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Supplemental Information

A Versatile Strategy for Isolating a Highly Enriched Population of Intestinal Stem Cells

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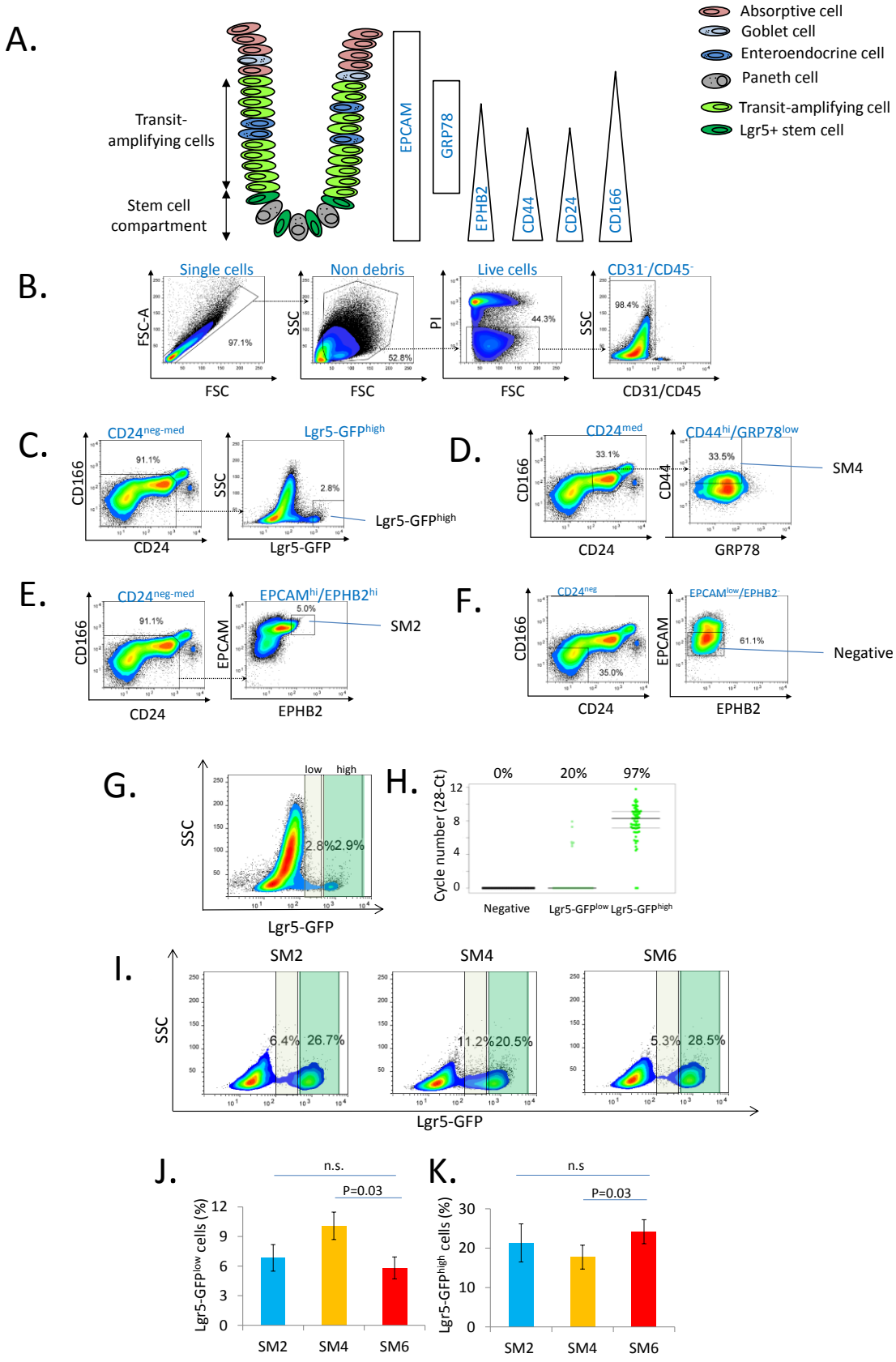
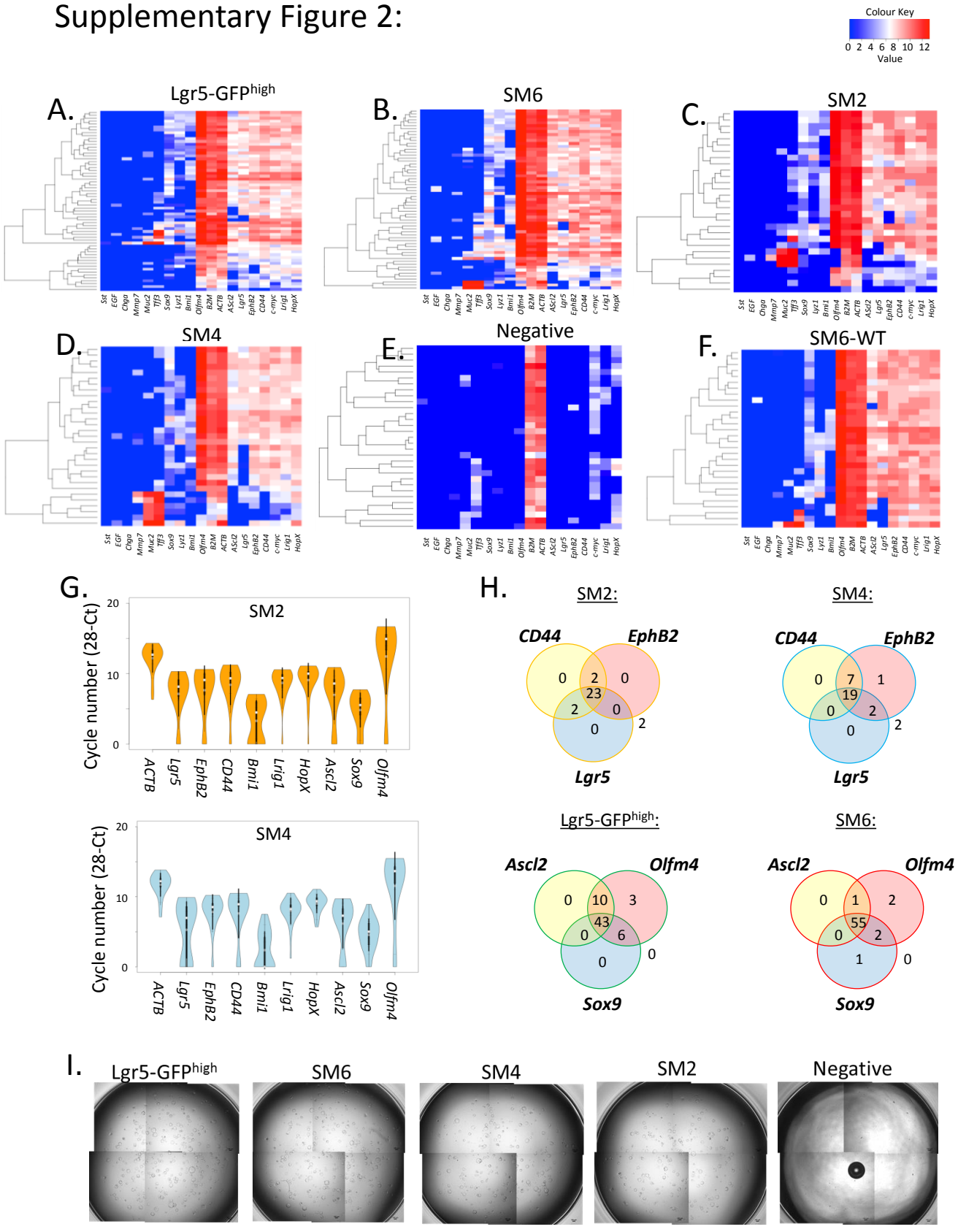
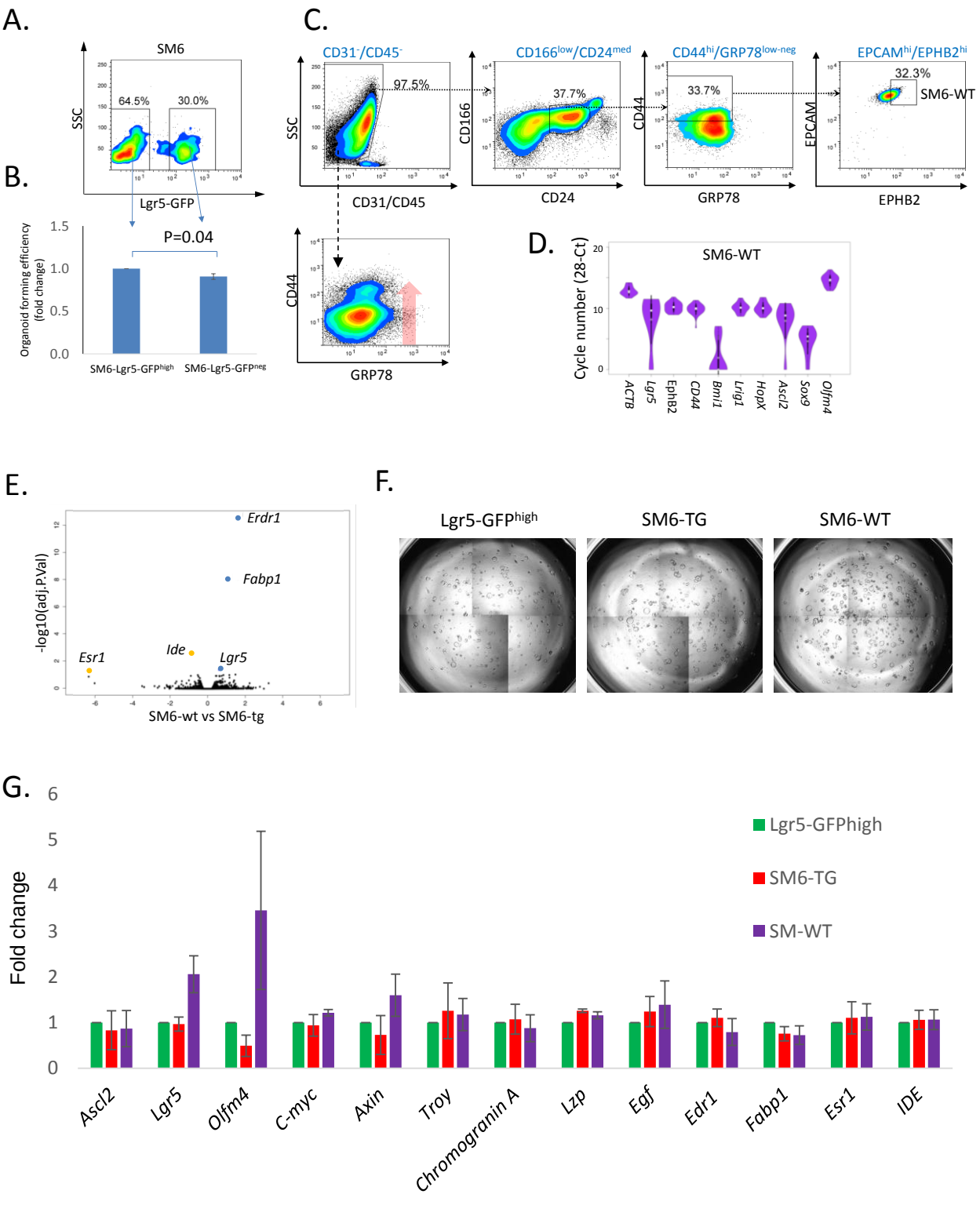
Supplementary Figure 1:



Supplementary Figure 2:



Supplementary Figure 3:



Supplementary figure 1: **Gating strategies, related to Figure 1.** (A) Localisation of cell surface markers in the intestinal crypt. (B) Depletion for aggregates, debris, PI positive events and CD31/CD45 positive cells before gating in on the (C) Lgr5-GFP^{high} (depleted for CD24 positive Paneth cells), (D) SM4, (E) SM2 and (F) Negative populations. (G) Representative FACS blots depicting Lgr5-GFP^{low} and Lgr5-GFP^{high} cells from an *Lgr5-Gfp* reporter animal. (H) Beeswarm plot for single cell Lgr5 expression for Negative, Lgr5-GFP^{low} and Lgr5-GFP^{high} cells; percentage values of cells with detectable Lgr5 transcripts are indicated above the blot (Negative 31 cells from one experiment, Lgr5GFP^{low} 30 cells from one experiment, Lgr5GFP^{high} 62 cells pooled from 2 independent experiments). (I) Representative FACS blots depicting Lgr5-GFP^{low} and Lgr5-GFP^{high} cells for SM2, SM4 and SM6. Quantification of (J) Lgr5-GFP^{low} cells and (K) Lgr5-GFP^{high} cells for SM2, SM4 and SM6 strategies; a paired Wilcoxon test was performed (mean±s.e.m, n=6, experimental replicates).

Supplementary figure 2: **Additional profiling data, related to Figure 3.** (A-F) Heat maps of the single cell data for Lgr5-GFP^{high}, SM6, SM2, SM4, Negative and SM6-WT*. (G) Violin plots for SM2 and SM4*. (H) Venn diagrams for SM2, SM4, Lgr5-GFP^{high} and SM6*. (I) Composite images of whole 96-wells at day 4 of culture for Lgr5-GFP^{high}, SM6, SM4, SM2 and the Negative population (scale bars, 100μm).

*replicates Single cell data: Lgr5GFP^{high} 62 cells pooled from 2 independent experiments, SM6/SM6-TG 61 cells pooled from 2 independent experiments, SM4 29 cells from one experiment, SM2 31 cells from one experiment, SM6-WT 30 cells from one experiment, Negative 31 cells from one experiment.

Supplementary figure 3: **Additional profiling data, related to Figure 3.** (A) Representative FACS blot depicting SM6-Lgr5^{negative} and SM6-Lgr5^{high} cells to subfractioning of SM6 according to Lgr5-GFP expression. (B) Organoid formation frequency (fold change compared to SM6-Lgr5^{high}) for SM6-Lgr5^{negative} and SM6-Lgr5^{high} cells; a 2-tailed unpaired Student's T test was performed (mean±s.e.m., n=3, experimental replicates). (C) Isolation of wild-type CBC cells using our combination of 6 cell surface markers (SM6, FACS plots 1st lane); robust shift in CD44 expression characterizes successful cell isolation (FACS plot, 2nd lane). (D) Violin plots for key ISC marker genes for SM6-WT cells (30 cells from one experiment). (E) Volcano plot depicting differentially expressed genes between SM6-TG and SM-WT (n=2, experimental replicates). (F) Organoid culture for prospective ISC populations. Composite images of whole 96-wells at day 4 of culture for Lgr5-GFP^{high}, SM6-TG and SM6-WT populations (scale bars, 100μm). (G) qPCR performed on day 4 organoid cultures (mean±s.e.m., n=3, experimental replicates).

Supplementary Table 1: **Cells surface marker profile of cell populations of interest, related to Figure 1**

	Negative cells	SM2	SM4	SM6	Lgr5-GFP
CD31/CD45	neg	neg	neg	neg	neg
CD24	neg	neg-med	med	med	neg-med
CD166	neg	n/a	low	low	n/a
CD44	neg	n/a	high	high	n/a
GRP78	neg	n/a	neg-low	neg-low	n/a
EPCAM	low	high	n/a	high	n/a
EPHB2	neg	high	n/a	high	n/a

Supplementary Table 2: **List of differentially expressed genes, related to Figure 2.**

SM2 versus Lgr5-GFP	SM4 versus Lgr5-GFP		
Gsdmc2	*Bcas1*	*Sst*	*Rep15*
Gsdmc4	*Chgb*	*Tph1*	*Plcb1*
Npc1l1	*Muc2*	*Rab3c*	*Srgap1*
Spink4	*Spink4*	*Ern2*	*Lrrc26*
Muc2	*Tff3*	*Mptx1*	*Trpa1*
Gsdmc3	*Ano7*	*Spdef*	*Gal3st2*
Slc43a2	*Creb3l1*	*Hpd*	*Tpsg1*
Anxa13	*Gsdmc4*	*Pcsk1*	*Gfi1*
Dclk1	*Gsdmc2*	*Lmx1a*	*Sphkap*
Apol10a	*Agr2*	*Vcan*	*Ptprr*
Sis	*Rgs2*	*Dbpht2*	*Myof*
Ano7	*Sytl2*	*Cacna1h*	*Rgs4*
Ms4a18	*Hepacam2*	*Cck*	*Insm1*
	Atoh1	*Cpe*	*Dnahc9*
	Atp2a3	*Kcnh6*	*Enpp2*
	Map1b	*Ptprn2*	*Fhl1*
	Pde2a	*Scn3a*	*Scnn1a*
	Serpina1b	*Cadps*	*Nfasc*
	Clca3	*Gch1*	*St8sia3*
	Rfx6	*Itga4*	*Capn9*
	Guca2a	*Fcgbp*	*Aplp1*
	P2ry4	*Kcnk6*	*Gnao1*
	Selm	*Pclo*	*Cacna2d1*
	Chga	*Afp*	*Map2*
	Gcnt3	*Bace2*	*Nkx2-2*
	Anxa13	*Gcg*	*Rcan3*
	Scin	*Adcy1*	*Sv2c*
	Tac1	*Gpr116*	*Gip*
	Gsdmc3	*Sec24d*	*Disp2*
	Zg16	*Klk1*	*Gfra3*
	Sct	*Pdia5*	*Kcnj5*
	S100a6	*Anxa2*	*Scg2*
	Ccl9	*Cbfa2t3*	*St18*
	Vim	*Rap1gap*	
	Pyy	*Amigo2*	

Supplementary Table 3: **Antibodies used in this study, related to detailed multi-step protocol.**

Antibody	Dilution Factor			Company	Clone	Catalog#	Excitation laser	Detection filter
	1°	2°	3°					
Mouse anti-EphB2	1:100			Genentech	2H9	courtesy of Genentech*		
rabbit anti-GRP78	1:100			Sigma	polyclonal	G9043		
donkey-anti-mouse-IGG AF555 antibody		1:200		Thermo-Fischer	polyclonal	A31570	561nm	555-633nm
goat-anti-rabbit-APC-Cy7		1:100		Santa Cruz Biotechnolog	polyclonal	sc-3847	635nm	750-810nm
rat-anti-Epcam-eFluor450			1:100	eBioscience	G8.8	48-5791-82	405nm	425-475nm
rat-anti-CD45-BV510			1:200	BDBiosciences	30-F11	563891	405nm	500-550nm
rat-anti CD31-BV510			1:200	BDBiosciences	MEC 13.3	563089	405nm	500-550nm
rat-anti-CD44-BV650			1:100	Biolegend	IM7	103049	405nm	640-680nm
rat-anti-CD24-PeCy7			1:100	eBioscience	M1/69	25-0242-82	561nm	750nm long pass
rat-anti-CD166-APC			1:100	eBioscience	eBioALC48	17-1661-82	635nm	655-685nm

*This antibody is now also available from BDBiosciences and is provided at the same concentration as used in this study.

Supplemental Experimental Procedures:

Crypt isolation and cell dissociation

Mice were culled by cervical dislocation. As previously described (Horvay et al., 2015; Jarde et al., 2013; Wang et al., 2013), the small intestinal tube was dissected out and flushed with PBS to remove faeces. Small intestinal tracts were opened longitudinally, scraped with a glass coverslip to remove villi, cut into 5-mm pieces and washed with PBS five times to remove unattached epithelial fragments, mucus and faeces. Following incubation for 30 min at 4°C in 3mM EDTA-PBS, intestinal crypts were released from small intestine tissue fragments by mechanically pipetting with a 10ml pipette in PBS and repeating this step three times. Isolated intestinal crypts were strained (70-μm cell strainer, BD Biosciences) and pelleted by centrifugation three times at 1500 rpm for 2 minutes at 4°C. The collected crypts were incubated for 30 minutes at 4°C in DMEM/F12 – 10% serum (Gibco) and then dissociated in TrypLE Express (Invitrogen) supplemented with 10 μM Rock inhibitor (Y-27632, Abcam) and 2.5μg/ml DNAse 1 (Sigma-Aldrich) for 5 minutes at 37°C. Cell clumps and mucus were removed using a 70-μm cell strainer (BD Biosciences) and the remaining dissociated cells were washed twice with PBS and collected by centrifugation at 4°C at 1500 rpm for 3 minutes.

Flow cytometry

All antibody labelling steps as well as the final resuspension of the samples were performed with PBS supplemented with 2mM EDTA, 2% FBS and 10 μM Rock inhibitor (Y-27632). Cellularised crypts were submitted to a three step sequential antibody labelling procedure: (I) mouse-anti-EPHB2 antibody (1:100 dilution, clone 2H9, courtesy of Genentech); (II) donkey-anti-mouse-IGG AF555 antibody (1:200, polyclonal, LifeTechnologies, cat# A31570) rabbit-anti-GRP78 antibody (1:100, polyclonal, Sigma, cat# G9043); anti-UEA-1-Biotin (1:1000,

Vectorslabs, cat# B-1065) (III) Strepdavidin-BUV395 (1:100, BD Biosciences cat# 564176), rat-anti-EPCAM-eFluor450 (1:100, clone: G8.8, eBioscience, cat# 48-5791-82), rat-anti CD31-BV510 (1:200, clone: MEC 13.3, BD Biosciences, cat# 563089) rat-anti-CD45-BV510 (1:200, clone: 30-F11, BD Biosciences, cat# 563891), rat-anti-CD44-BV650 (1:100, clone IM7, Biolegend, cat# 103049), rat-anti-CD24-PeCy7 (1:100, clone: M1/69, eBioscience, cat# 25-0242-82), rat-anti-CD166-APC (1:100, clone: eBioALC48, eBioscience, cat# 17-1661-82), and secondary antibody goat-anti-rabbit-APC-Cy7 (1:100, polyclonal, Santa Cruz Biotechnologies, cat# sc-3847). All antibody labelling steps were carried out (for the cells of one animal) in a 500µl volume for 15 minutes on ice; after each antibody labelling step, cells were washed with 10ml cold PBS and pelleted at 400xg for 3 minutes. The cells for each animal were then resuspended in a final volume of 1ml, passed through a 70um strainer and transferred into appropriate FACS tubes where propidium iodide (PI) was added to a concentration of 2ug/ml. Cell sorting was carried out with a 100 µm nozzle on an Influx instrument (BD Biosciences). The gating strategies to isolate SM2 and SM4 were adapted from Merlos-Suarez *et al.* and Wang *et al.* (Merlos-Suarez et al., 2011; Wang et al., 2013). For all populations of interest (SM2, SM4, SM6, Lgr5-GFP^{high} and Negative), aggregates, debris, dead cells (PI+) and CD45+/CD31+ hematopoietic/endothelial contaminates were depleted. Before isolating SM2 and Lgr5-GFP^{high} cells, Paneth cells were excluded by depleting for CD24^{hi} cells. For Lgr5-GFP^{high}, 2.5-3% of the Lgr5-GFP brightest cells were selected; for SM2 the top 5% EPCAM^{high}/EPHB2^{high} were selected. For SM4 and SM6, the CD24^{med}/CD166^{low} population was subgated into CD44^{high}/GRP78$^{neg\text{-}low}$ cells (the gate was set to encompass ~ 25% of the population). For SM6, an additional step was included where ~33% of the top EPCAM^{high}/EPHB2^{high} cells were collected (please note the % value of the final SM6 gate was set to approximate/emulate the position of the final SM2 gate). Purity of collected fractions

was confirmed by reanalysis of a small fraction of the sorted cells. For single cell applications cells were double sorted.

Multidimensional analyses of flow cytometry data

We used the Cytobank platform (Fluidgm, South San Francisco, California) to generate viSNE maps and SPADE trees from Flow Cytometry Standard files. Analyses were performed on live cells depleted for CD31 and CD45 positive cells and EPCAM negative cells. To generate viSNE maps, 10^5 events in total were used for sampling. SPADE trees were generated with a target number of 100 nodes; the down sampled events target was set to 100%. For both viSNE and SPADE six fluorescent channels were used for dimensional reduction (EPHB2, CD44, CD166, GRP78, CD24 and UEA-1).

RNA sequencing

RNA was extracted with Qiagen's RNeasy micro kit from 2-3 $X10^4$ FACS isolated cells as per instructions. For generation of sequencing libraries, 25ng of RNA (RIN value >9) were submitted to SPIA amplification (NuGen). Two biological replicates per condition were sequenced using the HiSeq 2000 sequencing platform (Illumina**,** San Diego, CA, USA). Each library was pair-end with a 100nt read length (350nt average insert size). The targeted number of sequencing reads per sample was 15 million. Raw sequencing reads were assessed for overall quality using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing specific adaptors and low quality reads (Phred score of 6 consecutive bases below 15, minimum read length of 36nt) were filtered and hard trimmed using Trimmomatic [v 0.30] (Bolger et al., 2014). Sample reads were aligned to the mouse genome [complete mm10 (UCSC version, December 2011)] using Tophat2 [v 2.0.13, default parameters] (Kim et al., 2013). Transcript quantification was performed using HTSeq [v 0.6.1, default parameters] (Anders et

al., 2015) and transcripts with more than ten sequencing reads in at least one sample were used for further analysis. Sample library size was normalized using the TMM method (Robinson and Oshlack, 2010). The sequences reported in this paper are available at the NIH Short Reads Archive, (www.ncbi.nlm.nih.gov/sra), accession number SRP066815.

Single Cell PCR

Single cell PCR was performed as previously described (Polo et al., 2012) with LifeTechnologies Single Cell to Ct kit. In brief, 96-well plates for qPCR were filled with 10ul lysis solution and single cells were deposited with a cell sorter into each well. As per kits instructions, cDNA was produced from the lysate and submitted to 18 cycles of pre-amplification with TaqMan probes (Life Technologies) of the 19 genes of interest (*Actb, Ascl2, B2M, Bmi1, c-myc, Cd44, Chga, Egf, EphB2, HopX, Lgr5, Lrig1, Lyz1, Mmp7, Muc2, Olfm4, Sox29, Sst, Tff3*). Pre-amplified templates that were positive for housekeeper *Actb* (manually tested with qPCR) were then used for Single-cell PCR data collection with a Biomark instrument (Fluidigm). Results are expressed as Log2Ex = LOD (Limit of Detection) Cq – Cq [Gene]. The limit of detection was set to 28. If Log2Ex value is negative, Log2Ex = 0. For SM2, SM4, SM6-WT, Lgr5GFP^{low} and Negative approximately 30 cells per group from one experiment were used for analysis. For key populations SM6 and Lgr5-GFP^{high} in total around 60 cells (from two separate experiments) were used for analysis.

Quantitative RT-PCR

After 4 days in culture, organoids generated from single SM6-TG, SM6-WT or Lgr5-GFP^{high} cells were homogenised and total RNA extracted using a RNeasy micro kit (Qiagen), as previously described (Jarde et al., 2015). RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Quantitative reverse transcriptase polymerase chain

reaction (qRT-PCR) was performed using Brilliant II SYBR Green QPCR Master Mix (Agilent technologies). Triplicate samples were analysed on a LightCycler 480 machine (Roche Diagnostics). Gene expression levels were calculated using the 2^{-DDCt} method using *Gapdh* as a normaliser. The following primer sequences (depicted 5'-3') were used: Ascl2 (F: CAGGAGCTGCTTGACTTTTCCA, R: GGGCTAGAAGCAGGTAGGTCCA), Axin2 (F: GCAGCTCAGCAAAAAGGGAAAT, R: TACATGGGGAGCACTGTCTCGT), Chromogranin A (F: TCCCCACTGCAGCATCCAGTTC, R: CCTTCAGACGGCAGAGCTTCGG), C-myc (F: CTAGTGCTGCATGAGGAGACAC, R: GTAGTTGTGCTGGTGAGTGGAG), Egf (F: GTTCAGTGCTTGGGAGAGATG, R: CCTGGGAATTTGCAAACAGTA), Esr1 (F: CCCGCCTTCTACAGGTCTAAT, R: CTTTCTCGTTACTGCTGGACAG), Erd1 (F: GGTCAAGATGTATGTGCCACC, R: GCTTCTACGTGTGTGCTTTCG), Fabp1 (F: GGAATTGGGAGTAGGAAGAGCC, R: TGGACTTGAACCAAGGAGTCAT), Ide (F: AATCCGGCCATCCAGAGAATA, R: GGGTCTGACAGTGAACCTATGT), Lgr5 (F: CCTTGGCCCTGAACAAAATA, R: ATTTCTTTCCCAGGGAGTGG'), Lzp (F: GAGACCGAAGCACCGACTATG, R: CGGTTTTGACATTGTGTTCGC), Olfm4 (F: AACATCACCCCAGGCTACAG, R: TGTCCACAGACCCAGTGAAA), Troy (F: GACTGCCTGCCAGGATTTTAC, R: CAGTGTGGTTCGTAGGGAGG), Gapdh (F: CTCGTCTCATAGACAAGATGGTGAAG, R: AGACTCCACGACATACTCAGCACC).

Cell culture

Following FACS isolation, single epithelial cells were collected in DMEM/F12 supplemented with 10% serum and 10 μM Y-27632 (Abcam). Intestinal cells were centrifuged at 4°C for 5 minutes at 1500 rpm. The cell pellet was resuspended in growth-factor reduced Matrigel (1000 cells per μl, BD Biosciences) containing 10 μM JAGGED-1 (Anaspec). 5000 cells were seeded per well in a 96 well plate. Following Matrigel polymerisation, 100 μl of crypt culture medium

per well was overlaid (DMEM/F12 supplemented with N2, B27, penicillin/streptomycin, glutamax, 10mM HEPES, fungizone, 50 ng/ml EGF (Peprotech), 100 ng/ml NOGGIN (Peprotech), 1 µg/ml R-SPONDIN 1 (R&D Systems), 10 µM Y-27632 (Abcam), 100 ng/ml WNT-3a (R&D) and 2.5 µM CHIR (Stemgent)). Intestinal cells were maintained in a 37°C humidified atmosphere under 5% CO2. After 3 days, the culture medium was entirely replaced by freshly made culture medium without Y-27632 and WNT-3a. After 4 days in culture, images of wells (5 wells per condition, 3-5 biological replicates) were taken and organoids were manually counted using FIJI image analysis cell counter software.

Statistical analysis and visualization

Descriptive statistics and plots were analyzed and produced using made4 (Culhane et al., 2005), caroline (Schruth, 2013), limma (Ritchie et al., 2015), gplots (Warnes et al., 2015) and beeswarm. Principal component and unsupervised hierarchical clustering (Pearson's correlation) analyses were performed using limma (Ritchie et al., 2015), bioDist (Ding et al.) and hclust (http://CRAN.R-project.org/package=gplots) respectively. Other statistical tests were performed as indicated in the figure legends.

Detailed multi-step protocol for SM6 isolation from C57/Bl6 wild type animals

Part A: Isolation of intestinal epithelial cells

1) Cull mice by cervical dislocation
2) Generously spray the animals abdomen with alcohol before removing the small intestine and collecting it in 30ml of ice cold PBS
3) Flush the intestinal tube with ice cold PBS with a 20ml Syringe to remove faeces.
4) Cut open the small intestinal tube longitudinally
5) With the inside of the intestinal tube facing up, very gently scrape the surface with a glass coverslip to remove villi

6) Cut the intestinal tract into 5mm long pieces and wash 5 times with 30ml PBS to remove unattached epithelial fragments, mucus and faeces (Note: the washing steps are crucial for the final quality of the preparation)
7) Incubate for 30 min at 4°C in 30mls of 3mM EDTA-PBS with gentle agitation
8) In 30ml fresh ice cold PBS, release intestinal crypts from small intestinal tissue fragments by mechanically pipetting them vigorously with a 10ml pipette and repeating this step three times
9) Strain isolated intestinal crypts through a 70-µm cell strainer (BD Biosciences) and pellet by centrifugation three times at 1500 rpm for 2 minutes at 4°C to enrich for crypt fragments
10) Incubate the collected crypts for 30 minutes at 4°C in 10ml DMEM/F12 plus 10% fetal bovine serum (FBS)
11) Fill tube to 30ml with ice cold PBS and pellet at 1500 rpm for 3 minutes at 4°C
12) Cellularize crypts in 1ml TrypLE Express supplemented with 10uM Rock inhibitor and 2.5µg/ml DNAse I for 5 minutes at 37°C
13) Immediately add 200 ul FBS and gently pipette up and down (~20 times) with a 1000ul pipette to break up clumps
14) Fill tube with 30ml ice cold PBS, pass through a 70um strainer and pellet for 3 minutes by centrifugation at 1500rpm

Part B: FACS purification of intestinal stem cells:

15) Resuspend cells in 10ml ice cold PBS and put aside 500 ul as unlabelled control (Note: the control cells are to be strained, supplemented with Propidium iodide @ 2ug/ml and 10 µM Rock inhibitor before use); pellet the remaining cells by centrifugation for 3 min at 1500rpm
16) Label epithelial cells via a 3-step labelling protocol (note: preparation of the Antibody labelling solutions is outlined in supplementary Table 3 and the methods section).
17) Resuspend the cell pellet in 500µl primary antibody labelling solution per mouse and incubate on ice for 15minutes.
18) Add 10ml ice cold PBS and pellet for 3 minutes at 1500rpm.
19) Repeat steps 17 and 18 for the secondary and the tertiary antibody labelling solutions
20) Resuspend the fully labelled pellet in 1ml of solution supplemented with Propidium iodide (2ug/ml), pass through a 70um strainer and transfer into appropriate FACS sample tubes
21) Note, compensation controls are essential for this multicolour protocol. Cells (from step 15) labelled with the individual, conjugated antibodies (or via a secondary approach for EPHB2/GRP78) are ideal, but antibody capture beads from BD Bioscience can also be used (except for the PeCy7 channel where the use of a labelled cell control is required).
22) Use the unlabelled cell sample (step 15) and the compensation tubes (step 21) to calibarate the cell sorter (100µm nozzle)

23) Gate out debris, aggregates and dead cells and set gates to capture the SM6 population as described in Figure 1D and Supplementary Fig 1B. (Crucial: Successful cell preparations with a high number of intestinal stem cells are defined by a robust shift of CD44 expression in a subset of all live cells as depicted in Supplementary Figure 3C)
24) Sort cells into collection tubes with DMEM/F12,10% FBS and 10 μM Rock inhibitor
25) Note that once the sorting process has commenced it is crucial that the gates for the $CD44^{high}/GRP78^{low}$ population and the $EPCAM^{+}/EPHB2^{high}$ population are checked on regular basis to ensure that only ≤33% of these populations are gated for. (Note: while sorting, if possible, display ⩾100000 live events, this will make it easier to establish relatively stable gates)
26) If sorting larger samples it is advisable to resuspend the sort sample every 15-20 minutes by gentle pipetting.

After sorting has been completed, it is important to routinely perform re-analysis on a small fraction of the sorted cells (20-40ul) to verify purity and viability of the target population.

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