Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration

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Although Apc is well characterized as a tumor-suppressor gene in the intestine, the precise mechanism of this suppression remains to be defined. Using a novel inducible AhcCre transgenic line in conjunction with a loxP-flanked Apc allele we, show that loss of Apc acutely activates Wnt signaling through the nuclear accumulation of β-catenin. Coincidently, it perturbs differentiation, migration, proliferation, and apoptosis, such that Apc-deficient cells maintain a “crypt progenitor-like” phenotype. Critically, for the first time we confirm a series of Wnt target molecules in an in vivo setting and also identify a series of new candidate targets within the same setting.

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The Apc gene encodes the adenomatous polyposis coli tumor-suppressor protein, the germline mutation of which characterizes Familial Adenomatous Polyposis [FAP], an autosomal syndrome characterized by multiple colorectal cancers [Kinzler et al. 1991]. Inactivation of APC is also recognized as a key early event in the development of sporadic colorectal cancers. By targeting Apc, mice bearing a lox-flanked Apc allele fl/ were given in Supplementary Fig. 1). To investigate the phenotype of conditional deletion of Apc, mice bearing a lox-flanked Apc allele were crossed onto a novel inducible cre transgenic background, which uses the Cyp1A promoter to deliver inducible cre expression in the intestine [details of this AhCre transgene are given in Supplementary Fig. 1]. CreApcfl/fl progeny were identified and were subjected to four daily injections of β-napthoflavone at 8–10 wk of age, resulting in virtually 100% intestinal recombination as scored through the Rosa26R allele (Supplementary Fig. 1). On day 5, CreApcfl/fl mice became visibly ill and were killed. In contrast, CreApc−/− mice showed no symptoms of illness.

Histological analysis of the organs from the naphthoflavone-induced CreApcfl/fl mice revealed altered crypt–villus architecture such that a discrete crypt was no longer identifiable, but morphologically atypical “crypt-like” cells now occupied the majority of the crypt–villus axis [Fig. 1a,b]. Cells were more densely packed than in control mice, and the upper extent of phenotypic change was readily identifiable from hematoxylin-and-eosin-stained material [Fig. 1a,b]. To confirm that the pattern of atypical histology was coincident with the pattern of overt preneoplastic lesions.

Cell renewal within the intestinal epithelium is highly regulated and position dependent. Absorptive cells generated within intestinal crypts migrate upward until they either die by apoptosis or are shed into the gut lumen, a process that takes 3–5 d [Potten et al. 1997]. A central role for Apc in controlling this process has been suggested by two recent studies. First, blockade of Wnt signaling in cultured cells has been used to implicate normal Wnt signaling in imposing a crypt progenitor phenotype [van de Wetering et al. 2002]. Second, Wnt signaling has been shown to influence paneth cell positioning in the crypt through transcriptional activation of EphB2 and EphB3 [Batlle et al. 2002]. Furthermore, a role for Apc in cell migration has been suggested based on its complex association with, and effect on, cytoskeletal proteins.

Here, for the first time, we determine the immediate consequences of Apc loss in otherwise normal murine epithelium. Inactivation of Apc leads to the rapid nuclear relocalization of β-catenin, a coincident gross change in the transcriptome, and a coordinated series of cellular changes, including failure to migrate and differentiate. Together, these effects allow evasion of the normal ablative fate.

Results and Discussion

Loss of Apc perturbs intestinal pathology and causes morbidity after 5 d

To investigate the phenotype of conditional deletion of Apc, mice bearing a lox-flanked Apc allele were crossed onto a novel inducible cre transgenic background, which uses the Cyp1A promoter to deliver inducible cre expression in the intestine [details of this AhCre transgene are given in Supplementary Fig. 1]. CreApcfl/fl and CreApc−/− progeny were identified and were subjected to four daily injections of β-napthoflavone at 8–10 wk of age, resulting in virtually 100% intestinal recombination as scored through the Rosa26R allele [Supplementary Fig. 1]. On day 5, CreApcfl/fl mice became visibly ill and were killed. In contrast, CreApc−/− mice showed no symptoms of illness.

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Apc inactivation, sections were stained for Apc expression [Fig. 1c,d]. In induced Cre’Apc+/+ mice, the pattern of loss of Apc directly overlaid the pattern of histological change. Analysis of Cre’Apc+/+ mice additionally transgenic for the Rosa26R reporter locus confirmed that 5 d after the first injection of the cre-inducing agent β-naphthovlone. [a,b] Hematoxylin-and-eosin-stained sections for control, induced Cre’Apc+/+ (a), and induced Cre’Apcfl/fl (b) mice, showing an enlarged crypt-like region in the induced Cre’Apcfl/fl mice. [c,d] Apc immunofluorescence in induced Cre’Apc+/+ (c) and in induced Cre’Apcfl/fl (d) mice. Loss of Apc protein was observed in all morphologically atypical cells [below the arrow] in Cre’Apc+/+ mice. Apc staining was observed in unrecombined morphologically normal cells in the villus [above the arrow]. [e,f] Wholemount preparations of intestines stained for lacZ activity to report cre-mediated recombination at the Rosa26R locus. Recombined control cells completely repopulate the crypt–villus axis (e) but fail to do so in the absence of Apc [f]. [g] Sectioned material from f confirming that the pattern of recombination scored by LacZ activity directly overlays the pattern of histological change. Note that the staining for β-galactosidase exactly complements the Apc staining, with only the morphologically atypical cells [below the arrows] staining for β-galactosidase. For all panels, arrows indicate the point of demarcation between normal and atypical histology.

**Figure 1.** Changes in intestinal crypt pathology 5 d after the first injection of the cre-inducing agent β-naphthovlone. [a,b] Hematoxylin-and-eosin-stained sections for control, induced Cre’Apc+/+ (a), and induced Cre’Apcfl/fl (b) mice, showing an enlarged crypt-like region in the induced Cre’Apcfl/fl mice. [c,d] Apc immunofluorescence in induced Cre’Apc+/+ (c) and in induced Cre’Apcfl/fl (d) mice. Loss of Apc protein was observed in all morphologically atypical cells [below the arrow] in Cre’Apc+/+ mice. Apc staining was observed in unrecombined morphologically normal cells in the villus [above the arrow]. [e,f] Wholemount preparations of intestines stained for lacZ activity to report cre-mediated recombination at the Rosa26R locus. Recombined control cells completely repopulate the crypt–villus axis (e) but fail to do so in the absence of Apc [f]. [g] Sectioned material from f confirming that the pattern of recombination scored by LacZ activity directly overlays the pattern of histological change. Note that the staining for β-galactosidase exactly complements the Apc staining, with only the morphologically atypical cells [below the arrows] staining for β-galactosidase. For all panels, arrows indicate the point of demarcation between normal and atypical histology.

**Loss of Apc alters the normal pattern of differentiation and proliferation**

We next examined how loss of Apc had perturbed differentiation using a number of histological markers [Fig. 2]. Apc-deficient cells were no longer capable of expressing the villus cell markers alkaline phosphatase [Fig. 2a,b] or Villin [Supplementary Fig. 2]. Figure 2d shows that Goblet cells are lost in the absence of Apc and that no binding is observed for the Pokeweed lectin. Despite this latter observation, lysozyme staining did identify Paneth cells, but these were now distributed throughout the crypt-like area, rather than being confined to their normal position at the crypt base [Fig. 2f]. These observations therefore reflect perturbed positioning and also possibly perturbed differentiation of the Paneth cells. Notably, the distribution of these Paneth-like cells directly parallels that observed in EphB3−/− mice (Batille et al., 2002) and is presumably a consequence of alterations in the EphB gradient. We also evaluated the relative proportion of enteroendocrine cells, finding them to be reduced in the aberrant areas [Supplementary Fig. 2; 1.5% in control crypts compared with 0.8% in induced Cre’Apcfl/fl crypts, p = 0.04, Mann Whitney U test]. Changes were also observed in the expression patterns of a number of extracellular matrix proteins, including laminin α1 and α2, laminin γ2, tenascin, and perlecan (Supplementary Fig. 2). These findings indicate that loss of Apc in the epithelium also has an immediate effect on the surrounding mesenchyme.

Previous studies have characterized a defined proliferative zone within the normal crypt. To test whether this zone was altered, mice were injected with BrdU and killed 2 h later [Fig. 3a,c]. The location and proportion of cells in S phase was then scored. In control mice, cycling cells were confined to the expected mid crypt region [Fig. 3a,e]. However, induced Cre’Apcfl/fl crypts showed a 45% increase in the proliferation index [Cre’Apc+/+ crypts 23.6% ± 2.3% standard error, Cre’Apcfl/fl crypts 34.4% ± 2.0%, p = 0.04, Mann Whitney, n = 3], with proliferation now occurring independently of position [Fig. 3c,f]. Staining with Mcm2 antibody further supported these observations, as it marked an expanded zone of proliferative competence in the mutants [Fig. 2g,h].

**Apc deficiency abrogates migration**

We next tracked the pattern of migration of BrdU-labeled cells by killing mice 24 h after BrdU exposure [BrdU is bioavailable for <2 h, effectively “tagging” those cells in S phase; Fig. 3b,d]. In induced Cre’Apc+/+ mice, the average distance of BrdU-positive cells from the base of the crypt increased by 60 µm at 24 h [control average distance at 2 h, 32.73 µm ± 3.31 µm S.E., and at 24 h, 98.64 µm ± 3.49 µm; Cre’Apc+/+ mice at 2 h, 92.35 µm ± 11.56 µm, and at 24 h, 92.86 µm ± 7.41 µm]. In the induced Cre’Apcfl/fl mice, the average value did not change, nor did the cumulative distribution of labeled nuclei [Fig. 3f]. We therefore show that inactivation of Apc completely abrogates migration along the crypt–villus axis. This change provides a ready mechanism both for the selective retention of somatically arising Apc mutant cells and for the acquisition of further genetic changes. It is, however, clear that the effect of Apc deficiency extends beyond migration, as deficiency also leads to the production of daughter cells that have many properties of premalignant cells, including both failed differentiation and increased proliferation.

The changes observed in proliferation were accompanied by enhanced levels of cell death, with elevated levels of apoptosis in the Cre’Apcfl/fl mice either scored...
in differentiation and migration. The enhanced perturbation associated with Apc loss may reflect differences in the timing, extent, or localization of β-catenin up-regulation or, indeed, β-catenin independent effects.

Loss of Apc leads to rapid nuclear localization of β-catenin and allows identification of Wnt targets in an in vivo setting

We next investigated β-catenin levels within the Cre’Apcfl/fl tissue at day 5. There was no increase in total β-catenin in the Cre’Apcfl/fl samples [Fig. 4a]. However, levels of dephosphorylated β-catenin were moderately elevated and, crucially, β-catenin relocalized to the nuclei in the Cre’Apcfl/fl tissue [Fig. 4c, Supplementary Fig. 3]. This analysis showed that relocalization occurred at day 5, and this was coincident with the observed onset of changes in morphology, proliferation, and apoptosis.

To test whether nuclear β-catenin was activating transcription of its known target genes, microarray analysis was performed using the af-

Figure 2. Perturbation of differentiation in the absence of Apc. (a,b) Immunohistochemical staining for alkaline phosphatase in control, induced Cre’Apc+/+ mice [e] and induced Cre’Apcfl/fl intestines. Apc mutant cells do not express the villus marker alkaline phosphatase, with the arrow indicating demarcation between the residual wild-type and recombined Apc-deficient cells. (c,d) Staining for Goblet cells [blue cells stained with alcian blue] and Paneth cells [red cells stained with Plokeweed lectin] in the crypt–villus axis of control induced Cre’Apc+/+ mice [c] and induced Cre’Apcfl/fl intestines [d]. Insets show detail of crypts. No Goblet or Paneth cell staining was observed in the induced Cre’Apcfl/fl mice. (e,f) Staining for lysozyme in induced Cre’Apc+/+ [e] and induced Cre’Apcfl/fl [f] mice. Lysozyme positivity is confined to the base of the crypt in the controls but is distributed throughout the crypt-like region in the induced Cre’Apcfl/fl mice. (g,h) Mcm2 staining with positivity restricted to the crypt within the induced Cre’Apc+/+ mice [g] but extending through the aberrant crypt structure in the induced Cre’Apcfl/fl mice [h].

histologically (control crypts, 3.5% ± 0.45%; S.E.; Cre’Apcfl/fl, 11.1% ± 1.2, p = 0.01, Mann Whitney, n = 5) or identified immunohistochemically with an antibody against active caspase-3 [Fig. 3a,b]. These findings contrast with previous studies [Bedi et al. 1995] that have reported reduced levels of apoptosis in colorectal cancer. This is presumably a reflection of our focus on the primary consequences of Apc loss within a naive epithelium, rather than on the adapted behavior of a neoplastic population. There appear three possible explanations for the increase we observe in apoptosis. First, this may be a direct consequence of c-Myc up-regulation in a manner analogous to that demonstrated in pancreatic β cells [Pelengaris et al. 2002]. Second, this may result from altered matrix interactions that subsequently stimulate an anoikis signal. In support of this, we see changes in the patterns of expression of many of the laminins, of MMP7, and of the antiadhesive molecule tenasin [Probstmeier et al. 1990]. Third, mitosis may be aberrant, as Apc has been implicated in the formation of mitotic spindles [Fodde et al. 2001; Kaplan et al. 2001]. Indeed, many of the apoptotic figures we observed were large, a phenomenon previously interpreted to reflect death of 4N cells at a G2/M checkpoint [Merritt et al. 1997].

Elevated apoptosis and enhanced proliferation have been described previously in studies that have altered β-catenin/TCF4 signaling in intestinal epithelium [Wong et al. 1998, 2002]. However, the changes we identify are far more extensive, including, in addition, gross changes in differentiation and migration. The enhanced perturbation associated with Apc loss may reflect differences in the timing, extent, or localization of β-catenin up-regulation or, indeed, β-catenin independent effects.

Loss of Apc leads to rapid nuclear localization of β-catenin and allows identification of Wnt targets in an in vivo setting

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Figure 3. Altered proliferation, migration, and apoptosis in the absence of Apc. (a–f) BrdU staining in control and Apc-deficient intestinal epithelium. (a) BrdU-positive cells in wild-type-induced Cre’Apc+/+ crypts 2 h following BrdU injection are confined to a proliferative zone within the crypt. (b) The position of BrdU-positive cells in crypt–villus axis of control, induced Cre’Apc+/+ mice 24 h after BrdU injection, indicating migration of labeled cells onto the villus. (c) BrdU staining in induced Cre’Apcfl/fl epithelium 2 h after BrdU injection show that positively stained cells are present throughout the entire aberrant crypt-like structure. (d) BrdU staining 24 h after injection of BrdU in induced Cre’Apcfl/fl intestinal epithelium. (e) Position of BrdU-positive cells within the control crypt–villus axis at 2 h [black bars] and 24 h [open bars]. Position 0 represents the base of the crypt. Note the increase in position at 24 h, reflecting migration. (f) Position of BrdU cells within the crypt-like structure in induced Cre’Apcfl/fl mice at 2 h [black bars] and 24 h [open bars]. Unlike in the controls, no migration of labeled cells is evident. (g,h) Increased apoptosis in Apc mutant crypts. Immunohistochemical staining of active caspase 3 in induced Cre’Apc+/+ mice [g] and induced Cre’Apcfl/fl mutant crypts [h]. This staining confirmed apoptosis counts from H&E-stained sections that showed significantly increased apoptosis in Apc deficient crypts (11.1% ± 1.2%) compared with control crypts (3.5% ± 0.45%; p = 0.01; Mann Whitney, n = 5).
of dysregulated genes. Up-regulation of CD44, C-Myc, laminin γ2, EphB2, and EphB3 was confirmed immunohistochemically in the Apc-deficient tissue (Figs. 4d–g, 5a–d; Supplementary Fig. 2). Expression of the EphrinB2 ligand, which is normally restricted to the top of the crypts and villi (Fig. 5f), was reduced in concordance with the reduction in villus differentiation in the Cre′Apc+/+ tissue (Fig. 5g). These data therefore confirm, in an in vivo setting, many of the targets of Wnt signaling that have been implicated from in vitro studies (e.g., Bienz and Clevers 2000; van de Wetering et al. 2002). These include up-regulation of CD44, C-Myc, MMP-7 (matrilysin), γ2 laminin, Sema3c [confirmed by RT–PCR; Supplementary Fig. 4] Ets-2, EphB2, EphB3, and GPR49. The array analysis also indicated up-regulation of a series of genes that either interact with CD44 or are targets of CD44. These include MMP-7, Tiam1, FGFR4, and its receptor, and FASR-2. The up-regulation of Tiam1 (confirmed by RT–PCR; Supplementary Fig. 4) is particularly interesting, as Tiam1 has been shown to mediate Ras signaling. Indeed, mice deficient in Tiam1 are resistant to Ras-induced skin tumors (Malliri et al. 2002).

Batlle et al. (2002) have shown that deficiency of EphB3 leads to abnormal Paneth cell positioning in the crypt. We show here that Wnt-mediated up-regulation of EphB3 yields a similar Paneth cell phenotype, confirming a pivotal role for the EphB/ephrinB mutual repulsion system in defining crypt–villus architecture. Our results are also consistent with the notion that Apc mutant cells express the same genetic program as cells at positions 1–2 of the crypt (van de Wetering et al. 2002), with notable increases in EphB3, MMP7, and Pla2g2a being characteristic of both Paneth cells (Stappenbeck et al. 2003) and the Apc-deficient cells described here.

In summary, we have shown that Apc is a critical determinant of cell fate in the murine small intestinal epithelium. Acute activation of Wnt signaling immediately produces many of the phenotypes associated with early colorectal lesions: failed differentiation, increased proliferation, and aberrant migration. Within a short time scale, multiple processes are affected: interactions with the cellular matrix, interactions with the basement membrane, increased proliferation, and failure of positional cues (EphB/ephrinB).

At present, many different strategies are being pursued to identify the genes controlled by Wnt signaling. However, these models are necessarily constrained by the experimental systems used. For example, analysis of Wnt blockade within immortalized cells might not be predicted to precisely model active Wnt signaling in a physiological setting. The ability to acutely activate Wnt signaling in normal epithelial cells has allowed us for the first time to examine many of the proposed targets of Wnt signaling in vivo. Remarkably, given the diverse nature of the models studied, we can validate virtually all the previously reported Wnt targets in an in vivo set-
leading edge of aberrance, where unrecombined villus cells stain for EphB2 staining throughout the aberrant crypt structure in the induced Cre+Apcfl/fl allele. Progeny from this cross were intercrossed to derive an outbred Rosa26R reporter allele. The loss of Ephrin B2 staining in crypt enterocytes, predominantly confined to the lower half of control crypts, produces a ranked list of genes, of which the probability that each individual gene is deregulated is 95%.

Materials and methods

Mouse colony

All experiments were performed according to UK Home Office regulations. Ahcre-positive mice were intercrossed with mice carrying a LoxP-flanked Apc allele: Apc<sup>Cre</sup> (Shibata et al. 1997) and the Rosa26R reporter allele. Progeny from this cross were intercrossed to derive an outbred colony, segregating for the C57BL6/J, 129/Ola, and C3H genomes at a ratio of 75%, 12.5%, and 12.5%, respectively.

RT-PCR

RT–PCR for Cre, EphB2, Semax, Pla2g2a, and T1AM1 were performed using the approach shown in Supplemental Material. Tissues were snap frozen, and RNA was extracted using the TRIzol method (Gibco).

β-Galactosidase analysis

To determine the pattern of recombination at the Rosa26R reporter locus, both whole mounts and sectioned material were analyzed as previously described (Ireland et al. 2004; see also Supplemental Material).

Tissue isolation

To induce recombination, mice were given daily intra-peritoneal injections of β-naphthoflavone (80 mg/kg). At each time point, mice were killed and the small intestine removed and flushed with water. Intestines were dissected as follows: The proximal 7 cm was mounted en face, fixed overnight in methacarn (methanol, chloroform, and acetic acid, 4:2:1 ratio), and paraffin embedded. The following 3 cm was opened and placed into RNA later (Sigma), ensuring that all mesentery and Peyers patches were removed. The following 5 cm was divided into 1-cm lengths, bundled using surgical tape, and then fixed in 4% formaldehyde at 4°C for no more than 24 h before processing. Samples for Western analysis, frozen sections, and electron microscopy were then taken from the next 5 cm of intestine, and the remainder was fixed in methacarn.

Assaying apoptosis, mitosis, and S-phase labeling in vivo

Apoptosis and mitotic index were scored from hematoxylin-and-eosin-stained sections as previously described (Merritt et al. 1997). Apoptosis was independently confirmed by immunohistochemical staining with an antibody against active caspase 3 (1:750; R&D systems, Marshman et al. 2001). For BrdU labeling, mice were injected with 0.25 mL of BrdU (Amersham), and staining was performed using an anti-BrdU antibody conjugate (Roche) at 1 part in 50.

Western analysis and immunohistochemistry

For a detailed description, see Supplemental Material. Immunohistochemistry was performed according to Batlle et al. (2002), Kongkanunt et al. (1999), and Van Noort et al. (2003). Western analysis was performed according to Hinck et al. (1994) and Van Noort et al. (2003).

RNA extraction and array

Eight-week-old male sibling mice were used for the array analysis. Three centimeters of the small intestine located 7 cm from the stomach was placed in RNAlater (after removing any mesentery and ensuring that no Peyers patches were present). The tissue was then homogenized in trizol reagent and RNA extracted using standard phenol-chloroform methodologies. Biotinylated target cRNA was generated according to http://www.paterson.man.ac.uk/facilities/mbed/protocols.jsp. Affymetrix Gene Arrays were run at the Cancer Research UK facility at the Paterson Institute for Cancer Research. Raw signal intensities were initially screened using the MaxD/View-Program [http://www.bioinf.man.ac.uk/microarray/maxd/index.html] to remove false-positive and false-negative signals (using a cutoff of at least one p value for each transcript <0.02). The data was then normalized by global normalization, again using the MaxD/View-Program. Data was finally analyzed using the SAM-Program (Tusher et al. 2001). A 0.8 value, which produced a false discovery rate of 5% was used to define the candidate genes. The output from this analysis therefore produces a ranked list of genes, of which the probability that each individual gene is deregulated is 95%.

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