Expression of the gene for Dec2, a basic helix–loop–helix transcription factor, is regulated by a molecular clock system

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Dec2, a member of the basic helix–loop–helix superfamily, is a recently confirmed regulatory protein for the clockwork system. Transcripts of *Dec2*, as well as those of its related gene *Dec1*, exhibit a striking circadian oscillation in the suprachiasmatic nucleus, and Dec2 inhibits transcription from the *Per1* promoter induced by Clock/Bmal1 [Honma, Kawamoto, Takagi, Fujimoto, Sato, Noshiro, Kato and Honma (2002) Nature (London) **419**, 841–844]. It is known that mammalian circadian rhythms are controlled by molecular clockwork systems based on negative-feedback loop(s), but the molecular mechanisms for the circadian regulation of *Dec2* gene expression have not been clarified. We show here that transcription of the *Dec2* gene is regulated by several clock molecules and a negative-feedback loop. Luciferase and gel retardation assays showed that expression of *Dec2* was negatively regulated by binding of Dec2 or Dec1 to two CACGTG E-boxes in the *Dec2* promoter. Forced expression of Clock/Bmal1 and Clock/Bmal2 markedly increased Dec2 mRNA levels, and up-regulated the transcription of the *Dec2* gene through the CACGTG E-boxes. Like Dec, Cry and Per also suppressed Clock/Bmal-induced transcription from the *Dec2* promoter. Moreover, the circadian expression of *Dec2* transcripts was abolished in the kidney of *Clock/Clock* mutant mice. These findings suggest that the Clock/Bmal heterodimer enhances *Dec2* transcription via the CACGTG E-boxes, whereas the induced transcription is suppressed by Dec2, which therefore must contribute to its own rhythmic expression. In addition, Cry and Per may also modulate *Dec2* transcription.

Key words: basic helix–loop–helix transcription factor, circadian rhythm, clock, Dec2, negative-feedback loop.

INTRODUCTION

A variety of organisms have circadian rhythms that control daily rhythms of physiology and behaviour, enabling them to adapt to recurring environmental conditions [1–3]. In mammals, the SCN (suprachiasmatic nucleus) of the hypothalamus acts as the master pacemaker that is essential for the generation of circadian rhythms and entrainment to the 24 h day. Light signals are perceived by the retina and transmitted to the SCN via the retinohypothalamic tract; synchronized oscillators in the SCN are transduced to peripheral oscillators through the output pathway.

Molecular and genetic studies have revealed that transcriptional regulation of multiple clock genes is crucial for the generation of circadian rhythms [4,5]. The molecular clock is composed of auto-regulatory feedback loops containing both positive and negative components. Clock and Bmal1, bHLH (basic helix–loop–helix) and PAS domain-containing transcription factors, form a heterodimer which activates transcription of the *Per* and *Cry* genes by binding to their CACGTG E-boxes [6,7]. A Per/Cry heterodimer suppresses its own transcription by interacting directly with Clock/Bmal1, although Per/Cry does not itself have DNA-binding capacity [8]. Thus the feedback loop generates rhythmic expression of *Per*, *Cry* and other genes. In addition to this core loop, several transcription factors, which are expressed in a circadian fashion in the SCN and in the peripheral tissues, are reported to affect clock gene transcription, suggesting that multiple feedback loops interact with the core loop. Rev-Erbα, for example, an orphan nuclear receptor, is up-regulated by Clock/Bmal1 through CACGTG E-boxes in its promoter [9], and the increased levels of Rev-Erbα protein repress Bmal1 transcription by binding to its response elements in the Bmal1 promoter [9,10]. The existence of such an interlocked feedback loop may be necessary for the maintenance of stable and precise circadian rhythms.

Dec1 (also called Stra13 [11] or Sharp-2 [12]) and Dec2 are structurally related to the Hes and Hey family proteins [13,14], with Sharp-1 being a minor or artificial frame-shift mutant of Dec2 [12,14]. While the Dec, Hes and Hey families share similar bHLH and Orange domains, Dec (unlike Hes and Hey) lacks the C-terminal WRPW or YRPW motif. We recently identified *Dec1* and *Dec2* as circadian regulatory genes [15,16]. Transcripts of *Dec2* and *Dec1* in the SCN oscillated in a circadian fashion under conditions of LD (light–dark) and DD (constant darkness), with peaks early in and in the middle of the subjective day respectively [15]. The phase of circadian rhythms for *Dec2* and *Dec1* was

Abbreviations used: bHLH, basic helix–loop–helix; DD, constant darkness; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HDAC, histone deacetylase; LD, light–dark; mDec2, mouse Dec2; RT-PCR, reverse transcription–PCR; SCN, suprachiasmatic nucleus; TK, thymidine kinase.

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The nucleotide sequence data for the mouse *Dec2* gene have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB126167.

similar to that for *Per1*, *Per2*, *Per3* [17], *Cry1* [18], *Dbp* [19] and *Rev-Erbα* [9]. Since Dec2 and Dec1 repressed Clock/Bmal1-induced transactivation of the *Per1* promoter through direct protein–protein interaction and/or competition for E-boxes [15], *Dec2* is probably a component of the mammalian clock system.

In the present study, we characterized the promoter of the *Dec2* gene in order to clarify the transcriptional mechanisms involved in the circadian-dependent expression of this gene. We show that the transcription of *Dec2* is up-regulated by Clock/Bmal through two CACGTG E-boxes in the *Dec2* promoter, and down-regulated by its own product through binding to the E-boxes. Furthermore, Cry and Per suppressed the Clock/Bmal1-mediated transactivation of the *Dec2* promoter. These findings suggest that *Dec2* expression is controlled by the clock genes, and that the autoregulatory feedback loop of *Dec2* transcription works together with the core loop to control circadian-dependent gene expression in mammals.

EXPERIMENTAL

Cell culture

NIH3T3 cells and C2C12 cells were supplied by the cell bank of the Institute of Physical and Chemical Research (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin (250 ng/ml). Human umbilical vein endothelial cells were obtained from Bio-Whittaker Inc. (Walkersville, MD, U.S.A.) and cultured in M199 medium supplemented with 20 % (v/v) fetal bovine serum, 60 μg/ml endothelial cell growth supplement (Collaborative Biomedical, Bedford, MA, U.S.A.) and 50 mg/ml heparin, as described previously [20].

Isolation of the *mDec2* (mouse *Dec2*) gene

A mouse genomic DNA bacterial artificial chromosome library was screened by PCR using *mDec2* specific primers: 5′-ACAGGACAGAAACCTCCAAATC-3′ and 5′-TCTTTCAGCTGAGCAATGCATTC-3′. Positive genomic clones were analysed further for restriction mapping by Southern blot analysis. A 12 kb *Hin*dIII fragment which hybridized to the *mDec2* cDNA probe was subcloned into the *Hin*dIII site of pBluescript (Stratagene, La Jolla, CA, U.S.A.). The entire nucleotide sequence was determined by using the BigDye terminator cycle sequencing kit with an ABI Prime 310 DNA sequencer (both from PE Applied Biosystems, Redwood, CA, U.S.A.). The fragment contained the complete *Dec2* coding region constituting five exons and 8 kb of the upstream region.

Luciferase reporter plasmid constructions

A 3.2 kb 5′-upstream fragment (− 3171 to − 83; + 1 indicates the translation initiation site) of the *mDec2* gene was amplified by PCR using a forward primer (5′-GGATCCACTGAACCATCTCTCCAACCCTAA-3′) and a reverse primer (5′-GGATCCGTGCGTCTCCAGGCTGTCTCGCTCT-3′), and ligated to the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). After confirming the sequence, the fragment was then subcloned into the *Bgl*II site of the promoter-less luciferase reporter plasmid pGL3-Basic vector (Promega), and named m-3171. Deletion constructs (p-1596-Luc, p-1388-Luc, p-795-Luc and p-303-Luc) were derived from m-3171 by digestion with restriction endonucleases (*Xho*I, *Apa*I, *Sma*I and *Pma*CI respectively), followed by ligation (see Figure 3).

pTK-Luc was obtained by subcloning a DNA fragment of the herpes simplex virus TK (thymidine kinase) region from plasmid phRL-TK into the *Hin*dIII–*Bgl*II site of pGL3-Basic vector. A 53 bp construct which contained three copies of the distal CACGTG E-box site (E-box1) with flanking sequence linked in tandem was made by annealing oligonucleotides 5′-CTAGTCCCGGCACGTGACCCGCCCGGCACGTGACCCGCCCGGCACGTGACCCG-3′ and 5′-TCGACGGGTCACGTGCCGGGCGGGTCACGTGCCGGGCGGGTCACGTGCCGGGA-3′. A 52 bp construct which contained three copies of the proximal CACGTG E-box site (E-box2) with flanking sequence linked in tandem was made by annealing oligonucleotides 5′-CTAGTTCCGCACGTGAGCTGTTCCGCACGTGAGCTGTTCCGCACGTGAGCTG-3′ and 5′-TCGACAGCTCACGTGCGGAACAGCTCACGTGCGGAACAGCTCACGTGCGGAA-3′. These fragments were ligated into the *Nhe*I and *Xho*I sites of pTK-Luc upstream of the TK promoter (pE1-TK-Luc and pE2-TK-Luc).

Expression plasmid constructions

Cry1 and *Per1* expression vectors were generously provided by M. Ikeda (Research Center for Genomic Medicine, Saitama Medical School, Saitama, Japan) and H. Tei (Laboratory of Functional Genomics, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan) [21]. Expression vectors for *Clock*, *Bmal1*, *Per2*, *Cry2* and *Bmal2* were described previously [15,22]. The coding region of *mDec2* was obtained by RT-PCR (reverse transcription–PCR) with primers 5′-GGATCCAGCCATTGAACATGGACGAAGGAAT-3′ and 5′-GGCACGCTTTAGAGGACGTTTGAA-3′, and subsequently subcloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, U.S.A.). The coding region of *mDec1* was obtained by RT-PCR with primers 5′-CTGCTCGCCGCATCATGGAACGGAT-3′ and 5′-CCAGAGTTTAGTCTTTGGTTTCTAAG-3′, and subcloned into the pcDNA3.1 vector. All constructs were verified by sequence analysis.

Transient transfection and luciferase assay

NIH3T3 cells were seeded at 1×10^4 cells/well in 24-well plates, and transfected with plasmids on the next day. The phRL-TK vector (0.5 ng) was co-transfected for normalization, and the total amount of DNA per well was adjusted by adding pcDNA3.1 vector. At 24 h after transfection, cells were harvested to determine luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). All experiments were repeated at least twice, and the results from representative experiments ($n > 3$) are shown with S.D.s.

Electrophoretic mobility shift assay

mDec1 and mDec2 were synthesized using the TNT Coupled Reticulocyte Lysate System (Promega). For preparation of the probes, the E-box1 oligonucleotides (5′-AGGCTCCCGGCACGTGACCCGCT-3′ and 5′-CTGGAGCGGGTCACGTGCCGGGA-3′) and the E-box2 oligonucleotides (5′-GGTACGTTCCGCACGTGAGCTGG-3′ and 5′-GCACCCAGCTCACGTGCGGAACG-3′) were annealed and then end-labelled with [^{32}P]dCTP using DNA polymerase I Klenow fragment (TAKARA, Kyoto, Japan). The ^{32}P-labelled probe (10^4 c.p.m.) was incubated for 20 min at room temperature in a buffer containing 10 mM Tris/HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 % (v/v) glycerol and 0.1 μg/μl poly(dI-dC) in the presence of the *in vitro*-translated products. For competition experiments, the *in vitro*-synthesized protein was incubated with competitors at 100-fold excess for 15 min at room temperature before adding the probe. The protein–DNA complexes were run on 6 % (w/v) polyacrylamide/TBE (Tris/borate/EDTA) gels and visualized by autoradiography.

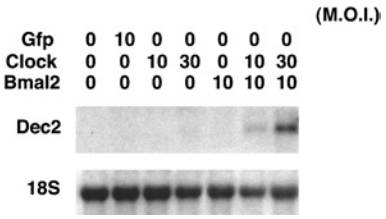

Figure 1 Induction of Dec2 mRNA expression by Clock/Bmal2

Human umbilical vein endothelial cells were infected with adenovirus expressing GFP, Clock and/or Bmal2 at the indicated multiplicity of infection (M.O.I.). Total RNA was isolated 48 h after infection and subjected to Northern blot analysis using ^{32}P-labelled human Dec2 cDNA as a probe. The blot was also hybridized with 18 S rRNA to normalize for loading.

RNA isolation and Northern blot analysis

Human umbilical vein endothelial cells were infected with adenovirus AdCMV.GFP, AdCMV.CLOCK or AdCMV.CLIF/BMAL2, as described previously [22]. Total RNA was prepared with the RNeasy kit (QIAGEN, Valencia, CA, U.S.A.) 48 h after adenovirus infection and subjected to Northern blot analysis. The probe for human Dec2 cDNA has been described previously [14].

RT-PCR analysis

First-strand cDNA was synthesized using ReverTra Ace (TOYOBO, Osaka, Japan) with 1 μg of total RNA. PCR was performed using an aliquot of first-strand cDNA as a template under standard conditions with Klentaq polymerase (Clontech, Palo Alto, CA, U.S.A.) for 28 cycles for *mDec2*. For normalization of RNA loading, RT-PCR of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also performed in each RT-PCR reaction as an internal control (25 cycles). The pairs of oligonucleotides 5′-ATGCTCGACAGGCTTAGGACA-3′ and 5′-GTGTGAGCTGAGACATGAAAC-3′ for *mDec2*, and 5′-GCTTCACCACCTTCTTGATG-3′ and 5′-GTCAAGGCCGAGAATGGGAA-3′ for *GAPDH*, were used as primers for the PCR. The PCR products were separated on 1 % (w/v) agarose gels.

Quantitative real-time RT-PCR analysis

Male wild-type and *Clock*/*Clock* mutant C57/BL6 mice (2 months old) were maintained under a 12 h/12 h LD cycle for 2 weeks before the day of the experiment. Mice kept under LD or DD conditions were killed by decapitation at different times of the day. Animals were cared for according to the Guidelines for the Care and Use of Laboratory Animals (NIH) in the Hokkaido University Graduate School of Medicine. Total RNA was extracted from the kidneys of the mice (three mice at each time point) at the indicated time points and reverse-transcribed with ReverTra Ace. Quantitative real-time RT-PCR analysis was performed using an ABI PRISM 7900HT Sequence Detection System instrument and software (PE Applied Biosystems, Redwood, CA, U.S.A.) as described [23]. The synthesized first-strand cDNA was amplified using the specific primers 5′-ATTGCTTTACAGAATGGGGAGCG-3′ and 5′-AAAGCGCGCGAGGTATTGCAAGAC-3′ for *mDec2*. The amplified cDNA was quantified using 6FAM-CGACTTGGATGCGTTCCACTCGG-TAMRA.

RESULTS

Induction of mDec2 mRNA by Clock/Bmal

Both Clock/Bmal1 and Clock/Bmal2 heterodimers act as positive components in the clockwork system, and are involved in the transcriptional regulation of a number of circadian genes. To investigate whether *Dec2* gene expression is up-regulated by Clock/Bmal, we overexpressed Clock and/or Bmal2 in endothelial cells using recombinant adenovirus vectors. As shown in Figure 1, co-expression of Clock and Bmal2 induced Dec2 mRNA expression, which robustly increased in a manner dependent on multiplicity of infection, whereas infection with Clock-, Bmal2- or GFP (green fluorescent protein)-expressing virus alone did not induce Dec2 mRNA expression. Thus expression of Clock/Bmal2 was sufficient to stimulate *Dec2* gene expression, indicating that *Dec2* is a target gene for Clock/Bmal in living cells.

To examine further whether *Dec2* expression is indeed controlled by Clock/Bmal *in vivo*, we compared the expression pattern of *Dec2* in *Clock*/*Clock* mutant mice [24] with that in wild-type mice (Figure 2). In wild-type mice, *Dec2* transcripts in the kidney exhibited a robust circadian rhythm, with peaks in the middle of the subjective day under both LD and DD conditions. In contrast, rhythmic Dec2 mRNA expression was severely blunted in *Clock*/*Clock* mutant mice under both LD and DD conditions,

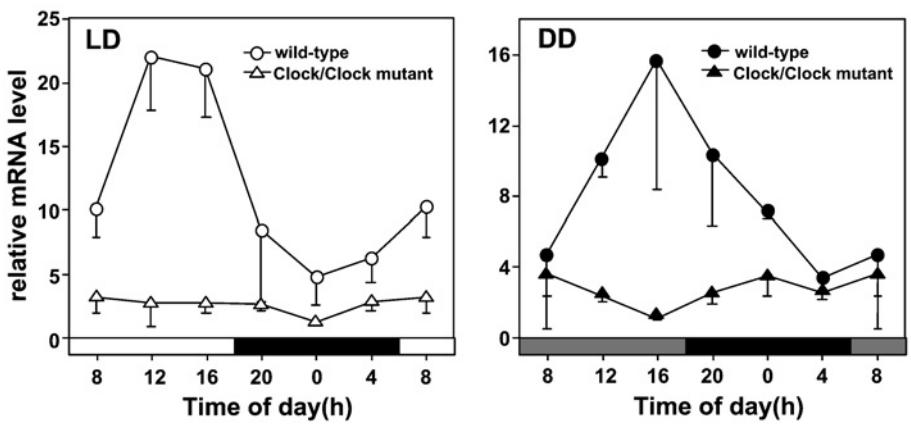

Figure 2 Reduction of Dec2 mRNA expression in the kidneys of *Clock*/*Clock* mutant mice

Relative mRNA levels of Dec2 in the kidneys of wild-type mice (○, ●) and *Clock*/*Clock* mutant mice (△, ▲) were determined by quantitative real-time RT-PCR under LD (left) and DD (right) conditions. The horizontal bar at the bottom of the left panel represents the light (white)/dark (black) cycle. Circadian time indicates the corresponding time in DD: grey and black bars in the right panel represent subjective day and night respectively. All data presented are means ± S.D. for three different experiments.

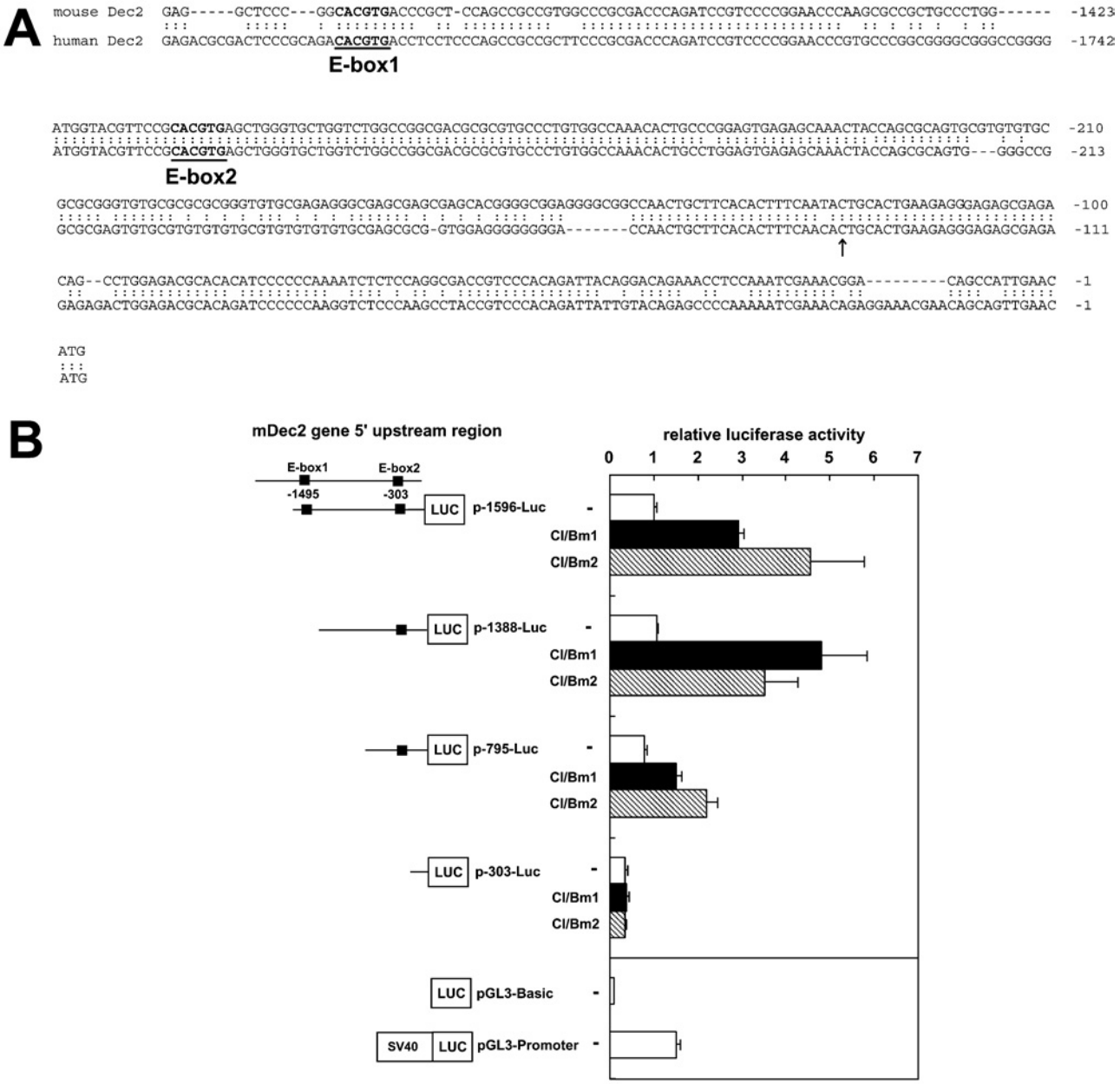

Figure 3 Enhancement of *Dec2* promoter activity by Clock/Bmal1 and Clock/Bmal2

(**A**) Comparison of nucleotide sequences of mouse and human *Dec2* gene upstream regions. Nucleotides are numbered beginning from the translation initiation site. The transcription initiation site of the human *Dec2* gene is shown by an arrow. The two CACGTG E-box elements present in both sequences are indicated by underlining. (**B**) Deletion analysis of *mDec2* promoter activity. Schematic diagrams of various *Dec2* promoter constructs are shown on the left, and the names of the plasmids are listed in the middle. The locations of the CACGTG E-boxes are indicated by closed boxes. Each construct was co-transfected with expression vectors for *Clock* (Cl), *Bmal1* (Bm1) and/or *Bmal2* (Bm2) into NIH3T3 cells. The total amount of transfected DNA was adjusted to a constant value with an empty vector, and promoter activity was normalized to the *Renilla* luciferase activity of a co-transfected internal control plasmid (phRL-TK). The promoter activity of p-1596-Luc in the absence of expression vector is given a value of 1. All data presented are means ± S.D. for four different experiments.

indicating that the rhythmic expression of *Dec2* depends on Clock *in vivo*.

Sequencing and promoter activity of the upstream region in the *mDec2* gene

It has been proposed that a CACGTG E-box element is necessary for Clock/Bmal1-mediated transcriptional activation [6]. To characterize the molecular mechanism of *Dec2* gene expression, we isolated and sequenced the *mDec2* gene, and compared its upstream region with that of the human *Dec2* gene (Figure 3A). There was no typical TATA box upstream of the coding region, and alignment of the approx. 1.5 kb mouse upstream region with the corresponding region from the human *Dec2* gene revealed some striking similarities, along with some interruption by deletions or insertions. The human *Dec2* and *mDec2* promoters each contain two CACGTG E-boxes, which are found at similar locations (at −1495 and −306 in the *mDec2* gene).

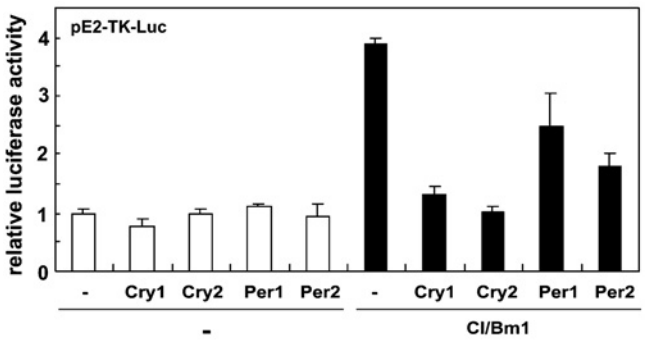

Figure 4 Inhibition of Clock/Bmal1-induced *Dec2* promoter activity by Cry and Per

pE2-TK-Luc and an expression vector for *Cry1*, *Cry2*, *Per1* or *Per2* were transfected into NIH3T3 cells with or without *Clock* (Cl) and *Bmal1* (Bm1) expression vectors. Promoter activities in the absence of expression vectors are given a value of 1. All data presented are means ± S.D. for four different experiments.

Enhancement of *Dec2* promoter activity by Clock/Bmal

To determine whether these putative Clock/Bmal1 binding sites in the *Dec2* promoter are functional, we performed reporter gene assays in NIH3T3 cells using a series of 5′ deletions of the mDec2–luciferase constructs. As shown in Figure 3(B), the promoter activity of the *mDec2* gene sequence p-1596-Luc was comparable with that of the simian virus 40 promoter. Deletion of the *mDec2* gene sequence from −1596 to −1388, or from −1388 to −795, had little effect on promoter activity, but deletion from −795 to −303 resulted in a 75% decrease in promoter activity, indicating the presence of positively acting elements in the region. Similar results were obtained in C2C12 cells and 10T1/2 cells (not shown). We also examined the effects of Clock/Bmal1 or Clock/Bmal2 on *Dec2* gene promoter activity. The promoter activities of p-1596-Luc (which contains two CACGTG sequences) and of p-1388-Luc and p-795-Luc (which contain one CACGTG sequence) were up-regulated by both Clock/Bmal1 and Clock/Bmal2 (Figure 3B), whereas Clock, Bmal1 and Bmal2 alone had no effect on the promoter activities (results not shown). Furthermore, Clock/Bmal-induced up-regulation was not observed with the p-303-Luc reporter, which contains no CACGTG sequence.

These results suggest that a proximal E-box (E-box2), at least, is functional for induction of *Dec2* transcription. To confirm the involvement of the CACGTG E-box in Clock/Bmal-mediated transactivation, we performed reporter gene assay using pE2-TK-Luc, in which three repeats of E-box2 and its flanking sequence were ligated into a luciferase reporter plasmid upstream of the TK minimal promoter. As shown in Figure 4, Clock/Bmal1 increased the promoter activities of the reporter gene, indicating that Clock/Bmal1 up-regulates *Dec2* transcription through the CACGTG E-box.

Effects of Cry and Per on *Dec2* promoter activity

The above findings suggest that the rhythmic expression of *Dec2* is positively regulated by Clock/Bmal. Since Clock/Bmal-mediated transcriptional activation of the *Per* gene is suppressed by Cry1, Cry2, Per1 and Per2 [8], we examined whether these clock gene products also regulate the expression of *Dec2*. Cry1, Cry2, Per1 or Per2 alone had no effect on the basal promoter activity of pE2-TK-Luc, but Cry1 and Cry2 abolished the Clock/Bmal1-induced promoter activity, and Per1 and Per2 also decreased the

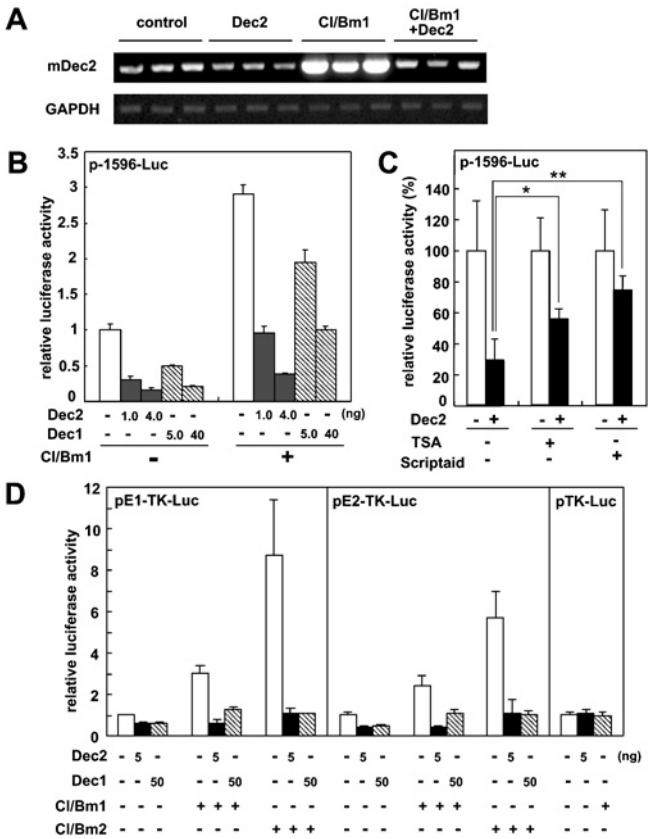

Figure 5 Repression of *Dec2* promoter activity by Dec2 and Dec1

(**A**) C2C12 cells were transfected with expression vectors for *Dec2*, *Clock* (Cl) and/or *Bmal1* (Bm1). At 48 h after transfection, total RNA was prepared to examine the expression levels of endogenous Dec2 mRNA by RT-PCR analysis. (**B**) Dose-dependent repression of *Dec2* promoter activity by Dec2 and Dec1. NIH3T3 cells were transfected with 50 ng of the p-1596-Luc construct and the indicated amounts of *mDec2* or *mDec1* expression vector, with or without *Clock* (Cl) and *Bmal1* (Bm1) expression vectors. The total amount of transfected DNA was adjusted to a constant value with an empty vector. Promoter activities in the absence of expression vectors are given a value of 1. (**C**) Effects of HDAC inhibitors on Dec2-mediated transcriptional repression. The p-1596-Luc reporter construct was co-transfected with or without 10 ng of *Dec2* expression vector. TSA (trichostatin A; 100 nM) or Scriptaid (8 μM) was added 3 h after transfection, and incubation was continued for 21 h. Luciferase activity is expressed as percentage of that in the absence of Dec2. (**D**) Involvement of E-box sites in Dec2- and Dec1-mediated transcriptional repression. An artificial promoter construct (pE1-TK-Luc or pE2-TK-Luc) containing three repeats of E-box1 or E-box2 was used as a reporter plasmid for the luciferase assay. Expression vectors were co-transfected into NIH3T3 cells. Promoter activities in the absence of expression vectors are given a value of 1. All data presented are means ± S.D. for four different experiments; $^{*}P < 0.02$; $^{**}P < 0.005$.

Clock/Bmal1-induced promoter activity, but to a lesser extent (Figure 4).

Suppression of *Dec2* promoter activity by Dec2 and Dec1

Stra13/Dec1 negatively autoregulates its gene expression [25] and also suppresses transcription from some artificial promoters by binding to CACGTG E-box sequences [26]. However, whether Dec2 negatively autoregulates its gene expression has remained unknown. To address this question, we examined the expression level of endogenous mDec2 mRNA in C2C12 cells by RT-PCR analysis with oligonucleotides located on the 3′ non-coding region of the mDec2 cDNA, which can be distinguished from exogenous Dec2 mRNA. As shown in Figure 5(A), the forced expression of Dec2 down-regulated basal and Clock/Bmal1-induced endogenous mDec2 mRNA expression. We also

examined the effects of Dec2 and Dec1 expression on the promoter activity of the *mDec2* gene by reporter gene assays. Both Dec2 and Dec1 repressed basal and Clock/Bmal1-induced *Dec2* promoter activity in a dose-dependent manner, and Dec2 seemed to be more potent than Dec1 (Figure 5B).

HDAC (histone deacetylase) is often involved in transcriptional repression by bHLH transcription factors, and some actions of Stra13/Dec1 or Sharp-1/Dec2 are suppressed by HDAC inhibitors [25,27]. We therefore examined the effects of the HDAC inhibitors trichostatin A and Scriptaid on Dec2-mediated repression. Treatment with trichostatin A or Scriptaid partly restored the Dec2-mediated repression for the p-1596-Luc reporter, indicating that Dec2 suppresses its own expression at least partly through an HDAC-dependent mechanism (Figure 5C).

Since Dec2 has high structural similarity to Dec1 in the bHLH region, which is involved in DNA binding and homo- and hetero-dimerization, Dec2 might also bind to the CACGTG E-boxes in the *Dec2* promoter to repress its own expression. To examine this hypothesis, we performed reporter gene assays using pE1-TK-Luc or pE2-TK-Luc. Both Dec2 and Dec1 repressed the basal and Clock/Bmal1-stimulated promoter activities of these reporter genes, whereas Dec2 and Clock/Bmal1 had little effect on the promoter activity of pTK-Luc, which contains no CACGTG sequence (Figure 5D). Thus the transcription of *Dec2* was positively regulated by Clock/Bmal1 and negatively regulated by both Dec2 and Dec1 via common CACGTG E-boxes.

Dec2 and Dec1 bind to E-boxes in the *Dec2* promoter

To determine whether Dec2 and Dec1 bind directly to the CACGTG E-box elements of the *Dec2* promoter, we carried out an electrophoretic mobility shift assay. Double-stranded oligonucleotides E-box1 (W1) and E-box2 (W2) were radiolabelled and used as probes. Incubation of each probe with *in vitro*-translated mDec2 yielded a shifted band (Figures 6B and 6C, lane 2). This binding was specific, since the shifted bands were successfully competed by a 100-fold excess of unlabelled probe (Figures 6B and 6C, lane 3), but not by mutated probes M1 and M2 (Figures 6B and 6C, lane 4). We also confirmed that Dec1, as well as Dec2, can bind to these E-box elements (Figures 6B and 6C, lanes 5–7). These findings suggest that Dec2 and Dec1 negatively regulate *Dec2* gene expression at least partly by direct binding to the CACGTG E-box in the *Dec2* promoter.

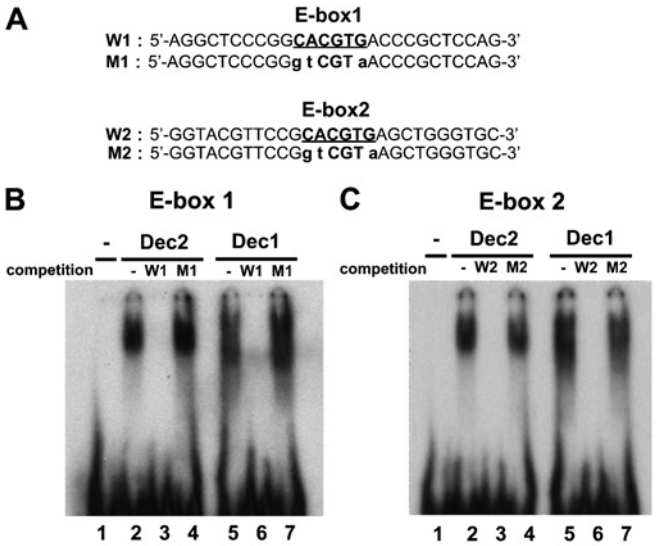

Figure 6 Both Dec2 and Dec1 bind to two CACGTG E-box sites in the *Dec2* promoter

(**A**) Sense-strand sequences of double-stranded oligonucleotides used as probes and competitors in electrophoretic mobility shift assays. The position of each E-box is underlined, and the mutant nucleotides are shown in lower-case letters. ^{32}P-labelled oligonucleotides encompassing the E-box1 (**B**) or E-box2 (**C**) sites were incubated with reticulocyte lysates in the absence (lane 1; negative control) or presence of Dec proteins. For competition assays, a 100-fold excess of unlabelled oligonucleotides was added. W1, wild-type oligonucleotide identical to the E-box1 probe; M1, mutant oligonucleotide of E-box1 probe; W2, wild-type oligonucleotide identical to the E-box2 probe; M2, mutant oligonucleotide of E-box2 probe.

DISCUSSION

We had shown previously that Dec2 mRNA is expressed in the SCN in a circadian fashion [15], and that the phase of the circadian rhythm for *Dec2* is similar to that for *Per1* and *Cry1*, whose gene expressions are known to be controlled by the clockwork system. The Clock/Bmal1 heterodimer, a positive regulator of the clock genes, induces expression of Per1 and Cry1, whose protein products form a complex that suppresses Clock/Bmal1-mediated transactivation. In the present study, we showed that *Dec2* expression is also regulated by the clock genes. The circadian expression of Dec2 mRNA was abolished in the kidneys of *Clock/Clock* mutant mice under LD and DD conditions, which is in accordance with our observation in the SCN of *Clock/Clock* mutant mice [28]. In living cells, *Dec2* transcription was up-regulated by Clock/Bmal1 and Clock/Bmal2. Bmal2 is expressed at high levels in some tissues, including blood vessels [29], and binds to Clock to form a heterodimer for gene activation [22]. A luciferase assay showed that Clock/Bmal1 and Clock/Bmal2 increased the promoter activities of pE2-TK-Luc and p-1596-Luc reporters, whereas Clock, Bmal1 and Bmal2 alone had no effect on the promoter activities. These findings suggest that both Clock/Bmal1 and Clock/Bmal2 directly regulate *Dec2* gene expression through interactions with CACGTG sequences. In addition, co-transfection of Cry or Per suppressed the Clock/Bmal1-mediated transactivation of the *Dec2* promoter.

We also show here that Dec2 could interact with its own promoter through the CACGTG E-boxes, and thereby repress its own expression. These findings, taken together, indicate that Clock/Bmal up-regulates *Dec2* expression, and that the increased Dec2 protein product may inversely down-regulate its own expression. Thus the expression of *Dec2* is probably controlled by a negative-feedback mechanism, which may contribute to its oscillatory expression, since autofeedback regulation is a common molecular mechanism for the clockwork system in a variety of organisms.

In accordance with the similar circadian profiles of Dec, Per and Cry mRNA levels, the transcriptional regulatory mechanism involved in *Dec2* expression may resemble that of *Per* and *Cry* expression. All *Dec*, *Per* and *Cry* genes are activated by Clock/Bmal and repressed by their own products through CACGTG E-boxes in their promoter regions. However, Dec2 binds directly to the CACGTG E-box, whereas Per and Cry have no DNA-binding capacity. Dec2 can compete with Clock/Bmal for DNA binding, whereas Per and Cry have to bind to Clock/Bmal in order to inhibit transcriptional activity [5]. In addition, Dec2 can bind to Bmal1 [15], which may also contribute to the repression of Clock/Bmal1-mediated transactivation.

The present study revealed that mDec1/Stra13, as well as mDec2, repressed the promoter activity of the *mDec2* gene, with mDec2 apparently more potent than mDec1 in suppressing transcription from the *mDec2* promoter. These findings are

consistent with a suppression of transcription from the human *Dec2* promoter by human Dec1 [30]. In addition, Stra13/Dec1 and Sharp-1/Dec2 suppressed *Dec1* promoter activity [31,32]. Thus Dec1 and Dec2 not only repress their own transcription, but each also mutually represses the expression of the other. Accordingly, mice lacking *Stra13*/*Dec1* showed up-regulation of Dec2/Sharp-1 [33].

Dec2 and Dec1 show a similar circadian rhythm in the SCN and other tissues [15,34]. It has recently been reported that *Dec1*/*Stra13* gene expression is up-regulated by Clock/Bmal, and that the induced activity is suppressed by Dec1 and Dec2 [31,33]. Thus the circadian expression of both *Dec2* and *Dec1* seems to be controlled by similar mechanisms. In addition, the Dec proteins have a similar bHLH domain, which may be required for binding to CACGTG E-boxes. These findings show a functional redundancy between these family members in the circadian system. In fact, mice lacking *Stra13*/*Dec1* exhibited up-regulation of Dec2/Sharp-1 and showed no significant changes in the expression patterns of clock genes, although these mice did show changes in expression levels of some genes, including several clock-controlled genes [33]. It is likely that Dec2 can compensate for the function of Dec1; however, *Dec1* expression is enhanced by a light pulse of 30 min in a phase-dependent manner, while *Dec2* expression is not [15], suggesting that the expression of these genes is regulated by different mechanisms in circadian light entrainment.

Besides being circadian clock genes, Dec(s) play roles in cell proliferation and/or differentiation. mDec1/Stra13, for example, inhibits cell proliferation and serum deprivation-induced apoptosis [25,35]; overexpression of *mDec1*/*Stra13* induces differentiation of nerve cells [11] and chondrocytes [36,37]; and *mDec1*/*Stra13*-deficient mice exhibit ineffective elimination of activated T and B cells [38]. Although it is unclear how these functions are associated with the rhythmical expression of *Dec* genes, a number of genes are controlled by circadian genes in a tissue-specific manner, and numerous biological phenomena, including cell proliferation and apoptosis, are associated with circadian rhythms. For example, *Per2*-mutant mice, which are deficient in circadian clock function, show cancer formation with an increase in c-*myc* expression, suggesting that Per2 plays a part in tumour suppression [39]. Interestingly, mDec1/Stra13 also represses the expression of c-*myc* [25], and is expressed abundantly in carcinomas [30,35], whereas Dec2 is highly expressed in adjacent normal tissue [30]. Further studies are needed in order to establish the relationship between the circadian control of *Dec* gene expression and cell proliferation/differentiation. The identification of downstream target genes and analysis of mice lacking *Dec2* should help in determining the physiological functions of Dec2. Furthermore, analysis of mice lacking both *Dec1* and *Dec2* will be required to elucidate the role of Decs in circadian rhythms.

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REFERENCES

1 Allada, R., Emery, P., Takahashi, J. S. and Rosbash, M. (2001) Stopping time: the genetics of fly and mouse circadian clocks. Annu. Rev. Neurosci. **24**, 1091–1119

2 Reppert, S. M. and Weaver, D. R. (2002) Coordination of circadian timing in mammals. Nature (London) **418**, 935–941

3 Roenneberg, T. and Merrow, M. (2003) The network of time: understanding the molecular circadian system. Curr. Biol. **13**, R198–R207

4 Dunlap, J. C. (1999) Molecular bases for circadian clocks. Cell **96**, 271–290

5 Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H. and Reppert, S. M. (2000) Interacting molecular loops in the mammalian circadian clock. Science **288**, 1013–1019

6 Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S. and Weitz, C. J. (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science **280**, 1564–1569

7 Hogenesch, J. B., Gu, Y. Z., Jain, S. and Bradfield, C. A. (1998) The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. U.S.A. **95**, 5474–5479

8 Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H. and Reppert, S. M. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell **98**, 193–205

9 Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U. and Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell **110**, 251–260

10 Ueda, H. R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S. et al. (2002) A transcription factor response element for gene expression during circadian night. Nature (London) **418**, 534–539

11 Boudjelal, M., Taneja, R., Matsubara, S., Bouillet, P., Dolle, P. and Chambon, P. (1997) Overexpression of Stra13, a novel retinoic acid-inducible gene of the basic helix-loop-helix family, inhibits mesodermal and promotes neuronal differentiation of P19 cells. Genes Dev. **11**, 2052–2065

12 Rossner, M. J., Dorr, J., Gass, P., Schwab, M. H. and Nave, K. A. (1997) SHARPs: mammalian enhancer-of-split- and hairy-related proteins coupled to neuronal stimulation. Mol. Cell. Neurosci. **9**, 460–475

13 Shen, M., Kawamoto, T., Yan, W., Nakamasu, K., Tamagami, M., Koyano, Y., Noshiro, M. and Kato, Y. (1997) Molecular characterization of the novel basic helix-loop-helix protein DEC1 expressed in differentiated human embryo chondrocytes. Biochem. Biophys. Res. Commun. **236**, 294–298

14 Fujimoto, K., Shen, M., Noshiro, M., Matsubara, K., Shingu, S., Honda, K., Yoshida, E., Suardita, K., Matsuda, Y. and Kato, Y. (2001) Molecular cloning and characterization of DEC2, a new member of basic helix-loop-helix proteins. Biochem. Biophys. Res. Commun. **280**, 164–171

15 Honma, S., Kawamoto, T., Takagi, Y., Fujimoto, K., Sato, F., Noshiro, M., Kato, Y. and Honma, K. (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature (London) **419**, 841–844

16 Alvarez, J. D. and Sehgal, A. (2002) Circadian rhythms: finer clock control. Nature (London) **419**, 798–799

17 Jin, X., Shearman, L. P., Weaver, D. R., Zylka, M. J., de Vries, G. J. and Reppert, S. M. (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell **96**, 57–68

18 Shearman, L. P., Jin, X., Lee, C., Reppert, S. M. and Weaver, D. R. (2000) Targeted disruption of the mPer3 gene: subtle effects on circadian clock function. Mol. Cell. Biol. **20**, 6269–6275

19 Yamaguchi, S., Mitsui, S., Yan, L., Yagita, K., Miyake, S. and Okamura, H. (2000) Role of DBP in the circadian oscillatory mechanism. Mol. Cell. Biol. **20**, 4773–4781

20 Maemura, K., Hsieh, C. M., Jain, M. K., Fukumoto, S., Layne, M. D., Liu, Y., Kourembanas, S., Yet, S. F., Perrella, M. A. and Lee, M. E. (1999) Generation of a dominant-negative mutant of endothelial PAS domain protein 1 by deletion of a potent C-terminal transactivation domain. J. Biol. Chem. **274**, 31565–31570

21 Hida, A., Koike, N., Hirose, M., Hattori, M., Sakaki, Y. and Tei, H. (2000) The human and mouse Period1 genes: five well-conserved E-boxes additively contribute to the enhancement of mPer1 transcription. Genomics **65**, 224–233

22 Maemura, K., de la Monte, S. M., Chin, M. T., Layne, M. D., Hsieh, C. M., Yet, S. F., Perrella, M. A. and Lee, M. E. (2000) CLIF, a novel cycle-like factor, regulates the circadian oscillation of plasminogen activator inhibitor-1 gene expression. J. Biol. Chem. **275**, 36847–36851

23 Suardita, K., Fujimoto, K., Oda, R., Shimazu, A., Miyazaki, K., Kawamoto, T. and Kato, Y. (2002) Effects of overexpression of membrane-bound transferrin-like protein (MTf) on chondrogenic differentiation *in vitro*. J. Biol. Chem. **277**, 48579–48586

24 Vitaterna, M. H., King, D. P., Chang, A. M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W. and Takahashi, J. S. (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science **264**, 719–725

25 Sun, H. and Taneja, R. (2000) Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. Proc. Natl. Acad. Sci. U.S.A. **97**, 4058–4063

26 St-Pierre, B., Flock, G., Zacksenhaus, E. and Egan, S. E. (2002) Stra13 homodimers repress transcription through class B E-box elements. J. Biol. Chem. **277**, 46544–46551

27 Garriga-Canut, M., Roopra, A. and Buckley, N. J. (2001) The basic helix-loop-helix protein, sharp-1, represses transcription by a histone deacetylase-dependent and histone deacetylase-independent mechanism. J. Biol. Chem. **276**, 14821–14828
28 Butler, M. P., Honma, S., Fukumoto, T., Kawamoto, T., Fujimoto, K., Noshiro, M., Kato, Y. and Honma, K. (2004) Dec1 and Dec2 expression is disrupted in the suprachiasmatic nuclei of clock mutant mice. J. Biol. Rhythms **19**, 126–134
29 Schoenhard, J. A., Eren, M., Johnson, C. H. and Vaughan, D. E. (2002) Alternative splicing yields novel BMAL2 variants: tissue distribution and functional characterization. Am. J. Physiol. Cell Physiol. **283**, C103–C114
30 Li, Y., Xie, M., Song, X., Gragen, S., Sachdeva, K., Wan, Y. and Yan, B. (2003) DEC1 negatively regulates the expression of DEC2 through binding to the E-box in the proximal promoter. J. Biol. Chem. **278**, 16899–16907
31 Kawamoto, T., Noshiro, M., Sato, F., Maemura, K., Takeda, N., Nagai, R., Iwata, T., Fujimoto, K., Furukawa, M., Miyazaki, K. et al. (2004) A novel autofeedback loop of Dec1 transcription involved in circadian rhythm regulation. Biochem. Biophys. Res. Commun. **313**, 117–124
32 Azmi, S., Sun, H., Ozog, A. and Taneja, R. (2003) mSharp-1/DEC2, a basic helix-loop-helix protein functions as a transcriptional repressor of E box activity and Stra13 expression. J. Biol. Chem. **278**, 20098–20109
33 Grechez-Cassiau, A., Panda, S., Lacoche, S., Teboul, M., Azmi, S., Laudet, V., Hogenesch, J. B., Taneja, R. and Delaunay, F. (2004) The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. J. Biol. Chem. **279**, 1141–1150
34 Noshiro, M., Kawamoto, T., Furukawa, M., Fujimoto, K., Yoshida, Y., Sasabe, E., Tsutsumi, S., Hamada, T., Honma, S., Honma, K. and Kato, Y. (2004) Rhythmic expression of DEC1 and DEC2 in peripheral tissues: DEC2 is a potent suppressor for hepatic cytochrome P450s opposing DBP. Genes Cells **9**, 317–329
35 Li, Y., Zhang, H., Xie, M., Hu, M., Ge, S., Yang, D., Wan, Y. and Yan, B. (2002) Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation. Biochem. J. **367**, 413–422
36 Shen, M., Kawamoto, T., Teramoto, M., Makihira, S., Fujimoto, K., Yan, W., Noshiro, M. and Kato, Y. (2001) Induction of basic helix-loop-helix protein DEC1 (BHLHB2)/Stra13/Sharp2 in response to the cyclic adenosine monophosphate pathway. Eur. J. Cell Biol. **80**, 329–334
37 Shen, M., Yoshida, E., Yan, W., Kawamoto, T., Suardita, K., Koyano, Y., Fujimoto, K., Noshiro, M. and Kato, Y. (2002) Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. J. Biol. Chem. **277**, 50112–50120
38 Sun, H., Lu, B., Li, R. Q., Flavell, R. A. and Taneja, R. (2001) Defective T cell activation and autoimmune disorder in Stra13-deficient mice. Nat. Immunol. **2**, 1040–1047
39 Fu, L., Pelicano, H., Liu, J., Huang, P. and Lee, C. (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response *in vivo*. Cell **111**, 41–50

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