METHODS

Mice

Previously, we established a conditional, inducible *Gata4* mutation model (Bosse et al. 2006) that consists of mice homozygous for the *Gata4*^{flox} allele (Pu et al. 2004) and heterozygous for a transgene expressing the tamoxifen-activated CRE recombinase under the control of the villin promoter (el Marjou et al. 2004). In the *Gata4*^{flox} allele, exon 2 is flanked by LoxP sites, and CRE-mediated recombination of the inserted LoxP sites results in excision of exon 2, and the synthesis of a truncated form of GATA4 devoid of its activation domains. This causes GATA4 activity in the small intestine to be reduced but not eliminated (Model 1, *G4Δex2*, supplementary fig S1) (Bosse et al. 2006). To inducibly delete *Gata4* in the mature small intestine, we established a new model (Model 2, *G4ap*, supplementary fig S1) using a previously published modification of the *Gata4* allele (Zhou et al. 2008). In Model 2, the coding region for the endogenous *Gata4* gene was replaced by a cassette encompassing a *Gata4* cDNA with an amino terminal epitope tag derived from the influenza HA protein (*HA-Gata4*), which is followed by a Stop repressor sequence and a placental alkaline phosphatase (PLAP) expression cassette. LoxP sites were inserted before the HA-tagged *Gata4* cDNA and after the Stop repressor cassette. CRE-mediated recombination results in excision of *HA-Gata4* and the Stop repressor cassette, and synthesis of PLAP under the control of the endogenous *Gata4* promoter. In both models, conditional recombination was targeted to the small intestinal epithelium by the Villin*Cre*ER^{T2} transgene (el Marjou et al. 2004; Bosse et al. 2006; Beuling et al. 2008). To induce recombination, and also to control for potential tamoxifen effects, all animals were treated with one intraperitoneal injection of tamoxifen (Sigma-Aldrich, St. Louis, MO) (100μl, 10mg/ml) per day for 5 consecutive days as described (Bosse et al. 2006; Beuling et al. 2008). Tissue was collected 14-17 days after the last tamoxifen injection. All mice were previously backcrossed into the C57BL/6 genetic background and only adult (6-12 wk of age)

males were used for study. Mice were fed ad libitum standard rodent chow containing approximately 5% w/w fiber and 60% carbohydrate, 28% protein, and 12% fat (% of calories), unless indicated otherwise. Genotypes were confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using previously validated primers (supplementary fig S2) (el Marjou et al. 2004; Pu et al. 2004; Bosse et al. 2006; Ma et al. 2008; Zhou et al. 2008). The genotypes for all control and test mice are indicated in supplementary table S1. Approval was obtained from the Institutional Animal Care and Use Committee.

RNA isolation and reverse transcriptase polymerase chain reaction

Mice were dissected and RNA was isolated from liver and small intestine using the RNeasy kit (Qiagen) as described previously (Bosse et al. 2006). Intestinal tissue samples 1 to 5 (approximately 1.0 cm in length) are indicated as the gray boxes in fig 1a. The tissue samples were taken from the most proximal region adjacent to the pylorus (segment 1), the 25% mark (segment 2), the geometric center (segment 3, also indicated as jejunum), the 75% mark (segment 4), and the most distal region adjacent to the ileocecal junction (segment 5, also indicated as ileum).

Messenger RNA (mRNA) abundances were determined by semi-quantitative and real-time RT-PCR (Bosse et al. 2006) using validated primer pairs (supplementary figs S2 and S3) designed with Beacon Designer™ software (PREMIER Biosoft International, Palo Alto, CA). Semi-quantitative RT-PCR was terminated in the linear range of amplification, and real-time RT-PCR was carried out using an iCycler and iQ SYBR GreenSupermix (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was measured for each sample and used to normalize the data.

Protein extracts and immunoblotting

After intestinal samples were obtained for RNA isolation, brush border membrane vesicles (BBMV) were prepared as described by Kessler et al (Kessler et al. 1978) on the remaining four quarters of intestine (fig 1a). Intestinal segments were placed on a glass plate on a bed of wet ice, rinsed in ice cold PBS containing protease inhibitors, and cut longitudinally along the antimesenteric border. Epithelial cells were then collected by scraping the mucosa with a glass microscope slide. The scrapings were suspended in 20 mL of buffer A (50 mmol/L mannitol, 2 mmol/L Tris, pH 7.2, 50 μg/mL benzamidine) and homogenized on ice with 10 strokes using a ground glass homogenizer. Solid $CaCl_2$ was added to a final concentration of 10 mmol/L, incubated on ice for 15 min, and centrifuged (3000 relative centrifugal force (rcf) for 10 min at 4°C). The supernatant was centrifuged again (24330 rcf for 20 min at 4°C) and the pellet was resuspended in 5 mL of buffer B (300 mmol/lL mannitol, 10 mmol/L Tris, pH 7.2, 50 ug/ml benzamidine). The homogenate was again centrifuged (24330 rcf for 30 min at 4°C), the pellet was resuspended in 200 µL of buffer B, and the concentration of proteins was quantified as described (Kalb and Bernlohr 1977). The proteins were stored at -80°C until used for immunoblotting.

Western analysis was conducted as described previously (van Wering et al. 2004) using 30 µg of BBMV. Primary antibodies included rabbit anti-ASBT (Wong et al. 1995) (1:3750) and mouse anti-β-actin (1:4000, Sigma). After probing with rabbit anti-ASBT, membranes were stripped and re-probed using mouse anti-β-actin (1:4000, Sigma). Horseradish peroxidase-linked secondary antibodies and chemiluminescence solution (Pierce West Femto Kit) were used to visualize the signals.

Bile acid measurements

Bile acid transport in everted gut sacs. The small intestine was divided into quartiles I – IV (depicted as white boxes in fig 1a), and the mucosal-to-serosal bile acid transport was measured using everted gut sacs as previously described (Rao et al. 2008). After removal of adherent fat, each segment was weighed, gently everted, filled with oxygenated Krebs Ringer Buffer (KRB), and closed using suture and weighed again. The closed sacs were incubated at 37^{o}C for 30 min in oxygenated KRB containing 25 μM [^{3}H]taurocholate (final specific activity = 55 mCi/mmol; Perkin-Elmer) and inulin carboxylic acid (2 mCi/mmol; Perkin-Elmer). After incubation, the sacs were removed and weighed, and the serosal fluid was recovered. The empty sacs were solubilized in sodium hydroxide, and aliquots of mucosal fluid, serosal fluid, and gut sac tissue extract were taken for radioactivity measurements. The amount of inulin [^{14}C]carboxylic acid associated with the sac or serosal fluid was similar for the different segments and genotypes, and was used to correct the sac-associated [^{3}H]taurocholate data for leakage and paracellular movement.

Bile acid abundance and composition in stools and tissues. Bile acid abundance and composition were determined in fecal material and in tissues. For total fecal bile acid excretion, mice were weighed, individually housed in wire-bottom cages for 72 h beginning 14 days after the completion of tamoxifen treatment, and the feces were collected. Bile acids were extracted from the fecal samples as described (Turley et al. 1996) and aliquots of the final methanol extract were taken to measure the radioactivity of the [^{14}C]cholic acid recovery control and to measure the bile acid content using an enzymatic assay (Mashige et al. 1981). For measurement of bile acid concentrations in segmental luminal contents, the small intestine was removed and divided into four quarters, the segments were cut longitudinally, and the luminal content of each segment was collected as described (Repa et al. 2002). Briefly, an internal standard (~50,000 dpm of [^{14}C]cholic acid) is added to the luminal contents. The samples are air-dried and extracted twice with methanol. Aliquots of the methanol extract are taken to

measure the radioactivity and to measure the bile acid content using an enzymatic assay (Mashige et al. 1981). Tissue-associated bile acids in each of the four quarters of small intestine were also measured. Briefly, an internal standard (~200,000 dpm of [^{14}C]cholic acid) is added to the tissue. The tissue is then minced and extracted twice with ethanol at 65° C. The ethanol extracts are pooled, dried, and resuspended in methanol. Aliquots of the methanol extract are taken to measure the radioactivity and to measure the bile acid content using an enzymatic assay (Mashige et al. 1981).

For bile acid pool measurements, mice were fasted for 5 h and the liver, gall bladder and small intestine were collected and bile acid extracted as described (Schwarz et al. 1998). Briefly, approximately 2.7 nmoles of nor-deoxycholic acid is added to the tissue as an internal standard. The tissue is homogenized in ethanol, and extracted twice with ethanol at 65°C. The ethanol extracts are dried, resuspended in water and applied to a C18 reverse phase column. The bile acids are eluted with methanol and analyzed using high-performance liquid chomatography and an evaporative light scatter detector (Alltech ELSD 800) (Torchia et al. 2001). Bile acids were identified and quantified by comparison to known amounts of authentic standards purchased from Steraloids (Newport, RI). In selected experiments, bile acid composition was also determined by high performance liquid chromatography for the luminal contents. The bile acid hydrophobicity index was calculated according to Heuman (Heuman 1989).

Surgery

Ten to twelve week old male mice were transferred to a liquid rodent diet (Bio-serve Inc, Frenchtown, NJ) two days prior to surgery. Mice were weighed and sham or ICR surgery was performed as previously described (Dekaney et al. 2007) (supplementary fig S4). In mice that underwent ICR, ~10 cm of ileum proximal to the ileocecal junction and cecum were removed, and the colon was anastomosed to the remaining small intestine. *Asbt* mRNA abundance in the

most proximal one-cm of the resected small intestine was low or undetectable, indicating that all of the native *Asbt*-expressing tissue was removed. In mice that underwent sham operations, transection and anastomosis occurred ~10 cm proximal to the ileocecal junction. Mice were given 2 ml intraperitoneal zosyn (100 mg/kg, Wyeth Pharmaceuticals, Philadelphia, PA) in phosphate-buffered saline and the abdomen was closed. The mice were incubated at 27°C for 2-4 h after surgery, and then transferred to their normal housing, with access to liquid diet. After a week of recovery, the mice were transitioned to solid food. Three weeks after surgery, mice were treated with tamoxifen and tissue was collected as described above. All mice treated with tamoxifen were fully recovered from surgery as indicated by normal activity, weight gain to pre-surgical body weights, and formed stools. The overall mortality rate was 59%; virtually all mice that did not recover from surgery died of obstruction.

Statistical analyses

Data are expressed as mean ± SD. Statistically significant differences were determined by the two-tailed Student's *t* test or analysis of variance followed by the Tukey-Kramer multiple comparison test. Differences were considered statistically significant at $P<0.05$.

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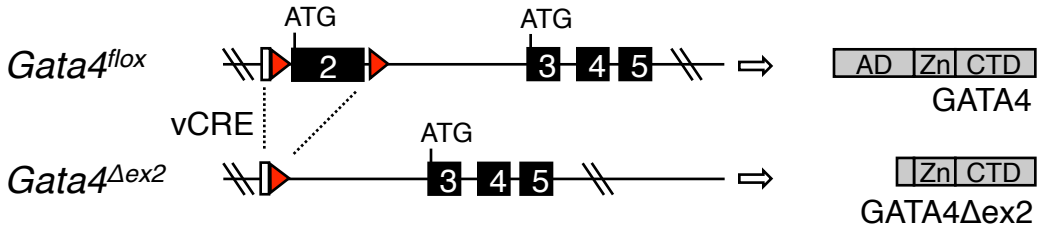
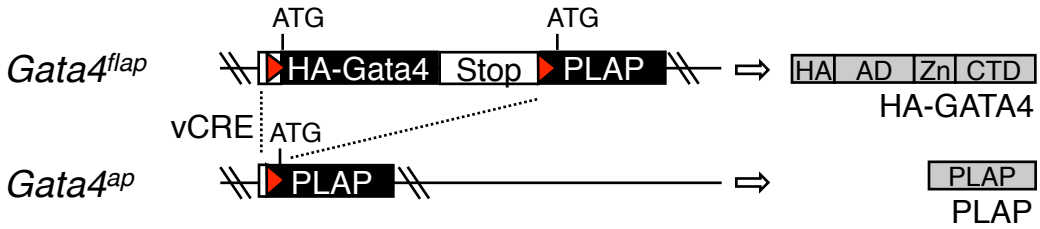
Model 1: *G4Δex2*



Model 2: *G4ap*



Supplementary figure S1 Schematic representation of conditional *Gata4* recombination models. In Model 1, the *Gata4flox* allele, in which exon 2 is flanked by loxP sites (red arrowheads), expresses WT GATA4 under control of the endogenous *Gata4* promoter. After intestine-specific CRE-excision, mediated by the Villin*Cre*ERT2 transgene (vCRE), the *Gata4flox* allele is recombined (*Gata4Δex2*), resulting in deletion of the GATA4 activation domains and the expression of a truncated form of *Gata4* (GATA4Δex2) in which an in-frame ATG in exon 3 is utilized. In Model 2, the *Gata4flap* allele contains a cDNA that encodes GATA4 tagged with influenza hemaglutinin (HA). After intestine-specific CRE excision, the *Gata4flap* allele is recombined (*Gata4ap*), resulting in excision of *HA-Gata4* and the expression of placental alkaline phosphatase (PLAP) under the control of the endogenous *Gata4* promoter. Filled boxes with numbers indicate exons. Grey boxes indicate peptides. AD, activation domains; Zn, zinc fingers; CTD, C-terminal domain.

Genotyping primers:

Cre: F: 5’-CGTATAGCCGAAATTGCCAG-3’

R: 5’-CAAAACAGGTAGTTATTCGG-3’

Gata4:

Flox1: F: 5’-GGTGGTTTCATTTGCTGTGTGGAAG-3’

Flox2: R: 5’-AATCGTGCGGGAGGGCGGACTCTATTC-3’

Flap1: F: 5’-CTTCGACAGCCCAGTCCTGCAC-3’

Flap2: R: 5’-GCACAGGTAGTGTCCCGTCCCATC-3’

Flap3: R: 5’-ACCAGTAGGCTACCCAGACATTGCTGG-3’

Semi-quantitative RT-PCR primers:

Gata4: F: 5’-AACCCTGGAAGACACC-3’

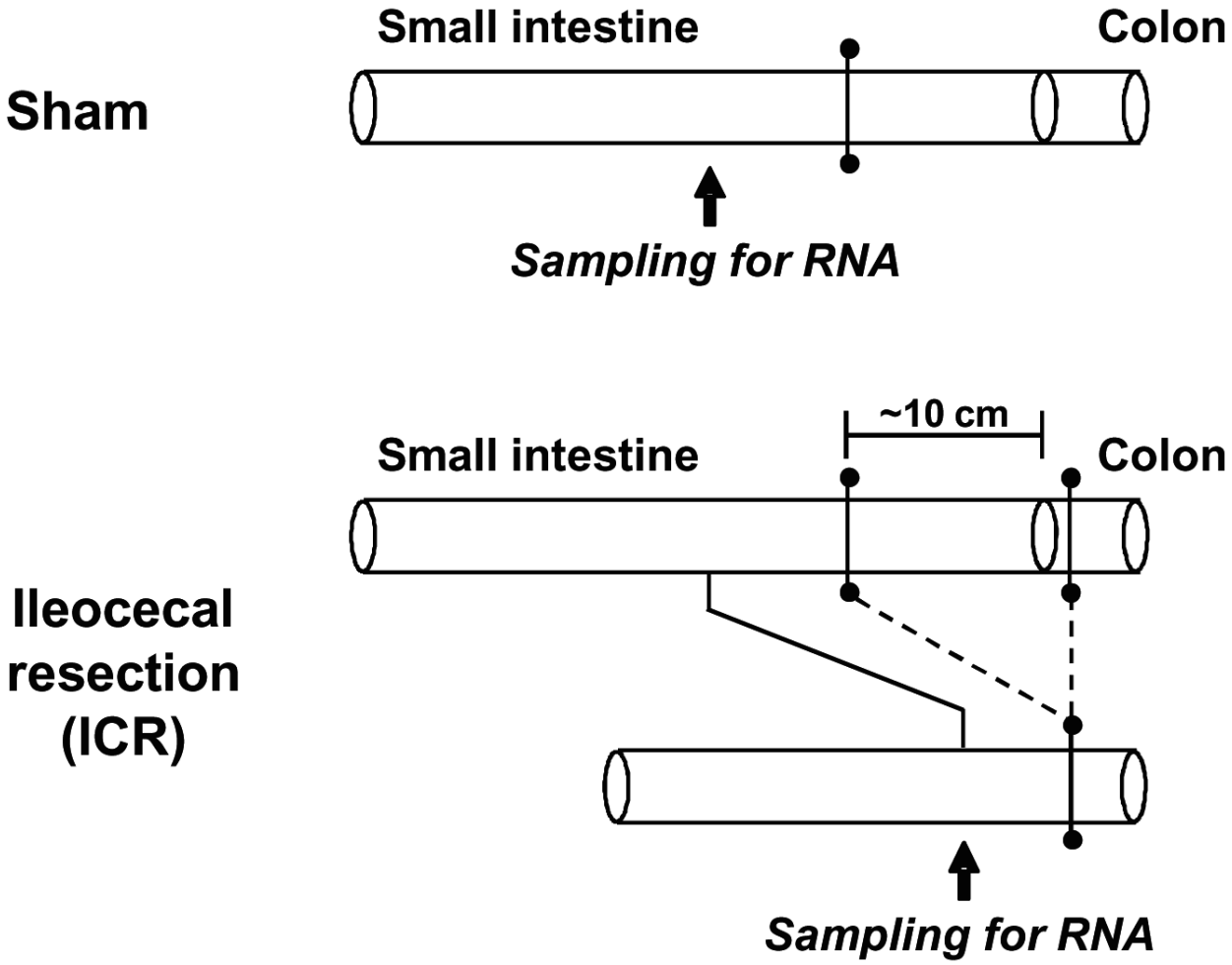
R: 5’-CATTGCTGGAGTTACCG-3’

Gapdh: F: 5’-GCCTTCCGTGTTCCTACCC-3’

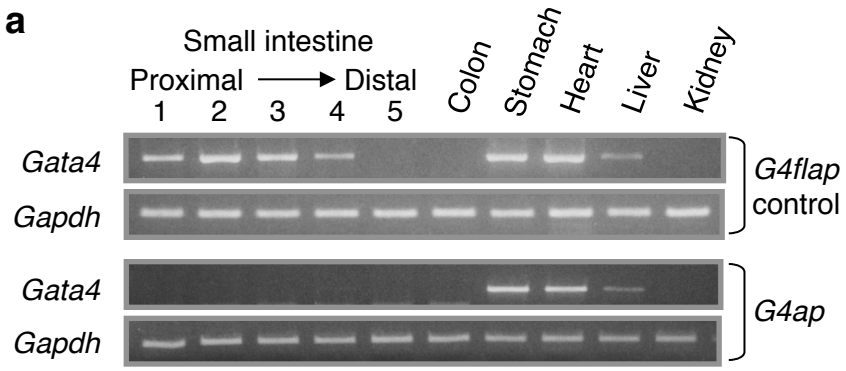
R: 5’-TGCCTGCTTCACCACCTTC-3’

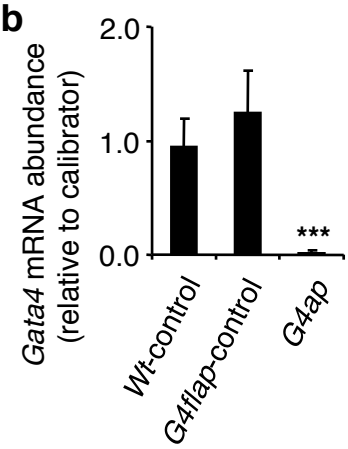
Real time RT-PCR primers:

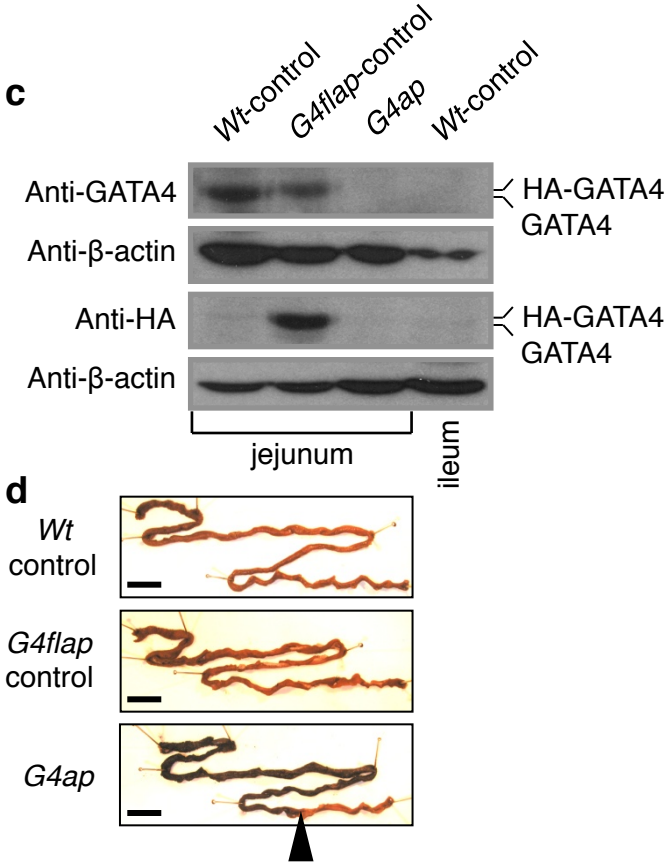
Asbt:	F: 5'-TTGCCTCTTCGTCTACACC-3'
	R: 5'-CCAAAGGAAACAGGAATAACAAG-3'
Ilbp:	F: 5'-TGGCAAAGAATGTGAAATG-3'
	R: 5'-CTCCGAAGTCTGGTGATAG-3''
Ostα:	F: 5'-TACAAGAACACCCTTTGCCC-3'
	R: 5'-CGAGGAATCCAGAGACCAAA-3'
Ostβ:	F: 5'-GTATTTTCGTGCAGAAGATGCG-3'
	R: 5'-TTTCTGTTTGCCAGGATGCTC-3'
Fgf15:	F: 5'-CCTGTTGTGTTAGTGGCTA-3'
	R: 5'-GAGTAAGTTCCCTATTAGTGG-3'
Cyp7a1:	F: 5'-GCCAGAGTCCAATGCTTAGG-3'
	R: 5'-ATCTCACACCAGGGTAAATGC-3'
Cyp7b1:	F: 5'-TAGCCCTCTTTCCTCCACTCATA-3'
	R: 5'-GAACCGATCGAACCTAAATTCCT-3'
Cyp8b1:	F: 5'-GCCTTCAAGTATGATCGGTTCCT-3'
	R: 5'-GATCTTCTTGCCCGACTTGTAGA-3'
Cyp27a1:	F: 5'-GGAGGGCAAGTACCCAATAAGA-3'
	R: 5'-TGCGATGAAGATCCCATAGGT-3'
Shp:	F: 5'-GCAACAGGAGGCTCACTG-3'
	R: 5'-ATGATAGGGCGGAAGAAGAG-3'
Gata4:	F: 5'-TTTGAGCGAGTTGGG-3'
	R: 5'-GAATGCGGGTGTGC-3'
Lph:	F: 5'-CAGCGATGCCCACAGGAAAG-3'
	R: 5'-ACGGAGCCCTTGACGAGAG-3'
Gapdh:	F: 5'-GCCTTCCGTGTTCCTACCC-3'
	R: 5'-TGCCTGCTTCACCACCTTC-3'

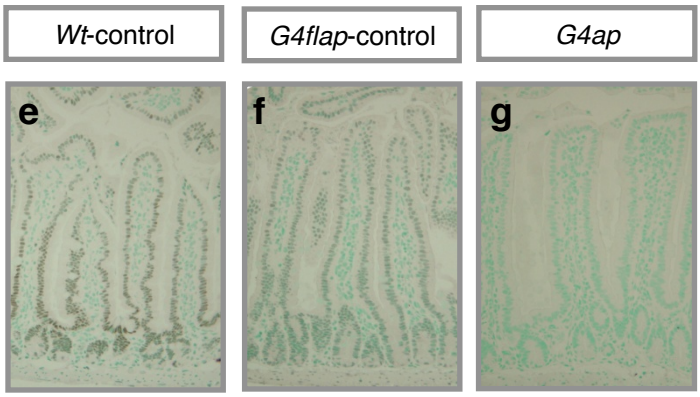


Supplementary figure S4. Schematic representation of sham and ileocecal resection (ICR) surgery. Arrows depict primary sampling location for RNA analyses.

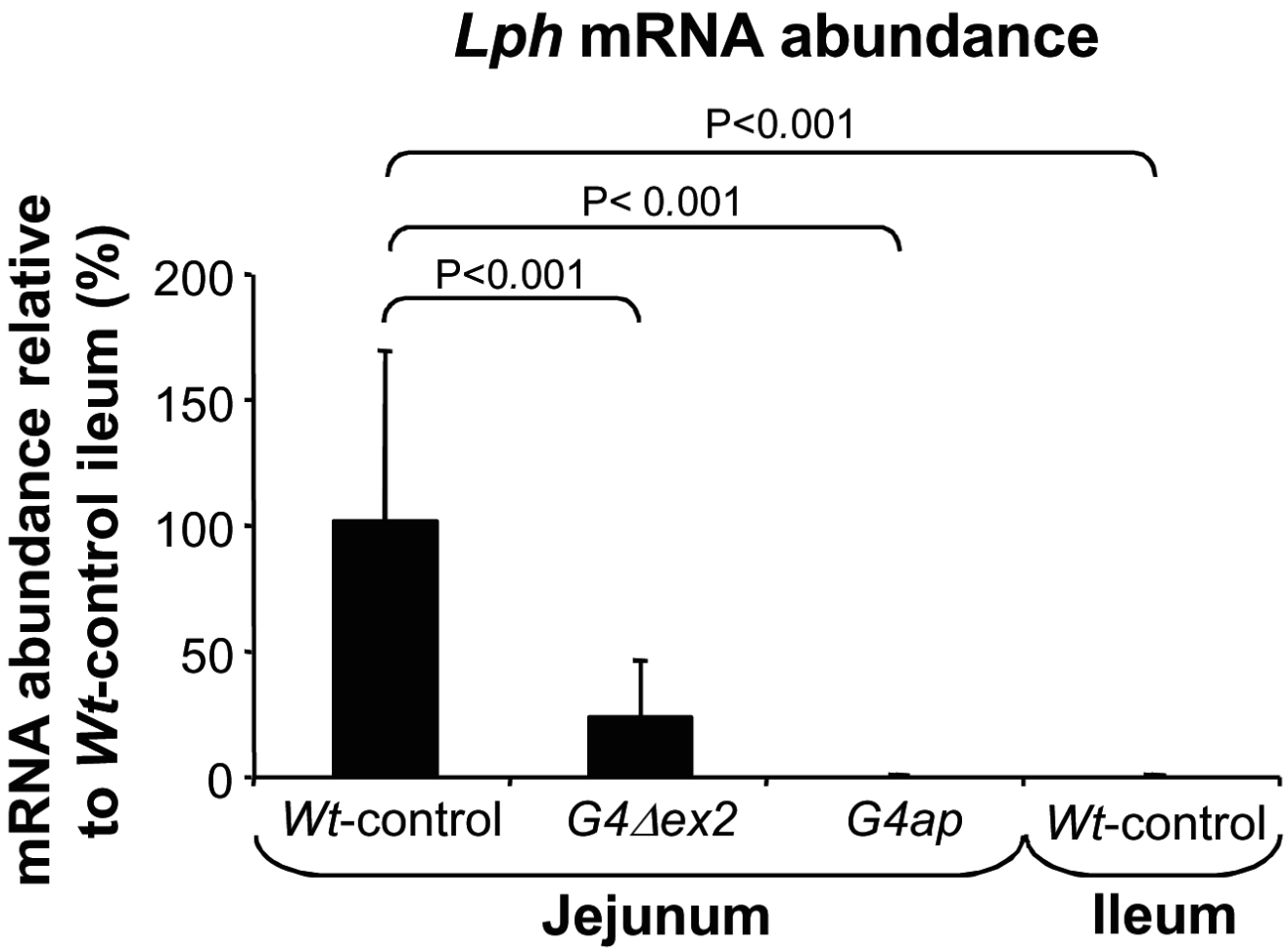








Supplementary figure S5 Villin*Cre*ERT2-mediated recombination of the *Gata4flap* allele results in conditional deletion of *Gata4* in the mouse small intestine. (a) Semi-quantitative RT-PCR analysis reveals a normal *Gata4* mRNA expression pattern in a representative *G4flap*-control mouse, and specific deletion of *Gata4* mRNA in the small intestine of a representative *G4ap* mouse. Small intestinal segments 1-5 represent 5 equidistant segments along the length of small intestine, as described in Materials and Methods. (b) Real-time RT-PCR analysis of mouse jejunum (segment 3) shows that *Gata4* mRNA is significantly reduced in *G4ap* mice as compared to *Wt*-control and *G4flap*-control jejunum. ***P<0.001, as compared to all other groups as determined by ANOVA and the Tukey-Kramer multiple comparison test, n=6 in each group. RNA from jejunum of a *Wt*-control mouse was used as the calibrator. (c) Western analysis of nuclear extracts isolated from small intestine demonstrates that GATA4 and HA-GATA4 are expressed in jejunum of *Wt*-control and *G4flap*-control mice, respectively, but neither is expressed in jejunum of *G4ap* mice. *Wt*-control ileum is shown as a negative control. (d) Whole mount staining reveals that placental alkaline phosphatase (PLAP) is expressed in the proximal 85 ± 2% (n=3) of small intestine of *G4ap* mice, but is not expressed in *Wt*-control and *G4flap*-control mice, confirming that the *Gata4flap* alleles are recombined specifically in the small intestine of *G4ap* mice. Arrowhead indicates the demarcation between the PLAP-expressing and PLAP-non-expressing region. (e-g) Immunostaining for GATA4 of *Wt*-control (e), *G4flap*-control (f) and *G4ap* (g) jejunum shows absence of GATA4 in the *G4ap* mice



Supplementary figure S6 Real-time RT-PCR of lactase phlorizin hydriolase (*Lph*) mRNA in *Wt*-control jejunum (segment 3) and ileum (segment 5), *G4Δex2* jejunum, and *G4ap* jejunum, shows a partial and more complete transformation to wild-type ileal levels in the jejunum of *G4Δex2* and *G4ap* mice, respectively. P<0.001, as compared to *Wt*-control jejunum, n= 6-10 in each group.

Supplementary table S1

Nomenclature for the different mouse lines used in this study.

Nomenclature	***Gata4* alleles**	**Villin*Cre*ER^{T2}**	**Tamoxifen**
Wt-control	*Gata4*$^{wt/wt}$	positive	treated
G4Δex2	*Gata4*$^{flox/flox}$	positive	treated
G4ap	*Gata4*$^{flap/flap}$	positive	treated

Supplementary table S2

Body weights, plasma triglycerides and plasma cholesterol in *Wt*-control and *G4ap* mice.

	***Wt*-control**	***G4ap***
Body weights (g)	28.0 ± 1.8	25.7 ± 2.4
Plasma triglycerides (mg/dl)	64 ± 25	87 ± 46
Plasma cholesterol (mg/ml)	45 ± 13	39 ± 10

Data are expressed as mean ± SD (n=4-6 in each group).