IL-17-driven intestinal fibrosis is inhibited by Itch-mediated ubiquitination of HIC-5

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Intestinal fibrosis is a major complication in inflammatory bowel diseases, but the regulatory mechanism that inhibits fibrosis remains unclear. Here we demonstrate that Itch$^{-/-}$ myofibroblasts express increased amounts of profibrotic collagen type I and α-SMA in response to IL-17. Mechanistically, we demonstrate that Itch directly binds to HIC-5 and targets it for K63-linked ubiquitination to inhibit IL-17-driven intestinal fibrosis. Reconstitution of Itch$^{-/-}$ myofibroblasts with wild-type Itch but not the Itch-C830A mutant normalized the expression of profibrotic genes. Similarly, shRNA-mediated inhibition of HIC-5 normalized the expression of profibrotic gene expression. Thus, we have uncovered a novel mechanism by which Itch negatively regulates intestinal fibrosis.

INTRODUCTION

Inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease are chronic inflammatory disorders of the gastrointestinal tract that affect about 1.6 million Americans.[1] Intestinal fibrosis is a common complication in IBDs that results in progressive tissue architectural distortion, loss of function, and luminal narrowing, which requires surgery.[2] Fibrosis is caused by the deposition of extracellular matrix (ECM) proteins, including collagen and fibronectin, which are produced by the intestinal myofibroblasts in response to chronic inflammation.[3]

IL-17 produced by Th17 cells and innate lymphoid cells plays a crucial role in the pathogenesis of IBD.[4–6] Development of Th17 cells requires the combined action of IL-6, TGF-β, IL-1β, and IL-23.[7] These cytokines, which are produced by the myeloid cells, promote the expression of the lineage-specific transcription factor ROR-γt, which is essential for IL-17 expression.[7,8] Although IL-17 is linked to pulmonary, liver, and intestinal fibrosis,[9] the mechanism by which IL-17 promotes fibrosis remains unclear.

Hydrogen peroxide-inducible clone 5 (HIC-5) is a member of the paxillin family protein that was originally identified as a TGF-β1 and H_2O_2-inducible gene.[10] The N-terminal region of HIC-5 is composed of four LD domains, which are rich in Leu and Asp. The C-terminal region is composed of four LIM domains with two zinc fingers.[11] Since both the LD and LIM domains are protein–protein interacting domains, HIC-5 acts as an adaptor and as a nuclear receptor coactivator.[12,13] HIC-5 plays a crucial role in fibrosis by regulating differentiation of myofibroblasts and expression of ECM proteins.[14–17]

Ubiquitin-mediated post-translational modification regulates a variety of signaling pathways.[18] Ubiquitination involves a cascade of biochemical reactions through ubiquitin activating (E1) enzymes, ubiquitin-conjugating (E2) enzymes, and ubiquitin ligase (E3) enzymes.[18] The E3 ubiquitin ligases are critical components of this system because they recognize, bind to, and recruit specific target proteins for ubiquitination.[18]

Itch is an E3 ubiquitin ligase that belongs to the HECT (homologous to the E6-AP C terminus) family. Itch contains a protein kinase C-related C2 domain, four WW domains – each of which contains two conserved tryptophan residues – and the HECT ligase domain.[19] The WW domains recognize the proline-rich Pro-Pro-X-Tyr (PPXY) consensus sequence (where X is any amino acid) in their substrate targets.[19] Itch deficiency in mice leads to multiorgan inflammatory disorders.[19] Similarly, a truncated mutation of human Itch results in inflammatory disorders, including enteropathy.[20] Here we demonstrate that Itch inhibits IL-17-driven fibrosis by targeting HIC-5 for ubiquitination. These findings could lead to advancement of therapeutic strategies for reducing fibrosis in IBD, which remains an unresolved clinical challenge.

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RESULTS

Itch deficiency leads to spontaneous colonic fibrosis

Recently, we demonstrated that Itch-deficient mice develop spontaneous rectal prolapse due to severe colonic inflammation.[21] Histological examination of Masson's trichrome-stained sections of colon tissues showed increased collagen fibers in the 6–8 month-old colon tissues of Itch $^{-/-}$ mice compared to age-matched control mice (**Figure 1a** and **b**). To investigate if fibrosis and inflammation coexist, we performed hematoxylin and eosin staining, and Masson's trichrome staining in the same sections. As shown clearly in **Supplementary Figure 1a** and **1b** online, we observed inflammation and fibrosis in adjacent areas. Since fibrosis is caused by pathological deposition of ECM proteins (collagens I–VI, α-SMA, etc), we analyzed the expressions of collagen I (Col I) and α-SMA by real-time polymerase chain reaction (PCR). As shown in **Figure 1c** and **d**, we found a marked increase in the expression of these fibrosis-associated genes in the Itch $^{-/-}$ colon compared to the Itch $^{+/+}$ colon.

Itch directly interacts with HIC-5

Next, we sought to identify the dysregulated signaling pathways that lead to fibrosis in Itch-deficient mice using a proteomics approach. We performed pull-down assays using either glutathione S-transferase (GST) alone or a fusion protein of GST and Itch (GST-Itch) with the lysate of colon tissue from Itch $^{-/-}$ mice (**Figure 2a**). The precipitated proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to mass spectrometry (MS) analysis following in-gel digestion with trypsin. By this approach, we identified several potential Itch binding proteins in precipitates of GST-Itch. Since the WW domains of Itch bind to the proline-rich PPXY motifs on its target proteins, we short-listed the PPXY motif-containing proteins from the list of proteins identified in the MS analysis (**Figure 2b**). HIC-5 was one of the potential Itch-interacting proteins that contained the PPXY motif. A representative MS/MS spectrum corresponding to ^{170}VQNHLPASGPPQPPAASPTR^{189} of the HIC-5 peptide sequence is shown in **Figure 3a**.

Since HIC-5 was shown to be involved in myofibroblast differentiation and fibrosis,[14,16,22–24] we investigated if Itch-mediated ubiquitination regulates colonic fibrosis. For this, we transiently transfected 293T cells with Flag-tagged HIC-5 (Flag-HIC-5) and Myc-tagged Itch (Myc-Itch). We immunoprecipitated proteins from cell lysates with control mouse immunoglobulin G (IgG), antibody to Flag (anti-Flag), or antibody to Myc (anti-Myc). Anti-Myc immunoprecipitated Flag-HIC-5 and anti-Flag immunoprecipitated Myc-Itch, which suggests that Itch interacts with HIC-5 (**Figure 3b**). Furthermore, we performed a pull-down assay using the lysates of 293T cells transfected with Flag-HIC-5 and bacterially expressed purified GST-Itch. GST-Itch but not GST alone precipitated Flag-HIC-5 (**Figure 3c**), confirming the Itch/HIC-5 interaction. To determine whether this interaction occurs endogenously in the colonic tissue, we performed co-immunoprecipitation experiments with mouse embryonic fibroblasts (MEFs) from Itch $^{+/+}$ mice. We found that Itch co-immunoprecipitated with HIC-5 and vice versa (**Figure 3d**). To test if this interaction occurs through WW-PPXY motifs, we generated a HIC-5 mutant (Flag-ΔHIC-5) in which the PPXY motif was deleted. We then transiently transfected 293T cells with Flag-HIC-5, Flag-ΔHIC-5 and Myc-Itch, and immunoprecipitated the cell lysates with anti-Flag. The immunoprecipitates were blotted with anti-Myc and then

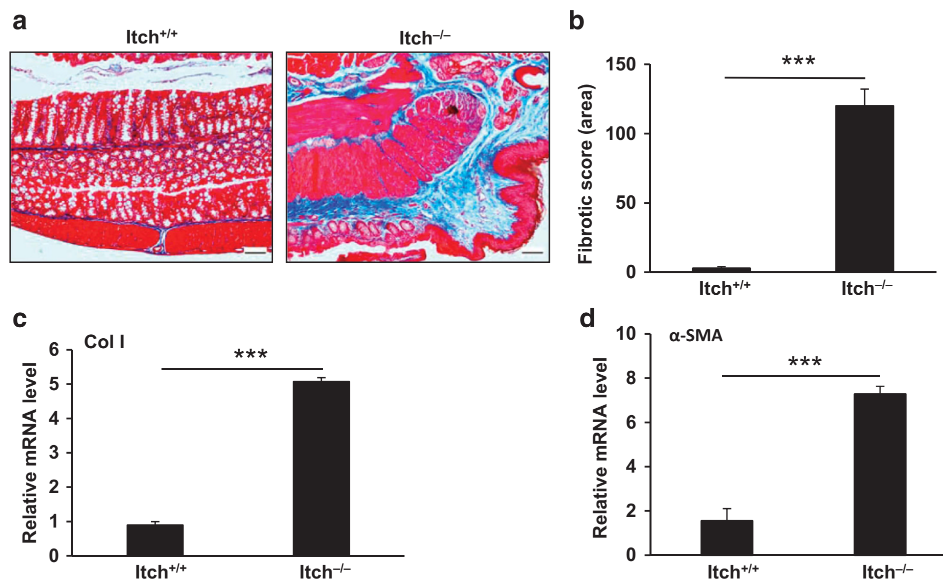

Figure 1 Severe fibrosis exists in the colon of Itch $^{-/-}$ mice. **(a)** Masson trichrome staining of collagen deposition in tissue sections of old Itch $^{+/+}$ and Itch $^{-/-}$ mouse colons. Collagen was stained blue against a red background. (**b**) Fibrotic scores for sections in part **a**. **(c, d)** Relative mRNA levels of (**c**) Col I and (**d**) α-SMA from old Itch $^{+/+}$ and Itch $^{-/-}$ colon tissues. The data are representative of three or more independent experiments. *** $P<0.0001$ based on paired two-tailed Student's *t*-test; the other values are not significant ($P>0.01$).

reprobed with anti-Flag. As shown in **Supplementary Figure 2a**, we found that HIC-5 and Itch were coprecipitated but the deletion of the PPXY motifs in the HIC-5 protein completely disrupted the Itch-HIC-5 interaction. Furthermore, deletion of all four of the WW domains on Itch (Myc-ΔItch) disrupted the Itch-HIC-5 interaction

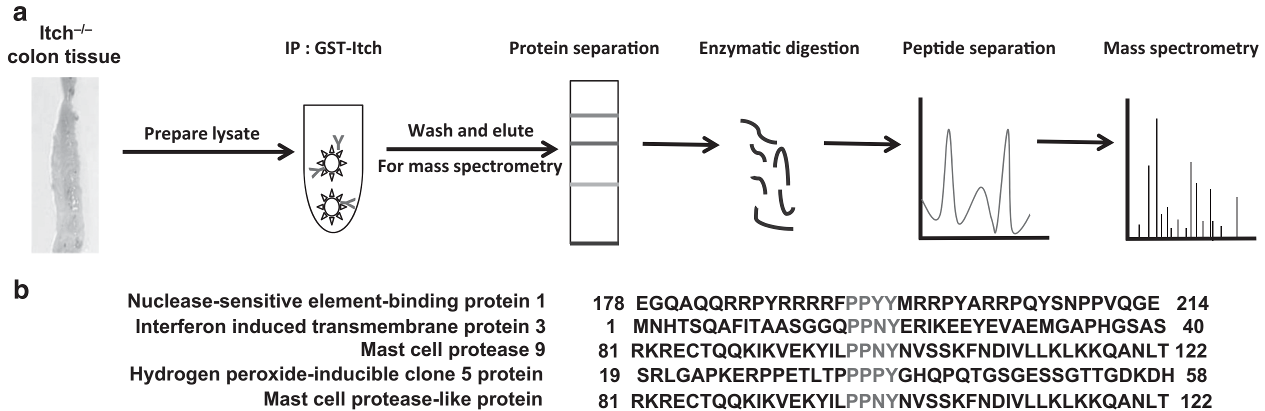

Figure 2 Itch-interacting proteins can be identified through MS analysis. (**a**) Schematic presentation of the MS analysis. (**b**) Proteins containing a PPXY motif identified in MS/MS analysis. A full color version of this figure is available at the *Mucosal Immunology* journal online.

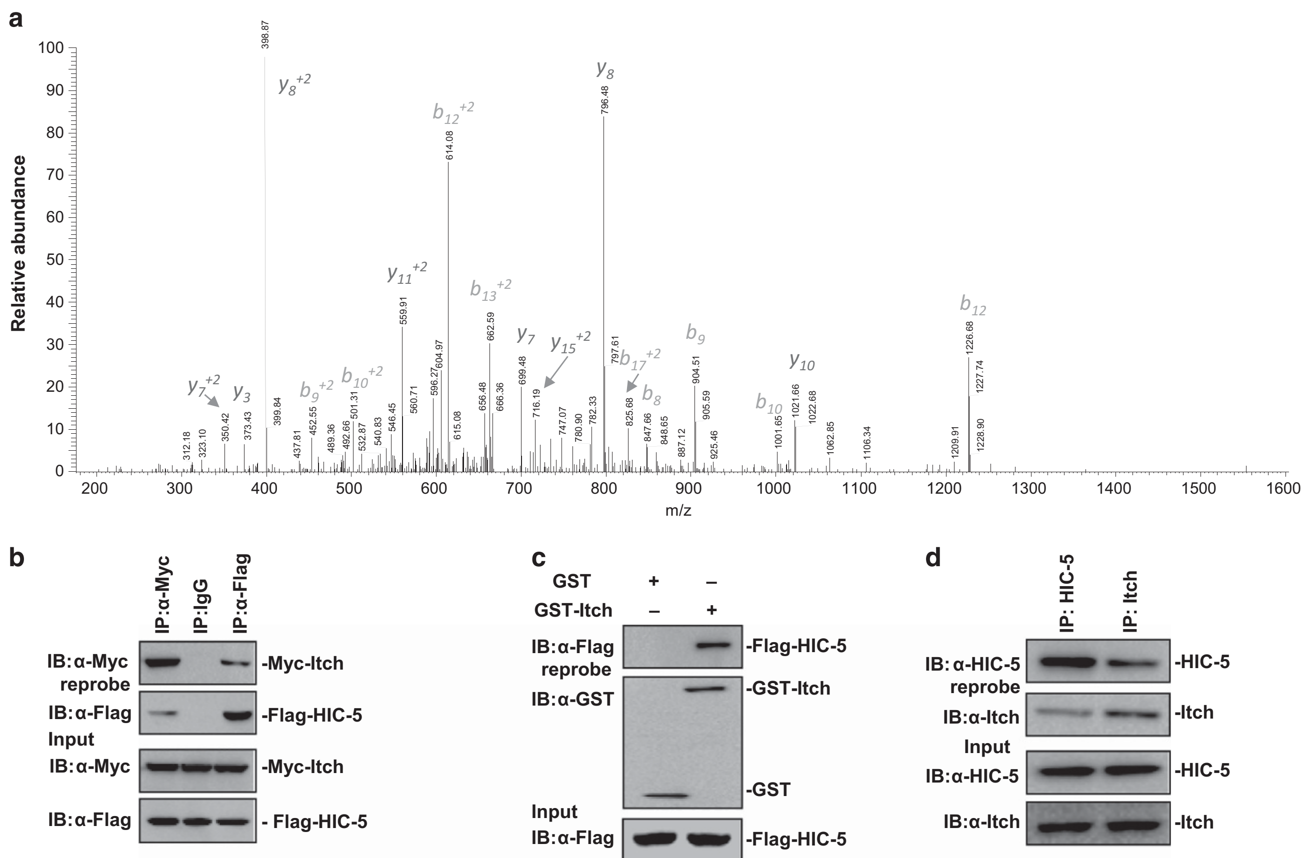

Figure 3 Itch interacts with HIC-5. (**a**) Itch $^{-/-}$ colon lysate was subjected to pull-down assay with either recombinant GST or GST-Itch. The precipitated proteins were subjected to SDS-PAGE and in-gel digestion. The resulting peptides were analyzed by high-resolution MS/MS. HIC-5 (SwissProt accession No. Q62219) was identified as a specific interactor of Itch protein. An MS/MS spectrum of the peptide 170VQNHLPASGPPQPPAASPTR189 ($[M+H]+3=674.69$ m/z) belonging to HIC-5 is shown. Observed b- and y-ions are indicated. (**b**) Immunoassay of lysates of 293T cells transfected with plasmids encoding Flag-HIC-5 and Myc-Itch. This was followed by immunoprecipitation (IP) with anti-Myc, anti-Flag, or the control antibody IgG (above blots) and immunoblot analysis (IB) with anti-Myc or anti-Flag (left margin). The Input rows show immunoblot analysis of the samples without immunoprecipitation (throughout). (**c**) Immunoassay of lysates from 293T cells transfected with Flag-HIC-5 and precipitated with GST or GST-Itch purified from *E. coli.* Reprobe blot shows GST and GST-Itch, and input shows Flag-HIC-5. (**d**) Immunoassay of lysates of Itch $^{+/+}$ colonic MEFs subjected to immunoprecipitation with anti-HIC-5 or anti-Itch and immunoblot analysis with anti-HIC-5 or anti-Itch. The data are representative of three or more independent experiments. A full color version of this figure is available at the *Mucosal Immunology* journal online.

(**Supplementary Figure 2b**). Taken together, these data suggest that Itch associates with HIC-5 through the 'WW-PPXY' motifs.

Itch targets HIC-5 for K63-linked ubiquitination

Next, we investigated whether Itch targets HIC-5 for ubiquitination to regulate fibrosis. We transiently transfected 293T cells to express Flag-HIC-5, Myc-Itch, and hemagglutinin (HA)-tagged ubiquitin and collected cells 36 h after transfection. We immunoprecipitated Flag-HIC-5 with anti-Flag and probed the membranes with anti-HA. We observed high-molecular-weight polyubiquitinated forms of HIC-5 that migrated slowly (**Figure 4a**). This finding suggests that Itch targeted HIC-5 for ubiquitination. To confirm the specificity of Itch as the E3 ligase, we generated an Itch mutant with a substitution of alanine for the cysteine residue at position 830 (Itch-C830A).[25] Expression of Itch-C830A with WT ubiquitin did not lead to ubiquitination of HIC-5 (**Figure 4a**).

Since Itch targets its substrates for polyubiquitination via Lys27 (K27)-, K29-, K33-, K48-, and K63-linked ubiquitin chains,[26–30] we sought to identify the linkage of the Itch-mediated ubiquitination of HIC-5 by using the ubiquitin expression constructs HA-Ub-K48, HA-Ub-K63, HA-Ub-K6, HA-Ub-K0, HA-Ub-K11, HA-Ub-K27, HA-Ub-K29, and HA-Ub-K33 (in which all of the lysine residues except K48, K63, K6, K0, K11, K27, K29, and K33, respectively, are replaced). As shown in **Figure 4b** and **Supplementary Figure 3**, Itch predominantly targeted HIC-5 for K63-linked ubiquitination. To further confirm that Itch targets HIC-5 for K63-linked ubiquitination, we used a ubiquitin mutant with a substitution

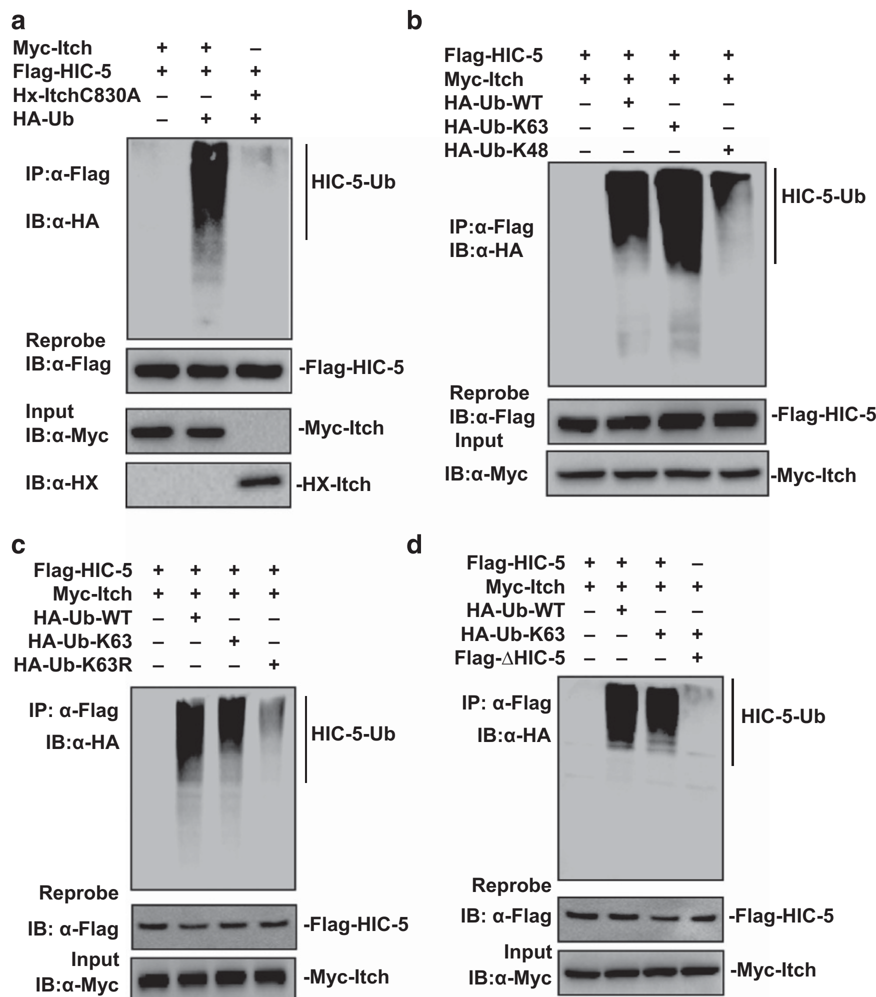

Figure 4 Itch targets HIC-5 via K63-linked ubiquitination. (**a**) Immunoassay of lysates of 293T cell transfected for 36 h with various combinations (above lanes) of plasmids encoding Myc-Itch, X-press (HX)-tagged Itch-C830A (HX-ItchC830A), Flag-HIC-5 and HA-tagged WT ubiquitin (HA-Ub WT). This was followed by immunoprecipitation with anti-Flag, immunoblot analysis with anti-HA, and reprobed with anti-Flag. (**b**) Immunoassay of 293T cells transfected with expression vectors for various combinations (above lanes) of Flag-HIC-5, Myc-Itch, (HA-Ub WT), Ub (K48) and Ub (K63), followed by immunoprecipitation of lysates with anti-Flag and immunoblot analysis with anti-HA. (**c**) Immunoassay of 293T cells transfected with expression vectors for various combinations (above lanes) of Flag-HIC-5, Myc-Itch, HA-Ub WT, Ub (K63), and Ub (K63R), followed by immunoprecipitation of lysates with anti-Flag and immunoblot analysis with anti-HA. (**d**) Immunoassay of 293T cells transfected with expression vectors for various combinations (above lanes) of Flag-HIC-5, Myc-Itch, Flag-ΔHIC-5, HA-Ub WT and Ub (K63), followed by immunoprecipitation of lysates with anti-Flag and immunoblot analysis with anti-HA. The data are representative of three or more independent experiments.

of arginine for the lysine residue at position 63 (Ub (K63R)). Co-expression of Ub (K63R) and Itch did not ubiquitinate HIC-5 (**Figure 4c**). To investigate whether the Itch-HIC-5 interaction is required for HIC-5 ubiquitination, we used the Flag-ΔHIC-5 mutant, which fails to interact with Itch. The Flag-ΔHIC-5 mutant was not ubiquitinated by Itch (**Figure 4d**), which suggests that the Itch-HIC-5 interaction was essential for Itch-mediated ubiquitination of HIC-5.

Itch negatively regulates IL-17-induced HIC-5 in myofibroblasts by lysosomal degradation

We have recently reported elevated expression of IL-17 in the colonic mucosa of Itch $^{-/-}$ mice. Since IL-17A has been shown to induce fibrosis in different organs by inducing profibrotic gene expression,[9,31,32] we tested if IL-17 regulates HIC-5 expression. We cultured MEFs from the colon tissues of Itch $^{+/+}$ mice. We then treated the cells with recombinant IL-17 and analyzed the expression of HIC-5 by real-time PCR and western blot. As shown in **Figure 5a** and **b**, IL-17 induced HIC-5 expression in MEFs.

Next, we tested if Itch regulates IL-17-induced HIC-5 in MEFs. We treated Itch $^{+/+}$ and Itch $^{-/-}$ MEFs with IL-17 and analyzed HIC-5 expression by immunoblotting. As shown in **Figure 5c**, we observed substantially elevated levels of HIC-5 in Itch $^{-/-}$ MEFs. To investigate the possibility of increased transcription of HIC-5 in Itch $^{-/-}$ MEFs, we performed real-time PCR experiments. However, no significant difference in HIC-5 transcription was observed between WT and Itch $^{-/-}$ cells (**Figure 5d**). Similar results were obtained when we analyzed HIC-5 levels following treatment with TGF-β (**Figure 5e** and **f**), which suggests that Itch regulates HIC-5 protein turnover.

Th2-biased differentiation was reported earlier in Itch $^{-/-}$ T cells. Since IL-13, a Th2 cytokine, is a major factor in colonic fibrosis,[33] we investigated the potential role of IL-13 in a polysaccharide dextran sodium sulfate (DSS)-induced colitis model in Itch $^{-/-}$ mice. We included 2.5% DSS in the drinking water of 6- to-10-week-old Itch $^{+/+}$ and Itch $^{-/-}$ mice. On day 8, we collected colonic mucosa and analyzed the expression of IL-13 by real-time PCR. As shown in **Supplementary Figure 4a**, DSS treatment induced IL-13 expression in both Itch $^{+/+}$ and Itch $^{-/-}$ mice, and the level of IL-13 was comparable in both sets of colons. Similarly, the expression of Tl1a, another well-documented regulator of colonic inflammation,[33] was not significantly altered in Itch $^{-/-}$ colonic mucosa in a similar experiment (**Supplementary Figure 4b**). This suggested that IL-13 and Tl1a did not play a major role in inflammation and fibrosis in Itch $^{-/-}$ mice.

To investigate the mechanism by which Itch regulates HIC-5 turnover, we treated WT MEFs with IL-17 and cycloheximide (CHX, a protein synthesis inhibitor), in combination with either chloroquine (a lysosomal inhibitor) or MG132 (a proteasomal inhibitor). As shown in **Figure 5g**, lanes 1 and 2, CHX treatment resulted in a substantially reduced level of HIC-5. Chloroquine but not MG132 prevented this effect of CHX on HIC-5 protein level. These results suggest that HIC-5 is degraded via the lysosomal pathway. To test if a defect in the degradation of HIC-5 results in increased abundance of HIC-5 in colonic mucosa of Itch $^{-/-}$ mice, we performed immunoblotting experiments. As shown in **Supplementary Figure 5**, we found increased HIC-5 in the colons of Itch $^{-/-}$ mice.

Inhibition of HIC-5 in Itch $^{-/-}$ mice decreases fibrotic gene expression

Next, we tested if inhibition of HIC-5 normalizes the expression of Col I and α-SMA by Itch $^{-/-}$ MEFs. We knocked down HIC-5 in Itch $^{-/-}$ MEFs using shRNA (**Figure 6a**), and the cells were then stimulated with IL-17. As shown in **Figure 6b**, knocking down HIC-5 substantially reduced Col I and α-SMA expression in the Itch $^{-/-}$ cells.

To further confirm that Itch-mediated HIC-5 ubiquitination regulates fibrosis, we reconstituted Itch $^{-/-}$ cells with either WT Itch or the Itch-C830A mutant (which lacks the ligase activity) and then stimulated with IL-17. As shown in **Figure 6c**, reconstitution of WT but not the Itch-C830A mutant inhibited Col I and α-SMA expression. Taken together, these data suggest that Itch regulates the expression of fibrosis-causing genes via ubiquitination of HIC-5.

Finally, we tested if attenuating the IL-17 response in Itch $^{-/-}$ mice prevented colonic fibrosis. For this, we utilized Itch $^{-/-}$ Rorc $^{-/-}$ (dKO) mice.[8] As shown in **Figure 7a**, Itch $^{-/-}$ Rorc $^{-/-}$ (dKO) mice were completely rescued from rectal prolapse and colonic fibrosis compared to Itch $^{-/-}$ mice (**Figure 7b** and **c**). The expression of Col I and α-SMA in Itch $^{-/-}$ Rorc $^{-/-}$ mice was significantly reduced compared to that in the Itch $^{-/-}$ mice (**Figure 7d** and **e**). Collectively, these results suggest that Itch plays an essential role in inhibiting colonic fibrosis.

HIC-5 in the colonic mucosa of ulcerative colitis patients

To investigate if a defect in Itch-mediated ubiquitination contributes to fibrosis in UC patients, we analyzed HIC-5 expression by immunoblotting the colonic mucosal tissue of control and UC patient samples. In the majority of the samples that we analyzed, the HIC-5 level was higher in the mucosa of UC patients (**Supplementary Figure 6a**). Next, we tested if Itch expression is reduced in UC patients and as a result causes the accumulation of HIC-5. Therefore, we reprobed the membranes with anti-Itch antibody. However, no significant change in Itch expression was observed between control and UC patient samples (**Supplementary Figure 6a**). Next, we investigated if Itch function is defective in UC patients. We immunoprecipitated Itch from both the normal control and UC patient samples, and tested the E3 ligase activity by an *in vitro* ubiquitination assay. As shown in **Supplementary Figure 6b**, the ligase activity of Itch protein precipitated from UC patients was substantially reduced, suggesting the possibility of a defect in Itch function in UC patients' mucosa.

DISCUSSION

Current therapies such as aminosalicylate, steroid and immunomodulator therapies, and anti-TNF-α monoclonal antibodies relieve inflammatory symptoms of IBD. However,

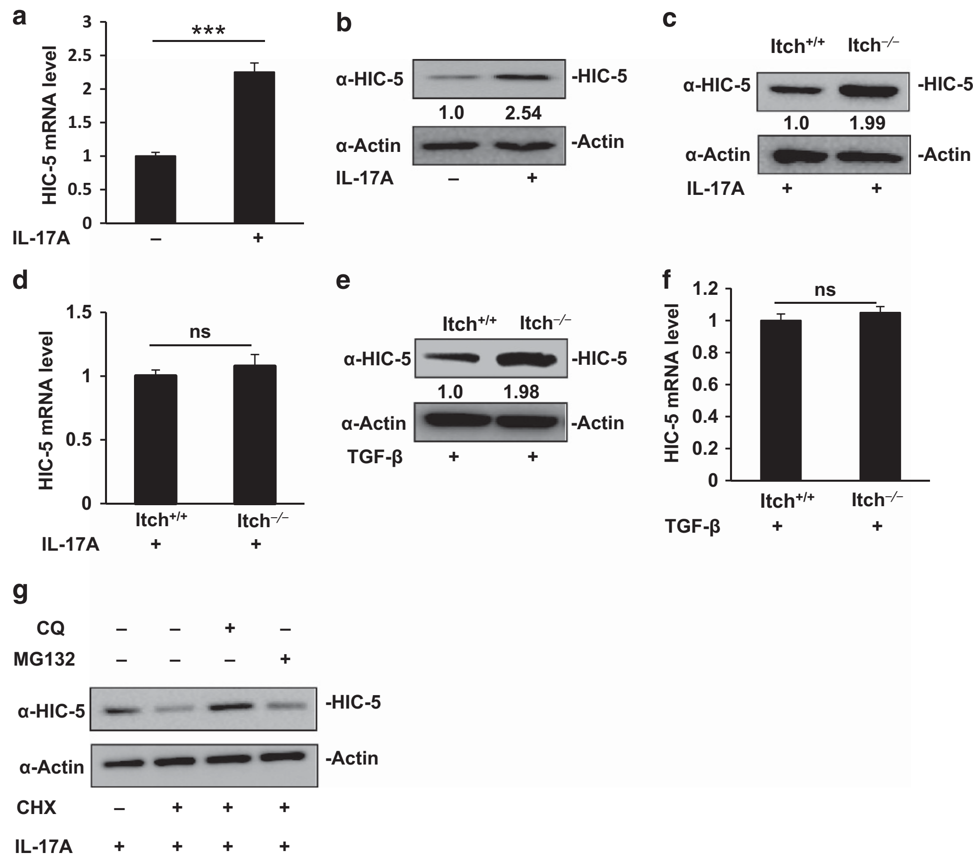

Figure 5 Itch negatively regulates IL-17–induced HIC-5 expression via lysosomal degradation. Itch $^{+/+}$ MEFs were either treated with 30 ng/ml of recombinant murine IL-17A (rIL-17A) or left untreated for 24 h. (**a**) Relative mRNA level of HIC-5 was measured in Itch $^{+/+}$ MEFs. The relative fold change in the mRNA levels of the gene has been normalized against untreated control of the respective Itch $^{+/+}$ MEFs. (**b**) Immunoblot analysis of HIC-5 protein level in lysates of Itch $^{+/+}$ MEFs. Itch $^{+/+}$ and Itch $^{-/-}$ MEFs were treated with 30 ng/ml of rIL-17A for 24 h. (**c**) Immunoblot analysis of HIC-5 protein level in lysates of Itch $^{+/+}$ and Itch $^{-/-}$ MEFs after rIL-17A treatment. (**d**) Relative mRNA level of HIC-5 was measured in Itch $^{+/+}$ and Itch $^{-/-}$ MEFs after rIL-17A treatment. Itch $^{+/+}$ and Itch $^{-/-}$ MEFs were treated with 10 ng/ml of recombinant TGF-β (rTGF-β) for 24 h. (**e**) Immunoblot analysis of HIC-5 protein level and (**f**) relative mRNA level of HIC-5 were measured in lysates of Itch $^{+/+}$ and Itch $^{-/-}$ MEFs after rTGF-β treatment. The relative fold change in the mRNA levels of the gene has been normalized against the untreated control of the respective Itch $^{+/+}$ MEFs. (**g**) Itch $^{+/+}$ MEFs were treated with 30 ng/ml of rIL-17 A for 24 h and further treated with 100 μg/ml of CHX for 6 h. Simultaneously, CHX-treated Itch $^{+/+}$ MEFs were also treated either with the lysosomal inhibitor chloroquine (50 μM) or with the proteasome inhibitor MG132 (5 μM) for 6 h, and the endogenous protein level of HIC-5 (top) was measured by immunoblot analysis. Untreated Itch $^{+/+}$ MEFs were used as a control. The data are representative of three or more independent experiments. *** $P<0.0001$, and the other values are not significant (ns; $>P<0.01$) using paired two-tailed Student's *t*-test. For all immunoblot analyses, β-actin was used as a loading control. Protein levels were normalized to β-actin and expressed as fold changes (mentioned below the blot) compared with WT controls.

these treatment strategies do not significantly improve fibrotic stricture lesions.[34,35] Therefore, intestinal fibrosis and its associated complications remain the major causes of surgical intervention in IBD patients.[35] The molecular mechanisms driving a profibrotic phenotype of the disease remain largely unknown. Currently, no criteria exist to stratify IBD patients into at-risk populations for developing fibrosis. In this report, we have uncovered a novel mechanism by which the E3 ligase Itch inhibits colonic fibrosis by attenuating the expression of profibrotic genes in myofibroblasts, which could be exploited therapeutically to inhibit intestinal fibrosis.

In contrast to the intensive investigation of the immunological mechanisms of intestinal inflammation in IBD, the pathophysiology of fibrosis has remained largely unexplored. Using a mouse model of spontaneous fibrosis, we show that Itch inhibits the expression of profibrotic Col I and α-SMA. Mechanistically, we demonstrate that Itch and HIC-5 interact through their conserved WW motif and PPXY motif, respectively, and that Itch targets HIC-5 for ubiquitination and subsequent proteolysis. Our results are consistent with a report that deletion of HIC-5 attenuated liver fibrosis[14] and also attenuated myofibroblast differentiation.[16]

HIC-5 (also called TGF-β1l1) was originally identified as TGF-β and H_2O_2-induced gene.[17] Our results show that HIC-5 expression is also induced by IL-17, suggesting a new role for HIC-5 downstream of IL-17 receptor signaling. IL-17 receptor signals by the recruitment of the adaptor Act1.[36] Act1 in turn recruits and ubiquitinates TRAF6, activating downstream NF-κB, CCAAT/enhancer-binding protein, and the mitogen-activated protein kinase pathway.[36] It was shown that IL-17 receptor signaling is negatively regulated by deubiquitinases A20 and USP25.[37] Our results show that Itch ubiquitinates a

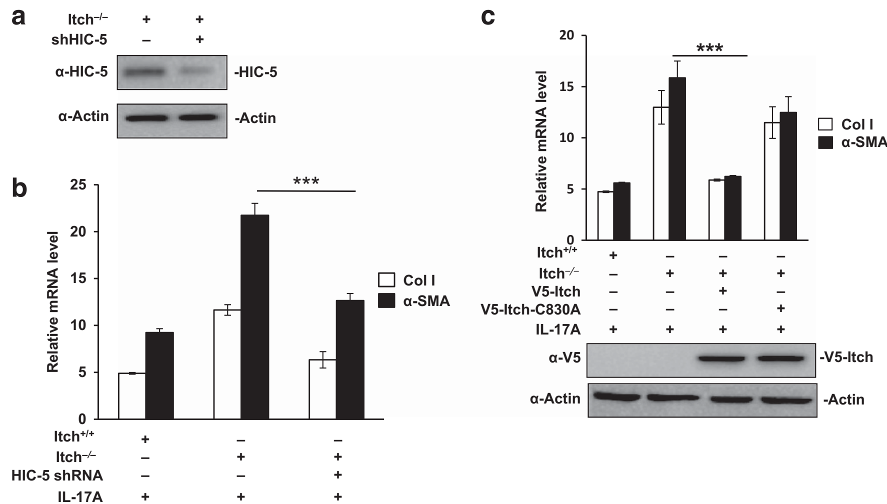

Figure 6 Inhibition of HIC-5 in Itch $^{-/-}$ mice decreases fibrotic gene expression. (**a**) Itch $^{-/-}$ MEFs were treated with HIC-5-specific shRNA (shHIC-5), and immunoblot analysis was done to assess knockdown of HIC-5 in those cells. (**b**) Real-time PCR analysis of Col I and α-SMA in Itch $^{+/+}$ and Itch $^{-/-}$ MEFs treated with shHIC-5 and then treated with 30 ng/ml of rIL-17A for 24 h. (**c**) Real-time PCR analysis of Col I and α-SMA in Itch $^{+/+}$ and Itch $^{-/-}$ MEFs transduced with lentivirus encoding V5-tagged WT Itch or Itch-C830A and treated with 30 ng/ml of rIL-17A for 24 h (top). Immunoblot analysis of lentiviral constructs (bottom). β-actin was used as a loading control. The relative fold change in the mRNA levels of the genes has been normalized against untreated control of the respective Itch $^{+/+}$ MEFs. The data are representative of three or more independent experiments. ***$P<0.0001$ based on paired two-tailed Student's *t*-test; the other values are not significant ($P>0.01$).

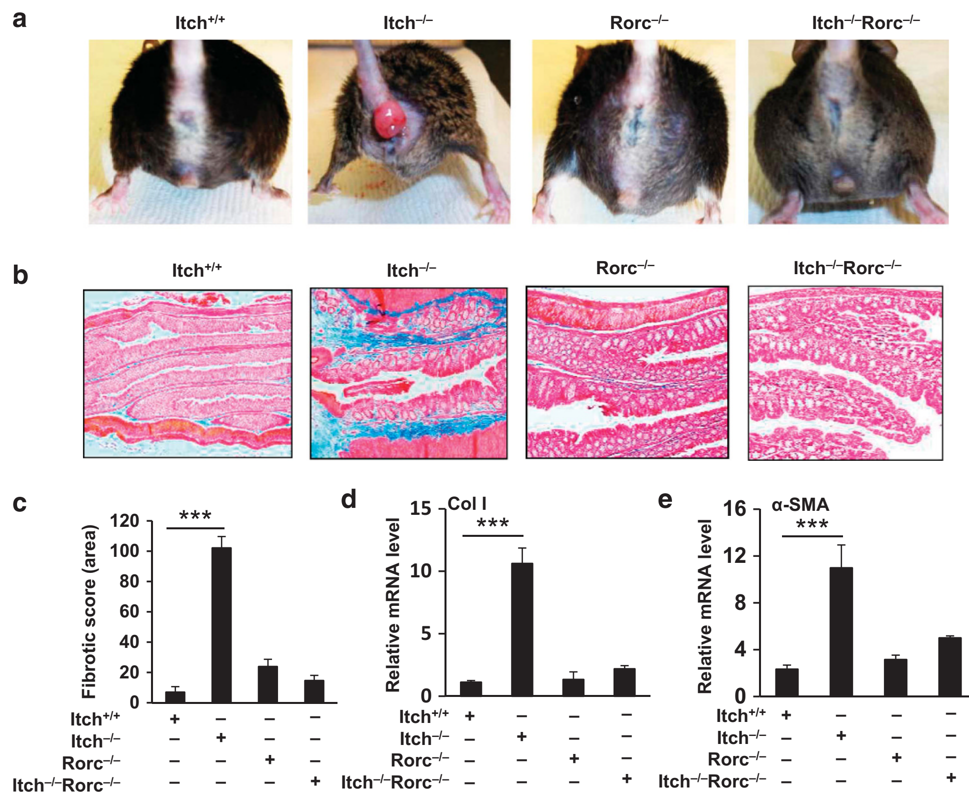

Figure 7 Impeding IL-17 response attenuates colonic fibrosis in Itch $^{-/-}$ mice. (**a**) Representative images of spontaneous rectal prolapse in old Itch $^{+/+}$, Itch $^{-/-}$, Rorc $^{-/-}$, and Itch $^{-/-}$ Rorc $^{-/-}$ mice. (**b**) Masson trichrome staining of collagen deposition in tissue sections of old Itch $^{+/+}$, Itch $^{-/-}$, Rorc $^{-/-}$, and Itch $^{-/-}$ Rorc $^{-/-}$ mice. (**c**) Fibrotic scores for sections from part (**b**). (**d,e**) Relative mRNA levels of (**d**) Col I and (**e**) α-SMA from old Itch $^{+/+}$, Itch $^{-/-}$, Rorc $^{-/-}$ and Itch $^{-/-}$ Rorc $^{-/-}$ colon tissue. The relative fold change in the mRNA levels of the genes has been normalized against Itch $^{+/+}$ control colon tissue. The data are representative of three or more independent experiments. ***$P<0.0001$ based on paired two-tailed Student's *t*-test; the other values are not significant ($P>0.01$).

downstream effector of IL-17 and negatively regulates IL-17–triggered fibrosis. Thus, our findings uncover a novel mechanism by which the IL-17-mediated pathology is inhibited by Itch.

Analysis of Itch expression in the mucosa of UC patients did not show gross change compared to healthy controls. This is consistent with a previous report that Itch expression was normal in UC patients' peripheral blood lymphocytes.[38] However, we observed that Itch protein isolated from UC patients had reduced ligase activity. The underlying mechanism currently remains unclear. It was demonstrated before that Itch ligase activity is regulated by JNK1-mediated phosphorylation.[39] Such phosphorylation-dependent regulation of Parkin by the tyrosine kinase c-Abl has been reported in Parkinson disease patients.[40] Additionally, Itch function was shown to be regulated by the adaptor protein NDFIP1.[41] Further studies investigating if these forms of regulation are altered in UC patients is required to fully understand the role of Itch in gut inflammation and fibrosis.

In addition to IL-17, Itch also regulates TGF-β-induced HIC-5 expression. Further studies on the physiological function of Itch-mediated regulation of TGF-β-induced fibrosis are needed. In addition to TGF-β and IL-17, additional mediators such as IL-13 and Tl1a are critically involved in fibrosis.[33] However, Itch does not seem to play any significant role in these pathways. Since Itch regulates multiple pathways in hematopoietic and nonhematopoietic cells, we cannot completely exclude the contribution of other cell types and additional mechanisms. Further detailed analysis using tissue/cell-specific conditional deletion of Itch will lead to a more complete understanding of how Itch regulates gut fibrosis. Nevertheless, our studies have uncovered a novel mechanism by which profibrotic gene expression in the colon is inhibited, which could be exploited therapeutically.

METHODS

Mice. Itch$^{-/-}$ mice were described previously.[25] Itch$^{-/-}$Rorc$^{-/-}$ mice were generated by crossing Itch$^{-/-}$ with Rorc$^{tm1Litt/J}$ mice.[21] All mice were housed in micro-isolator cages in the barrier facility of Baylor Institute for Immunology Research. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Baylor Research Institute.

Human tissue samples. Colonic surgical specimens from patients with UC who were admitted to Baylor University Medical Center for therapeutic bowel resection were collected. This study was approved by the Institutional Review Board of Baylor Research Institute.

Antibodies and reagents. The following antibodies were used in this study: anti-c-Myc (Santa Cruz, Dallas, TX), anti-Flag (Sigma, St. Louis, MO), anti-β-actin (Sigma), anti-HIC-5 (Santa Cruz), X-press (Invitrogen, San Diego, CA), anti-hemagglutinin (Santa Cruz), anti-Ub (Santa Cruz), anti-Itch (BD Biosciences, San Jose, CA), GST antibody (Santa Cruz), normal rabbit IgG (Santa Cruz), and normal mouse IgG (Santa Cruz). Recombinant murine IL-17 and recombinant murine TGF-β were purchased from PeproTech (Rocky Hill, NJ). HIC-5 shRNA (m) lentiviral particles, control shRNA lentiviral particles-A, polybrene, and puromycin dihydrochloride were purchased from Santa Cruz Biotechnology. Cycloheximide and chloroquine diphosphate were purchased from Sigma.

Cell culture. 293T cells were cultured in DMEM supplemented with 10% (vol/vol) FBS. Primary mouse colonic MEFs were prepared from 10-to-12-week-old mice as previously described.[42]

Protein identification by liquid chromatography (LC)-tandem MS. Immunoprecipitated proteins were separated by SDS-PAGE. In-gel digestion with trypsin, followed by protein identification using LC-tandem MS, was performed as described elsewhere.[43] Briefly, tryptic peptides were resolved on a nano-LC column (Magic AQ C18; Michrom Bioresources, Auburn, CA) and introduced into an Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). The Orbitrap was set to collect a high-resolution MS1 (FWHM 30,000@400 m/z), followed by the data-dependent collision-induced dissociation spectra on the "top 9" ions in the linear ion trap. Spectra were searched against a human protein database (UniProt release 2011_05) using the X!Tandem/TPP software suite.[44] Proteins identified with a ProteinProphet probability ≥ 0.9 false discovery rate $< 2\%$ were considered for further analysis.

Plasmid construction and cell transfection. Myc-Itch, HA-Ub (WT), Ub(K63), Ub(K48), GST-Itch, and HX-Itch (C830A) were described previously.[25,26,45] Flag-mHIC-5 and Flag-mHIC-5-ΔPPPPY were created from pMSCV mouse HIC-5 (Addgene, Cambridge, MA) and cloned into pCMV-Tag2B between the BamHI and XhoI sites. Transient transfection of 293T cells was performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. HIC-5 shRNA-mediated inhibition in Itch$^{-/-}$ MEFs was done according to the manufacturer's protocol (Santa Cruz Biotechnology).

Real-time PCR analysis. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA), followed by cDNA synthesis using a Verso cDNA Kit (Thermo Scientific). Quantitative real-time PCR was performed on a Mastercycler EP Realplex (Eppendorf). LightCycler 480 SYBR Green I Master Reaction Mix (Roche, Pleasanton, CA) was used in a 20 μl reaction volume. The expression of individual genes was normalized to the expression of actin. Cycling conditions were 95 °C for 2 min, followed by 50 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. The primer sequences for the genes are as follows:

Col I forward primer: 5′-TGCCGTGACCTCAAGATGTG-3′.
Col I reverse primer: 5′-CACAAGCGTGCTGTAGGTGA-3′.
α-SMA forward primer: 5′-GTGCTATGTCGCTCTGGACTTTGA-3′.
α-SMA reverse primer: 5′-ATGAAAGATGGCTGGAAGAGGGTC-3′.
HIC-5 forward primer: 5′-GTGCAGGGAATGCCTTGCGC-3′.
HIC-5 reverse primer: 5′-GTGCAGGTGAAGTGGTCTGGATC-3′.
IL-13 forward primer: 5′-GCAGCATGGTATGGAGTGTG-3′.
IL-13 reverse primer: 5′-TGGCGAAACAGTTGCTTTGT-3′.
Tl1a forward primer: 5′-GCTGCCTGTTGTCATTTCC-3′.
Tl1a reverse primer: 5′-TCTGGGAGGTGAGTAAACTTG-3′.
β-Actin forward primer: 5′-GAAATCGTGCGTGACATCAAAG-3′.
β-Actin reverse primer: 5′-TGTAGTTTCATGGATGCCACAG-3′.

Ubiquitination assay. 293T cells were transfected with Flag-HIC-5 and various constructs, as indicated. MG132 (10 mM) was added 4 h before cell lysis. Cells were lysed in Nonidet P-40 lysis buffer containing 50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, EDTA-free protease inhibitor cocktail (Roche), 2 mM sodium fluoride (Boston Bioproducts, Ashland, OR), 10 mM β-glycerophosphate (Boston Bioproducts), and 1 mM sodium orthovanadate (Sigma). Immunoprecipitation was performed using anti-Flag. HIC-5-associated Ub was analyzed by immunoblot using anti-HA, as described previously.[25,26,45] An *in vitro* ubiquitination assay was performed using the E2-Ubiquitin Conjugation Kit (Abcam, Cambridge, MA) with GST-HIC-5 protein as a substrate and Itch immunoprecipitated from either control and UC patients' tissue sample as the E3 ligase according to the manufacturer's protocol. GST-HIC-5 protein was prepared as described previously.[25]

Immunoprecipitation and immunoblot analysis. Cell lysates was prepared from colonic tissues of Itch$^{+/+}$ and Itch$^{-/-}$ mice after

homogenizing in NP-40 lysis buffer. Protein estimations were done using the Pierce BCA protein assay kit according to the manufacturer's protocol. Whole-cell lysates were precleared with 20 μl of Protein A/G plus agarose beads (Santa Cruz) for 1 h at 4 °C. Lysates were then incubated with 1 μg of the desired antibody overnight at 4 °C followed by a further 1 h of incubation at 4 °C with 25 μl of Protein A/G beads. The immunocomplexes were washed five times with lysis buffer and denatured using 4 × Laemmli buffer. Further, they were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Blots were visualized using Amersham ECL plus immunoblot analysis detection system (GE, Chicago, IL) on a Biorad ChemiDoc. For reprobing, membranes were stripped by incubation in a stripping buffer (62.5 mM Tris-HCl, pH 6.7; 100 mM 2-mercaptoethanol and 2% SDS) at 55 °C for 45 min and washed thoroughly before reprobing.

Histology and trichrome staining. Six-micrometer cryosections were fixed for 5 min in acetone and stained with a Masson trichrome staining kit (Sigma-Aldrich, St. Louis, MO) for detection of collagen deposition. Collagen deposition was quantified on trichrome-stained sections using ImageJ software (NIH, Bethesda, MD) on five fields/section.

Statistical analysis. The data were analyzed with GraphPad Prism 4 software (La Jolla, CA) to determine statistical significance using paired Student's *t*-test. The data are expressed as mean ± SD. A P-value < 0.05 was considered significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS
J.P., A.K.S., M.K., T.L.E., and M.Z. performed the experiments, analyzed the data, and assisted in manuscript preparation. V.B. performed MS analysis; A.T. assisted in manuscript preparation; and K.V. conceived the project, designed the experiments, and wrote the manuscript.

DISCLOSURE
The authors declare no conflict of interest.

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