Supplementary information

The Supplemental Data include supplemental experimental procedures and 6 figures and can be found with this article online at

Supplemental figures

Figure S1. Specificity of INT-777.

A. Activity of INT-777 on nuclear receptors related to lipid and xenobiotic metabolism, as described in material and methods section **B.** Relative activity of INT-777 in HEK293 cells transiently transfected with GPR40 relative to linoleic acid. Activity was assessed by measuring the intracellular level of inositol mono-phosphate (IP1). **C.** Activity of INT-777 in HEK293 cells transiently transfected with the cannabinoid 2 (CB2) receptor as compared to the CB2 ligand (GW405833). Activity was measured by the inhibition of cAMP levels in forskolin treated cells.

A

Figure S1

Nuclear Receptor Reference EC_{50}	FXR activation CDCA = 10-20µM	LXRα activation T0901317 = 0.18mM	LXRβ activation T0901317 = 10µM	PXR binding IC50 (µM)	PXR activation SR-12183 = 62nM	CAR activation CITCO = 5µM	PPARδ activation GW0742 = 4µM	VDR activation 1,25OH-VitD3 = 5µM	PPARγ activation GW1929 = 12µM	RARα activation ATRA = 1µM	PPARα activation GW7647 = 3µM	TR activation T3 = 0.1µM	PR activation Progesterone = 0.5µM	RXR activation 9cisRA = 4 µM
CDCA	20	>100	>100	>250	nd	>250	>100	>100	>100	>100	nd	nd	nd	>100
LCA	>100	>100	>100	23	14	>100	>100	>100	>100	>100	nd	nd	nd	nd
CA	>100	>100	>100	>100	nd	>100	>100	>100	>100	>100	nd	nd	nd	nd
UDCA	>100	>100	>100	>250	nd	>250	>100	>100	>100	>100	nd	nd	nd	nd
INT-777	175	>100	>100	110	>100	>250	>100	>100	>100	>100	>100	>100	>100	>100

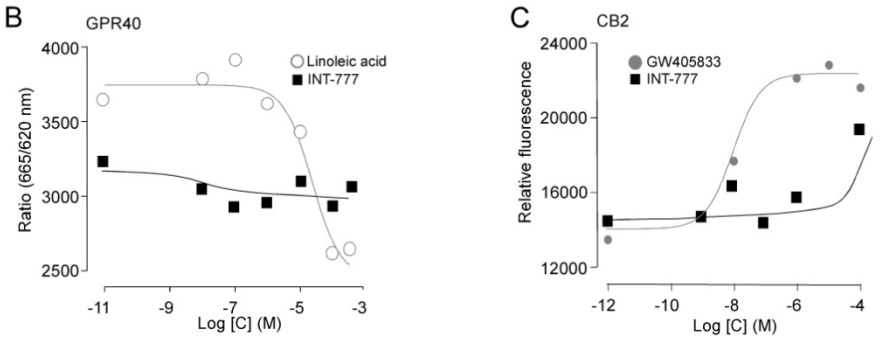


Figure S2. Correlation plots between the gene expression of TGR5 and subunits of the ATP synthase complex.

A. Relative TGR5 gene expression in the liver of different strains of male BxD mice as found in www.genenetwork.org (n=41). Large differences in TGR5 expression in the different BxD strains are evident. **B.** Correlation plots for liver mRNA expression of TGR5 and some subunits of the ATP synthase complex (Atp6v0b, ATPase H+ transporting V0 subunit B; Atpaf2, ATP synthase

mitochondrial F1 complex assembly factor 2; Atp1a3, ATPase Na+/K+ transporting alpha 3 polypeptide; Atp6v1b2, ATPase H+ transporting V1 subunit B isoform 2) in the mouse BxD genetic reference population as found in www.genenetwork.org (n=41).

Figure S2

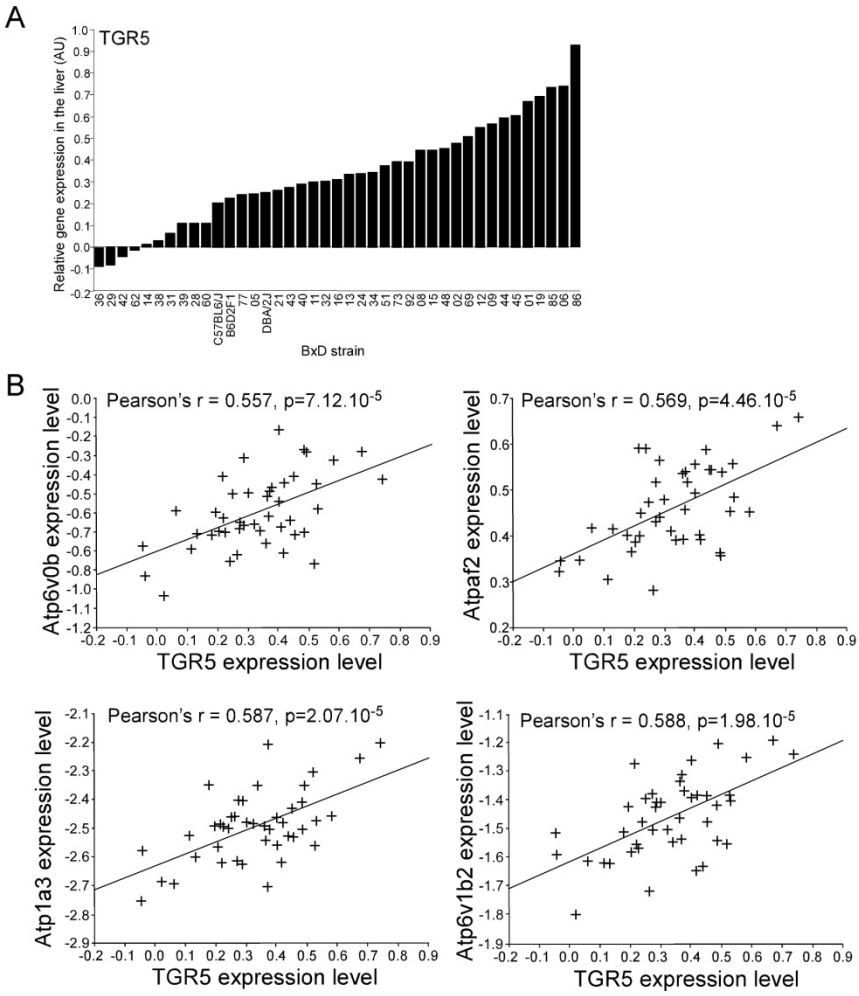


Figure S3. Effect of INT-777 of ATP production and GLP-1 release in human enteroendocrine NCI-H716 cells.

A. The human enteroendocrine cell line NCI-H716 was cultured in presence of 1g/dL of glucose and treated for 1hr with INT-777 (3µM) in presence of vehicle or the adenylate cyclase inhibitor MDL-12330-A (MDL) (10µM). ATP production was assessed by a bioluminescence assay (biovision) using ATP as a standard (100 to 0.01µM). **B.** Impact of 30 min INT-777 (3µM) treatment on GLP-1 release in NCI-H716 cultured with 1g/L of glucose in the presence of vehicle or 10 µM MDL-12330-A (MDL). MDL or vehicle were added 15 min prior to INT-777 treatment. A DPP4 inhibitor (Millipore) was added

to culture medium at 0.1% (n=3). The data are represented as mean ± SE. Student's unpaired *t*-test. * ($P < 0.05$) (n=3).

Figure S3

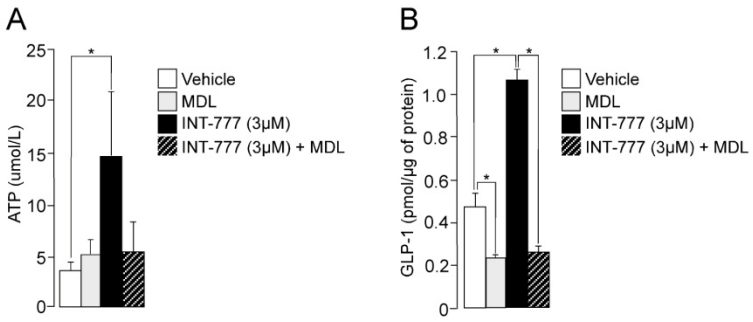


Figure S4. Genetically engineered mouse models for the study of the TGR5 signaling pathway.

A. Schematic representation of the BAC clone microinjected into oocytes in order to generate TGR5-Tg mice as described in the material and methods. **B.** Expression levels of TGR5 mRNA in TGR5-Tg and control littermates. **C-F.** Description of the strategy used for generating TGR5 floxed mice (as described in the material and methods) and target regions of southern blot probes and PCR primers used for identification of **D.** positive ES cell clones and **E.** targeted mice, respectively. **F.** PCR strategies used to genotype TGR5 L2/+ and TGR5 L-/+ mice. A+B PCR allows identifying Lox sites, which are only present in absence of recombination. A+D PCR allows to determine whether recombination and deletion of exon1 of the TGR5 gene occurred. In the absence of recombination, the region between the A+D primers was too large to be amplified, as illustrated by the absence of a PCR product. As mentioned in material and methods, TGR5 L2/+ mice were mated with CMV-Cre transgenic mice in order to delete the floxed allele in the germline. The resulting TGR5 L-/+ mice were further intercrossed to generate TGR5 L+/L+ ($TGR5^{+/+}$), TGR5 L-/+ ($TGR5^{-/+}$) and TGR5 L-/L- ($TGR5^{-/-}$) mice. na: non amplified. Drawings are not to scale.

Figure S4

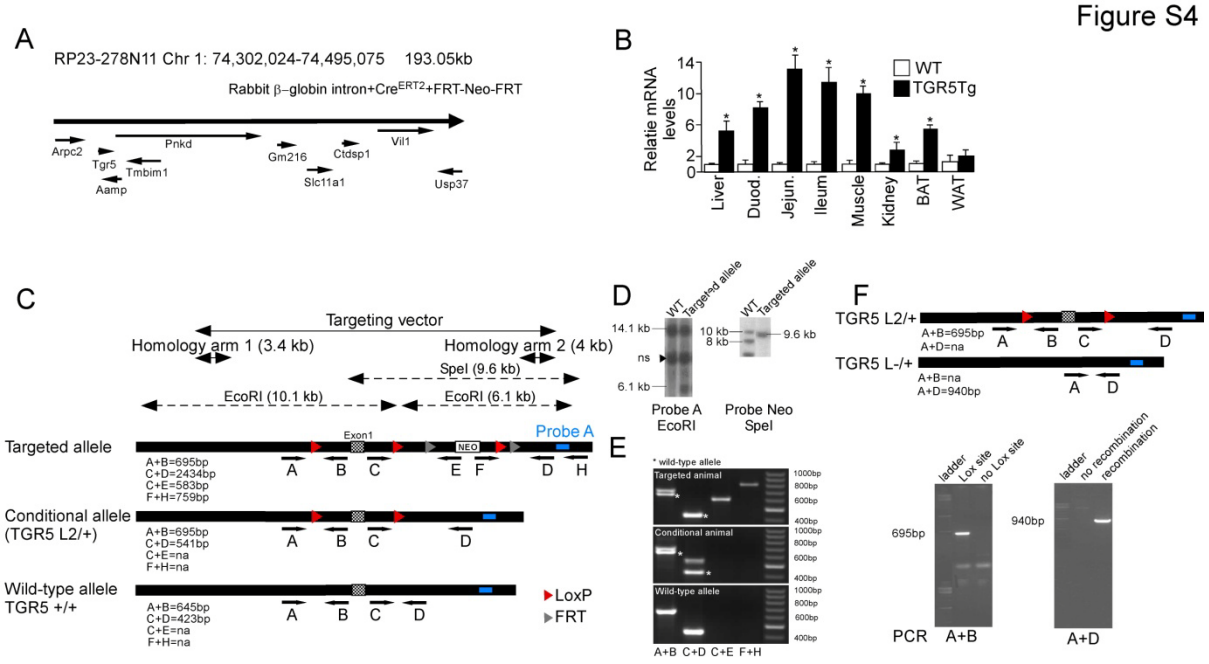


Figure S5. Impact of TGR5 over-expression in vivo on weight gain, energy expenditure and locomotor activity.

A. Body weight follow-up in 8 weeks-old TGR5-Tg and control littermate fed a chow (CD) or a high fat diet (HF) (n=8). **B.** Body composition was assessed by qNMR after 9 weeks of chow (CD) or a high fat diet (HF) feeding (n=8). **C-D.** Spontaneous horizontal activity and energy expenditure, evaluated by the measurement of oxygen consumption (VO_2) and carbon dioxide release (VCO_2), were monitored over a 18hr period, 13 weeks after the initiation of chow (CD) or a high fat (HF) diet. The respiratory quotient (RQ) was calculated as the ratio VCO_2/VO_2. Bar graphs represent the average AUC. For the RQ, bar graphs represent the average (n=8). **E-F.** 8 week-old male C57BL6/J mice were placed in cages equipped with an infrared beam system monitoring ambulatory activity. After a 6h acclimatization period, saline or exendin-4 (Ex-4) was intra-peritoneally administrated. Horizontal locomotor activity and food intake were monitored for an additional 24h period (n=12). Food intake was assessed by the number of pellet eaten. Pellets used were special pellets with a 1.5mm diameter. The data are represented as mean ± SE. Student's unpaired *t*-test. * ($P < 0.05$).

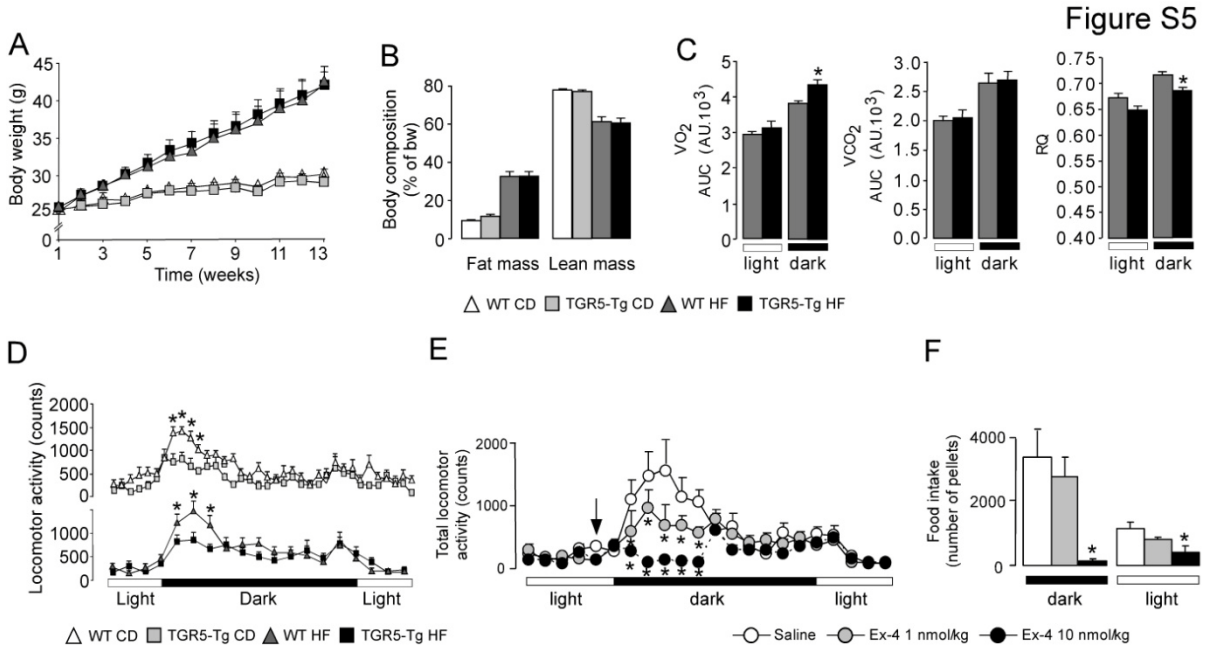
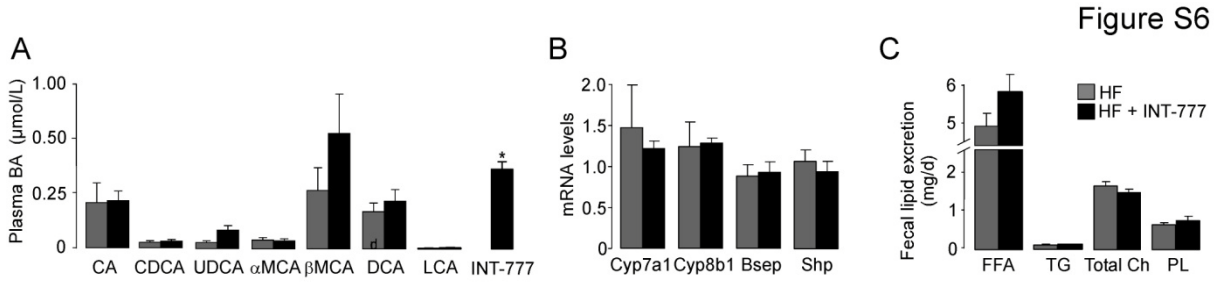


Figure S6. Impact of INT-777 treatment on BA and lipid metabolism

A. Plasma BA composition in DIO mice (initial period of 14 weeks of HF feeding) treated or not with INT-777 (30mg/kg/d) for 18 weeks (n=8). **B.** Expression levels of key genes of liver BA metabolism measured by quantitative real time PCR (n=8). **C.** Fecal lipid excretion measured on dried feces collected over a 24h period and then extracted according Folch's method and quantified by enzymatic assays (n=8). The data are represented as mean ± SE. Student's unpaired *t*-test. * ($P < 0.05$).



Supplemental Experimental Procedures.

Chemicals and reagents

All biochemical reagents were purchased from Sigma-Aldrich unless indicated. The DPP4 inhibitor (DPP4i) sitagliptin was a kind gift from Dr C. Ullmer (Hoffmann-La Roche).

Cell culture

STC-1 cells cultured and maintained in DMEM containing 15% (v/v) horse serum and 5% (v/v) fetal bovine serum. CHO cells were cultured as previously described (Sato et al., 2008; Watanabe et al., 2006). NCI-H716 were cultured in RPMI-1640 containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, supplemented with Fetal Bovine Serum 10%, penicillin 100000U/L, Streptomycin 100mg/L at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

ATP and ADP measurement

ATP/ADP ratio was measured as follow, 10^4 STC-1 cells per well were cultured in DMEM containing 15% (v/v) horse serum and 5% (v/v) fetal bovine serum on 96 well-plates for 24 hours. One hour prior the assay, medium was replaced by Kreb's ringer containing 4.5g/L of glucose and 0.1% BSA for 1h. Then, cells were treated for 1h with vehicle or INT-777, in presence or absence of 10 µM MDL-12330A. After 1h, medium was removed and 100 µl of Nucleotide Releasing Buffer for 5 minutes at room temperature with gentle shaking (ApoSENSOR^{TM} ADP/ATP Ratio Assay Kit, biovision). To measure the ATP levels in the cells, 1 µl of the ATP Monitoring Enzyme was added into the cell lysate. Luminescence was read during 1 second (Data A). Then, after 1h, luminescence was recorded again (for 1s) (Data B), then 1 µl of ADP Converting Enzyme was added in each well and luminescence produced was measured for 1s (Data C). Data C-Data B represents ADP levels. Data A represents ATP- Therefore ATP/ADP ratio = (C-B)/A

For ATP measurement, human enteroendocrine NCI-H716 cells were cultured cultured in RPMI-1640 containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, supplemented with Fetal Bovine Serum 10%, penicillin 100000U/L, Streptomycin 100mg/L. Ten thousands cells (10 µL) were stimulated with test compounds in Optimem for 60' at RT and then lyzed with 100 µl of Nucleotide Releasing Buffer for 5 minutes at room temperature with gentle shaking (ApoSENSOR^{TM} ADP/ATP Ratio Assay Kit, BioVision). The ATP level was measured after adding 1 µl of the ATP Monitoring Enzyme into the cell lysate. For ATP standard curve a 10-fold serial dilution series of ATP (SIGMA cat.A6419) were prepared in optimem (from 10 nM to 100 µM). The standard curve was set up in the same white Optiplate (perkinElemer) microplate that will be used

for the experimental samples in triplicate. Luminescence was measured by Victor light (Perkinelmer). ATP standard curve was analyzed by linear regression and ATP concentration in the samples was obtained interpolating their relative luminescence from standard curve.

Transfection studies

The mouse TGR5 coding sequence was inserted into the XhoI/EcoRI site of the pCMVSport6 vector using the following primer sequence: R-ACTCGAGtgatggccagagatttatttggtct (XhoI), F-AGAATTCaggagtccaggccaggccagagtctga (EcoI), after having determined the mouse full-length TGR5 cDNA sequence by 5'-RACE and 3'-RACE techniques and generating it by reverse transcription from mouse gallbladder mRNA. The mTGR5 expression vector was then transfected using JetPEI at a N/P ratio of 5 (Polyplus). Mouse TGR5 shRNA and related control shRNA vectors (Origene) were transfected with JetPEI, according to the manufacturer's instructions. pCMVsport6 hTGR5 was purchased from Invitrogen (clone MGC:40597 IMAGE:5221127). On-TargetPlus Smart pool hTGR5 and non-targeting siRNA were purchase from Dharmacon. For NCI-H716 cells were transfected using GenePulsarXCell electroporator (Biorad).

GPCR and nuclear receptor activation assays

The activity of CB2 ligands was tested by measuring inhibition of cAMP levels in HEK293 cells transiently transfected with the CB2 receptor. Briefly, transfected cells were stimulated with 0.1 μM Forskolin (determined in pilot experiments as the EC50 concentration) alone or in combination with increasing concentrations of the CB2 agonist GW405833 (Tocris) or INT-777 for 2hr at 21°C . Intracellular levels of cAMP were detected by the *Lance*^{TM} assay (Perkin Elmer), according to the manufacturer's protocol. The activity of GPR40 ligands was tested by measuring the intracellular level of inositol mono-phosphate (IP1), which accumulates into cells upon GPR40 activation. HEK293 cells were transiently transfected with GPR40 and stimulated with increasing concentrations of linoleic acid (Tocris) or INT-777 for 30' at 37°C. IP1 levels were measured by HTRF^{TM} IP-One *Tb* assay (Cisbio), according to the manufacturer's protocol. EC_{50} values were calculated using GraphPad Prism software. Coactivator Recruitment Assays were performed by time resolved-fluorescence resonance energy transfer (TR-FRET). The nuclear selectivity was assessed by using Lanthascreen assays (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, TR-FRET assays were

performed in 20µl of Lanthascreen assay buffer (Invitrogen, Carlsbad, CA), using a terbium-labeled anti-GST antibody, a fluorescein-labeled coactivator peptide, and a nuclear receptor ligand-binding domain tagged with glutathione-S-transferase, all from Invitrogen. Negative controls were carried out using vehicle alone (DMSO). A specific positive control was used for each nuclear receptor tested. Corticosterone (PR), 25-hydroxyvitamin D_3 (VDR), all-trans retinoic cid (RARα) and 9-cis retinoic acid (RXRα) were from Biomol; whereas progesterone (PR), calcitriol (VDR), SR-12813 (PXR), T0901317 (LXRα/β), W1929 (PPARγ), GW7647 (PPARα), GW0742 (PPARβ), triiodo-L-tyronine (THR), Citco (CAR), Estadiol (ER) and CDCA (FXR) were from Sigma. After a 1hr incubation in the dark, TR-FRET measurements were performed using an Envision plate reader (PerkinElmer). The emission ratio 520/495 was plotted against varying ligand concentrations. Dose-response curves were generated with increasing ligand concentrations ranging from $1x10^{-14}$ to $5x10^{-4}$ M ($n \geq 3$). A four-parameter dose-response curve (GraphPrism) was used to calculate EC50 values.

***In vitro*, *in vivo* and *ex vivo* GLP-1 measurements**

In vitro, GLP-1 release was measured in STC-1 cultured at 70% confluency in 96-well plates. Cells were grown in regular culture medium, then washed with PBS 1hr prior to the treatment with INT-777 or vehicle and transferred into culture medium without PBS and containing 0.1% BSA. Cells were then treated for 1hr in same medium supplemented 0.1% DDP4i. At the end of this period, medium was transferred into ELISA plates for GLP-1 measurement according to the manufacturer's instructions (Millipore). *In vivo*, as indicated in related sections, mice were administered an oral treatment or not with the DPP4i sitagliptin (3mg/kg) 30 min prior to retro-orbital blood collection (100-150µL) at the indicated time. Blood samples were collected in EDTA tubes containing a DPP4i (Millipore) and kept on ice. Plasma was collected by centrifugation and transferred into ELISA plates to proceed with GLP-1 measurement according to manufacturer's instructions (Millipore)

The assessment of GLP-1 release from ileal mucosa was performed in 4hr fasted TGR5-Tg mice or control littermates fed a HF diet for 20 weeks. Mice were sacrificed and an 8 cm section of ileum was collected for organ culture. The ileum was placed in cold HBSS containing 2% horse serum, opened longitudinally and cut into 5-mm-long pieces. These fragments were washed 5 times in HBSS/2% horse serum and then incubated for 10 min in HBSS/2% horse serum containing 1mM 1,4-dithiothreitol at 4°C to remove the excess mucus. After additional washing in HBSS/2% horse serum,

tissue pieces were equally distributed in a 24-well plate and incubated in DMEM containing 10% FCS at 37°C. After 4hr stabilization, medium was replaced with medium treated with 0.1% DPP4i (Millipore) and containing either the compound or vehicle (DMSO). After 1hr of treatment, the medium was removed and immediately frozen at -20°C. Ileal explants were rinsed with cold PBS, then immediately snap-frozen in liquid nitrogen. Protein content was assessed according to Bradford's method. GLP-1 content in culture medium was assayed by ELISA according to manufacturer's instructions (Millipore).

Generation of genetically engineered mouse models

TGR5-Tg mice were generated from the oocyte microinjection of mouse RP23-278N11 BAC clones. Prior to microinjection and for a genotyping purpose, a sequence containing the rabbit β-globin intron, cloned upstream to the coding sequence of the inactive Cre recombinase (CRE^{ERT2}) and a Neo cassette flanked with 2 FRT sites, was introduced by homologous recombination downstream to the vil1 promoter in this RP23-278N11 BAC. Positive clones were selected with G418. This modified BAC was subsequently microinjected to generate TGR5-Tg mice. Offspring chimeric mice were mated to female C57BL/6J mice that express the Flp recombinase under the control of the ubiquitous cytomegalovirus promoter (CMV) in order to remove the Neo cassette. The CRE^{ERT2} was used to identify the genotype of the resulting mice by PCR. TGR5-Tg mice were kept in a heterozygote state for the transgene and crossed with C57BL/6J mice. Only littermates were used for *in vivo* experiments. For the generation of TGR5 floxed (TGR5L2/L2) mice, genomic DNA covering the TGR5 locus was amplified from the 129Sv strain by using high-fidelity PCR. The resulting DNA fragments were assembled into the targeting vector (Institut Clinique de la Souris). The construct was then electroporated into 129Sv embryonic stem (ES) cells. G418-resistant colonies were selected and analyzed for homologous recombination by PCR and positive clones were verified by Southern blot hybridization. Thereafter, genomic DNA was prepared from ES cells, digested with EcoRI or SpeI, subjected to electrophoresis on a 0.8% agarose gel, and transferred to a positively charged nylon transfer membrane (Amersham Biosciences). The karyotype was verified and several correctly targeted ES cell clones were injected into blastocysts from C57BL/6J mice. These blastocysts were transferred into pseudo pregnant females, resulting in chimeric offspring that were mated to female C57BL/6J mice that express the Flp recombinase under the control of the ubiquitous cytomegalovirus promoter (CMV) in order to remove the Neo cassette. Offspring that transmitted the mutated allele, in

which the selection marker was excised, and that lost the Flp transgene (TGR5 L2/+ mice: heterozygote conditional animals containing the conditional allele with Lox sites) were selected and mated with CMV-Cre mice. Offspring with a deleted allele (TGR5 L-/+ mice) were then mated to C57BL/6J mice in order to remove the Cre-transgene. Resulting TGR5 L-/+ offspring without the Cre transgene were then further intercrossed to generate TGR5 L-/L- mice (here called $TGR5^{-/-}$). Breeding was only performed in the heterozygote state and only littermates ($TGR5^{-/-}$ and $TGR5^{+/+}$) were used for *in vivo* studies.

Correlation plots from BxD resource gene expression studies

The BxD resource is a mouse genetic reference population (GRP) (Abiola et al., 2003; Chesler et al., 2005; Chesler et al., 2004). The BXD set of recombinant inbred (RI) mouse strains were derived crossing F2 mice obtained from an intercross of C57BL/6J (B6) and DBA/2J (D2) mice and then inbreeding the resulting progeny for at least 20 generations to generate the BxD GRP (Argmann et al., 2005; Chesler et al., 2005; Chesler et al., 2004). This genetic reference panel is a remarkable resource because data for hundreds of interesting phenotypic traits, including clinical traits and molecular traits, i.e. microarray expression studies (in multiple tissues) have been acquired over a 25-year period. All these data are publically available on the www.genenetwork.com website. The web-based software on the genenetwork site furthermore allows the identification of all genes whose expression is significantly correlated with a gene of interest (TGR5 in our example) in a particular tissue (liver in our case). As illustrated in the supplementary figure S2A, TGR5 gene expression varies significantly in the liver of the different mice of the BxD RI panel (similar expression profiles can be generated for all probes contained in the microarrays). The software allows then the calculation of a correlation plot between TGR5 and these other genes generating a list that is classified according to its statistical significance.

Additional references

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