LETTERS

Crypt stem cells as the cells-of-origin of intestinal cancer

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Intestinal cancer is initiated by Wnt-pathway-activating mutations in genes such as adenomatous polyposis coli (*APC*). As in most cancers, the cell of origin has remained elusive. In a previously established *Lgr5* (leucine-rich-repeat containing G-protein-coupled receptor 5) knockin mouse model, a tamoxifen-inducible Cre recombinase is expressed in long-lived intestinal stem cells[1]. Here we show that deletion of Apc in these stem cells leads to their transformation within days. Transformed stem cells remain located at crypt bottoms, while fuelling a growing microadenoma. These microadenomas show unimpeded growth and develop into macroscopic adenomas within 3–5 weeks. The distribution of $Lgr5^+$ cells within stem-cell-derived adenomas indicates that a stem cell/progenitor cell hierarchy is maintained in early neoplastic lesions. When Apc is deleted in short-lived transit-amplifying cells using a different *cre* mouse, the growth of the induced microadenomas rapidly stalls. Even after 30 weeks, large adenomas are very rare in these mice. We conclude that stem-cell-specific loss of Apc results in progressively growing neoplasia.

The anatomy of the intestinal crypt is uniquely suited to study adult stem cells in their niche. The epithelium of the murine small intestine renews every 5 days[2,3]. We have recently identified slender $Lgr5^+$ cells located at crypt bottoms as the stem cells of the small intestine and colon[1]. Each crypt contains approximately six long-lived stem cells intermingled with Paneth cells in the small intestine and with goblet cells in colon (Fig. 1a). Counter-intuitively, these cells are not quiescent, but divide every day[1]. These cells have been called crypt base columnar cells[4,5]. Their daughter cells constitute the transit-amplifying crypt compartment. Transit-amplifying cells divide every 12–16 h, generating some 300 cells per crypt every day[6]; they reside within crypts for approximately 48 h, undergoing up to five rounds of cell division while migrating upwards[6]. When the committed transit-amplifying cells reach the crypt–villus junction, they rapidly differentiate while continuing their upward migration. Paneth cells escape this flow and reside for 3–6 weeks at the crypt base[7–9]. Initiating mutations in intestinal malignancies target Wnt pathway components, most frequently the negative regulator Apc[10,11]. This results in the constitutive activation of a Wnt target gene program that drives the formation of benign adenomas[12–15]. However, it remains unclear which cell type sustains the cancer-initiating mutation.

The cytochrome P450-promoter-driven *Ah-cre* mouse allows conditional deletion of floxed alleles in the intestinal epithelium by induction with β-naphthoflavone (β-NF). The *Ah-cre* allele is highly active throughout the epithelium, including the stem cells[16]. We have previously used a floxed allele of *Apc* (ref. 17) with the *Ah-cre* mouse line to demonstrate that acute loss of Apc throughout the adult intestinal epithelium after intraperitoneal injection of β-NF instantly transforms the epithelium[16]. This process depends on the Wnt target gene *c-myc*[18]. High-dose oral β-NF induces a more stochastic deletion of Apc (Supplementary Fig. 1), resulting in rapid adenoma formation throughout the intestine within 3 weeks[19]. Both of these high-dose induction protocols effect deletion in all compartments of the epithelium, including the stem cells at the crypt base.

We next sought the cell of origin of the adenomas. Careful titration revealed that after oral administration of 1 mg kg^{-1} β-NF, the efficiency of Cre activation in the stem cells at the crypt base was extremely low. In a group of 15 mice, only 5.0 ± 3.0 lineage tracing events (that is, stem cell hits) were seen in the entire small intestine at 100 days after induction. This dose still very efficiently induced Cre activity in the transit amplifying and villus compartments, as visualized 1 day after oral induction using the *Rosa26R* mouse as Cre reporter (Fig. 1b, c). Of note, most 5-bromodeoxyuridine (BrdU)-pulsed transit-amplifying cells migrate onto villi within 24 h and are lost into the gut lumen at 48 h (ref. 20). This indicated that blue-stained cells on the bottom half of the villi (Fig. 1b, c) resided in crypts as transit-amplifying cells at the time of deletion. At day 7, blue staining could no longer be detected (not shown).

Using this regimen on *Ah-cre/*$Apc^{flox/flox}$*/Rosa26R* mice, multiple β-$catenin^{high}$ foci became visible throughout upper crypt and villus at day 3 after induction (Fig. 1d–g). The foci were invariably $LacZ^+$, confirming that Cre-mediated deletion had occurred (Fig. 1f and Supplementary Fig. 2). These β-$catenin^{high}$ foci expressed the Wnt target gene *c-myc* and were actively proliferating (Supplementary Fig. 1). Of note, loss of Apc in a crypt cell induces maintenance of EphB expression in the resulting transformed clone[21,22]. As a consequence, the clone fails to migrate out of its crypt and eventually invades the subepithelium[22]. Figure 1h quantifies focus induction in the different compartments at day 3 after induction.

Most of the Apc-deficient villus cells were lost after 4–5 days. The remaining foci within the crypts demonstrated very limited expansion over a 24-day period (Supplementary Fig. 3) and no macroscopic adenomas occurred. Notably, the foci persisted over at least a 284-day period (Fig. 1i), and only very rarely progressed to adenomas (Fig. 1j, quantification in Fig. 1k; for histological definition see Supplementary Fig. 4). In 5 mice at >100 days after induction, we observed 3 ± 2.3 macroscopic adenomas, correlating with the inordinately low frequency of Cre activation in stem cells (see above). Thus, the long-term persistence of microadenomas derived from transformed transit-amplifying cells might, in very rare cases, result in a progressively growing neoplasia, possibly when additional hits are sustained.

We then crossed the stem-cell-specific *Lgr5-EGFP-IRES-*$creER^{T2}$ knockin mice to $Apc^{flox/flox}$ mice. In the resulting mice, Cre was

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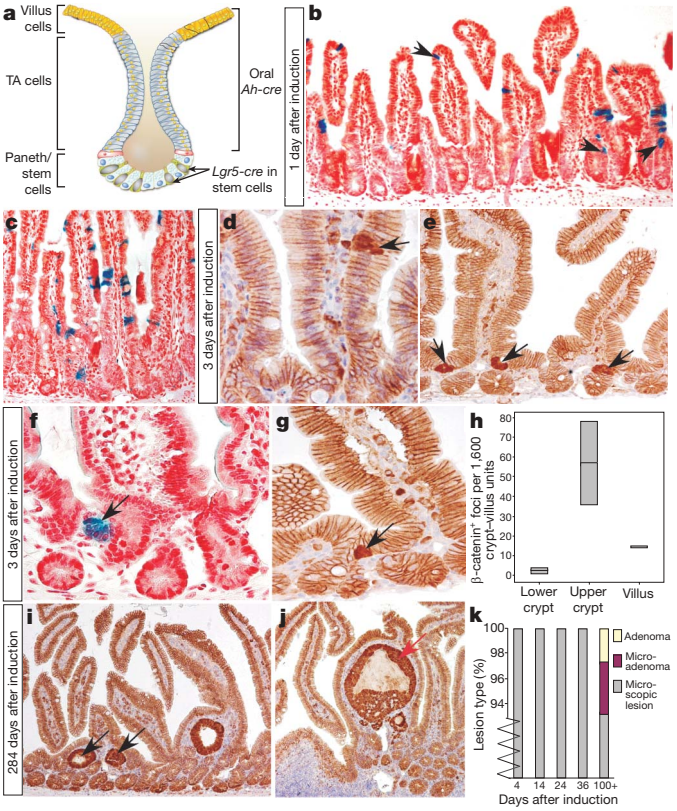

Figure 1 | Transformation of non-stem cell populations in *Ah-cre/Apc*$^{flox/flox}$ intestines through low-dose oral β-NF-induced Apc deletion fails to drive intestinal neoplasia. **a**, Cartoon depicting the general organization of intestinal crypts. *Lgr5-EGFP-IRES-creER*^{T2} (*Lgr5-cre* for brevity) is activated by intraperitoneal administration of tamoxifen only in the stem cells located at the crypt base. Low-dose oral administration of β-NF activates *Ah-cre* in the upper transit-amplifying (TA) compartment and villus. **b**, **c**, LacZ-stained sections of intestine from an *Ah-cre/Rosa26R* mouse treated with a single low-dose oral gavage of 1 mg kg^{-1} β-NF for 1 day. LacZ-positive cells were readily visible on the villi and upper crypts (black arrows) throughout the intestine, but were only very rarely observed at the crypt base. **d**, **e**, **g**, β-catenin immunohistochemistry performed on intestinal sections from *Ah-cre/Rosa26R/Apc*$^{flox/flox}$ mice 3 days after a single gavage of 1.0 mg kg^{-1} β-NF. Clusters of transformed cells with elevated levels of β-catenin (β-catenin^{high}) were frequently observed on the villus (**d**) and upper regions of the crypt (**e**, **g**). These clusters are highlighted with black arrows. **f**, The β-catenin^{high} clusters were invariably positive for LacZ (black arrow). **h**, Quantification of the location of the β-catenin^{high} cell clusters on intestinal sections from *Ah-cre/Rosa26R/Apc*$^{flox/flox}$ mice 3 days after a single gavage of 1.0 mg kg^{-1} β-NF. Box-plots show numbers of foci observed at the crypt base, the upper crypt and the villus in 1,600 crypt–villus units. Significantly more clusters/foci were seen in the upper regions of the crypt than any other region ($P = 0.04$, Mann–Whitney U-test, $n = 3$). **i**, **j**, β-catenin immunohistochemistry performed on intestinal sections from an *Ah-cre/Rosa26R/Apc*$^{flox/flox}$ mouse 284 days after induction. Note single adenomatous crypts (black arrows) with nuclear β-catenin in every cell which have not progressed and are histologically identical to single adenomatous crypts seen 14, 24 and 36 days after *cre* activation (see Supplementary Fig. 3). **k**, Classification of LacZ^{+} intestinal lesions in *Ah-cre/Rosa26R/Apc*$^{flox/flox}$ intestinal whole mounts (see Supplementary Fig. 4 and Methods for details). No adenomas and only one microadenoma were seen in mice at early time points (up to 36 days) after 1 mg kg^{-1} β-NF (at least four mice at each time point), whereas in older mice rare adenomas were observed (3 ± 2.3 for 5 mice counted). 'Microscopic lesions' were histologically equivalent to single adenomatous crypts. Original magnifications: **b**, **c**, **l**, 10 ×; **d**, **f**, **g**, 40 ×; **e**, 20 ×.

activated with a single intraperitoneal injection of tamoxifen. Accumulation of β-catenin first occurred in isolated EGFP^{+} stem cells after 3 days (Fig. 2a–c) and was not seen in control mice (Supplementary Fig. 5). Thirty-one transformed cells were counted in a total

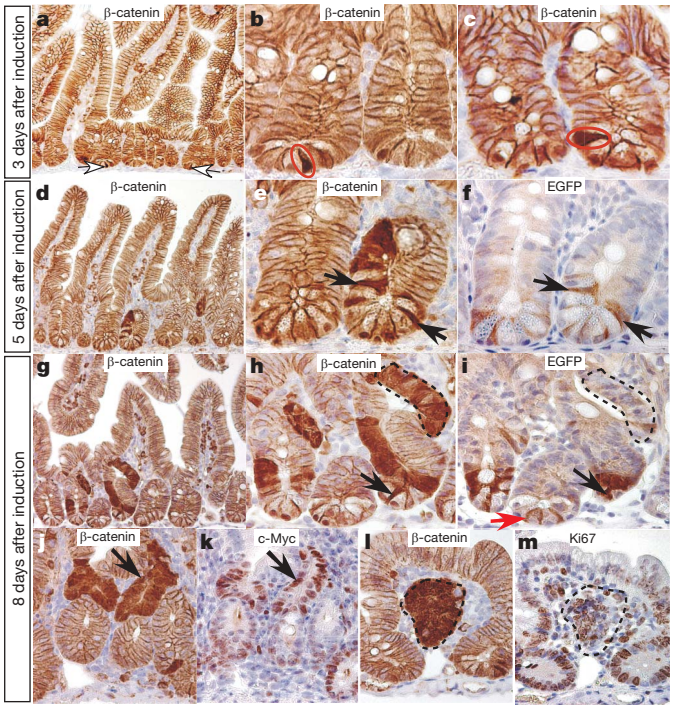

Figure 2 | Lgr5–EGFP^{+} intestinal stem cells transformed after loss of Apc persist and fuel the rapid formation of β-catenin^{high} microadenomas. *Lgr5-EGFP-creER*^{T2}*/Apc*$^{flox/flox}$ mice were treated with a single intraperitoneal injection of tamoxifen to activate the stem-cell-specific Cre and facilitate loss of Apc. **a**–**i**, The consequences of the resulting Lgr5–EGFP^{+} intestinal stem cell transformation and their subsequent fate was tracked over an 8-day period using β-catenin and EGFP as markers of transformed cells and Lgr5^{+} stem cells, respectively. **a**–**c**, Accumulation of the Wnt effector protein, β-catenin, was first observed in scattered Lgr5^{+} stem cells at the crypt base 3 days after Cre induction (**a**; white arrows). **b**, **c**, Representative examples of β-catenin^{high}Lgr5^{+} stem cells are circled in red. **d**–**f**, Five days after induction the transformed Lgr5–EGFP^{+} stem cells remained (compare black arrows in **e** and **f**) and were associated with clusters of transformed (β-catenin^{high}) cells within the transit-amplifying compartment. **g**, **h**, Eight days after induction the clusters of transformed cells had expanded to fill the entire transit-amplifying compartment (**h**). The transformed β-catenin^{high}/EGFP^{+} stem cells at the crypt base persisted (compare black arrow in **h** with **i**), but expression of the stem cell marker Lgr5–EGFP was lost in most of the transformed cells higher up the crypt (compare outlined regions in **h** and **i**). Lgr5–EGFP expression in transformed stem cells was significantly higher than in non-transformed stem cells (**i**; red arrow), reflecting elevated expression of the Wnt target gene *Lgr5* after loss of Apc. **j**, **k**, β-catenin^{high} lesions (**j**; black arrow) generated after transformation of the stem cells expressed high levels of the Wnt target gene *c-myc* (**k**; black arrow), reflecting aberrant activation of the Wnt pathway. **l**, **m**, The early β-catenin^{high} lesions expressed the Ki67 marker (compare outlined regions in **l** and **m**), confirming their highly proliferative status. Original magnifications: **a**, **d**, **g**, 10 ×; **b**, **c**, **e**, **f**, 60 ×; **h**–**m**, 40 ×.

of 500 crypts in a representative mouse (~6%), a frequency which remained constant over the next 8 days and was comparable to the deletion rate observed for the oral β-NF protocol (~5%, see Fig. 1h). After 5 days, multiple crypts harboured transformed (that is, β-catenin^{high}) EGFP^{+} stem cells associated with proliferative clusters of β-catenin^{high} cells within the transit-amplifying compartment (Fig. 2d–f). Thus, Wnt-transformed stem cells persisted at crypt bottoms, yet rapidly generated transformed progeny higher up in the crypts. Eight days after induction, the transformed cells had continued to expand (Fig. 2g–i). Microadenomas within the associated villus stroma became evident (Fig. 2j, l), expressing c-Myc and Ki67 (Fig. 2k, m). The lesions continued their aggressive expansion (Fig. 3). At day 14, a similar number (42 per 500 crypts, ~8%) of large, multi-villus adenomas was observed (Fig. 3a, b). At day 36, the mice had to be killed (Fig. 3g, h). Figure 4a gives a global view of the lesions.

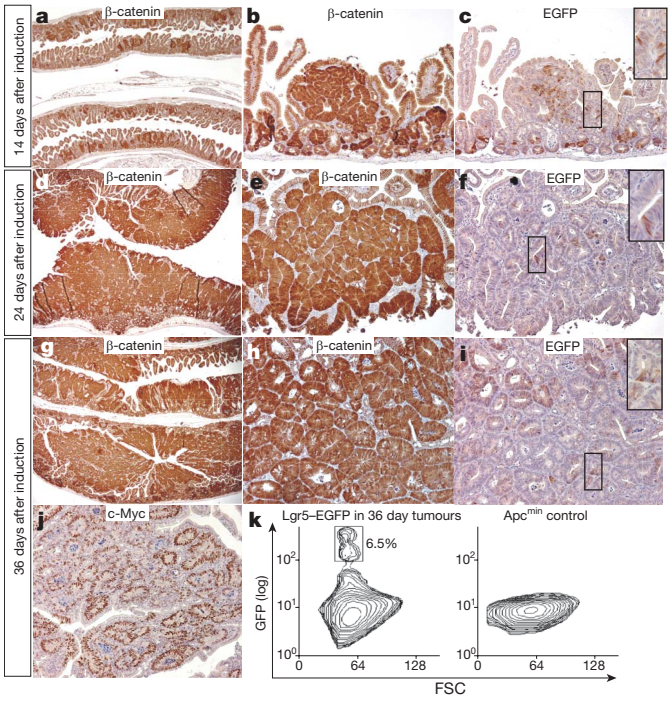

Figure 3 | Selective transformation of Lgr5–EGFP$^+$ stem cells after loss of Apc efficiently drives adenoma formation throughout the small intestine. **a–i**, The appearance and development of intestinal adenomas and expression of the Lgr5–EGFP stem cell marker within these adenomas was tracked over a 36-day period using β-catenin (adenoma marker) and EGFP (stem cell marker) immunohistochemistry respectively. **a**, **b**, Multiple small, β-catenin^{high} adenomas were readily visible throughout the intestine 14 days after Lgr5$^+$ stem cell transformation. **c**, Lgr5–EGFP expression was restricted to scattered cells in these small adenomas (inset box shows magnified examples). **d**, **e**, Multiple macroscopic adenomas (>100) were present after 24 days. **g**, **h**, After 36 days a lethal adenoma burden was reached, with massive neoplasia evident along the entire length of the intestine. **f**, **i**, Lgr5–EGFP expression in these macroscopic adenomas remained restricted to small populations of cells with characteristic crypt base columnar stem cell morphology (inset boxes show magnified examples). **j**, These adenomas expressed high levels of c-Myc, confirming aberrant activation of the Wnt pathway. **k**, Quantification of Lgr5–EGFP expression in 36-day post-induction adenomas and control Apc^{min} mouse adenomas by FACS analysis. Left panel: adenomas from 36-day post-induction mice contained, on average, 6.5% Lgr5–EGFP$^+$ cells. Right panel: no EGFP expression was detected in adenomas from Apc$^{min/WT}$ mice, confirming the specificity of the EGFP signal in the 36 day adenomas. Original magnifications: **a**, **d**, **g**, 2 ×; **b**, **c**, **e**, **f**, **h**, **i**, 10 ×; **j**, 4 ×; all insets, 40 ×.

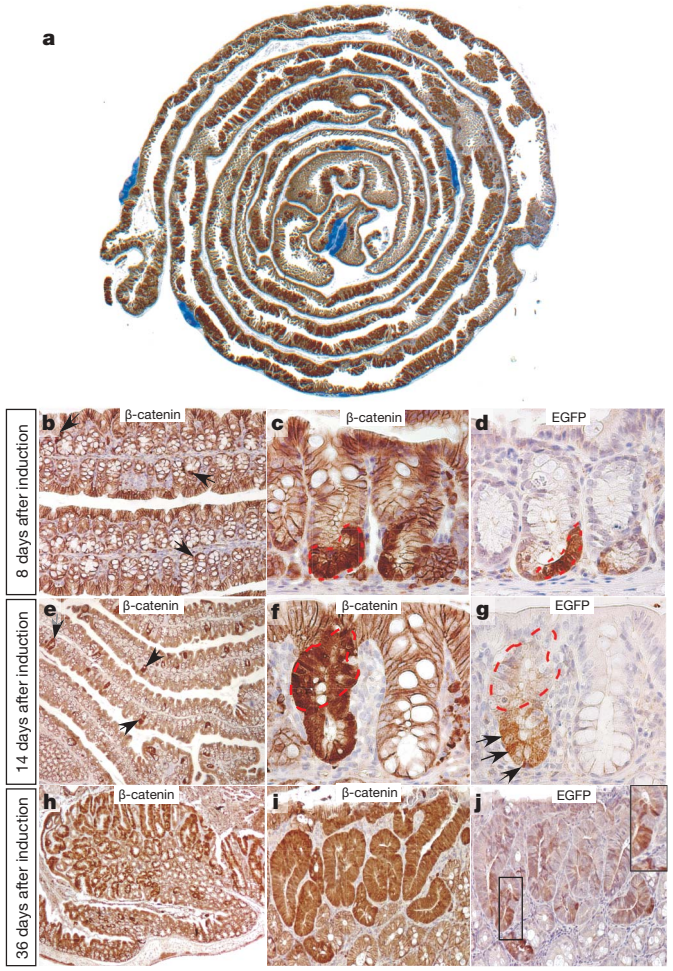

Figure 4 | Transformation of Lgr5–EGFP$^+$ stem cells drives intestinal neoplasia in both the small intestine and colon. **a**, A massive tumour burden is reached 36 days after transformation of the stem cells, with β-catenin^{high} macroscopic adenomas visible throughout the entire small intestine (Peyers patches are stained blue). **b–j**, The appearance and development of colonic adenomas and expression of the Lgr5–EGFP stem cell marker within these adenomas was tracked over a 36-day period using β-catenin (adenoma marker) and EGFP (stem cell marker) immunohistochemistry, respectively. **b**, **c**, Small clusters of β-catenin^{high} cells were first observed in the Lgr5$^+$ stem cell zone at the crypt base after 8 days (**b**, black arrows and **c**, outlined in red). **d**, High level expression of Lgr5–EGFP was apparent in the transformed stem cell compartment (**d**; outlined in red), confirming aberrant activation of the Wnt pathway after loss of Apc. **e**, **f**, Fourteen days after induction the clusters of transformed cells had expanded to fill the entire colonic crypt. The transformed, β-catenin^{high}/Lgr5–EGFP$^+$ stem cells persisted (black arrows), but expression of the stem cell marker Lgr5–EGFP was lost in most of the transformed cells higher up the crypt (compare outlined regions in **f** and **g**). **h**, **i**, Multiple β-catenin^{high} adenomas were observed throughout the colon 36 days after induction. **j**, Lgr5–EGFP expression in these colonic adenomas was restricted to small populations of cells with characteristic stem cell morphology (inset box shows magnified examples). Original magnifications: **b**, 4 ×; **c**, **d**, **f**, **g**, 40 ×; **h**, 2 ×; **i**, **j**, 10 ×; inset in **j**, 40 ×.

The *Lgr5* gene also marks stem cells in colon[1]. Indeed, transformed β-catenin^{high}Lgr5$^+$ stem cell populations at the base of colonic crypts became visible at day 8 after induction (Fig. 4b, c). The transformed crypts continued this steady expansion (Fig. 4e, f), forming micro-adenomas 3 weeks after induction (not shown). β-catenin^{high} colon adenomas of a considerable size were commonly observed at day 36 (Fig. 4h, i).

To investigate the hierarchy between the Apc-deficient stem cells and their transformed progeny, we studied Lgr5–EGFP during adenoma formation in our model. Non-transformed stem cells express Lgr5–EGFP[1]. Expression was maintained in the transformed stem cells at day 5 (Fig. 2f) and day 8 (Fig. 2i). Notably, the marker was absent in most transit-amplifying cells of the β-catenin^{high} clones (outlined regions in Fig. 2h and i). The marker was expressed in scattered cells within adenomas at later time points (Fig. 3c, f, i and Supplementary Fig. 6). In contrast, another Wnt target gene, *c-myc*, was uniformly expressed throughout the adenomas (Fig. 3j). Quantification by fluorescence-activated cell sorting (FACS) at 36 days after induction revealed that Lgr5$^+$ cells comprise 6.5% of the tumour population (Fig. 3k). A similar phenomenon was observed for the colonic adenomas (Fig. 4d, g, j).

Taken together, these data demonstrate that transformation of stem cells through loss of Apc is an extremely efficient route towards initiating intestinal adenomas. In a recent study, a *BmicreER* knockin allele visualized a potentially novel pool of intestinal stem cells by lineage tracing[23]. Lgr5 cells and Bmi-1 cells both produce offspring within days, yet persist for at least a year and are multipotent. At first glance, there are also some significant differences, particularly in the relative location of the two stem cell types. Bmi-1 cells are predominantly located at

position +4 directly above the Paneth cells and are restricted to the proximal small intestine. Thus, Lgr5 and Bmi-1 cells reportedly represent different pools of stem cells[23]. *BmicreER* mice were crossed with a conditionally activatable β-catenin allele (*loxP(ex3)*)[24]. Adenoma formation became visible 3–4 weeks after Cre induction. Although no comparison was made to *loxP(ex3)* activation in non-stem cells, these observations support the hypothesis that transformation of stem cells constitutes the principal route towards intestinal cancer.

Early studies on acute myelogenous leukaemia support the existence of tumour cells with stem-cell-like properties in this disease[25]. On the basis of many subsequent studies, the cancer stem cell concept[26] postulates that a small reservoir of self-sustaining cells is exclusively able to self-renew and maintain a tumour. These cancer stem cells may be refractory to current therapies and may also be the most likely cells to metastasize. Recently, cancer stem cells have been identified in solid tumours[27]. With the use of CD133 (refs 28, 29), a marker associated with stem and progenitor populations, or CD44 and EpCAM[30], human colon cancers have been demonstrated to contain cancer stem cells. Our observations on Lgr5 expression suggest that a stem cell/progenitor cell hierarchy is maintained in early stem-cell-derived adenomas, which would lend support to the cancer stem cell concept. Testing of the tumorigenic potential of these Lgr5^{+} cells awaits the development of a transplantation assay for intestinal adenomas.

METHODS SUMMARY

Mice. *Ah-cre*/*Apc*$^{flox/flox}$ mice were generated by interbreeding mice carrying a floxed *Apc* allele (*Apc*$^{580S/flox}$)[17] and the *Ah-cre* allele[9]. *Lgr5-EGFP-IRES-creER*^{T2}/*APC*$^{flox/flox}$ mice were generated by interbreeding mice carrying a floxed *Apc* allele, (*Apc*$^{580S/flox}$)[17] and the *Lgr5-EGFP-IRES-creER*^{T2} allele[1].

Oral β-NF induction. Mice aged 6–8 weeks were treated with a single oral gavage of 1 mg kg^{-1} β-naphthoflavone (β-NF) in corn oil. All experiments were performed according to UK Home Office guidelines.

Tamoxifen induction. Mice aged 6–8 weeks were injected intraperitoneally with a single 200 μl dose of tamoxifen in sunflower oil at 10 mg ml^{-1}.

LacZ analysis. To determine the pattern of recombination at the *Rosa26R lacZ* reporter locus, intestinal whole-mounts were prepared, fixed and exposed to X-gal substrate using a method previously reported (see Methods).

Tissue fixation and immunohistochemistry. Intestinal tissue was fixed and processed into paraffin blocks according to standard procedures. β-catenin, EGFP, c-Myc, CD44 and Ki67 immunohistochemistry was performed as previously described[14] (see Methods).

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

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1. Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* **449,** 1003–1007 (2007).
2. Barker, N., van de Wetering, M. & Clevers, H. The intestinal stem cell. *Genes Dev.* **22,** 1856–1864 (2008).
3. Potten, C. S. Kinetics and possible regulation of crypt cell populations under normal and stress conditions. *Bull. Cancer* **62,** 419–430 (1975).
4. Cheng, H. & Leblond, C. P. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am. J. Anat.* **141,** 537–561 (1974).
5. Cheng, H. & Leblond, C. P. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am. J. Anat.* **141,** 461–479 (1974).
6. Marshman, E., Booth, C. & Potten, C. S. The intestinal epithelial stem cell. *Bioessays* **24,** 91–98 (2002).
7. Bjerknes, M. & Cheng, H. The stem-cell zone of the small intestinal epithelium. II. Evidence from paneth cells in the newborn mouse. *Am. J. Anat.* **160,** 65–75 (1981).
8. Bjerknes, M. & Cheng, H. The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. *Am. J. Anat.* **160,** 51–63 (1981).
9. Ireland, H., Houghton, C., Howard, L. & Winton, D. J. Cellular inheritance of a Cre-activated reporter gene to determine Paneth cell longevity in the murine small intestine. *Dev. Dyn.* **233,** 1332–1336 (2005).
10. Jones, S. *et al.* Comparative lesion sequencing provides insights into tumor evolution. *Proc. Natl Acad. Sci. USA* **105,** 4283–4288 (2008).
11. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer. *Cell* **87,** 159–170 (1996).
12. Korinek, V. *et al.* Constitutive transcriptional activation by a β-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science* **275,** 1784–1787 (1997).
13. Morin, P. J. *et al.* Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275,** 1787–1790 (1997).
14. van de Wetering, M. *et al.* The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* **111,** 241–250 (2002).
15. Van der Flier, L. G. *et al.* The intestinal Wnt/TCF signature. *Gastroenterology* **132,** 628–632 (2007).
16. Sansom, O. J. *et al.* Loss of Apc *in vivo* immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev.* **18,** 1385–1390 (2004).
17. Shibata, H. *et al.* Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* **278,** 120–123 (1997).
18. Sansom, O. J. *et al.* Myc deletion rescues Apc deficiency in the small intestine. *Nature* **446,** 676–679 (2007).
19. Sansom, O. J. *et al.* Cyclin D1 is not an immediate target of β-catenin following Apc loss in the intestine. *J. Biol. Chem.* **280,** 28463–28467 (2005).
20. Muncan, V. *et al.* Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Mol. Cell. Biol.* **26,** 8418–8426 (2006).
21. Batlle, E. *et al.* β-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* **111,** 251–263 (2002).
22. Cortina, C. *et al.* EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. *Nature Genet.* **39,** 1376–1383 (2007).
23. Sangiorgi, E. & Capecchi, M. R. Bmi1 is expressed *in vivo* in intestinal stem cells. *Nature Genet.* **40,** 915–920 (2008).
24. Harada, N. *et al.* Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. *EMBO J.* **18,** 5931–5942 (1999).
25. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med.* **3,** 730–737 (1997).
26. Clarke, M. F. *et al.* Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* **66,** 9339–9344 (2006).
27. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J. & Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl Acad. Sci. USA* **100,** 3983–3988 (2003).
28. O'Brien, C. A., Pollett, A., Gallinger, S. & Dick, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445,** 106–110 (2007).
29. Ricci-Vitiani, L. *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* **445,** 111–115 (2007).
30. Dalerba, P. *et al.* Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl Acad. Sci. USA* **104,** 10158–10163 (2007).

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

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METHODS

Scoring of β-catenin$^+$ foci. This was performed on sections stained with anti-β-catenin (BD Transduction labs). A crypt–villus unit was defined on histological cross-section when the crypt exits on both sides into a villus. β-catenin^{high} foci were scored as clusters of cells that showed both increased nuclear and cytoplasmic β-catenin when compared to surrounding cells. The following categories were recognized: base of crypts (β-catenin-positive cells were below position 10 of the crypt), upper crypt (β-catenin-positive cells were between position 10 and the top of the crypt), and villus (β-catenin-positive cells were clearly located within the villus).

Scoring of LacZ$^+$ lesions on intestinal whole mounts. Mice were culled, their intestines removed, opened up and pinned out as intestinal whole mounts. They were then stained for LacZ. Using a dissecting microscope, microscopic lesions, microadenomas and adenomas were counted for the entire intestine and converted to a percentage. For all the intestines scored for this analysis, there were at least 100 microscopic lesions per animal on whole-mount analysis. Lesions occurring after Apc loss were divided into three categories: microscopic lesions, microadenomas and adenomas. Microscopic lesions were those that could only be seen using the dissecting microscope and were less than 0.5 mm × 0.5 mm. Microadenomas were judged as being visible without the dissecting microscope and ranged from 0.5 mm × 0.5 mm to 2 mm × 2 mm. Adenomas were greater than 2mm × 2mm. This categorization fitted with our previous histological criteria, where lesions were subdivided into single crypt lesions (equivalent to the microscopic lesion), complex lesions were greater than one crypt (2–3) and situated near the surface, and adenomas comprised numerous crypt-like structures and had reached the surface of the epithelium.

β-galactosidase (LacZ) staining protocol. Intestines were isolated, immediately flushed with ice-cold fixative (1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP40 in PBS0 (phosphate-buffered saline deficient in Mg^{2+} and Ca^{2+})) and incubated for 2 h in a 20-fold volume of the same ice-cold fixative at 4 °C on a rolling platform. The fixative was removed and the intestines washed twice in PBS0 for 20 min at room temperature on a rolling platform. The β-galactosidase substrate (5 mM $K_3FE(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}3H_20$, 2 mM $MgCl_2$, 0.02% NP40, 0.1% Nadeoxycholate, 1 mg ml^{-1} X-gal in PBS0) was then added and the tissues incubated in the dark overnight at room temperature. The substrate was removed and the tissues washed twice in PBS0 for 20 min at room temperature on a rolling platform. The tissues were then fixed overnight in a 20-fold volume of 4% paraformaldehyde (PFA) in PBS0 at 4 °C in the dark on a rolling platform. The PFA was removed and the tissues washed twice in PBS0 for 20 min at room temperature on a rolling platform.

The stained tissues were transferred to tissue cassettes and paraffin blocks prepared using standard methods. Tissue sections (4 μM) were prepared and counterstained with neutral red.

Immunohistochemistry protocol. Freshly isolated intestines were flushed with formalin (4% formaldehyde in PBS) and fixed by incubation in a tenfold excess of formalin overnight at room temperature. The formalin was removed and the intestines washed twice in PBS at room temperature. The intestines were then transferred to a tissue cassette and dehydrated by serial immersion in 20-fold volumes of 70%, 96% and 100% EtOH for 2 h each at 4 °C. Excess ethanol was removed by incubation in xylene for 1.5 h at room temperature and the cassettes then immersed in liquid paraffin (56 °C) overnight. Paraffin blocks were prepared using standard methods and 4 μM tissue sections generated. These sections were de-waxed by immersion in xylene (2 × 5 min) and hydrated by serial immersion in 100% EtOH (2 × 1 min), 96% EtOH (2 × 1 min), 70% EtOH (2 × 1 min) and distilled water (2 × 1 min). Endogenous peroxidase activity was blocked by immersing the slides in peroxidase blocking buffer (0.040 M citric acid, 0.121 M disodium hydrogen phosphate, 0.030 M sodium azide, 1.5% hydrogen peroxide) for 15 min at room temperature. Antigen retrieval was performed (see details below for each antibody), and blocking buffer (1% BSA in PBS) added to the slides for 30 min at room temperature. Primary antibodies were then added and incubated as detailed below. The slides were then rinsed in PBS and secondary antibody added (polymer HRP-labelled anti-mouse/rabbit, Envision) for 30 min at room temperature. Slides were again washed in PBS and bound peroxidase detected by adding DAB substrate for 10 min at room temperature. Slides were then washed 2× in PBS and nuclei counterstained with Mayer's haematoxylin for 2 min, followed by two rinses in distilled water. Sections were dehydrated by serial immersion for 1 min each in 50% EtOH and 60% EtOH, followed by 2 min each in 70% EtOH, 96% EtOH, 100% EtOH and xylene. Slides were mounted in Pertex mounting medium and a coverslip placed over the tissue section.

For c-Myc (Santa Cruz, SC-764), antigen retrieval involved 20 min boiling in Tris-EDTA pH 9.0; staining involved 1/500 dilution in blocking buffer (0.05% BSA in PBS) or 4 days at 4 °C. For β-catenin (BD Transduction Labs, 610154), antigen retrieval involved 20 min boiling in Tris-EDTA pH 9.0; staining involved 1/100 dilution in blocking buffer (0.05% BSA in PBS) for 2 h at room temperature. For Ki67 (Monosan, MONX 10283 clone mm1), antigen retrieval involved 20 min boiling in 0.01 M citrate buffer pH 6.0; staining involved 1/250 dilution in blocking buffer (0.05% BSA in PBS) for 1 h at room temperature. For EGFP (In-House), antigen retrieval involved 20 min boiling in 0.01 M citrate buffer pH 6.0; staining involved 1/8,000 dilution in blocking buffer (0.05% BSA in PBS) for 4 days at 4 °C. For CD44 (In-House), antigen retrieval involved 20 min boiling in 0.01 M citrate buffer pH 6.0; staining involved 1/200 dilution in blocking buffer (0.05% BSA in PBS) for 1 day at 4 °C.

Isolation of adenoma cells. Freshly isolated small intestines were incised along their length and adenomas were removed using forceps. The adenoma tissue was then incubated in PBS plus 5 mM EDTA for 10 min. Vigorous shaking removed remaining villi and the adenomas were subsequently incubated in PBS supplemented with trypsin (10 mg ml^{-1}) and DNase (0.8 units per μl) for 30 min at 37 °C. After incubation, cells were spun down, re-suspended in SMEM (Invitrogen) and filtered through a 40 μM mesh. The cells were analysed using a MoFlo cell sorter (DAKO).