Abstract SREBP-2 activates transcription of all genes needed for cholesterol biosynthesis. To study SREBP-2 function in the intestine, we generated a mouse model (Vil-BP2−/−) in which Cre recombinase ablates SREBP-2 in intestinal epithelia. Intestines of Vil-BP2−/− mice had reduced expression of genes required for sterol synthesis, in vivo sterol synthesis rates, and epithelial cholesterol contents. On a cholesterol-free diet, the mice displayed chronic enteropathy with histological abnormalities of both villi and crypts, growth restriction, and reduced survival that was prevented by supplementation of cholesterol in the diet. Likewise, SREBP-2-deficient enteroids required exogenous cholesterol for growth. Blockade of luminal cholesterol uptake into enterocytes with ezetimibe precipitated acutely lethal intestinal damage in Vil-BP2−/− mice, highlighting the critical interplay in the small intestine of sterol absorption via NPC1L1 and sterol synthesis via SREBP-2 in sustaining the intestinal mucosa. These data show that the small intestine requires SREBP-2 to drive cholesterol synthesis that sustains the intestinal epithelia when uptake of cholesterol from the gut lumen is not available, and provide a unique example of cholesterol auxotrophy expressed in an intact, adult mammal. —Rong, S., J. G. McDonald, and L. J. Engelking. Cholesterol auxotrophy and intolerance to ezetimibe in mice with SREBP-2 deficiency in the intestine. J. Lipid Res. 2017. 58: 1988–1998.

Supplementary key words  SREBP • Scap • cholesterol/biosynthesis • fatty acid/synthesis • Niemann-Pick Cl-like 1 • organoid

SREBP-2 is a membrane-bound transcription factor that controls cholesterol biosynthetic gene expression (1). It is a member of a larger family of transcription factors, also comprised of SREBP-1a and SREBP-1c. Together, SREBPs control the synthesis of both cholesterol and fatty acids. Whereas SREBP-2 preferentially activates genes required for cholesterol synthesis and SREBP-1c primarily activates genes required for fatty acid and triglyceride synthesis, SREBP-1a can activate genes in both biosynthetic pathways (2). All three isoforms are activated when cultured cells are depleted of sterols. Under low sterol conditions, SREBPs are transported by another integral membrane protein, Scap, from endoplasmic reticulum (ER) to Golgi, where they are processed proteolytically to yield active nuclear forms. When cells are sterol-replete, proteolytic activation of SREBPs is prevented by Insig, an ER retention protein that binds Scap and retains the Scap-SREBP complex in ER (3, 4).

Given that the liver is quantitatively the most important organ for cholesterol synthesis in rodents, the initial focus of studies on SREBP-2 function in intact animals was on the liver. As expected, the liver-specific disruption of SREBP-2 markedly reduced hepatic cholesterol synthesis in vivo (5). Surprisingly, fatty acid synthesis was markedly reduced as well. The latter was caused by a near complete loss of SREBP-1c, which was attributable to the loss of SREBP-2-dependent generation of sterol liver-X-receptor (LXR) ligands that maintain the transcription of SREBP-1c (6). However, the role of SREBP-2 and its relationship to SREBP-1 in extrahepatic tissues, such as the small intestine, is unknown.

The intestine is quantitatively second to the liver in terms of de novo sterol synthesis in mice and rats; in other species like rabbit, guinea pig, and possibly man, sterol synthesis in the intestine exceeds the liver (7–10). Most sterol synthesis in the small intestine resides in the epithelial cells of the lower villus and intestinal crypts (11), where it is thought to maintain rapid cellular division. Our previous studies on SREBP function in the intestine have suggested that SREBP-2 is a key regulator of epithelial sterol homeostasis, balancing cholesterol uptake from the gut lumen with de novo sterol synthesis. Ezetimibe, a cholesterol-lowering treatment, is most effective in intestinal tissues (12). Ezetimibe blocks the uptake of cholesterol from the gut lumen into enterocytes by the NPC1L1 transporter (13), mimicking the phenotype of SREBP-2−/− mice. There are many similarities between the effects of ezetimibe and the loss of SREBP-2 in intestinal epithelial cells, so we predicted that the combination would be lethal.

In this study, we investigated the role of SREBP-2 in regulating cholesterol synthesis in the intestine with a mouse model in which Cre recombinase ablates SREBP-2 in intestinal epithelia. Intestinal epithelial cells are the primary site of cholesterol uptake from the gut lumen. We found that SREBP-2 is critically needed for sterol synthesis and uptake in enterocytes. We examined the function of SREBP-2 in regulating cholesterol synthesis in the small intestine of intact animals and intestinal enteroids. We discovered that SREBP-2 is needed for cholesterol uptake and sterol synthesis in the intestine, as mice that lack SREBP-2 (Vil-BP2−/−) had reduced expression of cholesterol biosynthetic genes and sterol synthesis in vivo, and depletion of cholesterol in the intestinal mucosa. Intestinal enteroids, a useful tool for studying intestinal epithelial biology in vitro, were used to study the function of SREBP-2 in regulating sterol uptake and synthesis. We found that SREBP-2−/− enteroids required exogenous cholesterol without uptake of cholesterol from the gut lumen, similar to ezetimibe-treated mice. Furthermore, ezetimibe induced increased expression of SREBP-2 in enteroids, providing evidence that SREBP-2 is required for cholesterol uptake through NPC1L1. We conclude that SREBP-2 plays an essential role in regulating cholesterol uptake and sterol synthesis in the small intestine of intact animals.
drug that blocks apical cholesterol uptake in enterocytes by inhibiting the cholesterol transporter Niemann-Pick C1-like 1 protein (NPC1L1), potently activates proteolysis of SREBP-2 (12), and stimulates a compensatory increase in intestinal sterol synthesis (13, 14). In mice with intestinal deficiency of Insigs, feedback inhibition of SREBP-2 by cholesterol derived from the gut lumen was abolished, leading to massive increases in intestinal sterol synthesis (15).

We previously reported on mice with an inducible deficiency of Scap in the intestine (Vil-Scap<sup>−/−</sup> mice), in which all SREBP proteolysis was abolished, leading to reduced intestinal fatty acid and cholesterol synthesis (16). The relative changes in sterol synthesis outstripped those of fatty acid synthesis, suggesting that SREBPs, as a group, are quantitatively more important for sterol rather than for fatty acid synthesis in intestine. In addition, Vil-Scap<sup>−/−</sup> mice displayed a lethal, acute enteropathy with destruction of intestinal mucosa within a few days of induced Scap deletion. Inasmuch as all three SREBPs isoforms are expressed in the intestine and that modulating Scap or Insig levels affect the processing of all SREBP isoforms, these studies left unclear the relative roles of each individual SREBP isoform, with their differing preferences for sterol or fatty acid synthesis, in their essential function to maintain the integrity of the intestinal mucosa.

In the current studies, we explore the in vivo role of SREBP-2 in the intestine by generating and characterizing a line of mice with intestine-specific deletion of SREBP-2. Our data indicate that SREBP-2-mediated cholesterol synthesis is required for the maintenance of mucosal integrity in the intestine, and that when cholesterol synthesis is blocked, epithelial cells become dependent upon luminal cholesterol uptake via NPC1L1 to maintain their viability.

MATERIALS AND METHODS

Animals and diets

All mice were housed in cages with 12 h light/12 h dark cycles. All animal experiments were performed with approval of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center. Vil-BP2<sup>−/−</sup> mice were generated by intercrossing Vil-Cre transgenic mice and Srebp-2<sup>fl/fl</sup> mice (5, 17) and are in a mixed 129S6/BL6 background. Sibling littersmates were used as controls in all studies. Mice were maintained on a breeder chow diet (Harlan Teklad Global Diets, Cat. No. SRP04000e).

General supplies and measurements

Plasma concentrations of cholesterol, triglyceride, insulin, glucose, and tissue cholesterol and triglyceride contents were measured as previously described (15, 18).

Intestinal cell isolation and enteroid culture

Isolated small intestinal epithelial cells (IECs) were prepared as described (16) and cell pellets were used for protein and RNA extraction as well as for sterol and fatty acid synthesis assays. In the current study, intestinal organoids derived from crypt units from adult mice are termed enteroids (19). Intestinal crypt unit isolation, enteroid culture, enteroid recovery, microscopy, and ATP bioluminescence cell viability assay were performed as described (16) with the following modifications. After isolation, crypt units were plated to a density of 200 crypts per well in a 48 µl droplet of matrigel. Crypt-containing matrigel droplets were overlaid with IntestiCult complete organoid media (Stemcell Technologies, Vancouver, BC; cat. no. 06005) with penicillin/streptomycin. Some wells received 50 µm methyl-β-cyclodextrin cholesterol complexes (MβCD-cholesterol, prepared as described (20)) within the matrigel and 25 µM MβCD-cholesterol in the media. Other wells received equal concentrations of uncomplexed methyl-β-cyclodextrin (MβCD) in the matrigel and media. Media was changed on the third day and fifth day of culture and enteroids were imaged or recovered from matrigel for analysis on the sixth day.

Immunoblot analyses of IECs and organoids

Whole-cell extracts, membrane fractions, and/or nuclear extracts were prepared individually from organoids, IECs, or tissues of mice, and then equal amounts of protein from samples were pooled as indicated in figure legends and subjected to SDS-PAGE and immunoblot analysis as described (5, 16).

Quantitative real-time PCR

Total RNA was prepared from mouse tissues, IECs, or organoids and quantitative real-time PCR (QPCR) was performed as described (15).

Histology

Tissues were fixed in 4% (v/v) paraformaldehyde in PBS, embedded in paraffin and sectioned. Sectioning and Ki67, TUNEL, propidium iodide and H&E staining were performed by the UT Southwestern Richardson Molecular Pathology Core. Intestinal villus and crypt lengths visualized on the H&E sections were measured using Image J software (National Institutes of Health).

Fractional cholesterol absorption and lipid biosynthesis assay

Fractional cholesterol absorption was determined by a fecal dual-isotope ratio method as described (21). Rates of in vivo sterol and fatty acid synthesis were determined using <sup>3</sup>H-labeled water as described (22, 23).

RESULTS

Deletion of Srebp-2 in intestinal mucosa

Figure 1A diagrams the Vil-Cre-mediated disruption of Srebp-2 in intestinal mucosa. Mice with floxed Srebp-2 alleles were bred to Vil-Cre transgenic mice in which the Cre recombination is driven by the gut-selective Villin promoter (5, 17). Resultant Srebp-2<sup>fl/fl</sup>; Vil-Cre are designated as Vil-BP2<sup>−/−</sup> mice. Littermates lacking Vil-Cre but bearing one or two floxed Srebp-2 alleles are designated as control mice. In some studies, Srebp-2<sup>fl/fl</sup>; Vil-Cre littermates are used and are designated as Vil-BP2<sup>−/−</sup> mice. Vil-BP2<sup>−/−</sup> were born at the expected ratio but were smaller than control littermates (supplemental Table S1). Vil-BP2<sup>−/−</sup> mice were grossly indistinguishable from control littermates. When fed...
standard plant-based cholesterol-free chow diet, Vil-BP2−/− mice passed loose stools. The small intestine of Vil-BP2−/− mice was enlarged both in terms of mass and length. Other phenotypic parameters of Vil-BP2−/− mice are summarized in supplemental Table S1.

In Fig. 1B, isolated IECs were harvested from the small intestines of control, Vil-BP2−/−, and Vil-BP2−/− mice and precursor and nuclear SREBPs were measured by immunoblot analysis. As expected, precursor and nuclear SREBP-2 were reduced in Vil-BP2−/− mice and were undetectable in Vil-BP2−/− mice. In contrast to recent studies in liver-specific SREBP-2 knockout mice in which nuclear SREBP-1 was markedly reduced (5), nuclear SREBP-1 levels were actually increased in the Vil-BP2−/− mice (Fig. 1B, lane 6). Levels of calnexin, a control membrane protein, and cAMP response element binding protein, a control nuclear protein, were similar between the three groups of mice. mRNA levels from IECs were quantified by QPCR in Fig. 1C. As expected, SREBP-2 mRNA was reduced by 98% in Vil-BP2−/− mice, leading to a fall in the expression of four genes involved in sterol synthesis or LDL uptake [HMG-CoA synthase, (HMGS) HMG-CoA reductase (HMGR) squalene synthase (SS), and LDLR], which were decreased by 33% to 88%. SREBP-1c and SREBP-1a mRNA levels, as well as mRNA levels of Scap and ApoB, two genes whose expression is not directly regulated by SREBPs, were not different between control and Vil-BP2−/− mice. The expression of fatty acid biosynthetic genes whose expression is predominantly controlled by SREBP-1 declined to a lesser extent than that of those genes related to sterol synthesis (supplemental Table S2). To ensure tissue-specificity of the Vil-Cre-mediated disruption of SREBP-2, hepatic SREBP-2 protein and mRNA levels were measured and were not different between control, Vil-BP2−/−, and Vil-BP2−/− mice (supplemental Fig. S1). The data shown in Fig. 1B, C establish that Vil-Cre near-quantitatively abolishes SREBP-2 in the small intestinal epithelia of Vil-BP2−/− mice, leading to a marked reduction in cholesterol biosynthetic gene expression.

**SREBP-2 drives intestinal cholesterol synthesis**

To test whether the loss of SREBP-2 affects intestinal lipid synthesis in vivo, we measured the incorporation of intraperitoneally-injected [3H]-labeled water into digitonin-precipitable sterols and fatty acids in control and Vil-BP2−/− mice (Fig. 2A, B, D, E). Sterol synthesis in small intestinal IECs, on a per gram of protein basis, was decreased in the Vil-BP2−/− mice by 47% and 58% in proximal and distal small intestine, respectively (Fig. 2A). Sterol synthesis was also reduced by 54% in the colon but was not altered in a control tissue, the spleen. In contrast, intestinal fatty acid synthesis was not altered by deletion of SREBP-2 (Fig. 2B). Given increased size of the small intestines of Vil-BP2−/− mice compared with controls (4.2 ± 0.5 vs. 6.6 ± 0.5 g small intestine/100 g body weight, P < 0.01), when considered on a per organ basis, the relative fall in sterol synthesis in small intestine was blunted, being reduced to only 26% less than controls (Fig. 2D), and rates of fatty acid synthesis were actually increased by 43% (Fig. 2E). The fall in sterol biosynthetic rate in Vil-BP2−/− mice was associated with a 25% reduction in cholesterol content of small intestine IECs (Fig. 2C), whereas there was no difference in triglyceride content (Fig. 2F). The reduction in intestinal cholesterol synthesis was associated with reductions in levels of various cholesterol intermediates and oxysterol levels (supplemental Table S3) but was not associated with changes in plasma or hepatic cholesterol levels (supplemental Table S1).
Intestinal SREBP-2 deficiency provokes a lethal enteropathy “curable” by dietary cholesterol supplementation

The data shown in Figs. 1 and 2 indicate that SREBP-2 is required to maintain basal sterol synthesis in the small intestine. We next examined the phenotypic consequences of epithelial cholesterol deficiency in the Vil-BP2−/− mice. Figure 3A and B show the aforementioned growth restriction and intestinal hypertrophy of Vil-BP2−/− mice compared with control littermates. Intestines of Vil-BP2−/− mice were protuberant from the abdominal cavity (Fig. 3A, middle panel), being swollen with watery chyme and liquid stool. Solid stools, as seen in the colonic lumen of controls, were absent in the knockouts (Fig. 3A, bottom panel).

We then sought to correct the cholesterol deficiency of the Vil-BP2−/− mice by increasing cholesterol availability in the gut lumen, where cholesterol is derived from ingested food, biliary secretions, transintestinal cholesterol excretion (TICE), and the sloughing of mucosal cells (24, 25). Control and Vil-BP2−/− mice were placed on a cholesterol-free plant-based chow diet with or without supplementation of 0.2% (w/w) cholesterol and survival was measured through the first 7 weeks of life (Fig. 3C). On the cholesterol-free diet, 25 of 37 Vil-BP2−/− mice (68%) expired during this time-period, whereas on the cholesterol-supplemented diet, only 2 out of 18 (11%) expired. None of the control mice expired, irrespective of diet. Visibly, the stools of the Vil-BP2−/− mice tended to acquire solid form on the high-cholesterol diet, indicating an improvement in intestinal function (data not shown). Cholesterol feeding itself did not dramatically alter levels of nuclear SREBP-2 or the expression of its target genes in intestine (supplemental Fig. S2). To ensure that the biliary contribution of cholesterol to the gut lumen was similar between controls and Vil-BP2−/− mice, the cholesterol content of gallbladder bile was measured and was similar between groups (supplemental Table S1) as was the expression of a variety of genes involved in bile acid metabolism (supplemental Fig. S1, supplemental Table S2). Of note, Vil-BP2+/− mice displayed normal survival on the cholesterol-free diet (data not shown).

Figure 4 shows histological analysis of small intestine from control and Vil-BP2−/− mice fed a cholesterol-free diet. H&E-stained sections of intestinal sections (Fig. 4A, top row) revealed multiple abnormalities in the proximal and distal intestine of Vil-BP2−/− mice. Although the overall mucosal height was similar between control and Vil-BP2−/− mice, the villuscrypt ratio was reduced in Vil-BP2−/− intestines (Fig. 4B). Hyperplasia of crypts in Vil-BP2−/− intestines was confirmed with Ki67 immunostaining, which labels proliferating cells of the intestinal crypt (Fig. 4A, bottom panel). In the knockouts, villus morphology was irregular, with occasional branching morphology and sloughing of cells from villus tips. Cholesterol feeding, although it largely normalized survival of the Vil-BP2−/− mice, did not fully normalize the histological abnormalities of the small intestine.
In colon, crypt lengthening was noted in the Vil-BP2−/− mice but the abnormalities were in general less marked and diffuse than in the small intestine (supplemental Fig. S4). Figures 3 and 4 indicate that deficiency of SREBP-2 in the intestine produces a small bowel mucosal injury that is a hypomorphic version of the phenotype seen with intestinal Scap deficiency (16), in which inducible Scap deletion is followed acutely by diarrhea and death within a few days. Of note, similar to Vil-BP2−/− mice, mice with heterozygous deletion of Scap in the intestine (Scapf/+; Vil-Cre-ERT2 mice administered tamoxifen) display no overt phenotypes (supplemental Fig. S5).

Fig. 3. Disruption of intestinal SREBP-2 produces intestinal hypertrophy, enteropathy, and cholesterol auxotrophy. A: Gross appearance (top), abdominal organs (middle), and dissected gastrointestinal tracts (bottom) from control mice and Vil-BP2−/− mice are shown. Yellow arrow indicates protruberance of organs in Vil-BP2−/− mice. Green arrow indicates formed stools in colons of control mice absent in Vil-BP2−/− mice. B: Intestine length and weight (mice shown are the same as in Fig. 1B, C). C: Mice of the indicated genotypes (n per group is indicated in the key) and their dams were fed either a cholesterol-free chow diet or a diet containing 0.2% cholesterol from birth to 7 weeks of age. Survival percentage from age 10 days is shown. Statistical significance in B between groups of mice was assessed by the two-tailed Student’s t-test, *P < 0.05, and in C by Log-rank test (P value as indicated compares control and Vil-BP2−/− mice on cholesterol-free diet).

Fig. 4. Crypt hyperplasia and villus atrophy of small intestinal mucosa in Vil-BP2−/− mice. A: Representative histologic sections of proximal (Proximal S.I.) and distal (Distal S.I.) small intestine from 4- to 5-week-old male control and Vil-BP2−/− mice stained with H&E or immunostained for Ki67; magnification, 100×. B: Length ratio of villus to crypt of proximal (Prox. S.I.) and distal (Dist. S.I.) small intestine. Lengths of five villi and crypts on an H&E-stained section were measured and averaged for each mouse. Length ratios from groups of control and Vil-BP2−/− mice (four mice per genotype) are shown. Each bar represents mean ± SEM from groups of mice. *P < 0.05, **P < 0.01 denotes the level of statistical significance (two-tailed Student’s t-test) between control and Vil-BP2−/− mice.
Exogenous cholesterol rescues crypt growth in SREBP-2-deficient enteroids

The most direct mechanism by which dietary cholesterol might be "curing" Vil-BP2−/− mice (Fig. 3C) is that ingested cholesterol is taken up by enterocytes, where it replaces the cholesterol that would ordinarily be synthesized de novo through the activity of SREBP-2 in tissue-autonomous fashion. An alternative explanation is that dietary cholesterol, after absorption predominantly in the proximal small intestine, is distributed extra-intestinally, and its intestinal effects are secondary to altered extra-intestinal metabolism.

To differentiate between these two possibilities, the ability of exogenous cholesterol to rescue intestinal crypt growth ex vivo was tested using enteroid cultures (Fig. 5). Small intestinal crypts were isolated from control and Vil-BP2−/− mice, embedded in matrigel, and grown in culture for 6 days. To deliver cholesterol to the enteroids, cholesterol was complexed to MβCD (MβCD-cholesterol), and was added directly to the matrigel at the time of embedding and to the culture media. MβCD efficiently solubilizes cholesterol in aqueous solution and allows its delivery to the plasma membrane (26). Figure 5A shows photographically the growth rescue of enteroids from Vil-BP2−/− mice with exogenously-supplied cholesterol. Enteroids from control mice, irrespective of cholesterol addition, formed typical enteroid structures with crypt protrusions surrounding a villus core of apoptotic cells (Fig. 5A, panels 1–3). Vil-BP2−/− crypt units failed to grow if placed in basal medium without additions (panel 4), or if uncomplexed MβCD was added (panel 5), where only small clumps of dead cells representing the degenerating crypt units remain visible. However, Vil-BP2−/− crypt units grew into enteroids with indistinguishable morphology compared with those derived from control mice when cultured with MβCD-cholesterol (panel 6).

Cell viability of enteroids was confirmed by ATP bioluminescence assay (Fig. 5B). MβCD and MβCD-cholesterol had no effect on cellular ATP levels in enteroids from control mice, whereas ATP levels were virtually absent from Vil-BP2−/− enteroids grown without MβCD-cholesterol, indicating an absence of viable cells. ATP levels were nearly normalized compared with controls when grown with MβCD-cholesterol, indicating that cell viability in the SREBP-2-deficient enteroids was restored by exogenous cholesterol.

The results shown in Fig. 5A and B differ from recent studies in Scap knockout mice (16), in which both cholesterol and oleate (C18:1), a monounsaturated fatty acid, were required to restore normal enteroid growth. Inasmuch as monounsaturated fatty acids are a major products of SREBP-1 action and SREBP-1 abundance may be dependent on SREBP-2 (5), we measured levels of nuclear SREBPs (Fig. 5C) and mRNAs of cholesterol and fatty acid biosynthetic genes (Fig. 5D) in control and Vil-BP2−/− enteroids under the same culture conditions as in Fig. 5A and B. Precursor and nuclear SREBP-1 and SREBP-2 were not changed by the addition of MβCD and MβCD-cholesterol to control enteroids (Fig. 5C, lanes 1–3). In Vil-BP2−/− enteroids, as expected, SREBP-2 protein and mRNA was absent (Fig. 5C, lane 4, 5D). mRNA levels for SREBP-1a and SREBP-1c were slightly reduced, as were mRNAs for genes involved in fatty acid synthesis, such as acetyl-CoA carboxylase 1 and fatty acid synthase. Reductions in expression of genes involved in cholesterol synthesis and LDL uptake (HMG-CoA synthase, HMGR, and LDLR) were more pronounced, being reduced by 53% to 88%. Of note, MβCD-cholesterol treatment did not suppress SREBP-1 or SREBP-2 processing in control enteroids as might be expected (Fig. 5C) and in contrast to our previous studies (16), in which higher concentrations of MβCD-cholesterol and oleate together caused a partial suppression of SREBP-1 and SREBP-2 processing.

Blockade of cholesterol absorption with ezetimibe is acutely toxic with intestinal SREBP-2 deficiency

We next examined the consequence of SREBP-2 deficiency on NPC1L1, the primary cholesterol influx transporter of the brush border (27). Because NPC1L1 has been reported to be a transcriptional target of SREBP-2 (28), we first measured NPC1L1 by immunoblot analysis of lysates from control, Vil-BP2−/−, and Vil-BP2−/− IECs (Fig. 6A). NPC1L1 protein content was slightly reduced in Vil-BP2−/− IECs compared with controls, as was NPC1L1 mRNA as measured by QPCR (supplemental Table S2). Levels of LDLR protein, which obtain a minority of intestinal cholesterol in the form of LDL-cholesterol, were also modestly reduced. Despite this minor reduction in NPC1L1 expression, on a per microgram of protein basis, fractional cholesterol absorption (Fig. 6B) was actually increased and so overall NPC1L1 function is seemingly intact. This small increase in cholesterol absorption is explained by the compensatory hypertrophy of the intestine of Vil-BP2−/− animals, so that more mucosa is available for nutrient absorption, or possibly due to a modest reduction in the intestinal mRNA levels of ABCG5 and ABCG8, cholesterol efflux transporters that counteract the influx transporter NPC1L1 (supplemental Table S2) (29). Total fat absorption was normal in Vil-BP2−/− animals (supplemental Table S1) as was chylomicron secretion as estimated by the plasma excursion of radiolabeled triolein acutely after administration by oral gavage (supplemental Fig. S6).

To assess the effect of blocking cholesterol absorption in a setting where SREBP-2 cannot be activated, we administered the knockout mice ezetimibe, an inhibitor of the NPC1L1 (30). Mice were fed a cholesterol-free chow diet to which was added 0.01% (w/w) ezetimibe, a dose that is sufficient to block >90% of fractional cholesterol absorption, either without (Fig. 6C) or with 2% (w/w) cholesterol (Fig. 6D). A third group of mice was fed a cholesterol-free diet without additions (Fig. 6E). Ezetimibe without cholesterol caused no obvious toxicity in control and Vil-BP2−/− mice but was not tolerated by the Vil-BP2−/− mice, exacerbating their loose stools (data not shown) and leading to rapid mortality. The vast majority of Vil-BP2−/− mice (88%) expired after 2 days following exposure to ezetimibe, and none survived beyond 5 days. Cholesterol prevented the rapid mortality caused by ezetimibe (Fig. 6D), indicating that residual cholesterol absorption from the high cholesterol diet adequately

SREBP-2 deficiency in small intestine 1993
supplies the intestinal mucosa. Only two of nine Vil-BP2−/− mice (22%) fed the cholesterol-free diet without additions expired during the experiment (Fig. 6E), confirming that ezetimibe was reducing the survival of the Vil-BP2−/− mice in significant excess to what is observed solely on the cholesterol-free diet. A similar, slower rate of death on the cholesterol-free diet was also seen in Fig. 3C. Figure 6F shows the histological analysis of control and Vil-BP2−/− mice treated with ezetimibe in the cholesterol-free diet. H&E staining revealed worsening of the histological abnormalities of the Vil-BP2−/− intestines, with sloughing of the villi in both proximal and distal intestines (Fig. 6F, top).
SREBP-2 deficiency in small intestine

In addition, crypts of the distal small intestine were severely damaged, with necrotic material and apoptotic cells frequently noted in the residual crypt area. Increased apoptosis in distal small intestine of Vil-BP2−/− mice was confirmed by TUNEL staining (Fig. 6F, bottom row). The rapid demise and severe epithelial injury of ezetimibe-treated Vil-BP2−/− mice is reminiscent of Vil-Scap−/− mice (16), which experience rapid breakdown of the intestinal mucosa and bacteremia after inducible Scap disruption. The data shown in Fig. 6 indicates that the small intestinal mucosa suffers serious injury when the intestine’s major sources of cholesterol, absorption via NPC1L1 and de novo synthesis via SREBP-2, are dually inhibited.

DISCUSSION

The current study, combined with our previous studies of the liver and small intestine (5, 12, 15, 16), demonstrate...
that SREBP-2 is responsible for the majority of sterol synthesis in digestive organs, which together quantitatively account for the majority of the body’s sterol synthesis (7). Altogether, the data suggest that feedback inhibition of sterol synthesis in the intestine occurs via inhibition of SREBP-2 proteolysis by cholesterol derived from the gut lumen, which enters enterocytes via the brush border cholesterol transporter NPC1L1 and ultimately enters the regulatory pool of cholesterol in ER membranes that act on Scap and Insig. This inhibitory effect of NPC1L1 on SREBP-2 is only revealed by its elimination, as blocking cholesterol absorption with ezetimibe in turn stimulates SREBP-2 proteolysis (12).

The current study emphasizes the importance of these two factors in the maintenance of the integrity of intestinal mucosa. The data shown in Fig. 6 indicate that if the intestine is unable to upregulate cholesterol synthesis by stimulating SREBP-2 proteolysis when luminal cholesterol is made unavailable owing to blockade of NPC1L1, breakdown of the intestinal mucosa results. This finding clarifies the nature of sterol balance in enterocytes, which are unique in that they have at least three, rather than two, sources of cholesterol. Like all cells, enterocytes may synthesize cholesterol de novo or take up LDL-cholesterol from the plasma but they are unique in that they may also take up exogenous cholesterol from the gut lumen. Inasmuch as LDL receptors are largely intact in SREBP-2 deficient intestine (Figs. 1C, 6A), LDL uptake appears to be unable to compensate for the combined loss of both sterol synthesis caused by SREBP-2 deficiency and the loss of luminal cholesterol uptake caused by NPC1L1 blockade, as might be expected because, in mouse intestine, the rate of LDL-cholesterol uptake is only a few percent of the rate of sterol synthesis (7). On the other hand, in studies of enterocyte-like cultured cells, pharmacologic inhibition of sterol synthesis with lovastatin inhibited cell proliferation and microvillus formation, in general agreement with the result of Fig. 5, and the statin-induced block in cell proliferation was overcome by LDL-cholesterol in vitro (31). Likewise, nonLDLR-mediated basolateral plasma cholesterol uptake in enterocytes is a component of the TICE pathway (32, 33) but this pathway of cholesterol influx into the epithelia was seemingly not capable of sustaining the intestine in the setting of Fig. 6.

The critical link between SREBP-2 and NPC1L1 may also have therapeutic implications for Scap or SREBP inhibitors, which are under investigation for the treatment of dyslipidemia and other disorders of lipid excess (34–36). Coadministration of an inhibitor of SREBP or Scap with a blocker of cholesterol absorption, like ezetimibe, may precipitate intestinal toxicity. However, a 50% loss of Scap (supplemental Fig. S5) or of SREBP-2 (Fig. 6C) owing to heterozygous disruption in the intestine, the latter even in the presence of ezetimibe, is seemingly well-tolerated by the intestine, suggesting that the gastrointestinal toxicities of SREBPs or Scap inhibition are dose-dependent and are therefore potentially avoidable.

In comparison with recent studies on SREBP-2 function in the liver (5), two major differences come to light. First, the disruption of SREBP-2 in the liver was not associated with postweaning mortality as it is in the intestine. As such, in terms of the intact organism, SREBP-2 function in the intestine is essential for life whereas its function in the liver is not. This study joins a growing body of literature in which intestinal ablation of critical genes in a variety of pathways is associated with high rates of mortality in mice relatable to a reduction in intestinal function; i.e., fluid losses, malnutrition, and loss of barrier function (16, 37–39).

Second, intestinal SREBP-1 expression does not require SREBP-2, in contrast to the liver. The latter is partly explained by the relative abundance of isoforms SREBP-1a and SREBP-1c in the liver versus the intestine (40). In the liver, because SREBP-1c is the major isoform, SREBP-1 protein levels fall when SREBP-1c mRNA falls. SREBP-1c mRNA is reduced in SREBP-2-deficient hepatocytes owing to the loss of active synthesis of sterol liver-X-receptor agonists. SREBP-1a mRNA persists, but, as it is not abundant in the liver, is insufficient to sustain SREBP-1 protein levels. In the intestine, SREBP-1a is the major isoform, and its expression is not affected by SREBP-2 disruption. Accordingly, SREBP-1 precursor protein levels remain unchanged, and, of note, in IECs nuclear SREBP-1 levels actually increase (Fig. 1B). The latter implies enhanced SREBP-1 proteolysis as might be expected because the epithelial cholesterol content falls (Fig. 2C). Nevertheless, the increase in nuclear SREBP-1 is not associated with increased transcription of typical SREBP-1 target genes involved in fatty acid synthesis (supplemental Table S2), and so the overall rate of fatty acid synthesis, on a per gram of protein basis, is unchanged in the Vil-BP2+/− intestines (Fig. 2B). This finding raises the possibility that nuclear SREBP-1 is missing an unknown transcriptional cofactor in SREBP-2-deficient intestinal epithelia.

Similarly, in comparison with recent studies on Scap in the intestine, several key differences are noted. Whereas the Vil-BP2−/− mice could be rescued in vivo with dietary cholesterol, the intestinal Scap knockouts could not be rescued by the same treatment, despite similar reductions in the intestinal sterol biosynthetic rate (16). The less severe phenotype of the Vil-BP2+−/− mice may be attributable to the aforementioned persistence of SREBP-1, in contrast to the intestinal Scap knockouts in which nuclear SREBP-1 is also abolished and fatty acid synthesis is reduced. For reasons that remain unclear, the intestine can be maintained by exogenous cholesterol when sterol synthesis is impeded but cannot be sustained by exogenous fats and cholesterol when both sterol and fatty acid synthesis are impeded. However, as the reduction in intestinal fatty acid synthesis seen in Vil-Scap−/− mice is relatively minor (↓ by 14% for whole small intestine) (16), the large phenotypic differences between Scap- and SREBP-2 deficiency (i.e., survival vs. nonsurvival on a cholesterol-rich diet) may point to additional functions for Scap, apart from maintaining cholesterol and fatty acid synthesis, that are important for epithelial homeostasis of the intestine.

Another key difference between the Scap and SREBP-2 ablation is their effect on intestinal crypts. Scap ablation results in a mucosal injury characterized by loss of both
villus and crypt elements whereas SREBP-2 ablation results in crypt hyperplasia and villus loss. This crypt hyperplasia is possibly counterintuitive as results from the enteroendocrine studies (Fig. 5) confirm the requirement for SREBP-2 to provide cholesterol for crypt growth. Because cholesterol feeding does not reverse the crypt hyperplasia (supplemental Fig. S3), SREBP-2 deficiency may be stimulating crypt hyperplasia independently of its creation of a cholesterol deficit. However, because ezetimibe caused the rapid destruction of these enlarged crypts, it seems at least that they are dependent on absorbed cholesterol for survival (Fig. 6F).

Last, the Vil-BP2−/− model is an unusual example of cholesterol “auxotrophy” expressed in an intact, adult mammal. We use the term auxotroph here with some license, given that it has been most often used to describe microorganisms. However, it has also been used in the past to describe intact higher organisms (41, 42). Using somatic cell genetics, mammalian cell lines with cholesterol auxotrophy have been generated; their molecular defects were traced to Scap or to the proteases responsible for SREBP cleavage, site-1 protease and site-2 protease (43, 44). Mice with germline deficiencies in SREBP-2 or site-1 protease (45, 46), as well as for various genes encoding sterol biosynthetic enzymes, such as HMGR (47), display embryonic lethality, reflecting the essential developmental requirement for sterol and isoprenoid synthesis. Humans with deficient cholesterol synthesis, such as individuals with Smith-Lemli-Opitz Syndrome, in which 7-dehydrocholesterol reductase activity is deficient, are often treated with cholesterol supplementation with some therapeutic benefit, but these patients are not described as cholesterol auxotrophs (48, 49).

Mice with a liver-specific deletion of HMGR display multiple activity is deficient, are often treated with cholesterol supplementation with some therapeutic benefit, but these patients are not described as cholesterol auxotrophs (48, 49).

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