Glycosphingolipids are essential for intestinal endocytic function *

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Background: The intestine contains high concentrations of glycosphingolipids, but their function remained unclear.

Results: In newborn mice lacking glycosphingolipids, intestinal epithelia were indistinguishable from control littermates. However, a few days after birth, severe defects in epithelial differentiation occurred.

Conclusion: Glycosphingolipid expression in the intestinal epithelium is quintessential for maintenance of resorptive function.

Significance: Glycosphingolipids are essential for enterocyte function but primarily not for brush border formation.

SUMMARY

Glycosphingolipids (GSLs) constitute major components of enterocytes and were hypothesized to be potentially important for intestinal epithelial polarization. The enzyme UDP-glucose ceramide glucosyltransferase (Ugcg) catalyzes the initial step of GSL-biosynthesis. Newborn- and adult mice with enterocyte specific genetic deletion of the gene Ugcg were generated. In newborn mutants lacking GSLs at day P0, intestinal epithelia were indistinguishable from those in control littermates displaying an intact polarization with regular brush border. However, those mice were not able to absorb nutritional lipids from milk regularly. Between postnatal days 5 to 7 severe defects in intestinal epithelial differentiation occurred accompanied with impaired intestinal uptake of nutrients. Villi of mutant mice became stunted and enterocytes lacked brush border. The defects observed in mutant mice caused diarrhea, malabsorption, and early death.

In the present study we show that GSLs are essential for enterocyte resorptive function but primarily not for polarization; GSLs are required for intracellular vesicular transport in resorption-active intestine.

The intestine is the principal organ for digestion and absorption of nutrients. In the small intestine, enterocytes - columnar epithelial cells - cover the surfaces of villi, themselves containing thousands of densely packed apical microvilli which further
amplify the surface area for absorption of nutrients.

Glycosphingolipids (GSLs) are major constituents of enterocytes (1). Due to their high enterocytic expression, GSLs might be involved in several cellular processes in polarized intestinal epithelia. It could be shown that GSLs are integral constituents of lipid microdomains which seem to play important roles in membrane organization (2) and in endocytosis (3) of nutrients. In addition, GSLs might influence the biosynthesis (4) and intracellular transport of proteins (5). GSLs have been described to influence membrane receptors such as the insulin and EGF receptor (6-9).

The structural relevance of GSL synthesis in the intestine could be demonstrated in *C. elegans* mutants with significantly decreased glucosylceramide-based GSLs. Worms devoid of GSLs showed a pronounced growth arrest. The defect could be revoked by reexpression of glucosylceramide synthase in the digestive tract (10). This study highlighted the importance of intestinal GSLs while GSL expression - different from the mouse - was dispensable for other tissues including neuronal cells (10). In addition, a recent report suggested that GSLs were pivotal for epithelial polarization in *C. elegans*. With a marked reduction of GSL-synthesis, worms developed multiple ectopic lumens in their intestine and showed degraded microvilli (11).

In order to dissect the role of GSLs for intestinal function in mammals, we used a novel mouse model in which GSL-biosynthesis was genetically deleted in an enterocyte-specific manner by elimination of the gene *Ugcg* (glucosylceramide synthase), which encodes the key enzyme of the GSL biosynthesis pathway for all GSLs produced by enterocytes. To achieve specific deletion of GSLs in enterocytes, mice with loxP-flanked *Ugcg* gene (*Ugcg* **f/f**) were mated with mice expressing Cre-recombinase constitutively (VilCre, The Jackson Laboratories, Bar Harbor, MA, USA) (13). To investigate the consequences of the *Ugcg* deletion during adulthood, an inducible model was developed by crossing *Ugcg* **f/f** mice with animals expressing cre-recombinase constitutively (VilCreERT2-animals (15). Mice were genotyped as described (13,15). Resulting heterozygous mice (*Ugcg* **f/+**/VilCre or *Ugcg* **f/+**/VilCreERT2) were

EXPERIMENTAL PROCEDURES

**Mice** - Mice with loxP-flanked *Ugcg* gene (*Ugcg* **f/f**) were generated and genotyped by PCR and southern blot analysis as described (12). Cell-specific *Ugcg* deficient newborn mice were generated by crossing *Ugcg* **f/f** mice with animals expressing cre-recombinase constitutively (VilCre, The Jackson Laboratories, Bar Harbor, MA, USA) (13). To investigate the consequences of the *Ugcg* deletion during adulthood, an inducible model was developed by crossing *Ugcg* **f/f** mice with tamoxifen inducible VilCreERT2-animals (15). Mice were genotyped as described (13,15). Resulting heterozygous mice (*Ugcg* **f/+**/VilCre or *Ugcg* **f/+**/VilCreERT2) were
bred again with *Ugcg* f/f-mice and 25% of offspring, reflecting normal Mendelian inheritance, carried the homozygous genetic deletion of *Ugcg* (*Ugcg* f/f/VilCre and *Ugcg* f/f/VilCreERT2). Corresponding heterozygous- (*Ugcg* f/+VilCre and *Ugcg* f/+VilCreERT2) as well as ‘wildtype’ litters (*Ugcg* f/+ and *Ugcg* f/) showed no obvious phenotype and were used as controls. *Ugcg* gene deletion in adult *Ugcg* f/f/VilCreERT2 mice was initiated by intraperitoneal application of 1mg tamoxifen in 100µl sunflower seed oil (both, Sigma, Munich Germany) for three consecutive days. Respective controls were treated with tamoxifen in parallel. All animal experiments were approved by federal law.

**LacZ staining to evaluate cre-expression** - LacZ-Rosa26 reporter mice (kindly provided by G. Schütz, DKFZ-Heidelberg) bearing a beta-galactosidase gene were crossed with mice expressing the VilCre transgene. In the resulting offspring bearing both LacZ and cre, beta-galactosidase activity was measured as described (15).

**Sphingolipid and phospholipid analysis** - Freshly dissected tissue ~ 2 cm of length was frozen in liquid nitrogen and lyophilized. Tissues were powdered and dry weight was determined. Lyophilized samples were extracted with 2ml CHCl₃/CH₃OH/H₂O 10:10:1 v/v under sonication in a water bath at 50°C for 15 min. Supernatants were collected after centrifugation at 4000rpm for 10 min. The pellets were again extracted with 2ml CHCl₃/CH₃OH/H₂O 10:10:1 v/v and thereafter with CHCl₃/CH₃OH/H₂O 30:60:8 v/v as described above. All supernatants were combined and dried under a stream of nitrogen. For the determination of phospholipids, aliquots from the crude intestinal extracts corresponding to 0.05 mg dry tissue were loaded on thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany). TLC plates were developed in running solvent CHCl₃/CH₃OH/HAc 190:9:1 v/v. Visualization of ceramides with copper reagent was performed as described above. Glycosphingolipids were separated into neutral and acidic (sialic acid-containing) components by ion exchange chromatography. Glass pipettes were filled with 200µl DEAE Sephadex A25 (GE Healthcare, Uppsala, Sweden) and columns were preconditioned with 2ml methanol. The samples were solved in 2ml methanol and applied to the columns. Reagent tubes were rinsed 2 times with 1ml methanol and neutral GSLs were completely eluted from the columns with additional 2ml of methanol. Acidic GSLs were then eluted with 2ml of 0.5M KAc in CH₃OH. Acidic GSLs were dried and solved in 5ml H₂O and desalted via preconditioned RP18 columns as described above. Neutral GSLs were thoroughly dried and kept for several hours in a vacuum desiccator. GSLs were solved in 200µl dichloroethane (DCE) and acetylated by the
addition of 50 µl acetic anhydride and 50 µl 0.1% dimethylaminopyridine in DCE at 37°C for one hour. The solvents were evaporated under a N₂ stream by addition of 200 µl toluene, respectively for three times. Glass pipettes were filled with 400 µl Florisil (Merck, Darmstadt, Germany) and washed with 4 ml DCE/n-hexane 4:1 v/v. The acetylated neutral GSLs were solved in the same solvent mixture and applied to the columns followed by washing steps with DCE/n-hexane 4:1 and DCE, 4 ml each. Neutral GSLs were eluted from Florisil columns with 4 ml DCE/Acetone 1:1 v/v and dried. In order to remove acetyl groups from sugar moieties, neutral GSLs were solved in 0.1M KOH in methanol and incubated at 37°C for 2 hours. After neutralization with acetic acid, the purified neutral GSLs were dried down and desalted by RP18 column chromatography as described above. An amount corresponding to 0.25 mg dry intestine was loaded on TLC plates for the staining of neutral and acidic GSLs. Running solvent for neutral GSLs was CHCl₃/CH₃OH/H₂O 62.5:30:6 v/v and CHCl₃/CH₃OH/0.2% CaCl₂ 60:35:8 v/v was used for acidic GSLs. TLC plates were sprayed with 0.2% orcinol in 10% sulfuric acid at 120°C for ~10 min to visualize GSLs.

Quantification of fatty acids and cholesterol in stool - 1 cm of the distal rectum of sacrificed mice (controls, n=8 and Ugcg f/f/VilCre, n=6) was resected and the feces content carefully removed with a small forceps. Isolated stools were freeze-dried, the dry weight determined and extracted essentially as described (16). Lipids were quantified using a Shimadzu CS-9310PC TLC-scanner (Shimadzu Europe, Duisburg, Germany) at a wavelength of 580 nm. LC-MS/MS quantification of Ceramides and Hexosylceramides (GlcCer)-Aliquot of the above obtained, saponified, & desalted lipid extracts corresponding to 25 µg tissue dry weight were dissolved in 1 ml of 95% methanol containing a mixture of internal sphingolipid standards for LC-MS/MS analysis. Among these were the glucosylceramides GlcCer(d18:1;14:0), GlcCer(d18:1;19:0), GlcCer(d18:1;25:0), & GlcCer(d18:1;31:0), each in an amount of 2 pmol, as well as the ceramides Cer(d18:1;14:0), Cer(d18:1;19:0), Cer(d18:1;25:0), Cer(d18:1;31:0), & Cer(t18:0;14:0), each in an amount of 4 pmol. LC-MS/MS analysis was performed on a Xevo TQ-S tandem mass spectrometer coupled to an automated Aquity I class UPLC system using a ACQUITY UPLC® BEH C18 1.7 µm column (length 50 mm, diameter 2.1 mm) all from Waters Corporation. The column was equilibrated in buffer A (95% methanol, 0.05 % formic acid, & 1 mM ammonium acetate) and lipids were eluted with increasing percent of buffer B (99% 2-propanol, 1 % methanol, 0.05 % formic acid, & 1 mM ammonium acetate) at a flow rate of 0.45 ml/min (gradient see Table I). Sphingolipids were detected in SRM mode with their respective transitions. The decision basis for the incorporation of individual sphingolipids into the final SRM list was based on nanospray direct infusion in precursor ion scan m/z +264 mode identifying ceramides and hexosylceramides containing either a d18:1 or a t18:0 sphingoid base. For quantification peak areas of individual lipid species were divided by the respective average internal standard area, multiplied by the amount of this internal standard and finally divided by the tissue dry weight used. With the internal ceramide standards Cer(d18:1;31:0) and Cer(t18:0;31:0) an average response factor of 12.2±1.8 for the sensitivity of sphingosine-species over phytosphingosine-species was determined and was taken into account to quantify the phytosphingosine containing ceramides and hexosylceramides.
Isolation of milk sugars from feces and TLC - Feces were extracted essentially as described (16). An aliquot of the crude extract was directly loaded on a TLC plate and developed in running solvent pyridine/ethylacetate/glacial acetic acid/water 5:5:1:3 by vol.. Visualization with orcinol reagent was performed as described above.

Measurement of intestinal sialidase activity - Homogenates of total small intestines of mutant and control (n=4 each) were mixed with sialyllactose (Sigma, Munich, Germany) and the digest performed at 37°C either for 30 min or in a time dependent manner essentially as described (17). The cleavage product lactose was quantified by densitometry.

Fatty acid/glucose uptake - Animals 3-4 days and 6-7 days after birth (controls, n=12 and Ugcg f/f/VilCre, n=9) were anesthetized with isoflurane. The peritoneum was opened and a 1cm part of the intestinal jejunum of the same area was ligated. 100µl of a solution containing 10µM fluorescent labelled stearic acid (NBD-stearic acid, Avanti Polar Lipids, Alabaster, AL, USA) and 20µM fatty acid free BSA (Sigma-Aldrich, Munich, Germany) in PBS was injected and uptake performed for 10 min in the dark. For investigation of glucose uptake studies 60µM 2-NBD-glucose (Invitrogen, Darmstadt, Germany) in PBS was used. The ligated part of the jejunum was resected, washed with pre-warmed PBS and immediately frozen in liquid nitrogen. Cryo sections of 5µm thickness were air-dried for 30 min in the dark, embedded in Fluorumount G (SouthernBiotech, Birmingham, AL, USA) for detection of NBD-stearic acid or Vitro-Clud® (Langenbrink, Emmendingen, Germany) for NBD-glucose staining and scanned with a confocal laser microscope (TCS-SL, Leica, Wetzlar, Germany).

Liposome preparation and uptake - To investigate whether GSL depletion influenced the absorption of fat in macroscale amounts, liposomes were prepared for this uptake approach. Each lipid i.e. cholesterol, phosphatidylcholine (PC), and triolein was solved at a concentration of 10mg/ml in CHCl3/CH3OH 2:1 v/v. Fluorescent NBD-stearic acid (Avanti Polar Lipids, Alabaster, AL, USA) was solved in CHCl3 at a concentration of 1mg/ml. For the preparation of liposomes, 160µl triolein, and 20µl of cholesterol, PC, and NBD stearic acid each were mixed and dried under a stream of nitrogen. The lipid mixture was subsequently dried in a vacuum desiccator for 30 min. After addition of 500µl PBS, liposomes were formed by incubation of the mixture in an ultrasound water bath at 50°C for 15 min. For uptake studies of liposomes, mice were anesthetized and a 2cm part of the jejunum approximately 4cm caudally from the duodenum was ligated with clamps. 100µl of the liposome solution was injected (controls, n=8; Ugcg f/f/VilCreERT2, n=3. The liposome containing jejunum was excised after 15 min, rinsed with 5ml of PBS and immediately frozen on dry ice in O.C.T. Tissue-Tek embedding medium (Sukura Finetek, Alphen aan den Rijn, The Netherlands). Sections of 5µm were mounted with fluorescent mounting medium (DAKO, Hamburg, Germany) containing 1:2000 diluted DRAQ5 (Alexis, Shepshed, UK) for the nuclear staining and scanned with a confocal laser microscope (TCS-SL, Leica, Wetzlar, Germany).

Light microscopy and immunohistochemistry - Light microscopy at different time points (controls, n>40 and Ugcg f/f/VilCre, n>30) and immunohistochemistry/immunofluorescence (controls, n=4 and Ugcg f/f/VilCre, n=4) were performed as described (12). Paraffin embedded buffered paraformaldehyde (4%, pH 7.3) fixed sections were stained by hematoxylin/eosin (HE, Chroma, Köngen, Germany). Activity of alkaline phosphatase (ALP) was determined by overlay of sections with alkaline phosphatase substrate solution. For immunohistochemistry of rat anti-mouse Ki67, 1:200 (DAKO, Hamburg, Germany) an APAAP reaction was used (12). Goat anti-villin antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) were diluted 1:20. A 1:300
diluted biotinylated anti-goat antibody was used as secondary antibody (Dianova, Hamburg, Germany), followed by a streptavidin-HRP-complex (Vector SA-5004, Vector Laboratories, Burlington, CA, USA) at a dilution of 1:200. All antibodies and dilutions used in immunofluorescence are listed in Table II.

In vivo DNA labelling with EDU - Mice were injected intraperitoneally with 200µg EDU (Invitrogen, Darmstadt, Germany). After 24h mice were sacrificed and intestinal parts were embedded in paraffin. Sections of 5µm thickness were taken and EDU-labelled cells were stained by click-reaction as described by Salic et al. (18).

Electron microscopy - Intestinal sections were fixed with a mixture of 0.05% ruthenium red (AppliChem, Darmstadt, Germany), 2.5% glutaraldehyde, and 0.2 M cacodylate buffer pH 7.2 (both from Serva, Heidelberg, Germany), 1:1:1 v/v at room temperature for 2 hours. Samples were washed three times with cacodylate buffer and fixed for a second time with 0.05% ruthenium red, 0.2 M cacodylate buffer, and 2% osmiumtetroxide (Chempur, Karlsruhe, Germany), 1:1:1, by vol. at room temperature for 2 hours. Samples were washed again with cacodylate buffer, were dehydrated with a sequential ethanol gradient and embedded in araldite (Serva, Heidelberg, Germany). Alternatively to ruthenium red, samples were fixed according to Karnovsky (19). Araldite embedded tissue was cut (70nm) and viewed in a transmission EM (Leo 910, Zeiss, Oberkochen, Germany).

Immune electron microscopy - Intestinal sections were prepared for cryo immune-EM according to Tokuyasu et al. (20) to ensure that GSLs neither were extracted nor delocalized within the tissue. Sections of approximately 2 to 3mm in lengths were immersion fixed with 4% PFA in PBS for 2 hours. Subsequently, sections were allowed to sediment over night in 2.3 M sucrose and have thereafter been frozen until use. Frozen ultrathin sections were obtained using an ultracut (UC6, Leica, Nussloch, Germany) and were placed on carbon coated EM crids. The sections were blocked and labelled with antibodies as described (20). Primary antibodies and gold labelled secondary antibodies or protein A gold are listed in Table III. All primary antibodies were incubated at 4°C overnight. Gold labelled secondary antibodies or protein A were incubated at room temperature for 2 hours. Images of labelled sections were taken using a transmission EM (Leo 910, Zeiss, Oberkochen, Germany).

Nile red staining for lipids - For the staining of cryosections at P0, newborn mice were sacrificed after initial suckling approximately two to four hours after birth and one part of the intestinal jejunum with visible nutrient content was shortly perfused with 2ml of ice cold PBS and immediately frozen in Tissue-Tek embedding medium. Similarly, also one part of the jejunum has been prepared from newborns at P7. 0.05% Nile red was dissolved in acetone. 10µl of the stock solution was diluted 1:200 in glycerol/H2O, 3:1, v/v. 5µm cryo sections were covered with Nile red solution and scanned with a confocal laser microscope (TCS-SL, Leica, Wetzlar, Germany).

Western blotting - Proteins were isolated from freshly prepared small intestine from the jejunum essentially as described elsewhere (21). Antibodies used are listed in Table IV.

mRNA isolation and quantitative RT-PCR - One ~ 1cm piece of the intestinal jejunum was prepared from sacrificed animals. Adhering tissue was removed. Total mRNA was extracted as described (22). Quantitative real-time RT-PCR was done using the LC-fast DNA Master SYBR Green I kit PCR for the LightCycler (Roche, Mannheim, Germany) as described (12) and mouse 18s ribosomal RNA was used as reference (controls, n=8 and Ugcg /f/f/VilCre, n=8).

Lipase assay - Feces of newborn control- (n=7) and Ugcg /f/f/VilCre (n=6) mice were collected from rectum at day P6.
and immediately frozen and stored at -80°C until analysis. Feces were weighed and diluted 1:20 (weight/vol) with distilled water and dispersed. The suspension was centrifuged at 12000rpm and 4°C for 10 min. 5µl of the respective supernatants were used for lipase assay. The assay was performed according to Ma et al. (23).

Determination of triglycerides (TG) in plasma - Triglycerides were measured with a TG-Quantification Kit (BioVision, Mountain View, CA, USA) according to the manufacturer’s recommendations (controls, n=11; Ugcg f/f/VilCre, n=8).

Statistics - Data were analyzed using Student’s two-tailed unpaired t test. In all graphs mean values ± s.e.m. are shown. Significances, *P< 0.05; **P< 0.01; ***P< 0.001

RESULTS

Ugcg f/f/VilCre mice lack GSLs in the intestine of newborn mice - To investigate the role of GSLs for intestinal function, mice were generated in which the Ugcg gene - encoding the key enzyme Ugcg involved in the initial step of the glucosylceramide based GSL-synthesis pathway - was deleted in a cell-specific manner in enterocytes by using a villin-cre recombinase transgene (Figure 1A). Deletion of Ugcg can be expected to result in absence of GSLs in the epithelium of the intestine in newborn and adult mice (Figure 1B). A product of 383bp indicated the floxed Ugcg allele and a fragment of 216bp was indicative for the villin-cre transgene (Figure 1C). As shown by southern blot analysis, Ugcg gene deletion was restricted to the intestine (Figure 1D). The specificity of the Ugcg gene deletion for the complete intestinal tract but not for the stomach was indicated by a LacZ staining (Figure 1E). Consequently, all investigated intestinal parts of mutant mice showed GSL depletion (Figure 1F). In agreement with the Ugcg gene deletion, depletion of glucosylceramide (GlcCer) and its further synthesis products, e.g. ganglioside GM1 and GD1a (24) was seen in newborn mice at P0 and P7 (Figure 2A). The TLC data could be confirmed by LC-MS/MS. In our analysis from intestinal tissue at day P0 we could demonstrate that GlcCers with phytosphingosine- and α-hydroxylated fatty acid in their lipid anchor were by far the most prominent occurring species in the intestine (Figure 2B). Those species of GlcCers were 40 fold reduced in mutant intestine of newborn mice at P0 (Figure 2B). However, one minor GlcCer-species with sphingosine and non-hydroxylated fatty acids, representing less than 5% of the total GlcCers appeared unaltered in intestinal GSL extracts (Figure 2B). These GlcCers may have originated from cells not affected by the Ugcg-deletion such as muscle-, endothelial-, stroma- and goblet cells. Also the possibility that the remnant was not GlcCer but GalCer cannot be excluded. The total sphingomyelin content in mutant intestine appeared almost unaltered (Figure 2C) and ceramide increased ~ 20% in mutant tissue at P7 (Figure 2C). The phospholipid concentration in intestinal extracts was not changed in newborn mice at P0 (Figure 2D), but they significantly decreased in mutant intestines 5 to 7 days after birth (Figure 2E). The intestinal cholesterol concentrations were slightly but not significantly reduced in mutant jejunum at P7 (i.e. Ugcg-contr., 22.5 ± 1.1 nmol/mg dry; Ugcg f/f/VilCre, 20.0 ± 1.9 nmol/mg dry).

Newborn Ugcg f/f/VilCre mice at P0 displayed inconspicuous morphology and behaviour - Although newborn mice lacked enterocytic GSLs at P0 (Figure 2A), they and enterocytes were indistinguishable from their littermates in size and behaviour. Expression of villin, required for correct alignment of the microvillar actin core bundle to anchor the brush border, was normal in mutants at P0, implying a regular polarization of the epithelium (Figure 3A and 3E, anti-villin immunofluorescence). The ultrastructure of intestinal epithelium was similar in mutants and controls (Figure 3B and 3F, electron
micrographs). An increased proliferative property of the epithelium due to the absence of GSLs was not observed at P0 (Figure 3C and 3G). GSLs could be detected equally distributed in vesicular-like dots throughout the cytosol of enterocytes in control mice, as shown by anti-GM1 immunofluorescence (Figure 3D). In agreement with TLC GSL-analysis, a staining for GM1 was negative in mutant enterocytes (Figure 3H).

**Ugcg f/f/VilCre mice demonstrated loss of fat deposits** - As consequence of the intestinal GSL deficiency, mutant mice did not gain weight after birth with increasing age (Figure 4A and Figure 4A, inset), although they sucked milk normally (Figure 4C). The usual fat deposits in the abdomen and pelvis were not seen in mutants (Figure 4E). Mutant mice regularly defecated attesting a not significantly altered gut motility. However, stools of mutant mice appeared soft and lipid rich. Offsprings, depleted of intestinal GSLs succumbed between P5 and P8.

**Histology showed age-dependent severe structural alterations in mutant intestine** - In contrast to newborn mutant mice which showed an inconspicuous morphology at P0, postnatally, between days 5 to 7, the intestinal epithelium demonstrated massively altered structure throughout both the small and large intestines (Figure 5A). The morphology of mucosal villi in all parts of the small intestine was severely distorted in mutants one week after birth; they often appeared to be attached or “glued” to one another. In the colon, severe vacuolization was observed in particular in the uppermost layers of the enterocytes. Cell proliferation in the intestinal epithelium was significantly increased in mutant tissue, with proliferating cells extending to the intestinal lumen. In control litters proliferating cells were only located in the base of the intestinal crypts (Figure 5B, Ki67). Alkaline phosphatase (ALP), usually stringently and continuously located at the apical surface of the intestinal brush border, was detected in a patchy pattern in mutant mice implying a disorganization of microvillar structure (Figure 5B). In comparison to newborn Ugcg f/f/VilCre-mice at P0, in which the microvillus protein villin was regularly distributed, anti-villin failed to stain the apical brush border region in GSL-deficient enterocytes at P7 (Figure 5B, villin).

**Ugcg f/f/VilCre mice displayed severe disturbance in uptake of nutrients** - We thought that absence of GSLs in enterocytes would be associated with a decrease in the uptake of nutrients. And, indeed, the lipid marker Nile red indicated a drastic reduction of intracellular lipid deposits in Ugcg f/f/VilCre mutant mice at P0 (Figure 6B) and P7 (Figure 6E) whereas controls were strongly stained (Figure 6A and 6D; 6C and 6F, quantification). A reduced uptake of NBD-labelled stearic acid further demonstrated the restricted ability of the mutant epithelium to absorb lipids (Figure 6H and 6I, quantification).

Ultrastructurally, microvilli of enterocytes in control intestines form a regular array of finger-like protrusions (Figure 6J). Different from newborn mice with GSL depletion at P0, postnatally, one week after birth, mutant mice displayed only rudimentary stump-like microvilli on the apical surface of enterocytes. The enterocytes of GSL-deficient mice displayed fewer fat containing vacuoles (Figure 6K, LD) corroborating the Nile red lipid staining (Figure 6B and 6E). Caveolae like invaginations as seen in enterocytes of control mice were almost absent in newborn mutants. In addition, their lysosome-like structures appeared smaller than in controls (Figure 6K).

To investigate whether the observed reduced lipid uptake by mutant intestines led to increased fecal lipid excretion, feces of control and mutant mice were investigated for their lipid content. Fatty acids (Figure 7A and 7B) and cholesterol (Figure 7A and 7C) significantly accumulated in feces. Secretion of bile acids (Figure 7D) and pancreatic
lipases (Figure 7E) into the gut lumen were unaltered indicating that their production and release into the gut lumen were not affected in mutant mice. A subcutaneous fat layer was observed in newborn controls as well as in mutant mice at P0 (Figure 7F and 7H). Fat deposits were completely used up in mutants until P7 (Figure 7I) whereas controls increased significantly their subcutaneous fat layer (Figure 7G). This showed that the mutant mice metabolized their preterm subcutaneous fat deposits in compensation of the reduced intestinal lipid uptake. In concordance, plasma triglyceride concentrations significantly decreased in mutants (Figure 7J).

In order to verify whether absorption deficits in mutant intestines were restricted to lipophilic nutrients or occurred also in the uptake of hydrophilic components, the absorption of glucose was investigated. Although Ugcg-deficient mice were able to absorb glucose (Figure 7L), the extent was less than in wildtype animals (Figure 7K and 7M, quantification). The absorption of carbohydrates by the mutant intestine was maintained to an extent that lactose after digestion into galactose and glucose has been completely taken up (Figure 7N). However, sialylated lactose accumulated in feces of Ugcg f/f/VilCre mice (Figure 7N) as a consequence of reduced sialidase activity in the small intestine of mutant mice (Figure 7O, 7O’ and 7P, 7P’).

Quantitative rt-PCR was performed in order to investigate whether altered membrane protein expression negatively influenced the absorption of nutrients in mutant mice. The expression levels of the intestinal fatty acid binding proteins Fabp2 and CD36, the cholesterol transporters Abcg5 and Abcg8, the triacylglycerol synthesis and transfer proteins Dagt1 and Mttp, the glucose transporters Sglt and Glut5 as well as the small peptide transporter Pept1 were slightly but not significantly altered (Table V). Our data demonstrate that the functional defects observed were not primarily caused due to alterations of membrane protein expression with one exception, aquaporin 8 (Aqp8). As an indication for reduced water absorption in the small intestine, the mRNA levels of Aqp8 decreased.

Ugcg f/f/VilCreERT2 mice similar as newborn mutants at P5 to P7 had severe intestinal alterations shortly after induction of the Ugcg gene deletion - Besides glucosylceramide, newborn mice synthesize high levels of sialic acid-containing GSLs in the intestine (Figure 1F and 2A). In adult mice predominantly glucosylceramide and asialo GM1 (GA1, Figure 8A) can be found (25).

Upon induction with tamoxifen of the specific Ugcg gene deletion in the intestine, mice lacked GlcCer and its higher small intestinal synthesis product GA1 almost completely after four days (Figure 8A). In heterozygous control mice (Ugcg f/+VilCreERT2) intestinal GSL composition did not change. A reduction of GSL synthesis of 50% was already achieved at day one after start of the tamoxifen induction (Figure 8B). Reduction of 70% of intestinal GSL content accompanied with initial structural alterations was observed two days after start of the tamoxifen application (Figure 8B). An almost complete absence of GSL-synthesis and severe structural alterations in the villous epithelia were seen three days after beginning tamoxifen treatment (Figure 8B and 8B’). Our data obtained from the inducible model suggested a critical GSL reduction of ~50% to 70% to induce relevant structural and functional problems in the intestinal tract.

During the time dependent reduction of GSLs, a drastic decrease in bodyweight (Figure 8C), food and water consumption (Figure 8D and 8E) was observed in mutant mice.

To investigate whether endocytosis of large scale lipid was inhibited by GSL depletion similar as in newborn mice, fluorescence labelled liposomes were injected into the gut lumen of control- and Ugcg f/f/VilCreERT2 mice. In this approach we could show, that liposomes entered the apical
membrane of control mice epithelium unpimped (Figure 8F); in contrast, mutant Ugcg f/f/VilCreERT2 mice were not able to absorb liposomes (Figure 8F).

Morphologically, Ugcg f/f/VilCreERT2 mice resembled the phenotype of mutant newborns at P5 to P7 (Figure 9). However, the extent of changes was stronger as in newborns. Adult control- and mutant mice have been injected with EDU on day two after the tamoxifen triggered Ugcg gene deletion. One day later the proliferative zone was seen to be distributed almost throughout the complete epithelial layer, whereas proliferation in tamoxifen-treated controls was restricted to the crypts of the villi (Supplementary Figure (9B, EDU).

Ultrastructurally, similarly as seen in newborn mutant mice one week after birth, Ugcg f/f/VilCreERT2 mice showed loss of brush border three to four days after tamoxifen induction (Figure 10B and 10D), decreased phospholipid concentrations (Figure 10G), similar sphingomyelin levels as in controls (Figure 10H) and increased ceramide concentrations (Figure 10H).

Very similar to the GlcCer profile of newborns, GlcCers with a phytosphingosine containing ceramide anchor were found by LC-MS/MS to be the most prominent species in intestine (Figure 10I). In intestine of adult mutant mice GlcCers were drastically reduced (Figure 10I). As in newborns, GlcCers with (d18:1; n:0)-ceramide anchors were unaltered. The precursor ceramides increased in mutant tissue significantly (Figure 10H, TLC; Figure 10J, LC-MS/MS). In addition, sphingomyelins (SM) containing phytosphingosine residues were elevated about 5 fold (data not shown). However, these SMs represent only 2 % of the total SM content in control intestine. On the other hand, the most prominent SMs in intestine (i.e. d18:1; n:0) with sphingosine and non-hydroxylated fatty acid residues decreased roughly by 25% in the mutant tissue. In sum, the total sphingomyelin content in mutant intestine appeared unaltered (Figure 10H, TLC; Figure 10K, LC-MS/MS).

Increased ceramide levels may have triggered autophagy (Figure 10B and 10D) (26,27), which could be confirmed by elevated Lc3 II expression (Figure 10E and 10F, quantification). Concomitantly, a significantly higher number of TUNEL-positive cells were seen in tamoxifen-induced Ugcg f/f/VilCreERT2 mice (Figure 10M) as compared to newborn Ugcg f/f/VilCre animals (Figure 10L).

A detachment of enterocytes from the basal membrane was observed four days past tamoxifen induction. To investigate whether this phenomenon occurred due to a dislocation or reduction of adhesion molecules, expression of E-cadherin and β-catenin was investigated. Enterocytes of both control and mutant mice expressed E-cadherin in a basal and basolateral fashion throughout the whole epithelium (Figure 11A). β-catenin was stained strongly in basal and basolateral regions of control epithelium and in the lower compartments of villi of Ugcg f/f/VilCreERT2 mice. However, only partial β-catenin expression or complete absence was seen at the tips of the villi in mutant mice (Figure 11B). Immunofluorescence data could be confirmed by western blot analysis (Figure 11C and 11D).

Stromal immune cells in intestine

were not changed by the Ugcg-deletion. To investigate whether an infiltration of immune competent mononuclear cells would have triggered the hyperproliferation in mutant intestines both newborn and adult mutant mice and respective controls were stained for B-cells, T-cells and monocyte derived cells. The numbers of immune cells infiltrating intestinal tissue of newborn control mice was very low in general, but increased during adulthood (Data not shown). However, none of the stainings of mutant newborn and adult intestine showed significant increased
numbers of immune cells as compared to the respective control tissues.

The intestinal phenotype in Ugcg f/f/VilCreERT2 mice could not be restored by exogenous GlcCer feeding. Mutant mice have been fed with chow diet, supplemented with or without 2mg/g of glucosylceramide ad libitum and both groups were injected with tamoxifen as described. Mutant mice with GlcCer supplementation showed an almost identical decrease in body weight as mutants that received chow diet (Data not shown). Animals of both groups had to be sacrificed due to their severely compromised state of health three days after the initial tamoxifen induction. Morphologically, GlcCer treated and untreated Ugcg f/f/VilCreERT2 mice were undistinguishable (Data not shown) suggesting that exogeneous GlcCer-application could not compensate for the Ugcg-deletion.

Subcellular localization of enterocytic GSLs. - In order to verify the role of GSLs for the observed phenotype in mutant mice, their intestinal localization and possible involvement in intracellular transport was investigated. In this respect, stainings of GM1 together with the brush border protein villin as well as clathrin and Rab11 were performed in control and mutant mice postnatally at days 5 to 7.

GM1, ubiquitously distributed in vesicle-like structures in the cytosol of control mice postnatally at day P0 (see, Figure 3D), concentrated apically below the brush border one week after birth (Figure 12A, 12C and 12E). Only a small overlap could be seen between GM1 and villin in the microvillar region of wildtype intestine (Figure 12A). GM1 stained negative in mutant tissue and the brush border protein villin was detected only rudimentarily. A co-staining of GM1 with clathrin, one marker of coated pits and -vesicles (Figure 12C) as well as GM1 with the recycling endosomal marker protein Rab11 (Figure 12E) in control tissue indicated close proximity apically underneath the brush border. GM1 similar as at P0 was again not stained in mutant tissue (Figure 12D and 12F). The expression of clathrin and Rab11 was drastically downregulated in mutant tissue one week after birth (Figure 12D and 12F). In addition, also the lysosomal marker Lamp1 decreased in Ugcg f/f/VilCre intestine (Figure 12H).

The data obtained by immunofluorescence could be confirmed with western blot analysis. The expression of proteins involved in endocytic processes and in protein recycling such as clathrin, caveolin-1 and Rab11 was drastically downregulated (Figure 12G and 12J). However, the early endosomal marker proteins Rab4 and Rab5 were not significantly altered (Figure 12K).

GSLs cluster in vesicles and are located predominantly underneath or close to the brush border. Confocal immunofluorescence images have a limited resolution. To answer the question whether GSLs were involved in membrane events such as formation of caveolae and transport vesicles, immunogold EM-stainings have been performed to elucidate the exact intracellular localization of GM1 in newborn (P5) as well as GA1 in adult mice. GM1 was found to be predominantly located in vesicle-like structures (Figure 13A, arrows). These vesicles showed two opposite migration directions. The vesicular content including GSLs was shed into the intestinal lumen where GSLs have been found in the glycocalyx (Figure 13A, 13B) and also to be released intracellularly, associated with lipid droplets (Figure 13C). Absence of GM1 went along with an almost complete absence of lipid droplets in Ugcg f/f/VilCre mice (Figure 13D).

A costaining of GM1 with clathrin demonstrated that coated pits formed at the apical membrane surface (Figure 13E) mostly independent from GSLs; only few gold particles which labelled clathrin were associated with GSL-vesicles (Figure 13E). Specific staining for GM1 and clathrin did
not occur in mutant tissue. In a GM1 and Rab11 co-immune EM, Rab11 was - with a few exceptions (Figure 13G, inset) - not directly associated with GSL-vesicles (Figure 13G). Our immune electron microscopic data suggested that GSL-vesicle formation essentially not depended on clathrin and Rab11.

Similar to newborn mice, in adult animals GSL (GA1)-could be visualized in vesicle like structures (Figure 13I, arrows). Those vesicles appeared to be released into the intestinal lumen (Figure 13J) and again, attached to lipid depots (Figure 13K). 

DISCUSSION

The present study reports new insights into the role of GSLs for intestinal epithelial function. GSLs have been surmised to be important for epithelial polarization (28) and differentiation (29). Indeed, in C. elegans mutants in whom GSL-synthesis was inhibited by feeding of RNAi against glucosylceramide synthase (Ugcg), an impaired distribution of proteins involved in intestinal polarization such as ERM-1 and actin has been shown. A depletion rate of ~75% of glucosylceramide was sufficient to induce those dramatic defects (11).

Are these results also to be expected in mammals? Based on our data with GSL depletion in the liver in which bile canalicular function was maintained (16), we have postulated a different function of GSLs in mouse enterocytes but for epithelial polarity. Two mouse models, an inducible model in adult mice and a constitutive model in which GSLs were depleted during embryogenesis were applied. The latter model provided the advantage to investigate the morphology of the intestine shortly after birth when its absorptive activity was not yet fully developed. Such a state of functional quiescence cannot be tested in C. elegans and might be one reason that a similar phenotype has not been observed in worms.

Surprisingly, the intestine of newborn mutants at P0 could neither macroscopically nor by microscopy be distinguished from that of control littermates, but the mutants lacked GSLs in the intestine. Also epithelial proliferation did not differ from control intestine at P0, suggesting that GSL depletion had no immediate influence on the cell cycle of enterocytes. An epithelial-to-mesenchymal transition process (EMT) in which GSLs were described to play a critical role in vitro (30,31) also did not occur as enterocyte-morphology appeared normal at that stage and enterocytes seemed fully differentiated and polarized. However, newborn mutant offspring at P0 were not able to absorb milk fat in a similar amount as their control littermates. This was an initial hint that GSLs might be directly involved in lipid absorption and/or transport. The finding that villin - a marker of enterocytic polarization - was unchanged in GSL-depleted intestine at P0 and that the brush border was regularly formed suggested that GSLs were not immediately needed for microvillar generation and consequently for epithelial enterocytic polarization. This observation corroborated the results from mouse models with GSL depletion in liver (16) and in kidney (unpublished data). In these organs, neither hepatocytic autoplasmic microvillus-like protrusions into canaliculi of hepatocytic epithelial cells - nor microvilli of the proximal renal epithelium were altered upon GSL depletion. The latter findings implied the question of the cellular localization of GSLs in enterocytes. We surmised that GSL could be associated with proteins involved in intracellular transport. In control mice a few days after birth, we could demonstrate - by confocal microscopy and more clearly by immune electron microscopy - the existence of small GSL-containing vesicles at the apical membrane located underneath the brush border in the cytosol, similar to data reported earlier (3). These vesicles followed a transport route from the apical membrane to the enterocytic lipid depots and hence seemed to be involved in endocytosis and
intracellular transport of nutritional lipids. The GSL-dependent vesicle formation - essentially devoid of clathrin and Rab11 which are also involved in endocytosis and recycling processes (32-35) - might be unique for the intestine due to its high concentration of glycosphingolipids. The question whether GSL-vesicles require other proteins as the investigated ones for their formation and their nature still remain to be elucidated. Due to the disturbed lipid absorption, the intestine reacted with hyperproliferation; infiltration of immune cells which potentially may have triggered such a process could not be observed. As consequence of the epithelial hyperproliferation, a regular differentiation of the enterocytes was inhibited. Usually, in wildtype animals, the intestinal epithelium exhibits a small proliferative area located in the crypts of villi. Cells differentiate into enterocytes and while migrating to the apex of the villus, exert their absorptive function; they are then released into the gut lumen after three to five days (36). In our mutant mice, with fully developed phenotype, the proliferative zone was not restricted to the crypts of the villi and extended to half of the epithelium or even higher associated with immature differentiation of the epithelial enterocytes 5 to 7 days after birth, or 3 to 4 days after tamoxifen induced Ugcg deletion in adult animals. The altered epithelial differentiation could also be depicted by the rudimentary formation of the brush border. It should be stressed, though, that GSL depletion did not primarily induce hyperproliferation as in newborn mice with GSL-deficiency the proliferative rate of enterocytes was comparable to control mice.

Caveolin dependent invaginations have been proposed to be involved in several biological processes such as cell signalling, membrane traffic (2,37) and endocytosis (3). It cannot be definitively decided, whether a disturbed epithelial differentiation might have been accompanied with a reduced expression of caveolin or its intracellular recycling was affected. In our GSL-deficient mice a significant reduction of caveolae formation was indicated by electron microscopy and western blot.

Although the structural alterations in the intestine after Ugcg-deletion appeared very prominent, the mRNA expression levels of proteins, actively thought to be involved in lipid binding and intracellular transport were essentially unaltered. Neither the intestinal fatty acid binding proteins Fabp2 and CD36 nor enzymes important for triglyceride synthesis and transfer such as Dgat1 and Mttp were statistically significantly changed. Also the intestinal cholesterol transporters Abcg5 and Abcg8 as well as the short chain peptide transporter Pept1 and the glucose transporters Sglt1 and Glu5 were not significantly altered. However, the downregulation of the membrane integral protein Aqp8 which has been described to function on the apical side of duodenum, jejunum, and colon (38) was accompanied by a lower intestinal water absorption associated with a significant increase of hematocrit levels in Ugcg f/f/VilCreERT2 mice (not shown). Reduced expression and activity of Aqp8 water channels may have been one factor for the extreme soft stools observed in mutant mice.

Fat from nutrients, predominantly in form of triglycerides, is primarily cleaved into monoacylglycerols (MAG) and free fatty acids (FA) by secreted pancreatic lipases. The first step in lipid absorption, the movement of MAG and FA across the apical membrane of enterocytes is not well defined on a molecular level; it very likely involves different mechanisms. Several studies describe protein-dependent mechanisms, while others have reported protein-independent transport via diffusion through the apical membrane of the enterocytes (39,40) for which in particular bile salts appear to be necessary as they form micelles with the FA and facilitate thereby their absorption (41). Lipids also might be taken up via endocytosis. Ex vivo, in intestinal tissue sections it could also be shown that exposure of high lipid concentrations led to increased formation of clathrin containing invaginations and vesicles which were then transported and released to
intracellular lipid depots (42). This lipid uptake process appears to be particularly important when the lipid concentration in the intestine is extremely high such as in the situation of newborn suckling mice receiving high fat milk. Indeed in our in vivo studies we now could demonstrate that large scale fat from milk and in form of liposomes can be endocytosed. However, this process depended on GSL-expression. In support, caveolar endocytosis in ovary cells was significantly reduced after GSL-depletion by inhibition of GSL-synthesis (43).

Intestinal GlcCers predominantly express phytosphingosine with α-hydroxylated fatty acid in their ceramide anchor. In particular in the adult intestinal model, most of this GlcCer accumulated in form of its precursor ceramide and only about 1/5 of it was shuttled into phytosphingosine-containing SM. This is in contrast to what we had found previously in brain and liver, where almost all of the ceramide that could not be converted to GlcCer was shifted into sphingomyelin (12,16). On the other hand, to a similar extent as phospholipids, the major sphingosine-containing SM fraction decreased, probably as a consequence of loss of the brush border accompanied with loss of membrane. Increased ceramide accumulations particularly occurring in adult Ugcg-deficient mutants may have triggered the pronounced autophagy (26,27) and apoptosis.

β- and α-catenin form complexes with E-cadherin important to anchor epithelial cells to the lamina propria. In adult tamoxifen-induced mutant mice, a lowered expression of β-catenin has been observed which may have facilitated the detachment of enterocytes. Moreover it has been demonstrated that β-catenin binds GA1, which is the prominent GSL in adult enterocytes. GSL depletion may have indirectly also negatively influenced the epithelial binding to the basal lamina (31).

The phenotype observed in our mutant mice could not be alleviated by the uptake of exogenous GSL via milk or by adding GlcCer to the food. In this respect, it has been convincingly shown that exogenous applied GSL were almost completely degraded (44-47).

GSL-expression in the intestine very likely may not have been influenced by an uptake of shed liver-GSL via the blood as their concentration in the blood is very low. In addition, the blood-GSL pattern differs from the intestinal GSL and in particular the main GlcCer bands in the neutral GSLs showed a different migration speed as GlcCers from blood and milk (not shown), suggesting a different ceramide anchor as the ones mainly found in intestinal GSLs.

In summary, glycosphingolipid expression in the intestinal epithelium is quintessential for maintenance of a GSL-signature of absorption dependent intracellular transport and intestinal structure. However, GSLs are not primarily required for enterocyte polarized brush border formation.

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FOOTNOTES

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FIGURE LEGENDS

**Figure 1.** *Ugcg* cloning strategy, GSL synthesis pathway, genotyping and cre activity determination. (A) *Ugcg* gene deletion was initiated by generating mice expressing loxP-flanked *Ugcg* alleles together with cre-recombinase under the villin promoter. (B) GSL synthesis pathway in newborn and adult mice. The red boxes show GSLs expected to be absent in *Ugcg* floxed/VilCre
mice. Yellow labelled GSLs LacCer and GM2 in newborns as well as LacCer and GA2 in adult mice intestine represent intermediates which are immediately used to synthesize higher GSL products; for nomenclature of GSL, see (24). (C) Mutant mice were genotyped by PCR and Ugcg gene deletion could be confirmed by southern blot analysis (D, values from densitometry on top). (E) Cre-activity in the intestine has been indicated by a dark blue staining in mice expressing the villin-cre transgene in combination with a lacZ-reporter gene throughout the whole intestine. The stomach tested negative. (F) GSL analysis of intestinal compartments of Ugcg f/f/VilCre mice confirmed the results obtained by lacZ-staining and southern blot. Glycosphingolipids were depleted throughout the whole intestinal epithelium.

**Figure 2.** Sphingolipid analysis. (A) GSLs as synthesis products of Ugcg were depleted in mutant tissue already in newborn mice at day P0. (B) As revealed by mass spectrometry, the total GlcCer depletion rate in newborn Ugcg f/f/VilCre mice at P0 was > 90% (controls and mutant, n=3, respectively). A minor GlcCer fraction with sphingosine and non-hydroxylated fatty acids in its ceramide anchor was not affected by the Ugcg-gene deletion and might likely be originated from cells in which the cre-recombinase was not active. (C) Sphingomyelin in mutant intestine had a similar concentration as in control tissue and ceramide content increased slightly in Ugcg f/f/VilCre intestine as compared to controls. (D) Phospholipid content was not altered in newborn Ugcg f/f/VilCre mice at P0 (n=4, respectively). (E) However, intestinal phospholipid concentrations significantly decreased in mutant tissue at P5 to P7 (PE, phosphatidylethanolamine); PG, phosphatidylglycerol; PC, phosphatidylcholine); controls and Ugcg f/f/VilCre, n=6 each.

**Figure 3.** Newborn Ugcg f/f/VilCre mice, although depleted of GSLs, show no conspicuous phenotype at P0. (A/A’ and E/E’) The brush border marker protein villin was expressed in wildtype and mutant mice at postnatal day P0. (B and F) Presence of an intact brush border could be confirmed by electron microscopy. (C and G) As shown by anti Ki67, no differences in proliferation were apparent between control and mutant mice. (D/D’) The GSL GM1 was found ubiquitously distributed in vesicle-like structures within the cytosol of enterocytes. (H/H’) Ugcg f/f VilCre intestine stained negative for GM1. Scale bars: light microscopy, 100µm; EM, 1µM.

**Figure 4.** Ugcg deficient mice showed retarded postnatal weight gain and loss of body fat deposits. (A) Ugcg f/f/VilCre mice were of smaller size and displayed insignificant gain of weight (A, inset). (B and C) Control mice (B) and mutant mice (C) contained milk in their stomachs (arrows) one week after birth demonstrating successful suckling. (D and E) gonadal fat deposits observed in controls (D, arrows) were not observed in mutant mice (E). Stools in the rectum of mutant mice appeared soft and still displayed milk-like colour.

**Figure 5.** Ugcg f/f/VilCre mice exhibited major structural defects in the intestine and impaired distribution of intestinal proteins one week after birth. (A) Hematoxilin/eosin (HE) staining of intestinal segments as indicated. Pronounced structural defects were recognized in the mucosa of the small intestine of mutant mice with loss of villi and flattening of the mucosa as well as increased epithelial vacuolization. (B) Immunohistochemistry of polarization and proliferation marker of newborn mice at postnatal day P7. As exemplarily shown for sections of the jejunum, intestine from Ugcg deficient mice displayed high levels of cell proliferation (Ki67). Mutant mice showed a patchy distribution of alkaline phosphatase expression (ALP) and almost a complete absence of the structural protein villin, both of which were present in the microvillar region of intact intestinal mucosa in control mice; scale bars: 100µm.
Figure 6. Mice lacking glycosphingolipids in the intestine showed loss of lipid depots and drastically reduced lipid uptake. (A to F) Using Nile red, epithelium of mutant intestine at P0 (B) and P7 (E) showed a drastic reduction of lipid droplets as compared to respective controls (A and D). (C and F) For quantification of the mean fluorescence of lipids per villus. 10 to 15 villi per animal were taken (n=4, controls and n=2, mutant at postnatal day P0 as well as n=4, controls and n=3, mutant at postnatal day P6/P7). (G and H) Fatty acid uptake by the intestine was markedly reduced in mutant mice (H) as compared to controls (G). (I) Quantification of the mean fluorescence per villus of NBD-stearic acid uptake 10 min after intestinal administration; measured were 10 to 15 villi per animal. Shown is one out of three independent experiments with similar results and n=4 for each group, respectively. Scale bars: 100µm; **P< 0.01. (J and K) Electron micrographs stained with ruthenium red. Mutant Ugcg f/f/VilCre intestine (K) showed loss of microvillar brush border (mv) and lack of caveolae (arrows) as well as smaller and fewer fat containing lipid droplets (LD) in the cytoplasm of enterocytes as compared to wildtype control (J). l, lysosomes; scale bars: 1µm.

Figure 7. Ugcg f/f VilCre mice lost lipids by fecal excretion. (A, B, C) Lipids, fatty acids (A, TLC and B, quantification) and cholesterol (A, TLC and C, quantification) accumulated in feces of Ugcg-deficient mice; controls and Ugcg f/f/VilCre, n=6 each. (D, E) Bile acids (D) and lipase activity (E) in feces were unaltered indicating their intact secretion from bile duct and pancreas into the intestinal lumen. (F to I) Ugcg f/f/VilCre mice lost subcutaneous fat deposits. Histology showed that control (F) and mutant mice (H) had subcutaneous fat tissue (sf) on the day of birth which was no longer present in Ugcg-deficient animals one week later (I). (G) In contrast, control animals showed an increased fat storage. (J) As a consequence of the disturbed intestinal fat uptake, plasma triglyceride concentrations were significantly reduced in mutant mice at P7; controls, n=11 and Ugcg f/f/VilCre, n=8. (K to M) Ugcg f/f/VilCre mice displayed reduced intestinal glucose uptake. The absorption experiment of NBD-labelled glucose was performed according to uptake of NBD-stearate. Both control (K) and mutant newborn mice (L) absorbed NBD-glucose. However, the uptake was less in mutant intestine (L). (M) Quantification of the medium fluorescence per villus after NBD-glucose uptake, 10 min after intestinal administration; measured were 10 to 15 villi per animal (n=2, each). (N) Mutant mice were able to absorb lactose after its digestion into galactose and glucose completely but sialylated lactose accumulated in their feces. (O and P) Ugcg f/f VilCre mice showed reduced sialidase activity in the small intestine. Sialidase activity in intestinal cell lysates was measured by digestion of sialylated lactose for 30 min, n=4 for each group (O) or in a time dependent manner as indicated (P) and were quantified by a Shimadzu CS 9000 densitometer (O’ and P’); *P< 0.05; **P< 0.01; ***P< 0.001; scale bars: 100µm.

Figure 8. Ugcg f/f/VilCreERT2 mice showed severe structural alterations in the intestine shortly after induction of the Ugcg gene deletion. Ugcg-control- and mutant mice were treated for three consecutive days with 1mg tamoxifen, respectively. (A) GSLs in the intestine were absent four days past the initial tamoxifen application (left and right TLC’s represent neutral and acidic GSLs) and were remarkable reduced by ~50 and ~70% already one and two days after the initial induction (B). The remaining bands in extracts of mutant intestine migrating at the height of GM3 might be explained by admixtures of both intestinal muscle and stroma which were not affected by the Ugcg gene deletion. The degree of GSL depletion (B) correlated well with the dramatic alterations of the structure of the small intestine as shown by hematoxylin/eosin staining (B’). (C, D, E; controls and Ugcg f/f/VilCreERT2, n=5; heterozygous mice, n=3) Shortly after induction Ugcg f/f/VilCreERT2 mice showed reduction of bodyweight (C) as well as a lower food (D) and water consumption (E). Heterozygous mice developed similar to other controls. (F) The uptake of liposomes is impaired in Ugcg f/f/VilCreERT2 mice. Liposomes were rapidly endocytosed by
control intestine. In mutant intestine the uptake of liposomes is drastically diminished or absent; scale bars: 100µm.

**Figure 9.** Severe structural defects occurred in small and large intestine of *Ugcg* f/f/VilCreERT2 mice. (A) Major structural defects similar as in newborn *Ugcg* f/f/VilCre mice one week after birth were also recognized in the mucosa of the small and large intestine of *Ugcg* f/f/VilCreERT2 mutant mice three to four days upon tamoxifen induction. More obvious than in newborn mutants, in *Ugcg* f/f/VilCreERT2 intestines enterocytes detached from the basal lamina. (B) Highly increased proliferation (Ki67 and EDU) indicated by red nuclei, and drastically decreased expression of the brush border proteins alkaline phosphatase (ALP) and villin were observed in tamoxifen induced mutants devoid of GSL, similar as in newborn *Ugcg* f/f/VilCre mice; scale bars: 100µm.

**Figure 10.** Tamoxifen-induced *Ugcg* f/f/VilCreERT2 mice show loss of brush border as well as increased autophagy and apoptosis. (A to D) Electron micrographs of adult control mice (A and C, magnification) and tamoxifen-induced *Ugcg* f/f/VilCreERT2 mice (B and D, magnification). Sections were stained according to Karnovsky et al. (19). Adult mice lacking GSLs in enterocytes showed loss of brush border (B and D) similar as seen in newborn mutants. In addition, increased numbers of multi vesicular bodies (•) and marked autophagy (arrows) was detected in the cytoplasm of enterocytes of induced mutant mice (B and D); scale bars: 1µm. (E) Lc3 II expression, a marker of autophagy, was significantly elevated in tamoxifen-induced *Ugcg* f/f/VilCreERT2 mice as compared to their respective controls (F, quantification). (G) Similar as in *Ugcg* f/f/VilCre newborn mutant mice one week after birth, the phospholipid concentration in the intestine decreased significantly; controls, n=6 and *Ugcg* f/f/VilCre, n=4. (H) As revealed by TLC-analysis, ceramide concentration in jejunum of *Ugcg* f/f/VilCreERT2 increased significantly and stronger than in newborn mutant mice. The total sphingomyelin (SM) content was not altered; controls, n=6 and *Ugcg* f/f/VilCre, n=4. (I to K) LC-MS/MS quantification of GlcCers, ceramides and SM. (I) The total GlcCer depletion rate in adult *Ugcg* f/f/VilCreERT2 mutants reached similar levels (> 90%) as in newborn mice at P0 (controls and mutant, n=3, respectively). Similar as in newborns, a GlcCer fraction with sphingosine and non-hydroxylated fatty acids in its ceramide anchor was not affected by the *Ugcg*-gene deletion. (J) Further MS-analysis indicated that ceramide levels increased in mutant tissue and confirmed the data obtained from TLC-analysis. (K) Total SM levels were similar in control- and mutant intestine, however, the SM-composition changed qualitatively (not shown). (L and M) Increased ceramide concentrations in enterocytes may have contributed to the higher number of TUNEL-positive cells per villus in tamoxifen-induced adult mutant *Ugcg* f/f/VilCreERT2- (M) as compared to newborn *Ugcg* f/f/VilCre mice (L); n=4 each. *P< 0.05; **P< 0.01; ***P< 0.001.

**Figure 11.** *Ugcg* f/f/VilCreERT2 mice showed reduced expression of β-catenin in luminal epithelial cells at day four upon tamoxifen treatment. (A and B) Villous epithelial cells were stained with an anti E-cadherin- (A) or beta catenin antibody (B). The apical brush border was counterstained with anti-villin. E-cadherin accumulated in enterocytes (A). Particularly in the heads of villi of mutant mice, detachment of enterocytes from the basal membrane and consequently partial disruption of the columnar epithelium occurred and also β-catenin staining was reduced in *Ugcg* f/f/VilCreERT2 mice (B); scale bars, 100µm. (C and D) Results obtained by immunofluorescence could be confirmed by western blot analysis.

**Figure 12.** GM1 to a major extent was found apically in vesicular-structures in the cytosol and stained negative in mutant mouse tissue. All stainings shown were performed on intestinal jejunum sections collected five to seven days after birth. Animals of the same age (littermates) were
compared. (A and B) Costaining of GM1 together with villin. In contrast to newborn mice at day P0 in which GM1 was ubiquitously located in small dots in the cytosol of the epithelium, one week after birth GM1 located predominantly on the apical site of the enterocytes (A). The structural protein villin was stained in the brush border but only a small overlap could be seen together with GM1. GM1 was stained to a major extent apically, apart from the brush border. (B) In mutant tissue, as expected, GM1 could not be detected but also villin-expression was very weak. (C and D) Costaining of GM1 and clathrin. In control tissue GM1 and clathrin were found in similar regions apically in the enterocytes (C). Clathrin expression was much less pronounced in mutant epithelium but was still present in the intestinal zona muscularis propria (D). (E and F) Costaining of GM1 together with the recycling endosomal marker protein Rab11. GM1 could be located apically in similar regions as Rab11(E). Rab11 staining in mutant intestine was very weak (F). (G) Lamp1-positive lysosomes were stained and were regularly distributed in the cytosol of the intestinal epithelium of control mice. (H) In mutant tissue, Lamp1-staining appeared weaker and lysosomes were irregularly distributed within the cytosol of the enterocytes. (I to K) Western blot analysis revealed drastically decreased levels of clathrin and caveolin-1, proteins involved in endocytosis (I) as well as Rab11 as a marker for recycling endosomes important for protein recycling (J). The early endosomal marker proteins Rab4 and Rab5 showed similar expression in control and mutant tissue (K).

**Figure 13.** By immune electron microscopy, GSLs predominantly localize in vesicles close to the brush border. (A to C) Immunogold EM-stainings of GM1 in control intestine of newborn mice at P5. GM1 was visualized associated in vesicles, underneath the brush border (mv) and in the region of the glycocalyx (gc) (A, arrows). Only rudimentary staining was seen in the microvilli. (B) GSL-containing vesicles seemed to release their content at the apical membrane of the enterocytes into the intestinal lumen and intracellularly into lipid depots (LD) (A and C). (D) Only scarce unspecific gold particles were observed in enterocytes of Ugcg flox/VilCre mice. (E and F) Immunogold EM-costaining of GM1 (10nm gold particles) with clathrin (5nm gold particles) in newborn control- (E) and mutant mice (F) at P5. Clathrin coated pits (arrows) originate in wildtype mice mostly independent from GSLs at the apical membrane of the enterocytes (E). Only few clathrin-positive dots colocalized with GM1 vesicles (arrowheads). In mutant mice GM1 and clathrin were almost completely absent (F). (G) Similar as clathrin, also Rab11 (10nm gold particles, arrowheads) predominantly did not colocalize with GM1 vesicles (G, arrows, 5nm gold particles) with a few exceptions (inset). (H) Only a background staining for GM1 and a reduced Rab11-staining was seen in mutant tissue. (I to L) In adult animals, the GSL GA1 - similar as GM1 in newborns - also localized in vesicles. GSL (GA1)-associated vesicles (I, arrows) are present in a layer close to the brush border (J) or in lipid droplets (LD) (K) similarly to the location of GSLs in newborn animals one week after birth. (L) Ugcg flox/VilCreERT2 intestine showed only a few unspecific gold particles; m, mitochondria; scale bars: 200nm.
Table I: UPLC-gradient elution of Sphingolipids for tandem-mass spectrometrical detection.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Flow Rate [ml/µin]</th>
<th>Solvent A [%]</th>
<th>Solvent B [%]</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.45</td>
<td>100</td>
<td>0</td>
<td>Initial</td>
</tr>
<tr>
<td>0.1</td>
<td>0.45</td>
<td>100</td>
<td>0</td>
<td>linear</td>
</tr>
<tr>
<td>0.2</td>
<td>0.45</td>
<td>92</td>
<td>8</td>
<td>linear</td>
</tr>
<tr>
<td>5.0</td>
<td>0.45</td>
<td>20</td>
<td>80</td>
<td>7</td>
</tr>
<tr>
<td>5.75</td>
<td>0.45</td>
<td>20</td>
<td>80</td>
<td>linear</td>
</tr>
<tr>
<td>6.00</td>
<td>0.45</td>
<td>100</td>
<td>0</td>
<td>linear</td>
</tr>
<tr>
<td>7.00</td>
<td>0.45</td>
<td>100</td>
<td>0</td>
<td>linear</td>
</tr>
</tbody>
</table>
Table II: Antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>1st Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
<th>2nd AB</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.GM1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>rb</td>
<td>1:20</td>
<td>Matreya</td>
<td>Anti-rb IgG AF488</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>2.Villin</td>
<td>g</td>
<td>1:5</td>
<td>Santa Cruz</td>
<td>Anti-g IgG AF546</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>1.GM1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>m</td>
<td>1:10</td>
<td>Seikagaku</td>
<td>Anti-m IgM AF488</td>
<td>g</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>2.Clathrin</td>
<td>rb</td>
<td>1:100</td>
<td>Millipore</td>
<td>Anti-rb IgG AF546</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>1.Rab11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>m</td>
<td>1:20</td>
<td>Millipore</td>
<td>Anti-m IgG AF488</td>
<td>d</td>
<td>1:500</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>2.GM1</td>
<td>rb</td>
<td>1:20</td>
<td>Matreya</td>
<td>Anti-rb IgG AF546</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>1.E-cadherin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>m</td>
<td>1:25</td>
<td>BD</td>
<td>Anti-m IgG AF488</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>2.Villin</td>
<td>g</td>
<td>1:5</td>
<td>Santa Cruz</td>
<td>Anti-g IgG AF546</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>1.β-catenin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>m</td>
<td>1:20</td>
<td>BD</td>
<td>Anti-m IgG AF488</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>2.Villin</td>
<td>g</td>
<td>1:5</td>
<td>Santa Cruz</td>
<td>Anti-g IgG AF546</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Lamp1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>rb</td>
<td>1:50</td>
<td>Acris</td>
<td>Anti-rb IgG AF488</td>
<td>d</td>
<td>1:200</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

d, donkey; g, goat; m, mouse; rb, rabbit; <sup>a</sup>frozen sections; <sup>b</sup>paraffin sections;
Table III: Antibodies used for immune electron microscopy.

<table>
<thead>
<tr>
<th>1st Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
<th>2nd AB</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA1</td>
<td>rb</td>
<td>1:100</td>
<td>Acris</td>
<td>Prot.-A gold 10nm</td>
<td>-</td>
<td>1:70</td>
<td>CMC</td>
</tr>
<tr>
<td>GM1</td>
<td>rb</td>
<td>1:20</td>
<td>Matreya</td>
<td>Prot.-A gold 10nm</td>
<td>-</td>
<td>1:70</td>
<td>CMC</td>
</tr>
<tr>
<td>1.GM1</td>
<td>m</td>
<td>1:20</td>
<td>Seikagaku</td>
<td>Anti-m IgG/M 10nm gold</td>
<td>g</td>
<td>1:30</td>
<td>Aurion</td>
</tr>
<tr>
<td>2.GM1</td>
<td>rb</td>
<td>1:300</td>
<td>Millipore</td>
<td>Prot.-A gold 5nm</td>
<td>-</td>
<td>1:80</td>
<td>CMC</td>
</tr>
<tr>
<td>1.Rab11</td>
<td>m</td>
<td>1:10</td>
<td>Millipore</td>
<td>Anti-m IgG/M 10nm gold</td>
<td>g</td>
<td>1:30</td>
<td>Aurion</td>
</tr>
<tr>
<td>2.GM1</td>
<td>rb</td>
<td>1:20</td>
<td>Matreya</td>
<td>Prot.-A gold 5nm</td>
<td>-</td>
<td>1:80</td>
<td>CMC</td>
</tr>
</tbody>
</table>

g, goat; m, mouse; rb, rabbit; frozen sections
Table IV: Primary AB’s used for Western blot.

<table>
<thead>
<tr>
<th>1&lt;sup&gt;st&lt;/sup&gt; Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caveolin-1</td>
<td>m</td>
<td>1:1000</td>
<td>BD</td>
</tr>
<tr>
<td>Clathrin</td>
<td>rb</td>
<td>1:100</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Rab4</td>
<td>rb</td>
<td>1:500</td>
<td>GeneTex</td>
</tr>
<tr>
<td>Rab5</td>
<td>rb</td>
<td>1:200</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Rab11</td>
<td>m</td>
<td>1:1000</td>
<td>BD</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>m</td>
<td>1:2000</td>
<td>BD</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>m</td>
<td>1:3000</td>
<td>BD</td>
</tr>
<tr>
<td>Lc3</td>
<td>rb</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>β-actin</td>
<td>rb</td>
<td>1:500</td>
<td>Santa Cruz</td>
</tr>
</tbody>
</table>

g, goat; m, mouse; rb, rabbit; 2<sup>nd</sup> antibody: g anti-m or g anti-rb IgG, 1:1000, Santa Cruz
Table V: mRNA expression analysis of intestinal proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ugcg Contr.</th>
<th>Ugcg f/f/VilCre</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabp2</td>
<td>0.684 ± 0.177</td>
<td>0.543 ± 0.081</td>
<td>0.4612</td>
</tr>
<tr>
<td>CD36</td>
<td>0.064 ± 0.029</td>
<td>0.035 ± 0.006</td>
<td>0.2913</td>
</tr>
<tr>
<td>Abcg5</td>
<td>1.509 ± 0.208</td>
<td>1.914 ± 0.362</td>
<td>0.3972</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.701 ± 0.336</td>
<td>3.483 ± 0.701</td>
<td>0.0738</td>
</tr>
<tr>
<td>Dgat1</td>
<td>0.060 ± 0.023</td>
<td>0.048 ± 0.011</td>
<td>0.6409</td>
</tr>
<tr>
<td>Mttp</td>
<td>0.071 ± 0.037</td>
<td>0.142 ± 0.033</td>
<td>0.1818</td>
</tr>
<tr>
<td>Sglt</td>
<td>0.085 ± 0.043</td>
<td>0.113 ± 0.028</td>
<td>0.5716</td>
</tr>
<tr>
<td>Glut5</td>
<td>0.159 ± 0.034</td>
<td>0.132 ± 0.043</td>
<td>0.6489</td>
</tr>
<tr>
<td>Pept1</td>
<td>0.070 ± 0.038</td>
<td>0.035 ± 0.007</td>
<td>0.3117</td>
</tr>
<tr>
<td>Aqp8</td>
<td>0.759 ± 0.198</td>
<td>0.010 ± 0.006</td>
<td>0.0035 **</td>
</tr>
</tbody>
</table>
Figure 5

A

Duodenum  Jejunum  Ileum  Colon

Ugcg-contr.  Ugcg ff/VilCre

B

Ki67  ALP  Villin

Ugcg-contr.  Ugcg ff/VilCre
Figure 9

A

<table>
<thead>
<tr>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Duodenum" /></td>
<td><img src="image" alt="Jejunum" /></td>
<td><img src="image" alt="Ileum" /></td>
<td><img src="image" alt="Colon" /></td>
</tr>
<tr>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Ki67</th>
<th>EDU</th>
<th>ALP</th>
<th>Villin</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Ki67" /></td>
<td><img src="image" alt="EDU" /></td>
<td><img src="image" alt="ALP" /></td>
<td><img src="image" alt="Villin" /></td>
</tr>
<tr>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
<td>Ugcg-ff/fv/VIcCreERT2</td>
</tr>
</tbody>
</table>
Glycosphingolipids are essential for intestinal endocytic function
Richard Jennemann, Sylvia Kaden, Roger Sandhoff, Viola Nordström, Shijun Wang, Martina Volz, Sylvie Robine, Nicole Amen, Ulrike Rothermel, Herbert Wiegandt and Hermann-Josef Gröne

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