of the disease, but also to the development of new strategies for diagnosis and therapy.

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Conflicts of interest None

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Invited Mini Review

Long noncoding RNA involvement in cancer

*Reo Maruyama & Hiromu Suzuki**
Department of Molecular Biology, Sapporo Medical University, Sapporo 060-8556, Japan

Recent advances in genome and transcriptome analysis have enabled identification of numerous members of a new class of noncoding RNA, long noncoding RNA (lncRNA). lncRNAs are broadly defined as RNA molecules greater than 200 nt in length and lacking an open reading frame. Recent studies provide evidence that lncRNAs play central roles in a wide range of cellular processes through interaction with key component proteins in the gene regulatory system, and that alteration of their cell- or tissue-specific expression and/or their primary or secondary structures is thought to promote cell proliferation, invasion and metastasis. The biological and molecular characteristics of the large majority of lncRNAs remains unknown, and it is anticipated that improved understanding of the roles played by lncRNAs in cancer will lead to the development of novel biomarkers and effective therapeutic strategies. [BMB Reports 2012; 45(11): 604-611]

INTRODUCTION

Recent advances in genome analysis, including microarray and massively parallel sequencing, have shown that a much larger portion of the human genome is pervasively transcribed into RNA than previously recognized. Moreover, much of the evidence emerging in recent years has highlighted the biological and pathological importance of RNA molecules that lack protein-coding potential; these are collectively referred to as noncoding RNAs (ncRNAs) (1). Long ncRNAs (lncRNAs) are broadly defined as transcribed RNA molecules greater than 200 nt in length and lacking an open reading frame of significant length (less than 100 amino acids). Although there are no specific definitions, lncRNAs can be categorized into several subgroups based on their locations and characteristics. For instance, antisense RNAs are transcribed from the opposite strand of a protein-coding gene, while lncRNAs transcribed from intergenic regions are referred to as large intergenic ncRNAs (lincRNAs).

Much about the molecular and biological characteristics of lncRNAs is as yet unknown, but what is known suggests that many show expression patterns that are spatially and temporally specific and are generally poorly conserved among species. Cabili *et al.* recently used integrated RNA-seq data to construct a reference catalog of 8195 human lincRNAs (2). According to their reports, lincRNAs, like protein-coding transcripts, are transcribed by RNA polymerase II and are spliced and polyadenylated, but the maximum expression levels of lincRNAs are 10 times lower than those of protein-coding transcripts. lincRNAs are also smaller in size than protein-coding RNAs and have fewer exons: on average, lincRNAs are ~1 kb in length with 2.9 exons, whereas protein-coding transcripts are ~2.9 kb in length with 10.7 exons. In addition, lincRNAs are alternatively spliced more frequently than protein-coding mRNAs (2.3 isoforms per lincRNA locus, on average). It is also noteworthy that whereas 78% of examined lincRNAs exhibit tissue-specific expression patterns, only 19% of protein-coding transcripts do so. In fact, the tissue (cell or context) specificity of lincRNAs expression has been reported often enough (3, 4) to suggest that it is an important feature of lincRNAs.

Although the specific functions of the large majority of lncRNAs remain unknown, recent studies have begun to shed light on the critical roles played by these molecules in a variety of cellular processes, including differentiation, development and tumorigenesis. In this review, we will briefly outline the known functions of lncRNAs and their involvement in cancer.

FUNCTIONS OF lncRNA

Although, as mentioned, the function of most lncRNAs remains unknown, dozens of examples of biologically functional lncRNAs have already been reported, and the number of such examples is rapidly increasing (Table 1). Basically, lncRNAs appear to be involved in all aspects of gene regulation, including chromosome dosage-compensation, imprinting, epigenetic regulation, nuclear and cytoplasmic trafficking, transcription, mRNA splicing and translation. Through such gene regulation, lncRNAs are involved in a wide range of biological processes, including proliferation, cell cycle, apoptosis, differentiation and maintenance of pluripotency, among others. There is a good review of lncRNA's functions, focusing on biological and pathological processes in cancer (5); refer to that article for further details. On the other hand, it is also useful to look at

*Corresponding author. Tel: +81-11-611-2111; Fax: +81-11-622-1918; E-mail: hsuzuki@sapmed.ac.jp
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Table 1. Representative lncRNAs implicated in cancer

Name	Size	Cancer type	Biological function	Molecular function	References
Oncogenic					
HOTAIR	2.2 kb	Breast, hepatocellular, colorectal, pancreatic, GIST	Promotes invasion and metastasis, Modulates cancer epigenome	Scaffold (PRC2, LSD1), Guide (*in trans*)	(11, 26-29)
ANRIL	2.2 kb	Prostate, leukemia, neural system, melanoma	Suppresses senescence via INK4A	Scaffold (PRC1, PRC2), Guide (*in cis*)	(30-35)
MALAT1	8.7 kb	Lung, prostate, breast, colon, hepatocellular	Regulates alternative splicing of pre-mRNA	Scaffold (nuclear paraspeckle)	(36-40, 47)
PCAT-1	1.9 kb	Prostate	Promotes cell proliferation, inhibits BRCA2	Unknown	(42)
PCGEM1	1.6 kb	Prostate	Inhibits apoptosis, promotes cell proliferation	Unknown	(48, 49)
TUC338	0.6 kb	Hepatocellular	Promotes cell proliferation	Unknown	(50)
uc.73a	0.2 kb	Leukemia, colorectal	Inhibits apoptosis, promotes cell proliferation	Unknown	(51)
SPRY4-IT1	0.7 kb	Melanoma	Promotes cell proliferation and invasion, Inhibits apoptosis	Unknown	(52)
ncRAN	2.3 kb	Neuroblastoma, bladder	Promotes cell proliferation and invasion	Unknown	(53, 54)
PRNCR1	13 kb	Prostate	Promotes cell proliferation	Unknown	(55)
H19	2.3 kb	Breast, hepatocellular	Promotes cell proliferation, both oncogenic and tumor suppressive functions reported	Unknown	(56, 57)
Tumor suppressive					
GAS5	0.6-1.8 kb	Breast	Induces growth arrest and apoptosis	Decoy (glucocorticoid receptor)	(21, 44)
MEG3	1.6-1.8 kb	Meningioma, hepatocellular, leukemia, pituitary	Mediates p53 signaling Inhibits cell proliferation	Unknown	(58, 59)
PTENP1	3.9 kb	Prostate, colon	Inhibits cell growth	Decoy (PTEN-suppressing miRNAs)	(23)
LincRNA-p21	3.1 kb	Mouse models of lung, sarcoma, lymphoma	Induces apoptosis by repressing p53 targets	Guide (hnRNP-k, *in trans*)	(10)
Unknown, Biomarker					
HULC	0.5 kb	Hepatocellular	Unknown	Decoy (miR-372)	(60, 61)
PCA3	3.6 kb	Prostate	Unknown	Unknown	(62)
PANDA	1.5 kb	Unknown	Inhibits apoptosis by sequestering NF-YA	Decoy (NF-YA)	(4)

lncRNAs in the context of their molecular functionality (6). In this review we will focus on three molecular functions of lncRNAs: guide, scaffold and decoy.

Guide

One group of lncRNAs is able to bind specific proteins and then direct the localization of the resultant complex to specific targets. Such lncRNAs can guide proteins either *in cis* (on neighboring genes) or *in trans* (on distantly located genes). XIST, one of the most well-studied lncRNAs, guides PRC2 (polycomb repressive complex 2) to one of the two X chromosomes *in cis* to achieve X inactivation (7). Two other examples of lncRNAs that function as guides *in cis* are AIR and HOTTIP. AIR silences transcription of its target gene on the paternal chromosome by recruiting G9a and then mediating targeted histone H3 lysine 9 (H3K9) methylation and allelic silencing (8). HOTTIP, which is transcribed from the end of HOXA cluster, binds to WDR5 and recruits the MLL histone H3 lysine 4 (H3K4) methyltransferase complex to the HOXA cluster to support active chromatin confirmation (9).

Some lncRNAs, including lincRNA-p21 and HOTAIR, are able to alter and regulate epigenetic states and gene expression across multiple sites *in trans* (10, 11). LincRNA-p21 is transcribed upstream of CDKN1A gene and acts as a transcriptional repressor through its interaction with hnRNP-K, which it guides to target sites. Knocking down lincRNA-p21 alters the expression of over 1,000 genes, suggesting lincRNAs may be able to regulate numerous genes *in trans*. In addition, some lncRNAs may serve as cellular "navigation systems" for proteins lacking direct DNA binding capacity (12).

The mechanism by which lncRNAs specifically regulate their target genes remains unclear, and their binding sites throughout the genome are largely unknown. In one recent study, Chu and colleagues addressed this question using a novel assay they named ChIRP (Chromatin Isolation by RNA Purification)-seq, which is a method for genome-wide mapping of lncRNA binding sites *in vivo* (13). This analysis enabled them to obtain a high-resolution map of ncRNA occupancy

throughout the genome and to identify a set of 832 HOTAIR binding sites in human breast cancer cells. Interestingly, binding sites for HOTAIR are focal (<500 bp) and located in the midst of a broad polycomb binding domain, which suggests HOTAIR may act as a pioneering factor able to recruit polycomb to its target genes and then bilaterally spread the repressive regions outward. They also discovered an underlying DNA sequence motif enriched in HOTAIR binding sites, indicating the existence of a new class of regulatory element: lncRNA target sites. We now think that lncRNAs can function as sequence-specific transcription factors.

Scaffold

Another class of lncRNAs may possess distinct domains that bind different effector molecules. Such lncRNAs can mediate assembly of multiple molecular components in temporally and spatially specific manner. Two examples of lncRNAs that act as scaffolds are ANRIL, which interacts with components from PRC1 (polycomb repressive complex 1) and PRC2 (14, 15), and KCNQ1OT1, which binds both G9a and PRC2 (16). In addition, MALAT1 and NEAT1 serve as molecular scaffolds for proteins within nuclear speckles and paraspeckles, respectively (17). Depletion of NEAT1 is sufficient to cause loss of paraspeckles from within the nucleus, and overexpressing NEAT1, but not paraspeckle-associated proteins, leads to an increase in the number of paraspeckles, suggesting NEAT1 plays an essential role as a scaffold in the formation of paraspeckles.

A recent study revealed that HOTAIR interacts with two chromatin modifying complexes, the PRC2 complex ("writer" of a repressive mark, H3K27 trimethylation) and the LSD1/CoREST H3K4 demethylase complex ("eraser" of an activating mark, H3K4 trimethylation) (18). Using a series of deletion mutants, the PRC2 binding domain was mapped to the 5′ end (the first 300 nt) of HOTAIR, while the LSD1 binding site corresponds to the 3′ end. This suggests that HOTAIR that bridges between the PRC2 and LSD1 complexes, and that the resultant HOTAIR/PRC2/LSD1 complex can suppress gene expression via multiple mechanisms.

This finding is not applicable only to HOTAIR; many other lncRNAs also appear to interact with both the PRC2 and LSD1 complexes. For example, Khalil and colleagues performed RIP-chip assays (RNA coimmunoprecipitation combined with high throughput lincRNA microarray) using antibodies directed against several proteins involved in chromatin modifying complexes (PRC2, CoREST and SMCX) (19). They found that as many as 38% of lincRNAs expressed in the cell types studied reproducibly associate with one of these complexes. In mouse embryonic stem cells, moreover, a number of lincRNAs were found to be strongly associated with multiple chromatin modifier complexes (20). For example, eight lincRNAs bind to the PRC2 H3K27 and ESET H3K9 methyltransferase complexes and the JARID1C H3K4 demethylase complex. Similarly, 17 lincRNAs were found to bind to the PRC2, PRC1 and JARID1B complexes. Taken together, these results suggest the attractive hypothesis that lincRNAs bind to ubiquitously expressed chromatin modifying complexes in order to guide them to specific genomic regions.

Decoy

A third class of lncRNAs bind and then sequester a protein or RNA target, but do not exert additional effects. By acting as molecular decoys, these lncRNAs negatively regulate the expression of their targets. Examples of lncRNAs with decoy functionality include GAS5 (growth arrest-specific 5), which binds to the glucocorticoid receptor (GR) and represses GR-induced genes (21); PANDA (P21 associated ncRNA DNA damage activated), which binds to the transcription factor NF-YA to negatively regulate expression of pro-apoptotic genes (4); and TERRA (telomeric repeat-containing RNA), which interacts with telomerase via a repeat sequence to reduce enzyme activity (22).

Interestingly, several pseudogenes also reportedly act as molecular decoys. The 3′UTR of PTENP1, a tumor suppressor pseudogene, was found to bind the same set of regulatory miRNA sequences that normally target the tumor-suppressor gene PTEN, which reduces the downregulation of PTEN mRNA and allows its translation into the tumor-suppressor protein PTEN (23). It was also shown that the PTENP1 locus is selectively lost in human cancer, and that similar relationships also exist between other cancer-related genes and their pseudogenes (24). These findings attribute a novel biological function to expressed pseudogenes, as they can regulate coding gene expression and reveal a non-coding function in mRNAs.

DYSREGULATION OF lncRNAS IN HUMAN CANCER

HOTAIR

The lincRNA HOTAIR was originally discovered by Rinn and colleagues (25). It is encoded within the HOXC gene cluster and acts *in trans* to regulate HOXD genes through recruitment of PRC2 to induce trimethylation of H3K27 (H3K27me3). Remarkably, pull-down assays with PRC2 components demonstrated a direct and specific interaction with HOTAIR. This observation that HOTAIR binds PRC2 and induces epigenetic silencing of another HOX cluster on a different chromosome was an unexpected and novel finding.

In breast cancer, elevated expression of HOTAIR reportedly correlates with a poor prognosis and tumor metastasis (11). It is noteworthy that expression of a single lincRNA in primary tumors can be a powerful predictor of eventual metastasis and death. Enforced expression of HOTAIR induces genome-wide re-targeting of PRC2, leading to altered H3K27me3 and gene expression, and increased cancer invasiveness and metastasis. The link between HOTAIR and metastatic disease depends on both the direct interaction between the ncRNA and its protein partner and between the ncRNA and its target DNA sequence. Furthermore, several other studies have also shown that the level of HOTAIR expression correlates positively with metastasis and poor outcome in hepatocellular carcinoma, color-

ectal cancer and pancreatic cancer (26-28). We also discovered that upregulation of HOTAIR is strongly associated with aggressiveness in gastrointestinal stromal tumors (GISTs) (29). Interestingly, in malignant GISTs, HOTAIR is concurrently overexpressed with collinear HOXC genes and an oncogenic microRNA, miR-196a. We also observed enrichment of an active histone mark, H3K4me3, over a wide range of the HOXC cluster, suggesting the entire region is epigenetically activated in malignant GISTs. Taken together, these results suggest that lincRNAs play active roles in modulating the cancer epigenome and could be useful targets for cancer diagnosis and therapy.

ANRIL

ANRIL (antisense lncRNA of the INK4 locus) is transcribed antisense to The INK4 locus and mediates INK4a transcriptional repression *in cis* (30). Independent studies have shown that overexpression of ANRIL in prostate cancer results in the silencing of INK4b/ARF/INK4a and p15/CDKN2B due to heterochromatin formation (14, 31). ANRIL interacts with SUZ12 (suppressor of zeste 12 homolog), a subunit of PRC2 (15) and with CBX7 (chromo- box homolog 7), a subunit of PRC1 (14). ANRIL may recruit multiple sets of chromatin-modifying complexes to a target gene for silencing, serving as a molecular scaffold. Genome-wide association studies (GWAS) have linked ANRIL with increased susceptibility to coronary disease, intracranial aneurysm and type 2 diabetes, as well as to several types of cancer, including acute lymphoblastic leukemia, glioma, basal cell carcinoma, nasopharyngeal carcinoma, breast cancer and plexiform neurofibromas (32-35).

MALAT1

MALAT1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1) was initially identified in a screen for genes associated with metastasis (36). It is abundantly expressed in many human cell types and is highly conserved across several species. MALAT1 is reportedly upregulated in multiple malignancies, including lung cancer, uterine endometrial stromal sarcoma, cervical cancer and hepatocellular carcinoma (36-38). In lung metastasizing tumors, MALAT1 expression is three-fold higher than in non-metastasizing tumors (36), and it can serve as an independent prognostic parameter for patient survival in early stage lung adenocarcinoma (39). MALAT1 promotes the motility of lung cancer cells through transcriptional or post-transcriptional regulation of motility-related genes (40).

MALAT1 localizes to nuclear speckles, which contain several proteins known to be involved in alternative splicing (41). It appears that MALAT1 forms a molecular scaffold for several of the proteins present within nuclear speckles, and modulates the phosphorylation of SR proteins. Depletion of MALAT1 is sufficient to alter the patterns of alternative splicing of a subset of mRNAs.

LincRNA-p21

Recent studies identified a number of lincRNAs induced by the p53 tumor-suppressor gene (10). LincRNA-p21 is located upstream of the CDKN1A gene on mouse chromosome 17 and is directly activated by p53 in response to DNA damage. LincRNA-p21 acts as a transcriptional repressor in the canonical p53 pathway and plays a role in triggering apoptosis. Inhibition of lincRNA-p21 alters the expression of hundreds of genes normally repressed by p53, potentially explaining how p53 can activate large numbers of genes while simultaneously repressing many others. LincRNA-p21 also interacts with heterogeneous nuclear ribonucleoprotein K (hnRNP-K), a well-known RNA binding protein that acts as a transcriptional repressor. A 780-nt region at the 5′ end of lincRNA-p21 is necessary for interaction with hnRNP-K, which is required for proper genomic localization of hnRNP-K at repressed genes and for regulation of p53-mediated apoptosis. Although lincRNA-p21 has not been directly associated with disease, we would speculate that loss of lincRNA-p21 function could be an important factor contributing to cancer initiation.

PANDA

PANDA is also induced in a p53-dependent manner. After DNA damage, p53 directly binds to the CDKN1A locus to activate PANDA (4), which appears to possess decoy function. PANDA inhibits the expression of apoptotic genes by directly binding to and sequestering the transcription factor NF-YA away from target gene promoters. PANDA is overexpressed in a subset of human breast cancers, and its depletion can sensitize cells to chemotherapeutic agents.

PCAT-1

Prensner and colleagues used high throughput RNA-Seq with a large panel of clinical samples to comprehensively evaluate the ncRNAs dysregulated in prostate cancer (42). They identified approximately 1,800 lincRNAs in prostate tissue, of which 121 were transcriptionally dysregulated in prostate cancer. Among them, PCAT-1 (prostate cancer associated transcript -1) showed tissue-specific expression and was selectively upregulated in prostate cancer. Like HOTAIR, PCAT-1 functions predominantly as a transcriptional repressor by facilitating trans-regulation of genes preferentially involved in mitosis and cell division, including known tumor suppressor genes such as BRCA2. The discovery of PCAT-1 highlights the usefulness of unbiased transcriptome analysis when investigating the actions of lncRNAs in cancer.

GAS5

GAS5 was originally identified as a gene highly expressed in cells whose growth was arrested (43). The human GAS5 gene is transcribed from chromosome 1q25.1 and is alternatively spliced. GAS5 sensitizes cells to apoptosis by regulating the activity of glucocorticoids in response to nutrient starvation (21). GAS5 binds to the DNA-binding domain of the GR,

where it acts as a decoy preventing the GR from interacting with cognate glucocorticoid response elements (GRE). The binding of GAS5 to the GR is sufficient to repress GR-induced genes such as cIAP2.

Implicating GAS5 in breast cancer is the observation that levels of GAS5 transcript are significantly lower in breast cancer cells than in unaffected normal breast epithelium (44). In addition, genetic aberrations at the GAS5 locus have been found in many types of tumors, including melanomas and breast and prostate cancers (45, 46), though their functional significance has not yet been established. The GAS5 gene locus has also been linked to increased susceptibility to autoimmune disorders such as systemic lupus erythematosus in the mouse BXSB strain (21). Chromosomal translocations affecting the 1q25 locus containing the GAS5 gene have been detected in melanoma, B-cell lymphoma, and prostate and breast cancer.

CONCLUDING REMARKS

In recent years, technological advances in high-throughput sequencing have enabled us to identify numerous ncRNAs, which has improved our appreciation for the complexity of transcriptome and enabled us to demonstrate the dysregulated expression of a number of lncRNAs in various types of cancer. The affected molecules are now thought to function as oncogenes or tumor suppressors. However, the biological and molecular characteristics of most lncRNAs remain unknown, and much effort will be needed before a full understanding of their roles in normal and cancer cells is attained. This work will in part entail bioinformatics analysis, and secondary structure prediction will be important for identifying potentially functional motifs in lncRNAs. Elucidation of the structures of genes encoding lncRNAs, including the promoters, transcription start sites and enhancers will be essential for understanding the mechanisms governing their spatiotemporal expression patterns. Moreover, greater knowledge of the gene structures will lead to complete cloning of lncRNAs, which will facilitate functional studies using expression constructs and mouse models. Analysis of genetic and epigenetic alterations of lncRNA genes will also provide clues to understanding their pathological roles, and the mechanisms by which such alterations affect lncRNA function must be carefully characterized. Although the roles played by lncRNAs in cancer have just begun to be revealed, it is anticipated that advances in the study of lncRNAs will yield new diagnostic biomarkers as well as RNA-based therapeutic strategies.

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Dose-dependent roles for canonical Wnt signalling in *de novo* crypt formation and cell cycle properties of the colonic epithelium

Akihiro Hirata[1,*], Jochen Utikal[2,3,*], Satoshi Yamashita[4], Hitomi Aoki[5], Akira Watanabe[6], Takuya Yamamoto[6], Hideyuki Okano[7], Nabeel Bardeesy[2], Takahiro Kunisada[5], Toshikazu Ushijima[4], Akira Hara[8], Rudolf Jaenisch[9], Konrad Hochedlinger[2,‡] and Yasuhiro Yamada[6,10,‡]

SUMMARY

There is a gradient of β-catenin expression along the colonic crypt axis with the highest levels at the crypt bottom. In addition, colorectal cancers show a heterogeneous subcellular pattern of β-catenin accumulation. However, it remains unclear whether different levels of Wnt signalling exert distinct roles in the colonic epithelium. Here, we investigated the dose-dependent effect of canonical Wnt activation on colonic epithelial differentiation by controlling the expression levels of stabilised β-catenin using a doxycycline-inducible transgenic system in mice. We show that elevated levels of Wnt signalling induce the amplification of Lgr5+ cells, which is accompanied by crypt fission and a reduction in cell proliferation among progenitor cells. By contrast, lower levels of β-catenin induction enhance cell proliferation rates of epithelial progenitors without affecting crypt fission rates. Notably, slow-cycling cells produced by β-catenin activation exhibit activation of Notch signalling. Consistent with the interpretation that the combination of Notch and Wnt signalling maintains crypt cells in a low proliferative state, the treatment of β-catenin-expressing mice with a Notch inhibitor turned such slow-cycling cells into actively proliferating cells. Our results indicate that the activation of the canonical Wnt signalling pathway is sufficient for *de novo* crypt formation, and suggest that different levels of canonical Wnt activations, in cooperation with Notch signalling, establish a hierarchy of slower-cycling stem cells and faster-cycling progenitor cells characteristic for the colonic epithelium.

KEY WORDS: Wnt signalling, Notch signalling, Intestinal stem cell, Mouse

INTRODUCTION

The intestinal epithelium is characterised by rapid and continuous renewal throughout life. One of the major players involved in the renewal of the intestinal epithelium is the canonical Wnt signalling pathway. Experimental manipulation of Wnt signalling has been shown to influence epithelial proliferation in the intestines (Korinek et al., 1998; Pinto et al., 2003; Kuhnert et al., 2004; Sansom et al., 2004; Andreu et al., 2005; Fevr et al., 2007). For example, inactivation of Wnt signalling by transgenic or adenoviral expression of *Dickkopf1* (*Dkk1*), a secreted Wnt inhibitor, leads to marked inhibition of epithelial proliferation in the intestines (Pinto et al., 2003; Kuhnert et al., 2004). By contrast, two independent groups have demonstrated that loss of *Apc* results in a rapid and dramatic enlargement of the crypt compartment associated with abnormal cell proliferation in the small intestine (Sansom et al., 2004; Andreu et al., 2005). Together, these experiments provide definitive evidence for the importance of Wnt signalling in controlling intestinal epithelial proliferation.

In addition to controlling cell proliferation, a role for Wnt/β-catenin signalling in stem cell maintenance in the intestine has been suggested. Inactivation of Wnt signalling by either overexpression of *Dkk1* or conditional deletion of *Ctnnb1* (the gene encoding β-catenin) results in the loss of intestinal crypts, indicating that Wnt signalling is indispensable for stem cell maintenance (Pinto et al., 2003; Kuhnert et al., 2004; Fevr et al., 2007). In fact, the intestinal stem cell (ISC) marker *Lgr5* has initially been identified as a target of β-catenin/Tcf transcription (Barker et al., 2007), which is in accordance with the view that ISCs harbour a higher activity of canonical Wnt signals. In further support of this notion, nuclear accumulation of β-catenin has been observed at the crypt bottom in cells that potentially include ISCs (van de Wetering et al., 2002).

The number of ISCs has to be tightly regulated in the intestinal crypts in order to facilitate tissue turnover but prevent abnormal growth. ISCs are usually involved in a process of homeostatic self-renewal in the adult intestine but can also be rapidly recruited to repair tissues after injury. Indirect evidence for an involvement of Wnt signalling in stem cell amplification derives from a study showing that PTEN deficiency increases the frequency of crypt fission/budding and the number of cells expressing *Musashi1*, a putative ISC marker, through activated Wnt signalling (He et al., 2007). However, the underlying mechanism by which activated Wnt signalling may expand ISCs remains elusive, and direct evidence

[1]Division of Animal Experiment, Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu, 501-1194, Japan. [2]Massachusetts General Hospital Cancer Center and Center for Regenerative Medicine, Harvard Stem Cell Institute, 185 Cambridge Street, Boston, MA 02114, USA. [3]Skin Cancer Unit, German Cancer Research Center, Heidelberg, Germany and Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, Mannheim, Germany. [4]Division of Epigenomics, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan. [5]Department of Tissue and Organ Development and [8] Department of Tumor Pathology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu, 501-1194, Japan. [6]Center for iPS Cell Research and Application (CiRA), Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. [7]Department of Physiology, Keio University School of Medicine, 35 Shinanomach, Shinjuku-ku, Tokyo 160-8582, Japan. [9]Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge, MA 02142, USA. [10]PRESTO, Japan Science and Technology Agency, Saitama, Japan.

*These authors contributed equally to this work
‡Authors for correspondence (khochedlinger@helix.mgh.harvard.edu; y-yamada@cira.kyoto-u.ac.jp)

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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,

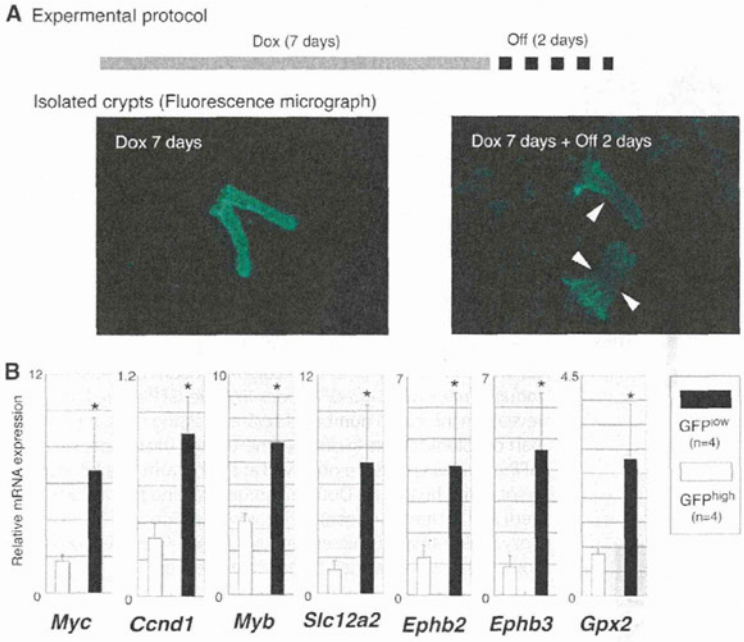

Fig. 1. Upregulation of canonical Wnt target genes in the proliferative compartments of colonic crypts. (**A**) Separation of proliferating cells from non-proliferative cells in the colon of histone H2B-GFP inducible mice. All crypt cells were labelled with nuclear GFP after Histone-GFP induction for 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

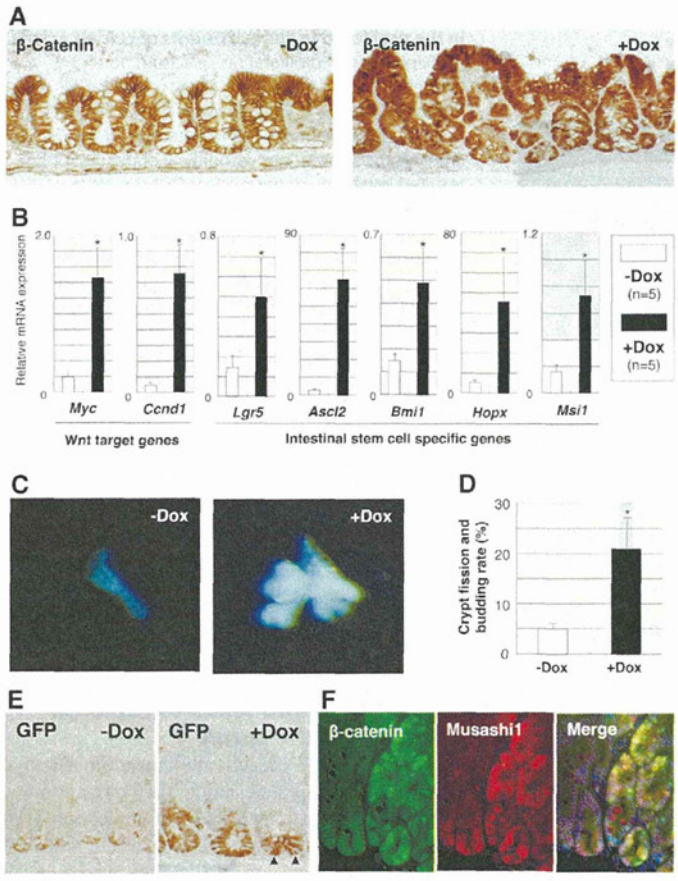

Fig. 2. β-Catenin induction leads to *de novo* crypt formation with increased expression of ISC markers in the colon. (**A**) β-Catenin immunostaining on colonic section of β-catenin-induced mice. Doxycycline treatment results in nuclear accumulation of β-catenin and frequent fission/budding of colonic 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

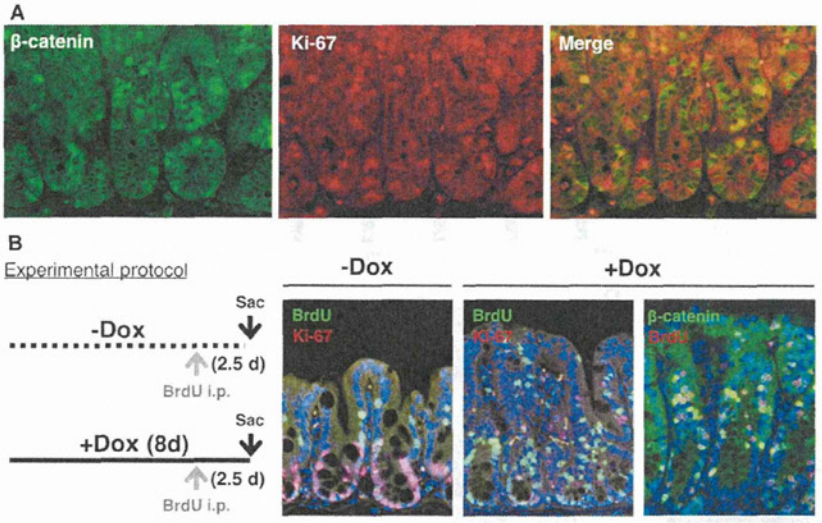

Fig. 3. Slow cycling properties of β-catenin-induced colonic cells. (**A**) Double immunostaining for β-catenin (green) and Ki-67 (red) on a β-catenin-induced colonic section. Majority of colonic cells with strong nuclear β-catenin expression are not coincident with Ki-67. (**B**) A scheme of the BrdU 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

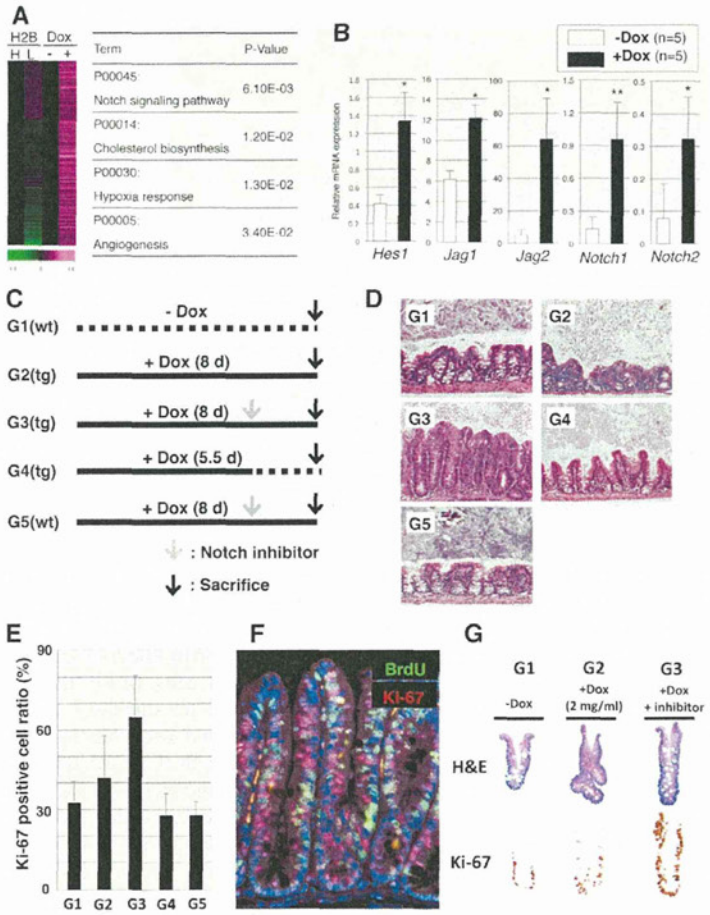

Fig. 4. Notch activation contributes to the maintenance of a slow-cycling state in β-catenin-induced colon. (**A**) Activation of Notch signalling pathway in β-catenin-induced slow-cycling colonic epithelium. Genes specifically upregulated in β-catenin-induced cells, but not in fast-cycling cells (GFP-Low 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

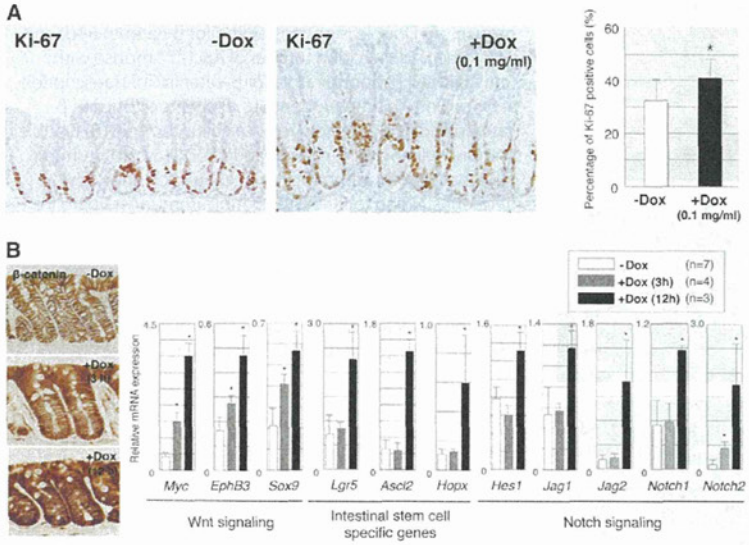

Fig. 5. Dose-dependent effect of Wnt activation on cell proliferation and gene expression in colonic epithelium. (**A**) Lower level of β-catenin induction promotes colonic epithelial proliferation. Ki-67 immunostaining and percentage of Ki-67-positive cells in colonic section from β-catenin-inducible 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

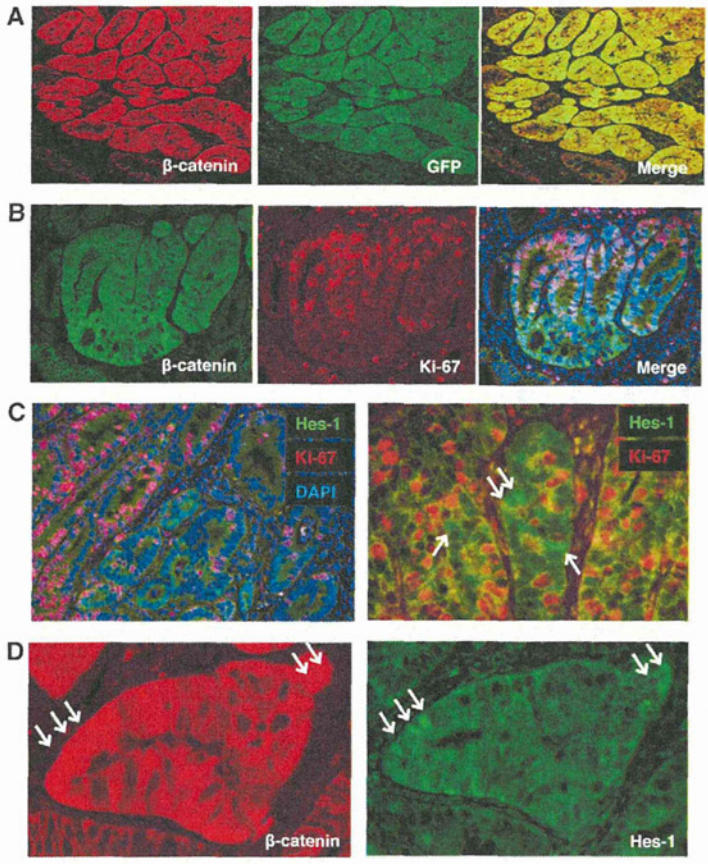

Fig. 6. Heterogeneity of colon tumour cells in $Apc^{Min/+}$ mouse. (**A**) Double immunostaining for β-catenin (red) and GFP (green) in the colon tumour of $Apc^{Min/+}$ mouse with transgenic GFP reporter allele for β-catenin/Tcf transcription activity. Note that heterogeneous expressions of both β-catenin 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.

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