Supporting Information

Louagie *et al.* 10.1073/pnas.0800340105

SI Materials and Methods

Measurement of Islet Area and Number of Islets [supporting information (SI) Fig. S1]. Pancreata from adult $fur^{flox/flox}$ or $fur^{flox/flox}$, *Pdx1-Cre* mice were fixed in 2% formaldehyde in PBS before paraffin-embedding and sectioning. Antibodies to insulin (NovoCastra, Newcastle, U.K.), glucagon (NovoCastra, Newcastle, U.K.), or both were used to stain islets, and the sections were counterstained with hematoxylin (Sigma). Micrographs of complete sections were assembled from pictures taken at 10x magnification. The relative size of islets was measured by overlaying the micrographs with a grid and counting the number of intersections with islets as a percentage of the number of intersections with all pancreas tissue. To count total pancreas islets, anesthetised mice were injected with 500 μl of 10 mg/ml dithizone (Sigma). Fifteen minutes after injection, mice were killed and the pancreas removed. After weighing, ≈100 mg of the pancreas was compressed between two microscope slides and scanned. The number of dark red dithizone stained islets was recorded.

Electron Microscopy (Fig. S1). Mice were perfusion-fixed with 3% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), the pancreas dissected and postfixed in 1% osmium tetroxide. Small pieces of the tissue (<1 mm in all dimensions) were embedded in Araldite (Agar Scientific Ltd., Essex, U.K.) according to standard procedures. Ultrathin sections were cut on a Ultracut UCT ultramicrotome (Leica), and stained with 2% aqueous uranyl acetate solution and 0.2% lead citrate in 0.1 M NaOH. The stained sections were examined in a Philips CM100 transmission electron microscope and photographed at ×21,000 magnification.

Radiolabeling of Islets of Langerhans and Immunoprecipitation (Fig. S2). Isolated islets were radiolabeled and immunoprecipitated as described (1) by using glucagon antiserum (Linco Research), CPE antiserum (provided by J. C. Hutton, Denver) (2) and 7B2 antiserum (kind gift from I. Lindberg, Baltimore) (3). However, radiolabeling of islets for CPE and 7B2 immunoprecipitation consisted of 1 h preincubation and 2 h labeling with 300 μCi [^{35}S]methionine/cysteine (1 Ci = 37 GBq; Easytag express protein labeling mix, PerkinElmer; specific activity, 1,175 Ci/mmol), after which islets were lysed.

***Fur*$^{flox/flox}$ *Pdx1-Cre* Mice: Detailed Information.** To confirm deletion of the floxed *fur* exon 2, DNA was extracted by incubating isolated pancreatic islets in 50 mM KCl/10 mM Tris-HCl (pH 8.3)/2 mM $MgCl_2$/0.1 mg/ml gelatin/0.45% Nonidet P-40/0.45% Tween 20/120 μg/ml proteinase K at 55°C for 1 h, followed by 10 min at 95°C. Standard PCR was carried out with primers, 5′-GACTCTCCAAGGAGCGACTG-3′, 5′-GAGGACCCACAAGAACATGC-3′.

Glucose Tolerance Test. Ten age-matched (2-month-old) and sex-matched (male) mice per genotype were fasted 16 h before glucose tolerance tests were performed. Glucose (1 mg/g body weight) was administered via i.p. injection. Blood was withdrawn from the tail vein at 0, 15, 30, 60, and 120 min. Blood glucose levels were measured with the OneTouch Ultra blood glucose monitoring system (Lifescan).

Immunofluorescence: Detailed Information. Cells were washed three times with Hanks' balanced salt solution (1.67 mM glucose). Indirect immunofluorescence was essentially performed as described (4). Briefly, cells were fixed 30 min in 4% formaldehyde, 4% sucrose in PBS, washed three times in PBS, permeabilized in PBS containing 0.5% TX-100 for 5 min, and washed again three times in PBS. Blocking was performed for 1 h in a humid chamber at 4°C after 5% goat serum (species secondary antibodies) was added to the described blocking solution. Primary and secondary antibodies were each applied for 1 h at room temperature. Coverslips were washed three times with PBS, once in distilled water, and were mounted in Mowiol (Calbiochem-Novabiochem).

Slides were analyzed at ×630 magnification on an Axiophot fluorescence microscope (Carl Zeiss) equipped with UV optics. Images were recorded with a CE200A CCD camera (Photometrics Inc.) by using smartcapture software (Digital Scientific) at a preset acquisition time. Image J software (http://rsb.info.nih.gov/ij) was used to quantify light intensity of single cells. The mean intensity of the cell without the fluorescence intensity of the nucleus was subtracted by the background mode. The average intensity value of WT cells was arbitrarily set at 100%, and the other intensities were adjusted by using the same correction factor.

Immunoelectron Microscopy: Detailed Information. The islets were washed with PBS, fixed with 4% formaldehyde/0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, and pelleted in an Eppendorf tube (13,000 rpm for 5 min). The fixative was aspirated and the cell pellet was resuspended in warm 10% gelatin. The cells were then pelleted (13,000 rpm for 5 min) and the gelatin-enrobed pellet was set on ice. The pellet was trimmed into 1 mm^3 blocks and infused with 1.7 M sucrose/15% polyvinyl pyrolidone for 24 h at 4°C. The blocks were subsequently mounted on cryostubs and snap-frozen in liquid nitrogen. Frozen ultrathin sections were cut by using a diamond knife in a cryochamber attachment (Leica), collected from the knife-edge with 50:50 2% methyl cellulose: 2.3 M sucrose (5) and mounted on formvar-carbon coated EM grids. Immunolabelling was performed by using the protein A-gold technique at room temperature (6). Sections were incubated with 50 mM NH_4Cl in PBS for 10 min to quench unreacted aldehydes, transferred to 2% gelatin in PBS for 10 min, and then 1% BSA in PBS for 10 min. Sections were incubated for 30 min with 5 μl of rabbit anti-DAMP diluted 1:100 in PBS containing 5% FCS and 0.1% BSA. The sections were washed with PBS/0.1% BSA (6 × 3 min) and incubated for 30 min with PBS/0.1% BSA containing protein A conjugated to 15 nm colloidal gold (Department of Cell Biology, University of Utrecht). The sections were washed with PBS/0.1% BSA (2 × 5 min) and PBS (4 × 5 min).

Finally the sections were rinsed with distilled water (5 × 3 min) and contrasted by embedding in 1.8% methyl cellulose/0.3% uranyl acetate. Sections were allowed to air dry before observation in a Philips CM100 transmission electron microscope at an operating voltage of 80 kV.

Quantitation of Gold Particles. Randomly sections were cut through the pellets of islets from each condition and labeled with anti-DAMP antibodies as described above. Fifty micrographs of each condition were collected by scanning systematically across the section and recorded by using a MegaView III CCD camera and iTEM software (Olympus). By using the software to record the number of gold particles labeling each granule 685 WT granules and 532 KO granules were circumscribed.

Quantitative Reverse Transcription PCR. Extraction of total RNA and cDNA synthesis were performed as previously described (7). Quantitative PCR reactions were performed on a Rotor-Gene 3000 instrument (Corbett Research) by using ABsolute QPCR SYBR Green mix (ABgene). Cycling conditions were 95°C for 15 min followed by 35 cycles at 95°C for 20 s, 60°C for 20 s, and 72°C for 30 s. Fluorescent signals were acquired at 72°C and 80°C. All samples were amplified in triplicate reactions and every experiment was repeated twice by using cDNA from independent tissue separations. Quantification of relative mRNA expression levels was done by using the method as described by Pfaffl (8) with normalization to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene. Design and sequences of GAPDH primers were described previously (9). Ac45 primer sequences were as follows: forward primer, 5′-GTCCCCGTAATGTACTGCTGTTC-3′; reverse primer, 5′-CCAAACACACCACCGTATGC-3′.

RNA Interference. Generation of the shRNA for RNAi-mediated knockdown of furin has been described previously (10). ShRNAs for knockdown of Ac45 were constructed similarly. Targeted sequences, selected by using siRNA Target Finder (Ambion) (www.ambion.com/techlib/misc/siRNA_finder.html), were 5′-GTCACAGGACCTAGCATCT-3′ and 5′-GAAAGGCAATCTCCTTGTG-3′. Efficacy was tested via cotransfection of recombinant protein and immunoblotting with anti-FLAG antibody M2 (Sigma).

Cell Lines and Transfections. RPE.40 and βTC3 cells were grown in Dulbecco's modified Eagles medium (Invitrogen) supplemented with 10% FCS at 37°C, 5% CO_2. RPE.40 cells were transfected by using Fugene6 (Roche), and βTC3 cells by using Lipofectamine and PLUS Reagent (both Invitrogen). Cotransfection was conducted in a 1:1 ratio except for testing RNAi-mediated knockdown of an expression construct. In this case, expression construct/shRNA was in a ratio 1:4. For RNAi-mediated knockdown, experiments were performed 48 h after transfection.

Radiolabeling of Cell Lines and Immunoprecipitation: Detailed Information. Transfected cell lines were preincubated in methionine/cysteine-free RPMI-1640 medium (Sigma) for 1 h at 37°C and then labeled in the same medium containing 100 μCi [^{35}S]methionine/cysteine (Easytag express protein labeling mix, PerkinElmer; specific activity: 1,175 Ci/mmol) for another hour. Cells were then chased in medium containing excess (0.4 mM), unlabeled methionine, and cysteine for 2 h at 37°C (1 h 30 min for proinsulin II immunoprecipitation). Cells were lysed on ice for 10 min in DIPA lysisbuffer [50 mM Tris-HCl (pH 7.4)/150 mM NaCl/1% Triton X-100 and protease inhibitors (Complete, Roche)]. For immunoprecipitation experiments by using M1 anti-flag 0.1 mM $CaCl_2$ was included in the lysisbuffer and EDTA-free Complete was used instead of Complete. Lysates were centrifuged at 4°C for 10 min at 15,000 rpm. in an Eppendorf centrifuge and precleared by using mouse serum bound to protein G Sepharose (GE Healthcare) at 4°C for 30 min. Immunoprecipitation was performed (2 h 4°C) by using anti-FLAG antibodies M1 or M2 (Sigma) bound to protein G Sepharose. After washing 3 times with the same DIPA buffer as described above (without protease inhibitors), immune complexes were directly eluted from the Sepharose by using sample buffer in case of proinsulin processing analysis. In case of Ac45 processing experiments, proteins bound to Sepharose were incubated in 11.5 μl 43 mM sodiumphosphatebuffer (pH 7.4)/0.13% SDS/0.65% β-mercaptoethanol for 5 min at 95°C. 1.2% Triton X-100 and 1 unit *N*-glycosidase F (Roche) were added to deglycosylate the proteins overnight at 37°C. Then, proteins were eluted by adding NuPage LDS sample buffer and reducing agent (both Invitrogen), and analyzed by SDS/PAGE (Criterion, Bio-Rad or NuPage, Invitrogen). Autoradiographic signals were obtained after fixing (30% methanol/10% acetic acid) and incubating in NAMP100V (GE Healthcare).

Calculation of Regulated Secretion. Regulated secretion was defined as the amount of AGRP secreted after stimulation with forskolin/3-isobutyl-1-methylxanthine (IBMX) minus tonic release and normalized for the total amount of AGRP in lysates. Regulated secretion in the absence of any silencing constructs was arbitrarily set at 1, and the other samples were adjusted by using the same correction factor.

1. Scheuner D, *et al.* (2005) Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. *Nat Med* 11:757–764.
2. Guest PC, *et al.* (2002) Proinsulin processing in the diabetic Goto-Kakizaki rat. *J Endocrinol* 175:637–647.
3. Zhu X, Lindberg I (1995) 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *J Cell Biol* 129:1641–1650.
4. Annaert WG, *et al.* (1999) Presenilin 1 controls gamma-secretase processing of amyloid precursor protein in pre-golgi compartments of hippocampal neurons. *J Cell Biol* 147:277–294.
5. Liou W, Geuze HJ, Slot JW (1996) Improving structural integrity of cryosections for immunogold labeling. *Histochem Cell Biol* 106:41–58.
6. Griffiths G (1993) *Fine Structure Immunocytochemistry* (Springer, Berlin), pp 1–323.
7. Vander Mierde D, *et al.* (2007) Glucose activates a protein phosphatase-1-mediated signaling pathway to enhance overall translation in pancreatic beta-cells. *Endocrinology* 148:609–617.
8. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
9. Roebroek AJ, *et al.* (2004) Limited redundancy of the proprotein convertase furin in mouse liver. *J Biol Chem* 279:53442–53450.
10. Jansen S, *et al.* (2005) Proteolytic maturation and activation of autotaxin (NPP2), a secreted metastasis-enhancing lysophospholipase D. *J Cell Sci* 118:3081–3089.

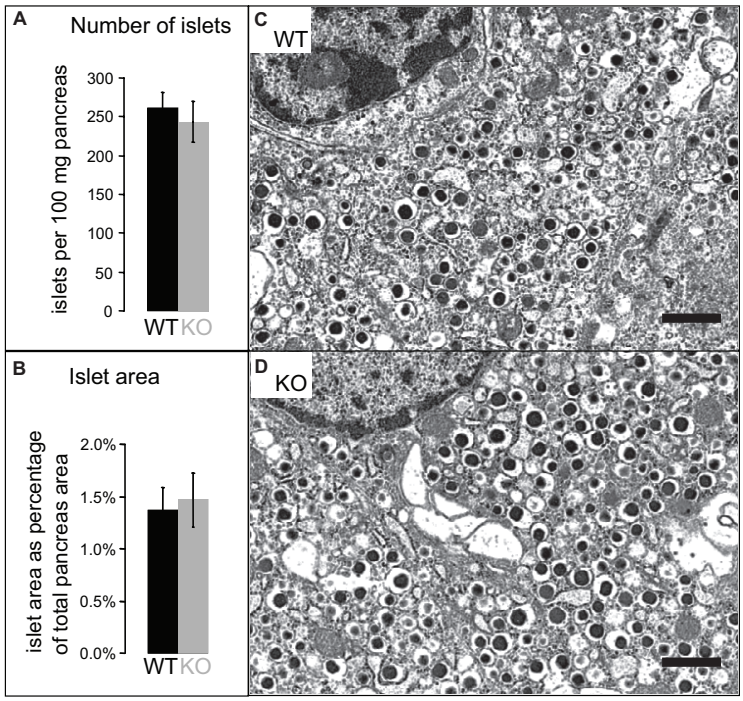

Fig. S1. Normal number, area and morphology of the furin KO islets. (*A*) Dark red stained islets per 100 mg pancreas were counted after injection of anesthetized mice with 5 mg dithizone. Data are mean values ± SEM, $n = 7$ mice per genotype. (*B*) Paraffin-embedded pancreas sections were stained with anti-insulin and anti-glucagon antibodies and counterstained with hematoxylin. Assembled micrographs were overlayed with a grid. The number of intersections with islets were compared to the total number of intersections with all pancreatic tissue. Data are mean values ± SEM expressed in percentage of total pancreatic tissue, $n = 6$ mice per genotype. (*C* and *D*) Electron microscopic analysis of large dense-core secretory vesicles in pancreatic β cells of WT mouse (*C*) and of KO mouse (*D*). (Scale bar, 1 μm.)

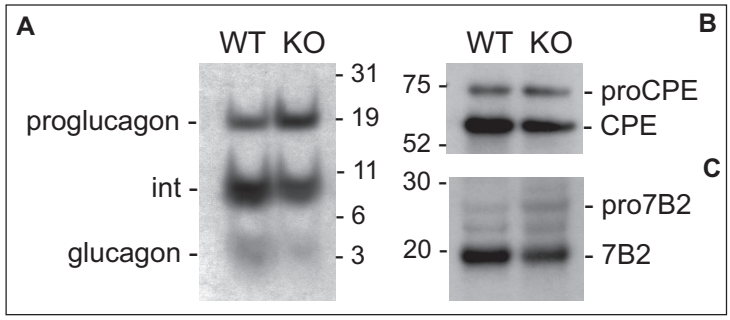

Fig. S2. Impaired processing of proproteins in the furin KO islets. Isolated islets of both genotypes were radiolabeled and the lysates were immunoprecipitated with the appropriate antiserum. The immunoprecipitated proteins were eluted, and processing was analyzed by SDS/PAGE. Representative autoradiograms are shown for glucagon (with int representing cleavage at the interdomain site which results in a double band of glicentin and glicentin-related polypeptide-glucagon; with the indication glucagon representing the double band of oxyntomodulin and glucagon) (*A*), CPE (*B*), and 7B2 (*C*). Molecular masses are indicated in kilodaltons.

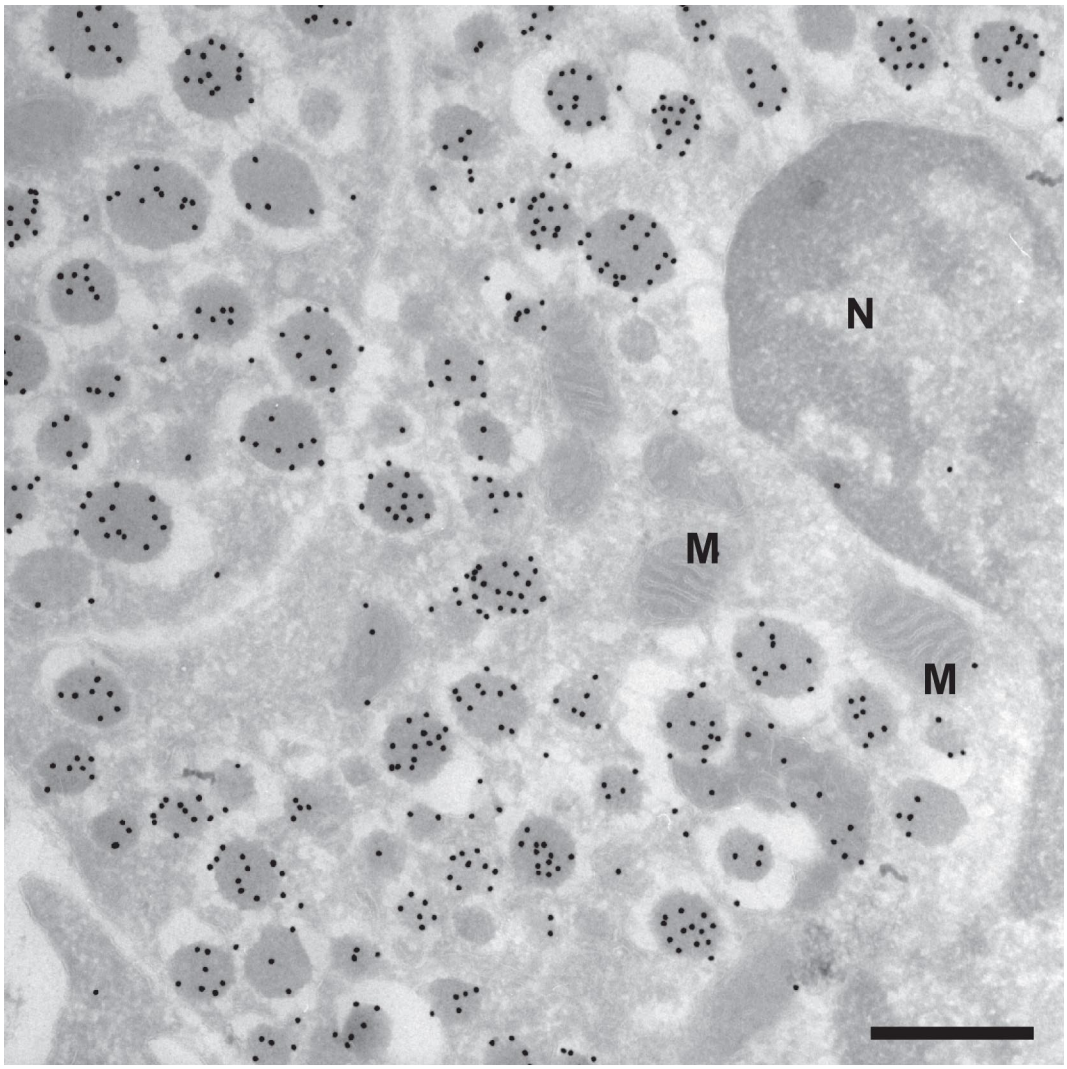

Fig. S3. DAMP accumulates in dense-core secretory granules of β cells. Immunoelectron microscopy by using protein A conjugated to 15 nm colloidal gold on cryosections of DAMP incubated islets. Overview of a WT β cell showing specific accumulation of DAMP in granules. Little labeling of the nucleus (N), mitochondria (M) and cytosol was observed. (Scale bar, 500 nm.)