Focal Adhesion Kinase Is Required for Intestinal Regeneration and Tumorigenesis Downstream of Wnt/c-Myc Signaling

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Focal Adhesion Kinase Is Required for Intestinal Regeneration and Tumorigenesis Downstream of Wnt/c-Myc Signaling

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SUMMARY

The intestinal epithelium has a remarkable capacity to regenerate after injury and DNA damage. Here, we show that the integrin effector protein Focal Adhesion Kinase (FAK) is dispensable for normal intestinal homeostasis and DNA damage signaling, but is essential for intestinal regeneration following DNA damage. Given Wnt/c-Myc signaling is activated following intestinal regeneration, we investigated the functional importance of FAK following deletion of the Apc tumor suppressor protein within the intestinal epithelium. Following Apc loss, FAK expression increased in a c-Myc-dependent manner. Codeletion of *Apc* and *Fak* strongly reduced proliferation normally induced following *Apc* loss, and this was associated with reduced levels of phospho-Akt and suppression of intestinal tumorigenesis in *Apc* heterozygous mice. Thus, FAK is required downstream of Wnt Signaling, for Akt/mTOR activation, intestinal regeneration, and tumorigenesis. Importantly, this work suggests that FAK inhibitors may suppress tumorigenesis in patients at high risk of developing colorectal cancer.

INTRODUCTION

A number of studies have shown that the integrin effector kinase pathway that involves Src and FAK is upregulated in colorectal cancer. Although levels of Src and FAK are deregulated at all stages of colorectal carcinogenesis (Cance et al., 2000; Theocharis et al., 2003), most studies have focused upon the importance of these proteins in colorectal cancer (CRC) invasion and metastasis (McLean et al., 2005; Serrels et al., 2006). Therefore, very little is known about the mechanism of Src/FAK deregulation at the early stages of cancer, including the functional importance of their elevated expression or indeed their role in normal tissue homeostasis.

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The intestinal epithelium provides one of the best model systems to study epithelial stem cells, differentiation, regeneration, and cancer. Unlike many other epithelia, the position of the intestinal epithelial stem cell is known, allowing characterization of the number and properties of these stem cells within the intestinal crypt-villus architecture (Potten, 1998; Barker et al., 2008). Indeed, elegant experiments have defined LGR5/GPR49 (a Wnt target gene) and Bmi1 as intestinal stem cell markers in murine small intestine and colon (Barker et al., 2007; Sangiorgi and Capecchi, 2008). Moreover, these cells may be the cell of origin for intestinal cancers (Barker et al., 2009).

The intestinal epithelium has a remarkable capacity to regenerate following DNA damage (Bach et al., 2000), injury (e.g., acute colitis), surgical resection (Bernal et al., 2005), and after Cre-mediated deletion of genes that are essential for intestinal homeostasis (Ireland et al., 2004). This latter process leads to rapid repopulation of the intestine from nonrecombined cells (i.e., those cells which have not undergone Cre recombinase-mediated excision of the LoxP flanked gene of interest and so have not deleted the gene that is essential for intestinal homeostasis). In all cases, the process of intestinal regeneration is characterized by a marked burst of proliferation within the crypt, with an associated transient crypt enlargement (Ijiri and Potten, 1986). Despite this wealth of biological information, very little is known about the molecular pathways important for stem cell maintenance and repopulation within the intestine. In previous studies we found high levels of β-catenin and c-Myc in regenerating intestinal crypts following loss of the genes important for intestinal homeostasis (Ireland et al., 2004; Muncan et al., 2006). Wnt pathway activation has been suggested as the mechanism that allows the intestine to regenerate following *Mdm2* deletion (Valentin-Vega et al., 2008). However, the functional significance of the enhanced Wnt signaling is unclear.

In the present work, we show that FAK is deregulated immediately following loss of the Apc protein and levels are increased during intestinal regeneration. Given that previous data have shown that FAK is important for preventing apoptosis (including progenitor cell apoptosis in the skin) (Frisch et al., 1996; Hungerford et al., 1996; McLean et al., 2004), and for wound healing in vitro (Essayem et al., 2006) (where there are often high levels of Wnt signaling), we addressed whether FAK was required for intestinal regeneration and Wnt-driven tumorigenesis in vivo. We established that FAK is essential for both of these processes, and contributes to Wnt-mediated induction of the Akt/mTOR pathway.

RESULTS

FAK Is Upregulated during Intestinal Regeneration and Transformation in a c-Myc-Dependent Manner

Given that FAK expression is upregulated in CRC and deregulated Wnt signaling is a key event in CRC, we addressed whether FAK expression was elevated following activation of Wnt signaling within the intestinal epithelium, either during intestinal regeneration or following acute activation of Wnt signaling due to Apc loss. We found that while FAK is weakly expressed in the normal murine intestinal epithelium (Figure 1A), it is clearly upregulated during intestinal regeneration (Figure 1B) and following *Apc* gene deletion (Figures 1D and 1E). Given our previous studies showing that c-Myc is an essential downstream target fol lowing Apc loss (Sansom et al., 2007), we next showed that c-Myc was essential for elevated expression of FAK either during intestinal regeneration after DNA damage or following Apc loss (Figures 1C, 1F, and 1G). To examine if this was due to a change in the transcriptional levels of FAK, we performed qRTPCR in wild-type, Apc-deficient, and Apc/c-Myc double-knockout intestines. FAK expression was increased by 2-fold in the Apc-deficient intestines and reduced back to wild-type levels in the double Apc/Myc mutant intestines (fold change relative to wild-type: Apc 2, Apc/Myc:0.82, Apc versus

Apc/Myc p value: 0.001). High FAK expression was maintained in intestinal adenomas from the $Apc^{fl/+}$ mouse suggesting that high FAK expression may play a role in Wnt-dependent tumorigenesis following Apc loss (Figure 1H).

Although we, and others, have previously shown that Wnt and c-Myc signaling is activated during intestinal regeneration, we had not proved that this was "driving" regeneration. Therefore, we tested whether c-Myc-deficient enterocytes could regenerate following gamma irradiation (Potten and Grant, 1998). Conditional deletion of the gene encoding *c-Myc* in the murine small intestine was achieved by crossing mice carrying LoxP-flanked *c-Myc* alleles with mice carrying the *Cre* recombinase transgene under the control of the Cyp1A1 aryl hydrocarbon-responsive promoter (*AhCre*) (Ireland et al., 2004; Muncan et al., 2006). Cre-mediated excision was induced by intraperitoneal injection of β-naphthoflavone. This induces Cre expression within the small intestinal crypt and also in hepatocytes (Ireland et al., 2004; Sansom et al., 2004, 2005). When using an induction regimen that yields maximal recombination (three injections spaced 4 hr apart) (Sansom et al., 2007), nearly 100% recombination is achieved in Cre^{+} mice as scored through β-galactosidase staining using the Rosa26R surrogate marker of recombination (Soriano, 1999).

Successful regeneration results in large hyperproliferative crypts indicated by the black arrows in Figure 2A (72 hr following the DNA damage insult). If this does not occur, crypts are either completely ablated or are in the process of dying (shown by the red arrows in Figure 2B). These "dying" crypts are composed of less than six consecutive epithelial cells and are often cystic. The number of surviving crypts can be counted by using hematoxylin and eosin (H&E)-stained sections from control and Myc-deficient intestine, to assess whether c-Myc is required for crypt survival (Potten, 1998). Figure 2 shows that $Cre^{+}Myc^{+/+}$ (control) intestinal crypts regenerate efficiently after treatment with 14 Gy gamma irradiation, while $Cre^{+}Myc^{fl/fl}$ crypts do not regenerate, with no c-Myc-deficient crypts being present 72 hr after irradiation (compare Figures 2A and 2B; see Figure S1A available online). The only surviving crypts had "escaped" the recombination event and showed high expression of c-Myc (and FAK) as shown by immunohistochemistry (IHC) (Figures 2D and 2E, see black arrows highlighting enlarged crypts with c-Myc staining, 2H enlarged crypt with FAK staining). From here on, we will describe these crypts as "escapers," which serve as excellent internal controls for immunohistochemical comparisons. Importantly, we can show using serial sections that the escaper c-Myc-positive crypts also have high levels of FAK when compared with the surrounding recombined Myc-deficient dying crypts (Figures S1B–S1E).

One of the difficulties with this analysis is that although c-Myc-deficient intestinal crypts can survive over the short term following c-Myc deletion, over the long term they are lost (Muncan et al., 2006). To deal with this problem, we intercrossed the *AhCre* $Myc^{fl/fl}$ mice with mice carrying a Lox-STOP-Lox *c-myc* allele targeted to the *Rosa26* locus (X.W., M. Cunningham, S. Tokarz, B. Laraway, X. Zhang, H. Arnold, M. Troxell, and R.C.S., unpublished data). This allele delivers physiological levels of c-Myc (equivalent to wild-type Myc) following Cre-mediated recombination, but as it is under the control of the *Rosa26* promoter (instead of its own promoter) it is not inducible by Wnt signaling. Following Cre induction, *AhCre* $Myc^{fl/fl}Rosa^{c\text{-}Myc/+}$ intestinal enterocytes proliferated as in wild-type mice and cells were not lost over the long term (Figure S1F). This allowed us to specifically address the role of c-Myc that is induced by Wnt following irradiation. We found that intestinal regeneration was markedly reduced in the *AhCre* $Myc^{fl/fl}Rosa^{c\text{-}Myc/+}$ mice with few surviving intestinal crypts, despite the presence of c-Myc (Figures 2F and 2G; Figures S1G–S1I). Importantly, FAK levels were much reduced in these *AhCre* $Myc^{fl/fl}Rosa^{c\text{-}Myc/+}$ poorly regenerating crypts (Figures 2H and 2I), suggesting FAK may be

an essential downstream target of c-Myc signaling during intestinal regeneration and oncogenic transformation in the mouse intestine in vivo.

FAK Is Redundant for Normal Intestinal Homeostasis but Is Required for Intestinal Regeneration

We next addressed whether FAK plays a role in intestinal homeostasis. Mice carrying LoxP-flanked *Fak* alleles (McLean et al., 2004) were intercrossed to mice carrying the *AhCre* transgene. To investigate the phenotype of loss of FAK from the intestinal epithelium in the adult mouse, experimental $AhCre^{+}Fak^{fl/fl}$ animals and control $AhCre^{+}Fak^{+/+}$ animals were induced and euthanized and intestines were analyzed at 4, 18, 150, and 350 days postinduction. At day 4 after cre induction, intestines were stained for activity of the LacZ recombination reporter transgene (Rosa26R), which showed that there was near 100% recombination in $AhCre^{+}Fak^{fl/fl}$ animals, and this level of recombination remained stable for over 350 days (Figure S2A). Using IHC, no appreciable staining was observed 4 days after gene deletion, and this protein loss was maintained indefinitely (Figure S2B). Immunoblotting for FAK protein also showed a loss of protein at day 4 (Figure S2C). Despite FAK deletion, intestinal homeostasis was not altered, with normal levels of proliferation, differentiation, apoptosis, and migration (Figure S2).

Next, we investigated whether FAK was important for intestinal regeneration. Therefore, 4 days after cre induction, $AhCre{+}Fak^{+/+}$ and $AhCre{+}Fak^{fl/fl}$ mice were irradiated with 14 Gy gamma irradiation. At 72 hr, mice were euthanized and the small intestines removed. We found an almost complete absence of hyperproliferative crypts in FAK-deficient animals (Figure 3A, right-hand panel and Figure 3B). Indeed, the few live crypts that remained were all escapers (described above), retaining high expression of FAK (judged by IHC and shown by black arrows in Figure 3C). Moreover, FAK deficiency also prevented regeneration following cisplatin treatment (Figure S3E).

Given the considerable amount of literature that links FAK to cell survival and suppression of apoptosis (Crouch et al., 1996; Frisch et al., 1996; Hungerford et al., 1996; McLean et al., 2004; Lim et al., 2008), it was possible that rather than affecting the regrowth of intestinal crypts, FAK-deficient crypts were dying due to increased apoptosis as a result of gamma irradiation. Therefore, we examined whether *Fak* deletion affected the ability of intestinal enterocytes to enter an apoptotic program following DNA damage. Experimental and control animals at day 4 after cre induction were exposed to 14 Gy gamma irradiation and tissues were harvested 6 and 24 hr after exposure. Histological sections from these animals were examined and levels of apoptosis scored. In both groups, irradiation caused a large increase in the number of apoptotic figures and a reduction in mitotic figures. However, FAK deficiency did not significantly alter either the levels of apoptosis or mitosis (Mann-Whitney $p \geq 0.5$, $n = 6$) (Figure S3A). Consistent with this, p53 and its target gene p21 were both induced irrespective of FAK status (Figures S3B–S3D). To investigate whether this was an agent-or dose-specific effect, we also investigated the levels of apoptosis and p53 and p21 induction following 5 Gy irradiation, or treatment with 10 mg/kg cisplatin. There were no significant differences between control and experimental animals (Figures S3A–S3C). Taken together, these findings show that FAK is not required for (nor does it exacerbate) p53-dependent apoptosis following high levels of DNA damage in the intestinal epithelium.

Although levels of apoptosis were equivalent at the early stages following DNA damage, during the regrowth phase of the intestinal epithelium at 48 hr, $AhCre^{+}Fak^{fl/fl}$ mice had higher levels of apoptosis and reduced levels of proliferation compared with wild-type regenerating intestines (Figures 3D and 3E). These data suggest that while FAK is not

required for homeostatic proliferation, it is required for the burst of proliferation during intestinal regeneration which is associated with high levels of Wnt signaling.

Our previous studies have shown that the AhCre expression is restricted to the epithelium of the murine small intestine, and so FAK should not be deleted in nonintestinal epithelial cells. Endothelial cells are also important for the regeneration process, but we found that conditional deletion of FAK within the intestinal epithelium did not affect apoptosis of endothelial cells following 14 Gy irradiation (median endothelial apoptosis per villus 5.12 [WT] v 5.22 [FAK] 6 hr following irradiation, Mann-Whitney $p = 0.66$, $n = 3$). However, given the *AhCre* transgene also yields recombination in other tissues (predominantly the liver), we also generated *VillinCre*^{ER}*Fak*$^{fl/fl}$ mice to ensure that our pheno-type was indeed due to deletion of FAK specifically within the intestine. Consistent with our studies in the *AhCre* mice, we again found that FAK was redundant for normal intestinal homeostasis, and did not affect short-term irradiation induced apoptosis (data not shown), but was absolutely required for intestinal regeneration following DNA damage (Figures 3F and 3G). Indeed, using *Villin-Cre*^{ER}, even fewer escaper, nonrecombined crypts survived. Taken together, our data show unequivocally that FAK is required for the hyperproliferative response and regrowth of the intestinal crypts (hence regeneration) after gamma-irradiation.

FAK Is Required to Induce Signaling to Akt/mTOR during Intestinal Regeneration

We next investigated what signaling downstream of FAK was linked to its requirement for intestinal regeneration. One of the key downstream effectors of FAK signaling demonstrated in vitro is the PI3 kinase-Akt pathway (Gabarra-Niecko et al., 2003; McLean et al., 2005; Thamilselvan et al., 2007). Therefore, we addressed whether Akt/mTOR signaling was activated during regeneration, and if so, whether this was FAK dependent. To examine signaling to Akt, we investigated the expression of phosphorylated active p-Akt^{Ser473}, and its targets p-GSK3β^{Ser9} and p-mTOR^{Ser2448}, as downstream readouts. Figures 4A–4D show the increased expression of p-Akt, p-GSK3β and p-mTOR in regenerating crypts, consistent with robust activation of the Akt signaling pathway in regenerating epithelium. To test whether this was required downstream of FAK signaling, we examined p-Akt in control and FAK-deficient intestines. p-Akt was not elevated in FAK-deficient crypts during the regenerative process when compared with controls, while in the few FAK-proficient escaper crypts, we found high levels of p-Akt, similar to levels in wild-type regenerating crypts (Figure 4A, black arrows indicate regenerating escaper crypts, red arrows indicate FAK-deficient crypts). p-Akt was also not visibly elevated in c-Myc-deficient crypts (Figure 4B, black arrow indicates regenerating escaper crypt, red arrows indicate c-Myc-deficient crypts), arguing that, Wnt/Myc signaling is upstream of signaling to Akt and is mediated by FAK.

In addition to signaling to Akt itself, two downstream targets, namely, GSK3β and mTOR, were also phosphorylated in wild-type regenerating crypts after irradiation (Figures 4C and 4D). Importantly, only regenerating FAK-proficient crypts showed upregulation of p-mTOR, whereas p-mTOR could not be visualized in FAK-deficient crypts (Figure 4D). We confirmed this upregulation of p-Akt and p-mTOR in regenerating FAK-proficient escaper crypts using serial sections (Figure 4E). Consistent with FAK being the key downstream target of c-Myc, *AhCre Myc*$^{fl/fl}$*Rosa*$^{c\text{-}Myc/+}$, intestines which failed to regenerate (or upregulate FAK) also did not display increased levels of p-Akt or p-mTOR (Figures S1J and S1K).

To test whether the lack of elevated Akt/mTOR signaling was functionally important, we treated wild-type mice with either PI103 (a PI3 kinase inhibitor (Raynaud et al., 2007)) or rapamycin (an inhibitor of the Akt-mediated phosphorylation of mTOR) and found both agents significantly reduced the number of surviving crypts following ionizing radiation

(Figure 4F). These data imply that a pathway linking Wnt signaling via c-Myc to Akt and mTOR is mediated by FAK and is critically required for intestinal regeneration. This provides a functional link between the Wnt and integrin signaling pathways in which FAK serves to connect Wnt- and integrin-dependent activation of Akt/mTOR signaling in vivo.

Activating Akt Signaling Independently of FAK Permits Regeneration

The finding that PI3 kinase or mTOR inhibition inhibits regeneration correlated with the failure of signaling to Akt/mTOR in the FAK-deficient intestinal enterocytes. To test causality, we activated Akt signaling, independently of FAK, to determine if this rescued regeneration in FAK-deficient intestines. One method known to stimulate Akt in the intestinal epithelium is to inject mice intravenously with IGF1 (insulin-like growth factor 1), and this has been shown to increase intestinal regeneration by stimulating Akt if given at the time of irradiation (Booth and Potten, 2001). Moreover, a recent study investigating the transcriptome of FAK-deficient mammary epithelial tumors showed a marked upregulation of IGFR (IGFI-receptor) (and GRB2) suggesting that FAK-deficient cells should remain responsive to IGF (Provenzano et al., 2008). Therefore, given our hypothesis that lack of Akt activation (due to FAK deletion) at the later stages of the regeneration process (48–72 hr) was contributing to failed regeneration, we injected mice with IGF1 24, 36, and 48 hr following irradiation (after the peak of DNA damage-induced apoptosis). This rescued the failed regeneration caused by FAK deficiency, with evidence of large hyperproliferative FAK-deficient crypts (Figures 5A–5C, red arrows indicate FAK-deficient regenerating crypts, black arrows indicate regenerating escaper FAK-proficient crypts). Moreover p-Akt and p-mTOR were now visible in all regenerating crypts (Figures 5D and 5E). These data demonstrate that FAK is required to link the Wnt and Akt/mTOR pathways during intestinal regeneration.

FAK Is Required for Intestinal Transformation following Apc Loss

We next wished to test if FAK was also required for oncogenic transformation downstream of Apc loss. Hence, we intercrossed *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice to *AhCre*$^{+}$*Fak*$^{fl/fl}$ mice to generate double-mutant *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ mice. Mice were induced with β-naphthoflavone as described previously, and the phenotypes of the intestines were analyzed 4 days after gene deletion. Apc-deficient intestinal enterocytes displayed a crypt progenitor cell-like phenotype, with marked hyperproliferation and nuclear β-catenin in every cell as described previously (Sansom et al., 2004). *AhCre*$^{+}$*Apc*$^{fl/fl}$*FAK*$^{fl/fl}$ intestinal crypts were smaller, showed an absence of FAK expression, and showed a significant reduction in proliferation (as measured by BrdU incorporation) 4 days after cre induction, when compared with *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice (Figures 6A–6C; Figure S4A). Moreover, they displayed increased levels of apoptosis (Figure 6C).

We next wished to test if the same Myc/FAK/p-AKT/mTOR pathway that was promoting regeneration was driving transformation following Apc loss. The double-knockout Apc/Fak (*AhCre*$^{+}$*Apc*$^{fl/fl}$*FAK*$^{fl/fl}$) intestines were used to test this hypothesis directly. Since we predict that FAK would be downstream of β-catenin and c-Myc, and upstream of Akt/mTOR, the Apc/FAK double-knockout intestines should display high levels of nuclear β-catenin and c-Myc (equivalent to Apc single knockouts; Figures 6D and 6E) but no increase in the levels of p-AKT/mTOR. IHC staining showed a marked increased in nuclear β-catenin and c-Myc staining in Apc/FAK double-knockout intestines compared with wild-types, which was similar to single Apc knockout intestines (Figures 6D and 6E). In contrast, much lower levels of p-Akt/p-mTOR were observed by both IHC and immunoblotting in the double knockout when compared with single Apc knockout intestines (Figures 6F and 6G; Figure S4B). This genetic experiment directly demonstrates that FAK is required for efficient activation of p-Akt/mTOR following Apc loss and induced c-Myc expression.

To test the long-term significance of *Fak* deletion to Apc-deficient cells, we next investigated whether FAK deficiency slowed tumorigenesis in mice heterozygous for *Apc* loss. Cohorts of 20 $AhCre^{+}Apc^{fl/+}Fak^{+/+}$ mice and 20 $AhCre^{+}Apc^{fl/+}Fak^{fl/fl}$ mice were given three injections of 80 mg/kg β-naphthoflavone at weaning to induce loss of one *Apc* allele, and mice were aged until they developed intestinal adenomas. As we have shown previously, adenomas arise following loss of the wild-type *Apc* allele and mice are culled when they develop signs of ill health due to intestinal tumorigenesis: anemia (as observed by white feet) in concert with weight loss and hunching (Sansom et al., 2006; Muller et al., 2009). All 20 $AhCre^{+}Apc^{fl/+}Fak^{+/+}$ mice developed symptoms of intestinal tumors, and on necropsy had numerous intestinal adenomas at 400 days (or earlier) following induction (consistent with our previous data; Sansom et al., 2006; see Figure 6H). However, none of the 20 $AhCre^{+}Apc^{fl/+}$ $Fak^{fl/fl}$ mice developed signs of intestinal disease, but began to die of nonrelated symptoms due to longevity (lymphoma and hepatocellular carcinoma; Figure S4E). β-galactosidase staining of $AhCre^{+}Rosa26R^{+}Apc^{fl/+}Fak^{fl/fl}$ whole mounts 500 days after induction showed retention of LacZ-positive recombined cells, showing that the suppression of tumorigenesis was not simply due to the loss of recombined enterocytes (Figure S4C). Consistent with the retention of the recombined cells, IHC for FAK showed that the majority of intestinal enterocytes still lacked FAK (Figure S4D). Of the 20 $AhCre^{+}Apc^{fl/+}Fak^{fl/fl}$ mice, 14 showed no signs of ill health and were culled at 500 days after induction (over 70 days after the last $AhCre^{+}Apc^{fl/+}Fak^{+/+}$ mouse had been sacrificed; Figure S4E for survival curve) and only 3 of 20 $AhCre^{+}Apc^{fl/+}Fak^{fl/fl}$ mice developed intestinal adenomas (with 2 of these only developing a single adenoma; Figure S4F). Therefore, deletion of *Fak* almost completely suppressed tumor formation following Apc loss, highlighting that FAK is required by cells with deregulated Wnt signaling within the intestinal epithelium for both oncogenic transformation and tissue regeneration after DNA damage.

DISCUSSION

In this study, we have provided in vivo evidence that crosstalk between the Wnt and integrin (via FAK) signaling pathways is required for both intestinal homeostasis and cancer. This pathway is critical for cell survival, as without FAK to link the Wnt and Akt/mTOR pathways, there is a switch from the proproliferative activity of the Wnt signaling pathway to an apoptotic response. This finding fits with recent data showing that mTOR is critical downstream of Wnt signaling for stem cell depletion in the skin and that rapamycin can slow tumorigenesis in the $Apc^{Min/+}$ mouse (Fujishita et al., 2008; Castilho et al., 2009).

Our previous studies have identified that the c-Myc oncoprotein is essential for all the phenotypes of Apc deficiency within the intestinal epithelium (Sansom et al., 2007; Wilkins and Sansom, 2008). Here, we now extend this c-Myc dependency to intestinal regeneration. Importantly, this is not simply a reflection of functionally compromised c-Myc-deficient intestinal enterocytes as mice carrying physiological levels of c-Myc, which is not Wnt inducible, are unable to undergo efficient intestinal regeneration. This reinforces how critical the levels of c-Myc are for biological outcome and is consistent with recent studies showing that the threshold of c-Myc activity governs apoptotic versus proliferative outcomes in vivo (Murphy et al., 2008). Indeed, following Apc loss, there is only a 4-fold induction of c-Myc mRNA and reducing this by even 2-fold can suppress proliferation and tumorigenesis (Athineos and Sansom, 2010; Yekkala and Baudino, 2007). It is thus essential to delineate the critical downstream pathways of c-Myc that are responsible for the effects of these relatively small changes in c-Myc expression. Our current identification of FAK and p-Akt/mTOR as key downstream effectors of this pathway is therefore important. Indeed, FAK is often viewed as a critical determinant of apoptosis in a number of different systems (Parsons, 2003; Mitra and Schlaepfer, 2006), and it is thus tempting to speculate that FAK

expression may be an important requirement of cells with high levels of c-Myc to permit survival. Moreover, FAK deletion has reduced tumorigenesis in a number of different cellular contexts (such as mammary and skin carcinogenesis) where *c-Myc* deletion (or mTOR inhibition) have also been shown to suppress tumorigenesis, suggesting that the pathway identified in the present study in mouse intestine may be relevant for numerous different cancer settings (Lahlou et al., 2007; Amornphimoltham et al., 2008; Provenzano et al., 2008). Therefore, we may have uncovered a more general role for FAK, downstream of activated Wnt signaling, in a number of biological contexts, such as development, tissue regeneration after damage, and tumorigenesis.

Although FAK is upregulated in numerous cancers, very little is known about the mechanism that causes elevated expression (Gabarra-Niecko et al., 2003; McLean et al., 2005). Of particular relevance to our study is that *FAK* can be coamplified with *c-MYC* in cancer (Agochiya et al., 1999) and that N-Myc can transcriptionally activate *FAK* in neuroblastoma (Beierle et al., 2007). Our finding that the precise c-Myc levels control FAK expression is therefore an important step in unraveling the precise molecular regulators of FAK levels in cancer. We are currently undertaking further work to assess if this is due to direct transcriptional control of FAK by c-Myc or whether this is through an indirect mechanism.

With regard to the lack of impact of *Fak* deletion on normal intestinal epithelium, we note that this phenotype differs somewhat from the phenotype of *ITGB1* (integrin β1) deletion in the intestine (Jones et al., 2006). Mice lacking β1-integrin solely within the intestinal epithelium show hyperproliferation and post-natal lethality (Jones et al., 2006). However, the latter study was performed using a constitutive Cre recombinase (Villin Cre) which is expressed in the embryonic mouse intestine. It is thus possible that there is developmental compensation between integrin family members that may explain these differences. Compensation does occur in some circumstances, as some components of integrin signaling are known to cause upregulation of others to drive proliferation (Reynolds et al., 2009). The existence of such compensatory mechanisms is further exemplified by the deletion of *Stat3*, using Villin Cre, causing activation of Stat1 (Musteanu et al., 2010).

Our finding that Wnt signaling is important for intestinal regeneration fits into the context of a generic role for Wnt signaling in epithelial tissue regeneration, as studies have identified activated Wnt signaling in both murine liver and zebrafish tail regeneration (Tan et al., 2006; Stoick-Cooper et al., 2007). However, given the development of Wnt inhibitors for cancer therapy (DeAlmeida et al., 2007), it raises the possibility that if these are used in combination with chemotherapeutic drugs, or radio-therapy, they may exacerbate gastrointestinal toxicity. Thus, it will be vital to study the impact of Wnt inhibitors on intestinal regeneration and stem cells following treatment with these genotoxic agents in preclinical models.

A recent study has suggested that one of the mechanisms by which FAK promotes cell survival is by control of p53 degradation via the FERM domain of FAK functioning in the nucleus (Lim et al., 2008). In particular, there is upregulation of p53 at E8.5 in the mesoderm of $Fak^{-/-}$ embryos. Although genetic knockout of p53 did not rescue the phenotype of FAK deficiency, the strong proliferation block that occurs in embryonic fibroblasts grown in culture was abolished in the double knockouts (Lim et al., 2008). Here, we failed to find any impact of *Fak* deletion on p53 levels, either in normal homeostasis or after DNA damage. This is not too surprising as the level of damage we have used in our study initiates a robust p53 response with high levels of apoptosis and arrest, and therefore we would be unlikely to see further enhancement of p53 accumulation. We therefore conclude

that FAK-mediated stabilization and accumulation of p53 does not occur in vivo in the intestine, and so may be cell type specific.

In summary, we have shown that FAK is essential downstream of activated Wnt signaling within the intestinal epithelium. Given the central role of Apc loss in colorectal cancer, this raises the potential that FAK may be a particularly attractive target for chemopreventive studies, especially given the redundancy of FAK for normal intestinal homeostasis and the number of novel FAK inhibitors which are in preclinical development (McLean et al., 2005).

EXPERIMENTAL PROCEDURES

Mouse Colonies

All experiments were performed under the UK Home Office guidelines. Outbred male mice from 6 to 12 weeks of age were used, which were segregated for the C57BL6 and S129 genomes. The alleles used were as follows: Apc^{580S} (Shibata et al., 1997), $Myc^{fl/fl}$ (Baena et al., 2005), *AhCre* (Ireland et al., 2004), $Rosa26R^{+}$ (Soriano, 1999), $Fak^{fl/fl}$ (McLean et al., 2004), and $VillinCre^{ER}$ (Andreu et al., 2005). Mice were exposed to gamma-irradiation 137Cs sources. This delivered gamma-irradiation at 0.423 Gy/min.

Rapamycin was made up as a 50 mg/ml stock solution in ethanol and diluted in PBS + 5% Tween 80, 5% PEG 400. PI103 was made up as a 25 mg/ml stock solution in DMSO and diluted in PBS + 20% 4-hydroxypropyl β-cyclodextrin.

Tissue Isolation

To induce recombination, mice were given three injections (i.p.), spaced 4 hr apart, of β-naphthoflavone (80 mg/kg). At the appropriate time point, mice were sacrificed and the small intestines were removed and flushed with water (at least three mice were used for each experiment).

β-Galactosidase Analysis

To determine the pattern of recombination at the *Rosa26R* reporter locus, intestinal whole mounts were prepared, fixed, and exposed to X-gal substrate using a method previously reported (Soriano, 1999).

Assaying Crypt Size, Apoptosis, and Mitosis

Apoptosis, crypt size, and mitotic index were scored from H&E-stained sections as previously described (Sansom et al., 2004). For each analysis, 25 full crypts were scored from at least three mice of each genotype. In this paper, data are presented as boxplots. All boxplots express the first (Q1) and third (Q3) quartiles within a given data set by the upper and lower horizontal lines in a rectangular box, inside which is a horizontal line showing the median. The whiskers extend upward and downward to the highest or lowest observation within the upper (Q3 + 1.5 × the interquartile range) and lower (Q1 − 1.5 × interquartile range) limits. Values outside the upper and lower limits are “outliers” and are shown by individual symbols.

RNA Extraction and Quantitative Real-Time PCR

Intestinal tissue was homogenized in Trizol reagent (Invitrogen) and RNA extracted using standard phenol-chloroform methodologies. Reverse transcription was performed using the SuperscriptII reverse transcriptase kit (Invitrogen) and Random hexamers (Invitrogen) as per the manufacturer's instructions. DyNAmo HS (hot-start) SYBR green supermix (Finnzymes)

was used and the PTC-200 Peltier thermal cycler and Chromo4 continuous fluorescence detector (both MJ Research) were used in conjunction with Opticon Monitor analysis software (Version 2.03, MJ Research) to calibrate and run the reaction. Primers sequences for FAK were CTATCAACAGGTGAAGAGT GAC (F), CTTGACAGAATCCAGTAAACTC (R).

Enrichment for Epithelial Cells

In order to obtain a population of cells enriched for epithelial cells, an epithelial extraction protocol based on that of (Bjerknes and Cheng, 1981) was performed on freshly harvested intestine. In brief, a 10 cm section of small intestine was flushed well with water before being tied off at one end and inverted over a 4 mm glass rod. Vibration was then applied to the glass rod, and the intestine was placed in 10 mM EDTA in Hanks' balanced salt solution (HBSS; GIBCO) at 37°C for 15 min. The intestine was then moved into a clean tube of 10 mM EDTA/HBSS and incubated in the same fashion for a further 15 min. Centrifugation (2700 × g, 4°C, 15 min) yielded a pellet containing predominantly epithelial cells.

Western Blotting

Protein was extracted from epithelial-enriched samples by standard methods using lysis buffer (20 mM Tris-HCl [pH 8.0], 2 mM EDTA [pH 8.0], 0.5% [v/v] NP-40) containing protease inhibitors (Complete Mini Protease inhibitor tablets, Roche) and phosphatase inhibitors (25 mM sodium β-glycerophosphate, 100 mM sodium fluoride, 20 nM Calyculin A, 10 mM sodium pyrophosphate). Solubilized proteins (30 μg) were separated by standard SDS-PAGE on a 10% polyacrylamide separating gel with 5% stacking gel, and subsequently transferred to PVDF membrane (Hybond-P, Amersham Biosciences) by standard methods.

Primary antibodies and conditions used to probe blots were rabbit anti-FAK (1:1000; Cell Signaling), mouse anti-β-actin (1:5000; Sigma), anti-p53 (1:1000; BD), and anti-pAkt^{S473}, anti-tAkt, and anti-tubulin (all 1:1000, Cell Signaling). Appropriate HRP-conjugated secondary anti-rabbit or anti-mouse antibodies were used (Amersham Biosciences).

Immunohistochemistry and In Situ Hybridization

IHC and ISH were performed as described previously (Sansom et al., 2004; Gregorieff et al., 2005). Primary antibodies used for IHC were anti-β-catenin (1:50; Transduction Labs), anti-FAK (1/200; Cell Signaling), anti-c-Myc (1:50; Upstate), anti-pAkt^{Ser473} (1/50; Cell Signaling), anti-pGSK3β^{Ser9} (1/50; Cell Signaling), anti-p-mTOR^{Ser2448} (1/100; Cell Signaling), anti-p21 M19 (1/400; Santa Cruz), anti-p53 CM5 (1/500; Vector Laboratories), and anti-lysozyme (1:100; Neomarkers).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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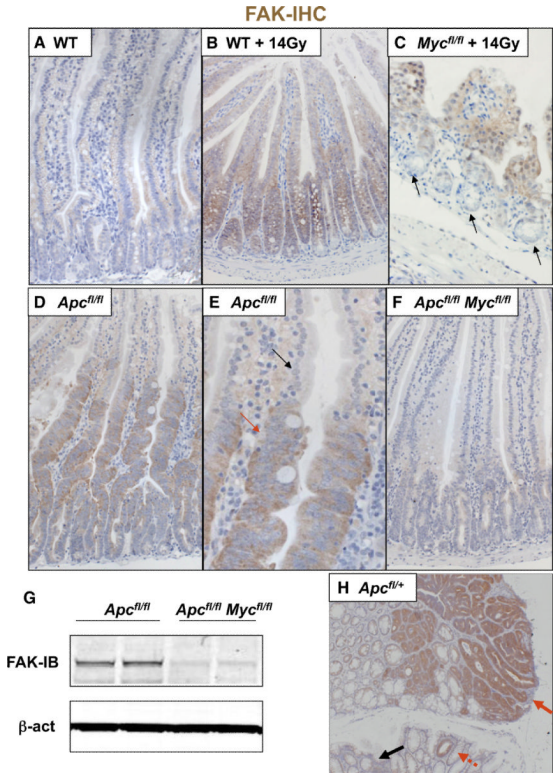

Figure 1. FAK Is Upregulated following Wnt Signaling Activation In Vivo

(A–C) FAK IHC in wild-type (A) and regenerating epithelium (B and C). Note low levels of FAK expression in normal intestinal epithelium (A) which markedly increase in regenerating epithelium (B) 3 days after gamma irradiation. (C) Lack of FAK expression in dying *c-Myc*-deficient crypts (arrows) 3 days after gamma irradiation.

(D and E) FAK IHC in $AhCre^{+}Apc^{fl/fl}$ (labeled $Apc^{fl/fl}$) intestines 4 days postinduction (PI). IHC shows a clear upregulation of FAK in *Apc*-deficient intestines (D). This is highlighted at the margin of the Apc-deficient cells where nonrecombined retained villi enterocytes have low levels of FAK protein (black arrow), while *Apc*-deficient cells (red arrow) have very high levels of FAK protein (E). The reason why there are still wild-type cells retained 4 days following Cre activation is that Apc-deficient cells do not migrate efficiently (see Sansom et al., 2004).

(F) FAK IHC in $AhCre^{+}Apc^{fl/fl}Myc^{fl/fl}$ (labeled $Apc^{fl/fl}Myc^{fl/fl}$) intestines 4 days after Apc loss. IHC shows that FAK is not upregulated in double-mutant *Apc Myc* intestines.

(G) Immunoblotting comparing FAK levels in epithelial extracts from $AhCre^{+}Apc^{fl/fl}$ and $AhCre^{+}Apc^{fl/fl}Myc^{fl/fl}$ intestines 4 days PI. Note there is clearly reduced FAK protein expression in the $AhCre^{+}Apc^{fl/fl}Myc^{fl/fl}$ intestines.

(H) FAK IHC of adenomas of $AhCre^{+}Apc^{fl/+}Fak^{+/+}$ mice 300 days following Cre induction. Note FAK is upregulated in both adenomas (solid red arrow) and single aberrant crypts (dashed red arrow) compared with normal epithelium (black arrow).

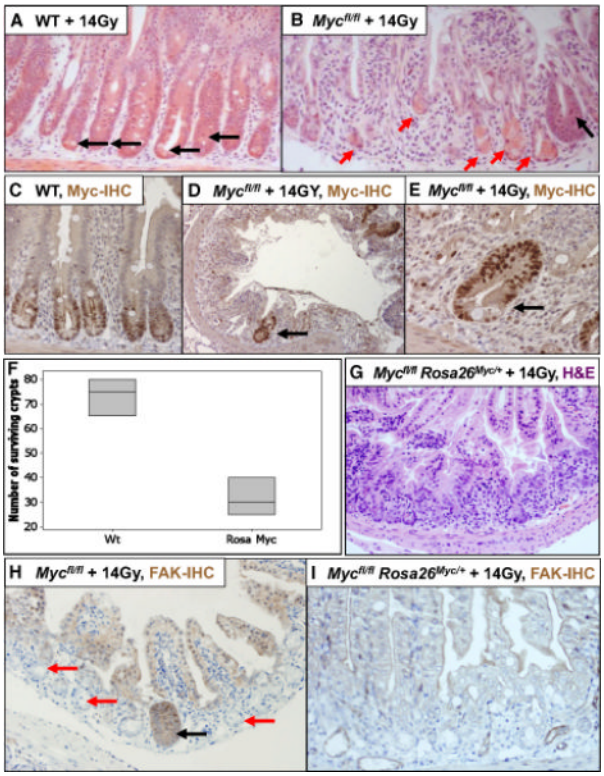

Figure 2. c-Myc Is Required for Intestinal Regeneration

(A) Wild-type regenerating crypts are enlarged following 14 Gy irradiation (black arrows).

(B) *AhCre*$^{+}$*Myc*$^{fl/fl}$ intestines day 6 PI, 3 days following 14 Gy irradiation. Note there are now large areas of intestine denuded of crypts with only occasional enlarged regenerative crypts (denoted by black arrow). Dying/cystic crypts are denoted by red arrows.

(C) IHC for c-Myc in wild-type intestine day 6 PI. Note c-Myc-positive cells are located at the base of the crypt.

(D and E) IHC for c-Myc in *AhCre*$^{+}$*Myc*$^{fl/fl}$ intestine day 6 PI, 3 days following 14 Gy irradiation. The regenerative intestinal crypts (black arrows) are all c-Myc proficient. These are thus defined as escaper crypts.

(F) Boxplot showing significant reduction in surviving crypts between *AhCre*$^{+}$*Myc*$^{fl/fl}$*Rosa*$^{Myc/+}$ (labeled Rosa Myc) and wild-type *AhCre*$^{+}$*Myc*$^{+/+}$*Rosa*$^{+/+}$ mice (Mann-Whitney p = 0.04, n = 4).

(G) H&E staining showing absence of functional crypts in *AhCre*$^{+}$*Myc*$^{fl/fl}$*Rosa*$^{Myc/+}$ (labeled *Myc*$^{fl/fl}$*Rosa*$^{Myc/+}$) mice, 72 hr following 14 Gy irradiation.

(H) FAK IHC showing FAK expression is generally absent from dying *AhCre*$^{+}$*Myc*$^{fl/fl}$ intestinal crypts 72 hr following irradiation (shown by red arrows). Black arrow denotes regenerating escaper crypt, which has high levels of FAK protein and would express c-Myc (see Figure S1).

(I) FAK IHC performed on *AhCre*$^{+}$*Myc*$^{fl/fl}$*Rosa*$^{Myc/+}$ intestinal epithelium 72 hr following 14 Gy irradiation showing absence of expression in the intestinal crypts.

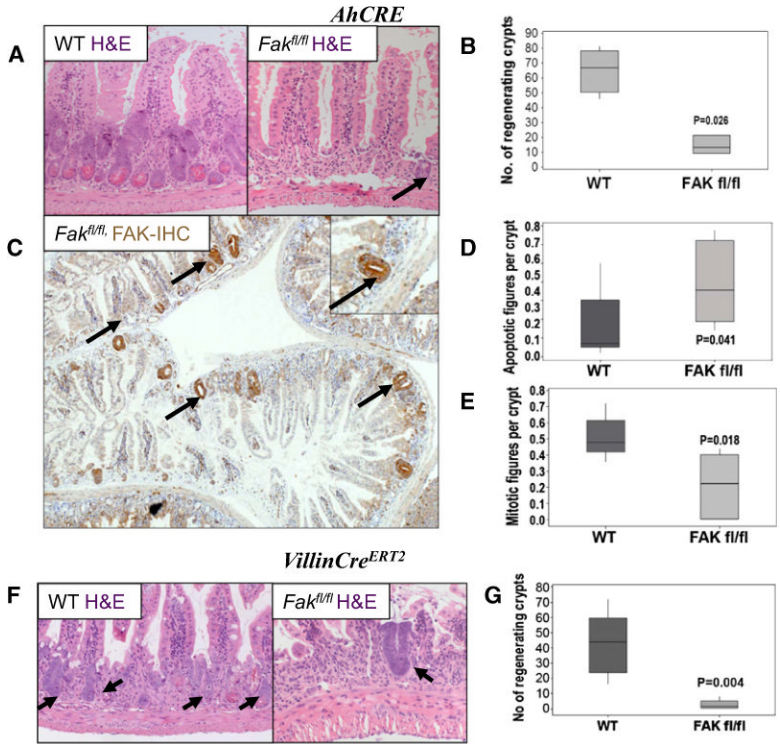

Figure 3. FAK Is Required for Intestinal Regeneration

(A) H&E staining of small intestine after 14 Gy irradiation showing multiple regenerating crypts in wild-type mice and only one regenerating crypt in *AhCre*$^{+}$*Fak*$^{fl/fl}$ mice (black arrow).

(B) Bar graph showing significant decrease in number of regenerating crypts in small intestine of *AhCre*$^{+}$*Fak*$^{fl/fl}$ mice compared with wild-type, 72 hr after 14 Gy irradiation.

(C) Corresponding high levels of FAK seen by IHC in FAK-proficient regenerating crypts 72 hr after 14 Gy irradiation. Black arrows show regenerating escaper crypts with high FAK expression. Inset shows close up regenerating crypt with high FAK expression and hence has escaped cre-mediated recombination and not deleted FAK.

(D and E) Boxplots showing significantly increased apoptosis and reduced mitosis in *AhCre*$^{+}$*Fak*$^{fl/fl}$ intestine compared with *AhCre*$^{+}$*Fak*$^{+/+}$ intestine 48 hr following gamma irradiation (Mann-Whitney $p > 0.05$, n = at least 4).

(F) H&E staining of small intestine after 14 Gy irradiation showing multiple regenerating crypts in wild-type mice, and only one regenerating crypt in *VillinCre*^{ER}*Fak*$^{fl/fl}$ mice 72 hr following irradiation (black arrows).

(G) Bar graph showing significant decrease in number of regenerating crypts in the small intestine of *VillinCre*^{ER}*Fak*$^{fl/fl}$ mice compared with wild-type, 72 hr after 14 Gy irradiation.

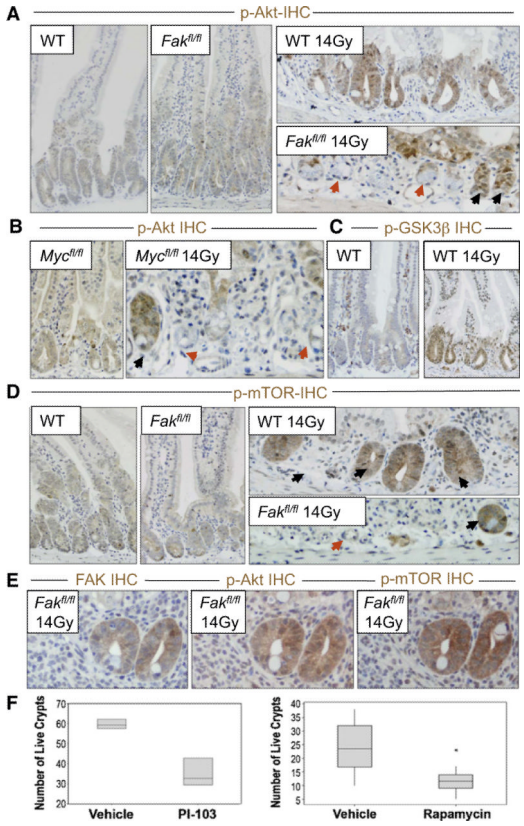

Figure 4. Akt Signaling via FAK Is Required for Efficient Regeneration

(A) IHC for p-Akt^{Ser473} shows weak staining in wild-type (first panel) and FAK-deficient crypts (second panel), but increased staining in regenerating crypts following 14 Gy irradiation (third panel upper). No upregulation was observed in FAK-deficient crypts (third panel lower: red arrows); however, in the rare escaper FAK-proficient crypts (black arrows), which efficiently regenerate, high levels of p-Akt were observed.

(B) IHC for p-Akt shows that Akt upregulation is c-Myc dependent. First panel shows weak p-Akt staining in nonirradiated c-Myc-deficient intestine. Second panel shows upregulation of p-Akt in a rare escaper c-Myc-proficient regenerating crypt (black arrow), while no discernable p-Akt staining was observed in the dying c-Myc-deficient crypts (red arrows).

(C) p-GSK3β (a target of Akt) is activated during regeneration. Left panel, nonir-radiated wild-type crypts showing low levels of p-GSK3β. Right panel shows high levels of p-GSK3β in wild-type regenerating crypts.

(D) p-mTOR is upregulated during regeneration but not in FAK-deficient crypts. p-mTOR IHC showing low levels of p-mTOR in wild-type (first panel) and FAK-deficient (second panel) crypts. Following irradiation, increased levels of p-mTOR are observed in wild-type regenerating crypts (third panel upper). In FAK-deficient crypts, p-mTOR is not upregulated (third panel lower: red arrow); however, in the rare escaper regenerating crypts, p-mTOR is upregulated (black arrow).

(E) “Back to back” IHC staining showing that the large escaper FAK-proficient regenerative crypts have high levels of p-Akt and p-mTOR expression.

(F) Boxplots showing that mice treated with either rapamycin or PI103 have significantly fewer surviving crypts than vehicle treated mice following 14 Gy irradiation (Mann-Whitney, p = > 0.04, n = 4).

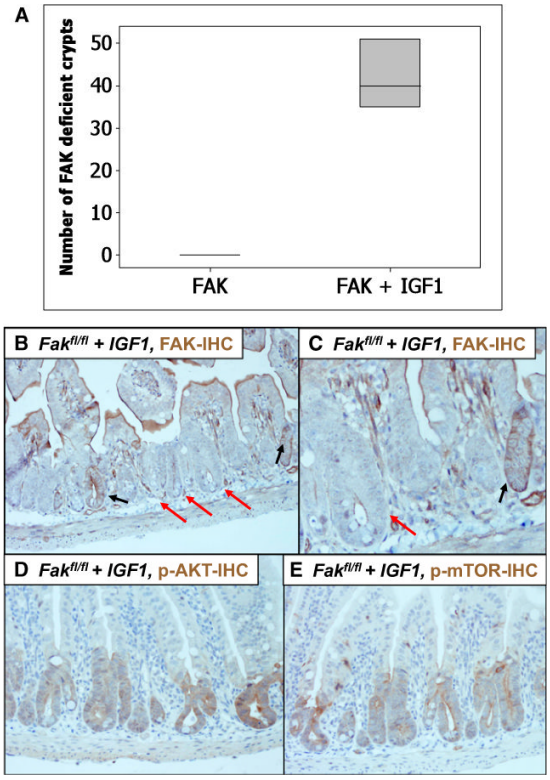

Figure 5. Activation of p-Akt by IGF1 Restores Intestinal Regeneration to FAK-Deficient Intestines

(A) Boxplot showing regenerating FAK-deficient crypts in *AhCre*$^{+}$*Fak*$^{fl/fl}$ intestines treated with IGF1 (Mann-Whitney, p = 0.04), compared with vehicle treated *AhCre*$^{+}$*Fak*$^{fl/fl}$ intestines.

(B and C) FAK IHC showing the presence of enlarged FAK-deficient crypts (red arrows), which are the same size as FAK-proficient crypts (black arrows).

(D and E) p-Akt and p-mTOR IHC showing all crypts from *AhCre*$^{+}$*Fak*$^{fl/fl}$ intestines treated with IGF1 now have high levels of both p-Akt (D) and p-mTOR (E).

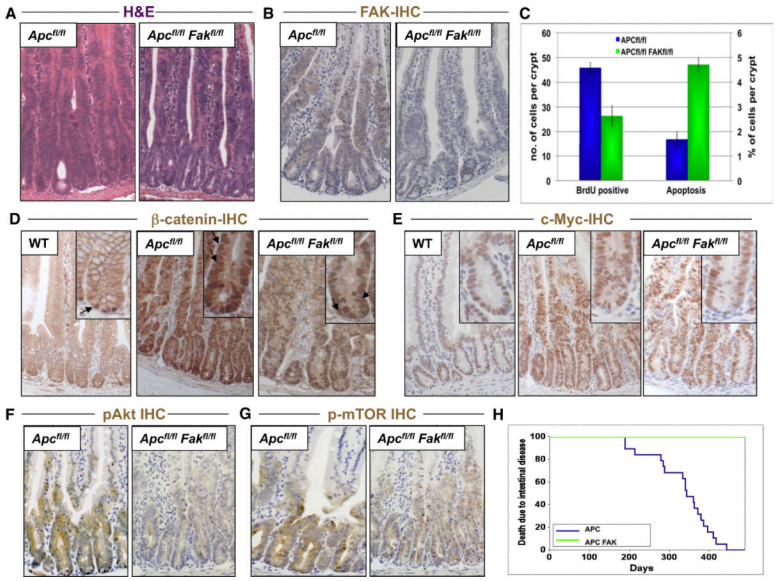

Figure 6. FAK Is Required for Intestinal Transformation following Apc Loss

(A) H&E-stained sections from *AhCre*$^{+}$*Apc*$^{fl/fl}$ (labeled *Apc*$^{fl/fl}$) and *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ (labeled *Apc*$^{fl/fl}$ *Fak*$^{fl/fl}$) intestines 4 days PI. Note that *Apc*-deficient crypts look much enlarged.

(B) FAK IHC in *AhCre*$^{+}$*Apc*$^{fl/fl}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ intestines 4 days after Apc loss. IHC shows that FAK expression is lost in doube-mutant *Apc Fak* intestines.

(C) Bar graph showing reduced BrdU positivity and increased apoptosis in *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ intestinal enterocytes compared with *AhCre*$^{+}$*Apc*$^{fl/fl}$ mice (Mann-Whitney $p = 0.04$, $n \geq 4$). Errors bars represent SD.

(D and E) β-catenin and c-Myc IHC performed upon *AhCre*$^{+}$*Apc*$^{+/+}$ (labeled WT), *AhCre*$^{+}$*Apc*$^{fl/fl}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ intestines 4 days PI. Note both *Apc* and *Apc Fak*-deficient intestines have increased levels of nuclear β-catenin and c-Myc compared with WT. Nuclear β-catenin is arrowed in the inset.

(F and G) p-Akt and p-mTOR IHC performed upon *AhCre*$^{+}$*Apc*$^{fl/fl}$ and *AhCre*$^{+}$*Apc*$^{fl/fl}$*Fak*$^{fl/fl}$ intestines 4 days PI. Note reduction in staining in the double-mutant *Apc Fak* intestines.

(H) Intestinal tumor-free survival of *AhCre*$^{+}$*Apc*$^{fl/+}$*Fak*$^{+/+}$ (blue line) and *AhCre*$^{+}$*Apc*$^{fl/+}$*Fak*$^{fl/fl}$ (green line) mice up to 500 days. No *AhCre*$^{+}$*Apc*$^{fl/+}$*Fak*$^{fl/fl}$ mice developed intestinal disease. All the *AhCre*$^{+}$*Apc*$^{fl/+}$ *Fak*$^{+/+}$ mice became ill with intestinal tumors with a median onset of 344 days $p \geq 0.005$ log rank.