Vol 459|14 May 2009|doi:10.1038/nature07935

LETTERS

Single Lgr5 stem cells build crypt–villus structures *in vitro* without a mesenchymal niche

Toshiro Sato[1], Robert G. Vries[1], Hugo J. Snippert[1], Marc van de Wetering[1], Nick Barker[1], Daniel E. Stange[1], Johan H. van Es[1], Arie Abo[2], Pekka Kujala[3], Peter J. Peters[3] & Hans Clevers[1]

The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. We have recently demonstrated the presence of about six cycling Lgr5$^+$ stem cells at the bottoms of small-intestinal crypts[1]. Here we describe the establishment of long-term culture conditions under which single crypts undergo multiple crypt fission events, while simultanously generating villus-like epithelial domains in which all differentiated cell types are present. Single sorted Lgr5$^+$ stem cells can also initiate these crypt–villus organoids. Tracing experiments indicate that the Lgr5$^+$ stem-cell hierarchy is maintained in organoids. We conclude that intestinal crypt–villus units are self-organizing structures, which can be built from a single stem cell in the absence of a non-epithelial cellular niche.

The self-renewing epithelium of the small intestine is ordered into crypts and villi[2]. Cells are newly generated in the crypts and are lost by apoptosis at the tips of the villi, with a turnover time of 5 days in the mouse. Self-renewing stem cells have long been known to reside near the crypt bottom and to produce the rapidly proliferating transit amplifying (TA) cells. The estimated number of stem cells is between four and six per crypt. Enterocytes, goblet cells and enteroendocrine cells develop from TA cells and continue their migration in coherent bands along the crypt–villus axis. The fourth major differentiated cell type, the Paneth cell, resides at the crypt bottom. We have recently identified a gene, *Lgr5*, that is specifically expressed in cycling crypt base columnar (CBC) cells that are interspersed between the Paneth cells[1]. Using a mouse in which a green fluorescent protein (GFP)/tamoxifen-inducible *Cre* recombinase cassette was integrated into the *Lgr5* locus, we showed by lineage tracing that the Lgr5$^+$ cells constitute multipotent stem cells that generate all cell types of the epithelium[1], even when assessed 14 months after induction of Cre[3].

Although a variety of culture systems have been described[4–7], no long-term culture system has been established that maintains basic crypt–villus physiology[2]. We attempted to design such a culture system by combining previously defined insights in the growth requirements of intestinal epithelium. First, Wnt signalling is a pivotal requirement for crypt proliferation[8–10] and the Wnt agonist R-spondin 1 induces marked crypt hyperplasia *in vivo*[11]. Second, signalling by epidermal growth factor (EGF) is associated with intestinal proliferation[12]. Third, transgenic expression of Noggin induces an expansion of crypt numbers[13]. Fourth, isolated intestinal cells undergo anoikis outside the normal tissue context[14]. Because laminin (α1 and α2) is enriched at the crypt base[15], we explored the use of laminin-rich Matrigel to support intestinal epithelial growth. Matrigel-based cultures have been used successfully for the growth of mammary epithelium[16].

Mouse crypt preparations were suspended in Matrigel. Crypt growth required EGF and R-spondin 1 (Supplementary Fig. 1a). Passaging revealed a requirement for Noggin (Supplementary Fig. 1b). The cultured crypts behaved in a stereotypical manner (Fig. 1a; Supplementary Movie 1). The upper opening rapidly became sealed, and the lumen filled with apoptotic cells. The crypt region underwent continuous budding events, reminiscent of crypt fission[17]. Paneth cells were always present at the bud site. Most crypts could be cultured (Fig. 1b). Further expansion created organoids, comprising more than 40 crypt domains surrounding a central lumen lined by a villus-like epithelium ('villus domain') (Fig. 1c–e). Staining with E-cadherin revealed a single cell layer (Supplementary Fig. 2). At weekly intervals, organoids were mechanically dissociated and replated at one-fifth of the pre-plating density. Organoids were cultured for more than 8 months without losing the characteristics described below. Expression analysis by microarray revealed that organoids remained highly similar to freshly isolated small-intestinal crypts, for instance when compared with fresh colon crypts (Supplementary Fig. 3). Moreover, no significant induction of stress-related genes was observed (Supplementary Table 1).

Culture of *Lgr5–EGFP–ires–CreERT2* crypts revealed Lgr5–GFP$^+$ stem cells intermingled with Paneth cells at the crypt base. Wnt activation, as demonstrated by nuclear β-catenin (Supplementary Figs 4a and 9) and expression of the Wnt target genes *Lgr5* (Fig. 1d) and *EphB2* (ref. 18) (Supplementary Fig. 4b), was confined to the crypts. Apoptotic cells were shed into the central lumen, a process reminiscent of the shedding of apopotic cells at villus tips *in vivo* (Supplementary Fig. 4c). Metaphase spreads of organoids more than 3 months old consistently revealed 40 chromosomes in each cell ($n = 20$) (Supplementary Fig. 4d). We found no evidence for the presence of myofibroblasts or other non-epithelial cells (Supplementary Fig. 5).

We cultured crypts from *Lgr5–EGFP–ires–CreERT2* mice crossed with the *Cre*-activatable *Rosa26–LacZ* reporter to allow lineage tracing. Directly after induction with low-dose tamoxifen, we noted single labelled cells (Supplementary Fig. 4e, g). More than 90% of these generated entirely blue crypts (Supplementary Fig. 4e–g), implying that the Lgr5–GFP$^+$ cells did indeed retain stem cell properties. Crypts from the *Cre*-activatable *Rosa26–YFP* reporter[19,20] mouse allowed lineage tracing by confocal analysis. Directly after treatment with tamoxifen, we noted single labelled cells that induced lineage tracing over the following days, both in freshly isolated crypts (Supplementary Fig. 6a–c) and in established organoids (Supplementary Fig. 6d). Supplementary Movie 2 represents four days of lineage tracing, revealing green Lgr5$^+$ cells and YFP$^+$ offspring (pseudocolour red) against the backdrop of a growing organoid.

Recently, mammary gland epithelial structures were established from single stem cells *in vitro*[21]. When single Lgr5–GFP^{hi} cells were sorted, these died immediately. The Rho kinase inhibitor Y-27632, which inhibits anoikis of embryonic stem cells[22], significantly

[1]Hubrecht Institute and University Medical Center Utrecht, Uppsalalaan 8, 3584CT Utrecht, The Netherlands. [2]Nuvelo, Inc., 201 Industrial Road, Suite 310, San Carlos, California 94070-6211, USA. [3]The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

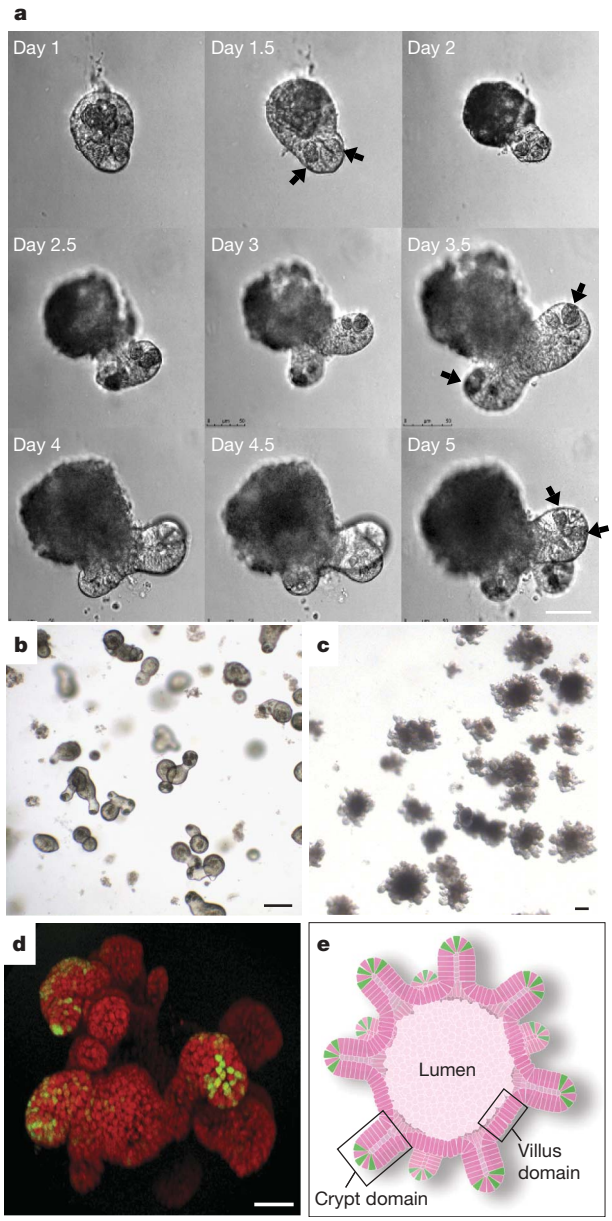


Figure 1 | Establishment of intestinal crypt culture system. **a**, Time course of an isolated single crypt growth. Differential interference contrast image reveals granule-containing Paneth cells at crypt bottoms (arrows). **b**, **c**, Single isolated crypts efficiently form large crypt organoids within 14 days; **b**, on day 5; **c**, on day 14. **d**, Three-dimensional reconstructed confocal image after 3 weeks in culture. Lgr5–GFP^{+} stem cells (green) are localized at the tip of crypt-like domains. Counterstain, ToPro-3 (red). **e**, Schematic representation of a crypt organoid, consisting of a central lumen lined by villus-like epithelium and several surrounding crypt-like domains. Scale bar, 50 μm.

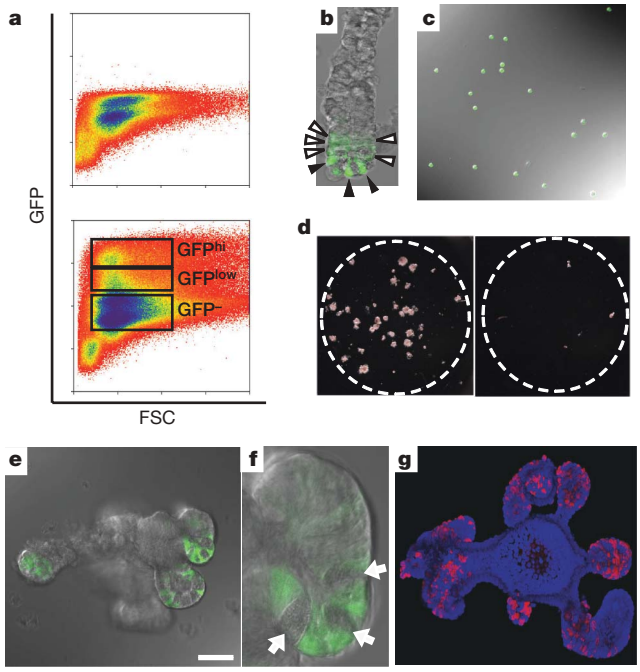


Figure 2 | Single Lgr5^{+} cells generate crypt–villus structures. **a**, Lgr5–GFP^{+} cells from an *Lgr5–EGFP–ires–CreERT2* intestine (bottom); wild-type cells (top). Two positive populations, GFP^{hi} and GFP^{low}, are discriminated. FSC, forward scatter. **b**, Confocal analysis of a freshly isolated crypt. Black arrowheads, GFP^{hi}; white arrowheads, GFP^{low}. **c**, Sorted GFP^{hi} cells. **d**, 1,000 sorted GFP^{hi} cells (left) and GFP^{low} cells (right) after 14 days in culture. **e**, **f**, Fourteen days after sorting, single GFP^{hi} cells form crypt organoids, with Lgr5–GFP^{+} cells and Paneth cells (white arrows) located at crypt bottoms. Scale bar, 50 μm. **f**, Higher magnification of **e**. **g**, Organoids cultured with the thymidine analogue EdU (red) for 1 h. Note that only crypt domains incorporate EdU. Counterstain, 4,6-diamidino-2-phenylindole (DAPI; blue).

decreased this cell death. Because cell-to-cell Notch signalling is essential to maintain proliferative crypts[23], we also provided a Notch-agonistic peptide[24]. Under these conditions, significant numbers of Lgr5–GFP^{hi} cells survived and formed large crypt organoids. Organoids formed rarely when GFP^{low} daughter cells were seeded (Fig. 2d). Multiple Lgr5–GFP^{hi} cells were intermingled with Paneth cells at crypt bottoms (Fig. 2e, f). Incorporation of 5-ethynyl-2′-deoxyuridine (EdU, a thymidine analogue) revealed S-phase cells in the crypts (Fig. 2g).

We sorted cells at one cell per well, visually verified the presence of single cells and followed the resulting growth. In each of four individual experiments, we identified and followed 100 single cells. On average, about 6% of the Lgr5–GFP^{hi} cells grew out into organoids, whereas the remaining cells typically died within the first 12 h, presumably as a result of physical and/or biological stress inherent in the isolation procedure. GFP^{low} cells rarely grew out (Fig. 3a). Figure 3b and Supplementary Fig. 7 illustrate the growth of an organoid from a single Lgr5–GFP^{hi} cell. By four days of culture, the structures consisted of about 100 cells, which is consistent with the 12-h cell cycle of proliferative crypt cells[25] (Fig. 3c). After 2 weeks, the organoids were dissociated into single cells and replated to form new organoids (Fig. 3d). This procedure could be repeated at least four times on a two-weekly basis, without apparent loss of replating efficiency.

The organoids derived from single stem cells were indistinguishable in appearance from those derived from whole crypts. Paneth cells and stem cells were located at crypt bottoms (Figs 2e, f and 4c, g). Fully polarized enterocytes, as demonstrated by villin^{+} mature brush borders and apical alkaline phosphatase, lined the central lumen (Fig. 4a, e, i). Goblet cells (Muc2^{+}, Fig. 4b; periodic acid–Schiff (PAS)$^{+}$, Fig. 4f) and enteroendocrine cells (chromogranin A^{+}, Fig. 4d; synaptophysin^{+}, Fig. 4h) were scattered throughout the organoid structure. Four types of mature cell were recognized by electron microscopy (Fig. 4i–l). Non-epithelial (stromal/mesenchymal) cells were absent, an observation confirmed by electron-microscopic imaging (Fig. 4i–p and Supplementary Fig. 8c–g). Both the crypts (Fig. 4m–o) and the central luminal epithelium (Fig. 4p) consisted of a single layer of polarized epithelial cells resting directly on the Matrigel support. High-resolution images of these electron-microscopic pictures are given in Supplementary Fig. 9. We frequently noted small intercellular vacuoles, possibly an indicator of culture-induced or fixation-induced stress (Fig. 4i–p and Supplementary Fig. 8).

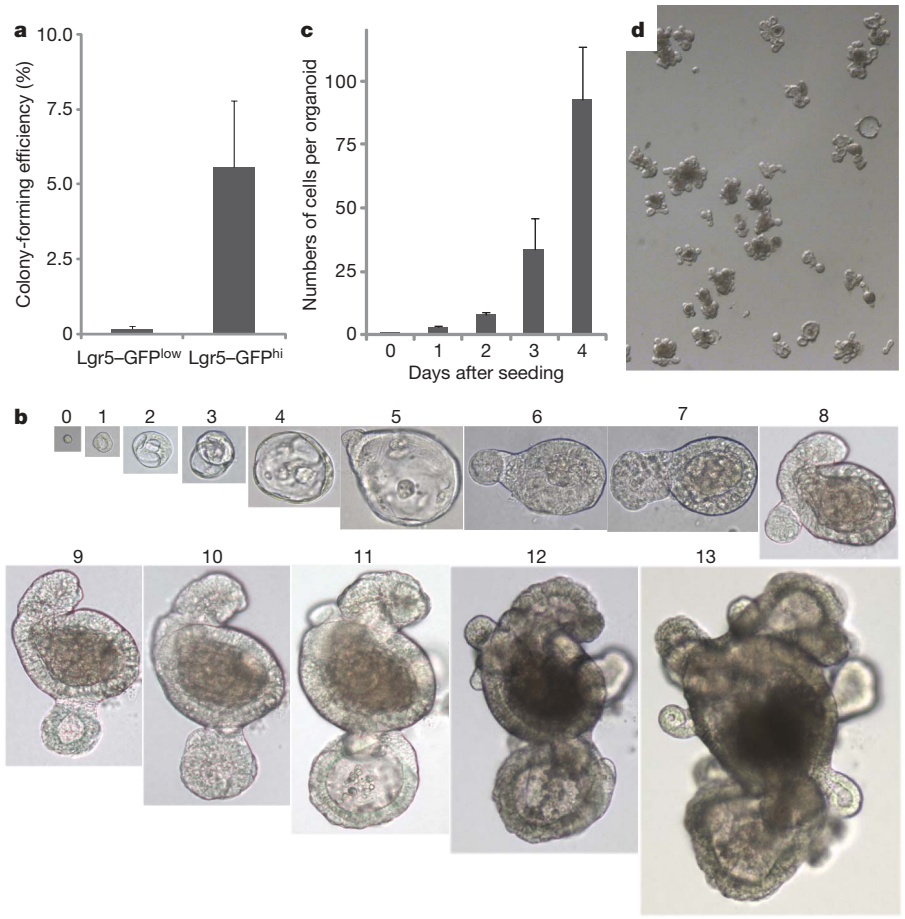
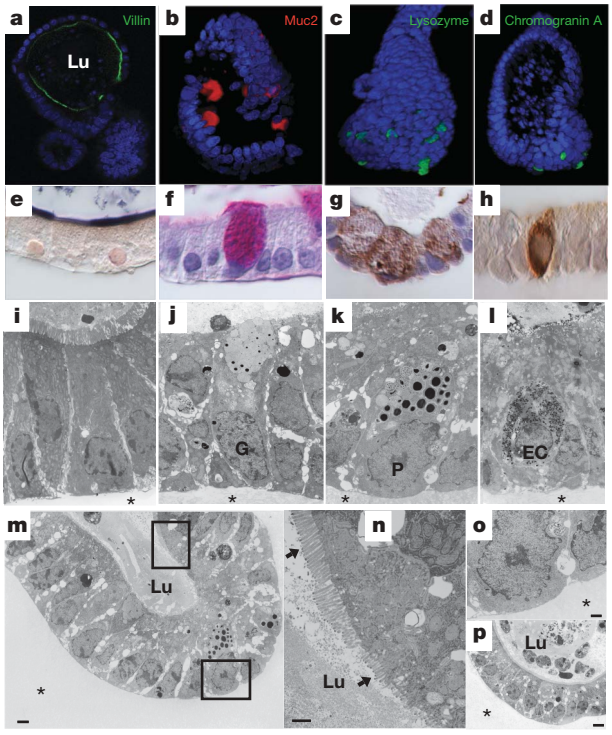


Figure 3 | Colony-forming efficiency of single cells sorted in individual wells. **a**, Colony-forming efficiency was calculated from 100 single sorted GFP^{hi} cells. **b**, An example of a successfully growing single GFP^{hi} cell. Numbers above the images are the days of growth. **c**, Numbers of cells per single organoid averaged for five growing organoids. **d**, A single-cell suspension derived from a single-cell-derived-organoid was replated and grown for 2 weeks. Error bars in **c** and **d** indicate s.e.m. Original magnifications in **b**: days 0–4, ×40; days 5–7, ×20; days 8–11, ×10; days 12 and 13, ×4.



It is well known that epithelial crypts are in intimate contact with subepithelial myofibroblasts[26–28], and it is generally believed that the latter cells create a specialized cellular niche at crypt bottoms[27,29,30]. Such a niche would create a unique environment to anchor and support the intestinal stem cells. We now show that a self-renewing epithelium can be established by a limited set of growth signals that are uniformly presented. Despite this, the isolated stem cells autonomously generate asymmetry in a highly stereotypical fashion. This rapidly leads to the formation of crypt-like structures with *de novo* generated stem cells and Paneth cells located at their bottoms and filled with TA cells. These crypt-like structures feed into villus-like luminal domains consisting of postmitotic enterocytes, in which apoptotic cells pinch off into the lumen in a manner reminiscent of cell loss at villus tips. The paradoxical observation that single cells exposed to a uniform growth-promoting environment can generate asymmetric structures is particularly evident

Figure 4 | Composition of single stem cell-derived organoids. **a**–**d**, Confocal image for villin (**a**, green, enterocytes), Muc2 (**b**, red, goblet cells), lysozyme (**c**, green, Paneth cells) and chromogranin A (**d**, green, enteroendocrine cells). Counterstain, DAPI (blue). **e**–**h**, Paraffin sections stained for alkaline phosphatase (**e**, green, enterocytes), periodic acid-Schiff (**f**, red, goblet cells), lysozyme (**g**, brown, Paneth cells) and synaptophysin (**h**, brown, enteroendocrine cells). **i**–**p**, Electron microscopy demonstrates enterocytes (**i**), goblet cells (**j**), Paneth cells (**k**) and enteroendocrine cells (**l**). **m**–**o**, Low-power crypt images. **n**, **o**, Higher magnifications of **m**. **n**, Maturation of brush border (black arrows). **p**, Low-power villus domain image. Lu, lumen with apoptotic bodies, lined by polarized enterocytes. G, goblet cells; EC, enteroendocrine cells; P, Paneth cells; *, Matrigel. Scale bars, 5 μm (m, p) and 1 μm (n, o).

on scrutiny of the Wnt pathway. Although all cells are exposed to R-spondin 1, only cells in crypts display hallmarks of active Wnt signalling; that is, nuclear β-catenin and the expression of Wnt target genes. Apparently, differential responsiveness to Wnt signalling rather than differential exposure to extracellular Wnt signals lies at the heart of the formation of a crypt–villus axis.

We conclude that a single $Lgr5^+$ intestinal stem cell can operate independently of positional cues from its environment and that it can generate a continuously expanding, self-organizing epithelial structure reminiscent of normal gut. The culture system described will simplify the study of stem-cell-driven crypt–villus biology. Moreover, it may open up new avenues for regenerative medicine and gene therapy.

METHODS SUMMARY

Mice. Outbred mice 6–12 weeks old were used. Generation and genotyping of the *Lgr5–EGFP–Ires–CreERT2* allele[1] has been described previously[3]. Rosa26–lacZ or YFP–Cre reporter mice were obtained from Jackson Labs.

Crypt isolation, cell dissociation and cell culture. Crypts were released from murine small intestine by incubation for 30 min at 4 °C in PBS containing 2 mM EDTA (Supplementary Methods). Isolated crypts were counted and pelleted. A total of 500 crypts were mixed with 50 μl of Matrigel (BD Bioscience) and plated in 24-well plates. After polymerization of Matrigel, 500 μl of crypt culture medium (Advanced DMEM/F12 (Invitrogen)) containing growth factors (10–50 ng ml^{-1} EGF (Peprotech), 500 ng ml^{-1} R-spondin 1 (ref. 11) and 100 ng ml^{-1} Noggin (Peprotech)) was added. For sorting experiments, isolated crypts were incubated in culture medium for 45 min at 37 °C, followed by trituration with a glass pipette. Dissociated cells were passed through cell strainer with a pore size of 20 μm. GFP^{hi}, GFP^{low} and GFP^- cells were sorted by flow cytometry (MoFlo; Dako). Single viable epithelial cells were gated by forward scatter, side scatter and pulse-width parameter, and by negative staining for propidium iodide. Sorted cells were collected in crypt culture medium and embedded in Matrigel containing Jagged-1 peptide (1 μM; AnaSpec) at 1 cell per well (in 96-well plates, 5 μl Matrigel). Crypt culture medium (250 μl for 48-well plates, 100 μl for 96-well plates) containing Y-27632 (10 μM) was overlaid. Growth factors were added every other day and the entire medium was changed every 4 days. For passage, organoids were removed from Matrigel and mechanically dissociated into single-crypt domains, and then transferred to fresh Matrigel. Passage was performed every 1–2 weeks with a 1:5 split ratio.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 16 July 2008; accepted 24 February 2009.
Published online 29 March 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank M. van den Born, J. Korving, H. Begthel and S. van den Brink for technical assistance, and N. Ong and M. van den Bergh Weerman for technical assistance with electron microscopy.

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METHODS

Reagents. Murine recombinant EGF and Noggin were purchased from Peprotech. Human recombinant R-spondin 1 (ref. 11), Y-27632 (Sigma), 4-hydroxytamoxifen (Sigma) and EdU (Invitrogen) were used for culture experiments. The following antibodies were used for immunostaining: anti-lysozyme (Dako), anti-Synaptophysin (Dako), anti-bromodeoxyuridine (Roche), anti-β-catenin (BD Bioscience), anti-E-cadherin (BD Bioscience), anti-smooth muscle actin (Sigma), anti-EphB2 and anti-EphB3 (R&D), anti-villin, anti-Muc2 and anti-chromogranin A (Santa Cruz) and anti-caspase-3 (Cell Signaling).

Crypt isolation. Isolated small intestines were opened longitudinally, and washed with cold PBS. The tissue was chopped into around 5 mm pieces, and further washed with cold PBS. The tissue fragments were incubated in 2 mM EDTA with PBS for 30 min on ice. After removal of EDTA medium, the tissue fragments were vigorously suspended by using a 10-ml pipette with cold PBS. The supernatant was the villous fraction and was discarded; the sediment was resuspended with PBS. After further vigorous suspension and centrifugation, the supernatant was enriched for crypts. This fraction was passed through a 70-μm cell strainer (BD Bioscience) to remove residual villous material. Isolated crypts were centrifuged at 150–200*g*. for 3 min to separate crypts from single cells. The final fraction consisted of essentially pure crypts and was used for culture or single cell dissociation.

Tamoxifen induction and staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). To activate CreERT2, crypts were incubated with a low dose of 4-hydroxytamoxifen (100 nM) for 12 h and cultured in crypt culture medium. X-Gal staining was performed as described previously[3]. No staining was seen without 4-hydroxytamoxifen treatment.

Electron microscopic analysis. As described previously[3], Matrigel including crypt organoids was fixed in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodiumm cacodylate, 2.5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4) for 5 h at room temperature (18–22 °C). The samples were embedded in Epon resin and were examined with a Phillips CM10 microscope.

Microarray analysis: gene expression analysis of colonic crypts, small-intestinal crypts and organoids. Freshly isolated small-intestinal crypts from two mice were divided into two parts. RNA was directly isolated from one part (RNeasy Mini Kit; Qiagen); the other part was cultured for 1 week, followed by RNA isolation. We prepared labelled antisense RNA in accordance with the manufacturer's instructions (Agilent Technologies). Differentially labelled cRNA from small-intestinal crypts and organoids were hybridized separately for the two mice on a 4 × 44k Agilent Whole Mouse Genome Dual Colour Microarray (G4122F) in two dye-swap experiments, resulting in four individual arrays. Additionally, isolated colonic crypts were hybridized against differentially labelled small-intestinal crypts in two dye-swap experiments, resulting in four individual arrays. Microarray signal and background information were retrieved with Feature Extraction (v. 9.5.3; Agilent Technologies). All data analyses were performed with ArrayAssist (5.5.1; Stratagene, Inc.) and Microsoft Excel (Microsoft Corporation). Raw signal intensities were corrected by subtracting local background. Negative values were changed into a positive value close to zero (standard deviation of the local background) to permit the calculation of ratios between intensities for features present in only one channel. Normalization was performed by applying a locally weighted linear regression (LOWESS) algorithm, and individual features were filtered if both intensities were changed or less than double the background signal. Furthermore, non-uniform features were filtered. Data are available at GEO (Gene Expression Omnibus, accession number GSE14594). Unsupervised hierarchical clustering was performed on normalized intensities (processed signal in feature extraction) of small-intestinal or colonic crypts and organoids using Cluster 3 (distance, city block; correlation, average linkage) and visualized with TreeView. Genes were considered significantly changed if they were consistently in all arrays more than threefold enriched in organoids or crypts.

Image analysis. The images of crypt organoids were taken by either confocal microscopy with a Leica SP5, an inverted microscope (Nikon DM-IL) or a stereomicroscope (Leica, MZ16-FA). For immunohistochemistry, samples were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature, and paraffin sections were processed with standard techniques[3]. Immunohistochemistry was performed as described previously[3]. For whole-mount immunostaining, crypt organoids were isolated from Matrigel using Dispase (Invitrogen), and fixed with 4% PFA, followed by permeabilization with 0.1% Triton X-100. EdU staining followed the manufacturer's protocol (Click-IT; Invitrogen). DNA was stained with DAPI or ToPro-3 (Molecular Probes). Three-dimensional images were acquired with confocal microscopy and reconstructed with Volocity Software (Improvision).