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Circadian expression of steroidogenic cytochromes P450 in the mouse adrenal gland – involvement of cAMP-responsive element modulator in epigenetic regulation of *Cyp17a1*

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The cytochrome P450 (CYP) genes *Cyp51*, *Cyp11a1*, *Cyp17a1*, *Cyb11b1*, *Cyp11b2* and *Cyp21a1* are involved in the adrenal production of corticosteroids, whose circulating levels are circadian. cAMP signaling plays an important role in adrenal steroidogenesis. By using cAMP responsive element modulator (*Crem*) knockout mice, we show that CREM isoforms contribute to circadian expression of steroidogenic CYPs in the mouse adrenal gland. Most striking was the CREM-dependent hypomethylation of the *Cyp17a1* promoter at zeitgeber time 12, which resulted in higher *Cyp17a1* mRNA and protein expression in the knockout adrenal glands. The data indicate that products of the *Crem* gene control the epigenetic repression of *Cyp17* in mouse adrenal glands.

Introduction

Cytochromes P450 (CYPs) constitute a large superfamily of enzymes that perform biotransformations of different endogenous and exogenous substrates [1]. Their tissue-specific and cell-specific expression is especially prominent in the adrenal gland [2]. Here, *Cyp51*, *Cyp11a1*, *Cyp17a1*, *Cyp11b1*, *Cyp11b2* and *Cyp21a1*

Abbreviations
ACTH, adrenocorticotrophic hormone; CRE, cAMP response element; CREB, cAMP response element-binding protein; CREM, cAMP-responsive element modulator; CYP, cytochrome P450; GC, glucocorticoid; ICER, inducible cAMP early repressor; KO, knockout; qPCR, quantitative PCR; WT, wild type; ZT, zeitgeber time.

participate in the production of glucocorticoids (GCs), mineralocorticoids, and adrenal androgens, three classes of steroid hormones that are important in the regulation of metabolism, water and salt balance, and reproduction [3]. Many CYPs, such as the *CYP17* family, also exhibit species-specific expression. For example, the human adrenal cortex expresses *CYP17A1* and can therefore produce cortisol, whereas rodents that do not express this gene in the adrenal glands produce corticosterone (Fig. 1) [4].

It is now widely accepted that the adrenal gland contains an intact circadian clock. Together with photic and nonphotic stimuli from the suprachiasmatic nucleus, this clock regulates the expression of about 5% of the adrenal genome. The circadian expression of genes involved in hormone synthesis results in the rhythmic expression of GCs observed in plasma of rodents and humans [5,6]. As GCs are known to regulate a wide array of different physiological processes, it has been suggested that they could play a central role as signaling molecules for the resetting of peripheral clocks [7,8]. If this is true, the deregulation of their synthesis could, in principle, lead to the disruption of peripheral circadian clocks and further contribute to the development of metabolic disorders, such as the metabolic syndrome [9,10].

The regulation of steroidogenic CYPs in the adrenal is under the tight control of adrenocorticotrophic hormone (ACTH). Binding of this hormone to the ACTH receptor elevates intracellular cAMP levels, leading to the activation of steroidogenic enzymes [11]. The upregulation of cAMP also leads to activation of protein kinase A and the subsequent activation of the cAMP response element (CRE)-binding protein (CREB)/cAMP-responsive element modulator (CREM)/ATF-1 family of transcription factors. Among them, CREM has been shown to be involved in circadian regulation of melatonin production [12] and cholesterol synthesis [13]. The role of CREM isoforms in circadian regulation of metabolic processes is well documented for melatonin synthesis in the pineal gland [14]. Using a *Crem* knockout mouse model, we show for the first time that *Crem* contributes to the circadian expression of steroidogenic *Cyp* genes in the mouse adrenal gland and is involved in epigenetic repression of *Cyp17*.

Results

Expression of steroidogenic *Cyp* genes in *Crem*$^{-/-}$ mice

The role of CREM in circadian expression of adrenal *Cyp* genes was evaluated by comparing the expression profiles of individual genes between wild-type (WT) and knockout (KO) mice (Fig. 2). A minimum of three

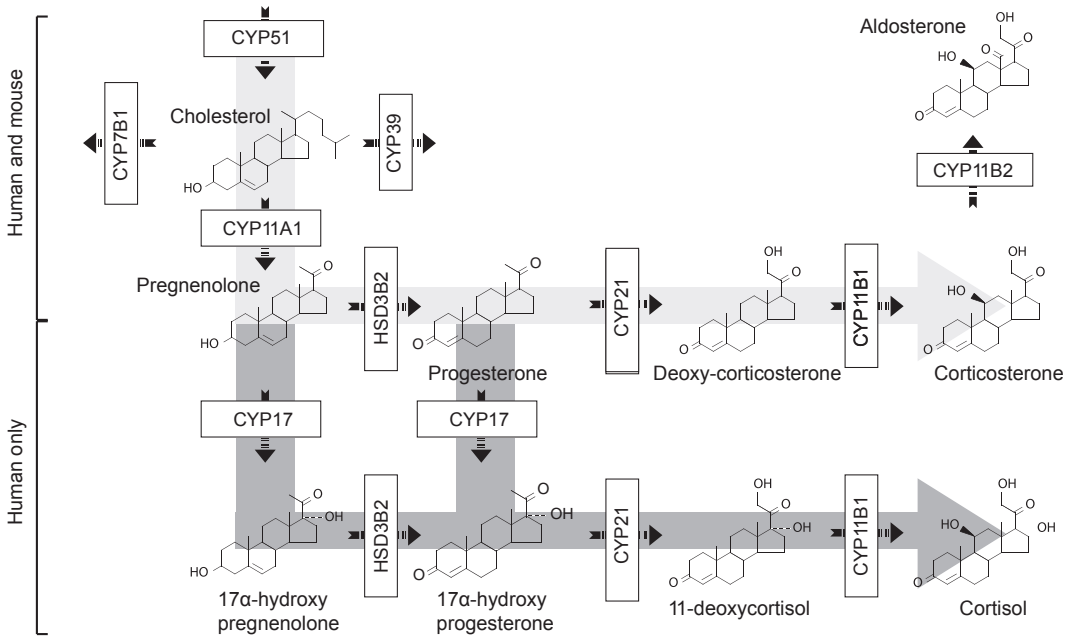

Fig. 1. Steroid synthesis in mouse and human adrenal glands. CYPs involved in adrenal steroidogenesis are shown, together with hydroxysteroid dehydrogenase HSD3B2. The light gray arrow indicates the main pathway in rodents, where *Cyp17a1* is methylated and CYP17 is not expressed. This leads in rodents to the production of corticosterone and not cortisol, which is the main GC in humans (dark gray arrow).

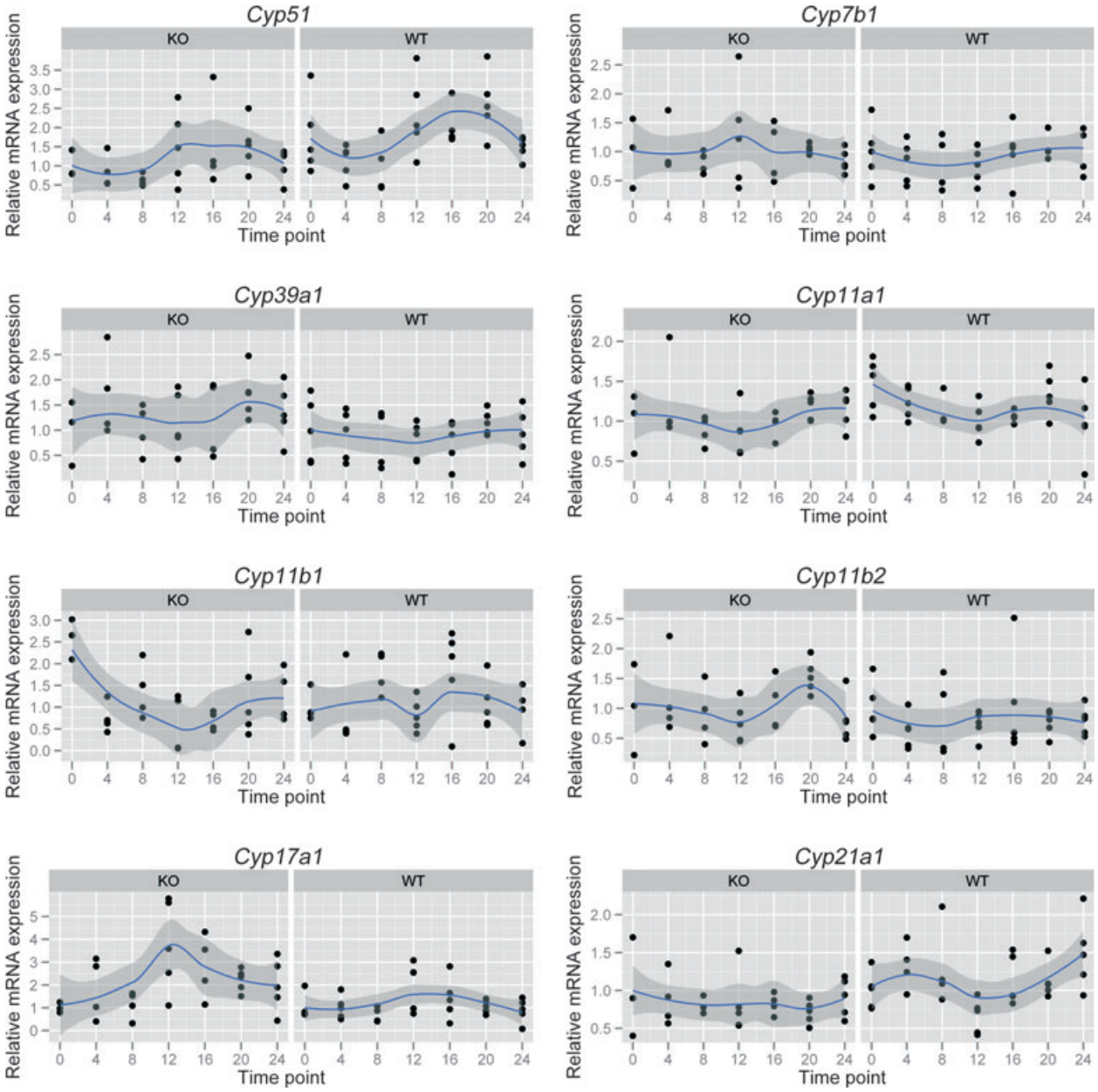

Fig. 2. Expression of *Cyp* genes measured by qPCR. The expression levels were measured at seven time points throughout a 24-h cycle. Dots represent biological replicates used at each time point. A weak circadian profile is present for *Cyp17a1*, *Cyp21a1*, and *Cyp51*. The largest difference in amplitude between KO and WT mice is seen for *Cyp17a1*. Time point represents the ZT.

biological replicates per time point were used. Although a clear and robust circadian pattern was seen for the core clock genes (data not shown), this pattern was less pronounced for adrenal *Cyp* genes (Fig. 2). The two-way analysis of variance showed a difference in expression between KO and WT animals for *Cyp17a1*, *Cyp51*, and *Cyp21a1*. The largest difference in amplitude was seen for *Cyp17a1* at zeitgeber time (ZT) 12.

Methylation pattern of *Cyp17* in *Crem* KO adrenals

DNA methylation is an important epigenetic factor that effectively prevents transcription of genes that are methylated [15]. According to the literature, *Cyp17* is methylated in rat adrenal glands and is therefore not expressed [4]. As we observed higher-level expression of *Cyp17* in mouse *Crem* KO adrenal glands, we

examined its methylation pattern. Two candidate CpG islands reside in the mouse *Cyp17* promoter. Their methylation status was evaluated in genomic DNA of *Crem* KO and WT mice by the use of methylation-sensitive *Bsr*FI endonuclease in at least three replicates (Fig. 3A). If DNA is methylated, DNA is not cleaved, and the PCR product is observed on the gel. Figure 3B shows representative gels. The average of the three replicates is shown in Fig. 3C. We found that DNA was less methylated in *Cyp17* CpG island 1 of the *Crem* KO mice, whereas no difference was observed in CpG island 2. Methylation in WT adrenals was not significantly different in either of the CpG islands. The up-regulated expression of *Cyp17a1* at ZT 12 was additionally confirmed by higher amounts of CYP17A1 as detected by western blotting (Fig. 3D).

CREM regulation of *Cyp17a1*

The *Cyp17* promoter contains CREs (Fig. 4A) and is transcriptionally regulated through cAMP signaling. Here, we found that CREM transcription factors were also able to influence *Cyp17* expression (Fig. 4B). The CREMτ activator, which is normally present only in the testis, includes the DNA-binding domains that appear in all *Crem* transcripts and bind to CREs. The data (Fig. 4B) suggest that the family of CREM proteins can activate *Cyp17* transcription. One of the CREM

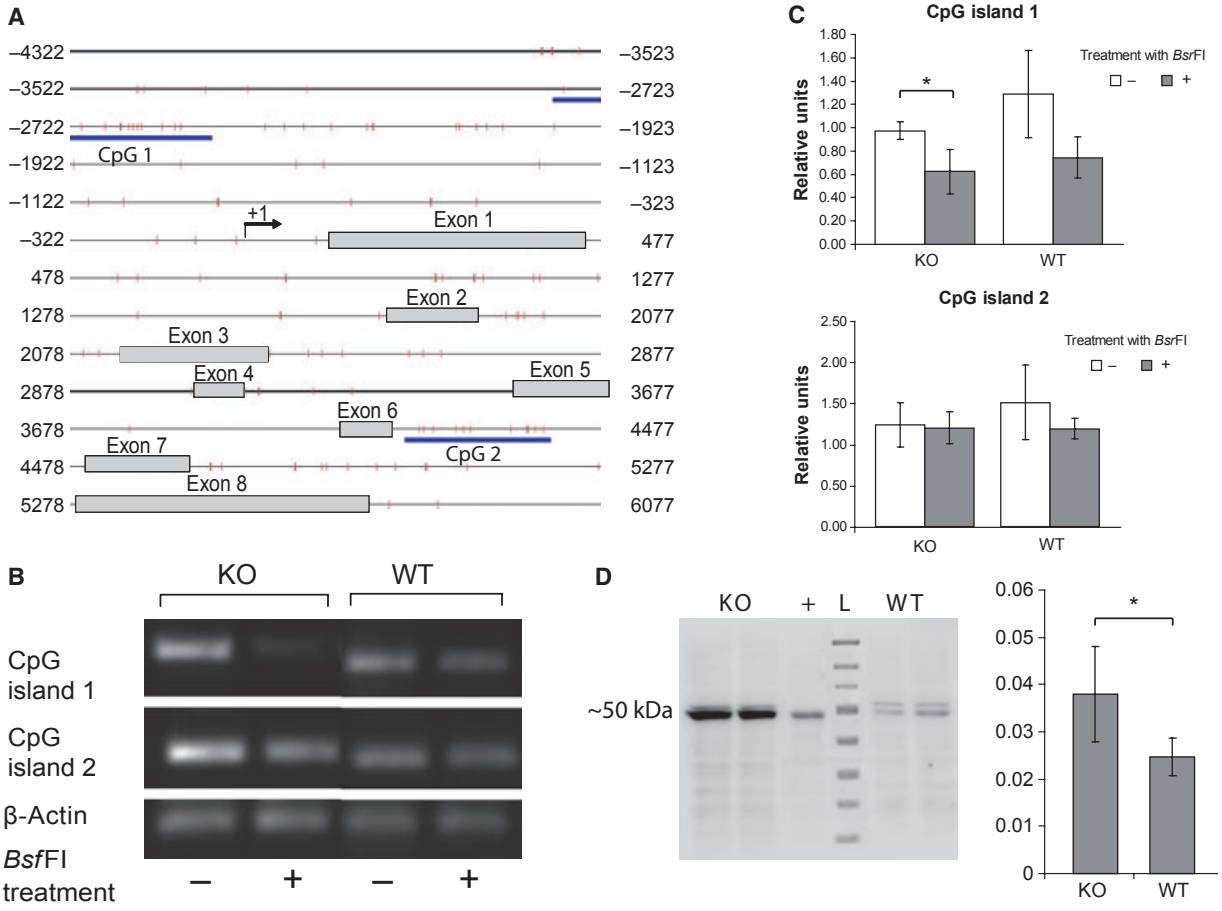

Fig. 3. Identification of methylation-targeted CpG islands in the mouse *Cyp17a1* gene and protein levels in KO and WT samples. (A) Scheme showing the CpG islands according to the transcription start site and position of exons. (B) Representative picture of methylation analysis by gel electrophoresis with methylation-sensitive digestion. Genomic DNA was isolated from *Crem* KO and WT adrenals, and PCR was performed on digested and undigested samples. (C) Quantification with β-actin normalization and *t*-tests revealed significant differences in the methylation pattern of *Crem* KO samples in CpG island 1. **P* = 0.027; the mean of five KO and three WT samples with standard deviation is presented in the plot. (D) CYP17A1 is present in higher amounts in KO adrenal samples than in WT samples. As a positive control (+), proteins isolated from WT testis were loaded. Quantification was according to β-actin. **P* = 0.023; the mean of six KO and six WT samples with standard deviation is presented in the plot.

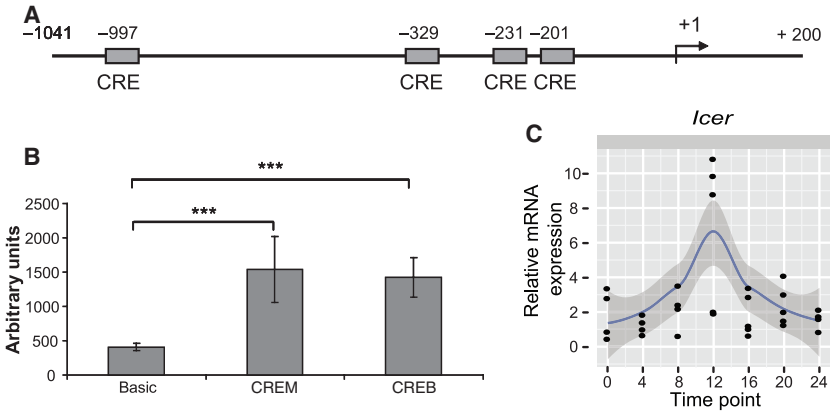

Fig. 4. *Cyp17* promoter and responsiveness to CREM. (A) Organization of CREs in the *Cyp17* promoter. (B) 'Basic' represents the basal activity of the *Cyp17* luciferase reporter in the Y-1 cell line; CREMτ overexpression activates the reporter, and CREB overexpression is used as a control ***$P < 0.001$. (C) qPCR measurements of *Icer*, one of the *Crem* repressive forms in WT samples. The peak of expression is at ZT 12.

isoforms is inducible cAMP early repressor (ICER), arising from one of the shortest *Crem* transcripts. It is considered to be the only cAMP-inducible transcription factor, and acts as a repressor. The maximum expression of *Icer* mRNA in WT animals was seen at ZT 12 (Fig. 4C). This was the same time when the difference in *Cyp17a1* expression was observed between *Crem* KO and WT animals (Fig. 2). However, overexpression of ICER in Y-1 cells did not diminish the activity of the *Cyp1a17* promoter-reporter (data not shown). Additional studies are required to clarify this issue and uncover the molecular mechanisms of the CREM-dependent regulation of mouse adrenal *Cyp17a1*.

Plasma corticosterone analysis

Because of elevated expression of *Cyp17a1* in *Crem* KO mice at ZT 12, a difference in plasma corticosteroid concentration was expected. The plasma corticosteroids were initially analyzed by ELISA, with antibodies that are most specific to corticosterone, and later also by LC with tandem MS. Figure 5A shows a clear circadian rhythm of corticosterone in both WT and KO animals, but also suggests a trend for KO animals to have higher corticosterone levels. The difference was statistically significant at ZT 0, ZT 8 and ZT 16 as measured by ELISA, where five samples per group were investigated. The LC-MS/MS analysis (three samples per group) shows a nonsignificant trend for KO animals to have higher corticosterone levels at ZT 0, ZT 4, ZT 8, and ZT 24 (Fig. 5B). The differences between ELISA and LC-MS/MS can be explained by antibody crossreactivity with other plasma sterols in ELISA assays. It is thus possible that, in addition to corticosterone, other steroids are overexpressed in *Crem* KO adrenal glands. To date, we are unable to confirm or to exclude the presence of cortisol in plasma of *Crem* KO mice.

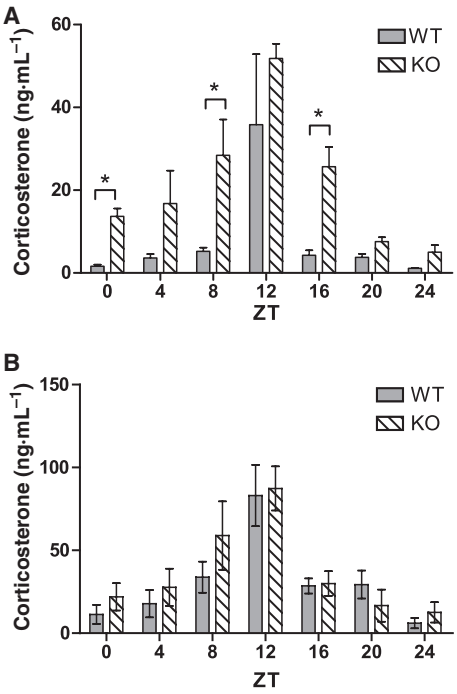

Fig. 5. Measurement of plasma corticosterone. Plasma corticosterone was measured in WT and KO mice by (A) ezyme immune assay (EIA) or (B) LC-MS/MS. Five and three animals, respectively, from each of the groups were used per time point. A trend of elevated corticosterone in plasma of KO mice was noted with both techniques; however, a statistically significant increase was only observed at time points 0, 8 and 16 with EIA. At present, it cannot be confirmed that this is solely attributable to corticosterone, as crossreactivity with other steroids is present in EIA. *$P < 0.05$.

Discussion

The adrenal gland seems to be the most important peripheral circadian oscillator in rodents. It mediates circadian signals from the suprachiasmatic nucleus to peripheral organs through production of GCs [7,8,16].

If the adrenal circadian rhythm is disrupted, the synchronization of peripheral organs is lost, and this can lead to the development of metabolic syndrome [10]. In the mouse, corticosterone is the major adrenal corticosteroid produced in a circadian manner in response to ACTH oscillation [17]. *Cyp51*, *Cyp11a1*, *Cyp17a1*, *Cyp11b1*, *Cyp11b2* and *Cyp21a1* are involved in the production of corticosteroids, where cAMP signaling is frequently involved in *Cyp* gene regulation [18]. Therefore, we examined the circadian expression of *Cyp* genes involved in steroid metabolism in adrenal glands of *Crem* KO and WT mice, in addition to *Cyp39* and *Cyp7b1* from the oxysterol pathways. Because light can influence the expression of genes through cAMP signaling [19], mice were killed in dark-room conditions, as explained in Experimental procedures. In this way, the direct influence of light is excluded, and only changes resulting from regulation by the core clock genes are observed. Three of the measured *Cyp* genes (*Cyp17a1*, *Cyp51*, and *Cyp21a1*) show low-amplitude circadian expression that differs between *Crem* KO and WT mice. The most evident change is the increase in *Cyp17a1* expression in *Crem* KO adrenal glands at ZT 12 (Fig. 2). The remaining *Cyp* genes (*Cyp11a1*, *Cyp11b1*, *Cyp11b2*, *Cyp39a1*, and *Cyp7b1*) fail to show a circadian expression profile in the WT mouse adrenal gland.

The reasons for the circadian upregulation of *Cyp17a1* in *Crem* KO mice might be transcriptional, post-transcriptional, or epigenetic. It is known that the rat *Cyp17* promoter is hypermethylated in adrenal glands as compared with the testis [4]. This is the reason why rodents synthesize corticosterone as the main GC, whereas the level of cortisol, the major human GC, is below the level of detection [20]. Methylation analysis indicates that the *Cyp17a1* promoter is also hypermethylated in the mouse WT adrenal gland. Hypomethylation of the promoter CpG island 1 in *Crem* KO mice is in line with the observed higher *Cyp17a1* mRNA and CYP17A1 expression in KO mice than in WT mice. We used the WT testis as a positive control, as CYP17A1 is expressed in Leydig cells, being involved in testosterone production [21].

The majority of *Cyp* genes from the adrenal gland are subject to cAMP regulation [18]. Human *CYP17* is activated by cAMP through four CREs in its proximal promoter [22,23]. CREM is a common name for a set of transcription factors that act as activators or repressors and can contain one or both DNA-binding domains encoded by the *Crem* gene [24,25]. CREMτ is normally not present in adrenal glands, but is known to be one of the strongest *Crem* activators [26]. To determine *in vitro* whether CREM isoforms can *trans*-activate mouse *Cyp17*, we applied CREMτ, as it contains both DNA-binding domains. The regulation of mouse *Cyp17a1* by CREM was proven to be activation, the intensity of the response being in the same range as for CREB. As *Crem* is alternatively spliced, it is difficult to design quantitative PCR (qPCR) assays to measure individual transcripts. However, we managed to measure the shortest transcript, *Icer*, the only cAMP-inducible form in the *Crem* family. ICER has been described as an early response gene and as a repressor, responding to cAMP stimuli through binding to CRE sequences and replacing other CRE_binding activators, such as CREB [27]. *Icer* is strongly expressed in various regions of the brain, whereas its expression and regulatory roles in peripheral organs are not well understood [28]. Our data show that the expression of *Icer* mRNA is circadian in adrenal glands and peaks at ZT 12. In addition to demethylation of the *Cyp17a1* promoter, this would aid in explaining the upregulation of *Cyp17* in *Crem* KOs at ZT 12. However, overexpression of ICER in transfection studies failed to repress *Cyp17a1*. We have to consider that *Icer* is prone to autoregulation [29], and that a small amount of basal transcription of *Icer* occurs in the Y-1 cell line that was used for transfection studies. Other possibilities include indirect actions of ICER by modulating factors that contribute to transcriptional, post-transcriptional or epigenetic regulation of *Cyp17*.

As discussed earlier, corticosterone is circadian in mouse plasma [17], and the maximum at ZT 12 [30] was confirmed in our study in WT and *Crem* KO mice. The two analytical techniques applied (ELISA and LC-MS/MS) showed a trend of elevated corticosterone in KO mice at certain time points; however, a statistical threshold was reached only at ZT 0, ZT 8 and ZT 16 when ELISA was used. As the antibodies applied in ELISA exhibit crossreactivity with other plasma steroids, we cannot conclude that corticosterone is the only elevated steroid. According to LC-MS/MS, it seems that the circadian concentration of plasma corticosterone is not significantly influenced by the absence of *Crem*. However, ELISA indicates that the circadian expression of corticosterone and/or the crossreacting steroids might be elevated by the absence of *Crem*, especially at certain time points. Detailed LC-MS/MS steroid profiling will be used in the future to address this issue.

The increased corticosteroid concentration in *Crem* KO mice cannot be explained solely by changes in the transcription of steroidogenic *Cyp* genes in the adrenal glands. It is known that cAMP regulation in humans leads to phosphorylation of CYP17 by protein kinase A [23]. This rapid modulation of activity could either

directly affect CYP17 enzymatic activity [31,32] or could change the activity of the transcription factors responsible for its regulation [33]. Another possibility is epigenetic regulation, as shown in our study, where the mouse *Cyp17* promoter was less methylated in the absence of *Crem*.

In conclusion, this is the first report revealing the *in vivo* role of CREM transcription factors in the circadian and epigenetic regulation of mouse adrenal steroidogenic *Cyp* genes, in particular of *Cyp17a1*.

Experimental procedures

Circadian sample collection and RNA isolation

Fifty-four WT and 45 *Crem* KO mice were used. Animals had free access to food and water, and were maintained under a 12 : 12-h light cycle (light on at 7:00 A.M.; light off at 7:00 P.M.). The experiment was approved by the Veterinary Administration of the Republic of Slovenia (license number 34401-9/2008/4) and was conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123), as well as in accordance with National Institutes of Health guidelines for work with laboratory animals. The process of sample preparation and total RNA isolation has been described previously [34]. In brief, mice were killed under dim red light every 4 h during a 24-h period starting at 7 A.M. (ZT 0). The liver and adrenal glands were excised, snap frozen in liquid nitrogen, and stored at −80 °C.

Primer design and qPCR analysis

Intron-spanning primers were designed for all *Cyp* genes (Table 1A). Primer specificity and amplification efficiency were also validated empirically by melting curve and standard curve analysis of a six-fold dilution series. Primers and probe for *Icer* were designed by PrimerDesign (Southampton, UK). Normalization was performed according to [34], with Eq-PCR Wizard. Real-time qPCR was performed in a

Table 1. Primer information for (A) qPCR and (B) methylation analysis. fw, forward; rv, reverse.

(A) qPCR

Gene	Accession number	Sequence (5′- to 3′)	Exon	Efficiency[a]
Cyp7b1	NM_007825.4	fw: aattggacagcttggtctgc	4	1.95
		rv: ttctcggatgatgctggagt	5	
Cyp11a1	NM_019779.3	fw: aagtatggccccatttacagg	1	1.99
		rv: tggggtccacgatgtaaact	2	
Cyp11b1	NM_001033229.3	fw: gccatccaggctaactcaat	5	1.78
		rv: cattaccaaggggttgatg	6	
Cyp11b2	NM_009991.3	fw: gcaccaggtggagagtatgc	2	1.78
		rv: gccattctggcccatttag	3	
Cyp17a1	NM_007809.3	fw: catcccacacaaggctaaca	6	1.97
		rv: cagtgcccagagattgatga	7	
Cyp21a1	NM_009995.2	fw: aggaattctccttcctcacttgt	4	1.99
		rv: tctgtaccaacgtgctgtcc	5	
Cyp39a1	NM_018887.3	fw: acctatgatgagggctttgagta	4	1.84
		rv: ccatcttttggattttgacca	5	
Cyp51	NM_020010.2	fw: acgctgcctggctattgc	5	1.86
		rv: ttgatctctcgatgggctctatc	6	
Icer		fw: gtaactggagatgaaactgctg	1	2
		rv: cagccatcaccacaccttg	2	
		MGB: cttaccagatcccagctcctactactgc	2	

(B) Methylation

Gene	Accession number	Sequence (5′- to 3′)	Amplicon length (bp)
Cyp17a1	CpG island 1	fw: acagctcactcaggtgtac	208
		rv: ggcaacttgaagaggaacg	
Cyp17a1	CpG island 2	fw: gcttcagtcgaacaccgtc	193
		rv: gcacagaacgggctaccta	
Actb	NM_007393.3	fw: cttcctccctggagaagagc	123
		rv: atgccacaggattccatacc	

[a] Number of duplicated amplicones per cycle. Maximum efficiency is 2.

384-well format on a LightCycler 480 (Roche Applied Science, Penzberg, Germany), with LightCycler 480 SYBR Green I Master Mix for *Cyp* genes and LightCycler 480 Probes Master Mix for the *Icer* gene (Roche Applied Science, Penzberg, Germany). The PCR reaction consisted of 2.5 μL of Master Mix, 1.15 μL of RNase-free water, 0.6 μL of 300 nM primer mix and 0.75 μL of cDNA in a total volume of 5 μL. Three technical replicates were performed for each sample. Cycling conditions were as follows: 10 min at 95 °C, followed by 40 rounds of 10 s at 95 °C, 20 s at 60 °C, and 20 s at 72 °C. Melting curve analysis for determination of the dissociation of PCR products was performed from 65 to 95 °C. Two-way analysis of variance was used for determination of statistically significant differences between KO and WT mice.

DNA methylation analysis

Mouse *Cyp17a1* (accession number AY594330) was analyzed for the presence of CpG islands with the CPG ISLAND SEARCHER program (http://cpgislands.usc.edu/). The CpG island was defined as a DNA sequence of 200 bp with a calculated percentage of CpGs higher than 50% and a calculated versus expected distribution of more than 0.6.

DNA was isolated from the same samples as RNA with Tri Reagent (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Five *Crem* KO and three WT samples were used. One microgram of DNA was incubated with the methylation-sensitive endonuclease *Bsr*FI (*Cfr*10I; Fermentas, St. Leon-Rot, Germany) for 48 h. Fresh enzyme was added to the reaction every 24 h. DNA was purified with the QIAquick PCR Purification kit (Qiagene, Hilden, Germany) and used for PCR with the CpG-specific primers. β-Actin primers were used as an internal control (Table 1B). Cycling conditions were as follows: 5 min at 95 °C, followed by 35 rounds of 1 min at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension for 5 min at 72 °C. PCR products were visualized on a 1.5% agarose gel with ethidium bromide staining and detected on an LAS4000 (FujiFilm, Tokyo, Japan). Bands on a gel were quantified with MULTI GAUGE software (FujiFilm, Tokyo, Japan). *T*-tests were used for calculation of differences between untreated and samples treated with the methylation-sensitive endonuclease.

Protein isolation and western blotting

Proteins were isolated from the same samples as RNA and DNA with Tri Reagent (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Six *Crem* KO and six WT adrenal samples were used. As a control, proteins were isolated from a single WT testis with the same protocol as used for the adrenal samples. Anti-CYP17 IgG (Abcam, Cambridge, UK) and secondary antibodies [donkey anti-(goat HRP-conjugated IgG)] (Santa Cruz Biotechnology, Heidelberg, Germany) were applied. For a loading control, monoclonal anti-β-actin IgG1 (Sigma, St. Louis, MO, USA) with secondary anti-mouse HRP-conjugated IgG (Amersham, Freiburg, Germany) were used. Detection of CYP17A1 was performed with West Femto substrate (Pierce, Rockford, IL, USA). For detection of actin, we used ECL substrate (Pierce, Rockford, IL, USA). Detection was performed on an LAS4000 (FujiFilm, Tokyo, Japan). Bands on a gel were quantified with MULTI GAUGE software (FujiFilm, Tokyo, Japan). *T*-tests were used to determine statistically significant differences between *Crem* KO and WT samples.

Cell cultures and transfection

Mouse adrenal cortex cell line Y-1 (ATCC no. CCL-79) was grown in DMEM (Sigma D7777 Sigmaaldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma F9665 Sigmaaldrich, St. Louis, MO, USA). One day before the transfection, cells were subcloned onto 96-well plates at a density of 0.5×10^6 cells·mL^{-1}. Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) was used for transient transfection, together with a luciferase reporter construct containing −1041 bp of the mouse *Cyp17* promoter (gift from C. E. Flueck [4] Universität Bern, Departement Klinische Forschung KiKl G3 812, Inselspital, CH-3010 Bern, Schweiz). Cells were cotransfected with expression vectors: pRSV Creb (gift from M. R. Waterman [35], Vanderbilt University Medical Center, 607 Light Hall, Nashville, TN 37232-0146, USA) and pCMV Crem Tau (gift from P. Sassone Corsi [35], Department of Pharmacology, 2115 Gillespie Neuroscience, University of California, Irvine, California 92697-4625, USA). For each transfection combination, 12 replicates were performed. After 48 h, the medium was removed, and cells were lysed with Promegas lysis buffer (Promega, Fitchburg, WI, USA). Luminiscence was measured with a ONE-Glo kit (Promega, Fitchburg, WI, USA) on a VictorX5 plate reader (PerkinElmer, Waltham, MA, USA). For evaluation of transfection efficiency, cells were cotransfected with pSV β-galactosidase plasmid as previously described [36], and absorbance was measured with Epoch (BioTek, Winooski, VT, USA).

Plasma corticosterone measurement

Blood was collected in EDTA-coated vials and centrifuged at 4000 ***g*** for 20 min at 4 °C. Plasma was removed and kept at −80 °C until the corticosterone concentration was determined with the Corticosterone EIA kit (Enzo Life Science, Farmingdale, NY, USA), following the manufacturer's instructions. Five samples per time point were used for each group (WT and KO). Plasma corticosterone was also measured by LC-MS/MS, as previously described [37]. We used three mouse samples per time point for each mouse group. Two-way analysis of variance was used to compare differences between KO and WT mice.

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