Figure S1 (related to Figure 1). Intestinal epithelial *Vil*^{CreER} and ISC specific *Lgr5*$^{CreER-GFP}$ alleles drive specific deletion of *Dlg1* in epithelium.

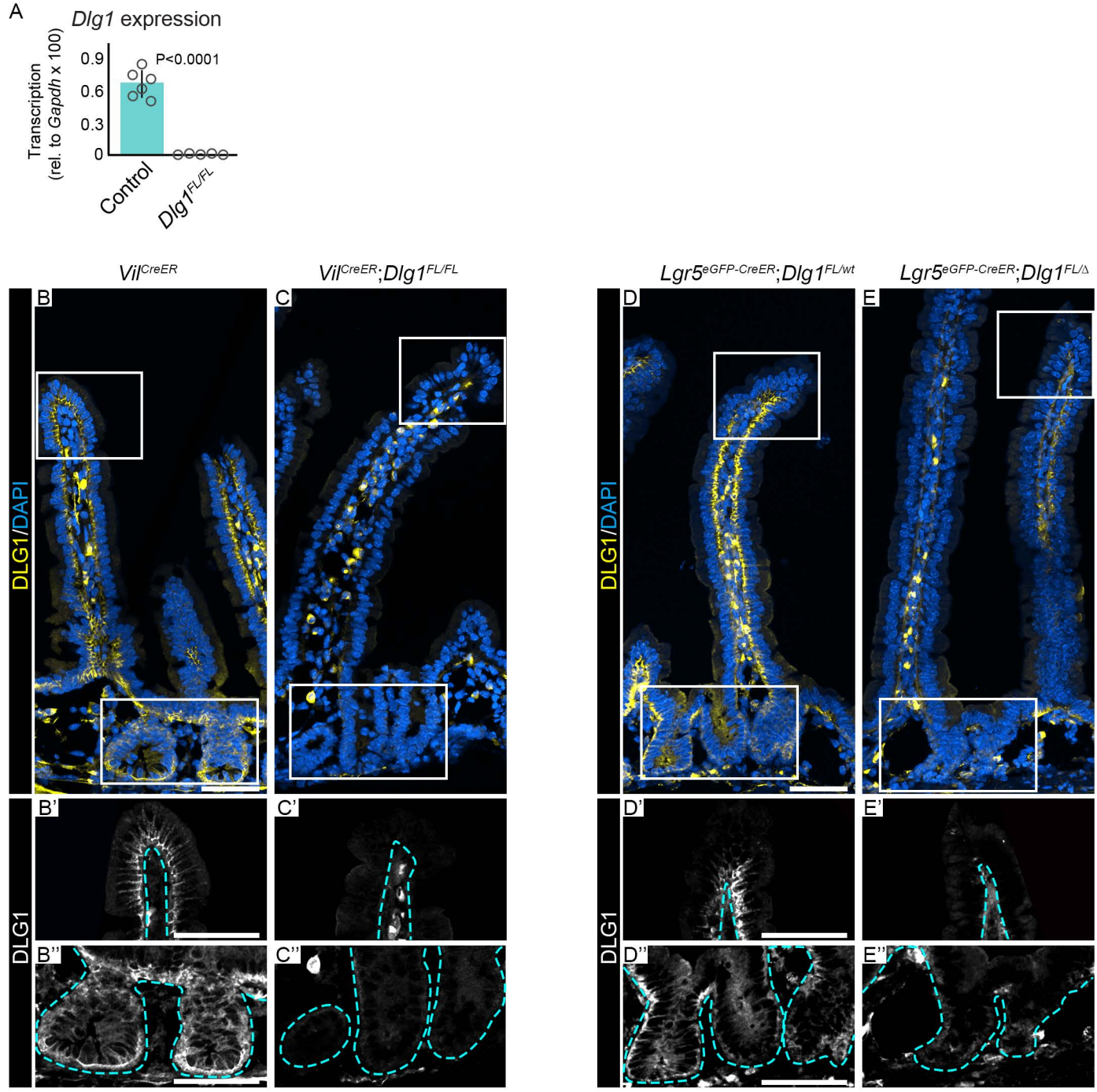

Figure S1 (related to Figure 1). Intestinal epithelial *Vil*^{CreER} and ISC specific *Lgr5*$^{CreER\text{-}GFP}$ alleles drive specific deletion of *Dlg1* in epithelium. Mice were injected with one dose of TAM, and the small intestinal epithelium was analyzed 7 days later. **(A)** Transcription levels of *Dlg1* were analyzed by qPCR on FACS-sorted intestinal epithelial cells ($DAPI^-/CD45^-/EpCAM^+$) isolated from control and $DLG1^-$ mice. N = 6 mice per condition, mean ± SD, unpaired t test with Welch's correction. (**B-E**) Immunofluorescence images of anti-Dlg1. (**B'-E'**) Higher magnification of villus tip of the boxed area in (B-E). (**B''-E''**) Higher magnification of crypt region of the boxed area in (A-D). Dash line indicates the epithelial basement membrane. Scale bars = 50 μm.

Figure S2 (related to Figure 2). Measurements of trametinib-induced Wnt signaling activation in intestinal stem cells and rotavirus shedding in stool.

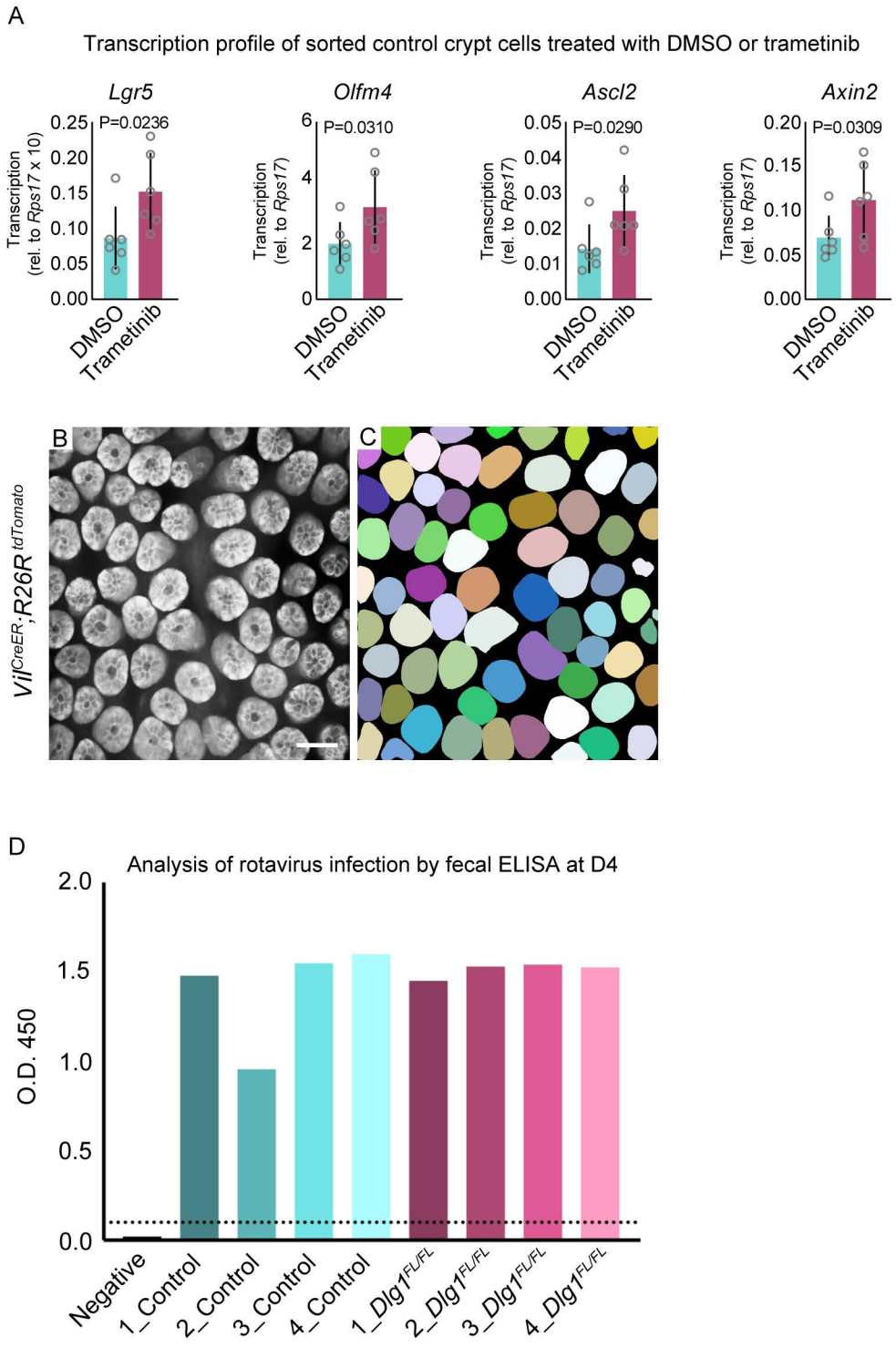

Figure S2 (related to Figure 2). Quantification of trametinib-induced Wnt signaling activation in intestinal stem cells and rotavirus shedding in stool. (A) Wnt target genes analyzed by qPCR on FACS-sorted live intestinal crypt cells ($DAPI^{-}/EpCAM^{+}/CD44^{+}$) from control mice treated with DMSO or trametinib. N = 6 mice per condition, mean ± SD, unpaired t test with Welch's correction. (**B**) Example of bottom view image of tdTomato expressed throughout the intestinal epithelium. (**C**) Pseudocoloring of crypt bottoms (B) for visualization. Images were used for crypt area quantification (see Fig. 2P and g). (**D**) Quantification of Rotavirus viral load using ELISA colorimetric assay. N = 4 mice per condition.

Figure S3 (related to Figure 3). DLG1⁻ ISCs are lost under high levels of WNT3A.

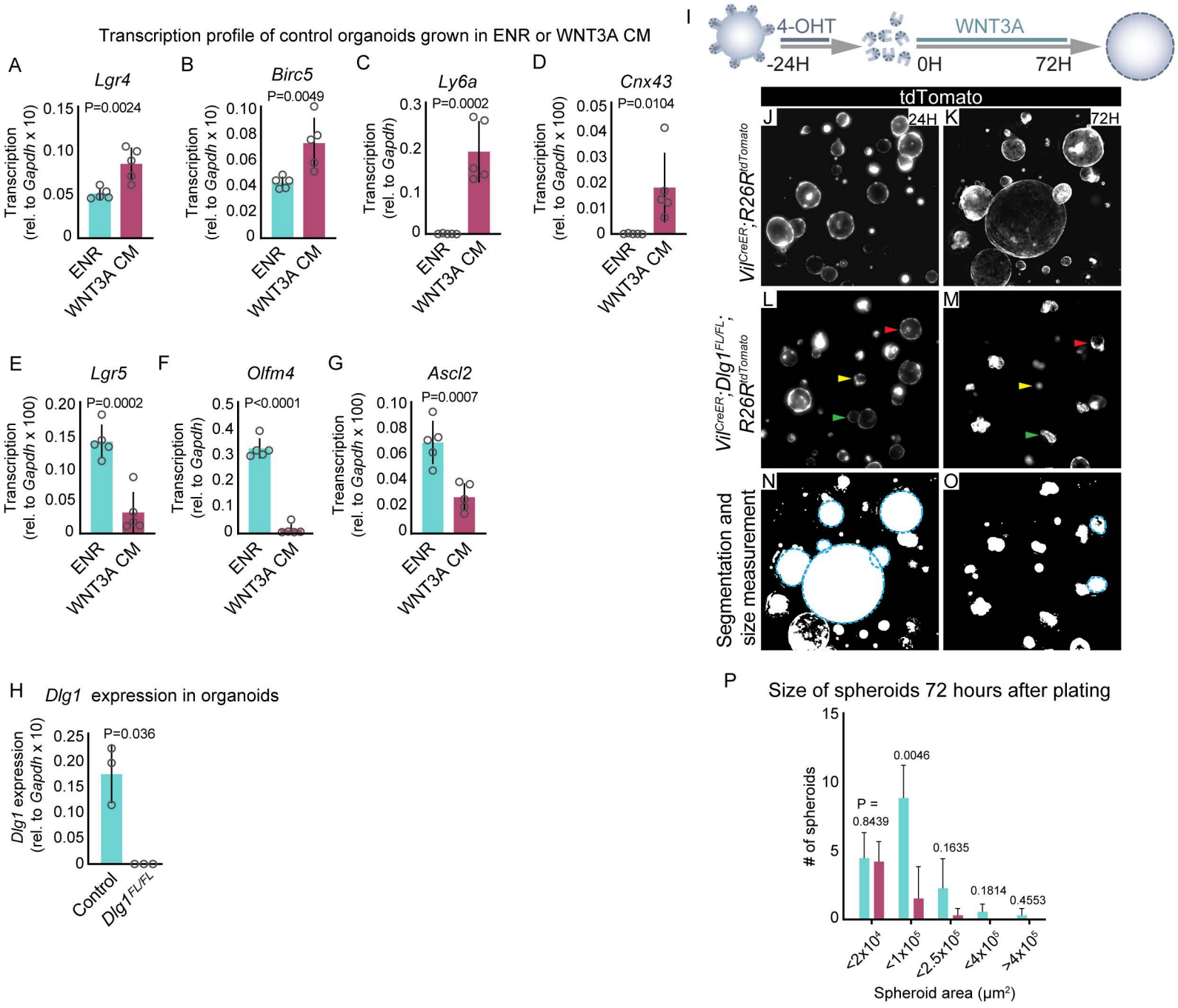

Figure S3 (related to Figure 3). DLG1$^-$ ISCs are lost under high levels of WNT3A. (**A-G**) Wnt target genes analyzed by qPCR on FACS-sorted live (DAPI$^-$) organoids established from control mice and grown in complete ENR medium or in ENR supplemented with 50% WNT3A CM. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**H**) Transcription levels of *Dlg1* analyzed by qPCR on FACS-sorted organoid live cells (DAPI$^-$). N = 3 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**I**) Experimental schematic for analyzing ISCs response to increased WNT3A levels. Control and DLG1$^-$ organoids were treated with 4-OHT prior mechanical passaging, crypts were plated into a Matrigel droplet and overlaid with ENR medium containing 200 ng/ml WNT3A. (**J-M**) 3D rendered Z-stack projections of spheroids at 24 (**J and L**) or 72 (**K and M**) hours after plating. Scale bar = 500 µm. (**N and O**) Segmentation of Z-stack projections of spheroids from (K and M). Blue circles indicate the organoid perimeter used for measuring the area. (**P**) Quantification of spheroid growth binned according to size at 72 hours after plating. N = 3 x 20 top sized organoids per condition, mean ± SD, unpaired t test with Welch's correction.

Figure S4 (related to Figure 3). DLG1⁻ ISCs are lost in increased levels of WNT3A and mesenchymal WNT, while their capacity to grow remains preserved in non-canonical WNT5A.

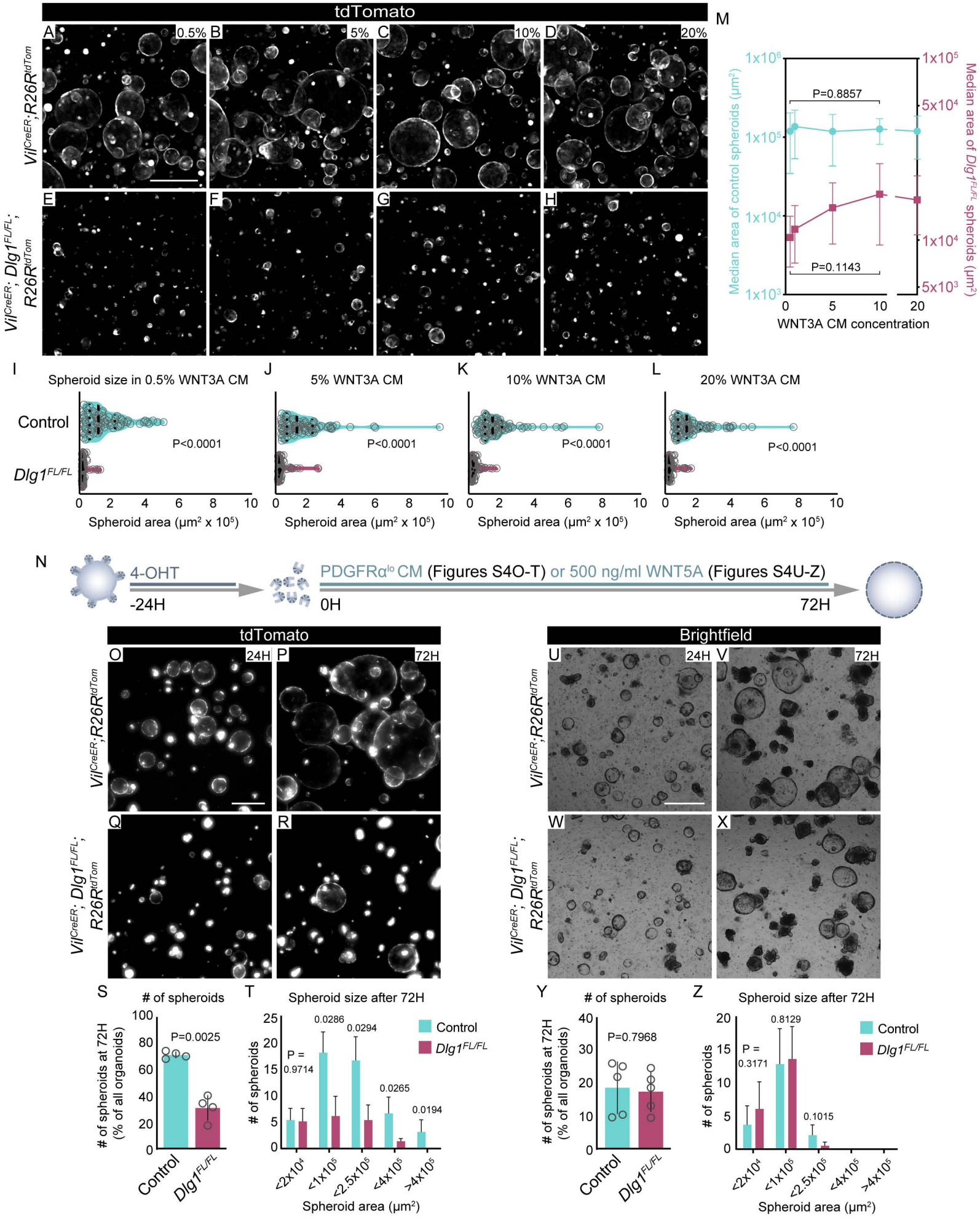

Figure S4 (related to Figure 3). DLG1$^-$ ISCs are lost in increased levels of WNT3A and mesenchymal WNT, while their capacity to grow remains intact in non-canonical WNT5A. Control and DLG1$^-$ organoids were enzymatically dissociated, plated into a Matrigel droplet and overlaid with ENR medium containing 4-OHT and WNT3A CM at different concentrations (for experimental details see Fig. 3A). (**A-H**) 3D rendered projection of organoids in (**A and E**) 0.5% WNT3A CM; (**B and F**) 5% WNT3A CM; (**C and G**) 10% WNT3A CM; and (**D and H**) 20% WNT3A CM. Scale bar = 500 µm. (**I-L**) Quantification of spheroid size at 144 hours after plating. N = 4 x 20 top sized organoids, mean ± SD, unpaired t test with Welch's correction. (**M**) Median spheroid size from (A-L) as a function of WNT3A CM concentration. N = 4 x 20 top sized organoids per condition, median ± SD, unpaired t test with Welch's correction. (**N**) Experimental schematic for analyzing ISCs response to increased mesenchymal WNT levels or WNT5A. Control and DLG1$^-$ organoids were treated with 4-OHT prior mechanical passaging, crypts were plated into a Matrigel droplet and overlaid with ENR medium containing 50% PDGFRα^{lo} conditioned medium (PDGFRα^{lo} CM) or 500 ng/ml WNT5A. (**O-R**) 3D rendered Z-stack projections of spheroids at 24 (**O and Q**) or 72 (**P and R**) hours after plating in PDGFRα^{lo} CM. Scale bar = 500 µm. (**S**) Quantification of formed spheroids in PDGFRα^{lo} CM as a ratio of all organoids at 72 hours after plating. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**T**) Quantification of spheroid size at 72 hours after plating. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**U-X**) 3D rendered Z-stack projections of spheroids at 24 (**U and W**) or 72 (**V and X**) hours after plating in WNT5A. Scale bar = 500 µm. (**Y**) Quantification of formed spheroids in WNT5A as a ratio of all organoids at 72 hours after plating. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**Z**) Quantification of spheroid size at 72 hours after plating. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction.

Figure S5 (related to Figure 3). DLG1⁻ ISCs are lost under homeostatic levels of WNT3A, while proliferation is maintained in increased WNT3A.

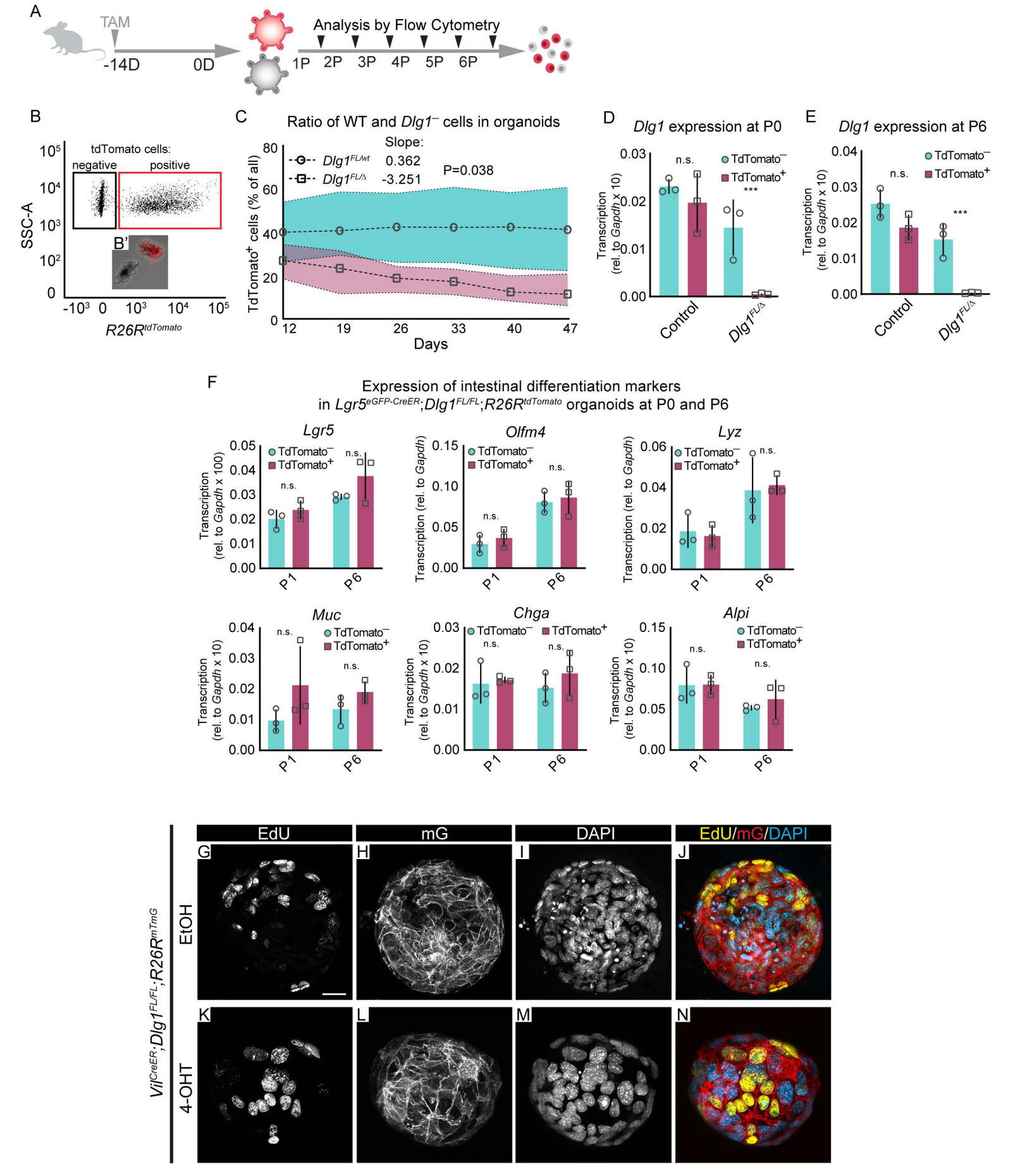

Figure S5 (related to Figure 3). Dlg1^{-} ISCs are lost under homeostatic levels of WNT3A, while proliferation is maintained in increased WNT3A. (**A**) Experimental schematic for analyzing organoid persistence in ENR medium, in which Paneth cells are the only source of Wnt. Control and *Lgr5$^{eGFP\text{-}CreERT2}$;Dlg1$^{FL/\Delta}$;R26R^{tdTomato}* (*Dlg1$^{FL/\Delta}$*) mice were injected with 1 dose of TAM, and two weeks later organoids were established. Organoids were passaged every 7 days, and the ratio of tdTomato^{-}/DLG1^{+} and tdTomato^{+}/DLG1^{-} cells was analyzed at day 5 at every passage by flow cytometry. (**B**) Flow cytometry plot of *Dlg1$^{FL/\Delta}$* organoids with highlighted tdTomato^{-} and tdTomato^{+} cells in rectangles and (**B'**) low resolution image of recombined (tdTomato^{+}) and non-recombined (tdTomato^{-}) organoid within the same Matrigel droplet. (**C**) Quantification of long-term persistence of tdTomato recombined organoids derived from control and *Dlg1$^{FL/\Delta}$* organoids as a ratio of all live cells. N = 6 organoid lines per condition, mean ± SD, simple linear regression. (**D and E**) Transcription levels of *Dlg1* analyzed by qPCR on FACS-sorted live (DAPI^{-}) tdTomato^{-} and tdTomato^{+} organoids at (**D**) passage 0; and (**E**) passage 6. N = 3 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**F**) Transcription levels of intestinal cell-type specific markers analyzed by qPCR on FACS-sorted live (DAPI^{-}) organoids established from *Lgr5$^{eGFP\text{-}CreERT2}$;Dlg1$^{FL/\Delta}$;R26R^{tdTomato}* mice. N = 3 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**G-N**) 3D rendered projection of organoid (**G and K**) stained with EdU; (**H and L**) expressing endogenous mG fluorescent protein; (**I and M**) stained with DAPI; and (**J and N**) multichannel overlay image composed of (P-R and T-V). Scale bar = 20 μm.

Figure S6 (related to Figure 4). Under homeostatic conditions ARHGAP31⁻ organoids grow at similar rate as control organoids.

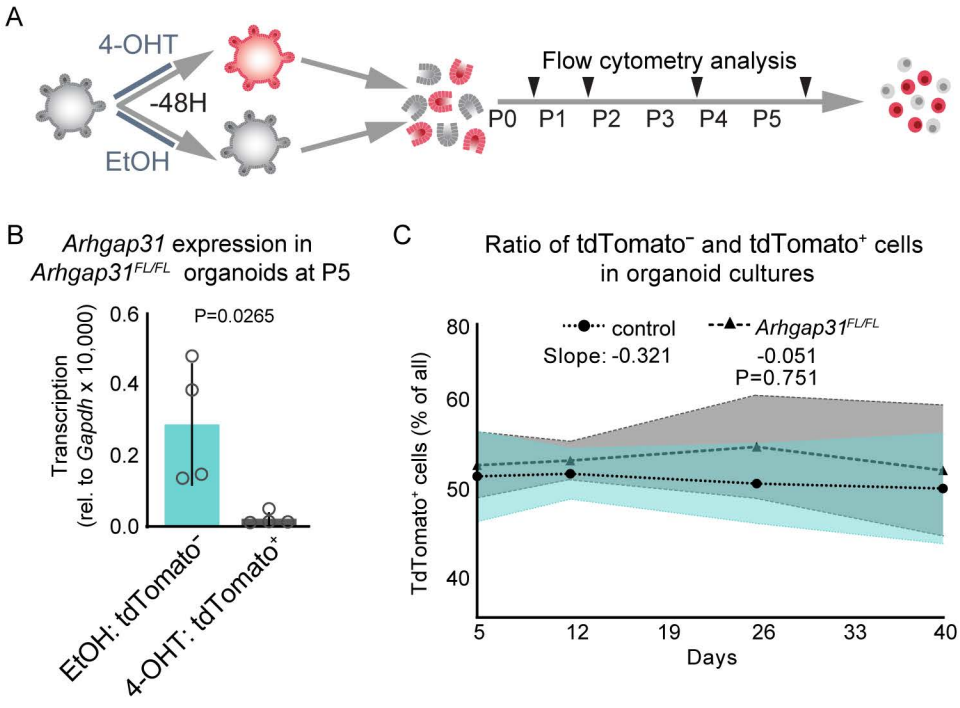

Figure S6 (related to Figure 4). Under homeostatic conditions ARHGAP31^{-} organoids grow at similar rate as control organoids. (**A**) Experimental schematic for analyzing organoid persistence in ENR medium, in which Paneth cells are the only source of Wnt. Organoids from control, and *Arhgap31*$^{FL/FL}$ mice were treated with EtOH or 4-OHT for 48 hours, and mixed at 1:1 ratio while passaged at 1:1 ratio. Organoids were passaged every 7 days, and the ratio of tdTomato^{-} (corresponding to non-recombined LoxP allele) and tdTomato^{+} (corresponding to recombined LoxP allele) cells was analyzed at day 5 of passages P0, P1, P3 and P5 by flow cytometry. (**B**) Transcription levels of *Arhgap31* in *Arhgap31*$^{FL/FL}$ analyzed by qPCR on FACS-sorted live (DAPI^{-}) tdTomato^{-} (EtOH) and tdTomato^{+} (4-OHT) organoids at passage 5. N = 4 organoid lines per condition, mean ± SD, unpaired t test with Welch's correction. (**C**) Quantification of long-term persistence of tdTomato recombined organoids derived from control, and *Arhgap31*$^{FL/FL}$ organoids as a ratio of all live (DAPI^{-}) cells. N = 4 organoid lines per condition, mean ± SD, simple linear regression.

Table S1 (related to STAR Methods). List of primers used for quantitative PCR.

Gene name	IDT Identifier	Forward primer (5'-3' orientation)	Reverse primer (5'-3' orientation)
Alpi	Mm.PT.58.41550204	GCTCAAAGAGGCCCATGA	ATGATCAGAACCTGGTGCAA
Arhgap31	Mm.PT.58.29080273	CTGACGGAGTATCTGGAAAGTTC	CGCTGGATGTTTGAGGTGAT
Arhgap31	Mm.PT.58.14024919	TCGAAGTCCAAGCTGAGTAGA	GAACACAGTGAGTCCATGCT
Ascl2	Mm.PT.58.30838854	GCTGCTTGACTTTTCCAGTTG	CACTAGACAGCATGGGTAAGG
Axin2	Mm.PT.58.8726473	AGTGTCTCTACCTCATTTTCCG	CTTTCCAGCTCCAGTTTCAGT
Birc5	Mm.PT.58.33055871	ATCTGCTTCTTGACAGTGAGG	CTGCTTTAAGGAATTGGAAGGC
Chga	Mm.PT.58.29862516	CGCTCCTTGGCACCTTG	TGTCAGCCCTGAGTGTCT
Clca3	Mm.PT.58.9995580	TGTAGCTTCAAACAGGTATGGA	CATCGTCATCGCCATTAGACC
Cnx43	Mm.PT.58.5955325	CCTTTGACTTCAGCCTCCAA	GACCTTGTCCAGCAGCTTC
Dlg1	Mm.PT.58.11608658	CACTGCTTTGAATGATCCACAC	GAAGTTCCATAGAGCGGGTTA
Gapdh	Mm.PT.39a.1	AATGGTGAAGGTCGGTGTG	GTGGAGTCATACTGGAACATGTAG
Lgr4	Mm.PT.58.31320048	CAGTACCCAGTGAAGCCATT	GTTGTCATCCAGCCACAGAT
Lgr5	Mm.PT.53a.15747338	CTCCAACCTCAGCGTCTTC	CATTTCCAGCAAGACGTAACTC
Lyz1	Mm.PT.58.7374112	CCCAAGATCTAAGAATGCCTGT	CCCATGCTCGAATGCCTT
Ly6a	Mm.PT.58.49069476	GATGGACACTTCTCACACTACA	GCAGGTAATTGATGGGCAAGA
Muc2	Mm.PT.56a.42107820.g	ACCACAATCTCTACTCCCATCT	TCCAGTCAGACCAAAAGCAG
Olfm4	Mm.PT.58.14228836	ACACAGCTCACATCCTTTCTC	GATGCTGTCCTTCTCCATGAC
Rps17	Mm.PT.56a	GCCCTAGATCAGGAGATCATTG	ATGCCAACTGTAGGCTGAGTG
Trop2	Mm.PT.58.12245282.g	TCAACCACTCTGACCTAGACT	TGCCGAAGCTCTATCTGAATG