Peroxiredoxins are conserved markers of circadian rhythms

Rachel S. Edgar1, Edward W. Green1, Yuwei Zhao1, Gerben van Ooijen1, Maria Olmedo1, Ximing Qin3, Yao Xu4, Min Pan4, Utham K. Valekunja1, Kevin A. Feeney1, Elizabeth S. Maywood7, Michael H. Hastings7, Nitin S. Baliga6, Martha Merrow5, Andrew J. Millar4, Carl H. Johnson3, Charalambos P. Kyriacou2, John S. O’Neill6 & Akhilesh B. Reddy1

Circadian rhythms are considered to be a feature of almost all living cells. When isolated from external stimuli, organisms exhibit self-sustained cycles in behaviour, physiology and metabolism, with a period of approximately 24 h. Circadian clocks afford competitive selective advantages that have been observed experimentally, and disturbance of circadian timing in humans, as seen in rotational shift work and jet lag, carries long-term health costs. For all organisms in which the molecular timing mechanism has been investigated, a common model has arisen, namely a transcription–translation feedback loop (TTFL). TTFL components are not, however, shared between organisms. For example, the cyanobacterial clock is modelled around three proteins: KaiA, B and C. In the fungus Neurospora crassa, a loop involving the protein FREQUENCY (FRQ) and the WHITE COLLAR (WC) complex is thought to drive cellular rhythms, whereas the plant TTFL involves elements including TOC1 and CCA1 (refs 1, 5). Furthermore, although some multicellular organisms such as Drosophila and humans possess homologous components (for example, the Period proteins), their functions seem to differ between organisms. Therefore, across phylogenetic kingdoms, there are apparently no common ‘clock’ components, suggesting that daily timekeeping evolved independently within different lineages. The converse, however, could equally be true, and the primary premise of this study was therefore to test the hypothesis that circadian clocks may instead have a common ancestry.

Conservation of peroxiredoxin in circadian systems

Recent studies show that the oxidation state of highly conserved peroxiredoxin (PRX) proteins exhibit circadian oscillations in cells from humans, mice and marine algae, probably reflecting an endogenous rhythm in the generation of reactive oxygen species (ROS). Because virtually all living organisms possess peroxiredoxins, we proposed that this marker for circadian rhythms in metabolism may be functionally conserved across all three phylogenetic domains: Archaea, Bacteria and Eukaryota. Peroxiredoxins are peroxidases, the activity of which is dependent on the oxidation of a key ‘peroxidatic’ cysteine residue (Cp) in the active site, that is absolutely conserved, as are neighbouring proline and threonine/serine residues: conforming to a PXXX(T/S)XXCp consensus. Crucially, the catalytic cysteine can become over- or hyperoxidized (PRX-SO2/3), rendering the peroxiredoxin catalytically inactive, but able to participate in ROS signalling and chaperone activity. Once overoxidized, peroxiredoxin can be recycled by sulphiredoxin.

We previously characterized circadian cycles of peroxiredoxin oxidation using antisera directed against the oxidized active site, which recognizes both over-(PRX-SO2) and hyper-(PRX-SO3) oxidized forms. To determine whether this antisera (raised against an oxidized DFTFVCPTEI peptide) could be used to assay over-/hyper-oxidation in diverse species, we performed several sequence alignments to compare peroxiredoxin protein sequences across a variety of circadian model organisms. This revealed a remarkable degree of conservation across all phylogenetic domains, especially within the active site (Fig. 1a, Supplementary Fig. 1 and Supplementary Tables 1–7). Even when we examined the structure of HyrA, the most distantly related peroxiredoxin orthologue in the archaeon Halobacterium salinarum sp. NRC-1, we found that amino acid substitutions would not perturb the geometry of the active site (Fig. 1b). Together, these findings suggested that the same antisera could be used to probe oxidation rhythms in potentially any organism expressing a peroxiredoxin protein. This was confirmed by gene knockout and peroxide treatment in several representative organisms (Supplementary Figs 3 and 7–9).

©2012 Macmillan Publishers Limited. All rights reserved
Using the PRX-SO$_{2/3}$ antiserum, we first examined circadian time courses from a range of eukaryotes under constant conditions (that is, in the absence of external timing cues). In mice, PRX-SO$_{2/3}$ and total PRX1 exhibited a daily cycle in liver tissue and also in the central pacemaker, the suprachiasmatic nuclei (SCN) of the hypothalamus (Fig. 2a). Interestingly, between the two tissues, peroxiredoxin oxidation rhythms were in distinct, and different, phase relationships with respect to total PRX1 and BMAL1 protein, suggesting a difference between brain and peripheral tissue (Fig. 2a), as observed previously for other clock components.$^{15}$ To extend these findings beyond vertebrates, we examined peroxiredoxin rhythms in the fruitfly Drosophila melanogaster. We pooled whole heads from insects maintained in constant darkness over two circadian cycles after they had been stably entrained to 12 h light, 12 h dark cycles. Again, circadian oscillations in PRX-SO$_{2/3}$ immuno-reactivity were observed, as well as in the clock protein Timeless (TIM) (Fig. 2b). Similarly, seedlings from the plant Arabidopsis thaliana exhibited robust PRX-SO$_{2/3}$ oscillations in free-running conditions of constant light, which were also seen in the filamentous fungus Neurospora crassa—another well-characterized clock model system (Fig. 2c and Supplementary Fig. 2). Therefore, just as in tissue from ‘complex’ vertebrates, this range of ‘simpler’ eukaryotic systems had robust peroxiredoxin oxidation cycles, with peaks tending to occur around anticipated dawn.

**Peroxiredoxin rhythms in euikaryotes**

Having observed ~24 h peroxiredoxin oxidation rhythms in organisms with nucleated cells, we next sought to examine representative prokaryotes from each major domain—Bacteria and Archaea.
For bacteria, we used the best characterized prokaryotic clock model system, *Synechococcus elongatus* sp. PCC7942 (ref. 16). The major proteins involved in the cyanobacterial clockwork are encoded by the KaiABC cluster from which, remarkably, circadian oscillations of phosphorylation can be reconstituted in vitro13. However, because all three Kai proteins are expressed together in only a small number of bacterial species, and in no known archaea18, this system cannot represent a general prokaryotic clock mechanism. We postulated that, regardless of the timekeeping mechanism, the peroxiredoxin oxidation cycles we observe reflect an absolutely conserved rhythmic cellular output. We tested this by assaying PRX-SO2/3 under free-running conditions (constant light) for 48 h, and observed cycles of cyanobacterial peroxiredoxin oxidation, peaking later than phosphorylated KaiC (Fig. 3a).

Furthermore, we extended our studies to the third phylogenetic domain, Archaea, assaying rhythms of peroxiredoxin oxidation in *H. salinarum* sp. NRC-1. Although no clock mechanisms have been identified for any archaeon, diurnal transcriptional rhythms were recently observed in *H. salinarum*19, and it thus represents an ideal platform to test whether peroxiredoxin oxidation constitutes a universal marker for cellular rhythms. After entraining the archaea for three cycles in 12 h light, 12 h dark cycles, we placed them into constant light at constant temperature. We observed robust, high amplitude circadian oscillations of PRX-SO2/3 for three cycles (Fig. 3b). Together, our findings in evolutionarily diverse prokaryotes provide compelling evidence that rhythmic peroxiredoxin oxidation is a conserved circadian marker across phylogenetic domains.

**Relations between peroxiredoxin cycles and TTFLs**

In all circadian model systems, the proposed clock mechanism revolves around a TTFL2,20,21. How the metabolic rhythms observable through peroxiredoxin oxidation relate to, and interact with, the known transcriptional clockwork in different organisms is unclear. Again, we used several model organisms, with available ‘clock’ mutants, to address this question.

The *Drosophila* transcriptional clockwork is structurally similar to the mammalian TTFL, although there are some important differences8. In flies, Clock and Cycle (orthologous to mammalian BMAL1, also known as ARNTL) comprise the positive limb, driving oscillatory expression of Period (PER) and Timeless (TIM). PER and TIM negatively regulate their own expression, closing the loop5,6. This circuit can be disrupted by many mutations21. Two mutants, *pe* and *clk*9, are behaviourally arrhythmic, and show non-cycling expression of circadian components, including PER and TIM22,23. To examine peroxiredoxin oxidation patterns in these mutants, they were entrained as described above for wild-type (Canton-S) flies. We observed two circadian rhythms of PRX-SO2/3 oscillation, with an altered circadian phase relative to wild type. This indicates the presence of an underlying capacity for circadian timing in both mutant strains, which was clearly perturbed by the absence of functional transcriptional feedback circuitry (Fig. 4a).

To establish the wider relevance of these findings, we also examined similar mutants in the fungus *Neurospora crassa*. The frequency (frq) locus encodes a critical element in the TTFL of Neurospora, in addition to the Per–Arnt–Sim (PAS)-containing WC transcription factors26. In the long-period *frq* mutant27, peroxiredoxin oxidation rhythms showed a similarly lengthened period, with an altered phase relative to rhythms in FRQ protein abundance (Fig. 4b). Deletion of the *frq* locus characterizes the *frq* strain, and measurable markers of clock output, such as its spore-forming (‘conidiation’) rhythm, are profoundly perturbed in these fungi, although apparently stochastic oscillations can re-emerge under various growth conditions27-29. Circadian rhythms of peroxiredoxin oxidation were, however, clearly seen in *frq* mutant sampled in constant darkness (Fig. 4b), with a delayed phase relative to wild-type (bd) fungi. This illustrates that peroxiredoxin rhythms represent an alternative readout for an oscillator that persists in the absence of a FRQ-dependent clock.

We next examined the phenotypes of mutant circadian transcriptional regulators in photosynthetic eukaryotes and prokaryotes. The transcriptional clockwork of the plant *Arabidopsis thaliana* and the alga *Ostreococcus tauri* are very similar and rely on circadian oscillation of TOC1. Accordingly, overexpressing *TOC1* in either species disrupts transcriptional rhythms10,31. In such strains, under constant light, we observed persistent oscillations of peroxiredoxin oxidation, albeit with altered amplitude and phase relative to controls (Supplementary Figs 2 and 3). Furthermore, in cyanobacteria we assayed peroxiredoxin oxidation in the arrhythmic *KaiA* deletion strain, AMC702 (ref. 32). Notably, an approximately 24-h rhythm of peroxiredoxin oxidation persisted despite a functional Kai-based oscillator being absent, again in an altered phase relative to wild type (Fig 5a). Taken together, these observations indicate that metabolic rhythms remain closely aligned to transcriptional feedback mechanisms when those mechanisms are present. Crucially, however, metabolic rhythms persist even when cycling clock gene transcription is abolished (summarized in Supplementary Table 9).

Having determined the TTFL influence on peroxiredoxin oxidation rhythms, we reciprocally tested whether rhythmic peroxiredoxin oxidation is required for timekeeping, using TTFL components as markers of the clockwork. We assayed reporter bioluminescence and delayed fluorescence in mutant *S. elongatus* and *A. thaliana* lines, respectively, that were deficient in 2-CysPRX (*Synechococcus* Δ2-CysPRX, GenBank accession AAP49028; *Arabidopsis* double mutant: Δ2-CysPRXA A2-CysPRXB, GenBank accession NM_111995 and NM_120712)33. In these mutants, circadian rhythms persisted with wild-type period, albeit significantly perturbed in either phase or amplitude, relative to controls (Fig. 5b and Supplementary Fig. 9). This suggests that peroxiredoxins are not required for oscillator

---

**Figure 3 | Peroxiredoxin oxidation cycles are conserved in prokaryotic models of the circadian clock.** a, b, Representative immunoblots probed for oxidized/hyperoxidized 2-Cys peroxiredoxin (PRX-SO2/3) are shown for bacteria (*S. elongatus* sp. PCC7942; a), and archaea (*H. salinarum* sp. NRC-1; b). Before sampling under free-running conditions (constant light), cyanobacteria were synchronized with a 12 h dark pulse; whereas archaea were stably entrained to 12 h light, 12 h dark cycles. Loading controls show Coomassie blue-stained gels loaded with identical samples used for immunoblotting. Immunoblot quantification by densitometry is shown below each panel (mean ± s.e.m.) for *n* = 3 biological replicates. See Supplementary Table 9 for cycle period estimates and detailed statistics. P, phosphorylated KaiC; NP, non-phosphorylated KaiC.
Successful organisms had to acquire ROS removal systems or were ancient known clock mechanism (the Kai oscillator) evolved. Around the time of the GOE, the same era during which the most catastrophic decline35. Evidently, organisms that survived the transition to an aerobic environment were those that respired and/or evolved metabolic pathways that could support aerobic metabolism in the most competitive early aerobes adapted to confer a selective advantage from the beginnings of aerobic life. The ability to survive cycles of oxidative stress may have contributed a selective advantage to the above model organisms, used commonly to study clock biology, we suggest that both peroxiredoxins and TTFL components of the circadian system are important, but potentially individually dispensable for circadian rhythms at the cellular level. Moreover, the phenotypes of these mutants suggest that the cellular ROS balance is important for robust clock function, as was described recently in Neurospora34.

**Implications for clock evolution**

We have observed ~24 h cycles of peroxiredoxin oxidation–reduction in all domains of life and consequently, the possibility that cellular rhythms share a common molecular origin seems increasingly plausible. Because the cellular role of peroxiredoxins principally involves the removal of toxic metabolic by-products (that is, ROS), we proposed that peroxiredoxin oxidation cycles we now observe in disparate organisms are ancient evolutionary adaptation are revealed by the conserved 2-Cys peroxiredoxin family, representing metabolism/ROS pathways, with the most ancient characterized clock mechanism: the three cyanobacterial Kai proteins. Because the Kai proteins are found exclusively in prokaryotes, we focused on these for our analysis18. All three components of the cyanobacterial Kai oscillator evolved and been introduced more recently (see Fig. 6).

If there was considerable pressure for the co-evolution of metabolic/ROS pathways with cellular timekeeping systems, then evidence for this should exist in the phylogenetic trees of their component enzymes. To substantiate this we used the Mirrortree algorithm41 to assess the degree of co-evolution between the 2-Cys peroxiredoxin family, representing metabolism/ROS pathways, with the most ancient characterized clock mechanism: the three cyanobacterial Kai proteins. Because the Kai proteins are found exclusively in prokaryotes, we focused on these for our analysis42. All three components of the cyanobacterial oscillator seem to have co-evolved with 2-Cys peroxiredoxins, as shown by the strong correlation between the distances of respective proteins within each phylogenetic tree (KaiA, r = 0.784; KaiB, r = 0.883; KaiC, r = 0.865; P < 1 × 10^-6 for all) (Fig. 5c and Supplementary Fig. 4). Notably, when evolution of KaiC (the most ancient member) was compared with other absolutely conserved protein families, the three highest correlations observed were for the other two clock components (KaiA and KaiB) and for ...
are intrinsically integrated with them in modern organisms. This suggests that similarities in the evolutionary profiles of these cellular mechanisms go beyond those that would be expected simply based on the time since a common ancestry, because even highly conserved proteins had considerably inferior correlations to peroxiredoxin (Supplementary Fig. 6 and Supplementary Table 8)\(^6\). Given that Kai proteins are not found in eukaryotic systems, but metabolic rhythms such as those observed in peroxiredoxin oxidation are, our results suggest that metabolic rhythms are at least as ancient as, and pre-date most, phylum-specific timekeeping mechanisms, but are intrinsically integrated with them in modern organisms.

**Concluding remarks**

It has long been recognized that oxygen-sensing PAS-domain proteins are involved in the clockwork of many eukaryotes, but the rationale behind this has remained elusive\(^23,41–45\). In light of our current findings, we speculate that sensing and responding to oxidative cycles in cellular environments could have driven the evolution of circadian rhythms, and maintained the intrinsic link between clocks and metabolism (Fig. 6). A direct prediction therefore, is that organisms that lack ROS detoxification systems will not have circadian rhythms. At least one such class of organism exists on Earth, an example being the hyperthermophilic archaea *Methanopyri* (NCBI taxonomy accession 183988). Given its distinct anoxic environmental niche and methanogenic metabolism\(^46\), there may never have been a selective evolutionary pressure to develop circadian timekeeping as we know it.

**METHODS SUMMARY**

**Organisms and strains.** *A. thaliana*, *D. melanogaster*, *H. salinarum* sp. NRC-1, *M. musculus*, *N. crassa*, *O. tauri* and *S. elongatus* were bred, grown or cultured in appropriate conditions, synchronized by specific methods normally used in each organism, and then sampled under constant conditions of either darkness or light, depending on the organism.

**Gel electrophoresis and western blotting.** Samples were lysed in LDS buffer and heated to 70 °C for 10 min with constant shaking (600 r.p.m.) in a thermomixer. Electrophoresis was performed using pre-fabricated 4–12% Bis-Tris gradient gels, using a non-reducing MES SDS buffer system, allowing characterization of proteins between 10 and 260 kDa. Immunoblotting was performed after protein transfer to nitrocellulose membranes. After blocking, membranes were incubated in antibody diluted in blocking buffer (0.5% milk in BSA) overnight at 4 °C. The next day, membranes were washed and bands visualized with chemiluminescence detection.

**Phylogenetic analyses.** We used the Mirrortree online server, using input FASTA sequences for human PRDX2 (GenBank accession CAG46588.1) and compared this serially with *S. elongatus* sp. PCC7942 proteins: KaiA (GenBank accession AAM82684.1), KaiB (AAM82685.1) and KaiC (AAM82686.1). Similar analyses were performed for KaiC comparisons with other conserved bacterial proteins. Interspecies plots were generated, which contain a simplified representation of the correlation between the interprotein distances in phylogenetic trees for each protein being compared.

**Image and statistical analysis.** Coomassie-stained gel images were obtained using a Licor Odyssey system, and immunoblot films were scanned using a flat-bed scanner. Densitometric quantification of images was performed using NIH ImageJ software. Signal was normalized against the respective loading control for each replicate at each time point for grouped data. Sine curve fitting was performed using Circwave software, using a harmonic regression method, and analysis of variance (ANOVA) was also performed as an independent measure of temporal variation. Statistical comparisons between Mirrortree correlation coefficients were performed as detailed above.
Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 22 July 2011; accepted 26 March 2012.

Published online 16 May 2012.

1. Dunlap, J. C. Molecular bases for circadian clocks. Cell 96, 271–290 (1999).
2. Welsch, M. A., Ouyang, Y., Phanvijitphong, K. & Johnson, C. H. The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. Curr. Biol. 14, 1481–1486 (2004).
3. Dodd, A. N. et al. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309, 630–633 (2005).
4. Barger, L. K., Lockley, S. W., Rajaratnam, S. M. & Landrigan, C. P. Neurobehavioral, health, and safety consequences associated with shift work in safety-sensitive professions. Curr. Neurol. Neurosci. Rep. 9, 155–164 (2009).
5. Wijnen, H. & Young, M. W. Interplay of circadian clocks and metabolic rhythms. Curr. Biol. 21, R614–R625 (2011).

1. O’Neill, J. S. et al. Circadian rhythms persist without transcription in a eukaryote. Nature 469, 554–558 (2011).
2. O’Neill, J. S. & Reddy, A. B. A circadian clock in human red blood cells. Nature 469, 498–503 (2011).
3. Hall, A., Karplus, P. A. & Polo, L. B. Typical 2-Cys peroxiredoxins—structures, mechanisms and functions. FEBS J. 276, 2469–2477 (2009).
4. Wood, Z. A., Polo, L. B. & Karplus, P. A. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science 300, 650–653 (2003).
5. Barranco-Medina, S., Lazaro, J. J. & Dietz, K. The oligomeric conformation of the circadian clock. PLoS Biol. 7, e189 (2009).
6. Lopez-Molina, L., Conquet, F., Daujat, D. & Rouyer, F. The homolog of mammalian timeless. Cell 93, 791–804 (1998).
7. Hardin, P. E. The circadian timekeeping system of Drosophila. Curr. Biol. 15, R714–R722 (2005).
8. Dunlap, J. C. & Loros, J. J. How fungi keep time: circadian system in Neurospora and other fungi. Science 292, 579–587 (2001).
9. Aronson, B. D., Johnson, K. A., Loros, J. J. & Dunlap, J. C. Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. Science 263, 1578–1584 (1994).
10. Granshaw, T., Tsukamoto, M. & Brody, S. Circadian rhythms in Neurospora crassa: farnesol or geraniol allow expression of rhythmicity in the otherwise arrhythmic strains frg-1 and wc-2. J. Biol. Rhythms 18, 287–296 (2003).
11. Corelli, F. et al. Clocks in the green lineage: comparative functional analysis of the circadian architecture of the picoeukaryote ostreococcus. Plant Cell 21, 3436–3449 (2009).
12. Mas, P., Alabada, D. M., Yanovsky, M. J., Oyama, T. & Kay, S. A. Dual role of TOC1 in the control of circadian and photomorphogenic responses in Arabidopsis. Plant Cell 15, 223–236 (2003).

13. Ditty, J. L., Canales, S. R., Anderson, B. E., Williams, S. B. & Golden, S. S. Stability of the Synechococcus elongatus PCC 7942 circadian clock under directed anti-phase expression of the kai genes. Microbiology 151, 2605–2613 (2005).
14. Pulido, P. et al. Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in Arabidopsis thaliana chloroplasts. J. Exp. Bot. 61, 4043–4054 (2010).
15. Yoshida, Y., Iigusa, H., Wang, N. & Hasunuma, K. Cross-talk between the cellular redox state and the circadian system in Neurospora. PLoS ONE 6, e28227 (2011).
16. Wang, M. & Kay, S. A. A universal molecular clock of protein folds and its power in tracing the early history of aerobic metabolism and planet oxygenation. Mol. Biol. Evol. 28, 567–582 (2011).
17. Nathan, C. & Ding, A. Snapshot: reactive oxygen intermediates (ROI). Cell 140, 951–951.e2 (2010).
18. Zelko, I. N., Mariani, T. J. & Foz, R. J. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2) and EC-SOD (SOD3) genes, structures, evolution, and expression. Free Radic. Biol. Med. 33, 337–349 (2002).
19. Mulholland, P. J., Houser, J. N. & Maloney, K. O. Stream diurnal dissolved oxygen profiles as indicators of in-stream metabolism and air-water exchange in aquatic ecosystems. Oecologia 153, 385–398 (2007).
20. Bamforth, S. S. Diurnal changes in shallow aquatic habitats. Limnol. Oceanogr. 7, 348–353 (1962).
21. Hora, D. & Pazos, F. Studying the co-evolution of protein families with the Mirotreer web server. Bioinformatics 26, 1370–1371 (2010).
22. Peixoto, A. A., Campesans, S., Costa, R. & Kyriacou, C. P. Molecular evolution of a repetitive region within the per gene of Drosophila. Mol. Biol. Evol. 10, 127–139 (1993).
23. McElroy, B. E., Bogenschutz, J. & Brandfield, C. A. Mammalian Per-Arnt-Sim proteins in environmental adaptation. Annu. Rev. Physiol. 72, 625–645 (2010).
24. Rutter, J., Reick, M. & McKnight, S. L. Metabolism and the control of circadian rhythms. Annu. Rev. Biochem. 71, 307–331 (2002).
25. Schibler, U. & McNeish, J. S. Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 293, 510–514 (2001).
26. Shima, S., Thauer, R. K. & Ermler, U. Hyperthermophilic and salt-dependent formyltransferase from Methanopyrus kandleri. Biochem. Soc. Trans. 32, 269–272 (2004).
27. Declercq, J. P. et al. Crystal structure of human peroxiredoxin 5, a novel type of mammalian peroxiredoxin at 1.5 Å resolution. J. Mol. Biol. 311, 751–759 (2001).
28. Schröder, E. & Schibler, U. Crystal structure of decameric 2-Cys peroxiredoxin from human retina: a new functional role for peroxiredoxins at 1.7 Å resolution. J. Mol. Biol. 236, 617–627 (2000).
29. Xu, Y., Mori, T. & Johnson, C. H. Cyanobacterial circadian clockwork: roles of KaiB and the kaiBC promoter in regulating KaiC. EMBO J. 22, 2117–2126 (2003).
30. Pazos, F. & Valencia, A. Similarity of phylegetic trees as indicator of protein-protein interaction. Protein Eng. 14, 609–614 (2001).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was primarily supported by the Wellcome Trust (083643/Z/07/Z and 093734/Z/12/Z), the European Research Council (ERC Starting Grant No. 271991, MetaCLOCK), and EMBO Young Investigators Programme, as well as the Medical Research Council Centre for Obesity and Related metabolic Disorders (MRC CORD), and the National Institute for Health Research (NIHR) Cambridge Biomedical Research Centre. C.P.K. and M.H.H. acknowledge European Commission grant EUCLICO (no. 018741) and Biotechnology and Biological Sciences Research Council (BBSRC) grant BB/C006941/1. SynthSys is funded by BBSRC and Engineering and Physical Sciences Research Council (EPSRC) award BB/D019621 to A.J.M. and others. N.S.B. was supported by ENIGMA, US Department of Energy, under contract no. DE-AC02-05CH11231, and by a grant from the National Institutes of Health (NIH; P50GM076547). C.H.J. was supported by the NIH (R01GM088595, R01GM067152 and R21HL102492). M.M. was supported by the Netherlands Organisation for Scientific Research (NWO; Dutch Science Foundation VICI award and Open Programma) and the University of Groningen (Rosalind Franklin Fellowship Program). We thank M. Jain, G. O’Neill and J. Chambers for discussion about the manuscript, and S. G. Rhee, F. Rouyer and R. Stanewsky for the gifts of antisera.

Author Contributions A.B.R. and J.S.O. conceived and designed the experiments, and wrote the manuscript. R.S.E., E.W.G., G.v.O., M.O., X.Q., Y.Y., Z.Y., M.P., U.K.V., K.A.F. and E.S.M. performed experiments. M.H.H., N.S.B., C.H.J., M.M., A.J.M. and C.P.K. provided reagents. R.S.E., E.W.G., G.v.O. and M.O. contributed equally to this work.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Reprints are welcome to be submitted on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.B.R. (aremdey@cantab.net) or J.S.O. (jso22@mdschl.cam.ac.uk).
METHODS

Arabidopsis thaliana. Surface-sterilized A. thaliana seeds (Ws) were plated on solid medium (1.2% agar plus 0.5% Murashige & Skoog medium (Duchefa Biochemie), pH 5.8), vernalized at 4 °C for 4 days, and grown for 7 days under 12 h light, 12 h dark cycles under cool-white fluorescent tubes (70–100 μEm−2 s−1) at 20 °C before transfer to constant light conditions at Zeitgeber time (ZT) 0. Plantlets were sampled every 4 h for 3 days by snap-freezing 15 seedlings per sample in liquid N2. Tissue was crushed in a Tissue Lyser (Eppendorf) using a 0.32-cm chrome ball (Spheric-Trafalgar), and tissue was thawed in extraction buffer (8 M urea, 300 mM NaCl, 100 mM Tris, pH 7.5, 10 mM EDTA, 4% poly(vinylpyrrolidone), 1% NP40) and 2% complete protease inhibitors (Roche) and incubated on ice for 15 min, vortexing every 2 min before centrifugation at 16,000g for 10 min. Supernatants were loaded onto gels after the addition of SDS loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris–HCl, pH 6.8) and heating to 100 °C for 5 min. Equal protein loading was confirmed by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each time point in each replicate set. Delayed fluorescence was performed as reported previously.

Drosophila melanogaster. Canton-S (CS) wild-type flies and congenic per0I and Ck0 I mutant strains were raised on standard medium at 25 °C in 12 h light, 12 h dark cycles, and their behavioural phenotypes were validated using DAMS activity monitors (TriKinetics). Adult male flies were entrained for a further 3 days in light boxes at 25 °C with a McIlwain Tissue Chopper. Slices were sorted and trimmed to contain principally SCN tissue and placed onto a Millipore Millicell-CM insert (0.4 μm pore size) in a glass-bottomed Petri dish sealed with a coverslip and vacuum grease. Total bioluminescence was recorded with Hamamatsu photomultiplier tube assemblies housed in a light-tight 7 °C incubator, and recordings were expressed as counts per second integrated over 6-min sample bins. SCN slices were collected ‘around the clock’ every 4 h according to the phase of the PER2–LUC bioluminescence cycle. Circadian time (CT) 0 was operationally defined as the nadir in bioluminescence signal, and CT12 was taken to be at the peak. Individual SCN slices (n = 3 per time point) were immersed in 50 μL 2× LDS sample buffer (Invitrogen), and heated to 70 °C for 10 min before loading on gels. For immunoblotting, 10 μL of lysate per lane was loaded. Equal protein loading was confirmed by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each time point in each replicate set.

Neurospora crassa. We used the following strains: wild type (bd, matA) Fungal Genetics Stock Center (FGSC) accession 1858, frq1 (bdfrq1, matA) FGSC accession 7490, frq2 (bdfrq2, matA) FGSC accession 4898 and PRX-KO (NRC06031, matA) FGSC accession 20012). All strains were maintained on Vogel’s minimal media with 1.5% sucrose as a carbon source. Strain manipulation and growth media followed standard procedures.

For circadian experiments, cultures were initiated by inoculating 107 conidia in 25 ml of Vogel’s medium containing 2% glucose, 0.5% arginine, 10 ng ml−1 biotin and 0.2% Tween 80. Plates were incubated under constant light for 36 h at 30 °C. Disks (1.2-cm diameter) were cut from the cohesive mycelial pad and three disks were placed in each of a series of 50 ml tubes (or 100 ml flasks) containing 30 ml of (or 50 ml) of Vogel’s medium with 0.03% glucose, 0.05% arginine and 10 ng ml−1 biotin. These were incubated at 25 °C under constant light for at least 2 h before staggered transfers to constant darkness (25 °C) with shaking at 150 r.p.m. Mycelia were then collected at 4 h intervals, dried on paper, frozen in liquid N2, and stored at −80 °C. Harvests were consolidated over 8 h to control for development/age of the tissue. All manipulations in the dark were performed under safe red light. For immunoblot analysis, tissue was ground in liquid N2 with a mortar and pestle and suspended in ice-cold extraction buffer (50 mM HEPES, pH 7.4, 137 mM NaCl, 10% glycerol, 0.03% glucose, 0.05% arginine and 10 ng ml−1 biotin). These were incubated at 25 °C for 10 min before loading on gels. For immunoblotting, an antiserum directed against human PRX6 (1-Cys) was used (at 1:5,000 dilution) because no typical 2-Cys PRXs are annotated in the N. crassa genome so far, and thus antibody specificity could not otherwise be assured. Equal protein loading was confirmed by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each time point in each replicate set.

Ostreococcus tauri. O. tauri cells were cultured as previously described and entrained in a 12 h light, 12 h dark cycle of blue light (17.5 μEm−2 s−1, 1 μg L−1 Lee lighting filter 724) at a constant temperature of 20 °C. Cultures of the arrhythmic TSL8 line (TOC1-overexpressing) and its parent line CCA1–LUCO6 were transferred into 12 h constant light for 4 h for 3 days. Every 3 h, cells were chilled on ice and pelleted at 4,500g for 4 °C for 10 min. The resulting pellet was resuspended in 50 μl sea water, and cells were lysed by adding 50 μl 2× extraction buffer (Sigma–Aldrich, LUC-1 kit) and then 100 μl SDS loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris–HCl, pH 6.8) while vortexing vigorously. Samples were heated to 100 °C for 5 min to denature proteins before loading on gels. Equal protein loading was confirmed by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each time point in each replicate set.

Synechococcus elongatus. The cyanobacterial strains used were S. elongatus sp. PCC7942 wild type (AMC149) and ΔKaIA mutant (AMC702). Cells were grown with aeration in constant light of 100 μEm−2 s−1 at 30 °C to an attenuation (D) at 730 nm of 0.3. The ΔKaIA strain was maintained between 0.27 and 0.45 by dilution with fresh BG-11 medium.66 The culture was exposed to 12 h of constant darkness to synchronize the circadian clock, and then returned to constant light. At 4-h intervals under constant light, cells were harvested immediately, frozen and then stored at −80 °C. Samples were prepared for immunoblotting as described previously.66 Equal protein loading was confirmed by gel electrophoresis and Coomassie staining of gels loaded with equal volumes of lysate from each time point in each replicate set. A single knockout for the 2-Cys S. elongatus sp. PCC7942 PRX gene66 was generated by inserting an expression cassette for the kanamycin resistance gene into the EcoRI site near the amino-terminal coding region of the gene. For bioluminescence recordings, psaBAlp:LexAAB was used.
Article

Gel electrophoresis and western blotting. We used NuPAGE Novex 4–12% Bis-Tris gradient gels (Life Technologies), and ran them using the manufacturer’s protocol with a non-reducing MES SDS buffer system, allowing characterization of proteins between 10 and 260 kDa. Protein transfer to nitrocellulose for blotting was performed using the iBlot system (Life Technologies), with a standard (programme P3, 7 min) protocol. Nitrocellulose was washed briefly, and then blocked for 30 min in 0.5% (w/v) BSA (Marvel) in TBS containing 0.05% Tween-20 (TBST). After three brief washes in TBST, membranes were incubated in antibody diluted in blocking buffer (0.5% milk in BSA) overnight at 4°C. The next day, membranes were washed in TBST for 5 min three times and then incubated with 1:10,000 horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich) for 30 min. Four more 10-min washes were then performed before performing chemiluminescence detection using Immobilon reagent (Millipore), or ECL Plus reagent (GE Healthcare). To check even protein loading, the gels were stained with Coomassie SimplyBlue (Life Technologies). Antisera against peroxiredoxins were obtained from Abcam and used in blocking buffer (PRX-SO2/3 1:10,000 dilution, and PRX1 1:2,000). Rabbit anti-Bmal1 antiserum was used as described previously. Rabbit anti-PRX antibody (Santa Cruz Antibodies) was used at 1:5,000 in 0.5% milk in BSA. Mouse anti-α-actin antibody (Santa Cruz Antibodies) was used at 1:5,000 in 0.5% milk in BSA. Mouse anti-FRQ antibody was used at a dilution of 1:40 in blocking buffer. Anti-KaiC antiserum was used as described previously.

Phylogenetic analyses. We used the Mirrortree online server, using input FASTA sequences for human PRX2 (GenBank accession ACG46888.1) and compared this to Synechococcus elongatus proteins: KaiA (GenBank accession AAM82684.1), KaiB (GenBank accession AAM82685.1) and KaiC (AAM82686.1). Similar analyses were performed for KaiC comparisons with other conserved bacterial proteins. Interspecies plots were generated, which contain a simplified representation of the correlation between the interprotein distances in phylogenetic trees for each protein being compared.

The Mirrortree web server implements the Mirrortree algorithm for calculating the tree similarity between two protein families, which has been demonstrated to be a good predictor of the interaction or functional relationships between them. Phylogenetic trees are obtained from these alignments with the neighbour-joining algorithm implemented in ClustalW using bootstrap (100 repetitions) and excluding gaps for the calculation. The distance matrices are obtained by summing the branch lengths that separate each pair of proteins in the tree. Instead of calculating the complete matrices, only the proteins of organisms present in both trees are used. The similarity of trees between the two families is calculated as the correlation between their distance matrices according to the equation:

\[ r = \frac{\sum_{i=1}^{n} (R_i - \bar{R})(S_i - \bar{S})}{\sqrt{\sum_{i=1}^{n} (R_i - \bar{R})^2} \sqrt{\sum_{i=1}^{n} (S_i - \bar{S})^2}} \]

in which \( n \) is the number of elements of the matrices, that is, \( n = (N^2 - N)/2 \); \( N \) is the number of common organisms; \( R_i \) is the element of the first distance matrix; \( S_i \) is the corresponding value of the second matrix; and \( \bar{R} \) and \( \bar{S} \) are the mean values of \( R_i \) and \( S_i \), respectively. Correlation coefficients obtained for analyses of KaiC distance versus other conserved bacterial proteins (including KaiB, KaiC and PRX2) were compared by converting them to a normally distributed metric using Fisher’s z-to-z transformation:

\[ z = \frac{r - r'_z}{\sqrt{\frac{1}{N_1 - 3} + \frac{1}{N_2 - 3}}} \]

in which \( r \) is the Mirrortree correlation coefficient; \( r'_z \) is the Fisher-transformed correlation coefficient. Transformed coefficients \( (r'_z) \) were then compared with each other to generate the test statistic, \( z \):

51. Gould, P. D. et al. Delayed fluorescence as a universal tool for the measurement of circadian rhythms in higher plants. Plant J. 58, 893–901 (2009).
52. Rosato, E. & Kyriacou, C. P. Analysis of locomotor activity rhythms in Drosophila. Nature Protocols 1, 559–568 (2006).
53. Reddy, A. B. et al. Circadian orchestration of the hepatic proteome. Curr. Biol. 16, 1107–1115 (2006).
54. Whitehead, K., Fan, M., Masumura, K., Borneau, R. & Buliga, N. S. Dually entrained anticipatory behavior in archaia. PLoS ONE 4, e5485 (2009).
55. Mori, T., Binder, B. & Johnson, C. H. Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. Proc. Natl Acad. Sci. USA 93, 10183–10188 (1996).
56. Yoo, S. H. et al. PERIOD2: LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl Acad. Sci. USA 101, 5339–5346 (2004).
57. House, S. B., Thomas, A., Kusano, K. & Gainer, H. Stationary organotypic cultures of oxytocin and vasopressin magnocellular neurons from rat and mouse hypothalamic. J. Neuroendocrinol. 10, 849–861 (1998).
58. Hastings, M. H., Reddy, A. B., McMahon, D. G. & Maywood, E. S. Analysis of circadian mechanisms in the suprachiasmatic nucleus by transgenesis and biolistic transfection. Methods Enzymol. 393, 579–592 (2005).
59. Maywood, E. S. et al. Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. Curr. Biol. 16, 599–605 (2006).
60. Davis, R. H. Neurosciences: Contributions of a Model Organism (Oxford Univ. Press, 2000).
61. Neurouro, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurosciences clock gene frequency. Nature 399, 584–586 (1999).
62. Olmedo, M. et al. A role in the regulation of translation by light for RCO-1 and ROM-1, the Neurosciences homologs of the yeast Tup1–Ssn6 repressor. Feral Genet. Biol. 47, 939–952 (2010).
63. Woo, H. A. & Rhee, S. G. in Methods in Redox Signaling (ed. Das, D.) Ch. 4 19–23 (Mary Ann Liebert, 2010).
64. Ishiura, M. et al. Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science 281, 1519–1523 (1998).
65. Qin, X. et al. Intracellular associations determine the dynamics of the circadian KaiABC oscillator. Proc. Natl Acad. Sci. USA 107, 14805–14810 (2010).
66. Xu, Y. et al. Intracellular regulation of phosphorylation status of the circadian clock protein KaiC. Proc. Natl Acad. Sci. USA 104, 7509 (2009).
67. Stork, T., Laxa, M., Dietz, M. S. & Dietz, K. J. Functional characterisation of the peroxiredoxin gene family members of Synechococcus elongatus PCC 7942. Arch. Microbiol. 191, 141–151 (2009).
68. Xu, Y., Mori, T. & Johnson, C. H. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC. EMBO J. 22, 2117–2126 (2003).
69. Ochoa, D. & Pazos, F. Studying the co-evolution of protein families with the circadian clock and intraorganismal regulation of phosphorylation status of the circadian clock protein KaiC. Proc. Natl Acad. Sci. USA 107, 14805–14810 (2010).
70. Pazo, F. & Valencia, A. Similarity of phylogenetic trees as indicator of protein–protein interaction. Protein Eng. 14, 609–614 (2001).
71. Oster, H., Damerow, S., Hul, R. A. & Eichele, G. Transcriptional profiling in the adrenal gland reveals circadian regulation of hormone biosynthesis genes and nucleoside assembly genes. J. Biol. Rhythms 21, 350–361 (2006).
CORRIGENDUM
doi:10.1038/nature11427

Corrigendum: Peroxiredoxins are conserved markers of circadian rhythms

Rachel S. Edgar*, Edward W. Green*, Yuwei Zhao*, Gerben van Ooijen*, Maria Olmedo*, Ximing Qin, Yao Xu, Min Pan, Utham K. Valekunja, Kevin A. Feeney, Elizabeth S. Maywood, Michael H. Hastings, Nitin S. Baliga, Martha Merrow, Andrew J. Millar, Carl H. Johnson, Charalambos P. Kyriacou, John S. O’Neill & Akhilesh B. Reddy

Nature 485, 459–464 (2012); doi:10.1038/nature11088

In the author list of this Article, the names of Gerben van Ooijen and Maria Olmedo should also have been asterisked, indicating their equal contributions. This error has been corrected in the HTML and PDF versions of the original paper.

*These authors contributed equally to this work.