrations of LDL. In contrast to the LDL receptor, whose expression was reduced by co-incubation with LDL, *Sort1* mRNA abundance increased over 400-fold with LDL incubation ($P < 0.05$; Figure 15a) and sortilin protein also increased significantly with LDL incubation (Figure 15b).

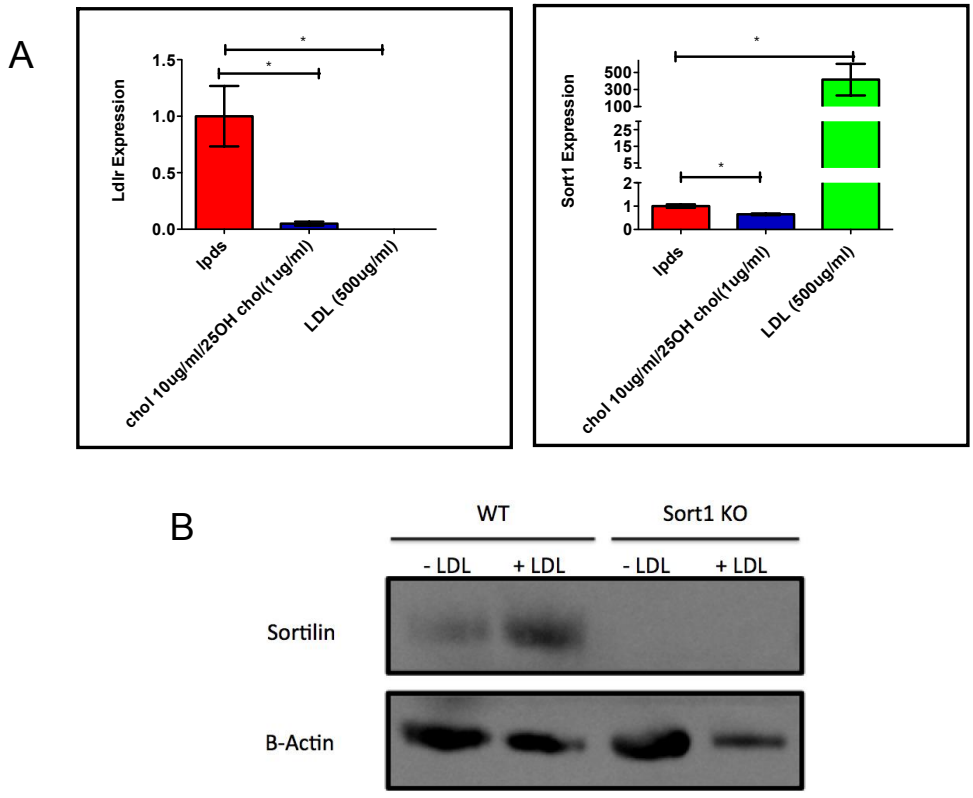


Figure 15 A-B: Sort1 and LDLr Expression in thio-elicited macrophages. A. After plating macrophages for 2 hours they were treated with conditions stated above for 24 hours **B.** Macrophages were treated with 500ug/ml LDL in LPDS media or LPDS media (control). (n=4 mice/group Experiment was replicated 2 times.)

To test the hypothesis that sortilin is able to promote the uptake of LDL into macrophages, ^{125}I-LDL uptake studies were performed in thioglycollate-elicited and bone marrow derived macrophages from *Sort1*+/+ and *Sort1*-/- mice. *Sort1* deficiency was

associated with a 48% and 33% percent reduction in LDL uptake, respectively (P <0.05 for both; Figure 15 c-d).

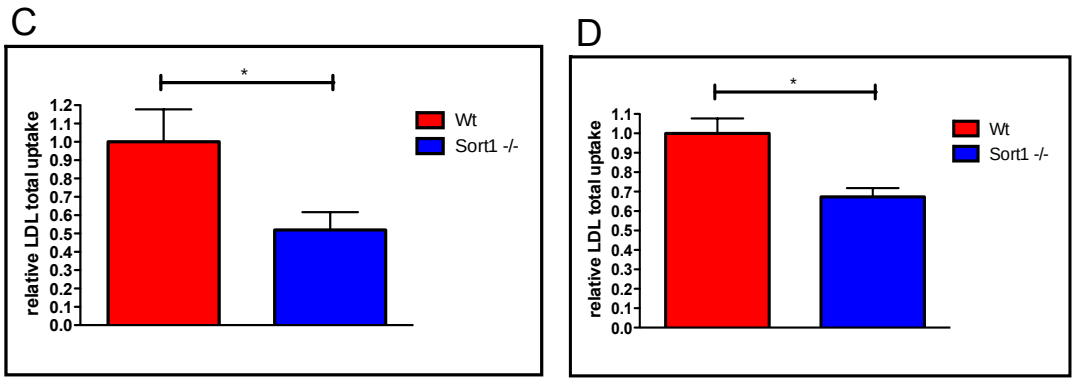


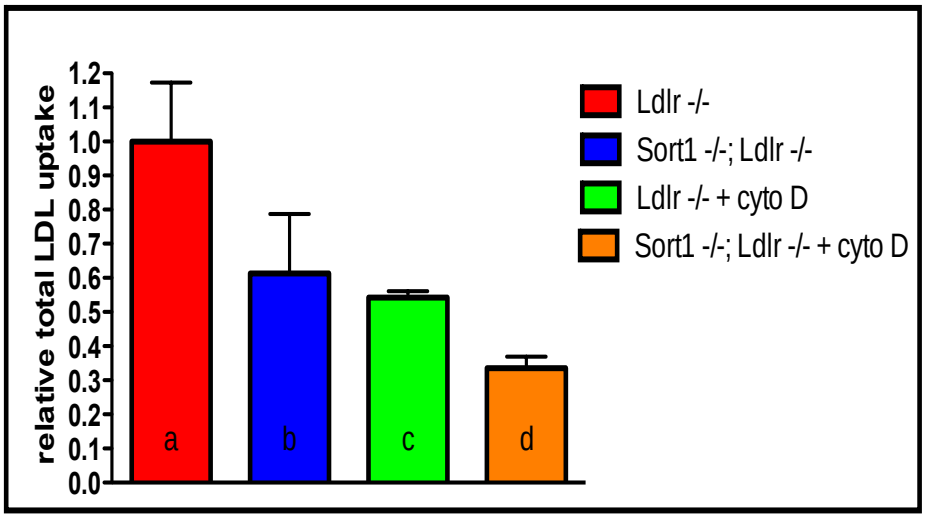
Figure 15 C-D: I-125 LDL uptake in thioglycollate elecited macrophages and bone marrow derived macrophages. C. Thioglycollate elecited macrophages and D. bone marrow derived macrophages were treated with 500ug/ml LDL for 24 hours. I-125 LDL was incubated for 5 hours. Cell associated and degraded LDL were individually assayed and the sum are represented as total uptake. (n=6 wells/per group Experiment was replicated 2 times)

We next tested if this effect on LDL uptake was independent of the LDL receptor. Bone marrow derived macrophages were isolated from *Sort1+/+;LDLr-/-* and *Sort1-/-;LDLr-/-* mice and ^{125}I-LDL uptake studies were performed. *Sort1* deficiency was associated with a 39% percent reduction in LDL uptake in the absence of the LDLR (P<0.05; Figure 15f).

To further confirm that sortilin deficiency confers atheroprotection by eliminating a receptor dependent pathway for LDL uptake and not by modulating macropinocytosis, LDL uptake studies were performed in bone marrow derived macrophages in the presence of cytochalasin D, a potent inhibitor of actin polymerization and macropinocytosis. Under these conditions, while LDL uptake is reduced, substantial residual LDL uptake still takes place.[71] *Sort1* deficiency was associated with a 38%

reduction in LDL uptake in the presence of cytochalasin D (P< 0.05; Figure 15e). These studies indicate that macrophage sortilin deficiency reduces macrophage uptake of LDL and formation of foam cells and this effect is independent of the LDL receptor and of macropinocytosis.

E



a		**39%(.02)**	**46%(7E-5)**	**67%(5E-4)**
b			**12%(9E-3)**	**45%(.01)**
c				**38%(.027)**

Figure 15 E: I-125 LDL uptake in M-CSF differentiated macrophages from LDLr -/- and Sort1-/-; LDLr -/- mice. Macrophages were pretreated with 500ug/ml LDL. 250ug/ml LDL was incubated and 4ug/ml cytochalasin D (as indicated) for 5 hours (n=6 wells/ per group. Experiment was replicated 2 times.).

Finally, to determine whether increased macrophage sortilin results in increased LDL uptake, J774 cells were transduced with lentivirus encoding GFP or *Sort1* and LDL

uptake studies were performed. *Sort1* overexpression in macrophages led to a 29% increase in LDL uptake (P< 0.05; Figure 15f).

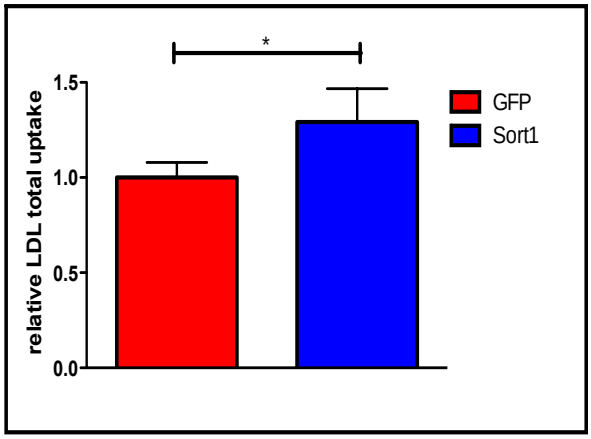


Figure 15F: I-125 LDL uptake in J774 cells overexpressing Sort1 and GFP. J774 cells were treated with doxycylcine for 48 hours to induce expression then I-125 LDL uptake study was performed as previously described. (N=6 wells/per group. Experiment was replicated 2 times.)

Methods:

Animals

Sort1-/- mice were obtained from Dr. Carlos Morales at McGill University and crossed onto the *Apobec1-/-; hAPOB* transgenic background. *LDLr-/-* mice for the bone marrow transplant studies. The University of Pennsylvania IACUC approved all animal protocols.

Total body sortilin deficient mouse atherosclerosis studies

Female *Sort1-/-;Apobec1-/-; hAPOB* Tg mice (n=10) and *Sort1+/+;Apobec1-/-; hAPOB* transgenic littermates (n=10) at 8 weeks of age were started on a western-type

diet. Mice placed on a western diet (21% fat, 50% carbohydrate, 20% protein Research Diets D12079B), which was continued for 18 weeks. Mice were bled at weeks 0, 2, and 9 and after 18 weeks on the diet were sacrificed and assessed for atherosclerosis in the aortic roots and the entire aorta by en face quantitation (see below).

Hematopoietic sortilin deficient mouse atherosclerosis studies

Donor bone marrow was isolated from male *Sort1-/-;LDLr -/-* and *LDLr -/-* mice by flushing femurs and tibias with sterile PBS. Female *LDLr -/-* recipient mice (8-10 weeks old) were irradiated with 900 rads from a cesium g source prior to transplantation. Each irradiated mouse was then injected with 4 E6 donor bone marrow cells via tail vein injection. The recipient *LDLr-/-* mice were given water with sulfamethoxazole and trimethoprim for 2 weeks post bone marrow transplantation. For bone marrow engraftment, Sort1 mRNA was quantified in spleen using quantitative PCR. Six weeks post transplantation mice were placed on a western diet for 18 weeks. Mice were bled at weeks 0, 4, and 14, and after 18 weeks on the diet were sacrificed and assessed for atherosclerosis in the aortic roots and the entire aorta by en face quantitation (see below).

Atherosclerosis quantitation and assessment

Mice placed on a western diet (21% fat, 50% carbohydrate, 20% protein Research Diets D12079B) for 18 weeks were anesthetized with isoflurane followed by a cervical dislocation after a four-hour fast. Aortas were collected from the base of ascending aorta and to the iliac bifurcation, whereas aortic roots with heart were harvested and both are fixed in 4% paraformaldehyde. Aortas for en face were stained with Oil Red O. Aortic

roots were dehydrated and paraffin embedded and used for lesion area quantification. Images were captured with Leica MZ12 microscope at a 40x magnification and quantification was performed with Image Pro Plus Software. For en face atherosclerotic lesion area was quantified in reference to the total surface area of the aorta as previously described. For aortic root atherosclerosis, lesion area was measured over the hematoxylin and eosin stained sections prepared from paraffin embedded hearts. In aortic roots atherosclerosis in either *Sort1-/- or Sort* +/+ *on Apobec1-/-; hAPOB* Tg model, we quantified representative section where captures 3 aortic valve leaflet equally observed in the section slice (same section as described below).[20] In the quantification of aortic roots atherosclerosis in irradiated / bone marrow transplanted experiment; we quantified lesion area of 5 serial sections (80um between sections) in each mice. For choosing 5 sections, we first defied a "zero-point" where meets all 3 leaflet of the aortic valve moving from aortic vessel towards internal lumen of the vessel. Then 2 serial sections towards aortic arch and another 2 distal serial sections to the zero point into the ventricle chamber were quantified. For each mouse, the data was represented as average lesion area (average area across the 5 points).

Monocyte recruitment assay

Sort1+/+ and *Sort1* -/- (n=10 per group) were injected i.p. with thioglycollate (3%) and three days later macrophages were isolated and counted by hemocytometer.

Assessment of cytokine response to LPS injection

Sort1+/+ and *Sort1* -/- (n=10 per group) were injected i.p. with LPS (3mg/kg). Mice were bled retroorbitally 2 and 5 hours post injection. Serum was isolated and run on the Bioplex Pro Mouse Cytokine 23-plex Immunoassay (#M60-009RDPD).

Studies of macrophage foam cell formation

For in vitro studies, M-CSF differentiated bone marrow macrophages from *Sort1-/-;LDLr -/-* and *LDLr -/-* mice ((n=3 per group; for procedure see below) on day 7 were incubated with 1 mg/ml LDL for 5 hours. The cells were fixed with paraformaldehyde and stained with Oil Red O and hematoxylin. For in vivo foam cell formation studies, *Sort1-/-;Apobec1-/-; hAPOB* Tg mice and *Sort1*+/+*;Apobec1-/-; hAPOB* Tg littermates (n=3 per group) were placed on a western diet for 18 weeks. Thioglycollate (3%) was injected i.p. and 3 days later cells were peritoneal macrophages were isolated, plated, and stained with Oil Red O and hematoxylin.

Studies of macrophage LDL uptake

For studies of macrophage LDL uptake, both thioglycollate-elicited peritoneal macrophages and bone marrow derived macrophages were used. For isolation of thioglycollate-elicited peritoneal macrophages, *Sort1*+/+ and *Sort1* -/- (n=6 per group) were injected i.p. with thioglycollate (3%) and three days later macrophages were isolated. Thioglycollate-elicited peritoneal macrophages after plating were incubated with 500ug/ml of LDL in 10% LPDS overnight. The next day the LDL uptake assay was performed with 25ug/ml I-125 LDL. For isolation of bone marrow derived macrophages,

bone marrow was isolated from mice femurs from *Sort1+/+* and *Sort1 -/-* (n=2 per group). Monocytes were differentiated for 7 days in M-CSF media into macrophages and on day 7 an LDL uptake study was performed with 250 ug/ml I-125 LDL. For lentiviral expression of *SORT1* in J774 macrophages, lentivirus encoding human was generated as previously described.[10] J774 cells were transduced with viral supernatant (Control: GFP + rtTA, Experimental SORT1 + rtTA). The cells were incubated at 37°C overnight, viral supernatant was removed, and cells were grown in RPMI medium. LDL uptake assay was performed with 25 ug/ml I-125 LDL.

LDL uptake assay

The macrophages (thioglycollate-elicited peritoneal or bone marrow derived) were incubated with ^{125}I-LDL for five hours. In some experiments, cytochalasin D (4ug/ml) was added to bone marrow derived macrophages as indicated right before LDL incubation. Cells were dissolved in 0.2M NaOH. The values were standardized to protein content of the dissolved cells in NaOH by bicinchoninic acid (BCA) assay (Thermo). After incubation the media was removed. Trichloroacetic acid was added to precipitate out unreacted LDL, followed by chloroform extraction of free iodine. Total Uptake values represent the sum of LDL associated and degraded.

Statistics

Statistical analyses were done using 2-tailed paired student's t test for total body knockout atherosclerosis, bone marrow transplant atherosclerosis, LDL uptake, macrophage recruitment, cellular cholesterol experiments. A 1 way ANOVA with a

Bonferroni correction was done for the LPS experiment. The error bars on graphs are representations of the standard error.

Discussion:

Genetic variation at the 1p13 *SORT1* locus is strongly associated both with CAD, as well as with plasma LDL-C levels. We have previously shown that sortilin is a cell surface receptor for LDL on hepatocytes and its elevated expression in liver reduces LDL-C at least in part by facilitating LDL clearance from blood. Sortilin is expressed in macrophages, which actively take up LDL, leading us to investigate the role of macrophage sortilin in LDL uptake, foam cell formation, and atherosclerosis. After a series of studies of atherosclerosis in mice and LDL uptake in macrophages, we conclude that macrophage sortilin promotes LDL uptake, foam cell formation, and atherosclerosis and that deficiency is protective against atherosclerosis at least in part by reducing LDL uptake.

Macrophage uptake of modified LDL can be mediated by scavenger receptors such as SRA and CD36. However, deletion of SRA or CD36 does not reduce macrophage uptake of native LDL[71] nor does it ameliorate atherosclerosis in hypercholesterolemic mice.[88] Even CD36-/-; SRA -/- mice still contain abundant lipid laden macrophages in vessel wall and develop atherosclerosis.[75] Kruth has shown that macrophages can take up native LDL through fluid-phase macropinocytosis, but there remains substantial LDL uptake even when this pathway is inhibited[71]. Our data establish macrophage sortilin as the first receptor-mediated pathway of uptake of native

LDL leading to foam cell formation and promoting atherosclerosis development. We also found that increasing concentrations of extracellular LDL causes an upregulation of macrophage *Sort1* mRNA and protein. Because a function of macrophages is to phagocytose LDL, it is very possible that increased exposure of macrophages to LDL triggers the transcriptional upregulation of sortilin, which then mediates increased LDL uptake. The mechanisms of this upregulation of macrophage *Sort1* by LDL require further exploration.

Mortensen et al. reported that whole body sortilin deficiency reduced atherosclerosis in the ApoE -/- mouse model[89]. While the fundamental observation is consistent with our data, these authors suggested a different mechanism, namely that decreased proinflammatory cytokines may have been responsible for the reduced atherosclerosis. We performed our cytokine assays prior to initiation of atherosclerotic disease, while Mortensen et al measured the cytokine profile after disease was present. In addition, these authors did not see a reduction in LDL uptake by sortilin deficient macrophages, although they used an ATTO dye conjugated to the LDL that may have influenced the interaction with sortilin. We also used a different mouse model, the *Apobec1-/-; hAPOB* Tg mouse, in which human apoB-100 containing LDL is the dominant lipoprotein, in a human-like lipoprotein profile, while Mortensen et al used the *Apoe-/-* mouse, which is characterized primarily by mouse apoB-48 containing remnant lipoproteins. Overall, the top-line results of the two studies, which used very different mouse atherosclerosis models, are highly comparable, whereas the mechanisms responsible for the reduced atherosclerosis may be complex and multifactorial.

In summary, our findings indicate that *SORT1* deficiency in macrophages reduces LDL uptake and macrophage cholesterol loading independent of the LDL receptor or macropinocytosis, and protects against the development of atherosclerosis. The macrophage sortilin pathway is a novel pathway of macrophage cholesterol loading that quantitatively contributes to atherosclerosis.

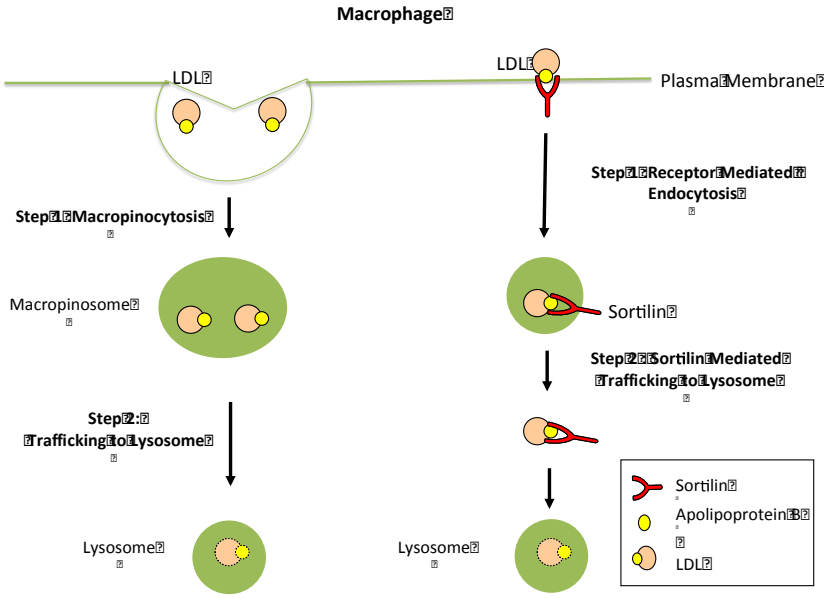


Figure 16: Macrophage sortilin mediates LDL uptake. Sortilin causes foam cell formation by mediating the uptake and degradation of LDL in macrophages independent of macropinocytosis.

Chapter Five: Conclusions and Future Directions

Despite decades of research in the metabolism of LDL and the pathogenesis of atherosclerosis, there remain major gaps in our knowledge. It is well-established that a major factor influencing plasma levels of LDL-C is the rate at which the liver secretes VLDL, the metabolic precursor to LDL. Yet the detailed molecular mechanisms by

which this large complex macromolecular particle containing apoB and multiple lipid constituents is assembled and secreted remain poorly understand. As another example, while the uptake of LDL by macrophages to form foam cells is virtually a sine qua non of atherogenesis, the molecular mechanisms by which macrophages internalize LDL remain very poorly understood. Unbiased human genetics can point to novel pathways involved in traits and disease and help to fill in mechanisms of incompletely understood processes. It is remarkable that the *SORT1* locus on chromosome 1p13 was one of the first novel loci to be identified in association with LDL-C levels as well as with coronary heart disease. In my thesis work, I investigated the role of the protein sortilin (encoded by the gene *SORT1*) in both VLDL secretion (affecting LDL-C levels) as well as in macrophage LDL uptake (affecting atherosclerosis). In doing so, I helped to elucidate the complex role that hepatic sortilin plays in both facilitating VLDL secretion through a probable chaperone-type function as a well as, at higher levels of expression, reducing VLDL secretion probably through targeting newly-synthesized VLDL from the Golgi to the lysosome. In addition, I established that macrophage sortilin plays a key role in mediating macrophage uptake of LDL, contributing to foam cell formation and atherosclerosis. These studies extended our previous knowledge of sortilin biology, provide examples of how human genetics can reveal new pathways relevant to human traits and disease, and provide a potential novel target for therapeutic intervention to reduce LDL-C levels and risk of CHD.

Our laboratory had previously shown that hepatic overexpression of sortilin can promote the presecretory lysosomal degradation of VLDL and reduce its secretion rate; in

addition, our lab and another group showed that complete ‘whole body’ sortilin deficiency is also associated with a reduction in VLDL secretion. One major aim of my thesis was to try to resolve these seemingly paradoxical observations. I proved that the explanation is not due to tissue specific differences in the overexpression and deficiency studies. By using two different approaches to knock down or delete sortilin specifically in the liver, I found that VLDL secretion was still reduced. We developed a hypothesis that there is a duality in the biology of sortilin: that it can act as both a chaperone and degrader of VLDL depending on factors such as level of expression and other unknown factors (Figure 17). We reasoned that the ability of sortilin to serve as a degrader of intracellular newly synthesized VLDL requires intact endolysosomal Golgi to lysosome trafficking, whereas the ability of sortilin to serve as a VLDL chaperone requires intact Golgi to plasma membrane trafficking. I carried out a series of experiments to test these hypotheses. In one of my most important findings, a mutant form of sortilin that is incapable of trafficking to the lysosome but retains the ability to traffic to the plasma membrane was shown to recover VLDL secretion in the sortilin knockout mouse (unlike wild-type sortilin which has the ability to traffic to the lysosome). These and other data help to support the working model shown in Figure 17.

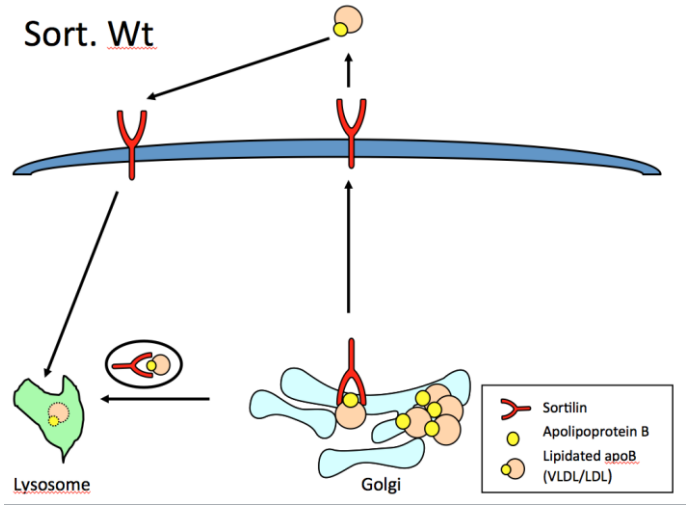


Figure 17: Working model of the dual role of sortilin in modulating VLDL secretion. At low levels of expression, sortilin promotes the secretion of VLDL, possibly through a chaperone-type function. At higher levels of expression, sortilin promotes the presecretory transport of VLDL from Golgi to lysosome thus reducing VLDL secretion.

The data presented in this thesis are consistent with a model in which hepatic sortilin facilitates VLDL export and thus its complete or virtually complete deletion from liver results in reduced VLDL secretion. Studies in primary hepatocytes lacking sortilin established that this is a cell-autonomous process that is not dependent on signals from other tissues or cell types. However, at increased levels of hepatic sortilin expression (such as those seen in individuals homozygous for the minor allele haplotype at 1p13), sortilin begins to serve a role of trafficking presecretory VLDL to the endolysosomal system for degradation. The molecular basis for this functional switch was an interest of my thesis work, but remains highly uncertain. I hypothesized that cleavage of sortilin by ADAM10 is a key determinant of the chaperone vs degradation function: that ADAM10 cleaves sortilin intracellularly and the soluble sortilin lacking the transmembrane domain is targeted for cellular secretion and serves as a VLDL chaperone. In this model, when

sortilin expresssion increases ADAM10 becomes rate-limiting and the increased amount of full-length sortilin transports presecretory VLDL to the lysosome. However, our *in vivo* and *in vitro* studies failed to show that Adam10 overexpression and knockdown affected VLDL secretion both in the context of endogenous and overexpressed sortilin. While these studies have not formally eliminated this hypothesis, I was unable to support this attractive working model.

I turned my attention to other factors that regulate the intracellular itinerary of sortilin, with particular attention to plasma membrane targeting. Cell surface sortilin levels are tightly regulated, with only 10% of cellular sortilin at the cell surface at a given time. It has recently been shown that NRH2 controls cell surface sortilin trafficking, and that its overexpression increases sortilin levels at the cell surface and its deficiency prevents cell surface sortilin expression[39]. NRH2 is only weakly expressed in liver, so it is possible that it would also become limiting with increasing sortilin expression, meaning that the excess sortilin would be unable to traffic to the plasma membrane and would instead only be capable of trafficking to the endolysosomal system to drive the presecretory lysosomal degradation of VLDL. Our laboratory is currently designing an *Nrh2* AAV and shRNA to test this attractive hypothesis *in vivo*.

Another possible explanation for the reduced secretion seen in sortilin deficient mice is that sortilin deficiency leads to ER stress, which Caviglia et al recently showed leads to global reductions in protein secretion as well as VLDL secretion[90]. As sortilin is a Golgi to lysosome trafficking receptor and the yeast sortilin homolog VPS10 has been shown to play a critical role in quality control, its deficiency would likely result in ER

stress due to the missorting of lysosomal enzymes as well as loss of quality control. Kjolby et al reported that apoB/VLDL accumulated in the ER of *Sort1-/-* mice[16], and accumulation of such a large hydrophobic protein could easily precipitate ER stress. To test the hypothesis that *Sort1* deficiency is associated with ER stress and that this is responsible for the reduced secretion seen in the *Sort1-/-* mouse, ER stress markers such as 78 kDa glucose-regulated protein (Grp78), C/EBP homologous protein (CHOP), and eukaryotic initiation factor alpha (eIF2a) phosphorylation can be determined by immunoblot in *Sort1+/+* and *Sort1-/-* mice. If there is evidence of ER stress, it could be alleviated through treatment with phenylbutyric acid (PBA) and the effect on VLDL apoB-100 secretion could be determined. Based on the model, I would hypothesize that PBA treatment should increase VLDL apoB-100 secretion in *Sort1-/-* mice. Such studies are planned as the project moves forward.

Interestingly, though our lab and another lab have reported reduced VLDL apoB secretion in 'primary' *Sort1* deficient systems, there have also been a number of reports of 'secondary' reduced expression of *Sort1* being associated with *increased* apoB secretion[77-79]. All of these systems have compromised autophagy or defects in mTOR signaling. It is tempting to speculate that in systems with compromised autophagy such as those used in the above studies, the VLDL not chaperoned by sortilin is ultimately secreted by a low affinity backup pathway to alleviate cell stress that would undoubtedly accrue as a result of the increased intracellular apoB and lack of autophagy mediated degradation (Figure 18). I measured apoB secretion from *Sort1-/-* primary hepatocytes treated with the autophagy inhibitors E64d and 3-methyladenine and found that inhibition

of autophagy rescues VLDL secretion in *Sort1-/-* hepatocytes, consistent with a role for autophagy in the degradation of VLDL not secreted by sortilin. The autophagy hypothesis as a basis for the paradoxical effects of sortilin deficiency on VLDL secretion could be tested by using siRNAs to knock down sortilin alone or in parallel with the conserved autophagy gene *Atg7:* the hypothesis is that that *Sort1* knockdown alone will reduce VLDL secretion whereas *Sort1* knockdown in parallel with *Atg7* knockdown will increase secretion. Complementary studies could also be performed in liver-specific *Atg7-/-* mice.

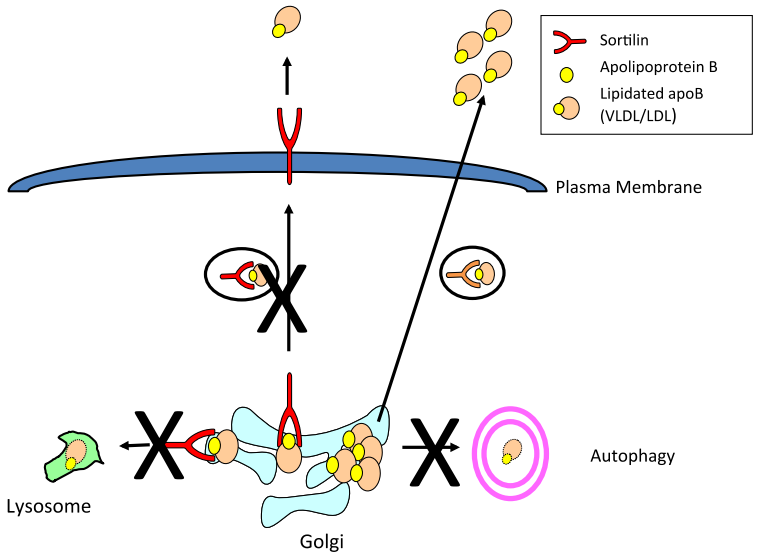


Figure 18: Compromised apoB autophagy and *Sort1* knockdown may increase apoB secretion. ApoB undergoes final lipidation in the Golgi. Once completely lipidated, VLDL can be bound by sortilin and trafficked to the lysosome or plasma membrane. When the two pathways are are blocked in conjuction with autophagy inhibition, a backup pathway may secrete apoB or it may buildup in the cell.

No matter what the exact mechanistic basis, it is clear that sortilin has a complex dual role in modulating VLDL secretion. This duality likely serves an evolutionary function, with sortilin levels tightly regulated to accommodate the cell's metabolic needs.

Specifically, it is tempting to hypothesize that at low *Sort1* expression levels such as those seen in obese mice and in systems in which there is a surplus of lipids, sortilin serves predominantly as a secretory receptor, facilitating lipid export from cells and preventing cellular lipotoxicity. Conversely, when cells are lipid depleted and sortilin is upregulated, its predominant function is as an endolysosomal trafficking receptor, allowing cells to conserve lipid by trafficking it to the lysosome for catabolism. This regulatory cycle also makes evolutionary sense in considering sortilin's role as a cell surface LDL receptor. When lipids are in surplus, sortilin would be downregulated to halt cellular uptake of LDL, whereas in the context of cellular lipid depletion sortilin would be upregulated to promote LDL uptake from exogenous sources.

Interestingly, we have demonstrated that sortilin expression is regulated differently in the macrophage, with cholesterol loading increasing sortilin protein. Given that sortilin is a high affinity cell surface receptor for LDL, this mechanism of cholesterol regulation suggests that sortilin on the macrophage could play an important role in atherosclerosis development. Indeed, we have shown that sortilin expression in macrophages is associated with increased atherosclerosis and that its deficiency in bone marrow-derived cells is atheroprotective. Specifically, we have found that sortilin in macrophages promotes the development of atherosclerosis by acting as a cell surface receptor for LDL, facilitating LDL uptake into macrophages and subsequent foam cell formation. While my bone marrow transplantation studies were unequivocal in their atherosclerosis results, further evidence for the important role of macrophage sortilin in influencing atherogenesis could be generated by breeding the *Sort1* flox/flox mouse with

the LysM-Cre mouse to delete sortilin in the macrophage and then performing atherosclerosis studies. These studies were established by me and are currently ongoing in our lab.

Macrophage sortilin plays a critical role in foam cell formation and atherogenesis; however, it is probably not the only cell type in which sortilin deficiency modulates atherosclerotic disease. For example, whereas total body *Sort1* deficiency resulted in a 67% reduction in plaque burden, myeloid specific *Sort1* deficiency resulted in a 40% reduction in atherosclerosis, suggesting a contribution by a non-hematopoetic cell type. The Aikawa lab at Harvard recently reported a role for vascular smooth muscle cell sortilin expression in atherogenesis[92]. They found that sortilin was strongly expressed in the calcified regions of human atherosclerotic plaques, and that C-terminal sortilin phosphorylation promoted smooth muscle cell calcification. Thus, sortilin deficiency in vascular smooth muscle cells also appears to be atheroprotective, which may account for the difference in protection with total body *Sort1* deficiency versus macrophage specific *Sort1* deficiency. This model could be tested by generating a mouse deficient in *Sort1* in vascular smooth muscle cells and performing atherosclerosis studies.

Although there are many currently available therapies that reduce LDL-C (statins, ezetimibe) and more in development (PCSK9 antibodies), coronary heart disease is still a major cause of morbidity and mortality in the US and around the world. Given the very strong human genetics linking sortilin to LDL-C levels and CHD risk coupled with the consistent preclinical data that I and others have generated, sortilin is an interesting potential novel therapeutic target for reducing LDL-C and risk of CHD. Determining the

molecular mechanisms by which sortilin affects LDL metabolism and atherosclerosis and dissecting the nuances of its expression-dependent functions in different tissues will be critical before sortilin can be seriously targeted. Overexpression of *Sort1* in the human liver would be expected to lower plasma LDL-C levels and risk of CHD. Even though gene therapy is booming (as evidenced by the recent initial public offerings of Spark Therapeutics, Bluebird Bio, and Avalanche), sortilin will not be an early target for gene therapeutic expression.

One could also imagine a therapeutic benefit to targeting sortilin expression in macrophages. Though liver directed therapies would aim to increase *Sort1* expression, *Sort1* inhibition in macrophages would be the preferred approach based on my data. By using nanoparticles specifically directed to macrophages, which are cleared by the kidneys, the liver could be bypassed enabling macrophage specific *Sort1* inhibition. The nanoparticles could deliver small molecule inhibitors, monoclonal antibodies, or genetic knockdown or deletion vectors like siRNAs or CRISPR/Cas systems. Much work remains to be done to unravel the nuances of the sortilin pathway, and there is much discovery to look forward to in the near future.

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