of the other member of the pair was suitable for long-distance transmission in optical fibre.

Mapping of photonic entanglement into and out of a quantum memory has been demonstrated already with trapped-atom systems[7]. However, the new work[1,2] is the first to achieve it using a solid-state memory. The use of the solid state offers certain practical advantages. For example, Saglamyurek and colleagues[2] formed the memory in an optical waveguide, which could enable integrated devices to be built. More significantly though, the authors' approach[1,2] to quantum memories using cryogenic rare-earth-ion-doped crystals is rapidly developing and has already surpassed the storage bandwidths[2], capacities[8], efficiencies[9] and storage times[10] of other approaches.

Such cryogenic rare-earth-ion-doped systems have already been studied for classical optical signal processing because of these systems' large ratio of inhomogeneous to homogeneous broadening[11] for their optical absorption lines. That is, the optical absorption linewidth of each dopant atom is very narrow, whereas the linewidth of the ensemble of dopants can be very large. This makes them very suitable systems for photon echoes, which is where the dopants emit a pulse of light (echo) in response to earlier applied light pulses. In particular, photon echoes allow signal processing with simultaneous large bandwidth, determined by ensemble linewidth, and high resolution, determined by single-dopant linewidth. The rapid advances of rare-earth quantum memories have been, in large part, due to the development of photon-echo techniques, which are suitable for preserving quantum states of light.

Although the storage of entanglement in a solid is a significant step, the efficiencies and storage times in the entanglement-storage experiments of Clausen *et al.*[1] and Saglamyurek *et al.*[2] need to be improved; they are currently inferior to those that can be achieved in a small spool of optical fibre. And whereas good efficiency, storage time and bandwidth have all been demonstrated by others, in separate demonstrations, the next challenge awaiting researchers is to achieve all these performance metrics for the same memory. This should open up new capabilities and technologies that will stretch quantum mechanics in a way that we have not yet been able to. Who knows, it might break. ■

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CIRCADIAN RHYTHMS

Redox redux

Oscillations in gene transcription that occur in response to biological daily clocks coordinate the physiological workings of living organisms. But turnover in cellular energy may be sufficient to make the clock tick. SEE ARTICLE P.498 & LETTER P.554

JOSEPH BASS & JOSEPH S. TAKAHASHI

Last spring, a visitor at the biennial meeting of the Society for Research on Biological Rhythms in Florida approached the geneticist Sydney Brenner inquiring as to what it was that scientists studying circadian rhythms actually do. With a glimmer in his eye, Brenner responded that the meeting concerned "those things that only happen once each day". Indeed, all forms of life undergo circadian (roughly 24-hour) fluctuations in energy availability that are tied to alternating cycles of light and darkness. Biological clocks organize such internal energetic cycles through transcription–translation feedback loops. But two papers[1,2] in this issue show that, in both humans and green algae, rhythmic cycles in the activity of peroxiredoxin enzymes can occur independently of transcription.

Biological circadian oscillators have long been recognized as a self-sustained phenomenon, their 24-hour length being both invariant over a wide range of temperatures and responsive to light. Early indications that genes underlie the clocks came[3] from the isolation of mutant fruitflies carrying altered, and yet heritable, circadian rhythms. This and subsequent work[4,5] established that endogenous molecular clocks consist of a transcription–translation feedback loop that oscillates every 24 hours in cyanobacteria, plants, fungi and animals.

Although the specific clock genes are not evolutionarily conserved across distinct phyla, their architecture is similar. The forward limb of the clock involves a set of transcriptional activators that induce the transcription of a set of repressors. The latter comprise the negative limb, which feeds back to inhibit the forward limb. This cycle repeats itself every 24 hours (Fig. 1).

Energetic cycles are one type of physiological process that shows transcription-dependent circadian periodicity[6,7]; such cycles include the alternating oxygenic and nitrogen-fixing phases of photosynthesis, and the glycolytic and oxidative cycles in eukaryotes (organisms with nucleated cells). The idea that biochemical flux per se may couple circadian and energetic cycles was first suggested by McKnight and colleagues[8], who showed that varying the redox state of the metabolic cofactor NAD(P) affects the activity of two clock proteins, and it gained further support from subsequent studies[9–14]. But exactly how transcriptional and non-transcriptional cycles may be interrelated was still not fully understood.

To address this relationship, O'Neill and Reddy[1] (page 498) examined the rhythmic properties of human red blood cells (RBCs). In their mature form, these cells lack both a nucleus and most other organelles, including energy-producing mitochondria. They function mainly as oxygen shuttles, utilizing the protein haemoglobin as the delivery vehicle.

Some of the most abundant proteins in mature RBCs are the evolutionarily conserved enzymes of the peroxiredoxin family, which can inactivate reactive oxygen species (ROS). Class-2 peroxiredoxins contain a cysteine amino-acid residue in their active site that undergoes oxidation when ROS accumulate. This results in the enzyme's transition from a monomeric to a dimeric state. Excess ROS accumulation induces the formation of even higher-order oligomers. Peroxiredoxin function is essential for RBC survival, as defects in the expression or activity of these enzymes lead to the breakdown of the cells.

A previous survey[15] searching for proteins that show circadian rhythms of expression in liver identified peroxiredoxins. In their study, O'Neill and Reddy[1] monitored the monomer–dimer transition of these proteins in RBCs from three humans. They observed two main circadian features in these enucleated cells. First, the oligomerization pattern was self-sustained over several cycles within an approximate 24-hour period and was not affected by temperature. Second, peroxiredoxin oxidation cycles were synchronized in response to temperature cycles, a property called entrainment that is a hallmark of circadian oscillators.

These results, which should be confirmed by replication in larger numbers of individuals, clearly show that circadian patterns of peroxiredoxin oxidation persist even in the absence of gene transcription. To rule out the contribution of other, nucleated, blood cells, the authors show that inhibitors of translation (cycloheximide) and transcription (α-amanitin) do not interfere with the peroxiredoxin oxidation rhythm.

In seeking to connect the observed peroxiredoxin oxidation rhythm with the broader physiological functions of RBCs, O'Neill and Reddy also examined the oligomeric transitions of haemoglobin. They detected a rhythmic pattern in the dimer–tetramer transition of haemoglobin, which suggests that the oxygen-carrying capacity of an RBC also exhibits circadian variation. Whether cycles of haemoglobin oxidation similarly show temperature compensation and responsiveness — and the robustness of such oscillations — remains unknown.

What drives the rhythmic cycles of oligomerization for peroxiredoxin and haemoglobin? One possibility is flux in metabolic cycles such as glycolysis — the only source of energy in RBCs. Indeed, O'Neill and Reddy report weak oscillations in the levels of ATP, the cellular energy molecule that can be generated by glycolysis. Nonetheless, further analyses are necessary to determine the exact relationship between oxidation-state transitions and energy production.

Rhythmic variation in levels of NAD(P)H, albeit of low amplitude, also corresponded with the variation in oligomerization state of both peroxiredoxin and haemoglobin[1] — a finding that points to this reduced form of NAD(P) as being a cofactor coupling energy flux with changes in the oxidation of these proteins. This observation echoes those of previous studies[8].

RBCs do not represent a special case for gene–energy coupling. O'Neill and colleagues[2] (page 554) frame their inquiry in a broader context by examining the rhythmic activity of the same family of peroxiredoxins in one of the most primitive eukaryotes known — the green alga *Ostreococcus tauri*.

The authors took advantage of a curious observation — that simply maintaining *O. tauri* out of the light suspends all gene transcription. Shifting this microorganism back into the light reinitiates transcription and restarts the clock. However, the clock does not simply reset following transfer into light at any time of day. Instead, it begins ticking again according to the time when the lights were switched off initially. In other words, the alga seems to keep track of time even in the dark, when transcription has ceased. This implies that other mechanisms in the cell provide a 'sense of time' independent of gene transcription.

To investigate how *O. tauri* senses time, O'Neill *et al.* tested the idea that the persistence of oscillations in peroxiredoxin oxidation may offer a clue to the 'invisible' factor responsible for keeping time in the absence of transcription. In contrast to the previously established 'transcriptional' oscillator of *O. tauri*, peroxiredoxin oscillation was still detected in the dark, further proving pharmacologically that the enzyme's rhythm is independent of new gene or protein synthesis. Exploring the interrelationship between transcriptional-feedback oscillators and post-transcriptional mechanisms, the authors show that drugs that inhibit transcription affect circadian oscillation within restricted phases of the daily cycle.

So, are RBCs and *O. tauri* exceptions to the generally accepted idea that the origins of biological clocks can be traced to genetic mechanisms? Intriguingly, O'Neill and Reddy[1] find that peroxiredoxin rhythms were altered in mouse embryonic connective-tissue cells harvested from mutant animals possessing a genetically disrupted clock. This indicates that, in nucleated cells, transcriptional and non-transcriptional oscillators are normally coupled. Similarly, cyclic phosphorylation of the protein KaiC, which can occur in the absence of transcription[16], is coupled with transcriptional rhythms in intact cyanobacteria[17].

The provocative models provided by these studies[1,2] return us to the question of the interdependence of circadian and energetic systems: just how do these processes communicate reciprocally? The cofactor signalling molecules that link the two systems remain of great interest, especially given the potential role of circadian disruption in metabolic disorders. Besides, the fact that oscillators exist in the absence of transcription does not negate the selective advantage that circadian genes confer. At the very least, these genes enhance organismal adaptation to the energetic environment. ■

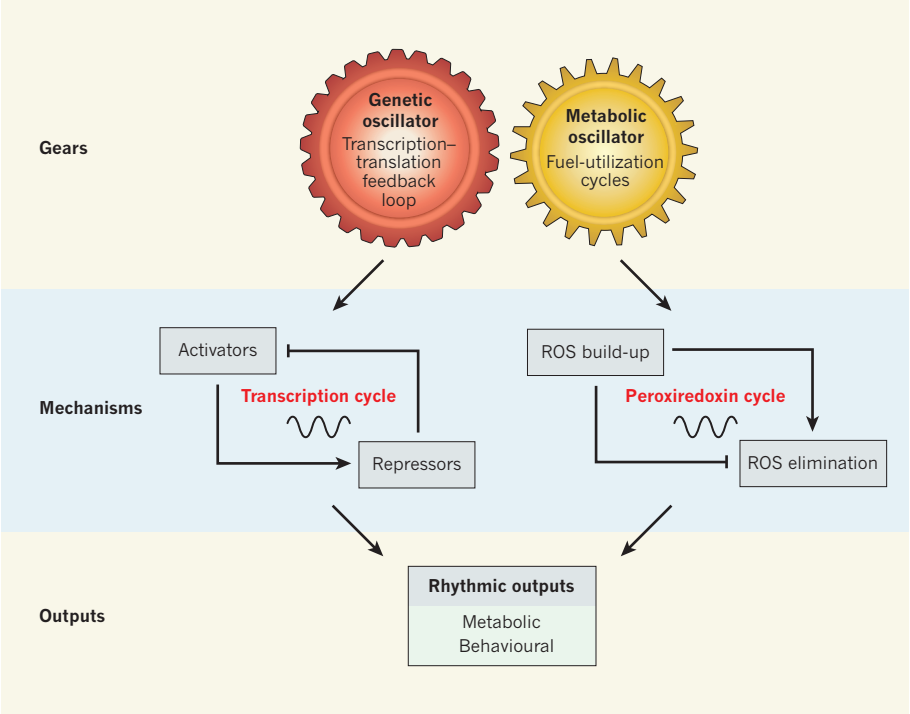

Figure 1 | Coupling of genetic and metabolic clocks. Two types of circadian oscillator maintain synchrony between the light–dark environment and internal biochemical processes. These are genetic oscillators, which consist of a transcription–translation feedback loop, and — as two new studies[1,2] show — metabolic oscillators, which are involved in fuel-utilization cycles and consist of the cycle of oxidation and reduction of peroxiredoxin enzymes. The two oscillator types are coupled, both driving rhythmic outputs (such as photosynthetic reaction cycles in plants and the feeding–fasting cycle in animals) in synchrony with Earth's rotation. ROS, reactive oxygen species.

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J.B. declares competing financial interests. See online article for details.

MEDICINE

Diabetes in India

With the spread of fast-food outlets and more sedentary lifestyles, the prevalence of diabetes in India is rising alarmingly. But the subpopulations at risk and the symptoms of the disease differ from those in the West.

JARED DIAMOND

India, the world's second most populous country, now has more people with type 2 diabetes (more than 50 million) than any other nation. The problem has been well documented in a battery of recent papers[1–6]. These publications were foreshadowed by studies of previously Westernized Indian populations elsewhere, and they illuminate distinctive features of diabetes in India.

Type 2 diabetes results from a genetic predisposition and from lifestyle factors, especially those of the so-called Western lifestyle, characterized by high calorie intake and little exercise. Also known as non-insulin-dependent or adult-onset diabetes, this form of the disease is far more common than type 1 (insulin-dependent or juvenile-onset) diabetes. Until recently, type 2 diabetes — henceforth simply 'diabetes' — was viewed as a disease of overfed, sedentary people of European ancestry. But it is now exploding around the world owing to the spread of Western habits.

Hints of trouble ahead came from observations of diabetes epidemics in emigrant Indian communities that achieved affluence long before Indians in India[1–3,7]. Those communities include ones in Fiji, Mauritius, Singapore, South Africa, Surinam, Tanzania and Britain. For instance, in the 1830s, Indians were brought to Mauritius for physically demanding work on sugar plantations. By the 1980s, the decline in world sugar prices had led the Mauritian government to promote industrialization and the export of manufactured goods, which in turn led to increasing affluence and decreasing physical activity for the local population.

As a result, between 1982 and 1986 deaths due to diabetes tripled, and by 1987 reached 13% in the Mauritius Indian community[7,8]. (By contrast, prevalence remained much lower in the even more affluent Mauritius European community, illustrating the role of genetic factors.) Today, Mauritius enjoys a per capita income four times that of India but suffers from the world's second highest national prevalence of diabetes, 24%. Those developments led Zimmet[8] to prophesy in 1996: "If over the next few decades the people in India become modernized to a similar level of those in Mauritius and other countries inhabited by Asian Indians, one could expect dramatically increased diabetes rates in India."

That prophecy has already been grimly fulfilled. In 2010, the average age-adjusted prevalence of diabetes in India was 8%, higher than that in most European countries[1]. By contrast, surveys in 1938 and 1959, in large Indian cities that are today diabetes strongholds, yielded prevalences of just 1% or less. Only in the 1980s did those numbers start to rise, first slowly and now explosively[5,6,9,10].

The reasons are those behind the diabetes epidemic worldwide. One set of factors is urbanization, a rise in living standards and the spread of calorie-rich, fatty, fast foods cheaply available in cities to rich and poor alike. Another is the increased sedentariness that has resulted from the replacement of manual labour by service jobs, and from the advent of video games, television and computers that keep people seated lethargically watching screens for hours every day. Although the specific role of TV has not been quantified in India, a study in Australia[11] found that each hour per day spent watching TV is associated with an 18% increase in cardiovascular mortality (much of it associated with diabetes), even after controlling for other risk factors such as waist circumference, smoking, alcohol intake and diet. But those factors notoriously increase with TV watching time, so the true figure must be even larger than the 18% estimate.

In India, a wide range of outcomes for different groups[9,10] is buried within the average diabetes prevalence of 8%. Prevalence is only 0.7% for non-obese, physically active, rural Indians. It reaches 11% for obese, sedentary, urban Indians; and it peaks at 20% in the Ernakulam district of Kerala, one of India's most urbanized states. Among lifestyle factors predicting the incidence of diabetes in India, some are familiar from the West, whereas others turn expectations upside down[9,10]. As in the West, diabetes in India is associated with obesity, high blood pressure and sedentariness. But prevalence of the disease is higher among affluent, educated, urban Indians than among poor, uneducated, rural people: exactly the opposite of trends in the West, although similar to the situation in other developing countries. For instance, Indians with diabetes are more likely to have undergone higher education, and less likely to be illiterate, than their healthy compatriots. In 2004, the prevalence of diabetes averaged 16%

D. SARKAR/AFP PHOTO/GETTY

Figure 1 | Raising awareness of diabetes. Participants on a 'walkathon' in Bangalore, India, in November 2010.

LETTER

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Circadian rhythms persist without transcription in a eukaryote

John S. O'Neill[1,2]*, Gerben van Ooijen[1]*, Laura E. Dixon[1], Carl Troein[3], Florence Corellou[4,5], François-Yves Bouget[4,5], Akhilesh B. Reddy[2] & Andrew J. Millar[1,3]

Circadian rhythms are ubiquitous in eukaryotes, and coordinate numerous aspects of behaviour, physiology and metabolism, from sleep/wake cycles in mammals to growth and photosynthesis in plants[1,2]. This daily timekeeping is thought to be driven by transcriptional–translational feedback loops, whereby rhythmic expression of 'clock' gene products regulates the expression of associated genes in approximately 24-hour cycles. The specific transcriptional components differ between phylogenetic kingdoms[3]. The unicellular pico-eukaryotic alga *Ostreococcus tauri* possesses a naturally minimized clock, which includes many features that are shared with plants, such as a central negative feedback loop that involves the morning-expressed *CCA1* and evening-expressed *TOC1* genes[4]. Given that recent observations in animals and plants have revealed prominent post-translational contributions to timekeeping[5], a reappraisal of the transcriptional contribution to oscillator function is overdue. Here we show that non-transcriptional mechanisms are sufficient to sustain circadian timekeeping in the eukaryotic lineage, although they normally function in conjunction with transcriptional components. We identify oxidation of peroxiredoxin proteins as a transcription-independent rhythmic biomarker, which is also rhythmic in mammals[6]. Moreover we show that pharmacological modulators of the mammalian clock mechanism have the same effects on rhythms in *Ostreococcus*. Post-translational mechanisms, and at least one rhythmic marker, seem to be better conserved than transcriptional clock regulators. It is plausible that the oldest oscillator components are non-transcriptional in nature, as in cyanobacteria[7], and are conserved across kingdoms.

Over the past two decades, great progress has been made towards delineating the molecular basis of eukaryotic circadian rhythms using model organisms such as *Arabidopsis thaliana* (plant), *Mus musculus* (mammal) and *Drosophila melanogaster* (insect)[5,8]. In each case, mechanistic models of the cellular clock have relied heavily on networks of transcriptional/translational feedback loops and can successfully account for a wide range of experimental data[9]. Although the identified 'clock genes' differ widely across taxa, a growing number of ubiquitous post-translational mechanisms, such as casein kinase II activity[5,10,11], have been shown to contribute to timing. Similarly, signal transduction pathways, for example, Ca^{2+}/cAMP, previously viewed as clock inputs have been shown also to be clock outputs, thus becoming indistinguishable from the 'core' mechanisms[5,12]. As a result it is presently unclear whether transcription, per se, is necessary to sustain the eukaryotic cellular clock[13,14], especially in light of observations that prokaryotic timekeeping can be reconstituted *in vitro* using the gene expression products of the cyanobacterial *kaiBC/kaiA* operons[7]. We hypothesized that non-transcriptional mechanisms would be competent to sustain cellular rhythms without a transcriptional contribution, and so set out to test this using the pico-eukaryote *Ostreococcus tauri*. This single-celled eukaryote has several advantages. It is readily cultured, possesses a small genome (~12 Mb), and yet its light-entrainable clock shares the transcriptional architecture of the clock in higher plants, namely a negative feedback loop between the morning-expressed *CCA1* and evening-expressed *TOC1* genes[4].

Recently, bioluminescent luciferase (LUC) reporter lines for transcription and translation of *O. tauri* clock genes were developed to enable non-invasive interrogation of clock mechanisms[4]. After entrainment in 12 h light/12 h dark cycles, circadian rhythms of bioluminescence from a translational (CCA1–LUC) and transcriptional (pCCA1::LUC) reporter were observed to persist for >4 days in constant light (Fig. 1a), indicating the presence of an underlying circadian clock, able to keep time without reference to any external time cues. Although many cellular processes in photosynthetic organisms are light-dependent[4,15,16], the cyanobacterial clock was recently shown to persist in darkness[7]. We therefore determined whether circadian rhythms might similarly persist in *O. tauri* without light. When placed in constant darkness, bioluminescent traces rapidly damped to background levels (Fig. 1a). After 96 h in constant darkness, no incorporation of [α-^{32}P]UTP was observed (Fig. 1b), meaning that no nascent RNA was being transcribed. Upon transfer of these transcriptionally incompetent cultures into constant light, circadian rhythms in bioluminescence began at a phase that was not dictated solely by the time of transfer into light (Fig. 1c and Supplementary Fig. 1a, b). If no cellular oscillation had persisted in the dark, we would expect the clock to restart with its phase determined solely by when it was transferred into the light (that is, complete phase resetting). In contrast, the cultures' new phase suggested that the response to light was modulated by a pre-existing oscillation, instead of being completely reset by light (Fig. 1c)[17]. These observations indicate that *O. tauri* is competent to keep time in the absence of transcription.

To confirm this, we used a novel post-translational biomarker for rhythmicity: peroxiredoxin oxidation. The peroxiredoxins (PRXs) are a ubiquitous family of antioxidant enzymes that scavenge reactive oxygen species, such as hydrogen peroxide, catalysing their own oxidation at a conserved redox-active cysteine (Cys) group to sulphenic acid followed by hyperoxidation through to sulphonic acid[18]. In plants, a subtype of peroxiredoxins (the 2-Cys group) is targeted to chloroplasts where they protect the photosynthetic membrane against photo-oxidative damage[19]. Oxidation of PRX drives the formation of higher molecular mass multimers with reported chaperone and signalling functions[18]. Circadian cycles of post-translational modification of PRX have previously been reported in mouse liver[6] and recently shown to persist in human red blood cells *in vitro*[20]. *Ostreococcus tauri* expresses at least one 2-Cys PRX[15] (GenBank accession CAL55168.1) sharing 61% sequence identity with human PRX2 and 100% sequence identity around the catalytic cysteine residue (Supplementary Fig. 2a, b). Immunoblots using an antibody targeting this highly conserved region[20] revealed diurnal regulation of PRX oxidation that was highest during

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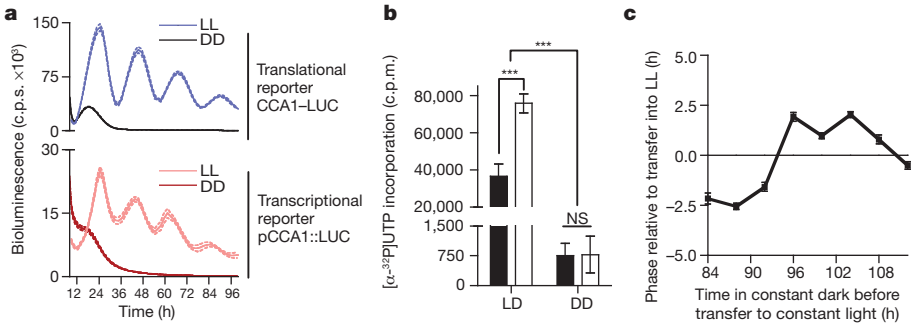

Figure 1 | Transcriptionally inactive cells show a phase-dependent response to re-illumination. **a**, Grouped data showing bioluminescent transcriptional (pCCA1::LUC) and translational (CCA1–LUC) reporter activity in constant darkness (DD) or constant light (LL) ($n = 16$, dotted lines ± s.e.m.). c.p.s., counts per second. **b**, After 96 h in darkness there is no significant (NS) incorporation of radiolabelled UTP; 10 min UTP treatment (black, ± s.e.m.) compared with 30 min treatment (white, ± s.e.m.) (2-way ANOVA interaction, $P < 0.001$ for time, condition and interaction, $n = 3$; Bonferroni post-tests: ***$P < 0.001$; for DD groups, $P = 0.95$). c.p.m., counts per minute. **c**, Upon transfer from darkness, the phase of CCA1–LUC (±s.e.m.) deviates significantly from the time of transfer into light (2-way ANOVA interaction, $P < 0.001$, $n \geq 16$).

subjective day, in advance of ribulose 1,5-bisphosphate carboxylase–oxygenase (RUBISCO) large chain expression (RbcL; a highly expressed plant/algal protein) (Fig. 2a). Moreover, in constant darkness, circadian rhythms persisted without transcription (Fig. 2b). PRX oxidation rhythms even persisted in constant darkness in the presence of inhibitors of cellular RNA synthesis (cordycepin) and cytosolic translation (cycloheximide), at concentrations that abolish clock reporter bioluminescence (Fig. 2c and Supplementary Fig. 3c, d), providing strong evidence that new RNA and/or protein synthesis is indeed not required for sustained rhythmicity. RbcL was used as a loading control, because although this protein was rhythmically expressed in a diurnal cycle, its levels were high and stable under constant conditions. Furthermore, rhythms in PRX oxidation are altered in a long period mutant (TOC1–LUC)[4], relative to controls (CCA1–LUC), under constant light; this indicates that post-translational oscillations are coupled with transcriptional/translational cycles under more physiological conditions (Supplementary Fig. 2c, d). Thus, PRX oxidation constitutes the first example, as far as we are aware, of a post-translational circadian biomarker shared between the animal (mouse/human) and green (plant) lineages.

Although experimentally useful for dissecting the algal clockwork, constant darkness potentially represents an exotic environmental

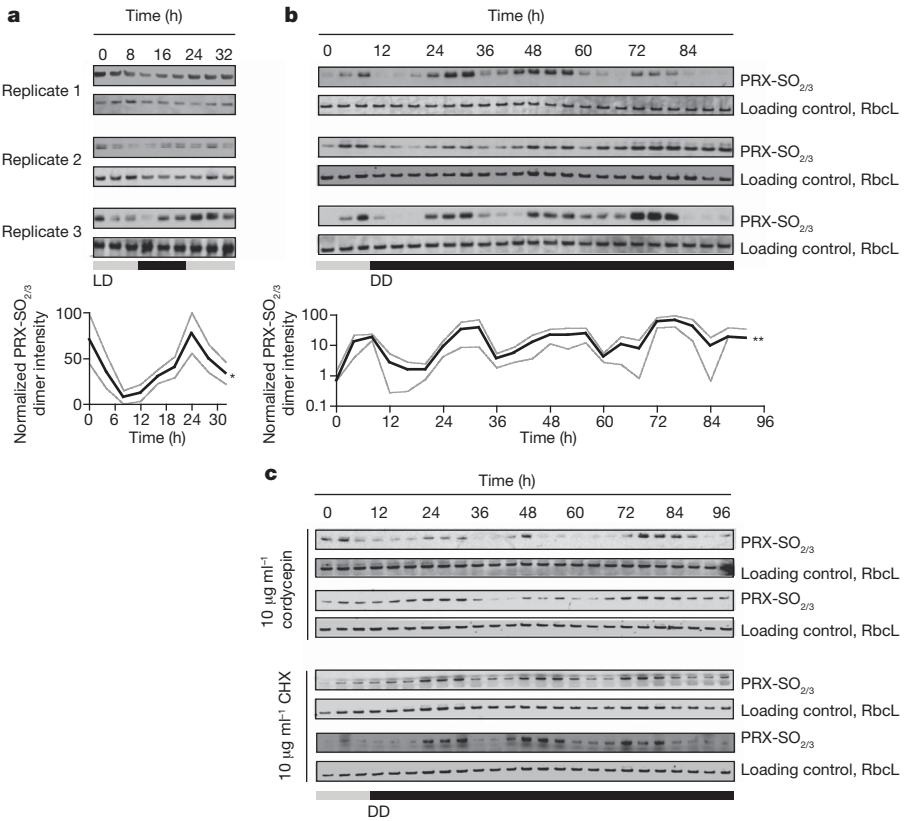

Figure 2 | Circadian cycles of PRX oxidation are detected during light/dark cycles and in constant darkness, and persist during drug inhibition of gene expression. **a**, Individual blots and grouped mean intensities for three *O. tauri* time series sampled under 12/12 h light/dark cycles (*Friedman test, $P = 0.04$ for time effect). **b**, Individual blots and grouped mean intensities for three *O. tauri* time series sampled under constant darkness (**Friedman test, $P = 0.005$ for time effect). **c**, PRX-$SO_{2/3}$ immunoblots of *O. tauri* time series sampled under constant darkness in the presence of inhibitors of transcription (cordycepin) and translation (cycloheximide (CHX)).

challenge to *O. tauri*, and hence we sought to also examine non-transcriptional rhythms in constant light using real-time bioluminescence reporter assays. CCA1–LUC and pCCA1::LUC reporter lines were incubated with a range of concentrations of cordycepin and cycloheximide during bioluminescent recordings, to assay the effects of inhibiting cellular RNA synthesis and cytosolic translation, respectively. At lower concentrations we observed dose-dependent damping of rhythmic amplitude with both drugs, and a robust increase in circadian period with increasing cordycepin concentration (Supplementary Fig. 3a, b), in agreement with observations in the marine mollusc *Bulla*[21]. At saturating doses, both drugs resulted in immediate damping and arrhythmia in the transcriptional reporter lines (Supplementary Fig. 3c). Notably, the translational reporter exhibited an additional cycle of CCA1–LUC synthesis in the presence of saturating transcriptional inhibitor (Supplementary Fig. 3d), which was not observed in the transcriptional reporter line. We interpret this to mean that when CCA1–LUC messenger RNA is present, post-transcriptional mechanisms are sufficient to drive an additional cycle of correctly timed protein accumulation.

Clearly in the context of a living cell, transcription is ultimately required for any biological process, including circadian rhythms, as the mRNAs have limited half-lives and can only be replaced through transcription. In a biological clock context, it seems natural that some mRNAs are cyclically expressed in anticipation of cellular need. Microarray studies in several organisms have shown that >10% of the transcriptome is regulated on a daily basis[6,15,22]. This implies that circadian cycles in transcription factor activity are a normal feature of cell physiology. Some of this transcriptional activity will contribute to timekeeping, directly or indirectly. If the natural state of a eukaryotic cellular clock revolves around reciprocal interplay between post-translational oscillations and established transcriptional feedback loops, it becomes of great interest to identify at what phases this interconnection is regulated.

Ostreococcus cultures are amenable to drug treatment that can be reversed by wash-off, because *O. tauri* grown in liquid culture forms aggregates at the bottom of microplate wells (Fig. 3a). To ascertain at which phases of the circadian cycle gene expression exerts priority over non-transcriptional mechanisms, we performed a 'wedge' experiment[21], in which transcription or translation was reversibly inhibited starting at 4-h intervals across the circadian cycle, for increasing durations, in constant light. Resultant phases were determined by the timing of CCA1–LUC expression peaks over the interval following removal of the drug (Fig. 3b). As with the earlier experiments using constant dark, our null hypothesis was that if the clock was immediately arrested by drug treatment then phase would be set by the time of drug wash-off. Phase is described relative to the zeitgeber, or time giver, during entrainment where ZT0 is dawn and ZT12 is dusk (ZT denotes zeitgeber time).

A general trend towards the anticipated wedge shape was observed (Supplementary Fig. 4a, b). However, there were significant exceptions to this pattern: (1) the clock was insensitive to transcriptional inhibition for up to 24 h in treatments starting from ZT8 (Fig. 3b, cordycepin treatment); (2) transcriptional inhibition outside ZT0–ZT8 did not affect phase; (3) after treatments spanning this window, the clock resumed at dusk if treatment began during the subjective night, or at dawn if treatment began during the subjective day (Fig. 3c and Supplementary Fig. 4a, c, d); (4) translational inhibition (cycloheximide treatment) outside ZT4–ZT12 did not affect phase (Fig. 3c and Supplementary Fig. 4b–d).

The simplest interpretation is that transcription of mechanistically relevant clock genes is licensed by post-translational mechanisms and occurs around the first half of the subjective day. These transcripts are translated around the second half of the subjective day and non-transcriptional mechanisms keep time during the subjective night. Presumably, when inhibition of transcription occurs at midday, for example, the resumption of stalled gene expression after wash-off overrides the phase of the non-transcriptional oscillations and the clock resumes from the nearest expected light/dark transition to when inhibition began. This runs contrary to current understanding of clocks in eukaryotes, in which transcription of key clock genes is active almost continuously around the circadian cycle[23]. Even in *O. tauri*, transcription of *TOC1* and *CCA1* would span the full cycle except for the interval ~ZT2–ZT8, after the peak of *CCA1* mRNA and before the rise in *TOC1* mRNA (ref. 4).

The final question of importance is what non-transcriptional mechanisms are involved in sustaining the clock? We hypothesized that the components that sustain these post-translational rhythms are

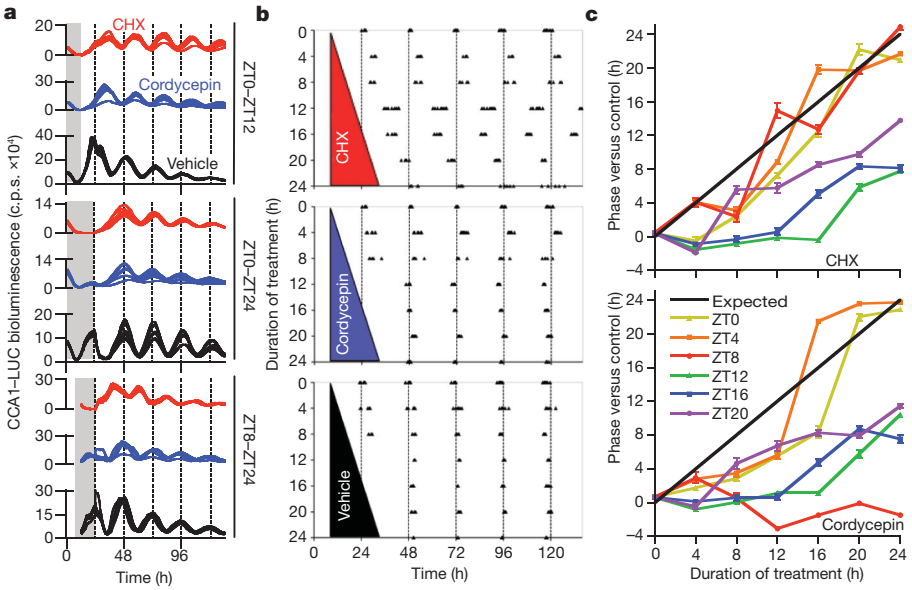

Figure 3 | Circadian timing can survive the inhibition of cellular transcription, or cytosolic translation. a, Treatment (from top, at ZT0–ZT12, ZT0–ZT24, ZT8–ZT24; shaded) with inhibitors of transcription (10 μg ml^{-1} cordycepin, blue lines) or translation (1 μg ml^{-1} cycloheximide (CHX), red lines) may shift the phase of reporter expression, compared with vehicle (0.04% DMSO, black lines), depending on the treatment phase and duration. **b**, Peak times of CCA1–LUC expression from individual wells ($n > 5$) are plotted, after treatments of different durations, starting at ZT8. **c**, Summary of phase shifts (±s.e.m., $n > 5$) relative to vehicle-treated controls, for all treatment durations (*x* axis) and starting times (see legend in lower panel). Black line represents the expected result, assuming total resetting by wash-off.

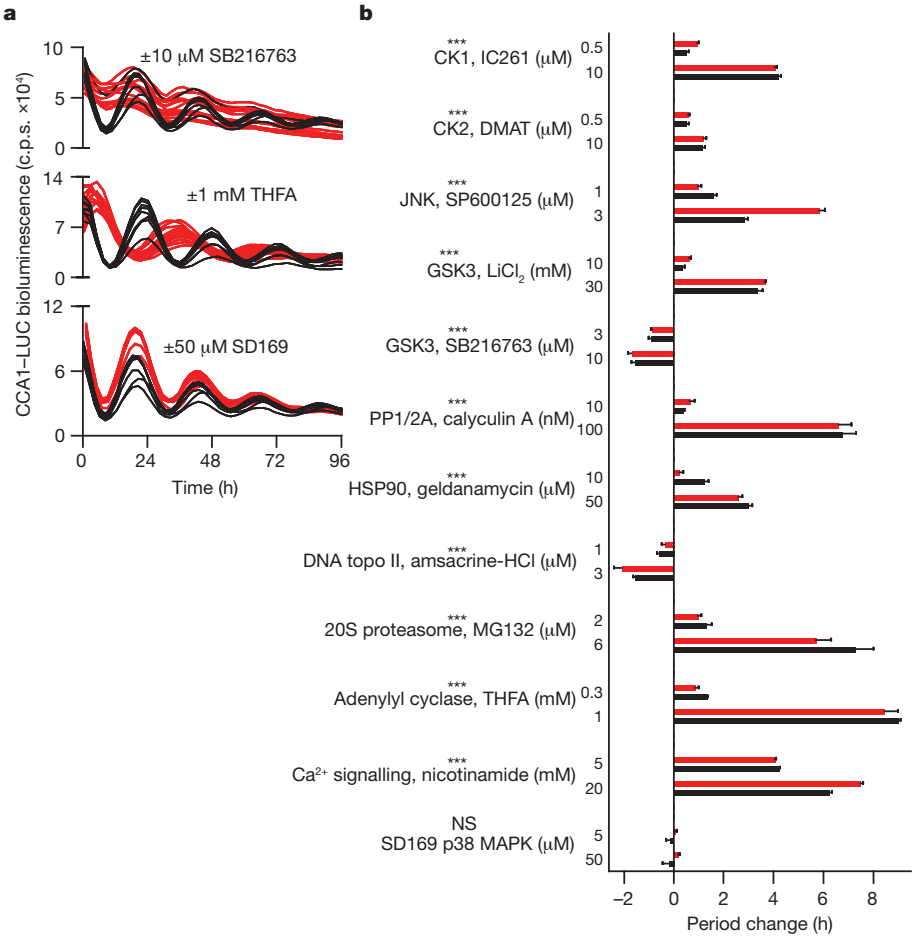

Figure 4 | Circadian period in *O. tauri* can be modulated pharmacologically in a dose-dependent manner by the application of inhibitors that have been previously validated in other taxa. a, Examples of drug effects (red) on CCA1–LUC bioluminescence compared with vehicle controls (black): SB216763 shortens period, 9-(tetrahydro-2-furyl)-adenine (THFA) increases period, SD169 has no effect. **b**, Summary showing grouped dose–responses on circadian period of transcriptional (red) and translational (black) reporter lines for selected drugs with previously demonstrated action in other taxa (±s.e.m., $n \geq 8$, $^{***}P < 0.0001$, 2-way ANOVA for concentration × reporter; not significant (NS) for SD169 negative control, $n = 8$, $P = 0.90$).

likely to be ubiquitous and highly conserved. Certainly the *O. tauri* genome encodes close homologues of enzymes such as casein kinase II that tend to exhibit greater sequence conservation across kingdoms than canonical transcriptional clock genes (Supplementary Table 1). In the last two years a number of high-throughput chemical biology screens on mammalian cellular rhythms in culture have been published, identifying a number of potent modulators of free-running period[12,24–27]. Because many such inhibitors target an enzyme's active site, it seemed plausible that drug action might be similarly conserved. The effects of specific pharmacological inhibitors that have been demonstrated to significantly affect free-running period in mammalian cells, and/or other model organisms (Supplementary Table 2), were therefore tested in *O. tauri*. In all cases, dose-dependent effects were observed on circadian period that correlated with their action in other species such as mouse or *Neurospora* (Fig. 4a, b and Supplementary Fig. 5a). Critically, where tested, such pharmacological perturbations also delayed the timing of transcriptionally incompetent cells, first with respect to the additional cycle of CCA1–LUC expression observed during transcriptional inhibition in constant light (Supplementary Fig. 6a). Second, the period of rhythmic PRX oxidation in constant darkness was also lengthened by the treatments tested (Supplementary Fig. 6b). Although drugs can have pleiotropic effects on cell biology, the drug effects on the clock are conserved across taxa. The parsimonious interpretation is that, both with and without transcription, conserved post-translational mechanisms are necessary to keep biological time.

Although the importance of transcription to circadian rhythms is self-evident, our observation that eukaryotic rhythms persist in the absence of transcription challenges the general model for eukaryotic clocks, indicating a functional equivalent to cyanobacterial timekeeping[28], although undoubtedly more complex. This is supported by increasing numbers of observations in diverse organisms[5,13,20,29]. Most prominently, the observation of a rhythmic post-translational marker that persists in the absence of transcription in species as diverse as a unicellular green alga and humans[20] raises exciting prospects for our understanding of how circadian clocks evolved. We note that both PRX and cyanobacterial KaiB are clock-relevant members of the thioredoxin-like superfamily[28] that associate into higher molecular mass complexes with catalytic function. We speculate that this may reflect conserved remnants of a proto-clock in the last common ancestor of eukaryotes and prokaryotes.

METHODS SUMMARY

All materials were purchased from Sigma-Aldrich unless otherwise stated. Transgenic *Ostreococcus tauri* lines[4] were cultured in Keller media-supplemented artificial sea water (Km) under 12/12 h blue (Ocean Blue, Lee lighting filter 724) light/dark cycles (17.5 $\mu E\, m^{-2} s^{-1}$). For recording, cultures were transferred to 96-well microplates (Lumitrac, Greiner Bio-one) at a density of ~15×10^6 cells per ml and entrained for 7–10 days. No density effects on clock output were observed under relevant density ranges, and cell division in microplates was found to be close to zero. One day before recording, 150 μl Km was replaced with 150 μl Km containing 333 μM luciferin (Km+). Drugs were made up in DMSO or Km+, diluted in Km+ and added to replicates of 8 or 16 wells immediately before

recording. For incubations in constant darkness, Km+ was supplemented with 200 mM sorbitol and 0.4% glycerol to increase cell viability. Bioluminescent recordings were performed on a TopCount (Packard) under constant darkness or constant red + blue LED light (5–12 $\mu E\ m^{-2}$). For wash-off of reversible inhibitors, cell aggregates formed in the bottom of the wells were quickly and gently washed twice with Km+, using multi-channel pipettes, and returned to recording conditions. Analysis of period was performed with FFT-NLLS (BRASS 3[30]) using time windows ≥3 days; mFourfit (BRASS 3) was used to assess phase and confirmed manually. Statistical analysis was performed using GraphPad Prism. For *de novo* RNA synthesis analysis by [α-^{32}P]UTP uptake, 1 ml cell aliquots were either incubated in darkness or light/dark cycles for 4 days. 0.2 MBq of [α-^{32}P]UTP was added, and after incubation cells were collected and washed twice with Km. Incorporation was measured using scintillation counting. Immunoblots were performed as described elsewhere[20]. Sequence alignments were performed using EBI Jalview. BLAST searches were performed using NCBI BLASTp under the default BLOSSUM62 settings.

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

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Author Contributions J.S.O'N., G.v.O. and L.E.D. designed and performed the experiments; J.S.O'N., G.v.O., L.E.D., C.T., A.B.R. and A.J.M. analysed data. F.-Y.B. and F.C. generated essential protocols and biomaterials. All authors contributed to writing. J.S.O'N. and G.v.O. contributed equally to this paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.B.R. (areddy@cantab.net) or A.J.M. (Andrew.Millar@ed.ac.uk).

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Putative *O. tauri* drug target	Accession	Closest *H. sapiens* homologue	Accession	Sequence Identity (%)	Sequence Similarity (%)	E-value (NCBI Blast, BLOSSUM 62)
DNA topoisomerase II	CAL56339	DNA topoisomerase II	AAA61209.1	54	70	0
HSP90	CAL56087	HSP90	NP_005339.3	67	85	6.00E-170
PP2A	CAL51458.1	PP2A	NP_060931.2	51	62	4.00E-127
CK1	CAL52491	CK1	NP_001884.2	72	82	2.00E-126
GSK3	CAL51449.1	GSK3	NP_001139628.1	60	74	6.00E-125
MAPK	CAL55559.1	MAPK	NP_620407.1	54	71	6.00E-103
CK2	CAL52182	CK2	CAI18393.2	58	71	1.00E-62
Proteasome beta subunit	CAL50436	Proteasome subunit	NP_002786.2	50	67	8.00E-51
Adenylyl cyclase	CAL54153	No relevant hits[1]	-	-	-	-
***O. tauri* clock gene**	**Accession**	**Closest H. sapiens homologue**	**Accession**	**Sequence Identity (%)**	**Sequence Similarity (%)**	**E-value (NCBI Blast, BLOSSUM 62)**
CCA1	AAU14271	MYB-like[2]	BAB67808.1	38	75	2.00E-05
TOC1	AAU14274	No relevant hits	-	-	-	-
***H. sapiens* clock gene**	**Accession**	**Closest *O.tauri* homologue**	**Accession**	**Sequence Identity (%)**	**Sequence Similarity (%)**	**E-value (NCBI Blast, BLOSSUM 62)**
Period2	ABM64216	DNA repair and transcription factor XPB1[3]	CAL53063.1	25	40	0.3
Bmal1b	BAA19935	Unnamed protein product[3]	CAL56979.1	36	56	0.18

Table S1. A comparison of protein sequence identity/similarity, for several clock-relevant genes, between *Ostreococcus tauri* and *Homo sapiens*.

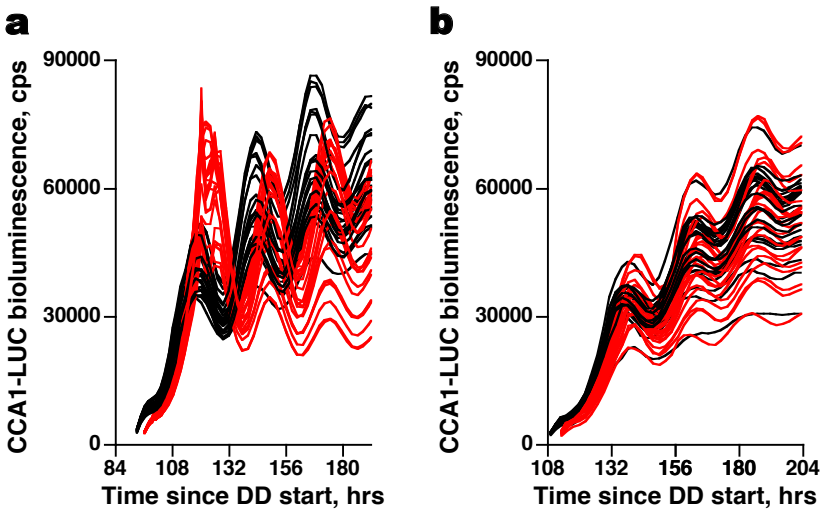
[1] *O. tauri* encodes two proteins (accessions: CAL50189.1, CAL54153.1) that are annotated as members of the class III nucleotidyl cyclase superfamily. These have little significant sequence homology with mammalian adenylyl cyclases (also class III). Due to the mode of action of 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine, 9-THF-Ade (THFA) - a post-transition state, non-competitive p-site ligand that binds the catalytic site by mimicking cAMP, it is plausible that this drug is preferentially active against adenylyl cyclase in *O. tauri* despite an clear divergence of primary protein sequence.

[2] Not implicated in the mammalian clockwork.

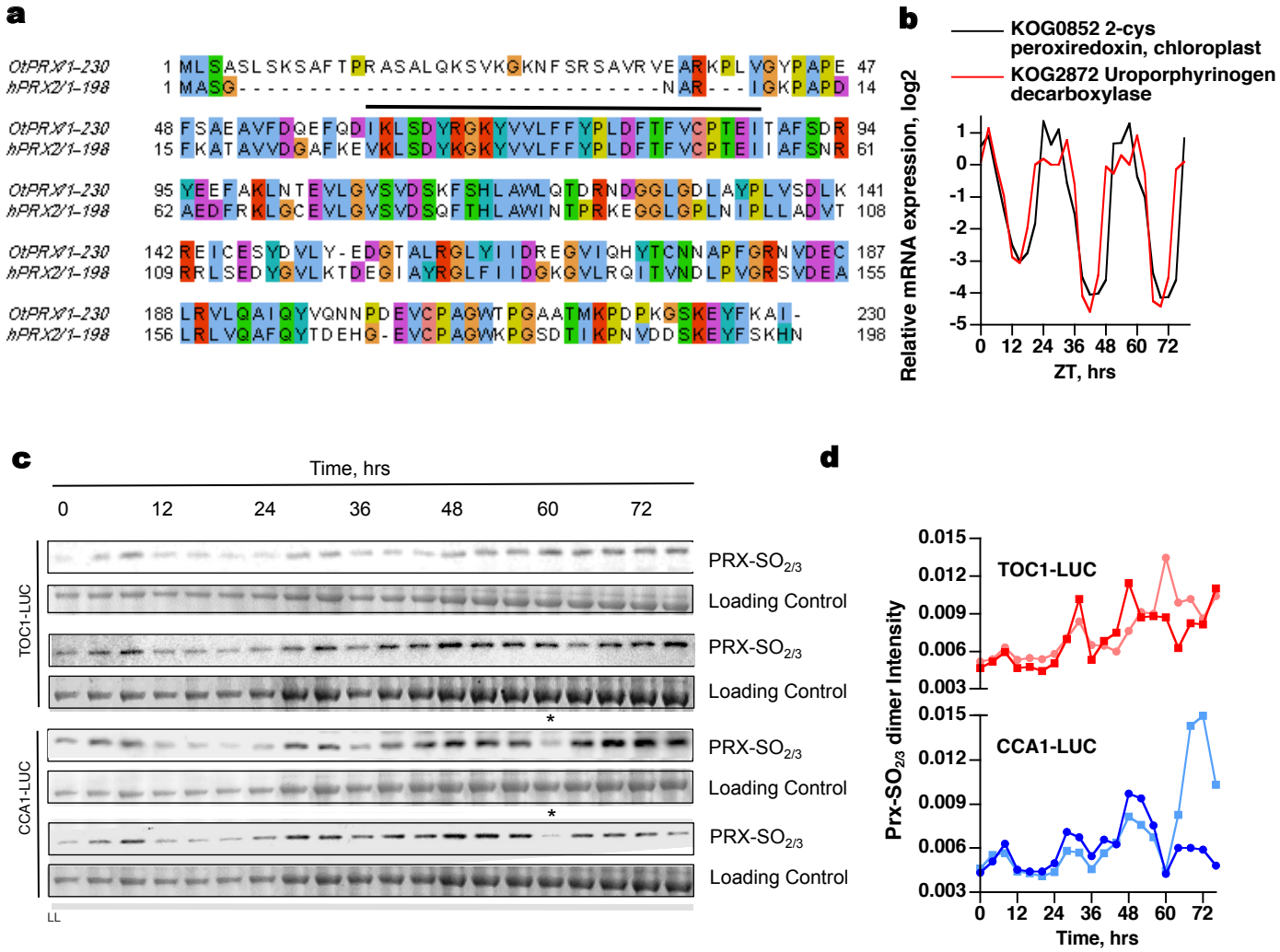
[3] Not implicated in the plant clockwork.

Compound	Target	Notes	Reference(s)
amsacrine-HCL	DNA topo II	Shown to shorten period in mammalian cells	Isojima et al, PNAS, 2009
SB216763	GSK3	Shown to shorten period in mammalian cells, GSK3 also implicated in the *Drosophila* clock	Isojima et al, PNAS, 2009 Hirota et al, PNAS, 2008
$LiCl_2$	GSK3	Increases period in a wide range of organisms, shown to act through GSK3 in mammalian cells	Iitaka et al, J Biol Chem, 2005 Yin et al, Science, 2006
IC261	CK1	Lengthens period in mammalian cells and in *Neurospora*	Eide et al, Mol Cell Biol, 2005; Querforth et al, Cold Spring Harb Symp Quant Bio, 2007;
D4476	CK1	Lengthens period in mammalian cells	Reischl et al, J Biol Rhythms, 2007
DMAT	CK2	Increases period in mammalian tissue *in vitro*.	Maier et al, Genes Dev, 2009 Mizoguchi et al, Int Rev Cytol, 2006
TBB	CK2	CK2 is also heavily implicated in *Arabidopsis*, *Neurospora* and *Drosophila* clock mechanisms	Miyata, Mol Cell Biol, 2004
nicotinamide	Ca^{2+} signaling &/ or NAD metabolism	Nicotinamide increases period in *Arabidopsis* and mammalian cells	Dodd et al, Science, 2007; Asher, Cell, 2008
BAPTA	Ca^{2+} signaling	Ca^{2+} signaling is also heavily implicated in *Drosophila* and mammalian clock mechanisms	Harrisingh et al, J Neurosci, 2007; Lundkvist et al, J Neurosci, 2005; Ikeda, Neuron, 2003
SP600125	JNK	Shown to increase period in mammalian tissues and cells *in vitro*, also inhibits CK1, *in vitro*	Chansard et al, Neuroscience, 2007; Isojima et al, PNAS, 2009 Zhang et al, Cell, 2009
THFA	adenylyl cyclase	Increases period in mammalian tissues *in vitro* and *in vivo*	O'Neill et al, Science, 2007
9-CPA	adenylyl cyclase	Increases period in mammalian tissues *in vitro*	O'Neill et al, Science, 2007
calyculin A	PP2A	Increases period in mammalian tissues *in vitro*	Eide et al, Mol Cell Biol, 2005;
geldanamycin	hsp90	Increases free-running period in *Drosophila* (w^{1118})	Hung et al, J Biol Rhythms, 2009
MG132	proteasome	Increases period in mammalian tissues *in vitro*	Eide et al, Mol Cell Biol, 2005;
trichostatin A	Histone deacetylase inhibitor	Regulates clock gene expression in mammalian cells and SCN *in vitro*, reversible histone modification also implicated in other organisms.	Naruse et al, Mol Cell, Biol, 2004. Perales and Mas, Plant Cell, 2007.
SD169	P38α MAPK	Implicated as an output mechanism in *Neurospora* (negative control)	Vitalini, PNAS, 2007

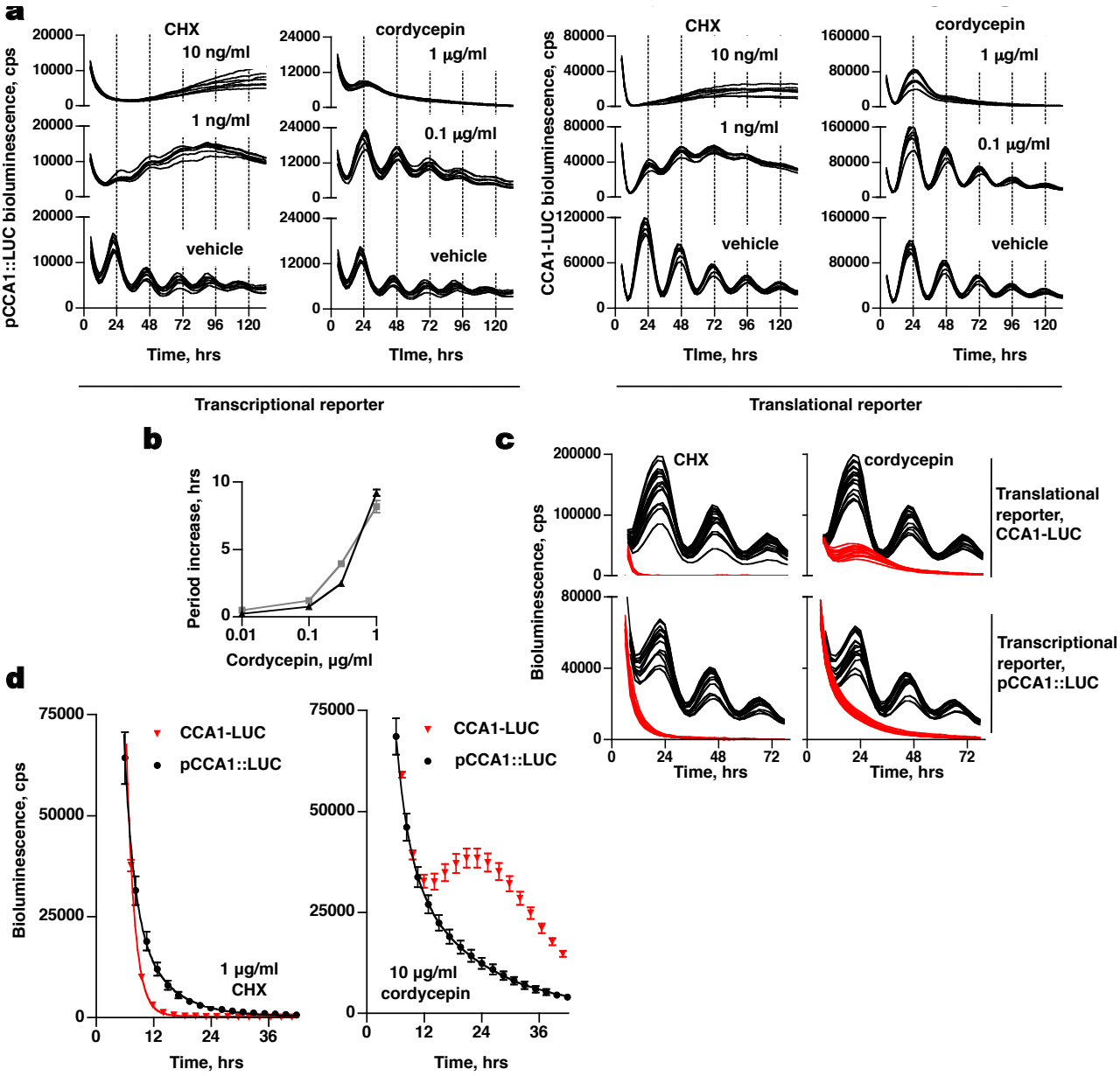
Table S2. A summary of literature reports in other taxa for the reported targets of drugs that modulate free-running period in *Ostreococcus tauri*.



Supplementary Figure 1. Representative plots of circadian rhythms of gene expression in *O. tauri* following constant darkness. Two representative plots showing recovery of rhythmic bioluminescence upon transfer at 4 hour intervals from constant darkness into constant light. An additional 4 hours in constant darkness separate the red from black traces in each case. Whilst plot **a** shows a clear difference in circadian phase, plot **b** does not, a departure from absolute phase resetting by light.

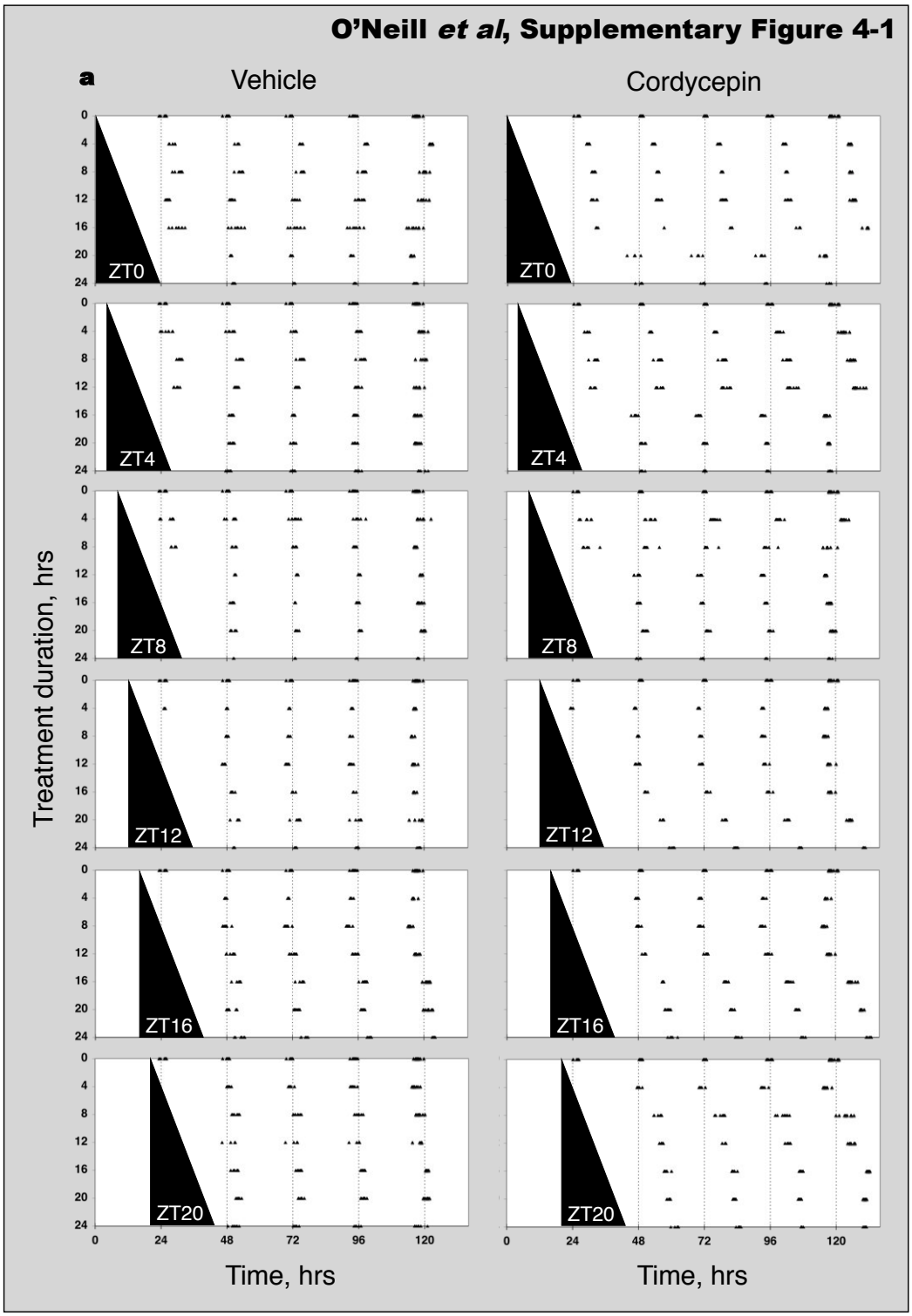


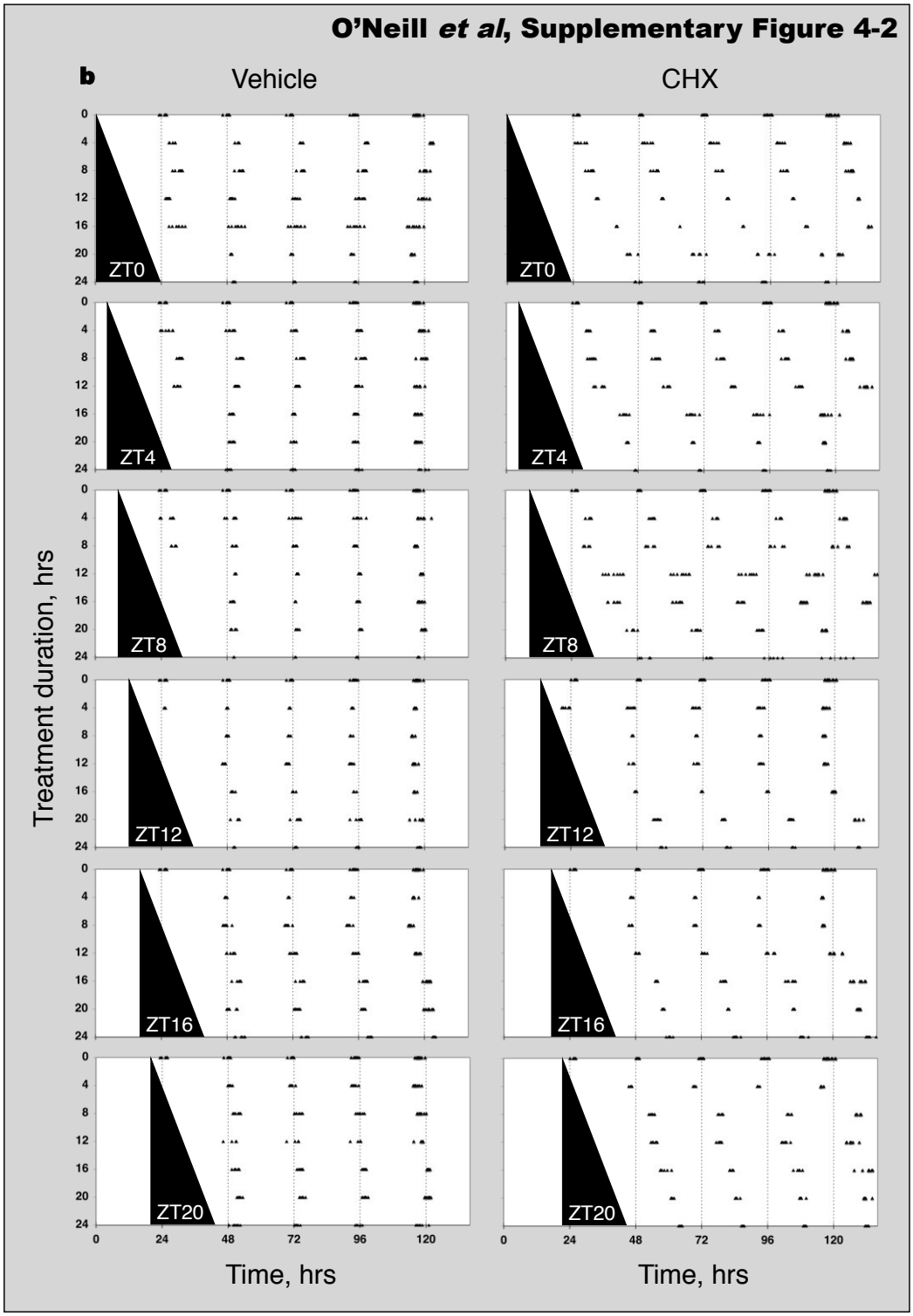
Supplementary Figure 2. Sequence, expression and post-translational modification of PRX in *O. tauri*. **a**, Sequence alignment of *O. tauri* thioredoxin peroxidase and human PRX2, black line indicates the highly conserved region surrounding the catalytic cysteine residue that is pertinent to the following western blots and those in Figure 2. **b**, *O. tauri* PRX is rhythmically transcribed under 12:12 hour light:dark cycles in phase with other afternoon-expressed transcripts (adapted from Monnier *et al*, 2010, BMC Genomics). **c**, Immunoblots show altered rhythms of hyperoxidised peroxiredoxin (PRX-$SO_{2/3}$) in constant light for mutant *O. tauri* lines that express an additional genomic copy of TOC1 (TOC1-LUC), previously shown to display longer period under constant conditions compared with lines expressing an extra copy of CCA1 (CCA1-LUC), indicated by the decrease in signal at 60 hours in the controls (*). **d**, Plots of individual intensities from **c.**

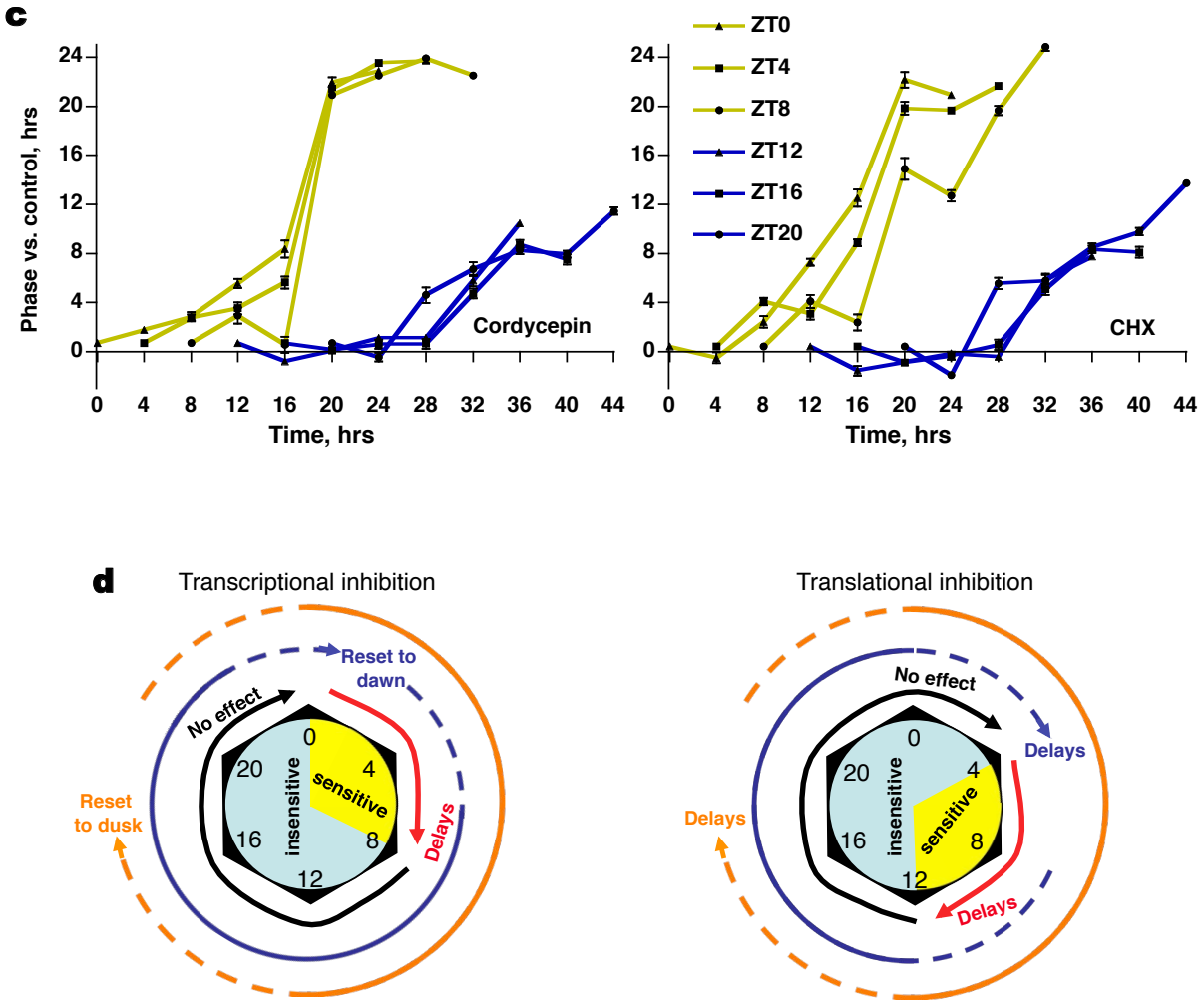


Supplementary Figure 3. The effect of inhibition of cellular RNA synthesis or cytosolic translation upon transcriptional and translational bioluminescent clock reporters under constant light.

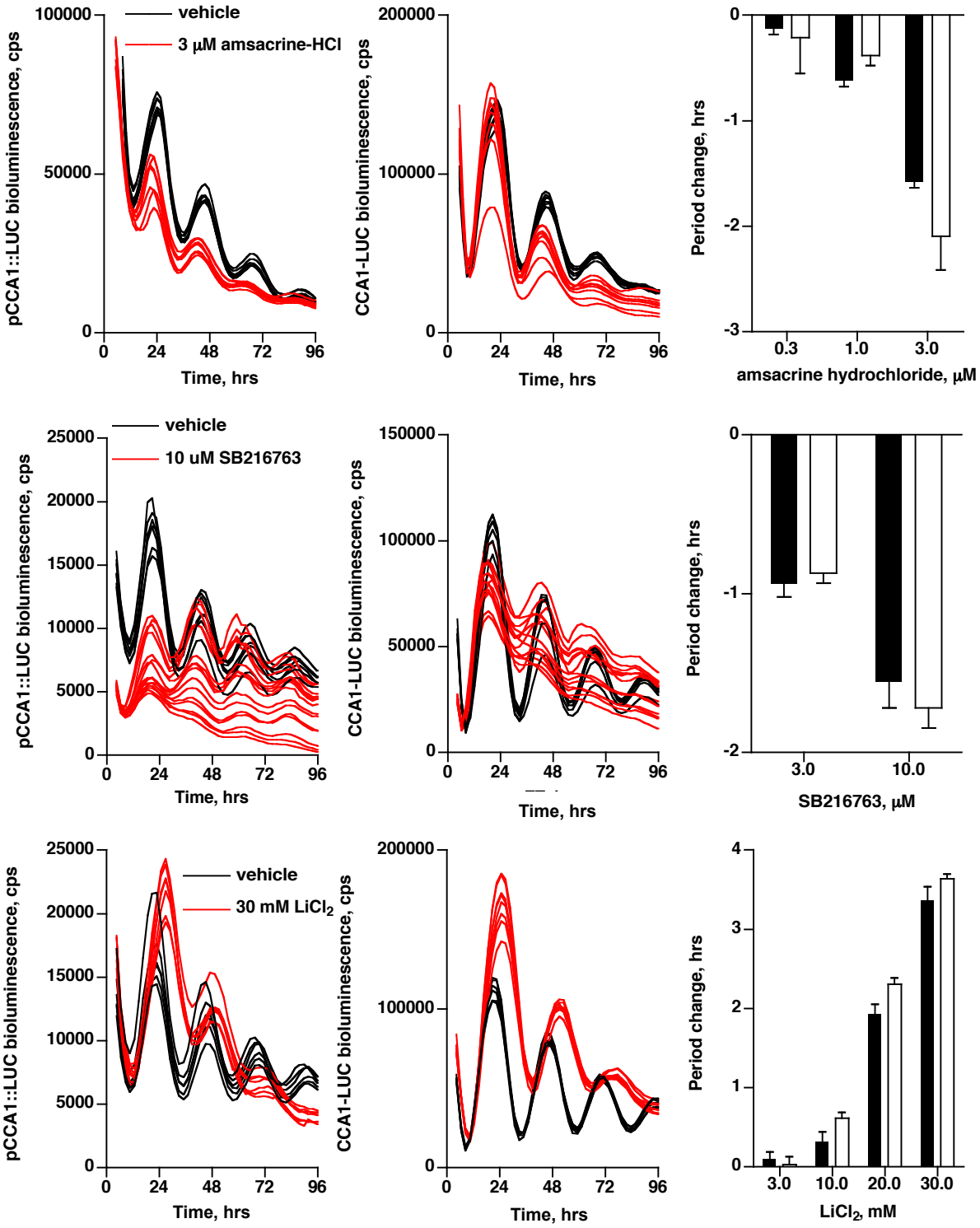
a, Representative plots showing the effect of increasing concentrations of CHX and cordycepin on transcriptional and translational reporters (n = 8). **b**, The effect on circadian period due to reduced cellular transcription rates (black, translational reporter; grey, transcriptional reporter; error bars ± SEM; 2-way ANOVA, $p < 0.0001$ for concentration effect, n = 8). **c**, The effect of maximal inhibition of transcription (cordycepin) or cytosolic translation (CHX) upon transcriptional or translational bioluminescent clock reporters (black, vehicle; red, drug treatment; n=16). **d**, Expanded from **c**, grouped data from lines expressing the translational CCA1-LUC reporter in the presence of 10 µg/ml cordycepin deviate from the exponential decay exhibited by the transcriptional pCCA1::LUC reporter under the same conditions, or either reporter in the presence of 1 µg/ml CHX ($R^2 \geq 0.98$ for all three curve fits; error bars ± SEM, n = 16).

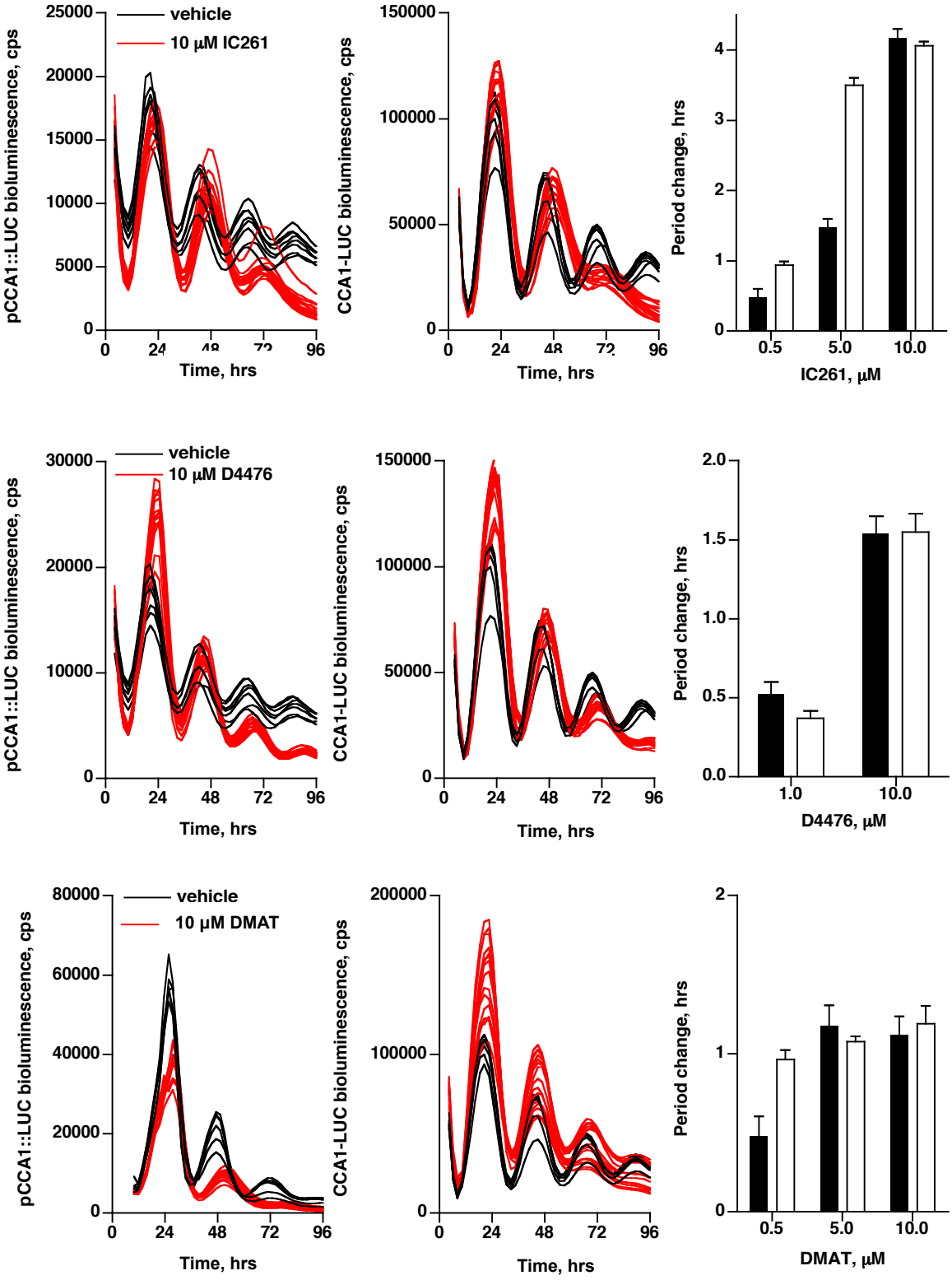

O'Neill *et al*, Supplementary Figure 4-1
a
Vehicle
Cordycepin
Treatment duration, hrs
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
0
4
8
12
16
20
24
0
24
48
72
96
120
Time, hrs
Time, hrs

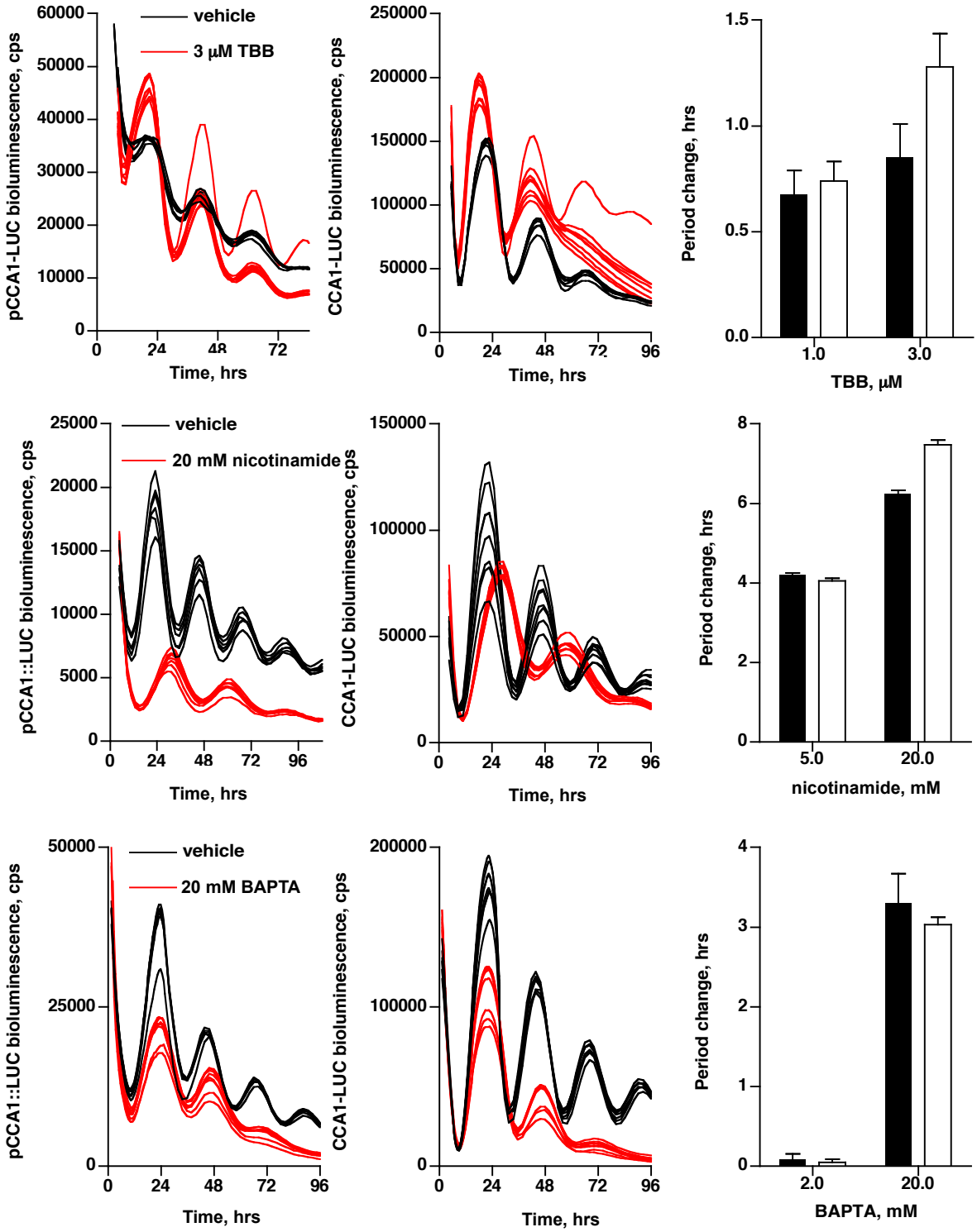

O'Neill et al, Supplementary Figure 4-2
b
Vehicle
CHX
Treatment duration, hrs
ZT0
ZT4
ZT8
ZT12
ZT16
ZT20
0
4
8
12
16
20
24
0
24
48
72
96
120
Time, hrs
Time, hrs

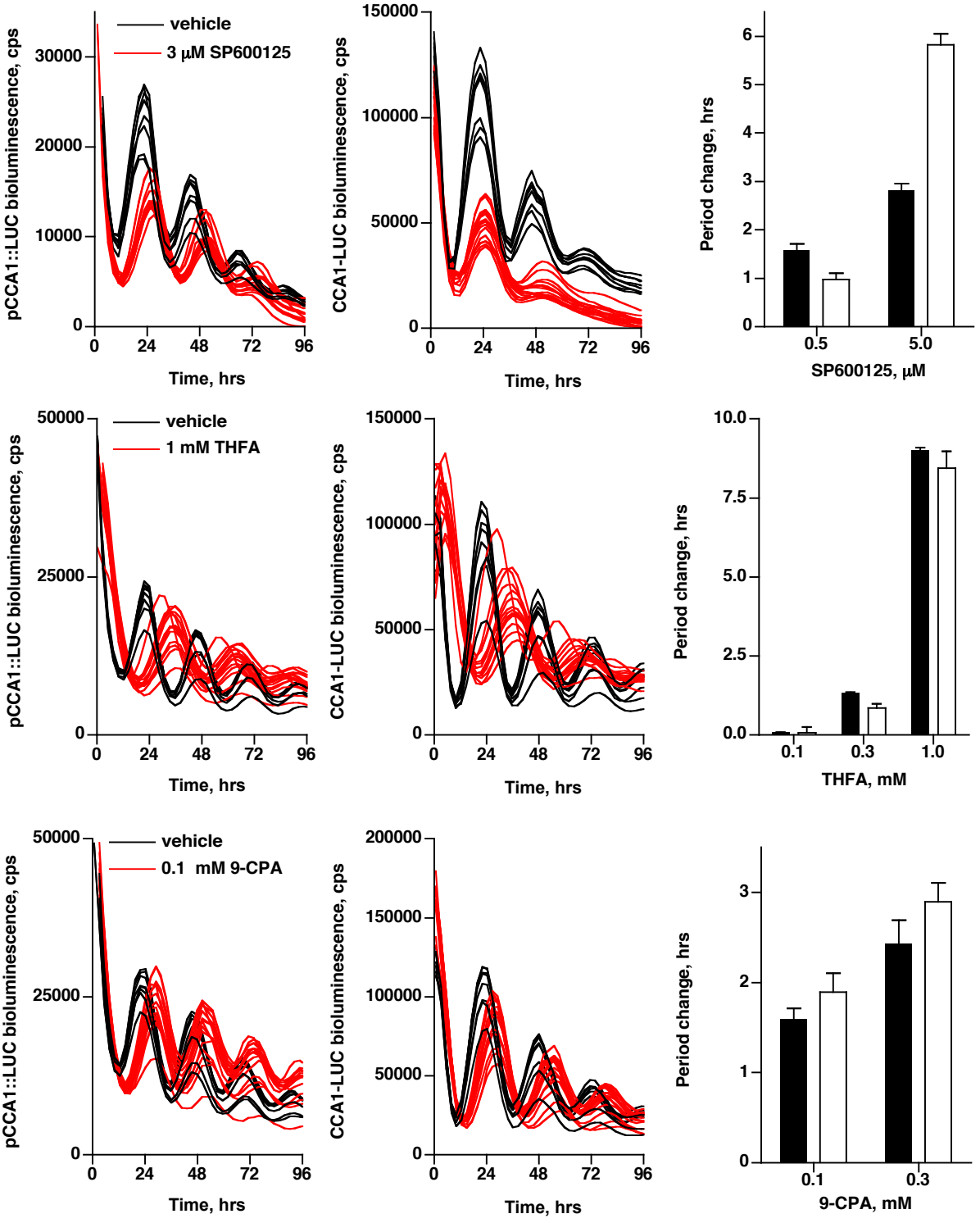


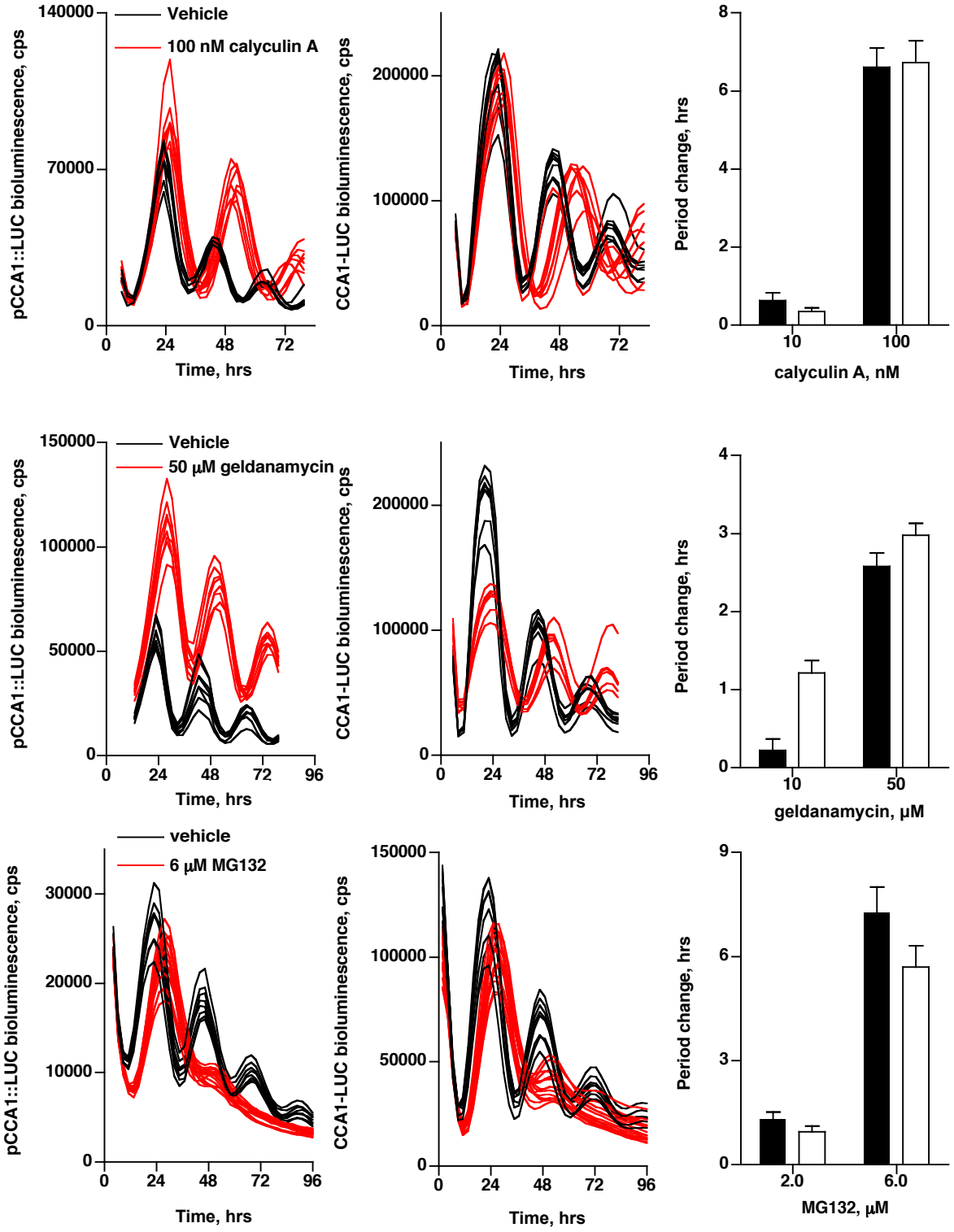
Supplementary Figure 4. Individual and grouped data from chemical 'wedge' experiment. a, Peak times of CCA1-LUC expression in constant light for individual replicates entrained under LD, following treatment with vehicle or cordycepin starting from ZT0, 4, 8, 12, 16, or 20 extending for 4-hour increments up to 24 hours (n>5). **b**, Peak times of CCA1-LUC expression in constant light for individual replicates entrained under LD, following treatment with vehicle or CHX starting from ZT0, 4, 8, 12, 16, or 20 extending for 4-hour increments up to 24 hours (n>5). **c**, An alternate plot of Figure 3c shows experimentally derived phases relative to vehicle controls (n > 5, error bars ± SEM). Yellow and blue lines indicate treatments beginning during subjective day and night, respectively. **d**, Cartoon schematic summarizing the experimental data shown in **a**, **b** and **c**; dotted lines represent different windows for treatment start times, solid lines represent continued treatment, arrows represent end of treatment with text describing the effect on phase relative to controls.

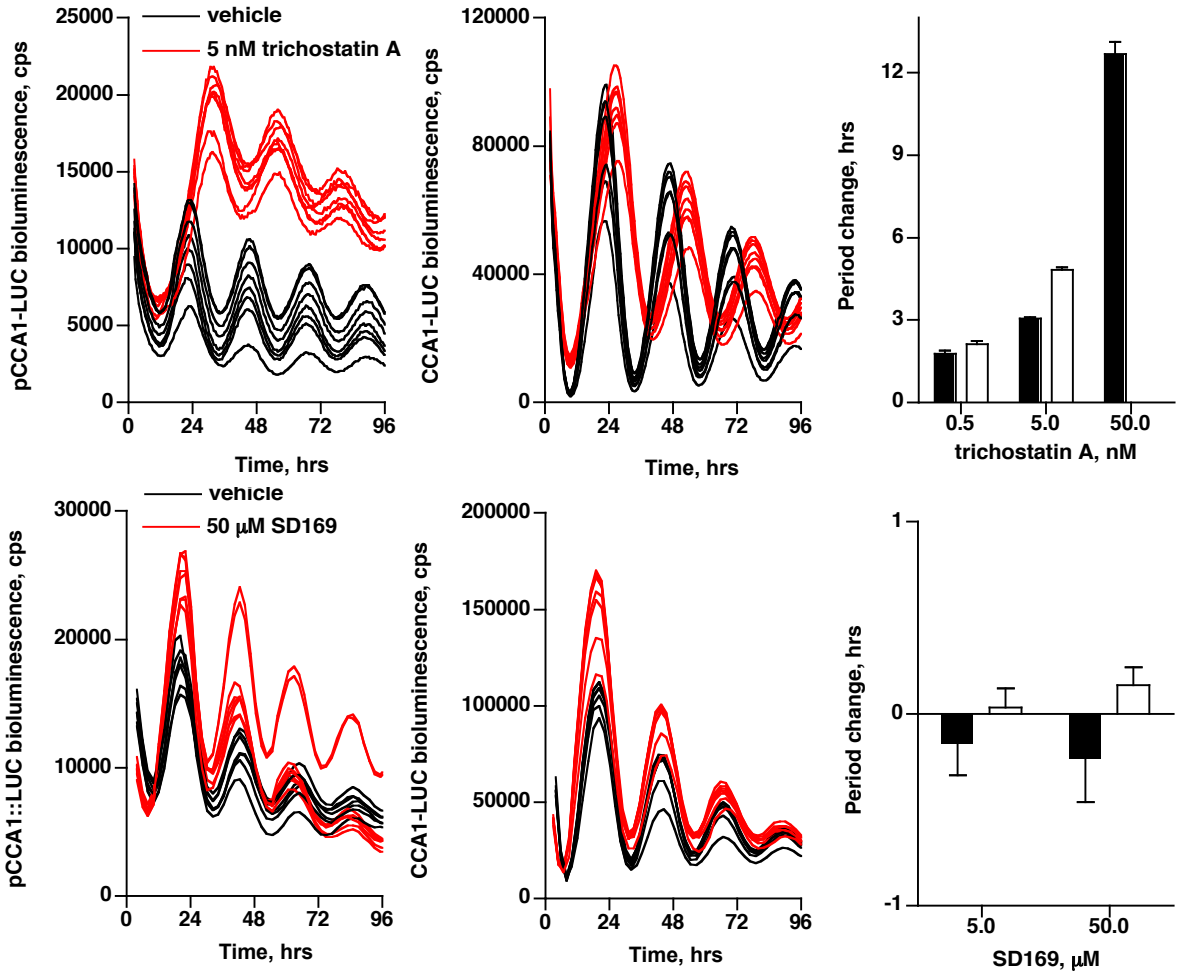

vehicle
3 μM amsacrine-HCl
pCCA1::LUC bioluminescence, cps
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
amsacrine hydrochloride, μM
vehicle
10 uM SB216763
SB216763, μM
vehicle
30 mM LiCl2
LiCl2, mM


vehicle
10 μM IC261
pCCA1::LUC bioluminescence, cps
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
IC261, μM
vehicle
10 μM D4476
D4476, μM
vehicle
10 μM DMAT
DMAT, μM

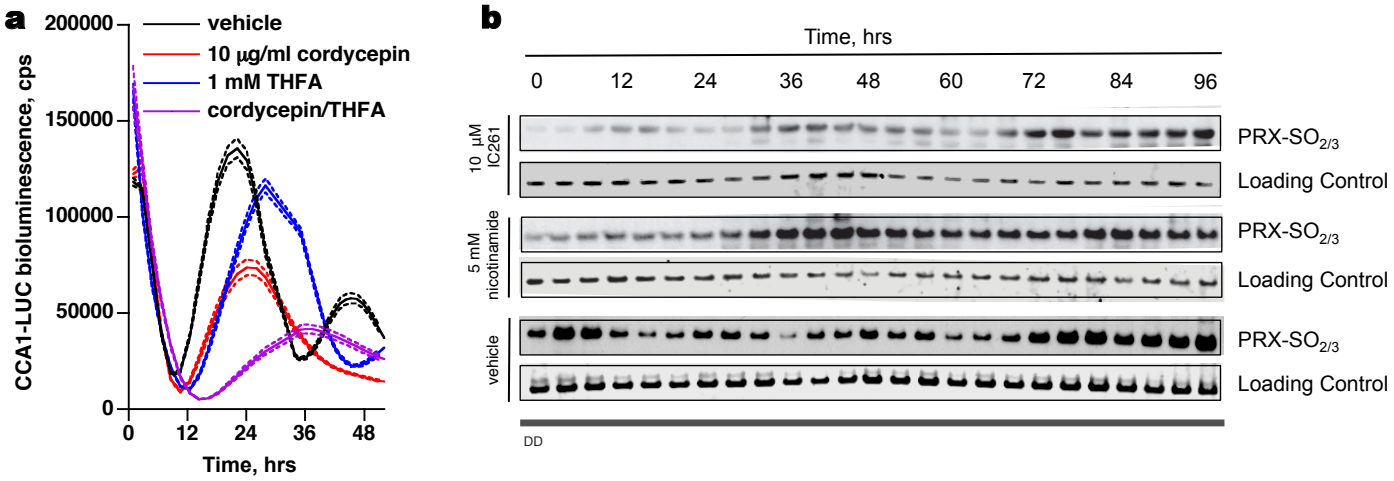

vehicle
3 μM TBB
pCCA1-LUC bioluminescence, cps
Time, hrs
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
TBB, μM
vehicle
20 mM nicotinamide
pCCA1::LUC bioluminescence, cps
Time, hrs
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
nicotinamide, mM
vehicle
20 mM BAPTA
pCCA1::LUC bioluminescence, cps
Time, hrs
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
BAPTA, mM


vehicle
3 μM SP600125
pCCA1::LUC bioluminescence, cps
CCA1-LUC bioluminescence, cps
Time, hrs
Period change, hrs
SP600125, μM
vehicle
1 mM THFA
THFA, mM
vehicle
0.1 mM 9-CPA
9-CPA, mM


Vehicle
100 nM calyculin A
pCCA1::LUC bioluminescence, cps
140000
70000
0
0 24 48 72
Time, hrs
CCA1-LUC bioluminescence, cps
200000
100000
0
0 24 48 72
Time, hrs
Period change, hrs
8
6
4
2
0
10 100
calyculin A, nM
Vehicle
50 μM geldanamycin
pCCA1::LUC bioluminescence, cps
150000
100000
50000
0
0 24 48 72 96
Time, hrs
CCA1-LUC bioluminescence, cps
200000
100000
0
0 24 48 72 96
Time, hrs
Period change, hrs
4
3
2
1
0
10 50
geldanamycin, μM
vehicle
6 μM MG132
pCCA1::LUC bioluminescence, cps
30000
20000
10000
0
0 24 48 72 96
Time, hrs
CCA1-LUC bioluminescence, cps
150000
100000
50000
0
0 24 48 72 96
Time, hrs
Period change, hrs
9
6
3
0
2.0 6.0
MG132, μM



Supplementary Figure 5. Representative and grouped data showing similar pharmacological actions on circadian period in *O. tauri* to those reported in other taxa. Representative plots from transcriptional and translational reporter lines with grouped data showing period change relative to vehicle controls (white bars, pCCA1::LUC; black bars, CCA1-LUC; ±SEM, 2-way ANOVA for concentration effect $p < 0.0001$ for all compounds except control compound SD169, $p = 0.90$, $n \geq 8$).



Supplementary Figure 6. Representative and grouped data showing the effects of pharmacological modulators of circadian period in *O. tauri*, in the absence of transcription. a, Grouped data showing CCA1-LUC reporter line under LL in the presence of cordycepin, THFA or both (n=8, mean ±SEM, dotted line). **b**, Immunoblots showing the effect of 5 mM nicotinamide or 10 μM IC261 on rhythms in PRX-$SO_{2/3}$ vs. vehicle in constant darkness.