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Disruption of the biorhythm in gastric epithelial cell triggers inflammation in *Helicobacter pylori*-associated gastritis by aberrantly regulating NFIL3 via CagA activated ERK-SP1 pathway

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Abstract

Helicobacter pylori (*H. pylori*) associated gastritis, marked by chronic gastric inflammation, heightens gastric cancer risk by fostering a malignancy-prone microenvironment. Disruption of the biorhythm contribute to the onset of various gastrointestinal disorders, such as gastric dyspepsia, gastric ulcers, and cancer. We aimed to investigate the functional roles and regulatory mechanisms of key biorhythm molecules in *H. pylori* associated gastritis. We investigated biorhythm gene expression in *H. pylori*-infected human gastric tissues and found significant impact on NFIL3 expression. Animal studies confirmed that *H. pylori* controls NFIL3 biorhythm. Clinical samples indicated a correlation between NFIL3 and gastritis severity, suggesting a regulatory role. Then, we found that *H. pylori* disrupt NFIL3 expression rhythm in gastric epithelial cells (GECs) through the CagA-activated ERK-SP1 pathway. Additionally, cytokines IL1β and TNFα enhance this disruption. RNA-seq and Gene set enrichment analysis (GSEA) indicated that NFIL3 positively regulates the inflammatory response during *H. pylori* infection. Our research highlights the crucial role of the biorhythm molecule NFIL3 in *H. pylori* associated gastritis. Modulating biorhythm molecules could be a promising therapeutic approach to manage disease progression, given their impact on gastrointestinal pathology.

Keywords *Helicobacter pylori*, NFIL3, Biorhythm, Gastric epithelial cells, Gastritis

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Introduction

Chronic gastritis, a common disease of the digestive system, clinically marked by persistent inflammation of the gastric mucosa. Many factors can contribute to chronic gastritis, of which the most common aetiological agent is *Helicobacter pylori* (*H. pylori*), which globally accounts for over 90% of all gastritis types [1, 2]. *H. pylori* is an infectious pathogen that infects the gastric mucosa and is estimated to have infected approximately 4 billion individuals globally [3]. Nearly all individuals with *H. pylori* infection will have chronic, non-self-limiting inflammation of the gastric mucosa, commonly known as *H. pylori*-associated gastritis. This condition induces an inflammatory microenvironment that results in persistent damage to the gastric mucosa and facilitating the pathological progression from chronic gastritis to gastric atrophy, intestinal metaplasia, dysplasia, and eventually gastric cancer (GC) [4, 5]. Consequently, *H. pylori*-associated gastritis has been identified as a significant risk factor for GC [6]. Despite extensive research elucidating the roles of certain key signaling pathways (such as NF-κB and MAPK pathway) [7] and targets (such as LTβR and TINAGL1) [8, 9] in *H. pylori*-associated gastritis, our comprehension of the disease's pathogenesis remains incomplete, and the identification of potential targets for druggable molecular is still insufficient. Therefore, further analysis and identification of key players in the pathogenesis of pathogenic bacterium infection is crucial for improving the treatment of chronic gastritis and the prevention of GC.

Biorhythm are cycles of physiological and behavioral events, created and kept by independent cellular transcription-translation feedback loops in peripheral tissues which are commanded by a master pacemaker in the suprachiasmatic nucleus [10]. Disruption of biorhythms, resulting from aberrant regulation of rhythm molecule (typically transcription factors, control approximately 10% of all genes), can adversely affect multiple physiological functions, including cognition, metabolism, and immunity [10, 11]. This dysregulation may contribute to the onset of various gastrointestinal disorders, such as gastric dyspepsia, gastric ulcers, inflammatory bowel diseases, and cancer [12, 13]. Within the stomach, circadian cycles influence the mucosal homeostasis and can cause or promote disease [14]. For example, the rhythm of trefoil factor family 2 (TFF2) is disrupted in individuals experiencing gastric symptoms and compromised mucosal protection [15]. Furthermore, circadian rhythms modulate stress-induced gastric mucosal injury through the actions of melatonin and prostaglandin E2 (PGE2) [16]. Studies indicate that *H. pylori* infection significantly disrupts stomach rhythms (such as gastric acid secretion and motility) and alters biorhythm molecules, as a result, impacting disease status [17–20]. For instance, *H. pylori* infection increases gastric mucosal inflammation by regulating biorhythm molecule BMAL1 [18] and creates an immunosuppressive microenvironment via another biorhythm molecule NR1D1 [19]. While some research has explored *H. pylori* infection's impact on gastric biorhythms, the molecular mechanisms are not well understood, necessitating further molecular-level studies.

Nuclear factor interleukin 3 regulated (NFIL3) is a basic leucine zipper transcription factor (TF), and it is also a biorhythm protein [21]. NFIL3 participates in one of the three classical regulatory loops of the biological rhythm. Specifically, NFIL3, in conjunction with DBP, as well as CLOCK and BMAL1, orchestrates the rhythmic regulation of the NR1D1, which in turn repress NFIL3 transcription [22]. NFIL3 is found in an extensive range of tissues and cells, including heart, liver, gastrointestinal tissues and immune cells [23–25]. As a biorhythm molecule with transcriptional regulation (activation or inhibition), it participates in a diverse array of physiological processes, including circadian rhythm homeostasis, energy metabolism and immune cell differentiation (especially NK cells), and its aberrant expression is associated with cancer, metabolic diseases and inflammation [24–26]. Previous research indicates that NFIL3 is involved in regulating intestinal mucosal homeostasis, and mice lacking NFIL3 (*Nfil3*$^{-/-}$ mice) will develop spontaneous colitis [27, 28]. Further studies showed that *NFIL3* gene in intestinal epithelial cells is rhythmic and contributes to the modulation of intestinal metabolism, and found that its rhythmic expression was regulated by intestinal microbiota [29]. However, the function and regulatory mechanisms of NFIL3 within the gastric mucosa, especially, in the context of *H. pylori* infection, remain inadequately understood.

Here, a novel mechanistic link between pathogenic bacterium infection and biorhythms in gastritis was reported, specifically the disruption of the biorhythm molecule NFIL3 expression in *H. pylori* infected gastric mucosa tissues, which promotes an inflammatory response. The results demonstrate that *H. pylori* CagA-induced NFIL3 activates the NF-κB signaling pathway, thereby exerting pathological effects in *H. pylori* infected gastric epithelial cells (GECs). These findings suggest biorhythm molecule NFIL3 as a potentially druggable target in *H. pylori* associated gastritis.

Materials and methods

Patient samples

Gastric biopsy samples were obtained from individuals who underwent upper esophagogastroduodenoscopy due to dyspeptic manifestations, including symptoms such as bloating, episodic epigastric discomfort, and general dyspepsia. A total of 38 biopsy specimens were collected, comprising 30 samples from patients infected

with *H. pylori* and 8 samples from uninfected volunteers. The histopathological evaluation was conducted by two seasoned histopathologists utilizing the modified Sydney System [30]. The presence of *H. pylori* infection was confirmed through the ^{14}C urea breath test. All experimental protocols received approval from the Ethical and Experimental Committee of The General Hospital of Western Theater Command and written informed consent was obtained.

Cell culture

The AGS (human gastric cell line) was sourced from the American Tissue Culture Collection (ATCC) and was maintained in DMEM/F12 (Hyclone, USA) medium, which was augmented with 1% penicillin/streptomycin solution (Beyotime, China) and 10% fetal bovine serum (FBS) (PAN Biotech, Germany). The process of isolating single cells ($EpCAM^+$) from human tissues, specifically human primary gastric epithelial cells (GECs), was conducted in accordance with previously established protocols [31]. These primary GECs were cultured in RPMI1640 (Hyclone, USA) medium, supplemented with 10% FBS (PAN Biotech, Germany) and 1% penicillin/streptomycin solution (Beyotime, China). All cell cultures were maintained in a mycoplasma-free environment at 37℃, in a 5% CO2 atmosphere, and under conditions of humidity.

H. pylori strains

We utilized several strains of *H. pylori*, specifically the *cagA* and *vacA*-positive strain 26,695 (ATCC 700392), the *vacA* knockout mutant strain 26,695 (referred to as Δ*vacA* herein), the *cagA* and *vacA*-positive strain NCTC 11,637 (ATCC 43504, designated as *H. pylori* 11637 in this manuscript), the *cagA* knockout mutant strain NCTC 11,637 (termed Δ*cagA* in this article), and the *cagA* and *vacA*-positive strain PMSS1 (pre-mouse Sydney strain 1) [32, 33]. These strains were cultured in Skirrow medium supplemented with 10% rabbit blood at 37 °C in a microaerobic humidified environment, following previously established protocols [32, 33].

Cell stimultion

Cellular stimulation was conducted using the aforementioned *H. pylori* strains at specified multiplicities of infection (MOI) and durations, as previously detailed [32]. For experiments aimed at inhibiting signaling pathways, cells were pre-treated with inhibitors, specifically SP1 inhibitor Mithramycin A (MedChemExpress, USA) or MEK1/2 inhibitor U0126 (Calbiochem, Germany), for a duration of 2 h prior to infection with *H. pylori* for either 3–24 h. The final concentration for all inhibitors was maintained at 10 mmol/L. For cytokine stimulation, cells were exposed to *H. pylori* and/or various cytokines for a period of 24 h. The cytokines employed in this study included IL17A, IFNγ, IL22, IL6, IL23, IL12, TGFβ, IL1β, and TNFα, with a final concentration of 100 ng/mL.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the cells using the EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech, Shanghai, China), while RNA from tissues was isolated using RNAiso Plus (Takara, Japan). cDNA synthesis for each sample was performed utilizing the PrimeScript™ RT reagent Kit (Takara, Japan). The qRT-PCR assays were conducted according to previous [32]. The primers were synthesized by Tsingke Biotech (Beijing, China), with the sequences detailed in Supplementary Table S1.

Immunohistochemistry (IHC) analysis

Gastric tissue sections underwent a series of preparatory steps including deparaffinization and rehydration, followed by boiling in a citrate buffer. The sections were then incubated overnight at 4 °C with a primary anti-NFIL3 antibody (1:100, Proteintech, China), and subsequently treated with the appropriate secondary antibodies (1:500, ZSGB-Bio, China). Following these procedures, the sections were stained with DAB (ZSGB-Bio, China) and counterstained using hematoxylin (Sangon Biotech, China). Photomicrographs were obtained utilizing a bright field microscope (Nikon Eclipse 80i, Japan).

Western blot (WB) analysis

Proteins were resolved in SDS-PAGE gels and subsequently transferred to PVDF membranes (Bio-Rad, USA). The membranes were incubated overnight at 4 °C with specific antibodies, followed by treatment with corresponding secondary antibodies. Visualization of the results was performed using a ChemiDoc™ Touch Imaging System (Bio-Rad, USA) in conjunction with the Super ECL plus WB Kit (Bioground, China). The primary anti-NFIL3 antibody (1:1000) was sourced from Proteintech (China), while anti-SP1 (1:1000), anti-p-ERK (1:1000), anti-ERK (1:1000), and anti-GAPDH (1:1000) antibodies were obtained from Cell Signal Technology (USA). HRP-conjugated secondary antibodies (1:10000) were purchased from ZSGB-Bio (China). The analysis of protein expression levels was conducted using Image Lab™ Software (version 5.1, BioRad, USA).

Immunofluorescence (IF) assay

Cells were cultured in μ-Slide 8 Well ibiTreat slide chambers (Ibidi, Martinsried, Germany) until reaching 80% confluency, followed by infection with *H. pylori* 11,637 for a duration of 24 h. The cells were then fixed using 4% paraformaldehyde, permeabilized with 0.5% Triton-X100 in PBS, and incubated with 5% BSA. Staining was

performed with anti-NFIL3 antibody (1:100, Proteintech, China), followed by the application of corresponding secondary antibodies (ZSGB-Bio, China). DAPI (Beyotime, China) was employed to stain the cell nuclei. Confocal fluorescence microscopy (LSM 510 META, Zeiss, Germany) was used to capture the photomicrographs.

Small interfering RNA (siRNA) transfection

Cells were transfected with either control siRNA (negative control, NC) or siRNA targeting specific genes for a period of 6 h using Lipofectamine 2000 (Thermo Fisher Scientific, USA). Following transfection, the cells were washed and incubated in fresh medium for 18 h before being infected with *H. pylori* for an additional 24 h. Post co-culture, cells were harvested for qRT-PCR, WB, or RNA-seq assays. All siRNA was procured from GenePharma (Shanghai, China). The sequences of the siRNA were presented in Supplementary Table S2.

Luciferase reporter assay

Promoter constructs encompassing the region from − 250 to 0 of the NFIL3 gene, along with their corresponding mutated sequences, were synthesized by Genechem (Shanghai, China). In brief, cells were plated in 24-well plates and subsequently transfected with the engineered luciferase reporter vector alongside the internal control vector pRL-TK (Promega, USA) utilizing lipofectamine 2000 (Thermo Fisher Scientific, USA) for a duration of 4 h. Following transfection, cells were exposed to *H. pylori* (with prior treatment using or without an inhibitor before *H. pylori* exposure) for 24 h. The luciferase activity was quantified using the Dual Luciferase Reporter assay system (Promega, USA).

RNA-seq

Total RNA was extracted from the cells using the EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech, Shanghai, China). The resultant samples were dispatched to Guangzhou Epibiotek Corporation Ltd. (Guangzhou, China) for sequencing on the Illumina NovaSeq 6000 platform. GSEA 4.3.3 application was derived from the Broad Institute (http://www.gsea-msigdb.org/gsea/login.jsp), with gene sets deemed significant when the false discovery rate (FDR) was below 25%. Gene set enrichment was visualized using ridge plots generated by the R package ggridges. Differential expression analysis (|Log2Fold Change|> 1; $P < 0.05$) was conducted using the R package DESeq2. Gene Ontology (GO) analyses were performed with the R package clusterProfiler and visualized using OmicShare tools at www.omicshare.com/tools. Volcano plots were created using the R package ggplot2, while heatmaps were generated through the OmicShare tools at https://www.omicshare.com/tools/.

Enzyme linked immunosorbent assay (ELISA)

The concentrations of CCL20, IL-23, and IL-8 in the supernatants of cell cultures were quantified using the Human CCL20 ELISA Kit (mlbio, Shanghai, China), Human Interleukin 23 (IL-23) ELISA Kit (mlbio, Shanghai, China), and Human Interleukin 8 (IL-8) ELISA Kit (Jianglai Biology, Shanghai, China), following the protocols provided by the manufacturers.

Public data analysis

The GEO series utilized in this investigation, specifically GSE60427, GSE27411, GSE10262, GSE70394, GSE60661, and GSE181917, were obtained from the GEO database at https://www.ncbi.nlm.nih.gov/gds. Data analysis was conducted using the OmicShare tools available at www.omicshare.com/tools. Transcription factor predictions were performed utilizing PROMO (https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The promoter sequences spanning − 2000 to 0 were sourced from The Eukaryotic Promoter Database at https://epd.epfl.ch//index.php.

Animal model

Six-week-old female wild-type C57BL/6 mice were acquired from Charles River (China). The mice were housed in a facility that adhered to specific pathogen-free standards, maintaining controlled environmental conditions, including a temperature range of 20–22 °C, humidity at 55%, and a 12-hour light/dark cycle, with access to sterile food and water. All animal care and experimental procedures complied with the Ethical and Experimental Committee of The General Hospital of Western Theater Command. For the purpose of infecting the mice, *H. pylori* PMSS1 were scraped from the agar plates and subsequently transferred to Brucella broth supplemented with 5% FBS, where they were gently shaken continuously at 37 °C under microaerobic conditions. Prior to inoculation, the mice were subjected to overnight fasting and were then orogastrically inoculated with the bacterial suspension (6×10^8 CFU) once every two days for a total of eight. Following an eight weeks post-infection period, the mice were euthanized, and gastric tissues were collected.

Statistical analysis

Quantitative data shown represent the mean ± SEM. Comparisons between two experimental groups were conducted using a Student's t-test, except in instances where the variances were differed, in which case the Mann-Whitney U test was employed. Data analysis was performed utilizing GraphPad Prism (version 9.5.1). $P < 0.05$ was defined as statistical significance.

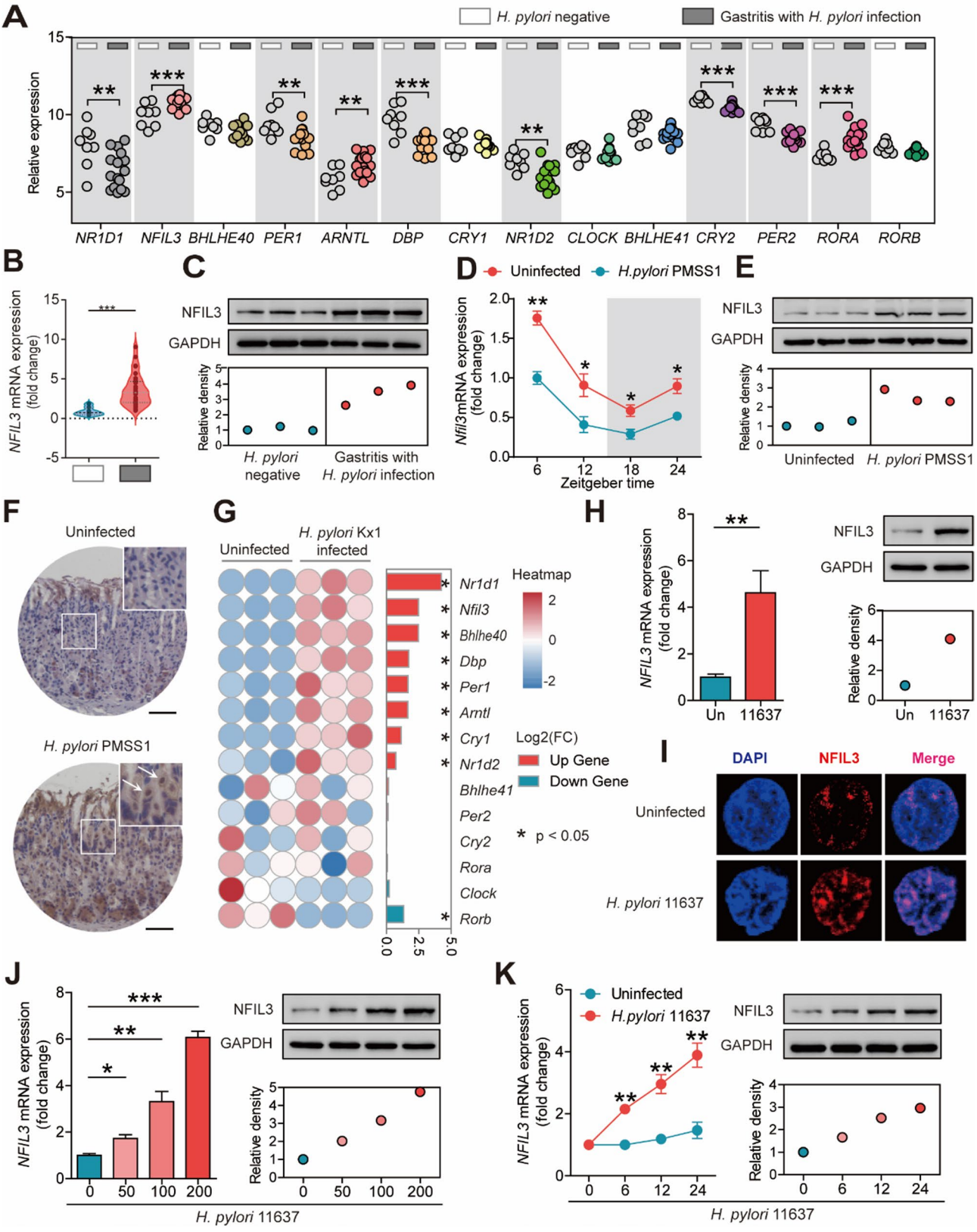

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Fig. 1 The biorhythm of NFIL3 is disrupted in *H. pylori*-infected GECs. (**A**) The expression levels of biorhythm genes in human gastric mucosa were evaluated using the GEO database (GSE60427), comparing two cohorts: *H. pylori*-negative individuals ($n=8$) and gastritis due to *H. pylori* infection ($n=16$). (**B**) The mRNA expression of NFIL3 was analyzed across gastric mucosa samples from uninfected individuals ($n=8$) and patients ($n=30$) infected with *H. pylori*. (**C**) The presence of NFIL3 protein in the gastric mucosa was determined in both *H. pylori*-uninfected and infected samples through WB. (**D**) The mRNA expression of *Nfil3* in the gastric mucosa of mice infected with *H. pylori* PMSS1 and uninfected controls was examined using qRT-PCR over a 24-hour light-dark cycle. (**E**) The protein expression of Nfil3 in the gastric mucosa of *H. pylori* PMSS1-infected and uninfected mice at 8 weeks post-infection (p.i.) was analyzed via WB. (**F**) Nfil3 protein expression in the gastric mucosa of *H. pylori* PMSS1-infected and uninfected mice at 8 weeks p.i. was evaluated using IHC. Scale bar: 50 μm. (**G**) The expression levels of biorhythm genes were evaluated in a mouse gastric epithelial progenitor-derived cell line following infection with *H. pylori* Kx1, utilizing data from the GEO database (GSE10262). (**H**) The presence of NFIL3 mRNA and protein was determined in human GECs infected with *H. pylori* 11,637, as well as in uninfected controls (MOI = 100, 24 h), employing qRT-PCR ($n=3$) and WB. (I) NFIL3 protein expression in AGS cells infected with *H. pylori* 11,637 and uninfected controls (MOI = 100, 24 h) was examined using IF. (**J**) The expression of NFIL3 mRNA and protein in AGS cells infected with *H. pylori* 11,637 at varying multiplicities of infection (MOI) over a 24-hour period was analyzed through qRT-PCR ($n=3$) and WB. (**K**) The expression levels of NFIL3 mRNA and protein in AGS cells infected with *H. pylori*11637 at different time intervals (MOI = 100) were assessed using qRT-PCR ($n=3$) and WB. *$P<0.05$, **$P<0.01$, and ***$P<0.001$

Results

The biorhythm of NFIL3 is disrupted in *H. pylori*-infected GECs

To explore the potential pathogenic role of gastric mucosal rhythm disorder, we first analyzed the relationship between *H. pylori* infection and the expression of gastric mucosa circadian rhythm genes [34]. The relative expression of biorhythm genes in public data from GEO database (GSE60427 and GSE27411) containing expression profile data of human gastric mucosa of *H. pylori*-infected (Gastritis with *H. pylori* infection) and uninfected (*H. pylori* negative) donors were obtained. Among these genes, compared to the uninfected, the expression of multiple genes was significantly downregulated in *H. pylori*-infected gastric mucosa, however, *NFIL3* was elevated in both datasets (Fig. 1A and Supplementary Fig. 1A). Curiously, we then collected clinical gastric mucosa samples, compare to uninfected patients, the level of NFIL3 was higher in gastric mucosa of *H. pylori*-infected patients (Fig. 1B and C). Then, an animal model of gastritis was established by infecting C57BL/6 mice with *H. pylori* PMSS1. After 8 weeks post-infection, the dynamic changes of Nfil3 expression were detected across a 24 h day-night light cycle. We found that *Nfil3* mRNA expression oscillated diurnally (Fig. 1D). Notably, comparison of Nfil3 expression levels across a circadian cycle in uninfected and *H. pylori*-infected mice revealed that *H. pylori* can regulate the level of *Nfil3* mRNA and thus govern the amplitude of *Nfil3* transcriptional rhythms (Fig. 1D). In addition, we found that *Nfil3* mRNA expression increased more at 8 weeks after infection than at 4 weeks after infection (Supplementary Fig. 1B). The protein expression of Nfil3 was also higher in gastric mucosa of *H. pylori* PMSS1 infected mice (Fig. 1E). Furthermore, IHC results also showed that the expression of Nfil3 was increased in GECs in *H. pylori* induced chronic gastritis model (Fig. 1F). The findings presented above provide compelling evidence that *H. pylori* disrupt the biorhythm of NFIL3, and NFIL3 potentially serving a distinct and essential function in H. pylori associated gastritis.

In consideration of the above experiments, it was found that NFIL3 expression was significantly upregulated in GECs in *H. pylori*-infected mouse gastric mucosa, subsequently, we further explored the relationship between *H. pylori* infection and biorhythm genes/proteins expression at the cellular level. The expression profile datasets for the mouse gastric epithelial progenitor-derived cell line infected with *H. pylori* Kx1 and Kx2 (GSE10262) and the human gastric AGS cell line infected with *H. pylori* 60,190 (GSE70394) were sourced from the GEO database. In the two datasets, we observed changes in the expression of multiple biorhythm genes, excitingly, consistent with the aforementioned clinical results, the *NFIL3* mRNA expression was significantly upregulated in GECs by directly infected with *H. pylori* (Fig. 1G and Supplementary Fig. 1C and D). In order to validate this experimental observation, we utilized human primary GECs expressing EpCAM to establish cell infection models. Our findings indicated that the infection of these human primary GECs with either *H. pylori* 11,637 or 26,695 resulted in an upregulation of NFIL3 expression (Fig. 1H and Supplementary Fig. 1E). IF results showed that AGS cells infected with *H. pylori* 11,637 would increase the expression of NFIL3 in the nucleus (Fig. 1I). This suggests that NFIL3 may play an important regulatory role during *H. pylori* infection. Additionally, AGS cells infected with either *H. pylori* 11,637 or 26,695 increased NFIL3 mRNA/protein levels in an infection dose (MOI) dependent manner (Fig. 1J and Supplementary Fig. 1F). And our investigation revealed that the duration of *H. pylori* infection correlates positively with the elevated levels of NFIL3 mRNA and protein expression (Fig. 1K). The results unequivocally demonstrated that *H. pylori* lead to the upregulation of NFIL3 expression in GECs.

H. pylori transcriptionally increased epithelial NFIL3 expression via ERK-SP1 pathway

The pathogenicity of *H. pylori* is influenced by various virulence factors, with CagA and VacA being the most significant among them [4]. Initially, we conducted a Transwell infection assay, and the findings indicated

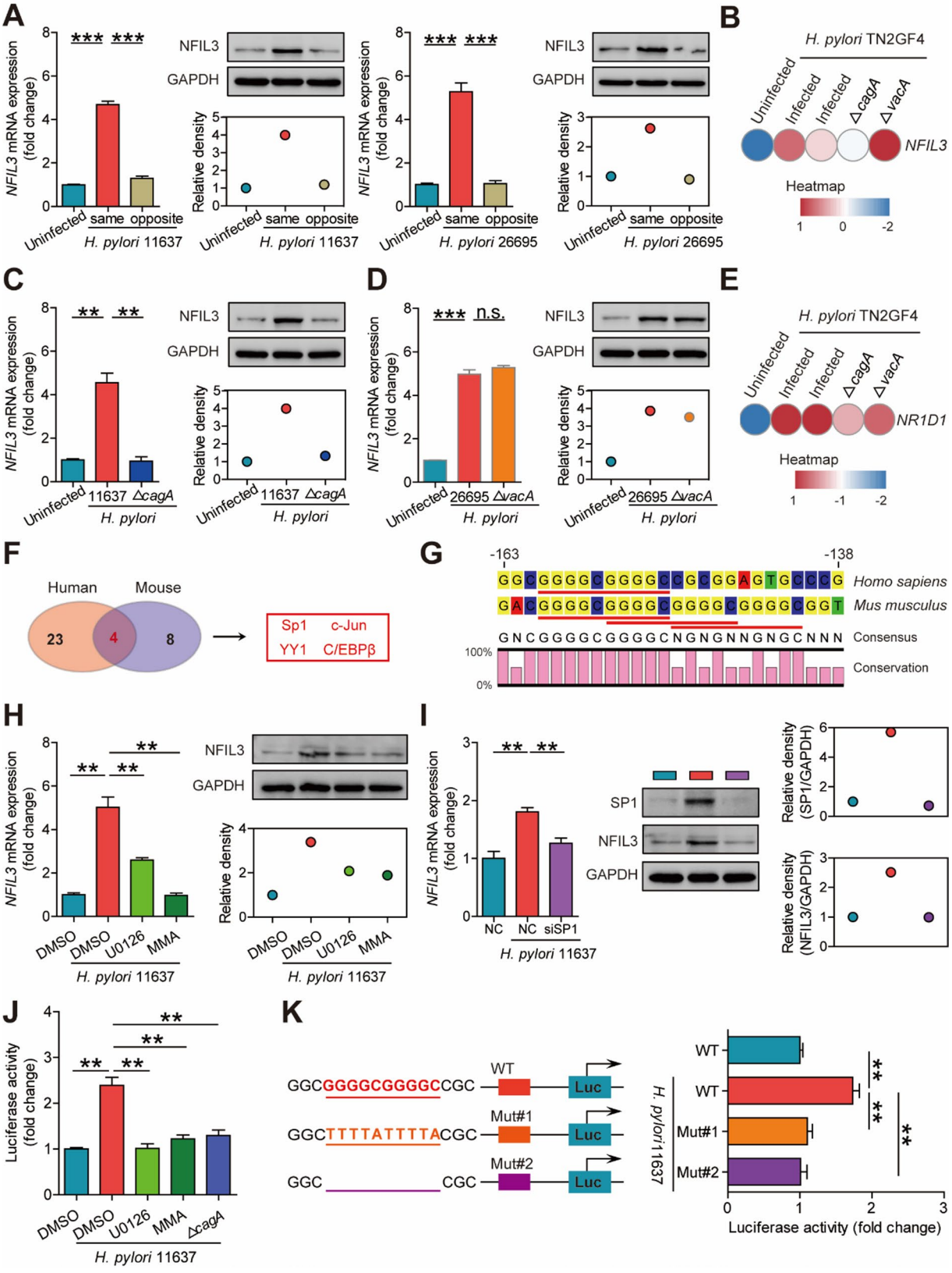

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Fig. 2 *H. pylori* transcriptionally increased gastric epithelial NFIL3 expression via ERK-SP1 pathway. (**A**) AGS cells were subjected to infection with *H. pylori* 11,637 or 26,695 (MOI = 100, 24 h) either in the same (lower) chamber or in a separate (upper) chamber of a Transwell apparatus. The expression levels of NFIL3 were assessed using qRT-PCR ($n = 3$) and WB. (**B**) The expression of NFIL3 in AGS cells infected with *H. pylori* TN2GF4 or its virulence mutant strains was evaluated through the GEO database (GSE60661). (**C**) NFIL3 expression was further examined in AGS cells infected with *H. pylori* 11,637 or the Δ*cagA* mutant, utilizing qRT-PCR ($n = 3$) and WB. (**D**) The expression of NFIL3 was also analyzed in AGS cells infected with *H. pylori* 26,695 or the Δ*vacA* mutant through qRT-PCR ($n = 3$) and WB. (**E**) The expression levels of NR1D1 were investigated in AGS cells infected with *H. pylori* TN2GF4 or virulence mutant strains utilizing the GEO database (GSE60661). (**F**) Potential transcription factor binding sites were predicted via the PROMO website, focusing on a 2000 bp segment of the NFIL3 promoter. (**G**) A conserved sequence of putative SP1 binding sites (red line) was identified in both human and mouse models. (**H**) WB and qRT-PCR ($n = 3$) analyses of NFIL3 were conducted in AGS cells post-treatment with *H. pylori* 11,637, U0126, and Mithramycin A (MMA). (**I**) Following a 24-hour transfection with SP1 siRNA, AGS cells were subsequently infected with *H. pylori* 11,637 for an additional 24 h. The expression levels of NFIL3 and SP1 were analyzed using qRT-PCR ($n = 3$) and WB. (**J**) NFIL3 promoter luciferase reporter assays were conducted in AGS cell lines treated with *H. pylori* 11,637, U0126, Mithramycin A (MMA), and the Δ*cagA* mutant. (**K**) Luciferase reporter assay for NFIL3-Luc and mutant-Luc. n.s. $P > 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

that direct contact is essential for *H. pylori* to stimulate the expression of NFIL3 in GECs (Fig. 2A). The VacA is a secretory virulence factor, while CagA affecting host cells requires direct bacterial contact and injection into cells via the type IV secretion system. Meanwhile, the dataset (GSE60661) of GEO database, containing expression profile data of AGS infected with *H. pylori* TN2GF4 and its mutant strains, also hints that cagA plays a regulatory role of NFIL3, but not vacA (Fig. 2B). Next, we also used mutant strains of virulence proteins for further validation. It is important to highlight that the levels of NFIL3 mRNA and protein exhibited an increase exclusively after infection with *H. pylori* 11,637, while no such elevation was observed in AGS cells infected with the Δ*cagA* (Fig. 2C). As anticipated, the VacA did not play a role in the induction of NFIL3 mRNA and protein expression (Fig. 2D). Taken together, these results unequivocally demonstrate that *H. pylori* CagA is responsible for the induction of NFIL3 expression in GECs.

Previous studies have shown that transcription factor NR1D1 has a negative regulatory effect on NFIL3 expression in intestinal epithelial cells under the control of microbiota [29]. However, the dataset (GSE60661) showed that *H. pylori* can induce upregulation of NR1D1 expression (Fig. 2E), which was also confirmed by our previous research [19]. These suggest that gastric epithelial NFIL3 expression is driven by *H. pylori* infection in a different regulatory mechanism than the intestinal epithelial cells. The promoter regions spanning from – 2000 to 0 of the NFIL3 were sourced from the Eukaryotic Promoter Database. Subsequently, an analysis of TF binding sites was conducted utilizing the PROMO Database, which revealed a total of 23 binding regions for human TFs and 8 binding regions for mouse TFs. Interestingly, there are four transcription factors (SP1, c-Jun, YY1 and C/EBPβ) that overlap in both humans and mice, and only SP1-binding sites in the conserved regions (Fig. 2F **and G**). Our previous research found that the ERK signaling pathway is an important upstream for the activation of SP1 [9], hence, we performed signaling pathway inhibition experiments with inhibitors. The results showed that inhibition of the ERK pathway by U0126 and inhibition of SP1 by Mithramycin A (MMA) remarkably suppressed NFIL3 expression during *H. pylori* infection, respectively (Fig. 2H). Additionally, we employed siRNA to inhibit the expression of SP1 during the infection with *H. pylori*. Consistent with our expectations, the induction of NFIL3 expression by *H. pylori* was effectively diminished following the suppression of SP1 (Fig. 2I). Then, we performed a luciferase reporter assay utilizing a plasmid that incorporates the NFIL3-luc promoter region (-250/0). When compared to the control group, the infection with *H. pylori* 11,637 led to a notable increase in luciferase activity. Conversely, the inhibition of the ERK signaling pathway, the suppression of SP1, or the application of the *H. pylori* Δ*cagA* does not lead to changes in luciferase activity (Fig. 2J). Subsequent luciferase reporter assay with mutations at the SP1 binding site also reconfirmed the direct regulation of SP1 on NFIL3 during *H. pylori* infection (Fig. 2K). Collectively, these results indicated that *H. pylori* induce epithelial NFIL3 expression via ERK-SP1 pathway.

Inflammatory cytokines from *H. pylori* infection exacerbate NFIL3 biorhythm disruption

Inflammatory cytokines play an irreplaceable role in *H. pylori*-associated pathology, so we investigated the influence of important cytokines on NFIL3 expression in GECs, especially in the *H. pylori* infection state. We showed that IL17, IFNγ, IL6, IL23 and IL12 had no effect on modulating NFIL3 expression in GECs; however, IL22 inhibits NFIL3 expression, TGFβ, IL1β and TNFα significantly upregulates NFIL3 expression in GECs (Fig. 3A). Surprisingly, in *H. pylori* infected state, IL6 slightly inhibits the expression of NFIL3, TGFβ has no effect on the expression of NFIL3, and IL1β and TNFα exerted a synergistic effect on *H. pylori*-mediated NFIL3 mRNA expression (Fig. 3A). We also validated that IL1β and TNFα exhibit a synergistic influence on the protein expression of NFIL3 induced by *H. pylori* (Fig. 3B). In the two datasets (GSE60427 and GSE27411), we observed that IL1β and TNFα had relatively high expression levels in the *H. pylori*-infected human gastric mucosa (Fig. 3C and D). And in another dataset (GSE181917) containing

expression profile data of mouse gastric mucosa (mice were subjected to infection with *H. pylori* PMSS1 for a duration of four weeks), the expression of *Il1β* and *Tnfα* was significantly elevated in the infected group (Fig. 3E). At the same time, the increased expression of *Il1β* and *Tnfα* was also detected in the model constructed by us, and the expression of *Il1β* and *Tnfα* oscillated diurnally during *H. pylori* infection (Fig. 3F). The dataset (GSE60661) showed that *H. pylori* CagA could induce IL1β and TNFα receptors to also be upregulated in GECs (Fig. 3G). In addition, we also found that IL1β and TNFα exerted a synergistic effect on *H. pylori*-mediated ERK pathway activation (Fig. 3H). These are the reasons why inflammatory cytokines may play a synergistic role during *H. pylori* infection. In summary, these results suggest that we should not ignore the remodeling effect of inflammatory cytokines on GECs, and inflammatory cytokines (IL1β and TNFα) could exacerbate the biorhythm disruption of NFIL3.

NFIL3 enhances the activity of NF-κB signaling and regulates inflammation during *H. pylori* infection

H. pylori serve as a significant etiological agent in the development of chronic gastritis and acts as the primary factor influencing the disruption of biorhythm expression of NFIL3 in GECs. Consequently, our primary focus is on investigating the functional role of NFIL3 in the context of *H. pylori* infection. To achieve this, we utilized siRNA to inhibit NFIL3 expression prior to subjecting the cells to *H. pylori* infection (Fig. 4A **and B**), and then carried out RNA-seq assay. GSEA was used to analyze the functional effects associated with NFIL3 based on the results of the RNA-seq. Ridge plot displaying gene set enrichment analysis in NC + *H. pylori* compared with NC and siNFIL3 + *H. pylori* compared with NC + *H. pylori* (Fig. 4C). The analysis results showed that "INFLAMMATORY_RESPONSE" and "TNFA_SIGNALING_VIA_NFKB" signature was significantly enriched in NC + *H. pylori* compared with NC (Fig. 4C and D). However, when comparing groups of siNFIL3 + *H. pylori* and NC + *H. pylori*, "INFLAMMATORY_RESPONSE" and "TNFA_SIGNALING_VIA_NFKB" signature was also significantly enriched in NC + *H. pylori* group (Fig. 4C and D). This suggests that NFIL3 promoted the inflammatory response and the activation of the NF-κB signaling pathway. We used heatmaps to present the gene-level expression of genes within the "INFLAMMATORY_RESPONSE" and "TNFA_SIGNALING_VIA_NFKB" gene set (Fig. 4E). Altogether, these results highlight the involvement of NFIL3 in activating the NF-κB signaling pathway during *H. pylori* infection.

For further analysis NFIL3-positive regulate genes during *H. pylori* infection, the differential expression analysis (|log2FoldChange|>1; $P<0.05$) was performed and the volcano plot of DEGs was showed (Fig. 5A). Analysis results showed that 164 genes were overlapped in NC + *H. pylori* compared with NC and siNFIL3 + *H. pylori* compared with NC + *H. pylori*. Among those genes, 42 protein coding genes were upregulated in NC + *H. pylori* and downregulated in siNFIL3 + *H. pylori* (including NFIL3) (Fig. 5B). The Gene-ontology analysis (top five GO terms under each category) of NFIL3-positive regulated genes were presented (Fig. 5C). We thought secreted proteins were more important for reshaping the microenvironment, so we analyzed the genes that NFIL3 could regulate, and ultimately, we focused on three genes, which are *CCL20*, *IL8* and *IL23* (Fig. 5D). The dataset (GSE60661) showed that *H. pylori* could induce *CCL20*, *IL8* and *IL23* expression in GECs, and the induced expression was CagA-dependent (Fig. 5E). Interestingly, these three genes are also downstream genes of the NF-κB signaling pathway during *H. pylori* infection [8, 35]– [36]. Subsequently, we verified using ELISA and found that NFIL3 can regulate the secretion of CCL20, IL8 and IL23 during *H. pylori* infection (Fig. 5F). In addition, we found that *Ccl20*, *Il8* and *Il23* expression increased more at 8 weeks after infection than at 4 weeks after infection (Supplementary Fig. 2).

According to the *H. pylori*-infected samples' histopathological evaluation, the mild, moderate, and severe gastritis were divided. Compared to uninfected patients, the level of NFIL3 was significantly correlated with the severity of gastritis (Fig. 6A). Based on clinical samples, we found that NFIL3 was positively correlated with the expression of CCL20, IL23 and IL8, respectively (Fig. 6B-D). Taken together, these results suggest that NFIL3, induced by *H. pylori*, amplifies the activity of NF-κB signaling pathways and modulates gastric inflammatory responses.

Discussion

Humans have inherent biological clocks, or biorhythms, that harmonize both internal and external signals to enhance the physiological operations of cells and tissues. Nevertheless, these clocks may be adversely affected by exogenous detrimental influences, such as bacterial infections [37]. *H. pylori* infection, the most prevalent bacterial infection globally, can significantly contribute to the development of chronic gastritis [3]. In the present investigation, utilizing clinical samples, animal models and cell-based assays, we investigated the pathological role of the key biorhythm molecule NFIL3 in *H. pylori*-associated gastritis (Fig. 6E). This could advance our fundamental molecular understanding of gastric biorhythm and inflammatory pathology and linked infectious chronic diseases.

Biorhythms are governed by a specific group of biorhythm proteins that modulate around 10% of the entire

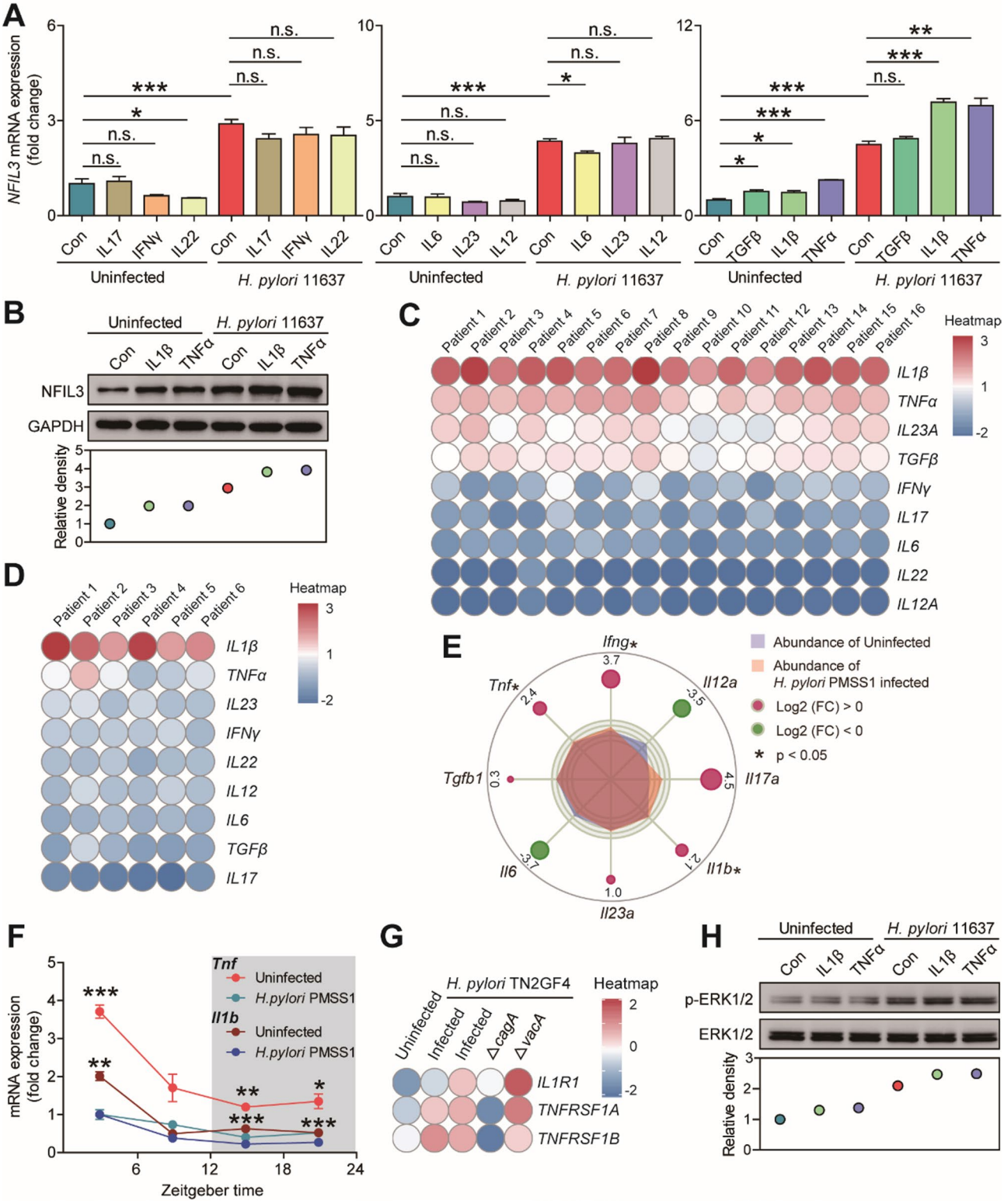

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Fig. 3 Inflammatory cytokines from *H. pylori* infection exacerbate NFIL3 biorhythm disruption. (**A**) The expression levels of NFIL3 were evaluated in AGS cells that were stimulated with *H. pylori* 11,637 (MOI = 100) and/or various cytokines including IL17A, IFNγ, IL22, IL6, IL23, IL12, TGFβ, IL1β, and TNFα (100 ng/ml) for a duration of 24 h using qRT-PCR ($n = 3$). (**B**) The protein expression of NFIL3 was assessed in AGS cells subjected to stimulation with *H. pylori* 11,637 (MOI = 100) and/or IL1β and TNFα (100 ng/ml) over a 24-hour period via WB. (**C**) The expression of inflammatory cytokine genes in human gastric mucosa was examined in the GEO database (GSE60427) for the cohort diagnosed with gastritis due to *H. pylori* infection ($n = 16$). (**D**) The expression of inflammatory cytokine genes in human gastric mucosa was analyzed in the GEO database (GSE27411) with gastritis associated with *H. pylori* infection ($n = 6$). (**E**) The expression of inflammatory cytokine genes in mouse gastric mucosa was investigated in the GEO database (GSE181917) for two distinct groups: uninfected and infected with *H. pylori* PMSS1. (**F**) The mRNA expression levels of Tnf and Il1b in the gastric mucosa of mice infected with *H. pylori* PMSS1 and uninfected controls were quantified using qRT-PCR throughout a 24-hour light-dark cycle. (**G**) The expression levels of IL1R1, TNFRSF1A, and TNFRSF1B were analyzed in AGS cells infected with either *H. pylori* TN2GF4 or its virulence mutant strains, utilizing the GEO database (GSE60661). (**H**) The protein expression of p-ERK1/2 and ERK1/2 was evaluated in AGS cells stimulated with *H. pylori* 11,637 (MOI = 100) and/or IL1β and TNFα (100 ng/ml) for 3 h using WB. n.s. $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

genome. Consequently, biorhythms exert a significant influence on various physiological and pathological processes, particularly in the context of inflammatory diseases [25, 38]. NFIL3 has also been reported to be involved in several inflammatory diseases. In rheumatoid arthritis, elevated levels of NFIL3 in peripheral blood mononuclear cells may be associated with the dysregulation of inflammatory cytokines, such as IL6, IL1β, IL8, and CCL2, as well as with the inflammatory responses that could play a significant role in the advancement of the disease [39]. In osteoarthritis, the analysis revealed that NFIL3 was a hub gene related to immune infiltration and correlated to the development and progression of disease [40]. However, in colitis, NFIL3 in macrophages induces an anti-inflammatory phenotype [41]. Experiments based on knockout mice have provided more insight. Nfil3-deficient mice (*Nfil3*$^{-/-}$ mice) exhibit spontaneous chronic colitis characterized by Th1/Th17 immune responses [28]. The absence of *Nfil3* (*Nfil3*$^{-/-}$ mice) has been shown to mitigate MOG35-55-induced experimental autoimmune encephalomyelitis by modulating various immune cell populations, notably Th17 cells [42]. Additionally, the lack of *Nfil3* (*Nfil3*$^{-/-}$ mice) significantly undermines the intestinal innate immune defense, particularly the type 3 innate lymphoid cells, in response to acute bacterial infections caused by *Citrobacter rodentium* and *Clostridium difficile* [27].

At present, most studies are based on the development and functional regulation of immune cells by NFIL3, while still know little about non-immune cells. In non-immune cell studies, NFIL3 has been shown to inhibit inflammation in LPS stimulated cardiomyocytes [43]. In the present study, we pay attention to the regulatory role of epithelial, not immune cells, and we found that NFIL3, which is induced by *H. pylori* to express, has a pro-inflammatory effect. GSEA analysis showed that NFIL3 can regulate the NF-κB pathway to mediate inflammation, and some reports also have mentioned the relationship between the two. In alzheimer's disease, rheumatoid arthritis and osteoarthritis, the association of NFIL3 and NF-κB pathway has been reported [39]– [40, 44]. As for the regulatory mechanism, in breast cancer, researchers have found that NFIL3 can inhibit the transcription of NFKBIA and thus increase the inflammation mediated by the NF-κB pathway [45]. In addition, there are no more reports on its specific regulatory mechanism, and further exploration is still needed. Notably, we also determined that CCL20, IL8 and IL23 showed an up-expression by NFIL3 regulation during *H. pylori* infection. These three genes are additionally considered downstream targets of the NF-κB signaling pathway in the context of *H. pylori* infection [8, 35]– [36]. Although several details of NFIL3 remain unanswered, the action of NFIL3 is very interesting during *H. pylori* infection.

Elucidating the regulatory mechanisms of essential molecules is vital for constructing a more comprehensive understanding of their physiological functions in both disease states and normal biological processes. Current studies have found that in the disease state, a variety of factors can interfere with the expression of NFIL3. In immune cells, melatonin triggers NFIL3 expression in Th17 via the MTNR1A receptor and Erk1/2 pathway [46], while commensal bacteria and their products induce NFIL3 in macrophages [47]. In non-immune cells, NFIL3 expression is influenced by various factors. miR-203 inhibits NFIL3 in LPS-stimulated cardiomyocytes [43]. Phosphorylated STAT3 activates NFIL3 transcription, leading to chemotherapy resistance in choriocarcinoma by preventing apoptosis [48]. Hypoxia upregulates NFIL3 via HIF1α in ovarian granulosa cells [49]. *Fusobacterium nucleatum* increases NFIL3 expression in apical papilla stem cells [50]. In breast cancer, NFIL3 self-regulates its expression [45]. Here, we observed that the pathogen *H. pylori* is capable of inducing the expression of NFIL3 in GECs. Mechanistically, the CagA protein activates the ERK signaling pathway, which subsequently facilitates the mediation of transcription factor SP1 to directly influence the transcriptional regulation of NFIL3. Significantly, we also revealed the cytokine regulation of NFIL3 in GECs. We showed that IL22 inhibits the expression of NFIL3, while TGFβ, IL1β and TNFα significantly upregulates the expression of NFIL3 in GECs. And during *H. pylori* infection, IL1β and TNFα exhibit a synergistic influence on the protein expression of NFIL3 induced by *H. pylori.*

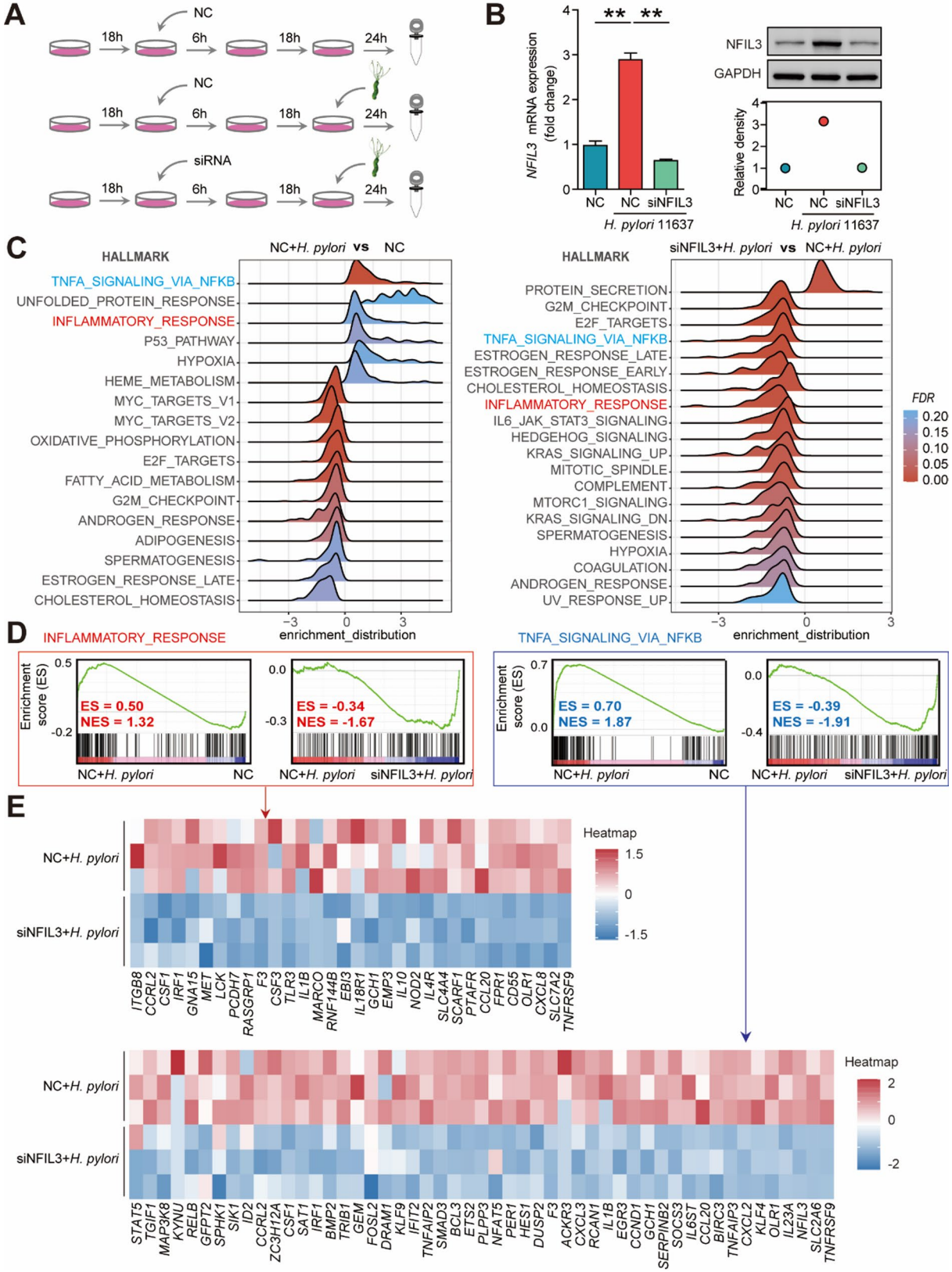

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Fig. 4 NFIL3 enhances the activity of NF-κB signaling during *H. pylori* infection. (**A**) A flowchart illustrating the suppression of NFIL3 in the context of *H. pylori* infection. (**B**) Following a 24-hour transfection with NFIL3 siRNA, AGS cells were subsequently exposed to *H. pylori* 11,637 for an additional 24 h. The expression levels of NFIL3 were assessed using qRT-PCR ($n=3$) and WB. (**C**) A ridge plot was generated to display the results of gene set enrichment analysis comparing *H. pylori* 11,637-infected NC with NC and *H. pylori* 11,637-infected siNFIL3 with *H. pylori* 11,637-infected NC based on RNA-seq. (**D**) An enrichment score plot for the HALLMARK sets "INFLAMMATORY_RESPONSE" and "TNFA_SIGNALING_VIA_NFKB" identified in part C. (**E**) A heatmap illustrating the expression levels of genes associated with the "INFLAMMATORY_RESPONSE" and "TNFA_SIGNALING_VIA_NFKB" HALLMARK sets presented in part D. $^{**}P<0.01$

However, the regulation of NFIL3 expression within the intestinal epithelium occurs through distinct mechanisms. Studies showed that NFIL3 in intestinal epithelial cells is rhythmic and its rhythmic expression was regulated by intestinal microbiota-ILC3-IL22-STAT3-NR1D1 axis [29]. Another study also confirmed that the role of IL22 in inducing NFIL3 expression is achieved by inhibiting the transcription of NFIL3 by NR1D1 in the intestinal epithelium [51]. In this study, we found that the expression of both NFIL3 and NR1D1 was increased, which indicated that the regulation mechanism was different from that in the intestine, and at the same time, it also reflected that the normal rhythm regulation mechanism was destroyed. Interestingly, under the state of infection, the expression of NFIL3 is still affected by some daily rhythms. However, over time (such as monthly and annual cycles) and with changes in bacterial colonization, this influence may gradually weaken. Further exploration of the dynamic changes and mechanisms of NFIL3 can better understand the pathological complexity of the disease.

In summary, our results highlight the significant importance of the biorhythm molecule NFIL3 in the pathogenesis of gastritis. *H. pylori* associated gastritis creates a microenvironment niche conducive to malignant transformation. Our research has expanded and deepened the understanding of NFIL3 as a pivotal molecular link among the bacteria, biorhythms, and inflammation. Considering the influence of biorhythms on gastrointestinal physiology and pathology, modulation of biorhythm molecules may represent a potential therapeutic strategy for controlling disease progression.

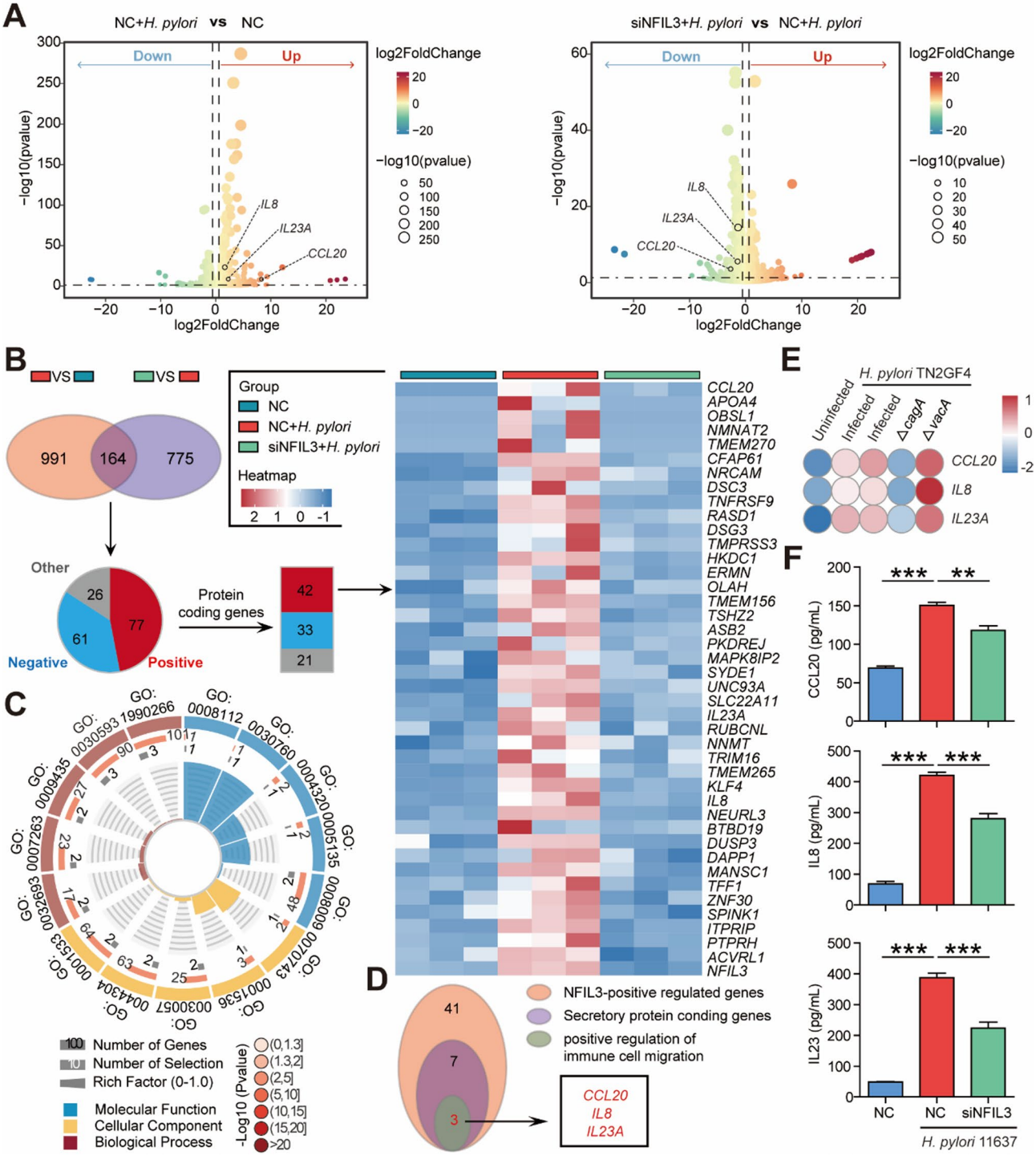

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Fig. 5 NFIL3 regulates inflammation during *H. pylori* infection. (**A**) The volcano plot illustrates DEGs in the context of *H. pylori* 11,637-infected NC compared to NC, as well as *H. pylori* 11,637-infected siNFIL3 cells in relation to *H. pylori* 11,637-infected NC. IL8, IL23A, and CCL20 are highlighted. (**B**) The RNA-seq analysis reveals that forty-two protein-coding genes exhibited upregulation in AGS cells transfected with NC and infected with *H. pylori* 11,637, while these genes were downregulated in AGS cells transfected with siNFIL3 and infected with the same strain. (**C**) Top five GO terms of Gene-ontology analysis (cellular component, molecular function and biological process) of target genes in B. Biological process: GO:0032693, negative regulation of interleukin-10 production; GO:0030593, neutrophil chemotaxis; GO:0007263, nitric oxide mediated signal transduction; GO:1,990,266, neutrophil migration; GO:0009435, NAD biosynthetic process. Cellular component: GO:0030057, desmosome; GO:0070743 interleukin-23 complex; GO:0001536, radial spoke stalk; GO:0044304, main axon; GO:0001533, cornified envelope. Molecular function: GO:0008112, nicotinamide N-methyltransferase activity; GO:0030760, pyridine N-methyltransferase activity; GO:0008009 chemokine activity; GO:0004320, oleoyl-[acyl-carrier-protein] hydrolase activity; GO:0005153, interleukin-8 receptor binding. (**D**) The analysis reveals overlapping genes among the categories of "NFIL3-positive regulated genes," "secretory protein-coding genes," and "positive regulation of immune cell migration." (**E**) The expression levels of CCL20, IL8, and IL23A were evaluated in AGS cells infected with either *H. pylori* TN2GF4 or virulence mutant strains, utilizing data from the GEO database (GSE60661). (**F**) Following a 24-hour transfection with NFIL3 siRNA, AGS cells were subsequently infected with *H. pylori* 11,637 for an additional 24 h. The concentrations of CCL20, IL8, and IL23A in the cell culture supernatant were quantified using ELISA. $^{**}P < 0.01$ and $^{***}P < 0.001$

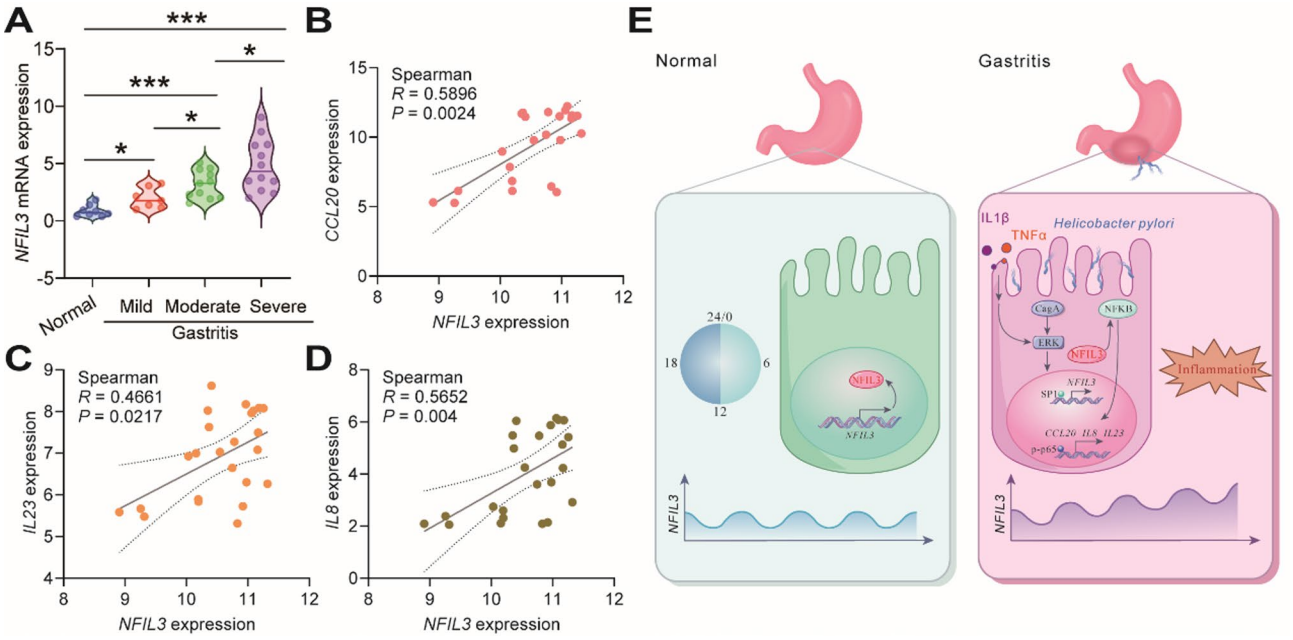

Fig. 6 The correlation between NFIL3 and inflammation in clinical samples. (**A**) The mRNA expression of NFIL3 was analyzed across gastric mucosa samples from uninfected individuals ($n=8$) and patients infected with *H. pylori*, categorized by the severity of inflammation as mild ($n=7$), moderate ($n=11$), and severe ($n=12$). (**B**) NFIL3 and CCL20 correlation analysis in the GSE60427 dataset. (**C**) NFIL3 and IL23 correlation analysis in the GSE60427 dataset. (**D**) NFIL3 and IL8 correlation analysis in the GSE60427 dataset. (**E**) A proposed model of disruption of the biorhythm in gastric epithelial cell triggers inflammation in *H. pylori*-associated gastritis by aberrantly regulating NFIL3. $^{*}P<0.05$ and $^{***}P<0.001$

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Yongsheng Teng, Hong Guo, Wenqing Tian and Yuan Zhuang designed the experiments. Yuan Zhuang, Quanming Zou, He Huang and Haiyan Li supervised the experiments. Yongsheng Teng, Yipin Lv, Wanyan Chen, Fangyuan Mao, Liusheng Peng and Liwei Shi collected the clinical data, performed the experiments, and analyzed the data. Yongsheng Teng, Wenqing Tian and Yuan Zhuang wrote the paper. All authors reviewed and approved the final manuscript.

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Data availability

The datasets supporting the conclusions of this article are included within the article (and its Additional files) and available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This research was approved by the Ethical and Experimental Committee of The General Hospital of Western Theater Command and written informed consent was obtained from all patients before enrolling in the research program. Similarly, the animal experiments conducted have been approved by the Ethics Committee of the Ethical and Experimental Committee of The General Hospital of Western Theater Command.

Consent for publication

All authors have read the manuscript and provided their consent for the submission.

Competing interests

The authors declare no competing interests.

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