PER2 mediates CREB-dependent light induction of the clock gene Per1

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Light affects many physiological processes in mammals such as entrainment of the circadian clock, regulation of mood, and relaxation of blood vessels. At the molecular level, a stimulus such as light initiates a cascade of kinases that phosphorylate CREB at various sites, including serine 133 (S133). This modification leads CREB to recruit the co-factor CRCT1 and the histone acetyltransferase CBP to stimulate the transcription of genes containing a CRE element in their promoters, such as Period 1 (Per1). However, the details of this pathway are poorly understood. Here we provide evidence that PER2 acts as a co-factor of CREB to facilitate the formation of a transactivation complex on the CRE element of the Per1 gene regulatory region in response to light or forskolin. Using in vitro and in vivo approaches, we show that PER2 modulates the interaction between CREB and its co-regulator CRCT1 to support complex formation only after a light or forskolin stimulus. Furthermore, the absence of PER2 abolished the interaction between the histone acetyltransferase CBP and CREB. This process was accompanied by a reduction of histone H3 acetylation and decreased recruitment of RNA Pol II to the Per1 gene. Collectively, our data show that PER2 supports the stimulus-dependent induction of the Per1 gene via modulation of the CREB/CRTC1/CBP complex.

Light perception is one of the most important mechanisms that provoke biological responses in organisms, from resetting the circadian clock to cell division, metabolism, and redox state regulation1–5. In mammals, light can entrain the circadian clock, regulate mood behaviors, and blood vessel relaxation6–8. Light is perceived by the retina by intrinsically photosensitive retinal ganglion cells (ipRGCs) and transduced via the retinohypothalamic tract (RHT) to many different brain regions. For instance, in the suprachiasmatic nuclei (SCN) located above the optic chiasm in the ventral part of the hypothalamus, the signal is responsible for the release of neurotransmitters such as glutamate and PACAP9. Light-evoked signals provoke Ca2+ influx in SCN neurons and activate a phosphorylation cascade, including protein kinase A (PKA), protein kinase G (PKG), Ca2+/calmodulin-dependent protein kinase (CaMK), and mitogen-activated protein kinases (MAPK) also known as extracellular-signal-regulated kinases (ERK). This cascade finally promotes phosphorylation of cAMP response element-binding protein (CREB) at serine 133 and 14210–12. CREB dimers recognize a specific motif of an 8-base pair palindromic sequence (TGA CGT CA) called cAMP response element (CRE) present on regulatory regions of target genes such as Per113,14. In neurons, cAMP-regulated transcriptional co-activator 1 (CRTC1) is required for efficient induction of CREB target genes during neuronal activity15,16. Upon stimulation of L-type voltage-gated calcium channels, CRTC1 is dephosphorylated by calcineurin. Subsequently, it translocates into the nucleus, where it interacts via its N-terminal CREB-binding domain (CBD) with CREB17. Finally, the histone acetyltransferase CREB binding protein (CBP) binds to phospho-Ser133 CREB (pS133-CREB)18. The subsequent stabilization of the CREB: CBP complex stimulates the expression of target genes by acetylating nucleosomal histone H3 at lysine 27 (AcH3K27), followed by recruitment of RNA polymerase II19,20. Within the wide range of light-responsive genes, the clock gene Per1 and the immediate-early gene cFos are targets of pS133-CREB dependent gene activation21–24.

Within the eukaryote domain, the filamentous fungus Neurospora crassa is the most used model system for studying light-inducible pathways, including entrainment and biosynthesis of photoprotective pigments, to name a few25–28. The NGF1: White Collar complex (WCC) regulates the light signaling system in this organism. The WCC consists of a heterodimer formed by the product of the white collar-1 (wc-1) and white collar-2 (wc-2) genes29–31. The blue light photoreceptor WC-1 interacts in the dark with the histone acetyltransferase NGF132,33.
via an LXXLL consensus sequence, a known motif responsible for the interaction between nuclear receptors and their co-activators\textsuperscript{31}. After a light pulse, WC-1 changes conformation and directs NGFI to the chromatin to acetylate histones. At the same time, it stabilizes the transcriptional factor WC-2 on the promoter of target genes, and target gene expression is promoted\textsuperscript{32}.

Structural similarities between the WC-1 and PER2 protein suggest that mammalian PER2 may act in a similar way as WC-1 in \textit{N. crassa}. Both proteins are similar in size and contain 3 PAS domains. They are both essential regulators of the circadian clock and can act as co-activators via LXXLL domains\textsuperscript{33,34}. PER2 accumulates in the SCN between zeitgeber time (ZT) 14 and 16, which corresponds to the time window of light-responsiveness that leads to induction of genes such as Per\textsuperscript{17}. Since WC-1 is involved in light-dependent responses, and due to the resemblance of PER2 with the fungal protein, we wondered whether the mammalian clock factor would modulate the light-mediated CREB signaling pathway to induce genes such as Per1 or cFos in a similar fashion. This resembles the function of WC-2, which is the transcription factor that modulates light responses in \textit{N. crassa}.

Here, we describe a novel function of PER2 as a mediator of the light-dependent CREB signaling in the early night. Using an \textit{in vitro} (forskolin-induced cell lines) and \textit{in vivo} system (light-stimulated mice), we show that PER2 modulates the light/forskolin mediated assembly of the pSer133-CREB/CRTC1/CBP complex. In particular, we show that PER2 gates the CREB: CRTC1 complex to the early night. Förster resonance energy transfer (FRET) analysis revealed that the complex formation between CREB and CBP depends on the presence of PER2. Consequently, PER2 is important for the histone H3 acetylation at lysine 27 by CBP, and hence induction of Per1 gene expression. We conclude that PER2 is a scaffold facilitating the formation of the CREB: CBP complex after a light pulse or forskolin stimulus.

**Results**

PER2 modulates light/forskolin-induced \textit{Per}1 and \textit{cFos} gene expression in an opposite manner. A light pulse (LP) applied to mammals in the dark phase of a 12 h light: 12 h dark cycle, where zeitgeber time (ZT) 0 is lights on and ZT12 is light off, elicits phase-shifts in locomotor activity\textsuperscript{11,29}, paralleled by induction of Per1 and Per2 genes in the SCN of wild type (wt) mice before and after an LP of 15 min duration at ZT14 (Fig. 1A). The pre-mRNA of Per1 was strongly induced after the LP, whereas the pre-mRNA of Per2 was significantly less responsive (Fig. 1A).

To corroborate this observation \textit{in vitro}, we stimulated NIH3T3 cells with forskolin, which mimics the effect of phase-shifting via activation of the Protein kinase A (PKA) signaling pathway\textsuperscript{40}. We observed that Per1, but not Per2, pre-mRNA was induced after 25 and 40 min of forskolin treatment (Fig. 1B). These results suggested that the Per2 gene is less inducible by light or forskolin than Per1. Because Per2 KO mice do not phase delay after a light pulse at ZT14\textsuperscript{41}, our general question was whether PER2 was an upstream factor rather than a downstream target of stimulus-mediated clock resetting.

To challenge this hypothesis, we analyzed Per1 pre-mRNA expression in SCN samples obtained from wt and Per2 knock-out (KO) mice\textsuperscript{42} collected either in the dark or 15 min after LP at ZT14. We observed that in Per2 KO mice the light inducibility of Per1 is dampened compared to wt (Figs. 1C, S1A upper panel). Although Per2 pre-mRNA was less light-inducible compared to Per1, it was not inducible in the Per2 KO mice (Figs. 1C, S1A lower panel). Subsequently, we analyzed Per1 and Per2 pre-mRNA expression in fibroblast cells derived from wt or Per2 KO animals. After forskolin treatment of the cells we observed induction of Per1 but not Per2 after 25 min in wild-type cells (Figs. 1D, S1B, upper panel). In contrast, Per1 was not induced in Per2 KO cells, but Per2 was slightly increased after 40 min (Figs. 1D, S1B, lower panel). These results are in line with the observation that in the SCN Per1 induction is affected by the loss of Per2. Taken together, our observations suggest that PER2 is important for the regulation of the light- and forskolin-dependent induction of Per1 expression. Of note, other clock genes did not respond in the same way as Per1 after both light and forskolin stimuli, \textit{in vivo} as well as \textit{in vitro} (Fig. S1C–D).

Because cFos is an immediate-early gene (IEG) and can be induced by light in the SCN\textsuperscript{23,24}, we wanted to test whether cFos induction may also be affected by the lack of Per2. In the SCN, we observed an induction of c-Fos after an LP in wt animals and surprisingly an even stronger induction in Per2 KO mice (Figs. 1E, S1E). Similarly, forskolin treatment caused stronger cFos induction after forskolin in Per2 KO fibroblasts than in wt cells (Figs. 1E, S1F). In order to corroborate our results, we tested the transcriptional activation of other CREB/CBP targets such as Icer, Nor1 and Nur77. We observed that Icer and Nor1 transcriptional activation after forskolin treatment was comparable to the activation of cFos in wt and Per2 KO cells (Fig. S1G, H). In contrast, transcriptional activation of Nur77 was comparable to the activation of Per1 (Fig. S1I).

These results suggest that PER2 plays an important role in regulating light/forskolin responsive genes, which is different from its role as a circadian regulator. Furthermore, Per2 can modulate light/forskolin responses in a positive (e.g., Per1, Nur77) or negative (e.g., cFos, Icer, Nor1) fashion.

**PER2 physically interacts with CREB and modulates its binding to a CRE element in the Per1 regulatory region.** Light and forskolin activate signaling pathways that lead to CREB phosphorylation at serine 133 (Ser-133) and subsequently evoke target gene expression such as \textit{cFos} and \textit{Per}1\textsuperscript{10–12}. The \textit{Per1} gene contains a CREB response element (CRE)\textsuperscript{14} (Fig. S2A) and is regulated by CREB\textsuperscript{32,34}. Since the induction of \textit{Per1} gene expression by light or forskolin is affected by the presence or absence of the Per2 gene (Fig. 1C, D), we set out to test whether the PER2 protein affects CREB binding to the \textit{Per1} promoter. For this purpose, we studied the CRE-element present in the \textit{Per1} regulatory region, including intron 1 (Fig. S2A) that was shown to be functional\textsuperscript{14}. We applied forskolin to wt and Per2 KO fibroblast cell lines and collected samples at various time points. Then, we performed chromatin immunoprecipitation (ChIP) using an antibody against CREB, followed by RT-qPCR to measure CREB occupancy at the CRE-element of \textit{Per1} and an unrelated element in...
Figure 1. Induction of Per1, Per2, and cFos gene expression after light or forskolin stimulation. The diagram top left indicates the light treatment protocol of mice (A, C, E) and the top right diagram shows the forskolin treatment of cells (B, D, F). (A) Fold change of Per1 (black bars) and Per2 (grey bars) pre-mRNA expression in the SCN after a 15 min light pulse (LP) applied at ZT14. Values are the mean ± SEM. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. (B) Fold change of Per1 (black bars) and Per2 (grey bars) pre-mRNA expression in NIH 3T3 cells after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. (C) Fold change of Per1 (top panel) and Per2 (bottom panel) pre-mRNA expression in the SCN of wild type (wt, black bars) and Per2 knock-out (KO, grey bars) mice after a 15 min light pulse (LP) applied at ZT14. Values are the mean ± SEM. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. (D) Fold change of Per1 (top panel) and Per2 (bottom panel) pre-mRNA expression in immortalized wt or Per2 KO fibroblast cells after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. (E) Fold change of cFos expression in the SCN of wt (black bars) and Per2 KO (grey bars) mice after a 15 min light pulse (LP) applied at ZT14. Values are the mean ± SEM. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. (F) Fold change of cFos expression in immortalized wt or Per2 KO fibroblast cells after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05.
the Per1 promoter as control (Fig. S2A). We found that forskolin modulated CREB occupancy at the intronic CRE-element of the Per1 gene in a time-dependent fashion with a peak at 25 min after the stimulus. On the other hand, this time-dependent change was absent in the control region (Fig. 2A, black columns). This transient CREB occupancy increase mirrored the Per1 pre-mRNA profile observed in Fig. 1B. Hence, it appeared that CREB was not recruited in a constant fashion to the Per1 promoter but bound the chromatin upon specific stimulation. Surprisingly, the lack of Per2 led to an increase of CREB binding to the Per1 promoter already after 10 min (Fig. 2A, grey columns), but did not further increase after 25 min. This indicated that the two profiles of the occupancy of the regulatory region were different between the two genotypes (two-way ANOVA, p < 0.01).

Taken together, these results indicate that PER2 modulates CREB recruitment onto the chromatin of Per1 in a stimulus-dependent manner.

Next, we asked whether PER2 could affect CREB recruitment to the Per1 gene regulatory region via modulation of CREB phosphorylation at serine 133 (Ser-133), which is associated with light or forskolin-induced phase shifts. Therefore, we performed a ChIP assay using the pSer133-CREB specific antibody as a bait. We observed that this phosphorylated form of CREB was recruited to chromatin in a similar manner as we observed when using the available CREB antibody for both wt (Fig. 2B, black columns) and Per2 KO (Fig. 2B, grey columns) derived chromatin. This result suggests that lack of Per2 did not alter the CREB binding profile to chromatin via changes in phosphorylation of Ser-133.

Above, we described that the lack of the PER2 protein affected the recruitment dynamics of CREB to the CRE-element of the Per1 gene regulatory region. This effect could be of direct or indirect nature. In order to test this, we performed a ChIP assay on the same genomic region using a PER2 specific antibody. Our experiments revealed that the PER2 profile of recruitment to the chromatin (Fig. 2C) was similar to the one for CREB (Fig. 2A) with a maximum at 25 min after forskolin. Interestingly, the circadian clock component BMAL1 was not recruited to the Per1 CRE element in response to forskolin (Fig. 2D left panel). This observation indicated that the recruitment of PER2 was not due to a general effect on the circadian clock.

Since CREB and PER2 show the same chromatin binding profile upon forskolin stimulation, we wondered whether they might physically interact. We stimulated fibroblast cells with forskolin and collected samples at various time points. Subsequently, we performed a Western blot (WB) and adjusted the protein loading to CREB accumulation. A proper forskolin induction was confirmed by monitoring CREB phosphorylation at serine 133, which was detected with a peak about 25 min after the forskolin stimulus (Fig. 2D left panel). An immunoprecipitation assay (IP) followed by WB revealed that PER2 binds CREB independently from the forskolin induction and that the interaction is specific (Fig. 2D, right panel, Fig. S2C). Next, we investigated whether we could observe this interaction in vivo in SCN tissue of mice. We immunoprecipitated CREB from SCN protein extracts collected in the dark or 15 min after LP, followed by immunodetection. We observed that PER2 specifically binds CREB (Fig. S2D) and that this was independent of the LP and of CREB phosphorylation (Fig. 2E), as evidenced by the PER2 signal in the absence of the pSer133 signal in the dark. Our in vitro and in vivo data suggest that PER2 physically interacts with CREB and it does so independently from the stimulus (forskolin or light). Therefore, we hypothesize that this interaction influences CREB occupancy in the Per1 regulatory region at the CRE element.

**PER2 modulates CREB: CRTC1 interaction upon light or forskolin stimulation.** CREB occupancy of CRE elements in various promoters has been described to involve a family of factors called CRTC.

It has been demonstrated that in particular CRTC1 localized to the nucleus and dimerized with CREB after exposure to cAMP or calcium. Interestingly, CRTC1 is the main CREB co-activator regulating light responses in the SCN. Upon an LP, CRTC1 was observed to translocate into the nucleus to support CREB activity. These observations prompted us to investigate whether the light or forskolin mediated effect on the induction of the Per1 gene involved CREB: CRTC1 dimerization and whether PER2 modulated this complex.

We applied forskolin to wt and Per2 KO fibroblast cell lines and collected samples at various time points. Then, we performed chromatin immunoprecipitation (ChIP) using an antibody against CRTC1, followed by RT-qPCR to measure CRTC1 occupancy at the CRE-element of Per1 and an unrelated element in the Per1 promoter as control (Fig. 3A). We observed that in wt cells, CRTC1 recruitment to the Per1 intronic chromatin after forskolin treatment displayed a profile comparable to CREB (Fig. 2A, black columns) with a peak at 25 min after forskolin treatment (Fig. 3A, black columns). Conversely, in Per2 KO cells, the recruitment of CRTC1 to the Per1 promoter after forskolin application was reduced (Fig. 3A, grey column) and comparable to the CREB profile (Fig. 2A, grey columns). Thus, PER2 appeared to influence CREB and CRTC1 recruitment to the Per1 CRE element in a similar way.

Next, we investigated whether CRTC1 abundance was affected by the lack of PER2. The WB with samples obtained from wt and Per2 KO cell lines collected before and 25 min after forskolin stimulation did not show any difference in CRTC1 levels between the two genotypes. The induction mediated by forskolin increased CRTC1 amounts in both genotypes in a similar manner (Fig. 3B). Interestingly, the interaction between CREB and CRTC1 appeared to be increased by the forskolin stimulus (about 20% of the total input), as shown by immunoprecipitation (IP) using an anti-CREB antibody as bait (Fig. S3A). In the absence of a good knock-out cell line as negative control, we validated the result repeating the IP using either CREB or rabbit IgG (negative control) or TORC-1(CRTC-1, positive reverse CoIP) antibodies as bait. We observed that the interaction between CREB and CRTC-1 is specific, because there was no signal in the IP when the rabbit IgG antibodies were used (Fig. S3B).

We wondered whether PER2 might affect CREB: CRTC1 interaction. IP with an anti-CRTC1 antibody as bait revealed that PER2 interacted with CRTC1 independently from the forskolin stimulus (Fig. 3C). Hence, PER2 binds to both CREB (Fig. 2D, E) and CRTC1 (Fig. 3C), although CREB and CRTC1 interact only after a stimulus45 (Fig. S3A). To test whether PER2 could modulate the CREB: CRTC1 interaction, we immunoprecipitated CREB in both wt and Per2 KO cell lines, without and 25 min after forskolin treatment. Surprisingly, CREB and CRTC1
Figure 2. Binding of CREB and PER2 to the CRE-element of the Per1 promoter and interaction between CREB and PER2. (A) Top panel: Chromatin immunoprecipitation (ChIP) of CREB on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Bottom panel: Control ChIP on an unrelated promoter region of Per1. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. Two-way ANOVA indicates a significantly different time profile between the two genotypes, **p < 0.01. (B) Top panel: ChIP of pSer-133 CREB on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Bottom panel: Control ChIP on an unrelated promoter region of Per1. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05. Two-way ANOVA indicates a significantly different time profile between the two genotypes, **p < 0.01. (C) Top panel: ChIP of PER2 on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Bottom panel: Control ChIP on an unrelated promoter region of Per1. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05. Two-way ANOVA indicates a significantly different time profile between the two genotypes, **p < 0.01. (D) Western blot (left panel) was performed on 10% of the total input used for the immunoprecipitation using a CREB antibody as bait (right panel). PER2 co-precipitates with CREB in NIH 3T3 cell extracts, the pSer-133 antibody was used to confirm the forskolin induction. kD = kilo Dalton. (E) IP with an antibody recognizing CREB in SCN extracts, which co-precipitated with PER2 before (D) and after a 15 min light pulse (LP) at ZT14. The pSer-133 antibody was used to confirm light induction. Input control with lamin B (bottom panel), kD = kilo Dalton. *Unspecific band recognized by the αPER2 antibody in tissue extracts.
Figure 3. Binding of CRTC1 to the Per1 promoter and interaction with PER2 and CREB. (A) Left panel: Chromatin immunoprecipitation (ChIP) of CRTC1 on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Right panel: Control ChIP on an unrelated promoter region of Per1. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. Two-way ANOVA indicates a significantly different time profile between the two genotypes, **p < 0.01. (B) Western blot of wt and Per2-/- (Per2 KO) cells before and 25 min after forskolin treatment. CRTC1 is induced by forskolin independently of Per2. CREB is phosphorylated (pSer133) in both genotypes after the stimulus. (C) Immunoprecipitation (IP) of CRTC1 pulls down PER2 independently of forskolin treatment. (D) IP of CREB pulls down CRTC1 after forskolin treatment in wt cells, but in Per2-/- cells, the interaction between CREB and CRTC1 is forskolin independent. (E) IP of CRTC1 in SCN extracts before and after a 15 min light pulse (LP) at ZT14. Co-IP of PER2 is independent of LP. (F) Western blot of wt and Per2-/- SCN tissue performed as in B on samples collected before and after LP, * unspecific band recognized by the αPER2 antibody in tissue extracts. CREB is phosphorylated in both genotypes after the light pulse. (G) IP of CREB pulls down CRTC1 in wt SCN extracts after LP, but in Per2-/- SCN the CREB: CRTC1 interaction is light-independent. kD = kilo Dalton.
interacted after this treatment only in wt cells. In contrast, in the Per2 KO cells (Per2−/−), the interaction was stimulus-independent (Fig. 3D), as evidenced by the co-IP of both components before forskolin treatment.

Next, we investigated whether our observations in cells were also manifested in the SCN of mice. Similar to what we observed in the fibroblast cell line, CREB interacted with CRT1 only after the light stimulus in SCN samples (Fig. S3C). Immunoprecipitation experiments showed that PER2 interacted with CRT1 in a specific manner (Fig. S3D). This interaction was independent of an LP, as revealed when sample loading between the two conditions was adjusted to equal amounts of CRT1 (Fig. 3E). Furthermore, light-induced CRT1 accumulation was not affected by the lack of PER2 (Fig. 3F), and CREB was still phosphorylated at Ser-133 after the LP, as revealed by WB (Fig. 3F). Although CREB phosphorylation and CRT1 accumulation were unaffected by PER2, the CREB-CRT1 interaction was modulated by the clock factor (Fig. 3G) in a comparable manner as observed in cells (Fig. 3D) when a stimulus (light or forskolin) was applied. Additionally, CREB and CRT1 interacted before the LP in Per2 KO mice (Fig. 3G) which was also observed in cells before the forskolin stimulus (Fig. 3D).

Altogether these results define PER2 as a modulator of CREB: CRT1 interaction. This modulation depends on specific triggers, such as forskolin for cell cultures or light for the SCN cells in animals.

**PER2 mediates CREB: CBP interaction upon light or forskolin stimulation.** CREB binding protein (CBP) is a histone acetyltransferase that can heterodimerize with CREB when CREB is phosphorylated at Ser-133. This CREB: CBP complex then promotes gene expression. To determine the CBP recruitment profile to the CRE-element of the Per1 regulatory region we performed a ChIP assay using an antibody against CBP, followed by RT-qPCR (Fig. 4A). In wt animals, recruitment of CBP to the CRE-element was very similar (Fig. 4A, left panel, black columns) to the profiles observed for CREB (Fig. 2A), pSer-133 CREB (Fig. 2B), PER2 (Fig. 2C), and CRT1 (Fig. 3A). Interestingly, CBP appeared to bind to the control region in the Per1 promoter as well (Fig. 4A, right panel, black columns) possibly due to interaction with BMAL1, since this region contained a BMAL1 binding site (Fig. S2B). In contrast, in Per2 KO cells, CBP did not bind to the CRE-element of Per1 and to a reduced extent to the control element after forskolin treatment compared to background signal at time point 0 (Fig. 4A, grey columns). These results suggested that PER2 affected CBP binding to both Per1 promoter regions.

Since we were interested in the CREB mediated regulation of the Per1 gene, we wondered how PER2 could affect CBP recruitment to the CRE element. We investigated whether PER2 could favor accumulation of CBP in the nucleus. We tested the cells for CBP expression before and after forskolin treatment (Fig. 4B). We saw that CBP accumulation in the nuclei was increased after forskolin treatment, but no distinct difference between the two genotypes was observed (Fig. 4B). We performed immunofluorescence on wt and Per2 KO fibroblasts to evaluate the specificity of our PER2 antibody (Fig. S4A). Next, we tested whether forskolin treatment could induce Ser133 phosphorylation of CREB in our assay using an anti-pSer133 CREB antibody. The phosphorylation of CREB was induced as expected in both wt and Per2 KO cells 25 min after but not before forskolin treatment (Fig. S4B). Since CBP can still accumulate in the nuclei after forskolin induction in the absence of Per2, we investigated whether PER2 could affect the interaction between CBP and CREB. This interaction is essential for the CBP acetyltransferase activity on chromatin. Therefore, we immunoprecipitated CREB at 0 and 25 min after forskolin treatment of cells and performed IP using an antibody against CREB followed by WB (Fig. 4C). We observed that CBP was co-precipitated with CREB 25 min after forskolin treatment.

In contrast, this co-precipitation was not observed in Per2 KO cells (Fig. 4C). Subsequently, we wanted to see whether a Per2-dependent CREB: CBP interaction could also be observed in the SCN of mice after the LP stimulus. Immunohistochemistry on SCN tissue showed that the amount of CBP was increased after LP in both wt and Per2 KO tissue, indicating that lack of Per2 did not affect the increase of CBP levels in response to LP (Fig. 4D, control Fig. S4C). Next, we immunoprecipitated CREB from SCN extracts collected in the dark and after LP at ZT14 of both genotypes (Fig. 4E). Similar to the observation in cells (Fig. 4C), CBP was co-precipitated with CREB in wt extracts but not in extracts of Per2 KO mice. Taken together, our findings indicate that PER2 is necessary for the formation of the CREB: CBP complex after a stimulus such as forskolin or a light pulse.

**FRET analysis indicates that PER2 supports the interaction of CREB and CBP domains.** The results presented above lend support to the notion that PER2 serves as a scaffold for CREB: CBP interaction. To further challenge this idea, we performed Förster resonance energy transfer (FRET) experiments, a widely used method to investigate molecular interactions between proteins such as CREB: CBP in living cells. We used a sensor called ICAP (an indicator of CREB activation due to phosphorylation). The sensor is composed of three different elements: 1) the KID domain of CREB containing the Ser-133, which is phosphorylated upon forskolin induction, 2) the KIX domain of CBP, which is responsible for the dimerization with phospho-CREB and 3) a short linker that separates the KID from the KIX domain. KID is flanked by a cyan fluorescent protein (CFP), while KIX is flanked by a yellow fluorescent protein (YFP). When KID is not phosphorylated, the ICAP conformation allows CFP to transfer energy to YFP, producing FRET resulting in yellow light emission. After a stimulus (forskolin), the serine in KID is phosphorylated and binds to KIX. The dimerization separates CFP from YFP, leading to decreased FRET resulting in blue light emission (Fig. 5A upper panel).

We hypothesized that depletion of the endogenous Per2 could affect the KID: KIX dimerization (Fig. 5A bottom panel). To test this hypothesis, we co-transfected ICAP and either scrambled (scr), or Per2 directed shRNA (shPer2) into NIH3T3 cells. Subsequently, we acquired the FRET signal. 3 min after forskolin treatment, which is the standard latency time for FRET signal detection, we noted that only about 10% of shPer2 transfected cells (Per2 knock-down (KD)) were responsive to the stimulus compared to about 90% of scr transfected cells (Fig. 5B). As evidenced by single-cell traces (Fig. S5A), most shPer2 transfected cells were not responsive, but some with a substantial delay, compared to the scr controls. These results suggested that the knock-down of Per2
Figure 4. Modulation of the interaction between CREB and CBP by PER2. (A) Left panel: Chromatin immunoprecipitation (ChIP) of CBP on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Right panel: Control ChIP on a promoter region of Per1 without a CRE-element. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. Two-way ANOVA indicates a significantly different time profile between the two genotypes, left panel: ****p < 0.0001, right panel: **p < 0.01. (B) Immunohistochemistry (IHC) of wt and Per2−/− cells before and after forskolin treatment. CBP (red signal) is induced after forskolin treatment in both genotypes. Scale bar: 17 µm. (C) IP of CREB pulls down CBP in wt but not Per2−/− cells after forskolin treatment, kD = kilo Dalton. (D) IHC of wt and Per2−/− SCN before and after a light pulse (LP) at ZT14. CBP (red signal) is induced after LP in both genotypes. Scale bar: 7 µm. (E) IP of CREB pulls down CBP in wt SCN extracts after LP, but not in Per2−/− SCN extracts. kD = kilo Dalton.
affected the KID: KIX interaction. Quantification of the FRET signal acquired over 30 min from 50 cells showed a difference in the profiles of scr and shPer2 transfected cells (Figs. 5C, S5B). 50% of the scr control cells responded to the forskolin treatment within about 3 min, while shPer2 cells needed over 10 min (Fig. 5D). Additionally,
the relative KID: KIX dimerization was significantly higher in scr control cells compared to shPer2 cells up to around 20 min after the stimulus (Fig. 5E). Altogether these results reinforce our notion that PER2 may support the CREB: CBP dimerization via their respective KID: KIX domains.

**PER2 modulates CBP-mediated chromatin acetylation at lysine 27 of histone H3 after forskolin or light stimulation.** CBP binds to chromatin by dimerizing with p53R3-CREB to exert its histone acetyltransferase (HAT) activity. Lysine 27 (K27) of histone H3 is the main target of CBP HAT activity to acetylate (Ac) K27 of histone H3. We wondered whether this epigenetic modification (AcH3K27) could be observed on chromatin containing the Per1 CRE-element in cells after forskolin treatment. A ChIP assay, using an anti-AcH3K27 antibody for IP, followed by RT-qPCR revealed that chromatin remodeling at that specific amino acid of histone H3 is forskolin-dependent (Fig. 6A, black columns). We observed highest acetylation of histone H3 in the Per1 CRE-element containing chromatin between 25 and 40 min after stimulus application (Fig. 6A, black columns). Interestingly, acetylation was dramatically reduced in the Per2 KO cells (Fig. 6A, grey columns). This observation suggested that CBP needed PER2 as a co-factor for assembling a functional complex allowing acetylation of histone H3 on the Per1 CRE-element. This observation is the first evidence indicating that acetylation at K27H3 is a result of PER2-dependent forskolin stimulation.

Acetylation of histone H3 leads to the assembly of the RNA polymerase II (RNA Pol II) complex to promote gene transcription. Therefore, we tested whether the formation of such an RNA Pol II containing complex on the Per1 CRE-element was promoted by forskolin treatment of cells and whether PER2 may be involved in this process. We performed a ChIP assay using an antibody against RNA Pol II as bait. We noted that RNA Pol II recruitment to chromatin of wt cells (Fig. 6B, black columns) paralleled the acetylation dynamics of histone H3 on K27 (Fig. 6A, black columns). In contrast, the recruitment of RNA Pol II was lower in cells lacking Per2 (Fig. 6B, grey columns), mirroring the absence of acetylation of H3K27 (Fig. 6A, grey columns). These results provide additional evidence for the importance of PER2 as a facilitating component for assembling the stimulus-dependent transcriptional complex on the intronic CRE-element of the Per1 gene.

PER2 can promote the forskolin-induced gene expression and it can repress that of cFos (Fig. 1). Therefore, we wondered how the chromatin of the CRE element in the cFos promoter was affected after forskolin treatment of cells. Therefore, we performed a ChIP assay using an anti-AcH3K27 antibody for IP, followed by RT-qPCR. Our results showed that the chromatin acetylation profile mirrored the one obtained on the Per1 CRE region (Fig. 6A, C). While we observed in wt samples the highest acetylation of histone H3 in the CRE-element containing chromatin between 25 and 40 min after stimulus application (Fig. 6C, black columns), the same acetylation was profoundly dampened in the Per2 KO samples (Fig. 6C, grey columns). As discussed before histone acetylation leads to the recruitment of RNA Pol II onto the chromatin. Therefore, we tested by ChIP whether forskolin treatment of cells affected the formation of the transcriptional complex including RNA Pol II on the promoter of cFos and whether PER2 could affect this formation. We found that RNA Pol II could be recruited onto the cFos chromatin of wt cells with a profile mirroring the gene expression (Fig. 6D, black columns). Surprisingly, the recruitment of RNA Pol II onto the cFos chromatin in Per2 KO samples was higher compared to wt (Fig. 6D, grey columns). This is consistent with the observation that the cFos pre-mRNA was more abundant in Per2 KO cells compared to wt (Fig. 1F). Together, our results indicate that the forskolin-mediated cFos gene expression is independent of the chromatin acetylation at lysine 27, although no difference was observed between wt and Per2 KO cells in CREB recruitment to the cFos chromatin (Fig. S6A).

A previous study that described light-dependent histone phosphorylation in the SCN of mice prompted us to ask whether acetylation of histone H3 at K27 in the SCN was light-dependent. We applied an LP at ZT14 to mice as described before and subsequently collected SCN tissue. We performed an immunofluorescence assay using an anti-AcH3K27 antibody. At ZT14 H3 in the SCN of wt mice displayed a basal level of chromatin acetylation, which was strongly increased after application of an LP (Fig. 6E upper row). In contrast, the immunofluorescence signal was partially dampened in the SCN of Per2 KO mice (Fig. 6E lower row). To confirm this observation, we performed WB on pooled SCN tissue collected in the dark or 15 min after LP from both genotypes (Fig. S6B). Quantification confirmed that AcH3K27 was increased right after the light stimulus in wt SCN tissue, whereas the induction was blunted in SCN extracts obtained from Per2 KO mice (Fig. 6F). Together, these data suggested that the light signaling cascade did regulate chromatin acetylation and that PER2 modulated this process in the SCN.

**Discussion**

In the present study, we report a role of the clock protein PER2 as a modulator of the light-dependent CREB signaling in the early night. In particular, we observed that PER2 acted as a positive factor in stimulus dependent Per1 and Nar1 gene expression, while it functioned as a negative regulator in cFos, Icer and Nor1 gene induction (Fig. 1C–I). This functional dichotomy indicated that the transcriptional complex involving PER2 is not identical on the Per1 and cFos promoters. That PER2 can act as a positive or negative regulator is consistent with previous observations that this protein has a modulatory influence on transcriptional regulation in both directions in mammals. Because deletion of cFos in mice attenuated behavioral responses to light only marginally, we focused on the role of PER2 in the stimulus-dependent activation of the Per1 promoter. Since light activates several signaling pathways in mammals culminating in the induction of the Per1 gene, we investigated the role of PER2 as co-factor in CREB mediated transcription, a common downstream target of protein kinase cascades.

ChIP analysis on the intronic CRE-element of the Per1 gene (Fig. S2A) revealed that CREB and its known co-factor CRTC1 are recruited in a time-dependent manner after a forskolin stimulus with a peak around 25 min. However, although CREB and CRTC1 bound to the same region in the Per2 KO cell lines, their binding was less pronounced and occurred earlier, with a peak around 10 min (Figs. 2A, 3A). On the other hand, the histone acetyltransferase CBP and the RNA Pol II were recruited to the same element only in wt, but not in Per2 KO cells.
Figure 6. Modulation of CBP-mediated chromatin acetylation of histone H3K27 by PER2. (A) Chromatin immunoprecipitation (ChIP) of acetylated histone H3 (AcH3K27) on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, **p < 0.01. Two-way ANOVA indicates a significantly different time profile between the two genotypes, **p < 0.01. (B) The ChIP of RNA polymerase II (RNA Pol II) on the CRE-element of the Per1 promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, **p < 0.01. Two-way ANOVA indicates a significantly different time profile between the two genotypes, ***p < 0.001. (C) ChIP with an antibody against AcH3K27 on the Cre-element of the cFos promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, ***p < 0.001. (D) ChIP of RNA Pol II on the CRE-element of the cFos promoter in wt (black bars) and Per2 KO (grey bars) fibroblast cell lines after forskolin treatment. Values are the mean ± SD. Student’s t-test with Welch’s correction, n = 3, *p < 0.05, ***p < 0.001. (E) Immunohistochemistry (IHC) on wt and Per2 KO SCN before and after a light pulse (LP) at ZT14. AcH3K27 (red signal) is stronger after LP in wt compared to Per2 KO SCN. Scale bar: 121 µm. (F) Quantification of 3 independent Western blot experiments of wt and Per2 KO SCN before and after an LP at ZT14. Acetylation of H3K27 is increased after an LP with a tendentially lower increase in Per2 KO. Two-way ANOVA, p = 0.059.
Our observations are in agreement with previous findings that describe CREB as an activator of \( \text{Per} \) transcription. Interestingly, \( \text{PER2} \) could be recruited to the CRE-element of the \( \text{Per1} \) promoter as well, with the same temporal profile as CREB (Fig. 2A, C). These results suggested that \( \text{PER2} \) could be part of the CREB-containing transcriptional complex. The hypothesis was further corroborated by the observation of a discrete reduction and temporal profile of CREB and CRTC1 binding to the \( \text{Per1} \) promoter in \( \text{Per2} \) KO cells. Immunoprecipitation experiments revealed that \( \text{PER2} \) co-precipitated with CREB (Fig. 2D, E) and with CRTC1 (Fig. 3C, E) in a stimulus-independent manner, suggesting that \( \text{PER2} \) could interact with both CREB and CRTC1 before the stimulus occurred (Fig. 7, left). Interestingly, CREB bound to CRTC1 independent of a stimulus when \( \text{PER2} \) was absent, however in the presence of \( \text{PER2} \) this interaction became stimulus-dependent (Fig. 3D, G). In contrast, interaction between CREB and CBP did not occur in absence of \( \text{PER2} \), but appeared in presence of it in a stimulus-dependent fashion (Fig. 4C, E). Hence, a stimulus led to rearrangement of the components CREB, CRTC1 and CBP with \( \text{PER2} \) most likely acting as a scaffold to facilitate this rearrangement (Fig. 7, right). Thus, we conclude that \( \text{PER2} \) appears to be the stimulus-dependent factor that mediates the stimulus-dependent build-up of the complex facilitating CRE element-dependent transcription of genes of the \( \text{Per1} \) type family (\( \text{Per1} \), \( \text{Nur77} \)). The limitation of our observation is, that we do not know whether this is a general mechanism or whether this mechanism is restricted to a subset of CRE elements that are flanked by specific uncharacterized regulatory sequences that are present in the \( \text{Per1} \) and \( \text{Nur77} \) genes. However, the conclusion that \( \text{PER2} \) appears to be the stimulus-dependent factor that facilitates gene activation in a subset of CREB dependent target genes seems reasonable.

Dynamic chromatin remodeling in the SCN has been suggested to occur in response to a physiological stimulus such as light. These findings are in line with our observation that stimulus-dependent activation of \( \text{Per1} \) transcription involved the acetylation of histone H3 at lysine 27 and that this acetylation depended on the presence of \( \text{PER2} \) (Fig. 6A). The acetylation profile on the \( \text{Per1} \) promoter was also mirrored by the recruitment of RNA Pol II and suggested a functional importance of this \( \text{Per2} \) dependent H3K27 modification (Fig. 6B). In contrast, recruitment of RNA Pol II on the \( \text{cFos} \) promoter was independent of \( \text{Per2} \) and the H3K27 modification.
European Mouse Mutant Archive (EMMA) strain ID EM: 10599, B6;129P2-Per2tm1Ual/Biat. using the KAPA probe fast universal master mix and the indicated primers on a Rotorgene 6000 machine. The transcribed using Superscript II with random hexamer primers (Thermo Fisher). Real-time PCR was performed ospin RNA II kit (Macherey & Nagel) and adjusted to 1 µg/µl with water. An amount of 1 µg was reverse-forskolin (50 mM stock in dimethyl sulfoxide) for the indicated time. Total RNA was extracted using the Nucleospin RNA II kit (Macherey & Nagel) and adjusted to 1 µg/µl with water. The amount of 1 µg was reverse-transcribed using Superscript II with random hexamer primers (Thermo Fisher). Real-time PCR was performed using the KAPA probe fast universal master mix and the indicated primers on a Rotorgene 6000 machine. The

Mice were housed with food and water ad libitum in transparent plastic cages (267 mm long × 207 mm wide × 140 mm high; Techniplast Makrolon type 2 126×4501) stainless-steel wire lid (Techniplast 126×45116), kept in light- and soundproof ventilated chambers. All mice were entrained to a 12:12 h light:dark (LD) cycle, and the time of day was expressed as Zeitgeber time (ZT; ZT0 lights on, ZT12 lights off). Two- to four-month-old males were used for the experiments. Housing and experimental procedures were performed following the guidelines of the Schweizer Tierschutzgesetz and the declaration of Helsinki. The state veterinarian of the Canton of Fribourg and the national approved ethics committee approved the protocol. The study was carried out in compliance with the ARRIVE guidelines. The floxed Per2 mice42 are available at the European Mouse Mutant Archive (EMMA) strain ID: 10599, B6;129P2-Per2tm1Ual/Biat.

Light pulse (LP, circa 500 lx) was given at ZT14, and mice were subsequently sacrificed within 15 min. As a control experiment, mice were sacrificed in the dark a ZT14. Brains were collected and SCN tissue isolated. For immunofluorescence experiments, mice were perfused with 4% PFA.

Mouse embryonic fibroblasts (MEFs) were obtained from mice without functional Per2 in all body cells42, or wild-type mice. Briefly, mice containing the ubiquitously expressed Cre recombinase transgene (European Mouse Mutant Archive (EMMA) EM:01149, B6.129-Cre-Deleter: B6.C(129)-Tg(CMV-cre)1Cgn/Cgn1bcm) crossed with a conditional Per2 allele45 were backcrossed over ten generations to obtain Per2 homozygous knock-out mice without Cre recombinase. The MEFs subsequently were serially diluted every three days over 30 passages to yield immortalized cells. All lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, stable glutamine, sodium pyruvate and 3.7 g/l NaHCO3 (PAN Biotech), supplemented with 10% fetal calf serum (FCS) and 100 U/mL penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. Forskolin stimulation (10–100 µM) was used to mimic in vitro the molecular pathway activated by light in mice. Samples were collected at specific time points mentioned in the text.

The following plasmids were used for the project:

- **ICAP-NLS** Vector carrying the two domains KID (CREB) and KIX (CBP) fused with fluorophores as described before, kindly provided by Prof. Dominique Glauser (University of Fribourg, CH)46.
- **Scramble Sh RNA vector** used as a control for the in vitro silencing of Per2 (Origene cat #TR30021).
- **Sh RNA Per2 Vector** used for silencing Per2 in cell lines. (Origene cat #TL501620).
- **Sequence**: 5′-ATGAGCAGCTGGCTCCAGCGGAAACGAGAA-3′.

NIH 3T3 cells were co-transfected with either ICAP-NLS/ Scramble shRNA or ICAP_NLPS/shRNA Per2 in 2 cm dishes at about 70% of their total confluence using linear polyethyleneimine (LINPEI25; Polysciences Europe). The amounts of expression vectors were adjusted to yield comparable levels of the expressed protein. Thirty hours after transfection, cells were induced with forskolin, and the appropriate experiment was performed.

Cells were grown to confluency on 6 cm Petri dishes and induced with 10 µM forskolin (50 mM stock in dimethyl sulfoxide) for the indicated time. Total RNA was extracted using the Nucleospin RNA II kit (Macherey & Nagel) and adjusted to 1 µg/µl with water. An amount of 1 µg was reverse-transcribed using Superscript II with random hexamer primers (Thermo Fisher). Real-time PCR was performed using the KAPA probe fast universal master mix and the indicated primers on a Rotorgene 6000 machine. The
**Table 1.** List of primers used in the paper (5′–3′; FAM: 6-fluorescin, BHQ1: black hole quencher 1).

| Name of the primer       | Sequence                                                                 | Use                                                                 |
|--------------------------|--------------------------------------------------------------------------|----------------------------------------------------------------------|
| Per1_ChIP_CREB_FW         | FW: CAG CCT CCC TGC CCC ACA TT<br>RV: GAG AGG GAG GTG AGC TCA AAG C<br>TAME: CCA GCC TGC TGC CCC GGC CT-BHQ1 | Amplification of a region within the first intron containing a CRE element |
| Per1_ChIP_control         | FW: AGG CAC CAG AAA CTC G TT<br>RV: GCC GTA GAT CTG ACA GGC TA<br>TAME: TGC CAC GAG AGT CTC CAA AGT ATG CCC AC-BHQ1 | Amplification of a region within the promoter |
| pPer1                    | FW: GCC ATG CTG CTG AC AGC<br>RV: GTG GAT GAT GTG CTC TGT GA<br>TAME: TGC CCC TCC TCC TCT ACC TTA GGC TGT TCC T-BHQ1 | Precursor of Per1 mRNA used for the RT-qPCR |
| pPer2                    | FW: CAC CCA CCC ACC CAC GTG AT<br>RV: GCC TGG GAA CTC GCA CTT CCT T<br>TAME: CCA GCC TGC TGC TGG AGG TAC CTC GAG AGC GC-BHQ1 | Precursor of Per2 mRNA used for the RT-qPCR |
| pcFos                    | FW: GTG AAG ACC GTG TCA GGA GGC A<br>RV: CCC AGC CCA CAA AGG TCC AGA A<br>TAME: GAC AGC GCA GAG CAT CCG AAG GGG C-BHQ1 | Precursor of cFos mRNA used for the RT-qPCR |
| pBmal1                   | FW: GAT CGG AGT GGG GGG G<br>RV: GCC AGC CAT GCC GAC ACT CA<br>TAME: CGG GGG GTC CTC GCA GGG AGC CA-BHQ1 | Precursor of Bmal1 mRNA used for the RT-qPCR |
| pCry1                    | FW: CTG TGT CGC CTC GTC CTC<br>RV: GCC AGC CCA CAAG AGG TCC AGA A<br>TAME: AGC AGG GGG ACT CGC TTA AGC AAG GCT-BHQ1 | Precursor of Cry1 mRNA used for the RT-qPCR |
| pCry2                    | FW: TGC TAC TGG CCT GTG GGC TT<br>RV: GGG AGG CTC CAG CAG GAA AGA<br>TAME: CCG GGC AGC TAG AGA CCC CAG TGG BCHQ1 | Precursor of Cry2 mRNA used for the RT-qPCR |
| pClock                   | FW: TGT AGT GGA GCC GGA AGC A<br>RV: GTG CCA AGC CAG GTT CTG A<br>TAME: CCA GCC GGC TGC TAG TGG TGC CCA G-BHQ1 | Precursor of Clock mRNA used for the RT-qPCR |
| Gsk3β                    | FW: CCA CCT CCT TGG CCG AGA GC<br>RV: CTG TGG TTA CCT TGG TGC CAT CT<br>TAME: TGC CAC GAG CAT CCG CAC TTA GCA TGG CTG TGC CAC CAG TGG TCC CCA G-BHQ1 | Probe for Gsk3β mRNA used as a normalizer for RT-qPCR |
| Atp5h                    | FW: TGG CCT GAA GAT TCC TGC TGC TGC TGA C<br>RV: ACT CAG CAC AGC TCT TCA CAT CCT<br>TAME: TGC CAT CCG TCC TCT CTC CCT GGC TGT TCC ACT A-TAG TGG TGC CAG TGG TCC CCA GGA GGC TGC CTC CTC CAA AGG TCC TGT GAG A-BHQ1 | Probe for Atp5h mRNA used as a normalizer for RT-qPCR |
| Sirt2                    | FW: CAG GCC AGA CGG ACC CCT TC<br>RV: AGG CCA CCT CCC TGT AAG CCC<br>TAME: TGA TGG GCC TGG GAG GTG GCA TGG A-BHQ1 | Probe for Sirt2 mRNA used as a normalizer for RT-qPCR |
| Nono                     | FW: TCT TTT TCT GCC AGG ATG GAG<br>RV: GTC TGC TGC CCA GTC TCT ACT<br>TAME: CCA GCC TGC TGG CTC CAA ATC CTC AGA GC-GC-BHQ1 | Probe for Nono mRNA used as a normalizer for RT-qPCR |

relative expression was calculated compared to the geometric mean of expression of the inert genes Nono, SirT2, Atp5h, and Gsk3β[15]. For a complete list of primers used in the paper, please see Table 1.

**RNA extraction from the SCN.** RNA from SCN samples was isolated using the Macherey-Nagel RNA Plus kit. Subsequently, 500 ng of purified RNA was used for producing cDNA by reverse transcription (Invitrogen SuperScript II). Real-time PCR was performed using the KAPA probe fast universal master mix and the indicated primers on a Rotorgene 6000 machine.

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation from cells was performed as described before[16]. Briefly, the cells were grown to confluence on 15 cm Petri dishes, induced with 10 µM forskolin (50 mM stock in dimethyl sulfoxide), and fixed at the indicated time with 1% formaldehyde/1 x phosphate-buffered saline buffer (PBS) for 10 min at 37 °C. Then the cells were washed twice with ice-cold 1 x PBS and scraped in 1 ml of 10 mM dithiothreitol/100 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 8.8. Cells were lysed in 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycol-bis(2-aminoethyl ether)-N, N’, N”-tetraacetic acid (EGTA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6, 0.2% Triton X-100 for 5 min on ice and the obtained nuclei washed with 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 10 mM HEPES, pH 7.6. The purified nuclei were sonicated in 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1% SDS using a BRANSON SLPe sonicator with a 4C15 tip for 6 cycles of 10 s each kept on
ice, and diluted 1:10 with 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1.1% Triton X-100. Equal amounts of chromatin were incubated with the indicated antibodies for 1 h at RT. (Table 2) and the immune complexes were captured with protein A agarose fast-flow beads (Sigma-Aldrich) for 1 h at RT. The beads were washed with 2 mM EDTA, 150 mM, 20 mM Tris, pH 7.5, 0.1% SDS, 1% Triton X-100, then 2 mM, 250 mM LiCl, 20 mM Tris, pH 7.5, 0.5% Na-deoxycholate, 0.5% NP40 substitute, and finally 2 mM, 250 mM LiCl, 20 mM Tris, pH 7.5, 1% SDS at 65°C overnight. The DNA fragments were eluted, and the crosslinks reversed in 2 mM EDTA, 150 mM NaCl, 20 mM Tris, pH 7.5, 1% SDS at 65°C overnight. The DNA fragments were purified using a MinElute PCR fragment purification kit (Qiagen). Real-time PCR reactions were performed using the KAPA probe fast universal master mix and the indicated primers on a Rotorgene 6’000 machine. The efficiency of the precipitations was calculated by comparing the amount of precipitated material to 1% of the starting material.

Protein extraction from cells. Total confluent cells plated in 10 cm dishes were washed two times with 1 × PBS (137 mM NaCl, 7.97 mM Na2HPO4 × 12 H2O, 2.68 mM KCl, 1.47 mM KH2PO4). Then PBS was added again, and plates were kept for 5 min at 37°C. Cells were detached and collected in tubes and frozen in liquid N2. They were subsequently resuspended in Ripa buffer (50 mM Tris-HCl pH7.4, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF) with freshly added protease or phosphatase inhibitors, and homogenized by using a pellet pestle. Homogenates were kept in ice for 15 min followed by sonication (10 s, 15% amplitude). Right after, the samples were centrifuged for 15 min at 16,100 g at 4°C. The supernatant was collected in new tubes and pellet discarded.

Protein extraction from SCN tissue. Isolated SCNs obtained from 5 different mice were pooled together according to the specific condition (either dark or 15 min after the light pulse). The pooled tissues were frozen in liquid N2, and resuspended in a brain-specific lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% SDS, 0.25% sodium deoxycholate, 1 mM EDTA). They were subsequently homogenized using a pellet pestle kept on ice for 30 min and vortexed five times for 30 s each time. The samples were sonicated (10 s, 15% amplitude) and subsequently centrifuged for 20 min at 16,000 g at 4°C. The supernatant was collected in new tubes and the pellet discarded.

Immunoprecipitation. Circa 400 µg of protein extract was diluted with the appropriate protein lysis buffer in a final volume of 250 µl and immunoprecipitated using the indicated antibody (ratio 1:50) at 4°C overnight on a rotary shaker. The day after, samples were captured with 50 µl of 50% (w/v) protein-A agarose beads (Roche) for 3 h at 4°C on a rotary shaker. Before use, beads were washed three times with the appropriate protein buffer and resuspended in the same buffer (50% w/v). The beads were collected by centrifugation and washed three times with NP-40 buffer (100 mM Tris-HCl pH7.5, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40). After the final wash, beads were resuspended in 2% SDS 10%, glycerol, 63 mM Trish-HCl pH 6.8, and proteins were eluted for 15 min at RT. Laemmli buffer was finally added, samples were boiled for 5 min at 95°C and stored at –20°C.

Western blot. The indicated amount of protein was loaded onto 10% SDS-PAGE gel and run at 100 Volt for two hours. Once the migration was completed, we performed a semidyed transfer (40 mA, 1 h 30 s) using Hybond™ ECL™ nitrocellulose membranes followed by red ponceau staining (0,1% of Ponceau S dye and 5%

| Product name | Catalog reference | Application | Dilution |
|--------------|-------------------|-------------|---------|
| Rabbit mab Per2 | PER21-A Alpha Diagn. (Lot # 869900A1.2-L) | ChIP | 1:5 |
| | | Western blot | 1:1000 |
| | | Immunofluorescence | 1:200 |
| Rabbit mab CREB | D76D11 cell signaling | ChIP | 1:500 |
| | | Western blot | 1:1000 |
| Rabbit mab CREB (phospho S133) | ab194687 Abcam | ChIP | 1:5 |
| | | Western blot | 1:1000 |
| | | Immunofluorescence | 1:100 |
| Rabbit mab TORC1 (CRTC1) | ab264144 Abcam | ChIP | 1:5 |
| | | Western blot | 1:1000 |
| Mouse mab Lamin B1 | sc-374015 SCBT | Western blot | 1:1000 |
| Rabbit mab CBP | PA5-27369 Thermofisher | ChIP | 1:5 |
| | | Western blot | 1:1000 |
| | | Immunofluorescence | 1:200 |
| Rabbit mab acetyl-histone H3 (Lys27) | D5E4 cell signaling | ChIP | 1:5 |
| | | Western blot | 1:1000 |
| | | Immunofluorescence | 1:100 |

Table 2. List of antibodies used in the paper and relative applications.
acetic acid) to validate the success of the transfer. The membrane was subsequently washed with TBS 1x/Tween 0.1% and blocked with TBS 1x/Milk 5%/Tween 0.1% for 1 h. After washing, the membrane was stained with the appropriate primary antibodies (Table 2) overnight. The day after, membranes were washed three times with TBS 1x/Tween 0.1% followed by secondary antibody immunoblotting for 1 h at room temperature. The densitometric signal was digitally acquired with the Azure Biosystem.

**Immunofluorescence.** Mice were cardiovascularly perfused with 0.9% NaCl followed by 4% PFA and brains were collected. They were subsequently cryoprotected by 30% sucrose solution, and sections of SCN were obtained (30 µm, coronal) using a cryostat. Selected sections were washed three times with 1 x TBS (0.1 M Tris/0.15 M NaCl) followed by 2 x SSC (0.3 M NaCl/0.03 M tri-Na-citrate pH 7). Antigen retrieval was performed with 50% formamide/2 x SSC by heating to 65 °C for 50 min followed by washing 2 x SSC and three times in 1 x TBS pH 7.5. Slices were blocked for 1.5 h in 10% fetal bovine serum (Gibco)/0.1% Triton X-100/1 x TBS at RT.

After the blocking, the primary antibodies (Table 2) were added to the sections and incubated overnight at 4 °C. The next day, sections were washed 1 x TBS and incubated with the appropriate fluorescent secondary antibodies diluted 1:500 in 1% FBS/0.1% Triton X-100/1 x TBS for 3 h at RT. (Lot: 132876, Alexa Fluor647-AffiniPure Donkey Anti-Mouse IgG (H + L) no. 715–605–150, Lot: 131725, Alexa Fluor647-AffiniPure Donkey Anti-Rabbit IgG (H + L) no. 711–602–152, Lot: 136317 and all from Jackson Immuno Research). Tissue sections were stained with DAPI (1:5000 in PBS; Roche) for 10 min. Finally, the tissue sections were rewashed twice in 1 x TBS and mounted on glass microscope slides. Fluorescent images were taken using a confocal microscope (Leica TCS SP5), and pictures were taken with a magnification of 10 ×, 20 ×, or 63 ×. Images were processed with the Leica Application Suite Advanced Fluorescence 2.7.3.9723, according to the study by64.

**Live FRET imaging.** Transfected NIH3T3 cells were starved for 4 h with 0.5% FBS DMEM. After starvation (0.5% FBS in DMEM), the medium was removed, and cells were washed twice with 1 x HBTS without CaCl2 and MgCl2 (25 mM HEPES, 119 mM NaCl, 6gr/L Glucose, 5 mM KCl) gently to avoid cell disturbance. One plate at the time was imaged. Therefore, the medium was changed shortly before the imaging started. NIH3T3 cells were imaged using an inverted epifluorescence microscope (Leica DMi6000B) with an HCX PL Fluotar 5x/0.15 CORR dry objective, a Leica DFC360FX CCD camera (1.4 M pixels, 20 fps), and EL6000 Light Source, and equipped with fast filter wheels for FRET imaging. Excitation filters for CFP and FRET: 427 nm (BP 427/10). Emission filters for CFP: 472 (BP 472/30) and FRET: 542 nm (BP 542/27). Dichroic mirror: RCU 440/520. One frame every 20 s was acquired for at least 90 cycles (0.05 Hz frequency), and the recording lasted at least 30 min. This acquisition rate was ideal to avoid cellular photobleaching and phototoxicity during long recordings. The baseline response in the presence of HBTS only was recorded for 2 min and 40 s. At minute 3:00, 100 µM Forskolin, 2 mM CaCl2, and 2 mM MgCl2 were added to the cells. To avoid buffer perturbations due to the addition of the drug stimulus, the latter was added between frames, so it had approximately 20 s to diffuse and activate a response in the cells.

**FRET imaging analysis.** The time-lapse recordings were analyzed using LAS X software (Leica), adapting a previously described method65. Briefly, two regions of interest (ROI) were selected for each cell, and 50 cells per plate were chosen randomly. A first ROI delimiting the background and a second ROI including the cell nucleus were chosen for each cell. In this case, ΔR is R–R0 and R0 is the average of R over the last 120 s prior stimulus.

**Statistical analysis.** Statistical analysis of all experiments was performed using GraphPad Prism8 software. Depending on the type of data, either an unpaired t-test with Welch’s correction or a paired t-test, when a sample was set to 1, were performed. Two-way ANOVA was performed on time and genotype-dependent experiments. Values considered significantly different are highlighted, *p < 0.05, **p < 0.01, or ***p < 0.001.

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Conceived and designed the experiments: A.B., J.A.R., U.A. Performed the experiments: A.B., G.S., J.A.R. Analyzed the data: A.B., G.S., J.A.R., U.A. Contributed reagents, materials, analysis tools: D.G., Z.Y., U.A. Wrote the paper: A.B., U.A. Edited the manuscript: G.S., J.A.R., D.G., Z.Y.

Competing interests
The authors declare no competing interests.

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