Non-coding RNA Research 12 (2025) 167–179

Contents lists available at ScienceDirect

Non-coding RNA Research

journal homepage: www.keaipublishing.com/en/journals/non-coding-rna-research

Original Research Article

Long non-coding RNA ANRIL/p65 negative feedback loop protects intestinal barrier function in inflammatory bowel disease

Keqi Yu [a], Hong Peng [a], Zhechuan Zhang [a], Lu Ye [b], Ke Zhan [b,c], Chuanfei Li [b], Li Gan [d], Yuru Lin [b], Yanhui Wang [b], Ya Song [b], Zhechuan Mei [b], Shengtao Liao [b,*], Jinjun Guo [a,**], Lin Lv [b,***]

[a] Department of Gastroenterology, Bishan Hospital of Chongqing Medical University, Chongqing, 402760, China
[b] Department of Gastroenterology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, 400010, China
[c] Department of Gastroenterology, The First Affiliated Hospital of Chongqing Medical University, Chongqing, 400042, China
[d] Department of Anatomy, and Laboratory of Neuroscience and Tissue Engineering, Basic Medical College, Chongqing Medical University, Chongqing, 400016, China

ARTICLE INFO

Keywords:
Inflammatory bowel disease
lncRNA ANRIL
YY1
p65
Intestinal barrier function

ABSTRACT

Patients with inflammatory bowel disease (IBD) demonstrate varying expression levels of long non-coding RNAs (lncRNAs) in their intestinal mucosa, which can potentially impact the function of the intestinal barrier. This impact may occur through the modulation of epithelial cell apoptosis, alteration of intestinal mucosal barrier permeability, and enhancement of inflammatory responses. The objective of this study was to explore the role and underlying mechanisms of the downregulated lncRNA ANRIL in modulating intestinal barrier function in IBD. Notably, ANRIL was found to be significantly downregulated in patients diagnosed with ulcerative colitis (UC), correlating strongly with disease progression. The overexpression of ANRIL in mice treated with dextran sulfate sodium (DSS) resulted in a significant reduction in colonic damage. This was accompanied by the suppression of pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β, and an improvement in intestinal barrier function. Transcriptome sequencing following overexpression of ANRIL revealed a significant enrichment of the NF-κB signaling pathway. In both DSS-induced mouse colitis and LPS-induced FHC cell models, the upregulation of ANRIL effectively suppressed the activation of the NF-κB pathway. Furthermore, our findings demonstrated that ANRIL competes with YY1 for binding, thereby inhibiting the interaction between YY1 and p65 subunit of NF-κB. This disruption in interaction results in the suppression of transcriptional activation of NF-κB p65, leading to a reduced expression of inflammatory cytokines and the promotion of intestinal barrier function in IBD. Additionally, we identified a negative feedback loop involving ANRIL and p65, wherein p65 binds to the ANRIL promoter, promoting ANRIL expression. In summary, the ANRIL/p65 negative feedback loop represents a potential therapeutic target for protecting intestinal barrier function in IBD.

1. Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis (UC), is a chronic and recurrent condition characterized by inflammation of the gastrointestinal tract [1]. The pathogenesis of IBD is intricate, involving dysregulated immune responses, genetic predisposition, environmental factors, and compromised intestinal barrier function [2]. The intestinal barrier serves as a crucial defense mechanism, preventing the uncontrolled passage of luminal antigens and commensal bacteria into the underlying tissue [3–5]. Tight junction

Peer review under the responsibility of Editorial Board of Non-coding RNA Research.

* Corresponding author. Department of Gastroenterology, The Second Affiliated Hospital of Chongqing Medical University, No. 74 Linjiang Road, Yuzhong District, Chongqing, China.

** Corresponding author. Department of Gastroenterology, Bishan Hospital of Chongqing Medical University, Chongqing, No.9 Shuangxing Avenue, Biquan Street, Bishan District, Chongqing, China.

*** Corresponding author. Department of Gastroenterology, The Second Affiliated Hospital of Chongqing Medical University, No. 74 Linjiang Road, Yuzhong District, Chongqing, China.

E-mail addresses: jianghe@cqmu.edu.cn (S. Liao), guojinjun1972@163.com (J. Guo), lin-miaomiao@cqmu.edu.cn (L. Lv).

https://doi.org/10.1016/j.ncrna.2025.03.002
Received 18 August 2024; Received in revised form 9 February 2025; Accepted 3 March 2025
Available online 14 March 2025

proteins (TJs) such as ZO, Occludin, and Claudin play a crucial role in the formation of the epithelial barrier and the maintenance of epithelial polarity. These proteins are essential for ensuring the integrity and function of the epithelial layer [6,7]. Disruption of this barrier integrity contributes to the perpetuation of mucosal inflammation in IBD. The NF-κB signaling pathway is a central regulator of intestinal barrier integrity and inflammatory responses in IBD. NF-κB activation in intestinal epithelial cells (IECs) drives the production of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β), which disrupt tight junction (TJ) proteins such as occludin and claudins, leading to increased paracellular permeability [8]. Conversely, NF-κB also plays a protective role by promoting epithelial repair through STAT3- and IL-22-dependent mechanisms during mucosal healing [9,10]. This dual role highlights the context-dependent regulation of NF-κB in IBD pathogenesis, where dysregulated activation exacerbates barrier dysfunction while controlled activity supports tissue restitution.

Long non-coding RNAs (lncRNAs) have emerged as pivotal participants in a variety of biological processes, encompassing transcriptional regulation, chromatin remodeling, and post-transcriptional modifications [11]. They are increasingly recognized as significant regulators of gene expression and cellular functions, often demonstrating tissue-specific expression patterns and dysregulation in disease states [12,13]. Recent studies have implicated lncRNAs (lncRNA MALAT1, DANCR, uc.173, H19, SPRY4-IT1) in the pathogenesis of IBD, suggesting their potential involvement in modulating intestinal barrier function and inflammatory responses [14–18].

lncRNA ANRIL (antisense non-coding RNA in the INK4 locus), situated within the 9p2.3 chromosomal region, has been recognized as a pivotal focal point for disease-associated SNPs. It plays a crucial regulatory role across a wide range of human diseases and cellular environments [19]. Mirza et al. [20], utilizing genome-wide transcriptome profiling, identified 438 and 745 lncRNAs that were differentially expressed in IBD, among which ANRIL was observed to be down-regulated. Xia et al. [21] presented evidence showing a notable decrease in ANRIL expression amongst patients diagnosed with IBD. They further established that a decline in ANRIL levels was correlated with an escalation in inflammation, enhanced disease activity, and an increase in the overall severity of the condition. Current research indicates that ANRIL plays a pivotal regulatory role in various conditions, including pancreatic cancer, acute myocardial infarction, colorectal cancer, allergic rhinitis, and chronic kidney disease [22–26]. However, the functional contribution and regulatory mechanisms of ANRIL in the progression of IBD are yet to be clearly defined.

The current study is centered on the role of downregulated ANRIL in intestinal barrier dysfunction associated with IBD. The study indicates that ANRIL downregulation in IBD is associated with disease progression and that its overexpression in a mouse model of colitis leads to reduced colonic damage, decreased expression of pro-inflammatory cytokines, and enhanced intestinal barrier function. In order to analyze the specific mechanism of ANRIL in IBD, we performed transcriptome sequencing after overexpressing ANRIL, which revealed significant enrichment of the NF-κB signaling pathway. Mechanistically, ANRIL competes for binding with the transcription factor YY1, thereby interrupting the interaction between YY1 and the p65 subunit of NF-κB. Additionally, p65 attaches to the ANRIL promoter, subsequently enhancing ANRIL expression. The negative feedback loop between ANRIL and p65 likely plays a role in preserving intestinal barrier integrity and could represent a potential therapeutic target for IBD. Collectively, this study offers valuable insights into the functional significance of ANRIL down-regulation in regulating intestinal barrier function in IBD.

2. Materials and methods

2.1. Human tissue collection

Colonic mucosal tissues were collected from 26 UC patients undergoing endoscopic examination and 26 healthy subjects undergoing routine colonoscopy at the Department of Gastroenterology, the Second Affiliated Hospital of Chongqing Medical University. Approval for this study was granted by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, and patients provided informed consent before undergoing the endoscopic procedure. The tissue samples obtained were confirmed as either inflammatory or normal tissue through pathological analysis, and promptly cleansed with saline post-retrieval. Subsequently, the specimens were placed in sterile 1.5 mL Eppendorf tubes, transferred in a chilled container, and ultimately preserved in a liquid nitrogen storage tank.

2.2. Cells, cell culture, and transfection

FHC cells, obtained from the American Type Culture Collection (ATCC) located in Manassas, VA, USA, were cultured in Dulbecco's Modified Eagle's Medium (Gibco #C11995500BT) sourced from Beijing, China. The medium was enhanced with 10 % fetal bovine serum (Natocor #NTC-HK009) from Argentina and supplemented with streptomycin (0.1 mg/mL) and penicillin (100 units/mL). The culturing conditions were maintained at 37 °C with a 5 % CO_2 environment. The ANRIL overexpression vector pcDNA3.1, and siRNA were obtained from GenePharma (Shanghai, China), were employed in the design of the ANRIL overexpression model (ANRIL-OV) and siRNA targeting human ANRIL (si-ANRIL). In parallel, a control vector, lacking targeted gene intervention, was also established. Subsequently, the FHC cells were exposed to a combination of viral particles and polybrene (10 mg/mL), provided by Sigma-Aldrich (St. Louis, MO, USA), for a period of 72 h. This was followed by a selection phase employing puromycin (also from Sigma-Aldrich), extending up to 7 days. Overexpression vectors of YY1 and p65, along with short hairpin RNA targeting YY1 in humans (si-YY1) and their corresponding control vectors, were encapsulated in plasmids, sourced again from GenePharma (Shanghai, China). FHC cells were then transfected with these plasmid particles alongside Lipofectamine™ 3000 Reagent (#L3000150; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and incubated for 48 h.

2.3. LPS-induced FHC inflammatory cell model

The Lipopolysaccharide (LPS, #L2880, Sigma-Aldrich, St. Louis, MO, USA) was used to create an inflammatory cell model in FHC, as described in our previous study [27]. In brief, LPS was dissolved in DMEM to achieve a concentration of 1 mg/mL. FHC cells were then subjected to 100 μg/mL of this LPS solution for a period of 24 h to stimulate inflammatory reactions.

2.4. Mouse models of colitis

The Animal Experimental Ethics Committee of Chongqing Medical University in China approved the animal studies. Eighty female C57 mice, aged 6–8 weeks and weighing between 18–22 g, were obtained from the Experimental Animal Centre of Chongqing Medical University (Chongqing, China). Prior to the studies, the mice were allowed to adapt for a minimum of three days in a regulated environment with 80 % humidity and a temperature of 22 ± 2 °C. They were housed separately in cages with access to standard food. Use distilled water to dissolve DSS (KeRui, Wuhan, China) to a concentration of 2.5 % (w/v). Mice in the colitis group were provided with free access to the DSS solution, which was replaced every other day. The mice's weight was measured daily, and their feces were monitored for characteristic changes. Occult blood in the feces was detected using occult blood test paper. In the control group, distilled water was used instead of the DSS solution. To investigate the role of ANRIL in DSS-induced colitis in mice, the mice were divided into the four groups: (1) normal control group, (2) DSS group, (3) DSS + ANRIL negative control group, and (4) DSS + ANRIL overexpression group. Plasmids (at a concentration of 1 μg/μL in PBS)

expressing ANRIL-OV and ANRIL-Crtl were procured from GenePharma (Shanghai, China). ANRIL-OV and ANRIL-Crtl, or ddH2O was injected intraperitoneally 72 h pre and post DSS treatment (50 μg each time).

2.5. Intestinal permeability assay

On the final day of the colitis model development, the mice underwent an overnight fast, followed by a single administration of FITC-dextran (60 mg/100 g body weight) via oral gavage. After a 4-h period, serum samples were harvested. The concentration of dextran in the serum was then quantified using a fluorescence microplate reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

2.6. RNA sequencing

We selected colonic tissues from both the ANRIL-OV and ANRIL-Crtl groups in DSS-induced mouse colitis for RNA isolation. Post extraction of the RNA, it was sent to Biopic for library preparation and sequencing on the Illumina HiSeq2000 platform. The process yielded between 40.5 and 47.1 million reads per sample, with a unique mapping rate exceeding 85 %. The software DEGseq was used to calculate differential gene expression. Fisher's exact tests, as implemented in the software, were used to determine P-values. To multiple testing corrections, P-values were adjusted to q-values using two alternative strategies.

2.7. CCK-8 assay

FHC cells were plated onto 96-well plates at a density of 5000 cells per well and incubated for 24 h at 37 °C in a 5 % CO_2 incubator. Following this, CCK-8 solution (#AR1160BOSTER, Wuhan, China) was introduced to each well and incubated for 1 h. The optical density at 450 nm (OD450) was then measured by Thermo Fisher Scientific in order to assess cell viability.

2.8. Real-time qPCR

Total RNA was extracted from FHC cells and colon tissues using TRIzol (#9109; Takara, Dalian, China), followed by reverse transcription with the PrimeScript™ RT kit (Takara #RR047A). Quantitative PCR (QCPR) was conducted using TB Green Premix of Taq™ II (Takara # RR 820a) in a CFX Connect™ thermal cycler (BR005222 Singapore BIO-RAD). GAPDH was utilized as a reference gene to determine the relative expression level. The PCR cycle steps included 40 cycles of denaturation at 95 °C for 30 s, followed denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, extension at 65 °C for 5 s, and final denaturation at 95 °C for an additional 5 s. Specific primer details can be found in S1 table.

2.9. ELISA

The levels of IL-6, TNF-α and IL-1β in colon tissues and cell culture supernatant were measured by ELISA kits (Jiubang, Fujian, China) according to the manufacturer's instructions.

2.10. Western blotting

Protein was extracted from cells and colon tissues using RIPA lysate (Beyotime Biotechnology, Shanghai, China) and then heated at 100 °C for 10 min. The protein concentration was measured using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). An equal amount of the protein sample (20 g) was electrophoresed on a 10 % sodium dodecyl sulfate-polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane (MilliporeSigma). The membrane was sealed in 5 % skim milk containing 0.1 % Tween for 2 h, then incubated overnight at 4 °C with primary antibodies GAPDH (1:5000; #10494-1-AP; Proteintech Group, Inc., Rosemont, IL, USA), ZO-1 (1:5000; #21773-1-AP; Proteintech Group), Occludin (1:5000; #27260-1-AP; Proteintech Group), Claudin-2 (1:2000; #AF0128; Affinity Biosciences), NF-κB p65 (1:1000; #8242; Cell Signaling Technology, Danvers, MA, USA), Phospho-NF-κB p65 (Ser536) (1:1000; #3033; Cell Signaling Technology), IκBα (1:1000; #4814; Cell Signaling Technology), Phospho-IκBα (Ser32) (1:1000; #2859; Cell Signaling Technology) and YY1 (1:1000; #46395; Cell Signaling Technology). Subsequently, the membranes were incubated with goat anti-rabbit antibodies (catalog no. SA000012, Proteintech Group, Inc.) at room temperature for 1 h. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection kit (EMD Millipore, Burlington, MA, USA) and analyzed using an ECL western blotting system (BioRad Laboratories, Inc., Hercules, CA, USA). The Image Lab software (Version 6.0.1; BioRad Laboratories, Inc.) was employed for semi-quantification, with normalization to GAPDH.

2.11. Luciferase reporter assay

The segments of the ANRIL promoter from the psiCHECK-2 vector (Promega, USA) were cloned, which have potential to bind with NF-κB p65, from wild-type ANRIL promoter segments (ANRIL-P-WT). Overlap PCR was utilized to introduce mutations into the ANRIL promoter binding site seeds (ANRIL-P-MUT) in the luciferase reporters. We seeded 2.0×10^4 293T cells (ATCC) in 100 mL of growth medium in 96-well plates. On the subsequent day, overexpression vectors of p65 were introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were collected for analysis using a dual-luciferase reporter assay kit (Promega, Madison, WI, USA) after incubating for 48 h post-transfection. Renilla luciferase activities were normalized to firefly luciferase activities.

2.12. Calculation of disease activity index (DAI)

The DAI score was calculated based on the criteria of weight loss, fecal viscosity, fecal occult blood, and bleeding in mice (refer to Table S2 for specific details) [28].

2.13. Histological analysis

After the euthanization of the mice, the colon was sectioned into 4 μm slices, fixed in 10 % paraformaldehyde, and embedded in paraffin. The sections were then stained with hematoxylin and eosin (H&E), followed by microscopic examination to determine the pathological score (refer to Table S3 for details). The remaining tissues were preserved by freezing at −80°C [29].

2.14. Co-immunoprecipitation(Co-IP)

All procedures adhered to the guidelines of the Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Scientific™). In summary, FHC cell lysates were treated with specified primary antibodies (2~4 μg) on a BeyoMag™ Magnetic Separation Rack (Beyotime, China) at 4 °C for a duration of 6 h. Subsequently, Pierce™ Protein A/G Magnetic Beads (Thermo Scientific™) were introduced and incubated for an hour. After a PBST wash, the immunoprecipitates were extracted, neutralized, and subjected to SDS degradation for subsequent immunoblotting.

2.15. RNA immunoprecipitation (RIP)

RNA immunoprecipitation assays were performed using the Magna RIP™ RNA-binding protein IP kit (Millipore, USA) following the manufacturer's guidelines. FHC cells were transfected with ANRIL-OV or negative control, and then lysed using RIP lysis buffer. The lysate was treated with Anti-IgG or YY1 antibody (1:200; #46395; Cell Signaling Technology) magnetic beads. After protein removal by protease K, RNA

was extracted using TRIzol reagent. The purified RNA was then subjected to RT-qPCR analysis utilizing the following primers: ANRIL, F-5′-GACTTGCCAGAAAGCCCTCC-3′ and R-5′-CGGAGCGGCTTTTAGTTCAA-3′.

2.16. Chromatin Immunoprecipitation (ChIP)

FHC cells were utilized for the ChIP experiment. ChIP assays were carried out using an Agarose ChIP Kit (Thermo Scientific), following the manufacturer's instructions. DNA/protein complexes were precipitated with 4 mg of NF-κB p65 antibodies (1:100; #8242; Cell Signal Technology) or IgG (ab171870, Abcam) overnight and then treated with Protein A/G agarose beads for 2 h. After reversing the protein-DNA crosslinks, DNA was purified, and the quantity of the ANRIL promoter was assessed by qPCR using the following primers: ANRIL, F-5′-AGCCTGGGCTAGAGACGAAT-3′ and R-5′-TACTGAGGAGC-CAGCGTCTA-3′.

2.17. Pull down

Biotinylated ANRIL RNA baits from Genepharma Corporation (Shanghai, China) were used for the RNA-protein pulldown assay. This procedure was executed in accordance with the specifications provided by the Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher, USA). Quantification of protein in the immunoprecipitated extracts was ascertained through Western blot analysis. For the RNA-RNA pulldown assay, Streptavidin-Dyna beads (Invitrogen) were utilized to isolate RNA samples. Following stringent washing steps, RNA complexes bound to the beads were subsequently eluted and purified using Trizol Reagent (Takara, Japan) in preparation for RT-qPCR investigation.

2.18. Immunofluorescence (IF)

The colon sections were submerged in xylene twice for 5 min each, followed by 100 % ethanol for 2 min, 95 % ethanol for a minute, 80 % ethanol for a minute, and 75 % ethanol for a minute. The slides underwent antigen retrieval in citrate buffer. The sections were then rinsed with 2 % phosphate buffer saline (PBS) and 0.3 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) for an hour at 25 °C. After rinsing, the sections were treated with antibodies against ZO-1 (1:1000; #21773-1-AP; Proteintech Group), Occludin (1:400; #27260-1-AP; Proteintech Group), Claudin-2 (1:200; #AF0128; Affinity Biosciences), NF-κB p65 (1:400; #8242; Cell Signaling Technology, Danvers, MA, USA) for 24 h at 4 °C. The sections were then rinsed thrice with PBS for 5 min each and treated with Cy3 conjugated anti-rabbit IgG (Abcam) for 30 min at 25 °C. DAPI (BOSTER, California, USA) was used for nuclear staining. Cell imaging was performed using a fluorescence microscope (Olympus, Tokyo, Japan).

2.19. Fluorescence In Situ Hybridization (FISH) and IF

ANRIL probes were obtained from GenePharma (Shanghai, China). Cells were stabilized with 4 % PFA and subsequently permeabilized. The RNA FISH procedure was carried out employing the FISH Kit (RiboBio). For immunofluorescent staining, cells were treated with a YY1 antibody (1:800; #46395; Cell Signaling Technology), followed by introduction to a secondary antibody. The co-localization of ANRIL and YY1 was visualized using a fluorescence microscope (Olympus, Tokyo, Japan).

2.20. Flow cytometry

FHC cells were harvested and resuspended in 1x binding buffer at a concentration of 1 × 10⁶ cells/mL. AnnexinV/FITC and PI (Thermo Fisher Scientific, Boston, MA, USA) were incubated in the dark for 5 min, followed by evaluation of apoptosis rate using flow cytometry (BD FACS Canto II, USA).

2.21. Statistical analysis

Statistical analysis was conducted with GraphPad Prism 9.5 (GraphPad Software, Inc) and SPSS 22.0 (International Business Machines Corporation). All experiments were conducted in triplicate, and the data are presented as mean ± standard deviation (SD). Patient samples analyzed was used Fisher analysis. Student's t-test was used to compare the two groups, while one-way ANOVA was utilized to assess differences between multiple groups. Post hoc Tukey testing was performed for pairwise comparisons. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. ANRIL expression correlated with IBD progression and protected mice against colitis

To verify the specific role of ANRIL in inflammatory bowel disease, we collected colonic mucosal tissues from 26 UC patients undergoing colonoscopic biopsy and 26 healthy subjects undergoing routine colonoscopy during the same period. We found that the expression level of ANRIL in the colonic tissues of UC patients was significantly lower than that in healthy individuals (Fig. 1A). Moreover, ANRIL expression was even lower in patients with high Calprotectin (CALP) (>62 μg/g), positive erythrocyte sedimentation Rate (ESR) (>20 mm/h), and moderate to severe UC (Fig. 1B–D) (See Table S4 for all clinical baseline information). These findings suggest that ANRIL is significantly underexpressed in UC patients and negatively correlates with disease progression, implying a potential protective role of ANRIL in UC.

To further verify the role of ANRIL in IBD, we induced colitis in mice with DSS (Fig. 1E). Overexpression of ANRIL significantly alleviated DSS-induced mouse colitis (Fig. 1F), accompanied by an increase in body weight (Fig. 1G), a decrease in the disease activity index (Fig. 1H), and an increase in colon length (Fig. 1I). Histological scoring also showed a reduction in the severity of DSS-induced colitis lesions after overexpression of ANRIL (Fig. 1J–K). RT-qPCR results also revealed a significant decrease in ANRIL expression in DSS-induced mouse colitis, consistent with results from human tissue samples (Fig. 2A). Meanwhile, ANRIL expression significantly increased after transfection with ANRIL-OV in DSS-induced mouse colitis (Fig. 2A). This suggests that upregulated ANRIL can provide protection in DSS-induced mouse colitis. Further investigation revealed that overexpression of ANRIL significantly suppressed the expression of inflammatory factors IL-6, TNF-α, and IL-1β in colonic tissues of DSS-induced colitis mice (Fig. 2B–G). In addition, overexpression of ANRIL also protected intestinal barrier function, as evidenced by the reduced intestinal permeability in the FITC-Dextran permeability test (Fig. 2H), increased expression levels of intestinal barrier-related proteins ZO-1 and Occludin, and decreased expression of Claudin-2 (Fig. 2I–O). Based on these analyses, we concluded that ANRIL could suppress the expression of inflammatory factors and enhance intestinal barrier function, thereby alleviating DSS-induced mouse colitis.

3.3. ANRIL suppresses activation of the NF-κB signaling pathway in DSS-induced mouse colitis

To analyze the specific mechanism by which ANRIL alleviates DSS-induced mouse colitis, we performed transcriptome sequencing after overexpressing ANRIL, which revealed significant enrichment of the NF-κB signaling pathway (Fig. 3A). WB results showed that overexpression of ANRIL significantly suppressed the phosphorylation level of p65 and IκBα, the key proteins of NF-κB signaling pathway (Fig. 3B–D). Immunofluorescence indicated that overexpression of ANRIL could significantly inhibit the expression and nuclear translocation of phosphorylated p65 (Fig. 3E). However, pull-down assays showed that ANRIL could not bind to p65 mRNA and protein (Fig. 3F–G). These

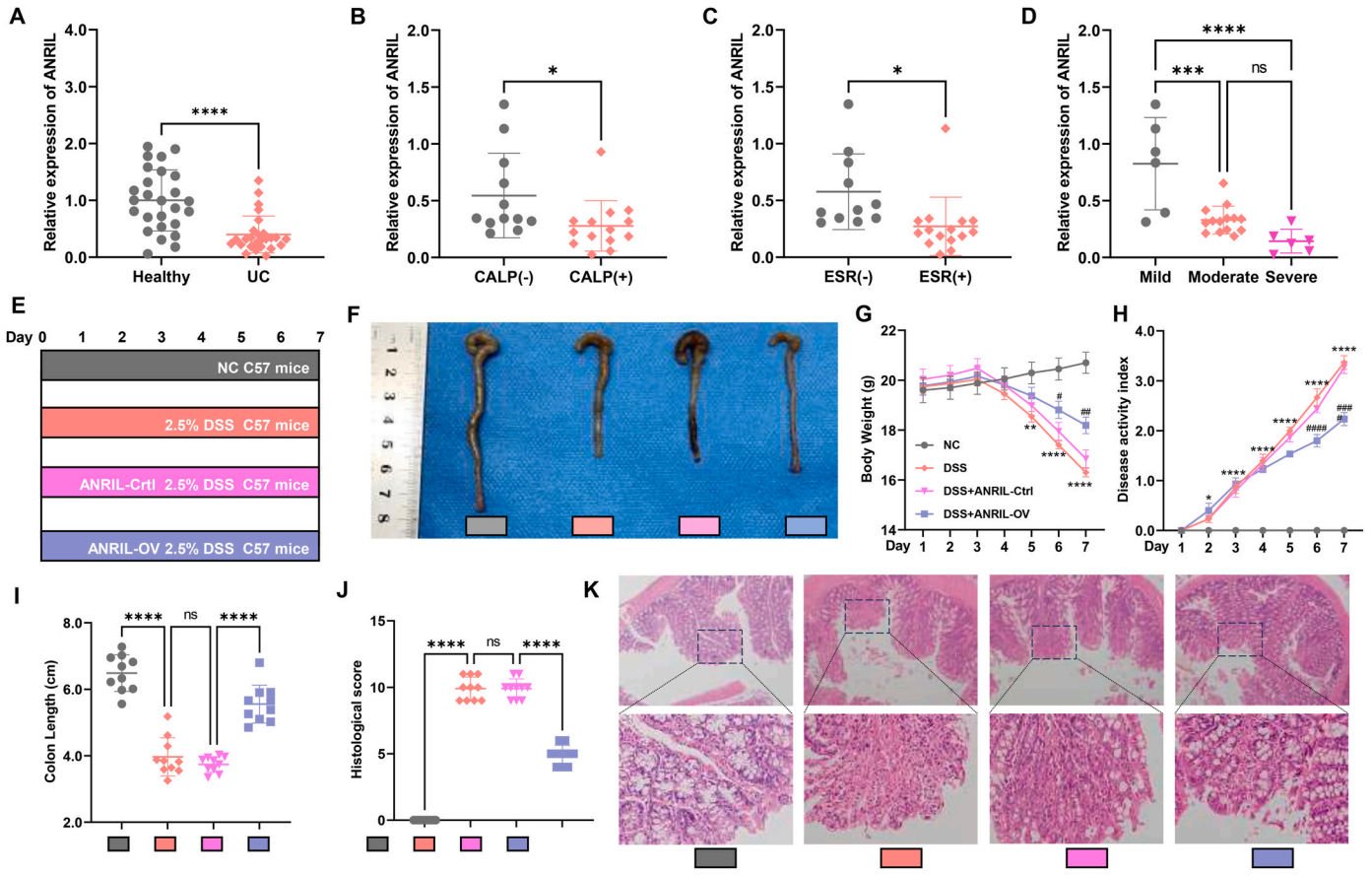

Fig. 1. ANRIL is significantly under-expressed in UC patients and correlates with disease progression, while overexpression of ANRIL alleviates DSS-induced mouse colitis. (A) RT-qPCR detection of ANRIL expression levels in colon tissues of healthy individuals and UC patients. (B–D) Expression levels of ANRIL in UC patients with CALP (Calprotectin) positivity (>62 μg/g), ESR positivity (>20 mm/h), and varying degrees of disease severity. (E) Schematic representation of DSS-induced mouse colitis and treatment with ANRIL overexpression. (F) Representative gross morphology and length of mouse colon post-ANRIL overexpression. (G–I) Weight, Disease Activity Index, and Colon Length in DSS-induced mouse colitis post-ANRIL overexpression. (J–K) Histological score shown by HE staining in DSS-induced mouse colitis. $^{*\&\#}p < 0.05$, $^{**\&\#\#}p < 0.01$, $^{***}p < 0.001$, $^{****\&\#\#\#\#}p < 0.0001$. Data are expressed as mean ± standard deviation.

results suggest that ANRIL might indirectly suppress the activation of the NF-κB signaling pathway, thereby exerting its protective role in IBD.

3.4. ANRIL binds to YY1 protein to regulate the biological functions of FHC cells

Based on the findings, we further investigated the mechanism of ANRIL in FHC cells and successfully constructed FHC cells with overexpressed ANRIL (Fig. 4A). In the LPS-induced FHC inflammatory cell model, the expression of ANRIL was downregulated (Fig. 4A). Overexpression of ANRIL in LPS-induced FHC inflammatory cell model promoted cell proliferation (Fig. 4B) and inhibited the expression of inflammatory factors IL-6, TNF-α, and IL-1β (Fig. 4C–E), as well as cell apoptosis (Fig. 4F–G). In addition, overexpression of ANRIL enhanced the expression levels of intestinal barrier-related proteins ZO-1 and Occludin and suppressed the expression of Claudin-2 (Fig. 4H–J), while also suppressed the phosphorylation level of p65 and IκBα (Fig. 4K–M). To verify the specific molecular mechanism of ANRIL in regulating the NF-κB signaling pathway, studies showed that ANRIL mainly binds to YY1, SUZ12, and EZH2 proteins to exert its biological functions [30,31]. Therefore, we performed a pull-down assay and found that ANRIL could bind to YY1, SUZ12, and EZH2 proteins in FHC cells, with most binding occurring with the YY1 protein (Fig. 4N). Overexpression of ANRIL did not significantly change the mRNA expression level of YY1 in FHC cells (Fig. 4O), and pull-down results also showed no binding between ANRIL and YY1 mRNA (Fig. 4P). However, RIP results showed that the YY1 protein could bind to ANRIL (Fig. 4Q). FISH further confirmed that ANRIL and the YY1 protein mainly colocalized in the cell nucleus (Fig. 4R). These results suggest that ANRIL can bind to the YY1 protein, thereby regulating the biological functions of FHC cells.

3.5. ANRIL competitively inhibits YY1 binding to p65

In our previous study, we successfully established an LPS-induced FHC inflammatory cell model [27]. In this model, the NF-κB signaling pathway was significantly activated and the expression of inflammatory factors IL-6, TNF-α, and IL-1β notably increased. Silencing YY1 significantly inhibited the activation of the NF-κB signaling pathway (Fig. 5A–C) and the expression of inflammatory factors (Fig. 5D–F) induced by LPS in FHC cell. Further mechanistic studies found that the YY1 protein could bind to the p65 protein (Fig. 5G), but overexpression of ANRIL significantly inhibited the binding of the YY1 protein to the p65 protein (Fig. 5H). In the LPS-induced FHC inflammatory cell model, the binding of the YY1 and p65 proteins was significantly promoted (Fig. 5I), which might be related to the downregulation of ANRIL expression in the LPS-induced FHC inflammatory cell model. These results indicate that ANRIL can competitively inhibit the binding of YY1 and p65, thereby suppressing the activation of the NF-κB signaling pathway.

3.6. ANRIL targets YY1 to alleviate IBD via inhibiting the NF-κB signaling pathway

To further substantiate that ANRIL exerts its biological functions by

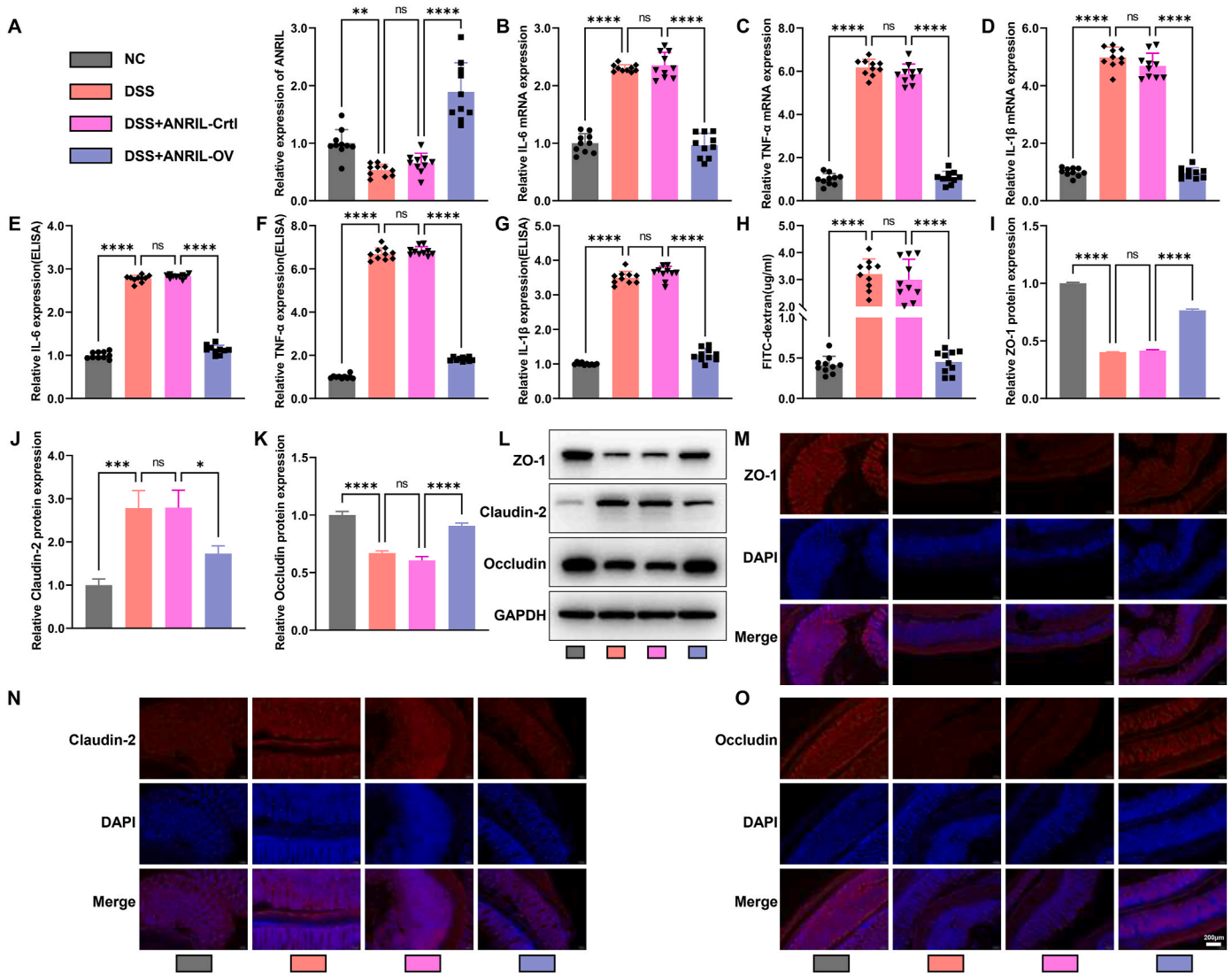

Fig. 2. ANRIL suppresses the expression of inflammatory factors and protects intestinal barrier function in DSS-induced mouse colitis. (A–D) RT-qPCR detection of expression levels of ANRIL, IL-6, TNF-α, and IL-1β in colon tissues of each mouse group. (E–G) ELISA detection of expression levels of IL-6, TNF-α, and IL-1β in colon tissues of each mouse group. (H) Assessment of intestinal permeability using the Fluorescein Isothiocyanate (FITC)-Dextran Permeability Assay. (I–L) Western blotting (WB) detection of expression levels of intestinal barrier-related proteins ZO-1, Claudin-2, and Occludin in colon tissues of each mouse group. (M–O) Immunofluorescence detection of expression of ZO-1, Claudin-2, and Occludin in colon tissues of each mouse group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are expressed as mean ± standard deviation.

binding to the YY1 protein, we co-transfected ANRIL and YY1 into the LPS-induced FHC inflammatory cell model. The results showed that the transfection of ANRIL alone could significantly inhibit the activation of the LPS-induced NF-κB signaling pathway, the expression of the inflammatory factors IL-6, TNF-α, IL-1β, and intestinal barrier function impairment. However, co-transfection of ANRIL and YY1 significantly reversed the inhibitory effects of ANRIL alone on the NF-κB signaling pathway (Fig. 6A–C), the inflammatory factors (Fig. 6D–F), and the protective effect on intestinal barrier function (Fig. 6G–J). In DSS-induced mouse colitis, co-transfection of ANRIL and YY1 further increased intestinal permeability (Fig. 6K), suppressed the expression levels of ZO-1 and Occludin, and promoted the expression level of Claudin-2 (Fig. 6L–N). This suggests that the co-transfection of ANRIL and YY1 can reverse the inhibitory effects of ANRIL on inflammatory factors and its protective role in intestinal barrier function in IBD. In summary, ANRIL can target YY1 then effectively preventing the activation of the NF-κB signaling pathway, reducing the expression of inflammatory factors, and protecting the intestinal barrier function, thereby alleviating IBD.

In addition, we knocked out ANRIL in FHC cells. Consistent with overexpression results, siRNA-mediated ANRIL knockdown (Figure S1A) significantly enhanced IL-6, TNF-α and IL-1β levels (Figure S1B-D), reduced ZO-1/Occludin expression and increased Claudin-2 (Figure S1E-H). Furthermore, si-ANRIL transfection significantly enhanced LPS-induced phosphorylation of p65 and IκBα (Figure S1I-K), confirming ANRIL's protective role in barrier integrity.

3.7. p65 binds to the ANRIL promoter to promote ANRIL expression

Research indicates that p65 plays a crucial role in regulating the expression of ANRIL [32]. In this study, when p65 was transfected into FHC cells, there was a significant increase in the expression level of ANRIL. Conversely, stimulation with LPS led to a marked down-regulation in the expression of ANRIL. Interestingly, overexpressing p65 in FHC cells that were stimulated with LPS was shown to boost the expression of ANRIL (Fig. 7A). Further investigation using ChIP revealed the ability of p65 to bind to the ANRIL promoter (Fig. 7B). When p65 was overexpressed, this interaction was significantly heightened.

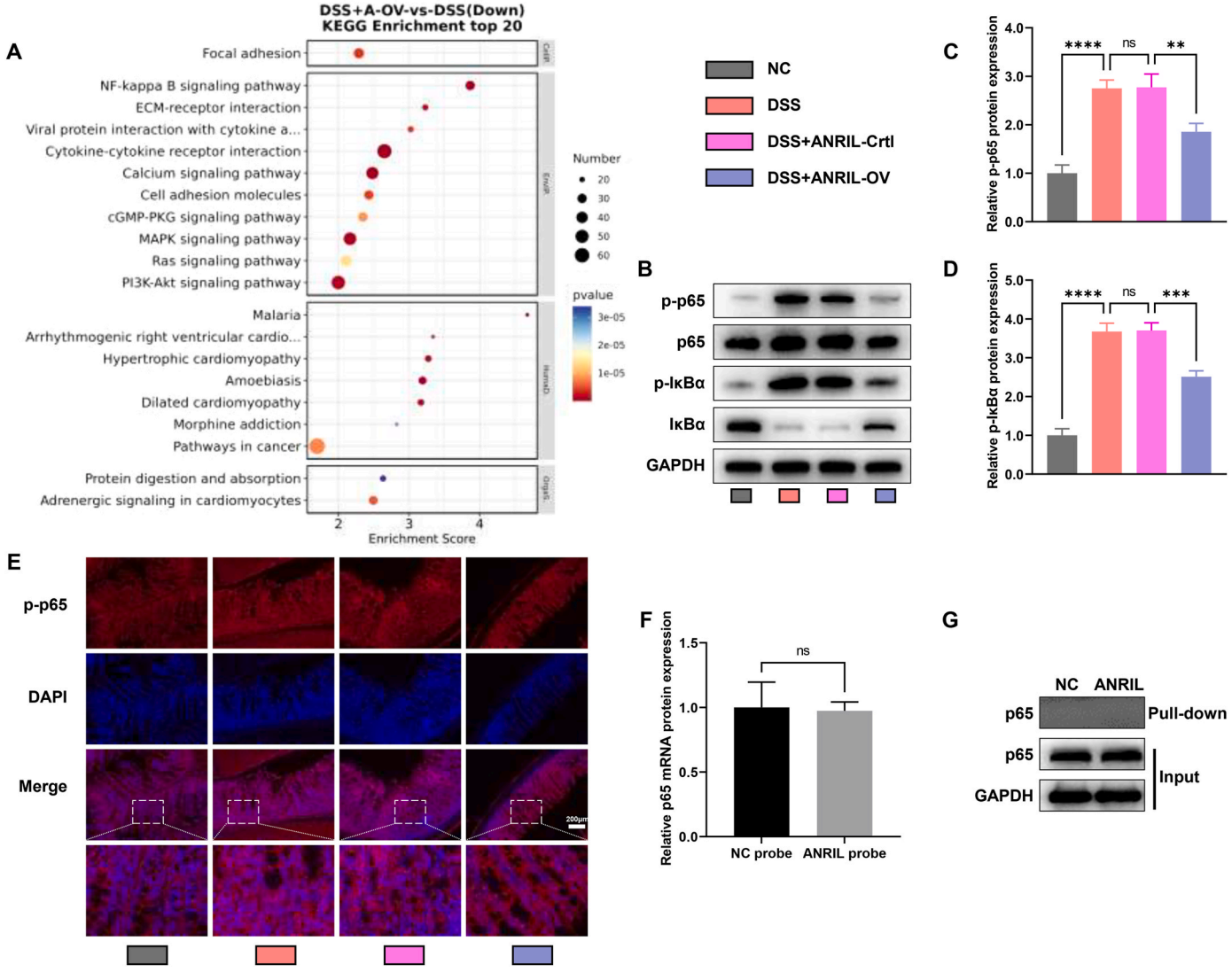

Fig. 3. ANRIL suppresses activation of the NF-κB signaling pathway in DSS-induced mouse colitis. (A) Transcriptome sequencing post-ANRIL overexpression in DSS-induced mouse colitis indicated significant enrichment of the NF-κB signaling pathway. (B–D) WB detection of the expression levels of NF-κB signaling pathway-related proteins p65, p-p65, IκBα, and p-IκBα in colon tissues of each mouse group. (E) Immunofluorescence detection of the expression of p-p65 in colon tissues of each mouse group. (F) RNA-RNA pulldown assay to detect the binding between ANRIL and p65 mRNA. (G) RNA-protein pulldown assay to detect the binding between ANRIL and p65 protein. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data are expressed as mean ± standard deviation.

However, this binding was disrupted when the ANRIL promoter underwent mutation, indicating a specific interaction between p65 and the ANRIL promoter (Fig. 7C). When YY1 and p65 were co-transfected, there was an even more significant increase in the binding of p65 to the ANRIL promoter compared to the single transfection of either p65 or YY1 (Fig. 7D). This observation implies the existence of a negative feedback loop involving ANRIL and p65. In essence, this ANRIL/p65 negative feedback loop appears to play a protective role in maintaining intestinal barrier function in IBD (Fig. 7E).

4. Discussion

IBD is an intricate condition characterized by persistent inflammation of the gastrointestinal tract, which is driven by dysregulated immune responses and impaired intestinal barrier function [33]. The present study focused on elucidating the role of the downregulated ANRIL in modulating intestinal barrier function and its underlying mechanisms in IBD. Our findings provide insight into the potential implications of ANRIL in IBD pathogenesis and its therapeutic significance. Consistent with previous studies [20,21,34], we observed ANRIL was significantly downregulated in patients with UC, a subtype of IBD, and its expression levels correlated with disease severity. We studied an inflammation model in which LPS/DSS is the initial factor of the IBD. ANRIL and p65 are dependent variables, as LPS/DSS-induced inflammation leads to their gene regulation disorders. Genes have different changing trends and functions in different diseases. In IBD, previous studies have reported the low expression of ANRIL in colon inflammatory tissues [20,35]. Likewise, LPS treatment reduced the expression of ANRIL in human colon epithelial cells [36]. These findings indicate the potential relevance of ANRIL as a biomarker for UC and suggest its functional importance in IBD.

To investigate the impact of ANRIL on intestinal barrier integrity, we utilized a well-established mouse model of DSS-induced colitis. Notably, the overexpression of ANRIL in DSS-treated mice resulted in a remarkable reduction in colonic damage, suggesting a protective role of ANRIL in mitigating intestinal inflammation. Moreover, Overexpression of ANRIL notably reduced key pro-inflammatory cytokines including IL-6, TNF-α, and IL-1β, while it elevated the levels of intestinal barrier-related proteins, thereby demonstrating its potential for anti-inflammatory action and mucosal barrier preservation in IBD. Zhou et al. [36]

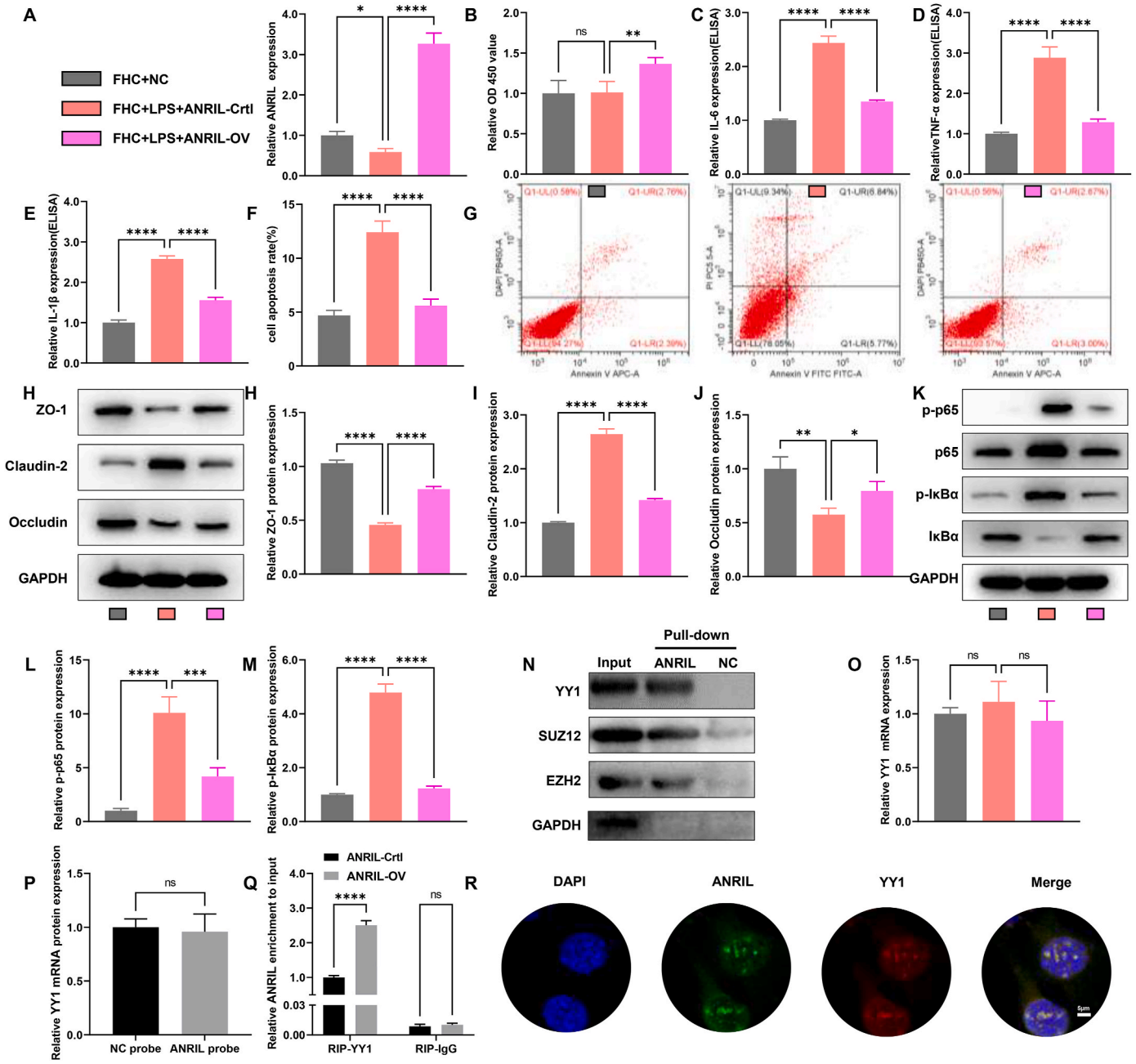

Fig. 4. ANRIL binds to YY1 protein to regulate the biological functions of FHC cells. (A) RT-qPCR detection of ANRIL expression levels in LPS-induced FHC inflammatory cell model after overexpression of ANRIL. (B) CCK8 assay to evaluate cell proliferation in LPS-induced FHC inflammatory cell model after overexpression of ANRIL. (C–E) ELISA detection of expression levels of IL-6, TNF-α, and IL-1β in LPS-induced FHC inflammatory cell model after overexpression of ANRIL. (F–G) Flow cytometry to examine cell apoptosis in LPS-induced FHC inflammatory cell model after overexpression of ANRIL. (H–J) WB detection of expression levels of intestinal barrier-related proteins ZO-1, Claudin-2, and Occludin in FHC cells after overexpression of ANRIL. (K–M) WB detection of expression levels of NF-κB signaling pathway-related proteins p65, p-p65, IκBα, and p-IκBα in LPS-induced FHC inflammatory cell model after overexpression of ANRIL. (N) RNA-protein pulldown assay to detect the binding between ANRIL and proteins YY1, SUZ12, and EZH2. (O) RT-qPCR detection of YY1 mRNA expression levels in FHC cells after overexpression of ANRIL. (P) RNA-RNA pulldown assay to detect the binding between ANRIL and YY1 mRNA. (Q) RIP assay to detect the binding between ANRIL and YY1 protein. (R) FISH-IF assay to detect the co-localization of ANRIL and YY1 protein. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data are expressed as mean ± standard deviation.

demonstrated that Resveratrol can alleviate DSS-induced mouse colitis and LPS-induced inflammation in FHC cells by upregulating ANRIL, which subsequently inhibits the release of inflammatory factors. LncRNA PSCK6-AS1 targets HIPK2 to activate the STAT1 signaling pathway, facilitating the differentiation of Th1 cells, and ultimately exacerbating mucosal barrier damage and tissue inflammation associated with chronic colitis [37]. LncRNA TUG1 mitigates DSS-induced mouse colitis by regulating the balance between HuR and miR-29b-3p [38]. FBXL19-AS1 diminishes the targeting of RHOB by miR-339-3p, thereby intensifying the defect in the intestinal epithelial barrier observed in DSS-induced colitis in mice [39].

To further elucidate the protective role of ANRIL in IBD, transcriptome sequencing analysis revealed a significant enrichment of the NF-κB signaling pathway upon ANRIL overexpression. NF-κB is a crucial transcription factor that regulates the expression of numerous pro-inflammatory genes and plays a central role in IBD pathogenesis [40].

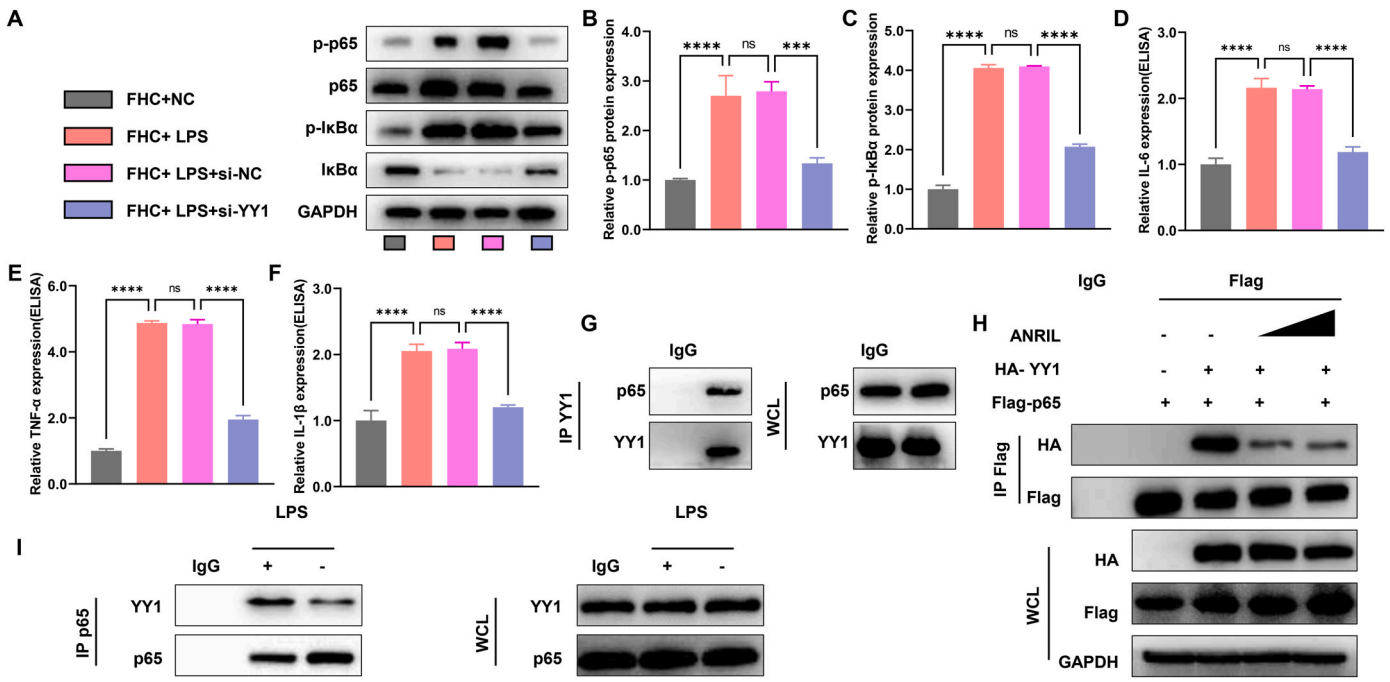

Fig. 5. ANRIL competitively inhibits YY1 binding to p65, thereby suppressing the activation of the NF-κB signaling pathway. (A–C) WB detection of NF-κB signaling pathway-related proteins p65, p-p65, IκBα, and p-IκBα expression levels in the LPS-induced FHC inflammatory cell model after YY1 silencing. (D–F) ELISA detection of IL-6, TNF-α, and IL-1β expression levels in the LPS-induced FHC inflammatory cell model after YY1 silencing. (G) Co-immunoprecipitation assay to detect the binding between YY1 and p65. (H) Co-immunoprecipitation assay to detect the binding between YY1 and p65 after overexpression of ANRIL. (I) Co-immunoprecipitation assay to detect the binding between YY1 and p65 in the LPS-induced FHC inflammatory cell model. **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are expressed as mean ± standard deviation.

In this study, ANRIL was found to downregulate p-p65 and p-IκBα, while simultaneously inhibiting the nuclear translocation of phosphorylated p65, thus suppressing the activation of the NF-κB signaling pathway. However, ANRIL could not bind with either p65 protein or mRNA, suggesting that ANRIL might inhibit the NF-κB signaling pathway indirectly. In the LPS-induced FHC inflammation cell model, ANRIL was shown to promote FHC cell proliferation, suppress the expression of inflammatory factors IL-6, TNF-α, and IL-1β, inhibit cell apoptosis, as well as enhance the expression levels of intestinal barrier-related proteins ZO-1 and Occludin and suppress the expression of Claudin-2. Previous studies have suggested that ANRIL can exert its regulatory roles on biological functions through YY1, SUZ12, and EZH2 [30,31]. In this study, RIP results showed that the YY1 protein could bind to ANRIL and FISH further validated that ANRIL and the YY1 protein primarily colocalized in the cell nucleus. These results suggest that ANRIL can bind to the YY1 protein, thereby regulating the biological functions of FHC cells.

YY1, a universally expressed zinc-finger DNA-binding transcription factor, plays a multifaceted role in a wide range of cellular processes such as cellular proliferation, apoptosis, differentiation, development, and tumorigenesis [41–43]. In this study, silencing YY1 significantly inhibited the activation of the NF-κB signaling pathway and the expression of inflammatory factors induced by LPS in FHC cell. Zhang et al. [44] demonstrated that YY1 forms a complex with p65, subsequently facilitating the transcription of IL-6 in BV2 microglial cells. Han et al. [45] confirmed that the formation of a complex between YY1 and p65/p300 enhances QKI transcription, thereby promoting the malignant progression of hepatocellular carcinoma. Further mechanistic studies found that the YY1 protein could bind to the p65 protein, and the binding of the YY1 and p65 proteins was significantly promoted in the LPS-induced FHC inflammatory cell model. Furthermore, overexpression of ANRIL significantly inhibited the binding of the YY1 protein to the p65 protein. Additionally, co-transfection of ANRIL and YY1 can reverse the inhibitory effects of ANRIL on inflammatory factors and its protective role in intestinal barrier function in IBD. Taken together, ANRIL can target YY1, thereby inhibiting the activation of the NF-κB signaling pathway, reducing the expression of inflammatory factors, and protecting the intestinal barrier function, thereby alleviating IBD.

NF-κB p65, also known as RELA, is a subunit of the NF-κB complex, a key transcription factor involved in regulating immune responses, inflammation, cell proliferation, and apoptosis [46,47]. The activation of NF-κB, particularly p65, has been observed in the mucosa of IBD patients, which is correlated with the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [48,49]. These cytokines can promote inflammation and damage in the intestinal epithelium. Zhou et al. [32] confirmed that p65 can bind to the promoter of ANRIL, promoting ANRIL expression, which in turn triggers an inflammatory response in endothelial cells. The regulatory role of LncRNA in various diseases is complicated. The apparent paradox between p65-induced ANRIL upregulation and its downregulation in IBD may reflect context-dependent regulatory mechanisms. While our data demonstrate that p65 directly binds to the ANRIL promoter to drive its transcription (Figure 7B-D), inflammatory signals such as LPS/DSS likely override this effect through post-transcriptional repression. Potential mechanisms include miRNA targeting (e.g., miR-34a, which destabilizes ANRIL in IBD and Parkinson's disease) or RNA-binding protein-mediated decay [50,51]. This dual regulation highlights the complexity of lncRNA dynamics in disease progression. ANRIL cannot directly regulate p65, but there are many research reports that support the viewpoint of our manuscript [31,32,52]. As for the opposite roles of ANRIL in regulating p65, those publications investigated other diseases, such as diabetic retinopathy [53], coronary artery disease [54] and neuroinflammation [55]. Our study focuses on inflammatory bowel disease. Furthermore, most of their publications do not involve mechanisms [56,57]. Our study focuses on how ANRIL inhibits p65 by targeting YY1. In this study, we identified a negative feedback loop involving ANRIL and p65, wherein p65 binds to the ANRIL promoter, promoting ANRIL expression. This

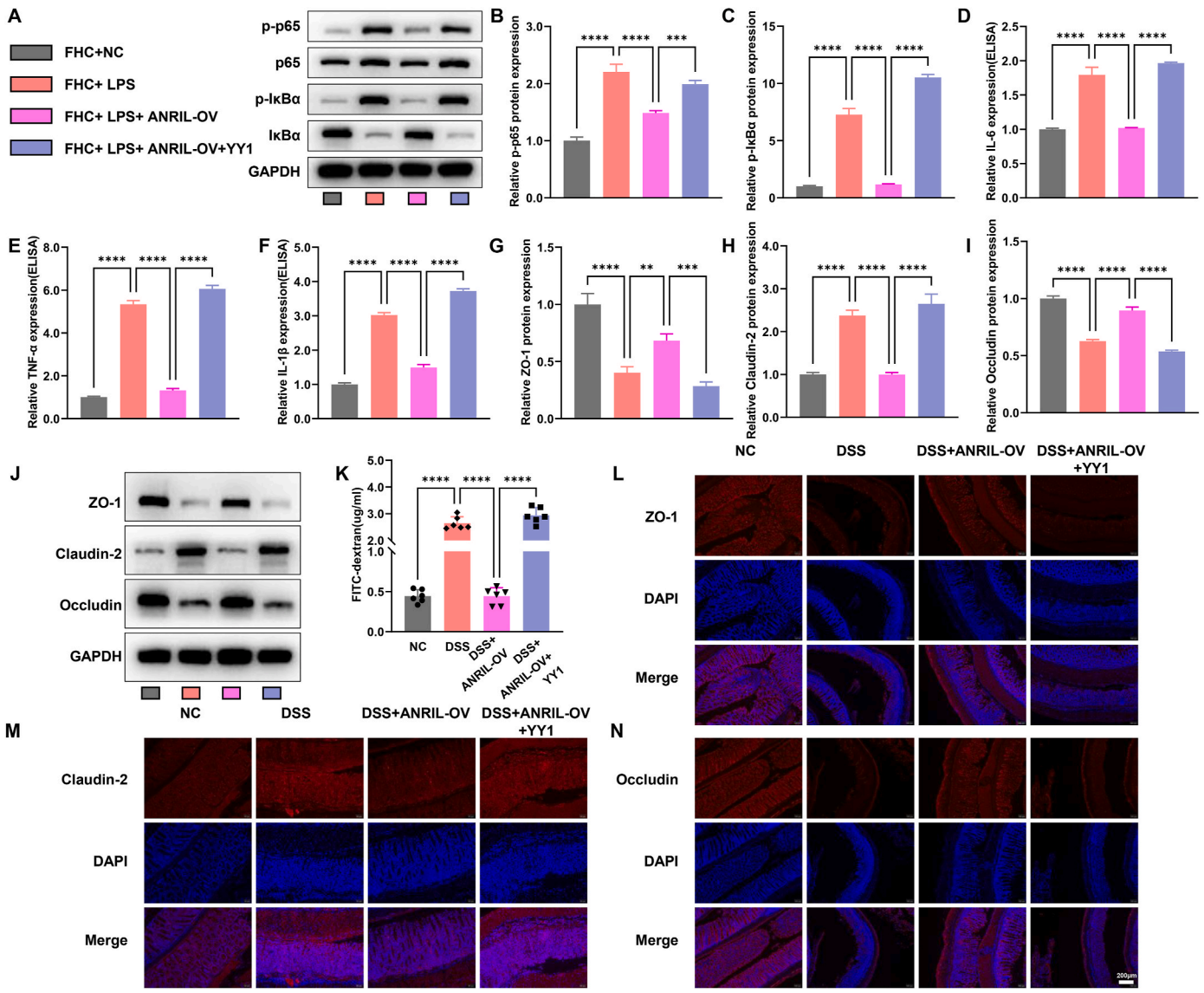

Fig. 6. ANRIL targets YY1 to reduce the expression of inflammatory factors and protecting intestinal barrier function via inhibiting the NF-κB signaling pathway. (A–C) WB detection of NF-κB signaling pathway-related proteins p65, p-p65, IκBα, and p-IκBα expression levels in the LPS-induced FHC inflammatory cell model after co-transfection with ANRIL-OV + YY1. (D–F) ELISA detection of IL-6, TNF-α, and IL-1β expression levels in the LPS-induced FHC inflammatory cell model after co-transfection with ANRIL-OV + YY1. (G–J) WB detection of the expression levels of intestinal barrier-related proteins ZO-1, Claudin-2, and Occludin in the LPS-induced FHC inflammatory cell model after co-transfection with ANRIL-OV + YY1. (K) Assessment of intestinal permeability in DSS-induced mouse colitis after co-transfection with ANRIL-OV + YY1 using the Fluorescein Isothiocyanate (FITC)-Dextran Permeability Assay. (L–N) Immunofluorescence detection of the expression of ZO-1, Claudin-2, and Occludin in colon tissues of DSS-induced colitis mice after co-transfection with ANRIL-OV + YY1. **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are expressed as mean ± standard deviation.

feedback loop likely contributes to the maintenance of intestinal barrier integrity and highlights a self-regulatory mechanism in IBD. The identification of this feedback loop provides further insights into the complex interplay between ANRIL and NF-κB signaling, emphasizing the multifaceted roles of lncRNAs in IBD pathogenesis.

The therapeutic implications of ANRIL in IBD are noteworthy. The restoration of ANRIL expression or the manipulation of its regulatory network, particularly the ANRIL/p65 negative feedback loop, may hold promise as a potential therapeutic strategy for IBD. By targeting this regulatory axis, it may be possible to restore intestinal barrier function, attenuate inflammation, and improve clinical outcomes in patients with IBD. However, further investigations are required to fully elucidate the specific mechanisms underlying ANRIL-mediated regulation and to evaluate the therapeutic potential of targeting this pathway in preclinical and clinical settings.

This study identifies the ANRIL/p65 feedback loop as a potential regulator of intestinal barrier integrity in IBD, yet key limitations must be addressed to advance its therapeutic relevance. The restricted cohort size and predominant focus on ulcerative colitis limit broader applicability, while reliance on simplified models (acute DSS colitis and fetal cell lines) inadequately mirrors human disease complexity. To bridge these gaps, future efforts must prioritize multi-center clinical cohorts encompassing diverse IBD subtypes, advanced models (e.g., patient-derived organoids, chronic immune-driven systems), and multi-omics approaches to map ANRIL's regulatory network. Equally critical is the development of targeted RNA delivery platforms to evaluate ANRIL modulation safely and effectively. By integrating these strategies, the translational potential of ANRIL can progress from conceptual validation to clinically actionable innovation in IBD management.

In conclusion, our study provides novel insights into the functional

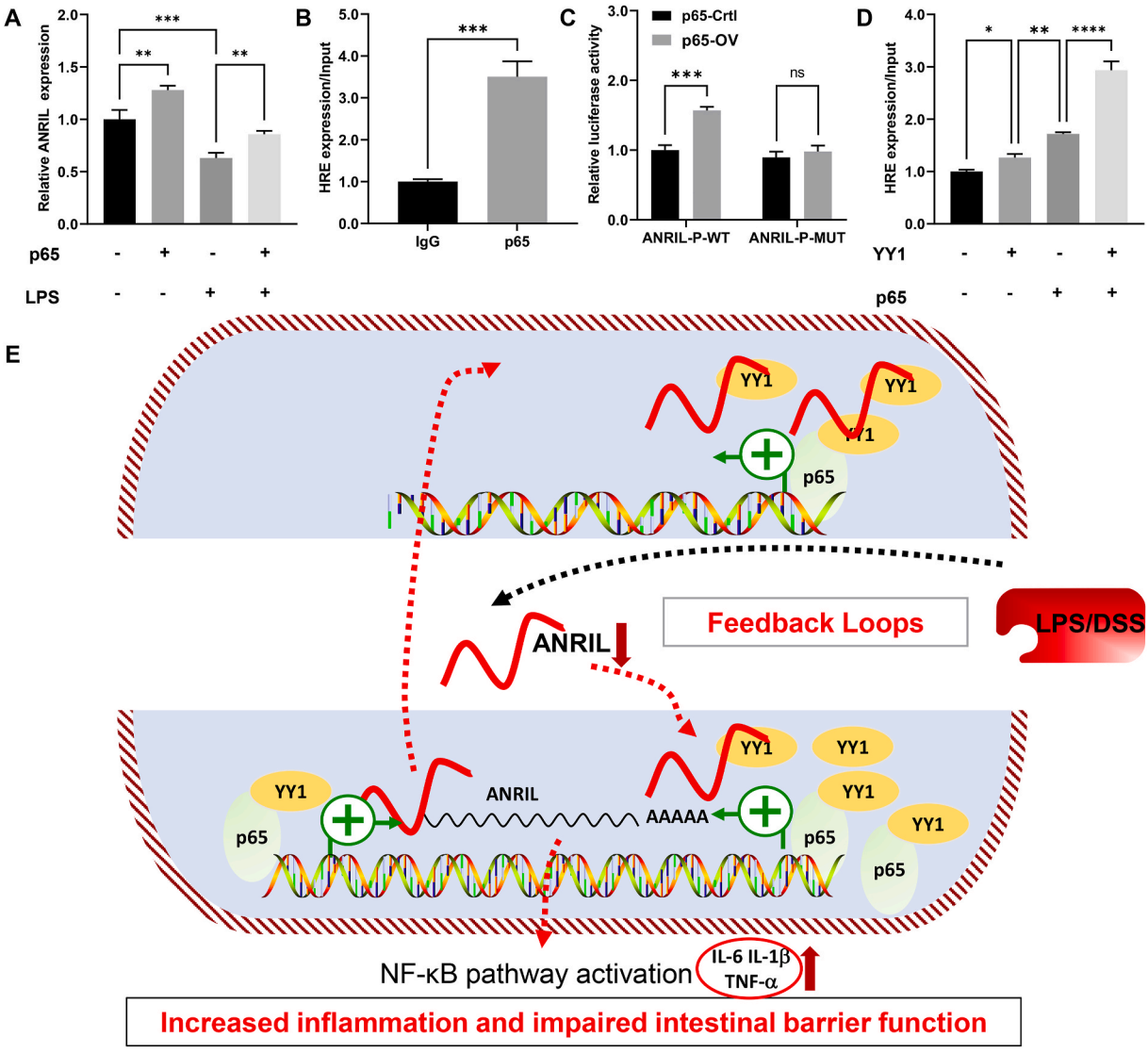

Fig. 7. p65 binds to the ANRIL promoter to promote ANRIL expression. (A) RT-qPCR detection of ANRIL expression levels in FHC cells after overexpression of p65 and/or LPS stimulation. (B–C) Chromatin Immunoprecipitation (ChIP) assay and dual-luciferase reporter assay to detect the binding between p65 and the ANRIL promoter (ANRIL-P). (D) ChIP assay to detect the binding between p65 and the ANRIL promoter after overexpressing YY1 and/or p65. (E) A schematic diagram showing that the ANRIL/p65 negative feedback loop protects intestinal barrier function in IBD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Data are expressed as mean ± standard deviation.

significance of the downregulated lncRNA ANRIL in modulating intestinal barrier function in IBD. The findings highlight the protective role of ANRIL in attenuating inflammation and preserving intestinal integrity through the modulation of NF-κB signaling. The identification of the ANRIL/p65 negative feedback loop further enhances our understanding of the intricate regulatory networks involved in IBD pathogenesis.

CRediT authorship contribution statement

Keqi Yu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Hong Peng:** Resources, Funding acquisition. **Zhechuan Zhang:** Software, Resources. **Lu Ye:** Resources. **Ke Zhan:** Software, Resources. **Chuanfei Li:** Validation, Software, Resources. **Li Gan:** Visualization, Resources. **Yuru Lin:** Resources. **Yanhui Wang:** Resources. **Ya Song:** Resources. **Zhechuan Mei:** Writing – review & editing, Funding acquisition, Data curation. **Shengtao Liao:** Writing – review & editing, Validation, Resources, Funding acquisition. **Jinjun Guo:** Resources, Methodology, Funding acquisition. **Lin Lv:** Writing – review & editing, Resources, Funding acquisition.

Ethical approval and participant consent

The study protocol and informed consent form were reviewed and approved by the Ethics Review Committee of The Second Affiliated Hospital of Chongqing Medical University (approval number: 2021089). Written informed consent was obtained from each patient.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 82173360), China Postdoctoral Science Foundation

(grant no. 2022M720606), Chongqing Science and Health Joint Medical Research Project (grant no. 2024QNXM053), Special support for post-doctoral of Chongqing (grant no. 2022CQBSHTB2064), Natural Science Foundation Project of Chongqing (grant no. CSTB2024NSCQ-MSX0689, CSTB2022NSCQMSX0137, CSTB2022NSCQ-MSX0105 and CSTB2024NSCQ-MSX0403), and Promotion project of health appropriate technology in Chongqing (grant no. 2025cgjstg003).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We sincerely thank Ms. Qingling (Department of Pathology, College of Basic Medicine, and Molecular Medicine Diagnostic & Testing Center, Chongqing Medical University) for her generous financial support and essential reagent contributions to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ncrna.2025.03.002.

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