Understanding systems-level properties: timely stories from the study of clocks

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Abstract | After several decades dominated by reductionist approaches in biology, researchers are returning to the study of complex biology with a litany of new and old techniques — this paradigm has been termed systems biology. Here we detail how systems biology is being used to uncover complex systems-level properties of the circadian clock. These properties include robustness, periodicity and temperature compensation. We describe how clock researchers are using systems-biology techniques, such as genetic perturbations, kinetic luminescence imaging, synthetic biology and mathematical modelling, to untangle these complex properties in mammals, fungi and bacteria. The strategies developed in the context of circadian clocks may prove useful for tackling similar problems in other systems.

Temperature compensation
In biological clocks, this is the property by which an increase or decrease in temperature fails to change the period length of the circadian rhythm.

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The old adage 'timing is everything' is just as true for biology as it is for comedy. In the past decade, research in biological rhythms has shown that many aspects of our biology occur with a daily rhythm (reviewed in REF. 1); we wake up, we are active and we sleep at similar times each day. Because the environmental changes that accompany the day are so predictable, nature devised an ingenious system, the circadian clock, to anticipate them and regulate physiology and behaviour accordingly.

For example, before we wake up, our heart rate and blood pressure increase in anticipation of this stressful event (reviewed in REF. 2). Our liver, fat cells and skeletal muscles anticipate that we will eat and maximize their efficiency in metabolizing, storing and utilizing energy produced from this food (reviewed in REFS 1–3). Our mental state and capacity for memory and learning, as well as our state of arousal and onset of sleep, are likewise clock-regulated (reviewed in REFS 3–6). Although these examples come from human studies, similar physiological and behavioural rhythms occur in most organisms, including cyanobacteria, fungi, plants and virtually all animals. Anticipation and adaptation to environmental change is a critical feature of biological systems[7].

To accomplish this timing, circadian clocks need: a light-input system that synchronizes the clock to day–night cycles; a biochemical and cellular oscillator to measure the passing of time; and output mechanisms to relay this timing information to the primary systems that regulate physiology and behaviour. Research in several model systems — *Synechococcus elongatus* PCC 7942 (cyanobacterium), *Neurospora crassa*, *Arabidopsis thaliana*, *Drosophila melanogaster*, *Mus musculus* and humans — uncovered many aspects of these basic mechanisms of oscillator function. In general, the light-input systems of bacteria, fungi, plants and animals all differ owing to their different photoreceptive systems. By contrast, the circadian clocks in these systems and their methods of regulating output are strikingly similar.

Clocks also have other interesting properties such as temperature compensation and genetic robustness. As temperature increases, the speed of most biochemical reactions also increases. However, the clock needs to keep 24-hour time in environments where the temperature may change tens of degrees during the day and even more between seasons. Poikilotherms need a circadian clock that accounts for this. Interestingly, clocks in homeotherms are also temperature-compensated, pointing to the conserved nature of this feature. A second interesting property is genetic robustness. As clocks are so crucial for life, organisms need genetic architecture that accommodates mutations without losing clock function.

What all of these properties have in common is that they are produced by systems of genes and proteins interacting together rather than being traits conveyed by single genes. In this Review, we document recent

Box 1 | **Omics data sets and integration**

Omics data

One of the key aspects of systems-biology approaches that distinguish them from traditional attempts is the availability of omics data and the opportunities for insight these data provide. Following an initial perturbation — which can take the form of RNA interference (RNAi), a genetic knockout or other genetic lesion, changing environmental conditions (for example, oxygen, time of day or temperature), or small molecule or metabolite perturbations — multi-parametric data are collected. These data can take the form of RNA or protein expression, analysis of post-translational modification, interaction data, metabolite profiling or phenotypic profiling by imaging or end-point analysis. For example, RNA sequencing (RNA-seq) and chromatin immunoprecipitation followed by high-throughput sequencing (ChIP–seq) are now robust and widely used to investigate changes in RNA levels, splicing and non-coding RNA expression (RNA-seq), or histone modifications and transcription factor binding (ChIP–seq). Quantitative mass spectrometry has, likewise, evolved to the point where genome-wide surveys of protein expression, protein modification and metabolite profiling can be conducted. High-throughput imaging technologies enable genome-wide screens of small interfering RNAs, cDNAs and small molecules at defined end points; they even enable kinetic studies. These studies are increasingly being applied at single-cell and even subcellular levels. After the data are collected, they can be analysed for patterns and other emergent properties. Two of these properties, parallel compensation and proportionality, are depicted in FIG. 1.

Data integration

A logical extension of having omics data is integrating them to gain insight into biology. This process involves multiple steps. Public databases are available to house many types of omics data — for example, gene expression data, short-read sequence data, proteomics data and even data for high-throughput screening. Most of these data sets require some form of conditioning before integration. This can involve specific analyses on each type of data — for example, protein–protein interaction data may require significant filtering and reciprocal protein–protein interactions before a novel interaction is considered valid. Also, each of these data sets often has its own analysis framework. Without understanding these frameworks (for example, which values are informative) you can contaminate downstream analyses and data integration. Even determining the identity of each gene or protein can be problematic. Although you might think this should be straightforward, genes and proteins have synonyms, and technology platforms often have their own identifiers, both of which can complicate integration. Integrating small molecule and metabolite profiling data may also not be straightforward — there are few one-to-one (small molecule or metabolite to gene) relationships. One-to-many relationships are more common and one-to-none findings are very frequent. Finally, there are many ways to integrate these data. Pathway approaches, Bayesian integration (REFS 74–75 for example), machine learning approaches (REF. 76 for example), including neural networks[77], and many other methods have been proposed and used in data integration. The choice of these methods often depends on the question — for example, for new members of a well-characterized pathway, Bayesian integration methods can be effective.

progress in understanding three clock properties: genetic robustness, periodicity and temperature compensation. To do so, we focus on three different model systems, namely mammals, cyanobacteria and *N. crassa*, in which significant progress has been made using a variety of systems-biology methods. These include perturbation analyses, genetics, biochemistry, imaging and conceptual and mathematical modelling.

Why is this a propitious time for this Review? Although reductionist approaches to biology have held the field for the past several decades, in the past few years researchers have begun to grapple with biological complexity. This has been aided by several developments. First, new technologies to collect omics data started in the 1990s and have been continuing to develop to this day. These multiparametric data — for example, DNA array, high-throughput sequencing and mass spectrometry — are enabling global analysis of biology following genetic or other perturbations (BOX 1). Second, old and new modelling approaches (BOX 2) are increasingly being used to analyse these data and gain insight into the governing principles of biology. Third, there has been a slow but inexorable realization that minimalist approaches to biology fail at addressing complexity. 'One-gene-at-a-time' research is giving way to global network approaches to study complex biology. The development of centres, institutes and departments of systems biology provides evidence that these approaches are reaching critical mass in academic institutions and elsewhere. It is our hope that lessons learned and strategies and techniques used in tackling complex systems-level properties in clocks will be useful for researchers interested in tackling similar problems in other systems.

Robustness

The clock takes a licking and keeps on ticking. A key property of clocks, biological or otherwise, is their resistance to intrinsic and extrinsic perturbation. What good would your watch be if it was reset by moving your arm? Instead, to set your watch, you need to use a precise mechanism that is difficult or impossible to do accidentally. This idea also holds true for biological clocks. For example, each day your circadian clock is reset by environmental cues, the most notable being light. This process of resetting is called entrainment, which acts to keep the clock running accurately to a 24-hour timescale. Entrainment also allows your body to become accustomed to new lighting schedules that accompany changes in seasons or geography (for example, after jet travel). In response to the change in light onset, the circadian clock shifts to the new light schedule by either advancing or delaying its phase. The clock is also not overly sensitive. The mammalian clock is most sensitive to blue light, but at reasonably high intensities and durations. A late-night snack with short-term refrigerator light is unlikely to substantially shift your circadian clock. Biological clocks have evolved balanced resetting mechanisms that allow synchronization to certain important environmental cues while ignoring others.

Although environmental changes such as light and temperature are one way to perturb a system, genetics provides another. Unlike consumer electronics, which are hardened to perturbation by precision manufacturing and engineering, our biological parts are subject to mutation and selection. Biological networks need to be robust to most of these mutations and allow sufficient genetic variation to exist in order to be acted on under selection. Here is a remarkable example: of the ~4,500 genes that have been knocked out in mice, only around 50 are heterozygous lethal. Mammals have evolved to do without 50% of the gene activity of 99% of the genome for all the pathways used between a fertilized egg and a reproductively fit adult mouse. Indeed, evidence of natural selection is especially evident in the fly clock system. Variation in two genes, *period* and *timeless*, has been shown to regulate temperature compensation and diapause, respectively[8].

Robustness
The resistance to perturbation by, for example, genetic or environmental factors.

Poikilotherms
Organisms that do not regulate their body temperatures.

Homeotherms
Organisms that regulate their body temperatures.

Box 2 | **Mathematical modelling**

Various mathematical models are used in the study of circadian clocks. Like Albert Einstein's famous quote, "Make everything as simple as possible, but not simpler", the value of a model can be measured by its simplicity of representation and its capability for explanation and prediction. One of the simplest ways to model circadian clocks is using geometry[78]. In this geometrical modelling, a state of the circadian clock is represented as a point on a circle, whereas the angle of a point and the radius of a circle represent a phase and amplitude of circadian oscillation. This simple representation can explain phase–response curves (the phase-dependent response of the clock to an external stimulus such as light) and can predict the experimental conditions to induce the singularity behaviour (transient stopping of circadian clocks). Various timings of circadian gene expression can also be modelled in geometry[52].

Another way to model circadian clocks is using algebra. For example, temperature compensation of circadian clocks can be formulated in an algebraic equation that explains the balance of the elementary processes involved in circadian oscillations[79]. There are two non-exclusive interpretations of this equation, the 'balance model' and the 'robust model', which are explained in the main text and shown in FIG. 3.

The analytical approach is a third way to mathematically model clocks. Many mathematical models of this type have been proposed that are based on ordinary differential equations. They range from simple abstractive models, which explain the basic kinetics of circadian clocks (for example, the van der Pol model[80], the Goodwin model[81], the Kuramoto model[82], and so on), to complex detailed models, which explain molecular details of living organisms (for example, the Goldbeter model[83], the Forger model[84], and so on). Recently, in order to grasp the probabilistic behaviour of circadian clocks, stochastic differential equations have also been used for the mathematical modelling of circadian clocks.

Bayesian integration
The use of the Bayesian inference — a statistical inference where experimental data is used to infer new or update prior hypotheses — to integrate large-scale, genomic data sets.

Diapause
The suspension of insect development after subjection to adverse environmental conditions.

Rhythmicity
In biological clocks, this is the property by which a molecular, cellular or physiological response recurs with regularity.

RNA interference
(RNAi). A biochemical system in cells that governs how dsRNA can interact with mRNAs to activate or inhibit their message levels or translation.

Autonomous oscillator
In biological clocks, this is a system in which cellular oscillations — rhythmic fluctuations with a definable period length — in gene transcription, reporter gene activity or physiology persist in isolated cells.

Approaches to study robustness: genetic manipulation. The clock community took a systems approach and systematically deleted clock components as single and combinatorial gene knockouts. In retrospect, these findings were remarkable — not only did they provide the genetic proof that these genes influenced mammalian behaviour (a requisite for a core clock gene in animals), they also illustrated the remarkable robustness of the circadian clock to genetic perturbation. Deletion of most clock genes in mice results in a clock with a short[11–18] or long[13,19] period length, and usually the discrepancy is less than 1 hour. Notably, in all cases, returning mice to a 12/12 hour light/dark schedule resulted in perfectly adapted mice. In other words, in laboratory conditions of constant darkness, complete loss of these genes had modest effects (1–4%) on clock function that would be difficult or impossible to see in normal lighting conditions.

These observations prompted researchers to investigate the genetic redundancy of clock components. In general, there are two or three mammalian orthologues for each clock gene conserved in flies (FIG. 1A). We note that other clock systems — *D. melanogaster*, *N. crassa* and cyanobacteria — do not have this level of clock gene redundancy. Mammals are more complex and also have clocks in many tissues rather than just one or a few. As clock gene expression is tissue specific, we speculate that orthologues may ensure that at least one member of each subfamily is present in a tissue to maintain circadian function. To test the effect of knocking out multiple orthologues, researchers generated compound knockouts and tested the rhythmicity of their locomotor activity in constant darkness. In order to see the endogenous period length of rhythmicity, laboratory conditions of constant darkness or light are necessary; in a normal lighting scheme, organisms maintain nearly precise 24-hour time keeping. Whereas cryptochrome 1 $(Cry1)^{-/-}$ and $Cry2^{-/-}$ mice generated short- and long-period-length phenotypes, respectively, $Cry1^{-/-}Cry2^{-/-}$ mice were arrhythmic[13]. Period homologue 1 $(Per1)^{-/-}$ and $Per2^{-/-}$ mice both had short-period-length phenotypes as single knockouts, but were also arrhythmic when combined[20]. Single-gene knockouts of *Clock* and neuronal PAS domain protein 2 (*Npas2*) both had short-period-length phenotypes, but again compound double-knockout animals were arrhythmic[21]. Moreover, none of these paralogous pairs of transcription factors was lethal either as single or double knockouts. As a comparator, 1 in 5 mammalian genes is lethal when both copies are knocked out (for further information, see the Mouse Genome Informatics website). Remarkably, deletion of only one clock gene, *Bmal1* (also known as *Arntl*), results in an arrhythmic clock by itself[22]. Deletion of a second gene, vasoactive intestinal peptide receptor 2 (*Vipr2*), also generates an arrhythmic mouse, but it is not considered a component of the clock as it neither interacts with clock components nor participates in the timekeeping mechanism[23]. Thus, deletion of most clock genes generates a modest phenotype, whereas only one, *Bmal1*, is required for oscillator function. The mammalian clock gene network, therefore, evolved to maintain extraordinary robustness to the loss of individual clock components.

Cellular approaches to studying robustness. In addition to regulating biology and organisms, the circadian clock is a cell-autonomous signal transduction pathway. As control of locomotor activity by the clock is definitely not cell-autonomous, cellular models provide a way to focus on the autonomous signal transduction pathway. We set out to explore this property of robustness by genetic perturbation using RNA interference (RNAi) in cells[24]. Because the clock functions autonomously as a cellular oscillator, we explored this property in a human cellular model of the clock, U2OS cells (an osteosarcoma cell line). These cells have robust oscillator function in culture, dozens to a hundred rhythmic genes, and are amenable to cell biology techniques including RNAi. Unlike behaviour, autonomous oscillator models — including dissociated fibroblasts and suprachiasmatic nucleus (SCN) neurons — indicated that cellular clock function was more fractious than the regulation of behaviour by the clock. For example, whereas $Cry1^{-/-}$ animals showed a short period length in locomotor activity, dissociated SCN neurons (and also lung, liver, cornea and fibroblasts) from these mice were arrhythmic[25]. Similar results were seen in lung and liver cells from $Clock^{-/-}$ animals[26].

We decided to test the requirement of clock factors for oscillator function in this model in dose–response — that is, sensitivity analysis in a biological system. With RNAi, by dilution, one can knock down an endogenous gene to any arbitrary level between 100% expression and somewhere near 10%. After knocking down these clock genes, we looked at the effect on circadian rhythm by using kinetic imaging to monitor a reporter gene driven by a clock gene promoter. These quantitative perturbations and phenotyping assays provided critical data for systems

A

Fly gene	Mammalian paralogues		
per	*Per1*	*Per2*	*Per3*
Clock	*Clock*	*Npas2*	–
cycle	*Bmal1*	*Bmal2*	–
cry	*Cry1*	*Cry2*	–

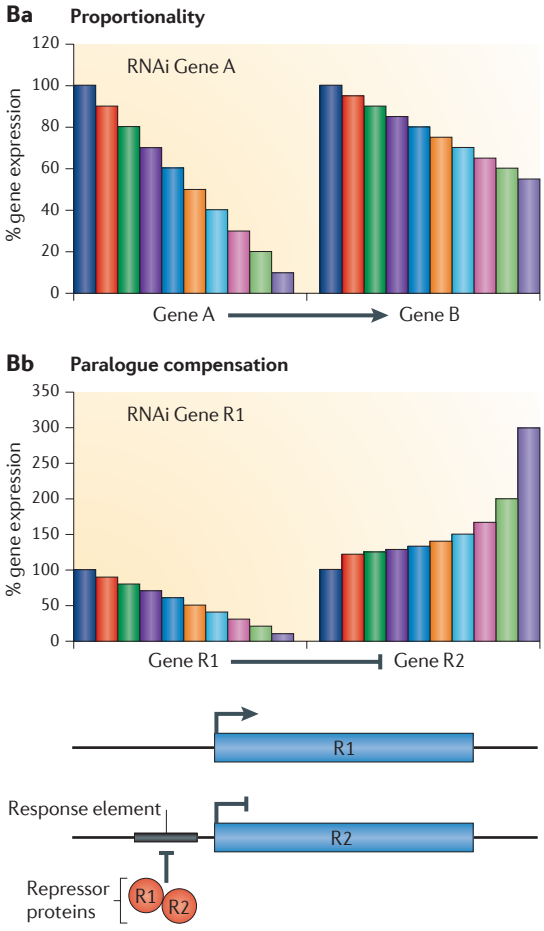

Figure 1 | **Perturbation modelling — paralogue compensation and proportionality.** Animal clock genes are conserved between flies and humans. In general, there are two or three mammalian paralogues (mouse genes shown) of each fly gene (**A**). Perturbation analysis has uncovered the principles of proportionality (**Ba**) and paralogue compensation (**Bb**). Here, proportionality is illustrated in an example in which knockdown (using RNA interference (RNAi), for example) of Gene A, a transactional activator, results in decreased levels of its target, Gene B. Paralogue compensation is shown by an example in which a transcriptional repressor, R1, is depleted by RNAi and expression of its target genes increase. In this case, one of these targets is its paralogue, R2. A mechanism that can explain paralogue compensation by repressors is shown below the graph. In this case, the repressors R1 and R2 both bind to the same response element. Knockdown of either gene, therefore, has the potential to regulate R2, which has this response element in its structural gene. R1 does not have this element, so knockdown of either repressor does not result in its induction. *Bmal1*, also known as *Arntl*; *Cry*, cryptochrome; *Npas2*, neuronal PAS domain protein 2; *Per*, period.

approaches. Similar to results seen in analysis of cells from knockout animals, knockdown of most clock components had a bigger effect on the U2OS model than behavioural rhythmicity in mice. Knockdown of *Bmal1*, *Clock*, *Cry1* and *Cry2,* and *Per1* all generated either arrhythmic cells or a clock with such low amplitude that it could not be observed. Unlike locomotor activity rhythms, analysis of kinetic luminescence imaging showed that knockout of most clock genes reduced the amplitude of oscillator function. Together, these results were consistent with previous reports which showed that cellular oscillations were not as robustly controlled as locomotor activity behaviour.

The U2OS cellular model was also advantageous for biochemical analysis; we looked at gene expression changes in clock genes after dose-dependent knockdown of clock components. We focused on conditions that generated an arrhythmic phenotype (knockdown of *Bmal1*, *Clock*, *Cry1* and *Cry2,* and *Per1*) and made two observations: most gene expression changes were linear and proportional[24] (FIG. 1Ba). A 50% knockdown of *Bmal1*, for example, resulted in a nearly 50% knockdown of its target gene nuclear receptor subfamily 1, group D, member 1 *Nr1d1* (also known as *Rev-erb alpha*). Some changes were still proportional, but nonlinear. For example, partial knockdown of the transcriptional repressors CRY1 and CRY2 resulted in a nearly tenfold induction of their target genes *Per2* and RAR-related orphan receptor G (*Rorg*). In addition to this property of proportionality, we saw several examples of paralogue compensation[24] (FIG. 1Bb). Knockdown of *Cry1* resulted in induction of its paralogue, *Cry2*. Knockdown of *Nr1d2* (also known as *Rev-erb beta*) resulted in induction of *Nr1d1*. Knockdown of *Per1* resulted in induction of both *Per2* and *Per3*. These relationships were unidirectional as knockdown of the other paralogues — for example, *Nr1d2*, *Cry2, Per2* or *Per3* — did not generate compensation. There were many other non-paralogous compensatory gene-expression changes as well.

Collectively, these results showed that nearly all perturbations generated complex changes in the clock-gene network. These properties of the network — proportionality and paralogue compensation — are mechanisms that explain the robustness of the clock. These properties are not clock specific; they have been noted in large-scale analysis of yeast gene knockouts and in meta-analysis of vertebrate knockout literature[27,28]. Although these properties have not yet been observed in other circadian models, their presence in several model organisms suggests that they will be. Whereas humans use materials and design strategies to engineer robustness, nature has even more ingenious means.

Finally, large-scale studies were recently performed to examine the response of the cellular oscillator to environmental perturbagens and synthetic small molecules[29,30]. These studies demonstrate that the vast majority of conditions and synthetic perturbations do little or nothing to cellular clock function. Future research will investigate how the clock can ignore most environmental changes to keep precise biological time. Moreover, this research will also uncover its sensitivity and specificity to appropriate environmental stimuli such as light, temperature

and activity. For example, in experiments similar to those described above, one could expose cells to various temperatures, then measure consequences for clock function in kinetic luminescence imaging and also measure transcriptional responses. The questions that such experiments could address include: how does the network rewire when the temperatures get low or high; which temperatures permit clock function and which do not; and are the same transcriptional circuits used or are there new ones?

Periodicity

A minimal model of circadian clock function — the cyanobacterial clock. The most basic property of the circadian clock is its oscillatory nature, including its frequency and amplitude. Although research in several models has identified clock genes and suggests biochemical mechanisms for oscillator function, none is as far advanced as *S. elongatus* PCC 7942, a cyanobacterial clock model[31–33]. In an astounding series of experiments, Kondo and colleagues took just three circadian clock proteins, KaiA, KaiB and KaiC, mixed them with ATP and observed circadian oscillations in KaiC phosphorylation[34]. Moreover, the system was temperature compensated (see more on this concept later) and entrained to large shifts in temperature. Therefore, this *in vitro* system constituted a circadian clock. This observation flew in the face of the dominant paradigm of the transcriptional–post-translational feedback loop (TTL). Most clock researchers (including ourselves) believed that it would be impossible to separate the transcriptional component of clock function from its biochemical mechanism. Moreover, O'Neill and Reddy[35] recently showed that human red blood cells, which lack a nucleus, still have rhythms in conserved antioxidant proteins called peroxiredoxins. These rhythms entrain to extrinsic stimuli, are temperature compensated and persist in the presence of transcriptional and translational inhibitors. In bacteria and humans, then, post-translational rhythms exist in the absence of transcription.

The development of an *in vitro* system simplified the study of the cyanobacterial clock. However, systems approaches to study this model revealed that it was far from simple.

The three Kai proteins and ATP produced robust oscillations in KaiC phosphorylation that lasted more than a week *in vitro*. Modelling coupled with experiments, a systems-biology paradigm, was used to address some of the interesting questions that came from this observation. First, how is it possible that oscillations happened at all (and that the system did not reduce to the steady state)? Second, how did the various biochemical complexes maintain synchrony with one another instead of becoming desynchronized and cancelling each other out? Models were, and continue to be, important in answering these questions by helping researchers to design, analyse and interpret experiments (BOX 2).

Biochemical studies powered these models. KaiC functions to both phosphorylate and dephosphorylate itself[36–38]. KaiA and KaiB modulate the function of KaiC and its phosphorylation status[36,39,40]. KaiA promotes the phosphorylation of KaiC at two adjacent residues near its ATP binding site[41,42]; one residue is phosphorylated, followed by the other. The phosphorylated form of KaiC has a higher affinity for KaiB, which promotes dephosphorylation in the same order: site one, then site two[39,40]. This process is termed the 'phosphoform cycle' (REF. 33) (FIG. 2A). However, despite having directionality and feedback, this structure would reach the steady state — wherein all forms of KaiC would be at similar levels of phosphorylation — without additional regulatory steps. The additional regulation is provided by a feedback mechanism in which KaiB has a higher affinity for KaiC in its phosphorylated state and inhibits the action of KaiA on KaiC[39,40]. Temporally, this cycle is maintained by having more KaiA activity at the beginning of the cycle, thus promoting the formation of KaiA–KaiC complexes and also phosphorylation of KaiC[43,44]. Later, as KaiB inhibits KaiA, the situation is reversed.

Molecular synchrony. The fact that three proteins and ATP can generate robust oscillations in phosphorylation is incredible. However, when considering populations of these complexes in a solution, not only are feedback events in individual complexes necessary, they need to act in synchrony. Otherwise, variability and noise would ensure that some complexes progressed faster or slower through the phosphoform cycle, or would be out-of-phase with other complexes, resulting in an asynchronous population. As the population cycles, individual complexes somehow maintain synchrony with one another through the phosphoform cycle. A remarkable example of this property is illustrated by an experiment from the Kondo laboratory[45], in which samples of Kai protein complexes at different initial phases were mixed. Samples in the dephosphorylation phase of KaiC remained in synchrony with the unmixed samples. However, phosphorylated KaiC samples either slowed their rate of phosphorylation or underwent dephosphorylation. Ito *et al.*[45] went on to thoroughly test the effect of initial phosphorylation status and showed that samples in the dephosphorylated state were dominant over samples in the phosphorylated state. In short, they showed that these molecular components could synchronize.

Modelling molecular synchrony. Models were put forward to explain this property of molecular synchrony. An early model, if early is just 3 years ago, was provided by Rust *et al.*[43]. In this model, KaiA is limiting — when it is inhibited later in the cycle by KaiB, it affects all KaiC molecules. In this model, KaiA would act as an allosteric activator of KaiC auto-phosphorylation, whereas KaiB would act as an allosteric inhibitor through action on KaiA. Synchrony, then, is an emergent feature of the cyanobacterial clockworks. A second idea, monomer shuttling, came from the Kondo laboratory experiment discussed above[45]. It was known that individual KaiC complexes formed hexamers, and this study showed that these KaiC hexamers exchanged monomeric subunits. They postulated that the shuttling occurred preferentially in the dephosphorylation phase of the cycle (FIG. 2B,C), a time in which these complexes are dominant. This monomer shuffling mechanism provides a

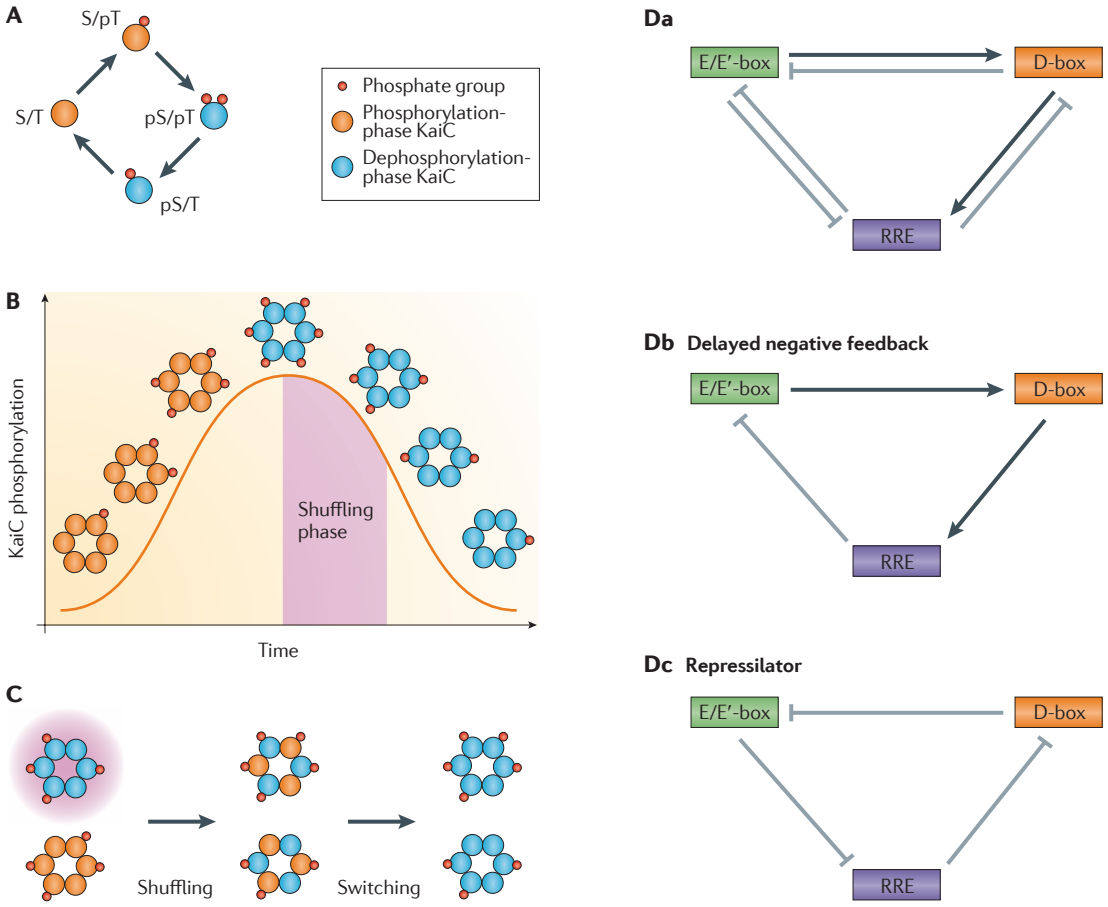

Figure 2 | **The phosphoform cycle and intermolecular synchronization in cyanobacteria circadian clocks and transcriptional network of mammalian circadian clocks. A** | A hypothetical KaiC phosphoform cycle for the cyanobacteria circadian clock, with four sequential reactions, is shown. KaiC first auto-phosphorylates T432 (shown as S/pT) and then S431 (shown as pS/pT). Double-phosphorylation (pS/pT) switches KaiC from an autokinase to an autophosphatase. KaiC auto-dephosphorylates T432 (shown as pS/T) and then S431 (S/T). The completely dephosphorylated form (S/T) switches KaiC from a phosphatase to a kinase. **B** | During the KaiC phosphorylation–dephosphorylation cycles, shuffling of KaiC monomers occurs only in the early dephosphorylation phase (pink shaded area). **C** | When a KaiC hexamer in a shuffling phase (pink shading) meets another KaiC hexamer, the monomers shuffle between the hexamers. After KaiC-monomer shuffling ('shuffling'), phosphorylated KaiC monomers are converted to dephosphorylated KaiC monomers ('switching'). **Da** | Here, a basic network structure of the transcription circuit of the mammalian circadian clock is shown, which consists of 'morning' (E/E′-box), 'daytime' (D-box) and 'night time' (RRE) transcriptional programs. **Db** | This shows one of the two oscillatory motifs, the 'delayed negative feedback' motif. **Dc** | This shows the second of the two oscillatory motifs, the 'repressilator' motif. Robust periodicity against internal noise might be conferred by internal coupling of these two oscillatory motifs in mammalian circadian clocks.

straightforward way for individual complexes to communicate their phosphorylation status with one another. These concepts are not mutually exclusive. Recently, they were combined to generate an allosteric transition and monomer shuttling (AMS) model that agrees with many experimental data[46].

What these studies show convincingly is the power of systems approaches — combining theoretical and quantitative models with experimental science. Although many of the biochemical and modelling details remain unresolved, systems approaches are providing information on mechanisms. Classical circadian principles such as synchronization and periodicity are being addressed in a more meaningful way in this model than in others. That being said, it is important to point out some of the limitations. Biological models are like politicians: they change their assumptions to fit the polls (or data). Moreover, most of these early models focus on *in vitro* dynamics and ignore the contributions from the rest of the cyanobacterial genome and entraining cues from the environment. In nature, cyanobacteria are not in constant darkness and the Kai proteins do not function in isolation with ATP.

Perhaps to address this issue, clock researchers have recently studied the cyanobacterial oscillator in the context of the cell cycle. In many species, the circadian clock 'gates' (regulates the timing of) the cell cycle. Single-cell imaging was used to study the behaviour of the circadian

clock and cell cycle simultaneously[47]. Normally, under moderate lighting conditions, cyanobacteria go through the cell cycle only once. When the amount of light (and available energy) was increased, cyanobacteria went through the cell cycle twice, mostly at defined phases of the clock. The authors speculated that the two systems, the clock and the cell cycle, work best when they resonate with one another. Why might this be? A possible answer is that de-synchrony between the two systems may be energetically inefficient. Alternatively, dividing at the wrong time may lead to an undesirably high mutation rate. Nevertheless, these results demonstrate the benefit of whole-organism systems approaches to understand complex issues such as coupling and gating.

However, minimal models, such as those used in cyanobacterial clock research, are generically useful in decoding complex systems properties. For example, Elowitz and Leibler[48] built a 'repressilator' in *Escherichia coli* to study oscillatory dynamics. This study, and the accompanying models, examined the system for principles that enable robust oscillations. These included aspects of transcription, as well as translation, and protein and mRNA decay rates[48–51]. Interestingly, a recent study revealed the existence of repressilator and 'delayed negative feedback' motifs in the transcriptional circuit of the mammalian circadian clock[52]. The basic transcription network of the mammalian circadian clock consists of 'morning' (E/E′-box), 'daytime' (D-box) and 'night time' (RRE) transcriptional programs, and forms two oscillatory motifs (FIG. 2Da). The first motif is a delayed negative feedback motif (FIG. 2Db), in which the morning transcriptional program activates the daytime, the daytime activates the night time and the night time represses the morning. The second motif is a repressilator motif (FIG. 2Dc), in which the morning transcriptional program represses the night time, the night time represses the daytime and the daytime represses the morning. Robust periodicity against internal noise might be conferred by internal coupling of these two oscillatory motifs in mammalian circadian clocks.

Temperature compensation

Changes in temperature occur on a daily basis — it is colder at night than it is during the day — and on a seasonal basis. Most organisms do not regulate their body temperature; therefore, their circadian clocks need to resist changes in temperature to keep 24-hour time. This property is called temperature compensation and it remains one of the three hallmarks of circadian clocks. Interestingly, human cells also display temperature compensation *in vitro*[53], which suggests that this property may be intrinsic to the mechanism of circadian clock function even in homeotherms. Mammals may be warm-blooded, but there are temperature gradients between the body's core and surface[54]. Temperature may also act as a universal cue in mammals to help reset peripheral oscillators[55].

The property of temperature compensation is arguably best understood in *N. crassa* (bread mould) which has a robust circadian clock that functions analogously to *D. melanogaster* and mammalian clocks[56,57]. As in mammals and flies, transcription factors function prominently in the *N. crassa* clock. Two genes, *white collar 1* (*wc-1*) and *wc-2*, act together to activate the transcription of the *frequency* (*frq*) gene (FIG. 3a). The FRQ protein and ancillary factors promote the phosphorylation of WC-1 and WC-2 to inhibit this activation and, thus, FRQ acts to repress its own transcription. To relieve this repression, FRQ is targeted for degradation by F-box/WD repeat-containing protein 1 (FWD-1), which physically interacts with FRQ to target its degradation in the proteasome. The net result of these biochemical actions is a 24-hour clock that drives rhythmic activity of the white-collar complex, the *frq* gene, the FRQ protein and its degradation, and circadian clock output.

Conceptual models of temperature compensation. As a fungus, *N. crassa* is exposed to large fluctuations in temperature, whereas the human brain varies in temperature by only a few degrees from its normal 37 °C (98.6 °F). A number of conceptual models have been put forward to explain temperature compensation. The simplest of these models considers two or more enzymatic reactions with opposing effects on clock function[58]. As the function of most enzymes speeds up with increasing temperature (within limits), reactions that 'speed up' the clock are counteracted by reactions that increase their rate to slow down the clock (FIG. 3b, upper panel). Theoretically, the more reactions that are involved, the more precise and robust compensation at this temperature can be. Initial research into the temperature compensation of *N. crassa* looked at regulation of *frq* and its product, FRQ. Levels of FRQ increase with temperature, whereas mRNA levels do not[59–61]. Two splice forms of FRQ, long and short, vary in their levels according to temperature; the long form is more prevalent at higher temperatures and the short form is more prevalent at lower temperatures[62]. These observations show the effects of temperature compensation on the *N. crassa* clock, but do not show its mechanism.

Systems analysis: genetics plus biochemistry. By incorporating classical genetics and biochemical analyses, Mehra and colleagues[63] have recently begun to take a systems approach to studying how *N. crassa* maintains constant clock function as temperatures change. They took advantage of gain-of-function genetics — the isolation of two mutant strains that were better at temperature compensation than wild-type *N. crassa*[64]. Although the circadian period length of wild-type *N. crassa* decreases as temperature increases from 18 °C to 30 °C, the *chrono* mutant displayed almost perfect compensation up to 30 °C. The second mutant, *period3*, is overcompensated with respect to wild-type *N. crassa*, as its period length increased rather than decreased when shifted from 18 °C to 25 °C. Cloning these strains revealed that the mutations are in two subunits of casein kinase 2 (CK-2), which has previously been implicated in clock function in other model systems. Reducing gene dosage of these components in wild-type strains produced phenotypes that were similar to the original mutants. Importantly, changing the dosage of several other kinases and phosphatases that have been implicated in clock function did not alter temperature compensation, so it is not just any phosphorylation event that can induce the effect.

Repressilator
This is a synthetic network that generates stable oscillations in GFP.

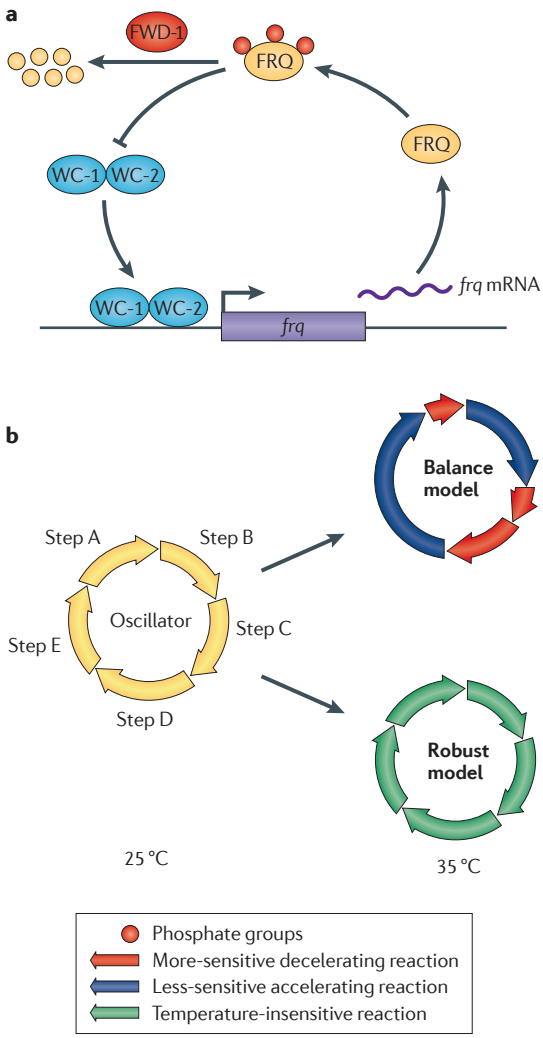

Figure 3 | **The *Neurospora crassa* circadian clock and two non-exclusive models for temperature compensation. a** | A hypothetical transcriptional circuit in *Neurospora crassa* circadian clocks. Two proteins, white collar 1 (WC-1) and WC-2, act together to activate transcription of the *frequency* (*frq*) gene. The FRQ protein and ancillary factors promote the phosphorylation of WC-1 and WC-2 to inhibit this activation (inhibitory arrow). Therefore, FRQ acts to repress its own transcription. To relieve this repression, FRQ is targeted for degradation by F-box/WD repeat-containing protein 1 (FWD-1), which physically interacts with FRQ to target its degradation (yellow fragments) in the proteasome. **b** | Two of the models that have been proposed to explain temperature compensation in circadian clocks are the 'balance' model and the 'robust' (or insensitive) model. The figure shows a hypothetical circadian oscillator at 25 °C and 35 °C. The balance model (upper right panel) proposes that there is a balance between more temperature-sensitive period-decelerating enzymatic reactions (blue) and less temperature-sensitive period-accelerating enzyme reactions (red). The robust model (right lower panel) proposes that a circadian oscillator consists of temperature-insensitive enzymatic reactions (green). These two models are not mutually exclusive with each other, so a hybrid model may be feasible.

To get at the biochemical mechanism, Mehra *et al.* investigated putative CK-2 phosphorylation sites on FRQ. They showed that, as well as phosphorylating FRQ *in vitro*, CK-2 interacts with, and phosporylates, FRQ *in vivo*. This phosphorylation was abrogated in strains that bore mutations in CK-2 components. Moreover, this phosphorylation was temperature-dependent and accompanied by changes in FRQ levels. In CK-2 mutants, the half-life of FRQ increased with temperature, becoming twice as stable compared with wild-type *N. crassa*. To establish the link between FRQ phosphorylation by CK-2 and temperature compensation, they constructed CK-2-phosphorylation-site mutants by inserting clusters of mutated sites into the endogenous *frq* locus. One of the two tested regions of mutated sites gave a similar phenotype to a CK-2 temperature compensation mutant and had a longer period length that did not change in a temperature range of 22–31 °C. *In vitro* analysis showed that CK-2 directly phosphorylated this region of FRQ, and that this phosphorylation event resulted in a more stable FRQ. These findings are consistent with the 'balance model' of temperature compensation postulated more than 50 years ago. As temperature rises, FRQ becomes more stable, but this is counteracted by increased CK-2 activity. Mutations in either protein can disrupt this relationship and lead to overcompensation.

Although it is possible, even likely, that additional proteins will also have a role in temperature compensation, these data provide convincing evidence that systems approaches can begin to reveal its mechanisms. Indeed, component identification is just the beginning of systems approaches. Perturbation, synthetic biology and mathematical modelling approaches in *N. crassa* may yet reveal regulatory and other mechanisms that contribute to robust temperature compensation.

Alternative models. Although the observations in *N. crassa* are consistent with the 'balance model', other observations from cyanobacteria[34,36,65] and mammals[66] are consistent with another model that explains temperature compensation: the 'robust model' (FIG. 3b, lower panel). In the robust model, all rate-limiting enzymatic reactions in the clock are temperature-insensitive. In fact, in the cyanobacterial clock, rate-limiting reactions such as phosphorylation, dephosphorylation and ATPase activities are all temperature-compensated[34,36,65]. In the mammalian clock, casein kinase 1 isoform-ε (CSNK1ε)-dependent and CSNK1δ-dependent phosphorylation of PER2 is temperature-compensated *in vitro* and *in cellulo*[66], but the temperature sensitivity of other rate-limiting reactions in the mammalian clock remains unknown. These two models may not be exclusive. For example, we note that CK-2-dependent activity is dispensable for temperature compensation of the *N. crassa* clock, as CK-2 hypomorphic mutants exhibited near-perfect temperature compensation[63].

Conclusions and future directions

When you think about it, trying to understand any dynamic process, such as circadian rhythmicity, forces you to take a systems approach. So, it is not too surprising

that when the appropriate tools were available, clock researchers used them in their studies. What lessons were learned? In tackling genetic robustness, the community took a systematic approach to component discovery and validation. Clock researchers knocked out all components and later used the concept of the dose–response to conduct sensitivity analysis on the circadian clock system. From the systematic study came the essential data on what broke or modified clock function, and from this came opportunities to see the rules that governed the network. In trying to understand the basic property of rhythmicity, cyanobacterial researchers defined a minimal system — three proteins and ATP — to study rhythms. Although a leap of faith was required to even start this research, studying a biological process in the simplest system possible is a central tenet of science. The Kondo laboratory and the cyanobacterial field have been reaping the rewards — conceptual and quantitative models of the circadian clock — ever since. And in understanding temperature compensation, *N. crassa* researchers went back to the basics: careful genetics and biochemistry, and the interplay between the two disciplines.

So, in a sense, success in taking systems approaches to understand complex biology starts with the desire to tackle the biological questions. What is clear is that after 15 years of (absolutely necessary) component discovery, the field is moving back to its roots — the understanding of the complex properties that underlie biological rhythms. Many, maybe the majority, of the most important questions remain unanswered. What governs period length? What are the molecular and systems details underlying entrainment? In multicellular organisms, what part does cell-to-cell communication play in rhythm generation and oscillator function? What are the transcriptional and post-transcriptional cascades that govern circadian output? Are the solutions used in one model system also used in others? Finally, how do we take this knowledge and translate it into therapies for people suffering from circadian sleep disorders, metabolic syndrome, cancer and other pathological conditions influenced by our biological clock?

To begin answering these questions, future research and new tools are needed. Kinetic imaging has been a boon to the field in providing high-resolution time-course data on oscillator function at both population and single-cell levels. At this point, single-cell imaging is slow and has a low throughput. It is also confined to *in vitro* conditions; it is not yet possible to image single cells in live organisms. New mathematical and computational models are also needed. Although optimal differential equation models exist that describe the biochemical events that are thought to comprise the clock, they are disconnected from network models that describe what happens when a clock gene is missing or loses function. Many of these mechanistic models are powered by sparse biochemical data. In many cases, this is because tools to measure things like cytoplasmic-to-nuclear translocation rates do not exist yet or are difficult to use at scale. New tools are needed that extend our knowledge of post-translational modifications and their dynamics.

How can these strategies and techniques be incorporated into other systems? Here is the good news: the problems of biological complexity and the approaches to handle them are generic. For example, yeast researchers have developed knockout panels of most genes in the yeast genome[67]; they have used these panels to study how *Saccharomyces cerevisiae* responds to environmental perturbation such as changing carbon source or oxidative and chemical stress[68,69]. Computational researchers are using these data to define networks and their properties that govern responses to these perturbations (for example, REFS 70–72). Recently, Forger[73] derived a useful mathematical formulation for genetic oscillators with delayed feedback repression. This mathematical formulation can be applied to the other biological oscillations of different timescale. In addition, Millar and colleagues developed the repository of models for circadian clocks (see the Circadian Modelling website). This model repository can easily be expanded to other biological models than circadian clocks; virtually all areas of biology have dynamic components and would benefit from many of the above concepts and tools. The research community's battle with complexity has just begun.

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Competing interests statement
The authors declare no competing financial interests.

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Circadian Modelling: http://millar.bio.ed.ac.uk/PEBrown/CircadianModelling/CircadianModelling.htm
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