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Hypoxia Induced Ubc9 promoter hyper-methylation regulates IL-17 expression in ulcerative colitis

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Summary

Dysregulated IL-17 expression is central to the pathogenesis of several inflammatory disorders including ulcerative colitis (UC). We have shown earlier that sumoylation of ROR-γt, the transcription factor for IL-17, regulates colonic inflammation. Here, we show that the expression of Ubc9, the E2 enzyme that targets ROR-γt for sumoylation is significantly reduced in the colonic mucosa of UC patients. Mechanistically, we demonstrate that hypoxia-inducible factor 1α (HIF-1α) binds to a CpG island within the Ubc9 gene promoter resulting in its hyper methylation and reduced Ubc9 expression. CRISPR-Cas9-mediated inhibition of HIF-1α normalized Ubc9 and attenuated IL-17 expression in Th17 cells and reduced diseases severity in Rag1$^{-/-}$ mice upon adoptive transfer. Collectively, our study reveals a novel epigenetic mechanism of regulation of ROR-γt which could be exploited in inflammatory diseases.

Introduction

Inflammatory bowel diseases (IBDs) are the chronic relapsing inflammatory disease of the gastrointestinal tract that includes ulcerative colitis (UC) and Crohn's disease (CD). Emerging evidence suggests a crucial role of IL-17 in gastrointestinal inflammation that is produced by Th17 cells, γδT cells and innate lymphoid cells (ILCs) (1). The orphan nuclear receptor ROR-γt is the major transcription factor that binds to ROR response elements within the IL-17a promoter to induce IL-17a transcription (2). However, the molecular mechanisms by which the expression of ROR-γt is regulated to prevent unrestricted inflammation remain unclear.

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Post-translational modification by small ubiquitin-like modifier (SUMO) proteins catalyzes a cascade of biochemical reactions that mediates E1, E2, and E3 enzymes. Ubc9 is the E2 enzyme that is used by the SUMO pathway as a conjugation enzyme to transfer SUMO to the substrate proteins (3–5). We have shown earlier that Ubc9 inhibits IL-17 expression in the colonic mucosa via the sumoylation of ROR-γt (6).

Hypoxia is a key feature of inflammatory conditions of the intestine via the transcription factor HIF (hypoxia-inducible factor) which regulates key target genes involved in inflammation (7). In this study, we report that HIF-1α binds to CpG island within the Ubc9 promoter is hypermethylated in the colonic mucosa of UC patients resulting in reduced Ubc9 expression causing elevated IL-17a expression.

Methods

Mice

C57BL/6 mice and Rag1$^{-/-}$ were purchased from Jackson Laboratory. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Baylor Research Institute and UT Southwestern Medical Center.

Antibodies and reagents

Antibodies used in these studies were anti-SUMO1 (1:500; #FL-101, Santa Cruz), anti–ROR-γt (1:800; BD Bioscience), anti-Ubc9 (1:1000, CST), anti-HIF-1α (1:200; BD Biosciences), mouse IgG (Santa Cruz Biotechnology), PE-CD45Rb (Biolegend), APC-CD4 (Biolegend), FITC-CD25 (Tonbo biosciences) and Clean-Blot™ IP detection reagent (HRP) (1:50; Pierce Biotechnology). Dulbecco's modified Eagle's medium (Gibco), fetal bovine serum (Gibco), QIAmp DNA mini kit (Qiagen, USA), EpiTect bisulfite kit (Qiagen, USA), EpiTaq HS polymerase (TaKaRa), ChIP assay kit (Millipore).

Patients and tissue samples

Colon tissue samples were collected from UC patients who underwent a colectomy at the Baylor University Medical Center. The study was approved by the Institutional Review Board of the Baylor Scott & White Research Institute.

Prediction of CpG Island in *Ubc9* gene

The CpG island of *Ubc9* gene was predicted by UCSC genome browser and by the MethPrimer software using an island size of 150 nucleotides, at least 50% GC percentage and observed/expected CpG ratio of 0.6. MethPrimer is used to design the primers for methylation specific PCR (MSP) and unmethylation specific PCR (USP) amplification of the target gene.

Methylation Analysis

Genomic DNA was isolated from the samples using the QIAmp DNA Mini Kit (Qiagen) according to manufacturer's protocol. 2 μg of genomic DNA were modified with sodium bisulfite using EpiTect bisulfite kit (Qiagen). Fragments were amplified using EpiTaq HS (TaKaRa). The primer sequences for the genes shown in supplementary table 1.

Luciferase assay

Jurkat T cells were transfected with HIF-1α and Ubc9 promoter plasmid using Lonza Cell Line Nucleofector kit. Cells were kept on hypoxia incubator chamber (2% O_2). After 24 h of transfection, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml). Lysates were prepared and luminescence was measured.

Adoptive transfer of colitogenic cells in Rag1$^{-/-}$ mice

$CD4^+$ T cells were isolated from the spleen of wild type mice using $CD4^+$ T cell isolation kit (Miltenyi Biotec). The $CD4^+CD25^-CD45RB^{hi}$ cells were FACS sorted and transduced with lenti-CRISPR-HIF-1α viral particles. Transduced cells were cultured in Th17 conditions (8) and then injected intraperitoneally (3×10^5 cells/mice) into Rag1$^{-/-}$ mice and monitored for disease severity up to eight weeks.

Statistical analysis

The data were analyzed with GraphPad Prism 8 software to determine statistical significance using the paired Student's *t*-test. The data are expressed as mean ± SD. A *p*-value < 0.05 was considered significant. *p < 0.05; **p < 0.01; and ***p < 0.001.

Results and discussion

Reduced Ubc9 level leads to decreased SUMOylation of ROR-γt in UC patients

To investigate the potential dysregulation of Ubc9-ROR-γt pathway (6) in UC patients, we tested Ubc9 expression by immunoblotting the surgically resected colon tissue samples from UC patients. As shown in Fig.1A, we observed reduced Ubc9 level in UC patients compared to control samples. Consistent with a previously published report (9), we also observed reduced level of Ubc9 transcripts in UC patient's samples (Supplementary Fig 1). Next, we analyzed the level of *IL-17a* expression in these samples and as shown in Fig 1B, we found a strong inverse correlation (Pearson coefficient, $P = -0.76$) between *Ubc9* mRNA expression and *IL-17a* level.

Next, we investigated if reduced Ubc9 expression resulted in reduced SUMOylation of ROR-γt in UC patients. We immuno-precipitated ROR-γt and analyzed SUMOylation by immunoblotting using anti-SUMO1 antibody. We observed a significant decrease in the SUMOylation of ROR-γt in UC samples (Fig 1C and 1D). These data suggest that reduced Ubc9 expression leads to decreased SUMOylation of ROR-γt in the inflamed colonic mucosa of UC patients.

CpG island hyper methylation within the Ubc9 gene in UC patients

Epigenetic modifications regulate gene expression without affecting genomic sequences. DNA methylation is the most well-established form of epigenetic regulation mediated by DNA methyltransferases (10). To investigate the potential mechanism for reduced Ubc9 expression in UC patients, we analyzed Ubc9 promoter within a 2.4 kb region upstream from first start codon. We observed a CpG island within the exon 1 and promoter of Ubc9 gene (GC content = 63.3 %, ratio of observed CpG vs expected CpG=1.11 and CpG count=167) (Fig 2A). We hypothesized that hyper methylation of this CpG island could be

the cause for reduced transcription of Ubc9 in UC patients. We performed methylation specific PCR (MSP) and unmethylation specific PCR (USP). Interestingly, we observed DNA methylation in most of the UC patients whereas no DNA methylation was observed in most of the controls (Fig 2B).

HIF-1α binds to promoter CpG islands of Ubc9 gene

Hypoxic conditions promote DNA methylation in IBD and other inflammatory diseases (11). Therefore, we hypothesized that hypoxia-induced transcription factors might promote DNA methylation of the CpG island. In support of this hypothesis, we found a consensus hypoxia response element (HREs) in human Ubc9 and mouse Ubc9 promoter (Supplementary Fig 2A and 2B). Since HIF-1α is the major factor that is induced during hypoxic conditions (7), we performed ChIP assays using colonic lamina propria lymphocytes (cLPLs) isolated from mice and kept under hypoxic conditions (2% O_2). As shown in Fig 3A, anti-HIF-1α antibody but not the control IgG precipitated Ubc9 promoter DNA suggesting that HIF-1α binds to HREs within the CpG island.

HIF-1α inhibits Ubc9 expression

To investigate if HIF-1α binding to Ubc9 promoter modulates Ubc9 expression, next, we performed a Ubc9 promoter-driven luciferase assays. We transfected cells with HIF-1α expressing construct along with pGL4-hUbc9 promoter construct. Cells were subjected to hypoxia (2% O_2) for 24 h, lysed, and luciferase activity was measured. As shown in Fig 3B, luciferase activity of Ubc9 promoter was decreased when WT-HIF-1α was expressed with pGL4-hUbc9 promoter construct.

To confirm that binding of HIF-1α to Ubc9 promoter inhibits Ubc9 expression, we knocked down HIF-1α expression in mouse cLPLs by using lenti-CRISPR viral particles specific to HIF-1α. The efficiency of knockdown was confirmed by western blotting (Fig 4A). We then stimulated these cLPLs with PMA and ionomycin and subjected to hypoxia (2% O_2). As shown in Fig 4B and 4C, knocking down HIF-1α substantially rescued the cells from reduced Ubc9 expression. Further, lentiviral mediated depletion of HIF-1α substantially reduced *IL-17a,* and HIF-1α target genes such as *PTGS1, PTGS2*, and *CXCR4* expression (Fig 4D and 4E). Also, hypoxia induced DNA methylation in *in vitro* generated Th17 cells but not in the cells in which HIF-1α was knocked-down (Fig 4F). These data collectively suggest that HIF-1α negatively regulates Ubc9 expression and modulates IL-17a expression. Previous studies have shown that hypoxia and HIF-1α induce promoter DNA-methylation and regulate gene expression (12–14). Several mechanisms have been proposed including inhibition of the activity of ten-eleven translocation (TET) enzymes (15) and by the induction of DNA methyltransferases (DNMTs) (16). It is likely that a similar mechanism is involved in HIF-1α-mediated regulation of Ubc9 promoter.

As has been shown earlier, the HIF-1α enhances the development of Th17 cells. Moreover, the murine T cells with HIF-1α-deficiency are resistant to induction of Th17-dependent experimental autoimmune encephalitis (17). Therefore, to investigate the physiological impact of HIF-1α-mediated regulation of Ubc9 and IL-17-mediated inflammation, we utilized the Th17 cell adoptive transfer colitis model (18, 19). We sorted

$CD4^+CD25^-CD45RB^{hi}$ cells and knocked down HIF-1α using lenti-CRISPR viral particles. The cells were differentiated under Th17-polarizing conditions for 6 to 7 days, and 3×10^5 cells were adoptively transferred into $Rag1^{-/-}$ mice (8). $Rag1^{-/-}$ mice that received Th17 cells with reduced HIF-1α showed reduced loss of body weight, lower fecal occult blood and diarrhea scores, and lower weight-to-length ratio of the colon compared to mice that received control T cells (Fig 5 A–E). These mice exhibited lower *IL-17a* mRNA levels in the colonic mucosa, and colonic cultures showed lower IL-17a secretion than control T cells (Fig 5 F–G). These mice showed increased *Ubc9* mRNA levels compared to mice receiving control T cells (Fig 5H); correspondingly, histologic analysis showed lower diseases scores in the $Rag1^{-/-}$ mice receiving Th17 cells with reduced HIF-1α (Fig. 5 I–J). Further, the expression of IL-17 induced genes such as *MMP1*, *MMP2*, *MMP3*, *MMP9* and *CXCL1* was substantially reduced in $Rag^{-/-}$ mice that received Th17 cells with reduced HIF-1α (Fig. 5K–O). Together, these findings suggest that HIF-1α negatively regulates Ubc9 expression and modulates inflammation in the colonic mucosa. Earlier studies have shown that conditional deletion of HIF-1α in epithelial cells, DC, and Tregs leads to increase in the intestinal permeability and more severe colitis (20–22). Whereas, knock-down of HIF-1α in myeloid cells ameliorates DSS induced colitis which was associated with reduced monocyte infiltration and attenuated IL-17 levels (23). These studies along with our findings further highlight important cell-specific pro-inflammatory roles of HIF-1α that should be taken into account when designing strategies to treat gut inflammation by targeting HIF-1α.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

- SUMOylation of ROR-γt is defective in the inflamed colon tissue of UC patients.
- HIF-1α inhibits Ubc9 expression via promoter DNA methylation.
- HIF-1α depleted Th17 cells are less colitogenic.

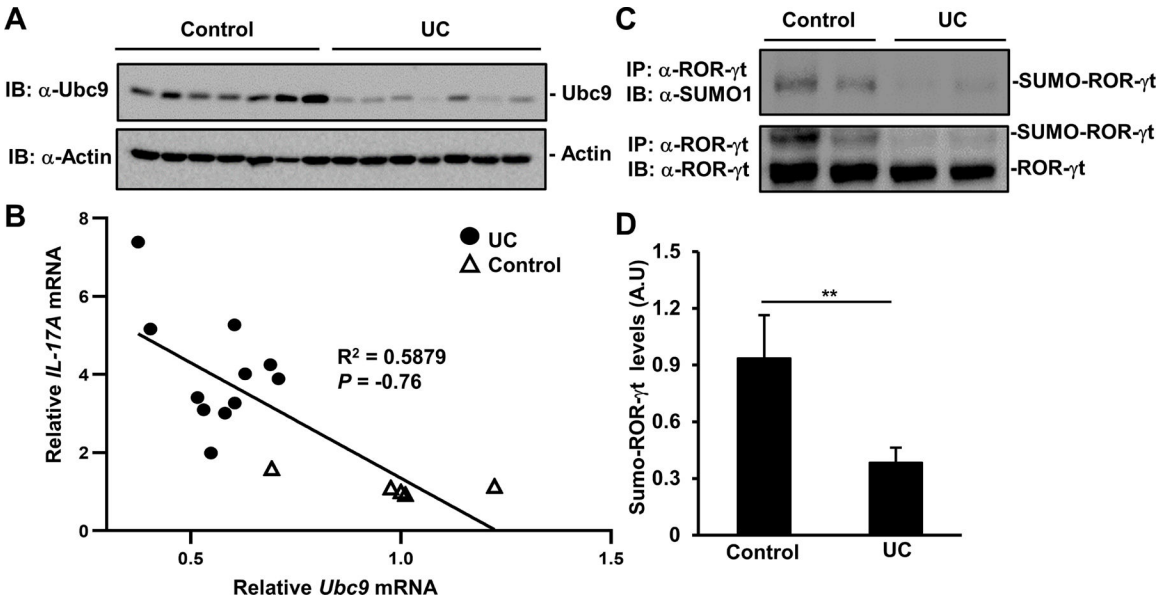

Figure 1. Reduced expression of Ubc9 in UC patients.

(A) Lysates from colonic tissue of inflamed UC and control were subjected to immunoblotting with anti-Ubc9 antibody (n=7, control and n=7, UC samples). (B) Expression of *IL-17a* mRNA and *Ubc9* mRNA was assayed by RT- PCR in UC and control samples (n=6, control and n=11, UC samples). Relative *IL-17a* mRNA was plotted against relative *Ubc9* mRNA of all samples linear regression curve was generated. (C) Colonic tissue lysates from UC and control samples were immunoprecipitated with anti-ROR- γt antibody and were immunoblotted with anti-SUMO1 antibody to detect the SUMOylated ROR- γt (D) Densitometry analysis of SUMOylated ROR- γt in UC and control samples (n=5, control and n=5, UC samples). The data are representative of three independent experiments, shown as mean ± SD.

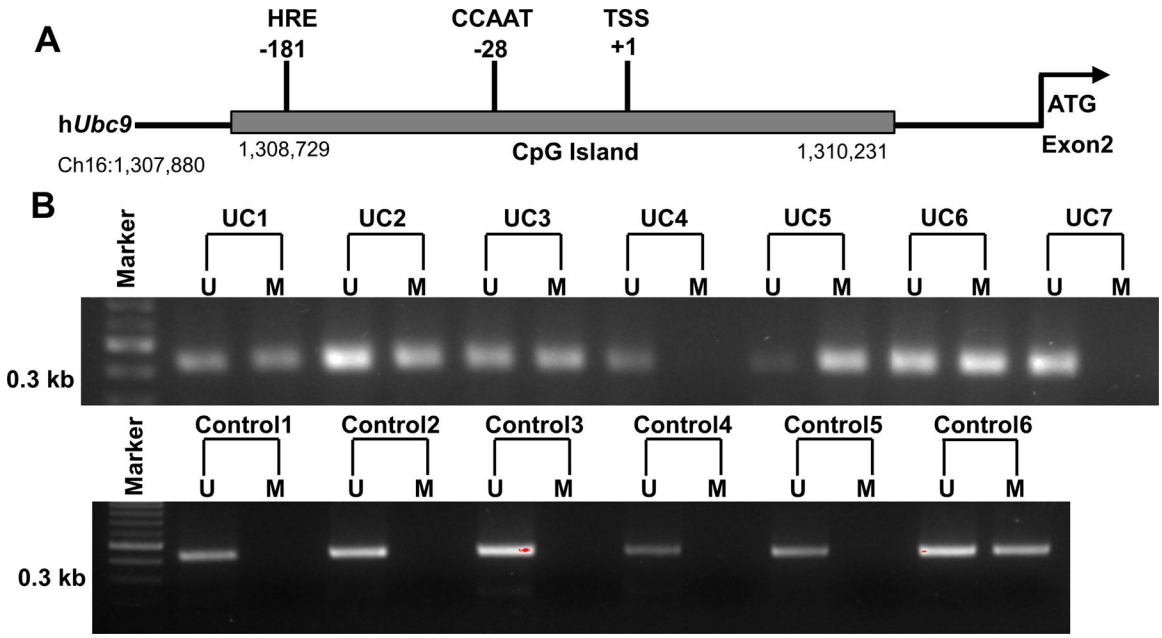

Figure 2. Hypermethylation of CpG Island on the promoter region of *Ubc9* gene

(A) Location of the promoter region, CpG Island and PCR amplicon of human *Ubc9* gene at chromosome 16. CpG Island is highlighted in dark filled bar. Position of transcription start site (TSS), CCAAT site and Hypoxia response element (HRE) are shown. (B) Tissue samples of UC and control were subjected to bisulfite conversion of DNA (n=6, control and n=7, UC samples). Representative results of methylation in inflamed and control tissues are shown. M, methylation specific PCR; U, unmethylation specific PCR. The data are representative of three independent experiments, shown as mean ± SD.

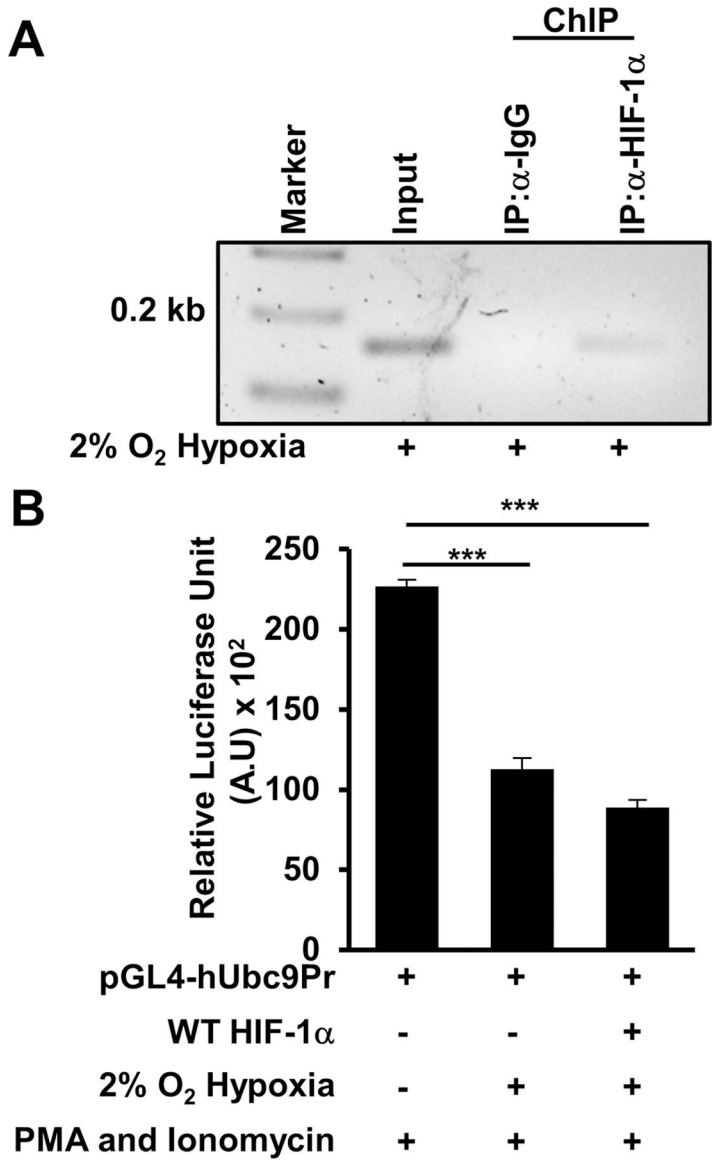

Figure 3. HIF-1α binds to the *Ubc9* gene promoter

(A) ChIP enriched DNA-protein complex from cLPLs of wild type mice was immunoprecipitated with anti-HIF-1α antibody (ChIP grade) and control IgG antibody followed by PCR. (B) Luciferase assay was conducted in Jurkat T cells transfected with various plasmid as indicated along with HIF-1α, and the results are shown as RLU. The data are representative of three independent experiments, shown as mean ± SD.

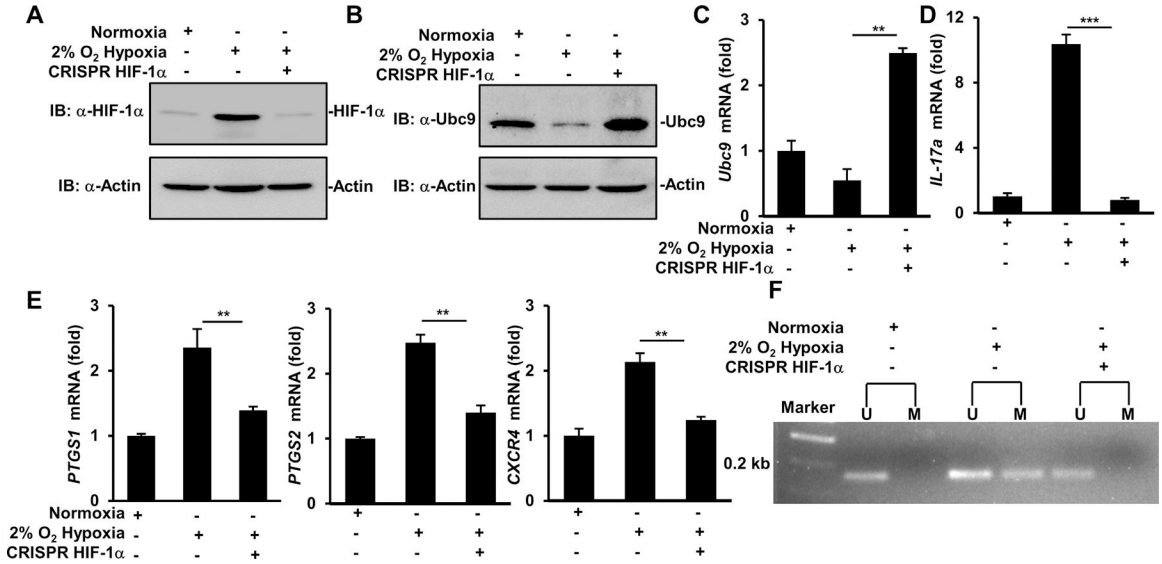

Figure 4. Knockdown of HIF-1α increases expression of *Ubc9* in hypoxic conditions

HIF-1α was knocked down using CRISPR in cLPLs isolated from mice and subjected to hypoxia. Immunoblotting of (A) HIF-1α (B) Ubc9 (C) RT-PCR of *Ubc9* (D) *IL-17a* (E) *PTGS1*, *PTGS2*, and *CXCR4*. List of primers are shown in supplementary table 1. (F) $CD4^+$ T cells transduced with LentiCRISPR HIF1-α were differentiated into Th17 inducing conditions. Cells were subjected to hypoxia (2% O_2) methylation analysis was performed. M, methylation specific PCR; U, unmethylation specific PCR. The data are representative of three independent experiments, shown as mean ± SD.

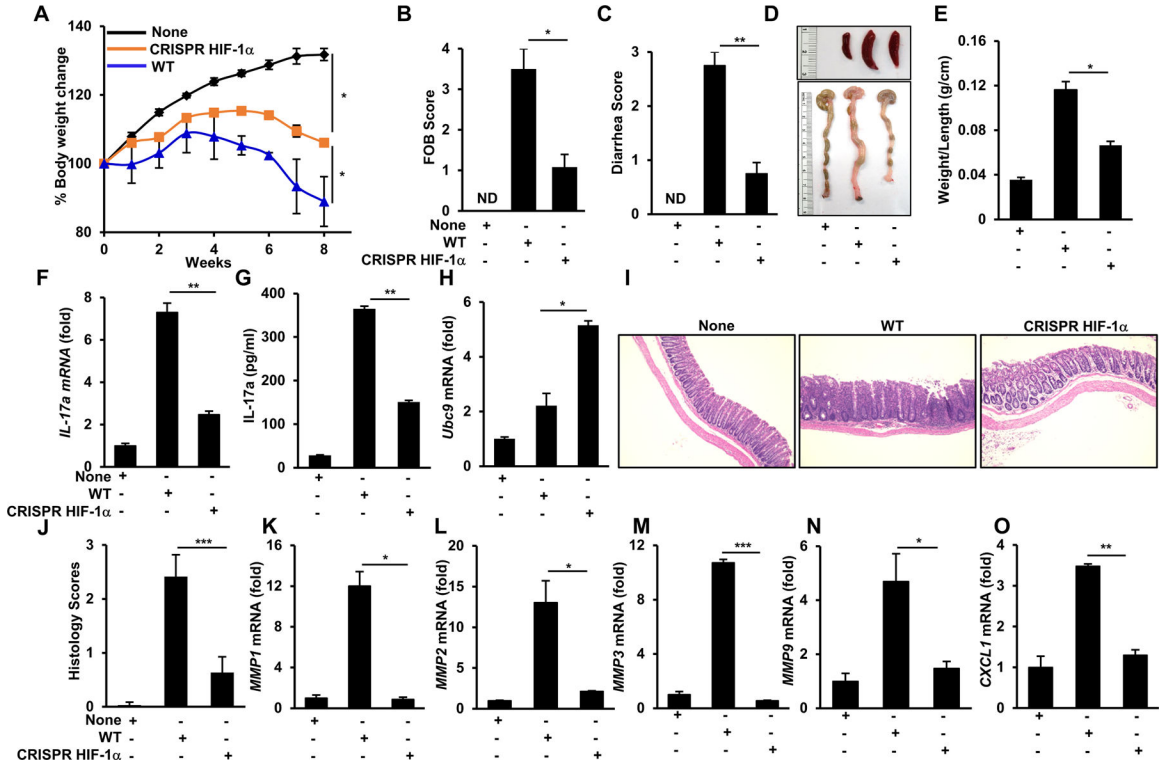

Figure 5. HIF-1α deficiency in Th17 cells reduces disease severity in Rag1$^{-/-}$ mice.

(A) Body weight change in percentage, (B) Fecal occult blood score, (C) Diarrhea score, (D-E) spleen size, colonic weight to length ratio given intraperitoneal injection of no cells (none) or WT Th17 cells or LentiCRISPR-HIF-1α transduced Th17 cells (CRISPR HIF-1α), and monitored for 8 weeks (n=5 mice, each group). (F) RT-PCR analysis of *IL-17a* (G) IL-17a ELISA using the explant culture of Rag1$^{-/-}$ mice. (H) RT-PCR analysis of *Ubc9*. (I) H&E sections of colon. (J) histology scores. (K-O) RT-PCR analysis of *MMP1*, *MMP2*, *MMP3*, *MMP9* and *CXCL1*. Data are from one experiment representative of three independent experiments with similar results. The data are representative of three independent experiments, shown as mean ± SD.