LETTERS

Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells

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The self-renewing epithelium of the small intestine is ordered into stem/progenitor crypt compartments and differentiated villus compartments. Recent evidence indicates that the Wnt cascade is the dominant force in controlling cell fate along the crypt–villus axis[1]. Here we show a rapid, massive conversion of proliferative crypt cells into post-mitotic goblet cells after conditional removal of the common Notch pathway transcription factor CSL/RBP-J (ref. 2). We obtained a similar phenotype by blocking the Notch cascade with a γ-secretase inhibitor. The inhibitor also induced goblet cell differentiation in adenomas in mice carrying a mutation of the *Apc* tumour suppressor gene. Thus, maintenance of undifferentiated, proliferative cells in crypts and adenomas requires the concerted activation of the Notch and Wnt cascades. Our data indicate that γ-secretase inhibitors, developed for Alzheimer's disease, might be of therapeutic benefit in colorectal neoplastic disease.

Notch genes encode large, single-transmembrane receptors that regulate a broad spectrum of cell fate decisions[2,3]. Interaction between Notch receptors and ligands results in proteolytic cleavages of the receptor within the plane of the cell membrane. The resulting free Notch intracellular domain translocates into the nucleus, where it binds to the transcription factor RBP-J (CSL or CBF1), thus activating target gene transcription[2,3]. The best-characterized Notch target genes are the hairy/enhancer of split (HES) and Achaete–Scute transcriptional repressors. These proteins in turn repress the expression of downstream genes[4,5].

A recent study describes increases in secretory cells at the cost of absorptive cells in the intestines of zebrafish that are mutant for *DeltaD* (a Notch ligand) and *mindbomb*[6]. Multiple Notch pathway components are expressed in murine crypts (M.E.v.G., unpublished data, and refs 7, 8). *Hes1* is a known Notch target gene in other tissues and is also expressed in crypts (Fig. 1a)[9,10]. Animals deficient in *Hes1* die perinatally from severe neurological abnormalities. Analysis of the developing fetal intestine of $Hes1^{-/-}$ mutant fetal mice revealed a relative increase in mucosecreting and enteroendocrine cells at the expense of absorptive enterocytes[10]. The crypt progenitor pool in the small intestine seemed unaffected, as judged by an analysis of proliferative activity. *Math1* is a target gene of Hes1-mediated repression in several organs, including the intestine[11,12]. $Math1^{-/-}$ mice die neonatally. Although the crypt–villus architecture was essentially undisturbed in the mutant mice, commitment towards the secretory lineage had entirely halted[12]. These results have been interpreted to indicate that *Hes1* and *Math1* are required to skew the fate of differentiating cells leaving the transit amplifying compartment towards an enterocyte or a secretory phenotype, respectively[10–12].

Indirect support for the control of intestinal cell fate by Notch stems from the use of γ-secretase inhibitors, as originally developed for Alzheimer's disease[13,14]. Notch is one of several known γ-secretase substrates. Proteolytic processing of Notch by γ-secretase is an essential step after activation of the pathway. As a consequence, γ-secretase inhibitors block Notch pathway activation[13,14]. Toxicological studies on rodents with these inhibitors have revealed increases in the size and number of mucosecreting goblet cells[15–17].

To directly assess the role of the Notch pathway in crypt homeostasis, we crossed mice carrying a floxed *Rbp-J* allele[18] with transgenic mice (P450-Cre) carrying the Cre enzyme under the control of the inducible *Cyp1A* promoter[19]. After intraperitoneal injection of β-naphthoflavone, this *cre* allele is activated in several internal organs, including the epithelium of the small intestine and colon. At 60 h after *cre* induction by β-naphthoflavone injection in 3-month-old mice, standard histological analysis did not reveal marked morphological changes in the crypts (Supplementary Fig. 1a, b). *In situ* hybridization revealed a decrease in *Hes1* expression (not shown). In accordance with observations made in

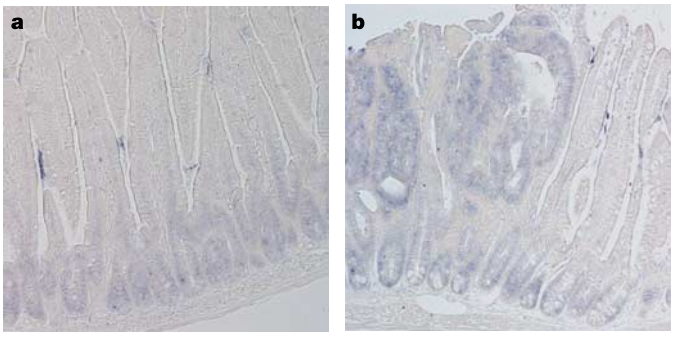

Figure 1 | Notch signalling pathway components are expressed in crypts of the small intestine. Expression of all Notch receptors and ligands in adult small intestine was analysed by *in situ* hybridization, essentially confirming refs 6 and 7 (M.E.v.G., unpublished data). **a**, **b**, Expression of the presumptive Notch target gene *Hes1* implies activity of the Notch cascade in the intestinal crypt (**a**) and in Apc^{Min} tumours (**b**). The sense control was negative (data not shown).

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Hes1 mutant mice, the *Math1* gene was derepressed. Nuclear Math1 protein, normally present only in secretory cells (Supplementary Fig. 1c), was abundant throughout the crypt compartment (Supplementary Fig. 1d; quantified in Supplementary Table 1). Moreover, there was a modest increase in goblet cell number as revealed by staining with periodic acid-Schiff (PAS) (compare Supplementary Fig. 1f with Supplementary Fig. 1e).

Histological analysis at day 5 after *cre* induction revealed a marked crypt phenotype. Phenotypic alterations occurred in more than 95% of all crypts of the small intestine and colon, underscoring the efficiency of the induction of the *cre* transgene by β-naphthoflavone. The following observations were made in the small intestine. Paneth cells still resided in near-normal numbers at the bottom of the crypts. However, the rapidly dividing transit amplifying compartment, which normally occupies the remainder of the crypt, was entirely replaced by post-mitotic goblet cells, as identified by morphology under haematoxylin/eosin staining (HE) (compare Fig. 2b with Fig. 2a) and PAS staining (compare Fig. 2d with Fig. 2c). This was confirmed by staining for the Math1 protein (compare Fig. 2f with Fig. 2e; quantified in Supplementary Table 1), whereas *Hes1* gene expression had become undetectable (Supplementary Fig. 2a, b). Staining for Ki67 (compare Fig. 2h with Fig. 2g) and analysis of bromodeoxyuridine (BrdU) incorporation (quantified in Supplementary Table 1) revealed that essentially all epithelial cell division had halted. No significant increases in apoptosis were observed in crypts, as revealed by staining for caspase-3 (compare Supplementary Fig. 2d with Supplementary Fig. 2c). Numbers of enteroendocrine cells were unchanged, as revealed by staining for synaptophysin

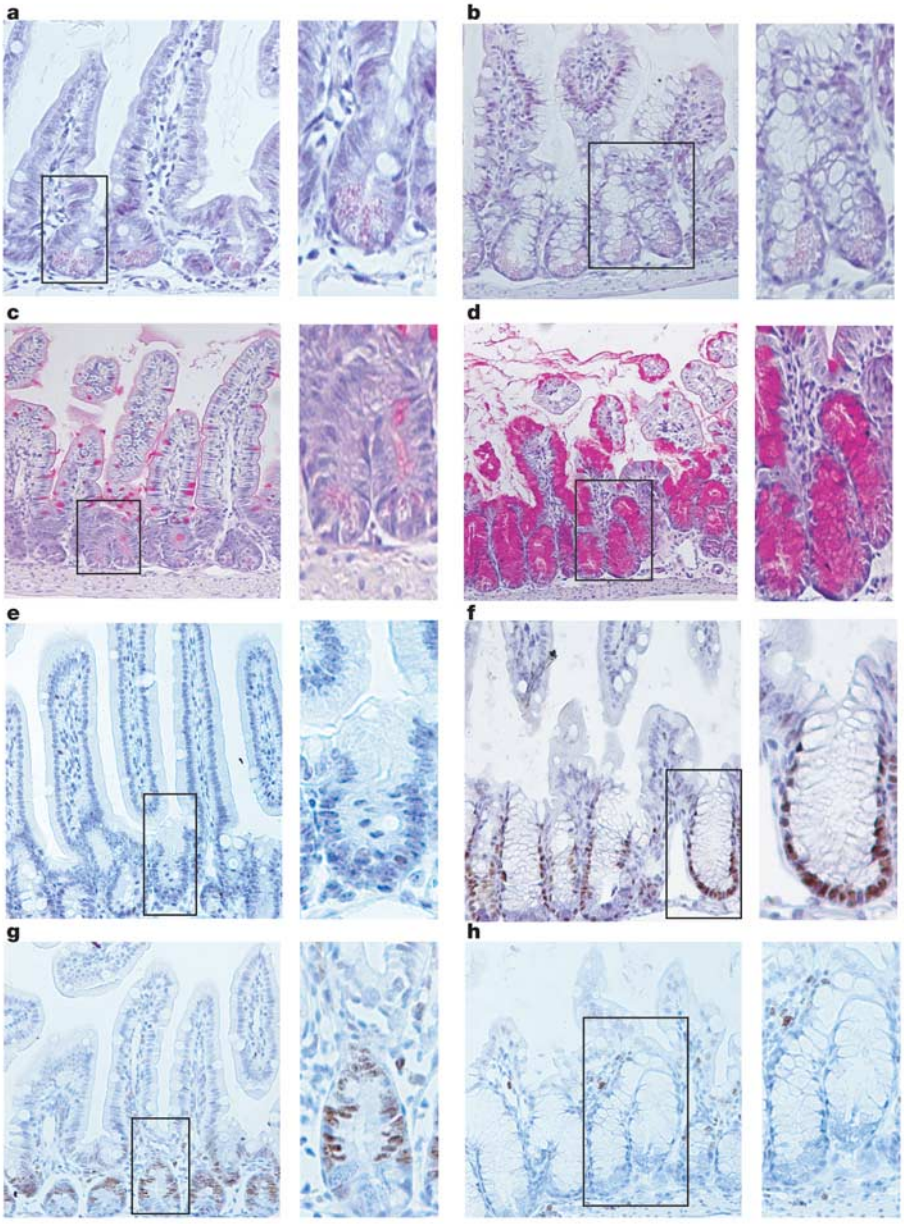

Figure 2 | Disruption of Notch signalling pathway induces goblet cell conversion of crypt proliferative cells. a–h, Immunohistochemical analysis of small intestine from RBP-J$^{floxed/floxed}$/P450-Cre mice (**b**, **d**, **f**, **h**) and control RBP-J$^{floxed/floxed}$ mice (**a**, **c**, **e**, **g**). Mice were injected at days 0 and 2.5 with β-naphthoflavone. The analysis shows a complete replacement of the transit amplifying compartment by goblet cells as shown by haematoxylin/eosin staining (HE) (**b** versus **a**) and PAS staining (**d** versus **c**). This notion was confirmed by staining for Math1 protein (**f** versus **e**). Staining for the proliferation marker Ki67 (**h** versus **g**) showed that essentially all epithelial cell division had halted. An enlargement of the boxed portion in the left-hand micrograph of each pair is shown in the right-hand micrograph.

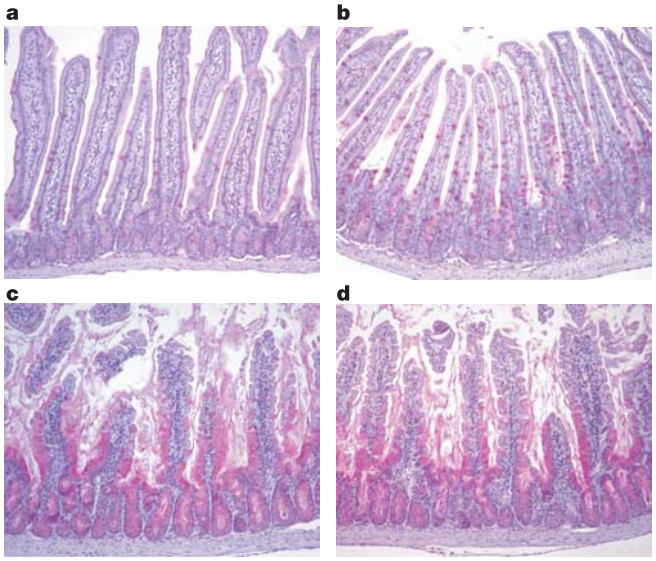

Figure 3 | Conversion of proliferative crypt cells into post-mitotic goblet cells by the γ-secretase inhibitor DBZ. C57BL/6 mice were injected intraperitoneally with 0 μmol kg^{-1} (**a**), 3 μmol kg^{-1} (**b**), 10 μmol kg^{-1} (**c**) and 30 μmol kg^{-1} (**d**) DBZ, daily for 5 days. At 3 μmol kg^{-1} DBZ, goblet cell numbers slightly increased as shown by PAS staining, whereas at 10 and 30 μmol kg^{-1} the conversion of proliferative crypt cells into post-mitotic goblet cells was complete.

(compare Supplementary Fig. 2f with Supplementary Fig. 2e). The Wnt signalling pathway remained active, as demonstrated by the presence of nuclear β-catenin (compare Supplementary Fig. 3h with Supplementary Fig. 3g). Essentially identical observations were made in the colon. Epithelial proliferation halted, whereas the numbers of goblet cells, already abundant in the colon, increased further (not shown).

We also inducibly inactivated the RBP-J gene by using a different Cre transgene, the tamoxifen-inducible vil-CRE-ERT2, which is expressed under the control of the villin promoter and is expressed exclusively in intestinal epithelium[20]. Twelve-day-old RBP-J$^{floxed/floxed}$/vil-Cre-ERT2 mice and RBP-J$^{floxed/floxed}$ littermate controls were injected intraperitoneally with tamoxifen on five consecutive days. The mice were analysed 6 and 12 days after the last injection. At both time points, histological analysis revealed goblet cell conversion. A nearly complete conversion of transit amplifying cells into post-mitotic goblet cells was observed 12 days after Cre induction (Supplementary Fig. 3a, b).

An explanation for the discrepancy between the observed phenotype in the RBP-J$^{floxed/floxed}$/P450-Cre mice and that of *Hes1* knockout mice might be the presence of other *Hes* genes. Indeed, we detected *Hes5* and *Hes6* expression in the crypts. Whereas *Hes6* expression seemed highest in the cells directly above the Paneth compartment, *Hes5* was expressed almost exclusively within the Paneth cell compartment (Supplementary Fig. 4a and b). *Hes6* expression was virtually abrogated after deletion of *Rbp-J*, whereas *Hes5* expression persisted in Paneth cells (Supplementary Fig. 4b and d, respectively).

As an alternative tool to block Notch signalling *in vivo*, we synthesized the γ-secretase inhibitor dibenzazepine (DBZ)[16] to more than 99.9% purity. DBZ blocked Notch cleavage in a cell-based assay with a half-maximal inhibitory concentration of less than

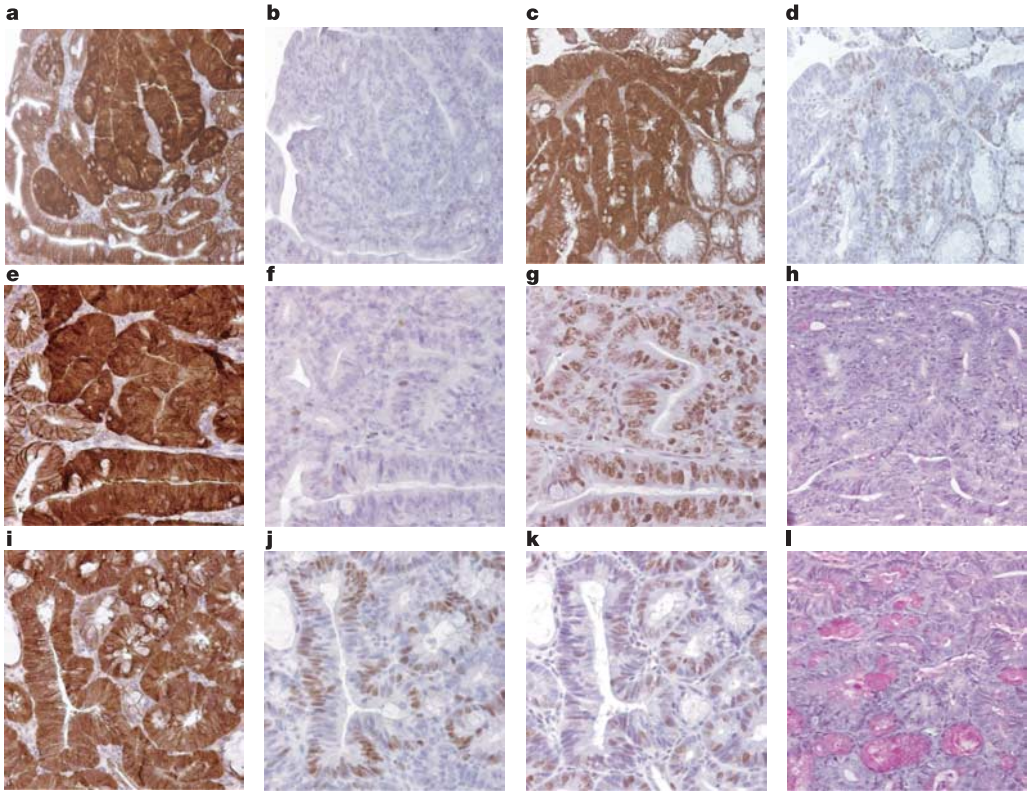

Figure 4 | Conversion of proliferative cells in the *Apc*^{Min} tumour into post-mitotic goblet cells by the γ-secretase inhibitor DBZ. **a**–**l**, *Apc*^{Min} mice were treated on alternate days without DBZ (**a**, **b**, **e**–**h**) or 10 μmol kg^{-1} DBZ (**c**, **d**, **i**–**l**) for 10 days, after which intestines were examined histologically by serial sectioning. Staining for β-catenin delineated the adenomas (**a**, **c**, **e**, **i**). DBZ treatment induced Math1 (**b** versus **d**; at a higher magnification, **f** versus **j**), decreased Ki67 expression (**k** versus **g**) and induced PAS expression (**h** versus **l**).

2 nM (not shown). After the pharmacokinetic studies of the original study[16], the compound was injected daily intraperitoneally at 0, 3, 10 and 30 μmol kg^{-1} into C57BL/6 mice for 5 days. At 10 and 30 μmol kg^{-1}, goblet cell conversion was complete within 5 days of starting intraperitoneal injections, as shown by PAS staining (Fig. 3c, d, respectively). Moreover, cell proliferation had entirely halted and histological markers (Ki67 and Math1) revealed that the tissue changes were indistinguishable from those observed after the deletion of *Rbp-J* (results not shown). At 3 μmol kg^{-1} DBZ, goblet cell numbers, as shown by PAS staining, increased slightly (Fig. 3b) in comparison with the intestine derived from the control mice (Fig. 3a).

We interpreted the combined genetic and pharmacological findings as indicating that Notch signalling is essential within the crypt compartment itself to maintain the undifferentiated state of the crypt progenitors. A complementary gain-of-function study yielded the reciprocal phenotype. Transgenic expression of the Notch intracellular domain in the intestinal epithelium results in a block of differentiation of secretory cells and an expansion of immature progenitor cells[21].

Intestinal adenomas result from mutational activation of the Wnt pathway, most commonly due to the loss of the intestinal tumour suppressor gene *Apc* (reviewed in ref. 22). We have recently reported a remarkable symmetry between colorectal cancer cells and proliferative crypt progenitors in terms of the expression of a Wnt target gene programme[23]. To investigate whether the symmetry between crypts and intestinal neoplasia extends to the Notch pathway, we studied the expression of various Notch pathway components and target genes in adenomas that spontaneously occur in multiple intestinal neoplasia (Min) mice, which carry a heterozygous mutation of the *Apc* gene (Apc^{Min})[24]. In general, expression of receptors and ligands in adenomas closely followed expression in crypts (M.E.v.G., unpublished data). In addition, *Hes1* expression, indicative of active Notch signalling, not only occurred in crypts but was also observed uniformly in adenomas of all sizes in the intestines of Apc^{Min} mice (Fig. 1b). This observation implied that, as in crypts, the Notch and Wnt pathways are simultaneously active in proliferative adenoma cells.

We then asked whether Notch pathway activity was essential for the maintenance of the proliferative phenotype of adenoma cells. We elected to inhibit the Notch pathway pharmacologically through the use of the γ-secretase inhibitor DBZ[16]. We initiated the treatment of 8-week-old Apc^{Min} mice, which at this age carry 30–60 macroscopically detectable adenomas (polyps) in the small intestine and 0–3 adenomas in the colon. Two mice each were treated with 0, 3, 10 or 30 μmol kg^{-1} DBZ for 10 days, after which intestines were examined histologically by serial sectioning. Staining for β-catenin delineated the adenomas, which were often embedded in an accumulation of hyperplastic yet untransformed normal crypts (Fig. 4a, c, e, i). DBZ at 10 or 30 μmol kg^{-1} readily induced Math1$^+$/PAS$^+$/Ki67$^-$ cells within adenomas (Fig. 4d, j–l versus 4b, f–h), whereas the effects at 3 μmol kg^{-1} were minimal, as were the effects on normal crypts (not shown). Different conversion rates were observed in individual adenomas, even within the same animal. To quantify this, 100 adenomas from mice treated with 10 μmol kg^{-1} DBZ were analysed by determining the percentage of Math1$^+$ nuclei. In 8% of the adenomas more than 50% of all epithelial cells were converted into Math1$^+$ cells. In 20% of the adenomas 10–50% conversion occurred; 28% showed 1–10% conversion, and 46% showed no conversion (examples are given in Fig. 4d, j). Goblet cell conversion was never observed in untreated Apc^{Min} mice: in each of 100 adenomas analysed, fewer than 1% Math1$^+$ goblet cells were observed. The treatment intensity and duration with this particular compound were limited by the concurrent changes in the normal epithelium. Nevertheless, the observations showed that adenoma cells can be forced to differentiate upon inhibition of the Notch pathway.

Inhibition of the Wnt/β-catenin/Tcf4 pathway induces a complete loss of crypt epithelial progenitors[25]. The combined observations indicate that multipotent crypt progenitors might be maintained only when both Notch and Wnt pathways are active. Specific inhibition of the Notch pathway drives the cells out of cycle towards a secretory fate, even while the Wnt cascade remains active. Conversely, inhibition of the Wnt cascade either by the deletion of *Tcf4* (ref. 25) or β-catenin[19] or by the transgenic expression of the soluble Wnt inhibitor Dickkopf-1 (ref. 26) drives the cells towards an enterocyte fate. Taken together, the Notch and Wnt signalling cascades synergize as gatekeepers of self-renewal in the intestinal epithelium. A wealth of evidence has indicated that the Wnt cascade might be the major driving force behind the proliferative potential of adenomas and adenocarcinomas of the intestine. The present data indicate that active Notch signalling might be equally important in maintaining the undifferentiated state of *Apc*-mutant neoplastic cells. Activating mutations occur relatively frequently in the *Notch1* gene in T-cell leukaemias[27]. It might be expected that such mutations occur also in colorectal cancer.

The Wnt cascade presents a rather unfavourable target for drug development, because the segment of the cascade downstream of the *Apc* tumour suppressor protein is driven entirely by protein–protein interactions[22]. The Notch pathway might provide an alternative targeted-drug strategy for the treatment of intestinal neoplastic diseases such as familial adenomatous polyposis or sporadic colorectal cancer. Multiple γ-secretase inhibitors of various chemical origins have been developed for the treatment of Alzheimer's disease[13,14,28]. Increases in intestinal goblet cell numbers in animal toxicity studies[15–17] have been noted as the principal unwanted side effect of these compounds. However, we suggest that the current experiments provide a proof of principle for the notion that these γ-secretase inhibitors could be developed into therapeutic modalities for colorectal neoplasia.

METHODS

Generation of RBP-J$^{floxed/floxed}$/P450-Cre and RBP-J$^{floxed/floxed}$/vil-Cre-ERT2 mice. The transgenic line Ahcre/P450-Cre (ref. 19) was crossed with floxed RBP-J mice[18] to generate RBP-J$^{floxed/floxed}$/P450-Cre mice. The Cre enzyme was induced at 3 months of age by a single intraperitoneal injection at day 0, or intraperitoneal injections at days 0 and 2.5, of 200 μl β-napthoflavone (10 mg ml^{-1}; Sigma Aldrich) dissolved in corn oil. The mice were killed on days 2.5 and 5 respectively. The transgenic line vil-Cre-ERT2 (ref. 20), in which Cre expression is specifically expressed in the intestine, was crossed with the floxed RBP-J mice[18] to generate homozygous floxed RBP-J/vil-Cre-ERT2 mice, as well as various genotypic controls. After tamoxifen injection the Cre enzyme becomes active. Tamoxifen was prepared as described[20]. Mice were injected intraperitoneally with tamoxifen (1 mg per 10 mg body weight).

Tissue sample preparation, immunohistochemistry and *in situ* hybridization. The intestinal tract was flushed gently with cold PBS followed by a flush with formalin. The small intestine was fixed in formalin at 21 °C for 16 h. The tissues were sectioned (2–6-μm slices). After dewaxing and hydration, sections were pretreated with peroxidase blocking buffer (120 mM Na_2HPO_4, 43 mM citric acid, 30 mM NaN_3, 0.2% H_2O_2 pH 5.8) for 15 min at room temperature. Antigen retrieval was performed by boiling samples for 20 min in 10 mM sodium citrate buffer pH 6.0. Antibodies used were mouse anti-Ki67 (1:100 dilution; Novocastra), mouse anti-β-catenin (1:50 dilution; Transduction Labs), mouse anti-bromodeoxyuridine (1:500 dilution; Becton Dickinson), rabbit anti-Math1 (1:50 dilution), rabbit anti-synaptophysin (1:200 dilution; Dako) and rabbit anti-caspase-3 (1:300 dilution; Cell Signalling). Incubation of antibodies was performed overnight in BSA in PBS at 4 °C for antibodies directed against caspase-3 and Math1, and for 1 h at room temperature for antibodies directed against Ki67, bromodeoxyuridine, synaptophysin and β-catenin. In all cases, the Envision$^+$ kit (Dako) was used as a secondary reagent. Stainings were developed with DAB. Slides were counterstained with haematoxylin and mounted. *In situ* hybridizations were performed as described[29]. The probes used for *in situ* hybridization were as described[7].

Treatment of animals with the γ-secretase inhibitor DBZ. DBZ[16] (3 g) was custom-synthesized to more than 99.9% purity by Syncom. DBZ was suspended finely in 0.5% (w/v) hydroxypropylmethylcellulose (Methocel E4M) and 0.1% (w/v) Tween 80 in water. Injections were performed intraperitoneally for the indicated periods with the indicated amounts.

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

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