Supplementary information

Chemerin regulates β-cell function in mice

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Mice and *in vivo* experiments

Mouse experiments were performed according to the guidelines of the Animal Ethics Committee of Kobe University Graduate Scholl of Medicine. Targeting strategy for the disruption of the chemerin gene locus is depicted in Supplementary Fig. 1A, and was performed in the ES cells of TT2 as described (http://www.cdb.riken.jp/arg/ Methods.html). We replaced all the coding regions of chemerin (exon 2) with a neomycin-resistant cassette. We analyzed chemerin-deficient mice derived from two independent clones of the ES cells (#33 and #86, Supplementary Fig. 1b, c) and both lines showed similar phenotypes. Chemerin expressions were completely ablated in the liver (Supplementary Fig. 1e), serum (Supplementary Fig. 1f) in the (-/-) mice. The results presented here are of studies conducted on mice backcrossed at least for six generations with C57Bl6 background and 7-15-week-old age-matched mice, unless otherwise specified. Genotyping was performed at weaning (21d) on genomic tail DNA. The primers (AC**1**CASP1; 5'- GAACTGGCACAGCCTAGGAG-3'), (AC**1**WSP3.1; 5'- CTACAGGTGGCTCTGGAGGA-3') and (PGKS1; TCCACTAAAATGGAAG TTTTTCCT) were used for 3 primer PCR, producing a 246 (wild type) or 386 (null) bp PCR product (Supplementary Fig. 1d).

A 0.6 kb human serum amyloid P enhancer/promoter fragment was used to drive expression of human chemerin in the *SAP-hChemerin* transgene[1]. Two transgenic lines, #9 (Tg1) and #27 (Tg2), were established in the C57Bl6 background. These lines exhibited similar glucose tolerance in IPGTT, and the lower-expressing line, Tg1, was used in subsequent studies. The primers (GLOBINsense1; GTGCTGGTTGTTGTGCTGTC), (hAC1sense1 ; CTGGAGGAATTTCACAAGCA), and (hAC1antisense1; CTTTGCACTCGGGTTTCTTC) were used for genotyping.

Animals were housed up to 5 per cage in a 12 h light/dark cycle (lights on at 0700), with free access to water and chow under specific pathogen-free conditions. Male C57BL/6J and *db/db* mice were purchased from CLEA Japan. For high-fat diet studies, 8-weeks-old male C57BL/6J mice were fed with a diet containing 45% fat, 10% protein and 35% carbohydrate (Oriental Bioservice, Japan) for 6 weeks. Streptozotocin (STZ)-diabetic mice were generated as previously described [2]. Briefly, for the analysis of chemerin and ChemR23 expression in pancreas, 100mg/kg of STZ (Sigma, St.Louis, MO, USA) in citrate sodium buffer (ph4.5) was administered intraperitoneally to 6-weeks-old male C57BL/6J mice in every week for twice in fasting status. Eight weeks after the first administration, mice were sacrificed and the pancreas was analyzed.

Glucose measurements and intraperitoneal glucose tolerance test

For the intraperitoneal glucose tolerance test, 50% glucose (1.5g/kg body weight) was injected intraperitoneally after a 15-h fasting. Blood was collected from the tail vein at 0, 30, 60, 90 and 120 min after the injection. For insulin measurements, blood was collected at 0, 15 and 30 min after the injection.

Hormone assays

The serum was separated by centrifugation and stored at -80℃ and assayed using mouse insulin ELISA kits (Shibayagi Inc. Japan), mouse leptin ELISA kits (R&D systems, USA), and mouse serum adiponectin kit (Otsuka assay, Japan). Serum total cholesterol, triglyceride, and NEFA were assayed using Cholesterol E test WAKO, Triglyceride E test WAKO, and NEFA C test WAKO kit from WAKO.

Euglycemic Hyperinsulinemic Clamp Studies

Euglycemic Hyperinsulinemic Clamp Studies were performed as previously described [3]. Briefly, five to seven days before the clamp, mice were anesthetized with sodium pentobarbital (80–100 mg/kg, ip) and a catheter was inserted into the right internal jugular vein for infusion. The analysis was performed under nonstressful conditions with conscious mice that had been deprived of food for 4 h. The [3-^{3}H]glucose was infused for 2 h at a rate of 0.05 μCi/min before initiation of the clamp, and a blood sample was collected at the end of this period to estimate basal glucose turnover. After a bolus injection of [3-^{3}H]glucose (10 μCi; NEN Life Science Products, Boston, MA) and the onset of subsequent continuous infusion of [3-^{3}H]glucose (0.1 μCi/min), a hyperinsulinemic-euglycemic clamp was applied for 120 min with continuous infusion of insulin at a rate of 2.5 and 4.0 mU/kg·min. Plasma glucose concentration was monitored every 10 min, and 30% glucose was infused at a variable rate to maintain plasma glucose concentration at about 110 mg/dl. Blood samples were collected 80, 90, 100, 110, and 120 min after the onset of the clamp for determination of the plasma concentrations of [3-^{3}H] glucose and ^{3}H$_2$O. The rates of glucose disposal and hepatic glucose production (HGP) were calculated as described [4].

Islet morphometry analysis, immunohistochemistry and immunofluorescence

The pancreas was isolated, fixed in 4% paraformaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and mounted on slides. The percentage islet area was quantified using Image J software as described previously [5,6]. 8-weeks-old male C57BL/6J mice were used for the analysis of chemerin and ChemR23 expression.

Immunohistochemistry and immunofluorescence were performed as previously described [7,8] using polyclonal pig anti-swine insulin antibody at a 1: 40 dilution (DakoCytomation, Denmark) as the primary antibody. A macrophage marker, Mac3 staining was performed as previously described[3].

Quantitative real time PCR and immunoblotting

Quantitative real-time PCR (QRPCR) was performed, as previously described [9,10], on a LightCycler system using the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Switzerland). Each value was normalized to 36B4 or β-actin expression. The sequence of primers is listed below. Immunoblotting analysis was performed as previously described [8,11].

Islet isolation and batch incubation

Mouse pancreatic islet isolation and batch incubation were performed as previously described [12]. Briefly, mouse pancreatic islets were isolated manually by the collagenase digestion method and cultured for 2 days in RPMI medium 1640 (Sigma) containing 10% FBS, streptomycin sulfate, and penicillin G under a humidified condition of 95% air and 5% CO_2. After preincubation (30 min) of the isolated islets with Hepes-KRB containing 2.8 mM glucose, 5 size-matched islets were collected in each tube and incubated for 30 min in the same buffer containing 2.8 and 16.7 mM glucose. Insulin concentration in the medium and content in the cells were measured by ELISA.

Perfusion experiments of mouse pancreata

Overnight (12-16 h)-fasted mice at 8–15 weeks of age were used in perfusion experiments as previously described [13]. A littermate in a same-gender pair was used for the experiments (n=5-6/genotype). Briefly, after anesthesia with 80 mg/kg sodium pentobarbital, the superior mesenteric and renal arteries were ligated, and the aorta was tied off just below the diaphragm. The perfusate was infused from a catheter placed in the aorta and collected from the portal vein. The perfusate was Krebs-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 4.6% dextran and 0.25% BSA and gassed with 95% O_2/5% CO_2. The flow rate of the perfusate was 1 ml/min. The pancreata were preperfused with KRBH buffer containing 2.8 mmol/l glucose for 10 min of equilibration period and stimulated by 16.7 mmol/l glucose for 5 min. The insulin levels in the perfusate were measured by ELISA kit.

MIN6 cells and knockdown of chemerin and ChemR23

Mouse β cell line, MIN6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose supplemented with 15% (v/v) heat-inactivated fetal calf serum under humidified conditions of 5% CO_2, 95% air at 37 °C [14]. After 6h of starvation, the indicated agents were added, incubated for 3 h and chemerin expression was analyzed by QRPCR. For the knockdown of chemerin or ChemR23 in MIN6 cells, shRNA expression vector, pcPURm6i [15] containing target sequence for mouse chemerin or ChemR23 was transfected by reverse transfection method into MIN6 cells using Lipofectamine 2000 reagent (Invitrogen) as previously described [16,17], according to the manufacturer's instruction. The target sequence for knock down of chemerin and ChemR23 are as follows; Chemerin, CKD-2:GGAGAAGGCGGAAATGCCCT, CKD-3:AGACCAACTGCCCCAAGAA, CKD -5:AAGCCGGAGTGCACAATCA and CHEMR23, CRKD-1:GCCACAACATGTACACCAG, CRKD-6:GTGCCAAGCTCTG TCTTCA, CRKD-8:GCATGAACCCCATTCTGTA. The vector plasmid was mixed with Lipofectamine 2000 in Opti-MEM I medium and incubated at room temperature for 20 minutes. MIN6 cells were stripped by trypsin-EDTA and incubated in antibiotic-free DMEM with 15% FBS before transfection. The transfection mixture was added to the cells in suspension status. The transfection efficiency was verified using GFP expression vector. Then, the cell were seeded to 96 well plates and incubated for approximately 8 h at 37°C and 5% CO_2 before being replaced with normal culture medium. Measurement of insulin secretion and content was performed as previously described [18], 48 h after the transfection. The values of insulin were normalized by protein content.

Statistics and data analysis

Results are represented as mean±SEM from at least 3 different experiments performed independently, unless stated otherwise. The data were analyzed using an unpaired 2-tailed Student's *t* test, analysis of variance (ANOVA), or Mann-Whitney u-test as appropriate. A *P* value of less than 0.05 was considered significant.

Primer sequence for quantitative real time PCR

	Sense primer	Antisense primer
Chemerin	GTGCACAATCAAACCAAACG	GGCAAACTGTCCAGGTAGGA
ChemR23	CTGATCCCCGTCTTCATCAT	AGGGCCACCTTGAATTTTCT
GPR1	GGAGCTCAGCATTCATCACA	GGCTCTTGGTTTCAGCACTC

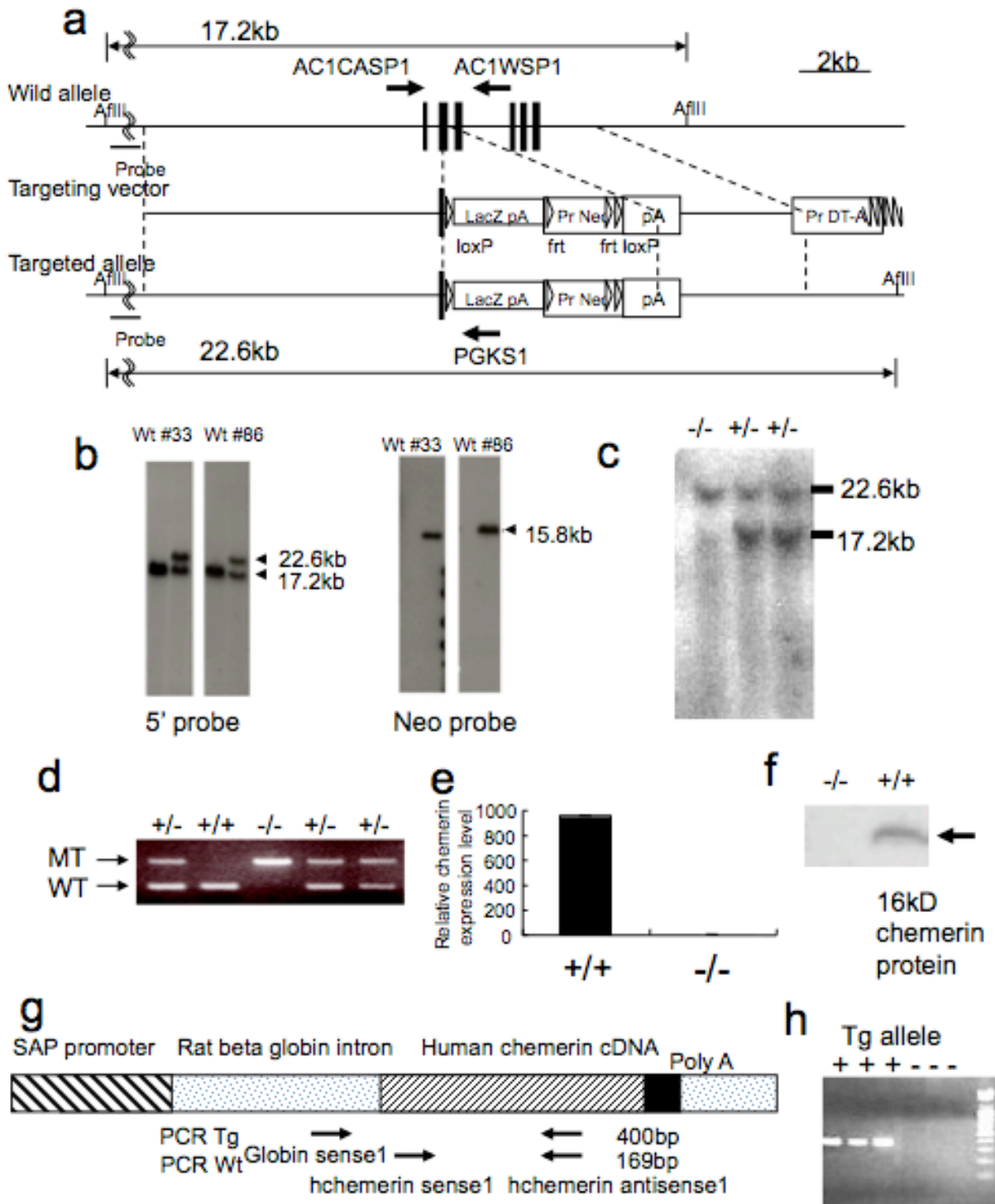|		
CCRL2	CTGGCGGTGTTTATCTTGGT	CAAGGAGGAGGATGTTGGAA
TNFa	CCGATGGGTTGTACCTTGTC	CGGACTCCGCAAAGTCTAAG
leptin	TGACACCAAAACCCTCATCA	TCATTGGCTATCTGCAGCAC
AP2	CATCAGCGTAAATGGGGATT	TCGACTTTCCATCCCACTTC
PPARg	CCCTGGCAAAGCATTTGTAT	GAAACTGGCACCCTTGAAAA
PGC1	AATGCAGCGGTCTTAGCACT	TTTCTGTGGGTTTGGTGTGA
SREBP1c	GATCAAAGAGGAGCCAGTGC	GTTTGTTCTAGGGGCTGCTG
CD68	CTTCCCACAGGCAGCACAG	AATGATGAGAGGCAGCAAGAGG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
HNF1α	GGTCAAGCTACGAGGACAGC	ATGTACTTGGCCCACTCGAC
HNF1β	GCACCTCAGATAAGCCGTTC	TTTCTCCCTTCTCGTTGCAC
HNF4α	GGTCAAGCTACGAGGACAGC	ATGTACTTGGCCCACTCGAC
NeuroD	GGAGGAGGAGGAAGATGAGG	TGGGTCTTGGAGTAGCAAGG
PDX-1	ACTTGAGCGTTCCAATACGG	AGAGGGGGAACGACTCTAGG
MafA	TTCAGCAAGGAGGAGGTCAT	TTCTCGCTCTCCAGAATGTG
Ins1	GGAGCGTGGCTTCTTCTACA	GGTGGGCCTTAGTTGCAGTA
Ins2	TTTGTCAAGCAGCACCTTTG	CACTTGTGGGTCCTCCACTT
Glut2	CATGCTGAGCTCTGCTGAAG	ACAGTCCAACGGATCCACTC
SUR	CTCTGATCCTCCCTTGCTTG	GGCCCTGGACTGTGTAAGAG
Nkx6.1	TCAGGTCAAGGTCTGGTTCC	CCGAGTCCTGCTTCTTCTTG
UCP2	CCTACAAGACCATTGCACGA	CATAGGTCACCAGCTCAGCA
ARNT	TCTCCCTCCCAGATGATGAC	CAATGTTGTGTCGGGAGATG

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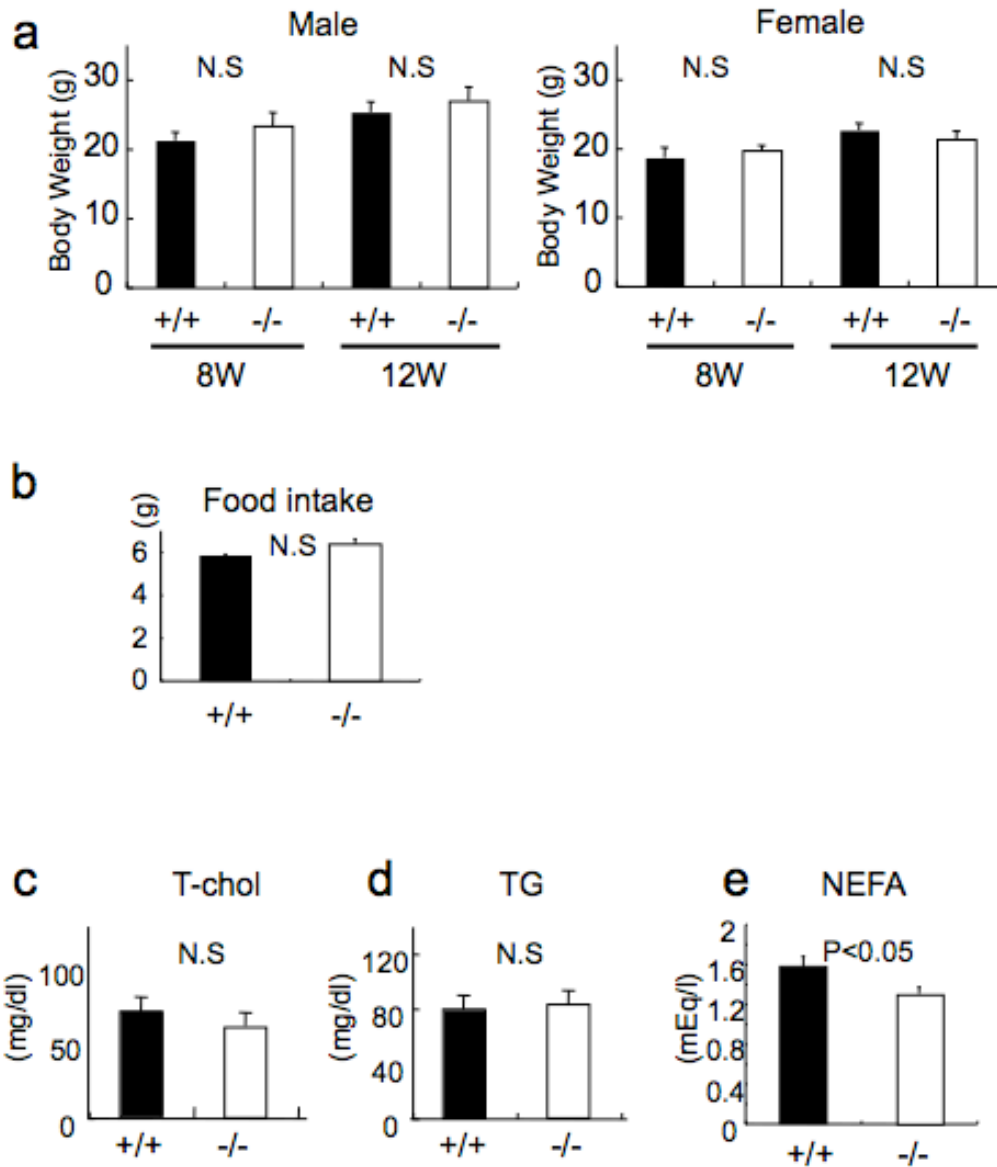
Supplementary Figure 1

a

17.2kb

AC1CASP1   AC1WSP1

2kb

Wild allele

AflII

Probe

Targeting vector

LacZ pA   Pr Neo   pA   Pr DT-A

loxP   frt   frt loxP

Targeted allele

AflII

Probe   22.6kb

PGKS1

AflII

b

Wt #33   Wt #86

22.6kb
17.2kb

5' probe

Wt #33   Wt #86

15.8kb

Neo probe

c

-/-   +/-   +/-

22.6kb
17.2kb

d

+/-   +/+   -/-   +/-   +/-

MT →
WT →

e

Relative chemerin expression level

1000
800
600
400
200
0

+/+   -/-

f

-/-   +/+

16kD chemerin protein

g

SAP promoter   Rat beta globin intron   Human chemerin cDNA   Poly A

PCR Tg
PCR Wt   Globin sense1 →   400bp
169bp

hchemerin sense1 →   hchemerin antisense1

h

Tg allele
+ + + - - -

9

Supplementary Fig. 1.   Targeted disruption of the *chemerin* gene and generation of chemerin transgenic mice

(**a**) Schematic representation of the targeting vector, and targeting strategy for disruption of the endogenous chemerin gene locus.   Closed boxes indicate exons. Arrows indicate the primers used for genotyping.   (**b**) Southern blot analysis of ES cells.   Two cell lines, #33 and #86 were obtained and successfully transmitted to the germline.   (**c**) Southern blot analysis of F1 offspring using the external probe indicated in *AflII* digestion yielded a 17.2-kb band for the wild-type allele and a 22.6-kb band for the mutant allele.   (**d**) An example of routine genotyping of F2 mice; the amplification with the primers AC1CASP1 and AC1WSP3.1 yielded a 264-bp product from the wild-type allele, and with primers AC1CASP1 and PGKS1 yields a 386-bp product from the mutant allele.   (**e**) QRPCR analysis of the chemerin expression in the liver from in chemerin (+/+) and (-/-) mice.   Adipose tissue analysis produced similar results (data not shown).   (**f**) Immunoblotting analysis of serum (10 μL) from chemerin (+/+) and (-/-) mice.   (**g**) Construction for serum amyloid protein (SAP) promoter-driven human chemerin transgenic mice.   (**f**) An example of routine genotyping for F2 mice; amplification with the primers Globin sense and hchemerin antisense 1 yielded a 400-bp product from the transgenic allele.
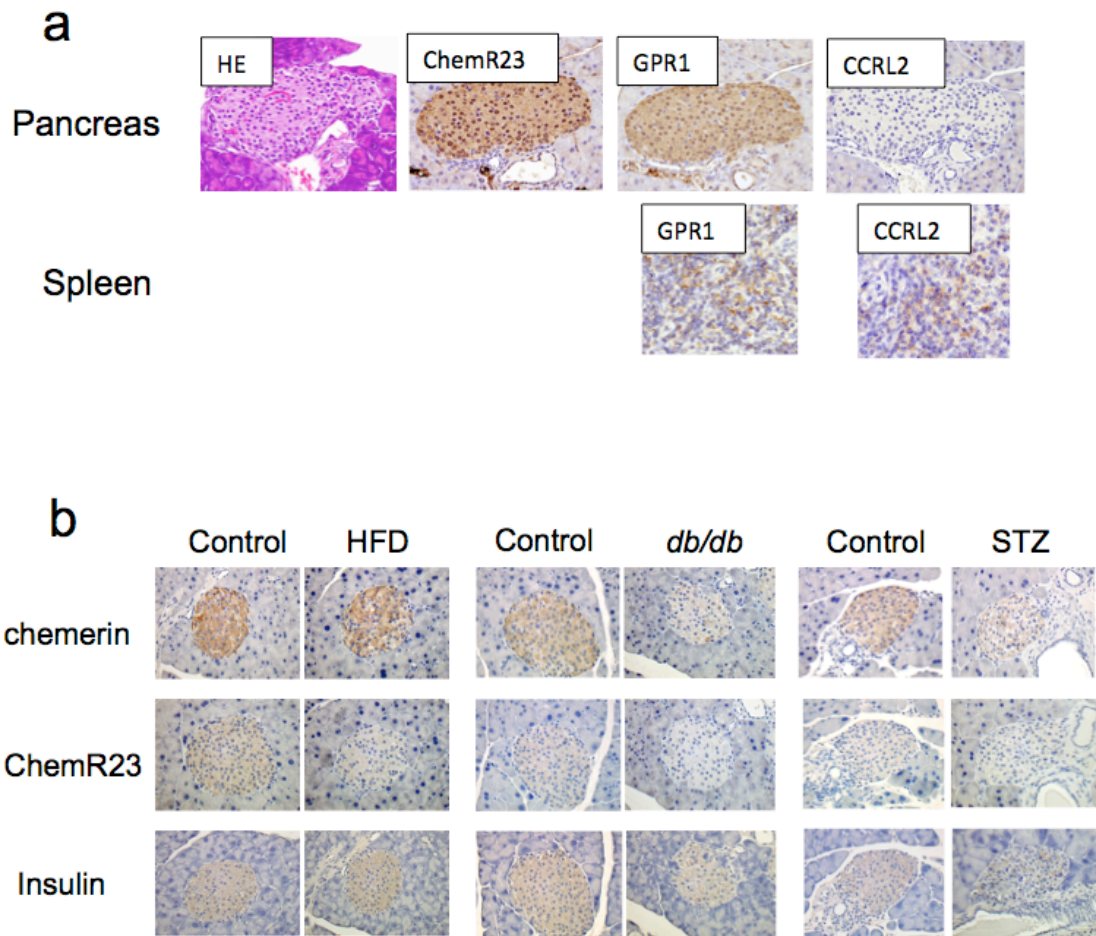
Supplementary Figure 2

Supplementary Fig. 2. Metabolic parameters in chemerin-deficient mice.
(a) Body weights of chemerin (+/+) and (-/-) mice were determined at 8 and 12 weeks of age. (n = 5-8/genotype). **(b)** Daily food intake of 10-week-old chemerin (+/+) and (-/-) mice was measured for 24 hr. (n = 5/genotype). **(c-e)** Serum T-cholesterol, triglyceride, and NEFA levels after 12-h fasting. (n = 5-8/genotype).
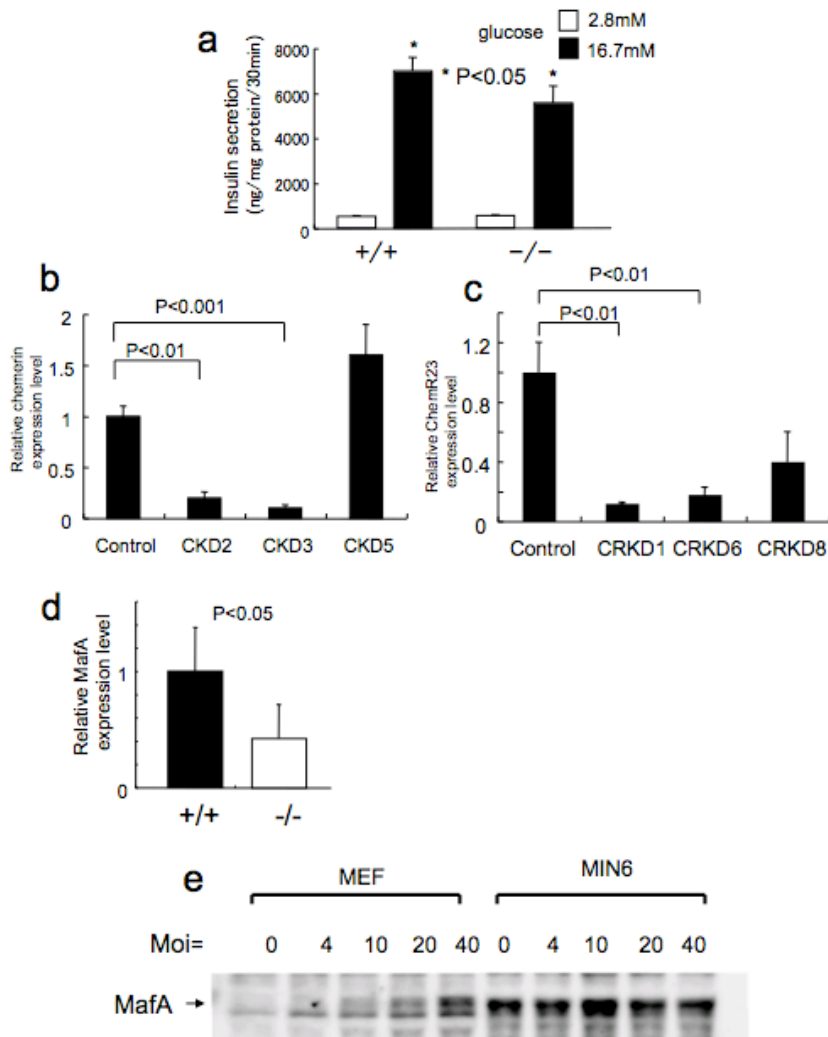
Supplementary Figure 3

Supplementary Fig. 3. Immunohistochemical analysis of chemerin, ChemR23, GPR1, and CCRL2.

(a) Immunohistochemical analysis of ChemR23, GPR1, and CCRL2 in pancreas and spleen. Tissues were obtained from male C57BL6 mice at 12-week-old. Spleen was used as the positive control for GPR1 and CCRL2 staining. (b) Immunohistochemical analysis of chemerin and ChemR23 expression in the pancreas of HFD-fed, *db/db*, and STZ-treated diabetic mice. Similar results were obtained from three independent experiments. Representative results are shown.

Supplementary Figure 4

Supplementary Fig. 4. **Knockdown of chemerin and ChemR23.**

(a) Insulin secretion in islets isolated from 7-8 week-old chemerin (+/+) and (-/-) mice. (n = 4 /genotype). **(b, c)** Knockdown of chemerin (**b**) or ChemR23 (**c**) in MIN6 cells. The knockdown efficiency was evaluated by QRPCR. In the GSIS assay, we used CKD3 for the knockdown of chemerin and CRKD1 for the knockdown of ChemR23, respectively. The sequences for knockdown are described in Supplementary Methods. **(d)** Quantitative analysis of MafA protein expression in islets from 7-8-week-old chemerin (+/+) and (-/-) mice. (n = 3/genotype). **(e)** Adenoviral expression of MafA in mouse embryonic fibroblasts (MEF; no endogenous MafA expression) and MIN6 cells (positive for endogenous MafA expression).