
Official URL: https://www.cell.com/cell-reports/fulltext/S2211-1247(12)00199-4
DOI: http://dx.doi.org/10.1016/j.celrep.2012.07.003
EPrint URI: http://eprints.glos.ac.uk/id/eprint/6060

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Luminal Iron Levels Govern Intestinal Tumorigenesis after Apc Loss In Vivo

Sorina Radulescu,1 Matthew J. Brookes,2 Pedro Salgueiro,1 Rachel A. Ridgway,1 Ewan McGhee,1 Kurt Anderson,1 Samuel J. Ford,2 Daniel H. Stones,2 Tariq H. Iqbal,2 Chris Tselepis,2,* and Owen J. Sansom1,*

1Birmingham Cancer Research UK Centre, School of Cancer Sciences, University of Birmingham B15 2TH, UK
2Beatson Institute of Cancer Research, Glasgow, G61 1BD, UK
*Correspondence: c.tselepis@bham.ac.uk (C.T.), o.sansom@beatson.gla.ac.uk (O.J.S.)
http://dx.doi.org/10.1016/j.celrep.2012.07.003

SUMMARY

It is clear from epidemiological studies that excess iron is associated with increased risk of colorectal cancer; however, questions regarding the mechanism of how iron increases cancer risk, the source of the excess iron (circulating or luminal), and whether iron reduction represents a potential therapeutic option remain unanswered. In this study, we show that after Apc deletion, the cellular iron acquisition proteins TfR1 and DMT1 are rapidly induced. Conversely, restoration of APC reduces cellular iron due to repression of these proteins. To test the functional importance of these findings, we performed in vivo investigations of the impact of iron levels on intestinal tumorigenesis. Strikingly, depleting of luminal (but not systemic) iron strongly suppressed murine intestinal tumorigenesis, whereas increased luminal iron strongly promoted tumorigenesis. Taken together, our data definitively delineate iron as a potent modifier of intestinal tumorigenesis and have important implications for dietary iron supplementation in patients at high risk of colorectal cancer.

INTRODUCTION

The Apc (adenomatous polyposis coli) gene is the most commonly mutated tumor suppressor gene in sporadic colorectal cancer (CRC; Bienz and Clevers, 2000). Loss of the Apc protein prevents the turnover of β-catenin, and hence β-catenin accumulates in the nucleus and causes the activation of TCF/LEF or Wnt target genes such as c-Myc and Cyclin D2 (Bienz and Clevers, 2000). Of importance, APC loss appears to initiate CRC, and thus one would predict that agents that can determine the survival of Apc-deficient cells could potently modify tumor risk. In this context, a number of epidemiological studies have shown that dietary factors can alter CRC risk (World Cancer Research Fund / American Institute for Cancer Research, 2011). However, whether these agents target Apc-deficient cells over normal cells, as well as their mechanism of action, is still unclear.

Iron is essential for all organisms, and iron-containing proteins catalyze a variety of crucial metabolic processes, such as oxidative phosphorylation and DNA synthesis, as well as cell-cycle progression and growth (Le and Richardson, 2002). In addition, excess iron can mediate reactive oxygen species through Fenton reaction chemistry, resulting in lipid, protein, and DNA damage (Toyokuni, 1996). A link between excess iron and cancer incidence in CRC has been reported (Chua et al., 2010; Kato et al., 1999; Lee et al., 2004; Mainous et al., 2005; Nelson, 2001; Toyokuni, 1999, 1996). Most notably, a meta-analysis of 33 epidemiological studies showed that 75% of studies supported an association between excess iron and CRC risk (Nelson, 2001). This is supported by the observation that patients with hemochromatosis (HFE) mutations, associated with the iron overload disorder hereditary hemochromatosis, have an increased risk of not only hepatocellular carcinoma but also CRC (Nelson et al., 1995; Shaheen et al., 2003). Conversely, several human epidemiological studies showed that a decrease in total body iron levels resulting from blood withdrawal decreases the risk of various cancers, including CRC (Edgren et al., 2008; Zacharski et al., 2008). However, despite this close association with tumorigenesis and disease, the role of iron in sporadic carcinogenesis and the mechanism by which it acts are still unclear. In particular, whether iron levels are a cause or a side effect of tumorigenesis has yet to be established, with previous studies in animal models reporting conflicting evidence regarding the impact of iron on colon cells (Lund et al., 1998; Soyars and Fischer, 1998). Our own previous studies demonstrated that stimulating colorectal cell lineages with iron results in amplification of Wnt signaling (Brookes et al., 2008), the major oncogenic signaling pathway in the colon (Bienz and Clevers, 2000). Of importance, though, iron could only amplify Wnt signaling in the background of a loss of the tumor suppressor Apc. These observations were further supported by a more recent study that highlighted a class of iron chelators (acyl hydrazones) as inhibitors of Wnt signaling and cell growth (Song et al., 2011). Furthermore, we previously reported a modulation in the expression of iron transport proteins in the malignant progression of disease (Brookes et al., 2006; Ward et al., 2008). In particular, in the process of human CRC, there was increased expression of the cellular iron...
import proteins (transferrin receptor 1 (TfR1) and divalent metal transporter 1 (DMT1)), and a loss of cellular iron export function. Although both the epidemiological evidence and results from our human cell line mechanistic studies are suggestive of a direct role of iron in CRC, it should be remembered that 25% of epidemiological studies have failed to find a link with iron consumption, and the increase in relative risk, although significant, is small. Thus, it is vital to test for a direct role of iron in sporadic carcinogenesis in vivo. In this study, we investigate how iron modulation affects intestinal tumorigenesis following Apc loss in vivo. Of importance, we show that the precise levels of iron dictate the proliferation and survival of Apc-deficient cells. This directly translates to suppressed tumorigenesis when luminal iron is depleted, and to increased tumorigenesis when luminal iron is in excess.

RESULTS

Modulation of Iron Transport Proteins as a Consequence of Wnt Signaling

To ascertain whether the expression of the iron import proteins TfR1 and DMT1, and the iron storage protein ferritin are modulated as a consequence of Wnt signaling, we selected and cultured two cell lines, RKO and SW480, in the presence and absence of iron. These two lines were chosen because they contain wild-type (WT) and mutant Apc, respectively. By activating Wnt signaling (via GSK3β inhibition using lithium chloride (LiCl)), a 3-fold c-MYC induction was also associated with significant increases in TfR1 (mean 1.8-fold, p = 0.021) and DMT1 (mean 1.7-fold, p = 0.036) protein expression compared with control (Figure 1A). No change was

Figure 1. Effect of Modulating Wnt Signaling on Expression of Iron Transport Proteins

(A) Culturing RKO cells with iron (IL) for 24 hr repressed TfR1 and DMT1, and increased ferritin protein levels. LiCl treatment increased the level of TfR1, DMT1, and c-Myc expression relative to control. Costimulation with LiCl and IL (IL+LiCl) further increased c-MYC, TfR1, and DMT1 levels and suppressed ferritin expression. p < 0.0001 c-MYC, DMT1, p = 0.0071 TfR1 in IL+LiCl versus IL alone; p = 0.003 c-MYC, p = 0.0017 TfR1, p = 0.0007 DMT1 in IL+LiCl versus LiCl alone, Student’s t test; * denotes statistical significance compared with control; | denotes statistical significance compared with LiCl alone. Data are presented as mean ± SEM.

(B) To assess cellular IL levels, a ferrozine assay was performed in RKO cells in control or in the presence of IL or LiCl, or both (IL+LiCl) for 24 hr. Wnt activation further increases IL content (p < 0.0001 IL+LiCl versus LiCl alone, p = 0.0006 IL+LiCl versus IL alone, Student’s t test; * denotes statistical significance compared with control; | denotes statistical significance compared with LiCl alone). Data are presented as mean ± SEM.

(C) Culturing SW480 cells for 24 hr with IL resulted in upregulation of c-MYC, TfR1, and DMT1, and repression of ferritin. Reconstitution of WT human Apc and culture with IL (IL+APC) significantly reduced c-MYC, TfR1 and DMT1 and ferritin expression (p = 0.002 TfR1, p = 0.003 DMT1, p < 0.0001 ferritin IL+APC versus IL, Student’s t test; * denotes statistical significance compared with control). Data are presented as mean ± SEM.

(D) SW480 cells reconstituted with WT human APC resulted in significantly decreased cellular IL content (ferrozine assay; control+APC versus control, p = 0.0053; control+APC versus IL+APC, p < 0.0048, Student’s t test; * denotes statistical significance compared with control; | denotes statistical significance compared with IL alone). Data are presented as mean ± SEM.

(E) To assess the association of c-MYC with TfR1, DMT1, and ferritin in vivo, mRNA expression levels were assessed in eight murine polyps from individual ApcMin/+ mice, 20 human colonic polyps, and 10 human colorectal carcinomas. Expression levels were assessed relative to matched normal control mucosa.
observed for ferritin protein expression. Costimulation of cells with the GSK3β inhibitor and iron further enhanced c-MYC, TIR1, and DMT1 levels by at least one-third above either GSK3 inhibitor or iron alone (p < 0.0001 c-MYC, DMT1; p = 0.0071 TIR1 in iron+LiCl versus iron alone; p = 0.003 c-MYC; p = 0.0017 TIR1; p = 0.0007 DMT1 in iron+LiCl versus LiCl alone; Figure 1A). Conversely, ferritin protein expression was statistically repressed by half or more compared with either GSK3 inhibitor (p = 0.013) or iron alone (p = 0.02). Of interest, without GSK3 inhibition, there was a dramatic decrease in TIR1 and DMT1 following treatment with iron, which was not observed following the activation of Wnt signaling (p < 0.0001). This made an important functional difference because when Wnt signaling was activated, the cells were now able to increase the expression of their cellular iron import proteins. This was evidenced by increased cellular iron deposition in cells challenged with iron and LiCl compared with cells challenged with iron (mean 3-fold, p = 0.0003) or LiCl alone (mean 40-fold, p < 0.0001; Figure 1B). To further solidify these observations, we performed a reverse set of experiments in which the expression of c-MYC and iron metabolism proteins was assessed in SW480 cells (a cell line with deregulated Wnt signaling) before and after restoration of APC (Figures 1C and 1D). We saw that challenging SW480 cells with iron further deregulated Wnt signaling targets such as c-MYC (mean 2.7-fold, p = 0.0004) and increased the expression of TIR1 (mean 2.7-fold, p < 0.0001) and DMT1 (mean 2.3-fold, p < 0.0001; Figure 1C). Functionally, this was associated with a 10-fold increase in cellular iron deposition (p < 0.0001; Figure 1D). When APC was reconstituted, TIR1 (p = 0.002) and DMT1 (p = 0.003) were dramatically reduced by more than half following iron treatment (Figure 1C). In addition, the cellular iron levels in SW480 cells with reconstituted APC (control+APC) were statistically decreased to one-fifth relative to sham transfected cells (p = 0.0053) and also moderately decreased when cultured in the presence of high iron (iron versus iron+APC, p = 0.0048; Figure 1D). As previously reported (Brookes et al., 2008), the iron-mediated induction of cellular proliferation was no longer observed when WT APC was reconstituted into these cells.

Association of Wnt Signaling with TIR1, DMT1, and Ferritin Expression

To further support the relationship between deregulated Wnt signaling and the expression of TIR1, DMT1, and ferritin, we assessed the expression of c-Myc (a key target of canonical Wnt signaling), TIR1, DMT1, and ferritin in ApcMin/+ mouse polyps (n = 8), human polyps (n = 20), and human colorectal carcinomas (n = 10), each matched with an adjacent nondiseased control mucosa (Figure 1E). The ApcMin/+ mouse model carries a germline mutation in the Apc gene and mimics familial adenomatous polyposis, a hereditary form of CRC (Su et al., 1992). The mice develop multiple adenomas and hence reconstitute an early stage of CRC. In all eight ApcMin/+ adenomas examined, we observed a marked induction of c-Myc mRNA (8/8 mean fold induction of 4.58), TIR1 (7/8 mean fold increase of 2.6) and DMT1 mRNA (7/8 mean fold increase of 3.2) compared with nondiseased adjacent tissue. A statistical analysis suggested a robust correlation of c-Myc with TIR1 (p < 0.004) and DMT1 (p < 0.001) expression; however, no relationship was observed with ferritin, which was overexpressed in five of the eight samples. Furthermore, this induction of c-Myc, TIR1, and DMT1 was also observed in human adenomas (16/20, 16/20, and 15/20, respectively) and carcinomas (10/10, 7/10, and 9/10, respectively). Ferritin mRNA was overexpressed in only 10/20 and 4/10 human adenomas and carcinomas, respectively.

Because previous studies have shown that c-Myc may regulate the expression of TIR1 by directly binding to its promoter region, as well as by indirectly regulating the iron regulatory protein 2 (IRP2) and ferritin, we overexpressed c-Myc in the RKO cell line using the well-characterized c-MycER system (Figure 2; Littlewood et al., 1995; O’Donnell et al., 2006; Wu et al., 1999). c-Myc induction mediated by tamoxifen treatment resulted in increased mRNA of TIR1 (mean 2.1-fold, p = 0.017) and DMT1 (mean 1.9-fold, p = 0.0005), whereas ferritin protein expression was repressed compared with non-tamoxifen-treated cells (p < 0.0001; Figures 2A–2D). Upon c-Myc activation in the presence of iron, an almost double cellular iron acquisition was observed compared with non-tamoxifen-treated cells challenged with iron (Figure 2E). To further confirm a direct regulation of these proteins by c-Myc, we deleted c-Myc in the adult murine intestine. To that end, we generated AHCre+c-Mycfl/fl mice. The AH Cre transgene yields highly penetrant Cre expression in the intestine following injection of beta-naphthoflavone and thus allows c-Myc to be inducibly deleted from the adult intestine (de Alborn et al., 2001; Sansom et al., 2007). Our previous studies showed that short-term deletion of c-Myc does not affect intestinal homeostasis, and that Wnt gene activation and the phenotypes of APC loss are dependent on c-Myc activity (Sansom et al., 2004, 2007). While investigating mRNA levels in vivo in WT versus c-Myc deleted intestines, we noted a significant downregulation of DMT1 by almost half (p = 0.0018) upon c-Myc loss. In contrast, TIR1 was not affected upon c-Myc deletion alone, but was significantly decreased to almost one-third in mice that had a combined deficiency for APC and c-Myc when compared with APC-deficient intestines alone (AHCre+ ApcMin/+ c-Mycfl/fl versus AHCre+ ApcMin/+ p = 0.02). Therefore, in vivo c-Myc levels are important for the expression of components of the iron import machinery.

Iron Modifies Intestinal Tumorigenesis

Given these clear mechanistic links between Wnt signaling and cellular iron protein machinery, and the upregulation of these proteins in the very early stages of CRC carcinogenesis, we next examined whether modifying the levels of iron could affect intestinal tumorigenesis in vivo. To that end, we examined whether a diet completely lacking iron [the iron-deficient diet (IDD)] could suppress tumorigenesis, and a diet with excess iron [the carboxyl iron diet (CID)] could stimulate tumorigenesis in the ApcMin/+ mouse (Su et al., 1992). In this mouse model, loss of the remaining WT Apc allele initiates tumorigenesis early in life and the mice develop hundreds of adenomas throughout their small intestine and colon by 100 days of age. ApcMin/+ mice were fed either an IDD (n = 16) or a control diet (CD; n = 17) from weaning and were euthanized at 80 days of age (Figures 3A–3C). We determined the tumor burden by assessing tumor number and size from whole-mount intestines. ApcMin/+ mice fed the IDD developed a greatly reduced tumor burden
by more than half (p < 0.0001), with a marked reduction in both tumor number (p < 0.0001) and size (p = 0.0006). WT mice treated with the IDD remained healthy and showed no intestinal phenotypes within the time course of the experiment. Furthermore, ApcMin/+ mice on the IDD were healthy and gained weight at the same rates as controls (Figure S1). In contrast, mice fed an iron-rich diet (CID; 2% carbonyl iron w/w; n = 13) rapidly began to show signs of intestinal neoplasia and thus had to be harvested at 60 days of age (Figures 3D and 3E). Due to the rapid development of intestinal tumorigenesis and the resulting ill health of the mice, we assessed tumor burden by scoring tissue sections for the number of tumors present within the first 10 cm of the small intestine. The CID resulted in a 2- to 3-fold amplification in tumorigenesis compared with mice on the CD sacrificed at the same 60-day time point (p = 0.002; Figure 3D). Overall, the 2% carbonyl iron resulted in higher levels of iron in both normal intestine and adenomas compared with the CD, as detected by Perls’ Prussian staining (Figure 3F; Figure S2). Of importance, previous reports indicate that in the short term, this level of dietary iron overload is not enough to cause liver toxicity, which suggests that the ill health was due to the formation of multiple intestinal adenomas (Pigeon et al., 1999).

Given that tumor formation in the ApcMin/+ mice occurred following loss of the remaining Apc allele, it is possible that iron affects both tumor growth and initiation by increasing the mutation rates of the WT allele. Therefore, we next assessed whether the IDD and CID could modify tumorigenesis following the loss of both copies of Apc. For these studies we used the Lgr5creERT2+ Apcfl/fl model, which has Apc deletion solely in stem cells (Barker et al., 2007). This represents a rapid model of intestinal tumorigenesis, with mice developing numerous adenomas within 50 days following Cre induction. Lgr5creERT2+ Apcfl/fl mice were placed on IDD (n = 18), CID (n = 17), or CD (n = 24), and following a 2-week dietary acclimatization were treated with tamoxifen to induce Cre-mediated recombination of Apc and allowed to age until signs of ill health were observed (Figure 4A). In this potent model of intestinal tumorigenesis, IDD strongly attenuated tumorigenesis, as indicated by the extended survival of mice on the IDD compared with the CD (median survival 89 days (IDD) versus 61 days (CD), p = 0.001, Kaplan Meier method, logrank). Again, the IDD did not have any adverse effects on the health of the mice as assessed by weight monitoring (Figure S3). Conversely, mice on the CID had a dramatically shortened survival period compared with mice on the CD (median survival 42 days (CID) versus 61 days (CD), p = 0.001, Kaplan Meier method, logrank).

To confirm that the cause of ill health in the CID group was due to increased tumorigenesis, we next performed a time-point experiment soon after Cre induction. For this experiment we again used the Lgr5creERT2+ Apcfl/fl mice, but this time

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**Figure 2. Effect of c-Myc Overexpression on Cellular Iron Metabolism**

RKO cells were transiently transfected with either pCDNA3.1/Zeo-MYCER or empty vector, and the chimeric protein was then activated by the addition of 4-OHT.

(A) Representative western blot demonstrating the expression of the chimeric protein.

(B) c-Myc overexpression resulted in increased TRR1 and DMT1 mRNA expression (p = 0.017 TRR1, p = 0.0005 DMT1, Student’s t test). Data are presented as mean ± SEM.

(C) Representative western blot indicating expression levels of TRR1, DMT1, and ferritin in control and c-Myc overexpressing cells. β-actin was employed for protein normalization.

(D) c-Myc overexpression resulted in increased TRR1 and DMT1, and decreased ferritin protein expression (p = 0.0025 TRR1, p = 0.046 DMT1, p < 0.0001 ferritin, Student’s t test). Data are presented as mean ± SEM.

(E) To assess the influence of activated c-Myc on cellular iron (IL) levels, cells were cultured in the presence and absence of 4-OHT and IL. Activated c-Myc ER and IL (c-Myc ER+4-OHT+IL) enhanced cellular IL loading compared with cells with no IL (c-Myc ER+4-OHT) and in nonactivated c-Myc ER cells challenged with IL (c-Myc ER+IL; p < 0.0001 c-Myc ER+4-OHT+IL versus c-Myc ER+4-OHT, p < 0.0001 c-Myc ER+4-OHT+IL versus c-Myc ER+IL, Student’s t test). Data are presented as mean ± SEM; * denotes statistical significance compared with control; † denotes statistical significance compared with c-Myc ER+IL.
administered a higher induction regime (four injections of tamoxifen over 4 days) to obtain more incipient adenomas, and took them at an early time point (day 15 post induction). At this stage, the mice on the CD (n = 3) had small lesions in the intestine visible on section but were otherwise without a phenotype. In contrast, the mice on the CID (n = 3) were already displaying signs of ill health at the same time point (day 15) and upon harvesting, they presented a thickened intestine, a hallmark of multiple adenomas. Figures 4B–4D show that the CID led to a drastic increase in both number and size at this early time point (p = 0.04). These lesions also show marked iron deposits in enterocytes, as detected by Perls’ Prussian staining, in contrast to CD lesions, in which such deposits are absent (Figure S3).

The Lgr5creERT2+ construct contains a green fluorescent protein (GFP) reporter knocked into the endogenous leucine-rich repeat containing a G protein-coupled receptor 5 (Lgr5) locus, which allows visualization of cells that express Lgr5 (Barker et al., 2007). Elegant studies by Merlos-Suárez et al. (2011) and van der Flier et al. (2007, 2009) showed that Lgr5 marks a stem cell population in the normal intestine that is retained within adenomas, and this may reflect a cancer stem cell population. A detailed characterization of the Lgr5-GFP population showed that this marks a particular subset of Wnt target genes that define the stem cell population. Therefore, examination of GFP expression may allow assessment of the Wnt stem cell gene expression, which is pertinent given our previous in vitro studies linking iron to activating Wnt signaling (de Lau et al., 2011). When we examined whole-mount intestines for the levels of GFP expression in the Lgr5creERT2+ Apcfl/fl mice on the CID, we noticed a marked increase compared with mice on the CD (Figure 4E). To assess whether this was specific to intestinal cells with modifications in the Wnt pathway, we crossed the ApcMin/+ mice with Lgr5creERT2+ mice. By using the GFP reporter in these mice, we were able to assess Lgr5 expression using multiphoton microscopy to create a 3D reconstruction of the intestinal crypts. In the normal epithelium, cells positive for GFP expression (and hence Lgr5 expression) are located at the bottom of the crypt. Of importance, we found a significant 1.5- to 2-fold increase in the area occupied by Lgr5-positive intestinal stem cells in mice carrying the ApcMin/+ allele (Figure 4F; Figure S4). This was in contrast to Lgr5creERT2+ Apc+/+ mice on the CID, which had the same volume of Lgr5 intestinal stem cells as mice on the CD. This demonstrates once more that iron sensitivity is strictly related to Apc status both in vivo and in vitro.

**Iron Levels Govern the Cellular Fate of Apc-Deficient Cells**

After determining that iron levels were potently modifying tumorigenesis, we next wanted to investigate the direct impact
of iron on intestinal enterocytes that have acutely lost both copies of the Apc gene. To that end, we generated AHCre* Apcfl/fl mice and studied the impact of iron modulation 4 days after Apc gene deletion (Sansom et al., 2004). Mice were placed on the IDD (n = 5), CID (n = 3), or CD (n = 5) for 2 weeks before induction. Figures 5A–5C show that the CID increased proliferation rates by 4- to 5-fold following Apc loss (p = 0.015), whereas the IDD promoted a marked increase in apoptosis by 2- to 3-fold (p = 0.015). This is in good agreement with the tumor burden outcomes obtained earlier in ApcMin/+ mice for the two diets, i.e., an increase in apoptosis gave rise to slow-growing, small tumors in mice on the IDD, whereas an increase in proliferation resulted in larger, more aggressive tumors in mice on the CID. We also looked at the effect of both diets on WT intestines (Figure S5), and we noted no change in the mitotic or apoptotic index for animals on the CID, but an increase in both mitosis and apoptosis in mice on the IDD. However, this increase in both apoptosis and mitosis did not alter the crypt size (compared with CD), indicating that WT intestines are able to cope with the lack of iron by maintaining a careful balance between proliferation and death. We also carefully monitored the health and potential anemia of the mice on the IDD by weighing the mice and obtaining blood cell counts at the end of the experiment (Figures S5 and S6).

We then scored apoptosis and mitosis in the tumors from Lgr5creERT2+ Apcfl/fl survival cohorts to see if these effects on proliferation and apoptosis were retained throughout tumorigenesis. Figures 5D–5F show that adenomas from the iron-deficient mice still retained a higher level of apoptosis (p = 0.006), and the tumors that developed on the CID had overall more proliferation (p = 0.006). Thus, the levels of iron directly affect the ability of Apc-deficient cells to survive and proliferate both immediately following Apc loss and within adenomas.

To confirm our in vitro results showing that Apc-deficient cells had high levels of TfR1 and DMT1 in vivo, and that these were not downregulated following iron administration, we examined the mRNA levels by quantitative reverse transcriptase (qRT)-PCR. In order to isolate a purer intestinal cell population, we used the laser capture microdissection (LCM) technique, which enabled us to microdissect specific parts of the intestinal crypt-villus structure and thus enrich in the epithelial cells of choice (Potter and Brunskill, 2012). We found that mice on the CID had dramatically increased levels of TIR1, DMT1, and ferritin following Apc gene deletion compared with WT mice on the same diet (Figure S7). Moreover, to further examine whether there was an additional expansion of the intestinal stem cell (ISC) signature following Apc loss, we looked specifically at the...
Figure 5. Iron Levels Modulate Proliferation and Apoptosis Immediately after Apc Loss In Vivo

(A) Mitotic index as percentage of cells in mitosis per crypt in Apc-deficient enterocytes from AHCre* Apc-/- mice on CD, IDD, or CID at day 4 post-induction (p = 0.015, CID versus CD, and CID versus IDD, Mann-Whitney).

(B) Apoptotic index as percentage of cells in apoptosis per crypt in Apc-deficient enterocytes from AHCre* Apc-/- mice on CD, IDD, or CID at day 4 post-induction (p = 0.015, IDD versus CD, and IDD versus CID, Mann-Whitney).

(C) Representative H&E pictures of intestines from AHCre* Apc-/- mice on CD, IDD, or CID at day 4 post-induction. Arrows indicate apoptotic bodies. Circles indicate mitotic figures. Scale bar: 10 μm.

(D) Representative H&E pictures of adenomas from Lgr5CreERT2* Apc-/- mice on CD, IDD, or CID at endpoint tumorigenesis. Arrows indicate apoptotic bodies. Circles indicate mitotic figures. Scale bar: 10 μm.

(E) Mitotic index as percentage of cells in mitosis per adenoma in Lgr5CreERT2* Apc-/- mice on CD, IDD, or CID at endpoint tumorigenesis (p = 0.006, CID versus CD, and CID versus IDD, Mann-Whitney).

(F) Apoptotic index as percentage of cells in apoptosis per adenoma in Lgr5CreERT2* Apc-/- mice on CD, IDD, or CID at endpoint tumorigenesis (p = 0.006, IDD versus CD, and IDD versus CID, Mann-Whitney). See also Figures S5 and S6.
stem cell compartment (bottom of the crypt) for the expression levels of the ISC signature genes olfactomedin 4 (Olmf4), Lgr5, tumor necrosis factor receptor superfamily member 19 (Troy) and RGM domain family member B (Rgmb; van der Flier et al., 2009). These genes were all significantly upregulated in Apc-deficient mice treated with high iron compared with WT mice on the same diet (Figure 6A). To verify that this was not simply a reflection of an increased ISC signature following Wnt activation (via Apc loss), we compared the expression in CID versus CD within the same stem/progenitor cell zone. Our results show that there was a significant increase in the ISC targets in the iron-treated mice. This was specific to the crypt base, as LCM of the top of the crypt did not show an increase in stem cell genes even though these cells are also Apc-deficient (Figure 6B). However, Wnt target genes such as c-Myc, Axin2, and Cyclin D2 were upregulated in both the crypt base and the top of the crypt in the CID group, which is consistent with Wnt pathway activation throughout the crypt and villus (Figure 6C).

Delineating the Pool of Iron that Is Responsible for Rescuing Tumorigenesis

Although our data clearly demonstrate a role for iron in tumorigenesis, they fail to delineate the pool of iron, as one would expect that feeding an IDD would result in decreased luminal iron levels and also chronically mediate a systemic iron deficiency. Thus, in an attempt to separate these two pools of iron, Apc<sup>Min/+</sup> mice were fed an IDD but maintained systemically iron replete (IDD+IDex) by subcutaneous injections of iron dextran (IDex; Theurl et al., 2005). Briefly, Apc<sup>Min/+</sup> mice were put on the IDD at weaning and given two subcutaneous injections of 50 mg IDex on two consecutive days to reach systemic iron overload. The iron load status was maintained with an additional subcutaneous injection of 50 mg IDex each month. The results of these studies again demonstrate a suppressed tumor burden compared with mice on the CD (p = 0.004; Figures 7A–7C). Furthermore, Perl’s Prussian blue staining of the intestines from IDD+IDex-treated mice does not show intestinal iron deposits, in contrast to mice on the CID, highlighting the fact that systemic iron overload does not lead to iron accumulation.
in intestinal tissue (Figure S2). However, these observations do not rule out the possibility that the systemic injections of IDex could have had an impact on tumorigenesis, so we further treated a separate cohort of $Apc^{Min/+}$ mice ($n = 9$), this time on a CD, and administered IDex as before. We failed to observe any influence on tumor burden at 80 days (Figure 7D).

**Figure 7. The Luminal Iron Pool Is Responsible for Intestinal Tumor Formation In Vivo**

(A–C) Tumor number, size, and burden comparison between CD, IDD, and IDD+IDex treatment (IDD+IDex) $Apc^{Min/+}$ mice at 80 days of age. Systemic iron overload via IDex treatment does not affect the tumor protection conferred by the lack of dietary iron.

(D) Tumor burden comparison between $Apc^{Min/+}$ mice with systemic iron overload (IDex), systemic iron chelation (DFO), or CD alone (CD).

(E) Overview representing the therapeutic opportunity offered by dietary iron modulation.
Furthermore, decreasing the level of systemic iron in \textit{Apc}^{Min/+} mice (n = 8) by administering daily injections of a potent iron chelator, desferrioxamine (DFO), also failed to affect tumorigenesis. Taken together, these data suggest that the systemic iron pool has little effect on intestinal tumorigenesis, but luminal iron is crucial.

**DISCUSSION**

In this study, we show that spontaneous intestinal neoplasia driven by the loss of the key intestinal tumor suppressor \textit{Apc} is highly dependent on iron. Moreover, we demonstrate that an excess of iron promotes intestinal tumorigenesis, and that tumorigenesis was markedly suppressed in mice fed an IDD. Our observations provide an explanation for previous results from human epidemiological studies that point to high dietary iron intake as a risk factor for CRC. Previous results from human epidemiological studies that point to high dietary iron intake as a risk factor for CRC (Kato et al., 1999; Shaheen et al., 2003; Weinberg, 1994). In addition, we demonstrate that high iron levels result in increased tumorigenesis in the background of an aberration in \textit{Apc}, we also identified the luminal iron pool (rather than the circulating iron pool) as the major driver of carcinogenesis.

One of the most exciting findings from our studies is that \textit{Apc}-deficient cells appear to require precise levels of iron for efficient tumorigenesis in vivo. Removal of iron drives apoptosis of \textit{Apc}-deficient cells, and raising the levels of iron increases proliferation, which suggests that levels of iron are critical for the cellular fate following \textit{Apc} loss. By altering the outcome of an \textit{Apc} mutation, one would predict that this could account for changes in the lifetime risk of iron exposure. Of importance, our studies investigating the impact of iron on WT intestinal enterocytes showed that even very high levels of iron did not lead to spontaneous tumorigenesis. Therefore, it is the impact of iron on cells that have deregulated Wnt signaling that is crucial. This is consistent with our in vitro data showing that downregulation of Wnt signaling (through \textit{Apc} addition) alters the proliferation of CRC cell lines, and our in vivo data showing deregulation of iron import proteins at the adenoma formation in both humans and mice. In support of the importance of a link among Wnt signaling, iron, and colorectal neoplasia, a recent study showed that a class of iron chelators, acyl hydrazones, abrogate Wnt signaling and growth of CRC cell lines with constitutive Wnt signaling (Song et al., 2011).

In the context of human CRC, one issue that has been difficult to resolve is the particular genotype-phenotype relationship of the sporadic \textit{Apc} mutations that occur. Rather than a complete loss-of-function mutation in the \textit{Apc} gene, specific mutations leading to a truncated protein that can bind \(\beta\)-catenin but not Axin may occur. These mutant proteins still cannot turn over \(\beta\)-catenin, but may still be able to more subtly modulate Wnt signaling activity. Knockin of the common human mutation \textit{Apc}^{T327T} in the mouse leads to faster tumorigenesis than the \textit{Apc}^{Min/+} mutation (Pollard et al., 2009). Wnt target gene activation in the model is modestly altered with a reduction in many Wnt targets but an increase in Lgr5 and other stem cell markers (Lewis et al., 2010). Given the increased levels of Lgr5 we observed in \textit{Apc}^{Min/+} mice following

**EXPERIMENTAL PROCEDURES**

**Ethics Statement**

All mouse experiments were performed according to the UK Home Office guidelines. The research was approved by the Glasgow University Ethics Committee. Work with human samples was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved by the ethics committee of the Birmingham and Black Country Comprehensive Local Research Network (LREC no. 10/H1202/40). All patients provided informed written consent. Samples (n = 10) of colorectal carcinoma and colorectal polyps (n = 20) were collected during endoscopy or surgery, and each tissue specimen was processed into mRNA and subjected to qRT-PCR.

**Mouse Colonies**

Outbred mice segregating for the C57BL6J and S129 genomes (five generations C57Bl6J) were used from 4 to 8 weeks of age. The following alleles were used: \textit{AH Cre} (Ireland et al., 2004), \textit{Lgr5-EGFP-iresCreERT2} (Barker et al., 2007), \textit{ApcMin} (Su et al., 1992), \textit{Apc^{S308T}} (Shibata et al., 1997), and \textit{c-MYC} (de Alboran et al., 2001). Only male mice were used in this study.

**Iron Modulation**

For luminal iron modulation, three diets were purchased from Harlan Laboratories UK: TD 80396 (IDD), TD 80394 (48 ppm added iron; CD), and TD 08496 (279 ppm added iron; CD). The research was approved by the ethics committee of the Birmingham and Black Country Comprehensive Local Research Network (LREC no. 10/H1202/40). All patients provided informed written consent. Samples (n = 10) of colorectal carcinoma and colorectal polyps (n = 20) were collected during endoscopy or surgery, and each tissue specimen was processed into mRNA and subjected to qRT-PCR.
(CID). Male ApcMin+/+ and WT littermates were placed on the respective diet at weaning (~30 days of age) and were aged to a time point of 80 days of age. Mice on the CID were taken due to illness at 60 days of age. To ensure that the diets had no adverse effects, three WT mice from each group were kept on each diet for 3 months. For systemic iron modulation, male ApcMin+/+ and WT littermates were injected with IDex (CosmoFer 50 mg/ml; Pharmacosmos, Holbaek, Denmark) or DFO (desferrioxamine mesilate 500 mg; Mayne Pharma, Warwickshire, UK) from weaning up to 80 days of age. IDex was administered subcutaneously (subcut) at 50 mg per dose on 2 consecutive days at the start of the treatment and once a month subsequently to keep the systemic iron levels high. DFO 200 mg/kg was injected intraperitoneally (i.p.) daily for the duration of the treatment to ensure effective systemic iron chelation. A combined IDI and IDex treatment was also administered from weaning, with mice receiving their first IDex injection the day they were put on the I. The IDex treatment regime was continued as above while mice were kept on the IDI until the 80-day time point.

Gene Deletion In Vivo
Adult male mice (6–8 weeks of age) were put on the IDI or CID for 2 weeks before gene manipulation to ensure diet acclimatization. For the survival study, Lgr5creERT2 ApcMin+/+ mice on IDI, CD, or CID were injected with a single dose of 3 mg tamoxifen (Sigma, Dorset, UK) and aged until they showed signs of illness. For time-point experiments, Lgr5creERT2 ApcMin+/+ mice on the IDI, CD, or CID were injected for 4 consecutive days with 3 mg (day 0), 2 mg (day 1), 2 mg (day 2), and 2 mg (day 3) tamoxifen i.p., and were taken at day 15 post-induction. AHCRe ApcMinW mice on IDI, CD, or CID were injected 3 times in 1 day with 80 mg/kg beta naphthoflavone (Sigma) i.p. and taken at day 4 post-induction. AHCRe ApcMin+/+, AHCRe c-MYCW, and AHCRe ApcMin+/+, c-MYCW mice on the CD were injected 3 times in 1 day with 80 mg/kg beta naphthoflavone i.p. and taken at day 4 post-induction.

Tumor Scoring
When ApcMin+/+ mice reached the 80-day time point, they were culled, and the intestine and colon were removed and scored for tumors on whole mount. WT littermates on the respective diets were also assessed for tumors. For the ApcMin+/+ mice on CID (taken at 60 days of age), this was not possible due to the small size of the tumors, so instead their tumors were assessed on hematoxylin and eosin (H&E)-stained sections. The tumors were counted in the first 10 cm of intestine near the stomach. As controls, a separate cohort of ApcMin+/+ mice on CD was culled at the same time point of 60 days and assessed for tumors in the same manner. For the Lgr5creERT2 ApcMin+/+ mice day 15 experiments, lesions were scored off sections stained for β-catenin (anti-β-catenin mouse monoclonal, 1:50; Transduction Labs, Oxford, UK). A strip of 10 cm of intestine taken from the same position was scored for each mouse three mice per group. A lesion was considered as ≥10 cells clumped together and displaying nuclear β-catenin. This was done to distinguish lesions from individual cells positive for nuclear β-catenin found at the bottom of the crypt. To measure the size of the lesions, the total number of nuclear β-catenin-positive cells was counted.

Assaying Apoptosis and Mitosis
The apoptotic and mitotic indexes were scored from H&E sections as previously described (Sansom et al., 2004). For the AHCRe Apc+/+ day 4 experiments, 25 full crypts were scored from at least three mice of each genotype. For the Lgr5creERT2 Apc+/+ survival cohorts, five mice were chosen at random for each diet (IDI, CD, and CID) and five tumors were scored per mouse. All of the cells in one tumor were included in the count.

In Vivo Imaging
In vivo imaging of GFP fluorescence within intestinal tissue was performed as follows: intestines were flushed with PBS, opened longitudinally, and imaged using the Olympus O100 molecular imaging system (Olympus, Southendon-Sea, UK). In Lgr5creERT2 Apc+/+ mice, the Lgr5-EGFP-IREScmERT2 construct drives the expression of GFP in cells that express the LGR5 stem cell marker (Barker et al., 2007).

Cell Culture
The human colorectal cell lines SW480 and RKO were selected as examples of lineages containing mutant and WT Apc molecule respectively and were both routinely cultured in Dulbecco's modified eagles medium (GIBCO, USA) with 10% fetal calf serum supplemented with 1% nonessential amino acids, 100 units/ml penicillin and 0.1 mg/ml streptomycin. For iron loading experiments cells were challenged upon reaching 70% confluence with either growth medium alone (control) or iron loaded medium (growth medium supplemented with 100 μM FeSO4 and 10 μM sodium ascorbate) for 24 hr and cellular iron loading assessed as previously described (Whitnall et al., 2006).

c-Myc Overexpressing Cell Line
RKO cells were transiently transfected with a c-Myc ER construct (pCND3.1-MYCER, a kind gift from Stella Peleangaris, University of Warwick), and 24 hr later some of these cells were treated with 100 nM 4-hydroxytamoxifen (4-OHT) for a further 48 hr. The cells were then lysed and processed into mRNA and protein.

Statistics
Statistical significance was calculated by use of the unpaired Student’s t test. All analyses were performed using SPSS version 10.0 (SPSS, Chicago). Significance was accepted at p < 0.05. The data presented are the mean of three independent experiments, each performed in triplicate.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.07.003.

LICENSING INFORMATION
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ACKNOWLEDGMENTS
We thank Colin Nixon for help with histology, and Derek Miller and Tom Hamilton for help with transgenic work. This work was funded by a Cancer Research-UK Programme Grant to O.S. and a Cancer Research-UK Discovery Committee and Development Grant and Experimental Cancer Medicine Centre funding to C.T. Author contributions: S.R. acquired, analyzed, and interpreted data; performed statistical analysis; and drafted the manuscript. M.J.B. provided samples; acquired, analyzed, and interpreted data; and performed statistical analysis. P.S. acquired, analyzed, and interpreted data. R.A.R. acquired, analyzed, and interpreted data. E.M.G. acquired, analyzed, and interpreted data, and performed statistical analysis. K.A. provided reagents. S.F. acquired and interpreted data. D.S. acquired, analyzed, and interpreted data, and performed statistical analysis. P.S. acquired reagents.

280 Cell Reports 2, 270–282, August 30, 2012 ©2012 The Authors
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