A Glis3-CD133-Wnt signaling axis regulating self-renewal of adult murine pancreatic progenitor-like cells in colonies/organoids

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LIST OF SUPPORTING INFORMATION:

A. **Supplementary tables**
B. **Supplementary figures and legends**
C. **Reference**

A. SUPPLEMENTARY TABLES:

Table S1. Taqman probes used for conventional and microfluidic qRT-PCR analyses.

Murine Gene	Assay ID from ABI
β-Actin	Mm02619580_g1
Amylase 2A	Mm02342487_g1
Axin2	Mm00443610_m1
CA2	Mm00501572_m1
c-myc	Mm00487803_m1
Ctnnb1	Mm00483033_m1
Elastase-1	Mm00712898_m1
Glis3	Mm00615386_m1
Glucagon	Mm00801712_m1
Hnf1b	Mm00447459_m1
Insulin II	Mm 00731595_gH
Krt7	Mm00466676_m1
Krt19	Mm00492980_m1
Lgr5	Mm00438890_m1
Lrp6	Mm00999795_m1
Mucin-1	Mm00449604_m1
Notch2	Mm00803077_m1

Prominin-1	Mm00477115_m1
Sox9	Mm00448840_m1

Table S2. Antibodies used for Western blot and co-immunoprecipitation analyses.

Primary Antibodies				
Antigen	**Species**	**Source**	**Clone; Catalogue Number**	**Dilution**
β-Actin	Mouse	GenScript	2D1D10; A00702	1:10,000
β-Catenin	Rabbit	Millipore	Polyclonal; 06-734	1:2,000
β-Catenin	Rabbit	Abcam	E247; ab32572	1:3,000
Non-phospho (Ser33/37/Thr41) β-Catenin	Rabbit	Cell Signaling Technology	D13A1; 8814S	1:1,000
p-Beta-catenin (Tyr142)	Rabbit	Abcam	Polyclonal; ab27798	1:500
p-Beta-catenin (Tyr654)	Rabbit	Abcam	Polyclonal; ab59430	1:500
AKT	Rabbit	Cell Signaling Technology	C67E7; 4691S	1:1,000
p-AKT (Ser473)	Rabbit	Cell Signaling Technology	Polyclonal; 9271 S	1:500
E-Cadherin	Mouse	Cell Signaling Technology	4A2; 14472	1:1,000 (WB) /1:100 (Co-IP)
Lgr5	Rabbit	Abcam	EPR3065Y; ab75850	1:1,000
Lrp6	Rabbit	Cell Signaling	3395S	1:1,000
p-Lrp6 (Ser1490)	Rabbit	Cell Signaling	2568S	1:1,000
Prominin-1 (CD133)	Rat	Millipore	13A4; MAB4310	1:1,000

Stat3	Mouse	Cell Signaling	9139	1:1,000
p-Stat3(Tyr705)	Rabbit	Cell Signaling	9131S	1:1,000
Secondary Antibodies				
Antigen	**Conjugation**	**Source**	**Clone; Catalogue Number**	**Dilution**
Mouse	Horseradish Peroxidase	Jackson ImmunoResearch	Ployclonal; 715-035-150	1:10,000
Rabbit	Horseradish Peroxidase	Jackson ImmunoResearch	Polyclonal; 711-035-152	1:10,000
Rat	Horseradish Peroxidase	Jackson ImmunoResearch	Polyclonal; 712-036-150	1:10,000

C. SUPPORTING FIGURES AND LEGENDS

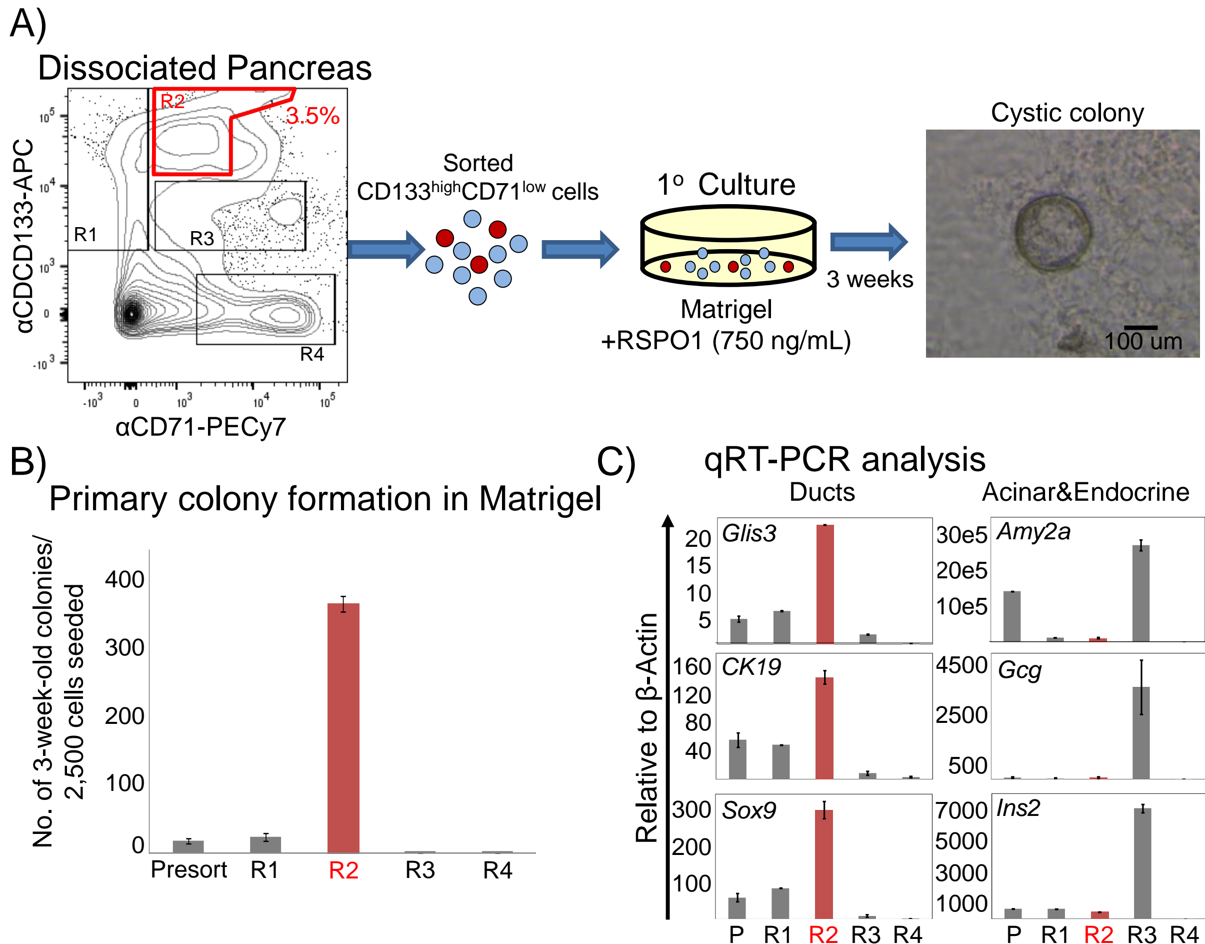

Figure S1. Sorted pancreatic CD133^{high}CD71^{low} cells express Glis3 and ductal cell markers and are enriched for PCFUs that form colonies in our standard colony assay containing Matrigel. A) Sorting gates and plating scheme of dissociated pancreatic cells isolated from adult (8-12-week-old) mice. Colonies that formed were generally referred to as cystic colonies. B) Colony frequency was highest in R2 (CD133^{high}CD71^{low}) cells, compared to other sorted cell populations. Data represent mean ± s.d. of 4 technical replicates from one experiment of a total of 3 experiments showing similar trends. C) Representative conventional qRT-PCR analysis of freshly sorted cells for various lineage markers. Cells in

region (R) 1 and R2 expressed higher levels of ductal markers, while R3 cells expressed higher endocrine and acinar markers. The presort (P) cells were used as controls. Data represent mean ± s.d. of 3 technical replicates from one experiment of a total of 3 experiments showing similar trends.

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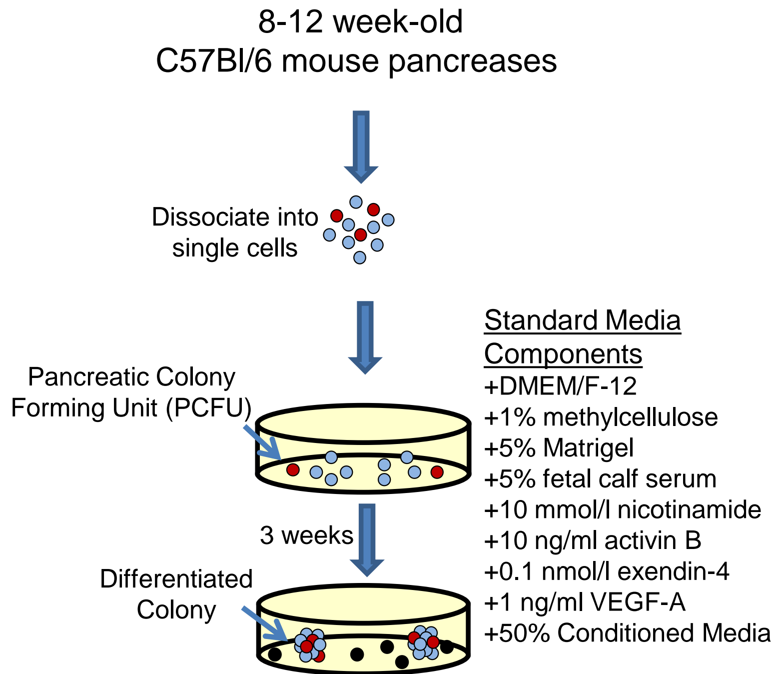

Figure S2. An *in vitro* quantitative assay for measuring pancreatic colony-forming units (PCFUs) isolated from adult murine pancreas. Single cell suspension was obtained from the whole pancreas by enzymic dissociation, labeled with antibodies against cell surface markers, and fractionated by a fluorescence-activated cell sorter to obtain CD133^{high}CD71^{low} cells, which are enriched for murine adult PCFUs (1). To obtain cystic colonies, sorted cells were plated into a "standard" colony assay in semisolid medium containing DMEM/F12, 1% (w/v) methylcellulose, 5% (v/v) Matrigel, 50% conditioned medium from murine embryonic stem cell-derived pancreatic-like cell culture, 10 mM nicotinamide, 0.1 nM exendin-4, 10 ng/mL Activin B and 10 ng/mL VEGF-A. To obtain endocrine/acinar colonies, 100 ug/mL laminin hydrogel was used to replace Matrigel in the standard colony assay. The semisolid medium restricted the movement and aggregation of PCFUs. Individual PCFUs gave rise to colonies, which were then numerated and analyzed by qRT-PCR and other techniques.

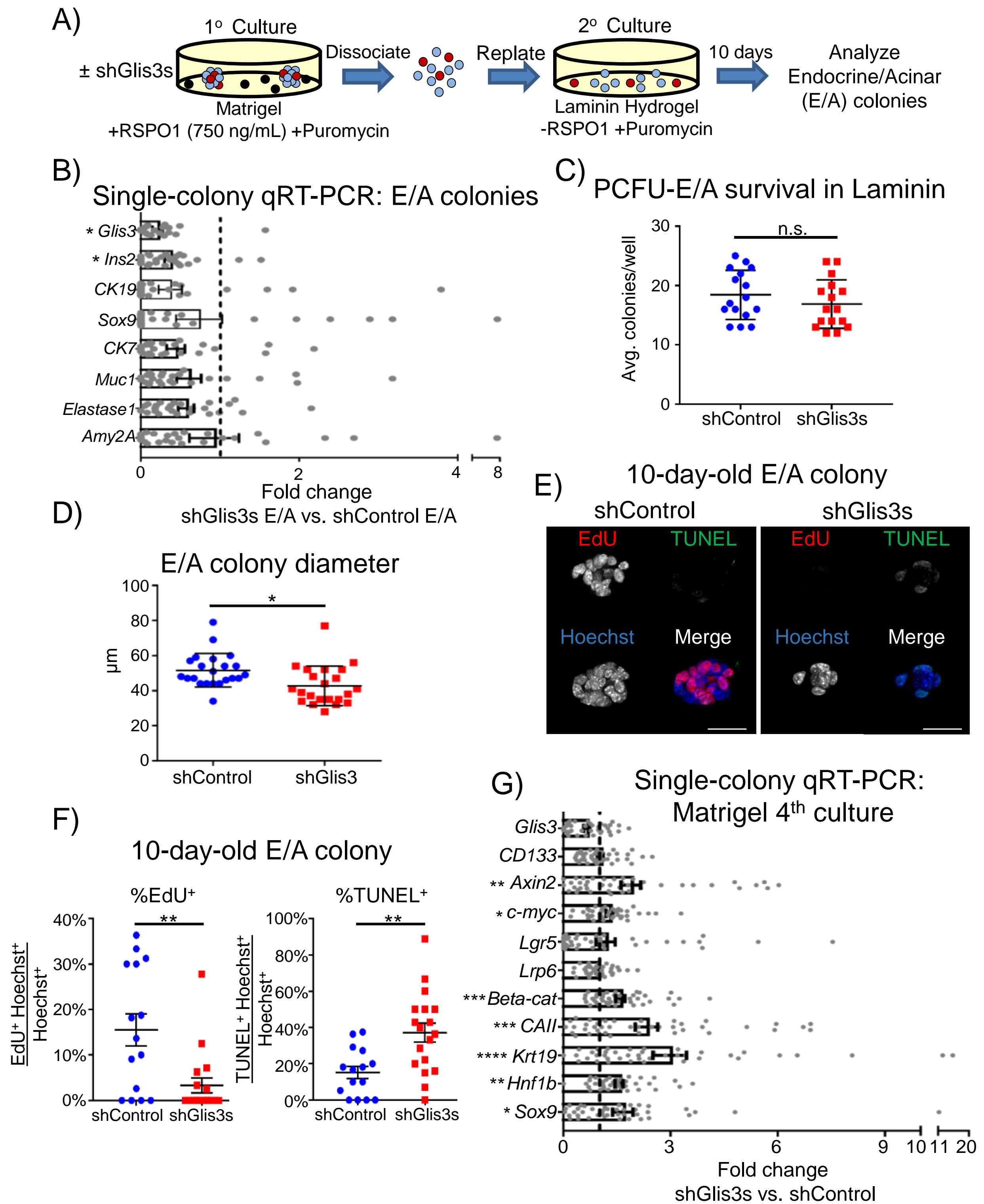
A)
1º Culture
± shGlis3s
Dissociate
Replate
2º Culture
10 days
Analyze Endocrine/Acinar (E/A) colonies
Matrigel
+RSPO1 (750 ng/mL) +Puromycin
Laminin Hydrogel
-RSPO1 +Puromycin
B)
Single-colony qRT-PCR: E/A colonies
* Glis3
* Ins2
CK19
Sox9
CK7
Muc1
Elastase1
Amy2A
0
2
4
8
Fold change
shGlis3s E/A vs. shControl E/A
C)
PCFU-E/A survival in Laminin
n.s.
Avg. colonies/well
30
20
10
0
shControl
shGlis3s
D)
E/A colony diameter
100
80
60
40
20
0
µm
*
shControl
shGlis3
E)
10-day-old E/A colony
shControl
shGlis3s
EdU
TUNEL
Hoechst
Merge
F)
10-day-old E/A colony
%EdU+
%TUNEL+
EdU+ Hoechst+ / Hoechst+
TUNEL+ Hoechst+ / Hoechst+
40%
30%
20%
10%
0%
100%
80%
60%
40%
20%
0%
**
**
shControl
shGlis3s
G)
Single-colony qRT-PCR:
Matrigel 4th culture
Glis3
CD133
** Axin2
* c-myc
Lgr5
Lrp6
*** Beta-cat
*** CAII
**** Krt19
** Hnf1b
* Sox9
0
3
6
9
10
11
20
Fold change
shGlis3s vs. shControl

Figure S3. Validation of the effects of lentiviral vectors carrying a pool of 5 short hairpin (sh) RNA against Glis3 in PCFU-derived colonies. A) Experimental scheme for generating "Endocrine/Aciniar (E/A)" colonies. Freshly sorted pancreatic CD133^{high}CD71^{low} cells, which enrich for PCFUs, were transduced with shGlis3s or shControl overnight, washed, and plated into Matrigel-containing culture with exogenous RSPO1 and puromycin for 3 weeks. Cystic colonies were collected, pooled, dissociated into single cell suspension and re-plated into laminin hydrogel-containing colony assay (without Matrigel and RSPO1) but with puromycin. E/A colonies form after 10 days in the laminin hydrogel culture. B) Microfluidic qRT-PCR analyses showed that *Glis3* and *Ins2* gene expression in individual 10-day-old E/A colonies were reduced by Glis3 knockdown. Data represent mean ± s.e.m. of a total of 25 colonies gathered from 3 independent experiments. *P-value<0.05. C) Glis3 knockdown did not affect the number of E/A colonies formed 10-days post-plating, suggesting that Glis3 was not required for the survival of PCFUs that gave rise to E/A colonies. Data are from 5 pooled biological samples run in 3 independent experiments with 4 technical replicates each and are represent as mean ± s.e.m. D) Glis3 knockdown decreased E/A colony diameter, suggesting that Glis3 was required for the growth of cells in E/A colonies. Data represent mean ± s.e.m. of a total of 25 colonies gathered from 3 independent experiments. E) Representative images for whole-mount immunostaining of 10-day-old E/A colonies that received shControl or shGlis3s and stained with makers of proliferation (EdU) and apoptosis (TUNEL). Hoechst dye was used to stain the nuclei. Glis3 knockdown caused reduced proliferation and enhanced apoptosis of cells growing in the E/A colonies. F) Quantification of EdU^{+}Hoechst^{+} or TUNEL^{+}Hoescht^{+} nuclei. Data represent mean ± s.e.m. from 15 E/A colonies treated with shControl and 18 E/A colonies treated with shGlis3s from 2 independent experiments. G) Microfluidic qRT-PCR analyses showed that the 4th generation of cells in long-term Matrigel and RSPO1-containing culture initiated with Glis3 knockdown have increased expression of ductal and Wnt genes. Data represented as means ± s.e.m. from 41 colonies collected from 3 independent experiments.

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CD133-KD decreased genes: top 10 upstream complexes			
Upstream Complexes	**z-score**	**p-Value**	**Predicted Activation**
PDGF BB	-4.365	2.22E-17	Inhibited
PI3K (complex)	-2.862	2.05E-09	Inhibited
Stat3-Stat3	-2.607	9.06E-09	Inhibited
NFkB (complex)	-3.316	9.69E-08	Inhibited
STAT1/3/5 dimer	-2.219	0.00000017	Inhibited
Cg	-3.322	0.000000338	Inhibited
FSH	-2.355	0.00000628	Inhibited
Immunoglobulin	-2.208	0.00000714	Inhibited
Ap1	-2.19	0.0000982	Inhibited
LDL	-2.208	0.000174	Inhibited

Figure S4. Genome-wide gene expression and bioinformatics analyses reveal that CD133 knockdown is predicted to inhibit PI3K/AKT and other signaling in colonies. A list of down-regulated protein complexes affected by CD133 knockdown. Results are from 2 independent experiments consisting of 5 pooled pancreases each.

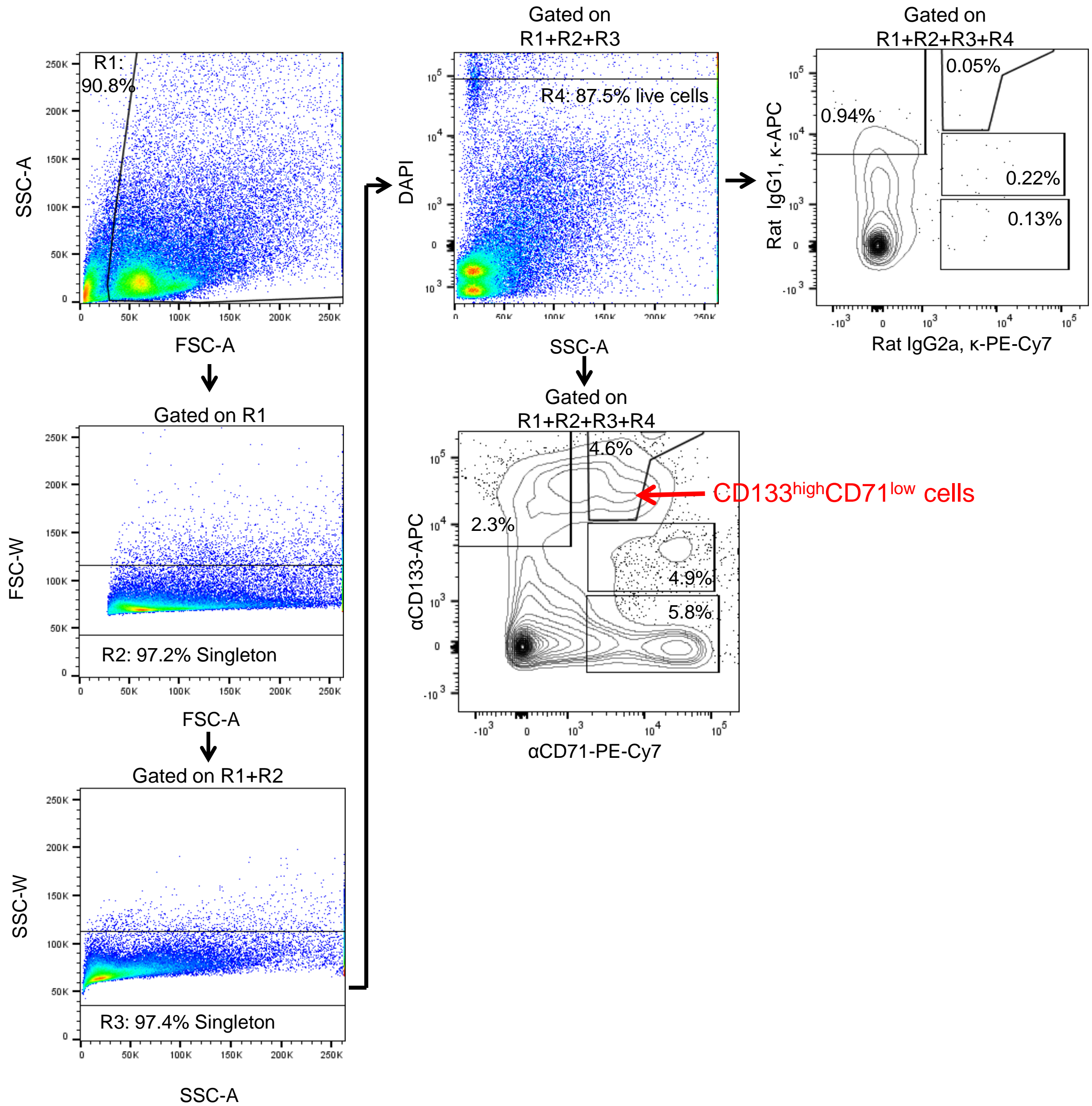

Figure S5. Representative gates used in flow cytometry for sorting cells. Pancreatic CD133^{high}CD71^{low} cells, a population that was previously determined to be enriched for PCFUs (1), were sorted after gating with forward scatter-area (FSC-A; indicative of cell size), side scatter-area (SSC-A; indicative of granularity), forward scatter-width (FSC-W; a high value of which is indicative of cell doublets), side scatter-width (SSC-W; a high value of which is indicative of cell doublets), and DAPI negative (indicative

of live cells). Cells stained with isotype control antibodies were used to indicate specificity of the CD133 and CD71 staining.

D. REFERENCES

1. Jin L, Gao D, Feng T, Tremblay JR, Ghazalli N, Luo A, Rawson J, Quijano JC, Chai J, Wedeken L, Hsu J, LeBon J, Walker S, Shih HP, Mahdavi A, Tirrell DA, Riggs AD, Ku HT: Cells with surface expression of CD133(high)CD71(low) are enriched for tripotent colony-forming progenitor cells in the adult murine pancreas. Stem Cell Res 2016;16:40-53