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Protein Mediators of Sterol Transport Across Intestinal Brush Border Membrane

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Abstract

Dysregulation of cholesterol balance contributes significantly to atherosclerotic cardiovascular disease (ASCVD), the leading cause of death in the United States. The intestine has the unique capability to act as a gatekeeper for entry of cholesterol into the body, and inhibition of intestinal cholesterol absorption is now widely regarded as an attractive non-statin therapeutic strategy for ASCVD prevention. In this chapter we discuss the current state of knowledge regarding sterol transport across the intestinal brush border membrane. The purpose of this work is to summarize substantial progress made in the last decade in regards to protein-mediated sterol trafficking, and to discuss this in the context of human disease.

Keywords

Intestinal cholesterol absorption; Niemann-Pick C1-Like 1; sterol-sensing domain; ATP-binding cassette transporters G5 and G8; scavenger receptor class B type I; animal model; cell model; ezetimibe

1. INTRODUCTION

As discussed in detail in previous chapters, cholesterol is essential for the growth and function of all mammalian cells. However, elevated low-density lipoprotein (LDL) cholesterol (LDL-C) represents a major risk factor for the development of atherosclerotic cardiovascular disease (ASCVD). For many years now, statin-mediated inhibition of endogenous cholesterol biosynthesis has been the major therapeutic means to lower LDL-C, yet ASCVD still persists in most of the world (Rosamond *et al.*, 2007, 2002). Therefore, additional LDL-C lowering is now recommended, and the search for therapeutic strategies that work in synergy with statins has now begun. As a result of this search, drugs that inhibit intestinal cholesterol absorption have become an attractive therapeutic strategy to use in combination with statins. However, only within the last decade have we begun to understand how intestinal sterol absorption occurs at the molecular level. Recently we have learned that intestinal sterol absorption is tightly regulated by key proteins located at the brush border membrane. One of these proteins, Niemann-Pick C1-Like 1 (NPC1L1), was recently identified to be essential for intestinal cholesterol absorption, and will be discussed in detail. In direct opposition of NPC1L1, the heterodimer of ATP-binding cassette transporters G5 and G8 (ABCG5/G8) has been shown to be critical for intestinal disposal of sterols. In addition, the scavenger receptor class B type I (SR-BI) has been implicated in modulating

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intestinal cholesterol absorption. The purpose of this chapter is to summarize the current state of knowledge regarding the structure and function of these apically-localized cholesterol transporters, and to provide discussion about how these proteins and others interact to regulate the complex process of intestinal sterol absorption.

2. INTESTINAL CHOLESTEROL ABSORPTION AND EZETIMIBE

Cholesterol in the intestinal lumen is mainly derived from bile and diet. A physiological process by which cholesterol enters intestinal or thoracic duct lymph across the small intestine is called intestinal cholesterol absorption (Wang, 2007) (Figure 1). Cholesterol absorption involves at least the following three phases: 1) intralumenal solubilization; 2) movement across the apical membrane of absorptive enterocytes; and 3) intracellular metabolism for incorporation into chylomicrons destined for lymph. Intestinal cholesterol absorption rates range widely from 29% to 80% in normal men and women consuming a moderately low cholesterol diet (Bosner *et al*., 1999). Molecular mechanisms underlying this large inter-individual variation remain to be elucidated.

Since a detailed review of intestinal cholesterol absorption and its potential protein mediators is beyond the focus of this chapter, readers interested in this topic are referred to many excellent reviews available (Wilson and Rudel, 1994, Dawson and Rudel, 1999, Davis and Veltri, 2007, Levy *et al*., 2007, Wang, 2007, Turley and Dietschy, 2003, Iqbal and Hussain, 2009, Hui and Howles, 2005).

Intestinal cholesterol absorption represents an attractive target for developing cholesterol-lowering drugs because it is a major pathway governing whole-body cholesterol homeostasis. Schering-Plough Research Institute successfully identified ezetimibe as a potent and specific inhibitor of intestinal cholesterol absorption using *in vivo* models of cholesterol absorption (Clader, 2004). The drug is now widely used in monotherapy or in combination with statins (inhibitors of cholesterol biosynthesis) to efficiently treat hypercholesterolemia in the general population (Davis and Veltri, 2007).

Intestinal cholesterol absorption was once thought to be a passive process. The fact that ezetimibe potently inhibits intestinal cholesterol absorption at very low doses (Van Heek *et al*., 1997, van Heek *et al*., 2001, Sudhop *et al*., 2002) suggests that specific protein(s) must be involved in cholesterol absorption (Turley and Dietschy, 2003). In a search for the ezetimibe-inhibitory protein(s), NPC1L1, a previously-identified protein of unknown function (Davies *et al*., 2000), was discovered in the ezetimibe-sensitive pathway because disruption of NPC1L1 in mice reduces intestinal cholesterol absorption to the level seen in ezetimibe-treated animals (Altmann *et al*., 2004, Davis *et al*., 2004). Whether NPC1L1 is the molecular target of ezetimibe has been under considerable debate (Smart *et al*., 2004, Kramer *et al*., 2005, Labonte *et al*., 2007, Knopfel *et al*., 2007). Ezetimibe can bind to intestinal brush border membrane vesicles from wild-type mice but not mice lacking NPC1L1 (Garcia-Calvo *et al*., 2005). Recently, Weinglass and associates from Merck Research Laboratories purified NPC1L1-ezetimibe complex from NPC1L1-expressing and ezetimibe-treated cells and found that NPC1L1 is the only protein to account for ezetimibe binding (Weinglass *et al*., 2008). These pieces of biochemical evidence, together with findings from animal, genetic and cell biology studies (Yu, 2008) (see Sections below), strongly supports that NPC1L1 is the molecular target of ezetimibe.

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3. NPC1L1

3.1 Structure: gene, mRNA, and protein domains

In human genome, NPC1L1 gene spans about 29 kb in chromosome 7p13 and contains 20 exons. It produces a predominant mRNA transcript that skips exon 15. This transcript, like that from rodents, encodes a 1332-amino acid protein, and has been used in most, if not all, studies (Davies *et al*., 2000, Yu *et al*., 2006, Altmann *et al*., 2004). Human NPC1L1 gene also produces two alternatively spliced transcripts (Davies *et al*., 2000). One contains a 27-amino acid insertion transcribed from the in-frame exon 15. The other skips exon 7 and terminates within intron 8, encoding a truncated protein of 724 amino acids. The physiological significance of these two alternatively spliced variants has yet to be defined.

The human NPC1L1 protein is a homolog of Niemann-Pick C1 (NPC1), having ~50% amino acid homology to NPC1 protein (Davies *et al*., 2000, Davies and Ioannou, 2006). Deficiency of human NPC1 causes an autosomal recessive lipid storage disorder, Niemann-Pick disease type C1 that is characterized by defective trafficking of intracellular cholesterol and lysosomal accumulation of free cholesterol, gangliosides and other lipids (Carstea *et al*., 1997, Loftus *et al*., 1997). NPC1L1, like its homolog NPC1, also shares similarity with the resistance-nodulation-division family of prokaryotic permeases that can pump out lipophilic drugs, detergents, fatty acids, bile acids, metal ions, and dyes from the cytosol of bacteria (Davies *et al*., 2000, Davies and Ioannou, 2006).

Based on the amino acid sequences, human NPC1L1 is predicted to have a typical signal peptide of 21 amino acids, 13 putative transmembrane domains (Figure 2A), and extensive potential *N*-linked glycosylation sites located within the extracellular loops of the protein facing intestinal lumen (Altmann *et al*., 2004, Davies *et al*., 2000) or within the luminal loops of the protein if endocytosed from plasma membrane into intracellular vesicles (Yu *et al*., 2006, Ge *et al*., 2008, Wang *et al*., 2009). The membrane topology and *N*-glycosylation of NPC1L1 have been examined experimentally and the findings are consistent with sequence-based prediction (Iyer *et al*., 2005, Temel *et al*., 2007, Altmann *et al*., 2004, Davies *et al*., 2000, Wang *et al*., 2009).

A sterol-sensing domain (SSD) is another signature of NPC1L1 protein (Davies *et al*., 2000, Altmann *et al*., 2004) (Figure 2A). This domain consists of ~180 amino acids that form five predicted membrane-spanning helices with short intervening loops (Radhakrishnan *et al*., 2004). The SSD is conserved in at least 7 other membrane proteins, all of which have relations to cholesterol (Kuwabara and Labouesse, 2002), including the aforementioned NPC1 (Davies *et al*., 2000, Carstea *et al*., 1997, Loftus *et al*., 1997); 3-hydroxy-3-methylglutaryl (HMG) CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (Brown and Goldstein, 1999, Goldstein and Brown, 1990); sterol regulatory element-binding protein (SREBP)-cleavage activating protein (SCAP), a protein that controls the endoplasmic reticulum (ER)-to-Golgi transport and proteolytic activation of membrane-bound transcription factors SREBPs (Brown and Goldstein, 1999, Horton *et al*., 2002, Goldstein and Brown, 1990); and Patched, a membrane receptor for the cholesterol-linked signaling peptide Hedgehog (Cooper *et al*., 2003). The function of NPC1L1 SSD remains unknown and may be implicated in cholesterol regulation of NPC1L1 protein trafficking (Yu *et al*., 2006).

3.2. Function: Lessons learned from animal models and human genetics

In mammals, NPC1L1 mRNA and protein are highly expressed in small intestine (Altmann *et al*., 2004, Davies *et al*., 2005). In the small intestine, NPC1L1 protein localizes at the apical surface of absorptive enterocyte (Altmann *et al*., 2004, Davis *et al*., 2004). Gene knockout studies in mice have unambiguously established an essential role of NPC1L1 in

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intestinal cholesterol absorption (Altmann *et al*., 2004, Davis *et al*., 2004, Tang *et al*., 2008, Tang *et al*., 2008, Temel *et al*., 2009). The cholesterol absorption inhibitor ezetimibe cannot further reduce intestinal cholesterol absorption in NPC1L1-deficient mice, demonstrating that NPC1L1 is in the ezetimibe-sensitive pathway (Altmann *et al*., 2004, Davis *et al*., 2004).

Interestingly, genetic ablation of NPC1L1 in mice also protects against obesity, insulin resistance and fatty liver induced by a nutrient-rich diet (Labonte *et al*., 2008, Davies *et al*., 2005) (Yu, L., unpublished observation). Ezetimibe treatment also improves dyslipidemia, hepatic steatosis, non-alcoholic fatty liver disease, and insulin resistance in several animal models (van Heek *et al*., 2001, Assy *et al*., 2006, Deushi *et al*., 2007, Zheng *et al*., 2008). Another interesting observation is that NPC1L1 ablation in mice greatly attenuates dyslipidemia, lipogenic gene overexpression, and hepatic steatosis induced by activation of nuclear receptor liver X receptors (LXRs) (Tang *et al*., 2008). LXR forms a heterodimer with retinoid X receptor to regulate expression of their target genes in response to fluctuations of cellular cholesterol content (Repa and Mangelsdorf, 2002). Given that rodent NPC1L1 is almost exclusively expressed in the small intestine and the primary defect of NPC1L1-null mice is the blockade of intestinal cholesterol absorption (Altmann *et al*., 2004), the observed phenotypes are likely attributable to reduced intestinal cholesterol absorption. Efficient intestinal cholesterol absorption may be essential for maximizing LXR activities in the liver and small intestine, and perhaps other tissues where cholesterol and its derivatives are likely used as endogenous LXR ligands. LXR target genes involve the regulation of many metabolic pathways, including metabolism of cholesterol, fatty acids, and carbohydrates (Kalaany and Mangelsdorf, 2006). Inhibition of NPC1L1-dependent intestinal cholesterol absorption may improve some metabolic disorders by down-regulating tissue LXR activities. Consistent with this notion, LXR knockout mice are protected against obesity induced by a high fat diet (Kalaany *et al*., 2005).

Although NPC1L1 mRNA and protein are very abundant in the small intestine of mammals examined, their levels in the liver differ remarkably among species. Rodents express only a negligible amount of NPC1L1 in the liver (Altmann *et al*., 2004, Tang *et al*., 2006). In contrast, human livers have readily detectable levels of NPC1L1 mRNA and proteins (Altmann *et al*., 2004, Davies *et al*., 2005, Temel *et al*., 2007). The reason for different expression pattern of NPC1L1 among species is unknown, but may result from differences in cholesterol metabolism among species (Dietschy and Turley, 2002, Yu, 2007). In the liver of nonhuman primates and humans, NPC1L1 localizes to the canalicular membrane of hepatocyte (Yu *et al*., 2006, Temel *et al*., 2007). When overexpressed in the mouse liver by transgenic technology, human NPC1L1 also concentrates to the canalicular membrane of hepatocyte (Temel *et al*., 2007). Whereas the function of NPC1L1 in human livers remains to be elucidated, transgenic overexpression of human NPC1L1 in the mouse liver dramatically reduces biliary cholesterol concentrations without altering hepatic expression levels of the cholesterol efflux transporter ABCG5/G8 (Temel *et al*., 2007). This finding implies that hepatic NPC1L1 may inhibit biliary cholesterol excretion by transporting cholesterol from the canalicular bile back into hepatoctyes (Yu, 2008). The inhibition of NPC1L1 overexpression on biliary cholesterol excretion can be rescued by ezetimibe treatment, suggesting that hepatic NPC1L1 is another target for ezetimibe, at least in mice (Temel *et al*., 2007).

Increased cholesterol concentrations in the bile can result in gallstone formation. If ezetimibe treatment results in an increase in biliary cholesterol concentration in humans by inhibiting hepatic NPC1L1, it may have a potential to promote gallstone formation particularly in subjects in whom NPC1L1 is more abundantly expressed in the liver than in the intestine. Currently, there is no evidence that ezetimibe increases the incidence of

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gallstone disease and it is unknown whether hepatic NPC1L1 regulates biliary cholesterol excretion in humans. In a small human study, inhibition of NPC1L1 for 30 days by ezetimibe treatment at 20 mg/day did not alter biliary cholesterol molar percentage, cholesterol to phospholipid ratio, and cholesterol saturation index in 5 overweight subjects without gallstones, but did reduce these parameters significantly in 7 patients with gallstones (Wang *et al*., 2008). Ezetimibe may have its predominant effect at the intestinal level, thereby reducing cholesterol that is transported from the gut lumen to the liver for biliary secretion. In animals that express NPC1L1 predominantly in the small intestine, inhibition of NPC1L1 by ezetimibe may reduce biliary cholesterol levels and prevent gallstone disease. This notion is consistent with the following observations: 1) NPC1L1 knockout mice have lower biliary cholesterol concentrations, even after being challenged with a high cholesterol diet (Davis *et al*., 2004); 2) In Golden Syrian hamsters, ezetimibe prevents high cholesterol diet-induced increase in biliary cholesterol (Valasek *et al*., 2008); and 3) In wild-type mice, ezetimibe treatment protects against lithogenic diet-induced increase in biliary cholesterol concentration and gallstone formation (Zuniga *et al*., 2008).

Human genetic studies have shown that sequence variations in NPC1L1 are associated with sterol absorption efficiency, LDL-C levels, and LDL-C response to ezetimibe therapy (Cohen *et al*., 2006, Wang *et al*., 2005, Hegele *et al*., 2005, Simon *et al*., 2005, Fahmi *et al*., 2008). These findings strongly support a key role of NPC1L1 and NPC1L1-depedent cholesterol transport in whole-body cholesterol homeostasis in humans.

3.3 Function: Lessons learned from cell model systems

In whole animals, NPC1L1 protein is asymmetrically enriched in the intestinal brush border membrane (Altmann *et al*., 2004, Garcia-Calvo *et al*., 2005, Iyer *et al*., 2005, Labonte *et al*., 2007, Sane *et al*., 2006) or hepatic canalicular membrane (Yu *et al*., 2006, Temel *et al*., 2007). In cultured cells, NPC1L1 proteins can localize to both the plasma membrane and intracellular compartments (Yu *et al*., 2006, Davies *et al*., 2005, Yamanashi *et al*., 2007, Iyer *et al*., 2005). Human NPC1L1 with a *C*-terminal green fluorescent protein tag predominantly localizes at the endocytic recycling compartment in actively growing McArdle RH7777 rat hepatoma cells (Yu *et al*., 2006). Intriguingly, the intracellular itinerary of NPC1L1 protein in these cells and in HepG2 hepatic carcinoma cells is under control of cellular cholesterol availability (Yu *et al*., 2006). Cholesterol depletion results in a redistribution of NPC1L1 from the intracellular endocytic recycling compartment to the cell surface likely via a mechanism involved in microfilament-associated myosin Vb/Rab11a/Rab11-FIP2 complex, and conversely, cholesterol reloading causes the proteins to transit from the cell surface back into the cell interior likely through clathrin-mediated enocytosis, which is coupled to NPC1L1-facilitated and ezetimibe-inhibitable cholesterol uptake (Yu *et al*., 2006, Ge *et al*., 2008, Brown *et al*., 2007, Chu *et al*., 2009, Petersen *et al*., 2008). This cholesterol regulated trafficking may explain why both intracellular and cell surface locations have been observed for NPC1L1 protein (Altmann *et al*., 2004, Garcia-Calvo *et al*., 2005, Iyer *et al*., 2005, Labonte *et al*., 2007, Sane *et al*., 2006, Knopfel *et al*., 2007, Yamanashi *et al*., 2007, Temel *et al*., 2007, Yu *et al*., 2006).

The establishment of NPC1L1-dependent and ezetimibe-sensitive cholesterol uptake assay in cell models allows an opportunity to examine if NPC1L1 differentiates plant sterols from cholesterol (Figure 3). Each day, a large amount of plant-derived sterols (mainly sitosterol and campesterol) are consumed. Although these phytosterols are structurally similar to cholesterol, in normal individuals phytosterols are poorly absorbed. The rank order of fractional intestinal sterol absorption is cholesterol (~45%) > campesterol (~20%) > sitosterol (~5%) (Lutjohann *et al*., 1995). In mammals, this discrimination for phytosterols may be protective because phytosterols can displace cholesterol in the cell membrane and interfere with cell functions (Wang *et al*., 1981, Su *et al*., 2006, Kruit *et al*., 2008, Kim *et*

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al., 2008). Mechanisms underlying the defense against phytosterols remain elusive. Although mutations in ABCG5 and/or ABCG8 cause accumulation of phytosterols in the body (Berge *et al*., 2000, Lee *et al*., 2001), the rank order of intestinal sterol absorption rates is maintained in mice lacking ABCG5 and ABCG8 (Yu *et al*., 2002), implying that ABCG5/G8 is a gatekeeper rather than a discriminator for phytosterols. Is NPC1L1 a discriminator of phytosterols? Studies in mice and humans suggest that NPC1L1 is a common transporter for all sterols because NPC1L1 and ezetimibe-sensitive pathway are essential for phytosterol absorption (Davis *et al*., 2004, Salen *et al*., 2004, Salen *et al*., 2006, Yu *et al*., 2005, Tang *et al*., 2008). However, cell culture studies suggest that NPC1L1 may not mediate cellular uptake of all sterols equally. NPC1L1-mediated uptake is 60% lower for sitosterol than cholesterol in intestine-derived CaCo2 cells overexpressing NPC1L1 (Yamanashi *et al*., 2007). Overexpression of NPC1L1 in McArdle RH7777 rat hepatoma cells facilitates cholesterol, but not sitosterol uptake (Yu *et al*., 2006, Brown *et al*., 2007, Yamanashi *et al*., 2007, Ge *et al*., 2008). These cell-based assays imply that NPC1L1 has lower affinity to sitosterol than cholesterol. The lower affinity of NPC1L1 to phytosterols has the potential to determine the rank order of intestinal sterol absorption rates. NPC1L1 might be the first genetic defense against phytosterol absorption. But this defense is not complete; otherwise, mutations in ABCG5/G8 would not cause sitosterolemia in the presence of NPC1L1.

3.4 Regulation of expression

Regulation of NPC1L1 gene expression is largely unknown and inconsistent. Since activation of many nuclear receptors, including peroxisome proliferators-activated receptor alpha (PPARα), PPARδ, liver X receptor (LXR), and retinoid X receptor (RXR), reduces intestinal cholesterol absorption (Knight *et al*., 2003, Repa *et al*., 2000, McNamara *et al*., 1980, Umeda *et al*., 2001, Vanhanen and Miettinen, 1995, Yu *et al*., 2003, Oliver *et al*., 2001, van der Veen *et al*., 2005), effects of these nuclear receptors on intestinal NPC1L1 expression have been examined in CaCo-2 cells and in mice. In 2005, van der Veen *et al* showed that PPARδ activation reduces intestinal cholesterol absorption by 43% in DBA mice after 8 days of treatment, coinciding with a significant reduction in intestinal NPC1L1 mRNA levels by 40% (van der Veen *et al*., 2005). PPARδ activation may explain why fish oil and docosahexaenoic acid treatments reduces NPC1L1 expression in CaCo-2 cells and in the proximal small intestine of hamsters (Mathur *et al*., 2007). In 2007, Valasek *et al* made an interesting observation that fenofibrate significantly reduces intestinal NPC1L1 mRNA and protein levels via a PPARα-dependent mechanism in mice, but NPC1L1 gene does not seem to be the direct target of PPARα because short-term treatment with fenofibrate does not reduce NPC1L1 mRNA expression and yet nuclear receptors generally enhance expression of their direct targets within several hours (Valasek *et al*., 2007). In the same study, Valasek *et al* also found that the effect of PPARα on intestinal NPC1L1 expression is LXR-independent because the similar effect was observed in the LXRα/LXRβ double knockout mice (Valasek *et al*., 2007).

Whether LXR directly modulates NPC1L1 expression is also uncertain. In a cell culture study, LXR activation by 1 μM of a synthetic agonist T0901317 or GW3965 for 24 h reduces NPC1L1 mRNA levels by ~30% or ~60%, respectively, in CaCo-2/TC7 cells (Duval *et al*., 2006). In studies with whole animals, LXR activation reduces NPC1L1 mRNA levels by ~35% in the duodena of apolipoprotein (apo) E2-KI mice (lacking endogenous apoE but expressing human apoE2 (Sullivan *et al*., 1998) fed a Western diet (Duval *et al*., 2006), and ~50% in the small intestine of ABCB4 knockout mice (Kruit *et al*., 2005). However, LXR activation by T0901317 or GW3965 does not alter intestinal mRNA levels significantly in the chow-fed wild-type mice (Yu L, unpublished data) (Kruit *et al*., 2005). NPC1L1 mRNA levels remains unaffected in the proximal small intestine of chow-

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fed LXRα/LXRβ double knockout mice (Valasek *et al*., 2007). Thus, it is unlikely that intestinal NPC1L1 is a direct target of LXR.

The human NPC1L1 promoter has a putative sterol regulatory element (Davies *et al*., 2000). In 2007, Alrefai *et al* reported that SREBP-2 (Horton *et al*., 2002) can bind to the two putative sterol regulatory elements in the human NPC1L1 promoter (Alrefai *et al*., 2007) that differ from the one predicted previously (Davies *et al*., 2000). They also showed that the NPC1L1 mRNA levels and promoter activities in CaCo2 cells are decreased by 25-hydroxycholesterol that suppresses SREBP activation, and are increased by mevinolin that induces SREBP activation (Alrefai *et al*., 2007). This study is consistent with that NPC1L1 mRNA levels are ~3-fold and ~2.4-fold higher in cholesterol-depleted CaCo2 cells induced by cyclodextrin and by taurocholate/phosphatidylcholine for 24h, respectively, and are significantly lower in CaCo-2 cells treated with cholesterol and 25-hydroxycholesterol (Field *et al*., 2007). Alrefai *et al* further showed that co-expression of the human NPC1L1 promoter-luciferase reporter and the active form of SREBP-2 dramatically increases human NPC1L1 promoter activity (Alrefai *et al*., 2007). Interestingly, SREBP-2 and the hepatocyte nuclear factor 4α (HNF4α) can synergistically increase human NPC1L1 promoter activity despite HNF4α alone having no effect, and the presence of HNF4α appears to be essential for cholesterol-dependent regulation of NPC1L1 expression (Iwayanagi *et al*., 2008).

Taken together, these studies suggest that NPC1L1 expression may be regulated by cellular cholesterol availability in a SREBP-dependent manner. Consistent with this regulation, we found that the hepatic NPC1L1 mRNA level is drastically increased in ABCG5/G8 transgenic mice (Yu *et al*., 2002) treated with lovastatin, a condition that causes a compensatory increase in hepatic mRNA levels of all cholesterol biosynthetic genes (Tang *et al*., 2006). Additionally, the intestinal NPC1L1 mRNA level is reduced by ~35% in mice lacking acyl-CoA:cholesterol acyltransferase-2 versus wild-type mice fed a synthetic diet containing 20% energy from palm oil and 0.17% cholesterol (Temel *et al*., 2005), and by ~45% in phospholipid transport protein-deficient versus wild-type mice fed a chow diet (Liu *et al*., 2007), which are two conditions under which free cholesterol is accumulated in the intestine. Further, in ezetimibe-treated miniature pigs fed a Western-type diet, intestinal and hepatic NPC1L1 mRNA levels are significantly increased (Telford *et al*., 2007).

Despite several pieces of evidence supporting regulation of NPC1L1 expression by cellular cholesterol availability, discrepancies exist in animal and cell culture studies. For example, high cholesterol diet feeding does not suppress intestinal NPC1L1 expression in mice (Valasek *et al*., 2007, Plosch *et al*., 2006), unless 0.5% cholate is added to the diet (Davis *et al*., 2004). Ezetimibe treatment that reduces cholesterol entry into enterocytes does not increase intestinal NPC1L1 expression in the chow-fed mice (Valasek *et al*., 2007). In one cell culture study, ezetimibe treatment for 16 h reduces rather than increases NPC1L1 mRNA by 65% in CaCo-2 cells (During *et al*., 2005). In another study, 24h treatment of CaCo-2 cells with ezetimibe causes no alterations in NPC1L1 mRNA levels or protein mass (Field *et al*., 2007). Currently, it is unclear if these obvious discrepancies in cholesterol regulation of NPC1L1 expression are related to differences in animal species, diet compositions, duration of drug treatment, or experimental systems used or other factors.

Other factors influencing NPC1L1 expression include estrogen receptors and diabetic state. Administration of high-doses of 17β-estradiol (6 μg/day) to ovariectomized AKR or C57L mice increases NPC1L1 mRNA expression in duodena and jejuna, but not ilea (Duan *et al*., 2006). Intestinal and hepatic NPC1L1 mRNA levels are significantly higher in streptozotosin-induced diabetic versus nondiabetic rats (Lally *et al*., 2007), and in Zucker diabetic fatty versus lean rats (Lally *et al*., 2007). NPC1L1 mRNA levels are also ~2-fold higher in the intestinal biopsy samples from type 2 diabetic patients than nondiabetic

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patients (Lally *et al*., 2006, Lally *et al*., 2007). Given the diabetes epidemic and clinical use of ezetimibe (NPC1L1 inhibitor), exploring mechanisms underlying the relationship between diabetes and NPC1L1 expression levels represents an important future direction and has enormous translation potential.

3.5 Cholesterol and ezetimibe binding studies

It is currently unknown if NPC1L1 protein binds cholesterol and other sterols. The purified SSD-containing membrane region of SCAP can directly bind cholesterol through receptor-ligand interaction and SCAP is thus considered as an ER receptor for cholesterol (Radhakrishnan *et al*., 2004). The binding between NPC1 (a homolog of NPC1L1) and photoactivatable cholesterol analog appears to be SSD-dependent (Ohgami *et al*., 2004). Unexpectedly, detailed cholesterol binding assays using purified NPC1 protein and its truncated versions localize the binding site of cholesterol and oxysterols to the luminal loop-1 (a 240-amino acid domain with 18 cysteines), instead of SSD (Infante *et al*., 2008, Infante *et al*., 2008). The *N*-terminal extracellular domain of NPC1L1 protein consists of 263-amino acids (the signal peptide of 21 amino acids excluded), 18 of which are also cysteines. NPC1L1, like its homolog NPC1, may also bind cholesterol via this *N*-terminal cysteine-rich domain (Figure 2A).

NPC1L1 protein has two large extracellular loops facing intestinal lumen (Figure 2A). A 61-amino acid region in the extracellular loop-1 has been recently shown to be critical for ezetimibe binding by Weinglass and associates (Weinglass *et al*., 2008). In this study, Weinglass *et al* purified an NPC1L1-ezetimibe complex from cultured cells and analyzed its constituents by mass spectrometry. They found that NPC1L1 is the only protein to account for ezetimibe binding. Taking advantage of the large difference in affinity between dog and mouse NPC1L1 for ezetimibe, they further identified two residues in this loop of NPC1L1 that are mostly responsible for the large differences in affinity between the two species. These residues reside adjacent to a hotspot of human NPC1L1 polymorphisms that are associated with reduced intestinal cholesterol absorption (Cohen *et al*., 2006). These findings indicate that the extracellular loop-1 of NPC1L1 plays an important role in mediating ezetimibe action and intestinal cholesterol absorption.

3.6 Potential mechanisms for NPC1L1 to mediate sterol uptake

Several lines of evidence suggest that NPC1L1 may mediate cholesterol uptake via the clathrin-mediated endocytic pathway (Yu, 2008). These include: 1) NPC1L1 cycles in a cholesterol-regulated manner to and from the cell surface (Yu *et al*., 2006); 2) NPC1L1 physically resides at both plasma membrane and intracellular compartments in cultured cells and in absorptive enterocytes of small intestine (Altmann *et al*., 2004, Garcia-Calvo *et al*., 2005, Iyer *et al*., 2005, Labonte *et al*., 2007, Sane *et al*., 2006, Knopfel *et al*., 2007, Yamanashi *et al*., 2007, Temel *et al*., 2007, Yu *et al*., 2006); 3) After extracted from cultured McArdle rat hepatoma cells, NPC1L1 co-immunoprecipitates with the μ2 (mu2) subunit of an adaptor protein complex AP2 and the clathrin heavy chain (Ge *et al*., 2008), two proteins that are involved in the clathrin endocytic pathway; 4) Potassium depletion, a condition known to arrest clathrin-mediated endocytosis (Larkin *et al*., 1983), inhibits NPC1L1-dependent cholesterol uptake (Brown *et al*., 2007); and 4) Mice lacking caveolin-1, a structural molecule of caveolae, display normal intestinal cholesterol absorption (Valasek *et al*., 2005), demonstrating that caveolin-mediated endocytosis (another important endocytic pathway) is not the cellular basis for NPC1L1-dependent cholesterol uptake. Clathrin-mediated endocytosis appears to be the cellular basis for intestinal fat absorption (Hansen *et al*., 2007, Hansen *et al*., 2003). Similar mechanism may operate for intestinal cholesterol absorption and perhaps hepatic cholesterol retrieval from canalicular bile (Temel *et al*., 2007). Ezetimibe appears to inhibit the sterol-induced

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internalization of NPC1L1 via clathrin-mediated endocytosis in cultured hepatoma cells (Ge *et al*., 2008, Chang and Chang, 2008, Petersen *et al*., 2008).

To definitively identify molecular mechanisms underlying NPC1L1-mediated cholesterol transport, many important questions have to be answered. For example, how does cholesterol regulate NPC1L1 trafficking? What sorting signals in the protein and sorting platforms in cells are involved in this regulation? Does NPC1L1 bind cholesterol? If it does, what is the physiological significance for cholesterol binding to NPC1L1? Do NPC1L1-cholesterol interactions function as a signal for inducing clathrin-mediated endocytosis of NPC1L1 or as a process to recruit and handle free cholesterol to NPC1L1-containing membrane microdomains? Does NPC1L1 bind cholesterol that resides in the plasma membrane or cholesterol present in extracellular spaces such as the gut lumen and hepatic bile canaliculus (Temel *et al*., 2007, Yu, 2008)? How does ezetimibe inhibit NPC1L1 internalization from the plasma membrane? Does ezetimibe interfere with NPC1L1-cholesterol binding? In addition, due to the dense and rigid microvillar cytoskeleton (Yamamoto, 1982), the base of the microvilli of small intestine is likely the only site for endocytic membrane traffic to occur (Hansen *et al*., 2003). Given that NPC1L1 abundantly localizes at the microvilli, a dilemma is how the microvillus-localized NPC1L1 and its cargos are endocytosed. If the microvillus-localized NPC1L1 has to move to the base of microvilli to mediate cholesterol uptake, what are signals triggering this translocation or movement? Elucidation of all these questions will greatly enhance our understanding of how cells such as enterocytes and hepatocytes handle extracellular unesterfied cholesterol.

3.7 Therapeutic perspectives

NPC1L1 is undoubtedly a gatekeeper of intestinal cholesterol absorption, thus positioning itself as an attractive drug target for prevention of cholesterol-driven diseases such as ASCVD and gallstone disease. Additionally, several studies have shown that NPC1L1 inhibition may provide benefits for important metabolic diseases such as hepatic steatosis, dyslipidemia, high fat diet-induced obesity and insulin resistance (Labonte *et al*., 2008, Davies *et al*., 2005, van Heek *et al*., 2001, Assy *et al*., 2006, Deushi *et al*., 2007, Zheng *et al*., 2008) (Yu, L., unpublished data). Further studies are needed to define molecular mechanisms underlying these intriguing observations. Intestinal cholesterol has been reported to regulate fat storage (Kalaany *et al*., 2005). Perhaps, simply by controlling how much cholesterol enters the body via small intestine, NPC1L1 can regulate responses of other metabolic pathways and physiological processes to overnutrition, thereby improving metabolic diseases induced by metabolic overload.

Although the NPC1L1 inhibitor ezetimibe can efficiently lower blood cholesterol, it is important to point out that inter-individual variations exist in response to ezetimibe treatment (Hegele *et al*., 2005). Comparison of multiple species NPC1L1 orthologs have shown that the *in vivo* responsiveness to ezetimibe correlates with NPC1L1 binding affinity (Hawes *et al*., 2007). Ezetimibe non-responders may have distinct NPC1L1 sequence variations, and these individuals may be responsive to other NPC1L1 inhibitors that work via distinct mechanisms. In addition, small molecule NPC1L1 inhibitors, after entering the body, have the potential to cause adverse effects. NPC1L1 localizes at the intestinal brush border membrane and its extracellular domains are exposed to contents in the gut lumen. This unique location and membrane topology makes NPC1L1 an ideal target for large molecule nonabsorbable NPC1L1 inhibitors (Davidson, 2009). Thus, NPC1L1 will remain an attractive drug target in the future.

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4. ATP-BINDING CASSETTE TRANSPORTERS G5 AND G8 (ABCG5/G8)

4.1 Discovery of ABCG5/G8: The power of human genetics

The discovery of the heterodimeric transporters ABCG5 and ABCG8 represents a powerful example of human genetics leading to mechanistic understanding of the underlying disease process. In this case, it has long been appreciated that cholesterol is structurally very similar to plant sterols, with only minor differences in side chain configurations (Schoenheimer, 1929) (Figure 3). However, it has been known for nearly a century that mammals consume large amounts of dietary plant sterols, yet these phytosterols are excluded from the body, primarily at the level of the intestine (Sudhop *et al*., 2005, Schoenheimer and Breusch, 1933, Borgstrom, 1968, Gould *et al*., 1969, Hernandez *et al*., 1954, Huang and Kuksis, 1965). A major breakthrough regarding this issue came when Bhattacharayya and Connor described a novel disease in which two sisters presented to the clinic with tendon xanthomas, and were surprisingly shown to have extremely elevated levels of plasma plant sterols (primarily β-sitosterol) (Bhattacharyya and Connor, 1974). Hence, the disease was named β-sitosterolemia, and the authors proposed that this disease was caused by a single genetic defect, which prevented the intestine from excluding plant sterols from being absorbed. It was later discovered that sitosterolemic patients have elevated fractional absorption of dietary sterols and diminished ability to secrete multiple sterols into bile (Miettinen, 1980, Lutjohann *et al*., 1995, Gregg *et al*., 1986). As a result, sitosterolemia manifests as the accumulation of both plant and animal sterols in the plasma, skin, tendons, coronary arteries and other tissues, and most affected individuals suffer from premature coronary heart disease (Salen *et al*., 1985, Kolovou *et al*., 1996, Mymin *et al*., 2003, Bjorkhem *et al*., 2001). Sitosterolemia locus was subsequently mapped to a single sitosterolemia locus on human chromosome 2p21 that contains two genes, ABCG5 and ABCG8 (Patel *et al*., 1998), and mutations in either of these genes is causative of sitosterolemia (Berge *et al*., 2000, Lee *et al*., 2001).

4.2 Structure: gene, mRNA, and protein domains

ABCG5 and ABCG8 genes are arranged in a head-to-head orientation with less than 400 base pairs between their respective start codons, and each has 13 exons and twelve introns (Berge *et al*., 2000). The two genes encode two distinct proteins known as sterolin-1 (ABCG5) and sterolin-2 (ABCG8), which are mammalian homologues of the *drosophila* gene *White*. In addition to being in the same genetic neighborhood, ABCG5 and ABCG8 are also in close proximity at the protein level. Both of these proteins contain an ATP-binding cassette near the *N*-terminus followed by six putative transmembrane domains (Figure 2B), and serve only as non-functional half-transporters when expressed alone (Graf *et al*., 2002, Graf *et al*., 2003, Graf *et al*., 2004). ABCG5 and ABCG8 must heterodimerize to transport sterols across membranes (Graf *et al*., 2002, Graf *et al*., 2003, Graf *et al*., 2004). This idea is supported by data demonstrating that, when expressed together, the proteins colocalize in the ER and the plasma membrane, they can be co-immunoprecipitated, and the exit of these proteins from the ER to the plasma membrane requires coexpression of both proteins (Graf *et al*., 2002, Graf *et al*., 2003, Graf *et al*., 2004). Even stronger evidence for obligate heterodimerization comes from the fact single mutations in either of these genes alone causes sitosterolemia (Berge *et al*., 2000, Lee *et al*., 2001). The current data support a model where ABCG5 and ABCG8 heterodimerize in the ER, traffic together through the Golgi apparatus, and subsequently target to apical subdomains in the plasma membrane. Both ABCG5 and ABCG8 undergo *N*-linked glycosylation, and glycosylation at Asn-619 in ABCG8 is critical for efficient trafficking of the heterodimer (Graf *et al*., 2004). The ABCG5/G8 heterodimer requires the molecular chaperones, calnexin and calreticulin for proper folding and trafficking out of the ER (Graf *et al*., 2004, Okiyoneda *et al*., 2006). Subsequent site-directed mutagenesis experiments demonstrated that the majority of mutants

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causative of sitosterolemia exhibit impaired transport of the heterodimer from the ER to the plasma membrane (Graf *et al*., 2004).

4.3 Function: Lessons learned from animal models

Since the discovery of the genetic basis for sitosterolemia, there has been intensive effort put forth to understand the function of the ABCG5/G8 heterodimer. Like NPC1L1, ABCG5 and ABCG8 are expressed almost exclusively on the apical membrane of enterocytes in the intestine and hepatocytes in the liver (Patel *et al*., 1998, Berge *et al*., 2000, Lee *et al*., 2001, Graf *et al*., 2003, Klett *et al*., 2004). It is generally accepted that the ABCG5/G8 heterodimeric complex serves as an efflux pump to remove sterols (cholesterol and phytosterols) from hepatocytes and enterocytes. In the intestinal brush border membrane, this function would allow for transport of intracellular sterols back into the lumen of small intestine for fecal excretion. If this were true, ABCG5/G8 would likely play an important role in intestinal sterol absorption by opposing the action of NPC1L1.

A potential role for ABCG5/G8 in intestinal cholesterol absorption was first discovered in sitosterolemic patients, who have elevated intestinal absorption of both plant sterols and cholesterol (Miettinen, 1980, Lutjohann *et al*., 1995, Gregg *et al*., 1986, Salen *et al*., 1992, Bhattacharyya *et al*., 1991, Salen *et al*., 1989). In fact, sitosterolemic patients absorb roughly 20–30% of dietary sitosterol, compared to <5% absorption in unaffected individuals (Bhattacharyya and Connor, 1974, Miettinen, 1980, Salen *et al*., 1992, Salen *et al*., 1989). Importantly, mice lacking either ABCG5 alone (Plosch *et al*., 2004), ABCG8 alone (Klett *et al*., 2004), or both transporters (Yu *et al*., 2002) exhibit sitosterolemia, which can likely in part be explained by increased intestinal absorption of phytosterols. In support of this concept, in mice lacking both ABCG5 and ABCG8 fed a chow diet, intestinal absorption of cholesterol is not significantly altered, yet the absorption of sitosterol, cholestanol, campesterol is significantly increased (Yu *et al*., 2002). In a recent study, Wang and colleagues demonstrated that the lymphatic transport rate of cholesterol and sitostanol is increased by ~40% and 500%, respectively in mice lacking ABCG8 (Wang *et al*., 2007). Furthermore, mice transgenically overexpressing ABCG5 and ABCG8 in the intestine and liver exhibit a 50% reduction in fraction cholesterol absorption, and fecal neutral sterol levels increase 3–6 fold (Yu *et al*., 2002). Collectively, studies in both sitosterolemic humans and animal models support an important role for ABCG5/G8 in intestinal sterol absorption. Although ABCG5 and ABCG8 heterodimer is thought to influence intestinal sterol absorption by serving as an efflux pump to deliver sterols from enterocytes back into the gut lumen for fecal disposal, direct experiment evidence of the concept is still lacking.

4.4 Function: Lessons learned from cell model systems

There is a strong body of work examining the subcellular trafficking and dimerization of ABCG5 and ABCG8, but characterization of the cellular sterol transport properties of the heterodimer is still incomplete. For unknown reasons, although ABCG5 and ABCG8 can be readily expressed and properly localized in mammalian cells, a standard reproducible functional assay for ABCG5/G8-dependent sterol transport has not been established. Recently, one group was able to show that overexpression of ABCG5 and ABCG8 in human kidney and gallbladder epithelial cells promotes the efflux of cholesterol and plant sterols (Vrins *et al*., 2007, Tachibana *et al*., 2007). In these studies it was shown that ABCG5/G8-dependent efflux is dependent on the presence of mixed bile salt micelles as an acceptor, whereas other cholesterol acceptors such as apoAI, high-density lipoprotein (HDL), or methyl-β-cyclodextrin do not efficiently promote ABCG5/G8-dependent efflux.

Given that mutations in either ABCG5 or ABCG8 cause abnormal accumulation of plant sterols in the body (Berge *et al*., 2000, Lee *et al*., 2001), most have assumed that the

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ABCG5/G8 heterodimer is the primary, if not the sole protein complex, responsible for sterol discrimination in the intestine. However, the current knowledge base does not firmly support this conclusion. For example, genetically sitosterolemic patients (Lutjohann *et al*., 1995), rats (Hamada *et al*., 2007), and mice (Yu *et al*., 2002) still possess the ability to discriminate between cholesterol, campesterol, and sitosterol at the level of intestinal absorption, indicating that ABCG5/G8 is a gatekeeper rather than the intestinal sterol discriminator. Given this information, caution should be taken when assuming that the ABCG5/G8 heterodimer is the sole mediator of sterol selectivity in the intestine. Much more work is needed in this area, and with recent progress using cell-based systems for ABCG5/G8-dependent sterol transport, we may gain further mechanistic insights into the critical question of substrate specificity.

4.5 Regulation of expression

Both ABCG5 and ABCG8 seem to be primarily controlled at the transcriptional level. The sterol-sensing transcription factors LXRα and LXRβ appear to be the primary regulator of ABCG5 and ABCG8 mRNA expression. In support of this, both dietary cholesterol and synthetic LXR agonists upregulate ABCG5 and ABCG8 mRNA expression in the small intestine and liver of wild type mice, but not LXR knockout mice (Berge *et al*., 2000, Repa *et al*., 2002, Plosch *et al*., 2002, Kaneko *et al*., 2003). This is indicative of a direct effect, but the presence of a *bona fide* LXR response element in the ABCG5/G8 intergenic promoter or surrounding areas has not been identified. The physiological relevance of LXR-driven upregulation of ABCG5 and ABCG8 mRNA has recently been highlighted in two separate studies. It was first shown that LXR-driven increases in hepatobiliary and fecal cholesterol excretion rely on functional ABCG5 and ABCG8, since ABCG5/G8 knockout mice could not elevate biliary and fecal sterol secretion in response to a synthetic LXR agonist (Yu *et al*., 2003). In agreement, using ABCG5/G8 knockout mice, Calpe-Berdiel *et al*. demonstrated that LXR-mediated induction of macrophage to feces reverse cholesterol transport requires functional ABCG5/G8 (Calpe-Berdiel *et al*., 2008).

Another transcriptional activator of the sitosterolemia locus is the liver receptor homolog-1 (LRH-1) (Freeman *et al*., 2004). However, it is important to point out that a functional LRH-1 binding site is only present in the human gene, and rodent orthologs do not possess LRH-1-sensitive transcriptional activation (Freeman *et al*., 2004). More recently, it was shown that three additional transcription factors known as HNF-4α, GATA binding protein 4, and GATA binding protein 6 act in a cooperative fashion to transactivate the human intergenic promoter (Sumi *et al*., 2007). There is also evidence that bacterial endotoxin can downregulate ABCG5 and ABCG8 mRNA levels (Khovidhunkit *et al*., 2003), yet the transcription factors involved in this response have yet to be clearly elucidated. Collectively, it is quite clear that ABCG5 and ABCG8 are coordinately regulated at the transcriptional level. More work in this area may prove to be critical for future ABCG5/G8-centered therapies.

Most recently, there has been support for hormonal control of ABCG5/G8 expression at both transcriptional and post-transcriptional levels. In support of this, hepatic insulin resistance promotes cholesterol gallstone formation, which is driven in part by transcriptional regulation of ABCG5/G8 (Biddinger *et al*., 2008). In this study it was shown that hepatocyte-specific deletion of the insulin receptor results in reduced insulin-driven inhibition of the transcription factor forkhead box O1 (FOXO1), and that FOXO1 is a direct transcriptional activator of ABCG5/G8. In addition to insulin-mediated regulation, ABCG5/G8 expression has also been linked to the leptin axis in the liver (Sabeva *et al*., 2007). In this case, the hepatic protein (not mRNA) abundance of ABCG5/G8 is reduced in mice lacking leptin signaling. Furthermore, hypophesectomized rats have dramatic reductions in hepatic ABCG5/G8 mRNA levels that are coupled to decreased fecal sterol loss, and these

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alterations can be normalized by thyroid hormone replacement (Galman *et al*., 2008). The mechanism by which thyroid hormone and leptin regulates ABCG5/G8 expression requires further exploration.

4.5 Biochemical studies on ABCG5/G8-dependent sterol transport

Although cellular functional assays have been somewhat elusive, ABCG5/G8-dependent sterol transport has been successfully reconstituted *in vitro* with either a recombinant or purified native ABCG5/G8, and the heterodimer can directly transport sterols, sterol esters, and phospholipids from donor vesicles to proteoliposomes in an ATP-dependent fashion and vanadate-sensitive manner (Wang *et al*., 2006, Wang *et al*., 2008). With these established *in vitro* assays and complimentary cell-based models (Vrins *et al*., 2007, Tachibana *et al*., 2007) for ABCG5/G8-dependent sterol transport we now have the tool to address important unanswered questions. For instance, does the ABCG5/G8-transported pool of sterols originate from a cytosolic pool, or does the heterodimer act primarily on a membrane-associated pool as a flippase? Are other proteins required for ABCG5/G8-dependent sterol transport? Additionally, what is the substrate specificity for ABCG5/G8?

4.6 Therapeutic perspectives for ABCG5/G8

ABCG5/G8 plays a crucial role in cholesterol excretion from the body and therefore it seems logical to assume that mice lacking the heterodimer should have increased atherosclerotic burden, while mice overexpressing ABCG5/G8 should be protected against atherosclerosis. Consistent with these hypotheses, transgenic overexpression of ABCG5/G8 in both the intestine and liver of LDL receptor knockout mice results in reduced LDL-C and significantly less atherosclerosis (Wilund *et al*., 2004). In contrast, hepatocyte-specific ABCG5/G8 overexpression, which elevates biliary sterol secretion by ~2-fold, does not protect against atherosclerosis in the LDL receptor or apoE knockout backgrounds (Wu *et al*., 2004). This result is very confusing, since it was anticipated that a 2-fold increase in biliary cholesterol output would result in atheroprotection. In a subsequent study, these same authors clarified this confusing outcome, and showed that ezetimibe treatment in mice overexpressing ABCG5/G8 specifically in the liver produces profound protection against atherosclerosis (Basso *et al*., 2007). These data strongly support the opposing roles of ABCG5/G8 and NPC1L1 (target of ezetimibe), and demonstrate that therapies that simply increase biliary sterol output may not be effective at altering sterol balance and atherosclerosis, since these sterols can re-enter the body via the action of intestinal NPC1L1 (Altmann *et al*., 2004). However, dual therapies that increase biliary sterol output and block re-uptake of biliary sterols by the intestine continue to hold promise.

It is well known that many sitosterolemic patients suffer from premature ASCVD (Salen *et al*., 1985, Kolovou *et al*., 1996, Mymin *et al*., 2003, Bjorkhem *et al*., 2001). It is however not known whether this ASCVD is caused by the massive accumulation of plant sterols in the blood or the associated hypercholesterolemia. Without a doubt hypercholesterolemia is a major driving force in ASCVD progression, but little is known about the consequences of elevated blood plant sterols because in organisms with intact ABCG5/G8 plant sterols are efficiently excluded from the body. Sitosterolemic patients and ABCG5/G8 knockout mice provide a unique research opportunity to ask the age-old question: How are phytosterols metabolized, and do phytosterols like their animal counterparts contribute to atherogenesis? Importantly, the plant sterol stigmasterol has been shown to regulate cholesterol metabolism by directly inhibiting SREBP-2 processing and activating LXR-driven gene expression (Yang *et al*., 2004). In addition sitosterol has recently been shown to drive macrophage cell death (Bao *et al*., 2006), an important feature of the late stages of ASCVD. These and many other findings have promoted a potential role for plant sterols in the progression of ASCVD in sitosterolemic patients. Recently, a study examined the relationship between blood

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sitosterol levels and atherosclerosis in both ABCG5/G8 knockout mice and in sitosterolemic patients (Wilund *et al*., 2004). It was found that plasma levels of plant sterols do not correlate with atherosclerosis extent in either of these sitosterolemic models, leading the authors to conclude that perhaps elevated plasma cholesterol levels are responsible for the development of premature ASCVD in sitosterolemic patients.

Inter-individual variations exist in responses to statins (Kajinami *et al*., 2004), and it was thought to be attributable to differences in the baseline of intestinal cholesterol absorption and/or endogenous cholesterol synthesis among individuals (Miettinen *et al*., 2000, Gylling and Miettinen, 2002). Stimulation of ABCG5/G8-mediated cholesterol excretion not only promotes fecal disposal of cholesterol, but also increases endogenous cholesterol synthesis (Yu *et al*., 2002, Yu *et al*., 2004). Coadministration of a statin and a yet-to-be developed ABCG5/G8 activator is expected to have a synergistic cholesterol-lowering effect. Consistent with this notion is that transgenic overexpression of ABCG5/G8 in the liver and intestine confers mice hypersensitivity to lovastatin in reducing plasma cholesterol (Tang *et al*., 2006).

Unlike the successful story for NPC1L1 and its inhibitor ezetimibe, a specific pharmacological activator for ABCG5/G8-dependent sterol transport has yet to be developed. Most current attempts at promoting ABCG5/G8-dependent sterol transport involve the use of synthetic LXR agonists, which robustly and transcriptionally upregulates heterodimer expression in the liver and intestine (Berge *et al*., 2000, Repa *et al*., 2002, Plosch *et al*., 2002, Kaneko *et al*., 2003). LXR activation, however, elicits the unwanted side effect of increased *de novo* lipogenesis, resulting in pronounced hepatic steatosis (Rader, 2007, Cao *et al*., 2004), which rules out synthetic LXR agonists as a safe way to promote ABCG5/G8 function. Alternative strategies for ABCG5/G8 modulation need to be pursued. ABCG5/G8 heterodimer may be directly targeted by small molecule activators. With *in vitro* and cell-based functional assays for ABCG5/G8-dependent sterol transport in place, it is now possible to screen for such compounds. Based on the limited data generated in mice transgenically overexpressing ABCG5 and ABCG8 (Yu *et al*., 2002, Tang *et al*., 2006), pharmacologic activators of this pathway could serve as powerful promoters of cholesterol removal from the body, and thus providing additional protection against atherosclerosis when given in combination with a statin.

5 SCAVENGER RECEPTOR CLASS B TYPE I (SR-BI)

5.1 Discovery of SR-BI

SR-BI was originally discovered based on its sequence homology to another scavenger receptor known as cluster determinant 36 (CD36) (Calvo and Vega, 1993, Acton *et al*., 1994). SR-BI is expressed in a wide variety of tissues, with the highest level of expression in tissues regulating cholesterol metabolism such as the liver, intestine, adrenal gland, testes, and ovary (Acton *et al*., 1994, Acton *et al*., 1996, Landschulz *et al*., 1996). SR-BI is widely accepted as an HDL receptor, and can bi-directionally transport sterols across biological membranes (Landschulz *et al*., 1996, Acton *et al*., 1996). SR-BI-driven selective uptake into the liver promotes biliary and fecal excretion of cholesterol (Kozarsky *et al*., 1997, Ji *et al*., 1999, Zhang *et al*., 2005). In addition, SR-BI-driven selective uptake into the adrenal gland is linked to steroid hormone production (Rigotti *et al*., 1996, Rigotti *et al*., 1997, Temel *et al*., 1997). Since the role of SR-BI in these processes has been the focus of previous reviews (Trigatti, 2005, Connelly, 2009), these concepts will not be discussed in detail here. The purpose of this chapter is to specifically discuss the structure and function of SR-BI in regards to intestinal sterol transport.

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5.2 Structure: gene, mRNA, and protein domains

SR-BI belongs to the class B scavenger receptor superfamily, which includes other proteins such as lysosomal integral membrane protein II (LIMP II), Drosophila melanogaster croquemort membrane protein, and Snmp-1 (Franc *et al*., 1996, Franc *et al*., 1999, Hart and Wilcox, 1993, Karakesisoglou *et al*., 1999, Vega *et al*., 1991). The human SR-BI gene contains 13 exons, and resides on chromosome 12q24.2-qter (Cao *et al*., 1997). The pattern of SR-BI full-length mRNA expression is similar in humans and rodents, with the highest level of mRNA in the adrenal gland, ovary, liver, intestine, and placenta (Acton *et al*., 1996, Landschulz *et al*., 1996, Cao *et al*., 1997). The full-length SR-BI mRNA encodes a 509 amino acid protein containing a short *N*-terminal cytoplasmic domain of 9 amino acids, a transmembrane spanning domain of 22 amino acids, a predominant extracellular domain of 408 amino acids, a second transmembrane domain of 23 amino acids, and a cytoplasmic *C*-terminus of 44 amino acids (Krieger, 1999, Williams *et al*., 1999). The predicted molecular weight of SR-BI is ~57 kDa, yet extensive *N*-linked glycosylation yields a protein that runs ~82–84 kDa on a Western blot (Krieger, 1999, Williams *et al*., 1999). SR-BI is also myristoylated and palmitoylated (Babitt *et al*., 1997), yet these post-translational modifications do not seem to impact SR-BI function. The extracellular domain of SR-BI is likely to form disulfide bridges since it contains six cysteine residues (Krieger, 1999). An alternatively spliced variant for of SR-BI mRNA was identified, and this protein is known as SR-BII (Webb *et al*., 1998, Eckhardt *et al*., 2004, Eckhardt *et al*., 2006). This alternatively spliced form has a slightly modified *C*-terminal tail, yet can also function to transport sterols across biological membranes (Webb *et al*., 1998, Eckhardt *et al*., 2004, Eckhardt *et al*., 2006). Most recently, the PDZ domain-containing adaptor protein PDZK1 has been shown to bind to the *C*-terminus of SR-BI and this protein-protein interaction controls SR-BI activity via a tissue-specific posttranscriptional mechanism (Silver, 2002, Yesilaltay *et al*., 2006, Kocher *et al*., 2003). In fact, mice deficient in PDZK1 have elevated plasma cholesterol levels due to liver-specific downregulation of SR-BI, implicating PDZK1 as a critical regulator of hepatic SR-BI function (Kocher *et al*., 2003).

5.3 Function: Lessons learned from animal models

Animal models of SR-BI deficiency or overexpression have shed light on the critical role of this protein in whole body sterol balance. Transgenic overexpression of SR-BI results in diminished very low density lipoprotein cholesterol, LDL-C, and HDL-cholesterol levels, increased biliary cholesterol secretion, and marked protection against atherosclerosis (Wang *et al*., 1998, Ueda *et al*., 1999, Ueda *et al*., 2000, Arai *et al*., 1999, Kozarsky *et al*., 2000, Ji *et al*., 1999). Conversely, mice lacking SR-BI accumulate cholesterol in the plasma, have decreased biliary sterol excretion and steroid hormone insufficiency, and develop severe atherosclerosis (Rigotti *et al*., 1997, Varban *et al*., 1998, Trigatti *et al*., 1999). Mice lacking SR-BI in the apoE-deficient background develop occlusive coronary artery atherosclerosis, myocardial infarction, and die at a very early age (Braun *et al*., 2002, Zhang *et al*., 2005). The hyperlipidemic and proatherogenic effects seen with SR-BI deficiency are thought to be primarily due to impairment of SR-BI's critical role in selective uptake of HDL cholesteryl esters into the liver. However, SR-BI is also abundantly expressed in the intestine, and has been proposed to play a role in intestinal cholesterol absorption. The primary focus of this chapter is to discuss the current state of knowledge regarding SR-BI's role in the intestine, which is still a matter of debate.

Using immunohistochemical and biochemical approaches SR-BI protein expression has been documented to be expressed in enterocytes, and can be found on both apical and basolateral membranes (Cai *et al*., 2001, Voshol *et al*., 2001, Hauser *et al*., 1998, Hatzopoulos *et al*., 1998). The intestinal gradient of SR-BI expression is found most prominently along the proximal regions of the gastrocolic axis, including the duodenum and

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jejunum where cholesterol absorption is thought to occur (Cai *et al*., 2001, Voshol *et al*., 2001, Hauser *et al*., 1998, Hatzopoulos *et al*., 1998),. The concept of SR-BI being involved in intestinal cholesterol absorption was first supported by *in vitro* studies demonstrating that cholesterol uptake in intestinal brush border membranes is reduced by anti-SR-BI blocking antibodies (Hauser *et al*., 1998). Further, early evidence showed that intestinal cholesterol absorption inhibitor ezetimibe could physically interact with SR-BI (Hatzopoulos *et al*., 1998). Also, it was shown that SR-BI is a high-affinity cholesterol binding protein in the intestinal brush border membrane (Altmann *et al*., 2002). However, mice genetically lacking SR-BI have either slightly elevated or normal intestinal cholesterol absorption (Altmann *et al*., 2002, Mardones *et al*., 2001, Nguyen *et al*., 2009). SR-BI-null mice still exhibit reduced fractional cholesterol absorption when treated with ezetimibe, ruling out SR-BI as the ezetimibe-sensitive transport protein (Altmann *et al*., 2002). In contrast to the results from SR-BI-deficient mice, intestine-specific transgenic overexpression of SR-BI promotes the appearance of radiolabeled cholesterol into the plasma following an oil-based gavage administration (Bietrix *et al*., 2006). This was interpreted as an increase in intestinal cholesterol absorption, yet this method may not provide an accurate quantitative measure of fractional cholesterol absorption. Collectively, there is evidence to support the ability of SR-BI to facilitate the binding of cholesterol from bile salt micelles donors into brush border membrane vesicles (Nguyen *et al*., 2009, Labonte *et al*., 2007, Knopfel *et al*., 2007), but data from knockout mice confirm that this phenomenon is not rate limiting in net cholesterol absorption (Altmann *et al*., 2002, Mardones *et al*., 2001, Nguyen *et al*., 2009).

5.4 Function: Lessons learned from cell model systems

Although SR-BI may have been implicated in the apical to basolateral transport process of intestinal cholesterol absorption, most cell models of SR-BI-dependent sterol transport suggest a quite different trafficking pattern of SR-BI-delivered sterols. Despite a matter of debate, in the steady state SR-BI seems to localize to both apical and basolateral membranes in polarized cells (Cai *et al*., 2001, Voshol *et al*., 2001, Hauser *et al*., 1998, Hatzopoulos *et al*., 1998, Silver *et al*., 2001, Burgos *et al*., 2004, Harder *et al*., 2007, Wustner *et al*., 2004, Sehayek *et al*., 2003). In polarized cells SR-BI undergoes sterol-dependent directional basolateral to apical transcytosis (Silver *et al*., 2001, Burgos *et al*., 2004, Harder *et al*., 2007, Wustner *et al*., 2004, Sehayek *et al*., 2003). This directional pattern of sterol transport is consistent with SR-BI's role as an HDL receptor promoting selective uptake of cholesteryl esters from circulation to liver for biliary disposal (Silver *et al*., 2001, Burgos *et al*., 2004, Harder *et al*., 2007, Wustner *et al*., 2004, Sehayek *et al*., 2003). This selective uptake involves the internalization of HDL-associated cholesteryl esters into the cell, without the net internalization and degradation of the lipoprotein itself. Additionally, SR-BI can also promote selective uptake of free sterols, phospholipids, triglycerides, and cholesteryl ethers (Stangl *et al*., 1999, Urban *et al*., 2000, Greene *et al*., 2001). Selective uptake may involve the "retro-endocytosis" pathway, which involves holoparticle uptake and the subsequent re-secretion of the cholesteryl-ester poor HDL (Silver *et al*., 2001, Rhainds *et al*., 2004, de la Llera-Moya *et al*., 1999). The precise molecular mechanism by which SR-BI facilitates selective sterol uptake remains incompletely understood, but purified SR-BI reconstituted into liposomes can recapitulate high-affinity HDL binding and selective uptake, indicating that these processes are intrinsic to the receptor itself (Liu and Krieger, 2002).

Although SR-BI is generally thought to be an HDL receptor, it can bind a wide variety of ligands. In fact, the first SR-BI ligands described were modified apoB-containing lipoproteins including acetylated-LDL and oxidized-LDL (Acton *et al*., 1994, Gillotte-Taylor *et al*., 2001). In addition to binding native and modified lipoproteins, SR-BI binds maleylated BSA (Acton *et al*., 1994), advanced glycated proteins (Ohgami *et al*., 2001), anionic phospholipids (Rigotti *et al*., 1995, Fukasawa *et al*., 1996), and β-amyloid (Paresce

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et al., 1996, Husemann *et al*., 2001, Husemann and Silverstein, 2001). The majority of SR-BI ligand binding studies have been carried out using native lipoproteins. Lipoprotein binding to SR-BI exhibits intrinsic nonreciprocal cross competition (Ashkenas *et al*., 1993, Gu *et al*., 2000). In this case, HDL binding efficiently blocks subsequent LDL binding, yet the ability of LDL to block HDL binding is relatively weak (Ashkenas *et al*., 1993, Gu *et al*., 2000). Therefore, it is generally accepted that LDL does not typically interfere with HDL binding *in vivo*, further supporting the claim that SR-BI is the HDL receptor (Acton *et al*., 1996, Landschulz *et al*., 1996). Importantly, not all HDL particles bind to SR-BI with the same affinity. Lipid-free apoA-I and pre-β HDL particles are poor substrates for SR-BI, whereas spherical large α-HDL particles bind with much higher affinity (Xu *et al*., 1997, Liadaki *et al*., 2000, de Beer *et al*., 2001, Williams *et al*., 2000). The interaction of HDL particles with SR-BI relies on interactions with intrinsic HDL apolipoproteins including apoA-I, apoA-II, and apoE (Li *et al*., 2002, Pilon *et al*., 2000, Bultel-Brienne *et al*., 2002, Liu *et al*., 2002).

In addition to facilitating cholesterol uptake from lipoproteins, SR-BI can serve as a bi-directional sterol transporter, facilitating cholesterol efflux from cells (Kozarsky *et al*., 1997, Ji *et al*., 1997, Jian *et al*., 1998, Yancey *et al*., 2000, Gu *et al*., 2000). This activity was originally established when SR-BI expression levels in a variety of different cell lines are highly correlated to HDL-mediated sterol efflux (Ji *et al*., 1997). Later it was shown in SR-BI gain of function experiments in cells that SR-BI promotes cholesterol efflux to HDL and phosphatidylcholine-containing liposomes (Jian *et al*., 1998). Studies done with mutated forms of apoA-I suggest that a direct interaction between apoA-I and SR-BI must occur for efficient cholesterol efflux (Liu *et al*., 2002), yet other studies have challenged the concept that physical binding of the HDL particle to SR-BI is required for sterol efflux (Kellner-Weibel *et al*., 2000, de la Llera-Moya *et al*., 1999). In the context of atherosclerosis, the process of cholesterol efflux is thought to be most important in immune cells present in the artery wall such as macrophages, but macrophage SR-BI does not seem to play a major role in macrophage cholesterol efflux or *in vivo* reverse cholesterol transport (Yu *et al*., 2004, Yvan-Charvet *et al*., 2008, Wang *et al*., 2007). The physiological implication of SR-BI-mediated sterol efflux has yet to be understood.

5.5 Regulation of expression

Since SR-BI is so critical to sterol balance and steroid hormone production, its expression is highly controlled at the transcriptional level. In fact SR-BI expression is regulated by trophic hormones, cholesterol, and fatty acids in a tissue specific manner (Rigotti *et al*., 1996, Wang *et al*., 1996, Cao *et al*., 1999, Towns *et al*., 2005, Fluiter *et al*., 1998, Spady *et al*., 1999, Li *et al*., 1998, McLean and Sandhoff, 1998, Mizutani *et al*., 1997, Mizutani *et al*., 2000, Reaven *et al*., 1998, Reaven *et al*., 1999, Azhar *et al*., 1998, Li *et al*., 2001, Svensson *et al*., 1999). In the case of hormone regulation, SR-BI expression is upregulated by adrenocorticotrophic hormone (Kozarsky *et al*., 1997, Rigotti *et al*., 1996, Wang *et al*., 1996, Cao *et al*., 1999), luteinizing hormone (Landschulz *et al*., 1996), human chorionic gonadotropin (Kozarsky *et al*., 1997, Li *et al*., 1998, McLean and Sandhoff, 1998), pregnant mare serum gonadotropin (Li *et al*., 1998, Mizutani *et al*., 1997, McLean and Sandhoff, 1998), cyclic AMP analogs (Azhar *et al*., 1998), and insulin (Li *et al*., 2001). It is generally accepted that trophic hormone-driven stimulation of SR-BI expression in steroidogenic cells relies on cAMP-dependent activation of protein kinase A, and subsequent promoter transactivation that depends on both steroidogenic factor-1 (SF-1) (Cao *et al*., 1997, Cao *et al*., 1999, Parker, 1998, Lopez and McLean, 1999) and CCAAT/enhancer binding proteins (Lekstrom-Himes and Xanthopoulos, 1998). SF-1 is critical for both basal and trophic hormone-stimulated transactivation of the SR-BI promoter, and directly promotes the

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expression of SR-BI during the development of several steroidogenic tissues (Cao *et al*., 1997, Cao *et al*., 1999, Parker, 1998, Lopez and McLean, 1999).

In addition to hormonal regulation, SR-BI expression is also sensitive to alteration in cellular cholesterol levels (Wang *et al*., 1996, Cao *et al*., 1999, Sun *et al*., 1999). In this case, a *bona fide* sterol response element has been identified in the SR-BI promoter (Lopez and McLean, 1999). This element confers SREBP-1α-dependent transactivation of the gene. Interestingly, in the ovary, gonadotropin treatment induces the expression of SREBP-1α and subsequent enhancement of SR-BI expression (McConihay *et al*., 2001). In line with this, depletion of plasma cholesterol levels results in induction of SR-BI expression in the adrenal gland, an effect that does not depend on plasma levels of adrenocorticotrophic hormone (Sun *et al*., 1999). In further support of sterol-dependent transcriptional regulation, SR-BI expression levels are dramatically altered in mouse models of altered cholesterol metabolism (Wang *et al*., 1996, Ng *et al*., 1997). For example, mice genetically lacking the steroidogenic acute regulatory (StAR) protein have elevated levels of adrenal SR-BI (Cao *et al*., 1999). Mice lacking either apoA-I, lecithin:cholesterol acyltransferase, or hepatic lipase all exhibit increased levels of adrenal SR-BI (Cao *et al*., 1999, Wang *et al*., 1996, Ng *et al*., 1997). Further, the human SR-BI promoter can be regulated by LXR in hepatocytes and adipocyte (Malerod *et al*., 2002).

Two transcriptional pathways are known to repress SR-BI expression, including YY1 (Yin Yang 1) zinc transcription factor and DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome, gene 1). YY1-mediated repression occurs in the basal state due to direct binding of the SR-BI promoter, and can alternatively bind directly to SREBP-1 functionally preventing its transactivation of the SR-BI promoter (Shea-Eaton *et al*., 2001). In contrast, DAX-1 represses SR-BI expression by directly interacting with the known transcriptional activators such as SF-1 and SREBP-1a (Lopez *et al*., 2001). In addition to these defined pathways, numerous additional modes of transcriptional regulation of the SR-BI promoter are being discovered including PPARγ, HNF4α, and the prolactin regulatory element-binding (PREB) transcription factor (Malerod *et al*., 2003, Murao *et al*., 2008). Collectively, the SR-BI promoter is under complex transcriptional control, and additional work is needed to more completely define physiological implications of these regulatory pathways.

5.6 Cholesterol binding studies

Although it is well accepted that SR-BI can bi-directionally transfer cholesterol across biological membranes, the precise molecular mechanism by which this is carried out is a matter of debate. The vast majority of work has focused on the direct interactions between SR-BI and apolipoproteins resident on HDL. However, there has been some recent evidence that SR-BI can directly bind cholesterol (Assanasen *et al*., 2005). In this work the authors tested whether SR-BI could bind a photoactivatable cholesterol analog, and indeed found direct binding between the *C*-terminal transmembrane domain of SR-BI and this cholesterol analog. The molecular base for SR-BI to bind cholesterol has yet to be determined. The *C*-terminal transmembrane region of SR-BI does not contain a cholesterol recognition/ interaction amino acid consensus (CRAC) or steroidogenic acute regulatory protein-related lipid transfer (START) domain found in other cholesterol-binding proteins (Li and Papadopoulos, 1998, Ponting and Aravind, 1999, Epand, 2006). However, SR-BI is not alone in its lacking of a canonical cholesterol-binding motif. Proteins in the tetraspanin and synaptophysin families also bind cholesterol through a CRAC-, or START-domain independent fashion (Thiele *et al*., 2000, Charrin *et al*., 2003). Recent evidence suggests that SR-BI may dimerize or form higher order oligomers, providing the potential for multiple *C*-termini to serve as a functional cholesterol binding pocket (Reaven *et al*., 2004, Sahoo *et al*., 2007, Sahoo *et al*., 2007). Additional work is needed to define this possibility.

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5.7 Therapeutic perspectives for SR-BI

The hyperlipidemic and proatherogenic effects seen with SR-BI deficiency are thought to be primarily due to loss of SR-BI's critical role in selective uptake of HDL cholesteryl esters into the liver, thereby reducing biliary and fecal sterol excretion. However, effects of SR-BI deficiency in the intestine and other tissues on whole-body cholesterol homeostasis should be considered as well. Several investigators have proposed that SR-BI facilitates intestinal cholesterol absorption (Altmann *et al*., 2002, Mardones *et al*., 2001, Nguyen *et al*., 2009, Bietrix *et al*., 2006, Labonte *et al*., 2007, Knopfel *et al*., 2007). The main argument for this is that SR-BI is present in intestinal brush border membrane vesicles, and an SR-BI antibody reduces cholesterol binding to brush border membrane vesicles. However these *in vitro* findings have not been substantiated in mice with targeted disruption of SR-BI. In fact, mice lacking SR-BI have normal or enhanced intestinal cholesterol absorption (Altmann *et al*., 2002, Mardones *et al*., 2001, Nguyen *et al*., 2009). The most thorough of these studies revealed that SR-BI deficiency significantly increases fractional cholesterol absorption, under several conditions of dietary cholesterol challenge (Mardones *et al*., 2001). Collectively, these studies have revealed that SR-BI is highly expressed in the intestine, but its role in intestinal cholesterol absorption has not been definitively established. In contrast to a role of SR-BI in the apical to basolateral transport process of cholesterol absorption, we propose that intestinal SR-BI may play a role in basolateral to apical transport of cholesterol in enterocytes. This directional process of transintestinal cholesterol excretion or non-biliary fecal sterol loss is a critical pathway to rid the body of excess cholesterol directly through intestinal secretion (Kruit *et al*., 2006, Brown *et al*., 2008, van der Velde *et al*., 2008, van der Velde *et al*., 2007). Based on a number of cellular trafficking studies, SR-BI-mediated selective uptake results in directional basolateral to apical trafficking of sterol cargo (Silver *et al*., 2001, Burgos *et al*., 2004, Harder *et al*., 2007, Wustner *et al*., 2004, Sehayek *et al*., 2003). This directional trafficking pattern for SR-BI cargo has been described *in vivo* in the liver (Silver *et al*., 2001), and investigations are now underway to examine whether a similar pathway exists in the proximal small intestine. At this point, given our incomplete and opposite understanding of SR-BI's role in intestinal sterol trafficking, it is difficult to know whether intestinal SR-BI remains a viable therapeutic target for ASCVD. More work is needed before this possibility can be realized.

6. OTHER PROTEINS INFLUENCING INTESTINAL CHOLESTEROL ABSORPTION

Other proteins thought to influence intestinal cholesterol absorption at the intestinal brush border membrane level include CD36 and aminopeptidase N (CD13). CD36 is a scavenger receptor. Deletion of CD36 in mice appear to reduce cholesterol absorption from the proximal small intestine, but increase cholesterol absorption from the distal small intestine, and therefore does not affect net cholesterol absorption (Nguyen *et al*., 2009, Nauli *et al*., 2006). CD13 localizes to the apical membrane of enterocyte and was reported to interact with ezetimibe (Kramer *et al*., 2005). Physiological evidence for a role of CD13 in intestinal cholesterol absorption is currently unavailable.

Cholesterol enters absorptive enterocytes as a free form. Cholesterol delivered to intestinal and thoracic duct lymph mainly exists as an esterified form. The enzyme that catalizes cholesterol esterification in absorptive enterocytes is acyl-CoA:cholesterol acyltransferase-2 (Anderson *et al*., 1998) (Figure 1). Thus, genetic inactivation of acyl-CoA:cholesterol acyltransferase-2 in mice significantly reduces intestinal cholesterol absorption (Buhman *et al*., 2000, Repa *et al*., 2004, Temel *et al*., 2005).

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Unesterified cholesterol, if not transported out back to the gut lumen by the apically-localized heterodimer of ABCG5/G8, can enter the circulation via the basolaterally-localized ABCA1 (Temel *et al*., 2005) (Figure 1) and this ABCA1-dependent pathway plays a significant role in HDL biogenesis because mice lacking ABCA1 display a reduced plasma HDL-cholesterol concentration (Brunham *et al*., 2006).

In addition to proteins discussed in this article, many other cellular proteins may also regulate intestinal sterol absorption, such as serine palmitoytransferase (Li *et al*., 2009) and those listed in a classic review on regulation of intestinal cholesterol absorption (Wang, 2007). Readers interested are referred to this review (Wang, 2007).

7. CONCLUDING COMMENTS

Within the last decade, our understanding of protein-mediated intestinal cholesterol transport has seen immense progress. We have gained important insights into the structure, function, and regulation of a dedicated apical sterol transporter NPC1L1, and its opposing heterodimeric protein complex ABCG5/G8. Furthermore, the development of small molecule inhibitors of intestinal cholesterol absorption has turned out to be important tools to dissect the pathway. Such rapid progress in our understanding of this complex physiological process has been a powerful integration of many scientific disciplines including human genetics, pharmacology, cell biology, biochemistry, genetically altered mouse models, and physiological approaches. Although we have made substantial progress in identifying several protein-mediators of sterol transport across intestinal brush border membrane, it is unlikely this list is complete in its current form. Importantly, mechanistic understanding of protein-mediated sterol transport across plasma membrane and subsequent intracellular trafficking of sterols in the absorptive enterocyte is still in its infancy. By taking advantage of this same multidisciplinary approach, we now have the tools in place to further our understanding of intestinal cholesterol absorption and its protein mediators.

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ABBREVIATIONS

ABC	ATP-binding cassette transporter
Apo	Apolipoprotein
ASCVD	Atherosclerotic cardiovascular disease
CD36	Cluster determinant 36
ER	Endoplasmic reticulum
HDL	High density lipoprotein
HNF4α	Hepatocyte nuclear factor 4 alpha
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-cholesterol
LXR	Liver X receptor
NPC1	Niemann-Pick C1

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NPC1L1	Niemann-Pick C1-Like 1
PPAR	peroxisome proliferators-activated receptor
SCAP	Sterol regulatory element-binding protein-cleavage activating protein
SR-BI	Scavenger receptor class B type I
SREBP	Sterol regulatory element-binding protein

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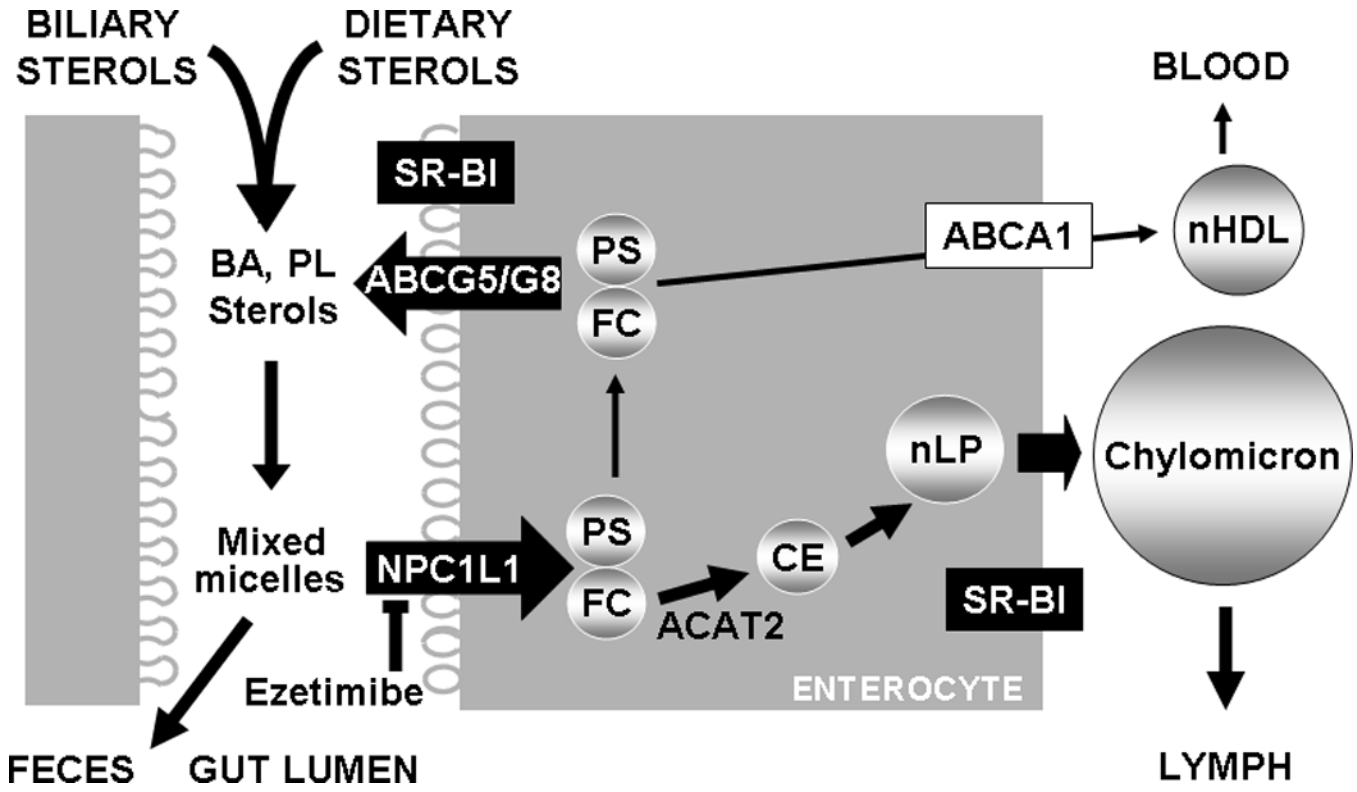

Figure 1.

Intestinal sterol absorption and secretion. Sterols including free cholesterol (FC) and free plant sterols (PS) from diet and bile are mixed with phospholipids (PL) and bile acids (BA) to form micelles. FC and PS solubilized in mixed micelles are transported into absorptive enterocytes via an NPC1L1-dependent and ezetimibe-inhibitable mechanism. FC is delivered to the ER for esterification by acyl-CoA:cholesterol acyltransferase-2 (ACAT2) to form cholesterol esters (CE) that is then packaged into nascent lipoprotein particles (nLP) and secreted as a constituent of chylomicron. PS and FC that escapes ACAT2 esterification may be directly transported to nascent HDL (nHDL) through basolateral ABCA1, or back to the gut lumen via ABCG5/G8.

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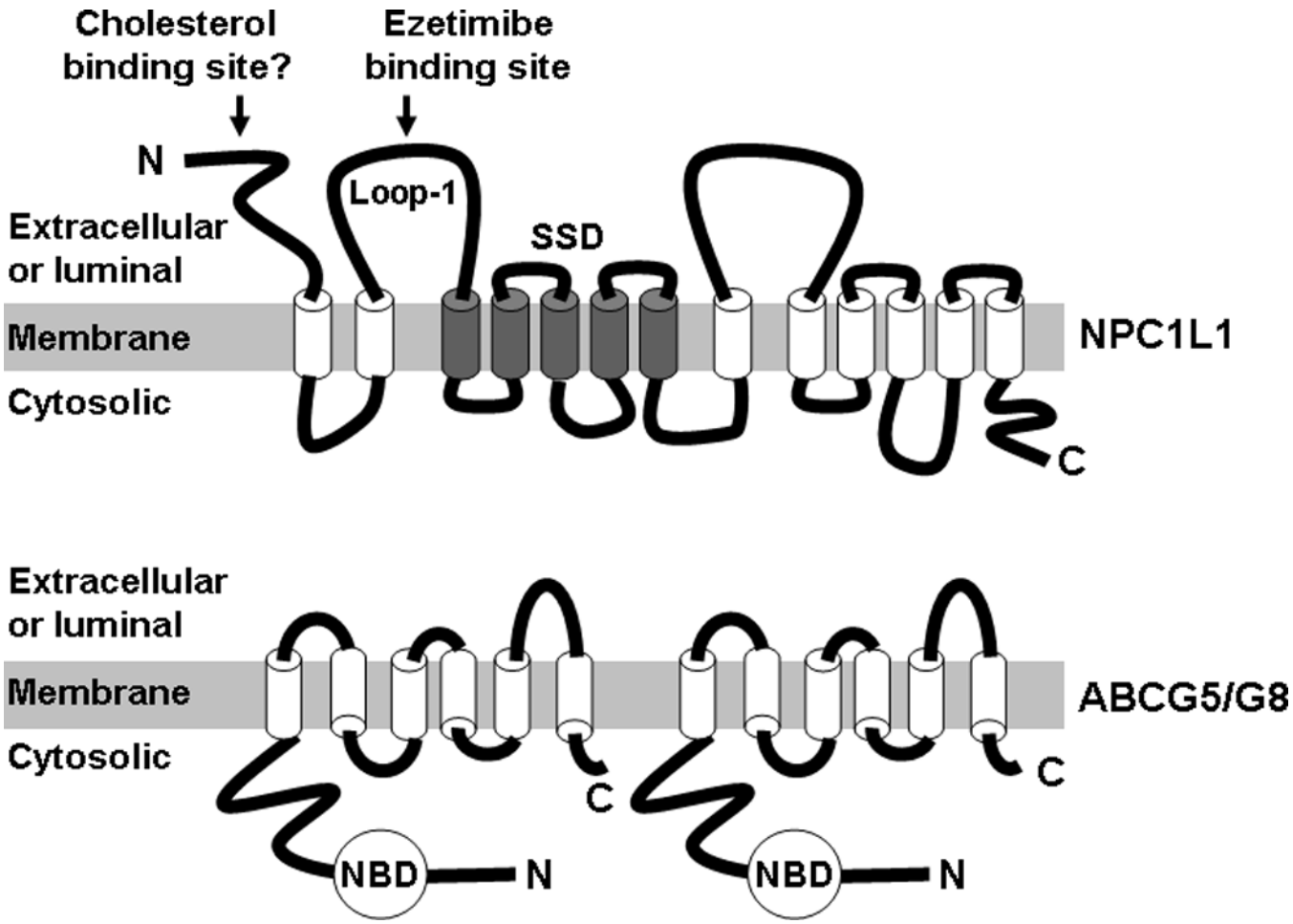

Figure 2.
Proposed membrane topologies and domains of NPC1L1 (A) and ABCG5/G8 (B) proteins. SSD, sterol-sensing domain; N, *N*-terminus; C, *C*-terminus; NBD, nucleotide-binding domain.

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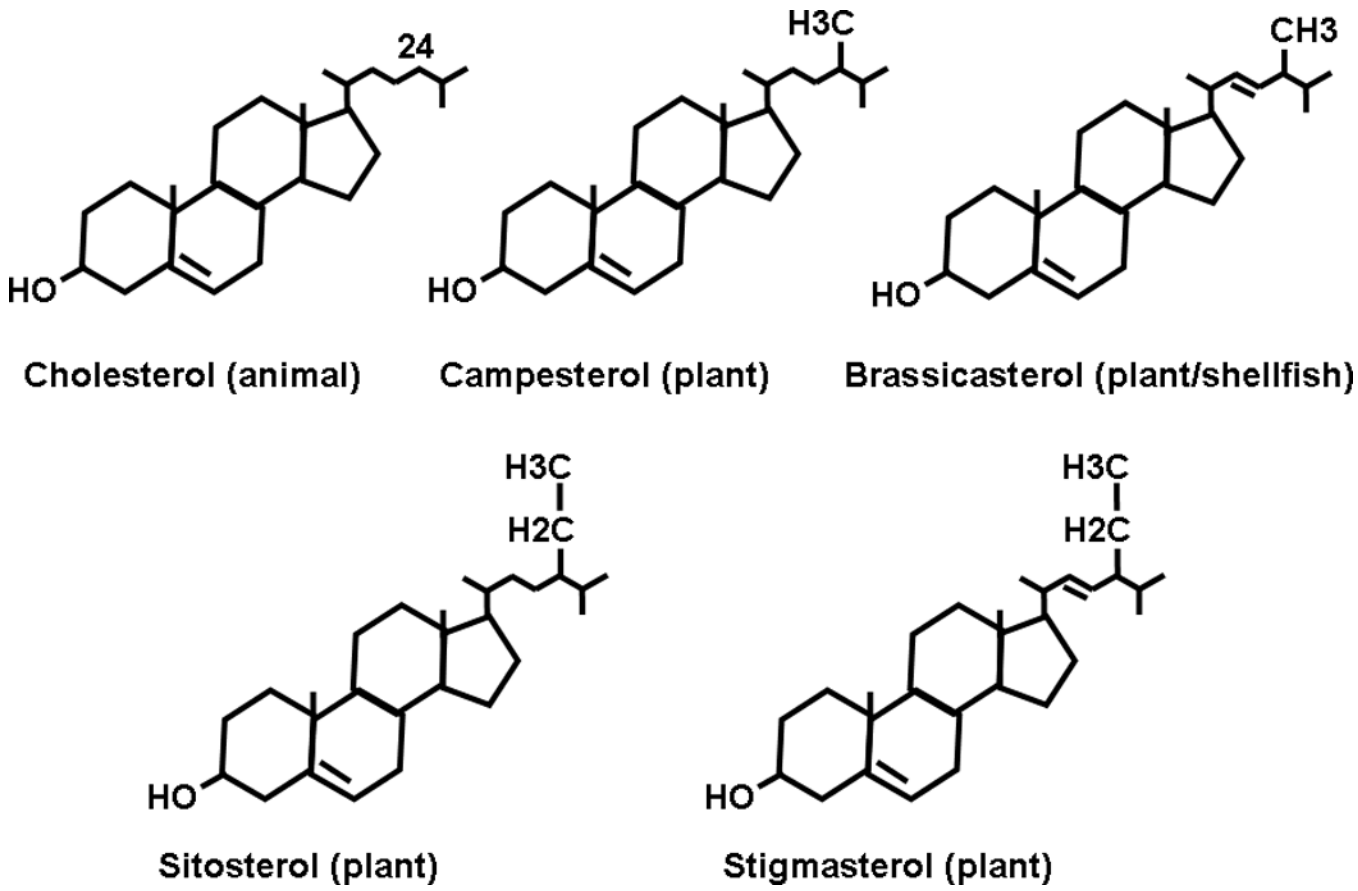

Figure 3.
Structures of sterols derived from animals and plants.