Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion


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INTRODUCTION

Zinc homeostasis is maintained by two types of proteins, namely metallothioneins and zinc transporters [1], the former being involved in the intracellular trafficking and storage of zinc. There are two classes of zinc transporters, the SLC39 (Zip) family that controls the cellular uptake of zinc and the SLC30 (ZnT) family that controls zinc efflux into the extracellular matrix and intracellular vesicles [1]. In mammalian cells the ZnT family comprises ten members that share a similar six transmembrane domain structure with a histidine-rich loop located between helices IV and V, with the exception of ZnT-6 which contains a serine-rich loop and ZnT-10 which contains a basic amino-acid rich loop [1].

Chimenti et al. [2] had suggested that expression of the Slc30a8 gene, which encodes the zinc transporter ZnT-8, is restricted to pancreatic islets, specifically insulin-secreting β-cells, but recent data suggest that it is also expressed in glucagon-secreting α-cells [3]. Recent GWA (genome-wide association) studies have shown that sequence variations in SLC30A8 are linked to an increased susceptibility to the development of Type 2 diabetes [4–7]. ZnT-8 has also recently been identified as an autoantigen in Type 1 diabetes in humans [8]. Interestingly, the same polymorphic variant in amino acid residue 325 of human ZnT-8 that is associated with altered susceptibility to Type 2 diabetes also affects ZnT-8 autoantibody epitope specificity in Type 1 diabetes [9]. Within β-cells ZnT-8 localizes to insulin secretory granules [10] and because insulin is stored as a hexamer bound to two zinc ions, it has been proposed that ZnT-8 is important for providing zinc to allow for the proper maturation, storage and secretion of insulin [11]. In the present study we examine the effect of a global Slc30a8-null mutation in vivo, a mouse model that is directly relevant to Type 2 diabetes susceptibility in humans. The results are consistent with a role for ZnT-8 in islet function, although surprisingly not in whole-body glucose metabolism.

MATERIALS AND METHODS

For details on the generation of the Slc30a8-targeting vector, generation and PCR genotyping of Slc30a8-knockout mice and the analysis of islet number, size and cellular composition in Slc30a8-knockout mice please see Supplementary Materials and methods (at http://www.BiochemJ.org/bj/421/bj4210371add.htm).

Animal care

The animal housing and surgical facilities used for the mice in these studies meet the American Association for the Accreditation of Laboratory Animal Care standards. All animal protocols were approved by the Vanderbilt University Medical Center Animal Care and Use Committee. Mice were maintained on standard rodent chow (LabDiet 5001: 23% protein and 4.5% fat; PMI Nutrition International) with food and water provided ad libitum.

Breeding strategy for Slc30a8-knockout mice

F1 chimaeric (129SvEvBrd−× C57BL/6J) mice were interbred to generate F2 wild-type, heterozygous and homozygous knockout mice. The F2 heterozygous mice were then bred with F1...
hybrid (C57BL/6 × 129SvEv$^{Brd}$) mice. Two male and two female heterozygous mice from this breeding, along with their heterozygous offspring, were then interbred to generate a mouse colony used in the phenotypic characterization of the effect of the Slc30a8-null mutation.

Immunohistochemical staining
Pancreas tissue was fixed for 1 h in 4% (w/v) paraformaldehyde in PBS and embedded for paraffin sectioning (8 µm). Primary antisera against insulin (guinea-pig 1:100; Dako), glucagon (mouse 1:100; Sigma) and somatostatin (rat 1:100; Abcam) were combined with a rabbit polyclonal antibody raised against a 102 amino acid C-terminal human ZnT-8 peptide (amino acids 268–369; used at 1:500) and were detected with species-specific secondary antibodies conjugated to Cy2 (carbocyanine), Cy3 (indocarbocyanine), Cy5 (indocarbocyanine) and AMCA (aminomethylcoumarin) (all from Jackson Immunoresearch Laboratories).

Timm’s staining analyses in Slc30a8-knockout mice
The determination of zinc content in wild-type and Slc30a8-knockout mouse pancreas was based on further modification of the revised Timm’s protocol described by Danscher et al. [12]. Briefly, pancreatic tissue sections (8 µm; frozen and paraffin) fixed in 4% (w/v) paraformaldehyde were placed on glass slides and immersed in 0.1% sodium sulfide in 0.15 M sodium phosphate buffer (pH 7.4) for 1 h in glass jars inside a chemical fume hood. The slides were briefly rinsed in PBS and immersed in AMG (autometallography) developer [pH 3.8; 60 ml of gum arabic, 10 ml of sodium citrate (25.5 g of citric acid monohydrate; 9:1) and warm water. AMG development was carried out at room temperature (22 °C), in the dark and with gentle shaking. The reaction was stopped after 45 min with 5% sodium thiosulfate solution for 10 min. The slides were briefly rinsed in warm water.

Measurement of islet zinc content
Freshly isolated islets from wild-type and Slc30a8-knockout mice were washed in Ca$^{2+}$-free Hank’s balanced salt solution and frozen down at −80°C in 20 islet aliquots. Islet pellets were lysed by re-suspension in 1 ml of lysis buffer [1% Triton X-100 in 10 mM Tris/HCl (pH 7.4)]. The Zn$^{2+}$ concentration in the lysate was measured using the Zn$^{2+}$-sensitive fluorescent dye FluoZin-3 (Invitrogen). In the presence of 1.181 µM FluoZin-3 the fluorescent signal at the emission peak (516 nm) was measured in the total sample lysate using a fluorometer (PTI Instruments). The fluorescent signal was compared with a standard curve generated from serial dilutions of ZnSO$_4$ in lysis buffer to obtain the lysate Zn$^{2+}$ concentration and thus the Zn$^{2+}$ content per islet. As a normalization factor, the protein content per islet was measured in the total sample lysate using the BCA (bicinchoninic acid) protein assay (Pierce). To minimize contaminating Zn$^{2+}$, all solutions were made in double-distilled water (18.2 MΩ), avoiding the use of any glassware. Blank samples were also prepared during the islet isolation to quantify any additional Zn$^{2+}$ contamination.

Phenotypic analysis of Slc30a8-knockout mice
Animals were fasted for 5 h, weighed and then 1 h later anaesthetized using isoflurane before collection of blood samples (~200 µl) from the retro-orbital venous plexus. Whole-blood glucose concentrations were determined using an Accu-Check Advantage monitor (Roche). EDTA (5 µl; 0.5 M) was then added before centrifugation (16000 g for 10 min at 4°C) to isolate plasma. Trasylol (aprotinin; 5 µl; Bayer Health Care) was added to the plasma to prevent proteolysis of glaucan. Cholesterol was assayed using the cholesterol reagent kit (Raichem), whereas triacylglycerol and glycerol were assayed using a serum triacylglycerol determination kit (Sigma). Insulin and glucagon levels were quantified using radioimmunoassays by the Vanderbilt Diabetes Center Hormone Assay Core.

Intraperitoneal glucose tolerance tests on overnight fasted conscious mice were performed as previously described [13].

Islet isolation and GSIS (glucose-stimulated insulin secretion) assays
Islets were isolated from ~5-month-old male mice as described previously [14]. After isolation, islets were rinsed in three 12 ml changes of RPMI-1640 medium containing 10% (v/v) FBS (foetal bovine serum), 100 units/ml penicillin, 100 µg/ml streptomycin and 11 mM glucose, and then cultured in 10 cm non-treated plates overnight at 37°C. The next day islets were transferred into medium with 5 mM glucose and allowed to equilibrate for 1 h at 37°C. Following the equilibration period, 50 IEQs (islet equivalents) were incubated in 5 ml of RPMI-1640 medium with 5 or 11 mM glucose for 30 min at 37°C. At the end of the static incubations, islets were collected into 1.5 ml tubes, washed three times with 1 ml of x PBS and insulin was extracted in 0.2 ml of acid alcohol for 48 h at 4°C. The medium from the static incubations was harvested into 15 ml conical tubes and centrifuged at 600 g for 1 min at 4°C. Islet insulin extracts and static incubation medium were stored at −80°C until assayed for insulin by radioimmunoassay.

Statistical analyses
Data were analysed using a Student’s t test; two sample assuming equal variance. The level of significance was as indicated (two-sided test).

RESULTS
Biochemical characterization of ZnT-8−/− mice
The human and mouse ZnT-8 genes contain eight exons ([2] and results not shown). A modified mouse Slc30a8 allele, in which 135/147 bp of exon 3 and the first 10 bp of intron 3 were replaced by a LacZ/Neo cassette, was generated by homologous recombination in 129/SvEv$^{Brd}$ (Lex-2) ES (embryonic stem) cells (Figure 1A). Deletion of exon 3 disrupts two putative ZnT-8 transmembrane domains [2]. Correct gene targeting was confirmed by Southern blot (Figure 1B) and PCR (results not shown) analysis prior to injection of ES cells into C57BL/6 (albino) blastocysts and subsequent generation of ZnT-8−/− mice on a mixed 129/SvEv$^{Brd} \times$ C57BL/6 background.

To confirm that the targeting strategy had abolished ZnT-8 expression, immunohistochemical staining was performed on pancreas sections prepared from a ZnT-8−/− mouse and a wild-type littermate. Figure 1(C) shows that ZnT-8 was detected in both α- and β-cells in wild-type, but not ZnT-8-knockout, mouse islets.
ZnT-8-knockout mice

Figure 1 Generation and biochemical characterization of ZnT-8−/− mice

(A) Strategy used to generate ZnT-8−/− mice by homologous recombination in ES cells. A schematic representation of the wild-type murine Slc30a8 locus and the targeting construct are shown. Exon 3 was replaced with a cassette containing an IRES (internal ribosome entry site), the LacZ gene and a TK (thymidine kinase)-neomycin selectable marker. Correctly targeted clones were identified by Southern blot analysis using the indicated probes and were confirmed by PCR using the primers indicated. The primers represented sequences in exon 3 (primer 1), intron 2 (primer 2), intron 3 (primer 4) and the Neo gene (Neo3a primer). (B) Southern blot analysis of the Slc30a8 locus using genomic DNA extracted from the indicated targeted ES cell lines, or wild-type ES cell genomic DNA, designated Lex-2, as a control, using 5′ and 3′ diagnostic probes (A). The sizes of the wild-type locus, targeted allele and DNA markers are indicated. Clone 2H8 was used to achieve germline transmission. (C) Immunohistochemical staining of wild-type and ZnT-8−/− mouse pancreas with antisera raised against insulin, glucagon, somatostatin and ZnT-8 was performed as described in the Materials and methods section. Representative pictures (200 × magnification) are shown. KO, knockout; WT, wild-type.

The size and number of islets in ZnT-8−/− animals were indistinguishable from wild-type littermates, as were the relative numbers of α- and β-cells (Supplementary Figure S1 at http://www.BiochemJ.org/bj/421/bj4210371add.htm). Histological analysis of zinc content on frozen pancreatic sections using a modified Timm’s staining procedure that involves silver enhancement of metal sulfide precipitation showed that, in wild-type mouse pancreas, islets contained abundant zinc relative to the exocrine tissue (Figure 2A). This contrasted with ZnT-8−/− mouse pancreas in which no difference was observed in Timm’s staining between islets and exocrine tissue, although gross islet morphology was preserved (Figure 2A). These results are consistent with analyses of zinc content in isolated islets using an assay that detects free and loosely bound zinc (Figure 2B). Figure 2(B) shows that zinc content was markedly reduced in islets isolated from Slc30a8-knockout mice relative to those isolated from wild-type mice. The concentration of zinc detected in wild-type islets was similar to that previously reported in the islet-derived INS1 cell line [10].

Phenotypic characterization of ZnT-8−/− mice

Genotype analysis of 383 3-week-old pups generated by cross-breeding heterozygous ZnT-8+/− mice demonstrated that 83 mice were ZnT-8+/+, 203 were ZnT-8+/− and 97 were ZnT-8−/−, a distribution close to the expected pattern for Mendelian inheritance. The ratio of male to female mice was 206:177. Cross-breeding experiments revealed that both male and female homozygous ZnT-8−/− mice are fertile.

The activity and behaviour of ZnT-8−/− mice were indistinguishable from their wild-type and heterozygous littermates at all ages, from birth up to 1 year in age. No gross anatomical changes were observed either externally or to major internal organs, and no differences were seen in the weights or lengths of ZnT-8−/− compared with wild-type mice (Table 1).

Table 1 summarizes metabolic parameters in these animals assayed at 16 weeks of age following a 6 h fast. No marked changes in plasma cholesterol, triacylglycerol or glycerol were observed in either male or female ZnT-8−/− mice relative to wild-type animals (Table 1). Blood glucose and glucagon concentrations were also unchanged in both male and female ZnT-8−/− mice relative to wild-type animals; however, a statistically significant difference in plasma insulin concentrations was observed (Table 1). This result suggests that, although the absence of ZnT-8 might affect islet function, it has a limited effect on whole-body glucose metabolism. In addition, since a statistically significant difference in plasma insulin concentrations was not observed between male or female ZnT-8−/− mice relative to wild-type animals, this suggests that loss of a single Slc30a8 allele is insufficient to affect islet function (Table 1). ZnT-8−/−
mice showed gender-related variation in the majority of these metabolic parameters that were in the same direction and of similar magnitude to the gender-related differences in wild-type mice. Thus in males compared with females, insulin, triacylglycerols, cholesterol and glucose were all higher, whereas glucagon was lower (Table 1).

Since some metabolic disturbances only become readily apparent under stimulatory rather than basal conditions intraperitoneal glucose tolerance tests were used to provide a measurement of dynamic islet function in vivo. Following glucose injection (2 g/kg of body weight) blood glucose was assessed over a 120 min period (Figure 3). The data show no impairment in glucose clearance between wild-type and ZnT-8−/− mice (Figure 3). This result again suggests that, although the absence of ZnT-8 might affect islet function it has a limited impact on whole-body glucose metabolism, at least under the conditions examined.

### DISCUSSION

In the present study we examine the effect of a global Slc30a8-null mutation in vivo, a mouse model that is directly relevant to Type 2 diabetes susceptibility in humans. The study addresses the hypothesis, based on GWA data, that changes in the activity or stability of the ZnT-8 protein may result in islet dysfunction, which contributes to the pathogenesis of Type 2 diabetes. The results indicate that deletion of the Slc30a8 gene results in a mild metabolic phenotype on a mixed 129SvEvBrd background. Plasma insulin is reduced in both male and female ZnT-8−/− mice following a 6 h fast (Table 1). Consistent with this observation, GSIS from isolated islets is impaired (Figure 4) and

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**Table 1 Phenotypic characterization of ZnT-8-knockout mice**

At 16 weeks of age mice were fasted for 5 h and then weighed. Mice were anaesthetized 1 h later, their length was measured and blood isolated. Blood glucose and plasma cholesterol, triacylglycerol, glycerol, insulin and glucagon levels were determined as described in the Materials and methods section. Results are means ± S.E.M. obtained from the number of animals indicated in parentheses. KO, knockout; WT, wild-type; −/+ heterozygote.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Genotype</th>
<th>Weight (g)</th>
<th>Length (mm)</th>
<th>Glucose (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Triacylglycerol (mg/dl)</th>
<th>Glycerol (mg/dl)</th>
<th>Insulin (ng/ml)</th>
<th>Glucagon (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>WT</td>
<td>23.8 ± 0.4 (29)</td>
<td>98.8 ± 0.5 (28)</td>
<td>110.1 ± 3.9 (29)</td>
<td>75.1 ± 4.0 (26)</td>
<td>49.2 ± 2.5 (27)</td>
<td>2.5 ± 0.1 (27)</td>
<td>0.45 ± 0.05 (19)</td>
<td>70.5 ± 5.6 (23)</td>
</tr>
<tr>
<td>Female</td>
<td>−/+</td>
<td>23.3 ± 0.3 (67)</td>
<td>98.7 ± 0.3 (65)</td>
<td>110.0 ± 1.8 (66)</td>
<td>87.8 ± 2.4 (64)*</td>
<td>49.5 ± 1.5 (62)</td>
<td>2.6 ± 0.1 (63)</td>
<td>0.34 ± 0.03 (38)</td>
<td>71.8 ± 3.4 (56)</td>
</tr>
<tr>
<td>Female</td>
<td>KO</td>
<td>23.5 ± 0.4 (32)</td>
<td>98.8 ± 0.4 (31)</td>
<td>116.9 ± 3.7 (32)</td>
<td>82.5 ± 2.6 (30)</td>
<td>46.1 ± 1.7 (30)</td>
<td>2.6 ± 0.1 (30)</td>
<td>0.31 ± 0.05 (30)</td>
<td>74.2 ± 6.3 (26)</td>
</tr>
<tr>
<td>Male</td>
<td>WT</td>
<td>32.4 ± 0.5 (30)</td>
<td>100.5 ± 0.4 (34)</td>
<td>135.7 ± 3.9 (35)</td>
<td>100.7 ± 4.4 (34)</td>
<td>68.4 ± 2.7 (32)</td>
<td>2.5 ± 0.1 (33)</td>
<td>1.50 ± 0.23 (12)</td>
<td>63.2 ± 5.6 (29)</td>
</tr>
<tr>
<td>Male</td>
<td>−/+</td>
<td>32.4 ± 0.4 (63)</td>
<td>104.7 ± 0.3 (60)</td>
<td>135.7 ± 3.0 (63)</td>
<td>103.6 ± 3.2 (58)</td>
<td>73.2 ± 2.0 (58)</td>
<td>2.7 ± 0.1 (59)</td>
<td>1.07 ± 0.22 (15)</td>
<td>66.4 ± 3.5 (54)</td>
</tr>
<tr>
<td>Male</td>
<td>KO</td>
<td>31.6 ± 0.7 (34)</td>
<td>105.3 ± 0.6 (32)</td>
<td>137.2 ± 3.4 (34)</td>
<td>111.7 ± 3.6 (33)</td>
<td>70.6 ± 2.9 (32)</td>
<td>2.5 ± 0.1 (31)</td>
<td>0.79 ± 0.11 (16)</td>
<td>52.0 ± 4.7 (26)</td>
</tr>
</tbody>
</table>

*P < 0.01, female wild-type compared with female heterozygote.
†P = 0.05, female wild-type compared with female knockout.
‡P < 0.01, male wild-type compared with male knockout.
§P < 0.05, male heterozygote compared with male knockout.
male wild-type and ZnT-8 individual animals were not statistically different between in normal blood glucose. Indeed, the insulin/glucagon ratios in decrease in both insulin and glucagon secretion could result from lower insulin sensitivity and GSIS [10]. Although the loss of ZnT-8 function only has a mild effect presumably accounts for the small contribution of INS-1 cells has the opposite effect, stimulating zinc accumulation and GSIS [10]. The SNP (single nucleotide polymorphism) that linked the human SLc30A8 gene to increased Type 2 diabetes susceptibility [4–7] is located in the C-terminus of ZnT-8 and represents a non-synonymous polymorphism that changes the sequence of amino acid residue 325 [2]. In theory this ZnT-8 variant could represent either a gain- or loss-of-function, but because overexpression of ZnT-8 enhances GSIS [10] and because deletion of the Slc30a8 gene in mice impairs GSIS (Figure 4) we would predict that this human sequence variant impairs ZnT-8 function. Future experiments will address this hypothesis.

**AUTHOR CONTRIBUTION**

Lynley Pound generated and maintained the ZnT-8-knockout mouse colony, performed mouse genotyping and metabolic phenotyping and the intraperitoneal glucose tolerance test assays. Suparna Sarkar performed Timm's staining and quantitation of mouse islet size, number and cellular composition. Richard K.P. Benninger performed quantification of islet zinc content. Yingda Wang performed LacZ staining and mouse genotyping. Adisak Suwanichkul was involved with the generation and analysis of ZnT-8-knockout mice. Richard Pintz performed real-time PCR primer design and data analysis. James Oeser performed mouse genotyping and metabolic phenotyping. Catherine E. Lee performed Timm's staining, and quantitation of mouse islet size, number and composition. David Piston performed quantification of islet zinc content. Owen McGuinness analysed the mouse phenotyping data. John Hutton performed Timm's staining, quantification of mouse
islet size, number and composition, and data analysis. David R. Powell was involved in the generation and analysis of ZnT-8-knockout mice. Richard O’Brien was involved in the mouse metabolic phenotyping and data analysis.

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SUPPLEMENTARY ONLINE DATA

Deletion of the mouse Slc30a8 gene encoding zinc transporter-8 results in impaired insulin secretion


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MATERIALS AND METHODS

Generation of the Slc30a8-targeting vector

The ZnT-8 mutant mice were generated and analysed in collaboration with Lexicon Pharmaceuticals. An Slc30a8-targeting vector was derived using the Lambda KOS system [1]. The Lambda KOS phage library, arrayed into 96 superpools, was screened by PCR using exon 3-specific primers (UTT047-1, 5′-GTGAGGATAGCCGACTCC-3′ and UTT047-2, 5′-CATGCTAATTCACAGCAACACGTTG-3′ and 5′-CTATATATGCATATCGAGGCATCAG-3′) were appended by PCR to a yeast selection cassette containing the URA3 marker. The yeast selection cassette and pKOS-36 were co-transformed into yeast, and clones that had undergone homologous recombination were isolated. DNA sequencing confirmed that recombination had replaced a 145 bp region, from base pair 13 of exon 3 extending to the first 10 bp of the third intron, with the yeast selection cassette. The PCR-positive phase superpools were plated and screened by filter hybridization using the 516 bp amplicon derived from primers UTT047-1 and UTT047-2 as a probe. A pKOS genomic clone, pKOS-36, was isolated from the library screen and the presence of the Slc30a8 gene was confirmed by sequence and restriction analysis. Gene-specific primers (5′-GATATTGTGCACTCTACAGCTTG-3′ and 5′-CTATATATGCATATCGAGGCATCAG-3′) were used to amplify a 61 bp product from the wild-type allele whereas primers C (5′-TCTTGATGCAAACCACAAG-3′) and B (5′-CTCTGAATATACCGATGTTG-3′) were used to amplify a 70 bp product from the wild-type allele. A Neo3a gene (Neo) was inserted by PCR into the wild-type allele and sequences were confirmed using primers that distinguished between the wild-type and targeted alleles. Primers A (5′-GAGTCACTTCTTTTAATTG-3′ and 5′-CTCTGAATATACCGATGTTG-3′) were used to amplify a 70 bp product from the wild-type allele whereas primers C (5′-TCTTGATGCAAACCACAAG-3′) and B (5′-CTCTGAATATACCGATGTTG-3′) were used to amplify a 70 bp product from the targeted allele. Tail DNA was isolated and purified by standard procedures [2]. The wild-type and targeted allele fragments were amplified using 2.8 ng of genomic DNA and iQ SYBR Green Supermix (Bio-Rad) under the following reaction conditions: 94°C for 30 s; 60°C for 30 s; and 72°C for 30 s (for 40 cycles). Standard-curve analyses were performed for each set of samples to determine the efficiencies of the two PCR reactions, which were both greater than 95%.

Generation of the Slc30a8-targeting vector

The NotI-linearized targeting vector was electroporated into 129 Svev (Lex-2) ES cells. G418/FIAU-resistant ES cell clones were isolated, and correctly targeted clones were identified and confirmed by Southern blot analysis using a 354 bp 5′ external probe (9/10), generated by PCR using wild-type Lex-2 ES cell genomic DNA as the template with primers (UTT047-9, 5′-GCTGCAAGCTTCTTTCATAGTA-3′ and UTT047-10, 5′-CTCTGAGGGACATATAAAGTGATGC-3′), and a 314 bp 3′ internal probe (11/12), amplified by PCR using primers (UTT047-11, 5′-CAGCTCTCTTAAAACCACAGGATG-3′ and UTT047-12, 5′-GATGACTACACAAAGGTGGAAGGTG-3′). Southern blot analysis using probe 9/10 detected a 8.0 kbp wild-type band and 11.1 kbp mutant band in PsI-digested genomic DNA, whereas probe 11/12 detected a 7.6 kbp wild-type band and 12.8 kbp mutant band in NheI-digested genomic DNA. Correctly targeted clones were also confirmed by PCR using the primers indicated below. The primers represented sequences: in exon 3, primer 1, 5′-GTGAGGATAGCCGACTCC-3′; intron 2, primer 2, 5′-CACGTAGTAATTCACAGCAACACGTTG-3′; and intron 3, primer 3, 5′-CTCTGAATATACCGATGTTG-3′. Cells from the correctly targeted ES cell clone, designated 2H8 (Figure 1B of the main paper), were microinjected into C57BL/6 (albino) blastocysts resulting in the generation of chimaeric mice.

PCR genotyping of Slc30a8-knockout mice

Mouse-tail DNA was genotyped using PCR in conjunction with primers that distinguished between the wild-type and targeted alleles. Primers A (5′-GAGTCACTTCTTTTAATTG-3′ and 5′-CTCTGAATATACCGATGTTG-3′) were used to amplify a 70 bp product from the wild-type allele whereas primers C (5′-TCTTGATGCAAACCACAAG-3′) and B (5′-CTCTGAATATACCGATGTTG-3′) were used to amplify a 70 bp product from the targeted allele. Tail DNA was isolated and purified by standard procedures [2]. The wild-type and targeted allele fragments were amplified using 2.8 ng of genomic DNA and iQ SYBR Green Supermix (Bio-Rad) under the following reaction conditions: 94°C for 30 s; 60°C for 30 s; and 72°C for 30 s (for 40 cycles). Standard-curve analyses were performed for each set of samples to determine the efficiencies of the two PCR reactions, which were both greater than 95%.

Analysis of islet number, size and cellular composition in Slc30a8-knockout mice

At least ten islets from a single or 20th consecutive pancreatic section were examined and scored for each of these groups of mice (n = 6). Images of individual islets co-immunostained for insulin and glucagon were recorded with an Olympus BX52 microscope using a Pixera 600 digital colour camera and analysed with Image-Pro Plus software (Media Cybernetics). Briefly, the islet perimeter was marked with a pen tracer tool and Cy3 (indocarbocyanine)-stained glucagon-positive cells and Cy2 (carboxycyanine)-stained insulin-positive cells with associated nuclei (stained by Hoestch 33258) were counted manually in a double-blind manner by two independent observers. Nuclei within the islet area that were not insulin-positive cells with associated nuclei (stained by Hoechst 33258) were counted manually in a double-blind manner by two independent observers. Nuclei within the islet area that were not associated with either insulin- or glucagon-positive cells were designated non-α/β-cells.
Pancreas tissue was isolated from male wild-type (WT) and ZnT-8 knockout (KO) mice. Fixation, preparation of mouse pancreatic slices, immunohistochemical staining with antibodies raised to insulin and glucagon and quantitation of islet size, islet number and α- and β-cell numbers were then performed as described in the Materials and methods section of this Supplementary material. Results are presented as means ± S.E.M.

Islet numbers and pancreatic area estimations were performed by scanning pancreatic sections immunostained for either insulin or glucagon using immunoperoxidase staining with diaminobenzidine as the pigment chromogen. The slides were counterstained with haematoxylin and scanned into ScanScope GL (Aperio). Using Imagescope viewing software (Aperio), total pancreatic and all individual islet areas (endocrine) visualized within the sections were quantified using a pen tracer tool. The percentage area was calculated as: 100 × (the sum of all individual islet areas)/(total area of the pancreatic section) and averaged (n = 6 each group).

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