Behavioral and molecular studies on membrane-bound transporters implicated in the circadian rhythm of Drosophila melanogaster.

伊藤, 太一
九州大学大学院システム生命科学府

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Behavioral and molecular studies on membrane-bound transporters implicated in the circadian rhythm of *Drosophila melanogaster* .

Taichi Itoh

Graduate School of Systems Life Sciences,

Kyushu University,

Hakozaki,

Fukuoka 812-8581, Japan

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Abstract

ATP-binding cassette transporters (ABC transporters) are a kind of membrane proteins, which bind ATP and use the energy to drive the transport of various molecules across all cell membranes. The functions are widespread and well-conserved among organisms. In *Drosophila melanogaster*, there are 56 genes encoding ABC transporters. In this study, we examined whether there is a clock-related ABC transporter by performing a genome-wide screen with tissue-specific RNA interference (RNAi). We obtained five candidates with *tim(UAS)-gal4* in which all clock-related cells virtually express GAL4. Since their phenotypes were principally reproducible even with *pdf-gal4* in which only a subset of pacemaker neurons express GAL4, those transporters were presumed to function in pacemaker neurons. Those five candidates can be categorized into two groups according to the phenotype of knockdown flies. In one group, *CG9281* and *CG15410* (*E23*), knockdown flies altered the circadian period. In the other group, *CG5944*, *CG6052* and *CG18633*, a part of the knockdown flies became arrhythmic while the remaining part kept an intact rhythmicity. Our results imply that at least these five ABC transporters have a significant function in the *Drosophila* circadian system. Especially, one of the genes, E*arly gene at 23* (*E23*) is induced by the molting hormone ecdysone. Pacemaker neurons in fly head express *E23*, and its knockdown flies lengthened circadian period with an increased expression of the clock gene *vrille*. *E23* and *vrille* responded to both ecdysone and clock signals, whereas E23 protein specifically suppressed the ecdysone response and is necessary for rhythmicity. Thus, E23 forms its own feedback loop in the ecdysone response to control circadian oscillation through ecdysone-mediated *vrille* expression. The ecdysone signaling

pathway involving E23 is essential not only in developmental stage but also for the circadian behavior in adult fly.

Introduction

Daily rhythms in physiology, metabolism and behavior are found in a diverse array of organisms including prokaryotic and eukaryotic microbes, plants and animals. These rhythms are not passively driven by environmental cycles, but are controlled by endogenous circadian clocks that can keep circadian time even in the absence of environmental time cues. It is well-known that the core components and the regulation mechanisms of the endogenous circadian clocks are highly conserved through a variety of organisms. This remarkable conservation of circadian clocks throughout evolution suggests that maintaining temporal order is of fundamental importance.

The first clock mutants of *Drosophila melanogaster* were reported in 1971 (Konopka and Benzer 1971). They isolated three mutants carrying a point mutation in *period* (*per*) gene. These *per* mutants showed a short *period* (per^S), longer period (per^L) and arrhythmicity (per^0) in eclosion and activity rhythm (Konopka and Benzer 1971). The *per* gene was cloned by two groups (Bargiello et al. 1984; Zehring et al. 1984). Initially PER was thought to be a proteoglycan because *per* contains threonine-glycine repeat sequences. However, this hypothesis was rejected because the transformed fruit flies with the *per* gene lacking the entire threonine-glycine repeat region showed normal circadian rhythm (Yu et al. 1987). In 1990, Hardin et al. showed that PER is a transcriptional regulator, which negatively regulates own transcription (Hardin et al. 1990) and then the clock mutants were screened and the clock genes were identified (Dubruille and Emery 2008). These study lead to an idea that the circadian molecular mechanism is composed of interlocked feedback loops in *Drosophila*. PERIOD/TIMLESS (PER/TIM) clock proteins constitute the core of negative

transcriptional feedback loop, in which these clock proteins inhibit its own transcription. The transcription factors VRILLE (VRI) and PAR DOMAIN PROTEIN 1ε (PDP1ε), along with PER and TIM, form two transcriptional feedback loops interlocked through CLOCK/CYCLE (CLK/CYC)-mediated transcriptional activation (Sehgal et al. 1994; Sehgal et al. 1995; Gekakis et al. 1995; Myers et al. 1995; Glossop et al. 1999; Blau and Young 1999; Cyran et al. 2003).

In mammal, Vitaterna et al. isolated the first clock mutant and identified the gene, *circadian locomotor output cycles kaput* (*Clock*) with the progeny of mice treated with N-ethyl-N-nitrosourea (ENU) (Vitaterna et al. 1994). Then it was found that many components of the *Drosophila* circadian oscillator have orthologs and/or functional equivalents in mammals and the circadian oscillator in mammals is composed of interlocked transcriptional feedback loops as in *Drosophila*,. CLOCK/BMAL1 replaces CLK/CYC, mPER/mCRY replaces PER/TIM, REV-ERBα replaces VRI, RORa replaces PDP1ε. mPER/mCRY functions to repress CLOCK/BMAL1 transcription, but mCRY is the major repressor as opposed to PER in flies (Reppert and Weaver 2002). Although CRY functions as a circadian photoreceptor in flies, its role as a transcriptional repressor has been retained in at least some fly peripheral tissues (Collins et al. 2006). REV-ERBα and RORa are nuclear receptors rather than bZIP transcription factors like VRI and PDP1ε (Bell-Pedersen et al. 2005). Although there are several differences in circadian rhythm between *Drosophila* and mammals, the core molecular mechanism of circadian rhythm in mammals is similar to that of *Drosophila*. While the framework of the molecular mechanism of circadian oscillation has been extensively elucidated, we still have not identified all the molecules required for feedback loop functions.

In the previous studies, high-density oligonucleotide probe arrays have been applied to evaluate how gene expression is regulated by the circadian clock on a genome-wide basis (Claridge-Chang et al. 2001; McDonald and Rosbash 2001; Ceriani et al. 2002; Lin et al. 2002; Ueda et al. 2002). Although hundreds of clock-controlled genes were identified in *Drosophila*, we need to select the core genes which encode critical components of the feedback loops. One method to select core clock genes is to observe the circadian phenotypes of mutants of these genes. Such a mutant screening strategy has significantly facilitated the analyses of the clock mechanism in *Drosophila* (Konopka and Benzer 1971; Sehgal et al. 1994; Allada et al. 1998; Price et al. 1998; Rutila et al. 1998; Blau and Young 1999). However, this strategy possibly reaches critical limit to identify new clock genes when we apply it at a genome-wide level. In particular, mutants are not available in most of the candidate genes to induce lethality or developmental anomaly because many genes are expected to be widely expressed and be involved in the regulation of development. Indeed, it is well-known that *vri* and *Pdp1* widely expressed during the development (Glossop et al. 1999; Blau and Young 1999; Cyran et al. 2003). Therefore, we applied a new strategy of functional genomics by using tissue-specific RNAi (Matsumoto et al. 2007; Fig. 1). This strategy allowed us to isolate five candidates of the core clock genes, *clockwork orange* (*cwo*), *Early gene at 23* (*E23), CG5273, Imitation SWI* (*Iswi*) and *proliferation disrupter* (*prod*). Among these genes, *cwo* has already been identified as the core clock gene (Matsumoto et al. 2007; Lim et al. 2007; Kadener et al. 2007; Richier et al. 2008). *cwo* is rhythmically expressed and is directly regulated by CLK-CYC through its E-box sequence (Matsumoto et al. 2007; Lim et al. 2007; Kadener et al. 2007; Richier et al. 2008). CWO is able to suppress its own expression to compose the third feedback loop interlocking

with other two loops (Matsumoto et al. 2007; Lim et al. 2007; Kadener et al. 2007; Richier et al. 2008). The competition between CLK-CYC activity and CWO activity keeps E-box-mediated transcription and thereby *Drosophila* circadian clock is capable of generating a high-amplitude oscillation (Fig.2).

The molecular mechanism of circadian clock in *Drosophila* has been elucidated on the transcriptional and posttranslational control mechanisms of clock genes and their products (Hardin 2006; Rosbash 2009; Tomioka and Matsumoto 2010). However, little is known about the intercellular clock mechanism, especially the signaling pathway between cells. In addition to the transcriptional/translational factors, membrane proteins have been postulated to be important in the circadian system. The *pdf receptor* gene (*pdfr*) encodes one of the clock-related membrane protein (Mertens et al. 2005; Lear et al. 2005b; Hyun et al. 2005). PDFR functions as a membrane-bound receptor for Pigment-Dispersing Factor (PDF), a neuropeptide that mediates the synchronization among pacemaker cells (Taghert and Shafer 2006). Except for the other receptors of neurotransmitters (Sheeba et al. 2008), *narrow abdomen* (*na*) encoding Na^+/Ca^{2+} channel is the first membrane protein gene reported to function in the *Drosophila* circadian system (Lear et al. 2005a). This channel is thought to be critical for the neural output of the pacemaker cells. The *slowpoke* (*slo*) encoding Ca^{2+} sensitive K^+ channel is also reported to function in the output pathway of the circadian system (Fernández et al. 2007). The third ion channel related to the *Drosophila* circadian system is *Shaker congnate w* (*Shaw*), voltage-gated K^+ channels (Hodge and Stanewsky 2008). This channel modulates circadian behavior by controlling the neural release of PDF in pacemaker neurons. In the visual system of *Drosophila* the cyclic expression of Na^+/K^+-ATPase under clock control has been reported although its function in the

circadian system has not been fully elucidated yet (Górska-Andrzejak et al. 2009). All these results in *Drosophila* suggest that ion transports across plasma membrane are essential for the circadian clock. As compared to such extensive studies on ion transportation and membrane-receptors of neurotransmitters in the circadian system, little is known about other membrane proteins, e.g. transporters, hormone receptors and cell adhesion proteins.

We isolated one of the ATP binding cassette transporters (ABC transporter), *E23* (Itoh et al. 2011b) by a genome-wide screen using RNA interference (RNAi) (Matsumoto et al. 2007; Fig.1)based on the expression profiling of whole genes by high-density oligonucleotide arrays (Ueda et al. 2002). The ABC transporter forms a large family of transmembrane proteins in many organisms (Higgins 1992; Childs and Ling 1994; Dean and Allikmets 1995). Most of t the eukaryotic ABC transporters transport various molecules either within the cell as part of a metabolic process or from a cell to other organs, or secret from the body. The ABC transporter principally contains two domains. One is the ATP-binding domain(s) known as nucleotide-binding folds (NBFs) to function with the energy of ATP hydrolysis and the other is the transmembrane (TM) domains, although some exceptions have been found (Dean et al. 2001). In addition, the eukaryotic ABC transporters are organized either as full types containing two NBFs and two TMs, or as half transporters (Hyde et al. 1990). The latter forms either homodimers or heterodimers to constitute a functional transport. In eukaryotes, the phylogenetic analysis of the amino acid sequence of ABC transporters reveals eight clades; thus eight sub-families are designated as ABCA to ABCH (Dean et al. 2001). Six of these contain both NBF and TM, suggesting that members of these groups are responsible for the membrane transport process. The remaining two groups,

ABCE and ABCF, lack TM, implying that they are unlikely to be involved in transport functions through membranes (Kerr 2004). Because eight subfamilies are classified by structural similarity, it is not known whether common functional features can be seen even among ABC transporters belonging to the same group. In *Drosophila*, a total of 56 ABC transporters have been identified and classified into seven subfamilies because there are no genes in the ABCF subfamily. It is totally unknown whether the other ABC transporters function in the circadian clock. In this study, we examined whether ABC transporters were involved in circadian rhythm by tissue-specific RNAi screen and identified five candidates. Then we classified them into two groups according to the phenotype revealed in knockdown flies.

One of the candidates, *Early gene at 23* (*E23*) encodes a membrane-bound ABC transporter induced by the molting hormone ecdysone (Hock et al. 2000). The function of ecdysone is well studied during larval–pupal development (Kozlova and Thummel 2000). Ecdysone is produced and secreted from the prothoracic gland (Kiriishi et al. 1990) and converted to 20-hydroxyecdysone (20HE) mainly in the fat body (Petryk et al. 2003). 20HE binds to Ecdysone receptor/ultraspiracle heterodimer (EcR/USP) and activates ecdysone-induced genes through ecdysone response elements (EcREs) in the genome (Buszczak and Segraves 1998). Although lines of evidence have implied that the ecdysone signal is associated with circadian behavior in insect, little is known about its molecular function in circadian system especially in adult, for example, 20HE titer cycles in a circadian manner in *Rhodnius prolixus* larva (Steel and Vafopoulou 2006). The circadian oscillations of 20HE levels were also observed in the hemolymph and testes of adult males in cotton leafwarm, *Spodoptera littoralis* (Polanska et al. 2009). The prothoracic gland in *Drosophila* larvae and pupae, where

ecdysone is secreted, is reported to strongly express clock genes (Plautz et al. 1997) and is necessary for rhythmic eclosion under the control of pacemaker neurons (Myers et al. 2003). Interestingly, the clock gene *vri* was identified as a 20HE-induced gene by genome-wide screenings using tissue culture (Beckstead et al. 2005) and cell line Kc167 (Gauhar et al. 2009). Although those inductions by 20HE might be only reflection of the pleiotropic function of *vri* in development (George and Terracol 1997), ecdysone signaling might also affect *vri* expression in adults. In this study, we show that the ecdysone signaling pathway functions in the adult circadian system and that E23 controls the circadian clock in adult flies.

Materials and Methods

Fly strains

Flies were raised on standard glucose-cornmeal medium under 12-h light:12-h dark cycles (LD) cycle at 25°C. The w^{1118} strain was used as wild-type. RNA interference (RNAi) was induced in a tissue-specific manner using Gal4-UAS system (Itoh et al. 2011a; Fig.1). *tim(UAS)-gal4* (Blau and Young 1999) and *pdf-gal4* (Renn et al. 1999) were used as a driver line. Knockdown flies were obtained by mating the *gal4* females to the *UAS-IR* males established in the National Institute of Genetics or *UAS-KK* males at the Vienna *Drosophila* RNAi Center. *UAS-IR* and *UAS-KK* transgenic strains carry short fragments of the target gene in the head to head manner (Pili-Floury et al. 2004). This inversed repeat downstream of the *UAS* sequence is driven by Gal4 to induce a tissue specific RNA interference after the genetic cross of the responder line, *UAS-IR* or *UAS-KK*, to the *gal4* driver line. Overexpression flies was obtained by mating the homozygous *pdf-gal4* females to *UAS-E23* males established by injection of *UAS-E23* plasmids to w^{1118} embryos in our laboratory. Double knockdown flies were obtained by mating as described elsewhere (Itoh et al. 2011a). The *4xEcRE-luc* and *E23G4.5-gal4* transgenic strains were established by injection of the corresponding plasmids to w^{1118} embryos (BestGene Inc.).

Recording of locomotor activity rhythms

Flies were kept on standard glucose–cornmeal medium under LD cycles at 25 °C. We measured the locomotor activity of the adult flies using *Drosophila* activity monitors 2 (Trikinetics Inc.) for 3 days in LD cycles and then over 10 days in constant

darkness (DD). A single fly was introduced into a measuring glass tube containing 1.5% agar with 10% glucose. The periods were calculated by v2 periodogram (Sokolove and Bushell 1978) programmed by the Matlab R2007b software (MathWorks Inc.).

Statistical analysis and criterion for clock gene candidates

The period of an individual fly was calculated by periodogram analysis (Sokolove and Bushell 1978) with Clocklab software (Actimetrics) using data of at least fourteen consecutive days under constant darkness. The period of knockdown flies was compared statistically to that of the parental *gal4* lines as a control by Dunnett's multiple range test. Additionally, in order to isolate plausible candidates beyond various genetic backgrounds (Sharma et al. 2005) which contaminated in mating steps to establish transgenic line, we formulated a stricter criterion to certify the effective change of period; whether the difference of mean periods between control and a knockdown line is over two times of the value of standard deviation (SD) in control. Thus, a knockdown line showing the mean period of from 22.93 to 25.06 h in *tim(UAS)-gal4* background was excluded from the candidate even if its period was significantly different from that of control. Similarly we excluded a knockdown fly in *pdf-gal4* background showing the period from 23.26 to 24.92 h. The ratio of arrhythmic flies in a knockdown line was compared to that of the parental *gal4* line as a control by residual analysis. We identified the target gene as a candidate when the phenotype of its knockdown line met either criterion.

Luciferase assay

Cultured *Drosophila* S2 cells were plated in 24-well tissue culture plates in

Shields and Sang M3 insect medium (Sigma) supplemented with 12.5% fetal bovine serum (Biowest, Canada) and antibiotics (12.5 U/mL penicillin, 12.5 mg/mL streptomycin; GIBCO). S2 cells were transfected by Effectene Transfection Reagent (QIAGEN, Hilden, Germany). 20HE (Sigma) was provided 24 h after transfection. The luciferase assay was performed 48 h after transfection at least three times. The luciferase signals were normalized by *Renilla luciferase* (*Rluc*) activity and then by the activity of an appropriate control in each experiment.

Quantitative PCR analysis

The flies entrained for at least 3 days under LD were sampled every 4 h. Total RNA was purified from 100 heads at each time point as described elsewhere (Ueda et al. 2002). cDNA was synthesized with Ready-To-Go T-Primed First-Strand Kit (Amersham) according to the standard protocol. In Fig. 1, QPCR was performed using Mx3000P and Brilliant II SYBER Green QPCR Master Mix (Agilent Technologies). In Fig. 2, Q-PCR was performed using Applied Biosystems 7300 and Power SYBR Green PCR Master Mix (Applied Biosystems). PCRs were carried out with samples containing Master Mix,

0.5 μM primers, and 0.1 μL of synthesized cDNA in a 20μL volume by the amplification procedure as follows: 10 min at 95 °C, then 40 cycles of 15 s at 95 °C 30 s at 60 °C, and 1 min at 72 °C. *Gapdh2* expression levels were quantified and used as the internal control. The sets of primers for PCR are described below.

per-forward 5’-TACCCGCATCCTTCGCTTTTCT-3’

per-reverse 5’-AATGCACCCGGCACCTTCT-3’

tim-forward 5’-ACTTTGCTGACAACTCCCACTTCC-3’

tim-reverse 5’-CTCCGCAGGGTCAGTTTAACGAA-3’

vri-forward 5’-CATCACTACAGCCAGCAGAAGC-3’

vri-reverse 5’-ATATTGGATAGCCGGACGTTGT-3’

Pdp1-forward 5’-GCAACTGGTAATGGAAATGGTG-3’

Pdp1-reverse 5’-CTGTTCAAATGGTTGTGATGCTC-3’

cwo-forward 5’-ATCTGCGCCCAAGTGTACCT-3’

cwo-reverse 5’-TGCTTCTCCTCCATTTCCATTAAC-3’

Clk-forward 5’-GTCAGTTCGCAAAGCCA-3’

Clk-reverse 5’-CGGCTCAAGAAATGTCG-3’

E23-forward 5’-AGTGTTGAGGCAGTCGATATGGGA-3’

E23-reverse 5’-TGAGGACGTTGAGCTGGAAGATGA-3’

luc-forward 5’-GCTCCCGCTGAATTGGAATCCAT-3’

luc-reverse 5’-AGACCT TTCGGTACTTCGTCCACA-3’

GAPDH2-forward5’-CTACCTGTTCAAGTTCGATTCGAC-3’

GAPDH2-reverse 5’-AGTGGACTCCACGATGTATTCG-3’

Immunostaining

A laser-scanning confocal microscope LSM710 (Zeiss) was used to detect the fluorescent signals. Whole heads were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature and were dissected in PBS. Dissected heads were immunostained with anti-PDF rabbit antibody (Abdelsalam et al. 2008) as primary antibody diluted at1:6000 for overnight at 4 °C and Alexa594-conjugated anti-rabbit IgG antibody (Invitogen) as secondary antibody diluted 1:200 for 4 h at 4 °C.

Construction of the reporter and expression plasmids

The chemically synthesized complementary single-strand DNAs of *4xEcRE* that contains four copies of hsp27 EcRE (Riddihough and Pelham 1987; Cherbas et al. 1991) were annealed, extended by Taq polymerase (Takara), and subcloned into *pCR2.1* plasmid (Invitrogen). To construct *4xEcRE-luc* plasmid, the 4xEcRE-fragment doubly digested by *Spe*I and *Xba*I (New England Biolabs) was purified and cloned into *Nhe*I site of *TATA-luc* plasmid, which contains a minimal promoter region of the *hsp70* gene (Matsumoto et al. 2007). *pCaSpeR4-4xEcRE-luc* for transgenic flies was constructed as follows. *4xEcRE-luc* plasmid for culture cell was doubly digested by *Bam*HI and *Kpn*I. This fragment was cloned into *Kpn*I and *Bam*HI site of the *pCaSpeR4* (Thummel and Pirrotta 1992). To construct *vriG-luc*, *vri* promoter divided into three fragments was amplified by PCR with w^{1118} fly genome as a template. The distal fragment of *vri* promoter (4.8 kb) amplified using following primers with BamHI restriction site (underlined), 5'-GGGATCCTAAGCTGCTTGTGTGTGCGAGAGAGTGAG-3' and 5'-GGGATCCTTCATTCTCGATTTCCCCCATTTCCTCCAG-3', was cloned into *pCR2.1*. The middle fragment of *vri* promoter (4.0 kb) amplified using following primers, 5'-GGCTTTGCAATCGATCATTGAACTTTGGTTGTG-3' and 5'-AGGTACCTGATACAGTGGTCCTTCGAGAGAAGGAACTGCT-3', in which underlined sequences correspond to *Kpn*I site, was cloned into *pCR2.1*. The distal fragment was cloned into the *Bam*HI site of *pCR2.1* carrying the middle promoter fragment. The 8.8 kb of joined promoter of *vri* was cloned into the *Kpn*I site of *vriG2.5-luc* (Matsumoto et al. 2007) to constructed *vriG-luc* (11.3 kb). The *E23-luc* was constructed as follows. *E23* promoter fragment (2.0 kb) amplified by PCR with w^{1118} fly

genome as a template using primers,

5’-GTCGAAGACCCGCATCAAGCACGCAAC-3’ and

5’-GGGTACCCTATGCACAGAGCAACAATTTAG-3’, in which underlined sequences correspond to *Kpn*I site, was cloned into *pCR2.1*. The fragment was cloned into *Kpn*I site of the *TATA-luc* plasmid (Matsumoto et al. 2007). *E23G4.3-luc* was constructed by the SA-cloning method (Matsumoto and Itoh 2011) with the following primer sets. The templates for SA-cloning were w^{1118} fly genome and *pGL3-Basic* (Promega).

E23pro4.3S	5’-GTGTTTGTACTGCTACAAACTG-3’
E23pro4.3S-EFC1	5’-GGAGACATGTGTTTGTACTGCTACAAACTG-3’
E23pro4.3A	5’-TATCACCTTTTTGTGAACGCT-3’
E23pro4.3A-EFC2	5’-CGGGAAAGTTATCACCTTTTTGTGAACGCT-3’
pGL3-60S	5’-GCATTCCGGTACTGTTGGTAAA-3’
pGL3-60S-EFC2	5’-ACTTTCCCGGCATTCCGGTACTGTTGGTAAA-3’
pGL3-59A	5’-CAAGCTTACTTAGATCGCAG-3’
pGL3-59A-EFC1	5’-ATGTCTCCCAAGCTTACTTAGATCGCAG-3’

To construct *pAc5.1B-E23*, *E23* coding region amplified by PCR with a cDNA of w^{1118} fly using following primers with *Xba*I restriction site (underlined),

5’-CTCTAGACGAAAAAATGCCAATGAATTTG-3’ and

5’-TTCTAGACACGTCTTTTTGGGGCATCGC-3’, was cloned into *pCRII* (Invitrogen). The *E23* cDNA fragment digested by *Xba*I (New England Biolabs) was cloned into *Xba*I site of *pAc5.1B* (Invitrogen). To construct *UAS-E23* plasmid, *E23* coding region amplified by PCR with a cDNA of w^{1118} fly using following primers 5’-AAAATGCCAATGAATTTGATGAT-3’ and 5’-CGTCTTTTTGGGGCATCGCAG-3’ was cloned into *pIB/V5-His-TOPO* (Invitrogen). The *E23* fragment in *pIB/*

V5-His-TOPO was amplified by PCR using following primer sets with *Spe*I site (underlined), 5’-GACTAGTAAAATGCCAATGAATTTGATGAT-3’ and 5’-GACTAGTTCAATGGTGATGGTGATGATGAC-3’, and it was cloned into *pCRII* (Invitrogen). This fragment was digested by *Spe*I (New England Biolabs) and then cloned into *Xba*I site of *pUAST* (Brand and Perrimon 1993).

Results

Knockdown screening in ABC transporters with *tim(UAS)-gal4*.

We focused on ATP binding cassette transporters (ABC transporter) to screen for membrane proteins involved in circadian rhythm. *tim(UAS)-gal4* (Blau and Young 1999) was used as a driver strain to express double strand RNA which induces RNA interference (RNAi) for a target gene (Pili-Floury et al. 2004). Because *tim* expresses in virtually all clock-related cells (Kaneko and Hall 2000), this allowed us to screen for candidates which contribute to the circadian system, regardless of the tissue specificity of the target gene expression. The *tim(UAS)-gal4* flies exhibited a normal period (Table 1 and Fig. 3). We knocked down 54 out of the total of 56 ABC transporter genes in *Drosophila* while two could not be done because *UAS-IR* strains have not been established (Table 1, 2 and 3).

Our statistical analysis revealed that there are seven knockdown lines, *CG8523* (*Multi drug resistance 50*: *Mdr50*), *CG9270*, *CG9281*, *CG17632* (*brown*: *bw*), *CG32091*, *CG4314* (*scarlet*: *st*), and *CG15410* (*Early gene at 23*: *E23*) whose periods were significantly different from *tim(UAS)-gal4* (Dunnett's multiple range test, $p<0.05$). Additionally, in three knockdown lines, *CG5944*, *CG6052* and *CG18633*, arrhythmic flies significantly increased (residual analysis, $p<0.05$). Of those ten lines, based on our criterion (see Materials and Methods) we further picked up the following five genes as more plausible candidates for a clock-related gene; *CG5944*, *CG6052*, *CG9281*, *CG18633* and *CG15410* (*E23*) (Fig. 3).

Knockdown screening in ABC transporters with *pdf-gal4*.

Next, we used *pdf-gal4* to induce RNAi only in a subset of pacemaker neurons

(Kaneko and Hall 2000). The result is shown in Tables 1, 2 and 3. *pdf-gal4* flies showed normal rhythmicity while five knockdown lines, *CG11897*, *CG9281*, *CG17632* (*bw*), *CG32091* and *CG15410* (*E23*) showed a longer period (Dunnett's multiple range test, $p<0.05$). The ratio of arrhythmic flies was significantly higher in three knockdown lines, *CG5944*, *CG6052* and *CG18633* (residual analysis, $p<0.05$). As a result, the following five genes met our criterion as the clock-related gene candidates, *CG5944*, *CG6052*, *CG9281*, *CG18633* and *CG15410* (*E23*). The typical phenotypes of these lines are illustrated in Fig. 4. Interestingly, these candidates are completely coincident with those obtained in the previous screen with *tim(UAS)-gal4* (Fig. 3). Since RNAi is commonly induced in pacemaker neurons with these two *gal4* drivers, it is possible that *CG5944*, *CG6052*, *CG9281*, *CG18633* and *CG15410* (*E23*) function at least in PDF positive/ TIM positive cells. These cells correspond to small lateral neuron ventrals (s-LNvs) and large lateral neuron ventrals (l-LNvs), which are thought to be important to controlling locomotor rhythmicity (Helfrich-Förster 2005).

_E23_ affects the circadian rhythm in pacemaker neurons.

To knockdown the expression of *E23* specifically in brain pacemaker neurons, we established transgenic flies carrying *pdf-gal4* as a pacemaker neuron-specific driver (Renn et al. 1999) and two copies of *E23-IR* to express the double-stranded RNA of *E23*. Half of the flies became arrhythmic and the remaining flies had a longer period (Fig. 5A and Table 4). Homozygous knockdown flies showed a more drastic phenotype in that all flies tested lost circadian rhythmicity. When we overexpressed *E23* in pacemaker neurons with two copies of *UAS-E23* transgene, three-quarter of flies became arrhythmic (Table 4). These results suggest that the levels of *E23* expression are

important for maintaining circadian oscillator function in adult pacemaker neurons. In fact, the quantitative PCR (Q-PCR) analysis of *E23* knockdown flies revealed that the expression level of the clock genes *per*, *tim*, *Clk* and *Pdp1ε* decreased with one exception, *vri*, whose level increased (Fig. 5B).

***E23* expression and 20HE response oscillate in a circadian manner.**

The expression level of *E23* revealed circadian oscillation with the peak at ZT17 when we quantified it by Q-PCR using whole bodies in wild-type as a template (Fig. 6A). Although the minor peak is seen at ZT9, the value is not significantly different from that at the trough phase (Tukey-test, $p > 0.05$). Because *E23* is reported to be induced by ecdysone during developmental stages (Hock et al. 2000), we checked if a daily change could be detected in the 20HE response. We established the transgenic strain carrying *4xEcRE-luc* reporter (Poels et al. 2004) which contains four ecdysone response elements (4xEcRE) upstream of a *luciferase* (*luc*) gene. The relative levels of the 20HE response were assessed through measurements of *4xEcRE-luc* expression levels by Q-PCR using whole body in wild-type as a template. We observed an obvious rhythm peaking at ZT17, similar to that of *E23* expression in whole body (Fig. 6B). The rhythmic change in the 20HE response we observed is very consistent with previous results obtained with a different method (Ishimoto and Kitamoto 2010). To examine whether the 20HE response relates to the rhythm, we knocked down two transcription factors in the 20HE signaling pathway using *pdf-gal4*. The *EcR* knockdown flies showed a slightly longer period while half of the *usp* knockdown flies were arrhythmic (Table 4). Two thirds of the double knockdown flies of *EcR* and *usp* became arrhythmic while the remaining lengthened the circadian period to 27 hours (Table 4). Additionally over a half of the

knockdown flies of *phantom* (*phm*), one of hydroxylases for ecdysone biosynthesis (Huang et al. 2008), also showed arrhythmicity and the remaining revealed a slightly longer period (Table 4). Based on these results the 20HE signaling pathway is involved in the generation of circadian oscillations in adult pacemaker neurons. We then measured the mRNA expression rhythm of *E23* in wild-type heads. Interestingly, it oscillated with the peak at ZT1 (Fig. 6C), almost antiphase to that in whole body (Fig. 6A) and the 20HE response rhythm (Fig. 6B). Considering that ecdysone ought to rapidly spread throughout whole body, this result implies that the expression of *E23* in adult heads is not limited to the 20HE response.

***E23* expresses in pacemaker neurons in adult head.**

In searching for homologous sequence to the canonical EcRE where EcR/USP binds (Reddihough and Pelham 1987; Cherbas et al. 1991) in a ~23kb genomic region spanning *E23*, we found seven EcRE-like sequences (EcRE-Ls). Interestingly, three were in close proximity to one another and within 1.8 kb of the third intron along with two E-boxes (Darlington et al. 1998) (Fig. 7A). As an intronic enhancer can regulate gene expression (Ott et al. 2009), a *E23G4.5-gal4* transgene containing 4.5 kb of the genomic region around this intron was established and crossed with the *UAS-mCD8::GFP* line (Lee and Luo 1999). GFP expression was detected in pacemaker neurons of the resulting progeny, as revealed by co-staining with an anti-PDF antibody that marks pacemaker neurons (Fig. 7B). In contrast, using 4.3 kb of the upstream genomic region to the border of the neighboring gene, no evidence of gene regulation was obtained *in vivo* and *in vitro* (Fig. 8). Thus, *E23G4.5-gal4* is expressed in at least a subset of brain pacemaker cells, which likely represents a portion of the *E23* expression pattern in the adult brain.

E23 protein suppresses 20HE response.

E23 protein is reported to modulate the 20HE response in cultured larval tissue (Hock et al. 2000). This modulation was confirmed in cultured *Drosophila* S2 cells using *4xEcRE-luc* as a reporter. The activity of *luc* increased as a function of the concentration of 20HE, which ranged from 1 to 200 nM (Fig. 9A). The induction of *4xEcRE-luc* with 100 nM of 20HE decreased in proportion to the amount of co-transfected plasmid expressing *E23* (Fig. 9B). A similar result was obtained with *E23G2.0-luc*, which carries the genomic region around the third intron of *E23* gene as described above. When 20HE was provided to S2 cells, the activity of *E23G2.0-luc* increased about 10-fold (Fig. 9C). This induction was sharply reduced when the E23 protein was co-expressed. Next, we investigated whether the two E-box sequences in *E23G2.0-luc* respond to CLK/CYC (Darlington et al. 1998) (Fig. 9C). The *luc* activity increased about 30-fold when CLK was induced in S2 cells. When both 20HE and CLK were provided, a remarkable increase was observed, suggesting that this region can be synergistically regulated by the 20HE signaling and circadian clock, at least *in vitro*. When the E23 protein was provided with 20HE and CLK, *E23G2.0G-luc* activity stayed at the same level as when only CLK was provided (Fig. 9C). Taken together, *E23* can respond to both the 20HE signal and circadian regulation, while the E23 protein specifically negates the 20HE response.

***vri* respond to 20HE and CLK/CYC.**

We also identified eight EcRE-Ls around the genomic region of *vri* (Fig. 10). The *vriG-luc* reporter plasmid carrying five out of eight EcRE-Ls under CLK

co-expression became higher when 20HE were provided, and this induction was negated by further providing the E23 protein (Fig. 9D). The regulatory region of *per*, *tim*, *Pdp1ε* and *cwo* neither have EcRE-Ls nor show the 20HE response (Fig. 11). In the case of *vriG-luc*, an additive effect of the 20HE response and clock regulation was observed, but the 20HE response was specifically negated by providing the E23 protein (Fig. 9D). Thus, *vri* also responds to both the 20HE signal and circadian regulation while the E23 protein can specifically negate this 20HE response.

Discussion

In this study, we focused on whether ABC transporters are involved in the circadian rhythms in *Drosophila* adult flies. To avoid lethality, we performed tissue-specific RNAi with two different Gal4 lines and classified the five candidates into two groups according to the phenotypes of knockdown flies. In one group, the knockdown flies in *CG9281* and *CG15410* (*E23*) showed a longer period but never become arrhythmic regardless of the *gal4* drivers (Table 2 and 3). In the other group, the knocking down of a target gene only induced arrhythmicity even though the ratios of arrhythmic flies were different among the *gal4* drivers, and the circadian period was intact if there were some rhythmic flies. For example, about 30% and 50% were arrhythmic in *CG5944* knockdown flies with *tim(UAS)-gal4* and *pdf-gal4*, respectively, and the remaining flies exhibited normal behavior (Table 1). In the knockdown flies of *CG6052*, the ratio of arrhythmic flies was almost 100% with *tim(UAS)-gal4* and 38% with *pdf-gal4* (Table 1). In the case of *CG18633*, all flies became arrhythmic with *tim(UAS)-gal4* while 69% of knockdown flies showed arrhythmicity with *pdf-gal4* driver. Even in those lines, the mean period of rhythmic flies was not significantly different from that of the parental *gal4* driver.

The phenotypes of knockdown flies with *tim(UAS)-gal4* are qualitatively similar to but quantitatively slightly different from those with *pdf-gal4* in *CG5944*, *CG6052* and *CG18633*. This difference might be caused by the tissue specificity where the knocking down of the target gene is induced, the efficiency of dsRNA expression level or both, depending on the *gal4* driver. Regarding the efficiency of knocking down, we reported that the expression level of *cwo* reduced to one third at the peak level in the

head of *tim(UAS)gal4*; *UAS-cwoIR* knockdown flies (Matsumoto et al. 2007). Because the tissue specificities of *cwo* and *tim* expressions are thought to be consistent in fly head, this efficiency is assumed to reflect the knockdown efficiency in each cell. In the case of *pdf-gal4*, there is no report that has shown the general knockdown efficiency in each PDF positive cell. Moreover because PDF expresses in a limited number of cells in the brain as compared to *tim*, it is difficult to estimate an exact knockdown efficiency when the target gene expresses in broader area of fly head than PDF. More detailed analysis is necessary to further discuss the quantitative difference of knockdown effect among *gal4* drivers.

There are eight subfamilies of ABC transporters in *Drosophila*. *CG15410* (*E23*) and *CG18633* belong to the G subfamily, which contains half type transporters (Dean et al. 2001). Since half type transporters generally dimerize to function to each other, one possibility is that CG15410 (E23) and CG18633 proteins heterodimerize to function in pacemaker neurons although our preliminary data using a two hybrid system for membrane proteins (Johnsson and Varshavsky 1994; Stagljar et al. 1998) do not support this hypothesis (data not shown). Thus the alternative possibility is that CG15410 (E23) and CG18633 proteins independently form a homodimer. We recently found that E23 protein controls circadian rhythm through mediating a molting hormone ecdysone signaling (Itoh et al. 2011b). Because there has been no report suggesting that *CG18633* contributes to the ecdysone signaling pathway, *CG15410* (*E23*) and *CG18633* possibly function in the circadian system independently. *CG9281* belongs to E subfamily lacking transmembrane domains (Dean et al. 2001). This means that *CG9281* encodes a soluble protein rather than a membrane protein. Although the function of *CG9281* in the circadian system is completely unknown, we might be able to get some

hints to elucidate its function from the fact that members of soluble ATP transporters play a role in translation initiation and ribosome biogenesis in *Drosophila* (Coelho et al. 2005; Andersen and Leevers 2007) as well as in other organisms (Kerr 2004). Further analyses are awaited to clarify whether CG9281 protein affects a post-transcriptional regulation of known clock genes.

CG5944 and *CG6052* belong to the A subfamily. There is also no information on the function of their proteins except that they are membrane-bound ABC transporters. Since ABC transporters basically transport various substrates across extra- or inter-cellular membranes (Dean et al. 2001), the transported molecules are probably important in controlling circadian behavior.

We focused on E23 and showed that *E23* and *vri* are dually regulated by the 20HE signal and circadian clock while the 20HE regulation is suppressed by E23 protein (Fig. 9C and D). Thus *E23* is capable of affecting circadian oscillation through the regulation of *vri*. Based on this hypothesis, the phenotypes observed in *E23* knockdown flies (Fig. 5A and Table 4) are explained as follows. The depression of 20HE response caused by a loss of E23 protein induced a higher expression of *vri* as observed in our Q-PCR analysis (Fig. 5B). A higher expression of *vri* lengthened or abolished the circadian period with suppression of *Clk* expression as previously reported (Blau and Young 1999).

In vertebrates, melatonin, which is a kind of hormone, is rhythmically produced and released by the pineal gland. First melatonin was thought to be a coupling factor but has no effect on the generation or maintenance of the circadian oscillator in the suprachiasmatic nucleus (Stehle et al. 2003). This function of melatonin contrasts with that of 20HE we found in this study. The 20HE response is necessary for circadian

oscillation in adult pacemaker neurons because the double knockdown flies of *EcR* and *usp*, which are the 20HE signaling pathway genes, revealed the abnormal circadian phenotype (Table 4). In addition, behavior arrhythmicity was also induced even in a pacemaker neuron-specific knocking down of *phm*, which encodes one of hydroxylases for ecdysone biosynthesis (Huang et al. 2008). This implies that ecdysone, which is necessary for circadian oscillation in adult fly, is synthesized in pacemaker neurons themselves although we cannot exclude the possibility that *phm* affects circadian rhythm through a metabolic pathway other than the ecdysone biosynthesis.

Since ecdysone and its metabolites are lipophilic, a membrane-bound transporter like *E23* might not be necessary to import and export them. Considering that E23 protein depresses the 20HE response, this ABC transporter might be associated with the reduction of an amount of intra-cellular 20HE. The E23 transporter might directly discharge 20HE to extra-cell or isolate it into intra-cellular vesicles. If this is the case, the excess of E23 protein causes to decrease an amount of intra-cellular 20HE and it makes flies arrhythmic through depression of the 20HE response as observed in double knockdown flies of *EcR* and *usp* (Table 4). In fact, we observed that *E23* overexpression abolished the circadian rhythmicity *in vivo* (Table 4).

The expression rhythm of *E23* in head was almost antiphase to that in whole body (Fig. 6A and C). The peak of the latter was consistent with that of the 20HE response (Fig. 6A and B). Given that *E23* mainly expresses in pacemaker neurons as observed using *E23G4.5-gal4* (Fig. 7B), the phase in head possibly reflects the expression profile in pacemaker neurons which rhythmically express clock genes. Because the peak phase of *E23* expression in head is close to those of CLK abundance and phospholylation (Lee et al. 1998), the phase angle difference between *E23*

expressions in head and body is probably caused by the synergy activation of *E23* by the 20HE response with CLK as observed *in vitro* (Fig. 9C). In mammals, a combination of two cis elements, one is activated in day-time and the other in night-time, reported to shift the expression phase to evening-time and such kind of phase modulation is required for mammalian clock function. (Ukai-Tadenuma et al. 2011). The antiphase expression of *E23* in head and body likely has a significant physiological role in *Drosophila* clock function.

We can propose three possibilities regarding the significance of the rhythmic expression of *E23* in head and body. First, E23 protein may just maintain the 20HE response within the range necessary for the function of the circadian clock both in head and body, then the oscillation of *E23* expression itself may not be essential for generating circadian rhythm. Second, only the rhythmic expression of *E23* in head is necessary to generate circadian rhythm in pacemaker neurons. Third, the rhythmic expressions of *E23* in head and body might be important to organize the *Drosophila* circadian system, implying that ecdysone works as an inter-cellular signaling molecule to synchronize the *Drosophila* circadian system as discussed below. A future study will allow us to address the latter two possibilities through examining the circadian rhythm of flies where *E23* is knocked down only in the peripheral tissues. Despite of a rhythmicity of the amount of intra-cellular 20HE, since *Clk* is rhythmically expressed in clock related cells, there might be many genes rhythmically regulated by the synergy effect of the 20HE response with CLK. Such ecdysone-induced genes possibly associate a broad spectrum of physiological phenomena. One of typical examples rhythmically regulated by ecdysone is the sleep-arousal behavior (Ishimoto and Kitamoto 2010).

There are two reports to discuss an existence of a humoral factor in the

Drosophila circadian system, which spreads throughout the whole body, based on transplantation experiments. In one report, a brain of per^s transplanted into an abdomen of an arrhythmic per^0 host could restore the circadian locomotor rhythm in the host, suggesting that *Drosophila* has a humoral factor to the output of circadian clock (Handler and Konopka 1979). In the other report, Malpighian tubules transplanted from wild-type fly into the oppositely entrained wild-type host showed the molecular oscillation of *per* and *tim* expression out of phase with the host despite sharing the same hormonal milieu (Giebultowicz et al. 2000). This appears to deny an existence of a humoral factor in *Drosophila* circadian system. Our present study suggests that the circadian oscillation in *Drosophila* is maintained by not only the transcriptional-translational feedback loops of clock genes but also the ecdysone signaling pathway. Since 20HE is a typical mediator of an inter-cellular signal to spread throughout whole body, an attractive hypothesis is that 20HE acts as a coupling factor as well. In this case, *E23* is important not only to maintain the amount of intra-cellular 20HE in pacemaker neurons but also that of inter-cellular one. Identification of the molecular mechanism of the humoral control of *Drosophila* circadian clock is the next major challenge in circadian biology.

In this study, we identified four candidates and one core clock gene of the clock-related ABC transporter by using the tissue-specific RNAi method. Although we do not have enough information about the relationship between their structural homology and their functions especially in regard to what molecules they transport, this study should open a new window to analyze circadian rhythm by focusing on small molecules transported across cell-membranes by membrane-bound transporters.

Tables

Table 1 The circadian periods of single knockdown flies in A and B subfamilies.

Sub-family	*Gene*	*tim(UAS)-gal4*			*pdf-gal4*		
		Period (mean ± SEM) (h)	N_R	N_A	Period (mean ± SEM) (h)	N_R	N_A
control	*promoter-gal4*	24.00 ± 0.12	21	0	24.09 ± 0.11	14	0
A	*CG8473*	23.98 ± 0.06	16	0	24.33 ± 0.08	12	0
	CG1819	24.26 ± 0.11	13	0	24.34 ± 0.14	12	0
	CG1718	23.90 ± 0.03	24	0	24.31 ± 0.12	17	0
	CG1801	24.26 ± 0.05	23	0	24.33 ± 0.10	23	0
	CG1494	24.46 ± 0.07	14	0	24.28 ± 0.15	23	1
	CG7491	24.22 ± 0.14	11	0	24.06 ± 0.09	7	0
	CG8908	24.13 ± 0.09	17	0	24.15 ± 0.08	8	0
	CG5944	23.52 ± 0.14	11	4*	24.49 ± 0.41	8	7*
	CG6052	23.80	1	18*	23.80 ± 0.39	13	8*
	CG4794	23.98 ± 0.05	16	0	23.98 ± 0.07	14	1
B	*CG4794*	24.10 ± 0.06	24	0	23.88 ± 0.14	12	0
	CG1824	23.82 ± 0.06	5	0	23.98 ± 0.05	8	0
	CG17338	n.e.	-	-	n.e.	-	-
	CG10441	24.31 ± 0.15	15	0	24.11 ± 0.08	10	0
	CG3879 (*Mdr49*)	24.14 ± 0.06	8	0	24.44 ± 0.05	8	0
	CG8523 (*Mdr50*)	24.66 ± 0.05[a]	8	0	24.05 ± 0.08	8	0
	CG7955 (*ABCB7*)	23.95 ± 0.07	8	0	24.07 ± 0.16	6	0
	CG10226	24.21 ± 0.12	11	0	24.54 ± 0.10	22	1
	CG10181 (*Mdr65*)	24.20 ± 0.10	8	0	24.05 ± 0.13	8	0
	CG4225 (*Hmt-1*)	24.13 ± 0.08	8	0	24.44 ± 0.20	20	2

N_R and N_A represent number of rhythmic and arrhythmic flies, respectively.

a: Significantly different from the period of control (Dunnett's multiple range test, $p<0.05$).

*: Significantly different from the ratio of control (residual analysis, $p<0.05$).

n.e.: not established *UAS-IR* line.

Table 2 The circadian periods of single knockdown flies in C, D and E subfamilies.

Sub-family	*Gene*	*tim(UAS)-gal4*			*pdf-gal4*		
		Period (mean ± SEM) (h)	N_R	N_A	Period (mean ± SEM) (h)	N_R	N_A
control	*promoter-gal4*	24.00 ± 0.12	21	0	24.09 ± 0.11	14	0
C	*CG7806*	24.06 ± 0.03	36	0	24.39 ± 0.08	12	0
	CG7627	24.18 ± 0.06	24	1	24.45 ± 0.13	22	0
	CG5772 (*Sur*)	23.88 ± 0.05	16	0	23.81 ± 0.05	15	0
	CG6214 (*MRP*)	23.98 ± 0.07	13	1	24.12 ± 0.09	13	0
	CG9270	24.63 ± 0.07[a]	7	0	24.06 ± 0.17	9	1
	CG8799 (lethal(2)03659)	24.12 ± 0.06	15	0	24.13 ± 0.08	8	0
	CG10505	24.17 ± 0.12	12	1	24.16 ± 0.07	10	0
	CG14709 (*Mrp4*)	24.04 ± 0.04	16	0	24.15 ± 0.08	15	1
	CG4562	24.28 ± 0.10	8	0	24.43 ± 0.14	14	0
	CG5789	24.34 ± 0.47	5	0	24.07 ± 0.09	6	1
	CG11898	24.31 ± 0.11	15	0	24.45 ± 0.08	10	0
	CG11897	24.41 ± 0.13	15	0	24.86 ± 0.27[a]	8	1
D	*CG12703*	n.e.	-	-	n.e.	-	-
	CG2316	24.15 ± 0.11	10	0	24.22 ± 0.06	12	0
E	*CG1703*	24.21 ± 0.14	10	1	24.31 ± 0.05	7	0
	CG9281	25.37 ± 0.08[a,*]	25	0	25.63 ± 0.15[a,*]	8	0
	CG5651 (*pix*)	23.98 ± 0.06	13	0	24.38 ± 0.20	5	0
	CG9330	24.25 ± 0.27	6	0	24.10 ± 0.11	10	0

N_R and N_A represent number of rhythmic and arrhythmic flies, respectively.

a: Significantly different from the period of control (Dunnett's multiple range test, $p<0.05$).

*: The period is out of range in mean ± 2SD in control.

n.e.: not established *UAS-IR* line.

Table 3 The circadian periods of single knockdown flies in G and H subfamilies.

Sub-family	*Gene*	*tim(UAS)-gal4*			*pdf-gal4*		
		Period (mean ± SEM) (h)	N_R	N_A	Period (mean ± SEM) (h)	N_R	N_A
control	*promoter-gal4*	24.00 ± 0.12	21	0	24.09 ± 0.11	14	0
G	*CG2759* (*w*)	23.98 ± 0.13	6	0	24.31 ± 0.06	16	0
	CG3164	24.26 ± 0.11	12	0	24.35 ± 0.14	16	0
	CG4822	24.22 ± 0.18	6	0	24.31 ± 0.14	15	0
	CG17646	24.52 ± 0.32	6	0	23.73 ± 0.13	6	0
	CG9892	24.18 ± 0.06	24	0	24.47 ± 0.13	6	0
	CG9664	24.18 ± 0.07	29	0	24.20 ± 0.06	15	0
	CG9663	24.27 ± 0.17	9	2	24.50 ± 0.07	12	0
	CG2969 (*Atet*)	24.34 ± 0.09	8	0	24.03 ± 0.14	14	0
	CG5853	24.25 ± 0.08	11	0	24.41 ± 0.09	15	0
	CG17632 (*bw*)	24.92 ± 0.11[a]	16	0	24.85 ± 0.11[a]	16	0
	CG32091	24.48 ± 0.12[a]	13	0	24.90 ± 0.10[a]	22	0
	CG4314 (*st*)	24.52 ± 0.25[a]	11	0	24.18 ± 0.08	14	0
	CG18633	n.d.	0	16[b]	24.84 ± 0.55	5	11[b]
	CG11069	24.34 ± 0.05	24	0	24.32 ± 0.32	6	0
	CG15410 (*E23*)	25.18 ± 0.13[a,*]	41	0	25.30 ± 0.13[a,*]	40	0
H	*CG6162*	24.43 ± 0.16	11	0	24.70 ± 0.27	15	0
	CG9990	24.50 ± 0.15	5	0	24.16 ± 0.10	12	0
	CG11147	24.36 ± 0.12	7	0	24.22 ± 0.14	6	0

N_R and N_A represent number of rhythmic and arrhythmic flies, respectively.

a: Significantly different from the period of control (Dunnett's multiple range test, $p<0.05$).

b: Significantly different from the ratio of control (residual analysis, $p<0.05$).

*: The period is out of range in mean ± 2SD in control.

n.d.: not determined.

Table 4 Free-running periods of knockdown flies of *E23* and 20HE signaling pathway genes.

Strains	Period (mean ± SEM) (h)	N_R	N_A
wild-type	23.70 ± 0.07	39	2
pdf-gal4; Sco/+	24.15 ± 0.05	12	0
E23-IR1; E23-IR2	24.02 ± 0.08	9	1
pdf-gal4; E23-IR1/+; E23-IR2/+	27.65 ± 0.28[ab]	20	18[*]
pdf-gal4; E23-IR; E23-IR2	−	0	12[*]
UAS-E23	23.52 ± 0.06	12	4
pdf-gal4; UAS-E23/+	24.23 ± 0.06[b]	29	10
pdf-gal4; UAS-E23	24.19 ± 0.18[b]	9	26[*]
EcR-IR2	23.86 ± 0.05	12	1
usp-IR1	24.18 ± 0.06	15	0
pdf-gal4;; EcR-IR2/+	24.63 ± 0.07[ab]	12	0
pdf-gal4; usp-IR1/Sco	24.13 ± 0.29	8	11[*]
pdf-gal4; usp-IR1/+; EcR-IR2/+	26.97 ± 0.63[ab]	7	15[*]
pdf-gal4;phm-KK/Sco	24.88 ± 0.85[a]	4	7[*]

N_R and N_A represent number of rhythmic and arrhythmic flies, respectively.

a: Significantly different from the period of *pdf-gal4* (Dunnett's multiple range test, $p<0.05$).

b: Significantly different from the period of parental strain other than *pdf-gal4* (Dunnett's multiple range test, $p<0.05$).

[*]: Significantly different from the arrhythmic rate in each of parental strain other than *pdf-gal4* (χ^2test, $p<0.05$)

—: not determined.

Figures

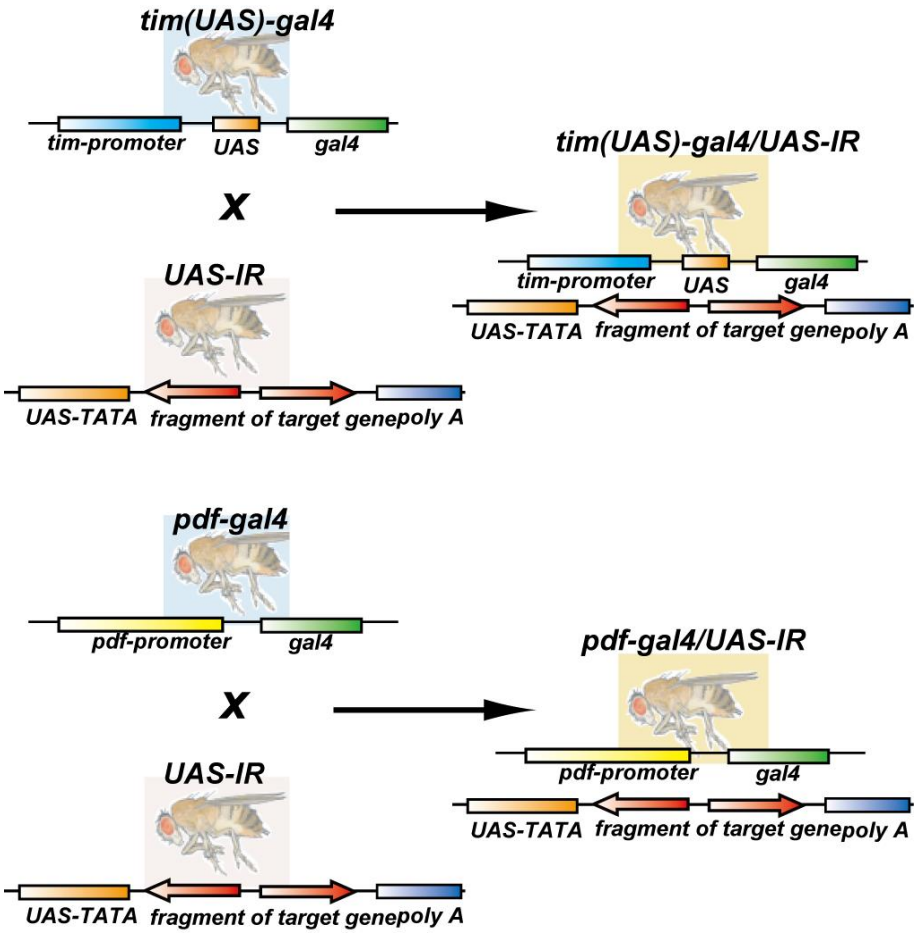

Figure 1 Tissue-specific RNAi screening

Double-stranded RNAs (dsRNA) of each target gene was induced by Gal4-UAS system in a tissue-specific manner to minimize unexpected side effects. Single knockdown in *tim*-positive cells and in *pdf*-positive cells. Gal4 is expressed under the control of each promoter (*tim(UAS)-gal4* and *pdf-gal4*, respectively). To strongly enhance the expression of Gal4-UAS sequence is inserted between *tim*- promoter and gal4 sequence. (*tim(UAS)-gal4*). The short fragments of the target gene are connected downstream of UAS sequence in head-to-head manner (UAS-IR). *tim(UAS)-gal4* and *pdf-gal4* lines are mated with *UAS-IR* lines. RNAi can be induced in tim positive cells of the progeny produced from the cross as described above.

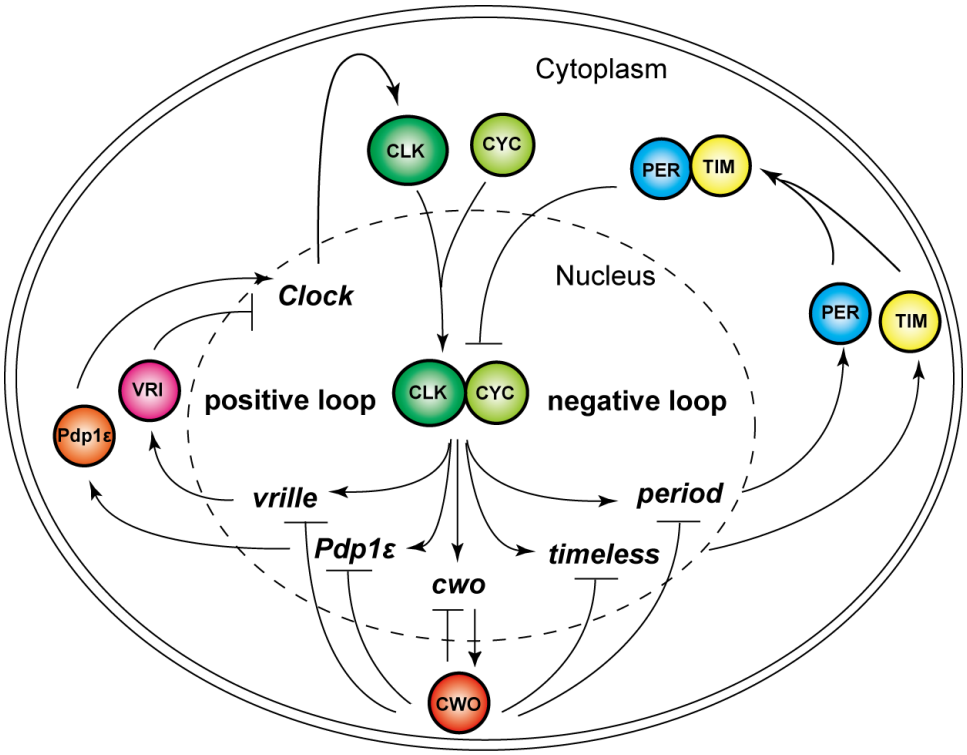

Figure 2　Circadian Molecular mechanism in *Drosophila.*

In *Drosophila*, circadian molecular mechanism is composed of triple feedback loops. Little is known about molecular mechanisms that control the *Drosophila* circadian clock beyond the transcriptional-translational feedback regulation of clock genes as an intra-cellular process.

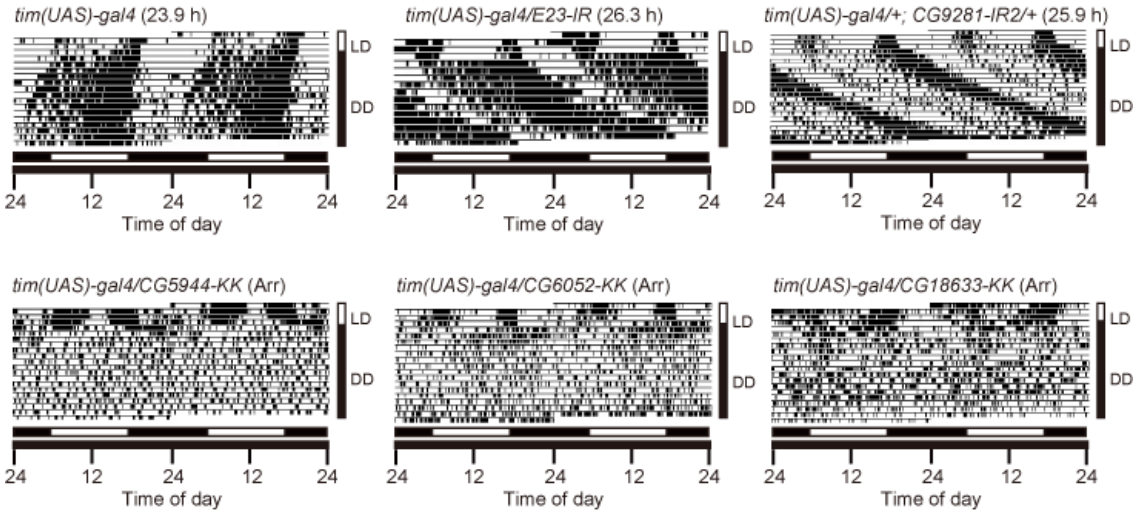

Figure 3　Actograms of knockdown flies of the candidate genes with *tim(UAS)-gal4.*

The genotype of knockdown fly is described at each actogram. The number in parenthesis represents the free-running period of the corresponding fly while Arr means arrhythmic. Adult flies were entrained in LD cycle for 3 days, and then kept in constant darkness (DD). The horizontal bars in white and black represent the light and dark regime of the LD cycle, respectively. White vertical bar indicates LD; black vertical bar indicates DD.

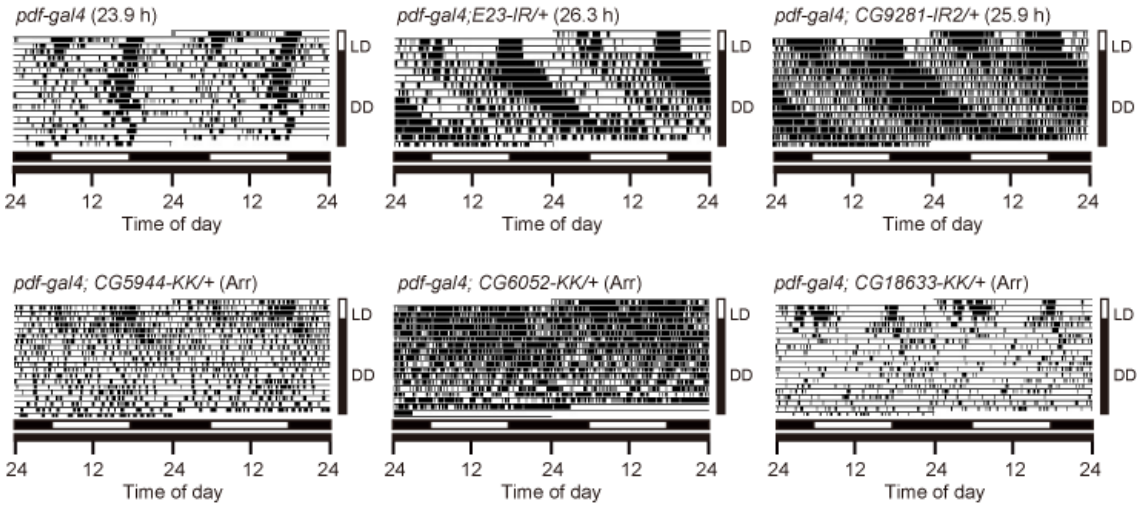

Figure 4 Actograms of knockdown flies of the candidate genes with *pdf-gal4*.

The genotype of knockdown fly is described at each actogram. The number in parenthesis represents the free-running period of the corresponding fly while Arr means arrhythmic. Adult flies were entrained in LD cycle for 3 days, and then kept in constant darkness (DD). The horizontal bars in white and black represent the light and dark regime of the LD cycle, respectively. White vertical bar indicates LD; black vertical bar indicates DD.

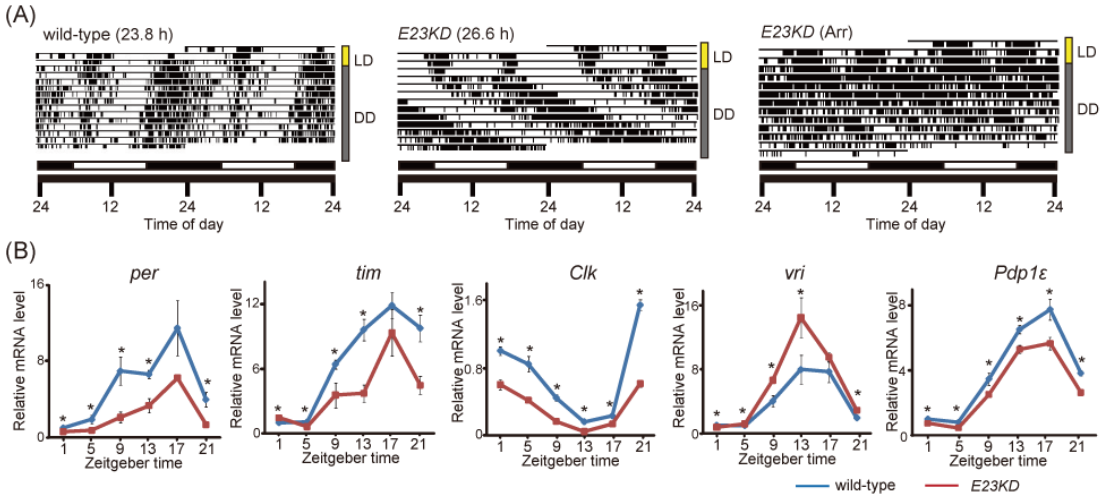

Figure 5 ***E23*** **knockdown in flies affects the circadian rhythm.**

(A) Typical locomotor activity in wild-type (upper) and *E23* knockdown flies (*E23KD*) showing a long period (middle) as well as an arrhythmic phenotype (lower). The number in parentheses represents the free-running period of the corresponding fly. Adult flies were entrained in a 12-hr light: 12-hr dark cycle (LD) for 3 days, and then kept in constant darkness (DD). Horizontal bar in white and black shows a light and dark regime in LD, respectively. Vertical bar in white: LD, vertical bar in black: DD. (B) Temporal expression profiles of *per*, *tim*, *Clk*, *vri* and *Pdp1ε* mRNA in wild-type (blue) and *E23* knockdown flies (red) under LD. Relative mRNA levels of the indicated genes were measured using a quantitative PCR assay. *Gapdh2* was used as an internal control. Data were normalized to the value of ZT1 in wild-type flies for each gene. Error bars represent S.E.M. (n=3). Asterisks denote significant differences in *E23* knockdown flies at each point compared with wild-type (t-test, $p < 0.05$).

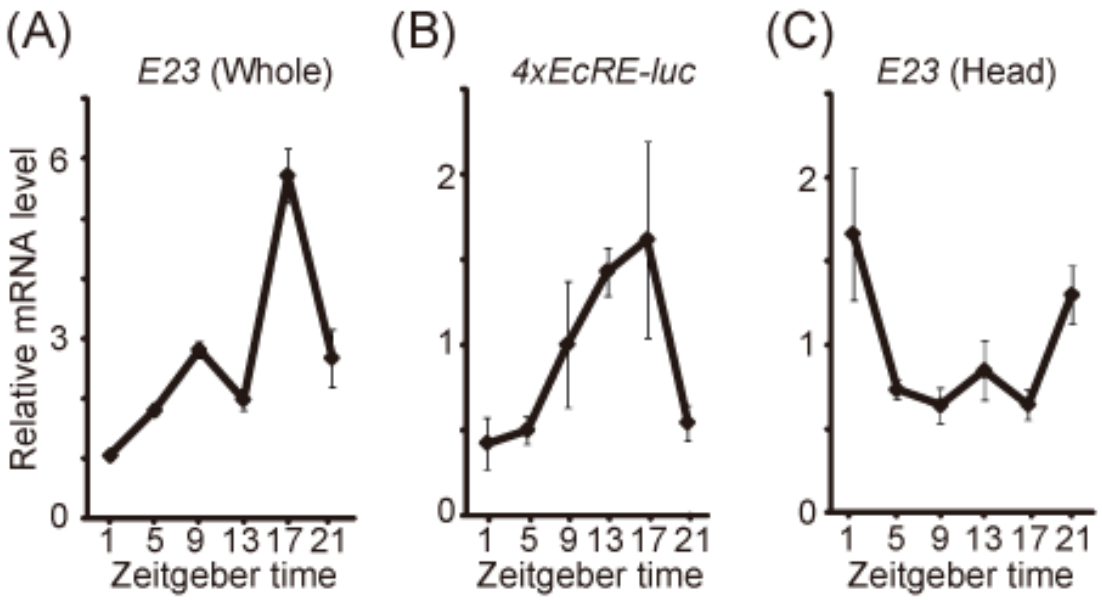

Figure 6　Temporal expressions of *E23* and the 20HE response in adult.

(A) Temporal expression of *E23* in whole bodies of wild-type under LD. (B) Temporal changes in the 20HE response in whole bodies of wild-type under LD measured as the relative *luciferase* expression level in *4xEcRE-luc* flies. (C) Temporal expression of *E23* in heads of wild-type under LD. Experiments were done as described in Fig.2B. Error bars represent S.E.M. (n=3).

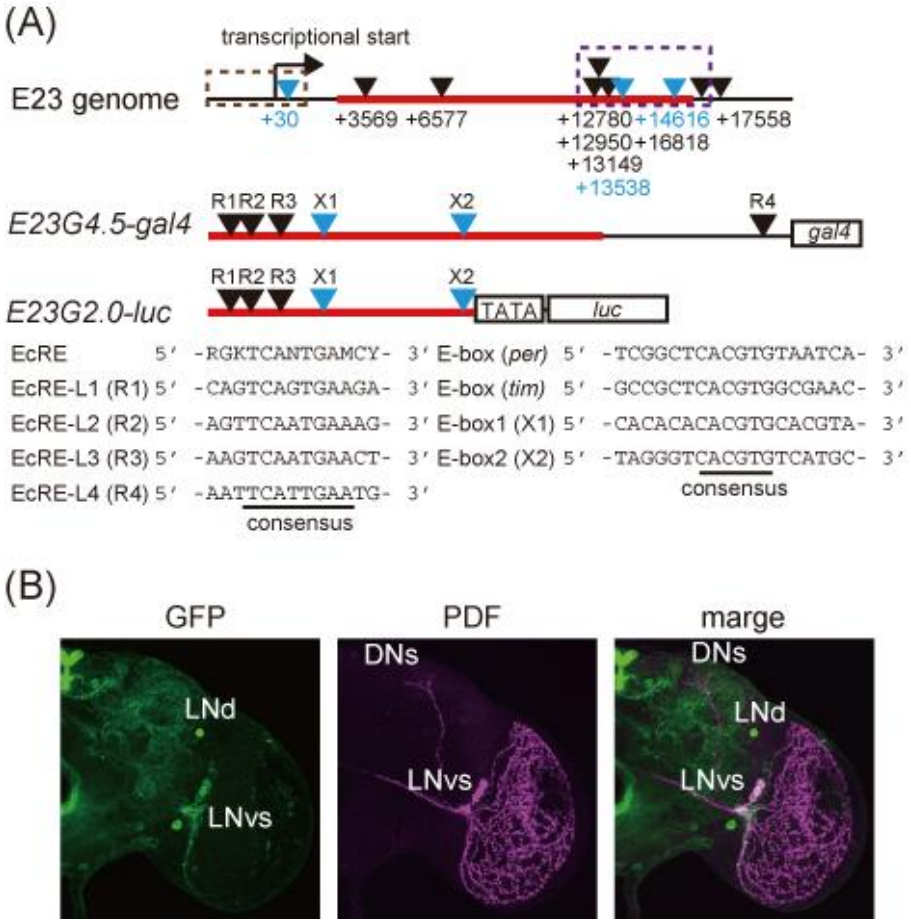

Figure 7　Spatial expression of *E23* in an adult fly.

(A) Mapping of ecdysone response element-like sequence (EcRE-L; black triangle) and E-box (blue one) around *E23* genome. Base pairs relative to the start site of the transcription (black arrow) are shown. The red line represents the third intron. Two regions fused to *gal4* are represented as brown and magenta dashed boxes, respectively. R and X with numbers represent EcRE-L and E-box in *E23G4.5-gal4* and/or *E23G2.0-luc*, respectively. Consensus sequences for EcRE-L and E-box are underlined in their alignments with the canonical EcRE (Riddihough and Pelham 1987; Cherbas et al. 1991) and E-boxes in the regulatory region of *per* and *tim* (Darlington et al. 1998), respectively. (B) The tissue specificity of *E23* expression in the frontal view of the adult head in *E23G4.5-gal4; UAS-mCD8::GFP*. GFP signals monitoring *E23* expression (green) were observed with PDF signals (magenta) in lateral neuron ventrals (LNvs) and solely in the lateral neuron dorsal (LNd), but not in dorsal neurons (DNs), all of which composes pacemaker neurons.

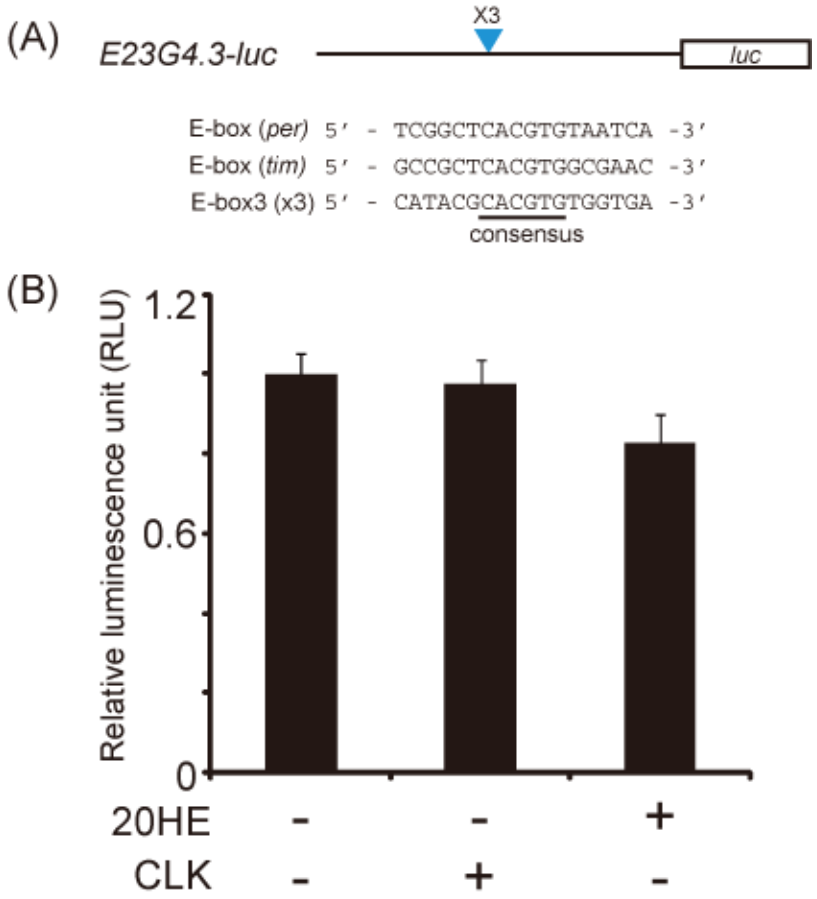

Figure 8 The upstream genomic region of *E23* responds to neither the 20HE signal nor to clock regulation.

(A) Schematic map of *E23G4.3-luc*, which contains 4.3 kb of the genomic region of *E23* represented as a brown dashed box in Fig.2c. In this region, there is one E-box at the site where a blue triangle named X3 indicates. The consensus sequence was represented as underlines in the alignment of the nucleic sequences around E-boxes of *per*, *tim* (Darlington et al. 1998) and X3. (B) Relative luciferase activities of *E23G4.3-luc* in the presence of *pAct-Clk* (100 nM) or 20HE (100 nM). Signals were normalized by *Rluc* activity and then by activity in the absence of both 20HE and *pAct-Clk* as a control. Error bars represent S.E.M. (n=3).

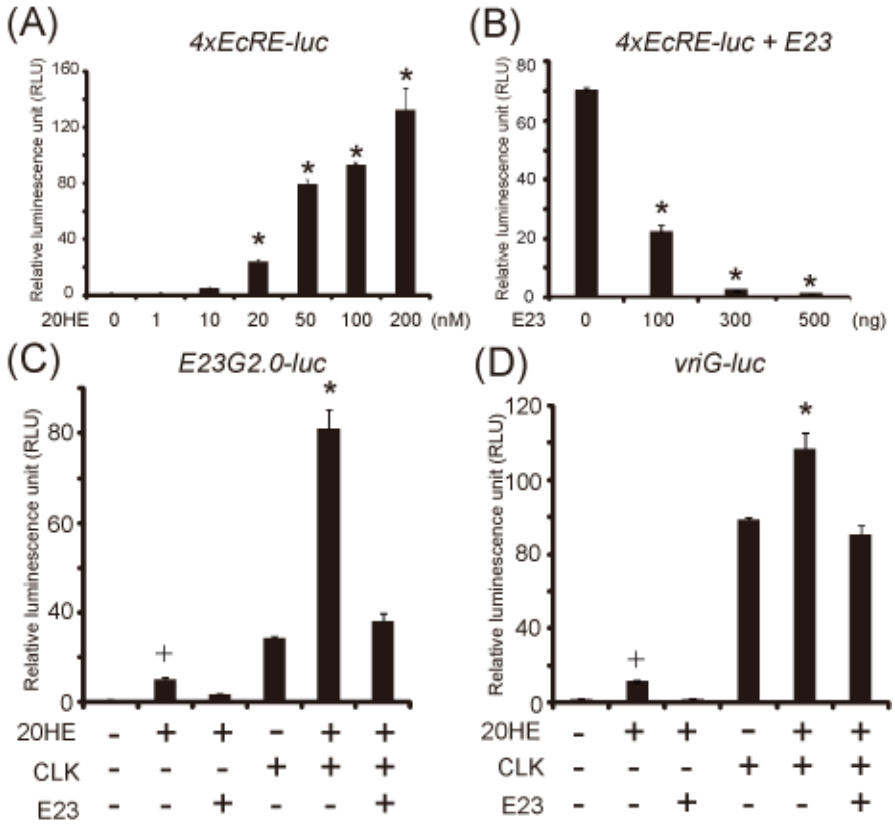

Figure 9 *E23* and *vri* respond to 20HE and CLK/CYC, while E23 protein suppresses 20HE response.

(A) Expression of *4xEcRE-luc* at 20HE concentrations ranging from 0 to 200 nM. Relative signals were normalized by the activity in the absence of 20HE as a control. (B) Expression of *4xEcRE-luc* in the presence of *pAc5.1B-E23* (0–500 ng) at 100 nM of 20HE. Relative signals were normalized by the activity in the absence of *pAc5.1B-E23* as a control. In Fig. 4A and 4B, asterisks show that the value was significantly different from that of the control (t-test, $p < 0.05$), and error bars represent S.E.M. (n=3). (C) Expression of *E23G2.0-luc* with 20HE and *Clk.* 20HE (100 nM), *pAct-Clk* (100 ng) and *pAc5.1B-E23* (100 ng) were provided together. Relative signals were normalized by activity in the absence of all three factors as a control. (D) Expression of *vriG-luc* with 20HE and *Clk.* The experimental conditions were similar to those in Fig.6C. In Fig.6C and 5D, a cross indicates that the value was significantly different among three transfections without *pAct-Clk* (Tukey's test, $p < 0.05$). An asterisk indicates that the value was significantly different among three transfections with *pAct-Clk*, respectively (Tukey's test, $p < 0.05$).

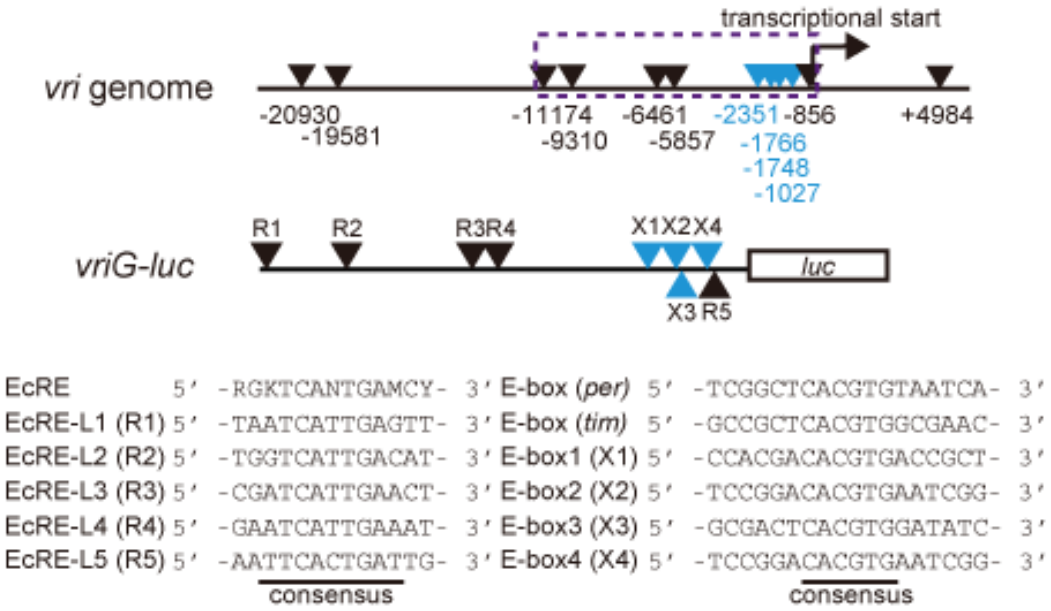

Figure 10 Mapping of ecdysone response element-like sequence and E-box around *vri* genome.

Base pairs relative to the start site of the transcription (black arrow) are shown. R (Black triangle) and X (Blue one) with numbers represent EcRE-L and E-box in *vri* genome and/or *vriG-luc*, respectively. Consensus sequences for EcRE-L and E-box are underlined in their alignments with the canonical EcRE (Riddihough and Pelham 1987; Cherbas et al. 1991) and E-boxes in the regulatory region of *per* and *tim* (Darlington et al. 1998), respectively.

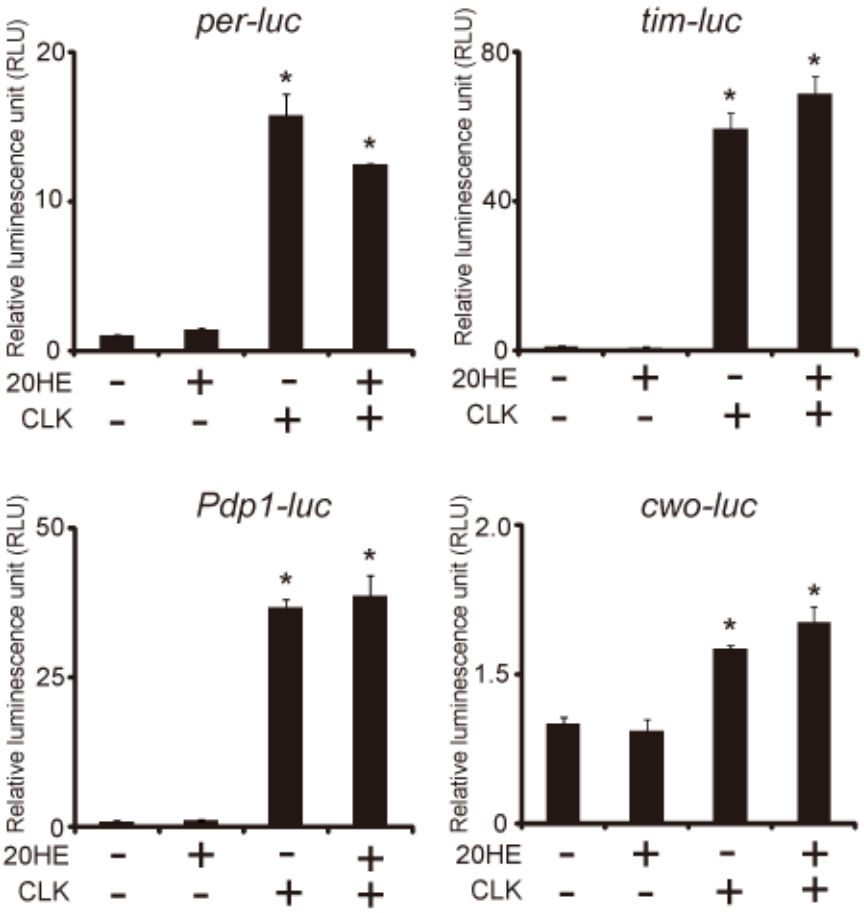

Figure 11 Clock genes controlled by E-box showed no 20HE response.

Relative luciferase activities of *promoter-luc* for each of the clock genes *per*, *tim*, *Pdp1* and *cwo*. 20HE (100 nM) and *pAct-Clk* (100 ng) were provided together. Signals were normalised by *Rluc* activity and then by the activity in the absence of both 20HE and *pAct-Clk* as a control. Asterisks show that the value was significantly different from that of the control (t-test, $p < 0.05$) and error bars represent S.E.M. (n=3).

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