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Involvement of urinary bladder Connexin43 and the circadian clock in coordination of diurnal micturition rhythm

Hiromitsu Negoro^{1,2}, Akihiro Kanematsu^{1,3}, Masao Doi⁴, Sylvia O. Suadicani^{5,6}, Masahiro Matsuo⁴, Masaaki Imamura¹, Takeshi Okinami¹, Nobuyuki Nishikawa¹, Tomonori Oura⁷, Shigeyuki Matsui⁸, Kazuyuki Seo⁴, Motomi Tainaka⁴, Shoichi Urabe⁴, Emi Kiyokage⁹, Takeshi Todo¹⁰, Hitoshi Okamura^{4,*}, Yasuhiko Tabata², and Osamu Ogawa^{1,*}

¹Department of Urology, Graduate School of Medicine, Kyoto University, Sakyo, Kyoto, 606-8507, Japan

²Department of Biomaterials, Institute for Frontier Medical Sciences, Sakyo, Kyoto University, Kyoto, 606-8507, Japan

³Department of Urology, Hyogo College of Medicine, Nishinomiya, Hyogo, 663-8501, Japan

⁴Department of Systems Biology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo, Kyoto, 606-8501, Japan

⁵Department of Urology, Albert Einstein College of Medicine, Bronx, NY, 10461, USA

⁶Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, 10461, USA

⁷Department of Biostatistics, Kyoto University School of Public Health, Sakyo, Kyoto, 606-8501, Japan

⁸Department of Data Science, The Institute of Statistical Mathematics, Tachikawa, Tokyo, 190-0014, Japan

⁹Department of Anatomy, Kawasaki Medical School, Kurashiki, Okayama, 701-0192, Japan

¹⁰Department of Radiation Biology and Medical Genetics, Graduate School of Medicine, Osaka University, Suita, Osaka, 565-0871, Japan

Summary

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*Correspondence should be addressed to H.O. (okamura@pharm.kyoto-u.ac.jp) or O.O. (ogawao@kuhp.kyoto-u.ac.jp).

AUTHOR CONTRIBUTIONS

H.N. and A.K. designed the experiments, analysed data and prepared the manuscript. H.N. performed most of the experiments. M.D. and H.O. contributed to the study design and manuscript preparation. S.O.S. provided *Cx43^{+/-}* mice and contributed to the microinjection of Lucifer yellow. M.M. contributed to the analysis of rhythm-associated experiments. M.I., T. Okinami, N.N., K.S., M.T. and S.U. helped with *in vitro* experiments. T. Oura and S.M. analysed microarray data. E.K. contributed to morphological analysis. T.T. provided *Cry*-null mice. H.O., Y.T. and O.O. supervised the study.

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Nocturnal enuresis in children and nocturia in the elderly are two highly prevalent clinical conditions characterized by a mismatch between urine production rate in the kidneys and storage in the urinary bladder during the sleep phase. Here we demonstrate, using a novel method for automated recording of mouse micturition, that connexin43 (Cx43), a bladder gap junction protein, is a negative regulator of functional bladder capacity. Bladder *Cx43* levels and functional capacity show circadian oscillations in wild-type mice, but such rhythms are completely lost in *Cry*-null mice having a dysfunctional biological clock. Bladder muscle cells have an internal clock, and show oscillations of Cx43 and gap junction function. A clock regulator, *Rev-erba*, upregulates *Cx43* transcription as a co-factor of Sp1 using Sp1 *cis*-elements of the promoter. Therefore, circadian oscillation of *Cx43* is associated with the biological clock and contributes to diurnal changes in bladder capacity, which avoids disturbance of sleep by micturition.

Nocturnal enuresis is the involuntary loss of urine during sleep in childhood, and nocturia is the undesired waking at night for micturition later in life. Studies show that at least 10% of school children have nocturnal enuresis^{1,2}, and 60–90% of the elderly over 60 years suffer from nocturia^{3,4}. These conditions are detrimental to the quality of life by interfering with patients' self-esteem or sleeping habits, and they are the major diseases found in urology clinics. Nocturnal enuresis and nocturia are characterized by a mismatch between urine production rate in the kidneys and storage in the urinary bladder^{5,6}. During the sound sleep of a healthy person, a smaller volume of urine is produced than that during the daytime, and more urine is stored during the sleep phase than during the active phase^{7–9}. Although it is unknown how such temporal variation is generated, these phenomena could be related to biological rhythms because behaviour, physiology and metabolism in mammals are subject to a well-controlled daily rhythm, generated by an internal self-sustained molecular oscillator referred to as the circadian clock^{10–13}.

Circadian oscillations are driven by a transcription-translation feedback loop consisting of PER and CRY as negative components and CLOCK and BMAL1 as positive components. Rhythmic oscillations of this core loop are followed by the clock-associated oscillations of *Dbp* and *Rev-erba*, whose products regulate oscillations of a number of clock controlled genes regulating cell- or organ-specific physiology through D-box and RORE sites, respectively^{14–16}. These molecular oscillators exist in most body cells and organs¹³, but little is known about the role of the clock in urinary bladder function.

Micturition occurs by the contraction of smooth muscles of the urinary bladder upon a sensation of fullness, which is precisely controlled by regulation of the central and peripheral nerves^{17,18}. We and others have reported that an increase in connexin43 (Cx43), a gap junction protein in the urinary bladder, enhances intercellular electrical and chemical transmission and sensitizes the response of bladder muscles to cholinergic neural stimuli. This results in a decrease in functional bladder capacity and an increase in micturition frequency in rats^{19–22}. These phenomena mimic some aspects of an overactive bladder, a human pathological condition characterized by urinary urgency and increased micturition frequency²³; however, the involvement of Cx43 in regulating normal bladder function remains unclear.

To further elucidate the role of Cx43 in bladder function, we investigated the effect of genetic ablation of *Cx43* on micturition behaviour in mice and the implication of *Cx43* for circadian micturition rhythm. The circadian micturition rhythm in free-moving mice still remains elusive, since the urine volume voided per micturition (UVVM) in mice is so minute (sometimes <50 μ l)^{24,25}. To overcome this problem, we designed a novel system, called the automated voided stain on paper (aVSOP) method, which can accurately record micturition of mice for several days. Using this system, we demonstrated the role of *Cx43* and the circadian clock as regulators of functional bladder capacity in mice. We also showed that bladder muscle has internal rhythms of the clock and Cx43, which are correlated with oscillation in gap junction function. Further, we propose a novel paradigm that links the circadian clock with Cx43, in which Rev-erba protein transactivates the *Cx43* promoter through interaction with Sp1.

RESULTS

Cx43 is involved in control of functional bladder capacity

We began our study by developing an automated machine called aVSOP (Fig. 1a). The conversion of UVVM by mice from a drop area on filter paper has been reported to be an accurate method^{24,26}, and this principle was applied to the automated system by using a laminated filter paper pre-treated to turn the edge of urine stains deep purple (Supplementary Fig. S1a). This modification enabled us to record the micturition of free-moving mice fed *ad libitum* for several successive days, for a UVVM as little as 10 μ l (Supplementary Fig. S1b).

To assess the effect of genetic *Cx43* ablation on micturition, we compared heterozygous *Cx43*^{+/-} and wild-type (WT) *Cx43*^{+/+} littermate mice by the aVSOP method (Fig. 1b–d) because homozygous *Cx43*^{-/-} mice die shortly after delivery²⁷. *Cx43* mRNA and protein levels in the urinary bladder of *Cx43*^{+/-} mice were decreased compared with those in *Cx43*^{+/+} mice (Fig. 1e,f). In both genotypes, UVVM showed temporal variation under 12-hour light and 12-hour dark (LD) conditions (Fig. 1b–d), consistent with the nocturnal characteristics of the mice²⁸. UVVM was higher in *Cx43*^{+/-} mice than that in *Cx43*^{+/+} mice (Fig. 1b–d), while total urine volume was not significantly different (Supplementary Fig. S1c).

These data demonstrate that the expression level of Cx43 is crucial for determining the functional capacity of the urinary bladder. This finding and the temporal variation in the micturition behaviour of the mice led us to focus on its association with *Cx43* expression in the bladder and the circadian clock.

Association of bladder clock with bladder capacity and Cx43

It has been reported that micturition shows a diurnal change in rodents as well as in humans^{8,22,28,29}, although the day-night change is inverted because of the difference in diurnal versus nocturnal habits. However, it is unknown whether these changes are induced by an endogenous oscillator or only by a reflection of external light-dark changes. To address this in mice, we first examined micturition of WT mice under both LD and constant dark (DD) conditions. A distinct rhythm of UVVM was recorded, peaking at zeitgeber time

(ZT) or circadian time (CT) 8–12 (Fig. 2a,b). Chi-square analysis showed a circadian periodicity under both LD and DD conditions (Supplementary Fig. S2a). No significant difference between LD and DD was found in the circadian amplitude (0.042 cycles per hour) quantified as relative power calculated by Fourier transform³⁰ (Supplementary Fig. S2b). This demonstrates that the mouse could be a model animal for micturition rhythmicity, and that the rhythm could be related to an internal biological rhythm that is also operational in the absence of environmental change in light/dark cycles. The involvement of the circadian clock in micturition behaviour was tested in mice with a dysfunctional circadian clock by deletion of *Cryptochrome-1* (*Cry1*) and *Cryptochrome-2* (*Cry2*) (*Cry*-null mice); accordingly, these mice have completely arrhythmic behaviour and metabolism^{11,31}. We placed our aVSOP system in a box with an infrared activity sensor and measured the micturition and locomotor activity simultaneously. Similar to behavioural locomotor rhythms (Supplementary Fig. S3a), circadian characteristics of UVVM observed in WT mice were abolished in *Cry*-null mice (Fig. 2c,d) analysed by Chi-square and Fourier transform (Supplementary Fig. S3b) as well as those of total urine volume and frequency (Supplementary Fig. S3c,d). These findings further support the notion that the circadian clock regulates UVVM.

We next examined whether the molecular machinery of the circadian clock is present in the urinary bladder. We found that the core oscillating machinery was present in the urinary bladder, as core clock genes including *Per2* and *Bmal1* (Fig. 2e), *Per1*, *Cry1*, *Clock* and *Dbp* (Supplementary Fig. S4) showed characteristic circadian expression profiles by real-time RT-PCR of circadian sampling of the urinary bladder every 4 hours (6 time points of the day) in WT mice. Dysfunction of the bladder circadian clock in *Cry*-null mice was demonstrated by a disturbed rhythm of *Per2* and *Bmal1* (Fig. 2e). We performed DNA microarray analysis to investigate the genes showing circadian rhythm in the urinary bladder more extensively. Besides the clock genes, there are thousands of oscillating genes in the bladder, as in other organs^{32,33}. Notably, our target gene, *Cx43* (also known as *Gja1*), was among the 184 genes with clear circadian rhythmicity (defined as greater than the max correlation of 0.85 from the cosine curve with a 1.5-fold amplitude of expression level^{34,35}) (Supplementary Data 1).

Cx43 mRNA showed a clear circadian rhythm with a peak at CT12 and a trough at CT0 by real-time RT-PCR (Fig. 2f). *Cx43* protein levels remained low during the sleep phase (CT4–12), began to elevate 4 hours after the peak of mRNA expression, and formed a plateau during the active phase (CT16–24) (Fig. 2g). Immunostaining of *Cx43* in the muscle layer of the urinary bladder at CT4 and at CT16 also showed a clear difference in immunoreactivity (Fig. 2h). In rats, in which day-night difference of micturition behaviour has been described^{22,29}, a similar correlation was observed between micturition rhythm (Fig. 3a), temporal variations of clock genes (*Per2* and *Bmal1*) and *Cx43* mRNA expressions in the urinary bladder (Fig. 3b), *Cx43* protein levels (Fig. 3c) and *Cx43* immunoreactivity (Fig. 3d).

The circadian change of mRNA was reflected in the protein level, and the increase/decrease in *Cx43* protein expression correlated with the decrease/increase of UVVM, respectively, in WT mice and rats. Similarly, *Cry*-null mice tended to show a constantly low level of *Cx43*

mRNA (Fig. 2f) with a high level of UVVM (Fig. 2d). This inverse expression can be accounted for by altered bladder sensitivity caused by a differential level of gap junction formation by Cx43 because the half-life of connexin proteins is up to 5 hours and expression largely follows transcript levels^{36,37}. These findings suggest that the bladder circadian clock coordinates UVVM via circadian regulation of *Cx43* gene expression.

Internal oscillations of bladder clock and Cx43 function

We investigated if the circadian clock oscillates in the bladder without systemic control. Therefore, we investigated its oscillation in *ex vivo* bladder in culture. We used mice carrying a PER2::LUC fusion protein, which has been engineered to produce bioluminescence when the clock gene *Per2* is activated in the presence of luciferin³⁸. The *ex vivo* slice culture of the bladder from *Per2::luc* mice demonstrated a robust oscillation of bioluminescence in the muscle layer of the bladder for at least four cycles (Fig. 4a and Supplementary Movie 1). The oscillation continued for approximately 2 months in the medium changed every 5 days. This oscillation was not observed in *Per2::luc* mice with the *Clock*-mutation (*Clk*⁻¹⁹/*Clk*⁻¹⁹) (Supplementary Fig. S5). These findings clearly demonstrate that a functional circadian clock exists in the smooth muscle of the urinary bladder.

We then examined cultured bladder smooth muscles cells (BSMC) under serum shock, an *in vitro* model of genetic oscillation³⁹. After serum shock in BSMC, autonomous oscillation of clock genes (*Per2* and *Bmal1*) was observed (Fig. 4b). Concurrently, *Cx43* levels also showed autonomous rhythmicity (Fig. 4b), which was followed by a change in protein levels (Fig. 4c,d), as observed in the bladder *in vivo* (Figs. 2f–h and 3b–d). This change occurred in parallel with a change in cell-cell communication rate as shown by a dye-transfer experiment with lucifer yellow microinjected intracellularly (Fig. 4e). At 24 h after the initiation of rhythm when *Cx43* protein levels were close to their nadir, the amount of dye-transferred cells was at its minimum. However, at 36 h when *Cx43* protein levels were close to their peak, the amount of dye-transferred cells was considerably increased (more than 2-fold compared with the minimum level at 24 h). These findings clearly demonstrate that bladder muscle cells have an internal rhythm generating system, which elicits oscillation in gap junction function.

Activation of Cx43 promoter by a clock component Rev-erba

We next investigated the molecular mechanism of *Cx43* regulation by the clock. We searched for E-box, D-box and RORE sequences in the 5' prime region of *Cx43*, because clock genes are known to regulate clock-controlled genes by binding to these sequences^{15,40,41}. However, we identified no such canonical sequences in a species-conserved manner within 10,000 bases from the transcription start site* (*for RORE, there are 3 atypical RORE sequences detected from -720 to -467, but their transcriptional role for *Cx43* was negated as shown in Supplementary Fig. S6 and Supplementary Discussion). Therefore, we suspected that the circadian clock might regulate the *Cx43* promoter by a novel mechanism.

We examined the effect of clock genes on the *Cx43* promoter by promoter-reporter assays in HEK293T cells using a pGL-2-mouse *Cx43*-promoter-reporter construct containing $-1686/+165$ ⁴². There was little effect of Clock/Bmal1 and Cry1 (positive and negative regulators of genes with E-box elements as shown for the *Per1* promoter) or of Dbp and E4bp4 (regulators of genes with D-box elements as shown for the *Per1* promoter) on the *Cx43* promoter (Supplementary Fig. S7a,b). A RORE-binding protein, Rora, also did not have any effect on *Cx43* promoter activity. However, Rev-erba, which has been reported as a negative competitor of Rora⁴³ (as shown for the *Bmal1* promoter, Supplementary Fig. S7c), markedly increased *Cx43* promoter activity (Supplementary Fig. S7a) in a dose-dependent manner (Fig. 5a), while the application of various concentrations of a mutant Rev-erba (Rev-erba truncated mutant deleted with 127–206 amino acids) failed to activate the *Cx43* promoter (Fig. 5b).

Additionally, in BSMC, Rev-erba dose-dependently upregulated *Cx43* promoter activity (Fig. 5c). Conversely, inhibition of Rev-erba by siRNA decreased *Cx43* mRNA and protein expression (Fig. 5d), while the same siRNA enhanced expression of Bmal1. These results suggest that *Cx43* activation is elicited through an unreported positive transcriptional control by Rev-erba. *Rev-erba* mRNA showed a clear circadian rhythm in mouse and rat urinary bladders with a peak time at CT/ZT 4–12 and a trough at CT/ZT16–24 (Fig. 5e,f). In *Cry*-null mice, *Rev-erba* mRNA stayed arrhythmic at a lower level (Fig. 5e). These two patterns are consistent with the role of Rev-erba as a positive regulator for *Cx43*, because the *Cx43* expression profile showed a peak at CT/ZT12–20 and a trough at CT/ZT0–8 in WT mice and rats, but stayed arrhythmic at a lower level in *Cry*-null mice (Figs. 2f and 3b).

Sp1 dependent activation of *Cx43* transcription by Rev-erba

To clarify the novel molecular mechanism of Rev-erba on promoter activity, we first attempted to identify the site with effects on the *Cx43* promoter by a deletion experiment because the 5' region of *Cx43* contains several *cis*-elements including AP-1, AP-2, Sp1, half ERE, and cAMP-response element⁴⁴. Truncated $-700/+165$, $-300/+165$ and $-147/+165$ constructs did not affect the activation of the *Cx43* promoter by Rev-erba. In contrast, the transcription activation of *Cx43* by Rev-erba was markedly diminished in the $-54/+165$ and $-44/+165$ constructs (Fig. 6a).

Between -147 and -54 of the *Cx43* promoter, there are *cis*-elements evolutionally conserved among humans, rats and mice including three putative GC-rich Sp1-binding sites (Fig. 6b)^{45,46}. Exogenous expression of a transcription factor Sp1 activated the *Cx43* promoter in a dose-dependent manner, and its effect was dramatically increased by the addition of exogenous Rev-erba (Fig. 6c). Protein expression of *Cx43* was also upregulated by exogenous Sp1 and Rev-erba (Fig. 6d). The promoter activation induced by Sp1/Rev-erba was inhibited by mutations of the three Sp1 *cis*-elements, in which the proximal was most crucial (Sp1C in Fig. 6e), and was completely abolished by deletion of all Sp1 sites (Fig. 6e). Sp3, another activator bound to Sp1 sites of *Cx43* promoter⁴⁵, also enhanced *Cx43* promoter activity in the presence of Rev-erba (Supplementary Fig. S8), supporting the involvement of Sp1 sites for enhancement of *Cx43* transcription by Rev-erba.

In conclusion, unlike the negative transcriptional role of Rev-erba using RORE sites, the novel positive transcriptional role of Rev-erba requires Sp1 *cis*-elements on the *Cx43* promoter.

Rhythmic assembly of Rev-erba and Sp1 at *Cx43* promoter

We hypothesized that Rev-erba acts as a co-factor of Sp1, because Rev-erba dose-dependently enhanced *Cx43* activation by Sp1. To determine whether Rev-erba interacts with Sp1 at Sp1 sites of the *Cx43* promoter, a co-immunoprecipitation assay and chromatin immunoprecipitation assay (ChIP) were performed in HEK293T cells transfected with HA-Rev-erba and DDDDK-Sp1 (protein expression of these constructs are shown in Supplementary Fig. S9). We observed a DDDDK-Sp1 band in immunoprecipitates with an anti-HA antibody, and HA-Rev-erba was detected in immunoprecipitates with an anti-DDDDK antibody (Fig. 7a), indicating that Rev-erba can form a complex with Sp1. The ChIP assay revealed that, in immunoprecipitates of the chromatin fragments using antibodies for HA and DDDDK, specific enrichment was obtained by primers targeted on Sp1 *cis*-elements of the human *Cx43* promoter (Fig. 7b). These findings demonstrate that Rev-erba interacts with Sp1 at Sp1 sites of the *Cx43* promoter.

We next examined whether the endogenous Rev-erba/Sp1 complex is rhythmically formed on Sp1 sites of the *Cx43* promoter. It is likely that Rev-erba, but not Sp1, could contribute to the rhythmic formation of the Rev-erba/Sp1 complex, because *Sp1* mRNA showed no marked rhythm (Fig. 7c), in contrast with the strong rhythm of *Rev-erba* mRNA in the bladder (Fig. 5e,f). To verify the rhythmic role of Rev-erba estimated *in vivo*, we applied rat BSMC under serum shock *in vitro* for the ChIP assay on the *Cx43* promoter using an anti-Rev-erba antibody. After confirming the circadian expression of *Rev-erba* mRNA and protein (Fig. 7d), we harvested the cells before serum shock and 14 h and 26 h after serum shock. At 26 h the level of Rev-erba detected on Sp1 sites of the *Cx43* promoter was higher than that at 14 h (Fig. 7d), when *Cx43* mRNA expression showed a peak and nadir, respectively (Fig. 4b). Therefore, we conclude that this rhythmic formation of the Rev-erba/Sp1 complex on the Sp1 sites of *Cx43* promoter is one mechanism that the clock induces for circadian oscillation of *Cx43* expression (Fig. 7e).

DISCUSSION

Day-night micturition rhythm in humans enables a sound sleep during the sleep phase. This rhythm is not simply caused by a higher water intake during the day, because temporal variation in urine production is maintained in subjects under constant routine, when food and drink are taken equally during 24 hours⁴⁷. In physiological conditions, nocturnal micturition is prevented not only by a decrease in urine production rate from the kidneys, but also by an increase in storage capacity of the urinary bladder⁷⁻⁹. Day-night differences in bladder capacity are experienced in the daily life of humans and their disturbance can be seen in disorders such as nocturnal enuresis and nocturia. The treatment of these patients in clinics is often limited to palliation because the precise mechanism underlying the micturition rhythm is unknown.

In the beginning of the current study, we aimed to use a mouse model with/without genetic modification for investigating this mechanism. A day-night difference in micturition is known to exist in humans and rodents^{8,22,28,29}, but circadian analysis of micturition in mice has been precluded by the difficulty in diachronic measuring of urine volume with high accuracy. To overcome these problems, we designed the aVSOP system, which enabled an accurate record of minute micturition volume as little as 10 μ l for several days. This method clearly recorded circadian rhythm of micturition in mice (also avoiding micturition during the sleep phase), suggesting that this rhythm should have a mechanism conserved among species. The aVSOP method also enabled us to use genetically engineered mice for unravelling the molecular events responsible for controlling micturition. In *Cx43*^{+/-} mice, this method showed the importance of Cx43 for determining functional bladder capacity, and in *Cry*-null mice, it showed a novel function of the circadian clock: circadian regulation of functional bladder capacity. Oscillation of the internal clock was demonstrated in the urinary bladder muscle layer *ex vivo* and in cultured BSMC, in parallel with the functional change in gap junctions, compatible with the rhythm of Cx43 levels. Collectively, these findings constitute the key concept of this study: the clock elicits oscillation in Cx43 levels and sensitivity of BSMC, which contribute to altered functional bladder capacity and micturition frequency during the 24 hour cycle.

Micturition during the sleep phase is undesirable for humans, in terms of arousal from sleep, hygiene or maintenance of body temperature, which could also be applicable to rodents. By focusing on Cx43 in the urinary bladder and the circadian clock in the present study, we showed a novel aspect in normal and pathological physiology of the diurnal micturition rhythm. However, we should also note that Cx43 in the bladder is not the only determinant of this rhythm. Enuresis and nocturia are caused not only by decreased functional bladder capacity, but also by impairment of cortical arousal level in the brain, urine production rhythm in the kidneys⁴, and interaction between each other⁴⁸. In the bladder, there are also many other candidate molecules with diurnal oscillation, as listed in our microarray data (Supplementary Data 1), such as genes associated with smooth muscle contraction (*Cacna1g*, *Ednrb* and *ucy1a3*) or response to pain (*Slc6a2*, *Ednrb* and *Grik1*). These genes could also be contributing to micturition rhythm. Future studies may elucidate the association of these factors with the circadian clock, and the aVSOP method could serve as an important tool for that purpose.

The molecular mechanism of *Cx43* oscillation that may underlie these phenomena is also noteworthy. Rev-erba protein, known as a constitutive repressor^{43,49}, acts as a transcriptional activator for *Cx43* by luciferase-reporter assay. Intriguingly, for *Cx43* activation, Rev-erba does not require a canonical RORE site, but acts indirectly on Sp1 sites by interacting with Sp1 protein. This is similar to previous reports showing the physical interaction of Sp1 and nuclear receptors, such as RAR, RXR, ERs and PPAR γ ⁵⁰⁻⁵². The identification of a co-activator-like function of Rev-erba advocates a novel paradigm for controlling circadian gene expression: circadian clock components can modulate the activity of transcription factors coded by non-clock genes by functioning as transcriptional co-factors. Genome-wide screening of putative clock-associated sequences has revealed that many genes showing circadian oscillation do not have binding sequences for clock

proteins^{14,15}, and our finding suggests that these genes could possibly be regulated by clock proteins acting as co-factors.

In summary, the circadian clock is associated with the oscillating expression of *Cx43* in BSMC via a previously unknown regulatory mechanism, and it contributes to changes in bladder capacity with an increase during the sleep phase and a decrease during the active phase. This study warrants chronobiological approaches for the investigation and treatment of nocturnal enuresis and nocturia.

METHODS

Animals

Female *Cx43* heterozygote KO mice (*Cx43*^{+/-})⁵³ aged 16–21 weeks, their female WT littermates (*Cx43*^{+/+}), and female *Cry1*^{-/-}*Cry2*^{-/-} mice (*Cry*-null)¹¹ aged 10 weeks were used. C57BL/6 mice and Sprague-Dawley (SD) female rats were purchased from Japan Lab Animals Co., Ltd and Japan SLC. Animals were treated in accordance with NIH animal care guidelines, and the Kyoto University Animal Experiment Committee approved all animal experiments.

Micturition analysis in mice

Micturition assessment machines for aVSOP were manufactured by Real-designs Co., Ltd (Kyoto, Japan). Rolled laminated filter paper, pre-treated to turn the edge of urine stains deep purple, was wound up at a speed of 10 cm/h under a water-repellent wire lattice. Urine stains were counted and traced to convert micturition volume by the formula of a standard curve, calculated by the correlation of normal saline and the stained area ranging from 10 to 800 μ l. *Cx43*^{+/-} and *Cx43*^{+/+} mice were kept in a cage with the dimensions of 110×160×75 mm (height × depth × width), measured under LD conditions for 4 days and sacrificed for RNA extraction. The male WT mice were measured for 8 days under LD and followed by 5 days under DD conditions. The female WT and *Cry*-null mice were kept in a cage with dimensions of 75×160×75 mm, and measured with a simultaneous actogram under DD conditions for 5 days. Total urine volume per hour was estimated by dividing the volume by the time interval between the given and preceding voiding (filling time) when the filling time was more than 1 hour⁷.

Micturition analysis in rats

Micturition of SD rats was recorded by an electronic balance system²² under LD conditions for 2 days.

Real-time RT-PCR analysis

Female C57BL/6 mice aged 8 weeks and *Cry*-null mice aged 10 weeks were sacrificed every 4 hours at six time points during the day under a dim light (n=3 for each time/strain) after acclimation for 2 weeks under LD conditions followed by DD conditions. Female SD rats aged 7 weeks were acclimated for a week under LD conditions and sacrificed at every 4 hours (n=5 for each time), followed by DD conditions, and then sacrificed in the same manner (n=2 for each time). Complementary DNA was synthesized from 1 μ g of RNA

extracted from the bladder and cultured cells using a Superscript VILO cDNA Synthesis Kit (Invitrogen). Primers used are listed in Supplementary Table S1. Real-time RT-PCR was performed using SYBR Green PCR Master Mix with 7500 Fast Real-Time PCR system (Applied Biosystems)²². Each sample was normalized against an internal 18s ribosomal RNA control (Takara). Maximal correlation coefficients (MaxCorr) were calculated using Mathematica ver. 5.1 according to a modified program³⁵.

Immunoblotting

Whole cell lysates from bladder tissues and cultured cells were lysed with radioimmunoprecipitation assay (RIPA) buffer containing proteinase inhibitors, which were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. The membranes were incubated with antibodies against Cx43 (Zymed, 1:200; Sigma-Aldrich, 1:1000), Rev-erba (Cell Signaling Technology [CST], 1:500; Abcam, 1:600), Bmal1 (Santa Cruz, 1:200), Sp1 (Millipore, 1:2000), α SMA (Sigma-Aldrich, 1:5000), Sp3 (BioLegend, 1:500), HA (Abcam, 1:8000), DDDDK (MBL, 1:2000) and GAPDH (CST, 1:2000). The immunoreactivities were visualized with enhanced chemiluminescence using HRP-conjugated anti-rabbit or mouse IgG antibody (Pierce)²².

Bioluminescence recording

Slice cultures of bladder were obtained from adult *Period2^{Luciferase}* knock-in (*Per2::luc*) mice³⁸ (Jackson Laboratories), and *Per2::luc* mice with a *Clock* mutation (*Clk^{19/Clk¹⁹}*), generated by crossing *Per2::luc* mice to *Clock* mutant (*Clk^{19/Clk¹⁹}*) mice (Jackson Laboratories). The slice cultures were kept at 36°C with culture medium containing 1 mM luciferin⁵⁴. Bioluminescence was measured with a highly sensitive cryogenic CCD camera (Spectra Video SV16KV/CT; Pixelvision) equipped with a microscope (Carl Zeiss)⁵⁵.

Immunostaining of the urinary bladder

Mice bladder tissues and slice cultures were fixed with 4% paraformaldehyde in 0.1M phosphate buffer (0.1MPB, pH 7.4) for 24 hours⁵⁴. After embedding bladder tissues in paraffin, we cut 5 μ thick sections with a microtome. After being treated with 1% BSA, sections were incubated with Cx43 rabbit polyclonal antibody (Invitrogen, 1:50) or α SMA (Sigma-Aldrich, 1:400). Immunoreactions were visualized by donkey anti-rabbit Alexa 594 (Molecular Probes), and observed by a fluorescent microscope (Carl Zeiss).

Serum shock analysis of BSMC

Primary BSMC, isolated from female SD rats aged 9 weeks^{22,56}, were cultured after two passages until sub-confluent in DMEM with 10% FCS and 1% penicillin streptomycin followed by 72 hours incubation in DMEM with 0.5% FCS. The cells were treated with 50% horse serum (GIBCO-BRL) in DMEM for 2 hours³⁹, and washed twice with DMEM and then maintained in DMEM with 0.5% of FCS for a maximum of 72 hours.

Immunostaining of BSMC

BSMC were serum-shocked and fixed with 4% formaldehyde, permeabilized with 0.4% Triton X-100, and blocked with 10% goat serum (Invitrogen). The cells were incubated with

Cx43 rabbit polyclonal antibody (Sigma-Aldrich, 1:500) followed by goat anti-rabbit Alexa 594 (Molecular Probes) and counter stained with DAPI⁵⁷.

Lucifer yellow microinjections

Nuclei of serum-shocked BSMC were pre-stained by 16 μ M of Hoechst 33342 (Invitrogen) for 15 minutes to identify the site of injection. A single cell was impaled with a microelectrode and Lucifer Yellow was injected by an electrometer (model 3100; A-M systems) for 1 minute with a continuous current of 0.1 μ A. Images were acquired using a CoolSNAP-HQ2 CCD camera (Photometrics)⁵⁷.

Promoter-reporter assay

The promoter-reporter constructs used were, mouse pGL-2-Cx43 (pCx43 -1686/+165-luc)⁴², mouse pGL-3-Per1 (pPer1-luc), pGL-3-Bmal1 (pBmal1-luc), and pGL-2 basic, pRL-TK (Promega) as controls. The expression vectors used were, Sp1 and Sp3 (fully lengthened according to a previous report)^{58, 59}, Clock, Bmal1, Cry1, Dbp, E4bp4, Rev-erba and Rora (Open Biosystems). Site-directed mutagenesis, deletion and addition of aimed sequences were performed using a mutagenesis basal kit (Takara). Corrected mutants were all verified by sequencing. For luciferase assays, reporter plasmids (100 ng) with various expression vectors (total 250 ng) were transfected to HEK293T cells in 24-well plates and those (total 100 ng) to BSMC in 96-well plates using Fugene6 (Roche) in DMEM with 10% FCS. Plasmid dosage was kept constant by the EGFP-N1 vector. Lysates were harvested 48 hours post-transfection, and the luciferase activity was measured using a dual luciferase assay reagent (Promega).

RNA interference assay

Three sets of Stealth Select RNAi targeted against rat Rev-erba (Rat Nr1d1 1330003) and Stealth RNAi Negative Control (low, medium and high GC, Invitrogen) were used. After two passages, BSMC were plated on 6-well plates and kept for 24 hours in DMEM with 10% FCS. This was followed by transfection of siRNA (75 pmol/well) in serum-reduced α MEM for 5 hours using Lipofectamin RNAiMAX (Invitrogen), followed by medium-change to DMEM with 10% FCS. After 48 hours of the transfection, total RNA and cell lysates were extracted.

Co-immunoprecipitation assay

Rev-erba with N-terminal MYPYDVPDYA-tag (HA-Rev-erba) and Sp1 with N-terminal MDYKDDDDK-tag (DDDDK-Sp1) were constructed using a mutagenesis basal kit (Takara). Nuclear extracts were prepared from HEK293T cells transfected with expression vectors for 48 hours, using a Nuclear Complex Co-IP kit (Active Motif,). A total of 100 μ g of nuclear extracts were incubated with antibodies for 0.27 μ g of Rev-erba (CST), 4 μ g of HA (Abcam), DDDDK (MBL) and control rabbit IgG (Zymed), in 500 μ l of low IP buffer overnight at 4°C with rotation followed by the addition of 30 μ l of Dynabeads Sheep anti-Rabbit IgG (Veritas) for 1 hour. After washing with low IP buffer with/without BSA three times each, the binding protein was eluted in 40 μ l of RIPA buffer for immunoblotting. A total of 2 μ g of nuclear extracts were used as input.

Chromatin immunoprecipitation

Formaldehyde-cross-linked chromatin was obtained from HEK293T cells and serum-shocked BSMC, which were sheared using Chip-IT Express Enzymatic (Active Motif). Sheared chromatin was immunoprecipitated with antibodies to 0.23 µg of Rev-erba (Lifespan Biosciences, LS-C37817), 2 µg of HA, DDDDK and control rabbit IgG overnight at 4°C with rotation followed by the addition of 15 µl of Dynabeads Sheep anti-Rabbit IgG for 1 hour. The Chip-IT control kits Rat and Human (Active Motif) were used as an experimental control. The enrichment of Sp1-binding sequences in eluted DNA was de-crosslinked by incubation at 65°C for 4 hours followed by digestion of protein by proteinase K. Purified DNA, using a QIAquick PCR purification kit (Qiagen), was quantified by real-time RT-PCR and normalized by the quantity of input DNA⁶⁰. Samples of BSMC after real-time RT-PCR were visualized by electrophoresis. Primers used are listed in Supplementary Table S1.

Microarray analysis of the mouse bladder

Whole bladder RNA of female C57BL/6 mice sacrificed at CT 0, 4, 8, 12, 16 and 20 as described above was used (n=2 for each time point). A total of 250 ng of qualified RNA was employed for the synthesis of Cy3 labelled cRNA, which was qualified and hybridized to the Whole Mouse Genome Oligo Microarray (44K; Agilent Technologies) according to the manufacturer's protocol (entrusted to DNA Chip Research, Inc., Yokohama, Japan). Statistical analysis of microarray data was performed using R statistical software (version 2.8.1, 2008-12-22) with BioConductor (version 2.3). Signal processing was performed by Agilent Feature Extraction (version 9.5.3). Normalization was performed based on the Agi4x44PreProcess package (version 1.2.0). The mgug4122a.db package (version 2.2.5) was used for annotation of each probe. Selection of circadian-oscillated genes was based on the modified procedure of Yamada et al³⁵ and McDonald et al³⁴. Specifically, we calculated correlation coefficients between each six-point time course and 60 different-phased cosine curves with six time points. For each probe, we selected one cosine curve with MaxCorr from the 60 different-phased cosine curves. The false-positive proportion was estimated on the basis of the null distribution of maximum correlations derived from randomly generated expression profiles.

Statistical analysis

For the micturition experiments, we used one-way ANOVA with Bonferroni's *post hoc* test to evaluate differences among time points. Two-way repeated measures ANOVA was used to compare differences between the two groups of mice tested, and time points were compared with Bonferroni's *post hoc* test. For the experiments in which three or more test groups were compared, we used one-way ANOVA, and for those including two factors, we used two-way ANOVA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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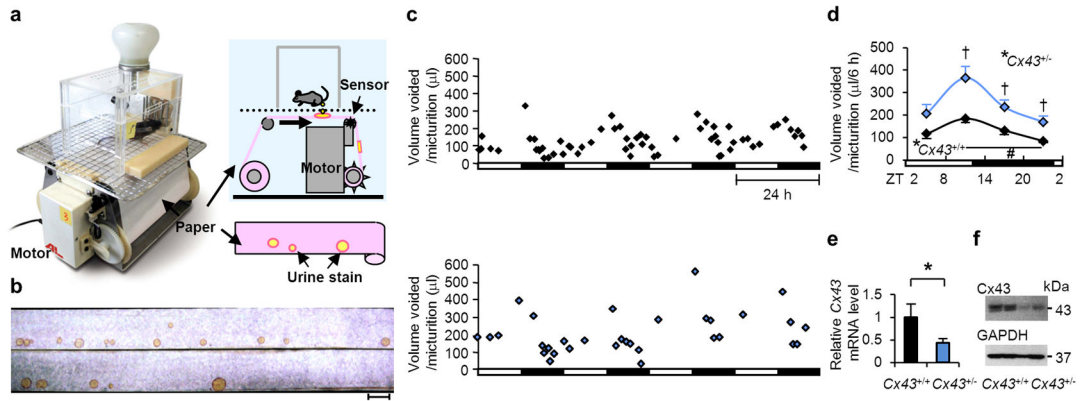


Figure 1. aVSOP reveals an association between functional bladder capacity and *Cx43*

(a) A photograph and diagram showing the aVSOP method. Each stain was traced, scanned and quantified by Image J 1.42 software. (b–d) Female *Cx43*^{+/-} mice had larger functional bladder capacity than sex-matched *Cx43*^{+/+} littermates. (b) A photograph of urine spots on paper made by *Cx43*^{+/+} (top) and *Cx43*^{+/-} (bottom) mice. The scale bar indicates 10 cm, corresponding to 1 hour. (c) Representative charts of UVVM of *Cx43*^{+/+} (top) and *Cx43*^{+/-} (bottom) mice under light/dark conditions for 4 days. UVVM, urine volume voided per micturition. (d) UVVM per 6 hours in *Cx43*^{+/+} and *Cx43*^{+/-} mice had diurnal variation ($F(3[\text{degrees of freedom (DF)} \text{ for the time factor}], 9[\text{error DF}]) = 12.3$ and 10.9 , respectively; $*P < 0.005$ by one-way repeated measures ANOVA; $\#P < 0.05$ in the late light [sleep] phase vs. late dark [active] phase, followed by Bonferroni's *post hoc* test). Maximal correlations from a cosine curve (MaxCorr) of *Cx43*^{+/+} and *Cx43*^{+/-} mice were 0.949 and 0.989, respectively. ZT, zeitgeber time: light-on at ZT0 and off at ZT12. UVVM was significantly different between *Cx43*^{+/+} and *Cx43*^{+/-} mice ($F(1[\text{DF for the strain factor}], 6[\text{error DF}]) = 11.2$, $P < 0.05$ by two-way repeated measures ANOVA; $\dagger P < 0.05$ vs. *Cx43*^{+/+} by Bonferroni's *post hoc* test; $n=4$ for each group, with a total of 296 micturitions). Error bars represent s.e.m. (e) Relative *Cx43* mRNA levels of the urinary bladder in *Cx43*^{+/-} and *Cx43*^{+/+} mice used in the micturition analysis by real-time RT-PCR. Error bars represent s.d., $n=4$ for each mice. The value of *Cx43*^{+/+} was set as 1. $*P < 0.05$ by Student's *t*-test. (f) *Cx43* protein expression of the urinary bladder in *Cx43*^{+/+} and *Cx43*^{+/-} mice.

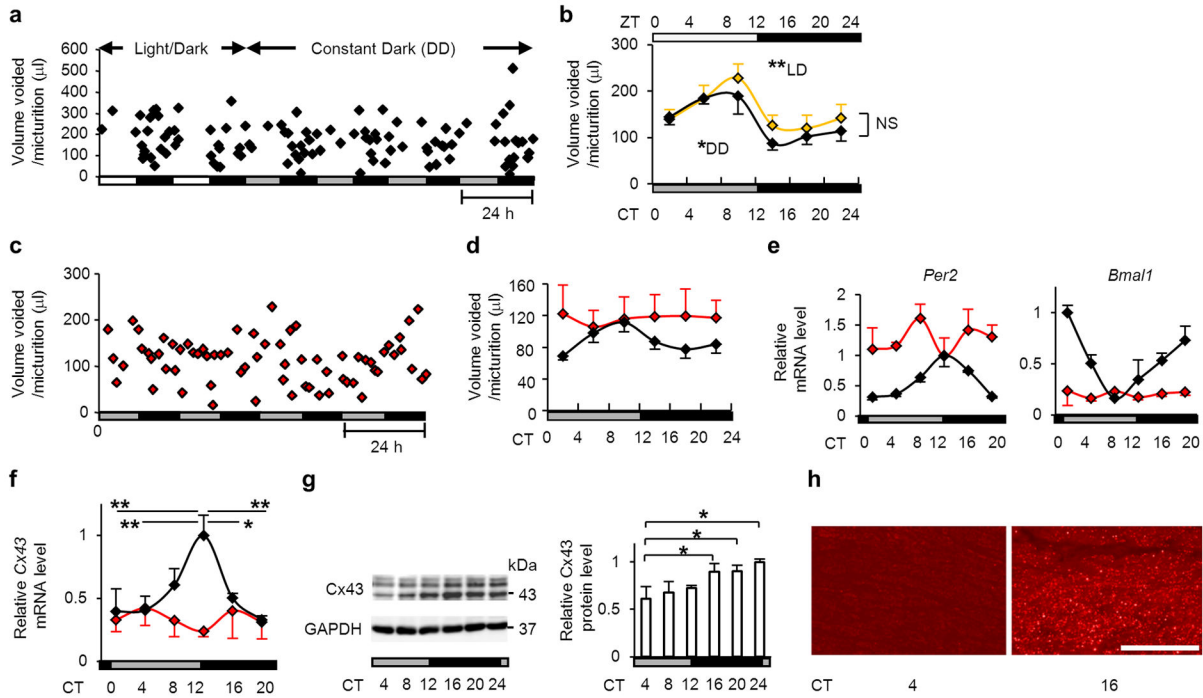


Figure 2. Rhythmicity of micturition, clock genes and *Cx43* expression in wild-type mice is disturbed in *Cry*-null mice

(a) A representative chart of UVVM of WT C57BL/6 mice under light/dark (LD) conditions followed by constant dark (DD) conditions. (b) Temporal UVVM every 4 hours in WT mice (n=5), for 8 days under LD conditions (940 micturitions) and 5 days under DD conditions (556 micturitions). Diurnal variation of UVVM in LD conditions (F(5[degrees of freedom (DF) for the time factor],20[error DF])=17.28, $**P < 0.005$ by one-way repeated measures ANOVA) was also observed in DD conditions (F(5,20)=8.23, $*P < 0.05$), with no significant difference among times in LD vs. DD by two-way repeated measures ANOVA. (c, d) Loss of circadian rhythm of UVVM in *Cry*-null mice under DD conditions. Age-matched female WT, 1493 micturitions; *Cry*-null, 1009 micturitions, n=5 each. (c) A representative chart of UVVM of *Cry*-null mice. (d) Temporal UVVM every 4 hours in *Cry*-null (red-diamond) and WT (black-diamond) mice. Diurnal variation detected in WT mice (F(5,20)=8.21, $P < 0.05$ by one-way repeated measures ANOVA) was not observed in *Cry*-null mice. (e) Temporal *Per2*, *Bmal1* and (f) *Cx43* mRNA accumulation in the bladder in WT and *Cry*-null mice (n=3 for each time point). MaxCorrs of *Per2*, *Bmal1* and *Cx43* were (0.96, 0.93 and 0.85) in WT and (0.19, 0.42 and 0.38) in *Cry*-null mice, respectively. There was no significant difference in temporal *Cx43* mRNA levels in *Cry*-null mice by one-way ANOVA. (g) Immunoblots showing temporal changes in protein levels of Cx43 in WT-mouse bladder (three independent samples for each time point). (h) Immunostaining of the muscle layer in mouse urinary bladder showing a difference in immunoreactivity with a decrease in Cx43 at CT4 compared to CT16. Representative photographs of three replicated experiments with similar results are shown. Bar, 50 μm. $*P < 0.05$ and $**P < 0.01$ by one-way ANOVA with Tukey's *post hoc* test in f and g. Error bars represent s.e.m. in b and d,

and s.d. in **e-g**. For the relative levels, the maximal values of WT were set as 1 in **e** and **f**. $F(x,y)$, $x=DF$ for the time factor; $y=error\ DF$ in **b** and **c**. CT, circadian time.

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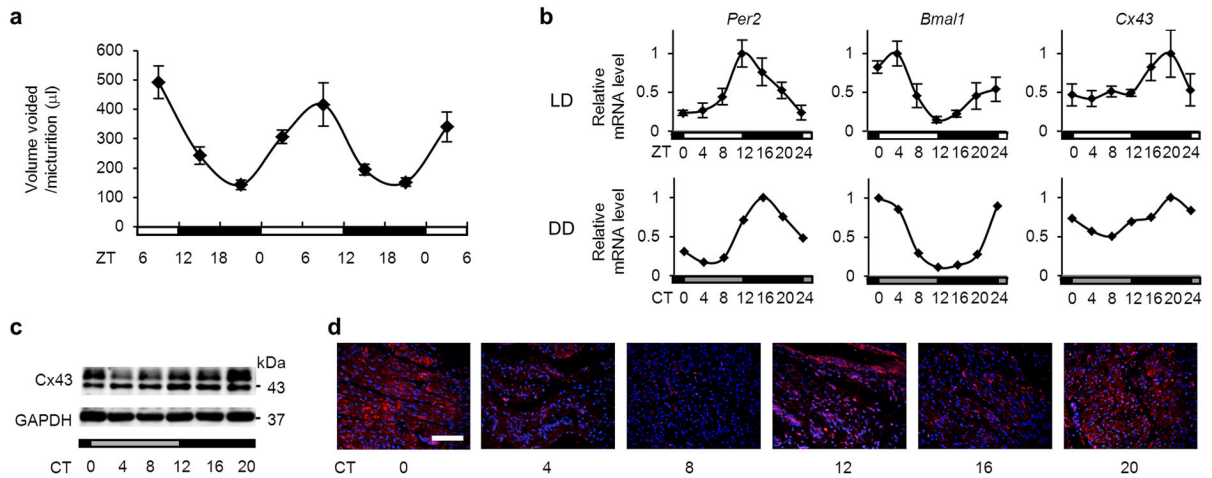


Figure 3. *Cx43* and clock-gene expression rhythms in rats and their correlation with micturition rhythm

(a) Patterns of UVVM in female Sprague-Dawley rats under LD conditions for 2 days (n=15, 1001 micturitions; $F(2.7[\text{degrees of freedom [DF]}, 38.3[\text{error DF}])=11.9$; $*P < 0.005$ by one-way repeated measures ANOVA with a Greenhouse-Geisser correction). (b) Temporal mRNA accumulation of *Per2*, *Bmal1* and *Cx43* in the rat bladder under LD and DD conditions (n=5 and n=2 for each time point, respectively). MaxCorrs were 0.87, 0.90 and 0.84 in LD conditions, respectively, and 0.98, 0.95 and 0.93 in DD conditions, respectively. (c) Temporal *Cx43* protein accumulation in the rat bladder under DD conditions as shown by immunoblotting. (d) Immunostaining of *Cx43* in the rat bladder under DD conditions (red, *Cx43*; blue, DAPI). The scale bar indicates 100 μm . Error bars represent s.e.m. in a and s.d. in b. For the relative expression, maximal values were set as 1 in b.

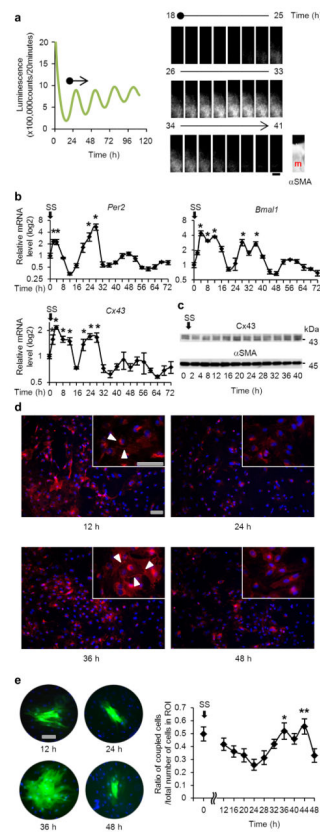


Figure 4. Oscillation of the circadian clock, *Cx43* and gap-junction function in bladder muscle cells without systemic control

(a) Oscillation of luminescence in bladder *ex vivo* slice culture obtained from *mPer2^{Luciferase}* knock-in (*Per2::luc*) mice. The period of oscillation was 24.92 ± 0.56 (mean \pm s.d.) (n=10). The muscle layer of the bladder is shown by alpha smooth muscle actin (α SMA) immunostaining. m, muscle. The scale bar indicates 100 μ m. The oscillation of luminescence is also shown by a movie in Supplementary Movie 1. (b) Temporal variation of *Per2*, *Bmal1* and *Cx43* mRNA levels in serum-shocked rat bladder smooth muscle cells (BSMC). * $P < 0.01$ against the nadir of each genes' mRNA levels (time 12 for *Per2*, time 48 for *Bmal1* and time 64 for *Cx43*) by one-way ANOVA with Dunnett's *post hoc* test (n=3–6). SS, serum shock. For relative levels, the values before serum shock were set as 1. (c) Immunoblots showing temporal changes in Cx43 protein levels with α SMA as a loading control in serum-shocked rat BSMC. (d) Immunostaining of Cx43 at times 12, 24, 36 and 48 hours in serum-shocked rat BSMC (red, Cx43; blue, DAPI). Arrow heads indicate typical plaques of gap junctions. Representative images of two replicate experiments with similar results are shown in c and d. (e) Oscillation of gap junction function evaluated by Lucifer yellow (LY) microinjection in serum-shocked rat BSMC. One representative photograph of each time point (green, LY; blue, Hoechst 33342) and overall quantification of the degree of dye-coupling (n=6–9, a total of 71 injections) is shown. * $P < 0.05$ and ** $P < 0.01$ vs. time 24 hours by one-way ANOVA with Tukey-Kramer's *post hoc* test. Similar significant differences were obtained in two independent experiments. Error bars represent s.e.m. Scale bars in d and e indicate 100 μ m.

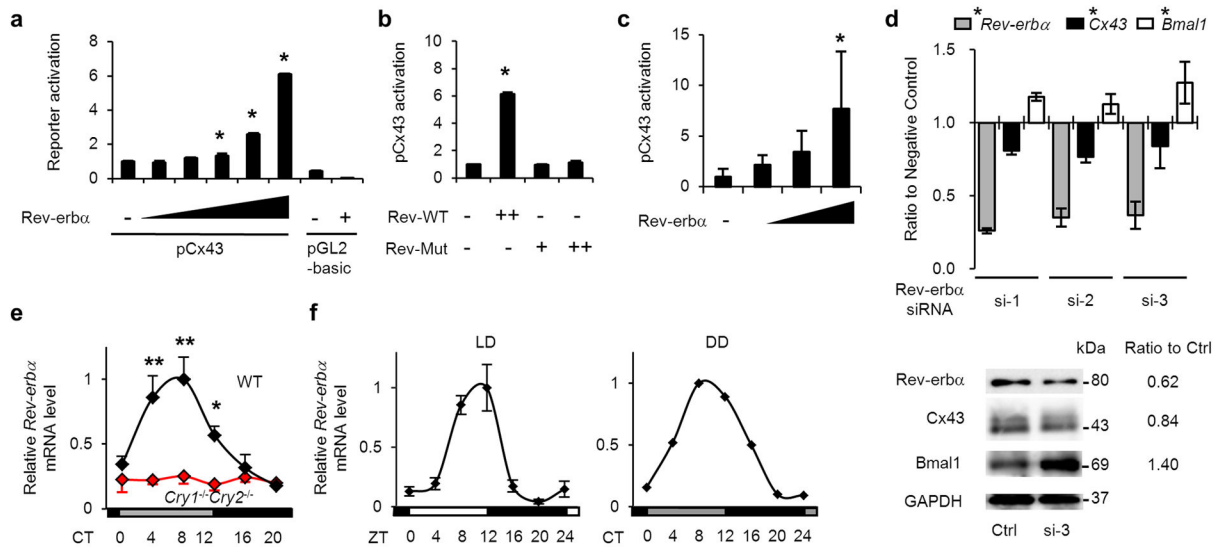


Figure 5. Rev-erba upregulates Cx43 expression

(a) Dose-dependent activation of *Cx43* transcription by Rev-erba in HEK293T cells (n=3 for each dose). (b) Impaired activation of *Cx43* transcription by a mutant of Rev-erba without 127–206 amino acids from the N-terminal (Rev-Mut) in HEK293T cells (n=3 for each dose). (c) Activation of *Cx43* transcription by Rev-erba in rat BSMC (n=6 for each dose). * $P < 0.01$ vs. Rev-erba (–) by one-way ANOVA with Dunnett’s *post hoc* test in a–c. Similar data obtained in three independent experiments for a and b, and in two independent experiments for c. (d) Suppression of Cx43 expression by knock-down of endogenous Rev-erba in BSMC. Three types of *Rev-erba* siRNAs, containing high (si-1), middle (si-2) and low (si-3) GC ratios or their controls containing corresponding GC ratios were transfected. (n=4). Messenger RNA and protein expression (data of si-3) was normalized by 18s ribosomal RNA and GAPDH, respectively. Interference of *Rev-erba* mRNA significantly decreased mRNA expression of *Cx43* and increased *Bmal1* compared with their corresponding controls (F(1[DF for the treatment factor],18[error DF])=324 for *Rev-erba*, 9.7 for *Cx43* and 11.7 for *Bmal1*. * $P < 0.01$ by two-way ANOVA). Temporal bladder *Rev-erba* mRNA accumulation in WT and *Cry*-null mice (n=3). (e) and in rats under LD (n=5) and DD (n=2) conditions (f). * $P < 0.05$ vs. CT8 and ** $P < 0.01$ vs. CT0, 16 and 20 in WT by one-way ANOVA with Tukey’s *post hoc* test. No significant difference in *Cry*-null mice. MaxCorrs were WT, 0.98; *Cry*-null, 0.31; rats in LD, 0.84; in DD, 0.93. The maximal value of WT was set as 1. Error bars represent s.d. in a–f. For relative levels, Rev-erba (–) was set as 1 in a–c. CT, circadian time; ZT, zeitgeber time.

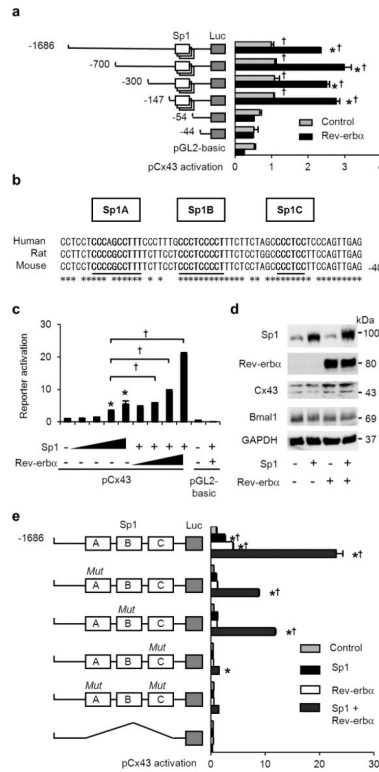


Figure 6. Sp1-dependent activation of Cx43 expression by Rev-erba

(a) Sequences including Sp1 sites are indispensable for *Cx43* promoter activation by Rev-erba. $*P < 0.001$ vs. the control of each construct and $\dagger P < 0.001$ vs. -54 (without Sp1 sequences) construct by two-way ANOVA with Bonferroni's *post hoc* test ($n=3$ for each). (b–e) Rev-erba and Sp1 activate *Cx43* expression using Sp1 sites. (b) Diagram of *Cx43* promoter sequences including three Sp1 sites, labelled as Sp1A, B and C. The asterisk indicates corresponding nucleotide sequences among humans, rats and mice. (c) Dose dependent activation of *Cx43* transcription by Sp1 and Rev-erba with Sp1. $*P < 0.001$ vs. the value -without Sp1 and Rev-erba, and $\dagger P < 0.001$ by one-way ANOVA with Tukey's *post hoc* test ($n=3$ for each). (d) Immunoblot analysis of the effect of Sp1 and Rev-erba on expression of *Cx43* and *Bmal1* (control of negative regulatory effect by Rev-erba). (e) Impaired activation of pCx43 with the Sp1 sites mutation by Sp1 and Rev-erba. $*P < 0.001$ vs. the controls of each construct, and $\dagger P < 0.001$ vs. the *MutC* construct by two-way ANOVA with Bonferroni's *post hoc* test ($n=3$ for each group). Error bars represent s.d. in a, c and e. Cells used were HEK293T in all transfection experiments. The control without Rev-erba and Sp1 was set as 1 in a, c and e. One representative of two experiments with similar results is shown in a, c, d and e.

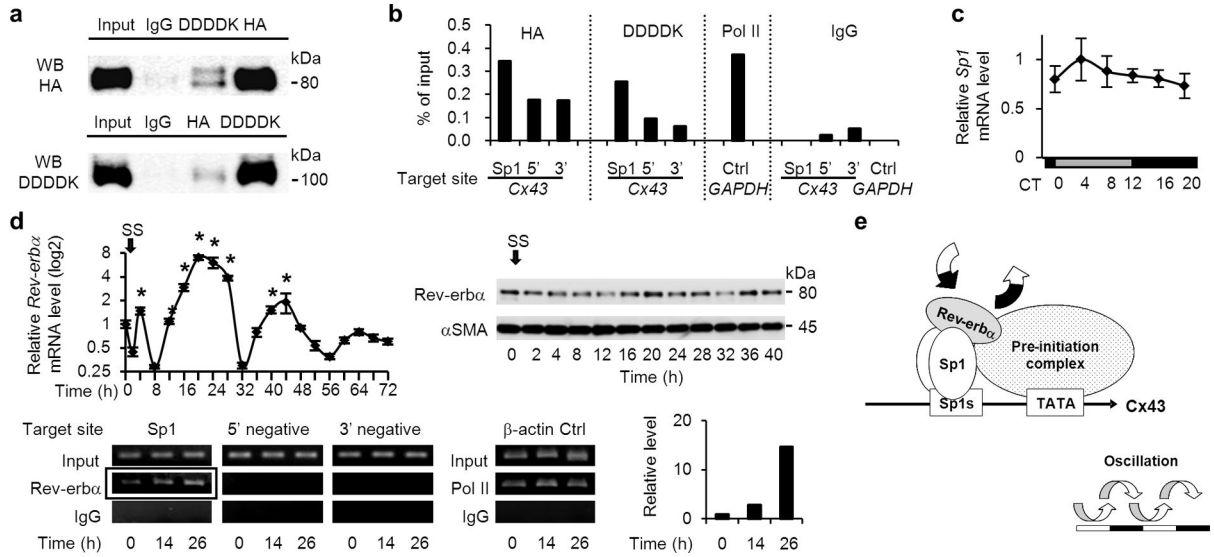


Figure 7. Rhythmic assembly of Rev-erba and Sp1 at Sp1 sites of the *Cx43* promoter

(a) Co-immunoprecipitation showing a complex formation between HA-tagged Rev-erba and DDDDK-tagged Sp1 transfected in HEK293T cells, using antibodies for HA and DDDDK. One representative of three experiments with similar results is shown. (b) Chromatin immunoprecipitation (ChIP) assay using antibodies for HA and DDDDK in HEK 293T cells transfected with HA-Rev-erba and DDDDK-Sp1. Analyses by real-time RT-PCR are shown, targeted against endogenous Sp1 sites of the human *Cx43* promoter and its negative control sites, which are approximately 7 kbp up-(5') and 10 kbp down- (3') stream from the transcription start site. A ChIP assay using an antibody for RNA polymerase II and primers for human *GAPDH* promoter was used as a positive control. One representative of two experiments with similar results is shown. (c) Temporal *Sp1* mRNA accumulation in the mouse bladder (n=3 for each time point). There were no significant differences among time points by one-way ANOVA. (d) Oscillations of *Rev-erba* mRNA (left) and protein expression (right) in serum-shocked rat BSMC (top row). * $P < 0.01$ vs. the nadir value (time 8) by one-way ANOVA with Dunnett's *post hoc* test (n=3–6). SS, serum shock. The ChIP assay, using an antibody for endogenous Rev-erba, was analysed by RT-PCR targeted against endogenous Sp1 sites of the rat *Cx43* promoter and negative control sites, which are approximately 8 kbp up- (5' negative) and down- (3' negative) stream (bottom row). β -actin is a positive control. Results of real-time RT-PCR are added; it was targeted against Sp1 sites of the *Cx43* promoter, which was immunoprecipitated using an antibody for Rev-erba (corresponding to the framed bands, bottom). One representative of two experiments with similar results is shown. (e) A mechanistic scheme of *Cx43* oscillation, controlled by the Rev-erba and Sp1 complex binding to Sp1 sites of the *Cx43* promoter. Error bars represent s.d. in c and s.e.m. in d. For relative levels, the maximal value was set as 1 in c and the values before serum shock (time 0) was set as 1 in d.