Supporting Information

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SI Materials and Methods

Cell Culture. Murine βTC-6 cells (American Tissue Culture Collection) were used for this experiment because (*i*) these cells are easy to culture and transduce; (*ii*) they have a high proliferation rate; and (*iii*) they show high levels of zinc finger BED domain-containing protein 6 (ZBED6) expression in their nuclei. βTC-6 cells at passage numbers 20–40 and human pancreatic cancer cell line PANC-1 cells were maintained in DMEM (Gibco) supplemented with 10% (vol/vol) FCS (Sigma Chemicals), 2 mM L-glutamine, streptomycin (0.1 mg/mL), and benzylpenicillin (100 U/mL).

Human islets from two donors were precultured for 3–6 d free-floating in Sterilin dishes in CMRL 1066 medium (ICN Biomedicals) containing 5.6 mM glucose, 10% (vol/vol) FCS, and 2 mM L-glutamine. All cells were kept at 37 °C in a humidified atmosphere with 5% (vol/vol) CO_2. Only organ donors who had agreed to donate for scientific purposes were included. Informed written consent to donate organs for medical and research purposes was obtained from donors, or relatives of donors, by the National Board of Health and Welfare (Socialstyrelsen), Sweden. Permission to obtain pancreatic islet tissue from the Nordic Network for Clinical Islet Transplantation was reviewed and approved by the local ethics committee (Regionala etikprövningsnämnden, Uppsala).

Generation of Stable ZBED6–shRNA βTC-6 and PANC-1 Cell Lines. Short-hairpin sequences against the *ZBED6* gene and the scrambled shRNA sequences were cloned into the lentiviral vector pLKO.1–TRC (Addgene). The target sequences selected are as follows: ZBED6–sh1, 5′-CTTCAACACTTCAACGACA-3′; ZBED6–sh2, 5′-TGTGGTACATGCAATCAAA-3′; and shMock, 5′-GAACCCTATATCCAACACT-3′. Viral particles were obtained by cotransfection of HEK-293FT cells with the pLKO.1–shRNA, envelope (pMD2.G), and packaging (psPAX2) vectors. βTC-6 and PANC-1 cells were transduced with the shRNA lentiviral particles (10 multiplicity of infection), and cells with stable expression of shZBED6 were selected by incubation in a medium containing puromycin (10 μg/mL) for at least 2 wk. *ZBED6* mRNA expression levels were determined by real-time RT-PCR, and protein expression was confirmed by immunoblotting.

RNA Isolation and cDNA Synthesis. Cells were washed once with PBS, and total RNA was isolated either by UltraspecTM RNA Isolation system (Biotecx Laboratories) or RNeasy Mini Kit (Qiagen) according to the manufacturers' instructions. The cDNA synthesis was performed by using M-MulV reverse transcriptase Rnase H- (FINNZYMES) and random nonamer primers. The synthesis reaction was terminated by heating at 95 °C for 5 min.

Human Islet RNA Sequencing. For whole-transcriptome sequencing, input total RNA was quality controlled by using a RNA 6000 Pico chip on a Bioanalyzer (Agilent Technologies); only RIN values > 7 were accepted. The RiboMinus Eukaryote Kit (Life Technologies) was used for removal of rRNA, followed by fragmentation of the RNA with RNaseIII according to manufacturer's protocols. RNA libraries were constructed by using the AB Library Builder Whole Transcriptome Core Kit (Life Technologies) and amplified (13 cycles). Emulsion PCR was performed by using the EZ Bead System (Life Technologies). The RNA libraries were sequenced on the SOLiD5500XL system, generating at least 49 million reads of 75-bp length for each of the six samples. Alignment of reads to the human reference sequence (hg19 assembly) and splicing analyses were performed by using LifeScope software (Version 2.1). Distributions of spliced and nonspliced reads over the ZBED6/ZC3H11A region were visualized in the UCSC Genome Browser (PMID 12045153).

ZBED6 Antibodies. An anti-mouse ZBED6 antibody and an anti-human ZBED6 antibody were both used. A polyclonal anti-mouse antibody was produced by immunizing rabbits with a recombinant protein corresponding to residues 90–384 (Fig. 1*E*) of mouse ZBED6 (1); the antibody was affinity purified before use. The antibody against human ZBED6 was made by immunizing rabbit with a peptide, (C)LSWDPEQNEVVQSSEKEILP, found at the extreme C-terminal part of the protein. Peptide reactive antibodies were affinity purified on SulfoLink beads (Pierce), coupled with the 21-amino acid residue peptide. Preincubation of the antibody with the peptide used for immunization blocked the reactivity in Western blots.

ChIP-Sequencing Analysis. Human islets (1,000 islets per sample) were cross-linked with 1% formaldehyde for 10 min, quenched with glycine, and stored at −70 °C. After thawing and treatment with cell lysis buffer, chromatin was sonicated in radioimmunoprecipitation assay (RIPA) buffer pH 7.4 by using a BioRuptor. Two separate ChIPs were prepared by using 2 μg of ZBED6 antibody bound to 20 μL of magnetic beads coated with protein A and G (Invitrogen). Illumina libraries were prepared by using NEXTflex adaptors (BIOO Scientific) and enzymes from Fermentas (Fast End Repair, Klenow exo-minus DNA polymerase, fast ligase). Paired-end sequencing was done on Illumina HiSeq 2000 instruments. In total, 16.8 million read pairs could be aligned to the hg18 human reference (BWA v0.5.9). Read pairs aligned with a distance of <500 bp and a combined mapping quality of at least 60 were used to calculate overlaps of the corresponding ChIP fragments to identify peaks of enrichment. A minimum of 12 unique overlapping fragments was required, and the list of peaks was filtered to remove recurrent false-positive regions such as Satellite repeats.

The official gene symbols for genes with ZBED6 peaks were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (Version 6.7; http://david.abcc.ncifcrf.gov/) for functional annotation clustering analysis. The default setting with high stringency was used for the GO analysis.

Semiquantitative Real-Time RT-PCR. The mRNA transcripts were measured by semiquantitative PCR analysis using the SYBR Green Taq Readymix (Sigma) on a Light Cycler 2.0 instrument (Roche). The value was then normalized against the value of GAPDH. To ensure the function of GAPDH as a housekeeping gene, GAPDH levels were compared with those of β-actin and 18S rRNA. GAPDH mRNA levels were unaffected by all treatments in βTC-6 cells, which verifies that GAPDH is a suitable housekeeping gene. The sequences of the primers used can be obtained from the corresponding authors. The PCR products were analyzed by a 2% (wt/vol) agarose gel to ensure that the fragments had the correct sizes.

Preparation of Cytosolic and Nuclear Fractions. Human islets (100 per group) or βTC-6 cells were washed once in cold PBS and resuspended in solution A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protein inhibitor mixture) and incubated on ice for 10 min. Following a brief centrifugation, the cells were again resuspended in solution A and lysed with an electric homogenizer. The lysates were then centrifuged for 5 min at 960 × *g* at 4 °C, and proteins in the supernatant fraction were

precipitated by using 70% (vol/vol) acetone for 10 min on ice followed by a 5-min centrifugation at 16000 × *g* at 4 °C. After removal of all acetone, the precipitated proteins were resuspended in SDS–sample buffer [2% (wt/vol) SDS, 0.15 M Tris, pH 6.8, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, bromophenol blue, and 2 mM phenylmethylsulfonyl fluoride] and used as cytosolic fractions. The pelleted fractions of the lysates were resuspended in SDS–sample buffer and used as nuclear fractions.

Immunoblot Analysis. Cells were washed in cold PBS and lysed on ice in SDS–sample buffer pH 6.8. The samples were boiled for 5 min and separated on 9% or 12% (wt/vol) SDS/PAGE gels. Proteins were transferred to Hybond-P membranes (GE Healthcare). The membranes were preblocked in 2.5% (wt/vol) BSA for 1 h and then incubated with anti-mouse ZBED6 (1:1,000) (1), anti-human ZBED6 C-terminal (1:1,000), pancreatic and duodenal homeobox 1 (PDX1) (1:1,000; Cell Signaling Technology), GADD153 (1:200; Santa Cruz), ZC3H11A (L-19) (1:200; Santa Cruz), Bim (1:1,000; Cell Signaling Technology), and polypyrimidine tract binding protein (Abcam) antibodies overnight at 4 °C. A horseradish peroxidase-labeled goat anti-rabbit, anti-mouse antibody (1:2,000) (GE Healthcare) or rabbit anti-goat (1:10,000; Sigma-Aldrich) was used as secondary antibody. The bound antibodies were visualized with the Kodak Image Station 4000 MM using ECL plus (GE Healthcare). The band intensities were calculated by using Carestream Molecular Imaging Software (Version 5.0.6). Total protein loading and transfer onto the membranes was visualized by amidoblack staining.

Immunoprecipitation of ZBED6 from βTC-6 Cells. βTC-6 cells (10^8) were washed three times in ice-cold PBS and resuspended in RIPA buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Igepal CA-630, supplemented with PMSF and Halt protease and phosphatase inhibitor mixture (Thermo Scientific)] on ice for 30 min. The lysed cells were cleared by centrifugation, and remaining extracts were first precleaned with 50 μL of Protein A Sepharose (GE Healthcare) and then incubated with 5 μg of anti-mouse ZBED6 rabbit polyclonal antibody or 5 μg of rabbit serum (control) for 1 h on ice. Immune complexes were purified by binding to 50 μL of Protein A Sepharose for 30 min on ice and thereafter washed three times with RIPA buffer and once with H_2O. The Sepharose beads were resuspended in SDS–sample buffer, and immunoprecipitates were loaded to Mini-PROTEAN precast gel (4–20%) (Bio-Rad) and separated by electrophoresis.

Identification of Proteins by Mass Spectrometry. After silver staining, the relevant bands were cut out and treated for in-gel digestion as follows: after removing the silver stain by Farmer's reagent (50 mM sodium thiosulfate/15 mM potassium ferricyanide) and extensive washing with water, gel plugs were treated with 50 mM ammonium bicarbonate (ambic) and then dried by neat acetonitrile (ACN). Porcine trypsin (modified, sequence grade from Promega) was added, and incubation continued at 37 °C overnight. Digestion was terminated by using 10% (wt/vol) trifluoroacetic acid, and peptide retrieval was facilitated by mechanical vortex and sonication. The samples were desalted and concentrated by using a micro-C18 ZipTip (Millipore) and eluted directly onto the target plate by using alpha-cyano-4-hydroxycinnamic acid in 75% (vol/vol) ACN as matrix. Mass spectra for peptide mass fingerprinting were acquired in positive reflectron mode on an Ultraflex III TOF/TOF (Bruker Daltonics). Searches for peptide identities were done by using the engine ProFound (The Rockefeller University and National Centre for Research Resources) and according to the following conditions: taxonomy mouse with mass tolerance 0.03 Da. Alternatively, the target proteins (band 3) were inserted in GPMAW (Lighthouse Data) and analyzed with regard to the obtained peptide masses.

Total Insulin Content Measurement. Stable ZBED6–shRNA βTC-6 cells and control cells (shMock) (3×10^4 cells per well; 24-well plate) were seeded and precultured in DMEM for 2 d. The cells were washed once with Hepes-balanced Krebs–Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM Hepes, pH 7.4) and then harvested and homogenized by sonication in 200 μL of redistilled water. A fraction of the homogenate was mixed with acid–ethanol, and insulin was extracted overnight at 4 °C. Samples were then stored at −20 °C until insulin analysis. After appropriate dilution, insulin concentrations were measured by Mercodia Insulin ELISA Kit (Mercodia AB). The amount of insulin was normalized by the cellular DNA content, which was measured by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific).

Determination of Cell Viability by Flow Cytometry. Stable ZBED6–shRNA βTC-6 cells and control cells (shMock) were exposed to 0.5 mM palmitate [0.5% BSA, 10% (vol/vol) FCS] for 16 h or incubated with 100 U/mL IL-1β and 1,000 U/mL IFN-γ for 24 h; or treated with 1 mM diethylenetriamine/NO for 24 h. Then cells were vital stained with 10 μg/mL propidium iodide (PI) for 10 min at 37 °C. Free-floating cells and cells attached to the culture dish were then washed separately with PBS, pooled, and examined by flow cytometry using the FACSCalibur (BD Biosciences). In each experimental group, 10,000 cells were examined and gated by PI intensity and FSC signal. The percentage of dead cells was analyzed by using CELLQUEST software (BD).

Cell Proliferation and Cell-Cycle Analysis. The proliferation properties of stable ZBED6–shRNA βTC-6 and PANC-1 cells and control cells (shMock) were investigated by using flow cytometry. A total of 10,000 cells of each type of cell clones were seeded to 24-well culture plate and number of cells in each well was counted by flow cytometry at days 1, 2, 3, and 4. For cell-cycle analysis, βTC-6 cells were trypsinized, washed with cold PBS, fixed in ice-cold 70% (vol/vol) ethanol, and stored at −20 °C until analysis. Before analysis, cells were subsequently washed twice with PBS, incubated for 30 min at room temperature with RNase A (100 μg/mL), and stained with PI (2.5 μg/mL) for 30 min. Flow cytometry was performed with the FACSCalibur instrument, and data were analyzed with the ModFit LT software (Version 3.3.11).

Immunohistochemistry, Immunofluorescence, and Confocal Microscopy. βTC-6 cells were cultured on poly-lysine–coated coverslips for 48 h before staining. Intact human islets were cytospinned (120 × *g* for 2 min) to poly-lysine microscopy slides. In some experiments, islets were trypsinized into cell clusters before cytospinning. Cells were fixed in 4% (wt/vol) paraformaldehyde for 10 min at room temperature, permeabilized with 0.2% Triton X-100 on ice for 10 min, and blocked with 5% (vol/vol) FCS for 30 min in PBS. Cells were incubated for 1 h with rabbit anti-ZBED6, rabbit anti–PDX1, guinea pig antiinsulin (Fitzgerald), mouse anti-human cytokeratin 19 (Dako), and mouse antiglucagon–NL557 conjugated (R&D Systems) antibody at room temperature. The cells were then washed four times with PBS to remove unbound antibodies and then treated with Alexa Fluor 488-labeled goat anti-rabbit, Alexa Fluor 594-labeled goat anti-guinea pig, and Alexa Fluor 594-labeled goat anti-mouse secondary antibodies (20 μg/mL each) (Life Technologies) for 1 h. Cells were washed four times with PBS and mounted with VECTASHIELD Hard Set mounting medium with DAPI (Vector Laboratories) or Fluoromount-G (Southern Biotechnology) and inspected with a Nikon Eclipse fluorescence/confocal C1/TE2000U microscope (Nikon).

Pancreata from 6.5-wk-old C57BL/6 mice were fixed in 10% (vol/vol) formalin and embedded in paraffin. Sections of 5-μm thick were cut and deparaffinized. After antigen retrieval, the sections were washed with PBS and incubated for 10 min with freshly prepared 3% (vol/vol) H_2O_2 in methanol. Consecutive sections were blocked by using the blocking solution (TSA Biotin System, Perkin-Elmer Life Sciences) for 30 min and incubated with primary antibody (antiinsulin, anti-ZBED6, and antiglucagon) for 1 h. The sections were subsequently stained for 30 min with biotinylated anti-rabbit IgG (Dako) or biotinylated anti-mouse IgG (Vector Laboratories), followed by incubation for 30 min with Streptavidin–horseradish peroxidase (Perkin-Elmer). Peroxidase activity was revealed by using the AEC peroxidase substrate kit (Vector Laboratories). Alternatively, a tyramide amplification step (TSA-Biotin System; Perkin-Elmer) was included. Microscopy was done by using an inverted Nikon Eclipse C1/TE-2000U.

Reexpression of ZBED6 in shMock, sh1, and sh2 βTC6 Cells. Full-length mouse ZBED6 was reexpressed in shMock, sh1, and sh2 βTC6 cells by using the pEGFP C1 vector with GFP fusion at the N-terminal by Lipofectamine 2000 transfection. GFP-negative and -positive cells were sorted by fluorescence-activated cell sorting (FACS) using the FACSCalibur flow cytometer instrument (BD Biosciences). Reexpression of ZBED6 was verified by real-time PCR and immunofluorescence staining.

BrdU Incorporation and Flow Cytometry Analysis. Two days after lipofection, shMock, sh1, and sh2 βTC-6 GFP-negative and -positive cells were incubated with 10 μM BrdU for 2 h. After fixation and permeabilization, cells were stained with anti-BrdU fluorescent antibodies by using the APC BrdU Flow Kit (BD Pharmingen). After gating GFP-negative and -positive cells separately, BrdU positivity was then assessed in the FL_4 channel.

Silencing of ZBED6 in GFP–ZBED6 and GFP–ZC3H11A Overexpressing C2C12 Cells. The GFP–ZBED6 and GFP–ZC3H11A BAC constructs were made by using a mouse BAC (MSM mouse BAC clone MSMg01-157F14; DNA Bank, Gene Engineering Division, RIKEN BioResource Center) containing the complete ZC3H11A/ZBED6 chromosomal region. In these two constructs, GFP was introduced N-terminally and in frame either to the ZC3H11A reading frame or to the ZBED6 reading frame. Briefly, a LAP cassette was inserted as a N-terminal fusion by recombination to ZBED6 or ZC3H11A, and the isolated BAC DNA was transfected and selected for stable integration in C2C12 myoblast cells as described (2). Positive transfected cells were FACS sorted, and individual clones were selected. The GFP–ZBED6 and GFP–ZC3H11A overexpressing C2C12 cells were transduced with sh1 lentivirus, and stable clones were selected by using the same method as with the βTC-6 cells. Expression of GFP–ZBED6 and GFP–ZC3H11A and endogenous levels of ZBED6 and ZC3H11A were detected by immunoblot analysis.

Statistical Analysis. Data are presented as means ± SEM. Statistical significance between two experimental conditions was analyzed by using Student's paired *t* test.

1. Markljung E, et al. (2009) ZBED6, a novel transcription factor derived from a domesticated DNA transposon regulates IGF2 expression and muscle growth. *PLoS Biol* 7(12):e1000256.

2. Poser I, et al. (2008) BAC TransgeneOmics: A high-throughput method for exploration of protein function in mammals. *Nat Methods* 5(5):409–415.

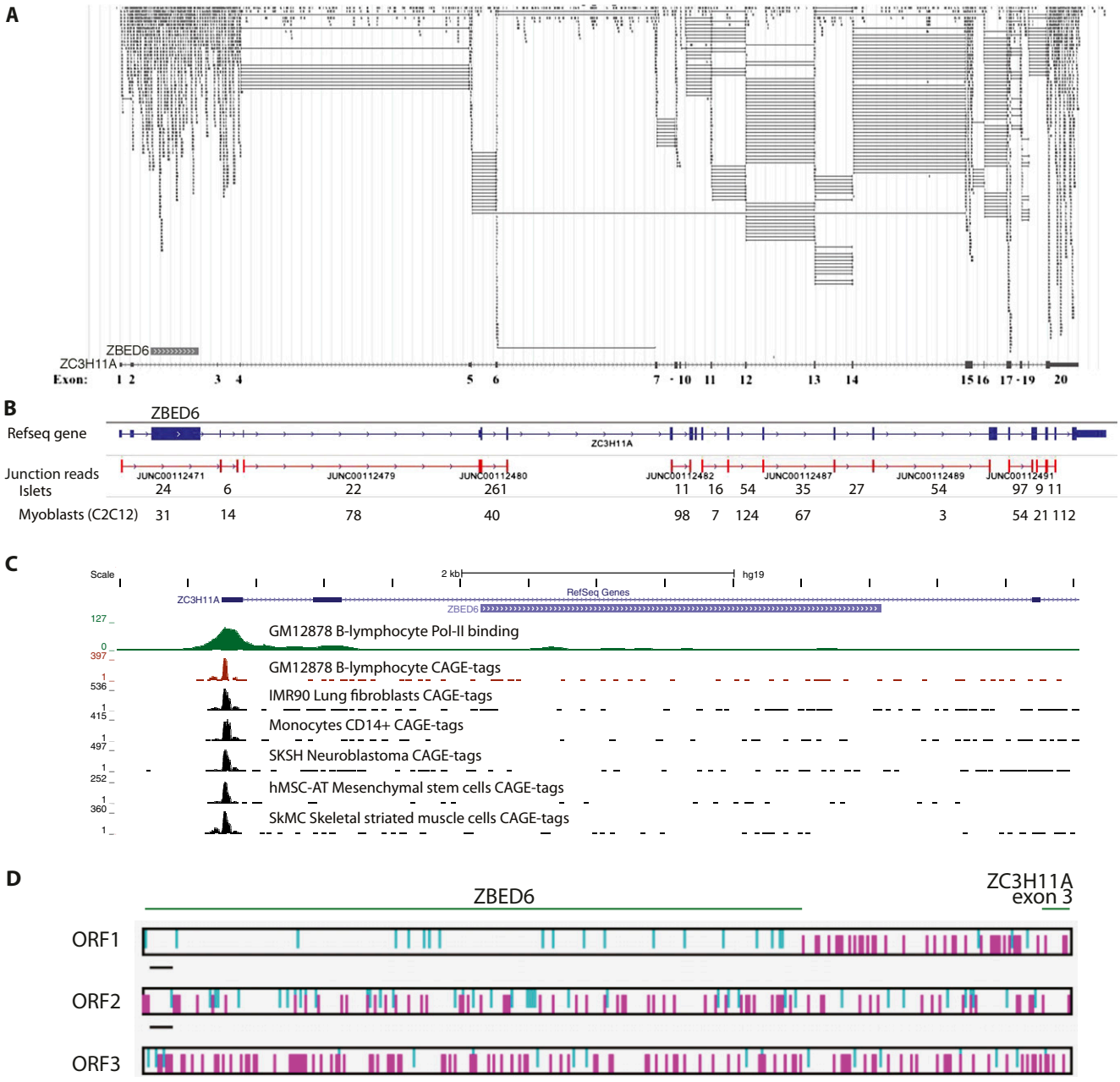

Fig. S1. (*A*) Mapping and splicing analysis of RNA sequencing reads to the *ZBED6* and *ZC3H11A* genes in human islet cells. The LifeScope software (Version 2.1) and the UCSC Genome Browser were used to visualize the distribution of spliced and nonspliced reads from one human islet sample over the *ZBED6*/*ZC3H11A* region. Nonspliced reads appear as dots present at 5′ UTR, exons, and 3′ UTR. The abundance of nonspliced reads mapping to introns 1–3 demonstrates frequent retention of intron 2 harboring the ZBED6 coding sequence. Spliced reads appear as horizontal lines connecting exons. The location of *ZBED6* (in intron 2) and the 20 *ZC3H11A* exons are given at the bottom of the image. The distribution of RNA sequencing reads shown is representative of a total of six human islet samples from two different donors. By using the LifeScope software, no splicing of the second intron harboring ZBED6 was seen, and only one read covering the first splice site (intron 1) was found (horizontal line covering intron 1), indicating incomplete splicing of intron 1 and 2. (*B*) However, we further analyzed these data by using the TopHat aligner (1), which is more sensitive to detect spliced reads, on the six human islet samples and reads from mouse C2C12 cells produced on the same platform. The number of reads for each junction supported by at least six reads in total was extracted. There were 24 reads supporting the transcript without *ZBED6* (denoted as Junc00112471) and at most 261 reads supporting a splice site present in both forms of *ZBED6*/*ZC3H11A* transcripts (Junc00112480). Thus, *ZBED6* was spliced out after transcription in at least 24/261 (9.2%) of the *ZBED6*/*ZC3H11A* transcripts in human islets. For C2C12, 31/124 (25%) of the spliced reads support ZC3H11A transcript without ZBED6. (*C*) ENCODE Pol II localization data (here exemplified with the GM12878 B-lymphocyte cell line) indicates that only one major transcription start site (TSS) is present at the *ZC3H11A*/*ZBED6* locus. This interpretation is further supported by the distribution of 5′ ends of capped analysis of gene expression mRNAs (CAGE tags) for multiple cell lines with different origins, which also indicate a single TSS in the vicinity of exon 1 of *ZC3H11A*. (*D*) All potential start and stop codons in the three possible reading frames (ORF1, 2, and 3) in the sequence from the 5′ end of *ZBED6* to *ZC3H11A* exon 3. The transcript with ZBED6 has retained 1,100 intronic bases after the coding part of ZBED6, with no ORF. Putative start codons are in green, and stop codons are in red.

1. Trapnell C, Pachter L, Salzberg SL (2009) TopHat: Discovering splice junctions with RNA-Seq. *Bioinformatics* 25(9):1105–1111.

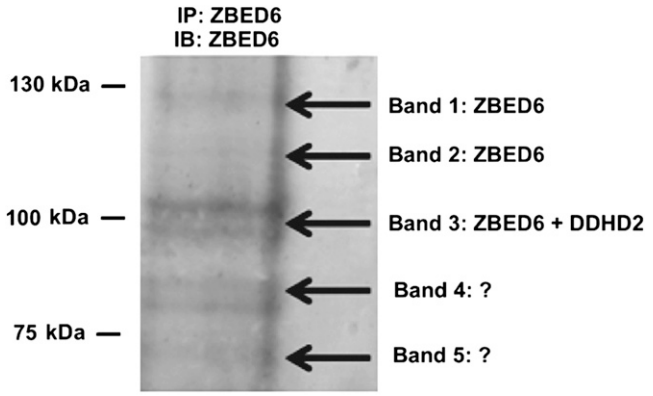

Fig. S2. Silver staining of ZBED6 immunoprecipitates. βTC-6 cells were lysed and immunoprecipitated by using anti-mouse ZBED6 antibody or rabbit serum as control. The immunoprecipitates were separated on a 4–20% gradient gel. The gel was then silver stained. Bands 1, 2, 3 (double band), 4 (double band), and 5 were cut out and processed for identification by mass spectrometry. Bands 1 and 2 were identified as ZBED6, and band 3 was identified as a mixture of ZBED6 and DDHD2.

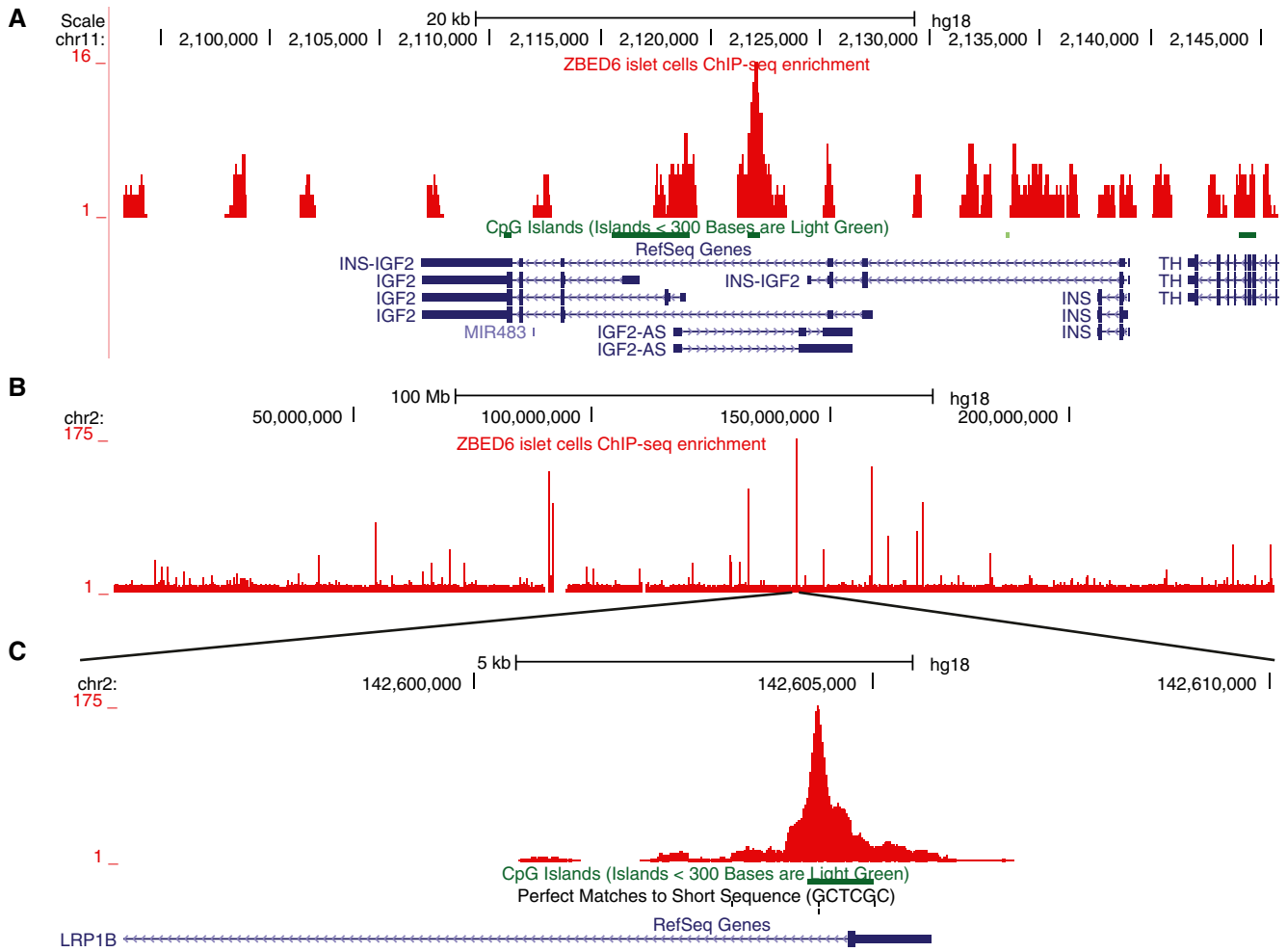

Fig. S3. ZBED6 ChIP-sequencing (ChIP-seq) enrichment. (*A*) The *IGF2* site where ZBED6 binding was identified in myoblasts is enriched also in islet cells. (*B*) ZBED6 genome-wide signals are exemplified on chromosome 2 with many highly enriched binding sites. (*C*) The highest peak on chromosome 2 is found at the LDL-receptor family member LRP1B. The binding site is located in a CpG island downstream of the TSS, with two matches to the ZBED6 consensus binding sequence close to the summit.

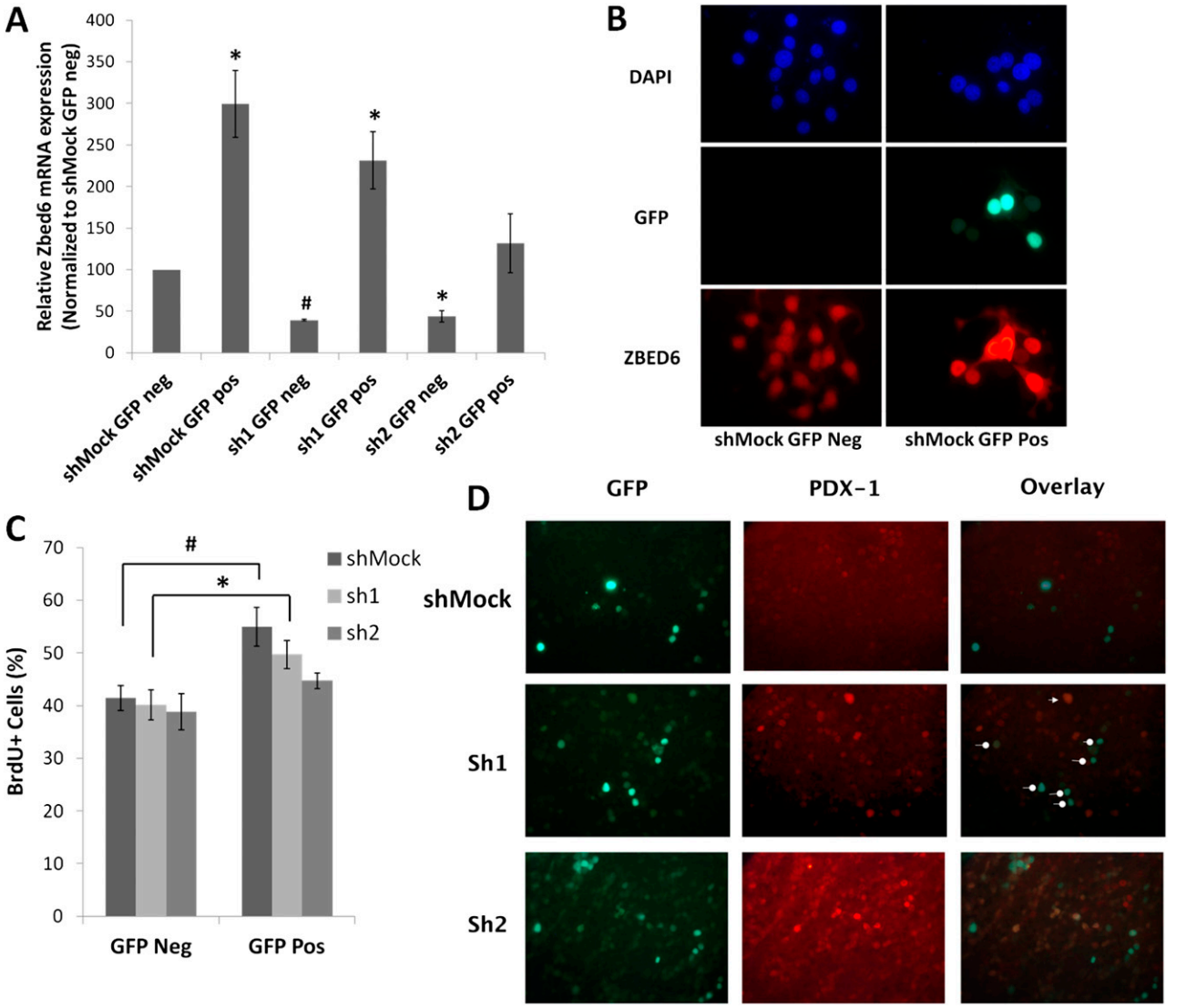

Fig. S4. Effects of ZBED6 reexpression in shMock, sh1, and sh2 βTC6 cells on BrdU incorporation and PDX1 expression. Full-length mouse ZBED6 was transiently expressed in shMock, sh1, and sh2 βTC6 cells by lipofection of the pEGFP C1 vector with GFP fused to the N-terminal of ZBED6. (*A*) Real-time RT-PCR semi-quantitative ZBED6 mRNA determination. GFP-negative and -positive cells were sorted 2 d after lipofection by FACS according to FL1-signal intensity. Results were normalized to GAPDH levels and expressed as percent of shMock GFP negative cells. Results are means ± SEM for three observations. $^{*}P < 0.05$; $^{\#}P < 0.01$ compared with shMock GFP-negative cells. (*B*) Representative immunostaining of ZBED6 in lipofected shMock βTC6 cells to confirm ZBED6 overexpression in GFP-positive cells. (*C*) Percentage of BrdU positive cells after a 2-h BrdU labeling period. GFP-positive and -negative cells were analyzed separately 2 d after lipofection by flow cytometry, and results are given as means ± SEM for five observations. $^{*}P < 0.05$; $^{\#}P < 0.01$. (*D*) PDX1 staining (red) of GFP-negative and -positive shMock, sh1, and sh2 cells. Circle head: Six cells with strong GFP signal and no PDX1 positivity; arrowhead, one cell with intermediate GFP signal and PDX1 positivity. To quantify PDX1 positivity, as indicated by a clear nuclear staining for PDX1, among GFP-positive and -negative cells, 7–16 different photographical images from all experimental conditions with at least 200 cells in each were analyzed. Only cells with strong GFP signal were counted as GFP-positive. Among GFP-positive shMock cells, 0% were PDX1-positive. Among the GFP-negative cells, however, 4.2 ± 0.5% shMock cells were PDX1-positive. When combining sh1 and sh2 cells, 2.7 ± 1.7% of the GFP-positive cells were PDX1-positive. Among the GFP-negative sh1+sh2 cells, 6.5 ± 0.5% were PDX1-positive, which was significantly higher than in the GFP-positive cell group ($P < 0.05$). These results indicate that reexpression of ZBED6 results in a loss of PDX1 expression. Moreover, the finding that reexpression of ZBED6 in sh1 cells resulted in an increased BrdU positivity, indicating an enhanced proliferation rate, also supports the notion that the phenotype induced by the sh1/sh2 lentiviral vectors is specific for ZBED6 knockdown and not the result of nonspecific effects. Thus, both the decreased proliferation and the increased PDX1 expression were reversed by ZBED6 reexpression in sh1 cells.

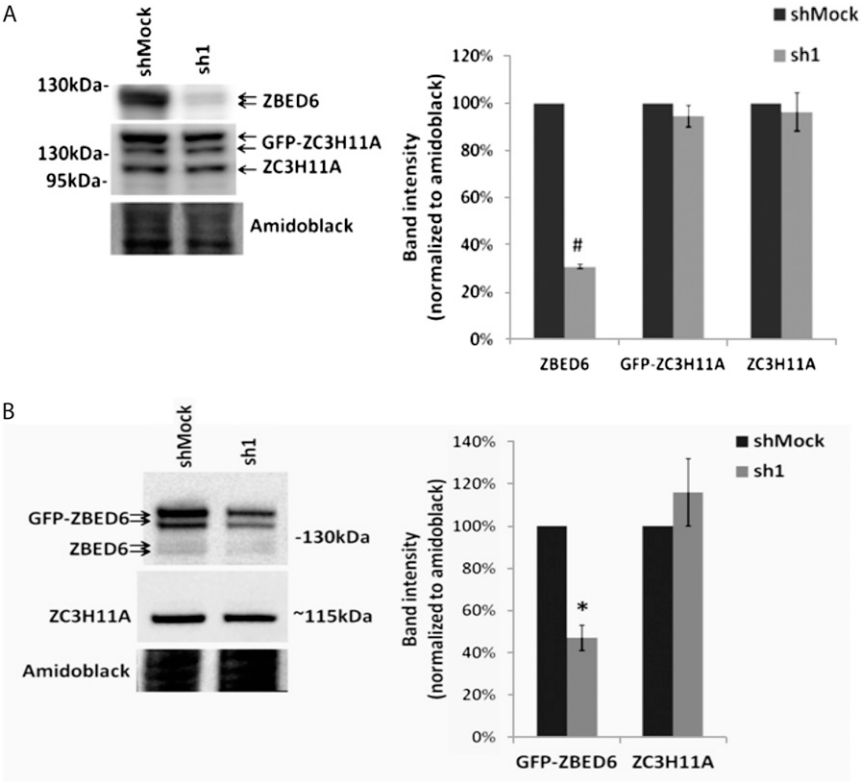

Fig. S5. Silencing of ZBED6 using mouse BAC clones expressing GFP–ZC3H11A (*A*) or GFP–ZBED6 (*B*). The two constructs were made by using the same BAC clone containing the entire ZC3H11A/ZBED6 genomic region. The results show that the lentiviral shRNA directed against ZBED6 does not affect ZC3H11A expression. (*A*) Silencing of ZBED6 by the sh1 lentiviral vector does not affect GFP–ZC3H11A expression in C2C12 cells. Samples were separated on a 4–20% gradient gel. The endogenous ZBED6 was detected as a double band with the approximate molecular mass of 120 kDa, and the endogenous ZC3H11A was observed at ~110 kDa in C2C12 cells. The GFP–ZC3H11A band migrated as a 140- to 150-kDa band and was verified by incubation with an anti-GFP antibody. Results are means ± SEM for three observations. $^{\#}P < 0.01$. (*B*) Silencing of ZBED6 by the sh1 lentiviral vector decreased GFP–ZBED6, but not ZC3H11A, expression in C2C12 cells. Samples were separated on a 4–20% gradient gel. Endogenous ZBED6 was detected as a double band with the approximate molecular mass of 120 kDa, and the endogenous ZC3H11A was observed at 110-kDa in C2C12 cells. GFP–ZBED6 migrated as a 150- to 160-kDa double band and was verified by incubation with an anti-GFP antibody. Results are means ± SEM for four observations. $^{*}P < 0.05$.

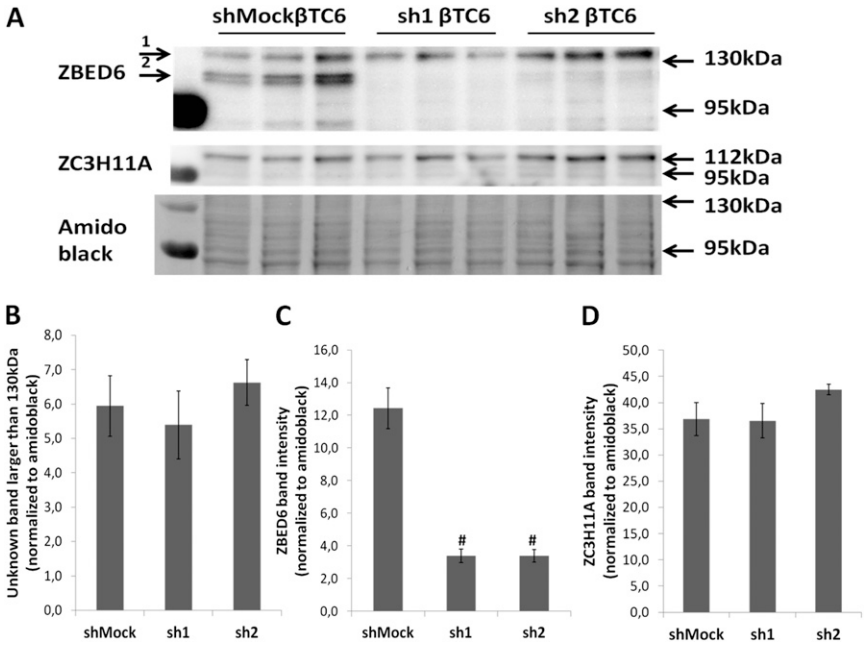

Fig. S6. The 130-kDa protein that is nonspecifically recognized by the ZBED6 antibody is not affected by sh1/2 lentiviral vectors. (*A*) Triplicate samples of shMock, sh1, and sh2 βTC6 cells were loaded onto a 4–20% gradient gel. Arrow 1, the unknown protein slightly above the 130-kDa marker; arrow 2, the three different ZBED6 forms; a ZC3H11A immunoreactive band was observed at 110 kDa. (*Lower*) Amidoblack staining of total protein between 95 and 130 kDa is shown. Because of a different gel setting, the unknown 130-kDa protein (arrow 1) was observed slightly higher than the 130-kDa molecular-mass standard. (*B*) Quantification of the unknown protein. (*C*) Quantification of ZBED6. $^{\#}P < 0.01$. (*D*) Quantification of ZC3H11A. In conclusion, neither the unknown 130-kDa protein nor ZC3H11A were significantly affected by sh1 or sh2.

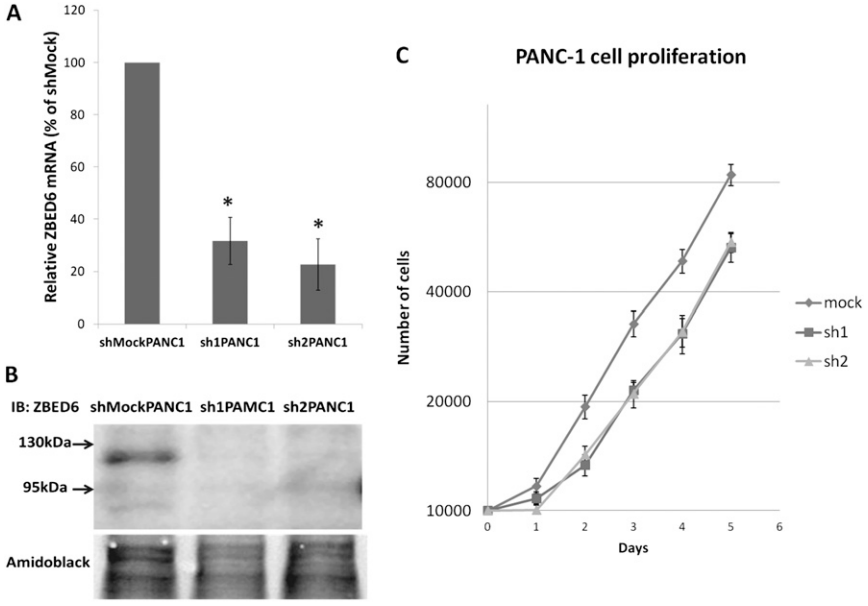

Fig. S7. ZBED6 knockdown in the human pancreatic duct cell line PANC-1 results in decreased proliferation. (*A*) Real-time RT-PCR results from the PANC-1 cell line. Results were normalized to GAPDH levels and expressed in percent of shMock. Results are means ± SEM for three observations. $^{*}P < 0.05$ (Student *t* test). (*B*) Immunoblot showing levels of ZBED6 protein in human PANC-1 cells after stable transduction with ZBED6-targeting shRNA. ZBED6 immunoreactivity was obtained using the anti-mouse ZBED6 antibody. The positions of the 130- and 95-kDa molecular mass markers are given on the left. (*C*) Cell numbers on days 1–5 were determined by flow cytometry. Results are means± SEM for three observations.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOC)