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Structural studies of apolipoprotein A-I and ATP-binding cassette A1 and their roles in nascent high density lipoprotein biogenesis

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STRUCTURAL STUDIES OF APOLIPOPROTEIN A-I AND ATP-BINDING
CASSETTE A1 AND THEIR ROLES IN NASCENT HIGH DENSITY LIPOPROTEIN BIOGENESIS

by

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DEDICATION

This dissertation is dedicated to my father Dr. Shuye Liu,

and my mother Ms. Lihua Zhang.
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STRUCTURAL STUDIES OF APOLIPOPROTEIN A-I AND ATP-BINDING CASSETTE A1 AND THEIR ROLES IN NASCENT HIGH DENSITY LIPOPROTEIN BIOGENESIS

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ABSTRACT

Apolipoprotein A-I (apoA-I) and ATP-Binding Cassette A1 (ABCA1) transporter play important roles in nascent high density lipoprotein (nHDL) biogenesis – the first step in the reverse cholesterol transport pathway. Based on the crystal structure of a C-terminally truncated form of apoA-I (apoA-I(1-184)) determined in the laboratory, structurally designed and naturally occurring mutants of apoA-I were conformationally characterized in solution. The function of these mutants in nHDL formation was assessed in ABCA1-transfected HEK293 cells. An apoA-I mutant designed to destabilize the N-terminal helical bundle at the first hinge region, 38/40G, exhibited a locally reduced \(\alpha\)-helical content, destabilized overall structure, and increased lipid binding ability in solution, indicating a destabilized N-terminal helical bundle. In the cellular system, 38/40G showed significantly enhanced nHDL forming ability, suggesting that a destabilized N-terminal bundle will facilitate nHDL formation. Other designed N-terminal mutants (Q41A, P66G, G65A, V67P, T68P, 65/67/68P) and the naturally occurring mutants (R153P, L178P, and insertion mutant apoA-I_Nashua) all showed either unchanged or destabilized overall structure, unchanged lipid binding abilities in solution.
and unchanged nHDL formation and cholesterol efflux promotion from the cells. Mutants designed to progressively extend the C-terminus (1-184, 1-198, 1-209, 1-220, 1-231) yielded progressively increased nHDL formation and cholesterol efflux, suggesting that the C-terminus of apoA-I is critical for these two activities. Central Helix 5 triple glycine mutation (H5 3xG) designed to lock the monomer conformation of apoA-I resulted in reduced nHDL formation but unaffected cholesterol efflux, suggesting that hindering apoA-I monomer to dimer conversion could retard nHDL formation. Remarkably, studies of cholesterol efflux and nHDL particle formation indicated that the two processes might be two uncoupled events. Analysis of the nHDL particles revealed the presence of ganglioside (GM1) in the complexes.

Cross-linking data demonstrated binding of apoA-I to ABCA1-expressing cells. The binding level of apoA-I mutants to ABCA1-expressing cells was positively correlated with nHDL forming ability of these mutants.

ABCA1 was isolated from FreeStyle™ HEK293-F cells in suspension by detergent solubilization and was shown to have ATPase activity. A direct interaction between apoA-I and amphipol solubilized- ABCA1 in solution was detected for the first time. Furthermore, the successful purification of ABCA1 has laid the foundation of structure determination of this protein in the future.
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LIST OF ABBREVIATIONS

AA: amino acid

ABCA1: ATP-Binding Cassette A1

ApoA-I: apolipoprotein A-I

ACAT: acetyl-coenzyme A acetyltransferase

BODIPY: boron-dipyrromethene

CAD: coronary artery disease

CD: circular dichroism

CE: cholesteryl ester

CETP: cholesterol ester transfer protein

CM: chylomicron

CMC: critical micelle concentration

CTB: cholera toxin B

DDM: n-Dodecyl-β-D-Maltoside

DMEM: Dulbecco's modified Eagle's medium

DMPC: dimyristoyl phosphatidylcholine

DMSO: dimethyl sulfoxide

ECD: extracellular domain

EM: electron microscopy

FA: free fatty acid

FC: free cholesterol

GM1: monosialotetrahexosylganglioside
HDL: high density lipoprotein
IDL: intermediate density lipoprotein
LCAT: lecithin cholesterol acyl-transferase
LDL: low density lipoprotein
LPL: lipoprotein lipase
MTP: microsomal triglyceride transfer protein
NBD: nucleotide binding domain
nHDL: nascent HDL
PA: phosphatidic acid
PBS: phosphate-buffered saline
PC: phosphatidylcholine
PDB: protein data bank
PE: phosphatidylethanolamine
PL: phospholipid
PS: phosphatidylserine
RCT: reverse cholesterol transport
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC: size exclusion chromatography
SM: sphingomyelin
SR-BI: scavenger receptor class B type I
TBST: Tris-buffered saline, 0.1% Tween 20
TG: triglyceride, triacylglycerols
TLC: thin layer chromatography
TMD: transmembrane domain
TMH: transmembrane helix
VLDL: very low density lipoprotein
WT: wild type
CHAPTER 1 Introduction

1.1 Lipoproteins

Lipids are an essential part of the human body. However, lipids, particularly triacylglycerols (TG) and cholesterol, are insoluble in the aqueous circulation and thus must be transported in the form of macromolecular assemblies called lipoproteins. Lipoproteins emulsify the hydrophobic lipids and also function as regulators that transfer specific lipids from or to target tissues.

Lipoproteins are lipid and protein assemblies that are held together by non-covalent forces (Atkinson & D. M. Small 1986; Miller & D. M. Small 1983). Lipids include TG, cholesterol, cholesteryl esters (CE), phospholipids (PL), and the proteins include apolipoprotein A to E. Among these apolipoproteins, only apoBs are the non-exchangeable apolipoproteins and the rest, apoAs, apoCs, apoDs and apoEs, are all exchangeable apolipoproteins. ApoAs can be subdivided into apoA-I, apoA-II, apoA-IV, apo-AV (Swaney et al. 1974; Fidge & McCullagh 1981; Beckstead et al. 2005), apoBs into apoB100 (Cladaras et al. 1986) and apoB48 (S. H. Chen et al. 1987), apoCs into apoC-I, C-II, C-III, C-IV (Allan & Taylor 1996), apoEs into apoE-2, apoE-3, apoE-4 (Weisgraber et al. 1981).

Based on their sizes and densities, lipoproteins can be classified into five groups (Figure 1-1): chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL), and high density lipoproteins (HDL) (D. W. Small 1992).
Diseases of lipid metabolism, including cardiovascular disease, remain the leading cause of health problems in Western society (Murray & Lopez 1997). Atherosclerosis is a coronary artery disease (CAD) in which plaque composed of mostly fat and cholesterol builds up in the arterial walls. Since lipoproteins play an essential role in lipid transport and metabolism, lipoprotein abnormalities are a key reason for the development of atherosclerosis (Kane & Havel 2001; Olofsson & Boren 2005; Lewis & Rader 2005).

1.2 Lipoprotein metabolism

A diagram of lipoprotein metabolism pathways is depicted in Figure 1-2. In these pathways, the liver and the intestine are the most important organs that regulate the delivery or removal of lipids to or from the peripheral tissues.
Figure 1-2 Lipoprotein metabolism pathways. Adapted from (Lusis & Pajukanta 2008).

1.2.1 Chylomicron metabolism

Dietary TG are hydrolyzed into monoacylglycerols and free fatty acids by pancreatic lipase and emulsified by bile salts in the intestinal lumen. These products, in the form of bile salt micelles, are then absorbed by enterocytes and resynthesized into TG. Dietary cholesterol is also esterified into cholesteryl esters (CE) in the enterocytes. Enterocytes synthesize apolipoprotein B48 (apoB48) and microsomal triglyceride transfer protein (MTP). With the help of MTP, apoB48 packs TG, CE and PL into chylomicrons (CM)
particles (90-1000 nm) (Atzel & Wetterau 1993). The density of CM is very low (< 0.93 g/ml). CM are first secreted to the lymph and then the systemic circulation. CM can acquire other exchangeable apolipoproteins such as apoCs and apoEs in the circulation. Lipoprotein lipase (LPL), with the help of its co-factor apoC-II on CM, hydrolyzes TG into free fatty acids (FAs) and glycerol for adipose tissue uptake, and converts CM into the cholesterol-rich and TG-poor CM remnants, which are about 40-100 nm in diameter. The released FAs are picked up by adjacent tissues such as the adipocytes. There, FAs either undergo β-oxidation to produce energy, or get re-esterified and stored as TG. The CM becomes higher in surface tension as it loses TG in the core and shrinks, leading to the transfer of PL and exchangeable apolipoproteins to HDL. CM remnants are then recognized by the CM remnant receptor in the liver, whose ligand is apoE (Herz et al. 1988; Krieger & Herz 1994).

1.2.2 VLDL metabolism

VLDL is initially assembled in the liver with components apoB100, apoE, CE, PL and vitamin E. MTP is also required for the assembly of VLDL (Wetterau et al. 1992). After secretion into the circulation, VLDL acquires apoCs from HDL. VLDL is very similar in composition to CM, also high in TG content. In VLDL the TG comes from de novo synthesis in the liver, rather than from dietary fat; and the cholesterol content is also higher than CM. The protein component on VLDL is apoB100 (synthesized in the liver) rather than apoB48 (synthesized in the intestine) on CM. The density of VLDL is < 1.006
g/ml. Similar to CM, TG in VLDL is hydrolyzed by LPL with apoC-II as its co-factor. As VLDL loses TG in the core, it shrinks to IDL which undergoes further lipolysis to become the cholesterol-rich LDL. In the meantime, as the surface tension increases, PL and exchangeable apolipoproteins are pushed off of the surface of LDL. Therefore, LDL only contains the non-exchangeable apolipoprotein apoB100 as its protein component. LDL is then recognized by LDL receptor on peripheral cells and on the liver.

1.2.3 LDL metabolism

LDL is the major cholesterol carrier in the plasma and regulates de novo synthesis of cholesterol. The density of LDL ranges from 1.019 to 1.063 g/ml. It contains a hydrophobic core of about 1500 molecules of CE. The surface of LDL consists of a PL monolayer, a small amount of cholesterol, and one apoB100 molecule. The function of LDL is to deliver cholesterol to peripheral tissues. This function is exerted through LDL Receptor (LDLR) – mediated endocytosis, which was first revealed by Brown and Goldstein in 1977 (Anderson et al. 1977). LDLR-mediated endocytosis can be divided into 4 steps shown in Figure 1-3:

1. Binding: LDLR are clustered in a specific region called clathrin-coated pits on both peripheral and liver cell membranes. ApoB100 on LDL binds to the cysteine-rich ligand binding region of LDLR.
2. Internalization: The LDLR-LDL complex is then internalized by endocytosis: clathrin-coated pits pinch off a membrane invagination from the plasma membrane to make endocytic vesicles.

3. Lysosomal hydrolysis: Once internalized, these LDL-containing vesicles fuse with the lysosomes. In the lysosome, LDL dissociates from LDLR because of low pH. The protein component of LDL is degraded into free amino acids and CE is hydrolyzed into cholesterol and FAs. The LDLR is recycled back to the plasma membrane.

4. Regulation: Released free cholesterol can be used directly for membrane biosynthesis, or be re-esterified into CE and be stored in the cell.

Figure 1-3 The LDL receptor pathway. Adapted from (Goldstein & Brown 2009)
Cellular cholesterol homeostasis is achieved by checking the balance between endogenous cholesterol synthesis and cholesterol uptake through LDLR. LDLR synthesis and cholesterol synthesis are both subject to feedback regulation by the Sterol Regulatory Element-Binding Proteins (SREBPs) pathway. When cholesterol level is low, SREBP can reach the nucleus and turn on LDLR and HMG CoA Reductase encoding genes. As a consequence, both cholesterol uptake through LDLR and cholesterol biosynthesis are up-regulated. When the cholesterol level is high, the SREBP pathway is blocked, and LDLR and HMG CoA Reductase synthesis shut down to stop further elevating the cellular cholesterol level.

It is now known that other than the SREBP pathway, LDLR is also regulated by Inducible Degrader of the LDLR (IDOL) (Zelcer et al. 2009) and Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) (Hall et al. 2013).

The Framingham Heart Study has revealed that plasma LDL level is positively correlated with the risk of CAD (Kannel et al. 1987). Therefore, LDL is also called “the bad cholesterol”.

### 1.2.4 HDL metabolism

High Density Lipoproteins (HDL) are a heterogeneous population of particles consisting of PL, cholesterol, CE, TG, and various exchangeable apolipoproteins, the major ones being apoA-I and A-II and the minor ones being apoA-IV, V, apoCs, apoD and apoE. The density range of HDL is between 1.063 to 1.21 g/ml.
Using 2D gel electrophoresis, HDL can be subclassified into \( \alpha-1 \) to \( \alpha-4 \) HDL, pre-\( \alpha \) HDL, and pre-\( \beta \) HDL. Among these, \( \alpha-2 \) and \( \alpha-3 \) contain both apoA-I and apoA-II, while \( \alpha-1 \), \( \alpha-4 \), pre-\( \alpha \) and pre-\( \beta \) contain only apoA-I. Using ultracentrifugation, HDL can be divided into HDL\(_2\) and HDL\(_3\) by density. HDL\(_2\) are larger and richer in CE- than HDL\(_3\). Although total HDL cholesterol (HDL-C) is routinely tested in clinical laboratories to predict CAD risk and is called “the good cholesterol”, evidence suggests that it is certain HDL subcategories that are atheroprotective. For example, both the Framingham Study and the Veterans Affairs’ HDL Intervention Trial show that \( \alpha-1 \) and \( \alpha-2 \) HDL are better markers for predicting CAD risks than total HDL-C is (Asztalos et al. 2004; Asztalos et al. 2005). Also, HDL\(_2\) have been shown to be more atheroprotective than HDL\(_3\) (Patsch et al. 1983).

1.2.4.1 Reverse Cholesterol Transport (RCT)

The major function and beneficial property of HDL lies in its role in the Reverse Cholesterol Transport (RCT) pathway (Tall 1998) (Figure 1-4), whereby excess cholesterol in peripheral tissues is moved back to the liver. During RCT, the liver synthesizes lipid-free apoA-I and secretes it into the circulation. Once apoA-I gets to peripheral cells (especially macrophages), it interacts with ATP Binding Cassette A1 (ABCA1) on the cell membrane and recruits cholesterol and PL transported by ABCA1 to form discoidal nascent HDL (nHDL) particles (Ji-Young Lee & Parks 2005), which can further take up cholesterol and PL through ABCG1 (DiBlasio-Smith et al. n.d.). Cholesterol on nHDL is then esterified into CE by lecithin cholesterol acyl-transferase (LCAT). CE is sequestered into the core of nHDL and nHDL first becomes small
spherical HDL$_3$ and then larger mature HDL$_2$. ApoA-I on larger HDLs is then recognized by Scavenger Receptor B1 (SR-B1) on hepatocytes and the CE is selectively taken up by the cell; cholesterol and CE are used to make bile salts in the liver. Bile salts and cholesterol are then secreted into the bile together with phospholipid.

![Diagram of HDL and Reverse Cholesterol Transport (RCT) pathway](image)

Figure 1-4 HDL and Reverse Cholesterol Transport (RCT) pathway. Adapted from speech by David Atkinson, “Crystal Structure of Apo A-1 and Models of HDL Formation” at the Arteriosclerosis, Thrombosis and Vascular Biology Scientific Sessions, 2014, Toronto, Canada.

Other than the direct removal of CE by SR-B1, there is an indirect pathway whereby HDL$_2$ and HDL$_3$ exchange CE for TG from CM and VLDL by Cholesterol Ester Transfer Protein (CETP) (Morton & Greene 1997), and CE is later cleared from the circulation.
through LDLR-mediated endocytosis. During contact with CM and VLDL, HDL₂ and HDL₃ can also recruit other exchangeable apolipoproteins such as apoCs, apoD and apoEs from them.

1.3 Apolipoprotein A-I (apoA-I)

1.3.1 Structure of apoA-I

1.3.1.1 Primary and secondary structure

In 1974, Segrest et al proposed ten tandem 11/22mer helices separated by proline residues in the exon-4 encoded region of apoA-I: H1 (44-65), H2 (66-87), H3 (88-98), H4 (99-120), H5 (121-142), H6 (143-164), H7 (165-187), H8 (188-208), H9 (209-219), H10 (220-243) (Segrest et al. 1974). Later, they classified these amphipathic alpha-helices into different types based on charge distribution (Segrest et al. 1992); class A, class G* and class Y helix (Figure 1-5). The class A helix is characterized by positively-charged residues that are localized at the interface of hydrophilic and hydrophobic faces and negatively-charged residues localized at the center of the polar face. The class A helix has the highest lipid binding affinity and is the major lipid binding motif in apoA-I. The N-terminal Exon 3-encoded region (1-44) of apoA-I contains a class G* helix, which is characterized by a random distribution of positively- and negatively-charged residues at the polar face. The class Y helix is characterized by a “Y” shape cluster of positively-charged residues at the polar face.
In 1992, Nolte and Atkinson determined that apoA-I, along with apoA-IV and apoE-3, consists of regions with variable homology to the consensus sequence unit A (PLAELRARLR) and consensus sequence unit B (AQLEELRERLG). AB together adopts a typical class A helix arrangement (Nolte & Atkinson 1992)(Figure 1-6).
Thus, the 10 helices could be classified into A or B consensus sequence units (for 11-mer helices) or combination of A and B (for 22-mer helices). The central region of apoA-I consists of five tandem AB repeats (Figure 1-8). Nolte and Atkinson also proposed secondary structures in different domains of lipid-free apoA-I using circular dichroism data: The N-terminal region (1-57) has the most ambiguous structure that is composed mostly of random coil or β-sheet and a short alpha-helix (9-13). The C-terminal region (185-243) was assigned as helices in 187-223 and 231-243; residues 224-230 were predicted to be very hydrophobic and exist as random coil. The central region, as mentioned above, was composed mostly of class A helices made by AB repeats (Figure 1-7).
In 2009, Phillips et al probed the secondary structure of plasma apoA-I using hydrogen-deuterium exchange experiments and suggested that residues 116-146 (which spans the whole H5 region) and 179-243 (which covers the whole C-terminal region) are random coils (Chetty et al. 2009).
1.3.1.2 Tertiary and 3D structure

The folding and function of domains in apoA-I have been probed by extensive mutation studies. Studies of the N-terminal truncated apoA-I Δ(1-43) and the C-terminal truncated apoA-I Δ(187-243) showed that Δ(187-243) has a similar folding to that of WT, while Δ(1-43) exhibits a drastically different conformation (Rogers et al. 1998), suggesting that the N-terminus and the C-terminus belong to different folding domains and the N-terminus is critical for stabilization of the overall folding of WT apoA-I in solution. The C-terminus was later shown to be responsible for self-association and phospholipid binding (Beckstead et al. 2005; Ji & Jonas 1995; Laccotripe et al. 1997). Point mutations, deletion mutations and peptide analysis are summarized in Figure 1-9. These studies have confirmed the two-domain model and demonstrated that the central H5 (121-143) region may act as a hinge region that connects the N-terminal and the C-terminal domain and
pivot the “folded-back” and the “extended” conformation (Gorshkova et al. 2000; Gorshkova et al. 2002; Gorshkova et al. 2006).

In 2003, Phillips et al examined the tertiary structures of the N- and C-terminal folding domains with a series of deletion mutations (Saito et al. 2003). By using CD, tryptophan and ANS fluorescence, they suggested that the N-terminal domain (1-189) forms a helical...
bundle, whereas the C-terminal domain has a poorly-defined structure, just as the arrangement in apoE. They also suggested that in the presence of lipid, it is the C-terminus that initiates lipid binding after which the N-terminal helical bundle opens and wraps around the lipid surface (Figure 1-10).

The first crystal structure of apoA-I was solved at 4 Å resolution by Borhani et al in 1997. In this crystal structure, the N-terminal residues 1-43, were truncated. As shown in Figure 1-11, two molecules of apoA-I Δ(1-43) are aligned antiparallel to form a dimer. Forces stabilizing the dimer include both salt bridges and hydrophobic interactions. Two dimers are aligned antiparallel again at their hydrophobic faces to form a tetramer. The overall structure is “horseshoe” shaped four-helix-bundle ring. The horseshoe shape comes from proline kinks at the junctions between helices, and this shape positions the N-terminus only 23 Å apart from the C-terminus within the same molecule. The overall alpha-helical content is over 90%, and the structure is believed to be in a lipid-bound conformation, despite being in a lipid-free environment. This might be caused by the high salt concentration (1.2 M sodium citrate, 4 °C) that the crystal was grown in. In a highly
hydrophilic environment, the hydrophobic interactions between monomers were enhanced, thus pushing the monomers to adopt a lipid-bound conformation. However, the low resolution (4 Å), poor quality of diffraction data and final model (R=37%) hinder a deeper understanding of the molecular details of apoA-I.

In 2011, Mei and Atkinson reported the high-resolution (2.2 Å) structure of the C-terminally truncated apoA-I Δ(185-243) (Mei & Atkinson 2011). The crystal structure was solved showing a dimer in a half-circle shape (Figure 1-12). The backbone of the dimer is two elongated antiparallel amphipathic helices. The N-terminal H1-H4 of one molecule forms a four-helix bundle with the C-terminal H6/H7 of the symmetric molecule. The N-terminal helical bundle is stabilized mainly by hydrophobic interactions from two aromatic clusters: W8, F71, W72 at one end of the bundle, and F33, F104, W108 at the other end of the bundle, and by π-cation interactions between W8-R61 and K23-W50. The backbone is stabilized by salt bridges. This crystal structure also showed a H5/H5 registration, and this central H5 had a high temperature factor, suggesting a hinging function of this region. A “folded-back” conformation in the central H5 may render a monomeric apoA-I, with the substitution of the C-terminal H6/H7 of the symmetric molecule with H6/H7 of the same molecule (Figure 1-13).
1.3.1.3 Structure of apoA-I on discoidal HDL

In 1977, Tall et al showed that nascent HDL and reconstituted HDL are discoidal (Tall et al. 1977). Based on this finding, several models of apoA-I structure on discoidal HDL have been proposed. The first was the “picket fence” model by Tall et al shown in Figure 1-14A. In this model, amphipathic alpha-helices of apoA-I are arrayed parallel to the PL
acyl chains in an alternating antiparallel manner. The idea came from the fact that the height of the 22-mer alpha-helices (~33 Å) is similar to the height of the lipid bilayer (~27-32 Å). But this similarity in height might just be a coincidence because mounting evidence has shown that apoA-I exists as extended alpha-helices on discoidal HDL.

Later, in 1999, inspired by the crystal structure of the apoA-I Δ(1-43), Segrest et al proposed the “double belt” model (Segrest et al. 1999). In this model, two molecules of extended apoA-I are aligned antiparallel and perpendicular to the PL acyl chain. Molecular dynamics simulation data suggested that the H5/H5 registration has the same curvature and orientation as shown in the apoA-I Δ(1-43) crystal structure. The “double belt” model has been supported by studies using other methods such as FTIR (Koppaka et al. 1999), FRET (Koppaka et al. 1999) and nitroxide spin labeling (Maiorano & Davidson 2000).

The “hairpin” model shown in Figure 1-14C was originally proposed by Tricerri et al who showed that the N-terminus and the C-terminus were found to be proximal using the fluorescence resonance energy transfer (FRET) method (Tricerri et al. 2000) and then supported by Maiorano et al using nitroxide spin labels (Maiorano et al. 2004).

Figure 1-14 Models of apoA-I on discoidal HDL. A. picket fence, B. double belt (B), C. hairpin. Adapted from (Martin et al. 2006)
Since apoA-I can form nHDL particles of different sizes, it is assumed that apoA-I adopts different conformations on nHDL of different sizes. Phillips et al have proposed models that will accommodate this variation in conformation (Sevugan Chetty et al. 2012). Using POPC rHDL particles of 7.8 nm and 9.6 nm, hydrogen-deuterium exchange (HX) and mass spectrometry (MS) methods, they suggested the model depicted in Figure 1-15. The overall structure adopts a double-belt conformation with a H5/H5 registration, agreeing with both crystal structures. In larger particles (9.6 nm), the region encompassing residues 125-158 could either form an amphipathic helix that directly interacts with lipid acyl chains, or exist as a disordered loop dangling in the aqueous surrounding. On the opposite side, 6 or 7 residues at the N-terminal and C-terminal ends are also in a loop state. In the smaller rHDL (7.8 nm), more residues are in loops: in addition to 125-158 (115-158 in this case), residues 130-174, 133-146 also form flexible loops. On the opposite side, loops are found between residues 212-225 and 45-57. These loops are in agreement with mutation- analysis of lipid-free apoA-I conducted by Aktinson et al in 2006 (Gorshkova et al. 2006). Apparently, segments that favor a loop structure in aqueous solution also tend to form loops on HDL particles. Notably, the structure is quite dynamic, rather than static. The alpha-helices unfold and refold in the time frame of a few seconds. Particle sizes larger than 10 nm diameter are predicted to contain more than two apoA-I molecules.
1.3.2 *Functions of ApoA-I*

The functions of apoA-I include:

1. lipid binding and HDL stabilization (Jonas et al. 1986)
2. lipid acceptor of ABCA1 (Fielding et al. 1972)
3. LCAT activation (Steyrer & Kostner 1988)
4. SR-BI binding (Acton et al. 1996)

These functions are likely to be regulated by different domains or segments of apoA-I.
It is well established that the N-terminal domain (1-44) of apoA-I has a stabilizing effect on lipid-free apoA-I (Beckstead et al. 2005; Ji & Jonas 1995) and that R149, R153, R160 in H6 and E110, E111 in H6 are responsible for LCAT activation (Sorci-Thomas et al. 1998; Roosbeek et al. 2001; Chroni et al. 2004). The C-terminal domain has the highest lipid binding affinity and thus may initiate lipid binding (Saito et al. 2003). The C-terminal domain is also thought to be involved in cholesterol efflux promotion mediated by ABCA1 (Chroni et al. 2004). A summary of putative domain functions of apoA-I is presented in Figure 1-16.

![Figure 1-16 Summary of apoA-I domain functions (Mei & Atkinson 2015).](image)

Scavenger receptor class B type I (SR-BI) is a 509 aa integral membrane protein that resides on liver cells and steroidogenic cells. In 1996, Krieger et al identified SR-BI as an HDL receptor that mediates CE uptake selectively (Acton et al. 1996). Interestingly, SR-BI can work in the opposite direction of CE uptake and mediate free cholesterol (FC) efflux to HDL down a concentration gradient (Ji et al. 1997). SR-BI consists of N- and C-terminal cytoplasmic domains, two transmembrane helices and a large glycosylated extracellular domain which has 6 cysteine residues. Recently, the structure of the
extracellular domain of a homologous protein of SR-BI, LIMP-2, was determined by X-ray crystallography (Figure 1-17) (Neculai et al. 2013). The overall structure showed an antiparallel β-sheet core with short alpha-helices. Two disulfide bonds (C274-C329 and C312-C318) hold the protein fold, which is believed to be the case in SR-BI as well (C274-C329 and C312-C318) (Papale et al. 2011). The helical bundle at the apex of the extracellular domain is the ligand binding site. For the transmembrane domains of SR-BI, Sahoo and others (Reaven et al. 2004; Sahoo et al. 2007) have shown that there is a GXXXG motif and a putative leucine zipper motif at the C-terminal transmembrane domain that are responsible for receptor oligomerization, a phenomenon which also occurs in ABCA1. The oligomerization of SR-BI was shown to be essential for HDL-CE uptake (Reaven et al. 2004). Because of their shared function in efflux promotion, the structure of SR-BI homologous protein LIMP-2 and motifs on SR-BI C-terminal transmembrane domain may provide some insights on ABCA1 structure.
However, unlike the well-understood LCAT activation, the interactions between apoA-I and SR-BI are much less clear. Various lipid-bound apolipoproteins, including apoA-I, apoA-II, apoE and apoC-III have been shown to interact with SR-BI. When complexed with 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), even a class A amphipathic alpha-helix could bind to SR-BI with high affinity (Williams et al. 2000). These results suggested that the recognition motif by SR-BI is class A alpha-helices, which is a common feature in all of the exchangeable apolipoproteins, explaining the lack of specificity of SR-BI substrate recognition.
1.4 Other apolipoproteins on HDL

1.4.1 ApoA-II

ApoA-II is the second major protein (next to apoA-I) on HDL. It forms homodimers (17.4 KDa) via one disulfide bond between residue Cys6 on each monomer. CD measurements suggest 70% alpha-helical structure in lipid-bound apoA-II (Benetollo et al. 1996). ApoA-II, like other exchangeable apolipoproteins, is also built by 11-mer amphipathic helical repeats, mostly class A helices (Segrest et al. 1992). Although most HDL particles contain apoA-I, there is a minor population of HDL that contain only apoA-II (Blanco-Vaca et al. 2001).

The function of apoA-II has been quite mysterious since its discovery. Multiple functions have been proposed, but since most of the functions are either supplemental to other major apolipoproteins, or have contradicting consequences, it is hard to attribute clear-cut assignments to apoA-II. For example, proposed functions of apoA-II include: remodeling HDL particles (Boucher et al. 2004), inhibiting CETP (Lagrost et al. 1994), activating LCAT (Jonas et al. 1984), stimulating or inhibiting hepatic lipase (HL) (Escolà-Gil et al. 1998; Hime et al. 2001), etc. However, given that apoA-I and other apolipoproteins can also carry out these functions, what exactly is the role of apoA-II remains hard to evaluate.
1.4.2 ApoC

ApoCs, including apoC-I, apoC-II and apoC-III, were first isolated from VLDL by Gustafson et al in 1966 (Gustafson et al. 1966). Other than on VLDL, apoCs are also protein constituents on CM and HDL. ApoCs are generally involved in the regulation of TG-rich lipoproteins, therefore mutations in apoCs are related to hypertriglyceridemia. ApoC-I can activate LCAT, at about 78% efficiency of apoA-I (Steyrer & Kostner 1988). ApoC-II is the activator of lipoprotein lipase (LPL), which hydrolyzes TG into FAs and glycerol. ApoC-III is the most abundant isoform in the apoC family and has been shown to inhibit lipolysis of TG in CM and VLDL by interfering with the interaction between the lipoprotein and the heparan sulfate proteoglycan (HSPG)/LPL complex (Jong & Havekes 2000).

1.4.3 ApoE

ApoE is a 299-amino acid protein (34 KDa) present on almost all types of lipoprotein particles: CM, CM remnants, VLDL, IDL and HDL, except for LDL which only contains apoB100. It has three isoforms apoE2, apoE3, and apoE4, with apoE3 being the most abundant, “parent” isoform. ApoE3 has Arg at position 112 and Cys at position 158; apoE2 has both Cys at position 112 and 158; and apoE4 has both Arg at position 112 and 158 (Weisgraber et al. 1981). ApoE3 is the ligand for LDLR (binding to residues 139-146 of apoE3) and LDLR related proteins.
ApoE consists of two structurally distinct domains with potentially different functions (Wetterau et al. 1988). The crystal structure of the N-terminal domain shows a four-helix bundle (Wilson et al. 1991), which contains the LDLR binding region. It is well established that apoE has the two-domain arrangement, with the N-terminus being a helical bundle and the C-terminus folded separately (Saito et al. 2003) (structure of the C-terminus of apoA-I is poorly defined (Mei et al. 2016)). Therefore, the NMR structure of the full-length apoE3 monomer may provide some structural insights into the full length apoA-I monomer and into the possible mechanism of apoA-I conformational change from the lipid-free to lipid-bound state. Consistent with the crystal structure of the N-terminal domain alone, the NMR structure also showed a four-helix bundle at the N-terminus (J. Chen et al. 2011). Additionally, the C-terminal domain (three helices) and the segment between N- and C-terminal domain (the hinge domain) forms helical structures as well, making the overall structure a six-helix bundle (Figure 1-18; left panel). Moreover, the C-terminal domain of apoE presents a large hydrophobic surface which is supposed to initiate the interaction with lipid. Chen et al proposed that firstly, lipid binding interrupts domain interactions and dissociates the C-terminal domain and the hinge domain from the N-terminal domain. Secondly, the C-terminal domain swings to the top of the N-terminal domain (Figure 1-18; right panel); finally, the N-terminal domain opens. Based on structural and functional similarities to apoE, apoA-I may employ a similar mechanism as well.
1.5 ATP Binding Cassette transporters

ATP Binding Cassette (ABC) transporters belong to the largest protein superfamilies that exist in both prokaryotic and eukaryotic organisms (Holland et al. n.d.). They transport substrates across cellular membranes in an ATP-dependent manner. The substrates and physiological processes associated with ABC transporters are extremely diverse.

Based on their directionality, ABC transporters can be divided into importers which move substrates from trans- to the cis-side (the side where ATP is consumed), and exporters which move substrates in the opposite direction. Whereas ABC exporters are ubiquitously present in all organisms, ABC importers are found exclusively in bacteria. Importers can be further divided into 3 subtypes, type I, type II and type III (ECF importers) (Figure 1-19). Exporters were thought to belong to the same type, but the
recent determination of the crystal structure of ABCG5/G8 added diversity to this family (Jyh-Yeuan Lee et al. 2016) (Figure 1-26). There are a few exceptions to the importer/exporter dichotomy: CFTR (ABCC7), an ATP-gated chloride channel in essence, and SUR1, a potassium channel regulator (Theodoulou & Kerr 2015).

![Figure 1-19 Representation of ABC importers and exporters. Adapted from (Theodoulou & Kerr 2015).](image)

Based on sequence homology, human ABC transporters can be classified into 7 subfamilies: ABCA – ABCG, consisting of ~ 50 proteins and ~ 20 of which are associated with diseases (Rees et al. 2009). Table 1-1 lists some clinically relevant ABC
transporters. (Linton 2007).

<table>
<thead>
<tr>
<th>ABC Protein</th>
<th>Pseudonym</th>
<th>Ligand(s)/Function</th>
<th>Associated Disease(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC1</td>
<td>ABCA1</td>
<td>Cholesterol</td>
<td>Tangier disease</td>
</tr>
<tr>
<td>ABCR</td>
<td>ABCA4</td>
<td>Retinal</td>
<td>Various eye diseases</td>
</tr>
<tr>
<td>TAP1/2</td>
<td>ABCB2/B3</td>
<td>Peptides</td>
<td>Bare lymphocyte syndrome</td>
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<td>ABCB7</td>
<td>Iron</td>
<td>Anemia and XLSA</td>
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<td>MRP6</td>
<td>ABCC6</td>
<td>?</td>
<td>Pseudoxanthoma elasticum</td>
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<td>ABCC1</td>
<td>VLCFA</td>
<td>Adrenoleukodystrophy</td>
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<td>Sterols</td>
<td>Sitosterolemia</td>
</tr>
<tr>
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<td>ABCB4</td>
<td>Phosphatidylcholine</td>
<td>Liver disease: PFIC3, OC</td>
</tr>
<tr>
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<td>ABCB11</td>
<td>Bile acids</td>
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<td>ABCC2</td>
<td>Conjugated bilirubin</td>
<td>Liver disease: D-J syndrome</td>
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<td>ABCB1</td>
<td>Hydrophobic drugs</td>
<td>Failure of chemotherapy</td>
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<td>ABCB2</td>
<td>Hydrophobic drugs</td>
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<td>SUR</td>
<td>ABCC8</td>
<td>Regulation of K&lt;sub&gt;a&lt;/sub&gt; channel</td>
<td>PHHI, persistent hyperinsulinemic hypoglycemia of infancy</td>
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<td>mRNA trafficking</td>
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Table 1-1 Clinically relevant ABC transporters and their functions. Adapted from (Linton 2007)

Figure 1-20 shows the architecture of human ABC transporters. ABCE and ABCF are not involved in substrate transport, instead, they are involved in translational control. All of the ABCA, some of the ABCB, and some of the ABCC family proteins are full transporters in which all the 12 transmembrane helices and two nucleotide binding domains (NBD) are encoded in one polypeptide chain. Some of the ABCB, all of the ABCD and the ABCG proteins are half transporters which contain 6 transmembrane helices and one NBD, but form homodimers or heterodimers with partners within the subfamily. Some of the ABCC family proteins have a unique topology that includes an
additional 5 transmembrane helices at the N-terminus. This arrangement positions the N-terminal end on the extracellular side of the membrane, while all other human ABC proteins have both N- and C-terminal ends on the cytoplasmic side. A characteristic of the ABCG subfamily is that their NBD is located at the N-terminus, before the transmembrane helices, whereas others all have the NBD at the C-terminus, following the transmembrane helices.

Figure 1-20 Architecture of human ABC transporters. Adapted from (Theodoulou & Kerr 2015)

1.5.1 Structure

The structure determination of ABC transporters has always been challenging because of the complicated steps involved in membrane protein overexpression, purification and
crystallization. The first structure of a complete ABC importer (Ecoli vitamin B12 importer BtuCD7) was reported in 2002 (Locher et al. 2002), and the first exporter structure (Staphylococcus aureus multidrug exporter Sav1866) was reported in 2006 (Dawson & Locher 2006). Since then, the crystal structures of a plethora of ABC transporters have been solved, most of which are from prokaryotes, with a few exceptions being the multidrug transporter P-glycoprotein from mouse (Aller et al. 2009) and C. elegans (Jin et al. 2012), and ABCB10, a mitochondrial transporter from human (Shintre et al. 2013). The structure of the ER membrane-resident ABC Transporter associated with antigen processing (TAP) was solved at low resolution (6.5 Å) by cryo-EM (Oldham et al. 2016). The first and only human plasma ABC transporter structure is ABCG5/ABCG8 in 2016 (Jyh-Yeuan Lee et al. 2016).

All ABC transporters share a core 4-domain architecture (Figure 1-21A): two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). In the case of half transporters that only have one TMD and one NBD, homodimers or heterodimers are formed to make a full transporter. However, not all ABC transporters consume two ATPs in a single working cycle. While the NBDs are highly conserved in both sequence and structure, there are extensive variations in both sequence and structure of TMDs, which is to be expected considering the specificity TMDs need to interact with their corresponding substrates (Jones & George 1999). In ABC importers, NBDs and TMDs are separate subunits, whereas in exporters, NBDs and TMDs are typically fused together.
The “Coupling helix” is a short alpha-helix that protrudes from each of the TMDs on the cytoplasmic side and fits into a groove on an NBD. In this way, nucleotide binding, ATP-hydrolysis, or substrate binding induced conformational changes are transduced between TMDs and NBDs. Soluble substrate-binding protein (SBP) is a soluble component on the trans-side that captures hydrophilic substrates and delivers them to the translocation pathway of ABC importers (Beek et al. 2014).

Figure 1-21 Schematic of ABC transporter domain arrangement and functional motifs. Adapted from (Beek et al. 2014). A. Side view; B. Top view; C. Relative positions of functional motifs in sequence. In A and B, red represents coupling helices and green represents ATP molecules. In C, colors codes are designated as illustrated.
1.5.1.1 NBDs

Seven highly conserved motifs have been identified in NBDs (Figure 1-21C) (Beek et al. 2014; Theodoulou & Kerr 2015):

(1) The A loop: an aromatic residue within this region forms π-stacking with the adenine ring of the ATP molecule;

(2) the Walker A motif (or the P loop): the conserved lysine residue within the sequence GXXGXGK(S/T) interacts with the γ-phosphate of ATP;

(3) The Walker B motif: the glutamate residue in conserved sequence øøøøDE (ø represents an aromatic residue) plays a critical role in ATP hydrolysis;

(4) The D loop: in an NBD dimer, one aspartate in the D loop (sequence SALD) cross-communicates with the other aspartate in the other monomer;

(5) The H loop: also called switch region; the conserved histidine residue interacts with aspartate of the Q loop, glutamate of Walker B motif, γ-phosphate of ATP and helps to position the attacking water molecule;

(6) The Q loop: is at the interface of RecA-like domain and α-helical domain. The glutamine in the Q loop allows for the interaction with TMDs;

(7) The ABC signature motif: LSGGQ, which is not present in other ATPases, is the characteristic feature of ABC transporters; it helps to induce the dimerization of two NBDs.
1.5.1.2 TMD fold and Coupling helices

The two TMDs of type I importers are identical (in homodimers) or at least are structurally similar. Each TMD contains 6 TM helices including an N-terminal helix that wraps around the symmetric TMD. The coupling helix is usually between TM H4 and H5. Type I importers usually import small hydrophilic molecules that are in bulk solution, such as ions, sugars, amino acids, etc.

![Topology and structure of type I ABC importers](image)

*Figure 1-22 Topology and structure of type I ABC importers. Adapted from (Locher 2009) and (Hollenstein et al. 2007).*

Type II importers have two identical 10-helices TMDs, making a total of 20 transmembrane helices in a full transporter. The helices do not cross over to the symmetric TMD. The Coupling helix is located between H6 and H7. Type II importers usually import metal chelates, which are generally larger but in smaller quantities than...
the substrates of type I importer, and the size of type II importers themselves are also bigger than type I importers.

Each of the two TMDs of ABC exporters have 6 transmembrane helices. Two helices from one TMD cross over to the other TMD. One notable feature of exporters is that the helices extend well into the cytoplasmic side of the membrane, making the NBDs 25 Å away from the membrane. Unlike importers, exporters usually do not have high substrate specificity; for example, multidrug resistance transporters can translocate a large variety of drugs. And the substrates for exporters are usually hydrophobic, rather than hydrophilic for importers.
The newly-determined structure of ABCG5/G8 revealed a new folding architecture of ABC exporters (Figure 1-26). G5/G8 share only 28% sequence identity, but exhibit high degree of structural similarity. Notably, ABCG subfamily members are unique in that they are the only ABC transporters that have the NBDs located at the N-terminus (as illustrated in Figure 1-20), rather than the C-terminus. Therefore, it is not surprising that the overall packing of the TMDs of G5G8 shows a different arrangement from other already known structures. Unlike most other ABC exporters, the transmembrane helices (TMHs) in G5G8 from each half do not cross over, nor do they extend to the cytoplasmic side of the membrane. Other than the prototypical coupling helices (CpH) between

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Figure 1-24 Topology and structure of type I ABC exporters. Adapted from (Locher 2009) and (Hohl et al. 2012).
TMH2 and TMH3, there is an additional orthogonal alpha-helix (named “connecting helix” (CnH)) that connects the NBD and the TMD in each half.

Figure 1-25 Topology of type II ABC exporters ABCG5/G8. Adapted from (Jyh-Yeuan Lee et al. 2016)

Because the substrate of ABCG5/G8 is sterols (G5/G8 heterodimer mediates neutral sterol – bile salt excretion from the liver into bile duct and from intestine enterocytes into the intestinal lumen), the structure of this transporter may provide some structural and mechanistic implications for ABCA1, whose substrates also include sterols (cholesterol).
The crystal structure of G5/G8 is the first human plasma membrane ABC transporter structure. It was solved in a lipid bilayer in a nucleotide-free, inward-facing state. In addition to the unique architecture discussed earlier, there are some important features of this sterol transporter (Figure 1-26): The two halves dimerize through the NPXDF motifs, which are conserved in ABCG subfamily at the C-terminus. The extracellular domain (ECDs) between TMH5 and TMH6 forms an alpha-helical structure that acts as a ceiling of the vestibule formed by TMH1-2 and TMH4-6 from each TMD. This alpha-helical ceiling is predicted to be important for sterol exit, because 3 mutations leading to sitosterolaemia are located at the apices of TMH2 and TMH5, where the ceiling alpha-helix is in contact. Cholesterol transport is likely to happen upon ATP binding and hydrolysis through a network of conserved polar residues that extend from CnH, CpH to the TMDs (Figure 1-27). This polar relay, the authors proposed, might be more energetically favorable than a buried hydrophobic core when deformation is needed to transfer cholesterol.
The ECDs of G5/G8 (~70 aa), although much smaller than those of ABCA1 (~600 aa and ~300 aa), may have some mechanistic implications on cholesterol exit in ABCA1. However, in G5/G8, the sterols are neutral bile salts, thus can be directly released into the aqueous surrounding; whereas in ABCA1, the sterol is free cholesterol thus has to be solubilized by PL and apoA-I. The large size of ECDs in ABCA1 may come from their additional role of interacting with apoA-I.

### 1.5.2 Mechanism of ABC transporters

ABC transporters move substrate against a concentration gradient and it is important to prevent leakage in the energetically favorable direction. Therefore, most ABC transporters, both importers and exporters, have been proposed to employ the “alternating
access” model (Jardetzky 1966; Locher et al. 2002; Dawson et al. 2007), in which the TMDs of the transporter alternate between outward-facing and inward-facing conformations in which ATP binding and hydrolysis induced conformational changes that alternately expose the substrate binding site to the outside or inside of the membrane. For importers, the outward facing conformation has higher a binding affinity for the substrate than the inward facing conformation and for exporters, it is the other way around.

The general mechanism proposed for ABC exporters works in the following four steps (Zolnergiks et al. 2011):

1. Ligand binding to TMDs brings the two NBDs close enough to bind to ATP
2. ATP binding brings the two NBDs together and the motion is transduced to TMDs through the coupling helices; TMDs adopt an outward-facing conformation and the ligand is released.
3. ATP hydrolysis changes the conformation in TMDs and triggers the dissociation of the two NBDs
4. Release of ADP and Pi reverts the TMDs back to the inward-facing conformation, ready for the next ligand to bind
Figure 1-28 Proposed general mechanism of ABC exporters. Adapted from (Zolnerciks et al. 2011)
However, in 2015, Locher et al reported the structures of PglK, a lipid-linked oligosaccharide flippase captured in different states and proposed a distinct mechanism.

In their model, flipping occurs as such:

1. The substrate, lipid-linked-oligosaccharide (LLO) interacts with PglK via its lipid tail; ATP replaces ADP, TMDs open and expose the binding cavity.
2. The head group of LLO is drawn directly to the outward facing cavity in the TMDs through electrostatic forces.
3. ATP hydrolysis-induced conformational changes in the TMDs push the head group of LLO out of the cavity; TMDs close.
4. LLO lipid tail releases.
5. ADP releases and PglK reverts to the apo, inward-facing conformation.
Table 1-2 Proposed mechanism for the lipid-linked oligosaccharide flippase PglK. Adapted from (Perez et al. 2015)

Because ABCA1 has been shown to be a flippase (Hamon et al. 2000a), and the substrate PL also has a long hydrophobic tail and a hydrophilic head group, this newly-proposed model may shed some light on the mechanism of ABCA1.

1.5.3 ABCA1

1.5.3.1 Structure and activities of ABCA1

In 1961, a new disease of HDL deficiency was identified in Tangier Island, Virginia, thus was named Tangier disease (Fredrickson & Altrocchi 1961). The symptoms included < 5% normal plasma HDL level and premature CAD, enlarged spleen, liver and yellow tonsil (Civeira et al. 1994). ApoA-I had been blamed for this disease until 1999 when three groups identified mutations in the ABCA1 gene from Tangier disease patients (Rust et al. 1999; Brooks-Wilson et al. 1999; Bodzioch et al. 1999).

ABCA1 is a 2261 aa protein with apparent molecular weight of ~250 KD. No 3D structure of ABCA1 or individual domains is available. Sequence analysis has predicted its topology on the membrane as shown in Figure 1-29: it is a full transporter with two TMDs and two NBDs; each TMD has 6 transmembrane helices; there are two large extracellular domains (ECD) between H1 and H2 (~600 aa), H7 and H8 (~300 aa). These two highly glycosylated ECDs are the supposed apoA-I interacting regions, and are believed to be connected via two intramolecular disulfide bonds (Hozoji et al. 2009).
has also been proposed that ABCA1 may dimerize (Nagata et al. 2013) or even oligomerize (Denis et al. 2004) when carrying out its duty.

It is well established that ABCA1 can mediate cholesterol and PL efflux, interact with apoA-I to make nascent HDL particles (Fielding et al. 2000), and remodel membrane lipid arrangement (Landry et al. 2006; Rigot 2002), but the mechanisms remain to be revealed.

More than 70 ABCA1 mutations have been found in Tangier disease patients, most of which are clustered in the ECDs and NBDs (Singaraja et al. 2003). These mutations can be categorized into four groups:

1. defective localization to the plasma membrane, such as R587W, Q597R, S1506L (Singaraja 2006);
2. defective apoA-I binding ability: mutant C1477R is capable of promoting cholesterol efflux to sodium taurocholate and translocating phospholipid, but has significantly reduced apoA-I binding ability (Rigot 2002; S. Wang et al. 2013);
3. defective lipid translocation activity: mutant W590S exhibits normal apoA-I binding ability yet lacks lipid translocation ability (Fitzgerald 2002);
4. defective NBDs: although correctly localized in plasma membrane, these mutants such as K939M and K1952M lack both phospholipid translocation and apoA-I binding ability (Hamon, Broccardo, Chambenoit, Luciani, Toti, Chaslin, Freyssinet, Devaux, McNeish, Marguet & Chimini 2000b; Nagao et al. 2011).
ABCA1 has two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). Each TMD consists of 6 transmembrane helices. Between transmembrane helix 1 and 2, 7 and 8 are two extracellular loops, which are highly glycosylated and are believed to be connected by one or more disulfide bonds (Hozoji et al. 2009).

1.5.3.2 Mechanism of ABCA1-mediated nascent HDL biogenesis

Although it has been known for decades that ABCA1 and apoA-I are the major players in nascent HDL biogenesis, a detailed mechanism underlying this process still remains unclear. Several models have been proposed to date.

The two-step model depicted in Figure 1-30 is one of the earlier models. In this model, ABCA1 first mediates PL efflux to apoA-I, and this apoA-I/PL complex then picks up passively-diffused FC in an ABCA1-independent manner. This model was based on
observations that FC efflux was inhibited by vanadate and okadaic acid but PL efflux was not (Fielding et al. 2000), and that conditioned media from ABCA1-expressing cells pretreated with cyclodextrin was able to promote FC efflux from ABCA1-free cells (Wang et al. 2001), suggesting that PL efflux and FC efflux could be two separate steps. However, a later experiment from the same group that added an additional wash step after cyclodextrin incubation showed that the conditioned media could not promote FC efflux (Wang et al. 2003). Furthermore, Smith et al. showed that FC and PL efflux happen concurrently and that inhibiting ABCA1 by glyburide and decavanadate also inhibits both FC and phosphatidylcholine efflux (Smith et al. 2004).

Figure 1-30 A two-step mechanism of nHDL biogenesis by ABCA1 and apoA-I, proposed by Fielding et al. Adapted from (Fielding et al. 2000)

In 2006, Landry et al. showed that ABCA1 disrupted membrane rafts and created a loosely packed raft domain that could facilitate apoA-I binding (Landry et al. 2006).
Later, inspired by the work of Chimini (Rigot et al. 2002) and Rothblat (Hamon et al. 2000a) which demonstrated that translocation of phosphatidylserine (PS) by ABCA1 from the inner leaflet to the outer leaflet of the membrane facilitates apoA-I binding, Phillips et al proposed a 3-step model (Figure 1-31): 1. A small regulatory pool of apoA-I activates ABCA1’s PL translocase activity and PL is translocated from the inner leaflet to the outer leaflet, causing lateral compression of PL in the outer leaflet. 2. An exovesiculated domain is formed to relieve the strain on the membrane. This high-curvature structure creates high-affinity binding sites for a larger pool of apoA-I. 3. ApoA-I solubilizes the exovesiculated domain and leaves cell surface. The key feature of this model is the high-curvature structure on the membrane created by ABCA1. According to this model, binding of apoA-I to the cell surface should be impaired if ABCA1 is defective in PL translocase activity because it cannot create the bulge on the cell membrane. However, the observation that W590S, a mutant of ABCA1 that is not able to translocate PL, could
still induce apoA-I binding at the same level as WT ABCA1 (Fitzgerald 2002) calls this model into question.
Ueda et al then showed that ATP-hydrolysis and translocation of substrates by ABCA1 are independent of apoA-I binding, but ATP-hydrolysis results in conformational changes in the ECDs of ABCA1, which are required for apoA-I binding. They also proposed that lipidated apoA-I loses affinity to ABCA1 and dissociates from the cell surface in the form of nHDL particles. However, according to Fitzgerald et al, dissociation of apoA-I was shown to be independent of lipid transfer (Fitzgerald 2003).

In 2013, Wang et al (S. Wang et al. 2013) mutated L38 and M112 into cysteines and covalently linked a self-quenching fluorophore BODIPY-tetramethylrhodamine (TMR) to each of the mutated cysteines. They showed that these two residues, which are in proximity when apoA-I forms a helical bundle at the N-terminus, were physically pushed away upon interacting with ABCA1-expressing cells, even with ABCA1 isoforms that are defective in membrane remodeling (W590S), suggesting that ABCA1 can mediate the unfolding of apoA-I helix bundle.

In addition to the cell-surface formation of nHDL, Smith et al showed that apoA-I could be engulfed by macrophages in an ABCA1-dependent manner and be resecreted in a process called retroendocytosis. They also showed that blocking endocytosis can reduce lipid efflux to apoA-I (Smith et al. 2004). The retroendocytosis pathway is believed to happen only when cells are rich in cholesterol, because when internalization of ABCA1 is blocked, cholesterol efflux from cells with high level cholesterol is decreased, whereas efflux from cells with basal level cholesterol is increased (Azuma et al. 2009). Although
retroendocytosis might be involved in nascent HDL biogenesis, it is most likely that nHDL formation can occur on the plasma membrane as well, since Faulkner et al have shown that the majority of apoA-I taken inside the cells is degraded and the intact apoA-I resecreted is not enough to account for cholesterol efflux into the media. Therefore, efflux must have happened at the cell membrane (Faulkner et al. 2008).
1.6 Aims and outline of studies

Despite being studied extensively, molecular details of the structure and function of relationships of apoA-I and ABCA1 during nHDL biogenesis still remain enigmatic. Many questions remain to be answered, such as

1. Is there direct interaction between apoA-I and ABCA1? Although crosslinking studies have suggested that apoA-I and ABCA1 could be cross-linked with a 3 Å crosslinker (Chroni et al. 2004), it is still possible that apoA-I is not directly interacting with ABCA1 but with the lipids around it.

2. Which motifs on apoA-I and ABCA1 are responsible for the interaction, if there is any, between these two proteins?

3. What structural feature of apoA-I and ABCA1 controls cholesterol efflux and nascent HDL formation?

To answer these questions, detailed structures of apoA-I and ABCA1, as well as the functional implications of these structures, are needed.

This thesis work first adopts the mutation method to approach these questions by probing biophysical characteristics of apoA-I mutants in solution and how these mutations translate into functional differences in the cellular system. The mutants include:

1. Destabilization of the N-terminal hinge region (38/40G, Q41A, P66G)

5. Locking the monomer conformation (H5 3xG)

Furthermore, this work investigates the interactions between apoA-I and ABCA1, both in the cells and in a cell-free system, for sometimes it is hard to avoid all the interfering factors that could potentially hinder the detection of direct interactions between ABCA1 and apoA-I in a cellular system. To achieve this aim, ABCA1 expression and purification were carried out and the interaction between ABCA1 and apoA-I in solution was probed.

To ultimately determine the structure of ABCA1 and understand the molecular details of this protein, this work also explores sample-preparation methods for ABCA1 in electron microscopy.
CHAPTER 2 Materials and Methods

2.1 Protein concentration determination

2.1.1 OD280 nm

Protein concentration can be measured by absorbance of ultraviolet (UV) at 280 nm. Amino acids with aromatic rings contribute mostly to the absorbance at 280 nm. The molar extinction coefficient of a certain protein can be calculated from its amino acid sequence using the following formula:

\[ E(Pr) = \text{# of Tyr} \times \text{Ext (Tyr)} + \text{# of Trp} \times \text{Ext (Trp)} + \text{# of Cys} \times \text{Ext(Cys)} \]

In this formula, Ext represents the molar extinction coefficient at 280 nm, which is 1490 for Tyr, 5500 for Trp, and 125 for Cys.

The absorbance at 280 nm, Abs(Pr) equals \( E(Pr)/MW(Pr) \). For WT apoA-I, the extinction coefficient is 32430 and absorbance is 1.155.

Protein concentration can be calculated using the formula:

\[ [Pr] \text{ (mg/ml)} = \text{Abs(Pr)} \times \text{dilution factor} \times \text{path length (cm)} \]

Because this method is based on absorbance by the side chains of certain amino acid residues, OD280 nm can be affected by secondary, tertiary, and quaternary structure of the protein. In order to exclude the possibility that some mutants of apoA-I may have a conformational change which exposes or buries these residues and lead to abnormal reading in OD280 nm, concentrations of mutants and WT apoA-I were measured under both non-denaturing and denaturing conditions (in 6 M guanidine hydrochloride).
2.1.2 Colorimetric assay (based on the Lowry assay)

Protein concentration can also be determined colorimetrically according to Lowry’s method (Lowry et al. 1951). Under alkaline conditions, Cu$^{2+}$ is reduced Cu$^{+}$ when forming a complex with peptide bonds. Cu$^{+}$ and aromatic rings of the Try, Trp and Cys residues react with Folin reagent and give a blue color, which can be measured by absorbance at 750 nm using a spectrophotometer. The absorbance signal at 750 nm is proportional to protein concentration in the range of 0.01–1.0 mg/ml. Bovine serum albumin (BSA) was used to generate a standard curve. Because the Lowry assay measures protein concentration under an alkaline-denatured condition, the result is less affected by secondary, tertiary, and quaternary structure of the protein. In our studies, the Pierce™ Modified Lowry Protein Assay Kit was used. Procedures were carried out according to the manufacture’s instruction.

2.2 ApoA-I refolding

A 1 ml aliquot of purified apoA-I and the mutants at ~ 2 mg/ml stored at -80 °C was thawed on ice slowly and dissolved in 9 ml 6M GdnHCl in 50 mM PO$_4$ buffer. This solution was kept on ice for > 2 hour in order for the protein to unfold completely. After unfolding, the solution was transferred into dialyzing membranes (Molecular Weight Cut Off =14 KD) and dialyzed against three tandem 4L 5 mM PO$_4$ buffer (pH 7.4) at 4 °C. In some cholesterol efflux essays and nHDL formation experiments, apoA-I and the mutants were heated to 60 °C for 30 min to thermally unfold the proteins and then cooled down to room temperature to standardize protein conformation (Mulya et al. 2007).
2.3 Circular dichroism (CD) spectroscopy

2.3.1 Principle of CD

When a plane-polarized light beam passes through a chiral medium, such as a biological molecule, the right-circularly polarized component and left-circularly polarized component are absorbed at different levels. The different absorbance of the two circular components results in elliptically polarized light. The ellipticity is expressed by the angle θ, whose tangent is the ratio of the resultant ellipse of the minor axis to that of the major axis. A CD experiment measures the ellipticity of the sample. Far-UV CD spectra (250-185 nm) of proteins are affected by peptide bond geometry, and different protein secondary structures give rise to different characteristic CD spectra, as depicted in Figure 2-1. The α-helix spectrum has two negative peaks at 222 nm and 208 nm, and a positive peak at 191-193 nm. The β-sheet spectrum has one negative peak between 210-225 nm and one positive peak between 190-200 nm. The random coil spectrum has one positive peak at 212 nm and one negative peak around 195 nm. Since the CD signal at 222 nm is almost solely determined by α-helical content, we can use this signal to calculate α-helical content and monitor conformational changes of a given protein.
2.3.2 CD experiments

In both Far-UV wavelength scan and thermal unfolding experiments, WT and mutant forms of apoA-I were used at 0.02 mg/ml to 0.2 mg/ml in 5 mM PB pH=7.4. Previous studies in our lab have demonstrated that apoA-I does not aggregate at this concentration (< 0.3 mg/ml) (Gorshkova et al. 2000). Refolded proteins were placed in 1 or 2 mm quartz cuvettes for the experiments. Data were recorded in the Aviv 62DS or Aviv 215 spectropolarimeter (Aviv Associates, Lakewood, NJ).

For Far-UV spectra, wavelengths 250 nm-185 nm were scanned with 1 nm bandwidth, 1 nm step size and 5 s accumulation time at 25 °C. Each sample was scanned 3 times. The
PO₄ buffer baseline was subtracted for later calculations. All data from our measurements were expressed as the normalized mean residue molar ellipticity [θ] in deg cm² dmol⁻¹:

\[ [\theta] = \frac{100 \theta}{C \times n \times l} \]

where:
\( \theta \) = raw output in mdeg
\( C \) = protein concentration in mM
\( n \) = # amino acid residues
\( l \) = cuvette path length in cm

The α-helical content can be calculated from mean residue molar ellipticity at 222nm ([\( \theta_{222} \)]) using the following formula (Woody 1996):

\[ \alpha\text{-helix}\% = \frac{-[\theta_{222}] + 3000}{39000} \]

For thermal unfolding experiments, the ellipticity of protein samples at 222 nm was monitored from 5 to 95 °C with 1 °C step size and 90s accumulation time for each data point. Melting temperature (Woody 1996) was derived from the peak of the first derivative of the thermal unfolding curve calculated in the Origin Software (Microcal).

2.4 DMPC clearance assay

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is a 14-carbon saturated phospholipid that is not soluble and appears turbid in aqueous dispersion. Upon binding to apoA-I, DMPC forms lipid-protein complexes and the turbid solution turns clear. Using this method, one can evaluate the kinetics of lipid binding of a protein. DMPC (10 mg) was dissolved in a glass tube using chloroform/methanol (2:1) and then dried under nitrogen and left in a desiccator at 4 °C overnight to remove the residual solvent. An
aliquot of 2 ml of phosphate-buffered saline (PBS) was added to the tube and the solution was vigorously vortexed. An aliquot of 50 µl stock solution (5 mg/ml) was mixed with 1 ml 0.1 mg/ml WT or mutant forms of apoA-I pre-equilibrated at 24 ºC. This gave rise to a 2.5 : 1 (w : w) lipid to protein ratio. The rate of DMPC clearance by WT and mutant forms of apoA-I was monitored by the change of the turbidity of the solution (absorbance at 325nm) at 24 ºC for 1 hour. The plot of Abs\textsubscript{325} versus time (min) was recorded.

### 2.5 Cholesterol efflux assay

BODIPY-cholesterol is a cholesterol compound that has the boron dipyromethene difluoride fluorophore linked to sterol carbon-24 (Figure 2-2). BODIPY-cholesterol has been extensively used to study cholesterol trafficking in living cells (Wüstner et al. 2016). It has been reported that BODIPY-cholesterol closely mimics partitioning of cholesterol in the membrane (Höltätä-Vuori et al. 2008). Use of BODIPY-cholesterol for cholesterol efflux study has also become more popular since the establishment of this method (Sankaranarayanan et al. 2011; Cho et al. 2015; Zhang et al. 2015).
This essay was developed based on the previously published method by Sankaranarayanan et al with slight modifications (Sankaranarayanan et al. 2011). HEK293 cells were plated in a 24-well collagen-coated plates at around 60% confluence in Dulbecco's modified Eagle's medium (DMEM) (low glucose) supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. After overnight incubation, the cells were transiently transfected with pcDNA1 containing human ABCA1 cDNA or pcDNA1 alone (as negative control). For each well, 0.8 µg of DNA and 2.4 µl of Genjet transfection reagent (version II) was diluted in 40 µl of DMEM (high glucose) separately. Genjet in DMEM was transferred to the tube containing DNA immediately after dilution. The Genjet/DNA mixture was incubated at room temperature for 10 min and then added to the cells drop-wise. Twenty-four hours post transfection, the media were aspirated and 1 µM dimethyl sulfoxide (DMSO) solubilized BODIPY-cholesterol or methyl-β-cyclodextrin complexed cholesterol (cholesterol: methyl-β-cyclodextrin = 1:30 molar ratio) was added to the cells and incubated for 1 hour. DMSO solubilized BODIPY-
cholesterol was used to label cells with basal level cholesterol; methyl-β-cyclodextrin complexed cholesterol was used to load cells with cholesterol. Cells were then washed once with DMEM and incubated with 5 µg/ml to 10 µg/ml apoA-I or mutants. Media were collected after overnight incubation. For kinetic studies, 20 µl of media were collected at time points 0h, 2h, 4h, 8h, 12h and overnight, or 0h, 2.5h, 5h, 7.5h, 10h, and overnight.

The media were then filtered through a 0.22 µm membrane to remove cell debris and the cells were lysed with 1% sodium cholate at room temperature for 4 hours. Fluorescence in both media and cells were then measured with a Tecan Infinite M1000 plate reader at excitation wavelength 490 nm (bandwidth 10 nm) and emission wavelength 520 nm (bandwidth 20 nm). Readings from empty-vector transfected cells were subtracted as baseline. The percentage efflux was calculated by taking the fraction of fluorescence in the media over total fluorescence in both media and the cells.

Figure 2-3 Flowchart of cholesterol efflux assay. Step 1, transfect HEK293 cells with cDNA encoding ABCA1, incubate for 24 hours. Step 2, label cells with BODIPY-Cholesterol for 1 hour. Step 3, wash excess BODIPY-
Cholesterol with DMEM. Step 4, add apoA-I and mutants at 5-10 µg/ml to the media and incubate overnight. Step 5.1, measure fluorescence in media and in the cells; 5.2, analyze nHDL particle formation using 4-15% native gel.

2.6 Gel electrophoresis

2.6.1 SDS-PAGE

Purified ABCA1 was analyzed on 5% or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 volts for 1 hour. Purified apoA-I was analyzed on 12.5% SDS-PAGE at 150 volts for 1 hour. The running buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. Proteins were then visualized by staining with Denville Blue Protein Stain.

2.6.2 Native-PAGE

Native gradient gels (4%-15%) were purchased from Bio-Rad (#4561096). For nHDL particle analyses, the gels were run at 180 volts for 75 min or at 200 volts for 1 hour. The running buffer was 25 mM Tris, 192 mM glycine, pH 8.3.

2.7 Western blot and dot blot analysis

For western blotting, proteins were transferred from a gel to a methanol-wetted polyvinylidene difluoride (PVDF) membrane by electrophoresis at 100 volts for 1 hour. The transfer buffer was 25 mM Tris, 192 mM glycine, pH 8.3.

For dot blotting, 2 µl of samples were directly applied to a methanol-wetted PVDF membrane.
The PVDF membrane was then blocked with TBST (Tris-buffered saline, 0.1% Tween 20) supplemented with 5% milk proteins for 20 min at 37 °C. Primary antibody in the same buffer was then added and incubated for 1 h at room temperature. After 3 x 5 min washes with TBST, the membrane was then incubated with HRP-conjugated secondary antibody for 1 h at room temperature and washed 3 x 5 min. Enhanced chemiluminescent (ECL) substrate was then added. The light emitted was captured on an X-ray film and protein bands were visualized using an automatic developer.

For membranes that needed to be probed multiple times with different antibodies or cholera toxin B (CTB), they were stripped by incubating with stripping buffer (0.1 M glycine pH 2) for 2 h at room temperature on a shaker.

2.8 Lipid analysis

2.8.1 Density separation of efflux media by ultracentrifugation

Efflux media from cell culture were collected and filtered as described in section 2.5. For density gradient separation, solid KBr was added to the media and mixed thoroughly to reach a density of 1.25 g/ml in 15 ml tubes. After KBr was completely dissolved, the solution was transferred to centrifuge tubes (from Beckman for SW41 rotor). Lower density KBr solution (1.05 g/ml in water) was overlaid on the top of the samples. The samples were spun on Beckman RC-95M with SW41 rotor at 39k rpm at 11 °C for at least 24 hours. Fractions of 0.5 ml or 1 ml were collected from the top. Fluorescence was measured in a Tecan Infinite M1000 plate reader for cholesterol (BODIPY-cholesterol)
content; protein and GM1 was analyzed by dot blot or by 4%-15% native PAGE followed by immuno-blotting.

For non-gradient separation, efflux media were adjusted to 1.21 g/ml with KBr and centrifuged on Beckman RC-95M with SW41 rotor at 39k rpm at 11°C for 24 hours.

After ultracentrifugation, all lipid-containing particles should be in the top 2 ml fraction. Lipid composition of this fraction was analyzed by Thin Layer Chromatography (TLC).

2.8.2 Thin Layer Chromatography (TLC)  
After ultracentrifugation, lipid-containing fractions were first transferred to glass conical tubes. Four volumes of methanol/chloroform (2:1) was added and the solution was vortexed vigorously. Water was then added to the mixture to reach a 1:1:0.9 (methanol : chloroform: water) ratio. The solution was vortexed again and was centrifuged at 1000xg for 30 minutes to separate phases. The methanol/water phase on the top was removed and the chloroform phase in the bottom was dried under nitrogen. The lipid was then dissolved in 40-50 µl chloroform to be loaded on a TLC plate. After sample loading, the plate was first developed in the polar system with solvent composition CHCl₃: MeOH: H₂O: Acetic acid (65:25:4:1) until the front line of the solvent reached to ~25% of the plate. The plate was air-dried after the polar phase separation and then run in the neutral system with solvent composition Hexane: Ether: Acetic acid (70:30:1) until the front line of the solvent reached close to the top edge of the plate. To visualize the lipids, the plate was either iodine stained or charred after air-drying. When charring, sterol-based lipids would first turn purple and then black.
2.9 ABCA1 expression and purification

2.9.1 Rho1d4 tagging system

The rho1d4 epitope is the last 9 amino acids of the C-terminus of bovine rhodopsin. Rho1d4 and its antibody were first characterized in the 1980s (Hodges et al. 1988). Highly specific, high affinity rho1d4 antibody immobilized onto a matrix can be used for purification of rho1d4-tagged proteins. Eluting rho1d4 tagged proteins with the rho1d4 peptide allows for the avoidance of harsh conditions such as extreme pH usually used for eluting antibodies. Since its discovery, the rho1d4 tagging system has been successfully used in the purification of several of membrane proteins, including GPCRs and ABC transporters (X. Wang et al. 2011; Zhong & Molday 2010; Takayama et al. 2008).

![Diagram of the rho1d4 epitope on bovine rhodopsin](figure2-4.png)

Figure 2-4 Illustration of the rho1d4 epitope on bovine rhodopsin. Adapted from cube-biotech.com.
2.9.2 Construct generation

PcDNA.1 containing the human ABCA1 gene was from Dr. Freeman’s lab at Harvard. A rho1d4 tag (T-E-T-S-Q-V-A-P-A) was added to the C terminus of ABCA1 using the Q5® Site-Directed Mutagenesis Kit from the New England Biolab. Primers used for making this construct:

Forward: GTGGCGCCGCGGTGAATCCTGTTCATACGGGG
Reverse: CTGGCTGGTTTCGGTTAATACATAGCTTTCTTTCACTTTT

In pcDNA1

Figure 2-5 ABCA1 construct in pcDNA1 with Rho1d4 tag at the C terminus.

2.9.3 ABCA1 expression in mammalian cells grown in suspension

HEK293F cells were grown in Freestyle Expression Media (Invitrogen) in 1 L rotating flasks at 130 rpm, 8% CO₂, 37 °C. Once cell density reached 2.5 x 10⁶/ml, the cells were spun down and resuspended in the same volume of fresh Free Expression Media. After resuspending for 5 min, 3 µg ABCA1-Rho DNA per 1 ml of cell culture were added to the culture. After 5 min incubation, 9 µg PEI (25KDa linear, Polysciences) per 1 ml of cell culture was added. Twenty-four hours post-transfection, the culture was supplemented with 2 mM valproic acid to help stabilize protein expression. Cells were harvested 2 days later.
2.9.4 ABCA1 Purification

Cells were spun at 1,000xg and lysed with ice-cold 10 mM HEPES pH 7.5 hypotonic buffer supplemented with Roche protease inhibitor cocktail using a Dounce homogenizer. Cell lysate was first centrifuged at 1,000xg to remove cell debris and nuclei, and then centrifuged at 100,000xg for 40 minutes to pellet down membranes using the Beckman RC-95M ultracentrifuge. The supernatant was discarded and the pellet was re-suspended in 50 mM HEPES and 150 mM NaCl pH 7.5 with 1% (w/v) n-Dodecyl-β-D-Maltoside (DDM (critical micelle concentration (CMC) = 0.009%)) and gently agitated for 1 hour. This suspension was centrifuged again at 100,000xg for 40 minutes and the supernatant was collected. The supernatant was then incubated with rho1d4 antibody-conjugated agarose beads overnight. The beads were washed twice with 10x bed volume of wash buffer (50 mM HEPES, 150 mM NaCl pH 7.5, 0.02% DDM) and incubated with elution buffer (50 mM HEPES and 150 mM NaCl pH 7.5, 0.02% DDM with 500 µM rho1d4 peptide) for 1 hour. Three fractions were collected. Purified protein was stored at 4 °C for short-term use, or snap-frozen in liquid nitrogen and stored at -80 °C for long-term use.

2.9.5 Amphipol exchange

Amphipol 8-35 is reported to bind to membrane proteins with very high affinity; in other words, with very low dissociation rate (Popot et al. 2011). Therefore, unlike detergents, excess amphipol micelles can be removed, leaving only those bound to the membrane protein in the solution. Figure 2-6 illustrates a typical amphipol exchange process. In our experiments, DDM- solubilized-ABCA1 was substituted with amphipol 8-35 (ABCA1:
amphipol = 1:3 w:w) for 1 h at room temperature followed by 1 h at 4 °C, either after the protein was eluted from the beads or while it was still attached to the agarose beads. In the former situation, 200 mg polystyrene beads (BioRad SM-2 beads) per mg DDM were used to remove excess DDM in the solution; in the latter situation, excess DDM and amphipol were washed away with wash buffer (50 mM HEPES, 150 mM NaCl pH 7.5) and amphipol solubilized-ABCA1 was then eluted from the rho1d4-antibody-conjugated agarose beads with 500 µM rho1d4 peptide.

Figure 2-6 Diagram of the amphipol exchange process. Adapted from (Zoonens & Popot 2014)
2.10 ATPase activity assay

The ATPase activity assay, developed by Innova Biosciences, measures the content of Pi released from ATP molecules using a colorimetric method. The principle of this method is depicted in Figure 2-7. Released Pi from the ATP-hydrolyzing reaction forms a green complex with an orange dye provided in the kit. The intensity of this green color is measured and is used to calculate the Pi concentration in the samples using a standard curve. The experiment was performed according to the manufacturer’s instruction with slight modifications. For a 25-well reaction, substrate/buffer (SB) mix was prepared as follows: 500 µl 0.5 M pH 7.5 Tris buffer, 125 µl 0.1M MgCl₂, 250 µl 10 mM ATP, and 1625 µl water. 100 µl SB mix was added to 100 µl 25 µg/ml purified ABCA1 in 50 mM HEPES 150 mM NaCl pH 7.4 with 0.02% DDM and incubated at room temperature for 1 hour. An aliquot of 50 µl PiColorLock™ mix was then added to stop the reactions. After 2 minutes, 20 µl of stabilizer was added and the color would reach maximal intensity after 30 minutes. The plate was read at any wavelength between 590 nm to 660 nm in a Tecan Infinite M1000 plate reader.

![Figure 2-7 Principle of the Innova Biosciences ATPase assay. Adapted from manufacturer’s manual.](image)
2.11 Cross-linking of apoA-I to HEK293 cells expressing ABCA1

HEK293 cells were plated and transfected in 24-well collagen-coated plates as described in section 2.5. Forty-eight hours post transfection, cells were incubated with 5 µg/ml apoA-I for 30 min at 37 °C followed by 30 min at 4 °C. For cholesterol-loaded cells, 1 µM cholesterol/Methyl-β-cyclodextrin complex (Sigma) were incubated with the cells for 1 h at 37 °C before apoA-I addition. ApoA-I containing media were then aspirated and the cells were washed twice with ice cold PBS. 2.5% paraformaldehyde was then added and incubated at room temperature for 30 min. The cells were washed with PBS 3 times and blocked with 5% non-fat milk in PBS at 37 °C for 30 min. Goat anti-apoA-I polyclonal IgG was added at 1:10,000 dilution and incubated at room temperature for 1 h. After washing 3 times with PBS, HRP-conjugated antibody to Goat IgGs was added and incubated again at room temperature for 1 h. After another 3 washes with PBS, TMB substrate (3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA from Sigma) was added and incubated in the dark for 30 min. After TMB incubation, the solution was supplemented with 3 M H₂SO₄ (final concentration 1.5 M) to stop the reaction. The solution then turned yellow. Absorbance at 450 nm was read in a Tecan Infinite M1000 plate reader.
2.12 Interactions between apoA-I and ABCA1

2.12.1 Interactions between DDM solubilized-ABCA1 and apoA-I

DDM solubilized-ABCA1 in (50 µg/ml) elution buffer (50 mM HEPES 150 mM NaCl pH 7.4 with 0.02% DDM and 500 µM rho1d4 peptide) with or without Dithiothreitol (DTT) was incubated with equimolar amounts of apoA-I and mutants (5 µg/ml) at room temperature for 1 hour. This apoA-I concentration was the same as in efflux essay and cross-linking experiments described previously in sections 2.5 and 2.11. Samples were then run on a 4%-15% native gels at 180 V for 90 min, transferred to PVDF membrane and probed with anti-apoA-I antibody. The same blot was then stripped with stripping buffer (0.1 M glycine pH 2) and probed again with ABCA1 antibody.

2.12.2 Interactions between amphipol solubilized-ABCA1 and apoA-I

After exchange for amphipol on the beads, 100 to 150 µg/ml eluted ABCA1 in elution buffer (50 mM HEPES 150 mM NaCl 2 mM TCEP pH 7.4 with 500 µM rho1d4 peptide) was incubated with 10 to 15 µg/ml of apoA-I (1:1 molar ratio to ABCA1) at room temperature for 1 hour. Native-PAGE analysis was the same as DDM solubilized-ABCA1 described above.

2.13 Electron Microscopy

2.13.1 The regular method

Carbon-coated grids were glow-discharged before use. An aliquot of 4 µl of sample was loaded onto the grid and incubated for 30 s. For ABCA1, sample concentration was ~ 20...
μg/ml in 25 mM HEPES, 75 mM NaCl, pH 7.4 with 0.02% DDM or amphipol. Excess sample was blotted away with filter paper. The grid was rinsed with pure water drops for 10 times. Freshly prepared uranyl acetate (1%) stain was applied and incubated for 30s. Excess stain was blotted away with filter paper and the grid was air-dried for 3 min. The grids were observed in the CM12 electron microscope (Philips Electron Optics, Eindhoven, The Netherland).

2.13.2 Native-gel blotting method

This method was developed in 2012 by Nickell et al (Knispel et al. 2012). Protein samples of 1 μg were loaded in duplicate onto 5% native gel. The gel was run at 200V for 1 hour. Half of the gel was stained as “the reference lanes” (lane M, 1, 2 in Figure 2-8), and the other half was used as “the blotting lanes” (lane 2’, 1’ in Figure 2-8). When locating the corresponding band position of the blotting lane, 5%~10% swell of the reference gel was taken into account. The band position of the blotting lane was wetted by a drop of electrophoresis running buffer and roughened by tweezers. Glow-discharged grids were placed on these bands for 1 min and rinsed with pure water for 10 times. The rest of procedure was the same as the regular method as described above.
Figure 2-8 The grid-blotting procedure. Figure adapted from (Knispel et al. 2012). Lane 1 and 2 were “the reference lanes”. Lane 2’ and 1’ were “the blotting lanes”.
CHAPTER 3 Characterization of ApoA-I Mutants in Solution

3.1 Overview

Based on the crystal structure of the C-terminus truncated apoA-I(1-184) (Mei & Atkinson 2011), a series of mutants were designed and isolated to study the structure-function relationships of apoA-I. The following four groups of mutations were studied to investigate the properties of the N-terminal domain, the C-terminal domain, and the monomer-dimer conversion of apoA-I. All mutations were introduced in WT apoA-I rather than to the C-terminus truncated apoA-I(1-184), except the C-terminal extension mutations.

1. Mutants designed to destabilize the N-terminal hinge (38/40G, Q41A)

Buried in the N-terminal helical bundle is a cluster of hydrophobic residues. These aromatic clusters (W8, F71, W72 from one apoA-I molecule and F33, F104, W108 from the symmetric molecule) and π-cation interactions (K23-W50 and W8-R61) are major forces that hold the helical bundle together. We propose that opening of the helical bundle happens at the two hinge regions (G65-T68, A37-Q41), which show high flexibility (temperature factor) as revealed by the crystal structure (Mei & Atkinson 2011). The first group of mutations was designed to perturb the first hinge region at A37-Q41. By mutating each residue flanking G39 into a Glycine residue (L38G/K40G), we were able to introduce a tandem 3x Gly, which, due to highly rotatable ψ, φ angle, can act as a helix breaker in this hinge region. One noticeable feature in the proximity of the 3xGly helix breaker is two hydrogen bonds between Q41 in the hinge region and Y115, R123 on the backbone Helix 4 and Helix 5 (Figure
3-1). Whether these two hydrogen bonds are critical for holding the “closed” conformation is unclear, therefore, mutant Q41A was also produced to investigate this question.

In addition, because previous studies in the laboratory have demonstrated entropic stabilization by proline substitutions in apoA-I (Gorshkova et al. 2000), P66G was designed to destabilize the second hinge region (Figure 3-2).

We hypothesize that disrupted helical structure in the N-terminal hinge regions will facilitate the opening of the N-terminal helical bundle, therefore facilitate lipid binding to the buried hydrophobic core and subsequently nHDL formation, or facilitate the conversion from smaller nHDL particles to larger ones.
Figure 3-1 Structural details of the first N-terminal hinge region of apoA-I. PDB: 3R2P.
2. Mutants designed to stabilize the N-terminal hinge (G65A; V67P, T68P, 66/67/68P)

The second hinge region is located at position G65-T68. Again, because of the stabilizing effect of proline substitutions in apoA-I (Gorshkova et al. 2000). V67P, T68P and 65/67/68 3xP were designed to stabilize the loop in the second hinge region. An alanine mutation was introduced at G65 to eliminate the flexibility brought about by the glycine residue. Again, our hypothesis is that a stabilized hinge region will hinder the opening of the N-terminal helical bundle and therefore, inhibit lipid binding to the hydrophobic core and ultimately hamper nHDL formation, or result in nHDL particles with a different size distribution (in favor of smaller particles).
3. Mutants designed to extend the absent C-terminus (1-198, 1-209, 1-220, 1-231, WT)

As described in the introduction, apoA-I has two folding domains: a well-folded N-terminal domain (1-184) and a less defined C-terminal domain (185-243). The structure of the N-terminal domain is revealed in great detail by the high-resolution crystal structure, however, the C-terminal domain that is missing in the crystal structure, is proposed to be responsible for initial lipid binding and promoting cholesterol efflux mediated by ABCA1. Sequence analyses have predicted B-B-A-A-B repeats in the C-terminal domain. Therefore, we designed 4 constructs with one-
consensus-repeat increments from the 1-184 form: 1-198, 1-209, 1-220, 1-231, and 1-243 (full-length, WT). Using these mutants allows us to pinpoint which consensus repeat in the C-terminus is most critical for apoA-I’s function of making nHDL particles.

Figure 3-3 C-terminal extension sequence schematics.

Figure 3-4 Crystal structure of apoA-I (1-184) and model of full-length apoA-I. Adapted from (Mei & Atkinson 2011).

4. Mutants designed to lock the monomer conformation (Helix5 triple Gly)
Two C-terminus truncated mutants of apoA-I, apoA-I (44-186) and apoA-I (1-192) were shown to exist as both monomers and dimers in solution (Beckstead et al. 2005; Ji & Jonas 1995). In the crystal structure of apoA-I (1-184), the protein was dimeric. Therefore, what controls the monomer-dimer conversion, and what role monomer or dimer plays in nHDL formation become important questions to answer. In the crystal dimer, the central region (121-142, AB3, H5) clearly shows high flexibility (high temperature factor), leading to our hypothesis that in the monomer, the AB4/5 (H6/7) repeats in one apoA-I molecule may fold back to substitute for the corresponding region of the other apoA-I molecule in the dimer. In order to shift the equilibrium of monomer-dimer conversion to monomer, point mutations A130G and R131G were introduced to make a tandem 3xGly helix breaker (loop) as shown Figure 3-5. A loop in this region will be more energetically favorable in the “folded-back” conformation than as a rigid, extended helical backbone in H5.
In addition, three naturally-occurring apoA-I mutants were also characterized

1. **R153P** (Esperón et al. 2008)

   Heterozygous carriers of the R153P mutation do not exhibit hypercholesterolemia symptoms, but are severely HDL-C and apoA-I deficient and prone to premature CAD. One representative patient in the original case report had only 4.3 mg/dl HDL-C, compared to the normally > 60 mg/dl level. The authors suggested this mutation might decrease protein stability or interfere with LCAT interaction.

2. **L178P** (Hovingh et al. 2004)

   L178P carriers exhibit about 52% reduction in blood apoA-I level and 62% reduction in HDL-C level, but LDL-C, TG levels remain unaffected. CAD risk is 24-fold higher than non-carriers.
Notably, R153P and L178P are two of the very few cases that show such symptoms; unaffected LDL-C and TG but higher risk for CAD.

3. **Insertion mutation** (Esther Y Lee et al. 2013)

Another naturally-occurring mutation is an 18-nucleotide in-frame insertion mutation at nucleotide position 1553. Sequence analysis revealed that this insertion is a duplication of nucleotides 1535 to 1552 resulting in a 6-amino-acid (GARAHL) insertion after amino acid position 157. This insertion mutation is designated apoA-I_{Nashua}.

Heterozygous carriers of this insertion mutation showed an elevated TG level (447 mg/dl), very low HDL-C (< 5 mg/dl) and apoA-I level (23.5 mg/ml, about 80% reduction), and unaffected LDL-C level. The patient had been having a long history of CAD since age 42. No statistical analysis was available for this mutation. The authors of the original case report suggested abnormal lipid binding ability of this insertion mutant of apoA-I.

Figure 3-6 Naturally occurring point mutation R153P, L178P and insertion mutation apoA-I_{Nashua}.
In this chapter, biophysical characterization of these apoA-I mutants in solution is discussed. In chapter 4, the function in cholesterol efflux of these apoA-I mutants in the cellular system will be discussed.

3.2 Mutants designed to destabilize the N-terminal hinge region: 38/40G, Q41A, P66G

3.2.1 Secondary structure

Figure 3-7 and Figure 3-8 show the Far-UV CD spectra of mutants designed to destabilize the N-terminal hinge region. Freshly refolded proteins at a concentration ~ 0.02 mg/ml to 0.2 mg/ml (below the self-aggregation concentration of apoA-I as characterized by previous studies in our lab (Gorshkova et al. 2000)) in 10 mM phosphate buffer pH 7.4 at 25 °C were used in these experiments. Similar conditions were used for other mutants unless otherwise indicated. All mutants and WT apoA-I showed negative peaks at 222 nm and 208 nm, characteristic of α-helical structure. Based on the [θ]222 signal, WT α-helical content was calculated at 54 ± 2%, consistent with previously reported helical content of WT apoA-I ((Gorshkova et al. 2000)). Helical content of P66G, Q41A remained the same as WT. Helical content in mutant 38/40G slightly decreased to 51 ± 2%. A 3% drop helical content in apoA-I, which consists of 243 amino acids, translates into 9 amino acids changed from helical to non-helical, more than the 2 glycine mutations we introduced, suggesting that this mutation is likely to have affected...
the helical content in the proximal region, rather than just affecting the 3 residues themselves.

Figure 3-7 Far-UV CD spectra of apoA-I mutant 38/40G and Q41A. Protein concentration used in this experiment was between 0.02 mg/ml to 0.2 mg/ml.
3.2.2 Thermal stability

Melting temperatures (Tm) were calculated from the peak of the first derivative of the melting curve (Figure 3-10 and Figure 3-12). (Tm of other mutants were calculated in the same way). Again, Tm of mutant Q41A, showed no significant difference from WT, both of which were about 62 ± 2 °C. Interestingly, 38/40G, when freshly refolded, had the same Tm as WT (Figure 3-9). However, when stored at 4 °C degree for over 1 month without being protected from oxygen (referred to as “non-freshly refolded”), it showed a significantly lower Tm, of about 57 ± 2 °C, while mutant Q41A and WT remained at 62 °C, as shown in Figure 3-11. In addition, the thermal unfolding cooperativity also decreased in the non-freshly-refolded 38/40G (flattened curve, rather than a sigmoidal
shape in WT), suggesting that the protein may have become less compact. ApoA-I has been well characterized to be prone to methionine oxidation (Anantharamaiah et al. 1988). After close examination of the crystal structure again, we noticed that M112 is in close proximity of residues 38 to 40 in space (Figure 3-13). Previous studies have shown that M112 oxidation renders the protein less stable during thermal unfolding (Sigalov & Stern 1998) and more susceptible to protease digestion (Roberts et al. 1997). Therefore, we speculate that because of a disrupted helical structure at residues 38 to 40 (possibly extending to further residues based on the result of secondary structure analysis that at least 9 helical residues were made non-helical), the side chains of these residues were no longer in position to shield M112 from the surrounding environment, thus making M112 more susceptible to oxidation. In other words, a disrupted helical structure at residues 38 to 40 probably had resulted in a more opened cleft at the end of the helical bundle. We hypothesized that in this way, the N-terminal bundle would be more readily open and the buried hydrophobic core would be better accessible to lipid molecules when they are present in the solution.
Figure 3-9 Thermal unfolding of apoA-I mutant 38/40G, freshly refolded. Protein concentration was between 0.02 mg/ml to 0.2 mg/ml.
Figure 3-10 First derivative of the melting curve of mutant 38/40G. Freshly refolded samples.

Figure 3-11 Thermal unfolding of apoA-I mutant 38/40G and Q41A, non-freshly refolded. Protein concentration used in this experiment was between 0.05 to 0.2 mg/ml.
Figure 3-12 First derivative of the melting curve of mutant 38/40G and Q41A, non-freshly refolded samples.

Figure 3-13 Methionine 112, which has been characterized to be prone to oxidation, is in proximity of the residues 38 to 40 in space.
On the other hand, mutant P66G also showed a decreased Tm of 57 °C (Figure 3-14), despite the fact that α-helical content remained almost unchanged, which is not surprising given the fact that P66 is already in a loop in the crystal structure (Figure 3-2). But since proline has a more restricted ϕ ϕ angle that is not favorable in α-helices, WT apoA-I may energetically favor a closed conformation (loop in this region) rather than an open conformation (straightened helix with a proline kink in this region) when not bound with lipid. Mutating P66 into a glycine, could increase the propensity of the loop structure to form a rigid, extended helical structure that opens the hinge, therefore making the mutant P66G more susceptible to heat-dependent unfolding.

![Figure 3-14](image-url)  
**Figure 3-14** Thermal unfolding of P66G. Protein concentration used in this experiment was between 0.1 to 0.2 mg/ml.
3.2.3 DMPC clearance

Clearance of DMPC multilamellar vesicles (MLV) provides a measure of the kinetics of apoA-I and mutants to form a DMPC-apoA-I complex. This assay reflects the apoA-I lipid binding ability (method described in section 2.4). As shown in Figure 3-15, the turbidity of DMPC MLV was reduced faster in 38/40G than in WT apoA-I, suggesting that 38/40G had better lipid binding ability. Because mutating the leucine residue at position 38 and lysine residue at position 40 to glycines does not add hydrophobicity to apoA-I, we attribute this increased lipid binding ability to the more-easily-opened N-terminal helical bundle, where the hydrophobic core is buried. This improved DMPC clearance validated our mutation design and agreed with our hypothesis that an open N-terminal helix bundle will facilitate lipid binding based on Far-UV CD spectra and thermal unfolding data.

The P66G mutant, however, did not demonstrate faster clearance of DMPC MLV (Figure 3-16), despite having the same Tm as the 38/40G mutant (both at 57 °C), possibly because at 24 °C (the temperature at which the DMPC clearance assay was conducted), the destabilizing effect of P66G was not very strong, but as the temperature increases, the propensity to form helical structure in the hinge region (G65-T68) made the protein more easily opened, therefore more unstable.
Figure 3-15 DMPC clearance assay of apoA-I mutant 38/40G.
3.3 Mutants designed to stabilize the N-terminal hinge region

3.3.1 Secondary structure

Figure 3-17 and Figure 3-18 show the far-UV CD spectra of these mutants, single G65A, V67P, T68P and the triple proline mutation. Based on these spectra, no secondary structure change in the single proline or glycine mutant was observed. Surprisingly, even in the triple proline mutant 65/67/68P (together with P66, there is a tandem 4xPro sequence), α-helical content remained the same, of about 54% helix. This result indicated that, at the concentrations used in this experiment (ranging from 0.1 mg/ml to
0.2 mg/ml), the hinge region (G65 to T68) of apoA-I in solution was most likely to be a loop structure that is not perturbed by the proline residues, rather than a helical structure in which proline residues are unfavorable (Ramachandran et al. 1963).

Figure 3-17 Far-UV CD spectra of apoA-I mutants G65A, V67P, T68P. Protein concentration used in this experiment was between 0.1 to 0.2 mg/ml.
3.3.2 Thermal stability

Thermal unfolding curves of single proline mutants designed to stabilize the N-terminal hinge (G65A, V67P, T68P) all yielded a Tm of 61 ~ 62 °C, and an equally cooperative sigmoidal shape, compared to WT (Figure 3-19). It appeared that these single proline mutations did not affect apoA-I structure locally or globally in the way we designed them to, based on the unchanged helical content and thermal unfolding profile.

Triple proline mutation (65/67/68) destabilized the protein, resulting in a Tm of 57 °C (Figure 3-20), which was not surprising considering that it may be hard for all four tandem prolines to adopt the lowest energy trans/cis conformation. Collision between bulky cis groups may have given rise to the instability observed in the melting curve.
Figure 3-19 Thermal unfolding of apoA-I mutants G65A, V67P, T68P. Protein concentration used in this experiment was between 0.1 to 0.2 mg/ml.
3.3.3 DMPC clearance assay

The 65/67/68P triple proline mutant cleared DMPC at a rate similar to WT apoA-I (Figure 3-21), despite having a destabilized overall structure (indicated by significantly decreased Tm), suggesting that hydrophobic lipid-binding region within the helical bundle in this mutation was not made more accessible to DMPC MLVs at 24 °C. In other words, this mutation did not help to open or keep the N-terminal helical bundle closed, at least not at 24 °C, which is optimal in creating disruptions in the vesicles.
A DMPC clearance assay was not conducted with G65A, V67P and T68P because these mutants showed no signs of a stabilizing effect.

3.4 Naturally occurring apoA-I mutants

3.4.1 Secondary structure

Figure 3-22 and Figure 3-23 show the secondary structure of the three naturally occurring mutants of apoA-I. Based on the signal at 222 nm, helical content did not change significantly in L178P, which remained at ~54%, slightly decreased in R153P (~52%), and remarkably decreased in the Insertion mutant 49%). The fact that both of the two single proline mutations are located at the junction of consensus sequence unit A and B
may help explain why helical structure of L178P and R153P was minimally affected: at the end of a consensus sequence repeat, or at the junction of A and B consensus repeat, the residues might have already existed as a bend or dislocation, which was more tolerant to proline than the α-helices. Alternatively, the proline mutations could have just created an extra kink and changed the curvature of the helices there, rather than directly disrupting the AB helices themselves. In contrast, the Insertion mutant, added six amino acids between aa positions 156 and 157 in the middle of the 11-mer consensus repeat B in H6. This insertion directly disrupted repeat B helical structure and the 5% drop in helical content ensued.

![Figure 3-22 Far-UV CD spectra of apoA-I mutant R153P, L178P. Protein concentration used in this experiment was about 0.2 mg/ml.](image-url)
3.4.2 Thermal stability

Figure 3-24 and Figure 3-25 show the melting curves of naturally occurring R153P, L178P and Insertion mutants recorded from 5 °C to 95 °C at 0.1 mg/ml concentration. Tm of all the mutants decreased significantly: 49 °C for R153P, 44 °C for L178P despite the observation that α-helical content seemed to be unaffected, and 48 °C for the Insertion mutant. The unfolding cooperativity of all mutants also decreased dramatically, indicating that the tertiary structure might have become less compact than WT. Thus, these proteins might be very unstable in the circulation at 37 °C, in good agreement with the phenotype exhibited by the carriers of this mutation, very low plasma apoA-I level and HDL-C level.
Figure 3-24 Thermal unfolding of naturally occurring apoA-I mutants R153P and L178P. Protein concentration used in this experiment was between 0.1 to 0.2 mg/ml.
Figure 3-25 Thermal unfolding of naturally occurring insertion mutant apoA-I_{Nashua}. Protein concentration used in this experiment was between 0.1 to 0.2 mg/ml.

3.4.3 DMPC clearance assay

DMPC clearance data for the naturally occurring mutants are shown in Figure 3-26 and Figure 3-27. All the mutants showed no change in DMPC clearing rate, suggesting an unchanged lipid binding ability. For the Insertion mutant, in the original case report, the authors proposed abnormal lipid binding properties of this mutant. Our DMPC clearance assay seemed to disagree with this proposal but our results here could only represent DMPC, a non-physiological lipid species. Thus, it would be imprudent to overturn the
original proposal based solely on this assay. Nascent HDL lipid (mainly PC and cholesterol) binding will be discussed in the next chapter.

![OD325nm vs Time (min) for DMPC clearance of naturally occurring mutants R153P and L178P.](image)

Figure 3-26 DMPC clearance of naturally occurring mutants R153P and L178P.
3.5 Summary

Based on the crystal structure of the C-terminus truncated apoA-I, a series of mutants were designed. Together with three naturally occurring mutants, these mutants were isolated and characterized. The mutants characterized are in 5 overall categories:

1. destabilization of the N-terminal hinge region (38/40G, Q41A, P66G)
2. stabilization of the N-terminal hinge region (G65A, V67P, T68P, 65/67/68P)
3. naturally occurring mutants (R153P, L178P, Insertion mutation apoA-I<sub>Nashua</sub>)
4. extension of the truncated C terminus (1-198, 1-209, 1-220, 1-231, 1-243 (WT))
5. to lock monomer conformation (H5 3xG)

This chapter discusses the biophysical properties of mutants in the first three categories in a lipid-free state in solution. Studies of mutants in the last two categories are in...
collaboration with Dr. Mei (Mei et al. 2016). Solution characterization was carried out by him therefore is not discussed in here. Characterization of these mutants in the cellular system will be discussed in chapter 4.

A summary of α-helical content and the thermal unfolding temperature of apoA-I mutants is given in Table 3-1.

Among all the designed mutants, some did not show any change in biophysical properties (Q41A, G65A, V67P, T68P), suggesting that those residues are probably not critical in maintaining the secondary structure, stability, and possibly lipid binding ability of apoA-I. Some retained secondary structure and lipid binding ability but became destabilized (65/67/68P and all of P66G). The only mutant that behaved as we expected is mutant 38/40G. The 3 tandem glycines from position 38~40 were designed to perturb the helical structure in the hinge region and to facilitate the opening of the N-terminal helical bundle. Far-UV CD data at 222 nm revealed a locally disrupted helical structure and thermal unfolding data indicated a structure that was more susceptible to methionine oxidation. DMPC clearance data demonstrated better lipid binding ability. Thus, all the results for this mutant point to a prone-to-open N-terminal bundle, validating our structure-based mutation design, and our hypothesis that the opening of the N-terminal bundle facilitates lipid binding.

The naturally occurring mutants, R153P and L178P have single proline mutations at the junction of A and B consensus repeat. The α-helical content remained unchanged in these two mutants, but Tm decreased dramatically (49 °C and 44 °C respectively). The
Insertion mutant showed a decreased α-helical content (49%) and thermal stability (Tm 48 °C) due to direct disruption in the H6 B repeat. However, all retained lipid binding ability as demonstrated in the DMPC clearance assay. It is noteworthy that both α-helical content and DMPC clearance rate was measured at 24~25 °C. At this temperature, the overall tertiary structure of these mutants might still be intact, therefore the secondary structure and lipid binding ability could remain unaltered. However, these mutants seemed to be less stable when exposed to high temperature. Thus, at physiological temperature, 37 °C, these mutants might be very unstable and could be quickly catabolized.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>α-Helical Content</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>54 ± 2%</td>
<td>62 ± 2 °C</td>
</tr>
<tr>
<td>38/40G</td>
<td>51 ± 2%</td>
<td>62 ± 1 °C</td>
</tr>
<tr>
<td>38/40G (non-freshly refolded)</td>
<td>-</td>
<td>57 ± 2 °C</td>
</tr>
<tr>
<td>Q41A</td>
<td>54%</td>
<td>62 °C</td>
</tr>
<tr>
<td>P66G</td>
<td>54%</td>
<td>57 °C</td>
</tr>
<tr>
<td>G65P</td>
<td>54%</td>
<td>61 °C</td>
</tr>
<tr>
<td>V67P</td>
<td>54%</td>
<td>61 °C</td>
</tr>
<tr>
<td>T68P</td>
<td>56%</td>
<td>61 °C</td>
</tr>
<tr>
<td>65/67/68P</td>
<td>54%</td>
<td>57 °C</td>
</tr>
<tr>
<td>R153P</td>
<td>52%</td>
<td>49 °C</td>
</tr>
<tr>
<td>L178P</td>
<td>54%</td>
<td>44 °C</td>
</tr>
<tr>
<td>Insertion</td>
<td>49%</td>
<td>48 °C</td>
</tr>
</tbody>
</table>

Table 3-1 Summary of α-helical content and thermal unfolding temperature of apoA-I mutants.
CHAPTER 4 The Function of ApoA-I Mutants in the Cellular System

It is well documented that apoA-I can promote cellular lipid efflux through ABCA1 and produce nHDL particles (Oram & Heinecke 2005). In this chapter, ABCA1-transfected HEK293 cells were used as a model system to study how structural alterations in apoA-I translate into functional changes in both designed and naturally occurring mutants.

4.1 Nascent HDL formation from ABCA1-transfected cells by apoA-I mutant

4.1.1 Mutants designed to destabilize the N-terminal helical bundle: 38/40G, Q41A, P66G

In these experiments, ABCA1- and empty vector (EV) mock-transfected cells were incubated with BODIPY-cholesterol/cholesterol/cyclodextrin complexes or DMSO-solubilized BODIPY-cholesterol according to method described in section 2.5; 4 µg/ml to 10 µg/ml of refolded mutants or WT apoA-I were then added to the cell culture media and incubated overnight (most experiments were done with 10 µg/ml apoA-I, experiments done with 4 µg/ml will be indicated as such). A native 4-15% gel was run at 200V for 1 hour (same method was used in later gels unless otherwise indicated). After transfer of proteins, the membrane was probed by anti-apoA-I polyclonal antibody.

Figure 4-1 shows the nHDL particles formed by mutant 38/40G and WT apoA-I (two parts were cropped from different regions of the same film). ABCA1-transfected groups were in triplicate. Both 38/40G (lane 1-3) and WT (lane 5-8) apoA-I formed nHDL particles from ABCA1-transfected HEK293 cells, but not from EV-transfected cells (lane 4, 8) or when no acceptor was added (lane 9), meaning that this process of particle
formation was specifically mediated by ABCA1 and apoA-I. Based on size, nHDL particles could be classified into the following species: pre-β1 (7.1 nm), pre-β2 (8.2 nm), pre-β3 (10.4 nm), pre-β4 (12.2 nm) and pre-β5 (17 nm) (Nichols et al. 1976). As shown in lanes 1-3 and lanes 5-8, all five species were formed by both mutant 38/40G and WT apoA-I, and the migration positions of particles were the same between the two groups. These data suggested that mutant 38/40G and WT apoA-I probably adopted the same conformation on each nHDL species. However, quantitation of the bands using ImageJ, as shown in Figure 4-2, revealed a 50% increase in particles from mutant 38/40G compared to WT, suggesting that this mutation increased particle formation.

Figure 4-1 nHDL particle formation by apoA-I mutant 38/40G (4 µg/ml). ABCA1-transfected groups were in triplicate; EV: empty vector mock-transfected cells. No acceptor: neither mutants nor WT apoA-I was added.
Figure 4-2 Quantitation of western band signal of nHDL particles formed by mutant 38/40G and WT apoA-I using ImageJ.

Even when proteins were not refolded (Figure 4-3) and WT apoA-I did not make nHDL particles very efficiently, mutant 38/40G could still form nHDL particles very competently. The contrast between 38/40G and WT in making particles was larger under this experimental condition.
Figure 4-3 nHDL particles formed by non-refolded mutant 38/40G and WT apoA-I. ABCA1-transfected groups were in triplicate. EV: empty vector mock-transfected cells. Nc: mutant or WT apoA-I not incubated with cells.

The nHDL particle distribution in mutant 38/40G followed the same pattern as in WT apoA-I (ratio between pre-β1: pre-β2: pre-β3: pre-β4: per-β5 is almost the same in 38/40G and in WT, refolded)(Figure 4-4), meaning that the facilitation of particle formation by 38/40G did not favor a specific pre-β HDL species. This observation suggests two possibilities: 1. Only the formation of the first nHDL species, pre-β1, is the rate-limiting step; once apoA-I has overcome this energy barrier, the transition from pre-β1 to pre-β2-5 happens spontaneously and rapidly; 2. Different pre-β HDL species are
not formed in a sequential manner, which is not likely the case considering lipid-free apoA-I has to adopt different initial conformations in order to make this situation happen.

Figure 4-4 Quantitation of nHDL particle species formed by 38/40G and WT apoA-I, refolded.

The increase in the quantity of nHDL particles formed by mutant 38/40G is consistent with solution studies shown in Chapter 3: mutating amino acid position L38 and K40 into glycine renders a 3x G helical breaker; disrupted local helical structure in the hinge region makes it harder to stabilize the N-terminal helical bundle; within the helical
bundle resides a hydrophobic cluster which will bind to either DMPC in solution or to cholesterol and phospholipid transported by ABCA1 from the cells. Collectively, these results agree with our hypothesis that nHDL formation by apoA-I through ABCA1 requires the opening of the N-terminal helical bundle, and by disrupting the N-terminal hinge region, we are able to facilitate this opening step, thus facilitating nHDL particle formation.

Figure 4-5 shows the studies in which mutant Q41A was tested for nHDL particle formation. Compared to WT, no changes in either the species or number of nHDL particles made were observed. This is also consistent with solution studies which showed that mutation Q41A was not able to destabilize the N-terminal hinge region, therefore was not different from WT apoA-I in nHDL formation. Taken together, mutant 38/40G and Q41A data affirmed that it is the rigid helical structure in amino acid position 37~41, rather than the H-bonds between Q41/Y115 and Q41/R123 that is critical for holding the N-terminal bundle together in free apoA-I.
The mutant designed to perturb the N-terminal bundle at the second hinge, P66G, was also able to make nHDL particles with no apparent difference from WT apoA-I: both migration position and signal intensity of lipidated apoA-I were the same in P66G as in WT. However, free P66G migrated faster than free WT, indicating a conformational change caused by this mutation. Unfortunately, it is hard to evaluate what kind of conformational change has happened based purely on migration position. Solution studies showed retained \( \alpha \)-helical content, DMPC binding property and destabilized overall structure (lower Tm).

In these experiments, DMSO was used to solubilize and deliver BODIPY-cholesterol to cells. 10 µg/ml of apoA-I were used. As shown in Figure 4-7 and Figure 4-8, mutations designed to stabilize the N-terminal hinge region did not change the ability of apoA-I to make nHDL particles. HEK293 cells with basal level cholesterol were used for these experiments. Every nHDL species made by WT apoA-I was also made by the mutants: in this case, pre-β1, pre-β2, and pre-β3. It is tempting to say that there were slight differences in the number of nHDL particles and in the distribution of particle species.
between mutants and WT shown in Figure 4-7 and Figure 4-8, however, unlike in 38/40G, which showed a consistent increase of particle quantity, the differences in G65A, V67P, T68P, and 65/67/68P were averaged out in repeated experiments.

The species of nHDL particles formed in these experiment were slightly different from those previously discussed, because the cholesterol loading method was different from previous experiments. The cholesterol effect on nHDL species will be discussed in Section 4.6.

Figure 4-7 nHDL particles formed from G65A, V67P, T68P. Low exposure. ABCA1-transfected groups were in duplicate. EV: empty vector mock-transfected cells. Nc: mutants or WT apoA-I not incubated with cells.
Surprisingly, 4 tandem prolines from position 65 to 68 did not seem to impair the ability of apoA-I to bind the effluxed lipids to form nHDL. Triple proline mutant 65/67/68P was just as efficient as WT in producing nHDL particles with the same size and in the same quantity. Even free apoA-I migrated at the same position as WT.
Figure 4-9 nHDL formation of mutant 65/67/68P. EV: empty vector mock-transfected cells.

From data for P66G, G65A, V67P, T68P, and 65/67/68P, it seems that the second hinge from aa position G65 to T68, is not crucial in holding the N-terminal bundle together. While the first hinge, A37 to Q41, is most critical in controlling the open-closed conformation of the N-terminal bundle. A rigid helical structure in this region is critical for maintaining a “closed” conformation; while a flexible loop introduced by 38/40G tends to open-up the helical bundle. We speculate that mutations in the hinge region G65~T68, do not break the interaction between the G helix (1~43) and the backbone central Helix 5. In other words, the other end (A37~Q41) of the N-terminal helical bundle is still holding the bundle together. But once the latter is disrupted, the helical bundle is prone to open up.
4.1.3 Naturally occurring mutants R153P and L178P, apoA-I\textsubscript{Nashua} Insertion mutation

Naturally occurring proline mutants R153P and L178P, were also able to make nHDL particles with no noticeable difference from WT apoA-I (Figure 4-10), probably because the destabilizing effect introduced by a proline residue at the junction of an AB repeat was re-stabilized upon lipid binding.

![Image of gel electrophoresis](image.png)

<table>
<thead>
<tr>
<th>ABCA1</th>
<th>EV</th>
<th>ABCA1</th>
<th>EV</th>
<th>ABCA1</th>
<th>EV</th>
</tr>
</thead>
<tbody>
<tr>
<td>R153P</td>
<td></td>
<td>L178P</td>
<td></td>
<td>WT</td>
<td></td>
</tr>
</tbody>
</table>

IB: anti-apoA-I antibody

Figure 4-10 nHDL formation by naturally occurring apoA-I mutants R153P, L178P. ABCA1-transfected groups were in triplicate/quadruplicate. EV: empty vector mock-transfected cells.

The Insertion mutant, on the other hand, exhibited different migration in both lipidated protein and free protein (Figure 4-11). It could form pre-\(\beta\)1 and pre-\(\beta\)2 nHDL particles with similar, if not better efficiency to WT. This result appeared to contradict the
hypothesis of the original case report that this insertion mutation has impaired lipid binding properties (Esther Y Lee et al. 2013). However, our data could only prove that this mutant was able to bind to nHDL lipid (mainly PC and cholesterol), but could not evaluate whether on CE and TG-rich particles, such as mature HDL and chylomicron, it will or will not retain its lipid binding ability.

Figure 4-11 nHDL formation by naturally occurring apoA-I insertion mutant. ABCA1-transfected groups were in triplicate. EV: empty vector mock-transfected cells.
4.1.4 Extending the C-terminus upon 1-184 (1-198, 1-209, 1-220, 1-231, WT)

In these experiments, 5 constructs, each adding one consensus A or B repeat (B-B-A-A-B) upon the previous one, were generated and isolated. Figure 4-12 shows a 12% SDS gel of purified proteins. Migration shift clearly indicated the size differences.

![Figure 4-12 12.5% SDS gel of C-terminal extension mutants stained with Denville Blue Protein Stain.](image)

Previous studies have shown that 1-184 does not promote cholesterol efflux nor make nHDL particles from ABCA1-expressing cells (Chroni et al. 2003). In the following experiment, DMSO was used to solubilized and deliver BODIPY-cholesterol to the cells. nHDL particles were formed with basal level cholesterol in the cells. Figure 4-13 and Figure 4-14 show low and high exposure images of the blot probed with antibody to apoA-I. All the proteins were added at 10 µg/ml concentration; mutant 1-231 was also added at 20 µg/ml (lane 19, 20) and 40 µg/ml (lane 21, 22). 1-184 almost did not get lipidated at all, even in high-exposure image. 1-198, 1-209 and 1-220 barely showed any
lipidation either in low-exposure image, and only minimal lipidation in high-exposure image. In 1-231, slight lipidation started to emerge in low-exposure image, but the band shape was vastly different from pre-β1 particles formed by WT apoA-I. The streaky band looked as if an undefined number of lipid or cholesterol molecules were “associated” with apoA-I (1-231), rather than “packed” into a discrete particle by apoA-I. Increasing the concentration of 1-231 did not help to make more particles, but this limitation was not caused by cholesterol or ABCA1 saturation because WT apoA-I (lane 23, 24) clearly formed much more particles under the same condition. In the high-exposure image, pre-β2, 3, 4 particles formed by 1-231 could be seen. However, the ratio between different species of particles appeared to have changed: with WT, pre-β1 was the dominant species, and the bigger the particles were progressively, the less abundant. With 1-231, the different particle species seemed to be more evenly distributed.
Figure 4-13 nHDL formation by C-terminal extension mutants. Low exposure. ABCA1-transfected groups were in duplicate. EV: empty vector mock-transfected cells. Mutant 1-231 were loaded at different concentrations: 10, 20, 40 µg/ml.

IB: anti-apoA-I antibody
Figure 4-14 nHDL formation by C-terminal extension mutants. High exposure. ABCA1-transfected groups were in duplicate. EV: empty vector mock-transfected cells. Mutant 1-231 were loaded at different concentrations: 10, 20, 40 µg/ml.

IB: anti-apoA-I antibody
Figure 4-15 shows the quantitation of the blot above. This bar graph revealed that the C terminus was necessary for the formation of nHDL particles, and the last 11 amino acids were most critical, because mutants 1-184, 1-209 and 1-220 barely made any particles; mutant 1-231 started to make some, but much less efficiently than WT apoA-I.

4.1.5 Locking monomer conformation (H5 3xG)

The H5 3xG mutant was designed to shift the equilibrium of monomer ⇌ dimer conversion to the monomeric form. Previous studies, including the crystal structure of the dimeric form, were consistent with the double-belt model of HDL. But evidence has suggested that apoA-I could also exist as a monomer (Beckstead et al. 2005; Ji & Jonas...
We hypothesized that when interacting with ABCA1-expressing cells, apoA-I monomers first dimerize and then make particles. With the H5 3xG mutant, which was shown to favor a monomeric state (unpublished data by Dr. Mei), we were able to monitor whether the monomer-favoring mutant would retard nHDL particle formation. Figure 4-16 depicts a blot of H5 3xG mutant and WT probed with anti-apoA-I antibody. Proteins not incubated with cells but incubated in DMEM media at 37 °C overnight were designated as nc; proteins in DMEM media that were neither incubated with cells nor at 37 °C overnight, but were rather kept at 4 °C, were designate as nc*. H5 3xG EV and WT EV are negative controls. Lane 8 and lane 13 of the low exposure image shown in Figure 4-17 demonstrated that same amount (10 µg/ml) of H5 3xG and WT apoA-I were loaded. In the high-exposure image in Figure 4-16, it was clear that H5 3xG (lane 6, 7) formed fewer nHDL particles than WT (lane 11, 12). Quantitation of repeated experiments revealed a 50% decrease in western blot signal of total particles (Figure 4-18). Species of the particles formed by H5 3xG compared to WT were the same: both formed pre-β1 to pre-β5. The distribution of different pre-β species did not seem to change. These results agree with our hypothesis that the monomer-favoring mutant retards nHDL formation. In this experiment, 1-184 was used as a negative control for nHDL formation and also as a marker for monomer and dimer migration position on the gel, since it is already known that 1-184 only dimerizes but does not aggregate and therefore will migrate as two sharp bands on the native gel. Unpublished data by Dr. Mei in the lab have suggested that higher temperature tends convert apoA-I to the monomeric form. Therefore, pure apoA-I and mutants at 10 µg/ml incubated at 37 °C but not with the cells (lane 4, 9, 14), and
those neither incubated at 37 °C nor with the cells (lane 5, 10, 15) were also run on the gel. The C-terminus truncated mutant, 1-184, as predicted, totally formed monomer after incubation at 37 °C (from lane 5 to lane 4). However, with the C-terminus present, even without the cells, both H5 3xG and WT free apoA-I showed streaky bands, indicative of self-association or non-uniform conformation. Therefore, it was challenging to resolve monomer and dimer bands on the gel. Nevertheless, H5 3xG’s tendency to remain as a monomer did have a negative impact on particle formation, possibly by inhibiting opening of monomeric apoA-I to adopt the double-belt dimer conformation needed to make nHDL particles.

![Image of gel showing apoA-I banding patterns](image)

**Figure 4-16 nHDL formation by apoA-I mutant H5 3xG.** High exposure. ABCA1-transfected groups were in duplicate. EV: empty vector mock-transfected cells. Nc: mutants or WT apoA-I not incubated with cells, but at
37°C overnight. Nc*: mutants or WT apoA-I neither incubated with cells nor at 37 °C, but rather were kept at 4 °C.

Figure 4-17 nHDL formation by apoA-I mutant H5 3xG. Low exposure. ABCA1-transfected groups were in duplicate. EV: empty vector mock-transfected cells. Nc: mutants or WT apoA-I not incubated with cells, but at 37 °C overnight. Nc*: mutants or WT apoA-I neither incubated with cells nor at 37 °C, but rather were kept at 4 °C.
4.2 Cellular lipid efflux promotion by mutant forms of apoA-I

In addition to probing nHDL particles, cholesterol efflux was measured for all the mutants. Here BODIPY-cholesterol, a fluorescently-labeled analog of cholesterol, was used as a marker for cholesterol efflux. The efflux of lipids promoted by the mutants were normalized to WT level (as 100%). The method for this assay is described in section 2.5.
4.2.1 Mutants designed to perturb the N-terminal hinge region: 38/40G, Q41A, P66G

Previous in-solution studies shown in Chapter 3 have suggested that mutation Q41A and P66G did not affect alpha-helical content and DMPC clearance rate of apoA-I; in-cell studies also suggested that these two mutants formed the same level of nHDL particles as WT did. All of these data suggested that mutation Q41A and P66G did not seem to have perturbed the N-terminal hinge region or changed nHDL forming ability of apoA-I. Therefore, it was not surprising that these two mutants did not show any changes compared to WT in the cholesterol efflux assay. However, it was surprising that 38/40G, which was able to facilitate nHDL formation, promoted the same cholesterol efflux as WT (Figure 4-19). This important and striking observation will be investigated further in detail in later sections.

Figure 4-19 Cholesterol efflux from apoA-I mutants 38/40G, Q41A, P66G.
4.2.2 Mutants designed to stabilize N-terminal helical bundle G65A, V67P, T68P, and 65/67/68P

For mutants G65A, V67P, T68P, 65/67/68P, because there was no change in nHDL formation, it was expected that the cholesterol efflux level would remain the same, which was indeed the case. As shown in Figure 4-20, all of these mutants yielded the same cholesterol efflux as WT.

![Figure 4-20 Cholesterol efflux from apoA-I mutants G65A, V67P, T68P, and 65/67/68P.](image)

4.2.3 Naturally occurring mutants R153P, L178P, and Insertion mutant apoA-I\textsubscript{Nashua}

Again, for naturally occurring mutants R153P, L178P, cholesterol efflux remained the same, consistent with the nHDL particle formation data of the two mutants. The insertion
mutant proved to be more complicated. Although it seemed to show a slightly better particle forming ability as shown in Figure 4-11, cholesterol efflux level seemed to be the same as WT (Figure 4-21).

4.2.4 Extending the C-terminus upon 1-184 (1-198, 1-220, 1-231, WT)

For the C-terminal extension mutants, cholesterol efflux followed the trend of nHDL formation: longer mutants resulted in more particles and promotion of efflux. These results were consistent with solution studies that longer mutants had faster DMPC clearance rate ((Mei et al. 2016)). It also excluded the possibility that this fluorescence-based efflux detection is not sensitive enough to resolve efflux differences between different mutants.
4.2.5 Locking monomer conformation (H5 3xG)

Similar to 38/40G, H5 3xG showed significant changes in nHDL formation (50% decrease), but promoted the same cholesterol efflux (Figure 4-23). Comparison of cholesterol efflux and nHDL formation by both 38/40G and H5 3xG together in Figure 4-24, demonstrated that the two sets of data did not follow the same trend. Conventional theory holds that cholesterol efflux and nHDL particles are two coupled events: whatever lipid effluxed out by ABCA1 is simultaneously picked up by apoA-I and made into nHDL particles. But counterintuitively, our results disagreed with this understanding. Instead, they suggested that cholesterol efflux and nHDL particle formation might be two separate processes.
Figure 4-23 Cholesterol efflux from H5 3xG

Figure 4-24 Comparison between nHDL particle and cholesterol efflux level of apoA-I mutant 38/40G, H5 3xG and WT. Data were normalized to WT level. In the nHDL particle quantitation graph (right panel), western bands were quantified in ImageJ. Both 38/40G and H5 3xG were significantly different from WT apoA-I. * P<0.05, N=3. *** P<0.001, N=3.
4.3 Presence of ganglioside (GM1) on nHDL particles

Previously, Phillips et al have reported the existence of apoA-I-free “microparticles” from ABCA1-upregulated J774 cells (Duong et al. 2006). We speculated that the microparticles, which, according to Phillips’ work, contains GM1 (a raft domain marker), might explain the seemingly paradoxical phenomenon in Figure 4-24. The same gel that was probed for apoA-I was stripped in 0.2 M glycine pH 2.5 overnight and re-probed with HRP-conjugated cholera toxin B (CTB), which specifically binds to GM1. Mutant 1-184 (as a negative control), H5 3xG and WT apoA-I were used in this experiment. But unexpectedly, instead of finding microparticles containing GM1, we found GM1 on all nHDL particles. As shown in Figure 4-25, free apoA-I was free of GM1, but all lipidated apoA-I particles contained GM1 and the intensity of the GM1 signal in H5 3xG and WT followed the pattern of apoA-I signal intensity: Weaker apoA-I signal (H5 3xG) correlated with weaker GM1 signal (H5 3xG).

Because particles at the same migrating position should have the same size and charge, thus the same number of protein (likely the same conformation) and lipid molecules, probing GM1 with CTB is a further validation that the number of particles formed did change in H5 3xG; differences in apoA-I signal was not the result of antibody sensitivity issues.
Plasma HDL were also probed for GM1 to test whether the presence of GM1 was an artifact (Figure 4-26). 1 µg (1x), 2 µg (2x) and 20 µg (20x) plasma HDL and 15 µl of cell culture media after overnight incubation with WT apoA-I were resolved by a 4-15% native gel, transferred onto a membrane and probed with anti-apoA-I antibody and with CTB to detect GM1. In this 1D gel, plasma HDL ran as a streaky band spanning from ~7 nm to ~10 nm position. When probed with apoA-I antibody, the signal was very strong relative to the cell culture media. When probed with CTB, the signal still appeared at the same position, but with much lower intensity relative to cell culture media. These observations showed that GM1 does exist on plasma HDL particles and the GM1 we detected in the cell culture media were not an artifact. However, apoA-I to GM1 ratio was much lower in plasma HDL than in cell culture media. Since the cellular system we used produces only pre-β HDL, while plasma HDL is mostly α-HDL and the lipids and
proteins have gone through many rounds of exchanges with other lipoprotein particles, it is possible that only nascent HDL, when first originating from the raft domain on the cell membrane, carries GM1 with it.

![Image](image.png)

**Figure 4-26 Presence of GM1 on plasma HDL.**

### 4.4 Lipid analysis of efflux media promoted by 38/40G and WT

As shown in previous sections, there appeared to be a discrepancy between nHDL formation and cholesterol efflux. To resolve this paradox, we further investigated how mutant 38/40G is different from WT in making nHDL particles. First, a time-course experiment was carried out for 38/40G and WT apoA-I. Following addition of acceptor
proteins media were collected at time points 2.5h, 5h, 7.5h, 12h and 24h. As shown in Figure 4-27 (the two parts were cropped from different areas of the same film), For mutant 38/40G, some particles were observed at 2.5h, while in WT no particles were visible. At 12h, the majority of 38/40G was lipidated, but the WT, was largely unlipidated. After 24h incubation time, almost all 38/40G was made into nHDL particles, but WT still had a significant amount of free protein left. Overall, consistent with previous results, 38/40G formed more nHDL particles than WT did after 24h. Moreover, 38/40G had a faster kinetics in making particles, further supporting our hypothesis that destabilization of the N-terminal hinge facilitates opening of N-terminal helical bundle, thereby facilitating nHDL particle formation.

It is also noteworthy that different species of nHDL particles were formed concurrently. All species of particles started to emerge at the same time. With time, the quantity of each species of particle appeared to increase at the same rate.
Figure 4-27 Time course experiment of apoA-I mutant 38/40G (4 µg/ml).

Figure 4-28 is quantitation of the blot in Figure 4-27. The plot of lipidation level versus time fitted almost into a straight line, suggesting that lipidation was happening at a steady rate during the 24 h incubation time. 38/40G had a steeper average slope and higher end point compared to WT.

Surprisingly, the cholesterol efflux versus time (Figure 4-29) suggested that efflux almost plateaued as soon as 8 hours. Summarizing, after 8 h cholesterol efflux almost maximized and remained constant, but nHDL particles continued to form until 24 h. Apparently, there was a lag time between nHDL formation and cholesterol efflux. In other words,
maximum cholesterol efflux preceded nHDL particle formation. This discrepancy in cholesterol efflux kinetics and nHDL formation kinetics could partially explain the seemingly paradoxical finding that different levels of apoA-I lipidation could occur with the same cholesterol efflux in the media. Because cholesterol efflux and nHDL formation might be uncoupled processes, they might also be controlled by different segments of apoA-I. Therefore, it is possible for apoA-I mutant forms to retain the ability to promote cholesterol efflux, yet affect the nHDL formation (e.g. 38/40G, H5 3xG).

Figure 4-28 Time course of nHDL formation from apoA-I mutant 38/40G. Quantitation of western bands was done in ImageJ. For both 38/40G and WT apoA-I, intensity of total band signal, $I_{tot}$ (including both lipided apoA-I and free apoA-I) after 24h incubation was taken as 100%. % Lipidated apoA-I was calculated as intensity of lipided apoA-I (nHDL particles) at each time point ($I_p$) over intensity of total signal after 24h: $\%$ lipidated apoA-I=$I_p/I_{tot}$x 100%.
Figure 4-29 Time course of cholesterol efflux promoted by apoA-I mutant 38/40G. % Cholesterol efflux was calculated as fluorescence in media at each time point ($F_m$) over total fluorescence after 24h-incubation (fluorescence in media and in cells): $\%\text{efflux} = F_m/(F_m + F_c)$. %Efflux at each time point was then normalized to maximum efflux level at 24h (taken as 100%). Data of each time point were in triplicate.

Lipids from efflux media after 24 hours were extracted and analyzed by thin layer chromatography (Figure 4-30).

As shown in lane 2 and lane 3, cholesterol efflux promoted by 38/40G and WT was the same, consistent with our fluorescence measurement. This is a further validation that the fluorescence-based essay is a valid method and that cholesterol efflux and apoA-I lipidation could be two separate processes. However, although cholesterol levels were the same for 38/40G and WT, PC and sphingomyelin (SM) levels might have changed, indicating that a pool of lipid that differs from nHDL in lipid composition might also exist in media. We also observed that even without apoA-I as the lipid acceptor, ABCA1
was still able to transport some cholesterol from the cells into the media, which is consistent with previous literature documenting “apoA-I-independent, ABCA1-depend cholesterol efflux” (Chroni et al. 2003) This is also consistent with our efflux data showing that ABCA1-transfected cells with no apoA-I as lipid acceptor always yielded higher signal than empty vector transfected cells. This apoA-I-free, cholesterol-containing lipid population, might explain the discrepancy between cholesterol efflux level and nHDL particle quantities.

Figure 4-30 TLC plate of total lipids from efflux media after 24h incubation. Marker 1 (top to bottom: phosphatidylethanolamine(PE), PC, phosphatidic acid(PA)); marker 2 (top to bottom: CE, FC, SM). Lipids were made visible by iodine stain.
To investigate this apoA-I-free lipid fraction in more detail, efflux media after 24 hours was subjected to gradient-ultracentrifugation to fractionate particles by density. The method of gradient ultracentrifugation was described in section 2.8.1. Figure 4-31 shows the density distribution in 1 ml fractions after ultracentrifugation. The 1.05 g/ml to 1.3 g/ml range covers the density of different nHDL particles. There was no difference in density distribution between mutant 38/40G- and WT apoA-I-containing efflux media.

![Density Distribution Graph](image)

Figure 4-31 Gradient ultracentrifugation fractions of efflux media after 24h incubation plotted by density.

Figure 4-32 shows the western blot of density fractions probed by anti-apoA-I antibody. The last lane was efflux media before ultracentrifugation as a reference. The density of
each fraction agreed with apoA-I or nHDL species of that fraction observed on the western blot. From the high to low density: fractions 9 to 13 had densities from 1.22 g/ml to 1.3 g/ml and showed only lipid-free apoA-I on the blot; fractions 6 to 8 had densities from 1.15 g/ml to 1.21 g/ml and mainly showed pre-β1 on the blot; fraction 3 and 4 had densities from 1.07 g/ml to 1 g/ml and showed pre-β2 and pre-β3 on the blot. Fraction 1 and 2 barely showed any apoA-I and had a density lower than 1.06 g/ml.

![Figure 4-32 Gradient ultracentrifugation fractions of WT efflux media probed by apoA-I antibody.](image)

In order to better understand the composition in different fractions, each fraction was then analyzed by dot blot and probed for apoA-I and GM1. Figure 4-33 shows the dot blot probed for apoA-I. In this blot, media from cells transfected with ABCA1 but not incubated with apoA-I was designated as “no acceptor” and did not show any signal. In empty vector transfected groups, both 38/40G (row 3) and WT (row 5) showed signal from fraction 7 to fraction 13, consistent with the finding that these fractions contained
lipid-free apoA-I (see blot Figure 4-32). In media collected from ABCA1-transfected cells (row 2 and 4), the apoA-I started to show up from fraction 2 or 3. The signal in fraction 2 to 6 of row 2 and 4 (compared to row 3 and 5) suggested that apoA-I had become less dense as a result of lipidation. Notably, fraction 1 had absolutely no apoA-I.

Figure 4-33 Dot blot of gradient ultracentrifugation fractions probed by anti-apoA-I antibody. No acc: no accepter added.
The blot was stripped and probed with CTB to identify GM1 (Figure 4-34). Unlike the distribution of apoA-I, GM1 mainly resided in lower-density fractions. From fraction 10+ (density > 1.23 g/ml), there was barely any GM1 content. Although empty vector transfected groups (row 3 and 5) also showed some signal, the signal intensity was much lower than in ABCA1-transfected groups (row 2 and 4). This empty vector signal was subtracted as baseline in the later calculations. Importantly, the first fraction, where there was no apoA-I at all, showed very significant GM1 content, meaning that there was indeed an apoA-I-free lipid population.
Fluorescence in each fraction was also read to measure cholesterol (BODIPY-cholesterol) level (Figure 4-35). The no acceptor group showed no cholesterol from fraction 2+, but considerable cholesterol in fraction 1, the apoA-I free fraction. Mutant 38/40G and WT showed slightly higher cholesterol content in fraction 1 than no acceptor, and peak cholesterol level in fraction 3, 4, 5. Fractions 2, 6, 7 had some cholesterol. Almost no cholesterol could be detected in fractions 8-13.
Figure 4-35 Cholesterol distribution in gradient ultracentrifugation fractions. Fraction densities (g/ml): f1: <1.06; f2: 1.060; f3: 1.073; f4: 1.097; f5: 1.129; f6: 1.153; f7: 1.179; f8: 1.184; f9: 1.219; f10: 1.228; f11: 1.247; f12: 1.271; 1.293.

In Figure 4-36, a dot blot probed for apoA-I (Figure 4-33) and GM1 (Figure 4-34) were quantified (both calculated as a fraction of total apoA-I or GM1 after baseline subtraction of 38/40 EV and WT EV signal), plotted and aligned with cholesterol distribution in each fraction. Fraction 3 to 7 had peaks in cholesterol, apoA-I and GM1, agreeing with the western blot data (Figure 4-32) that these fractions contained nHDL particles. Fraction 8 to 13 contained mainly lipid-free apoA-I, and therefore, were low in cholesterol and GM1 content. However, surprisingly, fraction 1 contained no apoA-I, but was cholesterol- and GM1- abundant, indicating that this low-density fraction was a protein-free lipid mixture. Furthermore, this population was also present in ABCA1-transfected cell culture media.
without apoA-I addition, but not in empty vector transfected cell culture media with apoA-I addition, indicating that the generation of this lipid population was ABCA1-dependent but apoA-I independent.

This method, however, was not quantitative enough to resolve the difference in cholesterol level in apoA-I-free lipid fraction of 38/40G and WT.
Figure 4-36 Comparison of apoA-I, cholesterol and CTB distribution from gradient ultracentrifugation fractions from efflux media of mutant 38/40G and WT apoA-I. Fraction densities (g/ml): f1: <1.06; f2: 1.060; f3: 1.073; f4: 1.097; f5: 1.129; f6: 1.153; f7: 1.179; f8: 1.184; f9: 1.219; f10: 1.228; f11: 1.247; f12: 1.271; f13: 1.293.
4.5 Effect of apoA-I concentration on nHDL formation and cholesterol efflux promotion

Figure 4-37 compares the level of apoA-I lipidation when cells are incubated with high (10 µg/ml) or low (4 µg/ml) concentration of apoA-I (most of previous experiments were carried out using 10 g/ml apoA-I). This blot (two parts were cropped from different areas of the same film) shows that for cells loaded with cholesterol (pre-treated with 1 µM cholesterol/β-methyl-cyclodextrin for 1 h), at 4 µg/ml, the majority of apoA-I were lipidated; while at 10 µg/ml, the majority of apoA-I remained unlipidated, and the quantity of nHDL particles was similar to that in the 4 µg/ml group, maybe only slightly elevated. ApoA-I concentration did not have an impact on nHDL species being made, either.
Similar results were observed in cholesterol efflux measurement. When apoA-I < 4 \( \mu g/ml \), efflux increased as apoA-I concentration increased; > 4 \( \mu g/ml \), efflux remained the same even when apoA-I concentration reached 10 \( \mu g/ml \).
4.6 Effect of cholesterol level on nHDL formation and cholesterol efflux promotion

Cholesterol level within the cells also affects apoA-I lipidation level. Figure 4-39 (two parts were cropped from different regions of the same film) depicts an experiment in which the lipidation state of apoA-I under different cellular cholesterol levels was analyzed. In cells loaded with cholesterol (pre-treated with 1 μM cholesterol/β-methyl-cyclodextrin for 1 h) (designated as “Ch-loaded”), more nHDL particles were formed,
with all apoA-I (3 µg/ml) being lipidated (lane 4, 5), while in cells with basal level of cholesterol, fewer nHDL particles were formed and a significant amount of apoA-I remained unlipidated (lane 1, 2). Furthermore, cells loaded with cholesterol yielded larger nHDL species. In addition to pre-β1 and pre-β2 formed from basal cholesterol cells (lane 1, 2), pre-β3, pre-β4 and pre-β5 were also formed from cholesterol-loaded cells (lane 4, 5).

Figure 4-39 Effect of cholesterol level on nHDL formation. Ch-loaded: cells were pre-treated with 1 µM β-methyl-cyclodextrin for 1h.
From the data in Figure 4-39, Figure 4-37 and Figure 4-27, it appears that the species of nHDL particles formed were only affected by cellular cholesterol level (the more cholesterol, the more species formed), but not affected by apoA-I concentration, or incubation time. As shown in Figure 4-39, although the overall lipidation level was lower in the basal level cholesterol group (lane 1, 2) than in the cholesterol-loaded group (lane 4, 5), pre-β1 seemed to be as abundant as in the basal level group. Without enough cholesterol, nHDL particles seemed to have been stuck in pre-β1 stage, suggesting that there might be a relatively high energy barrier for pre-β1 to transition into pre-β2 nHDL.

DMSO is not as efficient as cyclodextrin in delivering cholesterol (or BODIPY-cholesterol) to the cells. Therefore, DMSO solubilized BODIPY-cholesterol was used to test efflux level from cells with basal level cholesterol, and cyclodextrin/biodipy-cholesterol complex was used to measure efflux from cells loaded with cholesterol. The relative efflux level between mutant forms of apoA-I did not change upon changing cholesterol loading method, but the absolute value of efflux did change. As expected, with DMSO-BODIPY-cholesterol treated cells, cholesterol efflux, calculated as fluorescence in the media / total fluorescence (signal in the media + signal in the cells) was about 8% in WT; with cyclodextrin/BODIPY-cholesterol treated cells, the absolute efflux level could be up to 40%.
Figure 4-40 Effect of cholesterol level on efflux level.
4.7 Summary

One of the major functions of apoA-I is to take up lipids (mainly cholesterol and PC) transported by ABCA1 and pack them into nHDL particles. In this chapter we discussed nHDL formation and cholesterol efflux promotion by structurally-designed and naturally-occurring mutants of apoA-I. We also discussed various factors other than apoA-I mutation, such as apoA-I concentration, cholesterol level in the cells, that may affect nHDL formation and cholesterol efflux.

Out of all the designed mutants tested, Q41A, P66G, G65A, V67P, T68P and 65/67/68P did not show significant changes in either nHDL formation or cholesterol efflux promotion, consistent with solution studies shown in Chapter 3 that all of the mutants above cleared DMPC at the same rate, indicating equal lipid binding abilities. The following mutants have shown significantly different nHDL forming ability or cholesterol promoting ability:

- 38/40G designed to perturb the N-terminal hinge region: Better nHDL forming ability (50% increase in the quantity of particles), but counterintuitively, unchanged cholesterol efflux promoting ability. Time-course experiments revealed that 38/40G had a faster kinetics in formation nHDL particles.

- H5 3xG designed to lock monomer conformation: nHDL particle formation dropped 50% compared to WT, and again, unchanged cholesterol efflux.

- C-terminal extension mutants: the progressively-elongated constructs exhibited progressively increased efflux and nHDL formation, and the last 11 amino acids (B consensus repeat) were the most critical part in the C-terminus.
For naturally occurring mutants, surprisingly, none of them (R153P, L178P, Insertion mutant) seemed to have impaired nHDL forming or efflux promoting ability. Thus, the phenotype caused by these mutants might be associated with their instability and consequently high turnover rate in the circulation, or with dysfunctions in activities downstream of nHDL biogenesis in the reverse cholesterol pathway.

Both nHDL formation and cholesterol efflux were positively correlated with cholesterol levels in the cells, and higher cholesterol level tends to yield larger particles, which is to be expected.

ApoA-I concentration, under our cholesterol-loaded condition, reached maximum functionality at 4 µg/ml; after 4 µg/ml, increasing apoA-I concentration did not increase nHDL particle formation or cholesterol efflux anymore.

We also found that there was GM1 (raft domain marker) on all pre-β HDL particles, and that GM1 was also present on plasma HDL, which only contains trace amount pre-β nHDL, indicating that nHDL might originate from the raft domain.

One of the most surprising and important findings in this study is that nHDL formation, did not always follow cholesterol efflux level, as was shown for both 38/40G and H5 3xG: 38/40G was able to make more nHDL particles and H5 3xG fewer particles than WT was, yet both promoted the same efflux as WT. Also in the time-course experiment that compared the kinetics of nHDL formation and cholesterol efflux promotion, we found that there was a lag time between maximum efflux and maximum nHDL formation, which means cholesterol efflux preceded nHDL formation. This phenomenon
does not agree with the conventional idea that nHDL formation and cholesterol efflux are
two coupled processes; all the cholesterol that is pumped out by ABCA1 is taken by
apoA-I to make nHDL particles. Therefore, we further analyzed the efflux media to track
cholesterol, apoA-I, and GM1 distribution after gradient ultracentrifugation. We found
that, in the lowest density fraction, there was a lipid population that contains both
cholesterol and GM1, but no apoA-I. We speculate that this apoA-I-free lipid fraction
was what could fill the gap between cholesterol in nHDL and total cholesterol efflux.
Unfortunately, we were not able to quantitatively determine the difference in this apoA-I-
free lipid fraction promoted by mutant 38/40G and WT apoA-I.
CHAPTER 5 Interactions Between ApoA-I and ABCA1

5.1 Interactions between apoA-I and ABCA1 in cells

WT apoA-I was cross-linked to HEK293 cells using 2.5% paraformaldehyde as described in section 2.11. A “no-apoA-I-added” group was subtracted as baseline and WT apoA-I cross-linked to ABCA1-expressing, cholesterol-loaded cells was taken as 100%. Figure 5-1 shows that in both basal level cholesterol and cholesterol-loaded groups, more apoA-I was cross-linked to ABCA1-transfected cells than to empty vector-transfected cells, suggesting that cross-linking of apoA-I to cells was dependent on ABCA1 expression. Furthermore, in ABCA1-expressing cells loaded with cholesterol (e.g. treated with 1 µM cholesterol/Methyl-β-cyclodextrin complex for 1 h), the amount of apoA-I cross-linked to the cells was approximately 2 fold the amount of apoA-I cross-linked to ABCA1-expressing cells with basal level cholesterol. It has been reported that cholesterol level regulates ubiquitination and degradation of ABCA1 (Hsieh et al. 2014). In order to rule out the possibility that the difference in cross-linking level was the result of different ABCA1 levels in the cells, we probed for ABCA1 in cells with different cholesterol levels. In a parallel experiment, instead of being cross-linked to apoA-I by paraformaldehyde, cells with different cholesterol levels were lysed and probed for ABCA1. As shown in Figure 5-2, the ABCA1 level was the same in basal level cholesterol and cholesterol loaded groups. Therefore, the difference in cross-linking was not caused by different ABCA1 levels, but rather caused by different binding efficiencies.
Figure 5-1 ApoA-I cross-linking to HEK293 cells containing different level of cholesterol. No-apoA-I-added group was subtracted as baseline and WT apoA-I cross-linked to ABCA1-expressing cells with basal cholesterol level was taken as 100%.

Figure 5-2 Cholesterol level does not affect ABCA1 level in the cells. Ch loaded: cells were pre-treated with 1 μM cholesterol/Methyl-β-cyclodextrin complex for 1 h.
This increase in cross-linking level was not seen in EV-transfected cells (Figure 5-1), suggesting that the result was not caused by cholesterol level alone, but that it was ABCA1-dependent. However, there are two possibilities to account for this result: 1. ApoA-I was directly cross-linked to ABCA1, and because of the higher abundance of cholesterol, ABCA1 was more actively interacting with apoA-I in cholesterol-loaded cells; 2. ABCA1 was causing localized changes in the cholesterol-rich membrane that could bind apoA-I, e.g. the creation of a highly curved membrane surface, as proposed by Phillips (Vedhachalam et al. 2007).

Figure 5-3 illustrates the results of studies in which apoA-I mutants 1-184, 38/40G and H5 3xG were used for cross-linking. Empty vector-transfected with “no-apoA-I-added” group was subtracted as baseline and WT apoA-I cross-linked to ABCA1-transfected cells was taken as 100%. All mutants were cross-linked to ABCA1-transfected cells, but at different levels. The mutant 38/40G was most efficiently cross-linked to the cells, followed by WT, H5 3xG, and 1-184, which was the least efficiently cross-linked to cells. This trend was positively correlated with the trend in nHDL particle formation of these mutants, which was not surprising considering that apoA-I is likely to be cross-linked to the cells when they are forming nHDL particles. However, the difference in cross-linking between mutants and WT (about 20% increase in 38/40G and 15% decrease in H5 3xG) was smaller than the difference in nHDL particle formation (about 50% increase in 38/40G and 50% decrease in H5 3xG), probably because nHDL particles were measured after overnight incubation and thus represented the accumulation of many rounds of
apoA-I/cell interactions, whereas the cross-linking results were just a snapshot of a momentary event. Interestingly, 1-184, which could barely form any nHDL particles due to its inability to promote efflux, showed significant amount of binding determined by cross-linking (about 60% of WT).

These results, however, could not distinguish between apoA-I directly binding to ABCA1 and apoA-I binding to membrane macrodomains remodeled by ABCA1. In a co-immunoprecipitation (Co-IP) experiment where ABCA1 was pulled down with antibodies to ABCA1 and probed for apoA-I, we were unable to detect apoA-I binding to ABCA1. Other cross-linking studies have shown apoA-I cross-linked to ABCA1 by a 3 Å cross-linker (Chroni, Liu, et al. 2004). However, they still cannot exclude the possibility that apoA-I is binding lipid in close proximity to ABCA1. To further investigate this
issue, we next isolated ABCA1 from cells and probed for its interaction with apoA-I in solution.

5.2 Expression and function verification of ABCA1-Rho1d4

In order to isolate ABCA1, a Rho1d4 tag (Hodges et al. 1988) was introduced to the C terminus of ABCA1 in pcDNA1 (ABCA1-rho1D4). The resulting sequence was verified by Genewiz. We confirmed that the new construct was correctly folded and properly functioning when expressed in HEK293 cells. As shown in Figure 5-4, nHDL particles made by ABCA1-rho1D4 were identical in size and distribution to those formed by ABCA1 without the tag, suggesting that the addition of the rho1D4 tag did not affect the function of ABCA1 in making nHDL particles.

Figure 5-4 Western blot of nHDL particles formed from ABCA1-Rho1d4-expressing HEK293 cells after overnight incubation.
5.3 Purification of ABCA1

A large-scale mammalian expression system was established. Purification procedures were carried out according to the method described in the section 2.9. SDS-PAGE of purified ABCA1 treated with reducing agent (DTT) illustrated in Figure 5-5 showed a sharp single band with apparent MW of slightly more than 250 kD, consistent with the size of ABCA1 monomers based on the calculated molecular weight and previous literature (Takahashi et al. 2006). This band was confirmed to be ABCA1 by mass spectrometry. As shown on the gel, the elution fractions were very clean after this single-step affinity purification.

![Image of SDS-PAGE](image.png)

Figure 5-5 SDS-PAGE of rho1d4-antibody-purified ABCA1 from HEK293F cells. Two fractions were eluted from the rho1d4-antibody-conjugated beads by 500 µM rho1d4 peptide.

However, in the absence of reducing reagent, even in SDS-PAGE, it was much harder to observe ABCA1 monomers. Instead, faint bands appeared at upper areas (circled in yellow) of the gel (Figure 5-6 left panel, lanes 7, 8). In samples treated with DTT (lanes
10, 11), the bands shifted to monomer position (circled in red), indicating the shifted migration was probably the result of the formation of intermolecular disulfide bonds. We suspected that the upper bands were ABCA1 dimers (unfortunately the maximum size in the marker was 250 kD, therefore could not indicate the dimer position).

Western blots probed with anti-ABCA1 antibody confirmed the observations from the gels. As shown in Figure 5-6 right panel, eluted fractions treated with DTT (lane 10, 11) migrated as single bands at 250 kD position, corresponding to ABCA1 monomers. In elution fractions not treated with DTT (lane 7, 8), monomers did not exist. Instead, the majority seemed to be dimers. Moreover, higher order oligomers or bigger aggregates that could not enter the gel were observed. These oligomers or aggregates were formed from the beginning cell lysate (lane 1) of the purification process, and existed until the end. Previous studies have shown that ABCA1 forms intramolecular disulfide bonds (Bungert et al. 2001), but no evidence has suggested intermolecular disulfide bonds. Previous studies have also suggested that ABCA1 may function as a dimer (Nagata et al. 2013) or even oligomers (Denis et al. 2004). However, these dimer or oligomers should not be covalently linked because they were shown to be reversible. Considering that there are 39 cysteine residues in ABCA1, it is possible that the oligomers and aggregates were the result of SDS denaturing, which artificially exposed cysteine residues and made them available to form intermolecular disulfide bonds under non-native conditions.
Amphipol 8-35 was then used to replace DDM for solubilizing ABCA1. Both amphipol and DDM solubilized ABCA1 were analyzed on a 5% native gel (Figure 5-7). When no reducing agent was added during the purification process, as shown in lane 2 and 3, both solubilization methods yielded multiple discrete bands, corresponding to different oligomeric states. The lowest band showed an apparent MW of more than 480 kD indicated by the marker. However, since the marker represents the size of perfect globular proteins, and a native gel is usually more sensitive to highly glycosylated proteins like ABCA1, it is unlikely that monomer ABCA1 will run at 250 kD on a native gel. Furthermore, ABCA1 were bound to amphipol and DDM molecules which could
further add mass to the protein. Therefore, we suspect that the lowest band, although appearing to be the size of a dimer, was actually a monomer. Unlike in the SDS gels, adding reducing agent after the purification process did not help to break higher order oligomers into monomers (compare lane 5 and 6 to lane 2 and 3), suggesting that these oligomers were not formed via disulfide bonds, or that they were formed by disulfide bonds, but these bonds were not exposed at the surface and thus were not accessible to reducing agent. Amphipol solubilized ABCA1 tended to form higher order oligomers with better defined sizes compared to DDM solubilized ABCA1, which tended to keep the protein as monomers, but with less defined sizes. These observations agree with the suggestions that amphipols are not good for solubilizing membrane proteins, but good at keeping them in a stable state (Zoonens & Popot 2014).

Figure 5-7 5% Native-PAGE of purified ABCA1 in amphiphil and in DDM.
When 2 mM TCEP, a reducing agent, was added throughout the purification process, the majority of ABCA1 appeared as monomer on the native gel, as shown in Figure 5-8. This could indicate that the oligomers shown in Figure 5-7 were formed through disulfide bonds, but once formed during an oxidative purification process, these bonds were buried inside and thus were not accessible to reducing agent any more.

Figure 5-8 5% Native-PAGE of purified ABCA1 in amphipol. Lane 1, 2, 3 are three fractions eluted from the rho1d4-antibody-conjugated beads by 500 µM rho1d4 peptide.

Figure 5-9 shows the ATPase activity of the ABCA1 oligomer mixture solubilized in 0.02% DDM. It is evident that compared to the control group, ABCA1 in DDM exhibited significant ATPase activity, affirming that the protein was correctly folded during
purification. However, we were unable to tell exactly which specific oligomer was responsible for this activity.

![Figure 5-9 ATPase Activity of ABCA1 in 0.02% DDM. ATP hydrolysis driven by ABCA1 was taken as 100%. Basal level: ATP self-decay.](image)

5.4 Interaction between ABCA1 and apoA-I in solution

5.4.1 Interactions between DDM-solubilized ABCA1 and apoA-I

To investigate the interaction between apoA-I and ABCA1, an in-solution binding experiment was carried out. In addition to WT apoA-I, mutants 1-184 and 38/40G were also used. ApoA-I and the mutants were added at 5 µg/ml, similar to the concentration used in our cell-based experiments. ABCA1 solubilized in 50 mM HEPES 150 mM NaCl pH 7.4 with 0.02% (~ 2x cmc) DDM with or without DTT was added at ~50 µg/ml
(about 1:1 molar ratio to apoA-I) and incubated at room temperature for 1 hour. In all groups 1 mM ATPMg\(^{2+}\) was supplemented. The solution was then run on a 4-15% native gel and probed with apoA-I and ABCA1 antibody sequentially. As shown in Figure 5-10 lane 1-3, before adding DDM-containing buffer, both mutants and WT apoA-I showed monomer and dimer bands on the blot (low exposure on the left). When DDM-containing buffer was added (lane 4-6), all of the protein appeared at the dimer position. We suspected that these bands were apoA-I dimers rather than apoA-I monomers with DDM molecules bound, because monomeric apoA-I should not have sufficient exposed hydrophobic surface to bind to that many detergent molecules. Therefore, DDM seemed to have stabilized apoA-I as dimers. In lane 7-9 and 10-12, ABCA1 in DDM with or without DTT was incubated with apoA-I and mutants. The right panel shows the same blot probed with anti-ABCA1 antibody (lane numbers are marked with apostrophe). Lanes 7'-12' indicated the position of ABCA1 on the gel. However, at the corresponding positions, no apoA-I signal was detected (lane 7-12 on the left panel), suggesting apoA-I was not in complex with ABCA1. DTT nor apoA-I mutations made a difference in terms of binding.
Figure 5-10 Native-PAGE of apoA-I incubated with ABCA1 solubilized in 0.02% DDM. Lane number with apostrophe represents the same blot probed with anti-ABCA1 antibody. Lane 1-3, apoA-I and mutants in PB buffer. Lane 4-6, apoA-I and mutants in PB buffer with 0.02% DDM. Lane 7-9, apoA-I and mutants in 0.02% DDM solubilized ABCA1. Lane 10-12, apoA-I and mutants in 0.02% DDM solubilized ABCA1 supplemented with reducing agent DTT.

5.4.2 Interactions between amphipol-solubilized ABCA1 and apoA-I

To exclude the interference of detergent micelles, DDM was exchanged with amphipol, which is supposed to bind membrane proteins with such a high affinity that no amphipol micelles were needed in the surrounding solution. This behavior allowed us to minimize apoA-I’s interaction with anything else but ABCA1. In this way, we were able to show a direct interaction between apoA-I and ABCA1 in solution for the first time. In these experiments, ABCA1 in elution buffer (50 mM HEPES, 150 mM NaCl, 500 µM rho1d4 peptide, pH 7.5) was added at around 150 µg/ml and apoA-I in elution buffer was added
at 15 µg/ml (molar ratio 1:1). Figure 5-11 shows a blot probed with antibodies to apoA-I followed by antibodies to ABCA1.

All the samples were incubated at room temperature for 1 hour. When probed with anti-apoA-I antibody (left panel), apoA-I alone (lane 1) only appeared at the lower half of the blot, showing the same migration pattern as apoA-I in PB, suggesting that the rho1d4 peptide did not affect apoA-I conformation, and that apoA-I was likely to be in a monomer-dimer equilibrium. After incubation with ABCA1, a small fraction of apoA-I was seen at ABCA1’s position (lane 6). Recent work by Gulshan (Gulshan et al. 2016) suggested that PIP2 could induce apoA-I binding to ABCA1 in the cells. Therefore, we added PIP2 to apoA-I and ABCA1 mixture to test whether PIP2 would enhance the binding in solution. Instead of an enhanced binding, we found that PIP2 impaired apoA-I binding to ABCA1 (lane 7) in solution. We suspect that this observation could be caused by a “detergent-like” effect from PIP2 sodium salt used in the experiments. Because apoA-I appeared at the position of a supposed ABCA1 monomer (slightly above the 440 kD), it is likely that ABCA1 monomer was able to bind to apoA-I, rather than as proposed by Ueda’s group that it is ABCA1 dimerization that drives apoA-I binding (Nagata et al. 2013). However, due to the low resolution of the gel, we were not able to evaluate whether it was apoA-I monomer or dimer that was binding to the ABCA1 monomer based on shift in migration.
Figure 5-11 Native-PAGE of apoA-I incubated with amphipol solubilized ABCA1. ApoA-I was at 15 µg/ml, ABCA1 at 150 µg/ml, PIP2 was added at 57 µg/ml (100:1 molar ratio to apoA-I). All the samples were in elusion buffer. Lane number with apostrophe represents the same blot probed with anti-ABCA1 antibody. Lane 1, apoA-I alone. Lane 2, ABCA1 alone. Lane 3, PIP2 alone. Lane 4, apoA-I + PIP2. Lane 5, ABCA1 + PIP2. Lane 6, apoA-I + ABCA1. Lane 7, apoA-I + ABCA1 + PIP2.

Binding of mutants of apoA-I to amphipol-solubilized ABCA1 were also tested. Similar conditions were used as described earlier. As shown in Figure 5-12, when incubated with ABCA1, all mutants of apoA-I showed up at the position where ABCA1 migrated (above 440 kD) (lane 3, 6, 9, 12), with mutant 1-184 having the weakest binding level. This result is in accordance with our cell-based cross-linking experiments, in which mutant 1-184 also showed the lowest cross-linking to ABCA1-expressing cells. Quantitative binding levels of 38/40G and H5 3xG were hard to estimate based on the blot. One noteworthy observation was that amphipol was having an effect on apoA-I. When 15 µg/ml apoA-I was incubated with 150 µg/ml amphipol, apoA-I shifted from dimer to monomer (lane 4 to 5, lane 7 to 8, lane 10 to 11). The same pattern applied to all
the mutants except for 1-184. This pattern, however, did not seem to indicate that amphipol was binding to apoA-I, because the molecular weight of apoA-I monomer did not increase when incubated with amphipol (lane 5, 8, 11), but rather, amphipol seemed to have remodeled apoA-I into its monomeric state.

Figure 5-12 Native-PAGE of apoA-I mutants incubated with amphipol solubilized- ABCA1. ApoA-I was added at 10 µg/ml in elusion buffer, amphipol 10 µg/ml, ABCA1 100 µg/ml. Lane number with apostrophe represents the same blot probed with anti-ABCA1 antibody. Lane 1, apoA-I mutant 1-184. Lane 2, 1-184 + amphipol. Lane
Previous studies by Ueda have suggested that ATP hydrolysis-induced conformational changes in the extracellular loop of ABCA1 is required for apoA-I binding to ABCA1 (Nagao et al. 2011). In order to test whether ATP affects binding in solution, we also added ATP into the incubation mixture and found no significant difference from no-ATP group (Figure 5-13).

Figure 5-13 Effect of ATP on apoA-I binding to ABCA1 solubilized in amphipol. Lane number with apostrophe represents the same blot probed with anti-ABCA1 antibody. ApoA-I was added at 10 µg/ml, ABCA1 at 100 µg/ml, ATP at 20 µM. Lane 1, ABCA1 alone. Lane 2, apoA+ABCA1. Lane 3, apoA-I+ABCA1+ATP.
5.5 Analysis of purified ABCA1 by negative-stain-EM

Another goal we aim to achieve with the purified ABCA1 is structure determination of the protein. After elution (with or without substitution with amphipol), ABCA1 was centrifuged in a 100 kD cut-off Amicon concentrator to remove rho1D4 peptides. The sample was then diluted to ~20µg/ml in 20 mM HEPES, 75 mM NaCl pH 7.4 with 0.02% DDM. After this step, ABCA1 was negatively stained and observed under electron microscopy (method was described in section 2.13). Figure 5-14 shows a electron micrograph of negatively-stained DDM-solubilized ABCA1. Particles were well dispersed and the background was clean. Consistent with the analysis by native PAGE, the sample exhibited heterogeneity in particle size. The smallest particles were ~ 8-10 nm in diameter. Larger sized particles were also visible. However, it was hard to assign different oligomers to these particles at this resolution. The large chunks of aggregates in the sample might be caused by the centrifugation step before loading the protein onto the grid.
An electron micrograph of Amphipol-solubilized ABCA1 under EM is shown in Figure 5-15. The sample was processed the same way as DDM solubilized ABCA1. The particle size distribution exhibited in the EM image was consistent with that shown by native gel. In both cases amphipol-solubilized ABCA1 tended to have larger particles, probably dimers or tetramers. And because amphipol is thought to bind to membrane proteins with higher affinity, thus keeping ABCA1 more stable during the centrifugation step, less large aggregates were seen under EM.
Additionally, the “native gel blotting” method (described in section 2.13.2) was used to acquire a homogeneous population of ABCA1 on the grid (Knispel et al. 2012). The presumed dimer of amphipol-solubilized ABCA1 was transferred from native gel (Figure 5-16 left panel, circled in red) to the grid. The EM image showed a uniform population of particles. The size of these particles were ~15 nm, about 2x the size of the smallest particles shown earlier, agreeing with the presumption that they were blotted from a “dimer” band.
Figure 5-16 EM image of ABCA1 in amphipol using native gel blotting method.
5.6 Summary

In this chapter, interaction between apoA-I and ABCA1 was probed both in a cell environment and in solution.

Cross-linking of apoA-I to HEK293 cells showed that ABCA1 could induce apoA-I binding to the cell surface, and high cholesterol level enhanced the binding. Cross-linking of apoA-I mutants to ABCA1-expressing cells was positively correlated with the trend of nHDL formation from these mutants: Mutant 38/40G could form more nHDL particles and was better cross-linked to the cells. H5 3xG, in contrast, formed fewer particles and was not cross-linked to the cells as efficiently as WT apoA-I. For 1-184, which could not form nHDL particles, the cross-linking level was lowest.

In order to conduct an in-solution binding assay, a large scale mammalian expression and purification system for ABCA1 was established. Rho1D4 tag was added to the C-terminus of ABCA1. Immunoaffinity chromatography was used to isolate ABCA1-rho1D4. Pure ABCA1 was obtained and the protein was in a different oligomeric state when the purification process was not supplemented with reducing agent. Nonetheless, the mixture of oligomers solubilized in detergent DDM retained the ATPase activity. Addition of reducing agent TCEP throughout the purification process helped to convert oligomers into monomer.

Binding of apoA-I to DDM solubilized- ABCA1 was not detectable under the conditions where we performed the experiment. But amphipol solubilized- ABCA1 was shown to bind to apoA-I.
The size distribution of purified ABCA1 revealed by EM confirmed the pattern seen on the native gel. And the “native gel blotting” method yielded a uniform population of particles on the grid, providing a promising method for structure determination of ABCA1 in the future.
CHAPTER 6 Discussion and Future Directions

6.1 Solution studies and nHDL particle formation

Combining the solution studies of apoA-I and the data on nHDL formation for the designed N-terminal mutants of apoA-I, we found that mutations located at the second hinge (G65-T68) did not result in major change in either biophysical characteristics or nHDL particle forming abilities from the cells, whereas the mutant carrying 38/40G in the first hinge changed dramatically at both aspects. These results suggest that the first hinge region might be more critical than the second hinge in controlling the open or closed conformation of apoA-I (Figure 6-1).

![First hinge (A37-Q41) seemed to be more critical in holding a “closed” conformation of apoA-I than the second hinge (G65-T68) is.](image)

For the naturally occurring mutants, all demonstrated an overall destabilized structure but unaffected ability to form nHDL. The extra proline in R153P and L178P might have created an extra kink that changed the curvature of the particles. This change, although
too small to be detected on the gel, could potentially make the particles unrecognizable to downstream enzymes or cofactors. Because R153 has been shown to be critical in LCAT activation (Hoang et al. 2003), and the insertion mutant also has a disrupted Helix 6 which is involved in LCAT activation, it is likely that pre-β nHDL made by these mutants could not activate LCAT, and thus could not become mature HDL.

6.2 ABCA1-mediated efflux and nHDL formation

When we first observed that mutants 38/40G and H5 3xG both promoted the same efflux but formed different nHDL particles, concerns were raised around the use of BODIPY-cholesterol. However, mounting evidence has emerged to support this method and an increasing number of laboratories have adopted this method recently (Cho et al. 2015; Mao et al. 2014; Zhang et al. 2015; Rohatgi et al. 2014).

One of the concerns was that this fluorescent cholesterol analogue would simply yield the same efflux uniformly from different mutants because the resolution was not high enough to distinguish between mutants. However, the C-terminal extension mutants validated the specificity and sensitivity of the method by showing well-resolved differences in efflux level. Although the original developers of this method noted that BODIPY-cholesterol desorbs more readily from the membrane than \(^3\)H-cholesterol (Sankaranarayanan et al. 2011), it is unlikely that BODIPY-cholesterol molecules are simply passively diffusing into the media, because in the no acceptor control experiments, fluorescence in the media was only \(\sim 1/10\) of that when apoA-I was added, and the same observation was shown with \(^3\)H-cholesterol labeling (Chroni, Liu, et al.)
The addition of the BODIPY group did not seem to affect the incorporation of BODIPY-cholesterol into pre-ß nHDL particles since the gradient ultracentrifugation data (Figure 4-32 and Figure 4-36) have shown that BODIPY-cholesterol co-localizes with nHDL particles in the same fractions.

The other concern was in the time course experiments. It is possible that when natural cholesterol is loaded into the cells, some of it is esterified into CE and is stored in the cell as the reservoir for cholesterol supply while the cells continuously make nHDL particles. BODIPY-cholesterol cannot be esterified into CE because it is not recognized by Acetyl-Coenzyme A acetyltransferase (ACAT). Therefore, BODIPY-cholesterol could be rapidly effluxed to give rise to a maximum efflux reading, whereas natural cholesterol is slowly released from CE as the cells continuously make nHDL particles. We consider this unlikely because high fluorescence readings in the cell lysate after 24 hours clearly indicated an oversupply of BODIPY-cholesterol that was ready to be effluxed. ACAT inhibitor may be used to investigate this problem in the future.

In analyzing the data from all the mutants we have used, we observed a pattern in the efflux and nHDL formations results. It appeared that in all the mutants with an intact C-terminus (185-243), cholesterol efflux was not affected, but nHDL particle formation could vary. However, in mutants with a partially truncated C-terminus (1-198, 1-209, 1-220, and 1-231), both cholesterol efflux and nHDL formation were affected. Therefore, we speculate that it is the C-terminus that controls cholesterol efflux promotion, while the
opening of N-terminus controls nHDL formation and the two processes are not necessarily coupled.

Previous studies and our lipid analysis data have demonstrated the existence of apoA-I-free particles (Duong et al. 2006). Thus, cholesterol efflux in the media is actually a heterogeneous combination of apoA-I-free lipids and apoA-I-containing lipids, namely nHDL particles. We speculate that because mutants 38/40G, H5 3xG, and WT apoA-I showed the same total cholesterol efflux level but different nHDL particle levels, they may differ in apoA-I-free lipids as well in the form that exists in the apoA-I-independent cholesterol efflux pathway. Unfortunately, quantitation of this lipid population is hard to achieve using current methods. We attempted to separate apoA-I-containing and apoA-I-free particles by apoA-I antibody pull-down experiments, but did not succeed possibly due to low-affinity binding of antibodies to apoA-I with flexible conformations on particles of different sizes. We speculate that for apoA-I mutant H5 3xG, which has retarded nHDL formation, this apoA-I-free lipid fraction was more abundant than that in WT apoA-I, whereas for mutant 38/40G, which has enhanced nHDL formation, this apoA-I-free lipid was less abundant than that of WT apoA-I. Since the C-terminus was intact on both mutants, the overall cholesterol efflux in the media remained the same. One possible explanation is that the cholesterol efflux promoting activity of ABCA1 reached saturation upon interacting with the C-terminus of apoA-I and the variation in the N-terminal activity of different mutants gave rise to differences in nHDL particle formation.
6.3 Mutants that affect nHDL formation

One thing should be noted is that the mutants we studied only shift the equilibrium of open ⇌ close conformation, in other words, the mutants only facilitate or retard the conversion between the closed and open conformation. However, after the protein has opened and bound to lipids, the properties of these lipidated mutants are not likely to be much different from lipidated WT apoA-I. Because nHDL particles made from these mutants all migrated to the same position on the gel, they are likely to be of the same size and charge. In addition, because the size of the nHDL particles is determined by apoA-I conformation, these mutants are likely to adopt the same conformation and encompass the same amount of lipid molecules within them.

6.3.1 Mutant 38/40G

When designing this mutant, we originally expected that the size distribution of nHDL particles formed by 38/40G would change. We hypothesized that the N-terminal bundle would open hinge by hinge and that facilitating the opening of one hinge would favor a certain particle size. However, instead of a change in a certain species of nHDL, formation of all species was enhanced, suggesting that opening of this hinge might be the rate-limiting step in the overall nHDL formation reaction.
6.3.2 Mutant H5 3xG

H5 3xG was designed to shift the equilibrium between monomer-dimer conversion to the monomer form. We proposed that 3 tandem glycines in H5 would energetically favor a loop in the “folded-back” conformation to an extended helical structure in the open conformation. However, the monomer-dimer conversion is more complicated than we expected.

6.3.2.1 ApoA-I monomer ⇌ dimer conversion

One noteworthy observation between experiments was that on the western blots sometimes the lipid-free apoA-I was very close to pre-β1 nHDL, but sometimes very far apart from it. Although the gel percentage may vary a little bit, the running conditions were kept constant, so we do not think this different migration was purely caused by gel issues. For example, in one experiment, if we assign the distance between pre-β1 and pre-β2 as $a$, then the distance between pre-β1 and the top end of lipid-free apoA-I was usually ~0.4$a$, (Figure 6-2). Whereas in another experiment, if we assign the distance between pre-β1 and pre-β2 as $b$, the distance between pre-β1 and the single lipid-free apoA-I band was 1.6$b$ (Figure 6-3). If we measure the distance between pre-β1 and the bottom end of lipid-free apoA-I in Figure 6-2, it happened to be 1.6$a$. The lower bands are unlikely to be degradation that occurred during incubation overnight, because protein without incubating with the cells at 37 °C overnight (designated as nc*, lane 4 and 4’ in Figure 6-2) already appeared at those positions. Furthermore, SDS gels of these apoA-I mutants did not indicate heavy degradation (Figure 6-5). Unfortunately, there is not a commercially available native marker good enough to reference apoA-I monomer and
dimer position on the gel due to charge and shape differences between the marker and apoA-I. Considering the relative positions of monomer and dimer of the truncation mutant apoA-I(1-184) in Figure 4-16, and the relatively defined two bands shown in some experiments (Figure 6-4), we speculate that the bottom and top end of the streaky band could actually be the ends of monomer and dimer bands and the signal in between may come from different conformation caused by the poorly defined C-terminus. It is quite surprising that apoA-I at such a low concentration (10 µg/ml) could still appear as dimers on the native gel (lane 4' in Figure 6-2), because the reported self-associating concentration of lipid-free apoA-I in solution is ~0.3 mg/ml (Gorshkova et al. 2000).

Figure 6-2 Relative position of apoA-I monomer, dimer, and pre-β1 on 4-15% native gel. 1

nc*: not incubated with cells nor incubated at 37 °C. ApoA-I concentration =10 µg/ml
Figure 6-3 Relative position of apoA-I monomer, dimer, and pre-β1 on 4-15% native gel. 2
ApoA-I concentration = 4 µg/ml

Figure 6-4 Relative position of apoA-I monomer, dimer, and pre-β1 on 4-15% native gel. 3
ApoA-I concentration = 10 µg/ml

Figure 6-5 SDS-PAGE of apoA-I and mutants. An aliquot of 50 µg proteins were loaded and no significant level of degradation was observed.

We have excluded the following factors as being the sole determinant of the monomeric or dimeric state of lipid-free apoA-I. The monomer or dimer state is not solely determined by apoA-I concentration, because both high and low concentration of apoA-I yielded a supposed apoA-I dimer in Figure 6-6. Nor is it solely determined by cholesterol level, because both high and low cholesterol level gave rise to apoA-I monomer (evidence was shown in Figure 4-39). It might be a combination of temperature, apoA-I concentration, apoA-I initial conformation, cholesterol level in the cells, cell conditions, etc. Throughout these experiments, one consistent observation was that no matter what condition was used, monomeric or dimeric state of apoA-I was always the same in empty
vector-transfected cells and in ABCA1-transfected cells. Therefore, monomer - dimer conversion of lipid-free apoA-I was unlikely to be induced by ABCA1, but rather by apoA-I itself and the cells.

Possible factors that could contribute to different oligomeric states of apoA-I may include: 1. Albumin level in the medium; we noticed that adding higher concentration of albumin (0.2% w/v) to the media tended to give rise to higher oligomers of apoA-I, although no literature has reported any interaction between albumin and apoA-I. 2. Post-translational modification of apoA-I, such as methionine oxidation which has been shown to affect the hydrophobicity in model peptide analogs (Anantharamaiah et al. 1988).

Unfortunately, we were unable to determine exactly what condition was determining apoA-I monomeric or dimeric state in solution. In future studies we will further investigate into this question.
Monomeric or dimeric state of lipid-free apoA-I is not solely determined by apoA-I concentration. At both high (≈10 µg/ml) and low (≈4 µg/ml) concentrations, free apoA-I appeared to be dimer.

Nonetheless, one thing should be noted is that lipid-free apoA-I dimer on the gel may not necessarily be the double belt apoA-I dimer on nHDL particles. On the contrary, we speculate that dimerization of apoA-I occurs when apoA-I interacts with ABCA1, but as soon as this ABCA1-induced dimeric apoA-I is formed, it is readily made into nHDL particles and thus will not be shown on the gel as lipid-free apoA-I dimers.
6.3.2.2 *H5 3xG in apoA-I monomer ↔ dimer conversion*

Unpublished data from Dr. Mei suggests that apoA-I (1-184) H5 3xG in solution favors a monomeric state. However, with an intact C-terminus, it seemed that H5 3xG could still form dimers to a similar extent as WT. In Figure 6-7, which is the lower exposure image of Figure 4-16, remaining lipid-free H5 3xG (lane 6, 7) and WT apoA-I (lane 11, 12) from both ABCA1-transfected cells and EV-transfected cells (lane 8, 13) seemed to appear as dimers. But fewer nHDL particles were formed by H5 3xG than by WT. These data demonstrated that the formation of a dimeric apoA-I may not necessarily guarantee the formation of nHDL particles. As discussed earlier, the lipid-free apoA-I dimer and the double belt apoA-I dimer on nHDL particles may not form through the same mechanism. While the former is likely to be related to the protein itself, the latter could involve ABCA1-apoA-I interaction.

![Image](image_url)
We speculate that the dimeric, lipid-free apoA-I, could be an intermediate state shown in the 1-184 crystal structure (Mei & Atkinson 2011) and proposed in the newly published paper by our lab (Mei et al. 2016). Mutant H5 3xG, although designed to favor a “folded-back” conformation, could still reach this intermediate dimer conformation (Figure 6-8), possibly through the C-terminus. It is then trapped in this intermediate state because the triple glycine mutation at the central hinge region could somehow hinder the opening of the N-terminal bundle, or affect its interaction with ABCA1, thus hampering the recruitment of lipid into the N-terminal hydrophobic core to make nHDL particles. The exact mechanism underlying this process requires a knowledge of the structure of the full-length apoA-I.

We speculate that although monomer-dimer conversion seemed to be independent of ABCA1, the opening of the N-terminal bundle, which is required for nHDL formation,
does require ABCA1. This speculation agrees with the cross-linking result that H5 3xG was cross-linked to ABCA1-expressing cells less efficiently than WT.

![Figure 6-8](image)

**Figure 6-8** A model for the formation of nHDL particles proposed by Mei et al. Adapted from (Mei et al. 2016)

### 6.4 nHDL particle

#### 6.4.1 Species of nHDL particles

Nascent HDL particles formed in the cellular system usually fall into 5 species: pre-β1 to pre-β5. As mentioned in section 4.6, the species of nHDL particles seemed to be affected only by the cholesterol level in the cells. Cholesterol-loaded cells tended to yield larger nHDL species. The time course experiments on the kinetics of nHDL formation (Figure 4-27) and studies conducted by Parks et al (Mulya et al. 2007) have shown that different species of nHDL particles are formed almost concurrently, indicating that once a small particle was made, it was immediately converted to a larger one, provided the cholesterol
was abundant. When the cholesterol level was relatively scarce, as in cells with basal level cholesterol, apoA-I and ABCA1 were in relative excess, the cells could only produce the smaller species. A possible explanation for this observation is that whereas smaller particles were formed on ABCA1, larger particles could be formed via a different mechanism, such as passive diffusion of lipids from the cell membrane, or the retroendocytosis pathway, both of which are supposed to be up-regulated in cholesterol-loaded cells. Considering a hard-to-detect interaction between apoA-I and ABCA1 discussed earlier, we suspect that the former is more likely.

6.4.2 GM1 on nHDL particles

For the first time, we demonstrated the presence of GM1 in nHDL particles. The ratio of apoA-I to GM1 between different species tended to remain constant between apoA-I mutants and WT within a certain experiment, but varied between experiments. For example, in Figure 6-9 left panel, it is clear that the apoA-I to GM1 ratio decreased as the nHDL particles grew larger, whereas in the right panel, the trend was less evident, indicating that unlike other lipids which may have a fixed number of molecules on a certain nHDL species, as determined by mass spectrometry (Sorci-Thomas et al. 2012), GM1 may not be an essential lipid on nHDL particles and thus may vary in quantity. And the abundance of GM1 on nHDL particles is only dependent on the availability of it on the cells. Given the wide range of substrates typical for ABC exporters, it is hard to
evaluate whether GM1 is directly pumped through the ABCA1 transporting channel, or simply comes from the remodeled lipid rafts where ABCA1 resides (Mendez et al. 2001).

Figure 6-9 GM1 abundance on nHDL particles. IB: immunoblotting; AB: affinity blotting. The panels represent two different experiments.

6.4.3 A model for nHDL formation

Here we propose a model of nHDL formation by apoA-I and ABCA1 (Figure 6-10):

When apoA-I is absent, ABCA1 works at basal activity and translocates cholesterol and other lipids from the inner leaflet of the membrane to the outer leaflet. Strain accumulates in the outer leaflet, and is then relieved as vesicles pop off in the form of microparticles, as suggested by Phillips (Duong et al. 2006). These microparticles represent the apoA-I-independent lipid efflux which has been described by us and other groups (Chroni et al. 2004).

When apoA-I is present, the apoA-I-independent efflux pathway still exists. In addition, apoA-I interacts with ABCA1 and turns on the high-capacity efflux activity of ABCA1.
Upon interaction with ABCA1, the N-terminus of apoA-I unfolds to accept lipids transported by ABCA1 and form pre-β1 nHDL particles, which then pick up more lipids from microparticles produced in the apoA-I-independent pathway or from membrane bulges via passive diffusion to form larger pre-β nHDL species.

6.5 Interactions between apoA-I and ABCA1

Researchers have been looking for evidence for a direct interaction between apoA-I and ABCA1 for a long time. In a co-immunoprecipitation (Co-IP) experiment that pulled...
down ABCA1 from the cells, we were not able to detect Co-IP of apoA-I. In fact, the interaction is hard to detect without cross-linking even when very sensitive methods such as I^{125}-apoA-I labeling was used (Chroni, Liu, et al. 2004). There are several possible reasons for this phenomenon in the cellular system; 1. the interaction itself may be very transient and weak, therefore apoA-I dissociates from ABCA1 in washing steps during sample preparation; 2. apoA-I loses affinity as soon as it becomes lipidated, as Parks and Ueda have suggested (Mulya et al. 2007) (Nagao et al. 2011); 3. there could be no direct interaction at all, and apoA-I simply binds to a membrane macrodomain remodeled by ABCA1, as Laundry et al have proposed (Landry et al. 2006). Although Zannis et al have shown that apoA-I and ABCA1 could be cross-linked with a 3 Å cross-linker (Chroni, Liu, et al. 2004), it is still possible that apoA-I is not directly interacting with ABCA1 but with the lipid macrodomains where ABCA1 resides. Thus, the better way to detect a direct interaction is in a cell-free system.

Reboul et al have isolated ABCA1 and used CHAPS solubilized-ABCA1 to bind apoA-I and found no interaction between the two (Reboul et al. 2013). In our study, we saw similar results with DDM solubilized-ABCA1. But with amphipol solubilized- ABCA1, we observed, for the first time, a direct interaction between apoA-I and ABCA1 in solution. From the gel that showed apoA-I/ABCA1 complex (Figure 5-11), it appeared that apoA-I was binding to ABCA1 monomer, instead of dimer as claimed by Ueda’s group (Nagata et al. 2013). However, from this result, we were not able to tell whether it was apoA-I monomer or dimer that was binding to the ABCA1 monomer. We will further investigate into this question in the future.
Although there were no free amphipol molecules in the binding solution and the exposed surface of ABCA1-bound amphipol molecules should in theory be hydrophilic and thus should not interact with apoA-I in solution, we could not exclude the possibility that apoA-I was binding to amphipol molecules that were bound to ABCA1.

Another observation is that although apoA-I and ABCA1 were added at 1:1 molar ratio, only a small fraction of apoA-I was bound to ABCA1, which could potentially be attributed to the following reasons:

First, not all ABCA1 was in the conformation that was ready for apoA-I binding. Based on the mechanism proposed for PgIK (Perez et al. 2015), it is possible that only after substrate binding (PL and cholesterol) could apoA-I binding happen, and when extracted from the cell, only a small fraction of ABCA1 carries these lipid components with it.

Second, it has been reported that ABCA1 contains at least two intracellular disulfide bonds that are needed for apoA-I binding (Hozoji et al. 2009). However, ABCA1 contains 39 cysteine residues and we have no idea which ones are buried or exposed. As shown in Figure 5-7, without adding reducing agent, the protein existed as oligomers. Therefore, we added low concentration (2 mM) of a mild reducing agent TCEP in order to retain the right disulfide bonds while keeping the other cysteines free. But this goal might still be hard to achieve. We could have broken some disulfide bonds that are needed for apoA-I interaction, leaving the binding experiment less efficient.
6.6 Future directions

6.6.1 Further characterization of the interaction between apoA-I and ABCA1 in detergent-free solution

Phospholipid (PL) reconstituted vesicles will be used to maximally retain ABCA1 in its native state. Binding stoichiometry and binding kinetics of apoA-I will be studied in both amphipol solubilized-ABCA1 and PL reconstituted-ABCA1. Substrates of ABCA1, such as PC, PS and cholesterol, and an energy regeneration system could also be incorporated into the PL vesicles to simulate the active substrate pumping conditions.

We will also explore which specific regions of apoA-I are responsible for ABCA1 interaction in more detail.

6.6.2 Structure determination of ABCA1

The expression and purification methods will be refined to obtain ABCA1 in large quantity. In order to acquire enough protein for size exclusion chromatography (SEC) and for crystallography, different solubilizing methods will be used to generate a more uniform conformation of ABCA1. Different types of amphipol will be investigated. If large quantities of ABCA1 with uniform conformation can be obtained, crystallization of ABCA1 or co-crystallization of apoA-I and ABCA1 will be carried out for high-resolution structural studies. Alternatively, cryo-EM could be used. One advantage of cryo-EM is that multiple conformations of ABCA1 could be automatically sorted out by
the newly developed software, allowing for structure-determination of multiple conformations with a single preparation.


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