Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium

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The HMG-box transcription factor Sox9 is expressed in the intestinal epithelium, specifically, in stem/progenitor cells and in Paneth cells. Sox9 expression requires an active β-catenin–Tcf complex, the transcriptional effector of the Wnt pathway. This pathway is critical for numerous aspects of the intestinal epithelium physiopathology, but processes that specify the cell response to such multipotential signals still remain to be identified. We inactivated the Sox9 gene in the intestinal epithelium to analyze its physiological function. Sox9 inactivation affected differentiation throughout the intestinal epithelium, with a disappearance of Paneth cells and a decrease of the goblet cell lineage. Additionally, the morphology of the colon epithelium was severely altered. We detected general hyperplasia and local crypt dysplasia in the intestine, and Wnt pathway target genes were up-regulated. These results highlight the central position of Sox9 as both a transcriptional target and a regulator of the Wnt pathway in the regulation of intestinal epithelium homeostasis.

Introduction

The intestinal epithelium is constantly and rapidly renewing throughout the lifespan of vertebrates, thereby representing a major target for tumorigenesis. This epithelium can be divided into two functionally distinct compartments. The crypt of Lieberkühn constitutes the proliferative compartment and contains stem/progenitor cells, as well as, in the small intestine, terminally differentiated Paneth cells. Multipotent stem cells, located near the bottom of crypts, generate new cells, which migrate upwards while differentiating into enterocytes, goblet, and enteroendocrine cells. Proliferation stops at the crypt–villus junction, and terminally differentiated cells are located on the neighboring villus, which constitute the differentiated compartment. In the small intestine, a fourth cell type, the Paneth cell, migrates downward and settles at the bottom of the crypts as postmitotic, differentiated cells. The balance among proliferation, differentiation, migration, and cell death must be tightly regulated to maintain homeostasis of this epithelium.

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We reported the expression of Sox9, an HMG-box transcription factor, specifically, in the rapidly proliferating stem/progenitor cells found at the bottom third of Lieberkühn crypts throughout the length of the intestine and in the Paneth cells of the small intestine, as well as in human tumors of the intestinal epithelium (Blache et al., 2004). Sox9 was first identified as a key regulator of cartilage and male gonad development. Heterozygous *Sox9* mutations are responsible for the campomelic dysplasia syndrome, a skeletal dysmorphology syndrome characterized by skeletal malformation of endochondral bones and by male-to-female sex reversal in the majority of genotypically XY individuals (Foster et al., 1994; Wagner et al., 1994). Sox9 has also been implicated in the development of cranial neural crest derivatives (Spokony et al., 2002), in the neural stem cell switch from neurogenesis to gliogenesis (Stolt et al., 2003) and in heart (Akiyama et al., 2004a), hair (Vidal et al., 2005), and pancreas (Seymour et al., 2007) development. In each of these tissues, Sox9 expression is restricted to specific cell types, suggesting a complex transcriptional regulation. In addition, the currently identified Sox9 target genes, for instance, in the cartilage and in the gonad, display tissue-specific expression (Ng et al., 1997; de Santa Barbara et al., 1998), indicating that Sox9 may

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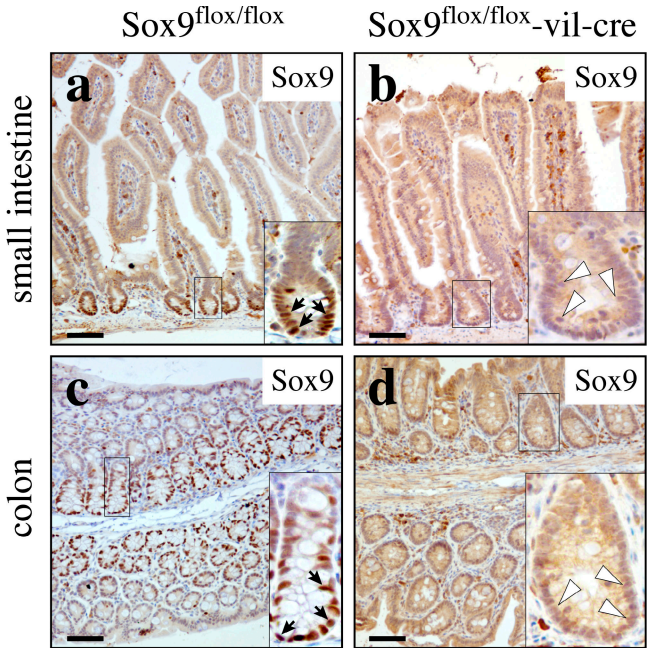

Figure 1. **Absence of Sox9 protein expression in the intestinal epithelium of Sox9-deficient mice.** In $Sox9^{flox/flox}$ mice, the Sox9 protein is expressed in the bottom of small intestinal (a) and colon (c) crypts. The absence of specific Sox9 staining in the small intestine (b) and colon (d) from $Sox9^{flox/flox}$-vil-Cre mice demonstrate efficient vil-Cre–mediated recombination. Arrows and arrowheads indicate Sox9-positive and -negative nuclei, respectively. Bars: (panels) 150 μm; (insets) 50 μm.

regulate distinct sets of genes in the different tissues in which it is expressed.

In the intestinal epithelium, the function of Sox9 remains unresolved, although in vitro studies suggested a role in the control of cell differentiation (Blache et al., 2004). In vitro and in vivo data indicate that *Sox9* is a transcriptional target of Wnt signaling. For instance, Sox9 expression is abrogated in Tcf4-null embryos, and it is strongly expressed in colorectal carcinoma cell lines containing activating mutations in components of the Wnt pathway (Blache et al., 2004).

The Wnt pathway plays a central role among the extracellular signals required to maintain the homeostasis of the intestinal epithelium. In particular, deletion of the gene encoding Tcf4, another HMG-box transcription factor (Korinek et al., 1998), or overexpression of the inhibitor Dickkopf (Pinto et al., 2003; Kuhnert et al., 2004), resulted in a loss of the proliferative compartment and in impaired differentiation of secretory cell lineages. Conversely, mutation of the gene encoding Apc, a negative regulator of the pathway, resulted in crypt expansion, abrogation of cell migration, and amplification of the Paneth cell population (Sansom et al., 2004; Andreu et al., 2005). In addition, deletion of the Wnt receptor Frizzled-5 revealed an essential role of the Wnt–Frizzled-5 pathway in the maturation of Paneth cells (van Es et al., 2005). The sorting process of epithelial cells along the crypt–villus axis also depends on the Wnt pathway, via a modulation of Ephrin–Eph receptor interactions (Batlle et al., 2002). The Wnt signaling pathway can thus induce diverse cellular responses in the intestinal epithelium. In addition to these physiological functions, the Wnt pathway is centrally implicated in cancer, as mutations in components of this pathway have been identified in the majority of human colorectal carcinoma (Morin et al., 1997). Such mutations mimic activation of the pathway by Wnt ligands (i.e., stabilization of β-catenin) and result in constitutive transcriptional activity of the β-catenin–Tcf4 complex and in aberrant expression of its target genes (Korinek et al., 1997). Despite the central importance of this pathway in the physiopathology of the intestinal epithelium, little is known about the molecular mechanisms involved in restricting this wide spectrum of potential functions to elicit a specific and adequate response from Wnt-stimulated cells.

The fact that *Sox9* is transcriptionally regulated by the β-catenin–Tcf4 complex (Blache et al., 2004), together with the particular expression of Sox9 in the compartment of the intestinal epithelium that contains Wnt-stimulated cells, suggests distinct functions in proliferating stem/progenitor cells and in the postmitotic Paneth cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200704152/DC1). To address the different aspects of Sox9 function during the turnover of the intestinal epithelium, including its possible role in specifying the cell response to Wnt signals, we specifically inactivated the corresponding gene in the intestinal epithelium.

Results

Generation of mice with a Sox9-deficient intestinal epithelium

To analyze the function of Sox9 in the turnover of the adult intestinal epithelium, Villin-Cre (vil-Cre) mice, in which the Cre recombinase is expressed specifically in the intestinal epithelium from 10.5 d postcoitum onward (el Marjou et al., 2004) were crossed with $Sox9^{flox/flox}$ (Kist et al., 2002) mice, which have both *Sox9* alleles flanked by loxP sequences. This generated $Sox9^{flox/flox}$-vil-Cre mice, with an intestinal epithelium lacking Sox9 protein, indicating effective vil-Cre–mediated recombination of the $Sox9^{flox}$ allele (Fig. 1). The control vil-Cre mice had no detectable phenotypic defect. $Sox9^{flox/flox}$-vil-Cre mice developed as their control littermates ($Sox9^{flox/flox}$ or $Sox9^{flox/wt}$-vil-Cre) to become healthy and fertile adult mice. No evidence for intestinal bleeding was found.

Sox9 inactivation causes aberrant morphology and decreased goblet cell lineage in the colon

Histological analysis of the intestine from 2–6-mo-old adult $Sox9^{flox/flox}$-vil-Cre mice revealed that, although the overall morphology of the small intestine seemed, at first sight, unaffected (Fig. 2, a and b), that of the colon was aberrant. The most striking feature of the $Sox9^{flox/flox}$-vil-Cre mice colon was the folding of the epithelium into villus-like structures, protruding into the colon lumen, reminiscent of the small intestine morphology (Fig. 2, c–f; and Fig. S1, B–D). Proliferation, however, was adequately restricted to the bottom half of the crypts in $Sox9^{flox/flox}$-vil-Cre mice, as assessed by Ki67 staining (Fig. 2, g and h).

We then examined the differentiation pattern of the $Sox9^{flox/flox}$-vil-Cre mice colon epithelium into the three main types of differentiated colon epithelial cells. Among these,

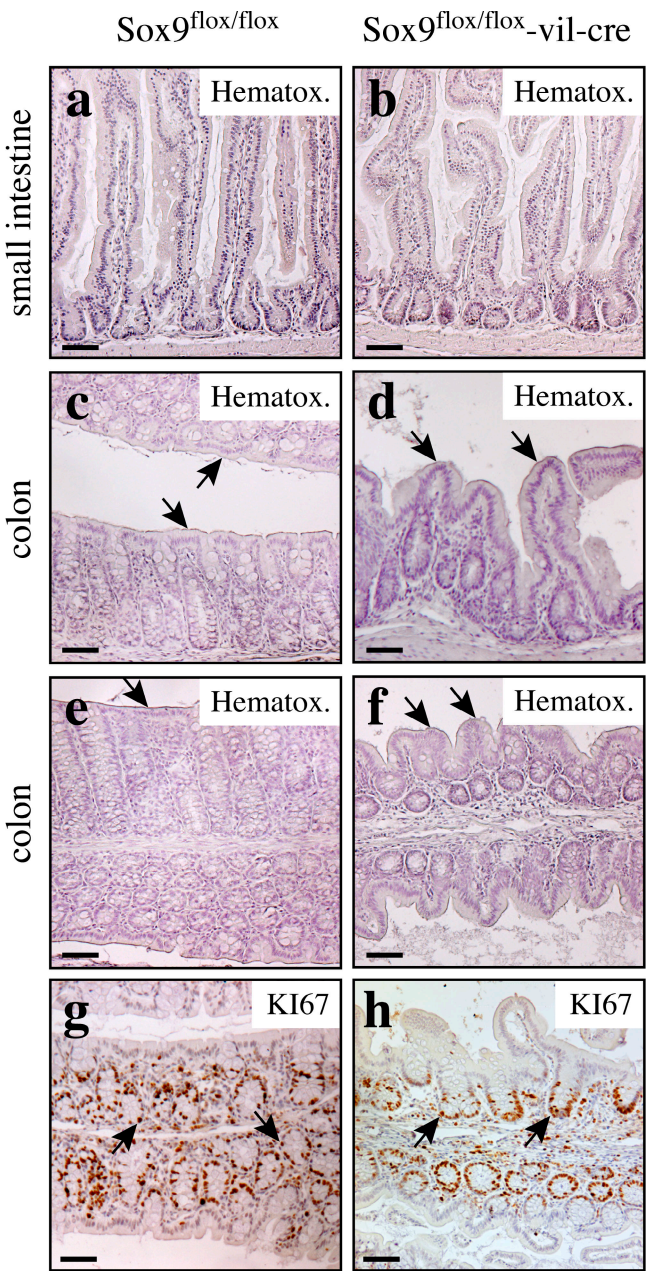

Figure 2. **Morphological alterations in the colon of Sox9-deficient mice.** Histological analysis (hematoxylin staining) of the intestine of Sox9$^{flox/flox}$ (a, c, and e) and Sox9$^{flox/flox}$-vil-Cre mice (b, d, and f). The gross morphology of the small intestine of the Sox9$^{flox/flox}$-vil-Cre mice is not affected (a and b), whereas that of the colon is strongly altered. The surface of the colon is normally flat (c and e, arrows), but villus-like structures protrude into the lumen of the colon of Sox9$^{flox/flox}$-vil-Cre mice (d and f, arrows). Immunohistochemical staining with the Ki-67 proliferation in Sox9$^{flox/flox}$ mice (g) and Sox9$^{flox/flox}$-vil-Cre mice (h) indicates appropriate crypt-restricted proliferation in the colon of Sox9-deficient animals. Bars, 150 μm.

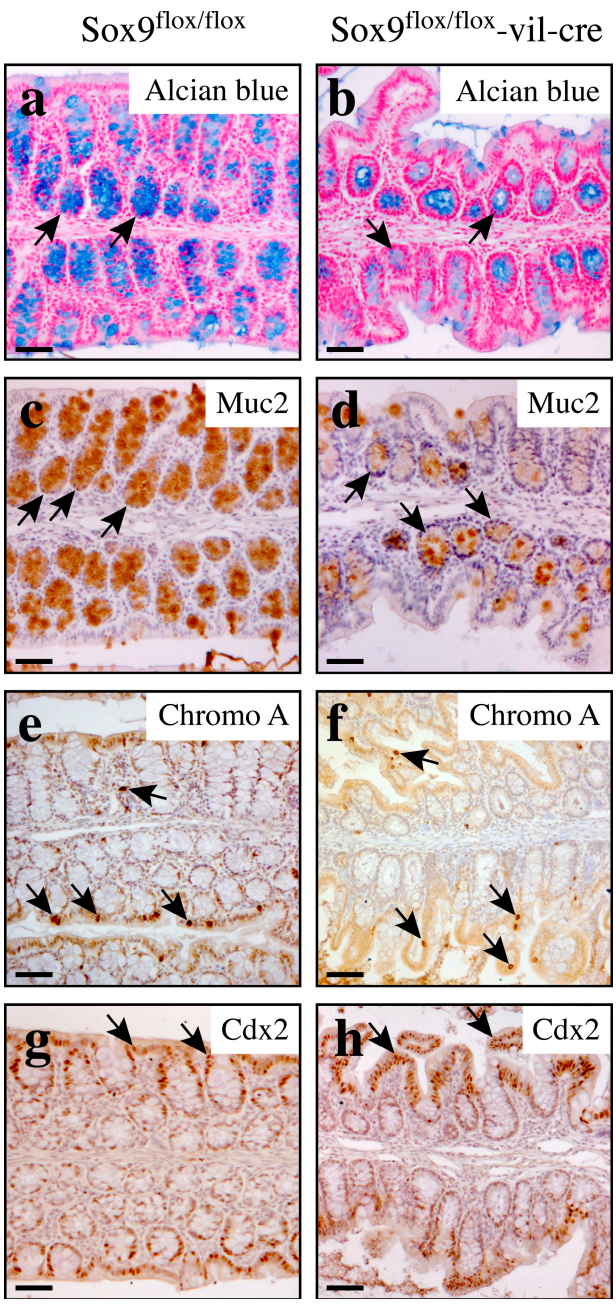

Figure 3. **Altered goblet cell differentiation in the colon of Sox9-deficient mice.** Immunohistochemical analysis of Sox9$^{flox/flox}$ mice (a, c, e, and g) and Sox9$^{flox/flox}$-vil-Cre mice (b, d, f, and h). Alcian blue staining (a and b) and Muc2 immunoreactivity (c and d) reveal a strong reduction of the goblet cell population in the colon epithelium of Sox9$^{flox/flox}$-vil-Cre mice. Sox9$^{flox/flox}$ and Sox9$^{flox/flox}$-vil-Cre mice have comparable populations of chromogranin A–positive enteroendocrine cells (e and f) and Cdx-2–positive enterocytes (g and h). Bars, 150 μm.

mucus-producing goblet cells represent the largest population and are responsible for epithelium protection and lubrication (Velcich et al., 2002). Most of the other cells are enterocytes, with few interspersed enteroendocrine cells. Alcian blue and Muc2 stainings showed that the goblet cell population was strongly decreased in the colon of Sox9-deficient animals (Fig. 3, a–d). No differences were detected in the morphology or staining intensity between individual alcian blue–positive goblet cells in Sox9$^{flox/flox}$ and Sox9$^{flox/flox}$-vil-Cre mice, and no changes in cellular representation were found either for the scarce enteroendocrine cell population (Fig. 3, e and f) or for the Cdx2-expressing enterocyte population (Fig. 3, g and h). Thus, Sox9 is involved in defining the colon epithelium morphology and plays a specific role in the differentiation of the goblet cell lineage in the colon.

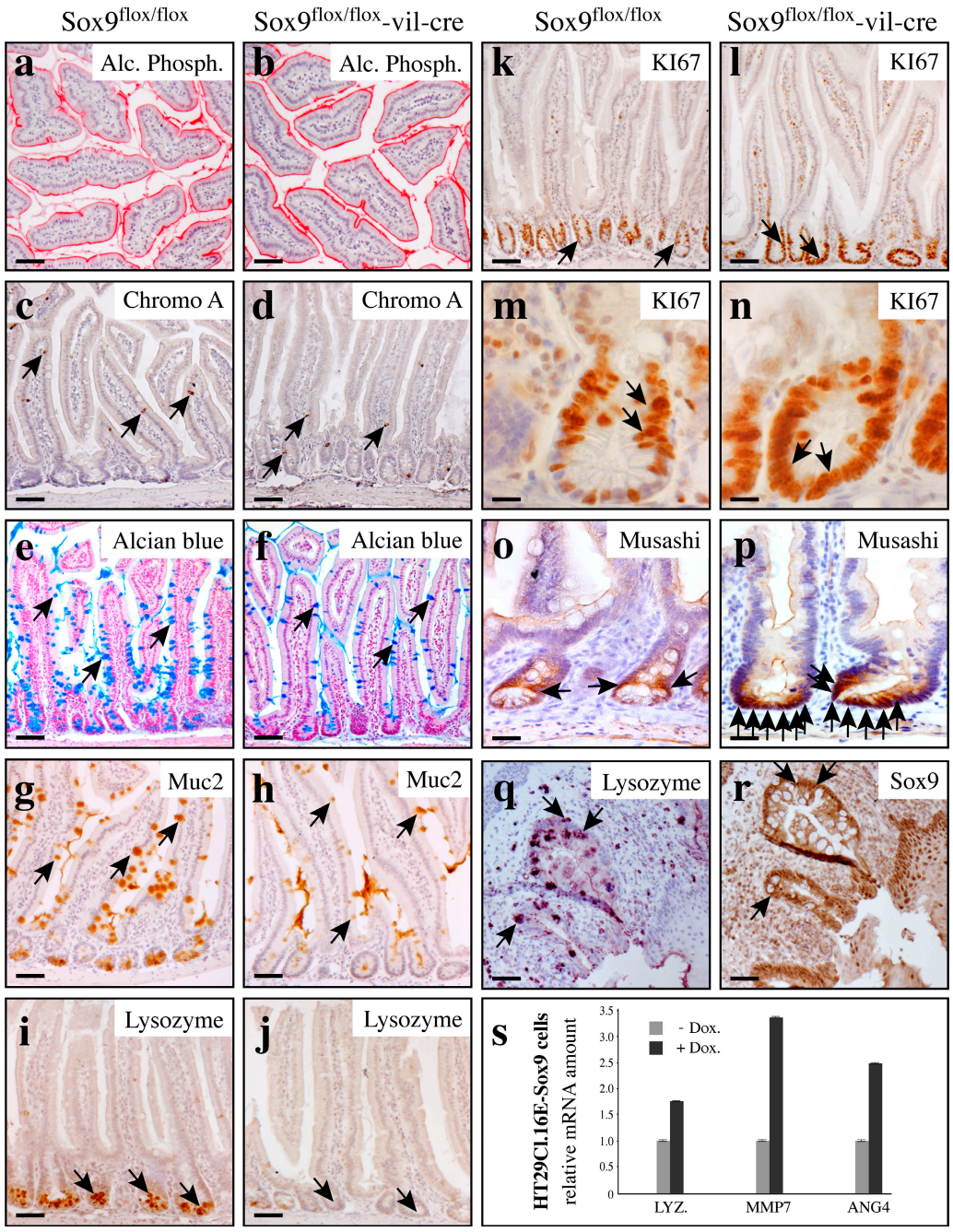

Figure 4. **Sox9 controls differentiation of the Paneth and goblet cell lineages.** Sox9$^{flox/flox}$ (a, c, e, g, i, k, and m) and Sox9$^{flox/flox}$-vil-Cre (b, d, f, h, j, l, and n) mice are compared. Differentiation along the enterocyte (a and b) and enteroendocrine (c and d) lineages is not affected. The goblet cell population, revealed by alcian blue staining for acidic mucins (e and f) and Muc2 immunostaining (g and h), is reduced in the small intestinal epithelium of Sox9-deficient mice. Paneth cells (arrows in control mice) are completely absent (arrows) in Sox9-deficient crypts (i and j). Instead, the proliferative compartment (k–n, arrows) expands to occupy the whole crypt bottom in mutant animals, where more Musashi-1–expressing putative stem cells are found (o and p, arrows). Ectopic Paneth cell found in a human patient with Barrett's esophagus (intestinal metaplasia in the esophagus) also express Sox9, as shown by staining of adjacent sections with lysozyme (q) and Sox9 (r) antibodies. Expression analysis of the Paneth cell markers lysozyme, MMP7, and Angiogenin-4, in the HT29Cl.16E-Sox9 cell line, before and after doxycycline induction of exogenous Sox9 expression (s). Bars: (a–l, q, and r) 120 μm; (m and n) 30 μm; (o and p) 40 μm.

Sox9 is required for Paneth cell differentiation

When we examined the expression of markers representative of the four main cell lineages constituting the small intestinal epithelium, no major differences were found in enterocyte and enteroendocrine cell numbers, as shown by alkaline phosphatase and chromogranin A staining (Fig. 4, a–d). However, both the goblet and Paneth cell lineages were considerably affected. Alcian blue–positive goblet cells were found in the Sox9$^{flox/flox}$-vil-Cre intestinal epithelium and appropriately expressed Muc2, but their number was reduced by 40% compared with control mice (Fig. 4, e–h; and Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200704152/DC1).

Paneth cells represent the fourth cell type found in the small intestine. They secrete a variety of products, including antimicrobial peptides, growth factors, phospholipase A2, and matrilysin. These cells are involved in regulating the interactions between epithelial cells and the indigenous microorganism population, which, in turn, is essential to elaborate the microvasculature underlying the epithelium (Wilson et al., 1999; Stappenbeck et al., 2002). Remarkably, morphological identification coupled with staining of small intestinal sections from Sox9$^{flox/flox}$-vil-Cre mice for lysozyme, an early marker of Paneth cell differentiation, revealed that almost all the crypts were completely devoid of Paneth cells (Fig. 4, i and j). In Paneth cell–depleted crypts, the proliferative compartment expanded to occupy the whole crypt base, including the normal Paneth cell compartment (Fig. 4, k–n). As Paneth cells are located next to the putative stem cells, the replacement of Paneth cells by proliferating cells raises the possibility that the number of stem cells is altered in Sox9-deficient mice. Indeed, the number of cells positively stained with Musashi-1, a putative marker of stem cells in the nervous system and the intestinal epithelium (Sakakibara et al., 1996; Potten et al., 2003), was increased in Sox9-deficient mice (Fig. 4, o and p). Sox9 is thus required for differentiation of the Paneth cell lineage, is involved in differentiation of the goblet cell lineage, and might be involved in the regulation of the stem cell number. The observed decrease in Paneth and goblet lineages in Sox9-deficient mice was not due to increased apoptosis, as no differences were found in the apoptotic rates between Sox9$^{flox/flox}$-vil-Cre and Sox9$^{flox/flox}$ mice (Fig. S2 C).

In the healthy human body, Paneth cells are also found uniquely in the small intestinal crypts of Lieberkühn. In some pathological situations, such as intestinal metaplasia, ectopic Paneth cells can also be found in the esophagus (Barrett's esophagus) or in the stomach (Schreiber et al., 1978). Thus, if Sox9 is required for the differentiation of the Paneth cell lineage, it should be expressed in Paneth cells found in such aberrant structures. To test this, we analyzed biopsy sections from a patient with Barrett's esophagus. Paneth cells were detected using lysozyme expression, and Sox9 expression was analyzed on an adjacent section. Sox9 expression was found in most cells constituting crypt-like structures in the metaplasic area, including Paneth cells (Fig. 4, q and r). Thus, Sox9 expression also seems to be associated with Paneth cells in the pathological context of human intestinal metaplasia.

To gain insight into the mechanism underlying the Sox9-dependent differentiation of Paneth cells, we screened by real-time PCR colon carcinoma cell lines for expression of Paneth cell markers. All the tested cell lines (SW480, HT29Cl.16E, HCT116, and DLD-1) had detectable expression of such markers, and this expression was highest in HT29Cl.16E cells (unpublished data), which were chosen for further analyses. A moderate (fivefold) overexpression of Sox9 in these cells resulted in an up-regulation of expression of several Paneth cell markers, which was most prominent for lysozyme, the matrix metalloproteinase MMP7, and Angiogenin-4 (ANG-4) mRNAs (Fig. 4 s). Thus, Sox9 may regulate the differentiation of Paneth cells, at least in part, through the transcriptional regulation of

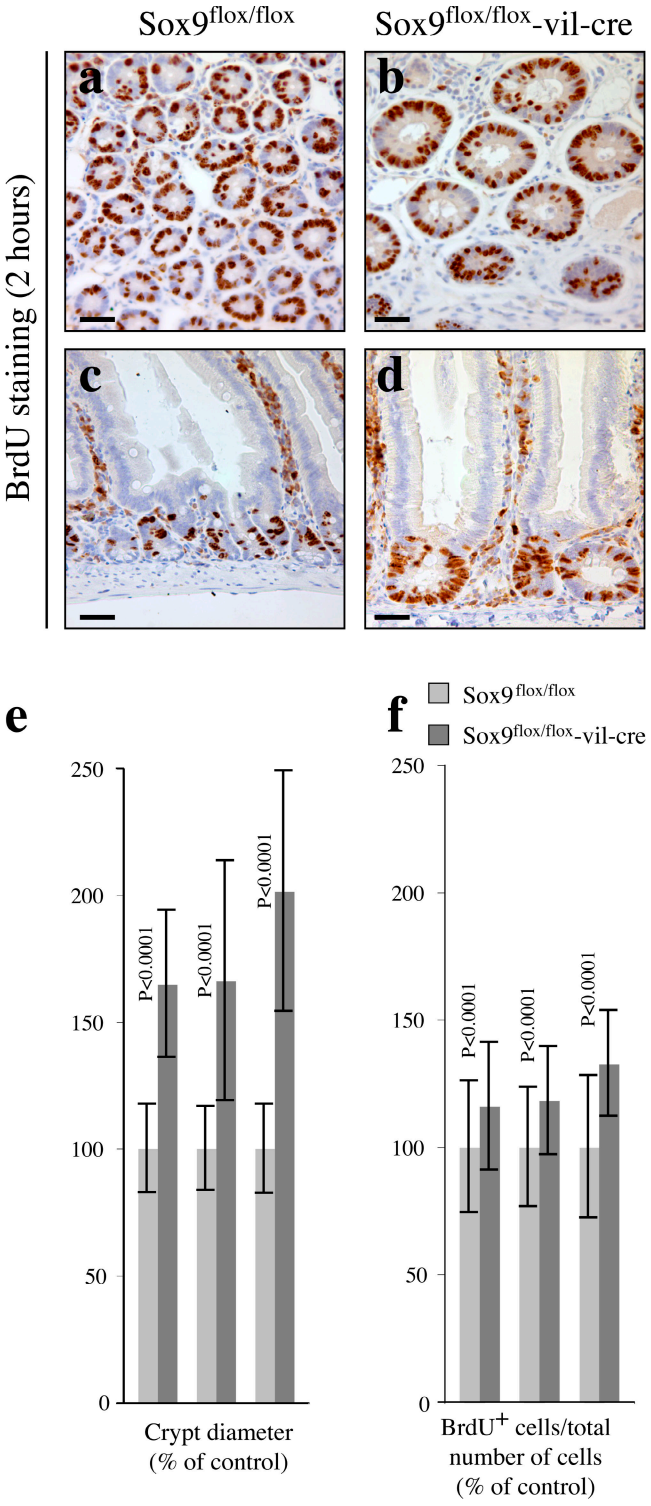

Figure 5. **Generalized hyperplasia develops in the absence of Sox9.** Difference of crypt size in the small intestines of Sox9$^{flox/flox}$ (a and c) and Sox9$^{flox/flox}$-vil-Cre (b and d) mice. (a and b) Crypt cross sections; (c and d) crypt longitudinal sections. Bars, 75 μm. (e) Histogram showing mean crypt diameters along the small intestines of three Sox9$^{flox/flox}$ and three Sox9$^{flox/flox}$-vil-Cre mice. Standard deviations are indicated. $P < 0.001$ (*t* test). (f) Histogram showing the BrdU incorporation rates (number of BrdU-positive cells/total number of cells) in crypts from three Sox9$^{flox/flox}$ and three Sox9$^{flox/flox}$-vil-Cre mice. Standard deviations are indicated. $P < 0.001$ (*t* test).

several markers of these cells. This regulation might be direct or indirect, but it likely contributes to the absence of identifiable Paneth cells in the intestinal epithelium of Sox9-deficient mice.

Increased cell proliferation and hyperplasia throughout the intestinal epithelium of Sox9-deficient mice

We then asked whether the homeostasis of the epithelium would be conserved despite the extension of the proliferative compartment into the usual Paneth cell area and found that, in fact, the crypt size of $Sox9^{flox/flox}$-vil-Cre mice seemed increased compared with $Sox9^{flox/flox}$ control mice (Fig. 5, a–d). When crypt diameters and BrdU incorporation rates were measured, an unambiguous increase of crypt size was found in Sox9-deficient mice (Fig. 5 e), and the ratio between BrdU-labeled cells and the total number of cells found in a crypt circumference was slightly, but reproducibly, increased in the Sox9-deficient mice (Fig. 5 f). This was statistically significant ($P < 0.0001$). The total number of cells in any crypt circumference increased according to the crypt size, indicating that the cell size was not affected (unpublished data). This indicates that the absence of Sox9 resulted in increased cell proliferation, leading, in turn, to crypt hyperplasia throughout the small intestine. The epithelium from the proximal colon was also found to display hyperplastic features, but

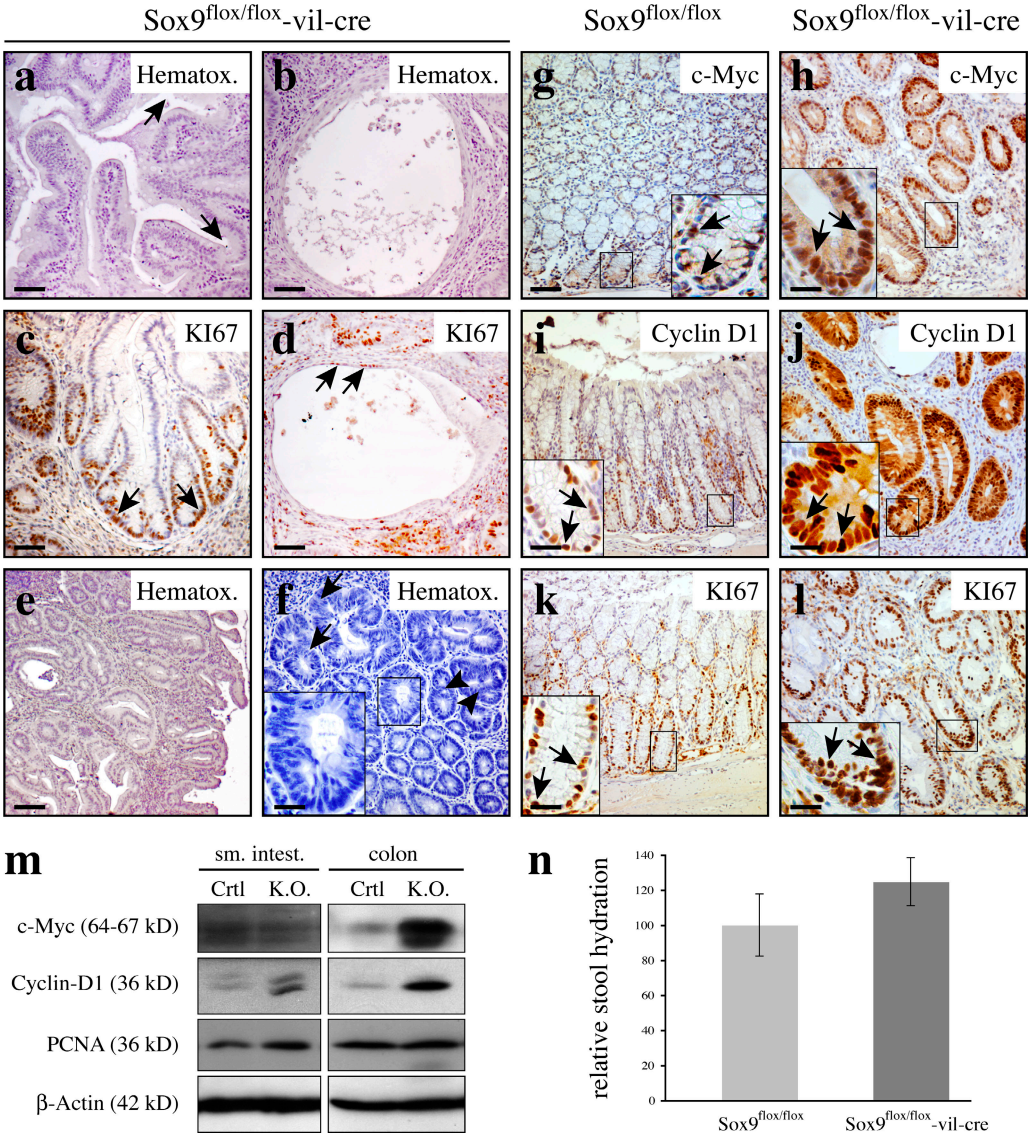

Figure 6. **Sox9 deficiency causes hyperplasia and dysplasia.** In the colon epithelium of $Sox9^{flox/flox}$-vil-Cre mice, hyperplastic lesions are found (hematoxylin staining) with branched and enlarged crypts (a). Some crypts have a cystic appearance (b). Such structures proliferate (c and d, arrows). Tubulovillous microadenomas spontaneously developed in hyperplastic areas of $Sox9^{flox/flox}$-vil-Cre mice (e). Typical example of the dysplastic-looking crypts (arrows) found in the distal half of the colon of Sox9-deficient mice. Arrowheads point at mutiadenoid crypts (f). Compared with normal tissue (g, i, and k), hyperplastic and dysplastic crypts of Sox9-deficient mice (h, j, and l) overexpress Wnt pathway target genes such as c-Myc (compare g and h) and cyclin D1 (compare i and j) and, accordingly, have elevated staining for the Ki67 proliferation marker (compare k and l). Insets in panels g–l show enlarged pictures of the indicated area of the panel, and arrows point at typical staining pictures. Western blot analysis of c-Myc, cyclin D1, and PCNA in the small intestine and colon of $Sox9^{flox/flox}$ and $Sox9^{flox/flox}$-vil-Cre mice. β-Actin expression is shown as a loading control (m). Stool hydration is increased in Sox9-deficient mice (n). Bars: (panels) 150 μm; (insets) 50 μm.

we were unable to perform accurate measurements because of its severely altered morphology in Sox9$^{flox/flox}$-vil-Cre mice.

Focal dysplastic crypts spontaneously develop in the colon of Sox9$^{flox/flox}$-vil-Cre mice

In addition to the general mild hyperplasia found throughout the intestine of Sox9$^{flox/flox}$-vil-Cre mice, extensive hyperplasia occurred, with occasional glandulocystic features, in the distal half of Sox9$^{flox/flox}$-vil-Cre mice colon. Numerous crypts were enlarged and branched, and some were extensively dilated with a cystic appearance (Fig. 6, a and b). Proliferation was correctly restricted to the bottom of hyperplastic crypts (Fig. 6 c). Cells constituting cystic crypts also proliferated, albeit modestly (Fig. 6 d), and were poorly differentiated (Fig. S2, D–F). In addition, tubulovillous microadenomas occasionally developed in hyperplastic areas of the epithelium with atypical tissue architecture (Fig. 6 e). Crypts with dysplastic features, including poor differentiation, pseudostratified nuclei, multiadenoid structures, and numerous mitosis, spontaneously developed in several locations along the colon of Sox9-deficient mice (Fig. 6 f). Interestingly, slight crypt hyperplasia/dysplasia was also detectable in the colon of 3-wk-old Sox9-deficient mice, indicating that these defects appear early but become more severe with time, likely as a consequence of increased cell proliferation (Fig. S2, G–J).

Some Sox9$^{flox/flox}$-vil-Cre mice had no detectable lesions but also had a normal colon morphology and had Paneth cells in their small intestine (unpublished data). In such mice, Sox9 staining was invariably identical to that of wild-type mice (unpublished data), indicating inefficiency of the Cre recombinase. Thus, dysplastic-like lesions were always found in the colon of true Sox9-deficient mice. That we never observed true carcinoma in the intestine of Sox9-deficient mice up to 6 mo old suggests, in turn, that Sox9 deficiency may not be sufficient, per se, to induce cell transformation.

Hyperplastic- and dysplastic-like crypts were found to strongly overexpress Wnt pathway–related genes, such as c-Myc and cyclin D1, suggesting an increase of Wnt-dependent transcriptional activity (Fig. 6, compare g with h, and i with j). This overexpression resulted from both an increase in the number of c-Myc– and cyclin D1–expressing cells and increased staining intensities in individual positive cells (unpublished data). Expression of the Ki-67 proliferation marker was also affected (Fig. 6, compare k with l). This finding was confirmed by Western blot analysis of extracts from Sox9$^{flox/flox}$ and Sox9$^{flox/flox}$-vil-Cre mice. Although few variations were found in the small intestine, likely because the few proliferating crypt cells are not sufficiently represented in the whole epithelial cell population, a clear increase of c-Myc and cyclin D1 was evident in the Sox9-deficient colon (Fig. 6 m). The increase in cell proliferation rate in Sox9-deficient mice (estimated as 15% from BrdU incorporation rates) was probably not sufficient to be clearly visualized with an anti-PCNA Western blot (Fig. 6 m). An alternative explanation is that, although crypt hyperplasia and increased BrdU intake are evident, the density of crypts is reduced in Sox9-deficient animals, which may compensate the increased proliferation observed in each crypt. That the overexpression of Wnt target genes is much more visible in the colon samples may

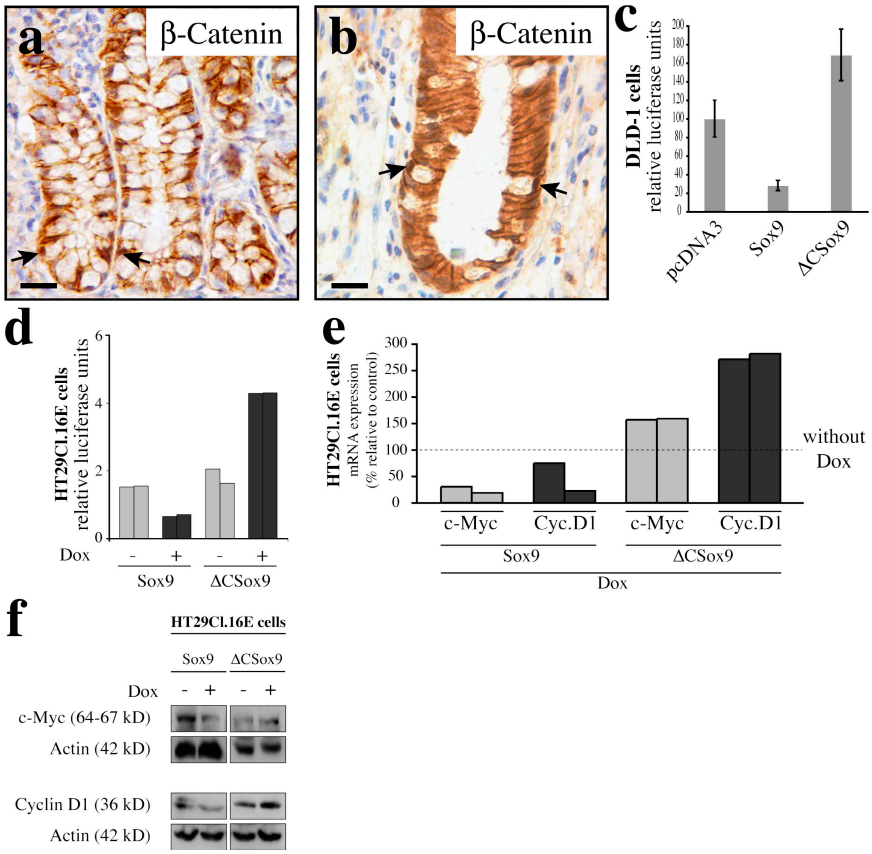

Figure 7. **Sox9 fine-tunes the activity of the β-catenin–Tcf4 transcriptional effector of the Wnt signaling pathway.** (a and b) No alteration of the number of cells containing nuclear β-catenin (arrows) is found in Sox9-deficient animals. Bars, 40 μm. (c and d) Analysis of the transcriptional activity of the β-catenin–Tcf4 complex, using a luciferase reporter system (Morin et al., 1997). Endogenous transcriptional activity of the β-catenin–Tcf4 complex. Transient overexpression of Sox9 inhibits the β-catenin–Tcf4 activity, whereas overexpressing ΔCSox9 increases it (c). Similarly, induction of Sox9 overexpression in the HT29-16E-Sox9 cell line causes inhibition of β-catenin–Tcf4 transcriptional activity, whereas inducing overexpression of ΔCSox9 in the HT29-16E-ΔCSox9 cell line resulted in a considerable increase of this activity (d). Regulation of the β-catenin–Tcf4 transcriptional activity impacted c-Myc and cyclin D1 mRNA expression. The amounts of mRNA after doxycycline induction are indicated as a percentage of the noninduced state (e, dashed line). These variations at the mRNA level were reflected at the protein level (f).

reflect either the bigger size of the crypts relative to the entire epithelium in the colon compared with the small intestine or the presence of dysplastic-like lesions, which strongly overexpress Wnt target genes, in the colon samples.

We then asked whether these alterations in the small intestine and colon structure may impact the mouse physiology. Indeed, the weight of $Sox9^{flox/flox}$-vil-Cre animals was always reduced (unpublished data), compared with the related $Sox9^{flox/flox}$ control mouse. This reduction was modest (mean 17%; $n = 10$) but, despite the heterogeneity in the age and sex of the pairs of animals tested, reached statistical significance ($P < 0.05$, *t* test). In addition, stools from $Sox9^{flox/flox}$-vil-Cre mice were more hydrated than those of control mice, indicating a partial impairment of the colonic epithelium function in Sox9-deficient animals (Fig. 6 n).

Sox9 modulates Wnt pathway activity in vitro

To understand the molecular bases of the observed up-regulation of Wnt target genes, we compared the expression of nuclear β-catenin, the hallmark of Wnt signaling, in Sox9-deficient versus control mice. Comparable results were found in both situations, with a typical nuclear staining of some cells scattered through the crypt bottom (Fig. 7, a and b; and Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200704152/DC1), indicating that the overexpression of Wnt target genes found in Sox9-deficient animals was not due to increased levels of β-catenin, in the crypt nuclei of $Sox9^{flox/flox}$-vil-Cre mice.

We and others have shown that the level of Sox9 expression regulates the transcriptional activity of the β-catenin–Tcf4 complex in cultured HEK293 cells (Akiyama et al., 2004b; Blache et al., 2004). Physical interaction between Sox9 and β-catenin has been reported, resulting in a competition between Sox9 and Tcf4 for binding to β-catenin. Formation of the Sox9–β-catenin complex results in degradation of the two proteins (Akiyama et al., 2004b). We thus hypothesized that the absence of Sox9 in crypt cells of $Sox9^{flox/flox}$-vil-Cre mice, where Wnt signaling is physiologically active, might result in increased availability of the nuclear pool of β-catenin for binding to Tcf4. To test this, we analyzed the transcriptional activity of the β-catenin–Tcf4 complex after manipulation of Sox9 expression in colon carcinoma cell lines containing a constitutive activation of the β-catenin–Tcf transcriptional complex (Korinek et al., 1997). The basal β-catenin–Tcf4 activity present in DLD-1 cells was efficiently inhibited by Sox9 and increased after overexpression of a dominant-negative version of Sox9 (Fig. 7 c). Comparable results were obtained in other colon carcinoma cell lines, such as SW480, HCT116, and HT29Cl.16E (Fig. S3, C–E), demonstrating that even in colon carcinoma cells, in which nuclear β-catenin accumulates constitutively, the level of Sox9 expression critically modulates the level of β-catenin–Tcf transcriptional activity.

We used the HT29Cl.16E-Sox9 and HT29Cl.16E-ΔCSox9 cell lines, inducibly overexpressing full-length or C-terminally truncated Sox9, respectively, to test whether Sox9 overexpression would result in a down-regulation of expression of endogenous Wnt pathway target genes. Doxycycline induction of Sox9 or ΔCSox9 expression also resulted in down- or up-regulation, respectively, of the β-catenin–Tcf complex activity (Fig. 7 d). Using real-time PCR, we found that induction of Sox9 expression resulted in a down- regulation of c-Myc and cyclin D1 mRNA expression, whereas that of ΔCSox9 resulted in an up-regulation of these two Wnt target genes (Fig. 7 e). C-Myc and cyclin D1 protein expression changed accordingly (Fig. 7 f).

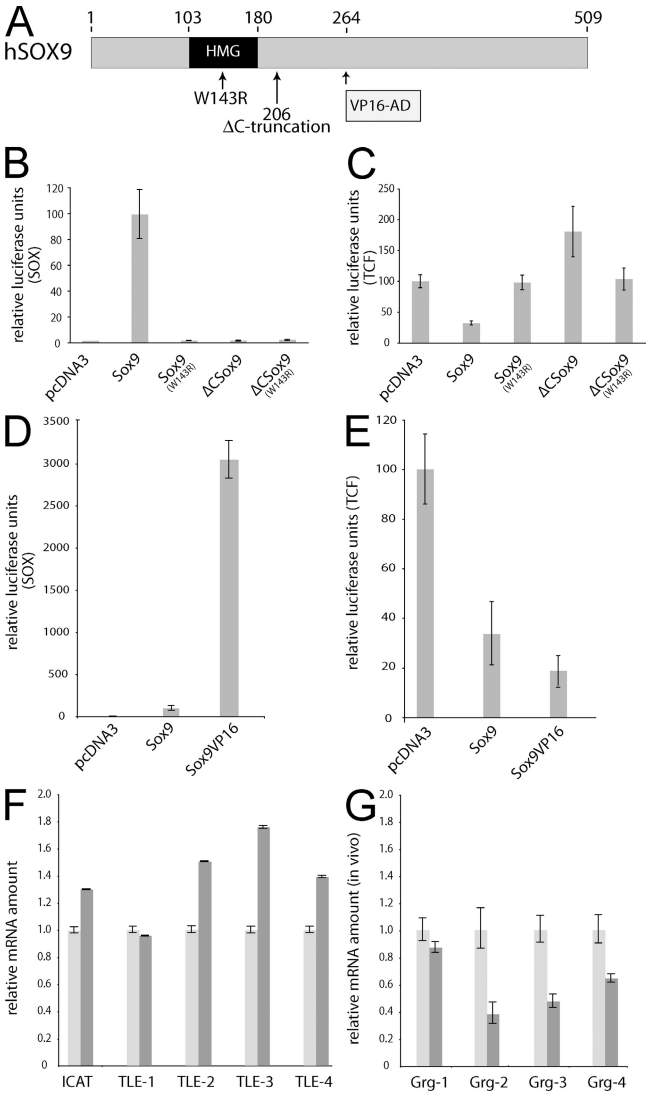

Figure 8. **Sox9 transcriptionally activates expression of inhibitors of the β-catenin–Tcf activity.** Structure–function analysis of the inhibitory function of Sox9 on the β-catenin–Tcf activity (a–e) in DLD-1 cells. (a) Diagram showing the different constructs used in this study. (b) Transcriptional activity of Sox9 and its mutated or truncated versions on a Sox-luciferase reporter system. (c) Inhibition of the β-catenin–Tcf activity by Sox9 and its truncated or mutated versions. (d) Transcriptional activity of the Sox9-VP16 chimeric protein compared with that of Sox9 on a Sox-luciferase reporter system. (e) Inhibition of the β-catenin–Tcf activity by Sox9 and Sox9-VP16. In panels b and d, the activity of the wild-type Sox9 construct is arbitrarily set to 100. In panels c and e, the endogenous β-catenin–Tcf activity is arbitrarily set to 100. (f and g) Real-time RT-PCR analysis of the expression of the inhibitor of β-catenin and Tcf (ICAT) and Groucho-related inhibitors of the β-catenin–Tcf activity in HT29Cl.16E-Sox9 cells before and after induction of exogenous Sox9 expression (f) and in the intestinal mucosa of $Sox9^{flox/flox}$ versus $Sox9^{flox/flox}$-vil-Cre mice (g). Results are expressed relative to the noninduced or nonrecombined states. Standard deviations are indicated.

This result demonstrates modulation of expression of key β-catenin–Tcf target genes by Sox9, but the molecular mechanism underlying this regulation remained unclear, as the ΔC-Sox9 construct does not contain the domain thought to interact with β-catenin (Akiyama et al., 2004b).

The previously reported physical interaction between Sox9 and β-catenin had been detected after overexpression of tagged Sox9 and β-catenin in COS cells (Akiyama et al., 2004b). We reasoned that if this interaction was important to modulate Wnt signaling in colon carcinoma cells, then the endogenous complex should be readily detectable in these cells. Despite repeated efforts, no β-catenin–Sox9 complex could be immunoprecipitated, although Tcf4 coimmunoprecipitated with β-catenin (unpublished data). We concluded that β-catenin–Sox9 complexes are probably not abundant in colon carcinoma cells. In addition, the subcellular localization of both β-catenin and Tcf4 was unchanged after transient overexpression of Sox9 in SW480 cells (Fig. S4, A–F, available at http://www.jcb.org/cgi/content/full/jcb.200704152/DC1), and the level of β-catenin expression was not decreased after induction of Sox9 expression in the HT29Cl.16E-Sox9 cells (Fig. S4 G).

To identify another possible mechanism, we performed mutational analysis of the Sox9-mediated inhibition of the β-catenin–Tcf activity. The W143R point mutation (Fig. 8 a) was originally identified in a campomelic dysplasia patient and abolishes the DNA binding properties of Sox9 (Meyer et al., 1997). We introduced this mutation in both the Sox9 and ΔCSox9 constructs. The resulting products did not have any transcriptional activity using Sox-luciferase reporters (Fig. 8 b) and failed to modify β-catenin–Tcf activity (Fig. 8 c). This indicates that Sox9-mediated inhibition of the β-catenin–Tcf activity requires an intact DNA binding domain of Sox9, which raises the possibility that this inhibition might be at least partly due to transcriptional regulation. To test this, we used a Sox9 construct in which the C-terminal domain of Sox9, involved in transactivation, and in the interaction with β-catenin (Akiyama et al., 2004b), is removed and replaced by the unrelated VP16 transactivating domain (Kamachi et al., 1999; Fig. 8 a). Transient transfection of this construct in colon carcinoma cells showed that the Sox9-VP16 chimeric protein potently activates transcription of a Sox-luciferase reporter gene (Fig. 8 d) and inhibits the β-catenin–Tcf activity even more efficiently than wild-type Sox9 (Fig. 8 e). We conclude that Sox9-mediated inhibition of β-catenin–Tcf activity involves transcriptional regulation.

We then aimed to identify potential Wnt pathway inhibitors, such as the inhibitor of β-catenin and Tcf (ICAT; Tago et al., 2000) and Groucho-related (Grg/TLE) corepressors (Cavallo et al., 1998; Roose et al., 1998), which might be transcriptionally regulated by Sox9. In vitro, the induction of Sox9 expression in HT29Cl.16E-Sox9 cells resulted in increased expression of the ICAT and TLE2-4 genes, whereas the expression of TLE1 was unaffected (Fig. 8 f). We then analyzed the expression of the mouse homologues of these genes in $Sox9^{flox/flox}$-vil-Cre mice and $Sox9^{flox/flox}$ mice, and we found that, again, the expression of Grg2, -3, and -4 was obviously down-regulated in Sox9-deficient mice, whereas that of Grg1 remained unchanged (Fig. 8 g). Icat expression seemed unchanged in Sox9-deficient mice, but this result varied with the samples analyzed (unpublished data). Although additional Wnt pathway inhibitors may be involved, this result provides a basis to explain the increased expression of Wnt target genes observed in Sox9-deficient mice, despite the absence of an increase in nuclear β-catenin expression.

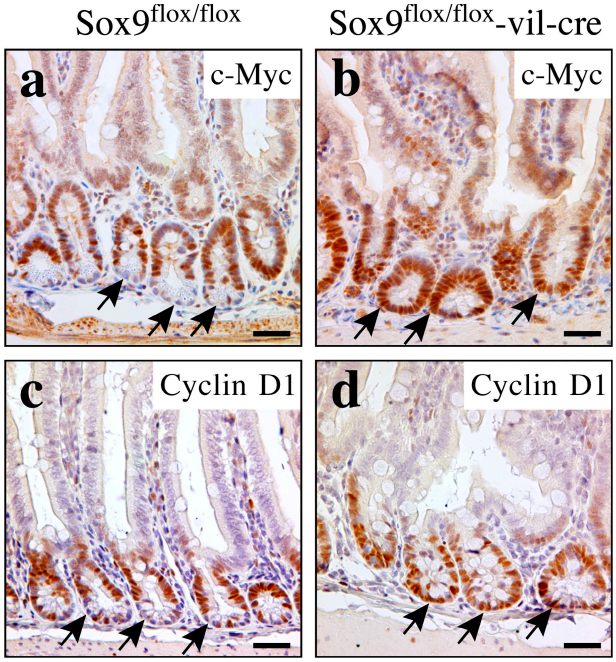

Figure 9. **Alteration of c-Myc and cyclin D1 expression in the bottom of Sox9-deficient crypts.** C-Myc and cyclin D1 expression was analyzed by immunohistochemistry in the intestine of $Sox9^{flox/flox}$ (a and c) and $Sox9^{flox/flox}$-vil-Cre (b and d) mice. Arrows indicate the Paneth cell compartment in $Sox9^{flox/flox}$ control mice (a and c) and the equivalent location in the $Sox9^{flox/flox}$-vil-Cre mice (b and d). Bars, 50 μm.

Sox9 expression defines compartmentalization of the crypt

When we stained small intestinal tissue for c-Myc and cyclin D1 protein expression, we found that both genes were expressed as expected in the stem/progenitor cell compartment but that their expression was strongly decreased in Paneth cells (Fig. 9, a and c). In contrast, the absence of Sox9 resulted in uniform expression of both c-Myc and cyclin D1 in the whole crypt bottom (Fig. 9, b and d), which then lacked Paneth cells.

Thus, Sox9 expression may define a compartment in which nuclear β-catenin expression results in a genetic program that includes low c-Myc and cyclin D1 expression, and in which differentiation of Paneth cells occurs. In the absence of Sox9, Paneth cells do not develop, and the genetic program characterized by high c-Myc and cyclin D1 expression expands to include the whole crypt base.

Discussion

This study shows that loss of Sox9 function affects the intestinal epithelium physiopathology at the level of (1) cell differentiation, (2) tissue homeostasis, and (3) colon tissue morphology. These different aspects of the phenotype were sex independent.

Control of cell differentiation by Sox9—relationship with the Wnt pathway

Interference with the Wnt pathway results in depletion of the Paneth cell lineage (Pinto et al., 2003) and, conversely, loss of the key negative regulator of the pathway, Apc, results in an expansion of the Paneth cell lineage (Sansom et al., 2004; Andreu et al., 2005). In addition, the terminal maturation of Paneth cells requires Wnt signaling through the Frizzled-5 receptor (van Es et al., 2005). As we previously showed that Sox9 is a transcriptional target of Wnt signaling, the Sox9 requirement for the differentiation of the Paneth cell lineage is likely to reflect its role to specify this specific aspect of the Wnt pathway. In this view, Sox9 function would be expected to occur at an earlier stage than the Wnt–Frizzled-5 pathway, during the course of Paneth cell differentiation, as this pathway was previously shown to be involved in the terminal differentiation of already committed Paneth cells (van Es et al., 2005). Although less likely, an alternative in which Sox9 and Wnt signaling, as two distinct pathways, would control Paneth cell differentiation cannot be formally excluded.

The functional consequence of the loss of the Paneth cell lineage, a component of the innate immunity, is still a matter of debate. In this study, we did not find an increased mortality in the Sox9-deficient mice lacking Paneth cells. In agreement with this, a previous experiment with ablation of Paneth cells concluded that the turnover of epithelial cells was not grossly perturbed. In addition, no major alteration of microorganism population along the crypt–villus axis was detected (Garabedian et al., 1997). On the other hand, a role of Paneth cells in regulating the villus angiogenesis (Stappenbeck et al., 2002) and in clearing bacterial infections (Wilson et al., 1999) have been described, although no major impact was reported at the physiopathological level.

The reduction of goblet cell numbers indicates that Sox9 is also involved in the differentiation of this lineage. This quantitative impact of the Sox9 deficiency on the goblet cell population is probably physiologically relevant given the key role of mucus in protecting the epithelium against the luminal content, well illustrated, for instance, by the colon epithelium tumorigenesis resulting from a deletion of the *Muc2* gene (Velcich et al., 2002). This function of Sox9 in regulating the differentiation of goblet cells might also play a part in the cellular response to Wnt signals, as altered goblet cell differentiation was also found in mice in which the Wnt pathway has been impaired (Pinto et al., 2003). That Sox9 deficiency results in the complete absence of Paneth cells but leads only to a reduction of the goblet cell lineage might indicate different Sox9 requirements for the differentiation of these two lineages, and suggests that additional factors might be involved in this process, at least for goblet cell lineage differentiation.

What is the function of Sox9 in goblet cell differentiation?

In a previous report, we showed that overexpression of Sox9 in the LS174T colon carcinoma cell line repressed the *Muc2* gene (Blache et al., 2004). This apparently contrasts with the present finding that the absence of Sox9 in the intestinal epithelium causes an obvious reduction of the number of goblet cells in vivo. The remaining goblet cells express the *Muc2* gene, which indicates their maturation into functional mucus-producing cells. The apparent discrepancy between the two experiments likely lies in the fundamentally different nature of the experimental models used (i.e., LS174T human colon carcinoma cells grown in vitro versus mouse intestinal epithelium in its physiological tissue context). We conclude that Sox9 definitely plays a role in the differentiation of the goblet cell lineage, and we hypothesize that this role occurs primarily at the level of cell fate choice, similar to what was shown previously in the developing nervous system (Spokony et al., 2002). In addition, as suggested by in vitro data and the respective expression patterns of Sox9 and Muc2, down-regulation of Sox9 expression might be necessary to allow terminal maturation of committed goblet cells, although this hypothesis is difficult to test using the present gene inactivation model.

"Gatekeeper" role of Sox9 in modulating Wnt pathway activity?

Our in vitro and in vivo results indicate that Sox9 down-regulates the transcriptional activity of the β-catenin–Tcf4 complex on endogenous target promoters, such as the *c-Myc* and *cyclin D1* gene promoters. Although previous studies indicated that Sox9 could inhibit the activity of the β-catenin–Tcf4 complex in HEK293 cells transiently transfected with artificial luciferase reporters (Akiyama et al., 2004b; Blache et al., 2004), the observed regulation of the β-catenin–Tcf4 complex transcriptional activity on endogenous target gene promoters, in cultured colon carcinoma cells known to have a constitutive β-catenin–Tcf4 activity, was somewhat unexpected. In this study, although down-regulation of an artificial luciferase Tcf reporter gene was obvious, that of endogenous β-catenin–Tcf target genes was slighter and necessitated optimization of the cell culture condition. Indeed, Sox9-mediated down-regulation c-Myc and cyclin D1, which was hardly detectable in low-confluence cell culture condition, became evident when a more confluent cell culture setting was used. In agreement with this, a down-regulation of the β-catenin–Tcf4 complex has been reported as cultured colon carcinoma cells reach confluence (Mariadason et al., 2001). Thus, in low- confluence culture conditions, the endogenous level of β-catenin–Tcf4 complex likely saturates the endogenous target gene promoters, even in the presence of exogenous Sox9. In contrast, the transfected luciferase Tcf reporter system exists in multiple copies in each transfected cell, which might explain its increased sensitivity to Sox9 regulation. When cells are more confluent, the endogenous β-catenin–Tcf activity likely decreases and allows regulation of endogenous Wnt target genes by Sox9. This also explains the apparent discrepancy between the results presented here and in our previous report, in which we showed that c-Myc was not regulated by Sox9 in low-confluence cell culture conditions (Blache et al., 2004). Thus, even in cells in which the Wnt pathway is constitutively active, Sox9 may play an important retroinhibition role.

We could show that the transcriptional activity of Sox9 is involved in this inhibition, and genes encoding inhibitors of the β-catenin–Tcf activity have been found to be regulated by Sox9

in vitro and in vivo. This finding might explain how many Sox proteins, which lack homology outside the HMG DNA binding domain, have the conserved property of inhibiting β-catenin–Tcf activity.

In vivo, inactivation of the Sox9 gene results in an increase in the rate of BrdU incorporation in crypt epithelial cells. This correlates with the development of hyperplastic- and dysplastic-like crypts, which overexpress Wnt target genes. The percentage of BrdU-positive cells obtained in this study with the $Sox9^{flox/flox}$ control mice (46–56% with a 2-h BrdU pulse) is consistent with previous analyses showing a ^3H labeling index of 29–33% after a 1-h treatment with ^3H-thymidine (Cheng and Bjerknes, 1982). The 16–32% increase in BrdU-positive cells in $Sox9^{flox/flox}$-vil-Cre mice is therefore likely to significantly impact epithelial homeostasis, given the rate of renewal of the intestinal epithelium. This increased proliferation rate likely explains the occurrence of Wnt target genes overexpressing hyperplastic- and dysplastic-like crypts in Sox9-deficient mice, whereas this was never observed in $Sox9^{flox/flox}$ animals. In agreement with this, an apparent increase of Musashi-expressing cells was found in Sox9-deficient mice, suggesting that Sox9 might regulate the number of stem cells in the intestinal epithelium.

A role of Sox9 in regulating cell proliferation has already been proposed in chondrocytic cell lines, in which Sox9 promoted differentiation (Panda et al., 2001), as well as in breast and prostate tumor cell lines (Afonja et al., 2002; Drivdahl et al., 2004). Here, however, Sox9 is specifically expressed, in a normal physiological situation, in the proliferative compartment of the intestinal epithelium, and activity of the protooncogenic Wnt pathway is required for its expression (Blache et al., 2004). Thus, Sox9 might function as a gatekeeper in intestinal crypts to prevent overactivity of the Wnt-dependent transcriptional program.

Role of the Sox9-mediated inhibition of the Wnt pathway in the differentiation of Paneth cells

Wnt signaling is known to be important for the maintenance of the proliferating compartment (Korinek et al., 1998; Pinto et al., 2003; Kuhnert et al., 2004), as well as for the differentiation and maturation of Paneth cells (Pinto et al., 2003; Andreu et al., 2005; van Es et al., 2005). Here, we found that c-Myc, a central effector of the Wnt pathway, as well as cyclin D1, was expressed in the proliferative compartment of the small intestinal epithelium but not, or at a much lower level, in the Paneth cell compartment of wild-type mice. This was unexpected given that Paneth cells have been reported to express the highest levels of nuclear β-catenin (van Es et al., 2005). This suggests that distinct genetic programs are set, downstream to the Wnt pathway, in the stem/progenitor versus Paneth cell compartments, and this was reflected by a recent transcriptomic study (Van der Flier et al., 2007). The absence of Sox9 results in a single proliferative stem/progenitor compartment, in which all the cells express c-Myc and cyclin D1. This suggests that Sox9 is required to specify the Paneth cell compartment, in which the presence of nuclear β-catenin results in a "Paneth cell" genetic program instead of a "stem/progenitor" program that includes c-Myc expression.

This also raises the question of the role of Sox9 in Wnt pathway–triggered tumorigenesis. Our data suggest that loss of Sox9 might promote tumorigenesis, either through the reduction of mucus protection or through up-regulation of the β-catenin–Tcf activity. It should be noted, however, that up-regulation of the β-catenin–Tcf complex activity by loss of Sox9 function is expected to occur only in cells in which Wnt pathway activity preexists, and Sox9 deletion might not be sufficient to cause neoplasia. Consistent with this, no evidence for spontaneous neoplasms was found in Sox9-deficient mice up to 6 mo old.

Conclusion

This study reveals the dual role played by Sox9 in relation with the Wnt pathway. (1) It likely mediates part of the Wnt-dependent program, involved in Paneth and goblet cell differentiation. Sox9 thus provides the first example of a transcription factor involved in specifying the cellular response to Wnt signals, which potentially trigger very diverse cellular behaviors, including proliferation, differentiation, sorting of the cells along the crypt–villus axis, etc. (2) Sox9 fine-tunes the transcriptional output of the canonical Wnt pathway in vitro, and inactivation of this gene causes an increase of BrdU incorporation rate and leads to hyperplasia and dysplasia of the intestinal epithelium and increased putative stem cell numbers. (3) Finally, Sox9 may also act independently of the Wnt pathway to control colon epithelium morphology, a role that had not been previously reported for the Wnt pathway, although architectural degeneration was described after Dickkopf-1 overexpression in the colon epithelium (Kuhnert et al., 2004).

Materials and methods

Mouse lines

The vil-Cre strain (el Marjou et al., 2004) was in a nearly pure C57BL/6 background (at least 14 backcrosses to this background). To generate $Sox9^{+/flox}$ mice (Kist et al., 2002), exons 2 and 3 of the Sox9 gene were flanked by LoxP sequences. Cre recombination results in the deletion of the last two exons, which encode part of the HMG DNA binding domain and the transactivation domain, thus resulting in a likely null allele. A peptide might still be produced from the first exon, but this would not contain any known functional domain. $Sox9^{+/flox}$ mice were originally on a 129P2/OlaHsd × C57BL/6 mixed genetic background and have been backcrossed to C57BL/6 for three generations. These N3 mice were made $Sox9^{flox/flox}$ by sister–brother mating before being crossed with the vil-Cre strain.

Cells and cell culture conditions

Colorectal cancer cell lines HCT116, HT29.16E, DLD1, and SW480 were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Eurobio), 1% L-glutamine, and penicillin/streptomycin. The HT29.16E-Sox9 and HT29.16EΔCSox9 cell lines were described previously (Blache et al., 2004; Jay et al., 2005).

DNA constructs, transient transfections, and luciferase assays

Full-length human Sox9 and C-terminally truncated (dominant-negative) human Sox9 constructs were described previously (Südbeck et al., 1996), as were the pTOP-FLASH and pFOP-FLASH reporter constructs (Morin et al., 1997). The Sox9-VP16 construct (Kamachi et al., 1999) was a gift from H. Kondoh (Institute for Molecular and Cell Biology, Osaka University, Osaka, Japan). The full-length human Sox9 (W143R) and the C-terminally truncated human Sox9 (W143R) expression constructs were constructed from a human Sox9(1–304) construct containing the W143R mutation, provided by P. de Santa Barbara (Institut National de la Santé et de la Recherche Médicale, Montpellier, France). DLD1, SW480, HCT116, and HT29-16E cells were cotransfected (EXGEN500; Euromedex) with 0.25 μg

pcDNA3, Sox9, or ΔCSox9 DNA constructs and 0.25 μg TCF/LEF-1 reporter (pTOP-FLASH) or control vector (pFOP-FLASH), using standard procedures. 0.025 μg pRLSV-Renilla was used as an internal control. Luciferase assays were performed with the Dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. Luciferase activities in cell lysates were normalized relative to the Renilla luciferase activity, and the indicated activities represent the TOPFLASH/FOPFLASH ratio, indicative of the Tcf binding site-specific activity. Each experiment was performed in duplicate and repeated several times, and representative examples are shown.

Quantitative RT-PCR

Total RNAs were prepared using the RNeasy minikit (QIAGEN) from HT29.16E-Sox9 or HT29.16E-ΔCSox9 cells grown to 100% confluence and treated or not for 6 d with doxycycline. For mRNA quantification, 2.5 μg of total RNA was pretreated with DNase RQ1 (Promega) for 30 min at 37°C and used for reverse transcription with M-MLV reverse transcriptase (Invitrogen). Quantitative PCR was performed using the LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics). Results were normalized with GAPDH expression.

Primers used are as follows: hGAPDH, forward (5′-GGTGGTCTCCTCTGACTTCAACA-3′) and reverse (5′-GTTGCTGTAGCAAATTCGTTGT-3′); hCYCLIN D1, forward (5′-CCGTCCATGGGGAAGATC-3′) and reverse (R-5′-ATGGCCAGCGGGAAGAC-3′); hc-MYC, forward (5′-CGTCTCCACACACATCAGAGCAA-3′) and reverse (5′-TCTTGGCAGCAGGATAGTCCTT-3′); hICAT, forward (5′-GCTCTGGTGCTTTAGTTAGG-3′) and reverse (5′-GCACTTGGTTTCTTTCTTTTC-3′); hTLE1, forward (5′-AAAGAGGAGGCGACAAGT-3′) and reverse (5′-TTCCGTTCCTCAATCCTACAA-3′); hTLE2, forward (5′-GCCCTGCCTAGGAACCGT-3′) and reverse (R-5′-TTTTATTTCCACGAGGTCCCC-3′); hTLE3, forward (5′-CTCCCTCAAGTTCGCCTAC-3′) and reverse (5′-TCCGCTGAAATGTCACAACTC-3′); hTLE4, forward (5′-TGTGGCAAATGGTTTGTAAGCAC-3′) and reverse (5′-CCCCAGAGCCAGTGACAATGTAT-3′); hLYSOZYME, forward (5′-AAACCCCAGGAGCAGTTAAT-3′) and reverse (5′-CAACCCTCTTTGCACAAGCT-3′); hMMP7, forward (5′-GACTTACCGCATATTACAGTG-3′) and reverse (5′-ATCCCTAGACTGCTACC-3′); hANG4, forward (5′-CTGGGCGTTTTGTTGGTC-3′) and reverse (5′-GGTTTGGCATCATAGTGCTGG-3′); mGapdh, forward (5′-ATTGTCZGCAATGCATCCTG-3′) and reverse (5′-ATGGACTGTGGTCATGAGCC-3′); mIcat, forward (F-5′-CCCGGGAAGAGTCCGGAGG-3′) and reverse (5′-GTCTTCCGTCTCCGATCTGG-3′); mGrg1, forward (5′-GCTGTGGTGGCCTATGGG-3′) and reverse (5′-CATTTGGCCATCAGCAGTAACAT-3′); mGrg2, forward (5′-GAGCTGGATCAGGGATTTACACT-3′) and reverse (5′-CCAGCCTGGTTTACATAGTTTCA-3′); mGrg3, forward (5′-CTGTGACATTTCAGCGGATGACA-3′) and reverse (5′-CCCTCCTTCTGCCGTCCT-3′); mGrg4, forward (5′-GCTGCAGCGACGGTAACATC-3′) and reverse (5′-CAGAGCTTGGTGCCATCATTAGA-3′).

Immunohistochemistry and Western blot

Immunohistochemistry was performed essentially as described previously (Blache et al., 2004). Sections of human intestinal metaplasia were provided by F. Bibeau (CRLC Val d'Aurelle Lamarque, Montpellier, France). In brief, for preparation of mouse intestinal sections, the intestinal tract was dissected as a whole from 2–6-mo-old mice and flushed gently with cold PBS to remove any fecal content. The small intestine and colon were rolled up into a compact circle and fixed in 4% PFA in PBS at RT for 4 h, dehydrated, embedded in paraffin, and sectioned at 5 μm, using standard procedures. Sections were dewaxed in a xylene bath and rehydrated in graded alcohols. Endogenous peroxidase activity was quenched with 1.5% H_2O_2 in methanol for 20 min and washed in PBS. Antigen retrieval was performed by boiling slides in 10 mM sodium citrate buffer, pH 6.0, except for anti–c-Myc and anti–cleaved Caspase3 antibodies, for which antigen retrieval was performed by boiling slides 20 min in 100 mM TRIS and 12.6 mM EDTA, pH 9.0. Nonspecific binding sites were blocked with 1% BSA, 3% NGS, and 0.2% Triton X-100 in PBS for 45 min at RT for all antibodies staining except for anti–c-Myc (1% BSA in PBS) and anti-Ki67 (no blocking). Slides were incubated with the primary antibodies overnight at 4°C in PBS with 0.1% BSA. In all cases, Envision+ (DakoCytomation) was used as a secondary reagent, stainings were developed with DAB (brown precipitate) or Vector Vip substrate (purple precipitate), and hematoxylin counterstain was used. After dehydration, sections were mounted in Pertex (Histolab). For alkaline phosphatase activity staining, sections were dewaxed and rehydrated as described, and the alkaline phosphatase substrate (Vector red; Vector Laboratories) was applied for 10 min. Sections were counterstained, dehydrated, and mounted as described. For BrdU countings, mice were injected with a solution (0.1 milligram per gram of mouse body weight) of Brdu diluted in PBS. Mice were killed 2 h after injection. For BrdU staining, the same method as explained above was used, except for an additional step in HCl 2N for 45 min, added after antigen retrieval. For the Musashi staining, an ABC kit (Vectastain) was used instead of the Envision+ system.

For Western blotting, an equal amount of protein, measured by the Bradford assay, was loaded on each lane of the gel. Protein lysates and immunoblotting were performed as described previously (Hollande et al., 2003). Proteins were visualized using ECL Plus (GE Healthcare), and the bands were quantified by densitometry using ImageJ 1.32J (NIH).

Quantification of c-Myc and cyclin D1 stainings

Immunofluorescence stainings were performed essentially as immunohistochemical stainings, but a goat anti–rabbit secondary antibody (Alexa Fluor 488; Invitrogen) was used, and nuclei were counterstained with Hoechst. Fluorescence quantifications were performed with ImageJ software. Nuclei were selected using the freehand selection tool, and integrated densities were measured.

Antibodies

The Muc2 antibody (1:250) was provided by I. Van Seuningen (Institut National de la Santé et de la Recherche Médicale, Lille, France), the Cdx2 antibody (1:200) was provided by J.-N. Freund (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France), the Musashi-biotin antibody (1:500) was provided by H. Okano (Keio University, Tokyo, Japan), and the anti-Sox9 was described previously (de Santa Barbara et al., 1998). β-Actin A5441 (Western blot; 1:5,000) was purchased from Sigma-Aldrich, anti-Ki-67 (1:200) and anti-lysozyme (1:1,000) were purchased from DakoCytomation, anti-PCNA (1:100) and anti–cyclin D1 (1:100) were purchased from Neomarker, anti-c-Myc (1:50) was purchased from Santa Cruz Biotechnology, Inc., anti–E-cadherin (1:150) and anti–β-catenin (1:50) were purchased from BD Biosciences, anti-Claudin2 (1:100) was obtained from Zymed Laboratories, anti–chromogranin A (1:300) was purchased from Immunostar, anti-BrdU (1:200) was obtained from Novocastra, and anti-Caspase3 (1:100) was purchased from Cell Signaling.

Analysis of stool hydration

To determine stool hydration, freshly isolated stool was weighted before and after overnight incubation at 50°C. Four mice of each genotype were analyzed. Mean and standard deviation values, as well as statistical significance (*t* test) were calculated using Excel (Microsoft).

Measurements of crypt diameter and of the number of BrdU-positive cells

Fields containing crypt transverse sections were selected randomly at several locations along the rostrocaudal axis of the small intestine. Only sections with several BrdU-positive cells and an apparent lumen were considered to avoid large variation in the position of the section in the crypt–villus axis. Six to nine fields, each containing 6–40 measured crypts, were analyzed by two individuals, who were blinded to the mouse genotypes, and two mice of each genotype were analyzed. Mean and standard deviation values, as well as statistical significance (*t* test) were calculated using Excel.

Image acquisition and manipulation

Immunohistochemistry images were acquired at RT using either an Axiophot microscope (Carl Zeiss MicroImaging, Inc.) with 10× 0.3 Plan Neofluar or 40× 1.0 Plan Apochromat lenses (Carl Zeiss MicroImaging Inc.) and a camera (DXM1200; Nikon) or an Eclipse 80i microscope (Nikon) with Plan Fluor 10× 0.3, 20× 0.5, 40× 0.75, and 60× 0.5–1.25 lenses (Nikon) and a camera (Q-Imaging Retiga 2000R with a Q-Imaging RGB Slider). Images were acquired with ACT-1 or Q-Capture Pro softwares (Nikon) and manipulated with Photoshop (Adobe), using the crop, levels, curves, brightness/contrast, and image size commands. For acquisition of immunofluorescence experiments, an Axiophot2 (Carl Zeiss MicroImaging, Inc.) microscope was used with a 63× 1.4 Plan Apochromat objective (oil) and a Coolsnap (Photometrics) camera driven by the Metavue software.

Online supplemental material

Fig. S1 shows Sox9 expression in Paneth cells fand a general view of a Sox9-deficient colon, showing the localization and extent of the described phenotypic features. Fig. S2 provides an analysis of the goblet cell population and of apoptosis rates in Sox9-deficient mice. Fig. S3 shows β-catenin expression in Sox9-deficient mice and consequences of Sox9 and ΔCSox9 overexpression on the β-catenin–Tcf4 activity in a panel of colon carcinoma cell lines. Fig. S4 shows no modification in the subcellular localization of β-catenin or Tcf4 or in the degradation of β-catenin after induction of Sox9 expression in the HT29Cl.16E-Sox9 cell line. Fig. S5 shows that in

Sox9-deficient mice, the c-Myc and cyclin D1 genes are expressed by an increased number of cells, and individual cells express higher levels of the c-Myc and cyclin D1 proteins. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704152/DC1.

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