# University of Alberta

Biochemical Characterization of ATP-Binding Cassette Transporter G1 Mediated Cholesterol Efflux

by

Xia Gao

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Medical Sciences-Pediatrics



Spring 2012
Edmonton, Alberta

## Dedication

This thesis is dedicated to my dearest parents who I missed so much.

## Abstract

ABCG1 mediates cholesterol efflux onto lipidated apolipoprotein A-I and plays an important role in macrophage reverse cholesterol transport, thereby preventing atherosclerosis. However, how ABCG1 mediates cholesterol efflux is unclear.

We found that Cys514 located within the third putative transmembrane domain is highly conserved. Replacement of Cys514 with Ala abolished ABCG1-mediated cholesterol efflux. Substitution of Cys514 with the more conserved amino acid, Ser or Thr, also significantly decreased cholesterol efflux. However, mutation C514A had no effect on ABCG1 stability, trafficking to plasma membrane or its dimerization.

Given the hydrophobicity of cholesterol and the abundance of cysteines, we proposed that ABCG1 is S-palmitoylated. Indeed, ABCG1 is S-palmitoylated at multiple N-terminal cysteines, but they are not functionally equivalent. Mutation at Cys311 dramatically impaired ABCG1-mediated cholesterol efflux, whereas mutations at other palmitoylation sites had minor or no effect. Moreover, defective palmitoylation had no effect on protein stability, trafficking, distribution pattern or the dimerization.

**Acknowledgements**

First, I want to take this chance to thank my family and all my friends for being there. Specially, I want to thank my senior high school teachers, also my life-long friends, Yalin, Zhongming and Mr. Tingyong Ma, without you all, I could never be here.

Second, I would like to extend my sincere gratitude and appreciation to my supervisor, Dr. Dawei Zhang, for kindly recruiting me into his laboratory, for his guidance, advice and encouragement throughout my graduate studies, and for creating a positive and pleasant work environment. I would like to thank my co-supervisor, Dr. Richard Lehner for his support, kindness and advice. I would also like to show my great gratitude to Dr. Luc Berthiaume for his brilliant suggestions and great support in my project, especially the technician assistance from Megan in his lab. I would also like to thank all the members of my supervisory committee: Drs. Richard Lehner, Luc Berthiaume and Dawei Zhang for their "tough love", their valuable comments, support and direction.

Next, I am thankful to all the current lab members, Hongmei Gu and Ge Li, for their technical support and collaboration. I am also in a great debt to the MCBL group, Dr. Dennis Vance's Lab, Dr. Jean Vance's Lab, Dr. Richard Lehner's Lab, Dr. Spencer Proctor's Lab, Dr. Rene Jacobs's lab and Dr. Simonetta Sipione's Lab, for the exciting and pleasant discussion in our journal club and for the suggestions and helps in daily lab work. Special gratitude should

be send to my friends here in campus, Ling Ji, Malanie Alpaugh, Shakib Rahman and Karen Kelly, for your kindness and friendship.

In addition, special acknowledgements should be extended to the following persons for their great contribution and cooperation for my thesis. I am very grateful to Dr. Matsuo at Kyoto University, Japan, for ABCA1 cDNA; Dr. Kerry-Anne Rye from Sydney for the acceptor of pHDL; Hongmei Gu and Ge Li for their help in making lipidated apoA-1 acceptor. Dr. Dawei Zhang made the cDNA constructs and stable cell lines used in Fig 4.1 and Fig 4.2; Hongmei Gu made great contribution to the finding that ABCG1 is S-palmitoylated (Fig 4.1).

Finally, I am very thankful to the following awards and funding agencies, which have graciously supported my graduate studies here: the Motyl Graduate Scholarship in Cardiac Sciences, the Mazankowski Alberta Heart Institute Graduate Studentship, 75th Anniversary Scholarship, Medical Sciences Graduate Program Graduate Research Assistantship and the FoMD/AHS Graduate Student Recruitment Studentship.

Overall, I am grateful to my family, my mentors, my friends and my colleagues, for their support throughout my graduate studies.

## Table of Contents

## List of Tables

## List of Figures

## List of Abbreviations

| | |
|---|---|
| 7-KC | 7-ketocholesterol |
| ABC | ATP-binding cassette transporter |
| ABCA1 | ATP binding cassette transporter A1 |
| ABCG1 | ATP binding cassette transporter G1 |
| Ala | Alanine |
| apoA-I | Apolipoprotein A-I |
| apoA-II | Apolipoprotein A-II |
| apoB-100 | Apolipoprotein B-100 |
| apoE | Apolipoprotein E |
| BSA | Bovine serum albumin |
| CCR5 | C-C chemokine receptor type 5 |
| CE | Cholesteryl ester |
| CETP | Cholesteryl ester transfer protein |
| CVD | Cardiovascular disease |
| Cys | Cysteine |

| | |
|---|---|
| DAPI | 4' 6-diamidino-2-phenylindole |
| DMEM | Dulbecco’s modified Eagle’s medium |
| DTBP | Dimethyl 3, 3’-dithiobispropionimidate |
| DTT | Dithiothreitol |
| ECs | Endothelial cells |
| eNOS | Endothelial nitric oxide synthase |
| ER | Endoplasmic reticulum |
| FBS | Fetal bovine serum |
| FC | Free cholesterol |
| GPS2 | G protein pathway suppressor |
| HDL | High density lipoprotein |
| HEK | Human embryonic kidney |
| HMG-CoAR | Hydroxymethylglutaryl CoA reductase |
| ICAM1 | Inter cellular adhesion molecule 1 |
| LCAT | Lecithin: cholesterol acyltransferase |
| LCFA | Long chain fatty acids |

| | |
|---|---|
| LDL | Low density lipoprotein |
| LDLR | Low density lipoprotein receptor |
| LXR | Liver X receptor |
| LXRE | LXR response elements |
| MCP-1 | Monocyte chemotactic protein-1 |
| NaDC-1 | Na(+)/dicarboxylate co-transporter |
| NBDs | Nucleotide-binding domains |
| NO | Nitric oxide |
| NPC1L1 | Niemann-Pick C1 Like 1 Protein |
| PBS | Phosphate buffered saline |
| PCSK9 | Proprotein convertase subtilisin/kexin type 9 |
| pHDL | HDL purified from human plasma |
| PLTP | Phospholipid transfer protein |
| PM | Plasma membrane |
| RCT | Reverse cholesterol transport |
| rHDL | Reconstituted HDL |

| | |
|---|---|
| S. cerevisiae | Saccharomyces cerevisiae |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Ser | Serine |
| SMCs | Smooth muscle cells |
| SR-BI | Scavenger receptor BI |
| SREBP-2 | Sterol regulatory element binding protein-2 |
| TCA | Trichloroacetic acid |
| TCR | T-cell-receptor |
| Tfr | Transferrin receptor |
| Thr | Threonine |
| TM | Transmembrane |
| TMD | Transmembrane domain |
| VLDL | Very low density lipoprotein |

# Chapter 1

## Introduction

## 1.1. Atherosclerosis and lipoproteins

### 1.1.1. Atherosclerosis

Atherosclerosis is characterized as a progressive disease with a gradually thickened arterial wall (1,2). It is the primary cause of cardiovascular diseases (CVD) such as coronary artery disease and stroke. Atherosclerosis and its complications are the leading cause of death in western society (2).

The progression of atherosclerosis is very complicated. Over the past sixty years, atherosclerosis has been greatly clarified by both epidemiologists and cellular and molecular biologists. A wide range of animal models have been developed and used, including rabbits, pigs, non-human primates and rodents (3-5). The two most commonly used models of atherosclerosis are mice deficient in apolipoprotein E (apoE) (6,7) or low-density lipoprotein receptor (LDLR) (8,9). ApoE deficient mice (*Apoe*$^{-/-}$) develop atherosclerosis when fed a chow diet (7,10), while the LDLR deficient mice (*Ldlr*$^{-/-}$) develop the atherosclerotic lesions when fed a high cholesterol or high fat diet (11). Etiological studies have identified several important environmental and genetic risk factors closely associated with atherosclerosis (2). Both environmental factors (e.g. smoking, a high fat diet) and genetic factors contribute to the development of atherosclerosis. Among all these elements, high plasma low density lipoprotein (LDL)-cholesterol levels show the highest correlation with atherosclerosis development in humans and animals (2).

### 1.1.2. Cholesterol and cholesterol metabolism

Cholesterol is an unique and essential metabolite in animal cells (12). It is abundant in mammalian cells, and plays an important role in membrane structure and metabolic system (13,14). Cholesterol, together with phospholipids (e.g. phosphatidylcholine) modulates membrane fluidity, permeability and membrane trafficking (15-17). On the plasma membrane (PM), cholesterol concentrates in

sphingolipids-rich membrane domains called lipid rafts. Lipid rafts accumulate a variety of signalling molecules, which are associated with a wide range of signalling transduction cascades (17-20) and physiological diseases (21). In addition, cholesterol is also a substrate for steroid hormone synthesis (22,23) and serves as the precursor of oxysterols and vitamin D (24). However, excess cholesterol can disrupt membrane integrity and promote cell toxicity and cell death (25). Uncontrolled cholesterol accumulation also contributes to physiological diseases (16,24,26), such as atherosclerotic cardiovascular diseases.

A complex network has been developed to regulate the availability and homeostasis of cholesterol in the human body. Cellular cholesterol comes from both *de novo* synthesis and dietary absorption (16,27,28). The major organ for cholesterol synthesis is the liver. The endoplasmic reticulum (ER) is specialized in cholesterol synthesis by harbouring some key enzymes in the biosynthesis pathway (28). Cholesterol is synthesized from acetate through ~30 enzymatic reactions. Hydroxymethylglutaryl CoA reductase (HMG-CoAR) is the rate limiting enzyme in this pathway (16). Dietary cholesterol uptake occurs in the enterocytes of the small intestine. The Niemann-Pick C1 Like 1 Protein (NPC1L1) is important for intestinal cholesterol absorption and NPC1L1 null mice are resistant to high cholesterol diet induced hypercholesterolemia (29,30). Cellular cholesterol balance is also maintained by various cholesterol transport pathways (24). Membrane receptors, LDLR and scavenger receptors, are involved in cholesterol uptake, while ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1) are responsible for cellular cholesterol export (16). Deficiency in either Niemann-Pick C protein, NPC1 or NPC2, which is responsible for intracellular cholesterol trafficking, causes Niemann-Pick type C disease (31). Moreover, the cellular cholesterol levels act as a highly sensitive indicator for transcriptional factors, including sterol regulatory element binding protein-2 (SREBP-2) and the liver X receptors (LXRs, LXRα and LXRβ). SREBP-2 is a transcription factor regulating cholesterol biosynthesis and homeostasis (28,32-

34). LXRs control cellular cholesterol homeostasis by regulating the expression of several cholesterol transporters like ABCA1 and ABCG1 (35,36).

Compared to the complicated regulation network in cholesterol synthesis and uptake, most peripheral cells cannot catabolise cholesterol. The major pathway for cholesterol excretion is through the conversion of cholesterol into bile acids in the liver (37,38). Although most of the bile acids will be reabsorbed in the intestine and recycled back into the liver, part of them can be eventually excreted out of our body (39). In addition, cholesterol can also be used to synthesize steroid hormones in the steroidogenic tissues (40).

### 1.1.3. Lipoproteins and atherosclerosis

In blood, cholesterol is transported by lipoprotein particles (1). According to the density and apolipoprotein compositions, lipoproteins are categorized into chylomicron, chylomicron remnant, very low density lipoprotein (VLDL), LDL, and high density lipoprotein (HDL).

Dietary cholesterol is first packaged into the large triglyceride-rich chylomicrons in the intestine. As chylomicrons flow through the blood to the peripheral tissues, such as muscle and adipose tissue, they are converted to chylomicron remnants by local lipoprotein lipases. Chylomicron remnants are subsequently cleared by the liver. VLDL is secreted from the liver as triglyceride-rich particles containing apolipoprotein B-100 (apoB-100) and apolipoprotein E (apoE). Similar to chylomicrons, VLDL releases fatty acids to the peripheral tissues through the action of lipoprotein lipases to produce VLDL remnants. VLDL remnants are further metabolized into LDL particles by removal of triglycerides and its apolipoproteins except for apoB-100. LDL can then be taken up by the liver through LDLR. HDL is responsible for the removal of excess cholesterol from peripheral tissues. The generation of HDL starts from the secretion of lipid-free apolipoprotein A-I (apoA-I) from the intestine and the liver.

The lipid free apoA-I receives cholesterol and phospholipids from both the liver and peripheral tissues and forms HDL.

In humans, the majority of serum cholesterol is carried by LDL (~2/3) and HDL (~1/3) particles. High plasma concentration of LDL-cholesterol is atherogenic, whereas high concentration of HDL-cholesterol is inversely correlated with atherosclerotic cardiovascular diseases (41).

### 1.1.4. HDL and its anti-atherosclerotic role

In comparison with LDL, the plasma HDL is comprised of a heterogeneous class of lipoprotein particles differing in their density (1.063-1.21g/ml), size, shape and electrophoretic mobility (42,43). The heterogeneity of HDL is intimately correlated with the relative contents of lipids and apolipoproteins (apoA-I, apoE and apoA-II) (44,45), especially the apoA-I, which accounts for ~70% of total HDL proteins (46). Extensive epidemiological data have consistently shown the inverse relationship between the circulating levels of HDL-cholesterol and the risk of atherosclerosis (47-50). According to the Framingham Heart Study, this correlation is independent of plasma LDL levels (48,51,52). Recent clinical trials also support that HDL stands by as an independent risk predictor of cardiovascular diseases (53).

Parallel with the heterogeneity, HDL are multifunctional. One major function is in the reverse cholesterol transport pathway (RCT), which mediates cholesterol transport from peripheral tissues to the liver for final elimination (54-56). As shown in Fig. 1.1 which is modified from figure 2 of (57), the RCT is tightly associated with the metabolism of HDL. The generation of HDL starts from the secretion of lipid-free apoA-I or nascent lipid poor pre-β HDL (58) from the intestine and liver. Such lipid free apoA-I/nascent HDL are not stable but ready to acquire additional cholesterol and phospholipids via ABCA1 to form small discoidal HDL (59). The discoidal particles can pick up apoA-II and other surface components generated from lipolysis of triglyceride-rich lipoproteins.

Meanwhile, the surface cholesterol can be converted into cholesteryl ester (CE) by lecithin: cholesterol acyl transferase (LCAT), creating an increasing cholesterol gradient from outside to inside by moving the surface cholesterol into the core of the particles as CE. These processes result in the generation of small spherical HDL. Such particles then act as acceptors for another ABC transporter, ABCG1, to pick up more cellular free cholesterol from peripheral tissues like macrophages (60). The newly recruited cholesterol can also get esterified by LCAT, which contributes to form the medium sized spherical HDL. In humans, these medium spherical HDL further exchange their CE for triglyceride with LDL or VLDL through cholesteryl ester transfer protein (CETP) to form large spherical HDL (61). Such particles undergo further modifications through hepatic lipase (62) and endothelial lipase, or remodelling by exchanging phospholipids with other plasma lipoproteins through the phospholipid transfer protein (PLTP) (42,59,63). In mice, HDL cholesterol can be selectively taken up by the liver through scavenger receptor BI (SR-BI), but in humans, it can also be taken up through LDLR after CE is transferred to LDL or VLDL. Eventually, the hepatic cholesterol can be secreted into bile after conversion into bile acids or directly as free cholesterol (64). The bile components are either reabsorbed in the intestine or excreted into feces. Since the accumulation of cholesterol loaded foam cells is the critical step in the development of atherosclerosis, HDL and peripheral RCT, especially macrophage RCT, have been receiving a considerable attention (46,54).

In addition, HDL subpopulations also possess other antiatherogenic properties, including anti-oxidative, anti-inflammatory, anti-thrombotic, vasodilatory and cytoprotective activities (42,65,66). All these activities favourably affect almost every step in atherogenesis (56). First, HDL and its apolipoproteins can compete with LDL for the proteoglycan-rich matrix binding (67), and prevent the aggregation of LDL (68). Second, HDL and its apoA-I can slow down the oxidation of LDL (69,70). Third, HDL can inhibit the monocyte recruitment and binding to endothelium by inducing endothelial cell nitric oxide (NO) synthesis (71-73), or reverse the up-regulation of cell adhesion molecules

and chemoattractants (74). Moreover, HDL is also involved in regulating the expression of inflammatory genes and chemokines in macrophages and the production of cell adhesions in ECs (75,76). Finally, HDL can help to preserve the macrophage phagocytosis (77,78) and inhibit the apoptosis of macrophages induced by oxidized LDL or free cholesterol loading (79). Phagocytosis plays a critical role in the clearance of excess cholesterol and foam cells and in preventing the extensive necrosis and inflammation (80). Both HDL associated macrophage phagocytosis and apoptosis are dependent on HDL mediated massive cholesterol efflux activity.

### 1.2. ABC transporters and atherosclerosis

#### 1.2.1 ATP-binding cassette (ABC) family

The ATP-binding cassette (ABC) superfamily is a family of membrane proteins involved in trafficking of a wide range of substrates. These substrates include phospholipids, sterols, sugars, bile acids, ions, peptides and various drugs (81,82). ABC proteins own a long evolutionary history, with over 250 family members expanding all the kingdoms of life (83). By coupling with ATP binding and hydrolysis, ABC members also facilitate ribosome assembly and DNA repair. In humans, 49 family members have been identified and subdivided into 7 subfamilies, from A to G, according to the domains and sequence (84). Among them, 17 family members are clinically linked with hereditary diseases, such as Tangier disease (85), adrenoleukodystrophy, cystic fibrosis and others (86).

A functional ABC protein typically contains two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs). With exceptions for subfamily E and F, both of them comprise of only two NBDs with no TMD (82). In eukaryotes, ABC proteins either function as a full transporter like ABCA1, with 2 TMDs and 2 NBDs expressed in one polypeptide, or as a dimer (hero- or homo-) of half transporter, like ABCG subfamily, which contains only 1 TMD and 1 NBD. The NBD, also called ATP-binding cassette, is comprised of around

180 amino acids and possesses three conserved motifs, namely the Walker A, the Walker B and the Signature motifs. The Walker A motif or P-loop (GXXGXGK(S/T), with X represents any amino acid) and the Walker B motif (XXXXD, where X resembles a hydrophobic amino acid) are characterized in all ATP-binding proteins (87), whereas the Signature motif or C-loop (LSGGQ) (88) presenting between the Walker A and the Walker B motifs distinguishes ABC transporters from other ATP binding proteins (81,89). The TMDs usually contain 6 putative transmembrane (TM) α-helices and a total of 12 TM α-helices are suggested to be necessary to form a central channel for substrate translocation. However, a few ABC proteins contain more TM α-helices, like ABCC6, which has 17 TM α-helices (90). The ABC motif localizing in the cytoplasm is well known to act as an engine for energy (ATP) supply, however, less is known about the function of TM α-helices, not to mention the additional ones. Besides, even with extensive studies over the past 30 years, there is little progress in either substrate identification or the mechanisms of substrate transport.

To uncover the molecular mechanisms of how ABC transporters facilitate substrates translocation through the membrane bilayer by ATP binding and hydrolysis, a wealth of studies have been proceeding around protein structure. Up until now, there is no available high resolution crystal structure for any mammalian ABC proteins (91), but the crystal structures of Sav1866 (92,93) and MsbA (94-96), two bacterial homologs of human ABCB1 (P-glycoprotein), have been determined. It appears that 12 TM helices form the TM core with an outward-facing conformation, while the ABC motifs are organized in a head-to-tail orientation with a shared interface. The authors propose that the ATP hydrolysis may cause a conformation transition from an outward-facing to inward-facing orientation, resulting in substrate binding and translocation (92,97). Due to the dynamic process and the vulnerable membrane protein extraction, more high resolution structures of ABC transporters will be required to evaluate the reported conformation changes. Besides, other biochemical, biophysical and

physiological studies will be mandatory in fully understanding ABC transporters and their functions (97).

### 1.2.2. ABC transporters in cholesterol metabolism and atherosclerosis

One of the major physiological functions of ABC transporters is to maintain lipid homeostasis by regulating cellular lipid transport. Defects in these proteins are associated with clinical diseases, such as ABCA1 in Tangier disease and ABCG5/G8 in sitosterolemia. To date, at least eight ABC transporters have been reported to be related with cholesterol/sterol trafficking, four of them (ABCA1, ABCG1, ABCG5 and ABCG8) display a significant impact on the lipoprotein and cholesterol metabolism, and two of them (ABCA1 and ABCG1) have tight correlation with RCT and atherosclerosis.

ABCA1, a 240-kDa full transporter, promotes both phospholipids and cholesterol to nascent/lipid-poor apoA-I, the initial step of HDL formation. The transcription of ABCA1 is regulated by LXRs (98), and post-transcriptional regulations are also important for protein stability and function (99,100). Mutations in both copies of *ABCA1* cause Tangier disease, which is characterized with a high deposition of cholesteryl esters and other lipids in a variety of tissues and low plasma HDL levels (101). Familial hypoalphalipoproteinemia patients, with heterozygous mutation in ABCA1, are characterized with only about half of the normal blood HDL levels (90). ABCA1 in the liver and intestine determines the biosynthesis of HDL (102,103), and ABCA1-mediated cholesterol efflux from the epithelial cells in the intestine is estimated to contribute to around one third of total HDL production (104). Macrophage ABCA1 is protective against atherosclerosis by mediating macrophage RCT (105), while deficiency of ABCA1 results in the accumulation of cholesterol, promoting an inflammatory response (105-107). Endothelial ABCA1 also plays a critical role in anti-atherosclerosis by preserving endothelial cells (ECs) integrity and functioning as well as HDL formation through regulating cellular cholesterol homeostasis (56,108). However, it is still unclear how ABCA1 functions as a cholesterol transporter. A large

amount of evidence suggests that ABCA1 translocates cholesterol and phospholipids simultaneously from the inner membrane leaflet to the outer leaflet, promoting the acceptor (apoA-I) binding and dissociation (109). Yet it is also possible that ABCA1 facilitates apoA-I lipidation and secretion through retro-endocytosis, a process including both endocytosis and exocytosis (110).

ABCG5 and ABCG8 belong to the G subfamily, and function as a hetero-dimer (111). Similar with ABCA1, ABCG5 and ABCG8 are also regulated by LXRs (112). Post-transcriptional glycosylation and hetero-dimerization are critical for proper protein localization and function (113). They localize at the apical membranes of both enterocytes and hepatocytes, where they limit the plant and shellfish sterol absorption and assist biliary sterol secretion (114,115). Mutations in any one of these two proteins result in a rare genetic disease, sitosterolemia (116). Patients in this disorder exhibit significant increase of plasma plant sterols and a modest rising of plasma cholesterol, and are vulnerable for development of premature CVD (112,117). In addition, hepatic ABCG5 and ABCG8 demonstrate an anti-atherosclerogenic role in mouse models (118,119).

### 1.2.3. ABCG1 general introduction and regulation

Like ABCG5 and ABCG8, ABCG1 belongs to the ABCG half transporter subfamily distinguished with the ABC motif localizing at the amino terminus followed by 6 putative α-helix TMD (the topology of ABCG1 shown in Fig 1.3). The G subfamily is widely present with 15 members in *Drosophila*, 9 in *Caenorhabditis elegans* and 24 in *Arabidopsis thaliana* (120). In mammals, a total of five members have been recorded (ABC G1, G2, G4, G5 and G8) (121), among which, ABCG1 shares 82% similarity in amino acid sequence with ABCG4. Human ABCG1, an encoded 74 kDa protein, is originally named after *Drosophila* White, with a 51% amino acid identity (122,123). White is a half transporter like ABCG1 and functions as a hetero-dimer with Brown or Scarlet, another two ABCG family members in *Drosophila* (124). White presents in the intracellular membranes of pigment granules, acting as a cargo for pigment precursors. *ABCG1*

used to be characterized as the ortholog of *White*, however, recent studies showed that overexpression of mammalian ABCG1 failed to restore the lost function of White, implying no functional overlapping between White and ABCG1 (89).

ABCG1 is expressed in multiple tissues, with relatively high levels in spleen, lung, brain and kidney (125). β-Galactosidase staining from *Abcg1*$^{-/-}$ *LacZ* knock-in mice shows that ABCG1 expression is cell type dependent, with high expression levels in macrophages, ECs, lymphocytes and neurons, but undetectable in hepatocytes and enterocytes (89,125). The human ABCG1 gene is mapped to chromosome 21q22.3, and ABCG1 cDNA was initially identified in 1996 (126,127). It is predicted to have 23 exons spanning over a 98kb genome (128). Earlier studies show that mRNA of macrophage ABCG1 is highly induced when macrophages are overloaded with modified LDL, oxysterols or treated with ligands for LXR (128-130). Following with the latter observations, a few multiple functional LXR response elements (LXREs) have been identified in the promoter region of both the murine and human *Abcg1* (128,131,132). A more detailed study shows a G protein pathway suppressor (GPS2) is required for the recruitment of LXR to the LXRE at the *ABCG1* locus (133). Due to the different splicing sites and transcriptional starting points, a few transcripts have been identified, but the meaning of the physiological existence for these transcripts is lacking (81). Except for one transcript that has the same length (666 amino acids) with mouse *Abcg1*, human macrophages also express another transcript encoding 12 more amino acids (131,134). Further studies show that these 12 amino acids in the second transcript are important for protein stability (81).

In addition to the complex transcriptional regulations, ABCG1 expression is also regulated at both mRNA and protein levels. MicroRNA miR-33a, a newly identified cholesterol homeostasis post-transcriptional regulator, can cause translational repression and/or destabilization the mRNA of murine *Abcg1* by binding to its complementary 3′-untranslated regions, but not to human *ABCG1*, which may be due to missing the binding sites (135,136). On the other hand,

activation of adenosine monophosphate activated protein kinase (AMPK) in human ECs can promote *ABCG1* expression by stabilizing its mRNA (137). Finally, no specific modification is predicted based on the sequence of ABCG1, but phosphorylation of ABCG1 due to the enhanced fatty acid oxidation is reported to accelerate protein degradation (138,139). Together, ABCG1 is modulated at multiple levels and the complexity of the regulations may be important for its cellular function.

### 1.2.4. ABCG1 and cholesterol homeostasis

ABCG1 is firstly proposed to function in sterol homeostasis due to its response to LXRs (140), which modulate a large genetic network in sterol homeostasis. Further *in vitro* studies indeed support this hypothesis that overexpression of ABCG1 promotes cellular cholesterol efflux to exogenous acceptors (60,131,141). Besides, after LXR induction, macrophage cholesterol efflux ability appears to be in an ABCG1 dependent manner (142). Unlike ABCA1, ABCG1 has less acceptor selectivity. LDL, HDL, reconstituted HDL, small phospholipids vesicles, cyclodextrin and bovine serum albumin (BSA) all function as an acceptor for ABCG1-mediated cholesterol efflux (131,141,143). Moreover, overexpression of ABCG1 is reported to increase cholesterol efflux to lipidated apoA-I obtained from ABCA1-expressing cells, implying that ABCG1 may work sequentially with ABCA1 in mediating cholesterol efflux (144,145). Conversely, down regulation of ABCG1 by siRNA suppresses cholesterol efflux (143), similar to human macrophages treated with antisense oligonucleotide for ABCG1 (129). The deficiency of ABCG1 in mice (*Abcg1*$^{-/-}$) causes high accumulation of cholesterol, oxysterols and CEs in various tissues, especially in the lung tissue (125). This deposition is reversed in *hABCG1*$^{Tg}$*Abcg1*$^{-/-}$ mice. These results are consistent with the decreased cholesterol efflux ability in cells derived from *Abcg1*$^{-/-}$ mice and more dramatic decrease in cells with combined deficiency of ABCG1 and ABCA1 (146,147). Together, both *in vivo* and *in vitro* data support that ABCG1 plays an important role in cholesterol homeostasis.

However, how ABCG1 mediates cholesterol export is still an enigma. ABCG1 has been reported to localize in the intracellular compartments and plasma membrane. Overexpression of ABCG1 leads to the translocation of cholesterol onto the outer leaflet of cell membranes (143), which is accessible for cholesterol oxidase. However, recent biotinylation and immunostaining studies show less or undetectable levels of ABCG1 at the cell surface (148,149). ABCG1 appears to be induced to redistribute to the PM by LXR ligands (142), while other studies disagree with this observation (150). ABCG1 has no specific acceptor(s) and unlike ABCA1, ABCG1 does not bind to HDL or other acceptors (60,141). ABCG1 also has less selectivity for the substrates. Except for cholesterol, the efflux of phospholipids (e.g. phosphatidylcholine and sphingomyelin) is also increased in cells overexpressing ABCG1 (151). Moreover, ABCG1 can promote the efflux of 7-ketocholesterol (7-KC) and 7β-hydroxycholesterol in both macrophages and 293 cells (79), and deficiency in ABCG1 results in significant deposition of 7-KC in ECs from aorta (79,152). Based on these available data, it has been proposed that ABCG1 may promote cholesterol translocation from intracellular compartments like the ER to the PM (142), simply facilitate cholesterol protrusion from the outer membrane, or change the PM organization pattern for the nonspecific acceptors (56) (see Fig. 1.2). Further studies are required to understand the underlying mechanisms for ABCG1-mediated cholesterol export.

Depending on its role in mediating cholesterol/sterol homeostasis, ABCG1 also plays other physiological roles in different cell types. ABCG1 is highly expressed in both macrophages and ECs (125). ABCG1 expression is up-regulated in foam cells and this up-regulation is correlated with the dramatic increase in the secretion of chemokines, cytokines and other proinflammatory factors (153,154). In ECs, deficiency of ABCG1 promotes a proinflammation phenotype with increased production of cell adhesions, chemoattractants and other proinflammatory factors (155), and disrupts the balance in endothelium vasoconstriction and relaxation by modulating the endothelial nitric oxide

synthase (eNOS) (156). ABCG1 may protect endothelial function by promoting the efflux of cholesterol and 7-KC to HDL (152), and the released cholesterol loading reduces the inhibitory binding of Caveolin1 to eNOS (157). ABCG1, co-expressed with ABCG4 in astrocyte and neurons, may affect brain cholesterol synthesis through SREBP-2 pathway (149,158). ABCG1 in glial cells regulates the lipidation of apoE containing lipoproteins (159). Recently, Hedrick and his colleagues (148) reported that ABCG1 in the insulin granules of pancreatic β cells appears to be critical in maintaining the granule morphology and insulin secretion (160). In addition, ABCG1 also seems to act as a special checkpoint for cell proliferation and cell death. ABCG1 in hematopoietic stem cells can repress cell proliferation and differentiation, particularly for monocytes (161). While, inactivation of ABCG1 promotes the proliferation of T cells by regulation of the oxysterol sulfotransferase enzyme SULT2B1 and LXR signaling (162). The lack of ABCG1 in macrophages has been reported to promote cell apoptosis (79,153).

### 1.2.5. ABCG1 and atherosclerosis

To date, there is no identified ABCG1 mutation related to any clinical disease. But, based on its significant contributions in RCT (around 50% together with ABCA1) (163), cell apoptosis, cell proliferation, inflammation and endothelial function, it is reasonable to propose that ABCG1 may play an important role in the progression of atherosclerosis (2,81). However, the current data from ABCG1 deficient mice are more controversial than consistent.

*Abcg1*$^{-/-}$ mice have similar plasma lipid levels with wild type mice, and do not develop atherosclerosis when fed a regular chow diet or a high fat diet (125). ABCG1-mediated RCT, especially macrophage-RCT, is well recognized to play an important role in reversing the development of atherosclerosis (54). Bone marrow transplantation was utilized to study the contribution of macrophage ABCG1 to atherosclerosis. Bone marrow transplantation from *Abcg1*$^{-/-}$ mice to *Ldlr*$^{-/-}$ mice resulted in either moderate increase (164) or significant decrease of the atherosclerotic lesion size. The reduced lesion size may have been due to the

compensatory up-regulation of ABCA1 or increased apoE secretion (165) or induced macrophages apoptosis (166). Meanwhile, Edwards's group reported that bone marrow transplantation from *Abcg1*$^{-/-}$ mice to *Apoe*$^{-/-}$ led to decreased lesion size (154). In addition to the macrophage RCT, ABCG1 is also highly expressed in ECs and ABCG1-mediated cholesterol export seems to be critical in maintaining normal endothelial function. To investigate the role of vascular ABCG1 in atherosclerosis, bone marrow transplantation has been carried out by transplanting bone marrow from wild type donor to recipient *Abcg1*$^{-/-}$*Ldlr*$^{-/-}$ or *Ldlr*$^{-/-}$ mice. The atherosclerotic lesion, especially in the aortic arch, is highly increased in *Abcg1*$^{-/-}$*Ldlr*$^{-/-}$ mice after 23 week western diet (156). Thus, ABCG1 in both macrophages and ECs seems to be involved in the development of atherosclerosis, but the above results can hardly achieve to a consistent conclusion on the role of macrophage ABCG1 in atherosclerosis.

To understand the role of ABCG1 in atherosclerosis, more extensive studies have been carried out in both *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice. Strategies include both deletion and overexpression of ABCG1. For *Ldlr*$^{-/-}$ mice, the further whole body deletion of ABCG1 (*Abcg1*$^{-/-}$*Ldlr*$^{-/-}$ mice) cause massive lipid accumulation and significant increase in atherosclerotic lesion size (Illiana et al. 2007). Under the background of *Apoe*$^{-/-}$ mice, the further whole body deletion of ABCG1 (*Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice) lead to decreased atherosclerosis, which may have been due to induced apoptosis (154). In addition, this group also reported that only hematopoietic ABCG1 deficiency by bone marrow transplanting from *Abcg1*$^{-/-}$ *Apoe*$^{-/-}$ mice to *Apoe*$^{-/-}$ mice impairs the development of atherosclerosis (154). But contrary results have been obtained from the *Ldlr*$^{-/-}$ recipient mice, which develop more dramatic and significant lesion size after receiving bone marrow from *Abcg1*$^{-/-}$*Apoe*$^{-/-}$ mice (167). Furthermore, overexpression of human ABCG1 in either *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice is not enough to reverse the progression of atherosclerosis (146,147). The observed difference may be caused by the difference in the methodologies, mice genetic backgrounds (81), the examined stage of atherosclerosis and/or other unidentified factors. Together, the above

conflicting data fail in clarifying the relationship between ABCG1 and atherosclerosis, and generation of different background mice models are required to explain these differences.

Due to the contradictory results in the above studies and the proposed sequential role of ABCG1 and ABCA1, double knockout mice (*Abcg1*$^{-/-}$*Abca1*$^{-/-}$) were generated to investigate the role of ABCG1 in atherosclerosis. As expected, *Abcg1*$^{-/-}$*Abca1*$^{-/-}$ mice displayed more severe lipid deposition phenotype, consistent with the impaired cellular cholesterol efflux activity (168-170). With bone marrow transplanted from donor *Abcg1*$^{-/-}$*Abca1*$^{-/-}$ mice, *Ldlr*$^{-/-}$ recipient mice have either accelerated (170) or similar atherosclerosis progression compared with wild type donor or *Abcg1*$^{-/-}$ mice donor (168).

### 1.3. Protein S-palmitoylation and its detection

#### 1.3.1. Lipidation and S-palmitoylation

Lipidation is protein modification by lipids (171), which includes the addition of long chain fatty acids (LCFA), glycosylphosphatidylinositols, isoprenoids and cholesterol. Together with phosphorylation and glycosylation, lipidation consists of one of the three major protein posttranslational modifications (172). Prenylation, myristoylation and palmitoylation are three common lipidations occurring in the cytoplasm (171). Prenylation involves the transfer of either a farnesyl or a geranyl-geranyl moiety to cysteine (Cys) residues, which localizes in the C-terminal CAAX box (A is an aliphatic amino acid) of the target proteins (173). Myristoylation and palmitoylation belong to protein acylations, in which proteins are modified by a wide range of fatty acids with different binding sites (171). Myristoylation (174), also called N-myristoylation, is an addition of myristate (a C14 saturated fatty acid) to the amino terminal glycine residue in the MGXXXS/T sequence motif (X is any amino acid), following the cleavage of N-terminal methionine (175). Initially, myristoylation was characterized as a co-translational modification, however, it has been well

established that myristoylation can also occur post-translationally on the N-terminal glycine after cleavage by caspases in apoptotic cells (174). Both prenylation and myristoylation have specific sequence requirements as described above.

A large amount of proteins are lipidated by LCFA due to their cellular abundance, especially palmitic acid (a C16 saturated fatty acid) (176,177). S-palmitoylation, documented over thirty years ago (178), is defined as a covalent attachment of palmitic acid to a Cys residue via a thioester linkage. S-palmitoylation is the most commonly observed protein lipidation in living cells. Palmitic acid can also bind to the target proteins via the stable amide linkage with Cys, which is named N-palmitoylation (179). N-palmitoylation is only recorded in secreted proteins like morphogen and Sonic Hedgehog (180). The amide group was presumed to be formed by transfer of the palmitoyl group from thioester intermediate (S-palmitoylation), while recently specific palmitoylacyltransferase Hhat has been reported to be required for the N-palmitoylation of Sonic Hedgehog (181). Physiologically, S-palmitoylation prefers palmitic acid, but other fatty acids, such as stearic, myristic or oleic acids, can also form a thioester linkage with Cys (182). So generally, S-palmitoylation should be called S-acylation (183). With the labile thioester bond, S-palmitoylation becomes the only reversible protein lipid modification (184). This is further confirmed by the recorded shorter half-life of palmitate compared with that of the palmitoylated proteins (183). Similar with reversible phosphorylation, S-palmitoylation is implied to play an important role in protein regulation. Later on in this thesis, S-palmitoylation is referred to palmitoylation.

### 1.3.2. Mechanisms of palmitoylation

Palmitoylation occurs in luminal, transmembrane and cytoplasmic proteins. Cys residues that localize in the cytoplasmic domains or close to the transmembrane regions of the target proteins are preferable candidate sites (183). But unlike myristoylation and prenylation, there is still no consensus sequence

motif for palmitoylation (171). Palmitoylation of some proteins, such as viral membrane glycoproteins, are stable for the whole life of the protein (185), while many other proteins undergo cycles between palmitoylated and depalmitoylated states (186). Like the function and regulation of protein phosphorylation, for which protein kinases and phosphatases are demanded, special enzymes may be required for protein palmitoylation and depalmitoylation.

To date, proteins appear to be palmitoylated by either autoacylation or enzymatic protein acyltransferases (PATs) (187). Due to the lag in the identification of PATs, the existence and the necessity of PATs have been under debate for years (188). Under physiological conditions, autoacylation has been reported in various proteins, including SNAP-25 (189), G proteins (190) and others (191). Meanwhile, Cys-containing proteins and peptides can be acylated *in vitro* when incubated with appropriate amount of palmitoyl-CoA. But the spontaneous reaction *in vitro* does not always happen under physiological pH or palmitoyl–CoA concentrations, and in some cases, it takes much longer than the observed protein turnover rate (183). Thus, for most of proteins, palmitoylation seems more likely to be an enzymatic process. The PATs, a family of multi-TM proteins characterized with a Cys-rich domain containing an Asp-His-His-Cys (DHHC) motif, were firstly discovered in *Saccharomyces cerevisiae* (192,193). Since then, 7 and 23 DHHC PATs have been identified in *S. cerevisiae* and mammals, respectively (191).

Parallel with the wide distribution of palmitoylated proteins, PAT activity has been found in Golgi, mitochondria and PM, especially in the cholesterol enriched microdomains (183). Loss of specific protein palmitoylation in combined deficiency of DHHC proteins in yeast (194) further supports the *in vivo* enzymatic palmitoylation. Recently, individual DHHC PAT has been reported to have variable substrate selectivity and some overlapping functions (194,195). On the other hand, palmitoylation in secreted proteins like ghrelin and Wnt, seems to require MBOAT family proteins (membrane-bound-O-acyltransferase) (196). In

Wnt proteins, fatty acids attach to two different positions, the O-linked acylation at serine is required for the following palmitoylation at Cys (197). A small set of family members have been known, such as Porcupine is responsible for Wnt (198) and ghrelin O-acyl-transferase (GOAT) for the acylation of Ghrelin (199).

In contrast to the fast progress in characterizing PATs, only one acyl protein thioesterase1 (APT1) has been found to function in depalmitoylation *in vivo* (200). APT1, originally discovered as a lysophospholipase (201), is a cytosolic protein. It is widely expressed among tissues, and preferably targets palmitoylated proteins (191). Several substrates have been identified *in vitro*, including eNOS (202), SNAP-23 (203), Ras and G protein α subunit (204). Co-overexpressing APT1 with eNOS increases the depalmitoylation of eNOS (191), and infusion of APT1 in platelets results in ~50% reduction in acylation of the platelet protein (205). APT1 seems to be promiscuous in substrates but with no consensus sequence and different deacylation efficiency. For example, Caveolin was not deacylated by APT1 under the same condition as eNOS (202). Moreover, disruption of APT1 in yeast affects the acylation of $G\alpha_{i1}$ but not H-Ras (206), implying the existence of other undefined thioesterase(s). In addition, another palmitoyl protein thioesterase1 (PPT1) has also been reported to remove the acyl group, however, this enzyme only localizes in lysosomes for protein degradation (191). Thus, only APT1 is identified as functioning in cytoplasmic deacylation, and the existence of other possible thioesterase(s) remains unknown.

### 1.3.3. Functions of palmitoylation

Palmitoylation has diverse functions. First, like all the other lipid modifications, it increases the hydrophobicity of target proteins and facilitates proteins (171), especially soluble proteins (183), tethering into phospholipids bilayer membranes and in some cases, clustering into different microdomains. For some proteins, palmitoylation enhances the weak membrane affinity induced by N-myristoylation or prenylation; while in several other cases, palmitoylation itself is sufficient for stable membrane attachment (186). Second, the role of

palmitoylation in protein trafficking and targeting has been well established (207). However, it is difficult to predict the location of palmitoylated proteins, and different palmitoylation sites may result in distinct trafficking and residence (207). For H-Ras and N-Ras proteins (208), palmitoylation is required for their trafficking. While monopalmitoylation of N-Ras is sufficient for its translocation to PM, the two palmitoylation sites in H-Ras, Cys181 and Cys184, are not functionally equivalent. Palmitoylation at Cys181 alone results in efficient protein distribution on the PM cholesterol-rich microdomain, while monopalmitoylation at Cys184 causes the retention of the majority of H-Ras in Golgi. Third, palmitoylation is implicated in protein stability (186). Defect palmitoylation may promote lysosomal protein degradation (209). Recent studies on SNARE in S. cerevisiae (210) and anthrax-toxin receptor in mammalian cells (211) suggest that palmitoylation may prevent protein degradation by avoiding ubiquitination (186). Finally, palmitoylation may also lead to alternation in the protein-protein interaction by regulating protein conformation or location change (212), or in signal transduction by affecting the association of proteins with lipid raft (183).

Palmitoylation plays an important role in maintaining various protein activities, and disruption of palmitoylation may contribute to various diseases (213). Palmitoylation of huntingtin protein (214) may decrease the neuronal toxicity in Huntington disease by reducing protein aggregation. Palmitoylation of eNOS (215,216) may be protective from hypertension and artherosclerosis by preserving normal endothelium function. Palmitoylation of oncoproteins, like H-Ras (217) and Hck (218), is directly relevant to oncogenesis. Palmitoylation may also be responsible for immune disorders, such as palmitoylation of T-cell-receptor (TCR) associated protein is necessary for TCR acitivation (219). Furthermore, several DHHC PATs have been reported to be linked with human diseases (184,187), such as DHHC2 with cancer (220), DHHC17 with Huntington disease (221), and DHHC9/15 with X-linked mental retardation (222,223). Together, palmitoylation is required for various protein functions, and regulation

of protein palmitoylation appears to be a good therapeutic target for various human diseases.

### 1.4. Rationale, hypotheses and research objectives

#### 1.4.1. Rationale

Due to the great contribution of atherosclerosis to cardiovascular diseases, large effort has been devoted to prevent the progression of atherosclerosis. The property of lowering plasma LDL-cholesterol leads to the revolutionized discovery of statins, but clinical treatment with statins only reduces 20~40% cardiovascular events depending on the reduction levels of LDL (56,224,225). So, other medical solutions are required to lower the remaining but still high incidence of atherosclerosis.

The inverse epidemiological relationship between HDL and cardiovascular diseases has drawn a huge attention, particularly, when the initial apoA-I infusion studies in human patients show some benefits in the regression of atherosclerosis (226), consistent with the observation in apoA-I transgenic mice (227,228). Extensive studies have been showing that the multiple properties of HDL (42) including anti-inflammation, anti-oxidation, anti-thrombotic activity and cytoprotective role are involved in almost every step of the generation and progression of atherosclerosis. Further studies have indicated that HDL mediated RCT, especially macrophage RCT, plays a major part in its anti-atherosclerosis roles (54,163). Accordingly, raising the HDL levels and promoting HDL mediated RCT may be the potential options. In the past few years, several HDL-raising drugs, including niacin and torcetrapib (a CETP inhibitor), have emerged with favourable effects (42). However, the failure of torcetrapib in the large clinical trial ILLUMINATE (56) impedes the further application. This may be due to the off-target toxicity of the drug (229,230). Considering the unique role in cholesterol homeostasis including absorption, synthesis, RCT (231) and secretion, LXR agonists are becoming another promising pharmacological target (232).

Synthetic ligands for LXRs are protective from atherosclerosis in different murine models (232-234). However, they also promote lipogenesis and cause severe triglyceride accumulation in the liver (235).

Both ABCA1 and ABCG1 are highly upregulated by LXR agonists (235,236), and both of them act as a cholesterol exporter. So drugs specifically targeting to increase ABCA1 and/or ABCG1-mediated RCT may be a prospective solution. However, the mechanism for ABCG1-mediated cholesterol efflux remains unclear, which slows down the potential drug development. Due to the lack of high resolution of ABCG1 structure, other biochemical and biophysical studies are required to understand the underlying mechanisms. Site mutagenesis, which has been widely used to characterize biochemical properties of proteins, is a valuable tool for understanding ABCG1 structure and its cholesterol efflux function. My project is designed to study the contribution of Cys residues in ABCG1 to ABCG1-mediated cholesterol efflux function.

Cys residues are special in the thiol side chain. The Cys S-hydroxylation, a post-translational modification, is a sensitive marker for cellular oxidative stress (237) and plays a significant role in the regulation of various signalling proteins related to both health and disease states (238). Disulphide bonds formed by oxidation of the thiol groups of Cys residues are one of the main types of linkage to crosslink proteins covalently (172), which can increase the protein rigidity and stabilize the tertiary structure and support for individual protein folding, secretion and stability. In addition, Cys is an important source of sulfide for the iron-sulfur clusters (239). The high affinity of the thiol group for heavy metals also facilitates the protein-metal binding (240,241), which is especially important for a wide range of enzymes such as zinc fingers (242,243). Beyond the above, Cys is also the potential site for lipid modifications, including myristoylation, prenylation and palmitoylation (171). Palmitoylation is the only reversible lipidation. Aside from the above mentioned lipidation and oxidation, rarely, Cys also involves in other protein modifications, like phosphorylation (244,245) and ubiquitination (246).

There are 16 Cys residues in the ABCG1 (see Fig. 1.3), of which, one is highly conserved in the 3rd predicted TM domain, two are located in the last extracellular loop between the fifth and sixth TM domain, and the other 13 are located in the N-terminal cytoplasmic region.

### 1.4.2. Hypothesis

ABCG1 is elegantly regulated at the transcriptional level as described above, but the mRNA levels are not always equal to the levels of protein expression. Moreover, less is known about ABCG1 translational modulation. Since the substrates of ABCG1 are lipids, we first hypothesize that ABCG1 is lipidated. Studies in ABCG5/G8 have demonstrated that ABCG5 is palmitoylated, but this palmitoylation is not required for its hetero-dimerization with ABCG8, normal protein trafficking and biliary sterol secretion (113). On the other hand, studies on ABCA1 have demonstrated that ABCA1 is palmitoylated at multiple Cys residues and this modification is required for its trafficking to PM and cholesterol export function (247). Furthermore, ABCG1 does not contain sequence motifs specifically required for other lipid modifications, like prenylation and myristoylation. Thus, we propose that ABCG1 is palmitoylated and this palmitoylation may be important in maintaining the cholesterol efflux function of ABCG1.

### 1.4.3. Research objectives

Site-mutagenesis strategy is applied to the 13 cytoplasmic Cys residues and the single TM Cys. This thesis is focused on the correlation of these Cys residues and cholesterol efflux function. The objective of my project is to investigate how ABCG1 facilitates cholesterol efflux, thereby providing basic knowledge for the future on-target drug design for treatment of atherosclerosis.

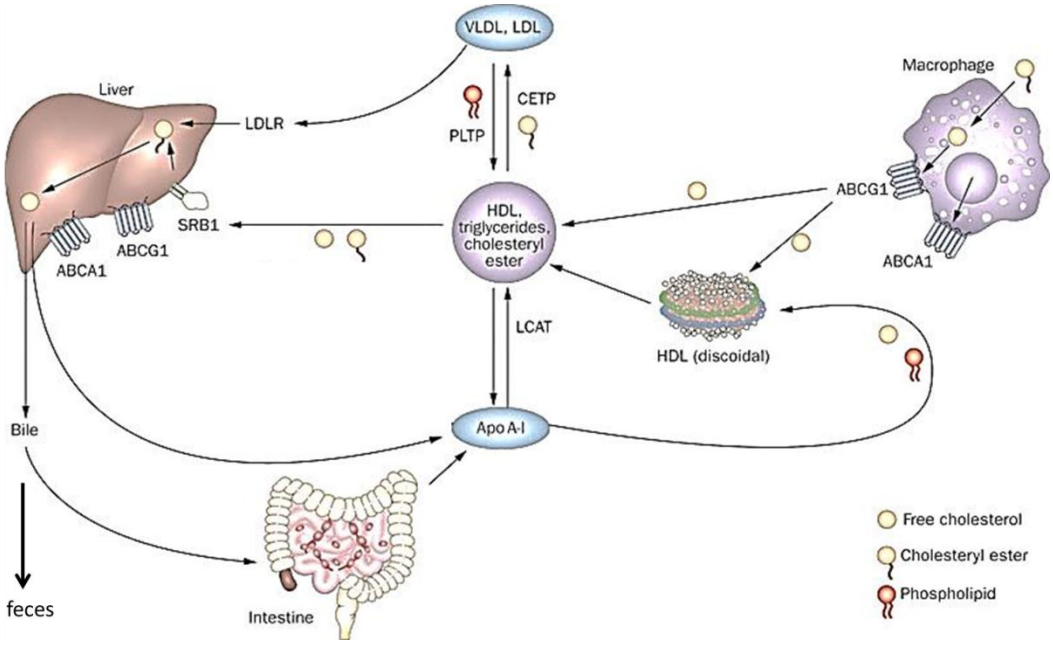



**Figure 1.1. HDL and HDL mediated RCT.** Modified from Figure 2 of (57). RCT is cholesterol transport from peripheral tissues to the liver for final elimination. The central antiatherogenic property of HDL is HDL mediated RCT. The generation of HDL starts from the secretion of lipid-free apoA-I or nascent lipid poor pre-β HDL from the intestine and liver. Such lipid free apoA-I/nascent HDL particles are ready to acquire additional cholesterol and phospholipids via ABCA1, forming small discoidal HDL. The received free cholesterol can then be converted into CE by LCAT, generating the spherical HDL. Both spherical HDL and discoidal HDL further promote cholesterol efflux from peripheral tissues such as macrophages via ABCG1. The newly recruited cholesterol can also get esterified by LCAT. Meanwhile, HDL particles can undergo further modifications through hepatic lipase and endothelial lipase or remodelling by PLTP. The HDL cholesterol can then be selectively taken up by the liver through SR-BI. In humans, spherical HDL can further exchange their CE for triglyceride with LDL or VLDL through CETP, and this part of cholesterol can then be taken up by the liver through LDLR. Eventually, the hepatic cholesterol can be secreted into bile after conversion into bile acids or directly as free cholesterol. The bile components are then either reabsorbed in the intestine or excreted into feces.

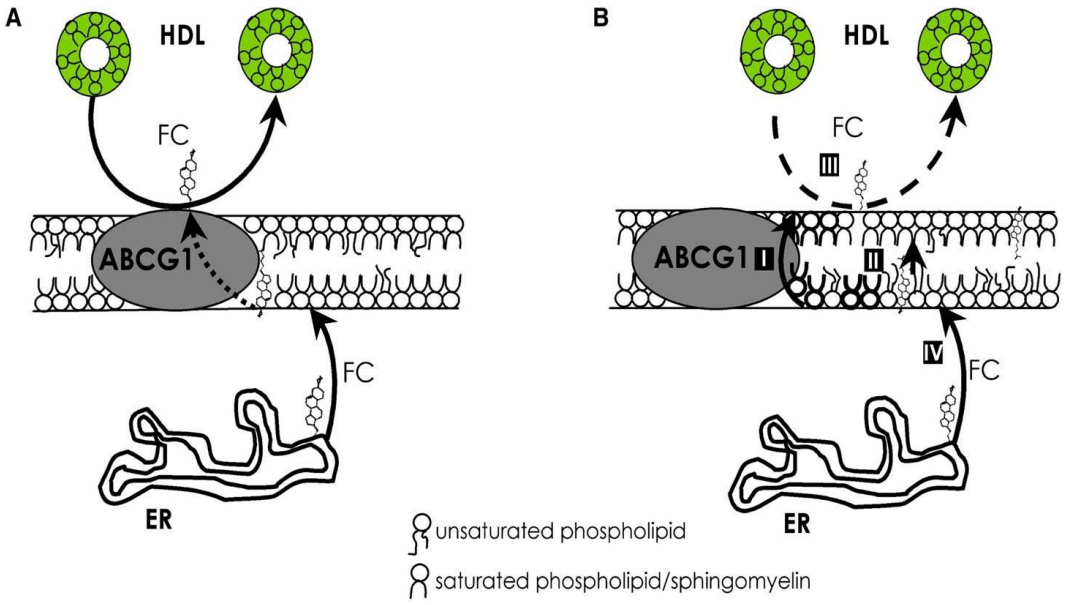



**Figure 1.2 Two proposed mechanisms of ABCG1-mediated cholesterol Efflux.** Modified from Figure 3 of (56). *Panel A:* One model suggests that ABCG1 may accomplish free cholesterol (FC) transfer by utilizing energy provided by ATP binding and hydrolysis at the NBDs to promote the protrusion of cholesterol molecule into hydrophilic water layer, followed by a transient collision with an acceptor (248) such as HDL. *Panel B:* In the second model, ABCG1 may facilitate FC transfer by changing the organization of PM pattern, following with nonspecific binding of acceptors (e.g. HDL) to the plasma membrane. In this model, the cholesterol export depends on the function of ABCG1 as a phospholipid flippase mediating the transfer of phospholipid from the inner to the outer leaflet (I) (151), which could lead to an extensive change in the equilibrium of membrane components such as an increased content of sphingomyelin or saturated phosphatidylcholine. These changes further induce the transbilayer diffusion of cholesterol toward the outer leaflet, which becomes more attractive to cholesterol after the above organization changes (II). Eventually, cholesterol can dissociate onto HDL, perhaps following nonspecific binding of HDL to the PM (III). In both models, the movement of cholesterol from the inner to the outer membrane may be followed by carrier-facilitated diffusion from cellular organelles, notably the ER (IV).

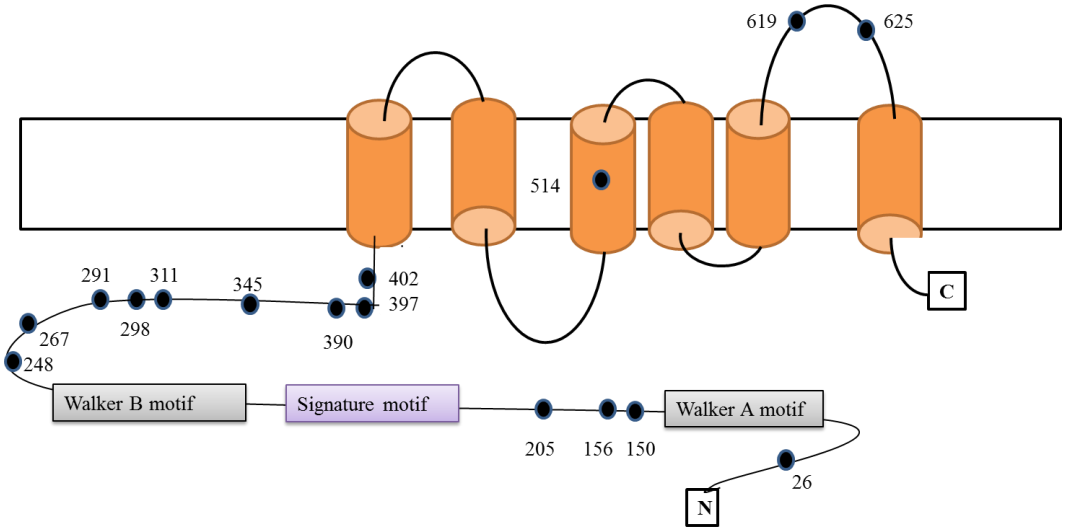



**Figure 1.3. Topology of ABCG1.** ABCG1 belongs to the ABCG subfamily. It contains 1 TMD and 1 NBD, with NBD locating at the N-terminal domain, followed by 6 putative TM α-helices. The NBD contains the ABC motif, namely, the Walker A motif, the Walker B motif and the Signature motif. There are sixteen Cys residues in the ABCG1, of which, one is highly conserved in the 3$^{rd}$ predicted TM domain, two are located in the last extracellular loop between the fifth and sixth TM domain, and the other thirteen are located in the N-terminal cytoplasmic region.

# Chapter 2

# Materials and methods

## 2.1. Materials

Lipofectamine 2000 and Directional pCDNA3.1 TOPO vectors were obtained from Invitrogen. Culture medium and antibiotic G-418 sulfate were purchased from Thermo Scientific and bovine serum albumin (BSA) was purchased from Invitrogen. Complete EDTA-free protease inhibitors were purchased from Roche. Poly-D-lysine and fetal bovine serum (FBS) were purchased from Sigma. [$^{3}$H]-cholesterol (54.2 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). Polyclonal anti-ABCG1 antibodies were purchased from Novus Biologicals and Santa Cruz Biotechnology, Inc. Polyclonal anti-Flotillin1 antibody (used at 1:1,000 dilution) and monoclonal anti-Caveolin1 antibody (D46G3, used at 1:1,000 dilution) were purchased from Cell Signaling Inc. Polyclonal anti-Calnexin antibody (used at 1:10,000 dilution) was purchased from BD Biosciences, and monoclonal anti-Tansferrin receptor (Tfr) antibody (used at 1:5,000 dilution) was purchased from Abcam. Horseradish peroxidase (HRP)-conjugated second antibodies (HRP-goat anti-mouse or HRP-donkey anti-rabbit IgG) were purchased from Sigma, and HRP-NeutrAvidin was purchased from Pierce. IRDye Secondary Antibodies (IRDye 800CW Goat anti-Rabbit IgG and IRDye 680 Goat anti-Mouse IgG) were purchased from Licor Biosciences. QuickChange Lightning Site-Directed Mutagenesis Kits were purchased from Agilent Technologies. Myc-DDK-tagged ORF clone of human apoA-I was purchased from Origene. ABCA1 cDNA was a gift from Dr. Matsuo (Kyoto University Graduate School of Agriculture, Kyoto, Japan). High-density lipoprotein purified from human plasma (pHDL) was purchased from EMD Millipore. Discoidal reconstituted HDL (rHDL) containing apoA-I and 1-palmitoyl-2-linoleoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was prepared by the cholate dialysis method as described previously (249). EZ-Link Sulfo-NHS-LC-Biotin and Streptavidin-agarose slurry were purchased from Thermo Scientific. PVDF (polyvinyl difluoride) membranes and nitrocellulose membranes were purchased from Thermo Scientific and GE Healthcare, respectively.

Antifade reagent was purchased from Vector Laboratories (Burlingame, CA). The ω-alkynyl-palmitate was synthesized as previously described (177), and ω-alkynyl-palmitate and azido-biotin were provided by Dr. Luc Berthiaume (University of Alberta, CA). Tris-(benzyltriazolylmethyl)amine (TBTA) was purchased from Sigma. Tris-carboxyethyl phosphine hydrochloride (TCEP-HCl) was purchased from Thermo Scientific. All other reagents were obtained from Fisher Scientific unless otherwise indicated.

The lipidated apoA-I used in these experiments contains a Myc-DDK tag at the C terminus, and was purified from human embryonic kidney (HEK) 293S cells as described (250). Briefly, HEK 293S cells stably expressing tagged human apoA-I were cultured in suspension in IS GRO serum-free medium (Irvine Scientific) supplemented with 10% FBS. ApoA-I was purified by anti-FLAG M2 affinity gel chromatography (Sigma) following the manufacturer's instruction. The lipidated fraction of apoA-I was isolated via size-exclusion chromatography on a Tricorn Superdex 200 10/300 column (GE Healthcare). Protein purity was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining using EZ-Run protein gel staining solution.

### 2.2. cDNA cloning of ABCG1 and site-directed mutagenesis

Human ABCG1 cDNA cloned in this study was same as the reported canonical sequence (Gene Bank accession number: NM_004915.3) except that the first four amino acid residues (MACL) were missing. The ABCG1 cDNA was cloned into Directional pcDNA3.1 TOPO vector alone (pcDNA3.1-ABCG1) or together with myc tag at the C terminus (c-myc tagged ABCG1). The human ABCG2 cDNA was also cloned into Directional pcDNA3.1 TOPO vector. The mutations were generated using the QuickChange Lightning Site-Directed Mutagenesis Kits according to the manufacturer's instructions. The template used was pcDNA3.1-ABCG1 or c-myc tagged ABCG1. Oligonucleotides bearing mismatched bases at the residue to be mutated were synthesized by IDT, Inc. (Coralville, IA) (see Appendix table1). The sample reactions were set up at 25 μl

volume, containing 2.5 μl of 10× reaction buffer, 50 ng of dsDNA template, 125 ng of oligonucleotide primer (forward), 125 ng of oligonucleotide primer (reverse), 0.5 μl of dNTP mix, 0.75 μl of QuikSolution reagent and 0.5 μl of QuikChange Lightning Enzyme. The reaction conditions are listed in table 2.1. The amplification reaction samples were then incubated with1 μl of *Dpn* I restriction enzyme at 37°C for 2 h. 1 μl of *Dpn* I treated DNA from each reaction sample was tranformed into 50 μl XL-Blue competent cells. DNA and competent cells were gently mixed and incubated on ice for 30min, and then cells were heat shocked at 42°C for 45 seconds, following with incubation on ice for 2 min. With the addition of 1 ml pre-warmed $NZY^+$ broth, the tranformants were incubated at 37°C for 1 hour with shaking at 225–250 rpm, and then selected on the agar plates containing antibiotic ampicillin (at 100μg/ml). The presence of the desired mutation and the integrity of each construct were verified by DNA sequencing (TAGC, Edmonton, Canada). The plasmids containing wild type (WT) or mutant ABCG1 used in the following experiments were ABCG1 without myc tag, unless otherwise indicated.

### 2.3. Cell culture and generation of stable cell lines

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (glucose, 4.5 g/liter) containing 10% (v/v) FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured under 37°C incubator with 5% $CO_2$ and 95% humidity.

To generate the stable cell lines, HEK 293 cells were transfected with pcDNA3.1 or plasmids containing wild type or mutant ABCG1, and then selected by antibiotic G418. Specifically, HEK 293 cells were seeded at $5\times10^5$ cells/well in a 6-well plate on day 1. After 24 h, the cells were transfected with empty vector, or cDNA construct containing WT or mutant ABCG1 (3.2 μg/well), using Lipofectamine 2000 (8 μl/well). After 4h, the medium were replaced with fresh DMEM containing 10% FBS. After reaching to 95% confluence, cells were split

and selected with 1 mg/mL antibiotic G-418 for over two weeks until there was no living cell observed in control cells (HEK 293 cells alone). The living cells were then limit diluted and planted into 96-well plate with DMEM medium containing 1 mg/mL antibiotic G-418. Several subclones were then isolated, and the expression of ABCG1 was confirmed by western blot analysis with a rabbit polyclonal anti-ABCG1 antibody (Novus). Those subclones stably overexpressing WT or mutant ABCG1 were maintained in DMEM containing 10% (v/v) FBS and 0.2 mg/mL G-418.

### 2.4. Cholesterol efflux assay

Cholesterol efflux assay was performed as described previously (60,151). For transient transfection, HEK 293 cells were seeded at $2.5\times10^5$ cells/well in a 12-well plate on day 1. After 24 h, the cells were transfected with plasmids expressing WT or mutant ABCG1 (1.6 μg/well) using Lipofectamine 2000 (4 μl/well). For the cells stably overexpressing wild type or mutant ABCG1, cells were seeded at $2.5\times10^5$ cells/well in a 12-well plate. After 48 h, the cells were directly labeled with [$^3$H]-cholesterol (2 μCi/ml) for 16 h. At the end of the labeling, cells were washed three times in 1 ml of DMEM containing 0.02% BSA. Then, cells were incubated with different acceptor, 5 μg/ml lipidated apoA-I, 25 μg/ml pHDL or 25 μg/ml rHDL in DMEM containing 0.02% BSA, for 5 h. Eventually, the media were collected and the cells were lysed in 0.5 ml of lysis buffer A (0.1 N NaOH, 0.01% SDS). The radioactive content of the media and cells was measured separately by scintillation counting (LS 6500 Multi-Purpose Scintillation Counting, Beckman Coulter). Sterol transfer was expressed as the percentage of the radioactivity released from the cells into the media relative to the total radioactivity in cells plus media. Data were analyzed with GraphPad Prism software and significance was defined as $p<0.05$.

### 2.5. Immunofluorescence of ABCG1

Confocal microscopy was carried out as described previously (251-253).

Approximately $5\times10^5$ HEK 293 cells were seeded in each well of a 6-well tissue culture dish on coverslips pre-coated with poly-D-lysine. After 24 h, the cells were transfected with plasmids expressing WT or mutant ABCG1 as described previously. 48 h later, the cells were washed and fixed with 3% paraformaldehyde in phosphate buffered saline (PBS). Cells were permeabilized using methanol for 20 min, and washed with a blocking solution of 1% BSA in PBS. Then cells were incubated overnight in a blocking solution containing an anti-ABCG1 polyclonal antibody, H-65 (used at 1:100 dilution). After washing with blocking solution for 10 min, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG for 1 h, following with another 3 times washing with the blocking solution, 10 min for each. Coverslips were mounted on the slides with one drop of Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Localization of ABCG1 in the transfected cells was determined using a Leica SP5 laser scanning confocal microscope (filters: 461 nm for DAPI, 488 nm for Fluor 488).

### 2.6. Biotinylation of cell surface protein

Biotin labeling of cell surface proteins were carried out exactly as previously described (250,253). For transient transfection, HEK 293 cells were set up in a 6-well plate and transfected with plasmids expressing WT or mutant ABCG1 as described previously. For the cells stably overexpressing WT or mutant ABCG1, cells were seeded at $5\times10^5$ cells/well in a 6-well plate. After 48 h, HEK 293 cells overexpressing WT or mutant ABCG1 were washed twice with ice-cold PBS buffer, and then biotinylated using EZ-Link Sulfo-NHS-LC-Biotin following the manufacturer's protocol (Thermo Scientific). Specifically, cells were incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin in amine free PBS buffer, gently rocking at 4°C for 30min. Cells were then washed with cold PBS buffer for twice, and the reaction was terminated by blocking with cold PBS containing 100 mM glycine for 20 min at room temperature (RT). After 3 times washing with PBS, cells were lysed in 200 μl of lysis buffer B (1% triton, 150

mM NaCl, 50 mM Tris-HCl, pH 7.4). A total of 45 μl of the cell lysate was saved and 150 μl of the lysate was added to 60 μl of 50% slurry of Streptavidin-agarose (Thermo Scientific) and another 200 μl of lysis buffer B. The mixture was rotated overnight at 4°C. After centrifugation at 7000 rpm for 5 min, the pellets were washed three times with lysis buffer B by centrifugation at 7000 rpm for 5 min and the cell surface proteins were eluted from the beads by adding 60 μl 2× SDS loading buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 50% glycerol, 0.005% bromophenol). A 15 μl 4× SDS loading buffer was added to the saved 45 μl cell lysate, and both cell lysate and cell surface proteins were incubated for 5 min at 95°C. Proteins were then subjected to SDS/PAGE (8%) and analyzed by immunoblotting with a rabbit anti-ABCG1 polyclonal antibody (H-65, Santa Cruz) and a mouse anti-Calnexin polyclonal antibody (BD Biosciences).

### 2.7. Preparation of membrane vesicles from stable cell lines

Membrane vesicles were generated through nitrogen cavitation as described previously (254) with minor modifications. HEK 293 cells stably overexpressing WT or mutant ABCG1were harvested in membrane buffer (250 mM sucrose, 50 mM Tris-HCl, pH 7.4). Cell pellets were stored at -80°C in membrane buffer containing 1× proteinase inhibitors. Cell pellets were thawed under hot water, followed with addition of EDTA to a final concentration of 0.5 mM. Cells were then disrupted by nitrogen cavitation (equilibration at 250 p.s.i. for 10 min) on ice. After centrifugation at 1800 rpm for 10 min, the supernatants were layered over 35% (w/v) sucrose in 20 mM Tris-HCl, 0.5 mM EDTA and then centrifuged at 25000 rpm for 2 h (Beckman, Sw32 Ti). The white interface (~7ml) was collected and washed once with diluted membrane buffer (4ml membrane buffer and 16ml dd$H_2O$) through another centrifugation at 25000 rpm for 1 h. The membrane pellets were resuspended in membrane buffer containing 1× proteinase inhibitors and the samples were further passed through a 27-gauge needle for 10 strokes to generate membrane enriched vesicles. The protein concentrations of these membrane vesicles were determined using the

BCA assay (Pierce). The membrane rich vesicles were aliquoted and temporarily stored at 80°C for future studies.

### 2.8. Protein distribution in sucrose gradient

Sucrose gradient was performed on discontinuous 4~45% sucrose following the previous report (157,255) with modifications. 500 μg of membrane enriched vesicles generated from HEK 293 cells stably overexpressing WT or mutant ABCG1 were solubilized in 1 ml of lysis buffer B containing 1× proteinase inhibitors and 10 mM dithiothreitol (DTT) on ice for 30min. The 1 ml whole cell lysate mixed with 3 ml 60% sucrose in MBS buffer (150 mM NaCl, 50 mM Hepes, pH 7.4) were loaded at the bottom of ultracentrifuge tubes. On the top was a 5–35% discontinuous sucrose gradient (4 ml of 5% sucrose / 4 ml of 35% sucrose, both in MBS buffer). The samples were centrifuged at 35000 rpm in a SW41 rotor (Beckman Instruments, Palo Alto, CA) for 18 h. The fractions were collected at 1 ml per fraction from the top and all fractions were precipitated with 200 μl 100% (m/v) trichloroacetic acid (TCA) overnight at 4 °C. After centrifuge at 14000 rpm for 10 min, the pellets were washed twice with cold acetone and dried at 85°C for 30 min. The dried pellets were dissolved in 60 μl 9 M urea. With the addition of 20 μl 4× SDS loading buffer, the samples were heated at 85°C for 10min before subjecting to SDS-PAGE and immunoblotting.

### 2.9. Gel filtration of ABCG1

Gel filtration chromatography was performed on an AKTA explorer system (GE Healthcare) with a Superdex-200 10/300 column. ~800 μg of PM vesicles generated as previously described were solubilized in 500 μl of lysis buffer B containing 1× proteinase inhibitors and 10 mM dithiothreitol (DTT). The lysates were centrifuged for 15 min at 14,000 rpm to clear insoluble materials. The supernatant was injected into the column pre-equilibrated with 2 column volumes of the same lysis buffer. The elution was collected at 0.5 ml per fraction and fractions were precipitated with TCA overnight at 4°C. The samples were

centrifuged for 10 min at 15,000 rpm. The pellets were then washed twice with cold acetone and dried at 85°C for 30 min. The dried pellets were dissolved in 60 μl 9 M urea. With the addition of 20 μl 4× SDS loading buffer, samples were subjected to SDS-PAGE and immunoblotting. The retention time of ABCG1 was determined by SDS/PAGE and immunoblotting of the collected fractions. Gel filtration protein markers including ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa) (GE Healthcare) were separated under the same condition and observed using a UV detector.

### 2.10. Palmitoylation assay

The palmitoylation of ABCG1 was detected by labeling cells overexpressing WT and mutant ABCG1 with ω-alkynyl-palmitate and followed by the click chemistry reaction as previously described (177,256) with minor modifications.

#### 2.10.1. Metabolic labeling of cells with ω-alkynyl-palmitate

HEK 293 cells transiently or stably expressing V5 tagged ABCG2, myc tagged WT or mutant ABCG1, were plated in 10 cm cell culture dishes. Prior to this labeling, cells were deprived of fatty acids by incubation in DMEM supplemented with 5% dextran-coated charcoal-treated FBS for 1 h. The ω-alkynyl-palmitate was dissolved in DMSO to generate 100 mM stock solutions. To facilitate cellular uptake, ω-alkynyl-palmitate was first saponified by incubation with a 20% molar excess of potassium hydroxide (KOH) at 65°C for 15 min and then conjugated with BSA. The saponified fatty acids were then dissolved in pre-warmed (at 37°C) serum-free DMEM medium containing 20% fatty acid-free BSA. The mixture was incubated at 37°C for an additional 15 min. For each 10 cm dish, a 20× labeling solution was prepared where 4 μl of 100 mM ω-alkynyl-palmitate was saponified by 4 μl 120 mM KOH and conjugated with 200 μl DMEM containing 20% fatty acid-free BSA. After deprivation of fatty

acids, cells were washed with pre-warmed PBS buffer and incubated in fresh 3.8 ml DMEM with a 200 µl supplement of the 20× fatty acid-BSA conjugate in serum-free media for each dish, so that the final concentration of BSA was 1%. Cells were incubated for 4 h, at 37°C in a 5% $CO_2$ humidified incubator.

When charactering the palmitoylation of ABCG1, HEK 293 cells transiently expressing V5 tagged ABCG2 or myc tagged WT ABCG1 were also labeled with palmitate. The labeling process and the following click chemistry reaction were performed exactly the same as when cells were labeled with ω-alkynyl-palmitate.

### 2.10.2. Detection of ω-alkynyl-palmitate labeled ABCG1 using click chemistry reaction

After labeling, cells were washed three times with ice-cold PBS and lysed in the lysis buffer B containing 1× Complete EDTA-free protease inhibitors, for 30 min on ice. The whole cell lysates were prepared and ABCG1 was immunoprecipitated with anti-c-myc antibody (9E10) conjugated beads for overnight at 4°C. Immunoprecipitated ABCG1 (as described above) were washed three times with lysis buffer B, and then adjusted to lysis buffer D (0.1% SDS, 150 mM NaCl, 50 mM Hepes, pH 7.4, 2 mM $MgCl_2$), containing 100 µM TBTA, 1 mM $CuSO_4$, 1 mM TCEP, and 100 µM azido-biotin. The beads were incubated under the above click reaction buffer at 37°C water bath in darkness for 30 min. Reactions were stopped by the addition of 10 volume of ice-cold lysis buffer D and washed for three times by centrifugation at 7000 rpm for 3 min. Precipitated proteins were eluted from the beads by addition of 1× SDS-PAGE sample buffer containing 20 mM DTT. Samples were heated at 95°C for 5 min, and then analyzed by SDS-PAGE and immunoblotting.

### 2.10.3. Potassium hydroxide treatment of PVDF membranes

Samples were subjected to SDS-PAGE in duplicate and then transferred to PVDF membranes by electrophoresis. For alkaline treatment of membrane, the membrane were rinsed briefly in pure methanol after transfer, and then cut into halves. One half was soaked in 50 ml of 0.1 M KOH in methanol [$H_2O$: methanol 1:9 (v/v)], and the other half was soaked in 50 ml 0.1 M Tris-HCl pH 7.0 in methanol [$H_2O$: methanol 1:9 (v/v)] (Tris buffer) at RT for 45 min with gentle shaking. Treated membranes were then briefly rinsed in pure methanol, followed by 6 washes with 1× PBS and 1× PBST (1×PBS buffer containing 1% Tween-20) before probing with HRP-NeutrAvidin, followed by enhanced chemiluminescence detection (ECL).

### 2.11. SDS-PAGE and immunoblot analysis of ABCG1

For cholesterol efflux, the cells overexpressing WT or mutant ABCG1 were collected and lysed in the lysis buffer B, containing 1× complete EDTA-free protease inhibitors for 30 min. The whole cell lysates were subjected to 8% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Immunoblotting was performed using an anti-ABCG1 polyclonal antibody (H-65, Santa Cruz).

For sucrose gradient and gel filtration, fractions were dissolved in 9 M urea and separated by either 8%, 8% ~12%, or commercial 4%~16% SDS-PAGE. After electrophoresis, membranes were analyzed by immunoblotting with a rabbit anti-ABCG1 polyclonal antibody (H-65, Santa Cruz), a rabbit anti-Caveolin1 monoclonal antibody, a mouse anti-Tfr monoclonal antibody and a rabbit anti-Flotillin1 polyclonal antibody,

For palmitoylation, samples were heated and centrifuged at 15000 rpm for 5 min before loading to 8% SDS-PAGE. After electrophoresis, PVDF membranes were rinsed thoroughly and then blocked overnight in 5% non-fat milk/TBST to reduce the background. After 4 times wash with PBST buffer (5 min for each), membranes were probed with HRP-NeutrAvidin (at 1:10,000 dilution) in 5%

BSA/TBS-T for 4 h, following with another 3 times washing with PBST before ECL, to assess the palmitoylation signal. The same membranes were stripped off the palmitoylation signal with stripping buffer (RT, shaking for 4 h) and reblotted with 9E10 antibody for protein expression.

For biotinylation, both whole cell lysates and the pellets were subjected to 8% SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Membranes were probed with both a rabbit anti-ABCG1 polyclonal antibody (H-65, Santa Cruz) and a mouse anti-Calnexin polyclonal antibody,

To assess the above first antibody binding, membranes were either blocked with HRP-conjugated goat anti-mouse or donkey anti-rabbit IgG (Sigma), followed by ECL and then exposed to Kodak BioMax MR films. Or membranes were probed with IRDye Secondary Antibodies (IRDye 800CW Goat anti-Rabbit IgG and IRDye 680 Goat anti-Mouse IgG), followed by near-infrared fluorescence detection in Licor, the Odyssey infrared imaging system. For quantification study in the gel filtration experiment, the latter fluorescence detection was applied and analyzed with the Odyssey infrared imaging software. While for palmitoylation assay, chemiluminescence detection (Pierce) was used and signals were exposed to Kodak BioMax MR films.

**Table 2.1. QuikChange amplification reaction conditions**

| Steps | Cycles | Temperature | time |
|---|---|---|---|
| 1 | 1 | 95°C | 2 minutes |
| 2 | 18 | 95°C | 30 seconds |
| | | 60°C | 10 seconds |
| | | 68°C | 270 seconds |
| 3 | 1 | 68°C | 10 minutes |

## Chapter 3

# Identification of an amino acid residue in ATP-binding cassette transport G1 critical for mediating cholesterol efflux

A version of this chapter has been published on *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* (July 2011)

### 3.1. Introduction

ABCG1 is ubiquitously expressed with relatively high expression levels in macrophages and ECs, and can be up-regulated by LXR agonists (131,132,142) and cholesterol loading (75,129), but suppressed by lipid efflux (257). ABCG1 can mediate the efflux of cellular cholesterol to HDL or to lipidated apoA-I but not to lipid-free apoA-I (60,129,131,143,151,258).

The precise role of macrophage ABCG1 in the protection against the development of atherosclerosis remains uncertain. Conflicting results have been reported from three independent groups (164-166). However, over-expression of ABCG1 in macrophages significantly increases macrophage RCT *in vivo*, whereas knockdown or knockout of *ABCG1* in macrophages markedly decreases macrophage RCT *in vivo* (163). Mice lacking ABCG1 also accumulate lipids in macrophages and hepatocytes fed a high-fat and high-cholesterol diet (125), and develop atherosclerotic lesions when fed an atherogenic diet (259). Recently, it has been reported that mice with vascular ABCG1 deficiency showed increased atherosclerotic lesion areas (156). Moreover, ABCG1 and ABCA1 have been shown to promote cellular cholesterol efflux synergistically (144,145). Knockout of both ABCA1 and ABCG1 in mice leads to dramatic foam cell formation and acceleration of atherosclerosis (168-170). On the other hand, transgenic mice over-expressing the human *ABCG1* gene are protected against dietary fat-induced lipid accumulation in the liver and lung (125). ABCG1-mediated cholesterol translocation also plays an important role in protection against endothelial dysfunction (152,157), interaction of monocytes with ECs (155), and regulation of insulin secretion (148,160). Most recently, Yvan-Charvet *et al.* demonstrated that ABCG1 together with ABCA1 could inhibit hematopoietic stem cell proliferation (161). Therefore, ABCG1-mediated cholesterol efflux plays various important physiological roles. However, how ABCG1 mediates cholesterol efflux onto lipidated lipoprotein particles is unclear.

ABCG1 belongs to the "G" branch of the ABC transporter superfamily. As mentioned previously (Fig. 1.3), the predicted topologies of the "G" subfamily of ABC transporters all contain one single hydrophilic NBD at the $NH_2$-terminus and one hydrophobic TMD consisting of 6 putative TM α-helices at the COOH-terminus. ABCG1 functions as a homo-dimer to mediate cholesterol efflux onto lipidated apoA-I (134). Studies in other ABC transporters have demonstrated that the TM α-helices are involved in substrate recognition, binding, and translocation (260-266). The crystal structure of p-glycoprotein revealed that the substrate-binding site is formed by TM helices (267). Since there are no available crystal structures for the "G" half transporter subfamily, site-directed mutagenesis has been widely used to identify amino acid residues important for transporter function. For example, mutational studies of ABCG2 have demonstrated that polar residues located in TMs 1 and 6 of ABCG2 are involved in conferring drug resistance (264,265). However, no such study has been done in ABCG1. We noticed that the TMD of ABCG1 contains a single Cys at position 514 (Cys514) within the putative TM 3. This Cys residue is completely conserved among various species except for Drosophila (the White protein) (Fig. 3.1). However, the white protein in drosophila transports precursors of eye pigments, such as guanine and tryptophan, in the eye cells of the fly (268). The mammalian ABCG1 protein is involved in cholesterol transport. Studies in other TM proteins have demonstrated that Cys residues located in TMs play a critical role in protein functions (269-277). To evaluate the role of Cys514 in ABCG1 function, we replaced this residue with alanine (Ala), threonine (Thr), and serine (Ser). These mutant proteins were then expressed in human embryonic kidney (HEK 293) cells, and the transfectants were characterized with respect to their ability to efflux cholesterol. We found that mutation of Cys514 to Ala, Thr, or Ser virtually abolished the efflux of cholesterol onto lipidated apoA-I. The findings identify Cys514 in ABCG1 as a critical component for mediating cholesterol efflux.

### 3.2. Materials and methods (see Chapter 2)

## 3.3. Results

### 3.3.1. Characterization of ABCG1 in HEK 293 cells

ABCG1 mediates cholesterol efflux onto lipidated lipoprotein particles and plays an important role in RCT, yet exactly how this occurs is unclear. To elucidate the underlying mechanism, we cloned human ABCG1 cDNA from HepG2 cells and performed the efflux assay. As shown in Figure 3.2A, when compared to cells transfected with empty vectors, HEK 293 cells transiently overexpressing ABCG1 significantly increased the efflux of cholesterol onto reconstituted HDL (rHDL), HDL purified from human plasma (pHDL), and lipidated apoA-I purified from HEK 293S cells stably expressing tagged human apoA-I. To further characterize ABCG1 function, we examined the efflux of cholesterol from ABCG1 or ABCA1 overexpressed cells onto different acceptors. ABCA1 was well expressed in transiently transfected HEK 293 cells (Fig. 3.2E). As shown in Figure 3.2D, ABCA1-mediated cholesterol efflux onto apoA-I, but not onto either lipidated apoA-I or rHDL. On the other hand, ABCG1-mediated cholesterol efflux onto lipidated apoA-I and rHDL, but not onto apoA-I (Fig. 3.2D). These findings are consistent with previous reports (100,151,278-280). Furthermore, the conserved lysine residue located in the Walker A motif of the NBD of ABCG1 was mutated to methionine (K120M). The protein levels of WT and mutant ABCG1 were comparable in transiently transfected HEK 293 cells (Fig. 3.2C). Mutation K120M virtually eliminated ABCG1-mediated cholesterol efflux onto lipidated apoA-I (Fig. 3.2B), a finding that is consistent with previous reports (151). Thus, ABCG1 cloned from HepG2 cells can efficiently mediate cholesterol efflux onto lipidated apoA-I.

### 3.3.2. Effect of mutations of Cys at position 514 in ABCG1 on cholesterol efflux

Next, we focused on structure and function studies of ABCG1. Amino acid residues located in TMDs of ABCG2 have been demonstrated to play an

important role in ABCG2-mediated drug resistance (264,265). We noticed that there is a single highly conserved Cys (Cys514) located in the putative TM 3 of ABCG1 (Fig. 3.1). TM Cys residues play a crucial role in protein functions (271-277). Thus, we mutated Cys514 in ABCG1 to alanine (C514A) to assess the role of this residue in ABCG1 function. The WT and mutant ABCG1 were transiently expressed in HEK 293 cells. The levels of mutant ABCG1 were comparable to that of WT ABCG1 (Fig. 3.3A, lanes 2 and 3). A cholesterol efflux assay was then performed to examine the effect of C514A on ABCG1-mediated cholesterol efflux. As shown in Fig. 3B, the mutation of Cys514 to Ala (C514A) dramatically decreased the efflux of cholesterol onto lipidated apoA-I.

To further confirm the effect of C514A on ABCG1 function, we made several HEK 293 stable cell lines. The pcDNA3.1 vector containing WT or mutated forms of full-length ABCG1 cDNAs was used to stably transfect HEK 293 cells. From the initial populations of transfectants, we isolated two subpopulations of mutant C514A by limiting cell dilution. Protein levels of one subpopulation of mutant C514 (C514A-1) were approximately equivalent to the levels of WT ABCG1 (Fig. 3.3C, lanes 2 and 3), which was used in the subsequent gel filtration experiment. The expression levels of C514 in another subpopulation (C514A-2) were higher than that of WT ABCG1 (Fig. 3.3C, lanes 2 and 4). Both subpopulations did not show any significant increase in cholesterol efflux onto lipidated apoA-I when compared with HEK 293 cells stably transfected with empty pcDNA3.1 (Fig. 3.3D). These findings demonstrated that Cys514 plays an important role in ABCG1-mediated cholesterol efflux onto lipidated apoA-I.

To investigate the requirement of the side chain of the amino acid residue at position 514, Cys514 was further mutated to Thr (C514T) and Ser (C514S). HEK 293 cells were transiently transfected with pCDNA3.1 vector containing WT or mutated forms of full-length ABCG1 cDNAs. The expression levels of WT and mutant ABCG1 were comparable (Fig. 3.4A). Mutations C514T and

C514S, like C514A, essentially abolished ABCG1-mediated cholesterol efflux onto the acceptor (Fig. 3.4B). These results demonstrate the specific requirement of the thiol group on the side chain of Cys at position 514 for ABCG1-mediated cholesterol efflux.

### 3.3.3. Effect of mutation C514A on ABCG1 trafficking

We next investigated how mutation C514A affected ABCG1 function. Mutation C514A appeared not to affect the stability of ABCG1 because the expression levels of C514A were comparable to that of WT ABCG1 in HEK 293 cells transiently or stably overexpressing ABCG1 proteins (Figs. 3.3A, 3.3C, and 3.4A). It is believed that ABCG1-mediated cholesterol efflux mainly occurs at the cell surface. The transporter redistributes cholesterol to a cell surface pool, from where cholesterol is then picked up by lipidated lipoprotein particles (143). To determine if the effect of C514A on cholesterol efflux might be attributable in part to changes in trafficking of ABCG1, we examined the localization of C514A as well as WT ABCG1 by confocal microscopy. As shown in Fig. 3.5A, cells expressing the mutant protein showed a staining pattern comparable with that of cells expressing WT ABCG1, indicating that the trafficking of the mutant protein was unaffected significantly.

To further confirm this finding, we monitored the cell surface amount of WT and mutant ABCG1 via labeling the cells with biotin. The cell surface proteins were precipitated using Streptavidin-agarose beads and then immunoblotted for the ABCG1. The amount of cell surface ABCG1 in cells expressing the WT or mutant protein was comparable (Fig. 3.5B, lanes 4 and 6). Taken together, our findings demonstrate that substitution of Cys514 with Ala does not affect trafficking of ABCG1 to the cell surface.

### 3.3.4. Effect of C514A on ABCG1 dimerization

ABCG1 is a half ABC transporter. Previous studies using chemical crosslinkers and co-immunoprecipitation have demonstrated that ABCG1 can form homo-dimers (134,151). Gel-filtration is a valid approach to give useful estimates of the molecular weights of proteins in solution and has been successfully used to characterize oligomerization of ABCG2 (281). Thus, we employed this technique to assess the native molecular size of ABCG1 in solution. To isolate enough PM enriched fraction for the gel filtration experiment, large quantities of cells were needed. Thus, we used HEK 293 cells stably overexpressing WT or mutant ABCG1 instead of cells transiently transfected with ABCG1 that were used in other experiments. PM enriched fractions isolated from HEK 293 cells expressing WT or mutant ABCG1 (~800 µg total proteins) were extracted in 500 µl of lysis buffer B containing 10 mM DTT. The extracts were fractionated by gel filtration chromatography on a Superdex 200 column, and fractions were analyzed by Western blotting with antibodies to ABCG1 and transferring receptor (Fig. 3.6). As shown in Figure 3.6A, the majority of WT ABCG1 (~75 kDa for a monomer) eluted in fractions 16 and 17 (retention volumes 8.0-8.5 ml) with an estimated molecular mass of 160 kDa, indicating that it was present predominantly as a dimer. We next performed chromatography of C514A on the same Superdex 200 column to investigate if the replacement of Cys514 with Ala disrupted the dimerization of ABCG1. The elution pattern of mutant ABCG1 was essentially identical as that of the WT protein. The majority of mutant ABCG1 protein also eluted as dimers in fractions 16 and 17 (retention volumes 8.0-8.5 ml) (Fig. 3.6 A and B). As a control, the elution patterns of Tfr, a well-studied membrane protein, extracted from cells transfected with control, WT ABCG1, or C514A were essentially the same, mainly eluted in fractions 20-25 (retention volumes 10-12 ml) (Fig 3.6C and 3.6D). The same experiment was also performed in the absence of DTT. Both WT ABCG1 and mutant C514A also mainly eluted in fractions 16 and 17 (Fig. 3.7B). Tfr mainly eluted in fractions 20-25 (Fig. 3.7A). There was no detectable ABCG1 signal in fractions 1-13 in the presence (data not shown) or absence of DTT (Fig. 3.7C). Therefore, mutation C514A does not affect dimerization of the half transporter.

### 3.4. Discussion

The major finding of this paper is that Cys514, located in the third putative TM α-helix of ABCG1, plays an important role in ABCG1-mediated cholesterol efflux. Substitution of Cys514 with Ala virtually eliminated the efflux of cholesterol onto lipidated apoA-I mediated by ABCG1. Replacement of Cys514 with a more conserved amino acid residue, Thr or Ser, also significantly decreased ABCG1-mediated cholesterol efflux. These findings demonstrate the specific requirement of the thiol group at position 514 within TM 3 of ABCG1 for the efficient efflux of cholesterol onto lipidated apoA-I.

Cys514 is conserved in human, mouse, rat, cow, chicken, turkey, dog, horse, chimp, fish (tilapia and zebrafish), and leishmania (Fig. 3.1). Since there are no available crystal structures for ABCG transporters, how Cys514 contributes to ACBG1 function is unclear. TM Cys residues play a role in protein trafficking and oligomerization. For instance, Cys residues in the TMDs of the Na(+)/dicarboxylate co-transporter (NaDC-1) is important for protein stability and trafficking to the PM (274). Removal of Cys residues in NaDC-1 led to very low total protein and cell surface protein expression. In the present study, we observed that the total protein levels of C514A in both transiently and stably transfected cells were comparable to that of WT ABCG1 (Figs. 3.3 and 3.4). Furthermore, the data from the biotinylation experiment and confocal microscopy clearly showed that the mutant half transporter trafficked to the PM as efficiently as the WT protein. Thus, simply replacing the Cys514 residue with Ala did not alter protein stability and trafficking, strongly suggesting that the mutations did not result in a major perturbation of the structure of the protein.

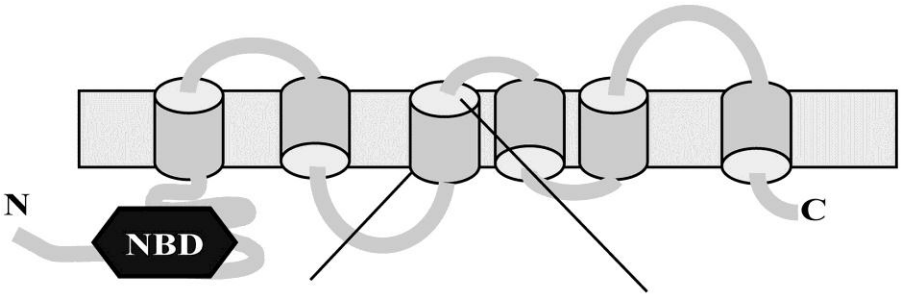
Studies in phospholamban demonstrated that the steric properties of three Cys residues located in the C-terminal TM domain are required for pentamer formation and stability (282,283). The Cys residue in the TM domain of synaptobrevin is also important for homo-dimerization of the synaptic vesicle protein (275,284). Mutation of Cys103 in synaptobrevin to Ala significantly

reduced homo-dimer formation (284). We observed that ABCG1 formed dimers in solution (Fig. 3.6A), consistent with previous results obtained from studies using chemical crosslinkers and co-immunoprecipitation (134,151). When the same experiment was carried out for C514A, we found that the elution patterns of mutant ABCG1 were the same as that of WT ABCG1. Thus, unlike phospholamban and synaptobrevin, mutation of Cys514 in ABCG1 does not appear to affect cholesterol transport through interrupting protein dimerization.

Crystal structure along with biochemical studies of *Escherichia coli* lactose permease, a 12 TM helix transporter, have revealed that Cys at position 148 within the fifth TM α-helix is located in the vicinity of a substrate-binding site and probably interacts with the hydrophobic face of galactosyl moiety (285,286). In addition, Cys144 in the third TM domain of creatine transporters is highly conserved and located in a substrate-binding site (287). Thus, it is possible that Cys514 within TM 3 of ABCG1 is located in or near a substrate-binding site and directly involved in substrate binding and/or translocation. However, in the creatine transporters, mutation of Cys144 to Ala essentially eliminated protein activity while replacement of Cys144 with the more conserved residue Ser had no significant effect. In the present studies, we observed that replacement of Cys514 with Ala, Ser, or Thr, all significantly impeded ABCG1-mediated cholesterol efflux. Thus, unlike the creatine transporters, ABCG1 specifically requires the sulfhydryl group at position 514 within TM 3 for its cholesterol transport function. It has been reported that the sulfhydryl group of Cys within TM regions tends to hydrogen bond to backbone carbonyl oxygens at position i-4 of the helix in the bilayer (288). Thus, the sulfhydryl group of Cys514 may not directly interact with substrates. Instead it appears to contribute to the establishment and/or stabilization of the substrate binding pocket in ABCG1 through hydrogen bonding to the i-4 carbonyl oxygen. Further studies will be required to determine this possibility.

To the best of our knowledge, this is the first report concerning the

identification of an amino acid residue that is critical for ABCG1-mediated cholesterol efflux. However, the detailed mechanism by which the thiol group, at position 514 within the putative TM 3 of ABCG1, contributes to the protein function is unclear. Further studies will be required to determine the underlying mechanism(s).




```
human    503 VPFQIMFP-VAYCSIVYWMT 521
chimpanzee   VPFQIMFP-VAYCSIVYWMT
mouse        VPFQIMFP-VAYCSIVYWMT
rat          VPFQIMFP-VAYCSIVYWMT
dog          VPFQIMFP-VAYCSIVYWMT
giant panda  VPFQIMFP-VAYCSIVYWMT
cattle       VPFQIMFP-VAYCSIVYWMT
horse        VPFQIMFP-VAYCSIVYWMT
tilapie      VPFQVVFP-VVYCSIVYWMT
zebrafish    VPFQIVFP-VAYCSIVYWMT
chicken      VPFQIMFP-VAYCSIVYWMT
turkey       VPFQIMFP-VAYCSIVYWMT
leishmania   FPVQILAVLVESC-ILYWTV
drosophila   LPLFLTVPLVFTA-IAYPMI
                          #
```

**Figure 3.1. Sequence alignment of TM 3 for 13 orthologs of ABCG1.** The sequence alignment was performed using ClustalW2, a multiple sequence alignment program for DNA or proteins. # indicated the highly conserved Cys residue in TM 3 of ABCG1.

**Figure 3.2. Efflux of cholesterol onto different acceptors.**

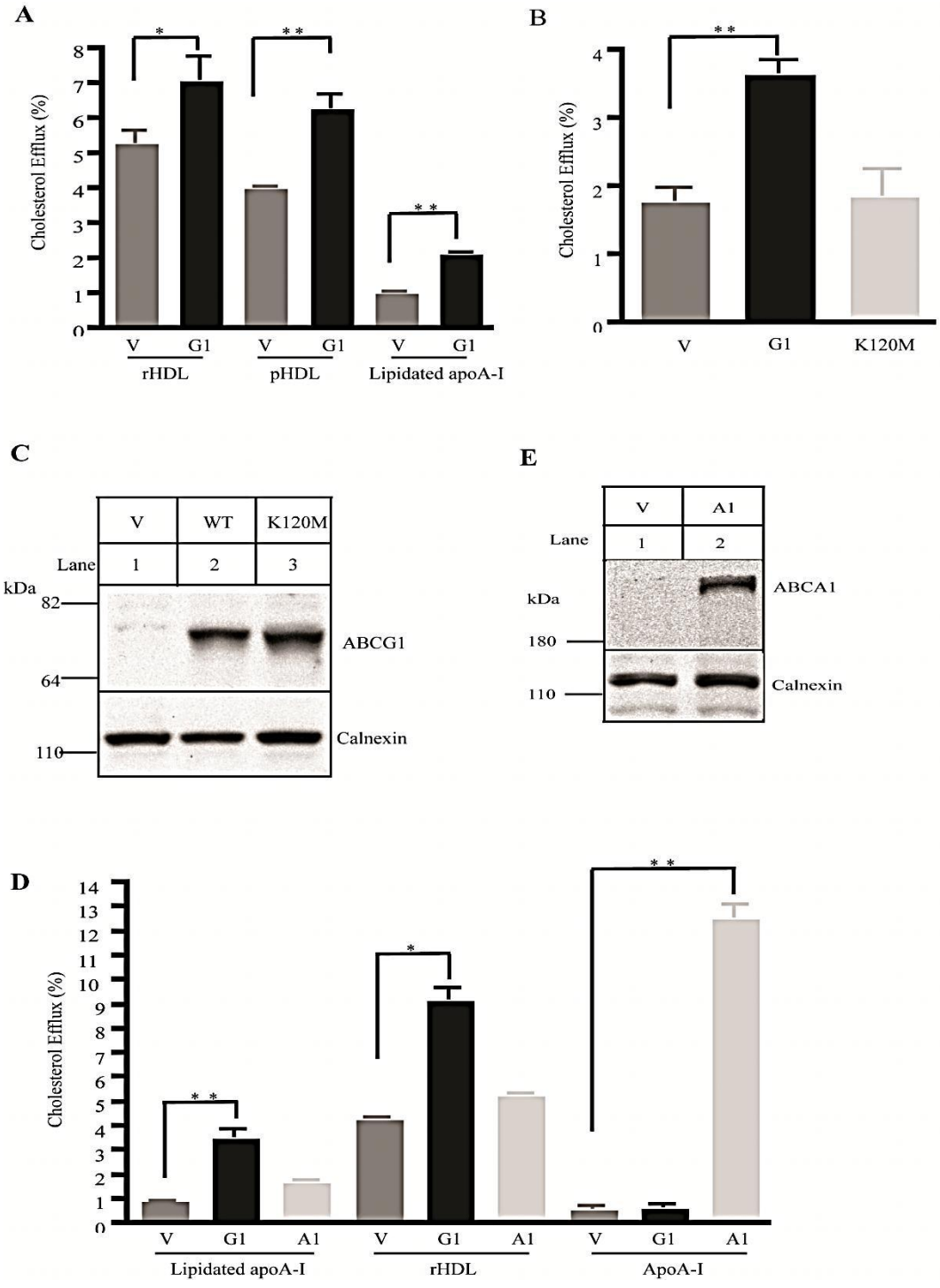


**Figure 3.2. Efflux of cholesterol onto different acceptors**. *Panels A and B*: Efflux of ABCG1-mediated cholesterol onto various acceptors was carried out as described. HEK 293 cells were transiently transfected with empty vector (V) or plasmid containing WT or mutant ABCG1 (K120M) cDNA and labeled with [$^3$H]-cholesterol. After washing, the cells were incubated with reconstituted HDL (rHDL), HDL purified from human plasma (pHDL) or lipidated apoA-I. The radioactive content of the media and cells was measured separately by scintillation counting. Sterol transfer was expressed as the percentage of the radioactivity released from the cells into the media relative to the total radioactivity in the cells plus media. Data were analyzed with GraphPad Prism software and significance was defined as $p<0.05$. *Panel C:* Expression of WT and mutant ABCG1 in HEK 293 cells transiently overexpressing WT and mutant ABCG1. Whole cell lysates were made from HEK 293 cells transiently transfected with empty vectors (V) or vectors containing WT or mutant ABCG1 (K120M) cDNA and subjected to SDS-PAGE and immunoblotting. Blots were probed with a polyclonal anti-ABCG1 antibody and a polyclonal anti-Calnexin antibody. *Panel D*: ABCG1- and ABCA1-mediated cholesterol onto various acceptors. The experiment was carried out as described in Fig. 3.2A. The cells tested transiently overexpressed ABCG1 (G1) or ABCA1 (A1). *Panel E:* Expression of ABCA1 in HEK 293 cells. The experiment was carried out as described in Fig. 3.2C except that the cells transiently overexpressed ABCA1 and that the blots were probed with a monoclonal anti-ABCA1 antibody. *: $p<0.05$; **: $p<0.01$. Values are mean ± S.D. of ≥3 independent experiments.

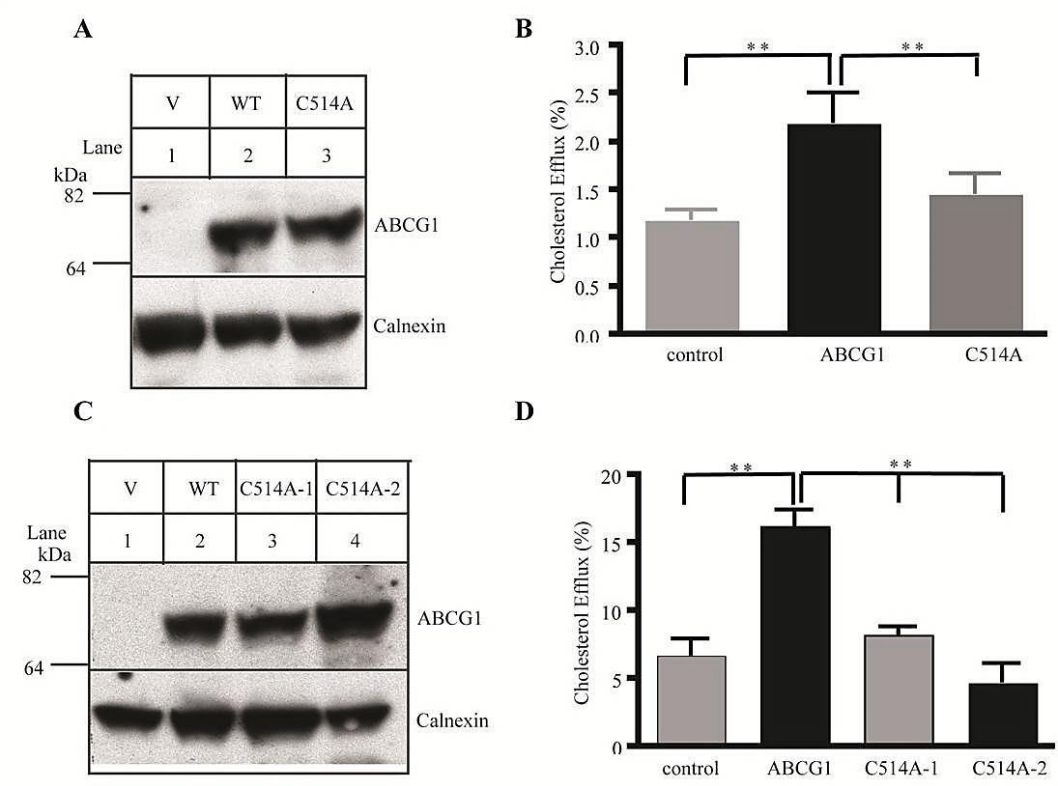



**Figure 3.3. Effect of mutation C514A on ABCG1-mediated cholesterol efflux.** *Panel A*: Expression of ABCG1 in HEK 293 cells transiently overexpressing WT and mutant ABCG1 was determined as described in Fig.2C. *Panel B*: Cholesterol efflux onto lipidated apoA-I was carried out as described in Fig. 3.2A. *Panel C*: Expression of ABCG1 in HEK 293 cells stably overexpressing ABCG1 protein. The ABCG1 expression levels were examined as described in Fig. 3.2C. *Panel D*: Cholesterol efflux onto lipidated apoA-I. The experiment was performed as described in Fig. 3.2A except that the cells stably overexpressed the WT or mutant ABCG1 (C514A). V, control: empty vector; WT: wild type ABCG1; C514; mutant ABCG1, in which Cys514 was mutated to Ala. *: $p<0.05$; **: $p<0.01$. Values are mean ± S.D. of ≥3 independent experiments.

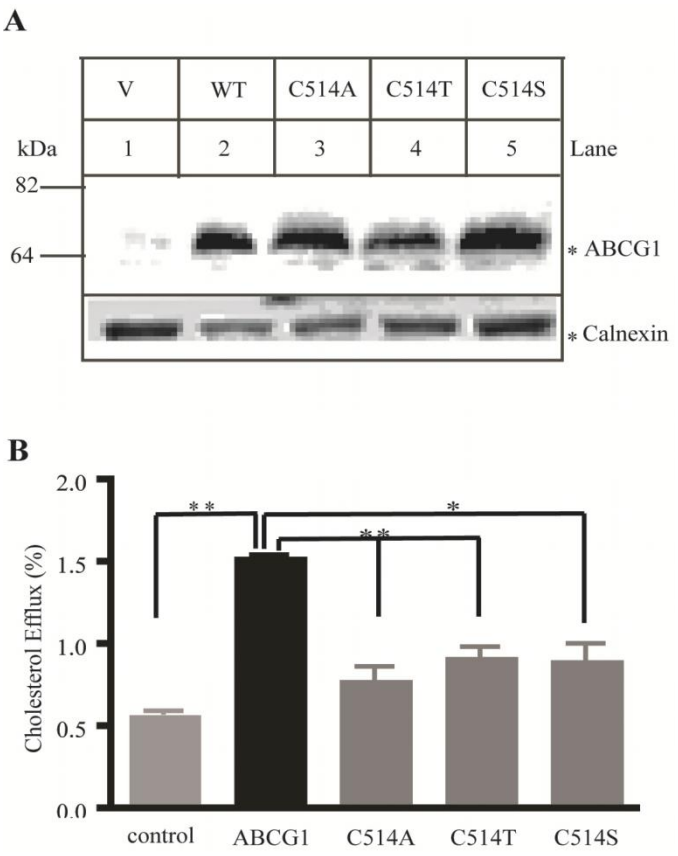



**Figure 3.4. Effect of mutations at Cys514 on ABCG1-mediated cholesterol efflux.** *Panel A*: Expression of WT and mutant ABCG1 in HEK 293 cells transiently overexpressing ABCG1 protein. Protein levels were determined as described in Fig. 3.2C. V: control; WT: wild type ABCG1; C514A, C514T, or C514S: mutant ABCG1, in which Cys514 was mutated to Ala, Thr, or Ser, respectively. *Panel B*: Cholesterol efflux onto lipidated apoA-I was carried out as described in Fig. 3.2A. *: $p<0.05$; **: $p<0.01$. Values are mean ± S.D. of ≥3 independent experiments.

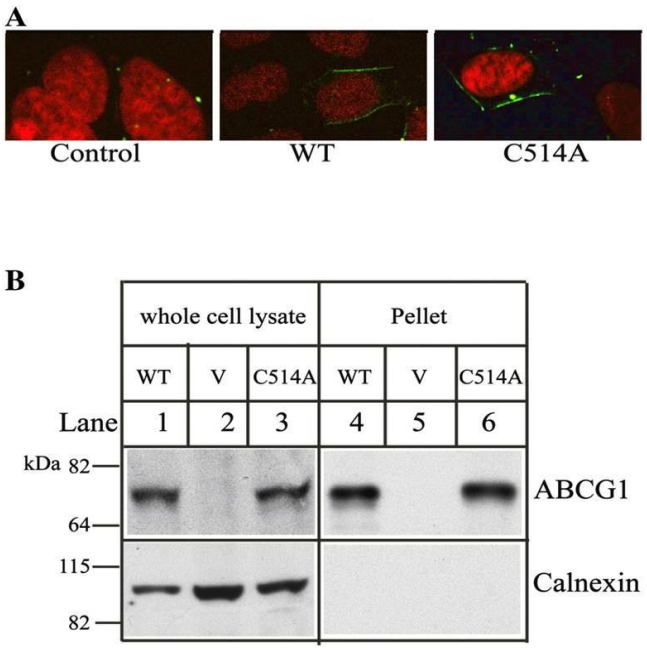



**Figure 3.5. Trafficking of WT and mutant ABCG1 in HEK 293 cells.** *Panel A:* The subcellular localization of WT and mutant ABCG1 was determined by confocal microscopy as described. ABCG1 was detected using polyclonal anti-ABCG1 antibody (H-65). Location of ABCG1 was indicated in green. Nuclei were stained with DAPI and were shown in red. Transfectants expressed empty vector (control), WT or mutant ABCG1 (C514A) were indicated in the figure. An x-y optical section of the cells illustrates the distribution of the WT and mutant proteins between the plasma and intracellular membranes. *Panel B:* HEK 293 cells transiently expressing WT or mutant ABCG1 were biotinylated exactly as described. Biotinylated cell surface proteins (indicated as pellet) were pulled down by Streptavidin-agarose slurry from the whole cell lysate. Both pellets and the whole cell lysate were subjected to SDS/PAGE and immunoblotting. ABCG1 was detected using a polyclonal anti-ABCG1 antibody and Calnexin was detected with a polyclonal antibody. Similar results were obtained in two independent experiments.

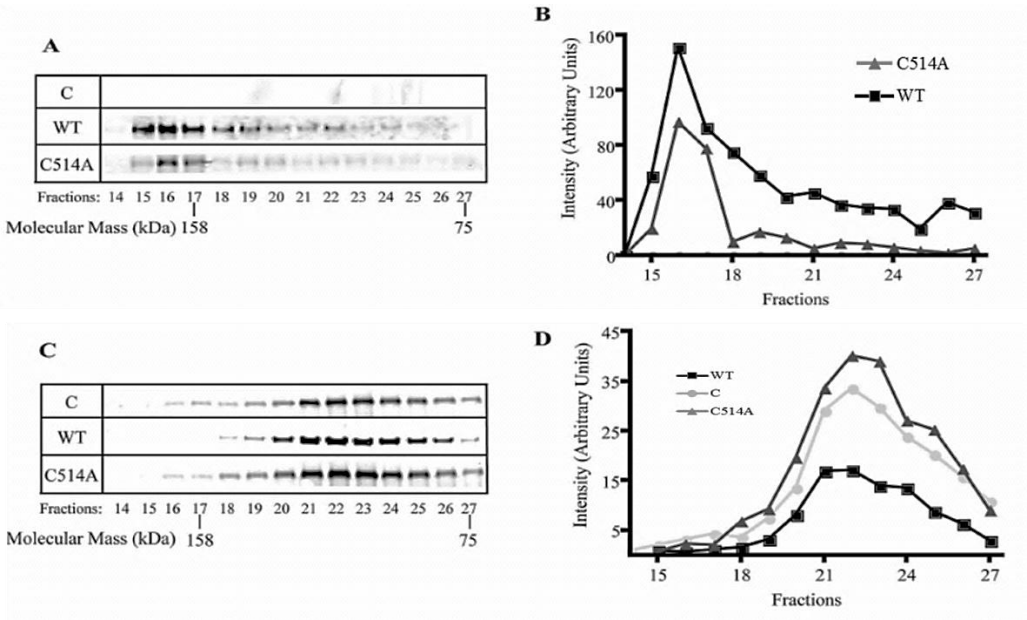



**Figure 3.6. Gel filtration of WT and mutant ABCG1 with DTT.** 800 μg of PM enriched fraction isolated from HEK 293 cells stably expressing WT or mutant ABCG1 were solubilized in 500 μl of lysis buffer B containing 1× proteinase inhibitor and 10 mM DTT. The lysates were centrifuged for 15 min at 14,000 rpm to clear insoluble materials. The supernatant was loaded onto a Superdex 200 10/300 GL column equilibrated with lysis buffer B. The elution was collected at 0.5 ml per fraction and precipitated with TCA overnight at 4°C. The pellets were washed twice with cold acetone and dried. The dried pellets were dissolved in 9 M urea and subjected to SDS-PAGE and immunoblotting. The retention time of ABCG1 was determined by SDS/PAGE and immunoblotting of the collected fractions. *Panels A and C*: Gel filtration fractions were blotted with a polyclonal anti-ABCG1 (*Panel A*) and a monoclonal anti-Tfr antibody (*Panel C*), respectively. The elution positions of the following protein standards are shown: aldolase (158 kDa) and conalbumin (75 kDa). Similar results were obtained in two independent experiments. *Panels B and D*: Densitometry of ABCG1 (*panel B*) and Tfr (*panel D*) in each fraction. The proteins were quantified by measuring the intensity of the Western blot using Licor software and plotted against fraction numbers.

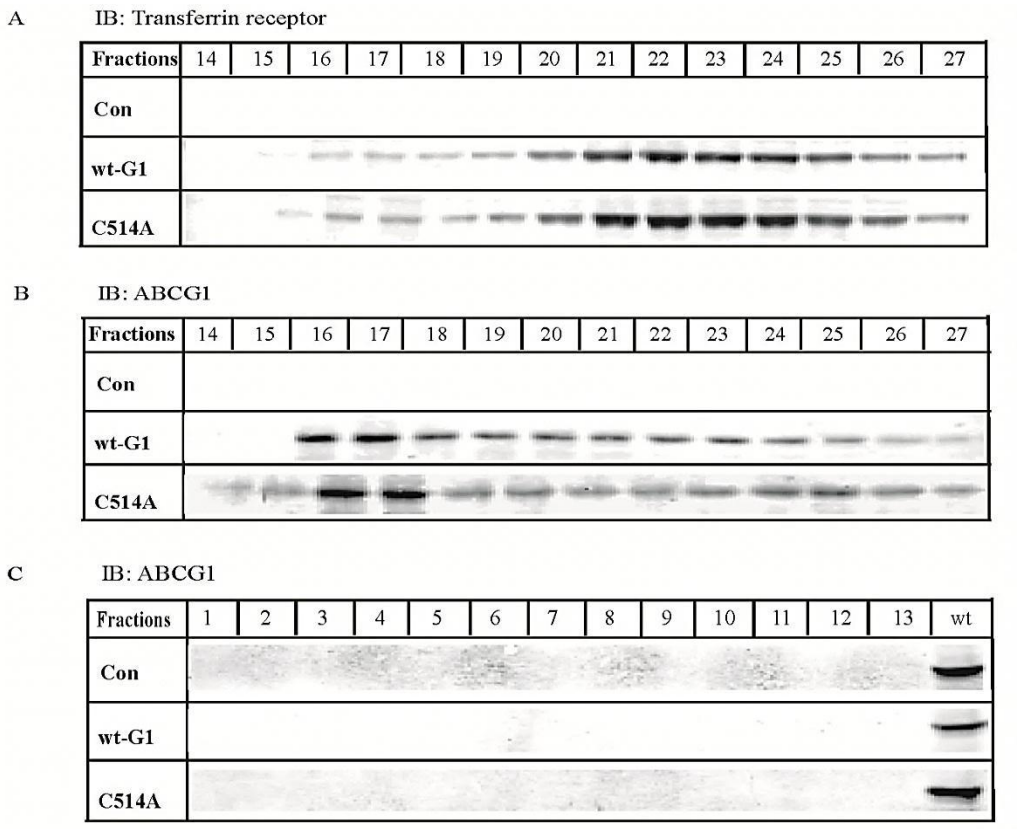



**Figure 3.7. Gel filtration of WT and mutant ABCG1 without DTT.** 800 μg of PM enriched fraction isolated from HEK 293 cells stably expressing WT or mutant ABCG1 were solubilized in 500 μl of lysis buffer B containing 1× proteinase inhibitor without DTT. The lysates were centrifuged for 15 min at 14,000 rpm to clear insoluble materials. The supernatant was loaded onto a Superdex 200 10/300 GL column equilibrated with lysis buffer B. The elution was collected at 0.5 ml per fraction and precipitated with TCA overnight at 4°C. The pellets were washed twice with cold acetone and dried. The dried pellets were dissolved in 9 M urea and subjected to SDSPAGE and immunoblotting. The retention time of ABCG1 was determined by SDS/PAGE and immunoblotting of the collected fractions. *Panel A:* Gel filtration fractions were blotted with a monoclonal anti-Tfr antibody. *Panels B and C:* Gel filtration fractions were blotted with a polyclonal anti-ABCG1 antibody (H-65, Santa Cruz). Extracts of WT ABCG1-overexpressed PM fractions (wt-G1) were used as a positive control in panel C.

## Chapter 4

# Palmitoylation of ABCG1 is required for ABCG1-mediated cholesterol efflux

Manuscript is in preparation.

### 4.1. Introduction

ABCG1 is ubiquitously expressed with relatively high levels in macrophages and ECs (131). It works sequentially with ABCA1 (144) and plays an important role in regulating cell and body sterol homeostasis (125). ABCG1 can mediate cholesterol efflux onto lipidated lipoproteins like HDL (143), thereby preventing the formation of foam cells, key cells in the initiation and progression of atherosclerosis (289). Due to the great contribution in RCT, especially macrophage RCT, ABCG1 is tightly associated with the progression and regression of atherosclerosis (46,290). Strategies to upregulate the expression of ABCG1 and enhance its cholesterol efflux function could have therapeutic potentials for reducing atherosclerosis.

Transcriptionally, ABCG1 is elegantly regulated by LXRs (132,142) as well as lipid loading (75). Synthetic LXR agonists have shown prospective benefits in the prevention of atherosclerosis (232,234). However, less is known of the protein co-translational and post-translational modifications. Unlike other ABCG family members, such as ABCG2 (291), ABCG5 and ABCG8 (292), ABCG1 is not glycosylated (134,151). Only recently, it was reported that ABCG1 is phosphorylated at Ser, Thr and tyrosine residues under a basal condition (138). More recently, 12/15-Lipoxygenase, a nonheme iron containing dioxygenase that is implicated in the pathogenesis of diabetes and atherosclerosis, has been demonstrated to be able to promote ABCG1 degradation by inducing protein serine phosphorylation in a p38- and JNK2-dependent manner (139). Thus, these pathways are potential novel approaches for maintaining cellular cholesterol homeostasis and prevention of atherosclerosis.

Palmitoylation, covalent attachment of palmitate to the side chain of Cys in proteins through a thioester linkage, is the most widely concurrent posttranslational protein lipid modification (171,186). Similar to other lipid modifications, such as prenylation and myristoylation, palmitoylation enhances the hydrophobicity of proteins and facilitates protein membrane targeting and

binding (171). Similar to the dynamic property of phosphorylation, palmitoylation is the only reversible posttranslational lipid modification (183). It acts as a switch in cellular signalling to police protein stability and trafficking (196) and plays an important role in maintaining the structure and function of a wide range of proteins (212,293). Given the hydrophobicity of ABCG1 substrates, sterols, and the abundance of cytoplasmic Cys residues, we hypothesized that ABCG1 is S-palmitoylated and palmitoylation of ABCG1 plays an important role in its cholesterol export function.

However, for a long period, the detection of palmitoylation has been relied upon the metabolic radioactive labeling, in which target proteins are labeled with [$^{3}$H]-fatty acids (294). The extremely long exposure time and the demanded large amount of work severely impede the investigation of the mechanism of palmitoylation and the identification of palmitoylated proteins. Although the later [$^{125}$I]-labeled fatty acids significantly accelerate the detection process, it still requires large supply and special handling of hazardous isotopes (177). Recently, the emergence of fatty acids analogues containing an azido group (295) and the following detection by Staudinger ligation has significantly increased the detection sensitivity (256). This approach has been applied in identifying the palmitoylated proteins in mitochondria (296) and the process of apoptosis (256). However, the above methods are not suited to characterize the subcellular localization of the lipid-modified proteins (297). The ω-alkynyl-fatty acids, including ω-alkynyl-palmitate, are another series of fatty acid analogues, which have been reported to be more specific and sensitive (298). These fatty acids enable the visualization of protein lipid modifications by microscopy and flow cytometry, and provide new opportunity to identify palmitoylated proteins and understand the functions and mechanisms of lipid modifications (297,298).

Here, by labeling cells with ω-alkynyl-palmitate, we found that ABCG1 was palmitoylated at multiple cytoplasmic Cys residues, and palmitoylation was required for ABCG1-mediated cholesterol efflux function. Our data also showed

that different palmitoylated sites were not functionally equivalent and Cys residue locating at position 311 (Cys311) played the most important role. In addition, the removal of palmitoylation by site-directed mutagenesis did not affect protein dimerization or protein trafficking. Mutation C311A alone appeared to cause a shift of ABCG1 from the non-lipid raft membrane domain to the lipid raft membrane domain. However, the replacement of all identified palmitoylated Cys residues in ABCG1 had no observable effect on ABCG1 distribution pattern at the PM. The current studies suggest that palmitoylation of ABCG1 is a novel target in modulating ABCG1 cholesterol efflux capability.

### 4.2. Materials and methods (see Chapter 2)

### 4.3. Results

#### 4.3.1. Palmitoylation of ABCG1 in HEK 293 cells

The alkynyl group (297), which is metabolically inert, is ready to be detected by probe-tagged azide, through a Cu (I)-catalyzed cycloaddition, namely "click chemistry reaction" (299-301). Recently, it has been exploited as a tag to study glycosylation (302) and acylation (297,303). To detect whether ABCG1 is palmitoylated, here we labeled cells with ω-alkynyl-palmitate as described previously (177), and then conducted the click chemical reaction with the biotin tagged azide to detect the labeling. Briefly, cells stably overexpressing ABCG1 tagged with myc at the carboxy terminus or ABCG2 tagged with V5 were labeled with either palmitate or ω-alkynyl-palmitate. The whole cell lysates were prepared and immunoprecipitated with anti-c-myc antibody (9E10) conjugated beads. The click reaction was then conducted on the beads, and the immunoprecipitated proteins were eluted and all samples were loaded in duplicate on an 8% SDS-polyacrylamide gel. After electrophoresis, membranes were cut into halves. Before probed with HRP-NeutrAvidin for protein palmitoylation, one half was treated with Tris buffer, the other half was treated with KOH. In the Tris-treated group, the palmitoylation band appeared at ABCG1 labeled with ω-alkynyl-

palmitate (Fig. 4.1A, lane 1), but not in either ABCG1 labeled with palmitate (Fig. 4.1A, lane 2) or ABCG2 (Fig. 4.1A, lanes 3 and 4).

The liable thioester bond formed in the palmitoylation is reversible and not stable under hydroxyl conditions, such as in hydroxylamine or KOH solutions (304). To examine the observed palmitoylation band is indeed palmitoylation, we blocked the other half of the membrane with KOH. After KOH treatment, the palmitoylation signal was diminished (Fig. 4.1A, lane 5), suggesting that ABCG1 is S-palmitoylated. To further confirm that the reduction of palmitoylation signal after treatment with KOH was due to the cleavage of the thioester bonds, not the loss of target proteins, the same membranes were stripped off the palmitoylation signal and detected to be signal free after ECL. The membranes were then re-blotted with 9E10 to detect the levels of myc-tagged ABCG1. The expression levels of ABCG1 were comparable between the ω-alkynyl-palmitate and palmitate labeled groups in Tris and KOH treated groups (Fig. 4.1B, lanes 1 and 2, 5 and 6). Together, the above findings demonstrate that ABCG1 is indeed S-palmitoylated.

### 4.3.2. Palmitoylation Cys residues in ABCG1

Until now, there is no identified sequence motif for palmitoylation. Cytoplasmic Cys residues in target proteins close to the interface with membrane appear to be more accessible candidates (305). For ABCG1, there are 16 Cys residues in total. Among them, 13 Cys residues that are predicted to be located in the cytoplasm (Fig. 4.2A) are the potential palmitoylation sites.

To identify the palmitoylation site(s), we made two multiple mutations (C26-248A and C298-402A) and several single mutations by mutating the cytoplasmic Cys residues to Ala. The palmitoylation assay was then conducted as described before. Cells overexpressing empty vector (V), WT or mutant ABCG1 were labeled with ω-alkynyl-palmitate and detected by the click chemistry reaction. Compared with WT (Fig. 4.2B, lane 2; Fig. 4.2C, lane 2), mutations

C26-248A, in which Cys residues at positions 298, 311, 345, 397, 402 were replaced with Ala, and C298-402A, in which Cys residues at positions 298, 311, 345, 397, 402 were mutated to Ala, dramatically decreased the palmitoylation signal (Fig. 4.2B, Lanes 3 and 6 and Fig. 4.2C, lanes 3 and 9), indicating the existence of palmitoylation sites among these Cys residues. To further define the palmitoylation sites in ABCG1, we replaced each single Cys residue localizing in these two regions with Ala individually. We observed that mutations C311A and C402A dramatically reduced the palmitoylation signal (Fig. 4.2B, Lanes 5, 9 and 11). Mutation at positions 26 or 150 also reduced palmitoylation in ABCG1 (Fig. 4.2C, Lanes 4 and 5), but to a lesser extent. Furthermore, replacement of Cys at position 390 also remarkably reduced the palmitoylation signal (Fig. 4.2B, Lane 5). On the other hand, mutations at other positions did not significantly reduce the palmitoylation signal in ABCG1 compared with WT ABCG1 (Figs. 4.2B and 4.2C). Taken together, our data suggest that ABCG1 is palmitoylated at five Cys residues located at positions 26, 150, 311, 390 and 402.

### 4.3.3. Effect of defect palmitoylation in ABCG1 on ABCG1-mediated cholesterol efflux

Next, we assessed the role of palmitoylation in ABCG1-mediated cholesterol efflux. Cells overexpressing WT or mutant ABCG1were incubated with lipidated apoA-I to examine the cholesterol efflux capability. However, the WT ABCG1 tagged with c-myc used in the above palmitoylation assay was functionally dead (data no shown). Replacement of the myc tag with His tag also caused loss of the cholesterol export function (data no shown), which is consistent with previous report (142). Thus, we deleted the myc tag in all pcDNA3.1-ABCG1 constructs. Both multiple mutations and single mutations of ABCG1 were prepared by replacement of the identified palmitoylation Cys residue(s) with Ala. There is no tag present in any of the ABCG1 cDNA construct used in the following studies.

Compared with cells overexpressing empty vector, cells overexpressing WT ABCG1 significantly increased the cholesterol efflux (Fig. 4.3A and Fig. 4.3B). While, all of the multiple mutations, M3 (replacement of Cys 311, 390 and 402 with Ala), M4 (replacement of Cys 26, 311, 390 and 402 with Ala) and M5 (replacement of Cys 26, 150, 311, 390 and 402 with Ala), dramatically decreased the cholesterol efflux onto lipidated apoA-I (Fig. 4.3A). Cells overexpressing mutant ABCG1, M4 or M5, showed similar cholesterol efflux ability with cells overexpressing M3 or empty vector (control group). However, this loss of function was not due to the decrease in the protein expression. On the contrary, the expression levels of these ABCG1 mutations (Fig. 4.3C, lanes 3, 5 and 6) were even higher than that of WT ABCG1 (Fig. 4.3C, lane 2). Thus, our findings demonstrate that palmitoylation of ABCG1 is important for its cholesterol efflux function.

To further investigate which palmitoylation site(s) is required for cholesterol transport, we monitored the protein function of mutations in which only single Cys was replaced with Ala. As showed in Figure 4.3B, mutation C311A essentially abolished ABCG1-mediated cholesterol efflux onto the apoA-I. Mutation C402A also decreased cholesterol efflux activity, but only to a lesser extent. In the meantime, we also observed that single mutations at positions 26, 150 or 390 had no significant effect on ABCG1 cholesterol efflux activity (Fig. 4.3B). As showed in Figure 4.3C, the protein levels of the above single mutations (Fig. 4.3C, lanes 7, 8, 9, 11 and 12) were comparable to that of WT ABCG1 (Fig. 4.3C, lanes 2 and 14). Thus, the above data demonstrate that palmitoylation in ABCG1 are not functionally equivalent, with palmitoylation at Cys311 playing the most critical role.

### 4.3.4. Effect of defect palmitoylation in ABCG1 on protein trafficking

We next investigated how palmitoylation of ABCG1 affected ABCG1-mediated cholesterol efflux. Palmitoylation, together with ubiquitination, has been shown to be important for protein quality control (210,211). Deletion of

palmitoylation sites can lead to either protein degradation (210) or ER retention (306). However, the expression levels of all mutant ABCG1 transiently overexpressed in HEK 293 cells were comparable to that of WT (Fig. 4.4A, lanes 1 and 3; Fig. 4.4B, lanes 2, 3, 4, 5). Thereby, palmitoylation appears to have no significant disturbance on the stability of ABCG1.

Palmitoylation also plays an important role in protein sorting (207) and trafficking (186). For ABCG1-mediated cholesterol efflux, it is proposed to mainly occur on the cell surface, where the transporter redistributes cholesterol to a cell surface pool that is picked up by HDL (143). To determine if the loss of function of ABCG1 mutations might be attributable in part to the changes in protein trafficking, we examined the cell surface amount of WT and mutant ABCG1 via biotin labeling. Cells were transiently transfected with the WT or mutant ABCG1. The cell surface proteins were labeled with EZ-Link Sulfo-NHS-LC-Biotin, then precipitated with Streptavidin-agarose beads and detected by immunoblotting with the anti-ABCG1 and anti-Calnexin (an integral ER protein as a negative control) antibodies. ABCG1 that were not biotinylated did not associate with Streptavidin-agarose beads (Data not shown). As shown in Figure 4.4A, the amount of cell surface ABCG1 in cells overexpressing mutant M5 (lane 6) was comparable to that of WT (lane 4). The same experiments were also performed on the single mutation at Cys311 (C311A), Cys402 (C402A) and double mutation C311, 402A. All these mutations had similar amount of cell surface proteins as WT ABCG1 (Fig. 4.4B, lanes 7, 8, 9 and 10).

To confirm these findings, we performed the same experiment on cells stably overexpressing ABCG1. Consistently, cells stably overexpressing mutant C311A had comparable ABCG1 levels with cells overexpressing WT ABCG1 in either whole cell lysates (Fig. 4.4C, lanes 2 and 3) or biotin labeled cell surface proteins (Fig. 4.4C, lanes 5 and 6). Similar results were also obtained in cells stably overexpressing WT ABCG1 or other ABCG1 mutations C402A or C311, 402A (data not shown). Taken together, these findings demonstrate that reducing

palmitoylation in ABCG1 appears to have no significant effect on protein trafficking to the cell surface.

### 4.3.5. Effect of defect palmitoylation in ABCG1 on protein distribution at PM enriched cell membrane

The lipid bilayer PM is heterogeneous with different microdomains responsible for various protein functions (307). Lipid rafts, defined as a sphingomyelin and cholesterol rich microdomains, is well accepted as a signalling platform (18,308). A large amount of palmitoylated proteins accumulate in lipid raft domains for normal function or efficient signal transduction (176). Since reducing palmitoylation in ABCG1 did not affect protein trafficking to the cell surface, next we questioned whether the loss of ABCG1 function was due to the changes in ABCG1 localization in cell membrane domains. Lipid rafts are detergent resistant and cannot be solubilized by non-ionic detergents such as Triton X-100 (308). Here, we first prepared PM enriched membrane vesicles from HEK 293 cells stably overexpressing empty vector (con), WT or mutant ABCG1 (M5, C311A, C402A and C311, 402A). The lipid rafts from the prepared membrane vesicles were extracted with 1% ice-cold Triton X-100. The whole extractions were then separated by centrifugation at a discontinuous 4~45% sucrose gradient as previously described (157,255) with minor modifications. 12 fractions were collected at 1 ml per fraction from the top to the bottom. All the fractions were then subjected to SDS-PAGE and immunoblotting. The PM enriched membrane vesicle lysates were clearly separated into light fractions (fractions 3~5) and heavy fractions (fractions 8~12) (Fig. 4.5 and Fig. 4.6).

In the control group, the majority of lipid raft marker Flotillin1 was co-fractionated with Caveolin1 at fraction 4 (Fig. 4.5C and 4.5D). Unlike the distribution pattern in endothelial (157) or adipocyte cells (309,310), the majority of Caveolin1 in HEK 293 cells was located in the non-lipid raft fractions 8~12 (Fig. 4.5D), consistent with previous observation (310). Overexpression of mutant ABCG1 (C311A, M5, C402A and C311, 402A) did not cause significant change

in this distribution of Flotillin1 or Caveolin1 (Fig. 4.5C, 4.5D and Fig. 4.6C, 4.6D). Consistent with previous report (311), Tfr, a non-lipid raft protein (312), was located at the heavy density fractions (9~12) at the bottom (Fig. 4.5B), with only a minor amount of Tfr in the light fractions 4 and 5. The majority of WT ABCG1 was co-fractionated with Tfr at the heavy fractions 9~12 (Fig. 4.5A) in the non-lipid raft membrane domain. The distribution pattern of mutation M5 was essentially the same as that of the WT ABCG1. The distribution patterns of other mutant ABCG1, C402A and C311, 402A, were also similar to that of WT ABCG1 (Fig. 4.6A). However, when compared with WT ABCG1, mutation C311A resulted in enhanced ABCG1 level in fraction 4 and reduced protein levels in fractions 9 to 11 (Fig. 4.5A). Taken together, our data show that the majority of WT ABCG1 locates in the non-lipid raft membrane domain with a minor part in the lipid raft domain. Substitution of all the identified palmitoylation sites with Ala (M5) does not disrupt this distribution pattern, however, mutation at position 311 alone leads to an increase in ABCG1 localization in the lipid raft membrane domain.

### 4.3.6. Effect of defect palmitoylation in ABCG1 on protein dimerization

ABCG1 is a half transporter. Previously, we have employed gel-filtration technique to assess the native molecular size of ABCG1 (313). The majority of WT ABCG1 is present predominantly as a homo-dimer. Here, we employed the same assay to examine whether the effect of mutations at palmitoylation sites in ABCG1 on ABCG1-medaited cholesterol efflux was due to disrupting the protein dimerization.

PM enriched vesicles isolated as described above (~800 µg total proteins) were extracted in 500 µl of lysis buffer B containing 10 mM DTT. The extracts were cleared by centrifugation and the supernatants were fractionated by gel filtration chromatography on a Superdex 200 column. Fractions were analyzed by immunoblotting with antibodies to ABCG1 and Tfr (Figs. 4.7 and 4.8). Consistent

with previous report, the majority of WT ABCG1 eluted in fractions 16 and17 as a dimer (Figs. 4.7A and 4.8A). We next performed chromatography of mutations M5 and C311A on the same Superdex 200 column to investigate if replacement of palmitoylated Cys residues with Ala affected the dimerization of ABCG1. The elution patterns of mutant ABCG1, M5 or C311A, were essentially identical to that of WT. The majority of mutant ABCG1 eluted in fractions 16 and 17 (retention volumes 8.0-8.5 ml) (Fig. 4.7A and 4.7B). As a control, the elution patterns of Tfr (~110 kDa) extracted from cells overexpressing empty vector (control), WT ABCG1, mutation M5 or C311A were essentially the same, mainly distributing in fractions 20-25 (retention volumes 10-12.5 ml) (Fig. 4.7C and 4.7D). The same experiment was also performed on mutations C402A and C311, 402A. Similarly, the two mutations did not significantly affect the elution patterns of ABCG1 (Fig. 4.8A and 4.8B) and Tfr (Fig. 4.8C and 4.8D). We did observe that there was almost 1 fraction delayed in mutations C402A and C311, 402A when compared with WT or other mutations (M5 and C311A). However, the elution fractions of ABCG1 were still earlier than that of Tfr (Fig. 4.8C and 4.8D). So, these two mutants still predominantly form homo-dimers. Thus, our findings show that substitution of palmitoylation Cys(s) in ABCG1 with Ala(s) appears to have no effect on ABCG1 dimerization.

### 4.4. Discussion

In the present study, we demonstrated that ABCG1 overexpressed in HEK 293 cells was palmitoylated at multiple Cys residues, namely Cys26, Cys150, Cys311, Cys390 and Cys402. Palmitoylation of ABCG1 was important for its cholesterol efflux function, but different palmitoylation sites were not functionally equivalent. Moreover, our data demonstrated that removal of palmitoylation sites in ABCG1 had no effect on protein dimerization or protein trafficking to the cell surface. Although mutation at Cys311 alone seemed to promote protein accumulation in the non-lipid raft membrane domain, mutation M5 in which all the five identified palmitoylation sites in ABCG1 were replaced with Ala had no

observable effect on the distribution pattern. More studies are needed to figure out how palmitoylation of ABCG1 contributes to ABCG1-mediated cholesterol efflux.

One single peptide might be palmitoylated at multiple Cys residues, which may function either differently or the same. H-Ras protein is palmitoylated at two Cys residues (Cys 181 and 184), monopalmitoylation at Cys 181 is sufficient for protein trafficking to the cholesterol rich PM domain, while monopalmitoylation Cys184 results in protein retention in Golgi (208). Palmitoylation of ABCA1 is critical for protein trafficking to the cell membrane and ABCA1-mediated lipid export. Removal of the four palmitoylation sites (Cys 3, 23, 1110, 1111) in ABCA1 did not have additive effect on protein function, but only causing 50% reduction of cholesterol export activity, same as that observed in the mutant ABCA1 with removal of only one single palmitoylation site (247). For ABCG1, five Cys residues at positions 26, 150, 311, 390 and 402 were palmitoylated (Fig. 4.2B and 4.2C). Palmitoylation at these five different palmitoylated Cys residues did not contribute equally to ABCG1-mediated cholesterol efflux function. Replacement of Cys311 with Ala dramatically decreased ABCG1-mediated cholesterol efflux to lipidated apoA-I (Fig. 4.3B). Mutation at Cys402, ~ 20 amino acids away from the 1$^{st}$ putative TM domain, also decreased the transport function, but to a lesser extent. However, palmitoylation of Cys390, which is only ten amino acids away from Cys402, seemed irrelevant to the cholesterol export function (Fig. 4.3B). Moreover, mutation at Cys26 or Cys150 did not cause significant difference in cholesterol efflux activity. The 638 amino acid transcript of ABCG1 that lacks of the first 40 amino acids in the amino terminal domain (143) is fully functional, which supports our observations that palmitoylation at Cys26 is not functionally important.

Palmitoylation may be involved in protein stability by targeting protein to lysosome for degradation. In yeast, unpalmitoylated Tlg1, analog of SNARE protein, is recognized by ubiquitin ligase for vacuole targeting (210). Using an

non-specific proteasome inhibitor Bafilomycin A1, palmitoylation deficient CCR5 (C-C chemokine receptor type 5) promotes protein accumulation in lysosome (314). Less is clear about how palmitoylation prevents protein degradation (209), but palmitoylation deficient mutations are reported to be correlated with ubiquitination (315,316), which may result in protein degradation. Palmitoylation deficient mutation of anthrax toxin receptor associates with membrane detergent resistant domain, promotes ubiquitination and subsequent endocytosis (211). In the present study, simultaneous mutation at all five palmitoylation Cys residues did not lead to reduced total protein levels in both transient transfection system (Figs. 4.3C and 4.4A) and the stable cell lines (Fig. 4.4C). These observations showed that reducing palmitoylation in ABCG1 does not dramatically affect protein stability, indicating that the mutations do not cause a major perturbation in the protein structure or protein misfolding.

Overexpressed ABCG1 *in vitro* is reported to localize at both intracellular compartments and the PM (142,150). In both human and mouse macrophages, LXR agonist induces the ABCG1 gene expression and promotes protein PM distribution (142). The distribution on PM is considered to be essential for the cholesterol export, probably through increasing the membrane cholesterol pool which is accessible for cholesterol oxidase or acceptors such as HDL (143). Palmitoylation participates in the regulation of many proteins trafficking and localization, such as ABCA1 (247), for which, palmitoylation is important for protein trafficking to the PM. Here, we observed that the mutant transporters, in which the palmitoylation sites were removed, trafficked to the PM as efficiently as WT, indicating palmitoyaltion of ABCG1 appears to have no effect on protein trafficking to the PM.

Cellular membranes are highly dynamic and heterogeneous, functioning differently in different microdomains. Lipid rafts, are special membrane microdomains enriched in sphingomyelin and cholesterol and play important roles in various physiological processes and pathological conditions such as cancer

(317). Recently, ABCG1 has been demonstrated to play an important role in maintaining normal endothelial functions by regulating the interaction of endothelial NO synthase with Caveolin1 (157). Deficiency of ABCG1 leads to the increased monocyte-endothelial interactions and promoted vascular inflammation in an IL-6 and STAT3 dependent manner (155). Here, using sucrose gradient centrifugation, we showed that WT ABCG1 overexpressed in HEK 293 cells mainly distributed in the non-lipid raft membrane domain, similar with Tfr, a well-studied membrane palmitoylated protein (212). Although palmitoylation deficient mutation C311A alone enhanced ABCG1 accumulation in the lipid raft domain, the majority of this mutant ABCG1 still localized in the non-lipid raft domain (Fig. 4.5A). Moreover, mutation M5, in which all the five identified palmitoylated Cys residues were replaced with Ala, and mutation C311, 402A did not affect ABCG1 distribution patterns in the PM-enriched membranes (Figs. 4.5A and 4.6A). Furthermore, mutations C311A, C311, 402A and M5 all dramatically reduced ABCG1-mediated cholesterol efflux. Thus, mutation C311A-induced change in ABCG1 distribution in the PM-enriched fractions might not contribute to the effect of the mutation on ABCG1-mediated cholesterol efflux. Our studies also demonstrated that removal of the palmitoylation sites in ABCG1 has no significantly effect on the protein dimerization.

It is clear that palmitoylation of ABCG1 is critical for cholesterol efflux function, but how deficiency of this lipid modification results in the loss of function remains unknown. It is proposed that the ATP binding and hydrolysis at the NBDs may cause a conformation transition of the TM helices from an outward-facing to inward-facing orientation, which facilitates substrate binding and translocation (92,97). The palmitoylation sites in ABCG1 are located in the amino terminal NBD. The NBDs work as an engine for ABC transporters. Substitution of the conserved lysine residue at the Walker A motif in ABCG1 with methionine results in loss of ATPase activity (319) and cholesterol efflux capability (313). Cys311 locates between the Walker B motif and the predicted 1$^{st}$ TM α-helix. So it is possible that the enhanced membrane affinity caused by

palmitoylation might be important to stabilize the ATP binding site. Cys402 is very close to the 1st TM domain, thus the attachment of palmitic acid at this position may function to maintaining the stability of the TMDs. Together, the defective palmitoylation may destabilize the ATP binding site and further block the ATP binding and hydrolysis, or may disturb the conformation transition of the TM domains, resulting in low efficiency of substrate binding and/or translocation. Further studies are needed to confirm these hypotheses.

To summarize, we demonstrated that ABCG1 is palmitoylated and palmitoylation of ABCG1 is required for its cholesterol export function. Accordingly, strategies to enhance the palmitoylation of ABCG1 may be the potential targets for enhancing ABCG1-mediated cholesterol export. Thus further studies in the palmitoylation and depalmitoylation of ABCG1 will be helpful to the future drug design for treatment of atherosclerosis.

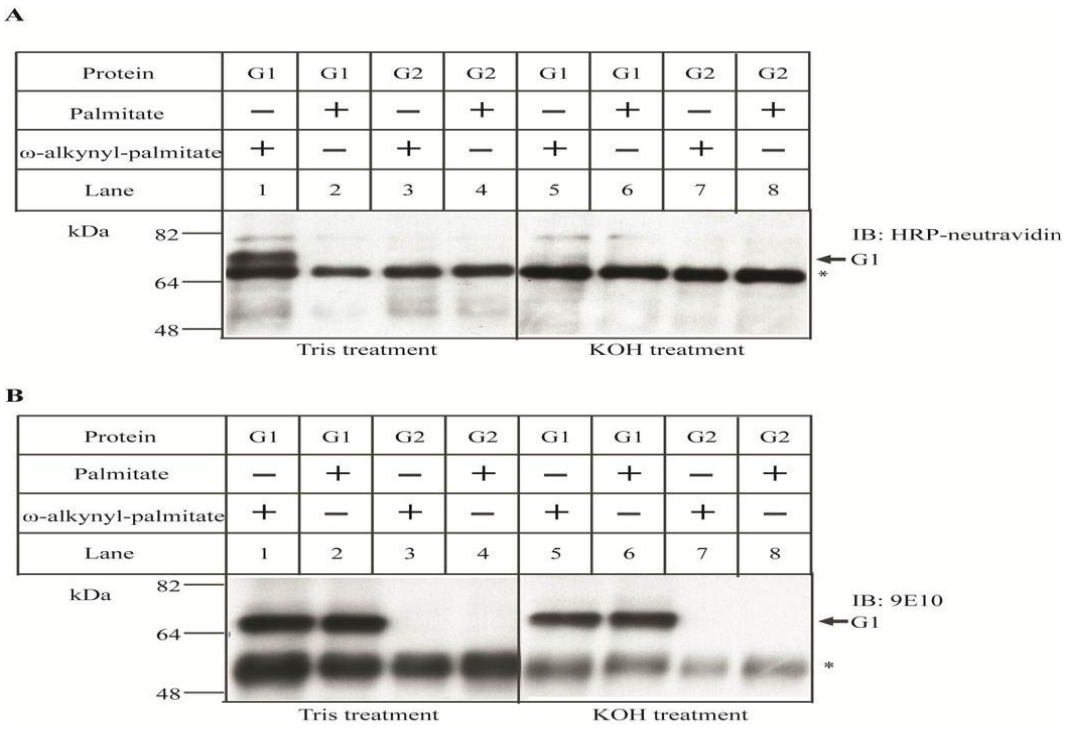



**Figure 4.1. Palmitoylation of ABCG1.** *Panel A:* Palmitoylation of ABCG1. Palmitoylation of ABCG1 was examined by labeling cells with ω-alkynyl-palmitate and followed by the click chemical reaction as described. Briefly, cells overexpressing ABCG1 tagged with myc at the carboxy terminus (c-myc) or ABCG2 tagged with V5 were starved with fatty acid free medium for 2 h, and then incubated with ω-alkynyl-palmitate for 4 h. Whole cell lysates were prepared, both ABCG1 and ABCG2 were immunoprecipitated with 9E10 conjugated beads. The click reaction was conducted on the beads. At the end of reaction, the immunoprecipitated proteins were eluted and subjected to SDS-PAGE. Membranes were treated with either Tris buffer or KOH buffer, and then probed with HRP-NeutrAvidin for protein palmitoylation signal. *Panel B:* Expression of ABCG1. The same membranes in Panel A were stripped off the palmitoylation signal, and re-blotted with 9E10 to detect ABCG1 protein expression levels. The arrow indicated the ABCG1 location, and * labeled the non-specific band. Similar results were obtained in three independent experiments.

**Figure 4.2. Palmitoylation sites in ABCG1.**

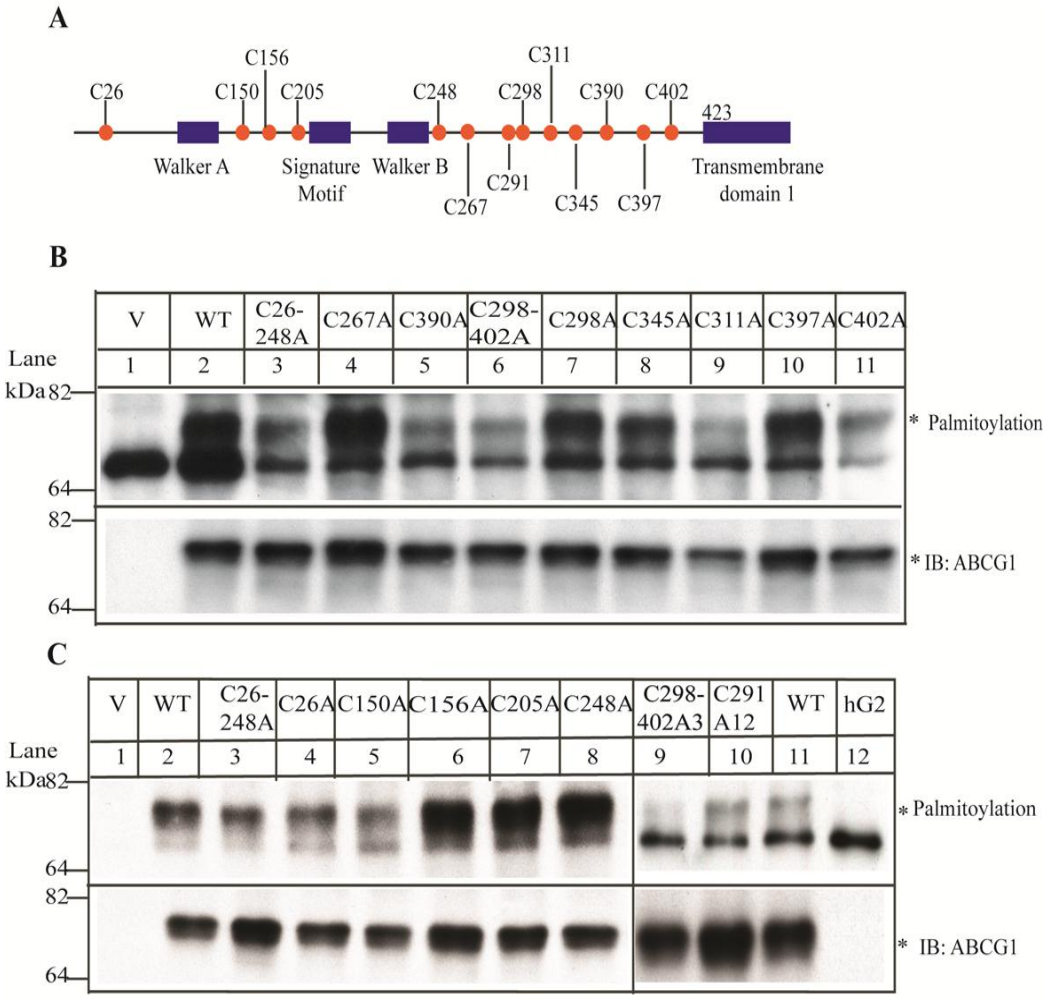


**Figure 4.2. Palmitoylation sites in ABCG1.** *Panel A:* The 13 Cys residues locating in the amino-terminal cytoplasmic region of ABCG1 are the potential palmitoylation sites. *Panels B and C:* Palmitoylation and protein expression levels of ABCG1. All candidate Cys residues were mutated to Ala individually or in combination. The WT and mutant ABCG1 were expressed in HEK 293 cells and the palmitoylation sites of ABCG1 were identified by applying the above palmitoylation assay used in the legend of Fig. 4.1. For each panel, the palmitoylation signal was showed at the top row and the protein levels were probed with 9E10, indicated with asterisk (*). V: control; WT: wild type ABCG1; C26-248A and C298-402A: multi-mutations of ABCG1, in which Cys residues located at Cys 26, 150, 156, 205, 248, or at Cys 298, 311, 345, 397, 402 were mutated to Ala respectively; CxA: single mutation of ABCG1, in which Cys residue located at position x was mutated to Ala, x= 26, 150, 156, 205, 248, 267, 291, 298, 311, 345, 390, 397, 402; C291A12: stable cell line overexpressing mutant ABCG1, C291A; C298-402A3: stable cell line overexpressing mutant ABCG1 with replacement of Cys residues at positions 298, 311, 345, 397, 402 to Ala; WT: stable cell line overexpressing WT ABCG1; hG2: stable cell line overexpressing human ABCG2. WT and mutant ABCG1 constructs were tagged with c-myc and ABCG2 was tagged with V5.

**Figure 4.3. Effect of defect palmitoylation in ABCG1 on ABCG1-mediated cholesterol efflux.**

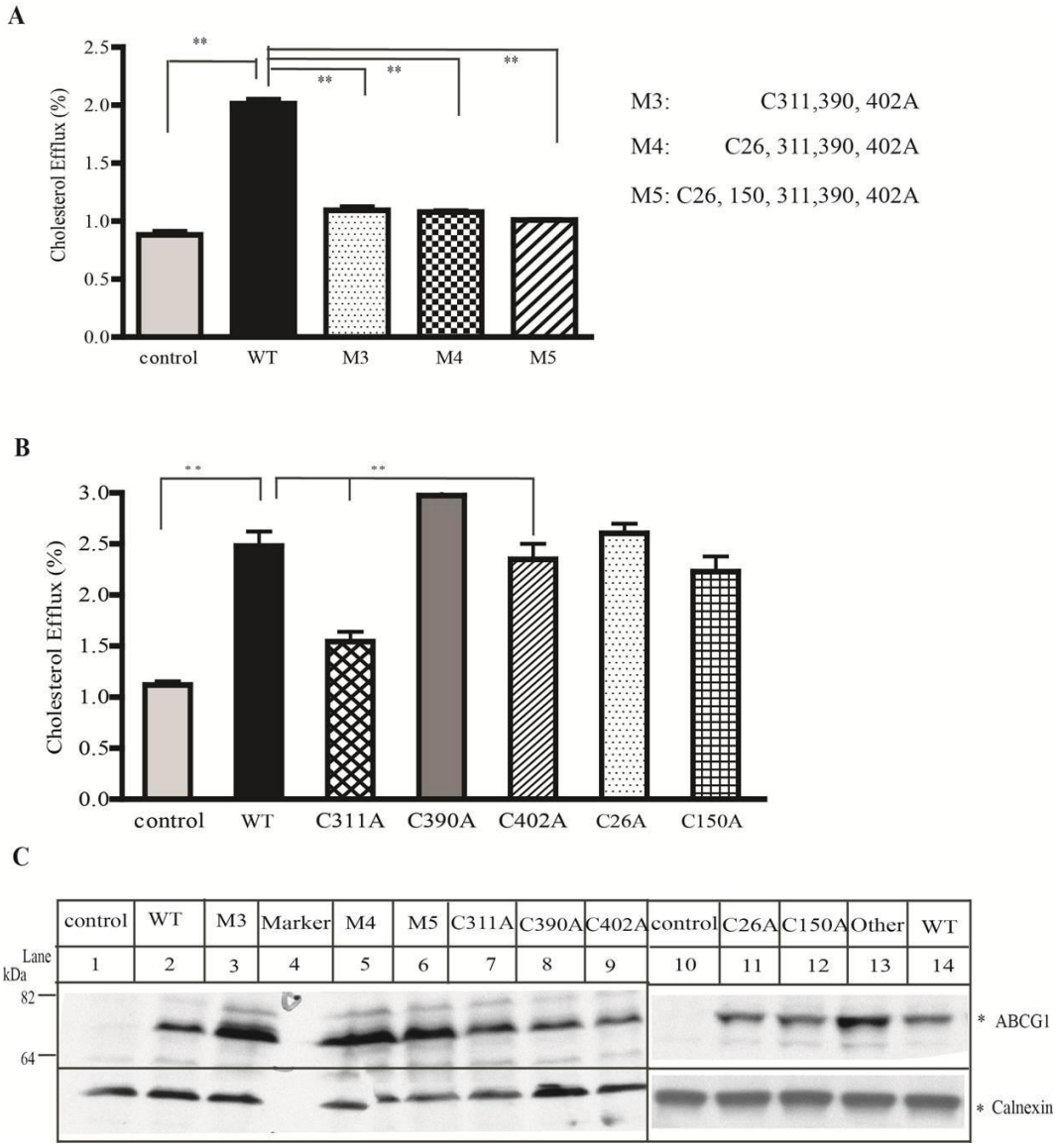


**Figure 4.3. Effect of defect palmitoylation in ABCG1 on ABCG1-mediated cholesterol efflux.** *Panels A and B*: Cholesterol efflux activity of WT and mutant ABCG1. HEK 293 cells were transiently transfected with empty vector (control) or plasmids containing WT or mutant ABCG1 cDNAs, and labeled with [$^3$H]-cholesterol for 18 h. After washing, the cells were incubated with 5 μg/ml lipidated apoA-I for 5 h. The radioactive content of the media and cells was measured separately by scintillation counting. Sterol transfer was expressed as the percentage of the radioactivity released from the cells into the media relative to the total radioactivity in the cells plus media. M3, multi-mutation of ABCG1, in which Cys residues located at positions 311, 390, 402 were mutated to Ala; M4, multi-mutation of ABCG1, in which Cys residues located at positions 26, 311, 390, 402 were mutated to Ala; M5, multi-mutation of ABCG1, in which Cys residues located at positions 26, 150, 311, 390, 402 were mutated to Ala. CxA: single mutation of ABCG1, in which Cys residues located at position x was mutated to Ala, x= 26, 150, 311, 390 or 402. *Panel C:* Protein expression levels of ABCG1 in HEK 293 cells transiently overexpressing ABCG1 WT and mutants. Whole cell lysates were prepared from HEK 293 cells transiently transfected with empty vectors (control) or vectors containing WT or mutant ABCG1 cDNAs, and subjected to SDS-PAGE and immunoblotting. Other was indicated as other irrelevant mutant ABCG1. Blots were probed with a polyclonal anti-ABCG1 antibody and a polyclonal anti-Calnexin antibody, indicated with asterisk (*). Data were analyzed with GraphPad Prism software and significance was defined as *: $p<0.05$; **: $p<0.01$. Values are mean ± S.D. of ≥ 3 independent experiments. All mutant and WT ABCG1 constructs used were not tagged.

**Figure 4.4. Effect of defect palmitoylation in ABCG1 on protein trafficking.**

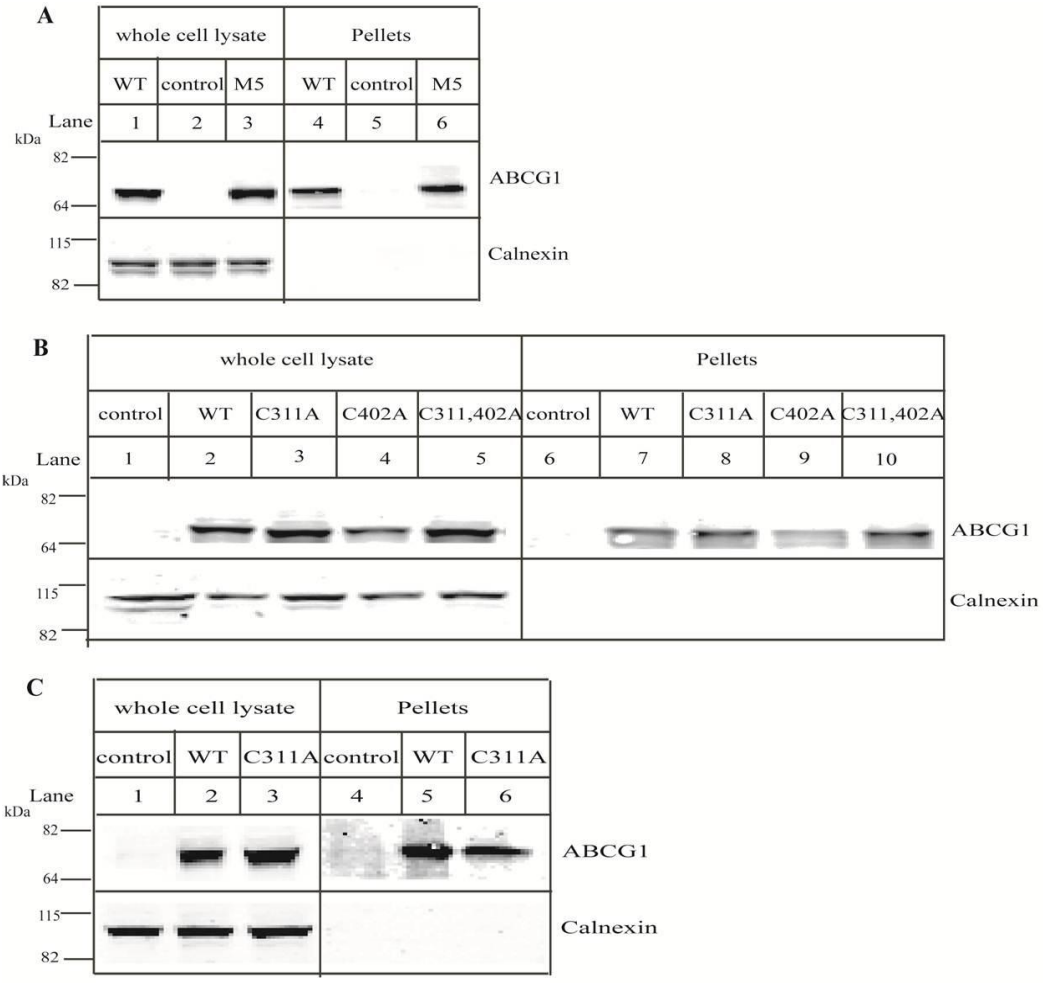


**Figure 4.4. Effect of defect palmitoylation in ABCG1 on protein trafficking.** *Panels A and B:* HEK 293 cells transiently transfected with empty vector (control), WT or mutant ABCG1 were biotin labeled exactly as described. The biotinylated proteins from the cell surface (pellets) and proteins from the whole cell lysates were analyzed by SDS/PAGE (8%) and immunoblotting with a polyclonal anti-ABCG1 antibody (top panel) and a polyclonal Calnexin antibody (bottom panel). *Panel A:* The biotinylation of WT and mutation M5. *Panel B:* The biotinylation of WT, single mutations C311A, C402A and double mutation C311, 402A. *Panel C:* The biotinylation of HEK 293 cells stably overexpressing empty vector (control), WT ABCG1 or mutation C311A. C311, 402A: double mutation of ABCG1, in which Cys residues at positions 311 and 402 were mutated to Ala. Other indicated mutations including M5, C311A and C402A, were the same as described in Fig. 4.3. Similar results were obtained in three independent experiments.

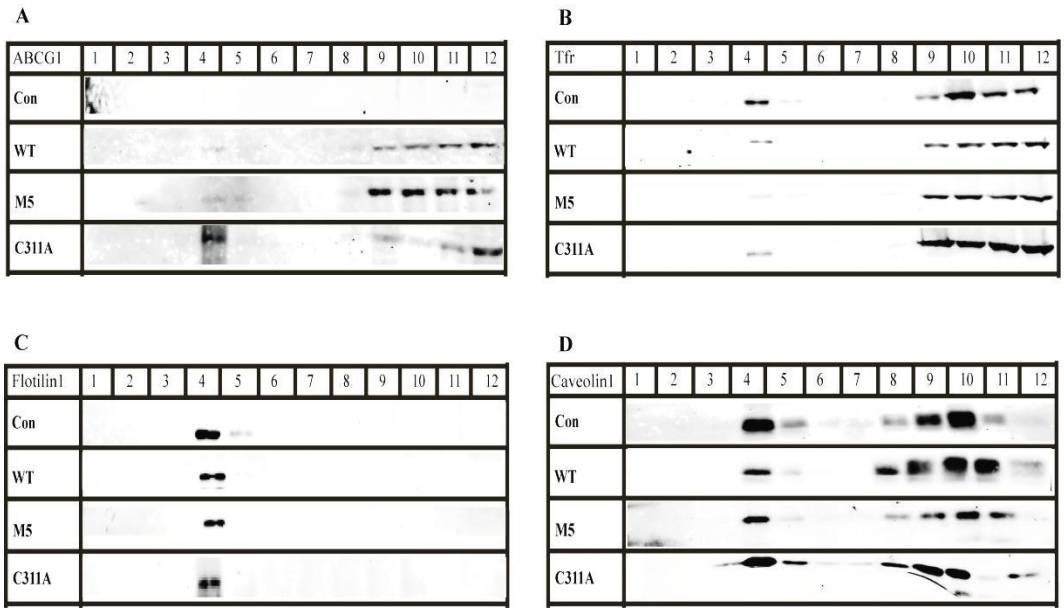



**Figure 4.5. Effect of palmitoylation deficient ABCG1 mutations M5 and C311A on protein distribution at PM.** Around 500 μg of PM enriched fraction isolated from HEK 293 cells stably overexpressing empty vector (control), ABCG1 WT or mutants (C311A and M5) were solubilized in 1 ml of lysis buffer B containing 1× proteinase inhibitor and 10 mM DTT. The lysates were loaded at the bottom of a discontinuous 4~45% sucrose gradient. After separation by centrifugation, fractions were collected from top to bottom at 1 ml per fraction. The fractions were precipitated and the pellets were washed with cold acetone. The dried pellets were dissolved in urea and subjected to SDS-PAGE and immunoblotting. Fractions were blotted with a polyclonal anti-ABCG1 (*Panel A*), a monoclonal anti-Tfr antibody (*Panel B*), a polyclonal anti-Flotillin1 antibody (*Panel C*), and a polyclonal anti-Caveolin1 antibody (*Panel D*), respectively. Similar results were obtained in three independent experiments.

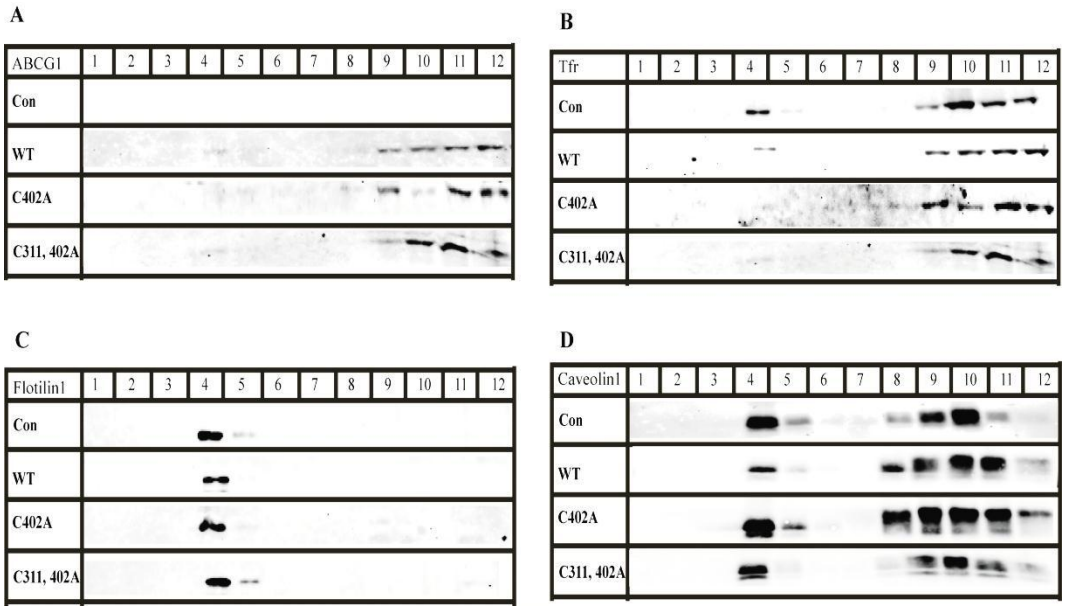



**Figure 4.6. Effect of palmitoylation deficient ABCG1 mutations C311A and C311, 402A on protein distribution at PM.** Membrane enriched vesicles prepared from HEK 293 cells stably expressing empty vector (con), ABCG1 WT or mutants (C402A and C311, 402A) were lysed and separated by a discontinuous 4~45% sucrose gradient as described in Fig. 4.5. Fractions were subjected to SDS-PAGE and membranes were immunoblotted with a polyclonal anti-ABCG1 (*Panel A*), a monoclonal anti-Tfr antibody (*Panel B*), a polyclonal anti-Flotillin1 antibody (*Panel C*), and a polyclonal anti-Caveolin1 antibody (*Panel D*), respectively. Similar results were obtained in two independent experiments.

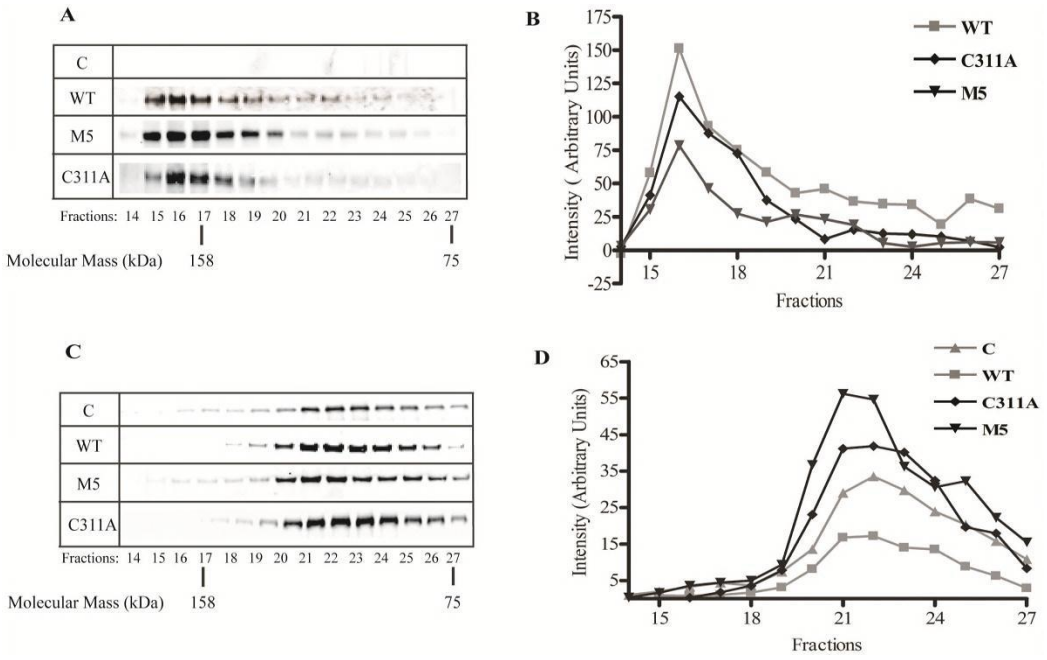



**Figure 4.7. Gel filtration of palmitoylation deficient ABCG1 mutations M5 and C311A.** 800 μg of PM enriched membrane vesicles isolated from HEK 293 cells stably expressing empty vector (control), ABCG1 WT or mutants (C311A and M5) were solubilized in 500 μl of lysis buffer B containing 1% Trition, 1× proteinase inhibitor and 10 mM DTT. The lysates were centrifuged for 15 min at 14,000 rpm to clear the insoluble materials. The supernatant was loaded onto a Superdex 200 10/300 GL column pre-equilibrated with lysis buffer B. The elution fractions were collected and subjected to SDS-PAGE. The retention time of ABCG1 was determined by immunoblotting. *Panels A and C*: Gel filtration fractions were blotted with a polyclonal anti-ABCG1 (*Panel A*) and a monoclonal anti-Tfr antibody (*Panel C*), respectively. The elution positions of the following protein standards are shown: aldolase (158 kDa) and conalbumin (75 kDa). Similar results were obtained in two independent experiments. *Panels B and D*: Densitometry of ABCG1 (*panel B*) and Tfr (*panel D*) in *panels A* and *C*, respectively. The proteins were quantified by measuring the intensity of the Western blot using Licor software and plotted against fraction numbers.

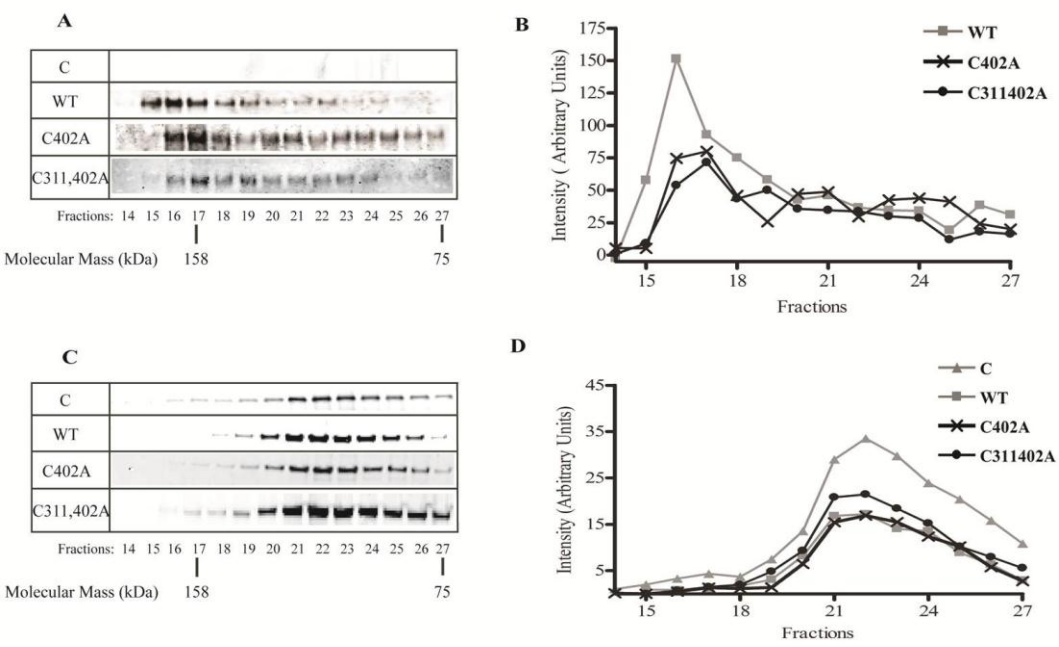



**Figure 4.8. Gel filtration of palmitoylation deficient ABCG1 mutations C311A and C311, 402A.** PM enriched vesicles isolated from HEK 293 cells stably expressing empty vector (control), ABCG1 WT or mutants (C402A and C311, 402A) were solubilized in lysis buffer B and separated on a Superdex 200 10/300 GL column as described in Fig. 4.7. The elution fractions were collected, precipitated, dissolved and subjected to SDS-PAGE. The retention time of ABCG1 was determined by immunoblotting. *Panels A and C*: Gel filtration fractions were blotted with a polyclonal anti-ABCG1 (*Panel A*) and a monoclonal anti-Tfr antibody (*Panel C*), respectively. The elution positions of the following protein standards are shown: aldolase (158 kDa) and conalbumin (75 kDa). Similar results were obtained in two independent experiments. *Panels B and D*: Densitometry of ABCG1 (*panel B*) and Tfr (*panel D*) in *panels A* and *C, respectively*. The proteins were quantified by measuring the intensity of the Western blot using Licor software and plotted against fraction numbers.

# Chapter 5

# General conclusion and future directions

## 5.1. General conclusion and discussion

ABCG1 mediates cholesterol export to lipidated lipoproteins and plays an important role in maintaining cholesterol homeostasis and protecting against the progression of atherosclerosis. However, the underlying mechanism remains unknown.

Mutations in ABCA1 transporter cause Tangier disease (320) and other Familial HDL Deficiencies (321). Mutations in ABCG5 or ABCG8 cause sitosterolemia (322), an autosomal recessive disease characterized with sterol accumulation, which contributes to premature atherosclerosis (323). Studies on the *ABCG1* polymorphism suggest the association of *ABCG1* with the plasma cholesterol level (324) and the coronary artery diseases (325,326). However, until now, there is still no ABCG1 mutation reported to be related with any physiological disorders.

Cys, with a special thiol side chain, is a multi-functional amino acid. It has high affinity for binding of various heavy metals (240-242). It is susceptible to be oxidized (243). Oxidation of the thiol groups of Cys residues can lead to the formation of the disulfide bonds, which are one of the main types of linkages to crosslink proteins covalently (172). Moreover, Cys residues are involved in a wide range of protein post-translational modifications, such as S-hydroxylation (237) and lipid modifications, such as prenylation and palmitoylation (171). ABCG1 has 16 Cys residues, in which, one is highly conserved in the 3$^{rd}$ putative TM α-helix domain, two in the carboxyl terminal last extracellular loop domain and the rest 13 in the cytoplasm.

Here, we first demonstrated that Cys514, which locates in the third putative TM α-helix of ABCG1, plays a critical role in ABCG1-mediated cholesterol efflux. Substitution of Cys514 with Ala virtually eliminated the efflux of cholesterol onto lipidated apoA-I. Furthermore, replacement of Cys514 with a more conservative Thr or Ser, also significantly decreased ABCG1-mediated

cholesterol efflux capability, implying the specific requirement for the thiol group at position 514. However, further studies showed that mutation C514A did not cause disturbance in protein stability or trafficking. Mutation C514A also had no effect on the dimerization of ABCG1. Taken together, our findings identify that the sulfhydryl group of Cys residue located at position 514 is required for ABCG1 function, but how the sulfhydryl group or this Cys residue participates in ABCG1-mediated cholesterol efflux remains unclear.

Cys residues in the reduced conditions cannot form the stable disulfide bond, but they are the potential sites for palmitoylation. By labeling with the ω-alkynyl-palmitate, a palmitate analog, we found that ABCG1 was palmitoylated. ABCG1 was multi- palmitoylated at 5 cytoplasmic Cys residues at positions 26, 150, 311, 390 and 402. Palmitoylation of ABCG1 was required for cholesterol efflux, however, the different palmitoylation sites were not functionally the same, with Cys311 playing the most essential role and Cys402 a minor role. Similar with mutation at Cys514, substituting palmitoylation site(s) in ABCG1 with Ala had no effect on protein stability, trafficking or the formation of ABCG1 homo-dimer. Moreover, when overexpressed in HEK 293 cells, the majority of WT ABCG1 protein localized to the non-lipid raft membrane domain using the sucrose gradient, with minor amount of protein in the lipid raft domain. Although replacement Cys311 alone to Ala led to more ABCG1 localized to the lipid raft membrane domain, substitution of all five identified palmitoylation sites with Ala (M5) had no observable effect on this distribution pattern. It is unclear if the mutation C311A caused retention of ABCG1 in the lipid raft membrane domain causes the loss of cholesterol efflux function. Together, ABCG1 is palmitoylated and palmitoylation of ABCG1 is critical for protein cholesterol efflux function. However, how palmitoylation of ABCG1 contributes to ABCG1-mediated cholesterol efflux function remains unknown.

### 5.2. Future directions

Both Cys514 and Cys311 are crucial for ABCG1-mediated cholesterol efflux. However, how the lack of the thiol group in these positions decreases cholesterol export remains unknown. To answer these questions, a few aspects need to be determined.

First, is the ATP binding and hydrolysis at the NBDs in ABCG1 affected by these mutations? The NBDs, especially the Walker A, Walker B and the Signature motifs, are strictly demanded for ABC protein function. It has been reported that mutation at one single conserved amino acid lysine residue in the Walker A motif completely abolishes ABCG1-mediated cholesterol export function (151,313). The loss of function in mutant ABCG1 may be due to the deficient ATP binding or hydrolysis, especially for mutation at Cys311, which localizes at the N-terminal NBD. We have attempted to address this issue by comparing the ATPase activity of membrane enriched vesicles prepared from HEK 293 cells overexpressing WT or mutant ABCG1. The vanadate-sensitive ATPase activity of the WT membrane rich vesicles has been observed (data not shown). However, it is inapplicable to detect the contribution of the mutant ABCG1, due to the high background ATPase activity in the control group (membrane enriched vesicles prepared from HEK 293 cells stably overexpressing empty vector). In fact, the vanadate-sensitive ATPase activity of ABCG1 has been determined in Sf9 insect cell overexpression system (319). Thus, further studies using Sf9 insect cells overexpressing WT or mutant ABCG1 may help to address this question.

Second point is extended from the finding that ABCG1 localizes in the non-lipid raft domain. ABCG1 is reported to redistribute cholesterol to the membrane domains, accessible to cholesterol oxidase or HDL (143). Under the stimulation of LXR agonists, ABCG1 is induced to redistribute to the cell PM in both human and mouse macrophages (142). So next question to be answered will be whether cholesterol is redistributed with ABCG1, such as from the intracellular membrane domain to non-lipid raft PM domain. Although ABCG1 is not a

cholesterol binding protein, other unidentified cofactor(s), probably cholesterol binding protein(s), may assist ABCG1 mediate cholesterol efflux. So, further studies should determine whether cholesterol is redistributed along with ABCG1 to the non-lipid raft membrane domain and which protein(s) can facilitate ABCG1-mediated cholesterol export. Moreover, an increased amount of lipid raft ABCG1 protein was observed in mutation C311A. Thereby, the loss of function in C311A may be due to the mislocalization of the minor amount of ABCG1 protein in the lipid raft membrane domain. Thus, more data will be needed to confirm this observation and determine whether indeed the retention of ABCG1 to the lipid raft will inhibit the cholesterol efflux function.

For both Cys514 and Cys311, it is possible that deletion of the thiol group results in the TMD conformation change, deficient substrate binding, and eventually the loss of cholesterol efflux function. According to the high resolution crystal structure of two bacterial homologs of ABCB1 (91,94,95), the TM α-helices are proposed to form the TM core for substrate binding and translocation, while the ATP hydrolysis at the NBDs may orient the TMDs conformation transition, from an outward-facing status to an inward-facing status (92,97). Cys514 may be part of the substrate binding pocket in ABCG1, while palmitoylation at Cys311 may indirectly involve in stabilization of the binding pocket structure through binding to the PM. However, the formation, maintaining and regulation of protein spherical structure are extremely complicated. Thus, to better understand how these two mutations abolish ABCG1-mediated cholesterol export, a high resolution crystal structure of ABCG1 will be invaluable.

Thus, here we propose that ABCG1 localizes in the PM non-lipid raft domain and functions as a homo-dimer (Fig. 5.1A). The TMDs form the cholesterol translocation pocket. The ATP binding and hydrolysis at the NBDs orient the conformation change of the translocation pocket, facilitating cholesterol export to lipidated lipoprotein particles such as HDL. While loss of function for both mutations at Cys514 and Cys311 may be caused by the twisted translocation

pocket. Since Cys311 localizes at the NBD, it is also possible that mutation C311A may abolish ABCG1 function by affecting the ATP binding or hydrolysis at the NBDs (Fig. 5.1B).

Furthermore, palmitoylation, especially at Cys311, is required for ABCG1-mediated cholesterol export. So enhancing ABCG1 palmitoylation may increase ABCG1-mediated cholesterol export and further support the on-target drug design for atherosclerosis. DHHC8 PAT is reported to catalyze ABCA1 palmitoylation and enhanced expression of DHHC8 PAT results in promotion of cholesterol efflux mediated by ABCA1 (247). Thus, it is demanded to investigate the underlying mechanisms for ABCG1 palmitoylation and its regulations.

First question to be answered will be: is ABCG1 palmitoylation stable or reversible? This can be evaluated through comparing the turnover rates of the palmitoylation of ABCG1 and ABCG1 protein itself. Then the following question will be: how ABCG1 is palmitoylated, including which PAT (s) is (are) required for ABCG1 palmitoylation and which enzyme is for ABCG1 depalmitoylation. 23 human DHHC PATs have been widely studied (327,328). Thus it will not be too difficult to identify the responsible enzyme(s) for ABCG1 palmitoylation. Besides, the successful detection of ABCG1 palmitoylation with addition of ω-alkynyl-palmitate, has already suggested that the existence of endogenous PAT(s) in HEK 293 cells for ABCG1 palmitoylation. In addition, ω-alkynyl-palmitate has been reported to be a useful tool in imaging palmitoylation process by fluorescence microscopy when the ω-alkynyl-palmitate conjugated proteins were detected with azido tagged fluorophore (297). This could be very helpful to identify the responsible enzyme(s) for palmitoylation according the locations, since the localizations of PATs are different (186,329). The third following concern will be: whether the induced protein palmitoylation by either increased PAT activity or inhibited acyl protein thioesterase will benefit cholesterol export function. Using the above palmitoylation assay and cholesterol efflux assay, this issue can also be easily addressed.

Eventually, studies of ABCG1 palmitoylation and underlying cholesterol export mechanisms should not be separated, but are complementary to each other. Further investigations of the mechanisms of ABCG1 palmitoylation and its regulations will benefit the future therapeutic drug design for atherosclerosis treatment.

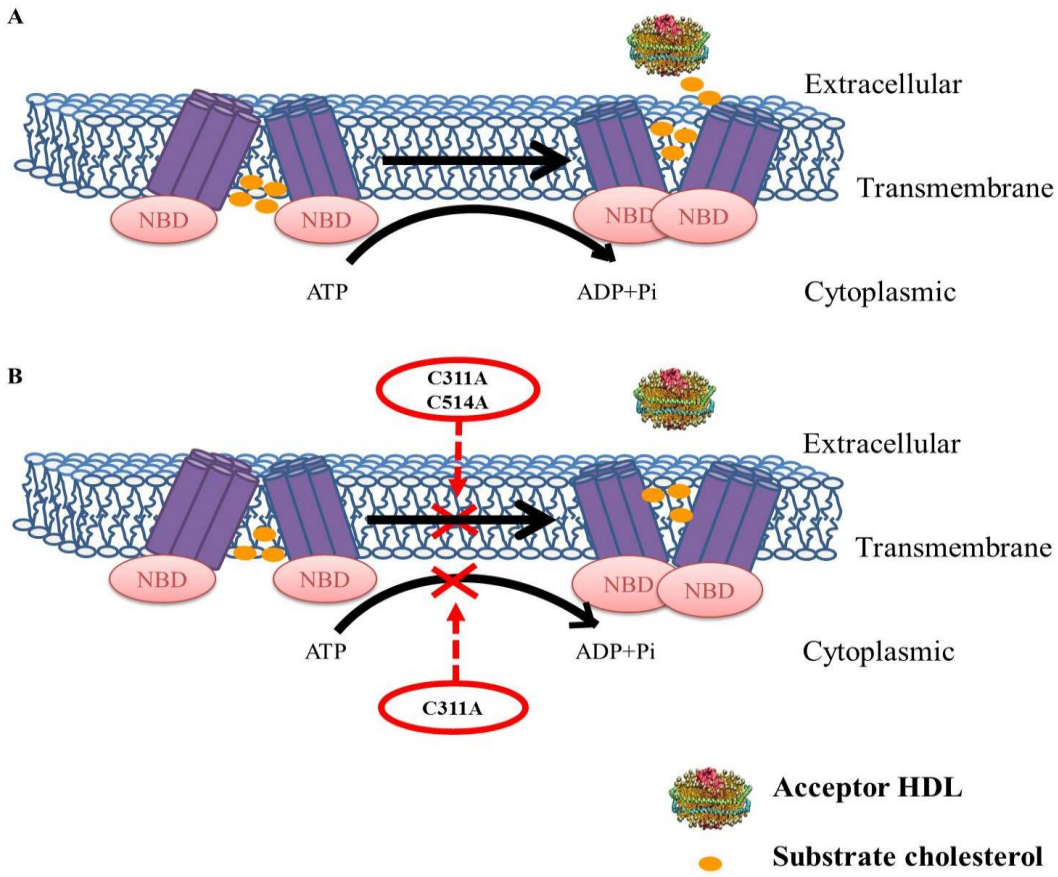



**Figure 5.1. Model for ABCG1-mediated cholesterol export.** *Panel A:* ABCG1 functions as a homo-dimer in the PM non-lipid raft domain. 12 TMDs form the cholesterol translocation pocket. The NBDs orient the conformation change of the translocation pocket with the supply of energy from ATP binding and hydrolysis, facilitating cholesterol export to lipidated lipoprotein particles such as HDL. *Panel B:* Mutation at Cys514 may cause the TMD conformation change, and further impair cholesterol export. Loss of function mutation at Cys311 may be caused by the twisted translocation pocket, or by blocked ATP binding or hydrolysis.

# Chapter 6

# References

1. Glass, C. K., and Witztum, J. L. (2001) *Cell* **104**, 503-516
2. Lusis, A. J. (2000) *Nature* **407**, 233-241
3. Daugherty, A. (2002) *Am J Med Sci* **323**, 3-10
4. Bodary, P. F., and Eitzman, D. T. (2009) *Curr Opin Hematol* **16**, 342-346
5. Narayanaswamy, M., Wright, K. C., and Kandarpa, K. (2000) *J Vasc Interv Radiol* **11**, 5-17
6. O'Neill, T. P. (1997) *Toxicol Pathol* **25**, 20-21
7. Meir, K. S., and Leitersdorf, E. (2004) *Arterioscler Thromb Vasc Biol* **24**, 1006-1014
8. Carmeliet, P., Moons, L., and Collen, D. (1998) *Cardiovasc Res* **39**, 8-33
9. Fazio, S., and Linton, M. F. (2001) *Front Biosci* **6**, D515-525
10. Coleman, R., Hayek, T., Keidar, S., and Aviram, M. (2006) *Acta Histochem* **108**, 415-424
11. Veniant, M. M., Withycombe, S., and Young, S. G. (2001) *Arterioscler Thromb Vasc Biol* **21**, 1567-1570
12. Maxfield, F. R., and van Meer, G. (2010) *Curr Opin Cell Biol* **22**, 422-429
13. Marsh, D. (2009) *Biochim Biophys Acta* **1788**, 2114-2123
14. Mukherjee, S., Zha, X., Tabas, I., and Maxfield, F. R. (1998) *Biophys J* **75**, 1915-1925
15. Simons, K., and Vaz, W. L. (2004) *Annu Rev Biophys Biomol Struct* **33**, 269-295
16. Ikonen, E. (2008) *Nat Rev Mol Cell Biol* **9**, 125-138
17. van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008) *Nat Rev Mol Cell Biol* **9**, 112-124
18. Simons, K., and Toomre, D. (2000) *Nat Rev Mol Cell Biol* **1**, 31-39
19. Lim, K. I., and Yin, J. (2005) *Biotechnol Bioeng* **90**, 694-702
20. Cherezov, V., Rosenbaum, D. M., Hanson, M. A., Rasmussen, S. G., Thian, F. S., Kobilka, T. S., Choi, H. J., Kuhn, P., Weis, W. I., Kobilka, B. K., and Stevens, R. C. (2007) *Science* **318**, 1258-1265
21. Simons, K., and Ehehalt, R. (2002) *J Clin Invest* **110**, 597-603

22. Hu, J., Zhang, Z., Shen, W. J., and Azhar, S. (2010) *Nutr Metab (Lond)* **7**, 47
23. Javitt, N. B. (2008) *Steroids* **73**, 149-157
24. Ikonen, E. (2006) *Physiol Rev* **86**, 1237-1261
25. Feng, B., Yao, P. M., Li, Y., Devlin, C. M., Zhang, D., Harding, H. P., Sweeney, M., Rong, J. X., Kuriakose, G., Fisher, E. A., Marks, A. R., Ron, D., and Tabas, I. (2003) *Nat Cell Biol* **5**, 781-792
26. Maxfield, F. R., and Tabas, I. (2005) *Nature* **438**, 612-621
27. Grundy, S. M. (1983) *Annu Rev Nutr* **3**, 71-96
28. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) *Cell* **124**, 35-46
29. Altmann, S. W., Davis, H. R., Jr., Zhu, L. J., Yao, X., Hoos, L. M., Tetzloff, G., Iyer, S. P., Maguire, M., Golovko, A., Zeng, M., Wang, L., Murgolo, N., and Graziano, M. P. (2004) *Science* **303**, 1201-1204
30. Davis, H. R., Jr., Zhu, L. J., Hoos, L. M., Tetzloff, G., Maguire, M., Liu, J., Yao, X., Iyer, S. P., Lam, M. H., Lund, E. G., Detmers, P. A., Graziano, M. P., and Altmann, S. W. (2004) *J Biol Chem* **279**, 33586-33592
31. Chang, T. Y., Reid, P. C., Sugii, S., Ohgami, N., Cruz, J. C., and Chang, C. C. (2005) *J Biol Chem* **280**, 20917-20920
32. Sun, L. P., Seemann, J., Goldstein, J. L., and Brown, M. S. (2007) *Proc Natl Acad Sci U S A* **104**, 6519-6526
33. Radhakrishnan, A., Ikeda, Y., Kwon, H. J., Brown, M. S., and Goldstein, J. L. (2007) *Proc Natl Acad Sci U S A* **104**, 6511-6518
34. Radhakrishnan, A., Goldstein, J. L., McDonald, J. G., and Brown, M. S. (2008) *Cell Metab* **8**, 512-521
35. Tontonoz, P., and Mangelsdorf, D. J. (2003) *Mol Endocrinol* **17**, 985-993
36. Zelcer, N., and Tontonoz, P. (2006) *J Clin Invest* **116**, 607-614
37. Bjorkhem, I. (1992) *J Lipid Res* **33**, 455-471
38. Rosen, H., Reshef, A., Maeda, N., Lippoldt, A., Shpizen, S., Triger, L., Eggertsen, G., Bjorkhem, I., and Leitersdorf, E. (1998) *J Biol Chem* **273**, 14805-14812

39. Charlton-Menys, V., and Durrington, P. N. (2008) *Exp Physiol* **93**, 27-42
40. Rigotti, A., Miettinen, H. E., and Krieger, M. (2003) *Endocr Rev* **24**, 357-387
41. Rader, D. J., and Daugherty, A. (2008) *Nature* **451**, 904-913
42. Camont, L., Chapman, M. J., and Kontush, A. (2011) *Trends Mol Med*, In press
43. Rosenson, R. S., Brewer, H. B., Jr., Chapman, M. J., Fazio, S., Hussain, M. M., Kontush, A., Krauss, R. M., Otvos, J. D., Remaley, A. T., and Schaefer, E. J. (2011) *Clin Chem* **57**, 392-410
44. Vaisar, T., Pennathur, S., Green, P. S., Gharib, S. A., Hoofnagle, A. N., Cheung, M. C., Byun, J., Vuletic, S., Kassim, S., Singh, P., Chea, H., Knopp, R. H., Brunzell, J., Geary, R., Chait, A., Zhao, X. Q., Elkon, K., Marcovina, S., Ridker, P., Oram, J. F., and Heinecke, J. W. (2007) *J Clin Invest* **117**, 746-756
45. Davidson, W. S., Silva, R. A., Chantepie, S., Lagor, W. R., Chapman, M. J., and Kontush, A. (2009) *Arterioscler Thromb Vasc Biol* **29**, 870-876
46. Lewis, G. F., and Rader, D. J. (2005) *Circ Res* **96**, 1221-1232
47. Castelli, W. P., Doyle, J. T., Gordon, T., Hames, C. G., Hjortland, M. C., Hulley, S. B., Kagan, A., and Zukel, W. J. (1977) *Circulation* **55**, 767-772
48. Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B., and Dawber, T. R. (1977) *Am J Med* **62**, 707-714
49. Lewington, S., Whitlock, G., Clarke, R., Sherliker, P., Emberson, J., Halsey, J., Qizilbash, N., Peto, R., and Collins, R. (2007) *Lancet* **370**, 1829-1839
50. Rhoads, G. G., Gulbrandsen, C. L., and Kagan, A. (1976) *N Engl J Med* **294**, 293-298
51. Castelli, W. P. (1988) *Can J Cardiol* **4 Suppl A**, 5A-10A
52. Franceschini, G. (2001) *Am J Cardiol* **88**, 9N-13N
53. Barter, P., Gotto, A. M., LaRosa, J. C., Maroni, J., Szarek, M., Grundy, S. M., Kastelein, J. J., Bittner, V., and Fruchart, J. C. (2007) *N Engl J Med* **357**, 1301-1310

54. Cuchel, M., and Rader, D. J. (2006) *Circulation* **113**, 2548-2555
55. Wang, X., and Rader, D. J. (2007) *Curr Opin Cardiol* **22**, 368-372
56. Tall, A. R., Yvan-Charvet, L., Terasaka, N., Pagler, T., and Wang, N. (2008) *Cell Metab* **7**, 365-375
57. Navab, M., Reddy, S. T., Van Lenten, B. J., and Fogelman, A. M. (2011) *Nat Rev Cardiol* **8**, 222-232
58. Rye, K. A., and Barter, P. J. (2004) *Arterioscler Thromb Vasc Biol* **24**, 421-428
59. Rothblat, G. H., and Phillips, M. C. (2010) *Curr Opin Lipidol* **21**, 229-238
60. Wang, N., Lan, D., Chen, W., Matsuura, F., and Tall, A. R. (2004) *Proc Natl Acad Sci U S A* **101**, 9774-9779
61. Chapman, M. J., Le Goff, W., Guerin, M., and Kontush, A. (2010) *Eur Heart J* **31**, 149-164
62. Santamarina-Fojo, S., Gonzalez-Navarro, H., Freeman, L., Wagner, E., and Nong, Z. (2004) *Arterioscler Thromb Vasc Biol* **24**, 1750-1754
63. Albers, J. J., Vuletic, S., and Cheung, M. C. (2011) *Biochim Biophys Acta*
64. Assmann, G., and Gotto, A. M., Jr. (2004) *Circulation* **109**, III8-14
65. Kontush, A., and Chapman, M. J. (2006) *Pharmacol Rev* **58**, 342-374
66. Kontush, A., and Chapman, M. J. (2006) *Nat Clin Pract Cardiovasc Med* **3**, 144-153
67. Saxena, U., Ferguson, E., and Bisgaier, C. L. (1993) *J Biol Chem* **268**, 14812-14819
68. Khoo, J. C., Miller, E., McLoughlin, P., and Steinberg, D. (1990) *J Lipid Res* **31**, 645-652
69. Navab, M., Hama, S. Y., Anantharamaiah, G. M., Hassan, K., Hough, G. P., Watson, A. D., Reddy, S. T., Sevanian, A., Fonarow, G. C., and Fogelman, A. M. (2000) *J Lipid Res* **41**, 1495-1508
70. Navab, M., Hama, S. Y., Cooke, C. J., Anantharamaiah, G. M., Chaddha, M., Jin, L., Subbanagounder, G., Faull, K. F., Reddy, S. T., Miller, N. E., and Fogelman, A. M. (2000) *J Lipid Res* **41**, 1481-1494

71. Yuhanna, I. S., Zhu, Y., Cox, B. E., Hahner, L. D., Osborne-Lawrence, S., Lu, P., Marcel, Y. L., Anderson, R. G., Mendelsohn, M. E., Hobbs, H. H., and Shaul, P. W. (2001) *Nat Med* **7**, 853-857
72. Gong, M., Wilson, M., Kelly, T., Su, W., Dressman, J., Kincer, J., Matveev, S. V., Guo, L., Guerin, T., Li, X. A., Zhu, W., Uittenbogaard, A., and Smart, E. J. (2003) *J Clin Invest* **111**, 1579-1587
73. Assanasen, C., Mineo, C., Seetharam, D., Yuhanna, I. S., Marcel, Y. L., Connelly, M. A., Williams, D. L., de la Llera-Moya, M., Shaul, P. W., and Silver, D. L. (2005) *J Clin Invest* **115**, 969-977
74. Libby, P. (2002) *Nature* **420**, 868-874
75. Hassan, H. H., Denis, M., Krimbou, L., Marcil, M., and Genest, J. (2006) *Can J Cardiol* **22 Suppl B**, 35B-40B
76. O'Connell, B. J., Denis, M., and Genest, J. (2004) *Circulation* **110**, 2881-2888
77. Cui, D., Thorp, E., Li, Y., Wang, N., Yvan-Charvet, L., Tall, A. R., and Tabas, I. (2007) *J Leukoc Biol* **82**, 1040-1050
78. Gerbod-Giannone, M. C., Li, Y., Holleboom, A., Han, S., Hsu, L. C., Tabas, I., and Tall, A. R. (2006) *Proc Natl Acad Sci U S A* **103**, 3112-3117
79. Terasaka, N., Wang, N., Yvan-Charvet, L., and Tall, A. R. (2007) *Proc Natl Acad Sci U S A* **104**, 15093-15098
80. Tabas, I. (2005) *Arterioscler Thromb Vasc Biol* **25**, 2255-2264
81. Tarling, E. J., and Edwards, P. A. (2011) *Biochim Biophys Acta*, In press
82. Dean, M., Rzhetsky, A., and Allikmets, R. (2001) *Genome Res* **11**, 1156-1166
83. Dean, M., Hamon, Y., and Chimini, G. (2001) *J Lipid Res* **42**, 1007-1017
84. Vasiliou, V., Vasiliou, K., and Nebert, D. W. (2009) *Hum Genomics* **3**, 281-290
85. Oram, J. F. (2002) *Trends Mol Med* **8**, 168-173
86. Stefkova, J., Poledne, R., and Hubacek, J. A. (2004) *Physiol Res* **53**, 235-243

87. Oswald, C., Holland, I. B., and Schmitt, L. (2006) *Naunyn Schmiedebergs Arch Pharmacol* **372**, 385-399
88. Schmitt, L., and Tampe, R. (2002) *Curr Opin Struct Biol* **12**, 754-760
89. Tarr, P. T., Tarling, E. J., Bojanic, D. D., Edwards, P. A., and Baldan, A. (2009) *Biochim Biophys Acta* **1791**, 584-593
90. Baldan, A., Bojanic, D. D., and Edwards, P. A. (2009) *J Lipid Res* **50 Suppl**, S80-85
91. Locher, K. P. (2009) *Philos Trans R Soc Lond B Biol Sci* **364**, 239-245
92. Dawson, R. J., and Locher, K. P. (2006) *Nature* **443**, 180-185
93. Dawson, R. J., and Locher, K. P. (2007) *FEBS Lett* **581**, 935-938
94. Weng, J. W., Fan, K. N., and Wang, W. N. (2010) *J Biol Chem* **285**, 3053-3063
95. Kaul, G., and Pattan, G. (2011) *Indian J Biochem Biophys* **48**, 7-13
96. Ward, A., Reyes, C. L., Yu, J., Roth, C. B., and Chang, G. (2007) *Proc Natl Acad Sci U S A* **104**, 19005-19010
97. Jones, P. M., O'Mara, M. L., and George, A. M. (2009) *Trends Biochem Sci* **34**, 520-531
98. Costet, P., Luo, Y., Wang, N., and Tall, A. R. (2000) *J Biol Chem* **275**, 28240-28245
99. Wang, N., Chen, W., Linsel-Nitschke, P., Martinez, L. O., Agerholm-Larsen, B., Silver, D. L., and Tall, A. R. (2003) *J Clin Invest* **111**, 99-107
100. Oram, J. F., and Vaughan, A. M. (2006) *Circ Res* **99**, 1031-1043
101. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J. C., Deleuze, J. F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) *Nat Genet* **22**, 352-355
102. Singaraja, R. R., Stahmer, B., Brundert, M., Merkel, M., Heeren, J., Bissada, N., Kang, M., Timmins, J. M., Ramakrishnan, R., Parks, J. S., Hayden, M. R., and Rinninger, F. (2006) *Arterioscler Thromb Vasc Biol* **26**, 1821-1827
103. Brunham, L. R., Kruit, J. K., Iqbal, J., Fievet, C., Timmins, J. M., Pape, T. D., Coburn, B. A., Bissada, N., Staels, B., Groen, A. K., Hussain, M. M.,

Parks, J. S., Kuipers, F., and Hayden, M. R. (2006) *J Clin Invest* **116**, 1052-1062

104. Attie, A. D. (2007) *Trends Biochem Sci* **32**, 172-179
105. Van Eck, M., Singaraja, R. R., Ye, D., Hildebrand, R. B., James, E. R., Hayden, M. R., and Van Berkel, T. J. (2006) *Arterioscler Thromb Vasc Biol* **26**, 929-934
106. Zhu, X., Lee, J. Y., Timmins, J. M., Brown, J. M., Boudyguina, E., Mulya, A., Gebre, A. K., Willingham, M. C., Hiltbold, E. M., Mishra, N., Maeda, N., and Parks, J. S. (2008) *J Biol Chem* **283**, 22930-22941
107. Aiello, R. J., Brees, D., Bourassa, P. A., Royer, L., Lindsey, S., Coskran, T., Haghpassand, M., and Francone, O. L. (2002) *Arterioscler Thromb Vasc Biol* **22**, 630-637
108. Soumian, S., Albrecht, C., Davies, A. H., and Gibbs, R. G. (2005) *Vasc Med* **10**, 109-119
109. Vedhachalam, C., Duong, P. T., Nickel, M., Nguyen, D., Dhanasekaran, P., Saito, H., Rothblat, G. H., Lund-Katz, S., and Phillips, M. C. (2007) *J Biol Chem* **282**, 25123-25130
110. Cavelier, C., Lorenzi, I., Rohrer, L., and von Eckardstein, A. (2006) *Biochim Biophys Acta* **1761**, 655-666
111. Graf, G. A., Cohen, J. C., and Hobbs, H. H. (2004) *J Biol Chem* **279**, 24881-24888
112. Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwiterovich, P., Shan, B., Barnes, R., and Hobbs, H. H. (2000) *Science* **290**, 1771-1775
113. Wang, J., Zhang, D. W., Lei, Y., Xu, F., Cohen, J. C., Hobbs, H. H., and Xie, X. S. (2008) *Biochemistry* **47**, 5194-5204
114. Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2002) *J Clin Invest* **110**, 671-680
115. Yu, L., Hammer, R. E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J. C., and Hobbs, H. H. (2002) *Proc Natl Acad Sci U S A* **99**, 16237-16242

116. Kidambi, S., and Patel, S. B. (2008) *J Clin Pathol* **61**, 588-594
117. Miettinen, T. A. (1980) *Eur J Clin Invest* **10**, 27-35
118. Basso, F., Freeman, L. A., Ko, C., Joyce, C., Amar, M. J., Shamburek, R. D., Tansey, T., Thomas, F., Wu, J., Paigen, B., Remaley, A. T., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2007) *J Lipid Res* **48**, 114-126
119. Tang, W., Ma, Y., Jia, L., Ioannou, Y. A., Davies, J. P., and Yu, L. (2009) *J Lipid Res* **50**, 293-300
120. Sugiyama, A., Shitan, N., Sato, S., Nakamura, Y., Tabata, S., and Yazaki, K. (2006) *DNA Res* **13**, 205-228
121. Schmitz, G., Langmann, T., and Heimerl, S. (2001) *J Lipid Res* **42**, 1513-1520
122. Curtiss, L. K. (2006) *Arterioscler Thromb Vasc Biol* **26**, 2175-2177
123. Savary, S., Denizot, F., Luciani, M., Mattei, M., and Chimini, G. (1996) *Mamm Genome* **7**, 673-676
124. Sullivan, D. T., Bell, L. A., Paton, D. R., and Sullivan, M. C. (1980) *Biochem Genet* **18**, 1109-1130
125. Kennedy, M. A., Barrera, G. C., Nakamura, K., Baldan, A., Tarr, P., Fishbein, M. C., Frank, J., Francone, O. L., and Edwards, P. A. (2005) *Cell Metab* **1**, 121-131
126. Chen, H., Rossier, C., Lalioti, M. D., Lynn, A., Chakravarti, A., Perrin, G., and Antonarakis, S. E. (1996) *Am J Hum Genet* **59**, 66-75
127. Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S., and Wu, R. L. (1997) *Gene* **185**, 77-85
128. Kennedy, M. A., Venkateswaran, A., Tarr, P. T., Xenarios, I., Kudoh, J., Shimizu, N., and Edwards, P. A. (2001) *J Biol Chem* **276**, 39438-39447
129. Klucken, J., Buchler, C., Orso, E., Kaminski, W. E., Porsch-Ozcurumez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R., and Schmitz, G. (2000) *Proc Natl Acad Sci U S A* **97**, 817-822

130. Laffitte, B. A., Repa, J. J., Joseph, S. B., Wilpitz, D. C., Kast, H. R., Mangelsdorf, D. J., and Tontonoz, P. (2001) *Proc Natl Acad Sci U S A* **98**, 507-512

131. Nakamura, K., Kennedy, M. A., Baldan, A., Bojanic, D. D., Lyons, K., and Edwards, P. A. (2004) *J Biol Chem* **279**, 45980-45989

132. Sabol, S. L., Brewer, H. B., Jr., and Santamarina-Fojo, S. (2005) *J Lipid Res* **46**, 2151-2167

133. Jakobsson, T., Venteclef, N., Toresson, G., Damdimopoulos, A. E., Ehrlund, A., Lou, X., Sanyal, S., Steffensen, K. R., Gustafsson, J. A., and Treuter, E. (2009) *Mol Cell* **34**, 510-518

134. Engel, T., Bode, G., Lueken, A., Knop, M., Kannenberg, F., Nofer, J. R., Assmann, G., and Seedorf, U. (2006) *FEBS Lett* **580**, 4551-4559

135. Rayner, K. J., Suarez, Y., Davalos, A., Parathath, S., Fitzgerald, M. L., Tamehiro, N., Fisher, E. A., Moore, K. J., and Fernandez-Hernando, C. (2010) *Science* **328**, 1570-1573

136. Bartel, D. P. (2009) *Cell* **136**, 215-233

137. Li, D., Zhang, Y., Ma, J., Ling, W., and Xia, M. (2010) *Arterioscler Thromb Vasc Biol* **30**, 1354-1362

138. Nagelin, M. H., Srinivasan, S., Lee, J., Nadler, J. L., and Hedrick, C. C. (2008) *Arterioscler Thromb Vasc Biol* **28**, 1811-1819

139. Nagelin, M. H., Srinivasan, S., Nadler, J. L., and Hedrick, C. C. (2009) *J Biol Chem* **284**, 31303-31314

140. Venkateswaran, A., Repa, J. J., Lobaccaro, J. M., Bronson, A., Mangelsdorf, D. J., and Edwards, P. A. (2000) *J Biol Chem* **275**, 14700-14707

141. Sankaranarayanan, S., Oram, J. F., Asztalos, B. F., Vaughan, A. M., Lund-Katz, S., Adorni, M. P., Phillips, M. C., and Rothblat, G. H. (2009) *J Lipid Res* **50**, 275-284

142. Wang, N., Ranalletta, M., Matsuura, F., Peng, F., and Tall, A. R. (2006) *Arterioscler Thromb Vasc Biol* **26**, 1310-1316

143. Vaughan, A. M., and Oram, J. F. (2005) *J Biol Chem* **280**, 30150-30157

144. Vaughan, A. M., and Oram, J. F. (2006) *J Lipid Res* **47**, 2433-2443

145. Gelissen, I. C., Harris, M., Rye, K. A., Quinn, C., Brown, A. J., Kockx, M., Cartland, S., Packianathan, M., Kritharides, L., and Jessup, W. (2006) *Arterioscler Thromb Vasc Biol* **26**, 534-540

146. Basso, F., Amar, M. J., Wagner, E. M., Vaisman, B., Paigen, B., Santamarina-Fojo, S., and Remaley, A. T. (2006) *Biochem Biophys Res Commun* **351**, 398-404

147. Burgess, B., Naus, K., Chan, J., Hirsch-Reinshagen, V., Tansley, G., Matzke, L., Chan, B., Wilkinson, A., Fan, J., Donkin, J., Balik, D., Tanaka, T., Ou, G., Dyer, R., Innis, S., McManus, B., Lutjohann, D., and Wellington, C. (2008) *Arterioscler Thromb Vasc Biol* **28**, 1731-1737

148. Sturek, J. M., Castle, J. D., Trace, A. P., Page, L. C., Castle, A. M., Evans-Molina, C., Parks, J. S., Mirmira, R. G., and Hedrick, C. C. (2010) *J Clin Invest* **120**, 2575-2589

149. Tarr, P. T., and Edwards, P. A. (2008) *J Lipid Res* **49**, 169-182

150. Xie, Q., Engel, T., Schnoor, M., Niehaus, J., Hofnagel, O., Buers, I., Cullen, P., Seedorf, U., Assmann, G., and Lorkowski, S. (2006) *Arterioscler Thromb Vasc Biol* **26**, e143-144; author reply e145

151. Kobayashi, A., Takanezawa, Y., Hirata, T., Shimizu, Y., Misasa, K., Kioka, N., Arai, H., Ueda, K., and Matsuo, M. (2006) *J Lipid Res* **47**, 1791-1802

152. Terasaka, N., Yu, S., Yvan-Charvet, L., Wang, N., Mzhavia, N., Langlois, R., Pagler, T., Li, R., Welch, C. L., Goldberg, I. J., and Tall, A. R. (2008) *J Clin Invest* **118**, 3701-3713

153. Wojcik, A. J., Skaflen, M. D., Srinivasan, S., and Hedrick, C. C. (2008) *J Immunol* **180**, 4273-4282

154. Tarling, E. J., Bojanic, D. D., Tangirala, R. K., Wang, X., Lovgren-Sandblom, A., Lusis, A. J., Bjorkhem, I., and Edwards, P. A. (2010) *Arterioscler Thromb Vasc Biol* **30**, 1174-1180

155. Whetzel, A. M., Sturek, J. M., Nagelin, M. H., Bolick, D. T., Gebre, A. K., Parks, J. S., Bruce, A. C., Skaflen, M. D., and Hedrick, C. C. (2010) *Arterioscler Thromb Vasc Biol* **30**, 809-817

156. Westerterp, M., Koetsveld, J., Yu, S., Han, S., Li, R., Goldberg, I. J., Welch, C. L., and Tall, A. R. (2010) *Arterioscler Thromb Vasc Biol*

157. Terasaka, N., Westerterp, M., Koetsveld, J., Fernandez-Hernando, C., Yvan-Charvet, L., Wang, N., Sessa, W. C., and Tall, A. R. (2010) *Arterioscler Thromb Vasc Biol* **30**, 2219-2225

158. Burgess, B. L., Parkinson, P. F., Racke, M. M., Hirsch-Reinshagen, V., Fan, J., Wong, C., Stukas, S., Theroux, L., Chan, J. Y., Donkin, J., Wilkinson, A., Balik, D., Christie, B., Poirier, J., Lutjohann, D., Demattos, R. B., and Wellington, C. L. (2008) *J Lipid Res* **49**, 1254-1267

159. Karten, B., Campenot, R. B., Vance, D. E., and Vance, J. E. (2006) *J Biol Chem* **281**, 4049-4057

160. Fryirs, M. A., Barter, P. J., Appavoo, M., Tuch, B. E., Tabet, F., Heather, A. K., and Rye, K. A. (2010) *Arterioscler Thromb Vasc Biol* **30**, 1642-1648

161. Yvan-Charvet, L., Pagler, T., Gautier, E. L., Avagyan, S., Siry, R. L., Han, S., Welch, C. L., Wang, N., Randolph, G. J., Snoeck, H. W., and Tall, A. R. (2010) *Science* **328**, 1689-1693

162. Bensinger, S. J., Bradley, M. N., Joseph, S. B., Zelcer, N., Janssen, E. M., Hausner, M. A., Shih, R., Parks, J. S., Edwards, P. A., Jamieson, B. D., and Tontonoz, P. (2008) *Cell* **134**, 97-111

163. Wang, X., Collins, H. L., Ranalletta, M., Fuki, I. V., Billheimer, J. T., Rothblat, G. H., Tall, A. R., and Rader, D. J. (2007) *J Clin Invest* **117**, 2216-2224

164. Out, R., Hoekstra, M., Hildebrand, R. B., Kruit, J. K., Meurs, I., Li, Z., Kuipers, F., Van Berkel, T. J., and Van Eck, M. (2006) *Arterioscler Thromb Vasc Biol* **26**, 2295-2300

165. Ranalletta, M., Wang, N., Han, S., Yvan-Charvet, L., Welch, C., and Tall, A. R. (2006) *Arterioscler Thromb Vasc Biol* **26**, 2308-2315

166. Baldan, A., Pei, L., Lee, R., Tarr, P., Tangirala, R. K., Weinstein, M. M., Frank, J., Li, A. C., Tontonoz, P., and Edwards, P. A. (2006) *Arterioscler Thromb Vasc Biol* **26**, 2301-2307
167. Lammers, B., Out, R., Hildebrand, R. B., Quinn, C. M., Williamson, D., Hoekstra, M., Meurs, I., Van Berkel, T. J., Jessup, W., and Van Eck, M. (2009) *Atherosclerosis* **205**, 420-426
168. Out, R., Hoekstra, M., Habets, K., Meurs, I., de Waard, V., Hildebrand, R. B., Wang, Y., Chimini, G., Kuiper, J., Van Berkel, T. J., and Van Eck, M. (2008) *Arterioscler Thromb Vasc Biol* **28**, 258-264
169. Out, R., Jessup, W., Le Goff, W., Hoekstra, M., Gelissen, I. C., Zhao, Y., Kritharides, L., Chimini, G., Kuiper, J., Chapman, M. J., Huby, T., Van Berkel, T. J., and Van Eck, M. (2008) *Circ Res* **102**, 113-120
170. Yvan-Charvet, L., Ranalletta, M., Wang, N., Han, S., Terasaka, N., Li, R., Welch, C., and Tall, A. R. (2007) *J Clin Invest* **117**, 3900-3908
171. Nadolski, M. J., and Linder, M. E. (2007) *FEBS J* **274**, 5202-5210
172. Walsh, C. T., Garneau-Tsodikova, S., and Gatto, G. J., Jr. (2005) *Angew Chem Int Ed Engl* **44**, 7342-7372
173. Zhang, F. L., and Casey, P. J. (1996) *Annu Rev Biochem* **65**, 241-269
174. Martin, D. D., Beauchamp, E., and Berthiaume, L. G. (2011) *Biochimie* **93**, 18-31
175. Hannoush, R. N., and Sun, J. (2010) *Nat Chem Biol* **6**, 498-506
176. Resh, M. D. (2006) *Sci STKE* **2006**, re14
177. Yap, M. C., Kostiuk, M. A., Martin, D. D., Perinpanayagam, M. A., Hak, P. G., Siddam, A., Majjigapu, J. R., Rajaiah, G., Keller, B. O., Prescher, J. A., Wu, P., Bertozzi, C. R., Falck, J. R., and Berthiaume, L. G. (2010) *J Lipid Res* **51**, 1566-1580
178. Mitchell, D. A., Vasudevan, A., Linder, M. E., and Deschenes, R. J. (2006) *J Lipid Res* **47**, 1118-1127
179. Mann, R. K., and Beachy, P. A. (2004) *Annu Rev Biochem* **73**, 891-923
180. Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K.,

Taylor, F. R., Wang, E. A., and Galdes, A. (1998) *J Biol Chem* **273**, 14037-14045

181. Buglino, J. A., and Resh, M. D. (2008) *J Biol Chem* **283**, 22076-22088
182. Resh, M. D. (1999) *Biochim Biophys Acta* **1451**, 1-16
183. Bijlmakers, M. J., and Marsh, M. (2003) *Trends Cell Biol* **13**, 32-42
184. Tsutsumi, R., Fukata, Y., and Fukata, M. (2008) *Pflugers Arch* **456**, 1199-1206
185. Schweizer, A., Rohrer, J., and Kornfeld, S. (1995) *J Biol Chem* **270**, 9638-9644
186. Linder, M. E., and Deschenes, R. J. (2007) *Nat Rev Mol Cell Biol* **8**, 74-84
187. Planey, S. L., and Zacharias, D. A. (2009) *Mol Membr Biol* **26**, 14-31
188. Dietrich, L. E., and Ungermann, C. (2004) *EMBO Rep* **5**, 1053-1057
189. Veit, M. (2000) *Biochem J* **345 Pt 1**, 145-151
190. Duncan, J. A., and Gilman, A. G. (1996) *J Biol Chem* **271**, 23594-23600
191. Zeidman, R., Jackson, C. S., and Magee, A. I. (2009) *Mol Membr Biol* **26**, 32-41
192. Lobo, S., Greentree, W. K., Linder, M. E., and Deschenes, R. J. (2002) *J Biol Chem* **277**, 41268-41273
193. Roth, A. F., Feng, Y., Chen, L., and Davis, N. G. (2002) *J Cell Biol* **159**, 23-28
194. Roth, A. F., Wan, J., Bailey, A. O., Sun, B., Kuchar, J. A., Green, W. N., Phinney, B. S., Yates, J. R., 3rd, and Davis, N. G. (2006) *Cell* **125**, 1003-1013
195. Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R. A., and Bredt, D. S. (2004) *Neuron* **44**, 987-996
196. Miura, G. I., and Treisman, J. E. (2006) *Cell Cycle* **5**, 1184-1188
197. Doubravska, L., Krausova, M., Gradl, D., Vojtechova, M., Tumova, L., Lukas, J., Valenta, T., Pospichalova, V., Fafilek, B., Plachy, J., Sebesta, O., and Korinek, V. (2011) *Cell Signal* **23**, 837-848
198. Zhai, L., Chaturvedi, D., and Cumberledge, S. (2004) *J Biol Chem* **279**, 33220-33227

199. Yang, J., Brown, M. S., Liang, G., Grishin, N. V., and Goldstein, J. L. (2008) *Cell* **132**, 387-396

200. Rocks, O., Gerauer, M., Vartak, N., Koch, S., Huang, Z. P., Pechlivanis, M., Kuhlmann, J., Brunsveld, L., Chandra, A., Ellinger, B., Waldmann, H., and Bastiaens, P. I. (2010) *Cell* **141**, 458-471

201. Sugimoto, H., Hayashi, H., and Yamashita, S. (1996) *J Biol Chem* **271**, 7705-7711

202. Yeh, D. C., Duncan, J. A., Yamashita, S., and Michel, T. (1999) *J Biol Chem* **274**, 33148-33154

203. Flaumenhaft, R., Rozenvayn, N., Feng, D., and Dvorak, A. M. (2007) *Blood* **110**, 1492-1501

204. Duncan, J. A., and Gilman, A. G. (1998) *J Biol Chem* **273**, 15830-15837

205. Sim, D. S., Dilks, J. R., and Flaumenhaft, R. (2007) *Arterioscler Thromb Vasc Biol* **27**, 1478-1485

206. Duncan, J. A., and Gilman, A. G. (2002) *J Biol Chem* **277**, 31740-31752

207. Greaves, J., and Chamberlain, L. H. (2007) *J Cell Biol* **176**, 249-254

208. Roy, S., Plowman, S., Rotblat, B., Prior, I. A., Muncke, C., Grainger, S., Parton, R. G., Henis, Y. I., Kloog, Y., and Hancock, J. F. (2005) *Mol Cell Biol* **25**, 6722-6733

209. Greaves, J., Prescott, G. R., Gorleku, O. A., and Chamberlain, L. H. (2009) *Mol Membr Biol* **26**, 67-79

210. Valdez-Taubas, J., and Pelham, H. (2005) *EMBO J* **24**, 2524-2532

211. Abrami, L., Leppla, S. H., and van der Goot, F. G. (2006) *J Cell Biol* **172**, 309-320

212. Charollais, J., and Van Der Goot, F. G. (2009) *Mol Membr Biol* **26**, 55-66

213. Draper, J. M., and Smith, C. D. (2009) *Mol Membr Biol* **26**, 5-13

214. Huang, K., Yanai, A., Kang, R., Arstikaitis, P., Singaraja, R. R., Metzler, M., Mullard, A., Haigh, B., Gauthier-Campbell, C., Gutekunst, C. A., Hayden, M. R., and El-Husseini, A. (2004) *Neuron* **44**, 977-986

215. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J. E., and Sessa, W. C. (1996) *Proc Natl Acad Sci U S A* **93**, 6448-6453

216. Kawashima, S., and Yokoyama, M. (2004) *Arterioscler Thromb Vasc Biol* **24**, 998-1005

217. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) *Nat Cell Biol* **3**, 368-375

218. Pecquet, C., Nyga, R., Penard-Lacronique, V., Smithgall, T. E., Murakami, H., Regnier, A., Lassoued, K., and Gouilleux, F. (2007) *Oncogene* **26**, 1577-1585

219. Kosugi, A., Hayashi, F., Liddicoat, D. R., Yasuda, K., Saitoh, S., and Hamaoka, T. (2001) *Immunol Lett* **76**, 133-138

220. Oyama, T., Miyoshi, Y., Koyama, K., Nakagawa, H., Yamori, T., Ito, T., Matsuda, H., Arakawa, H., and Nakamura, Y. (2000) *Genes Chromosomes Cancer* **29**, 9-15

221. Yanai, A., Huang, K., Kang, R., Singaraja, R. R., Arstikaitis, P., Gan, L., Orban, P. C., Mullard, A., Cowan, C. M., Raymond, L. A., Drisdel, R. C., Green, W. N., Ravikumar, B., Rubinsztein, D. C., El-Husseini, A., and Hayden, M. R. (2006) *Nat Neurosci* **9**, 824-831

222. Mansouri, M. R., Marklund, L., Gustavsson, P., Davey, E., Carlsson, B., Larsson, C., White, I., Gustavson, K. H., and Dahl, N. (2005) *Eur J Hum Genet* **13**, 970-977

223. Raymond, F. L., Tarpey, P. S., Edkins, S., Tofts, C., O'Meara, S., Teague, J., Butler, A., Stevens, C., Barthorpe, S., Buck, G., Cole, J., Dicks, E., Gray, K., Halliday, K., Hills, K., Hinton, J., Jones, D., Menzies, A., Perry, J., Raine, K., Shepherd, R., Small, A., Varian, J., Widaa, S., Mallya, U., Moon, J., Luo, Y., Shaw, M., Boyle, J., Kerr, B., Turner, G., Quarrell, O., Cole, T., Easton, D. F., Wooster, R., Bobrow, M., Schwartz, C. E., Gecz, J., Stratton, M. R., and Futreal, P. A. (2007) *Am J Hum Genet* **80**, 982-987

224. Baigent, C., Keech, A., Kearney, P. M., Blackwell, L., Buck, G., Pollicino, C., Kirby, A., Sourjina, T., Peto, R., Collins, R., and Simes, R. (2005) *Lancet* **366**, 1267-1278

225. Kearney, P. M., Blackwell, L., Collins, R., Keech, A., Simes, J., Peto, R., Armitage, J., and Baigent, C. (2008) *Lancet* **371**, 117-125

226. Nissen, S. E., Tsunoda, T., Tuzcu, E. M., Schoenhagen, P., Cooper, C. J., Yasin, M., Eaton, G. M., Lauer, M. A., Sheldon, W. S., Grines, C. L., Halpern, S., Crowe, T., Blankenship, J. C., and Kerensky, R. (2003) *JAMA* **290**, 2292-2300

227. Plump, A. S., Scott, C. J., and Breslow, J. L. (1994) *Proc Natl Acad Sci U S A* **91**, 9607-9611

228. Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G., and Clift, S. M. (1991) *Nature* **353**, 265-267

229. Nissen, S. E., Tardif, J. C., Nicholls, S. J., Revkin, J. H., Shear, C. L., Duggan, W. T., Ruzyllo, W., Bachinsky, W. B., Lasala, G. P., and Tuzcu, E. M. (2007) *N Engl J Med* **356**, 1304-1316

230. Barter, P. J., Caulfield, M., Eriksson, M., Grundy, S. M., Kastelein, J. J., Komajda, M., Lopez-Sendon, J., Mosca, L., Tardif, J. C., Waters, D. D., Shear, C. L., Revkin, J. H., Buhr, K. A., Fisher, M. R., Tall, A. R., and Brewer, B. (2007) *N Engl J Med* **357**, 2109-2122

231. Naik, S. U., Wang, X., Da Silva, J. S., Jaye, M., Macphee, C. H., Reilly, M. P., Billheimer, J. T., Rothblat, G. H., and Rader, D. J. (2006) *Circulation* **113**, 90-97

232. Joseph, S. B., and Tontonoz, P. (2003) *Curr Opin Pharmacol* **3**, 192-197

233. Joseph, S. B., McKilligin, E., Pei, L., Watson, M. A., Collins, A. R., Laffitte, B. A., Chen, M., Noh, G., Goodman, J., Hagger, G. N., Tran, J., Tippin, T. K., Wang, X., Lusis, A. J., Hsueh, W. A., Law, R. E., Collins, J. L., Willson, T. M., and Tontonoz, P. (2002) *Proc Natl Acad Sci U S A* **99**, 7604-7609

234. Bradley, M. N., Hong, C., Chen, M., Joseph, S. B., Wilpitz, D. C., Wang, X., Lusis, A. J., Collins, A., Hseuh, W. A., Collins, J. L., Tangirala, R. K., and Tontonoz, P. (2007) *J Clin Invest* **117**, 2337-2346

235. Nomiyama, T., and Bruemmer, D. (2008) *Curr Atheroscler Rep* **10**, 88-95

236. Levin, N., Bischoff, E. D., Daige, C. L., Thomas, D., Vu, C. T., Heyman, R. A., Tangirala, R. K., and Schulman, I. G. (2005) *Arterioscler Thromb Vasc Biol* **25**, 135-142

237. Seo, Y. H., and Carroll, K. S. (2011) *Angew Chem Int Ed Engl* **50**, 1342-1345
238. Stamler, J. S., Lamas, S., and Fang, F. C. (2001) *Cell* **106**, 675-683
239. Lill, R., and Muhlenhoff, U. (2006) *Annu Rev Cell Dev Biol* **22**, 457-486
240. Chang, C. C., Liao, W. F., and Huang, P. C. (1998) *Protein Eng* **11**, 41-46
241. Quig, D. (1998) *Altern Med Rev* **3**, 262-270
242. Nagahara, N., Matsumura, T., Okamoto, R., and Kajihara, Y. (2009) *Curr Med Chem* **16**, 4419-4444
243. Nagahara, N., Matsumura, T., Okamoto, R., and Kajihara, Y. (2009) *Curr Med Chem* **16**, 4490-4501
244. Feng, J., Zhu, M., Schaub, M. C., Gehrig, P., Roschitzki, B., Lucchinetti, E., and Zaugg, M. (2008) *Cardiovasc Res* **80**, 20-29
245. Guan, K. L., and Dixon, J. E. (1991) *J Biol Chem* **266**, 17026-17030
246. Pickart, C. M. (2001) *Annu Rev Biochem* **70**, 503-533
247. Singaraja, R. R., Kang, M. H., Vaid, K., Sanders, S., Vilas, G., Arstikaitis, P., Coutinho, J., Drisdel, R. C., El-Husseini, A. E., Green, W. N., Berthiaume, L., and Hayden, M. R. (2009) *Circ Res* **105**, 138-147
248. Small, D. M. (2003) *Proc Natl Acad Sci U S A* **100**, 4-6
249. Tabet, F., Lambert, G., Cuesta Torres, L. F., Hou, L., Sotirchos, I., Touyz, R. M., Jenkins, A. J., Barter, P. J., and Rye, K. A. (2011) *Arterioscler Thromb Vasc Biol*, In press
250. Zhang, D. W., Garuti, R., Tang, W. J., Cohen, J. C., and Hobbs, H. H. (2008) *Proc Natl Acad Sci U S A* **105**, 13045-13050
251. Zhang, D. W., Cole, S. P., and Deeley, R. G. (2001) *J Biol Chem* **276**, 34966-34974
252. Zhang, D. W., Cole, S. P., and Deeley, R. G. (2001) *J Biol Chem* **276**, 13231-13239
253. Zhang, D. W., Lagace, T. A., Garuti, R., Zhao, Z., McDonald, M., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2007) *J Biol Chem* **282**, 18602-18612

254. Loe, D. W., Almquist, K. C., Deeley, R. G., and Cole, S. P. (1996) *J Biol Chem* **271**, 9675-9682

255. Yu, P., Yang, Z., Jones, J. E., Wang, Z., Owens, S. A., Mueller, S. C., Felder, R. A., and Jose, P. A. (2004) *Kidney Int* **66**, 2167-2180

256. Martin, D. D., Vilas, G. L., Prescher, J. A., Rajaiah, G., Falck, J. R., Bertozzi, C. R., and Berthiaume, L. G. (2008) *FASEB J* **22**, 797-806

257. Abildayeva, K., Jansen, P. J., Hirsch-Reinshagen, V., Bloks, V. W., Bakker, A. H., Ramaekers, F. C., de Vente, J., Groen, A. K., Wellington, C. L., Kuipers, F., and Mulder, M. (2006) *J Biol Chem* **281**, 12799-12808

258. Gelissen, I. C., Cartland, S., Brown, A. J., Sandoval, C., Kim, M., Dinnes, D. L., Lee, Y., Hsieh, V., Gaus, K., Kritharides, L., and Jessup, W. (2010) *Atherosclerosis* **208**, 75-82

259. Out, R., Hoekstra, M., Meurs, I., de Vos, P., Kuiper, J., Van Eck, M., and Van Berkel, T. J. (2007) *Arterioscler Thromb Vasc Biol* **27**, 594-599

260. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2009) *J Biol Chem* **284**, 24074-24087

261. Daoud, R., Julien, M., Gros, P., and Georges, E. (2001) *J Biol Chem* **276**, 12324-12330

262. Zhang, D. W., Gu, H. M., Situ, D., Haimeur, A., Cole, S. P., and Deeley, R. G. (2003) *J Biol Chem* **278**, 46052-46063

263. Zhang, D. W., Gu, H. M., Vasa, M., Muredda, M., Cole, S. P., and Deeley, R. G. (2003) *Biochemistry* **42**, 9989-10000

264. Ni, Z., Bikadi, Z., Cai, X., Rosenberg, M. F., and Mao, Q. (2010) *Am J Physiol Cell Physiol* **299**, C1100-1109

265. Polgar, O., Ierano, C., Tamaki, A., Stanley, B., Ward, Y., Xia, D., Tarasova, N., Robey, R. W., and Bates, S. E. (2010) *Biochemistry* **49**, 2235-2245

266. Kogan, I., Ramjeesingh, M., Huan, L. J., Wang, Y., and Bear, C. E. (2001) *J Biol Chem* **276**, 11575-11581

267. Aller, S. G., Yu, J., Ward, A., Weng, Y., Chittaboina, S., Zhuo, R., Harrell, P. M., Trinh, Y. T., Zhang, Q., Urbatsch, I. L., and Chang, G. (2009) *Science* **323**, 1718-1722

268. Ewart, G. D., Cannell, D., Cox, G. B., and Howells, A. J. (1994) *J Biol Chem* **269**, 10370-10377

269. Wu, D. F., Othman, N. A., Sharp, D., Mahendra, A., Deeb, T. Z., and Hales, T. G. (2010) *J Physiol* **588**, 603-616

270. Castaneda-Bueno, M., Vazquez, N., Bustos-Jaimes, I., Hernandez, D., Rodriguez-Lobato, E., Pacheco-Alvarez, D., Carino-Cortes, R., Moreno, E., Bobadilla, N. A., and Gamba, G. (2010) *Am J Physiol Renal Physiol* **299**, F1111-1119

271. Wu, J., and Kaback, H. R. (1994) *Biochemistry* **33**, 12166-12171

272. Liu, D., and Sy, M. S. (1996) *J Exp Med* **183**, 1987-1994

273. Dodd, J. R., and Christie, D. L. (2001) *J Biol Chem* **276**, 46983-46988

274. Pajor, A. M., Krajewski, S. J., Sun, N., and Gangula, R. (1999) *Biochem J* **344 Pt 1**, 205-209

275. Roy, R., Laage, R., and Langosch, D. (2004) *Biochemistry* **43**, 4964-4970

276. Liu, D., and Sy, M. S. (1997) *J Immunol* **159**, 2702-2711

277. Gaddie, K. J., and Kirley, T. L. (2009) *Biochemistry* **48**, 9437-9447

278. Attie, A. D., Kastelein, J. P., and Hayden, M. R. (2001) *J Lipid Res* **42**, 1717-1726

279. Yvan-Charvet, L., Wang, N., and Tall, A. R. (2010) *Arterioscler Thromb Vasc Biol* **30**, 139-143

280. Hozoji, M., Kimura, Y., Kioka, N., and Ueda, K. (2009) *J Biol Chem* **284**, 11293-11300

281. Xu, J., Liu, Y., Yang, Y., Bates, S., and Zhang, J. T. (2004) *J Biol Chem* **279**, 19781-19789

282. Fujii, J., Maruyama, K., Tada, M., and MacLennan, D. H. (1989) *J Biol Chem* **264**, 12950-12955

283. Karim, C. B., Paterlini, M. G., Reddy, L. G., Hunter, G. W., Barany, G., and Thomas, D. D. (2001) *J Biol Chem* **276**, 38814-38819

284. Laage, R., and Langosch, D. (1997) *Eur J Biochem* **249**, 540-546
285. Jung, H., Jung, K., and Kaback, H. R. (1994) *Biochemistry* **33**, 12160-12165
286. Abramson, J., Smirnova, I., Kasho, V., Verner, G., Kaback, H. R., and Iwata, S. (2003) *Science* **301**, 610-615
287. Dodd, J. R., and Christie, D. L. (2005) *J Biol Chem* **280**, 32649-32654
288. Arkin, I. T., Adams, P. D., Brunger, A. T., Aimoto, S., Engelman, D. M., and Smith, S. O. (1997) *J Membr Biol* **155**, 199-206
289. Cho, H. J., Shashkin, P., Gleissner, C. A., Dunson, D., Jain, N., Lee, J. K., Miller, Y., and Ley, K. (2007) *Physiol Genomics* **29**, 149-160
290. Cuchel, M., Lund-Katz, S., de la Llera-Moya, M., Millar, J. S., Chang, D., Fuki, I., Rothblat, G. H., Phillips, M. C., and Rader, D. J. (2010) *Arterioscler Thromb Vasc Biol* **30**, 526-532
291. Maliepaard, M., Scheffer, G. L., Faneyte, I. F., van Gastelen, M. A., Pijnenborg, A. C., Schinkel, A. H., van De Vijver, M. J., Scheper, R. J., and Schellens, J. H. (2001) *Cancer Res* **61**, 3458-3464
292. Graf, G. A., Li, W. P., Gerard, R. D., Gelissen, I., White, A., Cohen, J. C., and Hobbs, H. H. (2002) *J Clin Invest* **110**, 659-669
293. Baekkeskov, S., and Kanaani, J. (2009) *Mol Membr Biol* **26**, 42-54
294. Schlesinger, M. J., Magee, A. I., and Schmidt, M. F. (1980) *J Biol Chem* **255**, 10021-10024
295. Hang, H. C., Geutjes, E. J., Grotenbreg, G., Pollington, A. M., Bijlmakers, M. J., and Ploegh, H. L. (2007) *J Am Chem Soc* **129**, 2744-2745
296. Kostiuk, M. A., Corvi, M. M., Keller, B. O., Plummer, G., Prescher, J. A., Hangauer, M. J., Bertozzi, C. R., Rajaiah, G., Falck, J. R., and Berthiaume, L. G. (2008) *FASEB J* **22**, 721-732
297. Hannoush, R. N., and Arenas-Ramirez, N. (2009) *ACS Chem Biol* **4**, 581-587
298. Charron, G., Zhang, M. M., Yount, J. S., Wilson, J., Raghavan, A. S., Shamir, E., and Hang, H. C. (2009) *J Am Chem Soc* **131**, 4967-4975

299. Rostovtsev, V. V., Green, L. G., Fokin, V. V., and Sharpless, K. B. (2002) *Angew Chem Int Ed Engl* **41**, 2596-2599
300. Speers, A. E., and Cravatt, B. F. (2004) *Chem Biol* **11**, 535-546
301. Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B., and Finn, M. G. (2003) *J Am Chem Soc* **125**, 3192-3193
302. Hsu, T. L., Hanson, S. R., Kishikawa, K., Wang, S. K., Sawa, M., and Wong, C. H. (2007) *Proc Natl Acad Sci U S A* **104**, 2614-2619
303. Charron, G., Wilson, J., and Hang, H. C. (2009) *Curr Opin Chem Biol* **13**, 382-391
304. Kaufman, J. F., Krangel, M. S., and Strominger, J. L. (1984) *J Biol Chem* **259**, 7230-7238
305. el-Husseini Ael, D., and Bredt, D. S. (2002) *Nat Rev Neurosci* **3**, 791-802
306. Abrami, L., Kunz, B., Iacovache, I., and van der Goot, F. G. (2008) *Proc Natl Acad Sci U S A* **105**, 5384-5389
307. Cheng, Z. J., Singh, R. D., Marks, D. L., and Pagano, R. E. (2006) *Mol Membr Biol* **23**, 101-110
308. Allen, J. A., Halverson-Tamboli, R. A., and Rasenick, M. M. (2007) *Nat Rev Neurosci* **8**, 128-140
309. Souto, R. P., Vallega, G., Wharton, J., Vinten, J., Tranum-Jensen, J., and Pilch, P. F. (2003) *J Biol Chem* **278**, 18321-18329
310. Liu, L., and Pilch, P. F. (2008) *J Biol Chem* **283**, 4314-4322
311. Sciorra, V. A., and Morris, A. J. (1999) *Mol Biol Cell* **10**, 3863-3876
312. Levental, I., Grzybek, M., and Simons, K. (2010) *Biochemistry* **49**, 6305-6316
313. Gao, X., Gu, H., Li, G., Rye, K. A., and Zhang, D. W. (2011) *Biochim Biophys Acta*, In press
314. Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J. L., Arenzana-Seisdedos, F., and Bachelerie, F. (2001) *J Biol Chem* **276**, 31936-31944
315. Haglund, K., and Dikic, I. (2005) *EMBO J* **24**, 3353-3359

316. Yin, H., Gui, Y., Du, G., Frohman, M. A., and Zheng, X. L. (2010) *J Biol Chem* **285**, 13580-13588

317. Quest, A. F., Leyton, L., and Parraga, M. (2004) *Biochem Cell Biol* **82**, 129-144

318. Hollenstein, K., Dawson, R. J., and Locher, K. P. (2007) *Curr Opin Struct Biol* **17**, 412-418

319. Cserepes, J., Szentpetery, Z., Seres, L., Ozvegy-Laczka, C., Langmann, T., Schmitz, G., Glavinas, H., Klein, I., Homolya, L., Varadi, A., Sarkadi, B., and Elkind, N. B. (2004) *Biochem Biophys Res Commun* **320**, 860-867

320. Oram, J. F. (2000) *Biochim Biophys Acta* **1529**, 321-330

321. Ordovas, J. M. (2000) *Nutr Rev* **58**, 76-79

322. Heimerl, S., Langmann, T., Moehle, C., Mauerer, R., Dean, M., Beil, F. U., von Bergmann, K., and Schmitz, G. (2002) *Hum Mutat* **20**, 151

323. Hubacek, J. A., Berge, K. E., Cohen, J. C., and Hobbs, H. H. (2001) *Hum Mutat* **18**, 359-360

324. Edmondson, A. C., Braund, P. S., Stylianou, I. M., Khera, A. V., Nelson, C. P., Wolfe, M. L., Derohannessian, S. L., Keating, B. J., Qu, L., He, J., Tobin, M. D., Tomaszewski, M., Baumert, J., Klopp, N., Doring, A., Thorand, B., Li, M., Reilly, M. P., Koenig, W., Samani, N. J., and Rader, D. J. (2011) *Circ Cardiovasc Genet* **4**, 145-155

325. Ma, L., Cheng, G. H., Wang, H., Li, L., Gong, Y. Q., and Liu, Q. J. (2010) *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* **27**, 506-511

326. Furuyama, S., Uehara, Y., Zhang, B., Baba, Y., Abe, S., Iwamoto, T., Miura, S., and Saku, K. (2009) *J Atheroscler Thromb* **16**, 194-200

327. Fukata, Y., Bredt, D. S., and Fukata, M. (2006) in *The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology* (Kittler JT, Moss SJ, ed), Chapter 5, CRC Press

328. Fukata, Y., Iwanaga, T., and Fukata, M. (2006) *Methods* **40**, 177-182

329. Fukata, Y., and Fukata, M. (2010) *Nat Rev Neurosci* **11**, 161-175

## Appendices

### Table A1 Primers for site mutagenesis

| | |
|---|---|
| C514A | F: 5'- CCC AGT GGC CTA CGC CAG CAT CGT GTA CTG -3' |
| | R: 5'- CAG TAC ACG ATG CTG GCG TAG GCC ACT GGG -3' |
| C514S | F: 5'- CCC AGT GGC CTA CTC CAG CAT CGT GTA CTG -3' |
| | R: 5'- CAG TAC ACG ATG CTG GAG TAG GCC ACT GGG -3' |
| C514T | F: 5'- CCC AGT GGC CTA CAC CAG CAT CGT GTA CTG -3' |
| | R: 5'- CAG TAC ACG ATG CTG GTG TAG GCC ACT GGG -3' |
| C26A | F: 5'- GAG CCC AAG TCG GTG GCT GTC TCG GTG GAT GAG -3' |
| | R: 5'- CTC ATC CAC CGA GAC AGC CAC CGA CTT GGG CTC -3' |
| C150A | F: 5'- CCC CGG GAC CTG CGC GCC TTC CGG AAG GTG TCC -3' |
| | R: 5'- GGA CAC CTT CCG GAA GGC GCG CAG GTC CCG GGG -3' |
| C156A | F: 5'- TTC CGG AAG GTG TCC GCC TAC ATC ATG CAG GAT -3' |
| | R: 5'- ATC CTG CAT GAT GTA GGC GGA CAC CTT CCG GAA -3' |
| C205A | F: 5'- CTG GGC TTG CTG TCT GCC GCC AAC ACG CGG ACC -3' |
| | R: 5'- GGT CCG CGT GTT GGC GGC AGA CAG CAA GCC CAG -3' |
| C248A | F: 5'- CTG GAC AGC GCC TCC GCC TTC CAG GTG GTC TCG -3' |
| | R: 5'- CGA GAC CAC CTG GAA GGC GGA GGC GCT GTC CAG -3' |
| C267A | F: 5'- CGC TCC ATC ATT GCC ACC ATC CAC CAG -3' |
| | R: 5'- CTG GTG GAT GGT GGC AAT GAT GGA GCG -3' |
| C291A | F: 5'- CTG AGT CAA GGA CAA GCT GTG TAC CGG GGA AAA -3' |
| | R: 5'- TTT TCC CCG GTA CAC AGC TTG TCC TTG ACT CAG -3' |
| C298A | F: 5'- TAC CGG GGA AAA GTC GCC AAT CTT GTG CCA TAT -3' |
| | R: 5'- ATA TGG CAC AAG ATT GGC GAC TTT TCC CCG GTA -3' |
| C311A | F: 5'- GAT TTG GGT CTG AAC GCC CCA ACC TAC CAC AAC -3' |
| | R: 5'- GTT GTG GTA GGT TGG GGC GTT CAG ACC CAA ATC -3' |
| C345A | F: 5'- GTT CGG GAG GGC ATG GCT GAC TCA GAC CAC AAG -3' |
| | R: 5'- CTT GTG GTC TGA GTC AGC CAT GCC CTC CCG AAC -3' |

| | |
|---|---|
| C390A | F: 5'- CGT CCA TGG AAG GCG CCC ACA GCT TCT CTG C -3'<br>R: 5'- GCA GAG AAG CTG TGG GCG CCT TCC ATG GAC G -3' |
| C397A | F: 5'- AGC TTC TCT GCC AGC GCC CTC ACG CAG TTC -3'<br>R: 5'- GAA CTG CGT GAG GGC GCT GGC AGA GAA GCT -3' |
| C402A | F: 5'- CTC ACG CAG TTC GCC ATC CTC TTC AAG AGG -3'<br>R: 5'- CCT CTT GAA GAG GAT GGC GAA CTG CGT GAG -3' |

**Table A2 Buffers**

| | |
|---|---|
| 2× SDS loading buffer | 62 mM Tris-HCl, pH 6.8<br>2% SDS<br>50% glycerol<br>0.005% bromophenol |
| 35% (w/v) sucrose | 35 g sucrose/100ml<br>20 mM Tris-HCl, pH 7.4<br>0.5 mM EDTA |
| Azido-biotin | 2 mM stock, dissolve in 0.1M sodium phosphate buffer (pH 7.4), store at -80°C |
| $CuSO_4$ | 50 mM stock, at room temperature |
| KOH (for palmitoylation) | 120 mM, at room temperature |
| Lysis buffer A | 0.1 N NaOH<br>0.01% SDS |
| Lysis buffer B | 1% triton<br>150 mM NaCl<br>50 mM Tris-HCl, pH 7.4 |
| Lysis buffer D | 0.1% SDS<br>150 mM NaCl<br>50 mM Hepes, pH 7.4<br>2 mM $MgCl_2$ |
| MBS buffer | 150 mM NaCl<br>50 mM Hepes, pH 7.4 |
| Membrane buffer | 250 mM sucrose<br>50 mM Tris-HCl, pH 7.4 |
| 0.1 N KOH<br>(for membrane treatment) | 1 N KOH in $H_2O$:methanol=1:9 (v/v) |
| 0.1 N Tris-HCl buffer<br>(for membrane treatment) | 1 N Tris-HCl, pH 6.8:methanol=1:9 (v/v), pH 7.0 |

| | |
|---|---|
| PBST | 8 g NaCl<br>0.2 g KCl<br>1.44 g $Na_2HPO4$<br>0.24 g $KH_2PO$<br>1 ml Tween-20<br>In 100 ml, pH 7.4 |
| Phosphate buffered saline (PBS) | 8 g NaCl<br>0.2 g KCl<br>1.44 g $Na_2HPO4$<br>0.24 g $KH_2PO$<br>In 100 ml, pH 7.4 |
| TBTA tris-(benzyltriazolylmethyl)amine | 2 mM stock, dissolve in tert-butanol: $H_2O$ (4:1 v/v), store at -80°C |
| TCEP | 50 mM stock, keep at room temperature |
| ω-Alkynyl-palmitate | 100 mM stock in DMSO, store at -80°C |

* All the buffers are made in MilliQ $ddH_2O$, unless otherwise indicated.