Avrahami et al., "Suppression of p57Kip2 allows successful replication of adult human β-cells"

Legends to Supplementary Figures

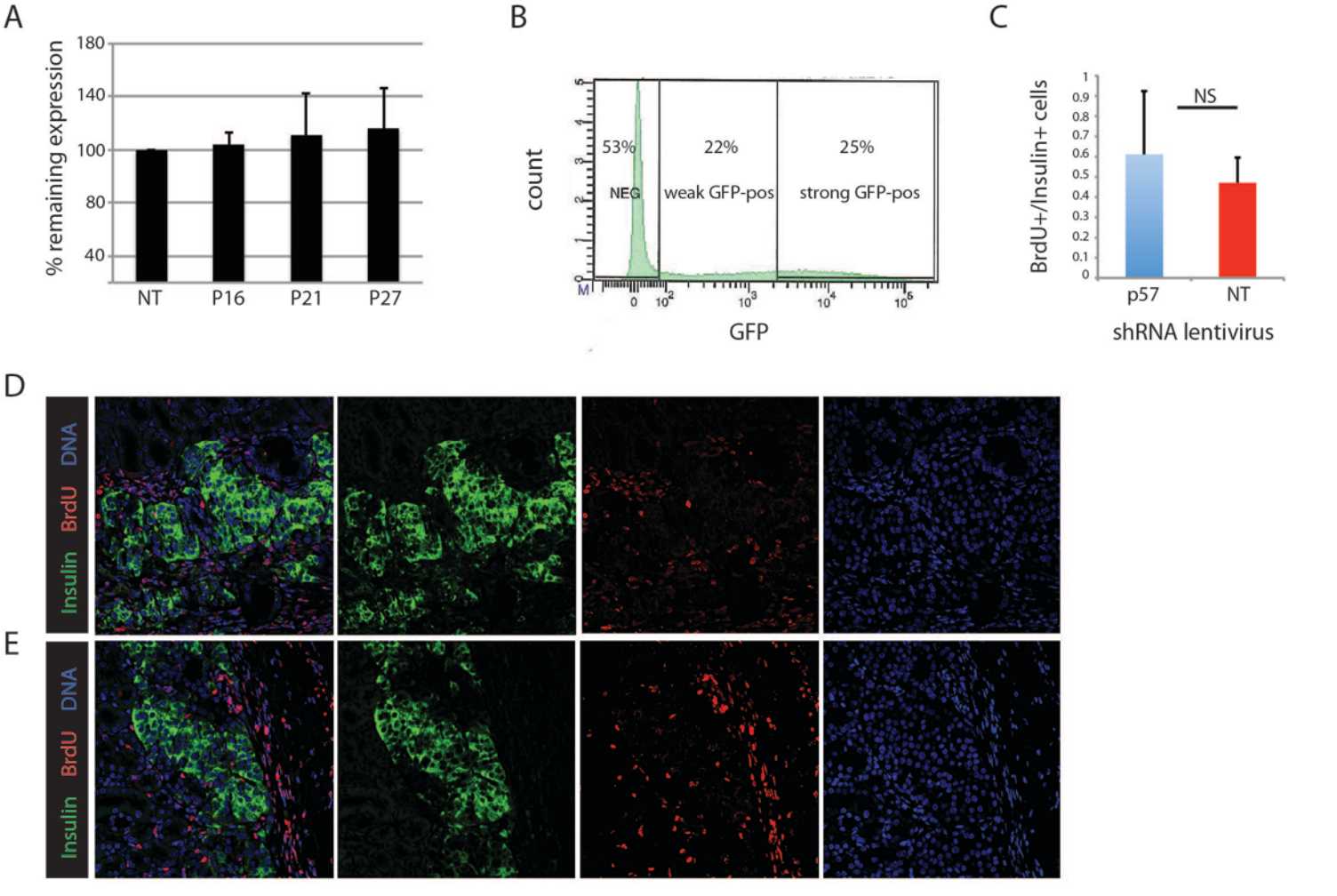
Supplementary Figure 1. A. Suppression of $p57^{Kip2}$ in human islets does not affect other cell cycle inhibitors. Quantitative RT-PCR showing the mRNA expression levels of p16, p21 and p27 (*CDKN2A*, *CDKN1A* and *CDKN1B,* respectively) in cultured human islets transduced with lentiviral particles containing pGIPZ-shRNA (clone p57-c) against $p57^{Kip2}$. 72 hours after transduction, the islet cells were dispersed and FACS sorted for GFP positive (transduced) and GFP-negative fractions for gene expression analysis. Expression levels are normalized to non-targeting (NT) shRNA control (n=3 human islet donors). **B. FACS analysis of cultured human islets transduced with lentiviral particles containing pGIPZ-shRNA against $p57^{Kip2}$.** 72 hours after transduction, we dissociated the islets and FACS sorted the cells for GFP positive (transduced) and GFP-negative fractions. Islet cells that express low levels of GFP are mainly from inner layers of the islets, about 22%. Cells that express strong levels of GFP are mainly from the outer layers of the islets (about 25%). **C, D and E. Transplantation of $p57^{Kip2}$-suppressed islets into normoglycemic immunodeficient mice does not enhance β-cell replication.** Transplanted mice were not rendered diabetic, and glucose levels remained normal throughout the transplantation period. **C.** Average percentage of BrdU-positive β-cells in $p57^{Kip2}$-suppressed islets (blue bars, 0.61%) was found not significantly higher than non-targeting (NT) shRNA transduced islets (red bars, 0.47%). n=3 donors. **D.** Co-staining of recovered grafts transduced with lentiviral particles containing shRNA against $p57^{Kip2}$ for insulin (green), BrdU (red) and DNA (blue). **E.** Co-staining of recovered grafts transduced with lentiviral particles containing NT shRNA control for insulin (green), BrdU (red) and DNA (blue). Images are representative, original magnification was 400x.

Supplementary Figure 2. A and B. The effect of $p57^{Kip2}$ suppression in human islet cells is specific to β-cells. Co-staining for insulin (green), glucagon (white in A) or somatostatin (white in B), BrdU (red) and DNA (blue). Yellow arrows point to glugacon^{+}/BrdU^{-} α-cells in (A) and to somatostatin^{+}/BrdU^{-} δ-cells in (B). White arrows

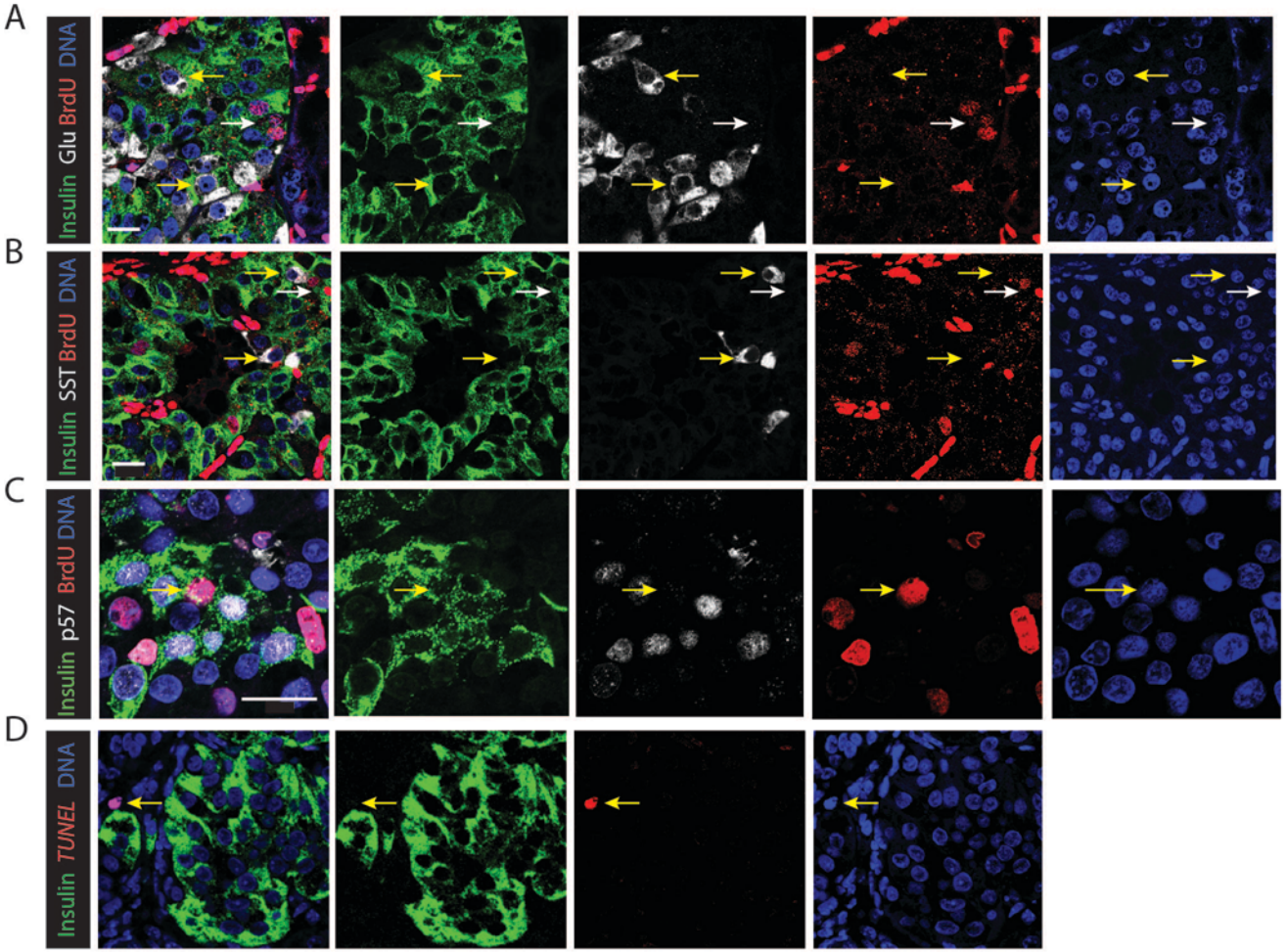
point to insulin^{+}/BrdU^{+} β-cells. **C.** Proliferating β-cells in control grafts transduced with NT-shRNA virus show negligible protein levels of p57^{Kip2}. Co-staining for p57^{Kip2} (white), insulin (green), BrdU (red) and DNA (blue). Arrows point to BrdU-positive/p57^{Kip2}-negative β-cells. **D.** Suppression of p57^{Kip2} in human islets does not cause apoptosis. Co-staining for insulin (green), *TUNEL* (red) and DNA (blue). Arrow points to a non-islet *TUNEL* positive cell. Images are representative, original magnification was 400x.

Supplementary Figure 3: Replicated β-cells following suppression of p57^{Kip2} express markers of mature β-cells. A. Co-staining for NKX6.1 (green), insulin (white), BrdU (red) and DNA (blue). Arrow points to a BrdU/insulin/NKX6.1 positive cell. **B.** Co-staining for PDX1 (green), BrdU (red) and DNA (blue). Arrows point to BrdU/PDX1 positive cells. Images are representative, original magnification was 400x.

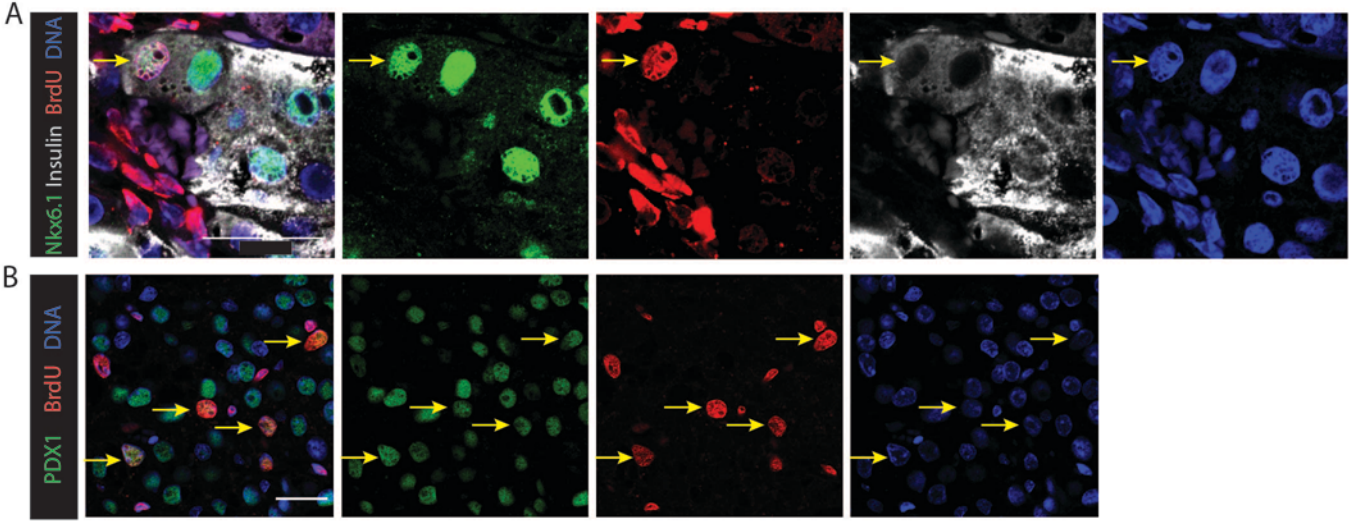
Supplemental Figure 1



Supplemental figure 2



Supplemental figure 3



METHODS

Lentivirus production

Lentivirus particles were produced by the Wistar Institute protein expression following[1]. Briefly, 293TN producer cells were co-transfected with the modified pGIPZ-shRNA expressing vector and 2nd generation packaging system (pCMV-dR8.91 and VSVg). After 48-72 hours, the cell culture medium containing the secreted viral particles was harvested. Cellular debris was removed from the cell culture media, and viral particles were concentrated by ultracentrifugation.

Culturing, transduction, and xenotransplantation of human cadaveric islets

Cadaveric human islets were supplied by the Diabetes Research Center of the University of Pennsylvania (NIH DK 19525), the National Disease Research Interchange (NDRI) (http://www.ndriresource.org), and the Integrated Islet Distribution Program (IIDP) (http://iidp.coh.org/). Islets were incubated in CMRL 1066 medium (Mediatech, Manassas, VA) containing 5.5 mm d-glucose, 0.5% human albumin (Talecris Biotherapeutics, Research Triangle Park, NC), 10 U/ml Heparin (Sagent Pharmaceuticals, Schaumberg, IL), 100 µg/ml penicillin/streptomycin, and 2 mM l-glutamine. About 500 islets were transduced with shRNA lentivirus (1.75e9 TU/mL by FACS titration) for 24h followed by washing. 200-400 islets were then xenotransplanted under the kidney capsule of streptozotocin-induced diabetic immunodeficient mice (*nude* or NOD/*SCID* gamma [NSG], The Jackson Laboratory, Stock Number:002019 and 005557, respectively). For the duration of the experiment mice received 1mg/ml bromodeoxyuridine (BrdU) (Sigma) in the drinking water. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Tissue processing

Mice were sacrificed ~20 days after engraftment and their transplanted kidney was removed. For histology, kidneys were immersed-fixed in 4% formalin buffer and embedded in paraffin. For the single cell calcium imaging assay, the grafts were retrieved, dispersed into single cells with 0.25% trypsin, and then cultured on coverslips in RPMI 1640 (Sigma) supplemented with 10 mM glucose, 10% fetal bovine serum, 100 µg/ml penicillin/streptomycin, and 2 mM l-glutamine.

Immunofluorescence analysis

Antigen retrieval was performed on Paraffin sections (5 µm) by pressure cooker heating (Prestige Medical, Northridge, CA) using citrate buffer (pH 6.0). Sections were blocked with CAS-Block (Invitrogen). Primary antibodies used in this study included: guinea pig anti-insulin (1:500; DAKO), rat anti-BrdU (1:500; Accurate Chemical), guinea pig anti-Pdx1 (1:2,500; a generous gift of Chris Wright), rabbit anti-Nkx6.1 (1:1000) from Beta Cell Biology Consortium, rabbit anti-turbo-GFP antibody (1:1000; Evrogen), rabbit anti-ki67 (1:200; Neomarkers, Fremont, CA, USA). For DNA counterstain we used DAPI (1:100; Sigma-Aldrich). Rabbit anti-Nkx6.1 required 48 hours incubation time. Secondary antibodies were all from Jackson Immunoresearch Laboratories (1:250; West Grove, PA, USA). For double staining, we used only affinity-purified secondary antibodies suitable for multiple labeling. All immunofluorescence images were captured on a Zeiss LSM 710 or Olympus FV1000 confocal microscope.

Evaluation of $p57^{Kip2}$ suppression

Evaluation of multiple shRNA constructs for suppression of $p57^{Kip2}$ mRNA expression was performed by transient gene silencing in human embryonic kidney (HEK) 293 cells using green fluorescence protein (turboGFP)-labeled shRNA lentiviral plasmids (pGIPZ-Lentiviral shRNA, Termo scientific). Two to three days following transfection, cells were sorted using FACS for GFP-positive and GFP-negative cell populations directly into TRI Reagent (Sigma-Aldrich,

USA) for subsequent gene expression analysis. For evaluation of $p57^{Kip2}$ suppression in cadaveric human pancreatic islets, about 500 cultured islets were transduced with lentiviral particles (1.75e9 TU/mL by FACS titration) containing pGIPZ-Lentiviral shRNA against $p57^{Kip2}$ or non-targeting shRNA control. Four to five days post transduction, islets were dissociated using trypsin and FACS-sorted for GFP-positive and GFP-negative cell populations directly into TRI Reagent (Sigma-Aldrich, USA) for subsequent gene expression analysis.

RNA isolation and quantitative real-time PCR

Total RNA was prepared using RNeasy minikit (Qiagen) according to the manufacturer's instructions. cDNA was prepared from 200 ng of total RNA using SuperScript II reverse transcriptase and oligo dT priming (Invitrogen). PCR reactions were performed using SYBR Green QPCR Master Mix (Agilent Technologies) or TaqMan gene expression assays (Applied biosystems) on an Mx3000 PCR cycler (Agilent Technologies). Reactions were performed in triplicate and GAPDH transcript levels were used to normalize between samples. The primers used with SYBR Green were $p57^{Kip2}$, 5′- GGC GAT CAA GAA GCT GTC C -3′ (forward), 5′- GAC ATC GCC CGA CGA CTT -3′ (reverse), GAPDH, 5’- GTC AGC CGC ATC TTC TTT TG -3’ (forward), 5’ -AGT TAA AAG CAG CCC TGG TG -3’.

Single-cell glucose-stimulated calcium influx

Ca^{2+} imaging of dispersed human islet cells retrieved from grafts was carried out following[2] with modifications. Briefly, at the end of the transplantation experiment, the grafts were retrieved from the kidney capsule, treated with trypsin, and single cells seeded for 48-72 hours on coverslips as mentioned above. Coverslips were first incubated with 15μM Fura-2 acetoxymethylester (Invitrogen) in Krebs-Ringer bicarbonate buffer (KRBB) with 5 mM glucose for 20 min, and then placed in a perfusion chamber mounted onto a Zeiss inverted microscope. The cells were then perifused in KRBB with 0.25% BSA at a flow rate of 1 mL/min containing

first 5 mM, then 10 mM glucose in order to assess glucose stimulation. Cytosolic calcium ($[Ca^{2+}]_i$) was measured by dual wave length fluorescence microscopy using a Zeiss AxioVision system as described previously[3]. After measurements of $[Ca^{2+}]_i$, without changing the field and position, cells were washed by PBS with 3% BSA, then fixed by 4% paraformaldehyde/PBS solution for 5 min, and then washed again and permeabilized with 1% Triton for another 5 min. Coverslips were incubated with mouse anti-BrdU antibody (BD Biosciences, Franklin Lakes, NJ) for 40 min, and after three washes by 3% BSA in PBS, a secondary antibody conjugated to Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) and DAPI (Molecular Probes, Eugene, OR) were applied for another 40 min. Next, BrDU, DAPI and bright field images were obtained, and each cell imaged during the Fura-2 staining for $[Ca^{2+}]_i$ measurement was identified by superimposing the BrDU, DAPI and bright field channels.

1 Tiscornia, G., Singer, O. & Verma, I. M. Production and purification of lentiviral vectors. *Nat Protoc* **1**, 241-245, doi:10.1038/nprot.2006.37 (2006).

2 Li, C. *et al.* Mechanism of hyperinsulinism in short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency involves activation of glutamate dehydrogenase. *J Biol Chem* **285**, 31806-31818, doi:10.1074/jbc.M110.123638 (2010).

3 Li, C. *et al.* Regulation of Glucagon Secretion in Normal and Diabetic Human Islets by Gamma-Hydroxybutyrate and Glycine. *J Biol Chem*, doi:10.1074/jbc.M112.385682 (2012).