Ou et al., JCI, Supplemental Data

Supplemental Figure 1. Coding sequence of the TALE-TET1 targeting the ICR2.

ATGTCGCGGACCCGGCTCCCTTCCCCACCCGCACCCAGCCCAGCGTTTTCGGCCGACTCGTTCTCAGACCTGCTTAGGCAGTT
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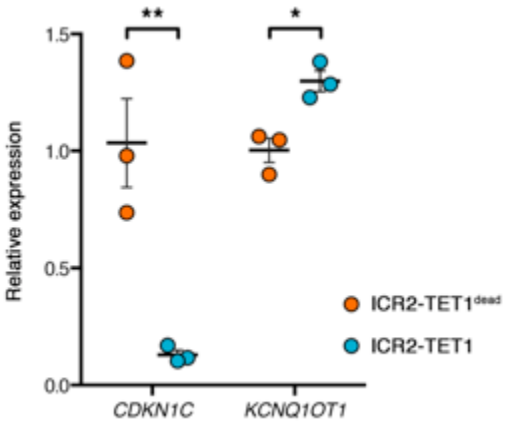
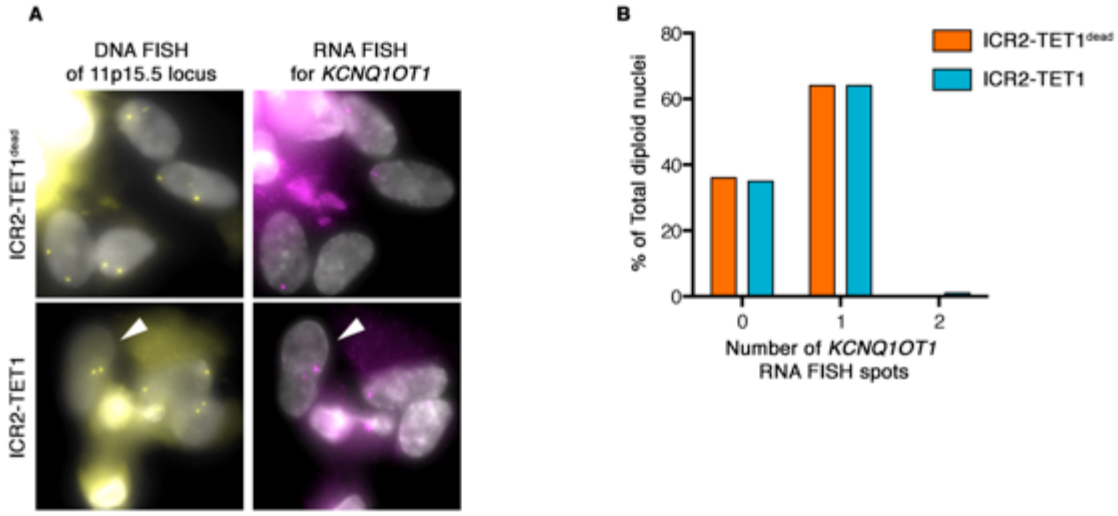
Supplemental Figure 2. Gene expression in sorted HEK293T cells.



Changes in gene expression in HEK293T cells overexpressing ICR2-TET1^{dead} or ICR2-TET1 (*, $p<0.05$; **, $p<0.01$)

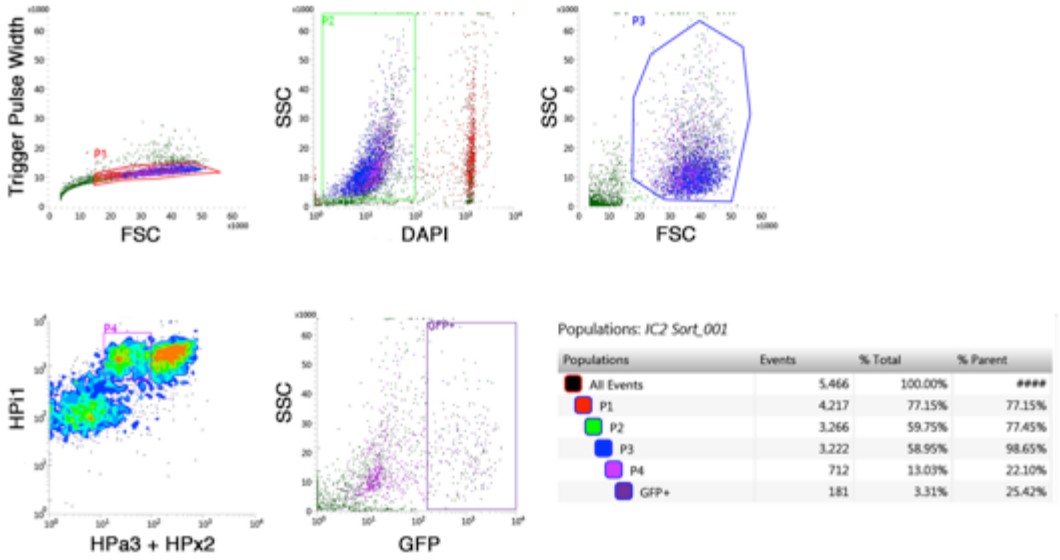
Supplemental Figure 3. RNA-DNA FISH for *KCNQ1OT1* on transduced fibroblasts.



(A) DNA and RNA fluorescent *in situ* hybridization of the chr11p15.5 genomic locus (yellow) and nascent *KCNQ1OT1* mRNA (magenta) in sorted fibroblasts. The nucleus (gray) containing bi-allelic expression of *KCNQ1OT1* is indicated by the white arrow.

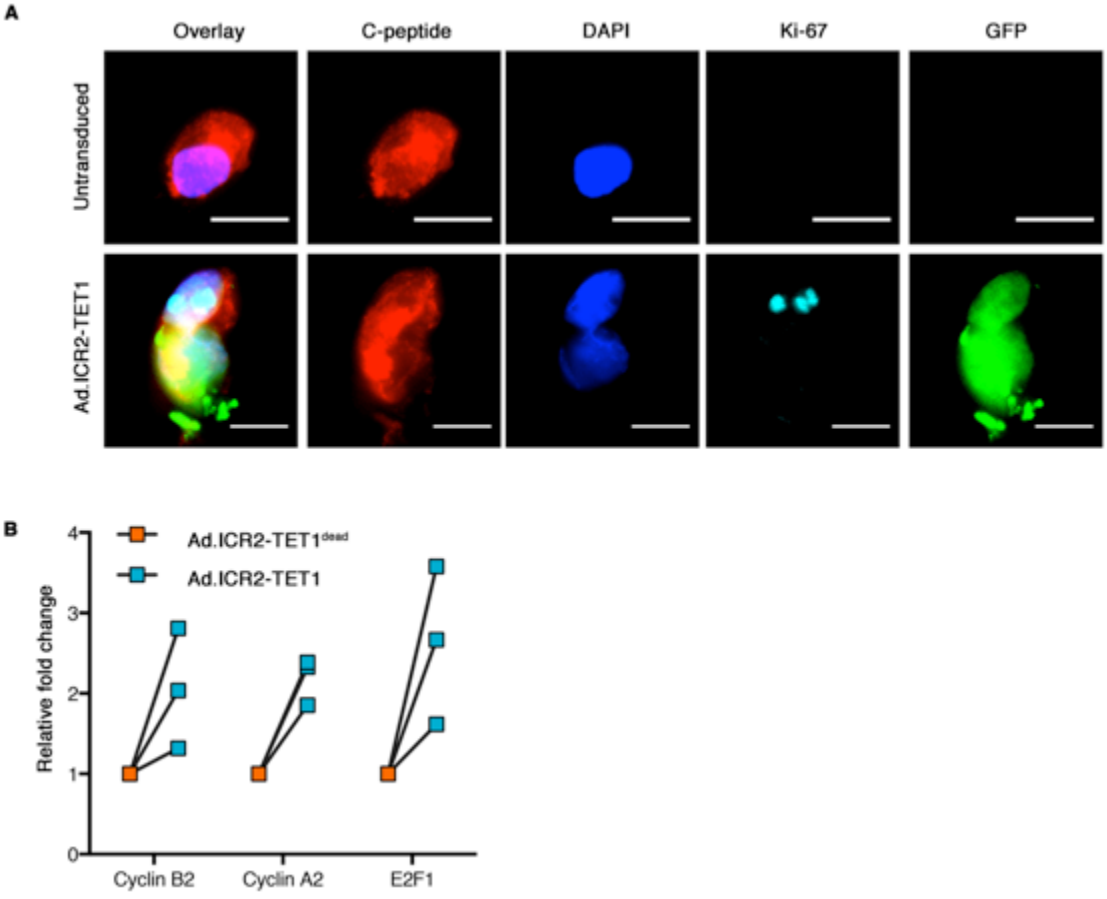
(B) Quantification of nuclei containing zero, one (mono-allelic), or two (bi-allelic) *KCNQ1OT1* RNA FISH spots (n=100 for both ICR2-TET1^{dead} and ICR2-TET1 groups).

Supplemental Figure 4. Transduction efficiency in human β cells.



Human islet samples were dispersed and antibody labeled for analysis by flow cytometry. Sequential gating was used to exclude doublets and cell clusters (subpanel 1), dead cells (subpanel 2), non-cell events (subpanel 3), and acinar / duct / endothelial / alpha cells (subpanel 4). GFP^+ cells were gated on the beta cell population.

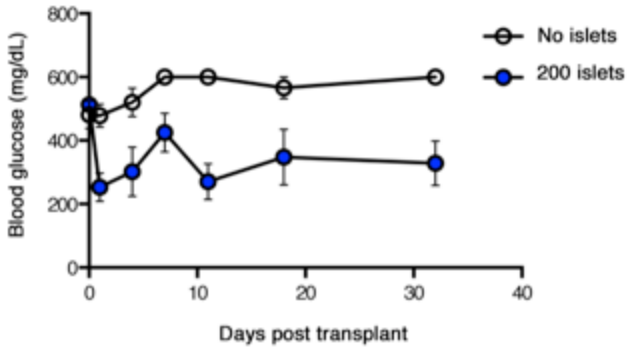
Supplemental Figure 5. Additional evidence for replication in β cells.



(A) Transduced islets were enzymatically dispersed on a coverslip and immunolabeled for C-peptide, Ki-67, GFP, and DAPI. Scale bar: 10 μm.

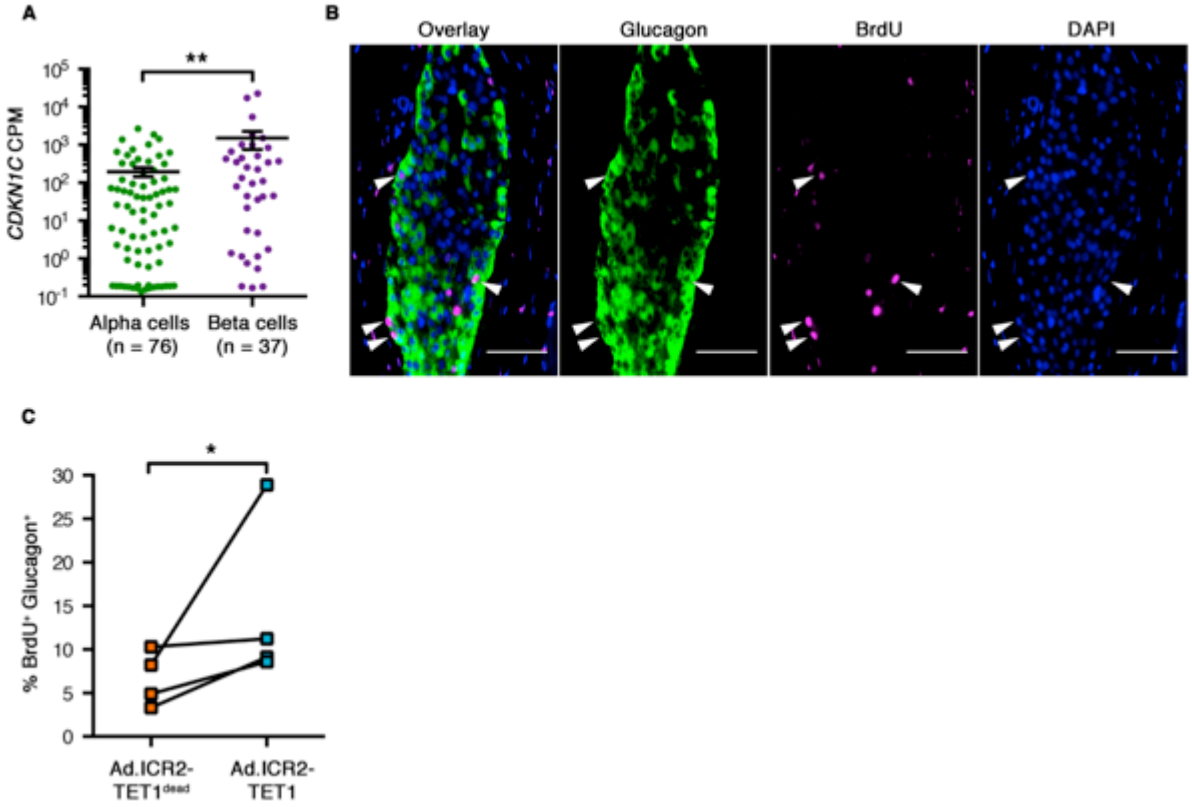
(B) mRNA levels of G2/M transition genes in transduced whole islets obtained from 3 non-obese, non-diabetic donors. mRNA levels were normalized to *HPRT1*.

Supplemental Figure 6. Blood glucose levels of streptozotocin (STZ)-treated NSG mice after human islet transplantation.



NSG mice were fasted overnight before STZ injection at a dose of 200 mg/kg of body weight. After 3 days, mice were subcutaneously transplanted with 0 islets or 200 total islets. Blood glucose was monitored during the duration of the study (n = 4 for each group).

Supplemental Figure 7. ICR2 demethylation induces α cell proliferation.



(A) *CDKN1C* mRNA levels from single cell RNA-seq data (**, p<0.01 using Mann-Whitney test).
(B) Example of a xenograft section immunolabeled for glucagon and BrdU. $BrdU^+$ alpha cells are inidicated with white arrows. Scale bar: 50 μm.
(C) Percentage of $BrdU^+$ alpha cells in sectioned xenografts. Each pair of points represents an individual donor. P-value was calculated using the ratio paired t-test after testing for normality using the Shapiro-Wilk test (*, p<0.05).

Supplemental Table 1. Primer sequences.

Primer Name	Primer Sequence (5'-3')	Application
CDKN1C_F	GCGGCGATCAAGAAGCTGT	RT-qPCR
CDKN1C_R	GCTTGGCGAAGAAATCGGAGA	RT-qPCR
KCNQ1OT1_F	TGGCAATAGGGATGGAGGGA	RT-qPCR
KCNQ1OT1_R	ACCTCAAGCCCATCACCAAG	RT-qPCR
MKI67_F	TGGGGCAGGTTCTTAGTTTG	RT-qPCR
MKI67_R	AGGGGAGCCAGAAACTCAAT	RT-qPCR
HPRT_F	GCAGACTTTGCTTTCCTTGG	RT-qPCR
HPRT_R	AACACTTCGTGGGGTCCTTT	RT-qPCR
ICR2_F1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTTTAGGTTAGGTTGTATTGTTG	Targeted bisulfite seq
ICR2_R1	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTCAACATAATTCTCCTC	Targeted bisulfite seq
ICR2_F2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGGTAGGATTTTGTTGA	Targeted bisulfite seq
ICR2_R2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCACCCAACCAATACCTCATA	Targeted bisulfite seq
ICR2_F3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTATTTATTTGGTAAAGGG	Targeted bisulfite seq
ICR2_R3	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAACACTATTTTTACAATCCAC	Targeted bisulfite seq

Supplemental Methods

TALE-TET1 construction

The TALE target sequence was selected by inserting the genomic sequence of the ICR2 (hg19 chr11:2720411-2722087) into the TAL Effector-Nucleotide Targeter 2.0 online tool (1). The binding site sequence 5'-TGCTGACCGTGTTCAAACCC-3' was selected due to predicted minimal off-target binding elsewhere in the genome. The coding sequence of the TALE repeat domain was jumbled as previously published (2) to minimize recombination events during lentivirus transfer plasmid propagation. The final sequence of the TALE repeat domain was synthesized by Eurofins Genomics and cloned into pTALE-TFv2 (Addgene #32188). The SV40-NLS-VP64 element was replaced by the SV40-NLS-TET1 active and inactive ($TET1^{dead}$) catalytic domain (amplified from Addgene plasmids 49239 and 49965) using restriction enzyme digest and ligation.

Lentivirus production

The complete ICR2 TALE-TET1 or TALE-$TET1^{dead}$ sequence was inserted into the pLU-EF1α-eGFP lentivirus transfer plasmid (Wistar Expression Core) by restriction enzyme digest and ligation. The transfer plasmid was then modified by removing the IRES-eGFP element to accommodate the limited packaging

size. Lentiviral particles were generated by co-transfecting HEK293T cells with pLU-ICR2-TALE-TET1 or pLU-ICR2-TALE-TET1^{dead}, psPAX2, and pMD2.G (Addgene). Lentiviral particles were harvested 24 and 48 hours after transfection, and concentrated by ultracentrifugation. Viral titers were estimated by ELISA using the LentiX p24 Rapid Titer Kit (Clonetech).

Adenovirus production

Adenovirus was generated using the AdEasy system (Agilent). Briefly, the ICR2 TALE-TET1 or TALE-TET1^{dead} sequences were sub-cloned into the pAdTrack-CMV shuttle vector (Addgene #16405). Due to the packaging constraints of adenoviral genome, the CMV GFP elements were replaced by IRES-GFP (amplified from pLU-EF1α-eGFP), which was cloned directly downstream of the stop codon of the TET1 catalytic domain. The purified pAdTrack-ICR2-TALE-TET1-IRES-GFP plasmid was linearized by PmeI digestion and electroporated into BJ5183-AD-1 cells (Agilent) containing the AdEasy1 destination adenoviral packaging plasmid. Recombinant plasmids were screened by restriction digest and Sanger sequencing. ElectroMAX DH10B cells (Thermo Fisher Scientific) were electroporated with the recombinant AdEasy1 plasmid for large scale production. Adenoviral particles were generated by transfecting HEK293T cells with linearized recombinant plasmid as previously described (3). After two rounds of amplification, adenoviral particles were isolated and concentrated using Fast-Trap Virus Purification and Concentration Kit (Millipore). Viral titers were determined by flow cytometry.

HEK293T transfection

HEK293T cells were seeded in 60mm dishes at 2.8x10^6 cells the evening prior to transfection. Cells were transfected with 10 μg of pAdTrack.ICR2-TET1, pAdTrack.ICR2-TET1^{dead}, or pAdTrack.TET1-cd plasmids using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. GFP$^+$ cells were collected by fluorescence-activated cell sorting 72 hours after transfection. RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA generated using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Changes in gene expression were assessed by real time quantitative PCR using Brilliant III Ultra Fast QPCR Master Mix (Agilent).

Human fibroblast transduction

Human foreskin fibroblasts were purchased from ATCC (CRL-2522) and cultured in 10% FBS in IMDM (Thermo Fisher Scientific). Fibroblasts were seeded at $1.5x10^5$ cells/well in a 6-well plate the evening before lentivirus or adenoviral transduction. Cells were infected at an MOI of 50 with Polybrene reagent (Sigma Aldrich). RNA and genomic DNA were harvested 72 hrs later using the AllPrep DNA/RNA Mini Kit (Qiagen). cDNA synthesis and RT-qPCR were performed as described above.

Targeted bisulfite sequencing

Targeted bisulfite sequencing primers were designed manually using MethPrimer (4) with partial adapter overhangs as described in Bernstein, DL *et al* (2). 100 ng of genomic DNA isolated from sorted cells were bisulfite converted using EZ DNA Methylation-Gold Kit (Zymo Research). Targeted ICR2 regions were amplified using the PyroMark PCR Kit (Qiagen), and gel purified using QIAquick Gel Extraction Kit (Qiagen). Libraries were barcoded using the Indexing Primer Set HT for Illumina (Takara), and library concentrations and fragment lengths were verified by Qubit (Thermo Fisher Scientific) and High Sensitivity DNA BioAnalyzer (Agilent), respectively. Next-generation sequencing and analysis was carried out as previously described (2). Primers are listed on Supplemental Table 1.

Western blot

Fibroblasts were seeded and transduced as described above. After 72 hours, cells were lysed with RIPA buffer (Cell Signaling Technology) supplemented with PMSF (Roche). Western blot was performed using the NuPAGE electrophoresis system (Invitrogen) with a 4-12% Bis-Tris acrylamide gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen) for 7 minutes. The membrane was blocked with 5% milk in tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with an anti-p57 primary antibody (Abcam ab75974, 1:100) overnight at 4 degrees. After washing with TBST, the membrane was incubated with Peroxidase AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch, 1:5,000) secondary antibody at room temperature for 1 hour. The membrane was washed and imaged with Luminata Crescendo Western HRP Substrate (Millipore). The membrane was then stripped and re-blotted for Vinculin (Cell Signaling Technology #4650, 1:1,000).

EdU incorporation assay

For proliferation studies, fibroblasts were seeded at 50% confluency on 13mm sterilized Nunc Thermanox Coverslips (Thermo Fisher Scientific) placed in a 24-well plate. Fibroblasts were transduced with lentivirus for 24 hrs. After transduction, fibroblasts were serum starved with 1% FBS in IMDM for 48 hours to arrest and sync the cell cycle. After replacing the serum-depleted media with regular growth media, fibroblasts were allowed to grow for an additional 48 hrs. During the last hour of growth, fibroblasts were pulsed with EdU to label proliferation. Cells were fixed with 4% paraformaldehyde and EdU incorporation was visualized using the Click-iT Plus EdU Alexa Fluor kit according to the manufacturer's protocol (Thermo Fisher Scientific). Cells were imaged on the Keyence All-in-One Fluorescence microscope using a 0.2 NA 4X objective (Nikon).

RNA fluorescent *in situ* hybridization

Human *KCNQ1OT1* Stellaris FISH probes with Quasar 670 Dye were purchased from LGC Biosearch Technologies. Fibroblasts were seeded and transduced with Ad.ICR2-TET1 or Ad.ICR2-TET1^{dead} for 72 hours in a 6 cm dish before fluorescence-activated cell sorting using the BD FACSAriaII. GFP^{+} cells are seeded onto Poly-L-Lysine treated glass slides for 24 hours before fixing in 4% paraformaldehyde. Hybridization steps were carried out as previously described (5). Images of cells were acquired using a 1.4 NA 63X oil-immersion objective (Leica) on a Leica wide-field fluorescence microscope. Coordinates of all captured images were saved and used for imaging DNA FISH.

Oligopaint DNA fluorescent *in situ* hybridization

Probe libraries were designed using the Oligopaint pipeline with the default parameters of OligoMiner (6, 7). Specifically, 42 nucleotide sequences with homology to the region of interest were mined from the hg38 genome build. The probe library targeted a 100 kb region of sequence. Oligopaints were synthesized as previously described (8). Slides that had already been imaged for *KCNQ1OT1* RNA FISH were pre-denatured in 2×SSCT/50% formamide at 92° for 2.5 minutes, then in 2×SSCT/50% formamide at 60° for 20 minutes. Oligopaint hybridization and visualization steps were carried out as previously described (8).

Human islet transduction

Human islets from non-obese, non-diabetic organ donors were obtained from the Integrated Islet Distribution Program. Islets were cultured in suspension using Prodo Standard Islet Media (Prodo Laboratories Inc). For whole islet gene expression and methylation analysis, 100 islets were picked for each group and transduced with Ad.ICR2-TET1 or Ad.ICR2-TET1^{dead} adenovirus at an MOI of 300. RNA and gDNA were isolated after 72 hours using the Qiagen Allprep DNA/RNA Mini Kit. Transduced GFP^{+} β cells were sorted by FACS as previously described (9).

Immunofluorescent staining of dispersed islets

Human islets were enzymatically digested with 0.05% trypsin and seeded on Poly-D-lysine treated glass coverslips placed in a 24-well plate. Dispersed cells were transduced with adenovirus (MOI = 300) for 72 hours after attachment to the coverslips. Cells were fixed with 4% paraformaldehyde, washed 3 times with cold PBS, permeabilized with PBST, and blocked with CAS-Block Histochemical Reagent (Thermo Fisher Scientific). Cells were stained for p57 (Abcam ab75974, 1:150), GFP (Aves Labs GFP-1020, 1:300), C-peptide (Millipore 05-1109, 1:300), and Ki-67 (Thermo Fisher SP6, 1:150) overnight at 4 degrees. After washing 3 times in PBST, cells were labeled with cyanine-conjugated secondary antibodies (Jackson ImmunoResearch, 1:600) and DAPI. After mounting onto microscope slides, cells were imaged with the Keyence All-in-One Fluorescence Microscope using a 1.4 NA 60X oil-immersion objective (Nikon).

Whole islet insulin secretion

Human islets were transduced with adenovirus for 72 hours prior to the perifusion procedure. 100 islets were picked and perifused as previously described (10) using Krebs-Ringer bicarbonate buffer (KRBB) media containing 3 mM glucose, 16.7 mM glucose, or 30 mM KCL in addition to 0.25% BSA. Fractions were collected at 1 minute intervals for 120 minutes, and insulin content was determined using a radioimmunoassay.

Single-cell glucose-stimulated calcium imaging

Human islets were enzymatically dispersed and seeded onto glass bottom wells. After recovering for 48 hours, dispersed cells were transduced with adenovirus for 72 hours. Cells were loaded with fura-2AM during a 40 min pretreatment at 37 °C in 2 ml KRBB supplemented with 5 mM fura-2 acetoxymethylester (Molecular Probes). Before perifusion with amino acid-supplemented media containing 3mM glucose, 16.7 mM glucose, or 30 mM glucose, an image was captured with the bright field and GFP channels to identify which single cells were successfully transduced with the virus. $[Ca^{2+}]_i$ levels were measured by dual wavelength fluorescence microscopy in response to different perifusion conditions as previously described (11).

Xenotransplantation experiments

Briefly, NSG mice (JAX) were fasted for 16 hours. The following morning, mice were intraperitoneally injected with pharmaceutical grade streptozotocin (Teva Pharmaceuticals) dissolved in 0.9% normal saline at a dose of 200 mg/kg of body weight. Three days after injection, hyperglycemic mice (blood glucose levels greater than 300 mg/dL) were shaved and anesthetized prior to making 2 small subcutaneous incisions at either side of the abdominal midline. For each group, 100 transduced islets were inserted subcutaneously in either incision, which were sealed by suturing.

To label all proliferation events, mice were given BrdU (Sigma) through their drinking water at a concentration of 0.8 g/L, which was replaced twice a week. Abdominal skin sections containing human islets were harvested 21 days after transplantation, fixed overnight with 4% paraformaldehyde, and processed by paraffin sectioning. Histological analysis of slides was performed as previously described (12) using BrdU (Abcam ab6326, 1:300), C-peptide (Millipore 05-1109, 1:300), and glucagon (Santa Cruz sc7779, 1:300). Images were captured on the Keyence All-in-One Fluorescence Microscope using the 0.95 NA 40X objective (Nikon).

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