Bcl9 and Pygo synergise downstream of Apc to effect intestinal neoplasia in FAP mouse models

Juliusz Mieszczanek1, Laurens M. van Tienen1, Ashraf E.K. Ibrahim1, Douglas J. Winton2 & Mariann Bienz1

Bcl9 and Pygo are Wnt enhanceosome components that effect β-catenin-dependent transcription. Whether they mediate β-catenin-dependent neoplasia is unclear. Here we assess their roles in intestinal tumourigenesis initiated by Apc loss-of-function (ApcMin), or by Apc1322T encoding a partially-functional Apc truncation commonly found in colorectal carcinomas. Intestinal deletion of Bcl9 extends disease-free survival in both models, and essentially cures Apc1322T mice of their neoplasia. Loss-of-Bcl9 synergises with loss-of-Pygo to shift gene expression within Apc-mutant adenomas from stem cell-like to differentiation along Notch-regulated secretory lineages. Bcl9 loss also promotes tumour retention in ApcMin mice, apparently via relocating nuclear β-catenin to the cell surface, but this undesirable effect is not seen in Apc1322T mice whose Apc truncation retains partial function in regulating β-catenin. Our results demonstrate a key role of the Wnt enhanceosome in β-catenin-dependent intestinal tumourigenesis and reveal the potential of BCL9 as a therapeutic target during early stages of colorectal cancer.
C olorectal cancer is the second most common cause for cancer mortality in the developed world (http://globocan. iarc.fr). The pathway to this cancer is usually initiated by the mutational inactivation of the Adenomatous Polyposis Coli (APC) tumour suppressor, both in sporadic and hereditary forms\(^1\). Individuals bearing APC germline mutations develop thousands of benign adenomas in their large intestine by their teenage years one of which, invariably, progresses to carcinoma within the next decades of their lives\(^2\). Progression to colorectal cancer requires a small number of additional driver mutations, e.g., the activation of the KRAS or PIK3CA oncogenes, and/or the inactivation of the P53 or ARID1A tumour suppressors\(^3\). Notably, APC germline mutations in the mouse also cause multiple intestinal neoplasia (Min), and various ApC loss-based models have been developed for pre-clinical studies\(^4\), whereby the original Apc\(^{Min}\) model\(^5\) has been used most widely.

At the molecular level, APC attenuates Wnt signal transduction through the ‘canonical’ branch, by cooperating with Axin to promote the proteasomal degradation of the key effector β-catenin. This process is inhibited by Wnt signals that promote the stabilisation of β-catenin, allowing it to access T cell factor (TCF) bound to cis-regulatory enhancers of Wnt-responsive genes to operate Wnt-dependent transcription. Key to this process is the Wnt enhancosome\(^6,7\), a multiprotein complex tethered via TCF to enhancers of lineage-determining master control genes whose Wnt-responsiveness is conferred by BCL9, or its paralog B9L (BCL9-Like, or BCL9-2)\(^8,9\): these factors (called BCL9 below unless a specific paralog is referred to) bind to and capture stabilised β-catenin, to convey it to TCF within the enhancosome via docking to Pygo, a chromatin-binding PHD finger protein\(^10\). Wnt/β-catenin signalling specifies numerous cell fate decisions during animal development, and controls self-renewal of virtually every adult tissue, including the intestinal epithelium\(^11\). This stem cell function could explain why β-catenin is a potent oncogene, as revealed by the prevalence, in many cancers, of activating mutations in β-catenin that render it refractory to degradation\(^1\).

Despite its considerable oncogenic potential, there are no well-validated inhibitors of activated β-catenin that could be developed as therapeutics, because of the lack of druggable downstream targets\(^12\). Nevertheless, several small molecules have been reported to inhibit activated β-catenin directly or indirectly, but most of these produce unspecific (off-target) cell toxicity: e.g., tankyrase inhibitors destabilise β-catenin by boosting the levels of Axin\(^13\), but also of other targets\(^14\), which may contribute to their toxicity in mice\(^15\). Furthermore, cells experiencing chronic Wnt signalling such as APC-mutant cancer cells proved refractory to tankyrase inhibition, mainly because they express high levels of LEFI (a TCF paralog) or B9L, which shield β-catenin from Axin-dependent destruction\(^13\).

The role of BCL9 as an essential co-factor of Drosophila β-catenin was demonstrated by genetic studies\(^16\). In mice, deletion of both Bcl9 paralogs causes embryonic lethality, while tissue-specific deletion in muscle leads to β-catenin-dependent regeneration defects\(^17\). Similarly, conditional deletion of both paralogs in the intestine reduces β-catenin-dependent transcription compartment in intestinal crypts, suggesting a role of Bcl9 in specifying ‘stemness’ in this self-renewing compartment\(^18,19\). BCL9 and B9L are often overexpressed in colorectal cancer cell lines and carcinomas, maintaining their β-catenin-dependent transcription\(^13,20\), and overexpressed B9L promotes intestinal tumourigenesis\(^21\).

BCL9 functions as a scaffold of the Wnt enhancosome\(^2\). Its binding to the Pygo PHD finger promotes its recognition of methylated histone H3 tail\(^10,22\). It also binds to the N-terminus of the Armadillo Repeat Domain (ARD) of β-catenin via a short helical domain called HD2\(^23,24\), an interaction that can be blocked by individual HD2 point mutations\(^16,25\). Binding of BCL9 to β-catenin can also be blocked by natural compounds\(^13,23\), stapled helices mimicking HD2\(^26\) or rationally-designed small molecules\(^27\), which attenuate β-catenin-dependent transcription in colorectal cancer cells and β-catenin-dependent tumourigenesis in mouse models. These studies have thus provided proof-of-concept for the druggability of BCL9-β-catenin interaction.

In the light of the well-documented role of BCL9 in facilitating β-catenin-dependent transcription, it was puzzling that the conditional double-knockout of Bcl9 and B9l in the intestinal epithelium (Bcl9/B9l DKO below) did not reduce the tumour numbers in mouse models based on colitis and chemically-induced β-catenin-dependent tumours\(^18\), or on weak attenuation of ApC function\(^19\). Here, we re-assess the role of Bcl9 in intestinal tumourigenesis in two mouse models bearing ApC mutations, namely Apc\(^{Min}\) which essentially abolishes ApC function, and Apc\(^{1322T}\) which retains partial function of ApC in regulating β-catenin and, importantly, mimics the most prevalent ApC mutations in human colon cancers\(^28\). Deletion of Bcl9 and Pygo extends the disease-free life in both models, especially deletion of Bcl9 which essentially cures Apc\(^{1322T}\) mice of their neoplastic disease, restoring a normal life span in these otherwise moribund mice. RNA profiling reveals that Bcl9 loss synergises with Pygo loss downstream of ApC loss to shift the adenomatous gene expression programme from stem cell-like towards differentiation along secretory cell lineages. Our study also uncovers a post-transcriptional effect of Bcl9 deletion in Apc\(^{Min}\) adenoma, namely a striking relocation of their nuclear β-catenin to their cell surface, likely increasing their cell adhesion and retention in crypts, which could account for the numerous tiny adenomas seen in this model. Importantly, this undesirable effect is not observed in Apc\(^{1322T}\) adenomas whose cell surface β-catenin appears normal, likely because the Apc\(^{1322T}\) truncation retains binding to, and partial regulation of, β-catenin. Our results from this model therefore illustrate the significant potential of BCL9 and Pygo as targets for therapeutic interference in colorectal cancer.

**Results**

**Loss of Bcl9 or Pygo extends the healthy life of Apc\(^{Min}\) mice.** Mice bearing the Apc\(^{Min}\) germline mutation develop some two dozen adenomas in their small intestines, detectable from ~100 days of age, each arising as a result of ApC loss-of-heterozygosity (LOH) in an individual intestinal epithelial cell\(^29\). Half of these mice succumb to their disease by ~120 days (Fig. 1a), turning anemic and losing weight. However, conditional deletion of Pygo or Bcl9 in the intestinal epithelium (with villin, Cre)\(^30\) prolonged the mean disease-free live of these mice by 60–90 days, or even more if all four paralogs were deleted simultaneously (QKO; Fig. 1a). The crypt-villus structure appeared normal in these strains, without any detectable changes in the numbers of dividing or apoptotic cells (Supplementary Figure 1, and A.E.K. Ibrahim, unpublished). Clearly, Apc\(^{Min}\) mice benefit from loss of Pygo or Bcl9 in terms of survival, with maximal benefit derived from the simultaneous loss of all four paralogs.

Next, we determined the RNA expression profiles of intestinal crypts from the DKO strains using Illumina Bead Arrays, but found only 96 significant (p < 0.05) gene expression changes compared to matched controls. Of these, 90% showed the same up- or downregulation trends in the two types of DKO (Supplementary Figure 2), as expected since Bcl9 and Pygo act in the same complex\(^3\). Notably, five histone H2A and four ribosomal protein genes were downregulated in these DKO samples, while five Defensin genes were upregulated, including
Defa1 encoding an early differentiation marker for Paneth cells. Thus, deletion of Bcl9 or Pygo may promote Paneth cell specification, although there was no detectable increase in the numbers of lysozyme-positive cells (marking fully differentiated Paneth cells) in DKO crypt sections (Supplementary Figure 1). Nevertheless, our RNA profiling data suggest that Bcl9 or Pygo loss biases cell fates from proliferative towards differentiated.

**Fig. 1** Loss of Bcl9 and Pygo extends the disease-free live of ApcMin mice. a Kaplan–Meier survival plots of ApcMin DKO or QKO cohorts and matched ApcMin control mice, as indicated on the right; statistical significance, p < 0.0001 (log-rank test). b Adenoma counts in small intestines from 120 day-old ApcMin DKO or QKO and matched ApcMin control mice, as indicated underneath graph; each dot represents one mouse; mean and SEM indicated by horizontal lines; ****, p < 0.0001 (Tukey’s multiple comparisons test). c Size distributions of adenomas shown in (b); tumours from each mouse (represented by dots) were grouped into three size classes, as indicated underneath graphs; lines as in (b). d–f IF of representative adenomas from ApcMin DKO and matched controls as indicated, after fixation and double-staining with α-β-catenin (red) and α-E-cadherin (green) antibodies; QKO adenomas look similar to Bcl9/B9l DKO adenomas (ad), of which the majority (> 95%) do not protrude above the villi (vi) and never progress to macroadenoma (unlike Pygo1/2 DKO and control adenomas); scale bar, 100 μm. ne, normal epithelium. See also Supplementary Figures 1–4.
Loss of Bcl9 or Pygo increases tumour numbers in Apc<sup>Min</sup> mice. In light of this, it was surprising that the tumour numbers were increased ~3x in small intestines of Pygo-deleted Apc<sup>Min</sup> mice compared to Apc<sup>Min</sup> littermates (Fig 1b). By contrast, there were far fewer large adenomas (>1 mm) in Bcl9-deleted Apc<sup>Min</sup> intestines, although these were embedded within a lawn of tiny adenomas (<1 mm; Fig 1c). In addition, histological analysis of ‘swiss rolls’ revealed numerous minute adenomas in these intestines buried below the intestinal villi and thus undetectable by surface inspection (Fig 1d), while the great majority of adenomas in Pygo-deleted and Apc<sup>Min</sup> control intestines protrude above these villi (Fig 1e, f). This striking change in size distribution from large to minute adenomas indicates that Bcl9 is required for tumour growth. Note that the increased tumour numbers in Apc<sup>Min</sup> DKO intestines were unlikely to be caused by elevated LOH, given that the numbers of γ-H2AX positive foci (marking double-stranded DNA breaks and other lesions underlying LOH) are similarly low as in Apc<sup>Min</sup> controls (Supplementary Figure 3). We conclude that loss of Bcl9 in the intestine, despite elevating tumour numbers, slows down the growth of these tumours, suggesting a role of Bcl9 in promoting their proliferation. This is consistent with the findings by Gay et al. (co-submitted) that Bcl9 is required for crypt regeneration following irradiation, and for efficient clonal expansion in the murine intestine. Furthermore, B9L also appears to be essential for the proliferation of APC-mutant cancer cells: its depletion in SW480 cells (expressing APC truncated at amino acid 1338) attenuated their proliferation substantially, and it proved impossible to recover proliferating cell colonies that lack B9L (Supplementary Figure 4).

The tumour phenotype of the Apc<sup>Min</sup> QKO intestines was virtually indistinguishable from that of Bcl9-deleted Apc<sup>Min</sup> intestines (Fig 1b, c), suggesting that Bcl9 loss may be epistatic over Pygo loss (but see below). Notably, the weight gain of DKO and QKO mice was normal during their extended life spans, and so their increased tumour loads were well tolerated. Indeed, the morbidity of Apc<sup>Min</sup> QKO mice is thought to be caused by their anaemia rather than their adenoma burden.

Synergy between loss of Bcl9 and Pygo in normalising tumours. Our RNA profiling data suggested that Bcl9 loss contributed rather more than Pygo loss to the QKO-dependent trend reversals, perhaps explaining why the tumour phenotypes of the QKO mice resembled those of Bcl9/B9l rather than Pygo1/2 DKO. To test this, and to address whether Bcl9 is epistatic over Pygo at the level of transcription, we conducted systematic Pearson’s correlations of the significant changes between QKO and control adenomas across all five cohorts.

While these correlations revealed some resemblance between the two types of DKO samples, the QKO samples more closely resembled the Bcl9/B9l DKO than the Pygo1/2 DKO (Fig. 3a). QKO and Bcl9/B9l DKO cohorts shared 531 changes (<15.6% of the Bcl9-regulated genes), QKO and Pygo1/2 DKO cohorts shared 43 changes (<1.3% of the Pygo-regulated genes; Fig 3b), and 22 changes were shared between all three cohorts (Supplementary Figure 7). Importantly, the vast majority of the QKO-dependent changes (80.7%) were unique and not shared with either DKO cohort (Fig 3c).

Two conclusions can be drawn: firstly, a large fraction of the Bcl9- and Pygo-regulated genes are downstream of Apc, consistent with Bcl9 and Pygo acting predominantly on Apc-regulated genes in these tumours. Secondly, although more Apc-regulated genes are affected by Bcl9 rather than Pygo loss, simultaneous deletion of both paralog pairs affects vastly more Apc-regulated genes than deletion of either alone. An important corollary is that Bcl9 loss strongly synergises with Pygo loss in shifting the adenoma gene expression programme towards that of normal crypts, and argues against an epistatic relationship between the two enhancosome factors.

Loss of Bcl9 or Pygo shifts cells towards differentiation. To test this apparent epistasis between Bcl9 and Pygo, we determined the RNA profiles in adenomas excised from Apc<sup>Min</sup> DKO and QKO small intestines (pooled from 2–3 RNA extractions). Comparing Apc<sup>Min</sup> adenomas to normal crypt samples, we found 8852 significant changes (p < 0.01), most of which likely represent indirect gene expression changes downstream of master transcription regulators (such as Ascl2, Math1 or c-myc; see below). They include upregulation of known Wnt targets (such as Lgr5), H2A and ribosomal protein genes, and downregulation of Defensin genes, e.g., Defa1 (Fig 2a and Supplementary Figure 2). Consistent with this, acute inactivation of Apc in the intestinal epithelium causes crypt expansions, and cell-fate shifts from differentiated to proliferative<sup>35</sup>. Hierarchical clustering based on Euclidean distance confirmed that profiles within samples of the same genotypic cohort were more similar to one another than to those from different cohorts (Fig 2b, top).

Comparing Apc<sup>Min</sup> to QKO adenomas, we found 2806 significant changes of which 62.3% were dependent on Apc, i.e., the corresponding gene probes showed differential expression in adenomas versus (vs) normal crypts; of these, 82.9% showed opposite trends in QKO vs Apc<sup>Min</sup> controls (Supplementary Figure 5). Furthermore, a substantial fraction of the QKO-dependent changes were trend reversals towards the RNA profile of normal crypts (Fig 2b), including most Wnt target genes (Fig 2c) except for Axin2, a gene constituting a negative feedback loop with elevated expression in Apc-mutant adenomas<sup>56</sup> which was unaffected by loss of Pygo and Bcl9. Indeed, these ‘normalisations’ also extended to previously identified stem cell signatures downstream of Wnt/Tcf3<sup>33</sup>, EphB2 or Lgr5<sup>38,39</sup> (Supplementary Figure 6), consistent with previous results<sup>18,19</sup>. Thus, simultaneous deletion of Bcl9 and Pygo shifts the transcription programme of Apc<sup>Min</sup> adenomas from stem-cell-like towards normal crypts.

Loss of Bcl9 or Pygo promotes differentiation towards secretory cell fates. Defa1 was our top hit amongst the changes shared between all three DKO and QKO cohorts (Supplementary Figure 7). Indeed, lysozyme and other Paneth cell markers behaved similarly, showing a tendency to be upregulated in both types of DKO compared to control adenomas (Fig 4a). We confirmed this for goblet cells, using Period-Acid Schiff (PAS) diastase (PAS-D) staining: we found about twice as many PAS-D-positive cells in Bcl9/B9l DKO vs control adenomas (Fig 4f–h), although the adenomatous PAS-D signals (labelling intracellular mucus) were smaller than those from normal goblet cells (Fig 4f, g: high-magnification views), suggesting that these metaplastic goblet cells are not fully differentiated. Thus, loss of Pygo or Bcl9 promotes secretory cell metaplasia, apparently at the expense of stem cell-like fates, given the downregulation of Lgr5 and stem cell signature genes in these adenomas (Supplementary Figure 6). In contrast, markers for absorptive enterocytes (constituting the bulk of intestinal villi) were not significantly changed (Fig 4a). This is consistent with the notion that enterocytes represent the
‘default’ cell fate within the intestinal epithelium\textsuperscript{31} whose specification requires neither β-catenin nor Math1\textsuperscript{32,33}.

**Redistribution of β-catenin in Bcl9-deleted Apc\textsuperscript{Min} adenomas.** The above-described RNA profile changes do not fully explain why the tumour phenotypes caused by Bcl9 loss differed from those in Pygo-deleted Apc\textsuperscript{Min} intestines. We thus surmised that the underlying cause may be post-transcriptional. One key determinant for tumour multiplicity is the β-catenin-dependent cell adhesion between tumour and adjacent normal cells which, if elevated, decreases the rate of tumour loss through the normal epithelial renewal process\textsuperscript{40,41} through upwards displacement along the crypt-villus axis\textsuperscript{31,}, thereby increasing tumour numbers.
Notably, APC regulates the junctional pool of β-catenin, and complete Apc loss-of-function causes a drastic increase of nucleocytoplasmic at the expense of junctional β-catenin. We thus examined the subcellular distribution of β-catenin in Bcl9-deleted ApcMin adenomas by immunofluorescence (IF).

As expected, ApcMin adenoma cells exhibited high levels of cytoplasmic and nuclear β-catenin (Fig. 5a, red signal), but low levels of junctional β-catenin compared to adjacent normal epithelial cells. In contrast, Bcl9/B9l DKO adenoma cells showed very little nucleocytoplasmic β-catenin, which explains the low...
Fig. 4 Loss of Bcl9 or Pygo causes a shift towards secretory cell fates in adenomas. a) Heat map (as in Fig. 2b) for selected differentiation markers, as indicated on the right. b–g) Sections through adenomas (genotypes indicated in panels) stained for (b–d) Paneth (brown, α-lysozyme) or (f, g) goblet cells (dark blue, PAS-D); right, magnified views of squares in (f, g), with strong PAS-D signals in villi (black square) or adenomas (red squares) indicating mature or immature goblet cells, respectively; purple, hematoxylin; scale bar, 100 μm. e, h) Quantification of (b–g).
levels of Wnt target gene expression in these Bcl9-deleted adenomas. Instead, we observed prominent β-catenin staining at the cell surface of these tumours, coinciding with strong E-cadherin staining (Fig. 5b, yellow). Indeed, in these sections, the membraneous β-catenin staining patterns barely differed between adenomas (which showed slightly elevated nucleocytoplasmic β-catenin, allowing their identification) and adjacent normal epithelium (Fig. 5b, insets). In contrast, the β-catenin staining patterns in Pygo-deleted adenomas looked similar to those in ApcMin controls (Fig. 5a, insets), and so even the tiniest nascent tumours stand out by their strong nucleocytoplasmic β-catenin signal43. The total levels of β-catenin and E-cadherin are comparable in DKO and ApcMin control adenomas (Supplementary Figure 8). It thus appears that, in the absence of its nuclear binding partner Bcl9, β-catenin cannot be retained in the nucleus and is thus sequestered at the cell surface by its alternative binding partner E-cadherin (see Discussion).

Loss of Bcl9 cures Apc1322T mice of their neoplastic disease.

While our analysis was in progress, a new model for colorectal cancer became available28, based on mice bearing an Apc allele (Apc1322T) that mimics one of the most commonly observed APC mutations observed in human carcinomas. The Apc1322T truncation is substantially longer than the ApcMin truncation and, unlike the latter, retains multiple β-catenin binding sites (Fig. 6a) and, thus, partial function in downregulating β-catenin. Notably, a wealth of genetic data from human colorectal cancers indicates a strong positive selection for the retention of β-catenin binding sites44, i.e., for the preservation of partial APC function (‘just-right signalling’). Indeed, colorectal carcinomas that express ApcMin-like truncations without any β-catenin binding sites are exceedingly rare44.

We thus repeated our analyses in the Apc1322T model. RNaseq analysis of adenomas from 100–120 day old ApcMin and Apc1322T mice revealed only 178 significant changes, a minute fraction of the > 8000 significant changes caused by the ApcMin mutation itself. We thus expect the above-described RNA profiling data from ApcMin adenomas to pertain also to Apc1322T adenomas, at least qualitatively. However, Wnt target genes tended to be less upregulated in Apc1322T compared to ApcMin adenomas (Supplementary Figure 9), consistent with the Apc1322T truncation retaining partial function in downregulating β-catenin.

Despite this, Apc1322T mice develop more rapid and severe polyposis, and succumb to their neoplastic disease earlier, than ApcMin mice28. Under our conditions, Apc1322T mice typically showed overt disease by ~110 days, but their disease-free life was extended to ~180 days upon Pygo deletion (Fig. 6b). Furthermore, the mean tumour numbers in Pygo-deleted Apc1322T intestines were < 10, compared to ~18 in control littermates.

![Fig. 5](https://example.com/fig5.jpg)

**Fig. 5** Loss of Bcl9 causes relocation of β-catenin to the cell surface of ApcMin adenoma cells. IF of nascent adenomas from a ApcMin or b Bcl9/B9l DKO small intestines, after fixation and double-staining as in Fig. 1d–f; arrows, boundaries between normal epithelium (ne) and adenoma (ad); underneath, magnified views of squares (in merges, right) containing comparable normal and adenomatous epithelial cells; blue, nuclei marked by DAPI (only in merges); scale bars, 50 μm. Note the high levels of nucleocytoplasmic β-catenin in ApcMin adenomas (strong red fluorescence in (a); see also Fig. 1e, f), relocated to the cell surface in Bcl9/B9l DKO adenomas (yellow fluorescence in b), leaving barely any discontinuities between adenomatous and adjacent normal epithelial cells.
Fig. 6 Loss of Bcl9 suppresses the neoplastic disease of Apc1322T mice. a Cartoon of human APC, with Axin (red) and β-catenin binding sites (15R1-3, blue; 20R1-7, green) in its central portion downstream of the ARD (grey); numbers, N-terminal limits of motifs; arrows, truncation endpoints of ApcMin and Apc1322T alleles; bracket, mutation cluster region (MCR) in human colorectal cancers (see text). b Kaplan–Meier survival plots of Apc1322T cohorts, as in Fig. 1a; statistical significance, p < 0.0001 (log-rank test). c Adenoma counts in small intestines from 77 day-old Apc1322T Pygo1/2 DKO and matched Apc1322T Pygo1 KO controls, as in Fig. 1b; statistical significance, p < 0.05 (t-test). See also Supplementary Figures 9 & 10

Discussion We used two mouse tumour models based on different Apc germline mutations (ApcMin and Apc1322T) to assess the roles of the Wnt enhanceosome components Bcl9 and Pygo in intestinal neoplasia. In both models, deletion of these factors suppressed tumourigenesis, and extended the disease-free life spans, possibly owing to a shift in the gene expression of Apc-mutant adenomas from stem cell-like towards differentiated. Importantly, simultaneous deletion of Bcl9 and Pygo proved vastly more effective in reversing the neoplastic transcription programme towards normal than deleting either of them alone. Our results underscore the notion that these factors synergise to facilitate the docking of β-catenin to the Wnt enhanceosome, to co-activate transcription of Apc target genes. In addition, we uncovered evidence for increased tumour retention in Bcl9-deleted ApcMin intestines, but barely delineating these tumours (Fig. 7a), consistent with previous results and with the notion that the Apc1322T truncation retains moderate function in downregulating β-catenin. As might be expected, there was no detectable discontinuity in cell surface β-catenin between adenomatous and adjacent normal epithelial cells in Apc1322T Bcl9/B9l DKO intestines (Fig. 7b), in stark contrast to the overt discontinuities in ApcMin adenomas (Fig. 7c).

Apc1322T adenomas show normal cell surface β-catenin. Given the similar RNA profiles between the two models, we surmised that the root cause for their different responses to Bcl9 loss might be post-transcriptional. Indeed, we detected only a subtle increase in nucleocytoplasmic β-catenin staining in Apc1322T adenomas, barely delineating these tumours (Fig. 7a), consistent with previous results and with the notion that the Apc1322T truncation retains moderate function in downregulating β-catenin. As might be expected, there was no detectable discontinuity in cell surface β-catenin between adenomatous and adjacent normal epithelial cells in Apc1322T Bcl9/B9l DKO intestines (Fig. 7b), in stark contrast to the overt discontinuities in ApcMin adenomas (Fig. 7c).

Discussion We used two mouse tumour models based on different Apc germline mutations (ApcMin and Apc1322T) to assess the roles of the Wnt enhanceosome components Bcl9 and Pygo in intestinal neoplasia. In both models, deletion of these factors suppressed tumourigenesis, and extended the disease-free life spans, possibly owing to a shift in the gene expression of Apc-mutant adenomas from stem cell-like towards differentiated. Importantly, simultaneous deletion of Bcl9 and Pygo proved vastly more effective in reversing the neoplastic transcription programme towards normal than deleting either of them alone. Our results underscore the notion that these factors synergise to facilitate the docking of β-catenin to the Wnt enhanceosome, to co-activate transcription of Apc target genes. In addition, we uncovered evidence for increased tumour retention in Bcl9-deleted ApcMin intestines, but barely delineating these tumours (Fig. 7a), consistent with previous results and with the notion that the Apc1322T truncation retains moderate function in downregulating β-catenin. As might be expected, there was no detectable discontinuity in cell surface β-catenin between adenomatous and adjacent normal epithelial cells in Apc1322T Bcl9/B9l DKO intestines (Fig. 7b), in stark contrast to the overt discontinuities in ApcMin adenomas (Fig. 7c).
this undesirable effect was not evident in the Apc\(^{1322T}\) model. Indeed, intestinal deletion of Bcl9 blocked tumourigenesis in these mice and essentially cured them of their neoplastic disease. Since this model recapitulates common human colorectal carcinomas in terms of their APC mutations\(^{28}\), this indicates the potential of BCL9 as a therapeutic target in this often lethal cancer.

Several of our results support the Wnt enhanceosome model and, indeed, can be explained by it even though this model, derived from biochemical and functional data from Drosophila and human cells (including APC-mutant colorectal cancer cells) has not been validated specifically for murine cells. For example, we observed a strong synergy between Bcl9 loss and Pygo loss in the suppression of neoplastic disease (Fig. 1a), and in the shift of adenomatous gene expression from neoplastic towards normal (Figs 2b and 3a), consistent with this model. However, we also uncovered clear quantitative differences between the two conditions in that the effects of Bcl9 loss on gene expression and tumour phenotypes were consistently stronger than those of Pygo loss, indicating that Bcl9 is more important than Pygo in enabling β-catenin to co-activate TCF-dependent transcription. This is consistent with its scaffold function within the Wnt enhanceosome\(^{7}\), whereby Bcl9 contacts Pygo as well as the Chip/LDB-SSDP (ChiLS) core complex of the enhanceosome and so only partially relies on Pygo for its association with the enhanceosome.

Also, the Wnt enhanceosome may retain partial integrity and function in the absence of Pygo owing to its linkage to the BAF chromatin remodelling complex\(^{46}\) whose two PHD fingers might substitute for Pygo’s PHD finger in conferring chromatin association. Crucially, Bcl9 confers Wnt responsiveness on this multiprotein complex as its only component that can bind to and recruit β-catenin. This, together with the scaffolding role of Bcl9, readily explains why the transcriptional profile of QKO tumours is largely determined by Bcl9 loss (Fig. 3b, c) and, by implication, why the latter has such a profound impact on β-catenin-dependent intestinal neoplasia in both Apc models (Figs 1a and 6b).

We also observed qualitative differences between Bcl9 and Pygo loss regarding the tumour phenotypes. The most striking one was a marked shift in adenoma size from large to tiny in Bcl9-deleted Apc\(^{Min}\) mice, while Pygo loss had no effect on the tumour size distribution, implying a role of Bcl9 but not Pygo in promoting tumour growth. However, this apparent qualitative difference might simply reflect a threshold effect: in the absence of Bcl9, expression of master regulators of tumour growth such as c-myc\(^{47}\) might be reduced sub-critically, which could translate into an off-switch of downstream effector programmes, while the effect of Pygo loss on these genes might be too weak to toggle this switch. This would explain why far fewer genes were differentially expressed in Pygo-deleted compared to Bcl9-deleted tumours. It is

---

**Fig. 7** The subcellular distribution of β-catenin in Apc\(^{1322T}\) adenoma cells is not sensitive to Bcl9 loss. IF of adenoma (ad) from (a) Apc\(^{1322T}\), (b) Apc\(^{1322T}\) Bcl9/B9l DKO or Apc\(^{Min}\) small intestines, and adjacent normal epithelia (ne), fixed and stained as in Fig. 5; scale bars, 50 μm. The nucleocytoplasmic β-catenin levels are barely elevated in Apc\(^{1322T}\) adenomas (compared to Apc\(^{Min}\) adenoma, red fluorescence in c) and their cell surface β-catenin is high (yellow fluorescence in a, b) regardless of Bcl9
also conceivable that some Apc target genes are controlled only by Bcl9 but not Pygo, but the synergy of their simultaneous deletion argues against this. Also, the qualitative differences in tumour phenotypes between the two DKO strains were only apparent in the ApcMin model (for reasons discussed below) while the Apc1332T mice responded to Bcl9 loss similarly albeit more profoundly than to Pygo loss.

Many of the Wnt enhancosome components including its ChiLS core and Groucho/TLE co-repressor were discovered as Notch signalling effectors in Drosophila56. ChiLS is recruited to target enhancers via direct binding to members of the basic helix-loop-helix (bHLH) proteins, such as the mammalian Notch effectors Ascl (Achaete/scute-like) and Math/Atoh (Atonal homolog); these positively activating enhancer-binding proteins compete with negatively-acting Hairy/enhancer-of-split (Hes) factors, Notch effectors whose expression is universally induced by Notch signalling, and which silence downstream enhancers by recruiting Groucho/TLE52. We thus proposed that the Wnt enhancosome integrates Wnt and Notch signalling inputs6, relying on Bcl9-Pygo and positively-acting Notch effectors for its activation, and on Notch-dependent Hes repressors for its repression.

This notion is reinforced by our finding that loss of Bcl9 or Pygo tends to shift cell fates from stem-cell like towards differentiation along all three secretory cell lineages of the intestinal epithelium. Each of these cell types requires opposing inputs from Wnt and Notch signalling50, and their fates appear to be specified stochastically in transit-amplifying cells located above the crypt base, descending from Lgr5-positive crypt cells51. Their specifications depend on β-catenin53 and the master regulator Math132, while their differentiation along specific secretory lineages requires repressive inputs from Hes1 and other redundant Hes factors52 whose localised accumulation depends on Notch signalling from neighbouring cells31. If Math1 were controlled by the Wnt enhancosome, a molecular device for integrating opposing inputs from β-catenin and Notch > Hes signalling, this would provide a mechanistic explanation for the results by Tian et al.50.

Paneth cells serve as stem cell niches that provide Wnt and Notch signals to reinforce Lgr5-posivity of adjacent cells53. This seemed at odds with the observed reductions of Lgr5 itself, and its downstream markers in Bcl9 or Pygo deletion. Our evidence suggests that the latter reflects sequestration of β-catenin at the cell surface by its alternative binding partner E-cadherin, following its leakage from the nucleus in the absence of Bcl9: it is well established that β-catenin at the cell surface and Notch signalling from neighbouring cells, regardless of its phosphorylation status, and that its partitioning between these subcellular compartments is determined by retention via its binding partners in these locations60-63. This redistribution of β-catenin from the nucleus to the cell surface owing to Bcl9 loss was not detectable in Pygo-deleted ApcMin adenomas, likely because the Apc1332T adenomas, which do respond to Bcl9 loss similarly to typical colorectal cancer cells59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription57 whereas the shorter ApcMin truncation does not, and so ApcMin is equivalent to a null allele with regard to β-catenin regulation. The tumours produced by two models are morphologically indistinguishable, and exhibit very similar RNA profiles38,60, although the Wnt and stem cell signature genes tended to be more upregulated in ApcMin than in Apc1332T adenomas (Supplementary Figure 9). This is consistent with results from the human colorectal cancer cell line COLO320 whose APC truncation is ApcMin-like and atypically short for human cancers59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription. The lower levels of junctional β-catenin in ApcMin tumours may be explained if their dysfunctional ApcMin truncation were unable to maintain junctional β-catenin. By contrast, the partially functional Apc1332T truncation may maintain near-normal junctional β-catenin in adenomas (Fig. 7a) which could boost their adhesion to neighbouring cells, thereby enhancing their retention within intestinal crypts41, and resisting their upward displacement to the villar apices and shedding into the gut lumen. It would explain why Apc1332T mice develop more severe, and faster, intestinal polyposis than ApcMin mice28, despite their lower Wnt and stem cell signatures.

The same could also explain why the Bcl9-deleted ApcMin intestines exhibited hundreds of tiny adenomas (Fig. 1): evidently, their growth was stunted, owing to their dysfunctional β-catenin-dependent transcription (Fig. 2 and Supplementary Figure 6), but they appeared to be retained more efficiently within intestinal crypts owing to their high levels of cell surface β-catenin (Fig. 6b).

Our evidence suggests that the latter reflects sequestration of β-catenin at the cell surface by its alternative binding partner E-cadherin, following its leakage from the nucleus in the absence of Bcl9: it is well established that β-catenin at the cell surface and Notch signalling from neighbouring cells, regardless of its phosphorylation status, and that its partitioning between these subcellular compartments is determined by retention via its binding partners in these locations60-63. This redistribution of β-catenin from the nucleus to the cell surface owing to Bcl9 loss was not detectable in Pygo-deleted ApcMin adenomas, likely because the Apc1332T adenomas, which do respond to Bcl9 loss similarly to typical colorectal cancer cells59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription57 whereas the shorter ApcMin truncation does not, and so ApcMin is equivalent to a null allele with regard to β-catenin regulation. The tumours produced by two models are morphologically indistinguishable, and exhibit very similar RNA profiles38,60, although the Wnt and stem cell signature genes tended to be more upregulated in ApcMin than in Apc1332T adenomas (Supplementary Figure 9). This is consistent with results from the human colorectal cancer cell line COLO320 whose APC truncation is ApcMin-like and atypically short for human cancers59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription. The lower levels of junctional β-catenin in ApcMin tumours may be explained if their dysfunctional ApcMin truncation were unable to maintain junctional β-catenin. By contrast, the partially functional Apc1332T truncation may maintain near-normal junctional β-catenin in adenomas (Fig. 7a) which could boost their adhesion to neighbouring cells, thereby enhancing their retention within intestinal crypts41, and resisting their upward displacement to the villar apices and shedding into the gut lumen. It would explain why Apc1332T mice develop more severe, and faster, intestinal polyposis than ApcMin mice28, despite their lower Wnt and stem cell signatures.

The same could also explain why the Bcl9-deleted ApcMin intestines exhibited hundreds of tiny adenomas (Fig. 1): evidently, their growth was stunted, owing to their dysfunctional β-catenin-dependent transcription (Fig. 2 and Supplementary Figure 6), but they appeared to be retained more efficiently within intestinal crypts owing to their high levels of cell surface β-catenin (Fig. 6b).

Our evidence suggests that the latter reflects sequestration of β-catenin at the cell surface by its alternative binding partner E-cadherin, following its leakage from the nucleus in the absence of Bcl9: it is well established that β-catenin at the cell surface and Notch signalling from neighbouring cells, regardless of its phosphorylation status, and that its partitioning between these subcellular compartments is determined by retention via its binding partners in these locations60-63. This redistribution of β-catenin from the nucleus to the cell surface owing to Bcl9 loss was not detectable in Pygo-deleted ApcMin adenomas, likely because the Apc1332T adenomas, which do respond to Bcl9 loss similarly to typical colorectal cancer cells59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription57 whereas the shorter ApcMin truncation does not, and so ApcMin is equivalent to a null allele with regard to β-catenin regulation. The tumours produced by two models are morphologically indistinguishable, and exhibit very similar RNA profiles38,60, although the Wnt and stem cell signature genes tended to be more upregulated in ApcMin than in Apc1332T adenomas (Supplementary Figure 9). This is consistent with results from the human colorectal cancer cell line COLO320 whose APC truncation is ApcMin-like and atypically short for human cancers59, and which exhibit 2-3 fold higher levels of β-catenin-dependent transcription compared to typical colorectal cancer cells60 whose longer APC truncations retain moderate β-catenin-dependent transcription. The lower levels of junctional β-catenin in ApcMin tumours may be explained if their dysfunctional ApcMin truncation were unable to maintain junctional β-catenin. By contrast, the partially functional Apc1332T truncation may maintain near-normal junctional β-catenin in adenomas (Fig. 7a) which could boost their adhesion to neighbouring cells, thereby enhancing their retention within intestinal crypts41, and resisting their upward displacement to the villar apices and shedding into the gut lumen. It would explain why Apc1332T mice develop more severe, and faster, intestinal polyposis than ApcMin mice28, despite their lower Wnt and stem cell signatures.

Therefore, this undesirable effect of Bcl9 deletion on tumour retention in the ApcMin model partially negates the beneficial effects of Bcl9 loss in curbing β-catenin-dependent neoplastic gene expression. Importantly, there was no detectable relocation of β-catenin in ApcMin adenomas, likely because the Apc1332T truncation retains partial activity in downregulating transcriptionally active β-catenin, keeping its nucleocytoplasmic levels relatively low and its cell surface levels normal. This truncation therefore buffers the negative effects of nuclear β-catenin efflux in the absence of Bcl9. The same is expected in the great majority of colorectal carcinomas whose APC truncations, like Apc1332T, retain binding to β-catenin and regulating its activity.
The Apc<sup>1322T</sup> model has allowed us to discover a crucial role of Bcl9 and Pygo2 in affecting intestinal neoplasia in a model with high relevance for colorectal cancer. Similar conclusions were reached by Gay et al. (co-submitted) who also found Bcl9 to be critical for tumorigenesis in different β-catenin-dependent murine models. However, our results are broadly consistent with evidence that excess Bcl9-2 or Pygo2 promote tumour progression in the intestine<sup>21,65,66</sup> and in other tissues<sup>67,68</sup>. Our study also revealed that Bcl9 is more critical for intestinal neoplasia than Pygo, although both proteins synergise to effect β-catenin-dependent transcription. Thus, BCL9 and B9L have significant potential as targets for therapeutic intervention in colorectal cancer. Since proof-of-concept has been obtained for the discovery of reagents that block their binding to β-catenin<sup>38,39,30,21</sup>, it might be possible to identify inhibitors of this interaction that can be developed into therapeutics.

**Methods**

**Animal procedures.** Animal care and procedures were performed in accordance with the UK regulations set by the United Kingdom Home Office. For tests in the Apc<sup>Min</sup>/−, model, Bcl9<sup>fl/fl</sup> Pygo2<sup>fl/fl</sup> villin.Cre or Pygo1<sup>−/−</sup> Pygo2<sup>fl/fl</sup> villin.Cre were back-crossed into a C57BL/6 background for 4 successive generations<sup>5</sup> whereby the final cross was with Apc<sup>Min</sup>−/+. To generate compound DKO Apc<sup>Min</sup> or Apc<sup>1322T</sup> strains, Apc<sup>Min</sup>/− or Apc<sup>1322T</sup>/− mice were crossed into a homozygous background of Bcl9<sup>LoxP/LoxP</sup> Pygo1<sup>−/−</sup> Pygo2<sup>LoxP/LoxP</sup> also bearing villin.Cre/<sup>+</sup>. Similarly, for the QKO Apc<sup>Min</sup>−/+ or Apc<sup>1322T</sup>−/+ mice were crossed into a homozygous background of Bcl9<sup>LoxP/LoxP</sup> Pygo1<sup>−/−</sup> Pygo2<sup>LoxP/LoxP</sup> also bearing villin.Cre/<sup>+</sup>. Only male bearing Apc alleles were used for breeding.

For genotyping, tissue from ear biopsies was used. DNA was extracted using TaqMan Sample-to-NP Kit (Thermofisher Scientific). For genotyping of Apc<sup>Min</sup>, the Custom TaqMan SNP Genotyping Assay with the primer set AHX1ET and GTPxress Taqman was used. All other alleles were genotyped by standard PCR with KOD DNA polymerase (Merck Millipore), using the following primers:

**Results**.

For IF and IHC, small intestines were fixed with 80% ethanol for <7 days. Swiss rolls were dehydrated by the following successive incubations: 1x in 70% ethanol for 45 min; 1x in 90% ethanol for 1 h; 4x in 100% ethanol for 75 min; 3x in xylene for 90 min. Subsequently, they were embedded in paraffin by incubating 3x in Tek Wax (SAKURA, no. 46599) for 90 min at 60 °C. Sections were cut at 3.5 μm with a Leica microtome RM255.

**IF and IHC.** For IHC, slides were de-paraffinized and rehydrated by overnight incubation at 65 °C and subsequently washed as follows: 2 x 3 min in 100% xylene, 2 x 3 min in 100% ethanol, 1 x 3 min in 95% ethanol, 1 x 3 min in 70% ethanol, 1 x 3 min in 50% ethanol, and 1x in water. For heat-induced epitope retrieval, slides were cooked in 10 mM sodium citrate (pH 6.0) containing 0.1% Tween in a microwaveable vessel for 20 min at 900 W, and subsequently cooled gently and rinsed in water and PBS containing 0.1% Tween. Tissue sections on slides were demarcated with an ImmEdge pen (Vector Laboratories, Peterborough, UK). For protein and RNA extraction, slides were heated in a microwave for 5 min at room temperature (RT) in a humid chamber. Slides were then washed twice in PBS containing 0.1% Tween.

For antibody staining, slides were treated with peroxidase-blocking reagent (Dako, Carpinteria CA, USA) for 10 min and washed 3x in PBS containing 0.1% Tween for 5 min. Slides were then blocked in PBS containing 10% BSA for 30 min at RT in a humid chamber, incubated with primary antibody in PBS containing 10% BSA for 90 min at RT (or overnight at 4 °C), and subsequently washed 3x for 5 min with PBS containing 0.1% Tween. For detection kit (Dako, α-lysozyme, α-phospho-histone H3, PH3) or Polymer Refine Detection System (Leica, α-BrdU) was used; BrdU detection was carried out at the Histopathology and in situ Hybridization Facility (CRUK Cambridge Institute). Hematoxylin (23% solution in water, Dako) was used for counterstaining (for 5 min), followed by two wash steps in water. For TUNEL staining, the DeadEnd Colormetric Apoptosis Detection System (Promega, Madison WI, USA) was used. For PAS-D staining, slides were incubated as follows: 2% amylase (Sigma Aldrich) solution in ultra-pure water for 15 min at 37 °C; tap water for 30 s; ultra-pure water for 30 s; 1% Alcian blue 8GX (TCS Biosciences, Buckingham, UK) in 3% acetic acid for 10 min; ultra-pure water for 30 s; 0.5% periodic acid (VWR, Radnor PA, USA) in ultra-pure water for 5 min; ultra-pure water for 1 min; Schiff’s reagent (TCS Biosciences) for 20 min; ultra-pure water for 1 min; tap water for 15 min; Harris Haematoxlin (CellPath, Newtown, UK) for 15 s; tap water for 1 min; 0.3% hydrochloric acid in 70% ethanol for 5 s for tap water for 1 min; 0.3% sodium borate for 15 s; tap water for 1 min; 0.3% hyaluronidase (Sigma Aldrich) in 100 mM Tris, pH 7.5, for <24 hours. Slides were dehydrated by the following incubations: 1 x 3 min in 50% ethanol; 1 x 3 min in 70% ethanol; 1 x 3 min in 95% ethanol; 2 x 3 min at 100 °C; 2 x 1 min in 100% ethanol. Staining was performed with the Genesis Kit (ThermoFisher Scientific) at the Human Research Tissue Bank, supported by the NIHR Cambridge Biomedical Research Centre. For detection of γ-H2AX, a commercial antibody (Cell Signalling Technology, Danvers MA, USA) was applied on an automated immunostainer (Bond-III, Leica Biosystems) using a working dilution of 1:50. Anti-γ-H2AX antibody was performed using the Bond Epitope Retrieval 2 Solution (Leica Biosystems) for 20 min. The Bond Polymer Refine Detection Kit (Leica Biosystems) was used for visualizing antigens. All IHC slides were dehydrated by the following incubations: 1 x 3 min in 50% ethanol; 1 x 3 min at 70 °C; 1 x 3 min in 95% ethanol; 2 x 3 min at 100 °C; 2 x 1 min in 100% ethanol. All slides were subsequently air-dried and mounted in Glycerol Mounting Medium (Dako). Imaging was done with a Zeiss microscope and Zeiss Axiocam MRC5 camera.

For IF, incubation with primary antibodies and washes were done as described above for IHC. Slides were subsequently incubated with fluorescently-labeled secondary antibodies (1:1000) for 1 h. Anti-γ-H2AX was performed using the Bond Epitope Retrieval 2 Solution (Leica Biosystems) with PBS containing 0.1% Tween and mounted in Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, no. H-1200). Images were acquired at identical settings with a Zeiss Confocal Microscope. Cells positive for TUNEL, γ-H2AX and Ph3 were counted manually with a cell counting grid. Cells positive for lysozyme were counted in a dark humid environment using a Trio25 (Omega Technologies), the adenoma surface area was determined using Fiji software (fiji.sc).

Wild type and CRISPR clones of SW480 cells were imaged with a Zeiss Confocal Microscope in 4 well glass bottom μ-slides (Biotix, Martinsried, Germany). Cultures were washed with PBS containing 0.5% FBS to remove adherent cells, donkey α-sheep and goat α-mouse secondary antibodies (Life Technologies), and Hoechst stain.

CRISPR/Cas9 genome editing. Genome editing in SW480 cells was essentially performed as previously described<sup>49</sup>, using single guide RNA encoding plasmid derivatives of pCPas9(BB)–2A-GFP (PX458). Cells were selected for high GFP expressing cells by flow cytometry and pooled. Genomic DNA was extracted from SW480 cells 48 h post-transfection, and sorted at a density of 1 cell well<sup>−1</sup> in a 96-well plate, to isolate single clones for subsequent screening by western blot analysis and DNA sequencing. For the proliferation assay (Supplementary Figure 4), GFP-positive cells were sorted in bulk at a density of 1<sup>0</sup> cells well<sup>−1</sup> in a 96-well plate, and the cell density at subsequent days was measured using an ECO20 flow cytometer (Sony Bio-technology, Weybridge, UK).

**RNA extraction.** Frozen adenomas and crypt samples were thawed on ice in RLT buffer (Qiagen, Hilden, Germany) containing 1% β-mercaptoethanol, and...
homogenized with a Qagen TissueRuptor followed by QIAshredder columns according to the manufacturer’s instructions. RNA was extracted from tissue lysates with the RNeasy Kit, and treated with RNase-free DNase I (Qiagen). Typically, 2–3 RNA extractions from one mouse were pooled for a single sample. RNA concentration and quality were determined with a NanoDrop 2000 (Thermo Scientific, Waltham MA, USA) and a 2100-Bioanalyzer (Agilent Technologies, Santa Clara CA, USA).

Gene expression analysis. Two batches of quality-controlled RNA samples were generated (Supplementary Table 1), for microarray analysis by Cambridge Genomic Services (University of Cambridge). RNA was linearly amplified with the Illumina TotalPrep RNA Amplification Kit, and the resulting cRNA was hybridized to the MouseWG-6 v2.0 Expression BeadChip (Illumina, San Diego CA, USA) overnight; subsequently, the BeadChip was washed, stained, and scanned with the Illumina BeadArray Reader. Sample and control probe profiles were exported from GenomeStudio software v2011.1 (Illumina) without background correction or normalisation, and subsequently loaded into the R environment. Relevant subsets of the data were imported separately for each comparison into a LumiBatch object, (Variance Stabilising Transformation, VST) and quantile-normalised with the lumi package. Differentially expressed genes were identified by the limma package73, and annotations were subsequently performed on rlog transformed read counts using previously published Wnt and stem cell marker gene sets (see Supplementary Figure 9, for references).

An FDR-adjusted p-value < 0.01 was used as a threshold for statistical significance during subsequent analysis. Quantitative set analysis for gene expression (QsSAGE)74 was performed on rlog transformed read counts using previously published Wnt and stem marker gene sets (see Supplementary Figure 9, for references).

Reagents and resources. The reagents and resources used in this study are listed in Supplementary Table 2.

Data availability

The microarray and RNA sequencing data that support the findings of this study are available in NCBI’s Gene Expression Omnibus and are accessible through GEO Series reference GSE121145.

Received: 29 May 2018 Accepted: 19 December 2018 Published online: 13 February 2019

References

1. Kinzler, K. W. & Vogelstein, B. Lessons from hereditary colorectal cancer. Cell 87, 159–170 (1996).
2. Lung, M. S., Trainer, A. H., Campbell, I. & Lipton, L. Familial colorectal cancer. Intern. Med. J. 45, 482–491 (2015).
3. Vogelstein, B. et al. Cancer genome landscapes. Science 339, 1546–1558 (2013).
4. Jackstadt, R. & Sansom, O. J. Mouse models of intestinal cancer. J. Pathol. 238, 141–151 (2016).
5. Moser, A. R., Pitot, H. C. & Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247, 322–324 (1990).
6. Fiedler, M. et al. An ancient Pygo-dependent Wnt enhancerosome integrated by Chip/LDB-SSDP. Elife 4, https://doi.org/10.7554/eLife.09073 (2015).
7. van Tienen, L. M., Mieszczanek, J., Fiedler, M., Rutherford, T. J. & Bienz, M. Constitutive scaffolding of multiple Wnt enhancerosome components by Leg/BL9/B9L. elife (2017).
8. Adachi, S. et al. Role of a BL9-related beta-catenin-binding protein, B9L, in tumorigenesis induced by aberrant activation of Wnt signaling. Cancer Res. 64, 8496–8501 (2004).
9. Brembeck, F. H. et al. Essential role of BL9-2 in the switch between betacatenin’s adhesive and transcriptional functions. Genes Dev. 18, 2225–2230 (2004).
10. Fiedler, M. et al. Decoding of methylated histone H3 tail by the Pygo-BL9 Wnt signaling complex. Mol. Cell 30, 507–518 (2008).
11. Nusse, R. & Clevers, H. Wnt/beta-catenin signaling, disease, and emerging therapeutic modalities. Cell 169, 985–999 (2017).
12. Polakis, P. Drugging Wnt signaling in cancer. EMBO J. 31, 2737–2746 (2012).
13. de la Roche, M., Ibrahim, A. E., Mieszczanek, J. & Bienz, M. LEFI and B9L shield beta-catenin from inactivation by Axin, desensitizing colorectal cancer cells to tankyrase inhibitors. Cancer Res. 74, 1495–1505 (2014).
14. Huang, S. M. et al. Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. Nature 461, 614–620 (2009).
15. Zheng, Y. et al. Tankyrase inhibition causes reversible intestinal toxicity in mice with a therapeutic index < 1. Toxicol. Pathol. 44, 267–278 (2016).
16. Kramps, T. et al. Wnt/wingless signaling requires BCL9/legless-mediated nuclear export of beta-catenin. Cell 109, 47–60 (2002).
17. Brack, A. S. et al. BL9 is an essential component of canonical Wnt signaling that mediates the differentiation of myogenic progenitors during muscle regeneration. Dev. Biol. 335, 93–105 (2009).
18. Deka, J. et al. Bcl9/Bcl9l are critical for Wnt-mediated regulation of stem cell traits in colon epithelium and adenocarcinomas. Cancer Res. 70, 6619–6628 (2010).
19. Mooij, A. E. et al. BL9/beta-catenin signaling is associated with poor outcome in colorectal cancer. ElBioMedicine 2, 1932–1943 (2015).
20. Mani, M. et al. BL9 promotes tumor progression by conferring enhanced proliferative, metastatic, and angiogenic properties to cancer cells. Cancer Res. 69, 7577–7586 (2009).
21. Brembeck, F. H. et al. BL9 promotes early stages of intestinal tumor progression. Gastroenterology 141, 1359–1370 (2011).
22. Miller, T. C., Rutherford, T. J., Johnson, C. M., Fiedler, M. & Bienz, M. Allotroeric remodelling of the histone H3 binding pocket in the Pygo2 PHD finger triggered by its binding to the B9L/BL9 co-factor. J. Mol. Biol. 401, 969–984 (2010).
23. de la Roche, M. et al. An intrinsically labile alpha-helix abutting the BL9-binding site of beta-catenin is required for its inhibition by carnosic acid. Nat. Commun. 3, 680 (2012).
24. Sampietro, J. et al. Crystal structure of a beta-catenin/BL9/Ctf4 complex. Mol. Cell 24, 293–300 (2006).
25. de la Roche, M., Worm, J. & Bienz, M. The function of BL9 in Wnt/beta-catenin signaling and colorectal cancer cells. Bmc Cancer 8, 199 (2008).
26. Takada, K. et al. Targeted disruption of the BL9/beta-catenin complex inhibits oncogenic Wnt signaling. Sci. Transl. Med. 4, 148ra117 (2012).
27. Zhang, M., Wang, Z., Zhang, Y., Guo, W. & Ji, H. Structure-based optimization of small-molecule inhibitors for the beta-catenin/B-cell lymphoma 9 protein-protein interaction. J. Med. Chem. 61, 2899–3007 (2018).
28. Pollard, P. et al. The Apc 1322T mouse develops severe polyposis associated with sub maximal nuclear beta-catenin expression. Gastroenterology 136, 2204–2213 (2009). e2201–2213.
29. Luongo, C., Moser, A. R., Gledhill, S. & Dove, W. F. Loss of Apc – in intestinal adenomas from Min mice. Cancer Res. 54, 5947–5952 (1994).
30. el Marjou, F. et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 39, 186–193 (2004).
31. Clevers, H. The intestinal crypt, a prototypic stem cell compartment. Cell 154, 274–284 (2013).
32. Yang, Q., Bermingham, N. A., Finegold, M. J. & Zoghbi, H. Y. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294, 2155–2158 (2001).
33. Fev, T., Robine, S., Louvard, D. & Huelsen, J. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol. Cell. Biol. 27, 7351–7359 (2007).
34. Lorenz, A. & Whitby, M. C. Crossover promotion and prevention. Biochem. Soc. Trans. 34, 537–541 (2006).
35. Sansom, O. J. et al. Loss of Apc in vivo immediately perturbs Wnt signalling, differentiation, and migration. Genes Dev. 18, 1385–1390 (2004).
36. Lustig, B. et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol. Cell. Biol. 22, 1184–1193 (2002).
37. Van der Flier, L. G. et al. The intestinal Wnt/Tcf7 signature. Gastroenterology 132, 628–632 (2007).
38. Merlos-Suarez, A. et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell. Stem. Cell. 8, 511–524 (2011).
39. Munoz, I. et al. The Lgr5 intestinal stem cell signature: robust expression of proposed quiescent ‘+ 4’ cell markers. EMBO J. 31, 3079–3091 (2012).
40. Huels, D. J. et al. E-cadherin can limit the transforming properties of activating beta-catenin mutations. EMBO J. 34, 2321–2333 (2015).
41. Song, J. H. et al. The APC network regulates the removal of mutated cells from colonic crypts. Cell Rep. 7, 94–103 (2014).
42. Bienz, M. & Hamada, F. Adenomatous polyposis coli proteins and cell adhesion. Curr. Opin. Cell Biol. 16, 328–335 (2004).
43. Metcalfe, C. et al. Dvl2 promotes intestinal length and neoplasia in the ApcMin mouse model for colorectal cancer. Cancer Res. 70, 6629–6638 (2010).
44. Laplum, H. et al. The type of somatic mutation at APC in familial adenomatous polyposis is determined by the site of the germline mutation: a new facet to Knudson’s ‘two-hit’ hypothesis. Nat. Med. 5, 1071–1075 (1999).
45. Albuquerque, C. et al. The ”just-right” signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. Hum. Mol. Genet. 11, 1549–1560 (2002).
46. Kadoch, C. & Crabtree, G. R. Mammalian SWI/SNF chromatin remodeling complexes and cancer: Mechanistic insights gained from human genomics. Adv. Sci. 1, e1500447 (2015).
47. Sansom, O. J. et al. Myc deletion rescues Apc deficiency in the small intestine. Nature 446, 676–679 (2007).
48. Bronstein, R. W. & Segal, D. Modularity of CHIP/LDB transcription complexes regulates cell differentiation. Fly (Austin) 5, 200–205 (2011).
49. Delidakis, C., Monastirioti, M. & Magadi, S. S. E(sp2): genetic, developmental, and evolutionary aspects of a group of invertebrate Hes proteins with close ties to Notch signaling. Curr. Top. Dev. Biol. 110, 217–262 (2014).
50. Tian, H. et al. Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis. Cell Rep. 11, 33–42 (2015).
51. Buczacki, S. J. et al. Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature 495, 65–69 (2013).
52. Ueo, T. et al. The role of Hes genes in intestinal development, homeostasis and tumor formation. Development 139, 1071–1082 (2012).
53. Sato, T. et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 470, 481–488 (2011).
54. van der Flier, L. G. et al. Interactions between Hes1 and Hes5 control Wnt signaling and increased expression of the stem cell marker Lgr5. Gut 59, 1680–1686 (2010).
55. Rowan, A. et al. APC mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. Proc. Natl Acad. Sci. USA 111, E4946–4953 (2014).
56. van de Wetering, M. et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111, 241–250 (2002).
57. Bienz, M. The subcellular destinations of APC proteins. Nat. Rev. Mol. Cell Biol. 3, 328–338 (2002).
58. Lewis, A. et al. Severe polyposis in Apc(1322T) mice is associated with potential risk stratification marker for PSA progression following radical prostatectomy. J. Clin. Pathol., https://doi.org/10.1136/jclinpath-2017-204718 (2017).
59. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308 (2013).
60. Lu, P., Kibbe, W. A. & Lin, S. M. lumi: a pipeline for processing Illumina microarrays. Bioinformatics 24, 1547–1548 (2008).
61. Ritchie, M. E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43, e47 (2015).
62. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14, 417–419 (2017).
63. Somes, C., Love, M. I. & Robinson, M. D. Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Res. 4, 1521 (2015).
64. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 (2014).
65. Yaari, G., Bolen, C. R., Thakar, J. & Kleinstein, S. H. Quantitative set analysis for gene expression: a method to quantify gene set differential expression including gene-gene correlations. Nucleic Acids Res. 41, e170 (2013).

Acknowledgements
We thank Michel Aguet, Jennifer Bulstine and Giese Ferrand for providing villin.CrePygo1/2 and Bcl6f/Bhlh DKO mice; Rogier ten Hoopen, Anna Nicholson and the CRUK Cambridge Institute Histopathology Core Unit for assistance with IHC; James Cruckshank and the Ares animal staff for mouse husbandry; Julien Bauer for assistance with microarrays and data analysis; Melissa Gammons for help with statistics; and Owen Sanson for discussion and sharing unpublished results. This work was supported by Cancer Research UK (C7379/A8709, C7379/A15291 and C7379/A24639 to M.B.) and the Medical Research Council (U105192713 to M.B.). A.E.K.I. was supported by a Clinician Scientist Fellowship from Cancer Research UK.

Author contributions
J.M. performed the tumour scoring and excisions, survival assays and microscopy; L.M.v. T. prepared the QKO samples and performed the biochemistry, RNA profiling and data analysis; A.E.K.I. generated the mouse strains, and prepared the DKO samples; D.J.W. contributed the Apc1332T model and helped with the histology; M.B. conceived and supervised the study, and wrote the manuscript with input from all authors.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-08164-z.

Competing interests: The authors declare no competing interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Journal peer review information: Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.