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The inhibition of HACE1 ameliorates inflammatory responses in citrobacter rodentium-induced murine colitis through Nrf2/ NLRP3 signaling pathway

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Abstract

This study was intended to elucidate the effects of HACE1 in Citrobacter rodentium-Induced Murine Colitis. Male C57BL/6 mice orally administered with 100 μLof *Citrobacter rodentium* DBS-100 (109 CFU mL^{-1}) for 14 days. The expression of HACE1 in Citrobacter rodentium-Induced Murine Colitis was increased. HACE1 promoted inflammation and ROS levels in macrophage through the inactivation of Nrf2/NLRP3 signaling pathway. Meanwhile, si-HACE1 reduced inflammation and ROS levels in macrophage via the inhibition of Nrf2/NLRP3 signaling pathway. Sh-HACE1 reduced inflammatory responses in Citrobacter rodentium-Induced Murine Colitis through the promotion of Nrf2/NLRP3 signaling pathway. IP showed that HACE1 protein catenated with Nrf2 protein to suppress ROS-induced NLRP3 in macrophage through the promotion of Nrf2 Ubiquitination. Altogether, our data demonstrate that the inhibition of HACE1 ameliorates

inflammatory responses in Citrobacter rodentium-induced Murine Colitis through the inhibition of ROS-induced mitochondrial damage by Nrf2/NLRP3. m6A promoted HACE1 stability by METTL3. Our study revealed the m6A methyltransferase METTL3 promoted HACE1 stability to promote NLRP3 activity in Citrobacter rodentium-Induced Murine Colitis by the inhibition of Nrf2.

Keywords: HACE1; NLRP3; m6A; METTL3; colitis.

Practical Application: HACE1 may lead to therapeutic potential of inflammation associated carcinogenesis for Colitis.

1 Introduction

Ulcerative colitis is a chronic disease characterized by persistent inflammation of mucosa and submucosa (Beukema et al., 2021; Yu et al., 2022). It is difficult to cure and easy to relapse due to the disorder of immune regulation (Flowers et al., 2021). At present, the disease has become the main cause of common digestive system diseases and chronic diarrhea, and most of the patients are young and middle-aged people With the development of economic level, the number of patients with infectious bowel diseases (IBD) is gradually increasing (Park et al., 2018). When the proportion of normal bacteria and pathogenic bacteria is out of balance, the body is prone to infection or inflammation. There are many factors affecting intestinal flora, among which environmental factors, especially diet, are the main factors affecting the structure of intestinal flora (Mao et al., 2021).

In the study of the pathogenesis of IBD disease, C. rodentium infection is one of the commonly used models, which can be used to observe the impact of intestinal host and environmental factors on its susceptibility (Wu et al., 2021; Zhang et al., 2021b). The ability of the host to resist C. rodentium infection is related to intestinal flora, intestinal epithelial cells and immune cells (Zhang et al., 2021c). Among them, intestinal flora is a recent research hotspot, which may provide new ideas and methods for clinical treatment. C. Rodentium and segmented filamentous bacteria (SFB) may enhance host resistance to C. rodentium by promoting cytokine production; Lachnospiraceae bacteria may promote the production of short chain fatty acids, thus improving the host resistance to C. rodentium infection; Akkermansia muciniphila may weaken the host's resistance to C. rodentium infection by degrading the intestinal mucus layer and destroying the intestinal epithelial barrier (Määttänen et al., 2020).

HACE1 is a tumor suppressor gene, which can inhibit tumor cell growth and promote tumor cell apoptosis (Li et al., 2019). HACE1 is not only an important tumor suppressor gene, which plays an important tumor suppressor role by mediating autophagy, ubiquitination of Rac1 and other mechanisms of action, but also involves many biological functions, playing a key role in cardiac protection, antioxidant stress and cytological dynamics (Mao et al., 2016; Tortola et al., 2016; Zang et al., 2022). This study was intended to elucidate the effects of HACE1 in i Citrobacter rodentium-Induced Murine Colitis.

2 Materials and methods

2.1 Colitis mice model

The present study was approved by the Animal Care and Use Committee of our hospital. Male C57BL/6 mice orally administered with 100 μLof *Citrobacter rodentium* DBS-100 (109 CFU mL^{-1}) for 14 days.

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2.2 Cell culture and cell transfection

RAW264.7 cells were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) in an incubator at 37 °C with 5% CO2. RAW264.7 cells were transfected with UAF1 plasmid, si-UAF1 plasmid using Lipofectamine 3000. After 48 h, RAW264.7 cells treated with 200 ng/mL of LPS for 4 h and then pulsed with ATP (1 mM, Sigma-Aldrich, MO, USA) for 30 min.

2.3 Histological analysis, immunohistochemistry, and immunofluorescence

Colon tissue samples were fixed in 4% paraformaldehyde, and executed histological analysis, immunohistochemistry, and immunofluorescence according to references (Pu et al., 2019; Zhang et al., 2021a).

2.4 Real-time PCR

Total RNAs were isolated with RNA isolator total RNA extraction reagent (Takara) and cDNA was synthesized using PrimeScipt RT Master Mix (Takara). qPCR were performed with the ABI Prism 7500 sequence detection system according to the Prime-ScriptTM RT detection kit. Relative levels of the sample mRNA expression were calculated and expressed as 2-DDCt.

2.5 ELISA kits

Tissue or cell samples in each group were collected at 2000 g for 10 min at 4 °C. IFNγ, TNFα, MPO, IL-17, IL-6 and IL-1β kits were used to measure the cytokine levels

2.6 Western blot

Western blot was performed as previously described (Pu et al., 2022). Membranes were incubated with HACE1, Nrf2, NLRP3 and β-Actin (BS6007MH, 1:5000, Bioworld Technology, Inc.) at 4 °C overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (sc-2004 or sc-2005, 1:5000, Santa Cruz, USA) for 1 h at 37 °C after washing with TBST for 15 min. Protein was measured using an enhanced chemiluminescence system (ECL, Beyotime) and analyzed using an Image Lab 3.0 (Bio-Rad Laboratories, Inc.).

2.7 Coimmunoprecipitation assay

The ChIP-qPCR experiment was performed as previously described (Xu et al., 2021). Cells were treated with RIP lysis buffer, supernatant was enriched with antibody- or rabbit IgG-conjugated protein A/G magnetic beads in IP buffer supplemented with RNase inhibitors and incubated overnight at 4 °C.

2.8 Statistical analysis

Data were represented as mean ± standard error of the mean (SEM). Student's t test and one way ANOVA test were used for statistical analyses of the data. A p-value less than 0.05 is considered with significant difference.

3 Results

3.1 HACE1 expression was up-regulated in citrobacter rodentium-induced murine colitis

This experiment investigated the expression level of HACE1 in Citrobacter rodentium-Induced Murine Colitis. HACE1 mRNA and protein expressions were up-regulated in citrobacter rodentium-induced murine colitis (Figure 1A, 1B). HACE1 mRNA and protein expressions were up-regulated in LPS+ATP-induced RAW264.7 cells (Figure 1C, 1D).

3.2 The inhibition of HACE1 ameliorates inflammatory responses in citrobacter rodentium-induced murine colitis

This experiment investigated the functions of HACE1 in Citrobacter rodentium-Induced Murine Colitis. HACE1$^{-/-}$ mice increased weight, reduced DAI score and ulcer area, expanded colon length, and inhibited inflammation levels in Citrobacter rodentium-Induced Murine Colitis (Figure 2).

3.3 The inhibition of HACE1 restored the function of intestinal epithelial cells in citrobacter rodentium-induced murine colitis

This experiment examined the effects of HACE1 participates in the function of intestinal epithelial cells of colitis. HACE1$^{-/-}$ mice increased goblet cell function and mucosal integrity in Citrobacter rodentium-Induced Murine Colitis (Figure 3A-3D). ACE1$^{-/-}$ mice promoted Muc-2 levels and crypt length, increased ratio of sulfomucin to sialomucin and the number of Sulfomucin positive goblet cells, and inhibited the mRNA of CXCL1 and CXCL2 in colon tissue of Citrobacter rodentium-Induced Murine Colitis (Figure 3E, 3I). These results strongly suggest that HACE11 might be participated in the disease progression of Citrobacter rodentium-Induced Murine Colitis.

3.4 HACE1 promoted inflammation and ROS- oxidative stress in vitro model

Further, we next investigated the function of HACE1 regulates inflammation and ROS- oxidative stress in vitro model. HACE1 levels was up-regulated in HACE1 group, and HACE1 up-regulation promoted inflammation levels and ROS- oxidative stress in vitro model (Figure 4A-4E). Si-HACE1 reduced HACE1 levels in si-HACE1 group, and down-regulation of HACE1 reduced inflammation levels and ROS- oxidative stress in vitro model (Figure 4F-4J). Data suggests that UAF1 promoted inflammation and ROS- oxidative stress in vitro model.

3.5 HACE1 suppressed Nrf2/NLRP3 signaling pathway

The study investigated that the pro-inflammation mechanism of HACE1 in colitis using Microarray analysis (Figure 5A). KEGG showed that Nrf2 might be one target spot for HACE1 (Figure 5B). The activation of Nrf2 protein expression, and the inhibition of Nlrp3 protein expression in HACE1$^{-/-}$ mice (Figure 5C). In general, data suggests that Nrf2 is one important targets of HACE1 in Citrobacter rodentium-Induced Murine Colitis.

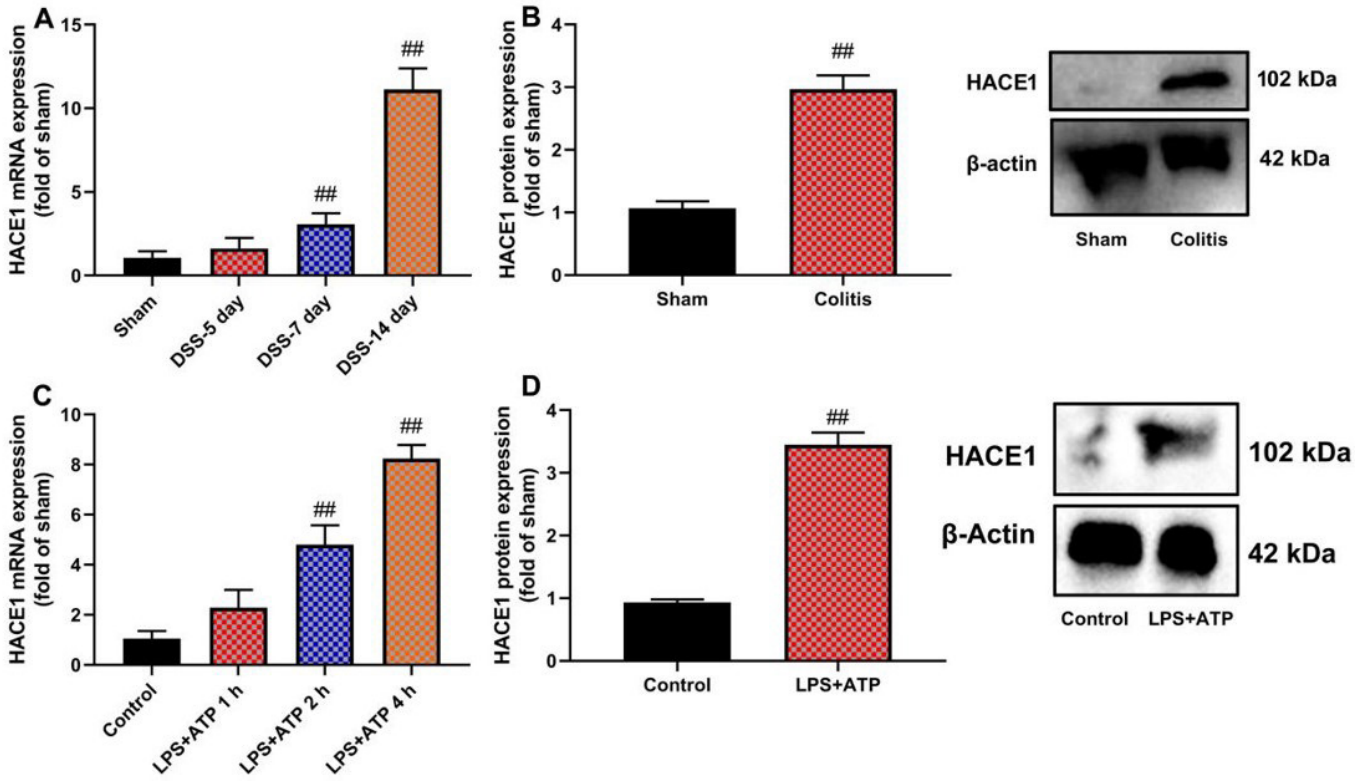

Figure 1. HACE1 expression was up-regulated in Citrobacter rodentium-Induced Murine Colitis. HACE1 mRNA and protein expression in colon tissue (A and B) in Citrobacter rodentium-Induced Murine Colitis; HACE1 mRNA and protein expression (C and D) in vitro model. ##p<0.01 compared with sham or control group.

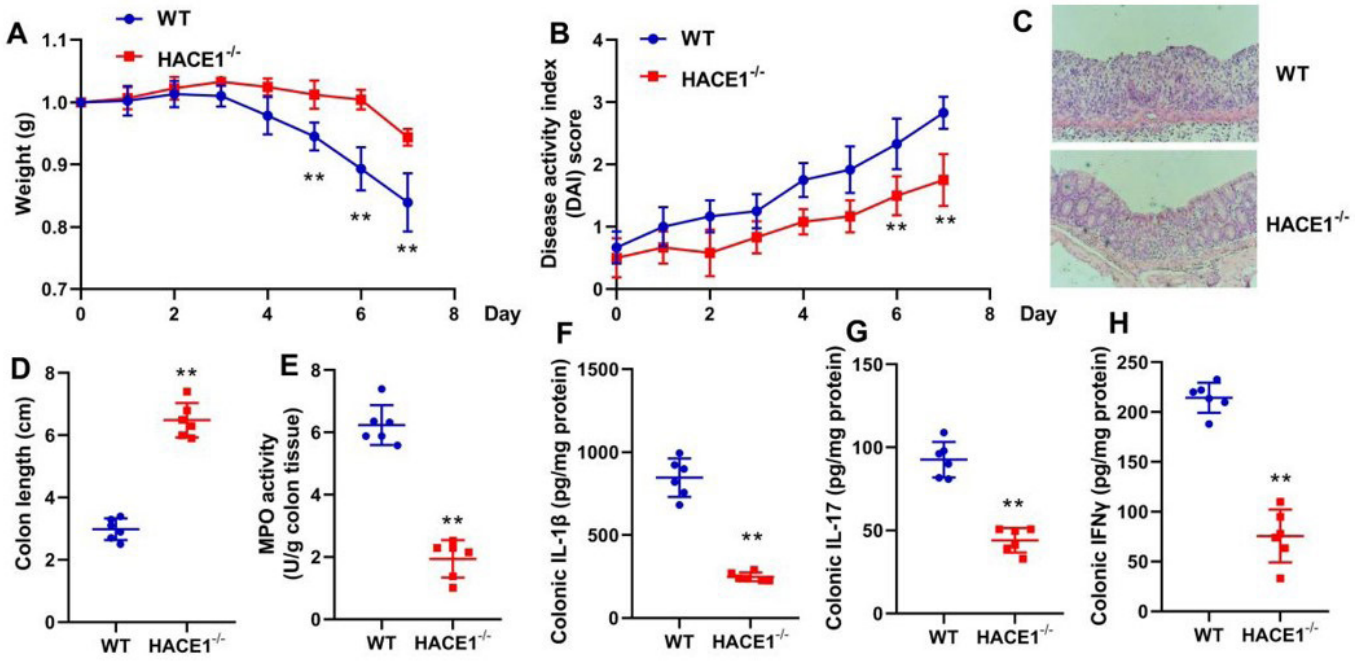

Figure 2. The inhibition of HACE1 ameliorates inflammatory responses in Citrobacter rodentium-Induced Murine Colitis. Weight (A), DAI score (B), Ulcer area (HE staining, C), crypt length (D), MPO activity level (E), IL-1β/IL-17/IFN-α levels (F, G, H) in mice model. **p<0.01 compared with WT group.

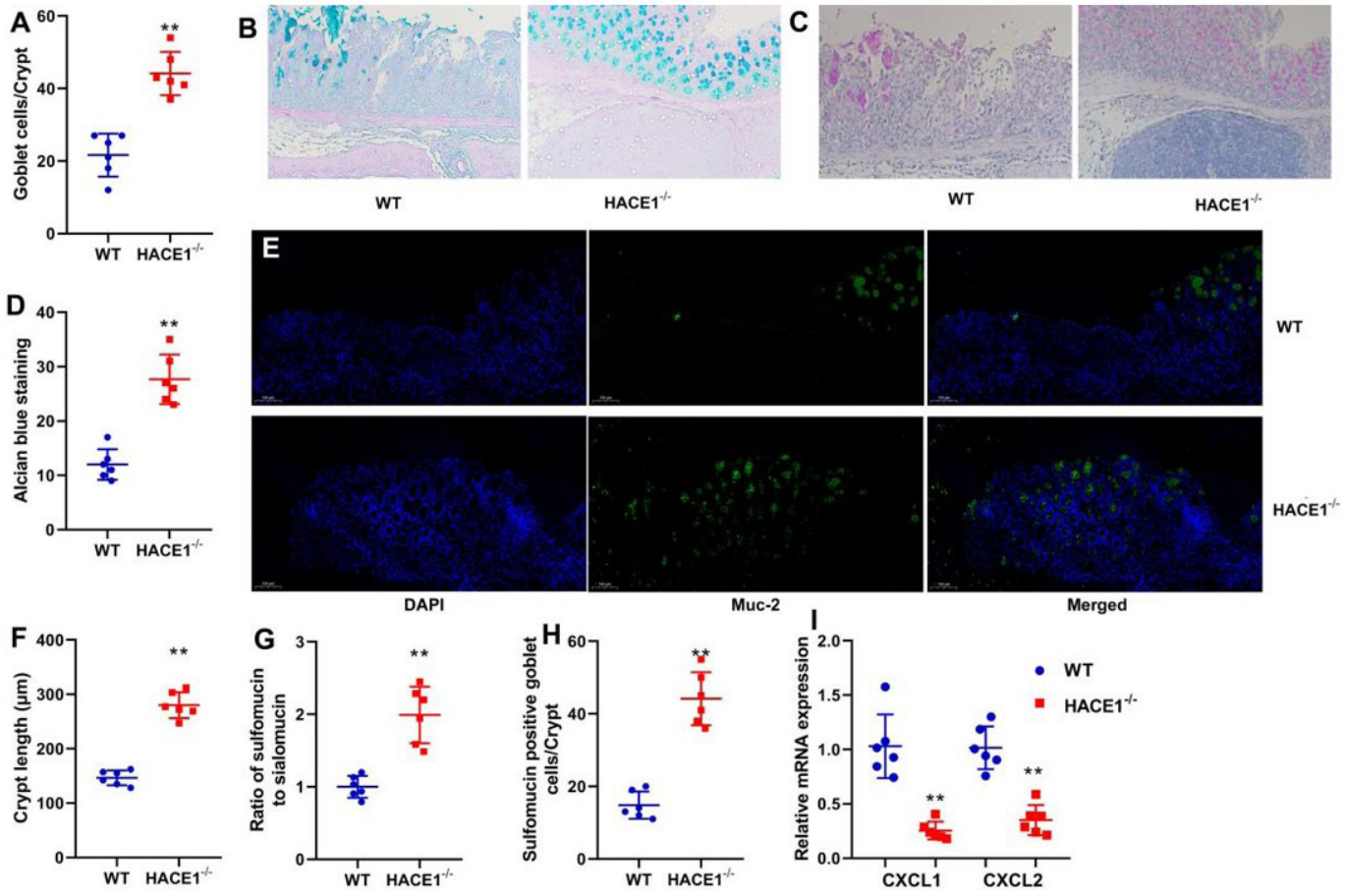

Figure 3. The inhibition of HACE1 restored the function of intestinal epithelial cells in Citrobacter rodentium-Induced Murine Colitis. Goblet cells (A), Alcian blue staining (B), colon tissue by Alcian blue/HE staining (C and D), MUC-2 levels in colon tissue (E), crypt length (F), sulfomucin ratio (G) and sulfomucin positive goblet cells (H), CXCL1 and CXCL2 mRNA expression levels (G). **p<0.01 compared with WT group.

3.6 HACE1 interlinked Nrf2 protein to promote Nrf2 ubiquitination

In vitro model, HACE1 up-regulation increased HACE1 and NLRP3 protein expressions, and suppressed Nrf2 protein expression (Figure 6A). Next, in vitro model, HACE1 down-regulation decreased HACE1 and NLRP3 protein expressions, and induced Nrf2 protein expression (Figure 6B). IP showed that HACE1 interlinked Nrf2 protein (Figure 6C). Confocal microscope showed that HACE1 up-regulation reduced Nrf2 expression (Figure 6D). HACE1 increased Nrf2 Ubiquitination, si- HACE1 reduced Nrf2 Ubiquitination in vitro model (Figure 6E). These results thus demonstrated HACE1 interlinking Nrf2 protein expression to promote Nrf2 Ubiquitination.

3.7 Nrf2 reduced the effects of HACE1 on inflammation and ROS- oxidative stress in vitro model

Nrf2 inhibitor (Brusatol, 0.05 μg/mL) suppressed the protein expression of Nrf2, and induced NLRP3 protein expression and IL-1β levels in vitro model by si-HACE1 (Figure 7A, 7B). Nrf2 agonist (compound O15, 1 μM) suppressed NLRP3 protein expression and IL-1β levels, and induced Nrf2 protein expression in vitro model by HACE1 (Figure 7C, 7D). Nrf2 inhibitor increased inflammation levels and ROS- oxidative stress in vitro model by si-HACE1 (Figure 7E, 8A, 8C). Nrf2 agonist reduced inflammation levels and ROS- oxidative stress in vitro model by HACE1 (Figure 7F, 8D-8F).

3.8 m6A promoted HACE1 stability by METTL3

Lastly, we examined the mechanism of methylation control HACE1 stability in Citrobacter rodentium-Induced Murine Colitis. HACE1 gene was multiple suspicious methylation modification sites near the stop codon (Figure 9A). Si-METTL3 reduced the stability of HACE1 mRNA in vitro model (Figure 9B). m6A-specific antibody suppressed HACE1 mRNA enrichment level in in vitro model by si-METTL3 (Figure 9C). Six m6A sites in the 3′-untranslated region (UTR) of HACE1, and m6A enrichment at sites 1, 2, 3, 4, 5 and 6 was decreased HACE1 levels (Figure 9D). Si- METTL3 reduced luciferase activity level by wild-type (WT) of UAF1, meanwhile, while the mutant (Mut) HACE1 did not (Figure 9E-9G). These results revealed that METTL3 regulates HACE1 expression in mice model.

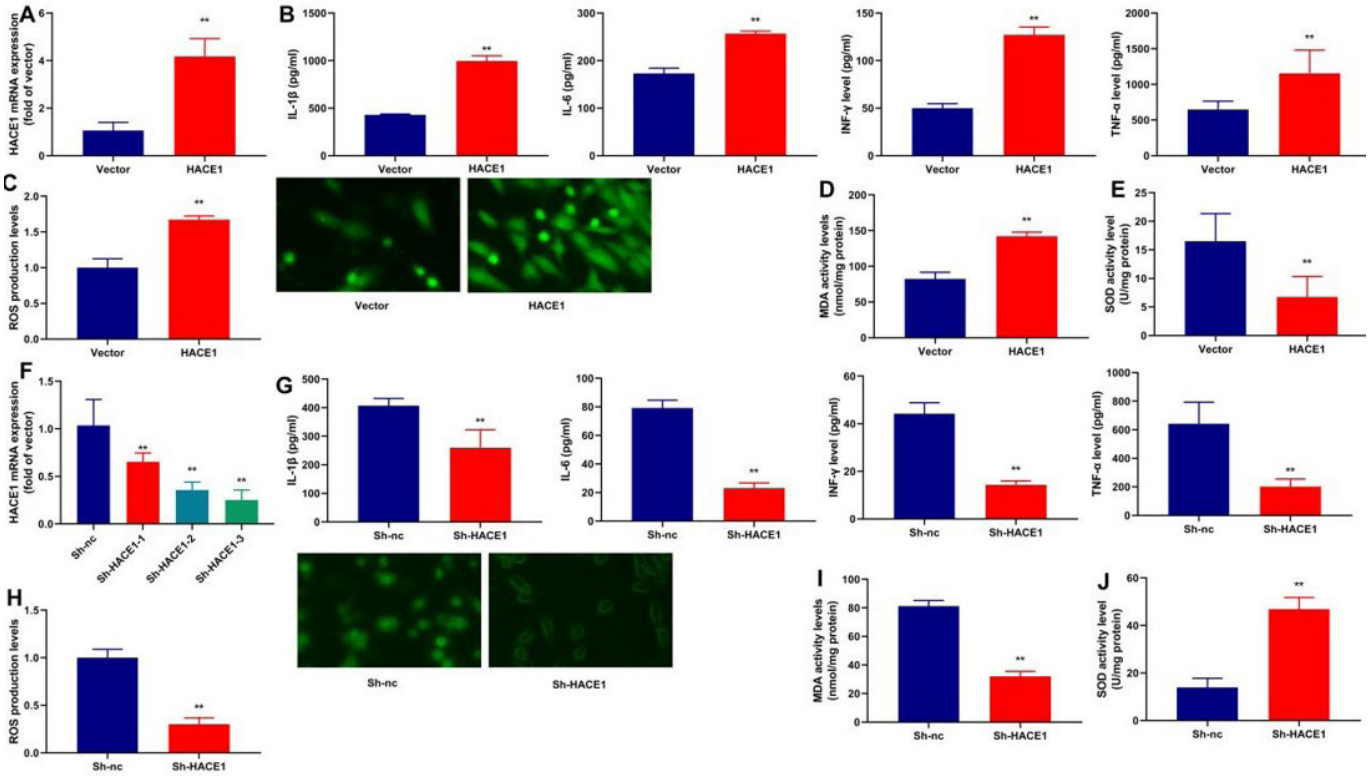

Figure 4. HACE1 promoted inflammation and ROS- oxidative stress in vitro model. HACE1 mRNA expression (A), IL-1β/IL-17/TNF-α/IFN-α levels (B), ROS production level (C), MDA level (D), SOD level (E) in vitro model by over-expression of HACE1; HACE1 mRNA expression (F), IL-1β/IL-17/TNF-α/IFN-α levels (G), ROS production level (H), MDA level (I), SOD level (J) in vitro model by down-regulation of HACE1. **p<0.01 compared with negative or sh-nc group.

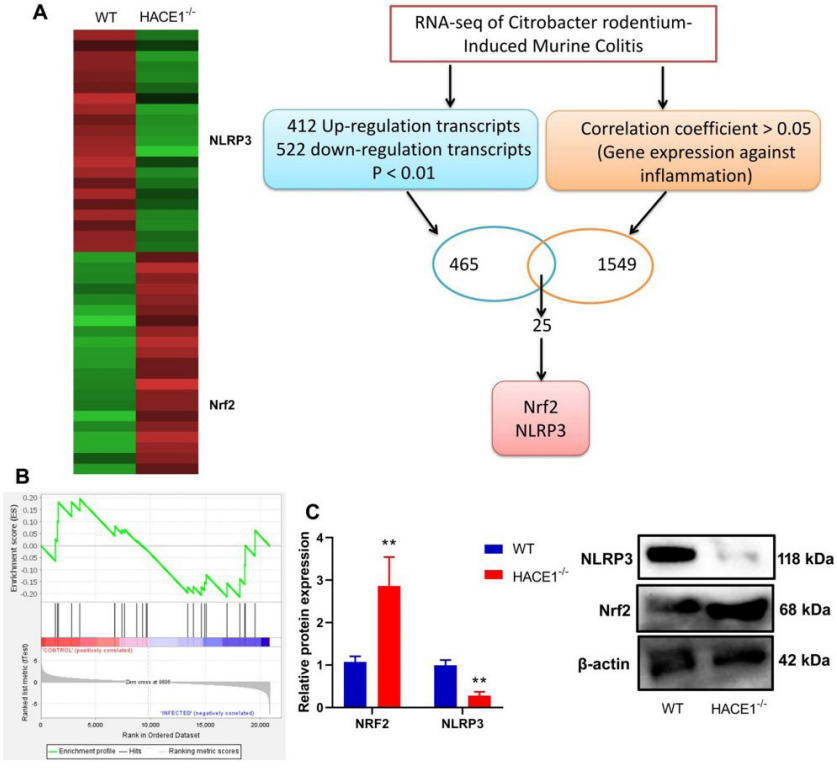

Figure 5. HACE1 suppressed Nrf2/NLRP3 signaling pathway. Microarray analysis (A), KEGG terms (B), Nrf2/NLRP3 protein expression (C). **p<0.01 compared with WT group.

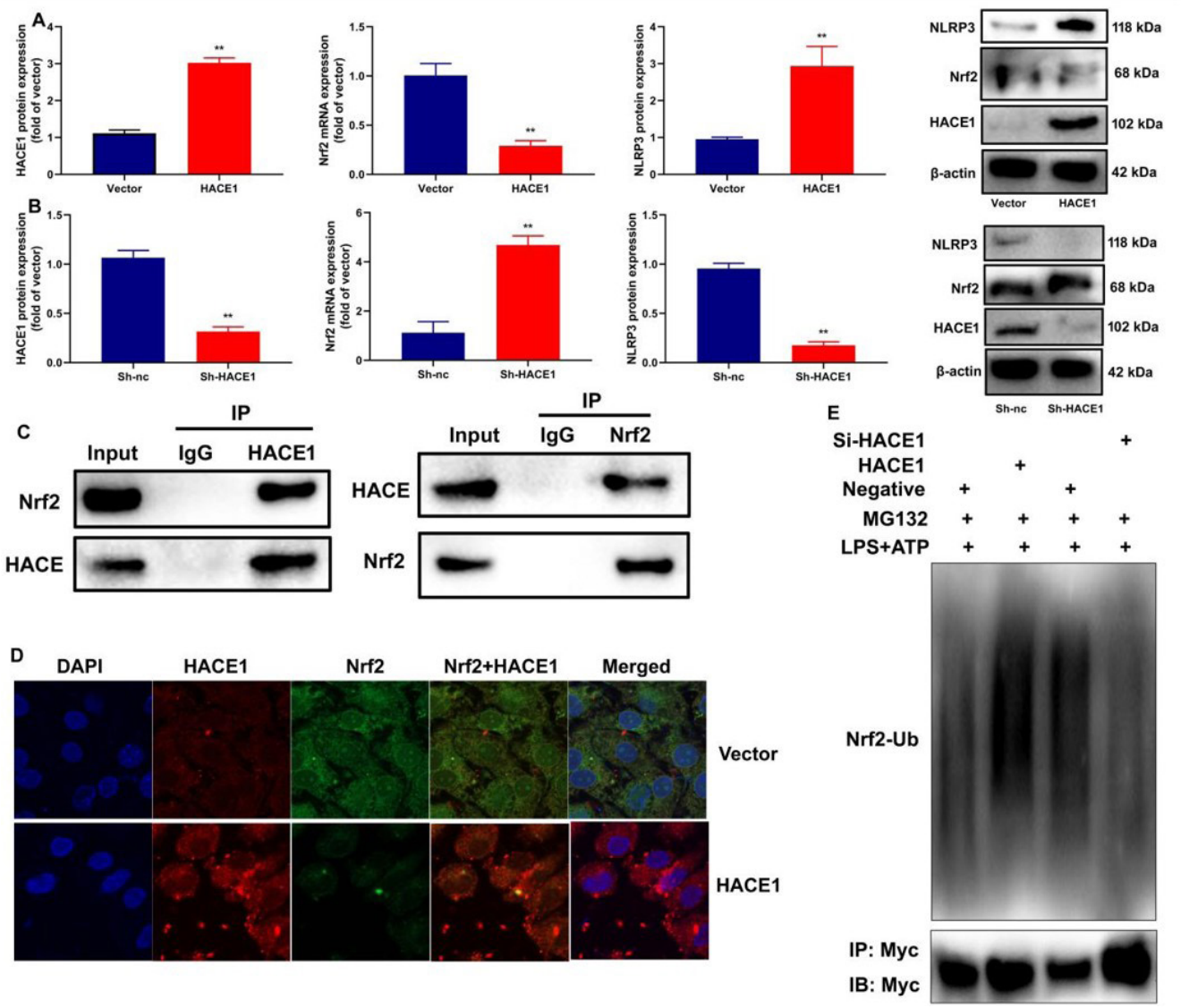

Figure 6. HACE1 interlinked Nrf2 protein to promote Nrf2 Ubiquitination. HACE1/Nrf2/NLRP3 protein expressions (A) in vitro model by over-expression of HACE1; HACE1/Nrf2/NLRP3 protein expressions (B) in vitro model by down-regulation of HACE1;HACE1 interlinked Nrf2 protein by IP (C), HACE1 and Nrf2 protein expression by IF (D), Nrf2 Ubiquitination (E). **p<0.01 compared with negative or sh-nc group.

4 Discussion

C. Rodentium is a gram-negative bacterium that can infect the distal colon of mice and cause colonic crypt hyperplasia (Patel et al., 2020). It has long been used to study the pathogenesis of clinically important enterogenetic Escherichia coli (EPEC) and enterohaemorrhagic E.coli (EHEC) infections (Ryu et al., 2016). Moreover, the mucositis caused by C. rodentium infection is similar to the symptoms of IBD, and can be used as a model of chronic intestinal inflammatory disease to study the relationship between host genes and intestinal flora and intestinal inflammation (Wu et al., 2021, Collins et al., 2014). In this study, HACE1 expression was up-regulated in Citrobacter rodentium-Induced Murine Colitis. The inhibition of HACE1 ameliorates inflammatory responses and restored the function of intestinal epithelial cells in Citrobacter rodentium-Induced Murine Colitis. HACE1 promoted inflammation and ROS- oxidative stress in vitro model. Zang et al. (2022) demonstrated that HACE1 negatively regulates neuroinflammation in Parkinson's disease models. Together, these data demonstrate that HACE1 exerts a regulatory role in Citrobacter rodentium-Induced Murine Colitis. This experiment for the first time evaluated the effects of HACE1 in model of Colitis, and which provided new research target for food chemical, and advanced application in food chemical.

Nrf2 signaling pathway is a key pathway to maintain cellular redox homeostasis, which is related to anti-inflammatory, antioxidant, reduction of mitochondrial damage, and regulation of iron death (Bai et al., 2019; Xu et al., 2022). Some studies have found that HO-1 is an essential enzyme for the occurrence of iron death, and the degradation product of HO-1 contains iron, which leads to iron overload in cells. Nrf2/HO-1 pathway can

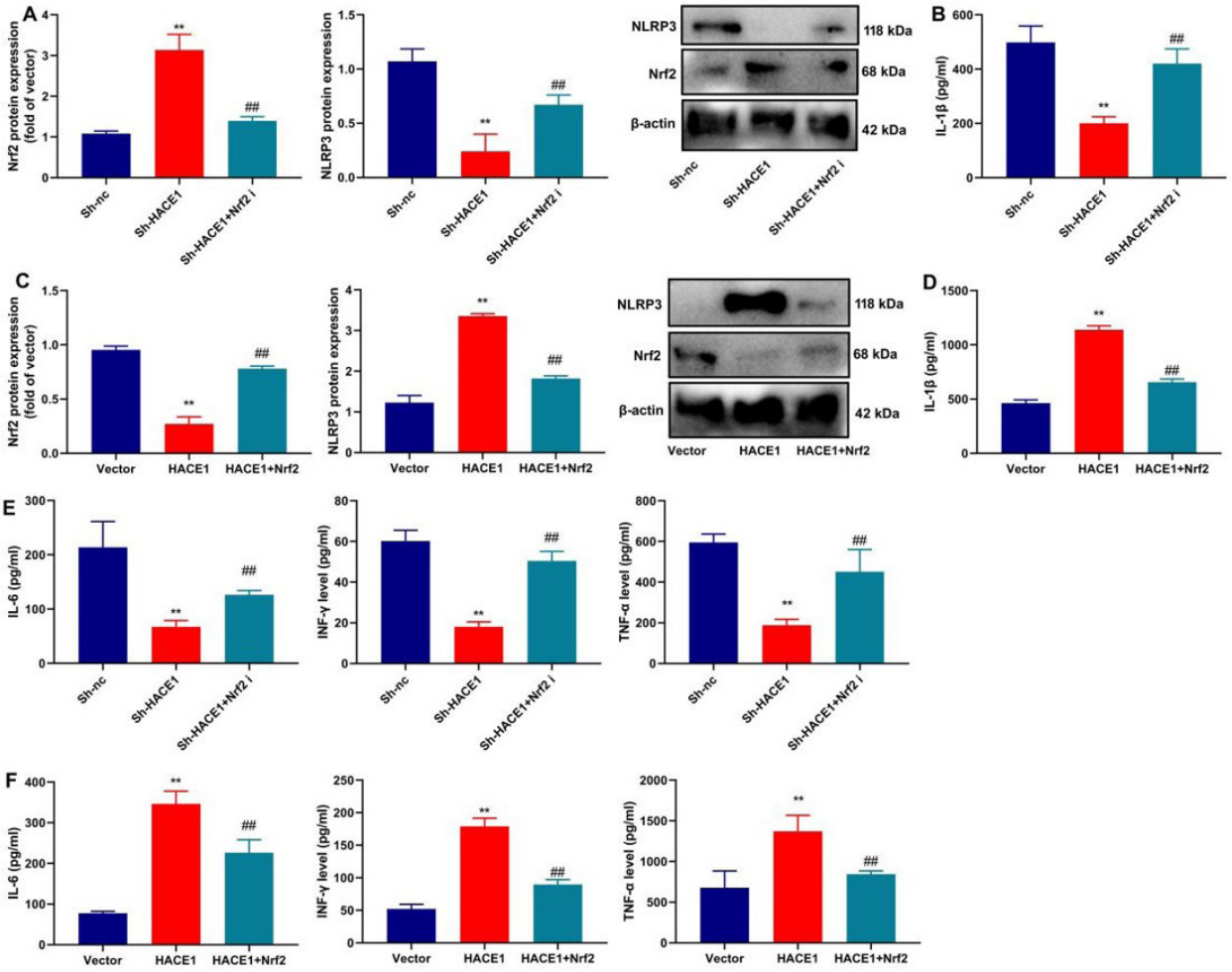

Figure 7. Nrf2 reduced the effects of HACE1 on inflammation in vitro model. Nrf2/NLRP3 protein expressions (A), IL-1β levels (B), IL-6 (E) in vitro model by si-HACE1 and Nrf2 inhibitor; Nrf2/NLRP3 protein expressions (C), IL-1β levels (D), IL-6 (F) in vitro model by over-expression of HACE1 and Nrf2; **p<0.01 compared with negative or sh-nc group. ##p<0.01 compared with si-HACE1 or HACE1 group.

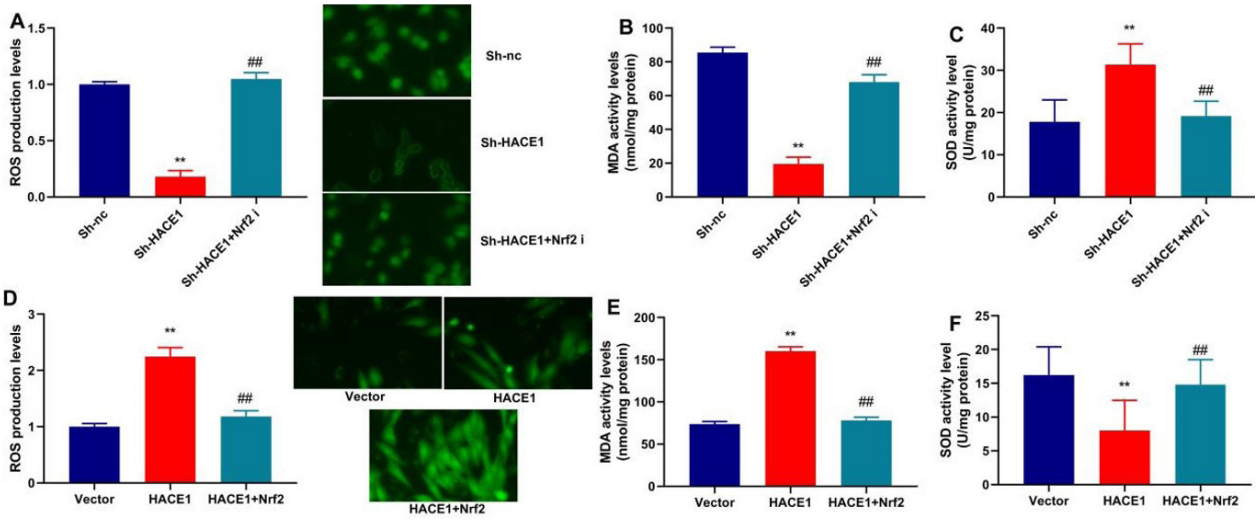

Figure 8. Nrf2 reduced the effects of HACE1 on ROS- oxidative stress in vitro mode. ROS production level (A), MDA level (B), SOD level (C) in vitro model by si-HACE1 and Nrf2 inhibitor; ROS production level (D), MDA level (E), SOD level (F) in vitro model by over-expression of HACE1 and Nrf2; **p<0.01 compared with negative or sh-nc group. ##p<0.01 compared with si-HACE1 or HACE1 group.

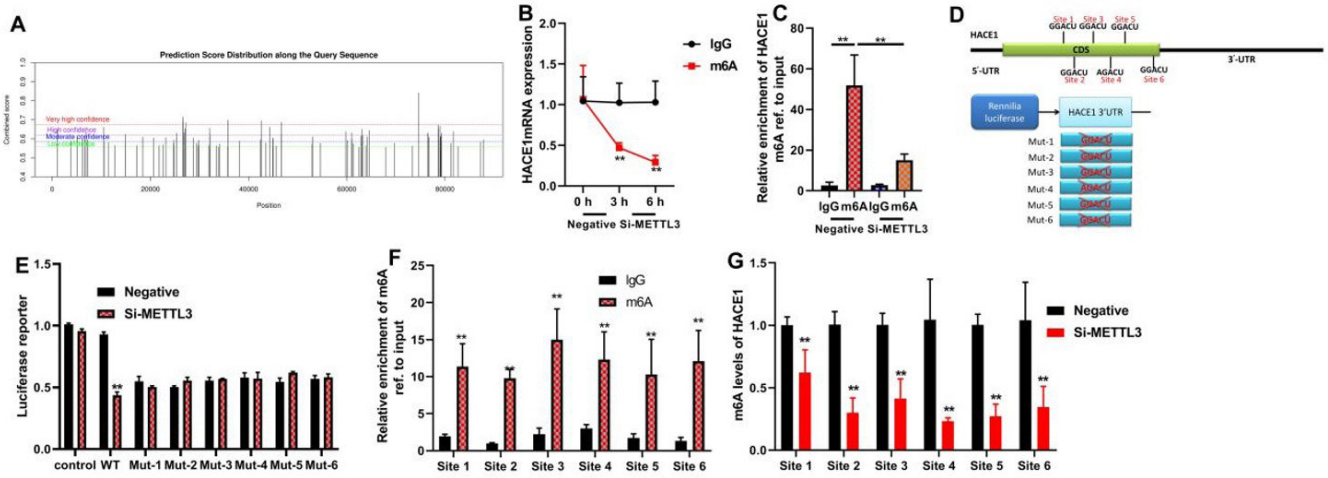

Figure 9. m6A promoted HACE1 stability. m6A modification site of HACE1 (A), METTL3-mediated UAF1 m6A modifications (B and C), the position of m6A motifs within HACE1 transcript sequence (D), luciferase reporter activity level (E), m6A levels of HACE1 (F and G). **p<0.01 compared with vector or negative or IgG group.

also negatively regulate the inflammatory factor TNF-α、IL-6, etc., alleviating the damage of oxidative stress and inflammatory factors to the body (Zhang et al., 2020, 2021a; Wang et al., 2019). We found that HACE1 interlinked Nrf2 protein to promote Nrf2 Ubiquitination. Nrf2 reduced the effects of HACE1 on inflammation and ROS- oxidative stress in vitro model. Da et al. (2021) demonstrated that HACE1 decreased radiosensitivity of glioma cells by activating NRF2. These data suggest that HACE1 interlinked Nrf2 protein to promote Nrf2 Ubiquitination in Citrobacter rodentium-Induced Murine Colitis.

NLRP3 inflammasome is mainly composed of NLRP3, ASC and caspase-1 (Pu et al., 2021; Lv et al., 2021). It plays an important role in maintaining intestinal homeostasis (Wang et al., 2022; Bauer et al., 2010,). Studies have shown that NLRP3 inflammasome is closely related to the occurrence and development of IBD, especially UC, and plays an important role in inflammatory response and intestinal immune barrier function (Zhang et al., 2021a; Wang et al., 2022; Bauer et al., 2010; Wu et al., 2021). Our study determined that HACE1 suppressed Nrf2/NLRP3 signaling pathway in model of colitis. Tortola et al. (2016) showed that HACE1 controls TNF-elicited cell fate decisions and anti-inflammatory activities (Zang et al., 2022). These data suggest that HACE1 increased NLRP3 inflammasome in Citrobacter rodentium-Induced Murine Colitis by the inhibition of Nrf2.

In eukaryotes, DNA methylation is a very important epigenetic marker, which can affect chromatin structure and gene expression (Emmett et al., 2017). Although DNA methylation does not change nucleotide sequence, it can regulate gene transcription and plays an important role in epigenetics (Guo et al., 2018). DNA methylation at the promoter can inhibit gene transcription, combine with transcription factors, change chromatin structure and even lead to inactivation of X chromosome (Howell et al., 2018). DNA methylation of gene ontology is ubiquitous and evolutionarily conservative, which has long been considered irrelevant to gene transcription (Li et al., 2020). However, with the deepening of research, people increasingly recognize the role of gene ontology methylation. Enhancers, silencers and transposons are the functional elements in the genome, and their methylation changes have a deeper impact on the entire genome, even leading to chromosome instability (Taman et al., 2021). M6A modification is catalyzed by m6A methyltransferase complex, which is composed of METTL3, METTL14 and Wilms tumor 1 binding protein (Du et al., 2018). METTL3 and METTL14 co locate in the nuclear spot and form a stable heterodimer, thereby synergistically increasing the methylation capacity (Han et al., 2019). In addition, the m6A methyltransferase complex also includes methyltransferase like 16, such as virus like m6A methyltransferase related protein, CCCH type zinc finger protein 13 and RNA binding motif protein 15/15B, which play a key role in regulating the stability of the complex and the methylation of m6A (Wang et al., 2018; Wu et al., 2020). This study showed that m6A promoted HACE1 stability by METTL3. Sakata et al. (2013) reported that methylation of the HACE1 gene is frequently detected in hepatocellular carcinoma. These data suggest that m6A promoted HACE1 stability by METTL3 to promote NLRP3 activity by the inhibition of Nrf2 in Citrobacter rodentium-Induced Murine Colitis.

In conclusion, our results here suggest that the m6A methyltransferase METTL3 promoted HACE1 stability to promote NLRP3 activity in Citrobacter rodentium-Induced Murine Colitis by the inhibition of Nrf2. Our findings provide insights into a novel tissue protective role of HACE1, and may lead to therapeutic potential of inflammation associated carcinogenesis for IBD or UC. by YTHDF2- dependent manner and in better understanding of pathogenesis of inflammation associated carcinogenesis is essential for colon homeostasis and defense system. Our findings provide insights into a novel tissue protective role of Kcnk6, and may lead to therapeutic potential of inflammation associated carcinogenesis for IBD or UC.

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