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ORIGINAL RESEARCH

NRF2 Deficiency in Bladder Epithelial Cells Owing to Ubiquitination by N6-Methyladenosine-Modified TRIM21 Induces Oxidative Stress and Inflammation to Aggravate IC/BPS

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Background: Interstitial cystitis/bladder pain syndrome (IC/BPS) has become a pressing clinical issue due to its unclear etiology and severe, persistent pelvic pain. Despite extensive research, the pathogenesis of IC/BPS remains unresolved, and current treatments primarily target symptom relief rather than addressing underlying disease mechanisms. This study aimed to investigate the effects of nuclear factor erythroid 2-related factor 2 (NRF2) on IC/BPS and the potential molecular mechanisms.

Methods: Bladder mucosal biopsies from IC/BPS patients were subjected to RT-qPCR and immunoblotting to quantify NRF2 mRNA/protein expression. In vivo modeling, WT and NRF2 gene knockout mice received intraperitoneal cyclophosphamide to induce cystitis. Bladder function was assessed via Void Spot Assays, and Urodynamic. In vitro validation, LPS-stimulated SV-HUC-1 cells were transduced with NRF2 knockdown or overexpression, and oxidative stress and inflammation levels were evaluated. Then, the molecular mechanism of NRF2 in IC/BPS was determined by conducting Western blot, mass spectrometry, co-immunoprecipitation, and RT-qPCR analyses.

Results: This study identified markedly reduced expression of NRF2 in the lesional bladder mucosa of patients with IC/BPS. By employing NRF2 knockout mice and cellular models of bladder inflammation, the essential role of NRF2 in modulating oxidative stress and inflammation was underscored. Furthermore, tripartite motif-containing 21 (TRIM21) interacted with NRF2, promoting its degradation via ubiquitination in bladder epithelial cell lines, thus elucidating TRIM21's regulatory role in bladder inflammation. Additionally, N6-methyladenosine (M6A) modifications recognized by IGF2BP2 enhanced TRIM21 expression by stabilizing TRIM21 mRNA.

Conclusion: This study positions the TRIM21-NRF2 axis as a key regulator of oxidative stress and inflammation in IC/BPS and suggests it as a promising therapeutic target for future IC/BPS interventions.

Keywords: IC/BPS, NRF2, TRIM21, ubiquitination, N6-methyladenosine

Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic bladder condition characterized by severe pelvic pain and frequent urination, significantly impairing patients' quality of life.[1] The prevalence of IC/BPS varies widely, from 0.01% to 17.3%, with women exhibiting notably higher rates.[2,3] Currently, the European Society for the Study of Interstitial Cystitis (ESSIC) distinguishes two IC/BPS subtypes based on Hunner's lesions: ESSIC Type 3 (with Hunner's lesions) and ESSIC Types 1 and 2 (without Hunner's lesions).[4] Despite substantial research efforts, the etiology and pathogenesis of IC/BPS remain

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elusive, complicating accurate diagnosis and treatment.[1] Current clinical management focuses on pain control, symptom relief, and quality-of-life enhancement for patients.[5] Several studies have demonstrated a close relationship between IC/BPS and chronic inflammation, glycosaminoglycan barrier deficiency, autoimmune dysregulation, uroepithelial dysfunction, infectious cystitis, and urinary cytotoxicity,[6,7] however, none of these factors fully explain the condition's complex clinical profile or sufficiently inform treatment strategies. Thus, identifying specific mechanisms underlying IC/BPS and developing novel therapeutic options remain essential. Oxidative stress, characterized by an imbalance between the production and clearance of reactive oxygen species (ROS), is pivotal in numerous inflammatory disorders.[8–10] Recent findings indicate that elevated ROS levels may exacerbate IC/BPS progression by altering bladder permeability and impairing normal bladder function.[11,12] Nuclear factor E2-related factor 2 (NRF2), a critical modulator of oxidative stress, regulates the transcription of antioxidant protein-encoding genes, thereby mitigating oxidative damage.[13] Among these genes, Heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase [quinone] 1 (NQO1) serve as key NRF2-targeted antioxidant proteins across various diseases.[14,15] Although evidence increasingly supports the roles of oxidative stress and NRF2 in IC/BPS development,[11,16] the precise mechanisms remain insufficiently understood, as previous studies primarily focused on alleviating symptoms in animal models through pharmacological NRF2 enhancement in vivo without thoroughly exploring the underlying mechanisms;[17] consequently, this study investigates the molecular mechanisms of oxidative stress imbalance by collecting bladder mucosal specimens from IC/BPS patients.

The ubiquitin-proteasome system plays a pivotal role in modulating post-translational protein degradation and transport.[18] E3 ubiquitin ligase dysfunction has been implicated in the onset and progression of numerous inflammatory diseases.[19,20] Tripartite motif-containing 21 (TRIM21), a RING E3 ubiquitin ligase within the TRIM protein family, has been linked to tumor progression through its disruption of tumor suppressor protein expression.[21] Recent studies also indicate that TRIM21 regulates glucose uptake, glycolysis, and oxidative stress, underscoring its role in various inflammatory conditions.[22,23] Nonetheless, the involvement of ubiquitination modifications and TRIM21 in inflammatory bladder disorders, particularly IC/BPS, remains unclarified.

This study demonstrates that TRIM21-mediated NRF2 ubiquitination is essential for bladder epithelial cells to counter inflammation and preserve oxidative stress equilibrium. Additionally, our findings indicate that the N6-methyladenosine (M6A) modification recognized by IGF2BP2 stabilizes TRIM21 mRNA, thereby enhancing its expression. This research unveils a novel IGF2BP2/TRIM21/NRF2 regulatory axis in IC/BPS, proposing potential new therapeutic strategies for this condition.

Methods and Materials

Patients and Tissues

This study was conducted in accordance with the Declaration of Helsinki. With the approval of the Ethics Committee and informed consent from patients, we collected paired lesional and non-lesional bladder mucosa specimens from 16 IC/BPS patients (same individual per patient) at the Second Affiliated Hospital of Nanjing Medical University between 2020 and 2022 (2017KY-102). According to the guidelines of the American Urological Association, the study participants are female patients aged 18 to 70 who were diagnosed with IC/BPS in the absence of infection.[24–26] The inclusion criteria and exclusion criteria for IC/BPS patients were shown in Box S1 and full information about these patients are summarized in Table S1.

Establishment of Animal Cystitis Model

8–10 weeks female wild-type (WT) and gene knockout (KO) C57BL/6J mice were chosen for intraperitoneal injection of cyclophosphamide (CYP, Sigma, USA) at a dose of 150mg/kg body weight.[27] The control group was injected with an equal dose of saline solution. All mice were subjected to the next step of the experiment 24 hours after injection. All animal experiments strictly adhered to the Guidelines for the Ethical Review of Animal Welfare (GB/T 35892–2018) and were approved by the Animal Ethics Committee of Nanjing Medical University (IACUC-2210019).

Void Spot Assays (VSA)

Urine frequency and volume were studied using VSA 24 hours after CYP injection.[28] Each mouse was gently placed into a circular metabolic cage (Yuyan, Shanghai, China) padded with filter paper (Whatman No. 1). The metabolic cage was

placed in a quiet room and the mice were provided with food and water during the experiment. The filter paper was recovered after 2 hours and dried before being imaged.

Measurement of Pelvic Pain Sensitivity

Pelvic pain sensitivity measurements were performed 24 hours after CYP injection. Each mouse was individually acclimatized in metal (Yuyan, Shanghai, China) with a porous bottom for at least 1 hour. Mechanical stimulation was applied vertically to the pelvic region near the bladder and using Dixon's up and down method to calculate the 50% withdrawal threshold.[29] We selected 0.008g, 0.02g, 0.04g, 0.07g, 0.16g, 0.4g, 1g, and 2g fiber filaments (North Coast, America) as a series for the experiment, and abdominal retraction or movement of position or licking of the stimulated site after stimulation was considered as a single positive response. We started the test with 0.07g of fiber, and if the mice showed a negative response to fiber stimulation, they were stimulated with a stronger fiber, and if they showed a positive response, they were stimulated with a smaller fiber.

Urodynamic Measurements

Mice anaesthetized with isoflurane (2%) nebulized 24 hours after CYP injection. After opening the lower abdomen to expose the bladder of the mice, a puncture needle connected to a pressure transducer (Taimeng, Chengdu, China) and a micro syringe pump (Silugao, Beijing, China) was threaded into the bladder. Saline was injected into the bladder at a rate of 2 mL/h to simulate the process of bladder filling and contraction, and the mice's micturition curves were recorded in real time by the software (Taimeng, Chengdu, China).[30]

Cell Culture and Transfection

Human normal bladder epithelial cell line (SV-HUC-1) and HEK293T cells were obtained from the Cell Bank of the Shanghai Institute of the Chinese Academy of Sciences. All these cell lines were cultured in a medium containing 10% FBS (Gibco, USA) and 1% antibiotics (Gibco, USA). At the cellular level, we established an in vitro inflammatory model by stimulating SV-HUC-1 cells with lipopolysaccharide (LPS, Sigma,USA) at 1μg/mL for 24 hours.

After transfection with Lipomaster 3000 (Vazyme, Nanjing, China) for 48–72 hours, the cells were collected for the next experiments. All plasmids used in the study were generated by Genebay (Nanjing, China). The shRNA sequences used are shown in Table S2.

RNA Extraction and Real-Time qPCR (RT-qPCR)

Total RNA was extracted using the FreeZol kit (Vazyme, Nanjing, China) and was reverse transcribed into cDNA for the next RT-PCR using HiScript II SuperMix (Vazyme, Nanjing, China) according to the instructions. Expression of the corresponding genes was analyzed and calculated by the comparative threshold cycling (2-ΔΔCt) method after normalization with β-Actin. The primers used for RT-PCR are shown in Table S3.

Western Blot

Cells and tissues were lysed for proteins using RIPA lysis buffer containing PMSF and protease inhibitors (NCM Biotech, Suzhou, China). Extracted proteins were subjected to SDS-PAGE and then transferred to PVDF membranes (Millipore, USA), and the membrane was incubated with primary antibodies at 4°C. After incubation with secondary antibodies at room temperature, membranes were treated with an ECL kit (Vazyme, Nanjing, China) for visualization. The antibodies used in the study are shown in Table S4.

ROS, Apoptosis and GSH Assay

After the treated cells were collected by centrifugation, they were incubated for 20 min at 37°C (Beyotime, Shanghai, China). Cells were washed and resuspended in phosphate-buffered saline and cellular ROS levels were assessed by flow cytometry.

For cell apoptosis analysis, cells were stained with Annexin V-FITC and PI (Vazyme, Nanjing, China) according to the supplier's protocol and were assessed by flow cytometry.

Relative GSH concentrations were measured spectrophotometrically at 412 nm using a GSH assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Immunoprecipitation (IP)

Cell supernatants lysed in IP Lysis Buffer (Beyotime, Shanghai, China) were incubated with the target antibody in a rotating culture at 4°C overnight. Protein A/G beads (Vazyme, Nanjing, China) were added and then continued to be spun at 4°C for 6 h. After thoroughly washing the A/G beads three times with wash buffer (1 mL), 1X SDS-PAGE buffer was added to the samples and incubated at 100°C for 10 minutes.

RNA Stability Assay

Briefly, 5 μg/mL of actinomycin D (Abmole, USA) was added to cells 48h after transfection with either plasmid, and RNA was collected at the indicated times. Specific RNA expression levels were analyzed and normalized to β-Actin.

Immunohistochemistry (IHC) and Immunofluorescence (IF)

Sections or cells were blocked with 5% BSA for 30 minutes. After incubation with the primary antibody overnight at 4°C, they were incubated with the secondary antibody for 1 h at room temperature, protected from light. IHC was performed using DAB staining, followed by staining of nuclei with hematoxylin, and IF was performed using DAPI to stain the nuclei. The antibodies used in the study are shown in Table S4.

Dot Blot Assay

In brief, the extracted RNA concentrations were diluted to 100ng/μL and 200ng/μL and added to a nylon membrane (Beyotime, Shanghai, China). It was then exposed to UV light for 30 min and blocked with 5% nonfat for 1h. When the membrane was incubated overnight with an M6A antibody, it was incubated in a secondary antibody for 1h and then exposed for imaging. Finally, the amount of total RNA was shown using 0.1% methylene blue.

Statistical Analysis

All data were analyzed using GraphPad Prism 9.0. Data are expressed as mean ± SD of at least three independent experiments. Differences between groups were measured by Student's *t*-test (for two-group comparisons) or one-way ANOVA followed by Tukey's post hoc test (for multi-group comparisons), with $P < 0.05$ considered statistically significant.

Results

NRF2 Protein Expression Is Significantly Decreased in the Lesional Bladder Mucosa of Patients with IC/BPS

To determine the association of NRF2 expression with IC/BPS, NRF2 levels were initially examined in the bladder mucosa of patients with IC/BPS. IHC and Western blotting analyses demonstrated a significantly lower expression of NRF2 in the lesional mucosa compared to non-lesional mucosa distal to the affected area (Figures 1A and B, S1A and S3A).

In Mice and Cellular Models of Inflammation, Lower Levels of NRF2 Expression Clearly Correlate with Poorer Performance

NRF2 function in bladder inflammation was further evaluated in vivo by comparing NRF2 KO and WT mice post-modeling. VSA results indicated that 24 hours following CYP injection, NRF2 KO mice exhibited increased urination frequency and decreased urine volume (Figure 1C). Urodynamic measurements revealed a notably shorter bladder filling phase and reduced urination pressure in NRF2 KO mice compared to WT mice following CYP injection (Figure 1D). The 50% withdrawal threshold assessment indicated heightened sensitivity to pelvic stimulation in the modeled NRF2 KO mice, suggesting more pronounced pelvic discomfort (Figure 1E). Observations of the bladder further revealed that modeled NRF2 KO mice displayed increased redness and swelling relative to WT mice (Figure 1F), and HE staining showed not only increased swelling but also structural damage in KO mouse bladders post-CYP injection (Figure 1G).

To elucidate the role of NRF2 in bladder inflammation in vitro, an inflammatory model was developed by stimulating the bladder epithelial cell line SV-HUC-1 with lipopolysaccharide (LPS). Based on prior studies linking NRF2 with

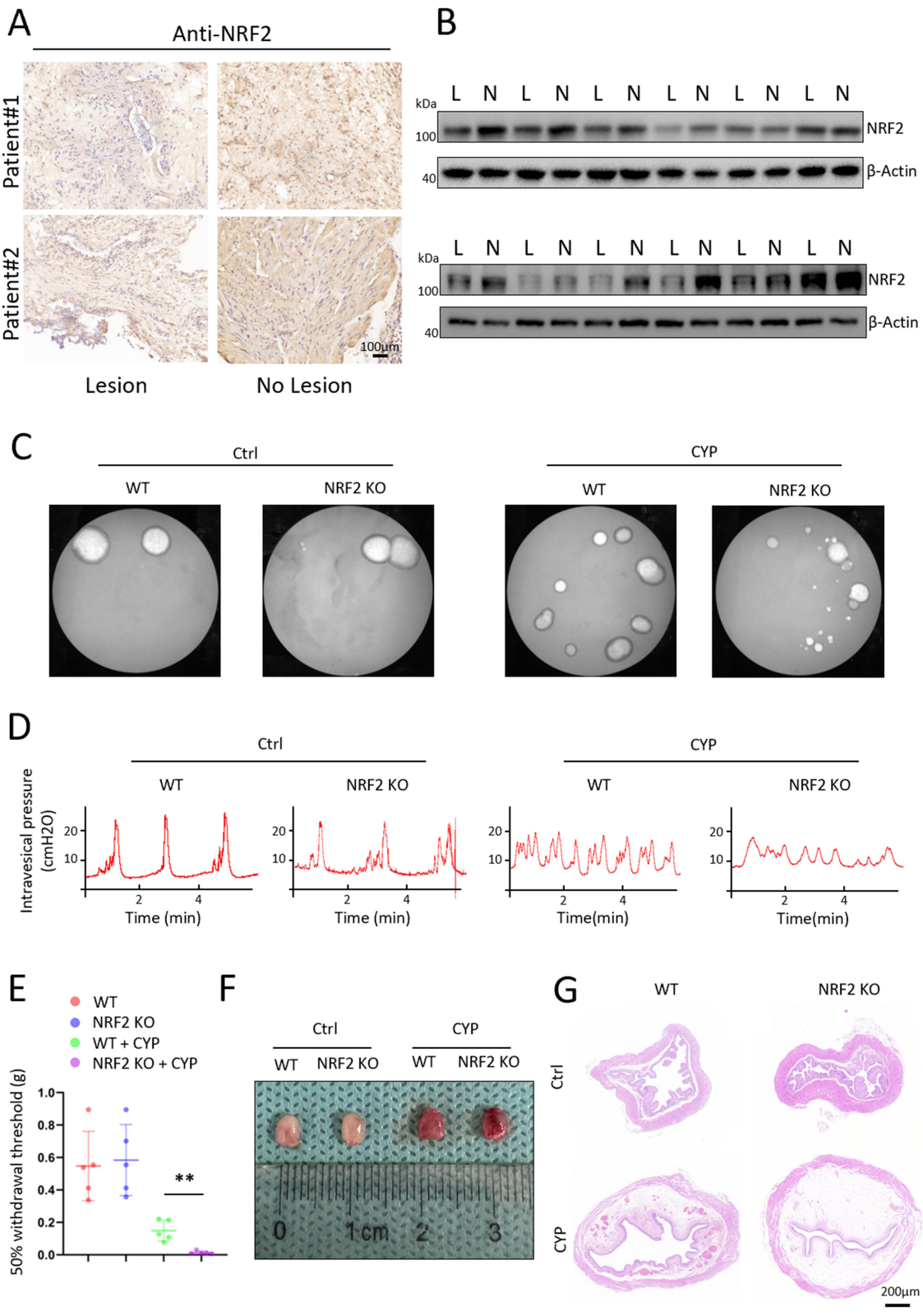

Figure 1 Effect of NRF2 expression on bladder function in the bladder inflammation model. (**A** and **B**) NRF2 protein levels in the lesional and non-lesional bladder mucosa of patients with IC/BPS (Each L and N constituted a paired sample.). (**C**) Urinary blots from WT and NRF2 KO mice, with each bright dot representing a urination event. (**D**) Diagram of the urodynamic examination, where each peak signifies a urination. (**E**) 50% withdrawal threshold of WT and NRF2 KO mice. (**F**) Representative bladder images. (**G**) HE staining of bladder tissues. ** $P < 0.01$.

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oxidative stress and apoptosis,[31,32] the effects of NRF2 knockdown and overexpression on related phenotypes were examined. Results indicated that NRF2 knockdown led to excessive ROS production and significantly reduced glutathione (GSH) levels in the bladder inflammation model, while NRF2 overexpression produced opposite effects (Figure 2A and B). Flow cytometry further revealed an increase in apoptosis upon NRF2 knockdown, whereas NRF2 overexpression reduced apoptotic cell proportions (Figure 2C). Western blotting analysis demonstrated that NRF2 overexpression enhanced HO-1 and NQO1 protein expression, thereby mitigating cellular inflammation and apoptosis, whereas NRF2 knockdown had the reverse impact (Figures 2D,E,S3B and C). Collectively, these results suggest that diminished NRF2 expression compromises its anti-inflammatory capacity at both cellular and organismal levels.

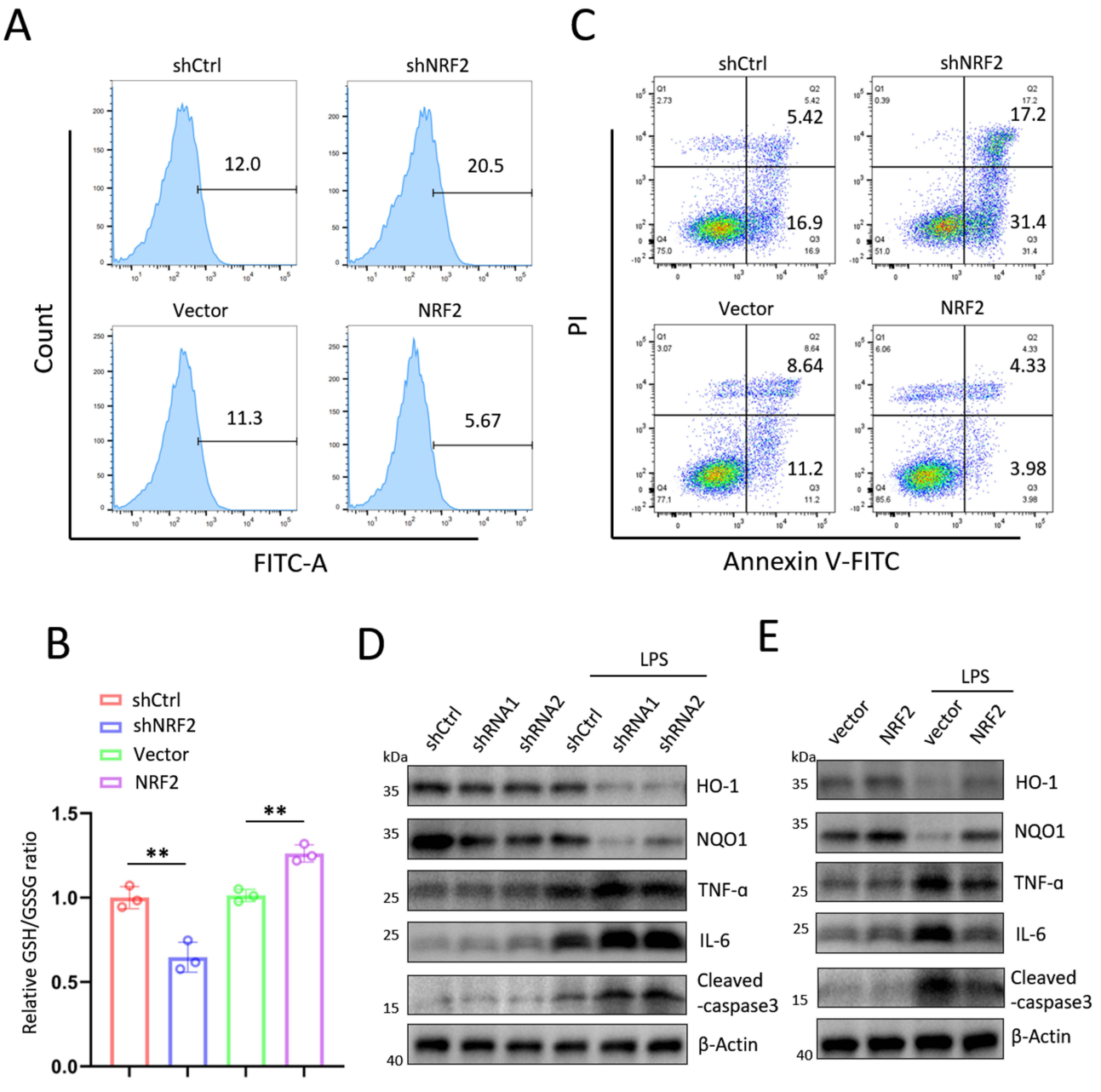

Figure 2 Effects of NRF2 expression on oxidative stress and inflammation in the cellular inflammation model. (**A**) ROS levels in cells transfected with vector, NRF2, shCtrl, or shNRF2. (**B**) Relative GSH/GSSG ratio of cells transfected as indicated. (**C**) Apoptosis rate of cells under the specified transfections. (**D** and **E**) Western blotting analysis of oxidative stress inflammatory and apoptotic indicators in SV-HUC-1 cells following indicated transfections and treatments. ** $P < 0.01$.

TRIM21 Interacts with NRF2

Given NRF2's critical role in mouse and cellular models of bladder inflammation, further exploration of its specific molecular mechanisms was pursued. Unlike protein levels, NRF2 mRNA levels showed no significant difference between lesional and non-lesional bladder mucosa (Figure S1B), suggesting that the reduction of NRF2 in lesional bladder mucosa was not due to altered gene expression but likely stemmed from post-translational protein modification. Previous research has indicated that changes in NRF2 protein expression across various diseases are largely influenced by ubiquitination modifications,[33–35] though less is known regarding inflammatory bladder conditions. To probe this, NRF2 protein synthesis was inhibited in SV-HUC-1 cells using cycloheximide (CHX), and protein expression was evaluated via Western blotting analysis. Findings revealed decreased NRF2 protein expression, which significantly increased following MG132 treatment (Figure S1C), supporting the hypothesis that NRF2 is regulated post-translationally by the ubiquitin-proteasome system.

To identify potential ubiquitin ligases associated with NRF2 degradation, IP and mass spectrometry (MS) analyses were performed. Silver staining and MS identified TRIM21 as a possible direct interactor with NRF2 in bladder epithelial cells (Figure 3A and B). Co-IP and Western blotting analyses further validated this interaction (Figure 3C and D), which was confirmed by fluorescence co-localization (Figure 3E). Molecular docking predictions using the ZDOCK platform suggested that the SPRY domain of TRIM21 was essential for binding (Figure 3F).[36] To verify this, various truncated mutants of Myc-TRIM21 were designed (Figure 3G). Transfection of these truncated mutants into HEK293T cells, followed by co-IP experiments, demonstrated that the SPRY domain is indeed critical for the TRIM21-NRF2 interaction (Figure 3H).

TRIM21 Regulates the Ubiquitination of NRF2

To investigate TRIM21's role as a ubiquitinase affecting NRF2 ubiquitination, HEK293T cells were transfected with Myc-tagged wild-type or ΔRING mutant TRIM21. The results indicated that wild-type TRIM21, but not the ΔRING mutant, reduced NRF2 protein levels in a dose-dependent manner, implying that TRIM21 mediates NRF2 regulation via ubiquitination (Figures S1D,4A and S3D). Additionally, deletion of TRIM21 led to an increase in NRF2 expression (Figure 4B and S3E).

Building on evidence of TRIM21's critical role in NRF2 degradation, further analysis assessed whether TRIM21 directly ubiquitinates NRF2. Co-transfection of HA-UB and Flag-NRF2 with either Myc-TRIM21 or Myc-TRIM21 -ΔRING in HEK293T cells, followed by immunoprecipitation after MG132 treatment, revealed that wild-type TRIM21, but not the mutant, significantly elevated NRF2 ubiquitination levels (Figure 4C). Furthermore, knockdown of TRIM21 using two independent shRNAs in bladder epithelial cells led to a marked reduction in NRF2 ubiquitination (Figure 4D), identifying TRIM21 as a specific ubiquitinase targeting NRF2 in bladder epithelial cells. Lysine 48 (K48) ubiquitination primarily targets proteins for proteasomal degradation, while Lysine 63 (K63) ubiquitination is not linked to proteasome-mediated degradation pathways.[37] Thus, a series of ubiquitin mutants with single Lys residues was generated for further analysis. Co-transfection of Flag-NRF2, Myc-TRIM21, HA-UB, and its mutants demonstrated that TRIM21 overexpression selectively enhanced Lys48 (K48)-linked ubiquitination of NRF2 without affecting Lys63 (K63)-linked ubiquitination (Figure 4E). Moreover, in cells overexpressing TRIM21, forced expression of a Lys48-resistant (K48R) ubiquitin variant led to increased NRF2 levels (Figures 4F, G and S3F). These results indicate that TRIM21 promotes NRF2 degradation through the formation of K48-linked polyubiquitin chains.

TRIM21 Exacerbates Inflammation in Cellular Models

Given the finding that TRIM21 mediates NRF2 degradation through ubiquitination, the role of TRIM21 in bladder inflammation models was further explored. In the bladder cell inflammation model, TRIM21 knockdown reduced excess ROS and increased GSH levels following LPS stimulation, while TRIM21 overexpression led to opposite outcomes (Figure 5A and B). Furthermore, TRIM21 knockdown in this cellular model decreased apoptosis rates induced by LPS stimulation, whereas TRIM21 overexpression increased apoptosis (Figure 5C). Western blotting analysis revealed that TRIM21 knockdown partially restored protein levels of HO-1 and NQO1, thereby attenuating cellular inflammation and

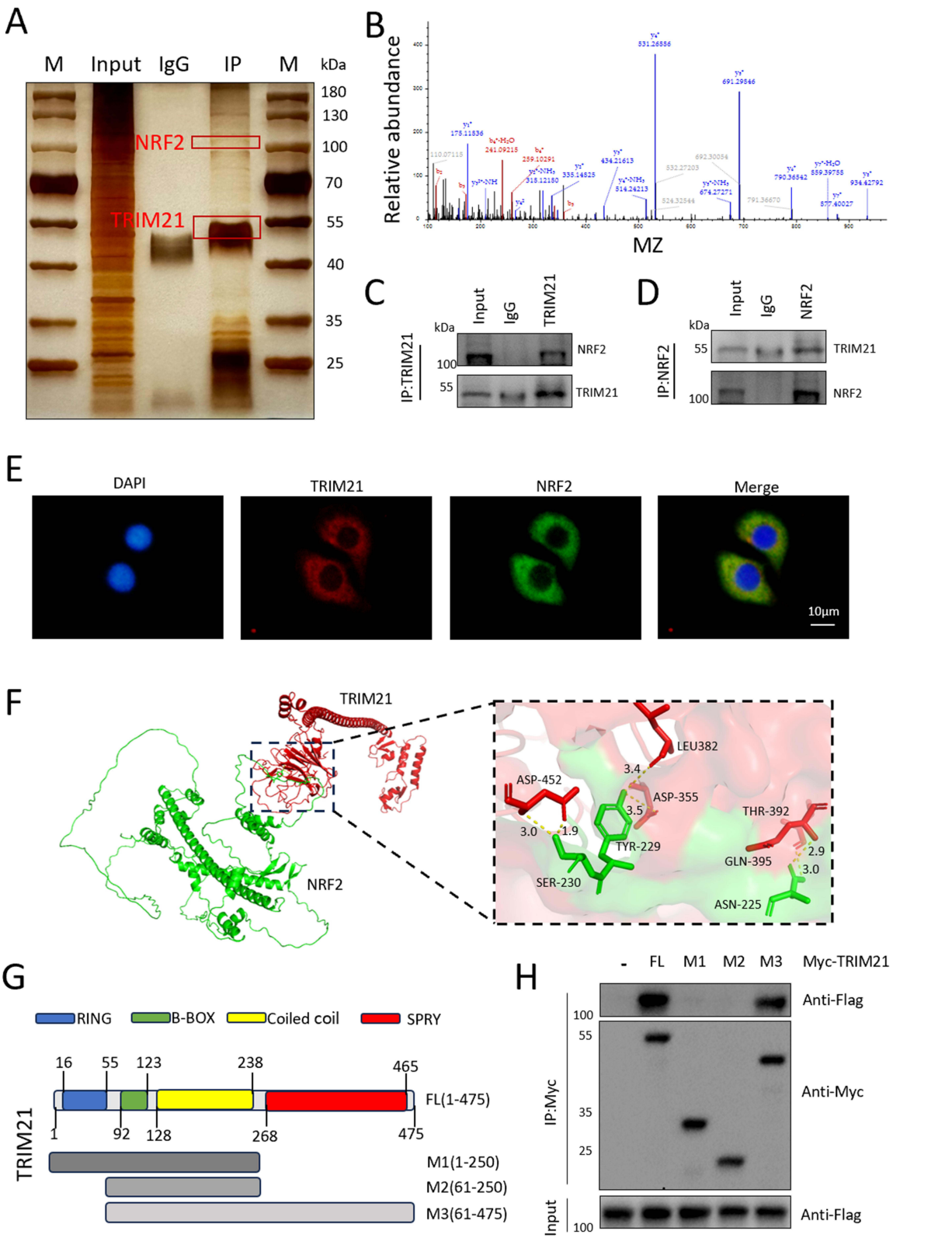

Figure 3 TRIM21 interacts with NRF2. (**A**) Silver staining of SDS-PAGE was performed through IP assays using either IgG or NRF2 antibodies. Red colored items: silver-stained protein bands (**B**) MS analysis confirmed the interaction between TRIM21 and NRF2. y-type ions: C-terminal fragments (blue peaks); b-type ions: N-terminal fragments (red peaks); Modified fragments: y^+-H_2O: Water loss peaks; y^+-NH_3: Ammonia loss peaks. (**C** and **D**) Cell lysates from SV-HUC-1 cells were subjected to IP using antibodies against TRIM21 and NRF2. (**E**) IF images demonstrate the colocalization of TRIM21 and NRF2 in SV-HUC-1 cells. (**F**) Structural domains illustrating the binding of TRIM21 and NRF2 are shown through molecular docking analysis. (**G**) Schematic representation of full-length (FL) Myc-labeled TRIM21 along with various deletion mutants. (**H**) HEK293T cells were co-transfected with the indicated plasmids, and cell lysates were analyzed by IP using Myc beads, followed by Western blotting analysis with specific antibodies.

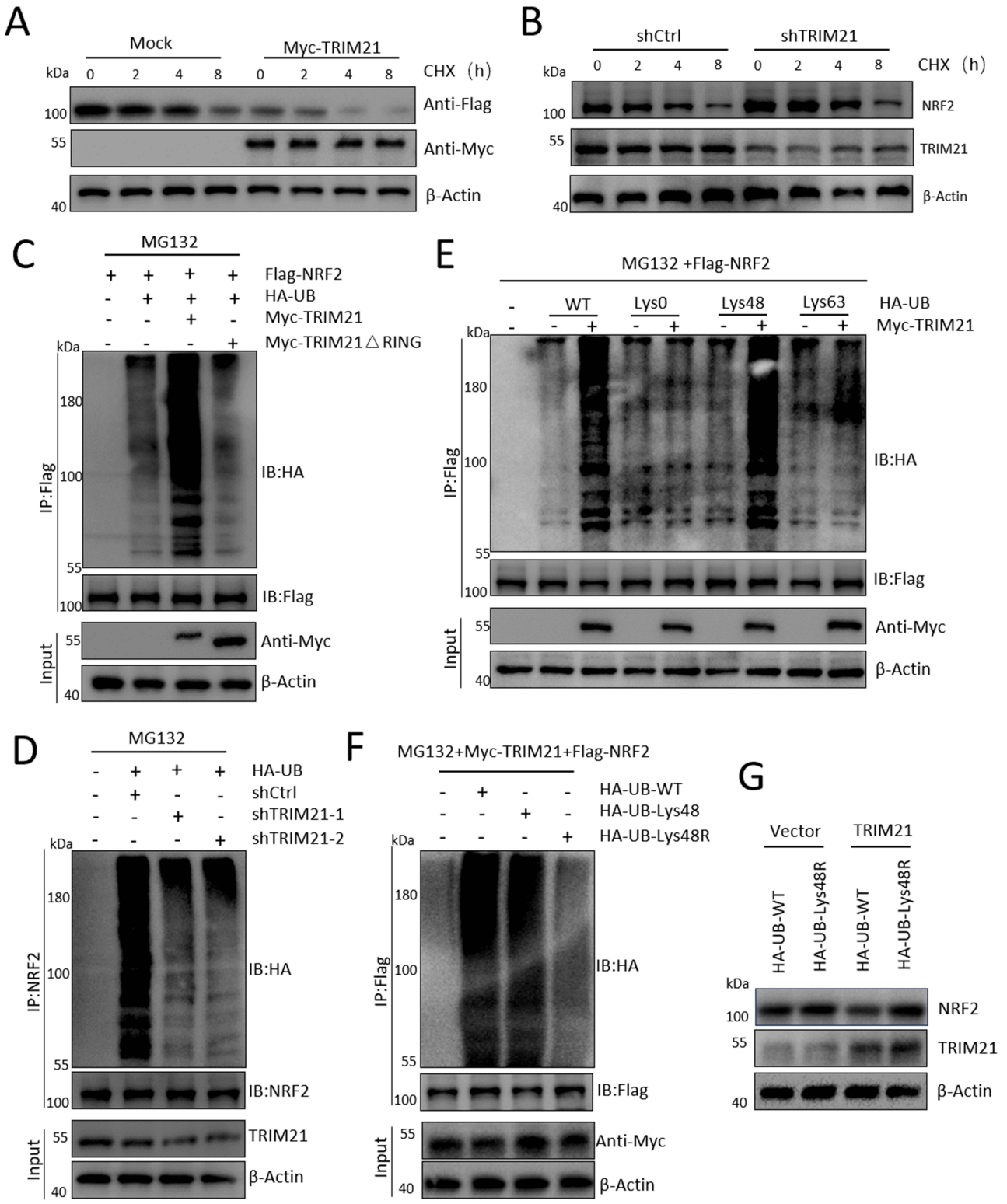

Figure 4 TRIM21 ubiquitinates NRF2. (**A**) HEK293T cells expressing vector or Myc-TRIM21 were treated with CHX (40 μg/mL) and collected at the indicated time, and analyzed by Western blotting. (**B**) SV-HUC-1 cells transfected with control shRNA (shCtrl) or TRIM21 shRNA were treated with CHX, collected at specified times, and subjected to Western blotting analysis. (**C**) HEK293T cells were cotransfected and treated as indicated; cell lysates underwent IP followed by Western blotting analysis. (**D**) SV-HUC-1 cells were cotransfected with the indicated shRNA and HA-ubiquitin (HA-Ub); cell lysates were subjected to IP and subsequent Western blotting analyses. (**E** and **F**) SV-HUC-1 cells were cotransfected as indicated, and NRF2 ubiquitination linkage was analyzed. (**G**) SV-HUC-1 cells transfected with wild-type Ub or Ub-Lys48R were cultured with either control vector or TRIM21; cell lysates were analyzed by Western blotting using specific antibodies. "+": Treated; "-": Untreated.

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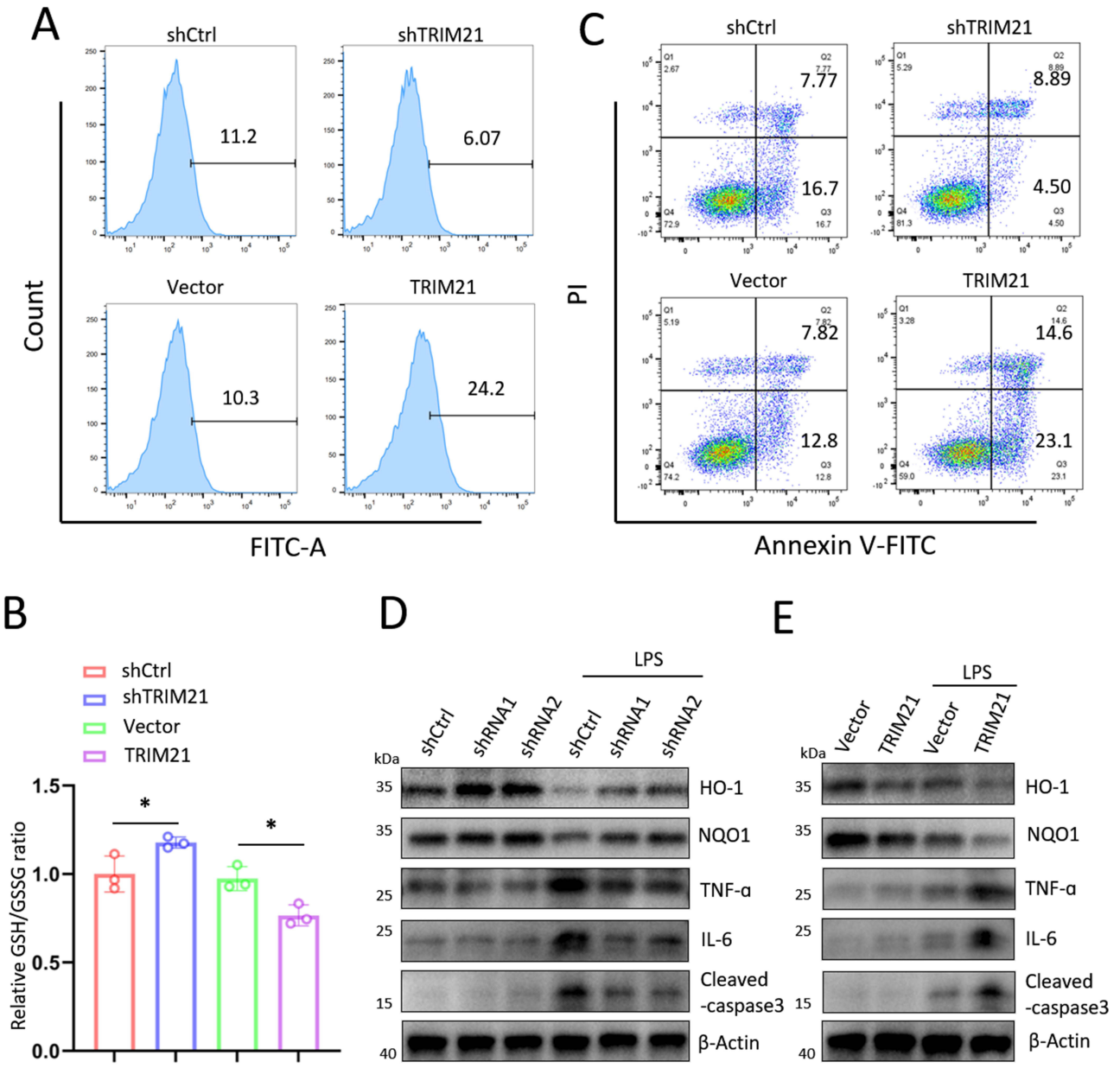

Figure 5 Effect of TRIM21 expression on oxidative stress, and inflammation in the cellular inflammation model. (**A**) ROS levels in cells transfected with vector, TRIM21, shCtrl, or shTRIM21. (**B**) Relative GSH/GSSG ratio of SV-HUC-1 cells transfection as indicated. (**C**) Apoptosis rates of cells transfected as indicated. (**D** and **E**) Western blot detected expression levels of oxidative stress inflammatory and apoptotic indicators in SV-HUC-1 after transfection and treated as indicated. * $P < 0.05$.

apoptosis, whereas TRIM21 overexpression exacerbated these effects (Figures 5D–E,S3G and H). Collectively, these results indicate that TRIM21 knockout mitigates the adverse effects of bladder inflammation on bladder function.

IGF2BP2 Maintains TRIM21 mRNA Stability Through m6A Modification

M6A modifications have been established as significant contributors to various inflammatory and bladder-related diseases.[38–40] However, their involvement in IC/BPS remains undocumented. In this study, dot blot experiments demonstrated elevated levels of M6A modifications in the lesional bladder mucosa of patients with IC/BPS (Figure 6A), accompanied by significant differences in TRIM21 mRNA levels between lesional and non-lesional tissues (Figure S2A). Further analysis utilizing the GEPIA database revealed a positive correlation between the expression of IGF2BP2 and YTHDF1, members of the M6A reader family, and TRIM21 at the mRNA level (Figure S2B and C).

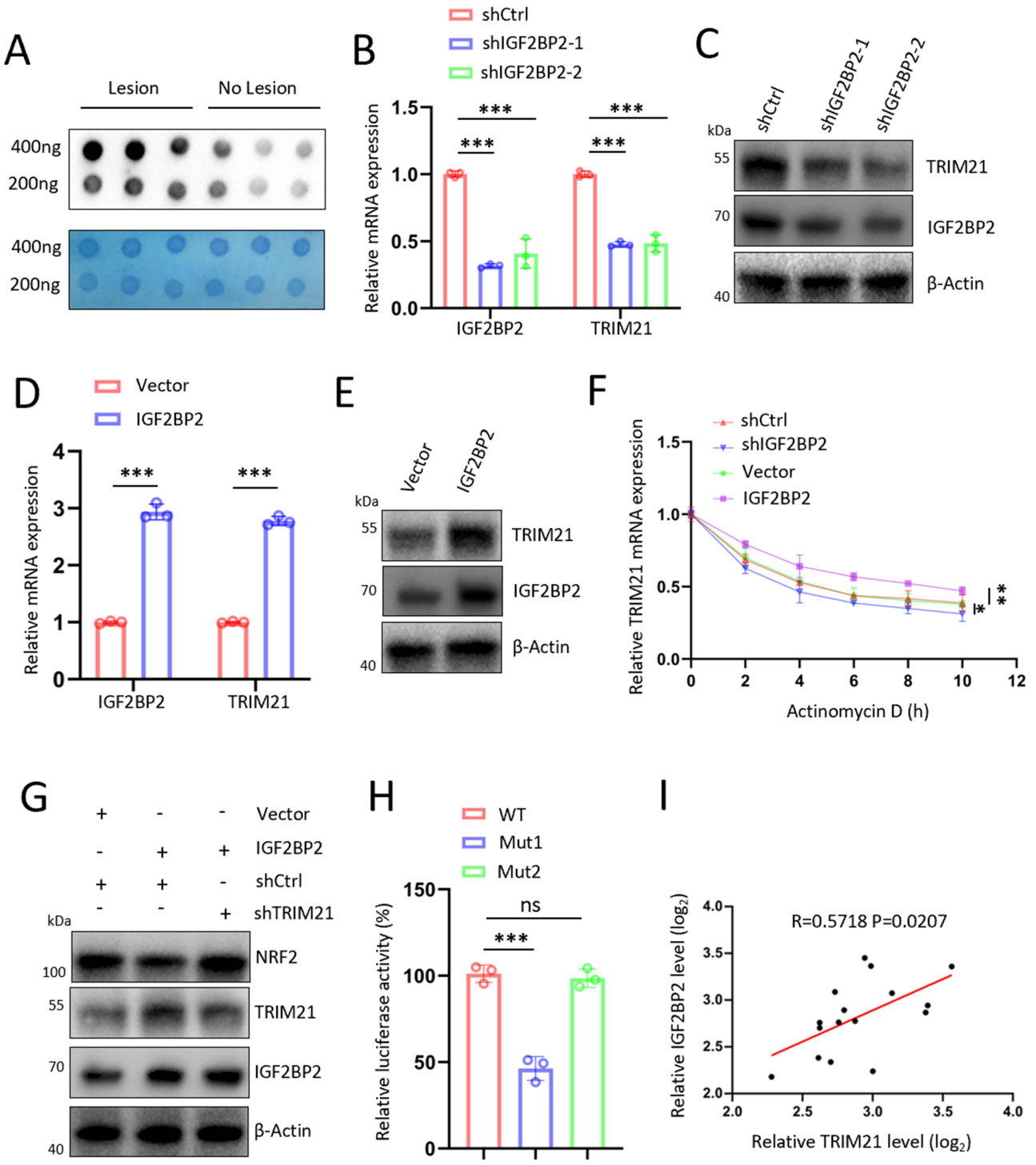

Figure 6 IGF2BP2 maintains TRIM21 mRNA stability through m6A modification. (**A**) Dot blot assay results from lesional and non-lesional bladder mucosa of patients with IC/BPS. (**B** and **C**) RT-qPCR and Western blotting analysis of TRIM21 expression following IGF2BP2 silencing in SV-HUC-1 cells. (**D** and **E**) RT-qPCR and Western blotting analysis of TRIM21 expression after IGF2BP2 overexpression in SV-HUC-1 cells. (**F**) IGF2BP2 enhances TRIM21 stability; SV-HUC-1 cells were treated with Actinomycin D at specified times, and the mRNA half-life of TRIM21 was assessed by RT-qPCR normalized to β-Actin. (**G**) Western blotting analysis of NRF2 following IGF2BP2 overexpression, with or without shTRIM21. (**H**) Luciferase activities of WT and mutated TRIM21 plasmids. (**I**) Positive correlation between IGF2BP2 and TRIM21 expression in the lesional bladder mucosa of patients with IC/BPS. "+": Treated; "-": Untreated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
Abbreviation: ns, not significant.

Detailed examination of IGF2BP2 and YTHDF1 mRNA levels in lesional versus non-lesional bladder mucosa indicated a significant difference for IGF2BP2, while YTHDF1 showed no such difference (Figure S2D and E). This observation led to the hypothesis that M6A modifications recognized by IGF2BP2 play a pivotal role in this disease. Subsequent

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experiments demonstrated that IGF2BP2 knockdown in SV-HUC-1 cells resulted in reduced mRNA and protein levels of TRIM21 (Figures 6B,C and S3I). Conversely, IGF2BP2 overexpression significantly elevated both mRNA and protein levels of TRIM21 (Figures 6D,E and S3J). To further assess TRIM21 mRNA stability, SV-HUC-1 cells were treated with actinomycin D. The results indicated that IGF2BP2 knockdown decreased TRIM21 mRNA stability, whereas overexpression of IGF2BP2 enhanced it (Figure 6F). Additionally, the decrease in NRF2 protein expression due to IGF2BP2 overexpression could be reversed by TRIM21 knockdown (Figure 6G and S3K). Predictive analysis of m6A binding sites using the RGB suite and SRAMP website suggested that IGF2BP2 likely binds to two specific sites in the first segment of the 3′-UTR of TRIM21. Based on this prediction, two mutants were successfully designed (Figure S2F–H). Comparison with the wild-type group revealed that luciferase activity for Mut1 was significantly reduced, while Mut2 remained unchanged, indicating that site 1 is the most critical binding site (Figure 6H). Finally, RT-qPCR analysis confirmed a positive correlation between TRIM21 and IGF2BP2 expression in the lesional bladder mucosa of patients with IC/BPS (Figure 6I). These results suggest that M6A modifications play a vital role in maintaining TRIM21 expression through IGF2BP2-dependent stabilization of TRIM21 mRNA.

Correlation Between IGF2BP2, TRIM21 and NRF2

The clinical relevance of our findings was evaluated through IHC detection of IGF2BP2, TRIM21, and NRF2. The results indicated a significant overexpression of both IGF2BP2 and TRIM21, accompanied by a marked reduction in NRF2 levels within the lesional bladder mucosa of patients with IC/BPS. In contrast, these expression patterns were reversed in the non-lesional bladder mucosa (Figure 7A). Collectively, these results demonstrate that IGF2BP2-dependent M6A modifications lead to elevated TRIM21 expression in patients with IC/BPS, with TRIM21 facilitating the progression of IC/BPS by ubiquitinating NRF2 (Figure 7B).

Discussion

IC/BPS, while classified as a benign bladder condition, has garnered increasing attention from both clinicians and patients due to its debilitating pelvic pain and the absence of a specific treatment.[25] Despite significant research efforts, few advancements have translated into tangible benefits for patients. Oxidative stress is known to play a critical role in various inflammatory diseases, including IC/BPS.[11] However, the precise molecular mechanisms underlying the development of IC/BPS due to oxidative stress imbalance remain unclear. This study identifies the oxidative stress-related protein NRF2 as essential for IC/BPS development, revealing that M6A-modified TRIM21 interacts with NRF2 to regulate its post-translational modification. Notably, this research marks the first identification of the involvement of the E3 ubiquitin ligase TRIM21 and the M6A reader IGF2BP2 in IC/BPS.

Dysregulation of NRF2 signaling has been strongly linked to numerous oxidative stress-related and inflammatory diseases, including liver fibrosis, neurodegeneration, cancer, and IC/BPS.[41–43] Previous studies have primarily focused on alleviating symptoms in animal models of IC/BPS by pharmacologically enhancing NRF2 expression in vivo, yet the underlying mechanisms remain largely unexplored,[17,44] however, in other inflammatory conditions or bladder pathologies, transcriptional regulation and post-translational modifications of NRF2 have been observed to exert pivotal influences on its expression, which are critically involved in disease pathogenesis and progression.[45,46] In this study, specimens collected from patients with clinical IC/BPS revealed significantly decreased NRF2 protein expression in the lesional bladder mucosa, while higher expression levels were noted in non-lesional tissues. This disparity likely contributes to the lesional bladder mucosa's diminished capacity to withstand damage from harmful substances, leading to scattered areas of bleeding and ulceration. Furthermore, through the establishment of mouse and cellular models of bladder inflammation, it was demonstrated that NRF2 plays a pivotal role in maintaining redox homeostasis and enhancing resistance to bladder inflammation. Additionally, this study elucidated that TRIM21, a key ubiquitinase, interacts with NRF2, subsequently reducing its protein expression and impairing its function in sustaining redox balance. Ubiquitination is a post-translational modification process that governs protein activity and function, playing a critical role in the progression of various inflammatory diseases.[47,48] TRIM21, a member of the TRIM protein family and a RING E3 ubiquitin ligase, regulates the ubiquitination of multiple proteins and has been implicated in several oxidative stress-related and inflammatory conditions. For instance, TRIM21 ubiquitinates NOX2 to amplify oxidative inflammation

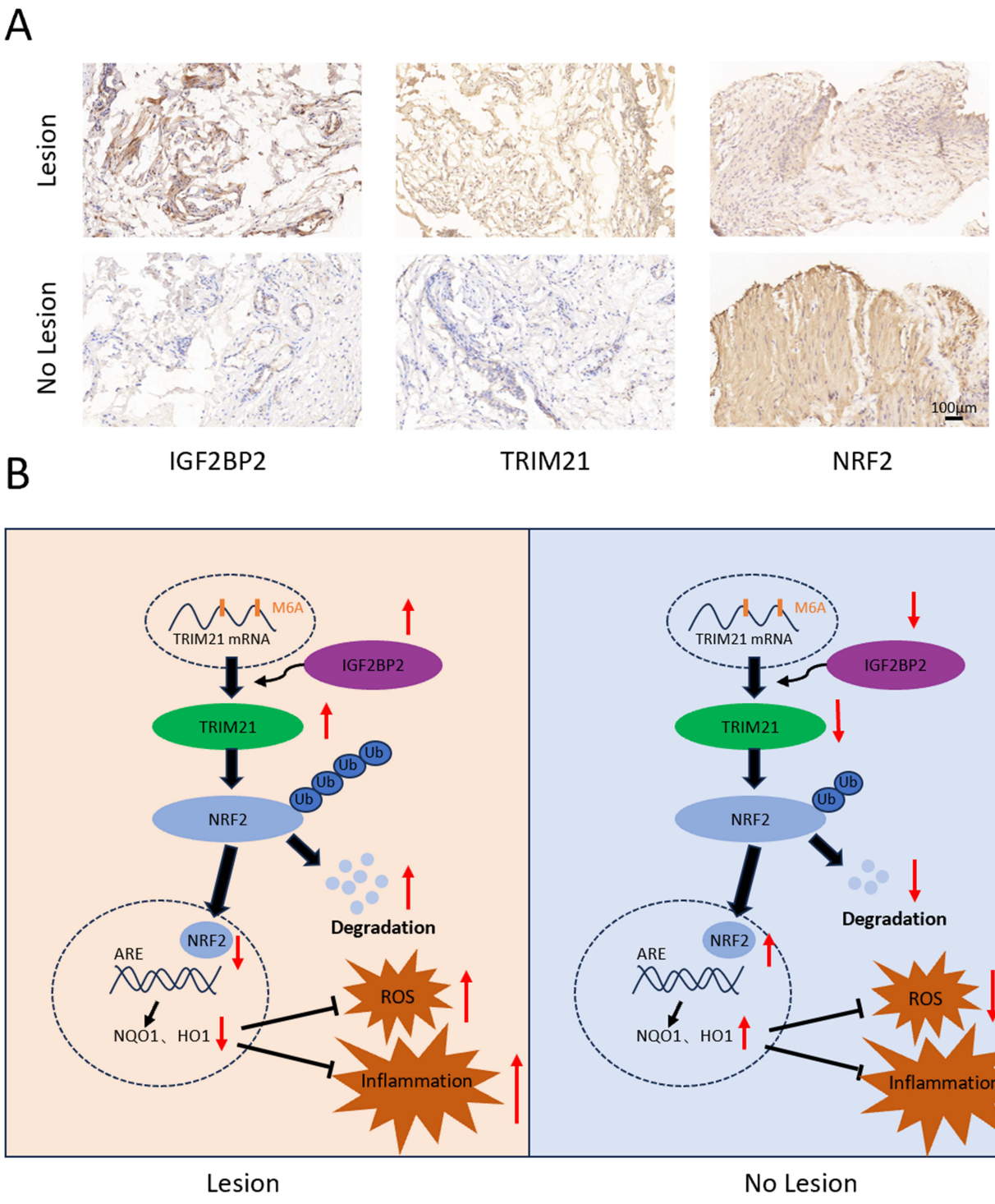

Figure 7 Correlation between IGF2BP2, TRIM21, and NRF2. (**A**) Representative IHC images of IGF2BP2, TRIM21, and NRF2 of clinical patients with IC/BPS. (**B**) The mechanistic scheme of our study.

in the heart, modifies TRPM2 to contribute to airway inflammation, and interacts with SIRT5 to modulate colonic inflammation.[49–51] This study identifies TRIM21 as a novel regulator of NRF2, facilitating NRF2 degradation through ubiquitination and thereby accelerating the development of IC/BPS.

As the most prevalent mRNA modification in humans, M6A modification plays a key role in regulating RNA structure and stability, significantly impacting the progression of various inflammatory diseases.[38,52] However, the role of M6A

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modification in inflammatory bladder diseases, particularly IC/BPS, has been underexplored. This study establishes a link between M6A modification and IC/BPS, revealing that IGF2BP2 is highly expressed in the bladder mucosa of patients with IC/BPS. IGF2BP2 enhances TRIM21 expression by stabilizing TRIM21 mRNA, resulting in decreased NRF2 levels.

However, our study has several limitations. First, the etiology and pathogenesis of IC/BPS remain incompletely understood, precluding the availability of disease-specific animal or cellular models. Consequently, we adopted surrogate inflammation models – CYP-induced cystitis in vivo and LPS stimulation in vitro – to validate the protective role of NRF2. While CYP-induced cystitis effectively recapitulates key IC/BPS clinical features (urinary frequency, pelvic pain, and bladder hyperemia),[53] and both CYP/LPS models induce ROS overproduction mirroring human disease mechanisms,[11] they primarily represent acute inflammatory states. Chronic IC/BPS involves complex neuroimmune interactions and tissue remodeling processes not fully captured here.[54] Our study focused on elucidating the IGF2BP2-TRIM21-NRF2 regulatory axis – a pathway conserved across acute/chronic inflammation – as validated by human tissue analyses. Future work will employ chronic models (eg, repeated low-dose CYP or autoimmune cystitis) to examine long-term adaptations of this axis. Additionally, the limited cohort size (n=16 patients) reflects inherent challenges in studying IC/BPS: low prevalence rates, stringent inclusion/exclusion criteria, and ethically constrained recruitment. We will continue collecting IC/BPS specimens for further validation.

In conclusion, our findings demonstrate a significant reduction in NRF2 expression in the lesional bladder mucosa of patients with IC/BPS, identifying NRF2 as a critical regulator in cellular and mouse models of bladder inflammation. Furthermore, this research marks the first identification of the roles of ubiquitination and M6A modifications in IC/BPS. Specifically, the M6A modification recognized by IGF2BP2 increases the stability and expression of TRIM21 mRNA, leading to excessive TRIM21-mediated degradation of NRF2 protein through ubiquitination. These insights provide new perspectives on the pathogenesis of IC/BPS and suggest potential strategies for therapeutic intervention.

Data Sharing Statement

All data necessary for confirming the conclusions are included in this article. The data employed in this investigation are accessible from the corresponding author upon justified request.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki. The collection of specimens was approved by the Ethics Committee of the second Affiliated Hospital of Nanjing Medical University (2017KY-102), and the samples involved in this study were obtained with written informed consent from patients or their families. All animal experiments strictly adhered to the Guidelines for the Ethical Review of Animal Welfare (GB/T 35892-2018) and were approved by the Animal Ethics Committee of Nanjing Medical University (IACUC-2210019).

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Author Contributions

Z.F., Q.G., and B.N. contributed equally to this work. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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