Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling

Vivian W. Y. Wong1,2,3,12, Daniel E. Stange4,12, Mahalia E. Page1,2,3,12, Simon Buczaki3, Agnieszka Wabik2, Satoshi Itami9, Marc van de Wetering4, Richard Poulson7,13, Nicholas A. Wright7,8, Matthew W. B. Trotter1,9,10, Fiona M. Watt2,5,11, Doug J. Winton5, Hans Clevers4 and Kim B. Jensen1,2,3,14

Maintenance of adult tissues is carried out by stem cells and is sustained throughout life in a highly ordered manner1,2. Homeostasis within the stem-cell compartment is governed by positive- and negative-feedback regulation of instructive extrinsic and intrinsic signals3,4. ErbB signalling is a prerequisite for maintenance of the intestinal epithelium following injury and tumour formation5,6. As ErbB-family ligands and receptors are highly expressed within the stem-cell niche7, we hypothesize that strong endogenous regulators must control the pathway in the stem-cell compartment. Here we show that Lrig1, a negative-feedback regulator of the ErbB receptor family8–10, is highly expressed by intestinal stem cells and controls the size of the intestinal stem-cell niche by regulating the amplitude of growth-factor signalling. Intestinal stem-cell maintenance has so far been attributed to a combination of Wnt and Notch activation and Bmpr inhibition11–13. Our findings reveal ErbB activation as a strong inductive signal for stem-cell proliferation. This has implications for our understanding of ErbB signalling in tissue development and maintenance and the progression of malignant disease.

The intestine constitutes an excellent system for studying stem-cell function. Intestinal stem cells (ISCs) reside at the bottom of crypts, where they are maintained in a multipotent and self-renewing state14. The ISC niche in the small intestine is composed of stem cells above and interspersed between Paneth cells and surrounded by mesenchymal cells7,15. This provides a unique microenvironment that enables a constant contribution from stem cells to sustain the high cell turnover of the differentiated compartment16–17. Lgr5 (leucine-rich repeat containing G protein coupled receptor 5)-expressing crypt-based columnar cells at the bottom of crypts are together with cells located immediately above this region responsible for the life-long steady-state maintenance of the epithelium14,15,18,19. How ISCs are maintained and which inductive signals are required for tissue maintenance is well established; however, very little is known with regard to the regulation of these pathways in vivo.

We recently demonstrated that Lrig1 (leucine-rich-repeats and immunoglobulin-like domains 1) controls stem-cell proliferation in the epidermis20. In situ hybridization now reveals that Lrig1 is highly expressed in the stem-cell niche of the small intestine and colon (Fig. 1a and Supplementary Fig. S1). Lgr5-expressing stem cells can be identified by their high levels of green fluorescent protein (GFP) in the Lgr5- eGFP - IRES - CreERT2 knock-in mouse21. GFP is subsequently diluted during successive cell divisions of stem cells (GFPhigh) and their early daughter cells (GFImid and GFPlow). Expression analysis of these different populations demonstrates that Lrig1 levels are highest within the ISCs (GFPhigh versus GFImid, 1.6-fold; GFPlow versus GFPlow, 2.2-fold). Immunofluorescence staining and flow cytometric analysis confirm the overlap of Lrig1 and Lgr5 expression at the protein level and that Lrig1 is expressed in a gradient with highest levels in ISCs and is absent from Paneth cells (Fig. 1b,c and Supplementary Fig. S2a–c). Overall, approximately one-third of all cells within the intestinal crypt express Lrig1 (Fig. 1d). This comprises the entire Cdc24low/mid stem-cell and progenitor compartment and includes all of the Lgr55+ ISCs (ref. 21; Fig. 1e–g). The percentage of Lgr5–GFP+ cells within the Lrig1-expressing population unfortunately cannot be determined owing to the mosaic nature of the Lgr5- eGFP - IRES - CreERT2

1 The Anne McLaren Laboratory for Regenerative Medicine, University of Cambridge, Cambridge CB2 OSZ, UK. 2 Wellcome Trust Centre for Stem Cell Research, University of Cambridge, Cambridge CB2 1QR, UK. 3 Department of Oncology, University of Cambridge, Cambridge CB2 0QQ, UK. 4 Hubrecht Institute, KNAW and University Medical Center Utrecht, Uppsalalaan 8, 3584CT Utrecht, The Netherlands. 5 Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Cambridge CB2 0RE, UK. 6 Department of Regenerative Dermatology, Graduate School of Medicine, Osaka University, 2-2, Yamadaoka, Suita-shi, Osaka 565-0871, Japan. 7 Cancer Research UK—London Research Institute, London WC2A 3LY, UK. 8 Barts and the London School of Medicine and Dentistry, London E1 2AD, UK. 9 Department of Surgery, University of Cambridge, Cambridge CB2 0QQ, UK. 10 Celgene Institute Translational Research Europe, Seville E-41092, Spain. 11 Department of Genetics, University of Cambridge, Cambridge CB2 3EH, UK. 12 These authors contributed equally to this work. 13 Present address: Centre for Digestive Disease, Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, UK. 14 Correspondence should be addressed to K.B.J. (e-mail: kbj22@cam.ac.uk)

Received 3 November 2011; accepted 13 February 2012; published online 4 March 2012; DOI: 10.1038/ncb2464
knock-in mouse, where a large fraction of Lgr5-expressing ISCs are GFP negative (http://jaxmice.jax.org/strain/008875.html; Supplementary Fig. S1). Characterization of Lrig1+ cells isolated by flow cytometry demonstrates that markers that define ISCs, such as Lgr5, Ascl2 and Msi1, are all enriched in this population (Fig. 1h; refs 19,22). We also observe differential expression of multiple transcripts for Lrig1 interaction partners, in particular epidermal growth factor receptor (Egfr) (6.2-fold, Fig. 1h). Expression of Lrig1 is associated with reduced proliferation in other tissues20. Therefore, we test whether Lrig1-expressing cells are mainly quiescent or proliferative. Pulse-chase studies show that 15–20% of the Lrig1-expressing cells incorporate 5-bromodeoxyuridine (BrdU) within 1 h following one injection and 60% are labelled after a three-day pulse with seven injections (Fig. 1i).

The BrdU label is subsequently lost or significantly reduced following a one-week chase (Supplementary Fig. S2d–e). We conclude that Lrig1 is highly expressed by all ISCs, that the majority of Lrig1-expressing cells are proliferating and that the expression of Egfr is enriched within the Lrig1+ population.

Work with Lrig1-knockout mice has until now been carried out on an outbred genetic mouse background20,23. To eliminate the contribution of genetic variability in the interpretation of the effects of Lrig1, we backcrossed Lrig1-knockout mice to an FVB/N background for more than seven generations. On this background, no differences are observed between heterozygous (Het) and wild-type littermates. Yet, Lrig1-knockout animals are smaller than their littermates from postnatal day 8 onwards and have to be killed around postnatal day 10.
owing to the severity of the phenotype. Although the sizes of most organs are in relative proportion to the reduced body weight, the relative size of the intestine is grossly enlarged in Lrig1-knockout mice (Fig. 2a–d; circumference, 7.3 ± 0.5 mm for wild type/Het (n = 5) and 11.0 ± 1.0 mm for knockout (n = 4); P = 0.01).

Histological examination of the intestine reveals that loss of Lrig1 causes a marked increase in crypt size along the entire length of the small intestine (Fig. 2e,f and Supplementary Fig. S3a). This is associated with an increased number of proliferating cells as measured by expression of phospho-histone H3 (Fig. 2g,h; pHistone H3 per crypt, 3.9 ± 0.9 fold knockout/control, P = 0.05). Phenotypically, crypts from knockout mice and littermate controls are indistinguishable until postnatal day 6 (Supplementary Fig. S3b). This coincides with stem-cell specification in the neonatal intestinal epithelium24. Crypt hyperplasia can be observed from this time onwards in Lrig1-knockout animals. Gene expression profiling reflects the phenotypic change associated with loss of Lrig1 as gene set enrichment analysis shows overrepresentation of crypt signature genes within the upregulated genes (P = 1.5 × 10^-177; ref. 25).

The enlarged crypt morphology can be explained by a specific increase in the transit-amplifying compartment, by a disproportionate increase in individual crypt-cell lineages or by increased numbers of ISCs with a proportionate increase in transit-amplifying and Paneth cells. The major intestinal cell lineages can be identified by their differential expression of Cd24 and binding of Ulex europaeus agglutinin 1 (UEA-1) by secretory cells (Supplementary Fig. S4). Loss of Lrig1 causes a profound increase in the proportion of the Cd24low/mid/UEA-1- stem-cell and progenitor compartment (D) and Cd24high/UEA-1+ Paneth cells (B), whereas the population comprising mature enterocytes is proportionally decreased (C) (Fig. 3a,b). Goblet (A) and enteroendocrine (E) cell populations show no significant change. In addition to their increased numbers, we observe increased expression of ISC markers within the Cd24low/mid/UEA-1- population, supporting an expansion of the stem-cell compartment following loss of Lrig1 (Fig. 3c). To confirm this finding, we carry out in situ hybridization for Olfm4 (olfactomedin 4), a marker of ISCs (ref. 22), and Cryptdin6 (also known as Defa6), a marker of Paneth cells66. Starting from postnatal day 6 we observe an increased number of Olfm4-positive cells in Lrig1-knockout samples and from postnatal day 10 an increased proportion of Cryptdin6-positive Paneth cells (Fig. 3d). The dynamic expansion of the stem-cell compartment is supported by flow cytometric analysis of dissociated cells from the intestinal epithelium (Supplementary Fig. S5a–e). The expression domain of the independent stem-cell marker Msi1 is also expanded following loss of Lrig1 (Supplementary Fig. S5f,g). Taken together, this supports a direct effect of Lrig1 on ISCs, which subsequently affects the size of the ISC compartment and the crypt.

Lrig1 interacts with the ErbB family, CRET and cMet in vitro and reduces signalling strength by negatively regulating both protein levels and the activity of the growth factor receptors8–10,27,28. Epithelial cells in the intestine express detectable levels of Egrf, ErbB2, ErbB3 and cMet (Figs 1h and 4a). ErbB signalling is mediated through ligand stimulation of Egrf and ErbB3, which can be stimulated by heterodimerization with each other or with the orphan receptor ErbB2 activates downstream signalling cascades29. cMet on the other hand is activated following hepatocyte growth factor stimulation. Loss of Lrig1 causes a pronounced increase in the protein levels of all of
Figure 3 Loss of Lrig1 causes crypt and stem-cell expansion. (a) Dissection of epithelial lineages by flow cytometry on the basis of the relative levels of Cd24 and binding of UEA-1. The five identifiable populations are enriched in A, goblet cells, (Cd24\(^\text{+}\)/UEA-1\(^-\)); B, Paneth cells, (Cd24\(^\text{+}\)/UEA-1\(^-\)); C, transit-amplifying cells/enterocytes, (Cd24\(^\text{+}\)/UEA-1\(^-\)); D, stem cells and progenitors, (Cd24\(^\text{low}\)/mid\(^\text{+}\)/UEA-1\(^-\)); E, enteroendocrine cells, (Cd24\(^\text{high}\)/UEA-1\(^-\)). (b) Loss of Lrig1 leads to a disproportionate increase in Cd24\(^\text{low}\)/mid\(^\text{+}\)/UEA-1\(^-\) stem cells and progenitors. Error bars, s.d. from four control and three Lrig1-knockout samples (Cd24\(^\text{+}\)/UEA-1\(^-\) transit-amplifying cells/enterocytes, \(P = 4.1 \times 10^{-3}\); Cd24\(^\text{low}\)/mid\(^\text{+}\)/UEA-1\(^-\) stem cells and progenitors, \(P = 5.3 \times 10^{-4}\); Cd24\(^\text{+}\)/UEA-1\(^-\) Paneth cells, \(P = 4.7 \times 10^{-4}\)). (c) Enrichment of stem-cell markers in the Cd24\(^\text{low}\)/mid\(^\text{+}\)/UEA-1\(^-\) stem cells and progenitors following loss of Lrig1. Error bars, s.e.m. from three Lrig1-knockout and four control samples (Lgr5, \(P = 2 \times 10^{-4}\); Olfm4, \(P = 0.02\); Msi1, \(P = 0.001\)). (d) Loss of Lrig1 causes a progressive expansion of the stem-cell niche from postnatal day 6 as detected by in situ hybridization for Olfm4 and Cryptdin6. Scale bars, 50 \(\mu\)m.
Figure 4 Lrig1 controls endogenous signalling through the ErbB pathway. (a) Loss of Lrig1 causes increased protein levels and activation of the ErbB pathway. pEgfr, Egfr, pErbB2, ErbB2, pErbB3, ErbB3 and cMet were detected by Western blotting in samples enriched for intestinal epithelium. β-actin is used a loading control. (b) Relative-expression analysis of the receptors by qPCR at P10 shows minor differences. Expression levels are shown relative to control samples (knockout/control). Asterisks indicate significant changes (ErbB2, P = 0.004; ErbB3, P = 0.04; knockout n = 4, control n = 3). (c-f) Increased activation of MAPK signalling and cMyc signalling following loss of Lrig1. Immunohistochemical analysis for p-MAP-kinase kinase (MEK)1/2 (c,d) and cMyc (e,f). (g-i) Altered Egfr activation dynamics in Lrig1-knockout samples. (i) Average normalized membrane intensity of pEgfr in intestinal samples from knockout (n = 6) and control animals (n = 10) for 6–18 individual crypts per sample. Error bars, s.e.m. (positions 1–4: P < 0.05). (j) Increased Myc activity in progenitors lacking Lrig1. Relative expression of Myc and Myc target genes in Cd24low/mid /UA1–1– transit-amplifying cells/enterocytes from control (n = 4) and Lrig1-knockout (n = 3) tissues. Error bars, s.e.m. (Myc, P = 0.001; Nsun2, P = 0.001; Ncl, P = 0.002). (k) Lrig1-knockout organoids mature in the absence of exogenous ErbB ligands. Maximum-intensity projection of confocal microscopy images of wild-type and knockout organoids shows incorporation of exogenous ErbB ligands. Maximum-intensity projection of confocal microscopy images of wild-type and knockout organoids shows incorporation of exogenous ErbB ligands. Red and green colours reflect increased and decreased deviation from the mean, respectively. The dendrogram indicates that control samples (Ctrl) grown for 6 days with (w) or without (wo) EGF. Error bars, s.e.m. (Myc, P = 0.009). (m) Loss of Lrig1 does not affect ErbB ligand expression. The relative levels of ErbB ligands were determined by qPCR on material from organoids grown for 6 days with (w) or without (wo) EGF. Red and green colours reflect increased and decreased deviation from the mean, respectively. The dendrogram indicates that control samples (Ctrl) grown for 6 days in normal conditions cluster with Lrig1-knockout samples (KO) grown with and without EGF. Scale bars, 50 μm (c–h) and 100 μm (k). Uncropped images of blots are shown in Supplementary Fig. S7.
Figure 5 Lrig1 controls ErbB activation in vivo. (a–d) Pharmacological inhibition of ErbB activation restores proliferation in the intestinal epithelium and the crypt size to normal in Lrig1-knockout animals. Crypt size and proliferation is visualized by the expression of Ki67. (e–l) Treatment with Gefitinib rescues the observed effect on the stem-cell niche in Lrig1-knockout animals. Paneth cells and stem cells are detected by in situ hybridization for Cryptdin6 and Olfm4, respectively. (m–p) pEgfr levels are reduced on treatment with the ErbB inhibitor Gefitinib. Detection of activated Egfr (pEgfr) in tissues from Lrig1 wild-type and knockout animals at P10 either untreated (m,n) or treated (o,p). (q-t) A loss-of-function Egfr mutant rescues the Lrig1-knockout phenotype. Morphologically, proliferation has been restored to normal levels in the rescued animals as detected by expression of Ki67 by immunohistochemistry. All Lrig1-knockout mice on an Egfr<sup>wt</sup>/wt background have the expected phenotype (q,r, 15 out of 15); however, a large proportion of Lrig1-knockout animals heterozygous for the hypomorphic Egfr allele (Egfr<sup>wt</sup>/wa<sup>-2</sup>) have normal intestinal morphology (t,u, 15 out of 37; P = 0.0095), although some still show hyperplasia (s). (u) Model of the role of Lrig1 in stem-cell homeostasis as a regulator of ErbB signalling. Scale bars, 50 μm (a–t).

10 phosphorylation can be observed on UEA-1<sup>+</sup> secretory cells and progenitor cells (Supplementary Fig. S6). In control animals, pEgfr levels are low at the crypt bottom and peak around cell position 8 (Fig. 4g,i). Of note, this is the reverse expression pattern of Lrig1. In Lrig1-knockout animals, Egfr activation is uniform within the crypt (Fig. 4h,i). Second, expression analysis of isolated cell populations signifies the increased activation of Myc within ISCs and progenitors following loss of Lrig1 (Fig. 4j). We conclude that loss of Lrig1 affects ErbB signalling within the ISC compartment.

Next, we functionally test in a defined three-dimensional intestinal culture system whether ErbB inhibition is the main function of Lrig1. ISC self-renewal and proliferation require stimulation of the Egfr
and the Wnt (wingless-related MMTV integration site) pathways, and inhibition of the Bmpr (bone morphogenetic protein receptor) pathway. In wild-type samples, stabilization of ErbB signalling with exogenous ligands is a prerequisite for maturation from spheres into budding organoids. Interestingly, Lrig1-knockout spheres mature into budding organoids in the absence of exogenous ErbB ligands (Fig. 4k,l). Nevertheless, ErbB signalling, similarly to stimulation of the Wnt pathway and inhibition of Bmpr signalling, is an obligate requirement for growth of both wild-type and knockout organoids, as inhibition of ErbB signalling with a number of ErbB inhibitors rapidly affects cell survival (data not shown). Importantly, loss of Lrig1 does not cause increased expression of ErbB ligands (Fig. 4m). We conclude that, following loss of Lrig1, endogenous ErbB ligands are sufficient to support organoid growth owing to reduced ErbB inhibition in ISC compartments. Moreover, as the phenotype observed in vitro can be recapitulated in vivo in a purely epithelial culture, the function of Lrig1 is likely to be crypt autonomous.

To address in vivo whether activation of the ErbB family is responsible for the observed phenotype, neonatal mice were treated daily with the ErbB inhibitor Gefitinib. Following administration of inhibitor in vivo, crypt proliferation, as well as ISC and Paneth cell numbers, is restored to normal levels in the Lrig1-knockout animals (Fig. 5a–l). Moreover, treatment with Gefitinib reduces pEgfr levels (Fig. 5m–p). Gefitinib-treated knockout animals are still significantly smaller than control animals, and we hypothesize that Lrig1 has important non-reversible functions in other tissues such as the stomach, where Lrig1 is expressed by a small sub-population of cells within the pyloric glands (M.E.P. and K.B.J., unpublished observations). The proposed mechanism of Lrig1 as an inhibitor of ErbB function in vivo is further substantiated by genetic rescue of the phenotype by crossing Lrig1-knockout animals to hypomorphic Egfr-wa/wa-knockout mice (Fig. 5q–t). The Egfr-wa/wa mouse strain harbours a missense mutation in the kinase domain of Egfr, which compromises the activity of the receptor on ligand stimulation. Homozygosity for Egfr-wa/wa, when crossed with Lrig1-knockout mice, is associated with perinatal lethality irrespective of Lrig1 status. Analysis of the Egfr-wa/wa heterozygous animals at postnatal day 10 demonstrates that the phenotypical changes associated with loss of Lrig1 are rescued in approximately 40% of the mice (n = 15 out of 37, P = 0.0095). Thus, the phenotypical changes observed on loss of Lrig1 can be rescued by both pharmacological and genetic modulation of endogenous ErbB activity, hereby demonstrating ErbB activity as the main target of Lrig1 in vivo.

Our data support a model whereby Lrig1 regulates proliferation within the ISC niche by inhibiting ErbB signalling. Expression of Lrig1 enables stem cells and progenitors to fine-tune their cellular response on ligand stimulation and ensures that tissue homeostasis is maintained (Fig. 5u). Proliferation and maintenance of the ISC compartment have until now been attributed to Wnt and Notch activation in combination with Bmpr inhibition. We show that ErbB signalling is a strong mitotic signal for ISCs, and when uninhibited leads to a rapid expansion of the stem-cell compartment. The prominent role of ErbB signalling in the ISC compartment is supported by recent evidence from Drosophila, where signalling through Egfr, the only ErbB receptor in Drosophila, is required for intestinal stem-cell activation. Moreover, the natural response to injury and tumour initiation in the mouse intestine, which includes stem-cell activation, is severely compromised following loss of Egfr and ErbB3 (refs 5,6). Analogous to our observations, eliminating one functional copy of Egfr significantly alters the injury response to sublethal doses of radiation. In the case of injury models, it remains to be shown whether Egfr and ErbB3 function is required for expansion of the stem-cell compartment or as a general mitotic stimulus.

Owing to the reported antiproliferative effect of Lrig1 in human and murine epidermis, we have assessed the proliferation status of Lrig1-expressing cells. As predicted from the overlap in Lrig1 and Lgr5 expression, we observe that the majority of Lrig1-expressing cells, similarly to Lgr5+ cells, are highly proliferative. We cannot rule out that mitotic heterogeneity exists within the Lrig1+ population; however, it is evident that Lrig1 does not define a population of quiescent cells in the intestine. The prominent effect on the stem-cell compartment on loss of Lrig1 does however highlight that ISCs are exposed to multiple signals that promote proliferation and that distinct inhibitory factors such as Lrig1 are required to control stem-cell behaviour.

We unambiguously demonstrate that Lrig1 is a key regulator of tissue homeostasis in the intestinal epithelium. As multiple tissue-specific stem cells rely on Lrig1 to regulate their behaviour, we propose that Lrig1 provides a general mechanism to control tissue homeostasis. On the basis of the study described here, and the non-overlapping expression pattern of Lrig1 and Lgr5 in stem cells of the epidermis, we anticipate that distinct cellular microenvironments, which can either converge as in the intestine or diverge as in the epidermis, specify stem-cell behaviour in vivo.

**METHODS**

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

**ACKNOWLEDGEMENTS**

We thank E. Wagner, M. Frey, S. Yaspa, B. Otto, R. Walker, R. Jeffery, H. Begthel, M. McLeish, the WTCSLR Facility and Cambridge Genomics Services for reagents, technical assistance and advice, and R. Fordham and R. Williams for critical comments on the manuscript. We acknowledge support from the UK Medical Research Council and Wellcome Trust.

**AUTHOR CONTRIBUTIONS**

V.W.Y.W., D.E.S., M.E.P., S.B., A.W., M.v.d.W., M.W.B.T. and K.B.J carried out experiments. S.I. and F.M.W. provided reagents. V.W.Y.W., D.E.S., M.E.P., F.M.W., M.W.B.T., D.J.W. and H.C. commented on the manuscript. We acknowledge support from the UK Medical Research Council and Wellcome Trust.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.
6. Roberts, R. B. et al. Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. *Proc. Natl Acad. Sci. USA* 99, 1521–1526 (2002).

7. Sato, T. et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 469, 415–418 (2011).

8. Gur, G. et al. Lrig1 restricts growth factor signaling by enhancing receptor ubiquitination and degradation. *EMBO J.* 23, 3270–3281 (2004).

9. Jensen, K. B. & Watt, F. M. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. *Proc. Natl Acad. Sci. USA* 103, 11958–11963 (2006).

10. Laederich, M. B. et al. The leucine-rich repeat protein Lrig1 is a negative regulator of ErbB family receptor tyrosine kinases. *J. Biol. Chem.* 279, 47050–47056 (2004).

11. Haramis, A. P. et al. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 303, 1684–1686 (2004).

12. Korinek, V. et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat. Genet.* 19, 379–383 (1998).

13. Van Es, J. H. et al. Notch1-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* 435, 959–963 (2005).

14. Barker, N. et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 449, 1003–1007 (2007).

15. Tian, H. et al. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 478, 255–259 (2011).

16. Lopez-Garcia, C., Klein, A. M., Simons, B. D. & Winton, D. J. Intestinal stem cell replacement follows a pattern of neutral drift. *Science* 330, 822–825 (2010).

17. Snippert, H. J. et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143, 134–144 (2010).

18. Takeda, N. et al. Interconversion between intestinal stem cell populations in distinct niches. *Science* 334, 1420–1422 (2011).

19. Sanger, E. & Capechi, M. R. Bmi1 is expressed in vivo in intestinal stem cells. *Nature* 459, 959–963 (2006).

20. Sato, T. et al. Single Lgr5 stem cells build crypt–villus structures *in vitro* without a mesenchymal niche. *Science* 345, 262–265 (2009).

21. Jaks, V. et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nature* 459, 959–963 (2009).

22. Van der Flier, L. G. et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 136, 903–912 (2009).

23. Suzuki, Y. et al. Targeted disruption of LIG-1 gene results in psoriasiform epidermal hyperplasia. *FEBS Lett.* 521, 67–71 (2002).

24. Schmidt, G. H., Winton, D. J. & Ponder, B. A. Development of the pattern of cell renewal in the crypt–villus unit of chimaeric mouse small intestine. *Development* 103, 785–790 (1988).

25. Chong, J. L. et al. E2F1–3 switch from activators in progenitor cells to repressors in differentiating cells. *Nature* 462, 930–934 (2009).

26. Van Es, J. H. et al. Wnt signalling induces maturation of Paneth cells in intestinal crypts. *Nat. Cell Biol.* 7, 381–386 (2005).

27. Shattuck, D. L. et al. Lrig1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. *Mol. Cell Biol.* 27, 1934–1946 (2007).

28. Ledda, F., Biaryangui, O., Fard, S. S., Vilar, M. & Paratcha, G. Lrig1 is an endogenous inhibitor of Ret receptor tyrosine kinase activation, downstream signaling, and biological responses to GDNF. *J. Neurosci.* 28, 39–49 (2008).

29. Citri, A. & Yarden, Y. EGF–ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol.* 7, 505–516 (2006).

30. Kim, J. et al. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell* 143, 313–324 (2010).

31. Sato, T. et al. Single Lgr5 stem cells build crypt–villus structures *in vitro* without a mesenchymal niche. *Nature* 459, 262–265 (2009).

32. Luetke, N. et al. The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. *Genes Dev.* 8, 399–413 (1994).

33. Biteau, B. & Jasper, H. EGF signaling regulates the proliferation of intestinal stem cells in *Drosophila*. *Science* 322, 822–825 (2010).

34. Iyer, R., Thames, H. D., Tealer, J. R., Mason, K. A. & Evans, S. C. Effect of reduced EGFR family receptor tyrosine kinases on tumor cell growth and metastasis. *Nat. Cell Biol.* 13, 915–920 (2008).

35. Sato, T. et al. Single Lgr5 stem cells build crypt–villus structures *in vitro* without a mesenchymal niche. *Nature* 459, 262–265 (2009).

36. Iyer, R., Thames, H. D., Tealer, J. R., Mason, K. A. & Evans, S. C. Effect of reduced EGFR family receptor tyrosine kinases on tumor cell growth and metastasis. *Nat. Cell Biol.* 13, 915–920 (2008).

37. Iyer, R., Thames, H. D., Tealer, J. R., Mason, K. A. & Evans, S. C. Effect of reduced EGFR family receptor tyrosine kinases on tumor cell growth and metastasis. *Nat. Cell Biol.* 13, 915–920 (2008).
METHODS

Tissue preparation. Intestinal samples were fixed and embedded in paraffin using standard protocols. Analysis of Lgr5-GFP/Lrig1 and UEA-1/pEgfr was carried out as recently described.

Antibodies. The following antibodies were used according to manufacturer’s instructions: rabbit anti-f-actin (Santa Cruz, sc-7199, 1:250), rabbit anti-phospho-histone H3(Ser 10) (Cell Signaling no 9701, 1:400), rabbit anti-phospho-ERK1/2(Ser 28/3, Tyr 187/8) (Cell Signaling, no 4285), mouse anti-Lrig1 (R and D Systems, AF3688, 1:100), rabbit anti-Myc (Santa Cruz, sc-757, 1:500), mouse anti-Akt (Cell Signaling, no 9272, 1:500), mouse anti-MAPK (Cell Signaling, no 9102, 1:500), rabbit anti-VE-cadherin (Chemicon, AB-265, 1:200), rabbit anti-phospho-Akt(Tyr 423/4) (Cell Signaling, no 4058, 1:100), rabbit anti-phospho-ErbB2(Tyr 1248) (Abcam, ab7755, 1:500), mouse anti-ErbB2 (Millipore, 05-1130, clone N3D10, 1:500), rabbit anti-phospho-ErbB3(Tyr 1289) (Cell Signaling, no 4791, clone 21D3, 1:250), mouse anti-ERβ (Millipore, 05-390, clone 2F12, 1:500), rabbit anti-Axin (Cell Signaling, 1:500), rabbit anti-β-catenin (Cell Signaling, cat 3824, 1:250), rabbit anti-phospho-α-catenin (Cell Signaling, 1:250), mouse anti-β-catenin (R&D Systems, AF3400, 1:100), rabbit anti-phospho-ErbB3(Tyr 1086) (Abcam, EP7774Y, 1:250), rabbit anti-Egr-2 (Cell Signaling, no 3267, clone D38B1, 1:250), rabbit anti-phospho-erbB2(Tyr 1248) (Abcam, ab7755, 1:500), mouse anti-erbB2 (Millipore, 05-1130, clone N3D10, 1:500), rabbit anti-phospho-ErbB3(Tyr 1289) (Cell Signaling, no 4791, clone 21D3, 1:250), mouse anti-ErbB3 (Millipore, 05-390, clone 2F12, 1:500), rabbit anti-c-Met (Santa Cruz, sc-162, 1:500), mouse anti-f-actin (Sigma, A5316, clone a-c-74, 1:5000) and Atto488-conjugated UEA-1 (Sigma, 10μl/10^6 cells) for flow cytometry, 1:1000 for near-native sections). For confocal microscopy, samples were imaged using an LSM700 confocal microscope or Leica SPS TCS. Z-stacks were acquired at optimal stack distance and at 1024×1024 dpi resolution. Maximum-intensity projections of Z-stacks were generated in ImageJ.

RNA extraction and qPCR. One centimetre pieces of the jejunum from postnatal day 10 Lrig1-knockout and control littermates were rinsed with ice-cold PBS and snap-frozen on dry ice. RNA was isolated from homogenized intestine or flow-sorted cells and used either directly for microarray experiments or for qPCR as described.

Gene-specific expression assays (Applied Biosystems) or SYBR Green analysis (Invitrogen) with optimized primer pairs were used for qPCR on an Applied Biosystems 7500HT RealTime PCR System (Applied Biosystems). Samples were normalized using the ΔΔCt method. For clustering analysis, expression average levels from at least three independent biological replicates were converted to z scores (z = (value – average value)/s.d.) and plotted using the heatmap.2 function from the Bioconductor ‘gplots’ library.

Microarray analysis. RNA was quality controlled for concentration, purity and integrity using spectrophotometry (BioSpec2000, Shimadzu) and Bioanalyser (Agilent). The amplification was carried out using the TotalPrep 96-RNA amplification kit (Ambion). Total RNA (~300 ng) was reverse transcribed into complementary DNA and amplified by in vitro transcription to generate biotin-labelled antisense RNA (cRNA). cRNA (1,500 ng) was hybridized to whole-genome bead arrays (MouseWG-6 v2.0 Expression BeadChip) according to the direct hybridization assay from Illumina and scanned using an Illumina BeadArray scanner. Bead-level data from all hybridizations were background corrected, using default parameters of the RMA algorithm, and summarized using the beadarray package for the Bioconductor suite (http://www.bioconductor.org) for the R statistical programming environment (http://www.r-project.org). Processed sample expression profiles were quantile normalized using the limma package for Bioconductor before analysis of differential regulation between sample groups with the moderated t statistic of the same package. To reduce errors associated with multiple hypothesis testing, the significance P values obtained were converted to corrected q values using the false discovery rate method, as implemented in the qvalue package for Bioconductor. Differential regulation between two sample groups was deemed significant at a threshold of q < 0.01 (false discovery rate 1%, Supplementary Table S1).

To generate a reference gene universe for gene-set enrichment analysis we defined the intestinal transcriptome as genes detected in three replicates from knockout and control samples (13,298 unique genes, 2,575 upregulated genes, 2,553 downregulated genes; Supplementary Table S2). For gene-set enrichment analysis using Fisher’s exact test we obtained a crypt gene signature (1,692 genes in reference list, 802 genes upregulated; Supplementary Table S3) and a Myc-dependent gene network (374 genes in reference list, 144 genes upregulated; Supplementary Table S4).

Western blot analysis and in situ hybridization. For protein isolation, intestinal epithelium was extracted as for organoid cultures and lysed in RIPA buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris at pH 8.0).

Digoxigenin in situ hybridization was carried out essentially as described before using IMAGE clones.

Flow cytometry. Intestinal epithelial cells were isolated as described. To generate a single-cell suspension, cells were incubated with trypsin or with 0.25 mg/ml thermolysin for 1 min at 37 °C in PBS supplemented with 1% BSA for trypsin-sensitive antigens such as Lrig1. Cell sorting was carried out using a FACSAria (BD Biosciences) for isolation of cells on the basis of C242 and UEA-1, and a MoFlo (Dako Cytomation) for isolation of Lrig1- and Lgr5-eGFP-expressing cells. Flow cytometric analysis was carried out on a CyAn ADP analyser (Dako Cytomation), and data were processed in FlowJo.

The Brdu-labelling analysis was carried out with an APC-Brdu flow cytometry kit (BD Biosciences).

Organoid cultures. Primary crypts were cultured according to ref. 31 using reduced concentrations of murine recombinant R-spondin1 (500 ng/ml) and varying concentrations of EGF (Peprotech). Organoid structures were imaged at day 6. Cell proliferation was measured by BrdU incorporation by incubation with 20μM BrdU (Roche) for 1 h at 37 °C before fixation.

Image analysis. Confocal microscopy images of intestinal samples stained for pEgfr were analysed in ImageJ to determine the levels of membrane-localized pEgfr. Intra-crypt intensities were normalized to the average intensity of positions 6–10. This corresponds to the peak in control samples.

Organoids in Matrigel were observed under phase contrast using an Axiovert 200M microscope (Zeiss) equipped with an AxioCam MRc (Zeiss). Images were interactively analysed using ImageJ to determine the perimeter and area of individual organoids and calculate the branching coefficient (1−4π(area/perimeter)^2) of the formed structures. Images were acquired from a minimum of eight organoids per condition derived from independent knockout and control samples.

Statistical analysis. Statistical significance of quantitative data was determined by applying a two-tailed Student t-test to raw values or to the average values obtained from analysis of independent organoid experiments. A two-tailed Fisher exact test was used to analyse the significance of the genetic rescue of the Lrig1 knockout phenotype, and to determine the significant overlap between different gene lists.

Accession numbers. Microarray data have been deposited at the EBI ArrayExpress under accession number E-MTAB-378.

37. Irizarry, R. A. et al. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31, e15 (2003).
38. Smyth, G. K. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, Article3 (2004).
39. Storey, J. D. & Tibshirani, R. Statistical significance for genomewide studies. Proc. Natl Acad. Sci. USA 100, 9440–9445 (2003).
40. Gregoire, A. et al. Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology 129, 626–638 (2005).
Figure S1 Lrig1 is expressed by stem cells in the colon. a) Lrig1 expression as detected by in situ hybridization in mouse colon. b) Lrig1 (red) expression in normal colon from an Lgr5-eGFP-ires-CreERT2 knock-in mouse. Nuclei are stained with ToPro (blue). Note the mosaic nature of the Lgr5-eGFP-ires-CreERT2 knock-in mouse, showing expression in 1 out of 5 crypts. c) Colonic crypt showing co-expression of Lrig1 (red) and Lgr5-GFP (green), nuclei are stained with ToPro (blue). c' and c'' show single colour images of c. 100µm (a), 50µm (b) and 25µm (c).
Paneth cells do not express Lrig1 and are mostly proliferating. a-c) Paneth cells and epithelial progenitors isolated based on CD45neg/CD31neg/EpCAMpos expression and defined by their level of CD24 and reactivity with the lectin, \textit{Ulex europaeus} agglutinin-1 (UEA-1). CD24mid/low/UEA-1neg progenitor cells and CD24pos/UEA-1pos Paneth cells were analysed for Lrig1 expression. Analysis of Lrig1 expression in Paneth and progenitor cells from WT animals (a), progenitor cells from Lrig1 KO and WT animals (b) and Paneth cells from Lrig1 KO and WT animals (c) unequivocally demonstrate that Lrig1 is not expressed by Paneth cells. d-e) Flow cytometric analysis of BrdU incorporation in Lrig1 expressing and non-expressing cells isolated from the small intestine. A large proportion of Lrig1 expressing and non-expressing cells incorporate BrdU into their genomic material following 7 doses applied every 12 hours. The graphs show that the majority of cells are initially labelled and that BrdU is subsequently lost over the course of one week.
**Figure S3** Loss of Lrig1 affects crypt size in the entire small intestine from postnatal day 8.  

a) Loss of Lrig1 has a pronounced effect on crypt morphology in the small intestine. Ki67 expression in the different regions of the intestine isolated from Lrig1 WT and KO littermates from postnatal day 10.

b) The hyperplastic phenotype is evident from postnatal day 8 and onwards. Ki67 stained sections from the proximal jejunum illustrates the onset of hyperplasia and the size of the crypts. Scale bar: 100µm (a) 50µm (b).
Figure S4 Dissection of intestinal cell lineages using flow cytometry.  
a) Recognition of goblet and Paneth cells by UEA-1 (red). Counterstained with dapi (blue).  
b) Dissection of intestinal cells into 6 distinct populations by flow cytometry based on expression of the UEA-1 reactive epitope and CD24 (A: Goblet cells (CD24neg/UEA-Ipos), B: Paneth cells (CD24pos/UEA-Ipos); F: Paneth cells (CD24pos/UEA-Ipos); C; transit-amplifying cells/enterocytes (CD24neg/UEA-Ineg); D: stem cells (CD24low/mid/UEA-Ineg); E: enteroendocrine cells (CD24high/UEA-Ineg).  
c-d) CD24mid/low/UEA-Ineg marks a stem cell enriched population.  
e) CD24 status differentiates goblet and Paneth cells in the UEA-I positive population. Scale bar: 50µm.
**Figure S5** ISC/Progenitor cell expansion precedes the effect on the Paneth cells compartment. 

**a)** The proportion of CD31neg/CD45neg/EPCAMpos/CD24pos/UEA-1pos Paneth cells and CD31neg/CD45neg/EPCAMpos/CD24mid/low/UEA-1neg ISCs/progenitors were determined for dissociated epithelial cells isolated from Lrig1 KO and ctrl animals (P4: n=6 Ctrl, n=2 Lrig1 KO; P6: n=3 Ctrl, n=6 Lrig1 KO; P8: n=9 Ctrl, n=4 Lrig1 KO; P10: n=8 Ctrl, n=8 Lrig1 KO). 

**b-c)** Expansion of progenitors is evident before the effect on the Paneth cell compartment at postnatal day 8. 

**d-e)** Stem cell expansion as detected by in situ hybridisation for Olfm4 precedes Paneth cell expansion. 

**f-g)** Increased expression domain of the independent intestinal stem cell marker, Msi1, in Lrig1 KO and control samples. Scale bar: 50µm.

Difference in progenitor cells p=7.0x10^{-4} (P8), 9.9x10^{-6} (P10); Difference in Paneth cells p=4x10^{-12} (P10).
Figure S6 Validation of the pEGFR antibody. a-b) Detection of activated Egfr (pEgfr) in tissues from Lrig1 WT and KO animals at P12. c) Intestinal sample from adult Egfr<sup>wa-2/wa-2</sup> hypomorphic Egfr mice and d-e) from Egfr KO and WT skin samples. a’-c’ show pEgfr the isolated green channel in inverted gray scale to signify the levels of the activated receptor and the gradient of activation observed in normal tissues. f-g) Egfr activation occurs on both UEA-1 positive and negative cells in the intestinal crypt. pEgfr (red) and UEA-1 (green) reactive cells in intestinal samples from postnatal day 10 WT and Lrig1 KO animals. Pictures represent maximum intensity projections of confocal z-stacks. Gray scale insert in f) and g) show activation levels of Egfr. 1-12: serial sections of z-stacks showing UEA-1 positive cells at high magnification. Arrows indicate the membrane between UEA-1 positive and negative cells. Scale bar: 100µm (a-e), 50µm (f-g).
**Figure S7** Full scans of the Western blots cropped for Figure 4a.