REV-ERBα integrates colon clock with experimental colitis through regulation of NF-κB/NLRP3 axis

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The roles of Rev-erbα and circadian clock in colonic inflammation remain unclarified. Here we show colon clock genes (including Rev-erbα) are dysregulated in mice with DSS-induced colitis. In turn, disruption of the circadian clock exacerbates experimental colitis. Rev-erbα-deficient mice are more sensitive to DSS-induced colitis, supporting a critical role of Rev-erbα in disease development. Further, Rev-erbα ablation causes activation of Nlrp3 inflammasome in mice. Cell-based experiments reveal Rev-erbα inactivates Nlrp3 inflammasome mainly at the priming stage. Rev-erbα directly represses Nlrp3 transcription through specific binding to the promoter region. Additionally, Rev-erbα represses p65 transcription and indirectly repressed Nlrp3 via the NF-κB pathway. Interestingly, Rev-erbα activation in wild-type mice by SR9009 attenuates DSS-induced colitis, whereas the protective effects are lost in Nlrp3−/− and Rev-erbα−/− mice. Taken together, Rev-erbα regulates experimental colitis through its repressive action on the NF-κB/Nlrp3 axis. Targeting Rev-erbα may represent a promising approach for prevention and management of colitis.
any aspects of physiology and behaviors in mammals are subjected to circadian rhythms (a 24-h oscillation)\(^1\). Disruption of circadian rhythms has been associated with various types of diseases such as cancers and metabolic disorders\(^2,3\). Circadian rhythms are driven by the mammalian clock systems that are organized in a hierarchical manner\(^4\). The central clock system (pacemaker), located in the suprachiasmatic nucleus of the hypothalamus, synchronizes peripheral clocks (present in peripheral organs) through neural and hormonal signals\(^5\). At the molecular level, circadian clock machinery consists of transcriptional activators ([circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1)](http://example.com), forming the positive limb) and repressors [e.g., PER (period) and CRY (cryptochrome), forming the negative limb]\(^6\). CLOCK and BMAL1 function as a heterodimer that activates transcription of clock-controlled genes, including PER and CRY. Once reaching a threshold level, PER and CRY proteins inhibit the activity of CLOCK/BMAL1, thereby repressing their own expressions. This type of transcriptional–translational feedback loop system generates circadian oscillations of clock-controlled genes\(^6\).

REV-ERBa/β (NR1D1/NR1D2) are two members of the nuclear receptor 1D subfamily, functioning as transcriptional repressors\(^7\). They repress transcription of target genes through specific binding to the response element (named “REVRE” or “REV-ERB response element”) in gene promoter and subsequent recruitment of co-repressors such as nuclear receptor corepressor-1 and histone deacetylase \(^3,8\). REV-ERBa is a core component of circadian clockwork as its deletion causes disruptions to circadian rhythms in mice\(^8\). In fact, REV-ERBa repres- sion of BMAL1 is an accessory feedback loop that consolidates the rhythms of circadian oscillators\(^8\). In addition to circadian genes, REV-ERBa regulates the expressions of metabolic genes, thereby integrating circadian rhythms with cell metabolism\(^10\). Therefore, it is not surprising that REV-ERBa has been implicated in control of various physiological processes, including cell differentiation, lipid metabolism, mitochondrial biogenesis, and inflammation, making it a potential therapeutic target for cancers, dyslipidemia, and inflammatory diseases\(^11–13\).

Ulcerative colitis (UC), one of two major types of inflammatory bowel diseases (IBD) (the other is Crohn’s disease), is an acute or chronic inflammation of the membrane that lines the colon\(^14\). UC is characterized by weight loss, diarrhea, rectal bleeding, and abdominal pain, affecting millions of people in the world\(^14,15\). Although the exact cause of UC is uncertain, activation of the mucosal immune system and consequent pathological cytokine production play a contributing role\(^16–18\). Dextran sulfate sodium (DSS) is frequently used to induce colitis in experimental animals to study the pathogenesis of UC because of model simplicity and high similarities with human UC\(^19,20\). Disruption of circadian rhythms is reported to increase the risks for developing IBD\(^21\). Circadian perturbance also has the potential to alter gut microbiota, potentially contributing to IBD pathogenesis\(^22–24\). However, the mechanisms for regulation of IBD and microbiota by circadian clock remain largely unknown.

NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is a large protein complex consisting of NLRP3, ASC, and caspase-1\(^25\). NLRP3 inflammasome plays a central role in innate immune responses to pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs)\(^25–27\). Activation of NLRP3 inflammasome involves two sequential steps (i.e., priming and assembling) triggered by two signals\(^28\). The priming step triggered by the first signal (e.g., a PAMP such as lipopolysaccharides (LPS)) activates nuclear factor-κB (NF-κB) signaling and induces the transcription of pro-interleukin (IL)-1β and Nlrp3. The second signal (e.g., a DAMP such as adenosine triphosphate (ATP)) triggers several signaling pathways, including potassium efflux, generation of reactive oxygen species, and lysosomal damage that induce the assembly of Nlrp3 inflammasome using the three components\(^29–32\). Activation of NLRP3 inflammasome promotes the cleavage of caspase-1 and maturation and secretion of proinflammatory cytokines IL-1β and IL-18\(^28\).

The mechanisms for regulation of colitis by circadian clock remain elusive. In this study, we investigate a potential role of the core clock component Rev-erba in colitis regulation. We first established a close relationship between colon clock system and DSS-induced colitis, identifying Rev-erba as a potential link between circadian rhythms and colitis. Further, we revealed a critical role of Rev-erba in development of experimental colitis through regulation of NF-κB and Nlrp3 inflammasome activities. Rev-erba directly repressed Nlrp3 transcription via specific binding to a RevRE site in Nlrp3 promoter. Additionally, Rev-erba repressed transcription of p65 (a subunit of NF-κB) and indirectly repressed Nlrp3 via the NF-κB pathway. Our data suggest Rev-erba as a drug target for prevention and management of colitis.

**Results**

**Dysregulated clock genes in mice with experimental colitis.** DSS-induced colitis caused persistent and genome-wide gene deregulation in mouse colon (Fig. 1a). As expected, the mRNA levels of inflammatory cytokines were upregulated in the colon (Supplementary Figure 1). Pathway analyses of differentially expressed genes (DEGs) revealed a significant enrichment in the circadian clock system in addition to the inflammation pathways, suggesting colitis-associated clock dysregulation (Fig. 1b and Supplementary Table 4). In fact, oscillations of core clock genes were blunted in mice with colitis (Fig. 1c and Supplementary Table 5). Dysregulation of clock genes (Nrd1d1/Rev-erba, Clock, Bmal1, Per2, Cry1, Npas2, Nrd1d2/Rev-erbβ, Rora, and Dbp) were further confirmed by quantitative polymerase chain reaction (PCR) analyses (Fig. 1d).

**Circadian clock disruption exacerbates experimental colitis.** We first examined the effects of jet lag (i.e., physiologic disruption of circadian clock) on the development of colitis. Jet-lagged mice were established with a jet lag schedule of 8 h light advance every 2–3 days following a published protocol\(^3,33\), and confirmed by a wheel-running test (Supplementary Figure 2A). Mice were subjected to jet lag for 8 weeks before DSS feeding. Compared with normal mice, jet-lagged mice were much more sensitive to DSS-induced colitis as evidenced by the inflammation index values (i.e., weight loss, disease activity index (DAI), histopathological score, colon length, and myeloperoxidase (MPO)) (Supplementary Figure 2B–G). We also examined the effects of Bmal1 knockout (i.e., genetic disruption of circadian clock) on colitis development. Bmal1 knockout mice were generated using the CRISPR/Cas9 technique, and validated by wheel-running test, PCR genotyping, and expression profiling (Supplementary Figure 3A–D). Similar to jet lag, Bmal1 ablation sensitized mice to DSS-induced colitis (Supplementary Figure 3E–J). Compared to wild-type mice, Bmal1-deficient mice showed aggravated weight loss, increased DAI and MPO values, a higher histopathological score, and shorter colons (Supplementary Figure 3E–J).

**Rev-erba ablation sensitizes mice to experimental colitis.** Disruption of circadian clock (under both situations of jet lag and Bmal1 knockout) led to marked downregulation of Rev-erba in the colon (Fig. 2a, b). We also observed diminished expression of Rev-erba in mice with experimental colitis (Fig. 1). Thus, we
predicted a potential role of Rev-erbα in regulation of experimental colitis. This prediction was first interrogated by genetic studies. Rev-erbα knockout mice were generated using the CRISPR/Cas9 technique and validated by expression profiling (Fig. 3b and Supplementary Figure 4a–c). DSS-induced colitis was much more severe in Rev-erbα-deficient than in wild-type mice, supporting a critical role of Rev-erbα in the disease development (Fig. 2c–h). Further, IL-1β was the primary cytokine elevated in Rev-erbα−/− mice at the early phase of DSS-colitis (Fig. 2i). Inflammation mediated by chemokine (C-X-C motif) receptor 1 (CXCR1) was also confirmed in the colons (Fig. 3b). However, the rhythmicity in Nlrp3 expression was dampened as a result of Rev-erbα knockout (Fig. 3b). These data suggest that Nlrp3 is a clock-controlled gene and a potential direct target gene of Rev-erbα.

Identification of Nlrp3 as a clock-controlled gene. Circadian expressions of Nlrp3 and colitis-related inflammatory cytokines were determined in the liver and colon. In addition to the core clock genes (e.g., Bmal1 and Rev-erbα), hepatic Nlrp3 displayed robust diurnal fluctuations (Fig. 3a and Supplementary Figure 5). Interestingly, Nlrp3 oscillated in antiphase to Rev-erbα (a pattern highly similar to that of Bmal1, a direct target of Rev-erbα), suggesting that Nlrp3 may be a target of Rev-erbα (Fig. 3a). IL-1β, IL-18, and IL-6 (a known Rev-erbα target) showed mild oscillations (Fig. 3a and Supplementary Figure 5). Circadian expression of Nlrp3 was also confirmed in the colons (Fig. 3b). However, the rhythmicity in Nlrp3 expression was dampened as a result of Rev-erbα knockout (Fig. 3b). These data suggest that Nlrp3 is a clock-controlled gene and a potential direct target gene of Rev-erbα.

Rev-erbα inactivates Nlrp3 inflammasome. Two treatment strategies alternating the order of SR9009 (a Rev-erbα agonist) and LPS were employed to explore the effects of Rev-erbα on Nlrp3 inflammasome. SR9009 prior to LPS treatment resulted in reduced Nlrp3 and IL-1β mRNAs and proteins in PMs, whereas SR9009 post LPS treatment showed no effects (Fig. 3c, d). Stimulation of PMs with LPS/ATP for a short period of time (30 min) led to activation of caspase-1 in PMs and SR9009 treatment had no effects on caspase-1 activation (Fig. 3e). These data indicated a main action of Rev-erbα on the priming rather than assembling step of Nlrp3 inflammasome.
Rev-erba represses Nlrp3 transcription. A series of experiments were performed to explore whether Rev-erba transcriptionally regulates Nlrp3. Additionally, we investigated a potential role of Rev-erba/NF-κB axis in transcriptional regulation of Nlrp3 because Rev-erba is a known repressor of NF-κB34 and NF-κB is a transcriptional activator of Nlrp335. In luciferase reporter assays with Raw264.7 and HEK293 cells, Rev-erba repressed the transcription activity of Nlrp3 (Fig. 5a, b). Promoter analysis of Nlrp3 revealed that a 210-bp region (−1310 to −1100 bp) was responsible for the repressor activity of Rev-erba (Fig. 5c). In silico prediction suggested one RevRE (Rev-erba response element) and two κB sites (NF-κB-binding sites) within the 210-bp region (Fig. 5d, e). The two κB sites have been validated in a previous study35. Mutation of either RevRE (1319/1129 bp) or κB sites attenuated but failed to abolish the transcriptional repression effect of SR9009 (Fig. 5d). A mutation of both RevRE and κB sites completely abrogated the effects of SR9009 (Fig. 5d).
The data suggested that both RevRE and κB sites were responsible for Rev-erba-mediated repression of Nlrp3. Electrophoretic mobility shift assay (EMSA) assays indicated direct binding of Rev-erba to the predicted RevRE site of Nlrp3 (Supplementary Figure 7A). Further, chromatin immunoprecipitation (ChIP) assays confirmed recruitment of Rev-erba to the RevRE site of Nlrp3, supporting direct interactions of Rev-erba with Nlrp3 promoter in vivo (Fig. 5f).

Next, we assessed the effects of Rev-erba activation on NF-κB signaling. SR9009 treatment markedly suppressed LPS-induced IκBa phosphorylation as well as phosphorylation of p65 (a NF-κB subunit) in Raw264.7 cells, indicating an inhibitory action of Rev-erba on NF-κB signaling (Fig. 6a). Inactivation of NF-κB signaling by Rev-erba was supported by decreased mRNA expressions of IL-1β, IL-18, and TNFa (Fig. 4a and Supplementary Figure 6A & D), and also confirmed by reporter assay, EMSA, and immunofluorescence confocal microscopy (Fig. 6b, c, e, f). Further, total, cytosolic and nuclear p65 proteins were reduced by SR9009, potentially accounting for inactivation of NF-κB signaling (Fig. 6a, d). Consistently, the mRNA level of p65 in Raw264.7 cells was Rev-erba-dependent and Rev-erba overexpression decreased both p65 mRNA and protein (Fig. 6g and Supplementary Figure 8A). These data indicated a repressive role of Rev-erba in p65 expression and NF-κB signaling. By performing luciferase reporter, EMSA, and ChIP assays, we showed that Rev-erba repressed p65 expression via its specific binding to a RevRE element (−474/−484 bp) in promoter region (Fig. 6h, i, Supplementary Figure 7B & 8B). Although Rev-erba regulates p65, it shows no effects on p50, the other subunit of NF-κB (Supplementary Figure 8A).

**Rev-erba activation alleviates experimental colitis.** SR9009 (or vehicle) was administered to mice once daily for 7 days prior to DSS challenge. Compared with vehicle-treated mice, SR9009-treated mice showed reduced body weight loss, a lower DAI score, a higher survival rate, and longer colons (Fig. 7a–d). Improvement of colonic inflammation was also evidenced by decreased histological score and MPO activity (Fig. 7e–g). These data suggested alleviation of DSS-induced colitis by Rev-erba activation. Consistently, SR9009 failed to alleviate DSS-induced colitis in Rev-erba-deficient mice (Supplementary Figure 9). Additionally, SR9009 administration post DSS challenge showed moderate protective effects on body weight loss (Supplementary Figure 10C). Further, SR9009 suppressed Nlrp3 inflammasome activation by...
mice, Nlrp3-deleted mice were pretreated with Rev-erbα plasmid for 24 h and then stimulated with LPS for 8 h. Data are mean ± SD (n = 3). *P < 0.05 versus DMSO-treated group (Mann-Whitney U test).

b Measurements of Nlrp3 mRNAs in Raw264.7 cells by qPCR. The cells were pretreated with DMSO or a series of concentrations of Rev-erbα agonist (SR9009, hemin, or GSK4112) for 1 h and then stimulated with LPS for 8 h. c Measurements of Nlrp3 mRNAs in Raw264.7 cells by qPCR. The cells were pretreated with DMSO or SR9009 (10 μM) for 1 h and then stimulated with LPS for indicated hours. d Protein expressions of Nlrp3, pro-IL-1β, ASC, and pro-caspase-1 in Raw264.7 cells measured by western blotting. The cells were treated with DMSO or SR9009 (10 μM) for 1 h and then stimulated with LPS for 12 h. e mRNA expressions of Nlrp3, IL-1β, ASC, and caspase-1 in Raw264.7 cells measured by qPCR. The cells were transfected with Rev-erbα siRNA or Rev-erbα plasmid for 24 h and then stimulated with LPS for 8 h. f Protein expressions of Nlrp3, Pro-IL-1β, ASC, and pro-caspase-1 in Raw264.7 cells measured by western blotting. The cells were transfected with Rev-erbα siRNA or Rev-erbα plasmid for 24 h and then stimulated with LPS for 12 h. g Western blotting of Nlrp3, cleaved caspase-1 (p20), cleaved IL-1β (p17), pro-caspase-1, and pro-IL-1β in the supernatants (Sup) or cell lysates (Lys) of PMs. PMs were pretreated with SR9009 or vehicle for 1 h and then stimulated with LPS for 12 h and ATP for 30 min (added last). The concentrations of LPS and ATP for cell treatment were 100 ng/ml and 2 mM, respectively. Each western blot is representative of three independent experiments (statistical differences between blot density levels were analyzed by Mann-Whitney U test, Supplementary Figure 12). In panels c and e, data are mean ± SD (n = 3), *P < 0.05 (Mann-Whitney U test). Casp1: caspase-1

Discussion
In this study, we first established a tight association between colon clock and experimental colitis. Based on loss-of-function studies, we showed the core clock component Rev-erbα was crucial in development of experimental colitis. Further, in vivo and in vitro experiments demonstrated that Rev-erbα inactivated Nlrp3 inflammasome by repressing NF-κB and Nlrp3 transcription, thereby integrating the clockwork to the colonic inflammation. It is noteworthy that Rev-erbβ, the paralog of Rev-erbα, did not show regulatory effects on Nlrp3 or p65 decreasing expressions of Nlrp3 and pro-IL-1β/pro-IL-18, reducing the formation of mature (cleaved) IL-1β/IL-18 in both PMS and colon (Fig. 7h–j). Contrasting with wild-type mice, Nlrp3-deficient mice were resistant to DSS-induced colitis (Fig. 7a–g). The protective effect of SR9009 on DSS challenge was lost in Nlrp3−/− mice (Fig. 7a–g), so were the repressive effects of SR9009 on IL-1β/IL-18 expressions (Fig. 7h–j). Taken together, Rev-erbα activation prevented DSS-induced colitis via suppression of NF-κB and inactivation of Nlrp3 inflammasome.
Luciferase or Mann PMs were pretreated with SR9009 or vehicle for 1 h, followed by treatment with LPS for 1 h. Data are mean ± SD (luciferase plasmids with normal or mutated its mutated version followed by treatment with SR9009 (or vehicle) for 1 h and LPS for 8 h. Data are mean ± SD (−Luciferase reporter assays with distinct versions of 1.31 kb transfected with the 2.0 kb, −1.1 kb, or −1.31 kb, flα−1.1 kb, or −1.31 kb, flα−1.31 kb, or −1.31 kb, flα−1.1 kb plasmid. Data are mean ± SD (n = 6). At the time of manuscript preparation, Pourcet et al. reported that Rev-erbα in mice from experimental colitis via a suppressive action on Nlrp3 inflammasome activity. Therefore, our study identified a mechanism for prevention and management of colitis. Further works are needed to establish optimal dose and dosing time for SR9009 in terms of drug development and clinical therapeutics.

Our data suggest Rev-erbα as a potential gatekeeper of intestinal inflammation. Rev-erbα reduces the severity of colitis in mice by repressing NF-κB and Nlrp3 expression, thereby down-regulating Nlrp3 inflammasome activity (Figs. 4–6). At the time of manuscript preparation, Pourcet et al. reported that Rev-erbα regulates circadian expression of Nlrp3 and Rev-erbα activation alleviates fulminating hepatitis in mice. This study and the present one consistently pinpoint a critical role of Rev-erbα/Nlrp3 axis in controlling inflammatory diseases. Both studies show that Rev-erbα directly represses Nlrp3 expression via its specific binding to a RevRE site (at the precise position of −1139/−1129 bp identified herein) within the promoter (Fig. 5d). However, we additionally demonstrated that Rev-erbα indirectly represses Nlrp3 expression via the transcription factor NF-κB (Figs. 5 and 6). Both direct and indirect regulation mechanisms play important roles in Rev-erbα repression of Nlrp3 because a mutation of both RevRE and κB site abolishes the repression effect of SR9009 on

Fig. 5 Rev-erbα binds to Nlrp3 promoter and inhibits Nlrp3 transcription. a Luciferase reporter assays showing that Rev-erbα represses Nlrp3 transcription in Raw264.7 cells. The cells were transfected with blank PGL4.11, 2.0 kb Nlrp3 reporter, or 2.0 kb Bmal1 reporter along with blank pcDNA or Nr1d1 plasmid. Data are mean ± SD (n = 4). b Luciferase reporter assays showing that Rev-erbα activation downregulates Nlrp3 transcription in HEK293 and Raw264.7 cells. HEK293 cells were transfected with the 2.0 kb Nlrp3 reporter followed by treatment with SR9009 (or vehicle) for 12 h. Raw264.7 cells were transfected with the 2.0 kb Nlrp3 reporter followed by treatment with SR9009 (or vehicle) for 1 h and LPS for 8 h. Data are mean ± SD (n = 6). c Luciferase reporter assays with distinct Nlrp3 reporters in Raw264.7 cells. Raw264.7 cells were transfected with blank PGL4.11 and Nlrp3 reporter (i.e., −2.0 kb, −1.31 kb, −1.1 kb, or −0.8 bp promoter reporter) followed by treatment with SR9009 (or vehicle) for 1 h and LPS for 8 h. Data are mean ± SD (n = 6). d Luciferase reporter assays with different versions of 1.31 kb Nlrp3 reporters in Raw264.7 cells. The cells were transfected with 1.31 kb Nlrp3 reporter or its mutated version followed by treatment with SR9009 (or vehicle) for 1 h and LPS for 8 h. Data are mean ± SD (n = 6). e Schematic diagram of Nlrp3 luciferase plasmids with normal or mutated Nlrp3 promoter. f ChiP assay, showing recruitment of Rev-erbα to Bmal promoter and Nlrp3 promoter in PMs. PMs were pretreated with SR9009 or vehicle for 1 h, followed by treatment with LPS for 1 h. Data are mean ± SD (n = 4). *P < 0.05 (t test or Mann–Whitney U test). The concentrations of SR9009, LPS, and ATP for cell treatment were 10 μM, 100 ng/ml, and 2 mM, respectively.
**Fig. 6** Rev-erbα activation inhibits NF-κB signaling and p65 transcription. 

**a** Protein expressions of p65, p-p65, and p-IKBα in Raw264.7 cells measured by western blotting. Cells were pretreated with indicated concentrations of SR9009 or vehicle for 1 h and then stimulated with LPS for 12 h. **b** Luciferase reporter assays with Raw264.7 cells, showing Rev-erbα-dependent activation of NF-κB pathway. Cells were transfected with the NF-κB-dependent reporter (containing four kB sites) followed by treatment with SR9009 or vehicle for 1 h and stimulated with LPS for 8 h. Data are mean ± SD (n = 6). *P < 0.05 versus the group treated with LPS alone (t test). **c** EMSA assays performed with biotin-labeled NF-κB probe (containing NF-κB consensus binding sequence) in the presence of nuclear extracts. Raw264.7 cells were pretreated with Bay11-7082 (10 μM) or SR9009 for 1 h and then stimulated with LPS for 8 h. **d** Protein expressions of p65, p-p65, β-actin, and Histone H3 in cytoplasm (Cyto) or nucleus (Nuc) of Raw264.7 cells measured by western blotting. Cells were pretreated with SR9009 or vehicle for 1 h and then stimulated with LPS for 12 h. **e** Immunofluorescence analysis of p-p65 localization. Scale bar = 10 μm. **f** Intensity levels of green fluorescence for p-p65 quantified in six different fields. Data are mean ± SD (n = 6). **g** Effects of Rev-erbα overexpression on p65 protein in Raw264.7 cells. Each western blot is representative of three independent experiments (statistical differences between blot density levels were analyzed by Mann-Whitney U test, Supplementary Figure 12). **h** Luciferase reporter assays in Raw264.7 cells with distinct p65 promoter reporters. Cells were transfected with blank PGL4.11 and p65 reporter [−2.0 kb, −1.2 kb, −0.8 bp, or RevRE (−474/-484 bp)_mutant], followed by treatment with SR9009 or vehicle for 1 h and stimulation with LPS for 8 h. Data are mean ± SD (n = 6). **i** ChIP assay with PMs, showing recruitment of Rev-erbα to Bmal1 or p65 promoter. PMs were treated with SR9009 or vehicle. Data are mean ± SD (n = 6). In panels **f, h** and **i**, *P < 0.05 (t test or Mann-Whitney U test). Concentrations of SR9009, LPS, and ATP for cell treatment were 10 μM, 100 ng/ml, and 2 mM, respectively.

**Nlrp3** expression, whereas mutations of either site cannot (Fig. 5d).

We observed increased IL-1β and IL-6 expressions in Rev-erbα-deficient mice at early stage of colitis (2 days of DSS feeding) (Fig. 2i). This agrees well with previous studies in which Rev-erbα represses transcription of IL-1β and IL-6. Consistent with Rev-erbα regulation, circadian expression of IL-1β and IL-6 showed a typical pattern of Rev-erbα target gene (e.g., Bmal1) (Fig. 3a). We argue for a much more important role of IL-1β in development of colitis compared to IL-6. First, IL-1β was the primary inflammatory cytokine altered the most in Rev-erbα-deficient mice at the early phase of DSS-colitis (Fig. 2i). Second,
the regulatory effect of Rev-erba on intestinal inflammation was lost upon deletion of Nlrp3 in mice (Fig. 7).

We provide strong evidence that Rev-erba regulates activation of Nlrp3 inflammasome mainly at the priming stage. First, treatment of SR9009 + LPS decreased Nlrp3 and IL-1β expressions in PMs, whereas treatment of LPS + SR9009 showed no effects (Fig. 3c, d). Second, rapid stimulation of PMs with LPS + ATP led to caspase-1 activation (independent on Nlrp3 expression) that was unaffected in the presence of SR9009 (Fig. 3e). Third, Rev-erba activation did not affect the expressions of ASC and caspase-1 (Fig. 4). Fourth, Rev-erba was identified as a negative regulator of NF-kB activation and signaling that plays a key role in the priming of Nlrp3 inflammasome.37 In the study of Pourcet et al., the authors observed an increased number of cells with ASC specks in Nrl1d1-deficient mice, thus proposed that Rev-erba modulates the formation (assembling) of Nlrp3 inflammasome complex though the regulatory mechanism was unexplored. Whether Rev-erba regulates assembly of Nlrp3 inflammasome complex awaits further investigations.

In line with a previous study,36 Rev-erba represses the expressions of IL-1β and IL-18 in addition to Nlrp3 (Fig. 4e and Supplementary Figure 6A). The rhythms of colonic IL-1β and IL-18 mRNAs were also altered upon Rev-erba deletion (Fig. 3b). This led to the speculation that downregulation of IL-1β and IL-18 mRNAs (as well as pro-IL-1β and pro-IL-18 proteins) also plays a role in Rev-erba repression of colonic inflammation. However, we believe that contributions of pro-IL-1β and pro-IL-18 proteins also play a role in Rev-erba repression of colonic inflammation. However, we believe that Rev-erba on inflammasome activation (i.e., activation of caspase-1 and maturation of IL-1β) and experimental colitis is highly Nlrp3-dependent (Fig. 7).

![Figure 7](https://example.com/figure7.png)

**Fig. 7** Rev-erba activation alleviated experimental colitis in mice. a Weight loss measurements of four groups of mice with DSS feeding. b DAI scores of four groups of mice with DSS feeding. In panels a and b, data are mean ± SD (n = 8). *P < 0.05 (t test). c Survival rates of SR9009-treated and control mice (log-rank test). d Colon length of four groups of mice treated with DSS. e Representative micrographs of colon H&E staining. Scale bar = 100 μm. f Histopathological scores of four groups of mice treated with DSS. g MPO activities of mice colons on day 8. h qPCR analyses of Nlrp3, IL-1β, and IL-18 expressions in whole colon tissues of mice with colitis on day 8. IL-18 ELISA measurements of colonic IL-18 protein on day 8 after DSS feeding. j Western blotting of Nlrp3, IL-1β, and β-actin in PMs or colons from mice with colitis on day 8. Each western blot is representative of three independent experiments (statistical differences between blot density levels were analyzed by Mann–Whitney U test, Supplementary Figure 12). SR9009 (50 mg/kg) was administered to mice via intraperitoneal injection once daily at ZT8 for 7 days prior to DSS treatment, and SR9009 dosing was continued along with DSS treatment. In panels d, f–I, data are mean ± SD (n = 5 for WT, n = 8 for other groups). *P < 0.05 (t test or Mann–Whitney U test). SR: SR9009.
Our results suggest a tight interconnection between circadian clock and immune system consistent with the literature\(^8,39\). Clock disturbance sensitized mice to experimental colitis by upregulating Nlrp3 inflammasome activity via the Rev-erba receptor. Accordingly, Rev-erba activation reduces clock functionality and reduces the severity of colitis (Fig. 7). On the other hand, experimental colitis led to perturbed clock in the colon (Fig. 1). However, why this occurred was not addressed in current studies. Nevertheless, control of circadian clock by immune system is highly possible because the immune mediators (e.g., cytokines) were shown to have strong effects on circadian rhythms\(^8,39,41\). In addition, DSS and SR9009 may modify the circadian behaviors\(^8,42,43\). Whether and how such circadian alterations affect inflammatory processes remain unexplored.

NLRP3 inflammasome has been implicated in the pathogenesis of a wide variety of diseases, including Alzheimer’s disease, atherosclerosis, and type 2 diabetes\(^44,45\). Here we additionally established a critical role of Nlrp3 inflammasome in colitis (an IBD that affects millions of people worldwide\(^46\)) consistent with previous findings with Crohn’s disease\(^46,47\). Current therapies for Nlrp3-dependent diseases typically modulate the final products of NLRP3 inflammasome (IL-1β and IL-18) or to target inflammasome components\(^49\). As stated by Poutrec et al., targeting the Rev-erba/Nlrp3 axis for management of inflammatory diseases is advantageous in its pleiotropic effects, including Ca2+ suppression\(^11\), macrophage infiltration, TLR4 regulation\(^46\), and inactivation of NF-kB signaling (Fig. 6). Another merit of this therapeutic target axis refers to the high druggability of Rev-erba whose functions can be readily modulated by small-molecule agonists or antagonists\(^2,4,23\).

It was noteworthy that SR9009 was dosed at ZT8 in animal efficacy studies. This dosing time was chosen as being in coincidence with the highest expression of Rev-erba protein in normal mice (Supplementary Figure 10A). However, circadian timing system may be altered in mice with DSS-induced colitis as suggested by dysregulated clock genes (Fig. 1). In particular, Rev-erba expression was downregulated and its rhythmicity was significantly blunted (Fig. 1d). It was therefore acknowledged that ZT8 perhaps was not the optimal dosing time for maximized efficacy in the disease model. Nevertheless, significant pharmacological effects elicited by SR9009 were sufficient to clarify the role of Rev-erba in colitis development. In summary, Rev-erba serves as an integrator of colon clockwork and experimental colitis. Activation of Rev-erba prevents DSS-induced colitis in mice through its repressive actions on NF-kB and Nlrp3 inflammasome. Targeting Rev-erba may represent a promising approach for prevention and management of colitis.

**Methods**

**Materials.** LPS, ATP, GSK4112, hemin, and thioglycolate broth were purchased from Sigma-Aldrich (St. Louis, MO). Macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech (Rocky Hill, NJ). DSS (molecular weight 36–50 kDa) was obtained from MP Biomedicals (Irvine, CA). SR9009 was purchased from MCE (Monmouth Junction, NJ). Bay11-7082, chemiluminescent EMSA kit and biotin-labeled NF-kB probe were purchased from Beyotime (Shanghai, China). Lipopolysaccharide was purchased from Invitrogen (Carlsbad, CA). Chk1 kit was purchased from Cell Signaling Technology (Beverly, MA). RNAsisco Plus reagent and PrimeScript RT Master Mix were purchased from Takara (Shiga, Japan). Dual-Luciferase® Reporter Assay system was purchased Promega (Madison, WI). IL-1β, tumor necrosis factor alpha (TNFα), and interferon-γ enzyme-linked immunosorbent assay (ELISA) kits were purchased from MBio (Shanghai, China). IL-18, IL-1α, and IL-6 ELISA kits were purchased from Meridian Biotechnology (Yancheng, Jiangsu, China). MPO kit was purchased from Jiancheng Institute of Biotechnology (Nanjing, Jiangsu, China). Murine Raw264.7 cells were purchased from American Type Culture Collection (Manassas, VA). Antibodies for western blotting are as follows: anti-rev-erba (WH00093572M2, Sigma-Aldrich, MO); anti-Nlrp3 (NB12-12466, CO); anti-IL1β (AF-401, R&D systems, MN); anti-pro-caspase-1 (14F468, Santa Cruz, CA); anti-caspase-1 p20 (22915-1-AP, Proteintech, Wuhan, China); anti-p65 (10745-1-AP, Proteintech, Wuhan, China); anti-p-p65 (30301, CST, MA); anti-p-IKBα (14D4, CST, MA); anti-Histone H3 (17168-1-AP, Proteintech, Wuhan, China); and anti-β-actin (ab8226, Abcam, Cambridge, UK). For ChIP assays, antibody against Rev-erba and normal rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA). For immunofluorescence analysis, Alexa Fluor 488-conjugated anti-mouse and anti-body was purchased from Invitrogen (Gaithersburg, MD). Plasmids: pGL4.11 and pRL-TK vectors were purchased from Promega (Madison, WI). Bmal1 (2 kb)-Luc, Nlrp3 (2000)-Luc, Nlrp3 (1310)-Luc, Nlrp3 (1100)-Luc, Nlrp3 (800)-Luc, p65 (2000)-Luc, p65 (1200)-Luc, p65 (800)-Luc, pDNA-Rev-erba, and pDNA-Rev-erba were synthesized by Bioray Technologies (Shenzhen, China). Small interfering RNA targeting Rev-erba was purchased from Genepharma reporter constructs containing mutated versions of RevRE sites were obtained from Bioray Technologies (Shenzhen, China). NF-kB-dependent reporter (containing four kB sites) was purchased from Beyotime (Shanghai, China).

**Animals.** Wide-type C57BL/6 mice were obtained from Beijing HFK Bioscience (Beijing, China). All genetic mice were on a C57Bl/6 background. Bmal1/− mice were generated using the CRISPR/Cas9 system (Bioray Laboratory, Shenzhen, China). Rev-erba/− mice (B6.129S6-Nlrp3tm1Bhk/J) were obtained from Jackson Laboratory. All mice were bred and housed in Institute of Laboratory Animal Science (Jinan University, Guangzhou, China). Eight- to fourteen-week-old mice (male) were used for in vivo experiments. All animal care and experimental procedures were in compliance with guidelines approved by the Institute of Laboratory Animal Science of Jinan University (Guangzhou, China). Mice were randomly allocated into experimental groups based on body weight. Sample size was determined according to preliminary experimental observations. No data were excluded.

**DSS-induced colitis model.** Acute colitis was induced by feeding mice with 2.5% (w/v) DSS (dissolved in drinking water) for 8 days. Mice were sacrificed on day 8, and colons were collected for biochemical analyses. The colon length was measured with a centimeter ruler. To evaluate the effects of SR9009 on colitis, SR9009 (50 mg/kg) was administered to mice via intraperitoneal injection once daily at ZT8 (corresponding to a peak expression of Rev-erba, Supplementary Figure 10A) for 7 days prior to DSS treatment, and SR9009 dosing was continued along with DSS treatment (Supplementary Figure 10B). Colon tissues were fixed in 4% paraformaldehyde and embedded in paraffin, followed by hematoxylin-eosin staining. Histological damage was scored based on goblet cell loss, mucosa thickening, inflammatory cells infiltration, submucosa cell infiltration, ulcers, and crypt abscesses. A score of 1–3 or 1–4 were given for each parameter (scoring criteria are provided in Supplementary Table 6) with a maximal total score of 20. DAI scores were determined based on body weight loss, occult blood, and stool consistency. A score of 1–4 was given for each parameter with a maximal total score of 12.

**Isolation of primary macrophages.** Mice were injected iperitoneally with 4% thioglycollate broth. Four days later, peritoneal fluid was collected and plated in 1640 supplemented with 10% fetal bovine serum (FBS). Two hours later, non-adherent cells were aspirated and adherent cells (PMs) were obtained. BMDMs were differentiated from total bone marrow aspirates of mice. Cells were cultured with 1640 supplemented with 10% FBS and 20 ng/ml recombinant murine M-CSF. Seven days later, adherent macrophages were obtained and plated in 12-well plates.

**Quantitative polymerase chain reaction.** Total RNA was isolated using RNAsisco Plus reagent and reverse-transcribed using the PrimeScript RT Master Mix. PCR amplification procedure consists of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s. 18s RNA was used as an internal control. Relative expression was calculated by using the 2−ΔΔCT method. Primer sequences are provided in Supplementary Table 1.

**Luciferase reporter assay.** Cells were transfected with 500 ng of luciferase reporter plasmids, 50 ng of pRL-TK vector (an internal control with renilla luciferase gene), and 500 ng expression plasmids (Rev-erba or Rev-erba). The transfection assays were performed using Lipofectamine 2000 according to the manufacturer’s protocol. On the next day, the medium was changed to phenol-free Dulbecco’s modified Eagle medium with or without SR9009. Luciferase activities were determined by the Dual-Luciferase® Reporter Assay System and GloMax™ 20/20 luminometer (Promega). The relative luciferase activity values of treated cells were normalized to that of control cells.

**EMSA and ChIP.** For EMSA assays, the nuclear proteins were prepared using a cytoplasm/nuclear protein extraction kit. The DNA–protein complex was loaded on 4% nondenaturing polyacrylamide gels. After 35 min electrophoresis in 0.25x Trisborate-EDTA buffer, the products in the gels were transferred to Hybond-N⁺

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10 NATURE COMMUNICATIONS | DOI: 10.1038/s41467-018-06568-5 www.nature.com/naturecommunications
membranes. The signals were visualized by Omega Lum G imaging system (Aplegen, CA). Oligonucleotides sequences are provided in Supplementary Table 2. CDNP assays were performed at a final concentration of 50 μM in 96-well plates coated with 96-well plates coated with anti-Histone H3 antibody (R&D Systems, St. Louis, MO). Plates were incubated at 4 °C overnight. Cells were probed with an Alexa Fluor 488-conjugated secondary antibody. The blots were visualized with enhanced chemiluminescence and imaged by Omega Lum G imaging system (Aplegen). Uncropped bands were quantified by densitometry using Quantity One software (Bio-Rad).

RNA-seq: Nine normal mice and nine mice with colitis were sacrificed at each circadian time point (ZT0, ZT8, and ZT16), and the colons were isolated, snap-frozen, and stored at −80 °C. Total RNA was extracted using RNeasy Plus reagent and quality was analyzed by 2100 BioAnalyzer Expert (Agilent). RNA-seq data have been deposited to Sequence Read Archive (SRA) with a SRA accession number SRP149957. Other data are available upon request.

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Author contributions
B.W. and S.W. designed the study; S.W., Y.L., X.Y., F.L. and L.G. performed experiments; S.W., Y.L. and X.Y. collected and analyzed data; B.W., S.W. and Y.L. wrote the manuscript. S.W. and Y.L. contributed equally to this work.

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