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A Potential Role for Sortilin in Lp(a) and Apo(a) Metabolism

By

MATTHEW GEMIN

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2017

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"A Potential Role for Sortilin in Lp(a) and Apo(a) Metabolism"

by

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January 25, 2017

DECLARATION OF CO-AUTHORSHIP

I hereby declare that this dissertation incorporates material that is the result of joint research, as follows:

This thesis incorporates the outcome of joint research in collaboration with Dr. Santica Marcovina, Dr. Nabil Seidah, Dr. Rocco Romagnuolo, Dr. Corey Scipione, Dr. Tanya Marar, Dr. Robert Hegele, and Dr. Zainab Bazzi under the co-supervision of Dr. Marlys Koschinsky and Dr. Michael Boffa. The majority of key ideas, experimental contributions, data analysis, and written examinations were performed by the author.

The recombinant-apo(a) stable HepG2 line, as well as the various apo(a) mutants used in this study were provided by Dr. Marlys Koschinsky, and generated as outlined in Chapter 2.

Multiple contributions are the work of Dr. Rocco Romagnuolo; Lp(a) internalization assays involving sortilin overexpression in HepG2 cells were co-performed by the author and Dr. Rocco Romagnuolo. Creation of the sortilin trafficking mutants used in this study (outlined in Chapter 2), as well as the corresponding internalization assays studying their effects on Lp(a) internalization in HepG2 cells, were solely performed by Dr. Rocco Romagnuolo. The agarose gel-based *in vitro* binding assay between sortilin and Lp(a)/LDL, as well as the generation of a soluble sortilin variant (outlined in Chapter 2), were also performed by Dr. Rocco Romagnuolo.

The isolation of primary mouse hepatocytes were co-performed by Dr. Rocco Romagnuolo and Dr. Corey Scipione. Internalization assays conducted in these cells were performed by the author.

Confocal microscopy was performed with the assistance of Dr. Zainab Bazzi.

Contribution from Dr. Santica Marcovina was through providing purified Lp(a) and anti-apo(a) antibody

Contribution from Dr. Nabil Seidah was through providing a wild-type sortilin construct

Contribution from Dr. Robert Hegele was through providing the deep sequencing analysis of *SORT1* and for identifying the SNPs present in individuals with elevated Lp(a) levels. Generation of expression vectors encoding the corresponding sortilin polymorphic variants used in this study were created by Dr. Tanya Marar as outlined in Chapter 2.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

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ABSTRACT

Elevated levels of lipoprotein(a) (Lp(a)) are an independent, causal risk factor for coronary heart disease. Lp(a) is structurally similar to low-density lipoprotein (LDL), but is distinguished by the covalent addition of apolipoprotein(a) (apo(a)), which is a polymorphic glycoprotein that shares homology with plasminogen. Genomewide association studies (GWAS) identified that mutations in the vicinity of the *SORT1* gene are associated with a ~5.8 mg/dL decrease in LDL cholesterol, 18% decrease in coronary artery disease risk, and 40% decrease myocardial infarction risk. Sortilin, the protein product of *SORT1*, was found to directly associate with apoB-100, and regulate apoB-100/VLDL secretion and LDL catabolism. The work in this thesis evaluates if sortilin plays a similar role in regulating Lp(a)/apo(a) metabolism. Pulse-chase analysis demonstrated that sortilin overexpression increased the accumulation of intracellular and secreted apo(a); the opposite effect was observed with reduced sortilin expression. Critical roles in mediating the ability of sortilin to increase apo(a) secretion were identified for the weak lysine bindings sites (LBS) in kringle IV type 7 and type 8, but not the strong LBS in kringle IV type 10, of apo(a). Sortilin overexpression also increased Lp(a) internalization, with data suggesting that this pathway occurs independently of the LDL receptor. Treatment with a lysine analog abolished the ability of sortilin to increase Lp(a) internalization. The effects of sortilin overexpression on both apo(a) secretion and Lp(a) catabolism were dependent upon the ability of sortilin to act as a trafficking receptor. Naturally occurring polymorphisms of sortilin were found to be expressed in individuals with elevated levels of Lp(a). Expression of these sortilin mutants resulted in altered apo(a) secretion and Lp(a) internalization compared to wild-type sortilin. Taken together, our data suggest that sortilin is involved in the regulation of Lp(a)/apo(a) metabolism.

DEDICATION

I dedicate this work to my family for all of their love and support

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I would also like to thank my committee members, Dr. Vacratsis and Dr. Hubberstey, as well as the remaining staff of the Chemistry and Biochemistry department, for contributing to my growth and education as a student throughout the years, and for allowing me to use the equipment required to complete my research. Thank you to the support staff of the Chemistry and Biochemistry department as well, for meeting with and assisting me whenever I required help.

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Thank you to all of the current and past students in the MBB and MLK labs who are associated with my time spent as a graduate student. Thank you to Dr. Rocco Romagnuolo, who made continuous contributions both through creative ideas and experimental contributions. This thesis would not be what it is today without his help and guidance. Thank you to Dr. Corey Scipione, Dr. Zainab Bazzi, and Dr. Tanya Marar for

their contributions to the work in this thesis. A further thank you to Dr. Tanya Marar, as well as Justin Garabon, Sera Sayegh, Jackson McAiney, and Scott Smith, for the constant laughs, encouragement, and coffee/food runs. These little moments helped to alleviate the stressful times. Thank you as well to current and past members: Mousa Gawanmeh (The Kid), Victor Igbokwe (Priest), Jen Balun, and Zach Adams, Abdullah Masoodi, Dapo Onabote, and Matthew Borrelli. I would also like to thank everyone in the Chemistry and Biochemistry department that helped to make my experience as a graduate student enjoyable. Particularly, thank you to members of the Otis lab, who were my first partners and teachers in research. This includes Dr. Christopher Bonham, Dr. Besa Xhabija, Dr. Quidi Geng, Justin Roberto, and Robert Gombar. I wish everyone the best in their future endeavors.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AB-ß	amyloid-beta peptide
AP-1	activator protein 1
apo(a)	apolipoprotein(a)
apoA-V	apolipoprotein A-V
apo(B)-75	apolipoprotein B-75
apo(B)-100	apolipoprotein B-100
apoE	apolipoprotein E
APP	amyloid precursor protein
ATCC	American Type Culture Collection
BACE1	ß-site ß-amyloid precursor protein (APP)-cleaving enzyme 1
BCA	bicinchoninic acid
BDNF	brain-derived neuronal factor
BiP	immunoglobulin heavy chain binding protein
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAD	coronary artery disease
CHD	coronary heart disease
CVD	cardiovascular disease
CELSR2	cadherin EGF LAG seven-pass G-type receptor 2
CO_2	carbon dioxide
DAPI	4′, 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DTT	dithiothreitol
ε-ACA	ε-aminocaproic acid
ECs	endothelial cells
ECM	extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EEA1	early endosome marker 1
EGF	epidermal growth factor
EGFA	epidermal growth factor-like repeat homology domain
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbet assay
ER	endoplasmic reticulum
ERAD	endoplasmic-reticulum-associated degradation
FBS	fetal bovine serum
FH	Familial hypercholesterolemia
FRET	Förster resonance energy transfer
GGAs	Golgi-localizing, Gamma-adaptin -containing, ARF-binding proteins
Glut4	glucose transporter type 4
GSV	Glut4 storage vesicles
GWAS	genome wide association studies
H_2O	water
HBS	HEPES-buffered saline
HDL	high density lipoprotein
HEK293	human embryonic kidney cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	human hepatocellular carcinoma cell
ICAM-1	intercellular adhesion molecule-1
IL	interleukin
IL-6	interleukin 6
IL-8	interleukin-8
IP	immunoprecipitation
K	kringle

KCL	potassium chloride
kDa	kiloDalton
KH_2PO_4	Potassium phosphate monobasic
KIV	kringle IV
KV	kringle V
LAMP1	lysosomal-associated membrane protein 1
LBS	lysine binding site
LDL	low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
Lp(a)	lipoprotein(a)
LPDS	lipoprotein-depleted serum
LPL	lipoprotein lipase
MPR	mannose-6-phosphate receptor
mAb	monoclonal antibody
MEM	minimal essential medium
MI	myocardial Infarction
MMP	matrix metalloproteinase
MTP	microsomal triglyceride transfer protein
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NGF	nerve growth factor
NT	neurotensin
OxPL	oxidized phospholipid
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PCSK9	proprotein convertase subtilin kinexin 9
PDI	protein disulfide isomerase
PEI	polyethylenimine
PMSF	phenylmethylsulfonyl fluoride

PSRC1	proline/serine-rich coiled coil 1
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
siRNA	small interfering ribonucleic acid
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SORT1	sortilin-1
SPR	surface plasmon resonance
SR-B1	scavenger receptor class B type I
TBST	Tris-buffered saline, 0.2% Tween 20
TGF	transforming growth factor
TGN	trans-Golgi network
THP-1	human acute monocytic leukemia cells
tPA	tissue plasminogen activator
Trk	tyrosine receptor kinase
UGGT	UDP-glucose:glycoprotein glucosyltransferase
uPA	urokinase-like plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VPS10	vacuolar protein sorting 10

CHAPTER 1: GENERAL INTRODUCTION

1.1 Cardiovascular Disease and Atherosclerosis

Cardiovascular disease (CVD) is a broad classification for a variety of disease states that affect the heart and associated blood vessels. It is recognized as the leading cause of mortality in the world (1). CVD can be subdivided into numerous categories, with the most common category known as coronary artery disease (CAD) (2). CAD is regulated by a plethora of complex factors such as genetic heritance, environmental elements, and lifestyle (3). CAD is a progressive disease, with atherosclerosis acting as the underlying, causal mechanism. Atherosclerosis is an inflammatory disease that is defined by the accumulation of lipids and other elements in the walls of the large arteries in the cardiovascular system (Fig. 1.1) (4, 5). This accumulation leads to the formation of atherosclerotic lesions. High density lipoprotein (HDL) does not accumulate in lesions, but rather acts as a scavenger that prevents lipid oxidation and removes excess cholesterol from circulation (4).

In normal physiology, endothelial cells (ECs) lining the lumenal side of the artery are bound together through tight junctions that function as a permeability barrier between the arterial lumen and the extravascular tissue (4, 5). However, factors such as infection, oxidation, and sheer stress can promote inflammation and induce EC injury. As a result, the actin and tubulin cytoskeletons of ECs are reorganized, thus leading to an increase in the permeability of this layer (6, 7). This process is called EC dysfunction, and it is associated with the beginning stages of atherosclerotic development. Lipids and fibrous materials diffuse passively from the blood into the intima through EC junctions, where they are retained in the vessel wall through interactions with the extracellular matrix (ECM).

Lipids undergo various modifications once in the sub-endothelial space, such as oxidation or lipolysis, which triggers an inflammatory response by ECs. Proinflammatory cytokines, such as interleukins (ILs) and transforming growth factors (TGFs), are released by ECs, and monocytes are recruited to the vessel wall by adhesion molecules, such as the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on the vascular surface (7). Monocytes are a type of white blood cell classified as leukocytes. They subsequently differentiate into macrophages and form foam cells in the arterial intima by ingesting modified lipids through various methods which include: phagocytosis, micropinocytosis, and receptor-mediated endocytosis (4). Foam cell accumulation leads to the formation of a lipid-rich core, which can become necrotic over time as foam cells undergo apoptosis. Furthermore, necrotic foam cells release pro-inflammatory elements that amplify plaque inflammation and further promote the recruitment of additional monocytes. Smooth muscle cells (SMCs) undergo migration and proliferation from the tunica media into the intima as a response to cytokine and growth factor release (4). This leads to the formation of a fibrous capsule that surrounds and stabilizes the growing inflammatory plaque. However, the integrity of this cap becomes compromised overtime through proteasomal degradation of ECM proteins by proteinases such as matrix metalloproteinases (MMPs) (8). A weakened fibrous cap, growing necrotic core, and the elevation of pro-inflammatory elements increases the risk of a ruptured atheroma. Acute thrombosis and vascular occlusion are the immediate result of a ruptured plaque, which can lead to ischemia, myocardial infarction (MI), and stroke (4). Individuals that possess elevated levels of plasma lipoproteins, such as low density lipoprotein (LDL) and lipoprotein(a) (Lp(a)), are associated with increased risk of developing CAD through their ability to contribute to the development and progression of atherosclerotic lesions.

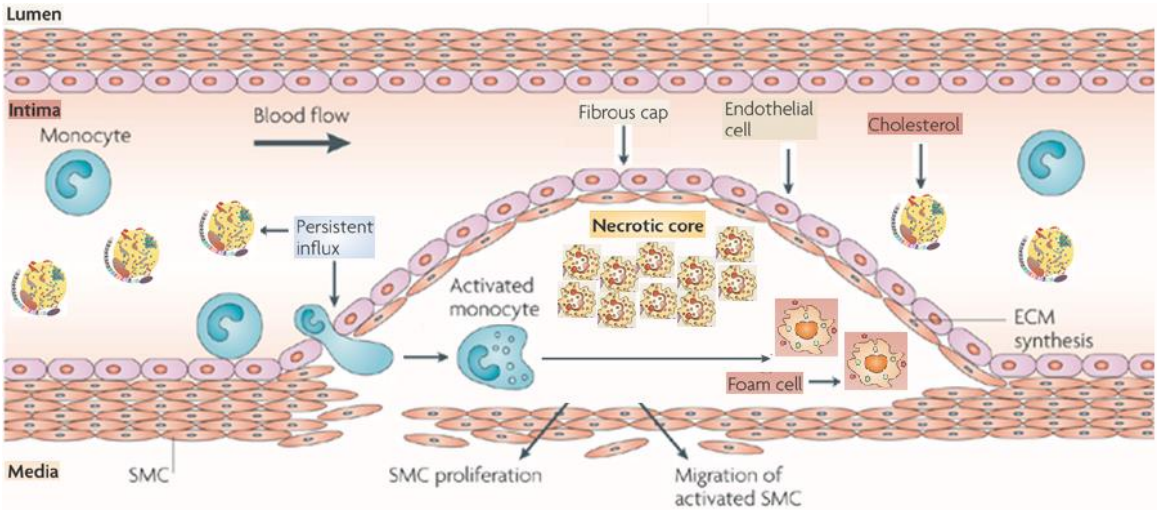


Figure 1.1: ***Progression of atherosclerosis.*** The initiation and progression of atherosclerosis is a complex process that incorporates several stages. In normal physiology, endothelial cells (ECs) act as a permeability layer on the lumenal side of the artery. EC dysfunction brought about by EC injury leads to a reorganization of the cytoskeletons of ECs, thus increasing the permeability of the EC layer. As a result, a persistent influx of lipids and fibrous materials occurs through passive diffusion of EC junctions. These materials are retained in the arterial wall, and undergo modification. This triggers an activation response by ECs, which recruits monocytes to the arterial wall. Activated monocytes differentiate into macrophages and engulf the modified materials, forming foam cells. Foam cell apoptosis over time leads to the formation of a necrotic core. Cytokine release by ECs stimulates the proliferation and migration of smooth muscle cells (SMCs), leading to the formation of a fibrous cap that surrounds the growing atheroma. Proteosomal degradation threatens the integrity of the cap over time, potentially leading to a rupture. Plaque rupture is immediately followed by acute thrombosis and vascular occlusion. Modified from reference 5.

1.2 Lipoprotein(a) and Apolipoprotein(a)

1.2.1 Structure of Lp(a) and Apo(a)

Lipoprotein(a) (Lp(a)), first discovered in 1963 by Kåre Berg, is a unique lipoprotein that is found only in humans, Old World primates, and hedgehogs (9-11). Lp(a) is an atherogenic lipoprotein that is structurally similar to low-density lipoprotein (LDL) in that it possesses a comparable lipid core associated with an apolipoprotein B-100 (apoB-100) moiety (12). However, it is distinguished from LDL by the presence of the polymorphic glycoprotein apolipoprotein(a) (apo(a)), which is covalently bound to apoB-100 through a single disulfide linkage (Fig. 1.2) (13). Apo(a) is encoded by the *LPA* gene located on chromosome 6q26-27 (14).

Structurally, apo(a) is composed of multiple triple-looped structures classified as kringle domains; each kringle is stabilized by three conserved intramolecular disulfide bonds (15). These domains are linked by short segments of serine/threonine- and proline-rich sequences called linker regions (16). Kringle domains are characteristic of many proteins that are involved in the coagulation and fibrinolytic cascades, and generally play a role in facilitating ligand binding (17). Analysis of the apo(a) cDNA sequence in 1987 revealed extensive homology between human apo(a) and the zymogen plasminogen, which is the precursor form of plasmin (18). Plasmin is an important enzyme involved in the process of fibrinolysis, and is composed of five distinct kringle domains followed by a serine protease domain (18). Apo(a), on the other hand, contains multiple plasminogen-like kringle IV (KIV) domains, followed by a kringle V (KV) and protease-like domains that are also highly similar to the corresponding domains in plasminogen (Fig. 1.3) (19). The

multiple copies of the KIV-like sequences in apo(a) can be further categorized into ten subtypes (KIV_{1-10}) based on amino acid sequence (18). Apo(a) contains only one copy of each KIV subtype with the exception of KIV_2, which can be present in 3 to >40 identically repeated copies (20). The mutable number of KIV_2 domains gives rise to the size heterogeneity observed within Lp(a), which can vary over 1000-fold between individuals (15), and can be attributed to allelic variation in the *LPA* gene (21, 22). The protease-like domain in apo(a) is catalytically inactive (16, 23), unlike the corresponding domain in plasminogen which can be activated through catalytic cleavage of the peptide bond between R^{560} and V^{561} (23).

Lysine-binding sites (LBS), present in both apo(a) and plasminogen, are binding pockets present within some of the kringles that bind lysine. The pocket is flanked by oppositely charged residues at either end of the site (24, 25). The anionic residues within the binding pocket interact with the ammonium groups of lysine residues, while the cationic residues in the binding pocket interact with the carboxyl groups of lysine residues during ligand binding. The trough of the pocket is lined by hydrophobic, aromatic residues that stabilize the aliphatic backbone of lysine (24). KIV_{5-8} of apo(a) are classified as weak LBS, while KIV_{10} is classified as a strong LBS (Fig. 1.3). The amino acid sequence of the LBS in apo(a) KIV_{10} is the most similar to that found in KIV of plasminogen, with the only difference being an arginine to lysine conversion at position 35 in KIV_{10} (18).

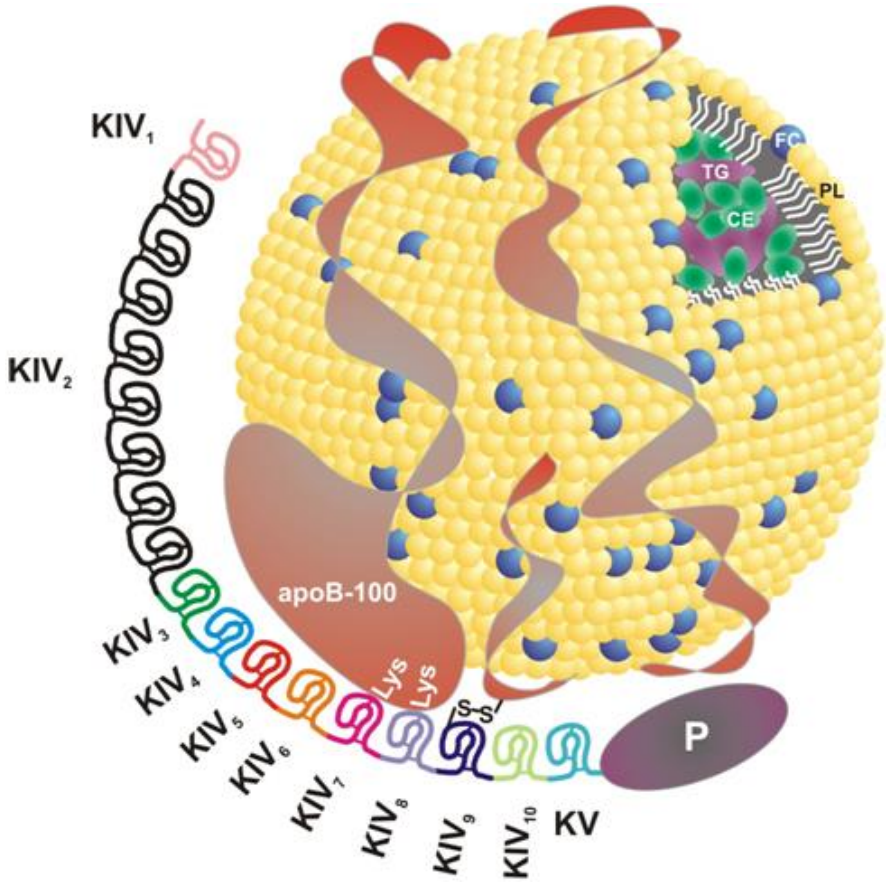


Figure 1.2: ***Structural representation of an Lp(a) particle.*** Lipoprotein(a) (Lp(a)) is composed of a lipid particle that is indistinguishable from low-density lipoprotein (LDL) with respect to lipid composition and the presence of an apolipoproteinB-100 (apoB-100) moiety. Lp(a) differs from LDL, however, by the covalent attachment of apolipoprotein(a). The lipid particle is composed of triglycerides (TG) and cholesterol esters (CE) that are contained by a layer of phospholipids (PL) and free cholesterol (FC) that surround the centralized core. Apo(a) contains multiple plasminogen kringle IV (KIV)-like domains, a single kringle V (KV)-like domain, and an inactive protease domain (P). The KIV domains are subdivided into 10 subtypes classified as $KIV_{1\text{-}10}$, and KIV_2 can be present in a variable number of tandemly repeated copies encoded by the *LPA* gene which gives rise to Lp(a) isoform size variation. Weak lysine binding sites present in apo(a) KIV_7 and KIV_8 are required to mediate a non-covalent interaction between apo(a) and apoB-100 (identified as Lys in the apoB structure) that precedes disulfide linkage between a free cysteine residue in apo(a) KIV_9 and the C-terminal region of apoB. Adapted from reference 12.

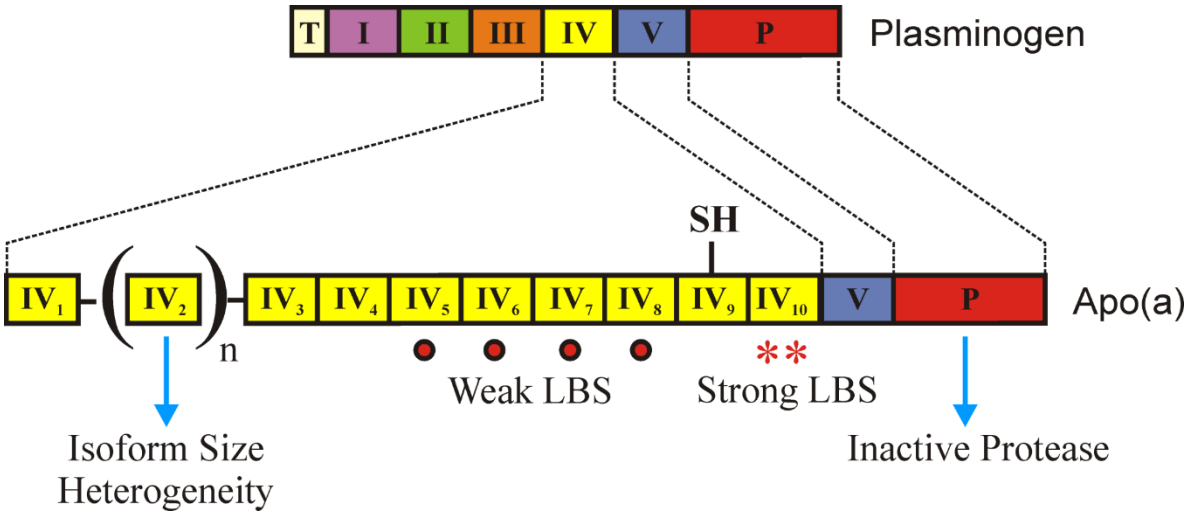


Figure 1.3: Apo(a) is homologous to plasminogen. The zymogen plasminogen is a precursor of plasmin. It is composed of an N-terminal tail sequence, followed by five distinct kringle domains classified as kringles I-V (KI-KV) and a serine protease domain. Apo(a) is composed of multiple kringle domains that share homology with KIV of plasminogen, as well as a single copy of a comparable KV domain and a protease-like domain that is catalytically inactive. The multiple copies of plasminogen KIV-like domains in apo(a) vary from each other with respect to their amino acid sequences. Therefore, they are classified into subtypes $KIV_{1\text{-}10}$. All of the KIV-like subtypes of apo(a) are present in one copy with the exception of KIV_2, where copy number can vary from 3 to greater than 40 tandemly repeated, identical copies. $KIV_{5\text{-}8}$ each possess weak lysine binding sites (LBS) whereas KIV_{10} contains a strong LBS. This kringle domain shares the most homology with KIV of plasminogen. KIV_9 contains a free cysteine residue that is critical in forming a disulfide bond with apoB-100. Adapted from reference 20.

1.2.2 Lipoprotein(a) Levels in Plasma

Varying levels of Lp(a) are present in the human population, with plasma concentrations ranging from undetectable to greater than 100 mg/dL (26). The *LPA* gene *LPA*accounts for up to 90% of the variation observed in plasma Lp(a) levels in the population (21). As mentioned above, the variable number of repeated KIV_2 domains gives rise to the size heterogeneity observed in Lp(a) (21, 22). A general inverse relationship exists between the number of KIV_2 copies present in apo(a) and plasma levels of the corresponding Lp(a) (15, 27). Smaller apo(a) isoforms have a reduced residence time in the endoplasmic reticulum (ER) (28) and, as such, are less susceptible to quality control mechanisms resulting in intracellular degradation of misfolded proteins (29). Various single nucleotide polymorphisms (SNPs) have also been found to greatly influence Lp(a) levels. Two in particular, rs10455872 and rs3798220, were found to account for 60% of the variability in plasma Lp(a) concentrations (30). These mutations were also associated with a significantly increased risk for coronary heart disease (CHD) (30).

1.2.3 Lipoprotein(a) Pathophysiology

Lp(a) has been identified through multiple genetic and epidemiological, genetic association, studies to be an independent, causal risk factor for CHD (30-35). A large meta-analysis indicated a continuous association between CHD risk and elevated levels of plasma Lp(a). This increase in risk is associated with a potential increase in the occurrence of coronary events (32). Lp(a) is now considered the strongest genetic risk factor for CHD (36). Mendelian randomization studies confirmed this association by demonstrating a strong correlation between Lp(a) isoform size and risk for MI (34). Lp(a) has also been

suggested to be a risk factor for stroke, peripheral vascular disease, and aortic valve stenosis (30, 32, 37-39).

1.2.4 Potential Mechanisms of Action

While the specific physiological role of Lp(a) remains enigmatic, it is generally accepted that this atherogenic lipoprotein greatly promotes the development of atherosclerosis. Numerous studies have revealed a variety of potential mechanisms by which Lp(a) can contribute to atherosclerosis and CVD (Fig. 1.4) (40). Since LDL is prominently related to increased risk of CVD and coronary events, it was postulated that the LDL-like component of Lp(a) plays an important proatherogenic role. Possession of an LDL-like component alludes to a similar mechanistic contribution for Lp(a) to that of LDL with respect to atherogenesis. This includes mediating pro-inflammatory effects, activating ECs, recruiting monocytes to the site of lipid retention and acceleration of foam cell formation. However, it is the presence of the unique apo(a) component that separates Lp(a) from LDL as a risk factor (13). Indeed, apo(a) provides distinction between Lp(a) and other atherogenic lipoproteins through its unique contributions to both the proatherogenic and prothrombotic properties of Lp(a) (Fig. 1.5) (13, 14, 40).

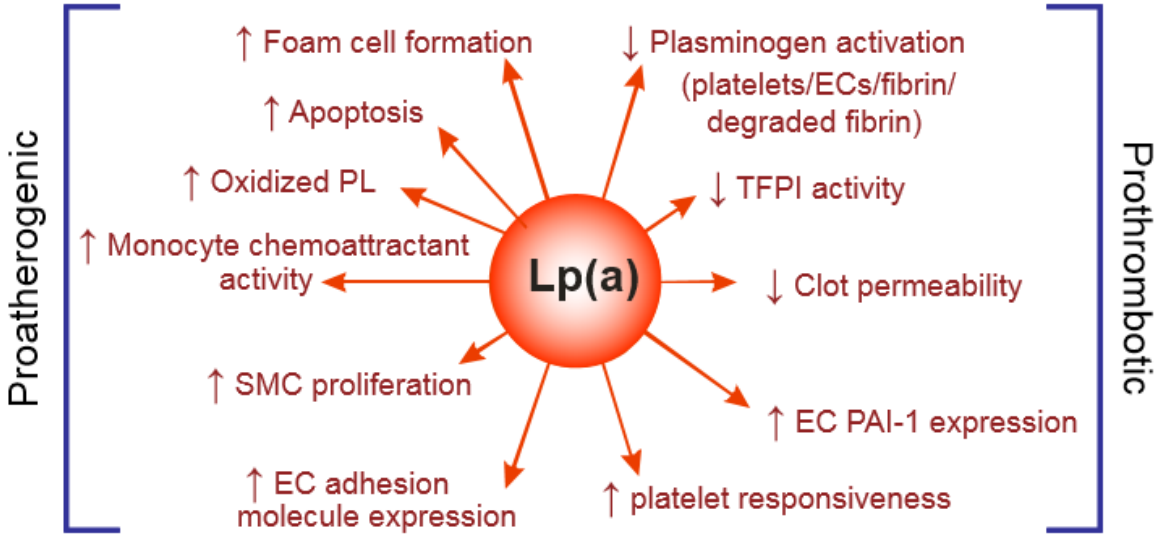


Figure 1.4: ***Postulated mechanisms of action for Lp(a).*** Lp(a) has been identified as the strongest genetic risk factor for coronary heart disease (CHD). Meta-analysis as well as numerous genetic studies have confirmed its role as an independent and causal risk factor for CHD. A role for Lp(a) in risk of myocardial infarction (MI), ischemic stroke, peripheral vascular disease, and aortic valve stenosis has also been determined. The proposed proatherogenic mechanisms of Lp(a) are listed on the left, while the prothrombotic mechanisms are listed on the right. PL, phospholipids; SMC, smooth muscle cells; EC, endothelial cells; TFPI, tissue factor pathway inhibitor; PAI-1, plasminogen activator inhibitor-1. Modified from reference 39.

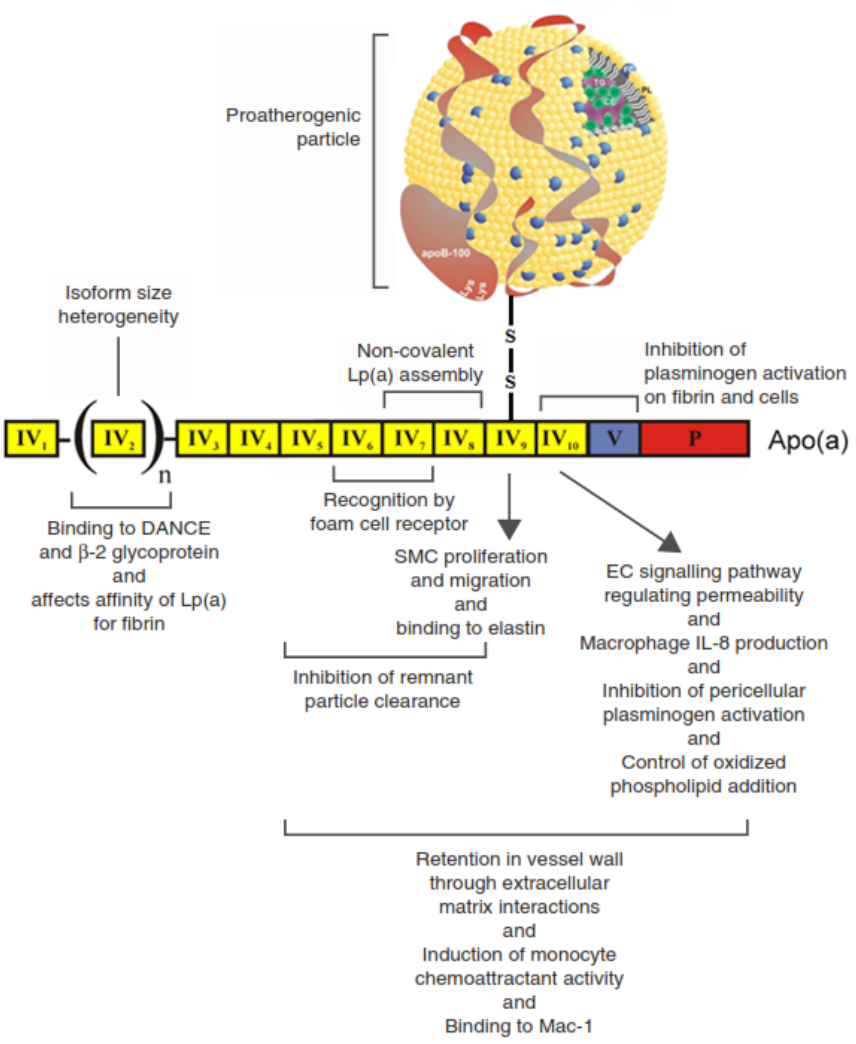


Figure 1.5: ***Apo(a)-driven mechanisms behind the proatherogenic and prothrombotic properties of Lp(a).*** Multiple mechanisms of action have been proposed for the prothrombotic and proatherogenic properties of Lp(a). Both the lipid particle, and apo(a) component, contribute to Lp(a) pathophysiology. The various proposed proatherogenic and prothrombotic properties of Lp(a) mediated by the apo(a) component are indicated here. The corresponding apo(a) kringle domains associated with these mechanisms are also indicated. DANCE, developmental arteries and neural crest epidermal growth factor (EGF)-like; SMC, smooth muscle cell; EC, endothelial cell; IL-8, interleukin-8. Modified from reference 13.

One of the major contributors to Lp(a) pathophysiology is the association between Lp(a) and inflammation. A key component in the pro-inflammatory capabilities of Lp(a) particles is the presence of an oxidized phospholipid (OxPL) modification. As previously mentioned, an elevated inflammatory response can lead to endothelial dysfunction, thus increasing arterial wall permeability (4). Interestingly, research conducted in human patients indicated preferential binding of pro-inflammatory OxPLs to Lp(a) in comparison to LDL (41). Previous research has implicated a role for apo(a) KV in facilitating covalent OxPL modification; however, the presence of OxPLs were observed in apo(a) variants that did not possess a KV domain (42, 43). Recent *in vitro* work indicates that the strong LBS in KIV_{10} plays a critical role in facilitating the addition of covalent OxPL to Lp(a) (44). Mutagenesis of an aspartic acid residue within the LBS led to the abolishment of the lysine binding capabilities of apo(a) KIV_{10}, and a concomitant inhibition of OxPL addition. This study also demonstrated that oxidation of apo(a) is associated with increased stimulation of interleukin-8 (IL-8), which is an important cytokine involved in the recruitment of inflammatory cells (44). These data demonstrate a novel mechanism through which the apo(a) component can stimulate atherogenesis. Interestingly, the aforementioned study is not the first to identify a role for Lp(a) in the stimulation and activation of various pro-inflammatory signalling cascades and cytokines. Previous *in vivo* and *in vitro* studies found that Lp(a) can stimulate monocyte chemoattractant protein-1 (Mac-1), which is a key chemokine involved in regulating the migration and infiltration of inflammatory cells such as monocytes (45, 46). This leads to an interesting role for Lp(a) as a chemoattractant and regulator of the inflammatory response within the vasculature. It is tempting to speculate that the covalent OxPL on apo(a) may be central in mediating many of the proinflammatory effects of apo(a)/Lp(a) that have been reported.

Homology between apo(a) and plasminogen led to the hypothesis that Lp(a) may also act as a prothrombotic factor in humans (18). Indeed, previous studies found that Lp(a) was able to compete with plasminogen for binding to endothelial cells and monocytes; this function was mediated by the apo(a) component (47, 48). Furthermore, the apo(a) component has also been shown to inhibit the activation of plasminogen. Generally, plasminogen is activated to plasmin through proteolytic cleavage induced by tissue plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) (49). Plasmin actively degrades fibrin, thus promoting the break-down of blood clots. Lp(a) was found to compete with both plasminogen and tPA in binding to fibrin (50, 51), thus leading to a decrease in both plasminogen activation and clot lysis. Furthermore, apo(a) KIV_{5-9}, as well as KV, were found to substantially inhibit the conversion of Glu-plasminogen to Lys-plasminogen, which is a reaction that enhances plasminogen activation (52). This was supported by a subsequent study that demonstrated inhibition of tPA mediated plasminogen activation *in vitro* in numerous cell types (53). Finally, Lp(a) was found to elevate the expression of plasminogen activator inhibitor-1 (PAI-1)(54). Therefore, Lp(a) may potentially contribute to the atherosclerosis through prothrombotic mechanisms, as well as through proatherogenic mechanisms.

1.2.5 Lp(a) Assembly

It is generally accepted that rate of assembly, and not the rate of catabolism, is the primary determinant of plasma Lp(a) levels (27, 55, 56). Both apo(a) and apoB are produced by hepatocytes in the liver (57, 58). Previous research has shown that, in the physiological setting, negligible amounts of free apo(a) exist as the majority of apo(a) associates with apoB following secretion (59). The formation of an Lp(a) particle occurs as

a two-step process, where an initial non-covalent association is subsequently followed by the formation of a disulfide bond linkage between apo(a) and apoB (60). It was initially determined that disruption of the weak LBS in apo(a) by lysine analogues led to a significant decrease in Lp(a) particle assembly (61). It was therefore postulated that the weak LBS in KIV_{5-8} were involved in mediating the initial non-covalent interaction between apo(a) and apoB (60). However, a study utilizing 17K apo(a), which is a physiologically relevant isoform of apo(a) that possesses eight KIV_2 repeats, demonstrated that only the weak LBS in apo(a) KIV_7 and KIV_8 are important for non-covalent assembly; these sites are required to mediate the initial non-covalent interaction with Lys^{680} and Lys^{690} in the amino-terminus of apoB-100 (62). Interestingly, it is also possible that ligand binding in the strong LBS of apo(a) KIV_{10} can induce a conformation change in apo(a), and accelerate covalent linkage with apoB (63). Since non-covalent assembly precedes the formation of the disulfide bond linkage, it has been postulated that this interaction may properly orient apo(a) and apoB to facilitate efficient disulfide bond formation (64). The covalent linkage has been reported to occur between Cys^{4057} of apo(a) in KIV_9 and Cys^{4326} in the C-terminal region of apoB-100 (65, 66). However, other studies have suggested that disulfide linkage occurs with Cys^{3734} in the C-terminal region of apoB (67-70).

Despite this generally accepted mechanism, the exact site of particle formation remains controversial. The majority of evidence suggests that the formation of the covalent bond between apo(a) and apoB-100 occurs extracellularly, or on the cell surface, of hepatocytes (71-76). Specifically, *in vitro* analysis in hepatocellular carcinoma (HepG2) cells utilizing r-apo(a) demonstrated that covalent Lp(a) particles were present in the medium, but absent in cellular lysates (73). This observation was further supported *in vitro*

through the discovery that extracellular disulfide bond formation occurs between apo(a) and apoB; this was proposed to be facilitated by Lp(a) oxidases secreted from HepG2 cells (74). Enzyme-linked immunosorbent assay (ELISA) analysis conducted with human liver tissue also established the absence of covalent Lp(a) particles in human liver homogenates (75). Conversely, multiple studies support an intracellular site of Lp(a) assembly (77-79); this data is supported by kinetic data in humans (80, 81). However, intracellular assembly of Lp(a) was only been observed in a previous study by Bonen *et al.*., where a very small, non-physiologically relevant isoform of apo(a) was used (79). While the kinetic data support the notion of intracellular assembly, it cannot exclude the possibility that the disulfide linkage between newly synthesized apo(a) and apoB-100 containing lipoproteins occurs extracellularly. It is possible that the non-covalent association between apo(a) and apoB-100 occurs intracellularly, with the subsequent covalent complex forming upon secretion of apo(a) and apoB from hepatocytes.

Interestingly, an association between the biosynthetic pathways of apo(a) and triglycerides has also been proposed, thus creating an intriguing link between the synthesis of apo(a) and apoB-100 containing lipids (77). Indeed, *in vitro* studies conducted in hepatoma cells demonstrated that a hypo glycosylated, immature, non-secretable form of apo(a) could associate with apoB-100, thus supporting the hypothesis that non-covalent assembly can occur intracellularly (82). Furthermore, a clinical study involving targeted therapy against the microsomal triglyceride transfer protein (MTP) further supports the concept that the secretion of apo(a) and apoB are linked. MTP is directly involved in the synthesis of apoB-containing lipoproteins (83). Inhibition of this protein leads to a significant reduction in apoB and a concomitant reduction in Lp(a) (84). Individuals with

abetalipoproteinemia, which involves a loss-of-function MTP, also possessed lowered levels of both apoB and Lp(a) (85). Finally, a kinetic study conducted in humans indicated that the production rate of apoB associated with Lp(a) particles was similar to apo(a), and different from apoB found in LDL (80). In support of this finding, Su *et al.* demonstrated that Lp(a) was composed of newly synthesized apo(a) and apoB moieties, and that the production rates of were similar for both components (81). Taken together, the previous studies suggest that the initial, non-covalent interaction between apo(a) and apoB may occur intracellularly, with the covalent assembly subsequently occurring on the cell membrane or in the extracellular milieu upon secretion of the complex.

Since the predominant mechanism dictating individual variation in Lp(a) levels is due to genetic differences within the *LPA* gene, it is important to understand the role of apo(a) biosynthesis in regulating Lp(a) production. Previous studies have established that the production of apo(a) is dependent upon the correct folding and processing of the protein in the ER, where small apo(a) isoforms are secreted more efficiently than large isoforms (28, 29, 86). While a variety of chaperones have been identified as playing a role in apo(a) maturation intracellularly, finding new players that are required for apo(a) maturation will help to further understand a potential regulatory mechanism involved in dictating plasma Lp(a) levels (76, 87, 88).

1.2.6 Production, Maturation, and Secretion of Apo(a)

The structure of apo(a), as well as the high levels of post-translational modifications required for its maturation, results in a complex biosynthetic pathway for this protein. As previously mentioned, apo(a) is synthesized in the liver, and it has been proposed that the rate of apo(a) synthesis is a major determinant of plasma Lp(a) concentrations (58, 88). The

production rate of the protein is dependent upon multiple factors. A previous study provided evidence that mRNA stability, as well as the rate of *LPA* gene transcription can alter the secretion rate of apo(a) (88). The isoform size of apo(a) also plays a role, with an inverse correlation observed between size and efficiency of synthesis (27, 28, 89). Previous studies have demonstrated that larger apo(a) isoforms are subject to increased ER retention times (27). This corresponds to increased time required for protein folding and modification of larger apo(a) isoforms (28). Pulse-chase studies conducted by White *et al.*. indicated that two forms of apo(a) were detectable in cell lysates, a hypo-glycosylated, immature form and a fully glycosylated, mature, secretable form. Furthermore, this study demonstrated that detection of mature apo(a) did not occur until approximately 30-60 minutes of chase time in baboon hepatocytes (28). This suggests that maturation of apo(a) is a rate-limiting step in apo(a) secretion, and that apo(a) is subject to complex folding kinetics (76). The general consensus is that small isoforms of apo(a) are more efficiently folded and secreted compared to large isoforms, the latter of which exhibits increased folding demands resulting in longer ER retention times, and thus, increased targeting to the endoplasmic-reticulum-associated degradation (ERAD) pathway (29, 86, 89). Interestingly, White and coworkers also found that 50% of apo(a) immunoprecipitated from the lysates of baboon hepatocytes was in the mature form (76), therefore suggesting that the intracellular transport of apo(a) through the biosynthetic pathway is another rate-limiting step in apo(a) secretion.

The proper completion of post-translational modifications is also required for the secretion of mature apo(a). Glycosylation is a post-translational modification that results in the addition of complex carbohydrate moieties to nascent proteins (90). Glycosylation plays

a role in the biosynthetic pathway of apo(a). Plasma-derived apo(a) possesses both N- and *O*-linked glycans, with the carbohydrate modifications accounting for approximately 28% of apo(a) by mass (91). Each individual apo(a) KIV domain contains at least one possible site for *N*-linked carbohydrate addition, while the linker regions contain multiple sites for *O*-linked glycosylation as the amino acid sequences in these regions are rich in serine/threonine residues (91, 92). Studies involving the inhibition of *N*-linked glycosylation in apo(a) resulted in aggregation of the protein in the ER, leading to increased levels of degradation (76, 93); this arises in part due to altered interactions between apo(a) and various chaperone proteins, thus increasing degradation rates of apo(a) (87-89).

Apo(a) has been found to directly interact with calnexin, BiP (immunoglobulin heavy chain binding protein), and protein disulfide isomerase (PDI). In general, these chaperone proteins act as regulators of quality control in the ER during protein synthesis, and can either retain misfolded proteins in the ER to facilitate corrective measures or target them to the ERAD pathway (94, 95). Furthermore, each of these chaperones have been proposed to regulate the intracellular, presecretory degradation rates of apo(a) (87, 89). Calnexin was found to transiently associate with apo(a) and facilitate movement of the immature, hypo-glycosylated form from the ER to the pre-medial Golgi compartment (87-89). Calnexin is an ER chaperone proteins that recognizes mono-glucosylated, *N*-linked carbohydrates on proteins, and it is an important component of the ERAD pathway (94). Generally, glucose molecules are sequentially cleaved from *N*-linked glycan precursors by glucosidases I and II in a process known as glucose trimming. Once removed, a protein can be shuttled to the Golgi for further modification and secretion (96). However, if a protein is improperly folded, a glucose residue is added back to the *N*-linked glycan motifs by

UDP-glucose:glycoprotein glucosyltransferase (UGGT) to re-establish an interaction with calnexin (97). Folded proteins that possess properly trimmed glycans cannot be recognized by UGGT, leading to secretion of the protein from the ER (97).

Studies conducted in baboon hepatocytes demonstrated that the presence of *N*-linked mono-glucosylated carbohydrates, as well as glucose trimming, were required for efficient secretion of apo(a) (89). Interaction with calnexin led to apo(a) retention in the ER, and prevented the improperly processed immature form of apo(a) from being exported to the Golgi or targeted to the ERAD pathway (89). Co-immunoprecipitation (co-IP) studies also demonstrated an interaction between apo(a) and the ER chaperones PDI and BiP (89). BiP targets improperly folded proteins to the ERAD pathway and retains misfolded proteins in the ER, while PDI has a well characterized role in catalysing the formation of disulfide bonds (89, 98). Upon completion of correct domain folding, apo(a) is transported out of the ER to the pre-medial Golgi compartment, where it undergoes further modification through the addition of *O*-linked glycans (91). Overall, the biosynthetic pathway of apo(a) is complex, and secretion of the mature protein is dependent upon multiple factors. However, further research is required to fully understand the biosynthetic pathway of apo(a), particularly with respect to the mechanistic details of apo(a) transport between intracellular compartments.

1.2.7 Lp(a) Catabolism

The catabolic pathways for Lp(a) removal from the circulation remains enigmatic despite numerous studies focused on elucidating mechanisms for particle clearance (reviewed in ref. 100). It is generally accepted that the liver is the predominant site of Lp(a) clearance, with a small role postulated for the kidneys in catabolism (99). Specifically,

individuals with impaired kidney function have been found to have higher levels of plasma Lp(a), which is correlated in part to a decrease in Lp(a) catabolism (100). However, in healthy individuals, the role of the kidneys in Lp(a) clearance was found to be minimal (101).

Numerous receptors have been implicated in Lp(a) catabolism. Based on the structural composition of Lp(a), it was initially postulated that the major catabolic pathway would be driven by the LDL component of Lp(a). As such, the LDL receptor (LDLR) was hypothesized to be the primary receptor in Lp(a) catabolism. However, evidence demonstrates a controversial role for the LDLR, as several studies support a role in Lp(a) catabolism (102-113), whereas multiple studies suggest that it does not mediate Lp(a) clearance (101, 114-117). The majority of these studies utilize *in vitro* methods, with the remainder using *in vivo* analysis in both mice and humans to analyze a potential role for the LDLR in Lp(a) catabolism. Furthermore, some studies focus on Lp(a) clearance in the context of Familial Hypercholesterolemia (FH) or proprotein convertase subtilisin kexin type 9 (PCSK9) activity. FH is an autosomal autosomal dominant disorder that is characterized by elevated levels of LDL cholesterol (LDL-C). It is caused by mutations in multiple genes that are associated with the production and catabolism of apoB-100 containing particles (118). PCSK9, on the other hand, is a serine protease zymogen that mediates the degradation of the LDLR (119).

In vitro analysis conducted in HepG2 and fibroblast cells demonstrates that Lp(a) can bind to and be internalized by the LDLR (102-105, 108). Furthermore, Lp(a) catabolism was found to be reduced in FH human fibroblasts (105, 109). This was supported by research conducted by our group, which demonstrated that PCSK9 treatment

led to a decrease in the amount of Lp(a) internalized in both HepG2 cells and human fibroblasts (112). Furthermore, Lp(a) internalization was reduced in HepG2 cells incubated with a monoclonal antibody that blocked LDLR activity, and in FH human fibroblasts (112). With respect to *in vivo* analysis, experiments conducted by Hofmann *et al.*. demonstrated that overexpression of the LDLR in a mouse model led to a significant increase in the clearance rate of injected Lp(a) when compared to control mice (110). Studies conducted in human patients revealed that Lp(a) plasma levels were significantly higher in individuals with FH as opposed to unaffected individuals (106, 107, 111). Furthermore, clinical trials studying evolocumab, a monoclonal antibody directed at PCSK9, showed reduced Lp(a) levels in treated patients compared to control patients. Taken together, these data provide strong evidence for an association between the LDLR and Lp(a) catabolism.

Conversely, *in vivo* studies conducted in a mouse model demonstrated that the catabolic rate of Lp(a) in *LDLR*$^{-/-}$ mice was similar to control mice (101). This was supported by a study conducted by Reblin *et al.*., which demonstrated no change in the uptake of radiolabelled Lp(a) in mice with defective LDLR when compared to control mice (117). Furthermore, in contrast to the aforementioned studies involving FH individuals, no significant difference in Lp(a) levels were observed between FH patients when compared to unaffected individuals (114-116). Studies conducted on statin therapy provide contradictory evidence for the role of the LDLR in Lp(a) clearance as well. Specifically, treatment with various statins was found to successfully lower LDL levels while concomitantly increasing, or having no effect on, Lp(a) levels (120). Overall, it is clear that further research is required to firmly establish if the LDLR plays a role in Lp(a) clearance.

Other receptors have been implicated in facilitating the internalization and degradation of Lp(a). These receptors include: the scavenger receptor class B type I (SR-B1), various plasminogen receptors, low-density lipoprotein-related protein (LRP-1), the very low density lipoprotein receptor (VLDLR), and megalin/glycoprotein 330 (gp330) (102, 108, 121-123). *In vitro* studies suggest that the apo(a) component is required to mediate an interaction with SR-B1, VLDL, LRP-1, and the plasminogen receptors, whereas the apoB component predominantly mediated an interaction with gp330. However, further research is required to characterize the exact mechanism of action for these receptors in Lp(a) catabolism. Overall, these data suggests that the catabolism of Lp(a) is complex, with the major pathway governing Lp(a) catabolism remaining enigmatic. Future work is required to further characterize receptors implicated in Lp(a) clearance, and to determine novel receptors that may play a role in mediating Lp(a) catabolism.

1.3 Sortilin: Defining Structure, Function, and Multi-Ligand Nature

Sortilin, also identified as neurotensin receptor-3, is a multi-ligand sorting receptor belonging to the vacuolar protein sorting 10 (VPS10) domain family (124, 125). Other members of this family include VPS10p, SorCS 1-3, and the LDLR-like SorLA (Fig. 1.6) (126, 127). Sortilin is classified as a type-1 transmembrane receptor and is characterized by the extracellular VPS10 domain. This domain, which constitutes the entire ectodomain of the protein, is composed of a 700 amino acid section containing a VPS10p-cysteine rich domain that binds ligands in the shape of a ten-bladed ß-propeller (128). The remaining structure consists of a single transmembrane segment, and a short, cytoplasmic tail that possesses multiple sorting/internalization motifs (129).

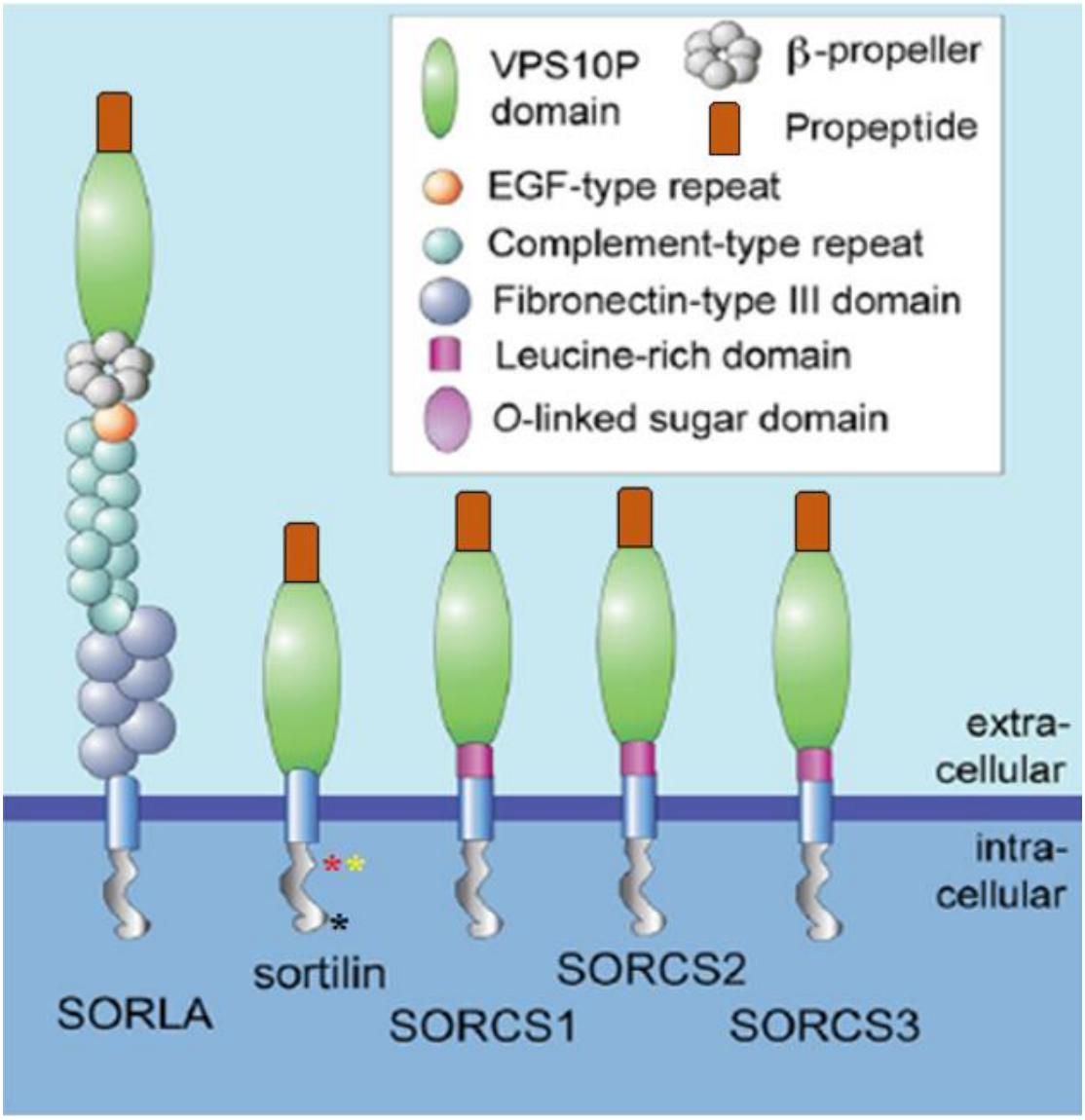


Figure 1.6: ***Structural representation of VPS10 domain family.*** The mammalian VPS10 domain family is represented by five proteins. They are all type-1 transmembrane proteins that are involved in cellular signaling and trafficking. Each receptor is initially synthesized as a propeptide. Removal of the prosegment, and activation of the receptors, is achieved in the Golgi through furin cleavage. Each receptor possess a unique VPS10 binding domain that is homologous to the VPS10P sorting receptor found in yeast. The VPS10 domain comprises the entire extracellular portion of sortilin, whereas the SORCS1-3 receptors contain an additional leucine-rich domain, and SORLA contains a variation of extracellular domains. The remaining structure is composed of a single transmembrane segment and short, cytoplasmic domains that possess multiple canonical sorting motifs. Location of the various sorting motifs of sortilin are indicated by asterisks, with red symbolizing the F-XXX-Y motif, yellow symbolizing the tyrosine motif, and black symbolizing the acidic dileucine motif. These motifs are also present in SORLA. Alternative splicing of SORCS1-3 leads to variation in the sorting motifs found in these domains. Image modified from reference 128.

Sortilin is initially synthesized as a propeptide, which is subsequently cleaved in the late trans-Golgi network (TGN) by the proprotein convertase furin (126, 130). This propeptide plays a crucial role in the early processing of sortilin by preventing premature ligand binding, and facilitating transport through the biosynthetic pathway. Upon cleavage, sortilin becomes an active protein receptor (126, 130). Research has demonstrated that sortilin is a multi-functional protein that interacts with a variety of ligands. Direct interactions have been documented with: neurotensin (131), the pro-form of nerve growth factor-ß (NGF) (132), the pro-form of brain-derived neurotrophic factor (BDNF) (133), tyrosine receptor kinases (Trk receptors) (134), amyloid precursor protein (APP) (135), progranulin (136),prosaposin and G_{m2} activator protein (G_{m2}AP) (137), apolipoprotein E (apoE) (138), lipoprotein lipase (LPL) (139), apolipoprotein A-V (140), apolipoproteinB-100 (141, 142), proprotein convertase subtilisin/kexin type 9 (PCSK9) (143) and receptor-associated protein (RAP) (124). Interestingly, the 44-amino acid propeptide that is generated by furin cleavage also exhibits high-affinity binding with the mature sortilin receptor. In general, these interacting partners compete for binding with the mature receptor (128, 130). This was specifically demonstrated between neurotensin, RAP, and the propeptide (130). This restriction in binding is attributed to restricted space within the 10-bladed ß-propeller domain (128).

Sortilin is highly expressed in neuronal tissues such as the brain, spinal cord, skeletal muscle, and thyroid; however, lower expression is also observed in the heart, liver, and kidneys (124, 129, 144, 145). With respect to the subcellular localization of sortilin, previous research found that the majority of sortilin localizes within the Golgi and endosomal compartments; however, sortilin is also able to localize at the cell surface (134,

135, 143, 146). With respect to physiological function, sortilin has been shown to play a role in a variety of biological processes that include: glucose metabolism (147, 148), regulation of neuronal cell proliferation (132, 140, 149), Golgi-to-lysosome trafficking (135, 139, 150, 151), and cell signaling and endosomal trafficking (134, 136, 140-143, 146, 152, 153). Overall, this research demonstrates that sortilin predominantly functions as an intracellular trafficking receptor.

Following activation by furin cleavage, the mature sortilin receptor facilitates sorting and trafficking through three main biology pathways. One pathway involves localization of sortilin to the cell surface through secretory vesicles, where it acts as an internalization receptor for a variety of ligands; sortilin internalizes through clathrin-mediated endocytosis (136, 139, 140, 142, 146, 150). A second pathway involves the transport of sortilin and its cargo from the cell surface, or the Golgi complex, to endosomes. Cargo either dissociates from sortilin in endosomal compartments, and is directed towards lysosomes, or is recycled with sortilin to the TGN (135, 137, 142, 146, 150, 154-158). The third pathway involves secretory conduits, where sortilin facilitates the trafficking of ligands from the Golgi to endosomes or secretory vesicles (129, 134, 141, 143, 152, 158, 159). In all cases, sortilin is recycled back to the TGN through retrograde transport mediated by the retromer adaptor complex (151, 160, 161). The retromer is a pentameric complex that was first identified in *Saccharomyces cerevisiae,* and previous research found that it played a role in recycling the VPS10p receptor, which is the bacterial orthologue of sortilin (162, 163). In mammals, the retromer is comprised of five subdomains that function in mediating the retrieval of several different cargo proteins from the endosome to the TGN (151).

Mechanistically, the functionality of sortilin is generally regulated by the intracellular domain of the protein, with the sorting and internalization motifs playing a major role. . This domain is involved in mediating receptor-cargo binding, degredation, and both the anterograde and retrograde transport of sortilin. The majority of the functionality of sortilin is dependent on interactions between the trafficking motifs of sortilin and various cargo adaptor complexes (Fig. 1.7) (146, 149, 151). In general, these trafficking motifs have been shown to be involved in adaptor protein binding, endocytosis, basolateral targeting and Golgi-endosome sorting (146). The intracellular domain of sortilin contains three canonical motifs: an acidic dileucine motif (HDDSDEDLLE), a tyrosine motif (YXXL or YSVL) and the consensus hexapeptide internalization motif (F-XXX-Y) (124). These motifs are also found in the cytoplasmic domain of the mannose-6-phoshpate receptor (MPR), which is an established sorting receptor in the TGN (164). Interestingly, the intracellular domain of sortilin is highly homologous to that of the MPRs, which suggests that these trafficking receptors may share similar trafficking pathways. Furthermore, immunogold labelling studies demonstrated that the subcellular distributions of both sortilin and MPRs were nearly identical in HepG2 cells, thus indicating that these receptors also share similar transport kinetics (161). This was further supported by co-localization studies, which demonstrated that sortilin and MPRs displayed strong co-localization, thus providing further evidence that these proteins function through similar pathways (124, 165). Previous research by Nielsen *et al.* supports these notions, as chimeric constructs consisting of the lumenal domain of MPR and the cytoplasmic domain of sortilin were able to rescue trafficking defects observed in cells lacking function MPRs (146). Overall, these studies indicate that sortilin and MPRs may act as redundant pathways for intracellular trafficking.

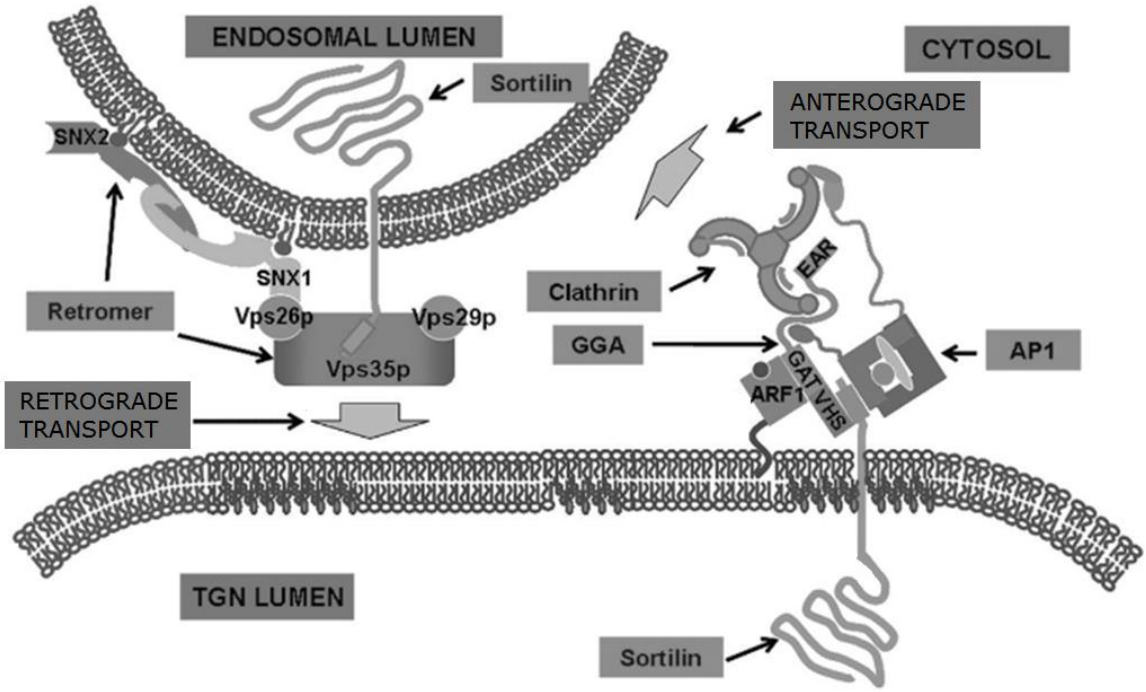


Figure 1.7: ***Intracellular interactions between the cytoplasmic domain of sortilin and various cargo adaptor proteins.*** Trafficking between the trans-Golgi network (TGN) and endosomal compartments is regulated by the interaction of sortilin with activator protein 1 (AP-1), Golgi-localizing, Gamma-adaptin -containing, ARF-binding proteins (GGAs), and the retromer complex. Sortilin interacts with clathrin through AP-1 and GGAs, which allows sortilin and its Golgi cargo to be internalized in clathrin-coated-cargo vesicles. Anterograde transport traffics these vesicles to endosomal compartments, where both the cargo and AP-1/GGAs are released. The exposed cytoplasmic domain interacts with the retromer complex, facilitating the retrograde transport of sortilin back to the TGN. Modified from reference 152.

Sortilin interacts with three types of adapter proteins (Fig. 1.7). One complex, the retromer, was mentioned previously. The remaining adaptor complexes include the activator protein 1 (AP-1) complex (166), and the Golgi-localizing, Gamma-adaptin ear-containing, ARF-binding proteins (GGA1, GGA2, and GGA3) (146, 167). Both AP-1 and the GGAs interact with clathrin, and mediate the trafficking capabilities of MPRs by facilitating the incorporation of the MPR-ligand complex into clathrin-coated cargo vesicles; this complex is subsequently transported to endosomes (151).

A similar mechanism is proposed for both AP-1 and the GGAs with respect to the intracellular trafficking of sortilin (Fig 1.7). Similar to MPRs, the interaction between sortilin and AP-1 is mediated through the tyrosine motif. Previous research involving the inhibition of AP-1 activity led to the increased accumulation of sortilin within the TGN (168). Multiple studies demonstrated that an intact tyrosine motif was required to mediate an interaction between sortilin and the retromer complex (161, 168). After sortilin binds to its ligand, it is proposed that this receptor-ligand complex is incorporated into clathrin-coated cargo vesicles and transported to the endosomes. While in the endosomes, AP-1 is released, thus exposing the cytoplasmic domain of sortilin, allowing the tyrosine motif to interact with the retromer complex. Mutation of either the tyrosine or leucine residue in the YXXL inhibited the interaction of the cytoplasmic domain of sortilin with the retromer. Consequentially, an accumulation of sortilin in the endosomal system, with a concomitant depletion in the Golgi complex, was observed as sortilin was unable to recycle back to the TGN (168). Therefore, the interaction between AP-1 and sortilin plays an important role in facilitating both the anterograde and retrograde transport of the sortilin and sortilin-ligand complexes. The dileucine motif is responsible for mediating an interaction between sortilin

and the GGAs. Similar to the studies on AP-1, mutation of the GGAs inhibited sortilin from participating in anterograde transport, as an increased accumulation of sortilin was observed in the perinuclear region (137, 146). Taken overall, sortilin functions as an intracellular trafficking receptor, and is functionally dependent upon interaction with various adaptor proteins.

Modification events of sortilin have also been implicated in regulating the functionality of the receptor (169). Previous studies found that MPRs possessed a casein kinase II phosphorylation motif, identified as ESEER, that was present immediately upstream of the dileucine sorting motif (170, 171). Phosphorylation of the serine residue within this motif increased binding affinity between GGAs and the cytoplasmic domain of MPRs, thus enhancing anterograde transport of the receptor-cargo complex (170, 171). The VPS29 subunit of the retromer was found to dephosphorylate this serine residue upon interaction of MPRs with the recycling complex, thus enhancing the interaction between the cytoplasmic domain of MPRs and the retromer (172, 173). These finding collectively indicated that phosphorylation of this motif plays a role in mediating the intracellular targeting of MPRs. Similar to MPRs, sortilin also contains a casein kinase II phosphorylation motif, identified as SGY. The similar subcellular distributions (161), as well as trafficking pathways (151), of sortilin and MPRs suggest that such a phosphorylation event may likewise regulate the intracellular targeting of sortilin. Palmitoylation, which is a reversible post-translationional modification involving the addition of a saturated palmitic acid on cysteine residues, was found to mediate levels of sortilin degradation. Previous studies found that a cysteine residue within the cytoplasmic domain of sortilin was palmitoylated, and that this modification was involved in controlling

the bioavailability of sortilin (169, 174). Removal of this modification led to the ubiquitination of sortilin, and subsequent degradation of the receptor in the lysosomal compartment (169). Taken together, these data demonstrate that both the bioavailability and activity of sortilin may be highly regulated within the cell.

1.3.1 Putative Role of Sortilin in Human Disease

A putative association between sortilin and a variety of human diseases has been suggested due to the multi-ligand and multi-functional nature of the trafficking receptor (reviewed in reference 170). First purified in human brain tissue when studying lipoprotein interactions, subsequent research led to the characterization of this receptor in various neurological and neurodegenerative diseases (175). A role for sortilin in promoting pro-apoptotic signalling and neuronal cell death has been established through its interaction with the proforms of various neurotrophin (NT) and neurological growth factors (NGF) (132-134, 159). Elevated levels of neuronal apoptosis are associated with numerous neurodegenerative diseases such as Alzheimer's and Parkinson's disease.

A putative role for sortilin in promoting the development of Alzheimer's disease has been postulated based on studies monitoring apoE and APP metabolism. Alzheimer's disease involves the accumulation of senile plaques within the hippocampus region of the brain, with development of these plaques precluding neuronal apoptosis (176). Experimental data demonstrate an ability for sortilin to act as a novel receptor for apoE in the brain. ApoE interacts with the amyloid-beta peptide (AB-ß), a cellular component of APP cleavage that is the main component of senile plaques in the brains of Alzheimer patients. This interaction facilitates the cellular catabolism of the complex through internalization by apoE receptors; thereby lowering the neurotoxic effects of AB-ß. Studies

conducted *in vivo* have demonstrated that increased sortilin expression is associated with a decrease in apoE/AB-ß accumulation, with sortilin promoting the lysosomal degradation of the apoE/AB-ß complex (138). Furthermore, sortilin was found to regulate the retrograde transport and activity of the ß-site ß-amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1). This protein catalyzes the initial phase of APP cleavage (158). Recent *in vitro* and *in vivo* work demonstrated that the intracellular domain of sortilin can directly interact with and promote the lysosomal targeting of APP. A functional hexapeptide motif was required for this interaction, as mutations in this domain led to a reduction in the ability of sortilin to mediate the lysosomal degradation of APP (135).

Associations between sortilin activity and the development of Diabetes mellitus have also been suggested through both *in vivo* and *in vitro* studies. Diabetes mellitus is a group of metabolic disorders that are characterized by hyperglycemia. Multiple studies have elucidated an association between sortilin and the intracellular trafficking of glucose transporter type 4 (Glut4) (147, 148, 165, 177). Glut4 plays a critical role in the regulation of blood glucose levels in adipose and muscle tissues. Elevated insulin levels lead to the translocation of Glut4 to the cell membrane and a concomitant increase in glucose uptake (148). Glut4 is contained and shuttled within Glut4 storage vesicles (GSVs) upon insulin response. Work conducted in adipocytes demonstrated that sortilin is a major component of GSVs (165, 177), and *in vitro* vesicle reconstitution assays indicated that the formation of GSVs can be regulated by sortilin activity (148). Furthermore, the VPS10 domain was found to facilitate translocation of GSVs to the cell surface upon response to elevated insulin levels (147). Therefore, these studies demonstrate that an increase in sortilin activity is associated with an increase in glucose uptake in adipocytes and muscle cells.

1.3.2 GWAS Studies and Identification of *SORT1* as a Risk Gene for Cardiovascular Disease and Its Role in LDL Metabolism

With multiple studies elucidating well-characterized roles for sortilin in neuronal cells, intriguing genome wide association studies (GWAS) led to an unexpected association between the 1p13 region and CAD (178-182). This suggested a role for sortilin in cardiovascular disease, particularly with respect to plasma cholesterol levels and risk of MI (183). Specifically, GWAS of European subjects possessing polymorphisms in the region near *SORT1* were associated with a ~5-8 mg/dL decrease in LDL cholesterol (LDL-C) and total cholesterol concentrations (153, 178). Furthermore, a 40% reduction in risk for myocardial infarction, and a 13% reduction in risk for CVD, was also associated with SNPs in the 1p13 region (153, 178). With respect to *SORT1*, these studies identified seven chromosomal loci to be associated with CAD; however, the gene cluster located in the 1p13 region appeared to have the most prominent role in affecting plasma LDL levels and MI risk (184).This locus contained a haplotype of three tightly linked genes in linkage disequilibrium, classified as *PSRC1*, *CELSR2*, and *SORT1*, that encode for the proline/serine-rich coiled-coil 1 (*PSRC1*) protein, the cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*) protein, and the sortilin protein (*SORT1*) respectively (183). These SNPs occurring in the 1p13 region, which include rs646776, rs599839, and rs12740374, were all found to alter the gene expression levels of *PSRC1*, *CELSR2*, and *SORT1*. The minor allele for rs646776 was found to increase mRNA levels. The minor G-allele of rs599839 increased *SORT1* gene expression without altering the expression of *PSRC1* and *CELSR2* (153). Fine mapping of SNP region also led to the discovery of the

minor T-allele at rs12740374, which was associated with increased *SORT1* gene expression and protein production (152, 185).

With the emergence of genetic analysis supporting a role for the 1p13 region in CAD, multiple studies were performed to determine a causal mechanism behind the association between this chromosomal region and CVD. Prior research demonstrated a role for sortilin in regulating lipoprotein catabolism; therefore, a similar role for sortilin in controlling LDL catabolism was postulated (124, 138-140). In a study monitoring the rs599839 SNP, HEK 293 cells were transfected with sortilin cDNA to determine if overexpression of the protein was related to increased LDL uptake. A significant increase was observed in LDL and RAP uptake in cells overexpressing sortilin (153). Therefore, it was proposed that individuals possessing the minor alleles for *SORT1* at this locus could lower plasma LDL levels by increasing the rate of LDL catabolism. This hypothesis was further supported in a subsequent study, where it was postulated that sortilin could act as an independent cell surface receptor for LDL. Indeed, an increase in the catabolic rate of LDL was observed *in vivo* in mouse models overexpressing hepatic sortilin (142). Furthermore, liver specific knockdown of sortilin expression in a mouse model was associated with a decrease in the catabolic rate of LDL (142). Interestingly, mutations involving the complete removal of the intracellular sorting domain, or mutagenesis of key leucine residues present in the acidic dileucine sorting motif, did not demonstrate a significant increase in LDL degradation. While the altered dileucine mutant had comparable LDL association rates to wildtype sortilin, the inability of sortilin to traffic from the cell membrane to endosomes inhibited this variant from mediating LDL degradation. The sortilin variant containing a removed cytoplasmic domain was unable to

associate with, or mediate the degradation of, LDL (142). In an attempt to determine if the regulation of LDL catabolism through sortilin expression was independent of the LDLR, knockout mice completely lacking the LDLR protein were studied the in setting of either sortilin knockout or overexpressing sortilin mice. Indeed, an increase in catabolism was also observed in mice lacking the LDLR, thus indicating an independent role for sortilin in LDL catabolism (142). Conversely, *in vivo* studies demonstrated that no difference in LDL catabolism was observed in a $SORT^{-/-}$ mouse model when compared to control mice (141).

In a study monitoring the rs12740374 SNP, humanized mouse models were utilized to study the effects of sortilin overexpression and sortilin knockdown on apoB and VLDL secretion (152). As previously mentioned, this study performed fine mapping of the 1p13 locus to determine the causal SNP variant involved in regulating LDL metabolism, as well as the effects upon gene expression brought about by this mutation. The presence of the minor T-allele, as opposed to the major G-allele, at this locus resulted in the generation of a C/EBP consensus site. Interestingly, the occurrence of the minor protective allele at this locus was found to be approximately 30% in European populations (152). Expression quantitative trait loci (eQTL) data demonstrated that a 5-6 fold increase in gene expression was observed with the *PSRC1* and *SORT1* genes for each copy of the minor allele (Fig. 1.8). (152). Interestingly, while sortilin is highly expressed in an abundance of human tissues, the increased gene expression brought about by the minor allele was liver specific, as no change in protein level was detected in adipose or lymphocyte tissues. This phenomenon could be explained by C/EBP, which is a liver-specific transcription factor. Generation of a consensus binding site would therefore lead to a hepatic-specific increase in sortilin production (152). With this knowledge, researchers conducting this study utilized

adeno-associated virus known to specifically alter gene expression in the liver to overexpress sortilin in humanized mice. Increased expression of hepatic sortilin was found to significantly reduce plasma LDL levels in these mice by 30-70%; this data agreed with the previous findings of the GWAS studies in human patients. To compliment these findings, siRNAs specific to the *SORT1* gene were utilized to knockdown hepatic sortilin production. Gene expression was reduced by 70-90% in humanized mice, and was accompanied by a 20-125% significant increase in LDL-C and VLDL-C levels (152).

With *SORT1* not implicated as the causal gene mediating the association between the SNPs in the 1p13 region and LDL-C levels, an attempt to elucidate a mechanistic basis for this association was performed through analysing the rate of VLDL secretion. Primary hepatocytes from a humanized mouse model were harvested from animals either overexpressing sortilin or siRNA directed against sortilin, and subjected to labelling experiments to monitor the rate of apoB secretion. A significant increase in apoB secretion was observed in hepatocytes that possessed siRNAs specific to *SORT1*. In support of this finding, the opposite trend was observed in mice overexpressing the *SORT1* gene, where higher sortilin levels correlated with a significant decrease in apoB secretion from hepatocytes. Since the gene expression of *PSRC1* was also greatly increased by the minor T-allele, identical overexpression analysis was performed to study the effects of altered *PSRC1* expression on LDL-C. However, no significant changes in LDL-C were observed, thus implicating *SORT1* as the sole causal variant associated with regulating LDL-C at the 1p13 locus. Therefore, it was proposed that individuals that were homozygous for the minor allele at SNP rs12740374 would possess a significantly lower risk rate for CAD due to a decreased rate of LDL production (152).

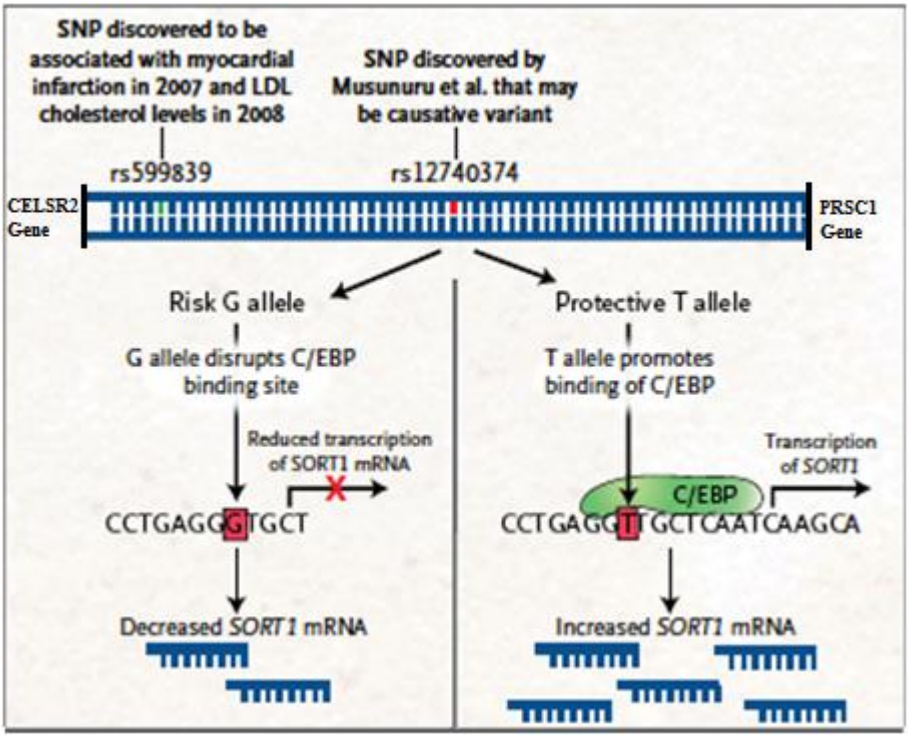


Figure 1.8: ***Schematic representation of SNP variation in the 1p13 gene and its effects on CAD.*** Various single nucleotide polymorphisms (SNPs) in the 1p13 gene region were identified through genome-wide association studies (GWAS) that significantly altered risk levels associated with coronary heart disease (CHD) and myocardial infarction (MI). The major SNPs were found to occur in a non-coding region between the *CELSR2* and *PSRC1* alleles. Initial studies demonstrated an association between the rs599839 SNP and MI and LDL cholesterol levels. Fine mapping of this intergenic region was conducted to identify a mechanism behind this association. The rs12740374 SNP was found to introduce a novel binding site for a liver-rich transcription factor identified as C/EBP. Increased binding of this factor lead to a significant upregulation in *SORT1* gene expression, as well as a concomitant elevation of the receptor sortilin. Image modified from reference 177.

With *SORT1* not implicated as the causal gene mediating the association between the SNPs in the 1p13 region and LDL-C levels, an attempt to elucidate a mechanistic basis for this association was performed through analysing the rate of VLDL secretion. Primary hepatocytes from a humanized mouse model were harvested from animals either overexpressing sortilin or siRNA directed against sortilin, and subjected to labelling experiments to monitor the rate of apoB secretion. A significant increase in apoB secretion was observed in hepatocytes that possessed siRNAs specific to *SORT1*. In support of this finding, the opposite trend was observed in mice overexpressing the *SORT1* gene, where higher sortilin levels correlated with a significant decrease in apoB secretion from hepatocytes. Since the gene expression of *PSRC1* was also greatly increased by the minor T-allele, identical overexpression analysis was performed to study the effects of altered *PSRC1* expression on LDL-C. However, no significant changes in LDL-C were observed, thus implicating *SORT1* as the sole causal variant associated with regulating LDL-C at the 1p13 locus. Therefore, it was proposed that individuals that were homozygous for the minor allele at SNP rs12740374 would possess a significantly lower risk rate for CAD due to a decreased rate of LDL production (152).

A subsequent study provided further evidence regarding an association between increased sortilin levels and a reduction in apoB secretion. *In vivo* studies in wild-type mice demonstrated that increased sortilin expression was associated with a decrease in apoB secretion. This association was replicated in LDLR knockout mice. Mechanistically, the ability of sortilin to lower apoB secretion appeared to be dependent upon lysosomal localization, as the introduction of an endolysosome inhibitor completely abolished this effect. Furthermore, while wildtype sortilin displayed an ability to co-localize with

lysosome markers, a sortilin mutant lacking the entire intracellular domain, as well as a mutant containing a mutated tyrosine and dileucine sorting motifs, were unable to replicate this localization with lysosomes. An inability to reduce apoB secretion was demonstrated in LDLR knockout mice expressing these sortilin variants, therefore supporting the hypothesis that sortilin reduced apoB secretion through facilitating the trafficking of the lipoprotein to lysosomes for degradation (142).

Conversely, an opposing study released shortly after demonstrated that increased sortilin expression was associated with an increase in LDL-C. This study demonstrated an approximately 30% reduction in plasma LDL levels in mice that were deficient in sortilin and LDLR, and was accompanied by a 48.2% reduction in apoB-100. To support this finding, overexpression of sortilin performed in hepatocytes yielded the opposite association, with an approximate 42% increase in total cholesterol as well as ~44% increase in apoB-100 (141). Interestingly, these findings only altered the levels of apoB-100, with apoB48 levels remaining consistent throughout, therefore implying a role for sortilin in regulating the metabolism of apoB-100 containing particles. To complement this hypothesis, interaction studies were performed to analyze a potential interaction between sortilin and apoB-100. Multiple experimental methods which included co-immunoprecipitation (co-IP), surface plasmon resonance, and Förster resonance energy transfer (FRET) demonstrated that an interaction occurs between sortilin and aboB100, with the highest association occurring in the Golgi compartments. The interaction between apoB-100 and sortilin, as assessed through SPR analysis, was found to be abolished when a sortilin mutant that was unable to undergo maturation by furin cleavage was used (141). A direct interaction between apoB and sortilin was also replicated in another study using

SPR analysis, where it was determined that the interaction between the two proteins was dependent upon a specific pH range. The highest rate of association was observed at a pH of 7.4, with dissociation rapidly occurring as the pH was lowered to 5 during analysis (142).

Similar to previous studies, an attempt to determine a mechanistic basis for the regulation of LDL-C was achieved through examining a potential role for sortilin in regulating apoB and VLDL secretion and LDL catabolism. However, in opposition to previous findings, metabolic labelling of hepatocytes demonstrated an increase in apoB-100 secretion with sortilin overexpression; sortilin knockout hepatocytes yielded an overall decrease in hepatic apoB-100 secretion. This relationship was reciprocated with respect to VLDL secretion. In support of these findings, experiments conducted by Strong *et al.*. further demonstrated that VLDL secretion rates were decreased in a $SORT^{-/-}$ mouse model when compared to control mice (142).

These data indicate that the relationship between sortilin expression and LDL metabolism is complex. However, it is clear that sortilin plays a role in regulating the metabolism of apoB-100, VLDL, and LDL particles. The enigmatic role of sortilin in regulating LDL-C levels may be dependent upon multiple conditions. The mechanism of regulation may be dependent upon the bioavailability of apoB and apoB-100 containing lipoproteins. Furthermore, the expression levels of sortilin may also play a role. Finally, environmental factors and experimental conditions may also regulate how sortilin affects LDL metabolism. Taken overall, it appears that the ability of sortilin to regulate LDL metabolism is dependent upon the metabolic milieu (183).

1.3.3 Potential Role of Sortilin in Atherosclerosis

It is universally accepted that LDL-C is involved in the development of atherosclerosis (4). With GWAS identifying a strong association between the 1p13 locus and LDL-C, and multiple mechanistic studies demonstrating *SORT1* as the causal gene responsible for this relationship, a role for sortilin was proposed in the process of atherosclerosis. Using apoE knockout mice that were deficient in sortilin expression, it was shown that reduced expression of sortilin protein did not alter plasma cholesterol levels, but led to a reduction in the development of atherosclerotic lesions (186). This result indicated a role for sortilin in promoting atherosclerosis through a mechanism independent of LDL-C regulation. Immune cells, as well as the production and recruitment of cytokines and proinflammatory molecules, play a crucial role in the development of atherosclerotic lesions (4). Studies conducted in sortilin deficient macrophages demonstrated a significant decrease in the secretion of the IL-6 and IFN-y cytokines. Furthermore, strong co-localization between the cytokines and sortilin was observed intracellularly in sortilin positive macrophages. Taken together, these data demonstrated that, in the absence of sortilin expression, an attenuation of the inflammatory response in immune cells was achieved with a corresponding reduction in the development of atherosclerosis in apoE knockout mice (186).

This association between sortilin expression and atherosclerosis was further demonstrated in macrophages. However, the mechanistic basis relied on variation in LDL internalization as opposed to cytokine regulation (187). In contrast to the previous study, no difference was observed in macrophage cytokine levels between sortilin knockout animals and control animals. However, sortilin deficiency was still shown to significantly

reduce the development of atherosclerotic lesions (187). With respect to mechanism, this study determined that sortilin overexpression led to a significant increase in the total cholesterol content of macrophages, which ultimately led to an increased rate of foam cell formation both *in vitro* and *in vivo* (187). This effect was significantly reduced by knocking down sortilin in macrophages. Furthermore, macrophages that were subjected to high concentrations of LDL demonstrated a significant increase in sortilin expression compared to cells with reduced cholesterol content (187). These data indicated that sortilin may be acting as a direct receptor for LDL clearance in macrophages. Since previous data indicated an ability for sortilin to regulate LDL-C levels independently of the LDLR receptor, a similar hypothesis was proposed for macrophage cholesterol uptake (141, 142). Indeed, *in vivo* experiments utilizing an inhibitor of actin polymerization and micropinocytosis demonstrated a significant reduction in LDL internalization in sortilin knock down macrophages, therefore supporting an LDLR independent mechanism for promoting LDL uptake and foam cell formation (187).

1.4 Proprotein Convertase Subtilin Kinexin 9

PCSK9 is a serine protease zymogen encoded by the PCSK9 gene found on chromosome 1p32.3 (188). First discovered in 2003, it is classified as the ninth member of the peptidase S8B family (189, 190). PCSK9 is ubiquitously expressed in a variety of tissues, with the highest levels of expression observed in the liver, intestines, and kidneys (190). Structurally, this enzyme is composed of a short, 30 amino acid signal peptide and a 121 amino acid pro-domain. These regions are followed by a catalytic domain possessing the D-H-S catalytic triad motif typically of serine proteases, a hinge region, and a C-terminal cysteine-histidine-rich domain (191). PCSK9 is initially synthesized as a 692-

amino acid propeptide. Maturation of the zymogen occurs after the removal of the signal peptide through autocatalytic cleavage of the prodomain between the amino acids Q_{152}-S_{153} in the ER. Similar to sortilin, the removal of the propeptide is a necessary step required for PCSK9 maturation (192). Furthermore, the newly formed propeptide can non-covalently bind to the catalytic domain of PCSK9 through hydrogen bond formation, leading to the catalytic inactivation of the mature enzyme (191). PCSK9 is ultimately secreted from the Golgi while complexed with its pro-domain (191).

The physiological function of PCSK9 is strongly associated with cholesterol homeostasis, as this enzyme directly binds to and promotes the degradation of the LDLR (119). This is achieved at the cell surface through an interaction between the catalytic domain of PCSK9 and the epidermal growth factor-like repeat homology domain (EGFA) of the LDLR (119). Under normal conditions, LDL will bind to its corresponding receptor, initializing clathrin-mediated endocytosis of the complex. Once in the acidic environment of the endosome, the LDLR undergoes a conformational change from an open state to a closed state and releases its LDL ligand. Unbound particles are transported to lysosomes for degradation while the LDLR is recycled to the cell surface (193, 194). However, in the presence of PCSK9, the LDLR is unable to adopt a closed state in endosomes. The interaction between PCSK9 and the LDLR is strengthened by the acidic conditions of endosomes. Salt bridge formation between the pro-domain of PCSK9 and the ß-propeller domain of LDLR, as well as a proposed interaction between the C-terminal domain of PCSK9 and the ligand-binding domain of LDLR, prevent the receptor from transitioning into a closed formation (195). As a result, the receptor is shuttled in complex with LDL and PCSK9 to the lysosome for degradation (196). Interestingly, a direct association between

LDL and PCSK9 can occur, where the lipoprotein particle has been observed to bind to the N-terminal region of PCSK9. This interaction prevents PCSK9 from binding to the LDLR, therefore abolishing its ability to degrade the receptor (197).

1.4.1 Regulation of PCSK9 Activity

PCSK9 activity and expression can be regulated in a variety of ways (reviewed in ref. 180). At the endogenous level, the secreted form of PCSK9 can undergo catalytic cleavage at an arginine residue present in the 218 amino acid position to produce in inactive protein product. Truncation of PCSK9 is achieved by other proprotein convertases, specifically furin and PC5/6A (198). Numerous natural mutations in the population have also been shown to moderate the functional ability of PCSK9 to degrade the LDLR. A well-studied gain-of-function mutation, D374Y, has been shown to have a 25-fold increase in affinity for the LDLR, which concomitantly leads to a significant increase in LDL-C levels (199). Data indicate that the presence of a tyrosine residue at this position enhances binding of PCSK9 to the extracellular domain (ECD) of the LDLR (191). Conversely, loss-of-function mutations have consistently demonstrated a negative correlation with LDL-C levels. Specifically, the L253F missense mutation was found to inhibit autocatalytic cleavage, leading to a significant reduction in circulating PCSK9 levels. A C679X nonsense mutation in the C-terminal domain leads to atypical folding of PCSK9, resulting in accumulation in the ER and an inhibition of secretion of the mature protein (200). Overall, greater than fifty PCSK9 variants have been characterized with respect to plasma cholesterol levels (199).

1.4.2 Association of PCSK9 with Sortilin

PCSK9 is a soluble protein that is incapable of interacting with adaptor proteins in cellular compartments, therefore indicating a role for chaperone proteins in facilitating its secretion from the ER and Golgi organelles. A recent study has demonstrated an interesting association between sortilin and PCSK9 secretion. SPR analysis demonstrated a pH dependent interaction between sortilin and PCSK9, with high affinity binding observed at pH 7.4 and an abolishment of this interaction observed at pH 5.5 (143). Co-IP studies in hepatic cells further supported this interaction, and further demonstrated that sortilin can bind the immature form of PCSK9 as well (143). Interestingly, a similar pH dependent interaction was observed between LDL and sortilin in hepatic tissues (141, 142). Mechanistically, the ability of sortilin to facilitate PCSK9 secretion was found to be dependent upon an interaction occurring late in the secretory pathway. A significant extent of co-localization was observed between the two proteins in the TGN (143). Experiments conducted with sortilin knock-out mice also demonstrated a significant alteration in subcellular localization for both the immature and mature forms of the zymogen, therefore suggesting that sortilin acts as a molecular chaperone for PCSK9 through the secretory pathway (143).

1.5 Rationale and Objectives

Elevated plasma levels of Lp(a) have been identified through recent meta-analyses to be an independent, causal risk factor for CHD (15, 31). Therefore, it is important to develop an understanding of factors that increase Lp(a) concentrations in humans. However, the metabolism of Lp(a) remains enigmatic. Furthermore, factors involved in

regulating the intracellular transport of apo(a) through its biosynthetic pathway remain unknown.

Lp(a) assembly occurs as a two-step mechanism. The majority of studies suggest that an intracellular, non-covalent interaction occurs between apo(a) and apoB, with covalent assembly of Lp(a) occurring extracellularly. Furthermore, previous studies indicate that the secretion of apoB and apo(a) with respect to Lp(a) particles are linked (80-82, 85). As such, increasing the rates of either apoB or apo(a) biosynthesis could potentially lead to a concomitant increase in plasma Lp(a). Previous studies suggest that apo(a) maturation and intracellular trafficking are the rate-limiting steps in apo(a) production (76), however, little is known about the intracellular transport of apo(a). The rate of Lp(a) catabolism also regulates plasma Lp(a) levels. Multiple receptors have been implicated in Lp(a) catabolism; however, the primary route of Lp(a) clearance remains undefined.

Previous research shows that sortilin can regulate lipoprotein metabolism (124, 138-140). In particular, studies have shown that sortilin is a novel regulator of apoB/VLDL secretion and LDL catabolism (141, 142, 152, 153). Furthermore, multiple studies establish sortilin as an intracellular trafficking receptor. Since Lp(a) is structurally similar to LDL, it is possible that sortilin may likewise regulate Lp(a) catabolism in a manner similar to that of LDL. Moreover, the possible association between apoB and apo(a) secretion indicates that sortilin may likewise affect apo(a) secretion.

Therefore, we hypothesize that sortilin can mediate Lp(a) clearance by acting as a novel cell surface receptor for Lp(a). Moreover, we hypothesize that sortilin may directly bind to apo(a), and regulate its rate of secretion by facilitating the intracellular trafficking of nascent apo(a). To address this hypothesis, the following specific aims were developed:

1) To assess if a direct interaction can be observed between sortilin and either Lp(a) or apo(a);

2) To define a role for hepatic sortilin in apo(a) secretion;

3) To determine if sortilin can increase the rate of Lp(a) or apo(a) internalization in hepatocytes

4) To identify if sortilin displays subcellular co-localization with Lp(a) or apo(a) in hepatocytes

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture – Established Cell Lines

All cell lines used in experiments were grown and cultured at 37ºC in humidified incubators containing an air:CO_2 ratio of 19:1. Human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC) and maintained in minimum essential medium (MEM; Gibco) supplemented with 10% FBS (ATCC) and 1% antibiotic-antimycotic (10 units/mL penicillin G sodium, 10 μg/mL streptomycin sulfate, and 25 ng/mL amphotericin B) (Gibco).

Human fibroblast cell lines established from patients with primary familial hypercholesterolemia (FH) were obtained from the Coriell Institute (catalogue numbers GM01386, GM01355, and GM00701) and maintained in MEM (Gibco) containing 10% (v/v) FBS (ATCC). Experiments with FH fibroblasts were performed between passages 8 and 20.

Human Embryonic Kidney (HEK293) cells were cultured in 100 mm tissue culture plates (Sarstedt) in MEM supplemented with 5% FBS (v/v) (Gibco) and 1% antibiotic-antimycotic.

HepG2 cells lines stably expressing a 17-kringle form (17K) of recombinant apo(a) (r-apo(a)) were constructed as follows. HepG2 cells were seeded at a density of 75,000 cells/well and transfected using MegaTran 1.0 transfection reagent (Origene) with 1 μg/well of expression plasmid (201) and 0.2 μg/plate of a plasmid encoding a neomycin resistance protein as per manufacturer's protocol. The transfection mixture was left on the cells for 24 hours, after which the cells were given fresh medium and allowed to recover

for 24 hours. The cells were then incubated in complete medium containing 400 μg/mL Geneticin® selective antibiotic (G418 sulfate; Thermo Scientific). Surviving colonies emerged after 3 weeks of selection and individual cell lines were obtained by dilution cloning.

2.2 Mouse Primary Hepatocyte Isolation and Culture

Wild-type mice of the background strain (C57BL/6) were obtained from Jackson Laboratories. Primary hepatocytes from wild-type mice were prepared by a modified collagenase perfusion method (202). Mice were subjected to deep anesthesia (pentobarbitol sodium diluted in saline at a dose of 50 mg/kg); mice were determined to have met surgical plane anesthesia when toe pinch reflex was absent. Following thoracotomy and catheterization of the hepatic portal vein, the livers were perfused with 50 mL of HEPES-buffered saline (HBS; 20 mM HEPES pH 7.4 containing 150 mM NaCl and 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) at a flow rate of 8 mL/min, followed by 50 mL of HBS containing 5 mM calcium chloride ($CaCl_2$) and collagenase (from *Clostridium histolyticum*, 0.5 mg/mL) at a rate of 6 mL/min. After isolation, viability of hepatocytes was assessed by trypan blue exclusion staining (viability of >80% was considered acceptable) and the cells were plated at a density of 2.0×10^5 cells/well into gelatin-coated 24-well plates in Williams E media (GIBCO) supplemented with 10% FBS (ATCC), 2 mL-glutamine (GIBCO), and 1% antibiotic-antimycotic (GIBCO). Approximately two hours after seeding, non-adherent cells were aspirated from the plate and fresh medium was added. The animal experiments described above were specifically approved by the University of Windsor Animal Use and Care Committee

(protocol #14-24) and mice were maintained following the Canadian Council on Animal Care guidelines at the University of Windsor.

2.3 Expression and Purification of Recombinant Apo(a)

The construction of expression plasmids encoding the r-apo(a)variants employed in this study (17K-pRK5, 17KΔLBS7,8-pRK5 -pRK5, or 17KΔLBS$_{10}$-pRK5) was previously described, as was creation of stably-expressing cell lines in Hek293 cells (23). Conditioned medium was harvested from lines stably-expressing the respective variants, and was supplemented with PMSF to a final concentration of 1mM. Lysine-Sepharose affinity chromatography was utilized to purify apo(a) from the conditioned media as previously described (23, 203). Protein concentrations were determined spectrophotometrically using pre-determined molar extinction coefficients (204). The purity of r-apo(a) was assessed through comparative analysis to a purified r-apo(a) standard. This was achieved using SDS-PAGE followed by silver stain analysis, where 20 μL of a 25 ng/μL solution was loaded for both the purified and standard samples. One band was detected for both samples at approximately 270 kDa, indicating that the protein solution was pure.

2.4 Construction of Recombinant Sortilin Expression Plasmids

A pcDNA 3.1C/Myc-His expression vector encoding wild-type sortilin was generously provided by Dr. Nabil Seidah. This vector contained the cDNA for human sortilin as well as a Myc-His tag at the carboxyl terminus. The cDNA of full-length, wild-type sortilin was amplified by polymerase chain reaction and inserted into pcDNA4A/Myc-His digested with *EcoRV* and *XbaI* sites. The sequences of the primer pairs are as follows: sortilin sense, 5'- CGG TCG AGA TGG AGC GGC CCT GGG GAG CT -3' (*EcoRV* site

underlined), sortilin anti-sense, 5'- CCT CTA GAT TCC AAG AGG TCC TCA TCT GAG TC -3' (*XbaI* site underlined). The pcDNA 3.1C/Myc-His expression vector containing the cDNA for human sortilin was used as the template.

Two different wild-type sortilin versions were constructed: a soluble wild-type sortilin (without the transmembrane region and cytoplasmic domain), and a wild-type sortilin including the transmembrane region but lacking the cytoplasmic domain (sortilinΔCT) in the pcDNA 4A/Myc-His expression vector. The pcDNA 4A/Myc-His expression vector containing the cDNA for human sortilin was used as the template. The same primer was used for the amino-terminus of the protein while the primer for the carboxyl terminal region differed. The sequences of the primer pairs are as follows: amino-terminus 5'- CGG TCG AGA TGG AGC GGC CCT GGG GAG CT -3' (*EcoRV* site underlined), soluble sortilin anti-sense, 5'- GGT CTA GAA TTT GAC TTG GAA TTC TG -3' (*XbaI* site underlined), sortilinΔCT anti-sense, 5'- ACT CTA GAC CTT CCC CCA CAG ACA TAT TTC -3' (*XbaI* site underlined). The resulting sequences were amplified by polymerase chain reaction and inserted into the pcDNA 4A/Myc-His expression vector.

For the sortilin trafficking variants, mutagenesis was carried out using the *Q5* Site-Directed *Mutagenesis* Kit (New England Biolabs) according to the manufacturer's protocol. The primers used are shown in Table 2.1. For the sortilin trafficking mutants, the template for the mutagenesis was the full-length, wild-type sortilin in pcDNA 4A/Myc-His expression vector. For the sortilin polymorphic variants, mutagenesis was carried out using the *Q5* Site-Directed *Mutagenesis* Kit according to the manufacturer's protocol. The primers used are shown in Table 2.2. For the sortilin polymorphic variants, the template for

the mutagenesis was the sortilin wild-type-pcDNA 3.1C/Myc-His expression vector. The presence of the mutations was verified by DNA sequence analysis.

Table 2.1 ***Primer sequences for construction of Sortilin Trafficking Mutants***[a]

Trafficking Mutants	Primer Sequence
Sortilin Y792A	5'-GTT CCT GGT GCA TCG A**GC** CTC TGT GCT GCA GCA G-3'
Sortilin L829A/L830A	5'-GAT GAC TCA GAT GAG GAC **GC**C **GC**G GAA CCT CGA GGT CAC CC-3'

Table 2.2 ***Primer sequences for construction of Sortilin Polymorphic Variants***[a]

Polymorphic Variant	Primer Sequence
SORT1-I124V	5'-CACTGGGGTC**GTT**CTAGTCTTG-3'
SORT1-K205N	5'-ATTTTGCGAA**T**AATTTTGTGCAAAC-3'
SORT1-K302E	5'-TATTGGTGTG**G**AAATCTACTCATTTG-3'
SORT1-F404Y	5'-GAGACGGAC**TAT**ACCAACGTG-3'
SORT1-E444Q	5'-GAGGAAGCCT**CAA**AACAGTGAATGTG-3'
SORT1-E447G	5'-GAAAACAGT**GG**ATGTGATGCTACAGC-3'
SORT1-V650M	5'-CAAGTCATCC**ATG**TGTCAGAATGG-3'

[a]Sense strand sequences only are shown. Mutated codons are double underlined; mutated nucleotides are in boldface type. The 5′ end of the antisense primer was designed at the base next to the 5′ end of the sense primer, and preceded in the opposite direction on the complementary strand. The antisense primer was complementary to the plasmid sequence.

2.5 Construction, Expression and Purification of Recombinant PCSK9

The construction and expression of PCSK9-pcDNA 4C in HEK293 is previously described (112). Conditioned medium was harvested from cells stably-expressing PCSK9 and supplemented with PMSF to a final concentration of 1mM. Nickel-Sepharose excel (GE Healthcare) affinity chromatography was utilized to purify PCSK9 from the conditioned medium as previously described (112). The bicinchoninic acid assay (BCA

assay; Pierce) was utilized to determine the concentrations of the purified protein samples. The purity of PCSK9 was assessed through comparative analysis to a purified PCSK9 standard. This was achieved using SDS-PAGE followed by silver stain analysis, where 20 μL of a 25 ng/μL solution was loaded for both the purified and standard samples. One band was detected for both samples at approximately 74 kDa, indicating that the protein solution was pure.

2.6 Transient Transfections

For internalization experiments, HepG2 cells grown to 70% confluence in 6 well tissue culture plates were transfected with 1μg of either pcDNA 4A/Myc-His empty vector (Invitrogen), sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, sortilin L829A/L830A-pcDNA 4A/Myc-His, sortilin I124V-pcDNA3. 1C/Myc-His, sortilin K205N-pcDNA3.1C/Myc-His, sortilin K302E-pcDNA3.1C/Myc-His, sortilin F404Y-pcDNA3.1C/Myc-His, sortilin E444Q-pcDNA3.1C/Myc-His, or sortilin V650M-pcDNA3.1C/Myc-His using linear PEI (Sigma) as per the manufacturer's protocol.

For pulse-chase experiments, HepG2 cells grown to 70% confluence in 100 mm tissue culture plates were transfected with 5 μg of either 17K apo(a)-pRK5, 17K$\Delta_{7,8}$ apo(a)-pRK5, or 17KΔLBS$_{10}$ apo(a)-pRK5 and either pcDNA 4A/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, or sortilin L829A/L830A-pcDNA 4A/Myc-His using linear PEI. HepG2 cells stably expressing 17K apo(a) grown to 70% confluence in 100 mm tissue culture plates were transfected with 5μg of either pcDNA3.1C/Myc-His empty vector or sortilin wildtype-pcDNA 3.1C/Myc-His with linear PEI.

For siRNA knockdown internalization experiments, HepG2 cells grown to 50% confluence in 6 well tissue culture plates were transfected with 80 pmol of either sortilin siRNA or scrambled control siRNA (Santa Cruz Biotechnology) as per the manufacturer's protocol. For siRNA-mediated sortilin knockdown pulse-chase experiments, HepG2 cells stably expressing 17K apo(a) cells grown to 50% confluence in 100 mm tissue culture plates were transfected with 440 pmol of either sortilin siRNA or scrambled control siRNA.

Human fibroblast cells were plated in 24-well tissue culture plates at 200,000 cells/well and were transfected 1-2 hours with 1μg of either pcDNA3.1C/Myc-His empty vector or sortilin wildtype-pcDNA 3.1C/Myc-His using Lipofectamine® 3000 (Invitrogen) as per manufacturer's protocol.

Primary mouse hepatocytes were grown to 90% confluence in 24-well tissue culture plate. The medium was changed approximately 1-2 hours after seeding. Approximately 1-2 hours following medium change, the cells were transfected with 1μg of either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, or sortilin L829A/L830A-pcDNA 4A/Myc-His using Lipofectamine® 3000.

2.7 Preparation of Lipoprotein-Deficient Serum

Lipoprotein-depleted serum (LPDS) was prepared by addition of 1.21 g/mL sodium bromide (NaBr) to FBS (ATCC) followed by ultracentrifugation at 45,000 *x* g for 18 hours at 4^{o}C. The top layer was removed by needle aspiration, and the infranatant was extensively dialyzed at 4^{o}C against HBS (20 mM HEPES pH 7.4 containing 150 mM NaCl) twice for

two hours, and once overnight. The dialyzed LPDS was sterilized by passing through a 0.22- μm filter prior to supplementation of cell culture medium.

2.8 Internalization Assays

HepG2 cells were seeded into 6-well tissue culture plates at 3 x 10^5 cells/well, and were allowed to attach overnight prior to transfection. Following overnight transfection with either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, sortilin L829A/L830A-pcDNA 4A/Myc-His, sortilin I124V-pcDNA3. 1C/Myc-His, sortilin K205N-pcDNA3.1C/Myc-His, sortilin K302E-pcDNA3.1C/Myc-His, sortilin F404Y-pcDNA3.1C/Myc-His, sortilin E444Q-pcDNA3.1C/Myc-His, or sortilin V650M-pcDNA3.1C/Myc-His, cells were trypsinized and seeded into 24-well tissue culture plates (pre-coated with 1 mg/mL gelatin type A) at 3 x 10^5 cells/well. The cells were grown in MEM containing 10% (v/v) LPDS for 16 hours prior to treatments. Cells were washed twice with Opti-MEM (Gibco) and subsequently treated with Lp(a) (10 μg/mL) or r-apo(a) variants (200 nM) in the absence or presence of either purified recombinant PCSK9 (20 μg/mL) or storage buffer (20 μg/mL) in Opti-MEM for 4 hours at 37^{o}C. For experiments utilizing ε-aminocaproic acid (ε-ACA), cells were treated with Lp(a) (10 μg/mL) or r-apo(a) variants (200 nM) in the absence or presence of 0.2M ε-ACA for 4 hours at 37^{o}C.

For experiments involving siRNA-mediated sortilin knockdown, cells were seeded into 6-well tissue culture plates at 2 x 10^5 cells/well, and were allowed to attach overnight prior to transfection. Following transfection with 80 ρmols of either sortilin siRNA or scrambled control siRNA, cells were trypsinized and seeded into 24-well tissue culture

plates (pre-coated with 1mg/mL gelatin type A) at 3 x 10^5 cells/well. The cells were sustained in MEM containing 10% (v/v) LPDS for 16 hours prior to treatments. Cells were washed twice with Opti-MEM and subsequently treated with Lp(a) (10 µg/mL) or r-apo(a) variants (200 nM) in the absence or presence of either purified recombinant PCSK9 (20 µg/mL) or storage buffer (20 µg/mL) in Opti-MEM for 4 hours at 37ºC.

Lysates were prepared from the cells as follows: the cells were extensively washed at 4ºC in the following order: three times with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, KH_2PO_4 at pH 7.4) containing 0.8% (w/v) bovine serum albumin (BSA; PBS-BSA), twice with PBS-BSA containing 0.2 M ε-ACA for 5 minutes each, once with acid wash (0.2 M acetic acid pH 2.5 containing 0.5 M NaCl) for 10 minutes, once more with PBS-BSA, once more with acid wash for 10 minutes, and finally twice for 5 minutes with PBS. The cells were then subsequently lysed in lysis buffer (50 mM Tris pH 8.0, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) SDS, 1 mM PMSF). Lysates were subsequently subjected to SDS-PAGE and western blot analysis as described below.

For internalization experiments in human fibroblasts, cells were seeded into a 24-well tissue culture plate at 3 x 10^5 cells/well, and were allowed to attach for approximately 1-2 hours prior to transfection. Following overnight transfection with either pcDNA3.1C/Myc-His empty vector or sortilin wildtype-pcDNA 3.1C/Myc-His, the cells were grown in MEM containing 10% (v/v) LPDS for 16 hours prior to treatments. Cells were washed twice with Opti-MEM and treated with Lp(a) (10 µg/mL) or apo(a) (200 nM) in the absence or presence of either purified recombinant PCSK9 (20 µg/mL) or storage buffer (20 µg/mL) in Opti-MEM for 4 hours at 37ºC. Cells were extensively washed as

described for HepG2 cells above, with some exceptions. Following the initial PBS-BSA wash step, the cells were washed twice with PBS containing 10 µg/mL heparin for 10 minutes each, followed by a single wash with PBS-BSA containing 0.2M ε-ACA for 5 minutes. Additionally, only one acid wash step was performed, which was followed by a neutralization wash (0.5 M HEPES pH 7.5 containing 100 mM NaCl) for 10 minutes and a single wash with PBS for 5 minutes. Lysates were prepared as described above.

For internalization experiments in primary mouse hepatocytes, cells were seeded into a 24-well tissue culture plate at 3 x 10^5 cells/well, and were allowed to attach for approximately 1-2 hours. Medium was changed, and cells were transfected approximately 1-2 hours following medium change. Following transfection with either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, or sortilin L829A/L830A-pcDNA 4A/Myc-His, the cells were grown in Williams' Medium E containing 10% for 16 hours prior to treatment. Cells were washed twice with Opti-MEM and treated with Lp(a) (10 µg/mL) in Opti-MEM for 4 hours at 37^{o}C. Cells were extensively washed as described for HepG2 cells above. Lysates were prepared as described above.

2.9 Secretion Studies

HepG2 cells stably expressing stably expressing 17K r-apo(a) were seeded into 6-well tissue culture plates at 3 x 10^5 cells/well and were allowed to attach overnight prior to transfection. Following overnight transfection with either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, sortilin I124V-pcDNA3. 1C/Myc-His, sortilin K205N-pcDNA3.1C/Myc-His, sortilin K302E-pcDNA3.1C/Myc-His, sortilin F404Y-pcDNA3.1C/Myc-His, sortilin E444Q-pcDNA3.1C/Myc-His, or sortilin V650M-

pcDNA3.1C/Myc-His, the medium was changed and the cells recovered for 24 hours. Following 24 hours, 200 µL samples of medium was harvested from each well, and lysates were prepared from the cells as follows: the cells were washed twice with ice cold PBS and subsequently lysed in lysis buffer (50mM Tris pH 8.0, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1 mM PMSF). Lysates were subsequently subjected to SDS-PAGE and western blot analysis as described below.

2.10 Pulse-Chase Studies

For overexpression studies, HepG2 cells were seeded into 100 mm tissue culture plates at 1.5 x 10^6 cells/plate, and were allowed to attach overnight prior to transfection. Following transfection with either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, SortilinΔCT-pcDNA 4A/Myc-His, sortilin Y792A-pcDNA 4A/Myc-His, or sortilin L829A/L830A-pcDNA 4A/Myc-His, the cells were trypsinized and seeded into 6-well plates at 6 x 10^5 cells/well. The cells were grown in MEM containing 10% FBS (ATCC) overnight prior to labelling. For siRNA-mediated sortilin knockdown studies, HepG2 cells stably expressing 17K apo(a) were seeded into 100 mm tissue culture plates at 1.75 x 10^6 cells/plate, and were allowed to attach overnight prior to transfection. Following transfection with either pcDNA3.1C/Myc-His empty vector, sortilin wildtype-pcDNA 3.1C/Myc-His, sortilin siRNA or scrambled control siRNA, the cells were trypsinized and seeded into 6-well plates at 6 x 10^5 cells/well. The cells were grown in MEM containing 10% FBS overnight prior to labelling.

On the day of the experiment, the cells were washed once with 1 mL of PBS, pre-incubated for 1 hour in 1 mL of methionine- and cysteine-free Dulbecco's Modified Eagle Medium (DMEM; Gibco) without serum and subsequently pulse-labelled in the same

medium containing 200 µCi/well of [^{35}S]-cysteine/[^{35}S]-methionine labeling solution (Perkin Elmer Life Sciences) for 1 hour. Following labelling, the wells were washed once with 1 mL of PBS, and chased in 1 mL of complete growth medium for 0, 30, 60, 120, 240, or 480 minutes. At each of these chase times, conditioned medium was collected and stored on ice, and the cells were washed once with ice-cold PBS and subsequently lysed in cold lysis buffer (50mM Tris pH 8.0, 1% (w/v) NP-40, 0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS, 1 mM PMSF). Media and lysates were clarified by centrifugation at 15,000 rpm in a microcentrifuge for 6 minutes to remove cellular debris, and the supernatants were subjected to immunoprecipitation as described below.

Both media and lysates (1 mL and 500µL respectively) were pre-cleared by incubation with 30 µL of gelatin-agarose (Sigma) for 2-3 hours at 4^{o}C while gently shaking. Samples were then immunoprecipitated with saturating quantities (1 µL) of anti-apo(a) antibody (a5) (205) overnight at 4^{o}C while gently shaking. After overnight incubation, 30 µL of protein G-agarose beads (Novex) was added and the mixtures incubated for 2-3 hours at 4^{o}C while gently shaking. The resulting pellets were washed three times with 500 µL of RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 20mM EDTA, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS), washed once with 500 µL of TE buffer (10 mM Tris pH 7.5, 1mM EDTA) and finally re-suspended in 2x SDS Sample Buffer (250 mM Tris pH 6.8, 4% (w/v) SDS, 0.001% (w/v) bromophenol blue, 40% (v/v) glycerol) supplemented with 7 µL of 100 mM dithiothreitol (DTT). Samples were briefly centrifuged to pellet the agarose, boiled for 7 minutes, and then pulse-centrifuged again.

Immunoprecipitates were subjected to SDS-PAGE on 7% polyacrylamide gels. Following electrophoresis, the gels were incubated in 100 mL fixing solution

(methanol:H_2O:glacial acetic acid, 40:50:10) while gently shaking for 20 minutes, briefly rinsed with milli-Q H_2O, and then incubated in 100 mL of Amplify solution (Amersham Biosciences) while gently shaking for 20 minutes. The gels were then incubated in 100 mL of milli-Q H_2O containing 5 drops of 100% glycerol while gently shaking for 10 minutes. Following the various washing steps, the gels were dried using a gel dryer (BioRad Model 583) on cycle 2 at 80ºC for 45 minutes. Finally, the gels were exposed to a phosphor K screen (BioRad) at room temperature for 90 hours before screens were imaged using a BioRad Molecular Imager FX phosphoimager. Densitometric quantification of resulting bands was performed using Alpha View software (Alpha Innotech). Lysate samples were determined by measuring the combined total density of the immature and mature forms of intracellular r-apo(a), as the two bands could not be reliably resolved for quantitative analysis.

2.11 Co-immunoprecipitation Assay Using Sortilin, Lp(a)/apo(a) and/or apoB Pull-Down

For co-immunoprecipitation (IP) studies involving sortilin, HepG2 cells were seeded into 6-well tissue culture plates at 4 x 10^5 cells/well. For co-IP studies involving Lp(a)/apoB, HepG2 cells were seeded into 100 mm tissue culture plates at 3 x 10^6 cells/plate. The cells were allowed to attach overnight, and were sustained in MEM supplemented with 10% LPDS and 1% antibiotic-antimycotic. The cells were washed twice with Opti-MEM, and treated with Lp(a) (10 µg/mL) in Opti-MEM for 4 hours at 37ºC.

For co-IP studies involving apo(a)/apoB, HepG2 cells stably expressing 17K apo(a) were seeded into 6-well tissue culture plates at 4.5 x 10^5 cells/well (sortilin pull-down) or into 100 mm tissue culture plates at 3 x 10^6 cells/plate (apo(a) pulldown). The cells were

allowed to attach overnight, and were sustained in MEM supplemented with 10% LPDS and 1% antibiotic-antimycotic.

For co-IP studies involving sortilin, the cells were washed once with PBS, and treated with 1mM of the reducible protein cross-linker dithiobis(succinimidylpropionate) (DPS; Pierce) in Opti-MEM for 30 minutes at room temperature (sortilin pull-down experiments only). The reaction was quenched through the addition of 1 mL of 100 mM Tris for 15 minutes at 4^{o}C. The cells were washed twice with PBS, and lysed with 1 mL of IP lysis buffer (50 mM Tris pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1.5% (v/v) NP-40, 0.4% (w/v) sodium deoxycholate, 5% (v/v) glycerol). Lysates (1 mL) were subjected to centrifugation at 15,000 rpm in a microcentrifuge for 10 minutes to clear cellular debris, and subsequently pre-cleared by incubation with 30 µL of gelatin-agarose for 2-3 hours at 4^{o}C while gently shaking. The lysates were divided into two tubes, each receiving 450 µL, and were incubated with either no antibody or goat-anti-human sortilin polyclonal antibody (R&D Systems) overnight at a final concentration of 2 µg/mL at 4^{o}C while gently shaking. The resulting pellets were washed three times with 500 µL of ice cold IP wash buffer (50 mM Tris pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) NP-40, 0.4% (w/v) sodium deoxycholate, 5% (v/v) glycerol). Samples were re-suspended in 2X SDS Sample Buffer supplemented with 7 µL of 100 mM DTT, and briefly centrifuged to pellet the beads. Samples were boiled for 7 minutes, and then pulse-centrifuged again before subjecting samples to SDS-PAGE and western blot analysis as described below.

For co-IP studies involving Lp(a)/apo(a) and/or apoB, the lysates were incubated with either 1 µL of an anti-apo(a) antibody (a5) (205) or 5 µL of rabbit-anti-human apoB

polyclonal antisera (Abcam) overnight at 4°C while gently shaking. After overnight incubation, 30 µL of protein G-agarose beads was added and incubated for 2-3 hours at 4°C while gently shaking. The beads were collected by brief centrifugation. The resulting pellets were washed three times with ice cold RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 20mM EDTA, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS) and once with TE buffer (10 mM Tris pH 7.5, 1mM EDTA). Samples were prepared as described above.

2.12 Western Blotting

For internalization studies, as well as secretion studies, cell lysates were subjected to SDS-PAGE on 5-15% (Lp(a)-treated cells) or 7-15% (apo(a) treated cells) polyacrylamide gradient gels. For co-IP studies, supernatants were subjected to SDS-PAGE on 5-12% polyacrylamide gradient gels. All samples were prepared as 50 µL aliquots, and re-suspended in 12 µL 2X SDS Sample Buffer supplemented with 7 µL of 100 mM DTT. Samples were boiled for 7 minutes, and then pulse-centrifuged again before subjecting samples to SDS-PAGE. The gels were electrophoretically transferred in ice cold transfer buffer (25mM Tris pH 7.8, 1.92 M glycine, 10% (v/v) Methanol) for 2 hours at 0.2 milliamps onto PVDF membranes (Millipore) and blocked in 15 mL of either 50 mM Tris pH7.6 containing 150 mM NaCl, 0.2% (v/v) Tween-20 (TBST) supplemented with 5% (w/v) powdered non-fat milk or 50 mM Tris pH 7.6 containing 6 mM EDTA, 150mM NaCl, 0.05% (v/v) TritonX-100 (NET; Lp(a)/apo(a) blots only) supplemented with 5% (w/v) powdered non-fat milk for 1 hour at room temperature while gently shaking. Following blocking, the membranes were incubated with either mouse-anti-human apo(a) a5 antibody (205), mouse-anti-human β-actin (Sigma), mouse-anti-human c-Myc epitope tag (N-EQKLISEEDL-C; Invitrogen), mouse-anti-human apoB-100 (Santa Cruz), or goat-anti-

human sortilin (R&D Systems) overnight in the same blocking buffer at 4ºC while gently shaking. After overnight incubation, the membranes were washed three times with 15 mL of TBST or NET for 10 minutes each while gently shaking. The membranes were then subsequently incubated with either sheep-anti-mouse secondary antibody (GE Healthcare) or bovine-anti-goat secondary antibody (Santa Cruz Biotechnology) in blocking buffer for 45 minutes at room temperature while gently shaking. The membranes were washed again three times with TBST or NET for 10 minutes each while gently shaking. Immunoreactive bands were visualized with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) using a FluorChem Q Imager (Alpha Innotech) and densitometric quantification of resulting bands was performed using Alpha View software (Alpha Innotech).

2.13 Immunofluorescence Studies

For apo(a) co-localization studies, HepG2 cells stably expressing 17K apo(a) were seeded at 1.5 x 10^5 cells/well onto coverslips (pre-coated with 1 mg/mL gelatin type A) placed in 24-well tissue culture plates. The cells were allowed to attach overnight in MEM supplemented with 10% LPDS. The cells were extensively washed at 4ºC as follows: three times in PBS-BSA, twice in PBS-BSA containing 0.2M ε-ACA for 5 minutes each, and three times with PBS. The cells were subsequently fixed with 3.7% paraformaldehyde (Sigma) for 20 minutes at room temperature. The cells were washed once with PBS, and then permeabilized with 0.2% (v/v) Triton X-100 in PBS for 5 minutes, and blocked with blocking buffer (PBS containing 5% (w/v) BSA and 0.1% (v/v) Triton X-100) for 30 minutes while gently shaking at room temperature. The cells were subsequently incubated with mouse-anti-human apo(a) antibody (a5) (205) (1:50), goat-anti-human sortilin

polyclonal antibody (R&D Systems) (1:100), and either rabbit-anti-human mannosidase II polyclonal antibody (Millipore) (1:100), rabbit-anti-human calnexin polyclonal antibody (Abcam) (1:100), rabbit-anti-human EEA1 polyclonal antibody (Abcam) (1:250), or rabbit-anti-human M6PR polyclonal antibody (Abcam) (1:100) in blocking buffer for 45 minutes at 37^{o}C.

Following primary antibody incubation, cells were washed three times with PBS for 5 minutes each at room temperature and then incubated with Alexa Fluor 488-conjugated donkey-anti-goat IgG (Invitrogen) (1:500), Alexa Fluor 568-conjugated donkey-anti-mouse IgG (Invitrogen) (1:500), and Alexa Fluor 647-conjugated donkey-anti-rabbit IgG (Invitrogen) (1:500) in blocking buffer for 30 minutes at 37^{o}C. Following secondary antibody incubation, the cells were washed 3x with PBS, with the second wash containing 1 µL/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma), for 5 minutes each and mounted to glass slides using anti-fade fluorescence mounting medium (Dako). Immunofluorescence microscopy was performed on an Olympus IX81 confocal microscope (Olympus America) using a 40x water immersion lens. Images were projected using Olympus software. Post-acquisition, brightness-contrast adjustments were made in Microsoft PowerPoint 2013 and were applied equally to all panels.

2.14 Sortilin Binding Assays

Purified soluble sortilin was dialyzed against 0.1M Na_2CO_3 pH 8.6 containing 0.2M NaCl. The protein was then incubated with a 5-fold molar excess of Alexa Fluor 488 carboxylic acid, succinimidyl ester mixed isomers dissolved in dimethyl sulfoxide at 10 mg/mL (Invitrogen). The reaction mixture was rocked at 4^{o}C for 4 hours to facilitate protein labelling. The reaction was quenched through addition of 0.01 volumes of 1 M Tris, pH 8.0

for 30 minutes at 4ºC. Unbound dye was removed through extensive dialysis against 25 mM HEPES, pH 7.5 containing 300 mM NaCl, 50 mM KH_2PO_4, 0.1 mM $CaCl_2$, and 10% (v/v) glycerol. Labeled sortilin was concentrated using an Amicon Ultra-4 centrifugal filter with a 10 kDa membrane cut-off (Millipore). The concentration of labeled-sortilin as determined spectrophotometrically as previously described (112).

Binding curves were generated by incubating LDL or Lp(a), at 0.5 mg/mL, with increasing amounts of soluble sortilin-Alexa 488 (25-800 nM) in binding buffer (25 mM HEPES pH 7.4 containing 150 mM NaCl, 2 mM $CaCl_2$, and 1% (w/v) BSA) for one hour at 37ºC. Glycerol was added to the samples to a final concentration of 10% (v/v) and the samples were subjected to electrophoresis on 0.9% agarose gels (UltraPure Agarose, Invitrogen) for 2 hours at 40V in 90 mM Tris pH 8.0 containing 80 mM borate and 2 mM calcium lactate. In-gel scanning and quantification of the amount of labeled sortilin bound to Lp(a) or LDL was performed with a FluorChem Q imager (Alpha Innotech). Intensity of bands corresponding to bound sortilin were plotted as a function total concentration of sortilin and the data were fit to a single site saturation ligand binding equation by non-linear regression analysis using SigmaPlot 11.

2.15 Statistical Methods

For all internalization experiments, secretion studies, and pulse-chase experiments involving 17KΔLBS$_{7,8}$ and 17KΔLBS$_{10}$ apo(a), comparisons between data sets were performed using two-tail Student's t-test assuming unequal variances

For pulse-chase experiments involving 17K apo(a), statistical analyses were performed with the use of GraphPad Prism software, version 7.0 (Graphpad Software, Inc.).

Comparisons between samples were performed by one-way ANOVA using a Tukey post-hoc analysis. Statistical significance was assumed at $p < 0.05$.

CHAPTER 3: RESULTS

3.1 Sortilin Overexpression Alters Wild-Type Apo(a) Secretion in HepG2 Cells.

Sortilin expression has previously demonstrated an ability to alter the hepatic secretion of VLDL (152, 206), apoB-100-containing lipoproteins (141, 206), or apoB-100 itself (142). We hypothesized that sortilin may likewise influence the secretion of apo(a), and therefore affect Lp(a) levels in plasma. A ^{35}S-Met/Cys pulse-chase protocol was utilized with hepatocellular carcinoma (HepG2) cells transiently expressing a recombinant 17K variant of apo(a) (17K r-apo(a)), which is a physiologically relevant apo(a) variant that possesses eight KIV_2 repeat domains. These cells were also transiently transfected with an expression vector encoding sortilin or the corresponding empty vector as a control to analyze increased sortilin expression on apo(a) secretion (Fig. 3.2 A-C).

As demonstrated in Fig. 3.2 B, there was a significant difference in the intracellular accumulation of 17K r-apo(a) in cells overexpressing sortilin between 0-120 minutes of chase time when compared to control cells. A significant difference in the accumulation of secreted 17K r-apo(a) in cells overexpressing sortilin was also observed between 30-480 minutes of chase time when compared to control cells (Fig. 3.2 C). The earliest appearance of mature apo(a) secreted into the medium was observed at 30 minutes for both control and sortilin expressing cells (Fig. 3.2 A,C). Qualitative analysis of the fluorograms indicated that there does not appear to be a notable difference in the rate of conversion from the immature to mature form of apo(a) between cells expressing sortilin and control cells. The rate of accumulation both intracellularly and in the medium is therefore altered by sortilin overexpression without an apparent effect in the dynamics of secretion. Apo(a) is initially

synthesized as a hypo-glycosylated precursor in the ER, with final maturation occurring in the *medial*- to *trans*-Golgi compartment (82). As a result, *N*-linked glycans are added to nascent apo(a) post-translationally, while *O*-linked glycosylation occurs in the Golgi compartment (92). As a result, two unique apo(a) species are detectable in immunoprecipitated, radiolabelled lysates that represent mature (top band) and immature (bottom band) apo(a) (28, 76). As demonstrated in the representative fluorographs (Fig. 3.2 A), two bands in the lysates representing 17K r-apo(a) are observed. The initial, intracellular r-apo(a) detected at 0 minutes of chase time (Fig. 3.2 A) is a higher mobility species that likely represents an immature, incompletely glycosylated form. Conversion of immature r-apo(a) to the mature, fully glycosylated, secretable form that is identical in mobility to the 17K r-apo(a) detected in the conditioned medium was observed over the time course of the experiment (Fig. 3.2 A). Radiolabelled immature 17K apo(a) was not detectable in the lysates after 120 minutes of chase, while mature apo(a) was first observed at 30 minutes of chase time. Small amounts of labelled mature apo(a) were still detectable by 480 minutes of chase. (Fig. 3.2 A, B). With respect to lysates, the intracellular r-apo(a) densitometric values are a representation of the total densities of the immature and mature forms depicted in Fig. 3.2 A. The two bands could not be reliably resolved for quantitative analysis, and were therefore analyzed in combination.

Multiple studies have identified sortilin as a novel mammalian sorting receptor. A previous study monitoring a relationship between sortilin expression and LDL metabolism found that some regulatory abilities were dependent upon a fully functioning cytoplasmic domain (142). Furthermore previous studies have found that mutation of the canonical YXXΦ tyrosine and DXXLL dileucine motifs (where X is any amino acid and Φ is any

bulky, hydrophobic amino acid) in sortilin inhibit its abilities to function as a trafficking receptor (142, 146, 168). To determine if the observed increase in apo(a) accumulation brought about by sortilin overexpression was dependent upon its ability to act as a trafficking receptor, we employed variants of sortilin in which this function was disrupted by site-directed mutagenesis. Three mutants of human sortilin were generated through site-directed mutagenesis. The tyrosine residue in the YXXΦ motif was substituted with an alanine residue, yielding Y792A. Likewise, the leucine residues present in the DXXLL motif were also substituted with alanine residues in combination, yielding L829/830A. Finally, a premature stop codon was introduced into the sequence subsequent to the transmembrane domain, yielding a sortilin variant, recognized as sortilinΔCT, which completely lacked a cytoplasmic domain (Fig. 3.1).

HepG2 cells were transiently transfected with 17K r-apo(a), as well as with an expression vector encoding either sortilinΔCT, sortilin Y792A, or sortilin L829/830A (Fig. 3.2 A-C). The three sortilin mutants expressed at similar levels in cell lysates, as determined through western blot analysis (data not shown). As demonstrated by asterisks in Fig. 3.2 B, there was a significant difference in the intracellular accumulation of 17K r-apo(a) in cells overexpressing sortilin Y792A between 30-120 minutes of chase time compared to control cells. No significant difference in intracellular accumulation was observed between control cells and cells expressing either sortilinΔCT or L829/830A. A significant difference in the accumulation of secreted 17K r-apo(a) in cells overexpressing both sortilin Y792A or L829/830A was only observed at 480 minutes of chase time when compared to control cells (Fig. 3.2 C, asterisks).

The defect in the ability of sortilin to increase both secreted and intracellular apo(a) was most profound in cells expressing the sortilinΔCT mutant. In fact, the amount of apo(a) in cells expressing sortilinΔCT was indistinguishable from that found in control cells. A significantly lower effect on secreted apo(a) was observed between 30-480 minutes of chase time, and a significantly reduced amount of intracellular apo(a) was observed between 30-120 minutes of chase time, in sortilinΔCT expressing cells when compared to cells expressing wild-type sortilin (Fig. 3.2 C, daggers). Reduction of the increasing effect on apo(a) secretion was not as prominent in cells expressing sortilin Y792A or L829/830A. The expression of either mutation led to lowered levels of both intracellular and secreted 17K r-apo(a) accumulation; however, levels of intracellular r-apo(a) were not significantly different from cells expressing wild-type sortilin (Fig. 3.2 B). This trend was also observed with secreted r-apo(a) accumulation, with the exception at 30 minutes of chase time, where a significantly lower amount of 17K r-apo(a) was secreted in cells expressing either sortilin Y792A or L829/830A when compared to sortilin expressing cells (Fig. 3.2 C, daggers). The earliest appearance of mature apo(a) secreted into the medium was again observed at 30 minutes for cells expressing both empty vector and sortilin (Fig. 3.2 A, C). Similar to cells expressing wild-type sortilin, qualitative analysis of the fluorograms indicated that there is no notable difference in the rate of conversion from the immature to mature form of apo(a) in cells expressing the sortilin mutants. The rate of accumulation in cells expressing the mutants is lowered both intracellularly and in the medium compared to cells expressing wild-type sortilin without an apparent effect in the dynamics of secretion.

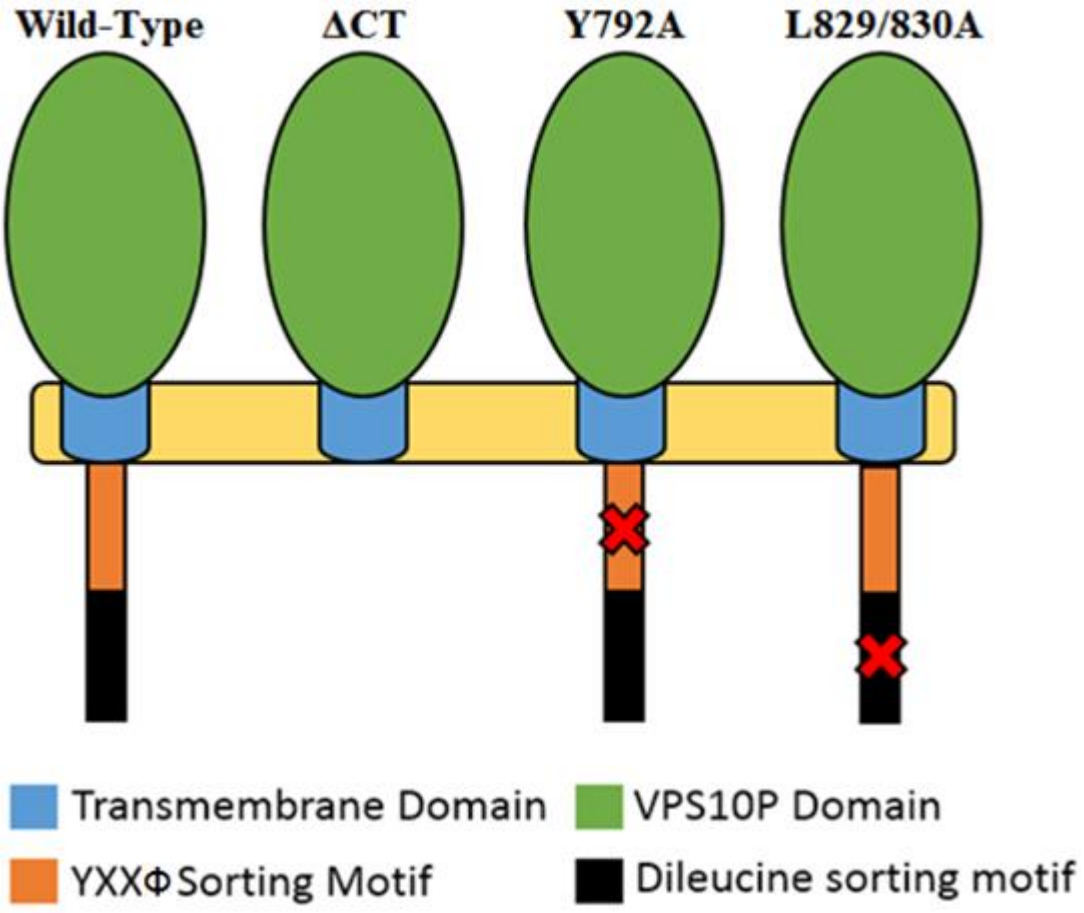


Figure 3.1: ***Recombinant sortilin constructs utilized in study.*** Sortilin is a type-1 transmembrane protein that is composed of an extracellular VPS10 domain, a single transmembrane domain, and a cytoplasmic tail containing multiple, canonical sorting motifs. From left to right: wild-type sortilin, sortilinΔCT (lacking a carboxyl terminal domain), sortilin Y792A (lacking a functional YXXΦ sorting motif), and sortilin L829/830A (lacking a functional DXXLL sorting motif).

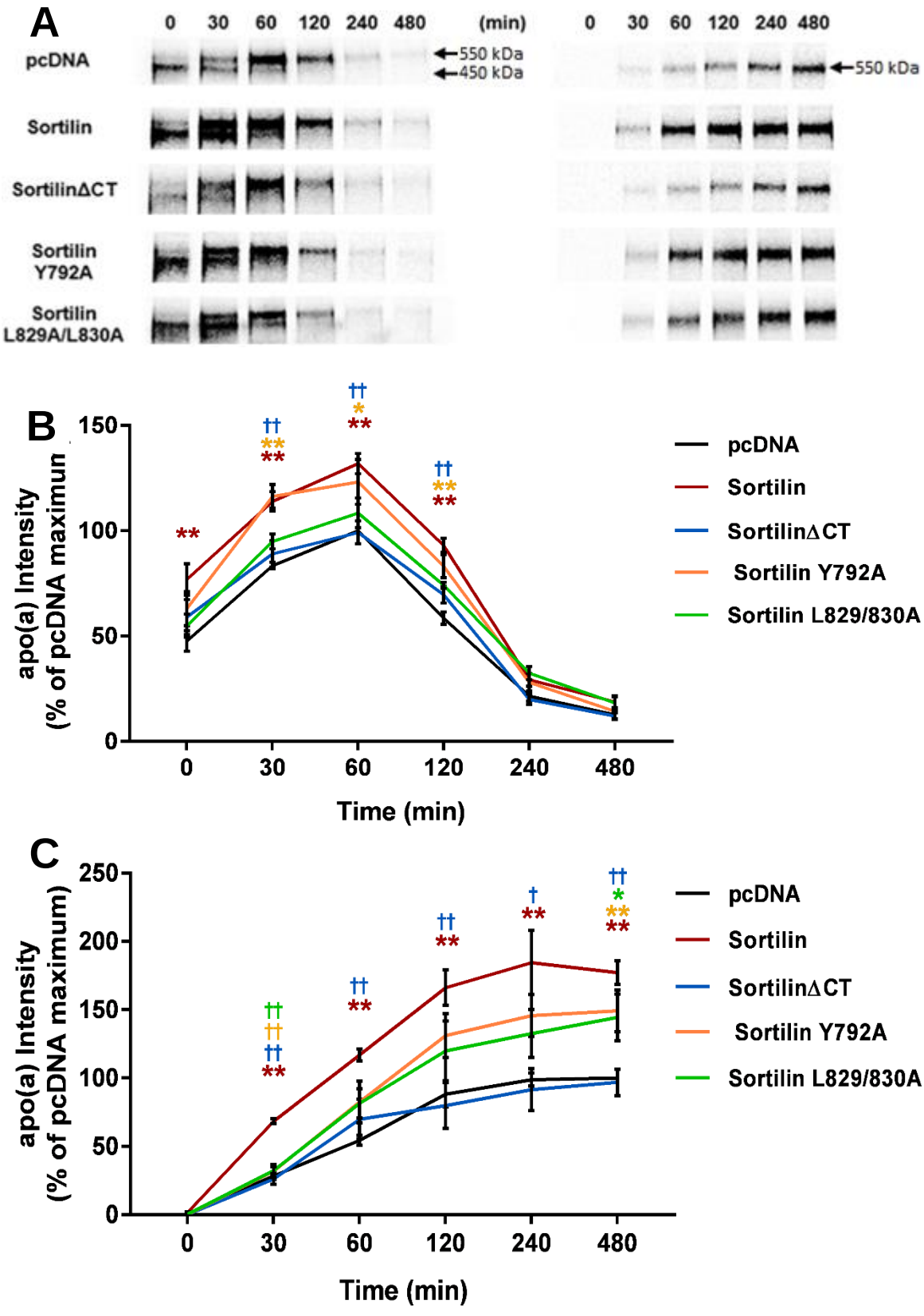
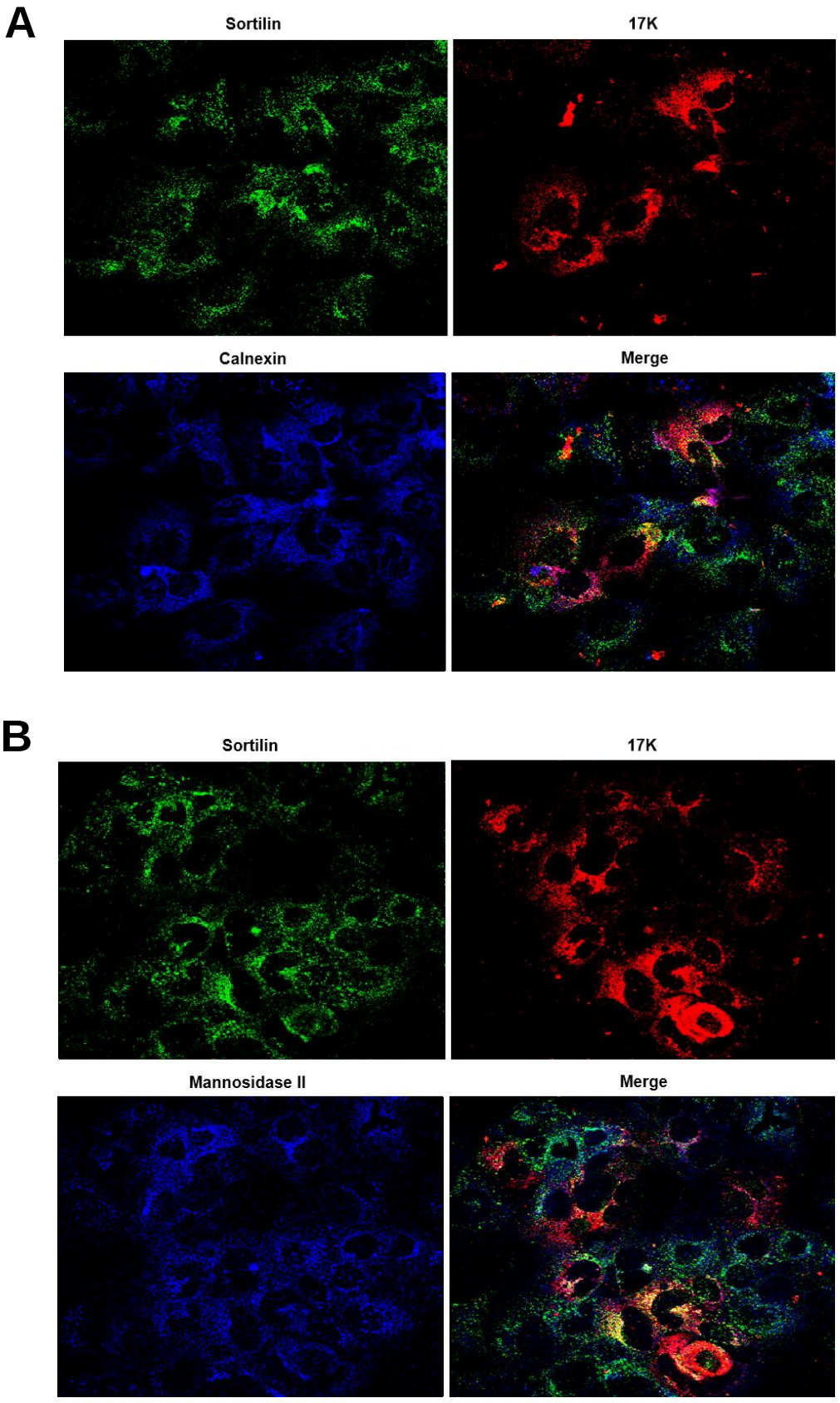
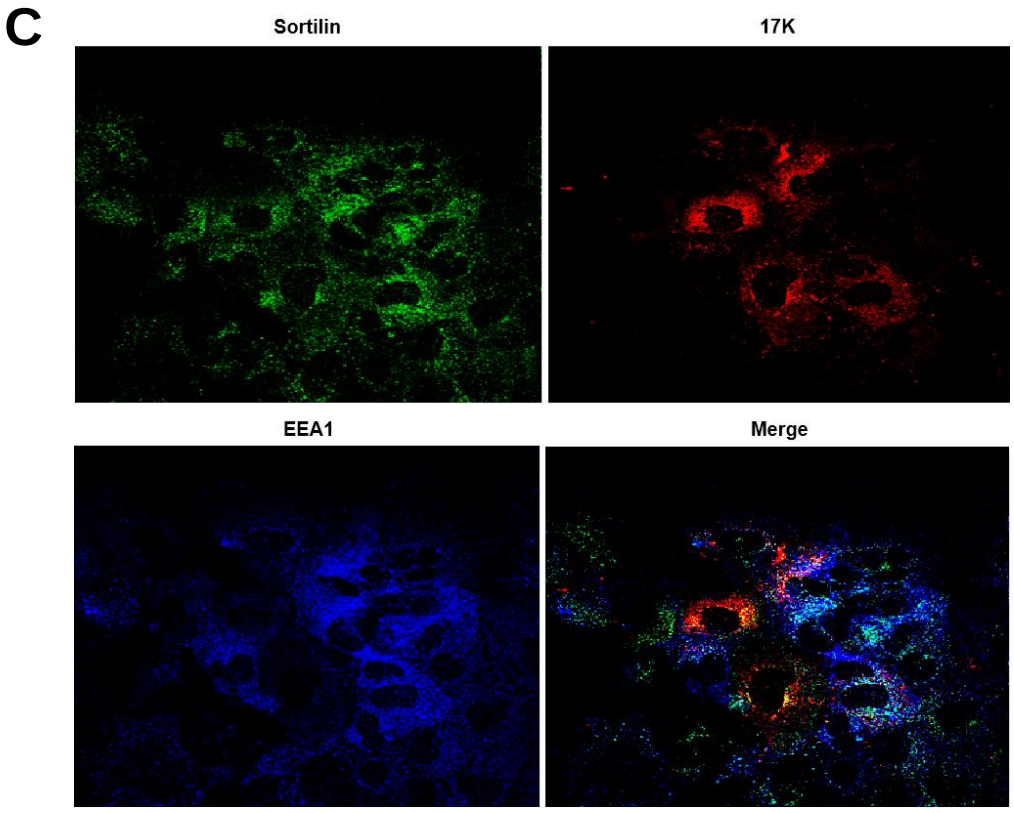

A
0 30 60 120 240 480 (min)
0 30 60 120 240 480
pcDNA
550 kDa
450 kDa
550 kDa
Sortilin
SortilinΔCT
Sortilin Y792A
Sortilin L829A/L830A
B
apo(a) Intensity (% of pcDNA maximun
150
100
50
0
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin
SortilinΔCT
Sortilin Y792A
Sortilin L829/830A
C
apo(a) Intensity (% of pcDNA maximum)
250
200
150
100
50
0
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin
SortilinΔCT
Sortilin Y792A
Sortilin L829/830A

Figure 3.2: ***Effect of sortilin overexpression on intracellular accumulation and secretion of apo(a).*** Pulse-chase analysis of HepG2 cells transiently expressing either a recombinant (**A**) 17K apo(a) as well as either sortilin, sortilinΔCT, sortilin Y792A, sortilin L829A/L830A, or the corresponding empty vector control (pcDNA). Cells were starved in –Cys/Met media for 60 minutes, pulse-labelled for 60 minutes with ^{35}S-Met/Cys, and subsequently chased in unlabelled media for 0-480 minutes. Cell lysates (left) and media (right) were collected at the indicated times of chase, apo(a) was immunoprecipitated, and analyzed by 7% SDS-PAGE and fluorography. Representative fluorograms are shown. The lower band (~450 kDa) represents immature, hypoglycosylated apo(a) while the upper band (~550 kDa) represents mature, secretable apo(a) **(B)** Resulting densities of intracellular 17K apo(a) accumulation (combination of immature and mature 17K apo(a) bands) at the indicated chase times respectively. **(C)** Resulting densities of secreted 17K apo(a) accumulation at the indicated chase times respectively. Densitometric analysis was conducted using AlphaView software, normalized to the maximum density observed for pcDNA vector, and plotted as a function of time. The data corresponds to the means ± SEM of at least 3 independent experiments. Significance compared to pcDNA is indicated through asterisks, where *$p<0.05$ and **$p<0.01$. Significance compared to wild-type sortilin is indicated through daggers, where $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. The colours correspond to the legend.

3.2 Sortilin Appears to Predominantly Co-Localize With Apo(a) in Endosomal Compartments

Sortilin predominantly localizes within the TGN and endosomal compartments, with <10% of the protein localizing at the cell surface (134, 135, 143, 146). Based on the concept that sortilin predominantly functions as an intracellular sorting protein, and that sortilin overexpression can alter apo(a) secretion (Fig. 3.2), we hypothesized that sortilin may co-localize intracellularly with apo(a). To explore this possibility, we studied if endogenous sortilin co-localized with apo(a) in HepG2 cells stably expressing 17K-r-apo(a) using immunofluorescence microscopy. As observed in Fig. 3.3, co-localization of apo(a) and sortilin was observed (yellow) in merge panels. Furthermore, Fig. 3.3 B-D demonstrated co-localization of sortilin and a Golgi specific marker (mannosidase II), an early endosome specific marker (EEA1), and a late endosome specific marker (M6P, teal). Triple co-localization of apo(a) and sortilin in both early and late endosomal compartments (white) is also observed in Fig. 3.3 C, D. Conversely, triple co-localization of apo(a) and sortilin with an ER specific marker (calnexin) or mannosidase II was not observed (Fig. 3.3 A, B).


A
Sortilin
17K
Calnexin
Merge
B
Sortilin
17K
Mannosidase II
Merge


C
Sortilin
17K
EEA1
Merge

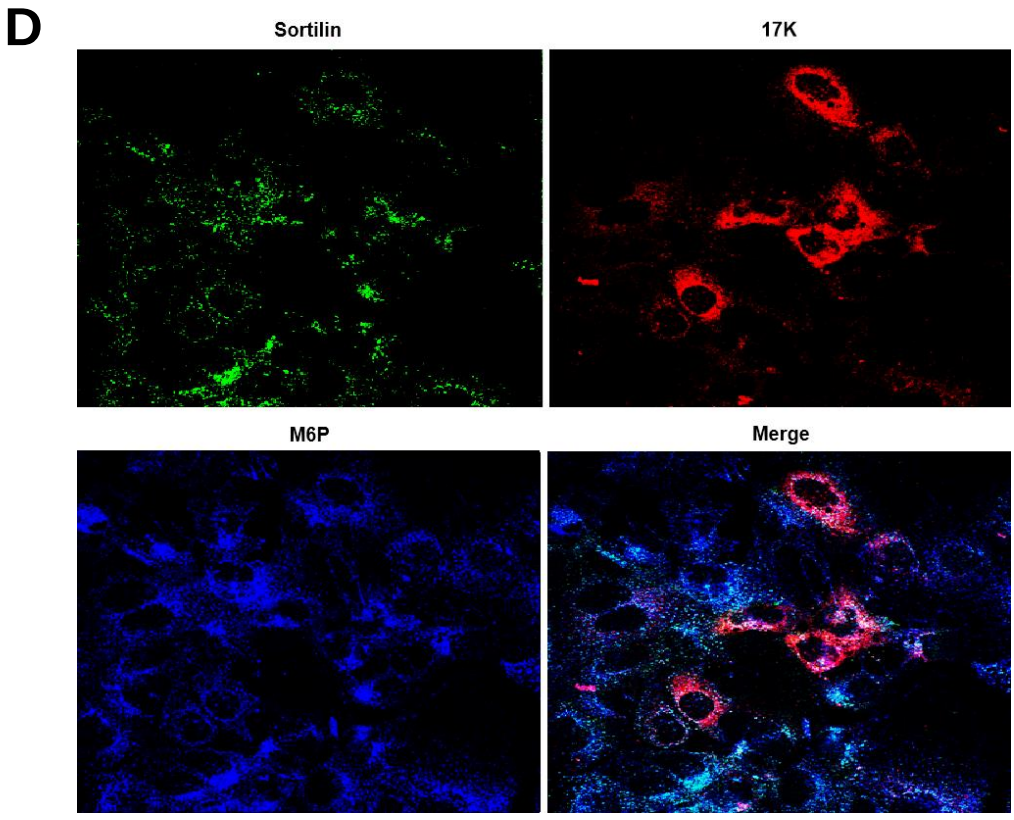
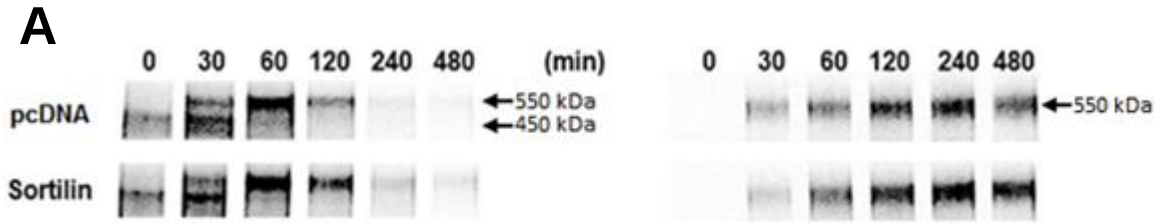

D
Sortilin
17K
M6P
Merge

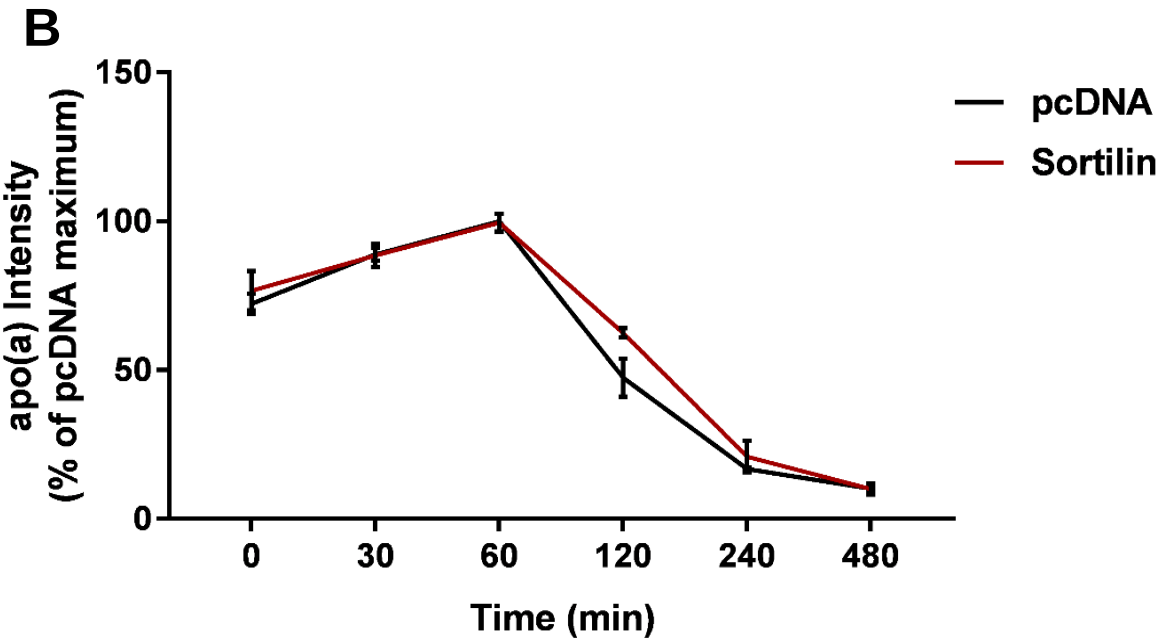
Figure 3.3: ***Sortilin and apo(a) display co-localization within endosomal compartments.*** HepG2 cells were extensively washed, fixed and immunostained with a mouse anti-apo(a), goat anti-sortilin, and either a rabbit anti-calnexin, anti-mannosidase II, anti-EEA1, or anti-M6P antibody. The cells were then incubated with the corresponding secondary antibodies that were linked to different fluorophores. (**A**-**D**) Co-localization is observed between sortilin, apo(a) and (**C**) an early endosome specific marker (EEA1) as well as (**D**) a late endosome specific marker (M6P). Co-localization between apo(a) and sortilin is observed as yellow. Co-localization between apo(a) and the various compartmental markers is observed as magenta, whereas co-localization between sortilin and the various compartmental markers is observed as teal. Triple co-localization between apo(a), sortilin, and compartmental markers is observed as white in all merge panels.

3.3 Sortilin Overexpression Does Not Alter Secretion of an Apo(a) Variant That Possesses Mutations in the Weak LBS of $KIV_{7,8}$.

Since apoB-100 secretion is regulated by sortilin expression (142), and apo(a) secretion has been suggested to couple with that of apoB (77, 82), a 17K apo(a) variant that is unable to non-covalently associate with apoB-100 was utilized to determine if sortilin overexpression could alter the secretion of this mutant in a manner similar to that of 17K r-apo(a). Previous work has demonstrated that the weak LBS of $KIV_{7,8}$ are required for the initial non-covalent interactions that occur between apo(a) and apoB-100 in Lp(a) assembly (62). Therefore, a ^{35}S-Met/Cys pulse-chase protocol was used with HepG2 cells transiently expressing a recombinant 17KΔ$LBS_{7,8}$-pRK5 apo(a) variant (17KΔ$LBS_{7,8\ r}$-apo(a)). These cells were also transiently transfected with an expression vector encoding sortilin or the corresponding empty vector to analyze the relationship between sortilin overexpression and secretion of 17KΔ$LBS_{7,8}$ r-apo(a) that cannot participate in non-covalent interaction with apoB-100 (Fig. 3.4 A-C). As demonstrated in Fig. 3.4 B, C, there was no significant difference observed in the accumulation of both intracellular and secreted 17KΔ$LBS_{7,8}$ r-apo(a) between cells overexpressing sortilin and control cells. The kinetics of intracellular maturation and secretion of 17KΔ$LBS_{7,8}$ r-apo(a) were similar to those of wild-type 17K r-apo(a) (Fig. 3.2):the earliest appearance of mature 17KΔ$LBS_{7,8}$ r-apo(a) was observed at 30 minutes for cells expressing sortilin and control cells (Fig. 3.4 A, C). Radiolabelled, immature 17KΔ$LBS_{7,8}$ r-apo(a) was barely detectable in the lysates after 60 minutes of chase, while mature apo(a) was first observed at 30 minutes of chase time. Small amounts of labelled, mature apo(a) were still detectable by 480 minutes of chase (Fig. 3.4 A, B).

However, the 17KΔLBS$_{7,8}$ r-apo(a) was completely resistant to the effects of overexpressing sortilin.


A
0 30 60 120 240 480 (min)
0 30 60 120 240 480
pcDNA
Sortilin
550 kDa
450 kDa
550 kDa


B
apo(a) Intensity
(% of pcDNA maximum)
150
100
50
0
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin

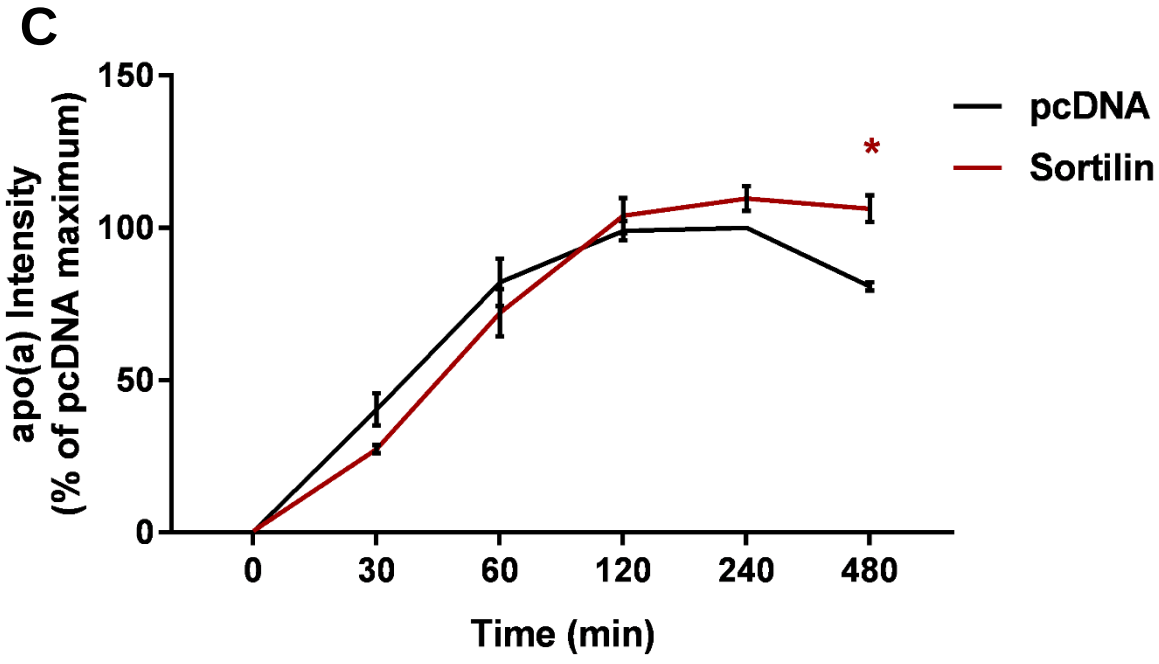

C
apo(a) Intensity
(% of pcDNA maximum)
150
100
50
0
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin
*

Figure 3.4: ***Sortilin overexpression does not alter the secretion of 17KΔLBS$_{7,8}$ apo(a).*** Pulse-chase analysis of HepG2 cells transiently expressing either a recombinant (**A**) 17KΔLBS$_{7,8}$ apo(a) as well as either sortilin or the corresponding empty vector control (pcDNA). Cells were starved in –Cys/Met DMEM for 60 minutes, pulse-labelled for 60 minutes with ^{35}S-Met/Cys, and subsequently chased in unlabelled media for 0-480 minutes. Cell lysates (left) and media (right) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 7% SDS-PAGE and fluorography. Representative fluorograms are shown. The lower band (~450 kDa) represents immature, hypoglycosylated apo(a) while the upper band (~550 kDa) represents mature, secretable apo(a) **(B)** Resulting densities of intracellular 17KΔLBS$_{7,8}$ apo(a) accumulation (combination of immature and mature 17K apo(a) bands) at the indicated chase times respectively. **(C)** Resulting densities of secreted 17KΔLBS$_{7,8}$ apo(a) accumulation at the indicated chase times respectively. Densitometric analysis was conducted as described in the legend to Figure 3.2. Significance compared to pcDNA is indicated through asterisks, where *$p<0.05$.

3.4 The Secretion of an Apo(a) Variant Lacking the Strong Lysine Binding Site in KIV_{10} is Increased by Sortilin Overexpression.

A variety of pathogenic mechanisms attributed to the apo(a) component of Lp(a) are associated with the proper functionality of the strong LBS in KIV_{10}, which mediates non-covalent, lysine-dependent interactions between Lp(a) and protein substrates (13). Therefore, a role for the LBS in KIV_{10} in facilitating the ability of sortilin to alter apo(a) secretion was assessed. Pulse-chase analysis was performed in HepG2 cells transiently transfected with a recombinant variant of apo(a) that lacked a functional LBS in KIV_{10}, identified as 17KΔLBS_{10} r-apo(a). These cells were also transiently transfected with an expression vector encoding sortilin or the corresponding empty vector (Fig. 3.5 A-C). Interestingly, a significant difference in the intracellular accumulation of 17KΔLBS_{10} r-apo(a) in cells overexpressing sortilin was only observed at the initial point of chase time when compared to control cells (Fig. 3.5 B). On the other hand, a significant difference in the accumulation of secreted 17KΔLBS_{10} r-apo(a) in cells overexpressing sortilin was observed between 120-480 minutes of chase time when compared to control cells (Fig. 3.5 C). Similar to previous results (Fig. 3.2, 3.4 A, C), the earliest appearance of mature apo(a) secreted into the medium was observed at 30 minutes for both empty vector and sortilin expressing cells (Fig. 3.5 A, C). Radiolabelled, immature 17KΔLBS_{10} r-apo(a) was detectable throughout the chase period; however, the majority was converted to mature apo(a) after 120 minutes of chase time. Mature 17KΔLBS_{10} r-apo(a) was first observed at 30 minutes of chase time, and small amounts of labelled mature apo(a) were still detectable by 480 minutes of chase (Fig. 3.5 A).

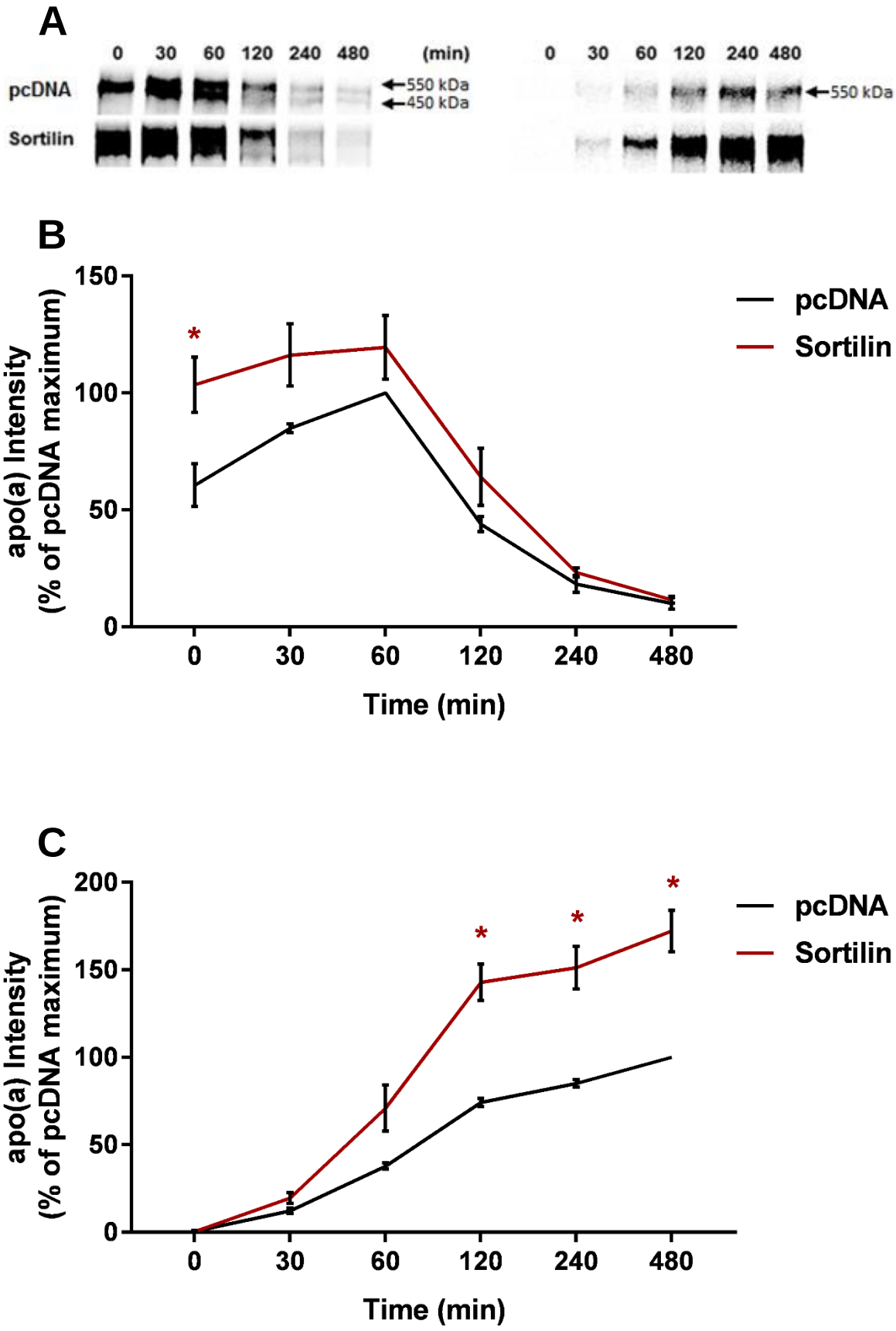

A
0 30 60 120 240 480 (min)
pcDNA
Sortilin
550 kDa
450 kDa
0 30 60 120 240 480
550 kDa
B
apo(a) Intensity
(% of pcDNA maximum)
150
100
50
0
*
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin
C
apo(a) Intensity
(% of pcDNA maximum)
200
150
100
50
0
* * *
0 30 60 120 240 480
Time (min)
pcDNA
Sortilin

Figure 3.5: ***Sortilin overexpression increases the secretion of a 17KΔLBS$_{10}$ apo(a) variant.*** Pulse-chase analysis of HepG2 cells transiently expressing either a recombinant (**A**) 17KΔLBS$_{10}$ apo(a) as well as either sortilin or the corresponding empty vector control (pcDNA). Cells were starved in –Cys/Met DMEM for 60 minutes, pulse-labelled for 60 minutes with ^{35}S-Met/Cys, and subsequently chased in unlabelled media for 0-480 minutes. Cell lysates (left) and media (right) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 7% SDS-PAGE and fluorography. Representative fluorograms are shown. The lower band (~450 kDa) represents immature, hypoglycosylated apo(a) while the upper band (~550 kDa) represents mature, secretable apo(a) (**B**) Resulting densities of intracellular 17KΔLBS$_{10}$ r-apo(a) accumulation (combination of immature and mature 17K apo(a) bands) at the indicated chase times respectively. (**C**) Resulting densities of secreted 17KΔLBS$_{10}$ r-apo(a) accumulation at the indicated chase times respectively. Densitometric analysis was conducted as described in the legend to Figure 3.2. Significance compared to pcDNA is indicated through asterisks, where *$p<0.05$.

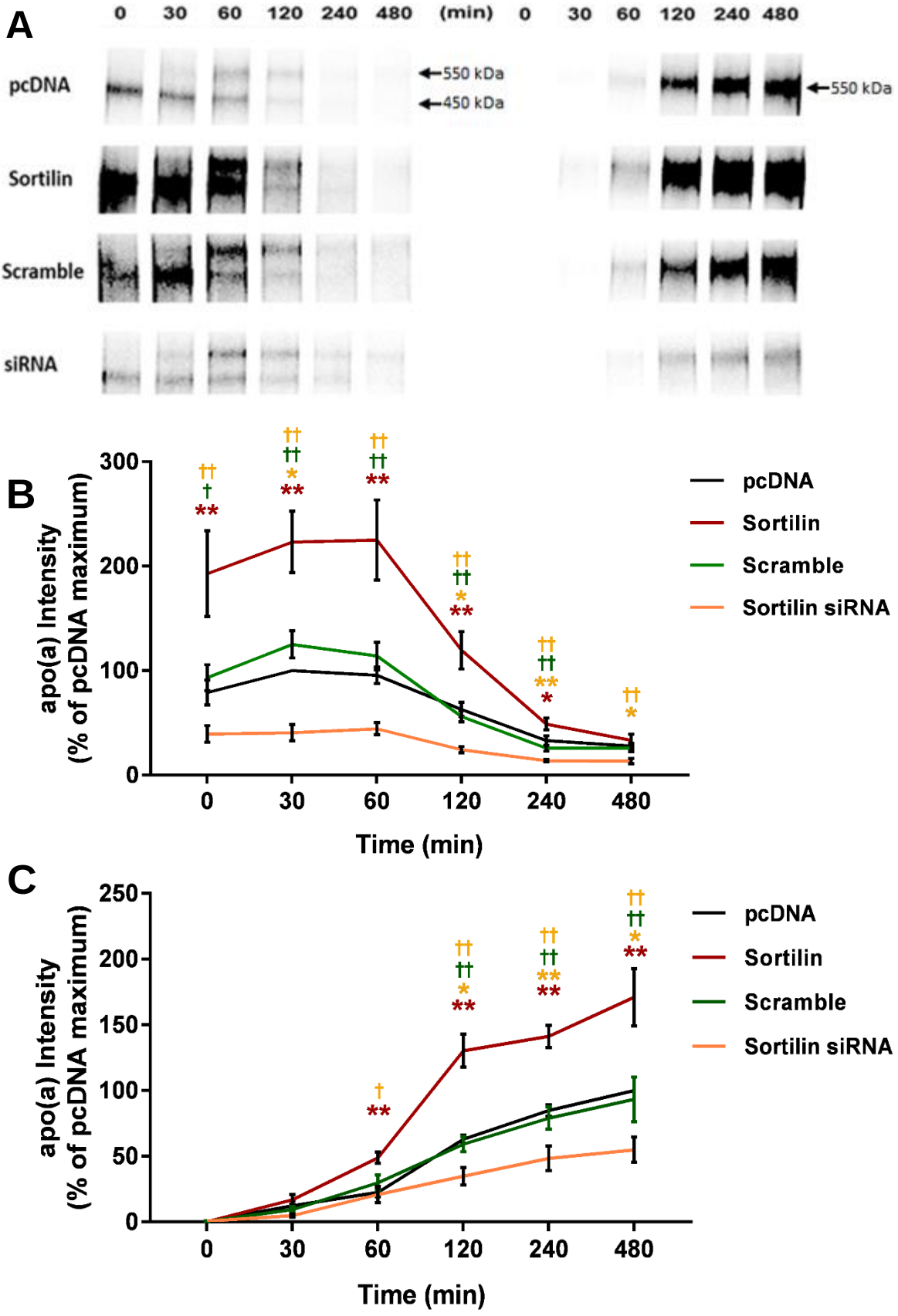
3.5 Knockdown of Sortilin Expression in HepG2 Cells Reduces Apo(a) Secretion

To provide independent evidence that sortilin promotes apo(a) secretion, we reduced sortilin expression through siRNA treatment directed towards *SORT1* mRNA. Knockdown treatment was conducted in HepG2 cells that were stably expressing a wild-type 17K apo(a) variant, thus providing a model that would better represent endogenous expression of both apo(a) and sortilin in hepatocytes. Pulse-chase analysis was performed as described above. Knockdown experiments utilized cells that were transiently transfected with either a scrambled siRNA that acted as a negative control for knockdown or siRNA directed towards *SORT1*. As demonstrated in Fig. 3.6 B, siRNA-mediated knockdown of sortilin expression reduced the intracellular accumulation of 17K apo(a), with significant differences observed at 30 and between 120-480 minutes of chase time when compared to control cells. Knockdown of sortilin expression also led to a concomitant reduction in the amount of 17K apo(a) secreted from cells, as a significant difference was observed between 120-480 minutes of chase time when compared to control cells (Fig. 3.6 C). No significant differences were observed in the accumulation of both intracellular and secreted apo(a) between cells transfected with the scrambled siRNA and control cells (Fig. 3.6 B, C).

In support of a role for sortilin in mediating apo(a) secretion, a significant increase in the accumulation of both intracellular and secreted apo(a) is observed in cells overexpressing wild-type sortilin compared to control cells. Specifically, a significant increase is observed in intracellular 17K r-apo(a) accumulation between 0-240 minutes of chase time, and between 60-480 minutes of chase time for secreted 17K r-apo(a). These data provide valuable confirmation for the ability of sortilin overexpression to regulate apo(a) secretion in the setting of hepatocytes that are stably expressing apo(a). In contrast

to HepG2 cells transiently expressing apo(a), the majority of immature to mature conversion of stably expressing apo(a) occurred at 60 minutes as opposed to 30 minutes of chase time. Significant differences in apo(a) secretion are also observed between cells expressing sortilin and cells transiently transfected with the scrambled siRNA or siRNA directed against sortilin. Fig. 3.6 B demonstrated significantly lower amounts of intracellular 17K r-apo(a) accumulation between 0-240 minutes of chase time, and between 120-480 minutes of chase time for secreted 17K r-apo(a) in cells transfected with the scrambled siRNA when compared to wild-type sortilin. Cells treated with siRNA directed against sortilin demonstrated significantly lower amounts of intracellular 17K apo(a) accumulation for all time points, and between 60-480 minutes of chase time for secreted 17K r-apo(a) when compared to cells overexpressing wild-type sortilin. (Fig. 3.6 C). Radiolabelled, immature 17K apo(a) was not detectable in the lysates after 240 minutes of chase, while mature apo(a) was first detected after 30 minutes of chase time for all samples. Small amounts of labelled, mature apo(a) were still detectable by 480 minutes of chase (Fig. 3.6 A, B)

Western blot analysis was used to determine the extent of sortilin knockdown in cells treated with siRNA compared to the scrambled control. SDS-PAGE analysis was performed on whole cell lysates 48 hours following siRNA treatment (Fig. 3.6 D). After correction using the expression of β-actin expression as an internal control, transfection with sortilin siRNA reduced sortilin expression by 53.7% compared to transfection with the scrambled siRNA.


A
0 30 60 120 240 480 (min) 0 30 60 120 240 480
pcDNA
Sortilin
Scramble
siRNA
550 kDa
450 kDa
550 kDa
B
apo(a) Intensity
(% of pcDNA maximum)
Time (min)
pcDNA
Sortilin
Scramble
Sortilin siRNA
C
apo(a) Intensity
(% of pcDNA maximum)
Time (min)
pcDNA
Sortilin
Scramble
Sortilin siRNA

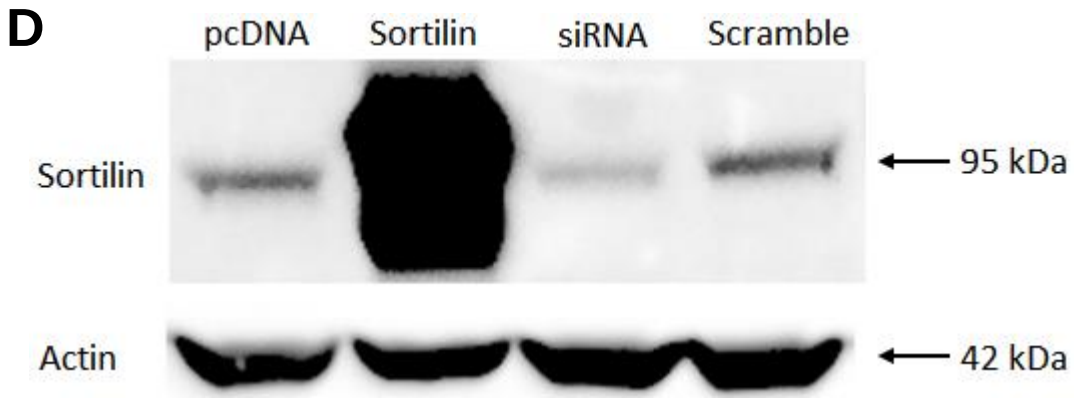


Figure 3.6: ***Sortilin overexpression increases the secretion of a stably expressed 17K apo(a) variant, while knockdown of sortilin expression reduces levels of secreted 17K apo(a).*** Pulse-chase analysis of HepG2 cells stably expressing a recombinant (**A**) 17K apo(a) and transiently transfected with either a plasmid vector encoding sortilin, the corresponding empty vector control (pcDNA), sortilin targeting siRNA, or a corresponding scrambled siRNA that acts as a negative control (scramble). Cells were starved in –Cys/Met DMEM for 60 minutes, pulse-labelled for 60 minutes with ^{35}S-Met/Cys, and subsequently chased in unlabelled media for 0-480 minutes. Cell lysates (left) and media (right) were collected at the indicated times of chase, apo(a) immunoprecipitated, and analyzed by 7% SDS-PAGE and fluorography. Representative fluorograms are shown. The lower band (~450 kDa) represents immature, hypoglycosylated apo(a) while the upper band (~550 kDa) represents mature, secretable apo(a) (**B**) Resulting densities of intracellular 17K apo(a) accumulation (combination of immature and mature 17K apo(a) bands) at the indicated chase times respectively. (**C**) Resulting densities of secreted 17K apo(a) accumulation at the indicated chase times respectively. Densitometric analysis was conducted as described in the legend to Figure 3.2. (**D**) Western blot analysis of sortilin expression in HepG2 cells ectopically expressing an empty vector control, wildtype sortilin, siRNA against sortilin expression, or a scrambled control respectively. Band intensities were corrected using the expression of β-actin, and knockdown percentage was determined by comparing band intensities of the scrambled siRNA with that of siRNA directed against sortilin. The data corresponds to the means ± SEM of at least 3 independent experiments. Significance compared to pcDNA is indicated through asterisks, where *$p<0.05$ and **$p<0.01$. Significance compared to sortilin is indicated through daggers, where $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. The colours correspond to the legends.

3.6 Sortilin Overexpression Alters Lp(a), but not Apo(a), Internalization by HepG2 Cells.

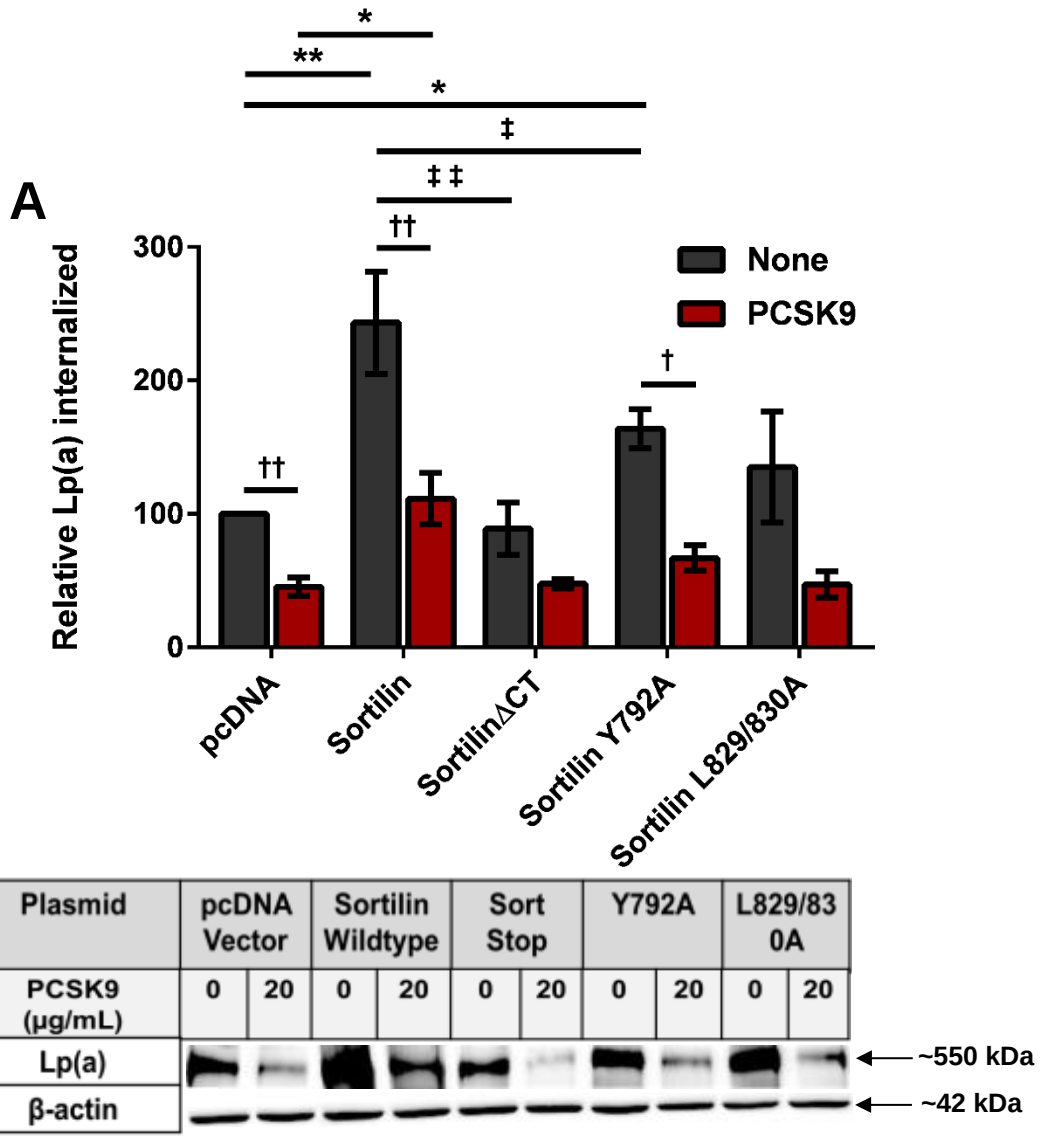
Sortilin can regulate the metabolism of LDL particles by acting as a *bona fide* internalization receptor (142, 153). Furthermore, this ability is dependent upon the ability of sortilin to act as an intracellular trafficking receptor (142). Previous research conducted by our group has demonstrated that exposure to exogenous PCSK9 can significantly reduce both Lp(a) and apo(a) internalization in HepG2 cells (112). We therefore evaluated the role of sortilin in regulating Lp(a) and apo(a) catabolism in HepG2 cells. We also sought to determine if the relationship between sortilin and PCSK9 was associated with regulating Lp(a) and apo(a) catabolism. Overexpression of sortilin resulted in a significant increase in the amount of Lp(a) internalized when compared to control cells (Fig. 3.7 A, asterisks). To confirm this finding, we generated internalization data in primary hepatocytes harvested from C57 BL/6 mice. Isolated primary hepatocytes from mice were transiently transfected with either an expression vector encoding sortilin or the corresponding empty vector as a control, and a similar increase in Lp(a) internalization was observed in cells overexpressing sortilin when compared to control cells (Fig. 3.7 B, asterisks). Conversely, no significant increase in internalization of 17K r-apo(a) was observed in HepG2 cells upon overexpression of sortilin (Fig. 3.9). Treatment of HepG2 cells with purified, recombinant PCSK9 resulted in a significant reduction in both Lp(a) and apo(a) internalization, either in the presence or absence of sortilin overexpression. Notably, a significant difference in Lp(a) internalization was seen between PCSK9-treated cells overexpressing sortilin compared to PCSK9-treated control cells (Fig. 3.7 A, daggers). No significant difference in apo(a) internalization was seen between PCSK9-treated cells either overexpressing sortilin or not (Fig. 3.9).

To determine if the effect of sortilin on Lp(a) internalization was dependent upon the ability of sortilin to act as a trafficking receptor, HepG2 cells were transiently transfected with the trafficking variants employed in section 3.1 (see above). While a significant increase in Lp(a) internalization was observed in cells overexpressing the Y792A mutant, no increase was observed in cells overexpression of the sortilinΔCT or L829/830A mutants when compared to control cells (Fig. 3.7 A). These findings were recapitulated in primary mouse hepatocytes transfected with expression plasmids encoding these sortilin mutants; however, a significant increase in Lp(a) internalization by cells overexpressing the Y792A mutant was not observed (Fig. 3.7 B, asterisks). The defect in the ability of sortilin to increase Lp(a) internalization in HepG2 cells was most profound in cells overexpressing either the sortilinΔCT or L829/830A mutants. Interestingly, significantly lower amounts of Lp(a) were internalized in cells overexpressing the sortilinΔCT or Y792A mutants when compared to cells overexpressing wild-type sortilin. A moderation reduction in Lp(a) internalization was observed in cells overexpressing the L829/830A mutant when compared to cells overexpressing wild-type, but the difference did not reach statistical significance (Fig. 3.7 A, double daggers). For internalization experiments in primary mouse hepatocytes, the defect in the ability of sortilin to increase Lp(a) internalization was most profound in cells overexpressing the sortilinΔCT mutant. No statistically significant difference in the amount of Lp(a) internalized was observed between cells overexpressing the Y792A or L829/830A mutants and cells overexpressing wild-type sortilin (Fig. 3.7 B, double daggers).

To determine if endogenous sortilin can regulate Lp(a) catabolism, sortilin knockdown experiments were conducted in HepG2 cells. Knockdown of human sortilin

did not significantly reduce the amount of Lp(a) internalized in comparison to a scrambled control (Fig. 3.8). After correction using the expression of β-actin as an internal control, transfection with sortilin siRNA reduced sortilin expression by 59.4% compared to transfection with the scrambled siRNA. Cells treated with PCSK9 demonstrated a significant reduction in the levels of internalized Lp(a) when compared to cells that were not treated with PCSK9 (Fig. 3.8, daggers).

To gain further insights into the molecular mechanism by which sortilin affects Lp(a) internalization, HepG2 cells were treated with a lysine analog, ε-ACA, to inhibit Lp(a) and apo(a) internalization by lysine binding receptors such as the plasminogen receptors. Addition of ε-ACA resulted in a significant reduction in Lp(a) internalization in HepG2 cells overexpressing either an empty vector control or wild-type sortilin. A significant difference between ε-ACA-treated cells expressing sortilin and an empty vector control was not observed (Fig. 3.10 E). As before, a significant difference in the amount of Lp(a) internalized was observed in untreated cells overexpressing sortilin when compared to the empty vector control (Fig. 3.7 A, B, Fig. 3.10 A). Similarly, no significant difference was observed in the amounts of 17K apo(a) internalized with sortilin overexpression; however, ε-ACA treatment led to a significant reduction in the levels of apo(a) internalization (Fig. 3.9, Fig. 3.10 B).


A
Relative Lp(a) internalized
300
200
100
0
None
PCSK9
*
**
*
‡
‡ ‡
††
††
†
pcDNA
Sortilin
SortilinΔCT
Sortilin Y792A
Sortilin L829/830A
Plasmid
pcDNA Vector
Sortilin Wildtype
Sort Stop
Y792A
L829/830A
PCSK9 (μg/mL)
0
20
Lp(a)
β-actin
~550 kDa
~42 kDa

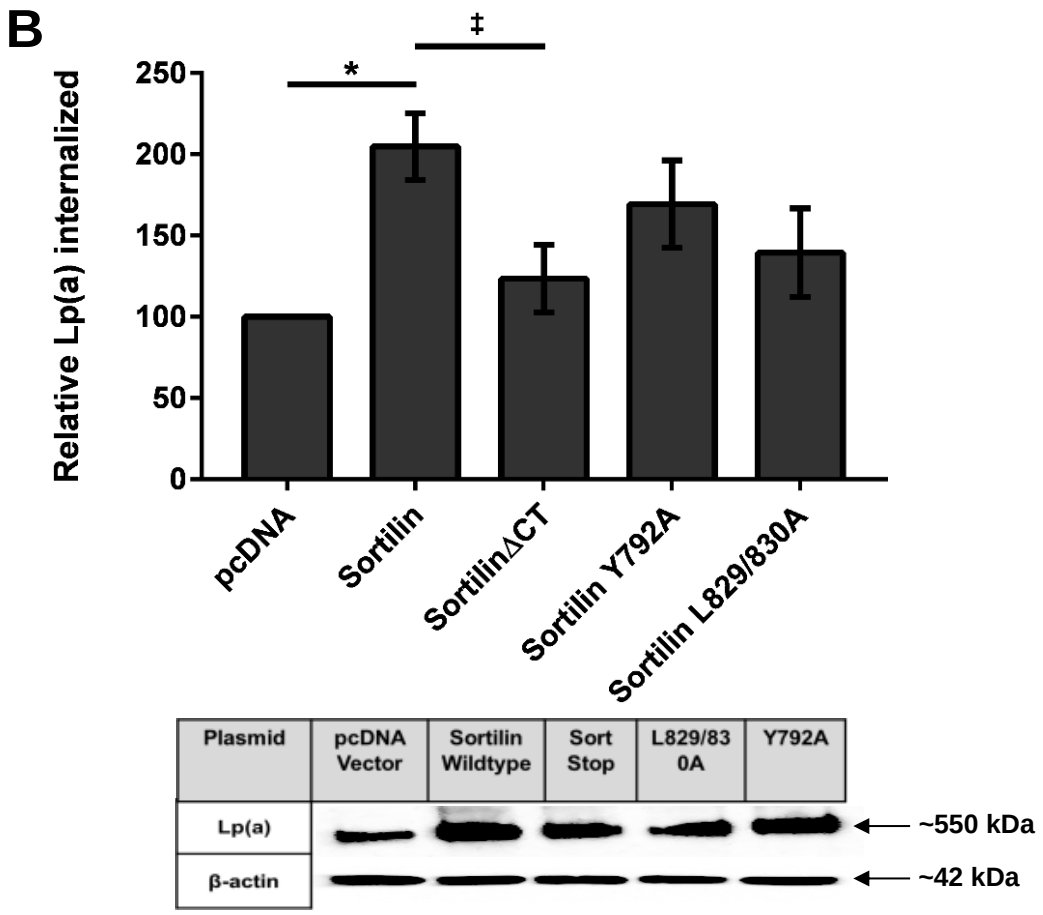
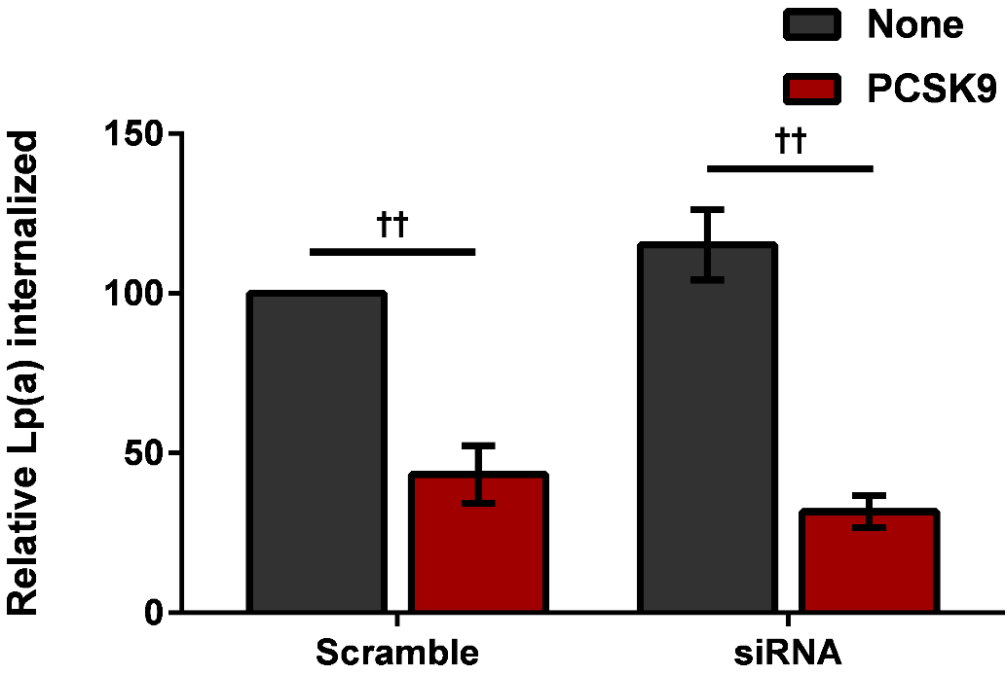


Figure 3.7: ***Sortilin overexpression increases Lp(a) internalization in hepatocytes.*** Internalization assays in (A) HepG2 cells and (B) primary mouse hepatocytes transiently transfected with a plasmid vector encoding sortilin or various sortilin trafficking variants. Control cells were transfected with the corresponding empty expression vector (pcDNA). Cells were grown for 16 hours in LPDS media and subsequently incubated with 10 µg/mL Lp(a) in Opti-MEM in the (A) presence or absence of 20 µg/mL PCSK9 for 4 hours. In all cases, the cells were extensively washed to remove any cell bound Lp(a)/apo(a), lysed, and subjected to western blot analysis to determine the amount of Lp(a)/apo(a) internalized. Densitometric analysis was conducted using AlphaView software. Values for Lp(a)/apo(a) were corrected using the signal for ß-actin, and normalized to the resulting density observed for pcDNA vector or scrambled siRNA control. The data corresponds to the means ± SEM of at least 3 independent experiments. Significance compared to pcDNA or scrambled siRNA is indicated through asterisks, where *$p<0.05$ and **$p<0.01$. Significance between cells treated with PCSK9 and non-treated cells is indicated through daggers, where $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$. Significance between cells overexpressing sortilin and cells overexpressing the various sortilin trafficking mutants is indicated through double daggers, where $^{\ddagger}p<0.05$ and $^{\ddagger\ddagger}p<0.01$.



Plasmid	Scramble Control		Sortilin siRNA	
PCSK9 ug/mL)	0	20	0	20
Lp(a)				
β-actin				
Sortilin				

Figure 3.8: ***Knockdown of sortilin expression does not alter Lp(a) internalization in HepG2 cells.*** Internalization assays in HepG2 cells transiently transfected with siRNA directed towards sortilin or a negative control scrambled siRNA. Cell treatments and data analysis were conducted as described in the legend to Figure 3.7. Significance between cells treated with PCSK9 and non-treated cells is indicated through daggers, where $^{\dagger\dagger}p<0.01$.

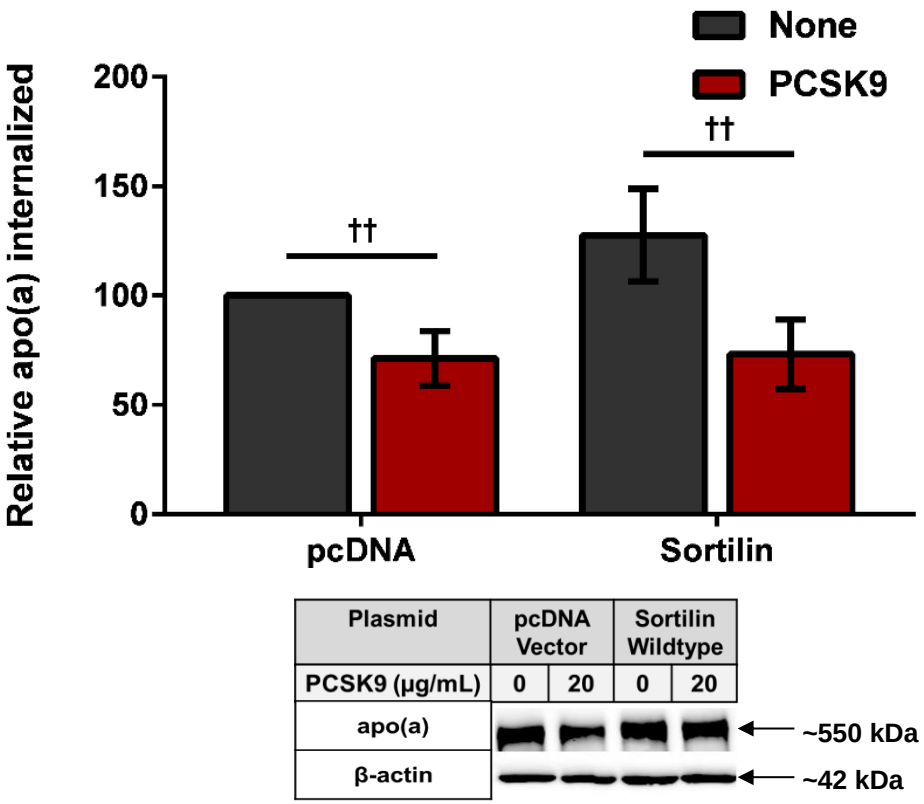


Figure 3.9: ***Sortilin overexpression does not alter apo(a) internalization in HepG2 cells.*** Internalization assays in HepG2 cells transiently transfected with a plasmid vector encoding sortilin or the corresponding empty vector control (pcDNA). Cells were grown for 16 hours in LPDS media and subsequently incubated with 200 nM apo(a) in Opti-MEM in the presence or absence of 20 µg/mL PCSK9 for 4 hours. Data analysis was conducted as described in the legend to Figure 3.7. Significance between cells treated with PCSK9 and non-treated cells is indicated through daggers, where $^{\dagger\dagger}p<0.01$.

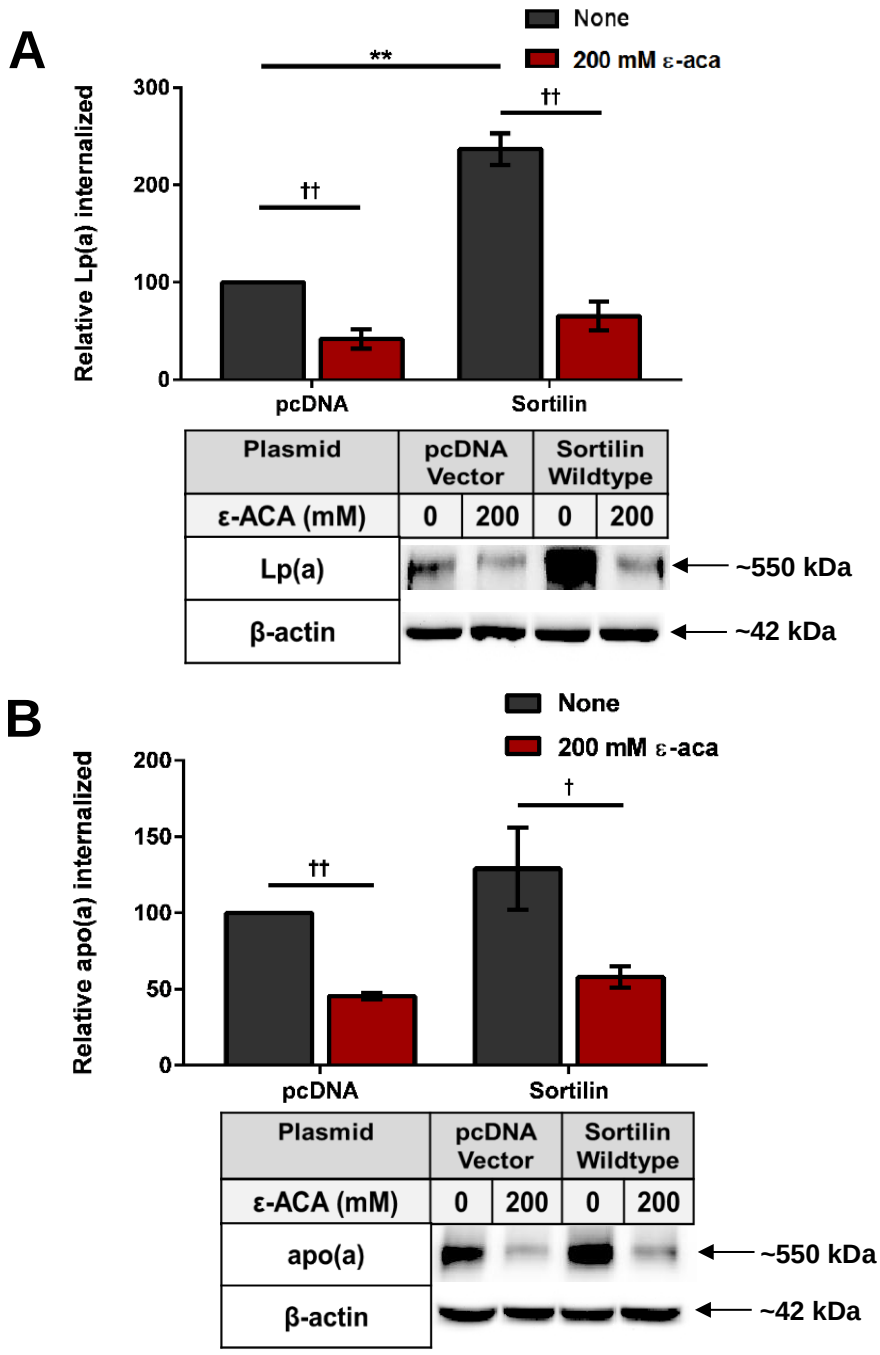


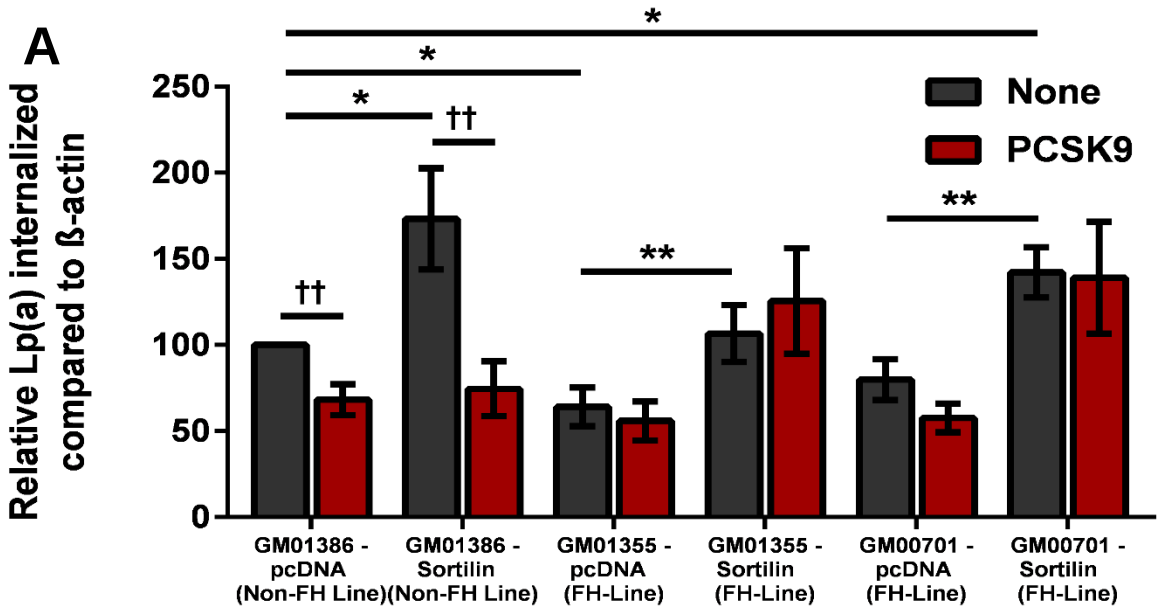
Figure 3.10: ***Treatment with ε-ACA inhibits the ability of sortilin overexpression to increase Lp(a) internalization in HepG2 cells.*** Internalization assays in HepG2 cells transiently transfected with a plasmid vector encoding sortilin or the corresponding empty vector control (pcDNA). Cells were grown for 16 housr in LPDS media and subsequently incubated with (A) 10 µg/mL Lp(a) or (B) 200 nM 17K r-apo(a) in Opti-MEM in the presence or absence of 200 mM ε-ACA for 4 hours. Data analysis was conducted as described in the legend to Figure 3.7. Significance between cells treated with PCSK9 and non-treated cells is indicated through daggers, where $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$.

3.7 Sortilin Overexpression Can Increase Lp(a), but not Apo(a) Internalization in Human Fibroblasts Lacking a Functional LDLR

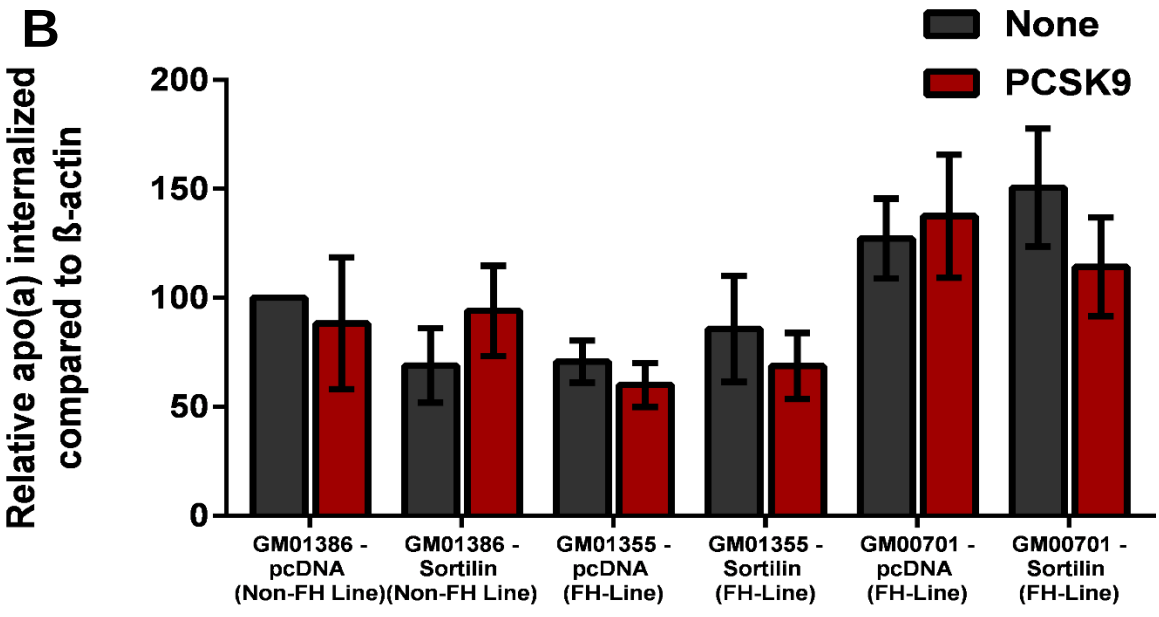
Research has demonstrated that sortilin can increase the catabolic rate of LDL in $LDLR^{-/-}$ mice, therefore identifying a novel LDLR-independent pathway for LDL catabolism (142). While controversial, evidence for a role for the LDLR in regulating Lp(a) catabolism has been previously demonstrated (102, 104, 110, 112). Therefore, we sought to determine if there was an association between LDLR activity and the increase in Lp(a) internalization mediated by sortilin overexpression. This was achieved through the utilization of primary human fibroblasts that were isolated from individuals with familial hypercholesterolemia (FH) or an unaffected control individual. The three cell lines used were GM01386, which was isolated from the control individual that possessed a fully functional LDLR; GM01355, which was isolated from an individual with severe hypercholesterolemia and partially negative LDLR activity; and GM00701, which was isolated from an individual that possessed <1% LDLR activity. Sortilin overexpression led to a significant increase in the amount of Lp(a) internalized in all three cell lines when compared to control cells transfected with the corresponding empty vector (Fig. 3.11 A). Conversely, sortilin overexpression did not significantly increase the amount of 17K r-apo(a) internalized by any of the cell lines (Fig. 3.11 B). These results are consistent with the findings obtained in HepG2 cells, where sortilin overexpression significantly altered Lp(a), but not apo(a), internalization (Fig. 3.7 A, B, Fig. 3.9).

A reduction in Lp(a) internalization was observed in the FH cell lines when compared to the non-FH cell line, thus agreeing with previous research that demonstrated a role for the LDLR in Lp(a) catabolism (102, 104, 112). Treatment of HepG2 cells with

PCSK9 resulted in a significant reduction in Lp(a) internalization in the GM01386 cell line only (Fig. 3.11 A). This is in agreement with the general accepted physiological function of PCSK9, which is to facilitate the degradation of the LDLR (188). With respect to apo(a) internalization, exogenous PCSK9 treatment did not significantly reduce internalization levels (Fig. 3.11 B). This matches our previous study, in which the same primary human fibroblast cell lines were utilized to study apo(a) internalization (112).



Cell Line	1386				1355				0701			
Plasmid	pcDNA		Sortilin		pcDNA		Sortilin		pcDNA		Sortilin	
PCSK9 (µg/mL)	0	20	0	20	0	20	0	20	0	20	0	20
Lp(a)												
β-actin												



Cell Line	1386				1355				0701			
Plasmid	pcDNA		Sortilin		pcDNA		Sortilin		pcDNA		Sortilin	
PCSK9 (µg/mL)	0	20	0	20	0	20	0	20	0	20	0	20
apo(a)												
β-actin												

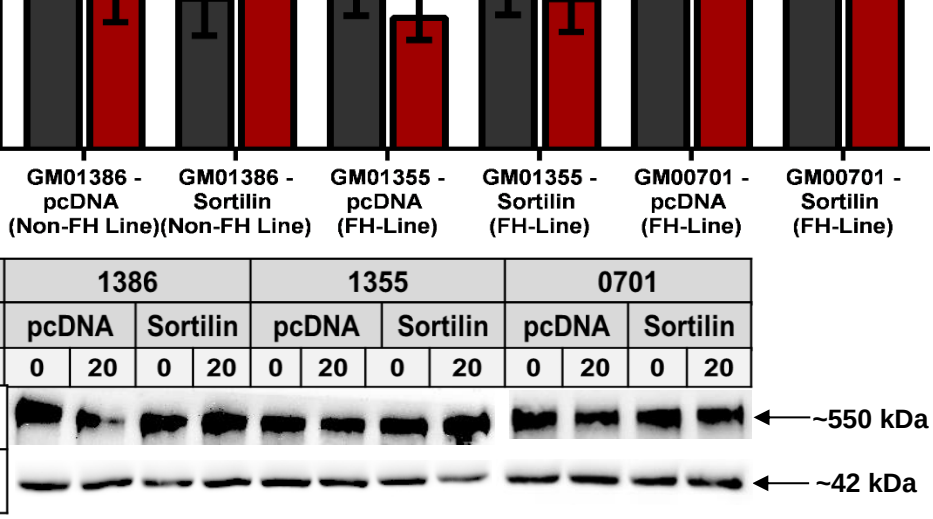


Figure 3.11: ***Sortilin overexpression increases the internalization of Lp(a) in primary human fibroblasts lacking functional LDL receptors.*** Non-FH and FH fibroblasts used were: GM01386 (normal LDLR function); GM01355 (partially negative LDLR activity); and GM00701 (<1% LDLR activity). Cells were transiently transfected with an expression vector encoding sortilin or the corresponding empty vector (pcDNA) were grown for 16 hours in LPDS. They were subsequently incubated with (**A**) 10 µg/mL Lp(a) or (**B**) 200 nM 17K in the presence or absence of 20 µg/mL PCSK9. The cells were extensively washed to remove any cell bound Lp(a) or apo(a) and subjected to western blot analysis to determine the amount of Lp(a)/apo(a) internalized. Data analysis was conducted as described in the legend to Figure 3.7. Significance compared to pcDNA is indicated through asterisks, where *$p<0.05$ and **$p<0.01$. Significance between cells treated with PCSK9 and non-treated cells is indicated through daggers, where ††$p<0.01$.

3.8 Sortilin Does Not Bind to Lp(a) or Apo(a) *in vitro*

Previous research has demonstrated that sortilin can directly bind to the apoB-100 component of LDL in hepatocytes (141, 142). This association allows for sortilin to act as a novel cell surface receptor for apoB-100 containing lipoproteins. Since Lp(a) contains an apoB-100 moiety, we investigated whether Lp(a), or 17K r-apo(a), could bind to sortilin *in vitro*. An expression plasmid encoding a soluble variant of sortilin was generated through the introduction of a premature stop codon prior to the transmembrane domain. The soluble variant was purified and then fluorescently labelled in order to perform *in vitro* binding analysis with Lp(a). LDL was utilized as a positive control. Band corresponding to LDL bound to sortilin were quantified and fit to a one site saturation ligand binding equation by non-linear regression analysis. We found that Lp(a) did not bind to sortilin *in vitro* (Fig. 3.12 A, C); however, sortilin was able to bind LDL with a K_D of ~ 435 nM (Fig. 3.12 B, C).

To further substantiate the inability of sortilin to physically interact with Lp(a), we performed co-immunoprecipitation of endogenous sortilin present in lysates of HepG2 cells with Lp(a), using an anti-apo(a) antibody. Co-immunoprecipitation of endogenous sortilin and apoB, with anti-apoB antiserum, was performed as a positive control. In these studies, a band of the appropriate size for sortilin was detectable in western blot analysis of fractions immunoprecipitated using the anti-apoB antibody following immunoblotting with an anti-sortilin antibody, indicating that an interaction occurred between apoB and sortilin in solution (Fig. 3.12 D). However, no observable sortilin band was present in HepG2 cell lysates containing added Lp(a). Furthermore, immunoprecipitates of HepG2 cell lysates stably expressing 17K r-apo(a) also lacked an immunoreactive band corresponding to

sortilin (Fig. 3.12 D). Similar co-immunoprecipitation experiments were conducted with an anti-sortilin antibody. However, neither Lp(a) nor apo(a) co-immunoprecipitated with sortilin present in HepG2 cell lysates (Fig. 3.12 E).

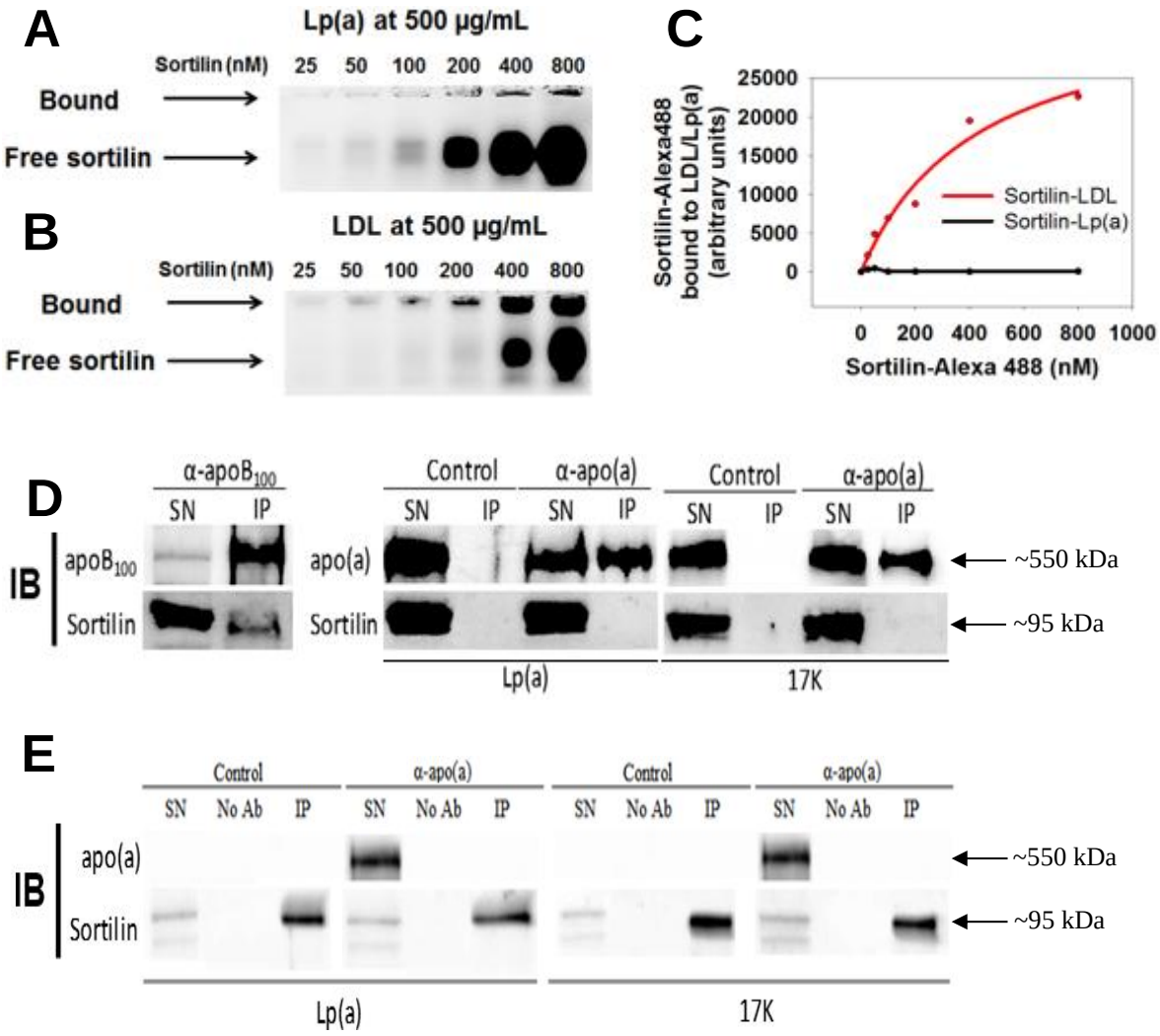


Fig 3.12: ***Sortilin binds to LDL in vitro but not to Lp(a) or apo(a).*** Various concentrations of sortilin-Alexa 488 were incubated with 0.5 mg/mL of purified LDL or Lp(a) for one hour at 37ºC. Samples containing soluble sortilin and either Lp(a) (**A**) or LDL (**B**) were resolved on 0.9% agarose gels. (**C**) Bands corresponding to either LDL-bound sortilin were quantified and fit to a one site saturation binding equation by non-linear regression analysis using SigmaPlot 11. K_D of LDL ~ 435 nM (n=2). (**D**) Lysates prepared from HepG2 cells either stably expressing 17K r-apo(a) or incubated with 10 µg/mL of Lp(a) were subjected to immunoprecipitation with either anti-apo(a) or anti-apoB antibodies, as indicated. Immunoprecipitates were analyzed by SDS-PAGE on 7-12% gradient polyacrylamide gels, followed by immunoblotting for apoB, apo(a), and sortilin (n=3). (E) Lysates from HepG2 cells either stably expressing 17K r-apo(a) or incubated with 10 µg/mL of Lp(a) were subjected to immunoprecipitation with an anti-sortilin antibody. Immunoprecipitates were analyzed by SDS-PAGE on 7-15% gradient polyacrylamide gels, followed by immunoblotting for apo(a), sortilin, or a no antibody control (n=3).

3.9 Naturally Occurring Sortilin Polymorphisms May Be Associated With Elevated Levels of Lp(a) in Human Patients

Deep sequencing of the *SORT1* locus was performed in a cohort of patients possessing elevated Lp(a) levels in an attempt to identify novel, common SNPs within the gene that might influence the role of sortilin in Lp(a) synthesis or catabolism. The resulting analysis led to the discovery of 19 rare, heterozygous missense or splicing variants within *SORT1*. Interestingly, 9 individuals possessed Lp(a) within the top 5^{th} percentile for the general population. The seven polymorphisms in these 9 individuals were: I124V, K205N, K302E, K404Y, E444Q, E447G, and V650M (Fig. 3.13).

To explore a potential relationship between these polymorphisms and elevated levels of plasma Lp(a), site-directed mutagenesis was performed to generate the variants of interest. Internalization assays were performed to determine if the SNPs could promote Lp(a) catabolism in a manner similar to sortilin overexpression. Any notable reduction in the amount of Lp(a) internalized could be suggestive of a possible mechanism contributing to elevated plasma Lp(a) levels. Therefore, HepG2 cells were transfected with expression vectors encoding either sortilin, or the various polymorphic variants, or the corresponding empty vector as a control. As demonstrated in Fig. 3.14 A, differences are observed in the amount of Lp(a) internalized in cells expressing the polymorphic *SORT1* variants when compared to either control cells or cells overexpressing sortilin. The amount of Lp(a) internalized in cells overexpressing the I124V and E444Q variants was comparable to cells overexpressing sortilin. A moderate decrease in the amount of Lp(a) internalized was observed in cells overexpressing the K205N and K302E variants when compared to cells overexpressing sortilin. A defect in the ability of sortilin to increase Lp(a) internalization

was most profound in cells overexpressing the F404Y and V650M variants (Fig. 3.14 A). The amount of Lp(a) internalized in cells overexpressing F404Y was comparable to control cells, while a modest reduction in Lp(a) internalization was observed in cells overexpressing V650M when compared to control cells. Overall, the data indicate that F404Y and V650M are possible loss-of-function sortilin variants with respect to Lp(a) internalization.

Since the data in this thesis have demonstrated the effect of sortilin expression and apo(a) secretion, we postulated that the *SORT1* polymorphisms may contribute to elevated Lp(a) levels by giving rise to an increased rate of apo(a) production and secretion. Overexpression of the sortilin variants in HepG2 cells had differential effects with respect to apo(a) secreted in the medium when compared to cells overexpressing sortilin (Fig. 3.14 B). The amount of apo(a) secreted into the media by cells overexpressing the I124V variant was comparable to cells overexpressing sortilin. A modest decrease in the amount of apo(a) secreted was observed in cells overexpressing the K205N and V650M variants when compared to cells overexpressing sortilin. Interestingly, large increase in apo(a) secretion was observed in cells overexpressing the F404Y variant when compared to cells overexpressing sortilin. Conversely, the amount of apo(a) secreted in cells overexpressing the K302E variant was comparable to control cells, while a reduction in apo(a) secretion was observed in cells overexpressing E444Q when compared to control cells (Fig. 3.14 B).

We have documented that sortilin overexpression not only increases apo(a) secretion, but the accumulation of intracellular apo(a) as well (Fig. 3.2). Therefore, HepG2 cells transiently transfected with expression vectors encoding either sortilin, or the various polymorphic variants, or the corresponding empty vector as a control were lysed to

simultaneously analyze intracellular apo(a) accumulation (Fig. 3.14 C). All of the polymorphic variants led to an increase in the intracellular accumulation of apo(a). Cells overexpressing K205N had the most robust increase in intracellular apo(a) accumulation when compared to control cells. Cells expressing I124V and F404Y demonstrated comparable levels of intracellular apo(a) accumulation to cells overexpressing sortilin (Fig. 3.14 C). A modest decrease in intracellular apo(a) accumulation was observed in cells overexpressing V650M when compared to cells overexpressing sortilin. Cells overexpressing K302E and E444Q demonstrated only a modest increase in the amount of intracellular apo(a) accumulated when compared to control cells (Fig. 3.14 C). Overall, the data indicate that certain variants show an altered function of sortilin with respect to apo(a) secretion. Western blot analysis of the expression of these plasmids demonstrated that the E447G variant was not expressed (data not shown). Data on this variant are therefore not available.

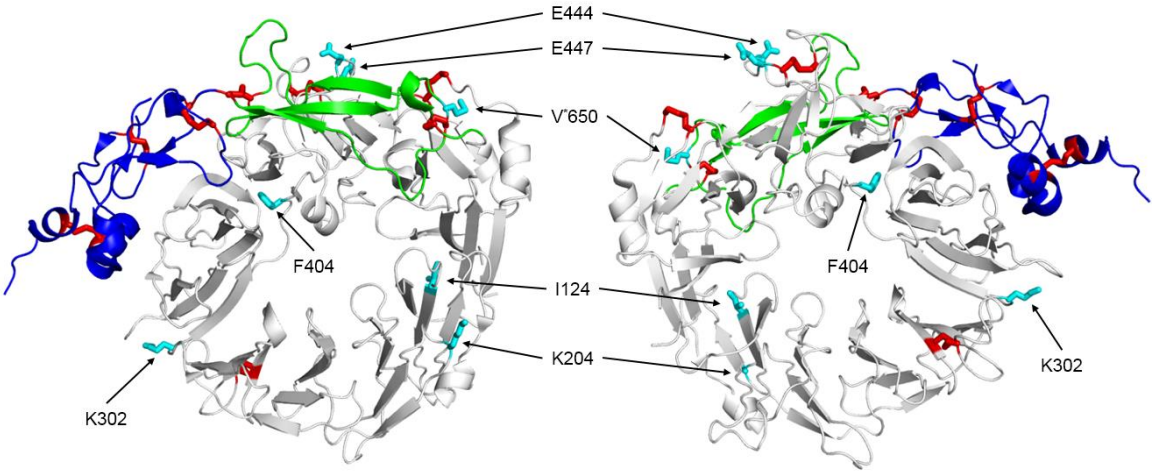
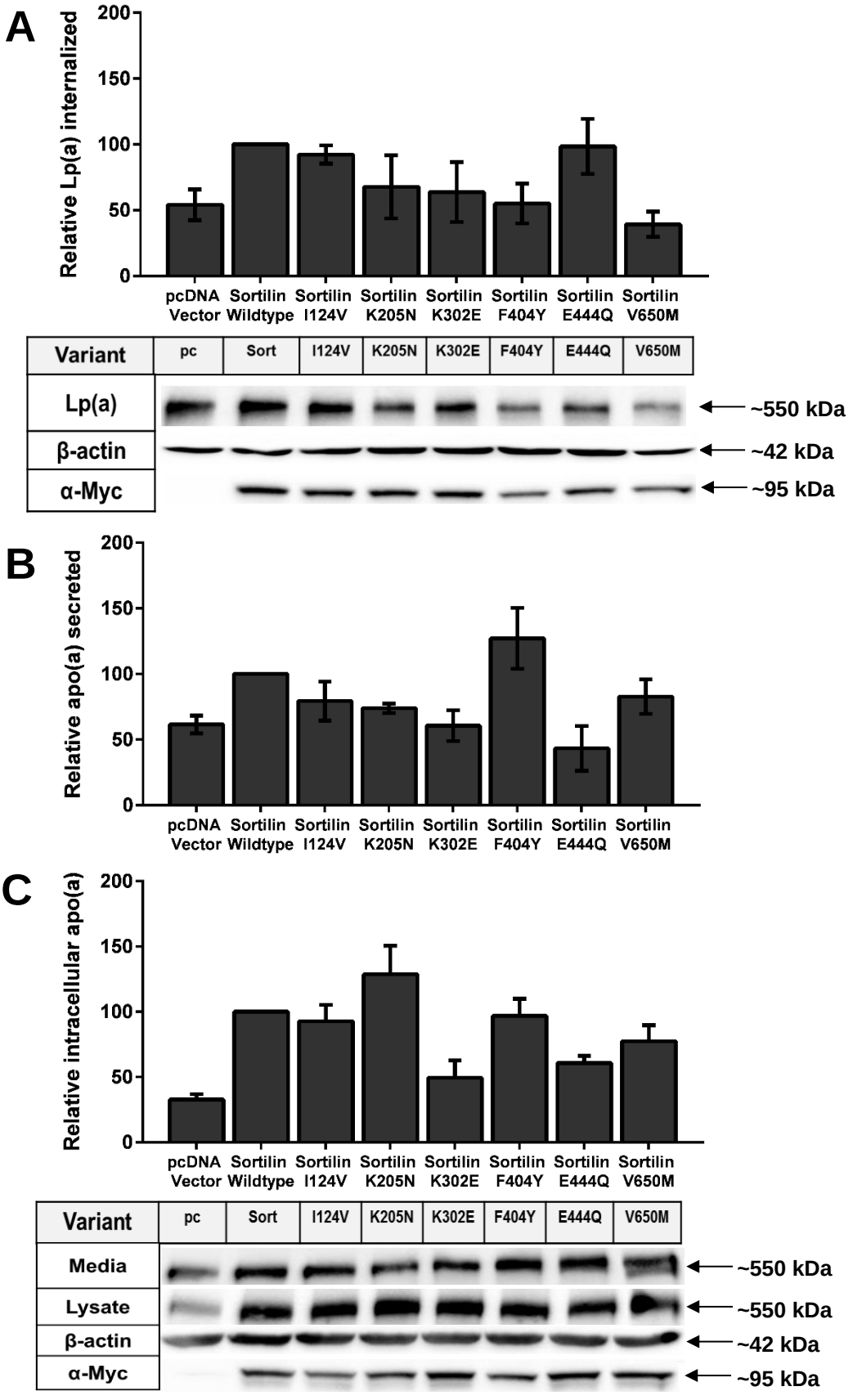


Figure 3.13: ***Polymorphisms within the VPS10 domain of sortilin.*** The crystal structure of the VPS10 domain of sortilin is rendered based on PDB #4PO7 (207) using Polyview (http://polyview.cchmc.org/polyview3d.html). Two views of the structure are shown, rotated 180° relative to each other along the vertical axis. The 10-bladed beta propeller is in silver, the 10CC-a subdomain is in blue, and the 10CC-b subdomain is in green. The conserved cysteine residues within the VPS10 domain are shown in red. Side chains of residues that were mutated are labelled and shown in cyan (Ile124, Lys204, Lys302, Phe404, Glu444, Glu447, Val*650). The residue in the 650 position is a methionine in the crystal structure; this mutation was noted in the corresponding PDB file. The allele frequency encoding either a valine or methionine at this position is not known.


A
Relative Lp(a) internalized
200
150
100
50
0
pcDNA Vector
Sortilin Wildtype
Sortilin I124V
Sortilin K205N
Sortilin K302E
Sortilin F404Y
Sortilin E444Q
Sortilin V650M
Variant
pc
Sort
I124V
K205N
K302E
F404Y
E444Q
V650M
Lp(a)
β-actin
α-Myc
~550 kDa
~42 kDa
~95 kDa
B
Relative apo(a) secreted
200
150
100
50
0
pcDNA Vector
Sortilin Wildtype
Sortilin I124V
Sortilin K205N
Sortilin K302E
Sortilin F404Y
Sortilin E444Q
Sortilin V650M
C
Relative intracellular apo(a)
200
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pcDNA Vector
Sortilin Wildtype
Sortilin I124V
Sortilin K205N
Sortilin K302E
Sortilin F404Y
Sortilin E444Q
Sortilin V650M
Variant
pc
Sort
I124V
K205N
K302E
F404Y
E444Q
V650M
Media
Lysate
β-actin
α-Myc
~550 kDa
~550 kDa
~42 kDa
~95 kDa

Figure 3.14: ***Sortilin polymorphisms may promote elevated levels of Lp(a) in human patients.*** Internalization assays in (**A**) HepG2 cells transiently transfected with a plasmid vector encoding sortilin or various polymorphic variants. Control cells were transfected with the corresponding empty expression vector (pcDNA). Cells were grown for 16 hours in LPDS media and subsequently incubated with 10 µg/mL of Lp(a) in Opti-MEM for 4 hours. the cells were extensively washed to remove any cell bound Lp(a), lysed, and subjected to western blot analysis to determine the amount of Lp(a) internalized. Secretion assays in (**B**)(**C**) HepG2 cells transiently transfected with a plasmid vector encoding sortilin, the various polymorphic variants, or pcDNA were grown for 24 hours in MEM media. Media was collected after 24 hours, and the cells were subsequently lysed and subjected to western blot analysis to determine the amount of both (**B**) secreted and (**C**) intracellular apo(a) accumulation. Values for Lp(a) internalization or apo(a) accumulation are corrected for cell number and sortilin overexpression, and are expressed relative to that observed when wild-type sortilin was overexpressed. Densitometric analysis was conducted using Image Lab software. The data corresponds to the means ± SEM of at least 3 independent experiments.

CHAPTER 4: DISCUSSION

GWAS studies have identified various SNPs at the 1p13.3 locus that are associated with total LDL cholesterol levels and risk for CAD and MI (183). While this locus incorporates a haplotype block of three genes, namely *SORT1*, *PSRC1*, and *CELSR2*, numerous mechanistic studies have identified a role for sortilin, encoded by *SORT1*, in regulating LDL-C levels (141, 142, 152, 153). However, these studies yielded disparate results with respect to the relationship between sortilin and LDL metabolism. Regarding LDL catabolism, Strong *et al.* found through *in vivo* studies in a wild-type mouse model that an increase in the catabolic rate of LDL is observed in animals overexpressing hepatic sortilin when compared to control animals (142). Furthermore, sortilin expression was found to increase LDL catabolism in an $LDLR^{-/-}$ mouse model, suggesting that sortilin can mediate LDL clearance independently of the LDLR (142). These findings are in agreement with previous i*n vitro* studies conducted by Linsel-Nitschke *et al.* which demonstrate that sortilin overexpression in Hek293 cells leads to an increase in the catabolic rate of LDL (153). Conversely, *in vitro* studies conducted in primary mouse hepatocytes found no difference in the endocytic uptake of LDL between control cells or sortilin-deficient cells (141).

Conflicting findings are also observed in the relationship between sortilin and apoB secretion. *In vivo* studies conducted by Musunuru *et al.* monitoring hepatic sortilin overexpression and knockdown found that overexpression of sortilin leads to a decrease in VLDL secretion, whereas knockdown of sortilin expression leads to an increase in VLDL secretion (152). These studies were conducted in *Apobec*$^{-/-}$; *APOB* TG mouse models, which possess a humanized lipoprotein profile. The lipoproteome of wild-type mice is

centered on higher circulating levels of HDL, whereas the lipoprotein distribution in humans is centered on LDL and VLDL. Therefore, *Apobec*$^{-/-}$; *APOB* TG mouse background is a more physiologically relevant model for human metabolism (183). These findings are in agreement with *in vivo* studies conducted by Strong *et al.*., which demonstrate that sortilin directly binds to apoB-containing lipoproteins and promotes their pre-secretory lysosomal degradation, thus inhibiting VLDL secretion (142). Furthermore, work conducted by Ai. *et al.* also support a role for sortilin in promoting VLDL degradation, as *in vivo* studies demonstrate that *SORT1* expression is associated with reduced VLDL secretion (208). In opposition to these findings, cell fractionation and pulse-chase experiments conducted by Kjolby *et al.*. in a *SORT1*$^{-/-}$ mouse model demonstrated a decrease in VLDL secretion when compared to control mice (141). Interestingly, studies conducted by Strong *et al.* in *SORT1*$^{-/-}$ mice of both the wild-type and *Apobec1*$^{-/-}$*; hAPOB Tg* background support this finding, as a reduction in VLDL secretion was observed in the absence of *SORT1* expression (142).

Overall, these disparate findings indicate that both sortilin deficiency and overexpression reduce VLDL secretion. Furthermore, some studies indicate that sortilin can regulate LDL catabolism, while another study demonstrates no role for sortilin no mediating LDL clearance. These data suggest that the hepatic functions of sortilin may depend on the metabolic milieu. Indeed, multiple hypotheses have been proposed to provide possible explanations for the contrasting results. One hypothesis suggests that sortilin may promote lysosomal degradation of VLDL in conditions of elevated intracellular apoB-100 levels, such as those observed in *Apobec1*$^{-/-}$ mouse models. Conversely, sortilin may facilitate VLDL biosynthesis and secretion in conditions of

reduced intracellular apoB-100 levels, such as those observed in a wild-type mouse model (183). Another hypothesis suggests that the regulation of VLDL biosynthesis through sortilin may be dependent upon the bioavailability of sortilin itself. This is reflected in the various mouse models utilized to study the effects of sortilin on LDL metabolism. A total body knockout of sortilin was associated with an increase in VLDL secretion (141, 142), whereas the specific knockdown of hepatic sortilin was associated with decreased VLDL secretion (142, 152, 208). In the latter studies, total expression of sortilin was unaffected with the exception of the liver. Finally, environmental factors may also influence the effects of sortilin on LDL metabolism, as the animals in the aforementioned studies were subjected to different diets (183). Indeed, future work is required to reconcile these disparate mechanisms. However, the overall consensus indicates that sortilin plays a role in regulating both VLDL and apoB-100 secretion as well as LDL catabolism.

Despite the novel association between sortilin, apoB-100 containing lipoproteins, and CAD, a role for this trafficking receptor in regulating Lp(a) metabolism has not been explored. Since Lp(a) is structurally homologous to LDL with the presence of a similar apoB-100 containing particle, it is plausible that sortilin may likewise regulate Lp(a) metabolism in a manner similar to LDL. In this thesis, we provide evidence in cultured hepatoma cells that increased sortilin expression is associated with an increase in the rate of apo(a) secretion, while decreased sortilin expression is associated with a decreased rate of apo(a) secretion; this effect is dependent upon functional LBS in apo(a) KIV_7 and KIV_8, but not KIV_{10}. We further demonstrated that sortilin overexpression increased the amount of Lp(a) internalized in hepatocytes and primary human fibroblasts either expressing or lacking functional LDLR activity. Interestingly, addition of ε-ACA, a lysine analog,

eliminated the ability of sortilin to increase Lp(a) internalization. We further demonstrated that both consequences of increased sortilin expression, namely increased apo(a) secretion and increased Lp(a) catabolism, required the functional activity of various sorting motifs in the cytoplasmic domain of sortilin. Regulation of Lp(a) catabolism and apo(a) secretion appears to be of an indirect nature, as no direct binding was observed between sortilin and either Lp(a) or apo(a). However, we demonstrated that sortilin co-localized with apo(a) in early and late endosomal compartments of the cell, which is suggestive of a role for sortilin in facilitating the intracellular trafficking of apo(a). Finally, deep sequencing of the *SORT1* gene identified 19 novel mutations that produce different polymorphic variants of sortilin. Seven of these mutations were found to occur in individuals that possessed extremely elevated levels of Lp(a). In an attempt to determine a potential mechanistic basis behind this association, we identified certain polymorphisms that altered Lp(a) catabolism and/or apo(a) secretion in hepatocytes when compared to wild-type sortilin.

4.1 Sortilin Enhances Apo(a) Secretion in HepG2 Cells

The rate of hepatic apo(a) production is an important determinant of plasma Lp(a) levels in humans (55, 56, 58). Previous research has demonstrated that the secretion of apo(a) is a complex process that is influenced by a variety of factors. Significant retention time of apo(a) both in the ER and Golgi compartments has been reported (76, 87). A direct relationship has been reported between isoform size and relative intracellular retention times (29). Studies conducted by White *et al.*. indicate that the observed variation in apo(a) secretion efficiency may be associated with the correct trimming of *N*-linked glycans, which occurs subsequent to folding events (28). Smaller isoforms of apo(a) are found to have increased rates of secretion compared to larger isoforms (27); therefore, smaller

isoforms may be more efficiently secreted through a reduced demand in *N*-linked glycan processing. Apo(a) is initially synthesized as a hypo-glycosylated precursor, with *N*-linked glycosylation occurring concomitantly to protein translation (82). The addition of *O*-linked glycans, as well as the completion of maturation of apo(a), occurs upon movement of the protein from the ER to the *medial*- to *trans*-Golgi compartments (91).

Overexpression of wild-type sortilin led to a significant increase in intracellular apo(a) accumulation in, and a significant increase in the rate of secretion of apo(a) from, HepG2 cells (Fig. 3.2). Furthermore, sortilin overexpression increased apo(a) secretion in HepG2 cells stably expressing 17K r-apo(a) (Fig. 3.6). HepG2 cells transiently transfected with siRNA directed towards *SORT1* expression demonstrated an average knockdown percentage of 53.7% in sortilin protein expression (Fig. 3.6 D). This reduction in sortilin expression is associated with a significant decline in the amount of apo(a) secreted from HepG2 cells stably expressing 17K r-apo(a) (Fig. 3.6 A, C). A significant difference in the intracellular accumulation of 17K r-apo(a) was also observed in cells transfected with sortilin directed siRNA (Fig. 3.6 B, C). Limits to the resolution of the bands meant that quantitative analysis could not be made about the rate of conversation from a hypo-glycoslyated to fully glycosylated form of apo(a); however, inspection of the fluorographs (Fig. 3.2, 3.4, 3.5, 3.6) demonstrates that there does not appear to be a notable difference. Mature apo(a) was first detectable in both the lysates and conditioned media at 30 minutes of chase time in both control cells and cells overexpressing the various forms of sortilin.

Maturation of apo(a) has been identified to be a rate-limiting step in the biosynthesis of the protein. However, it has also been postulated that the regulation of movement between intracellular compartments acts a second rate-limiting step in apo(a) production

(87). Our work indicates that sortilin may either prevent the presecretory degradation of apo(a), or potentially function in mediating the intracellular transport of apo(a), and that this effect is dependent upon the trafficking capabilities of sortilin. Analysis of the effect of various sortilin mutants supports this notion, as a reduced effect on apo(a) secretion compared to wild-type sortilin is observed for sortilin variants that possess mutations in canonical sorting motifs found in the cytoplasmic domain of the receptor (Fig. 3.1). Site-directed mutagenesis of key residues in the tyrosine and dileucine sorting motifs leads to a reduced effect for sortilin in increasing the intracellular accumulation and secretion of apo(a) (Fig. 3.2). Immunofluorescence data produced in this study further supports a trafficking role for sortilin in enhancing apo(a) secretion, as qualitative co-localization is observed between sortilin and apo(a) within endosomal compartments (Fig. 3.3 C, D). However, the co-localization observed with the endosomal compartments can reflect both the secretory and internalization pathways. Therefore, future work is required to optimize the immunofluorescence studies for quantitative analysis, and to explore the possibility that a sortilin-mediated regulation of apo(a) secretion is solely a representation of the secretory pathway. Maneuvers that inhibit apo(a) catabolism, such as ε-ACA addition, can be utilized to study this hypothesis.

Previous studies have demonstrated that the tyrosine and dileucine sorting motifs found in the cytoplasmic domain of sortilin are involved in mediating the intracellular targeting of the receptor (146, 160-162, 168, 209). Functionally, the tyrosine motif mediates sortilin trafficking through an interaction with adaptor protein-1 (AP-1), which is a member of the multimeric adaptor protein family. Inhibition of AP-1 activity leads to increased accumulation of sortilin within the TGN, suggesting that the tyrosine motif is involved in

the anterograde transport of sortilin and its cargo to other cellular compartments, as well as the cell surface (168). Our results support this notion, as a reduced effect on 17K r-apo(a) secreted compared to wild-type sortilin is observed for cells overexpressing the Y792A variant (Fig. 3.2 A, C). Interestingly, an increase in the intracellular accumulation of apo(a) is observed in cells overexpressing Y792A when compared to control cells (Fig. 3.2 A, B). Therefore, the increase in 17K r-apo(a) intracellular accumulation observed in cells overexpressing the Y792A variant is not reflected by an increase in secreted apo(a).

A possible explanation for the increase in intracellular apo(a) accumulation observed in cells overexpressing the Y792A variant may be attributed to the homology observed between the cytoplasmic domains of sortilin and mannose-6-phosphate receptors (MPRs). MPRs are well characterized sorting receptors involved in trafficking ligands to and from the *trans*-Golgi and endosomal compartments (151). Previous research has found that the cytoplasmic domain of sortilin is highly homologous to that of MPRs, with both domains possessing multiple, conserved sorting motifs that are involved in Golgi and endosomal trafficking (146, 168, 209). As a result, both receptors may share a conserved trafficking mechanism that exhibits functional dependence on interactions with adaptor proteins (151). Previous work conducted by Hirst *et al.* in fibroblasts demonstrated that, despite disrupting an interaction with AP-1, MPRs could still bind ligands, and participate in anterograde transport while complexed with cargo to endosomal compartments (210). This finding, as well as the homology between the cytoplasmic domains of sortilin and MPRs suggests that the Y792A variant of sortilin, which is unable to interact with AP-1, may be able to traffic to endosomal compartments in a manner similar to MPR. Indeed, previous research found that MPRs interact with Golgi-localizing, Gamma-adaptin Ear-

containing, ARF-binding proteins (GGAs), which mediate the anterograde transport of MPR and its cargo (211). Likewise, the dileucine motif mediates sortilin trafficking through an interaction with GGAs (146). Similar to the AP-1 studies, mutation of GGAs, which abolished their ability to interact with the dileucine sorting motif, inhibited the anterograde trafficking of sortilin resulting in the accumulation of the receptor in the perinuclear region (137, 146). Therefore, an intact and functional dileucine sorting motif in the cytoplasmic domain of sortilin is essential in facilitating the anterograde transport of the receptor and its cargo from the TGN to endosomes.

Similar to the results obtained for the Y792A variant, a reduced effect on 17K r-apo(a) secretion compared to wild-type sortilin is observed for cells overexpressing the L829/830A variant (Fig. 3.2 A, C). Moreover, no significant differences in intracellular apo(a) accumulation was observed between L829/830A and control cells (Fig. 3.2 A, B). This suggests that the dileucine motif may play a more essential in mediating the increase in the intracellular accumulation of apo(a) brought about through sortilin overexpression. Taken together, the pulse-chase data indicate that the tyrosine and dileucine motifs within the cytoplasmic domain of sortilin work in a coordinated manner to increase apo(a) secretion. Indeed, removal of the entire cytoplasmic domain yielded the greatest reduction in both intracellular and secreted apo(a) accumulation when compared to wild-type sortilin (Fig. 3.2). The individual mutation of these domains did not completely abolish an increase in apo(a) secretion brought about by sortilin overexpression, therefore, both motifs are essential in mediating this effect. This is in agreement with previous research, which indicated that the dileucine motif plays a bigger role in facilitating the intracellular trafficking of sortilin (146).

However, the tyrosine motif in sortilin is essential in binding the Vps35 subunit of the retromer complex, which is largely responsible for mediating the recycling of trafficking receptors from endosomes to the TGN (151, 168). Mutation of essential residues within this motif inhibits the interaction between sortilin and the retromer complex, thus leading to an accumulation of sortilin in the endosomal system along with a concomitant depletion in the Golgi complex (168). Therefore, the Y792A mutant receptors may be capable of trafficking nascent apo(a) from the TGN to endosomal compartments, thus leading to elevated levels of intracellular apo(a). However, this variant will become trapped in the endosomal compartments, therefore inhibiting its ability to continuously promote the secretion of newly synthesized apo(a). This may explain why cells overexpressing the Y792A yielded an increase in intracellular apo(a) without a concomitant increase in apo(a) secretion.

The overexpression of both the Y792A and L829/830A variants in HepG2 cells displayed a significant increase in the amount of apo(a) secreted after 480 minutes of chase. The homology with MPRs may again provide a possible explanation for this observation. A previous study by Nielsen *et al.* demonstrated that chimeric constructs consisting of the lumenal domain of MPR and the cytoplasmic domain of sortilin are able to rescue trafficking defects observed in cells lacking functional MPRs (146). These data suggest that sortilin and MPR may act as complimentary sorting receptors. Indeed, previous studies found that extensive co-localization is observed between sortilin and MPRs, which suggests that these proteins function in similar pathways (124, 165). Furthermore, immunogold labelling studies conducted by Mari *et al.* demonstrated that the relative subcellular distributions of sortilin and MPRs were nearly identical, demonstrating that these receptors

may not only share similar transport pathways, but similar transport kinetics as well (161). Taken together, these data suggest that the sorting pathways MPRs and sortilin may display redundancy. Future work is required to determine if MPRs are upregulated in cells overexpressing the sortilin trafficking mutants employed in this thesis, and if this upregulation is associated with an ability to rescue the impaired trafficking capabilities of sortilin.

Previous studies demonstrate that sortilin mediates apoB movement through the biosynthetic pathway through its ability to act as a trafficking receptor (142). Furthermore, sortilin was found to bind to apoB-100 with high-affinity, thus indicated that sortilin directly regulates apoB secretion (141, 142). As such, we conducted *in vitro* experiments to determine if sortilin can bind to apo(a) and directly facilitate transport of apo(a) through the biosynthetic pathway. We found, through the use of two different methods, that endogenous sortilin can bind to apoB-100, but not apo(a) *in vitro* (Fig. 3.12). This indicates that sortilin is indirectly enhancing apo(a) secretion as opposed to directly binding to and mediating apo(a) transport through intracellular compartments. The identification of co-localization in this thesis between apo(a) and sortilin (Fig. 3.3) indicates that sortilin may enhance apo(a) secretion as part of a complex. It is possible that increased sortilin expression may upregulate the expression, enhance the function, or alter the localization of other trafficking proteins that act to mediate apo(a) movement through the biosynthetic pathway.

Interestingly, pulse-chase analysis demonstrates that sortilin overexpression was unable to influence the secretion of 17KΔLBS$_{7,8}$, which is an apo(a) variant that lacks lysine binding capabilities in the weak LBS of KIV$_{7,8}$ (Fig. 3.4). The 17KΔLBS$_{7,8}$ apo(a) variant

possesses a glycine residue instead of an aspartate residue at position 56 in both KIV_7 and KIV_8, thus altering the charged environment of the lysine binding pocket. As a result, these kringle domains can no longer participate in lysine binding (62). Previous research of $17K\Delta LBS_{7,8}$ apo(a) demonstrates that intact, functional LBS in these KIV subtypes are required for Lp(a) assembly. Specifically, lysine residues within the amino-terminal segment of apoB-100 associate with the weak LBS of $KIV_{7,8}$, thus mediating the non-covalent interaction between apo(a) and apoB that is the initial step involved in Lp(a) particle assembly (212, 213).

The overall site of noncovalent, as well as final covalent, assembly remains controversial. Multiple studies suggest that an Lp(a) particle is formed extracellularly following the secretion of apo(a) and apoB from hepatocytes (73-76). However, research has also demonstrated that Lp(a) formation can occur intracellularly (77-79); this is supported by kinetic data in humans (80, 81). Interestingly, a study has found an association between triglyceride synthesis and apo(a) production in in hepatocytes (77). Furthermore, kinetic labelling studies in humans discovered that the production rates of both apolipoprotein moieties of Lp(a) are identical, and that particle formation incorporates newly-synthesized apo(a) and apoB as opposed to newly synthesized apo(a) associating with circulating LDL particles (81). *In vitro* studies in hepatoma cells also demonstrate that the hypo-glycosylated precursor of mature apo(a) can associate intracellularly with apoB-100 (82). Taken together, these data support the concept that a non-covalent association between apo(a) and apoB can occur intracellularly.

If apoB and apo(a) associate intracellularly as the evidence suggests, and previous evidence indicates that the secretion of these apolipoproteins are linked, it is therefore

possible that the increase in apo(a) secretion through sortilin overexpressing may be mediated by a "piggyback" effect with apoB. Sortilin overexpression may enhance the secretion of apoB, which indirectly promotes apo(a) secretion through the non-covalent association of the two lipoproteins. The data in this thesis are in agreement with this concept, as sortilin overexpression was unable to increase the secretion of $17K\Delta LBS_{7,8}$ apo(a).

However, this conclusion is complicated by the disparate results obtained in studies monitoring the effects of sortilin expression on the secretion of apoB and apoB-100 containing lipoproteins. As explained previously, multiple studies found that increased sortilin expression is associated with a concomitant reduction in apoB and VLDL secretion (142, 152, 208). Conversely, a study conducted by Kjolby *et al.* found that increased sortilin expression leads to enhanced apoB and VLDL secretion (141). Furthermore, multiple studies found that a reduction in apoB secretion is observed in *SORT1*$^{-/-}$ mice (141, 142). The pulse-chase analysis conducted in this thesis supports the latter findingsIf the ability of sortilin to regulate apo(a) secretion is dependent upon a non-covalent interaction with apoB, as well as its ability to directly regulate apoB biosynthesis, the observed increase in apo(a) secretion with sortilin overexpression would have to be accompanied with a concomitant increase in apoB secretion. The opposite effect would be observed in cells with reduced sortilin expression. Therefore, future work is required to examine the regulatory effects of sortilin expression on apoB secretion in the pulse-chase model utilized in this study. Overexpression and knockdown of sortilin can be studied in the context of apoB-100 secretion from HepG2 cells. The pulse-chase studies of apo(a) secretion can be repeated in the context of HepG2 cells that are treated with siRNA directed against the

apoB gene. It cannot be excluded that apo(a) $KIV_{7,8}$ are mediating interactions not with apoB, but instead with some other protein whose secretion is regulated by sortilin.

Pulse-chase analysis of 17KΔLBS_{10}, which is an apo(a) variant that lacks lysine binding capabilities in the strong LBS of KIV_{10}, demonstrated that overexpression of sortilin is able to significantly increase the intracellular accumulation and secretion of apo(a) in a manner similar to that observed for wild-type apo(a) (Fig. 3.5). The 17KΔLBS_{10} variant possesses an alanine residue at position 57 in KIV_{10} as opposed to an aspartic acid residue, thus altering the charged environment required for lysine recruitment and binding (214). A functional LBS in KIV_{10} is important for the lysine-dependent association of apo(a) with various protein substrates (13). It is possible that such interactions modulate apo(a) secretion. Since the secretion of this variant was upregulated by sortilin in a manner not dissimilar to wild-type apo(a), such interactions are not relevant to the effects of sortilin. Notably, the non-covalent interaction of apo(a) with apoB is not influenced at all by the KIV_{10} LBS. Taken together, the pulse-chase analysis (Fig. 3.2, 3.4, 3.5, 3.6), in combination with the *in vitro* binding analysis (Fig. 3.12), suggests that an intracellular association between apo(a) and apoB may be required to facilitate apo(a) secretion with sortilin overexpression.

4.2 Sortilin Increases Internalization of Lp(a), But Not Apo(a)

The rate of clearance also plays a role in dictating the plasma concentrations of Lp(a) (99). The main pathways of Lp(a) clearance remain controversial, as multiple studies implicate the involvement of a variety of receptors, such as plasminogen receptors, LDLRs, and VLDLRs, in facilitating the internalization and degradation of Lp(a) (99). The discovery of a novel association between sortilin and LDL cholesterol levels provides an

interesting insight into a potential role for sortilin in regulating LDL metabolism. As such, both *in vivo* and *in vitro* indicate that sortilin can affect plasma LDL-C levels by promoting the uptake and catabolism of LDL (142, 153), although a study by Kjolby and coworkers refutes this concept (141). The specific mechanisms by which sortilin regulates LDL catabolism appear to be dependent upon an interaction with the apoB component of LDL. Since Lp(a) possesses a similar apoB component, we hypothesized that Lp(a) catabolism may be regulated in a similar manner. As we report for the first time, overexpression of sortilin results in a significant increase in the amount of Lp(a) internalized in HepG2 cells, primary mouse hepatocytes, and primary human fibroblasts (Fig. 3.7, Fig. 3.11).

Recent evidence indicates that elevated levels of plasma Lp(a) can be significant reduced with the use of monoclonal antibodies directed against PCSK9 (120). Furthermore, previous data generated by our group demonstrates that treating HepG2 cells with wild-type, exogenous PCSK9 leads to a significant decrease in the amount of both Lp(a) and apo(a) internalized (112). Interestingly, work conducted by Gustafsen and coworkers provides evidence for a novel association between PCSK9 and sortilin (143). SPR analysis and co-immunoprecipitation studies indicate that PCSK9 can bind to sortilin in a pH dependent manner, and that this interaction was necessary for sortilin to act as a molecular chaperone for, and promote the secretion of, PCSK9 late in the secretory pathway (143). Based on this study, sortilin overexpression should lead to a concomitant increase in PCSK9 bioavailability. Furthermore, numerous studies support a role for the LDLR a role for the LDLR in in Lp(a) catabolism (102-113). However, this concept is controversial, as multiple studies indicate that the LDLR does not mediate Lp(a) catabolism (101, 114-117).

Previous work conducted by our group supports a role for the LDLR in mediating Lp(a) catabolism, as PCSK9-treated HepG2 cells demonstrated a significant decrease in the amount of Lp(a) internalized when compared to non-treated cells (112). In light of this evidence, we hypothesized that increased PCSK9 bioavailability may counter the increased rate in Lp(a) internalization brought about by sortilin overexpression. As observed in Fig. 3.7 A, PCSK9-treated HepG2 cells overexpressing wild-type sortilin demonstrate a significant increase in the amount of Lp(a) internalized compared to PCSK9-treated control cells. Therefore, sortilin overexpression is still able to promote Lp(a) internalization in HepG2 cells in the presence of elevated levels of PCSK9. Work conducted by Strong and coworkers found that sortilin expression in *LDLR*$^{-/-}$ mice led to a significant increase in the catabolic rate of LDL when compared to control mice, therefore suggesting that sortilin mediates LDL degradation through a pathway that is independent of the LDLR (142). As demonstrated in Fig. 3.11 A, sortilin overexpression significantly increases the amount of Lp(a) internalized in both FH and non FH human fibroblasts. This suggests that, similar to the effects on LDL catabolism, sortilin can increase Lp(a) internalization through an LDLR-independent pathway. This is supported by the results in PCSK9-treated HepG2 cells (Fig. 3.7 A). A similar result for PCSK9 treatment was not obtained in fibroblast cells (Fig. 3.11 A), however, this could be due to variation in LDLR availability. Fibroblasts do not produce apoB endogenously, and may therefore have reduced bioavailability of LDLR.

The ability of sortilin to increase LDL catabolism is dependent upon the proper trafficking capabilities of the receptor (142). Therefore, to determine if the increase in Lp(a) internalization brought about by sortilin overexpression is dependent upon the ability of sortilin to act as a trafficking receptor, we monitored the levels of Lp(a) internalization

occurring in HepG2 cells and primary mouse hepatocytes overexpressing the Y792A, L829/830A, and sortilinΔCT trafficking mutants of sortilin. The defect in the ability of sortilin to increase Lp(a) internalization in HepG2 cells was most profound in cells overexpressing either the sortilinΔCT or Y792A mutants, as a significant reduction in the amount of Lp(a) internalized is observed in cells overexpressing these variants when compared to cells overexpressing wild-type sortilin. Such a difference is not observed in cells overexpressing the L829/830A variant. However, as observed in Fig. 3.7, the amount of Lp(a) internalized is comparable in cells overexpressing either the L829/830A or Y792A variants. The SEM for the L829A/830A mutant is larger than the SEM of the Y792A or sortilinΔCT mutants. This may have skewed the statistical analysis, resulting in a lack of statistical significance observed between either control cells or cells overexpressing sortilin. Therefore, more trials are required for the L829/830A variant to confirm if impairment of the dileucine sorting motif leads to a reduced effect in Lp(a) internalization in HepG2 cells.

Interestingly, no significant difference in Lp(a) internalization is observed between primary mouse hepatocytes overexpressing either the Y792A or L829/830A mutant when compared to hepatocytes overexpressing wild-type sortilin. Only the sortilinΔCT mutant to demonstrated a defect in the ability of sortilin to increase Lp(a) internalization, as hepatocytes overexpressing this variant demonstrated a significant reduction in the amount of Lp(a) internalized when compared to cells overexpressing wild-type sortilin (Fig. 3.7 B).

Overall, the results obtained for Lp(a) internalization (Fig. 3.7 A, B) demonstrate some similarities with those obtained for LDL catabolism by Strong and coworkers (142). Mice expressing a sortilin variant similar to sortilinΔCT found that the absence of a

cytoplasmic domain completely abolishes the ability of sortilin to increase LDL catabolism (142). This is in agreement with the results obtained for HepG2 cells and mouse hepatocytes overexpressing sortilinΔCT (Fig. 3.7 A, B). The comparable amount of Lp(a) internalized between the control and the sortilinΔCT variant in both cell lines (Fig. 3.7 A, B) suggests that the effect of sortilin overexpression on Lp(a) internalization is dependent upon the ability of sortilin to act as a trafficking receptor, and that a fully intact cytoplasmic domain is required to mediate this effect. Interestingly, Strong *et al.* found that a sortilin variant that possessed mutations of conserved residues within the tyrosine and dileucine motifs was still able to increase the rate of LDL clearance from plasma. The internalization assays performed in primary mouse hepatocytes (Fig. 3.7 B) reflects this finding for Lp(a) internalization, as only a moderate reduction in the amount of Lp(a) internalized is observed in hepatocytes overexpressing both Y792A and L829/830A compared to wild-type sortilin. When taken together, these data indicate that the tyrosine and dileucine motifs within the cytoplasmic domain of sortilin may work in a coordinated manner to facilitate Lp(a) internalization in HepG2 cells.

Previous research found that sortilin directly trafficks LDL to the lysosome subsequent to internalization (142). Strong *et al.* found that the variant possessing mutagenized tyrosine and dileucine motifs was unable to effectively promote the lysosomal degradation of LDL in a manner similar to wild-type sortilin (142). LDL internalization by the LDLR occurs through clathrin-mediated endocytosis, where LDL is shuttled to the lysosome for degradation subsequent to internalization at the cell surface (215). Work conducted by our group demonstrates that Lp(a) is likewise internalized through clathrin-mediated endocytosis, and that Lp(a) is targeted to lysosomes following internalization

(112). Sortilin is able to bind a variety of ligands and traffic them from the Golgi apparatus to the lysosome. This ability is dependent upon the formation of clathrin-coated-cargo vesicles (151). Clathrin interacts with the multimeric and monomeric adaptor protein families which include AP-1 and GGAs. As previously mentioned, the tyrosine motif is responsible for binding AP-1, while the dileucine motif mediates interaction with the GGAs (137, 146, 168). Disrupting these motifs through mutation can inhibit their ability to interact with adaptor proteins, and thus clathrin. This may impair the ability of sortilin and its cargo to localize in clathrin-coated-cargo vesicles, leading to a concomitant reduction in the ability of sortilin to participate in lysosomal trafficking. This could lead to a reduction in the clathrin-mediated endocytosis of, and concomitant reduction in the lysosomal targeting of, LDL as observed by Strong *et al.* (142). Likewise, this disruption in clathrin interaction can providing a possible explanation for the decrease in Lp(a) internalization observed in HepG2 cells overexpressing the sortilin trafficking variants. Therefore, future work is required to determine if cells overexpressing the Y792A or L829/830A sortilin variants are unable to traffic Lp(a) to the lysosome for degradation, and if inhibiting localization within clathrin-coated vesicles is associated with decreased Lp(a) internalization in HepG2 cells. This can be achieved by studying the effects of lysosomal inhibitors on Lp(a) internalization in the context of sortilin overexpression. Likewise, co-localization studies between sortilin, Lp(a), and a lysosmal marker may also provide interesting insight with respect to this hypothesis.

To determine if sortilin could mediate Lp(a) internalization at the endogenous level, we reduced sortilin expression in HepG2 cells through siRNA-mediated targeting of the *SORT1* gene to determine if a reduction in sortilin expression would be associated with a

decrease in Lp(a) internalization. However, despite an average knockdown percentage of 59.36%, a significance decrease in Lp(a) internalization is not observed in cells treated with sortilin siRNA when compared to control cells (Fig. 3.8). These data indicate that sortilin may only play a role in regulating the catabolism of Lp(a) when it is expressed at elevated levels. GWAS studies identified an association between SNPs near the *SORT1* gene and LDL-C levels (178, 182). Fine mapping of this region demonstrates that a minor allele SNP, rs12740374, occurring in a non-coding region between *PSRC1* and *CELSR2* generates a novel binding site for C/EBP, which is a liver-specific transcription factor (152). Each copy of the minor allele is found to be associated with a 6-fold increase in *SORT1* expression. Therefore, individuals with copies of the minor allele would possess significantly elevated levels of sortilin. It is possible that Lp(a) catabolism is enhanced in such individuals, although this possibility has not been examined.

To gain further insights into the molecular mechanism by which sortilin expression regulates Lp(a) internalization, we conducted interaction studies to determine if sortilin is mediating Lp(a) internalization by acting as a cell surface receptor. We found, through the use of two different methods, that endogenous sortilin can bind apoB-100, but not Lp(a) (Fig. 3.12). Previous work conducted by Kjolby *et al.* found that sortilin can interact with apoB-100, but not apoB-48 (141). This suggests that sortilin interacts with the C-terminal region of apoB. Several studies suggest that Cys^{4326} in apoB-100 is involved in disulfide bond formation with apo(a) (65, 66). Conversely, other studies have implicated that the disulfide linkage occurs between apo(a) and Cys^{3734} ine apoB (67-70). Both of these cysteine residues are present in the C-terminal portion of apoB-100 (216, 217). Therefore, it is possible that the covalent attachment of apo(a) to apoB may impair the ability of apoB

to bind to sortilin. The interaction studies support this concept, as endogenous sortilin is able to interact with apoB-100, but not the apoB component of Lp(a) (Fig. 3.12). The lack of a direct interaction suggests that sortilin is not acting as a cell surface receptor for Lp(a).

It is possible that sortilin may affect the abundance of clearance receptors that require interaction with both apo(a) (in a lysine dependent fashion) and apoB. The reduced amounts of Lp(a) internalized in HepG2 cells overexpressing various trafficking mutants of sortilin supports this hypothesis (Fig. 3.7 A, B). Both the tyrosine and dileucine sorting motifs facilitate the anterograde transport of sortilin and its ligands, therefore indicating that disruption of these motifs can inhibit sortilin from trafficking potential Lp(a) clearance receptors to the cell surface. Interestingly, treatment with a lysine analogue, ε-ACA, leads to a significant reduction in the amount of both Lp(a) and apo(a) internalized in both control cells and cells overexpressing wild-type sortilin (Fig. 3.10 A, B). Indeed, the amount of Lp(a) internalized in ε-ACA-treated cells overexpressing sortilin is comparable to ε-ACA-treated control cells. This suggests that the ability of sortilin to increase Lp(a) internalization is dependent on lysine binding interactions, which implicates an important role for the apo(a) component of Lp(a) in mediating this effect.

The structural homology between apo(a) and plasminogen may be the potential driving force behind this effect, as plasminogen receptors contain carboxyl-terminal lysine residues that can interact with the LBS of apo(a) (102). Competitive binding between apo(a) and Lp(a) with plasminogen for plasminogen receptors supports this concept, as evidence suggests that Lp(a) can bind to and be internalized by these receptors (47, 48). This suggests that receptors with carboxyl terminal lysine residues, such as the plasminogen

receptors, may play a role in mediating the observed increase in Lp(a) internalization with sortilin overexpression.

Previous work demonstrates that the apo(a) component is necessary for Lp(a) to interact with the plasminogen receptor $\alpha_M ß_2$ (46). Furthermore, a study by Tam and coworkers indicate that the apo(a) component is responsible for binding to low affinity plasminogen receptors on the surface of HepG2 cells. Treatment with ε-ACA reduced the ability of plasminogen receptors to internalize apo(a), which are capable of binding and internalizing both free and LDL-complexed apo(a) (102). However, the apparent role of apo(a) is complicated by our results for apo(a) internalization, which demonstrate no significant change in the amount of apo(a) internalized with sortilin overexpression when compared to an empty vector control. (Fig. 3.9, Fig. 3.11 B). This may be reconciled by the methodology chosen to study the internalization of apo(a). These studies monitored the internalization of a free, recombinant apo(a). It is possible that sortilin is only capable of increasing the internalization of apo(a) when it is in a complex with apoB. In the physiological setting, negligible amounts of free apo(a) exists, as the majority of apo(a) associates with apoB in Lp(a) particles following secretion (59). A second possible explanation may involve the multiple pathways of apo(a) clearance. A greater amount of routes are available for the clearance of free apo(a) as opposed to apo(a) in the context of the Lp(a) particle. Indeed, a previous *in vivo* study demonstrated that apo(a) injected into mice was cleared by the liver greater than twice as fast as Lp(a), thus indicating that the lipid moiety may lower the affinity of Lp(a) for various apo(a) receptors (27). Therefore, future work is required to determine if an association exists between sortilin expression and various plasminogen receptors, as well as other receptors dependent upon lysine

interactions, on the cell surface of hepatic cells, and if this is relevant to the increase in Lp(a) internalization observed with sortilin overexpression. Measuring both the mRNA and protein levels of various plasminogen receptors under the context of sortilin overexpression may provide some interesting insight into this hypothesis.

It is also possible that sortilin is increasing the abundance of other receptors implicated in Lp(a) catabolism such as LRP-1, VLDL, gp330, and SR-BI, which are receptors implicated in Lp(a) catabolism (108, 121-123). The apo(a) component appears to mediate the abilities of VLDL, LRP-1 and SR-BI to internalize Lp(a); however, the exact mechanism of action remains unclear. Taken overall, our data indicate that sortilin is indirectly facilitating Lp(a) catabolism by potentially increasing the concentration of cell surface receptors responsible for binding to and internalizing circulating Lp(a). Future work is required to determine if sortilin overexpression leads to a concomitant increase is observed in the mRNA and protein levels of thesepotential Lp(a) receptors.

4.3 Naturally Occurring Polymorphisms Within the VPS10 Domain May Alter the Ability of Sortilin to Increase Apo(a) Secretion and Lp(a) Catabolism

Data presented in this study demonstrate that sortilin overexpression can promote Lp(a) catabolism and apo(a) secretion in hepatoma cells (Fig. 3.2, Fig. 3.7). Since nascent apo(a) appears to immediately associate with apoB upon secretion from hepatocytes (59), this suggests that an increase in apo(a) production should be associated with a concomitant increase in Lp(a) particle assembly. Therefore, our data suggest that sortilin expression may elevate Lp(a) plasma levels by increasing the synthesis of Lp(a). However, our findings with respect to Lp(a) catabolism indicate that an increase in Lp(a) internalization brought about by sortilin overexpression may likewise lower plasma levels of Lp(a). Indeed, it is

possible that both mechanisms are occurring simultaneously *in vivo*, and that the effects of sortilin on apo(a) secretion are cancelled out by the effects on Lp(a) internalization.

Therefore, in an attempt further understand how sortilin may contribute to Lp(a) levels, deep sequencing of *SORT1* was performed in patients possessing elevated levels of Lp(a). Sequencing analysis led to the identification of 19 rare, heterozygous missense or splicing variants within the *SORT1* gene. Interestingly, 9 individuals within the cohort of patients studied are found to possess plasma Lp(a) levels within the 5^{th} percentile for the general population. These individuals were found to be heterozygous for seven novel, polymorphic variants of sortilin lead to amino acid substitions, all of which occur within the VPS10 binding domain. In the interest of determining if an association can be made between the expression of these sortilin variants and elevated Lp(a) levels, site-directed mutagenesis was performed to generate plasmids for these variants, identified as I124V, K205N, K302E, K404Y, E444Q, E447G, and V650M (Fig. 3.13).

The VPS10 domain, which is representative of the entire lumenal section of sortilin, is a large domain composed of 716 amino acids (126, 128). This domain is present in all members of the VPS10 mammalian protein family. Modest sequence similarity is observed between the VPS10 domains for the VPS10 family members with the exception of the N-terminal propeptide and a ~120 amino acid sequence that contains ten conserved cysteine residues (126). This region, which is found closest to the transmembrane domain, is composed of amino acids 577-716 and is classified as the 10CC module. This segment can be further subdivided into two small subdomains categorized as 10CC-A and 10CC-B (residue 577-633 and 634-716 respectively) (128). Crystal structure analysis of the VPS10 domain found it to be oriented in the shape of a ten-bladed ß-propeller, with ligand binding

occurring in the centralized tunnel of the structure (Fig. 3.13) (128). The 10CC domains interact extensively with the ß-propeller, and the ten conserved cysteine residues were all found to participate in disulfide binding, thus resulting in the formation of five, highly conserved disulfide bond linkages within the 10CC module (Fig. 3.13) (126, 128). Interestingly, previous research found that the 10CC domains could also participate in ligand binding (126).

The I124V, K205N, K302E, F404Y, E444Q, and E447G polymorphisms all occur within the ß-propeller region of the VPS10 domain (residues 46-576). The V650M polymorphism is the only mutation that occurs within the highly conserved 10CC module, specifically occurring between two cysteine residues in the 10CC-B subdomain that are linked through a disulfide bond (Fig. 3.13). Overall, these mutations may contribute to ligand binding variability within the VPS10 domain through variable folding dynamics. This may consequentially lead to variation in the ability of sortilin to traffic cargo through the biosynthetic pathway, thus leading to variations in Lp(a) levels. Since these polymorphisms were found to occur in individuals that possessed elevated levels of Lp(a), understanding the effect of these variants on apo(a) secretion and Lp(a) metabolism might resolve whether sortilin has a greater impact on Lp(a) synthesis or catabolism. We overexpressed the various polymorphic variants in HepG2 cells to study the effects of these variants on apo(a) secretion and Lp(a) internalization. Varying trends are observed in HepG2 cells overexpressing the polymorphic variants (Fig. 3.14), that do not provide a definitive answer to this question.

The I124V variant appears to function in a manner similar to wild-type sortilin (Fig. 3.14), thus suggesting that the I124V variant may contribute to Lp(a)/apo(a) metabolism in

a manner similar to that observed for wild-type sortilin. A modest reduction in both apo(a) secretion and Lp(a) internalization is observed in cells expressing the K205N variant (Fig. 3.14 A, B). A large increase is observed in the intracellular accumulation of apo(a) for K205N expressing cells (Fig. 3.14 C). It is possible that this mutation may promote apo(a) biosynthesis or prevent the presecretory degradation of apo(a), yet be defective in its ability to promote apo(a) secretion. Overall, it appears that this variant may contribute to elevated Lp(a) levels through a modest reduction in catabolic rate accompanied with an increase in apo(a) biosynthesis. Similar to the K205N variant, a modest reduction in the amount of Lp(a) internalized in cells overexpressing the K302E variant compared to cells overexpressing wild-type sortilin (Fig 3.14 A). Conversely, a decrease in the accumulation of both intracellular and secreted apo(a) is observed in cells expressing this variant as well (Fig. 3.14 B, C). Therefore, K302E may only play a minor role in contributing to elevated Lp(a) levels, with this effect occurring at the level of catabolism as opposed to assembly. The F404Y variant also displays a reduction in Lp(a) catabolism (Fig. 3.14 A) when compared to cells overexpressing wild-type sortilin. Indeed, the amount of Lp(a) internalized in cells overexpressing this variant are comparable to control cells. The intracellular accumulation of apo(a) in cells overexpressing the F404Y variant is comparable to cells overexpressing wild-type sortilin. However, the amount of apo(a) secreted from cells overexpressing this variant is greater than that observed in cells overexpressing wild-type sortilin (Fig. 3.14 B, C). Unlike the previous three polymorphisms, the amount of Lp(a) internalized in cells expressing the E444Q mutant is comparable to that observed in cells expressing wild-type sortilin (Fig. 3.14 A). A reduction in both apo(a) secretion and intracellular accumulation is observed in cells overexpressing this variant when compared to cells overexpressing wild-type sortilin (Fig. 3.14 B, C).

These data suggest that this variant may not contribute to elevated Lp(a) levels. A reduction in the amount of Lp(a) internalized is observed in cells expressing the V650M variant when compared to cells expressing wild-type sortilin and control cells (Fig. 3.14 A). A moderate decrease in both apo(a) secretion and intracellular accumulation is observed when compared to cells overexpressing wild-type sortilin. However, the amount of both intracellular and secreted apo(a) in cells overexpressing V650M is increased compared to control cells, indicating that this variant can still promote apo(a) secretion (Fig. 3.14 B, C). Taken together, these data suggest that the V650M variant may contribute to elevated Lp(a) levels at the level of both catabolism and assembly.

The amino acid substitutions encompass conservative substitutions (e.g. I124V, where one aliphatic side chain is substituted for another); charge substitutions (e.g. K302E, where a positively charged side chain is substituted for a negatively charged one); and loss of a charged residue (e.g. E444Q). These substititons could alter the folding and thus tertiary structure of sortilin, or could result in changes to charged patches on the surface of the protein. Either of these changes could affect the ability of sortilin to bind to gargets through which it algers apo(a)/Lp(a) metabolism. The absence of overexpression for the E447G variant led to the exclusion of this SNP from the data. It is possible that an error occurred during the creation of the expression vector encoding this variant. Likewise, it is also possible that this SNP may lead to a non-functional sortilin protein, which would be a novel discovery. Therefore, future studies are required to characterize the E447G polymorphic variant.

A particularly interesting case is theV650M mutation, as it is the only SNP that occurs within the highly conserved 10CC module (Fig. 3.13). This region possesses 10

cysteine residues that are conserved in both location and spacing, and are involved in the formation of disulfide bonds. The 650 position is situated in the middle of two invariant cysteine residues that are linked through a disulfide bond (residues C642 and C655 respectively) (126). Both valine and methionine possess nonpolar, aliphatic side chains. However, the side chain of methionine is unique in that it possesses a sulfur atom. This evokes evokes the possibility of the methionine becoming oxidized to a sulfoxide, which would have a particularly notable effect on the structure of sortilin. Indeed, a previous study indicates that oxidized methione residues can lead to misfolded or dysfunctional proteins (218). Furthermore, a conserved disulfide bond occurs between C642 and C655; a sulfoxide modification could potentially destabilize or inhibit this linkage. Since the 10CC module is shown to extensively interact with and stabilize the ß-propeller (128), disruption within this conserved region may destabilize the ß-propeller and alter its ligand binding potential. Furthermore, the 10CC module can also participate in ligand binding (126). Therefore, variation in folding can not only affect ligand binding potential in the tunnel of the ß-propeller, but in the 10CC module itself. Our data suggest that sortilin may promote Lp(a) internalization by increasing the abundance of receptors involved in the clearance of Lp(a). The reduction in the amount of Lp(a) internalized in cells overexpressing the V650M variant suggests that this mutation impairs the ability of sortilin to increase the concentration of these receptors at the cell surface. Conversely, the methionine substitution in this region of the VPS10 domain does not appear affect the processing involved in mediating apo(a) secretion.

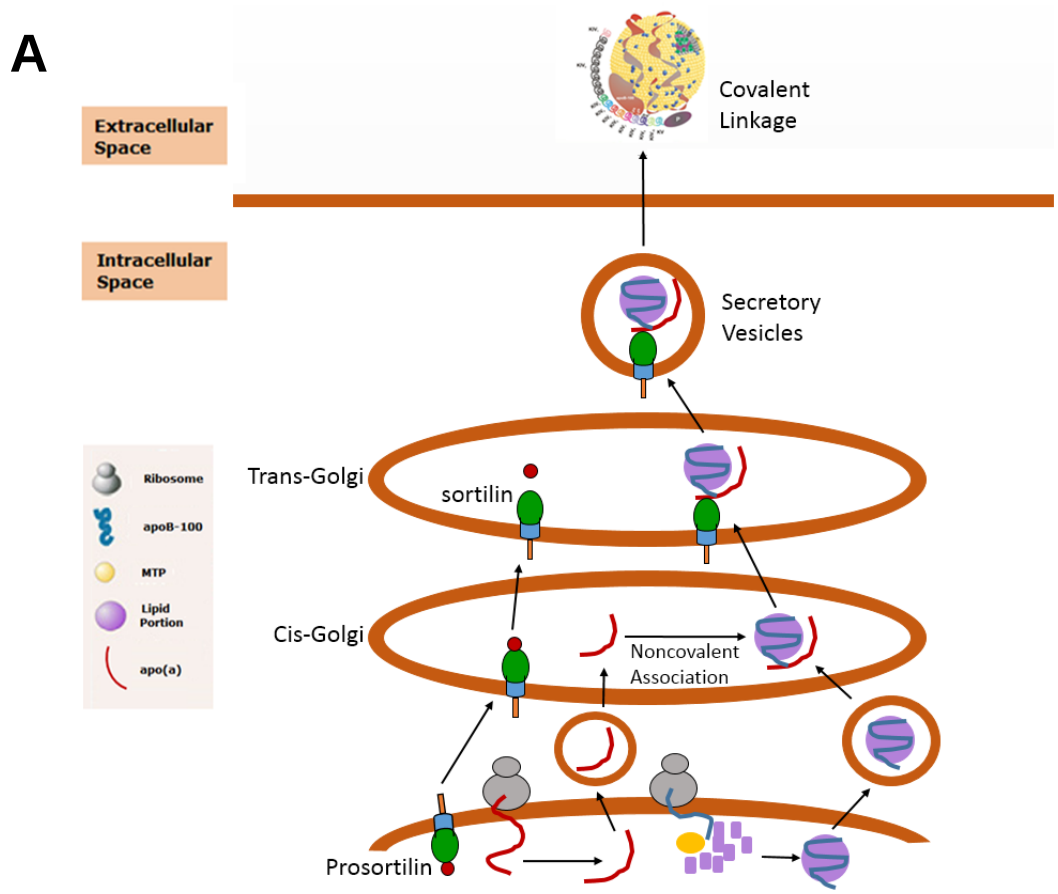
Previous data demonstrate that neurotensin binds in the tunnel of the ß-propeller domain. The C-terminal carboxylate group of neurotensin was found to be the major

contributor to this association, as it forms salt bridges with the guanidinium group of Arg292, as well as hydrogen bonds with the R groups of Ser283 and the aliphatic backbone of Tyr318 (128). An additional salt bridge occurs between Glu667 in the 10CC-B domain and Arg293 in the ß-propeller domain, suggesting that the 10CC-B domain is involved in mediating the binding of neurotensin (128). Interestingly, previously reported interacting partners of sortilin all demonstrate competitive binding, indicating that they share a similar binding site (126). Since treatment with ε-ACA abolished the ability of sortilin to increase Lp(a) internalization (Fig. 3.10), it is tempting to speculate that receptors with carboxyl-terminal lysine residues, such as plasminogen receptors, may bind to sortilin in a manner similar to that of neurotensin. This would allow sortilin to directly bind to and mediate the intracellular trafficking of these receptors, thus leading to a potential increase in cell surface concentrations and a concomitant increase in Lp(a) internalization. Taken together, our findings are consistent with the notion that some, but not all, variants may contribute to elevated Lp(a) levels by either increasing the rate of particle assembly or decreasing the rate of Lp(a) internalization.

4.4 Association Between Sortilin Expression and Plasma Lp(a) Levels

GWAS studies looking for biomarkers related to cardiovascular disease demonstrated an association between *SORT1* and a reduction in LDL-C, as well as risk for MI and CAD. Elevated levels of plasma Lp(a) is recognized as an independent, causal risk factor for CHD (15, 31). The association between CHD and Lp(a) as an independent risk factor, as well as the previous studies demonstrating an association between *SORT1* expression and LDL metabolism, led to our hypothesis that sortilin may play a role in regulating Lp(a) metabolism. Our data indicate that the effects of sortilin expression on

regulating Lp(a) levels is multifaceted. Certainly, there is precedence for a complex relationship between sortilin and aspects of Lp(a) metabolism, as evidenced by the ongoing controversy regarding the role of sortilin in apoB/VLDL secretion and LDL catabolism that was previously discussed. An additional layer of complexity is added by the significant size heterogeneity in Lp(a). It is possible that the effects on apo(a) secretion and Lp(a) metabolism vary depending on Lp(a) levels, which can differ up to 1000-fold between individuals (15), on the isoform size of apo(a), which also exhibits a high degree of variability between individuals (13), or on the expression levels of sortilin itself, as levels differ based on cell type (129, 144, 145). Fig. 4.1 provides an overall model depicting how sortilin may regulate Lp(a)/apo(a) metabolism based on the data in this thesis.


A
Extracellular Space
Intracellular Space
Covalent Linkage
Secretory Vesicles
Trans-Golgi
sortilin
Cis-Golgi
Noncovalent Association
Prosortilin
Ribosome
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MTP
Lipid Portion
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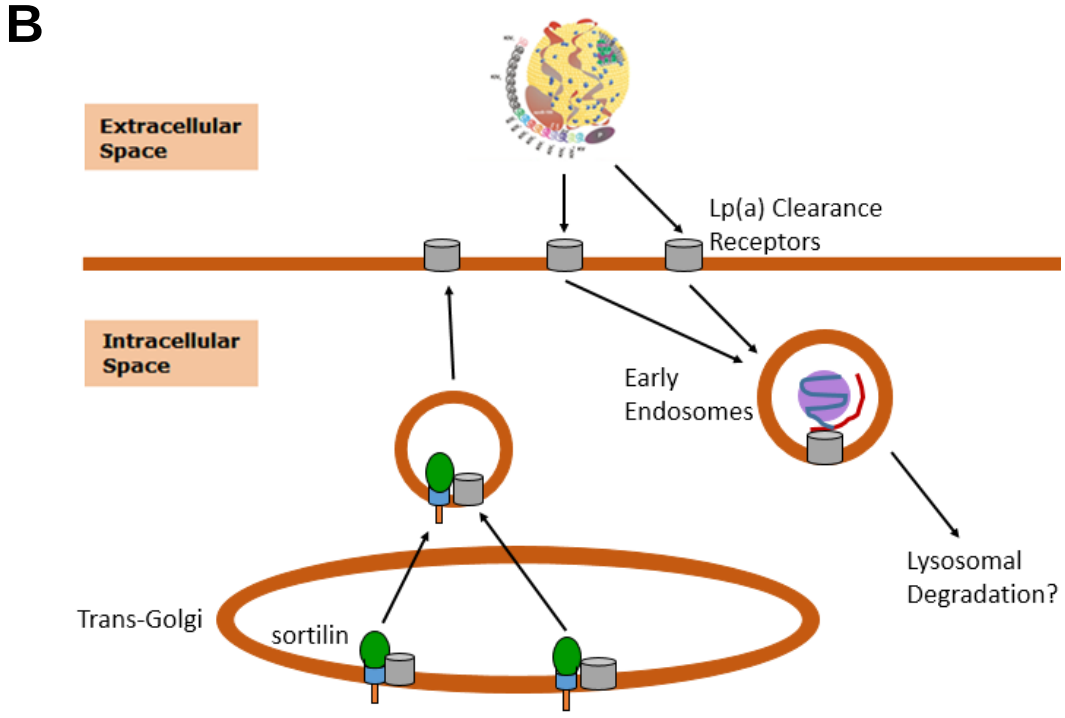
B
Extracellular Space
Intracellular Space
Lp(a) Clearance Receptors
Early Endosomes
Lysosomal Degradation?
Trans-Golgi
sortilin

Figure 4.1: ***Model of sortilin action in apo(a) secretion and Lp(a) catabolism.*** (A) Nascent apo(a) and apoB-100 are translated in the ER, where apoB undergoes lipidation by microsomal triglyceride transfer protein (MTP). Immature apo(a) and lipidated apoB-100 are transported to the Golgi, where they associate non-covalently. Sortilin is activated by furin cleavage in the trans-Golgi, where it might interact with, and facilitate the secretion of, the apo(a)-apoB complex. Upon secretion into the extracellular space, the final covalent attachment of apo(a) to apoB-100 occurs through the catalysis of a disulfide linkage. (B) Sortilin mediates the intracellular trafficking of receptors involved in Lp(a) clearance, thus increasing their bioavailability. Lp(a) is internalized through clathrin-mediated endocytosis, and incorporated into early endosomes. It may then be transported to the lysosome for degradation.

Ultimately, further research is required to elucidate if the effects of sortilin expression, or sequence variation within the protein, can lead to either an elevation or reduction in plasma Lp(a) levels. Data in this thesis indicates that overexpression of sortilin can regulate the pathways of both apo(a) secretion and Lp(a) catabolism. However, sortilin siRNA experiments suggest that sortilin may play a more important role in the secretion of apo(a). The discovery of the sortilin polymorphic variants in individuals with elevated Lp(a) levels provides the potential to further elucidate if sortilin promotes both Lp(a) assembly and catabolism in an equal manner. The available data on the polymorphic variants is not enough to validate the effects of sortilin on these two parameters as further repeats are required. However, the phenotypes of some variants, notably F404Y and V650M, indicate the potential for linkage analysis between sortilin expression and elevated levels of Lp(a). Analysis of individuals expressing these mutations may be the key to determining if sortilin polymorphisms are direcly linked withthe elevated Lp(a) phenotype. Furthermore, the polymorphism data demonstrate that some mutations only affect one of the parameters that contribute to plasma Lp(a) levels. Therefore, future work involving the analysis of structure vs. activity can be pursued to examine the mechanisms through which sortilin mediates apo(a) secretion and Lp(a) catabolism, and thus determine the major pathway in which sortilin regulates Lp(a) levels.

The GWAS studies may also be revisited to explore how sortilin affects Lp(a) metabolism. The discovered SNPs in the 1p13 locus can be studied in the context of Lp(a) levels. While LDL-C levels were studied, it would be interesting to determine differences in the plasma Lp(a) levels between individuals that are homozygous for the major and minor alleles in the 1p13 region. In particular, studying the Lp(a) levels in individuals that

are homozygous recessive (homozygous for the protective "T" allele) would be of great interest, as these individuals possess 10-12 fold higher hepatic sortilin levels compared to individuals that are homozygous dominant. Experiments conducted in animal models may also provide interesting insight into the effects of sortilin on Lp(a) levels *in vivo*. Purified Lp(a) could be infused into either wild-type or *SORT*$^{-/-}$ mice, therefore allowing for the comparison of Lp(a) clearance between the two murine models. Mice transgenic for the *LPA* gene (219) could also provide an interesting model to pursue, as these mice produce apo(a) *in vivo*. Therefore, a cross between *LPA* transgenic mice and *SORT*$^{-/-}$ mice could be used to determine if apo(a) levels are altered, or if differences in secretion can be observed in isolated primary hepatocytes. Finally, incorporating a mice that is transgenic for the *APOB* and *LPA* genes will allow for the production of Lp(a) *in vivo*. A comparison of Lp(a)/apo(a) levels could be determined between transgenic, wild-type mice and transgenic, *SORT*$^{-/-}$ mice. Therefore, the effects of sortilin on apo(a) secretion and Lp(a) clearance could be studied *in vivo* to determine if the effects are comparable to our *in vitro* models.

4.5 Conclusion

Multiple genetic and epidemiological studies have identified Lp(a) as an independent, causal risk factor for CHD. Lp(a) was first discovered in 1963, yet many characteristics of this lipoprotein remain enigmatic. The primary catabolic pathway of Lp(a) remains controversial. Furthermore, the synthesis and secretion of apo(a) remains a complex process with many unknowns, particularly with respect to the mechanisms involved in the intracellular trafficking of nascent apo(a). Although differences in synthesis, not catabolism, have been shown to explain the inverse correlation between

Lp(a) levels and apo(a) isoform sizes (27), the relative importance of control of synthesis or catabolism in dictating Lp(a) levels in general is not clear.

With the recent discovery of an association between sortilin, LDL-C, and risk for CAD, numerous groups worked to identify the mechanistic basis of this association. Sortilin was found to be a novel regulator of apoB/VLDL synthesis and LDL catabolism. Structural similarities between Lp(a) and LDL suggested that sortilin may likewise mediate a regulatory pathway for both Lp(a) and apo(a). The present study explored this possibility. We demonstrate that sortilin overexpression leads to a significant increase in the secretion of a recombinant 17K apo(a) variant in hepatocytes, and that this effect is dependent upon the ability of sortilin to act as a trafficking receptor. Moreover, siRNA-mediated knockdown of sortilin led to a significant decrease in the amount of apo(a) secreted from hepatocytes. Qualitative analysis of immunofluorescence studies indicated co-localization between sortilin and apo(a), with triple staining occurring with early and late endosomal markers. We indicate that certain kringle domains play a role in mediating this effect, as a decreased effect of sortilin on apo(a) secretion was observed in a variant that contained mutations in the weak LBS in $KIV_{7,8}$, rendering them inactive. This variant is unable to interact with apoB, suggesting that sortilin may increase apo(a) secretion in a manner that is affiliated with its effects on the secretion of apoB from hepatocytes. We further demonstrate that sortilin does not directly interact with apo(a) *in vitro*, thus indicating that sortilin expression indirectly affects apo(a) secretion.

This study also establishes that sortilin overexpression can significantly increase the amount of Lp(a), but not apo(a), internalized in hepatocytes and primary human fibroblasts. Similar to the effects observed for apo(a) secretion, this effect on internalization appears to

be dependent upon the ability of sortilin to act as a trafficking receptor. Knockdown of sortilin expression did not significantly influence Lp(a) internalization, suggesting that this effect is only relevant in individuals with elevated levels of sortilin expression. Treatment with ε-ACA significantly reduced Lp(a) internalization with sortilin overexpression, indicating that the increase in Lp(a) internalization brought about by sortilin overexpression may involve the apo(a) component of Lp(a), in addition to being dependent on the presence of apoB. Internalization assays conducted in human FH fibroblasts demonstrated that sortilin overexpression led to a significant increase in Lp(a) internalization in cells that lacked functional LDL receptors, suggesting that sortilin increase Lp(a) internalization through an LDLR-independent pathway. Since sortilin does not bind to Lp(a); therefore, sortilin expression acts to upregulate the intracellular trafficking and availability of cell surface receptors that bind to and internalize Lp(a).

Deep sequencing of *SORT1* in human patients with elevated Lp(a) levels identified seven novel polymorphisms, I124V, K205N, K302E, F404Y, E444Q, E447G, and V650M, occurring in the VPS10 domain of sortilin. These mutants displayed varying trends in their ability to regulate apo(a) secretion and Lp(a) metabolism. Of note are F404Y and V650M, which demonstrated a reduced ability to increase Lp(a) internalization and an enhanced or comparable ability to increase apo(a) secretion when compared to wild-type sortilin. Overall, these data implicate sortilin as a novel regulator of Lp(a) metabolism.

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