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Authors
Bellet, Marina M
Zocchi, Loredana
Sassone-Corsi, Paolo

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The RelB subunit of NFκB acts as a negative regulator of circadian gene expression

Marina M. Bellet, Loredana Zocchi and Paolo Sassone-Corsi*

Center for Epigenetics and Metabolism; School of Medicine; University of California, Irvine; Irvine, CA USA

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Abbreviations: CCGs, clock-controlled genes; *c/c*, *Clock* mutant; MEFs, mouse embryonic fibroblasts; LPS, lipopolysaccharide; TNFα, tumor necrosis factor-α; TSS, transcription start site

The circadian system controls a large array of physiological and metabolic functions. The molecular organization of the circadian clock is complex, involving various elements organized in feedback regulatory loops. Here we demonstrate that the RelB subunit of NFκB acts as a repressor of circadian transcription. RelB physically interacts with the circadian activator BMAL1 in the presence of CLOCK to repress circadian gene expression at the promoter of the clock-controlled gene Dbp. The repression is independent of the circadian negative regulator CRY. Notably, RelB$^{-/-}$ fibroblasts have profound alterations of circadian genes expression. These findings reveal a previously unforeseen function for RelB as an important regulator of the mammalian circadian system in fibroblasts.

Introduction

The clock system controls many physiological and metabolic functions in different organisms.[1] In mammals, a clock-driven transcriptional machinery is responsible for regulating the circadian gene expression of 10–20% of the genes within most tissues.[2-5] Aberrant regulation by the circadian machinery may lead to various pathological conditions, including neurodegeneration, insomnia, inflammation, obesity, diabetes and cancer.[6-12] The transcription factors CLOCK and BMAL1 are central to the positive transcriptional loop: after heterodimerization they bind to E-box promoter elements in the regulatory regions of many clock-controlled genes (CCGs). Among the CCGs, there are the *Per* and *Cry* genes, which encode negative regulators of CLOCK:BMAL1. These interplays are responsible for the oscillation of circadian gene expression.[13]

Accumulating evidence shows the presence of bidirectional links between circadian regulation and inflammatory response.[14-21] Previous studies have demonstrated that stimulation of fibroblasts with tumor necrosis factor-α (TNFα) represses circadian transcription.[22,23] Moreover, we have recently observed that circadian disruption is associated with acute bacterial infection in mice (unpublished data), whereas other reports indicate that circadian disruption is involved in the development of symptoms associated to the inflammatory state.[24-26]

The NFκB transcription factor plays a central role in the inflammatory response. It is composed by five different subunits that can homo- or hetero-dimerize to form a variety of transcriptionally active isoforms with widely different roles in the transcriptional activation or repression of inflammatory genes.[27-30]

Here we report on the interplay between the circadian clock and the NFκB transcriptional pathway. Cells with a disrupted clock system display an altered response to lipopolysaccharide (LPS) and aberrant levels of some specific components of the NFκB complex. We show physical and functional interaction between RelB and BMAL1. This results in the repression of CLOCK:BMAL1-driven transcription and in alteration of the circadian expression profile in mouse embryo fibroblasts lacking RelB. Our findings reveal a molecular link between two transcription pathways previously thought to be independent, providing a molecular framework to interpret the physiological relationship between the inflammatory response and circadian rhythms.

Results

Reduced inflammatory response in cells with a disrupted circadian clock. To explore whether the circadian clock could modulate the inflammatory response, we studied cultured cells with a disrupted clock system compared with their wild-type counterpart. We followed the timing of expression of various cytokines 1 h and 4 h after LPS stimulation of mouse embryonic fibroblasts (MEFs) derived from wild-type and *Clock* mutant mice (*c/c*), where a single point mutation determines a deletion of exon 19 within the CLOCK coding sequence,[31-34] thus generating a dominant negative mutant CLOCK protein that renders the CLOCK:BMAL1 heterodimers functionally defective.[35,36] The expression of the proinflammatory genes *Il-6*, *Cxcl1* and *Il-1β* (**Fig. 1**) was drastically reduced in *c/c* MEFs compared with

*Correspondence to: Paolo Sassone-Corsi; Email: psc@uci.edu
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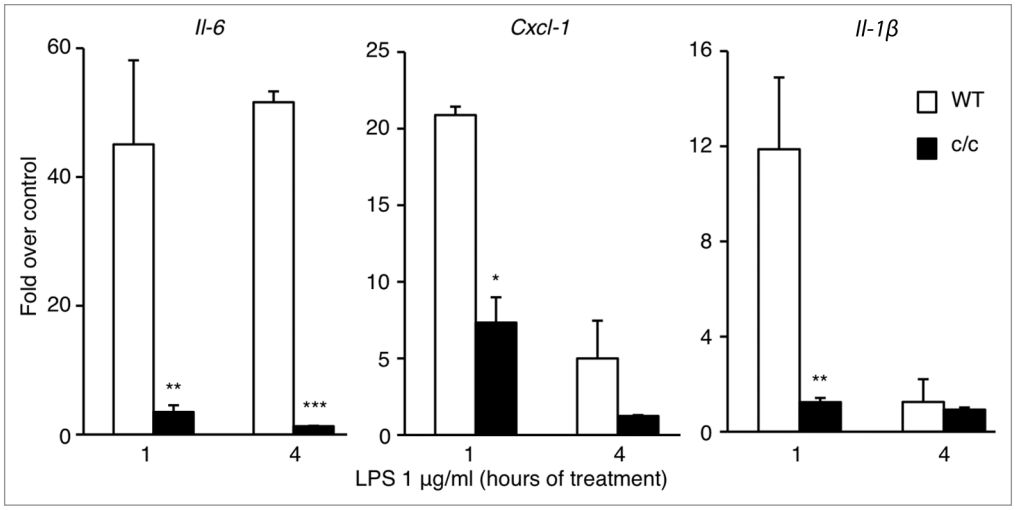

Figure 1. *Clock* mutant MEFs are less resposive to LPS stimulation. Time course of mRNA expression of different cytokines after LPS stimulation (1 µg/ml) of wt and *Clock* mutant (c/c) MEFs, measured by quantitative real time PCR. Shown are fold changes in gene expression compared with unstimulated cells. All the values are the mean +/- s.e.m. (n = 6); (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.

the wild-type cells. We also observed that these cells were only slightly responsive to stimulation with recombinant TNFα (**Fig. S1**), thus confirming that the low responsivity was independent of the stimulus applied to the cells to induce the inflammatory response. We also monitored the expression of circadian genes after TNFα stimulation (**Fig. S1**). As previously reported,[22,23] TNFα leads to a repressed expression of circadian genes in wild-type cells, while a constantly low level of *Per2* and *Dbp* mRNAs was detected in *c/c* MEFs. Thus, a normally functioning circadian clock is necessary to obtain an efficient inflammatory response.

Specific elelements of the NFκB pathway are overexpressed in *Clock*-mutant fibroblasts. Based on the differential to LPS and TNFα in cells with a disrupted clock (**Fig. 1**), we sought to explore the integrity of the NFκB signaling pathway. To do so, we monitored the protein levels of different NFκB subunits in wild-type and *c/c* MEFs, untreated or after LPS treatment. We observed a robust upregulation of the components of the non-canonical pathway RelB and p100/p52 in *c/c* fibroblasts as compared with isogenic wild-type cells. The upregulation appears independent from LPS stimulation (**Fig. 2A**). No differences in total levels of RelA and p50 were observed. The overexpression of RelB and p100/p52 is specific to *c/c* MEFs and not observed in cells carrying mutations in other clock components (**Fig. S2**).

These findings prompted us to investigate whether the expression of the transcriptionally active subunit RelB is regulated in a circadian manner. While the protein levels of RelB do not display a circadian oscillation in MEFs synchronized by serum shock, we observed a robust upregulation at all circadian time points in *c/c* MEFs compared with wild-type MEFs (**Fig. 2B**). Similarly, RelB mRNA showed only a marginal oscillation in both wild-type and *c/c* MEFs after serum-shock synchronization. Paralleling the protein levels (**Fig. 2B**), a significant upregulation of *RelB* transcript was present in *c/c* MEFs (**Fig. S3**).

Since RelB overexpression has been associated to repression of the inflammatory response in fibroblasts and induction of endotoxine tolerance,[37-42] we concluded from these experiments that RelB expression is upregulated in *c/c* MEFs, and that this is likely to contribute to the altered response of these cells to an inflammatory stimulus (**Fig. 1**).

Physical interaction between RelB and BMAL1. We sought to unravel the molecular mechanism by which the clock system may interplay with the NFκB pathway. Thus, we explored the possibility that one or several NFκB subunits could play a role in the regulation of circadian transcription by interacting with the components of the circadian machinery CLOCK and BMAL1. We ectopically overexpressed CLOCK and BMAL1, together with RelB, RelA, p50 or p52 in cultured cells. We found that RelB, but not RelA (**Fig. 3A**) or the other regolatory subunits (**Fig. 3B**), is able to efficiently interact with BMAL1, and weakly with CLOCK, thus suggesting a specific role of RelB in the circadian system.

Next, we determined whether native RelB interacts with BMAL1 and/or CLOCK expressed endogenously in cultured cells. A specific interaction was found with BMAL1 in wild-type MEFs (**Fig. 3C**), while we were not able to detect unequivocal interaction with CLOCK under the same conditions. To test whether CLOCK is dispensable for the interaction of RelB with BMAL1, we transfected CLOCK and BMAL1 alone or in combination, together with RelB. We found that RelB interacts with BMAL1 alone, and that the interaction is reinforced in the presence of CLOCK (**Fig. S4A**). No interaction with CLOCK alone was found (not shown).

We then explored whether mutations of the CLOCK protein may affect the RelB-BMAL1 interaction. We transfected full-length CLOCK or various deletion mutants, known to reduce or abolish CLOCK functions. RelB interaction with BMAL1 was more robust in the presence of a full-length CLOCK protein than in the presence of truncated forms of CLOCK, either

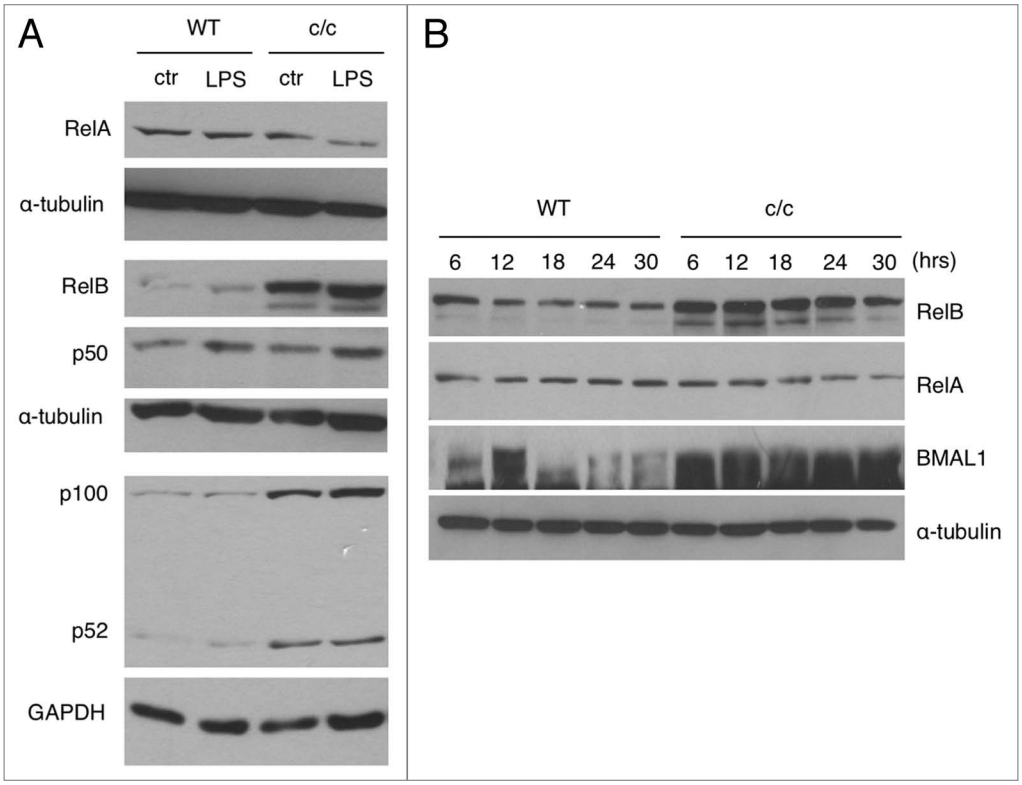

Figure 2. Expression of NFκB subunits in c/c MEFs. (A) Endogenous expression of RelA, RelB, p50 and p100/p52 in wild type (WT) and *Clock* mutant (c/c) MEFs, treated for 1 h with LPS (1 μg/ml) or left untreated (ctr), was determined by western blot analysis. The α-tubulin and GAPDH were used as loading controls. (B) Wild-type and c/c MEFs were synchronized by 2 h serum-shock treatment. Total lysates were prepared at the indicated times (hrs, hours) post-synchronization and resolved by SDS-PAGE. Levels of RelB, RelA, BMAL1 and α-tubulin were detected by western blot analysis using specific antibodies.

with a deletion in the N terminal region or with deletion of exon 19, domains affecting protein-protein interaction and transcriptional activation, respectively (**Fig. S4B**). Based on these results, we then tested the RelB-BMAL1 interaction in wild-type and *c/c* MEFs. The interaction of RelB with BMAL1 was significantly reduced in *c/c* MEFs as compared with wild-type cells (**Fig. 3C**). Stable ectopic expression of CLOCK in *c/c* MEFs rescued the efficacy of RelB-BMAL1 interaction at levels similar than wild-type MEFs (**Fig. 3C**). From these experiments we conclude that RelB readily interacts with BMAL1, and that this interaction is reinforced and dependent on the presence of a functional full-length CLOCK protein.

RelB represses CLOCK:BMAL1-driven trancriptional activation. The specific BMAL1 interaction with RelB prompted us to explore whether it could be functionally relevant in modulating CLOCK:BMAL1-driven transcriptional activation. To address this question we performed luciferase assays on transiently transfected cultured cells. First, we observed a significant repression of CLOCK:BMAL1-driven transactivation on the *Per1* promoter by RelB (**Fig. 4A**). Using a synthetic reporter vector carring only E-box promoter elements fused to the luciferase gene, we demonstrated that the repression was E-box-mediated (**Fig. 4B**). CLOCK:BMAL1-driven expression is known to be severely repressed by the circadian proteins CRYs.[43] As comparison, RelB induces a repression of comparable extent than CRY1 (**Fig. 4C**), whereas co-expression of CRY1 and RelB leads to an additive repression (**Fig. 4C**). Importantly, RelB-mediated inhibition of CLOCK:BMAL1 is maintained in *Cry1/Cry2*-deficient cells (**Fig. 4D**), thus indicating that RelB repression is independent on the negative circadian regulators CRYs. Since RelB requires heterodimerization to be transcriptionally active,[27-30] we asked whether the association with the regolatory subunit p52 could increase the repression. Interestingly, we observed increased repression in presence of p52 (**Fig. 4E**).

To better understand how the repression could take place, we performed a chromatin IP experiment at the promoter of the circadian gene *Dbp* in synchronized MEFs. Interestingly, RelB was immunoprecipitated together with CLOCK and BMAL1 on *Dbp* promoter transcriptionally active region (**Fig. 5A**) or on *Per2* TSS promoter region (**Fig. S5**), while no enrichment was observed at the 3' region of *Dbp* gene (**Fig. 5A**) or on *Bmal1* promoter (**Fig. S5**). We then asked if the presence of RelB at the promoter of circadian genes was dependent on CLOCK and BMAL1. We performed the same experiment in BMAL1 KO MEFs, where the circadian transcription is abolished, and the CLOCK:BMAL1 complex is not present at the chromatin level, and we could not either detect any presence of RelB (**Fig. S6**). These results indicate that RelB is within a chromatin complex with CLOCK and BMAL1 at the promoter of circadian genes.

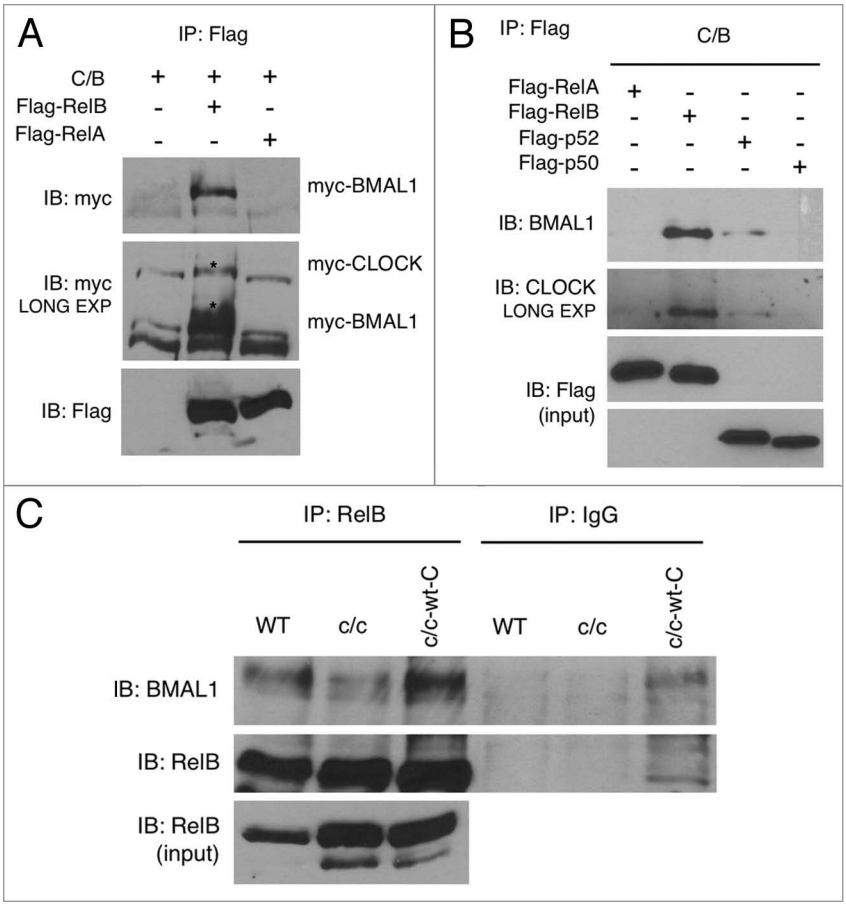

Figure 3. RelB interaction with BMAL1 and CLOCK. (A) HEK-293 cells were cotransfected with Myc-CLOCK and Myc-BMAL1 (C/B), without or with Flag-RelB or Flag-RelA. Flag-tagged proteins were immunoprecipitated by FLAG-Agar, and abundance of coimmunoprecipitated proteins was determined by western blotting with anti-Myc antibody and anti-Flag antibody. Asterisks indicate specific signals for BMAL1 and CLOCK proteins. (B) HEK-293 cells were cotransfected with Myc-CLOCK and Myc-BMAL1 (C/B) and series of expression vectors as described. Total lysates were prepared and subjected to immunoprecipitation using FLAG-Agar and coimmunoprecipitated proteins were detected by western blotting with anti-BMAL1 and anti-CLOCK antibodies. Lower panels show the expression of Flag-tagged proteins in total cell lysates as an input. (C) Cell extracts prepared from wild-type (WT), *Clock* mutant (c/c) and *Clock* mutant stably transfected with wild-type Myc-CLOCK (c/c-wt-C) were immunoprecipitated with RelB antibody or normal IgG, and immunoprecipitated BMAL1 and RelB were detected by probing with the BMAL1 and RelB antibody, respectively. Lower panel shows RelB expression in total cell lysates as an input.

RelB controls the amplitude of circadian transcription. Our findings strongly suggested that RelB modulates CLOCK:BMAL1 and thereby contribute to the normal oscillation of circadian transcription. This notion was confirmed by using a chromatin immunoprecipitation approach, which demonstrated that RelB is present at the promoter of the clock-controlled gene *Dbp* in parallel with CLOCK and BMAL1 (**Fig. 5A**). To address the question, we used MEFs in which the *RelB* gene is deleted and compared to the profile of circadian gene expression with equivalent wild-type MEFs cells. We followed the expression of various circadian genes during a 24 h cycle after serum-shock synchronization. The transcription of *Dbp* was enhanced in asynchronous cells (CT0), and the amplitude of the oscillation during the circadian cycle was significantly increased in the absence of RelB (**Fig. 5B**), consistent with a repressive role. Similar results were obtained for the circadian-regulated genes *Rev-erbα* and *Cry1* (**Fig. S7**). Interestingly, the transcription of the period genes was downregulated (**Fig. S7**), suggesting that RelB could play a more complex role in the maintainance of the functionality of the clock machinery.

Discussion

Previous reports showed as RelB expression is involved in the repression of the inflammatory response in fibroblasts.[39-42,44,45] Different mechanisms have been considered, including the recruitment of chromatin modifier enzymes,[37,46,47] thereby creating a repressive chromatin state, or stabilization of negative regulators, such as IkBα.[38,40] Importantly, RelB was also considered as the major mediator of the endotoxin tolerance status in fibroblasts.[42] This observation is of particular interest, since we observed increased levels of RelB in *Clock*-mutant MEFs compared with isogenic wild-type cells, and we also observed reduced induction of inflammatory genes expression in *Clock*-mutant macrophages (unpublished data) and MEFs (**Fig. 1**). We believe that these notions are important for future investigations.

Recent findings from another laboratory confirm that elements of the circadian clock may interplay with the NFκB pathway (M. Antoch, personal communication). Here we have shown that the NFκB subunit RelB directly partecipates in the regulation of circadian transcription, by interacting with the core clock factors BMAL1 and CLOCK at the chromatin level. Both interaction and presence of RelB at the promoter of circadian genes appears to be strictly dependent on a functional CLOCK:BMAL1 complex (**Fig. S4 and 6**). Moreover, RelB appears to directly control circadian transcription as shown by the use of *RelB*-deficient MEFs (**Fig. 5**; **Fig. S7**). It is still unclear whether RelB-induced repression of the circadian clock has a role during the inflammatory response. It is tempting to speculate that RelB might partecipate in mediating the repression of circadian genes during inflammation (**Fig. S1**), establishing an intriguing link between the clock system and the NFκB pathway. This possibility could be of great relevance during inflammatory responses and apoptosis and could establish the basis for the development of novel pharmacological strategies and therapeutic approaches.

Materials and Methods

Plasmids. C-terminal Flag-tagged RelB pcDNA3, RelA pcDNA3, p52 pcDNA3 and p50 pcDNA3 were purchased from

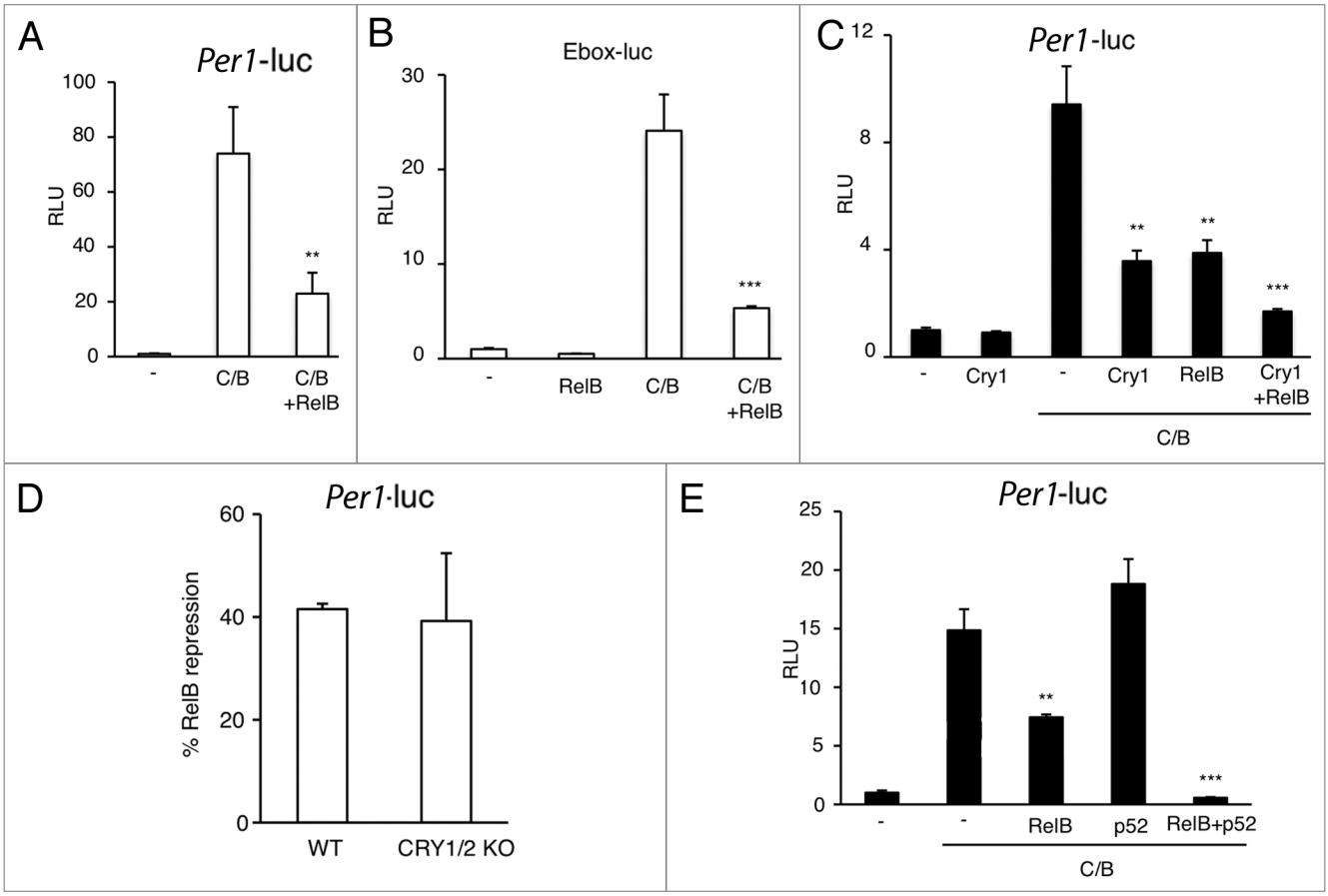

Figure 4. RelB represses CLOCK:BMAL1 transactivation potential. (A) Effect of RelB on CLOCK:BMAL1 dependent transcription. Vectors expressing CLOCK and BMAL1 (C/B) were cotransfected with a construct containing the mPer1-luc promoter, with or without RelB. The total DNA amount was kept constant by adding carrier plasmid DNA. After normalization for transfection efficiency using β-galactosidase activity, reporter gene activity was expressed as relative luciferase units (RLU) (activity of the control transfected only with non-expressing plasmid was set to 1). All the values are the mean +/- SD (n = 3); (**) $p < 0.01$ (B) The E box promoter element mediates RelB repression of CLOCK:BMAL1. Experimental condition was as in (A), except that a reporter construct containing three copies of the Ebox consensus sequence was used (Ebox-luc). All the values are the mean +/- SD (n = 3); (***) $p < 0.001$. (C) Additive effect of RelB and cry1 repression on CLOCK:BMAL1 dependent transcription. Experimental conditions are as in A except that Cry1 was cotransfected with C/B or C/B + RelB. All the values are the mean +/- SD (n = 3); (**) $p < 0.01$, (***) $p < 0.001$. (D) RelB repression is CRY-independent. CLOCK and BMAL1 were cotransfected as in A in wild-type (WT) and CRY1/2 KO MEFs. % of RelB repression of CLOCK:BMAL1 transactivation is shown. All the values are the mean +/- SD (n = 3). (E) Effect of RelB and p52 on CLOCK:BMAL1 dependent transcription. Vectors expressing CLOCK and BMAL1 (C/B) were cotransfected with a construct containing the Per1-luc promoter, with or without RelB and p52, as described. After normalization for transfection efficiency using β-galactosidase activity, reporter gene activity was expressed as relative luciferase units (RLU) (activity of the control transfected only with non-expressing plasmid was set to 1). All the values are the mean +/- SD (n = 3); (**) $p < 0.01$, (***) $p < 0.001$.

Addgene. N-terminal Myc-tagged plasmids Myc-mCLOCK/pSG5, Myc-mCLOCKΔ19/pSG5, Myc-mBMAL1/pCS2 and Myc-mCRY1 were previously described.[48] Myc-mCLOCKΔC/pSG5 and mCLOCKΔN/pSG5 were made by deletion of a DNA fragment from Myc-mCLOCK/pSG5, encoding the C-terminal part (571–855) and the N-terminal part (1–434) of mCLOCK, respectively. All the Myc-tagged proteins contain six copies of Myc epitope at the N and C termini. Plasmids expressing both β-galactosidase (pGL3-lacZ) for transfection control and luciferase (luc) for luminometry based expression (pGL3-mPer1-Luc promoter, pGL3-Ebox X3 luc) were described previously.[49]

Reagents and antibodies. LPS and mouse recombinant TNFα were purchased from Sigma-Aldrich. Antibodies against RelB for western blot and ChIP experiments were from Cell Signaling Technology and Santa Cruz Biotecnology, Inc., respectively. Antibodies against RelA, p50 and p100/p52 were from Cell Signaling Technology. Antibodies against CLOCK and rabbit IgG were from Santa Cruz Biotecnology; anti-BMAL1 was from Abcam; anti-Myc and anti-GAPDH were from Millipore, anti-flag and anti-α-tubulin from Sigma-Aldrich. Anti-Flag M2 affinity resin for immunoprecipitation experiments was from Sigma-Aldrich.

Cell culture. HEK-293 cells (ATCC) were mantained in DMEM (4.5 g/L glucose) supplemented with 10% newborn calf serum (NCS) and antibiotics and cultured at 37°C in 5% CO_2. MEFs from *c/c* mutants, *Cry1/2* -/- and *Bmal1* -/- were cultured in DMEM supplemented with 10% FBS and antibiotics. MEFs *c/c* + CLOCK were previously generated.[50] *RelB* +/+ and -/- were coltured with 10% BCS and antibiotics. Wild-type and

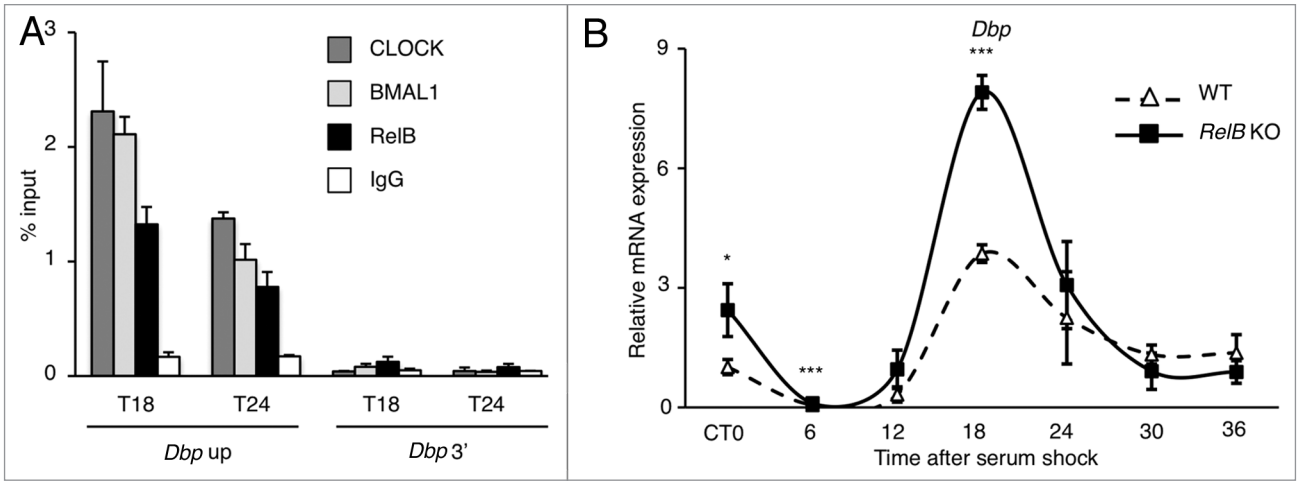

Figure 5. RelB negative regulation is required for proper circadian gene expression. (A) Cross-linked cell extracts were isolated at the indicated time points after serum shock from MEFs. The samples were subjected to ChIP assay with anti-CLOCK, anti-BMAL1, anti-RelB and anti-IgG and analyzed by quantitative PCR with primers for *Dbp* promoter (*Dbp* Up and Dbp 3'). Control IgG and *Dbp* 3'UTR were used as control for immunoprecipitation and PCR, respectively. All the values are the mean +/- SD (n = 3). (B) Circadian *Dbp* mRNA expression profile in wild-type (WT) and RelB KO MEFs, after serum-shock synchronization, analyzed by quantitative PCR. The values are relative to those of β-actin mRNA levels at each circadian time (CT). Time 0 (unsynchronized cells, CT0) in wt cells was set to 1. All the values are the mean +/- s.e.m. (n = 3), (*) $p < 0.05$, (***) $p < 0.001$.

RelB -/- MEFs were generous gift of Dr. Alexander Hoffmann. Wild type and *Cry* -/- MEFs were generous gift of Dr. Bert van der Horst.

Transient transfection and luciferase assay. Cells were transfected with BioT (Bioland Scientific LLC) according to the manufacturer's protocol. Cell extracts were subjected to a luminometry-based luciferase assay and luciferase activity was normalized by β-galactosidase activity.

RNA extraction and quantitative real-time RT-PCR. For circadian experiments, after 2 h of serum shock with media containing 50% horse serum, cells were incubated with serum-free medium for the indicated time. For gene expression analysis by real-time PCR, total RNA was extracted with TRIzol Reagent and processed according to the instructions of the manufacturer. Next, 2 μg of RNA from each sample was retrotranscribed (SuperScript II Reverse Transcriptase, Invitrogen) and 4-fold diluted cDNA was used for each real time reaction. For a 20 μl PCR, 50 ng of cDNA was mixed with primers to final concentration of 150 nM and 4 μl of RT[2] SYBR Green Fluor Fast master mix (QIAGEN). The reaction was first incubated at 95°C for 3 min, followed by 40 cycles at 95°C for 30 sec, and 60°C for 1 min. Each quantitative real-time PCR was performed using the Chromo4 real time detection system (BIO-RAD). The primers for ChIP and real-time qPCR for murine *Bmal1*, *Dbp*, *Cry1*, *Per1*, *Per2*, Per3, *Dbp* up promoter region, *Dbp* 3' UTR promoter region, *Per2* TSS promoter region, *Bmal TSS* were obtained from refs. 51.[51] The primers for *RelB*, *Il-6*, *Cxcl-1*, *Il-1*β, *Rev-Erb*α, β-*actin* and *Gapdh* are as follows: *RelB*: FW 5'-TGA TCC ACA TGG AAT CGA GA-3' RV CAG GAA GGG ATA TGG AAG CA; *Il-6*: FW 5'-TTC CAT CCA GTT GCC TTC TT-3' RV 5'-CAG AAT TGC CAT TGC ACA AC-3'; *Cxcl-1*: FW 5'-TGC ACC CAA ACC GAA GTC AT-3' RV 5'-TTG TCA GAA GCC AGC GTT CAC-3'; *Il-1β*: FW 5'-CTC TCC AGC CAA GCT TCC TTG TGC-3' RV 5'-GCT CTC ATC AGG ACA GCC CAG GT - 3' *Rev-erb*α: FW 5'-GGG CAC AAG CAA CAT TAC CA-3' RV 5'-CAC GTC CCC ACA CAC CTT AC-3'; β-*actin*: FW 5'-GGC TGT ATT CCC CTC CAT CG-3' RV 5'-CCA GTT GGT AAC AAT GCC ATG T-3'; *Gapdh*: FW 5'-TGT AGA CCA TGT AGT TGA GGT CA-3' RV 5'-AGG TCG GTG TGA ACG GAT TTG-3'.

Chromatin immunoprecipitation (ChIP) assay. Dual cross-linking ChIP assay[52] was used. Briefly, after 2 h of serum shock with media containing 50% horse serum, cells were incubated with serum-free medium for the indicated time. Then, cells were washed three times with room temperature PBS and PBS with 1 mM $MgCl_2$ was added. Disuccinimidyl Glutarate (DSG, Pierce) was added to a final concentration of 2 mM for crosslinking and incubated 45 min at room temperature, formaldehyde was added to a final concentration of 1% (v/v) and cells incubated for 15 min for dual crosslinking, and glycine was added to a final concentration of 0.1 M and incubated for 10 min to quench formaldehyde cross-linking. After harvesting, cells were lysed in 500 μL ice-cold cell lysis buffer (50 mM Tris/HCl pH 8.0, 85 mM KCl, 0.5% NP40, 1 mM PMSF, 1x protease inhibitor cocktail (Roche) for 10 min on ice. Nuclei were precipitated by centrifugation (3,000 g for 5 min) resuspended in 600 μL ice-cold RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycolate, 1 mM PMSF, 1x protease inhibitor cocktail) and incubated on ice for 30 min. Sonication was performed to obtain DNA fragments 100–600 bp in length.

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Supplemental Materials

Supplemental material may be downloaded here:
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