

Brg1 is required to maintain colorectal cancer stem cells

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Abstract

Tumor cells capable of self-renewal and continuous production of progeny cells are called tumor stem cells (TSCs) and are considered to be potential therapeutic targets. However, the mechanisms underlying the survival and function of TSCs are not fully understood. We previously reported that chromatin remodeling regulator Brg1 is essential for intestinal stem cells in mice and Dclk1 is an intestinal TSC marker. In this study, we investigated the role of Brg1 in Dclk1⁺ intestinal tumor cells for the maintenance of intestinal tumors in mice. Specific ablation of Brg1 in Dclk1⁺ intestinal tumor cells reduced intestinal tumors in *Apc*^{Min} mice, and continuous ablation of *Brg1* maintained the reduction of intestinal tumors. Lineage tracing in the context of Brg1 ablation in Dclk1⁺ intestinal tumor cells revealed that *Brg1*-null Dclk1⁺ intestinal tumor cells did not give rise to their descendent tumor cells, indicating that Brg1 is essential for the self-renewal of Dclk1⁺ intestinal tumor cells. Five days after Brg1 ablation, we observed increased apoptosis in Dclk1⁺ tumor cells. Furthermore, Brg1 was crucial for the stemness of intestinal tumor cells in a spheroid culture system. *BRG1* knockdown also impaired cell proliferation and increased apoptosis in human colorectal cancer (CRC) cells. Microarray analysis revealed that apoptosis-related genes were upregulated and stem cell-related genes were downregulated in human CRC cells by *BRG1* suppression. Consistently, high *BRG1* expression correlated with poor disease-specific survival in human CRC patients. These data indicate that Brg1 plays a crucial role in intestinal TSCs in mice by inhibiting apoptosis and is critical for cell survival and stem cell features in human CRC cells. Thus, *BRG1* represents a new therapeutic target for human CRC.

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Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the second most common cause of cancer-related death [1]. CRC is often diagnosed at an inoperable stage, with local invasion and distant metastases, and advanced CRC frequently demonstrates resistance to conventional chemotherapy. Thus, there is an urgent need to develop a novel therapeutic approach in addition to conventional cytotoxic drugs. Recently, in addition to mutations in certain oncogenes and tumor suppressor genes (*APC*, *KRAS*, *TP53*, *BRAF*), accumulation of epigenetic alterations has been shown to be involved in CRC [2]. Epigenetic modifications include DNA methylation, histone modifications, and chromatin remodeling, and play important roles in the initiation and

progression of CRC [3,4]. Therefore, drug discovery related to epigenetic modifications represents a promising field for the treatment of relapse after conventional cytotoxic chemotherapy.

SWI/SNF (switch/sucrose non-fermentable) complexes dynamically change chromatin structure and gene expression [5], with important roles in transcriptional regulation, DNA replication, and damage repair [6,7]. Mammalian SWI/SNF complexes are divided into three major assemblies: BAF, PBAF, and GBAF [8]. All of these complexes work in an ATP-dependent manner and contain one of two mutually exclusive catalytic ATPase subunits: SMARCA4 (BRG1, also known as Brahma-related gene 1) or SMARCA2 (BRM, also known as Brahma) [5]. Since silencing of *BRG1* occurs in a variety of human cancers, including CRC [9],

BRG1 has been considered as a tumor suppressor. Our group previously reported that Brg1 functions as a tumor suppressor in pancreatic cancer in the context of pancreatic-duct cell-derived tumorigenesis [10]. In contrast, BRG1 has recently been shown to be overexpressed in many tumor types and is associated with tumor aggressiveness and poor prognosis [11,12]. We showed that Brg1 is essential for acinar cell-derived pancreatic cancer formation [13]. Many human CRCs strongly express BRG1 [14,15], which is associated with recurrence, metastasis, and poor prognosis [14,16]. A recent report showed that Brg1 loss attenuates the aberrant Wnt signaling pathway and prevents Wnt-dependent tumorigenesis in the murine small intestine [17]. However, this report focused on the initiation of intestinal tumors and it remains unknown whether Brg1 is essential for their maintenance and progression.

The 'tumor stem cell' (TSC) theory has been widely accepted since cells with stem-like potential were first demonstrated in leukemia [18]. TSCs are thought to have the capacity to self-renew and produce descendant cells, and to be related to metastasis and resistance to conventional cytotoxic drugs [19]. Thus, TSCs have attracted attention as promising targets for anticancer therapeutics. We previously reported that Brg1 is essential for the development of normal intestines and controls intestinal stem cells by regulating the Notch signaling pathway in mice [20]. Another group also showed that Brg1 is essential for the maintenance of normal intestinal stem cells [21].

Doublecortin-like kinase 1 (Dclk1) is a microtubule-associated protein kinase that was originally known as a tuft cell marker in the intestine [22]. However, Dclk1 is also a marker of TSCs in many cancers, including human CRC [23–26]. We previously used lineage tracing to demonstrate the stem cell properties of Dclk1-positive ($Dclk1^{+}$) cells in intestinal tumors [27]. Ablation of Dclk1-expressing tumor cells resulted in the collapse of intestinal tumors and a reduction in their number in *Apc^{Min}* mice [27]. In this study, we sought to determine whether Brg1 plays a role in intestinal TSCs for maintenance of established intestinal tumors. We found that intestinal TSC-specific ablation of Brg1 resulted in suppression of intestinal tumors with increased apoptosis in mice, suggesting that Brg1 is essential for self-renewal of intestinal TSCs. BRG1 suppression also resulted in increased apoptosis and loss of stem cell features in human CRC cells.

Materials and methods

Detailed methods are provided in supplementary material, Supplementary materials and methods. Use of human material was approved by the ethics committee of Kyoto University Hospital and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects.

Animals

All mouse experiments were approved by the animal research committee of Kyoto University (Kyoto, Japan) and performed in accordance with Japanese government regulations. The following mouse strains were used: *Dclk1^{CreERT2-IRES-EGFP}* mice, which were generated by our group [27]; *Brg1^{fllox}* mice, which were kindly gifted by David Reisman (University of Florida, Gainesville, FL, USA), with permission from Pierre Chambon (University of Strasbourg Institute for Advanced Study, Strasbourg, France) [28]; *Hnf1b^{CreERT2}* mice, which were kindly gifted by Jorge Ferrer (Imperial College, London, UK) [29]; and *Rosa26^{LacZ}* mice (JAX Laboratory 003309) and *Apc^{Min}* mice (JAX strain 002020), which were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions at the animal facilities of Kyoto University. Mice were maintained on a C57BL/6 background. *Apc^{Min}* mice were maintained by crossing *Apc^{Min}* males with C57BL/6J females. For induction of CreER-mediated recombination *in vivo*, 100 or 200 μ l of 20 mg/ml tamoxifen (Sigma-Aldrich, St Louis, MO, USA) in corn oil was intraperitoneally injected.

Results

Brg1 expression in intestinal tumors and intestinal TSCs in mice, human adenomas, and CRCs

We first investigated Brg1 by immunohistochemical (IHC) analysis in normal intestinal epithelium and intestinal tumors in mice. Brg1 was ubiquitously expressed in normal intestinal epithelial cells of wild-type (WT) mice and in almost all of the intestinal tumor cells of *Apc^{Min}* mice (Figure 1A). Reverse transcription quantitative PCR (RT-qPCR) showed that *Brg1* mRNA was higher in intestinal tumors in *Apc^{Min}* mice than in the normal intestinal epithelium of WT mice (Figure 1B). In human tissues, BRG1 immunohistochemistry was positive in all adenomas (10/10) and human CRC specimens (50/50) (Figure 1C,D), and *BRG1* mRNA was higher in human CRCs than in the surrounding normal colorectal epithelium (Figure 1E).

We next performed co-immunofluorescence staining for Brg1 with the intestinal TSC markers, Dclk1 and Lgr5. Dclk1-expressing cells and Lgr5-expressing cells in intestinal tumors were positive for Brg1 in *Dclk1-EGFP;Apc^{Min/+}* and in *Lgr5-EGFP;Apc^{Min/+}* mice, respectively (Figure 1F). Previously, we reported the gene expression profile of $Dclk1^{+}$ cells in the intestinal tumors of *Apc^{Min}* mice [30]. The microarray data (GSE 99387) showed that *Brg1* was upregulated in $Dclk1^{+}$ compared with $Dclk1^{-}$ intestinal tumor cells (Figure 1G). These data suggest that Brg1 may have a crucial role also in intestinal TSCs in mice, besides other intestinal tumor cells.

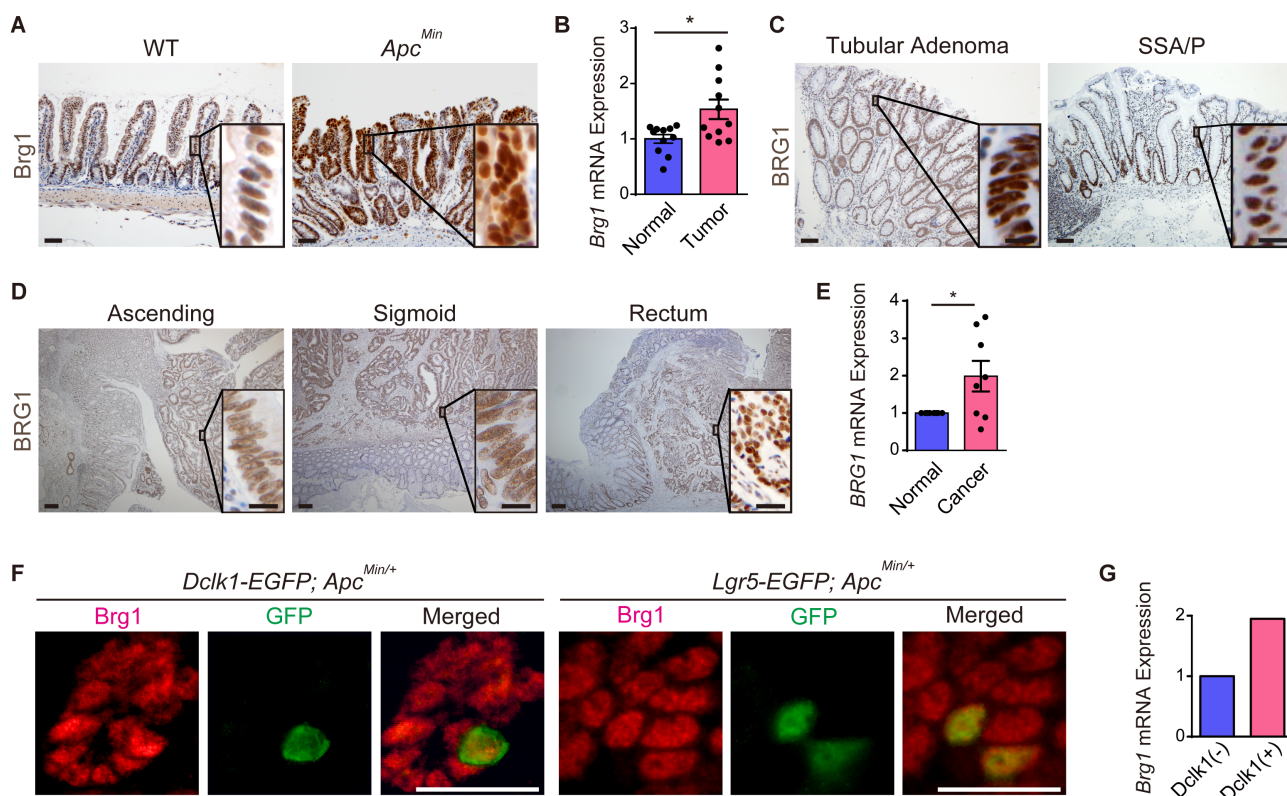


Figure 1. Brg1 is expressed in intestinal tumors and intestinal TSCs in mice and in human adenomas and colorectal cancer. (A) Immunostaining for Brg1 and (B) *Brg1* mRNA levels in the normal intestine of wild-type mice and intestinal tumors of *Apc*^{Min} mice (scale bars = 50 μ m; means $\pm$ SEM, $n = 11$, $p = 0.0115$, Student's *t*-test). (C) Immunostaining for BRG1 in human tubular adenoma and sessile serrated adenoma/polyp (SSA/P). Scale bars = 100 and 20 μ m (insets). (D) Immunostaining for BRG1 in human colorectal cancers (CRCs). Scale bars = 200 and 20 μ m (insets). (E) *BRG1* in human CRC and the surrounding normal epithelium ($p = 0.0466$; $n = 8$). (F) Co-immunostaining for Brg1 (red) and GFP (green) in *Dclk1*-EGFP;*Apc*^{Min/+} and *Lgr5*-EGFP;*Apc*^{Min/+} mice. Scale bars = 20 μ m. (G) Relative levels of *Brg1* in Dclk1-positive and Dclk1-negative cells in intestinal tumors of *Dclk1*-EGFP;*Apc*^{Min/+} mice (GSE 99387; one microarray session; RNA was collected from all existing small intestinal tumors of seven mice). * $p < 0.05$.

Brg1 loss in Dclk1⁺ intestinal tumor cells suppressed the maintenance of intestinal tumors in mice

To assess the ratio of Dclk1⁺ cells in the intestines of WT mice and intestinal tumors of *Apc*^{Min} mice, we performed IHC for Dclk1. Dclk1 was expressed in tuft cells in WT mice and intestinal tumor cells in *Apc*^{Min} mice (supplementary material, Figure S1A); 1.3% of normal intestinal epithelial cells and 1.8% of intestinal tumor cells were Dclk1⁺ (supplementary material, Figure S1B), compatible with previous data [27]. We also performed IHC for GFP in intestinal tumors of *Dclk1*-EGFP;*Apc*^{Min/+} mice, where GFP⁺ cells occupied 0.7% of intestinal tumor cells (supplementary material, Figure S1C,D). We also performed flow cytometry for intestinal tumor cells of *Dclk1*-EGFP;*Apc*^{Min/+} mice and 1.3% of intestinal tumor cells were positive for GFP (supplementary material, Figure S1E,F). RT-qPCR validated that GFP⁺ tumor cells expressed *Dclk1* (supplementary material, Figure S1G). These findings indicated that Dclk1⁺ tumor cells represent a small subset of intestinal tumors cells in *Apc*^{Min/+} mice.

To determine the efficacy of the tamoxifen-inducible Cre-loxP recombination system in *Dclk1*^{CreERT2-IRES-EGFP/+} mice, we generated *Dclk1*^{CreERT2-IRES-EGFP/+};

Rosa26^{LacZ/+} mice and treated them with either a single injection of high-dose tamoxifen (4 mg/body) or 5 days of continuous tamoxifen injections (2 mg/body per day) and assessed CreER efficiency in Dclk1⁺ cells by X-Gal staining to identify LacZ activity. More than 70% of Dclk1⁺ cells were positively stained (supplementary material, Figure S1H,I), indicating that the *Dclk1*^{CreERT2} allele was reasonably effective at Cre induction.

To investigate the effect of Brg1 ablation in Dclk1⁺ intestinal tumor cells, we generated *Dclk1*^{CreERT2-IRES-EGFP/+};*Apc*^{Min/+};*Brg1*^{flox/flox} (DAB) mice along with *Dclk1*^{CreERT2-IRES-EGFP/+};*Apc*^{Min/+};*Brg1*^{flox/wt} and *Dclk1*^{CreERT2-IRES-EGFP/+};*Apc*^{Min/+};*Brg1*^{wt/wt} (DA) mice (supplementary material, Figure S1J) and analyzed intestinal tumors on days 14 and 28 after 5 days of continuous tamoxifen injections (supplementary material, Figure S1K). Since abundant intestinal tumors and microadenomas were already observed in 6-week-old *Apc*^{Min} mice (supplementary material, Figure S1L), we initiated tamoxifen injections from 6 weeks of age. As the number of intestinal tumors were comparable between *Dclk1*^{CreERT2-IRES-EGFP/+};*Apc*^{Min/+};*Brg1*^{flox/wt} and *Dclk1*^{CreERT2-IRES-EGFP/+};*Apc*^{Min/+};*Brg1*^{wt/wt} mice at day 28 (supplementary material, Figure S1M), we regarded them as valid control DA mice. The number

of intestinal tumors in *DAB* mice treated with tamoxifen was lower than that of *DA* mice on day 14 (Figure 2A,B). The number of intestinal tumors had increased by day 28 in *DA* mice, whereas the number of tumors did not increase in *DAB* mice (Figure 2A,B). Analysis of the size of the tumors revealed that *DAB* mice displayed significantly smaller tumors than *DA* mice on day 28 (Figure 2C). These results suggested that *Brg1* loss in *Dclk1*⁺ intestinal tumor cells suppressed the maintenance and progression of intestinal tumors in mice.

IHC showed that intestinal tumor cells in both *DA* and *DAB* mice were positive for *Brg1* (Figure 2A) and there were no differences between *DA* and *DAB* mice in the level of *Brg1* mRNA in intestinal tumors on day 28 (supplementary material, Figure S1N). Immunofluorescence revealed that *Dclk1*⁺ intestinal tumor cells in *DAB* mice also expressed *Brg1* (Figure 2A). These results suggested that *Brg1*-deficient *Dclk1*⁺ intestinal tumor cells could not be maintained and therefore disappeared, and that only the tumors that escaped *Brg1* ablation remained.

Continuous ablation of *Brg1* in *Dclk1*⁺ intestinal tumor cells maintained the suppression of intestinal tumors in mice

To determine whether ablation of *Brg1* in *Dclk1*⁺ intestinal tumor cells suppresses intestinal tumorigenesis over a longer period of time, we analyzed tumors at day 56 after the last tamoxifen injection (supplementary material, Figure S2A). In contrast to days 14 and 28, we did not observe a significant difference in the number of tumors between *DA* and *DAB* mice on day 56 (Figure 2D,E). Again, the remaining tumors in both groups were positive for *Brg1* (supplementary material, Figure S2B). These results suggest that the effect of *Brg1* ablation on the suppression of intestinal tumors was temporary and did not persist.

To determine whether continuous ablation of *Brg1* in *Dclk1*⁺ intestinal tumor cells maintains the suppression of intestinal tumors, we assessed the tumor phenotype at 20 weeks after beginning the treatment at 6 weeks of age (supplementary material, Figure S2C). Notably, the ratio of intestinal tumors in *DAB* mice compared with *DA* mice was significantly lower at the 20-week time point (Figure 2F,G). Again, the remaining tumors in the *DAB* mice were positive for *Brg1* (supplementary material, Figure S2D). These results indicate that continuous *Brg1* ablation in *Dclk1*⁺ intestinal tumor cells suppresses intestinal tumors in mice.

Brg1 deletion in *Dclk1*⁺ intestinal tumor cells reduces self-renewal capacity

To clarify the effect of *Brg1* ablation on the stem cell activity of *Dclk1*⁺ tumor cells, we performed lineage tracing experiments. We generated *Dclk1*^{CreERT2-IRE5-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{flox/flox} (*DRAB*) mice along with *Dclk1*^{CreERT2-IRE5-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{flox/wt} or *Dclk1*^{CreERT2-IRE5-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{wt/wt} (*DRA*) mice

(Figure 3A) to trace the fate of *Dclk1*⁺ cells by the reporter protein LacZ (identified by blue staining with X-Gal). After administration of a single injection of high-dose tamoxifen (4 mg/body), lineage tracing showed that LacZ-labeled *Dclk1* lineage-positive tumor cells existed on day 2, and that expansion of these lineage-positive blue cells occurred around days 5–10 in *DRA* mice (Figure 3B). Consistent with a previous report [27], these blue-labeled tumor cells were maintained in *DRA* mice even on day 56 after the last tamoxifen injection (Figure 3B). In *DRAB* mice, lineage-positive cells also appeared on day 2 and expanded within the tumors at approximately days 5–10 (Figure 3C), consistent with the observations in *DRA* mice. However, these *Dclk1* lineage-positive tumor cells gradually decreased over time and finally disappeared in *DRAB* mice (Figure 3C), suggesting that *Brg1*-negative *Dclk1*⁺ intestinal tumor cells cannot maintain their lineages (Figure 3D). IHC identified both *Brg1*-negative and *Dclk1* lineage-positive tumor cells, and the proportion of such cells in all intestinal tumors increased 2–5 days after tamoxifen injection (Figure 3E,F). We confirmed that *Brg1*-negative cells also increased in *Dclk1* lineage-positive cells (supplementary material, Figure S3). However, the proportion of *Brg1*-negative blue-labeled cells decreased over time and almost disappeared in *DRAB* mice, suggesting the importance of *Brg1* in *Dclk1*-lineage cells.

These results suggest that ablation of *Brg1* in *Dclk1*⁺ intestinal tumor cells resulted in their loss of the capacity to self-renew, and that *Dclk1*⁺ intestinal tumor cells in which *Brg1* was ablated did not produce descendant cells in murine intestinal tumors.

Intestinal TSC-specific *Brg1* ablation increases apoptosis in intestinal TSCs and tumor cells

To investigate how specific ablation of *Brg1* in *Dclk1*⁺ intestinal tumor cells affects intestinal tumors over a short period of time, we induced *DA* and *DAB* mice with a single tamoxifen injection (4 mg/body). We observed no morphological changes in the intestinal tumors of *DA* mice on day 5 (Figure 4A). In contrast, the intestinal tumors in *DAB* mice collapsed on day 5 (Figure 4A). IHC showed that *Brg1*-negative tumor cells increased on day 5 (Figure 4B). In addition, co-immunofluorescence revealed that *Dclk1*⁺ intestinal tumor cells were negative for *Brg1* (Figure 4C,D). Flow cytometry also validated that *Brg1* was suppressed in GFP⁺ cells in *DAB* mice (Figure 4E). These findings confirmed the loss of *Brg1* in *Dclk1*⁺ intestinal tumor cells in *DAB* mice. Importantly, IHC showed that the ratio of cleaved caspase-3-positive (CC3⁺) cells in the intestinal tumors of *DAB* mice was higher than that in *DA* mice on day 5 (Figure 4F). IHC of CC3 and *Brg1* in serial sections revealed that most CC3⁺ tumor cells were *Brg1*[−] (Figure 4G), suggesting an essential role for *Brg1* in cell survival. Co-immunostaining for CC3 and *Dclk1* revealed that *Dclk1*⁺ intestinal tumor cells were CC3⁺ (Figure 4H). These results indicate that *Brg1* loss in *Dclk1*⁺ cells led to apoptosis of these cells.

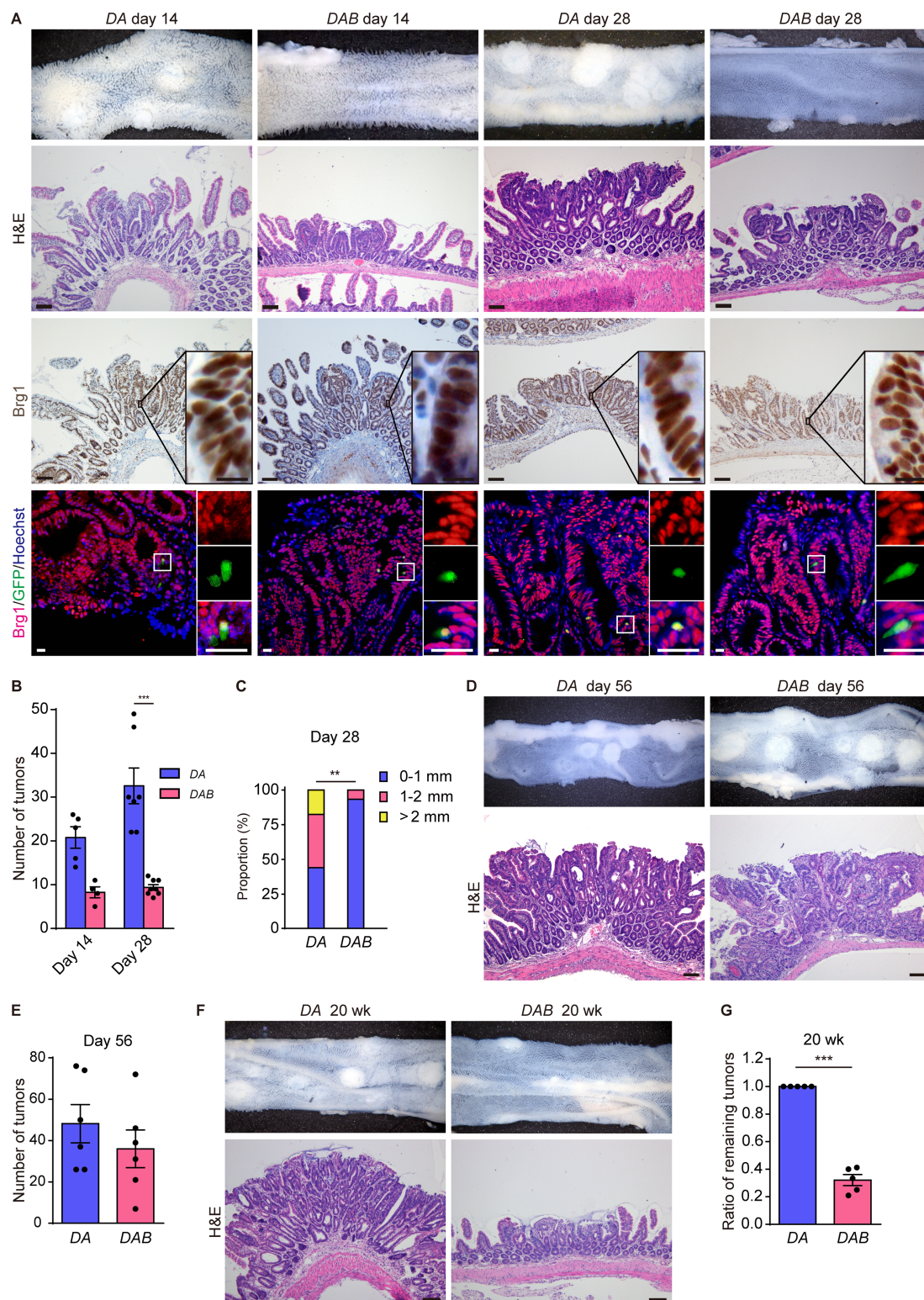


Figure 2. Continuous ablation of Brg1 in *Dclk1*⁺ intestinal tumor cells maintained suppression of intestinal tumors in mice. (A) Macroscopic images, H&E staining, and immunohistochemistry for Brg1 and co-immunostaining for Brg1 (red), GFP (green), and Hoechst (Blue) in *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{flox/wt} or *Dclk1*^{CreERT2-IRES-EGFP/+}; *Apc*^{Min/+}; *Brg1*^{wt/wt} (DA) and DAB mice at days 14 and 28 after the last tamoxifen (Tam) injection. Scale bars = 100 μ m (immunohistochemistry), 50 μ m (immunohistochemistry, insets), and 20 μ m (co-immunostaining). (B) The number of intestinal tumors in DA and DAB mice at days 14 and 28 after the last Tam injection [$n = 5, 4, 7, 8$; mean $\pm$ SEM, $p = 0.0003$, Student's t -test ($n \geq 5$)]. (C) Intestinal tumor size in DA and DAB mice at day 28 after the last Tam injection ($n = 130$ tumors from four DA mice, 15 tumors from three DAB mice; $p = 0.00133$; chi-square test). (D) Macroscopic images and H&E staining in DA and DAB mice at day 56 after the last Tam injection. Scale bars = 100 μ m. (E) Tumor numbers in DA and DAB mice at day 56 after the last Tam injection ($n = 6$, means $\pm$ SEM, $p = 0.3701$, Student's t -test). (F) Macroscopic images, H&E staining in DA and DAB mice 20 weeks after the last Tam injection. Scale bars = 100 μ m. (G) The ratio of remaining tumors in DA and DAB mice 20 weeks after the last Tam injection (mean $\pm$ SEM, $n = 5$, $p < 0.0001$; Student's t -test). ** $p < 0.01$, *** $p < 0.001$.

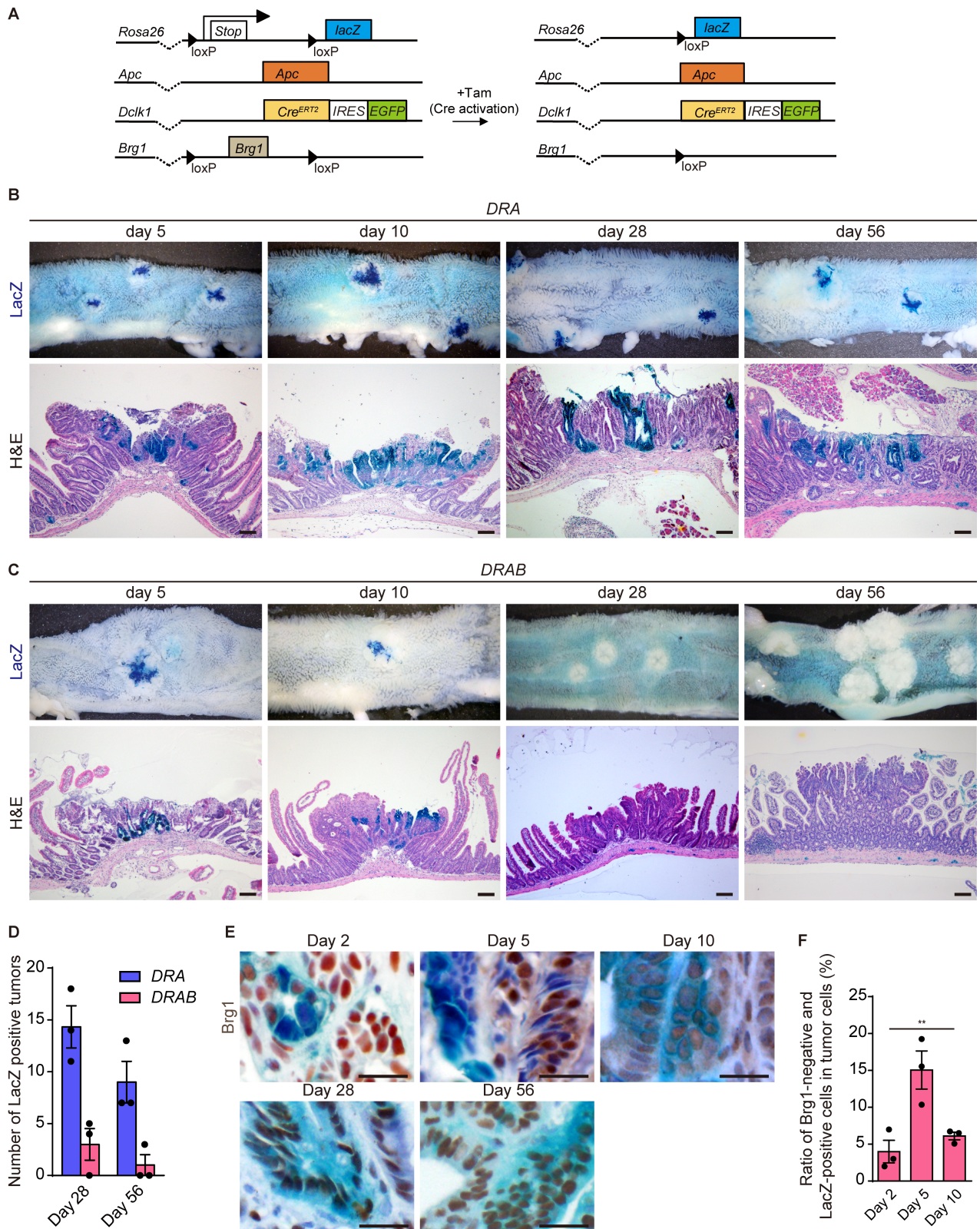


Figure 3. *Brg1* deletion in *Dclk1*⁺ intestinal tumor cells resulted in loss of self-renewal of *Dclk1*⁺ intestinal tumor cells. (A) Genetic strategy for lineage tracing of *Brg1*-deleted intestinal TSCs following tamoxifen (Tam) induction in *Dclk1*^{CreERT2-IRES-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{lox/lox} (DRAB) mice. (B, C) Macroscopic images and H&E staining of intestinal tumors with LacZ staining in (B) *Dclk1*^{CreERT2-IRES-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{lox/lox} or *Dclk1*^{CreERT2-IRES-EGFP/+}; *Rosa26*^{LacZ/+}; *Apc*^{Min/+}; *Brg1*^{wt/wt} (DRA) and (C) in DRAB mice at the indicated days after the last Tam injection (scale bars = 100 μ m). (D) The number of *Dclk1*-lineage positive tumors in DRA and DRAB mice on days 28 and 56 after the last Tam injection (mean $\pm$ SEM). (E) Immunostaining for *Brg1* with LacZ staining in the intestinal tumors of DRAB mice at the indicated days after last Tam injection (scale bars = 20 μ m). (F) The ratio of *Brg1*-negative and LacZ-positive cells in intestinal tumor cells ($n = 3$, $p = 0.0094$; one-way ANOVA). ** $p < 0.01$.

To determine the effect of Brg1 ablation on proliferative capacity, we performed IHC for Ki67 (Figure 4I,J). The ratio of Ki67⁺ cells was not significantly different between *DA* and *DAB* mice, and co-immunostaining revealed that Brg1[−] intestinal tumor cells were Ki67⁺ (Figure 4K), indicating that Brg1 loss did not affect the proliferative capacity of intestinal tumor cells.

Finally, we examined the proportion of Dclk1⁺ cells both in the normal intestine and in intestinal tumors of *DAB* mice on day 5 after tamoxifen injection (Figure 4L). There was no difference in the proportion of Dclk1⁺ cells in the normal epithelium between *Apc*^{Min} and *DAB* mice (Figure 4M), indicating that Brg1 ablation in tuft cells did not affect the proportion of tuft cells. In contrast, the proportion of Dclk1⁺ intestinal tumor cells was lower in *DAB* mice than in *Apc*^{Min} mice (Figure 4M). These results indicate that specific Brg1 ablation in Dclk1⁺ intestinal tumor cells resulted in increased apoptosis of both Dclk1⁺ intestinal tumor cells and other tumor cells, and that Brg1 is crucial for the survival of Dclk1⁺ intestinal tumor cells.

Brg1 was crucial for the stemness of intestinal tumor cells in a spheroid culture system

To directly investigate the cell-autonomous function of Brg1 in intestinal tumor cells without the impact of the microenvironment, we established spheroids from intestinal tumors with or without Brg1 expression. Given that almost all intestinal tumor cells expressed Hnf1b (Figure 5A), we generated *Hnf1b*^{CreERT2/+};*Apc*^{Min/+};*Brg1*^{flox/flox} (*HAB*) mice (Figure 5B) and established spheroids from intestinal tumors. Indeed, all spheroids from intestinal tumors in *Apc*^{Min} mice were positive for Hnf1b (Figure 5A). After passaging, we added 4-OHT (4-hydroxytamoxifen) to the culture media (Figure 5C). Notably, spheroids from the intestinal tumors of *HAB* mice incubated with 4-OHT rarely grew, whereas spheroids from the intestinal tumors of *Apc*^{Min} mice grew in media containing 4-OHT (Figure 5D,E). Levels of stem cell-related mRNAs were downregulated in 4-OHT-administered spheroids from *HAB* mice compared with controls (Figure 5F).

Next, we established tumor spheroids from intestinal tumors of *DAB* mice. To maintain Dclk1⁺ intestinal tumor cells and their capacity to give rise to their lineage cells, we added IL-13 and valproic acid to the culture media. After establishing spheroids from *DAB* mice and passaging them, we added 4-OHT to the culture medium to ablate *Brg1*. Notably, spheroids from *DAB* mice could not survive in 4-OHT-containing medium (Figure 5G,H). We validated that *Brg1* was suppressed in spheroids from *DAB* mice with 4-OHT-containing medium (Figure 5I). These findings further demonstrate the importance of Brg1 for the stemness of intestinal tumor cells in the spheroid culture system.

BRG1 was crucial for cell survival and stemness in human CRC cells

To investigate the role of BRG1 in human CRC, we next used a small interfering RNA (siRNA) in human CRC

cell lines (supplementary material, Figure S4A validated transfection efficiency). siBRG1 reduced *BRG1* mRNA to nearly 10% in both DLD-1 and HCT 116 cells compared with controls (supplementary material, Figure S4B). Western blotting and immunostaining showed that BRG1 was suppressed also at the protein level (Figure 6A and supplementary material, Figure S4C). Cell growth was repressed in *BRG1*-silenced DLD-1 and HCT 116 cells (Figure 6B,C), and annexin V staining and western blotting for CC3 revealed that apoptosis was increased in *BRG1*-silenced cells (Figure 6D–F).

To determine whether BRG1 is crucial for the stem cell properties of human CRC cells, we next performed a sphere formation assay. DLD-1 and HCT 116 cells easily formed spheres, whereas sphere formation was compromised when *BRG1* was silenced (Figure 6G). To determine the importance of BRG1 for the growth of human CRC cells, we next performed xenograft experiments. We generated *BRG1*-silenced human CRC cells by lentivirus transduction of shBRG1 and transplanted them in NOD/SCID mice. *Brg1* mRNA was downregulated by about 20% and IHC showed that shBRG1-transfected xenograft tumors had weak BRG1 staining (supplementary material, Figure S5A,B). Notably, *BRG1*-silenced human CRC cells grew more slowly than control cells (Figure 6H–J). These findings indicated that BRG1 suppression impaired the growth of human CRC cells.

Apoptosis-related genes were upregulated and stem cell-related genes were downregulated in human CRC cells by *BRG1* suppression

To elucidate the mechanisms through which BRG1 facilitates cell survival and stemness in human CRC cells, we performed microarray analysis of DLD-1 and HCT 116 cells silenced with control or siBRG1. Levels of stem cell-related and Wnt signaling pathway mRNAs were downregulated, whereas apoptosis-related mRNAs were upregulated in *BRG1*-silenced DLD-1 and HCT 116 cells (supplementary material, Figure S6). KEGG pathway analysis showed that *Brg1* suppression affected pathways related to cancer, apoptosis, and the Wnt signaling pathway (Figure 6K).

Of the 50 most highly upregulated genes in *BRG1*-silenced human CRC cells (Figure 6L and supplementary material, Table S3), *JUN*, *DDIT3*, *MAP3K14*, and *HMOX1* are genes related to apoptosis. Among the 50 most downregulated genes (Figure 6M and supplementary material, Table S4), *TNS4* and *AXIN2* are Wnt target genes, while *MYB*, *KRT23*, and *AXIN2* are related to stemness. Gene set enrichment analysis (GSEA) revealed negative enrichment for stem cell genes, Wnt signaling pathway genes, and MYC target genes, whereas there was a positive enrichment of gene sets related to apoptosis in *BRG1*-silenced cells (Figure 6N). RT-qPCR validated that Wnt signaling pathway genes and MYC target genes were downregulated

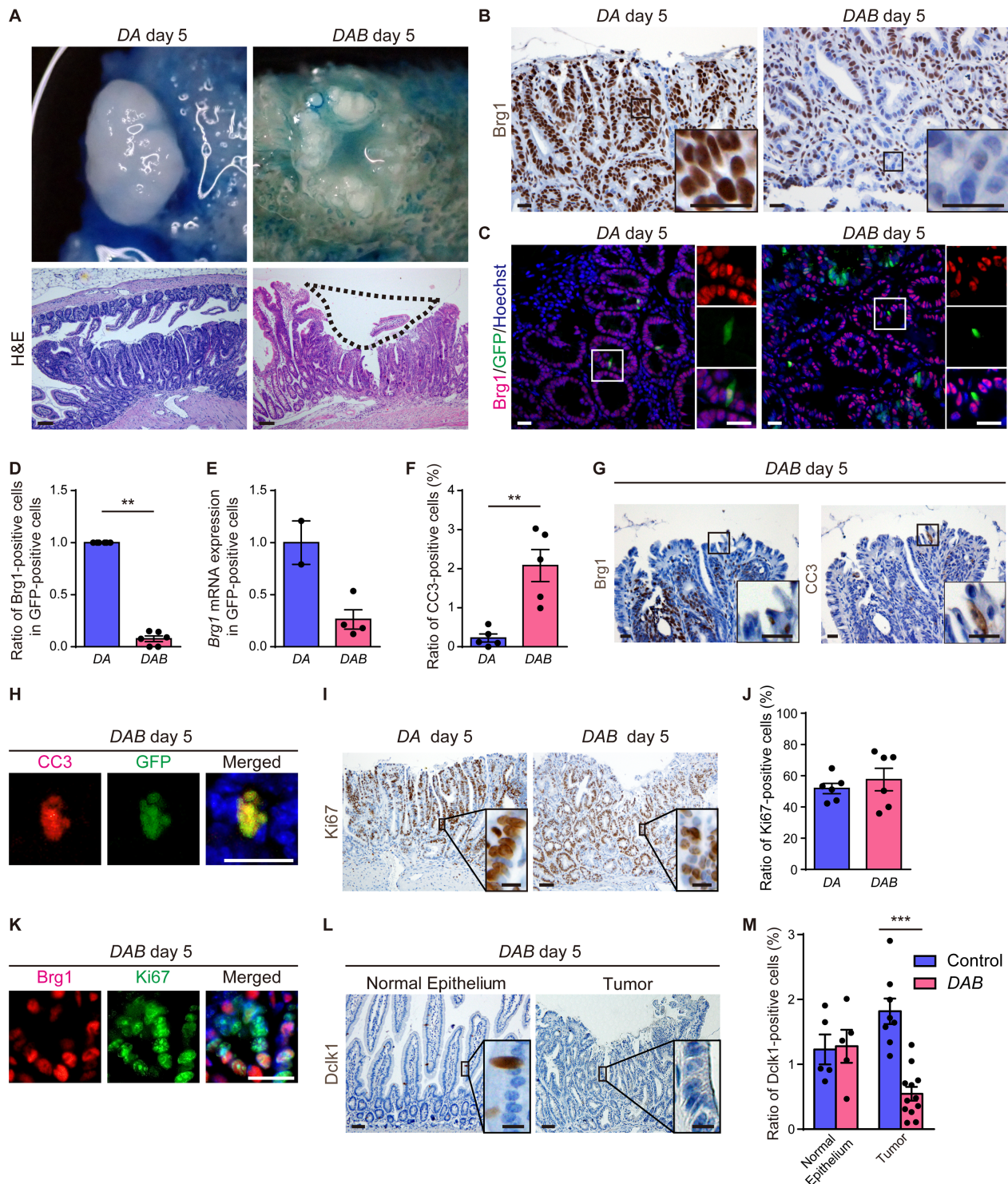


Figure 4. *Dclk1*⁺ intestinal tumor cell-specific Brg1 ablation results in increased apoptosis in *Dclk1*⁺ intestinal TSCs and other tumor cells. (A) Macroscopic images with indigo carmine, H&E staining (scale bars = 100 μ m); (B) Brg1 immunohistochemistry; and (C) co-immunostaining for Brg1 (red), GFP (green), and Hoechst (blue) in intestinal tumors of DA and DAB mice at day 5 after the last Tam injection (scale bars = 20 μ m). (D) The ratio of Brg1⁺ cells in GFP⁺ intestinal tumor cells in DA and DAB mice at day 5 after the last Tam injection (mean $\pm$ SEM, $n = 6$, $p = 0.0022$, Student's *t*-test). (E) *Brg1* mRNA expression in GFP⁺ intestinal tumor cells in DA and DAB mice at day 2 after the last Tam injection (mean $\pm$ SEM). (F) CC3⁺ cells in intestinal tumors in DA and DAB mice at day 5 after the last Tam injection (mean $\pm$ SEM, $n = 5$, $p = 0.0023$; Student's *t* test). (G) Immunohistochemistry for CC3 and Brg1 in serial sections and (H) co-immunostaining for CC3 (red), GFP (green), and Hoechst (blue) in intestinal tumors in DAB mice at day 5 after the last Tam injection (scale bars = 20 μ m). (I) Ki67 immunohistochemistry [scale bars = 50 and 20 μ m (insets)] and (J) the ratio of Ki67⁺ cells in intestinal tumors of DA and DAB mice at day 5 after the last Tam injection (mean $\pm$ SEM, $n = 6$, $p = 0.4882$, Student's *t*-test). (K) Co-immunostaining for Brg1 (red), Ki67 (green), and Hoechst (blue) in intestinal tumors in DAB mice at day 5 after the last Tam injection. Scale bars = 20 μ m. (L) Immunostaining for Dclk1 in normal small intestine and intestinal tumors in DAB mice at day 5 after the last Tam injection. Scale bars = 50 and 20 μ m (insets). (M) The ratio of Dclk1⁺ cells in the normal small intestine and intestinal tumors (mean $\pm$ SEM; $n = 5, 5, 8, 12$; normal epithelium: $p = 0.8879$; tumor: $p = 0.000188$; Student's *t*-test). ** $p < 0.01$, *** $p < 0.001$.

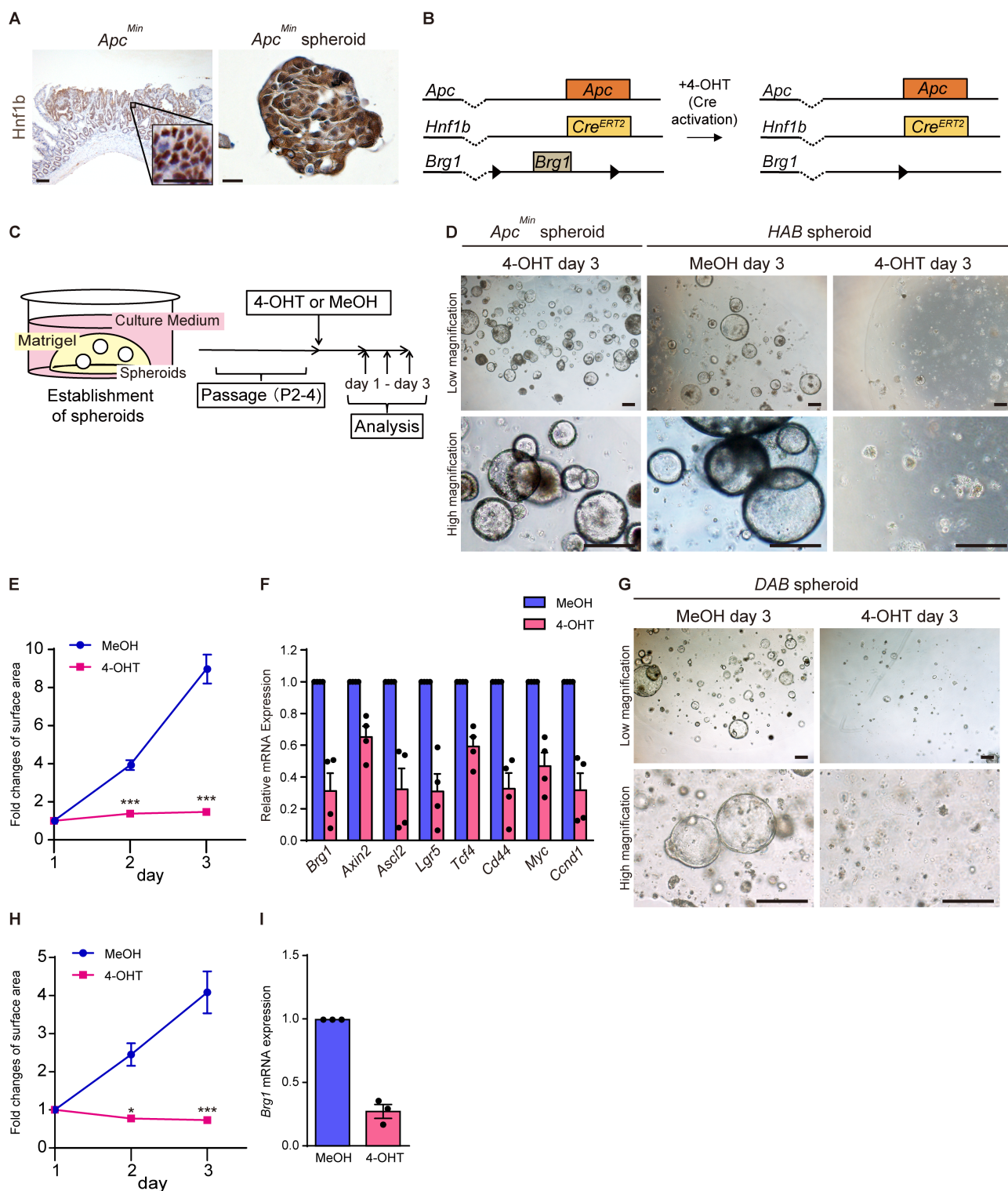


Figure 5. Brg1 is crucial for the stemness of intestinal tumor cells in a spheroid culture system. (A) Immunohistochemistry for Hnf1b in intestinal tumors and spheroids in *Apc*^{Min} mice [scale bars = 100 and 20 μ m (inset)]. (B) Strategy for *Brg1* ablation in intestinal tumor cells following tamoxifen (4-OHT) induction in *Hnf1b*^{CreERT2/+};*Apc*^{Min/+};*Brg1*^{flx/flx} (*HAB*) mice. (C) Schedule and (D) images of *Apc*^{Min} spheroids treated with 4-OHT and *HAB* spheroids treated with MeOH and 4-OHT at day 3 (scale bars = 200 μ m). (E) Fold-changes of the surface area of *HAB* spheroids treated with MeOH or 4-OHT (mean $\pm$ SEM, *n* = 22; day 2: *p* < 0.0001; day 3: *p* < 0.0001; Student's *t*-test). (F) RT-qPCR for stem cell-related genes in *HAB* spheroids treated with MeOH or 4-OHT (mean $\pm$ SEM; *n* = 4). (G) Images of *DAB* spheroids treated with MeOH and 4-OHT at day 3 after administration. Scale bars = 200 μ m. (H) Fold-changes of the surface area of *DAB* spheroids treated with MeOH or 4-OHT (means $\pm$ SEM, *n* = 23; day 2: *p* = 0.0182; day 3: *p* < 0.0001; Student's *t*-test). (I) RT-qPCR for *Brg1* in *DAB* spheroids treated with MeOH or 4-OHT at day 3 (mean $\pm$ SEM; *n* = 3).

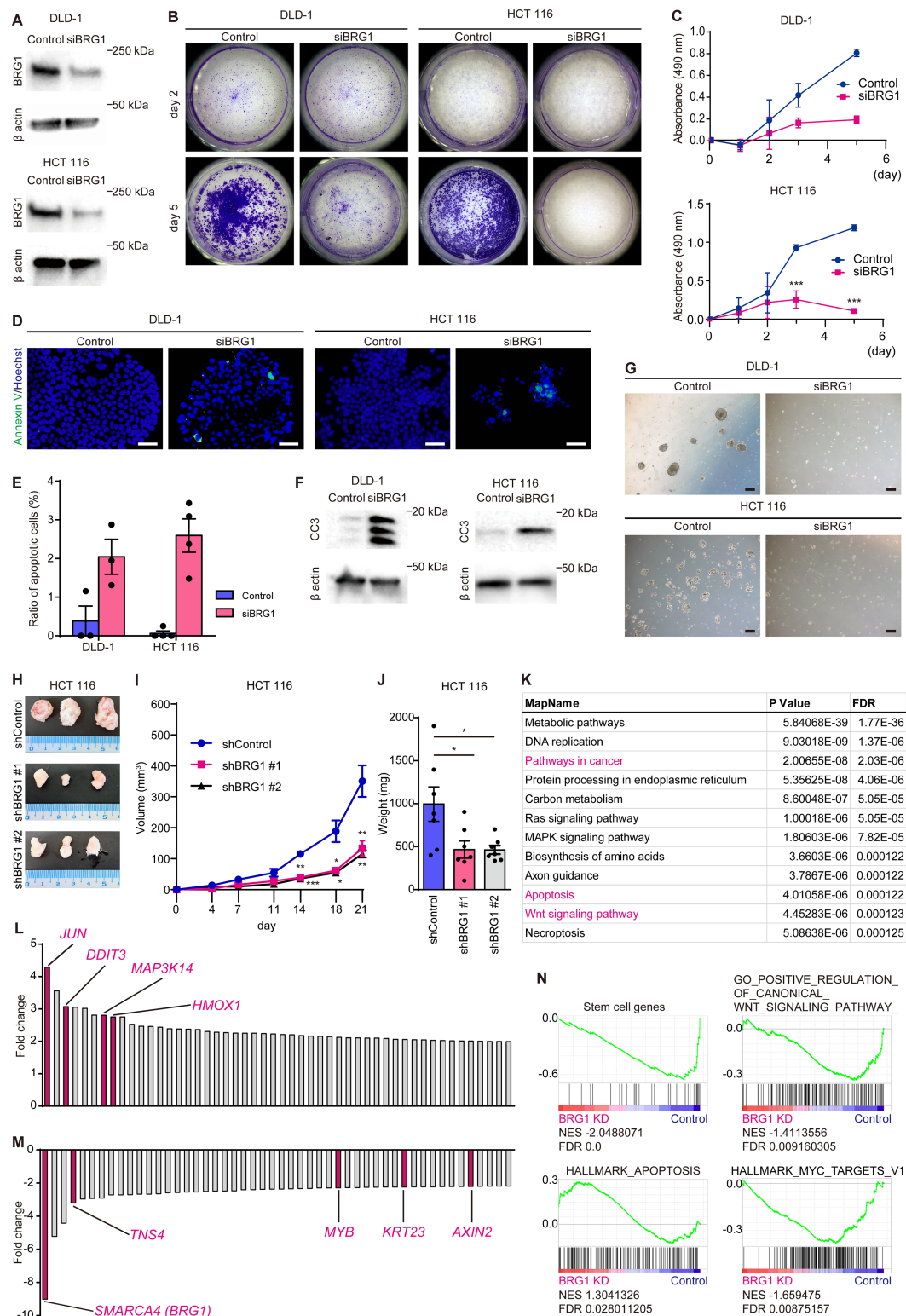


Figure 6. BRG1 suppression resulted in increased apoptosis and loss of stemness features concomitant with upregulated expression of apoptosis-related genes in human CRC cells. (A) Western blot for BRG1 in DLD-1 and HCT 116 cells 2 days after siControl or siBRG1. (B) Crystal violet staining of DLD-1 and HCT 116 cells on days 2 and 5 after siRNA treatment. (C) MTS assay of DLD-1 and HCT 116 cells silenced with control or siBRG1 (mean $\pm$ SEM; HCT 116: day 3, $p < 0.0001$; day 5, $p = 0.000475$; $n = 3, 5$; Student's t -test). (D) Annexin V (green) and Hoechst (blue) in DLD-1 and HCT 116 on day 2 after siRNA treatment (scale bars = 20 μ m). (E) Ratio of annexin V⁺ cells in DLD-1 and HCT 116 cells with control or siBRG1 (mean $\pm$ SEM, $n = 3, 4$). (F) Western blot for CC3 in DLD-1 and HCT 116 cells on day 2 after siRNA treatment. (G) Images of sphere formation of DLD-1 and HCT 116 cells on day 4 after siRNA treatment (scale bars = 100 μ m). (H) Representative images, (I) tumor volumes, and (J) weights of HCT 116 xenografts with control and shBRG1 on day 21 after transplantation (mean $\pm$ SEM; shBRG1 #1, $p = 0.0428$; shBRG1 #2, $p = 0.0376$; $n = 7$; Student's t -test). (K) KEGG pathway analysis of microarray data from DLD-1 and HCT 116 with control or siBRG1. (L) The 50 most upregulated and (M) the 50 most downregulated genes, and (N) gene set enrichment analysis (GSEA) of human CRC cells silenced with control and siBRG1. NES, normalized enrichment score; FDR, false discovery rate.

in *BRG1*-silenced human CRC cells (Supplementary material, Figure S7).

We also investigated molecular pathways or genes that correlated with *BRG1* mRNA levels in human CRC tissues using TCGA data. GSEA revealed that stem cell signatures, the Wnt/ β -catenin pathway, and MYC target genes were positively correlated, and that apoptosis-related genes were inversely correlated with *BRG1* (supplementary material, Figure S8). At the molecular level, intestinal TSC marker genes and Wnt pathway genes were positively correlated and apoptosis-related mRNA levels were inversely correlated with *BRG1* levels in human CRCs (supplementary material, Figure S9). In good agreement with the results from mouse intestinal tumors and human CRC cells, these findings suggested that *BRG1* expression was positively correlated with the expression of genes related to stem cell properties and inversely correlated with apoptosis-related genes in human CRCs.

Finally, we investigated whether *BRG1* mRNA correlates with survival in human CRC patients using a GEO dataset. Consistent with our data of mouse and human CRC cells, high *BRG1* expression was positively correlated with poor disease-specific survival (supplementary material, Figure S10), suggesting that BRG1 has a tumor-promoting function in human CRC.

Discussion

In this study, we demonstrated for the first time that Brg1 plays an essential role in intestinal TSCs for the maintenance of intestinal tumors in murine models. We found that Brg1 ablation in Dclk1⁺ intestinal tumor cells resulted in apoptosis of Dclk1⁺ intestinal tumor cells and other tumor cells, accompanied by loss of their capacity for self-renewal. Furthermore, we found that BRG1 plays a pivotal role in cell survival and stemness features in human CRC cells.

We revealed that Brg1 in Dclk1⁺ intestinal tumor cells is essential for maintaining intestinal tumors *in vivo*. In murine models, short-term ablation of Brg1 in Dclk1⁺ intestinal tumor cells suppressed intestinal tumors on days 14 and 28 after the last tamoxifen injection, although this effect did not persist. Brg1 was deleted in the Dclk1⁺ intestinal tumor cells of established intestinal tumors but not in newly developed tumors after Brg1 ablation, and Brg1 ablation in tuft cells did not affect the ratio of tuft cells. Therefore, our results suggest that Brg1 loss in Dclk1⁺ intestinal tumor cells suppresses the maintenance and progression, rather than initiation, of intestinal tumors in mice. Furthermore, continuous Brg1 ablation in Dclk1⁺ intestinal tumor cells sustained tumor suppression, even 20 weeks after the last tamoxifen injection. Notably, the remaining tumors in *DAB* mice were positive for Brg1, suggesting that these tumors developed from Dclk1⁺ intestinal tumor cells that had escaped Brg1 ablation due to incomplete Cre-loxP recombination. Another possibility is that the remaining tumors developed after Brg1 was ablated by tamoxifen injection. Moreover, a

previous report showed that once intestinal TSCs were temporally ablated, differentiated tumor cells dedifferentiated into cells with stem cell potential, which could restore the TSC pool and enable tumor re-expansion [31–33]. Although we could not detect the exact origin of these remaining tumors, this scenario is possible because of the high Brg1 ablation efficacy in Dclk1⁺ intestinal tumor cells.

Lineage tracing and spheroid experiments showed that Brg1 expression in Dclk1⁺ intestinal tumor cells is essential for maintaining their ability to self-renew and produce progeny for the maintenance of intestinal tumors in mice. We previously reported that both ablation of Dclk1⁺ intestinal tumor cells and deletion of the essential transcriptional factor (Hes1) in Dclk1⁺ intestinal tumor cells cause regression of intestinal tumors in *Apc^{Min}* mice [27,34], suggesting that this murine tumor model depends on stem cell properties and making it possible to monitor the function of intestinal tumor stem cells in a relatively short period. The present data that Brg1 ablation in Dclk1⁺ intestinal tumor cells resulted in tumor regression as rapidly as that of Dclk1⁺ tumor cells themselves or Hes1 indicate the critical role of Brg1 as an essential epigenetic modulator to maintain and regulate tumor stem cell survival, which is itself essential for the tumor to expand. Our present data that Dclk1⁺ cells represented 1–2% of all intestinal tumor cells are compatible with previous reports [27,30,33]. Lgr5⁺ cell ablation in spheroids of murine intestinal tumor caused regression *ex vivo* within 10 days [31], even though Lgr5⁺ cells represent a minor population of spheroid cells. Therefore, it is not surprising that a small subset of tumor cells is essential for maintenance of the whole tumor.

We and others previously reported that Brg1 is essential for the maintenance of normal intestinal epithelial stem cells and that *Brg1*-deficient intestine cannot produce epithelial cells and construct the normal crypt–villi axis [20,21]. We also reported that Arid1a, another subunit of the SWI/SNF complex, is also essential for the maintenance of intestinal epithelial stem cells [35]. The finding that the SWI/SNF complex, including Brg1, is essential for the maintenance of intestinal epithelial stem cells suggests that Brg1 also plays a critical role in the maintenance of intestinal TSCs.

Consistent with mouse data, *BRG1*-silenced human CRC cells were not able to form spheres and stemness-related genes were downregulated. We also showed that Brg1 plays a pivotal role in cell survival in both Dclk1⁺ intestinal TSCs and other tumor cells in mice and in human CRC cells. These findings are consistent with Brg1 deficiency increasing apoptosis in various organs and cell types [20,36], as well as in cancer cells [37]. In good agreement, *BRG1* mRNA correlated positively with stemness-related genes and inversely with apoptosis-related genes in the human TCGA.

In this study, Wnt signaling pathway and MYC target genes were downregulated in *BRG1*-suppressed human CRC cells and *BRG1* positively correlated with Wnt signaling pathway and MYC target genes in human CRC.

Previous reports suggested that Brg1 directly regulates the expression of Tcf and Myc [38,39], and that Wnt signaling and MYC target genes play important roles in the stemness of human CRC [40,41]. Therefore, it is conceivable that BRG1 positively regulates Wnt signaling pathway and MYC target genes. Further investigation is needed to fully understand the underlying molecular mechanisms by which Brg1 plays a pivotal role in the stemness and cell survival of murine intestinal TSCs and human CRC cells.

In summary, Brg1 plays an essential role in Dclk1⁺ intestinal tumor cells for the maintenance of established intestinal tumors in a mouse model. Ablation of Brg1 caused apoptosis of Dclk1⁺ TSCs and other intestinal tumor cells. Brg1 also plays a pivotal role in cell survival and stemness in human CRC cells. Thus, BRG1 represents a new therapeutic target for human CRC.

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Author contributions statement

TY and AF conceived and designed the study. MO, MN, MS, YF, TM, OA, MN, SO, KM, NG, YH, YM, MT, TM and YN conducted the experiments and analyzed the data. KK and ST contributed reagents, materials, and analytic tools. TY wrote the manuscript and AF and HS revised the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Brg1 loss in intestinal TSCs suppressed the maintenance of intestinal tumors in mice

Figure S2. The remaining tumors were positive for Brg1 after Brg1 ablation in *DAB* mice

Figure S3. The ratio of Brg1-negative cells in LacZ-positive intestinal tumor cells

Figure S4. *BRG1* expression was silenced by siBRG1 in human CRC cells

Figure S5. BRG1 expression in HCT 116 cells silenced with shBRG1

Figure S6. Heatmap of microarray analysis of DLD-1 and HCT 116 cells silenced with control or siBRG1

Figure S7. RT-qPCR analysis of Wnt pathway genes and MYC target genes in *BRG1*-silenced DLD-1 and HCT 116 cells

Figure S8. GSEA of CRC between the high and low *BRG1* expression group from the Cancer Genome Atlas (TCGA) RNA-seq data

Figure S9. Co-expression data between stemness-related genes, Wnt pathway genes, apoptosis-related genes, and *SMARCA4* in human CRC

Figure S10. Kaplan–Meier analysis of the human CRC patients between the *SMARCA4* high and low expression groups

Table S1. Primer sequences used for RT-qPCR (referred to in supplementary material, Supplementary materials and methods)

Table S2. The gene members of stem cell genes (referred to in supplementary material, Supplementary materials and methods)

Table S3. The 50 most highly upregulated genes in *BRG1*-silenced human CRC cells

Table S4. The 50 most highly downregulated genes in *BRG1*-silenced human CRC cells