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ORIGINAL PAPER

Light-induced COP9 signalosome expression in the Indian false vampire bat *Megaderma lyra*

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Abstract The COP9 signalosome (CSN) is a multi-subunit protein complex conserved in plants and animals. CSN subunits have been identified as light-mediated master regulators of eukaryotic circadian clocks from fungi to animals. The Indian false vampire bat *Megaderma lyra* is completely adapted to an anthropic biotope and behavioral studies have reported that *M. lyra* exhibits light-sampling behavior to assess environmental light. LC–MS–MS results for a 36 kDa protein were analyzed using the Sequest search engine, and COP9 signalosome subunit 5 (CSN5) was pinpointed as having the highest score with 6 matching peptides. To confirm the presence of CSN5, up-regulated cDNA was amplified, sequenced, and identified as CSN5. Furthermore, semi-quantitative RT-PCR analysis demonstrated that the level of induction of CSN5 was regulated by environmental light. We estimated the level of expression across a light–dark cycle and observed a higher level of expression at the end of the light phase. Similarly, when the animal was shifted from continuous dark to light, CSN5 expression was induced. Correspondingly, we detected the similar pattern of translated protein with JAB1 antibody. Knowledge about the circadian rhythm and its molecular mechanism in Chiroptera is very limited and this study suggests that CSN5 might be involved in the *M. lyra* light-signaling process.

Keywords COP9 · *Megaderma lyra* · Chiroptera · Mass spectrometry

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Introduction

The COP9 signalosome (CSN, also known as the COP9 complex) was discovered [1] as a component of a novel signaling complex and was described as a repressor of light-dependent growth in *Arabidopsis thaliana*. Subsequent identification and characterization of this complex in yeast, insects, and mammalian cells have described the complex as a general modulator of signal transduction [2]. The COP9 signalosome is a conserved protein complex in plants and animals and consists of eight subunits (CSN 1–8). The composition and sequences of these subunits are highly conserved both in plants and animals. More than any other of the CSN subunits, it has been found that CSN5 plays a multifactorial role. Based on the specific target, CSN5 exerts different effects on target stability. On the one hand, CSN5 stabilizes several of its protein-binding partners. On the other hand, CSN5 promotes the degeneration of other binding proteins [3]. Nonetheless, disruption of any of the subunits is able to cause a variety of developmental defects, because of the unique functions of each of these subunits [4]. The amino acid sequence identity of the various subunits varies from 32% (S7 and S8) to 60% (S2 and S5) in plants and mammals [5]. CSN5 has been identified as a Jun-activation-domain-binding protein 1 (JAB1), which has the capacity to bind with a number of regulatory proteins, and this affects various protein degradation and phosphorylation pathways [6].

He and his co-workers [7] demonstrated that the CSN is involved in the protein degradation pathway of the *PERIOD* (PER) and *FREQUENCY* (FRQ) genes, which are involved in the circadian rhythm. Generally, PER and FRQ are phosphorylated and dephosphorylated by homologous kinases and phosphatases. The phosphorylated proteins are the ubiquitinated by an ubiquitin ligase, depending on the environmental light–dark situation. This reaction was found to regulate the circadian rhythm of *Neurospora* through a pathway that is highly conserved from *Neurospora* to animals [8]. Recent studies in *Drosophila* have demonstrate that CSN5 is required for the normal degradation of the clock protein Timeless (TIM), a key regulatory molecule involved in the entrainment of the molecular clock during light–dark cycles [9]. In addition, they found an altered behavioral phase shift in individuals lacking normal CSN5 activity.

Many species of bats (Chiroptera), especially microchiropterans, use dark areas, for example the deeper recesses of caves, as their day roost. They mainly rely on acoustic signals (echolocation), rather than vision for orientation and for the detection/capture of prey [10]. Microchiropterans share a common visual pathway with all vertebrates, except primates [11]. It is well known that vision has developed for various different tasks in mammals, for example synchronizing biological rhythms and mediating the regulation of non-rhythmic physiological processes in response to day/night oscillations. Marimuthu [12] reported that a few minutes after sunset, bats move over to the entrance chamber of their caves, exhibit light-sampling behavior, and, when an optimal level of light intensity is reached, they begin to fly out to forage. Similarly, a few minutes before sunrise, all individuals of a colony return to their day roosts. The Indian false vampire bat *Megaderma lyra* is a common microchiropteran and is distributed across several parts of India [13]. It lives in caves, temples [14], and unused/ruined buildings (Marimuthu and Rajan, personal observations). Behavioral studies have revealed that *M. lyra* exhibits light-sampling behavior in order to assess the environmental light condition at the entrance of their day roosts. Specifically, local sunset and sunrise positively influence the time of emergence and return [14]. Although it seems that bats assess the external radiance level and this possibly regulates their activity cycle, knowledge of their circadian rhythm and the molecular mechanism of the process in the chiroptera is very limited. In this study we have confirmed that the CSN does exist in the Indian false vampire bat *M. lyra* and then tested the expression pattern of the CSN at different points in the light–dark (LD) cycle. Specifically, we identified a CSN5-like protein by use of mass spectrometry (MS) and examined whether this gene expression is regulated by environment light–dark conditions.

Materials and methods

Animal housing

Megaderma lyra individuals were captured using a nylon mosquito net as they emerged in flight from their day roosts. Four individuals were used for the LC–MS analysis and 15 individuals were used for quantification of CSN5 expression levels. After capture, they were released into a free flight room (bat chamber, 4.42 × 3.74 × 2.44 m; conditioned for temperature and humidity) at the animal house of the university and fed with frogs. In the flight room a 12 h light and 12 h dark cycle was maintained. The lighting cycle was produced by overhead cool fluorescent lamps with an intensity of approximately 120 lux. All the experimental protocols approved by Bharathidasan University Wild Animals Ethical Committee, Bharathidasan University, Tiruchirappalli.

MALDI-TOF LC–MS–MS analysis

The deep midbrain region was dissected out from *M. lyra* individuals and the total protein present in these samples was extracted by homogenizing the tissues in 2 mL phosphate-buffered saline (PBS; pH 7.2) that contained a cocktail of protease inhibitors (Sigma–Aldrich, USA). Next, the homogenates were precleared by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was then collected and stored at 4°C until use.

Total protein concentration was estimated by a standard protocol [15]. Next, protein (70 µg) was loaded on to a one-dimensional (12%) SDS-PAGE gel as described by Laemmli [16]. For determination of molecular mass, 5 µl of a standard protein marker (GeNei, Bangalore, India) was loaded in a parallel lane on the same gel. Protein bands were excised from the gel and destained in 200 µl destaining solution composed of 25 mM NH_4HCO_3, 50% (*v*/*v*) acetonitrile (ACN) in a 1:1 ratio. The proteins present were next digested with trypsin in 50 mM ammonium bicarbonate, pH 8.0 at 37°C for 12 h; after this, the peptides extracted with 5% (*v*/*v*) TFA (trifluoroacetic acid) in a 1:1 (*v*/*v*) water–ACN mixture. Following evaporation to dryness, the peptides were resuspended in 30 µl 0.1% FA (formic acid) in water and analyzed.

MS analysis was performed using an LTQ-Orbitrap (Discovery) hybrid tandem mass spectrometer with a nanoelectrospray ion source (Thermo Electron, San Jose, CA, USA) coupled to a nano flow LC system (Agilent Technologies 1200 series) and autosampler. The protein digests were separated using a 13.5-cm-long, 75-µm-inner diameter tip column and a 1.4-cm-long, 300-µm-inner diameter trap column packed in house with 5-µm C_{18} particles. The mobile phase consisted of 0.1% FA in water

(solvent A) and 0.1% FA in ACN (solvent B). The pump flow rate was set at 0.5 µl/min, and peptide elution was achieved by use of a linear gradient. The conventional MS spectra (survey scan) were acquired at high resolution over the acquisition range *m/z* 200–2000. The full-scan MS^2 spectra were analyzed in the linear trap. Peptides were analyzed in the data-dependent mode where for each 1-s survey scan the three most intense precursor ions were selected for MS^2 sequencing. To prevent the reacquisition of product ion spectra from the same precursor ion, a dynamic exclusion window of 0.050 Da was applied for 60 s. Mass calibration used either an internal lock mass (*m/z* 445.12057) or external calibration using Calmix (caffeine, MRFA, and Ultramark) and typically provided mass accuracy within 2 ppm for all nano-LC–MS experiments.

The MS dataset was analyzed using Xcalibur software (version 2.0 SR1). Product-ion scans obtained from tandem mass spectrometry experiments were analyzed by the database search software Sequest (Turbo). Peak lists were generated from product in scan data and used to search against the non-redundant protein sequence database obtained from the National Center for Biotechnology Information, NCBI. For proteolytic cleavage, only tryptic cleavage was allowed and the maximum number of missed cleavage sites was set to 2. Modifications at cysteines by carboxymethylation and methionine by oxidation were allowed. The mass tolerance for precursor peptide ions was set to 3.5 and fragment ion tolerance was set to 1. The Sequest results were filtered by use of criteria similar to those developed elsewhere [17]. Briefly, all accepted results had a DelCn (delta Cn) of 0.1 or greater, which is a value shown to lead to high confidence in a Sequest result. Cross-correlation (Xcorr) scores of singly charged peptides had to be higher than 1.9 and those of doubly and triply charged peptides higher than 2.2 and 3.75, respectively. In order to have high confidence in the protein identification, these criteria were always used in the database search. When a protein was identified by three or more unique peptides possessing Sequest scores that passed the above-mentioned criteria, no visual assessment of spectra was conducted and the protein was considered present in the sample.

Expression analysis of CSN5

In order to quantify the expression level of CSN5, a total of 15 *M. lyra* were divided into five groups consisting of three individuals each. Each group was kept in an environment with various different schedules of light and dark cycles. At an appropriate time, the individuals in a group were anesthetized by intra-peritoneal injection of urethane (3 mg/kg body mass) and then decapitated. The individuals forming group 1 were captured at their day roost and decapitation was done when a transition occurred from the natural light to darkness. The remaining four groups were kept under a LD cycle at the laboratory for 2 days. Decapitation of group 2 was carried out during the transition to darkness after 12 h of light on day two. The duration of light for group 3 was extended for an extra 12 h, and thus decapitation was carried out when the transition to darkness occurred after exposing the individuals to 24 h of light (LL). The duration of darkness for group 4 was extended for an extra 12 h, and thus decapitation was done when transition to light occurred after exposing the individuals to 24 h of darkness (DD). The duration of darkness for group 5 was extended for an extra 12 h and this was followed by exposing the individuals to 12 h of light. Decapitation was done at the end of the light phase.

Total RNA was isolated from the dissected brain tissue by homogenizing with 0.5 ml TRIzol (Invitrogen, USA) and then the RNA was purified using a Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The RNA concentration was estimated using optical absorbance at 260 nm by spectrophotometry (Optima, Japan). Total RNA (0.5 µg/sample) was reverse-transcribed for first-strand cDNA synthesis using the AMV reverse transcriptase (AccessQuick RT-PCR system; Promega, Madison, USA) for 1 h at 42°C. Then the COP9 subunit 5 (1100 bp) DNA fragment was amplified using the sense primer 5′-AT GGCGGCGTCCGGGAGCGGT-3′ and the antisense primer 5′-AGGACACTTCAGAGCACCTTA-3′. These primers were designed from the mouse CSN5 sequence (NM_013715). The PCR reaction was carried out by adopting the following conditions: initial denaturation at 94°C for 3 min, then denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min (MJ Mini Gradient Thermal Cycler; Bio-Rad, USA). The amplified product was cloned in the vector pTZ57R (InsTAclone; PCR Cloning Kit, Fermentas Life Sciences, Canada) and gene identity was confirmed by sequencing with universal M13 primers.

Semi-quantitative RT-PCR allows us to compare the levels of gene expression relative to a house-keeping gene and this was used to quantify the level of CSN5 expression compared with β-actin [18]; this used the same primers as the single PCR tube reaction. For semi-quantitative measurement, we amplified the CSN5 with β-actin and optimized the number of PCR cycles (27, 30, 33 cycles) to maintain amplification within a linear range. Furthermore, the degree of expression of a given CSN5 was established by dividing the *Toe1* mRNA expression by the level of the β-actin mRNA expression [19]. Each PCR product (20 µl) was electrophoresed on a 1.0% (*w/v*) agarose gel containing ethidium bromide (0.5 µg/ml). Images of the amplified products were acquired using a Molecular Imager

ChemiDoc XRS system. The intensity was estimated by use of image-analysis software (Quantity one; Bio-Rad). The intensity of the bands was expressed as relative peak density, and then the CSN5/β-actin product ratios were calculated and used as indices of CSN5 mRNA expression.

In order to assess the CSN5 translation, we performed western blot analysis. An equal concentration of protein (100 μg) was mixed with loading buffer (100% glycerol, 125 mM Tris–HCl pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% mercaptoethanol) then boiled for 5 min and resolved on a 12% polyacrylamide gel. The separated proteins were transferred electrophoretically on to a Hybond-XL NT membrane (Amersham Pharmacia Biotech, Bangalore, India) then the membrane was blocked in PBS (5% non-fat dry milk; 0.1% Tween 20) for 3 h at room temperature (RT) with gentle agitation. Immunodetection was performed by incubating the membrane with primary antibody (1:200; JAB-1 antibody, SC-13157, Santa-Cruz Biotech) for 12 h in PBS (3% non-fat dry milk; 0.1% Tween 20). The membrane was washed, and bound antibodies were detected by incubating for 6 h with goat anti-mouse antibody conjugated with alkaline phosphatase (1:2000, SC-2320; Santa-Cruz Biotech). After a final wash, alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-inolyl phosphate/nitroblue tetrazolium salt (BCIP/NBT) according to the manufacturer's instructions (Invitrogen).

Results

SDS-PAGE protein profiles of the brain samples showed expression of various proteins across a wide range of molecular weights ranging from 17 to 80 kDa. For the initial search, we selected proteins of low molecular mass with the objective of detecting the presence of non-retinal opsin as part of an ongoing project using LC–MS–MS analysis (unpublished). When 8 μl of the in-gel tryptic digested peptides from a 36-kDa protein were subjected to liquid chromatography–tandem mass spectrometry, the mass spectrum was generated and, interestingly, the outcome after Sequest search engine analysis was identification of CSN with a high score. The score for other proteins was very low. This identified CSN was then focused on. Further analysis clearly showed the presence of CSN5 with the highest score and overall, this protein matched CSN5 with a sequence coverage of 33.4% (Table 1). Analyses of the matching peptide coverage and the distribution pattern with regard to previously reported CSN subunit sequences indicated that the matching peptides are highly conserved in all reported CSN5 sequences (Fig. 1).

The designed degenerate primers based on the CSN5 sequence were then used to amplify a fragment of about 1100 bp and sequence analysis confirmed that we had cloned a fragment of CSN5 from *M. lyra.* The sequence obtained has been submitted to the GenBank and is available with accession number FJ713811. To investigate the light-regulated expression of CSN5, we measured the variation in the expression of CSN5 at different points in the light–dark cycle using semi-quantitative RT-PCR analysis. The level of CSN5 expression was estimated by comparing it against the expression of the housekeeping gene β-actin. In all the samples we measured the level of a 350-bp β-actin fragment and the 1100-bp CSN5 fragment. Importantly, the level of CSN5 expression varied depending on the environmental light–dark conditions before decapitation of the *M. lyra* individual (Fig. 2a). Initially, we estimated the level of CSN5 expression at completion of the light phase in free-ranging and captive *M. lyra.* The level of expression was higher in captive individuals that had been exposed to 12:12 h LD cycles than in the free-ranging individuals that had experienced a natural LD cycles of about 12:12 h (Fig. 2b). However, the difference was not statistically significant (one-way ANOVA, Bonferroni corrected, $F = 1.96$; $p = 0.234$). Further analysis showed that the level of CSN5 expression decreased compared with the control bats (12 h L:D cycle) when the light phase was extended by an additional 12 h compared with the regular LD cycle (Fig. 2). Furthermore, the level of expression of CSN5 significantly decreased under continuous light conditions (one-way ANOVA, Bonferroni corrected, $F = 20.64$, $p = 0.01$) and under continuous

Table 1 Sequence coverage and peptide masses of the COP9 constitutive photomorphogenic-like subunit 5

Subunit	No. of peptides	MH^+	Xcorr	Z	Peptide position	Sequence coverage (%)
	1	3139.5	2.88	2	12-TWELANNMQEAQSIDEIYK-30	
	2	2090.0	3.26	2	107-VNAQAAAYEYMAAYIENAK-125	
	3	2133.0	3.35	2	192-GYKPPDEGPSEYQTIPLNK-210	
5	4	2283.0	3.05	2	245-YWVNTLSSSSLLTNADYTTGQVFDLSEK-272	33.4
	5	1363.6	2.85	2	283-GSFMLGLETHDR-294	
	6	1640.9	3.36	2	310-TTIEAIHGLMSQVIK-324	

Matching peptides have been shown with their peptide position

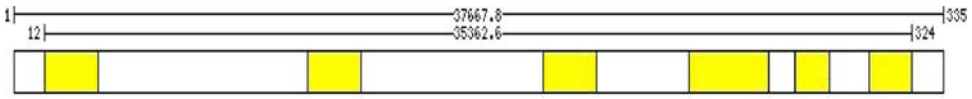

Fig. 1 The identified peptides from the *M. lyra* 36 kDa protein matched with various COP9 signalosome peptides; distribution of the subunit 5 peptides

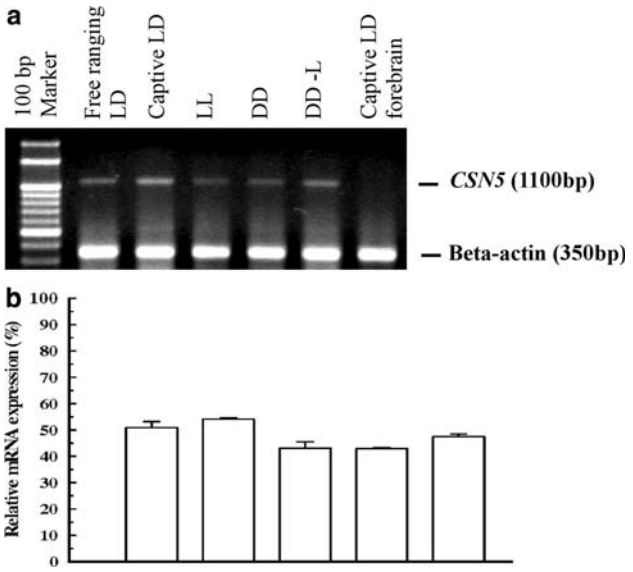

Fig. 2 Semi-quantitative analysis of CSN5 mRNA expression. **a** Gel electrophoresis analysis of the semi-quantitative RT-PCR products derived from bats exposed to different light–dark conditions; the amplification shows the differential expression of CSN5 and a constant level of β-actin expression. **b** The density of the CSN5 band was divided by the density of the band for β-actin mRNA to normalize the results. Average expression of CSN5 was obtained by calculating the mean ± standard error

dark conditions ($F = 264.95$, $p = 0.001$), when compared with the laboratory LD condition. Interestingly, when the bats were exposed to 12 h of light, after they had experienced 24 h of darkness, we observed reinstatement of CSN5 expression and this was significantly higher than in the continuous dark individuals ($F = 17.59$, $p = 0.014$). Nevertheless, this expression level was not equivalent to the level for 12:12 h LD. Finally, we found no expression of CSN5 in the forebrain region of bats that had been exposed to 12:12 h LD (Fig. 2a). Later, we performed immunoblot analysis with an antibody against JAB-1 to discover whether the light-induced mRNA translated as protein. The antibody recognized a protein with a molecular mass of 36 kDa (Fig. 3) in all groups. Observed variations reflected the pattern of CSN5 mRNA level.

Discussion

The COP9 signalosome represents one of the most evolutionarily conserved multifunctional protein complexes and

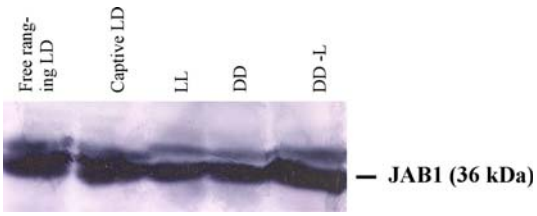

Fig. 3 Western blot analysis shows the light-induced expression of *CSN5* completely translated as protein. The JAB1 antibody recognized a protein of 36 kDa in all samples

we sought to identify its presence in a nocturnal mammal, the Indian false vampire bat *M. lyra.* On the basis of previous reports, we screened low-molecular-weight proteins and identified a protein similar to CSN5 with a molecular mass around 36 kDa. This agrees with the known molecular mass of CSN5 when expressed at high levels in thymus, spleen, and brain of mice [20, 21]. In addition, proteins highly similar to the components of the COP9 complex have been identified in animal systems that exhibit similar constitutive photomorphogenic phenotypes [2]. Genetic analysis clearly indicates that the COP9 complex is an important cellular component capable of modulating multiple signaling pathways that depend on environmental light. Furthermore, mutational analysis of the COP9 subunits in *Arabidopsis* was found to result in light-dependent phenotypes. A similar study in *Neurospora* found that the mutant had an abnormal circadian rhythm [22]. Recent reports have highlighted the fact that the COP9 signalosome is closely related to the 26S proteosome regulatory complex, which has been implicated in control of melatonin synthesis via the rate-limiting enzyme *N*-acetyltransferase. These findings suggest that the COP9 signalosome is sensitive to environmental light and is a possible clock component.

The daily activity of the Indian false vampire bat *M. lyra* depends on the environmental light–dark cycle because of the need to time emergence with dusk, which is set by the sunset, and the need to return at dawn to their roost, which is set by sunrise. Clearly sunlight intensity is important to these events. High levels of activity at night compared with day have been well documented for *M. lyra*, which clearly suggests that the intensity of light regulates their daily activity [14]. However, there is significant lack of knowledge about the molecular mechanism of the Chiroptera circadian rhythm. As an initial attempt, we exposed the

bats to the different light–dark conditions and estimated expression of CSN5 by using semi-quantitative RT-PCR analysis. The analyses clearly showed light-induced expression of CSN5 during the light phase and repression of CSN5 expression during the dark phase. Furthermore, the accumulation of CSN5 during the light phase possibly involved the TIM degradation pathway in order to reset the clock. Earlier studies support the idea that a $CSN5^{null}$ mutant will inhibit TIM degradation in *Drosophila*, whereas the CSN^{DN} mutant failed to inhibit TIM degradation [9]. Interestingly, we found higher levels of CSN5 expression when the bats were shifted from continuous dark to light. This might be explained by the fact that TIM degradation occurs in the presence of light. These results suggest that resetting of the clock occurs through the visual system and it is mediated by CSN5. Studies in *cry* double-mutants have suggested that the CSN is an element in a common pathway that connects photoreception by CRY to TIM degradation [23]. Surprisingly, in contrast with light-induced CSN5 expression, we observed a lower level of expression in bats exposed to continuous light. The role of the CSN seems to be limited to light-mediated TIM degradation and the lower level of CSN5 during continuous light suggests the presence of an alternative pathway that is able to rescue the clock mechanism in the absence of CSN or when there is low level of CSN. Additional western blot analysis revealed that all light-induced mRNA translated completely as protein. These results taken together suggest the presence of an additional mechanism that regulates CSN along with the environmental light signal. Chromosomal mapping studies in Smith–Magenis syndrome patients have provided some evidence for the role of CSN5 in environmental light signaling. Mutations in the human CSN5 fail to regulate the melatonin rhythm and this leads to sleeping disorders [24]. However, the role of melatonin in the *M. lyra* clock mechanism remains unexplored.

In conclusion, we have identified the presence of a CSN5 signalosome-like protein in the deep mid-brain of the Indian false vampire bat *M. lyra*. In addition, we have demonstrate that light influences CSN5 expression, which supports the hypothesis that CSN5 is involved in the light-signaling process of *M. lyra.* Although it is too early to claim a role for *M. lyra*'s CSN5 in the bat's daily activity rhythm, this would seem to be a strong possibility. In future, it will be of great interest to see how CSN5 interacts with the other clock components in *M. lyra* under various light–dark conditions.

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References

1. Wei N, Charmovitz DA, Deng XW (1994) Arabidopsis COP9 is a component of a novel signaling complex mediating light control of development. Cell 78:117–124
2. Oron E, Tuller T, Li L, Rozovsky N, Yekutieli D, Rencus-Lazar S, Segal D, Chamovitz DA (2007) Genomic analysis of COP9 signalosome function in *Drosophila Melanogaster* reveals a role in temporal regulation of gene expression. Mol Syst Biol 3:108–121
3. Peth A, Brerndt C, Henke W, Dubiel W (2007) Downregulation of COP9 signalosome subunits differentially affect the CSN complex and target protein stability. BMC Biochem 8:27–38
4. Busch S, Eckert SE, Krappmann S, Braus GH (2003) The COP9 signalosome is an essential regulator of development in the filamentous fungus *Aspergillus nidulans*. Mol Microbiol 49:717–730
5. Oron E, Mannervik M, Rencus S, Harari-Steinberg O, Neuman Silberberg S, Segal D, Chamovitz DA (2002) COP9 signalosome subunit 4 and 5 regulate multiple pleiotropic pathways in *Drosophila melanogaster*. Development 129:4399–4409
6. Tomoda K, Kubota Y, Kato J (1999) Degradation of the cyclin-dependent kinase inhibitor p27Kip1 is instigated by jab1. Nature 398:160–165
7. He Q, Cheng P, He Q, Liu Y (2005) The COP9 signalosome regulates the *Neurospora* circadian clock by controlling the stability of the SCF^{FWD-1} complex. Genes Develop 19:1518–1531
8. Eide EJ, Woolf MF, Kang H, Woolf P, Hurst W, Camacho F, Vielhaber EL, Giovanni A, Virshup DM (2005) Control of mammalian circadian rhythm by CKI-regulated proteasome-mediated PER2 degradation. Mol Cell Biol 25:2795–2807
9. Knowles A, Koh K, Wu J, Chien CT, Chamovitz DA, Blau J (2009) The COP9 signalosome is required for light-dependent timeless degradation and *Drosophila* clock resetting. J Neurosci 29:1152–1162
10. Neuweiler G (1989) Foraging ecology and audition in echolocating bats. Trends Ecol Evol 4:160–166
11. Barton RA, Harvey PH (2000) Mosaic evolution of brain structure in mammals. Nature 405:1055–1058
12. Marimuthu G (1984) Seasonal changes in the precision of the circadian clock of a tropical bat under natural photoperiod. Oecologia 61:352–357
13. Bates PJ, Harrison DL (1997) Bats of the Indian Subcontinent. Harrison Zoological Museum Publication, England, pp 80–101
14. Balasingh J (1990) The behaviour of the Indian false vampire bat *Megaderma lyra* (Geoffroy 1810):field ethological studies. Ph.D. thesis, Madurai Kamaraj University, India
15. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Ann Biochem 72:248–254
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
17. Qian WJ, Liu T, Monroe ME, Strittmatter EF, Jacobs JM, Kangas LJ, Petritis K, Camp DG, Smith RD (2005) Probability based evaluation of peptide and protein identifications from tandem mass spectrometry and Sequest analysis: the human proteome. J Proteome Res 4:53–62
18. McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush H Jr, Kreiger K, Rosengart T, Cybulsky MI, Silverman ES, Collins T (2000) High-level expression of Egr-1 and Egr-1–inducible genes in mouse and human atherosclerosis. J Clin Invest 105:653–662

19. Chamizo C, Rubio JM, Moreno J, Alvar J (2001) Semi-quantitative analysis of multiple cytokines in canine peripheral blood mononuclear cells by a single tube RT-PCR. Vet Immunol Immunopathol 83:191–202
20. Wei N, Deng XW (1998) Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. Photochem Photobiol 68:237–241
21. Wei N, Deng XW (1999) Making sense of the COP9 signalosome. Trends Genet 15:98–103
22. Wei N, Deng XW (2003) The COP9 signalosome. Annu Rev Cell Dev Biol 19:261–286
23. Helfrich-Förster C, Winter C, Hofbauer A, Hall JC, Stanewsky R (2001) The circadian clock of fruit flies is blind after elimination of all known photo-receptors. Neuron 30:249–261
24. Potocki L, Glaze D, Tan D, Park S, Kashork CD, Shaffer LG, Reiter RJ, Lupski JR (2000) Circadian rhythm abnormalities of melatonin in Smith–Magenis syndrome. J Med Genet 37:428–433