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RESEARCH ARTICLE

Brg1 plays an essential role in development and homeostasis of the duodenum through regulation of Notch signaling

Yutaka Takada, Akihisa Fukuda*, Tsutomu Chiba and Hiroshi Seno

ABSTRACT

Brg1, a core subunit of the SWI/SNF chromatin remodeling complex, is essential for development and homeostasis of various organs. However, the functional role of Brg1 in intestinal development and homeostasis, and the underlying molecular mechanism, remain unknown. We found that deletion of *Brg1* in the mouse intestine resulted in growth impairment and early death associated with abnormal crypt-villous formation, skewed differentiation into secretory lineage cells, markedly increased apoptosis, and stem cell loss in the duodenum. Furthermore, we found that the Notch signaling pathway was dramatically downregulated in *Brg1*-deficient duodenum. Remarkably, overexpression of the Notch1 intercellular domain (ICD) partially reversed the prognosis of intestinal *Brg1* mutant mice. Notch1 ICD overexpression rescued morphogenesis, prevented over-differentiation into secretory lineage cells, and restored apoptosis to normal levels in *Brg1*-deficient duodenum, although stem cell loss was not rescued. Our data demonstrate that Brg1 plays an essential role in development and homeostasis, including morphogenesis, stem cell differentiation and cell survival in the duodenum. Mechanistically, the rescue of the intestinal *Brg1* mutant phenotype by overexpression of the Notch1 ICD indicates that Notch signaling is a key downstream target that mediates the effects of Brg1.

KEY WORDS: Brg1, Notch signaling, Intestine, Homeostasis, Stem cell, Differentiation

INTRODUCTION

Tissue development and homeostasis are governed by activation or inactivation of multiple genes. Because chromatin is highly compacted, it must undergo structural changes to allow transcription factors to interact with nucleosomes and access binding sites on their target genes. ATP-dependent chromatin remodeling complexes alter local chromatin structure using energy from ATP hydrolysis. These structural alterations increase or decrease the accessibility of transcriptional factors and thereby activate or repress the expression of specific genes (Hargreaves and Crabtree, 2011; Helming et al., 2014; Martens and Winston, 2003; Wilson and Roberts, 2011). SWI/SNF (Switch/sucrose non-fermentable) complexes are evolutionarily conserved ATP-dependent chromatin remodeling complexes consisting of multiple protein subunits. Mammalian SWI/SNF complexes contain either of two mutually exclusive catalytic ATPase components: Brg1 (also known as Smarca4) or Brm (Smarca2) (Hargreaves and Crabtree, 2011; Helming et al., 2014; Martens and Winston, 2003; Wilson and Roberts, 2011). Brg1 is indispensable for embryonic development. *Brg1*-null mice die during the peri-implantation stage, whereas *Brm*-null mice develop normally (Bultman et al., 2000; Reyes et al., 1998). Recent studies showed that Brg1 is essential for development or homeostasis of various organs and cell types, including heart (Hang et al., 2010; Takeuchi et al., 2011), vasculature (Davis et al., 2013; Griffin et al., 2008, 2011), ureter (Weiss et al., 2013), smooth muscle (Zhang et al., 2011) and neurons (Li et al., 2013; Matsumoto et al., 2006; Seo et al., 2005). In the intestine, Brg1 is essential for maintenance of intestinal stem cells (Holik et al., 2013). However, the functional role of Brg1 in intestinal development and homeostasis and its molecular mechanism remain to be elucidated.

The intestinal epithelium is highly organized with multiple functions, including digestion, nutrient absorption and immunological defense. Structurally, it is organized into villi, which are finger-like projections and crypts of Lieberkühn, which are invagination structures adjacent to villi. The villous epithelium consists of differentiated epithelial cells, including absorptive-type enterocytes and secretory-type goblet cells, enteroendocrine cells and tuft cells (Gerbe et al., 2011). In contrast, crypts consist chiefly of undifferentiated proliferating progenitor cells. In the bottom of crypts, intestinal stem cells, termed crypt base columnar (CBC) cells, reside between secretory-type differentiated Paneth cells (Barker et al., 2007). CBC cells can self-renew and provide progenitors, called transit-amplifying (TA) cells, which can rapidly divide and differentiate into all types of mature cells in the intestinal epithelium; subsequently, they ascend from the crypt to the villi (Barker et al., 2007).

Diverse signaling pathways are involved in intestinal morphogenesis and maintenance of intestinal homeostasis (Medema and Vermeulen, 2011). In particular, Notch signaling plays a crucial role in the regulation of progenitor cell proliferation and differentiation (Sancho et al., 2015). Notch activity promotes differentiation into the absorptive-type cell lineage rather than the secretory cell lineage (Fre et al., 2005; Jensen et al., 2000; Milano et al., 2004; Ueo et al., 2012; van Es et al., 2005; VanDussen et al., 2012; Wong et al., 2004; Yang et al., 2001). Complete inhibition of Notch signaling in the murine intestinal epithelium using γ-secretase inhibitors results in significantly elevated differentiation into secretory lineages (Milano et al., 2004; van Es et al., 2005; Wong et al., 2004). On the other hand, persistent expression of the Notch intercellular domain (ICD) expands the proliferative zone and represses secretory cell differentiation (Fre et al., 2005; Stanger et al., 2005). Notch signaling is also required for maintenance of CBC cells (Pellegrinet et al., 2011; VanDussen et al., 2012). Notch inhibition inhibits cellular proliferation and induces apoptosis of CBC cells, thereby decreasing the overall number of these cells (VanDussen et al., 2012).

Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto 6068507, Japan.

*Author for correspondence (fukuda26@kuhp.kyoto-u.ac.jp)

A.F., 0000-0002-1940-596X

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Here, we show that Brg1 plays a pivotal role in crypt-villous remodeling, differentiation and cell survival in the duodenum. Furthermore, mechanistically, our data show that the effects of Brg1 are mainly mediated by the Notch signaling pathway.

RESULTS

Intestinal Brg1 deletion results in growth impairment, early death and abnormal villous formation in the duodenum

First, we ascertained the expression pattern of Brg1 in the murine intestinal epithelium. Brg1 was expressed in all intestinal epithelial cells from embryonic development to post-natal and adult stages (Fig. 1A-C). To investigate whether Brg1 plays a functional role in intestinal development and maintenance of intestinal homeostasis, we crossed transgenic mice carrying a *loxP*-flanked allele of *Brg1* (*Brg1*$^{f/wt}$) (Sumi-Ichinose et al., 1997) with *Villin-Cre* mice (Madison et al., 2002) to generate *Villin-Cre; Brg1*$^{f/f}$ mice. *Villin-Cre; Brg1*$^{f/f}$ mice were born at Mendelian ratios, but they exhibited early post-natal death and growth impairment relative to control littermates. Fifty percent of the *Brg1* mutant mice died within 4 days of birth (Fig. 1D-F). Histological analyses revealed that *Villin-Cre; Brg1*$^{f/f}$ mice had abnormal duodenal villous structure, shorter villi and disorganized intervillous pockets compared with control mice at post-natal day (P)4 (Fig. 1G,H,K). This abnormal villous structure was less striking toward the distal intestine and was much less prominent in the ileum (Fig. 1I,J,L). In accordance with Cre activity, almost all of the intestinal epithelial cells had lost Brg1 expression in *Villin-Cre; Brg1*$^{f/f}$ mice (Fig. 1M,N). At embryonic day (E)16.5, the duodenal villous structures of *Brg1* mutant and control mice were indistinguishable, but shorter villi could be observed in *Brg1* mutant mice at E18.5 (Fig. S1A-F).

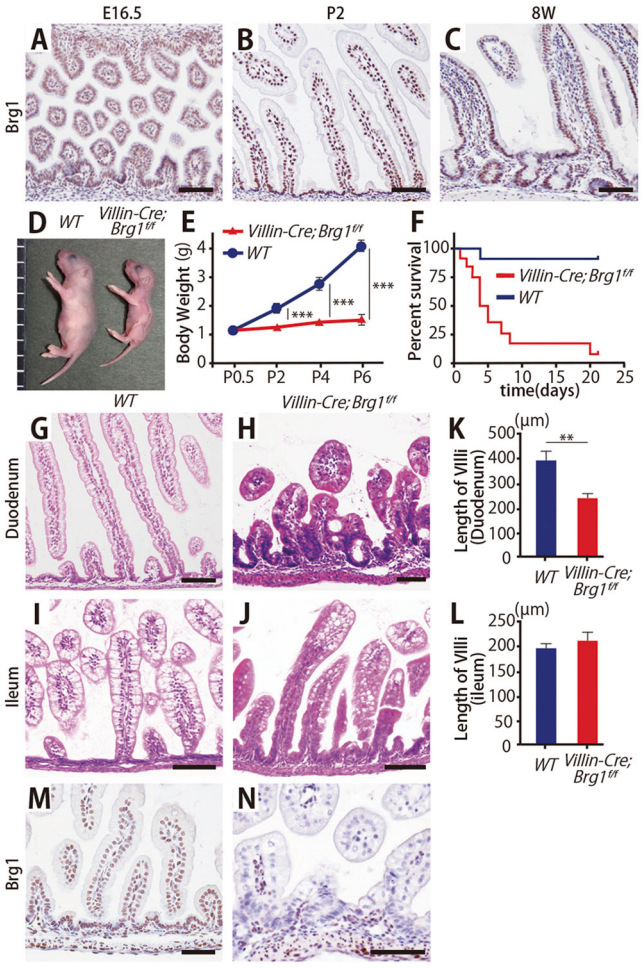

Fig. 1. Loss of Brg1 leads to growth failure, early death and abnormal duodenal villous structure. (A-C) Immunohistochemical staining for Brg1 in the duodenum of control mice at E16.5 (A), P2 (B) and 8 weeks (C). (D) Macroscopic view of control (left) and *Villin-Cre; Brg1*$^{f/f}$ mice (right) at P4. (E) Body weight at indicated time points for controls (blue, *n*=6) and *Villin-Cre; Brg1*$^{f/f}$ mice (red, *n*=6). ***P<0.001. (F) Kaplan–Meier curves show significantly shorter survival (P<0.0001) in *Villin-Cre; Brg1*$^{f/f}$ mice (red, *n*=12) compared with control mice (blue, *n*=12). (G-J) H&E staining of small intestine at P4. Abnormal villous structure was observed in the duodenum of *Villin-Cre; Brg1*$^{f/f}$ mice (H) relative to control mice (G), whereas no significant difference was seen in the ileum (I,J). (K,L) Length of villi of the duodenum (K) and ileum (L) in the indicated genotypes at P4 (*n*=4). **P<0.01. (M,N) Brg1 staining of the duodenum in the indicated genotypes at P4. Scale bars: 100 µm (A-C,G-J), 50 µm (M,N).

Brg1 depletion results in skewed intestinal differentiation into secretory lineage cells

We next investigated the effect of loss of Brg1 on differentiation of the duodenal epithelial cells at P4. Alcian Blue staining and quantitation revealed that the number of goblet cells was markedly higher in *Brg1*-deficient duodenum than in control duodenum (Fig. 2A,B,M). Chromogranin A immunostaining and quantitation revealed that the number of enteroendocrine cells was also markedly higher in *Brg1*-deficient duodenum (Fig. 2C,D,N). Among enteroendocrine cells, GIP (glucose-dependent insulinotropic peptide)-positive K cells were more numerous in *Villin-Cre; Brg1*$^{f/f}$ duodenum (Fig. 2E,F), whereas there were no significant differences between *Brg1* mutant and control duodenum in the numbers of other types of enteroendocrine cells, including serotonin-positive EC cells, gastrin-positive G cells, secretin-positive S cells, cholecystokinin-positive I cells and Glp1 (glucagon-like peptide-1)-positive L cells (data not shown). In addition, immunostaining of Dclk1 revealed that the number of tuft cells was also significantly higher in *Brg1*-deficient duodenum (Fig. 2G,H). Quantitative RT-PCR (q-PCR) analysis showed that *Math1* (*Atoh1*), a transcription factor that plays an essential role in the differentiation of intestinal secretory cell types (Yang et al., 2001), was upregulated in *Brg1*-deficient duodenum (Fig. 2O). Furthermore, immunostaining of the Paneth cell marker lysozyme (Sato et al., 2011) and Mmp7 (Wilson et al., 1999) revealed precociously differentiated Paneth cells in *Villin-Cre; Brg1*$^{f/f}$ duodenum, but not in control duodenum (Fig. 2I-L). In addition, *Gfi1* (Bjerknes and Cheng, 2010), a transcriptional factor that is crucial for the differentiation of Paneth cells, was more highly expressed in *Brg1*-deficient duodenum than in controls (Fig. 2P). These data indicate that loss of *Brg1* results in skewed differentiation into secretory cell types in the duodenum.

Loss of Brg1 increases the numbers of apoptotic and proliferative cells in the duodenum

Brg1 is involved in apoptosis and cell proliferation during organ development (Griffin et al., 2008; Hang et al., 2010; Li et al., 2013; Seo et al., 2005; Zhang et al., 2011). Therefore, we next investigated whether the abnormal villous structure in *Brg1*-deficient duodenum was associated with changes in apoptosis or cell proliferation. Remarkably, immunostaining and quantitation of cleaved caspase 3 revealed a dramatic increase in apoptotic cells in *Villin-Cre; Brg1*$^{f/f}$ duodenum relative to control duodenum at P4 (Fig. 3A-D). In addition, genes involved in apoptosis, including *Noxa* (*Pmaip1*) and *Bax* (Igney and Krammer, 2002), were significantly upregulated and the anti-apoptotic factor *Bcl2* (Igney and Krammer, 2002) was significantly downregulated, in *Brg1*-deficient duodenum relative to controls (Fig. 3E).

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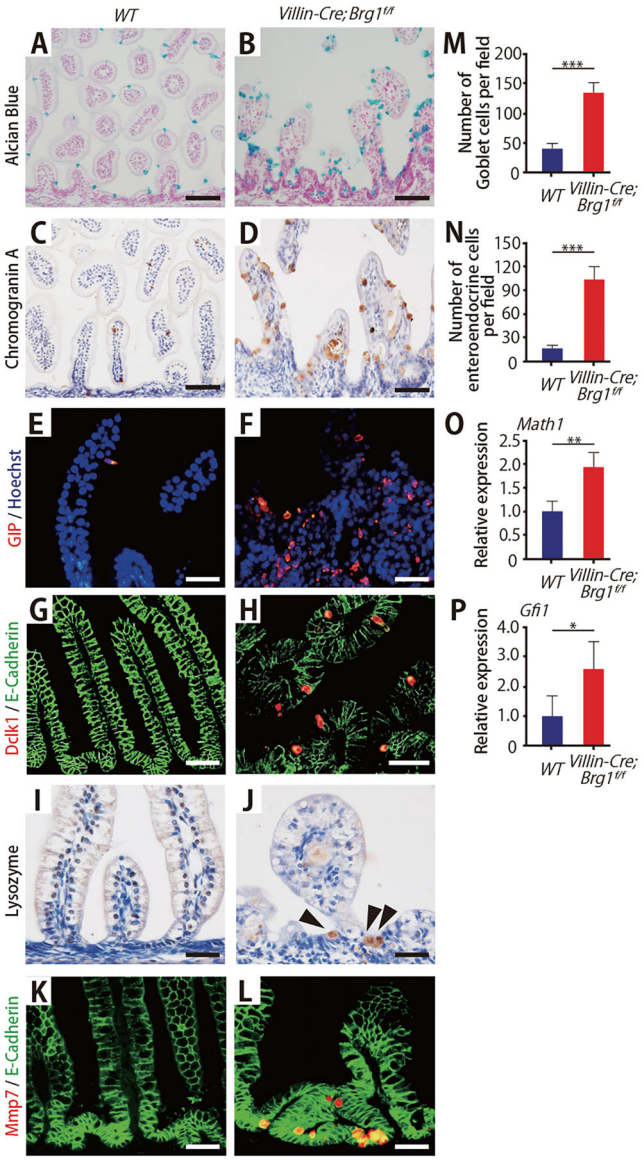

Fig. 2. Loss of Brg1 results in skewed differentiation into intestinal secretory lineage cells. (A-L) Histological analysis of the duodenum of control (A,C,E,G,I,K) and *Villin-Cre; Brg1$^{f/f}$* mice (B,D,F,H,J,L) at P4. Alcian Blue staining (A,B), chromogranin A staining (C,D), GIP/Hoechst co-staining (E,F), Dclk1/E-cadherin co-staining (G,H), lysozyme staining (I,J) and Mmp7/E-cadherin co-staining (K,L). Scale bars: 100 µm (A-D), 50 µm (E-L). Arrowheads indicate lysozyme-positive cells. (M,N) Numbers of goblet cells (M) and enteroendocrine cells (N) in the duodenum of the indicated genotypes. Means±s.d. (n=3); ***P<0.001. (O,P) Relative expression levels of *Math1* (O) and *Gfi1* (P) in the duodenum of the indicated genotypes at P4, as determined by q-PCR (n=4); *P<0.05, **P<0.01.

We next investigated whether cell proliferation was affected in *Brg1*-deficient duodenum. Ki67 staining revealed monostratal proliferative cells in the intervillous pocket in control duodenum at P4 (Fig. 3F). By contrast, in *Brg1*-deficient duodenum, stratified proliferative cells were observed not only in the intervillous pocket and crypt bottoms, but also in the middle of villi (Fig. 3G). Quantitation revealed that the number of Ki67-positive cells was significantly higher in *Brg1*-deficient duodenum than in control duodenum (Fig. 3H). Thus, loss of Brg1 increases the numbers of both apoptotic and proliferative cells in the duodenum.

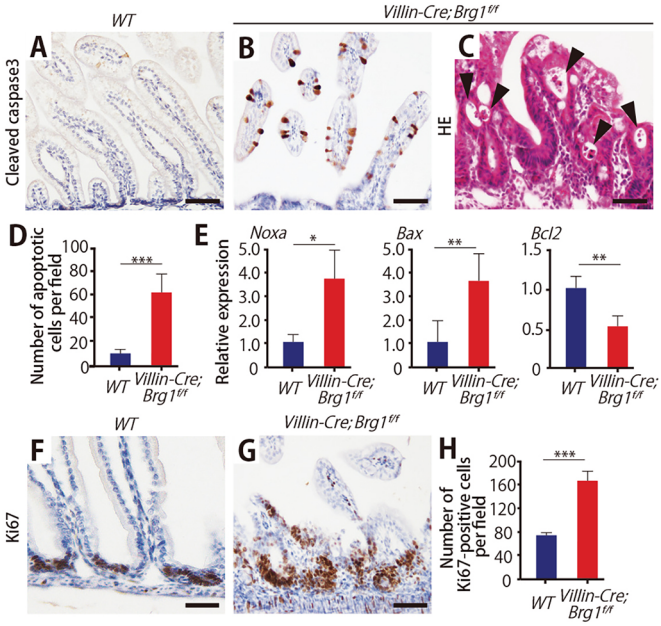

Fig. 3. Loss of Brg1 results in a dramatic increase in apoptosis and abnormal distribution of proliferative cells in the duodenum. (A,B) Cleaved caspase 3 staining of the duodenum of the indicated genotypes at P4. (C) H&E staining of the duodenum of *Villin-Cre; Brg1$^{f/f}$* mice at P4. Arrowheads indicate cells undergoing apoptosis with apoptotic bodies. (D) Number of apoptotic cells (n=3); ***P<0.001. (E) Relative expression levels of *Noxa*, *Bax* and *Bcl2*, genes related to apoptosis, as determined by q-PCR (n=4); *P<0.05, **P<0.01. (F,G) Immunostaining of Ki67 in the duodenum of the indicated genotypes. (H) Number of Ki67-positive cells in the duodenum of the indicated genotypes (n=3); ***P<0.001. Scale bars: 100 µm (A,B), 50 µm (C,F,G).

Loss of Brg1 results in depletion of intestinal stem cells

Brg1 is essential for maintenance of intestinal stem cells (Holik et al., 2013). To investigate the effect of loss of Brg1 on the development of intestinal stem cells, we used *Lgr5-GFP* mice, in which Lgr5^{+} CBC cells are GFP positive (Barker et al., 2007). Immunostaining of GFP revealed Lgr5^{+} CBC cells in the intervillous pocket in duodenum of *Lgr5-GFP; Brg1$^{wt/wt}$* mice at P4. By contrast, such cells were rarely seen in the duodenum of *Lgr5-GFP; Villin-Cre; Brg1$^{f/f}$* mice (Fig. 4A,B). Moreover, immunostaining of Msi1 (Musashi RNA-binding protein 1), a CBC cell marker (Kayahara et al., 2003; Potten et al., 2003) and a positive regulator of Notch signaling (Imai et al., 2001), revealed that Msi1-positive cells were very rare in *Brg1*-deficient duodenum, but they were present in the intervillous area in control duodenum at P4 (Fig. 4C,D). In addition, intestinal stem cell marker genes, including *Lgr5*, *Olfm4*, *Msi1*, *Ascl2*, *Bmi1* and *Tert* (Barker, 2014), were markedly downregulated in *Brg1*-deficient duodenum relative to control duodenum (Fig. 4E). These data indicate that Brg1 is indispensable for the development of intestinal stem cells in the duodenum.

Loss of Brg1 results in downregulation of Notch signaling

Notch signaling plays an important role in maintenance of intestinal stem cells and cellular differentiation of intestinal epithelial cells. In particular, Notch inhibition results in loss of CBC cells and over-differentiation into secretory cell types (Pellegrinet et al., 2011; VanDussen et al., 2012). Therefore, we next sought to determine whether loss of Brg1 would affect Notch signaling activity in the duodenum. Immunostaining of cleaved Notch1 revealed that it was

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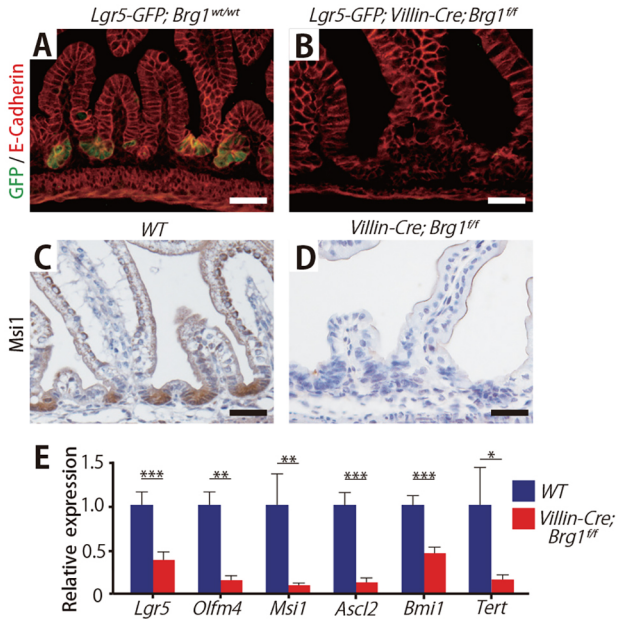

Fig. 4. Brg1 ablation leads to loss of intestinal stem cells. (A,B) GFP/E-cadherin co-staining in the indicated genotypes at P4. (C,D) Immunostaining of Msi1 in the indicated genotypes at P4. Scale bars: 50 µm. (E) Relative expression levels of intestinal stem cell marker genes in the duodenum of the indicated genotypes at P4, as determined by q-PCR (n=4); *P<0.05, **P<0.01, ***P<0.001.

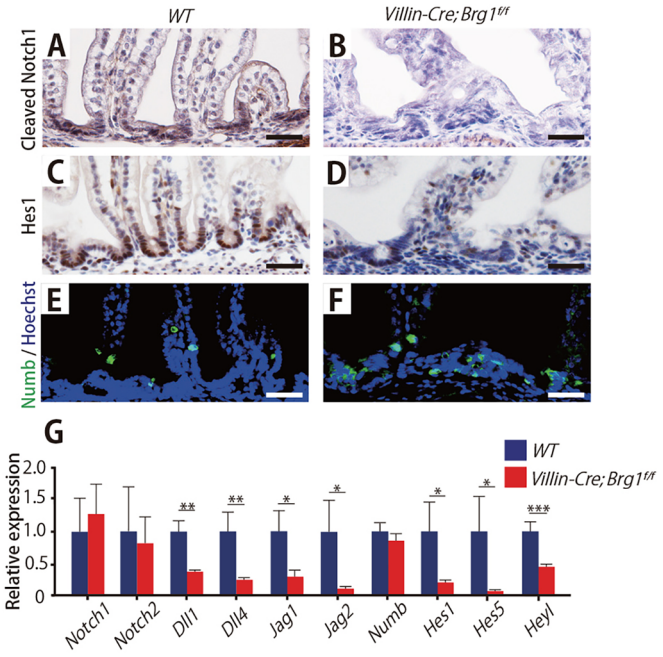

Fig. 5. Loss of Brg1 results in downregulation of the Notch signaling pathway in the duodenum. (A-F) Histological analysis of controls (A,C,E) and *Villin-Cre; Brg1$^{f/f}$* mice (B,D,F) at P4. Cleaved Notch1 staining of the duodenum (A,B), Hes1 staining of the duodenum (C,D) and Numb/Hoechst co-staining of the duodenum (E,F) in the indicated genotypes. Scale bars: 50 µm. (G) Relative expression levels of genes involved in the Notch signaling pathway in the duodenum at P4, as determined by q-PCR (n=4); *P<0.05, **P<0.01.

expressed in duodenal epithelial cells in intervillous pockets in control duodenum, whereas only a few cleaved Notch1-positive cells were observed in *Brg1*-deficient duodenum at P4 (Fig. 5A,B). Remarkably, Hes1, a target gene of the Notch signaling pathway, was barely expressed in *Brg1*-deficient duodenum, whereas it was expressed in intervillous pockets and the bottoms of villi in control duodenum at P4 (Fig. 5C,D). Furthermore, Numb, which acts as an inhibitor of Notch signaling by degrading the Notch1 ICD, was markedly overexpressed in intervillous pockets in *Brg1*-deficient duodenum relative to controls at P4 (Fig. 5E,F). Next, we performed q-PCR analyses to determine the expression levels of genes involved in the Notch signaling pathway in the duodenum of *Villin-Cre; Brg1$^{f/f}$* and control mice. Notch ligands, including *Dll1*, *Dll4*, *Jag1* and *Jag2*, were significantly downregulated in *Villin-Cre; Brg1$^{f/f}$* duodenum relative to control. Expression of *Numb*, which is translationally regulated by Musashi1 (Imai et al., 2001), was comparable between *Brg1* mutants and controls. Notch target genes, including *Hes1*, *Hes5* and *Heyl*, were markedly downregulated in duodenum of *Villin-Cre; Brg1$^{f/f}$* mice relative to controls (Fig. 5G). Downregulation of Notch target genes was also observed in *Brg1*-deficient duodenum at E17.5 (Fig. S2). These results indicate that the loss of Brg1 results in marked downregulation of the Notch signaling pathway in the duodenum.

The intestinal phenotype of *Brg1* mutant mice was manifested primarily in the duodenum, and was much less prominent in the ileum. Therefore, we next investigated whether Notch signaling activity is downregulated in *Brg1*-deficient ileum. Immunostaining revealed that Hes1 expression in the epithelial cells of the ileum was comparable between *Brg1* mutant and control mice at P4 (Fig. S3A,B). The relative expression level of *Hes5* was upregulated in *Brg1* mutant ileum (Fig. S3C). *Heyl* expression was comparable between *Brg1* mutant and control mice, whereas *Hes1* expression was slightly downregulated in *Brg1*-deficient ileum compared with the control (Fig. S3C). Thus, Notch signaling activity was not downregulated in *Brg1*-deficient ileum compared with *Brg1*-deficient duodenum.

Overexpression of the Notch1 ICD reverses growth failure and early death of *Brg1* mutant mice

Because the Notch signaling pathway was markedly downregulated in *Brg1*-deficient duodenum, we hypothesized that Notch1 ICD overexpression might rescue the phenotype of *Brg1* mutant mice. To test this hypothesis, we used *Rosa^{NotchIC}* mice (Murtaugh et al., 2003), in which the Notch1 ICD is constitutively overexpressed under the control of Cre recombinase. We crossed *Rosa^{NotchIC}* mice with *Villin-Cre* mice and *Brg1$^{f/wt}$* mice to generate *Villin-Cre; Brg1$^{f/f}$; Rosa^{NotchIC}* (VBN) mice (Fig. 6A). The body weight and survival phenotypes *of Villin-Cre; Brg1$^{f/f}$* (VB) were partially rescued in VBN mice (Fig. 6B-D). We confirmed that Brg1 was depleted and cleaved Notch1 was expressed in the intestinal epithelial cells in VBN mice (Fig. 6E,F). As expected, Hes1 was expressed at P4 in the duodenal epithelial cells of VBN mice (Fig. 6G). Moreover, expression levels of *Hes1* and *Hes5* were comparable, whereas *Heyl* was downregulated in VBN duodenum compared to wild-type (WT) control duodenum (Fig. 6I). As expected, Numb, an upstream regulator of the Notch1 ICD, was still overexpressed at P4 in intervillous pockets in VBN duodenum, as it was in VB duodenum (Fig. 6H). Thus, Notch1 ICD overexpression partially rescued the growth failure and early death phenotypes of *Brg1* mutant mice.

Notch1 ICD overexpression rescues abnormal villous formation, skews differentiation into secretory lineages and increases apoptosis

We next investigated whether the abnormal villous structure and skewed differentiation into secretory lineages observed in *Brg1*

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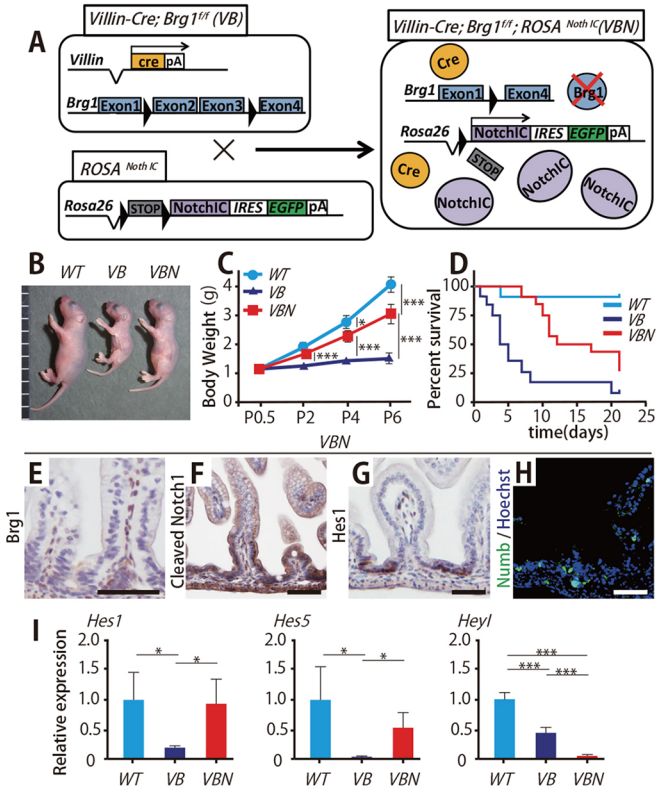

Fig. 6. Notch1 ICD overexpression rescues growth failure and early death of intestinal *Brg1* mutant mice. (A) Experimental strategy for generation of *Villin-Cre; Brg1*$^{f/f}$*; Rosa*^{NotchIC} (VBN) mice. (B) Macroscopic view of WT (left), *Villin-Cre; Brg1*$^{f/f}$ (VB) (middle) and VBN (right) mice at P4. (C) Body weight of WT (n=6), VB (n=6) and VBN mice (n=6) at the indicated time points; ***P<0.001. (D) Kaplan–Meier curves for overall survival of the indicated genotypes: wild type (n=12), VB (n=12) and VBN mice (n=12). Log-rank test revealed a significantly longer survival of VBN mice compared with VB mice (P=0.0075). (E-H) Immunostaining of the duodenum of VBN mice at P4: Brg1 (E), cleaved Notch 1 (F), Hes1 (G) and Numb/Hoechst (H). Scale bars: 50 µm. (I) Relative expression levels of *Hes1*, *Hes5* and *Heyl* in the duodenum at P4, as determined by q-PCR (n=4); *P<0.05, ***P<0.001.

mutant mice would be reversed in VBN mice. The length of villi in VBN duodenum at P4 was comparable to that of WT controls (Fig. 7I) and the structure of intervillous pockets was indistinguishable between VBN and WT (Fig. 7A). Alcian Blue staining and quantitation revealed that the number of goblet cells was significantly lower in VBN duodenum than in VB duodenum (Fig. 7B,J). Immunostaining of chromogranin A and GIP revealed that the numbers of enteroendocrine cells, including K cells, in VBN duodenum were comparable to those of WT controls (Fig. 7C,D,K). Furthermore, VBN duodenum contained fewer tuft cells than VB duodenum (Fig. 7E). Paneth cells were very scarce in duodenum of VBN mice, as in WT controls (Fig. 7F). In addition, expression levels of *Math1* and *Gfi1* were lower in duodenum of VBN mice than in VB mice (Fig. 7N). Thus, Notch1 ICD overexpression rescued impaired villous formation and skewed differentiation into secretory lineages of *Brg1*-deficient duodenum at P4.

We next investigated whether the elevation of apoptosis was rescued in VBN mice. Notably, immunostaining and quantitation of cleaved caspase 3 revealed that the number of apoptotic cells in the duodenum in VBN mice was significantly smaller than in VB duodenum and was comparable to that of WT duodenum at P4 (Fig. 7G,L). In addition, *Noxa* was significantly downregulated and

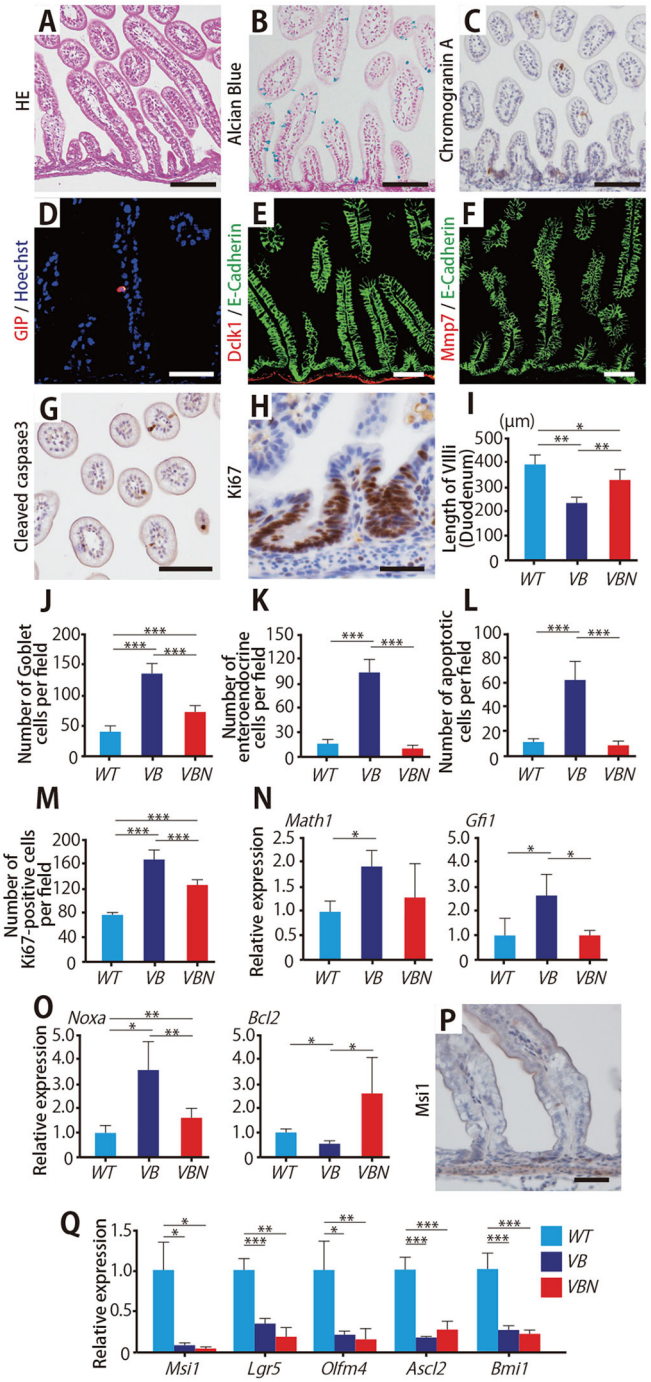

Fig. 7. Overexpression of Notch1 ICD rescues abnormal villous structure, skewed differentiation and increased apoptosis, but not loss of intestinal stem cells, in intestinal *Brg1* mutant mice. (A-H) Histological analyses of the duodenum of VBN mice at P4. H&E staining (A), Alcian Blue staining (B), Chromogranin A staining (C), GIP/Hoechst co-staining (D), Dclk1/E-cadherin co-staining (E), Mmp7/E-cadherin co-staining (F), cleaved caspase 3 staining (G) and Ki67 staining (H). (I-M) Length of villi (I) (n=4), number of goblet cells (J) (n=3), enteroendocrine cells (K) (n=3), apoptotic cells (L) (n=3) and Ki67-positive cells (M) (n=3) in duodenum of the indicated genotypes at P4. (N,O) Relative expression levels of *Math1*, *Gfi1* (N), *Noxa* and *Bcl2* (O) in the duodenum of VBN mice (red, n=4) at P4, as determined by q-PCR. (P) Immunostaining of Msi1 in duodenum of VBN mice at P4. (Q) Relative expression levels of *Msi1*, *Lgr5*, *Olfm4*, *Ascl2* and *Bmi1* in the duodenum of VBN mice (red, n=4) at P4, as determined by q-PCR. *P<0.05, **P<0.01, ***P<0.001. Scale bars: 100 µm (A-C,G,H), 50 µm (D-F,P).

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Bcl2 was upregulated, in VBN duodenum relative to VB duodenum (Fig. 7O). Thus, Notch1 ICD overexpression completely rescued increased apoptosis in VBN mice. In terms of the distribution of proliferative cells, Ki67 staining revealed that proliferative cells were monostratal but were still present in the middle of villi in VBN mice at P4 (Fig. 7H). The number of Ki67-positive cells was smaller in VBN duodenum than in VB duodenum, although it was still greater than that of WT controls at P4 (Fig. 7M). Thus, the number and distribution of proliferative cells was partially reversed by Notch1 ICD overexpression.

Furthermore, we investigated the phenotypes of *Villin-Cre; Rosa*^{NotchIC} (VN) mice. Misexpression of the Notch1 ICD reversed the phenotypes of VBN mice to the level of wild-type control but not to that of VN mice (Fig. S4A-J). The activity of Notch signaling (e.g. expression level of *Hes1*) was different between VBN and VN mice (Fig. S4A-J).

Taken together, these data indicate that the phenotype of *Brg1* mutant mice, including impaired morphogenesis, skewed differentiation into secretory lineages and elevated apoptosis, was due to downregulation of the Notch signaling pathway in *Brg1* mutant mice.

We next investigated intestinal stem cells of VBN mice. Immunostaining revealed that Msi1-positive cells were very scarce in VBN duodenum at P4 (Fig. 7P). In addition, expression levels of intestinal stem cell marker genes, including *Ms1*, *Lgr5*, *Olfm4*, *Ascl2* and *Bmi1*, were comparable in the duodenum of VBN and VB mice (Fig. 7Q). Thus, Notch1 ICD overexpression did not rescue intestinal stem cell loss in *Brg1*-deficient duodenum.

Stabilization of β-catenin does not reverse the phenotypes of *Brg1* mutant mice

Wnt signaling pathways also play a crucial role in development and homeostasis of the intestine (Sato and Clevers, 2013). Therefore, we investigated whether loss of Brg1 affected Wnt signaling activity in the duodenum. Q-PCR analyses revealed that the expression levels of genes involved in the Wnt signaling pathway were markedly downregulated at multiple levels in *Brg1*-deficient duodenum (Fig. S5A). Therefore, we next sought to determine whether activation of the Wnt signaling would reverse the phenotypes of *Brg1* mutant mice including duodenal stem cell loss. To this end, we used *Ctnnb1*$^{flox(ex3)}$ mice (Harada et al., 1999), in which Cre recombination removes exon 3 of *Ctnnb1* to stabilize β-catenin, and generated *Villin-Cre; Brg1*$^{f/f}$*; Ctnnb1*$^{f/f(exon3)}$ (VBB) mice. Interestingly, β-catenin stabilization did not rescue the phenotype of *Brg1* mutant mice, including impaired crypt-villous remodeling, skewed differentiation into secretory lineages, elevated apoptosis and cell proliferation (Fig. S5B-E,H-K). Moreover, of note, Lgr5^{+} CBC cells were rarely observed in the duodenum of VBB mice at P4, similar to their distribution in VB mice (Fig. S5F). Intestinal stem cell marker genes associated with the Wnt signaling, including *Lgr5* and *Ascl2*, were significantly downregulated in the duodenum of VBB mice (Fig. S5L). Of note, the Notch signaling pathway was still downregulated in VBB mice, although some of the Notch-related genes, including *Dll4*, *Jag2* and *Hes1*, were slightly upregulated in VBB mice compared with VB mice (Fig. S5G,M). Therefore, these data indicate that Brg1 plays a pivotal role for development and homeostasis of the duodenum through the regulation of Notch signaling in a manner that is independent of Wnt signaling.

DISCUSSION

In this study, intestinal *Brg1* mutant mice presented with early postnatal death associated with impaired crypt-villous formation, skewed differentiation into secretary lineage, markedly increased apoptosis and stem cell loss in the duodenum. Furthermore, the Notch signaling pathway was dramatically downregulated in *Brg1*-deficient duodenum. Notably, Notch1 ICD overexpression rescued impaired morphogenesis, over-differentiation into secretary lineage and elevated apoptosis in the absence of Brg1, demonstrating that Brg1 plays a pivotal role for intestinal development and homeostasis via a Notch signaling-dependent mechanism.

In terms of the functional relationship between Brg1 and the Notch signaling, we found that Notch ligands including *Dll1*, *Dll4*, *Jag1* and *Jag2* were strongly downregulated in *Brg1*-deficient duodenum. Reduced expression of these four Notch ligands can most likely explain the downregulation of Notch signaling. Misexpression of the Notch1 ICD reversed the phenotypes of VBN mice to the level of the WT control but not to that of *Villin-Cre; Rosa*^{NotchIC} (VN) mice. The other Notch cleaved components (e.g. Notch2, Notch3, or Notch4) might be involved in reversing the expression level of Notch target genes and consequently reverting the phenotypes of VBN mice to the level of VN mice. Regarding to the relationship with Wnt signaling, we found marked downregulation of the Wnt signaling in *Brg1*-deficinet duodenum. Of note, the Notch signaling activity was still downregulated in the model of β-catenin stabilization (VBB mice), although some Notch components, including *Dll4*, *Jag2* and *Hes1*, were slightly upregulated in VBB mice compared with VB mice. Thus, our data support the notion that the mechanistic link between Brg1 and Notch is not predominantly mediated by Wnt signaling. Our findings demonstrate that Brg1 plays an indispensable role in neonatal crypt-villous remodeling by regulating Notch signaling in a manner that is independent of Wnt signaling.

Brg1 is required for inhibition of apoptosis in the development of various cell types and organs, including neurons (Li et al., 2013), smooth muscle (Zhang et al., 2011) and blood cells (Griffin et al., 2008); consequently, knockout of *Brg1* increases the rate of apoptosis in these tissues. Our data showing that elevated apoptosis was reversed by Notch1 ICD overexpression in VBN mice demonstrate that marked downregulation of Notch signaling is responsible for increased apoptosis in *Brg1*-deficient duodenum. This finding is consistent with a previous report showing that Notch inhibition by conditional knockout of *Hes1*, *Hes3* or *Hes5* results in increased apoptosis in murine intestinal epithelial cells (Ueo et al., 2012). A previous study shows that apoptotic cells are increased only in crypt lesions in adult *Brg1* mutant mice (Holik et al., 2013). Similarly, crypt-restricted apoptosis increased in the adult Notch inactivation models (Milano et al., 2004). In contrast, in the embryonic Notch inactivation models, increased apoptosis is widely observed in the intestine (Ueo et al., 2012). This is similar to what we observed in our embryonic *Brg1* mutant mice. Our data, together with the previous study, underscore the specific requirement for Brg1 and Notch in preventing apoptosis during neonatal crypt-villus remodeling that has not been appreciated in previous adult studies.

Brg1 is essential for intestinal stem cell homeostasis (Holik et al., 2013), but the molecular details of the requirement for Brg1 in intestinal stem cell maintenance remain unknown. Notch signaling is essential for survival of intestinal stem cells (Pellegrinet et al., 2011; VanDussen et al., 2012) and treatment with the Notch inhibitor DBZ causes intestinal stem cell loss (VanDussen et al., 2012). However, our results show that intestinal stem cell loss was not reversed by Notch1 ICD overexpression in intestinal *Brg1* mutant mice. Furthermore, although Wnt signaling was also

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markedly downregulated in *Brg1* mutant mice, the activation of Wnt signaling by β-catenin stabilization did not reverse the stem cell loss of *Brg1*-deficient duodenum. The inability to completely rescue the intestinal phenotype by overexpression of Notch1 ICD or Wnt further suggests that the effects of Brg1 deletion are complex and not merely due to alterations of either signaling pathway. Future studies should seek to clarify the molecular mechanism by which loss of intestinal Brg1 expression results in stem cell loss in the duodenum.

Notably, we observed very different phenotypes between the duodenum and ileum in intestinal *Brg1* mutant mice. Brm was barely expressed in the duodenum in both *Brg1* mutant and control mice, but was widely expressed in the ileum of both *Brg1* mutant and control mice at P4 (Fig. S6A-D). Also, our results showed that the Notch signaling pathway was dramatically downregulated in the duodenum, but barely affected in the ileum. These data suggest that the difference in the Brm expression pattern might contribute to the difference in phenotype between the duodenum and ileum, through regulation of the Notch signaling pathway, in *Brg1* mutant mice. Future studies should seek to determine whether double loss of Brg1 and Brm in the intestine would lead to a severe phenotype in the ileum.

Brg1 acts as a tumor suppressor in the murine pancreas (von Figura et al., 2014). In addition, inactivating mutations in *Brg1* and other subunits of SWI/SNF complexes have been identified in various human cancers, including the pancreas and intestine (Helming et al., 2014; Wilson and Roberts, 2011). Furthermore, Brg1 is involved in cell proliferation during embryonic development (Hang et al., 2010; Seo et al., 2005; Li et al., 2013), a period in which Brg1 depletion increases the number of proliferative cells in neural plate (Seo et al., 2005). In line with these data, we found that the number of proliferative cells was elevated in *Brg1*-deficient duodenum. Although the precise mechanism is unknown, proliferative cells might be more abundant in *Brg1*-deficient duodenum in order to compensate for stem cell loss and a dramatic increase in apoptosis. The increase in cell proliferation caused by loss of Brg1 raises the possibility that Brg1 plays a tumor-suppressive role in the duodenum. Future studies are required to determine whether Brg1 functions as a tumor suppressor in murine and human duodenum.

MATERIALS AND METHODS

Mouse lines

Experimental animals were generated by crossing *Villin-Cre* mice (Jackson Laboratories #004586) (Madison et al., 2002), $Brg1^{flox}$ mice (gift from D. Reisman, University of Florida, with permission from P. Chambon) (Sumi-Ichinose et al., 1997), $ROSA26^{NotchICD}$ mice (Jackson Laboratories #008159) (Murtaugh et al., 2003), *Lgr5EGFP-IRES-CreERT2* mice (Barker et al., 2007) (Jackson Laboratories #008875) and $Ctnnb1^{lox(ex3)}$ mice (Harada et al., 1999). *Villin-Cre;* $Brg1^{f/wt}$ mice were normal and used as a parental line. *Villin-Cre* mice were also normal and used as a parental line or controls. To generate *Villin-Cre;* $Brg1^{f/f}$, we crossed *Villin-Cre;* $Brg1^{f/wt}$ mice with *Villin-Cre;* $Brg1^{f/f}$ mice. *Villin-Cre;* $Brg1^{f/f}$*;* $Rosa26^{NotchICD}$ mice were generated by crossing *Villin-Cre;* $Brg1^{f/wt}$ mice with *Villin-Cre;* $Brg1^{f/f}$*;* $Rosa26^{NotchICD}$ mice. All experiments were approved by the animal research committee of Kyoto University and performed in accordance with Japanese government regulations.

Histological analysis

Mouse tissues were fixed with 4% buffered paraformaldehyde solution, paraffin-embedded and sectioned (5 µm thickness). Sections were deparaffinized, rehydrated and stained with Hematoxylin and Eosin (H&E) or Alcian Blue and counterstained with Nuclear Fast Red (KPL). For immunohistochemistry, sections were incubated with primary antibodies overnight at 4°C and with biotinylated secondary antibody for 1 h at room temperature; immunoperoxidase labeling was performed with Vectastain ABC kit (Vector Laboratories) and then sections were colored with diaminobenzidine (DAB) substrate (Dako) and counterstained with Hematoxylin. For immunofluorescence, sections were incubated with primary antibodies overnight at 4°C and with fluorophore-conjugated secondary antibody (Invitrogen) for 1 h at room temperature. Primary antibodies used in this study were obtained from the indicated suppliers: rabbit anti-Brg1 (1:1000; sc-10768, Santa Cruz Biotechnology), rabbit anti-chromogranin A (1:200; ab15160, Abcam), rabbit anti-GIP (1:200; HPA021612, Sigma-Aldrich), rabbit anti-Dclk1 (1:200; ab 31704, Abcam), mouse anti-E-cadherin (1:100; 610182, BD Transduction Laboratories), rabbit anti-lysozyme (1:200; A0099, DAKO), rabbit anti-Mmp7 (1:100; 3801S, Cell Signaling Technology), rat anti-serotonin (1:100; sc-73025, Santa Cruz Biotechnology), goat anti-gastrin (1:100; sc-7783, Santa Cruz Biotechnology), goat anti-secretin (1:100; sc-22630, Santa Cruz Biotechnology), goat anti-cholecystokinin (1:100; sc-21617, Santa Cruz Biotechnology), mouse anti-GLP-1 (1:10,000; ab23468, Abcam), rabbit anti-cleaved caspase 3 (1:100; 9664, Cell Signaling Technology), rabbit anti-p53 (1:500; VP-P956, Vector Laboratories), rat anti-Ki67 (1:100; M7249, Dako), chicken anti-GFP (1:200; ab13970, Abcam), rat anti-Musashi1 (1:1000; gift from Prof. Hideyuki Okano, Keio University, School of Medicine, Department of Physiology, Tokyo, Japan), rabbit anti-Cleaved Notch1 (1:100; 2421, Cell Signaling Technology), rabbit anti-Hes1 (1:1000; sc-25392, Santa Cruz Biotechnology), rabbit anti-Numb (1:100; ab14140, Abcam), and rabbit anti-Brm (1:400; ab15597, Abcam). Antigen retrieval for all primary antibodies was achieved by boiling in 10 mM citrate buffer pH 6.0 for 15 min. For quantitative analysis, cell counting or measurement of villi length was performed in at least ten sections from three animals for each genotype.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from murine intestine using the RNeasy Mini kit (Qiagen). Single-stranded cDNA was prepared using Superscript III (Invitrogen). Quantitative RT-PCR was performed on a Light-cycler FastStart DNA Master SYBR Green 1 kit (Roche Diagnostic). Expression levels were normalized to *GAPDH* levels using the ΔCt method. Primers are listed in Table S1.

Statistics

Student's *t*-tests were performed to determine statistical differences, with $P<0.05$ considered to be statistically significant. Kaplan–Meier analysis was used to analyze percentage survival. Values are presented as means±s.d. unless otherwise noted.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Y.T. and A.F. conceived and designed the study. Y.T. performed the experiments. Y.T., A.F. and H.S. analyzed the data. Y.T., A.F., T.C. and H.S. wrote the manuscript.

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Supplementary information

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