The EMBO Journal (2010) 29, 1389–1400 | © 2010 European Molecular Biology Organization | All Rights Reserved 0261-4189/10
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THE EMBO JOURNAL

Interaction of MAGED1 with nuclear receptors affects circadian clock function

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The circadian clock has a central role in physiological adaption and anticipation of day/night changes. In a genetic screen for novel regulators of circadian rhythms, we found that mice lacking MAGED1 (Melanoma Antigen Family D1) exhibit a shortened period and altered rest–activity bouts. These circadian phenotypes are proposed to be caused by a direct effect on the core molecular clock network that reduces the robustness of the circadian clock. We provide *in vitro* and *in vivo* evidence indicating that MAGED1 binds to RORα to bring about positive and negative effects on core clock genes of *Bmal1*, *Rev-erbα* and *E4bp4* expression through the Rev-Erbα/ROR responsive elements (RORE). *Maged1* is a non-rhythmic gene that, by binding RORα in non-circadian way, enhances rhythmic input and buffers the circadian system from irrelevant, perturbing stimuli or noise. We have thus identified and defined a novel circadian regulator, *Maged1*, which is indispensable for the robustness of the circadian clock to better serve the organism.

The EMBO Journal (2010) **29,** 1389–1400. doi:10.1038/emboj.2010.34; Published online 18 March 2010
Subject Categories: signal transduction; neuroscience
Keywords: anticipation; circadian rhythms; locomotor activity; MAGED1; RORα

Introduction

Circadian clocks are endogenous oscillations of biochemical, physiological and behavioural phenomena with period length of around 24 h. The circadian clocks help a variety of systems anticipate or adapt to daily fluctuations (Albrecht and Eichele, 2003; Hastings, 2003). The current clock model comprises the basic helix-loop-helix transcription factors CLOCK and BMAL1 that activates the expression of the negative components *Per* and *Cry*. After several hours, PER and CRY proteins downregulate their own transcription by inhibiting BMAL1/CLOCK-mediated activation (Allada *et al*, 2001; Young and Kay, 2001; Reppert and Weaver, 2002; Lowrey and Takahashi, 2004; Schibler, 2005). The molecular mechanism for the robustness of the circadian clock is the core negative feedback loop with transcriptional and post-translational regulation, but there are also several accessory loops. The best characterized of these is mediated by the orphan nuclear receptors *Rorα* and *Rev-erbα*. Transcription of *Rorα* and *Rev-erbα* is activated during the circadian day by *Clock–Bmal1* complexes, after that they exert positive and negative transcriptional effects on Rev-erbα/RORα responsive elements (RORE) in the *Bmal1* gene (Preitner *et al*, 2002; Sato *et al*, 2004). The accessory loops thereby provide contrasting enhancements within the cycle, along with additional avenues for the transcriptional regulation of output genes, for example those with RORE sequences (Ueda *et al*, 2005).

A key feature of the circadian clock may be a robust ability to limit internal noise and external perturbation to keep clock stable (Liu *et al*, 2008). *Rorα*-deficient *staggerer* mice show dampened circadian rhythms of *Bmal1* transcription and aberrant locomotor activity with unstable rhythmicity (Akashi and Takumi, 2005). *Clock* mutant mice show a drastic increase in the phase-resetting effects of light, so this mutation has been implicated in the impairment of circadian amplitude (Vitaterna *et al*, 2006). Several factors have also been identified, which directly target the clock. The metabolic adaptor *Pgc-1α* and the haeme sensor *Rev-erbα* represent the molecular links between metabolism and the clock (Liu *et al*, 2007; Yin *et al*, 2007), and *Hsf-1* and *Sirt1* may integrate the internal redox status to the clock (Asher *et al*, 2008; Belden and Dunlap, 2008; Reinke *et al*, 2008). All these processes share a common character that they convey the daily fluctuated internal environment cues to the circadian oscillator, making it coupling between periodic external signals and innate oscillators. Whether these identified genes alone are sufficient for comprehensive clock coordination of physiological processes remains unknown (Nakahata *et al*, 2009). Nonetheless, the findings that a substantial proportion of sleep disturbances, metabolic disorders, tumours and bone growth abnormalities arise from dysfunction in the circadian system imply that the endogenous clock's role in homeostasis is more widely influential than previously thought (Toh *et al*, 2001; Fu *et al*, 2002; Preitner *et al*, 2002; Bunger *et al*, 2005; Tamanini *et al*, 2005; Turek *et al*, 2005; Xu *et al*, 2005, 2007; McDearmon *et al*, 2006). Thus, identification of the key components of the circadian clock remains an important goal.

To identify potential links from input signalling, through the core feedback loops, to the output system, we conducted a locomotor activity screen of the knockout mouse bank of the National Resource Centre for Mutant Mice in China, in an effort to find factors that coordinate control of the circadian clock and other pathways (described in Materials and methods). We identified a novel circadian regulating gene, *Maged1* (also known as *NRAGE* or *Dlxin-1*), that had been shown earlier to mediate multiple signalling pathways, including

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Received: 12 October 2009; accepted: 22 February 2010; published online: 18 March 2010

p75NTR-dependent apoptosis in sympathetic neurons, developmental apoptosis of motor neurons (Salehi *et al*, 2000, 2002; Kendall *et al*, 2005; Di Certo *et al*, 2007; Bertrand *et al*, 2008) and regulation of the transcriptional activities of several homeodomain-containing proteins (Masuda *et al*, 2001; Matsuda *et al*, 2003). *Maged1* knockout mice (*Maged1* KO) assayed for wheel running, and with the Comprehensive Lab Animal Monitoring System (CLAMS), showed that *Maged1* has an essential role in maintaining circadian periodicity and the rest–activity bouts. These phenotypes are proposed to be the result of effects on components of the core molecular clock network, including *Bmal1*, *Rev-erbα* and *E4bp4*.

MAGED1 binds to RORα and regulates the expression of *Bmal1*, *Rev-erbα* and *E4bp4*, whose promoters harbour the RORE elements. In contrast to the monotonic stimulation role of RORα on these E box, RORE and D box transcriptional factors, the presence of MAGED1 brings about positive and negative effects, respectively, indicating an enhanced potential for fine tuned circadian phase and robustness control. Furthermore, *Maged1* exhibits non-rhythmic expression; binds to RORα in non-circadian way and is not regulated by core clock genes. This critical feature of constancy allows *Maged1* to enhance rhythmic input and stabilize the circadian feedback loop.

Results

Maged1 KO exhibit distinct locomotor activity

We identified a short-period mutant mouse line by targeted disruption of the *Maged1* gene. The knockout strategy is illustrated in Supplementary Figure S1. Absence of MAGED1 was confirmed by RT–PCR and western blot analysis (Supplementary Figure S1). Two satellite markers on X chromosome (Dxmit213 and Dxmit186) were used to identify genetic changes in *Maged1* KO to assure their C57BL/6J background (Estill and Garcia, 2000) and results showed that both markers had been replaced by C57BL/6J (Supplementary Figure S1). For the first pilot study, *Maged1* KO were sixth generation, and expanded to more than 10 generations in the following experiments. Behavioural analysis showed high consistency across different generations.

Locomotor activities exhibit a significantly shortened period of 23.19 ± 0.21 h, (mean ± standard deviation (s.d.), $n = 42$) for *Maged1* KO males, in contrast with 23.72 ± 0.17 h, ($n = 40$) for wild-type (WT) male littermates ($P < 0.001$) (Figure 1A and B). Heterozygous male mice of this strain do not exist because the *Maged1* gene is on the X chromosome. Female mice lacking *Maged1* also exhibit a shortened circadian period in a *Maged1* copy number-dependent manner, although the differences were less dramatic than those of male mice (Supplementary Figure S2). (Mating strategies were shown in Supplementary Table S1.) The impairment in the free-running period provided the first indication that *Maged1* may be involved in regulation of the circadian clock.

As alteration of the circadian clock is most obvious in the rest–activity cycle, we analysed rest and activity bouts in detail using the CLAMS (Columbus Instruments, Columbus, OH) (Pack *et al*, 2007). Total rest bout numbers were similar in both genotypes (259 ± 10 for WT mice versus 285 ± 35 for *Maged1* KO mice, $P = 0.22$) and increased slightly in *Maged1* KO mice during light phase (152.3 ± 6.5 for WT mice versus

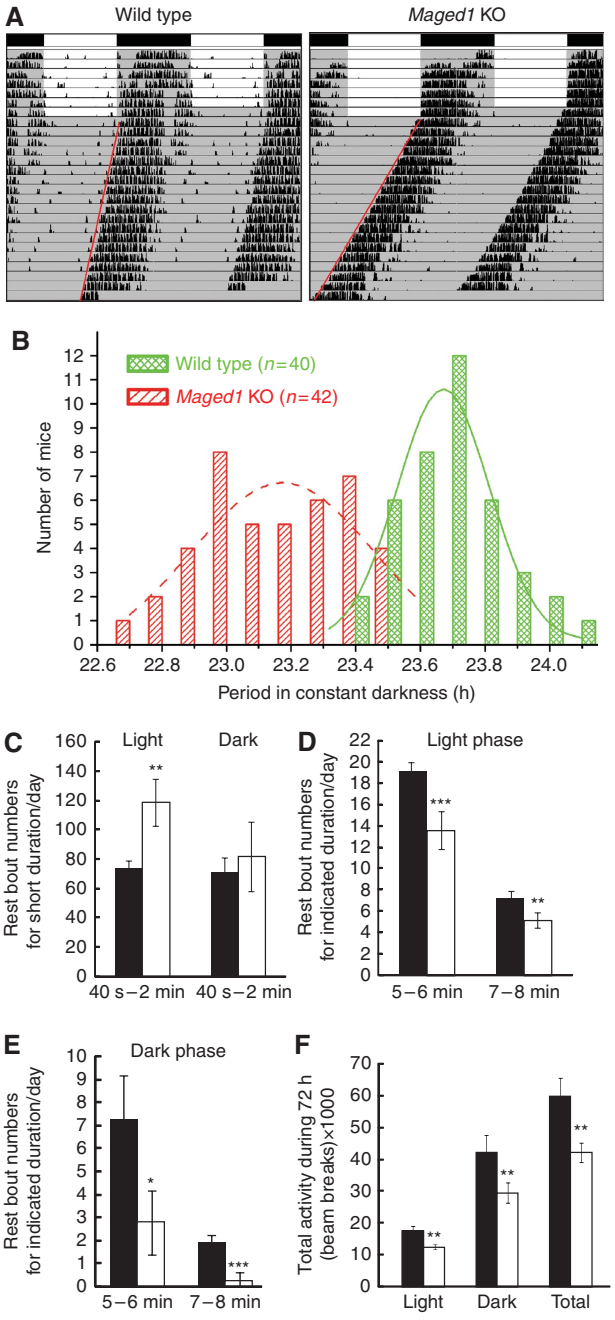

Figure 1 Locomotor activity of wild type and *Maged1* KO mice. (**A**) Voluntary locomotor activity was recorded as wheel-running activity from *Maged1* KO mice (23.19 ± 0.21 h, $n = 42$, male) and wild-type siblings (23.72 ± 0.17 h, $n = 40$, male). (Mean period ± s.d.) ($P < 0.001$, unpaired two-tailed Student's *t*-test). (**B**) Histograms for distributions of period length from the wheel-running recordings. (**C**–**F**) CLAMS was used to monitor rest–activity behaviour in wild type (black bars) and *Maged1* KO (white bars) mice. See 'Materials and methods' for detailed description. (**C**) Rest bouts of 40 s to 2 min per LD cycle. *Maged1* KO mice showed significantly increased percentages of short-duration rest episodes (40 s–2 min) compared with their wild-type littermates in the light phase. (**D**, **E**) Rest bouts of long duration in the light phase (**D**) and dark phase (**E**) per LD cycle. *Maged1* KO mice showed significant decreases in long-lasting rest episodes (5–6 and 7–8 min). (**F**) Total activity was reduced in *Maged1* KO mice compared to their wild-type siblings. ($n = 4$, mean period ± s.d. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, unpaired two-tailed Student's *t*-test). Three independent experiments were carried out.

185.2 ± 14 for *Maged1* KO mice, $P<0.05$) (Supplementary Figure S3). However, average rest bout duration was reduced in *Maged1* KO mice compared with their WT littermates (day time: 131.0 ± 12.4 s versus 168.0 ± 5.0 s, $P<0.01$; night time: 89.7 ± 16.5 s versus 124.3 ± 14.6 s, $P<0.05$; total: 116.6 ± 14.4 s versus 150.1 ± 8.8 s, $P<0.05$) (Supplementary Figure S3), implying possibly impaired rest maintenance. The rest bout numbers of 40 s–2 min duration were significantly greater in *Maged1* KO mice than in WT mice in light phase (Figure 1C), indicating an increase in the number of brief rest (118.7 ± 16.2 bouts versus 74.1 ± 5.3 bouts, $P<0.01$). Consistent with this observation, *Maged1* KO mice had a pronounced decrease in rest bout numbers for 5–6 and 7–8 min duration relative to their WT littermates between both day and night phase (Figure 1D and E; Supplementary Figure S3). This trend in *Maged1* KO mice caused a shift to a dispersed, fragmented rest pattern responsible for the effects of *Maged1* KO deficiency on rest quality. *Maged1* KO mice exhibited reduced total activity (Figure 1F), which suggested that the reduction in rest duration was not because of hyperactivity.

Maged1 has a global impact on circadian regulation

A biological clock is useful only if it can be set appropriately to local time. The primary synchronizing agent for circadian system is the environmental light–dark (LD) cycle. To define the role of *Maged1* more fully within the mammalian circadian system, we subjected *Maged1* KO mice and WT littermates to a simulated jet-lag environment, using a light regime-rescheduling experiment. During the initial LD cycle, WT and *Maged1* KO mice showed normally entrained, daily patterns of activity, and began their nightly bouts of activity at the beginning of the dark phase. However, in response to a 4 h advance shift of the LD cycle, *Maged1* KO mice immediately showed phase advance and were re-entrained within 3–4 days, whereas it took 6–7 days for WT littermates to reach complete re-entrainment (Figure 2A and B; Supplementary Table S2). No significant difference was observed in response to a phase-delay regime, or to short light pulses (data not shown). Thus, the faster phase advance in the *Maged1*KO could be caused by the short-period phenotype of *Maged1* KO mice. These phenotypes in *Maged1* KO mice may reflect the fact that downregulation of *Maged1* expression results in defects of circadian regulation.

Whether *Maged1* acts outside the nervous system was unknown. We therefore monitored PER2::LUC oscillations in lung tissues, testis and adrenal gland, by crossing *Maged1*$^{+/-}$ female mice to the homozygous *mPer2*^{Luc} knock-in reporter male mice (Yoo *et al*, 2004). Luminescence was continuously measured in real time with PMT detectors. The significant differences were observed in lung slices (KO: 23.7 ± 0.25 h, $n=9$ versus WT: 24.2 ± 0.1 h, $n=8$), adrenal gland (KO: 22.2 ± 0.1 h, $n=3$ versus WT: 22.7 ± 0.2, $n=3$) and testis (KO: 21.2 ± 0.08, $n=3$ versus WT: 21.9 ± 0.1 h, $n=3$) (Figure 2C; $P<0.01$ by two-tailed Student's *t*-test). It is also evident that there are advanced phase in *Maged1* KO lung slices, adrenal glands and testis compared with their WT controls (Figure 2C). To distinguish whether the phase difference is dependent of MAGED1, we monitored the acute effects of 50% horse serum shock on WT and *Maged1* KO embryonic fibroblasts (MEF). The first cycle of PER2::LUC in *Maged1*KO/*mPer2*^{Luc} MEF cells was significantly advanced by 2 h compared with WT MEF cells by peak alignment

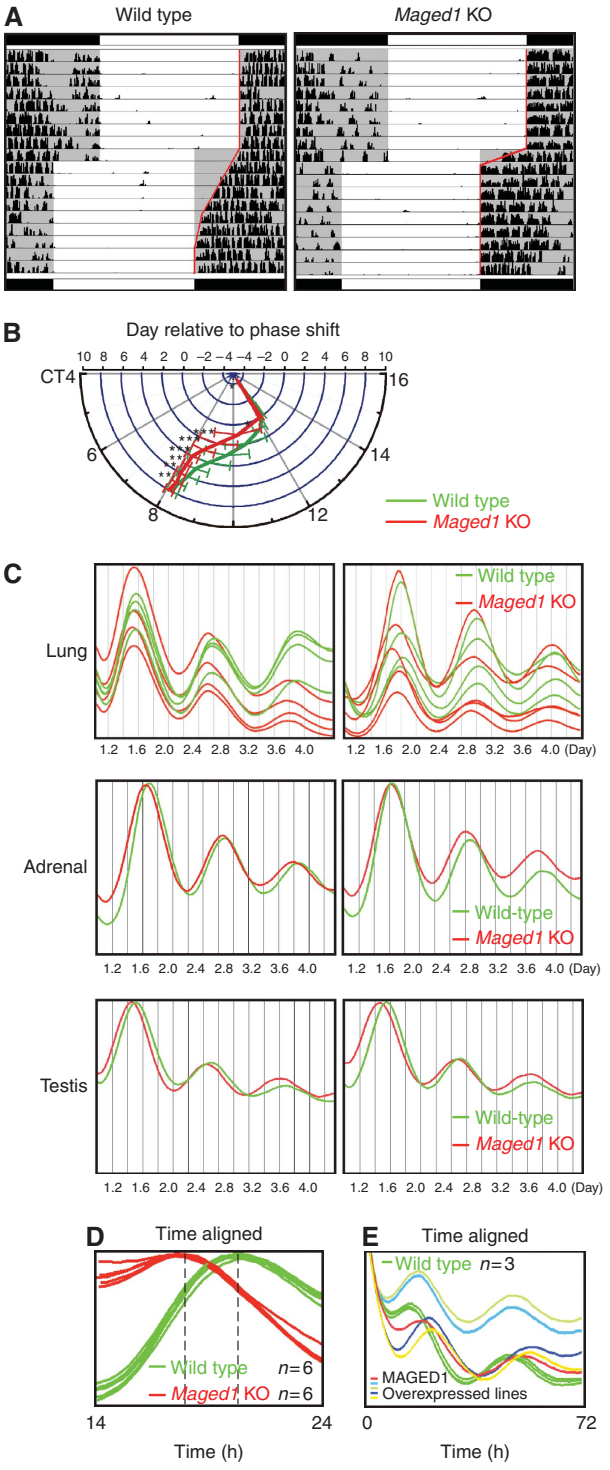

Figure 2 *Maged1* has a global impact on circadian stability. (**A**) Actograms from wild-type and *Maged1* KO mice that were subjected first to LD cycle, followed by a 4 h light phase advance. Red line indicates the onset of activity. (**B**) Re-entrainment traces from an average of wild type (green, $n=19$) and *Maged1* KO (red, $n=17$) mice. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, unpaired two-tailed Student's *t*-test (see Supplementary Table S2 for each recovery data). (**C**) Representative bioluminescence waveforms emitted by lung (upper panels), adrenal glands (middle panels) and testis (bottom panels) from wild-type/*mPer2*^{Luc} mice (green) and *Maged1*KO/*mPer2*^{Luc} mice (red). (**D**, **E**) Waveform alignments at the first peak after 50% serum shock in wild-type/*mPer2*^{Luc} MEFs (green) and *Maged1*KO/*mPer2*^{Luc} MEFs (red) (**D**), and overexpression MAGED1 rat *Per1-Luc* fibroblasts (**E**) (different colours indicate independent expressing lines) and control cells (green).

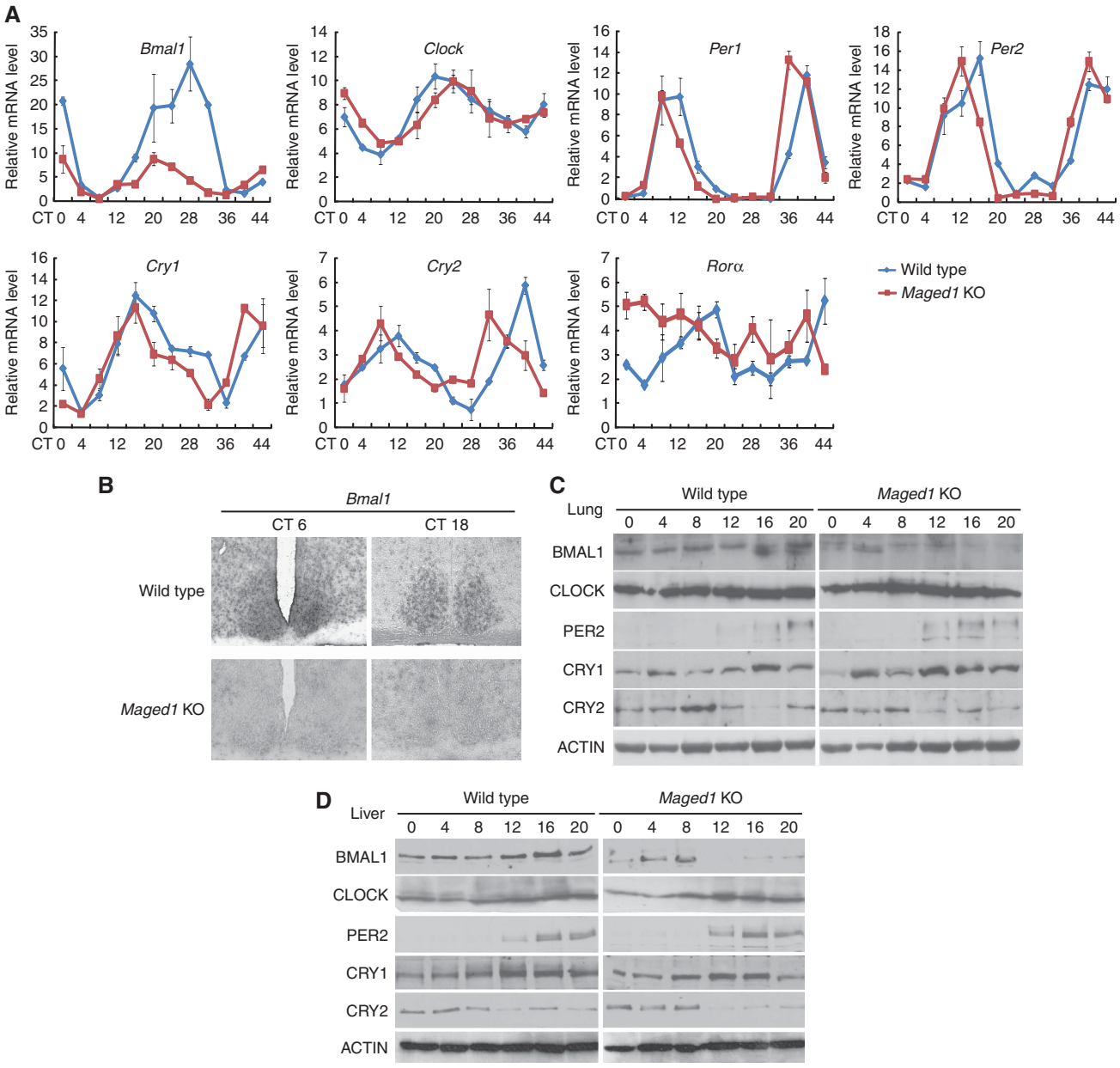

Figure 3 Expression levels of clock genes in *Maged1* KO mice. (**A**) Q–PCR analysis of expression of clock genes in liver tissues. All tissues were collected at 4 h intervals over the first day in DD for total 44 h. The relative levels of RNA were estimated by Q RT–PCR and normalized by *Gapdh*. Data represent mean ± s.d. ($n = 3$). (**B**) *In situ* hybridization showing *Bmal1* expression in the SCN of *Maged1* KO and wild-type mice. The expression level of *Bmal1* was severely reduced at both CT 6 and CT 18 in the SCN of *Maged1* KO mice. Two independent experiments were performed. (**C**, **D**) Representative protein oscillation profiles of clock genes from nuclear extracts at the indicated CTs over the first day in DD in wild type and *Maged1* KO lung (**C**) and liver (**D**) tissues. Three independent experiments, each with a time point from at least three mice, gave similar results for both mRNA and protein.

(Figure 2D). Then, we observed the effects of MAGED1 overexpression in rat *Per1-luciferase* (*Per1-Luc*) fibroblasts. A converse gap was detected between the empty vector and different MAGED1-overexpressing fibroblast lines (Figure 2E), indicating that *Maged1* may be responsible for phase difference in MAGED1-dependent manner.

Bmal1 expression is reduced in *Maged1* KO mice

To determine how MAGED1 affects circadian rhythm, we examined transcriptional level of the components of the circadian feedback loop. The mRNA oscillation patterns of the clock genes in WT mice were consistent with previous reports (Preitner *et al*, 2002; Albrecht and Eichele, 2003), showing detectable delay in *Clock*, and advance in *Per2* and *Cry2* of *Maged1* KO mice (Figure 3A). The most dramatic change was in *Bmal1* expression. In the absence of *Maged1*, the peak of *Bmal1* was reduced to <50% of the WT level in liver tissues ($P < 0.001$ by *t*-test). Moreover, *Bmal1* expression experienced its lowest value between circadian time CT 8 and CT 12 in WT mice, as described earlier (Preitner *et al*, 2002), whereas this trough was extended from CT 8 to CT 20 in *Maged1* KO mice (Figure 3A). As expected from the reduced expression of *Bmal1* in liver tissues of *Maged1* KO mice, *Bmal1* transcript levels in the SCN of *Maged1* KO mice

showed significant decrease at CT 6 and CT 18 on the first day of constant darkness compared with WT littermates (Figure 3B).

To obtain a clearer picture of the changes in clock components, we assayed the rhythmic translocation of clock proteins into the nucleus of both WT and *Maged1* KO lung and liver tissues. The mRNA changes were echoed in the cycles of protein expression, and CLOCK, PER2, CRY1 and CRY2 protein accumulation were very similar in *Maged1* KO and WT mice (Figure 3C). Although phosphorylated forms of BMAL1 were still detectable, BMAL1 protein levels were significantly downregulated in the *Maged1* KO lung tissues (Figure 3C) as well as liver tissues (Figure 3D). Considering *Bmal1* is a major transcription factor in the feedback loop, we propose that loss of *Maged1* may directly influence the transcription of *Bmal1*.

The ROR/REV/*Bmal1* loop clearly regulates the rhythm and amplitude of expression for many output genes. The magnitude of circadian phase shifting can be affected by the amplitude of the circadian oscillator (Vitaterna *et al*, 2006), and reduction of the general amplitude of the oscillator makes the clock more sensitive to phase-shifting stimuli (Brown *et al*, 2008) and circadian period defects (Preitner *et al*, 2002). Thus, it is reasonable to presume that alteration in *Bmal1* expression results in a short period in *Maged1* KO mice, as well as phase difference in MEF cells and peripheral tissues of *Maged1* KO mice.

MAGED1 is a coactivator of ROR proteins

Although *Maged1* does not possess a DNA-binding domain, it has been shown to bind to, and modulate the transcriptional activity of the homeodomain-containing *Dlx/Msx* family of proteins (Masuda *et al*, 2001). Therefore, we examined the possibility that MAGED1 is a new adaptor of known circadian transcriptional factors. The rhythmic expression of *Bmal1* is thought to be the result of the opposing effects of *Ror* activation and *Rev-erb* repression. Thus, the reduction of *Bmal1* in *Maged1* KO mice could arise from decreasing *Ror* activity and/or increasing *Rev-erb* activity. To distinguish between these possibilities, transcriptional reporter assays were performed. MAGED1 dramatically augmented the transcriptional activity of RORα and RORγ in the dose-dependent induction of *Bmal1* luciferase activity in HEK 293T cells (Figure 4A; Supplementary Figure S4A–E). The synergistic effects of ROR and MAGED1 are abolished when the two ROR-binding sites (RORE) on the *Bmal1* promoter were mutated (Figure 4B). To test specificity, we examined whether MAGED1 and other orphan receptor REV-ERBα have synergistic effects in *Bmal1* promoter, and checked MAGED1 effects on *Per2* and *Rorα* promoter. We found no significant effects (data not shown). Furthermore, overexpression of *Rorα* and *Maged1* in NIH3T3 cells significantly increased endogenous *Bmal1* expression (Figure 4C). Together with the low *Bmal1* expression level in *Maged1* KO mice, these data indicated that *Maged1* may mediate *Ror* activation of *Bmal1* transcription.

MAGED1 has been shown to relocalize in response to NGFR or the RTK receptor ROR2 (Salehi *et al*, 2000; Kani *et al*, 2004; Sasaki *et al*, 2005). We thus reasoned that MAGED1 may serve as an adaptor that binds to RORα, conveying signals from already identified or unknown membrane receptors to clock oscillators. To test for direct

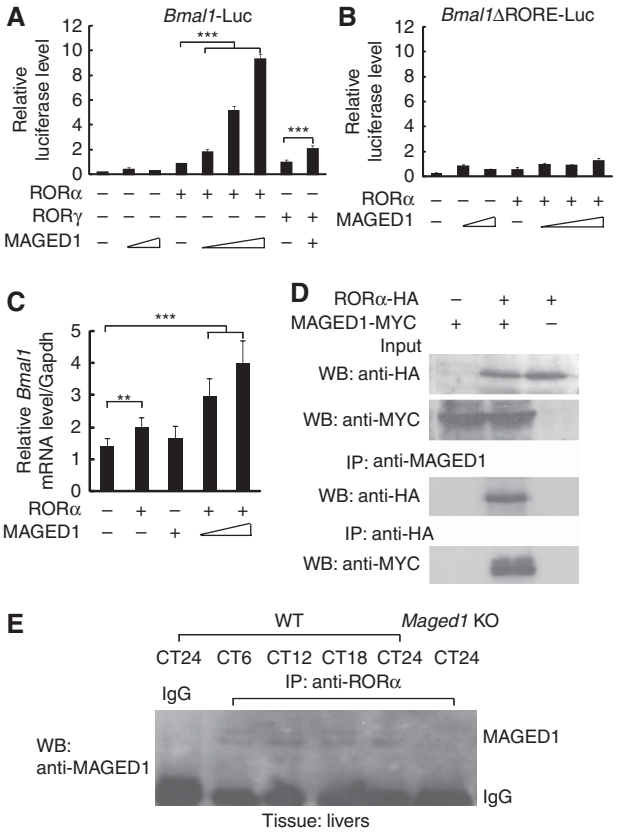

Figure 4 Activation of *Bmal1* transcription by *Ror*s and *Maged1*. Bar graphs depict relative luciferase activities mean ± s.d. of three replicates from a single assay. The results shown are representative of three independent experiments. (**A**) Effect of *Maged1* expression on the *Bmal1*-Luc promoter. (**B**) Effects of *Maged1* expression on the *Bmal1*-RORE mutant-Luc promoter. (**C**) Q–PCR analysis of endogenous *Bmal1* expression after overexpression of MAGED1 and/or RORα in NIH3T3 fibroblasts. (**D**) Co-immunoprecipitation assays of HEK 293T cells using epitope-tagged MAGED1 and RORα proteins as indicated. Each blot shows a representative example from three independent replicates. (**E**) Confirmation of interaction between RORα and MAGED1 in liver tissues. Liver tissues were collected at indicated times. IP was performed with anti-RORα. Immunoprecipitated proteins were further analysed by western blotting with anti-MAGED1 antibody. (**$P<0.01$, ***$P<0.001$, unpaired two-tailed Student's *t*-test).

interaction, we independently expressed MAGED1–MYC, RORα–HA or both in HEK 293T cells and found physical interaction between MAGED1 and RORα by immunoprecipitation (IP) (Figure 4D). To confirm that endogenous MAGED1 protein binds to RORα protein *in vivo*, we raised polyclonal antiserums against RORα and MAGED1. We pulled down endogenous RORα protein by anti-RORα at CT 6, 12, 18 and 24 using liver tissues, and immune complexes were then western blotted by anti-MAGED1 (Figure 4E). The endogenous MAGED1 binds to RORα in a time-independent manner, reinforcing the hypothesis that MAGED1 acts as a coactivator of RORs and suggesting that the interaction occurs in non-oscillation way.

As the primary structure of MAGED1 comprises a MAGE/necdin homology domain and a unique 25-hexapeptide repeat region, we then asked which domain was responsible for the activation of RORα and binding to RORα, and whether other members of the MAGE family also possessed the ability to activate *Bmal1*. Mutant proteins were constructed with

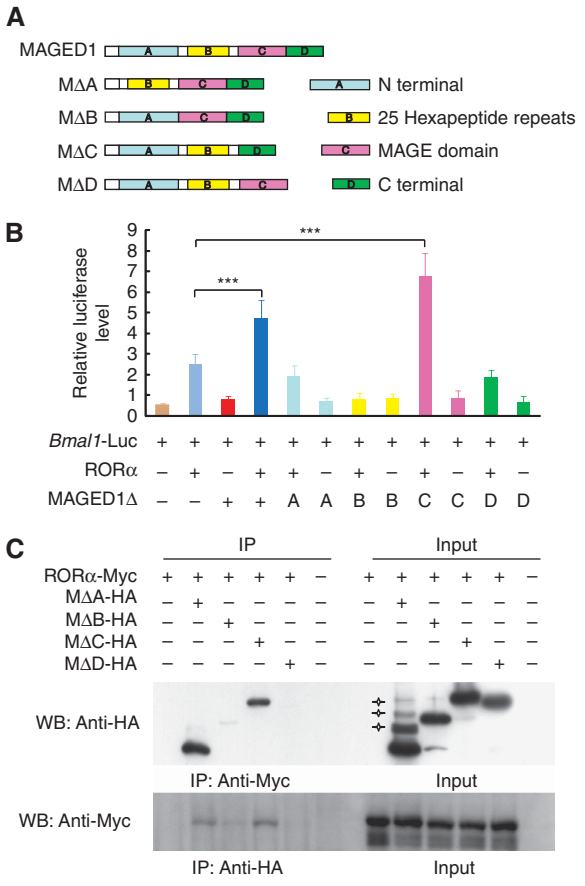

Figure 5 Characterizing MAGED1 functional domain. (**A**) Construct strategies for truncated HA-tagged MAGED1. (**B**) Effects of truncated MAGED1 on *Bmal1*-Luc activity in HEK 293T cells. MAGED1 lacking the MAGE/necdin domain (MAGED1ΔC) retains the ability to activate *Bmal1* promoter. (***$P<0.001$, unpaired two-tailed Student's *t*-test). (**C**) Co-immunoprecipitation assays of HEK 293T cells using Myc-tagged RORα and HA-tagged truncated MAGED1 as indicated. Deletion with the unique hexapeptide repeat domain or the C-terminal domain abolished the interaction between MAGED1 and RORα. Stars represent non-specific signals.

deletions in the characterized domains (Figure 5A). Deletion of the unique repeat region completely abolished the ability of MAGED1 to coactivate the *Bmal1* promoter, but constructs without the MAGE domain retained coactivation ability (Figure 5B). Truncated MAGED1 proteins were assayed by co-immunoprecipitation for interaction with RORα. Hexapeptide repeats and C-terminal were found to be responsible for the interaction with RORα (Figure 5C). These data further confirm the results of MAGED1 and RORα interaction and indicated that the activation of *Bmal1* observed in the promoter assays was dependent on their direct interaction.

Maged1 also functions in the other RORE elements

We hypothesized that *Maged1* may also function in the other circadian genes with Rev-Erb/ROR responsive elements (RORE). To test this hypothesis, the expression profiles of the circadian genes with RORE were examined in *Maged1* KO and WT liver tissues (Ueda *et al*, 2005). The accumulation of *Rev-erbα* mRNA rises significantly at CT 4, which is a peak during a 24 h period in *Maged1* KO liver tissues compared with WT littermates and trough value is not significantly altered (Figure 6A). The level of *E4bp4* is severely dampened in *Maged1* KO liver tissues compared with WT littermates (Figure 6A). However, *Clock*, *Dbp* and *Npas2* whose promoters all contain RORE sites showed no significantly alteration (Figures 3A and 6A). Then, to distinguish whether the alteration of *Rev-erbα* or *E4bp4* expression is a direct target by MAGED1 or secondary effect by transcriptional/translational feedback loop, transcriptional reporter assays were performed. In the *Rev-erba* promoter, two functional RORE sites have been well characterized in the proximity of the transcription initiation site (Adelmant *et al*, 1996; Delerive *et al*, 2002). Contrary to observations with the *Bmal1* promoter, transfection of increasing amounts of MAGED1 protein expression plasmid with RORα resulted in dose-dependent inhibition of *Rev-erbα* promoter activity (Figure 6B). Overexpression of MAGED1 in NIH3T3 cells suppressed endogenous *Rev-erbα* expression (Figure 6C). Thus, the endogenous *Bmal1* reduction in *Maged1* KO mice may be due not only to decreased activation of *Bmal1* expression by RORα and MAGED1, but also to increased *Rev-erbα* expression by RORα–MAGED1-mediated inhibition of *Rev-erbα* expression. Then, we constructed an *E4bp4*-promoter reporter for luciferase assays. Consistent with the above observations, co-transfection of *Maged1* and *Rorα* expression plasmids resulted in dose-dependent activation of an *E4bp4* reporter (Figure 6D). Overexpression of *Maged1* and *Rorα* in NIH3T3 cells increased endogenous *E4bp4* expression (Figure 6E). Finally, we assayed WT and *Maged1* KO hepatocytes by using *in vivo* dual cross-linking chromatin immunoprecipitation (ChIP), to detect chromatin proteins not directly bound to DNA (Nowak *et al*, 2005; Zeng *et al*, 2006). The results further support our conclusion that MAGED1 and RORα coexist in the liver at the *Bmal1*, *Rev-erbα* and *E4bp4* promoters, but without detectable signals on *Clock*, *Npas2* and *Dbp* promoters in the context of native chromatin (Figure 6F). These *in vitro* tests, combined with *in vivo* data, indicated MAGED1 may affect directly the expression of *Rev-erbα* and *E4bp4* gene through binding of RORs (see Discussion).

MAGED1 undergoes non-rhythmic expression

The significant effects of *Maged1* on circadian phenotypes prompted us to assay its spatial and temporal expression pattern. Real-time PCR of mRNA from various tissues of adult mice showed ubiquitous expression of *Maged1* (Supplementary Figure S5A). The relatively high expression level of *Maged1* in SCN seemed to be compatible with its role in the regulation of the circadian oscillator (Figure 7A; Supplementary Figure S5A). The temporal expression pattern of *Maged1* showed no robust oscillation across the circadian cycle in the liver or in SCN by northern blot and *in situ* hybridization (Figure 7A). Similar to results with *Maged1* mRNA, MAGED1 protein showed no obvious rhythmic expression in the liver or in the SCN (Figure 7A) or dynamic changes in nuclei over the course of the circadian cycle (Figure 7B).

Many circadian genes are themselves direct targets of transcriptional regulation by oscillator components, reflecting the general use of transcriptional/translation feedback loops. Therefore, we constructed and assayed a *Maged1*-LUC promoter to test whether known circadian proteins directly affected *Maged1* expression. We found no obvious effects on the *Maged1* promoter from overexpressing known clock genes (Supplementary Figure S5B). To further investigate

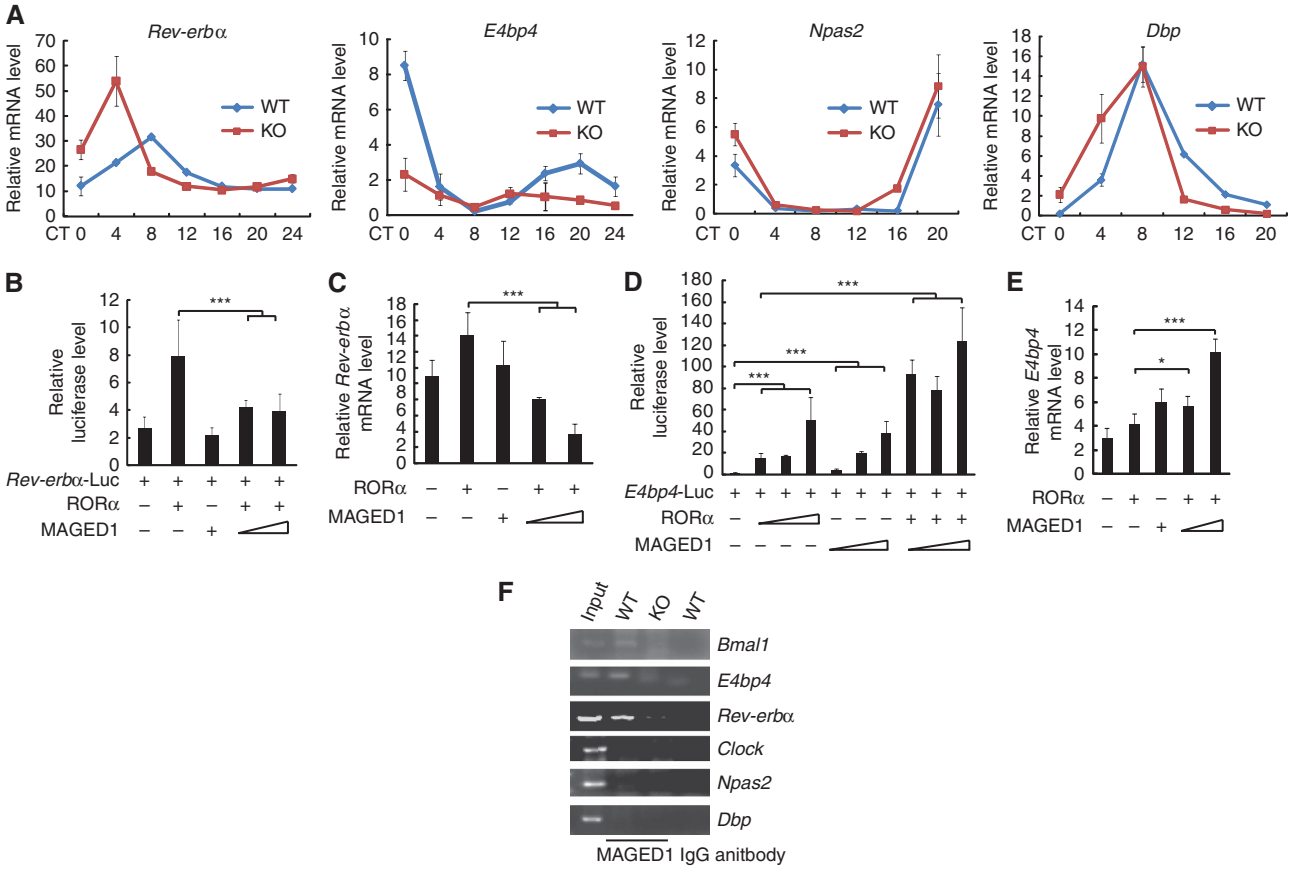

Figure 6 Identification of other circadian genes targeted by MAGED1. (**A**) Q–PCR analysis of endogenous *Rev-erbα* and *E4bp4* expression in WT and *Maged1* KO liver tissues. The relative levels of RNA were estimated by Q–PCR and normalized to *Gapdh*. Data represent mean ± s.d. ($n = 3$) and show a representative from three independent replicates. (**B**) Overexpression of MAGED1 and RORα inhibits the *Rev-erbα* promoter activity in HEK 293T cells. Bar graphs depict relative luciferase activities mean ± s.d. of three replicates from a single assay. The results shown are representative of three independent experiments. (**C**) Q–PCR analysis of endogenous *Rev-erbα* expression after overexpression of MAGED1 in NIH3T3 fibroblasts. (**D**) Effect of *Maged1* expression on the *E4bp4*-Luc promoter in HEK 293T cells. (**E**) Q–PCR analysis of endogenous *E4bp4* expression after overexpression of MAGED1 in NIH3T3 fibroblasts. (**F**) ChIP assay with MAGED1 antibody or control (IgG) in WT and *Maged1* KO hepatocytes. PCR was used to amplify a fragment flanking the proximal RORE on the indicated genes. (*$P<0.05$, ***$P<0.001$, unpaired two-tailed Student's *t*-test).

whether *Maged1* was regulated under the circadian feedback loop, we monitored *Maged1* mRNA levels at CT 6 and CT 18 in short-period *Cry1*$^{-/-}$, PER2S662G, *Rev-erbα*$^{-/-}$ and Part-time mice (Selby *et al*, 2000; Preitner *et al*, 2002; Xu *et al*, 2007; Siepka *et al*, 2007a) (Figure 7C); in long-period *Cry2*$^{-/-}$, *Clock*$^{\Delta 19}$, PER2S662D and Over-time mice (King *et al*, 1997; Selby *et al*, 2000; Xu *et al*, 2007; Siepka *et al*, 2007b) (Figure 7D); and in arrhythmic *Cry1*$^{-/-}$*Cry2*$^{-/-}$ double-knockout mice (Figure 7E). All liver *Maged1* mRNA levels were comparable, indicating that *Maged1* does not cycle, and its expression is unaffected by the clock. We also compared MAGED1 protein profiles in WT and *Clock*$^{\Delta 19}$ mice at different CT points in liver tissues. Consistent with the mRNA expression, the MAGED1 protein maintained a constant level and showed no change in *Clock* mutant mice compared with WT mice (Supplementary Figure S5C).

If the non-robust rhythmicity of MAGED1 externally influences the circadian loop by acting as a rheostat for transcriptional feedback loops, and stabilizes circadian rhythms, it should be able to receive incoming signals and alter their intensities. Many efforts have been made to identify signalling pathway that may mediate the regulation of *Maged1* expression. We found that *Maged1* showed an obvious reduction at 4 and 10 h after 2 h of 50% serum stimulation of cultured WT MEFs, but not by dexamethasone (Figure 7F). *Maged1* recovered its expression level at 22 h and did not induce oscillation over 52 h (Figure 7F).

Discussion

Synchronization or entrainment of biological clocks to environmental time is adaptive, and important for physiological homeostasis and proper species-specific behaviour (Wright *et al*, 2001), which is advantageous for survival in a competitive environment (Dodd *et al*, 2005; Wijnen and Young, 2006; Mackey and Golden, 2007). Our current findings on *Maged1* KO mice shed new light on the mechanism of the mammalian circadian regulation and enrich knowledge on the circadian framework. We have shown that *Maged1* KO mice impair circadian period that affects capacity to respond to environmental cues and also have abnormal rest–activity cycle that may affect sleep quality or be responsible for behaviour defects. We have provided *in vitro* and *in vivo* evidence that MAGED1 modulates the expression of *Bmal1*, *Rev-erbα* and *E4bp4* directly, by binding RORα to influence the robust capacity to respond to external cues.

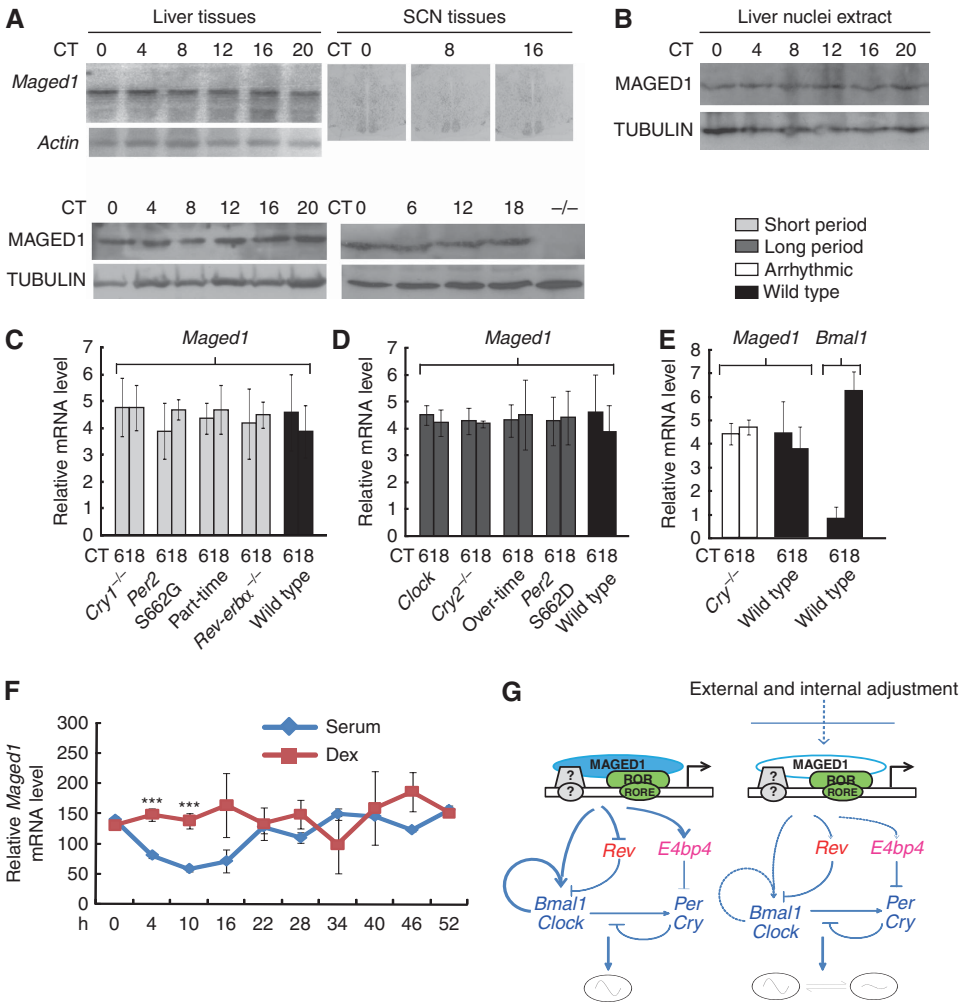

Figure 7 Expression of *Maged1* mRNA and protein. (**A**) Upper panel: temporal *Maged1* mRNA abundance in liver and SCN at indicated CTs. Bottom panel: protein expression profiles of MAGED1 in total liver and SCN area lysates at indicated CTs using MAGED1 antibody. (**B**) MAGED1 nuclear protein levels at the indicated CTs from liver tissues. (**C–E**) Q-PCR assays of *Maged1* mRNA expression at CT 6 and CT 18 in circadian mutant mice as indicated at the bottom. Right panel shows *Bmal1* expression in C57BL/6J mice as control ($n=3$ for each genotype). (**F**) Comparison of *Maged1* mRNA level from WT MEFs after serum shock or dexamethasone treatment at indicated time points, (***$P<0.001$, unpaired two-tailed Student's *t*-test). Three independent experiments were carried out. (**G**) A model for *Maged1* regulation in circadian rhythm. The complex of MAGED1 and ROR proteins regulates the amplitude of *Bmal1* by activating RORE in *Bmal1* promoter. MAGED1 may also participate in the inhibition of *Rev-erbα* and activation of *E4bp4* and thereby affect output pathway. The existence of other undefined transcriptional factors may contribute to the regulation preference and specificity of *Maged1*. The clock is thought to send an increasingly strong wake-promoting signal during the day, allowing wakefulness to be maintained. Similarly, during sleep, the clock may send a strong sleep-promoting signal, allowing sleep to be maintained. When the robustness of the circadian clock is impaired such as *Maged1* knockout or serum shock resulting in *Maged1* downregulation, the endogenous clock is entrained easily and increases sensitivity to respond to external cues.

Maged1 is an important circadian regulator

Although the altered period of *Maged1*-deficient mice is less dramatic like many clock genes (van der Horst *et al*, 1999; Zheng *et al*, 2001; Preitner *et al*, 2002; Debruyne *et al*, 2006; Liu *et al*, 2007), this does not undermine the significance of *Maged1* as a clock regulator. We characterized clock gene expression in *Maged1* KO mice and found that *Bmal1* levels were <50% of WT littermates in both central and peripheral tissues. Accordingly, the period of the whole animal activity and the bioluminescence of lung, adrenal and testis tissues were both shortened. *In vitro* and *in vivo*, co-immunoprecipitation assays demonstrated that MAGED1 interacts with RORα, and is capable of activating ROR-dependent *Bmal1* expression in transient transfection assays. Interestingly, the reduction of *Bmal1* mRNA level in the liver of *Maged1* KO mice is more dramatic than that of *staggerer* mice (*Rorα* mutant) or *Rorγ* KO mice. This discrepancy may reflect the redundant role of ROR proteins, whose adequate activations of *Bmal1* need MAGED1. An alternative explanation is that other regulation factors may reside in the *Bmal1* promoter to coordinate with ROR proteins by MAGED1. This is especially possible concerning the different responses of *Bmal1*, *Rev-erbα* and *E4bp4* promoters to loss of *Maged1*. Furthermore, although a substantial set of circadian gene promoters harbour the functional RORE sequence, only *Bmal1*, *Rev-erbα* and *E4bp4* show altered expression pattern in the liver of *Maged1* KO mice, whereas others including *Clock*, *Dbp* and *Npas2* are not affected. In parallel with this, only the promoter fragments of *Bmal1*, *Rev-erbα* and *E4bp4* were found in MAGED1 precipitates under our experimental conditions. Taken together, our data did show a preference of the RORE regulation by MAGED1. The underlying mechanism

suggests a model in which MAGED1 and unknown transcription factors binding on adjacent sites to the RORE led to increased/decreased function of MAGED1 and ROR on divergent RORE-containing promoters. With MAGED1 as an entry point, in contrast to the monotonic stimulation role of ROR proteins, the presence of MAGED1 brings about positive and negative effects, respectively; thus, it will be important in future work to focus attention on identifying MAGED1 regulatory module. *Bmal1*, *Rev-erbα* and *E4bp4* are *bona fide*, first-order clock genes; *Maged1* should efficiently induce clock-controlled genes and may contribute to the wide range of input and output pathways for appropriate anticipation. Future studies would be intriguing that show other MAGED1 co-regulation proteins. This may provide novel insights into the regulation preference, functional-specific and tissue-specific regulation of the circadian clock.

Comprehensive phenotyping shows impairment of the circadian capacity in Maged1 KO mice

Our results are compatible with a previous report that *Bmal1* mRNA expression is affected in *Rorα* mutant *staggerer* mice (Sato *et al*, 2004). The enhanced adaptability of *Maged1* KO mice to a 4 h phase advance is reminiscent of that observed in the *staggerer* mice (Akashi and Takumi, 2005), supporting our conclusion that MAGED1 is a coactivator for RORα, and may contribute to a shortened period in the same ways. Although the *Maged1* is a key regulator of *Bmal1*, *Maged1*KO did not recapitulate the phenotype of the *Bmal1*-deficient mice. The main reason for the difference is that *Bmal1* expression was downregulated but not abolished. Many data indicated that phenotypes of *Bmal1* KO mice were different from those of mice with *Bmal1* downregulation (Sato *et al*, 2004; Akashi and Takumi, 2005; Liu *et al*, 2007). Furthermore; we found that *Maged1* KO mice showed significantly reduced bout numbers for long-rest duration compared with WT mice in both light and dark phases. However, the bout numbers for short-rest duration were significantly higher for *Maged1* KO mice in the light phase, which is sleep time for nocturnal animal. The time of sleep and wake is a function of a homeostatic process that defines sleep need as being dependent on the previous amount of sleep and wake (process S), and on the circadian clock (process C) that modulates the timing and propensity of sleep (Borbely, 2001). Evidence has accumulated for a critical role for mammalian circadian clock genes in sleep–wake regulation. *Clock* mutant mice showed abnormal sleep (Naylor *et al*, 2000), and *Bmal1* knockout mice appeared an attenuated rhythms of sleep and wakefulness distribution across the 24 h period and increased in total sleep time (Laposky *et al*, 2005). Linking circadian genotypes with human sleep phenotypes are already being implicated in circadian sleep phase disorders (Toh *et al*, 2001; Xu *et al*, 2005, 2007). However, it is not known how the two processes actually contribute to the overall sleep need of the organism, or what role the circadian clock may have in other homeostatically regulated sleep–wake events with certainty. Determining whether the sleep-like behaviour in *Maged1*KO is linked to the sleep systems, or whether they extend to circadian feedback loop and non-circadian pathways, remains to be determined. Furthermore, it is also difficult to exclude the possibility that insufficient motor neuron apoptosis affect rest–activity cycle in *Maged1* KO mice at this stage (Bertrand *et al*, 2008). Especially, MAGED1 has been isolated as a novel *Dlx*/*Msx*-binding protein that binds not only to DLX5 but also to other Dlx/Msx family proteins, suggesting a common transcription regulator for DLX/Msx family protein (Sasaki *et al*, 2002). Interestingly, *Dlx* genes have shown to be linked with epilepsy and Rett syndrome (Cobos *et al*, 2005) (Horike *et al*, 2005). Thus, elucidation of the genetic cascades controlling *Dlx* gene expression through MAGED1 will enhance our knowledge of GABAergic interneuron development, as well as providing new insights into the understanding of important neurological disorders (Poitras *et al*, 2007). Measurements of time in REM/NREM sleep and rebound after sleep deprivation in *Maged1* KO mice should be the future direction to distinguish whether this abnormal behaviour is connected to sleep–wake cycle or motor neuron defects. We also noticed that the phase difference in peripheral tissues from *Maged1* KO is similar to that of fibroblast cells. The results reported here do not address the question whether MAGED1 are responsible for behaviour phase resetting defects or the phase advance in lung explants and MEF from *Maged1* KO mice is a circadian defect. However, given our additional experiment by overexpressing MAGED1 in *Per1-Luc* fibroblasts reverse the phase differences; we suggest that the phase differences in peripheral tissues or cells are dependent on MAGED1.

A model of MAGED1 function in the circadian clock of mammals

Two features are critical to the functions of a circadian clock. On the one hand, the clock ensures its circadian stability and robustness despite internal noise or external perturbations using mechanisms including positive feedback, intercellular coupling, gene redundancy and amplitude maintenance. On the other hand, the circadian clock adapts, using physiological processes that respond to changes in the external environment through photic or non-photic signal transduction pathways. This requires the clock to have a mechanism to reduce its stability and robustness. A previous study showed that *Clock* mutant mice exhibit increased response efficacy to resetting stimuli that reduce circadian amplitude (Vitaterna *et al*, 2006). Our phenotyping studies have implicated *Maged1* in maintaining period accuracy, and affecting the range to which the clock can entrain. Here, we propose that, as an ROR adaptor, *Maged1* mediates multiple regulations through coordinating with unknown transcript factors that activate *Bmal1* and *E4bp4* and suppress *Rev-erbα* genes simultaneously (Figure 7G). Such regulations by *Maged1* should efficiently induce genes controlled by E-boxes, D-boxes and RORE elements, enhancing robustness and limiting the capacity to respond to external cues. Our current evidence from mRNA and protein levels indicates that *Maged1* is not cyclical, and is not regulated by core clock genes. We acknowledge that *Maged1* may cycle in other ways, such as by modification. However, as yet, we have no evidence for this. Instead, our data indicated that *Maged1* buffers the circadian system from irrelevant perturbatory stimuli or noise.

Finally, the past decade has witnessed stunning advances in orphan receptor research, largely owing to the identification of dietary lipids and metabolites as the adaptors for a number of orphan receptors and establishing these adopted orphan receptors as lipid sensors that activate transcriptional

programmes for metabolic homeostasis (Chawla *et al*, 2001). MAGED1 identified here adds a new level of regulatory connection to the circadian clock and orphan receptors. A more profound understanding of the *Maged1* mechanism by which *Maged1* enhances transcriptional activity will definitely foster social applications of regulating circadian adaptable ability, such as controlling jet lag or providing therapeutic opportunities for clock-related pathologies.

Materials and methods

Animal care and behavioural analysis

Several international knockout programmes are underway that are aimed at developing a comprehensive spectrum of mouse models of human disease including the North American Conditional Mouse Mutagenesis Project (NorCOMM), the European Conditional Mouse Mutagenesis Program (EuCOMM), Knockout Mouse Project (KOMP), the Texas Institute for Genomic Medicine (TIGM) and a fifth initiative, the Chinese knockout consortium (ChCOMM). The ChCOMM aims to promote functional genomics and disease model studies, contribute to the standardization of mouse model quality, cryopreservation and phenotyping funding by Ministry of Science and Technology of China. The ChCOMM is generating conventional and conditional knockout mutations in selected genes of interest to the Chinese and international community, especially in metabolism, development, tumour, musculoskeletal and neurological-related diseases. The initial phenotype screens consist of an assessment of mouse wheel-running activity in a 12:12 hours LD cycle for 7 days followed by assessment in DD for up to 20 days launched in 2006. Animal studies were carried out in an Association for Assessment and Accreditation of Laboratory Animal Care International credited SPF animal facility and all animal protocols are approved by the Animal Care and Use Committee of the Model Animal Research Center, the host for the National Resource Center for Mutant Mice in China, Nanjing University.

Wheel-running activity assays were performed as described earlier using ClockLab (Actimetrics) software (Xu *et al*, 2005, 2007). Behavioural analysis for free activity and rest was performed using a CLAMS (Columbus Instruments, Columbus, OH) that consists of eight individual live-in cages for mice that allow automated, non-invasive data collection. In total, 16 infra-red beams intersect the *XY* plane providing 1/2″ beam spacing, and total activity was recorded as any movement producing a horizontal beam break. One consecutive 40 s with zero activity counts indicated rest status. A total of eight mice from 2–3-month-old WT and knockout male littermates were tested each time and have been backcrossed onto C57BL/6J more than 10 generations. Three independent experiments were carried out. All mice were maintained in a 12:12 LD cycle, and were recorded for at least 5 days. A time window of 72 h staring from light-on point on day 2 or day 3 was selected for analysing the rest and activity data.

Generation of Maged1 KO and backcross procedures

The targeting vector was constructed based on pNeotkloxp (kindly provided by Dr Philippe Soriano). The homologous arms (5′ arm: from CTGCTCACTCAGTCCTTTGCC to GGCTTGGAATGACACTAC TAAGGTC, 3′ arm: from ACCGAAGCTTGGCCTCCTCTTAG to AAAAAGCCCTTGGTCCTGTG) were amplified by PCR from 129S1 genomic DNA. About 2.4 kb of the *Maged1* genomic locus, from exons 3 to 8 was replaced by a pGK-Neo cassette in reverse orientation. The positive ES clone was selected by long-range PCR and injected into C57BL/6J blastocysts. Primers used for ES clone screen are listed in the Supplementary Table 2.

Chimaeric males were mated to C57BL/6J females. Then, heterozygous females were continuously backcrossed onto C57BL/6J males for at least six generations for behavioural analysis. Besides, Dxmit213 and Dxmit186 were used to check the exchange level in mouse chromosome X of *Maged1* KO mice as described earlier (Estill and Garcia, 2000).

Quantitative PCR and RT–PCR

All mice were individually housed in a 12:12 LD cycle for 7 days, and then released into constant darkness (DD). For tissue analysis, liver, brain and lung were collected at the selected CT points on the second day in DD. Total RNA was extracted using Trizol (Invitrogen) and random hexamers were used to prime reverse-transcription reactions with Superscript III (Invitrogen). Real-time quantitative PCR (Q–PCR) was performed using an ABI 7300 detection system (Applied Biosystems) with SYBR green I reagents (Takara). The real-time PCR primers were the same as reported earlier (Xu *et al*, 2007), and all other primers including primers for RT–PCR were listed in Supplementary data (Supplementary Table S4). Efficiency of amplification and detection by all primers was validated by determining the slope of CT versus dilution series. Transcript levels for each gene were normalized to *Gapdh* cDNA levels according to standard procedures.

In situ hybridization

Animals were killed by cervical dislocation at indicated time points. Coronal brain sections through the SCN were processed for *in situ* hybridization with a hamster *Bmal1* cRNA probe (from nucleotides 760–1470) as described (Shearman *et al*, 2000) and mouse *Maged1* cRNA probe (from nucleotides 70–888) (Bertrand *et al*, 2004). Hybridization steps were performed as described elsewhere (Lee *et al*, 2001).

Plasmid constructs

Coding regions of *Maged1* were amplified from C57BL/6J mouse cDNA by PCR using Platinum Taq polymerase and cloned into pCMV-Tag 2B (Stratagene) by *Eco*RI/*Xho*I, respectively. The vector was confirmed by sequencing. HA-tagged expression plasmids were constructed based on the pCGN vector. The pCGN vector was digested by *Xba*I and *Bam*HI, and the coding sequence of *Maged1* was then cloned in with two annealed DNA linkers: N-terminal linker: 5′-CTAGAAGGG-3′ and 5′-AATTCCCTT-3′; C-terminal linker: 5′-TCGAGGAGAG-3′ and 5′-GATCCTCTCC-3′; *Ror*α coding sequences were cloned into pCGN vector with the same C-terminal linker and a different N-terminal linker: 5′-CTAGAAGGA-3′ and 5′-AGCTTCCTT-3′. Construction of truncated *Maged1* used the same strategy as full length *Maged1* and *Nhe*I was used as the junction site for ligation. All primers were listed in Supplementary Table S3.

Cell culture, transfection and luciferase report assays

HEK 293T or NIH3T3 were cultured in DMEM containing 10% serum and penicillin–streptomycin in 96-wells, 35 mm or 10 cm dishes according to each experiment. Lipofectamine 2000 (Invitrogen) and Genescort (Wisegen) were used to transfect NIH3T3 and HEK 293T, respectively, according to the manufacturer's instructions. Reporter gene assays were performed with a Dual-Report assay system (Promega); 50 ng *Bmal1*-Luc, 20 ng RORα, 50 ng MAGED1, 1 ng *Renilla* pRL-TK *vector* were added for basic reaction and the pCMV-Tag2B was brought to the same amount.

Western blotting and IP

Tissue proteins were prepared as described previously using a nuclear extraction kit (Active Motif) (Xu *et al*, 2007). Rabbit antibodies against mouse BMAL1 (Abcam), CRY2 (Abcam), CLOCK (Abcam), CKIε (BD Biosciences), CRY1 (Acris Antibodies GmBH), MAGED1 (Oncogene) and mPER2 (a kind gift from the Fu and Ptáèek lab) were subjected to western blot according to the manufacturer's protocol. The rabbit anti-RORα and anti-MAGED1 antibodies were raised against the peptide CQEEIENYQNKQREV and CEAEARAEARNRMGIGDE. For IP, cells were lysed in RIPA buffer with protease inhibitor cocktail (Roche). According to a standard protocol, lysates were precleared with Protein A agarose beads (Amersham) and then IPed with rabbit polyclonal anti-HA antibodies (Santa Cruz Biotech) or anti-Myc antibodies (Sigma). After washing five times, the precipitates were resuspended in 2 × SDS–PAGE sample buffer, boiled for 3 min and run on an 8 or 10% SDS–PAGE gel followed by western blot analysis using mouse monoclonal anti-Myc antibody or anti-HA antibody (Sigma). Immunoreactive bands were detected by ECL (Amersham). These experiments were performed in triplicates.

Real-time bioluminescence recording

Homozygous PER2::LUC mice (Yoo *et al*, 2004) were mated with *Maged1*$^{+/-}$ mice. These mice were maintained in a 12:12 LD cycle. One hour before lights off, cultures of lung, testis and adrenal gland were prepared as described (Yamazaki and Takahashi, 2005). The MEFs were isolated from embryos at day E13.5 following standard procedures (Abbondanzo *et al*, 1993). Rat *Per1-Luc* fibroblasts in

35 mm culture dishes were transfected with 5 μg of linearized MAGED1 expression vector by Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection, G418 (400 μg/ml) was added to select transfected cells. After cells reached 100% confluency, the cells were treated with 50% horse serum (Invitrogen) for 2 h and the medium was replaced with assay medium (Izumo *et al*, 2003). Bioluminescence was recorded in real time with the LumiCycle (LumiCycle, Actimetrics) and photon counts were integrated over 10 min intervals (Yamazaki *et al*, 2000). Waveforms of rhythmic bioluminescence emission were analysed using the LumiCycle software package.

In vivo dual cross-linking ChIP

ChIP was performed as described (Nowak *et al*, 2005; Zeng *et al*, 2006). Isolation of mouse hepatocytes was performed according to the protocol (Guguen-Guillouzo *et al*, 1986). Cell yield and viability were determined by the trypan blue exclusion test; 1×10^7 cells were used for further ChIP assays. Anti-MAGED1 was used to identify genes regulated by MAGED1. Specific pairs of primers were listed in Supplementary Table S3.

Statistical analysis

Groups of data are presented as mean ± s.d. We performed statistical comparisons with the unpaired two-tailed Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Ueli Schibler for reading the paper and giving insightful comments and providing *Rev-erbα* mice. We appreciate JS Takahashi for *Clock*, *Part-time*, *Over-time*, *Per2* luciferase mice, J Hogenesch for his valuable discussions, and *Rorα* and *Rorγ* plasmids, S Reppert for the *Bmal1* and *Clock* expression plasmid, Fu & Ptáček labs for PER2 antibody and human *PER2* transgenic mice, Shi Yamazaki for rat *Per1-Luc* fibroblasts and technical support on LumiCycle, M Ikeda for *Bmal1* promoter, Laure Bernard and Vincent Laudet for *Rev-erbα* promoter. The Chinese knockout consortium (ChCOMM) was funded by Ministry of Science and Technology of China (2006BAI23B00 to YX and XG). This work was funded by the National Science Foundation of China, the Distinguished Young Scholar Foundation (30825024 to XG and 30725011 to YX); and Ministry of Science and Technology (2010CB945100 to YX).

Conflict of interest

The authors declare that they have no conflict of interest.

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