Genetic inactivation of NPC1L1 protects against sitosterolemia in mice lacking ABCG5/ABCG8

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Abstract Mice lacking Niemann-Pick C1-Like 1 (NPC1L1) (NPC1L1$^{-/-}$ mice) exhibit a defect in intestinal absorption of cholesterol and phytosterols. However, wild-type (WT) mice do not efficiently absorb and accumulate phytosterols either. Cell-based studies show that NPC1L1 is a much weaker transporter for phytosterols than cholesterol. In this study, we examined the role of NPC1L1 in phytosterol and cholesterol trafficking in mice lacking ATP-binding cassette (ABC) transporters G5 and G8 (G5/G8$^{-/-}$ mice). G5/G8$^{-/-}$ mice develop sitosterolemia, a genetic disorder characterized by the accumulation of phytosterols in blood and tissues. We found that mice lacking ABCG5/G8 and NPC1L1 [triple knockout (TKO) mice] did not accumulate phytosterols in plasma and the liver. TKO mice, like G5/G8$^{-/-}$ mice, still had a defect in hepatobiliary cholesterol secretion, which was consistent with TKO versus NPC1L1$^{-/-}$ mice exhibiting a 52% reduction in fecal cholesterol excretion. Because fractional cholesterol absorption was reduced similarly in NPC1L1$^{-/-}$ and TKO mice, by subtracting fecal cholesterol excretion in TKO mice from NPC1L1$^{-/-}$ mice, we estimated that a 25g NPC1L1$^{-/-}$ mouse may secrete about 4 µmol of cholesterol daily via the G5/G8 pathway. In conclusion, NPC1L1 is essential for phytosterols to enter the body in mice.—Tang, W., Y. Ma, L. Jia, Y. A. Ioannou, J. P. Davies, and L. Yu. **Genetic inactivation of NPC1L1 protects against sitosterolemia in mice lacking ABCG5/ABCG8.** *J. Lipid Res.* 2009. **50:** 293–300.

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The two ATP-binding cassette (ABC) half-transporters, G5 and G8, reside at the canalicular membrane of hepatocytes and the apical membrane of absorptive enterocytes where they function as heterodimers to transport cholesterol and noncholesterol sterols into the bile canaliculus

and the gut lumen for fecal excretion (1–4). Mutations in either ABCG5 (G5) or ABCG8 (G8) cause sitosterolemia, a rare autosomal recessive disorder (5, 6). The hallmark of sitosterolemia is the accumulation of sitosterol and campesterol, two major plant-derived sterols, in the blood and tissues (7–10). In sitosterolemic patients and mice, the plasma cholesterol concentrations follow fluctuations in dietary cholesterol intake (2, 11, 12). Many sitosterolemic individuals develop xanthomas and premature coronary heart disease as a result of sterol deposition in the skin, tendons, and coronary arteries (13–15).

Ezetimibe, a cholesterol absorption inhibitor (16), has been recently shown to effectively reduce the plasma phytosterol levels in sitosterolemic patients and mice (17, 18). It can also completely reverse xanthomatosis when used in combination with low-dose cholestyramine therapy in a sitosterolemic patient (19). These findings suggest two possibilities: *1*) there is a common pathway for intestinal absorption of cholesterol and phytosterols; or *2*) ezetimibe simultaneously targets two independent transporters: one for cholesterol and the other for phytosterols.

In search of the molecular target of ezetimibe, Altmann et al. (20) identified a polytopic transmembrane protein named Niemann-Pick C1-Like 1 (NPC1L1), which localizes at the brush border membrane of the small intestine and mediates intestinal absorption of cholesterol. Disruption of NPC1L1 in mice causes a substantial reduction in intestinal absorption of cholesterol, a phenotype identical to that seen in ezetimibe-treated animals (20). Currently, the majority of animal, genetic, and biochemical studies support the notion that NPC1L1 is a molecular target of ezetimibe (20–31).

Interestingly, NPC1L1 ablation also reduces intestinal absorption of phytosterols in mice, consistent with NPC1L1 being an intestinal phytosterol transporter (21). In

Abbreviations: ABC, ATP-binding cassette; NPC1L1, Niemann-Pick C1-Like 1; RAP, receptor associated protein; TKO, triple knockout; WT, wild-type.
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sitosterolemia, ezetimibe likely reduces plasma phytosterol concentrations by inhibiting the ability of NPC1L1 to transport dietary phytosterols across the apical surface of enterocytes in the small intestine. However, some studies have shown that multiple membrane proteins, other than NPC1L1, are the molecular targets of ezetimibe (32–34), although the physiological significance of these proteins is currently unknown. Additionally, in several cell-based studies, we and others have shown that NPC1L1 is a much weaker transporter for sitosterol than cholesterol (30, 31, 35). Although NPC1L1$^{-/-}$ mice have reduced intestinal sitosterol absorption and plasma phytosterol concentrations when compared with wild-type (WT) mice (21), the absolute differences between the two genotypes are minor because phytosterols do not accumulate in WT mice. To further clarify the physiological significance of NPC1L1 in phytosterol trafficking, we took advantage of G5/G8$^{-/-}$ mice that aberrantly accumulate phytosterols in the body. We genetically inactivated NPC1L1 in these animals and anticipated that mice lacking NPC1L1, G5, and G8 would not develop sitosterolemia. Consistent with our anticipation, these triple knockout (TKO) mice did not develop sitosterolemia, which supports the notion that NPC1L1 functions as an importannt phytosterol transporter. In addition, we also studied cholesterol trafficking in these TKO mice and genetically determined the relative contribution of NPC1L1 and G5/G8 pathways to fecal cholesterol output.

MATERIALS AND METHODS

Animals and diets

G5/G8$^{-/-}$ and NPC1L1$^{-/-}$ mice were reported previously (2, 36). G5/G8$^{-/-}$ mice have a mixed genetic background of C57BL/6 (75%) and 129SvEv (25%). NPC1L1$^{-/-}$ mice have a pure C57BL/6 background. To generate mice lacking G5/G8 and NPC1L1 (TKO mice) and the control mice of the same genetic background, G5/G8$^{-/-}$ mice were crossed with NPC1L1$^{-/-}$ mice. Their offspring were then crossed to each other to establish the following four mouse lines: WT, G5/G8$^{-/-}$, NPC1L1$^{-/-}$, and TKO mice. All mice were housed in a specific pathogen-free animal facility in plastic cages at 22°C, with a daylight cycle from 6 AM to 6 PM. The mice were provided with water and a cereal-based rodent chow diet, ad libitum, unless stated otherwise. All animal procedures were approved by the institutional animal care and use committee at Wake Forest University Health Sciences.

At 2 months of age, male mice were fed a synthetic diet containing 10% energy from palm oil, 0.02% (w/w) cholesterol, and 0.014% (w/w) phytosterols (56.6% sitosterol, 23.6% campesterol, and 19.8% stigmasterol), prepared by our institutional diet core. The synthetic diet is similar to a human diet, and the cholesterol content is similar to that used in a regular chow diet. After being fed the synthetic diet for 21 days, the mice were fasted for 4 h before being sacrificed during the daylight cycle. Blood, bile, and tissues were collected.

Measurement of fractional intestinal cholesterol absorption

On day 19 of the synthetic diet feeding, the mice were gavaged with 0.1 μCi of [4-^{14}C] cholesterol and 0.2 μCi of [22, 23-^{3}H] sitosterol (American Radiolabeled Chemical, Inc.) dissolved in 100 μl of soybean oil. Mice were individually housed for 3 days. The feces were collected for measuring fractional intestinal cholesterol absorption as described previously (37).

Determination of fecal sterol excretion

After being fed the synthetic diet for 18 days, the mice were individually housed for 3 days. The feces were collected, dried in a 70°C vacuum oven, weighed, and crushed into power. A measured mass (~100 mg) of feces was placed into a glass tube containing 103 μg of 5α-cholestane as an internal standard. The feces were saponified, and the lipids were extracted into hexane and analyzed by gas-liquid chromatography.

Analysis of lipid concentrations in plasma, liver, and bile

To measure plasma concentrations of various sterols, 50 μl of plasma was diluted to 800 μl with H_2O, followed by addition of 20 μg of 5α-cholestane as an internal standard. The lipids in this mixture were then extracted by adding 4 ml $CHCl_3$/MeOH (1:1) and 1 ml 20%N_aCl. The $CHCl_3$ phase was dried down and dissolved in hexane and analyzed for total and free sterols by gas-liquid chromatography (37).

For hepatic sterols, ~100 mg of liver was extracted with $CHCl_3$/MeOH (2:1) in the presence of 100 μg 5α-cholestane as an internal standard. The $CHCl_3$ phase was dried down and dissolved in hexane and analyzed for total and free sterols by gas-liquid chromatography (37, 38).

Lipid concentrations in the gallbladder bile were measured as described previously (39).

Preparation and Western blot analysis of small intestine homogenates

The entire small intestine was equally divided into five segments. The whole middle segment from each animal was homogenized in 0.5 ml lysis buffer (20 mM HEPES-HCl, pH 7.4; 5 mM $MgCl_2$; 1 mM EGTA; 10% glycerol; 1% Nonidet P-40; 0.12% cholesteryl hemisuccinate ester; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 5 μg/ml pepstatin A; and 1 mM DTT). The homogenates were then passed through a 221/2-gauge needle 15 times. After centrifuging at 1,000 g for 10 min at 4°C, the supernatant was collected and solubilized by rotating overnight at 4°C. Lysed proteins were immunoblotted as described previously (29) with the polyclonal rabbit anti-mouse NPC1L1 antiserum (40), the polyclonal rabbit anti-mouse ABCG5 antiserum (2), the polyclonal anti-mouse ABCA1 antibody (41), and the polyclonal rabbit anti-rat receptor associated protein (RAP) (42).

Statistical analysis

All data was reported as the mean ± the standard error of the mean (SEM). The differences between the mean values were tested for statistical significance ($P < 0.05$) by ANOVA (Tukey-Kramer honestly significant difference).

RESULTS

NPC1L1 ablation protects against sitosterolemia in G5/G8$^{-/-}$ mice

To clarify the role of NPC1L1 in phytosterol trafficking, plasma sterol levels were determined in WT, G5/G8$^{-/-}$, NPC1L1$^{-/-}$, and TKO mice. As expected, the G5/G8$^{-/-}$ mice developed sitosterolemia, characterized by a massive accumulation of sitosterol and campesterol in the plasma

TABLE 1. Plasma concentrations of total and unesterified sterols

	Total			Unesterified		
	Chol	Camp	Sito	Chol	Camp	Sito
	Mean ± SEM (mg/dl)					
WT	125.0 ± 2.8^a	1.1 ± 0.2^b	0.4 ± 0.2^b	32.9 ± 0.6^a	0.3 ± 0.1^b	UD
G5/G8$^{-/-}$	96.6 ± 6.3^b	14.9 ± 1.5^a	25.6 ± 1.5^a	27.1 ± 1.7^b	4.1 ± 0.4^a	8.0 ± 0.6^a
NPC1L1$^{-/-}$	95.9 ± 4.2^b	UD	0.2 ± 0.1^b	27.0 ± 1.3^b	UD	UD
TKO	89.5 ± 7.1^b	0.8 ± 0.2^b	2.7 ± 0.3^b	24.5 ± 1.7^b	0.1 ± 0.0^b	0.7 ± 0.1^b

NPC1L1$^{-/-}$, Niemann-Pick C1-Like 1; UD, undetectable amounts; WT, wild-type. The mice (n = 6) were fasted for 4 h prior to plasma analyses for total and unesterified cholesterol (Chol), campesterol (Camp), and sitosterol (Sito). The difference between values associated with different superscript letters in each column is statistically significant ($P < 0.05$).

(**Table 1**). These phytosterols were efficiently esterified in plasma, and only a small proportion existed as free sterols. The plasma concentrations of phytosterols did not differ between TKO and WT or NPC1L1$^{-/-}$ mice, indicative of the absence of sitosterolemia in TKO mice despite G5/G8-deficiency. Compared with WT mice, the plasma total and free cholesterol concentrations were significantly reduced in G5/G8$^{-/-}$ mice, consistent with previous publications (2, 18, 43). NPC1L1$^{-/-}$ and TKO versus WT mice also had a significant reduction in plasma cholesterol levels, which likely resulted from the blockade of intestinal cholesterol absorption, in agreement with our recent finding (44). Notably, phytosterol levels were undetectable by our methods in some of the WT, NPC1L1$^{-/-}$, and TKO mice that had been fed the synthetic diet for 21 days. The same diet apparently caused a massive accumulation of phytosterols in G5/G8$^{-/-}$ mice.

NPC1L1 ablation prevents phytosterol accumulation and restores cholesterol content in the liver of G5/G8$^{-/-}$ mice

To determine the effect of NPC1L1 deletion on tissue sterol content, the hepatic cholesterol and phytosterols were measured. We have previously shown that G5/G8$^{-/-}$ mice accumulate phytosterols in the liver, and that is associated with a reduction in tissue cholesterol (2, 18, 43). This observation was recapitulated in the current study (**Table 2**). Additionally, NPC1L1 inactivation abolished hepatic accumulation of sitosterol and campesterol, resulting in a significant recovery of hepatic cholesterol content. Interestingly, NPC1L1$^{-/-}$ versus WT mice showed a significant reduction in hepatic cholesterol, suggesting that there was cholesterol deprivation in NPC1L1$^{-/-}$ mice.

Whereas the majority of phytosterols were esterified in plasma (Table 1), they existed primarily as free sterols in the liver (Table 2). This is not surprising because it has been shown that the plasma lecithin:cholesterol acyltransferase can efficiently esterify cholesterol and sitosterol, while the hepatic acyl-Co-A:cholesterol O-acyltransferase 2 prefers cholesterol rather than sitosterol as its substrate (45).

NPC1L1 ablation restores fecal excretion of phytosterols not cholesterol in G5/G8$^{-/-}$ mice

Fecal excretion represents a major pathway by which the body eliminates sterols. Theoretically, when phytosterols accumulate in the body, their fecal excretion should be reduced because phytosterols cannot be synthesized endogenously. Indeed, the fecal excretion of phytosterols (sitosterol, campesterol, and stigmasterol) was decreased by ~37% in G5/G8$^{-/-}$ mice (2.64 versus 4.2 μmol/day/100 g BW for G5/G8$^{-/-}$ versus WT mice, respectively) (**Fig. 1A**). Blockade of intestinal sterol absorption by NPC1L1 disruption in these animals restored fecal phytosterol excretion to a level that is equivalent to those seen in WT and NPC1L1$^{-/-}$ mice. Fecal excretion of neutral sterols (cholesterol and its bacterial derivatives) was modestly reduced by 28% in G5/G8$^{-/-}$ mice (2.95 versus 4.1 μmol/day/100 g BW for G5/G8$^{-/-}$ versus WT mice, respectively) (Fig. 1B). NPC1L1$^{-/-}$ versus WT mice displayed a dramatic 7.3-fold increase in fecal neutral sterol excretion. NPC1L1 ablation in G5/G8$^{-/-}$ mice also caused a 4.9-fold increase (TKO versus G5/G8$^{-/-}$) in fecal neutral sterol excretion. Intriguingly, NPC1L1$^{-/-}$ mice had an additional 15.53 μmol/day/100g BW (~4 μmol each day for a 25 g mouse) of fecal neutral sterol excretion com-

TABLE 2. Hepatic total and unesterified sterol contents

	Total			Unesterified		
	Chol	Camp	Sito	Chol	Camp	Sito
	Mean ± SEM (mg/g wet weight)					
WT	4.0 ± 0.2^a	0.05 ± 0^b	0.01 ± 0.0^b	2.6 ± 0.1^a	0.07 ± 0^b	UD
G5/G8$^{-/-}$	1.9 ± 0.1^c	0.3 ± 0.03^a	0.42 ± 0.0^a	1.6 ± 0.1^b	0.44 ± 0.4^a	0.4 ± 0.02^a
NPC1L1$^{-/-}$	2.7 ± 0.1^b	UD	UD	$2.3 \pm 0.1^{a,c}$	UD	UD
TKO	3.0 ± 0.2^b	0.04 ± 0.0^b	0.04 ± 0.0^b	2.2 ± 0.1^c	0.03 ± 0.0^b	0.03 ± 0.0^b

UD, undetectable amounts. Hepatic total and unesterified cholesterol (Chol), campesterol (Camp) and sitosterol (Sito) were analyzed in individual male animals (n = 6), as described in Materials and Methods. The difference between values associated with different superscript letters in each column is statistically significant ($P < 0.05$).

A Phytosterols

B Neutral sterols

□ WT   ■ G5/G8⁻/⁻   ▨ NPC1L1⁻/⁻   ▨ TKO
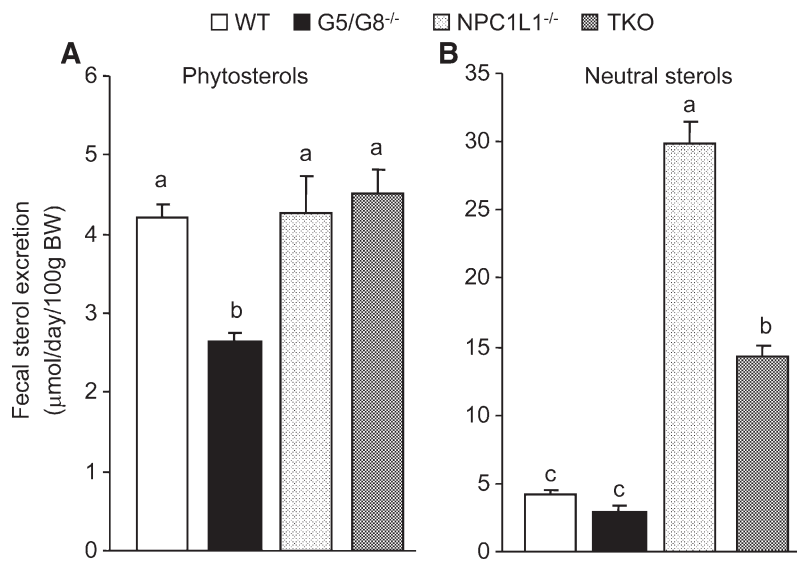
Fig. 1. Genetic inactivation of Niemann-Pick C1-Like 1 (NPC1L1) restores fecal excretion of phytosterols but not cholesterol in G5/G8$^{-/-}$ mice. Fecal sterol excretion was measured as described in Materials and Methods. A: Fecal phytosterols excretion. The amount of phytosterol is a sum of sitosterol, campesterol, and stigmasterol. B: Fecal neutral sterol excretion. The amount of fecal neutral sterols is a sum of cholesterol, coprostanol, and cholestanone. Each bar represents mean ± SEM of six samples, and the difference between values associated with different letters is statistically significant ($P < 0.05$).

pared with TKO mice, which represented 52% of total neutral sterol excretion in NPC1L1$^{-/-}$ mice. Since the only difference between TKO and NPC1L1$^{-/-}$ mice was that the TKO mice were lacking G5/G8, this additional neutral sterol excretion must be attributable to the G5/G8-mediated hepatobiliary and perhaps intestinal secretion of cholesterol.

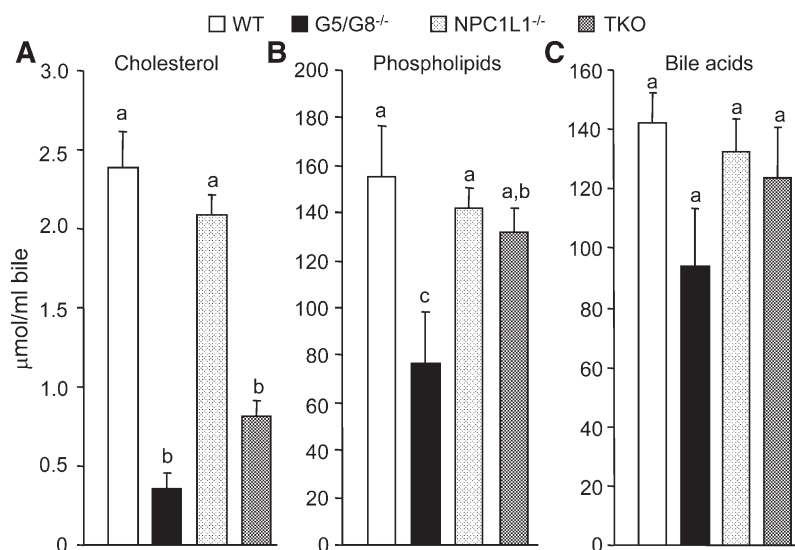### NPC1L1 ablation does not rescue biliary cholesterol secretion in G5/G8$^{-/-}$ mice

A major metabolic defect in G5/G8$^{-/-}$ mice is the blockade of sterol excretion into bile (2, 43). Because WT and NPC1L1$^{-/-}$ mice did not accumulate phytosterols in the body (Tables 1 and 2), we only examined the biliary secretion of cholesterol. The gallbladder cholesterol concentration was used as an indicator for biliary cholesterol excretion as we and others have previously shown that it is correlated well with hepatobiliary cholesterol secretion rates in mice (1, 2, 46–48). Compared with WT mice, the gallbladder cholesterol concentration was reduced by

~85% in G5/G8$^{-/-}$ mice, and unaltered in NPC1L1$^{-/-}$ mice (**Fig. 2A**). TKO versus WT or NPC1L1$^{-/-}$ mice still had approximately 66% or 61% reduction in the gallbladder cholesterol concentrations, respectively.

Phospholipids and bile acids, the other two major lipids in bile, did not change in parallel to cholesterol, and their concentrations in the gallbladder bile were largely maintained except that the phospholipids concentration was significantly reduced in G5/G8$^{-/-}$ mice compared with other genotypes (Fig. 2B, C). This phospholipids reduction was not observed previously in the chow-fed G5/G8$^{-/-}$ mice (2) and may be a diet-associated effect.

G5/G8 disruption does not increase fractional intestinal cholesterol absorption in the absence of NPC1L1

In the small intestine, G5/G8 is believed to transport cholesterol out to the gut lumen across the apical membrane of enterocytes. It, therefore, has the potential to indirectly reduce intestinal cholesterol absorption. To examine this possibility, we measured fractional intestinal

□ WT   ■ G5/G8⁻/⁻   ▨ NPC1L1⁻/⁻   ▨ TKO

A Cholesterol   **B** Phospholipids   **C** Bile acids

Fig. 2. Genetic inactivation of NPC1L1 does not rescue biliary cholesterol secretion in G5/G8$^{-/-}$ mice. The biliary lipid concentrations in the gallbladders were determined as described under Materials and Methods. A: Biliary cholesterol. B: Biliary phosphlipids. C: Biliary bile acid. Each bar represents mean ± SEM of six samples, and the difference between values associated with different letters is statistically significant ($P < 0.05$).

cholesterol absorption using a fecal dual-isotope ratio method. Compared with WT mice, G5/G8$^{-/-}$ mice did not exhibit an expected increase in fractional cholesterol absorption (**Fig. 3**), which is consistent with our previous report using the same method (2). It should be pointed out that G5/G8$^{-/-}$ mice excreted significantly reduced amounts of phytosterols in the feces (Fig. 1), which may have led to an underestimation of fractional cholesterol absorption in these animals when sitosterol was used as a nonabsorbable marker in this assay. However, our method is still valid in TKO mice despite the absence of G5/G8 because TKO mice excreted the same amounts of phytosterols in the feces as in the WT mice (Fig. 1). NPC1L1$^{-/-}$ and TKO mice both had a similar and dramatic reduction in fractional cholesterol absorption regardless of G5/G8 (Fig. 3).
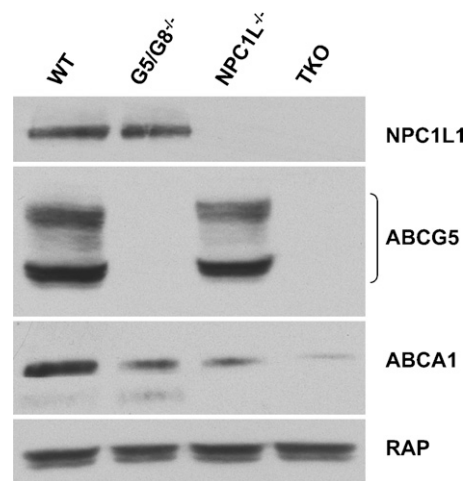
Intestinal protein levels of major sterol transporters

To define how the changes in intestinal cholesterol absorption were reflected by changes in major intestinal sterol transporters, and to determine whether G5/G8 deletion alters intestinal expression of NPC1L1 or vice versa, intestinal protein levels of NPC1L1, G5/G8, and ABCA1 were analyzed by immunoblotting. As shown in **Fig. 4**, the intestinal NPC1L1 protein level in G5/G8$^{-/-}$ mice was identical to that in WT mice. The intestinal G5/G8 protein level was also similar between WT and NPC1L1$^{-/-}$ mice. The receptor associated protein (RAP) (42) was included as a loading control, and its level was unchanged among all groups. Thus, genetic inactivation of G5/G8 or NPC1L1 did not affect each other's expression in the small intestine.

ABCA1 is a basolateral sterol transporter of enterocytes and has the potential to increase intestinal absorption of unesterified sterols (37). The intestinal ABCA1 mRNA level was reported to be approximately 75% lower in
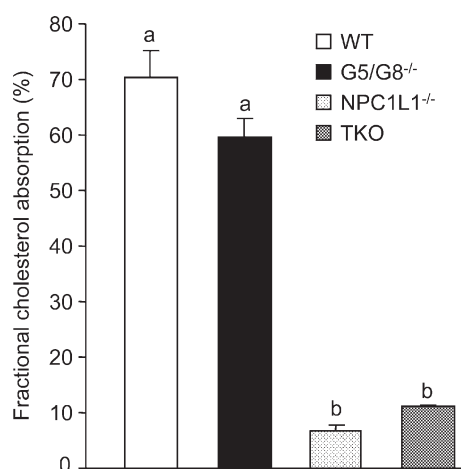
Fig. 4. Intestinal protein levels of sterol transporters. The homogenate of each jejunal sample was prepared as described in Materials and Methods. Equal amounts of homogenate protein from each sample were pooled. Fifty μg of pooled proteins in each group (n = 6) were immunoblotted with antibodies against NPC1L1, G5/G8, ATP-binding cassette (ABC)A1, and the receptor associated protein (RAP) as described in Materials and Methods. The same filter was stripped multiple times and used for all of the antibodies.

NPC1L1$^{-/-}$ versus WT mice (21, 44). Consistent with the reported mRNA changes, the intestinal ABCA1 protein level was dramatically reduced (Fig. 4). ABCA1 is a target gene of liver X receptor (49). It is conceivable that ABCA1 is lower in the small intestine of NPC1L1$^{-/-}$ mice because cholesterol cannot enter enterocytes from the gut lumen of these animals (21). Interestingly, ABCA1 protein level was also decreased in G5/G8$^{-/-}$ mice for an unknown reason and barely detectable in TKO mice (Fig. 4). Nevertheless, according to Figs. 3 and 4, it was NPC1L1 rather than ABCA1 or G5/G8 that dominated the efficiency of intestinal cholesterol absorption under our experimental conditions.

DISCUSSION

In this study, using genetically engineered mouse models, we have demonstrated that NPC1L1 inactivation fully protects against sitosterolemia in mice lacking G5/G8. Because the only pathway to obtain phytosterols is through the intestinal absorption of dietary phytosterols, NPC1L1 must be essential for this process. The best treatment for sitosterolemic patients would be the inhibition of NPC1L1-dependent transport of dietary phytosterols into the body, which is consistent with sitosterolemia patients and mice being very responsive to the treatment of ezetimibe (17–19), a cholesterol absorption inhibitor that targets the NPC1L1 pathway.

The finding that NPC1L1 ablation prevents sitosterolemia indicates that NPC1L1 plays a major role in phytosterols transport as well as that of cholesterol. This is consistent with a previous study showing that NPC1L1$^{-/-}$ mice absorb and accumulate reduced amounts of phytosterols (21). However, several cell-based studies suggest

Fig. 3. G5/G8 disruption does not increase cholesterol absorption in NPC1L1$^{-/-}$ mice. The fractional intestinal cholesterol absorption was measured as described under Materials and Methods. Each bar represents mean ± SEM of six samples, and the difference between values associated with different letters is statistically significant ($P < 0.05$).

that NPC1L1 may not transport phytosterols and cholesterol equally. In McArdle RH7777 rat hepatoma cells, we can detect the ezetimibe-sensitive NPC1L1-dependent uptake of cholesterol but not β-sitosterol (29, 35). In Caco-2 intestinal carcinoma cells, NPC1L1 can mediate the cellular uptake of cholesterol and β-sitosterol, but the efficiency for β-sitosterol is approximately 60% lower than for cholesterol (30). A fluorescent microscopic study of McArdle cells expressing NPC1L1 also showed that the NPC1L1-dependent phytosterol uptake was just 13% of the cholesterol uptake (31). Although phytosterols are structurally similar to cholesterol, in normal individuals, these sterols are poorly absorbed, and the rank order of fractional intestinal sterol absorption is cholesterol (∼45%) > campesterol (∼20%) > sitosterol (∼5%) (50). Mammalian cells have evolved to exclusively use cholesterol as an essential structural component. Phytosterols may displace cholesterol in the cell membrane and interfere with the cell function. Indeed, the accumulation of phytosterols has been reported to cause blood-cell abnormalities (51–53) and even some neurological disorder (54). Currently, the mechanisms underlying the defense against phytosterol accumulation remain elusive. The low capacity for phytosterols to bind the mixed bile acid micelles in the intestinal lumen (55) and the insufficient intracellular esterification by enterocytic acyl-Co-A:cholesterol O-acyltransferase 2 (45) may play a role. Interestingly, the rank order of sterol absorption is maintained in G5/G8$^{-/-}$ mice, though at a higher level (2). This finding suggests that the intestinal absorption specificities of different sterols are independent of G5/G8. The major function of G5/G8 is to eliminate cholesterol and the absorbed dietary phytosterols from the body. Given that NPC1L1 is a weaker transporter for phytosterols in cell-based assays (30, 31, 35), NPC1L1 might be the first genetic defense against phytosterol absorption. However, this defense must be leaky. Otherwise, G5/G8$^{-/-}$ mice would not develop sitosterolemia in the presence of NPC1L1.

Phytosterols cannot be synthesized in the body, and the only source for these sterols is from the diet. NPC1L1 disruption completely restored fecal excretion of phytosterols in G5/G8$^{-/-}$ mice (Fig. 1A), indicating that dietary phytosterols are not absorbed. Unlike phytosterols, cholesterol can be synthesized endogenously. Despite the blockade of its intestinal absorption in NPC1L1-deficent mice, there is still a large pool of endogenous cholesterol in the body, which can be excreted via the G5/G8 pathway. In the TKO mice, endogenous cholesterol cannot be excreted due to the absence of G5/G8, which explains why the TKO mice still had a dramatic reduction in the gallbladder cholesterol concentrations (Fig. 2A) and in fecal neutral sterol excretion (Fig. 1B) compared with NPC1L1$^{-/-}$ mice. Although we have previously shown that inhibition of hepatic NPC1L1 by ezetimibe restores biliary cholesterol excretion, it was only investigated using a liver-specific NPC1L1 transgenic mouse model (39). In rodents, NPC1L1 is almost exclusively expressed in the small intestine, and there are no detectable NPC1L1 proteins in the liver (20, 29, 36, 39). The direct effect of hepatic NPC1L1 on biliary cholesterol secretion would be minimal, if any, in rodents. In contrast, NPC1L1 is abundantly expressed in the small intestine and liver of humans and primates (20, 29, 36, 39), and localizes at the canalicular membrane of hepatocytes (29, 39). It has the potential to regulate biliary cholesterol excretion.

Because deletion of G5/G8 or NPC1L1 had no effect on each other's expression in the small intestine (Fig. 4), through the separate and simultaneous genetic manipulations of G5/G8 and NPC1L1, we were able to determine the contribution of G5/G8-mediated cholesterol excretion to the total fecal cholesterol excretion. Our data showed that approximately 4 μmol of cholesterol was excreted via the G5/G8 pathway each day in a mouse of 25 g BW (Fig. 1B) when the NPC1L1-dependent cholesterol absorption was eliminated, which represents approximately 52% of total fecal cholesterol excretion. Because mice lacking NPC1L1 have a compensatory increase in endogenous cholesterol synthesis (21), this number may be overestimated.

Humans with sitosterolemia have a greater reduction (>70%) in fecal excretion of neutral sterols (cholesterol and its bacterial metabolites) (50) than was seen in the sitosterolemic G5/G8$^{-/-}$ mice (∼30%) (Fig. 1B) (2). As discussed in the previous publication (2), this may be explained by a larger proportion of fecal neutral sterols being derived from bile in humans than in mice. Recently, the intestine has emerged as an important site for cholesterol excretion. For example, in mice lacking ABCB4 (mdr2), the biliary secretion of phospholipids and cholesterol is abolished, but the stimulation of fecal neutral sterol output by the activation of liver X receptor is largely preserved (56). To study the role of hepatic NPC1L1 in cholesterol metabolism, we have recently generated transgenic mice to overexpress NPC1L1 in the liver (39). NPC1L1 mice have approximately a 20-fold reduction in biliary cholesterol secretion (39), but their fecal cholesterol excretion remains unchanged (Ryan E. Temel and Liqing Yu, unpublished observation). These findings indicate that a large proportion of fecal sterols can be derived from nonbiliary sources, at least in mice. The most logical source would be from direct secretion by the small intestine. It would be interesting to compare this nonbiliary fecal sterol excretion between humans and mice. The difference in fecal neutral sterol excretion between sitosterolemic patients and mice may be attributable to distinctive "nonbiliary" pathways among species.▮▮

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