

POST-TRANSCRIPTIONAL REGULATION OF ABCA1

by

Martin Hubert Kang

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies

(Medical Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

December 2012

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Abstract

Epidemiological studies consistently demonstrate an inverse relationship between HDL levels and cardiovascular disease (CVD), independent of LDL and triglyceride levels. Due to the crucial role ABCA1 plays in HDL biogenesis, increasing ABCA1 expression is considered an attractive strategy to increase plasma HDL levels. In this thesis we attempt to identify novel post-transcriptional and post-translational mechanisms that regulate ABCA1 expression and/or function. Prior to translation, ABCA1 protein expression is regulated by non-coding RNA molecules known as microRNAs which bind and inhibit translation of mature mRNA transcripts in the cytoplasm. In this study we used bioinformatic prediction programs to identify potential microRNA regulators of ABCA1. Using reporter constructs, protein expression analysis by immunoblotting, and cholesterol efflux assays, we validated microRNA-145 as a novel repressor of ABCA1 translation. The inhibition of endogenous microRNA-145 in HepG2 cells increases both ABCA1 protein levels and cholesterol efflux activity. The inhibition of this microRNA in the liver is a potential strategy to increase HDL levels. Following translation, numerous post-translational modifications and protein-protein interactions are required for the ABCA1 protein to function properly. In this study we identified palmitoylation as a novel post-translational modifier of ABCA1. The majority of ABCA1-mediated cholesterol efflux and HDL biogenesis occurs at the cell surface. We show that palmitoylation is a crucial lipid addition for proper ABCA1 plasma membrane localization. We also identify a number of enzymes that mediate the incorporation of radio-labeled palmitate onto ABCA1, and demonstrate that the overexpression of the palmitoyl transferase enzyme DHHC8 increases ABCA1 palmitoylation and cholesterol efflux activity. The increase of ABCA1 palmitoylation in the liver is a novel strategy to increase HDL levels. In this thesis, we have contributed to the understanding of ABCA1 biology by the identification of two novel regulators of ABCA1 expression and/or function.

Preface

Publications arising in part or in whole from the work presented in this dissertation.

Chapter 1:

Kang MH, Singaraja R, Hayden MR. Adenosine-triphosphate-binding cassette transporter-1 trafficking and function. Trends Cardiovasc Med. 2010; 20(2):41-9. Review.

Contributions:

Martin H Kang and Michael R Hayden were responsible for the conception and outline of the review article. Martin H Kang wrote the manuscript. Roshni Singaraja provided corrections and suggestions for improvement.

Chapter 2:

Singaraja RR*, **Kang MH***, Vaid K, Sanders SS, Vilas GL, Arstikaitis P, Coutinho J, Drisdell RC, El-Husseini Ael D, Green WN, Berthiaume L, Hayden MR. Palmitoylation of ATP-binding cassette transporter A1 is essential for its trafficking and function. Circ Res. 2009; 105(2):138-47. (* co-first authors)

Contributions: Martin H Kang, Michael R Hayden, and Roshni Singaraja were responsible for the conception and design of the study. Martin H Kang was responsible for all experiments and data analysis with the exception of the following:

Figure 2.1.A-C RR Singaraja performed the 2 bromo-palmitate experiments.

Figure 2.2.A RC Drisdell and WN Green carried out the acyl-biotin exchange experiment.

Figure 2.2 RR Singaraja and SS Saunders carried out the site-directed mutagenesis.

Figure 2.2.E RR Singaraja carried out the ABCA1 palmitoylation metabolic labeling experiment in triplicate.

Figure 2.6.A K Vaid carried out the ABCA1/PAT metabolic labeling experiment.

Figure 2.7.A RR Singaraja performed the ABCA1/PAT cholesterol efflux assay.

Martin H Kang, Michael R Hayden, and Roshni Singaraja were responsible for the writing of the manuscript.

Chapter 3:

Kang MH, Sanders SS, Christopher N, Hayden MR. ATP-binding cassette transporter A1 palmitoylation and intracellular trafficking. 2012, Manuscript in preparation.

Contributions:

Martin H Kang and Michael Hayden were responsible for the conception, experiments and writing of the manuscript. Martin H Kang was responsible for all experiments and data analysis. Shaun Sanders and Nikki Christopher carried out the cloning in this study.

Chapter 4:

Wijesekara N, Zhang LH, **Kang MH**, Abraham T, Bhattacharjee A, Warnock GL, Verchere CB, Hayden MR. miR-33a modulates ABCA1 expression, cholesterol accumulation, and insulin secretion in pancreatic islets. *Diabetes*. 2012; 61(3):653-8.

Contributions:

Michael R Hayden and N Wijesekara were responsible for the conception, experiments and writing of the manuscript. Martin H Kang designed and tested the adenovirus expressing miR-33a used in this study.

Kang MH, Zhang LH, Butland S, Hayden MR. miR-145 regulates ABCA1 protein expression and cholesterol efflux activity. 2012, Manuscript in preparation.

Contributions:

Martin H Kang, Linhua Zhang, and Michael Hayden were responsible for the conception, experiments and writing of the manuscript. Martin H Kang was responsible for all experiments and data analysis with the exception of:

Linhua Zhang carried out the luciferase assays and expression analyses.

Stefanie Butland assisted in the bioinformatics.

Approval of Study by UBC Research Ethics Boards:

Studies involving animals in Chapter 4 required the approval of both the UBC Animal Care Committee and the UBC Biosafety Committee.

Both the Animal Care Committee (Certificate number: A10-0053) and the Biosafety Committee (Certificate number: B11-0050) granted approval for these studies.

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Abbreviations and Symbols

ABE	acyl-biotin exchange
Ad	adenovirus
apo A-I	apolipoprotein A-I
apo A-II	apolipoprotein A-II
apo B-100	apolipoprotein B-100
ABCA1	Adenosine-triphosphate binding cassette transporter-A1
ABCG1	Adenosine-triphosphate binding cassette transporter- G1
APT1	acyl protein thioesterase 1
bps	base pairs
BSA	bovine serum albumin
C _S	cysteine to serine
CAD	coronary artery disease
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
COOH	carboxyl
COS	CV-1 in Origin, SV40 transformation cell line
CVD	cardiovascular disease
DHHC	aspartate, cysteine, cysteine, histidine sequence
DMSO	dimethyl sulfoxide
DRM	detergent resistant membrane
DTT	dithiotreitol
ECD	extracellular domain
EL	Endothelial lipase
EndoH	endoglycosidase H
ER	Endoplasmic reticulum
FC	free cholesterol
FED	Fish-eye-disease
FHA	Familial hypoalphalipoproteinemia
FLD	Familial LCAT deficiency
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GC	Golgi complex
HAM	hydroxylamine
HEK293	human embryonic kidney-293 cell line
HepG2	human hepatocellular carcinoma cell line
HDL	High density lipoprotein
HL	Hepatic lipase
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HRP	horse-radish peroxidase
IDL	Intermediate density lipoprotein
IHD	ischemic heart disease
IMT	carotid intima media thickness
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein

LDLR	Low density lipoprotein receptor
LPL	Lipoprotein lipase
LXR	Liver X receptor
MES	2-(N-Morpholino) ethanesulfonic Acid buffer
miR	micro-RNA
miRNA	micro-RNA
MOI	multiplicity of infection
NBD	nucleotide binding domain
NH ₂	amino
OR	odds ratio
PAT	protein acyltransferase
PBS	phosphate buffered saline
PEST	proline, glutamic acid, serine, threonine sequence
PL	phospholipids
PLTP	Phospholipid transfer protein
PM	plasma membrane
PNGaseF	N-Glycosidase F
PPAR	Peroxisome proliferator-activated receptor
PSD-95	Postsynaptic density protein 95
PTE	protein thioesterase, palmitoyl thioesterase
RCT	reverse cholesterol transport
RT-PCR	reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
siRNA	small interfering ribonucleic acid
SNAP-25	Synaptosomal-associated protein 25kDa
SR-B1	Scavenger receptor class B, type 1
TD	Tangier disease
TG	triglycerides
UTR	untranslated region
U343	human neuronal glioblastoma cell line
VLDL	very-low-density lipoprotein
WT	wild-type

Acknowledgements

I offer my enduring gratitude to all the faculty, staff, students, and co-workers at UBC who have inspired and helped me to this point. I owe particular thanks to my supervisor Dr Michael R Hayden who helped me grow and develop, and who taught me so much about science and life. I am forever indebted to him for providing me with the opportunity to work in his lab.

I thank my committee members for their assistance and guidance. I thank Dr Elizabeth Conibear for enlightening me in the field of palmitoylation. I thank Dr Robert Molday for enlarging my vision on ATP transporters. I thank Dr Cheryl Wellington for her in-depth expertise in ABCA1 and lipid metabolism.

In my studies of ABCA1 palmitoylation I am grateful to Dr Roshni Singaraja, Kuljeet Vaid, Shaun Saunders, Dr Luc Berthiaume, Dr Gonzalo Vilas, Dr Alaa El-Husseini, Dr Pamela Arstikaitis, Dr Jonathan Coutinho, Dr Elina Ikonen, and Nikki Christopher.

In my studies in microRNA regulation I am grateful to Dr Linhua Zhang, Dr Nagat Bissada, Piers Ruddell, Dr Nadeeja Wijesekara, Stefanie Butland, Dr Angel Baldan, and Ryan Allen.

I thank all members of Dr Michael R Hayden's lab for their friendship, assistance, and expertise.

Special thanks are owed to my dad Seung Ho Kang, my mom Choon Eun Kang, and my brother Victor Kang for all their help and support, both moral and financial. I am eternally grateful.

1 Introduction

1.1 Background

In 2010, Health Canada estimates cardiovascular disease (CVD) claimed the lives of more than 70 000 Canadians (Health Canada). CVD is a disease of the heart and blood vessels (World Health Organization) and includes: coronary artery disease (CAD); ischemic heart disease (IHD); strokes; and heart attacks (Heart and Stroke Foundation). These disorders arise when a build-up of fatty deposits in the inner walls of blood vessels blocks the flow of blood to the heart or brain (World Health Organization). A number of controllable and non-controllable risk factors contribute to the risk of CVD. Non-controllable risk factors include a family history with CVD and advancing age. Controllable risk factors are behavioral or environment-related and include: inactivity; obesity; high blood pressure; cigarette smoking; and high cholesterol levels (Centers for Disease Control and Prevention).

The levels of cholesterol in the plasma are a major risk factor for CVD. As a lipid, the insolubility of cholesterol in plasma requires that it be transported complexed with proteins. The two major carriers of cholesterol in humans are low density lipoprotein (LDL) and high density lipoprotein (HDL) (Gurr, 2002). Epidemiological studies, genetic evidence, and clinical intervention trials have demonstrated the benefits of lowering LDL in the plasma to protect against CVD (Brautbar and Ballantyne, 2011). HMG CoA reductase inhibitors (statins) inhibit cholesterol synthesis, lower LDL levels, and because of these benefits, statins have been the most prescribed class of drugs in the United States since 2009 (IMS Health). However, the use of statins has reduced cardiovascular events by only 20% to 40%, and in the United States CVD remains the leading cause of death for both men and women (Centers for Disease Control and Prevention). The continued high mortality rate for CVD has encouraged the identification of novel approaches to therapeutically treat this disease.

Epidemiological studies demonstrate an inverse relationship between HDL and CVD (Francis, 2010). The protective role of HDL is hypothesized to center on its role in reverse cholesterol transport

(RCT). In RCT, excess cholesterol is effluxed out of cells by pumps like the adenosine-triphosphate binding cassette transporter-A1 (ABCA1) to lipid poor apolipoprotein A-I (apo A-I) proteins generating HDL particles. These HDL particles transport cholesterol to the liver for excretion in the bile either as cholesterol or after conversion of cholesterol into bile acids. Besides the role of removing excess cholesterol from the body, HDL is also thought to possess anti-inflammatory and anti-oxidative properties (Rader, 2006). Due to the importance of ABCA1 in HDL biogenesis, our focus is to identify mechanisms that regulate ABCA1 protein expression and cholesterol efflux function.

Potent transcriptional activators of ABCA1 exist (Repa et al., 2000); however, their potential therapeutic benefits are mitigated by their deleterious off-target effects such as increased plasma triglyceride (TG) levels and hepatic steatosis. Post-transcriptional mechanisms describe any regulatory step that occurs after the generation of the primary RNA transcript from DNA. This thesis will describe the work carried out to identify two novel post-transcriptional regulators of ABCA1: the regulation of ABCA1 messenger RNA (mRNA) by microRNA-145 (miR-145); and the post-translational modification of the ABCA1 protein by the addition of palmitate (Singaraja, 2009).

1.2 Introduction to Cholesterol

Cholesterol belongs to a class of lipids called sterols, which are common in eukaryotes but rare in prokaryotes (Gurr, 2002). The function of cholesterol in humans is not to provide energy to the host (Yokoyama, 2005), but to act as a component of the cellular membrane to modulate fluidity or rigidity of membranes (Maxfield and Tabas, 2005). Cholesterol provides the additional functions of acting as: a metabolic intermediate in the formation of bile acids to aid digestion (cholic acid); as a precursor for steroid hormones (cortisol, aldosterone, oestrogens); for the formation of nutrients (vitamin D3) (Gurr, 2002); and as a component in neuronal signaling pathways (Hegele, 2009).

Due to its functional importance, humans have developed elaborate and efficient mechanisms to increase cholesterol levels when they are low (Maxfield and Tabas, 2005). Almost all types of cells can

synthesize cholesterol with the majority of de novo synthesis occurring in the liver and small intestine. De novo synthesis of cholesterol contributes approximately 70% to our total cellular cholesterol needs (Ikonen, 2008). The remaining 30% of our cholesterol is obtained from our diet (Beckett, 2010). When cholesterol levels become too high, excess cholesterol must either be transported to the liver for excretion or converted into hormones or nutrients. Humans have no other mechanism to catabolize or break down cholesterol (Beckett, 2010). The metabolic pathways for cholesterol in humans highlights the efficient synthesis pathways in recognition of the importance of this molecule to proper cellular functioning, but it also shows how we are poorly adapted at managing excess cholesterol in our circulation (Yokoyama, 2005).

1.3 Low-Density Lipoprotein Cholesterol

The major cholesterol-carrying lipoprotein in humans is low-density lipoprotein (LDL) which transports two-thirds of the cholesterol in our bodies (Goldstein and Brown, 2001). LDL plays an important role in the delivery of cholesterol to our tissues and cells in a process called 'forward cholesterol transport' (Gurr, 2002). Cells expressing surface LDL-receptors (LDLR) binds to apolipoprotein B-100 (apo B-100) in LDL particles to internalize and supply cholesterol to the cell.

When the levels of cholesterol in our plasma and within intracellular storage deposits exceed our body's ability to remove cholesterol, LDL particles accumulate in the arterial walls and begin to oxidize. Oxidized LDL accumulation is an early event of atherogenesis (Maxfield and Tabas, 2005). Oxidized lipoproteins elicit a series of biological responses which includes the recruitment of monocytes and macrophages to ingest lipoprotein particles. Cholesterol from ingested LDL particles gathers in the cytoplasm, and generates macrophage foam cells which are major components of atherosclerotic plaques (Maxfield and Tabas, 2005).

The role of LDL in instigating CVD initiated the search for pharmacological strategies to manage cholesterol levels and in 1990 three cholesterol synthesis inhibitors or HMG-CoA reductase inhibitors

(i.e. lovastatin, simvastatin, and pravastatin) were approved for use in patients (Endo, 1992). Statins can lower LDL levels by up to 55% and statins are currently used as a first-line pharmacological therapy in patients at risk for CVD (Brautbar and Ballantyne, 2011). In 2008, statins were the third most prescribed class of drugs in the United States (IMS Health). However, statins reduce cardiovascular events by only 20-40% (Tall et al., 2008) and in 2008; heart disease was still the leading cause of death for both men and women in the U.S. (Centers for Disease Control and Prevention). Over the last 20 years, the rate of heart disease and strokes in Canada has declined by about 50% (Heart and Stroke Foundation). Despite this decline, in 2008 CVD still accounted for 29% of all deaths in this country (>69 500 people), and heart disease and strokes are two of the three leading causes of death in Canada (Heart and Stroke Foundation). Globally, more people die from CVD than from any other cause. In 2008, 30% of all worldwide deaths (17.3 million people) were estimated to be caused by CVD. The World Health Organization projects that 23.6 million people will die from CVD in 2030, and projects CVD to remain a leading cause of death on our planet (World Health Organization).

1.4 Very Low Density Lipoprotein, Intermediate Density Lipoprotein, and Chylomicrons

The precursor to LDLs are very low-density lipoproteins (VLDLs) secreted by the liver. VLDLs are composed of fatty acylated cholesterol (cholesteryl esters), TGs, and protein components such as apo B-100. The delivery of TGs to muscle and adipose tissues by VLDL, along with lipid exchange reactions, reduces the size of VLDL to LDL (Goldstein and Brown, 2001). Intermediate density lipoproteins (IDLs) are included as a class of lipoproteins between VLDL and LDL (Gurr, 2002). Chylomicrons are the largest lipoproteins and their function is to transport lipids obtained from our diet. Our primary dietary fat is TGs and they make up more than 80% of chylomicrons (Gurr, 2002).

1.5 High Density Lipoprotein Cholesterol

The second major cholesterol-carrying lipoprotein in humans after LDL is HDL (Eisenberg, 1984). HDL is not a homogenous population, but rather a heterogeneous, dynamic collection of lipoprotein particles that changes in size, shape, and composition due to a number of modifying factors (Lund-Katz and Phillips, 2010). Classification of the different HDL particles is based on density, electrophoretic mobility, and immunoaffinity methods (Rothblat and Phillips, 2010).

1.5.1 HDL and Reverse Cholesterol Transport

HDL removes cholesterol from our body in a process called RCT. In detail, RCT begins in the liver and to a lesser extent in the small intestine, where apo A-I acquires cholesterol and phospholipids from ABCA1 to generate pre- β or nascent HDL particles (**Figure 1.1**) (Rothblat and Phillips, 2010). Pre- β or nascent HDL acquires free cholesterol from peripheral tissues mediated by ABCA1 and ATP-binding cassette transporter G1 (ABCG1). The lecithin-cholesterol acyltransferase (LCAT) enzyme in HDL transforms free cholesterol into cholesteryl esters. Meanwhile, HDL continually recruits protein components including apolipoprotein A-II (apo A-II). In total, 75 to 100 different proteins reside in mature HDL particles. The addition of these lipid and proteins along with enzymatic remodeling activity generates α - or mature-HDL from pre- β or nascent HDL. In the mid-to-late stages of RCT, HDL remodeling by cholesteryl ester transfer protein (CETP) transfers HDL-associated cholesteryl esters to VLDL/ LDL, and VLDL/LDL-associated TGs to HDL. The phospholipid transfer protein (PLTP) mediates the transfer of phospholipids to HDL. The VLDL/LDL particles remove the newly acquired cholesteryl esters from the circulation through the indirect RCT pathway. When apo B-100 in VLDL/LDL binds to LDLRs on the surface of hepatocytes, endocytosis of VLDL/LDL particles occurs. The internalized VLDL/LDL particles are catabolized into component parts with the cholesterol excreted into the bile. Meanwhile, the remodeled HDL particles containing phospholipids and TGs are targeted by the hydrolytic enzymes

endothelial lipase (EL) and hepatic lipase (HL) respectively. The cholesterol in HDL is removed from the circulation by

Major components of RCT	Classification	Function/Role
Apolipoprotein A-I (apo A-I)	Protein	Major protein component of HDL particles
Apolipoprotein A-II (apo A-II)	Protein	Protein component in HDL that binds SR-B1 on hepatocytes
Apolipoprotein B-100 (apo B-100)	Protein	Protein component of VLDL/LDL that binds LDLR on hepatocytes
Adenosine-triphosphate binding cassette transporter-A1 (ABCA1)	Protein	Efflux of cholesterol and phospholipids from a variety of tissues to generate HDL
Adenosine-triphosphate binding cassette transporter-G1 (ABCG1)	Protein	Efflux of cholesterol and phospholipids from a variety of tissues to generate mature HDL
Scavenger receptor class B, type 1 (SR-B1)	Protein	Selective influx of cholesteryl esters from HDL in hepatocytes, can also efflux cholesterol from cells
Low density lipoprotein receptor (LDLR)	Protein	Mediates the uptake of cholesteryl esters through the endocytosis of the entire VLDL/LDL particle
Free cholesterol (FC)	Lipid	Minor fraction of cholesterol in the circulation
Cholesteryl esters (CE)	Lipid	Majority of cholesterol in the circulation
Triglycerides (TGs)	Lipid	Key energy source of free fatty acids linked to a glycerol backbone
Phospholipids (PLs)	Lipid	Lipid component of membranes and all lipoproteins
Lecithin-cholesterol acyltransferase (LCAT)	Enzyme/Protein	Present in lipoprotein particles where it converts free cholesterol into cholesteryl esters
Cholesteryl ester transfer protein (CETP)	Enzyme/Protein	Transfers cholesteryl esters from HDL to VLDL/LDL in a 1:1 exchange for triglycerides
Phospholipid transfer protein (PLTP)	Enzyme/Protein	Transfers phospholipids between unilaminar vesicles into HDL
Lipoprotein lipase (LPL)	Enzyme/Protein	Hydrolysis of triglycerides in chylomicrons, VLDL, and other triglyceride rich lipoproteins
Hepatic lipase (HL)	Enzyme/Protein	Enzyme that hydrolyses triglycerides
Endothelial lipase (EL)	Enzyme/Protein	Enzyme that hydrolyses phospholipids

Table 1.1. Major components of the Reverse cholesterol transport (RCT) pathway.

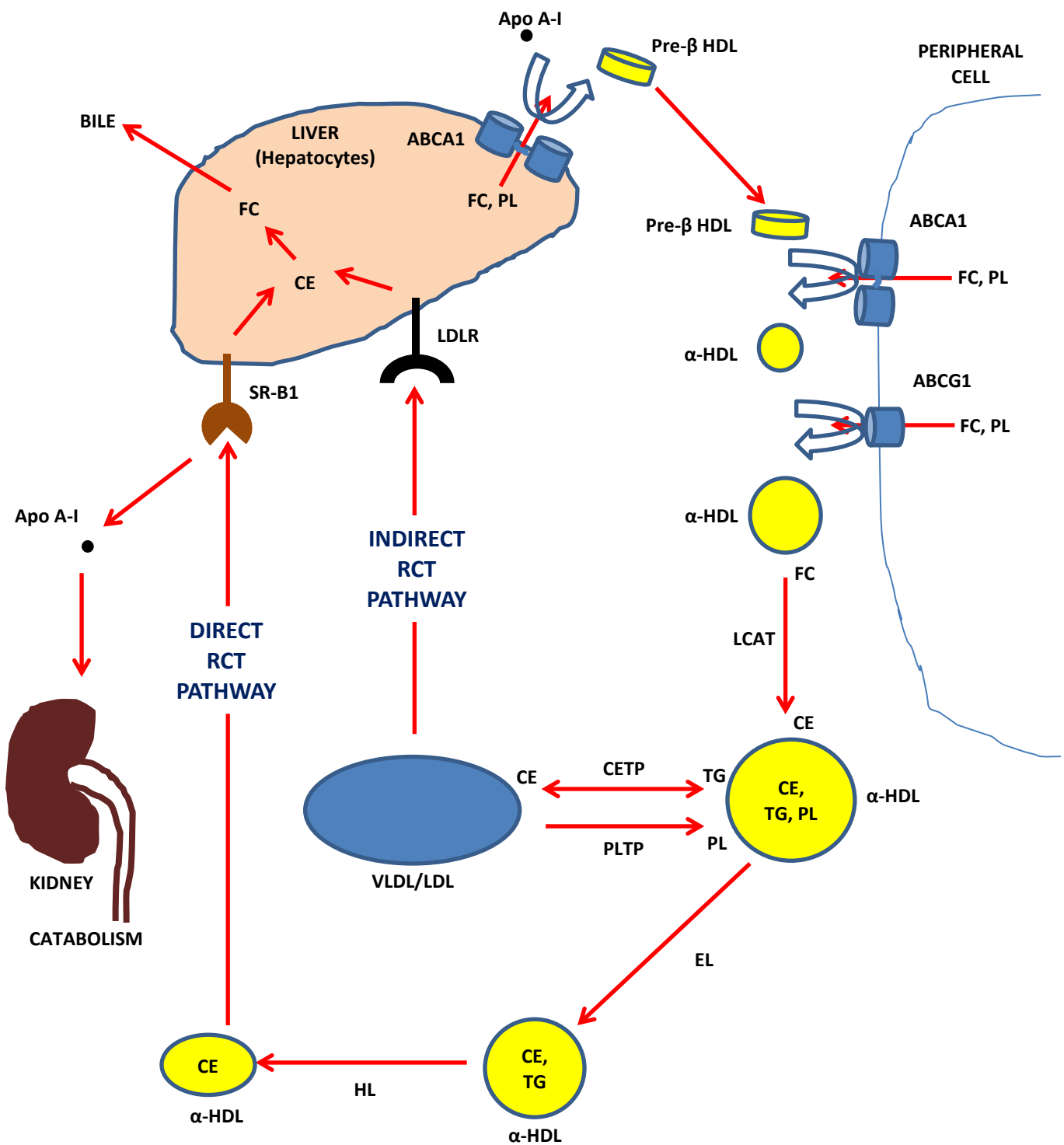


Figure 1.1. Schematic of the reverse cholesterol transport (RCT) pathway. For abbreviations see Table 1.2.

the direct RCT pathway. In direct RCT, cholesteryl esters are selectively removed from HDL when apo A-II in HDL binds to scavenger receptor class B, type 1 (SR-B1) receptors on the surface of hepatocytes. The cholesteryl esters imported by SR-B1 are converted into free cholesterol before being secreted into bile or converted into bile acids (**Table 1.1; Figure 1.1**) (Rothblat and Phillips, 2010; Asztalos et al., 2011; Gurr, 2002; Sissel Lund-Katz and Phillipis, 2010).

Besides RCT, HDL possesses anti-inflammatory proteins and anti-oxidative properties which are thought to contribute to its anti-atherogenic function (Navab et al., 2011; Gordon et al., 2011).

Paraoxonase/arylesterase 1 (PON1) is an HDL-associated protein that attenuates the expression of proinflammatory molecules like interleukin-8 (IL-8), and stimulates endothelial anti-inflammatory and repair-stimulating properties (van Lenten et al., 1995; Besler et al., 2011). HDL possesses the ability to inhibit monocyte chemoattractant protein 1 (MCP-1), which recruits innate and adaptive immune cells (e.g. monocytes, T lymphocytes) to sites of injury and inflammation (Navab et al., 1991). Besides lipids and proteins, HDL harbours small non-coding RNAs called microRNAs (miRNAs) (Vickers et al., 2011). miRNAs pair with the 3' untranslated region (3'UTR) of mRNAs to direct post-transcriptional repression of genes. Once incorporated into HDL, miRNAs are believed to be transported to the liver where they are removed from HDL by SR-B1. The miRNAs delivered by HDL have been shown to alter gene expression in recipient cells. In silico programs predict the miRNAs present in HDL primarily affect inflammatory and signaling pathways (Vickers et al., 2011).

1.6 Epidemiological Studies and HDL

Epidemiological studies support HDL as protective against CVD, independent of other lipids such as LDL and TG levels (**Table 1.2**) (Francis, 2010). In 1951, a study demonstrated that healthy men had higher levels of HDL than men with coronary heart disease (CHD) (Barr et al., 1951). Data from the Framingham Heart Study revealed that 43-44% of coronary events occurred in individuals with HDL levels below 40mg/dl, and that a 1% decrease in HDL levels can increase the incidence of CAD by 3-4%

Study	Sample size	Subject descriptions	Findings/ Conclusions	Reference
The Framingham Study	2815	Men and women aged 49 to 82 years	HDL had an inverse association with CHD ($p<0.001$), even when other lipids were taken into consideration	Gordon et al., (1977)
Follow-up to Lipids Research Clinics Prevalence Study	8825	Males and females at least 30 years of age	HDL had an inverse relationship with CVD mortality after controlling for age, LDL, TGs, body mass index, systolic blood pressure	Jacobs et al., (1990)
Tromso Heart Study	6595	Men ages 20-49 years living in Tromso, Norway	Coronary risk was inversely related to HDL independent of LDL, TG, blood pressure, body weight, smoking	Miller et al., (1977)
Prospective Cardiovascular Munster Study	26975	18460 males and 8515 females	Developed Weibull model for CHD prediction where HDL values $<35\text{mg/dl}$ are a major risk factor for coronary events within 10 years	Assmann et al., (2007)
Nurses' Health Study	48470	Post-menopausal women 30-63 years	Estrogen use decreased CHD and mortality from CVD, possibly due to estrogen induced increases to HDL	Stampfer et al., (1991)
Helsinki Heart Study	4081	Men aged 40-55 years with dyslipidemia. Half received fibrates, half received placebo	Fibrate use decreases CHD in men with dyslipidemias, possibly due to increases in HDL levels	Frick et al., (1987)
Veterans Affairs HDL Intervention Trial	2531	Men with a history of CHD and low HDL and low LDL. Half received fibrates, half received placebo	Fibrate use significantly reduced CHD, partially due to increases in HDL levels	Robins et al., (2001)
Quebec Cardiovascular Studies	2103	Middle-aged males from Quebec	HDL levels are an independent predictor of IHD. Reduced HDL levels have a greater impact than raised LDL levels on the atherogenic index	Despres et al., (2000)
The 10 year follow-up to the Atherosclerosis Risk In Communities Study	12339	Middle aged males and females free of CHD	HDL levels are strongly associated with decreased CHD risk independent of other lipids	Sharrett, 2001

Table 1.2. A sample of epidemiological studies and clinical intervention trials supporting an anti-atherogenic role for HDL.

Table 1.3 has been removed due to copyright restrictions. It was a table of the HDL values considered a major risk factor for heart disease, and HDL values considered protective against heart disease. Original source: American Heart Association website and Heart and Stroke Foundation of Canada website.

Table 1.3. Recommended HDL levels. To convert into mmol/l multiply mg/dl by 0.0259 (Singh et al., 2007). (References: American Heart Association; Heart and Stroke Foundation of Canada)

(Wilson, 1990). On the other hand, an increase in HDL levels by 1 mg/dl can decrease the risk of coronary-associated death by 6% (Gordon et al., 1989). Based on these epidemiological studies, the American Heart Association (AHA) states that HDL levels below 40mg/dl for men and 50mg/dl for women are major risk factors for heart disease (American Heart Association) (**Table 1.3**). Significant segments of the US population have low HDL levels and are considered at risk for developing CVD. In 2011, among Americans aged 20 and older, the following groups had HDL levels below 40mg/dl: 31.7% of Mexican American males; 12.2% of Mexican American females; 29.5% of white males; 10.1% of white females; 16.6% of black males; and 6.6% of black females (Roger et al., 2011).

1.7 Genetic Disorders and HDL

The genetic evidence that low HDL levels result in CVD is not as supportive as the epidemiological studies; however, the findings from the genetic studies are often confounded by other factors. HDL levels have a reported heritability of 40-60% (von Eckardstein, 2006; Sviridov and Nestel, 2007). The majority of low HDL cases are attributed to polygenic or multi-gene inheritance (Weissglas-Volkov and Pajukanta, 2010). However, monogenic or single gene causes of low HDL exist, and have been used to determine whether an association between HDL levels and CVD can be made. Three monogenic disorders result in very low HDL levels in humans. Mutations in apo A-I (HDL < 5mg/dl), ABCA1 (HDL < 5mg/dl), and LCAT (HDL < 5-10mg/dl) cause low to undetectable HDL cholesterol levels (von Eckardstein, 2006; Weissglas-Volkov and Pajukanta, 2010). Two monogenic disorders result in very high HDL levels. Mutations in CETP (HDL > 100mg/dl) and LIPC (HDL > ~70mg/dl) increase HDL to levels considered cardio-protective according to the AHA (**Table 1.3**) (Nordestgaard and Tybjaerg-Hansen, 2011). These five disorders show varied conclusions regarding the relationship between HDL and CVD (**Table 1.4**).

1.8 CETP Inhibitors

Animal studies provided convincing evidence that increasing HDL by inhibiting CETP was a promising therapeutic strategy (Honey, 2007). As noted, individuals with CETP mutations have high HDL levels (HDL>100mg/dl) (Inazu A et al., 1990). CETP is involved in the late stages of the RCT pathway and transfers cholesteryl esters from HDL to VLDL/LDL in a 1:1 exchange for TGs (**Table 1.1, Figure 1.1**) (Francis, 2010; Joy and Hegele, 2008). The inhibition of CETP blocks this exchange of lipids which increases the levels of HDL and decreases the levels of VLDL/LDL. CETP inhibitors were developed by Japan Tobacco (JTT-705) and Pfizer pharmaceuticals (torcetrapib). Clinical trials demonstrated that JTT-705 increased HDL levels by 34% and decreased LDL levels by 7% (deGroot et al., 2002). Clinical trials with torcetrapib showed a 72.1% increase in HDL and a 24.9% decrease in LDL (Barter et al., 2007).

Unfortunately, a phase III clinical trial with torcetrapib was halted when patients receiving this drug showed an increased risk of cardiovascular events (hazard ratio 1.25, 95% CI 1.09 to 1.44, $p=0.001$) as well as an increased risk of death (hazard ratio 1.58, 95% CI 1.14 to 2.19, $p=0.006$). The failure of torcetrapib has brought into question the rationale of raising HDL as a protective measure from CVD.

However, CETP is an important component of the indirect RCT pathway to remove cholesterol from the circulation. A number of warning signs concerning CETP inhibition as a treatment for CVD were overlooked and provided a foreshadowing of some of the negative outcomes:

- In early genetic studies of Japanese-American males with CETP deficiency, an increased risk for CHD was observed (Zhong et al., 1996).
- The majority of cholesteryl esters in humans are excreted in the bile and feces after being transferred to VLDL/LDL by CETP (indirect RCT) (Schwartz et al., 2004).
- If VLDL/LDL is effectively cleared from the plasma by the liver, increased CETP expression in animal studies actually enhances the RCT process by removing excess cholesterol from the circulation (indirect RCT) (Tanigawa et al., 2007).
- Metabolic studies show that CETP inhibition does not increase cholesterol or bile acid excretion in the feces (Brousseau et al., 2005).

A possibility as to why torcetrapib increased mortality is the rise in systolic blood pressure (SBP) following treatment (Toth, 2011; Joy and Hegele, 2008). After 12 months, patients on torcetrapib showed an elevated SBP of 4.5 mmHg. A 10 mmHg increase in SBP is associated with a 25% increase in risk for CVD and vascular mortality (Lewington et al., 2002). Recent animal and clinical studies suggests the rise in blood pressure was torcetrapib-specific. Although other CETP inhibitors have not affected blood pressure in clinical trials and are not expected to increase cardiovascular mortality (Ghosh and Ghosh, 2012), the enthusiasm supporting CETP inhibition as a therapeutic strategy has waned.

Gene Mutation	Disease	Evidence mutations results in CVD	Evidence mutations do not result in CVD (Possible reasons)	OMIM
APO A-I	Apo A-I deficiency	Thickening of IMT; increased risk of CAD (OR=18.9); disease often occurs before 50 yrs		#604091
ABCA1	Tangier disease (TD): homozygous mutations Familial HDL deficiency: heterozygous mutations	Increased risk of CAD (TD, OR=5.88) (FHA, OR=3.47); 31% of TD patients have CHD symptoms; ABCA1 mutations increase CAD risk 2-3 fold	The virtual absence of HDL predicts a greater risk of CVD. A confounding factor are low LDL levels in TD patients (40% of normal)	(TD, #205400) (FHA, hypoalphalipoproteinemia, #604091)
LCAT	Familial LCAT deficiency (FLD): completely absent LCAT activity Fish-eye-disease (FED): LCAT activity lost in HDL only, not LDL	LCAT mutations increase CAD (33% in homozygotes)	Most FED and FLD patients show no increased risk of CAD. A possible reason is that LCAT conversion of free cholesterol is not necessary for proper RCT.	(FLD, 245900) (FED, 136120)
CETP	Cholesteryl ester transfer protein deficiency	Some studies show CETP polymorphisms decrease risk of atherosclerosis.	Some studies show increased CHD risk. CETP transfer of cholesteryl esters to VLDL/LDL is a major pathway for cholesterol excretion (indirect RCT).	#143470
LIPC	Hepatic lipase (HL) deficiency		Does not reduce CVD, some patients show premature CAD development. HL hydrolysis of PLs and TGs are required for cholesteryl ester uptake from HDL by SR-B1 (direct RCT)	#614025

Table 1.4. Five monogenic disorders that alter HDL levels show varied association to cardiovascular disease. Abbreviations: CAD = coronary artery disease, CHD = coronary heart disease, CVD = cardiovascular disease, IMT = carotid intima media, OR = odds ratio, PLs = phospholipids, RCT = reverse cholesterol transport, SR-B1 = scavenger receptor class B, type 1, TGs = triglycerides. References: GK Hovingh et al., 2005; Sviridov and Nestel, 2007; von Eckardstein, 2006; Clee et al., 2000; Frikke-Schmidt et al., 2005; Frikke-Schmidt, 2010; Oram, 2000, Kees Hovingh et al., 2005; Weissglas-Volkov and Pajukanta, 2010; Schwartz et al., 1982; Ji et al., 1999; Inazu et al., 1990; Ordoas et al., 2000; Zhong et al., 1996; Hegele et al., 1993.

Despite the lack of genetic support and the failure of torcetrapib, the search for positive regulators of HDL remains an attractive goal due to the findings from epidemiological studies (**Table 1.2**), the continued high mortality rate from CVD, and the possibility that factors such as low LDL levels or improper cholesterol excretion in the monogenic disorders obscures the true association of HDL to CVD (Brautbar and Ballantyne, 2011). It is also important to note that genetic causes of high LDL cholesterol (familial hypercholesterolemia) are not always associated with premature CAD (Francis, 2010). Based on the current knowledge of HDL and CVD, the National Cholesterol Education Program (NCEP ATP III) guidelines urge physicians to aggressively manage low HDL in individuals at risk for CVD (Wang and Briggs, 2004).

1.9 Targeting ABCA1 to Increase HDL and Decrease CVD

A number of epidemiological and mechanistic studies in animal models support the up-regulation of ABCA1 expression as a strategy to raise HDL and decrease CVD (Liu and Tang, 2012). ABCA1 increases both HDL quantity and HDL functionality. The lack of a high CVD prevalence in Tangier disease (TD) patients may be confounded by low LDL levels in these individuals (Oram, 2000). Despite this lack of association, ABCA1 remains an attractive target due to concerns with targeting other components of the RCT pathway to alleviate CVD. Modulating RCT components such as SR-B1, EL, and HL have all been shown to increase HDL levels, but they can also interfere with cholesterol excretion (Toth, 2010). The mechanism that ABCA1 attenuates CVD is by its cholesterol efflux function and/or its ability to generate HDL and ABCA1 does not appear to interfere with the excretion of cholesterol through RCT.

1.9.1 ABCA1 Cholesterol Efflux Function

ABCA1-mediated cholesterol efflux function can inhibit CVD independent of its ability to generate HDL. In individuals with ABCA1 mutations, a negative correlation exists between apo A-I-mediated cholesterol efflux activity and intima media thickness ($r=-0.61$; $p=0.03$) (van Dam et al., 2002). ABCA1 cholesterol efflux function is especially relevant in macrophage cells. A strong inverse association

exists between cholesterol efflux capacity in macrophages, and sub-clinical atherosclerosis and obstructive CAD (OR=0.70; 95%CI 0.59 to 0.83; $p<0.001$). This relationship persists even after adjustment for HDL levels (Khera et al., 2011). ABCA1 along with ABCG1 are the two major transporters that efflux cellular cholesterol from cells (Vaughan and Oram, 2006). A deficiency of both ABCA1 and ABCG1 in macrophages accelerates atherosclerosis in LdLR^{-/-} mice as a direct result of deficiencies to cholesterol efflux activity (Yvan-Charvet et al., 2007; Wang et al., 2007).

1.9.2 ABCA1 and HDL Biogenesis

ABCA1 mediates the initial lipidation of apo A-I with cholesterol and phospholipids to form pre- β HDL, as well as lipidating pre- β HDL to generate α -HDL (Yokoyama, 2005; Francis, 2010). The almost complete absence of HDL in the plasma of TD patients points to the crucial role ABCA1 plays in HDL biogenesis (Nofer and Remaley, 2005). Hepatic ABCA1 is the single most important site for initial HDL generation. The intravenous injection of adenovirus-expressing ABCA1 into mice increases HDL levels by two to three fold ($p<0.05$) (Basso et al., 2003). The primary organ targeted following these saphenous vein injections is the liver. Targeted deletion of ABCA1 in the liver decreases plasma HDL levels in mice by 80% (Timmins et al., 2005). Besides the liver, the intestine is the second major contributor to HDL biogenesis. Intestinal-specific deletion of ABCA1 decreases plasma HDL levels by 30% (Brunham et al., 2006). ABCA1 in peripheral tissues plays a crucial role in HDL maturation. The intravenous injection of adenovirus-expressing ABCA1 into mice with a complete deletion of ABCA1 produces low levels of small HDL particles. The same injection into mice with a liver-specific deletion of ABCA1 results in mature HDL particles at a quantity comparable to that observed in wild-type mice (Singaraja et al., 2006). These results indicate hepatic ABCA1 is involved in initial HDL biogenesis, while extra-hepatic ABCA1 is important for the maturation of HDL particles. Human genetic studies in individuals with ABCA1 mutations provide evidence that HDL development decreases the risk of CAD. In heterozygous TD

patients (ABCA1+/-), there is a defect in the formation of larger α -HDL particles (Asztalos et al., 2001), which predicts an increased risk for CAD (Asztalos et al., 2000).

1.9.3 ABCA1 and CVD

However, an increased risk of CVD due to low HDL levels has not been definitively demonstrated in humans. Some ABCA1 mutations and variants increase the risk of ischemic heart disease (IHD) independent of low HDL cholesterol levels. In heterozygous carriers for an ABCA1 mutation (K776N), there is a two to three fold increase in the risk of ischemic heart disease (IHD) without a change in HDL cholesterol levels (Frikke-Schmidt, 2010). Three non-synonymous single nucleotide polymorphisms (SNPs) in ABCA1 (V771M, I883M, E1172D) increase IHD risk without changing HDL levels (Frikke-Schmidt et al., 2008). The mechanism as to how ABCA1 mutations and variants can increase risk of IHD independent of HDL levels is unclear but may be attributed to ABCA1-mediated cholesterol efflux activity. When human ABCA1 is expressed in a mouse model of atherosclerosis (apo E^{-/-} background), a slight increase in HDL levels is observed (from 0.20g/l to 0.25g/l; p=0.04), macrophage cholesterol efflux is increased by 38%, and aortic atherosclerosis is reduced 2.6 fold (Singaraja et al., 2002). Whether it is the modest increase in HDL levels, or the increase in macrophage cholesterol efflux activity that reduces lesion area in these mice is unclear. When ABCA1 is selectively inactivated in mouse macrophages, an increase in CVD is observed along with minimal changes to HDL levels. This suggests that anti-atherogenic properties of ABCA1 are attributable to its cholesterol efflux function (Aiello et al., 2002). However, another study with macrophage specific inactivation of ABCA1 on an alternate mouse model of atherosclerosis (LdLR^{-/-} background) demonstrated no increase in atherosclerosis and suggests ABCA1 cholesterol efflux activity in macrophages has little influence on atherosclerosis susceptibility (Brunham et al., 2009). Mice lacking hepatic ABCA1 on an apoE^{-/-} background showed a significant decrease in HDL cholesterol levels (from 29±6mg/dl to 14±1mg/dl; p=0.04), along with an increase in

atherosclerosis. These results suggest the anti-atherosclerotic properties of ABCA1 are due to its role in HDL biogenesis (Brunham et al., 2009).

Whether ABCA1 expression decreases CVD by the generation of HDL or through its macrophage cholesterol efflux activity remains a matter of contention. This may not matter as ABCA1 increases HDL quantity, improves HDL functionality, and decreases CVD risk (Liu and Tang, 2012). No mechanism post-ABCA1 can correct for the initial loss of ABCA1 function in HDL biogenesis, underlying the importance of this transporter to plasma HDL levels (Francis, 2010). If it is our goal to target ABCA1 in order to modulate HDL levels, it is crucial that we understand the mechanisms that regulate ABCA1.

1.10 Pre-transcriptional Regulation of ABCA1

Recent work has demonstrated epigenetic regulation of ABCA1 exists. Near the first exon in the ABCA1 gene promoter lays a differentially methylated region. Methylation of this site is negatively correlated with HDL levels ($r=-0.20$; $p=0.05$), and associated with increased risk of CAD (CAD=40.2% vs without CAD=34.3%; $p=0.003$) (Guay et al., 2012) (**Figure 1.2**).

1.11 Transcriptional Regulation of ABCA1

1.11.1 Liver X Receptors

Liver X receptors (LXRs) are transcription factors that partner with retinoid X receptors (RXRs) to form heterodimers (Duffy and Rader, 2009). Cholesterol-derived oxysterols are natural ligands for the LXR and RXR heterodimers, and this interaction induces the expression of many genes involved in RCT including ABCA1 and ABCG1 (Toth, 2009). The up-regulation of ABCA1 and ABCG1 increases cholesterol efflux, promotes RCT, decreases inflammation, and inhibits the progression of lesions in mouse models of atherosclerosis (both apoE^{-/-} mice and LdLR^{-/-} mice) (Toth, 2009). Synthetic LXR agonists which act as ligands for the LXR and RXR heterodimer have been developed and include T0901317 and GW 3965

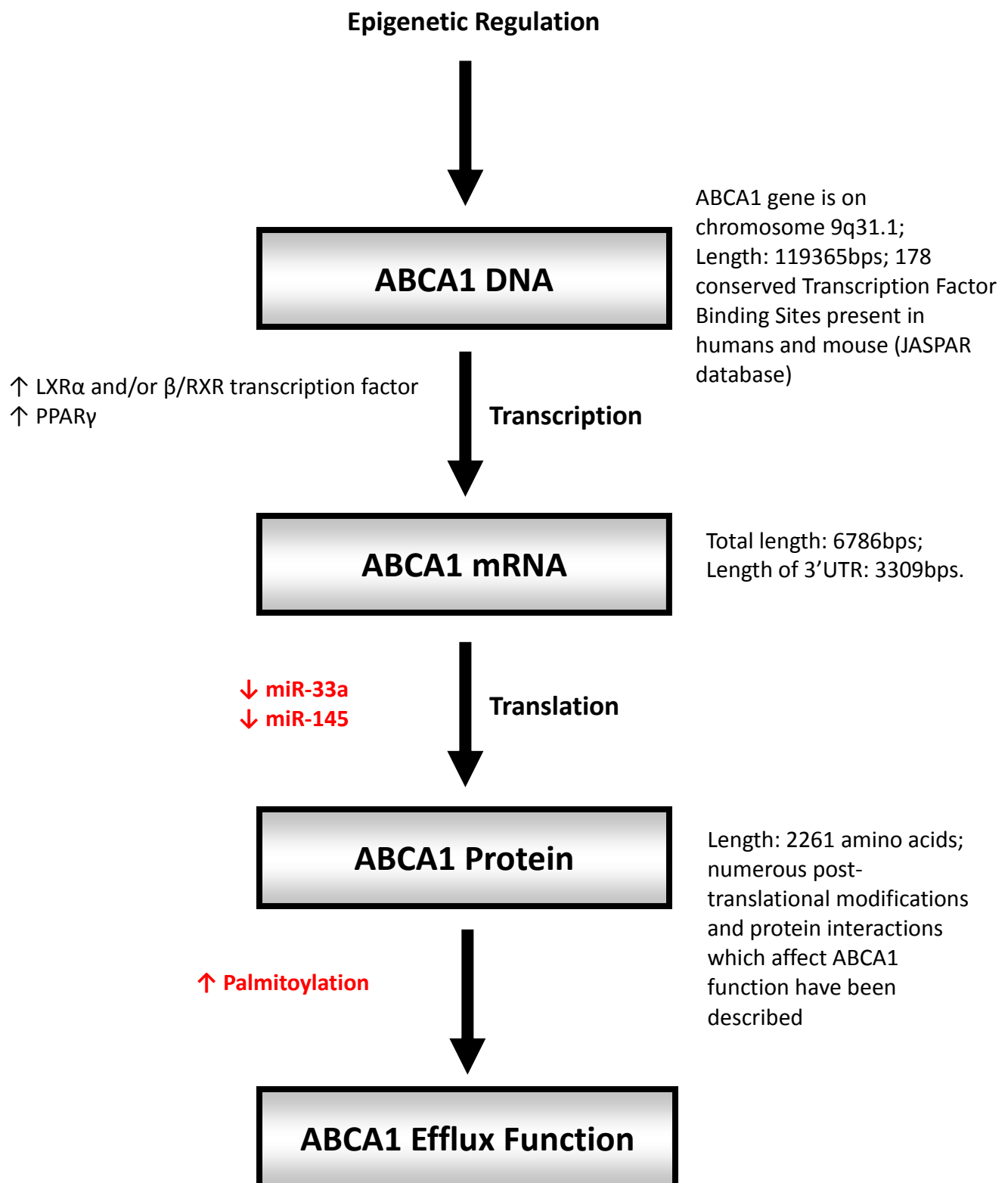


Figure 1.2. Multiple levels of regulation occurs in order to achieve a fully functional ABCA1 protein capable of cholesterol efflux activity. Regulatory events of ABCA1 described in this dissertation are in Red.

(Miao et al., 2004). The improvements in HDL levels, RCT, and decreases in atherosclerosis using LXR agonists are mitigated by their pleiotropic effects which also induce genes that stimulate lipogenesis. The induction of the sterol response element binding protein (SREBP1-c) and fatty acid synthetase (FAS) genes increases hepatic TG synthesis, fatty acid synthesis, hypertriglyceridemia, and hepatic steatosis (Duffy and Rader, 2009) **(Figure 1.2)**.

1.11.2 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear transcription factor that increases the expression of a number of components of RCT including: ABCA1; apo A-I; apo A-II; SR-B1; and lipoprotein lipase (LPL) (Nicholls and Nissen, 2007). By activating these genes, PPAR α promotes cholesterol efflux, increases HDL levels, induces RCT, and decreases the levels of inflammatory cytokines (Toth, 2009). Fibrates are PPAR α agonists that are currently used to raise HDL levels, but their effects are modest and the HDL levels raised by fibrates have not been demonstrated to appreciably reduce CVD (Duffy and Rader, 2009). Because of this, more potent agonists of PPAR α have been developed such as GFT505 and LY518674 which are 100 fold more potent (Toth, 2009) and several thousand times more potent respectively, than current fibrates (Duffy and Rader, 2009). Despite the potency of these PPAR α agonists, they have not demonstrated an ability to significantly raise HDL levels or decrease rates of CVD. Furthermore, potential safety concerns due to toxic adverse side-effects limits the appeal of PPAR α agonists as a therapeutic option (Nicholls and Nissen, 2007; Duffy and Rader, 2009) **(Figure 1.2)**.

1.12 Post-transcriptional Regulation of ABCA1

ABCA1 contains a longer than average 3309 base-pair (bp) 3' untranslated region (3'UTR). In an analysis of more than 1500 human genes, the average length of a 3'UTR was found to be only 755.4bps (Pesole et al., 2000). Because the interaction between a microRNA (miRNA) and the target mRNA occurs primarily in the 3'UTR of the mRNA, a longer 3'UTR increases the number of potential miRNA binding sites. This concept is supported in studies of genes that produce different sized transcripts based on

their different polyadenylation sites (addition of approximately 200 adenosines) to the 3' end of the UTR. Many genes have multiple polyadenylation sites that give rise to different sized 3'UTRs (Edwalds-Gilbert et al., 1997). The differential expression of these isoforms can have profound impacts on the final protein expression of a gene. A shorter 3'UTR of a given gene results in its increased protein expression, while the forced expression of a full-length 3'UTR of the same gene results in decreased protein expression (Sandberg et al., 2008). This reduction in expression can be reversed by the deletion of predicted miRNA target sites in some genes. The length of the ABCA1 3'UTR would suggest several miRNA binding sites likely exists to regulate ABCA1 protein expression.

In silico prediction programs estimate most mRNA targets are regulated by many different miRNAs (Rottiers and Naar, 2012). Depending on the prediction program, it is estimated that the 3'UTR of ABCA1 contains binding sites for 40 to over 100 different miRNA families (**Table 1.5**). The first microRNA (miRNA) gene was discovered in worms in 1993 (Berezikov, 2011). Today, over 1400 miRNA genes are thought to exist in humans. miRNAs are small non-coding RNAs that are estimated to regulate the translation of more than 60% of all protein coding genes (Esteller, 2011) including ABCA1. The mature miRNA is ~22 nucleotides and binds to the 3'UTR of a mature mRNA target in the cytoplasm (Bartel, 2009). This interaction either leads to mRNA degradation or inhibits the initiation of protein translation (Esteller, 2011).

Recently, three separate groups validated miR-33a as an inhibitor of cholesterol efflux activity and HDL biogenesis through the down-regulation of ABCA1 expression in the liver (Hani Najafi-Shoushtari et al., 2010; Rayner et al., 2010; Marquart et al., 2010). By inhibiting hepatic miR-33a, the groups demonstrated they could up-regulate ABCA1 protein expression in the liver, increase cholesterol efflux activity, and increase HDL levels in mice. The most common method to inhibit miRNAs are the use of antisense oligonucleotides (ASOs) which are complementary to endogenous miRNAs (Esteller, 2011).

miRNA Prediction Program (website address)	Number of miRNA binding sites in the 3'UTR of ABCA1
TargetScan Human 5.2 (www.targetscan.org)	40 miRNA family target sites broadly conserved among vertebrates
PicTar (pictar.mdc-berlin.de)	37 miRNA binding sites predicted in vertebrates based on conservation in mammals
microRNA.org (www.microrna.org)	76 miRNA binding sites predicted in humans
miRDB (mirdb.org/miRDB/)	115 miRNA binding sites predicted in humans
FindTar (bio.sz.tsinghua.edu.cn/)	2889 predicted miRNA target sites in ABCA1. Many targets are redundant for a single miRNA. (miR33a predicted with 2 binding sites; miR-145 predicted with 4 binding sites; miR-17 predicted with 9 binding sites)

Table 1.5. MicroRNA (miRNA) binding sites predicted in the 3'UTR of ABCA1 using five different algorithms: TargetScan Human 5.2; PicTar; microRNA.org; miRDB; and FindTar.

Anti-miR-33a ASOs introduced subcutaneously into a mouse model of atherosclerosis (LDLR^{-/-}) increased ABCA1 expression in the liver and macrophages, increased HDL levels, increased RCT, and reduced atherosclerotic plaque size (Rayner et al., 2011). miR-758 and miR-106b have also been validated as post-transcriptional regulators of ABCA1 expression and function (Ramirez et al., 2011; Kim et al., 2011). Like miR-33a, the introduction of miR-758 and miR-106b into HepG2 cell lines decreased

ABCA1 protein expression levels. However, both groups failed to compare the magnitude of ABCA1 regulation of the new miRNAs versus miR-33a. The manipulation of miR-758 and miR-106b levels on ABCA1 expression and HDL levels in vivo were also not assessed. Instead, both groups focused on the effect of these two new miRNAs in neuronal cell lines including miR-758 in glioblastoma H4 cells (Ramirez et al., 2011), and miR-106b in mouse neuroblastoma Neuro2a cells (Kim et al., 2012). Both groups suggest these new miRNAs may have roles in neurological processes through the regulation of ABCA1.

The role of miRNAs in regulating ABCA1 in different tissues may be an important process in health and disease. Studies have highlighted the importance of proper ABCA1 function in different tissues and cell types. ABCA1 expression in the liver is a crucial contributor to HDL biogenesis (Timmins et al., 2005). ABCA1 in the macrophage decreases macrophage foam cell formation and atherosclerotic plaque size (Zhao et al., 2010). ABCA1 in the beta-cell is required to maintain cholesterol homeostasis and ensure proper insulin secretion (Brunham et al., 2007). ABCA1 expression in neuronal cells is necessary for proper synaptic structure and motor function (Karasinska et al., 2009). Very few miRNAs are exclusive to a given tissue, and only a third of miRNAs analyzed in one study demonstrated high degrees of tissue-specific expression (Landgraf et al., 2007). miR-33a shows a ubiquitous tissue distribution (Najafi-Shoushtari et al., 2010; Rayner et al., 2010) and suggests the modulation of miR-33a can not only influence ABCA1 function in hepatocytes and macrophages, but in a variety of tissues. We manipulated ABCA1 expression in islet cells using adenoviral miR-33a or adenoviral miR-33a inhibitors and demonstrated changes in islet cholesterol levels and altered insulin secretion (Wijesekara et al., 2012). This thesis will describe the design and testing of adenoviral miR-33a on ABCA1 protein expression and function, and the impacts adenovirus-miR-33a or its inhibitor had on islet cell function.

Using a combination of in silico techniques and reporter constructs, we have identified several other potential miRNA regulators of ABCA1 besides miR-33a, miR-758, and miR-106b. Of these, we have

validated miR-145 as a direct regulator of ABCA1 expression and cholesterol efflux function in HepG2 cell lines. miRNAs are thought to fine-tune gene expression by acting as a 'rheostat' to micromanage protein levels (Bartel, 2009; Small and Olson, 2011; Berezikov, 2011). It is rare that miRNAs can influence gene expression by acting as a binary off-switch to completely dampen protein content in a cell (Bartel, 2009). In light of this concept, we investigated the impact of combining miR-33a with miR-145 on ABCA1 expression and function. This thesis will describe the work involved in identifying and validating miR-145 as a post-transcriptional regulator of ABCA1 (**Figure 1.2**).

1.13 Post-translational Regulation of ABCA1

In mice, both ABCA1 mRNA and protein show a ubiquitous tissue distribution. However, the level of ABCA1 mRNA expression does not always accurately predict ABCA1 protein levels and the overall concordance between mRNA and protein has been reported to be low ($r^2 = 0.199$). This suggests that post-translational mechanisms are important in regulating ABCA1 protein levels and function (Wellington et al. 2002) (**Figure 1.2**). A number of post-translational modifications, protein-protein interactions, and ABCA1 protein domains have been identified in the trafficking, regulation, and controlling of ABCA1 expression and function (Kang et al., 2010).

1.13.1 ABCA1 Sorting Through the Endoplasmic Reticulum and Golgi Complex

Following translation in the endoplasmic reticulum (ER), ABCA1 undergoes proper orientation into the membrane bilayer, N-glycosylation, dimerization and disulfide bond formation. Proper ABCA1 insertion into the ER requires an uncleaved 60 amino acid NH₂-terminal signal sequence that orients ABCA1 transmembrane regions so that its NH₂-terminus faces the cytosol and its extracellular domains (ECD) face into the ER lumen (**Figure 1.3**) (Fitzgerald et al. 2001). This particular orientation is critical as ABCA1 is highly glycosylated with oligosaccharide residues added to specific asparagine residues of the newly translated ABCA1 polypeptide chain exposed to the ER lumen (van Vliet et al. 2003, Tanaka et al. 2001). The glycosylation of proteins can be important in conferring proper protein folding, protein

stability and protein trafficking, however the role that this post-translational modification plays in ABCA1 function is presently unclear and may not significantly affect ABCA1 function. *Saccharomyces cerevisiae* (*S. cerevisiae*) lack the ABCA subfamily of ABC transporters. When the mouse ABCA1 gene was expressed in *S. cerevisiae*, ABCA1 appeared to function in yeast as evidenced by increased drug sensitivity and specific drug-sensitive growth profiles (Bocer et al., 2012). In yeast, plasma membrane proteins undergo limited glycosylation implying that glycosylation may not be required for proper ABCA1 function.

ABCA1 dimerization is initiated in the ER. Proper ABCA1 lipid efflux function is dependent on the oligomerization of ABCA1 into a homotetrameric complex. At equilibrium, ABCA1 primarily exists as a dimer whose formation is first observed in the ER. Upon ATP binding, the dimers associate into higher order structures such as tetramers, which are present at both the PM and at intracellular compartments (Trompier et al. 2006). The naturally occurring R1068H-ABCA1 mutation fails to demonstrate cholesterol efflux activity due to a failure in trafficking to the plasma membrane. This trafficking defect is thought to be due to impaired oligomerization of the mutant protein (Suetani et al., 2011).

The ER is the likely site for the formation of disulfide bonds in ABCA1 between its two ECDs. The ER possesses both the enzymes that oxidize disulfide bonds, as well as the optimal environment necessary for oxidative protein folding (Wakabayashi-Nakao et al. 2009). Two intramolecular disulfide bonds are formed in ABCA1 (between C75 and C309 in the first ECD and either C1463 or C1465 and C1477 in the second ECD), and the intact presence of both bonds are essential for cholesterol efflux function to apoA-I (Hozoji et al. 2009). ABCA1 exit from the ER is inhibited by its physical interaction with the serine palmitoyltransferase enzyme 1 (SPTLC1) protein. SPTLC1 localizes to the ER and physical interaction with ABCA1 leads to ABCA1 retention at this organelle. Although the reason for SPTLC1 negatively regulating ABCA1 ER exit is unclear, it is hypothesized that this mechanism is initiated when cellular demands for lipids are high. SPTLC1 catalyzes the synthesis of an intermediate in sphingomyelin

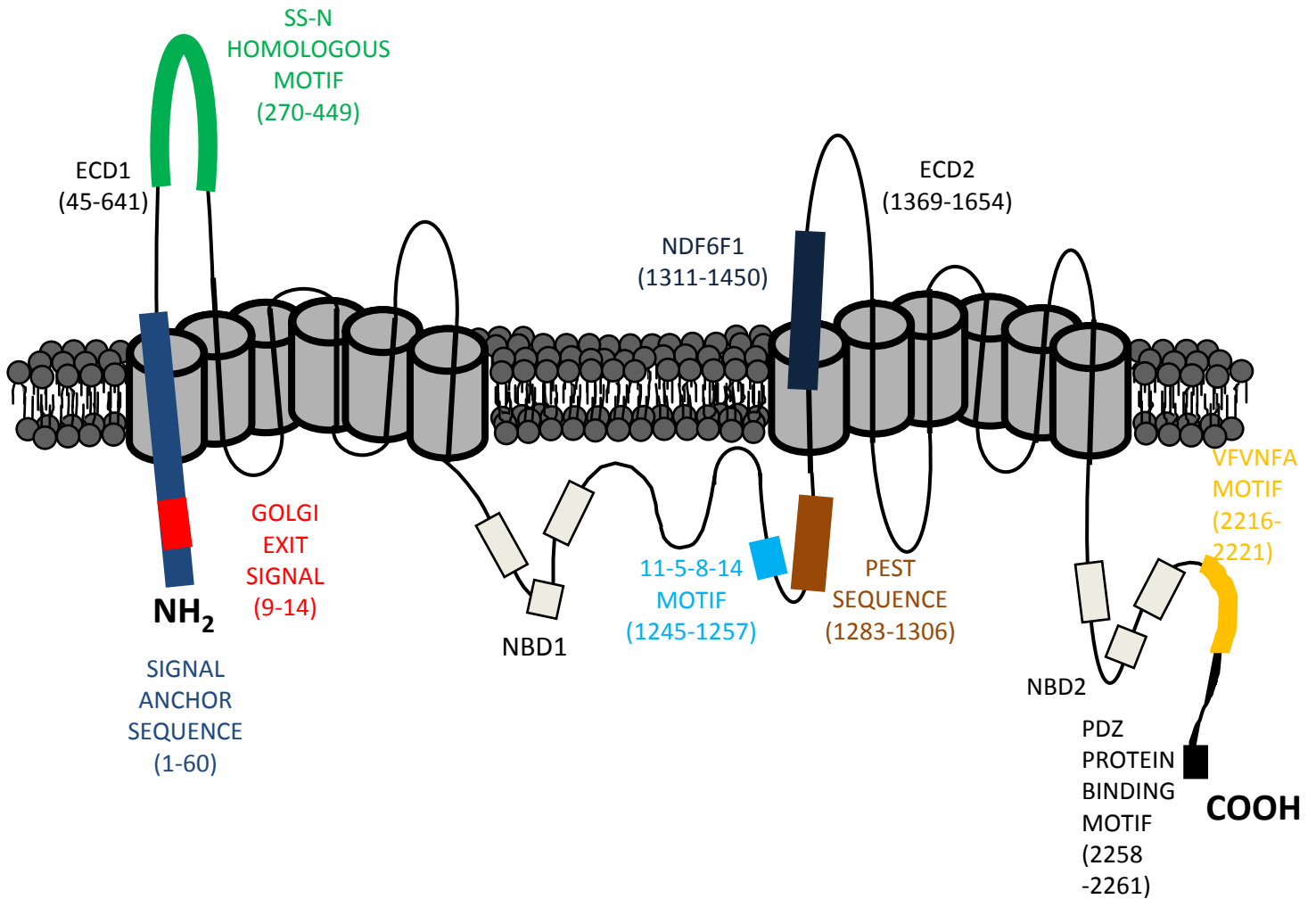


Figure 1.3. ABCA1 protein domains important in trafficking and/or function. The signal anchor sequence (aa 1-60) properly orients ABCA1 into the membrane bilayer in the ER. The Golgi exit signal (aa 9-14) directs ABCA1 to a post-Golgi vesicular sorting station. The 11-5-8-14 motif (aa 1245-1257) is a clamodulin binding site that when bound by calmodulin protects ABCA1 from calpain-mediated degradation. The PEST sequence (aa 1245-1257) is a target for calpain proteases. NDF6F1 (aa 1311-1450) is a sequence of amino acids used to generate an antibody against ABCA1. This antibody mimics properties of apo A-I and suggests this region is critical for apo A-I binding. The PDZ protein binding motif (aa 2258-2261) binds to class-1 PDZ proteins including α 1-syntrophin and β 1-syntrophin. The two NBDs bind and hydrolyze ATP to translocate substrates across membranes. The two large ECDs bind to apo A-I. The purpose of the homologous domain to SS-N (aa 270-449) is unknown. The VFVNFA motif (aa 2216-2221) is highly conserved among ABCA1 transporters and is important for cholesterol efflux activity although the mechanism is unknown.

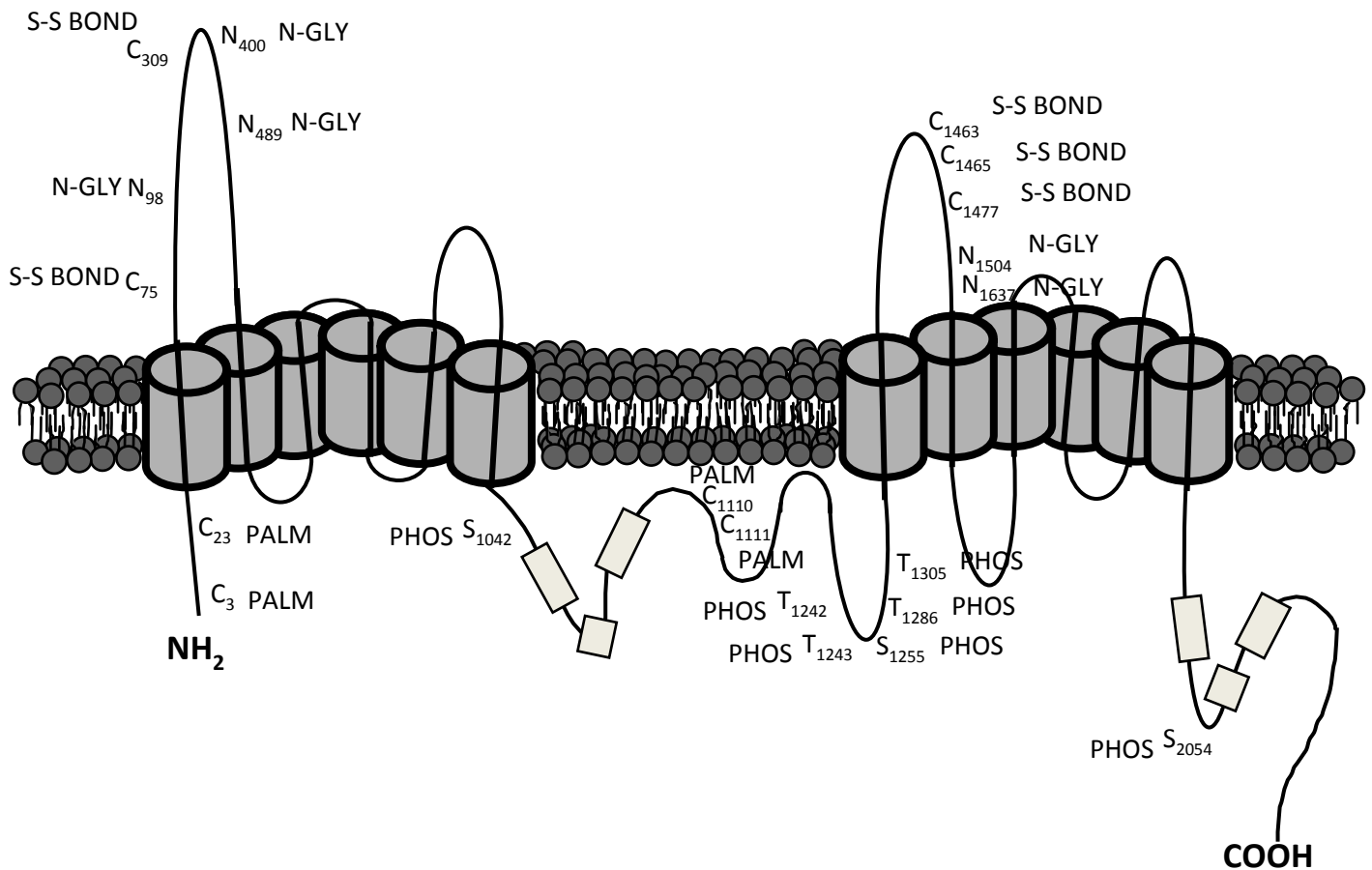


Figure 1.4 Known sites of post-translational modifications of ABCA1. ABCA1 palmitoylation (C3, C23, C1110, C1111) localize ABCA1 to the plasma membrane. Disulfide bond formation (between C75 and C309, and either C1463 or C1465 and C1477) are required for cholesterol efflux activity. The predicted sites of glycosylation (N98, N400, N489, N1504, N1637) have unknown effects on ABCA1. PKA mediates phosphorylation of ABCA1 at S1042 and S2054. The reduction of phosphorylation at S2054 reduces ABCA1 phospholipid efflux. The dephosphorylation of constitutively phosphorylated residues T1286 and T1305 inhibits calpain-mediated degradation. Binding of apo A-I to ABCA1 dephosphorylates T1286 and T1305. CK2 phosphorylates T1242, T1243, and S1255. The dephosphorylation of these residues increases apo A-I binding and cholesterol efflux function. PALM indicates palmitoylation; S-S BOND, disulfide bond; N-GLY, N-glycosylation; PHOS, phosphorylation.

(SM) production. SM along with cholesterol and phospholipids are key components of membrane bilayers and SPTLC1 may inhibit ABCA1 efflux function in order to maximize cellular lipid levels during membrane bilayer synthesis. Pharmacological inhibition of SPTLC1 with myriocin increases cholesterol efflux by 50% and cell surface ABCA1 by 30% (Tamehiro et al. 2008).

In the Golgi complex (GC), ABCA1 undergoes complex oligosaccharide processing as verified by its partial resistance to endoglycosidase H (EndoH). EndoH is an enzyme that cleaves oligosaccharide residues of the high mannose type added in the ER, but not of the complex type which occurs after Golgi processing. A portion of ABCA1 is also EndoH sensitive, which indicates that a pool of ABCA1 either remains in the ER or does not migrate through the Golgi complex (Singaraja et al. 2006). A number of naturally occurring mutations of ABCA1 (R587W; Q597R; Δ L693; N935H) acquire only the core and not the complex oligosaccharide chain and fail to exit the ER (Singaraja et al. 2006). In order to assess whether correcting the trafficking defect would restore cholesterol efflux function, Q597R-ABCA1 was localized to the plasma membrane using two different ER stress inducers: thapsigargin and dithiothreitol (DTT) (Tanaka et al., 2008). Thapsigargin inhibits calcium pumps in the ER, and has previously rescued the mislocalized delta F508 cystic fibrosis transmembrane conductance regulator (Δ F508-CFTR) mutant from the ER to the cell surface. The cell surface expression of Δ F508-CFTR partially recovered its chloride channel activity (Egan et al., 2002). Both thapsigargin and DTT rescued the trafficking defect and restored Q597R-ABCA1 to the cell surface. However, cholesterol efflux activity was not restored despite plasma membrane localization. The authors speculate Q597R-ABCA1 is a mutant defective for both trafficking, and an unidentified mechanism crucial for cholesterol efflux function. Previous studies have shown that Q597R-ABCA1 is able to oligomerize normally (Denis et al., 2004). ABCA1 trafficking from the ER to the Golgi likely involves Coat Protein II (COPII) coated vesicles (Tanaka et al. 2008) as it is generally assumed that most forward vesicle traffic from the ER to the Golgi is via COPII vesicle transport. As stated previously, the NH₂-terminus of ABCA1 contains an uncleaved 60 amino acid signal sequence

(Fitzgerald et al., 2001). Within this NH₂-terminal signal sequence lays a highly conserved xLxxKN motif common to the ABCA family. In ABCA1 this motif is represented by amino acid residues 9 to 14 (LLLWKN). The function of this motif is to serve as a Golgi exit signal and is believed to direct ABCA1 to a post-Golgi vesicular sorting station, where additional signals are necessary for delivery to the cell surface (Beers et al., 2011).

1.13.2 ABCA1 Trafficking to the Plasma Membrane

Until recently, how ABCA1 was transported to the cell surface from the GC was largely unknown. However, a critical regulator of ABCA1 PM localization has been identified in Rab8a. Once proteins arrive at the trans-Golgi network (TGN), they are sorted for delivery to multiple destinations including the PM, endosomes or involvement in retrograde transport. Large membrane carriers are required to transport proteins over long distances such as from the TGN to the PM (van Vliet et al. 2003). The regulation of ABCA1 transport to the cell surface involves the cytoplasmic small Ras like GTPase Rab8a. Rab proteins are involved in vesicle docking and fusion by tethering vesicles to their target membranes. Rab8a is involved in TGN to PM as well as endosome to PM trafficking and has previously been demonstrated to redistribute cholesterol from late endosomes to the PM (Linder et al. 2007). Rab8a also facilitates ABCA1 cell surface expression. Primary human macrophages expressing Rab8a results in a significant increase in ABCA1 protein levels but not mRNA levels. Along with increased ABCA1 protein expression at the PM, Rab8a expression increases cholesterol efflux to apoA-I by 20%. Targeting Rab8a with siRNA leads to a decrease of ABCA1 at the PM along with a decrease in cholesterol efflux activity (Linder et al. 2009). Whether Rab8 influences ABCA1 PM localization through a direct or indirect mechanism is unknown.

A second critical regulator of ABCA1 cell surface localization is the post-translational addition of the fatty acid palmitate to ABCA1. The addition of this saturated 16-carbon fatty acid is through a reversible covalent thioester linkage to select cysteine residues of proteins (Linder and Deschenes, 2007;

Conibear and Davis, 2010). Palmitoylation is a complex post-translational modification with numerous, varied, and occasionally unclear effects on the proteins it modifies. The addition of this lipid has been demonstrated to affect proteins in many ways.

- Palmitoylation provides a hydrophobic membrane anchor to soluble proteins to mediate stable membrane attachment (Baekkeskov and Kanaani, 2009).
- For some transmembrane domain (TMD) containing proteins, palmitoylation can increase the localization to specific membrane microdomains. However, palmitoylation can also prevent aggregation, and several palmitoylated integral membrane proteins are found outside of these microdomains (Linder and Deschenes, 2007; Baekkeskov and Kanaani, 2009).
- Palmitoylation can regulate protein-protein interactions (Salaun et al., 2010).
- Protein trafficking or sorting can be influenced by palmitoylation. The addition of this lipid can regulate whether a protein traffics to a distinct intracellular compartment, as well as influencing the trafficking of proteins between the ER and Golgi or in the endosomal/lysosomal system. Palmitoylation can influence protein sorting through a passive mechanism where palmitoylation acts as an anchor, while other domains within the protein regulate the trafficking. Palmitoylation can also actively influence protein sorting. For example, palmitoylation can partition proteins into cholesterol-rich membrane subdomains (Salaun et al., 2010).
- Palmitoylation of integral membrane proteins can tilt TMD segments to alter protein conformation (Conibear and Davis, 2010).
- Other post-translational modifications can be influenced by palmitoylation. Palmitoylation prevents ubiquitination which protects proteins from both proteasomal and lysosomal degradation. Depalmitoylation can increase the efficiency of neighbouring serine phosphorylation. Palmitoylation has also been associated with the post-translational

modifications nitrosylation and glycosylation (Linder and Deschenes, 2007; Salaun et al., 2010; Komekado et al., 2007).

We identified ABCA1 modification by palmitoylation. This thesis will describe the work in identifying and characterizing the importance of palmitoylation to ABCA1 plasma membrane localization.

1.13.3 Cell Surface ABCA1 Cholesterol Efflux Activity

ABCA1 localizes to the two largest pools of cholesterol in a cell: the plasma membrane (PM) and endosomes. This pattern of ABCA1 localization has been consistently demonstrated (Fitzgerald et al. 2001, Neufeld et al. 2001, Denis et al. 2008, Singaraja et al. 2009). These two sites are crucial for proper ABCA1 cholesterol efflux activity and HDL biogenesis.

The importance of PM localization for proper ABCA1 efflux function is confirmed by a number of independent studies. The single greatest pool of cholesterol is associated with the PM, with up to 90% of cholesterol present at the cell surface (Lange 1991). The importance of ABCA1 localization to the plasma membrane is observed in several mutations underlying TD (R587W; Q597R; Δ L693; M1091T), where mislocalization of ABCA1 away from the cell surface leads to a significant reduction in cholesterol efflux function (Singaraja et al. 2006).

In multiple cell types ABCA1 overexpression leads to a distinctive morphology of the PM (Vedhachalam et al. 2007, Linder et al. 2009). Confocal microscopy reveals spiky protrusions on the cell surface that resemble echinocytes in red blood cells. Echinocytes are formed when phospholipids are added to the outer membrane leaflet of the PM bilayer. ABCA1 at the cell surface modifies the local spatial arrangement of lipids by increasing exofacial phosphatidylserine (Chambenoit et al. 2001). The addition of apoA-I reduces these projections, suggesting that the change in morphology is a result of phospholipid extraction from the outer leaflet by apoA-I (Wang et al. 2000). Electron microscopy studies show that human fibroblasts and THP-1 macrophages expressing ABCA1 display 10-60 nm PM

projections that bind over 80% of exogenously added apoA-I. These protrusions are nearly absent from the fibroblasts of TD patients (Lin and Oram 2000), or in cells in which ABCA1 is not upregulated. These studies provide suggestive visual evidence that apoA-I lipidation is a cell surface event. This conclusion is supported by studies indicating that ABCA1 expression at the PM creates high capacity binding sites purported to be major lipid containing stores that facilitates the binding and lipidation of apoA-I at the cell surface (Vedhachalam et al. 2007).

Cell surface caveolae also appear to be sites of cholesterol efflux. HepG2 cells stably expressing the major structural component of caveolae, caveolin-1, increase cholesterol efflux to apoA-I by 45% (Fu et al. 2004). In aortic endothelial cells, ABCA1 binds directly with caveolin-1 at the cell surface (Chao et al. 2005) which highlights a role for ABCA1 in cholesterol efflux from the caveolae. Despite these findings, the interaction of ABCA1 with caveolin-1 and the effect that this may have on cholesterol efflux activity remains unresolved. Caveolae are considered to be classical Triton X-100 resistant membrane rafts, and caveolin-1 is a resident raft protein often used as a marker for such domains (Pike 2009). In multiple cell types however, it has been demonstrated that ABCA1 does not localize to Triton X-100 resistant rafts, and the localization of ABCA1 to these domains may vary with different cell types (Orlowski et al. 2007). Two recent studies further complicate this issue. Mutation to the oligomerization domain of caveolin-1 ($\Delta 62-100$) abolishes ABCA1 binding and suppresses HDL-mediated cholesterol efflux (Kuo et al., 2011). However, another group demonstrated that mouse embryonic fibroblasts deficient for caveolin-1 can efflux cholesterol 1.7 fold higher than wild-type cells. The authors suggest caveolin-1 is required for surface binding, internalization, and subsequent degradation of apo A-I and has little effect on cholesterol efflux processes (le Lay et al., 2011).

Alterations to ABCA1 cell surface expression can change cholesterol efflux activity. For example, elevated unsaturated fatty acids such as palmitoleate, oleate, linoleate or arachidonate reduces apo A-I binding to ABCA1, decreases ABCA1 levels at the plasma membrane, increases ABCA1 degradation, and

significantly decreases ABCA1 cholesterol and phospholipid efflux function (Wang and Oram, 2002). This effect is not observed with saturated fatty acids. The mechanism of ABCA1 degradation by unsaturated fatty acids is through phospholipase D (PLD) activation which increases the phosphorylation of serines in ABCA1 (Wang and Oram, 2005) resulting in ABCA1 destabilization. The kinase involved in serine phosphorylation following unsaturated fatty acid exposure is protein kinase C δ (PKC δ) (Wang and Oram, 2007). PKC δ inhibitors or small interfering RNA targeting PKC δ completely abolishes the destabilization effect of unsaturated fatty acids on ABCA1.

Ceramide, a potent lipid signaling molecule, increases cholesterol efflux up to threefold by increasing total and cell-surface ABCA1 and suggests that ceramide acts on ABCA1 post-translationally by either increasing ABCA1 trafficking to the PM, or decreasing the internalization of cell surface ABCA1. Ceramide has been previously linked to decreasing the internalization of LDL (Witting et al. 2003).

The ABCA1 protein contains a number of important domains necessary for proper cholesterol efflux function. Two domains homologous with all other ABC family members are the two nucleotide binding domains (NBDs) which bind and hydrolyzes ATP in order to translocate substrates across membranes (Rees et al., 2009; Linton, 2007). The NBD1 and NBD2 of ABCA1 each contains highly conserved motifs common to all ATP transporters including: the Walker A and B motifs; the ABC signature; and the stacking aromatic D, H, and Q loops. The hydrolysis of ATP at both NBD domains not only translocates cholesterol across the membrane, but also induces conformational changes in the extracellular domains of ABCA1 that allows apo A-I to bind (Nagao et al., 2012).

ABCA1 contains a total of six extracellular domains (ECDs) with two especially large domains (ECD1 [45-641] and ECD2 [1369-1654]). Epitope tagging studies have demonstrated the importance of amino acid residues 499-710 which overlaps with ECD1, and amino acid residues 1345-1466 which overlaps with ECD2 as important for apo A-I binding (Rigot et al., 2002). The residues 1311 to 1450 of ABCA1 which overlaps ECD2 were used to generate an antibody called NDF6F1. NDF6F1 competes with

apo A-I for binding to ABCA1. Like apo A-I, the presence of NDF6F1 protects ABCA1 from degradation, increases ABCA1 at the cell surface, and increases cholesterol efflux to apo A-I (Mukhamedova et al., 2007). This indicates that some of the residues that comprise ECD2 of ABCA1 (1311 to 1450) are crucial for apo A-I binding. In particular, positively charged amino acids in the ECDs of ABCA1 are likely responsible for binding to the negatively charged residues in apo A-I through electrostatic interactions. This is noticeable when the presence of highly charged molecules such as heparin and poly-L-lysine interferes with apo A-I binding to ABCA1. The introduction of reagents (NHS-acetate or sulfo-NHS-biotin) that specifically react with the primary amino groups of positively charged lysine residues completely abolished the interaction between apo A-I and ABCA1 (Nagao et al., 2012). This relationship is similar to the electrostatic interaction that mediates the binding between apo B-100 in LDL particles and LDLR.

1.13.4 Intracellular ABCA1 Cholesterol Efflux Activity

Although the majority of cholesterol resides at the PM, the second largest cellular cholesterol pool is at endosomes (Denis et al. 2008). ApoA-I conjugated to nascent lipids have been isolated from the endosomes in hepatocytes (Hamilton et al. 1991), suggesting that lipidation of apoA-I to form nascent HDL occurs intracellularly as well.

The two major cell-types that can synthesize and secrete apo A-I are hepatocytes and intestinal cells. In the HepG2-hepatic cell line, ~20% of synthesized apoA-I is lipidated intracellularly (Chisholm et al. 2002). Murine primary hepatocytes lacking ABCA1 show a dramatic reduction in the lipidation of endogenously synthesized apoA-I suggesting that this is an ABCA1 dependent process (Kiss et al. 2003). Macrophages may also be able to synthesize and secrete apo A-I although at a much lower rate than hepatocytes (Mogilenko et al., 2012). Apo A-I synthesized in macrophages do not appear to contribute to HDL biogenesis, but instead possess anti-inflammatory properties.

Most cells are unable to synthesize apoA-I and intracellular lipidation can only occur if apolipoproteins are endocytosed. ApoA-I can undergo endocytosis (Takahashi and Smith 1999) in an

ABCA1-dependent process (Lorenzi et al. 2008). In the RAW 264 macrophage cell line, immunogold labeled apoA-I are observed in coated pits. Upon endocytosis, apoA-I appears in endosomes where lipid transfer is postulated to occur, followed by sorting and recycling back to the PM where it is released extracellularly as a nascent HDL particle. Inhibiting endocytosis with either chlorpromazine or hypertonic media partially blocks cholesterol efflux activity (Takahashi and Smith 1999). Sequestering ABCA1 at the PM can lead to increased apoA-I binding to the cell surface but decreased cholesterol efflux, most likely due to a reduction in apoA-I internalization (Lorenzi et al. 2008). In this retroendocytosis model for ABCA1-mediated lipid secretion, ABCA1 generates a biophysical microenvironment favorable for apoA-I binding to the PM by providing a distinctive arrangement of phospholipids on the outer membrane leaflet (Chambenoit et al. 2001). Once apolipoproteins bind to these distinctive cell surface sites, they are endocytosed to intracellular lipid stores where ABCA1 mediates the efflux of cholesterol and phospholipids to these proteins. The resulting nascent HDL particle is recycled back to the PM for resecretion (Cavelier et al. 2006).

1.13.5 The Majority of ABCA1-mediated Cholesterol Efflux to Apo A-I Occurs at the Cell Surface

The PEST (proline, glutamic acid, serine and threonine) sequence of ABCA1 (amino acids 1283-1306) is a target for calpain protease activity which mediates ABCA1 degradation (Wang et al. 2003). Deletion of the PEST sequence (ABCA1-dPEST) significantly increases ABCA1 at the cell surface while decreasing localization to late endosomes. ABCA1-dPEST cells show an increase in apoA-I cell-surface binding, and a decrease in apoA-I internalization which is associated with a decrease in cholesterol efflux from late endosomes (Chen et al. 2005). However, overall cholesterol efflux function is increased two- to fourfold in ABCA1-dPEST expressing cells compared to wild-type ABCA1 expressing cells (Wang et al. 2003). This indicates that the deficiency in internal apoA-I lipidation is minor compared to the increase in PM apoA-I lipidation in ABCA1-dPEST expressing cells, and suggests that efflux function at the PM is the single most important site for generating nascent HDL particles.

In certain cell types such as baby hamster kidney (BHK) cells, ABCA1 colocalizes poorly with internalized apoA-I. Of the total internalized apoA-I, up to 80% ends up in lysosomes and is degraded while 20% is converted to HDL. More than 70% of the total apoA-I population remains at the cell surface and is not internalized. Based on these kinetic parameters it is estimated that internalized apoA-I contributes to a small proportion of total HDL production (~1.4%) (Denis et al. 2008). This model of apoA-I lipidation and secretion of a nascent HDL molecule may differ in different cell types.

These studies into sub-cellular ABCA1 function suggest lipidation of apoA-I occurs at both the PM and intracellularly, but that the PM is the most important site for ABCA1 mediated lipidation of apoA-I.

1.13.6 ABCA1 Recycling at the Plasma Membrane

A number of post-translational modifications increase the presence of ABCA1 at the cell surface by inhibiting ABCA1 internalization and degradation. The trafficking of cell surface GFP-ABCA1 in live cells reveals a complex intracellular pattern. Motile ABCA1-containing small vesicles in the cytoplasm dynamically traffic between the PM and large static endosomes (Neufeld et al., 2001). The trafficking pattern of biotin-labeled cell-surface ABCA1 was studied following the addition of apo A-I.

Like GFP-ABCA1, biotin-labeled cell-surface ABCA1 is rapidly internalized via clathrin-mediated endocytosis. The internalization of ABCA1 is rapidly followed by calpain-mediated degradation. However, exposure of ABCA1 to extracellular apo A-I before its endocytosis results in calpain inhibition and the rapid recycling of ABCA1 back to the cell surface (Lu et al., 2008). The putative mechanism is through the dephosphorylation of two constitutively phosphorylated threonine residues within the ABCA1 PEST sequence (T1286 and T1305). Apo A-I binding decreases the phosphorylation of residues T1286 and T1305 (Martinez LO et al., 2003). The kinases potentially responsible for T1286 and T1305 phosphorylation are casein kinase II (CKII), and protein kinase A (PKA) respectively (Schmitz and Grandl, 2009). However, these kinases have not been experimentally validated. The phosphatases responsible

for dephosphorylation of T1286 and T1305 upon apo A-I binding have not been identified (Liu and Tang, 2012) but may involve phosphatase 1 and/or phosphatase 2A (Haidar et al., 2004).

Apo A-I also leads to increased ABCA1 phosphorylation by both protein kinase C α (PKC α) and the cAMP/protein kinase A (PKA) dependent pathways. The binding and subsequent lipidation of apo A-I by ABCA1 generates diacylglycerol which activates PKC α . This kinase phosphorylates ABCA1 on an unidentified amino acid residue(s) which protects ABCA1 from calpain-mediated degradation (Yamauchi et al., 2003). The exposure of ABCA1 expressing cells to apo A-I also increases intracellular levels of cAMP which leads to ABCA1 phosphorylation by PKA. Several naturally occurring mutations of ABCA1 (C1477R, 2203X, and 2145X) have significantly reduced apo A-I mediated cAMP production and as a result, decreased ABCA1 phosphorylation (Haidar et al., 2004).

In the cytoplasmic loop of ABCA1 near the PEST sequence (1283-1306), the amino acid residues 1245 to 1257 comprise the 11-5-8-14 motif in ABCA1 which is a calmodulin binding site. Calmodulin is a Ca²⁺-binding protein and can regulate calpain-mediated proteolysis in proteins containing PEST sequences. In the presence of Ca²⁺, calmodulin can bind to and protect ABCA1 from calpain-mediated degradation (Iwamoto et al., 2010). Increasing calmodulin binding protects ABCA1 from degradation and increases ABCA1 cholesterol efflux activity.

ABCA1 degradation also occurs independently of the calpain pathway. ABCA1 is ubiquitinated on unidentified lysine residues (Wang et al., 2003). The COP9 signalosome (CSN) complex controls the ubiquitinylation and deubiquitinylation of ABCA1 (Azuma Y et al., 2009). Once ubiquitinated, a protein can undergo degradation by the ubiquitin-proteasome pathway or through lysosomal degradation. Ubiquitinated ABCA1 is associated with both pathways. Both proteasome inhibitors and lysosomal inhibitors increase ABCA1 expression and function in macrophage cells (Ogura et al., 2011; Mizuno et al., 2011). The ubiquitin-lysosomal degradation of ABCA1 occurs through the endosomal sorting complex

required for transport (ESCRT) pathway (Mizuno et al., 2011). The relative importance of each degradation pathway to ABCA1 function has yet to be determined.

The terminal four amino acids of ABCA1 (residues 2258 to 2261) contains a PDZ protein binding motif. PDZ is named after the initial members of the group (PSD-95, Dlg, ZO-1) that were identified as possessing a signature 90-amino acid domain that can bind to proteins that end in several distinctive consensus motifs. The final three amino acids of ABCA1 (SVY) conforms to the motif (S/T-X-V-COOH) bound by class-1 PDZ proteins. Two PDZ proteins, α 1-syntrophin and β 1-syntrophin physically interact with ABCA1 through the ABCA1 PDZ domain at the cell surface. ABCA1 co-localizes with α 1-syntrophin at the PM. Expression of α 1-syntrophin decreases ABCA1 degradation while increasing cholesterol efflux to apo A-I (Munehira et al., 2004). Another PDZ protein, β 1-syntrophin, was co-purified with ABCA1 from an antibody-affinity column and identified by liquid chromatography-mass spectrometric analysis. Inhibition of β 1-syntrophin decreases cholesterol efflux to apo A-I. An increase in β 1-syntrophin expression increases ABCA1 at the cell surface concomitant with an increase in ABCA1 cholesterol efflux function. Syntrophins appear to stabilize ABCA1 expression at the cell surface in a multi-protein complex (Okuhira et al., 2005) or may be involved in endocytic recycling and/or intracellular signaling that regulates ABCA1 stability at the plasma membrane. The Rho guanine nucleotide exchange factor 11 (PDZ-RhoGEF) physically interacts with the PDZ domain of ABCA1 as well. This interaction activates RhoA which can regulate cytoskeletal rearrangement via downstream effectors. The ABCA1 and PDZ-RhoGEF interaction increases ABCA1 stabilization and inhibits the degradation of ABCA1 (Okuhira et al., 2010). In Schwann cells, ABCA1 associates with Dp116/syntrophin complexes which localizes ABCA1 to the Cajal bands or the cytoplasmic channels just under the Schwann cell plasma membrane (Albrecht et al., 2008). This result suggests syntrophins in Schwann cells act in localizing ABCA1 to discrete subcellular regions.

The LXR β /RXR complex is commonly associated with the transcriptional regulation of ABCA1. A yeast-two-hybrid screen identified LXR β as associating with the COOH-terminal 120 amino acids of ABCA1. When cholesterol levels are low, immunoprecipitation studies confirmed that LXR β binds directly to ABCA1 at the plasma membrane to suppress ABCA1 function. When cholesterol levels increase, oxysterols binds to LXR β which dissociates from ABCA1 and results in ABCA1-mediated cholesterol efflux function (Hozoji et al., 2008). These results show that LXR β is involved in both the transcriptional and post-translational regulation of ABCA1.

Oxysterol-binding protein (OSBP) is a sterol sensor that regulates ceramide transport and sphingomyelin synthesis. OSBP has been found to post-translationally regulate ABCA1 by increasing ABCA1 degradation. The depletion of OSBP in cells increases ABCA1 protein expression and cholesterol efflux function by increasing ABCA1 stabilization (Bowden and Ridgway, 2008). A direct protein-protein interaction was not demonstrated between OSBP and ABCA1, but the presence of OSBP results in increased ABCA1 protease degradation.

1.13.7 Other ABCA1 post-translational modifications, domains and interactions of ABCA1

ABCA1 contains a number of post-translational modifications, domains, and protein-protein interactions whose significance has not been fully delineated. Phosphorylation is a major post-translational modification to ABCA1. As described, a number of kinases and phosphorylation sites are associated with either apo A-I binding or calpain-mediated degradation of ABCA1. ABCA1 also undergoes phosphorylation at a number of other sites. Protein kinase A (PKA) constitutively phosphorylates ABCA1 on two serine residues in NBD1 (S1042) and NBD2 (S2054). PKA phosphorylation of ABCA1 at these sites does not affect apo A-I binding, degradation, or subcellular localization of ABCA1 (See et al., 2002). However, mutation of S2054 (S2054A) significantly reduces ABCA1 phospholipid efflux function but has little effect on cholesterol efflux activity. PKA is a serine/threonine kinase that regulates a number of cellular events and PKA activation requires precise subcellular localization. Inhibition of PKA

anchoring by the inhibitor st-Ht31 increases cytosolic PKA activity. This significantly increases ABCA1-mediated cholesterol and phospholipid efflux function, as well as reversing foam cell formation, and restoring metabolic homeostasis to macrophages treated with acetylated LDL (Ma et al., 2011). The mechanism of how cytosolic PKA increases ABCA1 cholesterol efflux has not been determined.

Downstream of NBD1 and upstream of the PEST sequence in ABCA1 lies three protein kinase 2 (CK2) phosphorylation sites (T1242, T1243, and S1255). Phosphorylation of these sites does not affect subcellular localization or degradation of ABCA1. The prevention of CK2-mediated phosphorylation at these sites by CK2 inhibitors (4,5,6,7-tetrabromobenzotriazole and apigenin) or site-directed mutagenesis increases apo A-I binding as well as cholesterol and phospholipid efflux function (Roosbeek et al., 2004).

The COOH-terminus contains a number of important motifs and domains for proper ABCA1 function. The residues 2216 to 2221 contains a novel VFVNFA domain that is highly conserved among a subset of vertebrate ABCA transporters (Fitzgerald et al., 2004). Mutating this sequence does not alter trafficking of ABCA1 to the plasma membrane, but does eliminate binding to apo A-I and significantly diminishes cholesterol efflux activity. The mechanism as to how this cytoplasmic sequence affects apo A-I binding has not been determined.

The amino acid residues 270 to 449 of ABCA1, which comprise a portion of ECD1, are highly homologous to the autoantigen SS-N. SS-N is an epitope of Sjogren's syndrome. Interestingly, ABCA7, a cholesterol efflux transporter (Tanaka et al., 2011) is also highly homologous to the autoantigen SS-N in residues 186 to 360 (Tanaka et al., 2001). Sjogren's syndrome is an organ specific auto-immune disease. The significance or function of this highly homologous segment between ABCA1, SS-N, and ABCA7 is presently unclear.

A number of protein-protein interactions can regulate ABCA1 function. Phospholipid lipid transfer protein (PLTP) is a component of the RCT pathway (**Figure 1.1, Table1.1**) and mediates the

transfer of phospholipids from VLDL/LDL to HDL. PLTP has been found to physically interact with ABCA1 at similar or closely related sites as apo A-I. Like apo A-I, PLTP stabilizes ABCA1, and promotes cholesterol and phospholipid efflux activity likely by acting as a lipid acceptor (Oram et al., 2003). While apo A-I lipidation by ABCA1 is important for HDL biogenesis, the acceptance of cholesterol by PLTP from ABCA1 is unknown, but may function to shuttle lipids between lipoprotein particles or cells (Oram et al., 2008).

1.14 Research Objectives

The transcriptional regulation of ABCA1 induces the expression of genes with unwanted consequences on the liver leading to hepatic steatosis. This shifted my attention to identify novel post-transcriptional mechanisms that regulate ABCA1 expression and function. The subcellular localization of ABCA1 is critical for its proper functioning. In order to efficiently efflux cholesterol and generate HDL, ABCA1 must localize to the plasma membrane. The signals and processes necessary for ABCA1 localization to the cell surface have been unclear, especially once ABCA1 exits the Golgi. In Chapter Two, I outline the work carried out to identify a modification important for ABCA1 cell surface localization, namely palmitoylation. Palmitoylation is the covalent addition of the fatty-acid palmitate to specific cysteine residues of a protein. I demonstrated that palmitoylation is crucial for ABCA1 localization to the plasma membrane and full cholesterol and phospholipid efflux function. Palmitoylation is a dynamic event in which enzymes catalyze both the addition and removal of this fatty acid from proteins. We identified a number of palmitoyltransferase enzymes that mediates the addition of palmitate to ABCA1.

Proteins such as Rab8a (Ikonen et al., 2009), and lipid components such as ceramide (Witting et al., 2003) or unsaturated fatty acids have been found to alter levels of cell surface ABCA1. The mechanism that unsaturated fatty acids modulate ABCA1 at the plasma membrane is through the phosphorylation of serine residues on ABCA1 by PKC δ (Wang and Oram, 2007). However, the mechanisms behind Rab8a and ceramide induced localization to the cell surface are unknown. In

Chapter Three, my objective was to continue the work from Chapter Two and investigate the possibility that palmitoylation is the mechanism in which Rab8a or ceramide increases ABCA1 at the plasma membrane. I found that Rab8a and ceramide induce ABCA1 to the cell surface in a mechanism distinct from palmitoylation. I also investigated the effects of inhibiting endogenous DHHC8 on ABCA1 palmitoylation and cholesterol efflux function. DHHC8 is an enzyme we identified as mediating incorporation of radio-labeled palmitate onto ABCA1 (Singaraja et al., 2009). The increase of DHHC8 expression increased ABCA1 cholesterol efflux activity. We attempted to assess the effects of decreased DHHC8 expression on ABCA1 efflux activity. I also attempted to identify the thioesterase for ABCA1 by assessing whether the acyl-protein thioesterase-1 (APT1) enzyme catalyzes the removal of palmitate from ABCA1. Other studies included: investigating the link between glycosylation and palmitoylation; whether palmitoylation is a signal for ABCA1 raft domain localization; and whether palmitoylation is affected in naturally occurring mutations of ABCA1.

The 3' untranslated region (3'UTR) of ABCA1 at 3309 base pairs is longer than average. A long 3'UTR increases the number of potential binding sites for miRNAs (Gommons and Berezikov, 2012), and the size of the ABCA1 3'UTR suggests multiple miRNAs likely regulate ABCA1 translation. In silico prediction programs predict 37 to over 100 different miRNA binding sites in the 3'UTR of ABCA1 (**Table 1.5**). In Chapter Four, we independently identified a number of miRNAs including miR-33a as regulators of ABCA1 using in silico approach combined with a reporter assay. Subsequently, several groups validated miR-33a as a post-transcriptional regulator of ABCA1 expression and function (Najafi-Shoushtari et al., 2010; Rayner et al., 2010; Marquart et al., 2010). I designed and tested the effects of adenovirus-expressing miR-33a on ABCA1, and our group used this delivery system to manipulate ABCA1 expression in islet cells (Wijesekara et al., 2011). We also identified a novel miRNA regulator of ABCA1. We validated miR-145 as a modulator of ABCA1 protein expression and cholesterol efflux function in HepG2 cells. Our plan is to manipulate ABCA1 in vivo using miR-145 and assess its effects on HDL levels.

In light of this goal, I have designed and tested miR-145 expressing adenovirus. Chapter Four will describe the work carried out to test miR-33a on ABCA1 expression in islet cells, as well as the validation of miR-145 as a regulator of ABCA1 expression and function in HepG2 cells.

The final chapter summarizes my results, places my findings in the context of what is known about ABCA1 biology, details potential weaknesses in my research, and outlines future directions for this work along with expected findings.

2 Palmitoylation of ABCA1 is Essential for Its Trafficking and Function

2.1 Synopsis

The localization of ABCA1 to the plasma membrane is crucial for its cholesterol efflux activity. The identification of the processes regulating ABCA1 to the cell surface provides novel targets to increase ABCA1 function. Few components involved in the sorting, trafficking, and localization of ABCA1 to the cell surface have been identified. Our aim was to identify some of these processes.

Palmitoylation is the post-translational modification of proteins with the lipid palmitate, and has recently emerged as an important modification that regulates protein trafficking and function by targeting proteins to various cellular compartments or membranes (Linder and Deschenes, 2007). Palmitate is a 16-carbon saturated fatty acid that attaches to proteins post-translationally. This modification increases a protein's hydrophobicity, facilitates protein interactions with lipid bilayers, and can markedly alter protein sorting and function. Whereas other lipid modifications such as myristoylation or isoprenylation are permanent, the thioester bond that links proteins to palmitate is labile and reversible (Milligan et al., 1995; Mumby, 1997; Ross, 1995), and makes it a process that has the potential to constantly change and regulate a protein's function, as well as a process amenable to therapeutic regulation by influencing the addition or removal of this fatty acid.

The purpose of adding palmitate to soluble proteins is apparent. This lipid modification can mediate stable attachment to membranes by increasing the hydrophobicity of soluble proteins (Salaun et al., 2010). However, the purpose of adding palmitate to integral membrane proteins containing transmembrane domains (TMDs) like ABCA1 is not as apparent. A number of roles have been ascribed to how palmitoylation affects proteins with TMDs. For integral membrane proteins, palmitoylation has been shown to increase the solubility of TMD regions in cholesterol-rich domains, protect proteins from cellular quality control machinery, and alter conformation of proteins by tilting TMD segments in the membrane bilayer (Linder and Deschenes, 2007; Conibear and Davis, 2010). One of the major functional

consequences of palmitoylation on both soluble and TMD-containing proteins is influencing localization, sorting, and trafficking by increasing the interaction with hydrophobic membranes.

We investigated whether palmitoylation modifies ABCA1 localization and function by exposing cells to a chemical inhibitor of palmitoylation, 2-bromopalmitate. Exposure to 2-bromopalmitate resulted in mislocalized ABCA1, as well as significantly reduced ABCA1-mediated cholesterol efflux. This suggested that ABCA1 could be post-translationally modified by palmitate. However, to definitively demonstrate that ABCA1 was palmitoylated, we had to show that palmitate actually binds to ABCA1. We used three different labeling techniques to show that ABCA1 is palmitoylated on multiple cysteine residues. Mutagenesis of the palmitoylated cysteines resulted in decreased palmitate labeling of ABCA1, mislocalized ABCA1 away from the cell surface, and significantly reduced cholesterol and phospholipid efflux functions. Multiple enzymes, including DHHC8 were found to be involved in the palmitoylation of ABCA1. Upon increasing DHHC8 expression an increase in ABCA1-mediated cholesterol efflux activity was observed. The identification of palmitoylation as a post-translational modification of ABCA1 represents a novel regulatory process that is involved in ABCA1 cell surface localization.

2.2 Materials and Methods

2.2.1 Generation of ABCA1 Constructs

pcDNA3-ABCA1 was generated by RT-PCR amplification of human ABCA1 from liver RNA. ABCA1 was sequence verified and EGFP (Clontech) was added as a C-terminal fusion. This fusion did not cause mislocalization of ABCA1 nor alter its lipid efflux capabilities (Singaraja et al., 2006). All palmitoylation mutations in ABCA1 were generated by PCR based site-directed mutagenesis as previously described (Wellington et al., 2000) in the pcDNA-ABCA1-EGFP vector, and were fully sequenced. They were then cloned into the pcDNA5-FRT vector (Invitrogen) to generate stable cell lines. Amino-terminal (N-ter) and Carboxy-terminal (C-ter) ABCA1 constructs were generated by restriction enzyme digests and sub-cloning into the pcDNA3.1 vector.

2.2.2 Construction of Stable Cell Lines Expressing ABCA1 Variants

Polyclonal ABCA1 stable cell lines were generated in 293 Flp-In cells (Invitrogen) by co-transfecting pcDNA5/FRT-ABCA1 and pOG44 (Invitrogen) according to manufacturer's recommendations. Hygromycin-resistant colonies were evaluated for ABCA1 expression by Western blot analysis. A control hygromycin-resistant cell line was generated with the empty pcDNA5/FRT vector (Singaraja et al., 2006).

2.2.3 Protein Isolation and Antibodies

Cells were lysed in lysis buffer (150mM NaCl, 50mM Tris-HCl, pH 7.5, 1mM EGTA, 1mM EDTA, 1% tritonX-100, 0.2% SDS). A total of 40-60µg of protein was separated on 7.5% acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). ABCA1 was immunodetected using an anti-ABCA1 monoclonal antibody generated to its C-terminus (Wellington et al., 2000). In addition, anti-myc (in-house), anti-His (Roche), anti-GFP (gift from Dr. Luc Berthiaume, University of Alberta), anti-phalloidin (Invitrogen), anti-GAPDH (Chemicon) primary antibodies, anti-mouse and anti-rabbit HRP conjugated secondary antibodies (BioRad), and anti-mouse Alexa Fluor 488 (Molecular Probes) secondary antibody were used.

2.2.4 2-Bromopalmitate (2BP) Treatment of Cells to Inhibit Protein Palmitoylation

A 100mM stock of 2BP (Sigma) was prepared in dimethyl sulfoxide (DMSO) (Sigma) as per the manufacturer's instructions. The stock solution was diluted into media to a final concentration of 60µM. Cells were incubated from 5-8hrs with 2BP before preparation for immunofluorescence imaging or cholesterol efflux assays.

2.2.5 Palmitoylation Assays

Transiently transfected COS, HEK-293 or stable ABCA1 Flp-In cells were labelled with 1-2.5 mCi/ml [³H]-palmitic acid (57Ci/mmol, Perkin Elmer) for 3 hours and processed as previously described (Huang et al., 2004). For immunoprecipitation of full-length and C-terminal ABCA1, samples were

incubated with 50 μ L of anti-ABCA1 AC10 monoclonal antibodies for 24 hrs at 4°C. NH₂-terminal ABCA1 was immunoprecipitated by incubating samples with 50 μ L of anti-His monoclonal antibodies for 1 hr at 4°C. Upon addition of 50 μ L of Protein G Sepharose beads, samples were incubated for 24 hrs at 4°C. Alternately, a method using acyl-biotinyl exchange (ABE) chemistry (Drisdell and Green, 2004) was used where immunoprecipitated ABCA1 was subjected to three steps of ABE chemistry: (A) N-ethylmaleimide to block free protein thiols, (B) release of palmitoyl-modification through hydroxylamine-mediated cleavage of the thioester linkage, (C) biotinylation of exposed palmitoylation site thiols with biotin-conjugated 1-biotinamido-4-[4-(maleimidomethyl)cyclohexanecarboamide] butane (Biotin-BMCC). Finally, ABCA1 protein was eluted from antibody and subjected to Western analysis using streptavidin-HRP. In addition, metabolic labeling with ¹²⁵I-iodopalmitate was used. Synthesis of an iodopalmitate analogue was carried out as previously described (Berthiaume et al., 1995). Briefly, the radio-iodination of 16-iodo-hexadecanoic acid was carried out by incubation with Na¹²⁵I-iodide to generate the palmitate analogue 16-¹²⁵I-iodohexadecanoic acid (¹²⁵I-iodopalmitate). No oxidizing agents were required for this procedure and the final product was not HPLC purified. HEK 293 stable cell lines expressing ABCA1 were metabolically labeled with ¹²⁵I-iodopalmitate (25-50 μ Ci/mL) for 4hrs. Samples were separated on an 8% SDS-PAGE separating gel and transferred onto a polyvinylidene fluoride (PVDF) (Millipore) membrane. Incorporation of ¹²⁵I-iodopalmitate onto ABCA1 was visualized by phosphorimaging and autoradiography. The PVDF membrane was hydrolysed by soaking in 0.2N KOH (pH~13.0) for 24 hrs and re-examined by phosphorimaging and autoradiography. This membrane was subsequently processed for immunoblotting with anti-ABCA1 PEP4 polyclonal antibody.

2.2.6 Hydroxylamine Hydrolysis

Following metabolic labeling of cells expressing ABCA1 with [³H]-palmitic acid, cell lysates were immunoprecipitated and treated to either 1M Tris-HCl (pH7.5) or 1M NH₂OH (hydroxylamine) for 1-2hrs at 25°C.

2.2.7 Cell Surface Biotinylation

Transiently transfected COS cells were washed in ice cold (4°C) phosphate buffered saline (PBS) (20mM NaH₂PO₄, 0.9% NaCl, pH 7.4) and surface labeled with EZ-Link® Sulfo-NHS-SS-Biotin (Pierce) for 30mins at 4°C. The reaction was stopped and cells were lysed in lysis buffer (10mM Tris pH8.0, 1% Triton X-100). Immobilized NeutrAvidin™ gel (Pierce) was added to the lysate for 60mins at room temperature. The gel was washed with lysis buffer and samples were separated on an 8% SDS-PAGE separating gel and transferred onto a PVDF membrane (Millipore). The membrane was processed for immunoblotting with anti-ABCA1 monoclonal antibody and anti-GAPDH monoclonal antibody. GAPDH was run as a control to ensure internalization of the biotin label did not occur. Images were analyzed using quantitation software (Bio-Rad Quantity1™).

2.2.8 Lipid Efflux Assay

ABCA1 Flp-In cells were plated onto 24 well dishes in DMEM/10% FBS/50U/ml penicillin-streptomycin/20mM L-glutamine. 24 hours later, 10μCi/ml of choline chloride or 1μCi/ml cholesterol (Perkin-Elmer) was added. After 16 hours, cells were equilibrated in DMEM/0.2% defatted BSA, followed by efflux to 10-20μg/ml ApoA-I (Athens, Lee Biosystems). Lipids were extracted from the supernatants and cell lysates using chloroform:methanol and the radioactivity quantified. Efflux is expressed as a percent of the efflux induced by wild-type ABCA1.

2.2.9 Confocal Microscopy

Transiently transfected COS cells were washed in phosphate buffered saline (PBS) (20mM NaH₂PO₄, 0.9% NaCl, pH 7.4), fixed in 2% paraformaldehyde, 2% sucrose in PBS and permeabilized in 0.3% Triton X-100 (Sigma) in PBS. Cells were stained with GFP antibody for 24 hrs at 4°C, followed by incubation with a secondary antibody conjugated to Alexa 488 fluorophore for 24 hrs at 4°C, and by incubation with phalloidin conjugated to Alexa 568 fluorophore for 24 hrs at 4°C. Microscope cover glasses (Marlenfeld GmbH & Co) were mounted to microscope slides (Fisher) with fluorescent mounting

medium (DakoCytomation). Images were acquired on a Carl Zeiss Laser Scanning System LSM 510 META confocal microscope using a 63X/1.2 water objective. Images were handled using the Zeiss LSM Image Browser program Version 4,0,0,157.

2.2.10 Limited Tryptic Digest of ABCA1

Transiently transfected COS cells were washed in phosphate buffered saline (PBS) and resuspended in 5 volumes of lysis buffer (10mM Tris pH7.5, 1mM EDTA, 250mM sucrose, 1% n-Dodecyl β D-maltoside, complete protease inhibitors [Roche]) for 30mins on ice. Cellular debris was removed by centrifugation at 20000 X g for 20mins at 4°C. A total of 100 μ g of protein in each of the lysates was digested by 25 μ g/ml of trypsin on ice for 0mins, 15mins, and 45mins. Reactions were stopped by the addition of sample buffer containing 25mM DTT.

2.2.11 Statistics

All statistical analyses were performed using the two-tailed student's t test using GraphPad Prism (GraphPad Software). Results are plotted as percentage \pm standard deviation.

2.3 Results

2.3.1 Acute Inhibition of Palmitoylation Disrupts Normal ABCA1 Trafficking and Cholesterol Efflux Activity

Since localization of ABCA1 at the plasma membrane is vital for its lipid transport function, we hypothesized that palmitoylation may play a role in the targeting of ABCA1 to the plasma membrane. In order to determine if palmitoylation regulates ABCA1 transport and function, we treated ABCA1 over expressing cells with 2-bromo palmitate, a non-hydrolyzable analogue of palmitate that renders palmitoylation non functional (Resh, 2006). In the presence of 2-bromo palmitate, ABCA1 did not localize to the plasma membrane in COS and HepG2 cells (**Figure 2.1.A and Figure 2.1.B**) suggesting that palmitoylation may contribute to the proper subcellular localization of ABCA1.

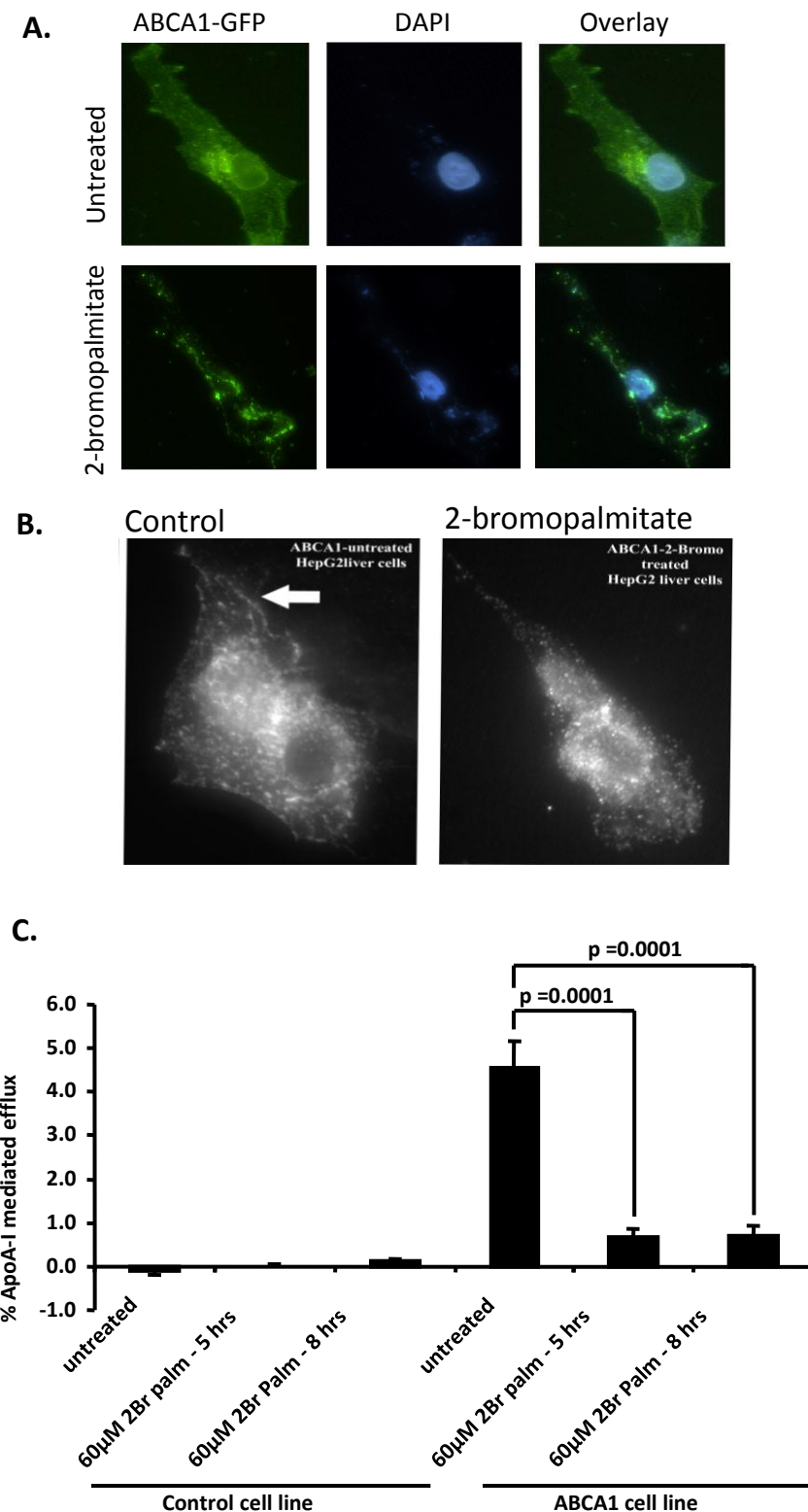


Figure 2.1. Effect of the non-metabolizable palmitate analogue 2 bromo-palmitate (2BP) on ABCA1 localization and cholesterol efflux activity. (A). COS cells transiently transfected with ABCA1 were treated with 2BP for 8hrs and stained with ABCA1 and DAPI. Cells treated with 2BP show altered localization of ABCA1 compared to untreated COS cells (DMSO only). (B). HepG2 cells treated with 2BP show altered localization of ABCA1 compared to untreated cells (DMSO only). (C). The addition of 60µM of 2BP for 5 or 8hrs results in a marked inhibition of ABCA1-mediated cholesterol efflux to lipid free apoA-1.

Since plasma membrane localization of ABCA1 was inhibited by the palmitoylation blocker 2-bromo palmitate, we next assessed if ABCA1 mediated cholesterol efflux to ApoA-I was affected by the acute inhibition of palmitoylation. In the presence of 2-bromo palmitate, ABCA1 mediated cholesterol efflux to ApoA-I was significantly reduced, indicating that abrogation of palmitoylation results in a reduction in ABCA1 efflux activity (**Figure 2.1.C**).

2.3.2 ABCA1 is Palmitoylated

Although the studies using 2-bromo palmitate suggested that palmitoylation of ABCA1 is important for its localization and efflux function, treatment with 2-bromo palmitate results in a general inhibition of palmitoylation of many substrates. These experiments do not directly or specifically address the role of palmitoylation of ABCA1. To directly determine if ABCA1 is palmitoylated, ABCA1 was immunoprecipitated from cells, and palmitoylation was assessed using biotinylated BMCC. In the presence of hydroxylamine (HAM), a reagent that specifically cleaves palmitate, a robust signal was observed, indicating that ABCA1 is palmitoylated (**Figure 2.2.A**).

To confirm ABCA1 palmitoylation, two additional methods were used, I^{125} -palmitate incorporation (**Figure 2.2.B**) or 3H -palmitate incorporation (**Figure 2.2.C**) by metabolic labelling, followed by immunoprecipitation, polyacrylamide gel electrophoresis and autoradiography. Using these labelling techniques, we show that ABCA1 is palmitoylated. Treatment with KOH (**Figure 2.2.B, panel 3**) or hydroxylamine (HAM [NH_2OH]) (**Figure 2.2.D**) resulted in a significant reduction in palmitoylation signal, indicating that the binding of palmitate to ABCA1 is specific, as both KOH and HAM hydrolyze the thioester bonds that covalently attach palmitate to cysteine residues (Kaufman et al., 1984).

2.3.3 ABCA1 is Palmitoylated at Cysteine Residues in Its N-Terminus

Palmitoylation occurs on intracellular cysteine residues, usually close to transmembrane domains (El-Husseini and Bredt, 2002). However, no consensus sequences for palmitoylation are currently known. ABCA1 contains 39 cysteines of which 23 are predicted to occur in intracellular regions.

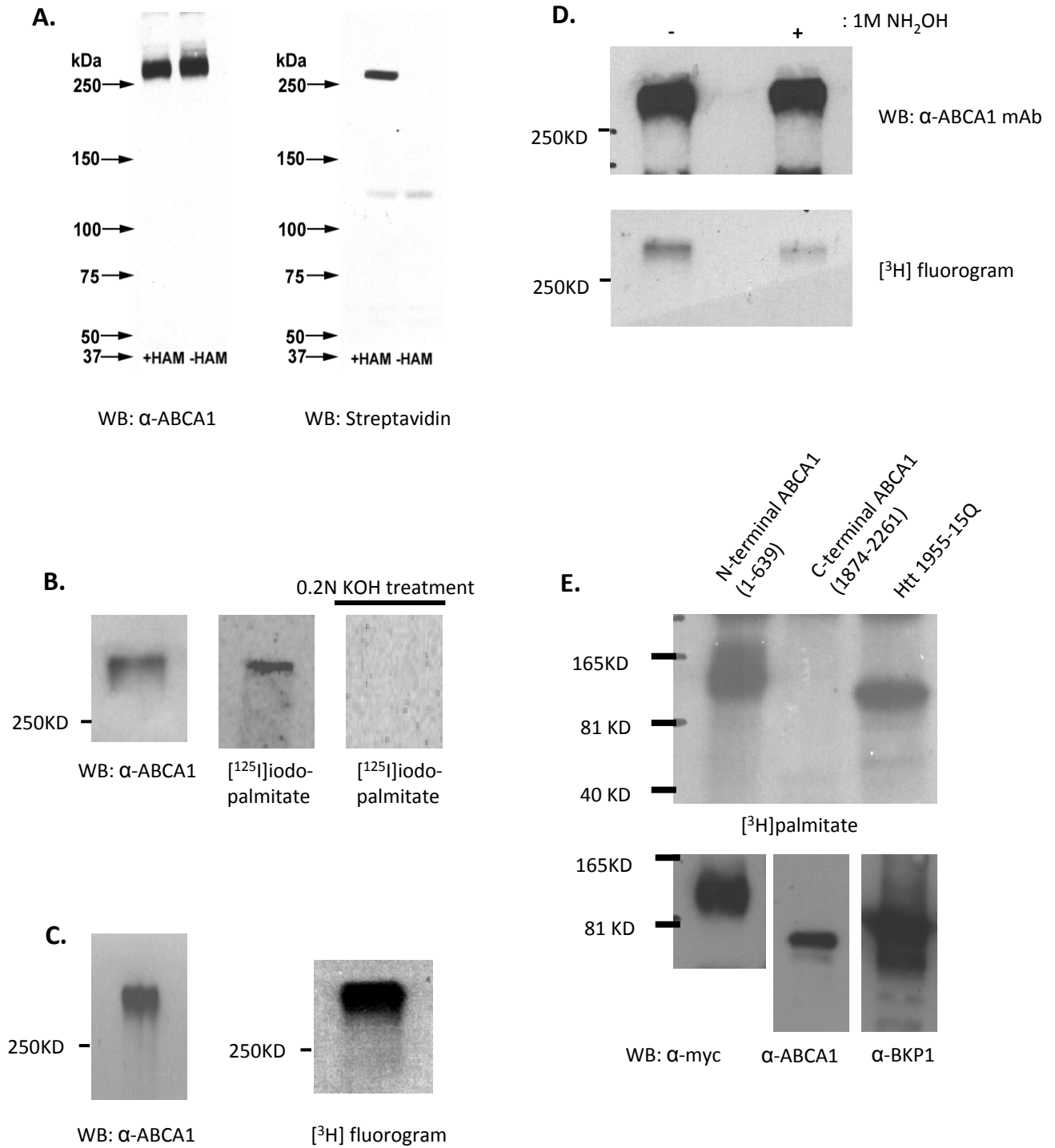


Figure 2.2. Assessment of ABCA1 palmitoylation by three methods. (A). HEK 293 cells were infected with adenovirus-expressing ABCA1 and cell lysates were either untreated or treated with 1M NH_2OH (hydroxylamine, HAM) which generates free sulfhydryl groups at sites of palmitoylation upon treatment. The addition of a sulfhydryl-reactive biotinylation reagent, Biotin-BMCC, was added to enhance the sensitivity of sulfhydryl detection by streptavidin-biotin. ABCA1 was immunoprecipitated and visualized by Western blotting with anti-ABCA1 PEP4 polyclonal antibodies or streptavidin-HRP. HAM treatment reveals full-length ABCA1 is palmitoylated as indicated by a band running at a molecular weight corresponding to ABCA1 in the streptavidin-probed immunoblot. (B). ABCA1 expressing HEK 293 stable cell lines were metabolically labeled with [^{125}I] iodopalmitate. A [^{125}I] iodopalmitate band corresponding to ABCA1 was detected by autoradiography. Treatment of the PVDF membrane with the weak base 0.2N KOH (pH~13.0) abrogated the radioactive signal, indicating that [^{125}I] iodopalmitate was chemically attached to ABCA1 through an ester bond at a cysteine residue. (C). COS cells were transiently transfected with ABCA1 and metabolically labeled with [^3H] palmitic acid. Immunoprecipitation of ABCA1 followed by fluorography indicated a [^3H] palmitic acid positive band corresponding to ABCA1. (D). COS cells were transiently transfected with ABCA1 and metabolically labeled with [^3H] palmitic acid. Immunoprecipitates of ABCA1 were treated with 1M Tris pH 7.5 (Control) or 1M NH_2OH . Radiolabeling was removed from ABCA1 following 1M NH_2OH treatment, indicating that [^3H] palmitic acid is coupled to ABCA1 through a thioester linkage. (E). COS cells were transiently transfected with an NH_2 -terminal fragment of ABCA1 (first 639 amino acids) tagged with Myc-His, and a COOH-terminal fragment of ABCA1 (last 387 amino acids of ABCA1) followed by metabolic labelling with [^3H] palmitic acid. A fragment of the huntingtin protein (Htt 1955-15Q) known to be palmitoylated was used as a positive control (Yanai A et al., (2006) Nat Neurosci 9:824-31). Metabolic labeling of the NH_2 -terminal fragment of ABCA1 suggests ABCA1 palmitoylation occurs in a cysteine residue present in this fragment.

In order to determine the site(s) of ABCA1 palmitoylation, we generated N-terminal (N-ter) and C-terminal (C-ter) constructs of ABCA1. The N-ter construct consisted of amino acids 1 to 639 and contains 11 cysteines, and the C-ter construct consisted of amino acids 1874 to 2261 and contains 5 cysteines. When metabolic labelling assays were performed with [^3H]-palmitate, the N-terminus of ABCA1 was palmitoylated (**Figure 2.3.E**). No palmitoylation was observed in the C-terminal ABCA1. These results indicate that ABCA1 is likely palmitoylated in its N-terminus.

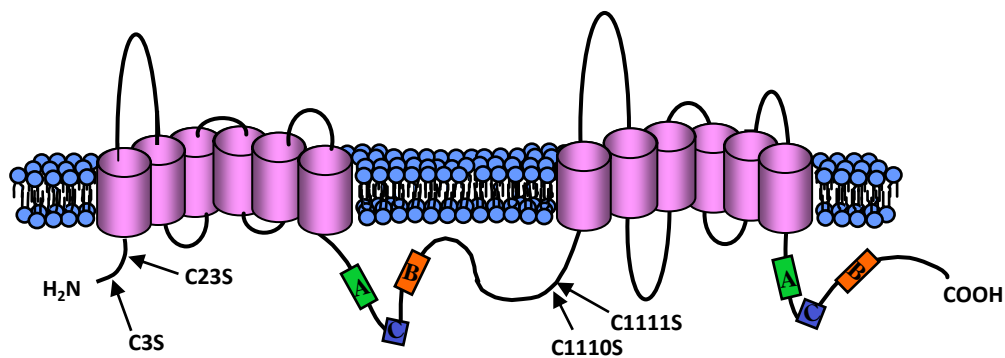
The N-terminal fragment of ABCA1 contains 11 cysteines. Of these, 2 cysteines are in the intracellular region, and 9 cysteines were localized to the large extracellular loop. Since palmitoylation occurs in intracellular regions, the two intracellular cysteines at amino acids 3 and 23 were replaced with serines by site-directed mutagenesis. Three mutant fragments were generated: C3S; C23S; and the double mutant C3S/C23S. *In vitro* metabolic labelling assays using [^3H]-palmitate showed that palmitoylation occurs on both the C3 and C23 residues, as ABCA1 palmitoylation levels were reduced in all 3 mutant constructs (**Figure 2.3.C**). However, compared with the C23S mutation, the mutation at C3 resulted in only a minor reduction in N-terminal ABCA1 palmitoylation, indicating that C23, the cysteine closest to the transmembrane domain is the major site of palmitoylation. Thus, ABCA1 is palmitoylated at amino acids 3 and 23.

2.3.4 ABCA1 is Also Palmitoylated at Other Intracellular Cysteines

Although palmitoylation occurs on cysteines 3 and 23 in the N-terminus of ABCA1, and no palmitoylation occurs in the very C-terminal portion of ABCA1, the possibility of palmitoylation between these two fragments (aa 640 to 1873) had not been assessed. To answer this question mutations in cysteines 3 and 23 were generated in full length ABCA1. Using [^3H]-palmitate metabolic labelling, we found that in the presence of the C3 and C23 mutations, full length ABCA1 still demonstrated palmitoylation (**Figure 2.3.D**), indicating additional sites of palmitoylation exist in ABCA1.

Since palmitoylation often occurs in intracellular cysteine residues within close proximity to transmembrane domains, and several proteins have been shown to be palmitoylated at double cysteine sites (el-Husseini and Bredt, 2002), cysteine to serine mutants of the adjacent amino acids 1110 and 1111 in ABCA1 were generated. Palmitoylation assessment of these sites in the context of full length ABCA1 showed that ABCA1 was still palmitoylated (**Figure 2.3.D, lane 5**). However, generation of a quadruple mutant C3S/C23S/C1110S/C1111S in full length ABCA1 resulted in a significant reduction of ABCA1 palmitoylation (**Figure 2.3.D, lane 6; Figure 2.3.E**). Thus, cysteines at residues 3, 23, 1110, and 1111 are the major sites of palmitoylation in ABCA1.

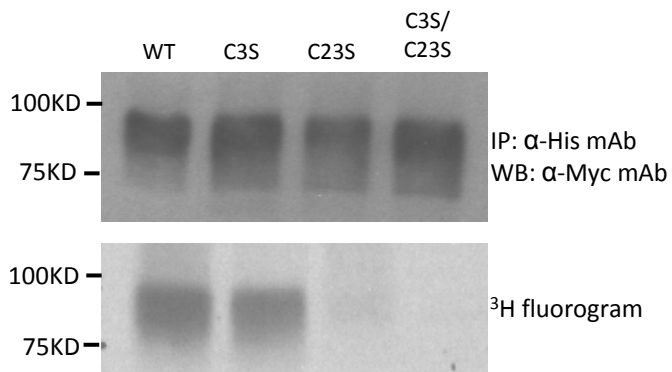
A.



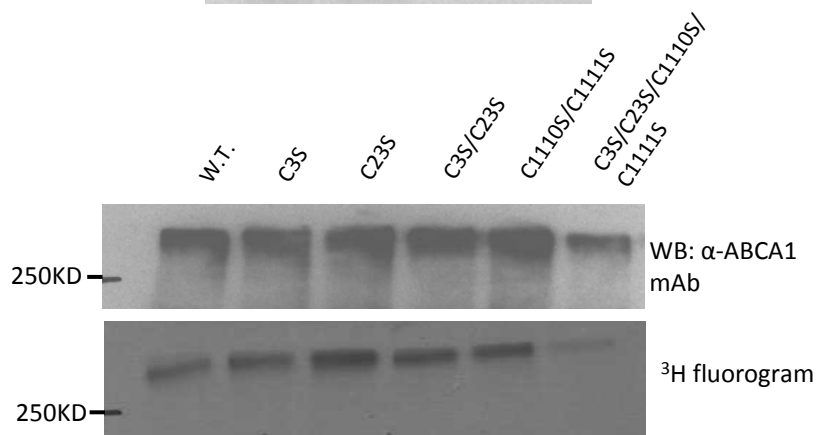
B.

	3	23	1110/1111
WT	MACWPQLRLLLWKNLTFRRRQT	CQLLL.....IAIISHGKL	CCVGSSL...
C3S	... <u>S</u>		
C23S		... <u>S</u>	
C1110S			... <u>S</u>
C1111S			... <u>S</u>
C3S/C23S	... <u>S</u> <u>S</u>	
C1110S/C1111S			... <u>SS</u>
C3S/C23S/C1110S	... <u>S</u> <u>S</u> <u>S</u>
C3S/C23S/C1111S	... <u>S</u> <u>S</u> <u>S</u>
C3S/C23S/ C1110S/C1111S	... <u>S</u> <u>S</u> <u>SS</u>

C.



D.



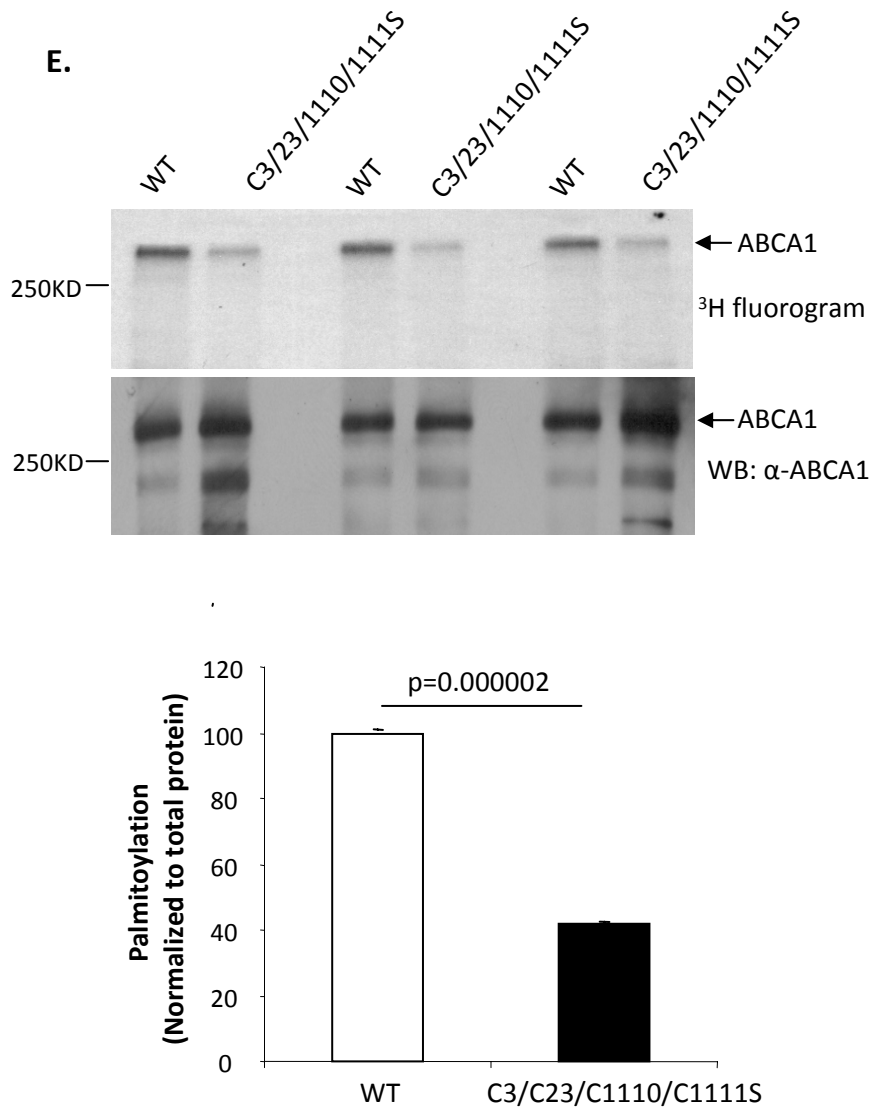


Figure 2.3. Identification of the cysteines responsible for ABCA1 palmitoylation. (A). Diagram of ABCA1 showing the general location of the four cysteines responsible for ABCA1 palmitoylation. (B). Site-directed mutagenesis on candidate cysteines in NH₂-terminal (1-639 amino acids; 11 total cysteines) and full-length ABCA1 (1-2261 amino acids; 39 total cysteines). Mutation of the cysteines present at the 3rd, 23rd, 1110th and 1111th amino acid positions into serines were carried out either singly or in combination. (C). COS cells were transiently transfected with NH₂-terminal ABCA1 (WT, C3S, C23S, C3S/C23S) and metabolically labeled with [³H] palmitic acid. Decreases in [³H] palmitic acid incorporation were observed for all mutants especially the C3S/C23S double mutant. (D). COS cells were transiently transfected with full-length ABCA1 (WT, C3S, C23S, C3S/C23S, C1110S/C1111S or C3S/C23S/C1110S/C1111S) and metabolically labeled with [³H] palmitic acid. Only the quadruple ABCA1 mutant (C3S/C23S/C1110S/C1111S) showed a decline in [³H] palmitic acid incorporation. (E). The palmitoylation of the quadruple ABCA1 mutant (C3S/C23S/C1110S/C1111S) is significantly reduced.

2.3.5 Palmitoylation at C3, C23, C1110, and C1111 are Essential for Proper ABCA1 Trafficking

ABCA1 is localized in the endoplasmic reticulum, in endocytic vesicles and at the plasma membrane. Since palmitoylation ensures the proper sorting and targeting of proteins, we assessed if the palmitoylation defective ABCA1 showed altered trafficking. We observed that each of the palmitoylation deficient mutants resulted in mislocalized ABCA1 that did not reach the plasma membrane, but instead localized to intracellular regions (**Figure 2.4**). As a control, cells expressing the TD clinical phenotype mutant in ABCA1, M1091T was used. This mutation has been previously described in patients and represents a severe loss of function mutation resulting in abrogation of ABCA1 expression at the plasma membrane (Singaraja et al., 2006).

2.3.6 Palmitoylation of ABCA1 Regulates Its Localization at the Plasma Membrane

In addition to the assessment of ABCA1 localization by immunofluorescence, we performed cell surface biotinylation on WT and C3/23/1110/1111S-ABCA1 to quantify the levels of plasma membrane localization of each construct. In agreement with our immunofluorescence data, the cell surface expression of palmitoylation deficient ABCA1 was reduced by >90% compared to WT ABCA1 (**Figure 2.5.A and Figure 2.5.B**) supporting the assertion that ABCA1 palmitoylation is required for proper plasma membrane localization. The intracellular protein GAPDH was used as a control. Biotin labeling of GAPDH (IP lane) indicates internalization of biotin.

2.3.7 ABCA1 Palmitoylation is Essential for Cholesterol and Phospholipid Efflux Activity

We next assessed if the palmitoylation of ABCA1 was essential for its function in efflux activity. We generated stable cell lines harboring each of the C3S, C23S, C1110S and C1111S mutants singly and in combination, and determined the extent of both cholesterol and phospholipid efflux to ApoA-I. In the presence of any of the palmitoylation mutations, ABCA1 mediated cholesterol (**Figure 2.5.C**) and phospholipid (**Figure 2.5.D**) efflux to ApoA-I was significantly decreased. The abrogation of palmitoylation at any one of these sites resulted in a similar magnitude (40-60%) of decrease in efflux, as

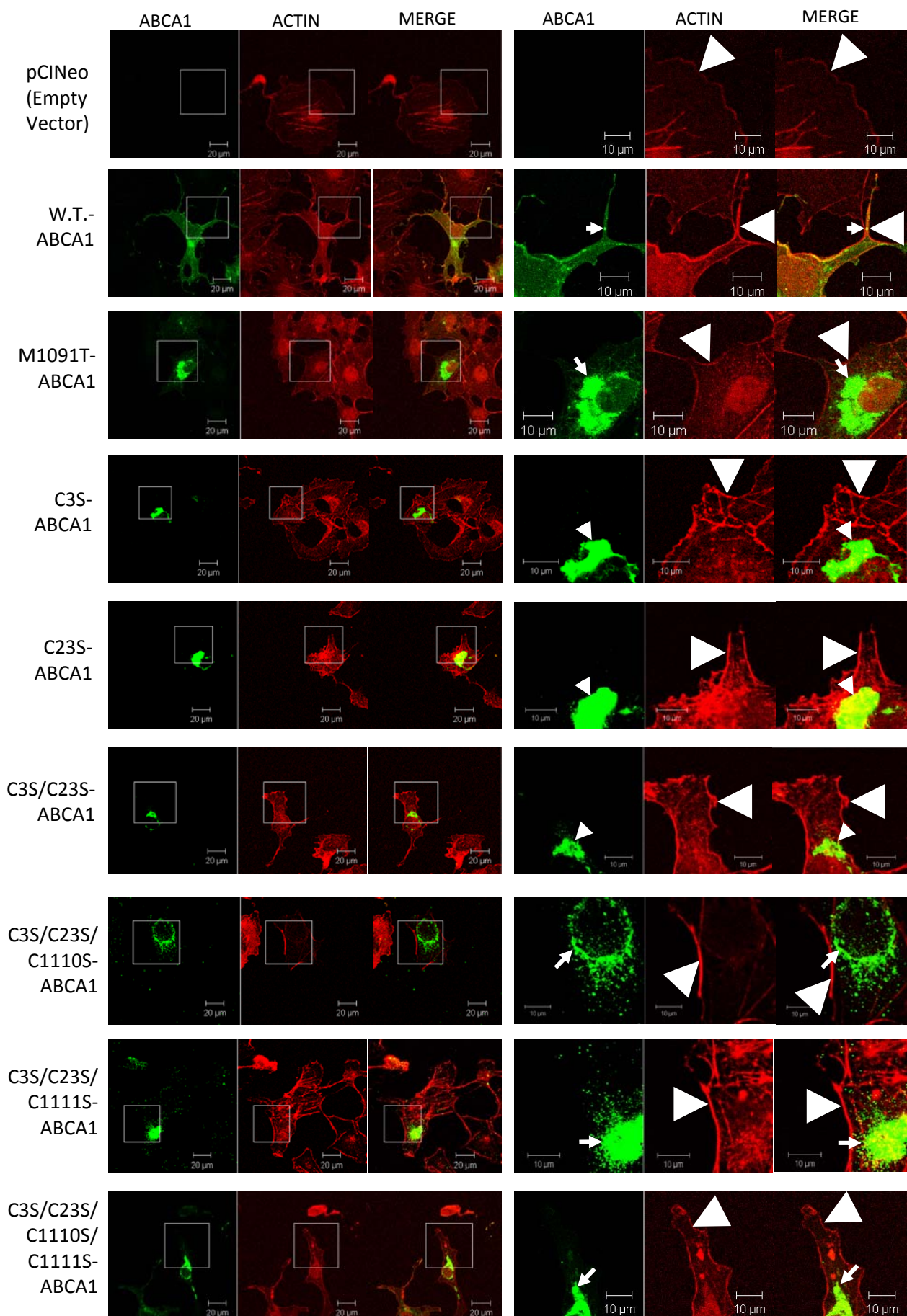
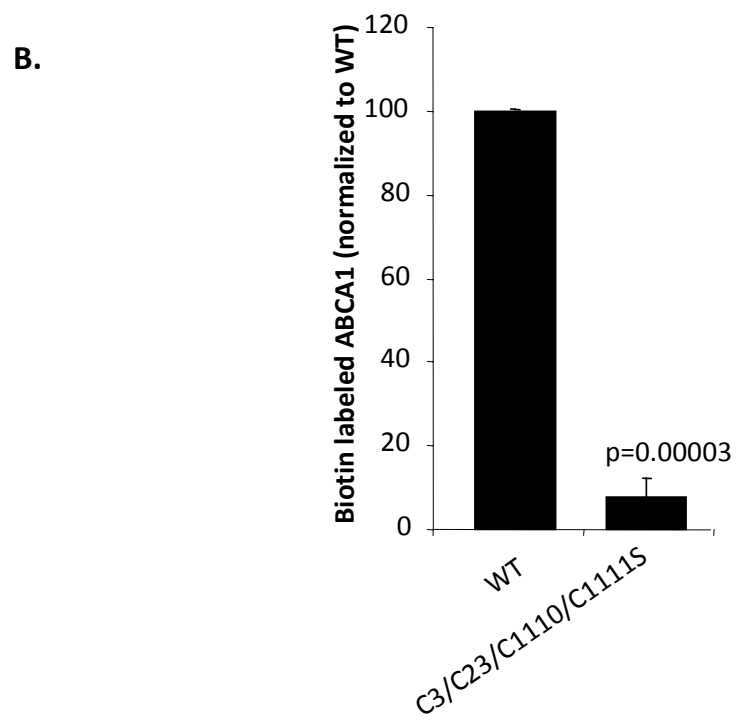
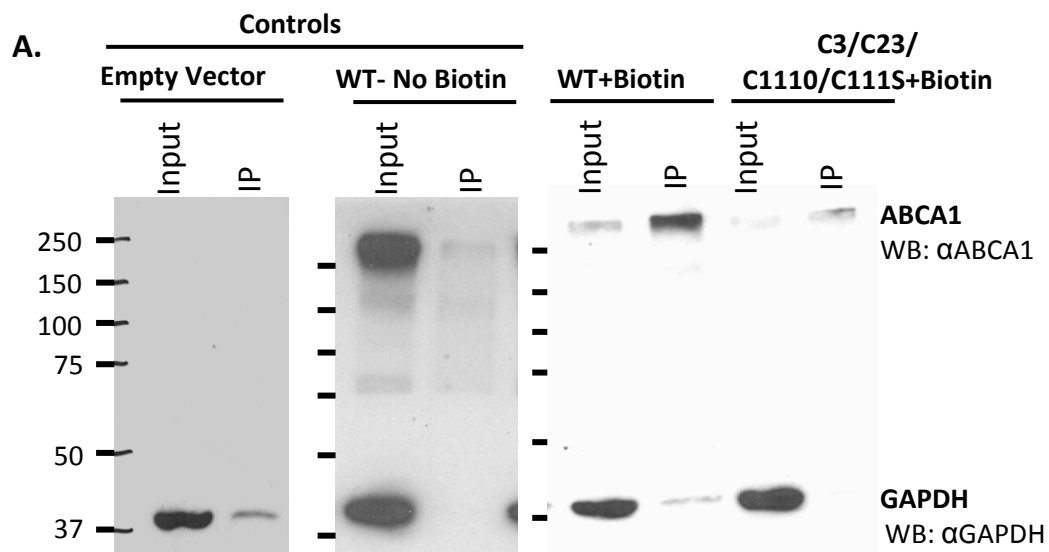


Figure 2.4. Altered distribution of palmitoylation-resistant ABCA1 mutants in transfected COS cells observed by confocal microscopy. COS cells were transiently transfected with full-length ABCA1-GFP constructs including: pCINeo (empty vector); M1091T- (TD clinical phenotype mutant) (Ref req); C3S- ; C23S- ; C3S/C23S- ; C3S/C23S/C1110S- ; C3S/C23S/C1111S- ; and C3S/C23S/C1110S/C1111S-ABCA1. Cells were immunolabeled with antibodies to detect ABCA1 (green) and actin (red). All palmitoylation resistant ABCA1 mutants show altered distribution. The three right-most panels (scale bars = 10 μ m) are enlarged images extracted from the three left panels (scale bars = 20 μ m) as marked by the white boxes. The small arrows indicate ABCA1 location and the large arrows indicate actin localization.



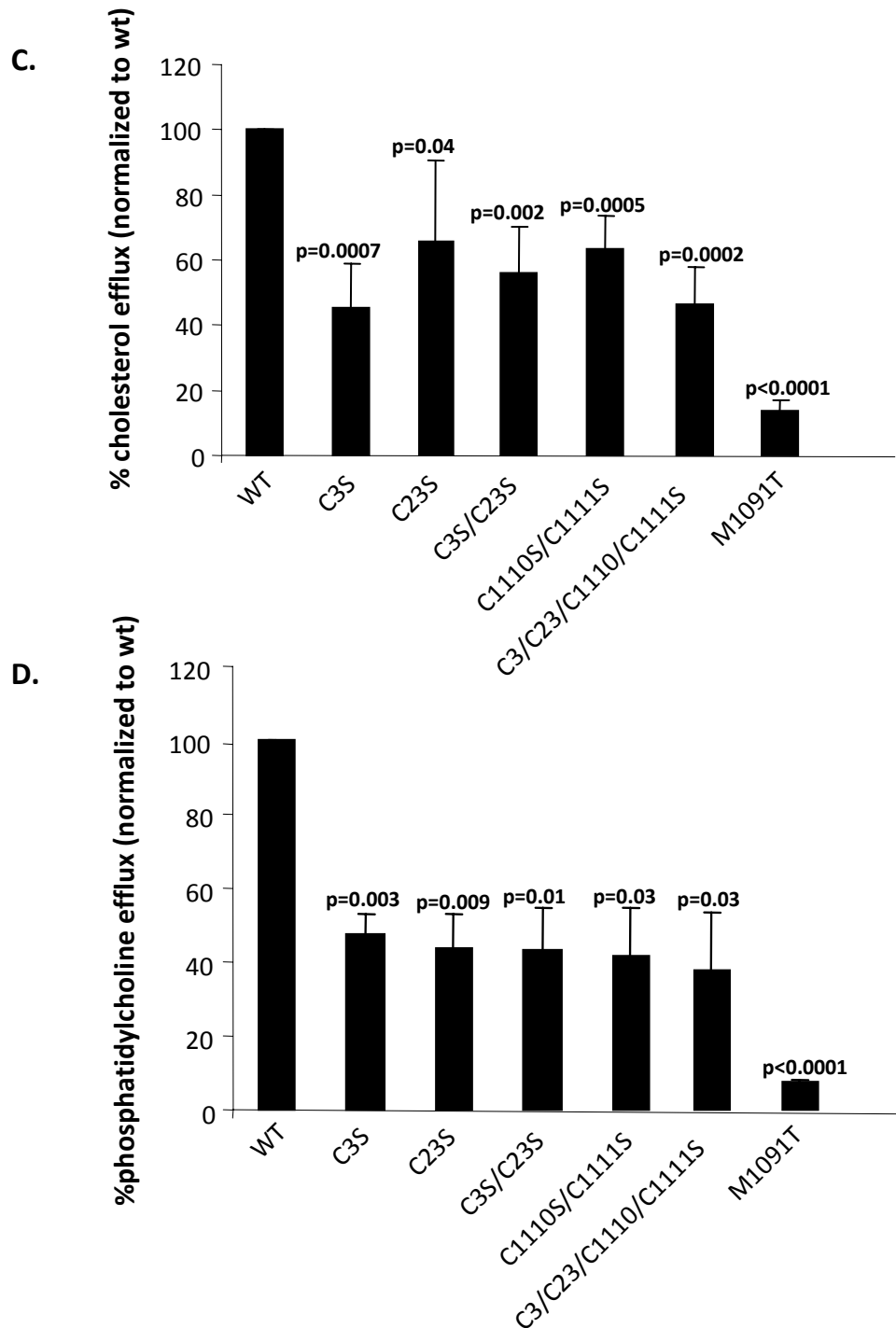


Figure 2.5. Palmitoylation is important for ABCA1 plasma membrane localization and phospholipid and cholesterol efflux function. (A) The amount of plasma membrane ABCA1 was assessed using cell surface biotinylation. A robust signal was detected for WT-ABCA1 (third blot, second lane). However, only a very modest signal was detected for C3/23/1110/1111S ABCA1 (third blot, fourth lane). (B) Quantification of cell surface ABCA1 showed a significant loss of ABCA1 at the plasma membrane. Stable cell lines were generated harbouring the cysteine mutants singly and in combinations. Efflux of cholesterol (C) and phospholipids (D) was reduced by ~50% in each of the palmitoylation-deficient ABCA1 mutants. As a positive control, efflux of lipids was reduced by ~80% in a cell lines harbouring M1091T, a naturally occurring loss-of-function TD patient mutation.

did the abrogation of palmitoylation at all four cysteines combined. This result is not surprising considering our finding that inhibiting ABCA1 palmitoylation at any individual cysteine site results in the mislocalization of ABCA1. In addition, our data indicate that palmitoylation at these sites, although important, is not the only factor regulating ABCA1 trafficking as ~50% of efflux function still remains. As a control, the efflux from a stable cell line harboring the naturally occurring ABCA1 mutation M1091T was reduced by ~80%.

2.3.8 DHHC8 is a Major Palmitoyl Transferase for ABCA1

In mammals, palmitoylation is catalyzed by a family of 23 DHHC domain containing palmitoyl acyl transferase proteins (PATs) (Ohno et al., 2006; Fukata et al., 2004). In order to determine the palmitoyl acyl transferase for ABCA1, we transiently transfected each of the PATs into an ABCA1 overexpressing cell line. Using ³H-palmitate metabolic labelling, we found that DHHC8 increased the palmitoylation of ABCA1 the most (**Figure 2.6.A**). DHHC12, 15, 20 and 21 were also able to increase ABCA1 palmitoylation, although these increases were modest compared to the effects of DHHC8 (**Figure 2.6.A**).

Treatment of palmitoylated ABCA1 with hydroxylamine removed much of the palmitate signal, indicating that the increase in palmitoylation caused by overexpression of the PATs was specific, and not caused by non-specific association of palmitate with ABCA1 protein (**Figure 2.6.B**).

2.3.9 ABCA1 Mediated Efflux is Increased in the Presence of Its PAT, DHHC8

Since DHHC8 is the major PAT for ABCA1, we analyzed its ability to increase ABCA1 mediated cholesterol efflux. When DHHC8 was transiently transfected into an ABCA1 expressing stable cell line, cholesterol efflux to ApoA-I was significantly increased (**Figure 2.7.A**). Transient transfection of DHHC2 and 12, two other PATs that had mild effects on ABCA1 palmitoylation, did not result in increased ABCA1 cholesterol efflux. Thus, increasing DHHC8 mediated palmitoylation of ABCA1 results in increased ABCA1 function.

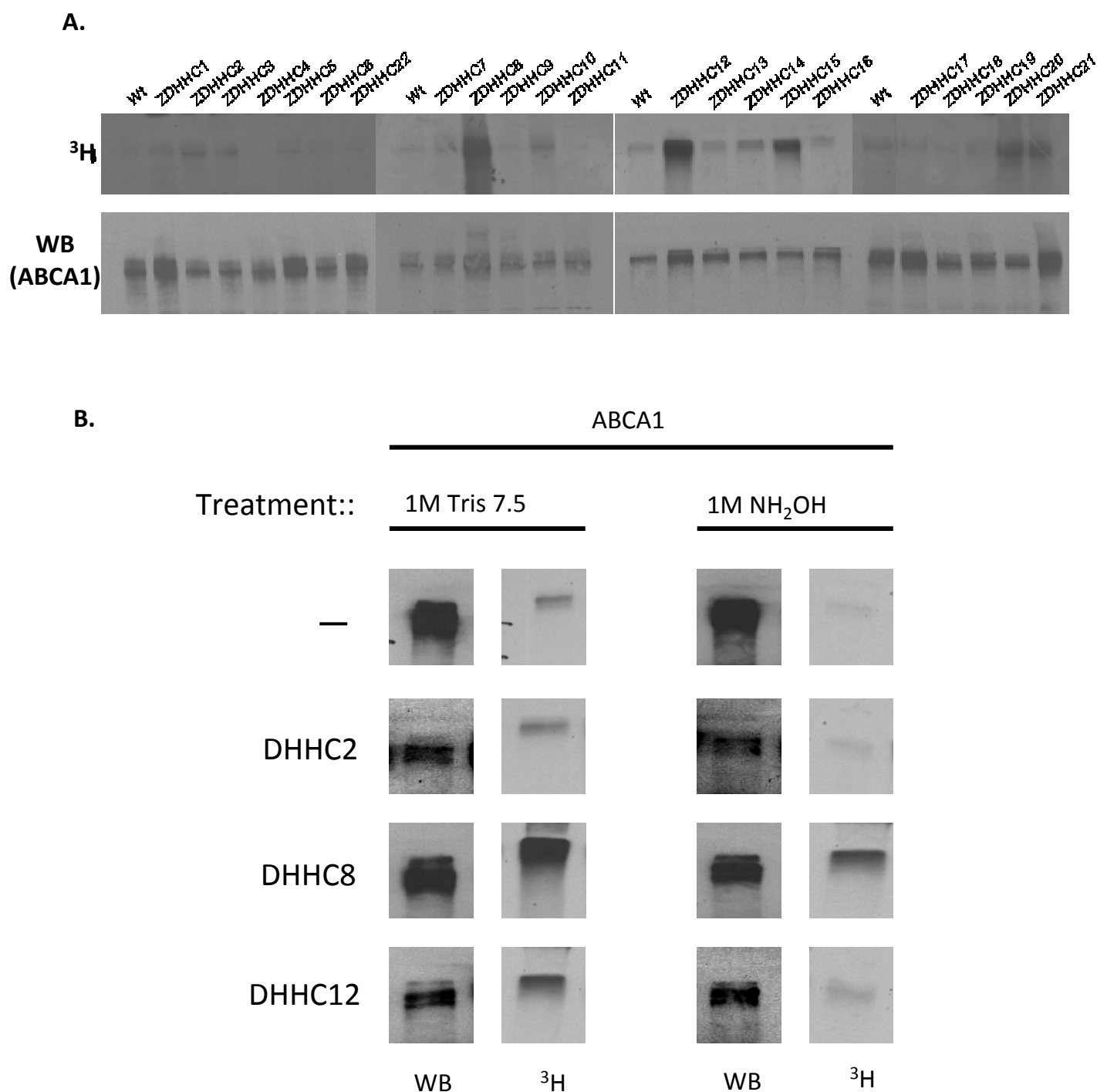


Figure 2.6. Screening for potential ABCA1 palmitoyl acyl transferases (PATs) enzymes that mediate ABCA1 palmitoylation. (A) COS cells were transiently co-transfected with ABCA1 and one of 22 individual PAT (DHHC) constructs and metabolically labeled with [³H] palmitic acid. ABCA1 palmitoylation was increased by the addition of several PATs. (B) Treatment with 1M NH₂OH reduced [³H] palmitic acid incorporation after treatment by DHHC2, -8 and -12, suggesting that the increase in ABCA1 palmitoylation following PAT expression is due to thioester linkage of ABCA1 cysteine residues with [³H] palmitic acid, and not a result of random non-specific radiolabeling.

Since it is possible that one other cause for the observed increase in cholesterol efflux may be an increase in ABCA1 expression modulated by the overexpression of PATs, we assessed ABCA1 protein in the presence of the PATs DHHC2, 8 and 12. ABCA1 protein levels were not altered following the expression of any of the three PATs (**Figure 2.7.B**). Thus the increase in efflux mediated by DHHC8 was not due to increased ABCA1 protein, but more likely due to increased ABCA1 palmitoylation and increased presence of ABCA1 at the plasma membrane.

PATs are widely thought to interact with their substrates. Immunoprecipitation studies found that ABCA1 and DHHC8 were able to interact with one another (**Figure 2.7.C**), fulfilling a feature of PAT/substrate specificity and interaction.

2.3.10 Trypsin Sensitivity of ABCA1

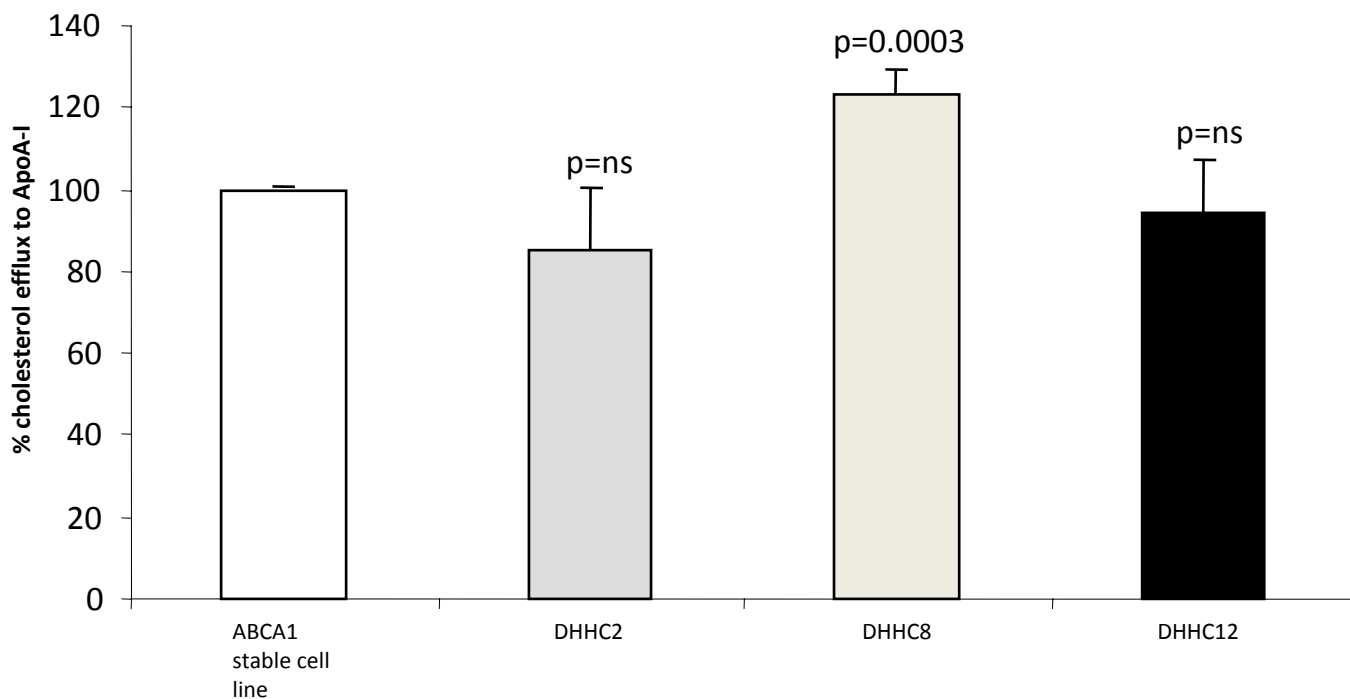
A major drawback in the interpretation of our results are that the cysteine-to-serine mutations may induce misfolding of ABCA1 which manifests as mislocalization and reduced cholesterol efflux activity rather than these findings being a result of reduced palmitoylation. To confirm that the ABCA1 palmitoylation mutants possess a similar conformation to wild-type (WT) ABCA1, we examined ABCA1 following trypsin exposure. Partial tryptic digest of ABCA1 produces distinct fragments including a 110-120kDa C-terminal fragment that contains the second nucleotide binding domain (NBD) of ABCA1 (**Figure 2.8.A**) (Takahashi et al., 2006). Our constructs have a 25kDa GFP tag attached to the C-terminus of ABCA1 so following tryptic digestion, the C-terminal fragment is sized at 135-145kDa. All our ABCA1 palmitoylation mutants including C3S/C23S/C1110S/C1111S produce the 135-145kDa fragment similar to WT-ABCA1 (**Figure 2.8.B**). This suggests that the cysteine mutations do not induce a conformation change in ABCA1.

2.3.11 ABCA1 May Contain More Than Four Sites of Palmitoylation

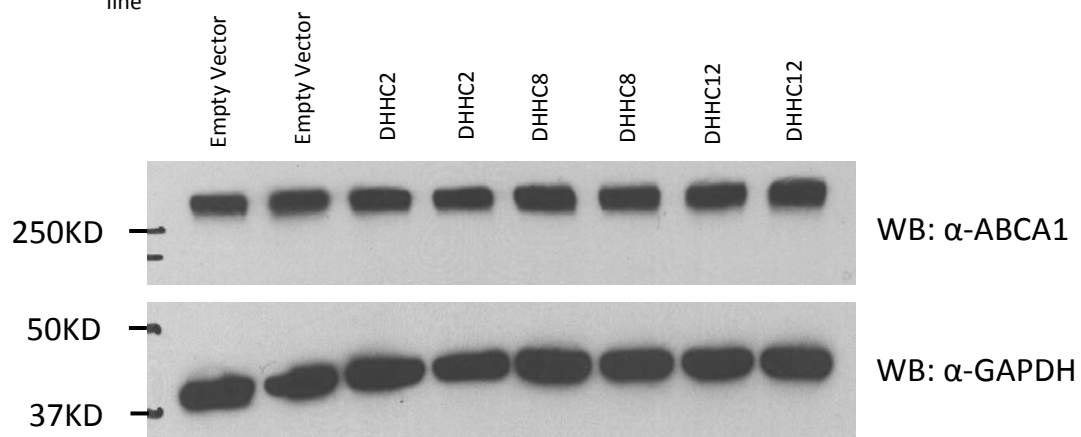
We have shown that ABCA1 is palmitoylated at four cysteine residues (**Figure 2.3.D**). Following cysteine-to-serine mutagenesis at positions 3, -23, -1110, and -1111, metabolic incorporation of [^3H]

palmitate is significantly reduced ($p < 0.001$) but not completely abrogated (**Figure 2.3.E**). To determine if the residual labeling was specific, we treated C3S/C23S/C1110S/C1111S-ABCA1 to 1M hydroxylamine (NH_2OH) which specifically cleaves the thioester bond that attaches palmitate to cysteine residues. We have found a further decrease in palmitoylation of C3S/C23S/C1110S/C1111S-ABCA1 following 1M NH_2OH treatment which suggests ABCA1 may be palmitoylated at other cysteine residues (**Figure 2.9**).

A.



B.



C.

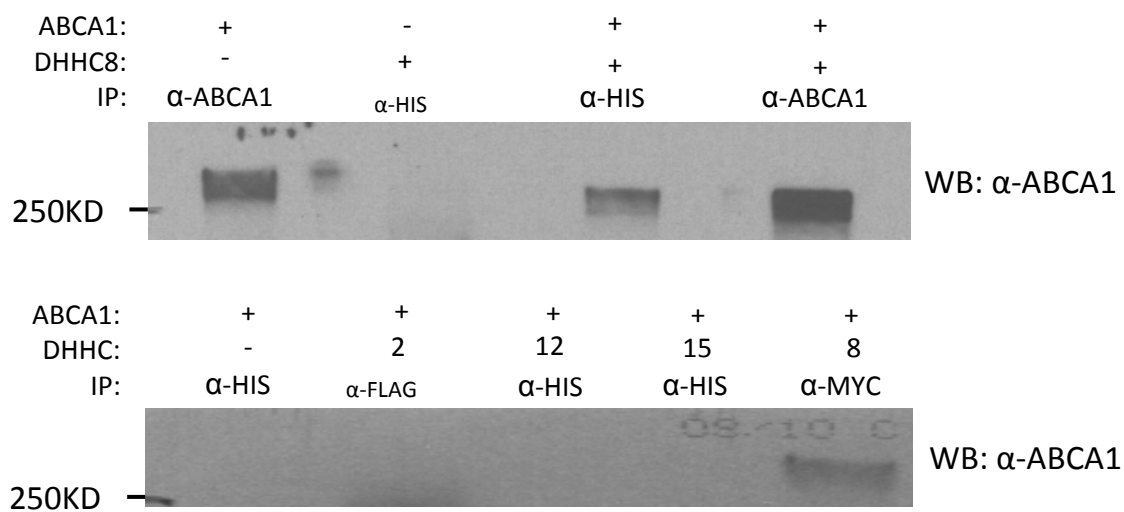


Figure 2.7. DHHC8 increases ABCA1-mediated cholesterol efflux activity. (A). Transient transfection of DHHC8 into HEK 293 WT-ABCA1 expressing stable cell lines reveals an ~20% increase ($p=0.0003$) in ABCA1-mediated cholesterol efflux. (B). A Western blot of ABCA1 shows no change in ABCA1 protein expression following DHHC8 expression. (C). The DHHC8 construct is Myc-His tagged. COS cells were transiently co-transfected with ABCA1 and different PATs. Lysates were immunoprecipitated with anti-Myc or anti-His monoclonal antibodies and immunoblotted with anti-ABCA1 monoclonal antibody. Co-immunoprecipitation is observed between ABCA1 and DHHC8.

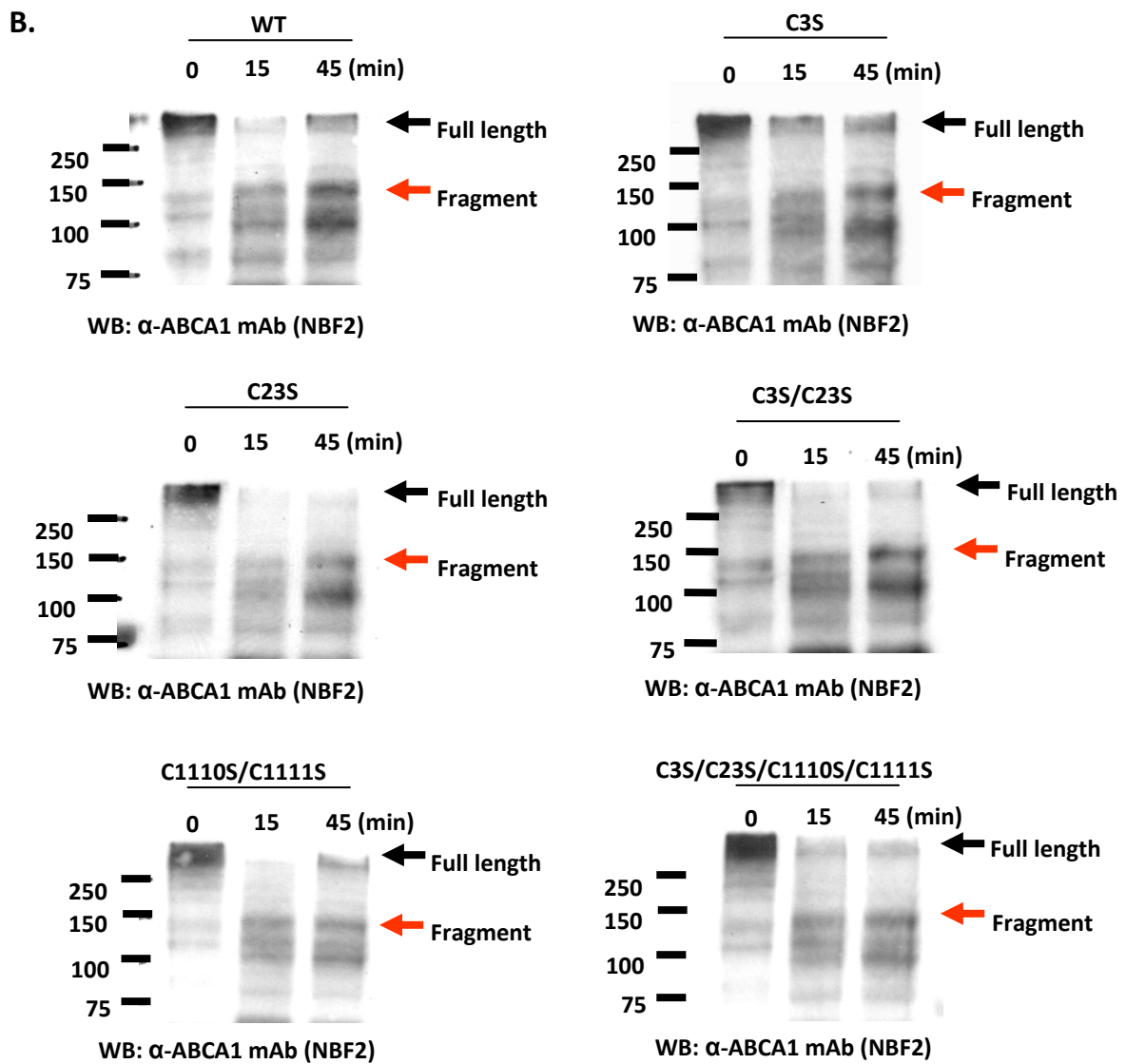
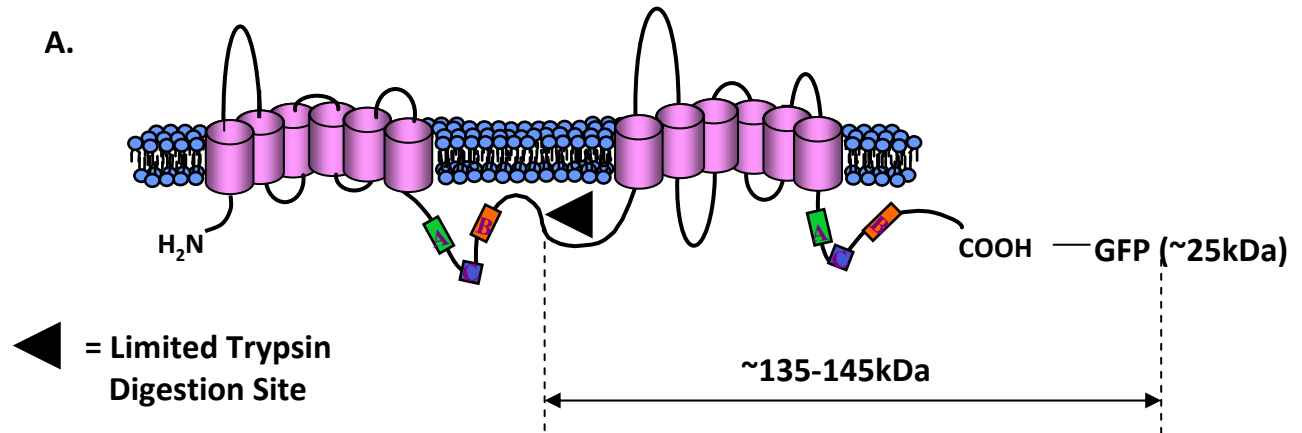


Figure 2.8. Trypsin-limited digestion of ABCA1. (A). Schematic diagram of trypsin-limited digestion of ABCA1 and the molecular size of the produced fragment (For details see Takahashi et al., JBC (2006) 281:10760-68). (B). COS cells transfected with WT-ABCA1 or various ABCA1 palmitoylation mutant constructs were treated with 25µg/mL of trypsin for 0, 15min and 45min and separated on a 7.5% SDS-PAGE gel. ABCA1 was detected by an anti-ABCA1 monoclonal antibody that recognizes the C-terminal NBD domain. Similar trypsin digestion pattern of mutant constructs to WT-ABCA1 suggests that they all have a similar conformation.

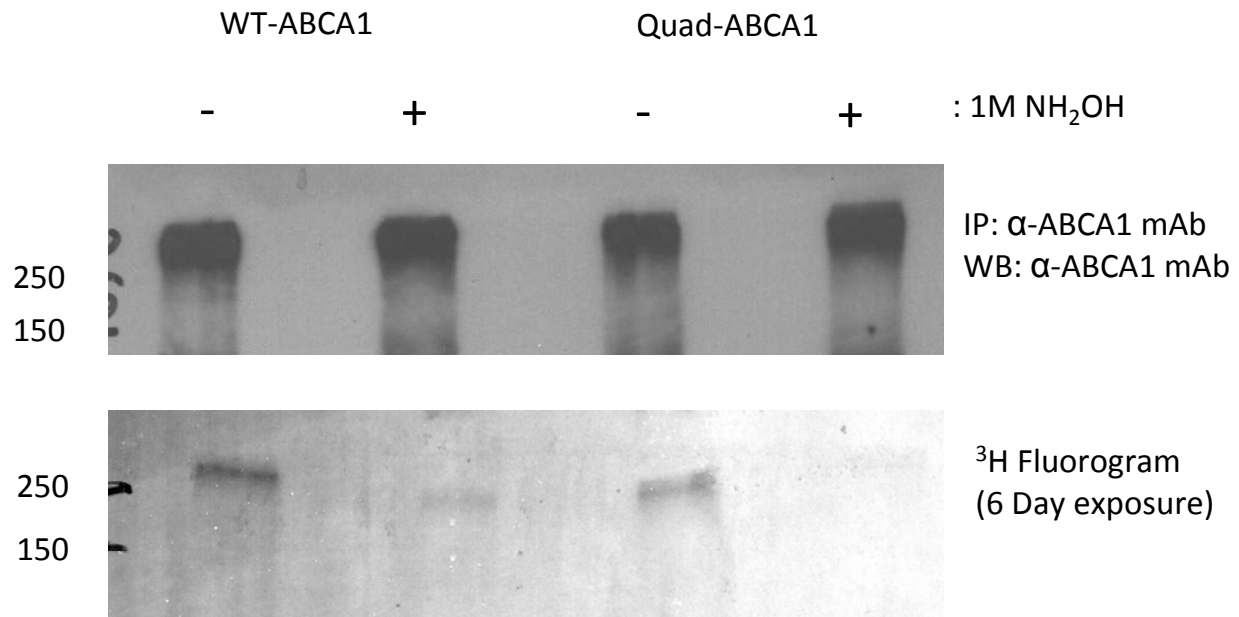


Figure 2.9. Assessment of ABCA1 palmitoylation in wild-type ABCA1 (WT) versus C3S/C23S/C1110S/C1111S ABCA1 (Quad) following 1M NH₂OH treatment. COS cells were transiently transfected with WT-ABCA1 and Quad-ABCA1 and metabolically labeled with [³H] palmitic acid. Immunoprecipitates of ABCA1 were treated with 1M Tris pH7.5 (Control) or 1M NH₂OH. Radiolabeling was removed from both WT-ABCA1 and Quad-ABCA1 following 1M NH₂OH treatment indicating [³H] palmitic acid remains coupled to Quad-ABCA1 through a thioester linkage to cysteine(s).

2.4 Discussion

We have demonstrated that ABCA1 is palmitoylated, and that the palmitoylation of ABCA1 regulates its localization at the plasma membrane and contributes to its efflux function. The palmitoylation of ABCA1 is mediated primarily by the palmitoyl transferase DHHC8, and increasing DHHC8 protein results in increased ABCA1 mediated cholesterol efflux.

ABCA1 localization at the plasma membrane is considered essential for its ability to transport lipids across membranes (Kang et al., 2010). How ABCA1 is targeted to the cell surface was previously unclear. Palmitoylation regulates the localization of many proteins to specific subcellular locations in a cell. We hypothesized that palmitoylation of ABCA1 was a contributor to plasma membrane targeting. Indeed, ABCA1 is palmitoylated at four cysteines, and we found that palmitoylation of ABCA1 is essential for its proper localization.

Although mutation of each individual cysteine reduced palmitoylation moderately, the mutation of all four cysteine residues reduced almost all ABCA1 palmitoylation. However, the magnitude of reduction in lipid efflux remained the same regardless of the level of reduction in palmitoylation. Similarly, each of the single mutations as well as the quadruple mutation all resulted in a loss of ABCA1 localization at the plasma membrane. One possibility for this is that even a slight disturbance in ABCA1 palmitoylation results in mislocalization and reduced ABCA1 function. Further loss of palmitoylation has no discernable effect on ABCA1 localization and activity. Thus, ABCA1 palmitoylation can be thought of as a binary switch rather than a fine-tuning mechanism that can modulate ABCA1 localization in increments.

Regardless of almost absent ABCA1 at the plasma membrane, a reduction in lipid efflux of only about 50% is observed. Thus our study provides further evidence for the fact that in addition to the direct lipidation of ApoA-I by ABCA1 at the plasma membrane, ABCA1 also contributes to the lipidation of ApoA-I using intracellular lipid pools. Indeed, as in the study using PEST-domain deleted ABCA1, which

showed that ABCA1 at the plasma membrane contributes to ~50% lipid efflux (Chen et al., 2005), our study also finds that removing plasma membrane ABCA1 reduces lipid efflux by ~50%.

Our findings also indicate that palmitoylation of ABCA1 is essential for its localization to the plasma membrane and not for its localization to intracellular sites. As controls for our efflux assays, we used cell lines harboring naturally occurring TD and FHA patient mutations. The subset of patient mutation containing cells that were chosen were previously characterized as loss of function mutants (Singaraja et al., 2006), and showed >80% reduction in efflux. This loss of function can be explained by a number of mechanisms including: the absence of ABCA1 at the plasma membrane; increased degradation of ABCA1; or the inability to bind apo A-1.

ABCA1 is palmitoylated at four different cysteine residues. This in itself is not unusual as many proteins contain multiple sites for palmitoylation. The palmitoylation at different sites on a protein can either signify different functional outcomes depending on which cysteine is modified, or the same functional role regardless of which cysteine is palmitoylated. Examples of each have been described. Regulator of G Protein Signaling 4 (RGS4), a member of a protein family that has GTPase activity protein (GAP) activity is palmitoylated at three cysteine residues (Tu et al., 1999). The addition of palmitate to C2 and C12 are required for RGS4 localization to membrane raft domains. Palmitoylation of the C95 internal residue is required for RGS4 GAP activity. Thus, RGS4 is a protein where palmitoylation at different residues modulates different functions. Palmitoylation of synaptosomal associated protein of 25kDa (SNAP-25) occurs at four sites. Unlike RGS-4, palmitoylation at all four cysteines share the singular aim of increasing membrane association of SNAP-25 (Lane and Liu, 1997). ABCA1 palmitoylation appears to follow the latter model where palmitoylation at all cysteines are required for ABCA1 localization at the plasma membrane.

Of the 23 mammalian DHHC domain containing palmitoyl transferase enzymes that catalyze the addition of palmitate, DHHC8 increased ABCA1 palmitoylation most significantly. DHHC8 transcripts are

expressed ubiquitously (Ohno et al., 2006). Our finding that increasing DHH8 levels increases ABCA1 mediated lipid efflux and suggests that raising ABCA1 palmitoylation through DHH8 activity may be a viable therapeutic strategy for raising plasma HDL-C. In addition, identification of the palmitoyl thioesterase enzyme that removes palmitate from ABCA1 would present as a novel therapeutic target to inhibit in order to maintain palmitate on ABCA1. Most studies to date have focused on therapeutics that increase ABCA1 transcriptionally, but have been fraught with difficulty due to deleterious off-target side effects by transcriptional agonists. Thus, post-translational modifications such as increasing ABCA1 palmitoylation represents a potential new target for therapeutic development.

A drawback to our findings is the possibility that the mutations of the cysteines-to-serines result in altered conformation, causing changes in ABCA1 function independent of palmitoylation. This possibility is unlikely, firstly, because treatment of ABCA1 expressing cells with 2-bromo palmitate, the non-functional analogue of palmitate also resulted in decreased efflux ostensibly without altering ABCA1 conformation. Secondly, each of the mutants still retains ~50% efflux activity, including the quadruple mutant, which would be expected to show very little efflux resulting from the most severe disruption of its 3-dimensional structure. Thirdly, trypsin sensitivity studies show a similar digest pattern between WT-ABCA1 and each of the palmitoylation mutants. The enzymatic cleavage of proteins can provide information about the sequence, amino acid composition, or structural conformation of a protein (Alberts et al., 2002). Trypsin is an enzyme that cleaves on the carboxyl side of lysine or arginine residues. Following trypsin digestion, the separation of peptides electrophoretically or chromatographically results in a unique pattern for each protein. By limiting the duration of trypsin exposure, information about the conformation of a protein can be obtained. A short exposure to trypsin results in cleavage at lysine or arginine sites that are only immediately accessible to the enzyme. When the natural conformation of a protein is altered, accessible cleavage sites can change resulting in different electrophoretic or chromatographic peptide patterns. Limited trypsin digestion of ABCA1

produces two major peptide fragments, a larger 155-165 kDa fragment and a smaller 110-120 kDa fragment observed by Western blotting using an antibody to the C-terminus of ABCA1 (Takahashi et al., 2006). Limited tryptic digests of naturally occurring ABCA1 mutations can result in changes to peptide fragment patterns. For example, the C1477R mutation results in proper localization of ABCA1 to the cell surface, but a complete loss of cholesterol efflux activity (Singaraja et al., 2006). This is due to changes in conformation of C1477R-ABCA1 which inhibits apo A-1 binding. Partial tryptic digestion of C1477R-ABCA1 results in a different peptide banding pattern from WT-ABCA1 (Hozoji et al., 2009). These results suggest that the cysteine mutations do not induce an altered conformation from WT-ABCA1. However, a definitive study to address this issue would be to compare ATPase activity of the palmitoylation mutants with WT-ABCA1. Similar conformations would manifest as equal ATPase activities.

There exists the possibility that we have not identified all the sites of palmitoylation on ABCA1. Treatment of the C3S/C23S/C1110S/C1111S-ABCA1 mutant with hydroxylamine further reduces [^3H] palmitate labeling from ABCA1. This suggests that other cysteines in ABCA1 may undergo metabolic labeling through thioester linkages. No canonical consensus sequence exists for palmitoylation (Xue et al., 2006). However the examination of palmitoylation sites in other proteins reveals recurring patterns. For example, many cytoplasmic cysteine residues proximal to membrane spanning regions are palmitoylated (Greaves et al., 2009). In ABCA1 the palmitoylated residues C3 and C23 are only 22 and 2 amino acids away from the membrane spanning segment respectively. A number of other cytoplasmic cysteine residues in ABCA1 are potential candidates for palmitoylation. Cysteine 860 is 17 amino acids away, cysteine 887 is 44 amino acids away, and cysteine 1281 is 67 amino acids away from the nearest membrane spanning segments. Neighboring amino acids can also influence whether a particular cysteine is palmitoylated or not. Palmitoylation sites are often enriched in leucine (L) and cysteine (C) residues (Xue et al., 2006). Non-polar aliphatic amino acids such as L, valine (V), isoleucine (I), and proline (P) often surrounds palmitoylated cysteines. Proteins such as ABCA1 (LCCV), PSD-95 (LCIV), the G

protein of VSV (LCI), MHV (LCI), and the prostacyclin receptor (VCCLCL) are examples of proteins with palmitoylated cysteine residues containing neighboring non-polar aliphatic amino acids. In ABCA1, the cysteine residues at 860 (PCT), 887 (ICM), and 1281 (SCL) have at least one non-polar aliphatic amino acid neighbor and are potential candidates for palmitoylation.

Together, our findings show that ABCA1 is robustly palmitoylated at cysteines 3,23, 1110 and 1111, and that this palmitoylation is modulated by the palmitoyl transferase DHHC8. We also find that palmitoylation of ABCA1 is essential for its proper plasma membrane localization and efflux function. How ABCA1 transport is regulated was previously unknown. We have shown here that palmitoylation is essential to this process.

3 ABCA1 Palmitoylation and Its Relationship to ABCA1 Biology

3.1 Synopsis

We have identified palmitoylation as a key post-translational modification that regulates ABCA1 localization and function. However, there are major gaps in our understanding of ABCA1 palmitoylation and its relationship to known aspects of ABCA1 biology, including the mechanism of how palmitoylation localizes ABCA1 to the cell surface. Palmitoylation can increase solubility of proteins into cholesterol-rich membrane domains (Melkonian et al., 1999). Many groups have positively identified ABCA1 as a resident of flotillin-1 positive detergent resistant membrane (DRM) domains (Nagao et al., 2011). Whether palmitoylation influences ABCA1 residence in DRM domains at the plasma membrane is unknown.

A number of processes are involved in the localization of ABCA1 to the cell surface. The lipid signaling molecule ceramide has been shown to increase ABCA1 efflux function by increasing levels of ABCA1 at the plasma membrane (Witting et al., 2003) through an unidentified protein-mediated pathway (Ghering and Davidson, 2006). More recently, the GTPase Rab8a has been demonstrated to increase ABCA1 efflux activity by increasing cell surface levels of ABCA1 (Linder et al., 2009). We hypothesize that ceramide or Rab8a-mediated increase in cell surface ABCA1 is through palmitoylation.

ABCA1 is post-translationally modified by glycosylation (Singaraja et al., 2006). The addition of sugars to proteins is important for a number of functions but the precise role of this oligosaccharide modification to ABCA1 is unclear. In other proteins, glycosylation plays numerous and varied roles including involvement in proper protein folding, providing protein stability, and influencing protein trafficking. Previous studies on the cysteine-rich secreted Wnt proteins demonstrated that they are modified by both palmitoylation and glycosylation. Wnt-3a requires initial glycosylation followed by palmitoylation in order to be secreted as an active protein (Komekado et al., 2007). Our preliminary

results using an N-terminal fragment of ABCA1 suggests a relationship between the oligosaccharide moiety attached to ABCA1 and the cysteine residue which undergoes palmitoylation.

Palmitoyl acyl transferases (PATs) catalyse the addition of palmitate to proteins, while protein thioesterases (PTEs) mediate the removal of palmitate from proteins. The identification and characterization of the enzymes that regulate ABCA1 palmitoylation would provide novel targets to regulate ABCA1 activity. DHHC8 along with several other enzymes were identified as increasing the incorporation of [^3H] palmitate onto ABCA1 (Singaraja et al., 2009). Increasing DHHC8 expression not only increases palmitate labeling of ABCA1, but significantly increases cholesterol efflux activity. However, the importance of endogenous DHHC8 to ABCA1 function has not been investigated. Using siRNA targeting of DHHC8, we examined the effects of endogenous DHHC8 depletion on ABCA1 function.

Despite an exhaustive search for the enzymes that depalmitoylate proteins, only three PTEs have been identified: acyl-protein thioesterase 1 (APT1), acyl-protein thioesterase 2 (APT2), and palmitoyl-protein thioesterase 1 (PPT1) (Zeidman et al., 2009). The inhibition of the PTE involved in the removal of palmitate from ABCA1 represents a potential strategy to increase ABCA1 function. Using a candidate approach, we investigated whether APT1 mediates deacylation of ABCA1.

No naturally occurring mutations have been associated with the four palmitoylated cysteine residues of ABCA1. However, alterations to palmitoylation are associated with many disease processes (Resh, 2012) and ABCA1 mutations found in TD patients may induce phenotypic alterations through a reduction in palmitoylation. To answer this we investigated whether select naturally occurring ABCA1 mutations have diminished palmitoylation labeling of ABCA1.

3.2 Materials and Methods

3.2.1 Protein Isolation and Antibodies

Cells were lysed in lysis buffer (10mM Tris pH8.0, 1% Triton X-100, Complete Protease Inhibitors [Roche]). 30-100µg of protein were separated on 6-10% acrylamide gels, depending on the size of the proteins and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). ABCA1 was immunodetected using an anti-ABCA1 monoclonal antibody generated to its C-terminus (Wellington et al., 2002). In addition, anti-transferrin receptor (Zymed), anti-flotillin (BD Biosciences), anti-Rab8a (BD Biosciences), anti-myc (in-house), anti-FLAG (in-house), mouse anti-GFP (kind gift from Dr Luc Berthiaume), rabbit anti-GFP (kind gift from Dr. Luc Berthiaume, University of Alberta) primary antibodies, and anti-mouse and anti-rabbit HRP conjugated secondary antibodies (BioRad, Jackson IR Laboratories) were used for immunoblotting.

3.2.2 Cell Culture

COS and HEK293 cells were maintained in DMEM (Gibco) with 10% fetal bovine serum, 4mM L-Glutamine, 100 units/ml penicillin, and 100µg/ml streptomycin. HEK293 stable cell lines were maintained in DMEM as above with the addition of 100µg/ml of Hygromycin B. Stable cell lines were generated as described in 2.2.2. All cell lines were maintained at 37°C with a humidified 5% CO₂ atmosphere.

3.2.3 Isolation of Detergent Resistant Membranes

Whole tissues or stable cell lines were washed in ice-cold 1X PBS. Five mls of homogenizing buffer (150mM NaCl, 20mM Na₂HPO₄, 2mM NaH₂PO₄, 20% glycerol, 2 mM sodium orthovanadate, protease inhibitors [Roche]) was added to the tissues or cell pellets and lysed using 30 passes in a 7mL Dounce homogenizer (Kontes) followed by sonication (Fisher) on ice 6 times for 30secs at 30% of maximum power. The nuclei and cellular debris were sedimented by centrifugation for 11min at 10K rpm at 4°C using a SW 41 Ti rotor (Beckman). The supernatant was subjected to 32.5K rpm for 90mins at

4°C to isolate the total membrane fraction. The supernatant was discarded and the pelleted total membrane was solubilized in 2mls of solubilizing buffer (0.5% Triton X-100, 25mM MES (Sigma) pH6.5/0.15M NaCl, 2mM sodium orthovanadate, protease inhibitors). The membranes were disrupted by 10 passes through an 18 gauge needle followed by 10 passes through a 21 gauge needle and allowed to solubilize for 15mins on ice. Protein content of the solubilized membranes was determined using a BCA Protein Assay Kit (Pierce). Equal concentrations of solubilized membranes were diluted with an equal volume of 80% sucrose and loaded into the bottom of a 14 X 89mm Ultra-Clear Centrifuge tube (Beckman). Four mls of 30% sucrose was layered on top of the sample and four mls of 5% sucrose constituted the top layer. The gradient was subjected to 35K rpm for 16 hrs at 4°C. Following centrifugation 1mL fractions were harvested from the top to the bottom of the gradient. The detergent insoluble pellet was resuspended in 25mM Mes pH6.5/0.15M NaCl buffer.

3.2.4 Rab8a Interference and Cholesterol Efflux Activity

Control and Rab8a-specific small interfering RNAs (siRNA) (Sigma) were a kind gift from Dr Elina Ikonen. siRNA targeting of Rab8a was carried out as described (Linder et al., 2009). Briefly, stable cell lines expressing either WT-ABCA1 or C3S/C23S/C1110S/C1111S-ABCA1 were seeded into 24 well plates. A total of 15pmol/well of siRNA was transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen). After 32hrs, cells were loaded with 1 μ Ci/ml of [³H] cholesterol and incubated for a further 16hrs upon which cholesterol efflux assays were carried out as described in 2.2.8.

3.2.5 Ceramide Treatment and Cholesterol Efflux Activity

Stable cell lines expressing C3S/C23S/C1110S/C1111S- or M1091T-ABCA1 were seeded into 24 well plates. Cells were loaded with 1 μ Ci/ml of [³H] cholesterol and incubated at 37°C. After 16hrs, cells were treated with or without 10 μ g/ml apo A-I, and with or without 20 μ M C₂-ceramide (D-erythro-Sphingosine, N-Acetyl-; Calbiochem) for a further 24hrs as described (Witting et al., 2003). Cholesterol efflux assays were carried out as described in 2.2.8.

3.2.6 APT1 Expression and Cholesterol Efflux Activity

Stable cell lines expressing WT- or C3S/C23S/C1110S/C1111S-ABCA1 were seeded into 24 well plates. Cells were transiently transfected with either APT1-FLAG construct or empty vector (pCINeo) overnight. After 24hrs, the cells were loaded with 1 μ Ci/ml of [3 H] cholesterol and incubated at 37°C. After 16hrs, cholesterol efflux assays were carried out as described in 2.2.8.

3.2.7 Glycosylation and Palmitoylation

Transiently transfected HEK293 cells were metabolically labeled with [3 H] palmitic acid as described in 2.2.5. Immunoprecipitated N-terminal ABCA1 from metabolically labeled cells were treated with either 1000 Units of PNGaseF (NEB) at 37°C overnight, or 1000 Units of EndoH (NEB) at 37°C overnight according to the manufacturer's instructions. Proteins were separated by SDS-PAGE on 7.5% gels and examined by Western blotting or [3 H] fluorography.

3.2.8 RT-PCR Analysis of DHHC8

RNA was isolated from fresh cells using the RNeasy® kit according to the manufacturer's instructions (Qiagen). Isolated RNA was quantified using a Pharmacia Ultrospec 3000 spectrophotometer, and 5 μ g of isolated RNA was used for reverse transcription using the Superscript™ First-Strand Synthesis system (Invitrogen). DHHC8 was amplified using the forward primer DHHC8-F2 (ATCATGTGGCCCTGCAGCCCCTGCG) and the reverse primer DHHC8-R1 (TTCACACCGAGATCTCGTAGGTGGTC) as previously described (Ohno et al., 2006). The PCR cycling conditions were 95°C for 5mins followed by 25 cycles of 94°C for 30secs, 65°C for 45secs, 72°C for 1min, and concluded with a 72°C extension for 10mins. The 18S rRNA was detected using the Classic 18S Internal Standards kit (Ambion) according to the manufacturer's instructions. The 18S competitor-to-primer ratio was 1:9. All amplified products were separated on a 2% agarose gel and densitometric quantification was performed using BioRad GelDoc100 and Quantity1™ software (BioRad). The relative abundance of DHHC8 transcripts were expressed as the ratio of quantified DHHC8 to 18S.

3.2.9 DHHC8 Interference

WT-ABCA1 stable cell lines were seeded into 24 well plates. A total of 30pmol/well of DHHC8-specific Stealth™ siRNA or control siRNA (Invitrogen) were transfected into cells for 48hrs using Lipofectamine RNAiMAX reagent (Invitrogen). After 48hrs, cells were loaded with 1μCi/ml [³H] cholesterol and incubated for a further 16hrs upon which cholesterol efflux assays were carried out as described in 2.2.8. After 48hrs of siRNA treatment, analysis of [³H] palmitate incorporation was carried out as described in 2.2.5.

3.2.10 Pulse-Chase Experiments

COS cells were transiently transfected with PSD-95-GFP or SNAP-25-GFP overnight. After 24hrs transfected cells were labeled for 3hrs in 1% bovine serum albumin (BSA) + DMEM medium containing 1mCi/ml [³H] palmitic acid. Following labeling, cells were incubated for 0, 30mins, 1hr, 2hrs, 3hrs, and 6hrs with 100μM unlabeled palmitic acid (Sigma). The cells were processed as described in 2.2.5. ABCA1 was co-transfected with either APT1-FLAG or an empty vector (pCINeo) into COS cells overnight. After 24hrs, transfected cells were labeled for 3hrs in 1% BSA + DMEM medium containing 1mCi/ml [³H] palmitic acid. Following labeling, cells were incubated for 0, 15mins, 30mins, 45mins, 1hr, and 2hrs with 100μM unlabeled palmitic acid (Sigma). The cells were processed as described in 2.2.5.

3.2.11 Statistics

All statistical analyses were performed using the two-tailed student's t test using GraphPad Prism (GraphPad Software). Results are plotted as percentage ± standard deviation.

3.3 Results

3.3.1 ABCA1 Palmitoylation and Detergent Resistant Membrane Targeting

Detergent resistant membrane (DRM) domains or rafts are 10-200nm cholesterol and sphingolipid enriched regions on the cell surface that compartmentalize cellular processes (Pike, 2009). Proteins such

as flotillin-1 or caveolin-1 are residents in these domains and these proteins are often used as positive markers for rafts. Different detergents can solubilize certain raft domains but not others and are thought to represent the heterogeneous population of DRM domains that exist in the plasma membrane. Using Triton X-100 and ultra-centrifugation (**Figure 3.1**) we isolated ABCA1 in the DRM domains of whole mouse brain, RAW Macrophages, and HepG2 cells (**Figures 3.2.A-C**). Flotillin-1 was used as a positive control, while transferrin-1 is a cell surface protein that resides outside of raft domains and was our negative control. Although other groups have not observed ABCA1 in Triton X-100 domains, they have found ABCA1 in flotillin-1 positive fractions (Nagao et al., 2011) which agree with our findings.

Palmitoylation is thought to be a DRM targeting signal and previous studies have shown that more than half of the proteins present in the DRMs of Madin-Darby canine kidney cells are potentially palmitoylated (Melkonian et al., 1999). To examine whether palmitoylation targets ABCA1 to DRMs, we performed Triton X-100 DRM isolation on HEK293 stable cell lines expressing various ABCA1 palmitoylation mutants. The ABCA1 palmitoylation mutants were mostly absent from the DRM fraction while WT-ABCA1 was present in the insoluble raft domains (**Figures 3.3.A-D**).

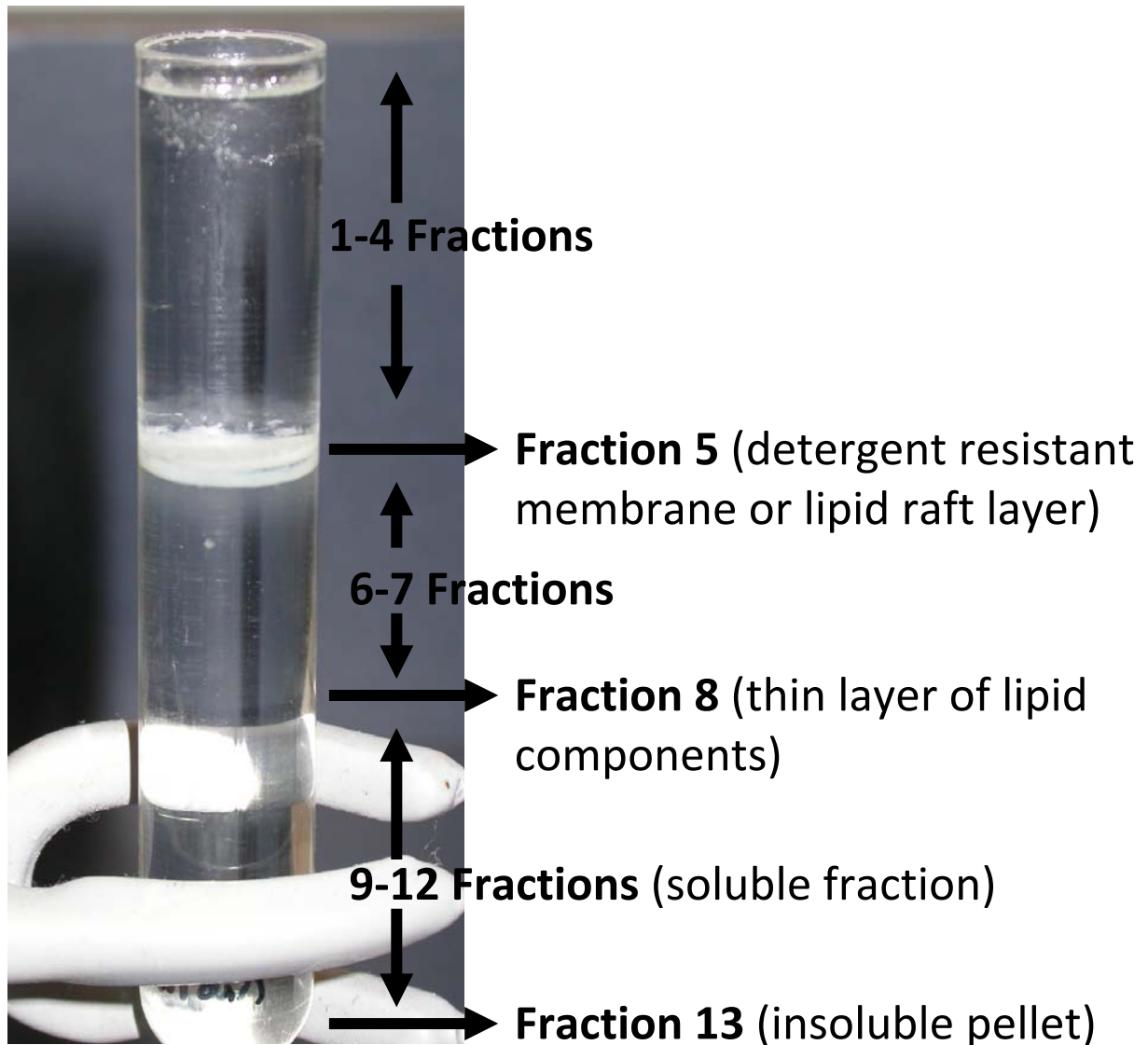


Figure 3.1. Photograph of TritonX-100 detergent resistant membrane isolation using ultra-centrifugation separation on a 5-40% sucrose gradient. This isolation is of a wild-type whole mouse brain.

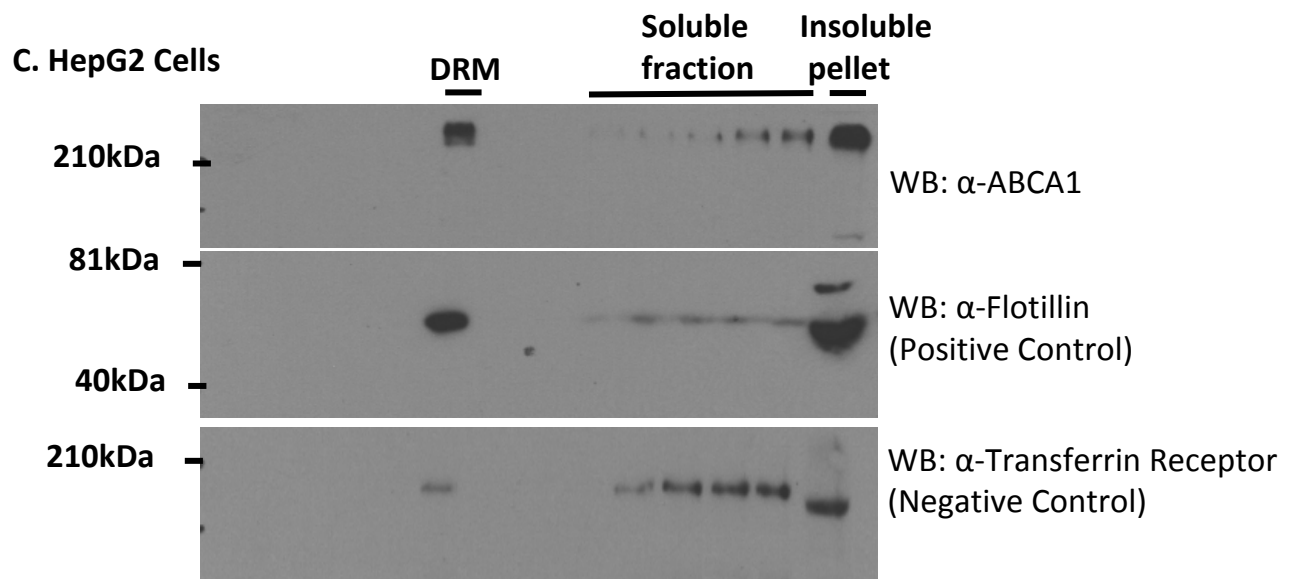
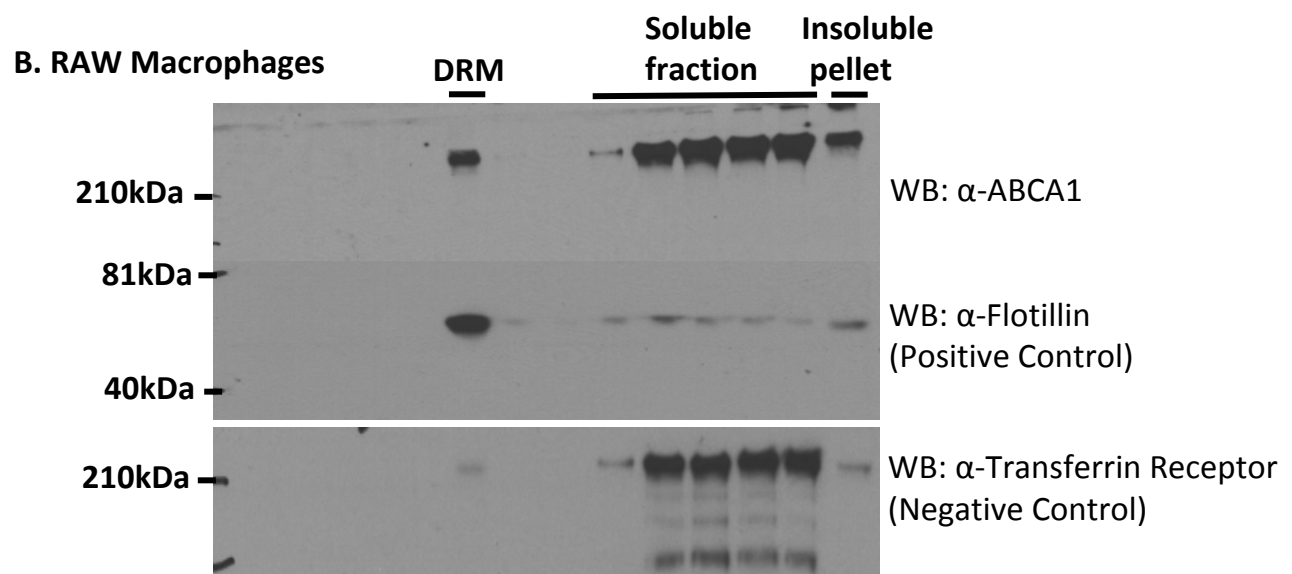
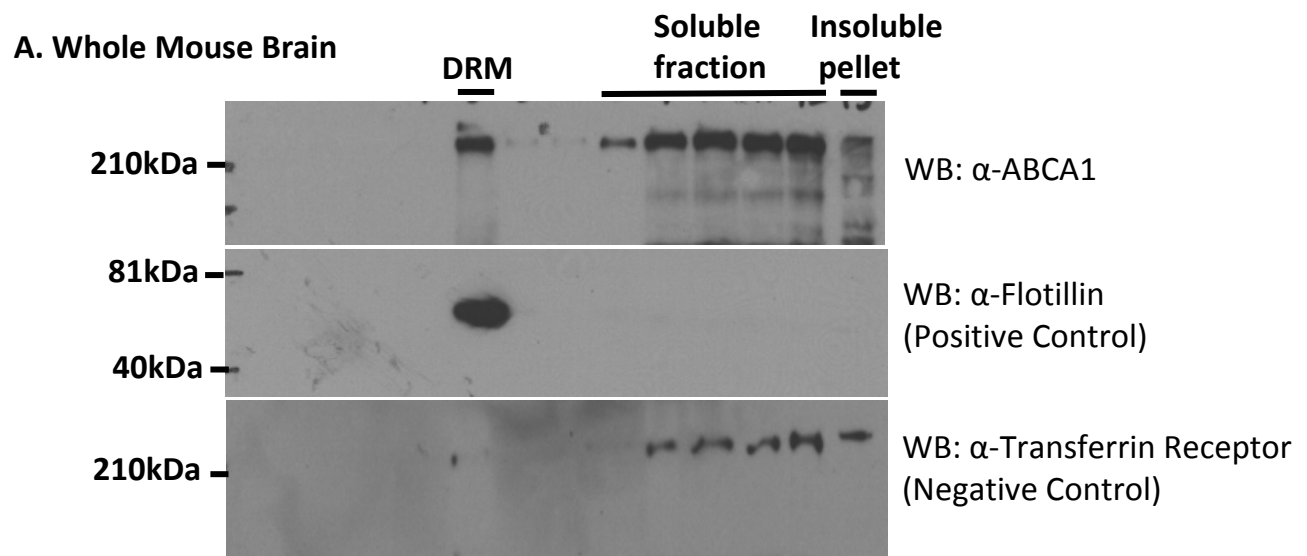


Figure 3.2. Distribution of ABCA1 in Triton X-100 detergent resistant membrane (DRM) and soluble membrane fractions. Membranes isolated from (A) wild-type mouse brain, (B) RAW macrophages, and (C) HepG2 cells were subject to sonication, Triton X-100 solubilization at 4°C, and separation on a 5-40% sucrose gradient. To induce ABCA1 protein expression, HepG2 cells were stimulated for 24 hours with 1µM 9-cis-RA and 1µM T-0901317 in DMEM+10%FBS. Western blots reveal ABCA1 localization to DRMs in all samples tested. Membrane fractions were also immunoblotted for the marker proteins Flotillin (insoluble in Triton X-100) and Transferrin receptor (soluble in Triton X-100).

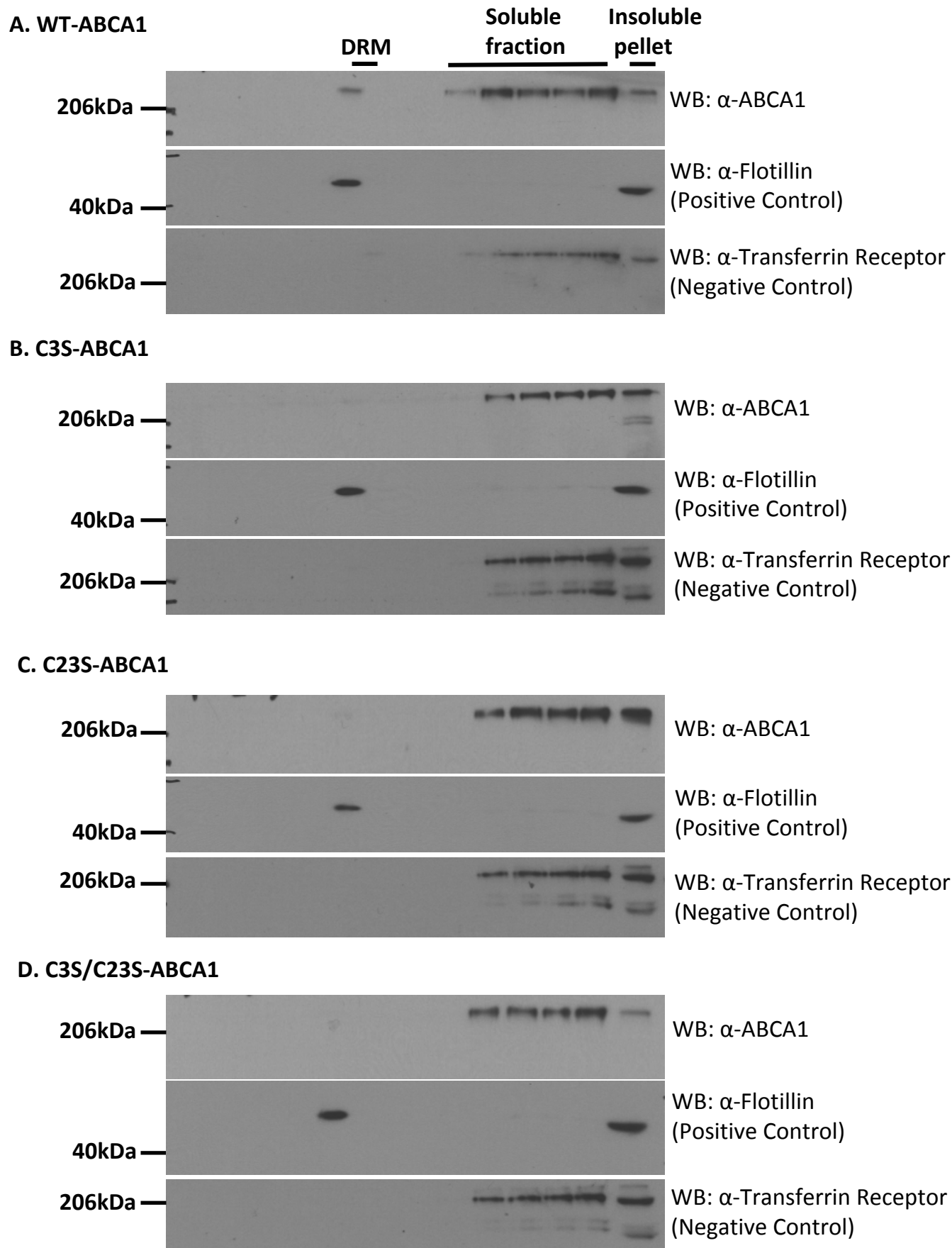


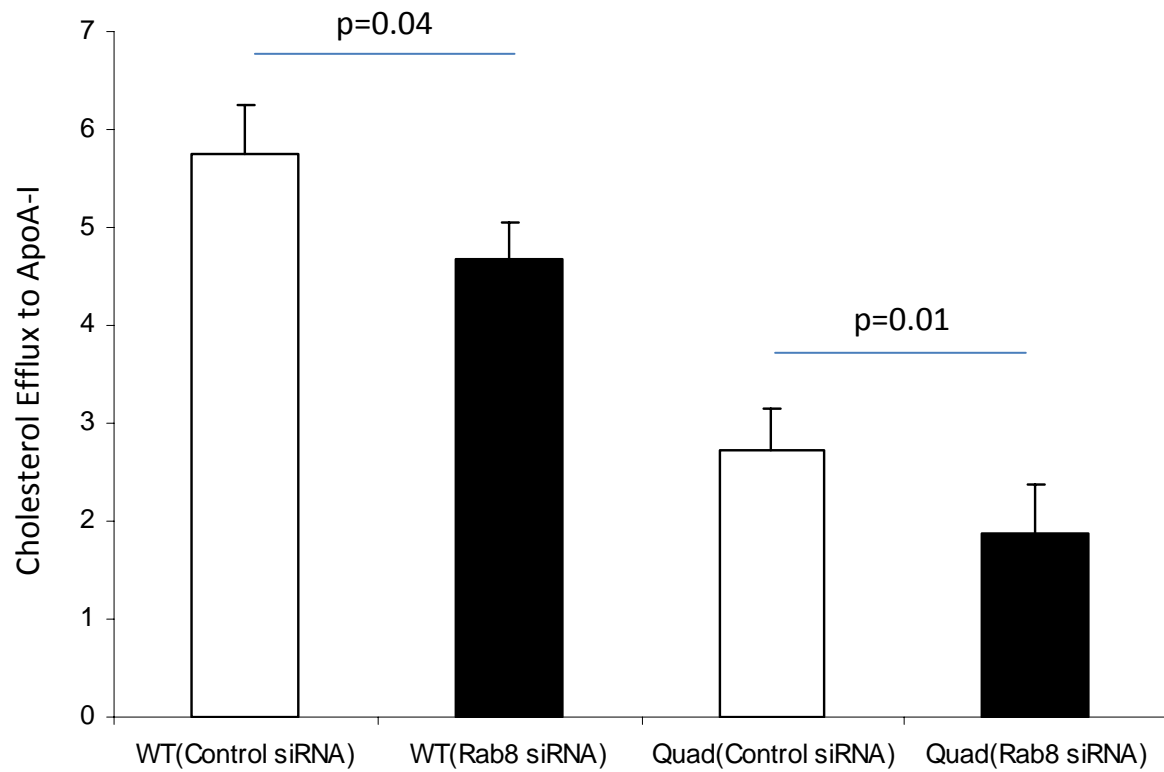
Figure 3.3. Distribution of ABCA1 palmitoylation mutants in Triton X-100 detergent resistant membrane (DRM) and soluble membrane fractions. Membranes isolated from HEK-293 stable cell lines expressing (A) wild-type ABCA1, (B) C3S-ABCA1, (C) C23S-ABCA1, and (D) C3S/C23S-ABCA1 were subject to sonication, Triton X-100 solubilization at 4°C, and separation on a 5-40% sucrose gradient. Western blots reveal ABCA1 localization to DRMs in WT-ABCA1 only. Membrane fractions were also immunoblotted for the marker proteins Flotillin (insoluble in Triton X-100) and Transferrin receptor (soluble in Triton X-100).

3.3.2 The Mechanism of Rab8a Regulation of ABCA1 Cell Surface Expression is Not Through Palmitoylation

Recently Rab8a, a RAB GTPase that is involved in vesicular transport, has been shown to regulate ABCA1 localization to the plasma membrane (Linder et al., 2009). The mechanism as to how Rab8a increases ABCA1 at the cell surface has not been determined. We examined whether Rab8a increases cell surface ABCA1 through increased palmitoylation.

We depleted endogenous Rab8a from HEK293 stable cell lines expressing WT-ABCA1 and C3S/C23S/C1110S/C1111S-ABCA1 and examined cholesterol efflux activity. A Western blot showed a reduction in Rab8a protein levels following Rab8a siRNA treatment compared to cells treated with Control siRNA (**Figure 3.4.B**). For the cholesterol efflux studies, WT-ABCA1 treated with Rab8a siRNA showed a significant decrease ($p=0.04$) in cholesterol efflux activity (**Figure 3.4.A**) in agreement with previously published data (Linder et al., 2009). The C3S/C23S/C1110S/C1111S-ABCA1 palmitoylation mutant also showed a significant decrease ($p=0.01$) in cholesterol efflux activity (**Figure 3.4.A**). This result suggests that ABCA1 palmitoylation and Rab8a work in separate pathways to regulate ABCA1 cholesterol efflux activity. If Rab8a mediated ABCA1 cell surface localization involved palmitoylation, the C3S/C23S/C1110S/C1111S-mutant treated with Rab8a siRNA should not show a further decrease in cholesterol efflux activity.

A.



B.

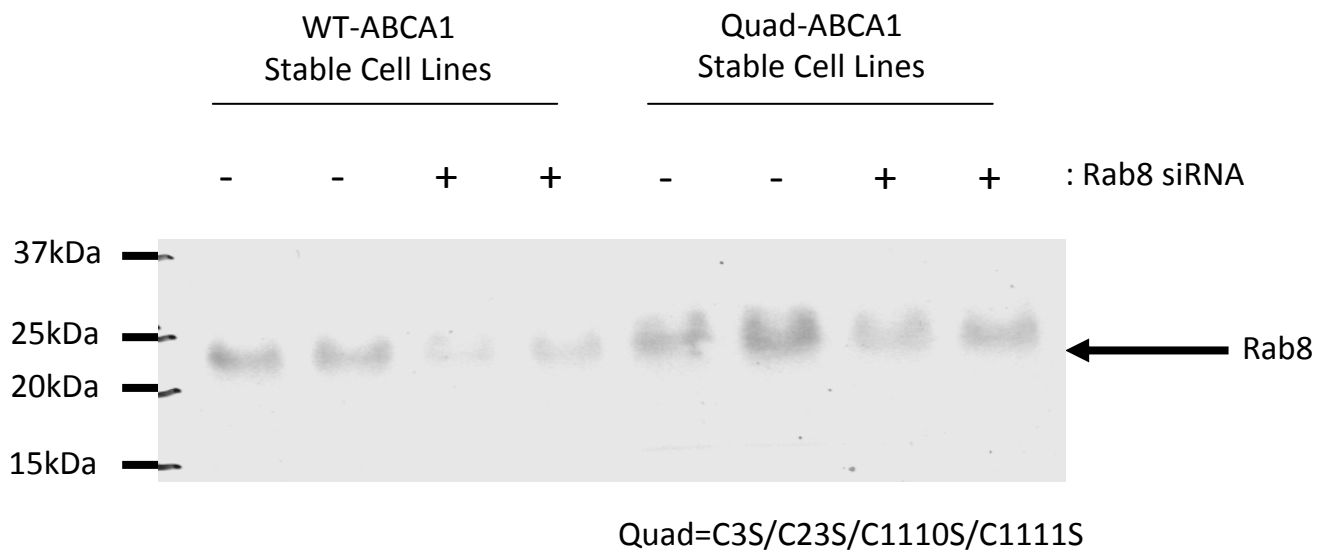


Figure 3.4. Relationship between Rab8a expression and ABCA1 palmitoylation. HEK293 stable cell lines expressing wild-type ABCA1 (WT) or C3S/C23S/C1110S/C1111S ABCA1 (Quad) were treated with siRNA targeting Rab8a or control siRNA. (A) Efflux of [³H] cholesterol to 10μg/ml apo A-I was measured after 4 hrs. The error bars represent standard deviation from measurements made in triplicate. The p value was calculated by Student's t test. (B) Western blot of Rab8a in stable cell lines treated with siRNA targeting Rab8a or control siRNA.

3.3.3 The Mechanism of Ceramide-Induced Increase in ABCA1 Cell Surface Expression is Not Through Palmitoylation

Ceramide is a component of sphingomyelin. Like cholesterol, sphingomyelin exists primarily at the plasma membrane and secondarily at endosomes and lysosomes. The digestion of sphingomyelin by sphingomyelinases yields ceramide, which is a potent lipid signaling molecule associated with regulating a variety of cellular events including cell growth, differentiation and stress responses (Witting et al., 2003). Ceramide treatment of cells has been shown to increase cholesterol efflux activity by increasing ABCA1 levels at the cell surface (Witting et al., 2003). The mechanism as to how ceramide does this is unclear but is thought to involve a protein mediated pathway (Ghering and Davidson, 2006). We examined whether ceramide increases cell surface ABCA1 through increased palmitoylation.

C3S/C23S/C1110S/C1111S-ABCA1 stable cell lines were loaded with [^3H] cholesterol for 16hrs before being treated with 20 μM ceramide and 10 $\mu\text{g/ml}$ apo A-I for a further 24hrs. Cells were treated with C₂-ceramide, a ceramide analog. A significant increase ($p=0.02$) in cholesterol efflux activity was observed in ABCA1 palmitoylation mutant expressing cells following treatment (**Figure 3.5.A**). In comparison, the naturally occurring M1091T-ABCA1 mutation showed no change in efflux activity following 20 μM ceramide treatment. Western blot analysis of C3S/C23S/C1110S/C1111S-ABCA1 with and without ceramide exposure shows no change to ABCA1 protein levels suggesting the increase in cholesterol efflux activity is a result of increased ABCA1 cell surface expression as previously published (**Figure 3.5.B**) (Witting et al., 2003). These results suggest that ABCA1 palmitoylation and ceramide work in separate pathways to increase ABCA1 cholesterol efflux activity. If ceramide increases cell surface ABCA1 through palmitoylation, the C3S/C23S/C1110S/C1111S-mutant would not show an increase in efflux activity following ceramide treatment as this mutant cannot be palmitoylated.

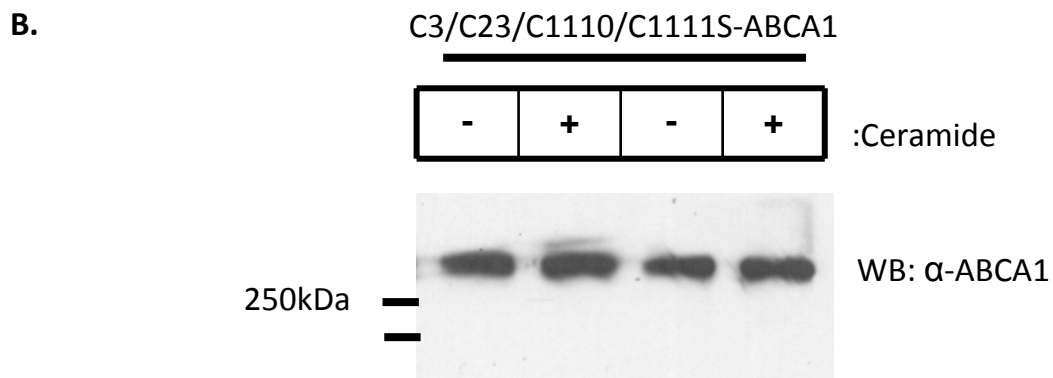
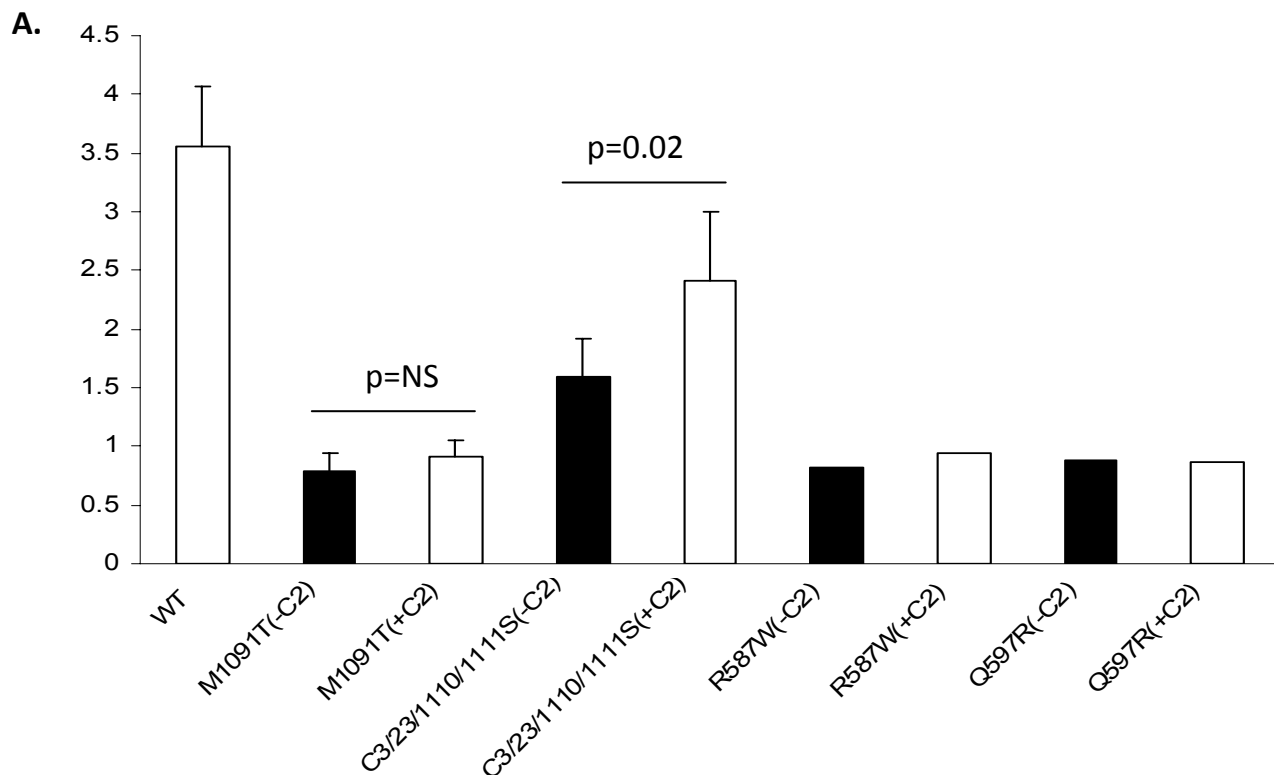


Figure 3.5. Effect of ceramide on cholesterol efflux from HEK293 stable cell lines expressing the ABCA1 palmitoylation mutant. (A) A HEK293 stable cell line expressing C3S/C23S/C1110S/C1111S-ABCA1 were treated with 20μM ceramide or vehicle only. Efflux of [³H] cholesterol to 10μg/ml apo A-I was measured after 24hrs. The error bars represent standard deviation from measurements made in triplicate. The p value was calculated by Student's t test. Stable cell lines expressing ABCA1 with mutations found in Tangier disease patients were also treated with 20μM ceramide.(B) Western blot of C3S/C23S/C1110S/C1111S-ABCA1 with 20μM ceramide or vehicle only.

3.3.4 The Site of Palmitoylation in an N-terminal Fragment of ABCA1 is Predicted by Glycosylation

Status

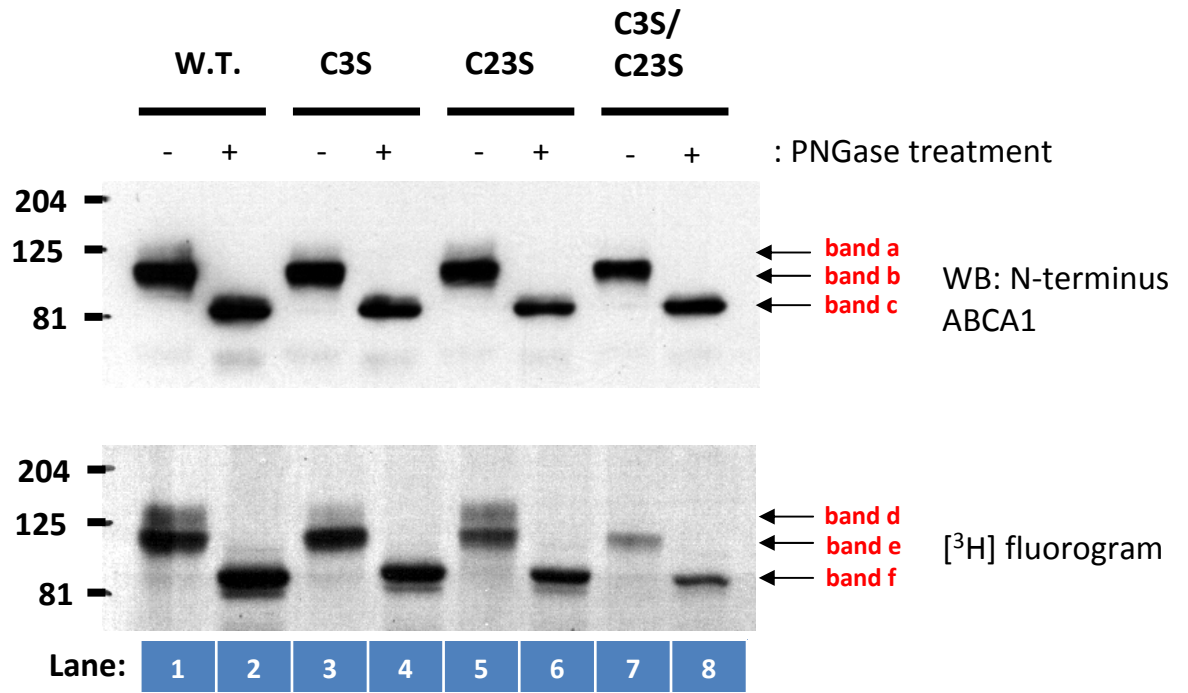
We have shown that ABCA1 is palmitoylated at two cysteine residues (C3 and C23) in an N-terminal fragment of ABCA1 (**Figure 2.2.E**). This fragment comprises the first 639 amino acids of ABCA1 and also contains the first large exocytosomal domain (ECD-1) of ABCA1. ECD-1 contains three asparagine residues (N98, N400, N489) with N-Linked glycosylation consensus sequences (NX(S/T)) (Bungert et al., 2001). Glycosylation is a post-translational modification initiated in the lumen of the endoplasmic reticulum (ER) where a core high-mannose oligosaccharide sugar is added to a newly translated protein. The sugar is trimmed and modified to a complex oligosaccharide by multiple enzymes as the glycoprotein traffics through the ER and Golgi complex (van Vliet et al., 2003). In the Golgi apparatus, a group of enzymes called glycosyltransferases exist in the various sub-compartments of the Golgi to carry out glycan synthesis and modify the original core oligosaccharide added to the protein in the ER (Tu and Banfield, 2010). The distribution of these enzymes in the Golgi reflects the sequential order in which they act on the glycoprotein. A number of clinical relevant mutations of ABCA1 (R587W, Q597R, Δ L693, N935H) acquire only the core and not the complex modified oligosaccharide chain and fails to reach the cell surface (Singaraja et al., 2006).

To differentiate between the core and complex modified forms of the oligosaccharide moieties attached to ABCA1, we subjected the N-terminal fragment to glycosidase activity of either PNGaseF or EndoH digestion. Endoglycosidases are enzymes that can release disaccharide or larger carbohydrate moieties from glycoproteins. EndoH and PNGaseF are two endoglycosidases with different glycosidase activities. PNGaseF demonstrates a much broader specificity than EndoH and can recognize and cleave almost all N-linked oligosaccharides from proteins (Maley et al., 1989). EndoH is more specific in its glycosidase activity and can recognize high mannose sugars added and modified in the ER and up to the

mid-Golgi, but not after the mid-Golgi where Golgi mannosidase II removes two mannose residues to generate mature N-glycan complexes (Ron and Horowitz, 2005).

N-terminal ABCA1 runs as a double band with a faint upper band (**Lane 1, band a, Figure 3.6A**) running just under ~125kDa, and a darker lower band (**Lane 1, band b, Figure 3.6A**) running at ~100kDa. When N-terminal ABCA1 is subject to PNGaseF treatment, both bands lose their oligosaccharide moieties and run at a molecular weight of 81kDa (**Lane 2, band c, Figure 3.6.A**). When N-terminal ABCA1 is subject to EndoH treatment, only the core sugar is lost and the dark lower band shifts to 81kDa (**Lane 9, band h to Lane 10, band i, Figure 3.6.B**), while the upper band which corresponds to a fragment with a complex sugar moiety remains at ~125kDa (**Lane 10, band g, Figure 3.6.B**). This suggests there are two populations of N-terminal ABCA1: a large population of N-terminal ABCA1 containing the core sugar that has not yet reached the mid-Golgi, and a smaller population with a sugar moiety modified in the mid-Golgi by Golgi mannosidase II. Previous studies have shown similar findings with full-length ABCA1 where EndoH treatment results in a population of ABCA1 sensitive to this enzyme, as well as ABCA1 containing EndoH resistant complex sugar side chains (Singaraja et al., 2006). Interestingly, the ABCA1 palmitoylation mutants revealed differences in which cysteine residue is palmitoylated depending on whether a core or complex sugar is attached to ABCA1. The dark lower band corresponding to the protein fragment with the core sugar (**Lane 11, band k, Figure 3.6.B**) is palmitoylated at C23 as C3 is mutated to a serine and cannot undergo palmitoylation. The light upper band corresponding to the protein fragment modified with the complex sugar (**Lane 13, band j, Figure 3.6.B**) is palmitoylated at C3 as C23 is mutated to a serine and cannot undergo palmitoylation.

A.



B.

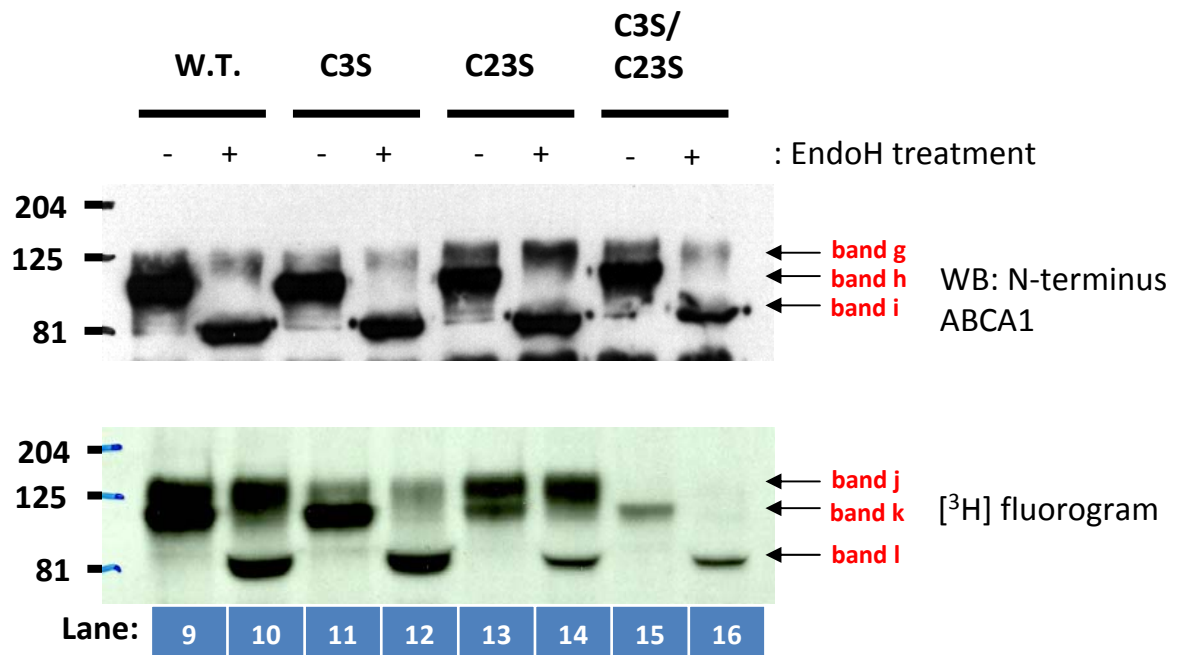


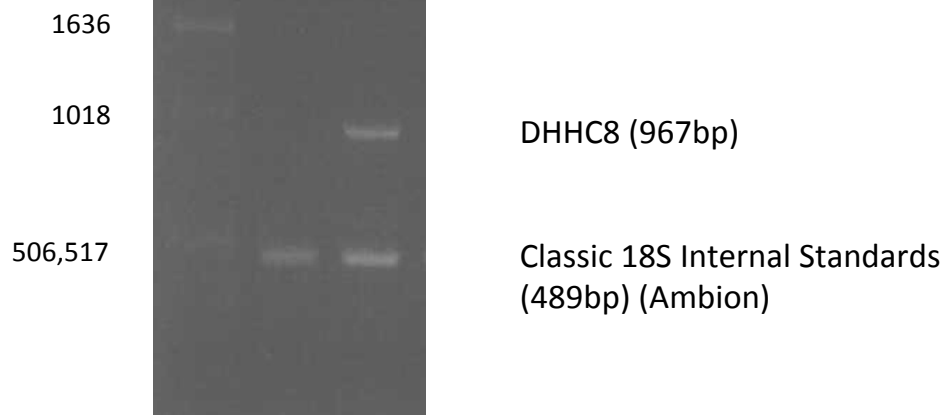
Figure 3.6. The relationship between palmitoylation and glycosylation in an N-terminal fragment of ABCA1 (1st 639 amino acids of full-length ABCA1) (A). N-terminus ABCA1 palmitoylation mutant constructs were treated with PNGase F. PNGase F can remove almost all oligosaccharide moieties, including initial glycosylation of proteins in the ER as well as Golgi complex modified forms. The Western blot indicates that N-terminus ABCA1 is highly glycosylated as observed by the significant bandshift following PNGase treatment (band b to band c in Lanes 1-8). In the Western blot of untreated samples there are two bands (Lane 1), a faint upper band and a dark, highly expressing lower band b. These two bands are much more noticeable in the [³H] fluorogram (bands d and e in Lane 1). Both light and dark bands represents ABCA1 modified with an oligosaccharide moiety. Only a single band remains after PNGase treatment (band c in the Western blot and band f in the fluorogram in Lane 2). **(B).** N-terminus ABCA1 palmitoylation mutant constructs were also treated with Endo H. Endo H can only remove oligosaccharide moieties attached and modified in the ER and up to the mid-Golgi. Any oligosaccharide chains processed beyond the mid-Golgi by Golgi mannosidase II are resistant to Endo H cleavage. Upon Endo H treatment in the Western blot and in the fluorogram, the faint upper band (g and j) remains and does not shift suggesting EndoH does not recognize this modified carbohydrate moiety. Thus, the upper band represents ABCA1 with a mid-Golgi modified oligosaccharide chain. In the [³H] fluorogram the upper band j is palmitoylated on the 3rd cysteine as the C23S mutant still shows radiolabeling. The lower band k is palmitoylated on C23 as the C3S mutant shows strong radiolabel incorporation.

3.3.5 Inhibition of DHHC8 Does Not Reduce ABCA1 Palmitoylation or Cholesterol Efflux Activity

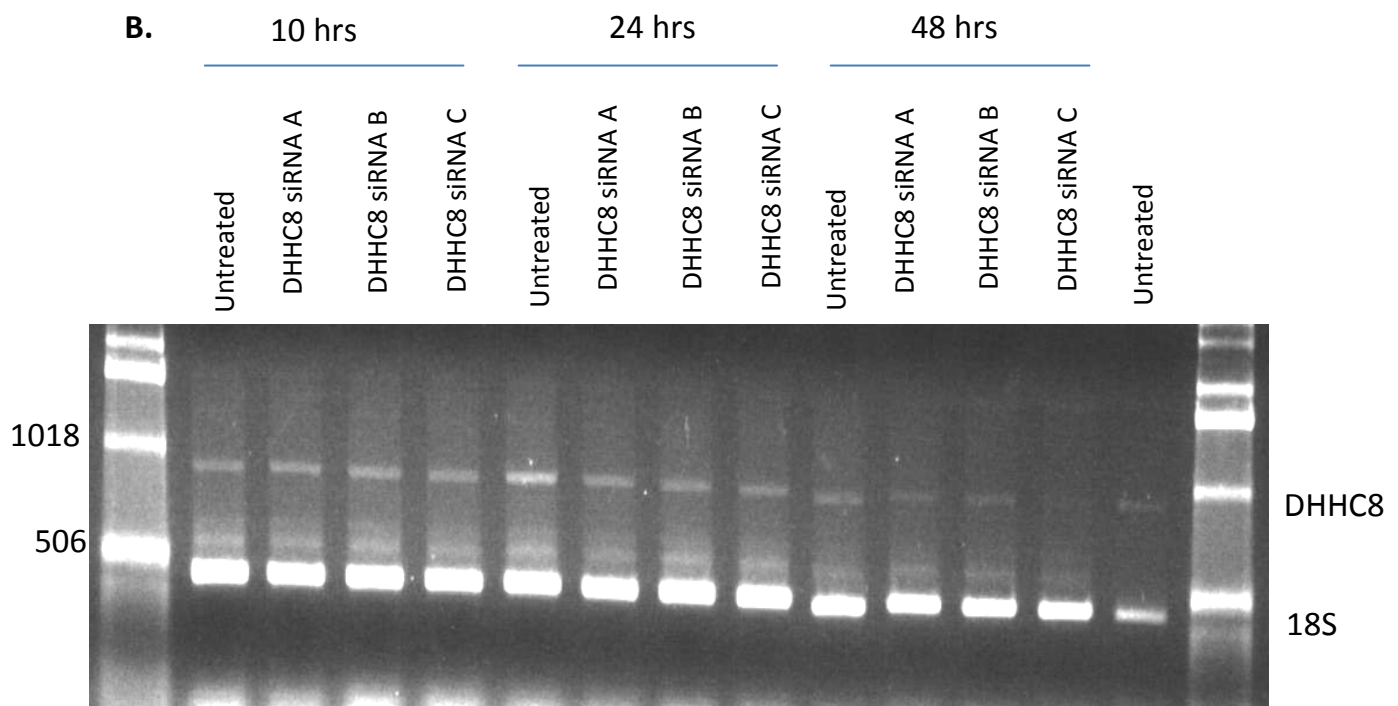
We have identified a number of palmitoyl acyl transferase (PAT) enzymes that increase ABCA1 palmitoylation (**Figure 2.6.A**). Of these, DHHC8 mediated the largest incorporation of [^3H] palmitate to ABCA1, and when DHHC8 was overexpressed in WT-ABCA1 stable cell lines, these cells had an increase in cholesterol efflux activity by ~20% ($p=0.0003$) (**Figure 2.7.A**).

In order to investigate the importance of endogenous DHHC8 to ABCA1 palmitoylation and function, we depleted DHHC8 using siRNA. DHHC8 levels were assessed using RT-PCR (**Figure 3.7.A**) based on primers previously published (Ohno et al., 2006). Treatment of different siRNA sequences targeting DHHC8 revealed one siRNA (DHHC8 siRNA C, Invitrogen) that decreased DHHC8 transcript levels by close to 70% ($p=0.005$) after 48hrs (**Figures 3.7.B and C**). However, the role that endogenous DHHC8 plays in proper ABCA1 function is unclear. [^3H] palmitate incorporation was not diminished following treatment of cells with DHHC8 siRNA C (**Figure 3.8.A**). WT-ABCA1 expressing cells treated with DHHC8 siRNA C showed no difference in cholesterol efflux activity from cells treated with Control siRNA (**Figure 3.8.B**). These findings may be due to a few different reasons. First, the 70% decrease in DHHC8 transcript level following siRNA treatment may not be sufficient to completely abolish activity, and the residual levels of DHHC8 can mediate ABCA1 palmitoylation and allow proper cholesterol efflux function. A second interpretation is that the redundancy of PAT enzymes for ABCA1 compensates for the loss of any single PAT, including DHHC8. Besides DHHC8, we identified DHHC12, -15, -20, and -21 as possible PATs for ABCA1 based on increased [^3H] palmitate incorporation (**Figure 2.6.A**). Other proteins also have multiple enzymes involved in their palmitoylation. Similar co-expression experiments showed that five different PAT enzymes (DHHC2, -3, -7, -8, -21) mediated the incorporation of radio-labeled palmitate onto endothelial nitric oxide synthase (eNOS) (Fernandez-Hernando et al., 2006). Inhibition of endogenous DHHC21 but not DHHC3 inhibited eNOS palmitoylation and function. These results suggest that although the overexpression of a single DHHC protein can increase [^3H] palmitate labeling of a

A. 1kb - + : DHHC8 primers



B. 10 hrs 24 hrs 48 hrs



C.

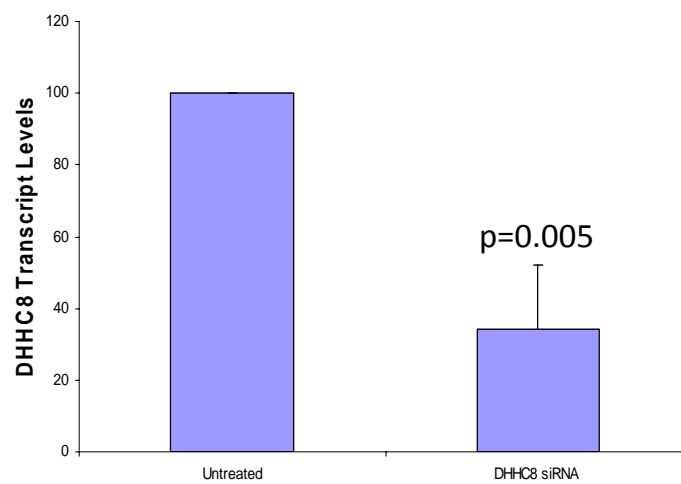


Figure 3.7. Knockdown of endogenous DHHC8 in HEK293 wild-type (WT) ABCA1 expressing stable cell lines. (A) Primers to amplify endogenous DHHC8 RNA transcripts were generated based on Ohno Y et al., (2006) BBA 1761:479-83. The forward primer DHHC8-F2 (ATCATGTGGCCCTGCAGCCCCTGCG) and reverse primer DHHC8-R1 (TTCACACCGAGATCTCGTAGGTGGTC) generates a 967bp band. (B) Invitrogen Stealth™ siRNA primers directed against DHHC8 were transfected into HEK293 WT-ABCA1 expressing stable cell lines. DHHC8 transcript levels were assessed at 10hrs, 24hrs, and 48hrs following siRNA treatment. (C) Quantification of DHHC8 siRNA C mediated knockdown of DHHC8 after 48hrs.

Figure 3.8. Assessment of ABCA1 palmitoylation and cholesterol efflux activity following knockdown of endogenous DHHC8. (A) COS cells transiently transfected with WT-ABCA1 were metabolically labeled with [³H] palmitic acid and treated with either control siRNA or siRNA targeting DHHC8. [³H] palmitic acid incorporations onto ABCA1 was assessed by fluorography. (B) Assessment of cholesterol efflux activity to 10μg/ml of apo A-I following siRNA-mediated suppression of DHHC8.

protein, the inhibition of endogenous DHHC enzyme may not always show a phenotype. The inhibition of each DHHC enzyme mediating ABCA1 palmitoylation should be attempted. Furthermore, if enzyme redundancy occurs in ABCA1 palmitoylation, it may be necessary to inhibit multiple combinations of enzymes. The effect of modulating endogenous PAT activity will provide the most crucial findings on the functional consequences of a particular enzyme on ABCA1 function.

3.3.6 APT1 is Not the Protein Thioesterase for ABCA1

Protein palmitoylation is a reversible process. Whereas other protein lipid modifications such as myristoylation (C14:0) are stable and permanent, the covalent thioester bond that links proteins to palmitate is labile. The removal of palmitate from proteins is thought to be enzymatically catalyzed by protein thioesterases (PTE). Currently, only three enzymes have been identified as PTEs and of these three, only one is validated in catalyzing palmitate removal from a number of different substrates in the cytoplasm, APT1 (Zeidman et al., 2009). Our initial approach in identifying the PTE for ABCA1 was to assess the ability of APT1 to remove palmitate from ABCA1 in a pulse-chase depalmitoylation assay (Duncan and Gilman, 1998).

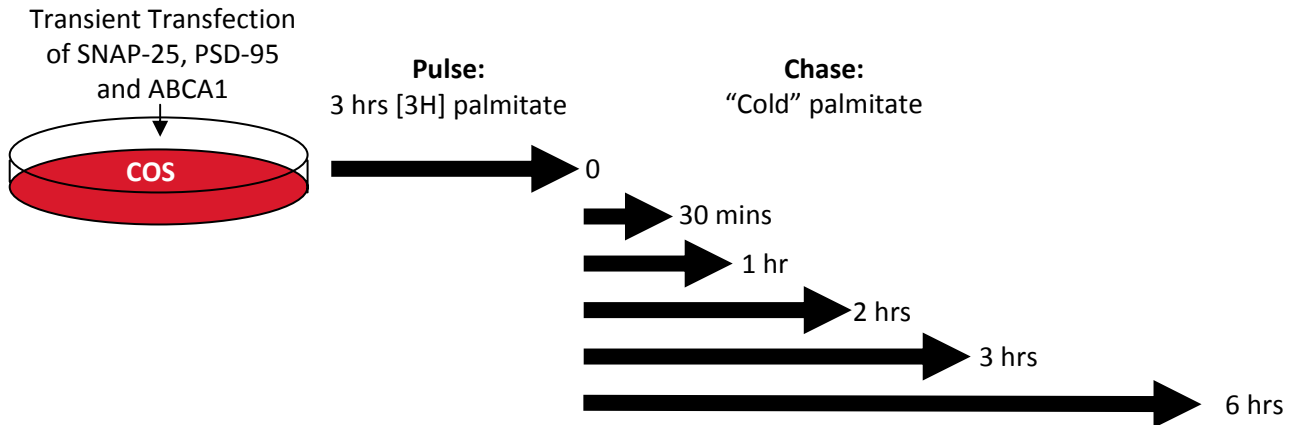
We initially performed pulse-chase studies on the neuronal proteins PSD-95 and SNAP-25 as controls. Transfected COS cells were labeled with [^3H] palmitate for 3hrs then chased with unlabeled palmitate for up to 6hrs (**Figure 3.9.A**). The half-life on the post-synaptic protein PSD-95 was between 3-6hrs, while no detectable change in the amount of SNAP-25 palmitoylation was found up to 6hrs post-chase (**Figure 3.9.B**). These results agree with previously published results (Kang et al., 2004).

To examine whether APT1 is the thioesterase for ABCA1, we co-transfected ABCA1 and either empty vector (pCINeo) or APT1 into COS cells overnight. The next day, the cells were labeled with [^3H] palmitate for 3hrs then chased with unlabeled palmitate for up to 2hrs. ABCA1 was immunoprecipitated and examined for protein expression by Western blotting and [^3H] palmitate labeling was examined by fluorography. The lysates were examined for APT1 expression by Western blot analysis (**Figure 3.10.A**).

We observed no difference in the levels of ABCA1 palmitoylation in the presence of APT1 or empty vector (**Figure 3.10.B**). This experiment was performed twice with equivalent results. These findings suggest APT1 is not the thioesterase for ABCA1. The findings from this study can be further strengthened by employing a negative control (PSD-95) and a positive control (H-Ras, Gα-protein) for APT1 depalmitoylation. A pulse-chase experiment using S^{35} to determine ABCA1 protein half-life should also be performed.

We previously demonstrated that DHHC8 overexpression can increase ABCA1 cholesterol efflux activity (**Figure 2.7.A**). To test whether APT1 expression decreases cholesterol efflux activity, we transfected empty vector (pCINeo) or APT1 into WT-ABCA1 expressing HEK293 stable cell lines and performed a cholesterol efflux assay. No change in ABCA1 cholesterol efflux activity was observed following APT1 expression compared to empty vector control (**Figure 3.11.A**). APT1 protein expression in the transfected cells was verified by Western blotting (**Figure 3.11.B**). These results suggest APT1 has no influence on ABCA1 cholesterol efflux activity.

A.



B.

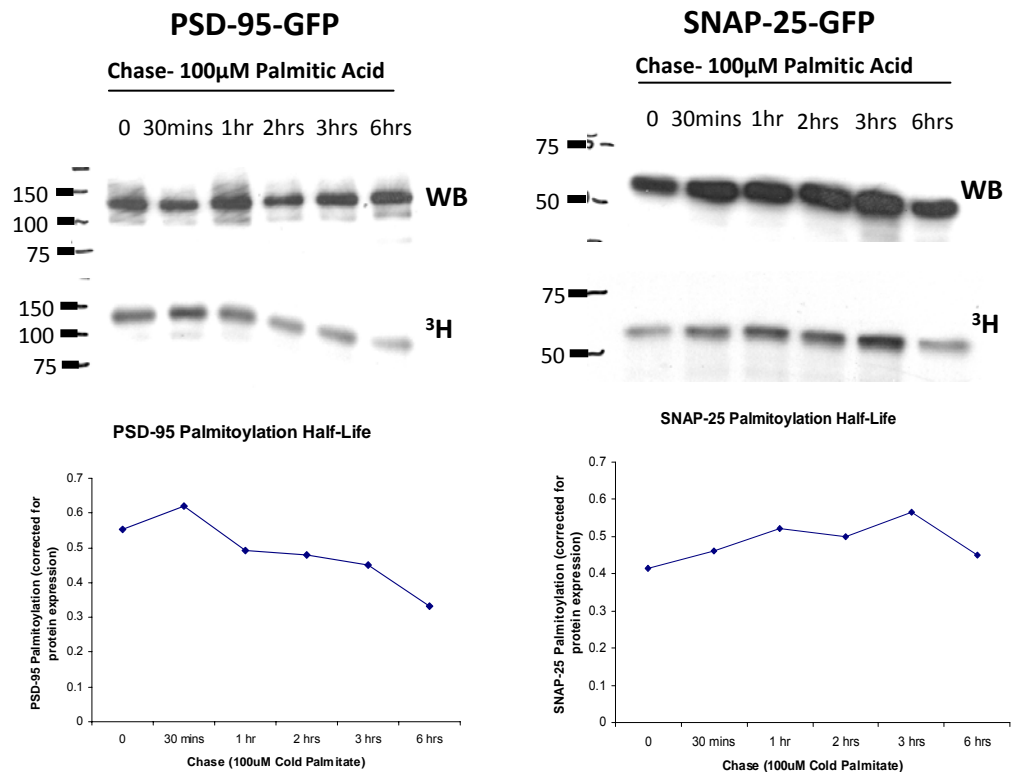


Figure 3.9. Pulse-chase study of SNAP-25 and PSD-95 palmitoylation. **(A).** COS cells transiently transfected with SNAP-25 (negative control), or PSD-95 (positive control) were labeled with [³H]palmitate for 3hrs and then chased for 0, 30mins, 1hr, 2hrs, 3hrs, and 6hrs with unlabeled palmitate. **(B).** PSD-95, and SNAP-25 were immunoprecipitated and subjected to SDS-PAGE and fluorography. Data show rapid reduction in the amount of palmitoylated PSD-95 within 1hr. In contrast, no change in the amount of palmitoylated SNAP-25 was observed during the 6hr chase period. These findings match previously published results (For details see Kang et al., JBC (2004) 279:50524-36).

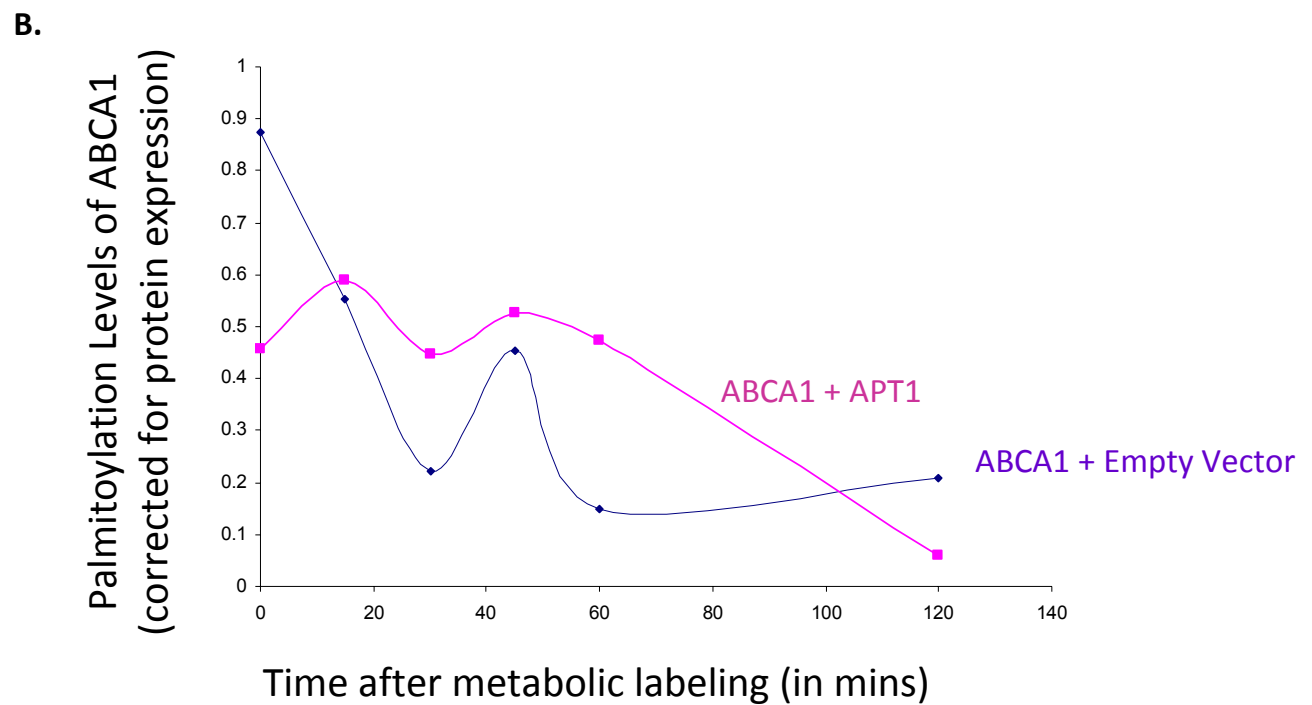
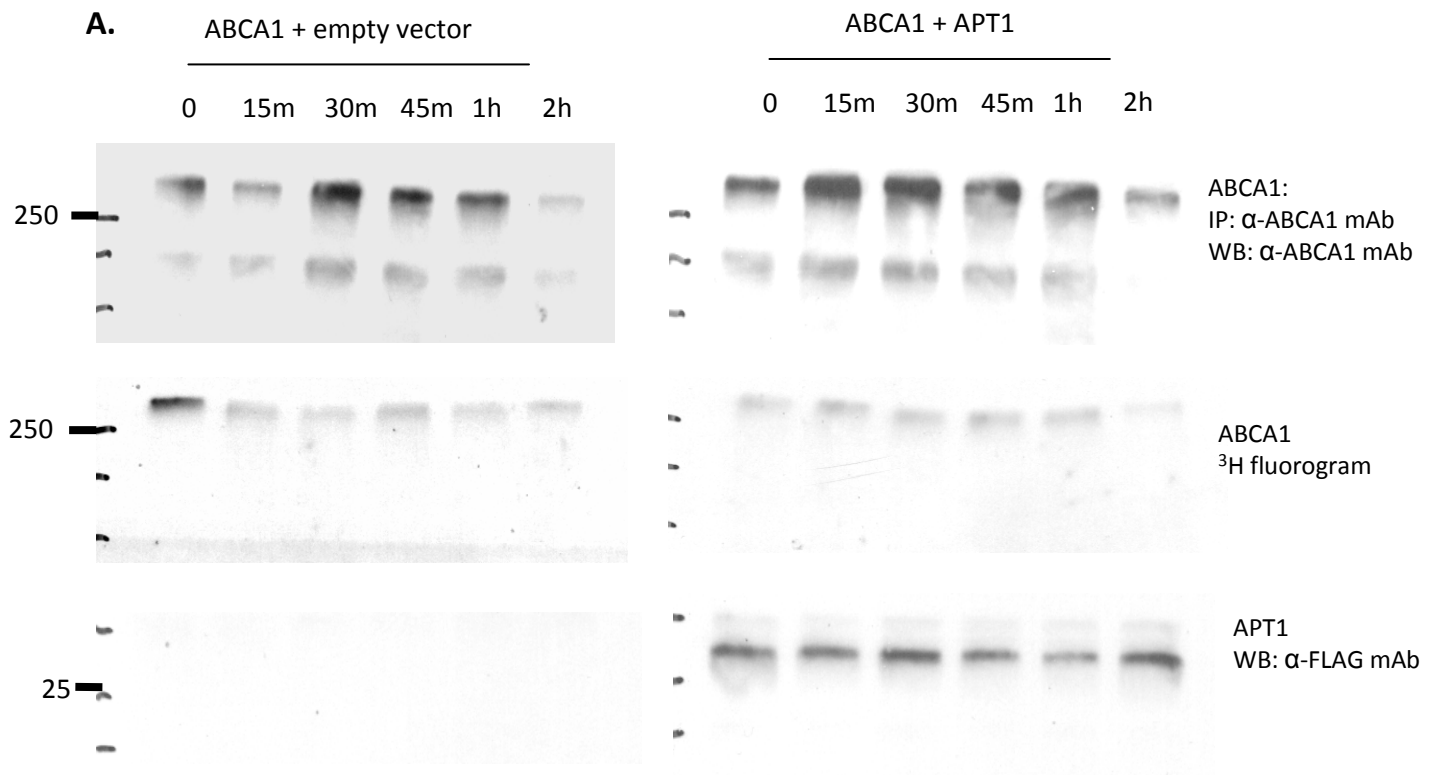


Figure 3.10. Assessment of depalmitoylation of ABCA1 in COS cells co-transfected with APT1 cDNA. COS cells were co-transfected with plasmids expressing ABCA1 and APT1 (■) or ABCA1 and control plasmid (◆) and metabolically labeled with [³H] palmitate for 3 hrs. Radiolabeled cells were washed in medium before incubation in medium containing 100μM unlabeled palmitate for 0m, 15 mins, 30 mins, 45 mins, 1 hr, and 2 hrs. (A) Western blot of ABCA1, fluorogram of radiolabeled ABCA1, and Western blot of APT1 in the pulse-chase study. (B) Chart of fluorogram corrected for ABCA1 protein expression. This experiment was performed twice with equivalent results.

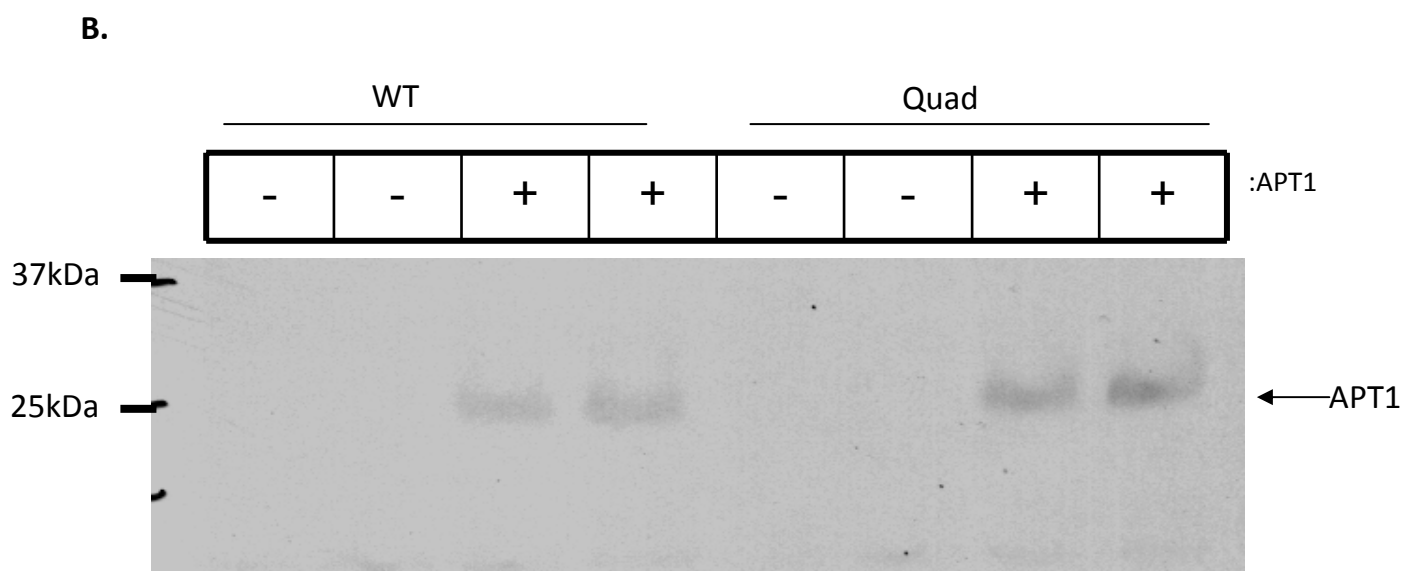
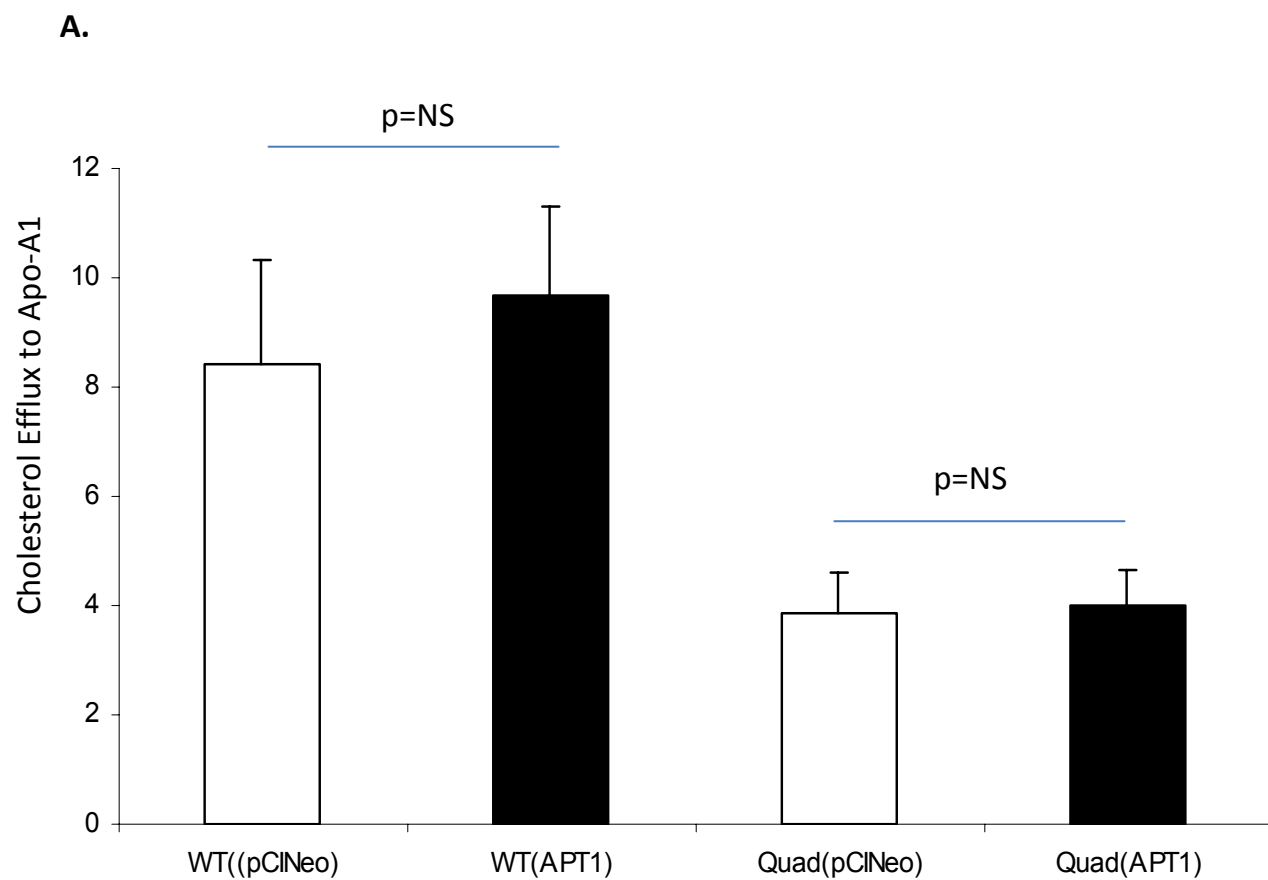


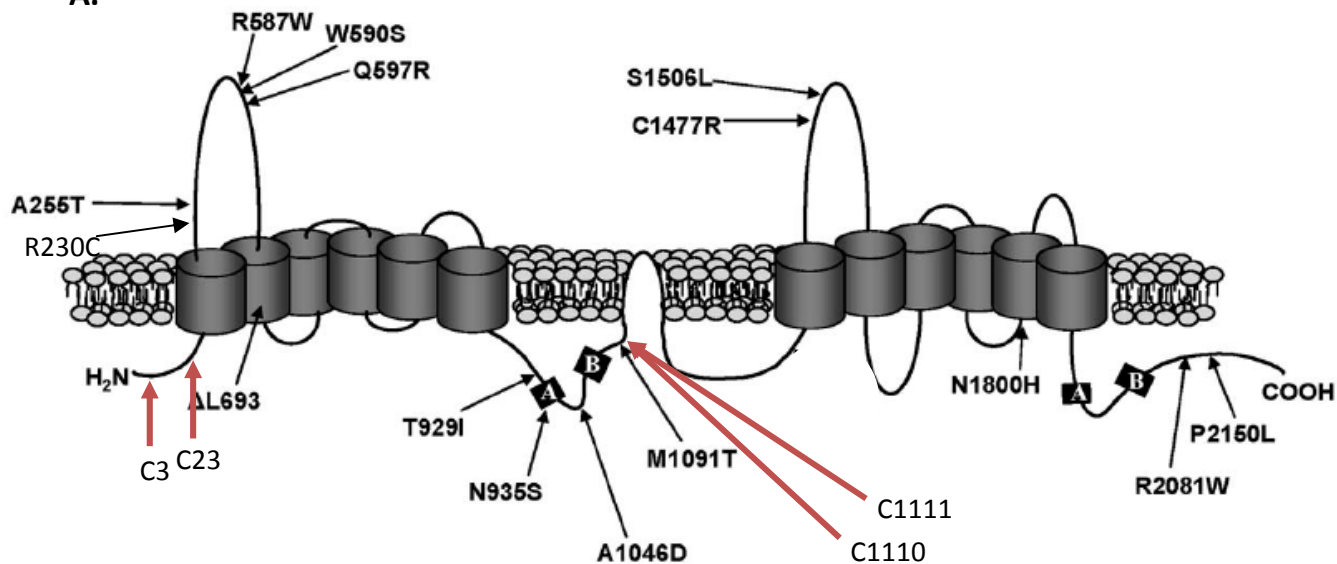
Figure 3.11. Effect of cholesterol efflux activity in ABCA1 stable cell lines transfected with APT1 cDNA. (A) HEK293 stable cell lines expressing wild-type ABCA1 (WT) and C3S/C23S/C1110S/C1111S-ABCA1 (Quad) were transfected with APT1 or pCINeo (empty vector). Efflux of [³H] cholesterol to 10µg/ml apo A-I was measured after 4 hrs. The error bars represent standard deviation from measurements made in triplicate. The p value was calculated by Student's t test. (B) Western blot of APT1 expression in stable cell lines.

3.3.7 Palmitoylation is Not Decreased in Eleven Naturally Occurring ABCA1 Mutations

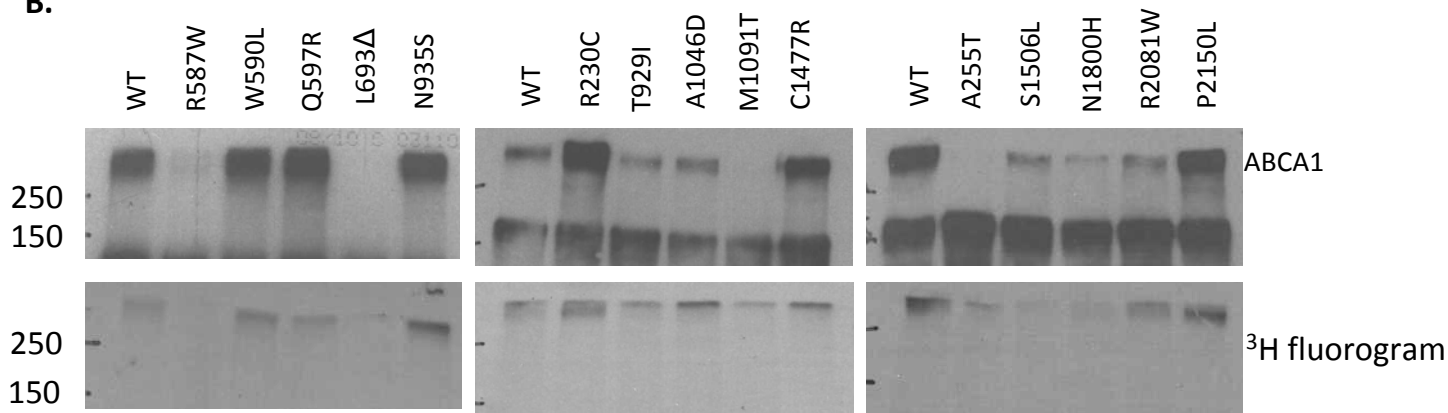
More than 80 different mutations in the ABCA1 gene result in Tangier disease (TD) (Brunham et al., 2006). To determine whether any of these naturally occurring ABCA1 mutations have altered palmitoylation, metabolic labeling of 15 TD clinical phenotype mutants was carried out on previously characterized constructs (Singaraja et al., 2006).

COS cells were transiently transfected with WT-, R587W-, W590L-, Q597R-, L693Δ-, N935S-, R230C-, T929I-, A1046D-, M1091T-, C1477R-, A255T-, S1506L-, N1800H-, R2081W-, and P2150L-ABCA1 (**Figure 3.12.A**). Transfected cells were labeled with [³H] palmitate for 3hrs before harvesting. ABCA1 was immunoprecipitated and subjected to Western blot analysis and [³H] fluorography. Only 11 of the 15 constructs expressed detectable ABCA1 protein levels by immunoblotting (**Figure 3.12.B**). [³H] palmitate-labeled products were normalized to the amount of immunoprecipitated protein and quantified (**Figure 3.12.C**). No decrease in palmitoylation was observed in our sample of naturally occurring ABCA1 mutations and suggests decreased ABCA1 palmitoylation is not associated with these 11 mutants.

A.



B.



C.

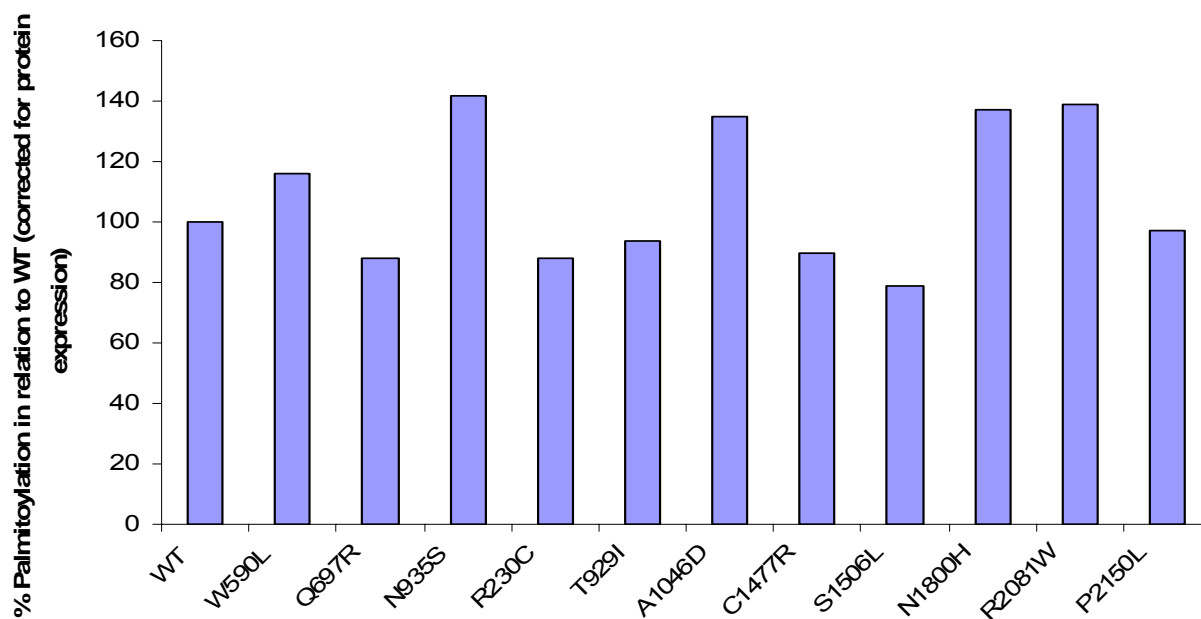


Figure 3.12. Palmitoylation status of 15 Tangier disease (TD) clinical phenotype mutants. (A) Schematic of the 15 naturally occurring ABCA1 mutations tested for palmitoylation status. The location of the four cysteines known to be palmitoylated in ABCA1 are noted (C3, C23, C1110, C1111). (B) ABCA1 constructs containing the individual TD mutations were transiently transfected into COS cells and metabolically labeled with [^3H] palmitate for 3 hrs. The cells were then washed with 1XPBS, lysed, and immunoprecipitated with α -ABCA1 antibodies. ABCA1 protein expression was detected by Western blotting and ABCA1 palmitoylation status was detected by [^3H] fluorography. (C) Quantification of palmitoylation was corrected for ABCA1 protein expression. Only 11 of the 15 constructs expressed enough ABCA1 protein to quantify palmitoylation levels. This quantification is based on a single experiment.

3.4 Discussion

The addition of saturated acyl chains such as palmitate is thought to provide proteins with an affinity for ordered lipid domains. WT-ABCA1 is palmitoylated and along with other groups, we have observed ABCA1 localization to flotillin-1 positive detergent resistant membrane (DRMs) domains. Although we demonstrate that ABCA1 palmitoylation mutants are not associated with DRM domains, their absence from these ordered structures is likely due to ABCA1 mislocalization away from the cell surface rather than a role for palmitoylation in targeting ABCA1 to DRMs.

We hypothesized that two contributors to ABCA1 plasma membrane localization, Rab8a and ceramide, utilize palmitoylation as a mechanism. ABCA1 palmitoylation mutants show a 50% decrease in lipid efflux activity (Sinagaraja et al., 2009). We depleted endogenous Rab8a with siRNA in these ABCA1 palmitoylation mutant expressing cell lines and re-measured cholesterol efflux activity. We observed a further decrease in efflux activity following siRNA Rab8a treatment in the ABCA1 mutants. This suggests that Rab8a invokes a separate mechanism from palmitoylation to facilitate ABCA1 plasma membrane localization, and that Rab8a and palmitoylation are involved in separate pathways.

Ceramide is a lipid molecule capable of increasing ABCA1 at the cell surface. Cells treated with ceramide result in increased ABCA1 cholesterol efflux activity (Witting et al., 2003). Treatment of ABCA1 palmitoylation mutants with ceramide also increased cholesterol efflux activity. If ceramide utilized palmitoylation to increase cell surface ABCA1, the C3S/C23S/C1110S/C1111S-mutant should not show improved efflux functions as the major sites of palmitoylation are lost. This suggests that ceramide does not increase ABCA1 to the cell surface through acylation. ABCA1 likely possesses a number of separate mechanisms and pathways to localize to the plasma membrane.

Working with an amino terminal fragment of ABCA1, we confirmed previous studies showing that ABCA1 expression produces two distinct populations: a population of ABCA1 with the core oligosaccharide sensitive to EndoH; and another population of ABCA1 with a modified oligosaccharide

moiety resistant to EndoH (Singaraja et al., 2006). We observed that NH₂-terminal ABCA1 with the core sugar (EndoH sensitive) is primarily palmitoylated at cysteine 23, while NH₂-terminal ABCA1 with the complex sugar (EndoH resistant) is primarily palmitoylated at cysteine 3.

What these results do not reveal is how or even if the modifications to ABCA1, glycosylation and palmitoylation influence one another. Following the covalent addition of a core sugar to a protein, a series of processing reactions trims the N-glycan in the ER before the glycoprotein continues on to the Golgi where further processing of the sugar chain occurs. In the Golgi, both trimming and addition of sugar residues generates a final complex sugar moiety (Varki et al., 2009). EndoH is a bacterial enzyme that releases oligomannose and hybrid N-glycans but not complex N-glycans from proteins. These complex N-glycans are generated when mannose residues are removed from the sugar chain by Golgi mannosidase II at the mid-Golgi (Ron and Horowitz, 2005). Thus, EndoH can differentiate between glycoproteins that have not yet been processed in the mid-Golgi from mature glycoproteins that have passed through the mid-Golgi.

There are multiple ways in which these post-translational modifications can interact with one another. One, the trafficking through the ER/Golgi glycosylation network can localize ABCA1 to membrane bound PATs. The majority of the DHHC enzymes are localized at either the ER or GC (Ohno et al., 2006). Of the five DHHC proteins we identified as palmitoylating ABCA1: DHHC8 is in the GC; DHHC12 is in the ER and GC; DHHC15 is in the ER and GC; and both DHHC20 and -21 are at the plasma membrane. This may suggest that DHHC12 or -15 palmitoylates cysteine 23 of ABCA1 in the ER, and DHHC8, -12, or -15 palmitoylates cysteine 3 of ABCA1 in the GC. Two, the palmitoylation of specific cysteine residues ensures proper trafficking of ABCA1 through the ER/Golgi glycosylation network so that proper modification to the sugar chain is dependent on proper cysteine palmitoylation. Three, these modification occur independently of one another.

We identified at least five different DHHC enzymes that increase ABCA1 palmitate labeling. Of these five, DHHC8 mediated the largest incorporation of palmitate onto ABCA1 and increasing DHHC8 expression significantly increased cholesterol efflux activity. However, the inhibition of endogenous DHHC8 using siRNA did not change either ABCA1 palmitoylation levels, or ABCA1 cholesterol efflux activity. This may be due to either inefficient siRNA-mediated depletion of DHHC8, or compensation by the other four enzymes that mediate ABCA1 palmitoylation. To demonstrate the relevance of endogenous PAT enzymes to ABCA1 function, it may be necessary to deplete all five PATs known to palmitoylate ABCA1.

Unlike the family of DHHC enzymes that mediate palmitoylation, the thioesterases that remove palmitate from proteins are not as well characterized and only three thioesterases have been identified. We tested the thioesterase APT1 which has been demonstrated to deacylate a number of proteins, and assessed whether it had an effect on ABCA1 palmitoylation levels or ABCA1 function. Our pulse-chase studies and cholesterol efflux assays involving APT1 suggests that it is not the thioesterase for ABCA1.

A number of disease processes are associated with altered palmitoylation levels (Korycka et al., 2012), but the clinical relevance of ABCA1 palmitoylation is unknown. A complete deficiency in one of the three known thioesterases, PPT1 results in infantile neuronal ceroid lipofuscinoses (NCL). PPT1 is the protein product of the *CLN1* gene and is a soluble lysosomal protein responsible for the depalmitoylation of proteins undergoing degradation in the lysosome (Hofmann et al., 2002). Individuals with infantile NCL present with psychomotor deficiencies, seizures, and loss of vision. For individuals with this disorder, the median age of death in a Finnish study was 6.5 years. Mutations in Porcupine (Porcn), a membrane bound O-acyl transferase causes Glotz syndrome or focal dermal hyperplasia. Porcn is responsible for the addition of the 16C monounsaturated fatty acid palmitoleate to Wnt proteins (Resh, 2012). Altered palmitoylation of proteins is believed to play a role in the development of a number of other diseases. Cells expressing oncogenic H-Ras treated with an inhibitor to APT1, the

thioesterase that depalmitoylates H-Ras, partially reverts transformed phenotypes (Resh, 2012). In individuals with diabetic vascular disease, the lack of eNOS palmitoylation results in a chronic inflammatory response (Karve and Cheema, 2011). The palmitoylation of thyrotropin-releasing hormone (TRH) receptor type 1 (TRH-R1) is required to maintain the receptor in an inactive state. The lack of TRH-R1 palmitoylation results in an active receptor that can lead to the over-secretion of the hormones thyrotropin and prolactin (Karve and Cheema, 2011).

To examine whether a loss of palmitoylation occurs in TD, naturally occurring mutations of ABCA1 were tested for their palmitoylation status. We observed no dramatic difference in the levels of ABCA1 palmitoylation in a sample of mutations found in TD patients. This is not surprising as we previously demonstrated that palmitoylation mutations decrease cholesterol efflux function by only 50%. In comparison, naturally occurring ABCA1 mutations usually show an almost complete deficiency in function and often decrease cholesterol efflux activity by nearly 90% (Singaraja et al., 2006). It remains a possibility that alterations to ABCA1 palmitoylation occur in patients with milder decreases in HDL levels or with familial hypoalphalipoproteinemia (FHA) but not TD. Besides TD, ABCA1 mutations also cause FHA (Sviridov and Nestel, 2007).

4. The Post-Transcriptional Regulation of ABCA1 by miRNAs

4.1 Synopsis

The regulation of ABCA1 by miR-33a was discovered by different groups using independent methods. Upon altering cellular cholesterol levels, Rayner et al. found approximately 20 miRNAs including miR-33a that underwent changes in their expression levels. The two other groups identified miR-33a using an *in silico* approach and noted the presence of the miR-33a gene in the intron of a gene involved in the regulation of cholesterol homeostasis, SREBF2 (Najafi-Shoushtari et al., 2010; Marquart et al., 2010). The introns of protein coding genes are often where miRNAs are found (Berezikov, 2011). miRNAs embedded within genes can either be co-transcribed with the host gene, or have their own intronic promoters (Gommans and Berezikov, 2012). miR-33a is co-transcribed with the SREBF2 gene when intracellular cholesterol levels are low (Rayner et al., 2012). The SREBP2 protein is a transcription factor that transcribes genes involved in sterol synthesis (HMGCR) and cholesterol uptake (LDL-R) (Marquart et al., 2010). The transcription of miR-33a inhibits efflux of cholesterol from cells through the post-transcriptional repression of ABCA1, and further increases cellular cholesterol content. The three groups demonstrated that miR-33a over-expression in hepatic cells and macrophages decreases ABCA1 protein expression and cholesterol efflux activity, while the inhibition of endogenous miR-33a up-regulates ABCA1. The generation of mice lacking miR-33a confirmed these studies and demonstrated that miR-33a deficient mice markedly increase ABCA1 levels in the liver and macrophages, which results in significantly higher serum HDL levels than in wild-type mice (Horie et al., 2010).

The regulatory role of miR-33a on ABCA1 in tissues and cells besides the liver and macrophages has not been assessed. Two other miRNAs, miR-758 and miR-106b were found to regulate ABCA1 in neuronal cell lines (Ramirez et al., 2011; Kim et al., 2012). The inhibition of miR-106b reduced the production of amyloid β (A β) peptides. Accumulation of A β peptides in the brain are associated with the pathogenesis of Alzheimer's disease (AD) (Hardy and Selkoe, 2002). This latter study highlights the role

of ABCA1 in maintaining proper cholesterol homeostasis in various tissues and cell lines, which may have an impact on health and disease processes. High levels of ABCA1 expression in the liver and macrophages can attenuate CVD (Tang and Oram, 2009); ABCA1 expression in the CNS can prevent pathological processes related to Alzheimer's disease (Fan et al., 2009); and ABCA1 in the pancreas is required for proper β -cell function (Vergeer et al., 2010). β -cell ABCA1 ensures proper islet cholesterol homeostasis and insulin secretion (Brunham et al., 2007). A variant of ABCA1, R230C is associated with early onset type 2 diabetes in the Mexican population (Villarreal-Molina et al., 2008). We investigated the effects of modulating miR-33a on β -cell function (Wijesekara et al., 2012). Some of the findings from this work will be presented in this dissertation.

miR-33a is only one of 40 to 100 miRNAs that are predicted to bind to the 3'UTR of ABCA1 (**Table 1.6**). Most miRNAs have a modest inhibitory effect on their target. In many cases, it is rare that the regulation by a single miRNA can produce a measurable biological response (Small and Olson, 2011). miR-33a is an exception, where modulating levels of this miRNA produces many observable phenotypes through the regulation of ABCA1 (Rayner et al., 2011; Rayner et al., 2011; Wijesekara et al., 2012). In this thesis, we have identified and validated miR-145 as a novel regulator of ABCA1 expression and function. Like miR-33a, miR-145 modulation induces a significant change in ABCA1 expression. Although miR-33a has a greater effect on ABCA1 protein levels and cholesterol efflux activity than miR-145 in cell lines, we found that miR-145 expression in the human liver is 3-4 times higher than miR-33a.

Due to the modest inhibitory effects of most miRNAs on a target, the inhibition of several different miRNAs for the same target often amplifies repression through combinatorial regulation (Small and Olson, 2011). This hypothesis where several miRNAs can regulate a single mRNA target is supported by studies in worms and mice. Studies in *C. elegans* suggest significant redundancy exists between miRNAs. In mice, no specific deletion of any one miRNA causes fully penetrant embryonic lethality and suggests redundancy exists between different miRNAs (Rottiers and Naar, 2012). The simultaneous

inhibition of multiple miRNAs that regulate ABCA1 may amplify the increase in efflux activity and HDL biogenesis. We assessed the simultaneous modulation of miR-33a with miR-145 on ABCA1 compared to miR-33a or miR-145 alone.

The attractiveness of regulating genes through miRNA modulation is the irrefutable evidence that miRNAs can be targeted and their levels can be changed (Small and Olson, 2011). The post-transcriptional regulation of ABCA1 by miRNAs represents a relatively novel strategy to influence HDL biogenesis.

4.2 Materials and Methods

4.2.1 Cell Culture

HepG2 (ATCC) and U343 cells (a kind gift from Dr Burton Yang) were maintained in DMEM (Gibco) with 10% FBS and 4mM L-Glutamine. All cell lines were maintained at 37°C with a humidified 5% CO₂ atmosphere.

4.2.2 Protein Isolation and Antibodies

Cells were lysed in lysis buffer (10mM Tris pH8.0, 1% Triton X-100, Complete Protease Inhibitors [Roche]). 30-100µg of protein were separated on 7.5% acrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). ABCA1 was immunoblotted using an anti-ABCA1 monoclonal antibody generated to its C-terminus (Wellington et al., 2002). In addition, anti-GAPDH (Millipore) primary antibody and anti-mouse-HRP conjugated secondary antibody (Jackson IR Laboratories) were used in immunoblotting.

4.2.3 3' UTR Luciferase Reporter Assays

Human ABCA1 3' untranslated region (3'UTR) (length: 3309 nucleotides, GenBank accession number NM_005502, position 17099-10412) was PCR amplified from genomic DNA and cloned into the luciferase reporter plasmid pmirGLO, downstream of the luciferase stop codon. All constructs were confirmed by sequencing. Luciferase vector plus vector containing ABCA1 3'UTR were transfected into

U343 cells. Dual luciferase (fire-fly luciferase and renilla luciferase as an internal control) expression was measured. Renilla luciferase activity was normalized to the corresponding fire-fly luciferase activity. Measurements were plotted as the relative fold change in luciferase activity in the vector containing the ABCA1 3'UTR with respect to empty vector.

4.2.4 miRNA Mimics and miRNA Inhibitor Transfection

HepG2 cells were transfected with 50nM of Pre-miR™ miRNA precursor (Ambion) or 100nM Anti-miR™ miRNA inhibitor (Ambion) using Lipofectamine RNAiMAX transfection reagent (Invitrogen) for 48hrs according to the manufacturer's instructions.

4.2.5 Adenovirus

Adenoviral expression vectors containing miRNA precursor or miRNA inhibitor in pHM10 vectors under control of the CMV promoter were obtained from Applied Biological Materials. The control virus expresses empty pHM10 vector. miRNA expressing adenovirus were designed with a single copy of human miR-33a or miR-145 with 150bps of respective upstream sequence and 150bps of respective downstream sequence. The inhibitor sequences of miR-33a and miR-145 were obtained from System Biosciences Inc and sub-cloned into adenoviral vectors. All amplification and purification steps (double cesium chloride gradient centrifugation) were carried out by Applied Biological Materials. HepG2 cells were transduced with adenovirus at a multiplicity of infection (MOI) of 100 in DMEM/10%FBS/4mM L-Glutamine. After 24hrs the media was changed to fresh DMEM/10%FBS/4mM L-Glutamine. After 24hrs cells were harvested and analyzed by Western blotting.

4.2.6 Cholesterol Efflux Assays

HepG2 cells were plated into 24 well dishes in DMEM with 10% FBS and 4mM L-Glutamine. 24hrs later cells were transfected as described in 4.2.4. 24hrs later 1μCi/ml of [³H] cholesterol (Perkin-Elmer) was added. After 16hrs, cells were equilibrated in DMEM (no supplements) for 1hr, followed by efflux to 10μg/ml apo A-I (Lee Biosystems) for 4hrs. Radioactivity was directly measured from

supernatant, while radioactivity in cell lysates was measured following lysis with 0.1N NaOH. Efflux is expressed as a percent of the efflux relative to WT-ABCA1.

4.2.7 Animal Studies

C57Bl6/J male mice were obtained from Jackson Laboratories. Mice were maintained on a standard chow diet. All procedures on mice were approved by the UBC animal care committee. Adenovirus was diluted in sterile PBS and delivered at a dose of 1×10^{10} pfu/mouse. A total of 100 μ l of diluted virus was tail-vein injected using a 1cc U-100 Insulin syringe 28G½ (Becton Dickinson). Pre-injection and post-injection blood samples were collected from mice through saphenous vein bleeds after a 4hr fast. Mice were weighed before and after injection to monitor weight gain and health. Mice were monitored daily following adenovirus injections. Mice were euthanized by carbon dioxide inhalation and blood and tissues were harvested.

4.2.8 Serum Lipid Analysis

After a 5hr incubation at 4°C, serum was isolated from blood by centrifugation at 3000Xg for 10mins at 4°C. Serum was stored at -20°C. HDL and total cholesterol concentrations were determined by enzymatic assays adapted to 96-well micro-titer plates using Infinity™ cholesterol measurement reagents (Thermo Scientific). Various dilutions of free cholesterol (100mg/dl) were used as standards (Wako Chemicals). Serum HDL cholesterol levels were determined following precipitation with 20% polyethylene glycol (PEG 8000). Data represent the mean and standard deviation from at least four mice. Statistical analysis was by the two-tailed student's t test.

4.3 Results

4.3.1 miRNAs Directly Binds the 3'UTR of ABCA1

To determine whether miRNAs regulate ABCA1, we generated a reporter gene construct containing luciferase and the entire 3'UTR of ABCA1 (3309bp). This construct was transfected into various mouse and human cell lines whereupon luciferase activity was measured. We observed that

luciferase activity was inhibited in all transfected cell lines (**Figure 4.1**) suggesting that the 3'UTR of ABCA1 may be a direct target for endogenous miRNAs in these cells. The 3'UTR is a region that can regulate translation of the open reading frame through miRNAs, alternative polyadenylation sites, or protein binding.

4.3.2 Identification of miRNAs That Regulate ABCA1

In silico programs predict 40 to 100 binding sites for different miRNAs in the 3'UTR of ABCA1 (**Table 1.6**). Prediction programs possess unique algorithms that predict which miRNAs targets a gene based upon different weighted factors including: the level of Watson-Crick base pairing between the miRNA seed region and the mRNA target; evolutionary conservation of the miRNA binding site; the nucleotide sequence surrounding the miRNA binding site; the free energy of the miRNA and mRNA heteroduplex; and the total number of binding sites for a given miRNA in the 3'UTR (Thomas et al., 2010; Alexiou et al., 2009). The most critical factor in determining whether a miRNA regulates a gene are the 6-7 nucleotides at the 5' end of the miRNA known as the seed sequence. But even this requirement can vary between different miRNAs and their mRNA targets. These variations in miRNA/mRNA targeting makes it very difficult to accurately predict targets in silico (Gommons and Berezikov, 2012) and validation studies are required to confirm miRNA regulation of a particular gene. The lowest number of miRNAs predicted to target ABCA1 is 37 by PicTar (**Table 1.6**). The validation of even 37 miRNAs is a daunting and expensive task. To narrow down the predicted number of potential miRNAs that regulate ABCA1, we imposed two criteria: one, the miRNA must be expressed in the liver owing to the importance of hepatic ABCA1 in HDL biogenesis; and two, at least four out of the five prediction programs we utilized must positively identify a miRNA as targeting ABCA1. The five prediction programs we used were freely available on-line algorithms (TargetScan 5.2, PicTar, microRNA.org, miRDB, and FindTar). Based on these two criteria, the candidates were narrowed down to eight miRNAs: miR-17; miR-145; miR-19a; miR-96; miR-33a/33b; miR-27a; miR-148; and miR-144 (**Table 4.1**).

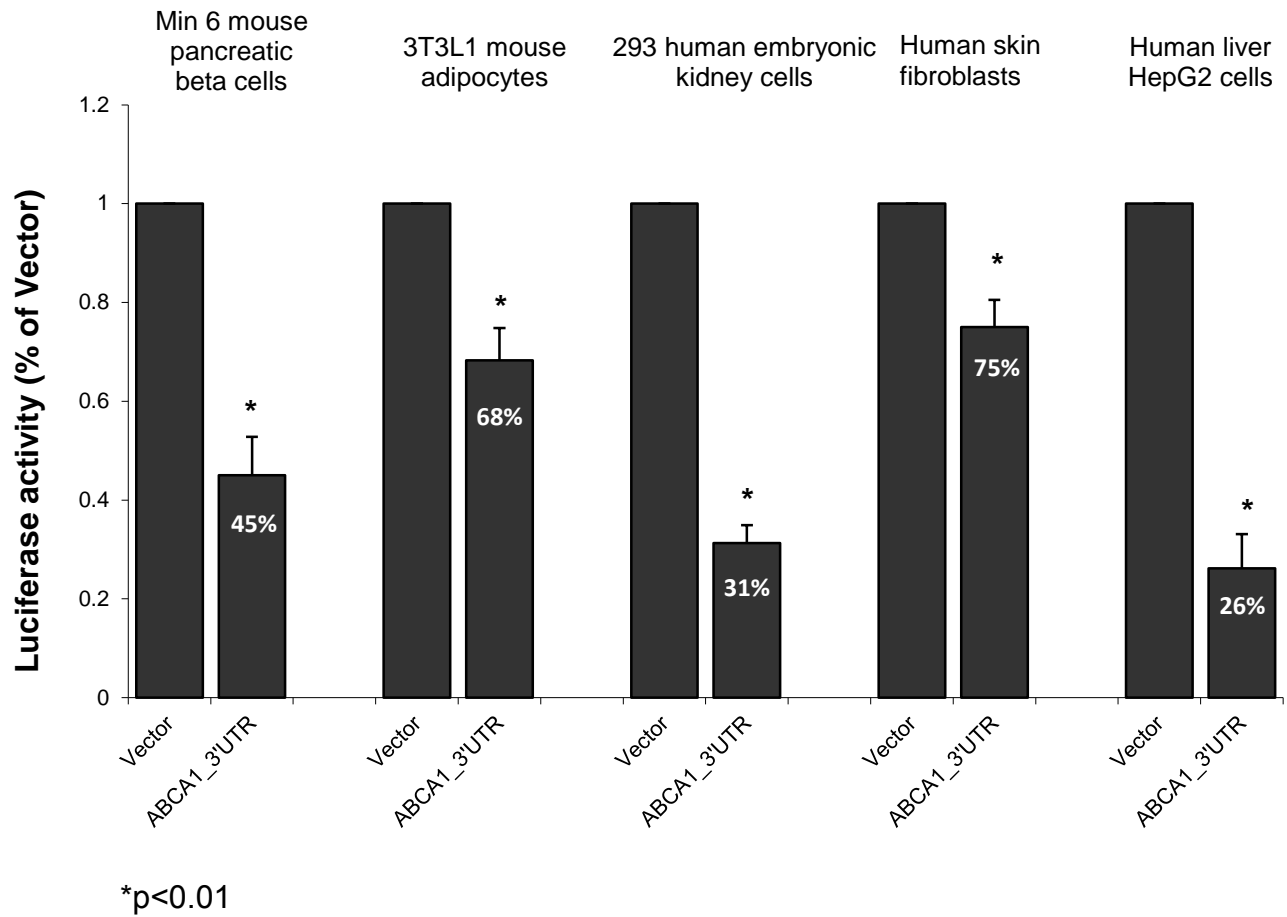


Figure 4.1. The 3' untranslated region (3'UTR) of ABCA1 may be regulated by endogenous microRNAs (miRNAs) present in various cell lines. The human ABCA1 3'UTR was amplified by PCR from genomic DNA and cloned into the luciferase reporter plasmid, pmirGLO, downstream of the luciferase stop codon. The reporter construct containing luciferase and the 3'UTR of ABCA1 was transiently transfected into MIN6 mouse pancreatic beta cells, 3T3L1 mouse adipocytes, 293 human embryonic kidney (HEK) cells, human skin fibroblasts, and HepG2 human hepatocytes. Following transfection, dual-luciferase (fire-fly luciferase, and renilla luciferase as an internal control) expression was measured. The relative fold change in luciferase activity for each cell line was plotted compared to pmirGLO without the 3'UTR of ABCA1. The error bars represent standard deviation from measurements made in triplicate.

	TargetScan5.2	PicTar	microRNA.org	miRDB	FindTar
miR-17	1 conserved site	2 sites	1 site	Not predicted	9 sites
miR-145	2 conserved sites	1 site	1 site	1 site	4 sites
miR-19	1 conserved site; 2 poorly conserved sites	3 sites	1 site	2 sites	4 sites
miR-96	1 conserved site	1 site	1 site	1 site	5 sites
miR-33ab	2 conserved sites; 1 poorly conserved site	Not predicted	2 sites	3 sites	2 sites
miR-27ab	1 conserved site; 1 poorly conserved site	2 sites	2 sites	2 sites	4 sites
miR-148ab	1 conserved site	1 site	1 site	1 site	3 sites
miR-144	2 conserved sites; 3 poorly conserved sites	5 sites	4 sites	4 sites	3 sites

Table 4.1. Predicted number of miRNA binding target sites in the 3'UTR of ABCA1. Five different prediction programs (TargetScan5.2; PicTar; microRNA.org; miRDB; FindTar) were used to narrow down candidate miRNAs that were bioinformatically predicted to regulate ABCA1. A total of eight different miRNAs (miR-17; miR-145; miR-19; miR-96; miR-33ab; miR-27ab; miR-148ab; miR-144) known to be expressed in the liver were identified by at least four out of five programs. The number of sites predicted by the five different programs to exist in the 3'UTR of ABCA1 for the eight different miRNAs are listed.

4.3.3 Validation of miRNAs

To validate which of the eight predicted miRNAs regulate ABCA1 expression, we co-transfected the reporter gene construct with precursor miRNA (pre-miRNA) mimics of the eight candidate miRNAs into U343 cell lines and measured luciferase activity (**Figure 4.2.A**). Five out of the eight predicted miRNAs demonstrated significant repression of luciferase activity ($p < 0.01$) (**Figure 4.2.B**). In agreement with previous findings, miR33a/33b expression resulted in significant repression of luciferase activity. miR-145, miR-27a, miR-148, and miR-144 also significantly ($p < 0.01$) repressed luciferase activity.

4.3.4 Manipulation of miR-33a in Islet Cells Alters ABCA1 Expression, Islet Cholesterol Levels, and Insulin Secretion

The post-transcriptional regulation of ABCA1 by miR-33a in hepatic cell lines, macrophages, and the liver has been extensively validated (Rayner et al., 2012). However, ABCA1 is important in maintaining cholesterol homeostasis in a number of different cell-types including the β -cell (Brunham et al., 2007). To assess whether the regulation of ABCA1 by miR-33a modulation in islet cells results in changes to cholesterol homeostasis and insulin secretion, we generated adenovirus expressing miR-33a and miR-33a inhibitors. We designed the adenovirus miRNAs to ensure stability of the final product, along with the ability to produce a precursor-miRNA (pre-miRNA) that would be recognized by the canonical miRNA processing pathway of the transduced cell. For details of the design see **4.2.5 Adenovirus**. The transduction of adenoviral-miR-33a into HepG2 cells repressed ABCA1 protein levels (**Figure 4.3.A**) and decreased cholesterol efflux to apo A-I (**Figure 4.3.B**). Adenoviral anti-miR-33a increased ABCA1 protein levels in HepG2 cells (**Figure 4.3.A**). Adeno-miR-33a transduction into wild-type mouse islets also repressed ABCA1 protein levels, while the miR-33a inhibitor increased ABCA1 protein expression (Wijesekara et al., 2012). This indicates that miR-33a can regulate ABCA1 in a variety of cell types including islet cells. In both human and mouse islets, the expression of miR-33a decreased insulin secretion. In cells from a mouse model of impaired insulin secretion and hypercholesterolemia (apoE-/-

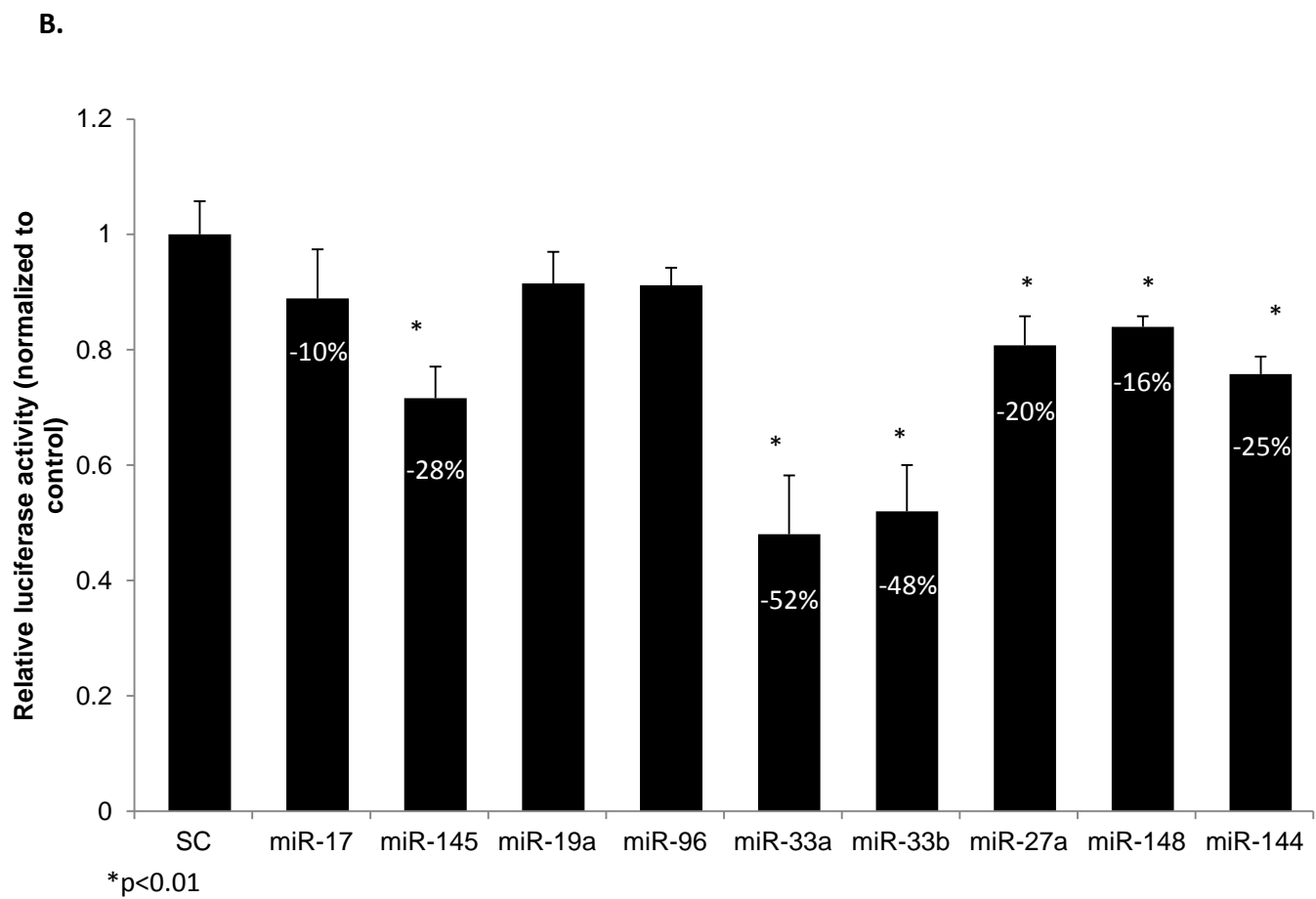
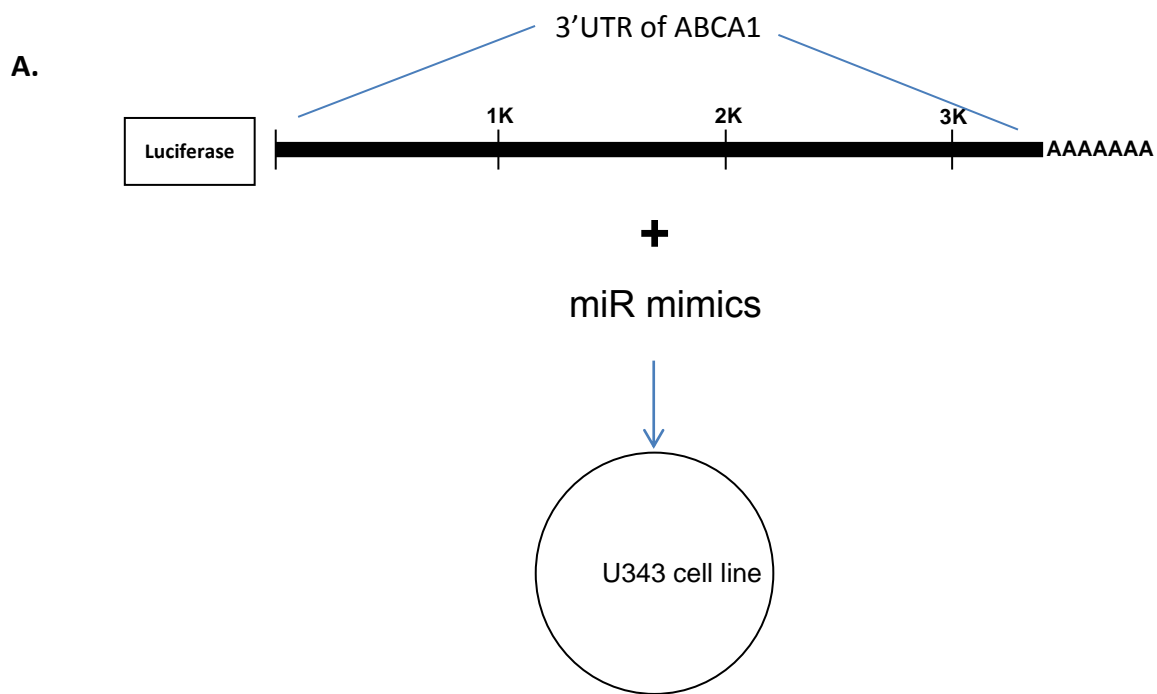


Figure 4.2. Validation of eight miRNAs predicted by 4 out of 5 miRNA prediction programs to bind to the 3'UTR of ABCA1. (A) A reporter construct containing luciferase and the 3'UTR of ABCA1 (3309bp long) was co-transfected into U343 cells along with 50nM of: control miRNA; miR-17; miR-145; miR-19; miR-96; miR-33a/33b; miR-27a; miR-148; or miR-144. (B) Dual-luciferase activity was measured with the error bars representing standard deviation from measurements made in triplicate.

mouse), the inhibition of miR-33a reduced cholesterol levels and restored insulin secretion to control levels (Wijesekara et al., 2012).

4.3.5 miR-33a and miR-145 Directly Binds to the 3'UTR of ABCA1 to Regulate Protein Translation

Besides miR-33a, miR-145 repression of luciferase activity was the strongest as measured by our reporter construct (**Figure 4.2.B**). miR-33a repressed activity by ~50% and miR-145 repressed activity by ~25%. To assess whether miR-145 directly binds to the 3'UTR to repress luciferase activity, we mutagenized at least two nucleotides in the TargetScan predicted seed sequences for miR-33a and miR-145 (**Figures 4.4.A and 4.4.C**). Mutagenesis at the sites reversed the inhibitory effect of these miRNAs (**Figures 4.4.B and 4.4.D**) and restored luciferase activity to control levels. This confirms that both miR-33a and miR-145 directly binds to the 3'UTR to regulate ABCA1.

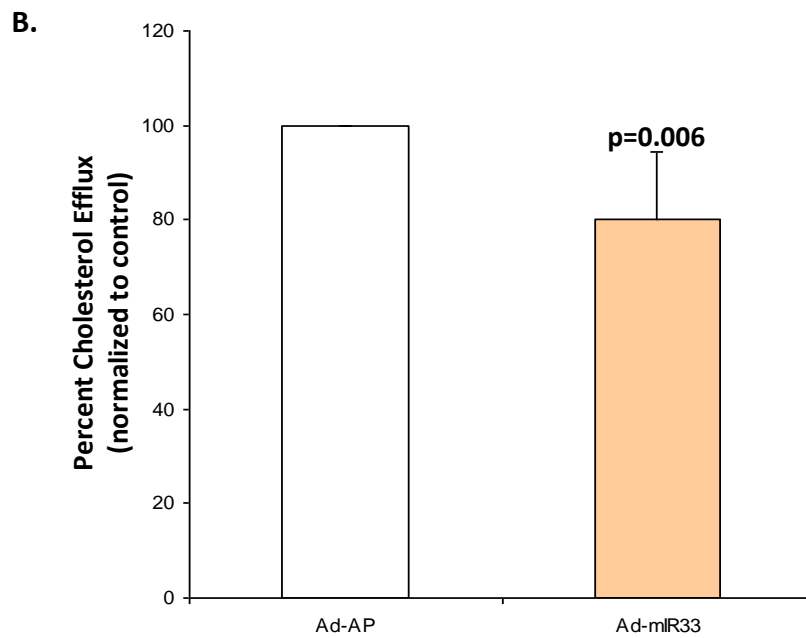
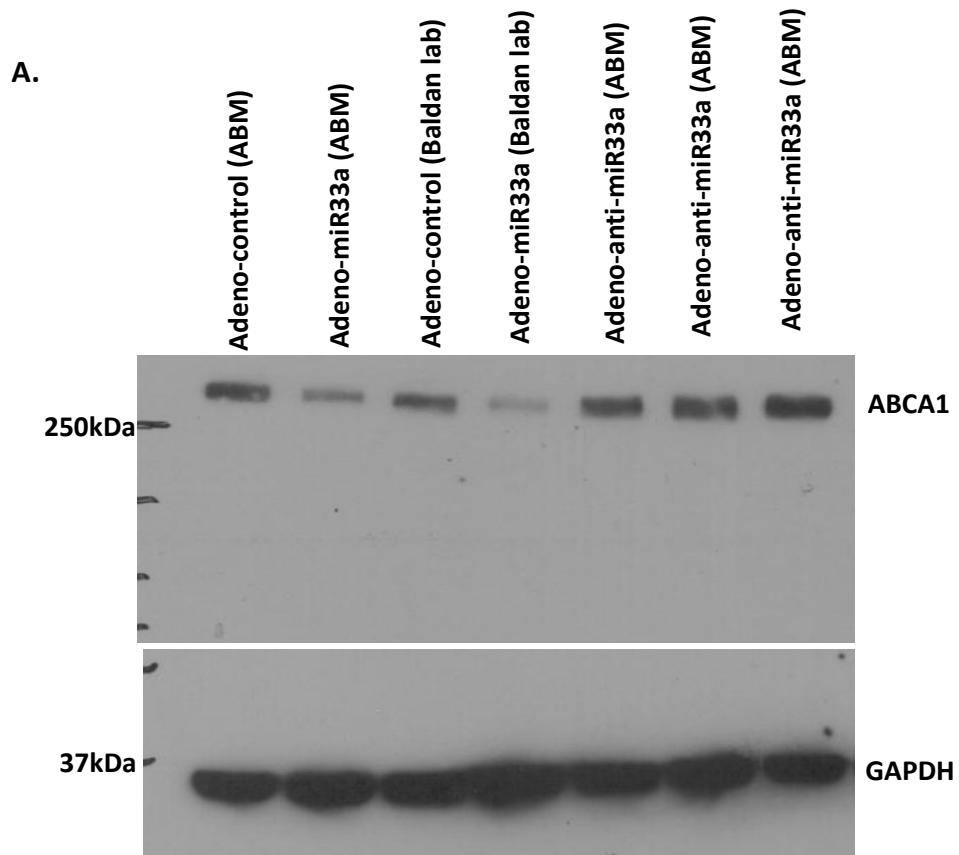


Figure 4.3 Generation of adenoviral-miR-33a and adenoviral-miR-33a inhibitor. To assess the effects of miR-33a modulation in islet cells and in vivo, we designed adenovirus expressing miR-33a and miR-33a inhibitor. A single copy of human miR-33a along with 150bps of upstream sequence and 150bps of downstream sequence was cloned into a pHM10 vector with a CMV promoter. The adenovirus was amplified followed by purification by cesium chloride gradient centrifugation by Applied Biological Materials. The miR-33a inhibitor sequence was obtained from System Biosciences Inc. and had been previously validated in vivo (Rayner et al., 2010). A single copy complementary to miR-33a was sub-cloned into a pHM10 vector using a PCR cloning strategy by Applied Biological Materials. (A) A Western blot of HepG2 cells transduced with adenovirus-control (ABM), adenovirus-miR-33a, adenovirus-GFP (a kind gift from Dr Angel Baldan), adenovirus-miR-33a-GFP (a kind gift from Dr Angel Baldan), and three independent experiments with adenovirus-miR-33a inhibitor (ABM) all at a multiplicity of infection (MOI) of 100 for 48hrs. (B) Cholesterol efflux to 10 μ g/ml of Apo A-I in HepG2 cells transduced with adenovirus-alkaline phosphatase (control) or adenovirus-miR-33a (ABM) at a MOI=100 for 48hrs.

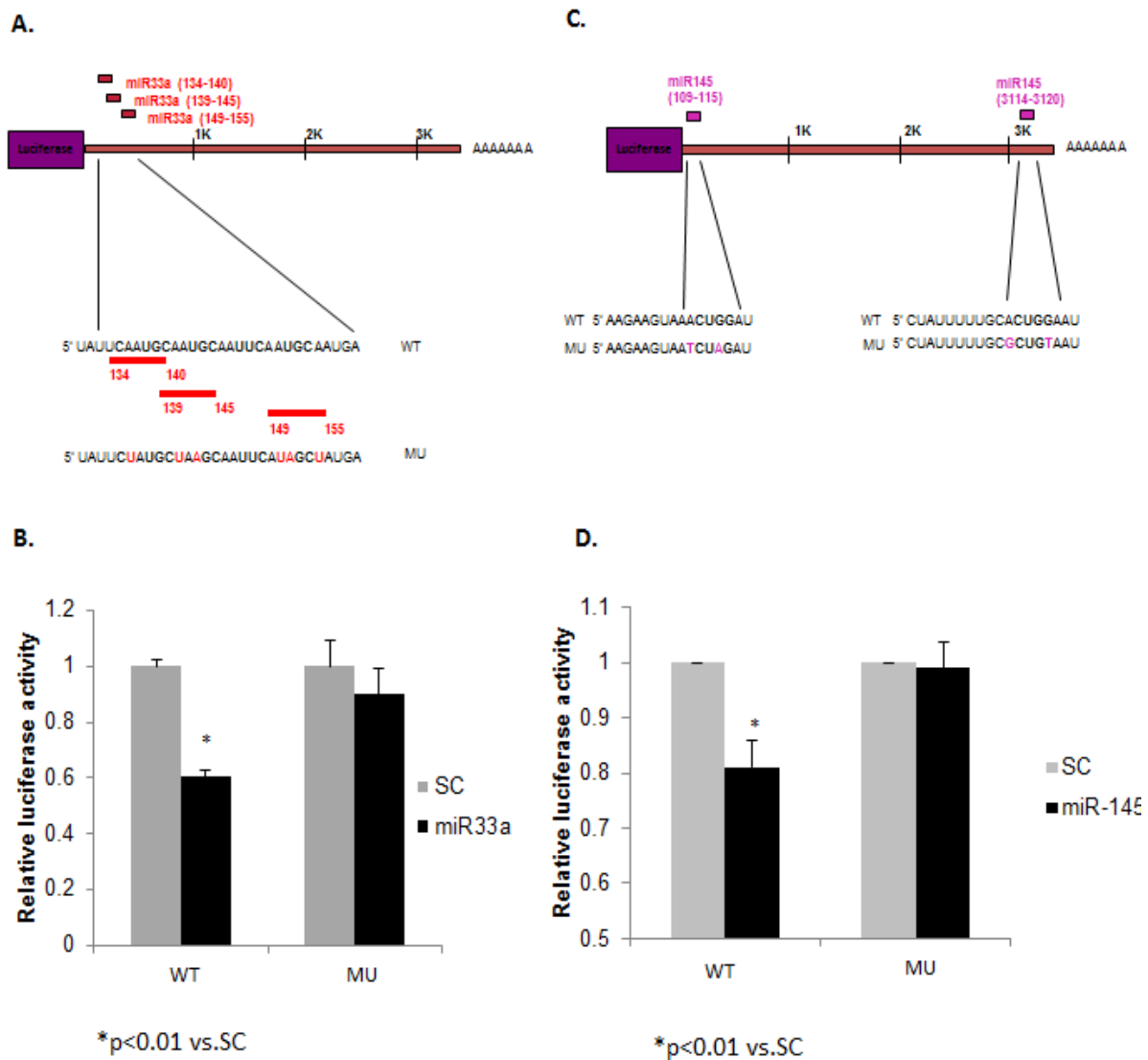


Figure 4.4. Luciferase activity following mutation in the binding sites of miR33a and miR-145. (A) A drawing of the human ABCA1 3'UTR reveals three potential of miR-33a at nucleotides: 134-140; 139-145; and 149-155 downstream of the ABCA1 stop codon (TargetScan 5.2 prediction; the seed sequences are shown only). At least two nucleotides in each of the seed sequences were mutated: 134-140 (135:A→U, 140:A→U); 139-145 (140:A→U, 142:U→A); and 149-155 (151:A→U, 152:U→A, 155:A→U). (B) The luciferase reporter containing the wild-type (WT) or the miR-33a binding site mutations (MU) 3'UTR of ABCA1 were transfected into U343 cells. The change in luciferase activity following miR-33a transfection is expressed in comparison to cells transfected with control miRNA for both WT and MU. (C) A drawing of the human ABCA1 3'UTR reveals two potential binding sites of miR-145 at nucleotides: 109-115; and 3114-3120 downstream of the ABCA1 stop codon (TargetScan 5.2 prediction; the seed sequences are shown only). Two nucleotides in each of the seed sequences were mutated: 109-115 (110:A→T, 113:G→A); and 3114-3120 (3114:A→G, 3118:G→T). (D) The luciferase reporter containing the wild-type (WT) or the miR-145 binding site mutations (MU) 3'UTR of ABCA1 were transfected into U343 cells. The change in luciferase activity following miR-145 transfection is expressed in comparison to cells transfected with control miRNA for both WT and MU.

4.3.6 Manipulation of miR-145 Expression Levels Alters ABCA1 Protein Expression and Cholesterol

Efflux Activity

To examine the effect of miR-145 expression on ABCA1 protein levels, we transfected 50nM of pre-miR-145 mimics into HepG2 cell lines and analyzed endogenous ABCA1 protein expression by Western blotting. We observed a decrease in ABCA1 protein expression following miR-145 expression. However, this decrease was not to the same level as achieved following miR-33a over-expression (**Figure 4.5.A**). Similarly, miR-145 significantly repressed cholesterol efflux activity ($p=0.04$), but not to the same magnitude as miR-33a (**Figure 4.5.B**). miR-17 is a miRNA expressed in the liver and was predicted to target ABCA1 by four out of the five prediction programs (**Table 4.1**). However our reporter assay did not show any effect following miR-17 expression. To ensure the results from our reporter assay are valid (**Figure 4.2.B**), we performed Western blot analysis and cholesterol efflux activity following miR-17 expression. miR-17 overexpression demonstrated no change in ABCA1 protein levels or efflux function (**Figures 4.6.A and 4.6.B**). miR-33a was utilized as a positive control, and control pre-miRNA was utilized as a negative control. Thus, these findings support our reporter assay results (**Figure 4.2.B**).

4.3.7 The Combined Effects of miR-33a and miR-145

It is generally believed that the majority of miRNAs have subtle effects on their mRNA targets (Small and Olson, 2011). miR-33a is an exception where the modulation of this single miRNA has profound effects on ABCA1 protein expression, cholesterol efflux activity, HDL levels, reduction in atherosclerotic plaque size, and lowering of VLDL TG levels (Hani Najafi-Shoushtari et al., 2010; Rayner et al., 2010; Marquart et al., 2010; Rayner et al., 2011; Rayner et al., 2011). However, it is believed that a combination of miRNAs known to target a single gene can enhance the effect over a single miRNA acting alone (Rottiers and Naar, 2012). The combination of miRNAs targeting ABCA1 may amplify the effects induced by miR-33a alone. To test this hypothesis, we co-transfected miR-33a and miR-145 into HepG2 cells and analyzed ABCA1 protein expression and cholesterol efflux activity. The combined effect of miR-

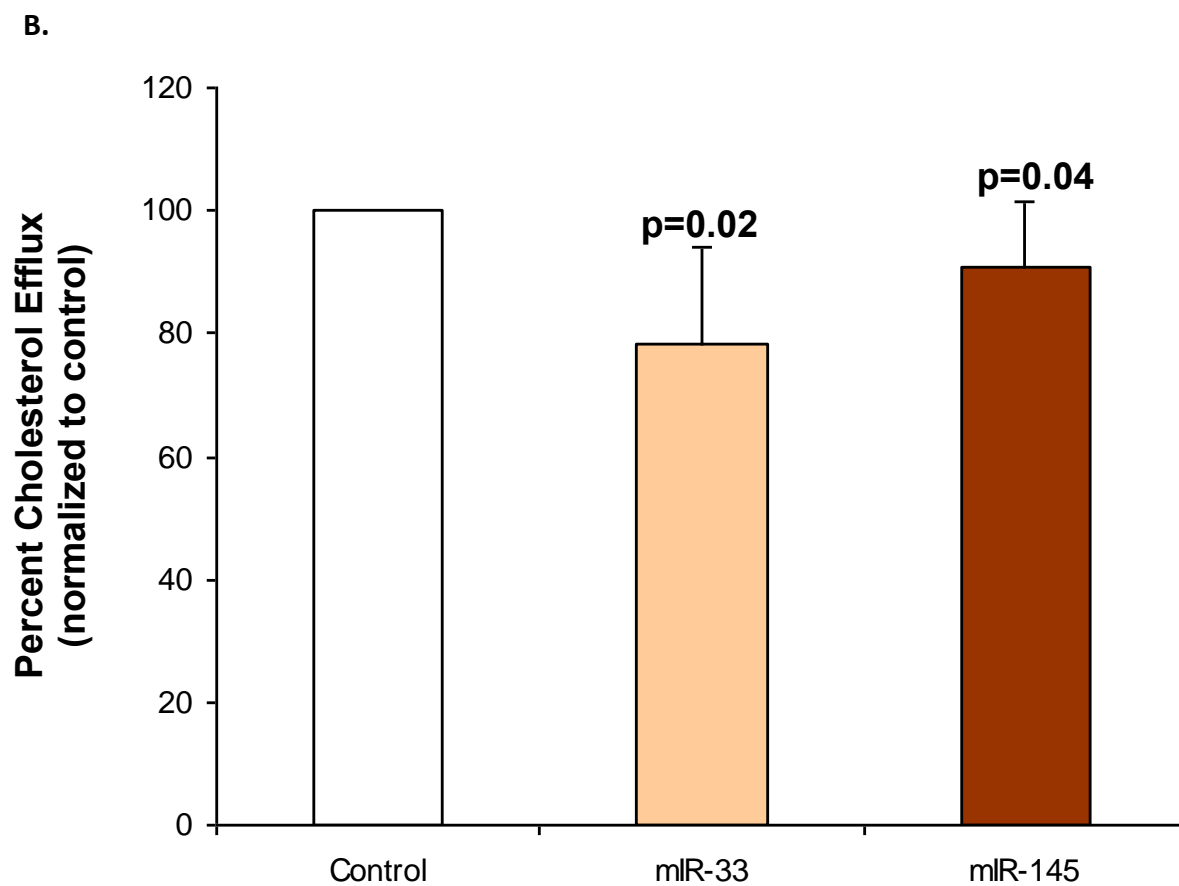
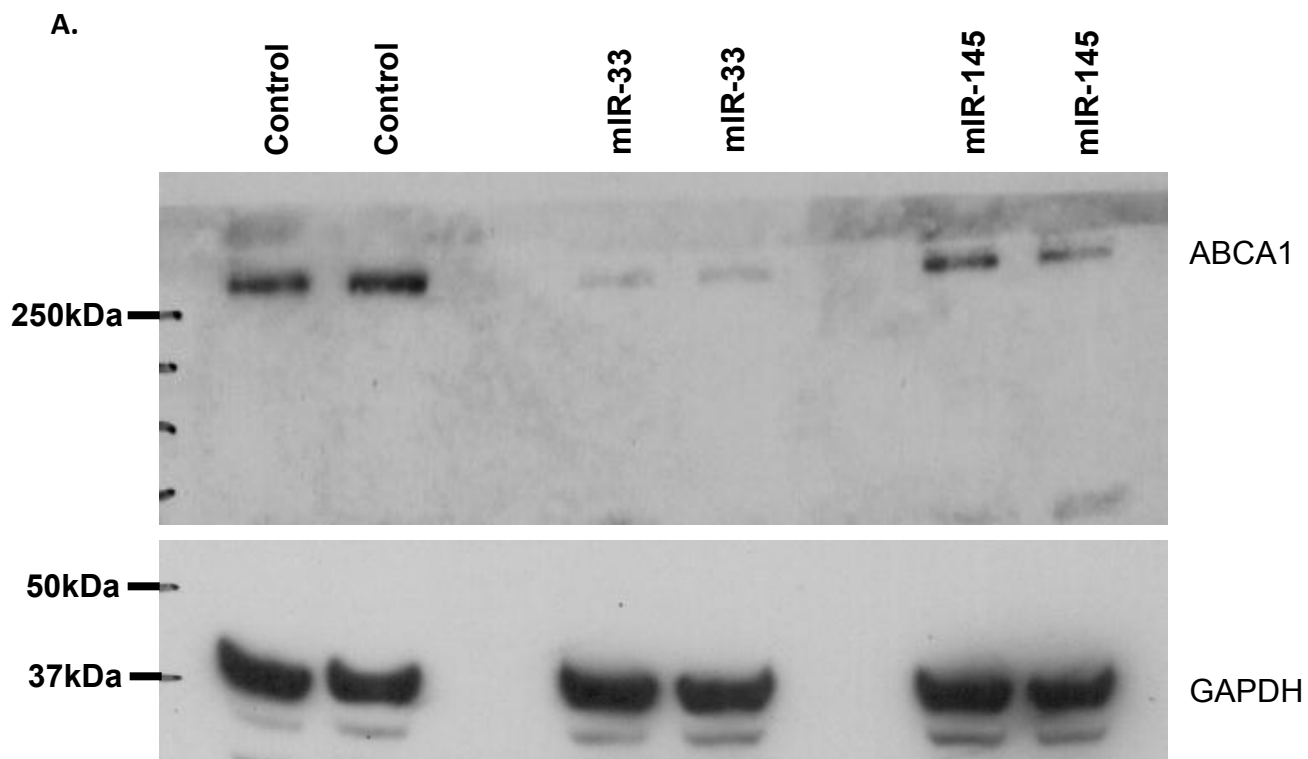
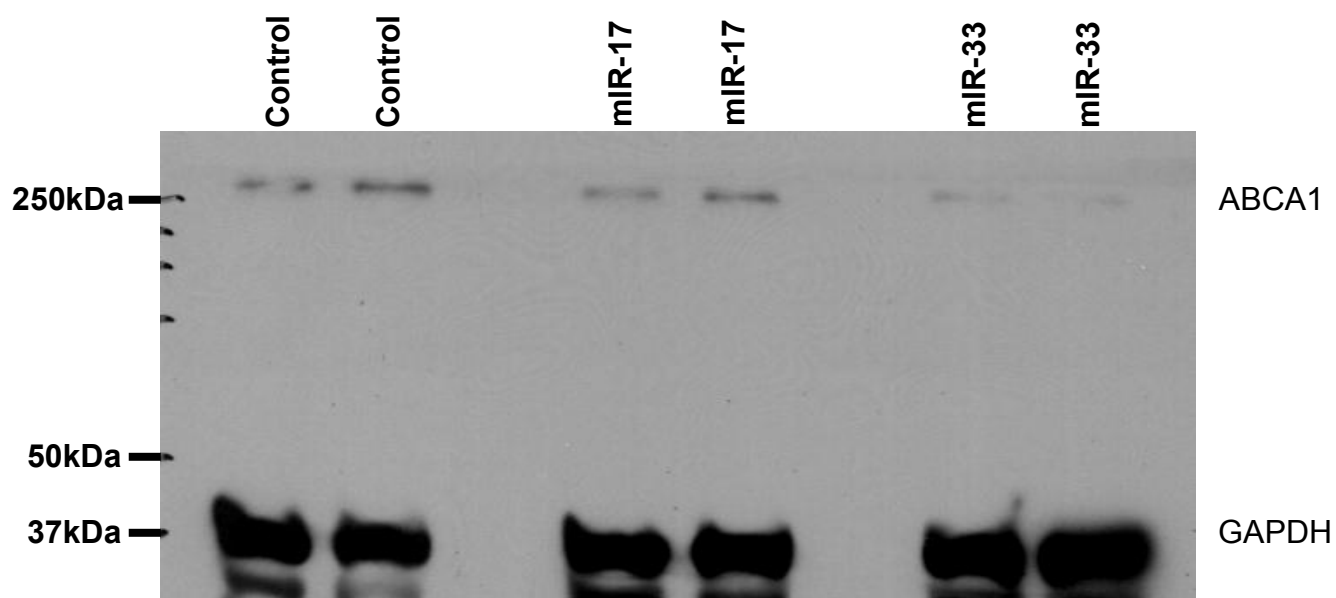


Figure 4.5. Assessment of ABCA1 post-transcriptional regulation by control miR, miR-33a, and miR-145. A. Western blot analysis of ABCA1 in HepG2 cells transfected with 50nM of Control miR, miR-33a, or miR-145 for 48 hrs. B. Cholesterol efflux activity to 10µg/ml Apo A1 in HepG2 cells transfected with 50nM of Control miR, miR-33a, or miR-145 for 48 hrs.

A.



B.

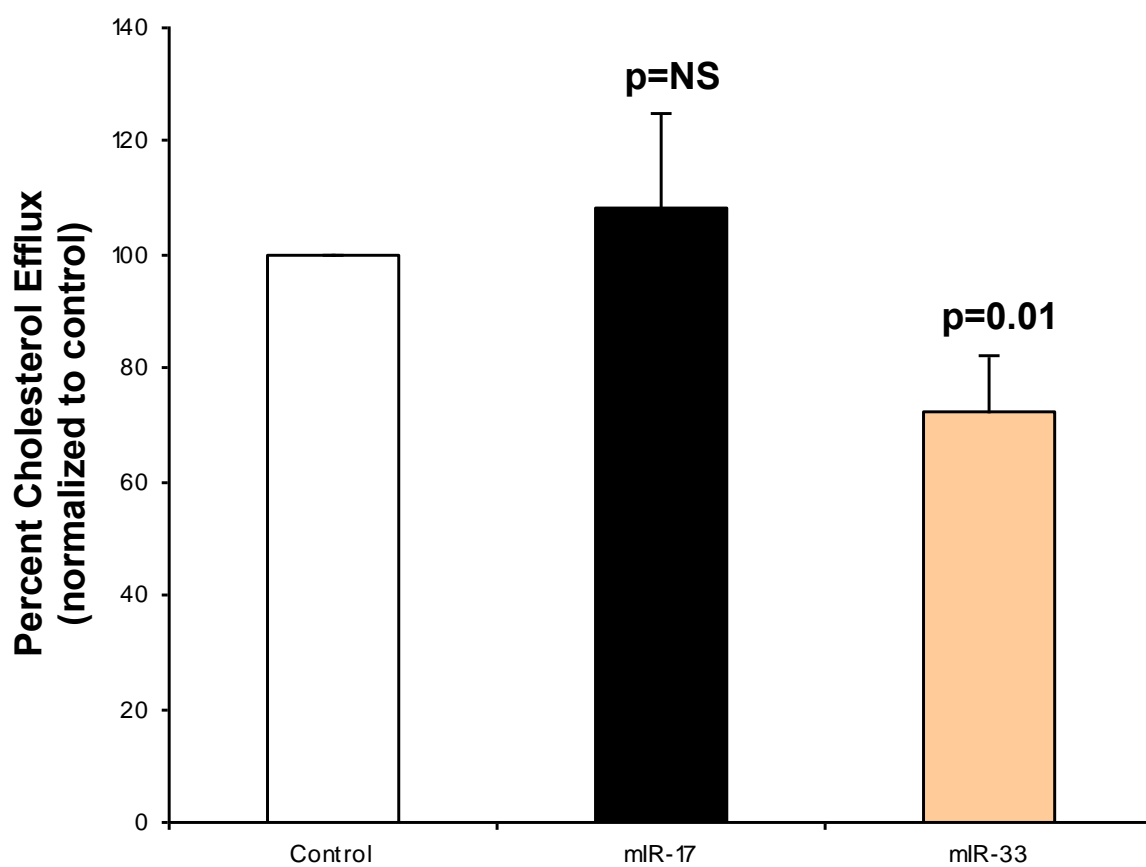


Figure 4.6. Assessment of ABCA1 post-transcriptional regulation by control miR, miR-17, and miR-33a. A. Western blot analysis of ABCA1 in HepG2 cells transfected with 50nM of Control miR, miR-17, or miR-33a for 48 hrs. B. Cholesterol efflux activity to 10µg/ml Apo A1 in HepG2 cells transfected with 50nM of Control miR, miR-17, or miR-33a for 48 hrs.

33a and miR-145 did not show a dramatic difference in protein levels (**Figures 4.7.A and 4.7.B**) or cholesterol efflux activity (**Figure 4.7.C**) over miR-33a alone. We also observed that the inhibition of endogenous miR-33a, miR-145, and a combination of miR-33a and miR-145 in HepG2 cells increases ABCA1 protein expression levels (**Figure 4.8**). Again, the combined effects of inhibitors to miR-33a and miR-145 did not significantly enhance ABCA1 expression over the single inhibitors alone.

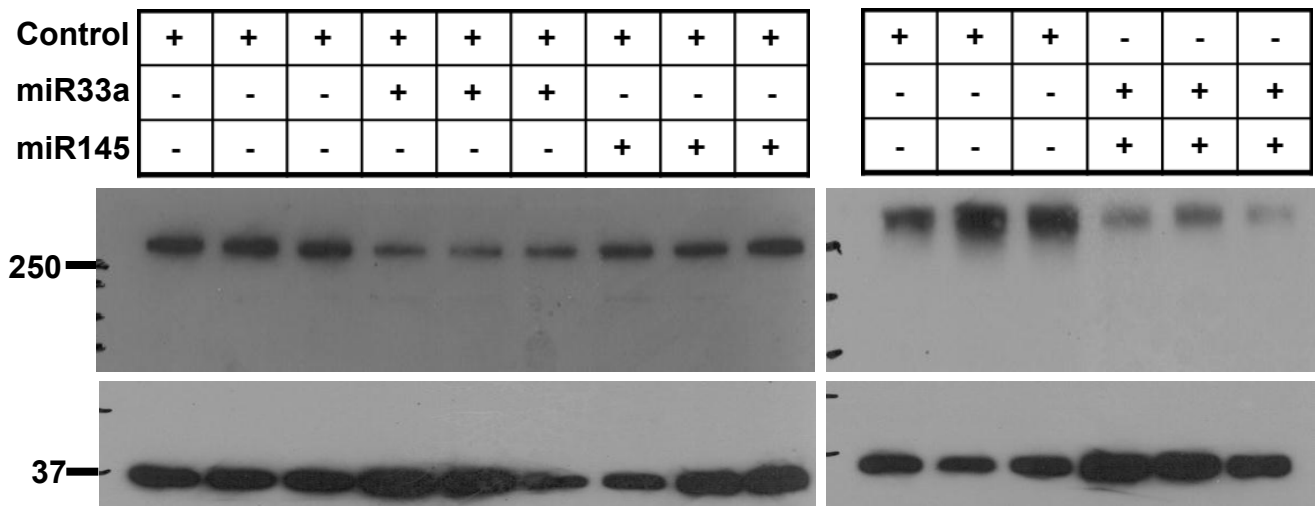
4.3.8 miR-145 Expression Levels

miRNAs have a wide range of expression in different cells ranging from less than 1 copy to greater than 10000 copies per cell. Target regulation of a gene is partly dependent on miRNA expression levels. Less than 100 copies per cell is considered too low for a miRNA to significantly repress a target (Brown and Naldini, 2009). Higher miRNA expression levels correlate with enhanced suppression of target transcripts. To determine the relative expression of miR-33a and miR-145 in various tissues and cell lines, we performed TaqMan analysis. In human liver tissues, miR-145 has greater than a three-fold expression level than miR-33a (**Figure 4.9**). This finding suggests that inhibition of miR-145 may have greater effects than miR-33a on ABCA1 expression in the human liver.

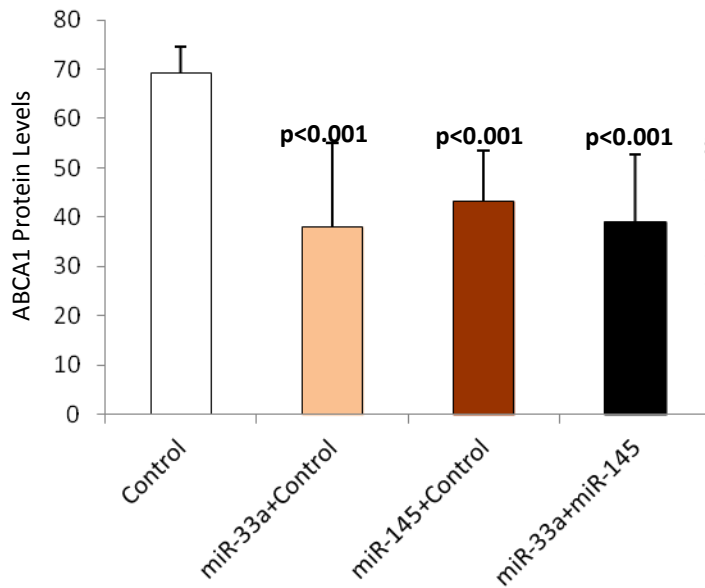
4.3.9 Adenovirus Expression

In order to test the in vivo effects of miR-145 modulation on HDL levels, adenovirus-control (empty vector), -miR-33a, -miR-145, -anti-miR-33a, and -anti-miR-145 were designed to express either one copy of the miRNA or one copy of the inhibitor. The adenoviral miRNAs or their inhibitors induced a similar effect on ABCA1 protein expression in HepG2 cells (**Figures 4.10 and 4.11**) as our studies using the pre-miR mimic oligonucleotides, or the anti-miR inhibitor oligonucleotides.

A.



B.



C.

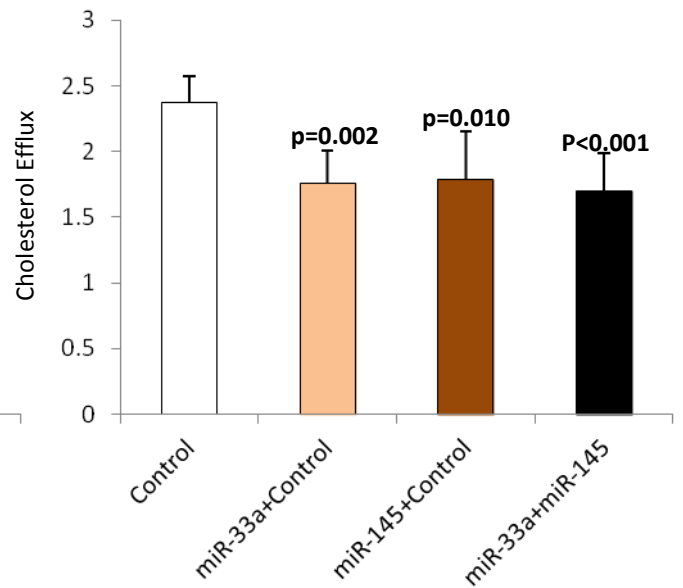


Figure 4.7. Assessment of ABCA1 post-transcriptional regulation by control miR, miR-33a, miR-145 and miR-33a + miR-145. A. Western blot analysis of ABCA1 in HepG2 cells transfected with Control miR (100nM), miR-33a (50nM) + Control miR (50nM), miR-145 (50nM) + Control miR (50nM), or miR-33a (50nM) + miR-145 (50nM) for 48 hrs. B. Quantification of Western blot using BioRad Quantity1™. C. Cholesterol efflux activity to 10µg/ml Apo A1 in HepG2 cells transfected with Control miR (100nM), miR-33a (50nM) + Control miR (50nM), miR-145 (50nM) + Control miR (50nM), or miR33a (50nM) + miR-145 (50nM) for 48 hrs. Statistical significance was measured using the Bonferroni method based on four comparisons of interest. Significance is set at $p \leq 0.0125$. $n=8$ for protein measurements and cholesterol efflux assays.

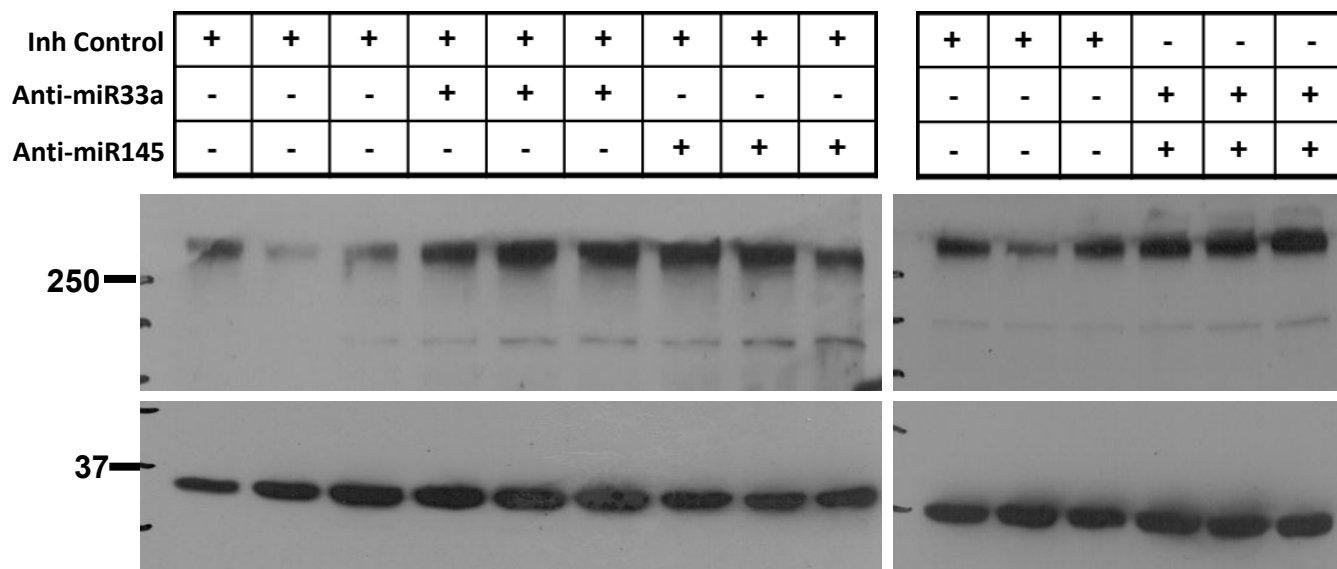


Figure 4.8. Assessment of ABCA1 post-transcriptional regulation by inhibition of endogenous miRNAs. Western blot analysis of ABCA1 in HepG2 cells transfected with Inhibitor Control miR (200nM), anti-miR-33a (100nM) + Inhibitor Control miR (100nM), anti-miR-145 (100nM) + Inhibitor Control miR (100nM), or anti-miR-33a (100nM) + anti-miR-145 (100nM) for 48 hrs.

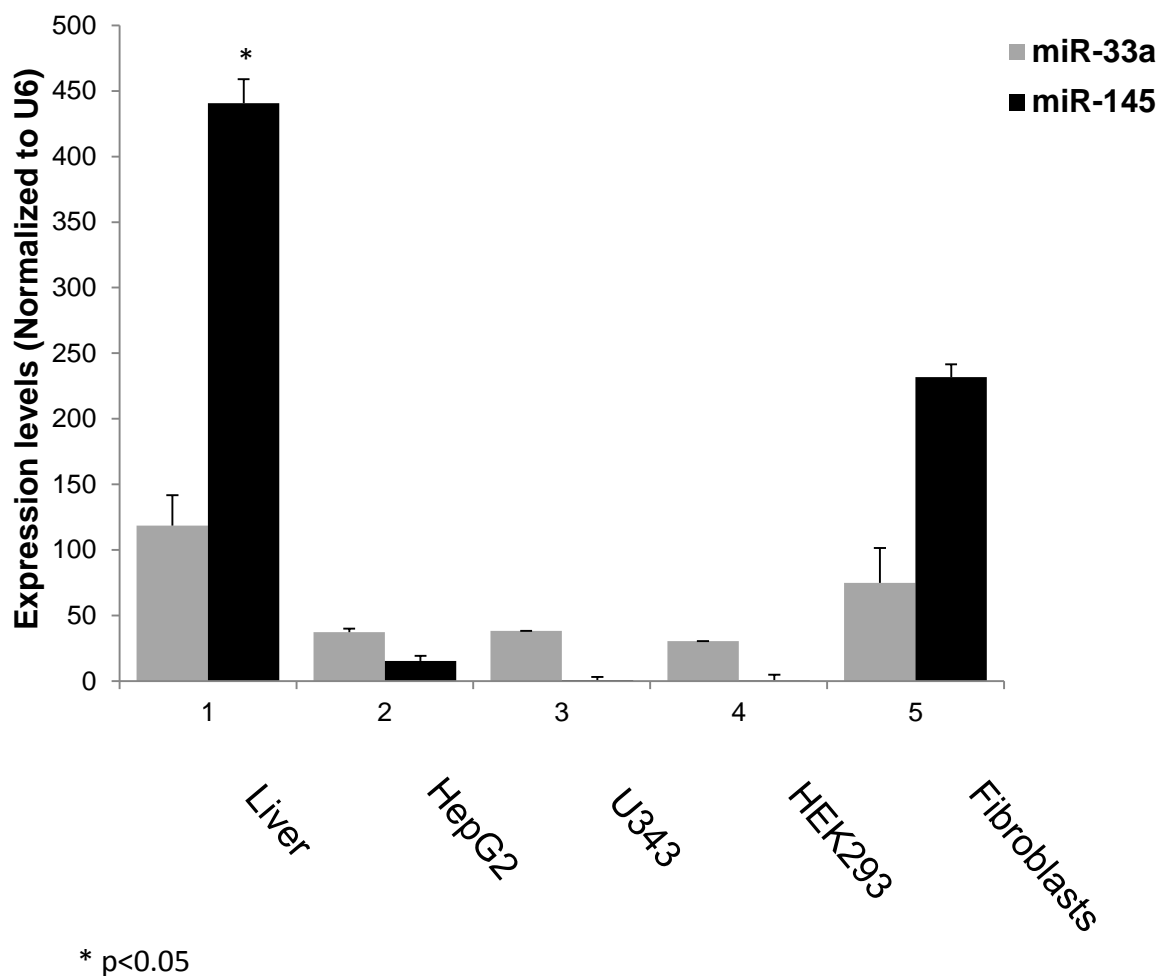


Figure 4.9. Expression of miR-33a and miR-145 in select tissues and cell lines. miR-33a and miR-145 levels were measured by TaqMan in human liver tissue, HepG2 human hepatocytes, U343 human neuronal glioblastoma cells, human embryonic kidney (HEK)-293 cells, and human skin fibroblasts. All expression levels are normalized to internal U6 control RNA.

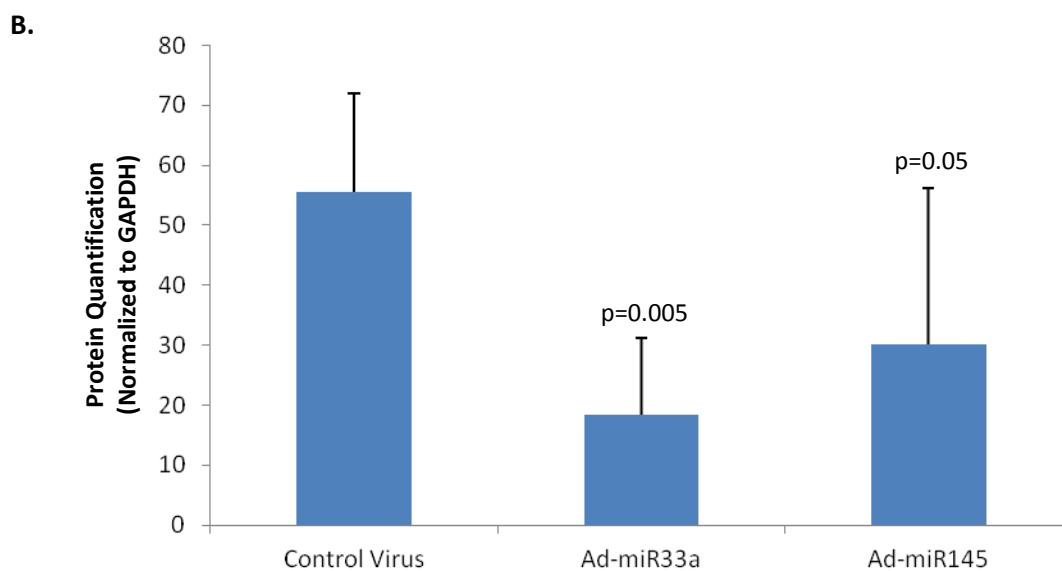
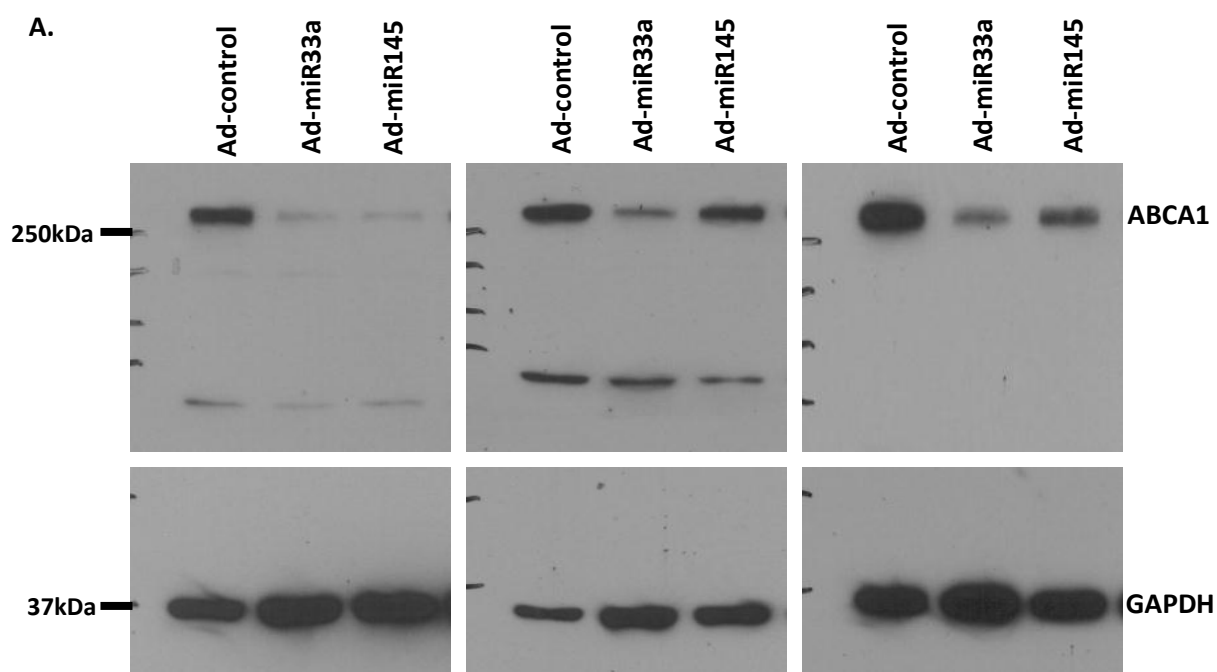


Figure 4.10. ABCA1 post-transcriptional regulation by adenovirus expression of control (empty vector), miR-33a, and miR-145 in HepG2 cells. A. Three independent experiments showing Western blots of ABCA1 in HepG2 cell lines transduced with adenovirus control, adenovirus miR-33a, and adenovirus miR-145, at a multiplicity of infection (MOI) of 100 and a 48 hour incubation. The loading control is GAPDH. B. Protein quantification of the three Western blot experiments was determined using BioRad Quantity1™. Results are normalized to respective GAPDH loading controls.

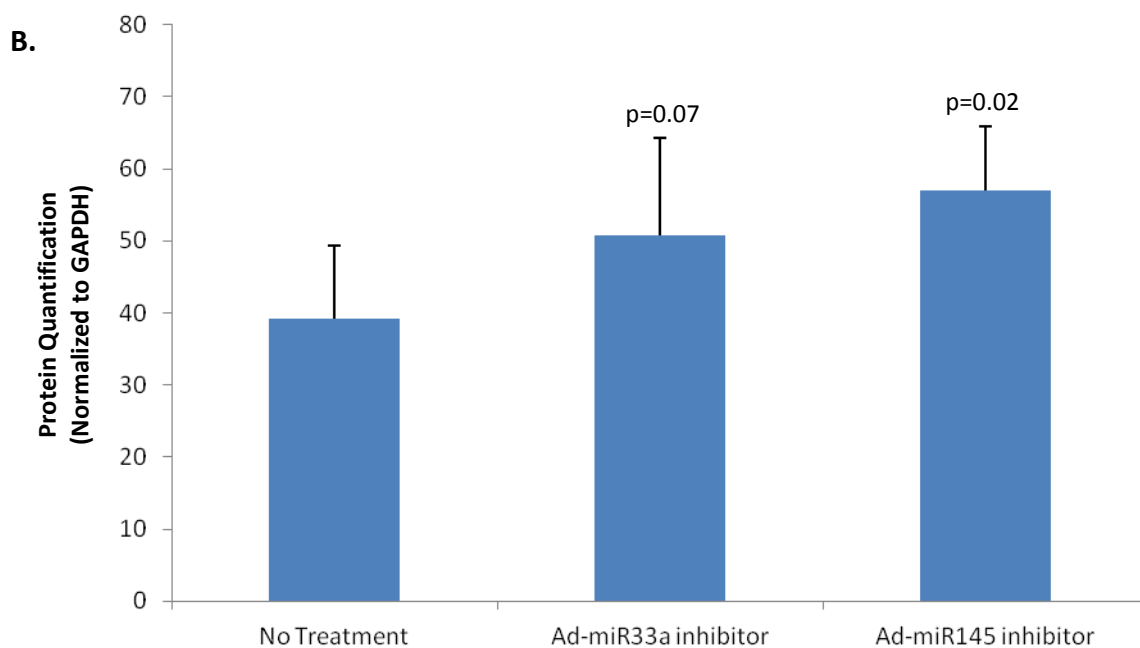
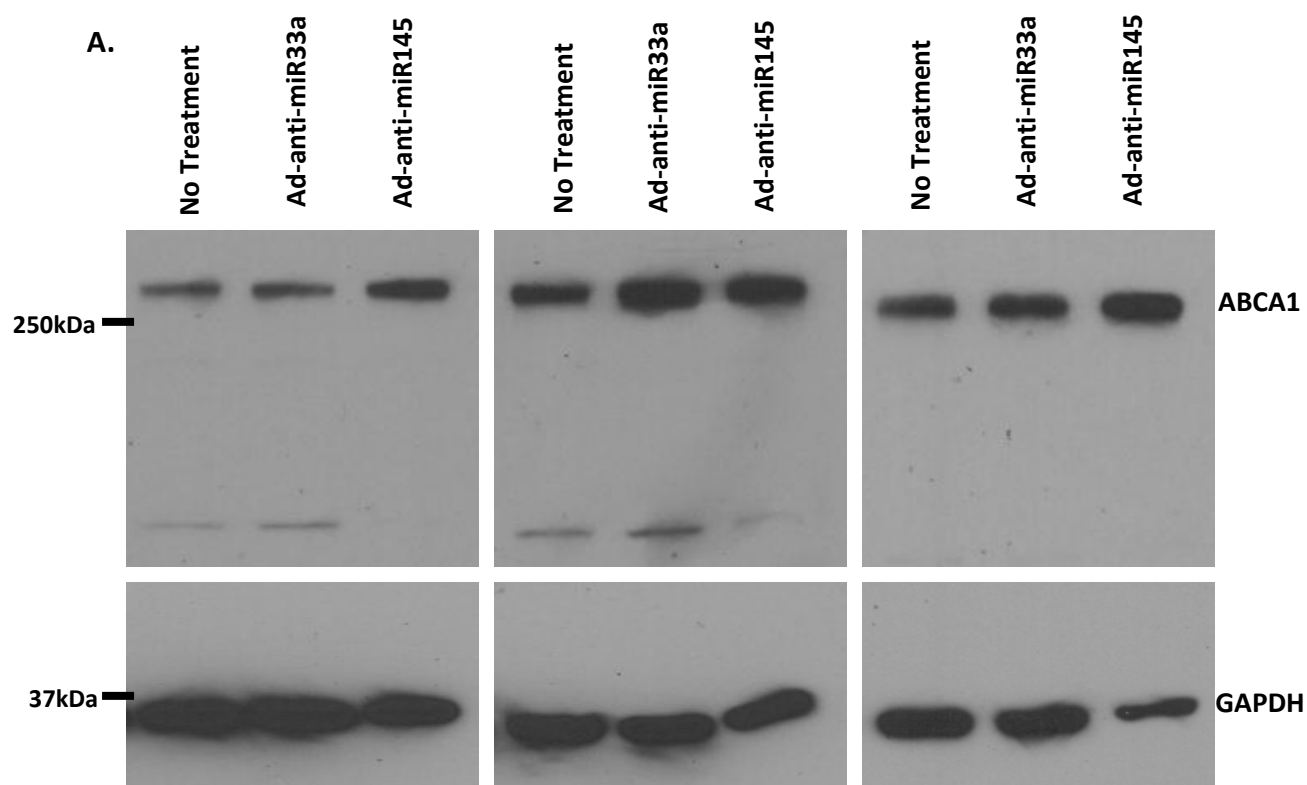


Figure 4.11. ABCA1 post-transcriptional regulation by adenovirus expression of control (empty vector), anti-miR-33a, and anti-miR-145 in HepG2 cells. A. Three independent experiments showing Western blots of ABCA1 in a HepG2 cell lines transduced with adenovirus control, adenovirus anti-miR-33a, and adenovirus anti-miR-145, at a multiplicity of infection (MOI) of 100 and a 48 hour incubation. The loading control is GAPDH. B. Protein quantification of the three Western blot experiments was determined using BioRad Quantity1™. Results are normalized to respective GAPDH loading controls.

Cholesterol efflux studies using the adenovirus-miRNAs also agreed with our previous results using the pre-miRNA oligonucleotides (**Figure 4.12**). These results suggest the expression of miRNAs or their inhibitors using different methods (oligonucleotide-based or adenoviral-based) are equally effective in modulating ABCA1 expression and function in vitro (HepG2 cells). The low costs and ease of reproducing adenoviruses encouraged our use of viral technology over miRNA oligonucleotide sequences for our in vivo studies.

4.3.10 In Vivo Studies

Previous studies have demonstrated the efficacy of delivering miR-33a by lentivirus (Rayner et al., 2010) or adenovirus (Marquart et al., 2010) in repressing circulating HDL levels by decreasing ABCA1 protein expression. Our ultimate goal is to study the effect of miR-145 modulation on ABCA1 expression and HDL levels in vivo. We initially carried out a proof-of-concept study to determine if we could replicate the findings from the previous groups. Lentiviral-miR-33a over-expression decreased HDL levels by 22% after six days (Rayner et al., 2010). The over-expression of miR-33a with adenovirus reduced HDL-cholesterol levels by 29% after five days compared to mice injected with adenovirus-GFP (Marquart et al., 2010). Both groups saw a decrease in hepatic ABCA1 protein expression in the miR-33a treated mice. We designed a similar study where 9-10 week old male C57Bl6/J mice were injected with 1×10^{10} pfu/mouse of miR-33a or control adenovirus (empty vector) (**Figure 4.13**). After seven days, miR-33a over-expression reduced hepatic ABCA1 protein levels (**Figure 4.14**) and reduced HDL cholesterol levels by 20% ($p=0.001$) (**Figure 4.15.B**) compared to mice injected with control adenovirus. This decrease in HDL levels agrees with the previous results from Rayner et al., and Marquart et al.

Adenovirus dosage was a critical issue in these studies. Low doses ($\leq 1 \times 10^9$ pfu/mouse) or high doses ($\geq 5 \times 10^{10}$ pfu/mouse) of adenovirus-miR33a showed no effects on HDL levels (results not shown). This finding has been observed with other groups working with adenovirus-miR-33a (personal communication with Dr Angel Baldan). Whether this is due to toxicity from the adenovirus, or to a

narrow effective range of miRNA expression is unknown. Studies involving miR-145 or the inhibitors to miR-33a and miR-145 will likely require a range of dosages to determine the effective therapeutic range. Studies with miR-145 will follow the design of the miR-33a proof-of-concept study (**Figure 4.13**).

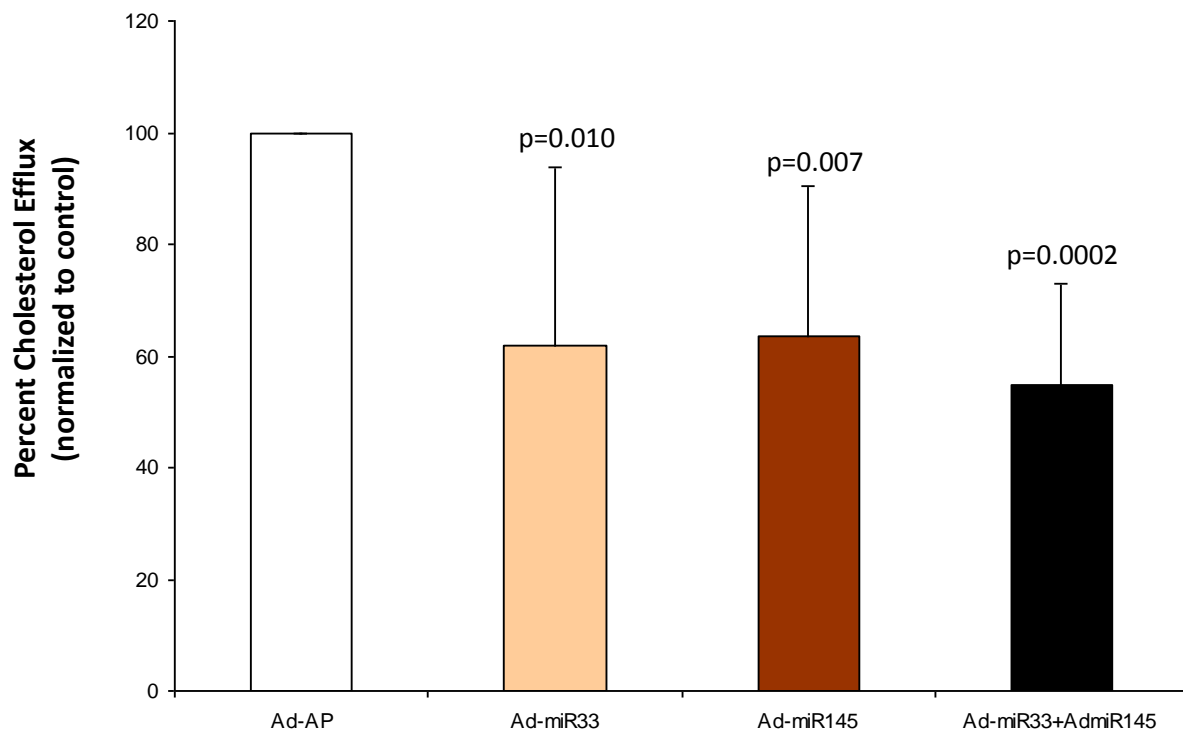


Figure 4.12. ABCA1 post-transcriptional regulation by adenovirus expression of control, -miR-33a, -miR-145, and miR-33a+miR-145 in HepG2 cells. Cholesterol efflux to apo A-I was assessed in HepG2 cells transduced with adenovirus at a MOI=200 for 48hrs. Statistical significance is based on the Bonferroni method ($p \leq 0.0125$).

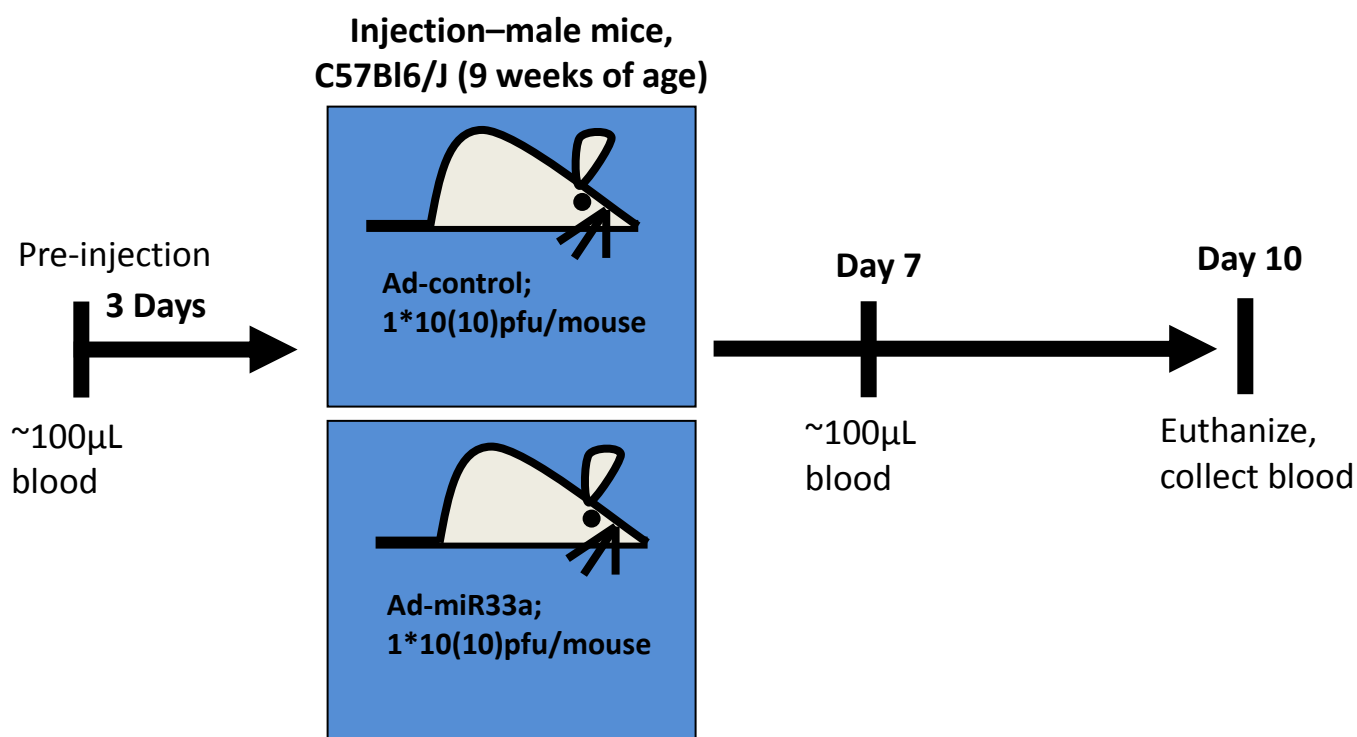


Figure 4.13. *In vivo* study design. Previous groups (Rayner et al., 2010; Marquart et al., 2010) identified the over-expression of miR-33a as decreasing HDL levels *in vivo*. We carried out a proof-of-concept study in order to replicate these findings. Three days before injection of adenovirus ~100µl of blood was taken from 9-10 week old C57Bl6/J male mice through the saphenous vein. HDL cholesterol levels were measured from serum to assure mice given the control or miR-33a expressing virus had equal HDL-cholesterol levels. On the day of injection, mice were tail-vein injected with either 1×10^{10} pfu of adenovirus-control or -miR-33a. Sterile PBS was used as a vehicle. Seven days post-injection, ~100µl of blood was taken from the saphenous vein and HDL cholesterol levels were measured from serum. Ten days post-injection, mice were euthanized with carbon dioxide. Blood and tissues (liver, small intestine, adipose, brain) were collected. HDL and total cholesterol (TC) levels were measured from serum.

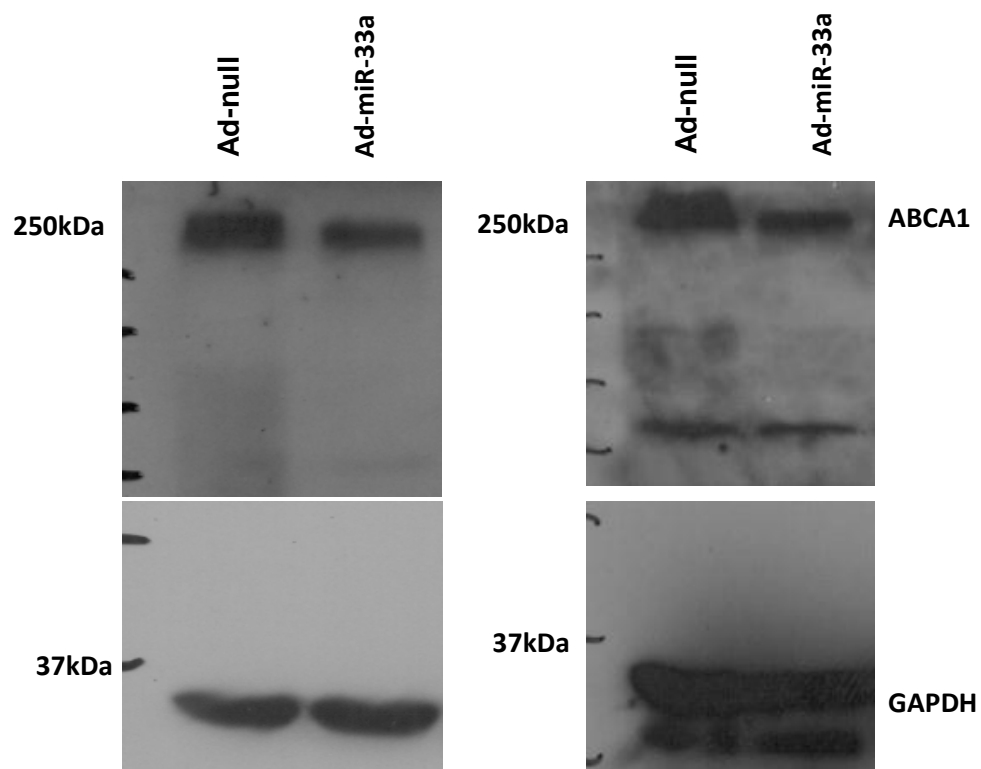
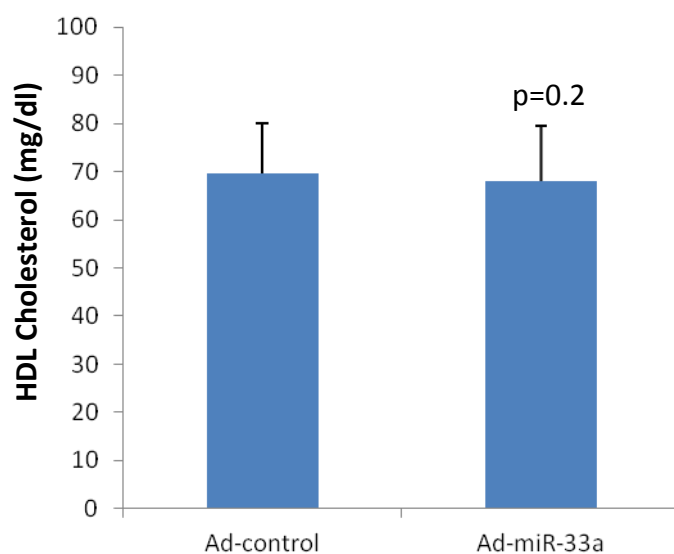
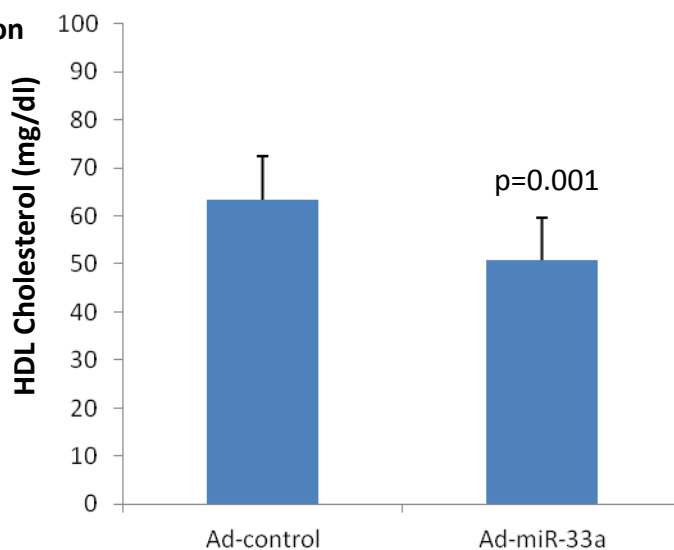


Figure 4.14. Overexpression of miR-33a decreases hepatic ABCA1 protein levels. ABCA1 protein expression in the liver was assessed by Western blotting in mice seven days after adenoviral control or miR-33a injection.

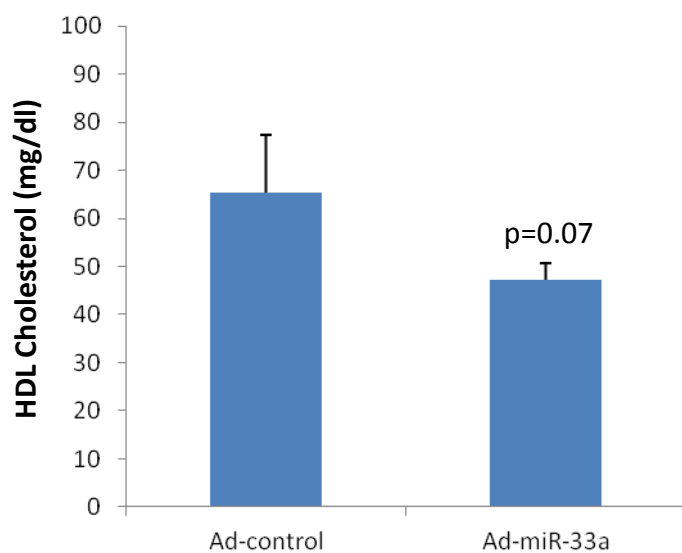
A. Pre-injection



B. Day 7 Post-injection



C. Day 10 Post-injection



D. Day 10 Post-injection

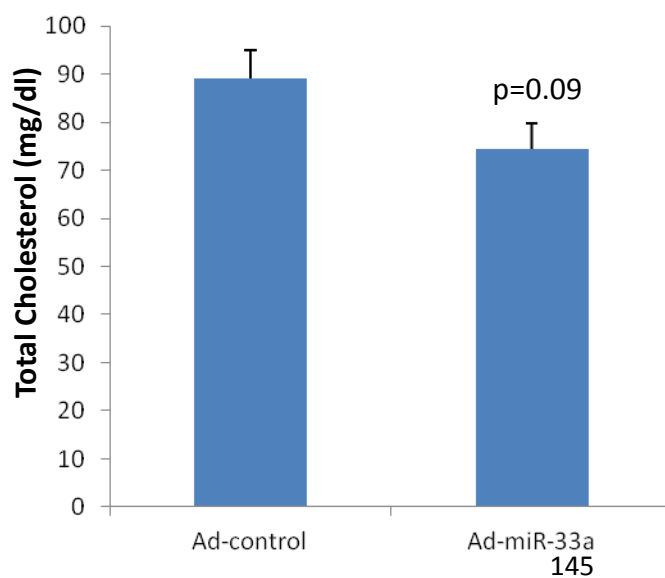


Figure 4.15. Over-expression of miR-33a decreases HDL levels. HDL cholesterol was measured in mice (A) before viral injection (n=7), (B) seven days after injection (n=7), and (C) ten days after injection (n=4). Total cholesterol was measured in mice (D) ten days after injection (n=4). HDL and total cholesterol were measured in the serum of mice using colorimetric enzymatic kits.

4.4 Discussion

The targeted deletion of ABCA1 in the different tissues of mice has highlighted the important role of maintaining cholesterol homeostasis in different organs. The inability to remove cholesterol from a cell can instigate pathological processes that may lead to diseases such as CVD, type 2 diabetes, and Alzheimer's disease (Tang and Oram, 2009; Fan et al., 2009). Because of this, the regulation of ABCA1 is a potential strategy to remove excess cholesterol from cells, and to maintain the proper functioning and health of different tissues. Multiple groups validated miR-33a as a regulator of ABCA1 expression in hepatic cells and macrophages, and found that the inhibition of miR-33a could increase HDL levels and reverse atherosclerotic plaque size in vivo by increasing ABCA1 expression (Rayner et al., 2012). We generated adenoviruses expressing miR-33a and miR-33a inhibitor to regulate ABCA1 in islet cells. miR-33a expression in pancreatic islets decreased ABCA1 expression, decreased insulin secretion, and increased islet cholesterol levels (Wijesekara et al., 2012). The inhibition of endogenous miR-33a in pancreatic islets increased ABCA1 protein expression, increased insulin secretion, and decreased islet cholesterol levels (Wijesekara et al., 2012). This validates the targeting of miR-33a as a potential strategy to regulate ABCA1 in β -cells.

Using an in silico approach we attempted to identify a number of miRNAs besides miR-33a that could regulate ABCA1. In silico approaches remain the only means to rapidly identify a potential miRNA target (Alexiou et al., 2009). Unfortunately the lack of clear miRNA target recognition rules means most bioinformatics predictions are false (Gommons and Berezikov, 2012). It has been calculated that commonly used prediction programs such as TargetScan and PicTar have a precision of ~50% and a sensitivity between 6 to 12% (Alexiou et al., 2009). To narrow down the number of miRNA predicted to

target the 3'UTR of ABCA1 we combined multiple algorithms. However, this approach is not always recommended as increased specificity is achieved at the cost of decreased sensitivity (Alexiou et al., 2009). Despite this caveat, our approach to combine algorithms identified multiple miRNAs including miR-33a as potential regulators of ABCA1. As miR-33a was validated by others in regulating ABCA1, we focused on miR-145 because this miRNA demonstrated the second greatest impact on the activity of our construct after miR-33a in our luciferase reporter assay. By mutating the two TargetScan5.2 predicted binding sites of miR-145, we rescued the repression of luciferase activity following miR-145 expression. This confirms that miR-145 directly binds to the 3'UTR to post-transcriptionally regulate ABCA1. We also demonstrated in a relevant cell line for HDL biogenesis, the HepG2 cell line, that miR-145 expression regulates ABCA1 protein levels and cholesterol efflux function. All our studies compared the regulatory effects between miR-145 and miR-33a to compare the magnitude of ABCA1 regulation between the two miRNAs.

Although miR-145 over-expression significantly diminishes ABCA1 protein and cholesterol efflux activity, it does not do so to the same extent as miR-33a. A likely reason for this is the context in which the binding sites for these miRNAs reside in at the 3'UTR of ABCA1. Features that boost miRNA site efficacy includes: proximity to the stop codon; possessing multiple sites for one miRNA; and two or more binding sites in close proximity to one another (Bartel, 2009). TargetScan predicts three miR-33a binding sites very close to one another (134-140, 139-145, 149-155) near the stop codon of ABCA1. Multiple sites often produce a multiplicative or synergistic effect compared to a single site alone. Two sites close together also tend to cooperate to increase repression compared to the independent contribution of two different miRNAs at two separate sites (Bartel, 2009). In comparison to miR-33a, miR-145 has a binding site close to the stop codon (109-115), and one binding site 3000bp away near the poly A tail of the ABCA1 3'UTR (3114-3120). The multiple neighboring sites of miR-33a is a likely reason why the repressive effects of this miRNA are much greater than miR-145 or the other validated miRNAs that

regulate ABCA1, miR-758 (Ramirez et al., 2011), and miR-106b (Kim et al., 2011). However, a direct comparison of the effects of miR-758 and miR-106b to miR-33a on ABCA1 levels or function were not performed by either group so whether the effects of miR-33a are much greater than miR-758 or miR-106b is unknown. Despite the difference in regulatory ability, miR-145 appears to be a potentially attractive target to modulate ABCA1 due to its high expression level in human liver samples compared to miR-33a. It is possible that miR-145 inhibition may have a greater effect on HDL levels than miR-33a inhibition in humans due to the differences in miRNA expression levels.

Few biological processes are strictly dependent on a single miRNA and often involve multiple members of a miRNA family, unrelated miRNAs, or miRNAs and transcription factors (Small and Olson, 2011). We investigated the possibility that miR-33a and miR-145 could cooperate to enhance ABCA1 repression. However, we did not observe a significant change in ABCA1 levels or function following miR-33a and miR-145 expression over either single miRNA alone. One group did report a slight additive effect of miR-758 and miR-33a expression on ABCA1 levels (Ramirez et al., 2011). The lack of a large cooperative phenotype observed following the combination of miR-33a with another miRNA may be due to the already large effect of miR-33a on ABCA1 levels and activity.

In order to assess the impact of modulating miR-145 on ABCA1 in vivo we generated adenovirus expressing miR-33a and miR-145 along with their respective inhibitors. A proof-of-concept study using adenovirus-miR-33a produced similar decreases in HDL levels as previous groups (Rayner et al., 2010; Marquart et al., 2010). We are currently assessing the impact of modulating miR-145 in vivo on ABCA1 expression and HDL levels.

There are a number of differences between miR-33a and miR-145. Unlike miR-33a which lies in intron 16 of the human SREBF2 gene (Marquart et al., 2010), miR-145 lies in an intergenic region on chromosome 5 in humans and chromosome 18 in the mouse. Besides ABCA1, miR-33a targets genes involved in fatty acid degradation including carnitine palmitoyltransferase 1A (CPT1A) and carnitine O-

octanoyltransferase (CROT) (Rottiers and Naar, 2012). According to TargetScan, miR-145 is not predicted to target many genes involved in lipid metabolism but is predicted to regulate many ATP transporters including: ABCE1 (OABP); CFTR (ABCC7); ABCC1; and ABCG4. Other potential targets of miR-145 include SR-B1, and the palmitoyltransferase responsible for mediating ABCA1 palmitoylation, zDHHC8 (Singaraja et al., 2009).

In this work we have identified a novel miRNA repressor of ABCA1 protein translation, miR-145. miR-145 represents a novel strategy to post-transcriptionally regulate ABCA1 levels.

5. Conclusions

5.1 Conclusions and Future Directions

Epidemiological studies provide the strongest evidence that high levels of HDL are cardio-protective (Francis, 2010). Because of the high mortality rate associated with CVD, increasing HDL is considered a valid strategy to lessen the burden associated with this disease. A complete deficiency in ABCA1 results in TD, a disorder whose primary phenotype is extremely low to absent HDL levels (Oram, 2000). The prominent role that ABCA1 plays in HDL biogenesis makes ABCA1 a logical target to increase HDL (Tang and Oram, 2009).

ABCA1 undergoes multiple levels of regulation before generating a fully functional protein. Epigenetic modifications, transcription factors, microRNAs, post-translational modifications, and protein-protein interactions have all been identified as crucial regulators of ABCA1 expression and/or function. The significant increase in ABCA1 gene expression following LXR/RXR-mediated transcription is offset by the increase in the expression of genes that result in hepatic steatosis. Because of this problem we focused our attention on identifying post-transcriptional mechanisms that regulate ABCA1.

Proper efflux of cholesterol to apo A-I is associated with ABCA1 localization to the cell surface (Kang et al., 2010). We identified the lipid modification palmitoylation as a post-translational modifier critical for ABCA1 plasma membrane localization (Singaraja et al., 2009). A number of enzymes were identified that mediated the addition of [³H]-palmitate onto ABCA1. The over-expression of one of these enzymes, DHHC8, significantly increased ABCA1 cholesterol efflux activity. Our findings suggest increasing palmitoylation of ABCA1 through DHHC8 is a potential strategy to elevate efflux activity.

We attempted to assess the impact of endogenous DHHC8 on ABCA1 palmitoylation and activity levels. Using siRNA targeting of DHHC8, we observed a decrease in DHHC8 mRNA but no changes to ABCA1 palmitoylation levels or efflux activity. A reason for this lack of change may be due to multiple enzymes capable of mediating ABCA1 palmitoylation. To assess the true impact of the PAT enzymes on

ABCA1 function, we may be required to simultaneously target all five enzymes identified in increasing the metabolic incorporation of [^3H]-palmitate onto ABCA1. In vivo studies on the importance of DHH8 on HDL levels in mice can be carried out. Adenovirus-DHH8 constructs injected into the tail-vein of mice will primarily target the liver (Descamps and Benihoud, 2009). Due to the important role liver ABCA1 plays in initial HDL biogenesis (Timmins et al., 2005), increasing DHH8 expression in the liver should increase HDL levels in vivo. These studies can reveal the in vivo impact of palmitoylation on ABCA1 function.

The deacylation of proteins is also mediated by enzymes. However, only three PTEs have been discovered to date (Korycka et al., 2012) and none appear to be promising candidates in the deacylation of ABCA1. We found that increasing the PTE APT1 had little to no effect on ABCA1 palmitoylation or cholesterol efflux activity. This is not completely surprising as APT1 primarily depalmitoylates soluble proteins such as Ras, eNOS, and G α subunits (Korycka et al., 2012), although APT1 has also been recently shown to act as a thioesterase for membrane-spanning calcium-activated potassium channels (Tian et al., 2012). Future work could include determining whether the two remaining PTEs, APT2 or PPT1 deacylates ABCA1. So far, the only substrate identified for APT2 is the neuronal growth-associated protein-43 (GAP-43) (Tomatis et al., 2010). PPT1 is a lysosomal enzyme involved in the degradation of palmitoylated proteins (Korycka et al., 2012). Although ABCA1 localizes to the lysosome (Kang et al., 2010), and is associated with lysosomal degradation (Mizuno et al., 2011), genetic evidence does not suggest PPT1 depalmitoylates ABCA1. PPT1 deficiency causes infantile neuronal ceroid lipofuscinosis (INCL), a disorder with a mean age of death of 6.5 years, and phenotypic manifestations localized almost completely to the central nervous system. Affected individuals have a cerebral cortex almost completely devoid of neurons, while their routine blood tests are normal (Hofmann et al., 2002). It would be expected that any loss of function of an ABCA1 associated PTE would improve ABCA1 function and increase HDL levels.

Although palmitoylation is crucial for ABCA1 plasma membrane localization, it is not crucial for complete ABCA1 efflux activity. Failure of ABCA1 palmitoylation results in only a 50% loss of efflux activity while naturally occurring ABCA1 mutants typically lose 80-90% of their efflux function (Singaraja et al., 2009). Confirming these results, we found that a number of naturally occurring ABCA1 mutants revealed no alterations in their palmitoylation status.

ABCA1 appears to possess multiple, independent pathways and mechanisms to facilitate trafficking to the cell surface. For example, ceramide is a lipid signaling molecule that increases ABCA1 at the plasma membrane through an unknown mechanism (Witting et al., 2003). Rab8a is a GTPase protein involved in vesicular trafficking that also increases ABCA1 to the cell surface (Linder et al., 2009). We found both pathways localize ABCA1 at the plasma membrane independent of palmitoylation. Currently, a number of other proteins and post-translational modifications are associated with localizing or anchoring ABCA1 at the plasma membrane. These include: the binding of PDZ proteins such as α 1- or β 1-syntrophin to ABCA1; the binding of Liver X receptor beta (LXR β) to ABCA1; the binding of phospholipid transfer protein (PLTP) to ABCA1; and various phosphorylation sites on ABCA1. Future work includes possibly determining if any of these pathways and signals invokes palmitoylation to influence ABCA1 cell surface localization.

ABCA1 is highly glycosylated, but the exact role that this post-translational modification plays in the function of ABCA1 is unknown. Preliminary work using an NH₂-terminal fragment of ABCA1 indicates the type of oligosaccharide moiety bound to ABCA1 is linked to which cysteine is labeled with [³H]-palmitate. Following the addition of an oligosaccharide moiety to proteins in the ER, the sugar chain becomes modified by numerous enzymes as the protein traverses the ER and Golgi complex (GC). Our results suggest ABCA1 undergoes palmitoylation at cysteine-23 by transferases localized at the ER or up to the mid-Golgi, or at cysteine-3 by transferases localized beyond the mid-Golgi. We do not know if glycosylation influences palmitoylation, or if palmitoylation influences glycosylation, or if these post-

translational modifications modify ABCA1 independent of one another. Future work includes replicating these findings in full-length ABCA1, and to demonstrate that PAT enzymes localized at the different organelles mediate the palmitoylation of different cysteine residues. The importance of glycosylation for ABCA1 palmitoylation may be determined by treating cells with tunicamycin or brefeldin-A. Tunicamycin inhibits the initial addition of the sugar to proteins in the ER by inhibiting the generation of lipid-linked oligosaccharide precursors (McDowell and Schwarz, 1988). Brefeldin-A interferes with anterograde transport from the ER to the GC (Pelham, 1991), and would be expected to fail in the palmitoylation of C3 (Golgi) but not C23 (ER).

The initial rationale for identifying novel post-transcriptional mechanisms of ABCA1 was the off-target effects induced by transcriptional activation of ABCA1. LXR agonists are potent activators of the two major transporters involved in cholesterol removal from cells, ABCA1 and ABCG1. The binding of LXR agonists to LXR elements (LXREs) have potent effects on their expression and function. For example, ABCG1 is increased by up to 1000-fold by LXR agonists (Wojcicka et al., 2007). Palmitoylation affects localization of ABCA1 rather than expression, and increasing ABCA1 palmitoylation through DHHC8 significantly increases cholesterol efflux activity by 20% (Singaraja et al., 2009).

A strong inverse relationship does exist between cholesterol efflux activity and intima media thickness ($r=-0.61$; $p=0.03$) (van Dam et al., 2002), and cholesterol efflux activity and subclinical atherosclerosis and obstructive CAD ($OR=0.70$; 95%CI 0.59 to 0.83; $p<0.001$) (Khera et al., 2011). The increase in cholesterol efflux activity following DHHC8 expression suggests palmitoylation of ABCA1 is still a potential cardioprotective strategy.

However, the same issues that limited LXR agonists as a useable therapeutic strategy may limit the value of increasing ABCA1 palmitoylation. No adverse effects have been reported from the overexpression of DHHC8, nevertheless DHHC8 affects numerous processes and how these processes may be affected from DHHC8 overexpression is unknown. DHHC8 is thought to be essential in the

development of the brain and for proper neuronal function (Korycka et al., 2012). Polymorphisms in the *ZDHHC8* gene have been associated with an increased risk of schizophrenia. Mice with *ZDHHC8* deletions have decreased prepulse inhibition, and lower exploratory activity. DHHHC8 may also be involved in the G2/M cell cycle checkpoint as cells with decreased DHHHC8 levels are susceptible to ionizing radiation. A number of other proteins have been found to be substrates for DHHHC8-mediated palmitoylation including: the spine formation protein paralemmin-1; the neuronal PDZ-protein PSD-95; endothelial nitric oxide synthase (eNOS); and another neuronal PDZ-protein GRIP1b (Korycka et al., 2012; Fernandez-Hernando et al., 2006; Thomas et al., 2012). An ideal regulator would be as specific as possible for ABCA1 with minimal off-target effects. Unfortunately DHHHC8 appears to regulate numerous targets.

Clinical trials are currently underway involving the regulation of the lipid modification farnesylation for the treatment of Progeria. Farnesyl transferase inhibitors are currently in trials to determine efficacy in treating Hutchison-Gilford Progeria syndrome, the premature aging disorder (Resh, 2012). As far as I know, no trials are currently underway involving the regulation of palmitoylation, although it is currently a target for agents that can manipulate this lipid modification. One example is the development of the APT1 inhibitor, palmostatin B. H-Ras is a palmitoylated protein whose thioesterase has been identified as APT1. H-Ras belongs to a family of GTPases that regulates cell growth. Altered Ras activity can lead to malignant growth of cells (Resh, 2012). The treatment of cells with palmostatin B did not produce the desired effect of localizing Ras to the plasma membrane. Instead, palmostatin B resulted in random distribution of Ras. The reasons for this maybe that palmostatin B are affecting the function of other thioesterases, or that multiple thioesterases depalmitoylate Ras (Resh, 2012).

A key experiment to address whether palmitoylation is a worthwhile target for increasing ABCA1 function is to assess the effect of this lipid modification on HDL levels and atherosclerosis in vivo.

Recently, it was discovered that ABCA1 is regulated post-transcriptionally by miRNAs (Moore et al., 2010). The inhibition of miR-33a in hepatocytes and macrophages increased ABCA1 protein expression, cholesterol efflux activity, in vivo HDL levels, and reduced atherosclerotic plaque size in a mouse model of atherosclerosis (Rayner et al., 2012). Besides the liver and macrophages, ABCA1 maintains cholesterol homeostasis and proper functioning in numerous tissues including the pancreas and central nervous system. By inhibiting miR-33a in islets, we increased ABCA1 protein expression, reduced islet cholesterol levels, and increased glucose-stimulated insulin secretion (Wijesekara et al., 2012).

ABCA1 also maintains cholesterol homeostasis in the brain, and plays a crucial role in proper neuronal function. Mice lacking ABCA1 in the CNS have reduced plasma HDL, disturbances in their motor activity, and reduced synapse and synaptic vesicle numbers (Karasinska et al., 2009). The major carrier of cholesterol in the CNS is apolipoprotein E (apoE). The lipidation of apoE by ABCA1 may be important in blocking the pathogenesis of Alzheimer's disease (AD) (Fan et al., 2009). A hallmark of AD diagnosis is the aggregation of A β peptides in amyloid plaques. The lipidation of apoE blocks the transport of A β across the blood-brain-barrier, initiates the proteolytic degradation of A β , and eliminates the formation of amyloid plaques in vivo (Fan et al., 2009). Based on this knowledge, the increase of ABCA1 expression and function by inhibiting miR-33a in mouse models of AD may increase ABCA1 protein expression, decrease A β levels, reduce amyloid plaque formation, and improve cognitive performances.

We also discovered a novel miRNA regulator of ABCA1 in miR-145. Like miR-33a, miR-145 directly binds to the 3'UTR of ABCA1 to decrease protein levels and cholesterol efflux activity in HepG2 cells. Although we show that miR-33a represses ABCA1 to a greater extent than miR-145 in cell lines, the differences are minor. We also observed that miR-145 has a three-fold higher level of expression than miR-33a in human liver samples. A key factor in the level of translational repression by miRNAs is the expression levels of miRNAs in a given tissue. Generally, the higher the expression of a miRNA in a cell,

the greater the miRNA is able to repress its target transcript (Brown and Naldini, 2009). Thus, the inhibition of miR-145 in the human liver may induce a greater increase in ABCA1 expression and function than the inhibition of miR-33a in the human liver. Future studies to assess this question would involve modulating miR-145 levels in mice and assessing ABCA1 protein expression in the liver and HDL levels in vivo. MiR-145 is also expressed in islet cells, the brain, and in adipose tissue. These are tissues in which the maintenance of cholesterol homeostasis by ABCA1 is important for their proper function (Brunham et al., 2007; Karasinska et al., 2009; Chung et al., 2011). The effects of modulating miR-145 in representative cell lines for these tissues could also be assessed.

MiR-33a has demonstrated itself to be a potent regulator of ABCA1 expression and function (Rayner et al., 2012). In hepatic cell lines, the regulatory effect of miR-145 on ABCA1 is not as strong as miR-33a. However, we show that in the human liver, miR-145 shows greater than threefold expression compared to miR-33a. This indicates that in humans, regulating miR-145 may have larger effects on ABCA1 than miR-33a.

A potential drawback to the manipulation of miR-145 is the many genes targeted by this miRNA. MiR-145 represses the translation of L-type calcium channels (Turczynska et al., 2012), and a number of genes involved in insulin-like growth factor (IGF) signaling (Law et al., 2012). MiR-145 is also underexpressed in cancer cell lines and is thought to be a tumor suppressor (Sachdeva and Mo, 2010). It will be important to monitor how miR-145 induced changes to these proteins affect normal cellular functions, and whether any pathological mechanisms arise upon doing so.

This dissertation summarizes novel post-transcriptional mechanisms of ABCA1 regulation by miR-33a in islet cells, miR-145 in hepatic cells, and palmitoylation for proper localization. The validity in targeting palmitoylation or microRNAs to increase ABCA1 and HDL levels will be dependent on the outcomes of in vivo trials in animals.

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