Supplemental Information

Overexpression of Hepatocyte Nuclear Factor- 4α initiates cell cycle entry, but is not sufficient to promote β-cell expansion in human islets

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A. Supplemental Figures and Legends:

Supplemental Figure 1 related to Fig. 1

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Supplemental Figure 3 related to Fig. 3

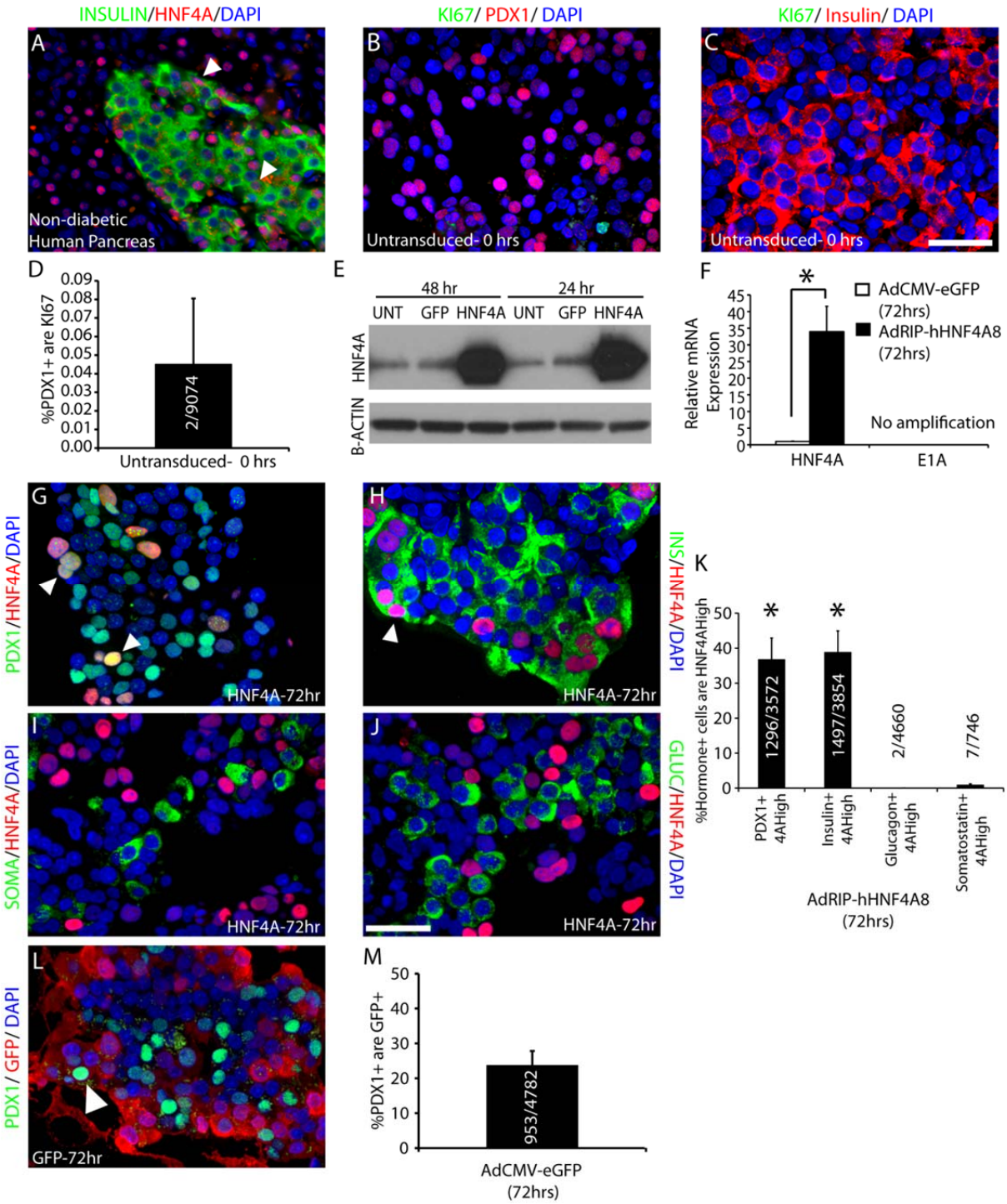
Supplemental Figure 4 related to Fig. 4

Supplemental Table 1 – Primary antisera employed for immunofluorescence analysis.

Supplemental Table 2 – Clinical information of human islet donations used in this study with contributions to Figures.

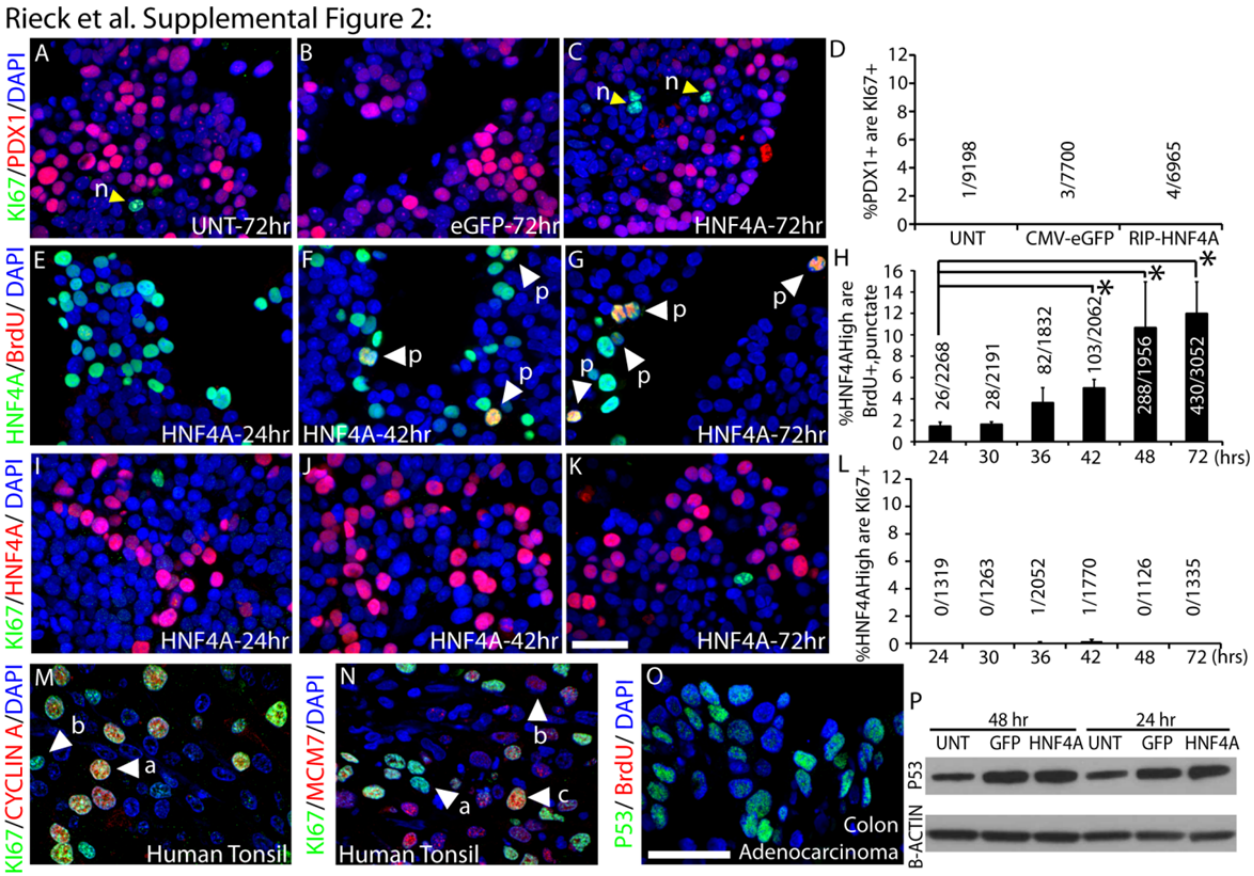
B. Supplemental References

Supplemental Figure 1:



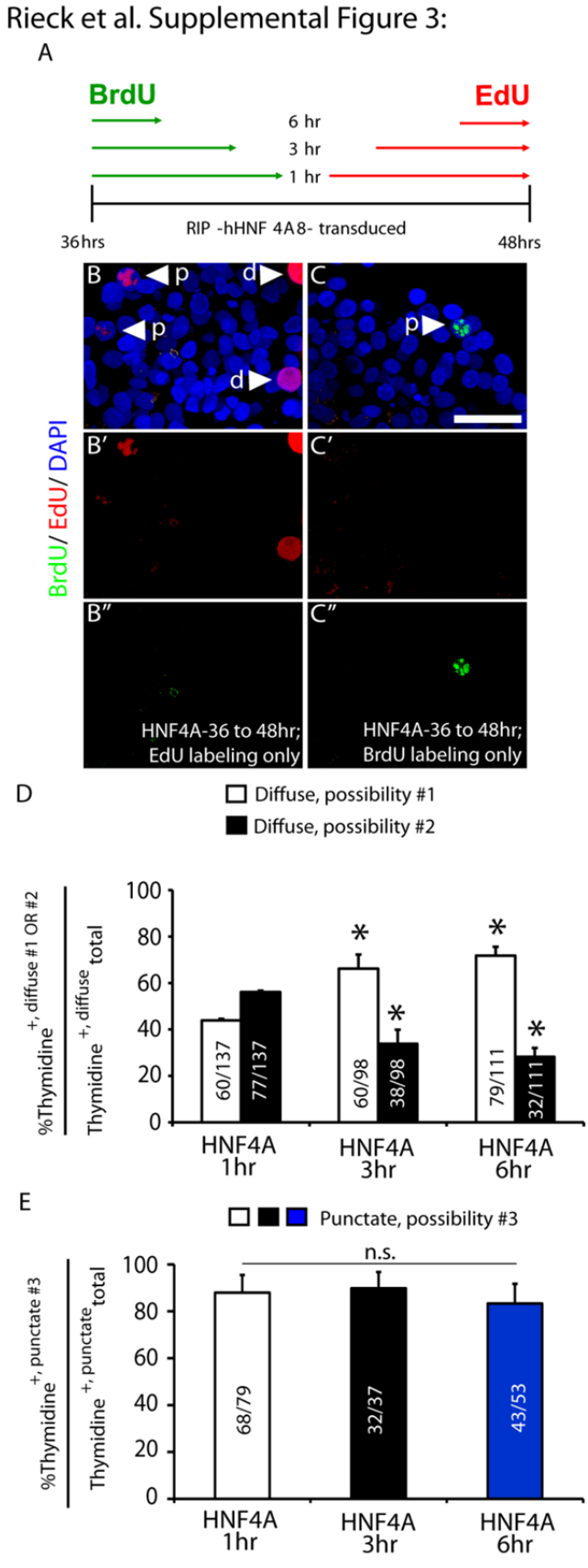
Supplemental Figure 1: Adenoviral overexpression of HNF4α8 in human β-cells. (A) Immunostaining of non-diabetic human pancreas with Insulin (green), HNF4α (red) and DAPI (blue). White arrows indicate examples of β-cells expressing HNF4α. Immunolocalization of Ki67 (green), DAPI (blue), (B) Pdx1 (red) and (C) Insulin (red) of untransduced primary human islets before transduction (0 hours). (D) Quantification of the percentage of $Pdx1^+$ that are $Ki67^+$ (n=3). (E) Western blot analysis of HNF4α expression in untransduced, eGFP-, and HNF4α8-transduced islets 24 hours and 48 hours after transduction with β-actin as a loading control (n=1 per time point). A replication-competent adenovirus, encoding *E1A*, can lead to misleading results when assessing β-cell proliferation (1). (F) Gene expression of *HNF4α* and *E1A* in AdCMV-eGFP and AdRIP-hHNF4α8 treated islets (n=5-6; *, $p<0.001$ versus CMV-eGFP condition). Dual immunofluorescence staining of HNF4α (red) and DAPI (blue) with (G) Pdx1 (green), (H) Insulin (green), (I) Somatostatin (green) and (J) Glucagon (green) in human islets incubated for 72 hours after transduction with AdRIP-hHNF4α8. White arrows show examples of $Pdx1^+$, $HNF4\alpha^{High}$ and $Insulin^+$, $HNF4\alpha^{High}$ cells. (K) Quantification of the percentage of hormone-expressing cells that are $HNF4\alpha^{High}$ (n=3 per $hormone^+$ group; Both $Pdx1^+$, $HNF4\alpha^{High}$ and $Insulin^+$, $HNF4\alpha^{High}$ groups are statistically significantly higher than either $Glucagon^+$, $HNF4\alpha^{High}$ and $Somatostatin^+$, $HNF4\alpha^{High}$, groups, *, $p<0.005$ as determined by one-way ANOVA with Tukey *post-hoc* test). (L) Immunodetection of Pdx1 (green), GFP (red), and DAPI (blue) in eGFP-transduced islets 72 hours after transduction. The white arrow indicates an example of a $Pdx1^+$ cell expressing eGFP. (M) Quantification of the percentage of $Pdx1^+$ cells that are GFP^+ 72 hours after transduction (n=4), statistically similar to the transduction efficiency seen when using AdRIP-hHNF4α8. The scale bars in (C, J) indicate 25μm.

Supplemental Figure 2



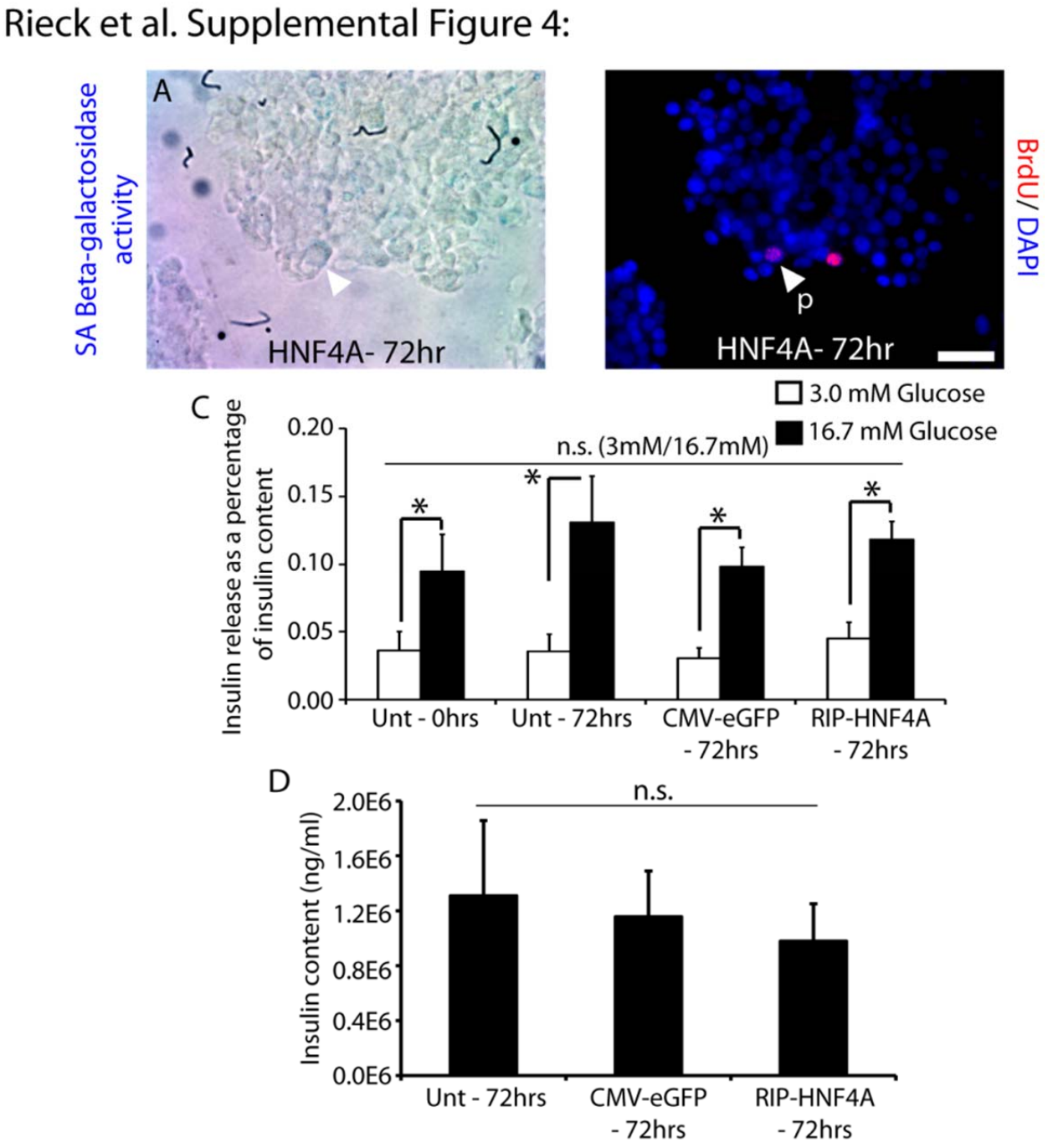
Supplemental Figure 2: HNF4α^{High} β-cells do not show signs of complete cell cycle progression. Immunolocalization of Ki67 (green), Pdx1 (red) and DAPI (blue) in (A) untransduced, (B) AdCMV-eGFP, and (C) AdRIP-hHNF4α8 transduced islets 72 hours after transduction. (D) Quantification of the percentage of Pdx1^{+} cells that are Ki67^{+} in untransduced, eGFP-, and hHNF4α8- transduced conditions 72 hours after transduction (n=4 for each group). Immunodetection of HNF4α (green), BrdU (red), and DAPI (blue) in AdRIP-hHNF4α8 transduced islets (E) 24 hours, (F) 42 hours, (G) and 72 hours after transduction. (H) Quantification of the percentage of HNF4α^{High} cells that are BrdU$^{+,punctate}$ at 24, 30, 36, 42, 48, and 72 hours after transduction (n=3-5 for each time point; 42, 48, and 72 hour time points are statistically significantly higher when compared to 24 hour time point individually, *, $p<0.03$ as determined by one-tailed Student's *t-test*). Assessment of Ki67 (green), HNF4α (red), and DAPI (blue) colocalization in primary human islets (I) 24 hours, (J) 42 hours, and (K) 72 hours after transduction with AdRIP-hHNF4α8. (L) Quantification of percentage of HNF4α^{High} cells that are Ki67^{+} (n=3-4 per time point). Immunostaining of Ki67 (green), DAPI (blue) with (M) Cyclin A and (N) Mcm7 in human tonsil as a positive control. (O) Immunodetection of p53 (green), BrdU (red) and DAPI (blue) in human colon carcinoma (positive control tissue). (P) Western blot analysis of total p53 protein expression in untransduced, eGFP-, and HNF4α8-transduced islets 24 hours and 48 hours after transduction with β-actin expression as a loading control. In (M) arrow 'a' indicates Ki67^{+}, Cyclin A^{+} cell and arrow 'b' Ki67^{+}, Cyclin A^{-} cell. In (N) arrow 'a' indicates Ki67^{+}, Mcm7^{-} cell, arrow 'b' Ki67^{-}, Mcm7^{+} cell, and arrow 'c' a Ki67^{+}, Mcm7^{+} cell. The scale bar in (K, and O) indicates 25 μm.

Supplemental Figure 3:



Supplemental Figure 3: Overexpression of HNF4α8 leads to activation of the DNA damage response associated with replication stress. (A) Schematic of the experimental protocol used in this study to assess repeated licensing of DNA replication at one origin. Immunodetection of BrdU (green), EdU (red), and DAPI (blue) in HNF4α8-transduced primary human islets exposed to (B) EdU-only, and (C) BrdU-only continuously from 36 to 48 hours after transduction. Individual red channel (B'-C') and green channel (B''-C'') are shown. (D) Quantification of the percentage of either thymidine$^{+,\text{diffuse \#1}}$ or thymidine$^{+,\text{ diffuse \#2}}$ over total thymidine$^{+,\text{diffuse}}$ cells in HNF4α8 transduced islets dual labeled with one hour, three hour, and six hour non-labeling times (n=3 per time point and BrdU incorporation pattern; both 3hr$^{\text{Diffuse,\#1}}$, 6hr$^{\text{Diffuse,\#1}}$ and 3hr$^{\text{Diffuse\#2}}$, 6hr$^{\text{Diffuse\#2}}$ are statistically significantly different then either 1hr$^{\text{Diffuse\#1}}$, 1hr$^{\text{Diffuse\#2}}$, respectively, *, $p<0.03$ as determined by one-tailed ANOVA with Tukey *post-hoc* test). (E) Quantification of the percentage of thymidine$^{+,\text{punctate \#3}}$ over total thymidine$^{+,\text{punctate}}$ cells in HNF4α8 transduced islets dual labeled with one hour, three hour, and six hour non-labeling times (n=3 per time point; each time point is statistically similar as determined by one-way ANOVA with Tukey *post-hoc* test). The scale bars in (C) indicates 25 μm.

Supplemental Figure 4:



Supplemental Figure 4: Analysis of senescence and β-cell function in human islets transduced with RIP-hHNF4α8. (A left) Brightfield image of AdRIP-hHNF4α8 transduced islets stained for SA β-galactosidase activity, and (A right) BrdU (red) and DAPI (blue). (B) Static glucose-stimulated insulin secretion assay (GSIS) of untransduced, eGFP-, and HNF4α8-overexpressing primary human islets 72 hours after transduction treated with 3mM and 16.7mM glucose. GSIS was also performed on untransduced primary human islets upon receipt (0 hours) (n=3-6; each glucose stimulation statistically significantly increases insulin secretion individually,*, $p<0.05$, as determined by one-tailed Student's *t-test*; All 3mM and 16.7mM groups are not statistically different as by one-way ANOVA with Tukey *post-hoc* test). (D) Insulin content measurements for untransduced, eGFP-, hHNF4α8- overexpressing islets 72 hours after transduction (n=3-5 per group; each group is time is statistically similar as determined by one-way ANOVA with Tukey *post-hoc* test). The white arrow in (A right) indicates a $BrdU^{+,punctate}$ cell that is positive for SA β-galactosidase activity. The scale bars in (A right) indicate 25 μm.

Supplemental Table 1 **Primary Antisera Employed for Immunofluorescence Analysis**				
Protein/Antibody	**Species**	**Company**	**Category Number**	**Dilution**
Pdx1	guinea pig	Dr. Christopher Wright	(Gift)	1 to 1000
Insulin	guinea pig	Millipore	4010-01L	1 to 250
HNF4alpha	mouse	R and D Systems	PP-H1415-00	1 to 500
HNF4alpha	rabbit	Santa Cruz	8987	1 to 500
BrdU	rat	Accurate Chemical	OBT0030G	1 to 1000
Glucagon	rabbit	Invitrogen	13091	1 to 200
Somatostatin	rabbit	Invitrogen	13099	1 to 200
Ki67	rabbit	Leica/Novacastra	NCL-Ki67p	1 to 500
Cyclin A	mouse	Thermo Scientific	ms-1061	1 to 200
Mcm7	mouse	Thermo Scientific	ms-862	1 to 200
gamma H2AX (Ser 139)	rabbit	Cell Signaling	2577	1 to 250
p53	mouse	Santa Cruz	126	1 to 200
phospho-Chk2 (Thr 68)	rabbit	Cell Signaling	2661	1 to 200
phospho-p53 (Ser 15)	rabbit	Cell Signaling	9284	1 to 200
GFP	goat	Abcam	6673	1 to 250
p16	mouse	Santa Cruz	1661	1 to 250
Nkx6.1	rabbit	BCBC	AB1069	1 to 4000 (TSA Amplification)
MafA	rabbit	Bethyl Laboratories	A300-611A	1 to 2000 (TSA Amplification)

Supplementary Table 2: Clinical Information of Human Islet Donations used in this study					
Islet Preparation	**Donor Age**	**Donor BMI**	**Islet Purity**	**Islet Viability**	**Used for following data (Figure #):**
Non-diabetic 1	49	24.5	85	94	1J, S1F, S2D, 3D, 4D, S4A, S4D
Non-diabetic 2	40	24.4	85	87	1J, 1O, 1P, S1M, S2D, S2H, S2L, 3D, 3I, 3J, 4G, 4J, S4A, S4D
Non-diabetic 3	48	25.6	80	89	1J, 1K, 1O, 1P, S1K, S1M,2D, 2R, S2D, S2H, 3D, 3I, 4D, 4G, 4J
Non-diabetic 4	36	20.5	70	94	1J, 1O, 1P, S1K, S1M, S2D, S2H, S2L, 3D, 4D, 4J
Non-diabetic 5	36	31.6	90	83	S1D, S2H, 2D,S4A
Non-diabetic 6	47	22.8	90	95	S1D, S1E, S2H, S2P, 2D, S4A
Non-diabetic 7	36	38.7	80	96	S1E, S2H, S2P, S2L, 2D, 2O, 2R, 4M, 4P
Non-diabetic 8	56	25.1	85	90	1O, 1P, S1K, S2D, S2H, S2L, 2D, 2G, 2J, 2O, 2R, 3D, 3I, 3J, 4J, 4M, 4P, S4A, S4D
Non-diabetic 9	54	27.8	85	98	S1K, S2D, S2L, 3I, 4D, 4G, S4A, S4D
Non-diabetic 10	36	40.8	95	90	S1D, S4A
Non-diabetic 11	31	28.7	90	90	1J, 1K, S1K, S2H, 2D, 2O, 2R
Non-diabetic 12	58	26.8	85	96	1K, S1M, 2D, 2R, S2H, S2L, 3I, 3J, 4D, 4G, 4J, 4M
Non-diabetic 13	61	22.9	88	95	S2H, S2L, 2D, 2G, 2J, 3I, 3J, 4G, 4J, 4M, 4P
Non-diabetic 14	28	29.2	80	83	1K, S1K, 2D, 2R, 3J, 4D, 4J
Non-diabetic 15	53	29.2	80	94	3I, 3J, 4G, 4J, S4A, 5C
Non-diabetic 16	22	22.4	85	90	2G, 2J
Non-diabetic 17	50	32.9	85	96	S2D, S3D, S3E, 5C
Non-diabetic 18	42	32.0	90	92	5C, 5F, 5G, 5J, 5K, 6C, 6F, 6G, 6I
Non-diabetic 19	40	Unavailable	95	95	2O, S3D, S3E, 5C, 5F, 5G, 5J, 5K, 6C, 6F, 6G, 6I
Non-diabetic 20	28	36.3	90	86	S3D, S3E, 4M, 5C, 5F, 5G, 5J, 5K, 6C, 6F, 6G, 6I
Non-diabetic 21	57	24.2	85	95	5C, 6C, 6F, 6G, 6I
Non-diabetic 22	38	27.8	90	95	S1F, S4A, S4D
Non-diabetic 23	40	37	95	95	S1F, 4M, 4P
Non-diabetic 24	20	35.2	85	94	S1F
Non-diabetic 25	51	23.5	90	89	S1F
Non-diabetic 26	26	Unavailable	Unavailable	Unavailable	S1A (No report of diabetes history)
Non-diabetic 27	40	28.4	95	94	S4A
Type 2 Diabetic 1	47	27.5	90	96	1P (2 year history of diabetes; HbA1c = 7.4%)
Type 2 Diabetic 2	52	29.2	80	80	1P (HbA1c = 6.3%)
Type 2 Diabetic 3	58	66.4	75	91	1P (15-20 year history of diabetes)

Supplemental References:

1. Lavine JA, Raess PW, Davis DB, Rabaglia ME, Presley BK, Keller MP, Beinfeld MC, Kopin AS, Newgard CB, & Attie AD (2010) *Mol Endocrinol* 24, 464-467.