Mutational inactivation of Apc in the intestinal epithelia compromises cellular organisation

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Summary statement

We have determined that APC control of intestinal epithelia structure and function is regulated through three independent effector pathways specifying: (i) cell proliferation; (ii) epithelial morphology and (iii) intracellular organisation.
Abstract

The adenomatous polyposis coli (Apc) protein regulates diverse effector pathways essential for tissue homeostasis. Truncating oncogenic mutations in Apc removing its Wnt pathway and microtubule regulatory domains drives intestinal epithelia tumorigenesis. Exuberant cell proliferation is one well-established consequence of oncogenic Wnt pathway activity however, the contribution of other de-regulated molecular circuits to tumorigenesis has not been fully examined.

Using *in vivo* and organoid models of intestinal epithelial tumorigenesis we find that Wnt pathway activity controls intestinal epithelial villi and crypt structure, morphological features lost upon Apc inactivation. While the Wnt pathway target gene c-Myc has critical roles in regulating cell proliferation and tumorigenesis, Apc specification of intestinal epithelial morphology is independent of the Wnt-responsive Myc-335 regulatory element.

We further demonstrate that Apc inactivation disrupts the microtubule cytoskeleton and consequently localisation of organelles without affecting the distribution of the actin cytoskeleton and associated components. Our data indicates direct control over microtubule dynamics by Apc through an independent molecular circuit.

Our study stratifies three independent Apc effector pathways in the intestinal epithelial controlling: (i) proliferation, (ii) microtubule dynamics and (iii) epithelial morphology.
Introduction

The intestinal tract (small intestine and colon) hosts a highly dynamic enterocyte monolayer that undergoes complete self-renewal every 3-5 days. The basic units of the intestinal epithelium are adjacent invaginations, termed crypts of Lieberkühn (Fig. 1A, B), each of which serves as a semi-autonomous cell production factory with a remarkably high proliferation rate - along the murine intestinal tract, crypts are composed of an average of 700 cells that produce up to 20 cells per hour in the small intestine or 7 cells per hour in the colon (de Rodriguez et al., 1978; Potten et al., 1982; Sunter et al., 1979). Throughout the enterocyte monolayer, each cell is spatially restricted, selective for homo- and hetero-typic cell-specific interactions, and is highly polarised with defined apical, lateral and basal faces, characteristics that are critical to epithelial barrier and transport functions. The hierarchal organisation of the enterocyte monolayer, from the stem cells at the base of crypts to differentiated cells types facing the gut lumen, is achieved through the highly regimented balance of rapid cellular proliferation, cellular organisation and morphology of the epithelial monolayer (Gehart and Clevers, 2019).

Malignant transformation as a result of mutational inactivation of the tumour suppressor gene adenomatous polyposis coli (APC) compromises tissue organisation of the intestinal epithelium (Dow et al., 2015; Kinzler, Kenneth W. and Vogelstein, 1996; Volgestein and Fearon, 1990). Somatic mutations in APC are widely regarded as the earliest genetic lesion in 80-90% of sporadic colon cancers (Groden et al., 1991). Perhaps surprisingly, mutational inactivation of APC reveals an oncogenic vulnerability largely restricted to the intestinal epithelium. Thus, individuals with familial adenomatous polyposis (FAP) that are heterozygous for a germline mutation inactivating one allele of APC (Su et al., 1992) exhibit spontaneous loss of heterozygosity that leads to hundreds of tumours, all of which are restricted to the intestinal epithelium. The well-established murine model of FAP, Apc<sup>Min/+</sup> (multiple intestinal neoplasia; Min), follows a similar pattern of tumour development—despite mono-allelic inactivation of Apc in every cell in the body, tumorigenesis is almost exclusive to the intestinal epithelium (Moser et al., 1990; Moser et al., 1995; Ren et al., 2019; Su et al., 1992).

Apc is a large multi-domain protein that governs a plethora of effector pathways regulating cellular and tissue homeostasis (Nelson and Näthke, 2013). Apc’s molecular roles are
generally ascribed to the regulation of Wnt pathway activity, a key determinant of stem cell multipotency and proliferation within the crypt. Pathway activity is sustained within the stem cell niche by redundant sources of Wnt ligands derived from adjacent Paneth cells and the underlying mesenchyme (Aoki et al., 2016; Farin et al., 2012; Gregorieff et al., 2005; Stzepourginski et al., 2017; Valenta et al., 2016; Zou et al., 2018) and potentiated by cellular engagement of LGR family receptors by R-spondins derived from specific mesenchymal cells (Yan et al., 2017).

Oncogenic APC inactivation in colorectal cancer follows a unique pattern of somatic changes – at least one APC allele harbours mutations that are largely confined to a short segment within exon 15 of the gene referred to as the mutation cluster region (MCR; Fig. 1C), resulting in the expression of truncated Apc. The other allele is most often silenced or incurs the same or a more severe truncating mutations (Crabtree et al., 2003; Lamlum et al., 1999; Rowan et al., 2000). Tumours arising from truncating mutations in exon 14 of the mouse Apc gene found in the Min mouse line (Fig. 1C), equivalent to human exon 15, display many features common with human colorectal cancer tumours.

The truncated Apc protein lacks regulatory protein-protein interaction domains for the Wnt pathway regulators β-catenin and Axin (Fig. 1C), explaining oncogenic Wnt pathway activation upon loss of heterozygosity in murine models. Extensive investigation of oncogenic Wnt pathway activity in cells lacking APC points to a key role in the regulation of intestinal epithelial cell proliferation through the Wnt pathway target gene c-Myc (Dave et al., 2017; He et al., 1998; Oskarsson and Trumpp, 2005; Sansom et al., 2007; Sur et al., 2012).

Truncated Apc protein also lacks the C-terminal microtubule end binding protein 1 (EB1) binding domain and a basic domain thought to bind directly to microtubules (Fig. 1C) (Deka et al., 1998). However, the molecular consequence of C-terminal Apc truncations and removal of the microtubule and EB1 binding domains is controversial. Apc mediated stabilisation of microtubules via its C-terminal domains supports the establishment of parallel arrays of microtubules in a polarised cell (Mogensen et al., 2002; Zumbrunn et al., 2001) and APC is known to regulate cytoskeletal rearrangements that accompany cell motility, cell division and tissue organisation through control of microtubule dynamics (Moseley et al., 2007; Munemitsu et al., 1994; Näthke et al., 1996; Smith et al., 1994).
Consistent with these observations is that APC localizes to the + end tips of microtubules, centrosomes, microtubule spindles and at the interface of microtubules and kinetochores during mitosis (reviewed in (Näthke, 2004a)). It is not clear if the truncating mutations in APC decrease its binding to microtubules or its capacity to stabilize the microtubule + ends (Karin Kroboth et al., 2007; Munemitsu et al., 1994; Smith et al., 1994; Zumbrunn et al., 2001). Furthermore, loss of Apc C-terminal microtubule and EB1 interaction domains in mouse embryonic fibroblasts and differentiated embryonic stem cells does not affect the distribution of β-tubulin, EB1 and Apc (Lewis et al., 2012; Smits et al., 1999).

In vivo mouse models have investigated whether loss of Apc’s C-terminal microtubule and EB1 binding domains are sufficient to drive intestinal epithelial tumorigenesis. Apc\textsuperscript{1638T/1638T} mice express a version of Apc lacking the C-terminal domains but retaining the ability to regulate Wnt pathway activity and do not present with intestinal epithelial tumours (Smits et al., 1999) (Fig. 1C). Conversely, Apc\textsuperscript{\DeltaSAMP/+} mice expressing a version of Apc unable to regulate Wnt pathway activity but retaining the microtubule and EB1 binding domains develop tumours with the same frequency and kinetics as the corresponding Apc\textsuperscript{1322/+} mice that express Apc lacking these domains (Fig. 1C) (Lewis et al., 2012). Thus, Apc’s ability to interact with microtubules and EB1 does not, on its own, drive intestinal epithelial tumorigenesis. Nonetheless, the potential contribution of loss of the Apc microtubule and EB1 binding domains to intestinal tumorigenesis has not been determined.

Herein, we stratify molecular pathways regulated by Apc in the murine intestinal epithelia by defining the molecular and phenotypic consequences in the small intestinal epithelia and corresponding organoids caused by Apc inactivation. In addition to deregulation of cell proliferation, we find that Apc inactivation disrupts the morphology of the intestinal epithelial monolayer and compromises the functional integrity of the microtubule cytoskeleton in component enterocytes. These emergent malignant properties are the direct consequence of Apc inactivation and are controlled by independent different molecular systems. Therefore, (i) enterocyte proliferation, (ii) microtubule dynamics and (iii) epithelial morphology are regulated by three separate effector pathways that, under the control of Apc, buttress normal intestinal epithelial homeostasis against malignant transformation.
Results

Compromised intracellular organisation and tissue morphology in Apc\textsuperscript{Min/-} tumours

Over the course of 110 days, Apc\textsuperscript{Min/+} mice develop 30-40 adenomas in the small intestine, the result of loss of heterozygosity of the wild type Apc allele (Moser et al., 1995; Su et al., 1992). Such Apc\textsuperscript{Min/-} tumours are composed of gland-like structures that maintain an epithelial monolayer yet lack the morphological hallmarks of crypt and villus compartments and the hierarchical cellular organisation of the wild type epithelia (Fig. 1A). For instance, Ki67\textsuperscript{+} proliferative stem cells and the transit amplifying cellular compartment, normally disposed basally within crypts, are instead interspersed throughout the monolayer of the tumour gland-like structures (Fig. 1B). We used fluorescently-labelled \textit{Ulex Europaeus} agglutinin (fUEA) to visualise secretory vesicles that, in wild type tissue, are found apically within the mechanically rigid, keystone-shaped Paneth cells in the crypts or columnar-shaped goblet cells in the villi (Fig. 1B) (Langlands et al., 2016). However, UEA-positive cells in Apc\textsuperscript{Min/-} tumours are interspersed throughout the glandular monolayer, are of variable shapes and fail to maintain their characteristic clustered apical localisation (Fig. 1B). We observed the identical phenotype using an antibody raised against lysozyme - Paneth cells no longer maintain their shape, size and clustered localisation to the crypt base and intracellular secretory vesicles are no longer apically restricted (Fig. 1B). In contrast, \(\beta\)-catenin maintains its characteristic localisation pattern at the cell periphery juxtaposed to cell-cell contacts in Apc\textsuperscript{Min/-} tumours cells (Fig. 1B). In the larger proportion of Apc\textsuperscript{Min/-} tumours cells, we observe high expression levels of \(\beta\)-catenin and localisation to the nucleus, an established consequence of Apc deficiency (Polakis, 2012). In general, tumour cells fail to maintain the consistent columnar cell shape and size of wild-type epithelial cells, yet they align as a monolayer to form the gland-like structures (Fig. 1A, B). We also note that, as opposed to wild-type enterocytes, Apc\textsuperscript{Min/-} tumour cells contain nuclei of variable shapes and sizes that fail to align along the plane of the monolayer. We conclude that, in addition to driving de-regulated epithelial cell proliferation and the disruption of tissue morphology, Apc inactivation compromises some aspects of intracellular organisation.
Defective regulation of microtubule function in Apc\textsuperscript{Min/-} tumours

The cytoskeleton provides the physical framework for intracellular organisation and cell polarity defined by localised dynamic polymerisation/depolymerisation of actin and tubulin monomers (Li and Gundersen, 2008; Rodriguez-Boulan and Macara, 2014). Apc harbours an array of protein-protein interaction domains with established roles in regulating F-actin and microtubule dynamics within intestinal epithelial cells (Fig. 1C)(Kawasaki et al., 2000; Munemitsu et al., 1994; Näthke, 2004b; Rosin-Arbesfeld et al., 2001; Tirnauer, 2004; Zumbrunn et al., 2001). We examined the localisation of the cytoskeleton in intestinal epithelial and Apc\textsuperscript{Min/-} tumour cells, using a series of fluorescent probes for F-actin, microtubules and known protein interactors. Consistent with a previous study (Fatehullah et al., 2013), all Apc\textsuperscript{Min/-} tumour cells maintain the correct disposition and configuration of actin cytoskeletal components - F-actin was concentrated along the apical face of the epithelial cells (Pelaseyed and Bretscher, 2018), β4-integrin, which anchors enterocytes to the underlying lamina propria is found at the cell base (Fatehullah et al., 2013), β-catenin localised adjacent to cell-cell contacts and the tight junction organiser ZO-1 was positioned apically at cellular junctions (Lee et al., 2018) (Fig. 1B, 2A; Table 1).

In contrast, components of the microtubule cytoskeleton in Apc\textsuperscript{Min/-} tumour cells were disorganised; microtubules, normally orientated along the apical-basal axis were instead disjointed and diffuse, often appearing as punctate (Fig. 2B). We used an antibody raised against the acetylated form of α-tubulin and found that the signal was concentrated at the apical domain of cells, in line with previously published data (Quinones et al., 2011). However, in tumour cells, acetylated α-tubulin was instead de-localised and diffuse (Fig. S1A). As a quantitative measure of the functional microtubule cytoskeleton, we determined the localisation of intracellular organelles whose location and positioning are dependent on microtubules. Predictably, we found that the normally strict basal positioning of nuclei, apical positioning of intracellular vesicles and the supra-nuclear localisation of the Golgi resident protein ZFLP1 and the centrosome marker pericentrin in wild type intestinal epithelia was lost in Apc\textsuperscript{Min/-} tumour cells. Instead we observe signals for the Golgi and centrosome split into multiple puncta and mis-localised (Fig. 2C; Table 1). To preclude mis-
localisation of the Golgi as the consequence of cells undergoing cell division (Thyberg and Moskalewski, 1999), we co-stained intestinal epithelial sections with an antibody to the mitotic marker phospho-histone 3 (PH3) - tumour cells displayed de-localised Golgi in the absence of detectable levels of PH3 (Fig. S1B). We quantified localisation data for components associated with the microtubule and actin cytoskeleton, presented in Table 1. Our results indicate that normal localisation of the actin cytoskeleton is maintained in Apc\textsuperscript{Min/-} intestinal epithelial tumour cells, whereas the localisation and functional integrity of the microtubule cytoskeleton is compromised.

We reasoned that the C-terminal microtubule and EB1 binding domains of Apc may be critical for the regulation of the microtubule cytoskeleton. The Apc\textsuperscript{1638T/1638T} mouse strain is homozygous for a truncating mutation in Apc that deletes the C-terminal microtubule and EB1 binding domains (Fig. 1C). However, as opposed to Apc expressed in Apc\textsuperscript{Min/+} mice, the Apc\textsuperscript{1638T} protein retains the Axin interaction domain and therefore retains regulatory control over Wnt pathway activity; Apc\textsuperscript{1638T/1638T} mice do not develop intestinal epithelial tumours (Fig. 1C)(Smits et al., 1999). Since the small intestine epithelia of Apc\textsuperscript{1638T/1638T} mice exhibit normal localisation of intact Golgi and fUEA-positive Paneth cell vesicles (Fig. S1C), we conclude that loss of Apc’s C-terminal microtubule and EB1 binding domains alone do not compromise regulation of the microtubule cytoskeleton or intestinal epithelial morphology.

Organoids accurately recapitulate the molecular and phenotypic consequences of APC inactivation in the intestinal epithelium

We generated organoid lines from wild-type, Apc\textsuperscript{Min/+} intestinal epithelia and Apc\textsuperscript{Min/-} tumour cells as an experimentally tractable model system for determining the molecular mechanisms linking Apc to microtubule integrity and epithelial morphology. Organoids derived from normal tissue form an epithelial monolayer, replete with crypts, that maintains the three-dimensional cellular organisation and hierarchy found in vivo. In contrast, tumouroids, organoids derived from Apc\textsuperscript{Min/-} tumour cells, form cystic structures lacking morphological features of the intestinal epithelial monolayer such as crypts (Sato et al., 2011).

Using a series of fluorescent probes, we found that F-actin and associated molecular components β4-integrin, β-catenin, and ZO-1 maintained their intracellular localisation in
both organoid and tumouroid cells (Fig. 3A). Note that in some cases, the middle portion of the spherical tumouroid collapses and is captured in some confocal sections. Consistent with our observations in intestinal epithelial tissue from $Apc^{Min/-}$ tumours, the organisation and function of the microtubule cytoskeleton was compromised: β-tubulin was no longer polarised in microtubules along the apical-basal axis of cells but was instead dispersed throughout tumouroid cells and acetylated α-tubulin was de-localised (Fig. 3B). We also found that nuclei varied in shape and size and did not follow the plane of the tumouroid monolayer and centrosomes and Golgi were split into multiple puncta distributed throughout the cell body (Fig. 3C). Quantification of the centrosome and Golgi localisation in tumouroids indicates they are mis-positioned in over 35% and 50% of cases, respectively (Fig. 3D).

Consistent with our in vivo results, our organoid data confirms that Apc inactivation in the intestinal epithelial monolayer leads to deregulation of microtubule dynamics and loss of intracellular organisation with the absence of detectable effects on the actin cytoskeleton. Apc deficiency directly compromises intracellular organisation and tissue morphology

It is possible that intestinal epithelial tumours from 110-day old $Apc^{Min/+}$ mice, and organoids derived from them, have acquired additional somatic changes that contribute to phenotype. To determine the immediate and direct effects of Apc inactivation we created a switchable organoid model of tumorigenesis that relies on the inducible expression of a previously-validated shRNA targeting Apc (Dow et al., 2015) (Fig. S2A). Induction of shApc in organoids depletes Apc mRNA concurrent with the expression of mCherry and leads to the intraconversion of organoids into a cystic tumouroid structure (Fig. S2B-D). Importantly, we observe a reversible increase in expression of the Wnt pathway target gene c-Myc (Fig. S2E).

Consistent with the appearance of $Apc^{Min/-}$ tumours and tumouroids, Apc depletion in organoids resulted in mis-localisation of UEA-positive intracellular vesicles as well as Golgi and centrosome fragmentation and mis-localisation (Fig. 4A). Importantly, all hallmarks of intracellular disorganisation and compromised tissue morphology were reversed upon Apc re-expression, leading to the appearance of ‘normal’ organoids (Fig. 4A). Our switchable in vitro tumorigenesis model confirms that compromised epithelial morphology and intracellular disorganisation are the direct consequence of Apc inactivation.
**Apc regulation of intestinal epithelial morphology and microtubule dynamics are discrete**

Ubiquitous activation of Wnt pathway activity in organoid cells by treatment with Wnt3A conditioned media leads to the intra-conversion of organoids into cystic tumouroid-like structures (Farin et al., 2012) that we refer to as Wnt-oids (Fig. 4B). Although the morphology of the Wnt-oid epithelial monolayer is compromised, they are distinct from tumouroids in that the Golgi and centrosome retain their normal apical position in component cells (Fig. 4B) – greater than 80% of Wnt-oid cells show apical localisation of the Golgi and centrosome as opposed to less than 65% in tumouroid cells (Fig. 4B). We conclude that Apc regulation of intestinal epithelial morphology through Wnt pathway regulation is not coupled to its function in regulating microtubule dynamics and intracellular organisation.

We carried out the complimentary experiment, selectively deregulating microtubule dynamics in organoids and determining the consequence on epithelial morphology. We treated organoids with a low concentration (100 nM) of the microtubule depolymerising agent nocodazole (Vasquez et al., 1997) for 48 hours, a timepoint sufficient for the conversion of organoids to Wnt-oids with Wnt3A treatment. Treated organoid and Wnt-oid cells displayed the characteristic mis-localisation of fragmented Golgi that was reversed after 24 hours post-nocodazole withdrawal (Fig. 4C, D). Importantly, throughout the experiments, nocodazole-treated organoids maintain intestinal epithelial crypts structures (Fig. 4C, D) indicating that maintenance of intestinal organisation and microtubule dynamics are not dependent on one another. Combined with our Apc loss-of-function studies, these data suggest that Apc-dependent control of intracellular organisation and epithelial morphology rely on independent molecular circuits.

**Loss of a Wnt-responsive enhancer element upstream of c-Myc does not impact intestinal epithelial morphology**

Previous studies have indicated that Apc inactivation in the intestinal epithelia compromising Wnt pathway-dependent regulation of c-Myc expression is the critical mediator of malignant transformation *in vivo* (Dave et al., 2017; Sansom et al., 2006; Sur et al., 2012). Removal of Wnt pathway-responsive enhancer element upstream of c-Myc, that
carries binding sites for the Wnt pathway effector transcription factor Tcf7l2, \((Myc-335^-)\) mice; Fig. S3) (Sur et al., 2012), while modestly reducing \(c-Myc\) expression, attenuates small intestinal epithelial tumorigenesis when combined with the \(Apc^{Min/+}\) allele, by approximately 70% multiplicity (Sur et al., 2012). We derived organoids from \(Myc-335^-\) mice to test whether de-regulated epithelial morphology, one phenotypic consequence of Wnt pathway activity imposed by Apc inactivation, was altered after deletion of the Wnt-responsive enhancer element upstream of \(c-Myc\).

Wnt3A conditioned media treatment of \(Myc-335^-\) and wild-type organoids indicated identical kinetics and frequency of Wnt-oid formation (Fig. 5A, B) that retained the normal Golgi apical localisation (Fig. 5C). Moreover, over the 7-day time course of Wnt3A treatment, we observed no differences in cell proliferation within wild-type and \(Myc-335^-\) organoids measured by Wnt-oid diameter (Fig. 5D). Taken together, our data suggest that regulation of intracellular organisation and epithelial tissue morphology by Wnt pathway activity is independent of regulation of \(c-Myc\) expression via \(Myc-335\).

**Discussion**

In this study, we unmasked individual molecular systems controlled by Apc in the intestinal epithelia through loss-of-function. Oncogenic Apc mutations are the principle driver of colon epithelial tumorigenesis and sufficient for malignant transformation of the colon and small intestinal epithelia. We stratify three emergent phenotypes in the murine intestinal epithelia that are the direct consequence of oncogenic Apc mutations: de-regulated proliferation, disrupted epithelial morphology and compromised microtubule dynamics leading to defective intracellular organisation.

In the intestinal epithelia, Apc activity restricts enterocyte proliferation through stringent control of the Wnt pathway-dependent transcriptional programme. In particular, regulated expression of the Wnt pathway target gene, \(c-Myc\), constrains proliferation to discrete, localised niches, providing a key molecular barrier to malignant transformation (Dave et al., 2017; Quyn et al., 2010; Sur et al., 2012); whereas oncogenic Apc mutations in the intestinal epithelia are sufficient to drive neoplastic growth, the absence of \(c-Myc\) expression attenuates all transforming properties of Apc inactivation, in vivo (Sansom et al., 2006). Less well understood is how oncogenic Apc mutations deregulate epithelial morphology and
intracellular organisation. We have established that organoids and their Apc-deficient counterparts, tumouroids, are a tractable model that effectively recapitulate the morphological and organisational hallmarks modelling the transition between intestinal epithelia and tumours.

Treatment of organoids with Wnt3A drives their intra-conversion into cystic tumouroid-like structure, termed Wnt-oids that, in contrast to tumouroids, maintain intracellular organisation of the component cells. Our interpretation is that Wnt3A treatment leads to selective inhibition of Wnt pathway regulation by Apc, compromising constraints on epithelial morphology, but retaining the integrity of the microtubule cytoskeleton and intracellular organisation – supporting the notion that regulation of epithelial morphology and cytoskeletal integrity are uncoupled. Conversely, selective destabilisation of microtubules compromises intracellular organisation in component organoid cells, yet normal morphology of the epithelia monolayer is retained. Taken together, our data support a model whereby Apc controls enterocyte proliferation and epithelial morphology through Wnt pathway regulation and regulates the microtubule cytoskeleton and intracellular organisation through other, separate pathways (Fig. 5E).

How then does Apc regulation of Wnt pathway activity impact the morphology of the epithelial monolayer? Our data support direct control of epithelial morphology by Wnt pathway activity rather than an inability of organisational constraints to cope with exuberant proliferation. In the intestinal epithelia, neoplastic growth is the result of precocious Wnt pathway target gene expression driving deregulated expression of the Wnt pathway target gene, c-Myc. Deregulated c-Myc expression is regarded as the major culprit in all transforming phenotypes attributed to Apc loss in vivo (Sansom et al., 2007). However, using the Myc-335−/− organoids, we find that the modest de-regulation of c-Myc expression observed in vivo (Sur et al., 2012), while sufficient to reduce transforming properties of Apc inactivation, does not alter Wnt pathway regulation of intestinal epithelial morphology or cellular organisation in our organoid system. Moreover, within the timeframe of our experiments, we did not observe any changes in the rate of proliferation accompanying the intra-conversion of organoids to Wnt-oids. Together, our data suggest that Apc control of epithelial morphology is not wholly dependent on Myc-335 mediated Wnt pathway
regulation of c-Myc expression nor is it the result of increased proliferative pressure on organisational constraints on the epithelial monolayer.

It will be important to identify Wnt pathway targets that control intestinal epithelial morphology – we anticipate that targeted modulation of such genes may provide therapeutic value for preventing or even reversing the compromised epithelial morphology accompanying malignant transformation of the intestinal epithelia. The intra-conversion between organoids and Wnt-oids is a ready-made assay system for rapidly testing sufficiency of Wnt pathway candidate target genes by their targeted loss of function; a list of such candidates has been previously identified by Sansom and colleagues (Sansom et al., 2007).

One striking observation was that Apc regulates the integrity of the microtubule cytoskeleton and consequently the intracellular location of organelles such as the nucleus, Golgi, centrosome and intracellular vesicles. Although control of the microtubule cytoskeleton may be mediated directly by the Apc C-terminal microtubule and/or EB1 binding domains (Morrison et al., 1998; Munemitsu et al., 1994), it is also possible that Wnt pathway regulatory components downstream of Apc or even Wnt pathway transcriptional targets contribute to microtubule integrity. For example, truncated Apc in Apc^{1638T/1638T} mice retains the ability to regulate Wnt pathway activity and maintain the integrity of the microtubule cytoskeleton (Smits et al., 1999). Our interpretation is that regulation of the Wnt pathway suppresses defects in the microtubule cytoskeleton, in vivo. It remains to be determined whether this is the case in the intestinal epithelial-autonomous milieu of in vitro organoid culture.

In colon cancer, oncogenic mutations that inactivate Apc are 10-fold more prevalent than oncogenic mutations in other Wnt pathway regulatory components suggesting that functions other than Wnt pathway deregulation contribute to disease aetiology. Although compromised microtubule integrity is a likely consequence of Apc truncations that delete C-terminal microtubule and EB1 binding domains, it is unlikely to impact tumorigenesis - the presence of C-terminal microtubule and EB1 binding domains in truncated versions of Apc has no impact on tumorigenesis (Lewis et al., 2012). However, one intriguing possibility is that compromised microtubule integrity in Apc mutant tumour cells contributes to chromosome instability (CIN). CIN is a feature of the evolution of aggressive colorectal...
adenocarcinoma right from the outset, evident in the smallest adenomas and multiple reports have directly linked oncogenic APC mutations in CRC with a predisposition to CIN (Dikovskaya et al., 2007; Fodde et al., 2001; Kaplan et al., 2001). Importantly, embryonic stem cells derived from \( Apc^{1638T/1638T} \) mice develop hallmarks of CIN (Fodde et al., 2001) and overexpression of truncated APC lacking the C-terminal domains in chromosomally stable colorectal cancer cells leads to mitotic defects, including errors in kinetochore attachment and alignment of chromosomes (Green and Kaplan, 2003; Tighe et al., 2004). However, the molecular relationship between Apc loss, microtubule deregulation and chromosome instability in the intestinal epithelia has yet to be established. The experimentally tractable organoid/tumouroid model system we have developed will be invaluable in determining the role of Apc in the loss of microtubule integrity and the impact of CIN in intestinal tumorigenesis.

Our results distinguish individual malignant properties of intracellular disorganisation, compromised tissue morphology and proliferation as direct, but independent consequences of Apc inactivation; we posit that the combination of these emergent properties creates a ‘perfect storm’ for malignant transformation of the rapidly dividing intestinal epithelia, explaining why this tissue is particularly vulnerable to oncogenic Apc mutations.

**Materials and Methods**

**Reagents, antibodies and molecular probes**

Doxycycline and nocodazole were sourced from Sigma-Aldrich and used at concentrations of 2 μg/ml and 100 nM, respectively. Wnt3A conditioned media was harvested from Wnt3A-expressing L-cells (ATCC, CRL-2647) according to a previously established protocol (Willert et al., 2003). The media was stored for up to two months at 4°C without any detectable loss of Wnt3A activity. Antibodies and molecular probes used for fluorescence microscopy are listed in Table 2.

**Tissue preparation and fluorescent labelling**

All procedures using mice were performed under the UK Home Office guidelines. Intestines obtained from wild-type CL57BL/6, \( Apc^{1638T/1638T} \), \( Apc^{Min/+} \) CL57BL/6, \( Apc^{fl/fl} \) LSL tdTom (a gift from Winton laboratory) and \( Myc-335^- \) (a gift from Taipale laboratory) mice were either
fixed in 4% formaldehyde and embedded in paraffin or fixed-frozen in 10% formalin and embedded in optimal cutting temperature (OCT) liquid, followed by snap freezing (fresh-frozen tissue).

Small intestinal epithelial sections (4% formaldehyde-fixed or fresh frozen) for molecular probe and antibody labelling were cut at a thickness of 4.5 μm onto slides. The exception was slides labelled with β-tubulin or acetylated-tubulin in which case 20 μm thick formaldehyde-fixed sections were cut onto poly-L-lysine coated slides.

For formaldehyde-fixed samples, epitope retrieval was performed in sodium citrate buffer (sodium citrate 10 mM, 0.05 % Tween-20, pH 6.0). Primary antibody incubations were carried out at 4°C overnight and secondary antibody incubation for 2 hours at room temperature, both in PBS containing normal goat serum (5%) and 0.1% Tween20. Samples were mounted in DAPI-containing Fluoromount-G (Thermo-Fisher).

**Organoid preparation and fluorescent labelling**

Murine small intestinal epithelial organoids were derived from the ileum of mouse small intestine according to Sato et al. 2013 (Sato and Clevers, 2013). Tumouroids were derived from tumours within the ileum of 110 day-old \( Apc^{\text{Min/+}} \) mice (Haigis et al., 2004). All organoids and tumouroids were cultured according to Urbischek et al. 2018 (Urbischek et al., 2019).

Organoids were seeded in Matrigel onto eight-well chamber slides (ThermoFisher) 48 hours prior to fluorescent labelling. Organoids were fixed in 92% methanol containing 8% formaldehyde and labelled following a published protocol (Goldspink et al., 2017). Organoids in primary antibody were incubated at 4°C overnight. The next day the slides were incubated at room temperature for 1 hour (allowing the Matrigel to harden), washed and then incubated for 1 hour at room temperature in the secondary antibody. Labelled organoid samples were then mounted in DAPI-containing Fluoromount-G.
Organoid and tissue imaging and data analysis

Fluorescent imaging of tissue was carried out using a Nikon C2 plus confocal microscope using 40X objective lens. Images were processed using ImageJ software. Fluorescent labelling of each antibody was repeated a minimum of three times.

Imaging of organoids was done using Nikon C2 plus confocal microscope using the 20X and 40X objectives and an automated spinning disc confocal microscope (YOKOGAWA Cell Voyager CV8000) using 40X objective. Z-stacks were taken at 1 μm steps. Images were processed and published using ImageJ software. All figures presented are representative images from a single plane within the Z-stack of the imaged specimen. For the quantification of organelle positioning within organoids, approximately 200 cells were counted per experiment manually. Statistical variation of phenotypes amongst treatment groups and genotypes was calculated by one and two-tailed student’s t-test using GraphPad Prism software.

Plasmids and organoid expression

The piggybac transposon and tet-on expression system was a kind gift from Bon-Kyoung Koo. The previously validated shRNA targeting mouse Apc (Dow et al., 2015) was inserted into the tet-responsive shRNA expression vector, pB-TRE-IRES-mCherry. The three plasmid system also consists of pB-CAG-rtTA, the vector for constitutive rtTA expression, and pPiggybac, the expression vector for constitutive expression of the piggybac transposase (Fujii et al., 2015).

The shApc organoid line was generated by transfection of the pB-TRE-shApc-IRES-mCherry, pPiggybac and pB-CAG-rtTA plasmids (Fig. S2A) using a NEPA21 electroporator according to a previously published protocol (Fujii et al., 2015). Organoids were selected for integration of constructs in organoid media containing Wnt3A conditioned media (Urbischek et al., 2019) supplemented with 150 μg/ml Hygromycin B (ThermoFisher) for 7 days, after which the media was switched to organoid media (Urbischek et al., 2019).
Validation of shApc organoid line

For western blotting, organoids were recovered from Matrigel using several rinses of ice-cold phosphate buffered saline (PBS) and the pellet was lysed with 50 μl 1X RIPA buffer (Millipore) containing protease (Sigma) and phosphatase inhibitors (Roche). Samples were loaded onto NuPAGE 3-8 % Tris-Acetate gradient gels (ThermoFisher) prior to transfer onto PVDF membrane. Antibodies used for probing membranes in PBS containing 0.2% Tween 20 and 5% non-fat milk are in Table 1.

Expression levels of Apc and mCherry were determined by qRT-PCR. RNA was isolated from organoids and tumouroids using the ReliaPrep RNA Cell Miniprep System kit (Promega) and cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (ThermoFisher) all according to manufacturers’ instructions. qRT-PCR was carried out using Fast SYBR Green Master Mix using a QuantStudio 5 real-time PCR system (both Applied Biosystems). B2m was used as a housekeeping gene and relative fold changes in Apc and mCherry expression were derived from ΔΔCT. The following primers were used: Apc (Forward: AGCCATGCCAACAAAGTCATCAGC; reverse: TTCCTTGCCACAGGTGAGGTAAAT), mCherry (Forward: CACGAGTTCCAGATCGAGGG; reverse: CAAGTAGTCGGGGATGTCGG) and B2m (Forward: ACCCCCACTCAGACTGATC; reverse: ATCTTCAGACGATCATGATG).

Genotyping of Myc-335 -/- mice

Primer pairs used for the genotyping of mice were U3An2 and UcSe2 for wildtype allele and U3An2 and U5Se2 for the Myc-335 null allele. The sequence of these primers are as follows:

U3An2 5’-TAT CTG CGG GTA GTA CAC CTG T-3’; U5Se2 5’-TAG TGA TTG GGT AAT AAA GAA TGA GGT C3’; UcSe2 5´-GCT GAC AGA GAT TGC TGA CAT AA-3´ (Sur et al., 2012). The expected amplicon sequences of the two alleles are:

Myc-335^wt (227 bp)
GCTGACAGAGATTGCTGACATAAAATGGTGTGCTCAAGGATGTGGGCTGACGCCAGGACAGA TGCAAGCTTCTTCTGATATCTCGAGGTATTTAGGGTCTCTTCTACTCTGGAAAAAGATAC CATGGGCAACATCATAGCACAGATGCTGATATTCCTGACAGAGTATCCCCACTGAGGACAGGGTGACTACCAG CAGATA and
Myc-335^null (311 bp)
TAGTGATTGCTGATATAAAGAATGAGGCTGACAGAGTTATGAGGGAACAGTGCCTGAGGTGACTAC CAGATA

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Competing interests

The authors declare no competing interests.
References


Fatehullah, A., Appleton, P. L. and Näthke, I. S. (2013). Cell and tissue polarity in the intestinal tract during tumourigenesis: Cells still know the right way up, but tissue


Morrison, E. E., Wardleworth, B. N., Askham, J. M., Markham, A. F. and Meredith, D. M. (1998). EB1, a protein which interacts with the APC tumour suppressor, is associated
with the microtubule cytoskeleton throughout the cell cycle. Oncogene 17, 3471–3477.


Figure 1. Compromised morphology of the monolayer and cellular organisation in Apc deficient intestinal epithelia. A. Haematoxylin and eosin stain of normal $Apc^{Min/+}$ murine intestinal epithelia and adjacent $Apc^{Min/-}$ tumour. Outsets - zoomed images of crypts from normal $Apc^{Min/+}$ murine intestinal epithelia and gland-like structures from $Apc^{Min/-}$ tumours. Scale bar, 200 μm. B. Fluorescent confocal microscope imaging of sections of $Apc^{Min/+}$ small intestinal epithelial (top panels) and $Apc^{Min/-}$ tumours (bottom panels). Left panels – Sections
are labelled with a fluorescent antibody to Ki67 (green), intracellular vesicles are labelled with fluorescent fUEA (red). *Middle panels* - sections are labelled with an antibody to lysozyme to mark Paneth cell vesicles. *Right panels* – sections are labelled with an antibody to β-catenin. Nuclei in all section are labelled with DAPI (blue). “A” marks the apical domain of cells and “B”, the basal domain. Scale bars, 50 μm. **C. Top** - Linear representation of the Apc protein showing constituent protein-protein interaction domains; oligo- oligomerisation domain; Arm – armadillo repeat domain; Axin-binding SAMP domains 1-3; MT/basic, the microtubule binding domain containing basic amino acids; EB1, EB1-binding domain; ovals refer to 15- and 20-amino acid β-catenin binding domains (grey and dark grey, respectively). MCR marks the corresponding mutational cluster region in human APC. **Bottom** - mouse models of Apc deficiency in the study. Domain structure of the expressed truncated forms of Apc alongside the genotype of the various models discussed in the manuscript. Also show is the propensity for intestinal epithelial tumorigenesis in the mouse models and the ability of the expressed truncated form of Apc to regulate Wnt pathway activity. See text for details and references.
Figure 2. Apc inactivation does not affect the localisation of the actin cytoskeleton yet compromises microtubule dynamics. A. Fluorescence confocal microscopy of small intestinal epithelia sections from Apc$^{Min/+}$ mouse, top panels and Apc$^{Min/-}$ tumours, bottom panels. Sections were labelled with fluorescent phalloidin (green; left panel), an antibody to β4-integrin (green; middle panel) and an antibody to ZO-1 (red; right panel). Scale bars, 50 μm. B. Confocal images of sections from Apc$^{Min/+}$ mouse, top panels and Apc$^{Min/-}$ tumours, bottom panels labelled with an antibody to β-tubulin. Top right panels are zoomed images of crypt and villi regions, bottom right panel, zoomed image of a region of the tumour. Arrowheads indicate position of individual microtubules aligning along the apical-basal axis of epithelial cells. Scale bars, 100 μm. C. Sections of small intestinal epithelia from Apc$^{Min/+}$ mouse, top panels and Apc$^{Min/-}$ tumours, bottom panels. Left panels - sections labelled with fluorescent UEA (red) and an antibody to ZO-1 (green). Middle panels - sections labelled with an antibody to pericentrin (red) to visualise the centrosome. Right panels - section labelled with an antibody to the Golgi resident protein ZFPL1 (green). All sections were co-labelled with DAPI (blue). In all panels, “A” marks the apical domain of cells and “B”, the basal domain. Scale bars, 50 μm.
**Figure 3.** Organoids recapitulate the consequences of Apc inactivation in the intestinal epithelia.  

**A.** Fluorescence confocal microscopy of small intestinal epithelial organoids (top panels) and Apc\(^{\text{Min/-}}\) tumouroids (bottom panels). All cells were labelled with DAPI (blue) and fluorescent phalloidin (green; *left panel*) or antibodies to \(\beta 4\)-integrin (green; *middle left panel*), \(\beta\)-catenin (red; *middle right panel*) and ZO-1 (red; *right panel*). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 \(\mu\)m.  

**B.** Organoids (top panels) and Apc\(^{\text{Min/-}}\) tumouroids (bottom panels) were labelled by immunofluorescence using antibodies to \(\beta\)-tubulin and acetylated-\(\alpha\)-tubulin as marked. All cells were labelled with DAPI (blue). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 \(\mu\)m.  

**C.** Immunofluorescent of small intestinal epithelial organoids (left panels) and Apc\(^{\text{Min/-}}\) tumouroids (right panels). *Top panels* show labelling with fUEA (red) and an antibody to \(\beta 4\)-integrin (green). *Middle panels* were labelled with antibodies to pericentrin (red) and an antibody to \(\beta 4\)-integrin (green). *Bottom panels* were labelled with antibodies to ZFPL1 (red) and an antibody to ZO-1 (green). All cells were labelled with DAPI (blue). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 \(\mu\)m.  

**D.** The cellular positioning of the centrosome and the Golgi was scored as apical or apical and clustered in organoids (orgs) or tumouroids (tums) according to the scheme on the right panels for >200 cells from three independent experiments. Values are represented with error bars, \(\pm\) SD and statistical differences in centrosome and Golgi localisation in tumouroids relative to organoids are shown at the \(p<0.01, *, \) or \(p<0.001, **, \) levels of significance calculated by one-tailed student’s t-test.
Figure 4. Switchable in vitro model of tumorigenesis recapitulates the consequences of Apc inactivation in the intestinal epithelia. A. An organoid line bearing pB-shApc, the tet-on inducible transgene system for induction of shApc expression (Fig. S2), untreated (top panels), treated with doxycycline for 10 days (middle panels), or the former followed by doxycycline withdrawal for an additional 6 days (lower panels). Left panels – fluorescence confocal microscopy of organoids labelled with fUEA (red). Middle panels – organoids labelled with antibodies to pericentrin (red). Right panels – organoids labelled with antibodies to pericentrin (red) and DAPI (blue). All specimens were co-labelled with an antibody to β4-integrin (green) and DAPI (blue). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 μm.

B. Fluorescence confocal microscopy of organoids (left panels) and tumouroids (right panels) treated with Wnt3A conditioned media for 72 hours and labelled with antibodies as marked. Top panels - specimens co-labelled with antibodies to pericentrin (red) and β4-integrin (green); bottom panels – specimens co-labelled with antibodies to ZFPL1 (red) and ZO-1 (green). All specimens were co-labelled with DAPI (blue).

Right panels – graphs quantifying apical localisation of centrosome and apical clustering of Golgi for the Wnt-oids and tumouroids treated with Wnt3A. Greater than 200 cells from three independent fluorescent sections were analysed according to the scheme in Figure 3D. Statistical differences amongst organoid, Wnt-oid and tumouroid phenotypes were evaluated using the two-tailed student’s t-test, error bars ± SD.

C. Top – scheme depicting the nocodazole treatment regime for organoid, Wnt-oid and tumouroid cultures. Fluorescence confocal microscopy of cultures treated as follows: control (I); treated with nocodazole (II); or nocodazole treatment followed by drug withdrawal for 72 hours (III). All panels show labelling with fluorescent phalloidin (green) and DAPI (blue) and probed with an antibody to ZFPL1 (red). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 μm.

D. Top – quantitation of apical Golgi clustering (according to the scheme in Fig. 3D). Bottom - percent organoid morphology amongst the nocodazole treatment groups quantified as the percent of organoids that maintained two or more crypts under the treatment conditions. Data is derived from analysis of 50 organoids from two independent experiments. Values are presented with error bars, ± SD and statistical differences in Golgi localisation for treated samples (II and III) relative to control (I) are shown for the p<0.001, *, level of significance using two-tailed student’s t-test. There were no significant differences in organoid morphology amongst the treatment groups.
Figure 5. The Wnt pathway target gene c-Myc does not specify tissue morphology and intracellular organisation upon Wnt pathway activation. A. Representable brightfield images of wild-type and Myc-335^{-/-} organoids grown in increasing concentrations of Wnt3A conditioned media for 7 days. Scale bars, 1000 μm. B. Quantification of Wnt-oid formation under conditions described above. Data displayed is derived from a minimum of 200 individual organoids from two independent experiments for each concentration of Wnt3A conditioned media. Data is presented as %Wnt-oid morphology, error bars ± SD. nd, no Wnt-oids detected. There were no statistical differences in frequency of Wnt-oid morphology between organoid genotypes, evaluated using the two-tailed student’s t-test. C. Fluorescence confocal microscopy of wild-type and Myc-335^{-/-} organoids grown in the
absence or presence of the maximal dose of Wnt3A conditioned media for 7 days. All sections were labelled with DAPI (blue), fluorescent phalloidin (green) and an antibody to ZFPL1 (red). Scale bars, 50 μm. D. Growth rate measured as average Wnt-oid diameter (μM) of wild-type and Myc-335⁻/⁻ organoids after 7 days growth in 100% Wnt3 A conditioned media. Data for the box plots was from greater than 50 organoids from two independent experiments for each organoid type. Data is presented as average diameter ± SD. There were no statistical differences in growth rate between organoid genotypes, evaluated using the two-tailed student’s t-test. E. Model for Apc regulation of proliferation, tissue morphology and intracellular organisation in the small intestinal epithelia. Red arrows represent Apc effector pathways stratified in the current study. See text for details.
Tables

Table 1. Phenotypic analysis of $APC^{Min/+}$ epithelial cells and $APC^{Min/-}$ tumour cells. Values represent the percent of cells that display the correct localisation and disposition for each organelle and cellular structure analysed. Nuclei are considered localised if they follow the plane of the monolayer; for UEA-positive vesicles, Golgi and centrosome, correct localisation is evaluated as supra-nuclear and clustered. Data is the mean of a minimum of three biological replicates ± SD. *, p<0.01 and **, p<0.001 levels of significance from $APC^{Min/+}$ epithelial cells, evaluated by one-tailed student’s t-test.

<table>
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<tr>
<th>Actin cytoskeletal-associated components</th>
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<th>$Apc^{Min/-}$ cells</th>
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</tr>
<tr>
<td>β-catenin</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ZO-1</td>
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<table>
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<th>$Apc^{Min/+}$ cells</th>
<th>$Apc^{Min/-}$ cells</th>
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<tr>
<td>Golgi</td>
<td>88 ± 5</td>
<td>29 ± 7**</td>
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<tr>
<td>Centrosome</td>
<td>95 ± 12</td>
<td>45 ± 9*</td>
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<tr>
<td>FUEA positive vesicles</td>
<td>99 ± 8</td>
<td>40 ± 11**</td>
</tr>
<tr>
<td>Nuclei</td>
<td>95 ± 10</td>
<td>60 ± 8*</td>
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Table 2. Antibodies and molecular probes used for fluorescence confocal microscopy.

Except for phalloidin and the antibody to β4-integrin that required the use of fresh-frozen tissue (see below), all fixation prior to labelling with antibodies and molecular probes was performed using 4% formaldehyde.

<table>
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<tr>
<th>Antibodies</th>
<th>Conjugate</th>
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<td>Acetylated tubulin</td>
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<td>Abcam</td>
<td>ab32072</td>
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<td>Ki67</td>
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<td>ThermoFisher</td>
<td>MA5-14520</td>
<td>1:200</td>
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<td>Lysozyme</td>
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<td>Dako</td>
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</tr>
<tr>
<td>Mouse IgG</td>
<td>HRP</td>
<td>Abcam</td>
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</tr>
</tbody>
</table>

Molecular probes

| DAPI                | SouthernBiotech | 0100-20         |
| Phalloidin          | Alexa Fluor 488 | ThermoFisher    | A12379           | 1:500    |
| UEA-1 (fUEA)        | Rhodamine       | Vector laboratories | RL-1062        | 1:2000   |
Figure S1. No loss of microtubule organisation and intestinal epithelial morphology in Apc^{M1638T/1638T} mice. A. Fluorescence confocal microscopy of sections of small intestinal epithelia sections from Apc^{Min/+} mouse (left panel) and Apc^{Min/-} tumours (right panel) using an antibody to acetylated α-tubulin (grey) and DAPI (blue). “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 50 μm. B. Apc^{Min/+} epithelial (left panel) and Apc^{Min/-} tumours (right panel) sections were labelled with antibodies to phospho-histone 3 (PH3, red), ZO-1 (green) and ZPLF1 (grey) and DAPI (blue). Scale bars, 50 μm. C. Confocal fluorescence images of small intestinal epithelia from a wild-type (top panels) and Apc^{1638T/1638T} (bottom panels) mouse. Panels, left to right, were labelled in order with – an antibody to ZPLF1, fUEA, an antibody to the Ki67 antigen and an antibody to β-tubulin. All sections were co-labelled with DAPI. “A” marks the apical domain of cells in the monolayers and “B”, the basal domain. Scale bars, 100 μm.
A. 

- Brightfield mCherry
- Post-dox (6 days)
- C.

- a-c-Myc
- a-vinculin
- + + - -

- pB-shAPCCTRL
- dox:

- D.

- Apc expression
- 5000
- 4500
- 4000
- 3500
- 25
- 20
- 15
- 10
- 5
- 0
- mCherry expression
- 1.0
- 0.8
- 0.6
- 0.4
- 0.2
- 0

B. 

- No treatment
- On dox (10 days)
- Post-dox (6 days)

C. 

- % spheroid/organoid morphology
- Days of doxycycline withdrawal

D. 

- pB-mCherry
- pB-shApc

E. 

- CTRL
- pB-shAPC

- dox:
- post-dox:

- α-c-Myc
- 0.3 0.7 1 0.6

- α-vinculin
Figure S2. The pB-shApc switchable model of in vitro tumorigenesis/tumour regression recapitulates the phenotypic consequences of oncogenic Apc mutations. A. Transgenes used for the construction of the control pB-mCherry or pB-shApc organoid lines. The shApc expression transgenic system includes pB-CAG-rtTA for constitutive rtTA expression and the plasmid for expression of the piggybac transposase for stable integration into organoids. In-built tet-on system enables inducible expression of shApc linked to mCherry by treatment of pB-shApc organoids with doxycycline. B. Time-course of doxycycline treatment (dox) of pB-shApc organoids; after ten days organoids convert to spheroids accompanied by mCherry expression. Subsequent withdrawal of doxycycline and growth for an additional 6 days (post-dox) restores the budding organoid morphology. Scale bar, 200 μm. C. Quantification of the conversion of spheroid morphology to budding organoids morphology upon withdrawal of doxycycline over the course of 6 days. Grey boxes represent the remaining spheroids as a percentage of the total; white boxes are the number of organoids that have intra-converted from spheroids. Data is derived from greater than 100 organoids from two independent experiments scored at each timepoint. Statistical differences between organoid and spheroid morphologies were evaluated using the two-tailed student’s t-test, error bars± SD. D. QRT-PCR quantification of relative Apc (top graph) and mCherry expression (bottom graph) in pB-mCherry control organoids (engineered for inducible expression of mCherry) and pB-shApc organoids (engineered for inducible expression of mCherry and shApc) after two days doxycycline treatment (dox) and at 6-days post-withdrawal (post-dox). Values for Apc and mCherry expression levels are presented with error bars, ± SD. Statistical differences in expression levels after 6-days post-withdrawal relative to doxycycline treated organoids at the p<0.001 level of significance is represented by **. E. Protein lysates from control (pB-Cherry) organoids or pB-shApc organoids with various treatments (no treatment, 2 days doxycycline treatment, 6 days post-doxycycline withdrawal; post-dox), shown above, were subject to western blot and probed with antibodies to c-Myc and vinculin as loading control. Quantified relative expression of c-Myc in the lysates were derived from densitometry of the corresponding western blot signals.
Figure S3. Genotyping of intestinal epithelia from Myc-335⁻/⁻ mouse used in this study (Sur et al., 2012) PCR genotyping of Myc-335⁻/⁻ intestinal epithelia using primers targeting the wild-type (WT; target amplicon length is 227 bp) or Myc-335 mutant allele (mut; amplicon length is 311 bp) (Sur et al., 2012).