Cited1 Deficiency Suppresses Intestinal Tumorigenesis

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Abstract
Conditional deletion of Apc in the murine intestine alters crypt-villus architecture and function. This process is accompanied by multiple changes in gene expression, including upregulation of Cited1, whose role in colorectal carcinogenesis is unknown. Here we explore the relevance of Cited1 to intestinal tumorigenesis. We crossed Cited1 null mice with ApcMin/+ and AhCreApcfl/fl mice and determined the impact of Cited1 deficiency on tumour growth/initiation including tumour multiplicity, cell proliferation, apoptosis and the transcriptome. We show that Cited1 is up-regulated in both human and murine tumours, and that constitutive deficiency of Cited1 increases survival in ApcMin/+ mice from 230.5 to 515 days. However, paradoxically, Cited1 deficiency accentuated nearly all aspects of the immediate phenotype 4 days after conditional deletion of Apc, including an increase in cell death and enhanced perturbation of differentiation, including of the stem cell compartment. Transcriptome analysis revealed multiple pathway changes, including p53, PI3K and Wnt. The activation of Wnt through Cited1 deficiency correlated with increased transcription of β-catenin and increased levels of dephosphorylated β-catenin. Hence, immediately following deletion of Apc, Cited1 normally restrains the Wnt pathway at the level of β-catenin. Thus deficiency of Cited1 leads to hyper-activation of Wnt signaling and an exaggerated Wnt phenotype including elevated cell death. Cited1 deficiency decreases intestinal tumourigenesis in ApcMin/+ mice and impacts upon a number of oncogenic signaling pathways, including Wnt. This restraint imposed by Cited1 is consistent with a requirement for Cited1 to constrain Wnt activity to a level commensurate with optimal adenoma formation and maintenance, and provides one mechanism for tumour repression in the absence of Cited1.

Introduction
Inactivation of the APC (adenomatous polyposis coli) gene marks one of the earliest events in colorectal tumorigenesis [1], an observation that has given rise to the concept of Apc as a ‘cellular gatekeeper’ protecting against tumorigenesis [2]. This role in suppressing tumour formation has been closely associated with its ability to regulate the level of β-catenin within cells. Thus, Apc normally forms part of the scaffold of proteins that phosphorylate β-catenin and target it for degradation. In the absence of Apc, β-catenin levels become elevated and translocates to the nucleus, where it drives increased transcription of Wnt target genes associated with cell proliferation and cell death [3].

To investigate the biological consequences of Apc loss and Wnt activation, we and others have previously used a conditional model of Apc loss. In this model, deletion of Apc is achieved through use of an inducible AhCre transgene, which is responsive to exposure to the xenobiotic β-naphthoflavone. Following Cre induction and loss of function of Apc, we observe a range of rapid phenotypic changes. These include promiscuous entry of cells into S phase, loss of differentiated cell types, loss of cell polarity and disorganisation of the crypt-villus structure to the point that discrete crypts are no longer discernable. Apc deficiency also reduces the normal migration of cells along the crypt villus axis, leading to the preferential retention of Apc deficient cells. These changes may all be considered pro-tumourigenic, however we also observe a considerable stress signal within Apc deficient cells, most clearly shown by a significant elevation in apoptosis. These phenotypic changes are accompanied by the expected elevation in levels of nuclear β-catenin and marked changes in the transcriptome [3].

One of the changes we observe in the intestinal epithelial cells of AhCreApcfl/fl mice is a strong induction of Cited1, a bifunctional transcriptional cofactor which is able to activate or repress transcription in association with other transcription factors [4,5]. We also found this induction to be dependent upon functional c-Myc as Cited1 expression returns to basal levels in the additional absence of c-Myc, which completely
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Author Summary

Colorectal cancer is the fourth leading cause of cancer related deaths worldwide, and a key genetic change associated with this disease is mutation of the gene APC. APC encodes a protein which plays a regulatory role in the Wnt signalling pathway. To better understand the mechanisms leading to colorectal cancer after APC loss, we have used a mouse model in which we deleted Apc in the bowel and which developed several characteristics of early stage cancers. Here, we show that after Apc loss, the expression of another gene, Cited1, is increased in mice and human colorectal tumours. To study the role of Cited1 in bowel cancer after loss of Apc, we generated mice mutant for Apc (Min) or mutant for Apc and Cited1 (MinCited1). We observed that MinCited1 mice developed fewer intestinal tumours and lived longer than Min mice suggesting that Cited1 is pro-tumourigenic. However, we also observed that Cited1 deficiency actually increased many of the aspects associated with loss of Apc, including deregulation of the Wnt pathway and cell death. To explain this apparent paradox, we propose a model whereby loss of Cited1, in the context of deregulated Wnt signalling, ‘over-stimulates’ the Wnt pathway, the net effect of which is to inhibit tumourigenesis.

We next assessed Cited1 levels in adenomas developing in the ApcMin/+ mouse model of human colorectal cancer, which allows evaluation of the effects of loss of Apc function over the course of polyp development. Again, the level of Cited1 expression was significantly increased in intestinal polyps from ApcMin/+ mice compared to normal tissue from the same mouse (p<0.01, Figure 1C; Figure S1A). Detection of high levels of Cited1/CITED1 expression in both human and murine tumours suggests that Cited1/CITED1 may play a role in intestinal tumourigenesis.

We performed in situ hybridization using a Cited1 probe designed against the deleted sequence in the Cited1+/- mouse (Figure 1D). We observed low levels of staining (compared to expression of the housekeeping gene P0l2a) throughout the crypt-villus structure of the ApcCreWT mouse intestine (WT) with a trend to higher levels within the crypt. There was no apparent specificity for the stem cell region at the base of the crypt or for any differentiated cell type. Consistent with QPCR data, we observed an increase in the level of staining throughout the crypt-villus structure of the ApcCreApCreP0l2a mouse.

Loss of Cited1 in ApcMin/+ mice increases survival and reduces the number of intestinal adenomas

As Cited1 is upregulated in colonic tumours we next asked whether deficiency of Cited1 could inhibit intestinal adenoma formation in the ApcMin/+ mouse. To achieve this, we crossed Cited1 null (Cited1−/−) mice onto ApcMin/+. Given that Cited1 is on the X-chromosome, we aged male cohorts of ApcMin+/−Cited1−/− and ApcMin+/− mice until they displayed symptoms of intestinal neoplasia (rectal bleeding and paleness). The median lifespan of ApcMin/+ mice was 230.5 days, which is increased to 515 days in the ApcMin+/−Cited1−/− mice (Log-Rank p = 0.001, Figure 2A). Cited1−/− mice had a survival rate that was not significantly different to that of WT mice (Figure 2A).

We next counted the number of adenomas in the small and large intestine (Figure 2B). ApcMin+/−Cited1−/− mice developed significantly less tumours compared to ApcMin+/− in both the small intestine (5 versus 18.5 adenomas p<0.05) and the large intestine (1 versus 6.3 adenomas, p<0.05). The tumour distribution in the small intestine and the colon was analysed at ill health (Figure

of a range of oncogenic signaling pathways, including the Wnt pathway.

Results

Cited1/CITED1 is up-regulated in the intestine of Apc deficient mouse models and human colorectal tumours

We have previously shown that deletion of Apc in the mouse intestine leads to nuclear β-catenin translocation and up-regulation of Wnt target genes, including Cited1, as scored by microarray analysis [3]. To confirm this upregulation, we analysed mouse intestinal epithelium from ApcCreApCreWT and ApcCreWT WT; wild type) mice which had been induced by intraperitoneal injection of β-naphthovalone 4 days previously [3]. Quantitative PCR analysis revealed significant upregulation of the Wnt targets c-Myc, Axin2 and Cd44 in the absence of Apc. Similarly, Cited1 showed a 15-fold increase in expression (p<0.05, Figure 1A).

To determine if CITED1 was also deregulated in human cancers, we performed a Tagman quantitative PCR on human colorectal tumour tissues. In comparison to paired normal tissues from the same patient, we observed over-expression of the human orthologues of the Wnt target genes c-MYC, AXIN2, CD44 and CITED1 (p<0.01, Figure 1B). These data demonstrate the potential transferability of our data from the acute Apc deletion mouse model to human colorectal carcinogenesis.
**Figure 1. Cited1 over-expression in Apc\textsuperscript{Min/+} mice, AhCre\textsuperscript{Apc\textsuperscript{fl/fl}} mice and human colorectal cancer.**

A: QPCR analysis of Wnt target genes 4 days after conditional deletion of Apc in the small intestinal. * p<0.05, Mann-Whitney U test, n = 3. B: Taqman qPCR analysis of gene expression in human CRC tumour tissue presented as fold change relative to adjacent normal tissue. The horizontal line indicates median fold change. p<0.01, Wilcoxon Signed Rank Test. C: Semi-QPCR analysis of Cited1 expression in Apc\textsuperscript{Min/+} adenomas. Three Apc\textsuperscript{Min/+} mice were used and 3–6 tumours were taken from each individual, n = 14. (p<0.01, Mann-Whitney U test). D: Cited1 in situ hybridization showing the increase in Cited1 expression in the AhCre\textsuperscript{Apc\textsuperscript{fl/fl}} compare to AhCre\textsuperscript{WT} mouse. The staining is represented by single dots which correspond to the Cited1 transcript. The low level of staining (compared to positive control probe Polr2a) of Cited1 probe is distributed throughout the crypt-villus structure in the AhCre\textsuperscript{WT} mouse and in AhCre\textsuperscript{Apc\textsuperscript{fl/fl}} mouse with an increase in staining after loss of Apc. Absence of staining is observed in the Cited1\textsuperscript{−/−} intestinal tissue and in the negative control probe DapB. Inset panels show magnifications of intestine of corresponding zone.

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There was no significant difference in the percentage of tumours found in the duodenum or jejunum of the small intestine, or in the large intestine. However, we did observe a significant increase in the percentage of tumours found in the last part of the small intestine which corresponds to the human ileum (Figure S1C). Total tumour burden of ApcMin/+ mice was not significantly different from that of ApcMin/+ (Figure 2C), and shared the same tubular morphology and degree of invasiveness, as assessed histologically by the frequency of invasion into the submucosa (52.8% High grade +47.16% Low grade in ApcMin/+ vs 42.8% High grade +57.4% Low grade in ApcMin/+Cited1−, Chi-square $\chi^2 = 2.1$, DF $= 1$, $p > 0.05$). These data suggest that mice became symptomatic of disease when they had developed an equivalent tumour burden, but that in the Cited1 mutant background this was significantly later and reflected fewer, but larger lesions at these later time points, hence implicating Cited1 in intestinal tumour initiation.

Cited1 deficiency modifies the phenotype observed immediately after Apc loss by increasing the number of BrdU positive cells and the size of the hyperplastic area

To address the mechanism underlying the reduction of adenoma formation in ApcMin/+Cited1− mice, we crossed Cited1− mice with conditionally mutant for Apc. We have previously demonstrated that we can achieve almost 100% recombination of the Apcfl/fl allele in the intestine using the b-napthoflavone inducible AhCre transgene to drive recombination [3]. Thus, AhCre+WT, AhCre+Apcfl/fl, AhCre+Cited1−, and AhCre+Apcfl/flCited1− mice were induced with b-napthoflavone and culled 4 days after the first injection to determine the role of Cited1 immediately following deletion of Apc. To confirm the level of Apcfl/fl recombination we used quantitative RT-PCR and again found that 100% of the PCR products obtained were from the recombined Apc allele (Figure S1B). We also confirmed Cited1 deficiency in Cited1− mice using RT-PCR. We observed a significant 3.81 fold difference decrease in Cited1 expression in AhCre+Cited1− compared to AhCre+WT mice (p < 0.05 Mann-Whitney U test). The small difference observed is most likely due to the low level of expression of Cited1 in the intestine [21]. Due to the increased level of Cited1 expression after loss of Apc, Cited1 deficiency is more noticeable in the intestinal epithelial cells of AhCre+Apcfl/flCited1− mice which showed a 277.81 fold decrease compared to AhCre+Apcfl/fl mice (p < 0.05 Mann-Whitney U test).

We have previously shown that the loss of Apc leads to an increase in proliferation and apoptosis and also to a loss of migration [3]. To analyse the effects of Cited1 deficiency after Apc loss, we first counted...
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**Figure A**

| WT | Cited1+ | Apcfl/fl | Apcfl/flCited1+ |
|----|---------|----------|----------------|

| 100μm | 50μm |

**Figure B**

Bar graph showing the number of BrdU positive cells per crypt-hyperplastic area.

**Figure C**

Cumulative frequency plots for different cell positions in different conditions.
the number of cells in S phase within the crypt or hyperplastic areas (formed after Apc loss). On day 4 after β-naphthoflavone induction, mice were injected with BrdU to label cells in S phase and culled 2 hrs later (Figure 3A). In Apcfl/fl WT and Apcfl/flCited1−/− mice the number of proliferating cells was not significantly different (Apcfl/fl WT: 18.97 vs Apcfl/flCited1−/−: 20.65 BrdU positive cells/ Crypt; p>0.05. Figure 3B). However, the number of cells in S-phase was significantly increased in the hyperplastic areas of Apcfl/flCre+ Apcfl/flCited1−/− mice compared to Apcfl/flCre+ Apcfl/fl (7.43 vs Apcfl/flCre+ Apcfl/flCited1−/−: 106.4 BrdU positive cells/area, p<0.05, Figure 3B) suggesting a role for Cited1 in controlling cell proliferation in the context of active Wnt signaling.

We next analysed the histology on HE sections of the intestinal tissue from all the genotypes after β-naphthoflavone induction. There were no gross changes in the crypt/villus architecture in Apcfl/fl WT compared to Apcfl/flCited1−/− mice, and induced Apcfl/flCre+ Apcfl/flCited1−/− mice had similar large aberrant crypts to those observed in Apcfl/flCre+ Apcfl/fl (Figure 4D).

Given our findings of decreased adenoma formation in Apc−/+Cited1−/− mice, we also examined the extent of the hyperplastic area within the crypts of Apcfl/flCre+ Apcfl/flCited1−/− mice compared to Apcfl/flCre+ Apcfl/flCited1−/− mice as determined by the extent of BrdU labelling. Surprisingly, the number of cells in the hyperplastic area was greater in Apcfl/flCre+ Apcfl/flCited1−/− mice compared to Apcfl/flCre+ Apcfl/fl mice (Apcfl/flCre+ Apcfl/fl: 78 cells/area vs Apcfl/flCre+ Apcfl/flCited1−/−: 106 cells/area. p<0.05. Figure 4E).

We next determined migration rates by comparing the position of cells 2 hrs and 24 hrs after BrdU labelling. The difference between the 2 hrs and 24 hrs distributions for a genotype was analysed with the Kolmogorov–Smirnov test. The distribution of BrdU positive cells from 2 hrs to 24 hrs varies significantly for all genotypes (p = 0.01) indicating cell migration. The migration of cells from 2 hrs to 24 hrs after BrdU labelling is similar in distributions for a genotype was analysed with the Kolmogorov–Smirnov test. The distribution of BrdU positive cells from 2 hrs to 24 hrs varies significantly for all genotypes (p = 0.01) indicating cell migration. The migration of cells from 2 hrs to 24 hrs after BrdU labelling is similar in

Cited1 deficiency enhances the apoptotic phenotype observed immediately after Apc loss

The increase in proliferation observed in the intestine following deletion of Apc is also associated with a dramatic increase in apoptosis [3]. We therefore examined if Cited1 was regulating apoptosis by counting apoptotic bodies in H&E sections and also scoring Caspase 3 staining. We observed no significant difference in apoptosis between Apcfl/fl WT and Apcfl/flCited1−/− mice, however, there was a significant increase in the number of apoptotic cells in Apcfl/flCre+ Apcfl/flCited1−/− mice compared to Apcfl/flCre+ Apcfl/fl mice, which was verified by both methods (Figure 4A).

As mentioned above, we observed an increase in the number of cells per hyperplastic area in the Apcfl/flCre+ Apcfl/flCited1−/− samples. To verify that the increase in cell death was not an artefact of the difference in the number of cells per area, we corrected for this difference between Apcfl/flCre+ Apcfl/fl and Apcfl/flCre+ Apcfl/flCited1−/− mice. The normalised data confirmed increased cell death in Apcfl/flCre+ Apcfl/flCited1−/− compared to Apcfl/flCre+ Apcfl/fl (p<0.05) after H&E counting (Apcfl/flCre+ Apcfl/fl: 11.33 apoptotic cells/area vs Apcfl/flCre+ Apcfl/flCited1−/−: 15.33 apoptotic cells/area, p<0.05) and after anti-cleaved-Caspase3 staining (Apcfl/flCre+ Apcfl/fl: 7.32 apoptotic cells/area vs Apcfl/flCre+ Apcfl/flCited1−/−: 9.53 apoptotic cells/area, p<0.05) (Figure 4B–C). These data indicate that the increase in cell death is not proportional to the increase in cell proliferation. Therefore, Cited1 deficiency in a Wnt perturbed background accentuates the apoptotic response.

Cited1 deficiency revealed no change in the number of apoptotic cells in the intestinal tumors of Apc−/+Cited1−/− mice compared to Apc−/+/+ mice at time of death (Figure 4F). Therefore, the increased apoptosis we observe in the absence of Cited1 is only manifested in the context of acute Wnt activation, which underlines the role of Cited1 in restraining tumour initiation, and also implies in those tumours that do develop in the absence of Cited1, they may have developed alternative mechanisms to restrain the Wnt pathway.
Cited1 regulates several pathways including the Wnt signaling pathway

We next wished to investigate the mechanism through which Cited1 may be modifying Wnt driven tumorigenesis. One possibility is a direct effect upon Wnt signaling, and in support of this, Cited1 has previously been shown to be able to bind to β-catenin and consequently inhibit Wnt induced transcription during Xenopus development [5]. Two potential TCF-4 sites were identified in the Cited1 promoter region (cttgt and cattga in the 2 kb prior exon1). This implicates Cited1 in the control of the Wnt pathway, however this is not the only pathway known to be altered by Cited1. Cited1 has been shown to bind to the p300/CBP coactivators and also to Smad4, thereby enhancing their transcriptional activity [11,25]. To analyse the effects of Cited1 deficiency on various transcriptional pathways we performed a microarray analysis using the Affimix Chip 430 2.0 and AffymetrixGUI software [26]. We then submitted our microarray data to ingenuity pathway analysis software (IPA) to identify pathways significantly affected by Cited1 deficiency.

In the AhCreWT after additional loss of Cited1, a number of signalling pathways identified by IPA analysis were found to be affected, amongst them: P53 (p = 6.87×10−5, ratio = 0.146), PI3K/AKT (p = 6.74×10−5, ratio = 0.114), Pten (p = 7.7×10−5, ratio = 0.097); Wnt (p = 1.08×10−3, ratio = 0.057); and TGFβ (p>0.1, ratio = 0.034). Several targets were analysed by QPCR including c-Myc, Asin2, CD44, Sox4, p53, Pten, Akt1, and Smad4 but none were found to be significantly deregulated (N = 6, p>0.05; Mann-Whitney).

Several signalling pathways identified by IPA analysis were affected in AhCreApcΔEarCited1 mice compared to AhCreApcΔEar mice, including: P53 (p = 3.5×10−5, ratio = 0.177); PI3K/AKT (p = 2.62×10−5, ratio = 0.107); Pten (p = 7.22×10−5, ratio = 0.097); Wnt (p = 5.4×10−3, ratio = 0.063); and TGFβ (p = 2.22×10−1, ratio = 0.056). The validity of the IPA analysis was subsequently verified by QPCR. We analysed several targets from these pathways by QPCR, and found significant upregulation of p53, Runx1, Sox4 (Figure 5C) and a number of Wnt targets known to be deregulated in the intestines of AhCreApcΔEar mice [3] or listed as Wnt target genes in the Nussel database (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes) (Figure 5A-B). 10 Wnt target genes, including c-Myc, Asin2, and CD44 were confirmed by QPCR to be significantly up-regulated in AhCreApcΔEarCited1 mice compared to AhCreApcΔEar mice (Figure 5A). Three additional transcripts were analysed by microarray analysis that have previously been identified as key players in the Wnt pathway [Nucleophosmin, Nucleolin, and β-catenin respectively: [27–29]]. These were also found to be up-regulated in AhCreApcΔEarCited1 mice compared to AhCreApcΔEar mice. These data indicate that Cited1 inhibits several signaling pathways, including the Wnt pathway following Apc loss.

Stem cell markers are upregulated after loss of Cited1

The Wnt signalling pathway has been shown to play a critical role in intestinal homeostasis which includes stem cells maintenance. Because Cited1 loss leads to a deregulation of the Wnt pathway and due to the potential role of Cited1 in the stem cell niche in the cap mesenchyme in the developing kidney [18], we analysed the effect of Cited1 deficiency in the intestine. RT-QPCR analysis revealed a significant upregulation of several stem cell markers (Gpr49, Axin2, Musashi and Olfm4) in AhCreApcΔEarCited1 tissues compared to AhCreApcΔEar controls (Figure 5C). We also performed ISH for the surrogate marker of Lgr5, Olfm4 (Figure 5D). In AhCreWT and AhCreCited1 mice the location of Olfm4 expressing cells is confined to the stem cell niche at the base of the crypts. In AhCreApcΔEarCited1 and Olfm4 expressing cells were distributed throughout the hyperplastic area. In AhCreApcΔEarCited1 mice Olfm4 expressing cells are also mislocalised throughout the aberrant crypts but expression is increased, consistent with our RT-QPCR data of the same tissue (Figure 5C).

Cited1 influences the level of the active form of β-catenin in the small intestine

Loss of Apc has been shown to drive an increase in total β-catenin and more importantly a re-localisation of the active form of β-catenin to the nucleus [3]. To test if Cited1 deficiency modified this phenotype, we analysed the localisation of total β-catenin in the small intestine by immuno-histochemistry (Figure 6A). We observed a normal pattern of localisation in both AhCreWT and AhCreCited1 mice consistent with previous findings [30]. Upon deletion of Apc, we observed nuclear translocation of β-catenin in the aberrant crypts of both AhCreApcΔEar and AhCreApcΔEarCited1 mice (Figure 6A) indicative of de-regulated Wnt signalling.

β-catenin regulates important cellular functions such as transcription and adhesion [31], and the cellular concentration and phosphorylation status of β-catenin has been shown to impact on these functions [31,29]. As we observe an increase in the transcription of several Wnt target genes in AhCreApcΔEarCited1 mice we examined the level of total β-catenin, the extent of phosphorylation at multiple sites and the ratio of transcriptionally active free β-catenin in purified intestinal epithelial cells (Figure 6B) as previously described [32]. First, we observed a significant increase in total β-catenin accompanied by an increase in the active form of β-catenin (dephosphorylation at s833, s37, pT41 sites) in AhCreApcΔEar and AhCreApcΔEarCited1 mice compared to AhCreWT and very importantly in AhCreApcΔEar mice compared to AhCreApcΔEarCited1 mice.

These data were confirmed by western blot analysis using an antibody raised against total β-catenin (Figure 6C) or against the active form of β-catenin (dephosphorylated sites s833, s37, pT41) (Figure 6D) and were verified with a second antibody against dephosphorylated β-catenin (Figure S2A-B). There was no
significant difference in the phosphorylated (inactive) form of β-catenin (phosphorylated β-catenin at pS33, pS37, pT41 sites is degraded as a mechanism of regulating Wnt signalling) between all genotypes (Figure 6B). β-catenin phosphorylated at pS45 (phosphorylated by casein kinase 1x as part of degradation pathway) is significantly increased in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} compared to AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} WT and AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}}.

Given that β-catenin can also be phosphorylated by Protein Kinase A (PKA) at Ser552 and Ser675 which acts to inhibit ubiquitination and therefore increase levels of active β-catenin [33], we also analysed levels of pS552 and pS675 and found phosphorylation at S675 significantly increased in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} compared to AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} WT and AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}}, demonstrating the ability of Cited1 to regulate β-catenin at multiple sites (Figure 6B).

We also measured the intracellular free β-catenin (active β-catenin) levels by pull-down with a GST-fusion protein of the Wnt regulator gene Apc in intestinal epithelial cells immediately following deletion of the Apc gene in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} mice measured by QRT-PCR, *p < 0.05 Mann Whitney U test. B: Microarray analysis showing up-regulation of Wnt target genes with Wnt key players in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} compared to AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}}. Fold changes (FC) are presented with correspondent P value and B value (B statistic is lod score). C: Fold change of stem cell markers expression in the small intestinal epithelium of AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} compared to AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} mice measured by QRT-PCR, *p < 0.05 Mann Whitney U test. D: In situ Hybridization (ISH) analysis of Olfm4 in intestinal epithelial cells of all 4 genotypes. Inset panels show magnifications of intestine of corresponding zone.

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**Discussion**

Colorectal cancer is driven by a multiplicity of different biochemical pathways, however, key amongst these is the Wnt pathway, which we and others have previously shown to activate a set of c-Myc dependent genes which are critical for the early stages of colorectal cancer [6,35]. One of these genes is Cited1, which has been found to interact at the protein level with β-catenin and thereby negatively regulate β-catenin transcription [5]. Its relevance to carcinogenesis has already been described as Cited1 up-regulation has been observed in various cancers [14,36,37]. Here, we have extended these observations and find that CITED1 is significantly up-regulated in colorectal tumours from patients and in intestinal adenomas developing in the Apc\textsuperscript{Min/+} mouse model [38]. We also previously found Cited1 to be over-expressed in intestinal epithelial cells immediately following deletion of the Wnt regulator gene Apc in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} mice in a c-Myc dependent manner [6]. These data establish Cited1 as an immediate Wnt target gene in the intestine.

On the basis of these data we hypothesised that Cited1 might control β-catenin activity and thereby modulate Wnt signaling activation and its effects on colorectal tumorigenesis. To investigate this, we used microarray analysis and quantitative PCR studies to show that loss of Cited1 on an Apc deficient background does indeed impact upon a range of oncogenic signalling pathways, including Wnt. Our array data therefore show multiple effects of Cited1 deficiency including negative regulation of the Wnt pathway.

To investigate the requirement of Cited1 during Wnt induced tumourgenesis, we analysed the effects of deletion of Cited1 in two well characterised mouse models of Wnt signaling activation; the Apc\textsuperscript{Min/+} mouse model of colorectal tumourigenesis and the AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} mouse, a conditional model of Apc loss in which the immediate phenotypic consequences of Apc deletion can be studied [3]. Surprisingly, we obtained the apparently paradoxical result that although Apc\textsuperscript{Min/+} Cited1\textsuperscript{-/-} mice developed fewer intestinal tumours (associated with an increased life-span) than Apc\textsuperscript{Min/+} mice, the phenotypes induced upon conditional loss of Apc (including perturbed cell proliferation, apoptosis, differentiation and migration) were enhanced, rather than diminished, with additional loss of Cited1. Of note, we observed reduced capacity to differentiate (reflected by a reduced number of goblet cells and enteroendocrine cells), but no difference in total paneth cell numbers, although we did observe a difference in the positioning of paneth cells, which may well reflect differences in the Wnt signalling environment.

Our studies, suggest that a possible explanation for this apparent paradox is that the hyper-activated Wnt phenotype that occurs in the absence of Cited1 includes increased apoptosis. Several studies in cell culture systems already support such a model. For example, it has been reported that overexpression of β-catenin when transfected into cell lines leads to a 3-4 fold increase in cell death [39]. In addition, it has been demonstrated that high levels of c-Myc induce apoptosis in vivo [40]. This is consistent with our observations that c-Myc is overexpressed immediately following deletion of Apc in the intestine and that levels are significantly increased further with additional absence of Cited1. We interpret our data to indicate that the increase in apoptosis may counteract the increase in proliferation to the extent that the overall effect is reduced development of Wnt transformed cells and consequently inhibition of tumourigenesis.

The mechanism underlying such hyper-activation of Wnt signaling appears to be at least in part mediated through increased levels of phosphorylated β-catenin, which we found to be up-regulated in AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}Cited1\textsuperscript{-/-}} tissue compared to comparable AhCre\textsuperscript{Apc\textsuperscript{Flox/Flox}} tissue at both the transcriptional and protein levels. Thus, we found increased levels of the phosphorylated forms of β-catenin (T41, S33, S37). These sites when phosphorylated are involved in the degradation of β-catenin by the proteasome pathway [31]. This is accompanied by an increase in the levels of phosphorylation at serine 675 which has been shown to be phosphorylated by protein kinase A (PKA) and which has been shown to lead to inhibiting of ubiquitination of β-catenin causing
AhCre showed no effect of Cited deficiency (Figure S3). Furthermore, to estimate intestine as similar analysis of kidney tumorigenesis in these mice with Wnt pathway to a level that is incompatible with maximum tumour growth (Figure 7). Notably, this relationship appears specific to the intestine as similar analysis of kidney tumorigenesis in these mice showed no effect of Cited deficiency (Figure S3). Furthermore, to define the precise relationship between Wnt levels and tumourigenicity will require mouse modelling experiments in which Wnt activity is precisely regulated at numerous levels.

Wnt/beta-catenin signalling plays a key role in the homeostasis of the intestinal epithelium and its role in the fate and maintenance of the stem cell compartment have been clearly demonstrated [44]. Our data clearly show that Cited1 is an immediate target of Wnt signalling and is an important regulator of the Wnt pathway. The loss of Cited1 has a direct impact on stem cell status in the small intestine as we have found several stem cell markers to be upregulated including Lgr5 (Qpr49), Musashi and Olfm4. These alterations in expression could be a direct consequence of the ‘hyper’ activation of the Wnt pathway we observe after combined loss of Apc and Cited1. This would implicate Cited1 as an important player in Wnt dependant stem cell maintenance in the small intestine. This has been already suggested in the developing kidney where Cited1 may contribute to the maintenance of the self-renewing capping mesenchyme [18]. By regulating the Wnt pathway, Cited1 may be an important regulator of the self-renewal compartment in the crypt of the small intestine.

Our data show that Cited1 deficiency suppresses tumorigenesis. The consequences of Cited1 deficiency are diverse, but in particular impact upon Wnt pathway activity. We propose a model whereby loss of Cited1, in the context of deregulated Wnt signalling, hyper-activates the Wnt pathway resulting in apoptosis of Wnt induced transformed cells and thus inhibits tumourigenesis. As Cited1 mice are fertile and viable this suggests that Cited1 represents a possible target for therapeutic intervention, where Cited1 inhibition induces cytotoxic effects due to very high Wnt signalling.

**Materials and Methods**

**Human colorectal cancer tissue RNA samples**

Total RNA samples from patient colorectal tumour tissues were obtained from the Cancer Tissue Bank Research Centre (CTBRC). All colorectal cancer tissues and adjacent uninvolved colonic mucosa were obtained from surgically removed specimens with informed patient consent. Uninvolved colonic mucosa was generally 5–10 cm away from the malignant tissue.

**Mouse colonies**

All experiments were performed under the UK Home Office guidelines. Mice were obtained and genotyped as follows: Cited1 null (Cited1−/−); Apcmin/+ (ApcMin/+); AhCre transgene (AhCre+); Apcmin/+ allele (AhCre+); β-catenin (β-catenin); Apcmin/+ and Apcmin/+Cited1 mice were maintained on an inbred C57BL/6j background and were confirmed as congenic for the C57BL/6 Mm Mm-1 allele via PCR analysis. Mice were sacrificed at ill-health. Intestine were fixed in Methacarn (methanol-chloroform-glacial acetic acid [4:2:1]), and the lesion numbers were scored macroscopically.

**Figure 6. Level of the active form of β-catenin in the small intestine.** A: Immunohistochemistry for total β-catenin in each genotype. Inset panels show magnifications of intestine of corresponding zone. B: Small intestine epithelial cell extracts from AhCre+WT (WT; n = 14), AhCre−/−Cited1 (AhCre−/−; n = 7), AhCre−/+Apcmin/+ (Apc−/−; n = 12); AhCre−/+Apcmin/+Cited1 (Apc−/−Cited1−/−; n = 7); AhCre−/+Apcmin/+Cited1−/− (Apc−/−Cited1−/−) were analysed for the status of β-catenin using a suspension bead array assay panel. Total β-catenin, dephospho β-catenin (S33, S37 and T41), phosphorylation at S33, S37, and T41, phosphorylation at S45, phosphorylation at S552 and phosphorylation at S675 were analysed by using respective capture antibodies in multiplexed sandwich immunoassays. Free β-catenin (non-complexed) was measured by μGST pull-down assays using GST-ICAT as bait protein. Signal intensities are displayed in relative fluorescence units (AU) (mean±SE). *p<0.05; All statistical tests were done using Mann-Whitney U test. C: Western blot analysis of the Total form of β-catenin in each genotype. The histogram represents the densitometry analysis of the total β-catenin immuno-blot normalised to the internal control α-tubulin, showing an increase in the level of total β-catenin in AhCre−/+Apc−/−Cited1 (Apc−/−Cited1−/−) compared to AhCre−/+Apc−/−Apc−/− (Apc−/−Cited1−/−) and AhCre−/+Apc−/−Cited1 (Apc−/−Cited1−/−) also show an increase compare to AhCre−/+WT. D: Western blot analysis of the active form of β-catenin using dephospho-β-catenin (dephosphorylated on Ser37/Thr41, Clone 8E7, Millipore) antibody in each genotype. The histogram represents the densitometry analysis of the dephospho-β-catenin immuno-blot normalised to the internal control β-actin, showing an increase in the level of dephos-β-catenin in AhCre−/+Apc−/−Cited1−/− (Apc−/−Cited1−/−) compared to AhCre−/+Apc−/−Cited1−/− (Apc−/−Cited1−/−). The sample of each genotype is pooled from 3 to 7 mice in the cohort. AhCre−/+Apc−/−Cited1−/− (Apc−/−Cited1−/−) is used as a negative control for dephospho-β-catenin.
To study the role of Cited1 after the early loss of Apc, AhCre WT, AhCre Apcfl/fl, AhCre Cited12 and AhCre Apcfl/flCited12 mice were generated and maintained on an outbred background. Cre activity was induced by three intraperitoneal injections of 80 mg/kg β-naphthoflavone within 24 h and mice were taken Day4 or Day5 later. Tissues analysed were from age (8–12 weeks), sex (males), background and genotype matched animals, however these were not always littermates.

Assaying apoptosis, number of cells per crypt, S-phase labelling in vivo and migration

Apoptosis was scored from H&E or after anti cleaved-Caspase3 immuno-staining as previously described [3]. For proliferation analysis, mice were injected with 0.25 ml of BrdU (Amersham) before culling and were taken either 2 hrs (day4) or 24 hrs (day5) after BrdU injection. Staining was performed as previously described [3]. The number of cells in AhCre Apcfl/fl and AhCre Apcfl/flCited12 hyperplastic area was scored using the position of the last BrdU positive cells in the hyperplastic area. For each analysis, 25 full crypts or areas were scored from at least 5 mice of each genotype and time point.

In situ hybridization (ISH)

In situ hybridization of Olfm4 and Cited1 in the small intestine was performed for all genotypes using sections embedded in paraffin sectioned at 5 μm. Olfm4 hybridization was performed as described in Gregoireff et al., 2005. [48]. Cited1 hybridization was performed using a probe against the sequence deleted in the

Figure 7. Schematic diagram of a “just right” model of Cited1 action on colorectal tumourigenesis. Loss of function of Apc is accompanied by multiple changes in gene expression, including upregulation of active β-catenin (dephospho-β-catenin), activation of Wnt and Cited1. Hence, immediately following deletion of Apc, Cited1 normally restrains the Wnt pathway at the level of β-catenin. We observe a range of rapid phenotypic changes. These include increase in proliferation and apoptosis and loss of differentiated cell types, also reduced migration, leading to the preferential retention of Apc deficient cells. These changes may all be considered pro-tumourigenic, leading to survival advantage of the tumour cell and decreased survival of the ApcMin/+ mouse compared to WT. Additional Cited1 deficiency leads to hyper-activation of Wnt signaling including upregulation of active β-catenin and an exaggerated Wnt phenotype including elevated proliferation, a further loss of cell differentiation, and most importantly increased cell death. The net effect of these changes is an increase in ApcMin/+ survival. This restraint imposed by Cited1 is consistent with a requirement for Cited1 to constrain Wnt activity to a level commensurate with optimal adenoma formation and maintenance, and provides one mechanism for tumour repression in the absence of Cited1. doi:10.1371/journal.pgen.1003638.g007
AhCre allele designed by Advanced cell Diagnostics inc (ACD). RNAscope 2.0 FFPE Reagent Kit – Brown kit was used according manufacturer instructions. Negative control Probe-DapB was used together with a positive control probe Polr2a from the ACD manufacturer.

Microarray data analysis

The DNA microarray were performed from three mice of each genotype using Mouse Genome 430 2.0 Affymetrix chips at Liverpool Microarray Facility according to the manufacturer’s instructions. The Microarray data were analyzed using Affymetrix GUI (Affymetrix linear modeling Graphical User Interface; http://bioinf.wehi.edu.au/affylinGUI/#citation) [26]. The p values have been presented for multiple testing using the BH method to control the false discovery rate. The B statistic is the log odds that the gene is differentially expressed and is adjusted for multiple testing using the assumption that 1% of genes are expected to be differentially expressed [26,49–51]. Microarray data were deposited in MIAME format at www.ebi.ac.uk/arrayexpress/ (Accession Number: E-MEXP-3202)

QPCR protocols, routine methods and a description of the statistical analyses used are provided in Protocol S1. List of primers for Taqman RT-QPCR, Sybr green RT-QPCR, and Cited1 semi quantitative RT-PCR are provided in Figure S4.

Analysis of signaling pathways

Ingenuity pathway analysis (IPA) software (www.ingenuity.com) was used to determine which signalling pathways were affected by the loss of Cited1 in AhCre+/- or AhCre+/- mice. The comparative (AhCre+/-WT vs AhCre+/-Cited1-) and AhCre+/-Apc+/- vs AhCre+/-Apc+/-Cited1-) data from the microarray analysis were filtered for a p value of less than 0.05 and imported into the IPA software. The significance of the association between the data set and the pathway was measured in 2 ways: by the ratio and by a p value. The ratio corresponds to the number of genes from our data set that map to the ingenuity pathway divided by the total number of genes that map to the Ingenuity canonical pathway. The p value is calculated by a right tailed Fischer’s exact test. The p-value associated with a pathway is a measure of the likelihood that the association between a set of focus genes in your experiment and a pathway is due to random chance.

β-catenin suspension bead array based assay

Analysis of biological function, localization, and posttranslational modification of the different forms of β-catenin were carried out as previously described [32]. Two additional assays were included in the analysis. Anti-dephospho S33/S37 and T41 (Cell Signalling Technologies) was used as an additional capture antibody to measure dephosphorylated β-catenin and GST-ICAT was employed as an additional bait protein to study free β-catenin.

Supporting Information

Figure S1 RT-PCR for Cited1 expression in ApcMo+m mice. A: RT-PCR products showing up-regulation of Cited1 in polyps (T) compared to normal intestinal tissue (N) in ApcMo+m. Note the absence of RT-PCR products in ApcMo+m/Cited1- condition confirming the loss of Cited1 expression. β-actin was used as internal positive control. B: RT-PCR of the Apc recombined cDNA in AhCre+/-Apc+/- and AhCre+/-Apc+/-Cited1-. The Apc non recombined cDNA (wild type Apc) gives a band of 383 bp whereas the Apc recombined cDNA (Apc Rec) gives a PCR product of 168 bp. C: Percentage tumour distribution varies in ApcMo+m mice compared to ApcMo+m/Cited1- mice. The percentage tumour distribution was analysed by counting tumour burden in each of 5 cm sections along the length of the small intestine and representing this as a percentage of total tumour burden per section. Sections 1–2 (duodenum), 3–5 (jejunal) 6–8 (comparable to human Beum). The colon was similarly divided into 1 cm sections. Section 1 corresponding to the rectum. Error bars represent standard errors. *p<0.05; statistical tests was done using Mann-Whitney U test.

Figure S2 Increased level of dephosphorylated-β-catenin and deregulated pathways in AhCre+/-Apc+/-Cited1- compared to AhCre+/-Apc+/. A: Western blot analysis of the active form of β-catenin (92 kD) using dephospho-β-catenin (Non-phospho-β-Catenin Ser33/37/Thr41, Cell signalling) antibody in AhCre+/-WT (WT), AhCre+/-Cited1- (Cited1-), AhCre+/-Apc+/- (Apc+/-) and AhCre+/-Apc+/-Cited1- (Apc+/-Cited1-). There is a strong up-regulation of dephospho-β-catenin in AhCre+/-Apc+/- compared to AhCre+/-WT and AhCre+/-Cited1- and the level of dephospho-β-catenin is further elevated in AhCre+/-Apc+/-Cited1- compared to AhCre+/-Apc+/-.

Figure S3 Cited1 deficiency does not modify the renal carcinoma phenotype induced after loss of Apc and activation of K-rasV12. Cre recombinease under the Cyp1a promoter has also been shown to be constitutively expressed in a proportion of cells in the renal epithelium [reference S1, in Protocol S1]. This drives loss of the Apc allele and the formation of dysplastic foci characterised by accumulation of nuclear β-catenin. Within 4 months, mice develop renal carcinoma [reference S1, in Protocol S1] which can be accelerated by an additional K-rasV12 oncogene [reference S2, in Protocol S1]. To study the role of Cited1 in renal cell carcinoma, mice AhCre+/-Apc+/-K-rasV12, AhCre+/-Apc+/-K-rasV12 Cited1- and AhCre+/-Apc+/-K-rasV12-Cited1- were generated and maintained on an outbred background. All experiments were performed under the UK Home Office guidelines. K-rasV12 allele was obtained and genotyped as previously described [reference S3, in Protocol S1]. Mice were sacrificed at ill health. We analysed expression of Cited1 in the renal carcinomas of AhCre+/-Apc+/-K-rasV12 compared to normal tissue. Histograms (A) are showing qRT-PCR delta CT values (left panel) and Fold change (right panel) for Cited1 expression in the kidneys of AhCre+/-WT, AhCre+/-Apc+/-K-rasV12 (tumours) and AhCre+/-Apc+/-K-rasV12-Cited1- (tumours) mice. There is a significant 19.48 fold increase in Cited1 expression in AhCre+/-Apc+/-K-rasV12-Cited1- mice compared to WT mice (p = 0.0041 Mann-Whitney U test) and 1007 fold change difference between AhCre+/-Apc+/-K-rasV12 and AhCre+/-Apc+/-K-rasV12-Cited1- mice (p = 0.0071 Mann-Whitney U test). (B) We generated cohorts of AhCre+/-Apc+/-K-rasV12, AhCre+/-Apc+/-K-rasV12-Cited1- and AhCre+/-Apc+/-K-rasV12-Cited1- mice and monitored them for signs of illness. The Kaplan-Meier shows no significant difference in survival between AhCre+/-Apc+/-K-rasV12 mice (n = 13) (dashed) versus AhCre+/-Apc+/-K-rasV12-Cited1- (n = 4) (Bold solid line) p = 0.732, Log-Rank test) and between AhCre+/-Apc+/-K-rasV12 versus AhCre+/-Apc+/-K-rasV12-Cited1- mice (n = 13) (dashed) versus AhCre+/-Apc+/-K-rasV12-Cited1- (n = 4) (Bold solid line).
AhCre 

Cited1 deficiency does not modify the survival of mice affected by renal carcinoma and that Cited1 does not play a role in renal carcinoma induced by a loss of Apc.

**Figure S4** Primers Table. Tables listing the primers and probes used for Taqman quantitative PCR in human (A) and in mice (B); Sybr Green quantitative real time PCR in mice (C) and semi-quantitative PCR in mice (D).

**Figure S5** Cited1 deficiency further represses the number of differentiated cell types after Apc loss. To determine if deficiency of Cited1 modifies cell differentiation along the crypt-villus axis, we analysed the presence and location of several secretory cell types using markers of cell lineage in mice intestinal epithelium of all 4 genotypes: goblet cells (A–B) [Alcian Blue staining and counting], enterocinocrine cells (C–D) [Grimeus staining and counting] and paneth cell (E–F) [Lysozyme staining and counting]. (G) Paneth cells position was analysed. Goblet cells and enterocinocrine cells were scored in 25 crypts (or hyperplastic areas)-villus (n = 6/genotype) (All statistical analysis were done using the MANN-Whitney U test and NS = Non significant). We found that the number of goblet cells and enterocinocrine cells were not significantly different in AhCre/Cited1 mice compared to AhCre- Cited1 mice (Goblet cells = AhCre WT: 9.8 cells/crypt-villus vs AhCre Cited1: 11.4 cells/crypt-villus, p = 0.1312; Enterocinocrine cells = AhCre WT: 1.56 cells/crypt-villus vs AhCre Cited1: 1.61 cells/crypt-villus, p = 0.5) (BD). However, the number of goblet cells and enterocinocrine cells per hyperplastic area-villus were both significantly reduced in AhCre/Apc/Cited1 mice compared to AhCre/Apc WT mice (Goblet cells = AhCre/Apc WT: 5.26 cells/area-villus vs AhCre/Apc Cited1: 3.71 cells/area-villus, p = 0.0463, Enterocinocrine cells = AhCre/Apc WT: 1.04 cells/area-villus vs AhCre/Apc/Cited1: 0.73 cells/area-villus, p = 0.0125) (BD). The position and the number of paneth cells were not modified in AhCre/Cited1 mice compared to AhCre WT mice (E–G). After loss of Apc, paneth cells lose their position at the bottom of the crypt and are mislocalized along the crypt-villus axis (G). We observe a change in position of the paneth cells in the hyperplastic areas of the AhCre/Apc/Cited1 compared to AhCre/Apc WT (E–G). Statistical test was done using Kolmogorov-Smirnov test (G). Inset panels show magnifications of intestine of corresponding zone. Taken altogether, these data suggest that loss of Cited1 does not perturb the pattern of differentiation when Apc is present. However in the absence of Apc, Cited1 deficiency appears to accentuate the phenotype, with a more extreme reduction in the number of differentiated cell types.

**Protocol S1** Statistical Analysis, Quantitative real-time PCR on mice samples, Cited1 status by Semi-quantitative PCR, First strand cDNA synthesis and taqman quantitative qPCR on human samples, Western Blot analysis, RNA and protein isolation from mice small intestine, Histology and Immunohistochemistry and Supplementary references are provided as supplementary information. Primers list for Taqman RT-QPCR, Sybr green RT-QPCR and semi quantitative RT-PCR are listed in Figure S4.

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**Author Contributions**

Conceived and designed the experiments: VM FS TP AW ARC. Performed the experiments: VM FS TP MY OP. Analyzed the data: VM FS TP LP JRJ GTW AW ARC. Contributed reagents/materials/analysis tools: SLD. Wrote the paper: VM FS TP AW ARC. Critical revision of the manuscript: JRJ SLD.

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