Brg1 Loss Attenuates Aberrant Wnt-Signalling and Prevents Wnt-Dependent Tumourigenesis in the Murine Small Intestine

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Abstract

Tumourigenesis within the intestine is potently driven by deregulation of the Wnt pathway, a process epigenetically regulated by the chromatin remodelling factor Brg1. We aimed to investigate this interdependency in an in vivo setting and assess the viability of Brg1 as a potential therapeutic target. Using a range of transgenic approaches, we deleted Brg1 in the context of Wnt-activated murine small intestinal epithelium. Pan-epithelial loss of Brg1 using VillinCreERT2 and AhCreER7 transgenes attenuated expression of Wnt target genes, including a subset of stem cell-specific genes and suppressed Wnt-driven tumourigenesis improving animal survival. A similar increase in survival was observed when Wnt activation and Brg1 loss were restricted to the Lgr5 expressing intestinal stem cell population. We propose a mechanism whereby Brg1 function is required for aberrant Wnt signalling and ultimately for the maintenance of the tumour initiating cell compartment, such that loss of Brg1 in an Apc-deficient context suppresses adenoma formation. Our results highlight potential therapeutic value of targeting Brg1 and serve as a proof of concept that targeting the cells of origin of cancer may be of therapeutic relevance.

Introduction

More than 90% of colorectal cancers (CRC) are characterised by aberrant activation of the canonical Wnt/β-catenin pathway, which is proposed to play a major role in the initiation and progression of CRC [1]. Despite this clear link between deregulated Wnt signalling and disease, therapies which target the Wnt pathway remain limited [2]. There is, therefore, a substantial demand for novel approaches to inhibit the Wnt pathway, preferably downstream of common aberrations such as mutations in APC, AXIN2 or β-catenin. The emerging plethora of epigenetic factors involved in DNA methylation, histone modifications and chromatin remodelling represent a set of new and relatively unexplored opportunities for such therapeutic intervention [3].

Brahma-related gene 1 (Brg1) or SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 4 (SMARCA4) is one of the two mutually exclusive ATPase subunits of the 2-MD family of SWItch/Sucrose Non-Fermentable (SWI/SNF) class of chromatin remodelling complexes. Brg1 has been implicated in a variety of biological processes, in both normal and neoplastic tissues [4,5]. The majority of these studies, both in vitro and in vivo, suggest that Brg1 acts as a tumour suppressor. For example, it has been found to be mutated in numerous cancer cell lines and primary cancers [6–9]. In support of this, studies using Brg1 knock-out mouse models have shown that heterozygous loss of Brg1 increases susceptibility to both mammary gland and lung tumourigenesis [10,11].

By contrast, BRG1 has been shown to interact with β-catenin and facilitate trans-activation of Wnt-dependent reporter assays and endogenous Wnt target genes in cancer cell lines [12,13]. Therefore better understanding of the role of BRG1 in aberrant Wnt signalling may allow the development of novel Wnt intervention strategies.

We have recently reported that loss of Brg1 in the small intestinal epithelium results in depletion of the intestinal stem cell population [14]. In this study we aimed to investigate the functional interaction between Brg1 and the Wnt pathway by generating mice, which carried floxed alleles of both Apc [15] and Brg1 [16,17] genes, thus placing Brg1 deficiency in the context of aberrant activation of the Wnt pathway.

Using three different conditional approaches, we find that additional loss of Brg1 from the small intestinal epithelium attenuates the Wnt-dependent phenotype resulting from Apc deletion.

Results

Brg1 loss attenuates aberrant Wnt signalling in the murine small intestinal epithelium

In order to assess the immediate consequences of Brg1 loss upon Wnt activation, we employed the (Tg(Vil-cre/ESR1)23Syr) transgene [18] driving expression of Cre-ER7 recombinase under
Aberrant Wnt signalling is responsible for the majority of colorectal cancers, the third leading cause of cancer-related mortality in the UK. However, no therapies directly targeting Wnt signalling are currently available. Using mouse models of intestinal cancer, we demonstrate that deleting chromatin remodelling factor BrgL1 in the context of Apc-deficient small intestinal epithelium attenuates Wnt-driven gene expression changes and prevents adenoma formation, which results in extended animal survival. We also demonstrate that BrgL1 loss impairs the small intestinal stem cell expansion associated with aberrant activation of Wnt signalling. These findings highlight BrgL1 as a potential therapeutic target in Wnt-driven intestinal tumorigenesis and illustrate the viability of targeting the somatic stem cell as the ‘cell of origin’ of cancer, which might be particularly valuable in patients with known predisposition to cancer.

control of the Villin 1 promoter, further abbreviated as VillinCreERT2. To achieve high penetrance inactivation of targeted genes, VillinCreERT2Apcfl/fl and VillinCreERT2Apcfl/flBrgfl/fl mice along with VillinCreERT2 controls were induced with four daily injections of 80 mg/kg Tamoxifen. We sacrificed the mice 4 days after the first induction and collected samples of the small intestinal epithelium.

Immunohistochemical analysis of β-catenin and BrgL1 expression in the jejunal epithelium at day 4 post induction revealed nuclear localisation of β-catenin in both VillinCreERT2Apcfl/fl and VillinCreERT2Apcfl/flBrgfl/fl mice as well as complete loss of BrgL1 in double knock-out animals (Figure 1A). Upon histological inspection, the small intestinal epithelium of both VillinCreERT2Apcfl/fl and VillinCreERT2Apcfl/flBrgfl/fl mice displayed an aberrant proliferative and apoptotic response synonymous with Wnt pathway activation [19] (Figure 1A). Although no difference in crypt and villus length was observed between VillinCreERT2Apcfl/fl and VillinCreERT2Apcfl/flBrgfl/fl mice (Figure 1B, p>0.05, n=4), quantitative analysis of other histological parameters such as apoptosis and mitosis differences between the experimental groups (Figure 1B–1C). At the same time, the majority of VillinCreERT2Apcfl/fl mouse epithelium developed into large cystic structures (Figure 2C) consistent with a previous report of organoid culture under Wnt activated conditions [20]. In contrast, crypts derived from VillinCreERT2Apcfl/flBrgfl/fl epithelium rarely developed into large cysts and instead the majority remained as simple spherical organoids (Figure 2C). At the same time, the majority of VillinCreERT2Apcfl/fl crypts developed beyond this time point. VillinCreERT2Apcfl/flBrgfl/fl mice along with VillinCreERT2 controls were induced by 3 daily intraperitoneal injections of 3 mg Tamoxifen and sacrificed at day 3 post induction. We therefore used the in vitro intestinal organoid system to investigate the fate of double knock-out small intestinal crypts beyond this time point. VillinCreERT2Apcfl/fl and VillinCreERT2Apcfl/flBrgfl/fl mice along with VillinCreERT2 controls were induced by 3 daily intraperitoneal injections of 3 mg Tamoxifen and sacrificed at day 3 post induction. Small intestinal crypts were isolated and placed in cystic structures (Figure 2C) consistent with a previous report of organoid culture under Wnt activated conditions [20].

To further investigate the small intestine-specific role of BrgL1 in the Wnt pathway, we assessed expression levels of known Wnt target genes in VillinCreERT2Apcfl/flBrgfl/fl and double knock-out mice. qRT-PCR analysis of CD44, c-Myc, CyclinD1 and Ascl2 expression levels revealed significant down-regulation of these genes in VillinCreERT2Apcfl/flBrgfl/fl mice compared to VillinCreERT2Apcfl/fl epithelium (Figure 2E, p<0.05, n=3). Notably, we did not observe a significant difference in expression levels of Axin2, which is commonly used as a readout of Wnt pathway activation [21], indicating possible differential recruitment of BrgL1 to distinct Wnt target genes.

In order to further investigate the consequences of BrgL1 loss on the Wnt pathway, we assessed expression levels of known Wnt target genes in the small intestinal epithelium of VillinCreERT2Apcfl/fl and double knock-out mice. qRT-PCR analysis of CD44, c-Myc, CyclinD1 and Ascl2 expression levels revealed significant down-regulation of these genes in VillinCreERT2Apcfl/flBrgfl/fl mice compared to VillinCreERT2Apcfl/fl epithelium (Figure 2E, p<0.05, n=3). Notably, we did not observe a significant difference in expression levels of Axin2, which is commonly used as a readout of Wnt pathway activation [21], indicating possible differential recruitment of BrgL1 to distinct Wnt target genes.

To further investigate the small intestine-specific role of BrgL1 in the Wnt mediated transcriptional program, we performed genome-wide expression analysis of Apc-deficient and double knock-out (DKO) epithelium. VillinCreERT2Apcfl/fl (n=3) and VillinCreERT2Apcfl/flBrgfl/fl (n=4) mice along with VillinCreERT2 controls (n=4) were induced with four daily injections of 80 mg/
kg Tamoxifen and whole epithelial extract was harvested at day 4 post induction. RNA was extracted from epithelial samples, labelled and hybridised to Mouse Ref8 v2 Illumina array. To determine the baseline effect of Brg1 loss, we performed similar analyses on wild type and induced VillinCreERT2+/Apcfl/flBrgfl/fl samples, which are described in our earlier publication [14].

Figure 1. Brg1 loss attenuates the effects of Apc deletion in the small intestinal epithelium. A. H&E, β-catenin and Brg1 staining of the small intestinal epithelium from control VillinCreERT2−/−, VillinCreERT2+/Apcfl/fl and VillinCreERT2+/Apcfl/flBrgfl/fl mice 4 days after high-dose induction revealed disturbed crypt architecture and nuclear localisation of β-catenin in both cohorts, as well as complete loss of Brg1 in VillinCreERT2+/Apcfl/flBrgfl/fl epithelium. Lysozyme immunostaining showed mis-localisation of Paneth cells in VillinCreERT2+/Apcfl/fl epithelium, which was restored to normal by inactivation of Brg1. Scale bars represent 100 µm. B. Scoring of the crypt and villus length revealed no differences between double knock-out mice (black) and VillinCreERT2+/Apcfl/flBrgfl/fl animals (grey). Scoring of the cleaved Caspase3 positive cells and Ki67 positive cells detected a decrease in both apoptosis and proliferation in VillinCreERT2+/Apcfl/flBrgfl/fl mice compared to VillinCreERT2+/Apcfl/fl animals. Data are shown as mean ± group’s standard deviation for Caspase3 quantification and as mean ± pooled standard deviation otherwise, p value was calculated by means of t test not assuming equal variance for Caspase3 data and by means of one way ANOVA otherwise, p value was adjusted for multiple testing. For all comparisons n=4. C. Analysis of cumulative frequency of Ki67 positive cells at each position along crypt-villus axis revealed expansion of the proliferative compartment in both double knock-out (green line) and VillinCreERT2+/Apcfl/fl animals. This expansion was less pronounced in double knock-out mice compared to VillinCreERT2+/Apcfl/fl controls (blue line). For all pair wise comparisons Kolmogorov-Smirnov test p<0.001, n=4.

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Analysis of gene expression between Wnt activated VillinCreERT2^+Apc^fl/fl and control epithelium (APCvsCTR gene set) detected 548 unique differentially expressed ‘Wnt target genes’ including a number of previously reported Wnt target genes, such as Cd44, Axin2, Lrig5, Tiam1, Ephb2, Ephr4 and Sox9 [22] (Figure 2G, Table S1) with four of these genes found to be down-regulated in DKO epithelium compared to Apc-deficient samples (Table S2). Cluster analysis of genes differentially expressed between Apc-deficient and control epithelium revealed that the same genes in VillinCreERT2^+Apc^fl/flBrg^ff epithelium largely assumed intermediate expression values between those of the other two groups. G. Venn diagram demonstrating overlap between pairwise comparisons of differentially expressed gene signatures within various cohorts.

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control epithelium revealed clustering of DKO mice with control samples (Figure 2F), indicating a greater similarity of the gene expression pattern between DKO and wild type samples compared to Apc-deficient intestine. Notably, the majority of gene expression values from DKO samples were intermediate between those of VillinCreER<sup>+/-</sup>Apc<sup>fl/fl</sup> and control samples (Figure 2F), indicating attenuation of the Wnt pathway transcriptional signature regardless of whether Wnt activation induced or suppressed gene expression. We also observed a strong negative correlation pattern when directions of gene expression changes between Apc-deficient and wild type epithelium were contrasted with changes between double knock-out and Apc-deficient mice (Figure S2A, r = -0.793, p < 0.0001).

In order to evaluate how many Wnt target genes were attenuated by additional Brg1 loss we compared the overlap between the sets of differentially expressed genes (Figure 2G). We identified 197 unique differentially expressed genes between VillinCreER<sup>+/-</sup>Apc<sup>fl/fl</sup> and VillinCreER<sup>+/-</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> samples (DKOvsAPC set) (Table S2). Of these, half (99/197) appeared to be ‘Wnt targets’ defined as those present in APCvsCTR gene set (Figure 2G, Table S1), representing 18% (99/548) of the total number of genes that were deregulated following Apc loss (Table S4, Figure 2G). Since Brg1 loss was able to significantly attenuate deregulation of these Wnt target genes, these genes could be designated as ‘highly dependent on Brg1’ (Table S7). This gene subset included such Wnt target genes from their expression levels in control intestine. With the samples prevented deregulation of 84% (458/548) of Wnt target genes differentially expressed between Brg1-deficient and control samples (DKOvsCTR gene set) identified 200 unique differentially expressed genes (Figure 2G, Table S3). Comparison of this gene set to the APCvsCTR set revealed that 16% (37/232) of Wnt target genes were present in both sets, but not in the DKOvsAPC set (Table S5). Since Brg1 loss failed to attenuate deregulation of these genes, they could be considered as ‘Brg1 independent Wnt targets’ (Table S7). Notably, Brg1 deficiency in double knock-out samples prevented deregulation of 84% (458/548) of Wnt target genes from their expression levels in control intestine. With the exception of the 99 highly Brg1 dependent Wnt targets these 66% (362/548) of genes could be designated as ‘Wnt targets with low Brg1 dependency’ (Table S7). This gene subset included such Wnt targets as Axin2, Ephh2 and Sox9. Overall, these data indicated that up to 84% of all Wnt target genes in the small intestinal epithelium are dependent to some extent on Brg1 for either their activation or suppression.

It could be argued that gene expression changes upon additional deletion of Brg1 in the Apc-deficient epithelium could arise from the global effect of Brg1 loss on gene expression rather than its specific relevance for the Wnt-driven transcriptional programme. To address this caveat we also analysed the genes differentially expressed between Brg1-deficient and wild type epithelium. Despite a drastic effect on epithelial homeostasis, Brg1 loss induced a relatively moderate perturbation in gene expression at day 4 post induction with 86 genes differentially expressed between Brg1-deficient and control epithelium (Table S8) [14]. Expectedly, a small proportion of the genes differentially expressed between Apc-deficient and double knock-out samples were also de-regulated between Brg1-deficient and wild type epithelium (23/197 genes (11.7%) Table S9, Figure S2B). This proportion was notably lower when Brg1 targets were compared to the list of Wnt targets with high Brg1 dependency (5/99 genes (5.1%), Table S9). Together, these observations strongly suggested that gene expression changes following Brg1 loss in the context of Apc deficiency were largely a result of a specific effect of Brg1 deficiency on the Wnt pathway transcriptional programme rather than an impact of Brg1 deletion on global gene expression.

16 genes which overlapped between the DKOvsCTR and DKOvsApc sets (Table S6, Figure 2G) appeared deregulated by Brg1 deficiency regardless of Wnt activation and therefore most likely represented direct Brg1 targets independent of Wnt signalling. Most of these genes (11/16) were also present among genes differentially expressed between Brg1-deficient and control samples (Table S9, Figure S2C) [14].

Notably, one of the Wnt targets, whose expression was attenuated following additional loss of Brg1, was the proposed intestinal stem cell marker Lgr5 [23]. We therefore decided to explore the effects of Apc deletion and subsequent Brg1 loss on the expression of genes associated with the intestinal stem cell compartment. To this end we compared gene expression changes between our cohorts to the extensive stem cell signature. Using 3 distinct genome-wide expression platforms Muñoz et al. identified 510 genes that were preferentially expressed in the murine small intestinal Lgr5<sup>high</sup> cells [24]. We identified 460 of these stem cell-specific genes in our array, which corresponded to 721 probes (Table S10).

We then employed ROAST function [25] from the limma Bioconductor package [26] to determine if stem cell-specific genes were enriched among genes differentially expressed between our cohorts. Consistent with the reported stem cell expansion following aberrant activation of Wnt signalling [27–29], 50.5% of stem cell-specific genes were found to be up-regulated in VillinCreER<sup>+/-</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice compared to the control cohort (p < 0.0001). Double knock-out epithelium also displayed increased expression of stem cell-related genes compared to control samples, however only in 32.3% of genes (p = 0.0002). In contrast, 41.2% of all stem cell genes were suppressed in double knock-out epithelium compared to VillinCreER<sup>+/-</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice (p < 0.0001).

We also queried sets of differentially expressed genes between our cohorts for the presence of stem cell-specific genes. While this approach was less sensitive than using ROAST function, we observed a substantial number of stem cell signature genes in our differentially expressed gene sets (Tables S11, S12, S13). In line with the proposed Wnt-driven stem cell expansion [27–29], 10.6% (58/548) of all genes differentially expressed upon Apc deletion were found to belong to the stem cell signature. Of these 98% (57/58) were found to be up-regulated. In contrast, genes differentially expressed between double knock-out and Apc-deficient intestine contained 11.2% (22/197) of genes from the stem cell signature, all of which were down-regulated (Tables S11, S12, S13). Similar to the pattern of gene expression changes across all genes, stem cell related genes displayed strong negative correlation, when expression changes between Apc-deficient and wild type epithelium were contrasted with ones between double knock-out and Wnt activated intestine (Figure S2D, r = -0.659, p < 0.0001).

Notably, the proposed Wnt-independent intestinal stem cell marker Olfm4 [27] was found to be down regulated in the double knock-out intestine when compared to both Apc-deficient and normal epithelium, indicating strong Brg1 control over Olfm4 expression.

**Brg1 loss prevents Wnt-driven adenoma formation in the context of the murine small intestinal epithelium**

The apparent requirement of Brg1 for the expression of Wnt target genes following aberrant Wnt activation in the small intestinal epithelium raises the possibility that Brg1 loss may also prevent Wnt-driven tumour development. To explore this possibility we assessed the effects of Brg1 loss on Wnt-driven adenoma formation by driving recombination of the floxed Apc and Brg1 alleles with the Tg(Cpfl1a1-cer/ESR1)1Dwi transgene, further abbreviated as AbCreER<sup>+</sup> [30]. This transgene encodes...
Cre-ER<sup>T2</sup> recombinase under the control of the Cyc1A promoter. In contrast to VillinCreER<sup>ERT2</sup> recombinase, which is expressed in the entirety of the intestinal epithelium, AhCreER<sup>T2</sup> transgene’s expression is confined to the stem cell and early progenitor compartments. Additionally, AhCreER<sup>T2</sup> recombinase requires exposure to both β-naphthoflavone and tamoxifen for its activation, which results in tighter control over its activity. We used this approach to inactivate Apc and Brg1 in the small intestinal epithelium at a lower frequency than above, thus extending animal survival and enabling us to analyse the long-term effects of Brg1 loss on Wnt-driven adenoma formation.

We induced 3 cohorts of mice (AhCreER<sup>T2</sup> controls, AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup> and AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup>) with 5 bi-daily intraperitoneal injections of 80 mg/kg of β-naphthoflavone and 50 mg/kg of tamoxifen. Animals were aged and sacrificed either at day 10 post induction (n = 4) or when they displayed signs of terminal illness (n ≥ 20).

Immunohistochemical analysis of β-catenin localisation in the small intestinal epithelium at day 10 revealed multiple aberrant foci with nuclear β-catenin in both AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup> and double knock-out mice, indicating successful activation of the Wnt pathway in those lesions (Figure 3A, left and central). At the same time, Brg1 immunostaining revealed clusters of Brg1 negative cells in AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> intestinal epithelium (Figure 3A, right). In contrast to the small intestine of VillinCreER<sup>ERT2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice, where the majority of cells after induction were deficient for Brg1 and displayed normal localisation of β-catenin, all the lesions with nuclear β-catenin in the AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> intestine were Brg1-positive and we failed to detect any overlap between Brg1-deficient clusters and Wnt-activated lesions (Figure 3A, central and right). This observation suggested that, when driven by AhCreER<sup>T2</sup> recombinase, Brg1 loss was incompatible with long term activation of the Wnt pathway and the development of aberrant crypt foci, consistent with inability of crypts from double knock-out epithelium to form aberrant organoids in vitro (Figure 2C).

Analysis of the ageing cohorts revealed that combined deletion of Apc and Brg1 deletion provided a survival advantage in comparison to single Apc deletion (Figure 3B). Whilst all the AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice became terminally ill within 20 days post induction (median survival 9 days), the majority of AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice survived substantially longer (median survival 38 days) with some mice surviving past 100 days (Figure 3B, Log-rank test p < 0.0001, n = 20 for each cohort). No control animals (n = 8) developed signs of ill health within the timeframe of the experiment.

Histological inspection of the small intestine of the AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice at late time points revealed numerous small lesions confined within the distended villi and identified as micro-adenomas (Figure 3C), as well as rare large adenomas. Immunohistochemical analysis of β-catenin expression showed that all of these lesions were positive for nuclear β-catenin, confirming aberrant Wnt signalling activation (Figure 3C, left). Notably, Brg1 staining demonstrated that all the lesions retained Brg1 expression (Figure 3C, right), indicating that they were likely to originate from cells that recombined at the Apc, but not at the Brg1 loci. Along with the apparent lack of double mutant lesions at day 10 post induction, this indicated that long-term progression of Wnt-driven neoplasia in the small intestine was incompatible with Brg1 deficiency. We therefore explored the possibility that Brg1 loss in the context of activated Wnt signalling could reduce tumour burden and thus improve animal survival. We induced AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice with two bi-daily injections of 80 mg/kg β-naphthoflavone and 80 mg/kg tamoxifen to achieve attenuated recombination and harvested the small intestinal epithelium at day 40 post induction. We then scored the number of lesions with nuclear β-catenin normalised to the number of normal crypt units contained within the analysed region of the small intestinal epithelium. Quantitative analysis of the tumour burden from two independent experiments revealed a 2.92-fold decrease in the number of lesions in the double knock-out small intestinal epithelium compared to AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice (p = 0.026, n ≥ 5) indicating reduced tumour burden upon Brg1 loss.

**Figure 3.** Brg1 loss is incompatible with Wnt-driven adenoma formation and improves animal survival upon Apc inactivation in the small intestine. A. Immunohistochemical analysis of β-catenin revealed numerous lesions with nuclear localisation of β-catenin in the small intestine of AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup> and AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice 10 days post induction. Brg1 immunostaining of serial sections showed clusters of Brg1-deficient cells in AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> intestine, but failed to detect any overlap between lesions with nuclear β-catenin and Brg1 negative cells. B. Survival analysis demonstrated significantly increased survival probability of AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice (blue line, median survival 58 days) compared to AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup> animals (green line, median survival 9 days), Log-rank test p < 0.0001, n = 20. No control animals died within the timeframe of the experiment (n = 8). C. β-catenin immunostaining of the small intestinal epithelium of AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice 100 days post induction revealed numerous micro adenomas enclosed within distended villi. Staining with Brg1 antibody showed that all the micro adenomas retained Brg1 expression. Scale bars represent 100 μm (A) and 200 μm (B).

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**Stem cell-restricted Brg1 loss suppressed Wnt-driven tumourigenesis**

Prevalence of micro-adenomas and lack of advanced adenomas in the small intestines of the AhCreER<sup>T2</sup>Apc<sup>fl/fl</sup>Brg<sup>fl/fl</sup> mice as late as 100 days post induction indicated that this genetic environment favoured the development of lesions with a limited growth potential. Barker et al. [28] suggested that adenomas originating from intestinal stem cells had a higher tumourigenic potential compared to those derived from transit amplifying cells. To test whether stem cell-specific Brg1 loss could attenuate Wnt-driven adenoma formation we intercrossed mice expressing the GFP-IRESCreER<sup>T2</sup> knock-in allele under control of the Lgr5 promoter (further abbreviated as Lgr5-GFP-CreER<sup>T2</sup>) [23] with animals
bearing targeted Apc and Brg1 alleles. Lgr5-GFP-CreERT2+/+Ap0/0/+ and Lgr5-GFP-CreERT2+/+Ap0/0/+Brg0/0/+ mice were induced with four daily intraperitoneal injections of tamoxifen (initial dose of 3 mg was followed by three doses of 2 mg). Animals were aged for 420 days after induction or until they developed signs of ill health. Survival analysis of the cohorts revealed a substantial increase in survival of double knock-out mice compared to their Lgr5-GFP-CreERT2+/+Ap0/0/+Brg0/0/+ counterparts (median survival 344 and 110 days post induction respectively, Figure 4A. Log-rank p<0.001, n = 14 for each cohort).

Immunohistochemical analysis of β-catenin expression in the small intestine of both Lgr5-GFP-CreERT2+/+Ap0/0/+ and Lgr5-GFP-CreERT2+/+Ap0/0/+Brg0/0/+ mice revealed a mixture of micro and macro-adenomas with nuclear β-catenin at a range of time points (Figure 4B). Immunohistochemical analysis of GFP expression in small intestinal tumours detected GFP positive cells in both macro and micro adenomas, implying activity of the Lgr5-GFP-CreERT2 transgene and stem cell origin of both types of lesions (Figure 4B). This observation suggested that not all stem cell-derived adenomas were able to progress to advanced stages. Equally, we observed numerous micro adenomas devoid of GFP expression (not shown). These lesions were likely to arise from early progenitor cells, which might have lost self renewal ability, but retained some Cre-ERT2 recombinase expression.

Similar to the pattern observed in the small intestine of AhCreERT+/+Brg0/0/+ mice, adenomas in the small intestine of Lgr5-GFP-CreERT2+/+Ap0/0/+Brg0/0/+ mice retained Brg1 expression (Figure 4B) providing further support to the notion that Brg1 loss is incompatible with Wnt-driven adenoma formation.

Discussion

Brg1 acts as a positive regulator of Wnt signalling and is required for Wnt-driven adenoma formation in the small intestinal epithelium

We have demonstrated that inactivation of Brg1 resulted in reduced expression of Wnt target genes following activation of the canonical Wnt pathway via Apc deletion in the small intestinal epithelium. A number of studies have previously suggested that Brg1 facilitates trans-activation of Wnt target genes by activated β-catenin in cancer cell lines [12,13], zebrafish [31] and during mammalian vascular development [32]. Our study provides the first evidence of the functional interaction between Brg1 and the Wnt pathway in the context of intestinal tumourigenesis using an in vivo system. Using transcriptome analysis, we identified sets of Wnt target genes which displayed differing levels of dependency on Brg1 function. Overall, we identified 548 genes that were deregulated upon Apc deletion in the small intestinal epithelium. Of these, 87 genes were Brg1 independent and 461 displayed a variable degree of dependency on Brg1 function, indicating that deregulation of the majority (85%) of Wnt responsive genes in the small intestinal epithelium depended (to differing degrees) on the presence of functional Brg1. This observation is in line with a previous report, which found a similar proportion (68%) of Wnt targets to rely on Brg1 for their response to Wnt activation in the HEK293T cell line [33].

Consistent with the requirement for Brg1 in the maintenance of the small intestinal stem cell population [14], we observed attenuated expression in a range of genes associated with the small intestinal stem cells following Brg1 loss in the context of aberrantly activated Wnt signalling. These genes included functionally validated stem cell markers such as Lgr5, Ascl2 and Olfm4, suggesting that Brg1 loss was able to impair the expansion of the ‘stem-like’ cell population characteristic of Wnt-driven intestinal tumourigenesis [27–29]. Furthermore, expression levels of Olfm4 in the small intestine of double knock-out mice were lower compared to that in both wild type and Apc-deficient epithelium, strongly suggesting a role of Brg1 in regulation of Olfm4 expression. Olfm4 is a secreted anti-apoptotic factor, which has been reported to be over-expressed in a variety of tumours [34]. Depletion of Olfm4 in gastric cancer cells has been reported to suppress proliferation and sensitise cancer cells to apoptosis [35,36]. Olfm4 thus constitutes a potentially attractive therapeutic target, especially so in view of its secreted nature, which makes it a feasible target for monoclonal antibody therapies.

In addition to suppression of Wnt target genes, Brg1 loss also attenuated the physiological manifestations of Wnt activation in the intestinal epithelium, most notably the increased cell proliferation and mislocalisation of Paneth cells. Both Paneth cell quantity and position were restored to normal levels in the double knock-out epithelium, suggesting that Brg1 deletion was able to preserve physiological levels of EphB/Ephrin signalling in the context of Apc deficiency [37]. Consistent with this
notion, our transcriptome analysis detected up-regulation of EphB2 expression in Apc-deficient intestine, but not in double knock-out epithelium, when compared to control samples. A similar effect of Wnt signalling attenuation on the Paneth cell mislocalisation has been previously demonstrated following loss of Mbd2 in the Apc-deficient small intestine [30]. Given the proposed role for Paneth cells as the intestinal stem cell niche [20], this effect of Brg1 loss on Paneth cell mislocalisation could contribute to the attenuated stem cell expansion in double knock-out intestine.

In the view of extensive role of Wnt signalling in tumourigenesis [1], Brg1 mediated modulation of the Wnt pathway may have implications for the development of novel therapeutic approaches. Here we report that loss of Brg1 in the context of Apc deletion improved animal survival by preventing the formation of double mutant adenomas. All adenomas observed in the small intestine of double knock-out mice retained Brg1 expression indicative of their origin from cells that had lost Apc but had escaped Brg1 deletion. This implies an absolute requirement of functional Brg1 for Wnt-mediated tumourigenesis in this tissue. A similar relationship between Brg1 inactivation and a loss of tumour suppressor Snf5 (Ini1) has been reported by Wang et al. [39]. Simultaneous inactivation of Brg1 and Snf5 under control of the T-cell lineage-specific Lck-Cre recombinase resulted in decreased tumour incidence and a longer disease onset. Similar to our observations, all the Snf5-deficient tumours, which developed in double-mutant animals, retained Brg1 expression [39].

Barker et al. [28] suggested a particular role for intestinal stem cells in Wnt-driven tumour initiation with non-stem cells giving rise to adenomas with limited growth capacity, while stem cell gene signature in human primary colorectal cancers was found to be associated with more aggressive phenotype [40]. In a similar fashion, Alcantara Laguno et al. [41] reported neural stem cells as the cell of origin for malignant gliomas. Furthermore, a recent study has demonstrated a substantial survival advantage of genetic targeting of glioblastoma cancer stem cells using a neural stem cell marker Nestin [42]. Consistent with these reports, and in line with negative impact of Brg1 loss on long-term small intestinal stem cell survival, we observed a markedly improved survival upon stem cell-specific Brg1 deletion in the context of aberrant Wnt activation. Notably, the above study made use of stem cell-specific Lck-Cre recombinase to target the sub-population of cancer cells bearing normal stem cell markers [42]. In contrast to this, we report successful tumour suppression by targeting a gene required for the physiological small intestinal stem cell maintenance, a relationship, which to our knowledge has not been previously reported. It should be noted that mice with stem cell-specific Apc loss alone in the present report exhibited longer survival times compared to those in the study by Barker et al. [28], which could be attributed to increased levels of silencing of the Lgr5-GFP-CreER2 transgene in our mice. Tumours in the small intestine of animals bearing the Lgr5-GFP-CreER2 transgene and targeted Apc alleles were mainly detected in the distal third of the small intestine with fewer lesions in the proximal part and very few tumours in between. Given that biallelic Apc deletion would fall within ‘high pathological Wnt’ scenario described in Leedham et al. [43], this tumour distribution was consistent with the gradient of the Wnt signal and stem cell density in the murine intestine.

It should be noted that given the concurrence of Brg1 and Apc deletion, results presented in this report pertain to cancer prevention rather than therapy. Additionally, Brg1 loss-driven elimination of intestinal stem cells is likely to be a major contributing factor to attenuated tumour burden in double knock-out mice and may therefore obscure the effects of Brg1 deletion on non-stem tumour cells. Importantly, this toxicity of Brg1 loss in respect to the small intestinal stem cell homeostasis constitutes a potential serious caveat to the use of Brg1 as a therapeutic target. However our data from the previous report [14] as well as from AhCreER2+/+Apcfl/flBrg1−/− and Lgr5-GFP-CreER2+/+Apcfl/flBrg1−/− models in the present study demonstrate that partial Brg1 loss is well tolerated by the small intestinal epithelium, which is gradually repopulated with wild type cells. At the same time, partial loss of Brg1 was sufficient to reduce the tumour burden, suggesting that a therapeutic window may exist that would allow targeting Brg1 in the intestinal polyps, while allowing repopulation of the normal intestinal epithelium. A strategy that would allow for the efficient Brg1 deletion in existing adenomas or the use of a Brg1 inhibitor would be required to further address the effects of Brg1 in non-stem cell portion of established Wnt-driven tumours, as well as the potential therapeutic window of targeting Brg1.

In summary, we demonstrate using mouse models of intestinal cancer that Brg1 is essential for Wnt-driven tumourigenesis in the murine small intestine with attenuation of Wnt target gene expression and elimination of transformed stem cells as two likely mechanisms. Brg1 therefore constitutes a potential therapeutic target in cancers with an abnormally activated Wnt pathway. Combined with our earlier observation that Brg1 is essential for stem cell maintenance in the small intestinal epithelium under the physiological conditions, these data may serve as a proof of concept that targeting the somatic stem cell as a cancer initiating cell may provide a valuable therapeutic approach, especially in the context of predisposition to Wnt-driven carcinogenesis, such as in Familial Adenomatous Polyposis patients.

Materials and Methods

Experimental animals

All experiments were carried out in accordance with Animals (Scientific Procedures) Act 1986 under project licenses 30/2246 and 30/2737 issued by UK Home Office. The study was approved by the Cardiff University Research Ethics Committee. Mice were maintained on an outbred background and genotyped as described previously for targeted Apc allele [15], Cre-EKT and Cre-EKT2 transgenes [19], targeted Brg1 allele [16]. Detailed induction protocols and dissection procedures are described in Text S1.

Histological procedures

Detailed description of protocols for tissue fixation, processing, immunohistochemistry, and quantitative analysis of tissue sections is available in Text S1.

Statistical analysis of histological data

All statistical tests except survival and cumulative distribution analyses were carried out using R software [44]. Scoring data were tested for normality using Shapiro-Wilk test and for equal variance using Levene’s test. Normally distributed data with equal variance were tested for difference between means using one-way ANOVA. Where appropriate, p values were adjusted for multiple testing using TukeyHSD function in R. In cases of unequal variance between groups the difference between those groups was tested using t-test not assuming equal variance. Unless otherwise
specified, pooled standard deviation from one-way ANOVA was used to represent error bars. Positional data were analysed for differences in distribution with Kolmogorov-Smirnov Z-test in SPSS (version 16.0.2). Kaplan-Meier survival curve and Log-rank survival analysis were carried out using GraphPad Prism (version 6).

RNA extraction, quantitative RT-PCR and microarray analysis

Detailed description of the protocols and statistical methods used for RNA extraction, qRT-PCR and transcriptome analysis is available in Text S1. Microarray analysis was carried out using beadarray [45] and limma [26] packages from Bioconductor project. Microarray data for the study are publicly available from the GEO repository (http://www.ncbi.nlm.nih.gov/geo) under the series record GSE46129.

Protein extraction and western blot analysis

A detailed protocol for obtaining epithelial-enriched population of cells for protein extraction and western-blotting is provided in Text S1. Protein was extracted from the epithelium-enriched small intestinal samples, separated, transferred and probed as described previously [46]. The primary antibodies used: mouse anti-active β-catenin (1:2000; Millipore) and mouse anti-β-actin (1:5000; Sigma). Anti-mouse horseradish peroxidase conjugated secondary antibody (1:3000; GE Healthcare) and ECL or ECL Plus reagents (Amersham Biosciences) were used to detect the signal according to the manufacturer’s manual.

In vitro organoid culture

Small intestinal crypts from VillinCreERTZ− controls, VillinCreERTZ+ Apcfl/fl and VillinCreERTZ+,Apcfl/fl,Brg1fl/fl mice were isolated and cultured as described in Sato et al. [47] with minor adjustments. Detailed procedures are described in Text S1.

Supporting Information

Figure S1 Brg1 loss attenuates Wnt-driven apoptosis and cell proliferation in the small intestinal epithelium. (A, B) Scoring of the apoptotic bodies (A) and BrdU positive cells 2 hours post labelling (B) showed significantly reduced apoptosis levels and BrdU incorporation in VillinCreERTZ+,Apcfl/fl,Brg1fl/fl mice compared to VillinCreERTZ− controls. Graphs are represented as mean ± group-wise standard deviation. Difference between means was tested by means of t-test for samples with unequal variance and adjusted for multiple testing. (C) Analysis of cumulative frequency of BrdU positive cells at each cell position along crypt-villus axis 2 hours after labelling revealed significant expansion of BrdU positive cells in VillinCreERTZ+,Apcfl/fl,Brg1fl/fl (green line) and VillinCreERTZ−,Apcfl/fl,Brg1fl/fl (orange line) mice compared to VillinCreERTZ− controls. This expansion was less pronounced in epithelium of VillinCreERTZ+,Apcfl/fl,Brg1fl/fl mice compared to VillinCreERTZ−,Apcfl/fl,Brg1fl/fl animals. For all comparisons Kolmogorov-Smirnov test p<0.001, n=4. (TIF)

Figure S2 Brg1 deletion specifically represses gene expression changes induced by Apc deletion. (A, D) Correlation analysis of changes in gene expression revealed a strong negative correlation in expression patterns of genes in Apc deficient and double knock-out epithelium. The same pattern was observed for genome-wide analysis (A) and when applied to the genes from intestinal stem cell signature (D). Biological correlation is distinguished from technical correlation using “genas” function from Limma Biocductor package [26]. (B) Genes deregulated by Brg1 loss in the control epithelium comprised a small fraction of genes affected by Brg1 deletion in the context of Apc loss (5/99 genes). (C) A small set of 16 genes that were disrupted by Brg1 loss regardless of Apc deletion were largely represented by direct Brg1 targets and were also misexpressed following Brg1 loss in normal intestinal epithelium (11/16 genes). (TIF)

Table S1 Genes differentially expressed between VillinCre+,Apcfl/fl and VillinCre− small intestinal epithelium. (XLS)

Table S2 Genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre+,Apcfl/fl small intestinal epithelium. (XLS)

Table S3 Genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre− small intestinal epithelium. (XLS)

Table S4 Overlapping and exclusive differentially expressed genes between VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre− (APCvsCTR) and VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre+,Apcfl/fl,Brg1fl/fl (DKOvsAPC) datasets. Colours correspond to the colours in venn diagram in Figure 2G. (XLS)

Table S5 Overlapping and exclusive differentially expressed genes between VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre− (APCvsCTR) and VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre− (DKOvsCTR) datasets. Colours correspond to the colours in venn diagram in Figure 2G. (XLS)

Table S6 Overlapping and exclusive differentially expressed genes between VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre− (DKOvsCTR) and VillinCre+,Apcfl/fl,Brg1fl/fl vs VillinCre− with Brg1 (DKOvsAPC) datasets. Colours correspond to the colours in venn diagram in Figure 2G. (XLS)

Table S7 Wat target gene sets with differing levels of Brg1 dependency. Colours correspond to the colours in venn diagram in Figure 2G. (XLS)

Table S8 Genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre− small intestinal epithelium. (XLS)

Table S9 Overlap of Brg1 targets (VillinCre+,Brg1fl/fl vs VillinCre−) and differentially expressed genes in various gene sets. Colours correspond to the colours in venn diagram in Figure 2G. (XLS)

Table S10 Small intestinal stem cell specific gene signature from Muñoz et al., (2012). Genes not detected in our array are highlighted in red. (XLS)

Table S11 Stem cell signature genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre− small intestinal epithelium. (XLS)

Table S12 Stem cell signature genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre− small intestinal epithelium. (XLS)

Table S13 Stem cell signature genes differentially expressed between VillinCre+,Apcfl/fl,Brg1fl/fl and VillinCre− small intestinal epithelium. (XLS)
References

1. Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: Wnt signaling in cancer. Biochimica et biophysica acta 1653: 1–24.
2. Gehre I, Gandhiraj RK, Kreuzer K-A (2009) Targeting the WNT/[beta]-catenin/TCF/LEF1 axis in solid and haematological cancers: Multiplicity of therapeutic options. European Journal of Cancer 45: 2759–2767.
3. Ellis I, Atadja PW, Johnstone RW (2009) Epigenetics in cancer: targeting chromatin modifications. Molecular Cancer Therapeutics 8: 1409–1420.
4. Khavari PA, Peterson CL, Tamkun JW, Mendel DB, Crabtree GR (1993) BRG1 contains a conserved domain of the SWI/SNF2 family necessary for normal mitotic growth and transcription. Nature 366: 170–174.
5. Trotter KW, Archer TK (2000) The BRG1 transcriptional coregulator. Nuclear Receptor Signaling 6:e0014.
6. Becker TM, Haferkamp S, Dijkstra MK, Scarr LL, Frausto M, et al. (2009) The chromatin remodelling factor BRG1 is a novel binding partner of the tumor suppressor p16INK4a. Molecular Cancer 8: 4.
7. Medina PP, Carretero J, Fraga MF, Esteller M, Sidransky D, et al. (2004) Genetic and Epigenetic screening for gene alterations of the chromatin-remodelling factor; SMARCAD1/BRG1, in lung tumours. Genes, Chromosomes and Cancer 41: 170–177.
8. Reisman DN, Scarrott J, Wang W, Funkhouser WK, Weisman BI (2003) Loss of BRG1/BRM in Human Lung Cancer Cell Lines and Primary Lung Cancers: Correlation with Poor Prognosis. Cancer Res 63: 560–566.
9. Wong AKC, Shanahan F, Chen Y, Lian L, Ha P, et al. (2000) BRG1, a Component of the SWI/SNF Complex, Is Mutated in Multiple Human Tumor Cell Lines. Cancer Res 60: 6171–6177.
10. Bulman SJ, Herschkowitz JL, Godfrey V, Gebuhr TC, Yaniv M, et al. (2008) Characterization of mammary tumours from Brg1 heterozygous mice. Oncogene 27: 456–468.
11. Glaros S, Cirrincione GM, Palanca A, Metzger D, Reisman D (2008) Targeted Activation of Cdk6 Marks a Subset of Colorectal Cancer Cells. Gastroenterology 137: 15–17.
12. Marks A, Jho E-h, Zhang T, Domon C, Joo C-K, Freund J-N, et al. (2002) Wnt/{beta}-Catenin Coactivators
13. Shibata H, Toyama K, Shioya H, Ito M, Hirota M, et al. (1997) Rapid Loss of BRG1/BRM in Human Lung Cancer Cell Lines and Primary Lung Cancers: Correlation with Poor Prognosis. Cancer Res 63: 560–566.
14. Holik AZ, Krzystyniak J, Young M, Richardson K, Jarde´ T, et al. (2013) Brg1 is a Wnt-Driven Intestinal Tumourigenesis
15. Park J-I, Venteicher AS, Hong JY, Choi J, Jun S, et al. (2009) Telomerase modulates Wnt signalling by association with target gene chromatin. Nature 457: 608–611.
16. Sumi-Ichinose C, Ichinose H, Metzger D, Chambon P (1997) SNF2beta-BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription. Nature 366: 170–174.
17. Indra AK, Dupe´ V, Bornert J-M, Messaddeq N, Yaniv M, et al. (2005) Functions of BRG1 in limb morphogenesis and skin barrier formation. Developmental Dynamics: An Official Publication of the American Association of Anatomists 235: 2722–2735.
18. Griffin CJ, Norris CD, Davis RB, Mukhambetov K, Magnuson T (2011) The chromatin-remodelling enzyme BRG1 modulates vascular Wnt signalling at two levels. Proceedings of the National Academy of Sciences 108: 2282–2287.
19. Mahmoudi T, Boj SF, Hatzi P, Li VSW, Taouatas N, et al. (2010) The Leukemia-Associated MLL1/IOl-Dct11 Are Tcf/{beta}-Catenin Coactivators Essential for Intestinal Homeostasis. PLoS Biol 8: e1000539.
20. Yu L, Wang I, Chen S (2011) Olfactomedin 4, a novel marker for the differentiation and progression of gastrointestinal cancers. Neoplasma 58: 9–13.
21. Jin Y, Yang M-h, Xiang H, Bao L-m, Yang H-a, et al. (2012) Depletion of OLFM4 gene inhibits cell growth and increases sensitization to hydrogen peroxide and tumor necrosis factor-alpha induced-apoptosis in gastric cancer cells. Journal of Biomedical Science 19: 38.
22. Liu R-h, Yang M-h, Xiang H, Bao L-m, Yang H-a, et al. (2012) Knockdown of miR-486 Regulates Tumor Progression and the OLFM4 Antiapoptotic Factor in Gastric Cancer. Clinical Cancer Research 17: 2657–2667.
23. Batlle E, Henderson JT, Beghtel H, van den Born MW, Sancho E, et al. (2002) beta-Catenin and TCF Mediate Cell Positioning in the Intestinal Epithelium by Controlling the Expression of Epha/Epibrin. Cell 111: 251–263.
24. Phese TJ, Parry L, Reed KR, Ewan KB, Dale TC, et al. (2008) Deficiency of Mbd2 Attenuates Wnt Signaling. Mol Cell Biol 28: 6194–6193.
25. Wang X, Samsam CG, Thom CS, Metzger D, Evans JA, et al. (2009) Oncogenic caused by loss of the SNF5 tumor suppressor is dependent on activity of BRG1, the ATPase of the SWI/SNF chromatin remodeling complex. Cancer research 69: 8094–8101.
26. Methos-Suarez A, Barriga FM, Jung P, Iglesias M, Cespedes MV, et al. (2011) Malignant Acroeyctomes Originate from Neural Stem/Progenitor Cells in a Somatic Tumor Suppressor Mouse Model. Cancer Cell 15: 45–56.
27. Chen J, Li Y, Yu T-S, McKay RM, Burns DK, et al. (2012) A restricted cell population propagates glioblastoma growth after chemotherapy. Nature 481: 522–528.
28. Leedham SJ, Roelen-Cuadrado P, Howarth K, Lewis A, Mallappa S, et al. (2013) A basal gradient of Wnt and stem-cell number influences regional tumour distribution in human and mouse intestinal tracts. Gut 62: 83–93.
29. R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL, http://www.R-project.org.
30. Dunning MJ, Smith ML, Ritchie ME, Tavare S (2007) beadarray: R classes and methods for Illumina microarray hybridisation services.
31. Authors Contributions
Conceived and designed the experiments: AZH BYS ARC. Performed the experiments: AZH MY JK BYS. Analyzed the data: AZH MY JK GTW. Contributed reagents/materials/analysis tools: DM. Wrote the paper: AZH MY ARC.
46. Marsh V, Winton DJ, Williams GT, Dubois N, Trumpp A, et al. (2008) Epithelial Pten is dispensable for intestinal homeostasis but suppresses adenoma development and progression after Apc mutation. Nat Genet 40: 1436–1444.

47. Sato T, Vries RG, Snippert HJ, van de Wetering M, Barker N, et al. (2009) Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459: 262–265.
