SUPPLEMENTARY DATA

Nardilysin is Required for maintaining Pancreatic β-Cell Function

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SUPPLEMENTARY INFORMATION INVENTORY

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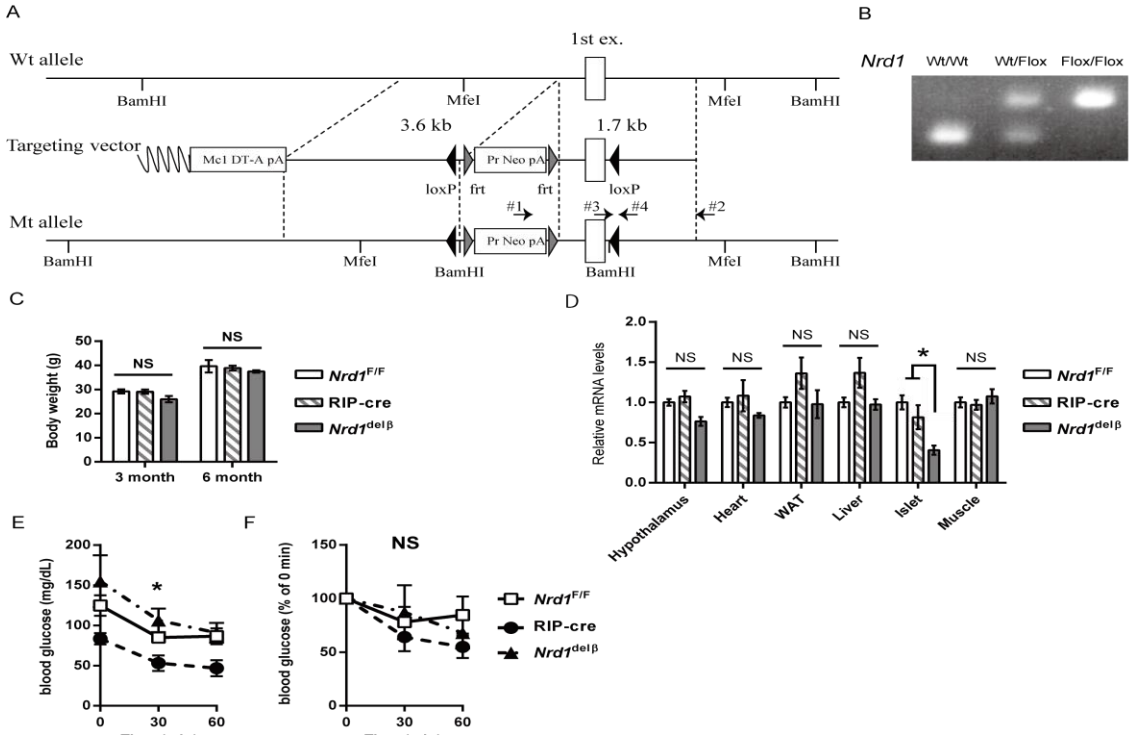
Supplementary Figure 1. (A) Structure of a part of *Nrd1*, a gene that encodes NRDc (top), the targeting vector (middle) and the mutated allele (bottom). The targeting vector was designed to insert loxP sites upstream and downstream of exon 1 of *Nrd1*. #1 (GTACTCGGATGGAAGCCGGTCTTGTC) and #2 (TCATTCAGGCAGGGTTTGAGTCACC) were the primers used for screening of the targeted ES cells. #3 (GCGATCCCAAGCAGTACCGGTG) and #4 (AAATTGCCCTGGCCGCCTCAC) were the primers used for routine genotyping of the mutant mice. (B) PCR genotyping of the *Nrd1*-floxed mice. The amplicon size is 174 bp for the wild-type allele and 234 bp for the floxed allele. (C) Body weight is similar between *Nrd1*$^{del\beta}$ and control mice (n=7-11 per genotype). (D) Relative mRNA levels of *Nrd1* in the indicated organs from 6-month-old *Nrd1*$^{del\beta}$ and control mice (n=4-5 per genotype). WAT: epididymal fat, Muscle: quadriceps femoris muscle. (E, F) Insulin tolerance test (i.p. injection of 2 U/kg insulin) reveals unaltered insulin sensitivity in 6-month-old *Nrd1*$^{del\beta}$ mice (n=5-6 per genotype). * $p<0.05$ for *Nrd1*$^{del\beta}$ vs. Rip-cre, NS: not significant



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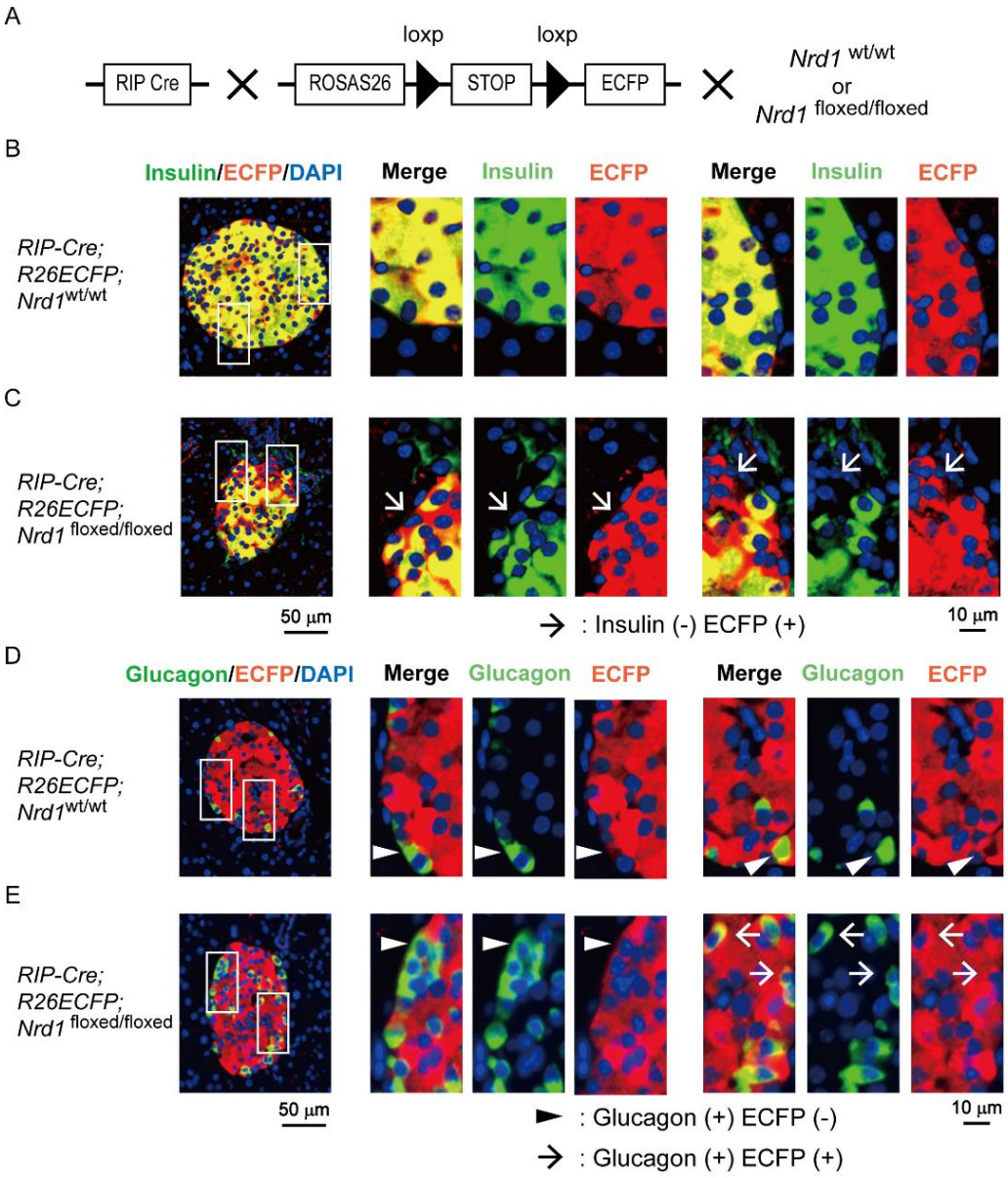
Supplementary Figure 2. (A) *Nrd1*$^{flox/flox}$ mice were crossed with RIPCre and R26ECFP mice to generate RIPCre;R26ECFP;*Nrd1*$^{flox/flox}$ and RIPCre;R26ECFP;*Nrd1*$^{wt/wt}$ mice. (B, C) Pancreata from 6- to 8-month-old RIPCre;R26ECFP;*Nrd1*$^{wt/wt}$ and RIPCre;R26ECFP;*Nrd1*$^{flox/flox}$ mice was stained with anti-insulin (green) and anti-ECFP (red) antibodies. The Arrow indicates insulin (-), ECFP (+) cell. (D, E) Pancreata from the mice described in (B, C) was stained with anti-glucagon (green) and anti-ECFP (red) antibodies. The Arrowhead and Arrow indicate glucagon (+), ECFP (-) and glucagon (+), ECFP (+) cells, respectively.



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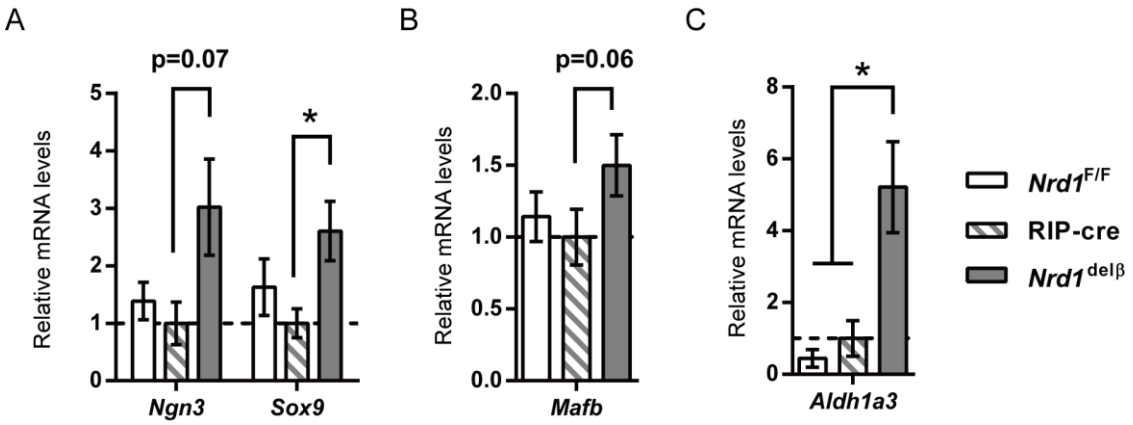
Supplementary Figure 3. Relative mRNA levels of *Ngn3, Sox9* (A), *Mafb* (B) and *Aldh1a3* (C) in isolated islets from 6- to 8-month-old *Nrd1*$^{del\beta}$ and control mice. Levels of RIP-cre control mice were normalized to 1 (n=4-5 per genotype). * p<0.05.



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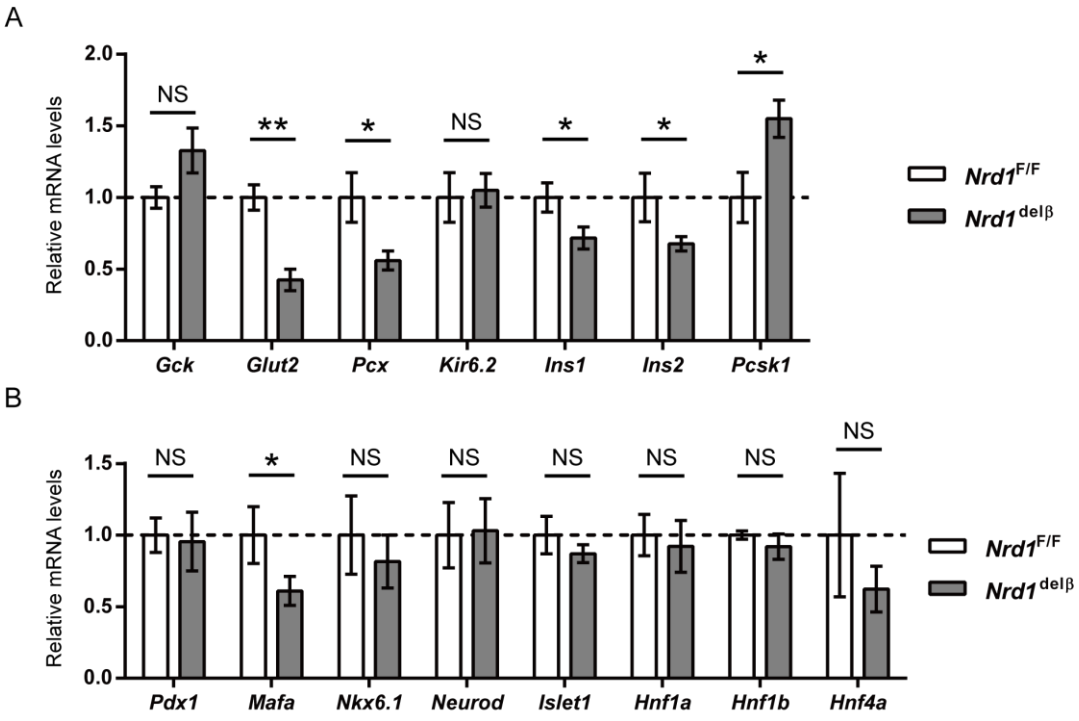
Supplementary Figure 4. Relative mRNA levels of genes involved in insulin secretion (A) and transcriptional factors (B) in isolated islets from 6- to 8-month-old *Nrd1*$^{del\beta}$ and *Nrd1*$^{flox/flox}$ mice, as measured by qRT-PCR. Levels of *Nrd1*$^{flox/flox}$ control mice were normalized to 1 (n=4-5 per genotype). * p<0.05, ** p<0.01.



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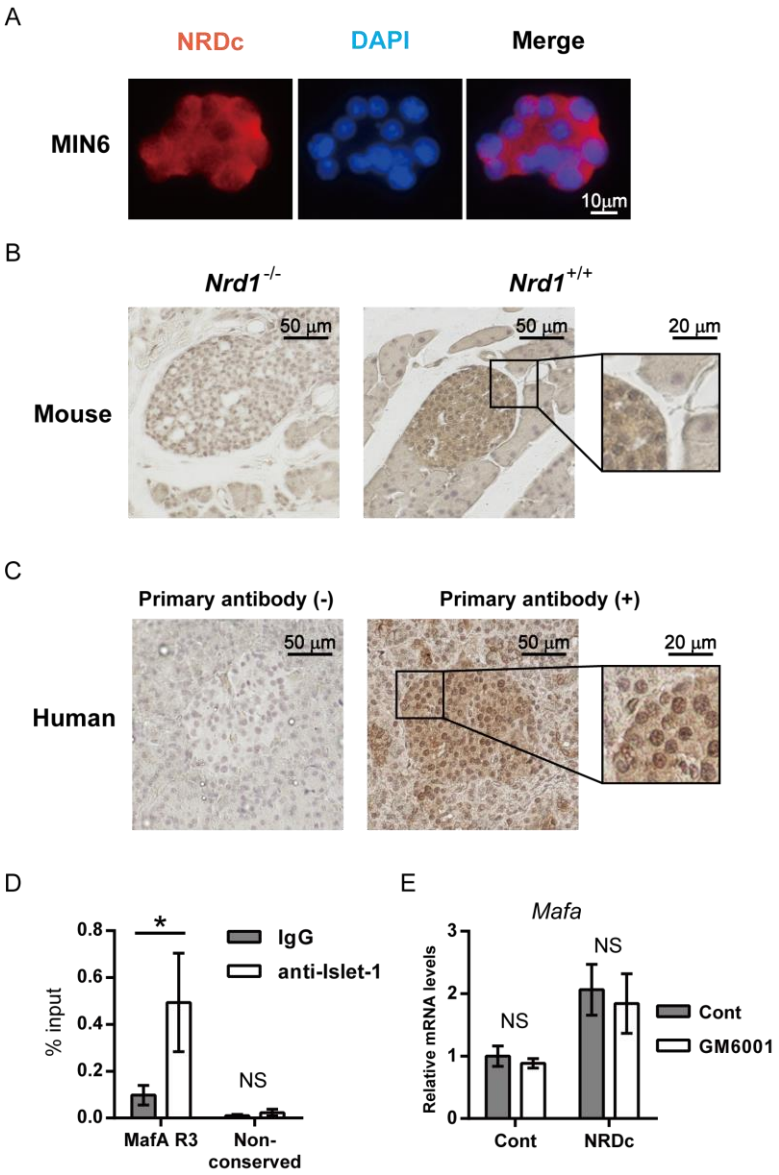
Supplementary Figure 5. (A) MIN6 cells were stained with anti-mouse NRDc antibody (red) and counterstained with DAPI (blue). (B, C) Pancreata from 3-month-old wild-type mice (B) and human (C) were stained with anti-mouse and human NRDc antibody, respectively, and counterstained with hematoxylin. (D) ChIP with anti- Islet-1 antibody followed by qRT-PCR demonstrates that Islet-1binds to the enhancer region of MafA. Results were normalized to input DNA (n = 6). MafA R3: -8118 to -7750 relative to the transcriptional start site (TSS) of *Mafa*; Non-conserved: -1678 to -1560 relative to the TSS of *Mafa*. (E) Relative mRNA levels of *Mafa* in control or NRDc-overexpressing INS 832/13 cells. Cells were treated for 24 hours with or without GM6001 (25μM) (n=5).



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Supplementary Table 1. List of primers used in qRT-PCR.

Gene	Forward	Reverse
M,R Nrd1	ATGGATGGCCTTTCCCTTG	CGCGAAGTTCAGCTTGTCAA
M Gck	TGTACAAGCTGCACCCGAGCTTCA	TGGATTTCACTGGCCCAGCATGCAA
M Glut2	CATTCTTTGGTGGGTGGC	CCTGAGTGTGTTTGGAGCG
M Pcx	GATGACCTCACAGCCAAGCA	GGGTACCTCTGTGTCCAAAGGA
M Kir6.2	AGGGGTCCTGTGCTTGCCATACAA	AGACAAAGTGACTCACAGCTGCCCA
M Ins1	GAAGCGTGGCATTGTGGAT	TGGGCCTTAGTTGCAGTAGTTCT
M Ins2	AGCCCTAAGTGATCCGCTACAA	CATGTTGAAACAATAACCTGGAAGA
M Pcsk1	GACCTGCACAATGACTGCAC	GGTCCAGACAACCAGATGCT
M Pdx1	CATCTCCCCATACGAAGTGC	GGGGCCGGGAGATGTATTTG
M Mafa	TTCAGCAAGGAGGAGGTCAT	CCGCCAACTTCTCGTATTTC
M NKx6.1	GAGTGATGCAGAGTCCGCCGT	GCTGTCCAGAGAACGTGGGTCTG
M Neurod	ATTCGCCCACGCAGAAGGCA	GACCTTGGGGCTGAGGCTCG
M Hnf1a	GTGGCGAAGATGGTCAAGTC	GCGTGGGTGAATTGCTGAG
M Hnf1b	GCCTGAACCAATCCCACCTC	TGACTGCTTTTGTCTGTCATGT
M Hnf4a	TAACACGATGCCCTCTCACCT	GGCAGGAGCTTGTAGGATTCA
M tIslet-1	CGGAGAGACATGATGGTGGTT	GGGCTGATCTATGTCGCTTTGC
M Sox9	GTACCCGCATCTGCACAACG	GATTGCCCAGAGTGCTCGC
M Gapdh	CCAGAACATCATCCCTGCATC	CCTGCTTCACCACCTTCTTGA
M β-actin	CTGACTGACTACCTCATGAAGATCCT	CTTAATGTCACGCACGATTTCC
R Glut2	TCAGCCAGCCTGTGTATGCA	TCCACAAGCAGCACAGAGACA
R Gck	AGTATGACCGGATGGTGGAT	CCGTGGAACAGAAGGTTCTC
R Pcx	TTGAAGGATGTGAAGGGCC	ACCTTTCGGATAGTGCCCTC
R Ins1	ACCTTTGTGGTCCTCACCTG	AGCTCCAGTTGTGGCACTTG
R Pcsk1	CAGGGGAGACAAGGAAAAG	AGGCACTGCTGATAGAGATGG
R Pdx1	GGATGAAATCCACCAAAGCTC	TTCCACTTCATGCGACGGT
R Mafa	CGAGTACGTCAACGACTTCG	AAGAGGGCACCGAGGAGCAG
R Nkx6.1	TCTTCTGGCCTGGGGTGATG	GGCTGCGTGCTTCTTTCTCCA
R β-actin	GATTACTGCCCTGGCTCCTA	TCATCGTACTCCTGCTTGCT
ChIPMafA R3 (-8118 to -7750)	CACTCAGCCTTGTTTAGGAGAGAA AAGA	CCGAGCTGCGTGGGGTTTAT
ChIPMafA Non-conserved (-1678 to -1560)	CCAGTTGCTTTTCACGGCCTC	CGGGGAGCCATTGGAATGTC

M = mouse; R = rat

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Supplementary Experimental Procedures

Plasmids

cDNAs encoding the full lengths of mouse PDX-1, Islet-1 and NeuroD were cloned into pME18s-FLAG. Deletion mutants of Islet-1, pME18s- Islet-1 $_{1\text{-}261}$ and pME18s- Islet-1 $_{158\text{-}349}$, were generated by PCR. pcDNA3.1-mNRDc-V5 was constructed as described previously (Nishi et al., 2006). Transfections were carried out using polyethylenimine (Polysciences) according to the manufacturer's instructions.

Antibodies and immunoblot analysis

Rat anti-mouse NRDc monoclonal antibodies (clone #1 and #135) were raised against recombinant mouse NRDc in our laboratory (Hiraoka et al., 2014). Mouse anti-human NRDc monoclonal antibody (#102) was raised against the GST fusion protein containing the N-terminal fragment (M^{50} to D^{129}) of human NRDc in our laboratory. For the production of mouse anti-mouse NRDc monoclonal antibody (2E6), *Nrd1*$^{-/-}$ mice were immunized with the C-terminal fragment of mouse NRDc (G^{348} to K^{1161}), which was synthesized by using silkworm protein expression system (Sysmex). Other antibodies were from the following sources: lamin A/C (Santa Cruz), β-actin (Santa Cruz), FLAG (Sigma), Islet-1 (Abcam), Glut2 (Santa Cruz), NeuroD (Santa Cruz), Pdx1 (Santa Cruz) and MafA (Bethyl).

Preparations of total cell extract and the immunoblot experiment were carried out as described previously (Hiraoka et al., 2014). In brief, cells were lysed in buffer containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma). Cell lysates were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. After blocking, filters were incubated with primary antibodies, followed by horseradish-peroxidase-conjugated secondary antibodies. The immobilized peroxidase activity was detected with an enhanced chemiluminescence system (Millipore). For the isolation of nuclei, cells were suspended in hypotonic buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma), followed by homogenization and centrifugation (3000 r.p.m., 5 min). Isolated nuclei were then resuspended in high-salt buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 400 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma), followed by centrifugation (13,200 r.p.m., 5 min).

Immunostaining and Histological analysis

Immunocytochemistry was performed as described previously (Nishi et al., 2006). Briefly, fixed MIN6 cells were incubated with rat monoclonal anti-mouse NRDc antibody (clone #135), followed by incubation with the secondary antibody (Alexa Fluor 488-conjugated goat antibody to mouse IgG) and counterstaining with DAPI. Immunohistochemistry was performed as described previously (Ohno et al., 2014; Ohno et al., 2009). For mouse islet, we used anti-mouse monoclonal NRDc antibody (clone #135), guinea pig antibody to insulin (Dako, 1:800), rabbit antibody to glucagon (Dako, 1:400) and rabbit antibody to MafA (Abcam, 1:500). After incubation with the primary antibodies, the sections were washed in PBS and incubated with secondary antibodies, Cy3-conjugated donkey antibody to guinea pig IgG (Millipore, 1:500) and Alexa Fluor 488-conjugated donkey antibody to rabbit IgG (Invitrogen, 1:200), for 1 h, followed by counterstaining with DAPI. For lineage tracing study, pancreatic sections from *Nrd1*$^{floxed/floxed}$; RIP-Cre; R26ECFP and *Nrd1*$^{wt/wt}$; RIP-Cre; R26ECFP mice were compared. Mouse antibody to glucagon (Santa Cruz, 1:800) and rabbit antibody to ECFP (Sigma, 1:100) were used as primary antibodies.

Pictures of immunostained sections were acquired using a BZ-9000 digital microscope (Keyence) and analyzed with dedicated software (Dynamic Cell Count System, BZ-HIC). For the estimation of β-cell mass, four pancreatic tissue sections (at least 50 μm apart each other) were analyzed in each mouse. Pictures of the whole pancreas were obtained by combining 48-108 pictures at magnification x4 in each section using the image stitching function of BZ-9000. The ratio of insulin-positive area to pancreatic area was calculated in each section,

and the proportions of four sections were averaged and multiplied by the pancreatic weight of each mouse. The ratio of α-cell area to α- and β-cell area was estimated by the calculation of the proportion of glucagon-positive area to insulin- and glucagon-positive area, which was calculated by using pictures at magnification x10 acquired from the same section used for the estimation of the β-cell mass.

For the immunostaining of human pancreata, non-cancerous regions from surgically resected specimens were obtained from patients with pancreatic cancer or neuroendocrine tumor admitted to Kyoto University Hospital. Written informed consent for the use of resected tissue samples was obtained from all patients in accordance with the Declaration of Helsinki, and the study was approved by Ethical Committee on Human Research of Kyoto University Graduate School of Medicine. For the primary antibody, we used mouse monoclonal anti-human NRDc antibody (clone #102).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was purified from freshly isolated islets, MIN6 cells and INS 832/13 cells using Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was carried out using the LightCycler 96 system (Roche) and THUNDERBIRD SYBR qPCR (TOYOBO, Japan) following the manufacturers' directions. The results were standardized for comparison by measuring the level of Gapdh or Actb mRNA in each sample. The primers used are listed in Table S2.