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SIRT1 Regulates Circadian Clock Gene Expression through PER2 Deacetylation

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SUMMARY

The mammalian circadian timing system is composed of a central pacemaker in the suprachiasmatic nucleus of the brain that synchronizes countless subsidiary oscillators in peripheral tissues. The rhythm-generating mechanism is thought to rely on a feedback loop involving positively and negatively acting transcription factors. BMAL1 and CLOCK activate the expression of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes, and once PER and CRY proteins accumulate to a critical level they form complexes with BMAL1-CLOCK heterodimers and thereby repress the transcription of their own genes. Here, we show that SIRT1, an NAD^+-dependent protein deacetylase, is required for high-magnitude circadian transcription of several core clock genes, including *Bmal1*, *Ror*γ, *Per2*, and *Cry1*. SIRT1 binds CLOCK-BMAL1 in a circadian manner and promotes the deacetylation and degradation of PER2. Given the NAD^+ dependence of SIRT1 deacetylase activity, it is likely that SIRT1 connects cellular metabolism to the circadian core clockwork circuitry.

INTRODUCTION

The physiology and behavior of mammals are subject to daily oscillations driven by an endogenous circadian clock (Albrecht and Eichele, 2003; Reppert and Weaver, 2002). In mammals, the circadian timing system is composed of a central pacemaker in the brain's suprachiasmatic nucleus (SCN) and subsidiary oscillators in most peripheral tissues. While light-dark cycles are the predominant *Zeitgebers* (timing cues) for the SCN pacemaker, cyclic feeding behavior is a strong *Zeitgeber* for clocks operating in many peripheral tissues (Albrecht and Eichele, 2003; Damiola et al., 2000; Reppert and Weaver, 2002; Stokkan et al., 2001). It is therefore likely that the SCN synchronizes peripheral oscillators by imposing rest-activity rhythms and thus feeding-fasting cycles.

The molecular oscillator in both master and subsidiary clocks (Balsalobre et al., 1998; Yagita et al., 2001) is thought to rely on a negative transcriptional feedback loop (Hardin et al., 1990; Lowrey and Takahashi, 2000; Reppert and Weaver, 2002). The PAS domain helix-loop-helix proteins BMAL1 and CLOCK (or its paralog NPAS2 [DeBruyne et al., 2007; Reick et al., 2001]) bind as heterodimers to regulatory elements of *Cry* and *Per* genes and stimulate the transcription of these genes. Once the repressor proteins CRY and PER have reached a critical concentration, they attenuate the activity of BMAL1-CLOCK heterodimers and thereby repress the transcription of their own genes. In addition, an interconnecting feedback loop involving orphan nuclear receptors of the REV-ERB and ROR families regulates the expression of *Bmal1* (Preitner et al., 2002; Sato et al., 2004).

Several lines of evidence suggest a strong interplay between metabolism and the circadian clock (Kaasik and Lee, 2004; Rutter et al., 2002; Tu and McKnight, 2006). The dominance of feeding cycles as a *Zeitgeber* for peripheral clocks implies that the circadian clock plays an important role in nutrient processing and energy homeostasis. Indeed, transcriptome profiling studies revealed that many genes involved in metabolism are rhythmically expressed (Akhtar et al., 2002; Duffield et al., 2002; Kornmann et al., 2007; Panda et al., 2002; Storch et al., 2002; Walker and Hogenesch, 2005). Furthermore, at least in vitro, the DNA-binding activity of BMAL1-CLOCK is strongly influenced by the ratio of reduced to oxidized NAD cofactors, which are often considered as a readout of the cellular metabolic state (Rutter et al., 2001).

SIRT1 is the mammalian homolog of yeast Sir2, an NAD^+-dependent deacetylase involved in transcriptional silencing, genome stability, and longevity (Blander and Guarente, 2004; Dali-Youcef et al., 2007). The SIRT1 catalytic reaction involves the breakdown of one NAD^+ molecule for each deacetylated acetyl lysine and the generation of nicotinamide and O-acetyl-ADP-ribose. SIRT1 was found to deacetylate not only histones but also several transcriptional regulatory proteins involved in the control of metabolism, including members of the FOXO protein family (Bordone et al., 2006; Brunet et al., 2004; Motta et al., 2004), peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC1α), and the nuclear receptor LXR (Li et al., 2007; Rodgers et al., 2005).

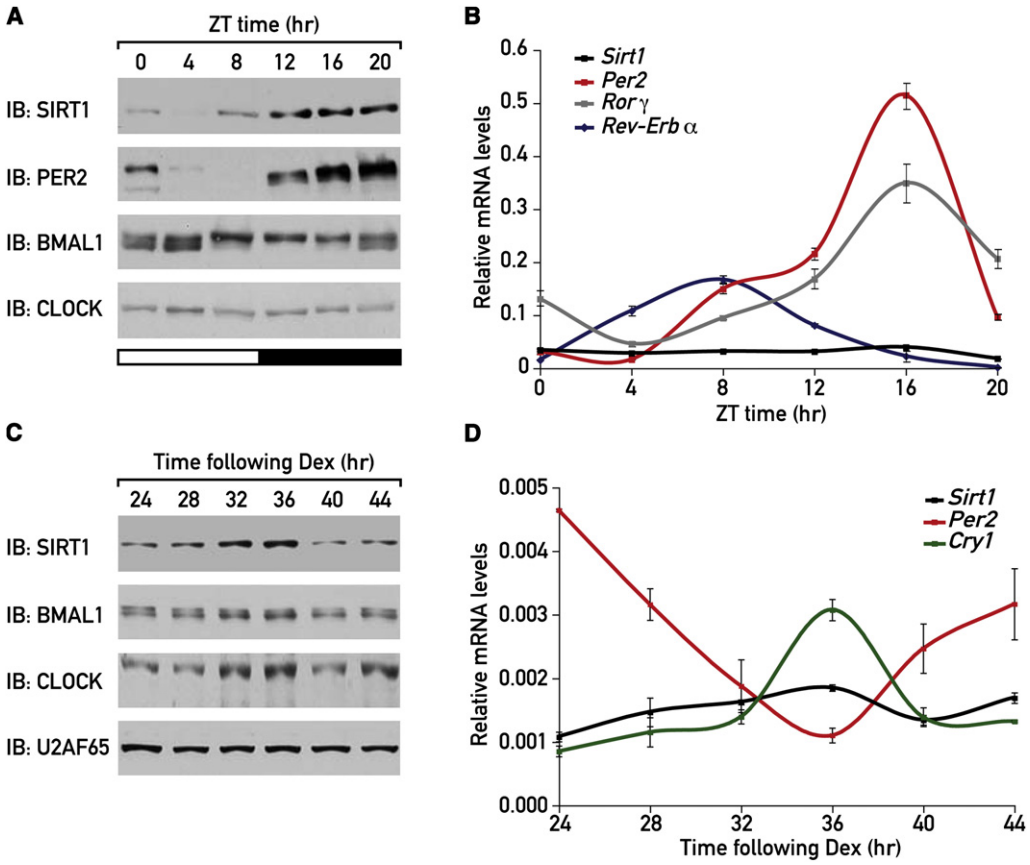

Figure 1. Circadian Expression of SIRT1 Protein

Mice were sacrificed at 4 hr intervals; liver nuclear protein extracts and total RNA were prepared.

(A) Protein extracts were analyzed by immunoblotting.

(B) RNA was analyzed by quantitative TaqMan real-time PCR using specific TaqMan probes.

MEFs were synchronized by a dexamethasone shock and samples were collected at 4 hr intervals, starting 24 hr after the shock.

(C) Protein extracts were analyzed by immunoblotting.

(D) RNA was analyzed by quantitative TaqMan real-time PCR using specific TaqMan probes.

Plotted values are the mean values ± standard deviation (SD) from three independent experiments.

Here we show that SIRT1 is expressed in a circadian manner and that it is required for high-magnitude circadian expression of several core clock genes. Moreover, we present evidence that SIRT1 binds to CLOCK-BMAL1 heterodimers and promotes the deacetylation and degradation of PER2.

RESULTS

Circadian Expression of SIRT1 Protein in Mouse Liver and in Cultured Fibroblasts

In order to investigate whether SIRT1 might be involved in circadian rhythm, we first examined the temporal expression of SIRT1 in mouse liver. Mice were sacrificed at 4 hr intervals around the clock, and liver nuclear proteins were prepared and analyzed. The results showed that SIRT1 accumulated in a circadian manner with maximal and minimal levels reached at around Zeitgeber time (ZT) 16 and ZT4, respectively (Figure 1A). In parallel, we also followed the temporal expression of known core clock proteins. As reported previously, circadian changes in PER2 protein expression as well as changes in BMAL1 phosphorylation were observed (Figure 1A) (Kornmann et al., 2007; Lee et al., 2001; Preitner et al., 2002; Ripperger and Schibler, 2006). To test whether the daily changes in SIRT1 protein expression were due to corresponding changes in *Sirt1* mRNA accumulation, we analyzed mouse liver RNA harvested around the clock by quantitative TaqMan real-time PCR. In contrast to several well-established circadian transcripts such as *Per2*, *Rorγ,* and *Rev-Erbα* mRNAs, the levels of *Sirt1* mRNA were nearly constant throughout the day (Figure 1B). Hence, posttranscriptional regulatory mechanisms must have accounted for the observed circadian changes in SIRT1 protein levels.

SIRT1 protein was also expressed in a circadian manner in dexamethasone-synchronized cultured mouse embryonic fibroblasts (MEFs) (Figure 1C) and NIH 3T3 cells (Figure S1A available online). Again, no significant changes in *Sirt1* mRNA accumulation were observed (Figure 1D). As expected *Per2* and *Cry1* mRNA levels were clearly rhythmic in these cells (Figure 1D). Finally, we examined temporal SIRT1 accumulation

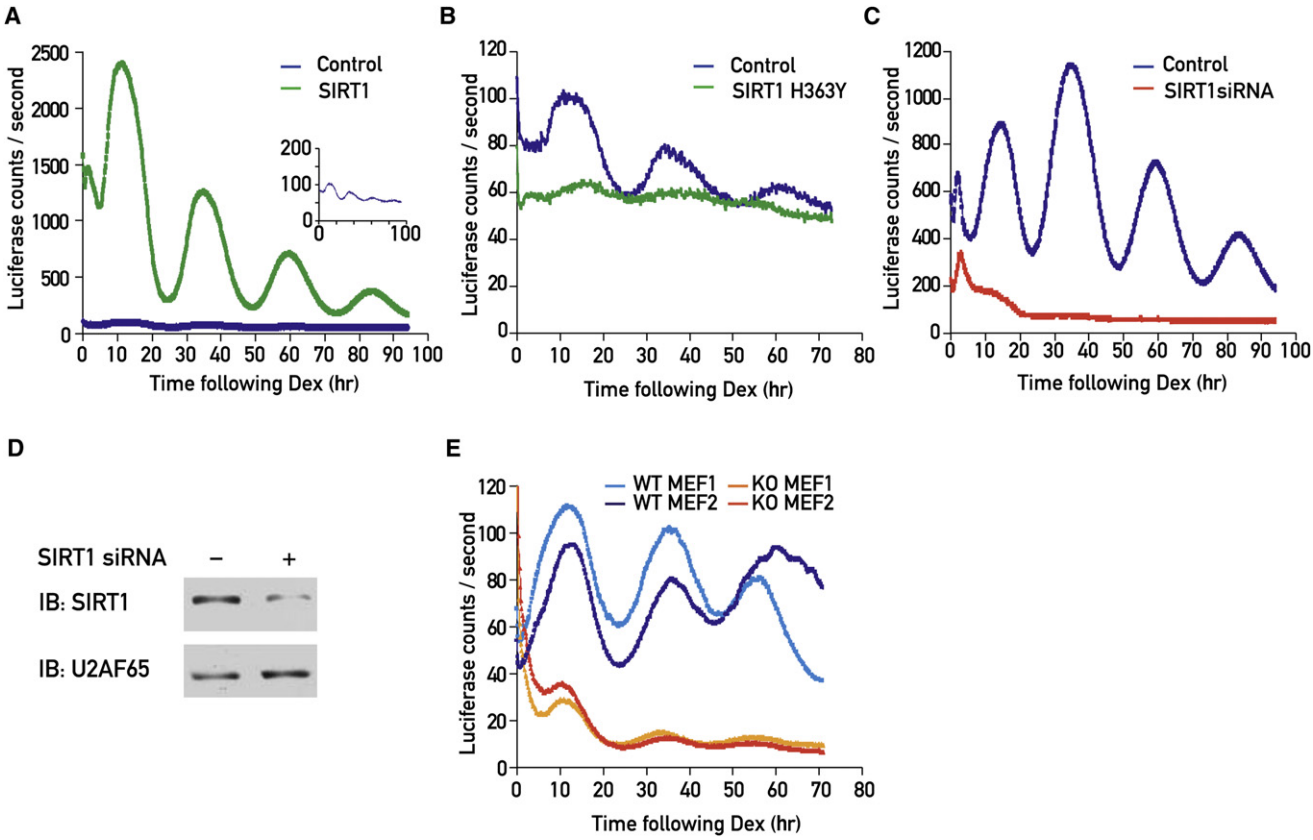

Figure 2. SIRT1 Deacetylase Activity Is Required for the High-Magnitude Oscillation of the *Bmal1*-Luciferase Reporter

(A) NIH 3T3 cells were transfected with a 200 ng/plate *Bmal1*-luciferase reporter gene either alone (blue) or together with an HA-Flag-SIRT1 expression vector (green). The small insert shows the cells transfected with the *Bmal1*-luciferase reporter gene alone at a higher magnification.

(B) NIH 3T3 cells were transfected with a 200 ng/plate *Bmal1*-luciferase reporter gene either alone (blue) or together with an HA-Flag-SIRT1 H363Y expression vector (green).

(C) NIH 3T3 cells were transfected with a 1 μg/plate *Bmal1*-luciferase reporter gene either with pU6 empty vector (blue) or together with pU6-*Sirt1* siRNA expression vector (red).

(D) Immunoblot analysis of protein extracts obtained from NIH 3T3 cells transfected with pU6 empty vector or pU6-*Sirt1* siRNA expression vector.

(E) Two WT MEF cell lines (dark and light blue) and two *Sirt1* KO MEF cell lines (dark and light orange), all obtained from different embryos harvested from the same pregnant female, were transduced with *Bmal1*-luciferase adenovirus.

Cells were synchronized by a dexamethasone shock and bioluminescence was recorded using photomultiplier tubes.

in synchronized NIH 3T3 fibroblasts by immunohistochemistry experiments. SIRT1 staining was mostly nuclear and more intense at 36 hr than at 24 hr following the dexamethasone shock (Figures S1C and S1D). Interestingly, even in knockout (KO) MEFs stably expressing human *SIRT1* from an expression vector containing the SV40 promoter and unrelated 5′- and 3′-untranslated regions, human SIRT1 protein was expressed differentially at two examined time points (Figure S1B). This experiment favors a mechanism involving changes in protein stability rather than translation rates in circadian SIRT1 protein accumulation.

SIRT1 Deacetylase Activity Is Required for High-Magnitude *Bmal1* Expression

In order to examine whether SIRT1 might influence circadian gene expression, we followed real-time bioluminescence recordings of a *Bmal1*-luciferase reporter whose expression is driven by the *Bmal1* promoter (Nagoshi et al., 2004). Cotransfections of a SIRT1 expression vector together with the *Bmal1*-luciferase reporter plasmid resulted in a dramatic increase in the magnitude of the bioluminescence oscillations (Figure 2A). The deacetylase activity of SIRT1 was required for this effect since cotransfection of SIRT1 H363Y, a catalytically inactive, dominant-negative SIRT1 mutant version (Vaziri et al., 2001), virtually abolished the circadian bioluminescence oscillations generated by the *Bmal1*-luciferase reporter gene (Figure 2B).

We also tested the effect of SIRT1 expression on reporter genes driven by other promoters, in particular *Per2*-luciferase, *Dbp*-luciferase, *Rev-Erbα*-luciferase, and *CMV*-luciferase. Whereas SIRT1 did not significantly affect *CMV*-luciferase or *Rev-Erbα*-luciferase expression, it increased the magnitude of the bioluminescence oscillations of *Per2*-luciferase and *Dbp*-luciferase (Figure S2).

Nicotinamide (NAM), a product of the SIRT1 deacetylation reaction, was reported to inhibit SIRT1 activity (Bitterman et al., 2002). Treatment of NIH 3T3 cells stably expressing the *Bmal1*-luciferase reporter gene with a moderate concentration

of NAM (10 mM) had no significant effect on the daily bioluminescence oscillations (Figure S3A). A higher concentration of NAM (50 mM) resulted in the dampening of circadian *Bmal1*-luciferase expression (Figure S3A). However, the most conspicuous effect of NAM treatment was a dramatic period lengthening (Figure S3A), a phenotype that was not observed in genetic loss-of-function experiments (see below) and, therefore, was unlikely to involve SIRT1. In contrast, Sirtinol, a more specific and potent inhibitor of SIRT1 deacetylation activity (Grozinger et al., 2001), strongly dampened the circadian *Bmal1*-luciferase reporter gene expression (Figure S3B) and thereby closely phenocopied the phenotypes observed in genetic *Sirt1* loss-of-function experiments.

To further scrutinize possible roles of SIRT1 on the clock function we performed loss-of-function experiments. Cotransfection of a *Sirt1* siRNA expression vector with the *Bmal1*-luciferase reporter gene attenuated the circadian oscillations (Figure 2C) in a dose-dependent manner (Figure S4A). The effect of *Sirt1* siRNA expression on endogenous SIRT1 protein accumulation was verified by analysis of protein extracts from the cells that were used for the *Bmal1*-luciferase recordings (Figure 2D). Since not all cells contributing to the protein extract were transfected, the downregulation of SIRT1 accumulation in transfected cells must have been very efficient. The expression of *Sirt1* siRNA also resulted in a decrease in the bioluminescence oscillations of *Per2*-luciferase and to a lesser extent of *Dbp*-luciferase oscillations (Figures S4B and S4C) but did not affect the bioluminescence of *CMV*-luciferase or *Rev-Erbα*-luciferase (Figures S4D and S4E). We also examined the effect of SIRT1 knockdown on *Bmal1*-luciferase reporter at a single-cell resolution. As can be concluded from a comparison of Movies S1 (control) and S2 (*Sirt1* siRNA) only a small proportion of cells displayed strong bioluminescence cycles when *Sirt1* expression was diminished. Experiments with MEFs from wild-type (WT) and *Sirt1* KO embryos, transduced with an adenoviral vector harboring the *Bmal1*-luciferase reporter gene, substantiated the observations made with *Sirt1* siRNA-expressing cells. Thus, the magnitude of bioluminescence cycles was considerably higher in WT MEFs than in *Sirt1* KO MEFs (Figure 2E). A closer inspection of our loss-of-function data also revealed a modest phase advance for the temporal expression of *Bmal1*-luciferase, *Per2*-luciferase, and *Dbp*-luciferase in the absence of SIRT1 (Figures 2E and S4). However, in none of these experiments were significant differences in period lengths noticed.

SIRT1 Influences the Expression of Endogenous Core Clock Genes

To examine whether SIRT1 also affected the expression of endogenous circadian genes, we analyzed the levels of various transcripts in WT and *Sirt1* KO MEFs by quantitative TaqMan real-time PCR. The levels of endogenous *Bmal1* mRNA were reduced to around 40% in nonsynchronized *Sirt1* KO MEFs compared to WT MEFs (Figure 3A). *Clock*, *Per1*, and *Cry1* mRNA accumulation was attenuated to a similar extent in *Sirt1* KO MEFs (Figure 3A), while *Per2* and *Rorγ* transcript levels in *Sirt1* KO cells only amounted to 20% and 10%, respectively, of those observed in WT cells (Figure 3A). In contrast, the mRNA levels of *Rev-Erbα*, *Dbp*, *βTrcp* (an F box protein targeting PER proteins for ubiquitination and degradation), and *Ckiε* (casein kinase Iε) were only slightly affected by the absence of SIRT1 (Figure 3A). The strong repression of *Rorγ* mRNA expression in the *Sirt1* KO MEFs incited us to examine the levels of the other two *Ror* paralogs, *Rorα* and *Rorβ* . However, no significant differences were observed for *Rorα* mRNA levels, and *Rorβ*, a neuron-specific *Ror* isoform (Dzhagalov et al., 2004), was undetectable in both WT and *Sirt1* KO MEFs (Figure 3B).

We also examined the accumulation of various clock proteins from nonsynchronized WT and *Sirt1* KO MEFs. In keeping with the changes observed for their mRNA levels, both BMAL1 and CLOCK protein levels were significantly downregulated in the *Sirt1* KO MEFs (Figure 3C). However, although the *Per2* mRNA level was strongly decreased in *Sirt1* KO MEFs (Figure 3A), PER2 protein accumulation was actually higher in these cells (Figure 3C). CRY1 protein accumulation was also slightly elevated in the absence of SIRT1 (Figure 3C), in contrast to the downregulation of its mRNA level (Figure 3A).

To verify whether the described changes in clock gene expression were indeed due to the absence of SIRT1 in the KO MEFs, we generated rescue cell lines from *Sirt1* KO MEFs that stably expressed a human *SIRT1* cDNA. The comparison of WT, *Sirt1* KO, and two human *SIRT1*-rescued KO MEFs cell lines showed that the mRNA levels encoding the different clock transcription factors and human *SIRT1* were restored to nearly normal levels in both rescued cell lines (Figure 3D). Similarly, the magnitude of the bioluminescence oscillations of *Bmal1*-luciferase reporter were reestablished in the human *SIRT*-rescued KO MEFs (Figure S5).

The observed changes in clock gene mRNA and protein levels were obtained in nonsynchronized cells and thus reflected average expression levels. Thus, we also wished to monitor the circadian expression of these genes in synchronized MEFs. In accordance with the observations made in the experiments with the *Bmal1*-luciferase reporter (Figure 2E), endogenous *Bmal1* mRNA was expressed at low and nearly invariable levels throughout the day (Figure 4A). Similarly, *Rorγ* mRNA accumulation was strongly repressed in the absence of SIRT1 (Figure 4A). *Per2* and *Cry1* mRNA were still expressed in a circadian manner in *Sirt1* KO MEFs, but with a significantly reduced magnitude (Figure 4A). Again, no significant changes in *Rev-Erbα* and *Dbp* mRNA accumulation were noticed (Figure 4A). These results were thus in keeping with the changes observed in our analysis of nonsynchronized MEFs (Figure 3A).

Similarly to the changes observed for *Bmal1* mRNA expression (Figure 4A), BMAL1 protein levels were significantly downregulated in the *Sirt1* KO MEFs (Figure 4B). In contrast, both PER2 and CRY1 protein levels were elevated and relatively constant in the absence of SIRT1 (Figure 4B), in spite of their diminished mRNA levels (Figure 4A). Again, SIRT1 accumulated in a circadian manner with maximal expression between 32 to 36 hr following the dexamethasone shock (Figure 4B).

SIRT1 Binds to CLOCK-BMAL1 and PER2 in a Circadian Fashion

SIRT1 was previously reported to deacetylate several transcriptional regulatory proteins (Blander and Guarente, 2004; Dali-Youcef et al., 2007), including the basic helix-loop-helix

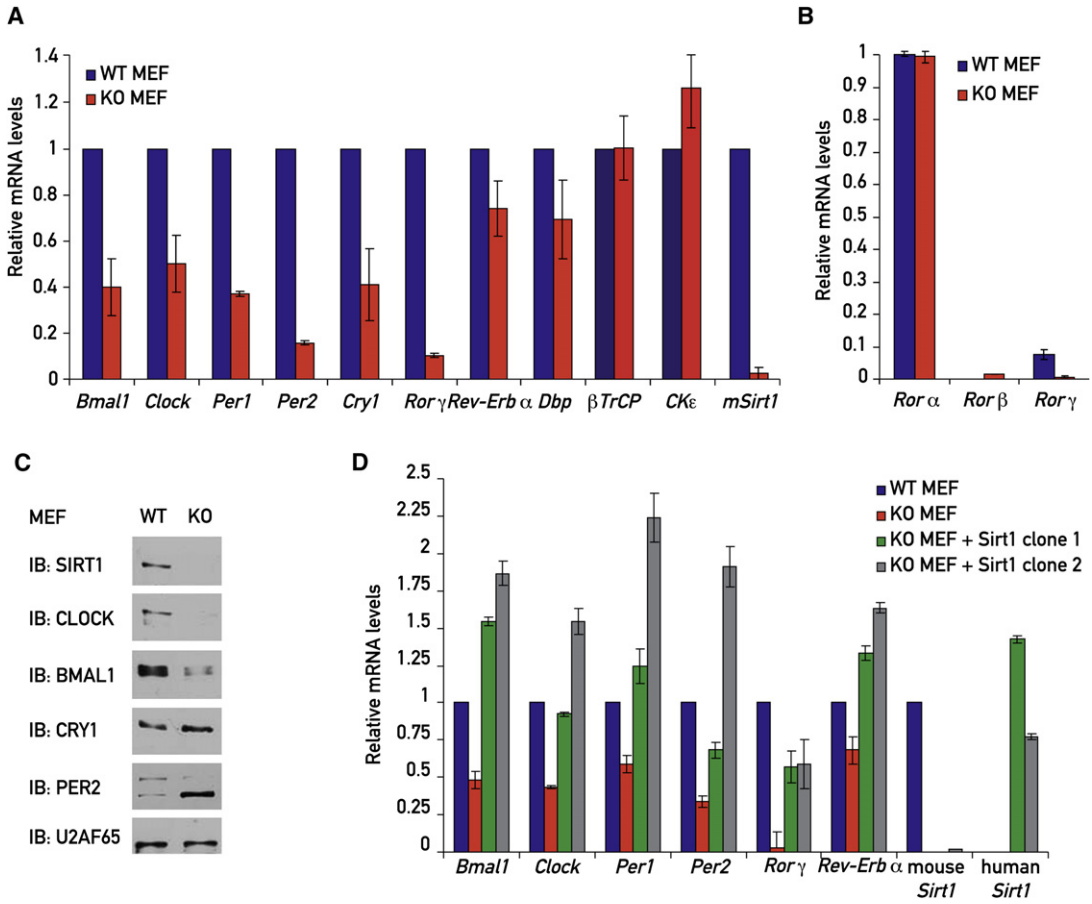

Figure 3. Analysis of mRNA and Protein Levels of Core Clock Proteins in Nonsynchronized WT, *Sirt1* KO, and *Sirt1* Rescue MEFs

(A) RNA extracts from nonsynchronized WT and *Sirt1* KO MEFs were analyzed by quantitative TaqMan real-time PCR using specific TaqMan probes.

(B) RNA extracts from nonsynchronized WT and *Sirt1* KO MEFs were analyzed for the mRNA expression of different ROR isoforms by quantitative TaqMan real-time PCR.

(C) Protein extracts from nonsynchronized WT and *Sirt1* KO MEFs were examined by immunoblotting.

(D) RNA extracts from nonsynchronized WT, *Sirt1* KO, and two different monoclonal *Sirt1* rescue MEF lines expressing human *SIRT1* were analyzed by quantitative TaqMan real-time PCR using specific TaqMan probes.

Plotted values are the mean values ± SD from three independent experiments.

repressors HES1 and HEY2 (Takata and Ishikawa, 2003). Since, similarly to HES1 and HEY2, both CLOCK and BMAL1 contain helix-loop-helix domains, we first examined whether SIRT1 might interact with these transcription factors. Both endogenous CLOCK and BMAL1 coimmunoprecipitated with SIRT1 in extracts obtained from mouse liver nuclei (Figure 5A) and from cultured NIH 3T3 fibroblasts (Figure 5B). In addition, coimmunostaining experiments for SIRT1 and CLOCK in NIH 3T3 cells showed that at least a fraction of these proteins colocalized in the nucleus (Figure 5C).

The binding of SIRT1 to CLOCK and BMAL1 prompted us to examine whether SIRT1 interacted with additional core clock proteins in a circadian manner. We thus analyzed the different binding partners of CLOCK around the clock in mouse liver nuclear extracts (Figure 5D). As expected, immunoprecipitaiton of CLOCK resulted in the coimmunoprecipitation of BMAL1, with maximal binding around ZT8 (Figure 5E). In contrast, maximal interactions of the repressors PER2 and CRY1 with CLOCK were observed around ZT0 (Figure 5E). After a longer exposure weak binding of PER2 to CLOCK was also detected around ZT4 (data not shown). Importantly, an immunoprecipitation experiment with SIRT1 antibody with the same protein extracts showed that SIRT1 bound to CLOCK in a circadian manner with maximal binding around ZT4 (Figure 5F). A similar experiment was conducted with whole-cell extracts from NIH 3T3 cells synchronized by a dexamethasone shock. The binding of CLOCK-BMAL1 to SIRT1 was circadian with maximal binding between 36 and 42 hr after the dexamethasone treatment (Figure 5G). Interestingly, PER2 could also be detected in SIRT1-associated complexes around 42 hr after the dexamethasone shock.

SIRT1 Deacetylates PER2

To examine whether CLOCK, BMAL1, or PER2 are acetylated and thereby potential substrates for SIRT1 deacetylation activity, we performed immunoprecpitation experiments with

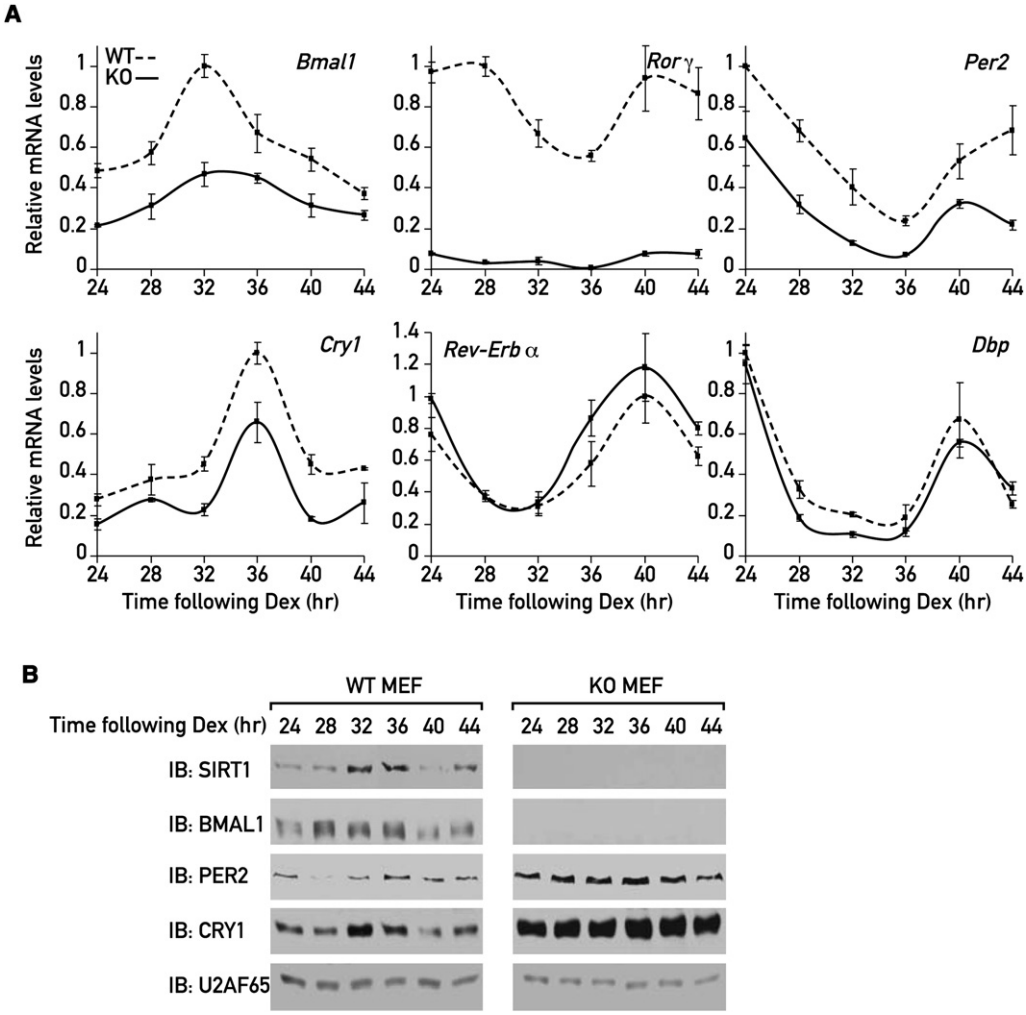

Figure 4. Analysis of Circadian mRNA and Protein Levels of Core Clock Proteins in WT and *Sirt1* KO MEFs

WT and *Sirt1* KO MEFs were synchronized by a dexamethasone shock, and protein and RNA were extracted at 4 hr intervals starting 24 hr after the shock.

(A) mRNA analysis was done by quantitative TaqMan real-time PCR using specific TaqMan probes. Plotted values are the mean values ± SD from three independent experiments.

(B) Protein extracts were analyzed by immunoblotting.

nonsynchronized WT and *Sirt1* KO MEFs, using a pan acetyl lysine antibody. The immunoprecipitated proteins were analyzed by immunoblotting using antibodies for various clock proteins. These experiments failed to reveal acetylated forms of CLOCK, BMAL1, CRY1, or SIRT1 (Figure 6A) but suggested that a fraction of PER2 was acetylated in *Sirt1* KO MEFs (Figure 6A). Low levels of acetylated PER2 were also detected in WT MEFs after a long exposure (data not shown). To corroborate PER2 acetylation we transfected NIH 3T3 cells with expression vectors for a tandem affinity purification (TAP) tagged PER2 (PER2-TAP) or a TAP-tagged luciferase (luciferase-TAP) (as a negative control). Immunoblot analysis of purified TAP-tagged proteins confirmed that PER2 was acetylated (Figure S6) and that the acetyl groups were removed in vitro by recombinant SIRT1 in an NAD^+-dependent manner (Figure 6B).

Next, we monitored the acetylation of PER2 in synchronized WT and *Sirt1* KO MEFs around the clock. Again, immunoprecipitation experiments were performed with a pan acetyl lysine antibody, and the immunoprecipitated proteins were analyzed by immunoblotting. In agreement with the previous experiments, no acetylation of BMAL1 or CLOCK was detected (Figure 6C). In WT MEFs, PER2 acetylation was maximal at around 32 hr after the dexamethasone shock (Figure 6C). The extent of PER2 acetylation was significantly higher in KO MEFs than in WT MEFs, and maximal acetylation in KO MEFs occurred between 24 and 28 hr after the dexamethasone shock. Our findings thus suggested that PER2 was acetylated in a circadian manner, and that SIRT1 deacetylated PER2 in vivo. While PER2 protein levels were elevated and relatively constant in the *Sirt1* KO MEFs (Figure 4B), PER2 acetylation and/or deacetylation was cyclic. This is in line with the circadian binding of SIRT1 to PER2 (Figure 5G). To examine whether PER2 was also acetylated in mice, we immunoprecipitated PER2 from mouse liver nuclear extracts prepared around the clock. Immunoblot analysis of

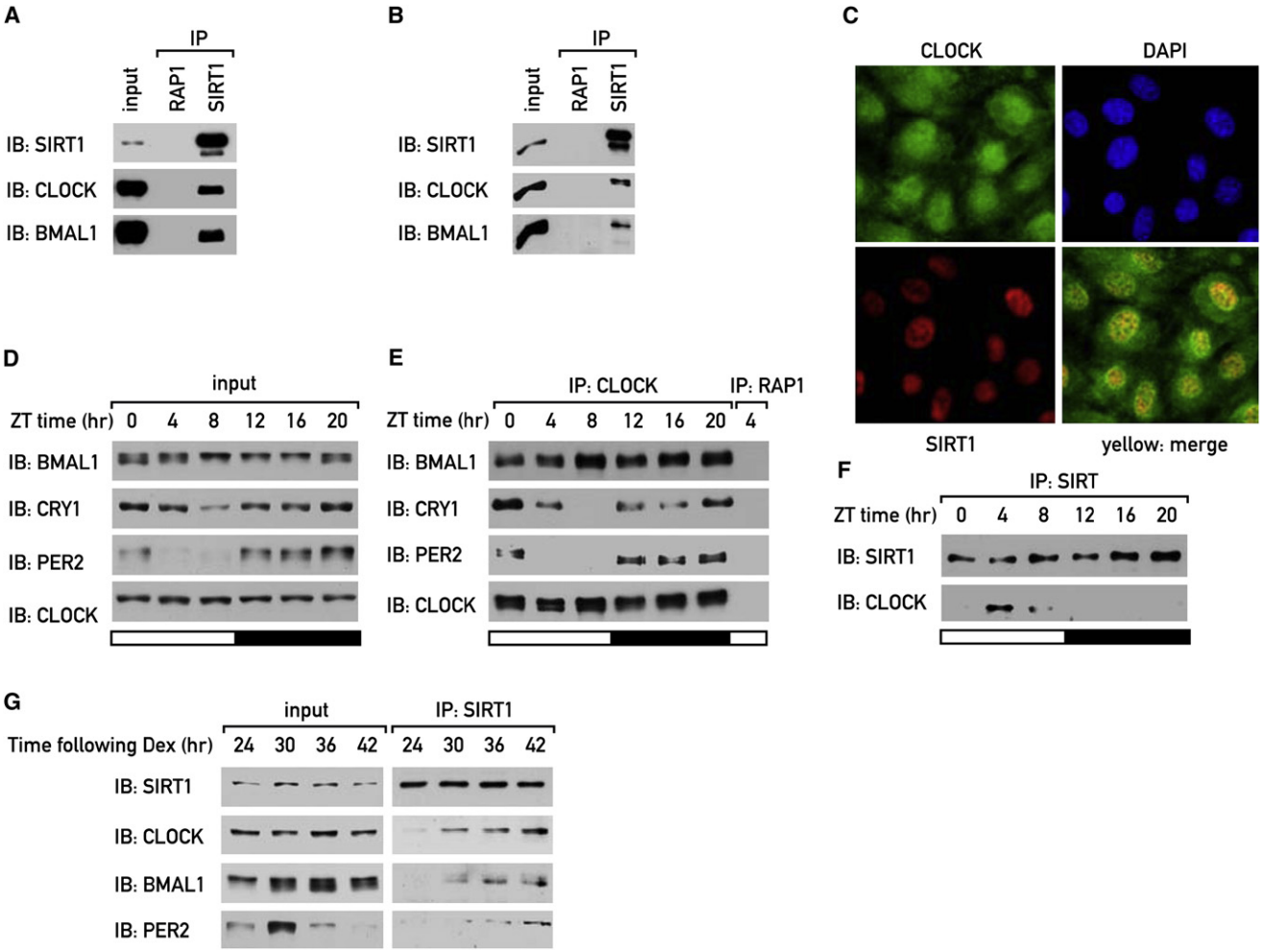

Figure 5. SIRT1 Binds to CLOCK, BMAL1, and PER2 in a Circadian Manner

SIRT1 was immunoprecipitated from mouse liver nuclear extracts (A) and from NIH 3T3 cells (B). The immunoprecipitated proteins were analyzed by immunoblotting. Rabbit yeast RAP1 antibody was used as a negative control.

(C) Immunostaining of SIRT1 (red) and CLOCK (green) in NIH 3T3 cells was performed with rabbit SIRT1 and CLOCK antibodies. In blue: DAPI staining. In yellow: merge of SIRT1 and CLOCK staining.

(D) Mice were sacrificed at 4 hr intervals, and liver nuclear extracts were analyzed by immunoblotting.

(E) CLOCK was immunoprecipitated from mouse liver nuclear extracts, and the immunoprecipitated proteins were analyzed by immunoblotting. Rabbit yeast RAP1 antibody was used as a negative control.

(F) SIRT1 was immunoprecipitated from mouse liver nuclear extracts, and the immunoprecipitated proteins were analyzed by immunoblotting.

(G) NIH 3T3 cells were synchronized by a dexamethasone shock, and protein extracts were prepared at 6 hr intervals, starting 24 hr after the shock. SIRT1 was immunoprecipitated from NIH 3T3 cells, and the immunoprecipitated proteins were analyzed by immunoblotting.

precipitated proteins with the pan acetyl lysine antibody showed that PER2 was acetylated also in mouse liver (Figure 6D).

SIRT1-Dependent Deacetylation of PER2 Determines PER2 Protein Stability

The elevated PER2 acetylation and accumulation in *Sirt1* KO MEFs on one hand and the reduced *Per2* mRNA levels on the other hand raised the possibility that acetylation of PER2 stabilized the protein. We thus compared the decay of PER2 protein in the presence and absence of SIRT1. To this end, WT and *Sirt1* KO MEFs were incubated with or without cycloheximide 24 hr after synchronization and PER2 protein levels were recorded during 4 hr. The results indicated that the protein half-life of PER2 was significantly prolonged in the absence of SIRT1 (Figures 7A and 7B). In keeping with these observations, the coexpression of SIRT1 together with PER2-TAP significantly reduced the accumulation (Figure 7C) and acetylation of PER2-TAP (Figure 7D), whereas knockdown of SIRT1 expression with *Sirt1* siRNA resulted in a significant increase in PER2 accumulation and acetylation (Figure 7E).

To further address the dependency of PER2 degradation upon deacetylation by SIRT1 we performed an in vitro assay with purified PER2-TAP and extracts obtained from WT and *Sirt1* KO MEFs. PER2 was deacetylated only in the presence of extracts

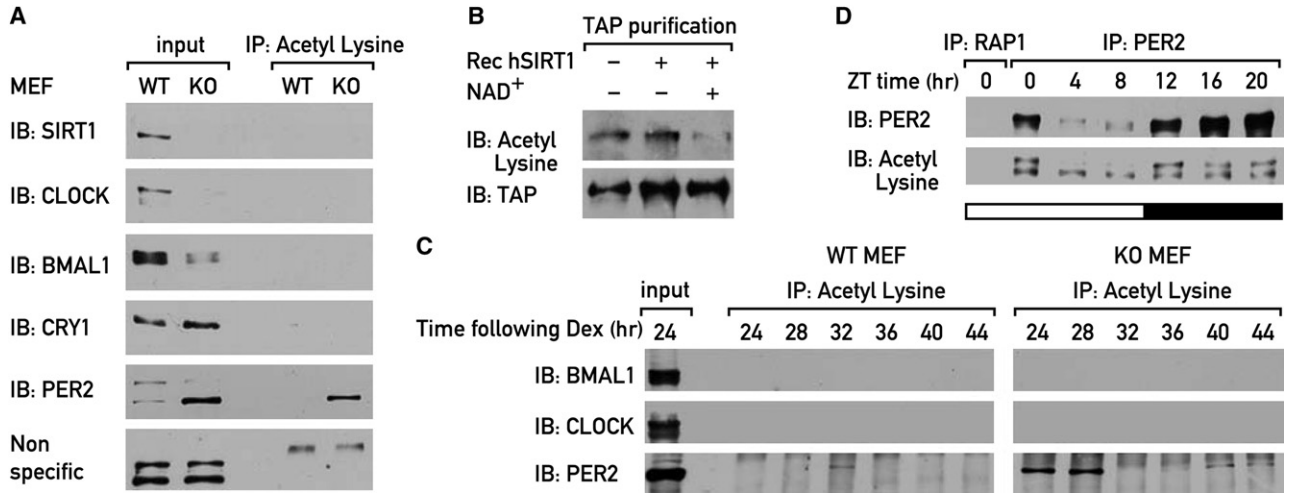

Figure 6. SIRT1 Deacetylates PER2

(A) Protein extracts from nonsynchronized WT and *Sirt1* KO MEFs were subjected to immunoprecipitation with rabbit pan acetyl lysine antibody, and the immunoprecipitated proteins were analyzed by immunoblotting.

(B) Purified PER2-TAP was incubated in the absence or presence of recombinant SIRT1 and NAD^+ for 3 hr at 30°, and samples were analyzed by immunoblotting.

(C) WT and *Sirt1* KO MEFs were synchronized by a dexamethasone shock, and protein extracts were prepared at 4 hr intervals, starting 24 hr after the shock. Immunoprecipitation experiments were performed with rabbit pan acetyl lysine antibody, and the immunoprecipitated proteins were analyzed by immunoblotting.

(D) Mice were sacrificed at 4 hr intervals and liver nuclear extracts were prepared. PER2 was immunoprecipitated, and the immunoprecipitated proteins were analyzed by immunoblotting. Rabbit yeast RAP1 antibody was used as a negative control.

from WT MEFs together with NAD^+. Deacetylation of PER2 resulted in PER2 degradation, which was blocked in the presence of the proteasome inhibitor MG132 (Figure 7F).

DISCUSSION

Modulation of Circadian Oscillator Function by Protein Acetylation and Deacetylation

We identified SIRT1 as a regulator of circadian gene expression. SIRT1 accumulates in a circadian manner in mouse hepatocytes and cultured fibroblasts and is required for high-magnitude circadian transcription of several core clock genes, including *Bmal1*, *Ror*γ, *Per2*, and *Cry1*. SIRT1 binds to CLOCK-BMAL1 and PER2 in a circadian manner and supports the deacetylation and degradation of PER2. In the absence of SIRT1, constitutively high protein levels of PER2 may lead to the repression of *Per1*, *Per2, Cry1*, and *Ror*γ mRNA expression. Repression of RORγ, an activator of *Bmal1* transcription, is likely to account for the dampening of *Bmal1* mRNA and protein expression in *Sirt1* KO cells (Figure 7G).

The enzyme(s) responsible for PER2 acetylation remain(s) to be identified, but the acetyltransferase activity of CLOCK (Doi et al., 2006) or p300, a coactivator associated with CLOCK-BMAL1 heterodimer (Etchegaray et al., 2003), are attractive candidates. PGC1α, a recently identified key player in circadian oscillator function (Liu et al., 2007), may also affect PER2 acetylation via stimulating the acetyl transferase activity of p300 (Wallberg et al., 2003).

Although our results cannot rigorously exclude a more complicated scenario, they suggest that SIRT1 deacetylates PER2 directly. Thus, SIRT1 is associated with CLOCK-BMAL1-PER2 complexes, purified PER2 is deacetylated in vitro by extracts obtained from wild-type but not from *Sirt1* KO MEFs, and recombinant SIRT1 deacetylates purified PER2 in vitro in a NAD^+-dependent manner. The latter result should, however, be interpreted with caution since the in vitro substrate specificity of recombinant SIRT1 is rather promiscuous (Blander et al., 2005).

Other posttranslational modifications such as phosphorylation, sumoylation, histone acetylation, and methylation have already been shown to play a key role in circadian gene expression (Gallego and Virshup, 2007). For example, sumoylation of BMAL1 has been shown to play an important role in BMAL1 accumulation and clock rhythmicity (Cardone et al., 2005). Likewise, phosphorylation of BMAL1 either by Casein Kinase I (CKI) (Eide et al., 2002) or by mitogen-activated protein kinases (MAPK) (Sanada et al., 2002) modulates BMAL1-CLOCK-dependent transcription. Recently, CLOCK was reported to acetylate BMAL1, thereby facilitating repression of BMAL1-CLOCK-dependent transcription (Hirayama et al., 2007). We suppose that due to the sensitivity of our immunoblot experiments, the acetylated fraction of endogenous BMAL1 in liver and fibroblast extracts was not revealed. CKI has been reported to phosphorylate PER2 protein, thereby regulating PER2 protein stability (Eide et al., 2005). Likewise, CRY degradation mediated by the F box protein SCF^{Fbxl3} has been demonstrated to be required for normal oscillator function (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007).

SIRT1 Affects Circadian Transcription in a Gene-Specific Manner

The extent to which SIRT1 affects circadian transcription appears to be target gene specific. For example, in the absence of SIRT1, *Ror*γ mRNA levels are strongly repressed and *Per2* mRNA levels are significantly downregulated, whereas *Rev-Erb*α

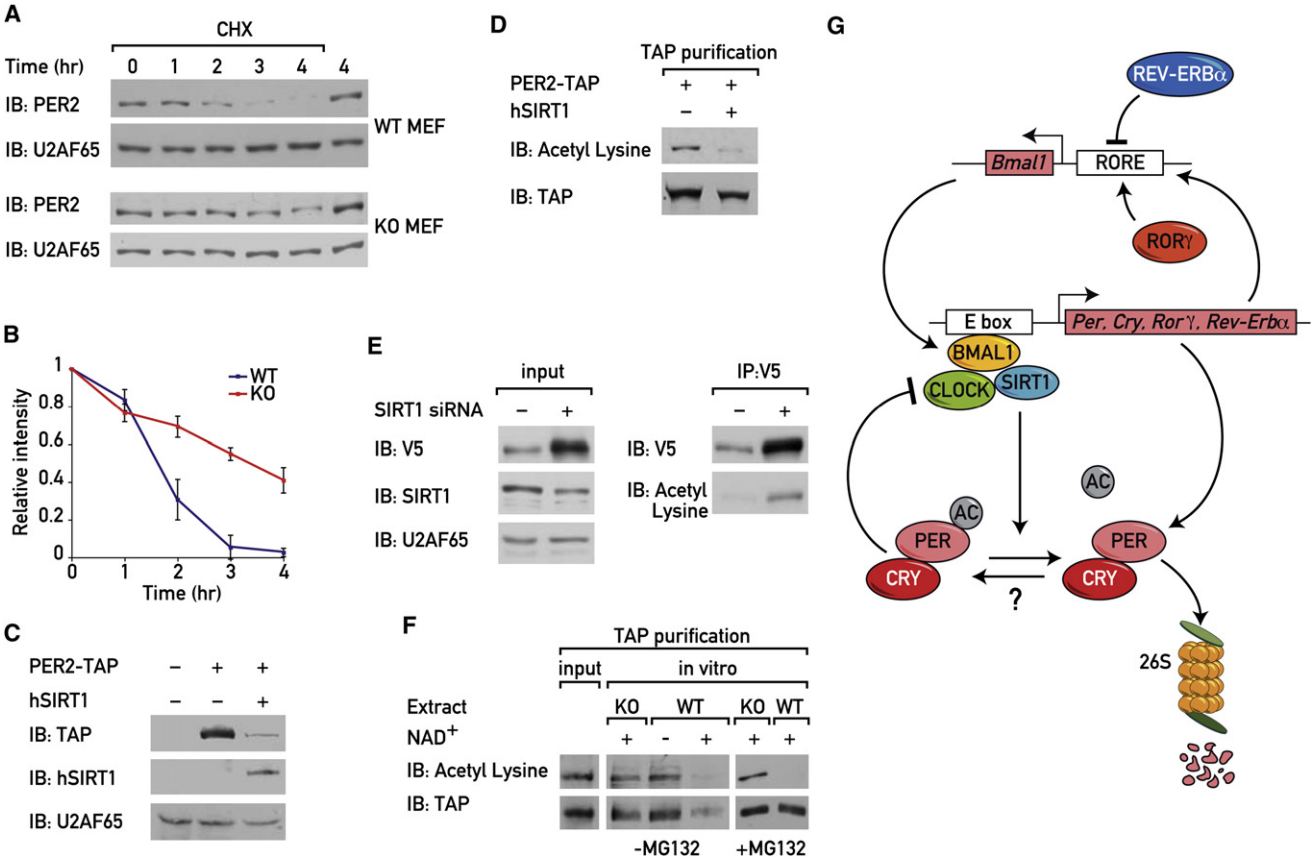

Figure 7. SIRT1-Dependent PER2 Deacetylation Determines PER2 Protein Stability

(A) WT and *Sirt1* KO MEFs were synchronized by a dexamethasone shock, and 24 hr after the shock cells were untreated or treated with cycloheximide. Cells were harvested 1, 2, 3, and 4 hr following the treatment, and protein extracts were analyzed by immunoblotting.

(B) The graph illustrates the quantification of PER2 by densitometry of triplicate experiments (mean ± standard error).

(C) NIH 3T3 cells were transfected with PER2-TAP expression vector either alone or together with HA-FLAG-human SIRT1 expression vector. Protein extracts were analyzed by immunoblotting.

(D) PER2-TAP was purified from NIH 3T3 cells transfected with PER2-TAP expression vector either alone or together with HA-FLAG-human SIRT1 expression vector and analyzed by immunoblotting.

(E) NIH 3T3 cells were transfected with the V5-PER2 expression vector either alone or together with the *Sirt1* siRNA expression vector. Protein extracts were prepared, and immunoprecipitation experiments were performed with mouse V5 antibody. The immunoprecipitated proteins were analyzed by immunoblotting.

(F) Purified PER2-TAP was incubated for 3 hr at 30° with protein extract obtained from WT or *Sirt1* KO MEFs in the absence or presence of 100 μM NAD^+ or 25 μM MG132, and samples were analyzed by immunoblotting.

(G) Hypothetical model showing the possible role of SIRT1 in circadian oscillator function. BMAL1-CLOCK heterodimers bind and activate transcription of the *Per*, *Cry, Rre-Erbα*, and *Rorγ* genes. Once the PER and CRY proteins accumulate to a critical level, they form complexes with BMAL1-CLOCK and thereby repress their own transcription. In addition, there is an interconnecting feedback loop in which REV-ERBα represses and RORγ activates *Bmal1* transcription. SIRT1 binds CLOCK-BMAL1 complexes and promotes PER2 deacetylation and degradation.

and *Dbp* mRNA levels are only slightly affected (Figure 3). Conceivably, BMAL1 and CLOCK bind their DNA cognate sites in *Rev-Erbα* and *Dbp* with a higher affinity than those present in *Per2* and *Rorγ*. The reduced BMAL1-CLOCK levels in *Sirt1* KO cells might then still support high-amplitude/magnitude *Rev-Erbα* and *Dbp* transcription.

PGC1α was found to be expressed in a circadian manner and to stimulate *Bmal1* transcription as a coactivator of the ROR family of nuclear orphan receptors (Liu et al., 2007). SIRT1 deacetylates PGC1α and thereby modulates its coactivator activity (Rodgers et al., 2005). Thus, it is possible that the downregulation of *Bmal1* expression in the absence of SIRT1 is caused by a combination of diminished RORγ expression and impaired PGC1α coactivation. Indeed, *Pgc1α* KO mice exhibit abnormal diurnal rhythms of activity, body temperature, and metabolic rate (Liu et al., 2007). Unfortunately, such studies cannot be performed with *Sirt1*-deficient mice because their postnatal survival rates are very poor and the few surviving mice exhibit many developmental defects (Cheng et al., 2003; McBurney et al., 2003).

Regulation of PER2 Protein Degradation

In mammals, the stability of PER proteins is regulated by the F-box-containing E3 ubiquitin ligase βTrCP (Gallego and

Virshup, 2007). PER phosphorylation by CKIε promotes the recruitment of βTrCP complexes, which in turn mediates the ubiquitination and proteasomal degradation of PER (Eide et al., 2005; Shirogane et al., 2005). Our results that both PER2 and acetylated PER2 levels are elevated in the absence of SIRT1 suggest that SIRT1-mediated deacetylation enhances the rate of PER2 degradation. Since ubiquitination and acetylation occur on lysine residues, it is conceivable that the same lysine residues can be either acetylated or ubiquitinated. If true, acetylated PER2 could no longer be ubiquitinated and degraded by the proteasome, which would explain the augmented PER2 levels in *Sirt1* KO cells. Interestingly, CRY1 protein levels are also elevated in the absence of SIRT1, in spite of reduced *Cry1* mRNA levels (Figure 4). It has been reported that PER2 inhibits the ubiquitination and degradation of CRY proteins (Yagita et al., 2002). Thus, the higher accumulation of PER2 in *Sirt1* KO cells may account for the elevated CRY1 levels in these cells.

The Circadian Accumulation and Activity of SIRT1 Protein

Circadian SIRT1 protein accumulation appears to be controlled by posttranscriptional mechanisms as no significant changes in *Sirt1* mRNA were observed (Figure 1). Surprisingly, temporal SIRT1 accumulation does not correlate with the circadian interaction of SIRT1 with its identified core clock binding partners. For example, in mouse liver nuclei, maximal SIRT1 protein expression is observed at around ZT16, whereas its maximal binding to CLOCK occurs at around ZT4, when SIRT1 levels are minimal. Similarly, in NIH 3T3 cells, maximal SIRT1 protein expression occurs between 32 and 36 hr after the dexamethasone shock, while its maximal binding to the core clock components is observed around 42 hr. Therefore, SIRT1 might also regulate the expression of circadian output genes expressed with a different phase, possibly through activation of coactivators and transcription factors such as PGC1α, FOXO, or LXR (Dali-Youcef et al., 2007; Li et al., 2007) or through the circadian deacetylation of histones in nucleosomes associated with clock-controlled genes (Belden et al., 2007; Curtis et al., 2004; Etchegaray et al., 2003; Naruse et al., 2004; Ripperger and Schibler, 2006). Future experiments with *Sirt1*-deficient and -proficient cells should shed light on the role of SIRT1 in these additional mechanisms involved in the regulation of circadian gene expression.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture

NIH 3T3 cells and NIH 3T3-*Bmal1*-luciferase cells stably expressing *Bmal1*-luciferase reporter were grown as previously described (Nagoshi et al., 2004). WT and *Sirt1* KO MEFs were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 8 mM nonessential amino acids (Sigma), 1 mM Na-Pyruvate, 0.006 mM β-mercaptoethanol, and 18 mM HEPES (pH 7.0) and cultured at 37°C in a humidified incubator with 5.6% CO_2. Cells were synchronized with 100 nM dexamethasone and real-time bioluminescence was recorded (Nagoshi et al., 2004). Nicotinamide and dexamethasone were prepared in H_2O and ethanol, respectively. Trichostatin a, cycloheximide, and sirtinol (Sigma) were dissolved in DMSO.

Plasmids and Transfections

The following plasmids were used: *Bmal1*-luciferase (Nagoshi et al., 2004), *Per2(E-BOX2)*-luciferase (Yoo et al., 2005), *Dbp*-luciferase (Brown et al., 2005), *Rev-Erbα*-luciferase, and *CMV*-luciferase; pCDNA HA-Flag-SIRT1 encoding WT human SIRT1, pCDNA HA-Flag-SIRT1 H363Y encoding inactive deacetylase mutant of SIRT1, pBabe human SIRT1, pU6-siRNA-*Sirt1*, and pU6-empty vector (Cohen et al., 2004); and pEF5/FRT7V5-PER2, pCMV PER2-TAP, and pCMV luciferase-TAP. Transient transfections of NIH 3T3 cells were carried out with FuGENE Transfection Reagent (Roche) according to the manufacturer's instructions.

Generation and Transduction of *Bmal1*-Luciferase Expressing Adenovirus

Bmal1-luciferase (Nagoshi et al., 2004) cassettes was cloned into pCV100 plasmid, first-generation adenoviral vector was amplified in N52.E6-producer cells, and viruses were generated and purified as previously described (Kreppel et al., 2002).

MEFs were incubated with *Bmal1*-luciferase expressing adenovirus at an multiplicity of infection (moi) of 5000 for 6 hr, cells were thoroughly washed with PBS, and the medium was replaced. Forty-eight hours after transduction cells were shocked with dexamethasone and real-time bioluminescence was recorded (Nagoshi et al., 2004).

Immunostaining

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in TBS and washed with TBS containing 0.1% Triton X-100 (TBS-T). Samples were blocked with 2% BSA in TBS-T followed by incubation with rabbit anti-SIRT1 and rat anti-CLOCK antibodies. Samples were washed with TBS-T and incubated with alexa 594-conjugated anti-rabbit and with FITC-conjugated anti-rat secondary antibodies. Nuclei were stained with DAPI. Microscopic images were obtained using a Leica SP2 confocal microscope.

RNA Analysis by Real-Time Quantitative PCR

RNA extraction and transcript quantification by TaqMan real-time PCR technology was performed as previously described (Preitner et al., 2003), using an ABI PRISM 7700 Sequence Detection System from PE-Applied Biosystems. The real-time PCR data were normalized to 45S pre-mRNA. Primers and probes are listed in Table S1.

Protein Extraction and Immunoblot Analysis

Proteins from mouse liver nuclei and cultured fibroblasts were prepared according to the NUN procedure (Lavery and Schibler, 1993). Trichostatin A was added during the extraction. SDS-PAGE and immunoblot analysis were performed according to standard protocols. Antibodies used were rabbit CRY1, PER2, BMAL1, and CLOCK (kindly provided by S. Brown and J. Ripperger) and rabbit SIRT1 (Upstate), human-SIRT1 (Santa Cruz), pan acetylated lysine (Cell Signaling), TAP (OPEN BIOSYSTEMS), mouse V5 (Invitrogen), and U2AF65 (Sigma).

Coimmunoprecipitation Experiments

Coimmunoprecipitation experiments were carried out with mouse liver nuclear extracts or with whole-cell NUN extracts. Extracts were incubated for 12 hr with the indicated antibodies at 4°C and further incubated with protein A beads (Roche) for an additional 2 hr at 4°C. The beads were collected by centrifugation and washed with NP40 buffer (100 mM Tris-HCL pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% NP40). Laemmli sample buffer was added and samples were heated at 95°C for 5 min and loaded on a polyacrylamide-SDS PAGE. Purification of the C-terminal TAP-tagged PER2 and TAP-tagged luciferase proteins were performed according to standard protocol as previously described (Puig et al., 2001).

In Vitro Deacetylation Assay

Purified PER2-TAP protein was incubated in deacetylation buffer (50 mM Tris-HCL pH 8, 50 mM NaCl, 4 mM $MgCl_2$) in the presence of purified recombinant human SIRT1 (BioMol, 5U) or in the presence of protein extracts from WT or *Sirt1* KO MEFs lysed in RIPA lysis buffer (150 mM NaCl, 1% NP-40 [vol/vol],

0.5% Na-deoxycholate [DOC vol/vol], 0.1% SDS [vol/vol], 50 mM Tris-Hcl pH 8, 1 mM dithiothreitol [DTT]). Reactions were carried out in the presence or absence of 100 μM NAD^+ for 3 hr at 30°.

SUPPLEMENTAL DATA

Supplemental Data include six figures, one table, and two movies and can be found with this article online at http://www.cell.com/cgi/content/full/134/2/317/DC1/.

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