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Tripartite interactions between Wnt signaling, Notch and Myb for stem/progenitor cell functions during intestinal tumorigenesis

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Abstract Deletion studies confirm Wnt, Notch and Myb transcriptional pathway engagement in intestinal tumorigenesis. Nevertheless, their contrasting and combined roles when activated have not been elucidated. This is important as these pathways are not ablated but rather are aberrantly activated during carcinogenesis. Using *ApcMin*/+ mice as a source of organoids we documented their transition, on a clone-by-clone basis, to cyst-like spheres with constitutively activated Wnt pathway, increased self-renewal and growth and reduced differentiation. We then looked at this transition when Myb and/or Notch1 are activated. Activated Notch promoted cyst-like organoids. Conversely growth and propagation of cyst-like, but not normal organoids were Notch-independent. Activated Myb promoted normal, but not cyst-like organoids. Interestingly the Wnt, Notch and Myb pathways were all involved in regulating the expression of the intestinal stem cell (ISC) gene *Lgr5* in organoids, while ISC gene and Notch target *Olfm4* was dominantly repressed by Wnt. These findings parallel mouse intestinal adenoma formation where Notch promoted the initiation, but not growth, of Wnt-driven *Olfm4*-repressed colon tumors. Also Myb was essential for colon tumor initiation and collateral mouse pathologies. These data reveal the complex interplay and hierarchy of transcriptional networks that operate in ISCs and uncover a shift in pathway-dependencies during tumor initiation.

Introduction

Linear transcriptional pathways that govern intestinal tumorigenesis have been extensively explored in mice using gene knock outs (KO) and to a limited extent hypomorphs as well as chemical inhibitors (Aoki and Taketo, 2007; Barker et al., 2009, 2010; Batlle et al., 2005; Cheasley et al., 2011;

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Fre et al., 2005, 2011; Malaterre et al., 2007; Muncan et al., 2006; Sansom et al., 2007; Taketo, 2006). Specifically these studies on the Wnt, Notch and Myb pathways confirm a dependency on their various downstream target genes but do not necessarily address the cross-talk between these pathways when they are instead activated. An additional consequence of gene deletion studies is that changes in disease course can be due to the actual loss of the very cells that are normally subject to transformation. To address these gaps we have focused on these three transcriptional programs that are activated in intestinal cancers and that have been shown to interact with each other (Cheasley et al., 2011; Ciznadija et al., 2009; Fre et al., 2009). To do this we have generated compound transgenic and mutant mice that modulate all three pathways and followed the earliest events in stem/progenitor cell (ISC) transformation in vitro and in vivo.

ISCs are at the nexus of the hierarchical organization of the intestinal epithelium, producing cells which give rise to differentiated enterocytes, enteroendocrine cells, Paneth cells, goblet cells, Tuft cells and Peyer's Patch M cells (Barker et al., 2010). To guarantee the lifelong maintenance of the tissue, the ISC/progenitor cell compartment has to be tightly regulated by several molecular pathways that control ISC self-renewal, proliferation and differentiation.

The Wnt pathway is active in ISCs and is essential for their self-renewal (Korinek et al., 1998) as well as Paneth cell differentiation (Andreu et al., 2005; Farin et al., 2012; van Es et al., 2005a). Activation of the Wnt pathway leads to stabilization of cytoplasmic and nuclear β-catenin, which interacts with other proteins to induce the expression of genes such as *Lgr5* and *Ascl2* in ISCs (Barker et al., 2007; van der Flier et al., 2009), *Myc* and *Ccnd1* (Andreu et al., 2005), as well as *Cryptidin*, and *Lysozyme* genes in Paneth cells (Andreu et al., 2005; van Es et al., 2005a). Similarly, the Notch pathway (Kopan and Ilagan, 2009) is essential for ISC self-renewal and differentiation (Pellegrinet et al., 2011).

Notch is clearly active in ISCs (Fre et al., 2011; Smith et al., 2012) and stimulates proliferation (Fre et al., 2005, 2009) while blocking secretory cell differentiation (Fre et al., 2005, 2011; van Es et al., 2005b). Furthermore, Notch is a direct activator of the ISC gene *Olfm4* (VanDussen et al., 2012) and the bHLH transcriptional repressor Hes1, which in turn blocks expression of the driver of secretory cell differentiation, Math1 (Jensen et al., 2000; Yang et al., 2001). The co-operation between Notch and Wnt in the intestine results in the amplification of Wnt-driven proliferation and Wnt-driven tumor formation is potentiated by constitutive Notch activation (Fre et al., 2009). Conversely, Wnt target genes are repressed by Notch signaling and negatively correlate with the Notch target gene *Nrarp* expression in CRC (Kim et al., 2012).

Myb is additionally active in ISCs where it is essential for self-renewal and blockade of goblet cell differentiation (Cheasley et al., 2011; Malaterre et al., 2007). Furthermore, we have demonstrated that Myb is crucially involved in the regulation of the Wnt target genes *Myc* and *Lgr5* in ISCs (Cheasley et al., 2011; Ciznadija et al., 2009). Myb is over-expressed in human CRC (Ramsay and Gonda, 2008) and *Myb* heterozygous deletion prolongs survival of intestinal cancer-prone *Apc* mutant mice (Ciznadija et al., 2009), indicating that Myb is involved in Wnt-dependent intestinal tumor formation.

Given these reported network interactions between Wnt, Notch and Myb in ISCs and CRC, we considered it important to explore their combined roles in controlling intestinal homeostasis and tumor formation. Thus far these three pathways have been investigated individually or perhaps in dual combinations and therefore this tripartite hierarchy remains uninvestigated. We found that Wnt, Notch and Myb activation had distinct effects on key ISC gene expression patterns, that the effects of full Wnt activation were dependent upon functional Myb expression, co-operated with activated Notch and were resistant to Notch inhibition. These data set the stage to hierarchically order the roles of these three pathways in ISC function and in intestinal transformation.

Materials and methods

Mice

VilCreERT2/NotchIC mice (Fre et al., 2009) and *ApcMin*/+ (Min) mice (Moser et al., 1990) have been described previously. Myb heterozygous knock-out mice and their intestinal defects have been described elsewhere (Ciznadija et al., 2009). MybER mice contain a transgene encoding a Tamoxifen-inducible fusion protein between Myb and the ERα-ligand binding domain driven by the intestinal-specific promoter, gpa33 (Malaterre et al., 2007). All mice were maintained on a C57/BL6 background. To activate MybER or VilCreERT2 proteins, mice were fed for seven days with chow containing Tamoxifen (Sigma, Castle Hill, Australia) to a total of 1 g/kg body weight. Min interbred mice were observed for any signs intestinal bleeding or ill health according the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee regulations. Sick mice were killed and their colon and SI were evaluated for the presence and size of adenomas and fixed in 10% neural buffered formalin.

Organoid culture

Briefly, 1000–2000 crypt nests were seeded in 50 μl Phenol red-free Matrigel (BD Biosciences, North Ryde, Australia) and over-layed with 500 μl of specific medium as described (Cheasley et al., 2011; Sato et al., 2009) including 100 nM of 4-Hydroxy-Tamoxifen (4-OHT, Sigma). Primary (1°) organoids were scored on day 1 and organoids (with at least one crypt-like projection) on day 7 after seeding. Organoid colony formation efficiency was calculated as ratio of day 1/day 7 scores. Organoid growth was measured using an MTT assay as described (Cheasley et al., 2011). Established organoids were extracted from Matrigel using Dispase (BD Biosciences) and dissociated using Accutase (Accumax, Merck Millipore, Kilsyth, Australia) followed by trituration with a fine polished glass pipette. Cell suspensions were seeded in Matrigel as described above in the presence of Rho-kinase inhibitor (Sigma) to prevent anoikis (Sato et al., 2009). Formation of secondary (2°) organoids was scored after 8 to 10 days. Self-renewal capacity was calculated as number of 2° organoids formed/number of dissociated 1° organoids used for 2° culture. To block Notch signaling, primary organoids were treated with Dibenzazepine (DBZ, Calbiochem, Merck Millipore) or vehicle alone (DMSO, Sigma) one day after seeding with addition of fresh DBZ or DMSO for the next three

consecutive days. 1° and 2° organoids were either fixed in 4% paraformaldehyde and embedded in agarose for immunohistochemistry or lysed in Trizol for RNA/DNA extraction.

Reporter assays

pCATbasic reporter constructs containing the human *Myb* or *Myc* promoters, as well as plasmids encoding for full-length MYB or Δ89-β-catenin have been described elsewhere (Ciznadija et al., 2009). The pBabe-N1IC vector was generated by cloning the cDNA (residues 1759–2556), encoding mouse intracellular Notch1 into the pBabePuro vector. HEK293 cells were transfected with plasmids using X-tremeGENE9 reagent and CAT expression was measured by a CAT ELISA kit (Roche, Castle Hill, Australia).

Immunohistochemistry and morphometric analyses

Immunohistochemical staining was carried out on sections of fixed, paraffin embedded tissues or organoids. Antibodies are listed in Supplemental information Table S1. Sections were dewaxed and blocked for endogenous peroxidase with 3% hydrogen peroxide prior to antibody incubation. Sections were counterstained with Periodic acid/Schiffs (PAS) reagent to mark secretory cells and with Hematoxylin and Eosin and images captured using an Olympus BH-2 microscope.

Real-time PCR and candidate gene expression

RNA was extracted from samples stored in Trizol (BioRad, Gladesville, Australia) according to the manufacturer's instructions. cDNA synthesis was carried out after DNase treatment of RNA (RQ1 DNase, Promega, Alexandria, Australia) using M-MLV, RNaseH-reverse transcriptase and random hexamer primers (Promega). Real-time PCR was carried out on a StepOne thermal cycler (Life Technologies, Mulgrave, Australia) using gene specific primers (Supplemental information Table S1) and Fast SYBR Green (Life Technologies).

Apc allelic distribution assay

Individual organoids were picked under a dissecting microscope, incubated in lysis buffer (5 mM Tris–HCl pH 8.8; 2.5 mM $(NH_4)2SO_4$; 1 mM $MgCl_2$; 0.5% Triton-X100 and 0.4 mg/ml Proteinase K) for 60 min at 55 °C, and PCR amplification was performed using cell lysates as template. Amplified products were digested with HindIII and separated on 10% acrylamide gels for detecting *ApcWT* and *ApcMin* alleles as described previously (Moser et al., 1992).

Statistical analysis

Statistical analysis was performed with GraphPad Prism Version 6 (GraphPad Software Inc., La Jolla, USA). Differences between groups were analyzed by t-test. When distribution of values per group did not pass the KS normality test, the Mann–Whitney test was used. When variances were significantly different (F-test) a t-test with Welch's correction was used.

The Supplementary information section accompanies the paper on the *Stem Cell Research* website.

Results

Demonstrating pathway activation in vitro

The SI organoid culture method has become an invaluable in vitro tool to investigate intestinal cell biology particularly when this approach is combined in parallel with animal studies (Sato et al., 2009). By doing so, it has been demonstrated by gene loss or mutation that the Wnt, Notch and Myb pathways are essential for crypt homeostasis, organoid formation and ISC function (Cheasley et al., 2011; van Es et al., 2012; VanDussen et al., 2012). Here, we investigated whether converse effects can be observed when these pathways are activated alone and then in combination. To do this we employed mice carrying a mutation in one allele of the *Apc* gene (Min) (Moser et al., 1990), an intestinal specific, inducible-form of Myb (MybER) (Ramsay et al., 2005; Malaterre et al., *manuscript submitted*) and an intestinal-specific, inducible model employing the Notch intracellular domain (NotchIC) (Murtaugh et al., 2003) as a source of crypts to establish organoid culture.

Establishing an in vitro model for progressive activation of Wnt signaling

To avoid any differences in ISC stromal influences from one mouse line to another we first addressed the effects of activated pathways on an individual basis using SI organoid cultures with the same reagents. Also note that all mice were breed onto a C57BL/6 background for at least 10 generations. As Wnt activation is generally accepted as a feature of most intestinal cancers and core to ISC function we began with the Wnt pathway finding as expected that primary (1°) SI organoids from Min mice were indistinguishable from matched WT cultures in terms of expression of Wnt targets *Ascl2* (van der Flier et al., 2009) and *Ccnd1* (Andreu et al., 2005) (Figs. 1A and B).

First we established an assay that allows differential RFLP PCR amplification of *ApcWT* and *ApcMin* alleles in crypts and adenoma DNA (Fig. 1C). We extracted DNA from individual organoids to show they were genotypically heterozygous for *Apc* alleles (Fig. 1D). However, it was also evident that a small proportion of crypt nests gave rise to cyst-like structures and these were most prominent when primary organoids (Min 1°) were dispersed and replated to form secondary organoids (Min 2°). Under these conditions most had lost the *ApcWT* allele (Fig. 1D). When total RNA was isolated from the secondary cultures and examined for *Ascl2* and *Ccnd1* expression it was now clear in these that the Wnt pathway was activated (Figs. 1A and B).

To explore the transition from 1° organoids to 2° cyst-like cultures in greater detail we performed morphological, immunohistochemical (IHC) and gene expression studies. A substantial number of Min 1° organoids displayed a cyst-like

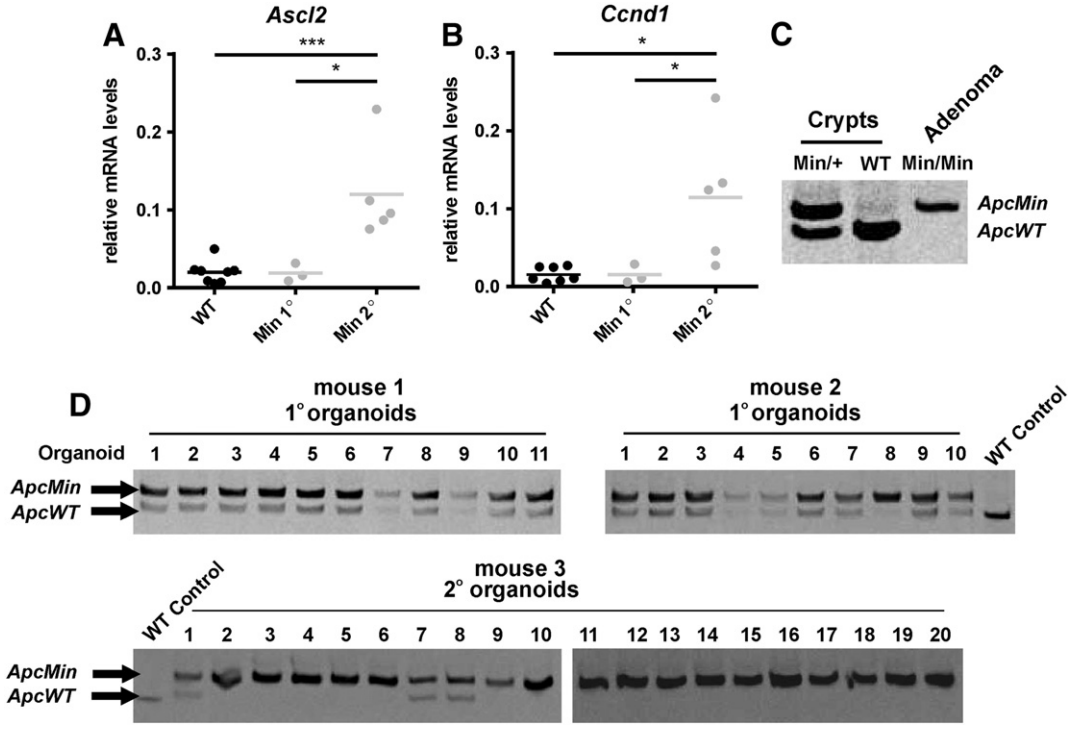

Figure 1 Loss of the *ApcWT* allele in Min organoids leads to immediate Wnt activation in vitro. (A–B) Passaging of primary (1°) into secondary (2°) SI intestinal organoid cultures leads to immediate expression of Wnt target genes, Ascl2 and Ccnd1 respectively compared to WT SI organoids. Mean ± SEM; *$p < 0.05$, $n > 3$. (C) A PCR-based assay identifies the *ApcMin* and *ApcWT* alleles in SI crypts from Min and WT mice and in adenomas from Min mice. (D) Individual organoids established from three Min mice were processed to determine the status of the *Apc* alleles.

structure (Fig. 2A). Normal organoids initially form small spheres and mainly grow by budding and expansion of crypt-like structures from these spheroids (Clevers and Nusse, 2012). By contrast and despite partial initiation of budding, cyst-like organoids did not contain crypt-like structures, but rather retained their spherical shape during growth (Figs. 2A and B).

Cyst-like organoids have previously been described to form from SI cells with imposed *ApcWT*-loss (Jarde et al., 2013), when the Wnt/β-catenin pathway was hyperactivated in vitro (Onuma et al., 2013; Sato et al., 2011b) or from intestinal adenoma derived cells (Sato et al., 2011a). Cyst-like organoids displayed strong staining for cytoplasmic and nuclear β-catenin and an absence of PAS+/goblet-like cells (Figs. 2C and D). This phenotype was more pervasive in Min 2° organoids and indistinguishable from organoids derived directly from adenomas in Min mice (Supplementary Fig. 1). We consistently observed some cyst-like organoids also in Min 1° organoid cultures (Fig. 2A). Nevertheless, in our hands, all single Min 1° organoids still contained cells harboring the *ApcWT* allele, but the overall abundance of the *ApcWT* allele was still lower than the *ApcMin* allele (Fig. 1D). This was based upon quantitative gel scanning where the ratio of the *ApcMin* allele to the *ApcWT* allele was mostly <1.2 in the primary organoid cultures while in the secondary organoids the *ApcMin* allele predominated and thus the ratio was >1.5.

After dissociation and passaging, the 2° organoids formed were predominantly cyst-like (Fig. 2A) and negative for the *ApcWT* allele (Fig. 1D). It was thus apparent that there was a selective advantage for cells initiating cyst-like organoids over cells initiating conventional-looking organoids on replating as secondary cultures (Fig. 2E). This assay is essentially a test of self-renewal capacity and was investigated in more detail as described below. In accord with the fact that *ApcWT*-loss results in constitutive Wnt pathway activation (Korinek et al., 1997; Sansom et al., 2004), Min 2° organoid cultures with a high percentage of cyst-like organoids displayed an increase in the Wnt target and ISC marker gene *Lgr5*, but a surprising and significant decrease in *Olfm4*, while the relatively low *Bmi1* or *mTert* expression remained unchanged (Fig. 3). Stem cell gene expression signatures in Min 1° organoids was indistinguishable from WT 1° organoids (*data not shown*).

Taken together our results suggest that cyst-like Min 2° organoids arise from Olfm4-negative cells with Wnt hyperactivation through loss of *ApcWT*. Furthermore, the general expansion of cyst-like organoids and cells with *ApcWT*-loss in 2° cultures implies that the cells initiating cyst-like growth have a very high organoid re-forming and, thus, self-renewal capacity. Hyperactivation of the Wnt pathway in concert with an expansion of ISC-like cells with high proliferative potential is also observed in intestinal adenomas formed following loss of *ApcWT* or constitutively active β-catenin in mice in vivo (Barker et al., 2009; Moser et al., 1992; Schepers et al., 2012; Schwitalla et al., 2013). Further, Apc-inactivated cyst-like organoids are tumorigenic when injected into mice (Onuma et al., 2013). Thus,

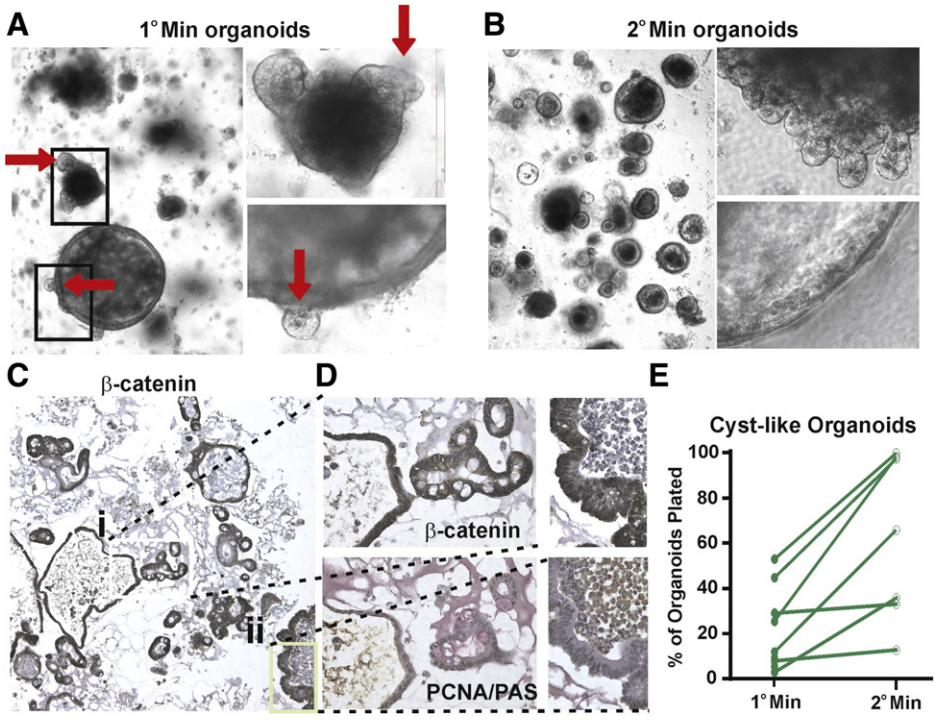

Figure 2 Characterization of primary (1°) and secondary (2°) organoids derived from Min mice. (A) Primary Min organoids generate either characteristic morphologies indistinguishable from those of WT organoids or cyst-like organoids. Budding was most evident in normal organoids and only to a minor extent in cyst-like organoids (red arrows). (B) Cyst-like organoids become predominant in secondary cultures. Few retain minimally budding structures (upper panel), but the majority grows completely as spheres (lower panel). (C–D) Cyst-like organoids show intense nuclear β-catenin staining compared to membrane staining in those organoids which also had persistent mucin staining detected by PAS histochemistry. (E) Cyst-like organoids arise preferentially when passaged individually to secondary cultures. Each dot represents organoid culture from one mouse and its derived secondary organoid culture, $p = 0.008$; paired t-test.

these cyst-like organoids in culture recapitulate the earliest processes of *ApcWT*-loss and adenoma formation in vitro.

Notch and Myb activation have distinct effects on organoid formation

Having defined the conditions under which Wnt pathway activation could be readily monitored we then explored the effects of activating Myb and Notch. To test the effect of Myb activation SI crypt nests derived from MybER and NotchIC:VilCreERT2 mice were cultured in the presence of 4-hydroxytamoxifen (4OHT). These were then compared in their respective abilities to form organoids and to WT and Min mice-derived SI crypt nests (1° organoids). MybER organoid formation was superior to the other genotypes (Fig. 4A) while only Min organoids showed significantly greater self-renewal (Fig. 4B) commensurate with the generation of cyst-like colonies which we showed above to be responsible for this increased 2° organoid formation. Interestingly, the combined genotypes in the *MybER/Min/+*organoids do not lead to increased organoid formation above that observed in WT cultures.

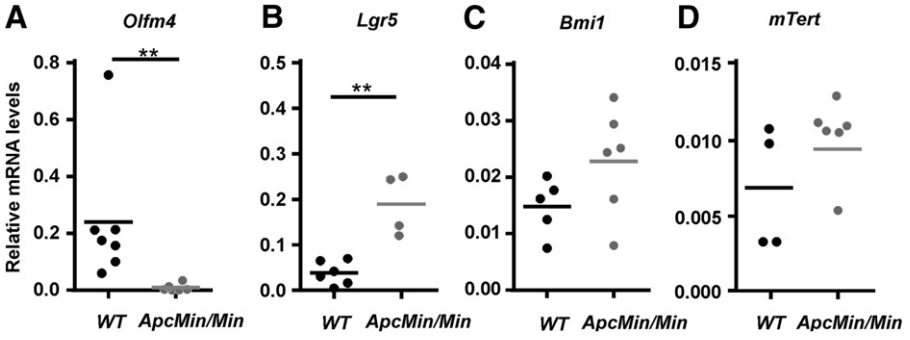

Figure 3 Activation and suppression of ISC genes occur following loss of the *ApcWT* allele in Min organoids. (A–B) mRNA levels of the ISC marker gene *Olfm4* reduced while *Lgr5* is increased in *ApcMin/Min* organoids compared to *WT* organoids. (C–D) Expression of other ISC genes *Bmi1* and *mTert* was not significantly changed (*: $p < 0.05$; **: $p < 0.01$).

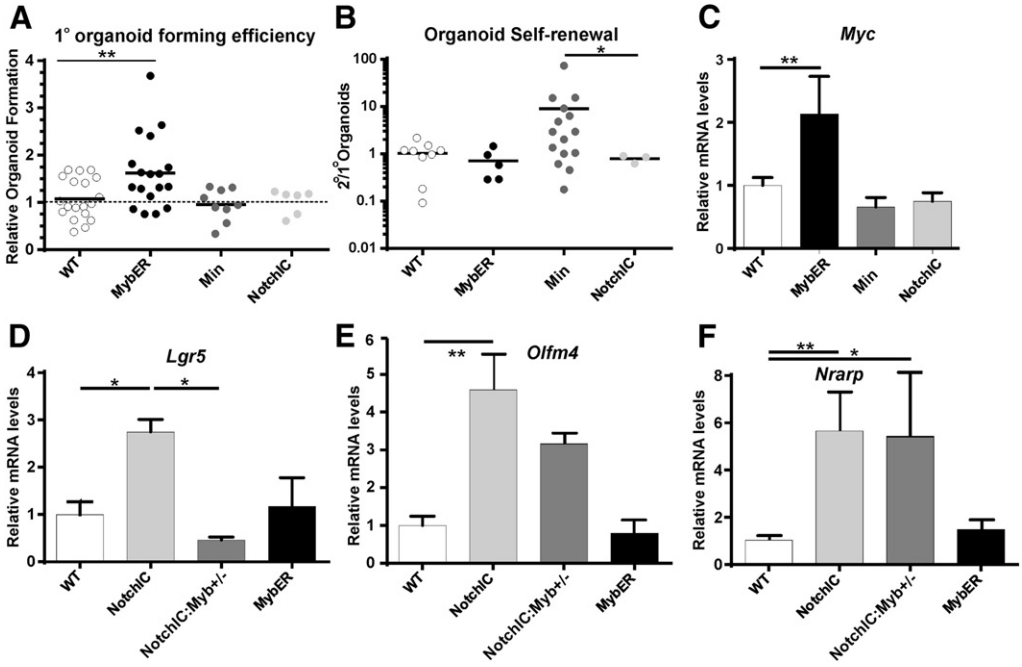

Figure 4 Activation of Myb and Notch differentially promotes organoid formation and target gene expression. (A) Activated MybER increases the relative organoid forming efficiency compared to WT, Min and NotchIC cultures; all cultures were exposed to 4OHT. Organoid formation was calculated as organoids formed on day 7 per crypt nest present on day 1 of culture. (B) 2° organoid formation is significantly increased in *Min* cultures. Self-renewal capacity of organoids determined by the number of 2° organoids formed when 1° organoids were dissociated into single cells and replated; each dot represents one mouse and values were derived from at least 5 independent experiments. (C) Expression of *Myc* increased significantly in primary MybER cultures. (D) NotchIC activates *Lgr5* expression and this is compromised when one allele of Myb is lost. (E–F) Notch target genes *Olfm4* and *Nrarp* are increased in NotchIC organoids but essentially unaffected by Myb heterozygosity. (*: $p < 0.05$: **: $p < 0.01$).

In 1° organoids expression of *Myc* was induced above WT only in the MybER cultures (Fig. 4C) even though *Myc* has been reported to be a NotchIC target gene (Efstratiadis et al., 2007; Klinakis et al., 2006) and of note a Wnt target gene (He et al., 1998). By contrast, NotchIC organoids showed significantly higher *Lgr5*, *Olfm4* and *Nrarp* expression compared to the others (Figs. 4D–F). As reported previously normal Myb function is required for *Lgr5* expression even when Wnt activation is robust (Cheasley et al., 2011) and similarly hyperactivated-Notch still appears to require normal levels of Myb expression to allow it activate *Lgr5* expression (Fig. 4D).

To begin to understand the interaction between Notch and Wnt in vitro we investigated the effect of the γ-secretase inhibitor DBZ in the context of Min organoid formation. We immediately noted that in the presence of the relatively low dose of 0.1 μM DBZ that normal-appearing organoids were essentially absent while cyst-like organoids were predominant (Figs. 5A and B). In these 1° organoid cultures it was also apparent that cyst-like organoids remained resistant to higher DBZ concentrations (Fig. 5C). We then confirmed that these DBZ-resistant organoids were of an *ApcMin/Min* genotype in three different mice (Fig. 5D) and were at a selective advantage compared to *ApcMin*/+organoids (Fig. 5E). They also retained the high self-renewal potential of unselected cyst-like organoids (Fig. 5F). In contrast to *ApcMin/Min* MybER did not circumvent Notch-dependence for self-renewal (Fig. 5F). Conversely, the presence of NotchIC doubled the percentage of cyst-like organoids (Fig. 5G). Collectively these data indicate that while normal Notch signaling is essential to normal organoid formation Min/Min cyst-like organoids are resistant to γ-secretase inhibition and therefore appear independent of Notch signaling. Conversely, the activation of Notch increases cyst-like organoid generation.

Finally, with regard to the effects of lineage specification in the context of different pathway activation we focused on goblet cells because they are readily quantifiable in SI tissue sections. Overall, we found that activation of Notch and Myb reduced goblet cell frequency in the SI (Supplementary Fig. 3), while *ApcMin*/+ are indistinguishable from WT until the second *ApcWT* allele is converted to the *ApcMin* allele.

Myb and Notch are involved in Wnt-mediated colon adenoma initiation

Activating mutations within the Wnt pathway are found in the majority of CRC patients (Kinzler and Vogelstein, 1996; Powell et al., 1992; Sparks et al., 1998). The Min/+ mouse model has been exploited and studied as an analogue to Familial Adenomatous Polyposis (FAP), a heritable disease where patients develop multiple colon polyps. Here we crossed several mouse lines to determine the interactions between the Wnt activation and the two other transcriptional programs in the intestines to evaluate how these might affect the course of tumorigenesis.

In agreement with our previous studies (Ciznadija et al., 2009; Fre et al., 2011) in Apc deficient mice, Myb deficiency

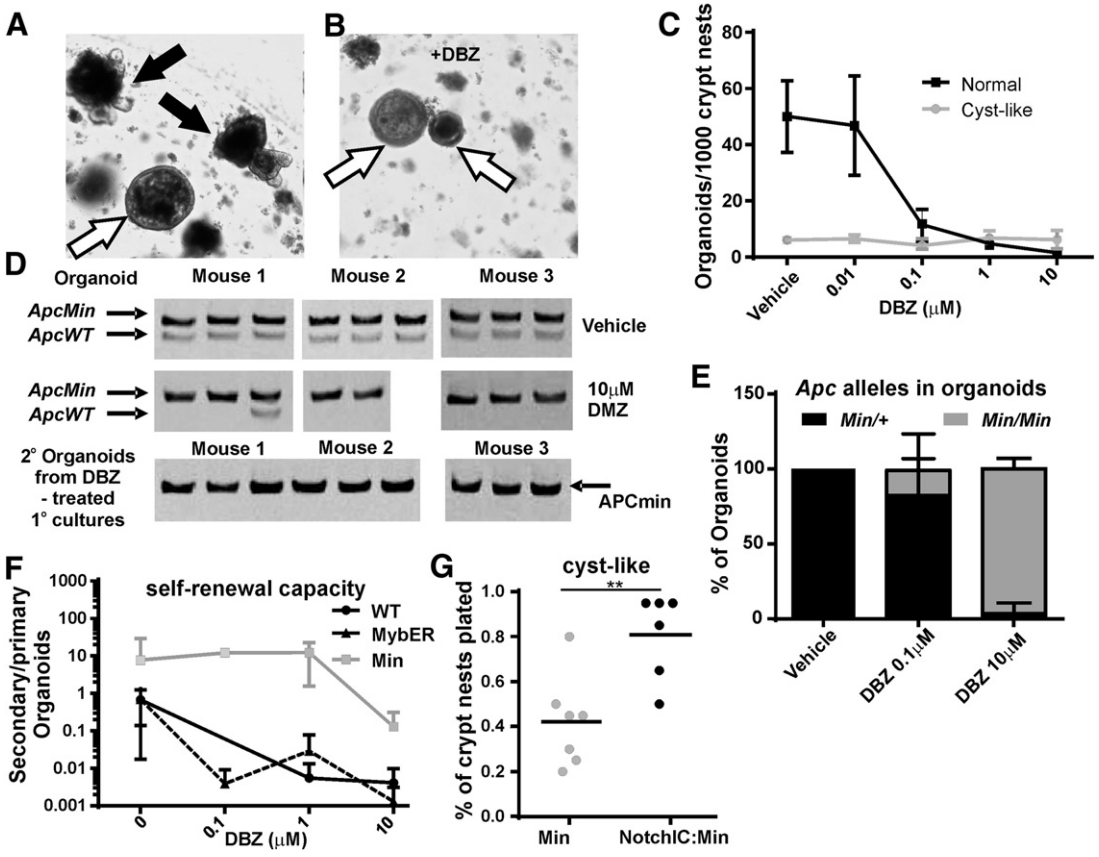

Figure 5 Notch activation selectively favors cyst-like organoid formation, but is dispensable for their propagation. (A) SI organoids from Min mice were treated with different concentrations of DBZ. Organoid formation and *Apc* allelic distribution were assessed at day 7 to day 8 after seeding. Normal (black arrows) and cyst-like (white arrows) organoids were observed in situ under vehicle and low dose (1 nM) DBZ treatment (B) but only cyst-like organoids were observed under a high dose (1 μM) DBZ treatment (n = 5 mice, 2 independent experiments). (C) Number of normal versus cyst-like organoids per 1000 crypt nests plated that formed under increasing concentrations of DBZ. (D–E) Allelic distribution was assessed in single Min organoids +/− DBZ treatment (mice: n = 3). (F) Self-renewal capacity of vehicle and DBZ treated WT, MybER and Min organoids. (G) NotchIC induces increased cyst-like growth in Min organoids (each dot represents one mouse and values were derived from at least 5 independent experiments *: $p < 0.05$; **: $p < 0.01$).

increased, while NotchIC activation decreased survival. Somewhat surprisingly, survival of *NotchIC*:*Min*:*Myb*+/− mice was not extended compared to *NotchIC*:*Min* mice (Fig. 6A), *Min*/+ mice spontaneously develop intestinal polyp-like adenomas and far more frequently in the SI compared to the colon (Moser et al., 1992). In this regard we found no differences in SI or cecal adenomas between the 4 lines investigated (*data not shown*) even though, the

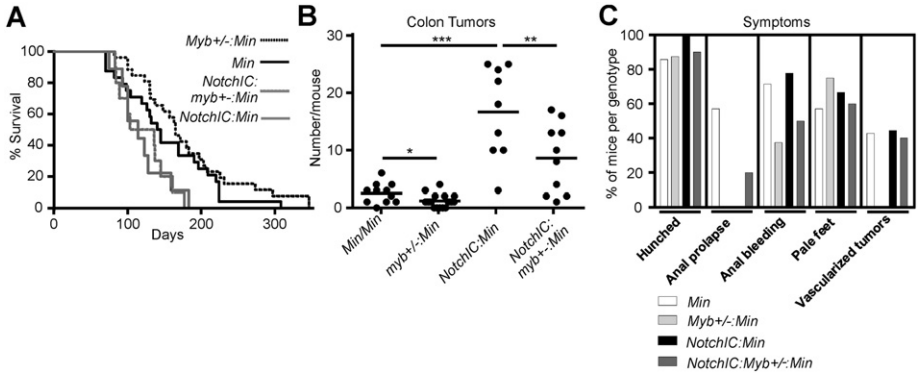

Figure 6 Myb and Notch status influences colon adenoma formation in Min mice. (A) Kaplan–Meir symptom-free survival of mice of the indicated genotypes is shown. (B) Number of colon adenomas at the time of sickness-related culling. (C) Percentage of mice of each genotype showing indicated symptoms of intestinal adenoma associated sickness at time of sickness related death. (*: $p < 0.05$; **: $p < 0.01$, ***: $p < 0.001$, ns: not significant).

number of colon adenomas was substantially increased from an average of three per *Min* mouse to an average of eleven per *NotchIC*:*Min* mouse. Analysis of markers of NotchIC-activating recombination events (Supplementary Fig. 2A) revealed that the majority of colon adenomas in *NotchIC*:*Min* mice originated from NotchIC-activated cells (Supplementary Figs. 2B and C). However, *NotchIC*:*Min* adenomas, as well as *Min* adenomas, displayed absence of *Olfm4* expression (Supplementary Fig. 2D). The opposite occurs in *Min*:*Myb*+/− mice where there was a significant reduction in colon adenomas (~1 per mouse) (Fig. 6B) while the number of SI adenomas remained essentially unchanged (*data not shown*) noting that such adenomas must have lost their *ApcWT* allele and canonical Wnt signaling is thus constitutively active (Korinek et al., 1997; Luongo et al., 1994). Nevertheless it would seem that Myb-heterozygosity has a specific or tissue tropism influencing colon adenoma formation (Fig. 6B). Interestingly the presence of a single colon adenoma often precipitates culling a sick mouse even though the same mouse may have multiple SI adenomas (*data not shown*) thus, the decreased tumor load in Myb-heterozygous mice did not translate into a significant survival benefit (Figs. 5A and B).

Investigating pathway cross-talk at a transcriptional level

In view of the apparent genetic interaction between Notch and Myb we investigated transcript levels of target genes of either pathway when the other transcription factor was activated. As described above, mRNA expression of the Notch target genes *Olfm4* and *Nrarp* was significantly increased in NotchIC organoids (Figs. 4E and F). This was independent of Myb+/− (Figs. 4E and F) or MybER and there was no significant effect on *Myb* mRNA expression itself or its target gene *Ccne1* (Malaterre et al., 2007) (Supplementary Figs. 4A–C). We then tested whether NotchIC influences the transcriptional activity on the human *Myb* promoter using a reporter assay approach similar to our previous studies (Cheasley et al., 2011; Ciznadija et al., 2009). To confirm that the comparable NotchIC vector was active we transfected HEK293 cells and assessed Notch-endogenous target gene *Hes5* expression which was significantly increased in a concentration-dependent manner (Supplementary Fig. 4D). By contrast, *Hes1* expression was not increased (*data not shown*). Under comparable conditions NotchIC did not activate a reporter construct containing the human *Myb* promoter (Supplementary Fig. 4E). Further, NotchIC did not activate the *Myc* promoter by itself and did not enhance its activation through co-operation with Myb and/or β-catenin (Supplementary Fig. 4F). Therefore, it appears that Myb is not a direct downstream target of Notch activation and that the apparent pathway cross-talk was not due to direct activation of respective transcription factor genes.

Clinical features in adenoma-bearing mice are influenced by Myb heterozygosity

The ethical determinants for humane management of mice mean that mice are sacrificed for a spectrum of reasons. When we assessed the clinical features that underpinned culling of mice on a Min background we found that mice that were heterozygous of Myb had reduced levels of, or were spared from, some pathological symptoms such as bleeding and the presence of vascularized tumors (Fig. 6C). Similarly, these same symptoms are exacerbated in MybER-mediated colon tumors (Malaterre et al., submitted).

Taken together, these results suggest that Notch and Myb are both crucially involved in initiation of Wnt-pathway-dependent colon adenomas, while their activation does not seem to be essential for their further growth and maintenance. This is in agreement with Notch being dispensable for the intestinal hyperplasia once established following *ApcWT* allelic loss (Peignon et al., 2011) and that fully functional Myb is required for intestinal stem cell activity (Cheasley et al., 2011) and finally clinical features typically observed in CRC patients (Malaterre et al., submitted).

Discussion

In summary, we have demonstrated that using an in vitro model of *ApcWT*-loss and using in vivo intestinal cancer model that activated Wnt, Notch and Myb are all essential to intestinal tumorigenesis. We systematically dissected within the same experimental milieu the influence of the Wnt, Notch and Myb pathways on optimal formation and growth of ISC intestinal organoids (or: self-renewal and proliferation of ISC) in vitro. Using this approach we deconstruct here how these three pathways interact during the earliest event of CRC, the initiation of intestinal adenomas, and report a remarkable shift in their functional and transcriptional interaction between ISCs and tumor-initiating cells.

Loss of the *ApcWT* allele in intestinal cells in organoid culture induces Wnt target genes and high self-renewal potential in these cells upon which they give rise to neoplastic growth. This manifests in cyst-like organoids with aberrant tissue architecture, cell differentiation and proliferation. In vivo loss of *ApcWT* in ISC leads to immediate induction of Wnt signaling and rapid expansion of ISCs resulting in aberrant tissue architecture and secretory cell differentiation typical of early intestinal adenomas in mice (Barker et al., 2009; Sansom et al., 2004). However, these studies used mice with an induced simultaneous loss of both *Apc* alleles, thus circumventing events that influence loss of Apc function first hand. Therefore, the spontaneous formation of cyst-like organoids genuinely reflects the initiating step in intestinal adenoma formation and represents a unique window to investigate cellular and molecular determinants of this first step in CRC. Our data suggest that the cyst-like *ApcMin*/*Min* organoids arise from an expansion of cells positive for the ISC marker Lgr5, but negative for the ISC marker Olfm4, a detail we find reflected in *ApcMin*/*Min* adenomas. This is in contrast to Olfm4-positive adenomas formed through *Apc* loss in Lgr5-positive cells (Myant et al., 2013; Schepers et al., 2012), suggesting that spontaneous adenomas in Min mice do arise through a mechanism differing from induced *ApcWT*-loss in ISCs.

A benefit of the organoid in vitro system is that it leaves out interactions of epithelial cells with stromal cells (Akcora et al., 2013; Barker et al., 2007), which can distort the detection of epithelial cell-specific signaling pathway interactions in vivo. Using this approach we wanted to know how the Notch

and Myb pathways interact with Wnt signaling during the transition of ISC to tumor-initiating cells. We confirm findings of previous studies that loss-of-function of Notch (Pellegrinet et al., 2011; VanDussen et al., 2012) and Myb (Cheasley et al., 2011) strongly disturbs ISC self-renewal in vivo and in vitro. Surprisingly, we find that gain-of-function in these pathways does not increase self-renewal, suggesting that either both pathways alone are not sufficient to drive self-renewal, or their maximal capacity to do so, is limited and cannot be further increased in organoids.

Importantly we found that Notch activation increases the number of isolated Min crypts that process to cyst-like organoids, while Notch activation or inhibition does not affect growth or self-renewal once the cyst-like growth has been initiated. This is a striking parallel to the situation in vivo, where Notch activation increases the number of Wnt driven adenomas, but does not affect colon adenoma growth thereafter (Fre et al., 2009; Peignon et al., 2011). Intriguingly, we did not find any significant differences in SI adenoma formation across the genotypes on an *Apcmin/+* background and we think this is because the activation of the Wnt pathway in the SI swamps out the effects of the other pathways. However, Notch activation and MybER had a significant effect in the colon in terms of adenoma formation and as colon cancer is approximately 10 times more common in humans we suggest that this was a more relevant matter to pursue. One explanation for this phenomenon is that Notch activation increases the number of tumor-initiating cells upon *ApcWT*-loss in the colon. This arguably can happen on three functional levels where we know aberrant Wnt activity affects initiation of intestinal adenomas: either by symmetric division of ISCs (Bellis et al., 2012), by the induction of a SC program in non-ISCs (Schwitalla et al., 2013) or by influencing the number of cells with *ApcWT*-loss able to clonally expand (Vermeulen et al., 2013). Notch activation leads to increased expression of the ISC markers Lgr5 and Olfm4, while the Lgr5-positive/Olfm4-negative phenotype of *ApcMin/Min* cyst-like organoids remains the same when Notch is activated and this is replicated in adenomas. This indicates that Notch-activation can affect the ISC phenotype, but does not affect the phenotype of tumor-initiating cells. Apparently the mechanism of how Notch influences adenoma initiation is complex and further studies will be necessary to elucidate it.

In contrast to Notch Myb activation does not affect cyst-like organoid formation, although MybER increases the percentage of isolated crypts capable of forming an organoid and increases adenoma formation in vivo (Malaterre et al., *manuscript submitted*). Interesting in this context is that MybER induces *Myc* expression in organoids, a gene which is essential for Wnt driven adenomas (Sansom et al., 2007). Myb deficiency on the other hand decreases the number of adenomas, both if Wnt alone or Wnt and Notch are hyper-activated, and decreases normal organoid formation (Cheasley et al., 2011). Although Myb expression in ISCs is clearly important for their function, it becomes evident that the role of Myb in intestinal tumor formation cannot be purely intrinsic to tumor cells and may involve cells within the tumor environment not present in organoid culture. In fact we recently found that Myb expression in intestinal tumor cells affects tumor vasculature (Malaterre et al., *manuscript submitted*), which further might explain the differences of symptoms associated with intestinal bleedings that we describe here for Myb-heterozygous tumor bearing mice.

We previously showed that Myb interacts with Wnt signaling to regulate Lgr5 expression (Cheasley et al., 2011). Here we demonstrate for the first time that Notch-activity also regulates Lgr5 expression and that Myb is involved in this. Our results nevertheless suggest that Notch does not achieve this through regulating Myb and Wnt activity, as in organoids Notch does not appear to influence other classic Myb and Wnt target genes or vice versa. Therefore, although all three pathways are known to activate distinct target genes, in organoids they combine on the regulation of two things: ISC function and activation of Lgr5 expression. Lgr5 itself is a co-regulator of Wnt signals (Carmon et al., 2011; Ruffner et al., 2012) and it is tempting to speculate that Lgr5 at least partially mediates the promotion of ISCs through Wnt, Notch and Myb. Furthermore, induction of Lgr5 expression through Wnt hyperactivation could result in a positive feedback loop, which might partially explain the independence from Notch and Myb signals in *ApcMin/Min* cells.

In conclusion, we demonstrate here that the Wnt, Notch and Myb pathways are essential for ISC function and co-regulate Lgr5 expression. Nonetheless, only hyperactivation of Wnt leads to the transformation of ISCs to tumor-initiating cells, at the same time rendering them independent of Notch and Myb signals (see Graphical abstract). The clinical consequence of this is that Notch targeting therapies, such as γ-secretase inhibitors, might at best have a preventive role for CRC, but may fail in later stages of the disease. Furthermore, our data show that blocking Notch in vitro selectively kills normal ISC, sparing adenoma-like cells. Thus these findings raise an important concern about the possible benefits of some Notch inhibiting therapies for established CRC. Myb, on the other hand, is over-expressed in the majority of CRCs (Ramsay and Gonda, 2008) and we have previously shown that the Myb oncoprotein is a valid therapeutic target both in preventive and adjuvant settings (Carpinteri et al., 2012; Williams et al., 2008). This discrepancy might be explained by the possibility that targeting Myb is not solely confined to tumor cells, but also on their interaction with the tumor environment. Collectively, the results presented here highlight that the Wnt pathway remains as the focal point of intervention to target tumor initiating and tumor sustaining cells, which should be considered for CRC therapy.

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Author contribution

MG: concept and design, collection and assembly of data, data analysis and interpretation and manuscript writing. HX: collection and assembly of data, data analysis and interpretation. JM: collection and assembly of data, data analysis and interpretation. SS: collection and assembly of data. MH: data analysis and interpretation. DC: data analysis and interpretation. SF: concept and design, provision of study material, manuscript writing. RGR: conception and design, provision of study material, collection and assembly of data, data analysis and interpretation and manuscript writing.

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References

Akcora, D., Huynh, D., Lightowler, S., Germann, M., Robine, S., de May, J.R., Pollard, J.W., Stanley, E.R., Malaterre, J., Ramsay, R.G., 2013. The CSF-1 receptor fashions the intestinal stem cell niche. Stem Cell Res. 10 (2), 203–212. http://dx.doi.org/10.1016/j.scr.2012.12.001.

Andreu, P., Colnot, S., Godard, C., Gad, S., Chafey, P., Niwa-Kawakita, M., Laurent-Puig, P., Kahn, A., Robine, S., Perret, C., Romagnolo, B., 2005. Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development 132 (6), 1443–1451. http://dx.doi.org/10.1242/dev.01700.

Aoki, K., Taketo, M.M., 2007. Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. J. Cell Sci. 120 (Pt 19), 3327–3335.

Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., Clevers, H., 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449 (7165), 1003–1007. http://dx.doi.org/10.1038/nature06196.

Barker, N., Ridgway, R.A., van Es, J.H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A.R., Sansom, O.J., Clevers, H., 2009. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 457 (7229), 608–611.

Barker, N., Bartfeld, S., Clevers, H., 2010. Tissue-resident adult stem cell populations of rapidly self-renewing organs. Cell Stem Cell 7 (6), 656–670. http://dx.doi.org/10.1016/j.stem.2010.11.016.

Batlle, E., Bacani, J., Begthel, H., Jonkheer, S., Gregorieff, A., van de Born, M., Malats, N., Sancho, E., Boon, E., Pawson, T., Gallinger, S., Pals, S., Clevers, H., 2005. EphB receptor activity suppresses colorectal cancer progression. Nature 435 (7045), 1126–1130. http://dx.doi.org/10.1038/nature03626 (nature03626 [pii]).

Bellis, J., Duluc, I., Romagnolo, B., Perret, C., Faux, M.C., Dujardin, D., Formstone, C., Lightowler, S., Ramsay, R.G., Freund, J.N., De Mey, J.R., 2012. The tumor suppressor Apc controls planar cell polarities central to gut homeostasis. J. Cell Biol. 198 (3), 331–341. http://dx.doi.org/10.1083/jcb.201204086.

Carmon, K.S., Gong, X., Lin, Q., Thomas, A., Liu, Q., 2011. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc. Natl. Acad. Sci. U. S. A. 108 (28), 11452–11457. http://dx.doi.org/10.1073/pnas.1106083108.

Carpinteri, S., Williams, B.B., Miao, R.Y., C. C., Malaterre, J., Norris, M.D., Haber, M., Anderson, R.L., Darcy, P.K., Ramsay, R.G., 2012. Optimizing DNA vaccines against nuclear oncogenes. Immuno-Gastroenterology 1 (2), 108–118.

Cheasley, D., Pereira, L., Lightowler, S., Vincan, E., Malaterre, J., Ramsay, R.G., 2011. Myb controls intestinal stem cell genes and self-renewal. Stem Cells 29 (12), 2042–2050. http://dx.doi.org/10.1002/stem.761.

Ciznadija, D., Tothill, R., Waterman, M.L., Zhao, L., Huynh, D., Yu, R.M., Ernst, M., Ishii, S., Mantamadiotis, T., Gonda, T.J., Ramsay, R.G., Malaterre, J., 2009. Intestinal adenoma formation and MYC activation are regulated by cooperation between MYB and Wnt signaling. Cell Death Differ. 16 (11), 1530–1538. http://dx.doi.org/10.1038/cdd.2009.94 (cdd200994 [pii]).

Clevers, H., Nusse, R., 2012. Wnt/beta-catenin signaling and disease. Cell 149 (6), 1192–1205. http://dx.doi.org/10.1016/j.cell.2012.05.012.

Efstratiadis, A., Szabolcs, M., Klinakis, A., 2007. Notch, Myc and breast cancer. Cell Cycle 6 (4), 418–429 (3838 [pii]).

Farin, H.F., Van Es, J.H., Clevers, H., 2012. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. Gastroenterology 143 (6), 1518–1529. http://dx.doi.org/10.1053/j.gastro.2012.08.031 (e1517).

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., Artavanis-Tsakonas, S., 2005. Notch signals control the fate of immature progenitor cells in the intestine. Nature 435 (7044), 964–968.

Fre, S., Pallavi, S.K., Huyghe, M., Lae, M., Janssen, K.P., Robine, S., Artavanis-Tsakonas, S., Louvard, D., 2009. Notch and Wnt signals cooperatively control cell proliferation and tumorigenesis in the intestine. Proc. Natl. Acad. Sci. U. S. A. 106 (15), 6309–6314.

Fre, S., Hannezo, E., Sale, S., Huyghe, M., Lafkas, D., Kissel, H., Louvi, A., Greve, J., Louvard, D., Artavanis-Tsakonas, S., 2011. Notch lineages and activity in intestinal stem cells determined by a new set of knock-in mice. PLoS ONE 6 (10), e25785. http://dx.doi.org/10.1371/journal.pone.0025785.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. Science 281 (5382), 1509–1512.

Jarde, T., Evans, R.J., McQuillan, K.L., Parry, L., Feng, G.J., Alvares, B., Clarke, A.R., Dale, T.C., 2013. In vivo and in vitro models for the therapeutic targeting of Wnt signaling using a Tet-ODeltaN89beta-catenin system. Oncogene 32 (7), 883–893. http://dx.doi.org/10.1038/onc.2012.103.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., Madsen, O.D., 2000. Control of endodermal endocrine development by Hes-1. Nat. Genet. 24 (1), 36–44. http://dx.doi.org/10.1038/71657.

Kim, H.A., Koo, B.K., Cho, J.H., Kim, Y.Y., Seong, J., Chang, H.J., Oh, Y.M., Stange, D.E., Park, J.G., Hwang, D., Kong, Y.Y., 2012. Notch1 counteracts WNT/beta-catenin signaling through chromatin modification in colorectal cancer. J. Clin. Invest. 122 (9), 3248–3259. http://dx.doi.org/10.1172/JCI61216.

Kinzler, K.W., Vogelstein, B., 1996. Lessons from hereditary colorectal cancer. Cell 87 (2), 159–170.

Klinakis, A., Szabolcs, M., Politi, K., Kiaris, H., Artavanis-Tsakonas, S., Efstratiadis, A., 2006. Myc is a Notch1 transcriptional target and a requisite for Notch1-induced mammary tumorigenesis in mice. Proc. Natl. Acad. Sci. U. S. A. 103 (24), 9262–9267. http://dx.doi.org/10.1073/pnas.0603371103 (0603371103 [pii]).

Kopan, R., Ilagan, M.X., 2009. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137 (2), 216–233. http://dx.doi.org/10.1016/j.cell.2009.03.045.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., Clevers, H., 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC−/− colon carcinoma. Science 275 (5307), 1784–1787.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J., Clevers, H., 1998. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat. Genet. 19 (4), 379–383. http://dx.doi.org/10.1038/1270.

Luongo, C., Moser, A.R., Gledhill, S., Dove, W.F., 1994. Loss of Apc+ in intestinal adenomas from Min mice. Cancer Res. 54 (22), 5947–5952.

Malaterre, J., Carpinelli, M., Ernst, M., Alexander, W., Cooke, M., Sutton, S., Dworkin, S., Heath, J.K., Frampton, J., McArthur, G., Clevers, H., Hilton, D., Mantamadiotis, T., Ramsay, R.G., 2007. c-Myb is required for progenitor cell homeostasis in colonic crypts. Proc. Natl. Acad. Sci. U. S. A. 104 (10), 3829–3834. http://dx.doi.org/10.1073/pnas.0610055104 (0610055104 [pii]).

Moser, A.R., Pitot, H.C., Dove, W.F., 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247 (4940), 322–324.

Moser, A.R., Dove, W.F., Roth, K.A., Gordon, J.I., 1992. The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J. Cell Biol. 116 (6), 1517–1526.

Muncan, V., Sansom, O.J., Tertoolen, L., Phesse, T.J., Begthel, H., Sancho, E., Cole, A.M., Gregorieff, A., de Alboran, I.M., Clevers, H., Clarke, A.R., 2006. Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. Mol. Cell. Biol. 26 (22), 8418–8426. http://dx.doi.org/10.1128/MCB.00821-06 (MCB.00821-06 [pii]).

Murtaugh, L.C., Stanger, B.Z., Kwan, K.M., Melton, D.A., 2003. Notch signaling controls multiple steps of pancreatic differentiation. Proc. Natl. Acad. Sci. U. S. A. 100 (25), 14920–14925. http://dx.doi.org/10.1073/pnas.2436557100 2436557100 (pii).

Myant, K.B., Cammareri, P., McGhee, E.J., Ridgway, R.A., Huels, D.J., Cordero, J.B., Schwitalla, S., Kalna, G., Ogg, E.L., Athineos, D., Timpson, P., Vidal, M., Murray, G.I., Greten, F.R., Anderson, K.I., Sansom, O.J., 2013. ROS production and NF-kappaB activation triggered by RAC1 facilitate WNT-driven intestinal stem cell proliferation and colorectal cancer initiation. Cell Stem Cell 12 (6), 761–773. http://dx.doi.org/10.1016/j.stem.2013.04.006.

Onuma, K., Ochiai, M., Orihashi, K., Takahashi, M., Imai, T., Nakagama, H., Hippo, Y., 2013. Genetic reconstitution of tumorigenesis in primary intestinal cells. Proc. Natl. Acad. Sci. U. S. A. 110 (27), 11127–11132. http://dx.doi.org/10.1073/pnas.1221926110.

Peignon, G., Durand, A., Cacheux, W., Ayrault, O., Terris, B., Laurent-Puig, P., Shroyer, N.F., Van Seuningen, I., Honjo, T., Perret, C., Romagnolo, B., 2011. Complex interplay between beta-catenin signalling and Notch effectors in intestinal tumorigenesis. Gut 60 (2), 166–176. http://dx.doi.org/10.1136/gut.2009.204719.

Pellegrinet, L., Rodilla, V., Liu, Z., Chen, S., Koch, U., Espinosa, L., Kaestner, K.H., Kopan, R., Lewis, J., Radtke, F., 2011. Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells. Gastroenterology 140 (4), 1230–1240. http://dx.doi.org/10.1053/j.gastro.2011.01.005 (e1237. S0016-5085(11)00039-4 [pii]).

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., Kinzler, K.W., 1992. APC mutations occur early during colorectal tumorigenesis. Nature 359 (6392), 235–237.

Ramsay, R.G., Gonda, T.J., 2008. MYB function in normal and cancer cells. Nat. Rev. Cancer 8 (7), 523–534. http://dx.doi.org/10.1038/nrc2439 (nrc2439 [pii]).

Ramsay, R.G., Ciznadija, D., Sicurella, C., Reyes, N., Mitchelhill, K., Darcy, P.K., D'Abaco, G., Mantamadiotis, T., 2005. Colon epithelial cell differentiation is inhibited by constitutive c-myb expression or mutant APC plus activated RAS. DNA Cell Biol. 24 (1), 21–29.

Ruffner, H., Sprunger, J., Charlat, O., Leighton-Davies, J., Grosshans, B., Salathe, A., Zietzling, S., Beck, V., Therier, M., Isken, A., Xie, Y., Zhang, Y., Hao, H., Shi, X., Liu, D., Song, Q., Clay, I., Hintzen, G., Tchorz, J., Bouchez, L.C., Michaud, G., Finan, P., Myer, V.E., Bouwmeester, T., Porter, J., Hild, M., Bassilana, F., Parker, C.N., Cong, F., 2012. R-spondin potentiates Wnt/beta-catenin signaling through orphan receptors LGR4 and LGR5. PLoS ONE 7 (7), e40976. http://dx.doi.org/10.1371/journal.pone.0040976.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., Clarke, A.R., Winton, D.J., 2004. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev. 18 (12), 1385–1390.

Sansom, O.J., Meniel, V.S., Muncan, V., Phesse, T.J., Wilkins, J.A., Reed, K.R., Vass, J.K., Athineos, D., Clevers, H., Clarke, A.R., 2007. Myc deletion rescues Apc deficiency in the small intestine. Nature 446 (7136), 676–679.

Sato, T., Vries, R.G., Snippert, H.J., van de Wetering, M., Barker, N., Stange, D.E., van Es, J.H., Abo, A., Kujala, P., Peters, P.J., Clevers, H., 2009. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459 (7244), 262–265. http://dx.doi.org/10.1038/nature07935.

Sato, T., Stange, D.E., Ferrante, M., Vries, R.G., Van Es, J.H., Van den Brink, S., Van Houdt, W.J., Pronk, A., Van Gorp, J., Siersema, P.D., Clevers, H., 2011a. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology 141 (5), 1762–1772. http://dx.doi.org/10.1053/j.gastro.2011.07.050.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., Clevers, H., 2011b. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469 (7330), 415–418. http://dx.doi.org/10.1038/nature09637.

Schepers, A.G., Snippert, H.J., Stange, D.E., van den Born, M., van Es, J.H., van de Wetering, M., Clevers, H., 2012. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. Science 337 (6095), 730–735. http://dx.doi.org/10.1126/science.1224676.

Schwitalla, S., Fingerle, A.A., Cammareri, P., Nebelsiek, T., Goktuna, S.I., Ziegler, P.K., Canli, O., Heijmans, J., Huels, D.J., Moreaux, G., Rupec, R.A., Gerhard, M., Schmid, R., Barker, N., Clevers, H., Lang, R., Neumann, J., Kirchner, T., Taketo, M.M., van den Brink, G.R., Sansom, O.J., Arkan, M.C., Greten, F.R., 2013. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. Cell 152 (1–2), 25–38. http://dx.doi.org/10.1016/j.cell.2012.12.012.

Smith, E., Claudinot, S., Lehal, R., Pellegrinet, L., Barrandon, Y., Radtke, F., 2012. Generation and characterization of a Notch1 signaling-specific reporter mouse line. Genesis 50 (9), 700–710. http://dx.doi.org/10.1002/dvg.22030.

Sparks, A.B., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res. 58 (6), 1130–1134.

Taketo, M.M., 2006. Mouse models of gastrointestinal tumors. Cancer Sci. 97 (5), 355–361.

van der Flier, L.G., van Gijn, M.E., Hatzis, P., Kujala, P., Haegebarth, A., Stange, D.E., Begthel, H., van den Born, M., Guryev, V., Oving, I., van Es, J.H., Barker, N., Peters, P.J., van de Wetering, M., Clevers, H., 2009. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 136 (5), 903–912. http://dx.doi.org/10.1016/j.cell.2009.01.031.

van Es, J.H., Jay, P., Gregorieff, A., van Gijn, M.E., Jonkheer, S., Hatzis, P., Thiele, A., van den Born, M., Begthel, H., Brabletz, T., Taketo, M.M., Clevers, H., 2005a. Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat. Cell Biol. 7 (4), 381–386. http://dx.doi.org/10.1038/ncb1240 (ncb1240 [pii]).

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., Clevers, H., 2005b. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435 (7044), 959–963.

van Es, J.H., Haegebarth, A., Kujala, P., Itzkovitz, S., Koo, B.K., Boj, S.F., Korving, J., van den Born, M., van Oudenaarden, A., Robine, S., Clevers, H., 2012. A critical role for the Wnt effector Tcf4 in adult intestinal homeostatic self-renewal. Mol. Cell. Biol. 32 (10), 1918–1927. http://dx.doi.org/10.1128/MCB.06288-11.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, A., Grosse, A.S., Gumucio, D.L., Ernst, S.A., Tsai, Y.H., Dempsey, P.J., Samuelson, L.C., 2012. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 139 (3), 488–497. http://dx.doi.org/10.1242/dev.070763.

Vermeulen, L., De Sousa, E.M.F., van der Heijden, M., Cameron, K., de Jong, J.H., Borovski, T., Tuynman, J.B., Todaro, M., Merz, C., Rodermond, H., Sprick, M.R., Kemper, K., Richel, D.J., Stassi, G., Medema, J.P., 2013. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat. Cell Biol. 12 (5), 468–476.

Williams, B.B., Wall, M., Miao, R.Y., Williams, B., Bertoncello, I., Kershaw, M.H., Mantamadiotis, T., Haber, M., Norris, M.D., Gautam, A., Darcy, P.K., Ramsay, R.G., 2008. Induction of T cell-mediated immunity using a c-Myb DNA vaccine in a mouse model of colon cancer. Cancer Immunol. Immunother. 57 (11), 1635–1645. http://dx.doi.org/10.1007/s00262-008-0497-2.

Yang, Q., Bermingham, N.A., Finegold, M.J., Zoghbi, H.Y., 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294 (5549), 2155–2158.