Supplemental methods

Cell Culture.

Isolated islets were maintained in RPMI 1640 medium (catalog number 61870; Invitrogen) containing 10% decomplemented fetal calf serum (catalogue number 10270; Invitrogen), 10 mM HEPES (catalog number H-3537; Sigma), 2 mML-glutamine (catalog number G-7513; Sigma), 1 mM sodium pyruvate (catalog number S-8636; Sigma) and penicillin (100 units/ml)/streptomycin (100 μg/ml) (catalogue number and 15140; Invitrogen) at 37 °C and 5%CO_2.

Chemicals and antibodies

The anti-phospho-(serine 473)-Akt rabbit polyclonal IgG antibody was from Cell Signaling Technology (catalogue number 9271). The antibody recognizing total Akt was from Santa Cruz Biotechnology (catalogue number A1205). The monoclonal antibody specific for the hemagglutinin (HA) tag was purchased as ascites from BabCo (Richmond, CA; catalogue number MMS-101R). This antibody was adsorbed on HeLa cell lysates to decrease nonspecific binding (1). The anti-RasGAP antibody directed at the Src homology domains of RasGAP was from Alexis (catalogue number ALX-210-860). The anti-insulin guinea pig polyclonal IgG antibody (catalogue number 4010-01) and the anti-glucagon rabbit polyclonal IgG antibody (catalogue number 4030-01F) were from Linco Research Inc. The anti-mouse Ki67 rat monoclonal IgG antibody, the EnVision+ system horse radish peroxidase (HRP)-labelled polymer anti-rabbit or anti-mouse antibodies and the liquid DAB+ substrate chromogen system were from Dakocytomation Denmark A/S (catalogue number M7249, K4003, K4001 and K3468, respectively). The Armenian hamster anti-CD3ε antibody (clone: 145-2C11) was from Santa-Cruz. The FITC-conjugated goat anti-guinea pig IgG polyclonal antibody (catalogue number 106-095-003), the Cy3-conjugated anti-Armenian hamster IgG polyclonal antibody (catalogue number 127-165-10), and the Cy3-conjugated goat anti-mouse IgG polyclonal antibody (catalogue number 115-165-003) were from Jackson ImmunoResearch Europe.
Glucose was from Merck (catalogue number 108342). Streptozocin, cisplatin, hydrogen peroxide and palmitate were from Sigma (catalogue number S0130, P4394, 349887 and P9767-5G, respectively). A 33 mM palmitate solution was prepared in a 33% ethanol solution (v:v in water). The dissolution required a 10 minute incubation at 65°C. The solution was then kept at 37°C and used within 5 minutes. Cytokines (TNFα, interleukin-1β, and interferon-γ) were from Alexis (catalogue number ALX-520-002-C010, ALX-520-001-C010, and PBL-11500-2, respectively).

Mouse islet isolation and dissociation

Islets were isolated after collagenase digestion as described (2). In some experiments, the islets were loosely dissociated as follows. The islets were washed with 1 ml of HBSS-Hepes containing 1 mM EGTA and 5 mM glucose, resuspended in 300 μl of the same buffer, and incubated at 37 °C for 3 min. The islets were then pipetted up and down until loosely dissociated. The reaction was stopped by the addition of 1 ml of culture medium. The islets were washed again with 1 ml of culture medium, resuspended in the same medium at a concentration of about 20 islets/ml before being placed in 6-well plates (2 ml/well).

Preparation of tissue sections and immunochemistry

Six to seven week old wild-type and RIP-N mice were deeply anesthetized (pentobarbital, 100 mg/kg i.p.) and transcardiacally perfused over a 10 min period with 150 ml of phosphate buffer saline (PBS; 116 mM NaCl, 10.4 mM Na_2HPO_4, 3.2 mM KH_2PO_4 [pH 7.25])/4% paraformaldehyde (PFA). Pancreatas and brains were isolated and post-fixed in PBS/4% PFA for 2 more hours.

For immuno-fluorescence labelling on frozen sections, pancreatas and brains were cryoprotected by overnight immersion in PBS/30% sucrose.

Pancreatas were then snap-frozen in isomethylbutane on dry ice (-30°C) and then kept at -80°C until sectioned. Frozen pancreatas were fully embedded in Tissue-Tek® Optimal Cutting Temperature Compound (Sakura Finetek Europe B.V.; catalogue number 4583) and 20 µm sections were obtained with a Leica CM3050 S Cryostat and mounted on SuperFrost® Plus slides (Menzel GmbH & Co KG; catalogue number J1800AMNZ). The slides were washed three times 5 minutes in PBS, followed by one 10 min-incubation in PBS, 0.0125% Triton X-100, and then incubated one hour in the antibody diluent solution 1 (ADS1; PBS containing 10% goat serum, 0.0125% Triton X-100, and 0.5% BSA) at room temperature, after which the appropriate dilution of the primary antibody was added and incubated over-night. The following day, the slides were washed three times with PBS for 15 minutes and incubated one hour with the secondary antibody in ADS1. The slide were then washed six times in PBS (last wash usually over-night), stained 10 minutes with Hoechst 33342 (2) and mounted (Vectashield mounting medium, Vector laboratories Inc).

Brains were frozen in liquid nitrogen and kept at -80° C until sectioned. Free-floating brain sections were washed three times 15 minutes with PBS and incubated one hour in the antibody diluent solution 2 (ADS2; PBS containing 10% goat serum, 0.3% Triton X-100, 2% BSA) at room temperature, after which the appropriate dilution of the primary antibody was added. The following day, slides were washed three times with PBS for 15 minutes and incubated one hour with the secondary antibody in ADS2. The slides were further washed three times for 15 minutes in PBS and mounted with a fluorescent protecting medium (Mowiol) on SuperFrost® Plus slides.

For HRP-labelled immunohistochemistry of RIP-N of pancreas slices, six to seven week old wild-type and RIP-N mice were deeply anesthetized (pentobarbital, 100 mg/kg i.p.) and transcardiacally perfused over a 10 min period with 150 ml of PBS/4% paraformaldehyde. Pancreatas were extracted and post-fixed in PBS/4% paraformaldehyde for 2 more hours. The tissues were then stored in formol until paraffin embedding. Eight µm sections were de-paraffined in xylene and re-hydrated in graded alcohols and distilled water. For the staining performed with the Ki67 antibody, antigen retrieval was performed by immersion of the slides in a vapour pot containing 1500 ml of Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA, 0.01% Tween 20, pH 9), boiled at 120°C for 2 minutes and finally washed with cold tap water. Endogenous peroxidase activity was blocked with methanol/hydrogen peroxide 1% for 15 minutes. Sections were blocked for 10 minutes with 10% goat serum diluted in 50 mM Tris-HCl pH 7.6, 1 mM EDTA. Primary antibodies were applied for 1 hour in 50 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.5% BSA. The secondary HRP-coupled antibody (EnVision+ System, DakoCytomation) was applied for 30 minutes according to the manufacturer's recommendations. A visualization reagent (3,3'-diaminobenzidine) was applied for 5 minutes and rinsed off in tap water. Sections were counterstained with Harris's haematoxylin, re-hydrated, and mounted (Vectashield mounting medium, Vector laboratories Inc). All the steps were performed at room temperature.

For immuno-histochemistry on paraffin-embedded sections, the sections were first de-paraffined as described in the previous paragraph and equilibrated 5 minutes in PBS and washed 3-4 times in the same buffer. The slides were then incubated 2 x 5 minutes with gentle agitation in PBS containing 0.025% Triton X-100. The slides were blocked in PBS containing 10% normal serum and 1% BSA for 2 hours at room temperature. They were then drained for a few seconds and the excess liquid removed with tissue paper. The slides were incubated with the primary antibody diluted in PBS, 1% BSA overnight at 4°C. The remaining steps were performed in the dark to avoid photo-bleaching. Following two washes with gentle agitation for 5 minutes in PBS, 0.025% Triton X-100, the fluorophore-conjugated secondary antibody, diluted in PBS, 1% BSA, was added to the slides and incubated for 1 hour at room temperature. The slides were then rinsed three times 5 minutes in PBS, stained with Hoechst 33342 (2), and finally mounted (Vectashield mounting medium, Vector laboratories Inc).
All pictures of fluorescent or HRP-labelled preparations were taken with a Zeiss AxioVis C1 microscope.

Transgene detection by PCR

The presence of the transgene was detected by PCR amplification of genomic DNA isolated from tail biopsies (see "Southern Blot" section). Sense (ACTCCAAGTGGAGGCTGAGA) and anti-sense (TCCTTCCACAAACCCATAGC) primers were used to amplify a transgenic-specific band of 204 bps. As an endogenous control for the PCR reaction, the sense (AGGAACACACCATCATCCAGG) and anti-sense (GGGGAGAACTTGTCAATGAAACA) primers were used to amplify an 804 bp-band from the MHC class II I-Eα gene. Touchdown PCR was performed as follows: one 5 minute-incubation at 94° C; 10 cycles using the following conditions: 30 seconds at 94° C, 30 seconds at 65° C for the first cycle or at a temperature decremented by 1°C in each subsequent cycle, and 45 seconds at 72° C; 25 cycles using the following conditions: 30 seconds at 94° C, 30 seconds at 55° C; 45 seconds at 72° C; a final elongation of 7 minutes at 72° C. The reactions were performed using *Taq* DNA Polymerase (Roche Applied Science, catalogue number 11647679001). The PCR products were analyzed on 1.5 % agarose gel containing ethidium bromide.

Quantitative PCR

Islets (50 per condition) were snap-frozen in liquid nitrogen and kept at –80°C until their RNA was isolated using the "High pure RNA tissue kit" (Roche; catalog n°12 033 674 001) as per the manufacturer's instructions. The RNA was then reverse-transcribed with the "Transcriptor high fidelity cDNA synthesis kit" (Roche; catalog n°05 091 284 001) following the manufacturer's instructions. Quantitative PCR assays were carried out on a real-time PCR detection system (iQ5; Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad catalog n° 170-8862), with 500 nmol/l primers (iNOS, sense: 5'-CAC CTT GGA GTT CAC CCA CT-3', antisense: 5'-ACC ACT CGT ACT TGG GAT GC-3'; S18, sense: 5'-GCA ATT ATT CCC CAT GAA CG-3', antisense: 5'-GGC CTC ACT AAA CCA TCC AA-3'), 1 □l of template per 20 □l of PCR and an annealing temperature of 55°C for S18 and 60°C for iNOS. Melting curve analyses were performed on all PCRs to rule out non-specific amplification. Reactions were carried out in triplicates. The iNOS mRNA levels were normalized against those of S18.

Insulin quantitation

Mice were euthanized by cervical dislocation and after a midline abdominal incision, pancreas was removed, placed in 2 ml of acid/ethanol (75% ethanol 1.5 % concentrated HCl) and homogenized using a Polytron PT 1200 homogenizer (Kinematica AG) until the pancreas was completely disaggregated. Once homogenized, 2 ml of acid/ethanol were further added to the samples and insulin was extracted by gentle rocking at 4° C overnight. Finally samples were centrifuged at 700 x *g* for 3 minutes and the supernatant was recovered. Insulin content, on the supernatant, was then quantified as previously described (2).

Western Blot Analysis

Cells were lysed in monoQ-c buffer (1). The primary antibodies were revealed with a 1/5'000 dilution of an Alexa Fluor 680–conjugated anti-rabbit antibody (Molecular Probes, Eugene, OR; catalogue number A21109) or an IRDye 800–conjugated anti-mouse antibody (Rockland, Gilbertsville, PA; catalogue number 610-132-121) and subsequently visualized with the Odyssey infrared imaging system (LICOR Biosciences, Bad Homburg, Germany).

Southern Blot.

DNA was purified from 2 to 5 mm mice tail biopsies. Briefly, tails were digested overnight at 56° C in 750 µl digestion buffer (100 mM NaCl; 10 mM Tris-HCl pH 8; 25 mM EDTA pH 8; 0.5 % SDS; 0.1 mg/ml proteinase K). The following day, samples were mixed 5 minutes at maximum speed in an Eppendorf Thermomixer Comfort mixer. Two hundred µl of a saturated solution of NaCl was added, the samples were mixed for 5 minutes at maximum speed in an Eppendorf Thermomixer Comfort mixer and centrifuged at maximum speed for 5 minutes in a benchtop Eppendorf centrifuge. The intermediate phase (~750 µl) was recovered and 500 µl of isopropanol was added. Samples were mixed and centrifuged as mentioned above. Finally the DNA pellet was washed with cold 70% ethanol and resuspended in 90 µl of Tris-EDTA buffer (10 mM Tris; 1 mM EDTA; pH 8.0).

DNA (30 µl) was digested with *Eco*RI overnight and separated on a 0.8 % agarose gel. The gel was later washed sequentially in 0.25 M HCl, for 10 minutes, 0.4 M NaOH for 30 minutes, in neutralizing solution (1.5 M NaCl; 0.5 M Tris-HCl pH 7.2; 1 mM EDTA pH 8) for 20 minutes and 5 minutes in 20x SSC (3 M Sodium Chloride; 0.3 M Sodium Citrate, pH 7.0).

The gel was transferred by capillarity overnight in 20x SSC to an Amersham Hybond™-N+ membrane (GE Healthcare; catalogue number RPN303B). The following day, the membrane was briefly washed in 2x SSC, air-dried and UV-fixed with 120 mJ (UV Stratalinker 2400; Stratagene). The membrane was then probed overnight in hybridization solution (0.5 M Sodium Phosphate pH 7.2; 7% SDS, 1mM EDTA and 1% BSA) with a probe corresponding to a 0.7 kb *Bam*HI/*Eco*RI fragment from the RIP-vmos.xf3 plasmid that contains the RIP1 promoter. The probe was labelled by random priming (Random Primed DNA Labelling Kit, catalogue number 1 004 760; Roche Applied Science). The probe recognizes a 2.8 kb transgene specific band and two 1.8 and 0.7 kb endogenous bands corresponding to the Ins1 and Ins2 promoters. Finally, the membrane was washed twice in washing Solution (40 mM Sodium Phosphate pH 7.2; 1% SDS; 5 mM EDTA and 0.5 % BSA) and visualized using a BioRad® Personal Molecular Imager FX™ System.

Nuclear protein extract preparation and electromobility shift assay (EMSA).

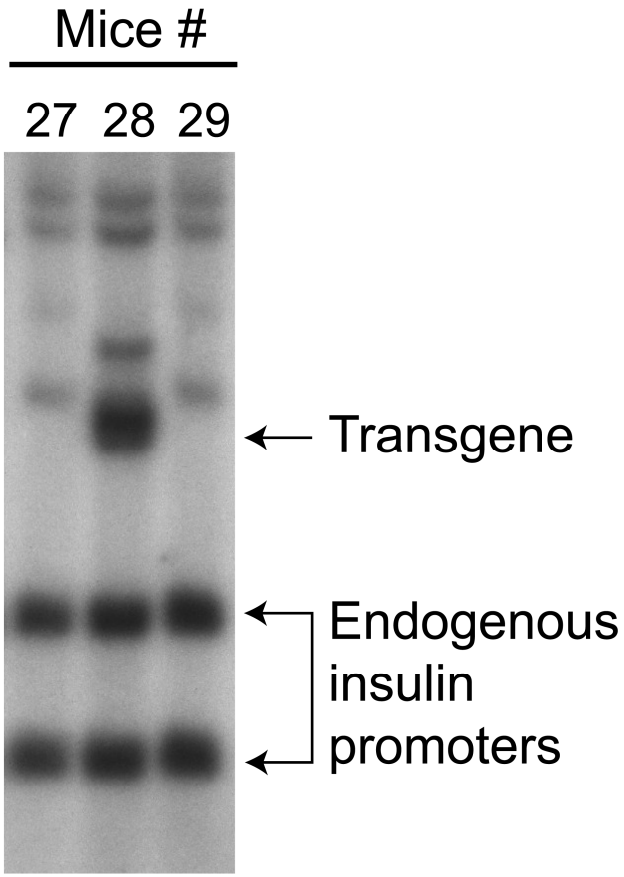
Nuclear extracts from islets were prepared as follows: 800 to 1'000 islets were collected in 1.5 ml Eppendrof tubes, washed with 500 µl of cold PBS, and centrifuged at 2'300 g for 5 minutes.

The pellet was resuspended in 400 µl ice-cold Buffer A (Hepes-KOH 10 mM pH 7.9, 1.5 mM $MgCl_2$; 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.2 mM PMSF, and 0.5% NP40). The islet cells were lysed using a Dounce homogenizer by applying ~15 strokes, rested on ice for 5 minutes and spun 10 minutes at 200 g.. The pellet containing the nuclei was resuspended in 50 µl of buffer C (Hepes-KOH 10mM pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA; 0.5 mM DTT, 25% glycerol, 0.2mM PMSF) and incubated on ice for 20 minutes (the tubes are flicked twice during this incubation period). Finally, the samples were spun 2 minutes at maximum speed in an Eppendorf centrifuge and the supernatant stored at -70°C until used.

The sequences of the oligonucleotides bearing the NF-κB binding elements were AGC TTC AGA GGG GAC TTT CCG AGA GGA GCT and CCT CTC GGA AAG TCC CCT CTG AAG CTA GCT. These oligonucleotides were hybridized and the created extremities filled using the Klenow fragment of DNA polymerase I (Roche Diagnostics) in the presence of deoxycytosine [□-^{32}P]-triphosphate (Amersham). Free nucleotides were separated by centrifugation through a G-50 column. Five □g of nuclear proteins in 20 □l of a solution containing 20 mM HEPES (pH 7.6), 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 250 mM KCl, and 2 □g of poly(dI-dC) were mixed with approximately 100 fmoles of double-stranded labeled oligonucleotides and incubated 20 min on ice. Samples were loaded onto a 6% non-denaturing polyacrylamide gel with 0.25× Tris-borate-EDTA buffer (0.5 mM EDTA, 22 mM boric acid, 22 mM Tris-base). The gels were fixed in a solution of 10% acetic acid and 30% methanol, dried, and exposed to Hyperfilm-MP (Kodak).

Supplemental Figures

Supplemental Figure 1: Identification of a second RIP-N founder. The progeny of the injected pseudo-pregnant mice were genotyped by Southern blot (see Material and Methods for details). Band A (2.8 kb) is specific for the transgene. Founder #2 corresponds to mouse #28.

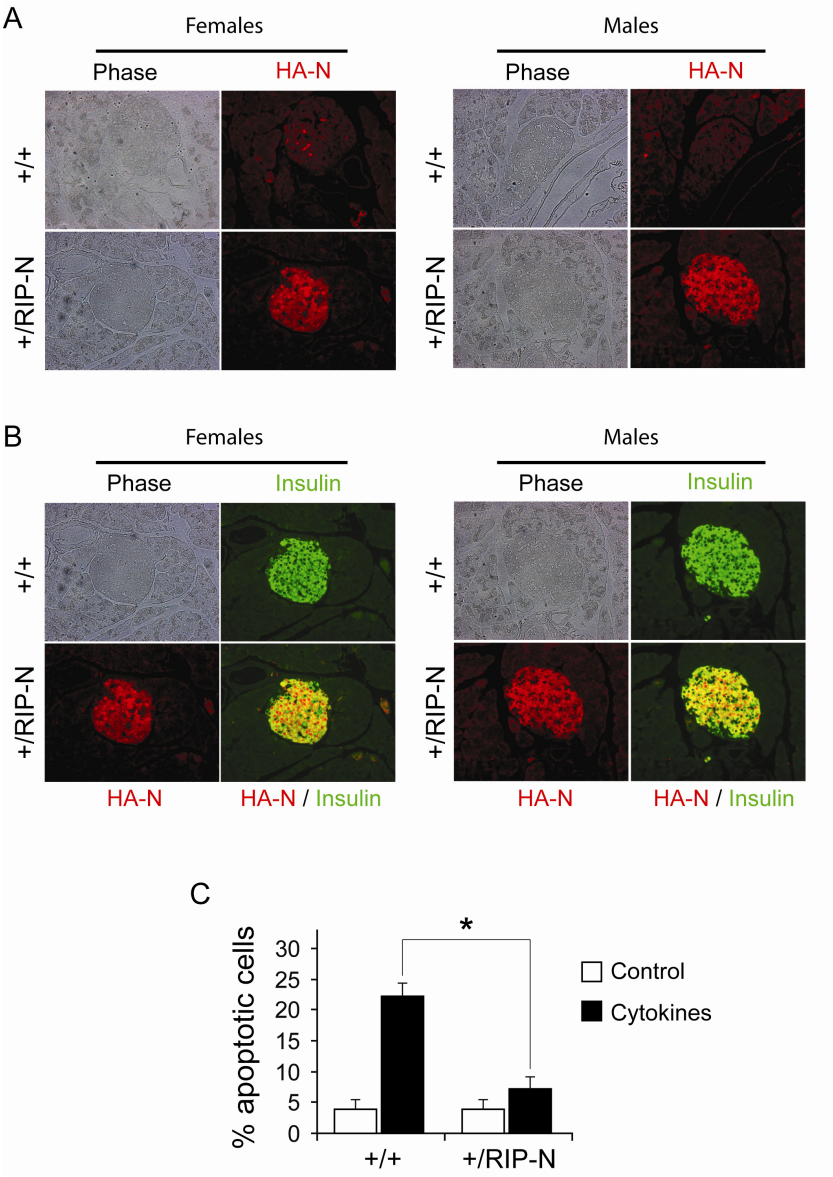


Supplemental Figure 2: Expression of fragment N in the islets of the progeny of founder #2 and resistance of these islets to cytokine-induced apoptosis.

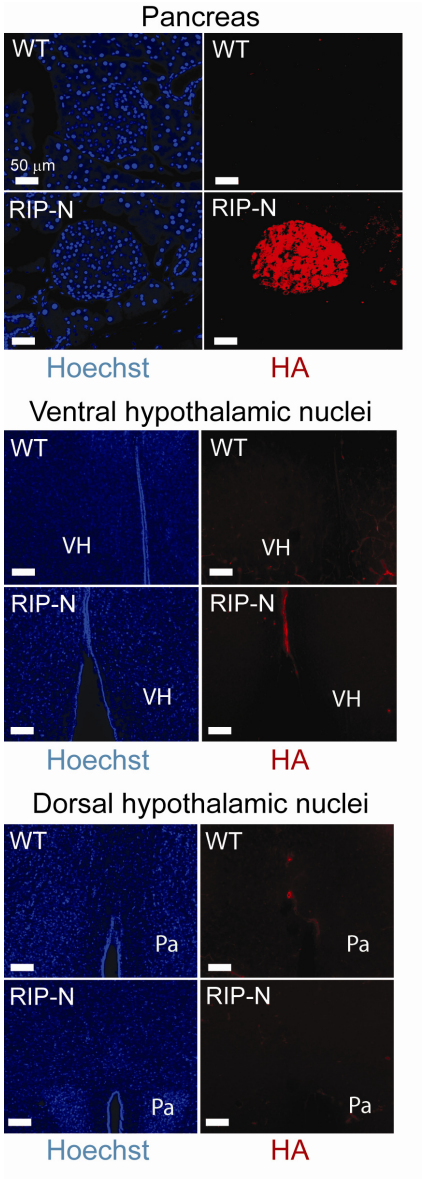
A. Expression of fragment-N in the pancreas. The presence of fragment N was assessed by immunofluorescense analysis of paraformaldehyde-fixed cryosections using an antibody recognizing the HA tag borne by fragment N.

B. Colocalization of insulin and fragment N. The specific location of fragment-N in pancreatic ß-cells was determined by immunofluorescence of paraformaldehyde-fixed cryo-sections from RIP-N mice using anti-insulin and anti-HA antibodies.

C. Freshly isolated islets were treated with inflammatory cytokines (1,000 units/ml TNFα, 1,000 units/ml interleukin-1□ and 50 units/ml interferon-γ) for 24 hours. The islets were then stained with Hoechst 33342 and apoptosis scored. The results correspond to the mean ± SD of 3 independent experiments (statistic analysis was performed for each condition between wild-type and RIP-N mice [3 comparisons]).



Supplemental Figure 3: No detectable expression of fragment N in the hypothalamus of RIP-N mice. The expression of fragment N was determined by immuno-histochemistry on frozen sections using an antibody directed against the HA tag of fragment N. Brain images are from areas of the hypothalamus in which the insulin promoter might be active (3). In the ventral hypothalamus, sections at corresponding levels in the wild-type (WT) and +/RIP-N (RIP-N) brains were taken in coronal sections around the level of Bregma -1.46 mm according to the atlas of Paxinos and Franklin (4), centred in the ventral hypothalamic nucleus (VH). In the dorsal hypothalamus, coronal sections of corresponding levels [around Bregma -1.06 mm, (4)] were centred on the paraventricular nucleus (Pa). The results are representative of three independent experiments performed on 9-12 week-old littermates originating from crossings between wild-type and +/RIP-N animals. Some non-specific staining of the endothelium and ventricular surfaces was observed in wild-type and +/RIP-N sections but no specific expression in neural tissues could be detected. Bar: 50 µm.



Reference List for the supplemental information

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