Supplementary Information for

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

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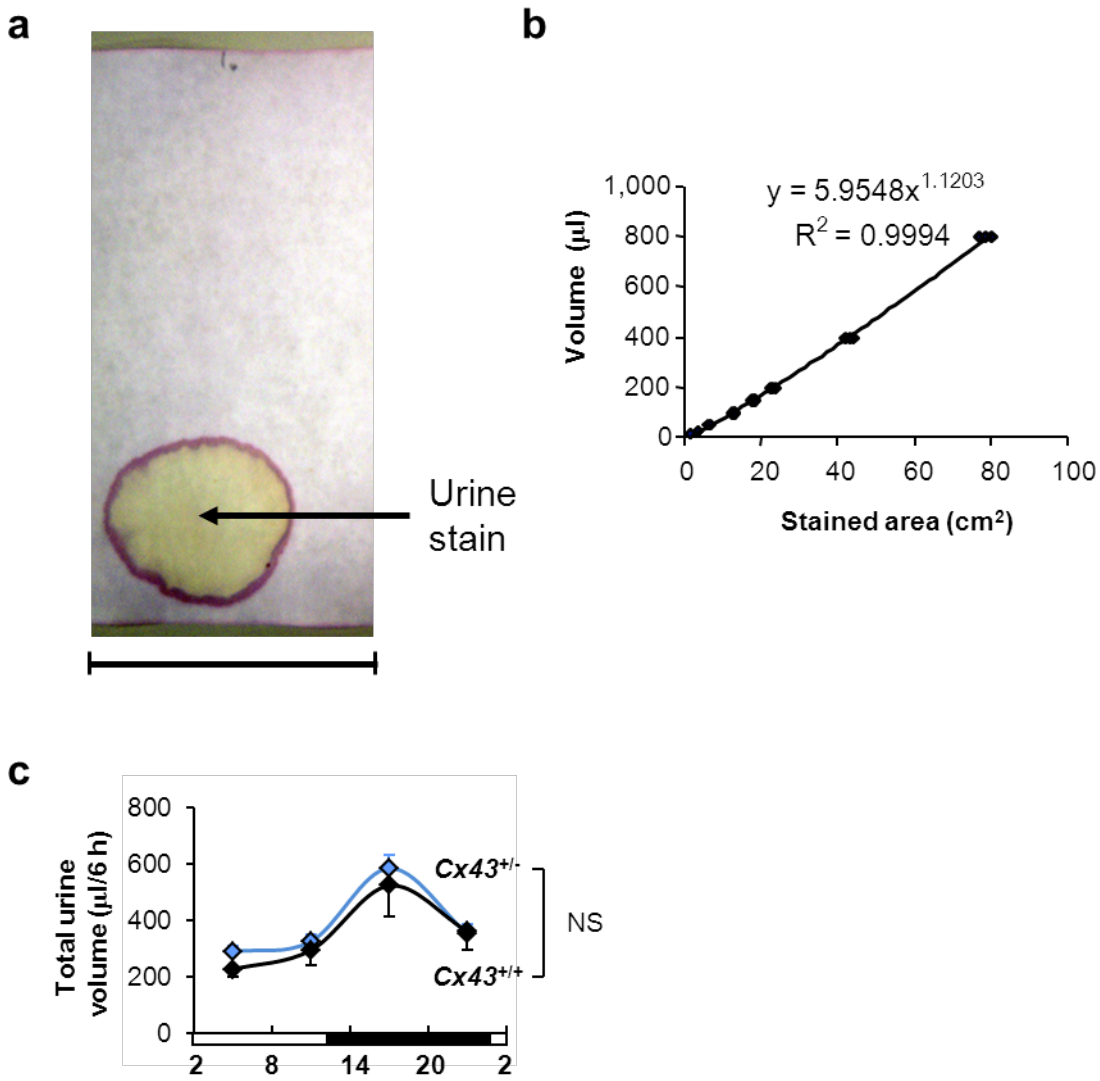
**Supplementary Information includes:**

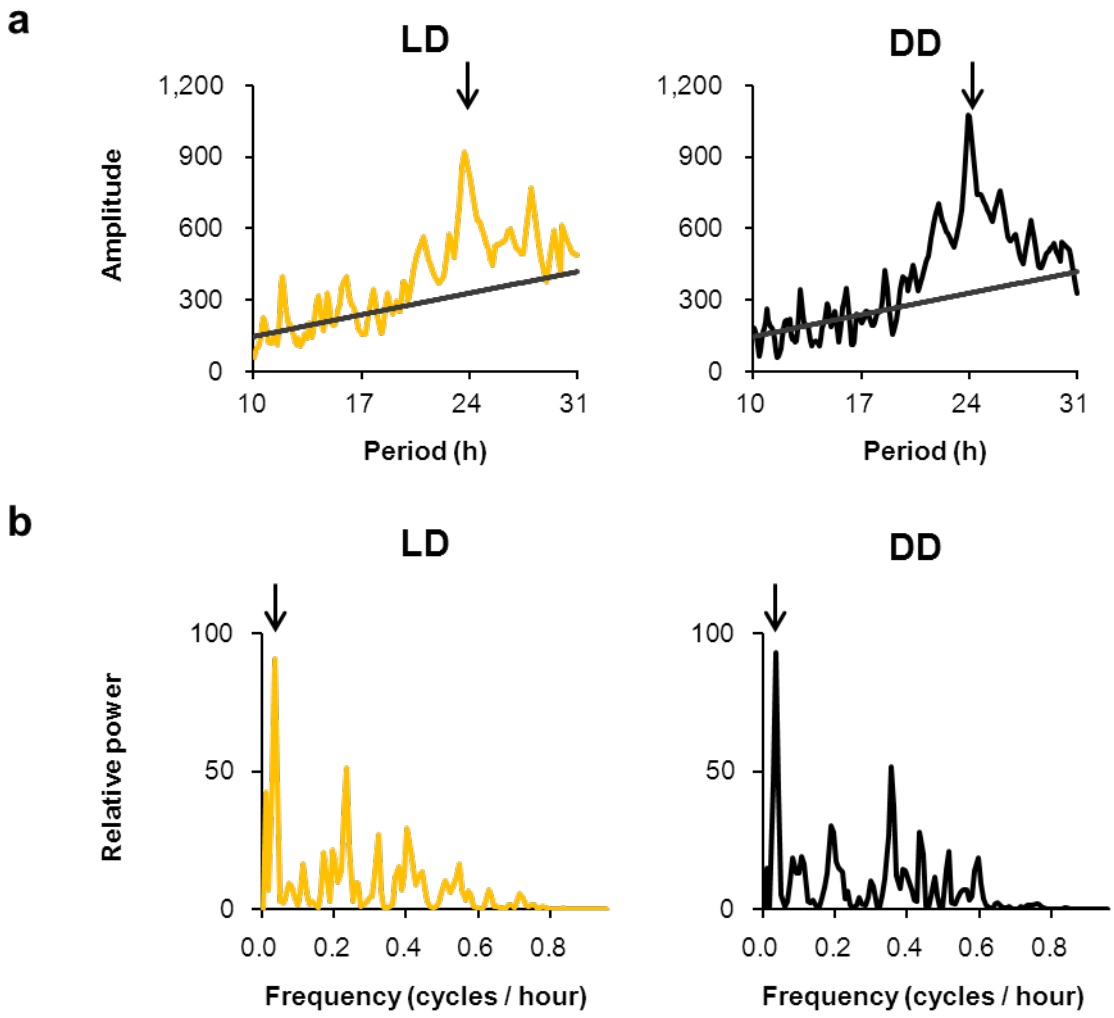
Supplementary Figures S1 to S9
Supplementary Table S1
Supplementary References

**Supplementary Figure S1**



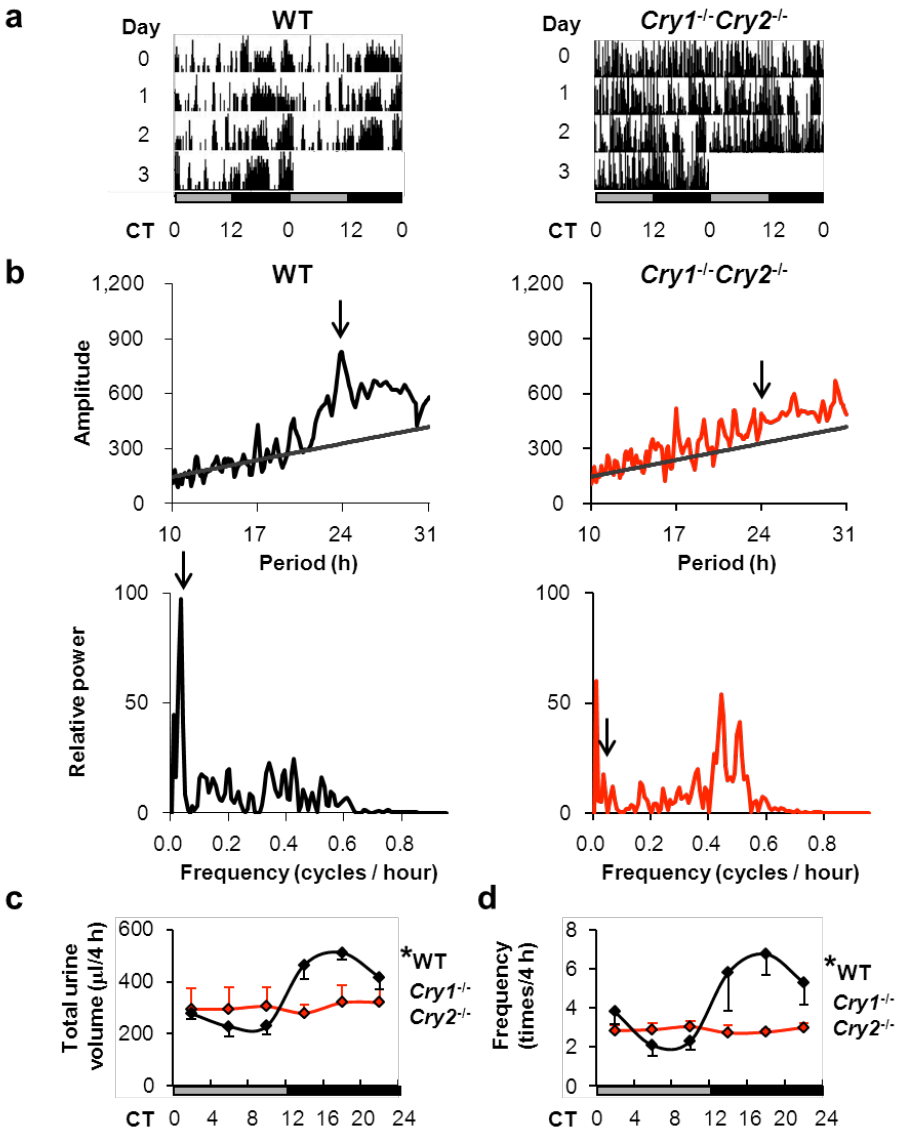

**Supplementary Figure S1 | Automated Voided Stain on Paper (aVSOP) method and characteristics of *Cx43*$^{+/+}$ and *Cx43*$^{+/-}$ mice.** (**a**) A representative urine stain with a deep purple edge. The scale bar indicates 10 cm, corresponding to 1 hour. (**b**) A standard curve was constructed from 10 to 800 µl of normal saline and their corresponding stained areas (n=3 for each volume). (**c**) There were no significant differences in total urine volume per 6 hours among *Cx43*$^{+/+}$ and *Cx43*$^{+/-}$ mice (two-way repeated measures ANOVA, n=4 for each mice). No marked difference was observed in body weights (23.4 ± 3.5 g in *Cx43*$^{+/+}$ mice and 22.9 ± 3.0 g in *Cx43*$^{+/-}$ mice, mean ± s.d., n=4 for each mice).

**Supplementary Figure S2**



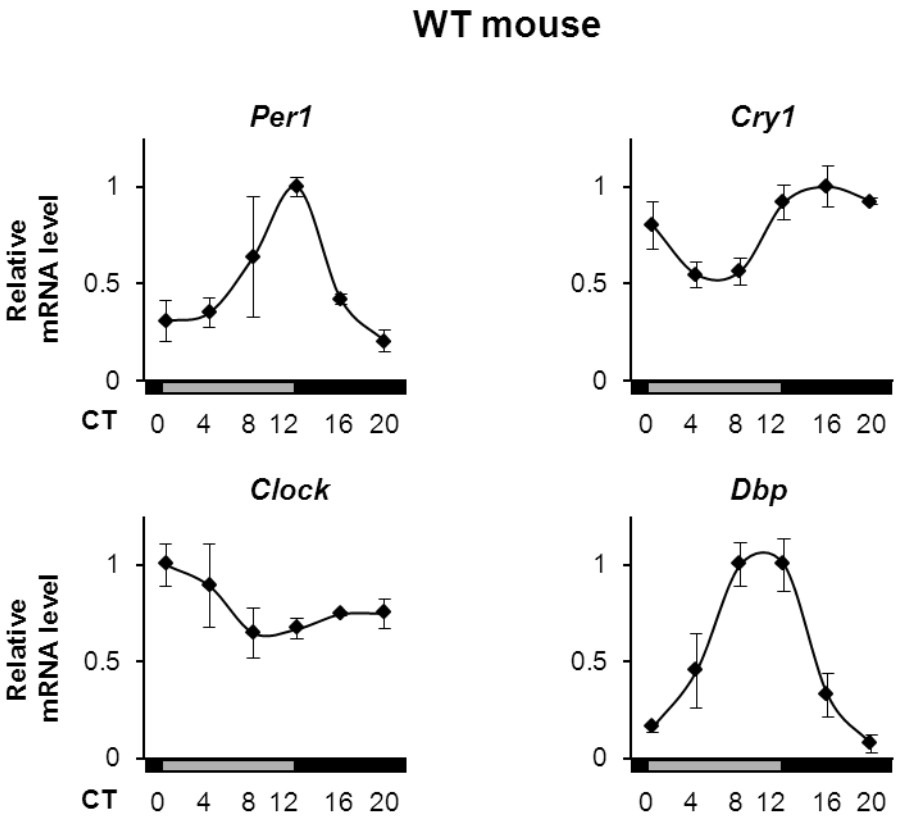

**Supplementary Figure S2 | Analyses of diurnal rhythm in UVVM under LD and DD conditions.** (**a**) Representative $\chi^2$ periodograms of UVVM under LD (left) and DD (right) conditions show a peak at 24 hours, indicated by arrow heads. (**b**) Circadian amplitudes in **a** were quantified as relative power calculated by Fourier transform (0.042 cycles per hour, indicated by arrow heads). Relative power spectral densities to 24 hours (rPSD) of five mice in LD and DD were 0.060 ± 0.026 and 0.053 ± 0.013, respectively (mean ± s.e.m.), with no significant difference by Mann-Whitney *U*-test.

**Supplementary Figure S3**



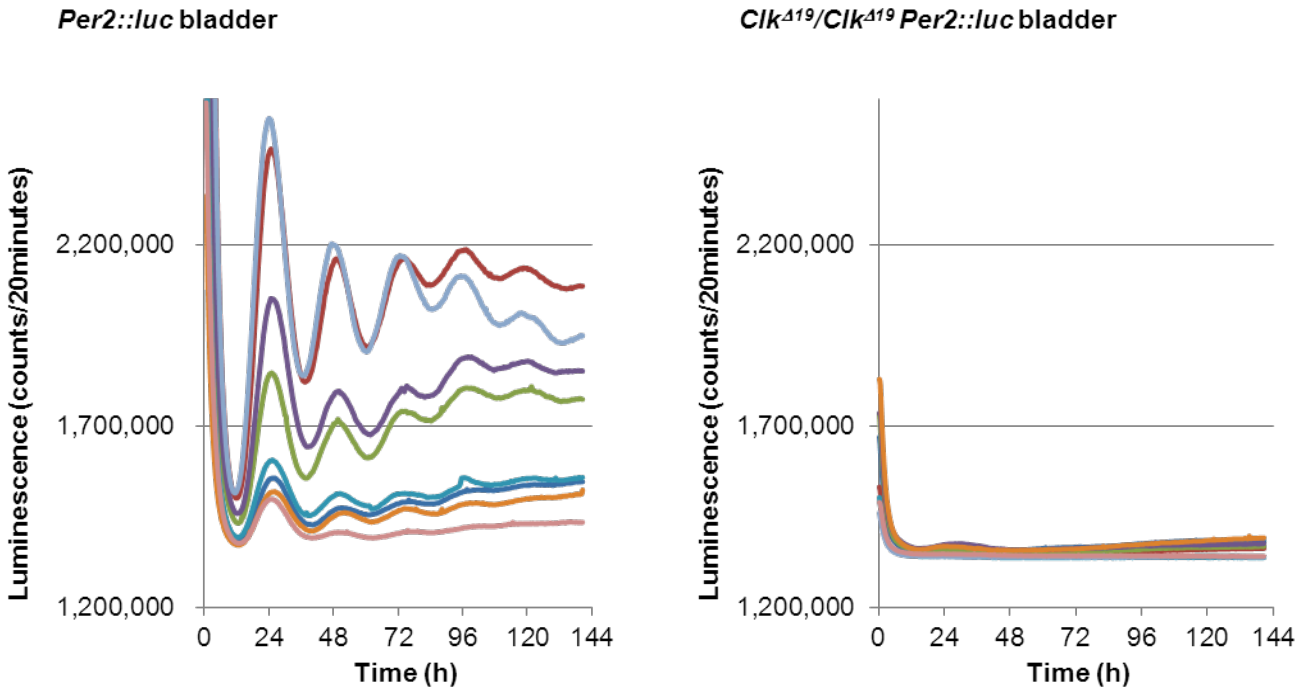

**Supplementary Figure S3 | *Cry*-null mice have disturbed rhythmicity in locomotor activity and micturition behaviour.** (**a**) Actograms of WT and *Cry*-null mice. Each following day was double-plotted. (**b**) One representative $\chi^2$ periodogram and circadian amplitude of UVVM in WT and *Cry*-null mice. Circadian periodicity (indicated by arrow heads) shown in the WT mice was disturbed in *Cry*-null mice. Circadian rhythmicity, demonstrated by rPSD, in *Cry*-null mice was significantly lower than that in WT mice (0.009 ± 0.002 vs. 0.039 ± 0.015 [mean ± s.e.m.], $P < 0.05$ by Mann-Whitney *U*-test; n=5 for each model). (**c**) Significant temporal changes in total urine volume per 4 hours observed in WT mice ($F(5,20)=9.8$, $^*P < 0.005$ by one-way repeated measures ANOVA) were not observed in *Cry*-null mice, and rPSD in *Cry*-null mice was significantly lower than that in WT mice (0.035 ± 0.013 vs. 0.135 ± 0.027, $P < 0.05$ by Mann-Whitney *U*-test, n=5 for each model). (**d**) Significant temporal changes in urinary frequency per 4 hours in WT mice ($F(5,20)=14.0$, $^*P < 0.005$ by one-way repeated measures ANOVA) were not observed in *Cry*-null mice, and rPSD in *Cry*-null mice was significantly lower than that in WT mice (0.004 ± 0.002 vs. 0.115 ± 0.025, $P < 0.005$ by Mann-Whitney *U*-test, n=5 for each model). Error bars represent s.e.m. F(x,y), x=DF for the time factor; y=error DF in **c** and **d**.

**Supplementary Figure S4**



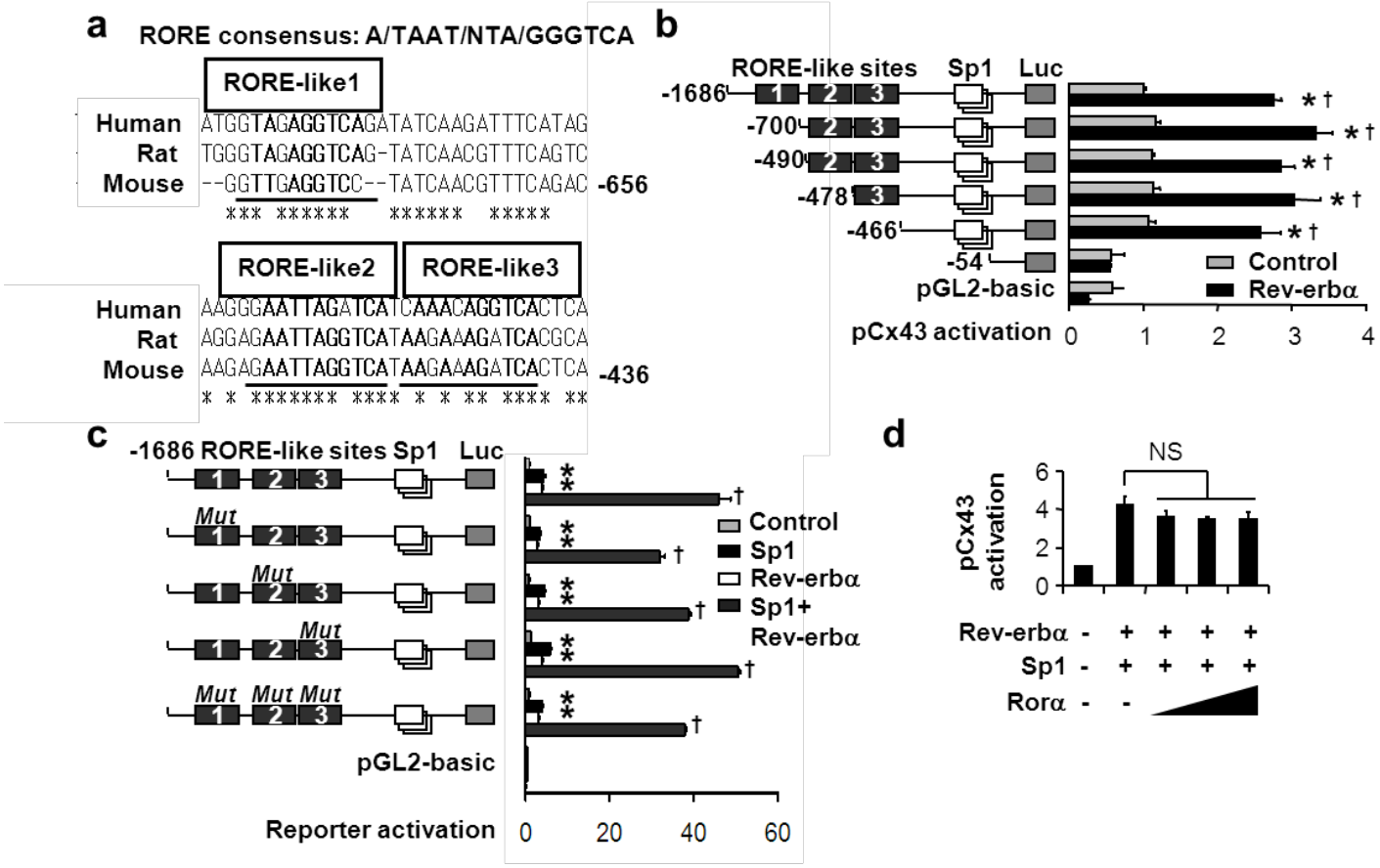

**Supplementary Figure S4 | Clock gene expression rhythms of the urinary bladder in WT mice.** Temporal mRNA accumulation of *Per1*, *Cry1*, *Clock* and *Dbp* in WT mouse bladder under DD conditions by real-time RT-PCR (n=3 for each time point). MaxCorrs of *Per1*, *Cry1*, *Clock* and *Dbp* were 0.89, 0.96, 0.86 and 0.98, respectively. Error bars represent s.d.. For the relative expression, maximal values were set as 1.

**Supplementary Figure S5**




**Supplementary Figure S5 | Disturbed oscillations of bladder internal clock in *Clock* mutant *Per2::luc* mice.** Oscillations of luminescence observed in bladder *ex vivo* slice culture obtained from m*Per2*$^{Luciferase}$ knock-in (*Per2::luc*) mice (left, n=8), and *Per2::luc* mice with the *Clock*-mutation (*Clk*$^{\Delta 19}$/*Clk*$^{\Delta 19}$)[61] (right, n=8). Note the bioluminescence of each bladder from *Clk*$^{\Delta 19}$/*Clk*$^{\Delta 19}$ mutant *Per2::luc* mice lacked a circadian rhythm.

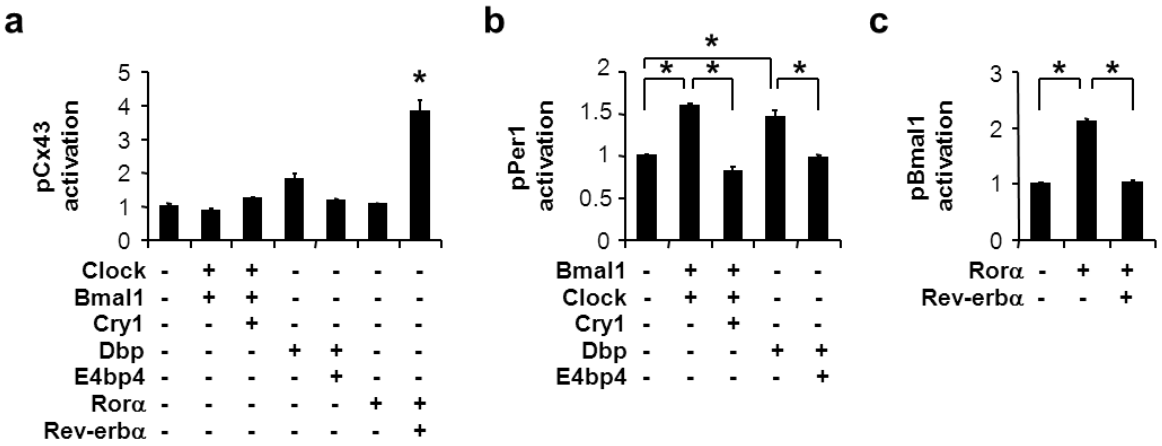
**Supplementary Figure S6**




**Supplementary Figure S6 | RORE-like site-independent activation of *Cx43* transcription by Rev-erbα.** (**a**) Schematic representation of *Cx43* promoter sequences including three RORE-like sites, numbered from the distal to proximal side as RORE-like 1, 2 and 3. The asterisk indicates corresponding nucleotide sequences among humans, rats and mice. (**b**) RORE-like sites are dispensable for *Cx43* promoter activation by Rev-erbα. Truncated mutants of pCx43-luc were transfected with and without Rev-erbα (n=3 for each). **P* < 0.001 vs. the control of each construct and †*P* < 0.001 vs. the -54 (without Sp1 sequences) construct by two-way ANOVA with Bonferroni's *post hoc* test. One representative of two experiments with similar results is shown. (**c**) No effect of mutations in the three predictable RORE-like sites (RORE-like 1, A**GG**TCC→A**CA**TCC; RORE-like 2, A**GG**TCA→A**CA**TCA; and RORE-like 3, A**G**ATCA→A**C**ATCA) on activation by Rev-erbα and Sp1 was observed. **P* < 0.001 vs. the control of each construct and †*P* < 0.001 vs. the control, Sp1 or Rev-erbα of each construct by two-way ANOVA with Bonferroni's *post hoc* test, n=3 for each group. One representative of three experiments with similar results is shown. (**d**) No significant competition by Rorα against Rev-erbα (one-way ANOVA, n=3 for each group). One representative of three experiments with similar results is shown. Error bars represent s.d.. Cells used were HEK293T in all transfection experiments. Similar results were obtained using the same expression vector without coding Rev-erbα or RORα. Five nanogram of pTK-RL was used in **b**-**d** as a transfection efficiency control. For relative expression, Rev-erbα (-) was set as 1 in **b**, Sp1 (-) Rev-erbα (-) in **c** and Rev-erbα (-) Rorα (-) Sp1 (-) in **d**. The RORE-like sites of the *Cx43* promoter are atypical (**Supplementary Fig. S6a**) because correspondence of RORE-like 1 to the RORE consensus is weak and the base pair number between tandem repeats of RORE-like 2 and 3 is not matched to the reported RORE site bound as a dimer of Rev-erbα[62]. However, the involvement of these RORE-like sites for activation of *Cx43* by Rev-erbα should have been tested, because the activation mechanism by Rev-erbα was contradictory to conventional notion. The truncation mutants of the *Cx43* promoter, whose truncated sites are more specific to the RORE-like sites, did not

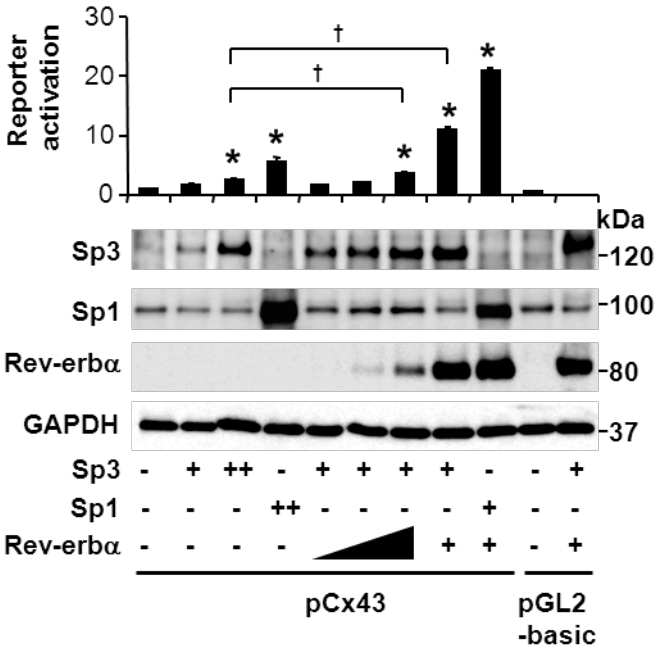
affect *Cx43* promoter activation (**Supplementary Fig. S6b**). Mutations of RORE-sites also had no effect on activation of the *Cx43* promoter by Rev-erbα, even with Sp1 (**Supplementary Fig. S6c**), which activates the *Cx43* promoter in association with Rev-erbα, as we will demonstrate later in **Fig. 6**. RORα, known as a positive competitor of Rev-erbα at the RORE site of the promoter, did not compete with Rev-erbα/Sp1 for the activation of *Cx43* (**Supplementary Fig. S6d**). In conclusion, RORE-like sites are dispensable for *Cx43* promoter activation by Rev-erbα/Sp1.

**Supplementary Figure S7**



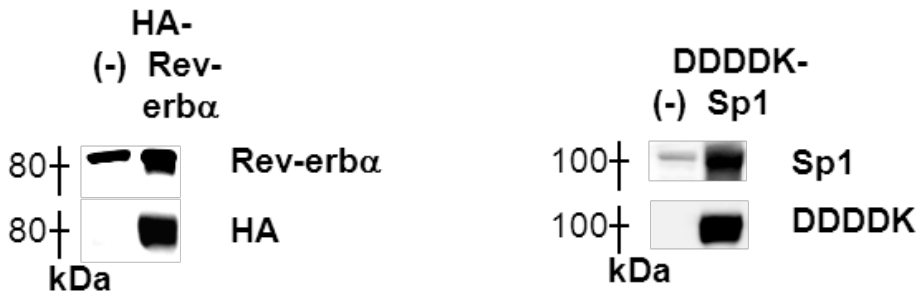

**Supplementary Figure S7 | Screening of clock genes for effect on pCx43-luc singled out *Rev-erbα*.** (**a**) Rev-erbα had a prominent effect on *Cx43* transcription among clock genes. **P* < 0.0001 vs. controls with no clock gene transfection by one-way ANOVA with Dunnett's *post hoc* test, n=3 for each group. (**b**) Activation of the *Per1* promoter-reporter (pPer1), containing both E-box and D-box elements, by Clock/Bmal1 and Dbp are blocked by Cry1 and E4bp4, respectively. Clock/Bmal1and Cry1 are positive and negative regulators of genes with E-box elements, respectively. Dbp and E4bp4 are positive and negative regulators of genes with D-box elements, respectively. **P* < 0.0001 by one-way ANOVA with Tukey's *post hoc* test, n=3 for each group. (**c**) Activation of the *Bmal1* promoter-reporter (pBmal1), containing RORE sites, by Rorα is blocked by Rev-erbα. **P* < 0.0001 by one-way ANOVA with Tukey's *post hoc* test, n=3 for each group. One representative of three experiments with similar results is shown in **a**-**c**. Error bars represent s.d. in **a**-**c**. The dosage of pPer1-luc and pBmal1-luc was 10 ng; cells used were HEK293T. Controls without clock-gene expression vectors were set as 1.

**Supplementary Figure S8**




**Supplementary Figure S8 | Sp3 is an activator of *Cx43* transcription.** The Cx43 transcription was dose-dependently activated by Sp3 and Rev-erbα in HEK 293T cells (n=3 for each group). **P* < 0.05 vs. controls without Sp1, Sp3 and Rev-erbα, and †*P* < 0.005 by one-way ANOVA with Tukey's *post hoc* test. One representative of two experiments with similar results is shown. Immunoblotting shows expression of Sp3, Sp1 and Rev-erbα. Error bars represent s.d. The relative expression of Sp3 (-) Sp1 (-) Rev-erbα (-) was set as 1.

**Supplementary Figure S9**




**Supplementary Figure S9 | Protein expressions of HA-tagged Rev-erbα and DDDDK-tagged Sp1 expression vectors.** Antibodies for Rev-erbα and HA or Sp1 and DDDDK detect proteins of HEK293T cells transfected with expression vectors of HA-tagged Rev-erbα or DDDDK-tagged Sp1, respectively. One representative blot of three independently performed experiments is shown.

## Supplementary Table S1

**Primers for real-time RT-PCR and ChIP assay**

| Species | Gene name | Forward (5'→3') | Reverse (5'→3') | Amplicon size (b.p.) |
|---|---|---|---|---|
| | | ***Primers for real-time RT-PCR*** | | |
| Mouse | *Cx43* (NM_010288) | CCATCCAAAGACTGCGGAT | GTAATTGCGGCAGGAGGAA | 138 |
| | *Per1* (NM_011065) | CACTTCGAAACCAGGACACCT | AAACACATCCCGTTTGCAAC | 110 |
| | *Per2* (NM_011066) | CACACTTGCCTCCGAAATAACTC | AGCGCACGGCTGTCTGA | 79 |
| | *Cry1* (NM_007771) | GGCGATTTTTGCTTCAGTGTC | CCATTCCTTGAAAAGCCTGG | 119 |
| | *Clock* (NM_007715) | TCAGCAGTCAGTCCATAAACTCC | AAACTGTGACATGCCTTGTGG | 104 |
| | *Bmal1* (NM_007489) | CCAAGAAAGTATGGACACAGACAAA | GCATTCTTGATCCTTCCTTGGT | 80 |
| | *Dbp* (NM_016974) | GAGACTTTTGACCCTCGGAGAC | TCATCCTTCTGTTCCTCAGGC | 107 |
| | *Rev-erb* $\alpha$ (NM_145434) | ACAGCAGCCGAGTGTCCC | ACACAGTAGCACCATGCCATTC | 70 |
| | *Sp1* (NM_013672) | ACCATGAGCGACCAAGATCA | CCCATTATTGCCACCAACATC | 81 |
| Rat | *Cx43* (NM_012567) | CATTAAGTGAAAGAGAGGTGCCC | GCAGCCAGGTTGTTGAGTGT | 210 |
| | *Per2* (NM_031678 ) | GATCTGATCGAGACCCCTGTG | GAATAATCGAAAGGCTGCCC | 110 |
| | *Bmal1* (NM_024362) | TCGGCGCTCTTTCTTCTGTAG | ACCCGTATTTCCCCGTTCA | 276 |
| | *Rev-erb* $\alpha$ (NM_145775) | AAGCTTAACGGCATGGTGCTACTG | TGGATGTTCTGCTGGATGCTCC | 125 |
| | *Sp1* (NM_012655) | ACCATGAGCGACCAAGATCA | CCCGTTATTGCCACCAACA | 78 |
| | | ***Primers for ChIP assay*** | | |
| Human | Sp1 site of *Cx43* (NM_000165) | TCCTCCCAGCCTTTCCCTT | AAAAGCTTTTTTGTAACTTGGAGCA | 136 |
| | Upstream sequence (5'neg)(-7027/-6922) | AACTTGACTGACCGCAGGG | GGCTTGACGTGTTTGGAAGA | 106 |
| | Downstream sequence (3'neg)(11770/11862) | AAGAGATCCCTGCCCACATC | ACCAAGGACACCACCAGCA | 93 |
| Rat | Sp1site of *Cx43* (NM_012567) | CTTCTCCCCGCCTTTTCTT | TCTAACTTGGAGCGCAGAGCT | 101 |
| | Upstream sequence (5'neg)(-8089/-7965) | TGCTCACTGTTCCATCAGAGAA | AGGCCACAAATGGAAGCTG | 125 |
| | Downstream sequence (3'neg)(8354/8461) | CCTTGTTGGACTGGAACATTGT | GTGGAAGCTCGTGCCTATAGTTTA | 108 |

*Cx43*, *Connexin43*

**Supplementary Table S1 | Primers for real-time RT-PCR and chromatin immunoprecipitation (ChIP) assay.** Primers were designed using Primer Express 2.0 software (Applied Biosystems) or according to previous reports[63,64].

**Supplementary References.**


61. King DP et al. Positional cloning of the mouse circadian clock gene. *Cell* **89,** 641-653 (1997).
62. Harding HP & Lazar MA. The monomer-binding orphan receptor Rev-Erb represses transcription as a dimer on a novel direct repeat. *Mol Cell Biol* **15,** 4791-4802 (1995).
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64. Fukuya H et al. Circadian expression of clock genes in human peripheral leukocytes. *Biochem Biophys Res Commun* **354,** 924-928 (2007).
65. Cockayne DA et al. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* **407,** 1011-1015 (2000).