Deficiency of the LIM-Only Protein FHL2 Reduces Intestinal Tumorigenesis in Apc Mutant Mice

Charlotte Labalette1,2*, Yann Nouet1,2*, Florence Levillayer1, Sabine Colnot3,4, Ju Chen5, Valerie Claude6, Michel Huerre7, Christine Perret3,4, Marie-Annick Buendia1,2, Yu Wei1,2*

1 Département de Virologie, Institut Pasteur, Paris, France, 2 Inserm U579, Paris, France, 3 Département d’Endocrinologie Métabolisme et Cancer, Institut Cochin, Paris, France, 4 Inserm U567, Paris, France, 5 Department of Medicine, University of California San Diego, La Jolla, California, United States of America, 6 Département d’Anapathologie, Hôpital Bégin, Saint Mandé, France, 7 Département d’Infection et Épidémiologie, Institut Pasteur, Paris, France

ABSTRACT

Background: The four and a half LIM-only protein 2 (FHL2) is capable of shuttling between focal adhesion and nucleus where it signals through direct interaction with a number of proteins including β-catenin. Although FHL2 activation has been found in various human cancers, evidence of its functional contribution to carcinogenesis has been lacking.

Methodology/Principal Findings: Here we have investigated the role of FHL2 in intestinal tumorigenesis in which activation of the Wnt pathway by mutations in the adenomatous polyposis coli gene (Apc) or in β-catenin constitutes the primary transforming event. In this murine model, introduction of a biallelic deletion of FHL2 into mutant Apc1410 mice substantially reduces the number of intestinal adenomas but not tumor growth, suggesting a role of FHL2 in the initial steps of tumorigenesis. In the lesions, Wnt signalling is not affected by FHL2 deficiency, remaining constitutively active. Nevertheless, loss of FHL2 activity is associated with increased epithelial cell migration in intestinal epithelium, which might allow to eliminate more efficiently deleterious cells and reduce the risk of tumorigenesis. This finding may provide a mechanistic basis for tumor suppression by FHL2 deficiency. In human colorectal carcinoma but not in low-grade dysplasia, we detected up-regulation and enhanced nuclear localization of FHL2, indicating the activation of FHL2 during the development of malignancy.

Conclusions/Significance: Our data demonstrate that FHL2 represents a critical factor in intestinal tumorigenesis.

Introduction

The four and a half LIM-only protein 2 (FHL2) contains only LIM domain (named after lin-11, mec-3 and Islet-1), which is a protein-protein interaction domain. FHL2 has recently emerged as a signalling protein that is critical in the transduction of signals from extracellular environment and in the control of gene expression program in response to different stimuli. In the cytoplasm, FHL2 interacts with α- and β-integrin subunits and focal adhesion kinase at focal adhesions where integrins bind extracellular matrix (ECM) [1–3]. FHL2 is involved in ECM-integrin receptor interaction, assembly of ECM proteins on the cell surface and bundling of focal adhesions [4,5]. In response to external stimuli, FHL2 is translocated to the nucleus where it plays the role of transcription cofactor by interacting with numerous transcription factors and coregulators including androgen receptor (AR), AP1, CREB, PLZF, WT1, SKI, β-catenin, FOXO1, Runx2, serum response factor (SRF), E4F1 and CBP/p300 [6–18]. Mouse deficient of FHL2 display defects in response to divers stimuli, including cardiac hypertrophy under β-adrenergic stimulation, healing defects in skin and intestinal wound and attenuated neovascularization after corneal injury [19–22].

Several lines of evidence suggest a role of FHL2 in carcinogenesis. FHL2+/− fibroblasts are temporarily resistant to oncogenic Ras-induced transformation [23]. FHL2 suppression inhibits anchorage-independent growth of cancer cell lines and tumor formation in immuno-compromised mice [24]. Up-regulation of FHL2 has been found in many human cancers [3,13,24–26]. Several studies have associated high level and nuclear expression of FHL2 with the aggressiveness of cancer and bad prognostic [6,27] [28]. Moreover, numerous FHL2-interacting proteins including AR, AP1, PLZF, SKI, WT1, β-catenin, BRCA1 and E7 of human papillomavirus 16 [29,30] have primary roles in various human cancers, implying that FHL2 may participate in transformation process through effects on the oncogenic activity of its partners.

We have previously demonstrated that FHL2 interacts with β-catenin and cooperates with CBP/p300 to stimulate transcript...
tional activity of the β-catenin/TCF4 complex [13,18]. β-catenin is a key effector of the Wnt signalling pathway that has central roles not only in embryogenesis and tissue homeostasis but also in tumorigenesis (for review, see [31]). In human colorectal cancer, an overwhelming majority of cases carry mutations in Wnt pathway components including the adenomatous polyposis coli gene (Apc), axin and β-catenin. All the mutations lead to constitutive activation of the Wnt signalling pathway characterized by the formation of constitutive nuclear β-catenin/TCF complex. Genetic modelling of activation of the Wnt signalling pathway in the intestine has been made possible by the generation of Apc mutant mice that recapitulate human colonic carcinogenesis. These mice, epitomized by the ApcM/+ mouse, have been used intensively to test genetically the ability of candidate genes to enhance or repress adenoma formation in vivo.

In this study, we used the Apc<sub>A14/+</sub> model to address the contribution of FHL2 activity to the intestinal transformation process. Apc<sub>A14/+</sub> mice in which exon 14 of the Apc gene is deleted in one allele spontaneously develop multiple polyps along their intestinal tract [32]. All tumors lose the Apc wild type (wt) allele, accumulate β-catenin in the nucleus and overexpress the β-catenin target genes cyclin D1 and c-myc [32]. Using a genetic approach, we demonstrate that loss of FHL2 significantly suppresses tumor multiplicity in Apc<sub>A14/+</sub> mice. Our analysis of FHL2 expression in murine and human intestinal lesions revealed its progressive up-regulation and enhancement of nuclear expression during disease development. These results provide unequivocal evidence of the implication of FHL2 activity in the transformation process.

Results

FHL2 deficiency reduces intestinal polyposis in Apc<sub>A14/+</sub> mice

To determine the in vivo effects of FHL2 in intestinal tumorigenesis, we took advantage of FHL2<sup>−/−</sup> and Apc<sub>A14/+</sub> mice to investigate how FHL2 affects the phenotypes associated with Apc loss [32,33]. Despite defects in bone formation [15,34], FHL2<sup>−/−</sup> mice live a normal lifespan [33]. Histological analysis of intestinal epithelium in FHL2-null mice revealed no major alterations in crypt-villus architecture of the small intestine and in the crypt structure of the colon (Fig. 1A). Moreover, FHL2-deficient cells remained in cycle in the crypt, as demonstrated by immunohistochemistry (IHC) for the proliferation marker Ki-67 (Fig. 1A), suggesting that FHL2 is dispensable for proliferation of intestinal epithelial cells. No significant difference was observed in the presence and localization of differentiated intestinal cell lineages (enterocytes, enteroendocrine, goblet cells and Paneth cells) in the FHL2 mutant mice (data not shown). We crossed the Apc<sub>A14/+</sub> strain on the C57Bl/6 background [32] into the FHL2<sup>−/−</sup> strain on the hybrid Black Swiss-129-SV/J background [33]. Intercrosses from the F1 generation produced Apc<sub>A14/+</sub> mice with three different FHL2 genotypes. Only the F2 generation was used for further analysis in order to rule out any influence of genetic background. In contrast to Apc<sub>A14/+</sub> mice on the C57Bl/6 background which die at 6-month-old [32], Apc<sub>A14/+</sub>/FHL2<sup>−/−</sup> mice on the mixed genetic background do not succumb to intestinal adenomas until 20-month-old, which is a late stage in a mouse life (the life span of wt animals on the same genetic background was about 24-month-old). Because most of Apc<sub>A14/+</sub>/FHL2<sup>−/−</sup> mice died at 24-month-old, the impact of FHL2 deficiency on time course of death was not significant in Apc<sub>A14/+</sub> animals of this study (data not shown).

Mice were first sacrificed at 3 months. Analysis of the intestine revealed similar lengths for Apc<sub>A14/+</sub> mice of each FHL2 genotype (data not shown). We divided the intestinal tract into small intestine and colon segments and scored for polyp number, location, and size. Despite few adenomas developed at this stage, tumors occurred more frequently in Apc<sub>A14/+</sub>/FHL2<sup>−/−</sup> animals (73%, n = 11) than in Apc<sub>A14/+</sub>/FHL2<sup>−/+</sup> littermates (40%, n = 10). We then examined the intestine at 11 months. Compared to Apc<sub>A14/+</sub>/FHL2<sup>−/+</sup> littermates, the polyp number in the small intestine decreased by 68% in Apc<sub>A14/+</sub>/FHL2<sup>−/−</sup> mice and 52% in Apc<sub>A14/+</sub>/FHL2<sup>−/+</sup> mice (Figs. 2A and 2B). Precisely, while the polyp number in Apc<sub>A14/+</sub>/FHL2<sup>−/+</sup> could reach more than 40, the great majority of Apc<sub>A14/+</sub>/FHL2<sup>−/−</sup> mice developed less than 9 tumors and none of the animals had more than 19 polyps (Fig. 2C). The results provide unequivocal evidence of the implication of FHL2 activity in the transformation process.

Figure 1. Histological analysis of normal intestine in FHL2<sup>−/−</sup> mice and intestinal adenomas in Apc<sub>A14/+</sub>FHL2<sup>−/−</sup> mice. A. Normal intestinal architecture in FHL2<sup>−/−</sup> mice. B. Intestinal adenomas in Apc<sub>A14/+</sub>FHL2<sup>−/−</sup> and Apc<sub>A14/+</sub>FHL2<sup>−/+</sup> mice. Original magnifications, X100 (A and B). doi:10.1371/journal.pone.0010371.g001
sample distribution between $Apc^{146/+}\text{FHL2}^{+/+}$ and $Apc^{146/+}\text{FHL2}^{-/-}$ mice in Fig. 2C was statistically significant ($p<0.0036$), indicating that FHL2 deficiency inhibits polyp formation. To test the effect of FHL2 deficiency on tumor growth, we measured the sizes of polyps in $Apc^{146/+}\text{FHL2}^{+/+}$ and $Apc^{146/+}\text{FHL2}^{+/-}$ mice (Fig. 2D) and carried out Wilcoxon Rank Sum test to determine if there was a difference in tumor size between the two genotypes. The results showed that tumor sizes in $Apc^{146/+}\text{FHL2}^{+/-}$ mice were not significantly different from those in $Apc^{146/+}\text{FHL2}^{+/+}$ mice ($p>0.2$, Wilcoxon Rank Sum test), suggesting that loss of FHL2 activity does not affect tumor growth. Histological evaluation of the adenomas revealed no difference in gross histological characteristics or grade between $Apc^{146/+}\text{FHL2}^{+/+}$ and $Apc^{146/+}\text{FHL2}^{+/-}$ mice (see Fig. 1B). No difference was observed in the distribution of polyps throughout the intestine between $Apc^{146/+}\text{FHL2}^{+/+}$ and $Apc^{146/+}\text{FHL2}^{+/-}$ mice (data not shown). We next analyzed the lesions in the colon. Few polyps developed in the colon at 11 months in $Apc^{146/+}$ mice with any of the FHL2 genotypes. However, while tumors were detected in 45% of $Apc^{146/+}\text{FHL2}^{+/+}$ (n = 11), only 17% of $Apc^{146/+}\text{FHL2}^{-/-}$ animals (n = 18) developed polyps, indicating that the frequency of tumors in the colon is also decreased by FHL2 deficiency. We therefore conclude that loss of FHL2 suppresses tumor formation in the intestine by probably acting on the initiation of tumors.

β-catenin and its targets cyclin D1 and c-myc are activated in adenomas independent of FHL2 genotype

We have previously shown that FHL2 interacts with β-catenin and is a crucial regulator of cyclin D1 expression in fibroblasts [5]. To investigate the molecular mechanisms of polyp formation in $Apc^{146/+}\text{FHL2}^{-/-}$ mice, we analyzed expression of β-catenin and its targets cyclin D1 and c-myc in tumors. Immunohistochemical staining revealed strong nuclear expression of β-catenin in polyps from both $Apc^{146/+}\text{FHL2}^{+/+}$ and $Apc^{146/+}\text{FHL2}^{-/-}$ mice (Fig. 3A, a and b), indicative of activation of the β-catenin/Wnt pathway.
As direct targets of β-catenin, cyclin D1 and c-myc are closely involved in intestinal tumorigenesis associated with activation of Wnt signalling [35–38]. To assess the impact of FHL2 deficiency on the targets, we examined expression of cyclin D1 and c-myc at both RNA and protein levels in the intestine of ApcD14/FHL22/2 mice. As shown in Fig. 3B, the mRNA levels of cyclin D1 and c-myc were indistinguishable in nontumor intestine between ApcD14/FHL2+/+ and ApcD14/FHL22/2 animals, and were strongly increased in adenomas from mice of both genotypes. Whereas expression of cyclin D1 in adenomas from ApcD14/FHL22/2 mice showed significant higher level than that in tumors from ApcD14/FHL2+/+ mice, no significant difference of c-myc expression was observed in adenomas between ApcD14/FHL22/2 and ApcD14/FHL2+/+ animals (Fig. 3B). In parallel, immunohistochemical analysis showed nuclear expression of cyclin D1 and c-myc in the proliferative compartment of normal crypts in both ApcD14/FHL22/2 and ApcD14/FHL2+/+ animals (Fig. 3A, c, f, g, and h). Moreover, in keeping with the proliferative property of tumor cells, cyclin D1 was highly expressed in all adenomas, regardless of the FHL2 genotype (Fig. 3A, c and d). In addition, we compared expression of other β-catenin targets such as Axin 2 and metalloproteinase matrilysin (MMP-7) between tumors and adjacent nontumorous tissues by real time RT-PCR [39,40]. Axin 2 and MMP-7 were uniformly activated in tumor samples, with no significant difference among ApcD14/FHL22/2 and ApcD14/FHL2+/+ animals (data not shown). We also performed IHC with the intestine of ApcD14/FHL22/2 mice for c-Jun and peroxisome proliferators-activated receptor δ (PPARδ), which have been shown...
to be the Wnt targets critical for the control of intestinal tumorigenesis [41,42]. The expression of c-Jun and PPARδ was similar between Apc<sup>14/+</sup>FHL2<sup>-/-</sup> and Apc<sup>14/+</sup>FHL2<sup>+/+</sup> mice in both normal and adenomatous tissues (data not shown). Taken together, these findings indicate that Wnt signalling remains constitutively active in the lesions of Apc<sup>14/+</sup>FHL2<sup>-/-</sup> mice and that the inhibitive effects of FHL2 deficiency on intestinal neoplasia are not associated with defects in the activation of c-myc and cyclin D1.

Loss of FHL2 increases cell migration

Cell migration represents a fundamental mechanism involved in the maintenance of epithelial homeostasis in the intestine. Impaired cell migration correlates with tumor growth and neoplastic progression during development of colorectal cancer [43]. The role of FHL2 in assembly of extracellular matrix prompted us to examine the impact of FHL2 deficiency upon cell migration along the crypt-villus axis. Apc<sup>14/+</sup>FHL2<sup>-/-</sup> mice were administrated with BrdU by intraperitoneal injection and sacrificed 2 h and 48 h later for BrdU-positive cell scoring in longitudinal sections with the base of crypt set as position 0. As shown in Fig. 4A, no difference was observed between Apc<sup>14/+</sup>FHL2<sup>-/-</sup> and Apc<sup>14/+</sup>FHL2<sup>+/+</sup> mice in the position and the number of BrdU-positive cells after 2 h exposure to BrdU. At 48 h, however, Apc<sup>14/+</sup>FHL2<sup>-/-</sup> cells moved to a higher position than Apc<sup>14/+</sup>FHL2<sup>+/+</sup> cells along the crypt-villus axis (Fig. 4A). Indeed, an average of 87.4% labelled Apc<sup>14/+</sup>FHL2<sup>-/-</sup> cells were located at positions between 0 and 20 in longitudinal sections versus 29.8% of labelled Apc<sup>14/+</sup>FHL2<sup>+/+</sup> cells (p<6.10<sup>-6</sup>. Student’s t test), and nearly 70% of BrdU-positive cells in Apc<sup>14/+</sup>FHL2<sup>-/-</sup> mice already migrated to positions higher than 20 against 12.6% in Apc<sup>14/+</sup>FHL2<sup>+/+</sup> animals (p<10<sup>-6</sup>), showing an accelerated migration rate (Figs. 4A and 4B). Moreover, the number of BrdU-positive cells in Apc<sup>14/+</sup>FHL2<sup>-/-</sup> mice was significantly increased compared to Apc<sup>14/+</sup>FHL2<sup>+/+</sup> littermates (p<0.005) (Figs. 4A and 4B). As we found no anomaly in the organization of proliferative and differentiated compartments in Apc<sup>14/+</sup>FHL2<sup>-/-</sup> intestine (see Fig. 1A and data not shown), the increased number of BrdU-labelled cells in these animals might be attributed to the rapid movement of the enterocytes from the proliferative compartment to the top of villi. The impact of FHL2 on cell migration was independent of Apc mutations, as the intestinal cells in FHL2<sup>-/-</sup> mice moved also faster than wt cells along the crypt-villus axis at 48 h post-BrdU injection (Fig. 4C). These data indicate that loss of FHL2 activity has positive effects on cell migration. As accelerated cell migration by chemopreventive agents has been reported to be beneficial for eliminating deleterious cells, the tumor suppression function of FHL2 deficiency may be achieved in part through acceleration of cell migration in Apc<sup>14/+</sup> mice.

Up-regulation of FHL2 in human intestinal adenoma and carcinoma

Next, we examined FHL2 expression in normal and tumorous tissues from Apc<sup>14/+</sup> mice by IHC with anti-FHL2 antibody, which does not cross-react with any other protein, since it detects no signal in Apc<sup>14/+</sup> FHL2<sup>-/-</sup> intestine (data not shown). In contrast to normal epithelium where FHL2 signal was barely detectable, strong nuclear staining of FHL2 was observed in tumors developed in Apc<sup>14/+</sup> mice (Fig. 5A). We then examined FHL2 expression in the intestine of Apc<sup>14/+</sup> animals by real time RT-PCR. As shown in Fig. 5B, the level of FHL2 transcript was significantly increased in tumor samples, compared to normal adjacent intestinal tissues. To assess the expression of FHL2 in human tumors, we analyzed five human colon adenomas with low-grade dysplasia, five human colon adenomas with high-grade dysplasia and five carcinomas by IHC for FHL2. Staining of adjacent normal crypts was used as internal control. In keeping with a previous report [24], FHL2 protein, undetectable in normal tissues, was expressed in all high-grade dysplasia and carcinomas analyzed (Fig. 5C, compare a with c and d). Interestingly, barely detectable in low-grade dysplastic tissues, FHL2 was significantly detected in high-grade dysplastic and adenomatous tissues, FHL2 was significantly
increased in high-grade dysplasia and cancer cells (Fig. 5C, compare b with c and d), showing a progressive expression pattern. Moreover, contrasting markedly with the predominant cytoplasmic localization in high-grade dysplasia, intense nuclear accumulation of FHL2 protein was observed in carcinomas (Fig. 5C, e). Taken together, these data suggest that FHL2 activation in intestinal cells may be associated with pre-cancerous and cancerous stages and that FHL2 protein may accumulate in the nucleus during neoplastic progression.

Discussion

In this study, we provide new insights into the FHL2 function in intestinal tumorigenesis. By employing a genetic approach in vivo,
we demonstrate that loss of FHL2 drastically suppresses tumorigenesis in the murine intestine, which may be partly due to the positive effects of FHL2 deficiency on cell migration in intestinal epithelium. In the study of human colorectal tumors, we found that FHL2 expression might be closely related to disease stages: the level of FHL2 in high-grade dysplasia is significantly elevated compared to normal tissues as well as low-grade dysplasia but clearly below that in carcinomas. Moreover, cellular localization of FHL2 switches from predominant cytoplasmic expression in high-grade dysplasia to enhanced nuclear accumulation in cancer cells. The \( \text{Apc}^{\text{14+/+}} \text{FHL2}^{-/-} \) mouse model underscores that cells deficient for FHL2 are less susceptible to oncogene-induced tumors. However, how FHL2 is involved in the proliferative and transforming functions of the Wnt signalling in the intestine is not clear. Previous report has shown that the D-type cyclins, in particular cyclin D1, are dramatically down-regulated in \( \text{FHL2}^{-/-} \) fibroblasts [5]. The results of this study indicate that cyclin D1 expression is not dependent of FHL2 in the context of intestinal epithelium. It appears that cyclin D1 expression was stronger in tumors developed in \( \text{Apc}^{\text{14+/+}} \text{FHL2}^{-/-} \) mice than those in \( \text{Apc}^{\text{14+/+}} \text{FHL2+/+} \) animals. As FHL2 plays an important role in cell proliferation, it is possible that high level of cyclin D1 may counteract FHL2 deficiency in the stimulation of tumor cell growth in \( \text{Apc}^{\text{14+/+}} \text{FHL2}^{-/-} \) mice. We also observed that the expression of other \( \beta \)-catenin targets including c-myc, Axin2, MMP-7, c-Jun and PPAR\( \alpha \) is not dependent of FHL2 in the intestine. Further analysis of the involvement of Wnt and FHL2 signalling in this specific tissue should provide insight into whether the Wnt and FHL2 signalling pathways function in an interdependent regulatory network.

The loss of FHL2 activity in intestinal epithelium clearly accelerates cell migration, which constitutes a fundamental mechanism in the control of tissue homeostasis. Apc mutation in \( \text{Apc}^{\text{min/+}} \) mice is associated with decreased cell migration, resulting in a prolonged residence time for enterocytes in the intestine [43]. Chemopreventive agents presumably exert their anti-tumor effects by increasing the rates of cell migration, thus eliminating rapidly deleterious cells [44–46]. The mechanism of the increase of cell migration in FHL2 mutant intestine remains to be elucidated. Because FHL2 is involved in focal adhesion, ECM-cell interaction and assembly of the extracellular matrix [4,5], a role for FHL2 in the regulation of cell mobility seems an attractive possibility. The tumorigenic effects of proteins that regulate cell migration have the regulation of cell mobility seems an attractive possibility. The tumorigenic effects of proteins that regulate cell migration have been previously illustrated by SPARC, a matricellular protein that associates with the extracellular matrix and modulates cell-ECM interaction [47]. Deficiency of SPARC augments enterocyte movement in \( \text{Apc}^{\text{min/+}} \) mice, thereby suppressing adenoma formation [47]. Moreover, the positive effects of FHL2 mutation on cell migration in \( \text{Apc}^{\text{11+/+}} \text{FHL2}^{-/-} \) mice correlate with the observation that FHL2 deficiency perturbs essentially adenoma initiation.

Consistent with an early report showing nuclear expression of FHL2 in gastrointestinal cancerous tissues [24], our data further indicate that high level expression and nuclear accumulation of FHL2 in human colorectal cancer might reflect disease progression towards the malignant state. This observation is correlated with recent discoveries that invasive breast cancers have much stronger expression of FHL2 than premalignant ductal carcinoma in situ samples [25] and that increases of nuclear FHL2 in prostate cancer are strongly correlated with differentiation of cancer cells and with high Gleason grade [6]. Altogether, these results support the view that the intensity and localization of FHL2 expression in cancer cells may serve as a biomarker in classifying tumor stage and predicting disease outcome [27]. Indeed, breast cancer patients with tumors expressing low amounts of FHL2 have a better survival compared to those with high intra-tumoral FHL2 expression [26] and activation of nuclear FHL2 signalling is linked to aggressiveness and recurrence of prostate cancer [27]. In colorectal cancer, further examination of FHL2 expression in a large panel of samples may permit to determine if FHL2 could be used as a marker for stage classification of the disease. Mechanistically, the sensor function and transcription coregulator activity of FHL2 can provide a ready explanation for the up-regulation and nuclear translocation of FHL2 in highly malignant cells. In prostate cancer cells, FHL2 is strongly induced by androgens [48]. As a coactivator of AR, FHL2 in turn robustly stimulates the AR activity that is critical for prostate cancer progression [48,49]. In colorectal cancer, the molecular hallmark is the accumulation of \( \beta \)-catenin in the nucleus. However, expression of the \( \beta \)-catenin target genes analyzed in this study seems not be affected by FHL2 deficiency. Nevertheless, up-regulation of FHL2 and its nuclear accumulation may stimulate transcription activity of not yet uncovered \( \beta \)-catenin targets or other transcription complexes, which may be required for carcinogenic progression.

Our data highlight FHL2 as important molecule in mediation of the transformation process and suggest that disruption of the FHL2 signalling may provide a viable and specific strategy for therapeutic intervention in colorectal cancer.

### Materials and Methods

#### Mice

Male \( \text{Apc}^{\text{14+/+}} \) animals on the C57BL/6 background were crossed with female \( \text{FHL2}^{-/-} \) mice on the hybrid Black Swiss-129-SV/J background [32,33]. F1 \( \text{Apc}^{\text{14+/+}} \text{FHL2}^{+/+} \) mice were intercrossed. Only the F2 \( \text{Apc}^{\text{11+/+}} \text{FHL2}^{-/-} \) animals were used for the study. Mice were housed under pathogen-free conditions. All experiments involving mice have been approved by Institut Pasteur.

#### Tumor scoring and histopathological analysis

Intestines were removed from F2 \( \text{Apc}^{\text{11+/+}} \text{FHL2}^{-/-} \) mice with different FHL2 genotypes at 3 or 11 months and fixed in 4% PFA. Polyps were counted and measured using a Nikon dissecting microscope at \( \times 6 \) magnification by an observer blinded to the genotype of the mice. Intestines were rolled in the “Swiss rolls” configuration and proceed for paraffin embedding [32].

#### Immunohistochemistry

Sections were cut from parafomaldehyde (PFA)-fixed paraffin-embedded “Swiss rolls”. Tissues were dewaxed in xylene and unmasked in a citric acid solution (H-3300, Vector Laboratories) at 96°C for 45 min. Normal horse serum (2.5%; S-2000, Vector Laboratories) was used as blocking solution. Sections were incubated at room temperature with primary antibodies for 60 min. Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide. The sections were then incubated with secondary antibodies (Vector Laboratories) for 30 min. The peroxidase reaction was developed using DAB Substrate Kit (SK-4100, Vector Laboratories). Hematoxylin was used for counterstaining. Antibodies against \( \beta \)-catenin and cyclin D1 (NeoMarkers), FHL2 (MBL), c-myc (Santa Cruz) and Ki-67 (Novocastra) were used as primary antibodies. Images were obtained on a FXA Microphot microscope equipped with a Nikon D1 camera controlled by Nikon capture.
Quantitative RT-PCR analysis

Total RNA was extracted from polyps and adjacent non-tumoral intestines removed from 11-month-old Apc−/− mice. Real-time PCR was performed as described previously [5].

Measurement of enterocyte migration

Mice at 10 weeks were administrated with Bromodeoxyuridine (BrdU) (50 mg/kg mouse body weight), followed by immunohistochemical analysis with anti-BrdU (Dako). Fields containing crypt transverse sections were selected randomly at several locations for BrdU positive cell counting in crypt-villus units.

Human polyp samples

Fifteen human colon specimens were obtained in Hôpital Bégin at Saint Mandé and fixed in PFA. Informed consent of patients was obtained at the hospital and the study was performed in accordance with European Guidelines for biomedical research.

Acknowledgments

We are grateful to Christine Neuveut and Béatrice Romagnolo for insightful discussion. We thank Jennifer Dahan for technical assistance.

Author Contributions

Conceived and designed the experiments: CL YN YW. Performed the experiments: CL YN FL YW. Analyzed the data: CL YN FL SC MH CP MAB YW. Contributed reagents/materials/analysis tools: SC JC VC MH CP. Wrote the paper: CL MAB YW.

References

1. Wixler V, Geerts D, Laplantine E, Westhoff D, Smyth N, et al. (2000) The LIN-only protein DRAL/FHL2 binds to the cytoplasmic domain of several alpha and beta integrin chains and is recruited to adhesion complexes. J Biol Chem 275: 35669–35678.
2. Samson T, Smyth N, Janetzky S, Wendler O, Muller JM, et al. (2004) The LIN-only proteins FHL2 and FHL3 interact with alpha- and beta-subunits of the muscle alpha/beta1 integrin receptor. J Biol Chem 279: 20641–20652.
3. Gabriel B, Mildenhberger S, Weisser GW, Metzger E, Gisch G, et al. (2004) Focal adhesion kinase interacts with the transcriptional coactivator FHL2 and both are overexpressed in epithelial ovarian cancer. Anticancer Res 24: 921–927.
4. Park J, Will C, Martin B, Gullotti L, Friedrichs N, et al. (2008) Deficiency in the LIN-only protein FHL2 impairs assembly of extracellular matrix proteins. Fasebj J 22: 2508–2520.
5. Labalette C, Noutet Y, Sobczak-Thépot J, Armengol C, Levillayer F, et al. (2008) The LIN-only protein FHL2 regulates cyclin D1 expression and cell proliferation. J Biol Chem 283: 15012–15018.
6. Muller JM, Metzger E, Girschik H, Bosserhofer AK, Mereep L, et al. (2002) The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. Embo J 21: 786–788.
7. Du X, Hoflitz Chr, Gunther T, Wilhelm D, Engler C, et al. (2002) The LIN-only coactivator FHL2 modulates WT1 transcriptional activity during gonadal differentiation. Biochem Biophys Acta 1577: 93–101.
8. Morson A, Sassone-Corsi P (2003) The LIN-only protein FHL2 is a smad-induced transcriptional regulator of AP-1. Proc Natl Acad Sci U S A 100: 3977–3982.
9. Finna GM, De Cesare D, Sassone-Corsi P (2000) A family of LIN-only transcriptional coactors: tissue-specific expression and selective activation of CREB and CREM. Mol Cell Biol 20: 8615–8622.
10. McLaughlin P, Ehler E, Carlig L, Licht JD, Schafer BW (2002) The LIN-only protein DRAL/FHL2 interacts with and is a coactivator for the pro-myelocytic leukemia zinc finger protein. J Biol Chem 277: 37045–37053.
11. Chen D, Xu W, Bales E, Colmenares C, Conacci-Sorrell M, et al. (2003) SKI inducible transcriptional coactivator of AP-1. Proc Natl Acad Sci U S A 100: 1402–1407.
12. Martin B, Schaeider R, Janetzky S, Wabler Z, Pandur P, et al. (2002) The LIN-only protein FHL2 interacts with beta-catenin and promotes differentiation of mouse myoblasts. J Cell Biol 159: 119–132.
13. Wei Y, Renard CA, Labalette C, Wu Y, Levy L, et al. (2003) Identification of the LIN protein FHL2 as a coactivator of beta-catenin. J Biol Chem 278: 5189–5194.
14. Yang Y, Hou H, Haller EM, Nicosia SV, Bai W (2005) Suppression of FOXO activity by FHL2 through SIRT1-mediated deacetylation. Embo J 24: 1021–1032.
15. Gunther T, Poli C, Muller JM, Carala-Lehnen P, Schinke T, et al. (2005) Fhl2 deficiency results in osteopetrosis due to decreased activity of osteoblasts. Embo J 24: 3049–3056.
16. Philippa U, Schratt G, Deierich C, Muller JM, Galgoczy P, et al. (2004) The SRF target gene FHL2 antagonizes RhoA/MLC-dependent activation of SRF. Mol Cell Biol 24: 867–880.
17. Paul C, Lacroix M, Iankova I, Julien E, Schafer BW, et al. (2006) The LIN-only protein FHL2 is a negative regulator of E4F1. Oncogene 25: 5475–5484.
18. Gabriel B, Redard CA, Neveu A, Bierada MA, Wei Y (2004) Interaction and functional cooperation between the LIN protein FHL2, CBP/p300, and beta-catenin. Mol Cell Biol 24: 10609–10702.
19. Kong Y, Shelton JM, Rodhimmel B, Li X, Richardson JA, et al. (2001) Cardiac-specific LIN protein FHL2 modifies the hypertrophic response to beta-adrenergic stimulation. Circulation 103: 2731–2738.
20. Wixler V, Hinner S, Muller JM, Gullotti L, Will C, et al. (2007) Deficiency in the LIN-only protein FHL2 impairs skin wound healing. J Cell Biol 177: 163–172.
41. Nateri AS, Spencer-Dene B, Behrens A (2005) Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development. Nature 437: 281–285.
42. Wang D, Wang H, Shi Q, Katiarki S, Walhi W, et al. (2004) Prostaglandin E2 promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. Cancer Cell 6: 285–295.
43. Mahmoud NN, Boobol SK, Bilinski RT, Martucci C, Chadburn A, et al. (1997) Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. Cancer Res 57: 5045–5050.
44. Mahmoud NN, Dannenberg AJ, Mestre J, Bilinski RT, Churchill MR, et al. (1998) Aspirin prevents tumors in a murine model of familial adenomatous polyposis. Surgery 124: 225–231.
45. Mahmoud NN, Bilinski RT, Churchill MR, Edelmann W, Kucherlapati R, et al. (1999) Genotype-phenotype correlation in murine Apc mutation: differences in enterocyte migration and response to sulindac. Cancer Res 59: 353–359.
46. Fenton JI, Wolff MS, Orth MW, Hord NG (2002) Membrane-type matrix metalloproteinases mediate curcumin-induced cell migration in non-tumorigenic colon epithelial cells differing in Apc genotype. Carcinogenesis 23: 1065–1070.
47. Sansom OJ, Mansergh FC, Evans MJ, Wilkins JA, Clarke AR (2007) Deficiency of SPARC suppresses intestinal tumorigenesis in APCMin/+ mice. Gut 56: 1410–1414.
48. Heemers HV, Regan KM, Dehm SM, Tindall DJ (2007) Androgen induction of the androgen receptor coactivator four and a half LIM domain protein-2: evidence for a role for serum response factor in prostate cancer. Cancer Res 67: 10592–10599.
49. Muller JM, Isle U, Metzger E, Rempel A, Moser M, et al. (2000) FHL-2, a novel tissue-specific coactivator of the androgen receptor. Embo J 19: 359–369.