Supplemental methods

Animals

Animal experiments were conducted under the approval of the animal care committee of the Royal Netherlands Academy of Arts and Sciences, (permit number HI 11.2503). All experiments and sample collection were performed on four- to five-month-old male mice. In vitro L-cell experiments and FAC-sorting was done on intestinal samples from GLU-Venus mice expressing YFP under transcriptional control of the proglucagon promotor (14). In vitro K-cell experiments were done on GIP-Venus mice expressing YFP under transcriptional control of the gastric inhibitory peptide (GIP) promotor (18). Both strains were bred on a C57BL/6 background in the Hubrecht animal facility. C57BL/6 mice for in vivo testing were obtained from the Hubrecht animal facility. Mice on normal diet, referred to as lean mice, were fed regular chow ad libitum. Mice on high fat diet (HFD) were given diet containing 60% Fat (824054 RM AFE (M) from Tecnilab-BMI, Someren, The Netherlands) at the age of 4-6 weeks for 12-14 weeks.

Human tissue

Surgically resected human intestinal tissues were obtained from the Diakonessen Hospital Utrecht. Informed consent was provided by all subjects. The study was approved by the ethical committee of the Diakonessen Hospital Utrecht.

Crypt isolation and organoid handling

Mouse and human ileal crypts were isolated as described previously (25, 26) and seeded into Matrigel [BD Biosciences], in which they grew into organoids (15, 25). Crypts were cultured in Advanced DMEM/F12 containing 100 U/ml penicillin/streptomycin, 10 mM HEPES, 2 mM Glutamax, supplements N2 (1X) and B27 (1X), 50ng/ml murine or human EGF (for mouse and

human crypt culture, respectively) [all from Life Technologies], 1 μM N-acetylcysteine [Sigma-Aldrich], and murine Noggin and human R-spondin-1 both as 10% conditioned medium (26). The culture medium for human crypts was additionally supplemented with 50% Wnt-3A conditioned medium (25), 10 nM gastrin [Sigma-Aldrich], 10mM nicotinamide [Sigma-Aldrich], 500 nM inhibitor of transforming growth factor beta type I receptor ALK5 kinase (A83-01) [Tocris], and 10 μM p38 mitogen-activated protein kinase inhibitor (SB202190) [Sigma-Aldrich] (25). The medium was refreshed every 2-3 days. Analysis of human organoids was done on 8-day-old cultures. Analysis of mouse organoids was done on 0.5- to 1-month-old cultures. For passage, every 5 days organoids were removed from the Matrigel, mechanically dissociated using a glass Pasteur pipette, pelleted and re-plated in fresh Matrigel in 24-well plates with 1:4 splitting ratio. To test the effect of DBZ on L-cell differentiation, 0.1 nM – 50 μM DBZ from DMSO-diluted stock was added to the culture medium on day 2 after splitting. The final DMSO content in the culture medium was 1%. Control organoids were treated with regular medium containing 1% DMSO. Short chain fatty acids (SCFA) were applied continuously as a combination of 5 mM acetate, 1 mM propionate and 1 mM butyrate.

qPCR analysis

Total RNA was extracted from organoids using Trizol (Invitrogen) and reverse-transcribed with Fermentas kit. Quantitative real-time PCR was performed on a real-time PCR System (Bio-Rad) using SYBR green assays. We tested Beta 2 microglobulin (B2M) as endogenous control gene (13). Markers of intestinal cell types and transcription factors were analysed in whole organoids. Primer sequences were published earlier (13).

***In vivo* DBZ treatment**

Mice were injected with either 50 mg/kg of DBZ once, or 10 mg/kg DBZ on two consecutive days, or 5 mg/kg DBZ on 4 consecutive days. DBZ was made into a suspension in sterile saline solution with 0.1% of hydroxypropylmethylcellulose and given intraperitoneally the volume of 10 µl/g of body weight (7). All testing was done 48 h after the beginning of DBZ treatment for 50 mg/kg group, 96 h for 2X10 mg/kg groups and 120 h for 5x4 mg/kg group. Measurement of GLP-1 and insulin after oral glucose gavage was performed at the time of maximal increase in L-cell density in a fragment of distal ileum. This fragment was used for L-cell density assessment as the naturally most L-cell enriched region of the small intestine. HFD mice and the lean mice were given 10x2 mg/kg because this dose was non-toxic and efficient in enrichment of L-cells (Results section). Vehicle controls for both diet groups were given saline solution with 0.1% hydroxypropylmethylcellulose in the same volume. After the tests, mice were sacrificed by cervical dislocation and their duodenum, ileum and colon was collected for L-cell count and morphological analysis.

Histology and immunostaining

Fragments of different parts of intestine were collected 96 h after DBZ treatment, fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 3 to 6 µm for hematoxylin and eosin (H&E) and PAS staining to identify enterocytes and Goblet cells, respectively. For immunostaining, frozen 10 µm sections were used as described earlier (13). The primary antibodies used were rabbit anti-GLP-1 (1:500, Phoenix Pharmaceuticals, cat no H-028-11) to identify L-cells, anti-lysozyme (1:500; DAKO) to identify Paneth cells, goat ChA (1:200, Santa Cruz, cat. no. sc-1488) as pan-endocrine marker, goat anti-serotonin (1:500, Immunostar, cat. No. 20079) as enterochromaffine cells marker, rabbit anti-GIP (1:500, Phoenix Pharmaceuticals, cat

no. H-027-02). Images were acquired on a confocal laser-scanning microscope (Leica, SP5) using LAS software. The number of different types of cells in the intestinal epithelium was determined based on the cell count per 1 millimeter of intestinal mucosa length in 30-40 longitudinally sectioned crypts and villi in 6 transverse sections.

Organoid GLP-1 and GIP secretion assay

Ileum organoids from human and Glu-Venus mouse were used for GLP-1 secretion test and duodenum organoids from GIP mouse were used for GIP secretion assay. Basic medium for static incubations of small intestine organoids contained Hanks Buffered Salt Solution (Life technologies) supplemented with 10mM HEPES, 0.1% fatty acid-free bovine serum albumin and no glucose, pH 7.4 (13). Organoids from 24-well plates were collected in 1.5 ml Eppendorf tubes (one well per tube) and incubated for two hours in basic medium. Organoids were then washed and incubated in 50 µl of basic medium containing DPP4 inhibitor (Millipore) for 1 hour, followed by incubation in 50 µl of 10 mmol/l glucose in basic medium with DPP4 inhibitor for 1 hour. The supernatant was collected from basal and stimulated fractions. GLP-1 concentrations were determined by Multi-Species GLP-1 total ELISA (Millipore) and GIP concentrations were determined by Mouse GIP ELIZA, Active form Assay Kit (IBL). Organoids were then lysed in CelLytic M buffer (Sigma-Aldrich) for DNA extraction (28) and quantification using the PicoGreen kit (Invitrogen) to normalize GLP-1 and GIP release.

Villus and crypt L-cell isolation and FAC-sorting

For comparison of control and DBZ-treated L-cells, freshly isolated small intestine crypts from GLU-Venus mice were dissociated with 0.05% trypsin-EDTA (Life Technologies) at 37°C into

single cells 96 h after they were injected with 10X2 mg/kg of DBZ or vehicle. Then they were centrifuged in 4% FBS in PBS at 300g and immediately sorted by flow cytometry. For mixed crypt and villus samples, villi were first collected from intestinal fragments prior to crypt isolation, washed in PBS containing 10% FBS, dissociated into single cells with 0.05% trypsin-EDTA and then combined with the crypt single cell sample. YFP-positive cells were separated by flow cytometry as described previously (14) into tubes containing Trizol for qPCR analysis or lysis buffer (Cell-Lytic M, Sigma) for hormone assay.

Assay for GLP-1 secretion in intestinal cultures

C57BL/6 mice were given an i.p. injection of DBZ (50mg/kg) or vehicle and killed by cervical dislocation after 48 h. Primary intestinal epithelial cells were isolated from the top half of the small intestine and cultured as previously described (29). At 24 h following seeding, a GLP-1 secretion assay was performed on the cells as previously described (29). The supernatant and lysate samples from each well were collected and total GLP-1 was assayed using a 'total-GLP-1' assay (Meso Scale Discovery, Gaithersburg, MD, USA).

Glucose tolerance test and acute insulin, GLP-1 and GIP response measurements

Mice were tested 96 h after the beginning of 2 X 10 mg/kg DBZ treatment. For oral glucose tolerance test (OGTT), glucose in the dose of 2g/kg of body weight was given to mice by oral gavage after 18-hour fasting with free access to water. Blood glucose was measured before and 5, 15, 30, 60, 90 and 120 minutes after the glucose challenge using a glucometer (FreeStyle Lite Blood Glucose Monitoring System, Abbot). In the experiments using the GLP-1 receptor

antagonist exendin 9-39, the vehicle-treated and DBZ-treated high fat diet mice received an intraperitoneal injection of 100 nmol/kg exendin 9-39 ten minutes before the oral glucose load. GLP-1, GIP and insulin levels were measured before and 5, 15, 30 and 60 minutes after the glucose challenge. For hormone measurements, 50 µl of blood was collected in a tube containing EDTA and DPP4 inhibitor (Millipore) and immediately centrifuged for plasma separation. Insulin was determined by Mouse Insulin enzyme-linked immunosorbant assay (Mercodia, Sweden), GLP-1 levels were measured by Multi-Species GLP-1 total ELISA (Millipore) and GIP concentrations were determined by Mouse GIP ELISA, Active form Assay Kit (IBL). Area under curve (AUC) for GLP-1, GIP and insulin were calculated during the first 15 minutes, and for glucose levels during 120 min by the Newton-Cotes method using IDL software. For the GLP-1 and GIP response, the AUC for GLP-1 and GIP were calculated without baseline subtraction, because we reasoned that absolute circulating levels of GLP-1 and GIP are the important parameters for potentiation of insulin response. For the insulin response, the $AUC_{insulin}$ was calculated with baseline subtraction by division because we expected marked insulin resistance in HFD mice and therefore the relative rise from basal is more important during a glucose challenge than absolute levels (30). AUC for glucose response was calculated with baseline subtraction because fasting levels were higher in HFD mice than in lean mice.

Insulin tolerance test

Insulin tolerance test was performed one day before OGTT after 2 h fasting, e.g. 72 h after starting DBZ treatment. Mice were given insulin intraperitoneally in the dose of 2U/kg of body weight. Glucose levels were measured before and 20, 40 and 60 min after the insulin injection. Inverted area under curve (30, 31) was calculated by Newton-Cotes method using IDL software.

Islet isolation and insulin secretion measurements

Islets were isolated from lean mice by collagenase (Sigma) digestion 48 h after treatment with 50 mg/kg DBZ or vehicle (control). Then islets were cultured overnight in RPMI Advanced (Invitrogen, Auckland, New Zealand) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/l L-glutamine, 1% penicillin–streptomycin and 15 mmol/l HEPES (27). All subsequent handling was performed in Hanks Buffered Salt Solution (Life technologies) supplemented with 10mM HEPES, 0.1% fatty acid-free bovine serum albumin, pH 7.4. Similar-sized islets (five islets per batch) were first incubated in 500 µl of HBSS buffer containing 3 mmol/l glucose for 30 min at 37°C. The medium was collected and islets were further incubated in 500 µl of HBSS containing 20 mmol/l glucose for 15 min for assessment of the first phase of insulin release and another 15 minutes in fresh 500 µl HBSS containing 20 mmol/l for the second phase. Insulin concentrations in the three fractions were measured as described above.

Transmission electron microscopy

Ileal fragments from two control and two DBZ-injected mice were collected 96 h after treatment with 10mg/kgX2 DBZ or vehicle, dissected in 1-2 mm rings and fixed in 1.5% glutaraldehyde and 1% PFA in PBS overnight at 4°C. Subsequently, the samples were washed twice with 0.1 M sodium cacodylate buffer (pH 7.4), then post-fixed for 1 hour with 1% OsO4 (osmium tetroxide) in cacodylate buffer. Samples were dehydrated and embedded in epon LX-112 and polymerized for 48 hours at 60°C. Sequential 100 nm sections were mounted on a copper slot grid covered with Formvar (1%) support film and a 7 nm carbon coating for TEM. TEM sections were stained with aqueous solutions of 7% uranyl acetate and Reynolds lead citrate and before visualization. Data were collected at an acceleration voltage of 120 kV, on a Tecnai 12 (BioTWIN) transmission

electron microscope, equipped with an Eagle CCD camera (FEI Company, the Netherlands). TEM images were extracted from virtual slides that allow a better overview of the sample, recorded with automated data acquisition and stitching software (32) with 9460x magnification at the detector plane, which corresponds to 3.2 nm pixel size at the specimen level.

Data collection and statistics. All experiments on organoids were replicated three times as indicated in Supplementary Table 1. In vivo experiments were done in two sets, each set performed on 3-5 mice from each DBZ and vehicle group. For HFD groups, mice with body weight below 45 g after 12 weeks of high fat diet were excluded from the experiment as not gaining enough weight. Littermates with similar body weight were randomly assigned to different experimental groups. The sample size is reported in each figure legend and description of the sample collection is reported in respective sections of Methods. The number of samples was selected based on the variability of the particular assay. Samples were excluded from analysis only when they were clear outliers (identified from hormone assays and confirmed with the Grubbs' test) or were erroneously generated due to a technical problem. The DBZ effect on L-cell numbers in organoids and mice in culture wells or slides was analyzed in a blinded manner. No blinding was done for *in vivo* experiments. Statistical tests are reported in Statistical analysis. Kolmogorov–Smirnov test and Q-Q plots were used to confirm normal distribution.

Statistical analysis

Results are expressed as mean ± SEM. Differences between vehicle control and DBZ groups, or DBZ groups with and without exendin 9-39, were assessed using non-paired two-tailed t-tests for equal or unequal variance determined by F-test. Data involving more than two groups were

assessed by analysis of variance (ANOVA) with post hoc Bonferroni's tests. Significance level was set at $p<0.05$.

Additional references

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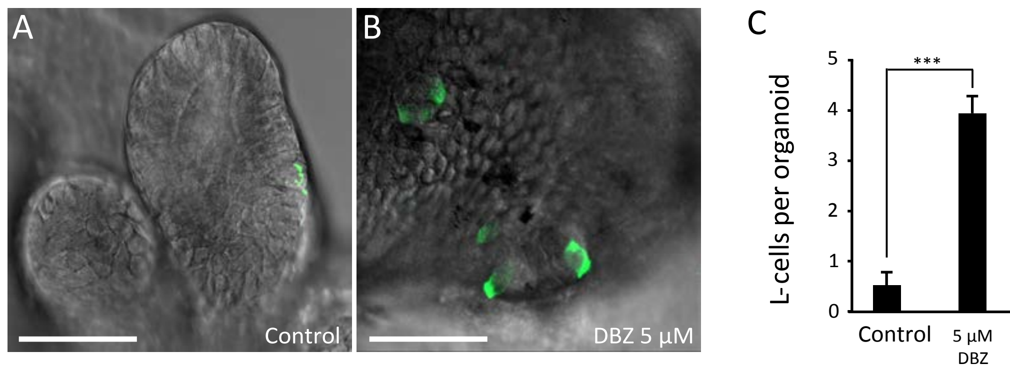

Figure S1. Modulation of L-cell development in human crypts. Immunostaining for GLP-1 in control human ileum organoids (**A**) and 96 h after a 3h-treatment with 5 µM DBZ. Images are representative of 33 control organoids and 28 of DBZ-treated organoids from 4 platings. (**B**). (**C**) L-cell numbers in control and DBZ-treated human organoids 96 hours after the pulse. Number of observations is the same as in panels **A** and **B**. Bars 50 µm. *P<0.05, ***P<0.001, by non-paired two-tailed Student's test.

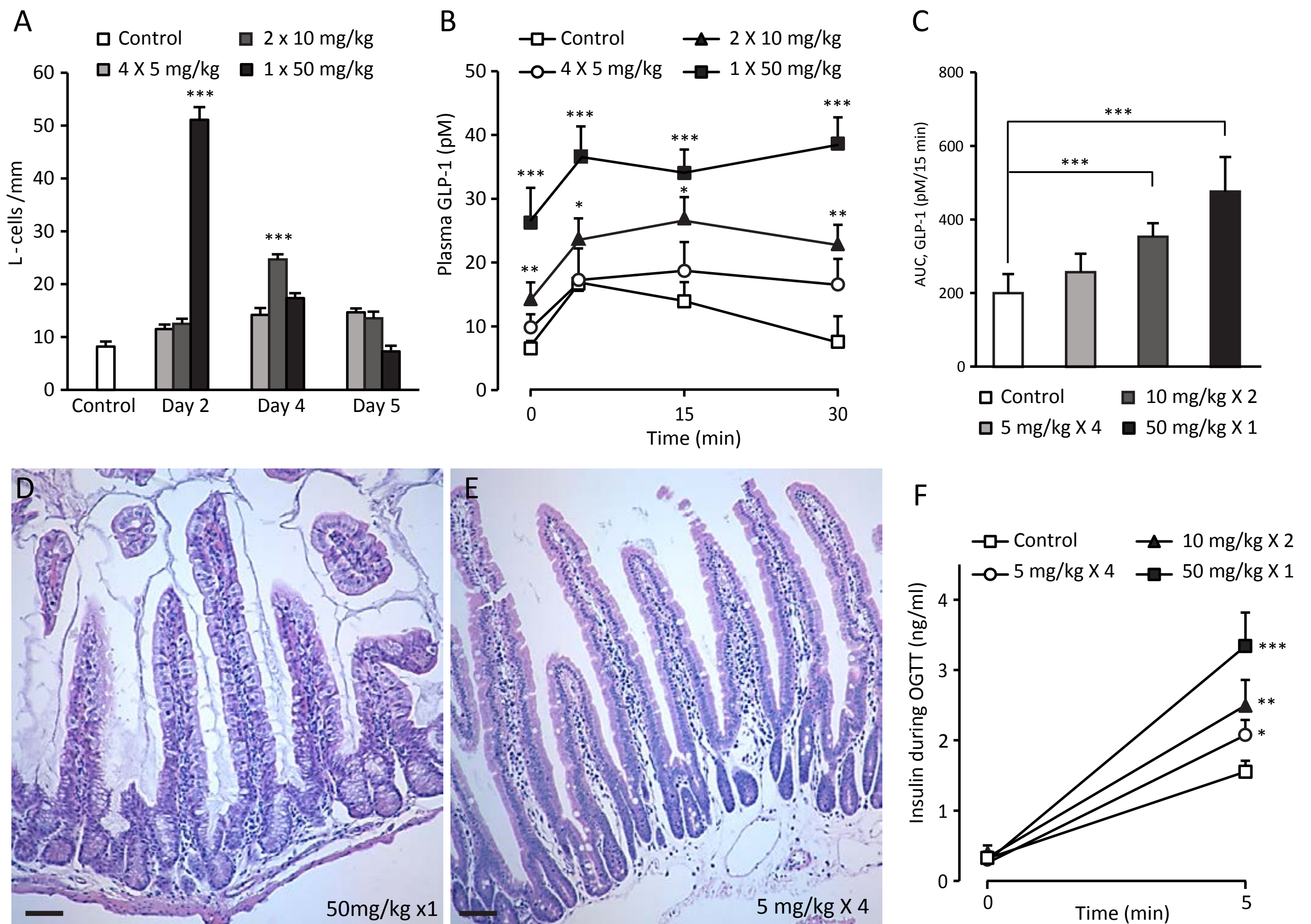

Figure S2. Effect of different DBZ treatment regimes on glucose-induced GLP-1 response, intestinal morphology and insulin secretion in vivo. (**A**) L-cell numbers over time in mouse ileum sections. Data from 6 sections, from 3 mice for each series. In panels **A-C** and **F**, *P<0.05, ***P<0.001, by one-way Anova with Bonferroni test. (**B**) GLP-1 response to 2 g/kg body weight glucose given by oral gavage in the treatment groups. N = 11 for controls, n = 4 for 5 mg/kg DBZ group, n = 10 for 10 mg/kg DBZ group, n = 4 for 50 mg/kg DBZ group. (**C**) Area under curve for GLP-1 responses. (**D-E**) Hematoxylin-eosin staining of the small intestine from mice treated with 50 and 5 mg/kg of DBZ. Bar 50 µm. Images representative of 6 sections from 3 mice in each series. (**F**) Acute insulin response to oral glucose gavage at 5 minutes in all DBZ dose groups compared to the vehicle control. N = 5 for control group, n = 5 for 5 mg/kg DBZ group, n = 6 for 10 mg/kg DBZ group, n = 4 for 50 mg/kg DBZ group.

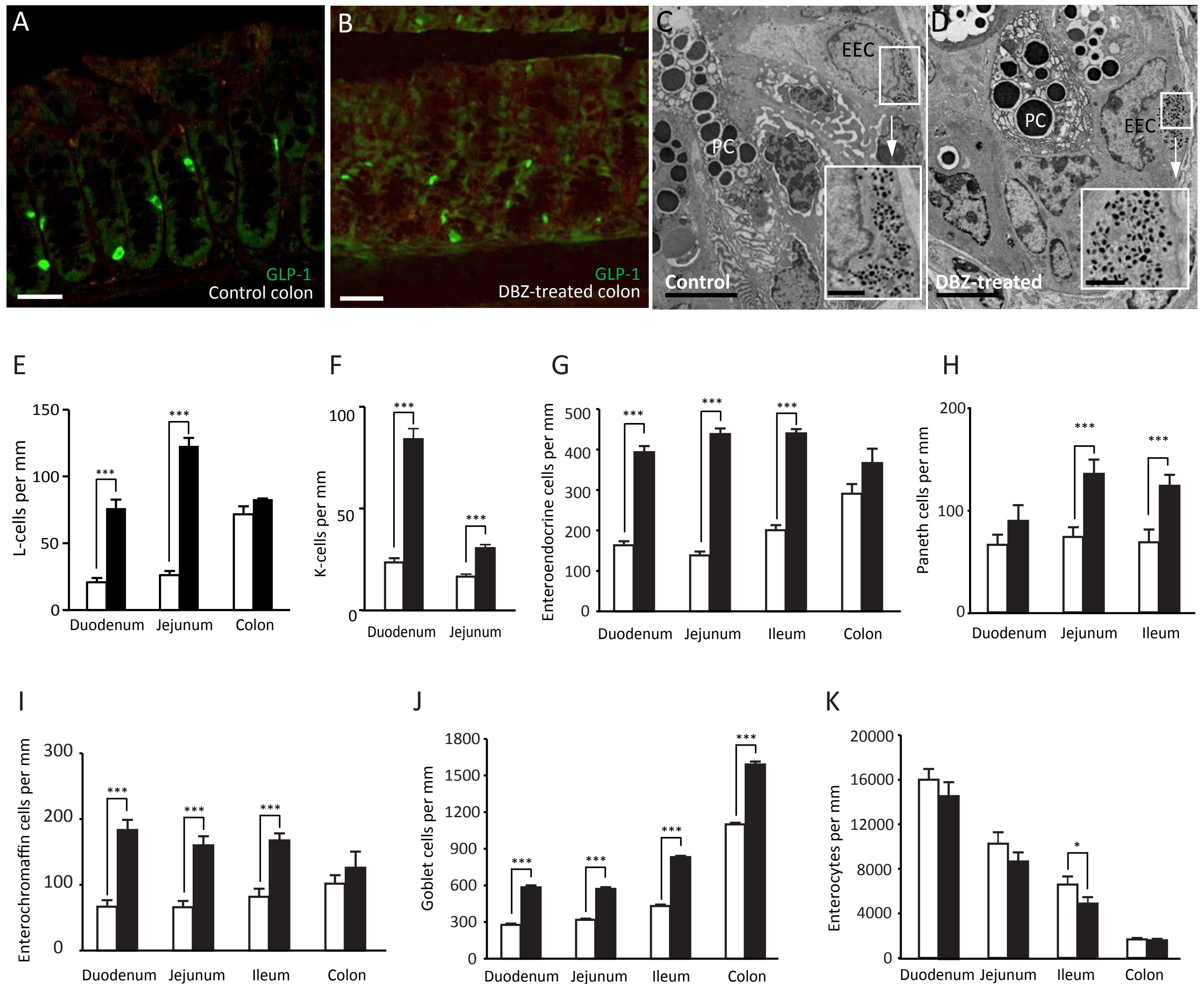

Figure S3. (**A-B**) Immunostaining for GLP-1 (green) in the colon of control and DBZ-treated mouse. Images representative of 8 sections from 4 mice in each series. Bar 50 µm. (**C-D**) Electron microscopy of ileal crypts. Bars 5 µm. Inset figures: a magnified view of granules in enteroendocrine cells. EEC, enteroendocrine cell, PC, Paneth cell. Bars 1 µm. Images representative of 4 sections from 2 control and 2 DBZ-treated mice. (**E**) Numbers of L-cells in different parts of intestine in mice treated with 2 X 10mg/kg DBZ. L-cells are identified by GLP-1 immunostaining in 10 µm sections and counted per mm of mucosal lining. Data from microscopy of 6 transverse sections for each series from 3 control and 3 DBZ-treated mice. White bars, control; black bars, DBZ-treated. In panels **E-K**, data are mean ± SEM. *$P<0.05$, ***$P<0.001$ by non-paired two-tailed Student's test. (**F-K**) Representation of different cell types in DBZ-treated mouse intestine: K-cells detected by GIP immunoreactivity (**F**), enteroendocrine cells detected by ChA immunoreactivity (**G**), Paneth cells detected by lysozyme immunostaining (**H**), enterochromaffine cells detected by serotonin immunoreactivity (**I**), goblet cells detected by PAS staining (**J**), and enterocytes detected by H&E staining (**K**).

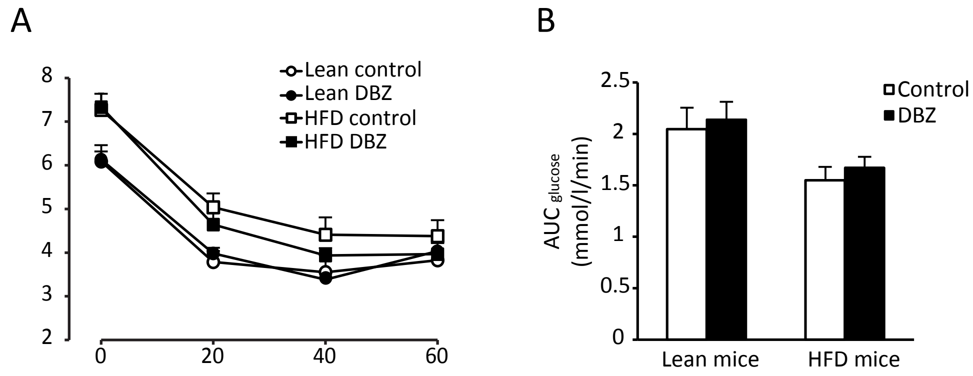

Figure S4. (**A**) Insulin-stimulated glucose disposal during an insulin tolerance test in lean control mice (n=11), DBZ-treated lean mice (n=10), control high fat diet (n=10) and DBZ-treated mice on high fat diet (n=13). Statistical difference was tested between vehicle control lean group versus DBZ-treated lean group and between vehicle control HFD group and DBZ-treated HFD group by non-paired Student's test, $^*P<0.05$. (**B**) Inverted area under the glucose curve ($AUC_{glucose}$) calculated from the data in panel **A**.

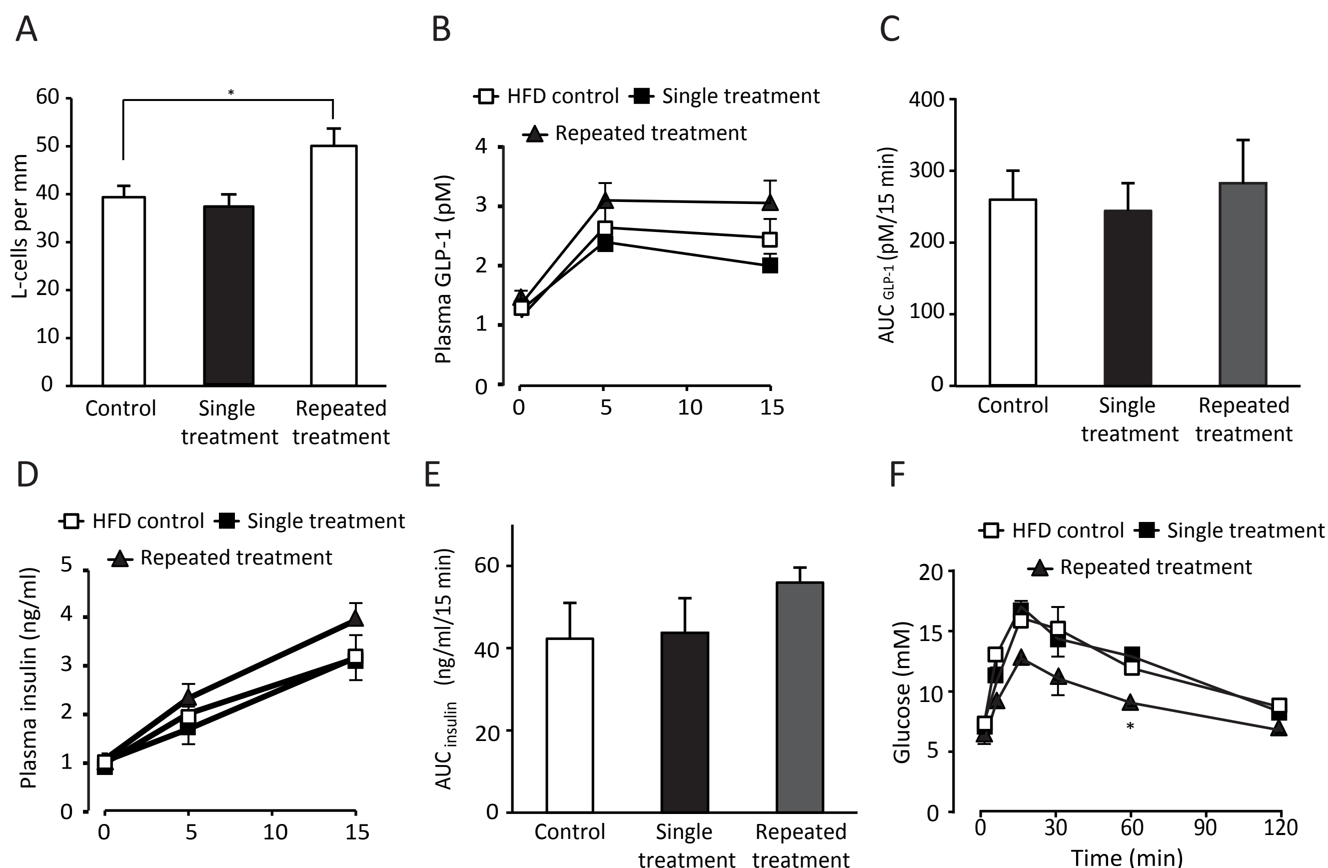

Figure S5. Cessation of DBZ effect in high fat diet mice on day 8 after the single DBZ treatment and effect of repeated DBZ treatment administered on day 5. (**A**) L-cell numbers in the ileum (detected by immunostaining). Data from microscopy of 6 transverse sections from 3 mice for each series. (**B**) Early GLP-1 response during an oral glucose tolerance test on day 8. (**C**) Area under curve for the GLP-1 response. (**D**) Early plasma insulin response from the same test. (**E**) Area under curve for the insulin response. (**F**) Blood glucose concentrations from the same test. Data are means ± SEM, n = 6 for control, n = 6 for single treatment group, and n=5 for repeated treatment group. *P<0.05 by one-way Anova with Bonferroni test.