

miRNA-129/FBW7/NF- κ B, a Novel Regulatory Pathway in Inflammatory Bowel Disease

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F-box and WD repeat domain-containing protein 7 (FBW7) has been documented to be implicated in nuclear factor κ B (NF- κ B) signaling and inflammation, but its role in the pathogenesis of inflammatory bowel disease (IBD) remains unknown. FBW7 was increased both in colon tissues from IBD patients and trinitrobenzene sulphonic acid (TNBS)-induced colitis mice. Immunoprecipitation assay identified that FBW7 as a novel inhibitor of κ B α (I κ B α)-binding partner. FBW7 upregulation promoted I κ B α ubiquitin-dependent degradation, NF- κ B activation, and subsequent intestinal inflammation in intestinal epithelial cells, whereas inhibition of FBW7 produced the opposite effects. Computational analysis revealed that microRNA-129 (miR-129) directly targets at 3' UTR of FBW7. The miR-129-suppressed proteasome pathway mediated the degradation of I κ B α by negatively regulating FBW7. The *in vivo* study demonstrated that upregulation of miR-129 ameliorated intestinal inflammation in TNBS-induced colitis mice through inhibition of the NF- κ B signaling pathway. In conclusion, FBW7 is a novel E3 ubiquitin ligase for I κ B α and thereby leads to NF- κ B activation and inflammation. miR-129 negatively regulates FBW7 expression, resulting in secondary inhibition of the NF- κ B pathway and amelioration of intestinal inflammation. Our findings provide new insight into the development of therapeutic strategies for the treatment of IBD.

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic debilitating disease referring to local inflammation that can affect all parts of the gastrointestinal tract.¹ Crohn's disease (CD) and ulcerative colitis (UC) are the two major common subtypes of IBD. It has been well documented that IBD results from immune dysregulation, although the exact etiology is unknown.^{2,3}

Nuclear factor κ B (NF- κ B), a transcriptional factor, is the hallmark of immune response.^{4,5} Several studies have demonstrated that overwhelming inflammatory responses, including NF- κ B activation and proinflammatory cytokine overexpression, contribute to colitis.^{6,7} Upon stimulation with proinflammatory cytokines, such as tumor necrosis factor α (TNF- α), interleukin (IL)-1 β , IL-6, and IL-8, the inhibitor of κ B (I κ B) kinase (IKK) complex occurs and activates, which lead to phosphorylation and ubiquitin-dependent degradation of NF- κ B inhibitory protein I κ B (e.g., I κ B α) by skp1-Cullin-F-box- β -transducin repeat-containing protein (SCF ^{β -TRCP}).^{8,9} This allows

NF- κ B to translocate into the nucleus and triggers a variety of target gene transcriptions.¹⁰

Protein degradation plays a critical role in various cellular functions and the pathogenesis of human diseases.¹¹ F-box and WD repeat domain-containing protein 7 (FBW7) is another type of SCF ubiquitin ligase that targets various mammalian oncoproteins for degradation, such as c-Jun, c-Myc, cyclin E, and Notch.¹²⁻¹⁵ Consistent with the anti-carcinogenic role of FBW7, it also suppresses the development of colorectal cancer,^{15,16} but the role of FBW7 in IBD has not been addressed. Interestingly, similar to β -TRCP, FBW7 governs the destruction of the p100 precursor, a recently identified inhibitor of NF- κ B, suggesting a key role of FBW7 in the inflammatory signaling pathway.^{4,17,18} Furthermore, our present study observed that FBW7 was increased both in colon tissues from IBD patients and the experimental mouse colitis model. However, whether and how FBW7 participates with IBD remain unknown. Here, the aim of this study is to investigate the involvement of FBW7 in the pathogenesis of IBD and the underlying mechanism. Our results suggest that FBW7 is an important regulator of the NF- κ B pathway and intestinal inflammation.

RESULTS

FBW7 Is Increased in Colon Tissues of IBD Patients and Experimental Colitis Mice

To unveil the role of FBW7 in IBD, the expression of FBW7 in colon tissues from healthy individuals, CD patients, or UC patients was determined (Table S1). As shown in Figure 1A, FBW7 mRNA expression was significantly increased in colon tissues from CD and UC patients compared with those from non-IBD individuals. FBW7 expression was elevated in 141/172 CD patients and 89/147 UC patients. Interestingly, the mRNA level of FBW7 was significantly connected with the CD endoscopic index of severity (CDEIS)

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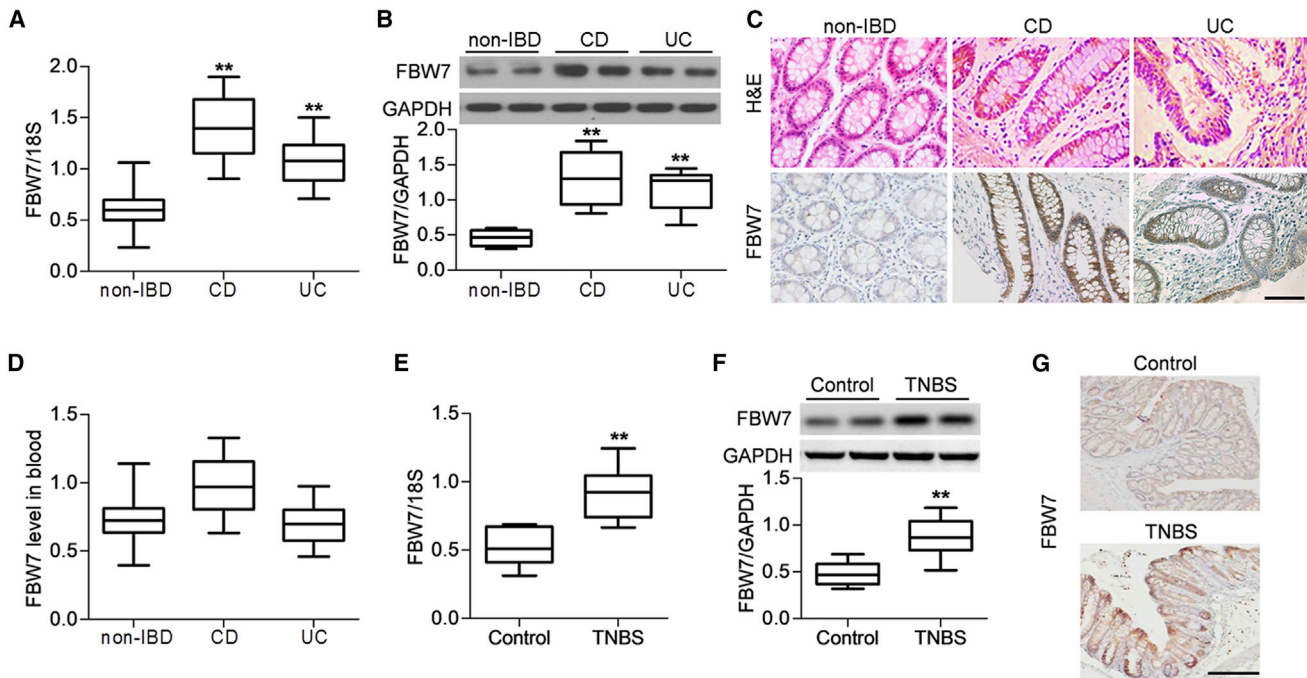


Figure 1. Increased Expression of FBW7 in Colon Tissues of Inflammatory Bowel Disease (IBD) Patients and the Trinitrobenzene Sulphonic Acid (TNBS)-Induced Mouse Colitis Model

(A) The mRNA expression of FBW7 in colon tissues from control individuals (non-IBD, $n = 189$) and patients with Crohn's disease (CD; $n = 172$) and patients with ulcerative colitis (UC; $n = 147$) was determined by RT-PCR. $**p < 0.01$ versus non-IBD. (B) Western blotting analysis of FBW7 protein expression in non-IBD individuals and patients with CD and patients with UC; $n = 40$ in each group. $**p < 0.01$ versus non-IBD. (C) Representative images of hematoxylin and eosin staining and immunohistochemistry for FBW7 expression. Scale bar, 50 μm . (D) The mRNA expression of FBW7 in blood from IBD patients was determined; $n = 40$ in each group. (E and F) A mouse acute experimental colitis was induced by rectal injection with TNBS. The expression of FBW7 in colon tissues was analyzed by RT-PCR (E) and western blotting (F). $**p < 0.01$ versus control, $n = 12$. (G) Representative images of immunohistochemistry for colonic FBW7 expression; $n = 4$ in each group. Scale bar, 50 μm .

for CD patients and Mayo score for UC patients (Table 1). Western blotting also showed upregulated protein expression of FBW7 in CD or UC colitis specimens (Figure 1B). Moreover, immunohistochemistry found increased membrane staining for FBW7 in colonic epithelial cells of CD and UC patients compared with non-IBD individuals (Figure 1C). However, no significant difference of FBW7 level was observed between the IBD group and the control group in the blood (Figure 1D). These results suggest that FBW7 expression in the inflamed tissues, but not in the blood, was correlated with the disease activity. To confirm the change of FBW7 expression in the development of IBD, we also examined FBW7 expression in an animal model of IBD. An experimental mouse colitis model was established by rectal injection with trinitrobenzene sulphonic acid (TNBS). Consistent with the observations in human specimens, FBW7 mRNA and protein expression were significantly increased in the TNBS-induced colitic mice (Figures 1E and 1F). Immunohistochemical staining further confirmed that TNBS injection enhanced colonic FBW7 expression compared with control mice (Figure 1G).

FBW7 Induces $\text{I}\kappa\text{B}\alpha$ Degradation and NF- κB Activation

To explore whether FBW7 was involved in the regulation of the NF- κB pathway, we used up- and downregulation of FBW7 expres-

sion approaches in Caco-2 cells. RT-PCR and western blotting confirmed the efficiency of FBW7 small interfering RNA (siRNA) transfection or FBW7 adenovirus infection, respectively (Figures 2A–2D). Downregulation of FBW7 markedly increased $\text{I}\kappa\text{B}\alpha$ expression, which in turn, inhibited $\text{I}\kappa\text{B}\alpha$ phosphorylation and p65 expression (Figure 2C). On the contrary, FBW7 overexpression reduced $\text{I}\kappa\text{B}\alpha$ expression and increased $\text{I}\kappa\text{B}\alpha$ phosphorylation and p65 expression (Figure 2D). Consequently, several proinflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-8, were reduced by FBW7 siRNA treatment, but increased by FBW7 adenovirus infection (Figures 2E–2H). To determine the mechanism of how FBW7 regulates $\text{I}\kappa\text{B}\alpha$ expression, we initially examined the mRNA expression of $\text{I}\kappa\text{B}\alpha$. No difference was detected in $\text{I}\kappa\text{B}\alpha$ mRNA expression after knockdown or overexpression of FBW7 (Figures S1A and S1B), indicating that the post-transcriptional regulation does not take place. Given that FBW7 is an E3 ubiquitin ligase that targets substrate proteins for degradation, we examined the effect of FBW7 on $\text{I}\kappa\text{B}\alpha$ protein stability by using a protein synthesis inhibitor cycloheximide (10 $\mu\text{g}/\text{mL}$). The results showed that cycloheximide caused a time-dependent decrease in $\text{I}\kappa\text{B}\alpha$ protein expression in Caco-2 cells. Compared with the negative siRNA group, FBW7 knockdown attenuated the degradation of $\text{I}\kappa\text{B}\alpha$, whereas overexpression of

Table 1. Correlation between FBW7 mRNA Level and Disease Severity of IBD Patients

Index	FBW7		
	n	r	P
CD (CDEIS)	172	0.513	<0.01
UC (Mayo)	147	0.304	0.017

IBD, inflammatory bowel disease; CD, Crohn's disease; CDEIS, Crohn's disease endoscopic index of severity; UC, ulcerative colitis.

FBW7 significantly increased the rate of I κ B α degradation (Figures 2I and 2J). The effect of FBW7 overexpression on I κ B α expression was dramatically restored by the proteasome inhibitor MG132 but not the lysosome blocker chloroquine (Figures 2K and 2L). This suggests that FBW7 inhibits I κ B α expression by enhancing I κ B α degradation through the proteasome degradation pathway. The endogenous immunoprecipitation assay clearly showed that I κ B α bound with FBW7 (Figure 2M). To validate this interaction further, Caco-2 cells were cotransfected with a construct encoding I κ B α with a hemagglutinin (HA) tag and an adenovirus encoding FBW7 with a GFP tag. Immunoprecipitation of HA-I κ B α with an HA antibody led to coimmunoprecipitation of FBW7 (Figure 2N). We next tested the effect of FBW7 on I κ B α protein degradation. By cotransfection with myc-ubiquitin, HA-I κ B α , and FBW7 siRNA or negative siRNA in Caco-2 cells, immunoprecipitation of HA antibody revealed that inhibition of FBW7 significantly inhibited I κ B α ubiquitination, followed with immunoblotting against the myc tag (Figure 2O). However, upregulation of FBW7 caused a significant increase in the ubiquitination of I κ B α (Figure 2P). These results suggest that FBW7 binds with I κ B α and causes I κ B α degradation and thus activates NF- κ B signaling.

Verification of FBW7 As a Target Gene of miR-129

microRNA (miRNA) regulation plays an important role in intestinal inflammation.¹⁹ Computational mRNA target analysis by bioinformatic software miRanda reveals that miR-129 contains a potential binding site in FBW7. A 7-bp fragment of FBW7 3' UTR is complementary to the miR-129 seed sequence. This binding site is conserved in human and mouse (Figure 3A). To investigate whether miR-129 directly binds to FBW7 and inhibits its translation, we increased or decreased the expression of miR-129 and measured the luciferase activity of FBW7 3' UTR. Successful overexpression or downregulation of miR-129 was evidenced by RT-PCR (Figures 3B and 3C). By cotransfection with miR-129 mimics and FBW7 3' UTR luciferase reporter into Caco-2 cells, the luciferase assay showed that overexpression of miR-129 dramatically decreased the luciferase activity of FBW7 3' UTR (Figure 3D). Conversely, the miR-129 inhibitor was associated with increased FBW7 3' UTR luciferase activity (Figure 3E). Furthermore, the effect of miR-129 on endogenous FBW7 protein expression was determined. Consistently, the protein expression of FBW7 after miR-129 mimics or inhibitor transfection showed the similar changes in the luciferase activity (Figures 3F and 3G). These data indicate that miR-129 directly targets at FBW7 3' UTR.

The Crosstalk between miR-129 and I κ B α

With the consideration that miR-129 negatively regulated FBW7 expression, we next examined whether miR-129 affected I κ B α expression and degradation. Western blotting showed that miR-129 mimics significantly increased I κ B α protein expression. However, when the cells were cotransfected with miR-129b mimics and FBW7 adenovirus, no significant difference was observed between miR-129 mimics and mimics negative control groups (Figure 4A). In addition, inhibition of miR-129 caused a marked decrease in I κ B α protein expression. Similarly, the inhibition of the miR-129 inhibitor on I κ B α expression was significantly attenuated by transfection of FBW7 siRNA (Figure 4B). Consistently, miR-129 upregulation was associated with reduced I κ B α ubiquitination. Following ectopic expression of FBW7, miR-129 failed to suppress the ubiquitination of I κ B α (Figure 4C). Furthermore, the increased ubiquitination of I κ B α in miR-129 inhibitor-treated cells was completely abrogated by FBW7 siRNA (Figure 4D). These results indicate that miR-129 suppresses I κ B α ubiquitination and increases I κ B α expression, and these effects are dependent on FBW7.

Restoration of miR-129 Expression Ameliorates TNBS-Induced Colitis by Inhibiting the NF- κ B Pathway

The results of RT-PCR showed that miR-129 expression was remarkably decreased in CD or UC patients compared with healthy individuals (Figure 5A). Similar to the results of IBD colon tissues, we also found that the expression of miR-129 was inhibited in TNBS-induced colitic mice (Figure 5B). To clarify the role of miR-129 in intestinal inflammation, mice were injected with miR-129 mimics or mimics negative control followed by TNBS challenge. miR-129 mimics treatment expectedly restored the TNBS-induced decrease in miR-129 expression (Figure 5C). Compared with mimics negative control, the survival rate of miR-129 mimics-injected mice was increased during the TNBS treatment period (Figure 5D). The TNBS-induced loss of body weight and the shorting of colon length were significantly less pronounced in the miR-129 mimics group than in the mimics negative control group (Figures 5E–5G). Hematoxylin and eosin staining revealed that TNBS induced marked disruption of the colon architecture, which was effectively improved by miR-129 mimics (Figures 5H and 5I). Moreover, TNBS induced the increase in myeloperoxidase (MPO) activity in colon tissues, which indicates neutrophil infiltration was markedly inhibited by miR-129 upregulation (Figure 5J). Overexpression of miR-129 was also associated with reduced mRNA expression of proinflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-8 (Figures 5K–5N). A subsequent western blotting showed that restoration of miR-129 significantly attenuated the TNBS-induced increase in FBW7 and p65 expression, concomitantly with increased I κ B α expression (Figures 5O–5Q). Additionally, immunoprecipitation of I κ B α in colon tissue lysate from TNBS-treated mice showed a significant increase in I κ B α ubiquitination, as revealed by the ubiquitin antibody immunoblotting. However, miR-129 upregulation was associated with reduced I κ B α ubiquitination (Figure 5R). These data suggest that miR-129 ameliorates intestinal inflammation by inhibiting I κ B α ubiquitination and NF- κ B activation.

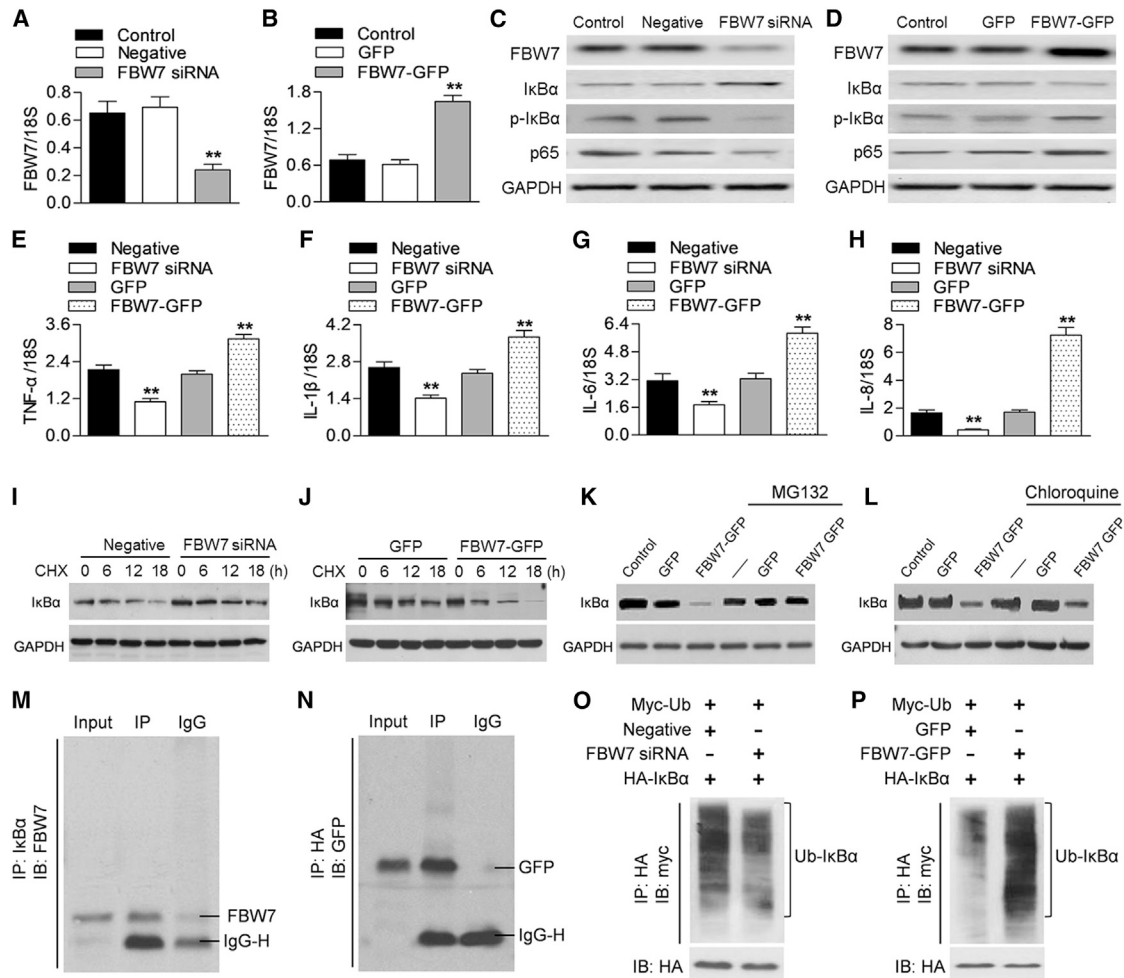


Figure 2. FBW7 Decreases IκBα Expression and Promotes NF-κB Activation

(A) Caco-2 cells were transfected with FBW7 siRNA (20 nM) or negative siRNA (negative) for 48 h. FBW7 mRNA expression was determined by RT-PCR. (B) The cells were infected with adenovirus encoding FBW7-GFP (FBW7-GFP, 50 MOI) or GFP for 48 h. RT-PCR analysis of FBW7 mRNA expression. ** $p < 0.01$ versus control, $n = 6$. (C and D) Western blotting analysis of FBW7, IκBα, and p65 expression and IκBα phosphorylation in cells treated with FBW7 siRNA (C) or FBW7-GFP adenovirus (D). FBW7 knockdown significantly increased IκBα expression and decreased IκBα phosphorylation and p65 expression, whereas FBW7 upregulation reduced IκBα expression and increased IκBα phosphorylation and p65 expression. Representative images from six independent repetitions were shown. (E–H) The mRNA expression of TNF-α (E), IL-1β (F), IL-6 (G), and IL-8 (H) was examined by RT-PCR. ** $p < 0.01$ versus negative siRNA or GFP adenovirus, $n = 6$. (I and J) Caco-2 cells were treated with FBW7 siRNA (I) and FBW7-GFP adenovirus (J) for 48 h, and then cycloheximide (CHX) was added at 10 μg/mL for the indicated times. IκBα expression was determined by western blotting. The degradation of IκBα-induced cycloheximide was significantly inhibited by FBW7 downregulation but enhanced by FBW7 overexpression; $n = 4$. (K and L) Western blotting analysis of IκBα expression in Caco-2 cells pretreated with MG132 (10 μg/mL) (K) or chloroquine (10 μg/mL) (L) for 30 min, followed by FBW7 siRNA for another 48 h. MG132, but not chloroquine, significantly reversed the inhibitory effect of FBW7 knockdown on IκBα expression. $n = 5$. (M) Cell lysates were immunoprecipitated with IκBα antibody, and immunoprecipitated proteins were blotted with FBW7 antibody; $n = 4$. (N) Caco-2 cells were cotransfected with HA-IκBα plasmid and FBW7-GFP adenovirus. HA-IκBα was immunoprecipitated (IP) and immunoblotted (IB) with GFP antibody; $n = 4$. (O and P) Cells were cotransfected with myc-ubiquitin (myc-Ub), HA-IκBα, and FBW7 siRNA (O) or FBW7-GFP adenovirus (P). After immunoprecipitation with HA antibody, proteins were immunoblotted with myc antibody to show IκBα ubiquitination; $n = 6$.

DISCUSSION

In this study, to the best of our knowledge, we provide the first evidence demonstrating that miR-129/FBW7/NF-κB is a novel and critical axis in the pathogenesis of IBD. The central findings of the present study were summarized as follows: (1) ubiquitin E3 ligase FBW7 is upregulated in colon tissues from IBD patients and experimental colitic mice; (2) we identify that IκBα, a suppressor of NF-κB, as a novel

substrate of the ubiquitin E3 ligase FBW7; therefore, FBW7 promotes ubiquitin-mediated degradation of IκBα, which enhances NF-κB activation and proinflammatory cytokines; and (3) miR-129 directly targets the 3' UTR of FBW7 and negatively regulates FBW7 expression. Overexpression of miR-129 ameliorates TNBS-induced intestinal inflammation and colitis, an effect that inhibited FBW7-mediated IκBα degradation and NF-κB activation.

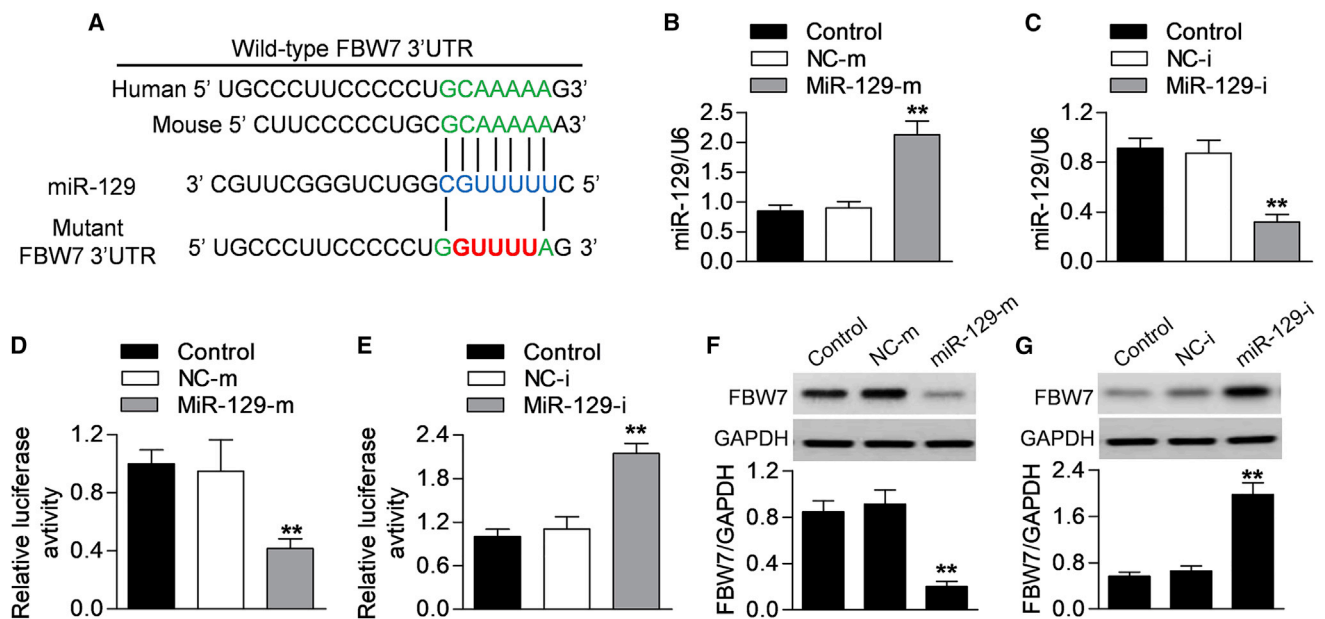


Figure 3. miR-129 Directly Targets FBW7 3' UTR

(A) Predicted miR-129 seed matches the sequence in the 3' UTR of FBW7 in human and mouse species. (B and C) Caco-2 cells were transfected with miR-129 mimics (MiR-129-m; 10 nM) or mimics negative control (NC-m) (B) or miR-129 inhibitor (MiR-129-i; 10 nM) or inhibitor negative control (NC-i) (C) for 48 h. The expression of miR-129 was determined by RT-PCR. (D and E) Dual luciferase activity assay was performed by cotransfection of luciferase reporter containing FBW7 3' UTR or the mutant one with miR-129 mimics (D) or miR-129 inhibitor (E) in Caco-2 cells. (F and G) Western blotting analysis of FBW7 protein expression in cells transfected with miR-129 mimics (F) and miR-129 inhibitor (G). All data were presented as mean value \pm SEM. ** $p < 0.01$ versus control; $n = 6$.

Elevated proteolytic activity has been observed in colonic specimens of IBD patients, suggesting that ubiquitin-related protein degradation may be implicated in immune recognition and inflammation.^{20,21} Protein ubiquitination is a highly conserved process that is characterized by binding a ubiquitin molecule to a lysine residue within a target substrate protein via a cascade formed by three enzymatic proteins: the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin ligase E3.²² E3 ubiquitin ligases control the specificity of the ubiquitination cascade, and several E3 ligases have been demonstrated to be implicated in IBD.^{23,24} Recently, the E3 ligases, such as Ndfip1, Itch, and RNF186, were shown to be critical for gastrointestinal inflammation. Knockout of Ndfip was associated with a gastrointestinal inflammation phenotype in mice, whereas Itch mutant mice showed much less severe inflammation in the gastrointestinal tract.²³ In addition, RNF186 was upregulated in colon tissues from IBD patients and targeted $\text{I}\kappa\text{B}\alpha$, which led to NF- κB activation and IBD progression.²⁵ FBW7, a member of the F-box protein family, functions as a substrate recognition component of SCF ubiquitin ligases.^{13,14} In keeping with the antitumor activity of FBW7, decreased FBW7 expression is significantly associated with gastrointestinal carcinomas, including gastric cancer and colorectal cancer.^{26,27} Although FBW7 has been reported to be a prognostic factor for patients with gastric cancer or colorectal cancer,^{15,16,25,26} information regarding to role of FBW7 in IBD is very limited. In this study, we found that FBW7 expression was increased in colon tissues from both IBD patients and TNBS-induced colitis mice.

Importantly, the increased FBW7 expression was parallel with the severity of inflammation in the colonic mucosa of CD or UC patients observed by endoscopy. This suggests that the change of FBW7 expression may serve as a mediator or a causal factor in the pathogenesis of IBD. However, in the blood, no significant difference was found in the FBW7 level between IBD patients and non-IBD individuals. The possible reason for this discrepancy is likely that FBW7 is not a circulating protein associated with an acute phase reaction, indicating that FBW7 may play an organ-specific role the inflammatory process of IBD.

NF- κB signaling pathway is one of the most important pathways involved in inflammation.⁵ The NF- κB family consists of proteins p50, p52, p65 (RelA), RelB, and cRel.¹⁰ The precursors of p50 and p52 are inhibitory proteins of p105 and p100, respectively, and function as inactivate factors of NF- κB . NF- κB signaling divides into two major pathways: canonical and noncanonical pathways.¹⁰ The canonical pathway is activated by proinflammatory agents, which allow the IKK complex formation, leading to phosphorylation and subsequent degradation of $\text{I}\kappa\text{B}$.^{17,28} For the noncanonical pathway, ligand binding activates the IKK complex and in turn, triggers ubiquitination of precursor p100. This leads the process into a mature p52 subunit, which with RelB, forms and activates the NF- κB dimer of the noncanonical pathway.⁴ Several E3 ligases have been reported to contribute to the regulation of NF- κB activation. For example, RNF186 targeted $\text{I}\kappa\text{B}\alpha$ for ubiquitination, and it is crucial for NF- κB activation.²⁴ SCF ^{β -TRCP}

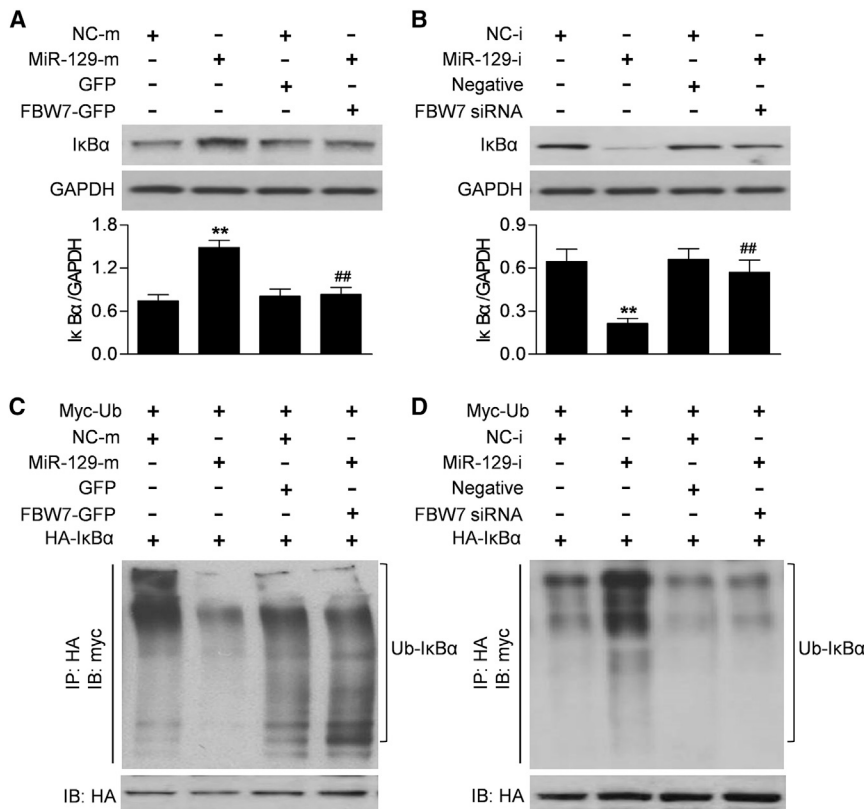


Figure 4. miR-129 Suppresses IκBα Ubiquitination and Increases IκBα Expression

(A) Caco-2 cells were transfected with miR-129 mimics (MiR-129-m) or mimics negative control (NC-m) in the presence or absence of FBW7-GFP adenovirus treatment for 48 h. IκBα protein expression was examined by western blotting. (B) miR-129 inhibitor (MiR-129-i; 10 nM) or inhibitor negative control (NC-i) was cotransfected with or without FBW7 siRNA into cells. IκBα protein expression was determined. ** $p < 0.01$ versus mimics negative control or inhibitor negative control; ## $p < 0.01$ versus miR-129 mimics or inhibitor; $n = 6$. (C and D) Caco-2 cells transfected with myc-ubiquitin (myc-Ub) and HA-IκBα were treated as above. HA-IκBα was immunoprecipitated (IP) and immunoblotted (IB) with myc antibody. Upregulation of FBW7 reversed the miR-129-induced decrease in ubiquitination of IκBα (C), while miR-129 inhibitor failed to induce ubiquitination of IκBα in FBW7 siRNA-treated cells (D); $n = 5$.

enhanced both p50 and p52 activation by ubiquitination of p105 and p100 precursor proteins, which ultimately leads to activation of both canonical and noncanonical NF-κB pathways.^{5,27} It is noteworthy that p100 ubiquitination was also governed by FBW7 in a glycogen synthase kinase 3 (GSK3)-dependent manner.¹⁷ Herein, we reported for the first time that IκBα is a novel FBW-interacting protein. Thus, FBW7 decreased IκBα expression by ubiquitin-mediated degradation, resulting in NF-κB-p65 activation and inflammation in intestinal epithelial cells. The enhanced effect of FBW7 on NF-κB and inflammation, revealed in our work, was consistent with the results of a previous study.¹⁷ By contrast, a report by Sterneck and coworkers¹⁸ showed that FBW7 suppressed inflammation in macrophages and tumor cells by targeting Toll-like receptor 4 (TLR4). Presumably, the discrepancy is probably related to different functions of FBW7 in different cell types, even under the same acute inflammatory stimulation, indicating the organ-specific role of FBW7 in the pathogenesis of IBD.

Another significant finding of our study was that miR-129 directly targeted the 3' UTR of FBW7 and subsequently regulated the IκBα/NF-κB signaling pathway. Accumulating evidence has demonstrated that miRNAs are strongly implicated in the pathogenesis of IBD for their important roles in the regulation of immune inflammation.^{19,29} miR-129, which is known to function as a tumor suppressor, has been suggested to be a biomarker for gastrointestinal and colorectal cancer.^{30,31} Although several miRNAs have been evidenced to control FBW7 through binding to specific target sequences in the 3' UTR of the

gene, as shown for miR-363, miR-223, and miR-27a,^{32–34} we found that miR-129 had the strongest inhibitory effect on FBW7 expression (Figures S2A and S2B). More importantly, we observed a significant decrease in miR-129 expression in colon tissues from both IBD patients and TNBS-induced colitis mice, but the expression of miR-363, miR-223, and miR-27a was unchanged. This indicates that the downregulation of miR-129 increases FBW7 expression and IκBα degradation, leading to intestinal inflammation and IBD (Figures S2C–S2H). Indeed, restoration of miR-129 ameliorated TNBS-induced colitis by attenuating intestinal inflammation through the IκBα/NF-κB signaling pathway. These results were consistent with the report that miR-129 upregulation also prevented inflammation in the neuron.³⁵ Here, we also investigated whether modulation of FBW7 expression could influence the effect of miR-129 on IκBα expression and ubiquitination. We found that elevation of FBW7 reversed the miR-129 overexpression-induced decrease in IκBα degradation. On the contrary, inhibition of FBW7 abolished the enhanced effect of miR-129 knockdown on IκBα degradation. These results further support that miR-129 inhibits IκBα degradation through targeting FBW7.

Conclusion

Our study demonstrates that FBW7, which is negatively regulated by miR-129, functions as a novel E3 ubiquitin ligase for IκBα and thereby leads to NF-κB activation and intestinal inflammation. This work suggests that miR-129/FBW7/NF-κB may be an important molecular mechanism in the pathogenesis of IBD.

MATERIALS AND METHODS

Materials and Reagents

Antibodies against FBW7, ubiquitin, GFP, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Abcam (MA, USA). IκBα, p-IκBα, p65, HA, myc, and immunoglobulin G (IgG)

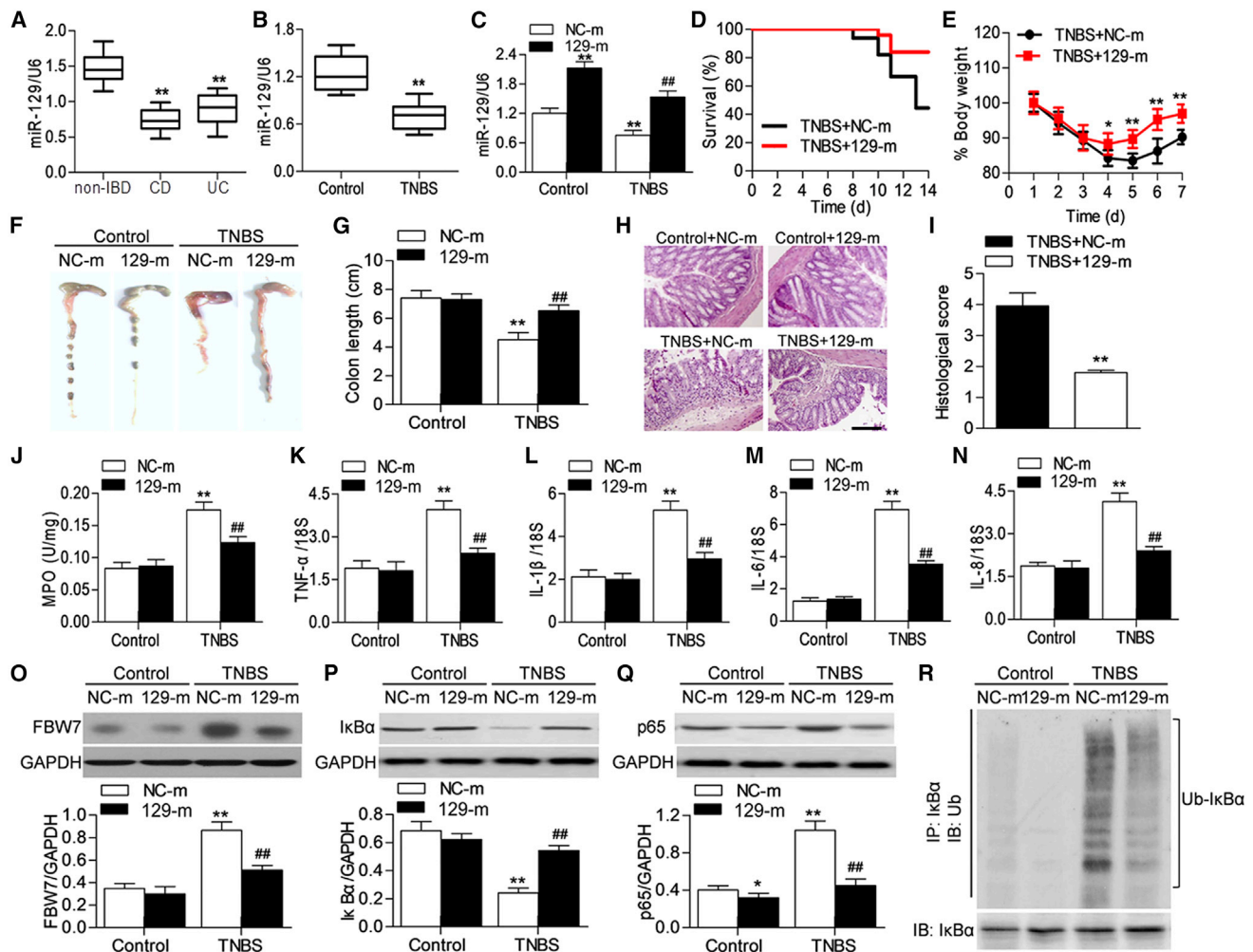


Figure 5. miR-129 Prevents TNBS-Induced Colitis in Mouse

(A) The expression of miR-129 in non-IBD individuals ($n = 189$) and patients with Crohn's disease (CD; $n = 172$) and patients with ulcerative colitis (UC; $n = 147$) was determined by RT-PCR. $**p < 0.01$ versus non-IBD individuals. (B) Colonic miR-129 expression in mice treated with TNBS was analyzed by RT-PCR. (C) Mice were treated with miR-129 mimics (129-m) or mimics negative control (NC-m) for 7 days prior to TNBS-induced colitis. The expression of miR-129 was determined. $**p < 0.01$ versus control; $###p < 0.01$ versus TNBS + mimics negative control; $n = 10$ in each group. (D) Survival curves were recorded until day 14 after the start of TNBS treatment; $n = 20$ in each group. (E) The change of body weight of mice. $*p < 0.05$, $**p < 0.01$ versus TNBS + mimics negative control; $n = 15$ in each group. (F and G) Mice were sacrificed on day 7 (F), and the colon length was measured (G). MiR-129 overexpression inhibited TNBS-induced the shorting of colon length. $**p < 0.01$ versus mimics negative control; $###p < 0.01$ versus TNBS + mimics negative control; $n = 6$ in each group. (H) Histopathological changes in colon tissue were examined by hematoxylin and eosin staining. Scale bar, 50 μ m. (I) Semi-quantitative scoring of histopathology was performed. $**p < 0.01$ versus TNBS + mimics negative control; $n = 6$ in each group. (J) The activity of myeloperoxidase (MPO) was assessed. (K–N) The colonic mRNA expression of TNF- α (K), IL-1 β (L), IL-6 (M), and IL-8 (N) was examined by RT-PCR. (O–Q) Western blotting analysis of FBW7 (O), I κ B α (P), and p65 (Q) protein expression in colon tissues. $*p < 0.01$, $**p < 0.01$ versus mimics negative control; $###p < 0.01$ versus TNBS + mimics negative control; $n = 6$ in each group. (R) Lysates of colon tissues were immunoprecipitated with I κ B α antibody, and proteins were blotted with ubiquitin antibody to detect the ubiquitination of I κ B α . $n = 5$.

antibodies were purchased from Cell Signaling Technology (MA, USA). miR-129 mimics, miR-363 mimics, miR-223 mimics, miR-27a mimics, mimics negative control, miR-129 inhibitor, inhibitor negative control, FBW7 siRNA, negative siRNA, and miR-129 and U6 primers were synthesized from RiboBio (Guangzhou, China). Mammalian expression plasmids for myc-tagged ubiquitin and HA-tagged I κ B α were constructed by BioVector NTCC (Beijing, China). Adenovirus encoding human FBW7 cDNA and GFP were provided by Sunbio Biotechnology (Shanghai, China). Dulbecco's minimum

essential medium (DMEM), OptiMEM I medium, fetal bovine serum (FBS), penicillin, streptomycin, and Lipofectamine 2000 were obtained from Invitrogen (CA, USA). All other reagents utilized were purchased from Sigma Chemical (MO, USA) unless otherwise specified.

Human Studies

The research protocols related to human specimens were approved by the Ethics Committee of Harbin Medical University and performed in accordance with the Declaration of Helsinki and Good Clinical

Practice. The IBD patients, including 172 CD patients and 147 UC patients, met the criteria of the Montreal Classification system and were recruited in this study between 2011 and 2015 in the First Affiliated Hospital of Harbin Medical University.³⁶ The disease severity of CD patients was evaluated by CDEIS, and the disease activity of UC patients was graded according to the Mayo score.³⁷ The normal group was non-IBD individuals (n = 189) undergoing colonoscopy for abdominal pain or screening purposes, for which IBD was excluded. Informed consent was obtained from all subjects in this study.

Animal Studies

The experimental protocol related to animals was approved by the Institutional Animal Care and Use Committee of Harbin Medical University and carried out in accordance with the guidelines for the Care and Use of Laboratory Animals. 8-Week-old male C57BL/6J mice (20 ± 2 g) were purchased from The Jackson Laboratory (ME, USA). Mice were treated with miR-129 mimics or mimics negative control for 7 days before TNBS-induced colitis. Briefly, 1 μL of 1 nM miR-129 mimics or mimics negative control was mixed with 99 μL phosphate buffer saline (PBS). 30 μL Lipofectamine 2000 was mixed with 70 μL PBS. The above solutions were placed at room temperature for 15 min and were mixed for further coincubation for 30 min. The mixture was injected into the tail veins of mice. After 7 days of systemic delivery of miR-129, mice were presensitized with TNBS at a dose of 40 mg/kg body weight through the skin for 7 days. Then, the presensitized mice received TNBS by rectal injection at a dose of 100 mg/kg body weight, as previously described.³⁹

RT-PCR

Total RNA from human colon tissues, mouse colon tissues, or Caco-2 cells was isolated using the QIAGEN RNAsasy Mini Kit (QIAGEN, CA, USA), according to the manufacturer's instructions. The concentration of the isolated RNA was determined by UV spectrometry. 1 μg of RNA was reverse transcribed to cDNA using a first-strand cDNA synthesis kit (Thermo Fisher Scientific, IL, USA). Blood DNA was isolated from 200 μL heparinized blood sample using the Blood & Cell Culture DNA Mini Kit (QIAGEN), according to the manufacturer's instruction. Levels of miR-129, miR-363, miR-223, miR-27a, and mRNAs were examined by Fast SYBR Green Master Mix Kit (Applied Biosystems, CA, USA) with the ABI 7500 RT-PCR System (Applied Biosystems). The relative mRNA expression index was normalized with U6 or 18S. The primer sequences for RT-PCR of gene expression were as follows: FBW7 (human), 5'-AGTCCGAAGGAGGAAGG-GAA-3' and 5'-GAACGGGCAGGTCCACAATA-3'; FBW7 (mouse), 5'-TTGGCTTGGGACAACAGACT-3' and 5'-ATAAGCAGCCC GTGTTTGGGA-3'; TNF-α (human), 5'-AGAATCACTGGGGC CTACA-3' and 5'-GCTCCGTGTCTCAAGGAAGT-3'; TNF-α (mouse), 5'-GATCGGTCCCCAAAGGGATG-3' and 5'-GGGAGGC CATTGGGAACTT-3'; IL-1β (human), 5'-CTGAGCTCGCCAG TGAATG-3' and 5'-TGTCATGGCCACAACAAC-3'; IL-1β (mouse), 5'-ATCTCGCAGCAGCATCAA-3' and 5'-ATGGG AACGTACACACCAG-3'; IL-6 (human), 5'-CTCAATATTAGA

GTCTCAACCCCCA-3' and 5'-GAGAAGGCAACTGGACCGA A-3'; IL-6 (mouse), 5'-AGTTGCCTTCTTGGGACTGA-3' and 5'-TC CACGATTTCCCAGAGAAC-3'; IL-8 (human), 5'-TTGCCAG CTGTGTTGGTAGT-3' and 5'-AACAAAATGCTAAGGGGAA GCAT-3'; IL-8 (mouse), 5'-AGTTGCCTTCTTGGGACTGA-3' and 5'-TCCACGATTTCCCAGAGAAC-3'; 18S rRNA (human), 5'-CG GCTACCACATCCAAGGAA-3' and 5'-CTGGAATTACCGCG GCT-3'; 18S rRNA (mouse), 5'-GCAATTATCCCCATGAACG-3' and 5'-GGCCTCACTAAACCATCCAA-3'.

Western Blotting and Immunoprecipitation

The colon tissues or Caco-2 cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing 1% protease and phosphatase inhibitors (Thermo Fisher Scientific). Protein concentration of each sample was determined by the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China), and equal protein was separated on 8%–10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked by 5% skim milk powder in Tris-buffered saline-Tween 20 (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 [pH 7.6]) for 1 h and incubated with primary antibodies at 4°C overnight. After washing and incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, the blots were visualized by the chemiluminescence system (Thermo Fisher Scientific) and quantified by ImageJ software. For immunoprecipitation, nonspecific binding was precleared with A/G agarose beads (Santa Cruz Biotechnology, CA, USA) and then the lysates were coincubated with indicated primary antibodies and A/G agarose beads at 4°C for 4 h. The beads containing the bound proteins were washed with PBS three times and boiled in lysis buffer at 95°C for 10 min. The supernatant was subjected for western blotting analysis.

Histological Analyses

Immunohistochemistry for FBW7 was performed on 4 μm paraffin-embedded sections from human or mouse colon tissues using the streptavidin-biotin-peroxidase complex system, according to the supplier's instructions (Dako Japan, Tokyo, Japan). The sections were heated for 30 min at 65°C, de-waxed in xylene, and rehydrated by 100%, 95%, 70%, and 50% alcohol at room temperature for 1 min. The endogenous peroxidase activity was blocked by 5% H₂O₂, and the nonspecific staining was prevented by 2% nonimmune serum solution. The sections were incubated with antibody against FBW7, diluted in nonimmune serum solution overnight at 4°C. After rinsing with PBS three times, the sections were treated with biotinylated secondary antibody and then incubated with the streptavidin-peroxidase reaction using 3,3'-diaminobenzidine. The sections were counterstained with hematoxylin. For histopathological examination, the slides of colon tissues were stained with hematoxylin and eosin. All slides were observed and examined using a light microscope (CKX41; Olympus, Tokyo, Japan). Histological score was calculated by a pathologist, as previously described.³⁸

MPO Activity Assay

The infiltration of neutrophil into the colon tissue was determined by MPO activity, as previously described.³⁹ The colon tissues were homogenized and centrifuged. Then, the pellet was resuspended and centrifuged. The supernatant was harvested and added to the substrate containing o-dianisidine hydrochloride (0.167 mg/mL), 0.0005% hydrogen peroxide, and KH_2PO_4 (50 mM). The absorbance was measured at 460 nm immediately and recorded as the optical density (OD) value. The second OD value was read after 5 min. The changes of OD value in 5 min were represented as MPO activity, which were normalized to total protein concentration.

Cell Transfection and Infection

The human colon cancer cell line Caco-2 was purchased from the American Type Culture Collection (ATCC; VA, USA) and cultured in DMEM, supplemented with 0.5% heat-inactivated FBS, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 100 U/mL penicillin. For siRNA or miRNA transfection, FBW7 siRNA or negative siRNA, miR-129 mimics or miR-129 inhibitor, mimics negative control, or inhibitor negative control were diluted with OptiMEM I medium and transfected into cells with Lipofectamine 2000, according to the supplier's instructions. For overexpression of FBW7, Caco-2 cells were cultured in FBS-free DMEM containing FBW7 adenovirus or GFP adenovirus. After 6 h incubation, the cells were transferred into complete medium and cultured for a further 48 h.

Luciferase Assay

The wild-type human 3' UTR of FBW7, which contains the predicted binding site for miR-129, was cloned into the pMIR vector (Promega, WI, USA). The mutant FBW7 3' UTR was generated from wild-type FBW7 3' UTR by site-directed mutagenesis. 2×10^5 Caco-2 cells were transfected with miR-129 mimics or miR-129 inhibitor or corresponding negative controls and wild-type or mutant 3' UTR of FBW7 dual luciferase reporter vector using Lipofectamine 2000. After 48 h transfection, luciferase activities were quantified using a dual luciferase reporter system (Promega) on a luminometer (Elesys 2010; Roche Diagnostics, Basel, Switzerland).

Statistical Analysis

Data were presented as mean value \pm standard error of mean (SEM). The number of samples in each experiment was indicated in figure legends. The differences between groups were analyzed by two-tailed Student's t test or one-way ANOVA, followed by the Bonferroni multiple comparison test. Statistical analysis was performed by SPSS 18.0 software (SPSS, IL, USA). $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2019.10.048>.

AUTHOR CONTRIBUTIONS

Data Curation, P.X.; Formal Analysis, L.L.; Investigation, Q.M. and W.W.; Methodology, W.W., J.X., and L.S.; Project Administration,

Q.M. and T.P.; Software, J.X. and D.L.; Supervision, Q.M. and T.P.; Validation, P.X.; Writing – Original Draft, T.P.; Writing – Review & Editing, Q.M. and T.P.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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Supplemental Information

miRNA-129/FBW7/NF- κ B, a Novel Regulatory

Pathway in Inflammatory Bowel Disease

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Table S1. Basic characteristic of all subjects included in this study

Parameter	non-IBD	CD	UC
All (n)	189	172	147
Male	107 (56.6%)	114 (66.3%)	95 (64.6%)
Age (years)	41.7 ± 9.5	49.4 ± 11.3	45.2 ± 8.2
Location			
Colon	102	89	78
Rectum	51	33	28
Terminal ileum	22	28	26
Ceca	14	22	15

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

Figure S1

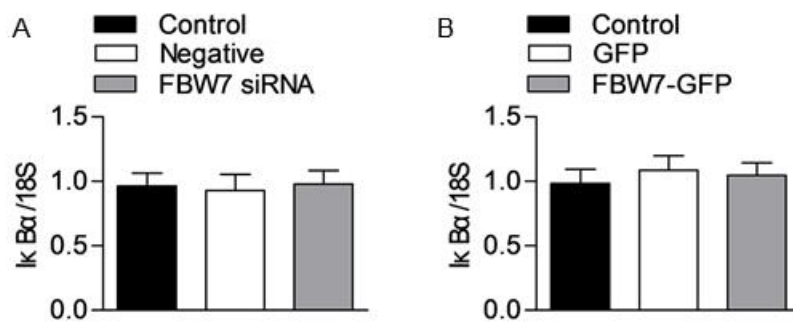


Figure S1. Knockdown or overexpression of FBW7 had no effect on IκBα mRNA level. (A and B) RT-PCR analysis of IκBα mRNA expression in Caco-2 cells treated with FBW7 siRNA (A) or FBW7-GFP adenovirus (B). n=6.

Figure S2

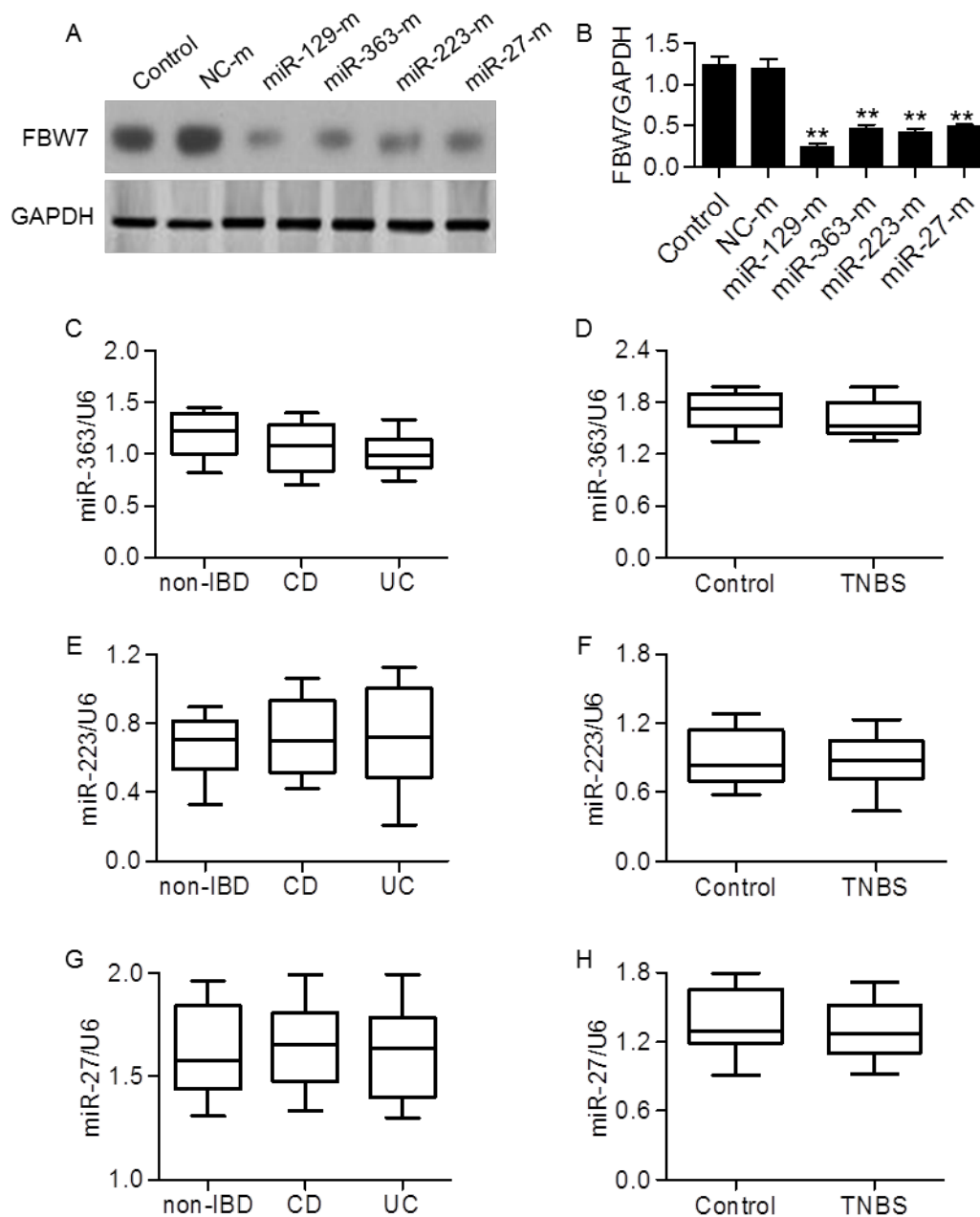


Figure S2. The expression of FBW7-binding miRNAs in colon tissues of inflammatory bowel disease patients and trinitrobenzene sulphonic acid (TNBS)-induced mouse colitis model. (A) Western blotting analysis of the extracts from Caco-2 cells transfected with indicated miRNA mimics. (B) Quantification of FBW7 protein level normalized to GAPDH. **P<0.01 vs. control, n=6. (C-H) The expression of miR-363, miR-223 and miR-27a in **non-IBD individuals** (n=189), patients with Crohn's disease (CD, n=172),

patients with ulcerative colitis (UC, n=147), control mice (n=12) and TNBS-treated mice (n=12) was determined by RT-PCR.