that elevated Wnt signalling is sufficient for stem cell expansion in the adult intestine is lacking.

Disruption of canonical Wnt signalling is involved in the vast majority of colon cancers. Mutation in *APC* or *CTNNB1* is the initiating event in the transformation of colonic epithelial cells, which lead to the constitutive activation of Wnt signalling. Importantly, despite the presence of the activating mutations for Wnt signalling, colorectal cancers show cellular heterogeneity of β-catenin accumulation within a tumour mass. Immunohistochemical studies have revealed that nuclear β-catenin accumulation, the hallmark of activated Wnt signalling, is observed in a subset of colon tumour cells (Brabletz et al., 2001; Jung et al., 2001; Fodde and Brabletz, 2007). Furthermore, a recent study indicates that colon tumour cells with high Wnt signalling activity show the properties of cancer stem cells (Vermeulen et al., 2010), which emphasises the need for further studies on the dose-dependent effect of Wnt signalling on intestinal epithelial cells.

Although a large body of literature has established that activation of the canonical Wnt signalling is the dominant force in the maintenance of intestinal homeostasis, other signalling cascades, such as the Notch, BMP and PI3 cascades, have also been implicated in the control of epithelial cell proliferation and stem cell turnover (Scoville et al., 2008). However, it remains poorly understood how these other signalling cascades integrate with Wnt signalling in the intestinal epithelium to control stem cell turnover and epithelial regeneration. It is assumed that the various signalling cascades act in a hierarchical manner, and regulate each other. A better understanding of how the coordinated activity of these signalling cascades maintains intestinal homeostasis is crucial for dissecting the mechanisms of ISCs as well as for attempts to utilise stem cells in regenerative medicine and to target them in diseases such as cancer.

Using a novel β-catenin-inducible mouse model, we show here that elevated levels of activated β-catenin induces *de novo* crypt formation but reduces epithelial cell proliferation among progenitors. However, combined β-catenin overexpression and Notch inhibition turns these slow-cycling cells into proliferating cells. These results imply that β-catenin signalling fulfils dual roles in the control of intestinal epithelial regeneration by (1) promoting crypt formation and (2) activating cell proliferation in cooperation with Notch signalling.

MATERIALS AND METHODS

Mice

Transgenic mice expressing histone H2B-green fluorescent protein (H2B-GFP) fusion protein under the control of a TRE were obtained from Jackson Laboratories [Bar Harbor, ME, USA; strain name: Tg(tetO-HIST1H2BJ/GFP)47Efu] and crossed with mice harbouring a ROSA26 promoter-driven M2rtTA allele (Beard et al., 2006). β-Catenin embryonic stem (ES) cell line was generated with stabilised β-catenin (S33 mutation) cDNA (Morin et al., 1977; van Noort et al., 2002) with use of KH2 ES cell line and injected into blastocysts to produce transgenic mice. Mice of 4 to 8 weeks of age were fed 0.1 or 2.0 mg/ml doxycycline in the drinking water supplemented with 10 mg/ml sucrose. *Lgr5-GFP* knock-in mouse were obtained from Jackson Laboratories (strain name: B6.129P2-*Lgr5*$^{tm1(cre/ESR1)Cle}$/J).

Crypt isolation

Crypts were isolated form the whole colon and caecum by incubation in Hanks' balanced salt solution containing 30 mM EDTA as described previously (Tsukamoto et al., 2001).

Flow cytometry

Isolated crypts were incubated in 1% collagenase type 1 for 15 minutes at 37°C and then 0.25% trypsin/1m M EDTA for 5 minutes at 37°C. Single-cell suspensions were obtained by transfer through nylon mesh to remove large clumps, washing, and resuspension in staining medium containing 0.5 μl/ml propidium iodide (Calbiochem-Novabiochem Corp., San Diego, CA, USA) to eliminate dead cells. The cells were sorted by fluorescence-activated cell sorting (FACS) using a Vantage SE flow cytometer (Becton Dickinson, San Jose, CA, USA).

Microarray analysis

Total RNA was extracted from isolated crypts or FACS-sorted cells as previously reported (Yamashita et al., 2003). Oligonucleotide microarray hybridisation and scanning using GeneChip Mouse Genome 430 2.0 Array (Affimetrix) were performed as previously reported (Yamashita et al., 2003). For the pathway analysis, 907 probe sets, which are specifically upregulated in β-catenin induced cells, but not in H2B-low fast-cycling cells, were selected. The gene enrichment analysis was performed with DAVID PANTHER annotation tool. Microarray data have been deposited in Gene Expression Omnibus database under accession number GSE41688.

Quantitative real-time RT-PCR

qRT-PCR was performed as described previously (Oyama et al., 2008). The expression level of each gene was normalised to the β-actin expression level using the standard curve method. Each experiment was done in either duplicate or triplicate, and then, the average was calculated. Primer sequences for qPCR were taken from PrimerBank. The primer sequences are listed in supplementary material Table S2.

Histological and immunohistochemical analysis

Normal and tumour tissue samples were fixed in 10% buffered formalin, proceeded by standard method and embedded in paraffin. Sections were stained with Haematoxylin and Eosin (H&E), and serial sections were used for immunohistochemical analysis. Immunostaining was performed as described previously (Oyama et al., 2008) using the following antibodies: anti-β-catenin (1:1000 dilution; BD Transduction Laboratories, San Diego, CA, USA), anti-Musashi-1 [1:500 dilution (Kaneko et al., 2000)], anti-BrdU (1:250 dilution; Abcam, Cambridge, UK), anti-Hes1 [1:100 dilution; a gift from Dr Sudo (Ito et al., 2000)], anti-GFP (1:1500 dilution; Invitrogen, Carlsbad, CA, USA), anti-Ki67 (1:250 dilution; Dako Corp., Carpinteria, CA, USA) and anti-chromogranin A (1:1500 dilution; Abcam). Photomicrographs show the distal part of the colon or caecum in the figures.

Bromodeoxyuridine (BrdU) assay

Mice were injected with BrdU intraperitoneally (i.p.) at a dose of 100 mg/kg body weight. Mice were sacrificed 2 or 48 hours after injection, and incorporated BrdU was detected by immunostaining with anti-BrdU antibody as described above.

Notch inhibitor

γ-Secretase inhibitor (MRK003-ONC) was kindly provided by Merck and administrated orally at 100 mg/kg 2 days before sacrifice.

RESULTS

Canonical Wnt signalling is physiologically active in proliferative compartment of colonic crypts

Previous studies have shown by experimental manipulation of the Wnt signalling cascade that canonical Wnt signalling regulates intestinal epithelial proliferation (Korinek et al., 1998; Pinto et al., 2003; Kuhnert et al., 2004; Sansom et al., 2004; Andreu et al., 2005; Fevr et al., 2007). However, whether canonical Wnt signalling is active in the proliferative compartment of normal colonic crypts remains unclear. To address this question, we separated actively proliferating progenitor cells (transit-amplifying cells) from non-proliferating cells in the colon by using transgenic mice that express a histone H2B-GFP fusion protein under the control of a tetracycline-responsive regulatory element (TRE) (Tumbar et al., 2004). H2B-GFP becomes incorporated or diluted in a cell cycle-dependent manner and thus facilitates the separation of frequently dividing cells from infrequently dividing cells in any given tissue,

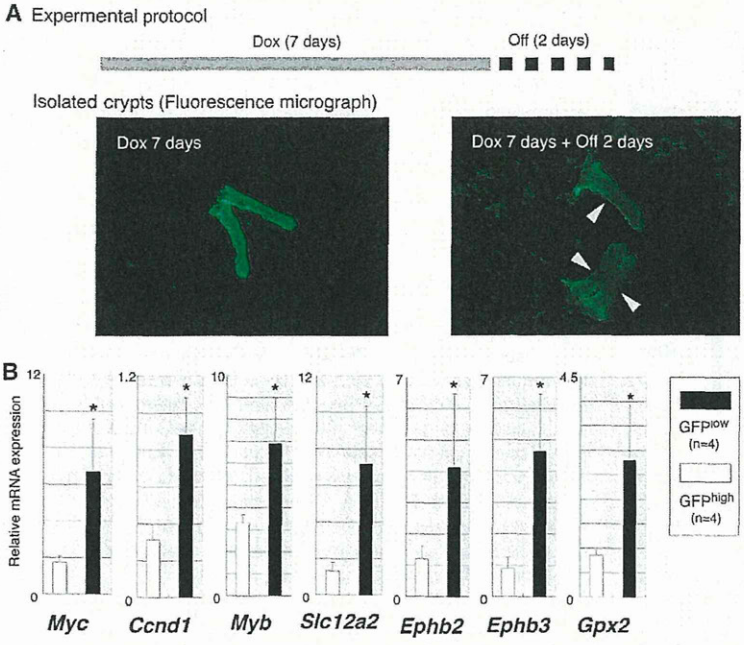

Fig. 1. Upregulation of canonical Wnt target genes in the proliferative compartments of colonic crypts. (**A**) Separation of proliferating cells from non-proliferative cells in the colon of histone H2B-GFP inducible mice. All crypt cells were labelled with nuclear GFP after Histone-GFP induction for 7 days, whereas the subsequent withdrawal of the induction resulted in dilution of the nuclear GFP signals in proliferating progenitor cells according to the cell divisions. Arrowheads indicate the decreased signal of the nuclear GFP at the proliferating compartments. (**B**) qRT-PCR for canonical Wnt target genes in GFP^{low} and GFP^{high} cells. After FACS sorting, the expression of canonical Wnt target genes was analysed by qRT-PCR. Expressions of *Myc*, *Ccnd1*, *Myb*, *Slc12a2*, *Ephb2*, *Ephb3* and *Gpx2* are significantly higher in GFP^{low} cells than in GFP^{high} cells. Data are mean ± s.d.; $^*P<0.05$, by Mann–Whitney *U*-test.

as has been successfully shown for the skin and haematopoietic system (Tumbar et al., 2004; Foudi et al., 2009). Specifically, H2B-GFP mice were crossed with mice harbouring a *Rosa26* promoter-driven M2 reverse tetracycline transactivator (M2rtTA) allele (Beard et al., 2006) to enable H2B expression in essentially all tissues. In the absence of doxycycline treatment, colonic epithelial cells exhibited no detectable GFP signals, thus excluding leaky expression of the transgene. By contrast, 7 days after doxycycline administration, all crypt cells exhibited a strong nuclear GFP signal (Fig. 1A). When doxycycline was withdrawn for 2 days after the initial labelling period, nuclear GFP signal was diluted in proliferating cells, consistent with rapid cell divisions of progenitor cells, whereas non-proliferating cells retained GFP (Fig. 1A). GFP^{high} non-proliferating and GFP^{low} proliferating epithelial cells were then sorted from the isolated crypts by FACS for subsequent molecular analyses (supplementary material Fig. S1A). To validate our approach to separate proliferating cells from non-proliferating cells using H2B-GFP dilution, we examined the expression levels of cell proliferation-related genes by microarray analysis. As expected, the expression of cyclins and Cdks, including *Ccna2*, *Ccnb1*, *Ccnd1*, *Ccnd2*, *Cdk2*, *Cdk4* and *Cdk6*, was higher in GFP^{low} cells than in GFP^{high} cells, whereas Cdk inhibitors, such as *Cdkn1a* and *Cdkn2b*, were found to be downregulated in GFP^{low} cells compared with GFP^{high} cells. Gene expression of candidates was validated by quantitative RT-PCR (supplementary material Fig. S1B). We also confirmed that GFP^{low} cells contained a higher number of Ki-67 (Mki67 – Mouse Genome Informatics)-positive cells than GFP^{high} cells by immunostaining colon sections of H2B-GFP mouse (supplementary material Fig. S1C). Importantly, we found that a number of canonical Wnt signalling target genes were upregulated in GFP^{low} proliferating cells compared with GFP^{high} non-proliferating cells using microarray analysis. qRT-PCR confirmed a significant upregulation of Wnt target genes (van de Wetering et al., 2002) (Fig. 1B), implying that canonical Wnt signalling is associated with active proliferation of progenitor cells in normal colonic crypts.

Forced induction of β-catenin leads to rapid *de novo* crypt formation in the colon

To investigate the effects of acute Wnt activation on adult intestinal homeostasis, we generated doxycycline-inducible β-catenin mice. This was achieved by targeting a constitutive active version of β-catenin (S33 mutation) under the control of a tetOP minimal promoter into the *Col1a1* locus in ES cells, which were subsequently injected into blastocysts to produce transgenic mice. Unless noted, homozygous transgenic mice were used in the experiment. When we fed adult mice doxycycline in the drinking water (2.0 mg/ml), β-catenin-induced animals became morbid after only 6-8 days. In the colon, 5 days of doxycycline treatment led to nuclear accumulation of β-catenin in the epithelium (Fig. 2A) and strong upregulation of canonical Wnt target genes such as *Myc* and *Ccnd1* (Fig. 2B). Notably, we frequently observed crypt fission and/or branching in β-catenin-induced colon sections, suggesting that the *de novo* crypt formation was induced by β-catenin induction (Fig. 2A). Immunohistological analyses of colon sections from doxycycline-induced chimeric mice demonstrated that the crypt fission/branching phenotype was only seen in β-catenin-induced crypts but not in host embryo-derived crypts, documenting a cell-autonomous effect of β-catenin induction (supplementary material Fig. S2A). We also observed an increase in crypt fission/branching in the crypts of the small intestine (supplementary material Fig. S2B). Analysis of isolated crypts confirmed that the fission and budding of crypts occurred at a significantly higher rate in β-catenin-induced colon than in non-induced colon (Fig. 2C,D). In addition, staining of sections for mucin with Alcian Blue-periodic acid-Schiff (AB-PAS) demonstrated a significant suppression of cellular differentiation towards goblet cells following β-catenin activation (supplementary material Fig. S3A). By contrast, chromogranin A-positive cells were found in both β-catenin-induced and non-induced crypts, showing a lesser effect on the enteroendocrine cell differentiation (supplementary material Fig. S3B). The numbers of chromogranin A-positive cells per crypt were 1.36±1.00 and 1.12±1.10 in β-catenin-induced and non-induced

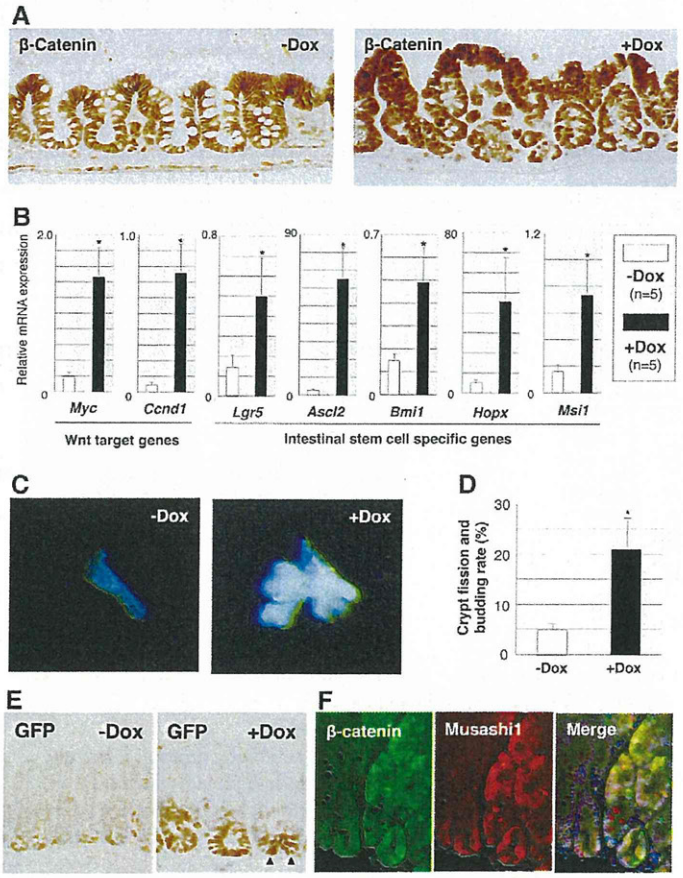

Fig. 2. β-Catenin induction leads to *de novo* crypt formation with increased expression of ISC markers in the colon. (**A**) β-Catenin immunostaining on colonic section of β-catenin-induced mice. Doxycycline treatment results in nuclear accumulation of β-catenin and frequent fission/budding of colonic crypts. (**B**) qRT-PCR for Wnt target genes and ISC-specific genes. The expression of Wnt target genes and ISC-specific genes are significantly upregulated by β-catenin induction. Data are mean ± s.d.; *P<0.05, by Mann–Whitney U-test. (**C**) Isolated colonic crypts from a doxycycline-treated mouse. A drastic crypt budding is observed in the crypt with β-catenin induction. (**D**) Fission/budding rate in isolated crypts from doxycycline-treated mice. Crypt fission/budding occurs at a significantly higher rate in doxycycline-treated mice than in non-treated mice. Data are mean ± s.d.; *P<0.05, by Mann–Whitney U-test. (**E**) Immunostaining for GFP on colonic sections of β-catenin-induced mice with *Lgr5-GFP* knock-in allele. GFP expression reveals an increased number of *Lgr5*-expressing cells at the lower part of colonic crypts in doxycycline-treated mice. Note that GFP-expressing cells are observed at the bottom of a bifurcating crypt (arrowheads). (**F**) Double immunostaining for Musashi1 (red) and β-catenin (green) on a colonic section of a doxycycline-treated chimeric mouse. Musashi1 expression is coincident with increased β-catenin expression.

colonic crypts, respectively, and no statistical significance was found between groups.

Barker et al. demonstrated that in the mouse gastrointestinal tract Lgr5 specifically labels active ISCs, which are located at the crypt base, cycle frequently and replenish the entire epithelium within a week (Barker et al., 2007). Consistent with the fact that *Lgr5* is a target of β-catenin/Tcf transcription (Barker et al., 2007), qRT-PCR demonstrated that β-catenin activation caused a significant increase in *Lgr5* expression (Fig. 2B). To determine whether the number of *Lgr5*-expressing cells has also increased in these mice, we crossed β-catenin-inducible mice with *Lgr5-GFP* knock-in mice, in which the *GFP* gene is regulated by the endogenous *Lgr5* promoter (Barker et al., 2007). Immunohistochemistry for GFP revealed that the number of *Lgr5*-expressing cells had indeed increased by 4.2-fold following β-catenin induction (Fig. 2E; supplementary material Fig. S4). Of note, although nuclear accumulation of β-catenin was observed throughout the crypt epithelium, expanded *Lgr5*-expressing cells were only observed at the lower part of the crypts (Fig. 2E; supplementary material Fig. S4A). This finding suggests that only existing ISCs, and possibly progenitor cells, respond to Wnt activation by producing more *Lgr5*-expressing cells whereas differentiated cells, located at the upper part of the crypts, are unresponsive to forced β-catenin expression. In addition to an increase in *Lgr5* expression, we also observed a strong upregulation of *Ascl2* (Fig. 2B), another active ISC-specific gene (van der Flier et al., 2009). As transgenic expression of *Ascl2* has been recently shown to induce ectopic crypt formation in the intestine (van der Flier et al., 2009), the increased levels of *Ascl2* might explain the observed crypt fission/budding phenotype in β-catenin-induced crypts. In addition to active ISCs, recent reports have indicated that quiescent ISCs are located at position 4 of the small intestine (Li and Clevers, 2010). Interestingly, β-catenin induction increased the expression of markers for the quiescent ISCs as well, including *Bmi1* and *Hopx* (Fig. 2B) (Sangiorgi and Capecchi, 2008; Takeda et al., 2011). Lastly, we examined the expression of Musashi1, a marker for putative stem and early progenitor cells (Potten et al., 2003), and found that β-catenin induction resulted in an upregulation of Musashi1 (Fig. 2B,F) in a cell-autonomous manner (Fig. 2F). Taken together, these data demonstrate that acute activation of β-catenin results in *de novo* crypt formation within a few days in a cell-autonomous fashion, accompanied by the amplification of ISC-like cells.

Colon cells with highest nuclear β-catenin do not actively divide

Previous studies have suggested that the canonical Wnt signalling plays a role in active cell proliferation of the intestine (Sansom et al., 2004; Andreu et al., 2005). In agreement, using the histone H2B-GFP mouse model, we show here that Wnt signalling is active in the proliferating progenitor compartment of normal colonic crypts under physiological conditions (Fig. 1B). To assess directly the effect of Wnt activation on the cell proliferation, we performed double-immunostaining with β-catenin and the proliferation marker Ki-67 on β-catenin-induced colonic sections. Unexpectedly, we found that the majority of cells with nuclear β-catenin failed to stain positively for Ki-67 (Fig. 3A). Instead, Ki-67 staining was

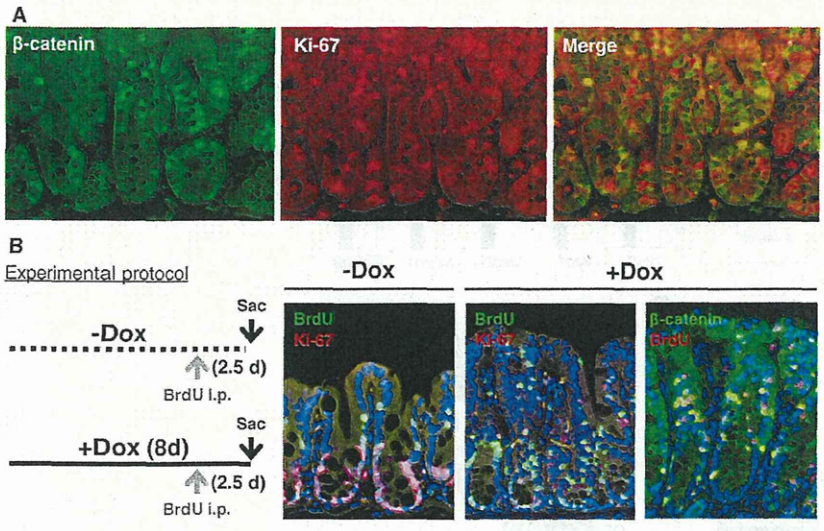

Fig. 3. Slow cycling properties of β-catenin-induced colonic cells. (**A**) Double immunostaining for β-catenin (green) and Ki-67 (red) on a β-catenin-induced colonic section. Majority of colonic cells with strong nuclear β-catenin expression are not coincident with Ki-67. (**B**) A scheme of the BrdU pulse-chase experiment and double immunostaining for Ki-67/BrdU and β-catenin/BrdU. In normal crypts, most proliferating progenitor cells have lost the BrdU retention according to the active cell divisions, and only a small number of cells retain BrdU. By contrast, β-catenin induction leads to an increased number of BrdU-retaining cells. Immunostaining for β-catenin (green) and BrdU (red) shows that BrdU-retaining cells frequently express nuclear β-catenin, indicating that colonic cells with strong nuclear β-catenin divide slowly. Sac, sacrifice.

predominantly observed in cells adjacent to cells with strong nuclear β-catenin signal (Fig. 3A). The majority of Ki-67-positive cells showed cytoplasmic and moderate β-catenin expression (76.7%) on the section, but some Ki-67-positive cells revealed nuclear and strong expression (23.3%). These observations were confirmed by a BrdU incorporation assay. When mice were injected with BrdU (100 mg/kg i.p.) 2 hours before sacrifice, the colonic cells with strong nuclear β-catenin showed less frequent BrdU incorporation (supplementary material Fig. S5A). We infer from this finding that intestinal cells with strong nuclear β-catenin expression did not actively divide. To investigate further the proliferation history of cells after β-catenin induction, we performed a pulse-chase experiment using BrdU (Fig. 3B). Mice were given a single BrdU injection (100 mg/kg i.p.) during the doxycycline treatment and were sacrificed 2 days later (Fig. 3B). β-Catenin induction caused an increased number of BrdU-retaining, i.e. non-dividing, cells near the crypt bottom, whereas non-induced crypts contained a small number of BrdU-retaining cells above the proliferative compartment (Fig. 3B). Furthermore, double-immunostaining for BrdU and β-catenin revealed that BrdU-retaining cells frequently expressed nuclear β-catenin (Fig. 3B). These results imply that, although forced β-catenin activation results in a net increase of cell proliferation in the colon, cells with strong nuclear β-catenin signal divide relatively slowly as measured by Ki-67 proliferation and BrdU label-retention assays. To support these findings, qRT-PCR revealed that the expression of the Cdk inhibitors *Cdkn1a*, *Cdkn1b* and *Cdkn1c* were significantly upregulated in β-catenin-induced colonic crypts (supplementary material Fig. S5B).

β-Catenin overexpression induces activation of Notch

In order to dissect further the molecular mechanisms underlying *de novo* crypt formation upon β-catenin induction, we compared the gene expression profiles of β-catenin-induced and non-induced colon crypts. Briefly, colonic crypts isolated from β-catenin-inducible control mice and from mice fed doxycycline for 5 days were subjected to microarray analysis. Consistent with our finding that β-catenin induction results in *de novo* crypt formation, microarray data confirmed the upregulation of ISC-specific genes, such as *Lgr5*, *Ascl2* and *Hopx*, as well as Wnt target genes in β-catenin-induced colon crypts (supplementary material Table S1).

Next, we wished to elucidate the apparent discrepancy between β-catenin-induced *de novo* crypt formation and the observed slow cycling properties of β-catenin-high cells. To this end, we compared gene expression profiles of β-catenin-induced cells and fast-cycling H2B-GFP low cells. Interestingly, pathway analysis revealed that genes in the Notch signalling pathway are specifically upregulated in β-catenin-induced colonic cells compared with fast-cycling normal crypt cells (Fig. 4A). qRT-PCR confirmed that *Hes1*, a well-established target gene of Notch signalling, is strongly induced by β-catenin activation with significant upregulation of Notch ligands (*Jag1* and *Jag2*) and Notch receptors (*Notch1* and *Notch2*) (Fig. 4B). Furthermore, we found that Notch ligands and Notch receptors were significantly upregulated as early as 12 hours after doxycycline treatment (Fig. 5B; see more details below). Consistent with this observation, immunohistochemical analysis revealed the strong nuclear expression of Hes1 on colonic sections of β-catenin-induced mice. (supplementary material Fig. S6). Our results suggest that β-catenin expression might activate Notch signalling through upregulation of its ligands and receptors.

Notch inhibition induces active cell proliferation in slow-cycling cells and blocks crypt fission and budding by β-catenin induction

In order to determine the relative contribution of activated Notch signalling to *de novo* crypt formation and the slow-cycling properties of colonic cells following β-catenin activation, we treated β-catenin-induced mice with a Notch/γ-secretase inhibitor (Fig. 4C). Surprisingly, treatment with a Notch inhibitor induced active proliferation of β-catenin-expressing, slow-cycling cells. Inhibitor-treated crypts were elongated with increased numbers of Ki-67 positive cells (Fig. 4D,E; supplementary material Fig. S7). Importantly, the simple withdrawal of doxycycline treatment (protocol G4) or the administration of Notch inhibitor alone (protocol G5) did not cause abnormal cell proliferation (Fig. 4E), indicating that constitutive Wnt activation is essential for active cell proliferation. To quantify the effect of Notch inhibition on cell proliferation in the presence of β-catenin activation, we performed a pulse-chase experiment with BrdU. Mice were given a single dose of BrdU (100 mg/kg i.p.) during the doxycycline treatment in the presence or absence of Notch inhibitor, and animals were sacrificed 2 days later. Immunohistochemical analysis showed that, in contrast

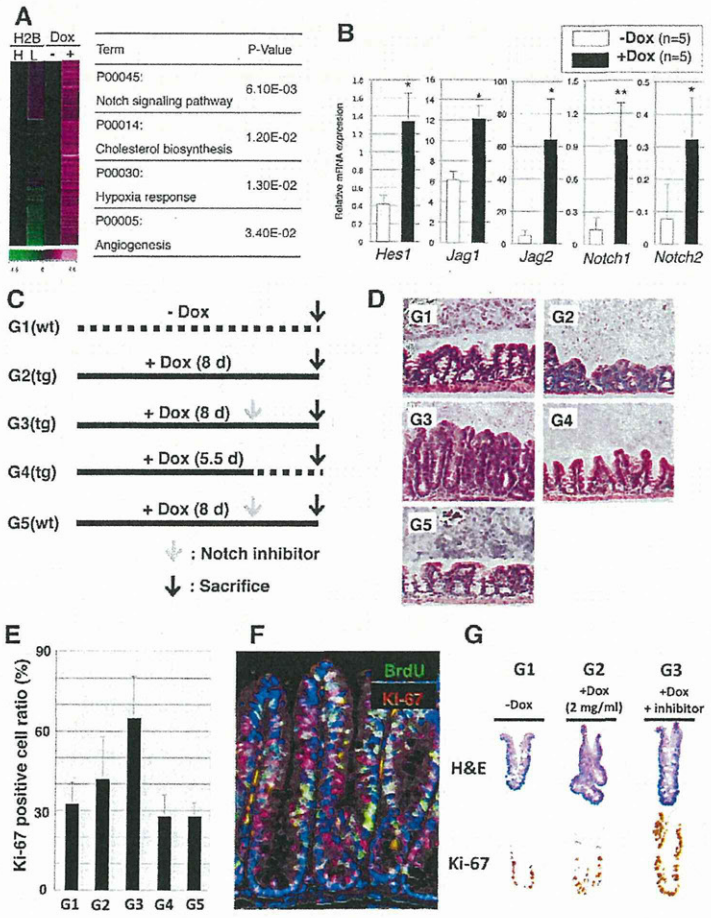

Fig. 4. Notch activation contributes to the maintenance of a slow-cycling state in β-catenin-induced colon. (**A**) Activation of Notch signalling pathway in β-catenin-induced slow-cycling colonic epithelium. Genes specifically upregulated in β-catenin-induced cells, but not in fast-cycling cells (GFP-Low cells in the H2B-GFP experiment) were selected. The heat map shows log2-fold changes in gene expression between β-catenin-induced and non-induced colon (right two columns in the left panel) and between histone-GFP-low and high cells (left two columns). The values for β-catenin non-induced colon and histone-GFP-high cells were used as normalisation for comparison, respectively. Subsequently, gene enrichment analysis were performed using DAVID on the selected genes and revealed that genes in a Notch signalling pathway are significantly concentrated in β-catenin-induced cells. All of the significantly enriched pathways in β-catenin-induced cells are listed in the table. H2B, histone H2B-GFP mouse; H, GFP^{high} cells; L, GFP^{low} cells; Dox, doxycycline treatment for β-catenin induction. (**B**) qRT-PCR analyses of Notch signalling related genes in β-catenin-induce colonic crypts. The Notch target *Hes1*, the Notch ligands *Jag1* and *Jag2*, and the Notch receptors *Notch1* and *Notch2* are strongly upregulated in β-catenin-activated crypts. Data are means ± s.d.; $^{*}P<0.05$, $^{**}P<0.01$, by Mann–Whitney *U*-test. (**C**,**D**) Experimental protocols for treatment with the Notch inhibitor (C) and the representative histology in each group (D). A Notch inhibitor was administrated orally at 2 days prior to sacrifice. (**E**) The Notch inhibitor induces active proliferation in β-catenin-induced colon. Ki-67-positive cell ratio (percentage of Ki-67-positive cells) is significantly higher in G3 than in other groups ($P<0.00001$ for G1, G4 and G5, and $P<0.0005$ for G2, by one-way ANOVA and Turkey's post hoc test, respectively). (**F**) BrdU pulse-chase experiment in mice treated with doxycycline and Notch inhibitor (protocol G3). Double immunostaining for BrdU (green) and Ki-67 (red) on a colon section. The Notch inhibitor reduces BrdU-retention in colonic crypts, whereas it increases Ki-67-positive cells throughout the crypt. (**G**) H&E staining and Ki-67 immunostaining of isolated crypts. The Notch inhibitor induces active cell proliferation and suppressed the *de novo* crypt formation in β-catenin induced crypts.

to the increased number of BrdU-retaining cells following β-catenin induction alone (Fig. 3B), combined treatment with doxycycline and the Notch inhibitor reduced the number of BrdU-retaining nuclei, whereas it increased the number of Ki-67-positive cells (Fig. 4F). These findings suggest that treatment with the Notch inhibitor induces proliferation of slow-cycling cells that have accumulated as a consequence of β-catenin expression. Importantly, treatment of β-catenin-induced mice with the Notch inhibitor also normalised crypt fission and budding rates (Fig. 4G; supplementary material Fig. S8A), which was accompanied by decreased nuclear β-catenin expression without a change in gene expression at the mRNA level (supplementary material Fig. S8B,C). These results indicate that Notch activation contributes to the maintenance of a slow-cycling state and to *de novo* crypt formation in β-catenin-induced colon, and, hence, Notch inhibition turns slow-cycling cells into fast-cycling cells in the context of transgenic β-catenin expression. However, in spite of the clear morphological changes, we could not detect a change in gene expression of the Notch target

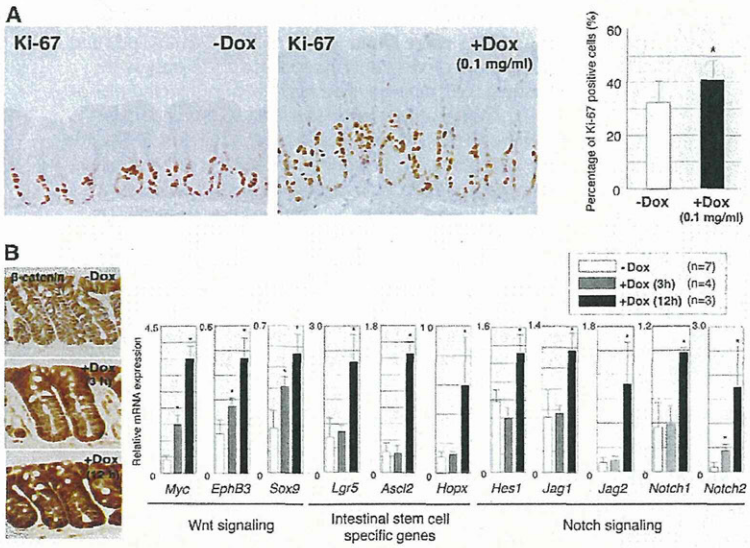

Fig. 5. Dose-dependent effect of Wnt activation on cell proliferation and gene expression in colonic epithelium. (**A**) Lower level of β-catenin induction promotes colonic epithelial proliferation. Ki-67 immunostaining and percentage of Ki-67-positive cells in colonic section from β-catenin-inducible mice treated with lower dose of doxycycline. Lower levels of β-catenin induction increase Ki-67 positive cell ratio and elongate the proliferating compartment of the crypts. Data are mean ± s.d.; *P<0.05, by Welch's t-test. (**B**) Expression of Wnt target genes, ISC-specific genes and Notch signalling-related genes in the colonic crypts with different levels of β-catenin. The different levels of β-catenin accumulation are shown in the left-hand panels. Data are mean ± s.d.; *P<0.05 compared with non-treated mice, by Kruskal–Wallis test followed by Steel test.

Hes1 in β-catenin-induced mice treated with the Notch inhibitor (data not shown). It is possible that the Notch inhibitor led to a transient inactivation of Notch signalling and thus the altered *Hes1* expression was not detectable at 2 days after treatment. However, given that the Notch/γ-secretase inhibitor has multiple substrates, we cannot completely rule out the possibility that the effect was partly independent of Notch inhibition.

Lower levels of β-catenin activation induce active proliferation of progenitor cells, but not stem cell expansion

In contrast to the well-established role of canonical Wnt signalling in activating cell proliferation in the intestine (Sansom et al., 2004; Andreu et al., 2005), our data show that the Wnt activation confers slow-cycling properties on colonic cells, which is accompanied by *de novo* crypt formation. In an attempt to consolidate these opposing results, we hypothesised that different levels of Wnt signalling may induce different biological outcomes with elevated levels of activation leading to the expansion of slow-cycling ISC-like cells and lower levels of activation inducing active cell proliferation. In order to determine the effects of different levels of β-catenin induction on colon homeostasis, we treated β-catenin-inducible mice with a lower dose of doxycycline than was used previously (0.1 mg/ml in drinking water) and analysed crypt sections. Colonic crypts did not show signs of increased crypt fission/branching rate in mice, suggesting that *de novo* crypt formation is not induced when β-catenin is expressed at low levels (Fig. 5A). However, low levels of β-catenin increased the number of Ki-67-positive cells, and led to an elongation of crypts (Fig. 5A), indicative of enhanced cell proliferation of progenitor cells. These results suggest that different strengths of canonical Wnt signalling result in different transcriptional outputs and, thus, biological effects.

To examine the effects of different levels of Wnt signalling on transcription, we performed gene expression analyses of colonic crypts with high and low levels of β-catenin accumulation. β-Catenin-inducible mice were intragastrically administered doxycycline (100 mg/kg) and sacrificed 3 and 12 hours later, leading to different levels of β-catenin accumulation in the colonic crypts (Fig. 5B). We found that *Myc*, *EphB3* and *Sox9*, well-known targets of canonical Wnt signalling, were upregulated in crypts with both higher and lower levels of β-catenin expression in a level-dependent manner (Fig. 5B). However, activation of the Notch target gene *Hes1* was detected only in crypts with high β-catenin, which is accompanied by the upregulation of ISC-specific genes including *Lgr5*, *Ascl2* and *Hopx* (Fig. 5B). We also examined the gene expression in colonic crypts isolated from β-catenin-inducible mice treated with a lower dose of doxycycline in drinking water (0.1 mg/ml) and found that the lower dose treatment significantly upregulated the expression of Wnt target genes such as *Myc*, but the same treatment did not induce *Lgr5* and *Hes1* in colonic crypts (supplementary material Fig. S9). Together, these results show that activation of the Notch signalling pathway and amplification of ISC-like cells require higher level of β-catenin accumulation. In addition, the expression of the Cdk inhibitors *Cdkn1a*, *Cdkn1b* and *Cdkn1c* were not altered by the lower level of β-catenin induction (supplementary material Fig. S9) in sharp contrast to the case of the higher level of β-catenin induction (supplementary material Fig. S5), suggesting that altered expression of Cdk inhibitors might be responsible for the different proliferative activities.

Colon tumors show heterogeneity in nuclear β-catenin expression and slow-cycling cells in the *Apc*$^{Min/+}$ mouse model

A large body of evidence indicates that accumulation of β-catenin is an initiating event in intestinal carcinogenesis (Harada et al., 1999; Yamada et al., 2002). The vast majority of colon cancers show accumulation of β-catenin and expression of elevated levels of β-catenin/Tcf target genes. However, strong nuclear accumulation of β-catenin is only observed in a subset of tumour cells, indicating heterogeneity of tumour cells within the tumour (Fodde and Brabletz, 2007). Similarly, we found that colon tumours in *Apc*$^{Min/+}$ mice, a well-established model for colon tumorigenesis, also show heterogeneous expression of nuclear β-catenin (Fig. 6A). To determine whether such heterogeneous expression of nuclear β-catenin affects downstream transcription of the canonical Wnt signalling, we examined colon tumours of *Apc*$^{Min/+}$ mice carrying a transgenic GFP reporter allele of β-catenin/Tcf transcription (Oyama et al., 2008). Double

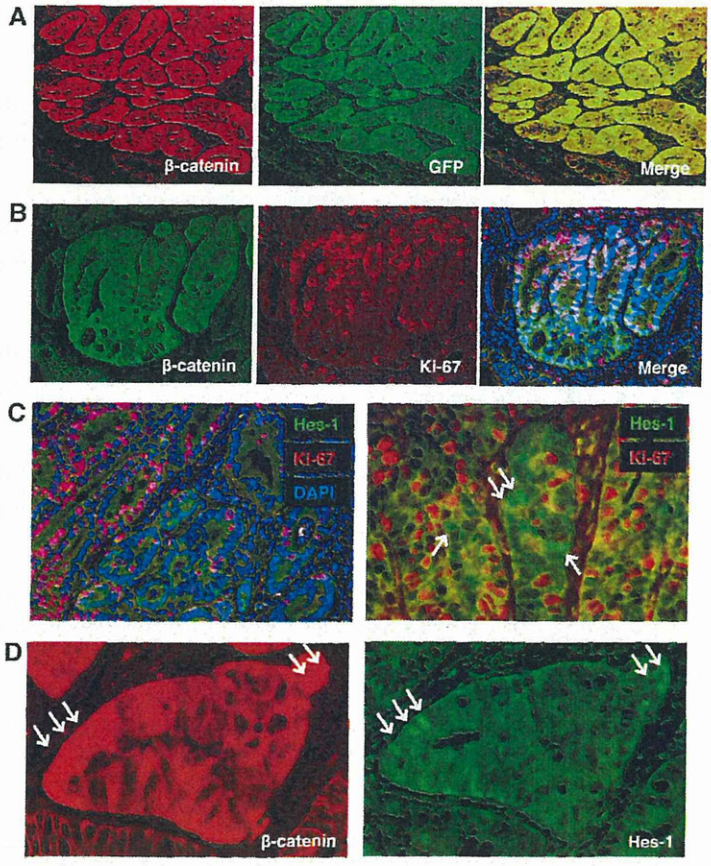

Fig. 6. Heterogeneity of colon tumour cells in $Apc^{Min/+}$ mouse. (**A**) Double immunostaining for β-catenin (red) and GFP (green) in the colon tumour of $Apc^{Min/+}$ mouse with transgenic GFP reporter allele for β-catenin/Tcf transcription activity. Note that heterogeneous expressions of both β-catenin and GFP are observed in a colon tumour. (**B**) Double immunostaining for β-catenin and Ki-67 in a colon tumour. Tumor cells with strong β-catenin expression show less frequent staining for Ki-67. (**C**) Double immunostaining for Hes1 (green) and Ki-67 (red). Distinct localisation of Hes1-expressing cells and Ki-67-positive cells are seen in colon tumour. Arrows indicate cells with positive nuclear staining for Hes1. (**D**) Immunostaining for β-catenin and Hes1 in serial sections. Colocalisation of higher levels of β-catenin and Hes1 expression is observed in the colon tumour.

immunofluorescence staining revealed that β-catenin levels were well correlated with GFP intensity, demonstrating that different levels of β-catenin accumulation directly affect β-catenin/Tcf transcription in colonic tumours (Fig. 6A). Importantly, most tumour cells with nuclear β-catenin did not express Ki-67 (Fig. 6B), recapitulating our observations in β-catenin-overexpressing mice. When the intensity and localisation of β-catenin expression were examined by immunofluorescence staining, the majority of Ki-67-positive tumour cells showed cytoplasmic β-catenin expression (93.6%) rather than strong nuclear expression (6.4%). In addition to the heterogeneous pattern of nuclear β-catenin accumulation, expression of Hes1 was detectable only in a small subset of colon tumour cells (Fig. 6C,D). Co-staining for Ki-67 revealed that tumour cells with high levels of Hes1 do not divide actively (Fig. 6C). Furthermore, we found that cells with a nuclear β-catenin signal often exhibited high Hes1 expression (Fig. 6D), as we have seen in β-catenin-induced crypts (supplementary material Fig. S4). These findings indicate that colon tumours, like our β-catenin inducible mouse model, consist of heterogeneous populations of cells displaying different activities of canonical Wnt signalling, Notch signalling and cell proliferation.

DISCUSSION

Previous studies using conditional *Apc* knockout mice demonstrated that acute loss of the *Apc* gene rapidly expands progenitor cells in the intestinal crypts (Sansom et al., 2004; Andreu et al., 2005) but does not lead to crypt fission/branching, suggesting that Wnt activation through loss of *Apc* is not sufficient to induce *de novo* crypt formation. In the present study, we showed that high levels of β-catenin activation are sufficient for *de novo* crypt formation of adult mice (Fig. 2). Our observation suggests that β-catenin activation amplifies ISCs, which is consistent with recent work carried out in *Drosophila* hindgut (Takashima et al., 2008). The discrepancy between previous reports and our study seems to arise from differences in the levels of Wnt activation. In fact, by titrating down the levels of activated β-catenin, we also failed to induce *de novo* crypt formation but instead expanded the proliferating progenitor compartment of the crypts (Fig. 5A). These combined findings strongly suggest that high levels of the canonical Wnt effector β-catenin are required for ISC expansion, whereas low levels of activation can induce the active proliferation of progenitor cells. This notion is consistent with a recent finding, which demonstrated that different levels of Wnt signalling exert distinct roles on the self-renewal and differentiation potentials of haematopoietic stem cells (Luis et al., 2011).

The notion that *de novo* crypt formation and cell proliferation are controlled by distinct levels of β-catenin activation is reminiscent of previous observations from our laboratory on the two-stage tumorigenesis of the $Apc^{Min/+}$ mouse (Yamada et al., 2002; Oyama et al., 2008). In the colon of $Apc^{Min/+}$ mice, we detected many microadenomas as early as 3 weeks of age, of which only a limited number progressed to large tumours. Although early

microadenomas already harboured frequent loss of *Apc* and increased β-catenin/Tcf transcription, larger tumours exhibited further elevations of β-catenin/Tcf transcriptional activity, thus suggesting that increased β-catenin/Tcf signalling is required for the development of larger tumours. The dose-dependent effect of Wnt activation on intestinal tumorigenesis has also been implicated in mouse models with different hypomorphic *Apc* mutant alleles, supporting the requirement for higher levels of Wnt activation for intestinal tumorigenesis (Gaspar and Fodde, 2004). A series of previous studies demonstrated that epigenetic modifications associated with DNA methylation are involved in the transition from microadenomas to large tumours in the $Apc^{Min/+}$ mouse (Yamada et al., 2005; Lin et al., 2006; Linhart et al., 2007). In human colorectal cancers, it has been shown that epigenetic silencing of SFRPs, negative modifiers of Wnt signalling, are frequently found, and such inactivation can further activate the canonical Wnt signals in colon cancer cell lines with *APC* or *CTNNB1* mutations (Suzuki et al., 2004). It is therefore possible that activation of the canonical Wnt signalling by both genetic and epigenetic alterations enables colonic stem cells to expand, leading to *de novo* crypt formation, which ultimately results in tumour growth.

A number of signalling cascades have been implicated in the maintenance of intestinal homeostasis (Scoville et al., 2008), but it remains unclear how the Wnt signalling pathway connects with other signalling cascades within the intestine to control homeostasis. Here, we showed that canonical Wnt signalling plays an important role in *de novo* crypt formation in the colon, and that a higher level of β-catenin activation is crucial for Notch activation. Our finding that Notch inhibition prevented crypt fission/branching in β-catenin-induced colon indicates the requirement for Notch activation in β-catenin-induced *de novo* crypt formation (Fig. 4G; supplementary material Fig. S8A). Interestingly, β-catenin activation rapidly induced transcriptional activation of the Notch ligands *Jag1* and *Jag2*, and the Notch receptors *Notch1* and *Notch2* (Fig. 4B, Fig. 5B), thus offering a possible direct link between these two pathways. Together with previous findings that β-catenin induces *Jag1* transcription, leading to Notch activation in human colon cancer cell lines (Rodilla et al., 2009), it is therefore likely that the increased expression of Notch ligands by β-catenin induction causes Notch activation in the colonic epithelium. Furthermore, a recent study clearly demonstrated that Notch1 and Notch2 receptors are expressed specifically in ISCs (Fre et al., 2011; Sato et al., 2011). The increased expressions of Notch receptors could play a role in the induction of ISC-like cells by β-catenin induction (Fig. 4B). It is also noteworthy that the constitutive activation of Notch results in no obvious effect on β-catenin nuclear localisation (Fre et al., 2005). These findings indicate a hierarchical relationship between the Wnt and Notch signalling pathways in the intestinal epithelium. This hierarchy might explain why genetic alterations in colon cancers are frequently detected in the Wnt signalling pathway, but not in the Notch signalling pathway.

The failure of most current therapies to cure cancer has led to the hypothesis that treatments targeted at malignant proliferation spare a slowly cycling cancer stem cell population. In this study, higher levels of Wnt activation induced *de novo* crypt formation and induced crypt cells to acquire slow-cycling properties. Interestingly, our observation of a β-catenin-induced slow-cycling property is consistent with previous reports in human colorectal cancers. Human colourectal cancers showed heterogeneous intracellular distribution of β-catenin, and tumour cells with nuclear accumulation revealed low cell proliferation rates (Brabletz et al., 2001; Fodde and Brabletz, 2007). Importantly, we also found that colon tumours in $Apc^{Min/+}$ mice consist of heterogeneous cells displaying different levels of β-catenin accumulation and downstream gene expression (Fig. 6A), and tumour cells with nuclear β-catenin are dividing more slowly than surrounding tumour cells, suggesting that such cells are similar to cells at the crypt bottom of the normal colon. Thus, we propose that a hierarchical control of cell proliferation in the colonic crypt epithelium is retained to some extent in colonic neoplasms. Accordingly, we found that tumour cells with nuclear β-catenin are accompanied by high Notch signalling (Fig. 6D), as has been reported in crypt bottom cells (Kayahara et al., 2003). It is interesting to note that a γ-secretase inhibitor turned slow-cycling cells into actively proliferating cells (Fig. 4C-G; supplementary material Fig. S7A). A previous study showed that Notch inhibitors turn undifferentiated, proliferating cells into quiescent cells in colorectal neoplasias (van Es et al., 2005), indicating that the Notch inhibitor might be of therapeutic benefit in colorectal cancers. The discrepancy in the effects of Notch inhibitor could be explained by differences in states of the affected cells between proliferating progenitor cells and ISC-like cells. Although the previous study showed effects on the transition of proliferating cells into terminally differentiated quiescent cells, our data suggest that a Notch inhibitor may promote the transition of slow-cycling ISC-like cells into progenitor cells in the colon. Considering the chemoresistance of slow-cycling cancer stem cells, the results also suggest that Notch inhibitors combined with chemotherapeutic agents and/or irradiation might be effective as treatments targeting slow-cycling cancer stem cells in the colon.

In summary, our results indicate that, although proliferating progenitor cells in colonic crypts physiologically express higher levels of β-catenin/Tcf transcriptions, a further activation of the canonical Wnt singalling leads to *de novo* crypt formation, consisting of relatively slow-cycling cells in the adult colon, which is accompanied by activation of Notch signalling with transactivation of Notch ligands and receptors. However, treatment with a Notch/γ-secretase inhibitor turns such slow-cycling cells into proliferating cells, although we cannot exclude the possibility that some of the observed phenotypes are the result of superphysiological β-catenin expression obtained with our transgenic system. These findings suggest that Wnt and Notch signalling act in a synergistic and hierarchical manner to control differentiation and proliferation of the colonic crypt epithelium *in vivo*.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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Research article

EWS/ATF1 expression induces sarcomas from neural crest–derived cells in mice

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Clear cell sarcoma (CCS) is an aggressive soft tissue malignant tumor characterized by a unique t(12;22) translocation that leads to the expression of a chimeric *EWS/ATF1* fusion gene. However, little is known about the mechanisms underlying the involvement of *EWS/ATF1* in CCS development. In addition, the cellular origins of CCS have not been determined. Here, we generated *EWS/ATF1*-inducible mice and examined the effects of *EWS/ATF1* expression in adult somatic cells. We found that forced expression of *EWS/ATF1* resulted in the development of *EWS/ATF1*-dependent sarcomas in mice. The histology of *EWS/ATF1*-induced sarcomas resembled that of CCS, and *EWS/ATF1*-induced tumor cells expressed CCS markers, including S100, SOX10, and MITF. Lineage-tracing experiments indicated that neural crest–derived cells were subject to *EWS/ATF1*-driven transformation. EWS/ATF1 directly induced Fos in an ERK-independent manner. Treatment of human and *EWS/ATF1*-induced CCS tumor cells with FOS-targeted siRNA attenuated proliferation. These findings demonstrated that FOS mediates the growth of *EWS/ATF1*-associated sarcomas and suggest that FOS is a potential therapeutic target in human CCS.

Introduction

Clear cell sarcoma (CCS) is an aggressive malignancy of adolescents and young adults that was first described by Enzinger (1). It typically arises in the deep soft tissues of the lower extremities closed to tendon, fascia, and aponeurosis (2). Chemotherapy and radiotherapy are not of any benefit (3–5), and a high rate of local and distant recurrence results in poor survival rates (3, 6, 7). CCSs harbor the potential for melanocytic differentiation and melanin synthesis (8). Gene expression profiles support the classification of CCS as a distinct genomic subtype of melanomas (9). These melanocytic features often make the distinction from malignant melanoma (MM) difficult. However, in contrast to MM, CCS is characterized by a chromosomal translocation, t(12;22)(q13;q12), that leads to the fusion of activating transcription factor 1 (*ATF1*) gene localized to 12q13 to Ewing's sarcoma oncogene (*EWS*) gene at 22q12 in up to 90% of cases, resulting in expression of the *EWS/ATF1* fusion gene (10–12). Given that CCS and MM have such similar characteristics, it has been proposed that CCSs may arise from a neural crest progenitor. However, the exact origin of CCS still remains to be determined.

The biological role of the EWS/ATF1 fusion protein is still unclear. EWS contains a transcriptional activation domain in the N-terminal region (13–15) and several conserved RNA binding motifs in the C-terminal region (16). Binding of the N-terminal region of EWS to the RNA polymerase II subunit hsRPB7 has been proposed to be important for transactivation of the target genes (17). In contrast, ATF1 is a member of the CREB transcription factor family, whose activity is regulated through phosphorylation of its kinase inducible domain (KID) by protein kinase A (18). ATF1 mediates the activation of cAMP-responsive genes through binding to a conserved cAMP-responsive element (CRE) as a dimmer (19, 20). However, the N-terminal activation domain of EWS replaces the KID in the EWS/ATF1 fusion protein, rendering it unable to support a typical inductive signal (21). Therefore, EWS/ATF1 can act as constitutive transcriptional activator in a cAMP-independent fashion with normal CRE DNA binding activity (14, 22, 23).

Previous studies have revealed some target genes of EWS/ATF1, but their true function in tumorigenesis is still not well understood (24). Expression of *MITF* is constitutively activated by *EWS/ATF1* in CCS in vitro (25). Consistent with this finding, several studies have identified the expression of MITF protein or mRNA in CCS (26–28). MITF is a master regulator of melanocyte development and plays a role in melanoma development (29, 30). Importantly, activation of MITF by EWS/ATF1 is required for CCS proliferation as well as for melanocytic differentiation of CCS in vitro (25).

Although previous studies have demonstrated that EWS/ATF1 is associated with oncogenic potential in CCS, the effect of in vivo expression of *EWS/ATF1* on sarcoma formation is still not known. In the present study, we established *EWS/ATF1* transgenic mice using a doxycycline-dependent expression system in order to investigate the role of *EWS/ATF1* on CCS development in vivo. Our results showed that forced expression of *EWS/ATF1* induced CCS-like sarcoma in the transgenic mice. This mouse model was used to identify the origin of *EWS/ATF1*-induced sarcomas as well as the direct target of EWS/ATF1 in these sarcomas.

Results

Inducible expression of EWS/ATF1 in mice. We first generated doxycycline-inducible *EWS/ATF1* ES cells, in which the human *EWS/ATF1* type 2 fusion gene (26, 31) can be induced under the control of

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