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Circadian clock in *Ciona intestinalis* revealed by microarray analysis and oxygen consumption

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The molecular mechanisms of the endogenous circadian clocks that allow most animals to adapt to environmental cycles have recently been uncovered. The draft genome of the ascidian, *Ciona intestinalis*, a model animal that is close to vertebrates, has been described. However, the *C. intestinalis* genome lacks the canonical clock genes such as *Per*, *Bmal* and *Clock* that are shared by vertebrates and insects. Here, we found the circadian rhythms at the physiological and molecular levels. The oxygen consumption rate was lower during the light phase and higher during the dark phase during a day, and the rhythm highly damped and continued under constant darkness. From the microarray analysis, the 396 spots (1.8% of the total; corresponding to 388 clones) were extracted as candidates for circadian expression. We confirmed the circadian expression of several candidate genes by northern blotting. Furthermore, three of four rhythmic expressed genes showed phase-shifts to prolonged light period. However, most of known clock genes did not oscillate. These data suggest that *C. intestinalis* have a unique molecular circadian clock and the daily environmental change is not such a strong effect for sea squirt in its evolution when compared to vertebrates and insects.

Keywords: Ascidian/*Ciona intestinalis*/circadian clock/microarray/northern blotting.

Ascidians are urochordates that are considered the closest living relatives of vertebrates (*1*, *2*). Due to a simple structure, rapid embryogenesis, its transparent body, a published draft of the genome sequence (*3*), a rich cDNA/EST database (*4*) and other advantages, *Ciona intestinalis* is regarded as an experimental model of chordates that comprise urochordates, cephalochordates and vertebrates. The draft genome indicates that the composition of the *C. intestinalis* genes that encode proteins is generally intermediate between those of protostomes and vertebrates (*3*).

Most organisms have endogenous circadian clocks that allow adaptation to daily environmental cycles, and ascidian behaviour is also somewhat time-related. The tropical colonial ascidian, *Diplosoma virens*, undergoes a circadian colony expansion rhythm under continuous darkness (DD) (*5*). *Halocynthia roretzi* periodically spawns under DD and continuous light (LL), and an endogenous circadian clock seems to control the process (*6*). However, the mechanisms of ascidians' clocks remain unknown.

Thus we performed this research as an initial step towards understanding the circadian clock system of ascidians. Because various molecular biological techniques including making transgenic animals are applicable for *C. intestinalis* (*7*) in addition to the advantages described earlier, we used *C. intestinalis* as a model animal of ascidian. A time-related behaviour of this animal is also reported. Light after dark adaptation triggers *C. intestinalis* spawning (*8*), but whether this behaviour is controlled by an endogenous circadian clock in this species remains unknown.

The molecular mechanisms of circadian clocks have been thoroughly investigated in mammalian (mouse) and insect (*Drosophila*) models. A negative feedback system in which 'clock gene' protein products regulate their own transcription controls the circadian clock in both mammals and insects. Since some of the canonical clock genes such as *Per* and *Clock* play important roles in the clocks of both vertebrates and arthropods, ascidians should possess and use such genes at the centre of the clock system. However, some canonical clock genes such as *Per*, *Clock* and *Bmal* (*cycle*) have not been identified in the *C. intestinalis* genome (*3*), despite the presence of homologues of some transcription factors containing the PAS domain such as *Sim*, *Ahr* and *Arnt* (*9*) (Table 1). Thus ascidians might have unknown clock mechanisms that differ from known systems of vertebrates and insects, and if so, understanding ascidians clock mechanisms might provide a new insight into animal clock mechanisms. To date, neither 'clock genes' nor behaviour controlled by a circadian clock has been identified in *C. intestinalis*. We, therefore, investigated circadian rhythms at the physiological and molecular levels. We measured the rhythms of oxygen consumption and searched for mRNAs with circadian expression. The results

Table 1. Clock gene homologues in *C. intestinalis*.

Mammal	*Drosophila*	*Ciona* homologues	*r*	Nb*
	timeless	no homologue found		
Per 1, 2, 3	*period*	no homologue found		
Clock	*clock*	no homologue found		
Bmal1,2	*cycle*	no homologue found		
Cry1,2	*cryptochrome*	no homologue found		
Hlf	*pdp1*	ciad045n22	−0.94	ryhthmic
Rxr		citb010c11	−0.90	arrythmic
Fwd1	*slimb*	cieg001c06	−0.85	arrythmic
Ck1 epsilon	*double−time*	cieg027g08	−0.58	arrythmic
Rev-erb alpha	*E75*	cilv001k03	−0.53	arrythmic
E4bp4	*vrille*	ciad100b09	−0.36	ryhthmic
Ror alpha	*HR3*	cilv010m12	−0.24	arrythmic

*Confirmation by northern blotting experiment. Rhythmic: Circadian expression with approximately 2-fold amplitude. Arrhythmic: Circadian expression undetectable.

suggested that an endogenous circadian clock does exist in *C. intestinalis*.

Experimental procedures

Respirometry

Adult ascidians were collected at Oarai fishing port (Ibaraki Pref., Japan) and entrained to 12 h light–12 h dark (LD) conditions for at least 5 Days. Thereafter, oxygen consumption rates were measured under LD and DD at 20°C using a semi-closed system equipped with an oxygen electrode (*10*). The experiments were performed in the isolated darkroom, and the water temperature was strictly controlled by a thermostat. Both in LD and DD experiments, five animals were placed in a respiration chamber that was closed with a pinchcock at each time point for 45 or 30 min (LD experiments) or 60 min (DD experiments) in each time course. Our preliminary experiments revealed that the length of pinchcock closing time does not affect the data (Supplementary Fig. 1). The initial and final concentrations of dissolved oxygen were measured using a B-505 dissolved oxygen meter (Iijima Electronics), and the difference was taken as the amount of oxygen consumed at each time point. The total oxygen consumption was divided by pinchcock closing time (30, 45 or 60 min), and the oxygen consumption rate was obtained. The LD experiments proceeded continuously for 3 and 4 Days, and we obtained data for a total of 7 days ($n = 7$, 4 and 3 of 7 experiments were performed with 45 and 30 min of closure, respectively). The DD experiments were repeated independently five times ($n = 5$). Since the animals were starved, the oxygen consumption decreased over time, so the values were corrected by extrapolating the curve of the reduction based on the average 24-h decrease. After correction for the time-dependent decrease, the maximal value in each time course was set to 1, and relative values were calculated. Time-dependent variations were statistically analysed using one-way analysis of variance.

Microarrays

Adult animals cultivated at the Maizuru Fisheries Station of Kyoto University (Maizuru, Japan) were entrained to LD for 1 week and then maintained under DD. At DD Days 2 and 3, we collected three animals every 6 h (CT2, 8, 14, 20 of DD Days 2 and 3) under dim red light. Pooled RNA from each group was used for microarray analysis. Total RNA was isolated from whole animals (tunic removed) using cesium trifluoroacetic acid ultracentrifugation (*11*). The RNA quality was verified by electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (5 μg) was labelled with either Cy3 or Cy5 using an Agilent Fluorescent Linear Amplification Kit (Agilent Technologies), mixed and then hybridized with the *C. intestinalis* Oligoarray ver. 1 that contains 21,939 probes (21,617 independent 60-mer oligonucleotides) (*12*). The microarray experiments, comprising anchored comparison and moving window analysis, were designed according to Akhtar *et al.* (*13*) with slight modification. The anchored comparison examined the CT2 RNA sample labelled with Cy3 and one of the other samples (CT 8, 14, 20 of Day 2, and CT 2, 8, 14 and 20 of Day 3) labelled with Cy5, thus seven chips were analysed in the anchored comparison. The other strategy of moving window analysis compared RNA levels at paired times that were 12 h apart (CT 20 of Day 2/CT 8 of Day 2, CT 2 of Day 3/CT 14 of Day 2, CT 8 of Day 3/CT 20 of Day 2, CT 14 of Day 3/CT 2 of Day 3 and CT 20 of Day 3/CT 8 of Day 3; labelled with Cy5/Cy3, respectively), thus five chips were analysed in the moving window analysis. The microarrays were hybridized and washed according to the manufacturer's instructions and then scanned using a GenePix 4000B DNA Microarray Scanner (Axon Instruments, Foster City, CA, USA). The resulting fluorescence intensity for each spot was quantified using GenePix Pro4.0 microarray analysis software (Axon Instruments) (*14*).

Data analysis

In the microarray analysis, seven anchored comparison and five moving window data were obtained for each spot. To define circadian expression, we compared the data from the anchored and moving windows. At first, Cy5/Cy3 ratio (in log2 units) of each spot was calculated. Next, we calculated the Pearson correlation coefficients (r) of the data (Cy5/Cy3 ratio) from the first five of anchored comparison (CT 8 of Day 2/CT 2 of Day 2, CT 14 of Day 2/CT 2 of Day 2, CT 20 of Day 2/CT 2 of Day 2, CT 2 of Day 3/CT 2 of Day 2 and CT 8 of Day 23/CT 2 of Day 2) and from the moving window (CT 20 of Day 2/CT 8 of Day 2, CT 2 of Day 3/CT 14 of Day 2, CT 8 of Day 3/CT 20 of Day 2, CT 14 of Day 3/CT 2 of Day 3 and CT 20 of Day 3/CT 8 of Day 3). When an identical gene that is rhythmically expressed with a 24-h period was assumed, the anchored data and 12 h apart moving window data negatively correlate ($r = -1.0$). From the analysis of frequency distribution for Pearson's correlation coefficients (r), we defined the cut-off value of $r \leqslant -0.7$ for circadian rhythmicity (for details, see Supplementary Figs. 2 and 3). The false discovery rate was also estimated by a random permutation test (1000 permutations).

Northern hybridization

Independent animal samples were northern blotted to confirm the results of the microarray analysis. Adult animals cultivated as described earlier were kept under LD for over 7 days, followed by DD after entrainment. On DD day 2, we collected six animals every 3 h using an infrared light scope. Pooled RNA from each group was northern blotted. Total *C. intestinalis* RNA was isolated by CsCl ultracentrifugation and poly A RNA was isolated using GenElute mRNA Miniprep Kits (Sigma). Northern blotting proceeded as described (*15*). The signal intensity of a target gene was divided by that of the internal control (cytoskeletal actin gene; NM_001032502) to determine relative expression.

Prolonged exposure to light

We prolonged the exposure of the animals to light to confirm whether the circadian clock regulates mRNA oscillations. Animals were maintained under DD after exposure to a further 12 h of light on the last day of the LD cycle, and six individuals were collected every 3 h on DD day 2. Northern hybridization proceeded as described earlier.

Search for homologues of canonical clock genes

We searched for *C. intestinalis* homologues of canonical clock genes using the tBLASTn algorithm at the JGI web site (http://genome.jgi-psf.org/). The amino acid sequences of *timeless*, *Per*, *Clock*, *Bmal* and *Cry* of *Drosophila*, mouse and zebrafish were used as query sequences, and with the following search parameters: expect = 1e-3 and scoring matrix = BLOSUM 45. The *C. savignyi* homologues of such genes were also searched at the *C. savignyi* genome database (http://www.broad.mit.edu/annotation/ciona/) with the same parameters.

Results

Rhythm of oxygen consumption in C. intestinalis

We measured oxygen consumption rhythms using a dissolved oxygen meter under LD and DD to understand the circadian rhythms of *C. intestinalis* at the

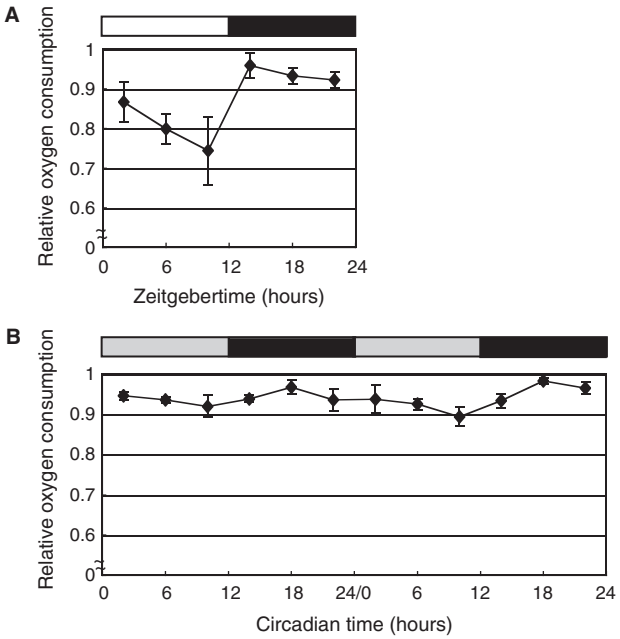

Fig. 1 Oxygen consumption rhythms of *C. intestinalis* measured as dissolved oxygen under (A) LD and (B) DD. Time-dependent decrease was corrected, and maximal value in each time course was set to 1. (A) Total of 7 days data were put together in the graph. (B) The 48 h experiment was repeated for five times. Data are expressed as means ± SEM. White and black bars indicate day and night, respectively (A); grey and black bars indicate subjective day and night, respectively (B).

physiological level. Less oxygen was consumed during the light period, and more was consumed during the dark period of LD ($n=7$; Fig. 1A). The oxygen consumption rate under LD was significantly time-dependent (F [5,36] $=3.53$, $P<0.05$). Under DD following LD entrainment, less and more oxygen was consumed under subjective light and subjective darkness, respectively ($n=5$; Fig. 1B). While the time-dependence was not significant on the first day of DD [F (5,24) $=0.94$, $P=0.47$], it was significant on the second day of DD [F (5,24) $=2.80$, $P<0.05$]. The trough of the oscillation was higher under DD than LD.

Circadian expression of genes in C. intestinalis

We examined circadian rhythms at the molecular level in adult animals collected at circadian times (CT) 2, 8, 14 and 20 on DD days 2 and 3. A microarray (*C. intestinalis* Oligoarray ver. 1) was applied to analyse the mRNA expression profile (*12*). The microarray chip contained 21,939 spots, but we excluded 244 of them from the analysis because of missing values. A correlation analysis of anchored and moving window data indicated that 5,635 of 21,695 analysed spots (26.0%) were apparently expressed in a circadian manner (Pearson correlation coefficient $r \leqslant -0.7$), and the false discovery rate (defined as the expected proportion of false positives among true circadian clones) obtained by a random permutation test (1,000 permutations) was 36.4%. Because the amplitude of the expression profiles of these clones was usually quite low, we defined circadian expression by considering the amplitude of each individual clone. Thus, we defined circadian expression as Pearson correlation coefficient $r \leqslant -0.7$ and at least 2-fold the maximal/minimal expression signal ratio both in the first (CT 8, 14 and 20 of day 2, and CT2 of day 3) and latter four (CT 2, 8, 14 and 20 of day 3) sampling points in the anchored comparison data. We extracted 396 (1.8% of the total) spots corresponding to 388 clones with circadian expression (supplementary table), and we defined these clones as candidates for circadian expression. We confirmed these findings by further investigating the expression of clock-related genes and of genes that are expressed in a circadian manner by independent northern blotting.

We initially analysed the expression profiles of homologues of known clock-related genes. However, the *C. intestinalis* genome does not include homologues of the core clock components, *Per*, *timeless*, *Clock*, *Bmal* (*cycle*) and *Cry* (*3*) (Table 1). We examined the expression profiles of other clock gene homologues of *Hlf*, *Rxr*, *Fwd1*, *Ck1 epsilon*, *Rev-erb alpha*, *E4bp4* and *ROR alpha*, and correlation analysis suggested the circadian expression of *Hlf*, *Rxr* and *Fwd1* identified in the microarray analysis (Table 1). However, low amplitude indicated that these genes were not expressed in a circadian manner which we defined. The amplitude of *Hlf* was >2-fold at the first four sampling points in the microarray analysis, and northern blotting showed that the amplitude of the circadian expression of *Hlf* was also ~2-fold (Fig. 2A). The profiles of *Rxr* and *Fwd1* were circadian but with very low amplitudes in the microarray analysis, and rhythmicity was not confirmed by northern blotting, indicating that *Rxr* and *Fwd1* did not show rhythmicity. Microarray analysis and northern blotting did not uncover circadian expression of *CK*1 *epsilon*, *Rev-erb alpha* and *Ror alpha*. The correlation efficiency of *E4bp4* in the microarray was $r=-0.36$ indicating the absence of circadian expression, but northern blotting indicated circadian expression with approximately 2-fold oscillation (Fig. 2B). We then used independent northern blotting to confirm the expression profiles of clones that were expressed in a circadian manner. We performed northern blotting for 59 of 388 clones, and obtained the data for 28 genes. In these 28 genes, 15 genes showed ~2-fold oscillation, so the false positive rate revealed by the northern blotting is 46.4%. Figure 3A shows the expression profiles of seven representative genes, named CT8-2, Amp2, Amp4, Amp30, Amp31, Amp37 and Amp56 (corresponding EST IDs: cibd034k24, citb064b04, ciad033o23, ciad002j17, cieg032o11, citb005m02 and cigd012m22, respectively), with high-amplitude oscillation and expression levels in adult *C. intestinalis*. The functional domains of four of these genes are addressed in the Discussion section.

Of the 396 candidates, 46 (11.6%), 52 (13.1%), 176 (44.4%) and 122 (30.8%) spots initially peaked at CT 8, 14 and 20 of Day 2, and CT 2 of Day 3, respectively. Most spots peaked at the end of subjective night (CT 20 of Day 2) and at the beginning of subjective day (CT 2 of Day 3), and peak analysis of the latter four sampling points (CT 2, 8, 14 and 20 of Day 3)

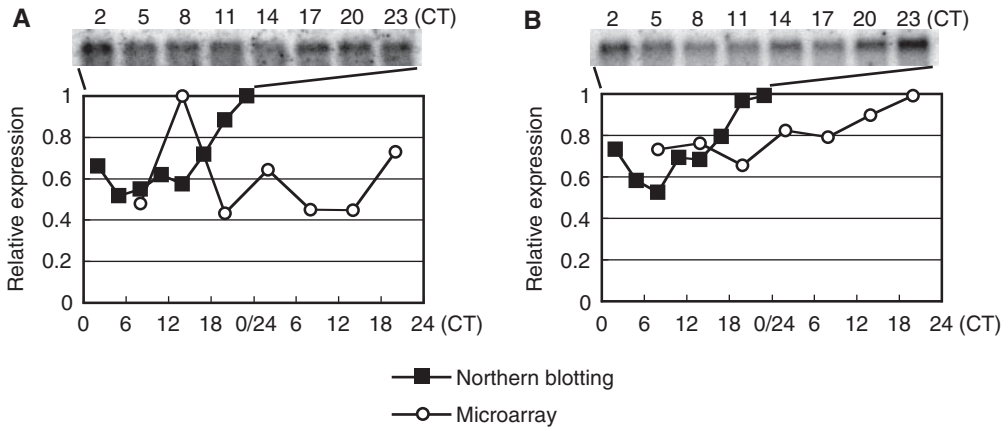

Fig. 2 Expression rhythms of mRNA of *C. intestinalis* homologues of *Hlf* and *E4BP4* under DD. Expression profiles of (A) *Hlf* and (B) *E4BP4* mRNA, revealed by microarray (open circles) and northern blotting (closed boxes). The highest value in each course was set to 1, and the linear relative expression amounts are plotted. The original photograph of the northern experiment is shown above each graph.

confirmed the same tendency (Fig. 4). The peak time and expression amplitude were related. While the spots with low amplitude tended to peak at CT 2 of Day 3, that is early during subjective day, high-amplitude clones tended to peak at CT 20 of Days 2 and 3, namely later during subjective night (Fig. 4).

The phase of circadian expression genes was shifted by prolonged exposure to light.

The phase of the circadian clock can be shifted by environmental cues such as light, and this feature is one important definition of the circadian clock. To examine this notion, the animals were exposed to light for a further 12 h on the last day of the LD cycle. Four representative oscillation genes obtained in this study served as reporters. Peak times of the CT8-2, Amp4 and Amp30 genes expression were delayed by 6–9 h. In contrast, the peak of Amp56 was similar to that of controls, but the trough was altered (Fig. 3B).

Functional annotation of circadian candidates

The candidates with circadian expression (396 spots) were functionally annotated using the KEGG Automatic Annotation Server (KAAS: http://www.genome.jp/kegg/kaas/), and 125 of 396 spots were automatically annotated by KAAS. We annotated 1,000 randomly selected control spots in the same manner and compared the results. A comparison of five categories of KEGG Orthology (KO) top-level annotation showed that the proportion of genes involved in 'genetic information processing' was significantly higher among those with circadian expression (Fig. 5A). Furthermore, second-level annotation revealed that the proportions of genes involved in 'translation', 'neurodegenerative disorders' and 'metabolic disorders' were significantly higher than predicted, whereas those of genes involved in 'nucleotide metabolism', 'replication and repair' and 'cell growth and death' were significantly lower (Fig. 5B–F).

Search for Ciona homologues of canonical clock genes

We searched for the homologues of canonical clock genes (*timeless*, *Per*, *Clock*, *Bmal* and *Cry*) in two *Ciona* species by tBLASTn algorism. All hits identified by the tBLASTn search apparently encoded homologues of other known genes, and the homologues of *timeless*, *Per*, *Clock*, *Bmal* and *Cry* were not identified in the *C. intestinalis* and *C. savignyi* genome.

Discussion

We found that *C. intestinalis* has circadian rhythms at molecular (mRNA expression) level and that these rhythms continued autonomously for 3 Days under DD. The rhythm at physiological level (oxygen consumption) was not clear; however, it showed significant rhythm at the DD Day 2. These results suggested that *C. intestinalis* possesses an endogenous circadian clock; however, it seems that the environmental daily changes did not strongly affect the rhythm of this animal in its evolutionary process: it may have affected by other rhythmic factors such as tidal rhythms. Some authors have described the circadian clocks of ascidians (*5*, *6*); however, we are the first to describe the circadian rhythms of *C. intestinalis*, which is a useful model animal of chordates.

Oxygen consumption values showed that the metabolic rate of *C. intestinalis* is lower during the light phase and higher during the dark phase of LD and even under DD though it was not statistically significant for the DD first day partly because of the low amplitude (Fig. 1). The data suggested that *C. intestinalis* has a circadian clock system. Furthermore, the results of prolonged exposure to light suggested that the clock-controlled genes phase-shifted according to light stimulation.

This animal inhabits shallow seas and grazes mainly on plankton and dissolved organic matter. Some zooplanktons migrate to the surface during the night time (*16*). These facts suggest that increased oxygen

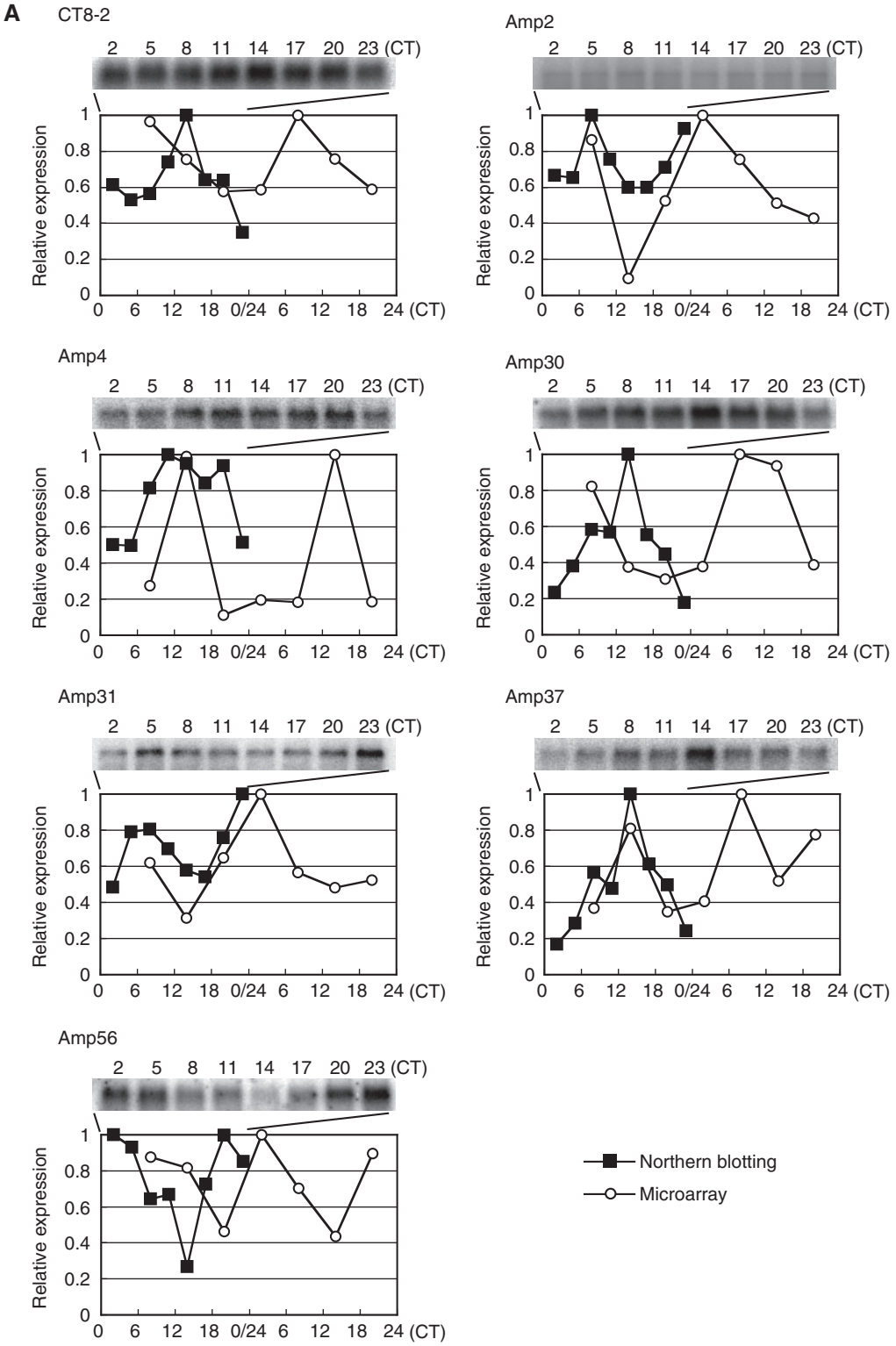

Fig. 3 Expression rhythms of mRNA under DD of seven representative *C. intestinalis* circadian genes and the phase shift of these mRNA expressions by light. (A) Expression profiles of CT8-2 (corresponding EST ID is cibd034k24), Amp2 (citb064b04), Amp4 (ciad033o23), Amp30 (ciad002j17), Amp31 (cieg032o11), Amp37 (citb005m02) and Amp56 (cigd012m22), revealed by microarray (open circles) and northern blotting (closed boxes). The highest value in each course was set to 1, and the linear relative expression amounts are plotted. The original photograph of the northern experiment is shown above each graph. **(B)** Upper part: effect of exposure to 12 h of prolonged light on expression of CT8-2, Amp4, Amp30 and Amp56 (open boxes). The highest value in each course was set to 1, and the linear relative expression amounts are plotted. Data from untreated group (closed boxes) are redrawn from panel A. Lower panel: the light-dark regime of the untreated (above) and prolonged light (below) groups. After LD entrainment, the control group was maintained under DD, while the prolonged light group was exposed to additional 12-h light. Arrowheads reveal the sampling times for the northern experiments.

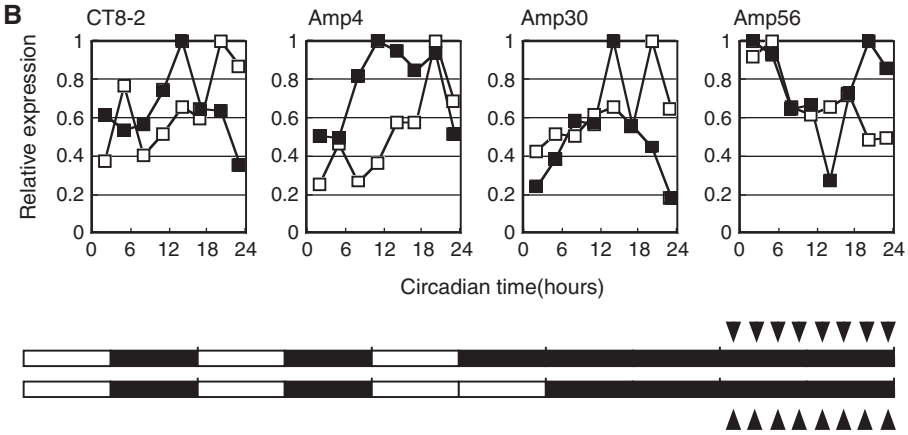

Fig. 3 Continued.

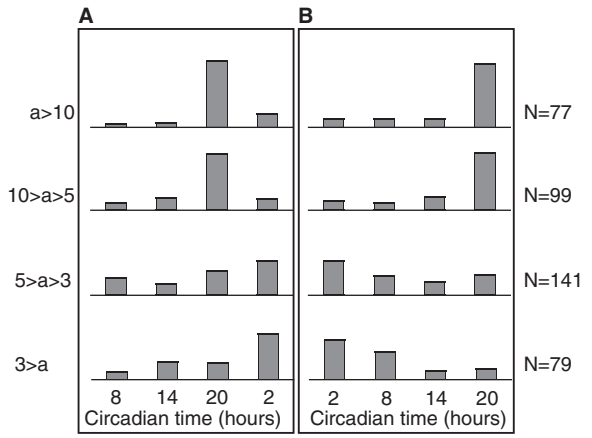

Fig. 4 Relationship between peak phase and amplitudes of 396 circadian candidate clones extracted with microarray analysis. Proportions of clones that peaked at each circadian time in (A) former (CT8, 14 and 20 of DD day 2, and CT 2 of DD day 3) and (B) latter (CT 2, 8, 14 and 20 of DD day 3) four sampling points are shown. Amplitude and group size are given as a and N, respectively.

consumption at night is related to grazing behaviour. The trough of the oxygen consumption rate was lower under LD, than under DD, suggesting that light suppresses oxygen consumption in *C. intestinalis*. In fact, *C. intestinalis* escapes from light at the latter larval stage (*17*).

The molecular mechanisms of the circadian clock are under investigation in cyanobacteria (*18*), *Neurospora* (*19*), plants (*20*), insects (*21*) and mammals (*22*, *23*). The clock mechanisms of all these organisms share the process of negative feedback loops of clock genes that cause oscillations. The canonical core 'clock genes' such as *Clock*, *Bmal* (*cycle*) and *Per* play important roles in these feedback loops from *Drosophila* to mammals. These canonical clock genes also have homologues in the lancelet (Cephalochordates) (http://genome.jgi-psf.org/Brafl1/Brafl1.home.html), mollusk and echinoderm (e.g., see the sea urchin genome at: http://sugp.caltech.edu/) genomes. Coelomates including all these animals and ascidians should possess the same or similar clock genes and clock mechanisms. We, therefore, intensively searched for *Ciona* homologues of canonical clock genes using the tBLASTn algorithm, and the homologues of *timeless*, *Per*, *Clock*, *Bmal* and *Cry* were not identified in the *C. intestinalis* and *C. savignyi* genome. Although not finding a homologue by BLAST search does not directly mean there is no homologue, the *C. intestinalis* genome has apparently undergone rapid evolution together with extensive gene loss (*24*), implying that these canonical clock genes might have been lost after diversification from vertebrates. Furthermore, we found that some known clock-related genes, which show circadian oscillation in insects and/or mammals, were not significantly expressed in a circadian manner in *C. intestinalis* (Table 1). We confirmed the expression patterns of seven clock-related genes by northern blotting. *Hlf* and *E4bp4* are the bZIP transcription factors, and known to regulate the circadian rhythm in vertebrates (*25*, *26*). *FWD*1 is a homologue of *Drosophila slimb* gene, which controls the level of *period* and *timeless* (*27*). *RXR*, *ROR alpha* and *Rev-erb alpha* are nuclear receptors that interact with clock proteins or regulate the circadian transcription of clock genes in vertebrates (*28*, *29*). *CK*1 *epsilon* is involved in degradation of PER proteins (*30*). All mRNAs of these genes excluding *CK1 epsilon* are known to oscillate in vertebrates and/or insects (*26*, *27*, *29*, *31*). Among the clock-related genes confirmed by northern blotting, only the mRNAs of *Hlf* and *E4bp4* were expressed at an ~2-fold amplitude, suggesting that these genes may be involved in circadian clock mechanisms. However, the peak expression phases of these two genes were similar in *C. intestinalis*, whereas they are expressed in an anti-phase manner in mammals (*26*). The present data together with these results suggest that the molecular clock components of *C. intestinalis* may differ from those of *Drosophila* and mammals, as observed in bacteria, fungi and plants (*32*).

The present data showed that some circadian expression genes had high-amplitude oscillations (Fig. 3A). The phases of some genes differed somewhat between microarrays and northern blotting. Because all animals used in this study were grown naturally, these phase differences might have been due to the age of the

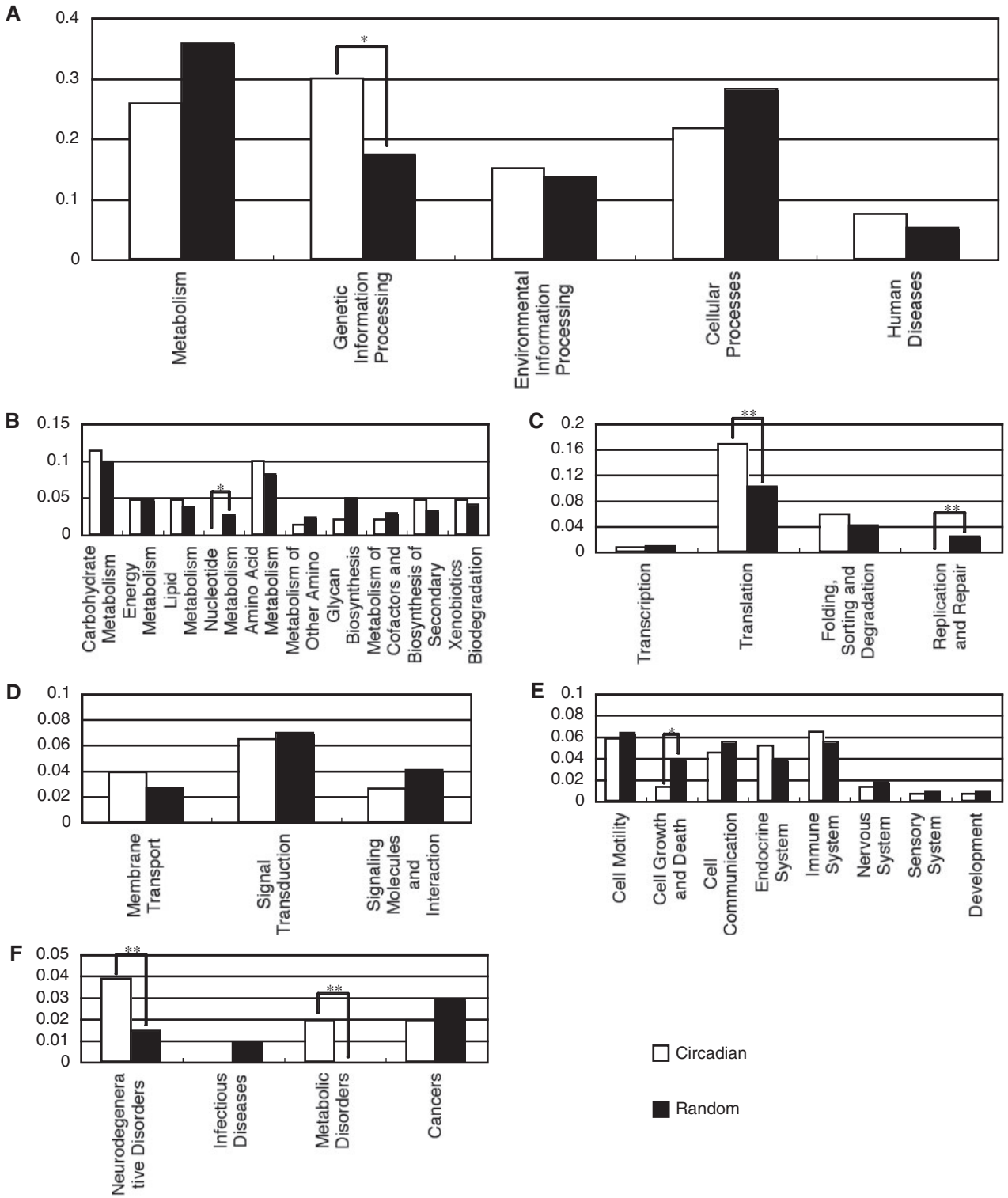

Fig. 5 Results of functional annotation of circadian candidate genes (open bars) and randomly selected genes (closed bars) by KAAS. (A) Proportions of genes classified to each KO top-level annotation class. (B–F) Proportions of genes classified to second-level annotation class classified in (A), (B) metabolism, (C) genetic information processing, (D) environmental information processing, (E) cellular processes and (F) human diseases. $*p<0.05$; $**p<0.01$.

samples or the seasons in which they were collected. A BLASTP search based on the deduced amino acid sequence from 5′ EST of each gene failed to reveal the homologue of many of seven representative genes (supplementary table). We further analysed the homologues of these genes using all available data such as 5′ EST, 3′ EST and full insert sequences as queries, and found that four of the seven representative genes had high similarity with known proteins. Amp4 (ciad033o23) had high similarity with chicken tolloid-like protein (Accession: Q9DER7), and possessed four CUB domains. Amp30 (ciad002j17) was identical to

Ci-meta2 (NP_001027777), which encodes a protein with a secretion signal and three thrombospondin type-1 repeats, and its expression is upregulated in metamorphosing juveniles of *C. intestinalis* (*33*). Amp37 (citb005m02) and Amp56 (cigd012m22) had the highest similarity with rabbit bactericidal permeability-increasing protein (Q28739), which is a mammalian lipid-binding serum glycoprotein, and ectoine hydroxylase (Q6QUY7) involved in bacterial salt tolerance. The functions of these genes in circadian rhythms are unknown in other animals. Because these genes show not only circadian expression but are also expressed at relatively high levels in the transparent bodies of *C. intestinalis* adults, the promoter regions of these genes might be a useful tool with which to construct transgenic circadian reporter animals for further studies of the molecular clock mechanism.

The results of prolonged exposure experiments (Fig. 3B) strongly suggested that the mRNA oscillations of rhythmic expression genes are regulated by the circadian clock of *C. intestinalis*. The expression peak of CT8-2, Amp4 and Amp30 among the four circadian expressed genes was delayed by 6–9 h (Fig. 3B), indicating that prolonged exposure to light delayed the circadian clock of *C. intestinalis*. The data also suggested that these three genes are useful as clock reporters of *C. intestinalis*. One gene (Amp56) did not clearly undergo a phase shift (Fig. 3B), suggesting that the oscillation of this gene is regulated by other mechanism rather than circadian clock.

Among the 396 candidates extracted in this study, we found homologues of some circadian oscillatory genes in other organisms (supplementary table). Opsins in vertebrates are candidate photoreceptors of circadian clocks and they are expressed in a circadian manner (*31*, *34*). Ci-Opsin1, a putative homologue of vertebrate opsins (*35*), also showed circadian expression in *C. intestinalis*. In addition, *C. intestinalis* also expressed the predicted homologues of cytochrome P450 genes in a circadian manner. This gene in mice shows circadian expression (*13*), and PAR bZIP proteins are thought to control circadian detoxification through regulation of the cytochrome P450 genes (*36*). Notably, many genes are assumed to encode ribosomal proteins that oscillate in a circadian manner. Six of the 388 clones isolated herein were putative ribosomal protein genes. A recent report indicates that nocturnal leaf changes in cottonwoods are controlled by cytoplasmic growth, which seems to be caused by the oscillation of ribosomal proteins (*37*). The expression of putative ribosomal protein genes in *C. intestinalis* peaked during subjective night as in the cottonwood, and might be related to higher oxygen consumption at this time, suggesting a similar mechanism between the two organisms.

At the first level of KO classification, a significant number of genes concerned with 'genetic information processing' showed circadian oscillation (Fig. 5A), and this was due to the large number of oscillating genes related to the 'translation' function (Fig. 5C). These data suggested that *C. intestinalis* produces oscillations at the translational as well as the transcriptional level.

We investigated the relationship between the time of peak expression and deduced gene function. We found that the expression of 86% of genes involved in 'genetic information processing' peaked later during subjective night (CT20). Most of the clones expressed at high amplitude (69% of clones with amplitude increased over 5-fold) peaked at late subjective night, whereas those expressed at a lower amplitude (52% of clones with amplitude increased below 3-fold) tended to peak at CT 2 of day 3, which is the beginning of subjective day (Fig. 4). Akhtar *et al.* (*13*) pointed out that genes upstream in the circadian signalling pathway might oscillate with high amplitudes, whereas those downstream might have lower amplitudes and delayed expression peaks. Thus the high-amplitude genes that peaked at CT20 might include some genes involved in clock functions

The present findings suggested that *C. intestinalis* possesses a circadian clock and that this species has clock genes that may differ from those of *Drosophila* and mammals. Although the clock mechanisms of ascidians remain unclear, our findings provide important clues towards understanding them.

Supplementary Data

Supplementary Data are available at JB Online.

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Conflict of interest

None declared.

References

1. Bourlat, S.J., Juliusdottir, T., Lowe, C.J., Freeman, R., Aronowicz, J., Kirschner, M., Lander, E.S., Thorndyke, M., Nakano, H., Kohn, A.B., Heyland, A., Moroz, L.L., Copley, R.R., and Telford, M.J. (2006) Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. *Nature* **444**, 85–88
2. Delsuc, F., Brinkmann, H., Chourrout, D., and Philippe, H. (2006) Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* **439**, 965–968
3. Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-Bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S.,

Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., and Rokhsar, D.S. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* **298**, 2157–2167

4. Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y., and Satoh, N. (2002) *Ciona intestinalis* cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. *Gene* **287**, 83–96
5. Ryland, J.S. (1990) A circadian rhythm in the tropical ascidian *Diplosoma virens* (Ascidiacea: Didemnidae). *J. Exp. Mar. Bio. Ecol.* **138**, 217–225
6. Numakunai, T. and Hoshino, Z.I. (1980) Periodic spawning of 3 types of the ascidian, *Halocynthia roretzi* (Drasche), under continuous light conditions. *J. Exp. Zool.* **212**, 381–387
7. Sasakura, Y., Nakashima, K., Awazu, S., Matsuoka, T., Nakayama, A., Azuma, J., and Satoh, N. (2005) Transposon-mediated insertional mutagenesis revealed the functions of animal cellulose synthase in the ascidian *Ciona intestinalis*. *Proc. Natl Acad. Sci. USA* **102**, 15134–15139
8. Lambert, C.C. and Brandt, C.L. (1967) The effect of light on the spawning of *Ciona intestinalis*. *Biol. Bullet.* **132**, 222–228
9. Satou, Y., Imai, K.S., Levine, M., Kohara, Y., Rokhsar, D., and Satoh, N. (2003) A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. I. Genes for bHLH transcription factors. *Dev. Genes. Evol.* **213**, 213–221
10. Oikawa, S., Itazawa, Y., and Gotoh, M. (1991) Ontogenetic change in the relationship between metabolic rate and body mass in a sea bream *Pagrus major* (Temminck & Schlegel). *J. Fish Biol.* **38**, 483–496
11. Azumi, K., Sasaki, T., Okouchi, K., Yamasaki, S., Saito, T., Takayama, H., and Yokosawa, H. (2005) Differential display analysis reveals the expression of glutathione S-transferase w and novel genes through an ITAM-containing receptor in ascidian immunocytes. *Immunogenetics* **57**, 444–452
12. Yamada, L., Kobayashi, K., Satou, Y., and Satoh, N. (2005) Microarray analysis of localization of maternal transcripts in eggs and early embryos of the ascidian, Ciona intestinalis. *Dev. Biol.* **284**, 536–550
13. Akhtar, R.A., Reddy, A.B., Maywood, E.S., Clayton, J.D., King, V.M., Smith, A.G., Gant, T.W., Hastings, M.H., and Kyriacou, C.P. (2002) Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr. Biol.* **12**, 540–550
14. Azumi, K., Takahashi, H., Miki, Y., Fujie, M., Usami, T., Ishikawa, H., Kitayama, A., Satou, Y., Ueno, N., and Satoh, N. (2003) Construction of a cDNA microarray derived from the ascidian *Ciona intestinalis*. *Zoolog. Sci.* **20**, 1223–1229
15. Oishi, K., Sakamoto, K., Okada, T., Nagase, T., and Ishida, N. (1998) Humoral signals mediate the circadian expression of rat period homologue (*rPer2*) mRNA in peripheral tissues. *Neurosci. Let.* **256**, 117–119
16. Bayly, I.A.E. (1986) Aspects of diel vertical migration in zooplankton, and its enigma variations in *Limnology in Australia (Monogr. Biol. 61)* (DeDecker P. and Williams W.D., eds.), pp. 349–368, Dr W. Junk, Amsterdam
17. Svane, I. and Young, C.M. (1989) The ecology and behaviour of ascidian larva. *Oceanogr. Mar. Biol. Annu. Rev.* **27**, 45–90
18. Iwasaki, H. and Kondo, T. (2004) Circadian timing mechanism in the prokaryotic clock system of cyanobacteria. *J. Biol. Rhythms.* **19**, 436–444
19. Dunlap, J.C. and Loros, J.J. (2004) The *Neurospora* circadian system. *J. Biol. Rhythms.* **19**, 414–424
20. Gardner, M.J., Hubbard, K.E., Hotta, C.T., Dodd, A.N., and Webb, A.A. (2006) How plants tell the time. *Biochem J* **397**, 15–24
21. Hardin, P.E. (2005) The circadian timekeeping system of *Drosophila*. *Curr. Biol.* **15**, R714–R722
22. Hastings, M.H. and Herzog, E.D. (2004) Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. *J. Biol. Rhythms.* **19**, 400–413
23. Ishida, N., Kaneko, M., and Allada, R. (1999) Biological clocks. *Proc. Natl Acad. Sci. USA* **96**, 8819–8820
24. Holland, L.Z. and Gibson-Brown, J.J. (2003) The *Ciona intestinalis* genome: when the constraints are off. *Bioessays* **25**, 529–532
25. Falvey, E., Fleury-Olela, F., and Schibler, U. (1995) The rat hepatic leukemia factor (HLF) gene encodes two transcriptional activators with distinct circadian rhythms, tissue distributions and target preferences. *EMBO J* **14**, 4307–4317
26. Mitsui, S., Yamaguchi, S., Matsuo, T., Ishida, Y., and Okamura, H. (2001) Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. *Genes Dev.* **15**, 995–1006
27. Grima, B., Lamouroux, A., Chelot, E., Papin, C., Limbourg-Bouchon, B., and Rouyer, F. (2002) The F-box protein Slimb controls the levels of clock proteins Period and Timeless. *Nature* **420**, 178–182
28. McNamara, P., Seo, S.-b., Rudic, R.D., Sehgal, A., Chakravarti, D., and FitzGerald, G.A. (2001) Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell* **105**, 877–889
29. Preitner, N., Damiola, F., Luis Lopez, M., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002) The orphan nuclear receptor REV-ERB alpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**, 251–260
30. Dey, J., Carr, A.J.F., Cagampang, F.R.A., Semikhodskii, A.S., Loudon, A.S.I., Hastings, M.H., and Maywood, E.S. (2005) The *tau* mutation in the syrian hamster differentially reprograms the circadian clock in the SCN and peripheral tissues. *J. Biol. Rhythms.* **20**, 99–110
31. Panda, S., Sato, T.K., Castrucci, A.M., Rollag, M.D., DeGrip, W.J., Hogenesch, J.B., Provencio, I., and Kay, S.A. (2002) Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science* **298**, 2213–2216
32. Young, M.W. and Kay, S.A. (2001) Time zones: a comparative genetics of circadian clocks. *Nat. Rev. Genet.* **2**, 702–715
33. Nakayama, A., Satou, Y., and Satoh, N. (2001) Isolation and characterization of genes that are expressed during *Ciona intestinalis* metamorphosis. *Dev. Genes Evol.* **211**, 184–189

34. Chaurasia, S.S., Rollag, M.D., Jiang, G., Hayes, W.P., Haque, R., Natesan, A., Zatz, M., Tosini, G., Liu, C., Korf, H.W., Iuvone, P.M., and Provencio, I. (2005) Molecular cloning, localization and circadian expression of chicken melanopsin (Opn4): differential regulation of expression in pineal and retinal cell types. *J. Neurochem.* **92**, 158–170
35. Kusakabe, T., Kusakabe, R., Kawakami, I., Satou, Y., Satoh, N., and Tsuda, M. (2001) Ci-opsin1, a vertebrate-type opsin gene, expressed in the larval ocellus of the ascidian *Ciona intestinalis*. *FEBS Lett.* **506**, 69–72
36. Gachon, F., Olela, F.F., Schaad, O., Descombes, P., and Schibler, U. (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab.* **4**, 25–36
37. Matsubara, S., Hurry, V., Druart, N., Benedict, C., Janzik, I., Chavarria-Krauser, A., Walter, A., and Schurr, U. (2006) Nocturnal changes in leaf growth of *Populus deltoides* are controlled by cytoplasmic growth. *Planta* **223**, 1315–1328