Biomedical Research **26** (2) 43-49, 2005

Expression of ubiquitin-related enzymes in the suprachiasmatic nucleus with special reference to ubiquitin carboxy-terminal hydrolase UchL1

Xin DONG[1], Kazuhiro YAGITA[1, 2], Jing ZHANG[1] and Hitoshi OKAMURA[1]

[1] Division of Molecular Brain Science, Department of Brain Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

(Received 4 January 2005; and accepted 11 January 2005)

ABSTRACT

There is growing evidence that ubiquitin-proteasome system plays an important role for the generation of circadian rhythms in mice as in *Drosophila*. Here we examined the expression of ubiquitin-related enzymes (Ubce5, UbcM4, Ube2v, Ube2d2, UchL1, UchL3, Ubp41, Ufd1L, β-TrCP) in the suprachiasmatic nucleus (SCN). At mRNA level, the expression of these enzymes were faint to moderate except ubiquitin carboxy-terminal hydrolase L1 (UchL1), a dominant deubiquitinating salvaging enzyme. Although strongly expressed in the SCN, UchL1 mRNA did not show the rhythm in the SCN in both light-dark and constant dark conditions.

In mammals as in many organisms, the circadian oscillation is driven by a transcription/translation-based core feedback loop of a set of clock genes which is dynamically regulated by clock proteins. As well as phosphorylation, ubiquitination of clock proteins (PER1, PER2, PER3, CRY1 and CRY2) constituting negative limbs of core loop plays the central role for this process. Indeed, it is already revealed that ubiquitination and proteasome-dependent degradation of mPER and mCRY proteins occur in mammalian cells (23). In *Drosophila*, the mutation of *Drosophila slimb*, an F-box/WD40-repeat protein functioning in the ubiquitin-proteasome pathway, is known to induce the constant accumulation of PER protein and behavioral arrhythmicity (4, 9). Although counterpart molecules of this protein is not discovered in mammals, the similar system might also exist since circadian oscillatory system and ubiquitin-proteasome system are both evolutionally conserved.

The ubiquitin-proteasome system is a major pathway for selective protein degradation (21). Ubiquitin is a 76-aa polypeptide that is conjugated to the epsilon-amino acid group of lysine residues. Ubiquitylation of a protein commonly serves to mark the modified protein for proteasome-mediated degradation. The attachment of ubiquitin to a substrate proceeds through a multi-enzyme cascade involving activating enzyme (E1), a conjugating enzyme (E2), and a protein ligase (E3), followed by the subsequent degradation by the proteasome (2). Ubiquitinated substrates were deubiquitinated by the deubiquitinating enzymes (DUB) essential for maintaining the ubiquitin pathway which release ubiquitin from its binding proteins. DUB is divided into two groups: smaller (10~30 kDa) ubiquitin C-terminal hydrolase (UCH) and larger (50~300 kDa) ubiquitin specific protease (UBP).

For revealing the involvement of ubiquitin-proteasome system in mammalian circadian oscillation, in the present study for the first step, we examined the expression of ubiquitin-related enzymes in the suprachiasmatic nucleus (SCN), the mammalian circa-

Address correspondence to: Dr. Hitoshi Okamura
Division of Molecular Brain Science, Department of Brain Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
Tel: +81-78 382 5340, Fax: +81 78 382 5341
E-mail: okamurah@kobe-u.ac.jp
[2] Present address: Unit of Circadian Systems, Department of Biological Sciences, Nagoya University Graduate School of Science, Nagoya, 464-8602, Japan

dian center which governs the characteristics of mammalian behavior (19). Ubiquitin-related enzymes were selected from the reported DNA microarray studies (15), and the studies in *Drosophila* (4, 9). We examine the expression of E2 (Ubce5, UbcM4, Ube2v and Ube2d2), E3 (β-TrCP), DUB (UchL1, UchL3 and Ubp41), and an ubiquitin-binding protein (Ufd1L). Among these, UchL1 (also called PGP9.5) which is highly expressed in the brain and testis (8, 22), shows the highest level of expression of its mRNA in the SCN. Thus, for this enzyme, we examined its circadian expression in the SCN in light-dark and constant dark conditions.

MATERIALS AND METHODS

Animals. For examining the expression of enzymes related to the metabolism of ubiquitin in SCN, we purchased commercially available male C57BL/6J mice at 6 weeks age (JAPS, Osaka, Japan). They were housed under standard 12 h : 12 h light-dark (LD) cycles at least for 2 weeks at 22 ± 2°C with freely provided diet and water. Twenty seven animals were examined for assessing the expression of ubiquitin-related enzymes at ZT4 (ZT stands for Zeitgeber time in a LD cycle; ZT0 is lights-on and ZT12 is lights-off and thus ZT4 means 4 hours after the light onset). The expression of UchL1 mRNA was examined in the LD cycle and in the second complete darkness (DD) cycle every 4 h (n = 5 at each time point for both experiments), starting at ZT0 or CT0 (CT stands for circadian time; CT0 is subjective dawn and CT12 is subjective dusk). All experimental procedures were approved by the committee for animal research at Kobe University.

Probes for in situ hybridization. UbcM4: UbcM4 cDNA fragment (positions 1549-2224 of *mus musculus* ubiquitin-conjugating enzyme E2L 3, Gene Bank accession number X97042) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-CCACTTGAAGCTCACTC AATATC-3') and reverse (5'-ACAGCTGGACTA TCTCTAGATTCAG-3'). This 675 bp fragment was then cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced to verify their identity and orientation.

Ube2v: Ube2v cDNA fragment (positions 192-871 of *mus musculus* ubiquitin-conjugating enzyme variant Kua, Gene Bank accession number BC002270) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-GTGTTCCGT GATCCTCTGTT-3') and reverse (5'-AGACATGC TACTTGATCTTCTGTG-3'). This 680 bp fragment was then cloned into the PCRII-TOPO vector (Invitrogen, Carsbad, CA) and sequenced to verify their identity and orientation.

Ube2d2: Ube2d2 cDNA fragment (positions 403-822 of *mus musculus* ubiquitin-conjugating enzyme E2D 2, Gene Bank accession number NM-019912) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-TCCACAAGGAATT GAATGACCTG-3') and reverse (5'-CATACTTCT GAGTCCATTCCCGAG-3'). This 420bp fragment was then cloned into the pBluescript II SK(+) vector (Stratagene, La Jolla, CA) and sequenced to verify their identity and orientation.

Ubce5: Ubce5 cDNA fragment (positions 181-686 of *mus musculus* ubiquitin-conjugating enzyme E2E 1, Gene Bank accession number NM-009455) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-ACCAGCTCCTCGT CATCTTCG-3') and reverse (5'-GCTCTGTTGGT CATGTACTGAGTG-3'). This 506 bp fragment was then cloned into the pBluescript II SK(+) vector and sequenced to verify their identity and orientation.

Ufd1L: Ufd1L cDNA fragment (positions 306-981 of *mus musculus* ubiquitin fusion degradation 1 like, Gene Bank accession number NM-011672) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-GAATTCAGATCGG ATGACACACTG-3') and reverse (5'-TCCT TCTCCAGAGAAAGCAACG). This 676 bp fragment was then cloned into the pBluescript II SK (+) and sequenced to verify their identity and orientation.

Ubp41: Ubp41 cDNA fragment (positions 85-936 of *mus musculus* ubiquitin-specific protease Ubp41, Gene Bank accession number AF079565) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-ATTCTTCAGTG CCTGAGCAACACC-3') and reverse (5'-AC TTCGGCAGTAGGCTGTATAGTG-3'). This 852bp fragment was then cloned into the PCRII-TOPO vector and sequenced to verify their identity and orientation.

UchL3: UchL3 cDNA fragment (positions 1-710 of *mus musculus* ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase), Gene Bank accession number NM-016723) was polymerase chain reaction amplified using oligonucleotide primers: forward (5' -AGCAGTCATGGAGGGTCAACGCTG-3') and reverse (5'-GCTATGCTGCCGAGAGAGCAATT G-3'). This 710bp fragment was then cloned into the PCRII-TOPO vector and sequenced to verify their identity and orientation.

UchL1: UchL1 cDNA fragment (positions 256-788 of *mus musculus* ubiquitin carboxyl-terminal hydrolase PGP9.5, Gene Bank accession number AF172334) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-AG CAGACCATCGGAAACTCCTGTG-3') and reverse (5'-AACGCAAGAAGACAGCTGTG-3'). This 533 bp fragment was then cloned into the PCRII-TOPO vector and sequenced to verify their identity and orientation.

β-TrCP: A β-TrCP cDNA fragment (positions 311-932 of *mus musculus* β-transducin repeat containing protein, Gene Bank accession number NM-009771) was polymerase chain reaction amplified using oligonucleotide primers: forward (5'-AA CTTGCCAATGGCACTTCCAGC-3') and reverse (5'-ACTATCTTCTGGTCGTCGTACTG-3'). This 622bp fragment was then cloned into the PCR2.1-TOPO vector (Invitrogen, Carsbad, CA) and sequenced to verify their identity and orientation.

These cDNA fragment-containing vectors were linearized with restriction enzymes and used as templates for sense and antisense cRNA probes. Radiolabeled probes for above enzymes were made using ^{33}P-UTP (New England Nuclear, Boston, MA) with a standard protocol for cRNA synthesis.

Methods for in situ hybridization. Mice were deeply anesthetized with ether, and intracardially perfused with 10 ml of autoclaved ice cold saline, followed by 20 ml of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). For the animals housed in darkness, anesthesia was performed under safe dark red light. The brains were removed, postfixed in the same fixative for 24 h at 4 °C and placed in 0.1 M PB containing 20% sucrose for 48 h. These brains were frozen using dry ice and stored at −80°C until use. Mouse brain sections were made 40 µm in thickness by a cryostat (Reichert-Jung, Heidelberg, Germany). To minimize technical variations throughout the hybridization procedure, sections from different experimental conditions were gathered into one group and processed simultaneously. In situ hybridization histochemistry was performed as described previously (17). Briefly, tissue sections were processed with 1 µg/ml proteinase K (0.1 M Tris buffer, pH 8.0, 50 mM EDTA, 10 min) at 37°C and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in the hybridization buffer (60% formamide, 10% dextran sulphate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.6 M NaCl, 0.2% N-laurylsarcosine, 500 µg/ml transfer RNA, 1 × Denhardt's and 0.25% sodium dodecyl sulphate) containing the ^{33}P-UTP-labeled antisense cRNA probes for 16 h at 60°C. After hybridization, these sections were rinsed in 2 × SSC/50% formamide for 45 min at 60°C, and rinsed in 2 × SSC/50% formamide for 15 min at 60°C and the sections were treated with a solution containing 10 µg/ml RNase A (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 M NaCl) for 30 min at 37°C. The sections were further rinsed in 2 × SSC/50% formamide for 15 min at 60°C and in 0.4 × SSC for 30 min at 60°C. Sections for free floating in situ hybridization were mounted onto gelatin-coated microscope slides, air-dried, and dehydrated. These sections together with ^{14}C-acrylic standards (Amersham, Buckinghamshire, UK) were exposed to BioMax film (Kodak, Rochester, NY) for five days and subjected to the image analysis process. For assessing the nine ubiquitin-related enzymes in the SCN, we used 405 brain sections from 27 mice (45 sections of 3 mice at ZT4 were hybridized simultaneously with each probe). The radioactivity of each SCN on BioMax film was analyzed using a microcomputer interface to an image analysis system (MCID, Imaging Research, St Catherines, Ontario, Canada) after conversion into the relative optical densities produced by the ^{14}C-autoradiographic microscales (Amersham). Data were normalized with respect to the difference between signal intensities in equal areas of the SCN and the corpus callosum. The intensities of the optical density of the sections from the rostral to the caudal most part of the SCN (8 sections per mouse brain) were then summed; the sum was considered to be a measure of the amount of UchL1 mRNA in this region. The intensity values are expressed as means ± SEM (n = 5). We use relative mRNA abundance, which means that the peak intensity value in the LD and DD conditions was adjusted to 100, respectively. For statistical analysis, one-way ANOVA followed by Scheffe's multiple comparisons was applied.

RESULTS

Expression of ubiquitin-related enzymes in the SCN

Since the SCN is the site for biological clock, we examined the expression of ubiquitin-related enzymes in the SCN. In situ hybridization examination using the cRNA antisense probes for Ubce5, UbcM4, Ube2v, Ube2d2, UchL1, UchL3, Ubp41, β-TrCP and Ufd1L have demonstrated a variety of signals (Table 1). High level of expression was detected for UchL1 (Fig. 1), moderate level for Ubce5,

Table 1 *Expression level of mRNAs of uniquitin-related enzymes in the mouse SCN*

TYPE	NAME	Expression Level in the SCN
E2	Ubce5	++
	UbcM4	+
	Ube2v	0
	Ube2d2	+
DUB (UCHs)	UchL1	+++
	UchL3	0
DUB (UBPs)	Ubp41	0
E3	β-TrCP	+
ubiquitin-binding protein	Ufd1L	+

In situ hybridization signals were judged as strong (+++), moderate (++), low (+), and very low to none (0).

and weak levels for UbcM4, Ufd1L and β-TrCP. The use of sense cRNA probes for these enzymes revealed no specific hybridization signals in brain sections. Even using antisense probes, Ube2v, UchL3 and Ubp41 were almost absent in the SCN.

Circadian expression of UchL1 mRNA in the SCN

Among these enzymes, UchL1 showed the highest level of expression. Since many of the substances expressed in the SCN showed a circadian expression, we tested whether the expression of UchL1 in the SCN shows circadian change at mRNA level. UchL1 mRNA was highly expressed in mice by using in situ hybridization (Fig. 2). Visual inspection of autoradiographic films revealed constant high expression of UchL1 mRNA in the SCN under conditions of both light-dark and constant dark (Fig. 2, inlets). When SCN hybridization signals were assessed quantitatively by image analysis, no significant variation of UchL1 signal was detected over the circadian cycle under both LD (one way ANOVA, $F_{5,24} = 1.83$, $P = 0.14$) and DD ($F_{5,24} = 0.52$, $P = 0.76$) conditions (Fig. 2).

DISCUSSION

By using the fibroblast cell lines in which expression of *mPer2* is controlled through tetracycline-regulatable promoter, we recently found that mPER2 protein accumulation in these cells showed clear circadian oscillation even in the presence of constitutive *mPer2* mRNA expression (25). This suggests that post-transcriptional regulation of *mPer2* plays an important role in generating the mPER2 accumulation cycle and following cycling of circadian feedback loops in mammals. This is similar to

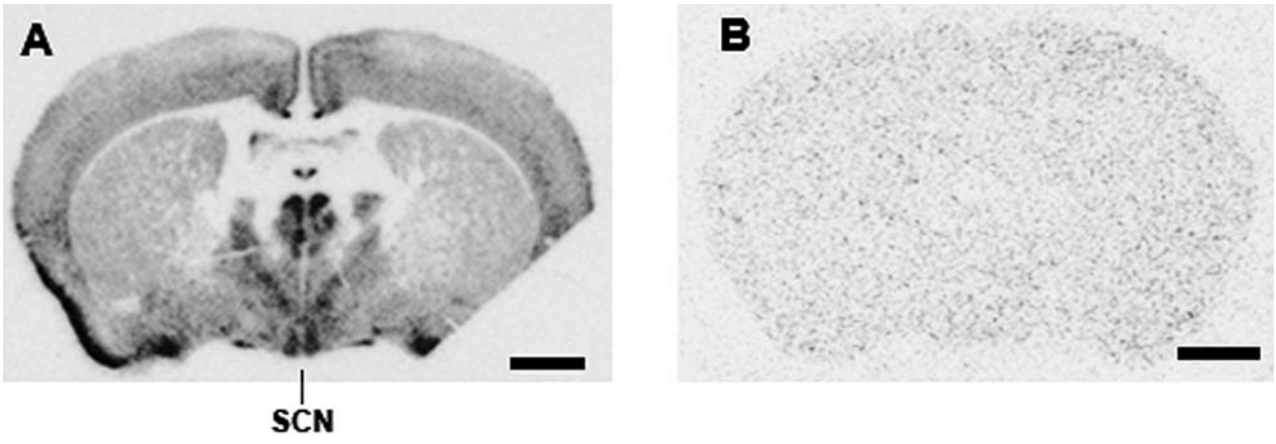

Fig. 1 In situ hybridization of UchL1 mRNA in the suprachiasmatic nucleus (SCN). Hybridization with antisense probe (A) and sense probe (B). Bar = 1 mm.

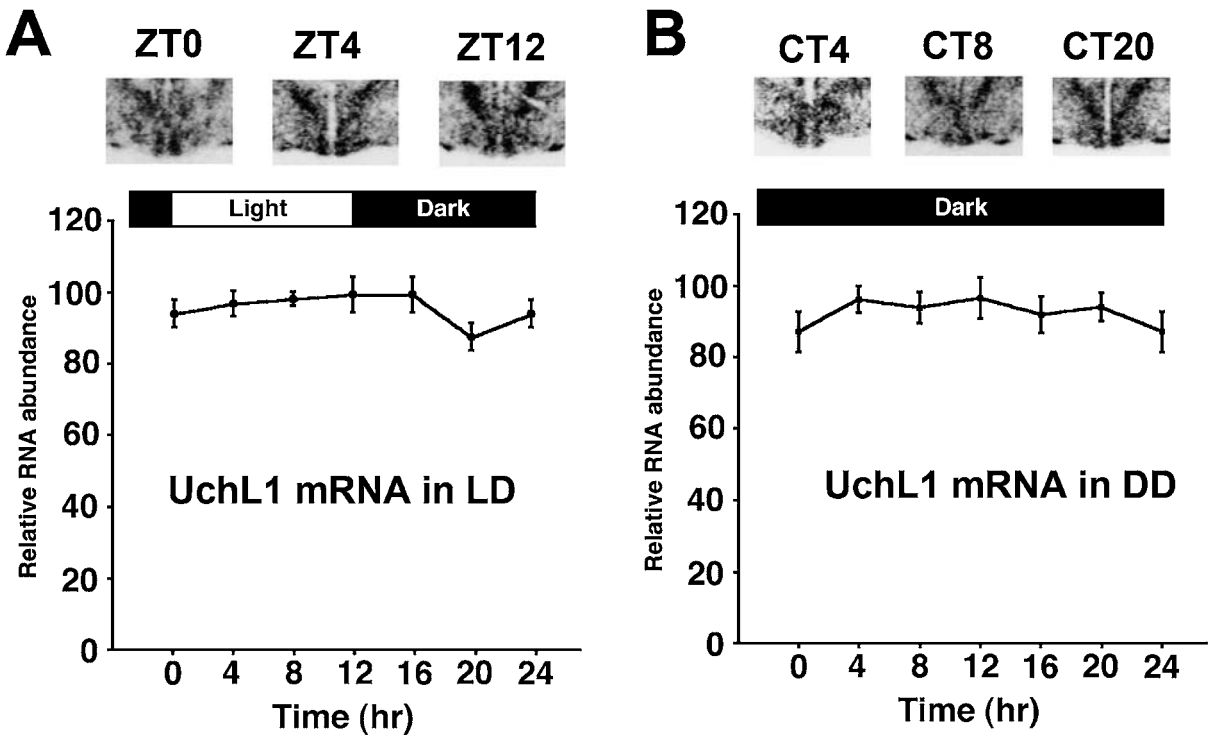

Fig. 2 The expression profiles of UchL1 mRNA in the SCN in LD 12 : 12 (A) and in DD (B). The relative RNA abundance was determined by quantitative in situ hybridization, with the peak value adjusted to 100%. Values are expressed as means ± SEM (n = 5). Representative autoradiograms for each time point are shown above each graph. Numbers on each autoradiogram indicated the sampling time (hr).

Drosophila per since cycling at protein level without accompanying mRNA cycling was also reported (1, 3, 20, 26). Interestingly, the application of proteasome inhibitor MG132 showed the attenuation of the reduction of mPER2, suggesting that proteasome-mediated proteolysis step has an important role for the generation of circadian rhythms. Ubiquitin-proteasome mediated degradation is functionally active in *Drosophila* circadian system, since the mutation of *Drosophila slimb*, an F-box protein constituting ubiquitin ligase, is known to induce the constant accumulation of PER protein and behavioral arrhythmicity (4, 9).

In *Neurospora*, a similar F-box protein negatively regulates the ubiquitination of FRQ, which is a negative element of circadian feedback loop in this species (5). Since it is speculated that the clock oscillatory machinery is evolutionally conserved, the similarity between the *Drosophila* and *Neurospora* suggests that mammalian counterpart of ubiquitin ligase plays the similar role. Here we examined the β-TrCP, which is a homologue of *Drosophila slimb*, but we only found the very weak expression of this gene in the main circadian center SCN. This speculates that β-TrCP will not have a dominant role for the generation of circadian oscillation in mammals. However, this study is only an expression analysis, and the exact role of this protein is not solved before the clarification of ubiquitin ligase of PER1 and PER2. The involvement of ubiquitin on mammalian circadian system should be examined from the broader spectrum of view.

Contrary to β-TrCP, in the SCN, we found the high levels of expressions of UchL1, an essential member of DUB. UchL1 recycles ubiquitin from ubiquitin-protein complexes or polyubiquitin chains by cleaving the amide linkage neighboring the C-terminal glycine of ubiquitin (10). A novel ubiquitin-ubiquitin ligase activity of UchL1 that may be important in Parkinson's disease pathogenesis was also discovered (12). UchL1 is one of major proteins in the brain, constituting 1–5% of total solute brain proteins.

The cell clock coordinates the timing of the expression of a variety of genes with specific cellular functions. Gene array studies have demonstrated that there are hundreds of genes controlled by the circadian clock (15, 18). Central clock oscillatory signals

are proposed to be transmitted by two routes: the first by E-box (CACGTG, CACGTT) and the second by D-box (RTTAYGTAAY: R, purine; Y, pyrimidine) (6, 7, 11, 13, 24).

In the present study, we examined circadian expression profiles of UchL1 in the SCN by the quantitative in situ hybridization, but there were no such change in the SCN. This finding strongly suggests that UchL1 is not a clock controlled gene. A previous DNA microarray study (15) reported the circadian change of their mRNA in the SCN with a peak in subjective day and a trough in subjective night. The difference of the present and their studies may be derived from the difference of the methods employed, but at least we can say that the real expression profiles of microarray data should be confirmed in more quantitative methods (e.g., Northern blotting and in situ hybridization). In the present study, we examined the expression of ubiquitin-related enzymes in the SCN only at ZT4 (4 hours after the subjective dawn) except UchL1. Thus, the reported circadian expression of some of ubiquitin-related enzymes (Ubce5, Ufd1L, UchL1 and Ubp41) by DNA microarray method should be re-examined in further semiquantitative in situ hybridization study.

Although not rhythmically expressed, this does not deny the possibility that UchL1 is involved in the genesis of circadian rhythms. Recently it is demonstrated that gracile axonal dystrophy (*gad*) mice carry the mutation of UchL1 gene (16): the *gad* allele encodes a truncated UchL1 lacking a segment of 42 amino acids containing a catalytic residue. In this mutation, neurodegeneration occurs progressively showing sensory ataxia at an early stage followed by motor ataxia at a later stage. Since *gad* mutation affects ubiquitin protein turnover (14), it is possible that *gad* mutation will be used for the analysis of the involvement of ubiquitin-proteasome system in mammalian circadian oscillatory system.

Acknowledgments

This work was supported by the Scientific Grants from the Ministry of Health, Welfare and Labor, The Special Coordination Funds and the Scientific Grants of the 21st Century COE Program from Ministry of Education, Culture, Sports, Science and Technology of Japan, SRF, and Hyogo Science Foundation.

REFERENCES

1. Cheng Y and Hardin PE (1998) Drosophila photoreceptors contain an autonomous circadian oscillator that can function without period mRNA cycling. *J Neurosci* **18**, 741–750.
2. Ciechanover A, Orian A and Schwartz AL (2000) Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* **22**, 442–451.
3. Frisch B, Hardin PE, Hamblen-Coyle MJ, Rosbash M and Hall JC (1994) A promoterless period gene mediates behavioral rhythmicity and cyclical per expression in a restricted subset of the Drosophila nervous system. *Neuron* **12**, 555–570.
4. Grima B, Lamouroux A, Chelot E, Papin C, Limboug-Bouchon B and Rouyer F (2002) The F-box protein slimb controls the levels of clock proteins period and timeless. *Nature* **420**, 178–182.
5. He Q, Cheng P, Yang Y, He Q, Yu H and Liu Y (2003) FWD1-mediated degradation of FREQUENCY in Neurospora establishes a conserved mechanism for circadian clock regulation. *EMBO J* **22**, 4421–4430.
6. Ishida Y, Yokoyama C, Inatomi T, Yagita K, Dong X, Yan L, Yamaguchi S, Nagatsu I, Komori T, Kitahama K and Okamura H (2002) Circadian rhythm of aromatic L-amino acid decarboxylase in the rat suprachiasmatic nucleus: gene expression and decarboxylating activity in clock oscillating cells. *Genes Cells* **7**, 447–459.
7. Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ and Reppert SM (1999) A molecular mechanism regulating rhythmic output from the suprachiasmatic nucleus. *Cell* **96**, 57–68.
8. Kim JH, Park KC, Chung SS, Bang OS and Chung CH (2003) Deubiquitinating enzymes as cellular regulators. *J Biochem* **134**, 9–18.
9. Ko HW, Jiang J and Edery I (2002) Role for slimb in the degradation of Drosophila period protein phosphorylated by Doubletime. *Nature* **420**, 673–678.
10. Larsen CN, Krantz BA and Wilkinson KD (1998) Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. *Biochemistry* **37**, 3358–3368.
11. Lavery DL, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquest F, Schibler U and Bonfils C (1999) Circadian expression of the steroid 15a-hydroxylase (*Cyp2a4*) and coumarin 7-hydroxylase (*Cyp2a5*) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. *Mol Cell Biol* **19**, 6488–6499.
12. Liu Y, Fallon L, Lashuel HA, Liu Z and Lansbury PT, Jr (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* **111**, 209–218.
13. 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 Develop* **15**, 995–1006.
14. Osaka H, Wang YL, Takada K, Takizawa S, Setsuie R, Li H, Sato Y, Nishikawa K, Sun YJ, Sakurai M, Harada T, Hara Y, Kimura I, Chiba S, Namikawa K, Kiyama H, Noda M, Aoki S and Wada K (2003) Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum Mol Genet* **12**, 1945–1958.
15. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS and Hogenesch JB (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109**, 307–320.
16. Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosa-

wa H, Harada T, Ichihara N, Wakana S, Kikuchi T and Wada K (1999) Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in *gad* mice. *Nat Genet* **23**, 47–51.
17. Shigeyoshi Y, Taguchi K, Yamamoto S and Okamura H (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**, 1043–1053.
18. Storch KF, Lipan O, Leykin I, Viswanathan N, Davis FC, Wong WH and Weitz CJ (2002) Extensive and divergent circadian gene expression in liver and heart. *Nature* **417**, 78–83.
19. Sujino M, Masumoto KH, Yamaguchi S, van der Horst GT, Okamura H and Inouye ST (2003) Suprachiasmatic nucleus grafts restore circadian behavioral rhythms of genetically arrhythmic mice. *Curr Biol* **13**, 664–668.
20. Vosshall LB and Young MW (1995) Circadian rhythms in Drosophila can be driven by period expression in a restricted group of central brain cells. *Neuron* **15**, 345–360.
21. Wilkinson KD (1995) Roles of ubiquitinylation in proteolysis and cellular regulation. *Annu Rev Nutr* **15**, 161–189.
22. Wilkinson KD (1997) Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* **11**, 1245–1256.
23. Yagita K, Tamanini F, Yasuda M, Hoeijmakers JHJ, van der Horst GTJ and Okamura H (2002) Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. *EMBO J* **21**, 1301–1314.
24. Yamaguchi S, Mitsui S, Yan L, Yagita K, Miyake S and Okamura H (2000) Role of DBP in the circadian oscillatory mechanism. *Mol Cell Biol* **20**, 4773–4781.
25. Yamamoto Y, Yagita Y and Okamura H (2005) Role of cyclic mPer2 expression in mammalian cellular clock. *Mol Cell Biol* **25**, 1912-1921.
26. Yang Z and Sehgal A (2001) Role of molecular oscillations in generating behavioral rhythms in Drosophila. *Neuron* **29**, 453–467.