Supporting Information

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SI Methods

Mice. All animal experiments were conducted in accordance with the Swiss Animal Welfare Law and were approved by the Veterinary Authority of the Kanton Bern, Switzerland. Mice had free access to water and standard chow diet (no. 3436; Provimi Kliba AG) and were maintained on a 12-h light/12-h dark cycle. Generation of genetrapped NHA2 mice by our group was described in detail (1). Targeted NHA2 KO mice were generated by a homologous recombination approach with help of PolyGene AG. A 7-kb SmaI fragment containing solute carrier 9 B2 (*Slc9b2*)/*NHA2* gene exons 5–9 of a Sv129 BAC clone (Geneservice Ltd) was subcloned into pBluescript KS. From this, a 600-bp PshAI fragment harboring exon 7 of the *Slc9b2*/*NHA2* gene was cloned into PolyGene plasmid D245, which contains a loxP-flanked neo-Tk (thymidine kinase) positive/negative selection cassette plus an additional loxP site. The PshAI fragment with exon 7 was cloned between the distal loxP site and the loxP-flanked selection cassette. The targeting vector was finally generated by replacing the PshAI fragment of pBlueskript KS with a PmeI/BstBI fragment, containing the loxP-flanked exon 7 of *Slc9b2*/*NHA2*. The linearized targeting vector was electroporated into ES cells. G418-resistant ES cells with correct homologous recombination were electroporated with the pMC–Cre plasmid to excise the selection cassette together with exon 7 and then injected into blastocysts. Resulting male chimeras were bred with C56BL/6J females to yield heterozygous mice. Unless stated otherwise, all experiments were performed on littermates with mixed C56BL/6J–Sv129 background bred from heterozygous mice. Backcrossed NHA2 KO and WT mice used for experiments were backcrossed for >10 generations into C56BL/6J background. Backcrossing was verified by polymorphic microsatellite marker analysis ($n = 96$) at PolyGene AG.

Cell Culture, Transfection, and Min6 Insulin Release Assays. MIN6B1 cells (2) were provided by Philippe Halban (University of Geneva, Geneva) with permission from Jun-ichi Miyazaki (University of Osaka, Osaka), who produced the maternal MIN6 cell line (3), and grown in high-glucose DMEM supplemented with 10% heat-inactivated FBS and 70 μM β-mercaptoethanol. INS-1E cells were obtained from P. Maechler (University of Geneva) and grown in RPMI 1640 medium supplemented with 10% FBS and 50 μM β-mercaptoethanol. Transfection of Min6 cells with cDNA or siRNA was performed by using Lipofectamine 2000 according to the manufacturer's instruction by a reverse-transfection protocol with 0.5×10^6 cells per 12-well plate (Invitrogen). The two siRNAs targeting murine NHA2 (4) and the control siRNA were reported and validated (5). The human WT NHA2–HA construct in the pMH vector (Roche) was described (6). To generate functionally dead (DD) NHA2, two conserved aspartic acid residues (D278 and D279) were mutated into cysteines by site-directed mutagenesis (7). The GFP–Rab4 construct was obtained from A. Ferrer-Montiel (Universidad Miguel Hernandez, Alicante, Spain). The synaptophysin–GFP construct was a gift from B. Odermatt (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom). All constructs were verified by sequencing.

At 72 h after transfection, cells were washed three times with Krebs–Ringer bicarbonate Hepes buffer (KRBH) containing (in mM): 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 2 glucose, 10 Hepes, pH 7.4, and 0.1% (wt/vol) BSA. After an equilibration period of 2 h in this buffer at 37 °C, cells were incubated for 1 h in fresh KRBH buffer containing either 2 mM glucose or indicated secretagogues; for KCl stimulation, incubation time was 15 min (2). After this period, supernatants were harvested, plates were put on ice, and total cellular insulin was extracted by the addition of acid ethanol [70% (vol/vol) ETOH, 1.5% (vol/vol) HCL conc. (=12.5%)]. Secreted and cellular insulin were determined by ELISA (CrystalChem).

Antibodies. Generation and validation of the polyclonal NHA2 antibody 1.3 was described in detail (1). Other antibodies used in the study were from the following sources: monoclonal anti-NHE1 (Millipore); anti-protein disulfide isomerase (PDI; Stressgene); monoclonal anti–F-ATPase α-subunit and monoclonal anti-transferrin receptor (Invitrogen); monoclonal anti–γ-adaptin and monoclonal anti-early endosomal antigen 1 (BD Biosciences); polyclonal anti-actin and polyclonal anti-carboxypeptidase E (Santa Cruz Biotechnology); polyclonal anti-insulin and polyclonal anti-LAMP1 (lysosomal-associated membrane protein 1) (Abcam); polyclonal anti-rab4 (Cell Signaling); and monoclonal anti-synaptophysin, polyclonal anti-HA, and polyclonal anti-NHA2 (Sigma-Aldrich).

Subcellular Fractionation. Sucrose gradient equilibrium centrifugation and glycerol velocity gradient centrifugation were performed essentially as described (1, 8). Cells of six confluent 10-cm dishes were scraped into 1 mL of centrifugation buffer (5 mM Hepes, pH 7.4, 0.5 mM EGTA) containing 1× protease inhibitor mixture (Roche) and lysed by Dounce homogenization on ice (30 strokes). The homogenate was centrifuged (10 min, $3{,}000 \times g$, 4 °C), and postnuclear supernatants were then loaded onto the top of a continuous sucrose density gradient (0.45–2 M sucrose in homogenization buffer). After centrifugation ($200{,}000 \times g$, 18 h, 4 °C), fractions were collected from the top to the bottom of the gradient. Protein content of individual fractions was analyzed by using the DC protein assay (Bio-Rad), and insulin content was determined by ELISA. Fractions were then concentrated by trichloroacetic acid/acetone precipitation, and equal amounts of each fraction were separated by SDS/PAGE, followed by immunoblotting with indicated antibodies. For glycerol velocity gradients, cell supernatants were prepared as described above for sucrose gradients, except that the homogenization buffer was (in mM) 150 NaCl, 10 Hepes, 1 EGTA, and 0.1 $MgCl_2$, and was loaded onto a 5–25% continuous glycerol gradient with a 50% sucrose cushion. After centrifugation ($280{,}000 \times g$, 1 h, 4 °C), fractions were collected from the top of the gradient and analyzed by Western blotting as described for sucrose gradients.

Immunofluorescence and Immunohistochemistry. Cells were fixed in 4% paraformaldehyde in 1× PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 3 min, and blocked by 1.5% BSA and 10% goat serum in 1× PBS for 1 h. Fixed monolayers were incubated with primary antibodies in 1.5% BSA and 5% goat serum overnight at 4 °C. Then, after three times washing in 1× PBS, cells were incubated with the appropriate Alexa 488- or 568-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Cells were then washed three times for 10 min in 1× PBS, counterstained with DAPI (Invitrogen), and mounted on glass slides. Images were obtained through a Nikon C1 confocal microscope. Immunohistochemistry was performed on a Bond automated staining machine with NHA2 antibody 1.3 (dilution 1:500) and the Polymer Refine Detection Kit (Leica Microsystems). Specificity of the antibody was tested by using a multitissue microarray, constructed from formalin-fixed paraffin

embedded tissue. Normal pancreatic tissue was obtained from surgical specimens.

RNA Isolation, RT-PCR, and Real-Time PCR. RNA isolation and RT-PCR were performed as described (6). Primers overlapping two exons were used to avoid artifacts due to genomic DNA contamination. The following primers were used: NHA2_forward: 5′-TTTATCCAGTACTTCCCGAGCAGG-3′; NHA2_reverse: 5′-TTTCTGCTCTGAGAGACACAATGG-3′; β-actin_forward: 5′-AACCGTGAAAAGATGACCCAG-3′; β-actin_reverse: 5′-CCATCTCCTGCTCGAAGT-3′ (1). Real-time PCR was performed by using presynthesized Assays-on-Demand (AoDs; Life Technologies/ABI) on an ABI ViiA 7 System. The following AoDs were used: NHEDC1 (Mm00512342_m1), 3-hydroxybutyrate dehydrogenase (BDH2) (Mm00459075_m1), and β-actin (Mm00607939_s1). Cycle threshold (Ct) values for triplicate technical replicates were averaged, and the amounts of mRNA relative to β-actin were calculated by using the ΔCt method.

Yeast Complementation Assay. Yeast assays were performed exactly as described with the exception that the AP medium was buffered to pH 4.0 with 20 mM citric acid/Tris (6, 7, 9). Salt-sensitive growth was assayed at 30 h. For immunoblot, 10 OD units of yeast were washed once with 1 mM EDTA/H_2O and lysed in 2 M NaOH for 10 min on ice. Proteins were isolated by trichloroacetic acid/acetone precipitation, resuspended in Lämmli buffer (pH 6.8), separated by SDS/PAGE, and analyzed by immunoblotting.

Measurement of Intracellular Ca^{2+} in Islets. For cytosolic Ca^{2+} measurements ($Ca^{2+}{}_i$), intact islets instead of single dispersed cells were used because β-cell $Ca^{2+}{}_i$ oscillations undergo profound changes upon islet dissociation (10). Islets were loaded in RPMI medium with 2 μM Fura-2-AM dye (Invitrogen), which was dissolved in DMSO containing 0.007% pluronic acid for 30 min at 37 °C. Single islets were then transferred to a perifusion chamber with a coverslip bottom and perfused with buffer at 1 mL/min. The buffer contained (in mM): 140 NaCl, 3.6 KCl, 5 Hepes, pH 7.4, 2 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 2.5 $CaCl_2$, and 2 (basal), 12, or 20 glucose. Fura-2 fluorescence was measured at 37 °C with an inverted Nikon Eclipse TiU epifluorescence microscope equipped with a 40× S Fluor oil immersion objective. Images were taken with a Hamamatsu Orca-EG cooled, monochrome CCD camera. Islets were excited alternatively at 340 and 380 nm, and emitted light was collected at 515 nm with 1-Hz frequency. Cells at the periphery of the islets were excluded from analysis to minimize contribution of non–β-cells. Image acquisition and analysis were performed with SimplePCI 6.2 from CImaging. The $Ca^{2+}{}_i$ was calculated as described assuming a K_d of 224 nm (11). The maximum ratio (R_{max}) was achieved by addition of 1 mM ionomycin in the presence of 10 mM $CaCl_2$.

Transferrin, Cholera Toxin B, and Fluid Phase Uptake Assay. Min6 cells or isolated β-cells were washed and preincubated for 1 h in serum-free DMEM, then chilled on ice and incubated for 30 min with 50 μg/mL Alexa Fluor 568–human transferrin, 2 μg/mL Alexa Fluor 488–cholera toxin B, or 1 mg/mL lysine-fixable Texas Red–dextran 3000 MW (Invitrogen). After three washes in ice-cold serum-free DMEM, cells were put back to 37 °C for the indicated time. At the end of the incubation time, cells were washed three times in ice-cold serum-free DMEM and fixed with 4% paraformaldehyde. Image analysis was performed with ImageJ software. The region of interest was set according to the cell shape, and averaged fluorescence intensity of randomly selected cells (n = 30 per experiment) was measured.

Measurement of Endosomal pH. Fluorescence ratio imaging with pH-insensitive Alexa Fluor 568–transferrin and pH-sensitive FITC–transferrin (Invitrogen) was performed to measure endosomal pH as described (12). Cells were starved for 1 h at 37 °C in serum-free DMEM and then incubated for 1 h at 37 °C in HBSS (in mM: 137 NaCl, 5.3 KCl, 1.3 Ca_2Cl, 0.82 $MgSO_4$, 0.34 Na_2HPO_4, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 5.6 glucose, and 15 Hepes–NaOH, pH 7.4) containing 75 μg/mL FITC–transferrin, 50 μg/mL Alexa Fluor 568–transferrin, and 0.1% BSA. After extensive washing of the cells, FITC and Alexa Fluor 568 fluorescence were acquired at 37 °C with a Nikon C1 confocal microscope equipped with a 40× S Fluor oil immersion objective. A pH calibration curve was constructed by using a calibration solution containing 125 mM KCl, 25 mM NaCl, 10 μM nigericin, and one of the following buffers at a concentration of 25 mM: Hepes (pH 7.4 or 6.8) or MES (pH 6.2 or 5.5). Fluorescence intensities of the perinuclear recycling endosomes were quantified with ImageJ software. In a single experiment, endosomal pH values of 24 randomly selected living cells were measured and averaged.

Measurement of GABA Content and Secretion. After isolation, islets were incubated overnight in RPMI medium with 11 mM glucose. The next day, islets were washed twice with KRBH containing 2 mM glucose and then placed in 12-well plates (200 islets per mouse per well) containing KRBH with 2 mM glucose and preincubated for 2.5 h at 37 °C. GABA secretion into KRBH containing 20 mM glucose was then measured for 2 h. After this time, supernatants were harvested, plates were put on ice, and total cellular GABA was extracted by addition of 0.01 M HCl. Secreted and cellular GABA were determined by a GABA ELISA (Labor Diagnostika Nord).

Statistical Analysis. Statistical significance measurements were performed by Student t tests or ANOVA with Tukey's post hoc analysis for multiple comparisons, as appropriate. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

1. Hofstetter W, Siegrist M, Simonin A, Bonny O, Fuster DG (2010) Sodium/hydrogen exchanger NHA2 in osteoclasts: Subcellular localization and role in vitro and in vivo. *Bone* 47(2):331–340.
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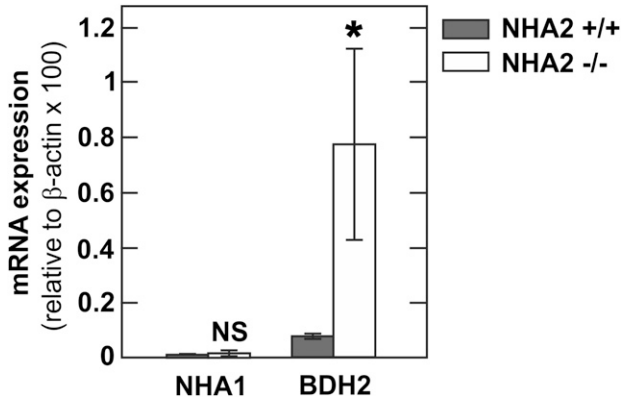

Fig. S1. Expression of NHA2-neighboring genes on murine chromosome 3 in isolated islets. Expression of NHA1 and BDH2 relative to β-actin was assessed by quantitative real-time PCR. Total RNA was isolated from NHA2 WT and KO islets (200 islets per mouse) and used for cDNA synthesis ($n = 3$ mice per genotype). Data are means ± SD. *$P < 0.05$ vs. WT; NS, not significant.

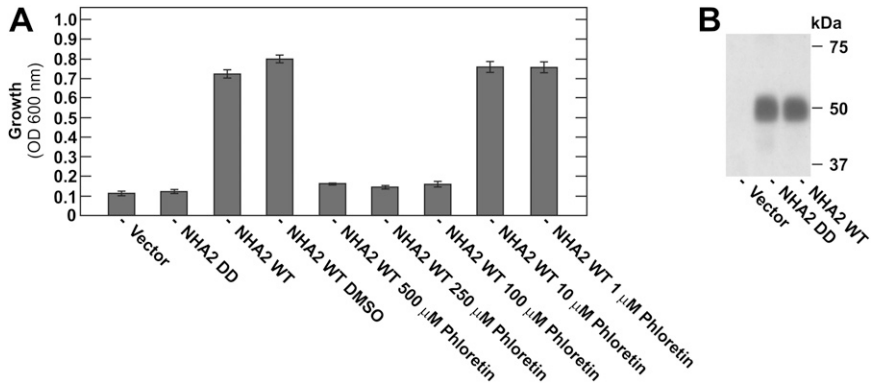

Fig. S2. NHA2 transport assay by functional complementation of salt-sensitive yeast. (*A*) The salt-sensitive yeast strain AXT3 was transformed with empty vector (Vector), C-terminally HA-tagged NHA2 (NHA2 WT), or the corresponding mutant with both D278 and D279 replaced by Cys (NHA2 DD). Yeast were grown in AP medium buffered with 20 mM citric acid/Tris to pH 4.0, supplemented with 25 mM LiCl and no additives, the vehicle DMSO, or indicated concentration of phloretin. Growth of yeast was determined by optical density of the culture at 600 nm (OD 600 nm) after 30 h at 30 °C ($n = 8$ per condition). (*B*) Immunoblot of yeast lysates transformed with indicated constructs. Equal amounts of yeast lysate of 10 OD units were loaded per lane.

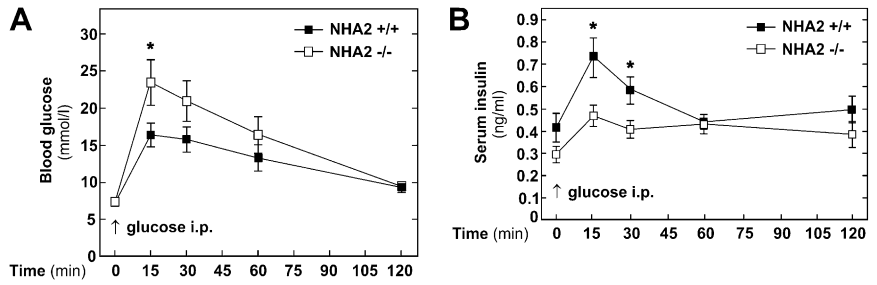

Fig. S3. Glucose tolerance test in backcrossed NHA2 KO and WT mice. (*A*) Blood glucose levels measured in whole blood following i.p. glucose challenge (2 g per kg of body weight) in backcrossed NHA2 KO and WT mice ($n^{+/+} = 13$; $n^{-/-} = 10$). Data are means ± SEM. *$P < 0.05$, WT vs. KO. (*B*) Insulin concentration measured in sera of backcrossed NHA2 KO and WT mice following i.p. glucose challenge (2 g per kg of body weight) ($n^{+/+} = 13$, $n^{-/-} = 10$). Data are means ± SEM. *$P < 0.05$, WT vs. KO.

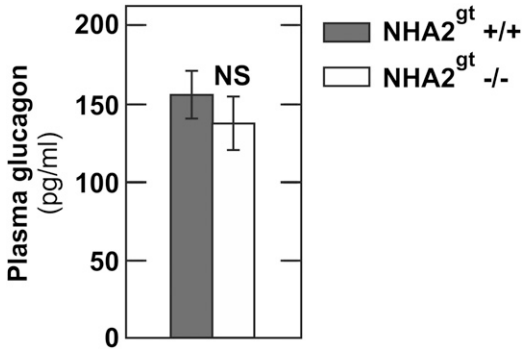

Fig. S4. Plasma glucagon concentrations of genetrap NHA2-deficient and WT mice after 6 h of food deprivation ($n = 6$ per genotype). Data are means ± SEM. NS, not significant.

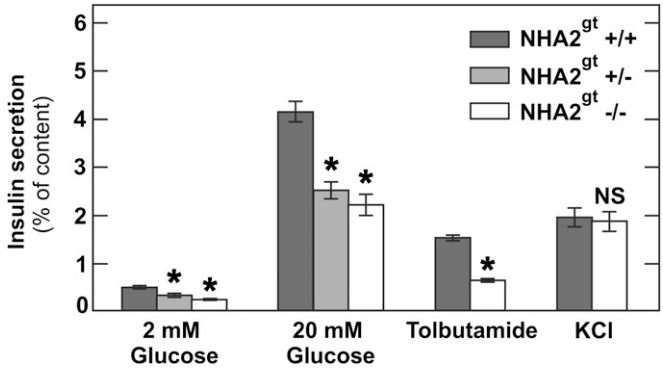

Fig. S5. Insulin secretion of NHA2 genetrap, heterozygous, and WT islets. Basal (2 mM) and glucose-induced (20 mM), tolbutamide-induced (250 μM), or KCl-induced (50 mM) insulin release by isolated islets normalized to insulin content ($n = 12$ per genotype and condition). Data are means ± SEM. *$P < 0.05$ vs. WT; NS, not significant.

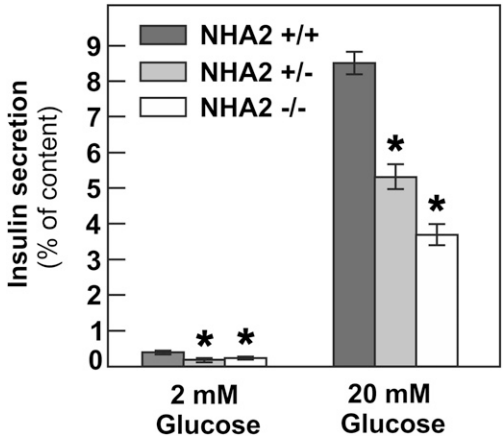

Fig. S6. Basal (2 mM) and glucose-induced (20 mM) insulin secretion of islets isolated from fully backcrossed NHA2 WT, heterozygous, and KO mice ($n = 10$ per genotype and condition). Data are means ± SEM. *$P < 0.05$ vs. WT.

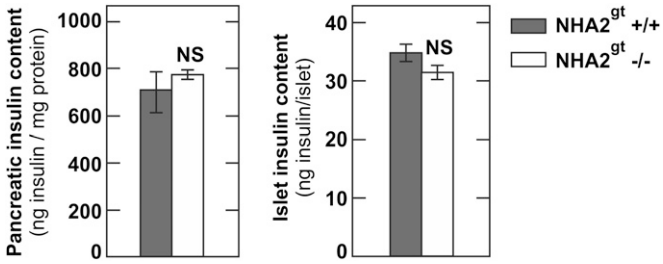

Fig. S7. Pancreatic ($n = 5$ WT and 3 genetrap mice; *Left*) and islet insulin content of NHA2 genetrap and WT mice ($n = 80$ per genotype; *Right*). Data are means ± SEM. NS, not significant.

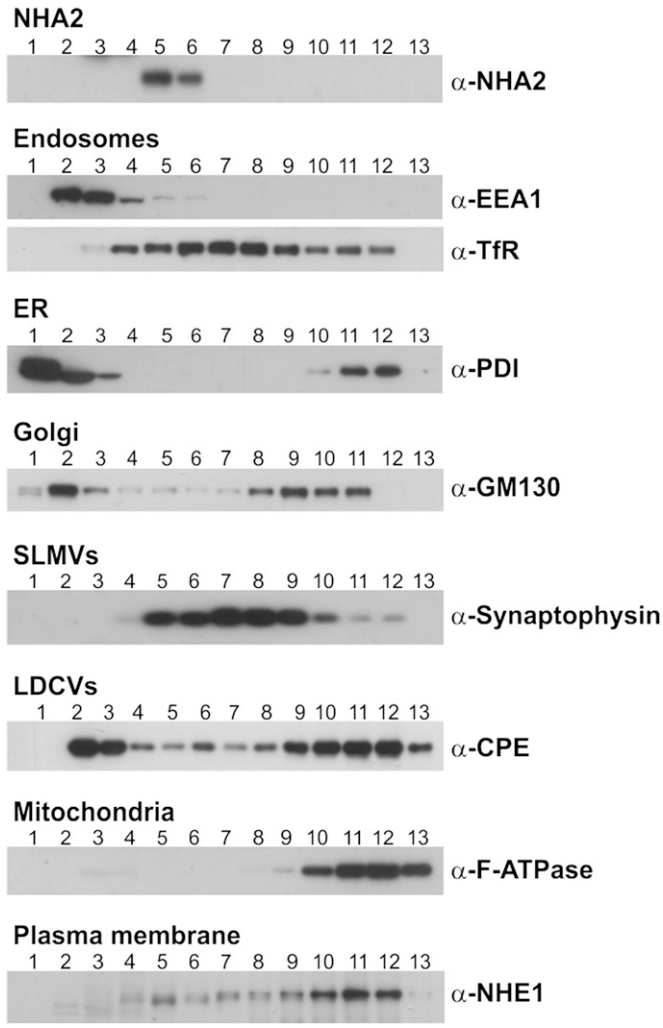

Fig. S8. Subcellular fractionation of rat INS-1E cells by linear sucrose gradient equilibrium density centrifugation. Data shown are representative of two independent experiments.

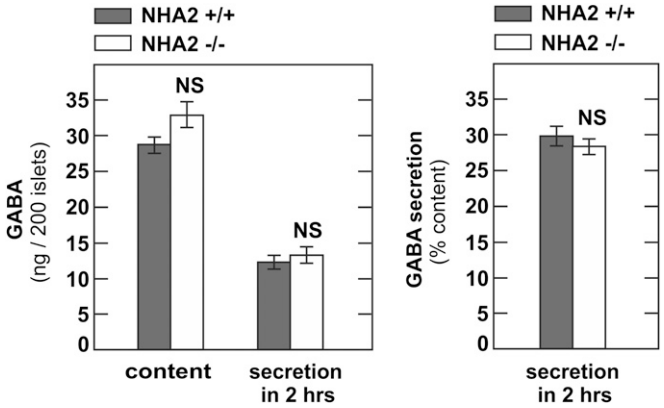

Fig. S9. GABA secretion and content of islets isolated from NHA2 WT and KO mice ($n = 6$ per genotype). Absolute content and secretion over 2 h in 20 mM glucose KRBH from 200 islets per mouse (*Left*) and secretion over 2 h in percentage of content (*Right*). Data are means ± SEM. NS, not significant.

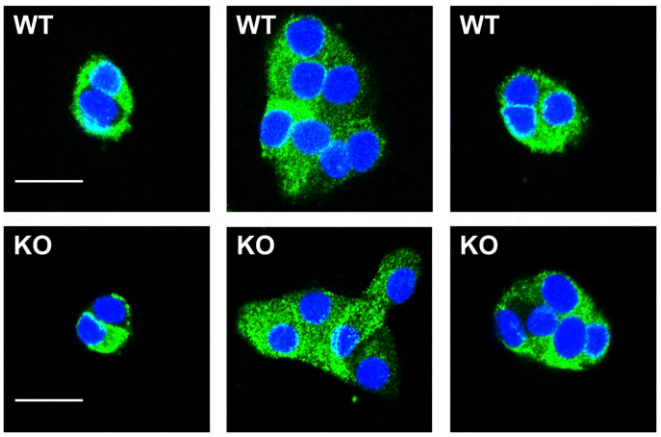

Fig. S10. Staining of primary β-cells isolated from NHA2 WT and KO mice and used for transferrin–Alexa Fluor 568 uptake with anti-insulin antibody. (Scale bars: 20 μm.)

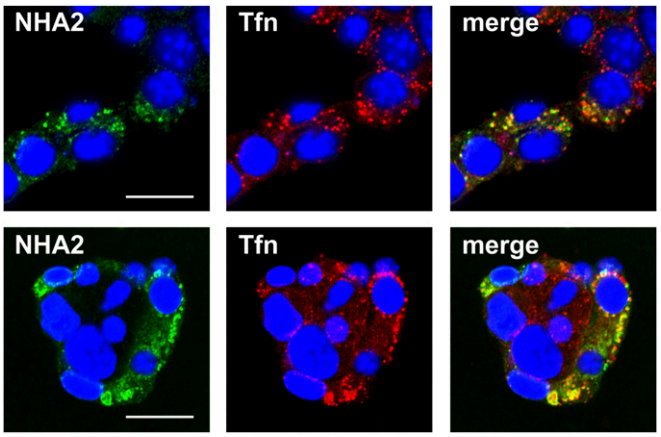

Fig. S11. Confocal images of Min6 cells transfected with HA-tagged NHA2 after 30-min incubation with transferrin–Alexa Fluor 568 at 37 °C. Colocalization is indicated by yellow in merged images. (Scale bars: 20 μm.)

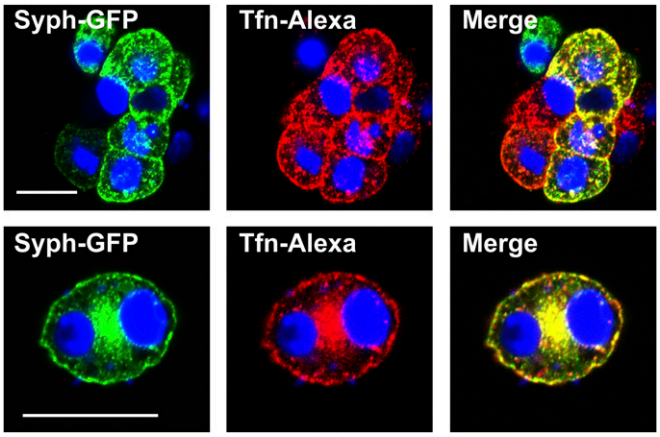

Fig. S12. Confocal images of Min6 cells transfected with GFP-tagged synaptophysin after 30-min incubation with transferrin–Alexa Fluor 568 at 37 °C. Co-localization is indicated by yellow in merged images. (Scale bars: 20 μm.)

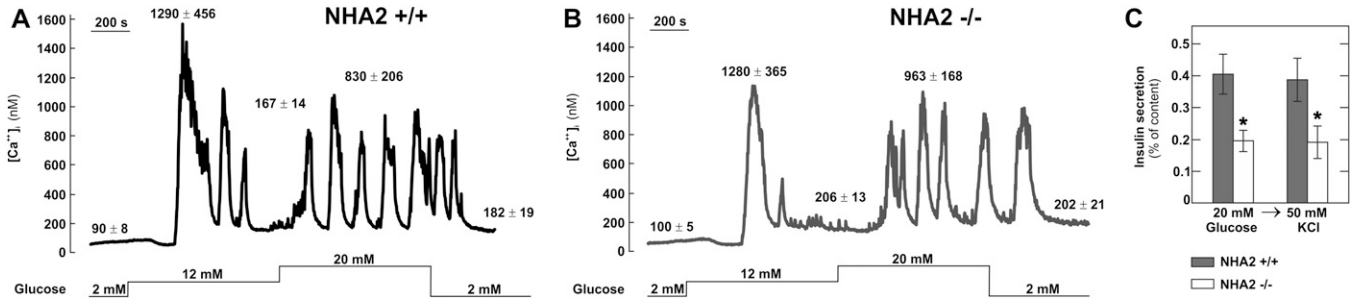

Fig. S13. Loss of NHA2 does not affect intracellular Ca^{2+} homeostasis in islets. (*A* and *B*) Representative recordings of the intracellular Ca^{2+} concentration in a WT and a KO islet. Glucose was changed as indicated. Values given above the traces are means ± SEM ($n = 8$ per genotype). No siginificant differences were observed between NHA2 WT and knockout islets. (*C*) KCl-induced (50 mM) insulin secretion of NHA2 WT and KO islets following a stimulation with high glucose (20 mM). Static insulin secretions were measured over a period of 15 min, separated by a period of 15 min with low glucose (2 mM) ($n = 10$ per genotype and condition). Data are means ± SEM. $^*P < 0.05$ vs. WT.

Table S1. Body weights of mice used for IPGTT and IPITT experiments in Fig. 3 and Fig. S2

Test	Transgenic line and genotype	Average weight ± SEM, g	*P* value
IPGTT	$NHA2^{+/+}$	24.88 ± 1.17	
	$NHA2^{+/-}$	23.19 ± 1.03	NS vs. $NHA2^{+/+}$
	$NHA2^{-/-}$	23.72 ± 1.16	NS vs. $NHA2^{+/+}$
IPITT	$NHA2^{+/+}$	24.36 ± 0.90	
	$NHA2^{-/-}$	23.05 ± 0.72	NS vs. $NHA2^{+/+}$
IPGTT	$NHA2^{+/+}$ backcrossed	23.78 ± 0.94	
	$NHA2^{-/-}$ backcrossed	23.68 ± 1.28	NS vs. $NHA2^{+/+}$ backcrossed
IPGTT	$NHA2^{gt\ +/+}$	24.08 ± 0.70	
	$NHA2^{gt\ -/-}$	23.08 ± 0.49	NS vs. $NHA2^{gt\ +/+}$
IPITT	$NHA2^{gt\ +/+}$	25.41 ± 0.57	
	$NHA2^{gt\ -/-}$	25.27 ± 0.69	NS vs. $NHA2^{gt\ +/+}$

IPGTT, i.p. glucose tolerance test; IPITT, i.p. insulin tolerance test; NS, not significant.