Discovering circadian clocks in microbes

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DOI:
10.33612/diss.170828314

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PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Monday 21 June 2021 at 11.00 hours

by

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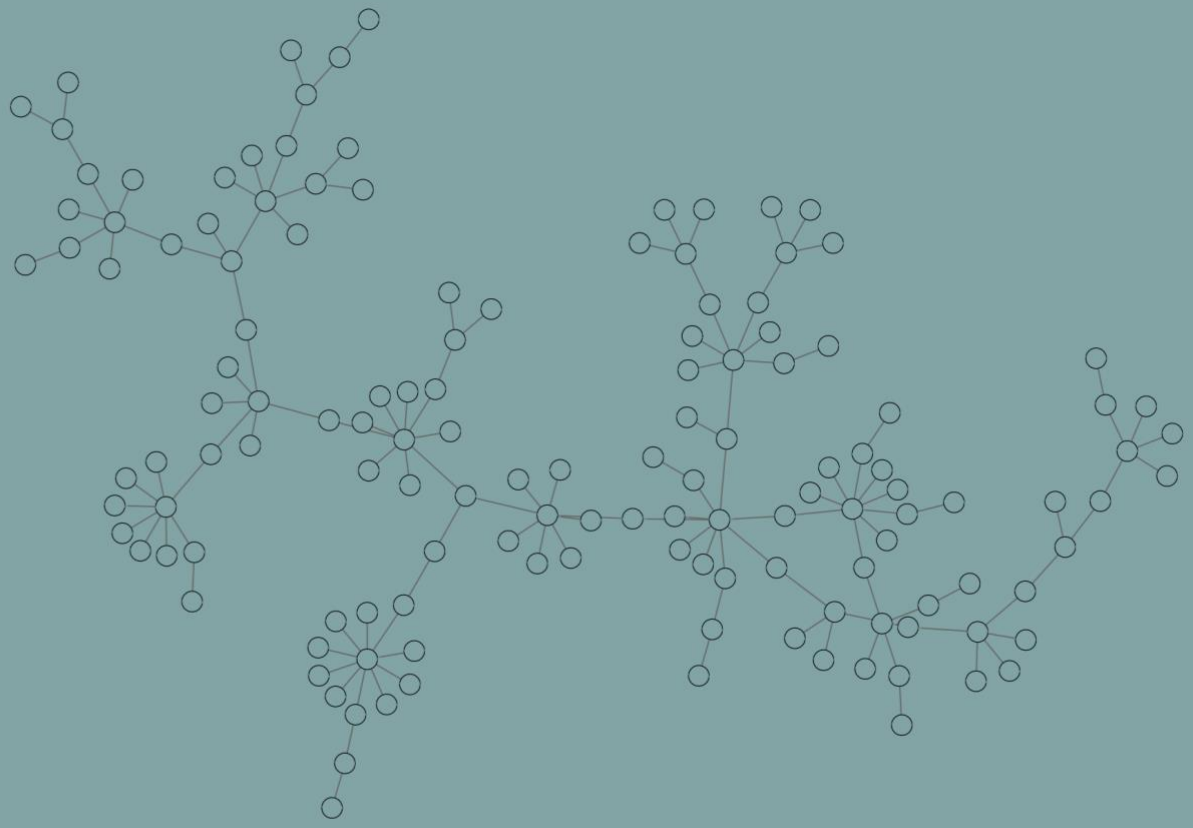
Table of Contents



Chapter 1

Introduction to this thesis

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Circadian biology

For millions of years the rotation of the earth on its axis has created the day and night-cycle of this planet. As a result of this, earth's environmental conditions such as light, temperature and humidity change predictably with the course of day with a 24-hour period, as well as with time of year. For life to flourish many organisms developed mechanisms to exploit these rhythmically changing conditions. These are known as circadian clocks, biological programs that provide a temporal structure to organisms giving them the ability to anticipate predictable environmental changes. Much can be gained having such a mechanism, regulating aspects of molecular biology, physiology and social interactions. For this reason, circadian clocks (from Latin "circa diem", about a day) are found in a multitude of organisms of all phyla[1] .

One of the earliest observations (1729) of a circadian clock at work was done by the French geophysicist, astronomer and, chronobiologist Jean-Jacques d'Ortous de Mairan[2] . He was intrigued by the daily opening and closing of the heliotrope plant and basically performed the first circadian experiment, removal of the external environment timing cue, the sun, by placing the plants in continuous darkness. Surprisingly, the self-sustained rhythmically opening and closing of the leaves persisted with a proximately a period of 24 hours. As time passed, chronobiological (from Latin "chronos" time, "bios" life, "logos" study) research continued. In the 1960s Pittendrigh suggested that deviations from the endogenous time-keeping system, as we experience during a jetlag, can be continuously aligned with the external cyclic environment. Making the system of endogenous timekeeping, entrainable by light and temperature, more robust and precise in controlling the timing/phase of the expressed rhythms. Clock systems of many organisms have been described; *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Neurospora crassa* even unicellular organisms such as *Synechococcus elongatus* and *Gonyaulax polyedra*[3,4] However, one of the most important and studied eukaryotic genetic model organisms, *Saccharomyces cerevisiae* is missing from this clock repertoire. Also, with respect to evolution and the conservation of clock systems across phyla, we expect that a similar mechanism is developed in bacteria. This thesis focusses on the circadian clock mechanisms of *S. cerevisiae* and the light sensitive bacterium *Bacillus subtilis* by exposing the cell to the two most powerful zeitgebers (German for "time givers"), temperature[5,6] and light[7–9].

Clock properties

By analysing and comparing the behavioural aspects of organisms, properties of their endogenous clock mechanisms can be identified. Common features and properties of these circadian clock systems are in general terms described[10–12]and include the following:

- A *rhythm*
  The biological oscillation should have a quantifiable amplitude range and should be robust enough to drive behaviour.
- A *period* (*tau*, τ) or *circadian range* of approximately 24 hours.
  The endogenous cycle oscillates with a duration/length of about 24 hours in the absence of an external zeitgeber.
- *Entrainable*
  The clock mechanism is able to align/ synchronize the endogenous timing with zeitgebers/ external cues i.e., light and temperature.
- *Self-sustained*
  Even after removal of the external temporal structure (constant conditions), the endogenous cycle continues with approximately a period of 24 hours. This is called the free-running period.
- *Temperature compensated*
  The period of the endogenous cycle is roughly unaltered by differences in the external temperature over a physiological range[5].

Clock mechanisms

The structure of molecular circadian clocks are complicated biochemical mechanisms, providing a temporal structure to the biological processes and gene regulation. The complexity increases since some components of the molecular circadian clock components slightly differ between animals, plants, fungi, cyanobacteria, while others are conserved. A basic, conceptual model of the circadian clock has been proposed by Eskin[13]. The model described a system for essentially any circadian clock. It consists of three main components. i. input pathway, ii. rhythm generator and iii. output pathway. A zeitgeber signals and activates the input pathway, signal-cascades are turned on and synchronize the rhythm generator. The rhythm generator regulates molecular functions (output pathway) and influences organism behaviour and physiological status. A second model, the Transcription-Transcript-Oscillator (TTO) is hypothesized in which transcriptional negative feedback loops of clock genes regulate their own expression[14]. The clock genes FREQUENCY and WHITE-COLLAR-COMPLEX-1 and -2 in *Neurospora crassa* show this behaviour[15]. A comprehensive model by Merrow and Roenneberg[12] shows that a freerunning period of 24 hours can be achieved by interlocking

several TTOs. This model also mimics all the clock properties mentioned above. Interestingly, self-sustained circadian cycles of KaiC phosphorylation have been observed *in vitro*[16]. To gain more insights into this phenomenon, a third model had been proposed named the "phoscillator"[16,8]. This model shows *in vitro* self-sustained temperature compensated circadian cycles of KaiC phosphorylation by incubating KaiC with KaiA, KaiB, and adenosine triphosphate[17]. The Kai-proteins are considered the core-clock protein of *Cyanobacteria*[18]. However, the phosphorylation cycles do not account for the dynamic properties of circadian clocks. For this yet another layer of regulation needs to be added, interconnecting with the phoscillator, such as a Transcription-Transcript feedback loop. However, this remains a poorly understood concept. These molecular properties together with cellular processes such as transport, post-transcription modifications, metabolic status, DNA-methylation and environmental conditions should all be taking into account to unravel new clock mechanism in "new" organisms.

Interaction between zeitgebers and the transcriptional-translational oscillator

Zeitgebers, e.g. light, temperature and food, synchronise the internal clock network with external time. They can reset the clock and also affect the rhythmic amplitude of clock outputs[19]. Signal transduction from external Zeitgebers/ timing cues are often mediated via extracellular signalling receptors followed by a signalling cascade influencing gene expression. Two examples *N.crassa* and mice:

The circadian clock in *N.crassa* is based on a transcription and translation loop (TTL). This TTL senses blue light. Basically, two feedback loops compose the system[20]. First, blue light is sensed by blue-light receptor WHITECOLLAR-1 (WC-1) and together with WC-2 forms the WHITECOLLAR complex (WCC), which in its turn regulates FREQUENCY (frq) transcription. FRQ complexes with frq-interacting helicase (FRH), regulating WC-1 transcription and inhibiting the WCC. The second loop involves VIVID (vvd), a flavoprotein serving as a blue light photoreceptor. VVD inhibits the WCC, while the WCC regulates vvd gene expression. WCC formed in the early morning induces frq gene expression leading to a higher concentration WCC-FRQ-FRH complex at darkness the complex gets phosphorylated by CK1-2, inactivating it and frq-mRNA expression is diminished. VVD gates the light input to the system[21].

In mice, light acts as a zeitgeber via the retina (melanopsin acts as a blue light photoreceptor) which conveys a glutamate-based signal to the Suprachiasmatic Nucleus (SCN)[22]. Within the SCN, the circadian clock is based on transcriptional/ translational feedback loop (TTL). The core TTL includes Circadian Locomotor Output Cycles Kaput (CLOCK), Brain and Muscle

ARNT-like protein 1 (BMAL1), Period (PER1-3) and Cryptochrome (Cry1/2). During daytime, the transcription factors CLOCK and BMAL1 activate the expression of the PERIOD- and Cry-genes resulting in the accumulation of PER and CRY proteins in the cytoplasm. At dusk, these proteins migrate to the nucleus where they inhibit CLOCK/BMAL1-mediated transcription and repress production of Per and Cry mRNAs. During the night, toward dawn PER/CRY complexes are slowly degraded causing the release of CLOCK/BMAL1 dimer from PER/CRY suppression, leading to reinitiating of the clock cycle and production of PER and CRY proteins[23]. High glutamate concentrations repress the PER2 expression, influencing the circadian clock mechanism which in its turn changes SCN neural activity (firing rate) high during the day and lower during night[24].

Biology of budding yeast Saccharomyces cerevisiae

Given the pervasiveness of circadian clocks throughout nature, we wished to explore yeast for clock properties. The budding yeast *S. cerevisiae* is classified in the fungal phylum *Ascomycota* and can be found in nature primarily on ripe fruits but also on oak bark, where it is subject to seasonal and daily cycles of tree sap flow[25,26]. This broad range of natural habitats, in which it can encounter i.e., nutrient depletion, temperature shifts, light cycles and osmotic shock, requires flexible survival strategies. Cells can adapt to their changing environment by reprogramming the cell, activating and repressing specific metabolic pathways and processes. Reprogramming the cell takes a considerable amount of time and resources, hence the lag phase in growth curves[27] during which cells function suboptimal. Yeast is able to utilise different carbon sources, such as glucose, glycerol and maltose, using three different cellular respiration systems. i. Aerobic respiration, in the presence of oxygen glucose is converted into ATP and CO_2. This occurs in the mitochondria using the metabolic pathway: glycolysis, TCA cycle and oxidative phosphorylation. ii. Anaerobic respiration (fermentation), in the absence of oxygen glucose is converted in ethanol and CO_2. This occurs in the cytoplasm using only glycolysis producing less energy. Yeast can survive in a range of oxygen concentrations and the switch from respiration to fermentation is not only mediated by oxygen levels, but primarily by glucose concentration[28]. Strikingly, fermentation is the favoured mechanism for energy production even in the presence of oxygen. Upon glucose limitation, the cells switch from glucose fermentation to aerobic respiration (iii. ethanol respiration), utilizing ethanol in the medium (diauxic shift) as a carbon source[29]. This metabolic switch between glucose and ethanol is enabled by the Ethanol Regulated Transcription factor 1 (ERT1)[30]. ERT1 is one of the four per-ARNT-sim (PAS) domain containing proteins in *S. cerevisiae* (RDS2, PSK1, ERT1, PSK2). PAS-domains (PAS), sensory modules that are found

in all kingdoms of life[31], are commonly found in proteins at the heart of circadian clocks in different organisms i.e. plants, molds, fruit fly, mice, and humans[32].

Yeast cells are either haploid or diploid and both forms reproduce by the means of mitosis (Fig. 1). However, under stress haploid cells tend to die, while diploid cells enter meiosis and producing four haploid spores (asexual reproduction). The process of meiosis in yeast is called budding. After each division cycle, a bud-scar remains on the mother cell, limiting the replicative lifespan to about 26 cell divisions. In optimal conditions, depending on the strain and environment, a yeast culture can double itself every 100 minutes. Sexual reproduction occurs between two haploid yeast cells of opposite mating type (a and α) to form diploid cells. This mating is activated through the mating pathway and employs a G protein-coupled receptor, G protein, RGS protein, and MAPK signalling cascade. Wild haploid cells are capable to switch their mating type while most laboratory strains cannot be due to the deletion of the HO gene.

Yeast have been used for centuries for food production such as wine, beer and bread and it may be the most studied model organism, thus creating a rich molecular toolbox. Unravelling and harnessing the power of the circadian clock of *S. cerevisiae* can contribute to process optimization in, for instance, the food industry and other biotechnological applications.

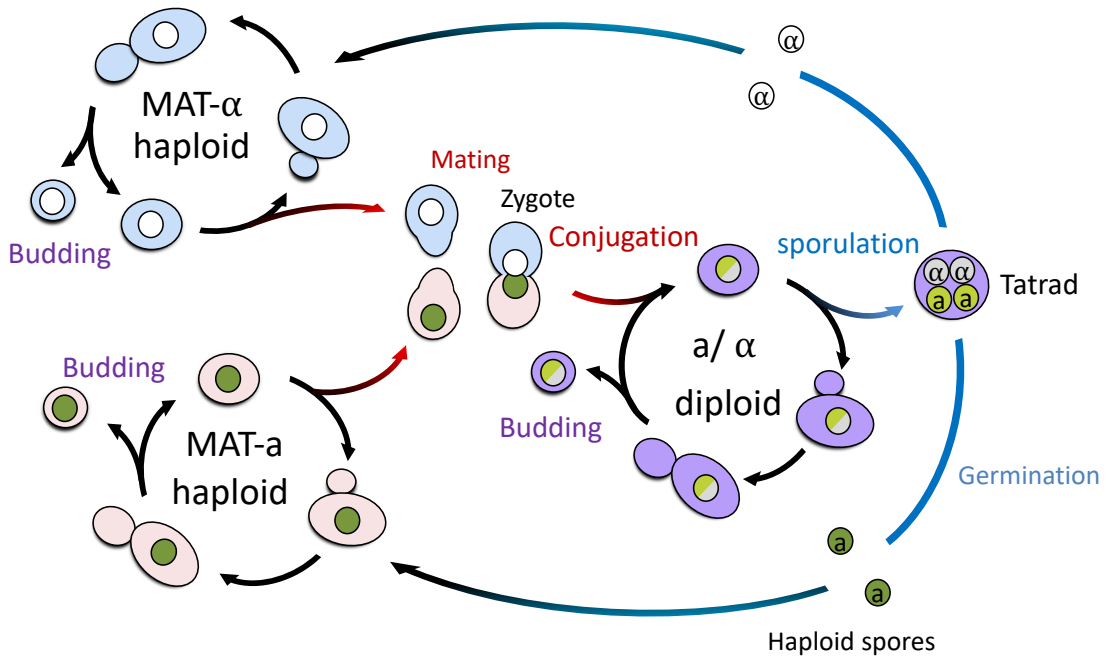


Figure 1. Life cycle of yeast. Yeasts can reproduce via asexual budding during which a mother cell forms a bud (daughter cell) which is genetically identical. Alternatively, they may procreate via mating a?n conjugation, forming diploid cells or tatrads following the typical Mendel's low of segregation.

Biology of Bacillus subtilis

The ground dwelling, non-photosynthetic, Gram-positive bacterium *Bacillus subtilis* is classified in the phylum *Firmicutes* and can found in soil and the gastrointestinal tract of ruminants and humans. As for yeast, its natural habitats suggest exposure to daily cycles of changing conditions. *B.subtilis* is a motile prokaryote and has a two life-cyles, resulting in different patterns of cell division (Fig. 2). The vegetative cycle and sporulation is followed by germination cycle[33]. Using these two strategies, *B.subtilis* is able to form biofilms on top of an extracellular matrix[34]. Biofilm formation and sporulation can be influenced by exposure to visible light, this due to cross talk between σB and σF pathways[35,36]. Activation of the σB pathway results in a general stress response (GSR) mediated by the blue-light receptor YtvA[37] and red-light receptor RsbP[35]. YtvA acts as a positive regulator of the stress response signaling pathway[35]. Based on this observation, we hypothesized that *B.subtilis* should also have evolved strategies to cope with the daily fluctuations in light exposure and temperature cycles, caused by our sun. YtvA contains a N-terminal Light-Oxygen-Voltage (LOV) domain. LOV domains are found in blue-light receptors, oxygen-sensors and voltage-gated potassium channel proteins and are a subfamily of the Per-ARNT-Sim (PAS) superfamily[38], RsbP also contains a PAS domain. *B.subtilis* has 16 proteins containing a PAS domain[39] which play an

important role as a sensory system for redox potential, oxygen tension, and light. Also, ytvA, shows significant homology with *N.crassa* vvd and wc-1, both of which encode PAS domains and are identified as clock genes[40].

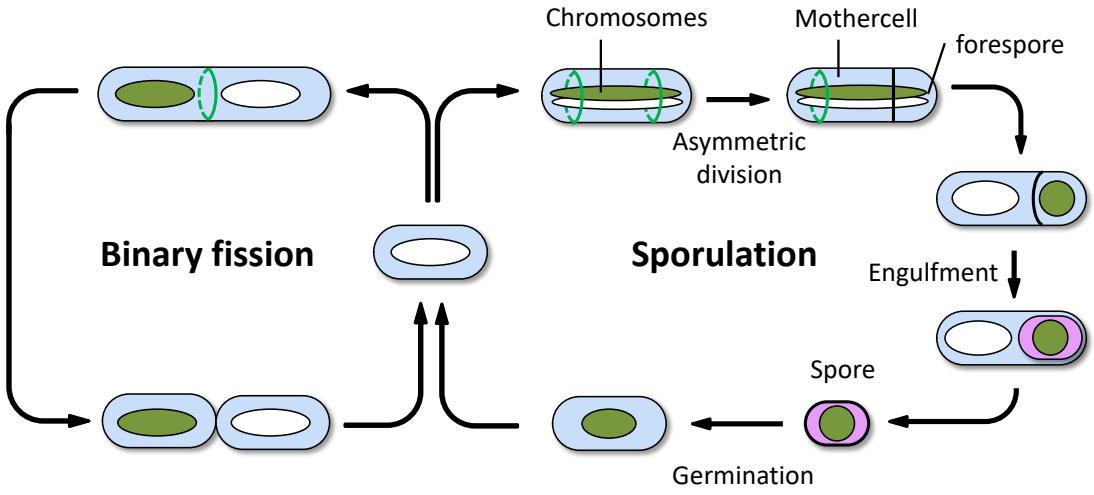


Figure 2. Life cycle of B. subtilis. B. subtilis has two alternative life cycles: binary fission and sporulation. During favorable conditions replication is mediated by binary fission, during which genetic material is replicated (green and white ellipses) and divided along the poles of the cell after which the cell is divided in half and two cells are formed. When nutritional resources are limited, B. subtilis can form highly resistant spores (pink ellipse). After engulfment a protective protein barrier is formed. When conditions improve, the spore germinates and B. subtilis re-enters the vegetative life cycle.

Prospect of this thesis

Although circadian clocks have been identified in organisms in different kingdoms and phyla, two important and powerful microbial model organisms, commonly are missing. In this thesis I've applied a bio-informatics approach to predict molecular clock structures and components. The method, PREMONition, uses publicly available data and a micro-array dataset (in this work), where yeast chemostat cultures were systematically entrained with temperature cycles. In addition, an extensive yeast knock-out broad-spectrum light sensitivity growth assay is performed in order to identify genes that causes a growth increase or decrease when exposed to light specifically in 24h structures. In the second half of this thesis work, we subjected *B. subtilis* to wide variety of circadian entrainment conditions, using temperature, light and culture medium variations in order to identify clock components in this non-photosynthetic Eubacterium.

In **Chapter 2** fermentor cultures of *S. cerevisiae* were subjected to temperature cycles with a period of 24 h, to generate a rhythmic environment while monitoring the pH and dissolved oxygen (dO2) in the culture. During entrainment, we observed systematic synchronization to environmental cycles. The results show that metabolism in yeast shows circadian entrainment, responding to cycle length and zeitgeber strength, and a free running rhythm. Sampling the cultures during the freerun and analyzing the mRNA concentrations of MEP2 and GAP1 using the pH oscillation readout as a reference we show a circadian cycle in the gene-expression of these two genes. The molecular mechanism behind the observed pH oscillations may thus involve the regulation of internal pH by plasma membrane H+-ATPase, PMA1p.

In **chapter 3** I've developed a method, PREMONition[41], PREdicting MOlecular Networks, that uses well annotated functional relationships to predict a fully connected Protein-Protein-Interaction (PPI) network to predict molecular circadian clock associations. After tuning PREMONition on the networks derived for human, fly and fungal circadian clocks, I deployed the approach to predict a molecular clock network for *Saccharomyces cerevisiae*, for which there are no readily identifiable clock gene homologs.

I**n Chapter 4** PREMONition is applied to reconstruct circadian PPI 118 of organisms annotated in the Circadian Gene DataBase (CGDB)[42]. The interactions networks are mined for significantly enriched biological properties in order to find Gene Ontology categories (Function, Process, Location), SMART proteins domains and KEGG biochemical pathways

characterizing circadian mechanism. The identified biological properties are validated using *M.musculus*, in which I was able to identify the core clock genes Per1, Per3, Arntl, Arntl2, Clock and Npas2. Next, this method is applied on *S. cerevisiae* and identified the serine/threonine protein-kinase SAK1 as potential regulatory unit of the circadian mechanism in yeast. The Gene Ontology enrichment analysis (biological process) of the 118 PPIs are subjected to GOGO semantic similarity score calculation and used for agglomerative clustering with MultiDimensional scaling. This approach allows us to visualize the distribution of circadian PPIs from organisms originating in different phyla and based on the formed clusters, to identify process-based common PPI networks amongst organisms.

In **Chapter 5** yeast cultures are exposed to the powerful zeitgeber: light. The hypothesis is that a day-night (i.e., light-dark) cycle might also be a crucial factor in controlling the circadian rhythm in yeast. Therefore, in this work I investigate the growth of yeast under light and dark conditions and the effect of specific gene knockouts on its growth. Dilutions series of yeast

KO-strains were subjected to broad-spectrum light, imaged and analyzed for colony/cell density. A linear model is applied to identity the colonies showing the highest growth repression of increase relative to the wild-type strain CEN-PK2. Using this approach, a serine-threonine protein kinase SNF1 is identified. Also, SCH9, IME2, PHO85, KSP1, IRE1 and RIM15 show a strong relationship between light sensitivity and growth.

Chapter 6 is a literature study regarding the existence of circadian rhythms in the Eubacteria. The presence of a circadian mechanisms in these bacteria, playing a crucial role in agricultural and industrial processes, could be a great importance. Clock dysfunction has been associated with disease states, and particular microbial populations may be involved in maintaining health or transition to disease status. Our survey of the literature suggests that non-photosynthetic prokaryotes are capable of generating rhythmic gene expression and we propose *Bacillus subtilis* as a potential model to investigate circadian mechanisms in non-photosynthetic bacteria.

In **Chapter 7** we subjected floating biofilm-forming *Bacillus subtilis* cultures to different ranges of 24-hour temperature cycles as well as light/ dark cycles. We hypothesized that this Eubacterium would entrain to its environment using light and/or temperature signals like all other circadian systems. Sixteen PAS-domain-encoding genes have been annotated in the *B. subtilis* genome. We selected YtvA and kinC and constructed bioluminescent reporter strains from them. The promoter YtvA encodes a blue light photoreceptor with a PAS domain

accompanied by a PAC domain. KinC is a histidine kinase involved in the regulation of differentiation processes, including biofilm development and sporulation. We found bacterial biofilm populations have a free-running rhythm of ytvA and KinC activity close to 24h upon release to constant dark and temperature conditions in B. subtilis. The free-running oscillations are temperature-compensated and have phase-specific characteristics of entrainment. These are key features for a circadian clock mechanism, making it highly probable for such a system to exist in *B. subtilis*.

In **Chapter 8** I reflect on the results obtained and hypothesize an integrated circadian clock mechanism in unicellular microbes with an emphasis on *S.cerevisiae*. This mechanism involves several metabolic pathways, and its key regulator is the stress-responsive transcriptional activator Msn2p. Msn2p is regulated by Snf1p (Sucrose NonFermenting), Tor1p (Target Of Rapamycin (TOR) pathway) and Hog1p (High Osmolarity Glycerol (HOG) response), candidates from our studies, and Msn2p itself regulates the Peroxiredoxin PRX1. Peroxiredoxins are highly conserved and show circadian oscillations in their oxidation state in

cells from humans, mice and marine algae[43]. Also, this mechanism allows glucose concentration feedback via the TOR pathway and via protein kinase A. The later are associated in the *Neurospora crassa* clock[44]. Besides protein kinase A, serine/threonine protein-kinases also play an important role, are highly conserved and critical components in known clock mechanisms[45].

Literature references

1. Gerhart-Hines, Z. & Lazar, M. A. Circadian metabolism in the light of evolution. *Endocrine Reviews* (2015) doi:10.1210/er.2015-1007.
2. Smoller, L. A. *et al.* Dortous de Mairan, Jean-Jacques. in *The Biographical Encyclopedia of Astronomers* (2007). doi:10.1007/978-0-387-30400-7_5160.
3. Bunning, E. Opening Address: Biological Clocks. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/sqb.1960.025.01.003.
4. Bell-Pedersen, D. *et al.* Circadian rhythms from multiple oscillators: Lessons from diverse organisms. *Nature Reviews Genetics* (2005) doi:10.1038/nrg1633.
5. Pittendrigh, C. S. On Temperature Independence in the Clock System Controlling Emergence Time in Drosophila. *Proc. Natl. Acad. Sci.* (1954) doi:10.1073/pnas.40.10.1018.
6. Merrow, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurospora clock gene frequency. *Nature* (1999) doi:10.1038/21190.
7. Pohl, H. Proportional effect of light on entrained circadian rhythms of birds and mammals. *J. Comp. Physiol. A* (1976) doi:10.1007/BF00612678.
8. Merrow, M., Mazzotta, G., Chen, Z. & Roenneberg, T. The right place at the right time: Regulation of daily timing by phosphorylation. *Genes and Development* (2006) doi:10.1101/gad.1479706.
9. Roenneberg, T. & Merrow, M. Entrainment of the human circadian clock. in *Cold Spring Harbor Symposia on Quantitative Biology* (2007). doi:10.1101/sqb.2007.72.043.
10. Gwinner E. *Adaptive Significance of Circannual Rhythms. In: Circannual Rhythms*. (1986). doi:https://doi.org/10.1007/978-3-642-82870-6_6.
11. PITTENDRIGH, C. S. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/SQB.1960.025.01.015.
12. Roertneberg, T. & Merrow, M. Molecular Circadian Oscillators: An Alternative Hypothesis. *J. Biol. Rhythms* (1998) doi:10.1177/074873098129000011.
13. A, E. Identification and physiology of circadian pacemakers. Introduction. *Fed Proc* 38, 2570–2572 (1979).
14. Lakin-Thomas, P. L. & Brody, S. Circadian rhythms in Neurospora crassa: Lipid deficiencies restore robust rhythmicity to null frequency and white-collar mutants. *Proc. Natl. Acad. Sci. U. S. A.* (2000) doi:10.1073/pnas.97.1.256.
15. Loros, J. J. & Dunlap, J. C. Genetic and molecular analysis of circadian rhythms in

Neurospora. *Annual Review of Physiology* (2001) doi:10.1146/annurev.physiol.63.1.757.

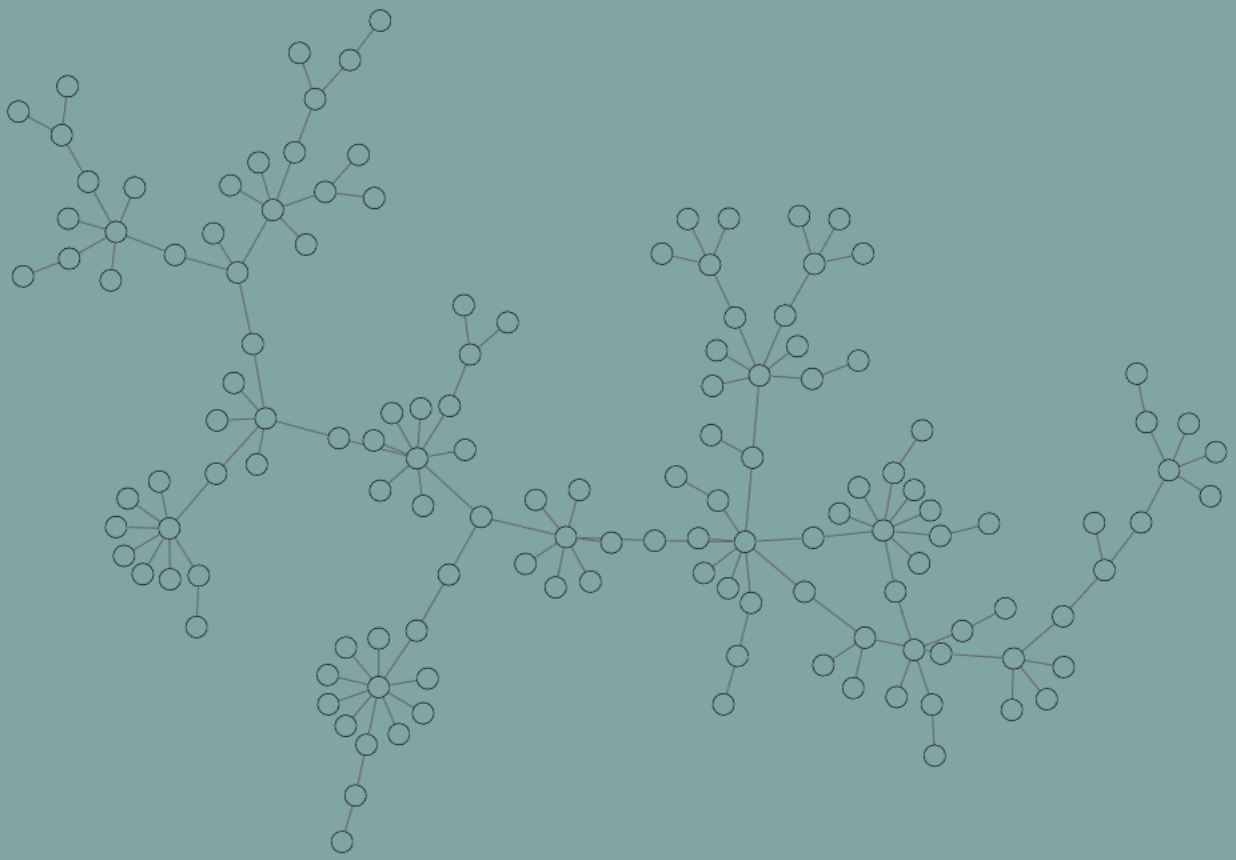
16. Nakajima, M. *et al.* Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science (80-.).* (2005) doi:10.1126/science.1108451.
17. Nakajima, M., Ito, H. & Kondo, T. In vitro regulation of circadian phosphorylation rhythm of cyanobacterial clock protein KaiC by KaiA and KaiB. *FEBS Lett.* (2010) doi:10.1016/j.febslet.2010.01.016.
18. Swan, J. A., Golden, S. S., LiWang, A. & Partch, C. L. Structure, function, and mechanism of the core circadian clock in cyanobacteria. *Journal of Biological Chemistry* (2018) doi:10.1074/jbc.TM117.001433.
19. Saini, R., Jaskolski, M. & Davis, S. J. Circadian oscillator proteins across the kingdoms of life: Structural aspects. *BMC Biology* vol. 17 (2019).
20. Brunner, M. & Káldi, K. Interlocked feedback loops of the circadian clock of Neurospora crassa. *Molecular Microbiology* vol. 68 (2008).
21. Hong, C. I., Ruoff, P., Loros, J. J. & Dunlap, J. C. Closing the circadian negative feedback loop: FRQ-dependent clearance of WC-1 from the nucleus. *Genes Dev.* 22, (2008).
22. Herzog, E. D., Hermanstyne, T., Smyllie, N. J. & Hastings, M. H. Regulating the suprachiasmatic nucleus (SCN) circadian clockwork: Interplay between cell-autonomous and circuit-level mechanisms. *Cold Spring Harb. Perspect. Biol.* 9, (2017).
23. Takahashi, J. S. Transcriptional architecture of the mammalian circadian clock. *Nature Reviews Genetics* vol. 18 (2017).
24. Colwell, C. S. Linking neural activity and molecular oscillations in the SCN. *Nature Reviews Neuroscience* vol. 12 (2011).
25. Liti, G. The fascinating and secret wild life of the budding yeast S. cerevisiae. *eLife* (2015) doi:10.7554/eLife.05835.
26. Fiora, A. & Cescatti, A. Diurnal and seasonal variability in radial distribution of sap flux density: Implications for estimating stand transpiration. *Tree Physiol.* (2006) doi:10.1093/treephys/26.9.1217.
27. Cerulus, B. *et al.* Transition between fermentation and respiration determines history-dependent behavior in fluctuating carbon sources. *Elife* (2018) doi:10.7554/eLife.39234.
28. Otterstedt, K. *et al.* Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. *EMBO Rep.* (2004) doi:10.1038/sj.embor.7400132.
29. Galdieri, L., Mehrotra, S., Yu, S. & Vancura, A. Transcriptional regulation in yeast

during diauxic shift and stationary phase. *OMICS A Journal of Integrative Biology* vol. 14 (2010).

30. Gasmi, N. *et al.* The switch from fermentation to respiration in Saccharomyces cerevisiae is regulated by the Ert1 transcriptional activator/repressor. *Genetics* (2014) doi:10.1534/genetics.114.168609.
31. Nambu, J. R., Lewis, J. O., Wharton, K. A. & Crews, S. T. The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* (1991) doi:10.1016/0092-8674(91)90292-7.
32. Hennig, S. *et al.* Structural and functional analyses of PAS domain interactions of the clock proteins Drosophila PERIOD and mouse period2. *PLoS Biol.* (2009) doi:10.1371/journal.pbio.1000094.
33. Claessen, D., Rozen, D. E., Kuipers, O. P., Søgaard-Andersen, L. & Van Wezel, G. P. Bacterial solutions to multicellularity: A tale of biofilms, filaments and fruiting bodies. *Nature Reviews Microbiology* (2014) doi:10.1038/nrmicro3178.
34. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: Building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology* (2013) doi:10.1038/nrmicro2960.
35. Ávila-Pérez, M., Van Der Steen, J. B., Kort, R. & Hellingwerf, K. J. Red light activates the σB-mediated general stress response of Bacillus subtilis via the energy branch of the upstream signaling cascade. *J. Bacteriol.* (2010) doi:10.1128/JB.00826-09.
36. Propst Ricciuti, C. & Lubin, L. B. Light induced inhibition of sporulation in Bacillus licheniformis. *J. Bacteriol.* (1976) doi:10.1128/jb.128.1.506-509.1976.
37. Ávila-Pérez, M., Hellingwerf, K. J. & Kort, R. Blue light activates the σB-dependent stress response of Bacillus subtilis via YtvA. *J. Bacteriol.* (2006) doi:10.1128/JB.00716-06.
38. Möglich, A. & Moffat, K. Structural Basis for Light-dependent Signaling in the Dimeric LOV Domain of the Photosensor YtvA. *J. Mol. Biol.* (2007) doi:10.1016/j.jmb.2007.07.039.
39. Letunic, I., Doerks, T. & Bork, P. SMART: Recent updates, new developments and status in 2015. *Nucleic Acids Res.* (2015) doi:10.1093/nar/gku949.
40. Schafmeier, T. & Diernfellner, A. C. R. Light input and processing in the circadian clock of Neurospora. *FEBS Letters* (2011) doi:10.1016/j.febslet.2011.03.050.
41. Bosman, J., Eelderink-Chen, Z., Laing, E. & Merrow, M. PREMONition: An algorithm for predicting the circadian clock-regulated molecular network. *bioRxiv* (2018) doi:10.1101/463190.
42. Li, S. *et al.* CGDB: A database of circadian genes in eukaryotes. *Nucleic Acids Res.*

(2017) doi:10.1093/nar/gkw1028.

43. Edgar, R. S. *et al.* Peroxiredoxins are conserved markers of circadian rhythms. *Nature* (2012) doi:10.1038/nature11088.
44. Liu, X. *et al.* Role for Protein Kinase A in the Neurospora Circadian Clock by Regulating White Collar-Independent frequency Transcription through Phosphorylation of RCM-1 . *Mol. Cell. Biol.* (2015) doi:10.1128/mcb.00709-14.
45. Mizoguchi, T., Putterill, J. & Ohkoshi, Y. Kinase and Phosphatase: The Cog and Spring of the Circadian Clock. *International Review of Cytology* (2006) doi:10.1016/S0074-7696(06)50002-6.



Chapter 2

A circadian clock in *Saccharomyces cerevisiae*

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Published: PNAS February 2, 2010 107 (5) 2043-2047

Introduction

The circadian clock is a cell-based, molecular regulatory network that controls processes from gene expression to behavior. Whether one looks for circadian clocks within a complex homeothermic organism (*e.g.* human) or a non-motile single cell (*e.g.* yeast), the environment is rhythmic with a period of 24 hours. This represents both opportunities and dangers and for this reason a mechanism for organizing cellular biology over 24h is beneficial and therefor found in diverse organisms. These daily clocks, share a set of signature properties[1]. One of these is a free-running, circa 24 h (circadian) oscillation in constant conditions. The phenomenon of self- sustained rhythm, however, has never been the "aim" of evolution. It is per se not a prerequisite for the timing system but rather a consequence of how a daily timing system has developed in an environment that is utterly predictable in its alternation of light and darkness, warmer and colder temperatures, and numerous other qualities[2]. Notably, many organisms do not show obvious free-running rhythms. For instance, the ascomycete, *Neurospora crassa*, suppresses daily, rhythmic circadian spore formation when CO_2 accumulates[3]. The accidental discovery of a mutant strain that makes "bands" of spores once every 22 h in constant darkness — without exchanging the air to decrease CO_2 levels — permitted development of *Neurospora* as a clock model system[4]. Even the banding strain of *Neurospora* appears arrhythmic in constant light, as do many animals. Yet, in the case of *Neurospora*, several transcript levels and the activity of the enzyme nitrate reductase are oscillating with a circa 24 h period despite no observable rhythms in spore formation[5,6]. When animals become arrhythmic in constant light, usually a decrease in irradiance will allow rhythmicity to emerge[7]. These examples suggest that the expression of a free-running clock very much depends on conditions or that it is not a universal property of circadian clocks. They furthermore suggest that organisms for which circadian rhythms have not been described could still possess them. In contrast to free-running rhythm, the major "task" of circadian clocks is to facilitate systematic synchronization of the organism with the cyclic environment (zeitgebers)[8]. This active process, called entrainment, results in a stable phase relationship between the endogenous clock (the multitude of clock-controlled processes) and the exogenous cycle (environment; additional information on entrainment in SI Materials), the multitude of clock- controlled processes and the exogenous (environment) cycle (additional). This entrained phase varies systematically according to conditions such as strength[9] or period (T)[10] of the zeitgeber and the proportion of night and day (*e.g.* photoperiod or even light pulse), which also allows for seasonal adjustments[11]. The process of entrainment remains poorly understood at the level of the cell although it organizes cellular biochemistry and metabolism to distinct temporal compartments. To this end, more genetic model systems that feature tools

for cell biology research are needed and among the best candidates for this purpose is *Saccharomyces cerevisiae*. There is *a priori* no reason to suspect that *S. cerevisiae* should be exempt from circadian regulation. Although yeast has been a denizen of laboratories for many decades, in nature, it is found in the soil and on many forms of biota and is thus subject to the same evolutionary pressures that have driven the development of circadian clocks in animals, plants, other fungi, and even in the rapidly dividing cyanobacteria[12,13]. The demonstration that circadian clocks confer an adaptive advantage in less than 10-20 generations is compelling[14,15]. It suggests that microbes will rapidly capture any spontaneous mutations that facilitate anticipation of environmental cycles.

In this report, we show circadian regulation in the budding yeast. We approach the problem first via entrainment, showing systematic synchronization to environmental cycles according to established principles that have been demonstrated in fungi, plants, and animals[16,17]. Using conditions and methods derived from entrainment experiments, we investigate free-running rhythms, both at the physiological and molecular levels. For these experiments, we developed a fermentor culture system that maintains cells in a nutritionally stable environment for weeks to months. Short (so-called ultradian) rhythms in metabolism and gene expression have been reported in similar cultures[18–21] when minimal medium is used and when pH levels are strictly controlled. However, when searching for a circadian regulation, it seems disadvantageous to clamp pH because it can serve as a read-out of daily metabolic fluctuations[22]. We, therefore, let the culture freely establish its own pH levels. Furthermore, we used a rich, complex culture medium that would support a higher level of metabolism than minimal medium.

Results and Discussion

Fermentor cultures were subjected to temperature cycles with a period of 24 h, to generate a rhythmic environment (11 h at 21 °C and 11 h at 28 °C — unless otherwise specified — with 1 h transitions between temperatures; Fig. 1A). Temperature cycles with an amplitude of 7 ºC were applied, because this amplitude is big enough to allow entrainment of the organism's circadian clock, while it minimizes masking effects. One hour transition period is used to maximize exposure to the "extreme" conditions (warm/cold), while not shocking the yeast culture with the temperature transitions. Dissolved oxygen (dO_2) in the media fluctuated with a period of 24 h, presumably reflecting daily alterations in metabolic rate. Under these conditions, ultradian oscillations were absent. Similar to dO_2, daily rhythms in pH were also observed. The incoming media (pH 6.3) was "conditioned" by the cells to a mean level of ≈pH 5. The pH oscillated around this set point, in synchrony with the temperature cycle and corresponding to fluctuations of roughly 10^7 H^+/day/cell.

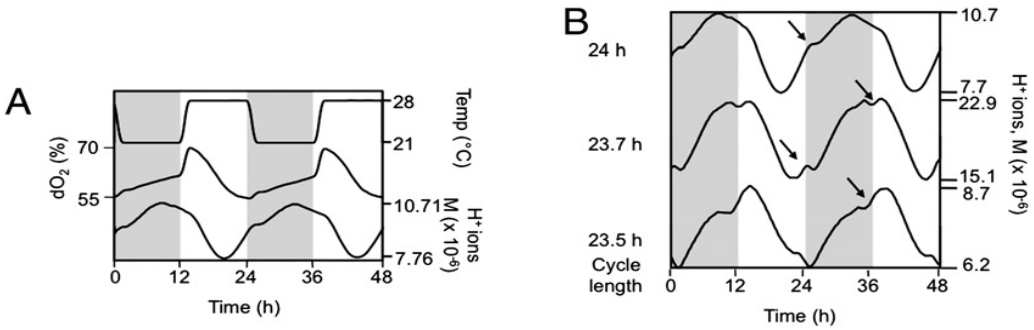


Figure 1. Temperature cycles induce oscillations in dO_2 and protons in media. Gray panels represent cool temperature; open panels represent warm temperature. (A) The experimental protocols used temperature cycles, shown here from 21 °C to 28 °C (Top), which support oscillations in dissolved O_2 (Middle) and pH (Bottom). Note that here and in the subsequent figures, the pH is converted to proton concentration. (B) In sub-24 h T-cycles, the oscillations in H^+ concentrations occur later within the temperature cycle. The top line shows the H^+ levels in a 24 h temperature cycle and the bottom line shows the oscillation in a 23.5 h cycle. The Middle tracing is a 23.7 h cycle. Arrows indicate where the shape of the curve changes, indicating passive changes in the oscillation because of the zeitgeber transition (see text).

The oscillations in dO_2 and protons could simply represent passive, temperature-dependent changes in metabolic rates (called masking)[23]. Alternatively, the temperature cycle could entrain a circadian system that actively regulates the timing of the observed oscillations. Both mechanisms have been reported for biological clocks in response to environmental cycles, and established protocols exist that can distinguish between these two[16]. During entrainment, the waveform of *e.g.* pH resembles a sinoid-like patterns of which the peaks and troughs are

out of phase with the temperature transition. The waveform during masking on the other hand, rapidly increase (cold to warm) or decreases (warm to cold) at temperature transition. Furthermore, zeitgebers can induce a mixture of masking and entrainment, evident in the responses of many organisms to daily light/ dark cycles. In *Drosophila*, a shock response is observed at light transitions, yet these are preceded by a gradual increase in activity that is controlled by the circadian clock[24]. The activity in mice is acutely suppressed at light onset, whereas their activity would have continued in dim light or sustained darkness[25]. Masking has even been noted at the molecular level, with RNA from the clock gene frequency being rapidly induced at all times when lights come on although protein is produced selectively depending on the elapsed time from midnight[11]. By simply changing the structure of the zeitgeber cycle, synchronization by circadian (active) versus masking (passive) processes can be discerned[16]. If the oscillations in the fermentors were passive responses, the phase relationship between the external (temperature) and the internal (metabolic) rhythms should be independent of conditions (*e.g.* of the zeitgeber's period). If the oscillations were actively produced by an entrained timing system, then these phase relationships should change systematically. And finally, if they were a product of both mechanisms, the waveform of the oscillations should change in addition to changing its phase angle. We, therefore, subjected cultures to shorter temperature cycles (T = 23.7 and 23.5 h), which should delay their phase in relationship to this slightly shorter temperature cycle[10,17]. In the circadian system, rhythmic events have a systematic relationship to the entraining cycle. If they are tested in entraining cycles of a different length, the phase relationship of the circadian rhythm changes: earlier in long cycles, later in short cycles. T 23.7h and T 23.5h gave the greatest phase shift. Consistent with the predictions for a circadian timing mechanism that actively entrains — and inconsistent with a passive response — we observed that the pH oscillation in the yeast cultures showed delayed phases relative to the 24 h temperature cycle (Fig. 1B). The delays were as much as 4-5 h, with a more or less preserved wave form: the delay was similar for the peaks, the troughs, as well as the halfway transitions between peaks and troughs. This is similar to observations in circadian systems where essentially opposite entrained phases can be achieved by changing the cycle length[17,26] and contrasts synchronization in noncircadian cycles (Fig. S1). There were, however, subtle changes in the curves' shapes between the different cycles, indicating that passive responses to the temperature changes also occur. We therefore used additional protocols to distinguish active versus passive mechanisms of synchronization. Circadian entrainment should also result in changed phase relationships when the zeitgeber strength is altered[9,16]. We changed zeitgeber strength by simply shifting the temperature so that it cycled between 18 °C and 25 °C. The structure of the cycle remained the same, with 11 h at 25 °C, a 1 hour transition to the lower temperature, 11 h at 18 °C, and then a 1-hour transition back

to 25 °C. The phase angle of the pH rhythm was delayed by 6 h relative to the 21/28 °C cycles, moving the pH peak from the cold to the warm phase (Fig. 2A). Again, these changes are more consistent with active entrainment than with passive responses. A high-amplitude cycle from 15 to 30 °C (Fig. S2), approximating what is experienced in nature, yielded a completely different wave form relative to Figs. 1 and 2. The stronger zeitgeber drove a steep increase in protons in the media with the onset of the warm phase of the incubation, followed by a relaxation back to lower levels. This appears to be more passive in its characteristics than the other entraining protocols. Although the dO_2 and pH rhythms shift their peaks in the same direction when the zeitgeber strength changes (Fig. 2B), their respective responses to the altered zeitgeber conditions are clearly different. Whereas the dO_2 rhythm shows a predominantly passive response with a strong increase at the transition to the warmer temperature and a drastic change in waveform, the pH rhythm shows the typical properties of an output of an entrained clock, shifting its entire waveform in response to zeitgeber strength with little change in shape.

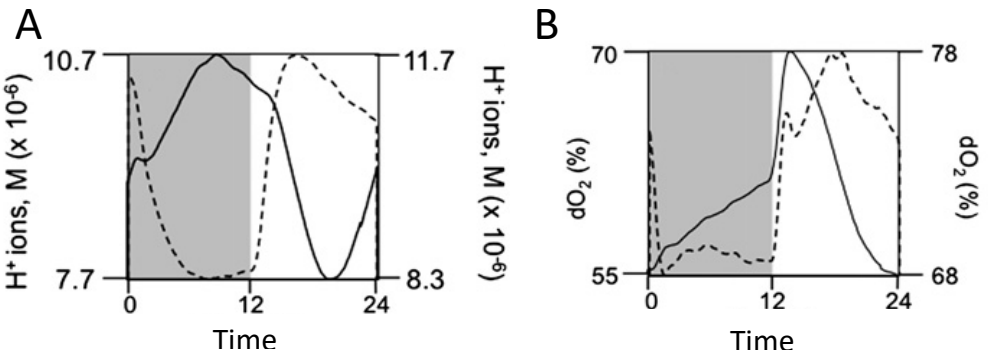


Figure 2. Phase relationships change with zeitgeber strength. (A) In lower temperature cycles (18 °C to 25 °C; dashed line), the peak of the H^+ oscillation moves into the warm phase, later than the peak in warmer cycles (21 ° C to 28 °C; solid line). (B) In the same cultures, the relationship between the peaks of the dO^2 oscillations and the temperature cycle is largely preserved (solid and dashed lines as for A).

Systematic circadian entrainment (different phase relationships in different T-cycles and zeitgeber strengths with a preservation of waveform) are qualities of both robust, self-sustained, free-running rhythms and of weak oscillators that rapidly damp in constant conditions[17]. To investigate whether the yeast timing system is a weak or a robust circadian oscillator, we released cultures after temperature entrainment to constant temperature. The oscillation in proton concentrations damped in under two cycles (Fig. 3; see also Fig. S3). The phenomenon of damping has been noted previously in, among others, plants[27,28] and in cell culture using mouse and rat fibroblasts and organ explants[29–32]. Some organisms even dispense of circadian rhythms if cycling environmental conditions recede, such as in an arctic

summer or winter[33]. The yeast timing system shows canonical properties of a circadian clock controlled by a weak, damped oscillator (at least under the culture conditions applied here).

The general explanation for damping under constant conditions is either loss of sustained rhythms at the level of the individual cell or desynchronization of a population of individual sustained cellular oscillators via small, stochastic changes in period. In the latter case, a change in the average free-running period is not anticipated. Here, the period lengthens as it is damping, suggesting that the former scenario is in play, namely that the timing system itself is impacted. A hallmark of the fermentor culture system — indeed, our goal in using it — is achieving a stable state for weeks or even months at a time with respect to the cell number, nutrition, etc. The same state is revisited from 1 day to the next. However, on release to the free run, the yeast culture is no longer stable as evidenced by increasing cell number, decreasing pH of the media, and increasing amplitude of the oscillation before it damps to non-rhythmicity. There may also be trivial reasons for lack of selfsustained rhythms in yeast, namely that we are following the wrong clock outputs. In *Neurospora*, several mRNA transcripts oscillate in the absence of any obvious circadian rhythm[5]. Furthermore, these transcripts can fail to entrain when the frequency of the circadian rhythm becomes long, as in the case of the mutant frq7, with a circa 29 h free-running period. This example is akin to a biological T-cycle, with one oscillatory system running at 29 h and another at circa 24 h. Each is outside the other's range of entrainment.

Circadian clocks are controlled by a transcriptional–translational feedback loop, posttranscriptional processes, or a mixture of the two[34–36]. *S. cerevisiae* has no clear orthologs of the transcription factors (clock genes) that mediate circadian regulation in fungi or animals, so we targeted likely circadianly regulated output pathways. Trafficking of ions in and out of cells is well understood in yeast, thus we have used this physiology to identify clock-controlled gene expression, a first step to understanding clock mechanisms in yeast. We focused on those genetic components involved in pH regulation, with consideration to the media used in our experiments, YPD. It supplies a rich source of amino acids but — in this form — nitrogen is expensive to metabolize. In comparison, ammonium is efficiently shuttled into the glutamine synthesis pathway, a gateway for production of multiple amino acids. Furthermore, yeasts have been shown to secrete ammonium during colony development on a time scale of day(s) as a means of intercellular communication[37]. MEP2p and GAP1p are ammonium and amino acid permeases, respectively, and both are similarly regulated by nitrogen catabolite repression[38]. We therefore measured their RNA concentrations over 48 h after release from 24 h temperature entrainment to constant conditions. Their RNAs show a high amplitude

oscillation in constant conditions with expression mirroring that of the pH oscillation (Fig. 4), with a peak in RNA concentration about 3 to 6 h before the media reaches the lowest pH. In entrained conditions, MEP2 and GAP1 RNAs precede the pH oscillation much as in the free-running condition (Fig. S4). The periodic transport of amino acids and ammonium to the cytoplasm would increase cytoplasmic pH, as they carry protons into the cell. The plasma membrane H^+-ATPase, PMA1p, maintains intracellular pH by controlled extrusion of protons in response to their increase in the cytoplasm[39,40] leading to secretion of excess protons and creating the observed oscillations. This may be a manifestation of clock regulation of metabolism in yeast, as cellular pathways are coordinated for optimal function. The general strategy of metabolic regulation is a fundamental property of clocks as demonstrated in higher organisms[41–44].

Although circadian clocks are found widely in nature, we recognize the hypothesis 'every organism will benefit from a clock because of the cycling environment' is weak, because it is not testable. On the other hand, the accumulating evidence is compelling, and it is hard to 'imagine' an organism that would not benefit from a mechanism for creating a temporal structure. Circadian clocks have not yet been scrutinized in *S. cerevisiae*. An extensive literature describes ultradian rhythms in yeasts (*e.g.* [18–21]), and it was recently suggested that these short rhythms could be used as building blocks for longer circadian rhythms[45]. Although this is formally possible, we see no evidence for ultradian oscillations under the conditions used for these experiments. Several decades ago, experiments purported to show circadian rhythms of cell division in bulk cultures of yeast[46], but these findings were never independently repeated. In these fermentor cultures, the cell division rate is approximately once every 9 h and there is no apparent rhythm in cell division, which indicates gating of this process to a specific time of day. The temperature cycle protocols applied here reveal a circadian timing mechanism in *S. cerevisiae* that can systematically entrain and that rapidly damps in constant conditions. Furthermore, we have shown clock-controlled molecular rhythms in gene expression of a key metabolic pathway that can be further used to investigate circadian behavior as well as to search for clock genes in yeast. These observations open the door for new approaches to elaborating circadian clock mechanisms and behaviors in eukaryotes. Budding yeast is especially attractive as it invites utilization of the multiple genome wide toolkits that facilitate high-throughput protocols.

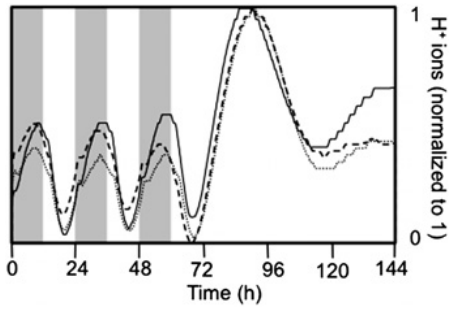


Figure 3. The yeast oscillator rapidly damps in constant temperature. Cultures were entrained to a 24-h temperature cycle (21 °C to 28 °C) and released to constant conditions (28 °C). The relative H^+ concentration of the cultures are shown.

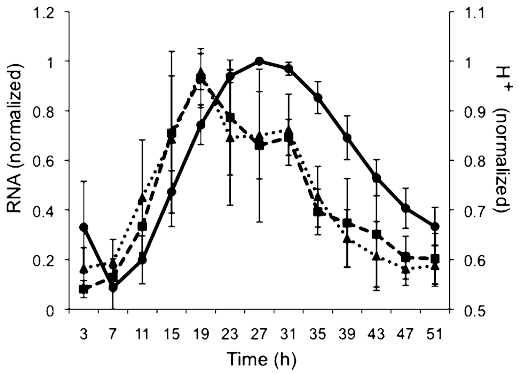


Figure 4. Oscillations in gene expression and in the pH of the media are synchronized. MEP2 and GAP1 RNA were measured (three independent, experimental replicates) in cell extracts from free-running cells in constant conditions. The solid line shows the H^+ oscillation, the dashed line is MEP2 RNA, and the dotted line is GAP1 RNA. The RNA values are normalized using tubulin RNA levels. Averages ± SD are shown.

Materials and Methods

Yeast Strain and Culture Conditions.

The strain used throughout this study was *S. cerevisiae* FY1679-2B (MATα ura3-52 leu2Δ1 TRP1 his3Δ200 GAL2; EURO- SCARF, Frankfurt am Main, Germany). All experiments were performed in fermentors (APPLIKON) to facilitate control and monitoring of the cultures. The 1 L culture vessels were inoculated with overnight cultures grown from single colonies in YPD (1% yeast extract, 2% peptone, 2% dextrose). The remainder of the experiment was then performed using YPD plus 10 mL l−1 Sigma Antifoam A. A batch culture at 30 °C lasted ≈36 h. When a rapid decrease in dO_2 was observed, the culture was starved for an additional 4 h. Cultures were then operated in continuous mode (a constant rate of media inflow and outflow) with agitation at 750 rpm, aeration at 150 mL min^{-1}, and dilution at 0.09–0.1 h^{-1} (unless otherwise specified). The dO_2 and the pH were monitored online; the dilution rate was monitored offline. The cell number was stable at around 2–3 × 10^9 cells/mL. It usually took about 2 weeks for the culture to become stable, such that it showed the same phase angle each day for weeks or months. Transitions from one cycle condition to another (*i.e.* from 24 to 23.5 h in length) would take up to a week to stabilize at a new entrained phase.

Zeitgeber Cycles

Half of each cycle was spent in high temperature (25 °C, 28 °C, or 30 °C), the other half in low temperature (15 °C, 18 °C, or 21 °C), with temperature transitions occurring over 60 min to decrease masking. The temperature of the room was maintained at 18 °C; the temperature of the cultures was maintained using a programmable water bath (Lauda).

RNA Preparation

A total of 1 × 10^9 yeast cells were collected and frozen in liquid nitrogen. Under free-running conditions, cells were harvested every 4 h over 2 days of a free run, starting 2 h after the temperature transition from cold to warm. Under entrained conditions, cells were harvested every 3 h over 24 h. Yeast total RNA was prepared using a modified version of the hot phenol RNA extraction protocol[47]. The frozen yeast pellet was suspended in 400 µL AE buffer (50 mM NaOAc pH 5.3 and 10 mM EDTA); 40 µL 10% SDS and 400 µL acidic phenol were added. The cells were disrupted by vortexing and then heated at 65 °C for 30 min. The samples were cooled, centrifuged, and the aqueous phase was reextracted with 400 µL acidic phenol followed by chloroform. RNA samples were purified and concentrated using a NucleoSpin RNA II kit (Macherey-Nagel).

RT-PCR Analysis

cDNA was prepared according to standard methods (ABI reagents). One microliter of template cDNA was analyzed in triplicate for each primer set. Primers were designed with Primer Express software (ABI). The sequences were:

GAP1-Fw, 5'-TTGTTCTGTCTTCGTCACCGC-3',
GAP1-Rv,5'-TACGGATTCACTGGCAGCAAG-3',
MEP2-Fw, 5'-CAGATGCGGAAGAAAGTGGAC-3',
MEP2-Rv, 5'-GGGTGATACCCACTAGGCCAG-3';
TUB1-Fw, 5'-TCCATTGCTGAGGCTTGGAA-3',
TUB1-Rv, 5'-ACCAGTGGACGAAAGCACGTT-3'.

RT-PCR was a SYBR green based assay using these cycle conditions: incubate samples 10 min at 95ºC, 15 sec at 95ºC (40 cycles), 1 min at 60ºC (40 cycles). Each plate included replicate samples for the standard curve and a negative control. 20 μl reactions we used which contained 10 μl 2 x PCR master mix, 0.4 μl (200 nM) forward primer and reverse primer, 8.2 μl RNAse free water and 1 μl of cDNA.

For each timepoint 3 biological repeats were sampled from two independent yeast fermentor cultures. Free running conditions were sampled for 48 hours (4h interval) and entrainment conditions for 24 hours (3h interval). Intervals were chosen for logistical purposes.

Data Analysis

The output files from the fermentors were analyzed with ChronosX software[48].

Acknowledgments

We thank Jack Pronk, Pascale Daran-Lapujade, Peter Phillipsen, Douglas Murray, Bert Poolman, and Andre Goffeau for advice, comments, and discussions on this work. Our work is supported by the Dutch Science Foundation (the NWO), The Hersenen Stichting, EUCLOCK (Entrainment of the Circadian Clock), a Sixth Framework Program of the European Union, and the Rosalind Franklin Fellowships of the University of Groningen.

Literature references

1. Pittendrich, C. S. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/SQB.1960.025.01.015.
2. Roenneberg, T. & Merrow, M. Life before the clock: Modeling circadian evolution. *J. Biol. Rhythms* (2002) doi:10.1177/0748730402238231.
3. Sargent, M. L. & Kaltenborn, S. H. Effects of Medium Composition and Carbon Dioxide on Circadian Conidiation in Neurospora . *Plant Physiol.* (1972) doi:10.1104/pp.50.1.171.
4. Sargent, M. L., Briggs, W. R. & Woodward, D. O. Circadian nature of a rhythm expressed by an invertaseless strain of Neurospora crassa. *Plant Physiol.* (1966) doi:10.1104/pp.41.8.1343.
5. Correa, A. *et al.* Multiple oscillators regulate circadian gene expression in Neurospora. *Proc. Natl. Acad. Sci. U. S. A.* (2003) doi:10.1073/pnas.2233734100.
6. Christensen, M. K. *et al.* A nitrate-induced frq-less oscillator in Neurospora crassa. *J. Biol. Rhythms* (2004) doi:10.1177/0748730404265532.
7. Steinlechner, S. *et al.* Robust circadian rhythmicity of Per1 and Per2 mutant mice in constant light, and dynamics of Per1 and Per2 gene expression under long and short photoperiods. *J. Biol. Rhythms* (2002) doi:10.1177/074873040201700303.
8. Hastings, J. W. THE ROLE OF LIGHT IN PERSISTENT DAILY RHYTHMS. in *Photophysiology* (1964). doi:10.1016/b978-1-4832-2798-6.50018-3.
9. Pittendrigh, C. S. & Daan, S. A functional analysis of circadian pacemakers in nocturnal rodents - IV. Entrainment: Pacemaker as clock. *J. Comp. Physiol. A* (1976) doi:10.1007/BF01417859.
10. Hoffmann, K. Zur beziehung zwischen phasenlage und spontanfrequenz bei der endogenen Tagesperiodik. *Zeitschrift fur Naturforsch. - Sect. B J. Chem. Sci.* (1963) doi:10.1515/znb-1963-0211.
11. Tan, Y., Dragovic, Z., Roenneberg, T. & Merrow, M. Entrainment dissociates transcription and translation of a circadian clock gene in Neurospora. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.02.035.
12. Johnson, C. H. Endogenous timekeepers in photosynthetic organisms. *Annual Review of Physiology* (2001) doi:10.1146/annurev.physiol.63.1.695.
13. Kondo, T. *et al.* Circadian rhythms in prokaryotes: Luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* (1993) doi:10.1073/pnas.90.12.5672.
14. Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K. & Johnson, C. H. The adaptive value of

circadian clocks: An experimental assessment in cyanobacteria. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.08.023.

15. Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Johnson, C. H. Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.15.8660.
16. Roenneberg, T., Dragovic, Z. & Merrow, M. Demasking biological oscillators: Properties and principles of entrainment exemplified by the Neurospora circadian clock. *Proc. Natl. Acad. Sci. U. S. A.* (2005) doi:10.1073/pnas.0501884102.
17. Merrow, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurospora clock gene frequency. *Nature* (1999) doi:10.1038/21190.
18. Robertson, J. B., Stowers, C. C., Boczko, E. & Johnson, C. H. Real-time luminescence monitoring of cell-cycle and respiratory oscillations in yeast. *Proc. Natl. Acad. Sci. U. S. A.* (2008) doi:10.1073/pnas.0809482105.
19. Adams, C. A., Kuriyama, H., Lloyd, D. & Murray, D. B. The Gts1 protein stabilizes the autonomous oscillator in yeast. *Yeast* (2003) doi:10.1002/yea.976.
20. Tu, B. P., Kudlicki, A., Rowicka, M. & McKnight, S. L. Cell biology: Logic of the yeast metabolic cycle: Temporal compartmentalization of cellular processes. *Science (80-.).* (2005) doi:10.1126/science.1120499.
21. Klevecz, R. R., Bolen, J., Forrest, G. & Murray, D. B. A genomewide oscillation in transcription gates DNA replication and cell cycle. *Proc. Natl. Acad. Sci. U. S. A.* (2004) doi:10.1073/pnas.0306490101.
22. Eisensamer, B. & Roenneberg, T. Extracellular pH Is under Circadian Control in Gonyaulax polyedra and Forms a Metabolic Feedback Loop. *Chronobiol. Int.* (2004) doi:10.1081/CBI-120027979.
23. Mrosovsky, N., Lucas, R. J. & Foster, R. G. Persistence of masking responses to light in mice lacking rods and cones. *Journal of Biological Rhythms* (2001) doi:10.1177/074873001129002277.
24. Rosbash, M. *et al.* A Drosophila circadian clock. in *Cold Spring Harbor Symposia on Quantitative Biology* (1996). doi:10.1101/sqb.1996.061.01.029.
25. Mrosovsky, N. Masking: History, definitions, and measurement. *Chronobiology International* (1999) doi:10.3109/07420529908998717.
26. Aschoff, J. A Survey on Biological Rhythms. in *Biological Rhythms* (1981). doi:10.1007/978-1-4615-6552-9_1.
27. James, A. B. *et al.* The circadian clock in Arabidopsis roots is a simplified slave version of the clock in shoots. *Science (80-.).* (2008) doi:10.1126/science.1161403.
28. Millar, A. J. & Kay, S. A. Integration of circadian and phototransduction pathways in

the network controlling CAB gene transcription in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* (1996) doi:10.1073/pnas.93.26.15491.

29. Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. & Menaker, M. Entrainment of the circadian clock in the liver by feeding. *Science (80-.).* (2001) doi:10.1126/science.291.5503.490.
30. Yoo, S. H. *et al.* PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc. Natl. Acad. Sci. U. S. A.* (2004) doi:10.1073/pnas.0308709101.
31. Balsalobre, A., Marcacci, L. & Schibler, U. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr. Biol.* (2000) doi:10.1016/S0960-9822(00)00758-2.
32. Hughes, M. E. *et al.* Harmonics of circadian gene transcription in mammals. *PLoS Genet.* (2009) doi:10.1371/journal.pgen.1000442.
33. Van Oort, B. E. H. *et al.* Circadian organization in reindeer. *Nature* (2005) doi:10.1038/4381095a.
34. Nakajima, M. *et al.* Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science (80-.).* (2005) doi:10.1126/science.1108451.
35. Mittag, M. Conserved circadian elements in phylogenetically diverse algae. *Proc. Natl. Acad. Sci. U. S. A.* (1996) doi:10.1073/pnas.93.25.14401.
36. Lowrey, P. L. & Takahashi, J. S. Mammalian circadian biology: Elucidating genome-wide levels of temporal organization. *Annual Review of Genomics and Human Genetics* (2004) doi:10.1146/annurev.genom.5.061903.175925.
37. Palková, Z. *et al.* Ammonia pulses and metabolic oscillations guide yeast colony development. *Mol. Biol. Cell* (2002) doi:10.1091/mbc.E01-12-0149.
38. Lorenz, M. C. & Heitman, J. The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae. *EMBO J.* (1998) doi:10.1093/emboj/17.5.1236.
39. Van Der Rest, M. E. *et al.* The plasma membrane of Saccharomyces cerevisiae: Structure, function, and biogenesis. *Microbiological Reviews* (1995) doi:10.1128/mmbr.59.2.304-322.1995.
40. Morsomme, P., Slayman, C. W. & Goffeau, A. Mutagenic study of the structure, function and biogenesis of the yeast plasma membrane H+-ATPase. *Biochimica et Biophysica Acta - Reviews on Biomembranes* (2000) doi:10.1016/S0304-4157(00)00015-0.
41. Nakahata, Y., Sahar, S., Astarita, G., Kaluzova, M. & Sassone-Corsi, P. Circadian Control of the NAD+ Salvage Pathway by CLOCK-SIRT1. *Science (80-.).* (2009)

doi:10.1126/science.1170803.

42. Ramsey, K. M. *et al.* Circadian clock feedback cycle through NAMPT-Mediated NAD+ biosynthesis. *Science (80-.).* (2009) doi:10.1126/science.1171641.
43. Dodd, A. N. *et al.* The Arabidopsis circadian clock incorporates a cADPR-based feedback loop. *Science (80-.).* (2007) doi:10.1126/science.1146757.
44. Brody, S. & Harris, S. Circadian rhythms in neurospora: Spatial differences in pyridine nucleotide levels. *Science (80-.).* (1973) doi:10.1126/science.180.4085.498.
45. Tu, B. P. & McKnight, S. L. Metabolic cycles as an underlying basis of biological oscillations. *Nature Reviews Molecular Cell Biology* (2006) doi:10.1038/nrm1980.
46. Dunlap, J. C. Cellular and Molecular Bases of Biological Clocks. Models and Mechanisms for Circadian Timekeeping . Leland N. Edmunds, Jr. . *Q. Rev. Biol.* (1989) doi:10.1086/416246.
47. Schmitt, M. E., Brown, T. A. & Trumpower, B. L. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Res.* (1990) doi:10.1093/nar/18.10.3091.
48. Roenneberg, T. & Taylor, W. Automated recordings of bioluminescence with special reference to the analysis of circadian rhythms. *Methods in Enzymology* (2000) doi:10.1016/s0076-6879(00)05481-1.

Supporting Information Materials

Entrainment of Oscillators.

The first "modern" record of entrainment of oscillators is attributed to the Dutch scientist, Christian Huygens[1]. He was attempting to solve the problem of keeping accurate time at sea and thus had multiple pendula swinging simultaneously. As he lay ill in bed one day (it was the custom to have one's bed off to the side of the living area), he noted that the pendula on the adjacent wall were synchronized. When he per- turbed them they would resynchronize after some minutes. This held only for pendula on the same wall. They could thus communicate with each other and entrain each other. This phenomenon is comparable to circadian rhythms synchronizing to the external zeitgeber cycle. The physical cycles need to communicate in some way with the biological ones (those of the circadian clock). Most entrainment mechanisms have been defined using light as a zeitgeber; it is an important zeitgeber for most circadian systems and it is experimentally facile to use. In the case of the work here, the external cycle uses temperature as a zeitgeber. There is virtually nothing known concerning the mechanisms by which temperature entrains circadian rhythms. It may be changes in biochemical reactions in the cell or it may be via specialized temperature sensors, akin to photoreceptors. Regardless of the mechanisms, temperature is apparently also a universal zeitgeber for circadian systems. In the case of homeotherms, low-amplitude temperature cycles are effective synchronizers[2]. In the case of poikilotherms, the experienced temperature cycles are typically much higher. Temperature acts to synchronize the metabolism of yeast in a highly systematic way like what was first demonstrated by Hoffmann, when he put lizards into temperature cycles of different length, showing that they would entrain later as cycles became shorter[3,4]. This phenomenon turns out to be one of the circadian rules that even contributes to the explanation of chronotype, the distinct entrained phase of an individual. In the general population, there is a distribution of chronotypes[5] and this is thought to be due to differences in genetic background resulting in differences in free-running period (and probably other circadian properties, such as zeitgeber input pathways, as well). Indeed, in the 1970s, the concept of zeitgeber strength as it regulates chronotype was demonstrated in hamsters[6].

Obviously, the environmental cycle is the cycle to which the biological one must adjust. If the biological clock would simply respond with a switch-like mechanism (becoming active when the sun comes up, for instance), then the organism would need no biological clock. A circadian clock, however, allows differential entrainment according to season, for instance, thus allowing gating of seasonal behaviors such as reproduction[7,8]. The experiments described in this paper

use these basic ideas, namely that the absence of a clock should yield driven responses and, conversely, the presence of a clock should yield entrainment in a similar manner as has been demonstrated for other circadian systems. We have applied short temperature cycles, which should show a biological clock entraining to a later phase[3,4,9,10]. We have altered the zeitgeber strength, which would be predicted to change the entrained phase of a circadian system[5,10].

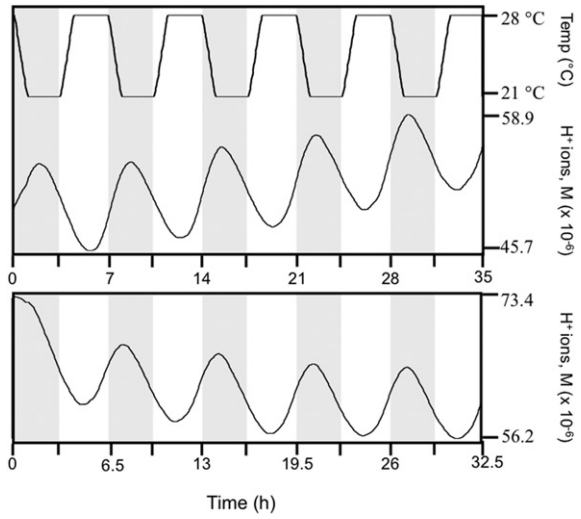


Figure S1. Short, non-resonating T-cycles show fixed (driven) phase angles in the concentration of H^+ ions in the media. Temperature cycles were imposed on the yeast fermentor cultures, with a cycle length of 7 h (Upper) or 6.5 h (Lower). The temperature cycle is indicated in two ways on the graph: the gray panels indicate cold phase and the open panels show the warm phase; the actual temperature cycle is plotted at the Top of the Upper graph, showing the gradual, 1 h transitions between the high (28 °C) and low (21 °C) temperature phases. The scale for the oscillations in H^+ ion concentration is indicated. The data are smoothed using a 2 h window and are not trend corrected.

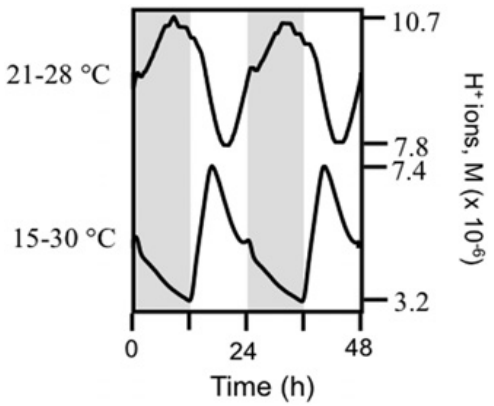


Figure S2. High-amplitude temperature cycles show an altered waveform and phase angle. A 24 h temperature cycle of 15 °C to 30 °C was applied to fermentor cultures. Here, the oscillation in protons from the high-amplitude cycle (Lower) is compared with that from the 21/28 °C temperature cycle from Figure. 1 (Upper). Two sequential days of stable entrainment are shown.

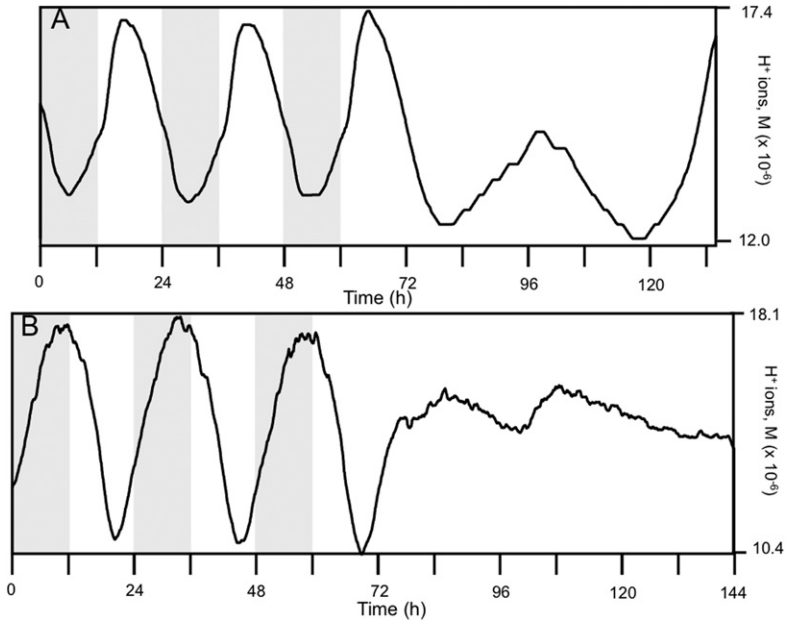


Figure S3. Oscillations following release to constant conditions. In two experiments, the pH did not change substantially on release from a 24 h temperature cycle to constant conditions. In these cases, the period of the nonentrained, freerunning oscillation was closer to 24 h and did not appear to be unstable (although it did damp rapidly). These two examples contrast what is more typically observed in the fermentor cultures (see Figure 3 and note the reproducibility therein between experiments). (A) 18/25 °C temperature cycle with a release to 25 °C. (B) 21/28 °C cycle with a release to 28 °C.

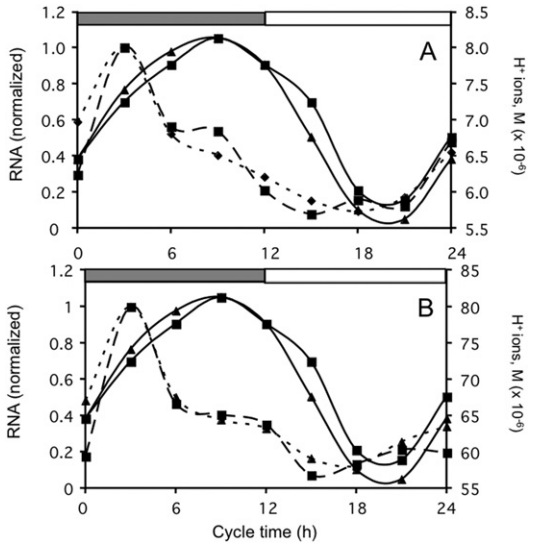
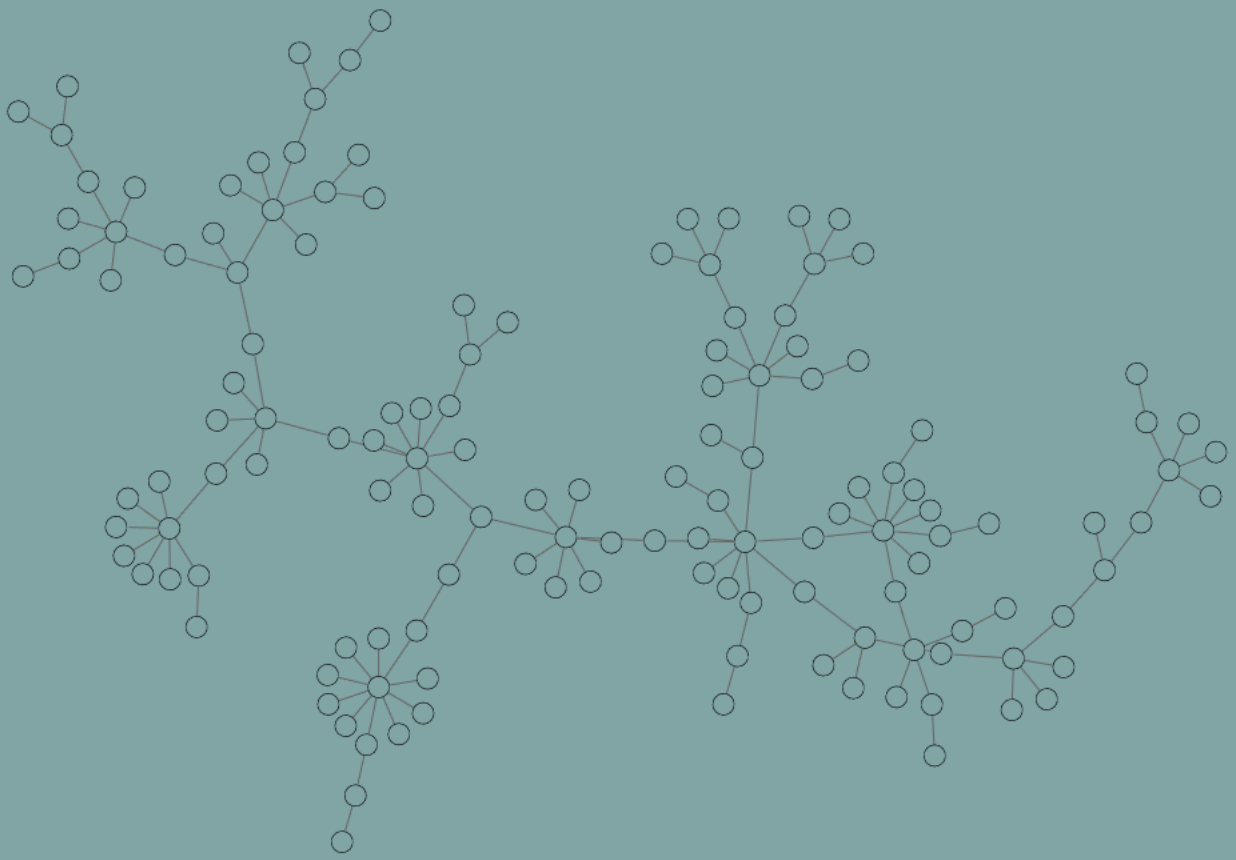


Figure S4. RNA expression during entrainment with temperature. MEP2 (A) and GAP1 (B) mRNA levels were determined in a 24 h 21/28 °C temperature cycle. In each graph, two separate experiments are shown, with MEP2/GAP1 RNA as dashed and dotted lines (representing the two experiments). The oscillations in proton concentrations are shown in the same graphs (solid lines). The lines with squares as markers correspond to the same experiment; the lines with triangles and diamonds are samples from the same experiment. The gray and open bars at the top of the graphs indicate cold and warm phases of the entraining cycle, respectively. Tubulin was used for normalization; for each time series, the high value is set to 1.



Chapter 3

PREMONition: An algorithm for predicting the circadian clock-controlled molecular network

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Published: Cold Spring Harbour BioRxiv November 05, 2018.

Introduction

The circadian clock is a common biological program in living organisms[1]. It creates a temporal structure that anticipates the regular and predictable changes in the environment that occur each day[2]. Circadian clocks are cell-based and are found in organisms from all phyla. The molecular theme of the eukaryotic circadian clock involves transcription factors that activate a set of genes that encode proteins capable of downregulating their own expression, forming a negative feedback loop. However, experiments suggest that circadian clock properties persist in the absence of identified clock genes. For instance, *Neurospora crassa* shows free-running rhythms and circadian entrainment without the clock gene *frequency*[3,4] and rhythms in the redox state of peroxiredoxin persist in many model genetic systems in the absence of transcription or clock genes[5]. Paracrine signaling can sustain circadian rhythms in pacemaker (neuronal) tissue that lacks clock genes and displays no endogenous rhythm[6]. One interpretation of this is that feedback integral to circadian clocks spans multiple molecular 'levels', from transcription to metabolism. We conjecture that this multi-level response can be captured through functional relationships, referred to as functional interactions. We know of no models that explicitly attempt to integrate these different levels to more fully describe the mechanisms of the circadian clock. Here, we describe the method PREMONition, PREdicting MOlecular Networks, to construct functional molecular clock networks using publicly available datasets and bioinformatics tools. After tuning the method on clock genetic model systems, we deployed PREMONition to predict a molecular clock network in *S. cerevisiae*, an experimental system with a circadian phenotype that is not suited to genetic screens[7]. We validated the results of the yeast experiment *in silico* and *in vivo*. Methods like PREMONition, that can span multiple levels in the cell, are essential to developing integrative models of complex biological processes.

Results

PREMONition applied to model circadian mechanisms

To construct circadian molecular networks PREMONition incorporates two sets of data: rhythmically expressed genes and evidence-based (physical and functional) interactions of molecules derived from published literature and stored in the STRING database[8]. The set of rhythmically expressed genes (Clock-Controlled Genes (CCGs)) is derived from transcriptome analyses. Clock genes - those that have been determined to be involved in the central clock mechanism – are also included here, when known. By logic, most clock genes must be rhythmically expressed at some level, whether it be RNA or protein expression or functional competence (*e.g*. determined by rhythmic post-translational modification). CCGs are genes that are controlled by the central clock mechanism, that are not thought to feed back into the clock mechanism (no evidence that their disruption affects the clock). For the second data set, interacting proteins catalogued and/or predicted by STRING are increasing in number based on the massive amount of genome-wide quantitative measurements that are now common.

Network construction

Within STRING molecular interaction networks are predicted and displayed. The network reflects the specified stringency of the statistical probability of interactions. One, can thus make a network that is more or less connected and/or one that is larger or smaller by specifying statistical parameters. To develop the PREMONition method, we first created a molecular interaction network formed of proteins encoded by canonical clock genes only. As expected, these proteins, known to be involved in circadian transcriptional feedback loops in *H. sapiens* cells, *D. melanogaster* and *N. crassa*, form small, highly connected networks (Figs. 1A, B and C). However, when combining clock genes together with CCGs derived from published transcriptome analyses[9–11] (Table S1), the derived networks comprised disjointed sub-networks rather than a cohesive network (Figs. 1D, E and F). For the human network, only two interactions between the clock genes and CCGs were identified.

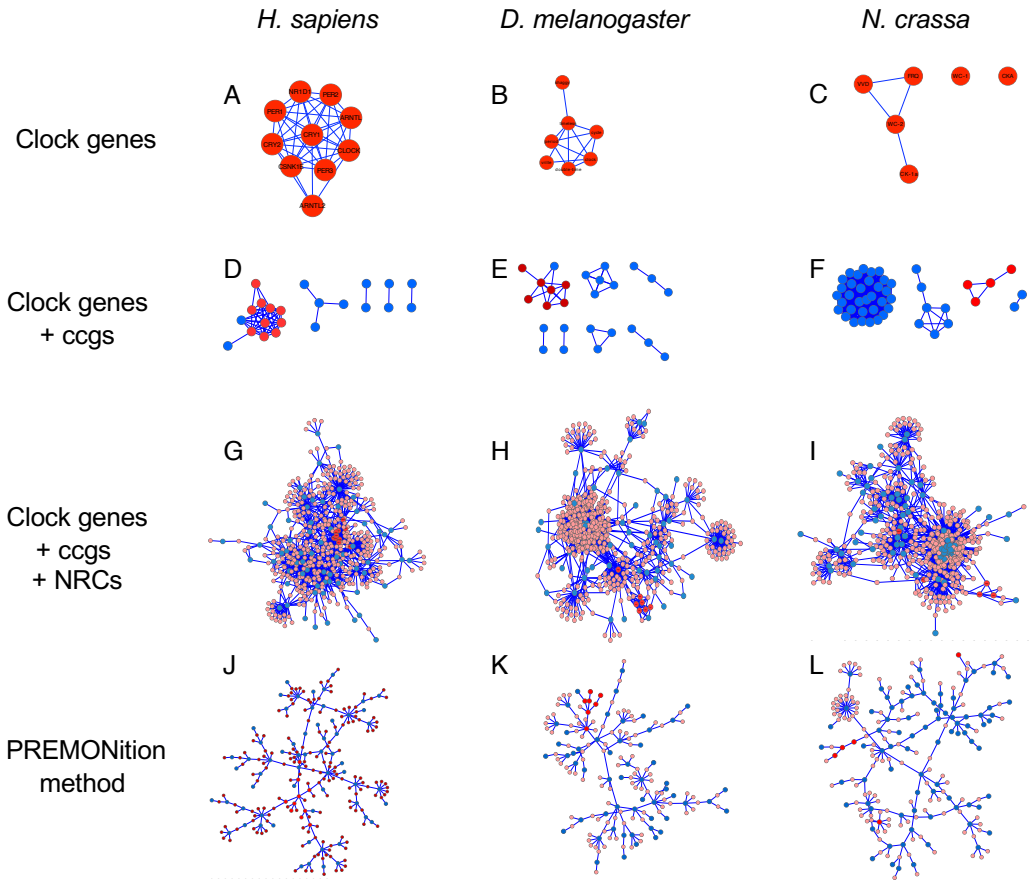


Figure 1. Molecular interaction networks comprising molecules encoded by human, Drosophila and Neurospora clock genes, Clock Controlled Genes (CCGs) and Non-Rhythmic Connectors (NRCs). Clock genes alone tend to form connections (A, B, C). Addition of CCGs does not lead to a cohesive network (D, E, F). Addition of NRCs makes a highly connected network (G, H, I). Removal of the least probable NRCs connecting two rhythmic nodes and removal of terminal NRCs (panels J, K and L). Molecules that fail to be incorporated into the network in panels D-L are not shown. Spheres marked with red indicate proteins encoded by clock genes; blue spheres indicate proteins encoded by clock-controlled genes; pink spheres indicate proteins encoded by non-rhythmic connectors.

Forming a molecular interaction network associating all clock/clock-controlled genes is a key step in moving beyond studies that concentrate on single genes and their functions. It is well known that rhythmic RNAs can lead to non-rhythmic proteins and non-rhythmic RNAs can lead to rhythmic proteins[12–14]. Furthermore, post-translational modifications can confer rhythmic function even in the absence of rhythmic protein expression[14,15]. We therefore allowed PREMONition to incorporate Non-Rhythmically expressed Connectors (NRCs) into the 'clock gene + CCG molecular interaction network. NRCs by definition are molecules that interact, functionally, with molecules associated with clock genes and/or CCGs; as their name suggests, NRCs are not themselves identified as rhythmically expressed at the gene expression level. In the experiments described here, a relatively stringent probability score of ≥ 0.7 was used for *Drosophila* and human and ≥ 0.6 for *Neurospora* within STRING. This cutoff was derived by

determining when highly interconnected networks would form, and when they would break (become fragmented) if higher scores were used. Networks were formed that incorporated 70%, 38% and 57% of CCGs from human, *Drosophila* and *Neurospora* gene sets, respectively (Figs. 1G, H and I). Networks were trimmed by allowing only a single NRC between two nodes (either clock gene or CCG; Table S2). The most probable connection was retained, and all others were discarded. Through this process, which selects for the most likely interactions, networks were reduced from 483 to 248, from 498 to 227, and from 566 to 196 nodes without decreasing CCG incorporation for the three species (*H. sapiens*, *N. crassa* and *D. melanogaster* respectively). In all three organisms, an extensive and inclusive network was generated (Figs. 1 J, K and L).

Network interpretation

The three PREMONition derived networks shared thirty-eight common Gene Ontology (GO) terms[16]. Ten of these terms were significantly enriched biological processes in the human, fly and *Neurospora* networks (Fig. S1 and Table S3). A thousand simulations using random sets of genes, representing random networks the same size as the PREMONition derived networks, yielded not a single over-represented GO category. Applying GO analysis to each of the collections of CCGs also yielded no significantly enriched categories. Conversely, the GO categories that were detected in the analysis of entire PREMONition networks represent a large percentage of categories for a given genome. As such, these categories are not predictive – they cover too much of the genome to be useful for identification of discrete genes and proteins involved in clock function.

Orthology between the molecular networks was assessed using the InParanoid8 database[17]. For human, fly and fungus the 283, 267 and 245 unique molecules, referenced by their corresponding gene name were submitted to the InParanoid8 database, respectively, for a cross comparison. Table 1 shows the number of orthologous protein pairs, including orthologous clusters identified by Inparanoid 8 (Table S4). In total 30, 23 and 21 human, fly and *N. crassa* genes were found to be in common between the respective networks.

Table 1. Identification of orthologs with InParanoid8. The number of *D. melanogaster* (fly) orthologs that occur *H. sapiens* (human) and *N. crassa* (fungus) and between human and fungus is shown. Several of the orthologs are included in the PREMONition networks for the specific organisms. q-values indicate the significance of ortholog enrichment in the networks.

	Orthologs	Networked	q-value
Fly - human	10711	19	2.08e-22
Fly – mold	4004	11	4.23e-28
Human - mold	5155	14	1.15e-33

A yeast PREMONition network

Although clock properties such as systematic entrainment, indicative of a *circa* 24 h oscillator, can be elicited in yeast[7], the clock phenotype is not robust enough to support a high-throughput genetic screen that could uncover candidate clock genes. There are no clock gene candidates in yeast that are predicted by homology searches or functional assays. In order to generate a putative circadian molecular interaction network for yeast, we used PREMONition. A yeast transcriptome was generated covering the first day following release into constant conditions (Fig 2A). One-hundred-and-thirty-seven genes were determined to be rhythmically expressed, with all phases of expression observed over the subjective day (Fig. 2B and Fig. 3). This set of CCGs did not form a fully interconnected network (probability score of >= 0.7) when submitted to STRING. Only 23 genes/proteins were connected. Allowing NRCs, however, resulted in a network of 477 interacting molecules, consisting of 123 rhythmic genes and 354 NRCs (Fig. 4). 14 rhythmic genes could not be integrated into the network. Referring to the networks described for human, *Drosophila* and *Neurospora* clock systems, 19, 11 and 19 genes, respectively, have orthologs in *S. cerevisiae* and 13 of those were identified in the yeast network.

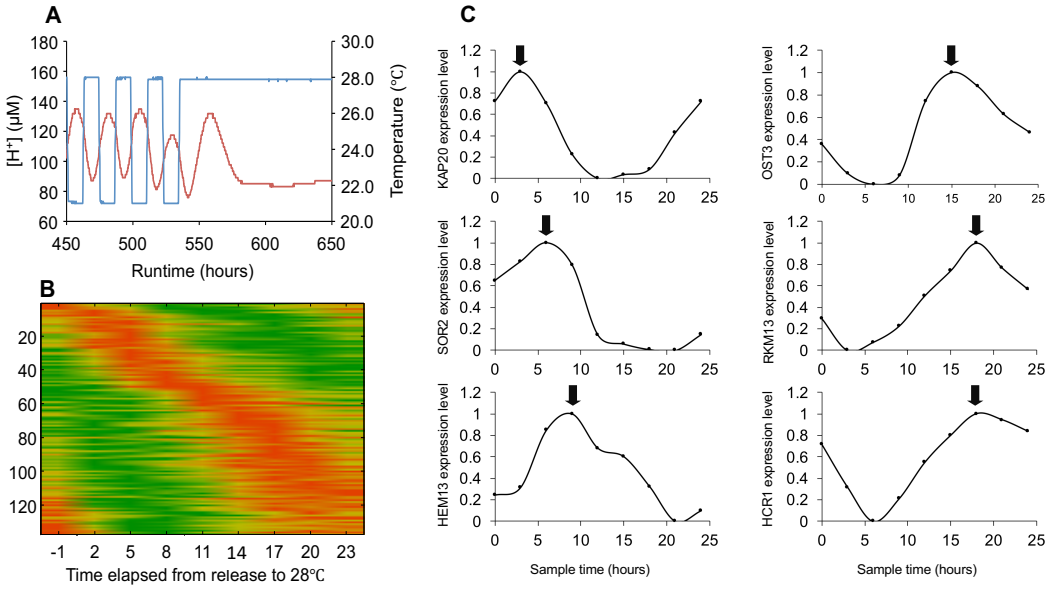


Figure 2. Rhythmicity of gene expression in yeast. Fermentor cultures were established using 24h zeitgeber cycles (21 °C / 28 °C, blue line) to synchronise the cells, as measured by pH of the culture medium (red line). Once stable synchronisation was observed, the temperature was held at 28 °C leading to one >24h oscillation in the pH (A). Cells were harvested every 3h for 24h, starting 1 hour before the last temperature transition (from 21 °C to 28 °C). The RNA transcriptome was analysed using microarrays, yielding a set of 137 rhythmic genes (q-value <0.01). The heat map shows the time of high expression in red and the time of low expression in green (B). Each individual signal is normalized between 0 (green) and 1 (red). They are plotted in order of peak phase. Rhythmic genes determined with CircWave[18] span 'all' circadian phases (C). The 137 rhythmic genes were grouped into 6 bins of 4 hours each based on their peak phase of gene expression. For each bin, the gene showing highest significance is shown. An arrow marks the peak phase of each gene. All time series were normalized such that their values fall between 0 and 1.

Validation of the yeast PREMONition network

We attempted to experimentally validate the putative yeast circadian molecular interaction network in two ways. First, we relaxed the stringent threshold used to identify CCGs in our yeast transcriptome from a q-value of 0.01 < to < 0.05. This increased the number of CCGs from 136 to 526. We found that thirty-three of the 354 original *S. cerevisiae* NRCs were considered to be rhythmically expressed with the relaxed q-value (Fig. 4). This number (33 of 354) is higher than expected by chance (hypergeometric test q-value = 2.53^{e-06}) and indicates that, as a method, PREMONition is able to identify clock associated molecules.

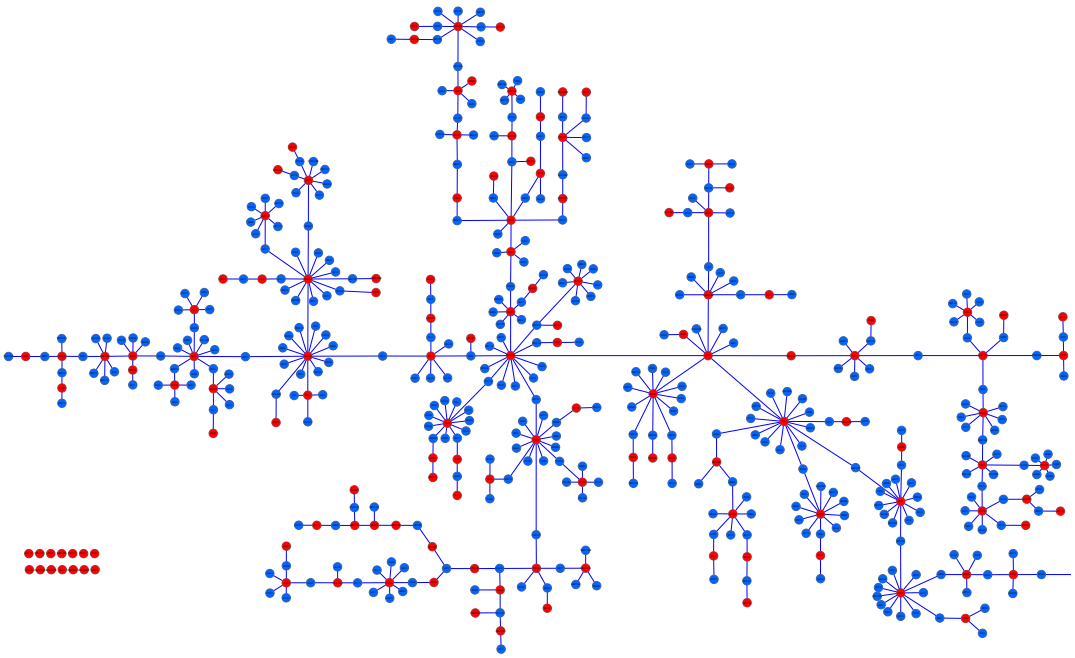


Figure 3. The PREMONition network for yeast. Proteins encoded by rhythmically expressed genes (red spheres) and their non-rhythmic connectors (blue spheres) are shown. The network is composed of 123 rhythmic genes and 354 NRCs, connected by 476 edges. 14 rhythmically expressed genes are disjoined from the network. The figure is scalable such that each gene/protein can be identified.

A second validation method was developed based on the observation that circadian clocks of surface-dwelling organisms are sensitive to light. Photobiology in yeast is unknown except for reports of suppression of growth in constant light conditions apparently via interaction with cytochromes[19,20]. We hypothesized that mutations in genes involved in circadian clock function will show either an increased or decreased sensitivity to light in a growth assay. We therefore assayed a selection of mutants carrying knockouts of genes from the network for their sensitivity to light. Six genes present in the PREMONition network were selected based on their relationship to known circadian systems across different phyla. SOD1 (YJR104C) encodes a cytosolic copper-zinc superoxide dismutase, having a rhythmic mRNA expression level in mouse liver[21]. Superoxide dismutase activity is rhythmic in the dinoflagellate *Gonyaulax polyedra*[22] and the *sod–1* mutant in *N. crassa* shows changes in the period of circadian rhythms and abnormal responses to light as a zeitgeber for entrainment[23]. The yeast FET3 (YMR058W) protein is a multicopper oxidase, a family that is conserved in many organisms and showing circadian rhythms in *A.thaliana*[24]. Additionally, copper functions as a redox active cofactor for many proteins like cytochrome-c-oxidase and copper-zinc-superoxide dismutase (Cu/Zn-SOD)[25]. Its expression is rhythmic and is important for circadian rhythms in the mammalian

pacemaker[26]. The SGS1 (YMR190C) protein is a RecQ-family nucleolar DNA helicase. RecQ mRNA is among the few transcripts that cycle independent of Kai clock proteins in cyanobacteria (*S. elongatus*)[27]. The CHC1 (YGL206C) gene encodes a Clathrin heavy chain protein broadly involved in intracellular protein transport and endocytosis. Clathrin-interacting proteins have been implicated as potential clock effectors or as clock regulated genes and proteins in flies and in mice[28,29]. HOG1 (YLR113W) is the homolog of mitogen activated protein kinase (MAPK). Many MAPK proteins are involved in circadian regulation in the mammalian clock[30]. In *Neurospora* it functions as a mediator of clock-controlled outputs[31]. HCR1 (YLR192C) is an elongation initiation factor regulating translation. This gene scored amongst the most highly rhythmic (q-value = 0.0001) and its protein performs an important cellular function that has relatively rarely been investigated for clock function[32]. We used knockouts in all of these genes in an assay for suppression of growth by light (Fig. 5). With the exception of HCR1, all knockouts tested showed a significant alteration in growth suppression by light. One of these, a DNA helicase homolog, was resistant to the effects of light. The other strains – knockouts for genes encoding SOD1, HOG1, CHC1 and FET3 – all showed more growth suppression in the presence of light than did the parental yeast strain.

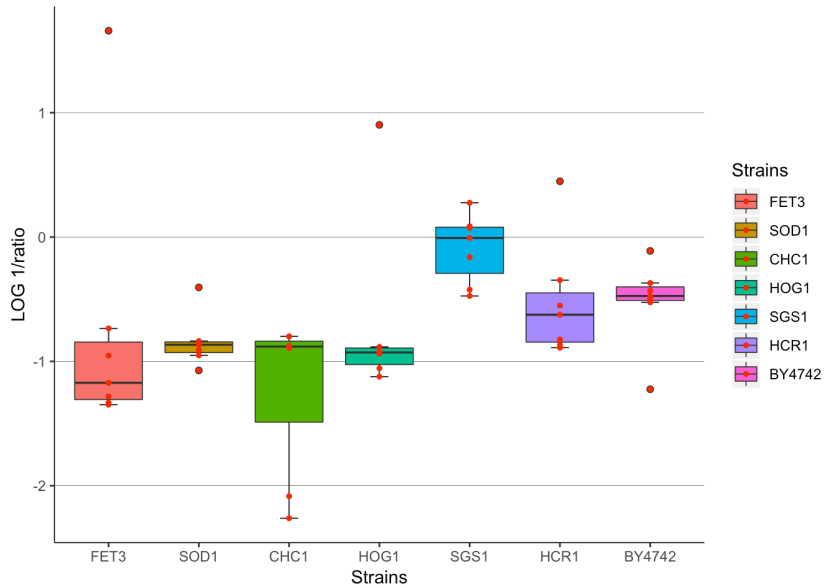


Figure 4. Light mediated growth repression of yeast. Growth in darkness was compared to growth in constant blue light at 15 °C. Single experiments are indicated by red spheres. All strains except YLR192C are significantly different relative to the control.

Discussion and conclusion

The circadian clock is often referred to as a success story for the field of behavioural genetics. Genetic screens and molecular genetic technologies provided breakthroughs in identification of clock genes[33–36], loosely defined as genes without which free-running circadian rhythms are disturbed or absent. The validated clock genes themselves encode highly networked transcription factors and kinases. In addition, collections of CCGs for many organisms and tissues have accumulated in public databases and can be used in diverse ways to generate hypotheses concerning clock regulation and clock regulated physiology[37]. The CCGs, by definition regulated directly or indirectly by the circadian clock mechanism, have rarely been directly linked to said mechanism. In addition, substantial experimental evidence has accumulated concerning non-transcriptional levels of circadian clock regulation. Indeed, puzzling evidence of non-transcription-based clocks have been published. One interpretation of these observations is that embedding circadian clocks at diverse levels will enable their function under diverse conditions, thus fulfilling the prediction that the circadian clock should be adaptive.

How to model this? Combining and integrating information from different levels can be done by using machine learning techniques. The putative clock protein CHRONO was identified by combining time course results, interactions, ubiquitous expression and sequence homology using Naïve Bayes learning[38]. Indeed, machine learning typically depends on well characterized datasets to train the algorithm, *e.g.* to predict protein interactions using text mining with support vector machines[39]. Based on the data resources used and contextual understanding of the algorithm, machine learning could also assign cross-level relationships. However, different data resources require different approaches in data processing because using a single data resource may bias results.

Some data repositories collect data from ‘all’ functional and/or association interactions, including proteins, genes, transcripts, metabolites, etc. and, as these resources mature, they can be mined for insights into how a given network functions in a given tissue. We thus developed an algorithm called PREMONition that uses a set of known clock genes and CCGs in conjunction with various resources encapsulated within the STRING database (*e.g.* SMART, KEGG, etc.) to construct a plausible circadian molecular interaction network that can be used as a springboard for hypothesis generation and validation.

The PREMONition tool

There are several algorithms that have been used to predict networks. Breadth First Search (BFS)[40] visits all child nodes before making extension nodes. Depth First Search (DFS)[41] first goes into depth of a single child before going on the next child. Depth Limited Search (DLS) uses DFS with a limited search space and a fixed search depth[42]. It increases the search depth until a solution is found, within the search space. Iterative Deepening Depth First Search (IDDFS)[42] is similar to DFS, but continually expands its search depth until a solution is found. PREMONition uses a DFS implementation, since it makes an uninformed (*i.e.* all edges have the same probability), fully interconnected network. However, as in DLS, it uses a limited search space (a fixed number of NRCs are allowed between the CCGs). When the search limit is reached, the algorithm reverts to its parental node and deepens other child nodes.

PREMONition exploits the power of curated, precomputed functional interaction data (*e.g.* protein-protein interaction inferred from screens or shown in targeted experiments). It is an approach, with similarities to Biana[43], POINeT[44], SNOW[45] and UnuHI[46], that can be used to construct a network. With the publication of genome-wide, protein interaction databases, several methods to predict the proteome-wide interactomes have been published[47,48]. PREMONition uses many of the same resources as other methods but aims not to predict an interactome that incorporates the entire proteome, on one extreme, nor one that involves only a single input protein, on the other. Rather, we wish to form an interaction network around the molecules encoded by clock and clock-controlled genes. We achieve this by allowing the insertion of single NRCs (linker molecules). Increasing the number of NRCs, similar to expanding the search limit in DLS, will also increase the “noise” (non-specific or less specific interactions that would thus be more likely to be incorrectly assigned) in the network. In contrast to BFS, DFS, DLS and IDDFS, PREMONition tries to decrease noise and to increase the representation of more likely network components by removal of all but the most statistically significant interactors via an iterative process of sequential edge removal of the least probable interactor. On one hand, this makes visualization and analysis of the interaction network simpler. On the other hand, NRCs and their pruning also effect network parameters such as connectivity, degree distribution, clustering coefficient, characteristic path length network diameter and between-ness. It is striking that the set of rhythmically expressed components – CCGs with their accompanying experimental evidence - fails to form a cohesive network unless NRCs, based on their own experimental evidence, are integrated. The resulting networks derived for different organisms are functionally similar based on GO analysis (Fig. S1 and Table S3) despite the fact that input datasets are distinct.

The PREMONition algorithm is adaptable. Of the two input components, both can be modified for stringency. The set of rhythmic genes can be increased or decreased according to statistical thresholds. It could also be limited to those that oscillate with a certain phase to predict time-of-day-specific molecular networks. Or based on a set of cell/ tissue/ organ specific expressed genes to predict clock-controlled processes that are relevant for the distinct function of cells/tissues/organs[49]. Similarly, the NRCs can be increased or decreased in number by changing statistical threshold of an interaction or by altering the allowed number of connections. Alternative or additional datasets could be used as source data, including those for non-circadian biology, to predict non-clock molecular mechanisms. (Circadian clocks modulate expression over time of day but in clock-less mutants these genes are typically also expressed.)

A limitation of the PREMONition method is that it requires a mature interaction database (we used STRING exclusively for these experiments). The formation of the network thus depends on the quantity and quality of the annotation of the interactions. In emerging model systems or those that are less studied, there will simply be less data available thus leading to a sparse or less accurate network. With respect to the models that we used, the STRING database contains 3,364 *N. crassa* proteins connected with 24,332 interactions with a probability score ≥ 0.70 and an average confidence score of 0.84 (on average 7 connections per molecule). *D. melanogaster* is represented with 5,598 proteins connected with 93,915 interactions and an average confidence score of 0.89 (on average 16 connections per molecule). This roughly reflects the 7 times more publications in PubMed for *D. melanogaster* relative to those for *N. crassa.* Further, the network composition will be biased toward the most successful research for that system: if an organism has been primarily used to study enzymatic pathways, then the archived data – and the network formed from it – will be fundamentally different than that for an organism that has been used to study development. A disadvantage of using the STRING database is that it merely provides a probability of interaction in a static network, whilst the true interaction probability may be time-of-day dependent[50], as well as condition or tissue dependent.

Insights into the clock in yeast based on PREMONition network topology

We identified the most densely connected components of the reconstructed yeast network, reasoning that they may suggest organism-specific regulatory mechanisms (Table S5). Several of these densely-connected proteins are involved in (generic) transcription and translation processes (TIF3, the translation initiation factor eIF-4B; HCR1, part of the eIF-3 complex; RPA135, the RNA polymerase; RPC37, a subunit of RNA polymerase complex;

TFA1, a recruiter of polymerase II; IKI3, a subunit of the elongator complex; and HAS1, an ATP dependent RNA helicase). These, *per se,* are not likely first-line clock components. HIR2 also regulates transcription, as part of a complex regulating nucleosome assembly. This protein recruits SWI-SNF, which plays an important role in nucleosome remodeling and carbon source switching[51]. SNF1, together with HOG1, whose homolog is clock regulated in *N. crassa*[52], and BMH2, regulates MSN2/4, which in turn regulates the expression of PRX1[53]. The PREMONition yeast network thus maps out a possible mode of how the clock regulates PRX, whose rhythmicity in oxidation state is observed in eukaryotes, prokaryotes and even in the archeae[54,55].

Insights based on comparing networks

We compared the genes which are common in the human, *D. melonogaster, N. crassa* and yeast networks (using GO and Inparanoid) revealing 13 genes common to all four networks (Table 2). Of these, two genes are rhythmic in yeast with a p-value < 0.05 (YDR188W (CCT6) and YPR173C (VPS4)). The remaining 11 would not have been included in this dataset without incorporating NRCs. Clustering all 13 genes using PANTHER[56] provides insights into their functions. Ten of these genes are involved in nitrogen compound transport: *CDC5, ACT1, RPD3, RPN11, HRR25, HOG1, EFB1, CDC19* and *TKL1*. We observed clock regulation of an ammonium and an amino acid permease in yeast under conditions permissive for circadian entrainment[7] and nitrogen metabolism and utilization is known to be regulated by the circadian system in prokaryotes (the cyanobacterium *Synechococcus* fixes nitrogen during the night[57]) and in eukaryotes at multiple levels in many organisms (*e.g.*[58]). Three genes shared by all of our networks are involved in vacuole organization: *YPT7, ACT1* and *HRR25*. Of these, *HRR25* encodes a Casein kinase I homolog with many diverse functions in most organisms. Casein kinase plays a major role in the mammalian circadian clock through phosphorylation of the PERIOD proteins and BMAL1[59] and core clock proteins in *D. melanogaster*[60].

Table 2. Yeast genes included in the yeast PREMONition network which have overlapping gene ontology terms and orthologs in the human, fly and fungus networks.

Systematic name	Standard name	Name description
YNL330C	RPD3	Reduced Potassium Dependency
YPL204W	HRR25	HO and Radiation Repair
YDR188W	CCT6	Chaperonin Containing TCP-1
YIL021W	RPB3	RNA Polymerase B
YML001W	YPT7	Yeast Protein Two
YFL039C	ACT1	ACTin
YAL003W	EFB1	Elongation Factor Beta
YLR113W	HOG1	High Osmolarity Glycerol response
YMR001C	CDC5	Cell Division Cycle
YPR173C	VPS4	Vacuolar Protein Sorting
YPR074C	TKL1	TransKetoLase
YAL038W	CDC19	Cell Division Cycle
YFR004W	RPN11	Regulatory Particle Non-ATPase

Four genes (*CDC5, HRR25, HOG1, CDC19*) encode proteins with kinase activity, a group of proteins known to be involved in the regulation and functioning of the circadian clock[61]. Yeast *HOG1* is an ortholog of *N. crassa cka* and hog-1, of which *cka* is known to be involved in its circadian clock[62]. When it is mutated Neurospora circadian rhythms are abolished[63]. *HOG1* is also a homolog of *OS-2* mitogen-activated protein kinase (MAPK). In *N. crassa* there is a feedback loop between the circadian clock and the MAPK pathway[31]. *N. crassa* encodes two other MAPK genes; MAK-2, a homolog of FUS3, and MAK-1, a homolog of SLT2 (YHR030C) and shaggy (*D. melanogaster*) which is a key player in the fly circadian clock through phosphorylation of TIMELESS[64]. *SLT2* is included in the yeast PREMONition network as an NRC (q-value for transcriptional rhythmicity = 0.74). *RPN11* is a homolog of Rpn11 (DmS13) in *D. melanogaster*. In *Drosophila*, light-dependent CRY degradation is regulated by the proteasome of which DmS13 is a subunit[65]. Genetic screens in mice have also yielded genes involved in the proteasome as key circadian clock regulators[66]. *CCT6* is homologous to the human *PSMD5*, a proteasome component, and acts as a chaperone during the assembly of the 26S proteasome. Interestingly, this protein shows interaction, via UBB and UBC, with CRY1. *CRY1* encodes a flavin adenine dinucleotide-binding protein, a key clock protein in mammals. *VPS4* is an ortholog of *VPS4B* in humans and *VPS4* (NCU06942) of *N. crassa*, both vacuolar protein sorting-associated proteins. Although no direct relationship between *VPS4* and the circadian clock have been reported, several suggestive physiologies are impacted by decreased function of VPS4. These include: increased heat sensitivity (possibly changing sensitivity to a zeitgeber), decreased replicative lifespan, chronological lifespan and decreased rate of respiratory growth (possibly related to the phenotype used to demonstrate circadian entrainment in yeast[67])[68–70]. The PREMONition NRCs shared by three networks thus point to conserved clock-relevant pathways. However, the number of candidate clock-involved genes regulating those pathways in yeast is drastically decreased by our algorithm.

The yeast PREMONition network in general

Whilst a large proportion of the PREMONition network components in yeast do not have a direct homolog in the networks of the other three species, they do have homologous function. The RAD23 and RAD14 proteins bind damaged DNA, reminiscent of a documented clock function in mammals and the green yeast, *Chlamydomonas*[71,72]. CNM67, a hub with 16 connections in the yeast PREMONition network, is involved in spindle body formation, a connection to the cell cycle machinery, linking this network to observations of gating of cell division[73,74]. A subunit of the ubiquitin ligase complex, SLX5, together with DOA1, brings this aspect of protein degradation pathway into daily temporal regulation in yeast[65]. Although most of these functional classes are more or less expected, if one were using function as a predictor it would be impractical to sort through the hundreds of candidates with similar function based on *e.g.* GO categories. The PREMONition network suggests which proteins/molecules could be involved in the yeast clock.

In summary, we have developed a template for building interconnected networks that span multiple functional levels. The PREMONition algorithm depends on combining defined input sets of function-specific experimental results and non-specific, evidence-based, curated database entries. By incorporating the totality of interaction data, not just genetic interactions, we can move beyond transcription to different functional levels, achieving a molecular network that reflects the complexity of biological mechanisms.

Materials and Methods

Network construction

The network reconstruction tool and GO analysis method were both written in the programming language Python (version 3.7.2). Reconstructed network visualizations were made in Cytoscape (version 3.2.1)[75]. The network construction method consists of reading in pairs of molecules (as referenced by protein/gene names) and their interaction (ppi) probabilities that meet a set threshold to generate a network. This is done in a recursive manner, starting with a single protein, a seed, which grows into a network until no additional connections can be made. Then, the next protein is seeded etc. During this process the method is able to integrate NRCs in order to reach out to molecules encoded by rhythmic genes.

For this study, only one NRC between two rhythmic genes/proteins was allowed hence a last step was added to remove excess connections. After network construction, some proteins are highly connected. This makes the visual investigation of specific pathways difficult. We thus developed a routine to reduce complexity whilst maintaining the overall integrity of the network. Hence, for each connection, we asked “are all proteins in the network still connected if this connection is removed?”. If all proteins remain connected, then the connection is redundant and not required for network integrity[76]. If not, the connection is maintained. The result is a network with a minimal number of connections. The remaining connections have the highest interaction probability score.

Known protein-protein and inferred functional interactions were obtained from the STRING interaction database (“protein.links.detailed.v9.1.txt”[77]). Each interaction is associated with an interaction probability score as derived from different information sources. To create a molecular interaction network, all interactions for a given organism that met a given interaction threshold were extracted. Taxonomy numbers used were; Hs:9606, Dm:7227, Nc:5141, and Sc:4932. The CCGs were used for network construction based on the interaction confidence scores provided by STRING. In this study, an interaction confidence score with a threshold of 0.6 was assigned for *Neurospora* and 0.7 for human, *Drosophila* and yeast networks.

Network analysis

In order to analyse the (re)constructed molecular interaction networks for functionality we assessed the enrichment of Gene Ontology (GO) annotations within the network using a hypergeometric test, with all p-values adjusted using the Benjamini and Hochberg method to correct for multiple testing. This is comparable to the GOrilla method[78]. A False Discovery Rate

of 0.05 was maintained. The most complete GO annotations for any organism typically reside in organism-specific databases supported by the community. Hence GO annotations were obtained from the Broad Institute for *N. crassa (https://www.broadinstitute.org),* Flybase for *D. melanogaster (http://flybase.org),* GOC for human (*http://www.geneontology.org*), and the *S. cerevisiae* Database *(https://www.yeastgenome.org).* For the 1,000 yeast randomized GO experiments, the YeastMine API was used[79]. All ORFs, both verified and uncharacterized, were included in the background. Holm-Bonferroni multiple-testing correction[80] was applied. All genes with a corrected p-value <= 0.05 (q-value) were considered significant.

Homology analysis

Homologous were identified using inParanoid annotated sequences. InParanoid is an algorithm designed with the aim to generate ortholog groups that include all inparalogs (orthologs duplicated after the speciation event) but no outparalogs (orthologs in two species, resulting from a gene duplication event prior to a speciation event). It uses Basic Local Alignment Search Tool (BLAST) comparisons of all protein sequences in two species followed by a number of clustering rules to build ortholog groups[17].

In order to use the InParanoid8 database, several ID-mapping steps were performed. Two hundred and sixty-nine *N. crassa* STRING identifiers were converted to NCBI Gene IDs (using NCBI genome.gff). These Gene IDs were subsequently mapped to Uniprot identifiers using the Uniprot mapping tool. For each organism (human, N.c and D.m) STRING identifiers had to be converted to Uniprot identifiers. For N.c, 269 STRING IDs were translated to NCBI Gene IDs; these were further converted by applying the Uniprot mapping tool to 283 yield unique refSeq protein IDs. 246 unique D.m. STRING IDs were converted to 219 refSeq protein ID (using Flybase) which then were converted to 267 unique Uniprot protein IDs. Finally, 258 unique H.s. STRING ID's were translated into 245 unique Uniprot protein ID's.

All orthologous protein pairs, including orthologous clusters identified by Inparanoid 8 (information downloaded on 25-March-2018), *i.e.*, no actual filter, was used to identify orthologs between all three species. Enrichment of orthologous molecules within the networks was assessed using Phyper[81] (Package stats version 3.2.2), a method to perform the hypergeometric test, from which a q-value < 0.05 indicates significant enrichment.

Yeast Fermentor Culture Conditions

To reveal genes and processes involved in the regulation of the circadian clock of *S. cerevisiae*, a microarray experiment was performed according to methods described earlier[7]. Samples were taken from fermenter cultures (1×10^9 yeast cells) every 3 hours over the first day of

constant conditions (free-run), starting 1h before the temperature transition from 21°C to 28°C. Cells were centrifuged for 1 minute at 4°C and 14000 rpm. Pellets were flash-frozen in liquid nitrogen and stored at -80°C until processing.

RNA Preparation

Total RNA was obtained using a modified version of the hot phenol method[82]. The flash-frozen yeast pellets were suspended in 400 µL AE buffer (50 mM NaOAc pH 5.3 and 10 mM EDTA) to which 40 µL SDS (10%) and 400 µL acidic phenol were added. Next, cells were disrupted by vortexing, followed by heating the sample to 65 °C for 30 min. The disrupted cells were quickly chilled on ice, centrifuged, and the aqueous phase was re-extracted using 400 µL acidic phenol followed by the addition of 400 µL chloroform, mixing, centrifuging and re-extraction of the liquid phase. The final step was purification and concentration of the RNA samples using the NucleoSpin RNA II kit (Macherey-Nagel). The RNA was used for transcriptome analysis (Service XS Leiden; Affymetrix yeast genome 2.0 arrays). Data can be accessed via Gene Expression Omnibus ID: GSE122152.

Gene expression

Microarray data was analysed using the functional programming language R (version 2.15) and Robust Multi-array Average (RMA) normalization[83] (Bioconduction “affy” package, version 1.34.0). The normalized expression values were analysed for rhythmicity with a 24 h period using CircWave[18]. This method performs an F-test on the fitted harmonics and calculates a p-value (with multiple testing correction). We used only highly significant genes with a p-value less than 0.01. The thresholds used for the selection of rhythmic genes (‘CCGs’) in human, fly, fungus and yeast are listed in Table S6.

Light sensitivity assay

The knockout library was obtained from EUROSCARF[84] (Frankfurt am Main, Germany). Selected knockout strains were individually cultured overnight in 150 µl YPD (1% yeast extract, 2% peptone, 2% dextrose) medium in one well of a 96 well plate. Plates were held at 30°C with shaking at 160 RPM. The following day, the cultures were placed at 4°C until further use. On the day before initiating the growth assay, 10 µl from each well of the 96-well plates were inoculated into 6 ml of YPD medium. The cultures were grown overnight at 30°C with shaking at 160 RPM. Prior to making the final dilution series, the cell number in the overnight cultures was adjusted such that the OD_{600} was 0.1. From this cell suspension, a series of five 1:3 dilutions were made in YPD. From each dilution, 2 µl was spotted on two independent YPD plates containing 2% agar. The knockout strains were compared to their parental wild-type

strain (BY4742). Of the two, replicate plates containing replicate sets of dilution series, one was wrapped in aluminum foil (dark control) and then both were placed in the experimental setup. The dark controls thus control for small temperature effects. The light source consisted of 500 lux blue light LEDs (Bartheleme product # 1484330, 12V DC). Incubations were carried out in an incubator with the temperature (measured at the level of the plates) at 15.0 °C. Plates were incubated for four days, after which their images were saved (BioRad, ChemiDoc MP Imaging System) for analysis.

The scans of the plates were analyzed using an in-lab developed Python routine that determines the amount of growth *i.e.* the pixel intensity and density of each spot within a circle of a fixed radius. All pixels within the circle having a pixel intensity higher than a given background BRG-value are counted and used as a quantitative measure for colony formation. Data were analysed by the following method. The dilution where 25% growth of BY4742 control strain was determined for both light and dark incubation conditions (the value was interpolated from the growth-curves). For each strain, the LOG(1/(light / dark ratio)) was calculated. This conversion leads to an intuitive (lower) value for growth repression relative to control values.

Acknowledgements

We thank members of the Merrow lab, Marco Grzegorczyk and John Hogenesch for comments on the manuscript. We thank Margriet van der Pol and Edwin Eelderink for technical assistance. This work was supported by the NWO (Open Programma, 820.02.014), the European Commission (FP6 EUCLOCK), the Rosalind Franklin Fellowships of the University of Groningen and LMU Munich.

Author Contributions

J.B. and M.M. conceived the project, analysed data and wrote the paper. J.B. and Z.C.-E. performed experiments. E. L. analysed data and contributed to writing the paper.

Literature references

1. Hogenesch, J. B. & Ueda, H. R. Understanding systems-level properties: timely stories from the study of clocks. *Nat. Rev. Genet.* 12, 407–416 (2011).
2. Merrow, M., Spoelstra, K. & Roenneberg, T. The circadian cycle: daily rhythms from behaviour to genes. *EMBO Rep.* 6, 930–5 (2005).
3. Merrow, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurospora clock gene frequency. *Nature* 399, 584–586 (1999).
4. Lakin-Thomas, P. L. & Brody, S. Circadian rhythms in Neurospora crassa: lipid deficiencies restore robust rhythmicity to null frequency and white-collar mutants. *Proc. Natl. Acad. Sci. U. S. A.* 97, 256–61 (2000).
5. Edgar, R. S. *et al.* Peroxiredoxins are conserved markers of circadian rhythms. *Nature* 485, (2012).
6. Maywood, E. S., Chesham, J. E., O'Brien, J. A. & Hastings, M. H. A diversity of paracrine signals sustains molecular circadian cycling in suprachiasmatic nucleus circuits. *Proc. Natl. Acad. Sci.* 108, 14306–14311 (2011).
7. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2043–2047 (2010).
8. Franceschini, A. *et al.* STRING v9.1: Protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res.* 41, (2013).
9. Hughes, M. E., Grant, G. R., Paquin, C., Qian, J. & Nitabach, M. N. Deep sequencing the circadian and diurnal transcriptome of Drosophila brain. *Genome Res.* 22, 1266–1281 (2012).
10. Hughes, M. E., Hogenesch, J. B. & Kornacker, K. JTK-CYCLE: An efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. *J. Biol. Rhythms* 25, 372–380 (2010).
11. Correa, A. *et al.* Multiple oscillators regulate circadian gene expression in Neurospora. *Proc. Natl. Acad. Sci. U. S. A.* (2003) doi:10.1073/pnas.2233734100.
12. Reddy, A. B. *et al.* Circadian Orchestration of the Hepatic Proteome. *Curr. Biol.* 16, 1107–1115 (2006).
13. Robles, M. S., Cox, J. & Mann, M. In-Vivo Quantitative Proteomics Reveals a Key Contribution of Post-Transcriptional Mechanisms to the Circadian Regulation of Liver Metabolism. *PLoS Genet.* 10, (2014).
14. Mauvoisin, D. *et al.* Circadian clock-dependent and -independent rhythmic proteomes implement distinct diurnal functions in mouse liver. *Proc. Natl. Acad. Sci.* 111, 167–172 (2014).

15. Robles, M. S., Humphrey, S. J. & Mann, M. Phosphorylation Is a Central Mechanism for Circadian Control of Metabolism and Physiology. *Cell Metab.* 1–10 (2016) doi:10.1016/j.cmet.2016.10.004.
16. Ashburner, M. *et al.* Gene Ontology: tool for the unification of biology. *Nat. Genet.* 25, 25–29 (2000).
17. Sonnhammer, E. L. L. & Östlund, G. InParanoid 8: Orthology analysis between 273 proteomes, mostly eukaryotic. *Nucleic Acids Res.* 43, D234–D239 (2015).
18. Oster, H., Damerow, S., Hut, R. A. & Eichele, G. Transcriptional Profiling in the Adrenal Gland Reveals Circadian Regulation of Hormone Biosynthesis Genes and Nucleosome Assembly Genes. *J. Biol. Rhythms* 21, 350–361 (2006).
19. Ułaszewski, S. *et al.* Light effects in yeast: Evidence for participation of cytochromes in photoinhibition of growth and transport in Saccharomyces cerevisiae cultured at low temperatures. *J. Bacteriol.* 138, 523–529 (1979).
20. Robertson, J. B., Davis, C. R. & Johnson, C. H. Visible light alters yeast metabolic rhythms by inhibiting respiration. *Proc. Natl. Acad. Sci.* 110, 21130–21135 (2013).
21. Jang, Y. S., Lee, M. H., Lee, S. H. & Bae, K. Cu/Zn superoxide dismutase is differentially regulated in period gene-mutant mice. *Biochem. Biophys. Res. Commun.* 409, 22–27 (2011).
22. Colepicolo, P., Camarero, V. C. P. C. & Hastings, J. W. A circadian rhythm in the activity of superoxide dismutase in the photosynthetic alga gonyaulax polyedra. *Chronobiol. Int.* 9, 266–268 (1992).
23. Yoshida, Y., Maeda, T., Lee, B. & Hasunuma, K. Conidiation rhythm and light entrainment in superoxide dismutase mutant in Neurospora crassa. *Mol. Genet. Genomics* 279, 193–202 (2008).
24. Puig, S. Function and regulation of the plant COPT family of high-affinity copper transport proteins. *Adv. Bot.* 2014, 1–9 (2014).
25. Horn, D., Al-Ali, H. & Barrientos, A. Cmc1p is a conserved mitochondrial twin CX9C protein involved in cytochrome c oxidase biogenesis. *Mol. Cell. Biol.* 28, 4354–4364 (2008).
26. Yamada, Y. & Prosser, R. A. Copper chelation and exogenous copper affect circadian clock phase resetting in the suprachiasmatic nucleus in vitro. *Neuroscience* 256, 252–261 (2014).
27. Ito, H. *et al.* Cyanobacterial daily life with Kai-based circadian and diurnal genome-wide transcriptional control in Synechococcus elongatus. *Proc. Natl. Acad. Sci. U. S. A.* 106, 14168–73 (2009).
28. Kilman, V. L., Zhang, L., Meissner, R. A., Burg, E. & Allada, R. Perturbing Dynamin

Reveals Potent Effects on the Drosophila Circadian Clock. *PLoS One* 4, (2009).

29. Pouly, D. *et al.* USP2-45 is a circadian clock output effector regulating calcium absorption at the post- Translational level. *PLoS One* 11, (2016).
30. Coogan, A. N. & Piggins, H. D. MAP kinases in the mammalian circadian system - Key regulators of clock function. *Journal of Neurochemistry* vol. 90 769–775 (2004).
31. Bennett, L. D., Beremand, P., Thomas, T. L. & Bell-Pedersen, D. Circadian activation of the mitogen-activated protein kinase MAK-1 facilitates rhythms in clock-controlled genes in Neurospora crassa. *Eukaryot. Cell* (2013) doi:10.1128/EC.00207-12.
32. Jouffe, C. *et al.* The Circadian Clock Coordinates Ribosome Biogenesis. *PLoS Biol.* 11, (2013).
33. Bargiello, T. A., Jackson, F. R. & Young, M. W. Restoration of circadian behavioural rhythms by gene transfer in Drosophila. *Nature* 312, 752–754 (1984).
34. Hardin, P. E., Hall, J. C. & Rosbash, M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature* 343, 536–540 (1990).
35. Zehring, W. A. *et al.* P-element transformation with period locus DNA restores rhythmicity to mutant, arrhythmic drosophila melanogaster. *Cell* 39, 369–376 (1984).
36. Aronson, B. D., Johnson, K. a, Loros, J. J. & Dunlap, J. C. Negative feedback defining a circadian clock: autoregulation of the clock gene frequency. *Science (80-.).* 263, 1578–1584 (1994).
37. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. & Hogenesch, J. B. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc. Natl. Acad. Sci. U. S. A.* 111, 16219–24 (2014).
38. Anafi, R. C. *et al.* Machine Learning Helps Identify CHRONO as a Circadian Clock Component. *PLoS Biol.* 12, (2014).
39. Relógio, A. *et al.* Ras-Mediated Deregulation of the Circadian Clock in Cancer. *PLoS Genet.* 10, (2014).
40. Moore, E. F. The shortest path through a maze. *Proc. an Int. Symp. Theory Switch. (Cambridge, Massachusetts, 2–5 April 1957). Cambridge Harvard Univ. Press* 285–292 (1959) doi:10.1177/0959354304043641.
41. Riansanti, O., Ihsan, M. & Suhaimi, D. Connectivity algorithm with depth first search (DFS) on simple graphs. in *Journal of Physics: Conference Series* vol. 948 (2018).
42. Korf, R. E. Depth-First Iterative-Deeping: An Optimal Admissible Tree Search. *Artif. Intell.* 27, 97–109 (1985).
43. Garcia-Garcia, J., Guney, E., Aragues, R., Planas-Iglesias, J. & Oliva, B. Biana: a software framework for compiling biological interactions and analyzing networks. *BMC*

Bioinformatics 11, 56 (2010).

44. Lee, S.-A. *et al.* POINeT: protein interactome with sub-network analysis and hub prioritization. *BMC Bioinformatics* 10, 114 (2009).
45. Minguez, P., Götz, S., Montaner, D., Al-Shahrour, F. & Dopazo, J. SNOW, a web-based tool for the statistical analysis of protein-protein interaction networks. *Nucleic Acids Res.* 37, (2009).
46. Chaurasia, G. *et al.* UniHI: An entry gate to the human protein interactome. *Nucleic Acids Res.* 35, (2007).
47. Sanz-Pamplona, R. *et al.* Tools for protein-protein interaction network analysis in cancer research. *Clinical and Translational Oncology* vol. 14 3–14 (2012).
48. Koh, G. C. K. W., Porras, P., Aranda, B., Hermjakob, H. & Orchard, S. E. Analyzing protein-protein interaction networks. *J. Proteome Res.* 11, 2014–2031 (2012).
49. Storch, K.-F. *et al.* Extensive and divergent circadian gene expression in liver and heart. *Nature* 417, 78–83 (2002).
50. Wallach, T. *et al.* Dynamic Circadian Protein-Protein Interaction Networks Predict Temporal Organization of Cellular Functions. *PLoS Genet.* 9, (2013).
51. Sudarsanam, P. & Winston, F. The Swi/Snf family: Nucleosome-remodeling complexes and transcriptional control. *Trends in Genetics* vol. 16 345–351 (2000).
52. Lamb, T. M., Finch, K. E. & Bell-Pedersen, D. The Neurospora crassa OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes. *Fungal Genet. Biol.* 49, 180–188 (2012).
53. Monteiro, G. & Netto, L. E. S. Glucose repression of PRX1 expression is mediated by Tor1p and Ras2p through inhibition of Msn2/4p in Saccharomyces cerevisiae. *FEMS Microbiol. Lett.* 241, 221–228 (2004).
54. Edgar, R. S. *et al.* Peroxiredoxins are conserved markers of circadian rhythms. *Nature* (2012) doi:10.1038/nature11088.
55. Olmedo, M. *et al.* Circadian regulation of olfaction and an evolutionarily conserved, nontranscriptional marker in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. U. S. A.* 109, 20479–84 (2012).
56. Mi, H. *et al.* PANTHER version 11: Expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.* 45, D183–D189 (2017).
57. Johnson, C. H., Stewart, P. L. & Egli, M. The Cyanobacterial Circadian System: From Biophysics to Bioevolution. *Annu. Rev. Biophys.* 40, 143–167 (2011).
58. Jeyaraj, D. *et al.* Klf15 orchestrates circadian nitrogen homeostasis. *Cell Metab.* (2012) doi:10.1016/j.cmet.2012.01.020.

59. Eide, E. J., Kang, H., Crapo, S., Gallego, M. & Virshup, D. M. Casein kinase I in the mammalian circadian clock. *Methods Enzymol.* 393, 408–418 (2005).
60. Chiu, J. C., Vanselow, J. T., Kramer, A. & Edery, I. The phospho-occupancy of an atypical SLIMB-binding site on PERIOD that is phosphorylated by DOUBLETIME controls the pace of the clock. *Genes Dev.* 22, 1758–1772 (2008).
61. Reischl, S. & Kramer, A. Kinases and phosphatases in the mammalian circadian clock. *FEBS Letters* vol. 585 1393–1399 (2011).
62. Baker, C. L., Loros, J. J. & Dunlap, J. C. The circadian clock of Neurospora crassa. *FEMS Microbiology Reviews* (2012) doi:10.1111/j.1574-6976.2011.00288.x.
63. Yang, Y., Cheng, P., He, Q., Wang, L. & Liu, Y. Phosphorylation of FREQUENCY protein by casein kinase II is necessary for the function of the Neurospora circadian clock. *Mol. Cell. Biol.* 23, 6221–8 (2003).
64. Harms, E., Young, M. W. & Saez, L. CK1 and GSK3 in the Drosophila and mammalian circadian clock. *Novartis Found. Symp.* 253, 267–77; discussion 102-9, 277–84 (2003).
65. Sathyanarayanan, S. *et al.* Identification of novel genes involved in light-dependent CRY degradation through a genome-wide RNAi screen. *Genes Dev.* 22, 1522–33 (2008).
66. Godinho, S. I. H. *et al.* The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* 316, 897–900 (2007).
67. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, (2010).
68. Schleit, J. *et al.* Molecular mechanisms underlying genotype-dependent responses to dietary restriction. *Aging Cell* 12, (2013).
69. Marek, A. & Korona, R. Restricted pleiotropy facilitates mutational erosion of major life-history traits. *Evolution (N. Y).* 67, 3077–3086 (2013).
70. Steinmetz, L. M. *et al.* Systematic screen for human disease genes in yeast - Nature Genetics. *Nat. Genet.* 31, 400–4 (2002).
71. Michael, A. K., Fribourgh, J. L., Van Gelder, R. N. & Partch, C. L. Animal Cryptochromes: Divergent Roles in Light Perception, Circadian Timekeeping and Beyond. *Photochemistry and Photobiology* vol. 93 128–140 (2017).
72. Matsuo, T. & Ishiura, M. Chlamydomonas reinhardtii as a new model system for studying the molecular basis of the circadian clock. *FEBS Letters* (2011) doi:10.1016/j.febslet.2011.02.025.
73. Brachat, A., Kilmartin, J. V, Wach, A. & Philippsen, P. Saccharomyces cerevisiae Cells with Defective Spindle Pole Body Outer Plaques Accomplish Nuclear Migration

via Half-Bridge-organized Microtubules. *Mol. Biol. Cell* 9, 977–991 (1998).

74. EDMUNDS, L. N., LAVAL-MARTIN, D. L. & GOTO, K. Cell Division Cycles and Circadian Clocks: Modeling a Metabolic Oscillator in the Algal Flagellate Euglena. *Ann. N. Y. Acad. Sci.* 503, 459–475 (1987).
75. Shannon, P. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* 13, 2498–2504 (2003).
76. Truong, C. D. & Kwon, Y. K. Investigation on changes of modularity and robustness by edge-removal mutations in signaling networks. *BMC Syst. Biol.* 11, (2017).
77. Szklarczyk, D. *et al.* The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkw937.
78. Eden, E., Navon, R., Steinfeld, I., Lipson, D. & Yakhini, Z. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10, 48 (2009).
79. Balakrishnan, R. *et al.* YeastMine-An integrated data warehouse for Saccharomyces cerevisiae data as a multipurpose tool-kit. *Database* 2012, (2012).
80. Holm, S. A Simple Sequentially Rejective Multiple Test Procedure. *Scand J Stat.* 6, 65–70 (1979).
81. Kachitvichyanukul, V. & Schmeiser, B. Computer generation of hypergeometric random variates. *J. Stat. Comput. Simul.* 22, 127–145 (1985).
82. Schmitt, M. E., Brown, T. A. & Trumpower, B. L. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Res.* (1990) doi:10.1093/nar/18.10.3091.
83. Irizarry, R. A. *et al.* Comprehensive high-throughput arrays for relative methylation (CHARM). *Genome Res.* 18, 780–790 (2008).
84. Winzeler, E. a. Functional Characterization of the S. cerevisiae Genome by Gene Deletion and Parallel Analysis. *Science (80-.).* (1999) doi:10.1126/science.285.5429.901.

Supporting Information Materials

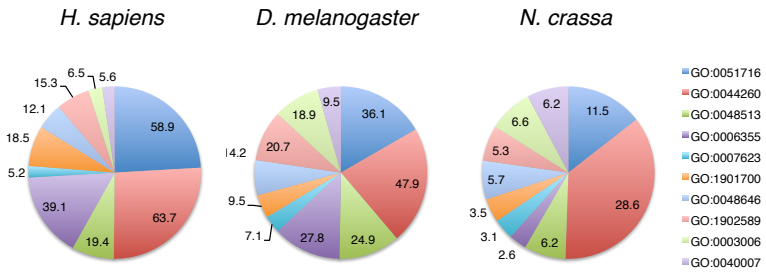


Figure S1. Pie charts showing the percentage of genes annotated to the ten child GO-terms that are significantly enriched in all three organism PREMONition networks. Percentages do not add up to 100% because one gene can be associated with multiple GO-terms.

Table S1. Sets of clock genes in *Homo sapiens*, *Drosophila melanogaster* and *Neurospora crassa* used for network construction.

Common/ abbreviated name	Gene name	String ID
	N. crassa	
frg	*frequency*	NCU02265.1
wc-1	*white collor-1*	NCU02356.1
wc-2	*white collor-2*	NCU00902.1
vvd	*vivid*	NCU03967.1
ck1a	*casein kinase I*	NCU04005.1
cka (prd3)	*casein kinase II*	NCU03124.1
	D. melanogaster	
per	*period*	FBpp0070455
tim	*timeless*	FBpp0077255
clk	*clock*	FBpp0099478
cyc	*cycle*	FBpp0074693
vri	*vrille*	FBpp0289297
dbt	*double-time*	FBpp0085106
sgg	*shaggy*	FBpp0070454
	H. sapiens	
ARNTL (BMAL1)	aryl hydrocarbon receptor nuclear translocator-like	ENSP00000374357
ARNTL2 (BMAL2)	aryl hydrocarbon receptor nuclear translocator-like 2	ENSP00000266503
CLK	clock circadian regulator	ENSP00000308741
CRY1	cryptochrome circadian clock 1	ENSP00000008527
CRY2	cryptochrome circadian clock 2	ENSP00000406751
PER1	period circadian clock 1	ENSP00000314420
PER2	period circadian clock 2	ENSP00000254657
PER3	period circadian clock 3	ENSP00000355031
NR1D1	nuclear receptor subfamily 1	ENSP00000246672
CSNK1E	casein kinase 1, epsilon	ENSP00000352929

Table S2. Incorporation of ccgs and NRCs into networks. Three methods of handling nodes are compared. #CGs is the number of clock genes incorporated in the indicated network; #ccgs is the number rhythmic or clock-controlled genes; #NRCs is the number of non-rhythmic connectors.

	H. sapiens			*N. crassa*			*D. melanogaster*		
	#CGs	**#ccgs**	**#NRCs**	**#CGs**	**#ccgs**	**#NRCs**	**#CGs**	**#ccgs**	**#NRCs**
No assumptions, outer nodes removed	10	65 (69.9% incl.)	408	5	83 (57.2% incl.)	401	7	54 (37.5% incl.)	505
Nodes with < 3 connections removed	10	60 (64.5% incl.)	129	5	80 (55.2% incl.)	242	7	48 (33.3% incl.)	327
Only 1 interaction allowed	10	74 (79.6% incl.)	164	5	82 (56.6% incl.)	140	7	60 (41.7% incl.)	102

Table S3. The ten child GO-terms that are significantly enriched in all three organism PREMONition networks. Percentages do not add up to 100% because one gene can be associated with multiple GO-terms

GO-term	Description	% Human	% Fly	% Fungus
GO:0016043	`	15.3	47.9	5.3
GO:0022414	reproductive process	12.5	44.4	6.6
GO:0032501	multicellular organismal process	37.9	68.0	2.6
GO:0040007	growth	5.6	39.1	6.2
GO:0044237	cellular metabolic process	77.4	55.0	42.7
GO:0044707	multicellular organismal process	42.7	49.7	7.9
GO:0044763	cellular process	77.0	7.1	30.8
GO:0044767	developmental process	41.9	27.2	5.7
GO:0048511	rhythmic process	8.5	39.1	3.1
GO:0048856	anatomical structure development	35.9	71.0	9.3
GO:0050896	response to stimulu`s	66.9	70.4	30.4
GO:0065007	biological regulation	79.0	113.6	18.1
GO:0071840	cellular component organization or biogenesis	39.5	65.1	11.5

Table S4. InParanoid8 identifiers of D.melanogaster (fly) orthologs with H.sapiens (human) and *N.crassa* (fungus) proteins found in the PREMONition networks and between human and fungus.

Fly – Human		**Fly - Mold**		**Human - Mold**	
D.melanogaster	***H.sapiens***	***D.melanogaster***	***N.crassa***	***H.sapiens***	***N.crassa***
Q9VCD8	Q14683	Q9W2H8	Q7SED5	P63261	P78711
Q9VW54	Q13200	P10987	P78711	Q9UN37	Q7S0H4
Q7KMM4	Q14697	P02572	P78711	P68400	Q8TG13
Q9VDE3	Q9Y297	P53501	P78711	P14618	Q7RVA8
Q9VDE3	Q9UKB1	Q94517	Q7SEB0	O75439	P11913
P10987	P63261	P15357	P14799	P51149	Q9C2L8
P02572	P63261	Q9VDE3	Q7RWG8	Q15181	Q6MVH7
O18413	P62195	P08646	P22126	Q9Y297	Q7RWG8
Q8INB9	P31749	O97183	Q1K5K1	Q9UKB1	Q7RWG8
Q9V3H2	O00487	Q9VHN7	F5HDV2	P53350	Q7SF52
O76324	P49674	P48598	Q7SEY8	P62987	P0C224
O61734	O00327			P27449	P31413
O61734	Q8WYA1			P60866	Q7S3G1
O61735	O15516			P30049	P56525
O77430	P63208				
Q9VL18	P29692				
P07663	O15534				
P07663	O15055				
P07663	P56645				

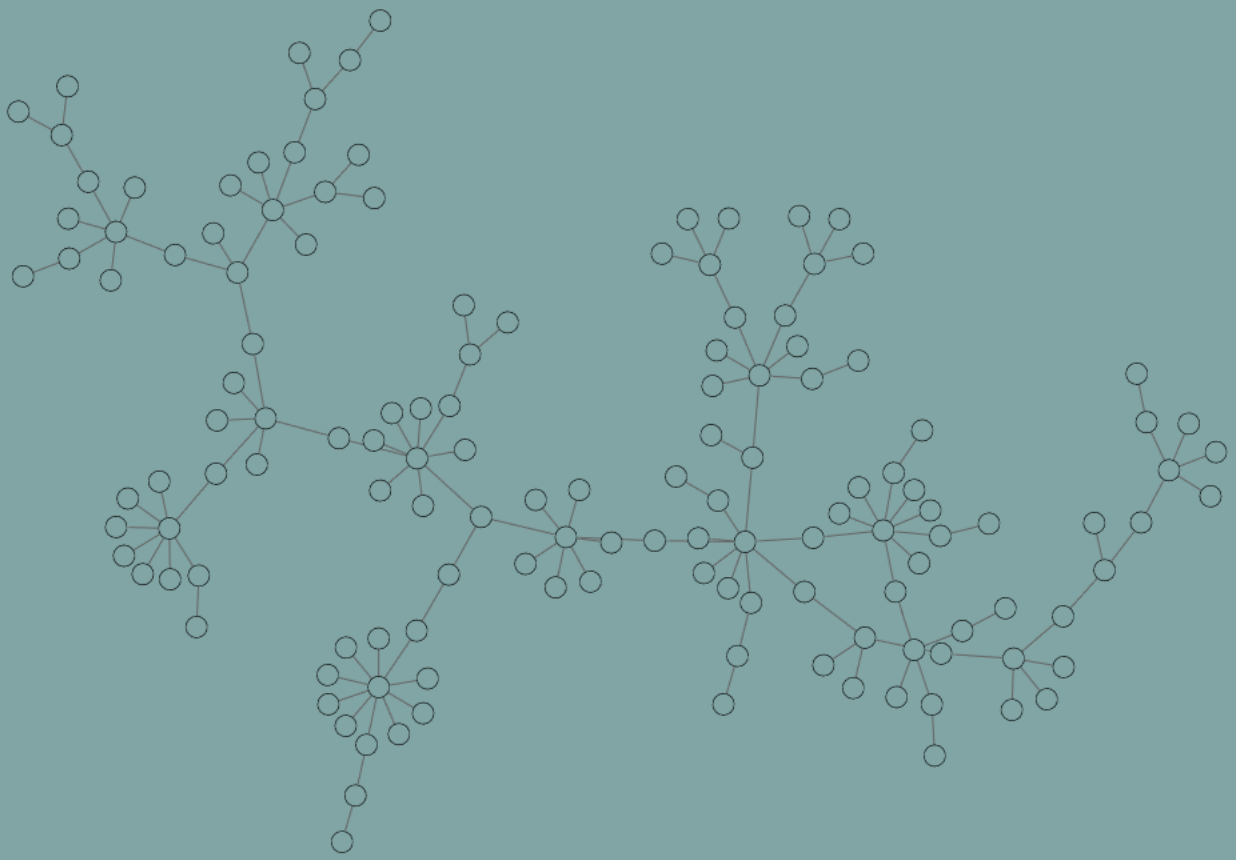
Table S5. Genes with more than 15 connections in the PREMONition networks for human, *Drosophila*, *Neurospora* and yeast.

Unique genes	Gene name	Count
H. sapiens		
ENSP00000225964	COL1A1	20
ENSP00000304592	FASN	19
ENSP00000254657	PER2	17
ENSP00000293379	ITGA5	16
ENSP00000352929	CSNK1E	16
ENSP00000358866	FLNA	16
D. melanogaster		
FBpp0086408	Strn-Mlck	18
FBpp0086993	CG8778	16
S. cerevisiae		
YPR010C	RPA135	32
YEL037C	RAD23	28
YPR163C	TIF3	27
YLR401C	DUS3	26
YOR038C	HIR2	25
YLR384C	IKI3	24
YKR025W	RPC37	21
YDR188W	CCT6	20
YMR290C	HAS1	20
YDL013W	SLX5	19
YMR201C	RAD14	19
YLR192C	HCR1	17
YKL028W	TFA1	17
YKL213C	DOA1	16

Unique genes	Gene name	Count
N. crassa		
NCU04553.1	ubi-3	34
NCU05804.1	NCU05804	34
NCU03757.1	93G11.150	33
NCU06226.1	NCU06226	33
NCU08500.1	B11B22.020	33
NCU08963.1	rpl-30	33
NCU01552.1	rps-23	32
NCU01776.1	rpl-15	32
NCU01949.1	NCU01949	32
NCU03806.1	rpl-28	32
NCU03988.1	NCU03988	32
NCU07182.1	B8G12.400	32
NCU00315.1	NCU00315	31
NCU00464.1	rpl-32	31
NCU00979.1	B20J13.300	31
NCU02509.1	rpl-11	31
NCU02708.1	B12J7.070	31
NCU06432.1	NCU06432	31
NCU06892.1	NCU06892	31
NCU09109.1	NCU09109.1	31
NCU00634.1	NCU00634	30
NCU01317.1	rpl-12	30
NCU01827.1	B23G1.190	30
NCU08620.1	rps-16	30
NCU01966.1	NCU01966	29
NCU05810.1	cpc-2	29
NCU04779.1	B15B10.040	28
NCU06661.1	100H1.050	28
NCU05032.1	NCU05032.1	27
NCU05275.1	NCU05032	27
NCU05599.1	rps-28	25
NCU09089.1	NCU09089	24
NCU03396.1	nop-58	18

Table S6. Rhythmic gene sets or ccgs. The ccgs used in our network construction for human, *Drosophila* and *Neurospora* are drawn from three publications. [1] = Fisher G-test, [2] = Adjusted

Organism	Publication	Number of rhythmic genes used	Statistical cutoff	Tissue
D. melanogaster	M.E. Hughes *et al.*	144	FG[1]. p-value < 0.05	Brain
H. sapiens	M.E. Hughes *et al.*	93	Adj.[2] p-value < 0.05	U2 OS cells
N. crassa	A. Correa *et al.*	145	From reference. Multiple parameters	Whole
S. cerevisiae	This article	137	q-value < 0.01	Whole



Chapter 4

Mining circadian protein-protein-interaction-networks

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Unpublished

Introduction

Survival of the fittest, a popular term in evolutionary biology, describes how well an organism adapts to its environment such that it leads to a higher survival and thus reproductive rate. Adaptation is mediated by mutation providing new functionalities to existing alleles. A hugely influencing environmental factor on life is the day and night cycle, changing environmental conditions such as light, temperature and humidity with the course of day in a continuous and predictable way. This rhythmic alternation of conditions is an important evolutionary force influencing species diversity on earth[1]. Developing a timing mechanism to anticipate these changes is certainly beneficial in terms of fitness[2–4]. These timing mechanisms are known as circadian clocks, biological programs providing a temporal structure to organisms. They are found in a multitude of organisms of all phyla[5].

Due to the evolutionary nature of these clocks, certain molecular structural properties such as Per-Arntl-Sim (PAS) domains are conserved and can be found throughout the tree of life[6]. Presumably, these represent functionally significant shared features also. Insights in these commonalities can be used to identify the circadian clock of organisms in which no circadian clock has been found yet. Here I present an approach to identify common circadian molecular clock features between a variety of organisms.

The Circadian Gene DataBase (CGDB) “is an online resource containing over 72,800 genes in more than 148 organisms that exhibit (or may exhibit) daily oscillation at the transcript level”[7]. Constructing fully connected protein-protein-interaction networks, using the PREMONition method[8] for each of the 148 organisms, analysing them for enriched biological properties and constructing a semantic similarity score matrix results in a functional circadian clock relatedness “chart” where species from the same phyla map closer together then evolutionary more distant ones. More importantly, by comparing the enriched biological properties, such as the Gene Ontology categories (Function, Process, Location), SMART proteins domains and KEGG biochemical pathways, a list of circadian network properties present across many organisms can be constructed. By projecting these properties on organisms in which no circadian clock mechanism has been fully described, hypothetical circadian clock genes can be identified. For validation purposes this method was applied to *Mus musculus*, which was excluded from the reference biological property list composition. 848 genes sharing at least 4 of the selected biological properties were identified. These 847 genes include well-known clock proteins in mice such as Per1, Per3, Arntl, Arntl2, Clock and

Npas2. Cry1, Cry2 and Per2 are missing from this list and they share 3 common biological properties which are missing in KEGG and SMART catalogues.

Here, I applied this approach on a *Saccharomyces cerevisiae* and identified several genes which are present in the molecular clock network. These genes encode proteins which comply to the identified biological properties and indicate that energy metabolism might be under circadian control in yeast.

Results

PPI enrichment analysis

Although the CGDB contains 148 organisms, only 116 were taken into account because 30 of these organisms have fewer than 30 circadian genes annotated and thus are assumed to have limited statistical power. PREMONition networks were constructed allowing a single Non-Rhythmically expressed Connector (NRC) between two 'circadian' (rhythmically expressed) genes. NRCs by definition are molecules that interact, functionally, with molecules associated with clock genes and/ or CCGs; as their name suggests, NRCs are not themselves identified as rhythmically expressed at the gene expression level. The interaction is predicted based on reports such a protein-protein interaction or demonstrated genetic interaction. Each organism's Protein-Protein Interaction (PPI) network has its own characteristics in terms of the number of circadian genes, the incorporated number of NRCs and the significantly enriched terms. Not all 116 constructed PPIs have enriched terms that reached significance. Forty-one networks are enriched for GO-term biological process, 44 with molecular functions, 38 with cellular components, seventy-nine networks contain enriched KEGG metabolic pathways and ninety-five networks are enriched with SMART annotated protein domains.

Functional similarity between circadian protein networks

The maturation of circadian clock mechanism must be an evolutionary process. Therefore, I expect similar biological functionalities within the PPI-networks of organisms belonging to the same phylum. To test this, the semantic similarity score between significantly enriched biological process GO-terms contained within the 41 organism networks was calculated and collected in a similarity scoring matrix used for agglomerative clustering with multi-dimensional scaling (Fig. 1). Organisms having similar enriched biological functions cluster together.

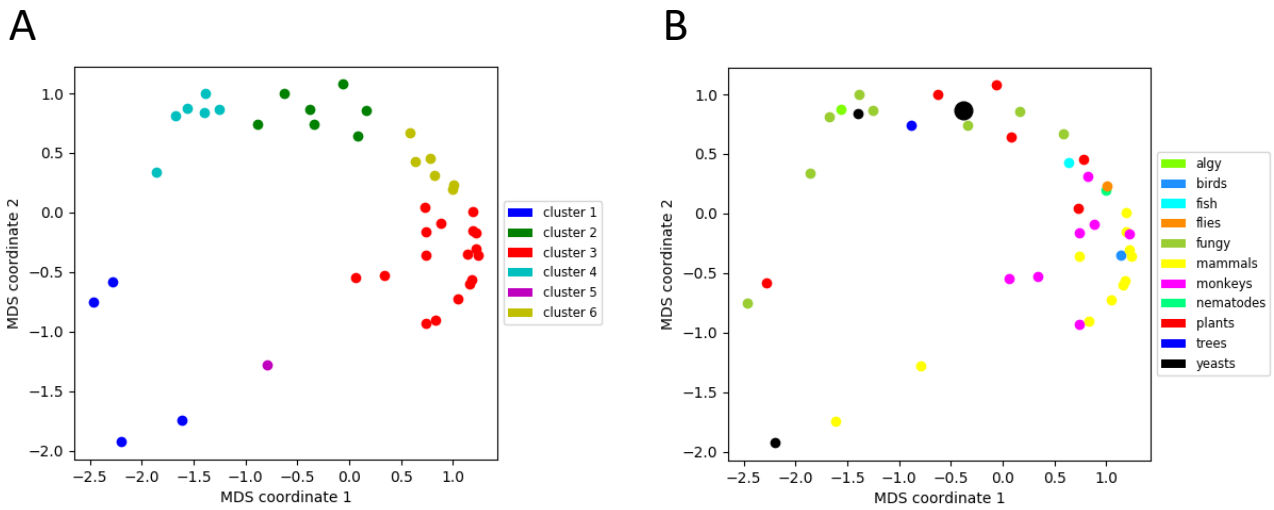


Figure 1. Multi-dimensional scaling (MDS) of semantic similarity scores calculated from the significantly enriched biological process GO-terms for 41 organisms. Agglomerative clustering (A) shows clusters of functional related circadian clock networks and organisms colored by taxonomy (B) depicts a gradual change in network functionality regarding of the organism. The black sphere with increased radius depicts *S. cerevisiae*; semantic similarity scores were calculated based on the PPI-network shown in Figure 2.

Method validation mapping Mus musculus

To verify the set of enriched biological aspects found in the circadian PPI networks (Table S1) I mapped them on all the *M. musculus* genes annotated in the STRING database. In total 16088 genes are associated with the biological aspects and 847 genes occur in at least 4 of the biological properties (GO-categories, SMART domains or KEGG pathways). Seven genes occur in all 5. However, none of these 7 genes are known clock genes. Since the PPI networks are based on rhythmically expressed genes, I wondered if the 7 genes are rhythmically expressed in some tissue or condition. Mining the CircaDB[9] shows that 6 of 7 genes are indeed significantly rhythmically expressed (Table 1). The probability (p-value) of obtaining 6 out of 7 randomly selected rhythmic genes in a mouse genome containing 19.788 genes of which 55% of the genes are rhythmic[10] is 0.015.

Table 1. Rhythmicity statistics from the 7 *M. musculus* genes that occur in all five biological properties (GO-process, -function and -localization, SMART protein domains and KEGG metabolic pathways). Statistics obtained from CircaDB[9].

Gene name	p-value	q-value	Period	Phase	Experiment
Cat	1.78E-03	3.02E-02	24	7	Mouse 1.OST Lung
Fads1	1.25E-04	1.52E-02	26	16	Mouse 1.OST Adrenal Gland
Fads2	2.36E-07	2.62E-04	24	15	Mouse 1.OST Adrenal Gland
Gck	1.44E-06	1.28E-04	24	15	Mouse 1.OST Liver
Pdhb	2.89E-09	2.60E-07	24	14	Mouse Liver 48-hour Hughes 2009
Tkt	No data available				
Tktl1	7.21E-04	1.79E-02	16	15	Mouse Distal Colon 2008

Predicting circadian clock genes in S. cerevisiae

In order to identify circadian clock genes in *S. cerevisiae*, I extracted all yeast genes annotated in the STRING database associated with the biological aspects found in the circadian PPI networks (Table S1). 294 genes are at least in 4 of the biological properties and by comparing these to a circadian microarray experiment which was performed according to methods described earlier by Chen *et al*[11], 22 genes have p-value < 0.05 for rhythmic gene expression (Table S2). On the other hand, one could use a more stringent threshold (p-value < 0.01) for having a circadian gene expressions profile with a period of 24 hours. This yields 137 rhythmically expressed genes, used to construct a PREMONition PPI network (Fig. 2). The resulting network contains 128 connected rhythmic genes and 82 Non-Rhythmic-Connectors (NRCs). Superimposing these 210 genes on the 294 genes associated with the significantly enriched terms in the 5 biological property categories, reveal 9 genes of which 4 also occur in the 22 genes obtained by mapping the 294 directly on the significant rhythmically expressed genes and 5 novel genes (NRCs) obtained by protein interaction evidences from the STRING database (Table 2).

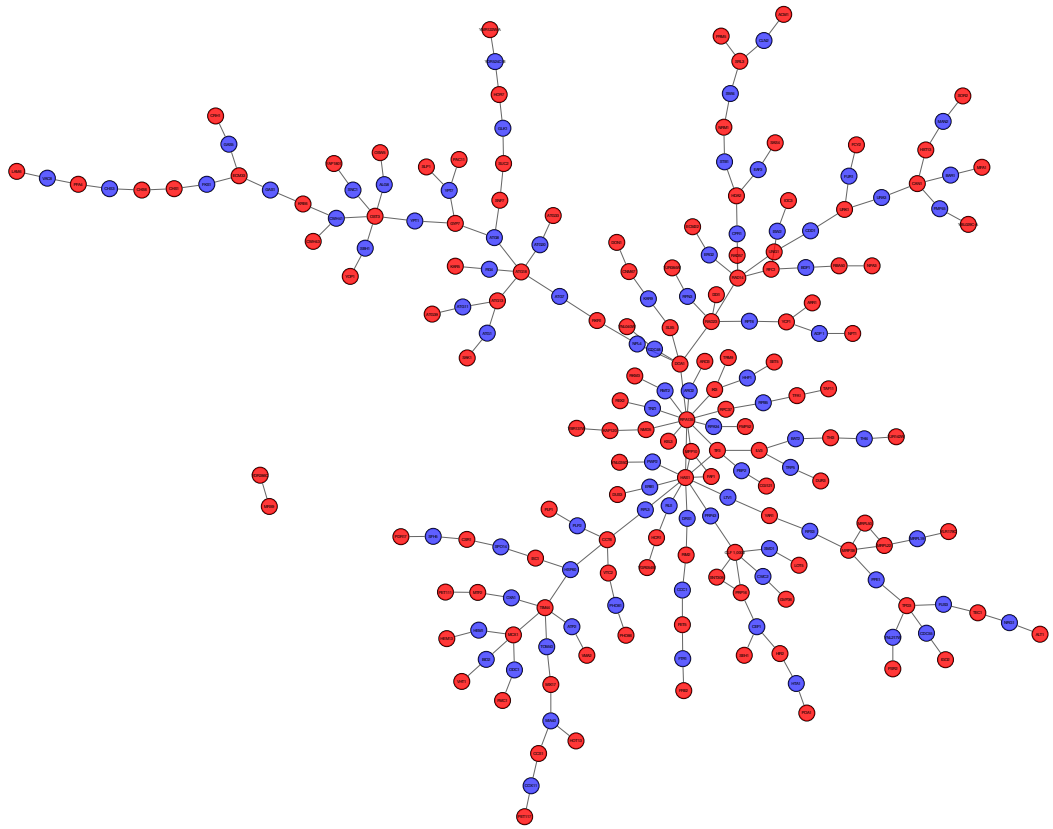


Figure 2. The PREMONition network for yeast, composed of 128 rhythmic genes and 82 NRCs, connected by 210 edges. Proteins encoded by rhythmically expressed genes (red spheres) and their non-rhythmic connectors (blue spheres) are shown. Two rhythmically expressed genes are disjoined from the network. The figure is scalable such that each gene/ protein can be identified.

Table 2. Nine *S. cerevisiae* genes identified by comparing 294 genes that occur at least in 4 of the 5 selected biological properties (GO-process, -function and -localization, SMART protein domains and KEGG metabolic pathways) to 210 genes forming a PREMONition network based on 137 genes showing a circadian gene expression profile with a p-value < 0.01 (analysed using CIRCWAVE[12]). Genes also included in the 22 *S. cerevisiae* genes identified by comparing 294 genes that occur at least in 4 of the 5 selected biological properties (GO-process, -function and -localization, SMART protein domains and KEGG metabolic pathways) and showing a circadian gene expression profile with a p-value < 0.05 (Table S2) are marked with an asterisk.

Systematic name	Standard name	p-value	Period	Phase
YHR207C *	SET5	0.001	24	21.93
YER129W *	SAK1	0.009	24	0.38
YPR199C *	ARR1	0.009	24	18.34
YLR089C *	ALT1	0.001	24	16.78
YBR023C	CHS3	0.251	24	5.06
YBL016W	FUS3	0.076	24	6.81
YCL040W	GLK1	0.171	24	6.92
YGL026C	TRP5	0.093	24	19.45
YJR148W	BAT2	0.095	24	19.55

Discussion and Conclusion

PPI enrichment analysis

Protein-Protein-Interaction networks were constructed with the PREMONition method. This method relies on the maturity or richness of the interaction database. Here, I used STRING. Also, PREMONition was developed to construct a coherent network, in which the interactions have the highest possible probability. PREMONition tries to find a coherent network solution in which the incorporation of novel nodes (NRCs) is minimal. In order to do so, the method only retains nodes with the highest interaction score. Since this process only depends on the actual score and not on a functional relationship it could be that interactions/ nodes acting in the same mechanism are removed while ones with unrelated mechanisms/ processes/ functions are kept. On the other hand, functional relationship is curated based on experimental data which is also reflected in the PPI-score. One could construct a new score, a combination of STRING interaction probability and GOGO semantic similarity score. The latter should be calculated for the entire genome, resulting in a m x n similarity matrix where m and n are the number of genes in the genome having GO terms annotated.

Functional similarity between circadian protein networks

Enrichment analysis, a method to identify classes, terms, genes or proteins that are over-represented in a large set of genes or proteins, was done using the STRING API focusing on the Gene Ontology annotations, SMART protein domain annotation and KEGG biochemical pathways. This combination allows balancing between being too lenient or stringent in gene selection. Taking only GO annotation (process, function and location) into account would be too lenient, while using only SMART protein domains would be too stringent. For a gene to be selected it should be listed in at least 4 of these 5 annotation types. One of the assumptions made is that the regulation of circadian mechanisms affects similar cellular processes across species. The PPI's should reflect these processes and their genes will be the main contributors to the network. However, a PPI network represents many processes, functions and metabolic pathways. To remove uninformative terms, only significantly enriched biological properties are taken into account for calculating the overall semantic similarity score between two PPI networks (GOGO[13]). Using this metric as a measure for biological functional relatedness, clusters of organisms with closely associated PPI-network functionality can be formed (Fig. 1A). Although the distribution/ pattern of organisms is irrelevant, I can observe grouping of organisms of the same phylum or closely affiliated phyla, indicating that indeed circadian PPI-networks based on rhythmically expressed genes show equivalent biological properties on a phylum level.

Method validation by mapping Mus musculus

M. musculus was used to validate the identified biological property terms, GO categories (biological process, molecular function and cellular component), metabolic pathways (KEGG) and protein domains (SMART). All *M. musculus* genes annotated in the STRING database were considered and in total 16088 genes are associated with at least 1 of the biological properties. After thresholding, 847 genes were identified as potential clock candidates and include known mouse clock proteins such as Per1, Per3, Arntl, Arntl2, Clock and Npas2. Note Cry1, Cry2 and Per2 are missing. Seven genes occur on all 5 biological properties of which 6 show rhythmic gene expression profiles (Table 1). These 7 genes represent two basic cellular processes, glycolysis/ gluconeogenesis and fatty acid metabolism. Glycolysis is one of the main processes involved in cellular respiration a mechanism located in mitochondria. Kremer *et al*[14] showed a strong relationship between the circadian clock and the glycolysis in muscle cells of mice. Also, fatty acid metabolism has been shown to be up regulated in mice having a myocyte-specific loss of BMAL1 muscle cells[15]. Metabolic disruptions are linked to diseases, such as type 2 diabetes[16]. While blood glucose levels exhibit a clear daily rhythm in humans and animals[17], shiftwork increases the susceptibility to many metabolic disorders[18] and increase circadian misalignment which in its turn induces upregulation of fatty acid metabolism pathways[19].

Predicting circadian clock genes in S. cerevisiae

Two strategies were applied to find candidate clock genes in *S. cerevisiae*. First, all yeast genes annotated in the STRING database associated with the biological aspects found in the circadian PPI networks (Table S1) were identified and include 294 genes associated with at least 4 of the biological properties. These 294 genes are enriched with (serine/threonine) protein-kinase domains, Cytochrome b5-like Heme/Steroid binding domains, basic-leucine zipper domains and their products are active in the following metabolic pathways: glycolysis (TCA cycle and pyruvate metabolism) and carbon-, starch- and sucrose metabolism. Phosphorylation and serine/threonine kinases play an important role on the circadian clock regulatory mechanisms. DOUBLETIME in *D. melanogaster*[20], casein kinase 1 in *M. musculus*[21] casein kinase 2 in *A. thaliana*[22] are just a few examples. It has been proposed by Merrow *et al*., that phosphorylation is “at the heart of the circadian oscillation” orchestrated by a process named the phoscillator[23]. 41 of the 294 genes are serine/threonine protein-kinases.

To further reduce the number of candidates, only the 22 showing a circadian pattern on the mRNA transcript level were considered (Table S2). Inspecting their biological functions, one finds serine/threonine protein kinases, bZIP transcription factor domains and metabolic

pathways gluconeogenesis and biosynthesis of amino acids. 4 of the 22 genes are serine/threonine protein-kinases (SAK1, KCC4, PSK2 and IME2). However, little circadian transcriptome data is available making it subjective to errors *e.g.* proteins containing PAS domains are in many animals associated with the circadian clock[24]. Yeast has 4 proteins containing a PAS domain annotated (RDS2, PSK1, ERT1, PSK2). None of them are rhythmic in their gene expression but they are present in 294 genes found in by biological property association.

The second strategy, constructing a PREMONition PPI-network for *S. cerevisiae* (Fig. 2) based on a micro-array experiment, sampled during the free run from a temporal temperature entrainment experiment provides a method to gain insights into hypothetical clock genes and the regulation therein of underlying biological mechanisms. Subjecting the networked yeast genes to the clustering strategy, a set of functions related to circadian mechanisms, resembling the ones from fungi and plants (cluster 2. Fig. 1A) can be obtained. However, for candidate selecting comparing the networked yeast genes with a collection of biological properties obtained from enrichment analysis of circadian PPI-networks of different organisms, a set of 9 genes is obtained (Table 2) which should reflect the functions related to the circadian clock in *S. cerevisae*. Two genes (FUS3 and SAK1) are serine/threonine protein-kinases of which SAK1 also shows a 24-hour circadian cycle in its gene expression pattern (Table 2). SAK1 is one of the three kinases phosphorylating SNF1 the yeast homolog of adenosine monophosphate-activated protein kinase (AMPK)[25]. AMPK is a cellular energy sensor and is activated when intracellular ATP levels decreases. The activity pattern of AMPK shows circadian rhythms in a multitude of organs and tissues and thus might play an important role in the circadian regulation of cellular metabolism[26]. Comparing these findings to the genes found in *M. musculus* suggest that energy metabolism is under circadian control and is conserved across different phyla [27,28].

Using PREMONition to construct coherent protein-protein-interaction networks to increase biological relevance, I identified a set of biological properties commonly associated with circadian gene networks. Clustering these networks based on a sematic information similarity score using biological function, cluster consisting of organism of the same phylum are formed. A constructed hypothetical circadian *S. cerevisiea* PPI-network shows biological similarities with fungus. Key aspects seem to be energy metabolism regulated by serine/threonine protein-kinases. The yeast genome has four genes encoding a PAS domain, all serine/threonine protein-kinases and all involved in energy metabolism. Our results could be biased towards these processes as yeast shows a opportunistic behavior under laboratory

conditions, rapidly depleting high energy carbon sources. In its natural environment, yeast is subjected to temperature and light cycles. Light causes photo-oxidation of the cytochromes and radical oxygen species form. A circadian clock in yeast would represent a cellular mechanism or strategy to anticipate these predictable and stressful environmental changes.

Material and Methods

Constructing PREMONition networks

For PREMONition protein-protein-interaction network construction, Circadian Gene DataBase Uniprot identifiers were translate to STRING protein interaction database identifiers[29] using the Uniprot[30] and STRING Application programming Interfaces (API's). For 118 of the organisms annotated in the CGDB (30 organisms have less then 30 "rhythmic" genes annotated and were excluded) a PREMONition network is constructed using a single Non-Rhythmically expressed Connectors (NRCs) between two rhythmically expressed genes. In the experiments described here, a relatively stringent probability score of ≥0.7 was used for most organisms, except for *H. sapiens*, *G. max* and *A. thaliana* a score of 0.95 was used.

Semantic similarity score calculation

Gene Ontology (GO) term enrichment analysis was performed on each of the constructed PREMONition networks using the STRING enrichment analysis tool, a 0.05 False Discovery Rate (FDR) threshold was maintained.

An overall semantic similarity score between two PPI networks (GOGO[13]) is calculated in two steps. First, a similarity score between all GO terms of network N_1 (having n enriched GO terms) and network N_2 (having m enriched GO terms), resulting in a m x n similarity matrix, was calculated. Next, dimensional reduction to a single similarity score between two networks ($Sim(N_1, N_2)$) is performed according to Equation 1. Where go_{1i} is the i^{th} GO term of N_1 and go_{2j} j^{th} GO term of N_2.

$$Sim(N_1, N_2) = \frac{\sum_{1 \le i \le m} sim(go_{1j}, N_2) + \sum_{1 \le j \le n} sim(go_{2j}, N_1)}{\mathrm{m} + \mathrm{n}} \qquad (Eq. 1)$$

This results in a similarity matrix for each of the three independent GO categories: molecular function, biological process and cellular component.

Visualising circadian functional relatedness

The three semantic similarity matrices hold information on the functional relatedness of the organisms of which significant enriched GO terms were found in the PREMONition networks. For visualization purposes each matrix was scaled using MultiDimensional scaling (MDS[31]) with 2 components. To identify groups/ clusters of functional similar circadian clocks, agglomerative clustering[31] is applied on the MDS distance matrices, using complete linkage, the Euclidian distance metric and six clusters.

Common biological properties

Some of the enriched biological property items *e.g.* GO molecular function; oxygen binding (GO:0019825) occur only a single time within the 44 enriched PPI-networks, limiting its statistical contribution. In order to reduce the noise of these sparse items an arbitrary threshold was set, >15 for GO-terms, ≥ 10 for SMART domains and > 45 KEGG pathways. The remaining properties are assumed to reflect the most important biological aspects of circadian PPI networks (Table S1).

Literature references

1. Connor, K. M. & Gracey, A. Y. Circadian cycles are the dominant transcriptional rhythm in the intertidal mussel Mytilus californianus. *Proc. Natl. Acad. Sci. U. S. A.* (2011) doi:10.1073/pnas.1111076108.
2. Johnson, C. H., Mori, T. & Xu, Y. A Cyanobacterial Circadian Clockwork. *Current Biology* (2008) doi:10.1016/j.cub.2008.07.012.
3. Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Johnson, C. H. Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.15.8660.
4. DeCoursey, P. J. & Krulas, J. R. Behavior of SCN-Lesioned Chipmunks in Natural Habitat: A Pilot Study. *J. Biol. Rhythms* (1998) doi:10.1177/074873098129000075.
5. Gerhart-Hines, Z. & Lazar, M. A. Circadian metabolism in the light of evolution. *Endocrine Reviews* (2015) doi:10.1210/er.2015-1007.
6. Henry, J. T. & Crosson, S. Ligand-binding PAS domains in a genomic, cellular, and structural context. *Annual Review of Microbiology* (2011) doi:10.1146/annurev-micro-121809-151631.
7. Li, S. *et al.* CGDB: A database of circadian genes in eukaryotes. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkw1028.
8. Bosman, J., Eelderink-Chen, Z., Laing, E. & Merrow, M. PREMONition: An algorithm for predicting the circadian clock-regulated molecular network. *bioRxiv* (2018) doi:10.1101/463190.
9. Pizarro, A., Hayer, K., Lahens, N. F. & Hogenesch, J. B. CircaDB: A database of mammalian circadian gene expression profiles. *Nucleic Acids Res.* (2013) doi:10.1093/nar/gks1161.
10. Zhang, R., Lahens, N. F., Ballance, H. I., Hughes, M. E. & Hogenesch, J. B. A circadian gene expression atlas in mammals: implications for biology and medicine. *Proc. Natl. Acad. Sci. U. S. A.* 111, 16219–24 (2014).
11. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, (2010).
12. Oster, H., Damerow, S., Hut, R. A. & Eichele, G. Transcriptional Profiling in the Adrenal Gland Reveals Circadian Regulation of Hormone Biosynthesis Genes

and Nucleosome Assembly Genes. *J. Biol. Rhythms* 21, 350–361 (2006).

13. Zhao, C. & Wang, Z. GOGO: An improved algorithm to measure the semantic similarity between gene ontology terms. *Sci. Rep.* (2018) doi:10.1038/s41598-018-33219-y.
14. Thurley, K. *et al.* Principles for circadian orchestration of metabolic pathways. *Proc. Natl. Acad. Sci. U. S. A.* (2017) doi:10.1073/pnas.1613103114.
15. Dyar, K. A. *et al.* Transcriptional programming of lipid and amino acid metabolism by the skeletal muscle circadian clock. *PLoS Biol.* (2018) doi:10.1371/journal.pbio.2005886.
16. Ding, G., Gong, Y., Eckel-Mahan, K. L. & Sun, Z. Central circadian clock regulates energy metabolism. in *Advances in Experimental Medicine and Biology* (2018). doi:10.1007/978-981-13-1286-1_5.
17. Kalsbeek, A., La Fleur, S. & Fliers, E. Circadian control of glucose metabolism. *Molecular Metabolism* (2014) doi:10.1016/j.molmet.2014.03.002.
18. Karlsson, B., Knutsson, A. & Lindahl, B. Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27 485 people. *Occup. Environ. Med.* (2001) doi:10.1136/oem.58.11.747.
19. Wefers, J. *et al.* Circadian misalignment induces fatty acid metabolism gene profiles and compromises insulin sensitivity in human skeletal muscle. *Proc. Natl. Acad. Sci. U. S. A.* (2018) doi:10.1073/pnas.1722295115.
20. Eide, E. J., Kang, H., Crapo, S., Gallego, M. & Virshup, D. M. Casein kinase I in the mammalian circadian clock. *Methods Enzymol.* 393, 408–418 (2005).
21. Etchegaray, J. P., Yu, E. A., Indic, P., Dallmann, R. & Weaver, D. R. Casein kinase 1 delta (CK1) regulates period length of the mouse suprachiasmatic circadian clock in vitro. *PLoS One* (2010) doi:10.1371/journal.pone.0010303.
22. Lu, S. X. *et al.* A role for protein kinase casein kinase2 a-subunits in the Arabidopsis circadian clock. *Plant Physiol.* (2011) doi:10.1104/pp.111.179846.
23. Merrow, M., Mazzotta, G., Chen, Z. & Roenneberg, T. The right place at the right time: Regulation of daily timing by phosphorylation. *Genes and Development* (2006) doi:10.1101/gad.1479706.
24. Hennig, S. *et al.* Structural and functional analyses of PAS domain interactions of the clock proteins Drosophila PERIOD and mouse period2. *PLoS Biol.*

(2009) doi:10.1371/journal.pbio.1000094.

25. Clement, S. T., Dixit, G. & Dohlman, H. G. Regulation of yeast G protein signaling by the kinases that activate the AMPK homolog snf. *Sci. Signal.* (2013) doi:10.1126/scisignal.2004143.
26. Huang, C. C. Y. *et al.* A new role for AMP-activated protein kinase in the circadian regulation of L-type voltage-gated calcium channels in late-stage embryonic retinal photoreceptors. *J. Neurochem.* (2015) doi:10.1111/jnc.13349.
27. Serin, Y. & Acar Tek, N. Effect of Circadian Rhythm on Metabolic Processes and the Regulation of Energy Balance. *Ann. Nutr. Metab.* (2019) doi:10.1159/000500071.
28. Poggiogalle, E., Jamshed, H. & Peterson, C. M. Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism.* (2018) doi:10.1016/j.metabol.2017.11.017.
29. Szklarczyk, D. *et al.* STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* (2019) doi:10.1093/nar/gky1131.
30. Nightingale, A. *et al.* The Proteins API: Accessing key integrated protein and genome information. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkx237.
31. Pedregosa, F. *et al.* Scikit-learn: Machine learning in Python. *J. Mach. Learn. Res.* (2011).
32. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* (2010) doi:10.1073/pnas.0907902107.

Supporting information materials

Table S1. Lists of terms associated with the 5 biological properties from which the enriched terms have multiple occurrences, >15 for GO-terms, ≥10 for SMART domains and > 45 KEGG pathways, within the circadian Protein-Protein-Interaction organism networks. Where "Function" indicates GO Molecular Function, "Component"; GO Cellular Component, "Process"; GO Biological process, "SMART"; SMART domains and "KEGG" KEGG pathways. Within prentices the number of enriched terms.

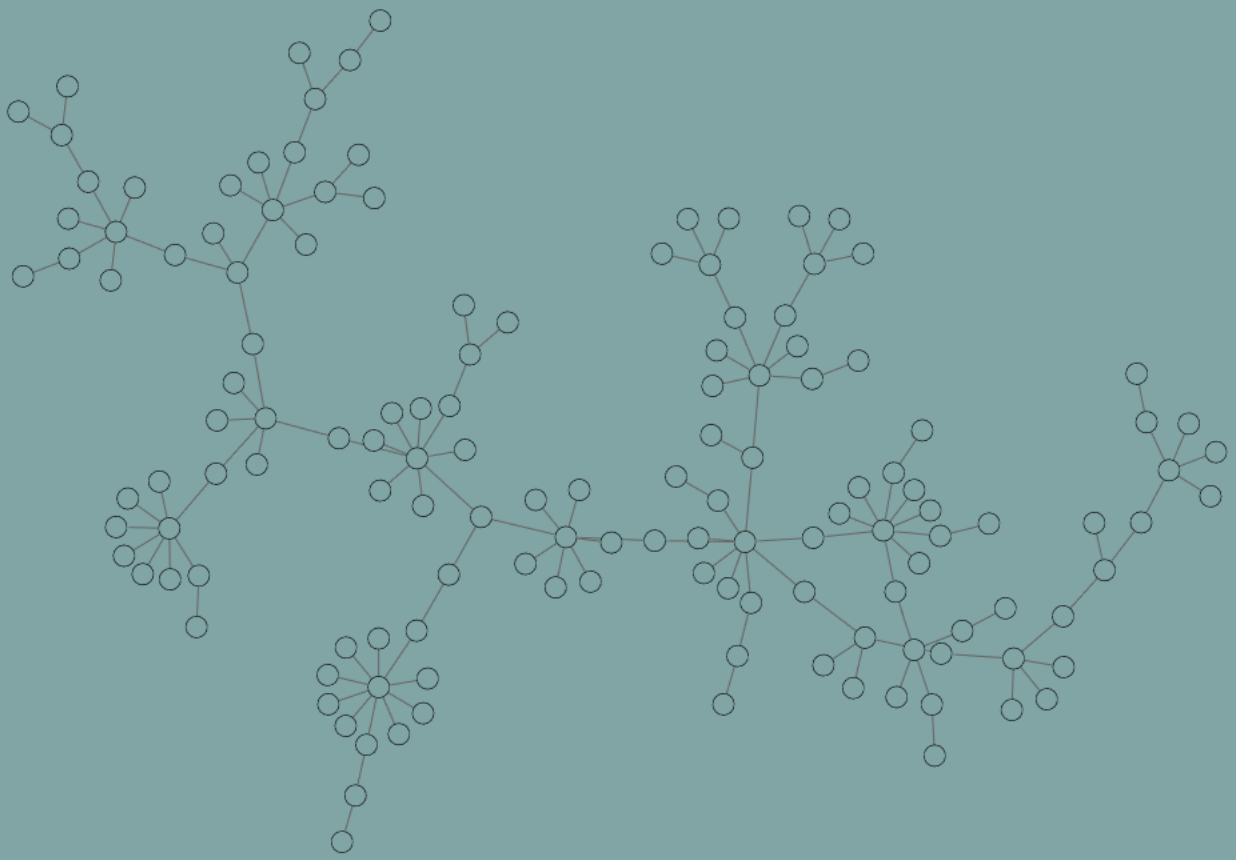
Function (19)	Component (26)	Process (83)		SMART (28)	KEGG (25)
GO:0005488	GO:0043231	GO:0009987	GO:0048583	SM00091	mmu01100
GO:0097159	GO:0044444	GO:0050794	GO:0051704	SM00086	mmu01200
GO:0003824	GO:0043227	GO:0051716	GO:0043170	SM00353	mmu01230
GO:1901363	GO:0043229	GO:0008152	GO:0042221	SM00338	mmu00270
GO:0016491	GO:0032991	GO:1901564	GO:0051254	SM00399	mmu00500
GO:0005515	GO:0044464	GO:0050896	GO:0019538	SM00430	mmu00900
GO:0043167	GO:0005737	GO:0055114	GO:0016310	SM00520	mmu00010
GO:0046872	GO:0044446	GO:0031325	GO:1901701	SM01060	mmu00630
GO:0043168	GO:0005634	GO:0030154	GO:0048856	SM01212	mmu00330
GO:0048037	GO:0044425	GO:0044238	GO:0009628	SM00220	mmu04146
GO:0098772	GO:0031090	GO:0044281	GO:0044260	SM00387	mmu00561
GO:0000166	GO:0016020	GO:0031323	GO:0006725	SM01065	mmu00380
GO:0016740	GO:0012505	GO:0060255	GO:0017144	SM00336	mmu00220
GO:0036094	GO:0005622	GO:0010033	GO:0051049	SM00448	mmu00260
GO:0008144	GO:0005886	GO:0065007	GO:0071702	SM00065	mmu00071
GO:0050662	GO:0005739	GO:0070887	GO:0051179	SM00219	mmu00250
GO:0042802	GO:0044424	GO:0032501	GO:0051252	SM00810	mmu00190
GO:0032555	GO:0016021	GO:0044237	GO:0006464	SM00388	mmu01212
GO:0005524	GO:0005615	GO:0007165	GO:0007154	SM01117	mmu01040
	GO:0005829	GO:0080090	GO:0006950	SM00439	mmu00100
	GO:0044459	GO:1901360	GO:0048513	SM00079	mmu03040
	GO:0005576	GO:0065008	GO:0051239	SM00918	mmu00564
	GO:0098796	GO:0071704	GO:0042592	SM00511	mmu00860
	GO:0005887	GO:1901362	GO:0044248	SM00133	mmu00910
	GO:0005783	GO:0051171	GO:0050793	SM00861	
	GO:0098590	GO:0010604	GO:2000112	SM00864	
		GO:1901700	GO:0006355	SM00317	
		GO:0065009	GO:0048584	SM00865	
		GO:0044249	GO:0050789		
		GO:1901576	GO:0009058		
		GO:0048523	GO:0033554		
		GO:0071310	GO:0010468		
		GO:0048522	GO:0009605		
		GO:0006807	GO:0016043		
		GO:0051173	GO:0034654		
		GO:0006810	GO:0071495		
		GO:0034641	GO:0009966		
		GO:0006139	GO:0044271		
		GO:0006796	GO:0006091		
		GO:0044283	GO:0051128		
		GO:0006811	GO:0009888		
		GO:0046483			

Table S2. Twenty-two *S. cerevisiae* genes identified by comparing 294 genes that occur at least in 4 of the 5 selected biological properties (GO-process, -function and -localization, SMART protein domains and KEGG metabolic pathways) and showing a circadian gene expression profile with a p-value < 0.05, analysed using CIRCWAVE[12], within a thermostat culture as described by Chen *et al*[32].

Systematic name	Standard name	p-value	Period	Phase
YHR207C	SET5	0.001	24	21.9
YLR089C	ALT1	0.001	24	16.8
YER129W	SAK1	0.009	24	0.4
YPR199C	ARR1	0.009	24	18.3
YOL028C	YAP7	0.010	24	21.0
YDL078C	MDH3	0.012	24	4.6
YOR231W	MKK1	0.015	24	1.6
YCL024W	KCC4	0.018	24	4.9
YLR027C	AAT2	0.020	24	14.6
YER052C	HOM3	0.024	24	23.3
YML085C	TUB1	0.024	24	19.1
YER086W	ILV1	0.025	24	20.3
YER045C	ACA1	0.026	24	4.9
YDL066W	IDP1	0.030	24	18.6
YGR254W	ENO1	0.031	24	4.4
YKL127W	PGM1	0.032	24	20.5
YER073W	ALD5	0.036	24	21.3
YFR055W	IRC7	0.038	24	20.8
YAL017W	PSK1	0.039	24	7.7
YGL202W	ARO8	0.041	24	19.8
YPR069C	SPE3	0.047	24	19.8
YJL106W	IME2	0.047	24	4.7

Literature references

1. Oster, H., Damerow, S., Hut, R. A. & Eichele, G. Transcriptional Profiling in the Adrenal Gland Reveals Circadian Regulation of Hormone Biosynthesis Genes and Nucleosome Assembly Genes. *J. Biol. Rhythms* **21**, 350–361 (2006).
2. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* (2010) doi:10.1073/pnas.0907902107.



Chapter 5

Shedding Light on Photobiology in *S. cerevisiae*

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Unpublished

Introduction

A circadian clock is an endogenous program that regulates functions from gene expression to behaviour according to the time of day. Almost all known organisms use light to entrain their circadian clock. We recently found that yeast shows circadian entrainment characteristics in 24h (and near 24h) temperature cycles[1]. This suggests that yeast also has a circadian clock. We were, however, unable to elucidate conditions where yeast has a free running circadian rhythm that persists for more than one day. As organisms with a circadian clock are usually sensitive to light, we assume that a day-night (*i.e.* light-dark) cycle might also be a crucial factor in controlling the circadian rhythm in yeast. Therefore, in this work I investigate the growth of yeast under light and dark conditions. I challenged this phenotype by assaying specific gene knockouts in this protocol.

Yeast lacks many of the obvious photoresponses of other organisms. It typically grows rapidly in artificial, rich media to facilitate lab experiments that require biological mass. However, when yeast cells are kept in starvation media and at low temperatures, they exhibit photoreception as seen by a markedly decreased growth rate[2]. Increasing the pH or the phosphate concentration of the growth medium increases sensitivity to light[3]. Experiments focusing on metabolism showed that the decreased growth rate coincides with a reduction of glucose and acetate uptake, and decreased protein synthesis and transport[4–6]. In addition, yeast undergoes characteristic changes in its respiration rate with light exposure[5]. This complex response to light by yeast has been characterised for spectral sensitivity. Yeast responds to discrete wavelengths in the near the UV (365 nm)[7] and blue (400-500 nm[8], 405 and 436 nm[9], 408 nm[10]) regions.

The effects of blue light on the cellular mechanisms in yeast are thought to arise from an interplay between physical and molecular processes. Blue light causes ROS formation, which leads to the activation of oxidative stress genes such as thioredoxin reductase (TTR1), cytochrome c peroxidase (CCP1) and cytosolic thioredoxin (TRX2). These genes are controlled by a transcription factor (YAP1), of which the knockout strain shows significantly more growth reduction when illuminated[11].

To characterise the physical and underlying molecular processes involved in the growth response of yeast to light, I first used DNA microarrays to identify genes that are either up- or down- regulated on exposure to light. Subsequently, I developed a simple growth assay to screen for function in response to light and found 28 light-regulated genes that give a phenotype in this assay. I further identified a set of candidate genes from the published literature whose expression was not altered when yeast is exposed to light. Using these hand-picked genes, an additional 14 genes were discovered to impact growth in light. The entire set of 42 genes was used to construct a hypothetical network involved in yeast photobiology, providing a basis for future work on yeast photosensitivity.

Results

Light regulated gene expression in yeast

In selected fungi, gene transcription has been shown to be acutely regulated by light[12]. Furthermore, in the fungus *Neurospora crassa*, both fast- and slow-regulated genes are described as part of the molecular response to light, and this system is furthermore integral to their circadian clock[13]. I therefore sampled yeast after 30 and 120 minutes incubation in blue light. RNA was isolated and DNA microarrays were performed showing 514 genes that changed expression (Fig. 1). Only 34 of these were significantly altered in expression after 30 minutes (T_1) and only three (IFM1, SOL1, SDP1) of these were down regulated. After 120 minutes (T_2), 214 genes were down-regulated and 266 were up-regulated in their mRNA concentration levels (Table S1). Gene ontology (GO) analysis reveals no significantly enriched biological processes (eBPs) at T_1, but I found 38 significant eBPs (FDR < 0.05) in the up regulated genes at T_2 and 129 significant eBPs at T_2 in the down regulated genes (Table S3). There is an overlap of 4 processes between the eBPs found in the up- and down-regulated gene collections.

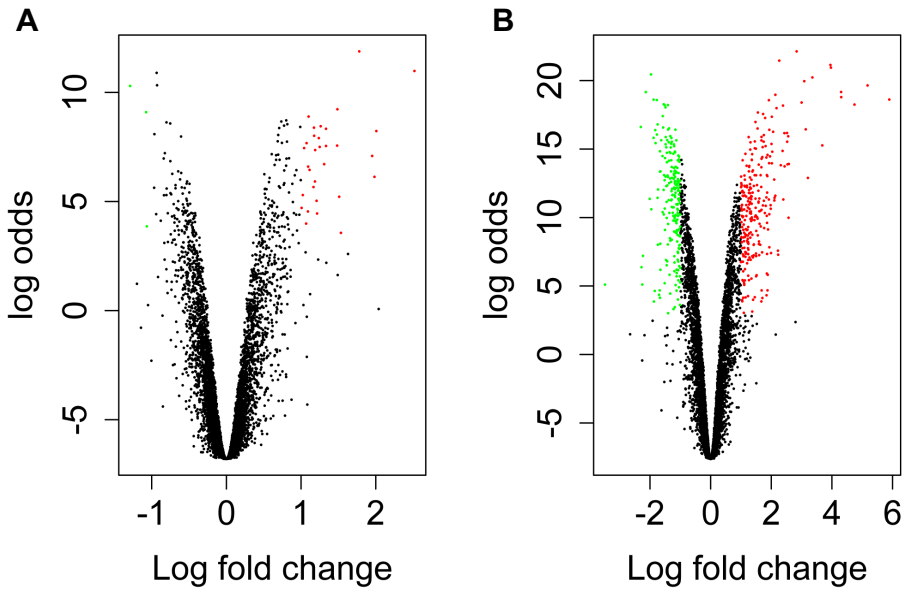


Figure 1. Volcano plots of differentially expressed genes from yeast cells exposed to blue light for (A) 30 minutes and (B) 120 minutes. Genes are considered significant when the Log Fold Change (LFC) ≥1 and the log-odds (LO) ≥ 2.944 (p-value ≥ 0.95). Significantly down regulated genes are marked green and up-regulated genes are marked red.

Phenotyping the collection of mutants in light regulated genes

The most obvious phenotype that has been reported concerning the effect of light on wild type yeast is a suppression of growth on poor media. I developed a simple, robust, high throughput and quantitative assay to screen the collection of light-regulated genes for how they contribute to the growth phenotype. From overnight yeast knockout strain cultures, a series of five, 1:1 dilutions were made. From each dilution, 2 µl was spotted on YPD plates (Fig. 2). After 48 hours, plates were imaged and the colony density for each dilution calculated and used to construct a growth curve in order to identify light sensitive mutants.

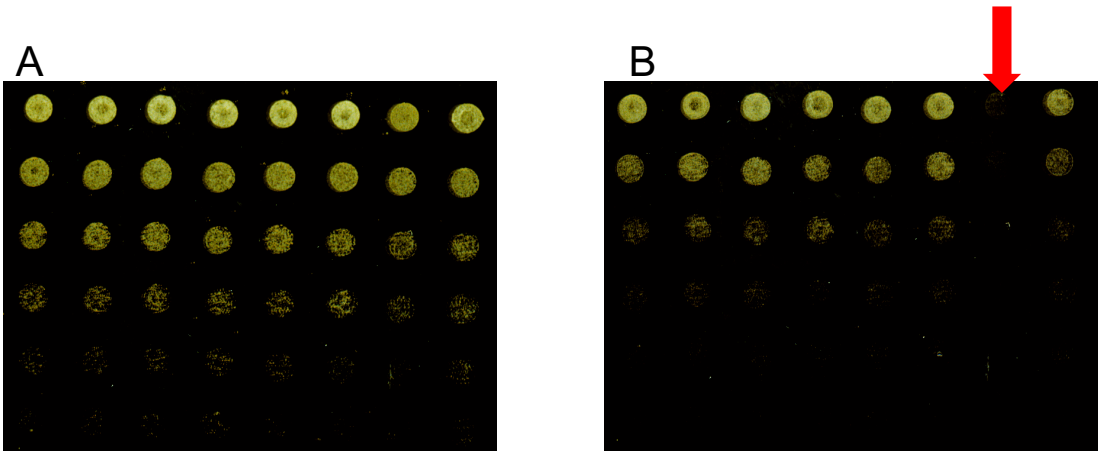


Figure 2. Imaged YPD-plates from the yeast knockout screen. 2 µl of a serial cell-suspension dilution from 8 knockout strains were spotted on YPD agar plates (dilution series for individual strains run from top to bottom) and grown for 48 hours in (A) darkness and (B) light. Red arrow indicates a yeast knockout strain (SNT1) which shows significant growth reduction.

Knockout strains of all 514 candidate light-regulated genes were obtained (Courtesy provided by Arjen Krikken and Ida van der Klei, University of Groningen, Molecular Cell Biology). These were screened for light sensitivity, yielding 28 genes that showed a reduced growth phenotype. It was striking that several of these genes are known to be involved in growth, sporulation and energy metabolism (Table 1, source column: “Micro”).

Candidate genes derived from previous studies

Among the 28 light-regulated genes derived from the DNA microarrays in combination with the phenotyping screen, I found few of the genes that would have been predicted from previous published work[2,3,9,14]. This suggests that previously identified candidate genes are regulated post-transcriptionally. I wished to determine if these proteins would be discovered using our growth assay or if they were part of a different light-regulated system in yeast. I assayed 124 of these post-transcriptional genes, encoding *e.g.* cytochromes and proteins predicted to contain flavins (Table S2). Of these, 14 showed a light-induced growth phenotype in our assay (Table 1, Source “Studies”); including VPH1, VMA4, VMA3 and VMA11 which

are involved with ATP hydrolysis coupled transmembrane transport, and together with FET3 and SOD1 regulate ion homeostasis and redox regulation. The former two proteins as well as SKN7, RIM15, URE2 and SNF1 function in the cellular response to stimuli. The FET3 (YMR058W) protein is a multicopper oxidase, a family that is conserved in many organisms, required for Cu homeostasis and involved in the circadian clock in *A. thaliana*[15]. Additionally, copper functions as a redox active cofactor for many proteins like cytochrome c oxidase and copper-zinc-superoxide dismutase (Cu/Zn-SOD). GEF1, FET3 and SOD1 are also involved in metal ion homeostasis in which SOD1 responds to superoxides and oxygen radicals. SKN7 is needed for responses to oxidative stress.

Table 1. 42 Significant, differentially expressed genes, which also show a difference in growth phenotype when exposed to white light. The column 'Source' indicates the origin of the gene; "Studies" (14 genes identified as candidates from the literature) or from the "Micro"-array (28 genes), Log fold change in the microarray (LFC) and Log Odds score (LO).

Identifier	Symbol	Name	LFC	LO	Source
YGL206C	CHC1	Clathrin Heavy Chain	-1.46	12.30	Micro
YMR198W	CIK1	Chromosome Instability and Karyogamy	-1.45	9.97	Micro
YLR087C	CSF1	Cold Sensitive for Fermentation	-1.49	12.99	Micro
YNL111C	CYB5	Cytochrome B	1.11	5.19	Micro
YBL043W	ECM13	ExtraCellular Mutant	-1.25	10.74	Micro
YLR056W	ERG3	ERGosterol biosynthesis	1.57	11.95	Micro
YMR058W	FET3	FErrous Transport	-0.17	-7.35	Studies
YMR215W	GAS3	Glycophospholipid-Anchored Surface protein	-1.43	6.80	Micro
YJR040W	GEF1	Glycerol Ethanol Ferric requiring	-0.48	3.44	Studies
YPL067C	HTC1	Histidine Triad with Channel	1.05	5.43	Micro
YLR309C	IMH1	shares with Integrins and Myosins significant Homology	-1.18	11.62	Micro
YMR163C	INP2	INheritance of Peroxisomes	-1.18	11.50	Micro
YHR079C	IRE1	Inositol REquiring	-1.18	12.66	Micro
YHR082C	KSP1	Kinase Suppressing Prp20-10	-1.18	10.07	Micro
YGR057C	LST7	Lethal with Sec Thirteen	1.03	10.52	Micro
YLR069C	MEF1	Mitochondrial Elongation Factor	-1.02	5.71	Micro
YDL233W	MFG1	Morphogenetic regulator of Filamentous Growth	1.17	10.91	Micro
YBR255W	MTC4	Maintenance of Telomere Capping	-0.59	4.11	Studies
YHR124W	NDT80	Non-DiTyrosine	1.30	11.78	Micro
YKL134C	OCT1	OCTapeptidyl aminopeptidase	-1.08	10.11	Micro
YDR323C	PEP7	carboxyPEPtidase Y-deficient	-0.21	-5.35	Studies
YDR529C	QCR7	ubiQuinol-cytochrome C oxidoReductase	0.11	-5.37	Studies
YFL033C	RIM15	Regulator of IME2	-0.92	8.50	Studies
YDR159W	SAC3	Suppressor of ACtin	-1.49	15.35	Micro
YJL080C	SCP160	S. cerevisiae protein involved in the Control of Ploidy	-1.04	7.96	Micro
YBL104C	SEA4	SEh1-Associated	-1.19	13.39	Micro
YMR190C	SGS1	Slow Growth Suppressor	-1.31	13.37	Micro
YLR398C	SKI2	SuperKIller	-1.38	15.49	Micro
YHR206W	SKN7	Suppressor of Kre Null	0.35	1.98	Studies
YDR477W	SNF1	Sucrose NonFermenting	-0.53	3.96	Studies
YCR033W	SNT1	SaNT domains	-1.20	15.21	Micro
YJR104C	SOD1	SuperOxide Dismutase	0.19	-3.20	Studies
YCR081W	SRB8	Suppressor of RNA polymerase B	-1.09	10.94	Micro
YNL229C	URE2	UREidosuccinate transport	0.04	-7.25	Studies
YPL234C	VMA11	Vacuolar Membrane Atpase	0.34	1.59	Studies
YEL027W	VMA3	Vacuolar Membrane Atpase	0.12	-5.49	Studies
YOR332W	VMA4	Vacuolar Membrane Atpase	0.15	-5.44	Studies
YOR270C	VPH1	Vacuolar pH	-0.21	-3.13	Studies
YLL040C	VPS13	Vacuolar Protein Sorting	-1.52	12.73	Micro
YHR048W	YHK8		1.11	5.09	Micro
YBR016W			1.81	15.22	Micro
YDL241W			1.94	10.24	Micro

A light-regulated molecular network in yeast

We combined the transcriptionally and post-transcriptionally regulated genes that showed a phenotype in the growth assay (Table 1) and submitted them to the STRING database as a way to validate and organize this gene set. STRING is a database that catalogs 'all' experimentally derived interactions between genes and their products. It thus is a useful resource for making connections across levels (*e.g.* transcription, translation, metabolism, etc.). The STRING resources for yeast are extensive, due to the rich history of this organism in genetic and cell biological studies.

Network reconstruction, using the STRING protein interaction database with a high confidence interaction score (>0.7), fails to form a cohesive network; only 4 genes are connected by 2 edges. In order to increase the amount of information, in a context specific way, I applied the PREMONition method[16]. This method allows a Protein Protein Interaction (ppi) network to expand beyond a specific protein set (42 candidates) by incorporating No-light Phenotype displaying Connectors (NPCs)) resulting in an interconnected network containing 38 phenotype-displaying genes and 101 NPCs connected by 138 edges, removal of non-informative nodes, i.e the most outer NPC nodes, and edges results in a network of 69 nodes (31 NPC) connected by 68 edges (Fig. 3).

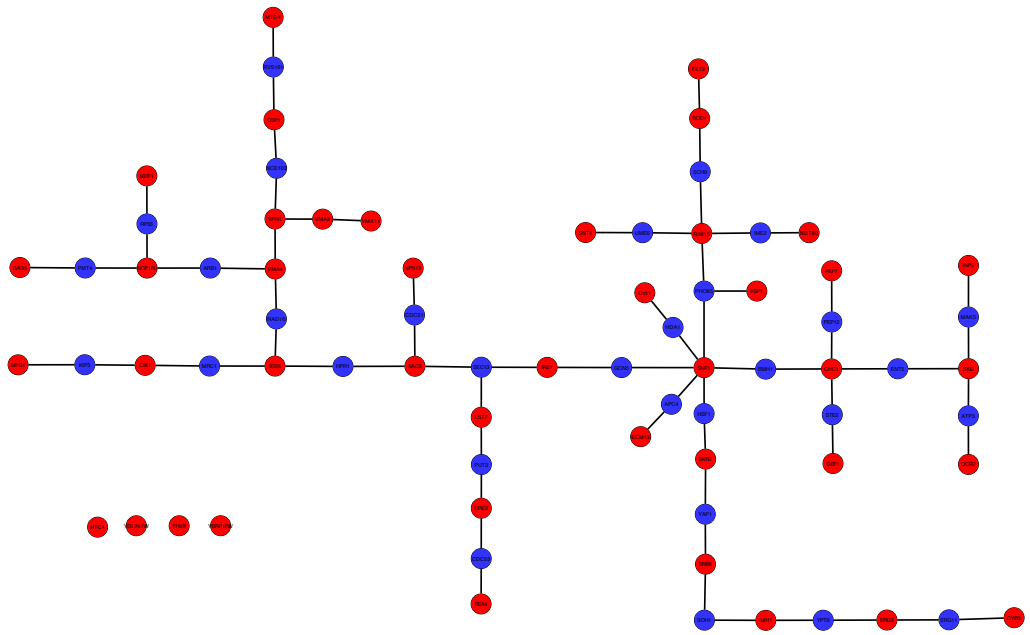


Figure 3. The PREMONition network for 42 phenotype displaying genes leading to a network that includes 38 genes. Proteins encoded by differentially expressed genes also showing a growth phenotype when exposed to light (red spheres) and their Non-Phenotype displaying Connectors (NPC) (blue spheres) are shown. The network is composed of 38 phenotype displaying genes and 31 NPCs, connected by 68 edges. 4 phenotype displaying genes are disjoined from the network. The figure is scalable such that each gene/protein can be identified. Annotations and node-type are shown in supplementary table S4.

Discussion

Previous work has shown that yeast cells are most susceptible to light in the blue to near-UV range. At the applied dosage of light, only 34 genes show differential expression after 30 minutes whereas 514 genes were altered after 120 minutes. This reperesents 9% of all protein coding genes. In *Neurospora crassa*, 24% of the predicted protein coding genes (2353 of 9728) are effected by exposure to light[17]. Of all 514 differentially expressed genes, 266 are up- (T_2U) and 214 are down- (T_2D) regulated relative to the dark sample.

Applying Panther[18] Gene Ontology (GO) enrichment analysis on T_2U, 38 biological processes (BPs) and 116 BPs in T_2D are identified (Table S2). Examining the 10 most specific child terms per GO-tree branch from the T_2U BPs (Table S3, terms marked with an asterisk), 2 processes are directly involved in cell wall formation and organization: Two processes are related to transport. Four BPs relate to protein and organelle organization and finally, 2 BPs are involved in the regulation of gene expression

Examining the 116 T_2U BPs, reveals 28 most specific child terms per GO-tree branch. Among these 28 BPs (Table S3, terms marked with an asterisk), 6 are directly involved in mitotic division (cell budding), an expected process since I selected for genes showing a repressed growth when exposed to light. Six BPs are involved in localization and organization, which are underlying processes of the cell division. Six BPs associate with cell signaling and transport. These signaling pathways include TOR, stress and response to external stimuli. The TOR kinase acts with in regulatory network, affecting growth according to nutrient availability, gene expression and protein stability, making TOR a global regulator of cellular activity[19]. TOR (Target Of Rapamycin)is also connected to the retrograde signaling pathway, which also senses mitochondrial disfunction[20], a phenomenon known to occur due to photo-oxidation/ destruction of the mitochondrial cytochrome a_1, a_3 and b[21]. Seven BPs are associated with managing DNA structure, on one hand DNA-organization for cell division, on the other hand chromatin remodeling for transcriptional activation or repression. The remaining 3 are regulation of cellular component biogenesis, regulation of biological quality and finally, trehalose biosynthetic process, which is involved on a range of processes including sporulation and glycolysis and so plays an important role in the glycolytic flux[22].

Analyzing the in total 42 candidate genes using PANTHER, only two significantly enriched biological process GO-terms are found; ATP hydrolysis coupled transmembrane transport and ion homeostasis. The later contains 8 genes and includes the four genes from the first GO-term (VPH1, VMA3, VMA4, VMA11, FET3, GEF1, OCT1, SOD1). Analyzing the PREMONition network (Fig. 3), comprising 69 genes, results in 103 significantly enriched GO-terms (Table S5) having 15 most specific child terms. The increased number of terms stems from the NPCs. Figure 3 shows the 15 child GO-terms and the number of occurrences of each protein associated with these terms. These 15 GO-terms show a high degree of overlap with the 28 T_2U BPs.

Most proteins are represented by multiple GO-terms making their relative contribution to the analysis larger (Fig. S4). SNF1 occurs in 13 out of 15 GO-term branches (having 57 associated GO-terms) and functions as a Serine-Threonine Protein Kinase. Ser/Thr kinases is an enzyme, known to regulate critical cellular functions such as signal transduction, metabolism, movement, circadian rhythm[23], various nutrient-responses, meiosis, sporulation, aging, haploid invasive growth, diploid pseudohyphal growth, environmental stresses, such as sodium ion stress, heat shock, alkaline pH, oxidative stress, and genotoxic stress[24]. The yeast genome encodes 110 Ser/Thr protein kinases[25]. Seven are captured in the PREMONition network (SNF1, SCH9, IME2, PHO85, KSP1, IRE1 and RIM15). SNF1 is activated by phosphorylation[26] in response to glucose and nitrogen limitation and involved in responses to other environmental stresses[24]. SNF1 is essential for the expression of glucose-repressible genes and SNF1 mutants do not store glycogen[27]. PHO85, is also involved in glycogen accumulation and storage and functions antagonistically to SNF1[28]. Glycogen storage is known to be under control of the circadian clock in cyanobacteria, fungus and mammals[29–31]. Also, Nuclear Magnetic Resonance, rhythms in glucose concentration levels were detected (unpublished data) in 1L fermentor cultures under a circadian temperature cycle. This suggests rhythms in glucose uptake and metabolism.

The protein encoded by SCH9 claimed the most terms in our GO-analysis (52 terms). SCH9 interacts with V-ATPase, influences vacuolar pH and thereby regulates / controls lifespan extension or shortening[32]. Although SCH9 is not assayed, it functions as a connector between RIM15 (a protein kinase involved in cell proliferation, involved in signal transduction during cell proliferation and the integration of glucose, nitrogen and amino acid availability[33], in response to nutrients) and SuperOxidase Dismutase 1 (SOD1) which plays a role in the activation of stress response genes due to the presence of radical oxygen species (ROS). ROS influence the intracellular pH of the yeast cells which affects the cellular homeostasis. Vacuolar $H(^+)$-

ATPase (V-ATPase) play an important role maintenance of intracellular pH homeostasis through vacuolar acidification, serving as a protection mechanism for the yeast cells against ROS species[34]. The PREMONition network contains VMA3, VMA4, VMA11 and VPH1. VMA3 has significant homology with vma-11 in *N. crassa,* a gene known to respond to light[17].

Screening the 514 knockout strains of the differentially expressed genes, identified using DNA micro-arrays, yielded only 28 genes showing a growth repression phenotype. In addition fourteen of the 124 genes predicted from previous published work[2,3,9,14] show a phenotype. These 42 genes plus 31 NPCs represent a set of biological processes suggesting adaptation of cellular physiology, either directly via a yet unidentified photoreceptor or indirectly via photo-oxidation, to the exposure of white light. Given the impact of light on yeast cells, it seems reasonable that yeast developed mechanisms minimize its damaging effects. Anticipating the light period would give yeast an advantage over organisms competing for the same nutrition's in its environment. The candidates identified here can serve as a set of candidate light-input genes to the yeast circadian clock.

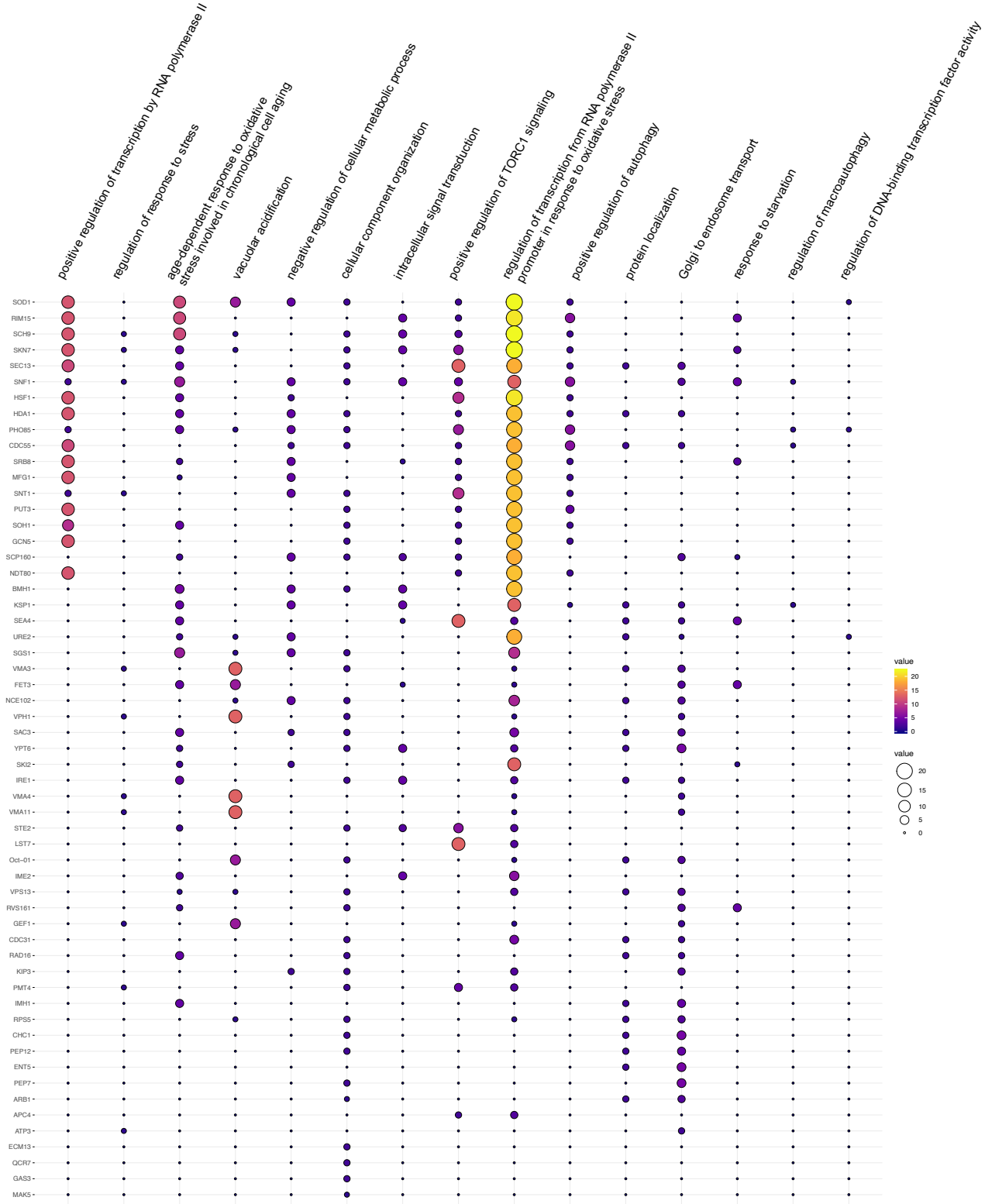


Figure 4. Fifteen significantly enriched gene ontology terms (child) and the number of gene occurrences of each gene within the parental terms. Some genes have a frequency of association then others.

Material and methods

DNA microarrays for light-regulated genes

Four individual FY1679-02b overnight cultures were grown in 4 ml liquid YPD at 30 °C, with shaking at 160 RPM. Each overnight culture was then pelleted at 4500 RPM and resuspended in 600 µl YPD. Using these cell suspensions, 12 YPD plates were inoculated with 200 µl cell suspensions, with the cells homogeneously distributed. Six plates were wrapped with aluminum foil (dark controls) and all 12 YPD plates were cultured for 2 days at 22°C in constant darkness.

Yeast cultures received a light pulse of either 30 or 120 minutes or no light pulse (dark control). The light-source consisted of a panel with blue LEDs (465 nm) emitting 5.6 µmole photons/m^2/s. Following light pulses, yeast cells, including dark controls, were collected, flash-frozen and stored until processing. RNA was extracted using the hot phenol method[1] and used for micro-array analysis (Service XS, Leiden NL) using the Affymetrix Yeast Genome 2.0 arrays. The microarray data was analysed using the Limma package[35] of the statistical programming language R[36]. A statistical threshold of a Log-fold change of ≥ 1 and a log-odds ≥ 2.944 (p-value ≥ 0.95) was applied.

The knockout library was obtained from EUROSCARF (Frankfurt am Main, Germany). Selected knockout strains were individually cultured overnight in 150 µl YPD (1% yeast extract, 2% peptone, 2% dextrose) medium in one well of a 96 well plate. Plates were held at 30°C with shaking at 160 RPM. The following day, the cultures were placed at 4 °C until further use.

On the day before initiating the growth assay, 10 µl from each well of the 96-well plates were inoculated into individual 100 ml Erlenmeyer flasks containing 20 ml of YPD medium. The cultures were grown overnight at 30 °C with shaking at 160 RPM. Prior to making the final dilution series, the cell number in the overnight cultures was adjusted such that the OD_{600} was 0.1. From this cell suspension, a series of five 1:1 dilutions were made (*e.g.* 100 µl of suspension in 100 µl YPD). From each dilution, 2 µl was spotted on two independent YPD plates containing 2% agar. The knockout strains were interspersed with five wild-type strains (FY1679-02b, CEN-PK2, BY4741, BY4742, BY4743), such that every 43 knockout-strains were followed by 5 wild-type strains. Of the two, replicate plates containing a given set of dilution series, one was wrapped in aluminum foil (dark control) and both were placed in the experimental setup. The dark controls are included to precisely control for small temperature effects. The light source consisted of 12 Philips 36W Master TL-D/835 Super 80 tubes (Fig.

S1). Incubations were carried out in a climate-controlled room with the temperature at the level of the plates 18.0 °C. Plates were incubated for two days at which point they were imaged (Scanmaker 9800 XL, Microtek; ScanWizard Pro 7 software).

Light induced growth repression analysis

The plate scans were analyzed using an in-lab developed Python routine that determines the amount of growth *i.e.* the pixel intensity and density of each spot within a square with fixed dimensions. All pixels within the square having a pixel intensity higher than a given background BRG-value (35) were counted and used as a quantitative measure for colony formation. Using this approach all 518 yeast knockout strains were screened for growth repression by light phenotype.

The intercept of a dilution series is used as quantitative growth measurement. Using the intercept, first a control strain showing the least growth repressed light response is determined, for internal (consistency) control. The wild-type strain CEN-PK2 appears most suitable, being the least sensitive to the light regime and having a narrow distribution (Fig. S2). The results suggest that the wild-type is (relatively) insensitive to the treatment (light or dark), justifying using the wild-type of each plate as base line, to be subtracted from each individual value. Using the CEN-PK2 light-light (LL) and the dark-dark (DD) intercept by subtracting it from the other strain intercepts.

Taking CEN-PK2 as reference *r*, the following question can be addressed: Is the difference between LL and DD larger for some strain *s* than for CEN-PK2 within a given experiment *e*? If the response be called $y_{se,LL}$ for strain *s* in experiment *e* under light conditions light-light and $y_{se,DD}$ under light conditions dark-dark, we can write the deviations from the reference values $\Delta_{s,LL} = y_{se,LL} - y_{re,LL}$, and $\Delta_{s,DD} = y_{se,DD} - y_{re,DD}$.

To investigate the relations between $\Delta_{s,LL}$ and $\Delta_{s,DD}$, principal component analysis was used on the 2 by 2 covariance matrix. This yields a view on the internal structure of the data set in which neither of the variables is given precedence, as is the case in regression analysis. The first principal component is expected to optimally capture the correlation between growth under light and dark conditions and reflect the overall effect of the knockout on growth regardless of these conditions. The deflection captured by the second principal component must then reflect a specific response to light or dark (Fig. S3). This deflection is further characterized as the Z-score of the projection on the second eigenvector. Strains having a z-score above 2 or below -2 were considered to have a significant specific light mediated growth

effect (Fig. 5). Strains outside of the core of the distribution and above this line grow more under dark conditions as expected based on the growth under light conditions and those strains that fall under this line grow less than expected.

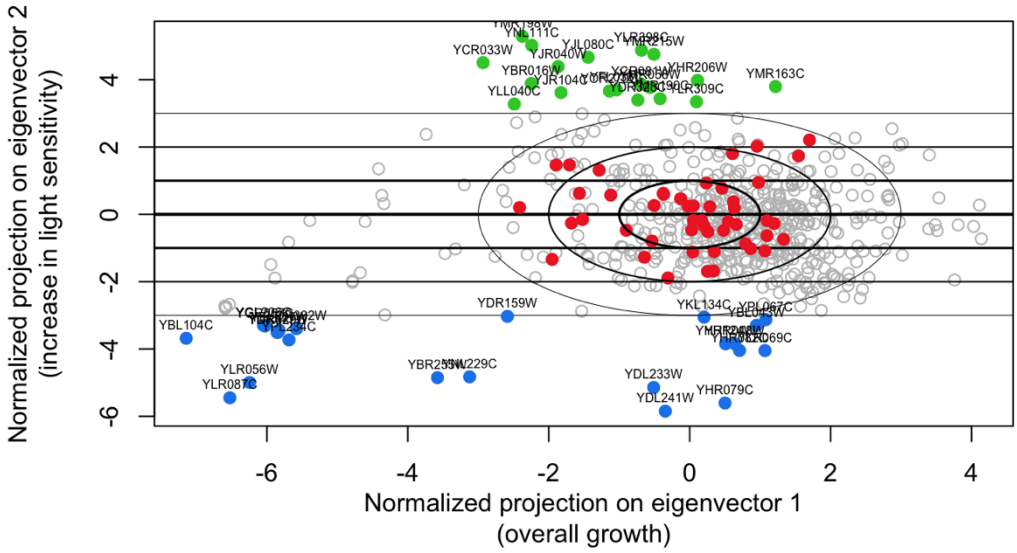


Figure 5. Normalized (z-scores) principal component plot. Confidence intervals (CI) 1, 2 and 3 standard deviations from the median (black lines / circles). Strains above the CI grow more under dark conditions as expected based on the growth under light conditions (green spheres) and those strains that fall under the CI grow less than expected (blue spheres). Red spheres indicate the control/ wild-type strains.

Acknowledgements

We thank members of the Merrow lab for comments on the manuscript. We thank Margriet van der Pol for technical assistance. Our work is supported by the NWO (Open Programma, 820.02.014), the European Commission (FP6 EUCLOCK), and the Rosalind Franklin Fellowships of the University of Groningen.

Literature references

1. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* (2010) doi:10.1073/pnas.0907902107.
2. Kippert, F., Engelmann, W. & Ninnemann, H. Blue light both synchronizes and inhibits growth of Schizosaccharomyces pombe at low temperatures. *Prog. Clin. Biol. Res.* (1990).
3. Elkind, M. M. & Sutton, H. Lethal effect of visible light on a mutant strain of haploid yeast. I. General dependencies. *Arch. Biochem. Biophys.* (1957) doi:10.1016/0003-9861(57)90176-5.
4. Sulkowski, E., Guerin, B., Defaye, J. & Slonimski, P. P. Inhibition of protein synthesis in yeast by low intensities of visible light. *Nature* (1964) doi:10.1038/202036a0.
5. FREEMAN, P. J. & GIESE, A. C. Photodynamic effects on metabolism and reproduction in yeast. *J. Cell. Physiol.* (1952) doi:10.1002/jcp.1030390209.
6. Matile, P. & Erey-Wyssling, A. Atmung und Wachstum von Hefe im Licht. *Planta* 58, 154–163 (1962).
7. Epel, B. & Butler, W. L. Cytochrome a3: Destruction by light. *Science (80-.).* (1969) doi:10.1126/science.166.3905.621.
8. Ehrenberg, M. Die Höhe des Lichteffektes auf Wachstum und Stoffwechsel von Saccharomyces cerevisiae in Abhängigkeit vom Phasenstatus der Vorkulturzellen. *Arch. Mikrobiol.* (1968) doi:10.1007/BF00704288.
9. Ninnemann, H., Butler, W. L. & Epel, B. L. Inhibition of respiration and destruction of cytochrome a3 by light in mitochondria and cytochrome oxidase from beef heart. *BBA - Bioenerg.* (1970) doi:10.1016/0005-2728(70)90116-7.
10. Ułaszewski, S. *et al.* Light effects in yeast: Evidence for participation of cytochromes in photoinhibition of growth and transport in Saccharomyces cerevisiae cultured at low temperatures. *J. Bacteriol.* 138, 523–529 (1979).
11. Robertson, J. B., Davis, C. R. & Johnson, C. H. Visible light alters yeast metabolic rhythms by inhibiting respiration. *Proc. Natl. Acad. Sci.* 110, 21130–21135 (2013).
12. Olmedo, M., Ruger-Herreros, C., Luque, E. M. & Corrochano, L. M. Regulation of transcription by light in Neurospora crassa: Amodel for fungal photobiology? *Fungal Biology Reviews* (2013) doi:10.1016/j.fbr.2013.02.004.
13. Koritala, B. S. C. & Lee, K. Natural Variation of the Circadian Clock in Neurospora. in *Advances in Genetics* (2017). doi:10.1016/bs.adgen.2017.09.001.
14. Ninnemann, H., Butler, W. L. & Epel, B. L. Inhibition of respiration in yeast by light. *BBA - Bioenerg.* (1970) doi:10.1016/0005-2728(70)90115-5.

15. Puig, S. Function and regulation of the plant COPT family of high-affinity copper transport proteins. *Adv. Bot.* 2014, 1–9 (2014).
16. Bosman, J., Eelderink-Chen, Z., Laing, E. & Merrow, M. PREMONition: An algorithm for predicting the circadian clock-regulated molecular network. *bioRxiv* (2018) doi:10.1101/463190.
17. Wu, C. *et al.* Genome-wide characterization of light-regulated genes in Neurospora crassa. *G3 Genes, Genomes, Genet.* (2014) doi:10.1534/g3.114.012617.
18. Mi, H. *et al.* PANTHER version 11: Expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. *Nucleic Acids Res.* 45, D183–D189 (2017).
19. Powers, T., Dilova, I., Chen, C. Y. & Wedaman, K. Yeast TOR signaling: A mechanism for metabolic regulation. *Current Topics in Microbiology and Immunology* (2003) doi:10.1007/978-3-642-18930-2_3.
20. Giannattasio, S., Liu, Z., Thornton, J. & Butow, R. A. Retrograde response to mitochondrial dysfunction is separable from TOR1/2 regulation of retrograde gene expression. *J. Biol. Chem.* (2005) doi:10.1074/jbc.M509187200.
21. Woodward, J. R., Cirillo, V. P. & Edmunds, L. N. Light effects in yeast: Inhibition by visible light of growth and transport in Saccharomyces cerevisiae grown at low temperatures. *J. Bacteriol.* (1978) doi:10.1128/jb.133.2.692-698.1978.
22. Gancedo, C. & Flores, C. L. The importance of a functional trehalose biosynthetic pathway for the life of yeasts and fungi. *FEMS Yeast Research* (2004) doi:10.1016/S1567-1356(03)00222-8.
23. Park, G. *et al.* Global analysis of serine-threonine protein kinase genes in neurospora crassa. *Eukaryot. Cell* (2011) doi:10.1128/EC.05140-11.
24. Hedbacker, K. & Carlson, M. SNF1/AMPK pathways in yeast. *Frontiers in Bioscience* (2008) doi:10.2741/2854.
25. Brinkworth, R. I., Munn, A. L. & Kobe, B. Protein kinases associated with the yeast phosphoproteome. *BMC Bioinformatics* (2006) doi:10.1186/1471-2105-7-47.
26. Ruiz, A., Xu, X. & Carlson, M. Roles of two protein phosphatases, Reg1-Glc7 and Sit4, and glycogen synthesis in regulation of SNF1 protein kinase. *Proc. Natl. Acad. Sci. U. S. A.* (2011) doi:10.1073/pnas.1102758108.
27. Wang, Z., Wilson, W. A., Fujino, M. A. & Roach, P. J. Antagonistic Controls of Autophagy and Glycogen Accumulation by Snf1p, the Yeast Homolog of AMP-Activated Protein Kinase, and the Cyclin-Dependent Kinase Pho85p. *Mol. Cell. Biol.* (2001) doi:10.1128/mcb.21.17.5742-5752.2001.
28. Huang, D., Farkas, I. & Roach, P. J. Pho85p, a cyclin-dependent protein kinase, and

the Snf1p protein kinase act antagonistically to control glycogen accumulation in Saccharomyces cerevisiae. *Mol. Cell. Biol.* (1996) doi:10.1128/mcb.16.8.4357.

29. Baek, M. *et al.* Circadian clock regulation of the glycogen synthase (gsn) gene by WCC is critical for rhythmic glycogen metabolism in Neurospora crassa. *Proc. Natl. Acad. Sci. U. S. A.* (2019) doi:10.1073/pnas.1815360116.
30. Doi, R., Oishi, K. & Ishida, N. CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2. *J. Biol. Chem.* (2010) doi:10.1074/jbc.M110.110361.
31. Pattanayak, G. K., Phong, C. & Rust, M. J. Rhythms in energy storage control the ability of the cyanobacterial circadian clock to reset. *Curr. Biol.* (2014) doi:10.1016/j.cub.2014.07.022.
32. Wilms, T. *et al.* The yeast protein kinase Sch9 adjusts V-ATPase assembly/disassembly to control pH homeostasis and longevity in response to glucose availability. *PLoS Genet.* (2017) doi:10.1371/journal.pgen.1006835.
33. Swinnen, E., Rosseels, J. & Winderickx, J. The minimum domain of Pho81 is not sufficient to control the Pho85-Rim15 effector branch involved in phosphate starvation-induced stress responses. *Curr. Genet.* (2005) doi:10.1007/s00294-005-0583-3.
34. Milgrom, E., Diab, H., Middleton, F. & Kane, P. M. Loss of vacuolar proton-translocating ATPase activity in yeast results in chronic oxidative stress. *J. Biol. Chem.* (2007) doi:10.1074/jbc.M608293200.
35. Ritchie, M. E. *et al.* Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* (2015) doi:10.1093/nar/gkv007.
36. R Core Team. R: A language and environment for statistical computing. http://www.R-project.org/. *R Foundation for Statistical Computing, Vienna, Austria* (2019).

Supporting Information Materials

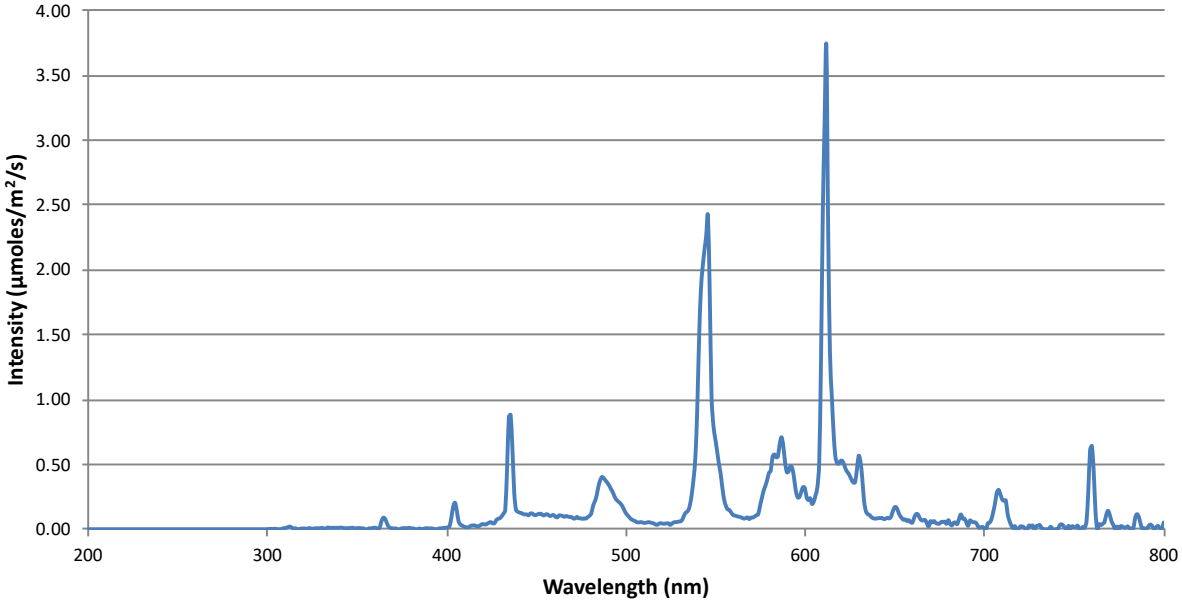


Figure S1. Spectrum of Philips 36W Master TL-D/835 Super 80 light tube collected using a 20-millisecond integration time.

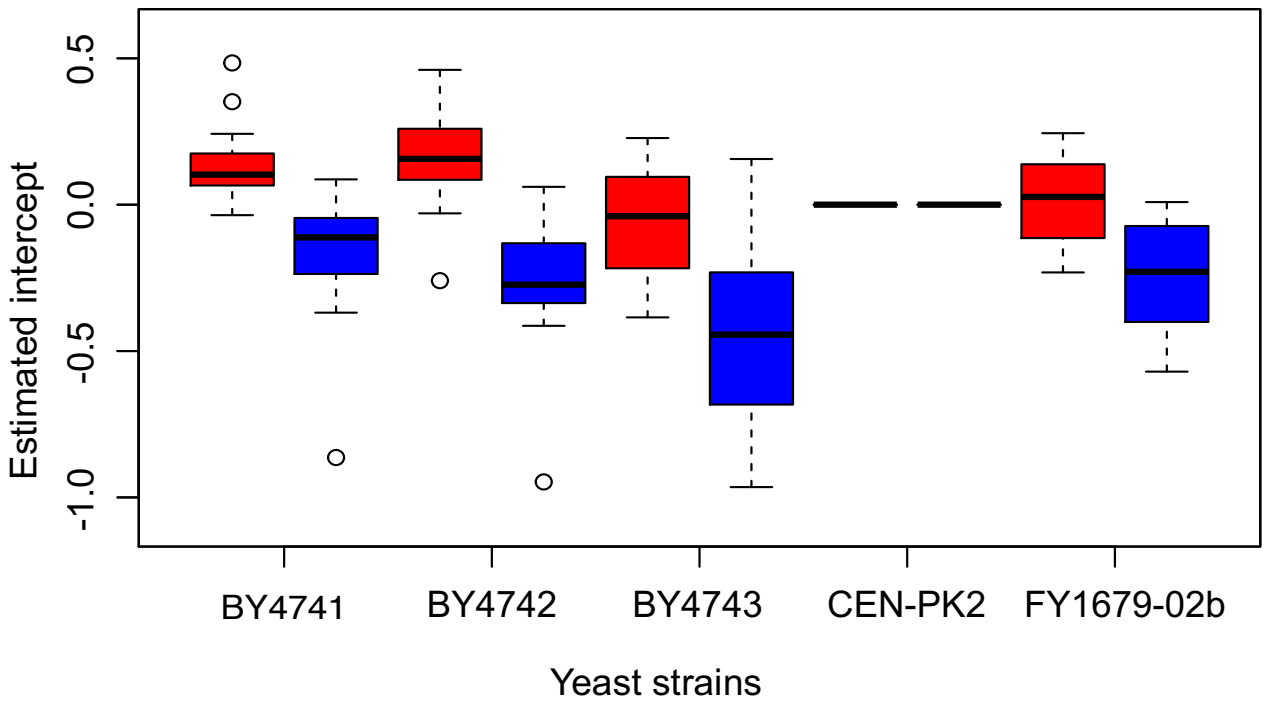


Figure S2. Distributions of the control strains for continuous light (blue) and continuous dark (red) conditions. Each distribution consists of 14 experiments and is calculated by subtracting the estimated intercepts of CEN-PK2 from the estimated intercepts of the control strains. Intercepts are calculated from the cell-densities from the dilution series spotted on the YPD-agar plates (main text Figure 2).

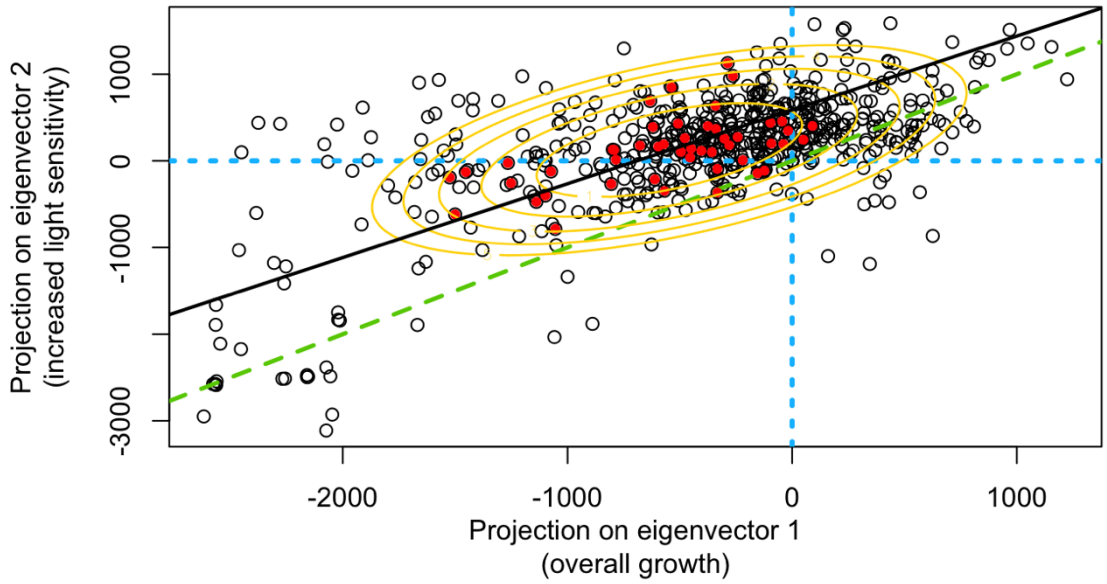


Figure S3. Principal component plot of all corrected knockout strains (open spheres) and control strains (red spheres) based on the 2 by 2 Δs,LL and Δs,DD covariance matrix. See main text, materials and methods light induced growth repression analysis for more details. Quadrans line passes through 0,0 (blue dotted); linear regression line through 0,0 (dotted green line); linear regression line through the dataset centre / median (solid black line) and confidence intervals 1 to 5 standard deviations from the median (blue circles).

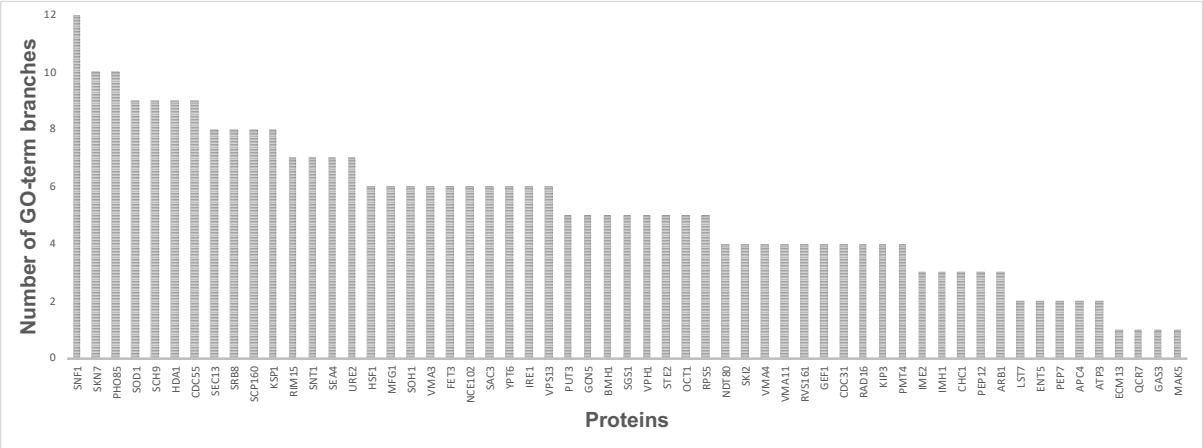


Figure S4. The number of GO-term branches in which each protein occurs.

Table S1. Differentially expressed genes from yeast cells exposed to blue light for 60 minutes (T1 up, T1 down and 120 minutes (T2 up, T2 down). Genes are considered significant when the Log-fold-change (LFC) ≥1 and the log-odds (LO) ≥ 2.944 (p-value ≥ 0.95).

T_1 up	T_1 down	T_2 up								
YOR394C-A	YOL023W	YAL053W	YCR102C	YEL073C	YGR161C	YJR150C	YLR099C	YMR016C	YNR032W	YOR342C
YPR015C	YGR248W	YAL064W	YDL010W	YER011W	YGR177C	YKL001C	YLR099W-A	YMR018W	YNR051C	YOR381W
YHR214C-D	YIL113W	YAL068C	YDL012C	YER020W	YGR204C-A	YKL029C	YLR100W	YMR040W	YNR059W	YOR385W
YGR177C		YAR007C	YDL059C	YER045C	YGR213C	YKL043W	YLR107W	YMR069W	YNR064C	YOR394C-A
YOL155W-A		YAR020C	YDL233W	YER046W	YGR268C	YKL046C	YLR108C	YMR084W	YOL014W	YPL027W
YOR049C		YAR027W	YDL241W	YER073W	YHL048C-A	YKL068W-A	YLR120C	YMR085W	YOL015W	YPL038W-A
YNL251C		YBL040C	YDL243C	YER081W	YHR022C-A	YKL071W	YLR121C	YMR094W	YOL016C	YPL056C
YLR056W		YBL042C	YDL243C	YER092W	YHR048W	YKL074C	YLR125W	YMR095C	YOL038C-A	YPL067C
YLR073C		YBL054W	YDL246C	YER095W	YHR124W	YKL096C-B	YLR136C	YMR177W	YOL123W	YPL088W
YOR306C		YBL055C	YDL247W	YER184C	YHR179W	YKL159C	YLR137W	YMR180C	YOL124C	YPL149W
YPL024W		YBL061C	YDR034W-B	YER185W	YHR214C-D	YKL166C	YLR162W-A	YMR238W	YOL155W-A	YPL171C
YOR009W		YBL101W-A	YDR043C	YER188C-A	YHR214C-E	YKL183C-A	YLR176C	YMR316W	YOL156W	YPL189W
YNL065W		YBL108C-A	YDR072C	YFL020C	YIL011W	YKR053C	YLR180W	YNL020C	YOL158C	YPL233W
YGR045C		YBR004C	YDR078C	YFL020C	YIL046W-A	YKR061W	YLR194C	YNL044W	YOL159C-A	YPL250C
YGR079W		YBR005W	YDR124W	YFL026W	YIL056W	YKR091W	YLR234W	YNL065W	YOL162W	YPR015C
YLR194C		YBR008C	YDR182W-A	YFL056C	YIL066C	YKR092C	YLR266C	YNL093W	YOL163W	YPR078C
YLR406C-A		YBR016W	YDR207C	YGL012W	YIL073C	YLL012W	YLR346C	YNL111C	YOL164W	YPR094W
YDR402C		YBR056W-A	YDR213W	YGL053W	YIL114C	YLL028W	YLR350W	YNL138W-A	YOR004W	YPR144C
YMR011W		YBR066C	YDR246W-A	YGL120C	YIL117C	YLL033W	YLR406C-A	YNL145W	YOR009W	YPR154W
YML058W-A		YBR070C	YDR253C	YGL127C	YIL121W	YLL049W	YLR412C-A	YNL146C-A	YOR032W-A	YPR167C
YOL014W		YBR071W	YDR259C	YGL157W	YIL134C-A	YLL056C	YLR414C	YNL155W	YOR049C	YPR174C
YNL162W-A		YBR114W	YDR398W	YGL166W	YIL176C	YLL057C	YLR417W	YNL157W	YOR072W-B	YPR198W
YBL054W		YBR182C	YDR476C	YGL183C	YIR011C	YLL060C	YLR460C	YNL192W	YOR134W	
YGR035C		YBR244W	YDR501W	YGL194C	YIR018C-A	YLL061W	YLR461W	YNL193W	YOR153W	
YOL124C		YBR284W	YDR502C	YGL263W	YJL106W	YLL062C	YLR466C-B	YNL210W	YOR174W	
YCR072C		YBR294W	YDR508C	YGR035C	YJL108C	YLL064C	YLR466C-B	YNL211C	YOR192C	
YER185W		YBR295W	YEL035C	YGR057C	YJL134W	YLL066W-B	YML018C	YNL277W	YOR197W	
YMR316W		YBR301W	YEL047C	YGR060W	YJL144W	YLL066W-B	YML058W-A	YNL312W	YOR220W	
YBR056W-A		YCL069W	YEL048C	YGR079W	YJL171C	YLR004C	YML116W	YNR010W	YOR237W	
YKR061W		YCR007C	YEL049W	YGR121W-A	YJR010C-A	YLR054C	YMR009W	YNR014W	YOR306C	
YLL012W		YCR020W-B	YEL072W	YGR131W	YJR078W	YLR056W	YMR011W	YNR019W	YOR338W	

T_2 down					
YAL024C	YDR222W	YHR079C	YKR039W	YMR198W	YPL253C
YAR018C	YDR258C	YHR080C	YKR054C	YMR215W	YPL255W
YBL003C	YDR301W	YHR082C	YKR066C	YMR218C	YPR008W
YBL023C	YDR326C	YHR099W	YKR077W	YMR231W	YPR055W
YBL034C	YDR334W	YHR120W	YLL006W	YMR246W	YPR083W
YBL043W	YDR420W	YHR155W	YLL040C	YMR261C	YPR095C
YBL066C	YDR443C	YHR158C	YLR069C	YMR273C	YPR117W
YBL069W	YDR457W	YHR164C	YLR084C	YMR278W	YPR119W
YBL104C	YDR475C	YHR165C	YLR087C	YMR287C	YPR127W
YBR038W	YDR520C	YIL031W	YLR153C	YMR288W	YPR138C
YBR059C	YDR536W	YIL119C	YLR168C	YNL037C	YPR157W
YBR067C	YER008C	YIL126W	YLR182W	YNL058C	YPR160W
YBR081C	YER013W	YIL129C	YLR190W	YNL068C	YPR184W
YBR097W	YER033C	YIL138C	YLR223C	YNL106C	YPR189W
YBR117C	YER093C	YIL149C	YLR309C	YNL172W	
YBR136W	YER103W	YIL158W	YLR382C	YNL242W	
YBR140C	YER164W	YIR023W	YLR398C	YNL271C	
YBR156C	YER166W	YJL005W	YLR419W	YNL300W	
YCL024W	YFL034W	YJL042W	YLR422W	YNR031C	
YCL063W	YFL036W	YJL056C	YLR424W	YOL004W	
YCR033W	YFR016C	YJL080C	YLR425W	YOL023W	
YCR081W	YFR019W	YJL087C	YLR454W	YOL034W	
YCR088W	YFR031C	YJL181W	YML006C	YOL078W	
YCR093W	YGL021W	YJL194W	YML027W	YOR018W	
YDL019C	YGL033W	YJL197W	YML034W	YOR025W	
YDL028C	YGL049C	YJR033C	YML052W	YOR093C	
YDL056W	YGL113W	YJR036C	YML064C	YOR129C	
YDL058W	YGL150C	YJR066W	YML065W	YOR160W	
YDL138W	YGL195W	YJR092W	YML072C	YOR161C	
YDL140C	YGL206C	YJR112W	YML091C	YOR216C	
YDL171C	YGR047C	YJR140C	YML100W	YOR290C	
YDL240W	YGR092W	YKL048C	YML120C	YOR291W	
YDR027C	YGR098C	YKL062W	YMR001C	YOR296W	
YDR033W	YGR116W	YKL105C	YMR001C-A	YOR304W	
YDR074W	YGR150C	YKL129C	YMR032W	YPL029W	
YDR146C	YGR217W	YKL134C	YMR105C	YPL040C	
YDR150W	YGR248W	YKL203C	YMR109W	YPL082C	
YDR159W	YGR271W	YKR013W	YMR162C	YPL085W	
YDR170C	YHL028W	YKR028W	YMR163C	YPL111W	
YDR180W	YHR023W	YKR031C	YMR190C	YPL137C	

Table S2. One-hundredth twenty-four genes predicted from previous published work, designated as post-transcriptional genes, screened for photo-responsiveness using the growth assay. Gene standard name is abbreviated as "Std.name" and it's systematic name as "Sys.name"

Std. name	Sys.name	Std. name	Sys.name	Std. name	Sys.name	Std. name	Sys.name
AIF1	YNR074C	COX9	YDL067C	PEP7	YDR323C	SKN7	YHR206W
AIM45	YPR004C	CUP5	YEL027W	PHO89	YBR296C	SLM6	YBR266C
ATP1	YBL099W	CYC1	YJR048W	PTP2	YOR208W	SNF1	YDR477W
ATP14	YLR295C	CYC2	YOR037W	PTP3	YER075C	SOD1	YJR104C
ATP15	YPL271W	CYC7	YEL039C	QCR10	YHR001W-A	SOD2	YHR008C
ATP17	YDR377W	CYS4	YGR155W	QCR6	YFR033C	SSK1	YLR006C
ATP18	YML081C-A	CYT1	YOR065W	QCR7	YDR529C	STE11	YLR362W
ATP19	YOL077W-A	FET3	YMR058W	QCR8	YJL166W	STE20	YHL007C
ATP2	YJR121W	FMO1	YHR176W	QCR9	YGR183C	STV1	YMR054W
ATP20	YPR020W	FOB1	YDR110W	RAS1	YOR101W	TFP1	YDL185W
ATP3	YBR039W	GEF1	YJR040W	RAS2	YNL098C	TFP3	YPL234C
ATP3	YBR051C	HAP2	YGL237C	REI1	YBR267W	TOS3	YGL179C
ATP3	YBR074C	HAP3	YBL021C	RIM15	YFL033C	TPK1	YJL164C
ATP4	YPL078C	HAP4	YKL109W	RIP1	YEL024W	TPK2	YPL203W
ATP5	YDR298C	HOG1	YLR113W	ROM2	YLR371W	TRK1	YJL129C
ATP7	YKL016C	HST2	YPL015C	RPL31A	YDL075W	TRX2	YGR209C
BSD2	YBR290W	IDH2	YOR136W	RPL6B	YLR448W	TSA2	YDR453C
CIR2	YOR356W	IRC14	YOR135C	RTG1	YOL067C	URE2	YNL229C
COQ6	YGR255C	LAT1	YNL071W	RTG3	YBL103C	VCX1	YDL128W
COR1	YBL045C	LOT6	YLR011W	SAK1	YER129W	VMA13	YPR036W
COX10	YPL172C	MAF1	YDR005C	SCH9	YHR205W	VMA2	YBR127C
COX11	YPL132W	MSN2	YMR037C	SDH1	YKL148C	VMA21	YGR105W
COX12	YLR038C	MTC4	YBR255W	SDH2	YLL041C	VMA22	YHR060W
COX13	YGL191W	NDE1	YMR145C	SDH4	YDR178W	VMA4	YOR332W
COX15	YER141W	NDE2	YDL085W	SDH9	YJL045W	VMA5	YKL080W
COX17	YLL009C	NHA1	YLR138W	SHH3	YMR118C	VMA6	YLR447C
COX5A	YNL052W	NHX1	YDR456W	SHH4	YLR164W	VMA7	YGR020C
COX5B	YIL111W	OSM1	YJR051W	SHO1	YER118C	VMA8	YEL051W
COX6	YHR051W	PAI3	YMR174C	SIR2	YDL042C	VPH1	YOR270C
COX7	YMR256C	PBS2	YJL128C	SIR3	YLR442C		YBR238C
COX8	YLR395C	PEP4	YPL154C	SIR4	YDR227W		YBR225W

Table S3. Significant enriched Gene Ontology (GO) PANTHER overrepresentation analysis results for *Saccharomyces cerevisiae* cells exposed to blue light for 120 minutes (T_2 up, T_2 down), using FISHER Exact test with a False Discovery Rate (FDR) correction to correct for multiple testing. Fold enrichment designates the overrepresentation compared to the background. Per GO-tree branch, alternating white/grey areas, most specific child terms are marked with an asterisk.

T_2 Up			
GO biological process	**Fold Enrichment**	**raw P value**	**FDR**
xenobiotic transport *	11.73	4.95E-05	9.39E-03
sterol metabolic process *	4.77	2.52E-04	3.93E-02
steroid metabolic process	4.67	2.89E-04	4.03E-02
organic substance metabolic process	0.73	2.03E-05	4.30E-03
metabolic process	0.75	1.24E-05	2.74E-03
primary metabolic process	0.70	3.26E-06	1.02E-03
fungal-type cell wall organization *	2.93	2.35E-05	4.80E-03
cell wall organization	2.73	1.21E-05	2.93E-03
cell wall organization or biogenesis	2.67	2.33E-06	8.25E-04
cellular process	0.81	1.11E-06	5.88E-04
external encapsulating structure organization	2.73	1.21E-05	2.80E-03
fungal-type cell wall organization or biogenesis	3.22	2.80E-07	2.47E-04
RNA metabolic process *	0.46	1.51E-04	2.50E-02
nucleic acid metabolic process	0.52	5.94E-05	1.09E-02
nucleobase-containing compound metabolic process	0.51	9.13E-06	2.55E-03
cellular nitrogen compound metabolic process	0.46	4.12E-09	2.19E-05
nitrogen compound metabolic process	0.65	4.05E-07	3.08E-04
cellular metabolic process	0.67	6.27E-08	1.11E-04
heterocycle metabolic process	0.51	3.39E-06	9.99E-04
cellular aromatic compound metabolic process	0.50	2.15E-06	8.76E-04
macromolecule metabolic process	0.58	7.65E-08	1.02E-04
protein localization *	0.43	2.09E-04	3.36E-02
macromolecule localization	0.47	2.74E-04	3.93E-02
organelle organization *	0.39	4.36E-08	1.16E-04
intracellular transport *	0.38	2.72E-04	4.01E-02
cellular localization	0.38	1.16E-05	2.93E-03
cellular component assembly *	0.37	7.20E-05	1.28E-02
protein-containing complex subunit organization *	0.25	2.10E-06	9.31E-04
translation *	< 0.01	1.18E-06	5.72E-04
cellular macromolecule metabolic process	0.58	2.15E-06	8.17E-04
protein metabolic process	0.55	7.35E-05	1.26E-02
organonitrogen compound metabolic process	0.65	2.59E-04	3.92E-02
peptide biosynthetic process	< 0.01	7.27E-07	4.82E-04
peptide metabolic process	0.13	3.40E-05	6.68E-03
cellular amide metabolic process	0.16	1.01E-05	2.69E-03
amide biosynthetic process	< 0.01	1.44E-07	1.53E-04
cellular nitrogen compound biosynthetic process	0.35	3.19E-06	1.06E-03
gene expression	0.40	9.57E-07	5.64E-04

T2 Down			
GO biological process complete	**Fold Enrichment**	**raw P value**	**FDR**
positive regulation of Ras protein signal transduction *	23.51	9.29E-04	4.41E-02
regulation of Ras protein signal transduction	7.84	7.81E-05	7.68E-03
regulation of small GTPase mediated signal transduction	7.31	1.13E-04	9.72E-03
regulation of intracellular signal transduction	4.27	5.25E-05	5.93E-03
regulation of signal transduction	3.02	6.17E-04	3.27E-02
regulation of cell communication	2.87	9.54E-04	4.40E-02
regulation of cellular process	1.93	2.20E-12	2.34E-09
regulation of biological process	1.89	1.09E-12	1.45E-09
biological regulation	1.77	4.86E-13	1.29E-09
regulation of signaling	3.02	6.17E-04	3.31E-02
positive regulation of small GTPase mediated signal transduction	23.51	9.29E-04	4.37E-02
positive regulation of intracellular signal transduction	5.93	3.52E-04	2.20E-02
positive regulation of signal transduction	5.22	6.92E-04	3.50E-02
positive regulation of biological process	2.07	4.55E-07	1.34E-04
positive regulation of cellular process	2.08	6.10E-07	1.70E-04
positive regulation of signaling	5.22	6.92E-04	3.53E-02
trehalose biosynthetic process *	17.91	2.47E-04	1.66E-02
trehalose metabolic process	11.40	9.25E-04	4.47E-02
cellular process	1.16	2.45E-05	3.25E-03
disaccharide biosynthetic process	15.67	3.61E-04	2.20E-02
oligosaccharide biosynthetic process	15.67	3.61E-04	2.23E-02
mitotic cytokinetic process *	11.40	9.25E-04	4.43E-02
mitotic cell cycle process	3.99	5.59E-13	9.89E-10
mitotic cell cycle	4.13	2.15E-14	1.14E-10
cell cycle	2.63	4.92E-12	4.35E-09
cell cycle process	2.83	1.80E-11	1.36E-08
mitotic cytokinesis	5.39	1.61E-05	2.25E-03
cytoskeleton-dependent cytokinesis	5.45	5.91E-06	1.01E-03
cytokinesis	5.03	5.26E-06	9.64E-04
cell division	2.81	5.94E-06	9.85E-04
actomyosin contractile ring organization *	9.79	3.65E-04	2.20E-02
cortical actin cytoskeleton organization	7.62	8.77E-06	1.33E-03
actin cytoskeleton organization	3.81	7.56E-05	7.72E-03
cytoskeleton organization	3.53	1.21E-07	4.02E-05
organelle organization	1.85	5.93E-09	2.86E-06
cellular component organization	1.61	1.14E-08	5.03E-06
cellular component organization or biogenesis	1.45	3.55E-06	7.54E-04
actin filament-based process	3.92	2.99E-05	3.87E-03
cortical cytoskeleton organization	7.62	8.77E-06	1.29E-03
actomyosin structure organization	9.79	3.65E-04	2.18E-02
chromosome separation *	9.22	4.60E-04	2.60E-02
positive regulation of mitotic nuclear division *	8.95	1.39E-04	1.15E-02
regulation of mitotic nuclear division	3.79	2.83E-04	1.86E-02
regulation of cell cycle process	3.13	7.55E-06	1.18E-03
regulation of cell cycle	2.82	3.54E-06	7.84E-04

regulation of nuclear division	3.31	2.71E-04	1.80E-02
regulation of organelle organization	3.02	5.17E-08	1.96E-05
regulation of cellular component organization	3.06	5.79E-11	3.84E-08
regulation of mitotic cell cycle	3.65	2.79E-06	7.05E-04
positive regulation of mitotic cell cycle	5.42	9.24E-05	8.76E-03
positive regulation of cell cycle	4.20	1.23E-04	1.04E-02
positive regulation of nuclear division	8.13	6.41E-05	6.68E-03
positive regulation of organelle organization	3.69	5.49E-05	5.95E-03
positive regulation of cellular component organization	3.28	5.95E-05	6.32E-03
positive regulation of cell cycle process	4.29	2.16E-04	1.51E-02
TOR signaling *	8.55	1.72E-04	1.25E-02
intracellular signal transduction	3.13	3.51E-05	4.33E-03
signal transduction	2.84	4.97E-06	9.78E-04
cellular response to stimulus	2.10	9.60E-10	5.67E-07
response to stimulus	1.95	4.70E-09	2.50E-06
signaling	2.84	4.97E-06	9.43E-04
cell communication	2.75	1.51E-07	4.72E-05
regulation of transcription involved in G1/S transition of mitotic cell cycle *	7.84	7.81E-05	7.83E-03
G1/S transition of mitotic cell cycle	5.32	1.05E-04	9.64E-03
mitotic cell cycle phase transition	5.29	3.20E-06	7.73E-04
cell cycle phase transition	5.29	3.20E-06	7.39E-04
cell cycle G1/S phase transition	5.32	1.05E-04	9.47E-03
regulation of cellular macromolecule biosynthetic process	1.65	9.49E-04	4.42E-02
regulation of cellular biosynthetic process	1.65	7.85E-04	3.90E-02
regulation of biosynthetic process	1.65	8.02E-04	3.94E-02
regulation of metabolic process	1.59	1.71E-04	1.26E-02
regulation of cellular metabolic process	1.63	1.09E-04	9.47E-03
regulation of macromolecule biosynthetic process	1.67	6.79E-04	3.50E-02
regulation of macromolecule metabolic process	1.60	1.82E-04	1.31E-02
regulation of primary metabolic process	1.60	4.00E-04	2.31E-02
regulation of nitrogen compound metabolic process	1.59	4.92E-04	2.70E-02
protein autophosphorylation *	7.23	3.74E-04	2.18E-02
protein phosphorylation	2.98	1.64E-04	1.23E-02
lipid transport *	5.22	2.08E-05	2.84E-03
lipid localization	4.93	3.39E-05	4.29E-03
macromolecule localization	1.73	1.09E-04	9.61E-03
cell budding *	5.04	1.53E-04	1.23E-02
reproduction of a single-celled organism	2.64	5.91E-04	3.20E-02
reproduction	2.16	1.63E-04	1.24E-02
asexual reproduction	5.04	1.53E-04	1.21E-02
regulation of mitotic sister chromatid segregation *	5.01	3.68E-04	2.17E-02
regulation of sister chromatid segregation	4.82	4.65E-04	2.57E-02
regulation of chromosome segregation	4.27	4.61E-04	2.58E-02
regulation of chromosome organization	3.48	5.42E-05	5.99E-03
exocytosis *	4.92	4.14E-04	2.36E-02
DNA packaging *	4.86	1.94E-04	1.37E-02
DNA conformation change	3.32	1.52E-04	1.24E-02

chromosome organization	2.23	2.48E-06	6.58E-04
establishment of cell polarity *	4.72	4.80E-05	5.66E-03
establishment or maintenance of cell polarity	4.27	5.97E-06	9.61E-04
negative regulation of mitotic cell cycle phase transition *	4.70	2.44E-04	1.68E-02
negative regulation of cell cycle phase transition	4.70	2.44E-04	1.66E-02
negative regulation of cellular process	2.34	4.96E-08	2.03E-05
negative regulation of biological process	2.22	5.31E-08	1.88E-05
negative regulation of mitotic cell cycle	3.56	8.32E-04	4.05E-02
positive regulation of cytoskeleton organization *	4.56	6.51E-04	3.42E-02
regulation of cytoskeleton organization	4.12	3.64E-05	4.40E-03
regulation of actin cytoskeleton organization *	4.48	1.58E-04	1.22E-02
regulation of actin filament-based process	4.48	1.58E-04	1.24E-02
mitotic cell cycle checkpoint *	3.97	7.45E-04	3.73E-02
negative regulation of organelle organization *	3.81	5.83E-06	1.03E-03
negative regulation of cellular component organization	3.76	3.56E-06	7.27E-04
response to external stimulus *	3.05	3.49E-04	2.20E-02
regulation of cellular component biogenesis *	2.82	3.02E-04	1.93E-02
nuclear division *	2.72	6.62E-04	3.45E-02
regulation of localization *	2.71	1.03E-03	4.72E-02
chromatin organization *	2.40	8.11E-05	7.83E-03
regulation of biological quality *	1.91	2.87E-04	1.86E-02
cellular response to stress *	1.87	1.01E-04	9.39E-03
response to stress	1.95	9.31E-06	1.34E-03
cellular localization *	1.75	5.17E-05	5.97E-03

Table S4. Seventy-three PREMONition network genes/nodes. Standard name is abbreviated as "Std.name" and systematic name as "Sys.name". Two node types have been defined, "PDG": Phenotype-Displaying-Gene and "NPC": Non- Phenotype-Connector.

Std.name	Sys.name	Annotation	Node type
APC4	YDR118W	Subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C)	NPC
ARB1	YER036C	ABC transporter ATP-binding protein ARB1	NPC
ATP3	YBR039W	Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase	NPC
BMH1	YER177W	14-3-3 protein, major isoform	NPC
CDC31	YOR257W	Cell division control protein 31	NPC
CDC55	YGL190C	Regulatory subunit B of protein phosphatase 2A (PP2A)	NPC
CHC1	YGL206C	Clathrin heavy chain	PDG
CIK1	YMR198W	Spindle pole body-associated protein CIK1	PDG
CSF1	YLR087C	Protein required for fermentation at low temperature	PDG
CYB5	YNL111C	Cytochrome b5	PDG
ECM13	YBL043W	Non-essential protein of unknown function	PDG
ENT5	YDR153C	Epsin-5	NPC
ERG11	YHR007C	Lanosterol 14-alpha-demethylase	NPC
ERG3	YLR056W	Delta(7)-sterol 5(6)-desaturase	PDG
FET3	YMR058W	Iron transport multicopper oxidase FET3	PDG
GAS3	YMR215W	Probable 1,3-beta-glucanosyltransferase GAS3	PDG
GCN5	YGR252W	Catalytic subunit of ADA and SAGA histone acetyltransferase complexes	NPC
GEF1	YJR040W	Anion/proton exchange transporter GEF1	PDG
HDA1	YNL021W	Histone deacetylase HDA1	NPC
HPR1	YDR138W	Subunit of THO/TREX complexes	NPC
HSF1	YGL073W	Trimeric heat shock transcription factor	NPC
IME2	YJL106W	Serine/threonine protein kinase involved in activation of meiosis	NPC
IMH1	YLR309C	Golgin IMH1	PDG
INP2	YMR163C	Inheritance of peroxisomes protein 2	PDG
IRE1	YHR079C	Serine/threonine-protein kinase/endoribonuclease IRE1	PDG
KIP3	YGL216W	Kinesin-like protein KIP3	NPC
KSP1	YHR082C	Serine/threonine-protein kinase KSP1	PDG
LST7	YGR057C	Protein LST7	PDG
MAK5	YBR142W	Essential nucleolar protein	NPC
MEF1	YLR069C	Mitochondrial elongation factor involved in translational elongation	PDG
MFG1	YDL233W	Regulator of filamentous growth	PDG
MRC1	YCL061C	S-phase checkpoint protein required for DNA replication	NPC
MTC4	YBR255W	Maintenance of telomere capping protein 4	PDG
NCE102	YPR149W	Non-classical export protein 2	NPC
NDT80	YHR124W	Meiosis-specific transcription factor	PDG
OCT1	YKL134C	Mitochondrial intermediate peptidase	PDG
PEP12	YOR036W	Syntaxin PEP12	NPC
PEP7	YDR323C	Adaptor protein involved in vesicle-mediated vacuolar protein sorting	PDG
PHO85	YPL031C	Cyclin-dependent kinase	NPC
PMT4	YJR143C	Dolichyl-phosphate-mannose--protein mannosyltransferase 4	NPC
PUT3	YKL015W	Proline utilization trans-activator	NPC
QCR7	YDR529C	Subunit 7 of ubiquinol cytochrome-c reductase (Complex III)	PDG
RAD16	YBR114W	Nucleotide excision repair (NER) protein	NPC
RIM15	YFL033C	Serine/threonine-protein kinase RIM15	PDG
RPS5	YJR123W	Protein component of the small (40S) ribosomal subunit	NPC
RVS161	YCR009C	Reduced viability upon starvation protein 161	NPC
SAC3	YDR159W	Nuclear mRNA export protein SAC3	PDG
SCH9	YHR205W	Serine/threonine-protein kinase SCH9	NPC
SCP160	YJL080C	Protein SCP160	PDG
SEA4	YBL104C	SEH-associated protein 4	PDG
SEC13	YLR208W	Protein transport protein SEC13	NPC
SGS1	YMR190C	ATP-dependent helicase SGS1	PDG
SKI2	YLR398C	Antiviral helicase SKI2	PDG
SKN7	YHR206W	Transcription factor SKN7	PDG
SNF1	YDR477W	AMP-activated S/T protein kinase	PDG
SNT1	YCR033W	Probable DNA-binding protein SNT1	PDG
SOD1	YJR104C	Cytosolic copper-zinc superoxide dismutase	PDG
SOH1	YGL127C	Subunit of the RNA polymerase II mediator complex	NPC
SRB8	YCR081W	Subunit of the RNA polymerase II mediator complex	PDG
STE2	YFL026W	Receptor for alpha-factor pheromone	NPC
UME6	YDR207C	Transcriptional regulatory protein UME6	NPC
URE2	YNL229C	Nitrogen catabolite repression transcriptional regulator	PDG
VMA11	YPL234C	Vacuolar ATPase V0 domain subunit c'	PDG
VMA3	YEL027W	V-type proton ATPase subunit c	PDG
VMA4	YOR332W	Subunit E of the V1 domain of the vacuolar H+-ATPase (V-ATPase)	PDG
VPH1	YOR270C	Subunit a of vacuolar-ATPase V0 domain	PDG
VPS13	YLL040C	Vacuolar protein sorting-associated protein 13	PDG
YAP1	YML007W	Basic leucine zipper (bZIP) transcription factor	NPC
YBR016W	YBR016W	Uncharacterized protein YBR016W	PDG
YDL241W	YDL241W	Uncharacterized protein YDL241W	PDG
YHK8	YHR048W	Probable drug/proton antiporter YHK8	PDG
YPL067C	YPL067C	Uncharacterized protein YPL067C	PDG
YPT6	YLR262C	GTP-binding protein YPT6	NPC

Table S5. Significant enriched Gene Ontology (GO) PANTHER overrepresentation analysis results from the genes retained in the reconstructed PREMONition interaction network, for *Saccharomyces cerevisiae* cells knockout strains showing an altered phenotype relative to the wild-type strains, using FISHER Exact test with a False Discovery Rate (FDR) correction. Per GO-tree branch, alternating white/ grey areas, most specific child terms are marked with an asterisk.

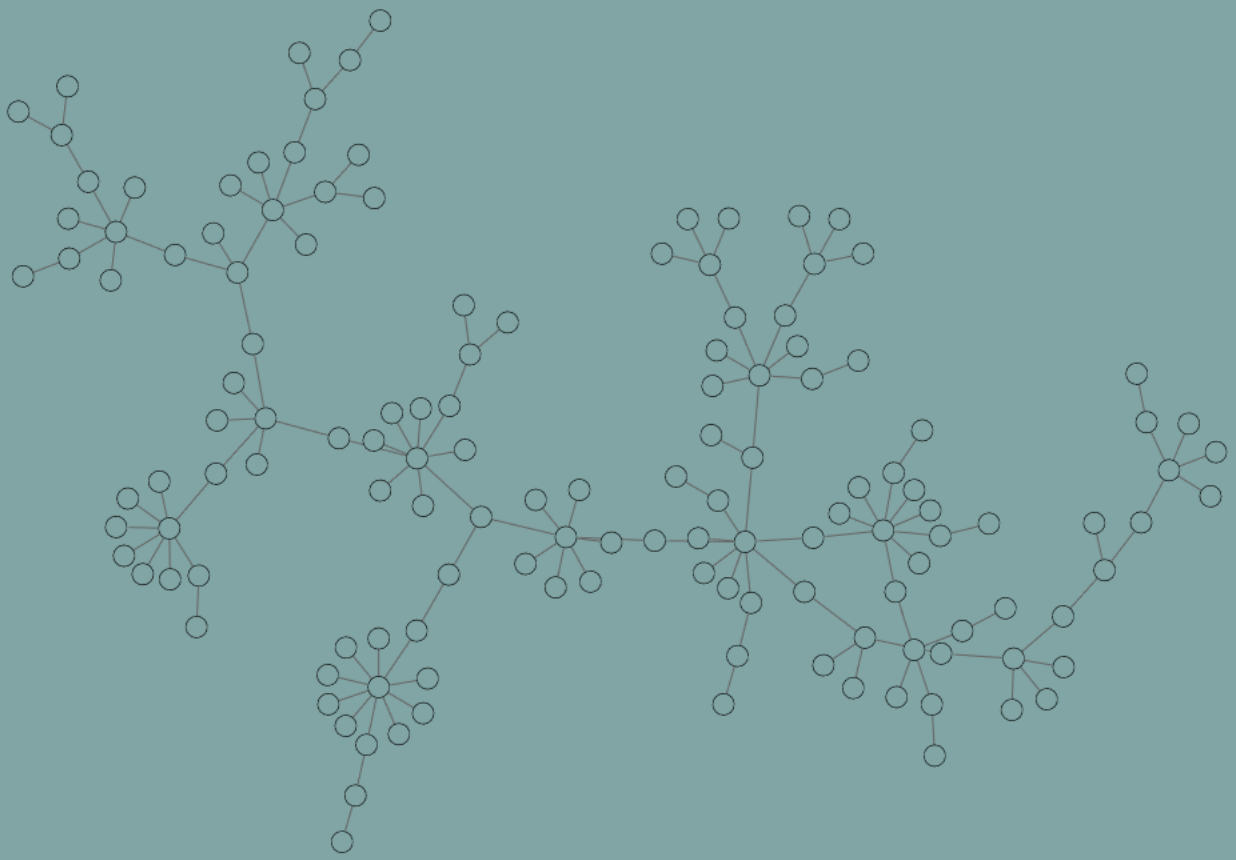
GO biological process complete	Fold Enrichment	raw P value	FDR
age-dependent response to oxidative stress involved in chronological cell aging *	48.01	8.39E-05	1.20E-02
age-dependent general metabolic decline involved in chronological cell aging	48.01	8.39E-05	1.17E-02
age-dependent general metabolic decline	36.01	1.62E-04	1.63E-02
developmental process	3.35	8.14E-04	4.32E-02
aging	7.68	1.67E-04	1.64E-02
chronological cell aging	12.00	4.72E-04	3.02E-02
cell aging	7.89	1.46E-04	1.55E-02
age-dependent response to oxidative stress	36.01	1.62E-04	1.66E-02
response to stress	2.54	6.02E-05	1.03E-02
response to stimulus	2.10	9.09E-05	1.24E-02
cellular response to stress	2.45	2.45E-04	2.13E-02
cellular response to stimulus	2.24	6.72E-05	1.12E-02
regulation of transcription from RNA polymerase II promoter in response to oxidative stress *	32.01	2.15E-04	1.93E-02
regulation of transcription from RNA polymerase II promoter in response to stress	7.50	6.71E-04	3.75E-02
regulation of DNA-templated transcription in response to stress	7.50	6.71E-04	3.79E-02
regulation of transcription, DNA-templated	2.85	2.07E-05	6.46E-03
regulation of cellular macromolecule biosynthetic process	2.59	1.58E-05	6.01E-03
regulation of cellular biosynthetic process	2.47	3.28E-05	7.58E-03
regulation of biosynthetic process	2.46	3.40E-05	7.53E-03
regulation of metabolic process	2.15	4.33E-05	8.21E-03
regulation of biological process	2.04	1.60E-06	2.83E-03
biological regulation	2.05	1.02E-08	5.42E-05
regulation of cellular metabolic process	2.16	1.06E-04	1.34E-02
regulation of cellular process	2.13	8.84E-07	2.35E-03
regulation of macromolecule biosynthetic process	2.55	1.96E-05	6.92E-03
regulation of macromolecule metabolic process	2.20	3.50E-05	7.44E-03
regulation of gene expression	2.63	4.01E-06	5.32E-03
regulation of nucleic acid-templated transcription	2.83	2.25E-05	6.30E-03
regulation of RNA biosynthetic process	2.83	2.25E-05	6.65E-03
regulation of RNA metabolic process	2.66	5.14E-05	9.10E-03
regulation of nucleobase-containing compound metabolic process	2.57	4.90E-05	8.98E-03
regulation of primary metabolic process	2.14	1.80E-04	1.67E-02
regulation of nitrogen compound metabolic process	2.21	8.00E-05	1.18E-02
regulation of transcription by RNA polymerase II	2.98	1.19E-04	1.41E-02
positive regulation of TORC1 signaling *	26.19	3.50E-04	2.58E-02
regulation of TORC1 signaling	17.46	1.29E-04	1.49E-02
regulation of TOR signaling	16.00	1.74E-04	1.68E-02

regulation of intracellular signal transduction	6.55	3.78E-04	2.71E-02
regulation of signal transduction	6.40	1.40E-05	5.70E-03
regulation of cell communication	6.09	2.05E-05	6.79E-03
regulation of signaling	6.40	1.40E-05	6.18E-03
regulation of response to stimulus	5.59	4.85E-06	5.15E-03
positive regulation of TOR signaling	24.00	4.35E-04	2.85E-02
positive regulation of intracellular signal transduction	10.38	7.80E-04	4.23E-02
positive regulation of response to stimulus	7.17	8.15E-04	4.29E-02
positive regulation of biological process	2.68	8.93E-06	6.77E-03
positive regulation of cellular process	2.75	5.91E-06	5.23E-03
regulation of DNA-binding transcription factor activity *	26.19	3.50E-04	2.55E-02
Golgi to endosome transport *	16.70	1.51E-04	1.57E-02
post-Golgi vesicle-mediated transport	7.11	2.48E-04	2.12E-02
establishment of localization	1.87	6.62E-04	3.78E-02
localization	2.05	1.18E-05	6.26E-03
cellular localization	2.47	1.19E-05	5.75E-03
vacuolar acidification *	14.22	2.62E-04	2.17E-02
intracellular pH reduction	14.22	2.62E-04	2.14E-02
regulation of intracellular pH	12.39	4.22E-04	2.88E-02
regulation of cellular pH	12.39	4.22E-04	2.91E-02
regulation of pH	12.00	4.72E-04	3.05E-02
cation homeostasis	4.80	2.95E-04	2.30E-02
ion homeostasis	4.39	5.24E-04	3.16E-02
regulation of biological quality	2.67	2.31E-04	2.04E-02
inorganic ion homeostasis	5.05	2.11E-04	1.93E-02
cellular cation homeostasis	5.19	1.77E-04	1.68E-02
cellular ion homeostasis	4.80	2.95E-04	2.33E-02
cellular chemical homeostasis	4.22	6.73E-04	3.72E-02
pH reduction	14.22	2.62E-04	2.11E-02
positive regulation of autophagy *	12.39	4.22E-04	2.84E-02
regulation of autophagy	9.41	2.53E-04	2.13E-02
positive regulation of cellular catabolic process	8.14	4.73E-04	2.99E-02
positive regulation of catabolic process	8.00	5.09E-04	3.10E-02
positive regulation of metabolic process	2.76	9.32E-05	1.21E-02
positive regulation of cellular metabolic process	2.80	7.89E-05	1.20E-02
regulation of macroautophagy *	10.38	7.80E-04	4.27E-02
response to starvation *	7.06	8.68E-04	4.52E-02
response to nutrient levels	5.33	3.96E-04	2.80E-02
response to extracellular stimulus	4.98	5.88E-04	3.51E-02
response to external stimulus	6.00	2.27E-05	6.04E-03
regulation of response to stress *	6.96	9.24E-04	4.76E-02
proton transmembrane transport	5.88	6.50E-04	3.75E-02

intracellular signal transduction *	4.80	1.19E-04	1.44E-02
signal transduction	3.83	1.40E-04	1.55E-02
signaling	3.83	1.40E-04	1.52E-02
cell communication	3.47	4.20E-05	8.25E-03
positive regulation of transcription by RNA polymerase II *	3.35	4.25E-04	2.82E-02
positive regulation of transcription, DNA-templated	3.44	9.18E-05	1.22E-02
positive regulation of gene expression	3.01	3.35E-04	2.58E-02
positive regulation of macromolecule metabolic process	2.61	4.87E-04	3.04E-02
positive regulation of nucleic acid-templated transcription	3.62	2.67E-05	6.43E-03
positive regulation of RNA biosynthetic process	3.62	2.67E-05	6.74E-03
positive regulation of cellular biosynthetic process	3.50	9.50E-06	6.31E-03
positive regulation of biosynthetic process	3.50	9.50E-06	5.61E-03
positive regulation of RNA metabolic process	3.49	3.97E-05	8.11E-03
positive regulation of nucleobase-containing compound metabolic process	3.31	6.98E-05	1.12E-02
positive regulation of nitrogen compound metabolic process	2.65	4.10E-04	2.87E-02
positive regulation of macromolecule biosynthetic process	3.17	1.10E-04	1.36E-02
negative regulation of cellular metabolic process *	2.74	7.91E-04	4.24E-02
negative regulation of metabolic process	2.61	4.87E-04	3.01E-02
negative regulation of biological process	2.35	6.49E-04	3.79E-02
negative regulation of cellular process	2.44	6.23E-04	3.68E-02
protein localization *	2.41	7.63E-05	1.19E-02
macromolecule localization	2.18	3.43E-04	2.56E-02
cellular component organization *	1.71	1.38E-04	1.56E-02
cellular component organization or biogenesis	1.61	3.37E-04	2.56E-02

Table S6. Number of occurrences per gene within each significantly enriched child-term and its corresponding branches

Child-terms	positive regulation of transcription by RNA polymerase II	regulation of response to stress	age-dependent response to oxidative stress involved in chronological cell aging	vacuolar acidification	negative regulation of cellular metabolic process	cellular component organization	intracellular signal transduction	positive regulation of TORC1 signaling	regulation of transcription from RNA polymerase II promoter in response to oxidative stress	positive regulation of autophagy	protein localization	Golgi to endosome transport	response to starvation	regulation of macroautophagy	regulation of DNA-binding transcription factor activity	Total
# parental terms	12	2	11	13	4	2	4	13	22	6	2	5	4	1	1	102
SOD1	12	0	11	7	4	2	0	2	22	2	0	0	0	0	1	63
RIM15	12	0	11	0	0	0	4	2	21	6	0	0	4	0	0	60
SCH9	12	1	11	1	0	2	4	3	22	2	0	0	0	0	0	58
SKN7	12	1	4	1	0	2	4	6	22	2	0	0	3	0	0	57
SEC13	11	0	4	0	0	2	0	13	18	2	2	3	0	0	0	55
HSF1	2	1	7	0	4	2	4	4	13	6	0	3	4	1	0	51
HDA1	12	0	4	0	2	0	0	9	21	2	0	0	0	0	0	50
SNF1	12	0	4	0	4	2	0	2	19	2	2	2	0	0	0	49
SRB8	2	0	4	1	4	2	0	7	19	6	0	0	0	1	1	47
CDC55	11	0	0	0	2	2	0	2	18	6	2	2	0	1	0	46
MFG1	12	0	2	0	4	0	1	2	19	2	0	0	3	0	0	45
SOH1	12	0	1	0	4	0	0	2	19	2	0	0	0	0	0	40
PUT3	2	1	0	0	4	2	0	9	19	2	0	0	0	0	0	39
SNT1	12	0	0	0	0	2	0	2	19	4	0	0	0	0	0	39
GCN5	9	0	4	0	0	2	0	2	19	2	0	0	0	0	0	38
NDT80	12	0	0	0	0	2	0	2	19	2	0	0	0	0	0	37
SCP160	0	0	2	0	4	2	3	2	18	0	0	3	1	0	0	35
BMH1	12	0	0	0	0	0	0	2	19	2	0	0	0	0	0	35
KSP1	0	0	5	0	4	2	4	0	19	0	0	0	0	0	0	34
SEA4	0	0	4	0	4	0	4	0	13	1	2	2	0	1	0	31
URE2	0	0	4	0	0	0	1	13	3	0	2	2	4	0	0	29
SGS1	0	0	2	1	4	0	0	0	18	0	2	1	0	0	1	29
VMA3	0	0	7	1	4	2	0	0	9	0	0	0	0	0	0	23
FET3	0	1	0	13	0	2	0	0	1	0	2	3	0	0	0	22
SKI2	0	0	4	7	0	0	1	0	1	0	0	3	4	0	0	20
VPH1	0	0	0	1	4	2	0	0	8	0	2	3	0	0	0	20
SAC3	0	1	0	13	0	2	0	0	1	0	0	2	0	0	0	19
VMA4	0	0	4	0	2	2	0	0	5	0	2	3	0	0	0	18
STE2	0	0	2	0	0	2	4	0	3	0	2	5	0	0	0	18
LST7	0	0	2	0	2	0	0	0	13	0	0	0	1	0	0	18
IRE1	0	0	4	0	0	2	4	0	3	0	2	2	0	0	0	17
YPT6	0	1	0	13	0	0	0	0	1	0	0	2	0	0	0	17
OCT1	0	1	0	13	0	0	0	0	1	0	0	2	0	0	0	17
IME2	0	0	2	0	0	2	3	6	3	0	0	0	0	0	0	16
GEF1	0	0	0	0	0	0	0	13	3	0	0	0	0	0	0	16
VPS13	0	0	0	7	0	2	0	0	1	0	2	3	0	0	0	15
RVS161	0	0	3	0	0	0	4	0	6	0	0	0	0	0	0	13
KIP3	0	0	1	1	0	2	0	0	3	0	2	3	0	0	0	12
IMH1	0	0	2	0	0	2	0	0	0	0	0	3	4	0	0	11
CDC31	0	1	0	7	0	0	0	0	1	0	0	2	0	0	0	11
RAD16	0	0	0	0	0	2	0	0	5	0	2	2	0	0	0	11
PMT4	0	0	4	0	0	2	0	0	0	0	2	2	0	0	0	10
RPS5	0	0	0	0	2	2	0	0	3	0	0	3	0	0	0	10
CHC1	0	1	0	0	0	2	0	4	3	0	0	0	0	0	0	10
PEP12	0	0	4	0	0	0	0	0	0	0	2	4	0	0	0	10
APC4	0	0	0	1	0	2	0	0	1	0	2	3	0	0	0	9
ENT5	0	0	0	0	0	2	0	0	0	0	2	5	0	0	0	9
PEP7	0	0	0	0	0	2	0	0	0	0	2	4	0	0	0	8
ARB1	0	0	0	0	0	0	0	0	0	0	2	5	0	0	0	7
ATP3	0	0	0	0	0	2	0	0	0	0	0	5	0	0	0	7
ECM13	0	0	0	0	0	1	0	0	0	0	2	3	0	0	0	6
MAK5	0	0	0	0	0	0	0	2	3	0	0	0	0	0	0	5
QCR7	0	1	0	0	0	0	0	0	0	0	0	2	0	0	0	3
GAS3	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
PHO85	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
VMA11	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	2
NCE102	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1



Chapter 6

Are there circadian clocks in non-photosynthetic bacteria?

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Published: Biology 2019, 8(2), 41

Introduction

Circadian clocks are molecular programs that impart temporal organization. These pervasive biological systems are responsible for distinct and characteristic patterns of daily activities that are observed throughout nature. Across the Kingdoms of life, circadian clocks have a broad influence upon cells, as evidenced by molecular rhythms in RNA abundance, protein abundance, protein modifications, metabolism; upon the organism, as evidenced by a set of stereotyped behaviors related to self-sustained rhythms and their synchronization; and upon species, as evidenced by circadian rhythms in populations. Although circadian clocks are seemingly ubiquitous, they have rarely been described in non-photosynthetic prokaryotes. Here, we review those descriptions and speculate on the potential existence and nature of circadian systems in this group of organisms.

What is a circadian clock? The single observation that is most often used as proof of a circadian clock is a self-sustained, *circa* 24h oscillation that continues in constant conditions (e.g., constant light or dark, constant temperature; Fig. 1A). This is a remarkable feat that occurs in mammals including humans, as well as in insects, plants, fungi and several photosynthetic bacteria. These so-called free-running rhythms are, however, conditional. A mouse that has a free-running rhythm in constant darkness is arrhythmic in constant bright light. A gradual increase in light levels from darkness to moderate illumination causes a gradually-lengthening period of the mouse free-running rhythm with a concurrent decrease in consolidation of activity/inactivity[1]. Similarly, a variety of rhythmic physiologies and underlying molecular processes cease to be rhythmic in Arabidopsis plants under constant darkness. Therefore, even model circadian clock systems with a robust and dependable circadian rhythm in a certain constant condition can be non-rhythmic in a modestly different environment. In the absence of a good understanding how an organism responds to its natural environment, it may be difficult to identify the correct set of conditions that would support robust, free-running rhythms.

Other rhythmic properties (Fig. 1B and C) that are considered reliable evidence of a circadian clock include temperature compensation of the period of the free-running rhythm and synchronization of the rhythm to a 24h environmental cycle or to appropriate stimuli (called zeitgebers, German for ‘time givers’). Temperature compensation refers to the stability of a free-running rhythm over a range of temperatures[2,3]. For circadian biology, the actual or imputed change in period length over 10 °C is calculated (the Q_{10}). For circadian systems, this number is close to 1 (higher than 1 in most poikilotherms and lower than 1 in cells or tissues

from homeotherms)[3–5]. For context, chemical reactions can have a Q_{10} of *ca*. 5 and cellular processes that are not temperature compensated (some enzymatic reactions) have a Q_{10} of 2-3.

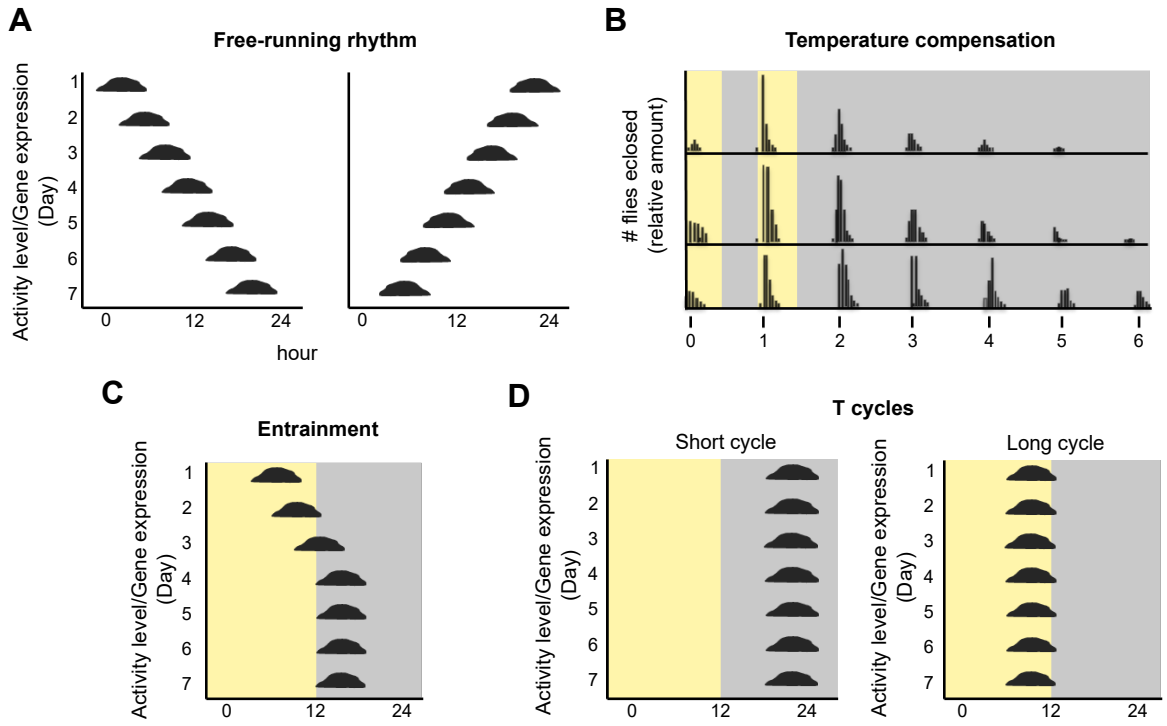


Figure 1. Characteristics of circadian clocks (see also6). (A) A free-running rhythm in constant conditions can have a period that is either slightly longer (left panel) or slightly shorter (right panel) than 24h. Graphing the activity/amount (y-axis) over the course of the day (x-axis), such that changes from day to day are visually obvious (seen from top to bottom), facilitates an implicit understanding of period and phase of the circadian rhythm. (B). Eclosion of *Drosophila* occurs once per ca. 24h in constant conditions following 2 days of entrainment in a 24h light dark cycle (depicted as yellow and grey). The top of the panel shows eclosion at 26 °C, the middle shows 21 °C and the bottom shows 16 °C. The timing of eclosion (and hence its period or frequency) remains almost the same over this broad range of physiological temperatures, hence it is temperature compensated. Data redrawn from Pittendrigh[6]. (C) The circadian rhythm synchronises (entrains) to exactly 24h in a zeitgeber cycle, shown here as a light dark cycle. The rhythm gradually finds a stable time of day (phase) relative to the zeitgeber. (D) The stable phase depends on the relationship between the biological rhythm and the zeitgeber cycle. Most circadian rhythms entrain to a later phase in a shorter cycle and earlier phase in a longer one.

Synchronization or entrainment of the circadian rhythm to its cyclic environment (Fig. 1 C) leads to the establishment of a specific phase of the biological rhythm relative to the zeitgeber (the entraining agent for a biological clock)[7]. The endogenous oscillation (the circadian rhythm) actively probes the environment until it finds the optimal time point in the zeitgeber cycle where it no longer advances or delays but is stable, remaining at the same phase, until either the biological or environmental oscillator changes. Indeed, it is entrainment that creates the concept of 'clock' for this temporal program because the oscillator combined with the zeitgeber cycle will show a characteristic, unique, entrained phase (chronotype). This property is thought

to reflect the biological function of the circadian clock, namely to adaptively match function to a certain time of day.

A circadian rhythm alone, in a single condition, is not generally taken as solid proof of the existence of a circadian clock. Likewise, synchronization or entrainment of said rhythm to a single zeitgeber cycle in the absence of a free-running rhythm is generally not taken as evidence of a daily clock. It could simply be a driven response to the zeitgeber[8]. When do we start to believe that an endogenous clock is present in an organism? A circadian rhythm that is temperature compensated is compelling evidence because it shows that the temporal program in question has a built-in mechanism to protect against temperature effects that invariably occur and would otherwise disturb its timing function. Systematic entrainment properties such as earlier or later phase angles in longer or shorter zeitgeber cycles (Fig. 1D; T cycles) are likewise considered as evidence of a clock because they demonstrate an endogenous biological rhythm that is capable of direction – in this case, capable of directing the system to a certain phase angle relative to the entraining zeitgeber cycle as a result of entrainment by an oscillator with a unique set of characteristics[9,10]. A driven (non-entrained) rhythm would not show a systematic set of phase angles relative to an entraining cycle but rather uniform ones. These concepts are exemplified by the circadian system in *Neurospora crassa.* Light drives rapid expression of the clock gene *frequency* RNA at any time of day whereas the FREQUENCY protein is produced at a phase that correlates with mid-dark or mid-light phase rather than the time that lights turn on or off[11]. The outward behavior (spore formation) also correlates with the midpoint of the dark or light phase rather than the transitions between them[12]. These proofs are rare outside of the eukaryotic kingdom.

Among the prokaryotes, certain species of cyanobacteria provide clear evidence for a robust circadian clock, with *Synechococcus elongatus* representing the primary model system[13]. *S. elongatus* has one of the best characterized molecular clocks of all experimental models. Invoking the power of a prokaryotic model system, three essential clock proteins have been identified (KaiA, KaiB and KaiC). These three proteins direct clock-regulated gene expression of almost the entire genome[14] (a much greater proportion of circadian transcription than with eukaryotic model systems). KaiA, KaiB and KaiC are capable of circadian clock function at a purely post-transcriptional level through cycles of phosphorylation and de-phosphorylation as demonstrated by remarkable clock-in-a-tube experiments; the three purified Kai proteins combined with ATP lead to temperature compensated circadian rhythms that are capable of synchronization to 24h temperature cycles[15]. When these proteins were transformed into *E. coli*, they underwent a circadian oscillation in the phosphorylation of KaiC[16]. The KaiC protein,

in the absence of KaiA and KaiB, is not rhythmically phosphorylated in *E. coli*, thus the rhythmicity is derived from several cyanobacterial clock genes. The *S. elongatus* circadian clock has a temperature compensated free-running rhythm[17] and entrains with phase angles in T cycles[18]. Finally, *S. elongatus* is one of the very few examples that has been used to demonstrate that a circadian clock contributes to adaptive selection[18,19]. Experiments that combined otherwise fit clock mutant strains (e.g., with a short or long free-running period) with a wild type strain were cultured in constant conditions as well as in T cycles. When the length of the T cycle matched the period of one strain better than the other, that strain had superior fitness, whether it was a mutant or not. Furthermore, mutagenesis of 'every' gene within the *S. elongatus* genome identified a set of genes that contribute to fitness under light/dark cycles[20]. This includes genes associated with managing carbon fluxes over the day-night cycle, those involved in responses to light-induced stresses during the transition from dark to light, and also the gene encoding the oscillator component KaiA[20]. Therefore, the clock rapidly shapes microbial population structure under light/dark cycles and cyanobacteria have specific mechanisms to adapt to the daily fluctuating environment.

Rhythmic processes have also been reported in purple bacteria; like *S. elongatus* these bacteria are capable of photosynthesis. For example, *Rhodospirillum rubrum* has rhythms with a period of 12-15 h in enzymatic activity of the uptake hydrogenase (Hup), which participates in redox metabolism during photosynthesis and nitrogen fixation[21]. Similarly, under aerobic conditions, there are oscillations of transcription of the *aidB* gene in *Rhodobacter sphaeroides* with a period of 20.5 h that, intriguingly, shorten to 10 – 12 h under anaerobic conditions[22]. *aidB* encodes an acyl-CoA dehydrogenase. The genome of *R. sphaeroides* harbors orthologues of *kaiB* and *kaiC*. It lacks a KaiA homolog, the protein which promotes KaiC phosphorylation[22]. In *R. sphaeroides*, the *kaiBC* operon includes a PAS-domain protein, which is intriguing because PAS-domain proteins are often associated with circadian clocks in a variety of species (see below). Although the rhythms in purple bacteria rhythms do not appear to meet the criteria for classification as a circadian clock, they demonstrate that other groups of bacteria can also possess endogenous temporal programs. We suggest that other microbes, even those that are non-photosynthetic, are subject to evolutionary pressure from zeitgeber cycles, leading to development of endogenous temporal programs.

It has been argued previously that a circadian rhythm might not be adaptive for organisms that have a doubling time shorter than the diel cycle, meaning that there might not be selection for circadian programs in a variety of micro-organisms[23]. This has been referred to as the "circadian-infradian rule"[23]. However, experiments with *S. elongatus* refute this notion.

Cyanobacteria with generation times of between 5 and 10 h have been found to retain circadian rhythms in gene expression[24]. In addition, a memory effect has been identified in non-photosynthetic bacteria, which spans over the cell cycle interval (*i.e*. the physiological state of a bacterium is sustained for several cell divisions)[25]. Therefore, we speculate that circadian clocks may exist within other bacterial species, including rapidly dividing non-photosynthetic bacteria, that are exposed to a rhythmic environment that imposes a selection pressure.

Here, we review the published evidence that suggests circadian rhythms can exist in non-photosynthetic eubacteria whose doubling times can be shorter than the diel cycle, review the bioinformatical hints that clocks might exist in such organisms, and provide reasoning for the historical and current challenges in identifying and describing a circadian timekeeper in these organisms.

Endogenous circadian rhythms in non-photosynthetic prokaryotes

The circadian-infradian rule is violated by the well-described circadian rhythms in *Synechococcus* but there are also reports of *circa* 24 h period rhythms in non-photosynthetic bacteria that extend as far back as 1930[26]. A 15 m long spiral growth tube filled with liquid media and a color indicator was inoculated at one end with *Escherichia coli*. The culture was incubated at 30 °C and the growth front was measured every about every hour over a period of 8 days. An “intermittent growth” (Fig. 2A) was noted. Rogers and Greenbank[26] did not calculate or comment on the periodicity of growth but the data shows long, circadian-like rhythms. Thirty years later, Halberg and Conner calculated the periodicity of the ‘growth’ rate at 20.6 h[27]. Comparable studies were performed by Sturtevant[28], who monitored the growth of *E. coli* in a linear tube of liquid culture, again using a color indicator to mark the growth front. During a 9.5 day experiment in darkness, with no apparent entrainment prior to or during the incubation, there was a 23.2 h rhythm in growth at 37 °C. A coincident 16 h rhythm was also reported, which might be a harmonic. When this experiment was repeated at 30 °C, rhythmic growth still occurred although the period length no longer appeared circadian. *E. coli* grown at 30 °C had a 4.8 h ultradian rhythm. Therefore, these rhythms fail the criterion of temperature compensation of a free-running circadian rhythm. The idiosyncratic nature of these rhythms might also be supported by Bibb[29] who found no evidence of periodicity in the growth of *E. coli* under similar conditions. In this case, however, the time resolution of measurements is unclear and the description suggests no active temperature control, which was reported as room temperature (24 °C - 32 °C).

Reports of rhythmic growth of non-photosynthetic prokaryotic bacteria are not limited to *E. coli.* For example, rhythms have been identified in growth of *Klebsiella pneumoniae*[30]. Rather than a tube configuration, this protocol used a fermenter that replaced the media at a rate of $0.25h^{-1}$ under constant temperature and a 12:12 (fluorescent) light-dark cycle (Fig. 2B). Following inoculation with *K. pneumoniae*, the optical density of the culture was measured with 15 or 30 m intervals. The pattern of optical density readings was fitted using a cosinor formula yielding a period of 24.1 h. No free-running rhythm was reported under constant conditions. Even under entrained conditions, the rhythm appeared to dissipate after about 4 days of the 13 day experiment.

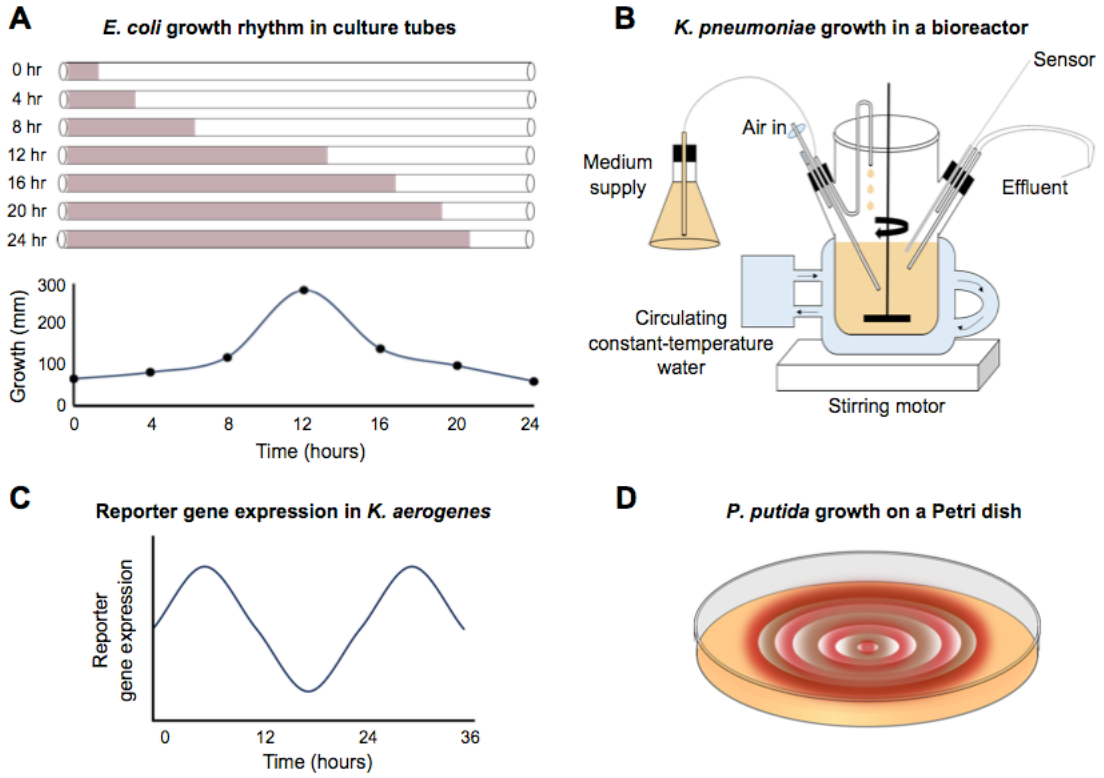


Figure 2. Schematic representations of the various rhythms observed for non-photosynthetic eubacteria. (A) Growth rhythms observed in E. coli by growth in a long culture tube filled with liquid media and inoculated at one end. A dye is included to measure the growth front as the culture moves down the tube free-running rhythm. The graph summarizes the growth with 4 h intervals over 24 h, corresponding to hours 40 to 64 of the original experiment by Rogers and Greenbank[26]. (B) Sturtevant[30] measured the growth rhythm in *K. pneuomoniae* in a bioreactor. Samples were taken out every 15 or 30 minutes and the optical density was measured. (C) Reporter gene rhythms detected in *K. aerogenes*[31]. A gene fusion between a motility gene (motA) and the reporter GFP allowed detection of circa 24h rhythms. Similar types of long rhythms of reporter genes have been observed in *B. subtilis*. (D) Morphological rhythms were observed by Soriano *et al*[32] when studying *P. putida* by incorporating dyes (Congo Red or Coomassie Brilliant Blue) that detect changes in the redox state or cell surface of *P. putida*. Growth "rings" were then observed that had circa 24h rhythms.

More extended and robust observations of *ca.* 24 h rhythms have been reported in an enteric bacterium. Paulose *et al*. identified rhythms in the swarming response of *Klebsiella aerogenes* (called *Enterobacter aerogenes* in the publication) in the presence of melatonin[31]. *K. aerogenes* growth seems uniquely sensitive to melatonin, in that several other species demonstrably lack sensitivity to the hormone and *K. aerogenes* lacks sensitivity to melatonin-related molecules such as tryptophan. A fusion of luciferase to a motility gene demonstrated that a pure culture of *K. aerogenes* has a *ca.* 24 h rhythm in constant conditions (Fig. 2C) and that the rhythm is synchronized by the presence of melatonin. At 34 °C, a free-running rhythm persisted for four cycles. Furthermore, the authors showed that the rhythm is temperature compensated; in the presence of melatonin the periodicity of the rhythm was about 25 h at temperatures ranging from 27 °C to 40 °C, with a reported Q_{10} of 0.96. This report is the clearest case of a classically defined circadian rhythm in a non-photosynthetic prokaryote: temperature compensated, free-running rhythms. It is noted by the authors that only about 35% of the cultures showed a rhythmicity from the *PmotA*::lux reporter, suggesting a conditionality of the rhythm.

There is some evidence for daily rhythms under *in vitro* conditions in bacteria that inhabit the soil and can interact with plant roots. For example, Soriano *et al.*[32] showed that *Pseudomonas putida* had rhythmic growth on agar plates containing either Congo Red or Coomassie Blue, both of which interact with the extracellular matrix (Fig. 2D). Under entrained conditions of 16L:8D and 12L:12D, the growth rhythms had a periodicity of about 24 h for both dyes (Congo Red, 24.1h, Coomassie Blue, 24.9h). When the cultures were shifted to constant conditions, the rhythm persisted for one cycle with a period of 19.2 h or 24 h (Congo red or Coomassie blue, respectively). No experiments were included concerning temperature compensation of the rhythms.

The dyes that were utilized to show rhythms in *P. putida* are differentially incorporated according to developmental state. Indeed, as they grow and develop, these organisms profoundly change their characteristics. Bacteria utilize complex regulatory pathways in these processes. Potentially, a circadian system might put a temporal stamp on the switch between different bacterial cell types, selecting the times of day that are most favourable and harvesting environmental information to make costly switches in cellular biochemistry most efficient.

Possible timekeeping components in non-photosynthetic Eubacteria

Given these reports of circadian rhythms in non-photosynthetic Eubacteria, is there evidence that there are timekeeping components in these organisms? It is tempting to make

assumptions about function based on gene homology. This is certainly justified in the circadian clocks field where the Period gene is broadly involved in circadian timing in metazoans. Therefore, we would be remiss to not explore the occurrence of orthologous genes or clock-associated protein domains in non-photosynthetic bacteria as an indication of potential circadian systems. Dvornyk et al.[33] and Loza-Correa et al.[34] searched for homologues of *kai* genes in other bacteria, finding that *kaiA* is only present in cyanobacteria. Homologues of *kaiC* are found in three taxa of Bacteria (Proteobacteria, Thermotogae and Chloroflexi). Interestingly, some Proteobacteria form close associations with organisms with identified circadian rhythms. One such example is the nitrogen-fixing rhizobacterium *Sinorhizobium medicae*, which possesses a *kaiC* homologue. *S. medicae* form root nodules on plants in the *Medicago* genus in the legume family[35,36]. *P. putida* also forms associations with plants, taking up secreted nutrients whilst providing protection against pathogens[37]. The *P. putida* genome has a *kaiC* homologue.

PAS domain family members play a prominent role in circadian biology. This family was originally described by its founding three members: Period, ARNT, and SIM[38–40]. More generally and with respect to timing, PAS domains are present as motifs within circadian clock-associated proteins in the eukaryotes. They are thought to be involved in the formation of protein-protein interactions between transcription factors that participate in clock function[41,42]. Significantly, the PAS domains appear to be functionally similar to the PYP domain within a bacterial blue-light sensing protein[42]. The FixL (oxygen receptor) and HERG (potassium gate) domains also show similarities to the PAS domain. PAS domain containing proteins are encoded in various phylogenetic groups of the Eubacteria, predicted to function in two-component regulatory systems[43]. When PAS domains are shown to mediate protein-protein interaction they are mostly involved in heterodimer formation, such as in CLOCK and CYCLE, PER and TIM and AHR and ARNTL proteins regulating circadian mechanisms[43]. As PAS domains add stability and specificity to the dimers, homodimers can be formed between PAS domain-containing proteins, as with the PER proteins and also homodimerization and nuclear targeting of plant phytochromes are enabled by PAS domains[44].

In addition to a molecular mechanism that support daily rhythms, circadian clocks must also have appropriate sensory pathways for processing zeitgeber inputs such as light and temperature for their entrainment. In free-living non-photosynthetic bacteria, one candidate zeitgeber molecule is bacteriophytochrome, a family that senses red and far-red light. These exist in a range of bacteria, including non-photosynthetic species such as *Pseudomonas aeruginosa, Agrobacterium tumefaciens* and *Deinococcus radiodurans*[45,46]. Many of the

Rhizobiaceae, which form plant symbionts, have bacteriophytochromes[47] despite their occupation of the soil environment. Perhaps they harvest light near the soil surface or, alternatively, might capitalize upon the light pipe function of some roots[48,49]. In either case, the potential exists for these bacteria to use light as a zeitgeber.

There is also evidence for functional blue light photoreceptors in non-phototrophic bacteria. The description of the light, oxygen, voltage (LOV) domain in *Bacillus subtilis* was the first documentation of this sensory motif outside of plants[50]. Since then, a repertoire of other blue light sensing photoreceptors has been identified in bacterial families, including the photoactive yellow protein (PYP) and BLUF (blue light sensing using FAD) domain proteins[51–53]. Many of these blue light sensing bacteria form associations with plant roots in the soil. Examples include the beneficial rhizobacterium *Burkholderia phytofirmans* that possesses genes with a PYP domain, and *B. subtilis* with a LOV domain within the blue light photoreceptor protein YtvA[54]. The pea-nodulating endosymbiont *Rhizobium leguminosarum* uses LOV domain light receptors to regulate exopolysaccharide production and the amount of root nodulation, however it is also hypothesised that light sensing could allow rhizobia to sense the time of day and position within the environment to optimise root infection[55].

We note the confluence of three phenomena: non-photosynthetic Eubacteria (e.g *E. coli*, *P. putida*, *K. pneumonia*, and *K. aerogenes*) with demonstrated rhythms do not yet meet classical circadian clock definitions, the prediction of potential clock components such as KaiC and LOV domain proteins in these organisms, and the association of these bacteria with hosts that have a well-described circadian clock. Perhaps a key to better understanding timekeeping in non-photosynthetic Eubacteria is a model system that goes beyond a monoculture of the bacteria.

Bacillus subtilis – a potential model to investigate circadian programs in non-photosynthetic bacteria?

The existence of circadian clocks in non-photosynthetic bacteria is currently speculative. However, the body of findings described above suggest that this might be worth revisiting. In considering promising model systems, we found several examples of cyclic changes in gene expression, with a periodicity of the order of 24 h, in the Gram-positive bacteria *B. subtilis*. This bacterium lives in the soil, where it interacts with other organisms including other bacteria, fungi, single cell predators (e.g. amoebae), worms and plants. In addition to biotic interactions, *B. subtilis* growth and differentiation is influenced greatly by changes in abiotic factors, including temperature. Under laboratory conditions, *B. subtilis* adaptation includes various

modes of motility (single cell swimming, multicellular swarming and sliding), natural competence for the ability to take up extracellular DNA that promotes genetic variability, protease production for degrading food sources, matrix production for biofilm formation that facilitates attachment and structure formation and, under adverse conditions, formation of highly resistant spores[56–59].

These differentiation processes are connected by complex regulatory pathways including feedback loops, protein phosphorylation and protein degradation. For instance, Süel et al.[60] used time-lapse fluorescence microscopy to follow the expression of genes determining the transient differentiation of *B. subtilis* into a competent state. This is the state where the bacterium can take up exogenous DNA from the environment. The entrance and exit from the competent state are controlled by an excitable core module containing positive and negative interactions determined by cross-regulated expression of the *comK*, *comG* and *comS* loci. ComK is the master regulator of competence state and its proteolytic degradation is inhibited by the small ComS protein. When ComK protein approaches a threshold level in the cells, an autoregulation boosts the production of ComK, which can activate later stage competence genes, including the *comG* operon. During competence, the activity of the promoters of *comK* and *comG* genes is highly correlated, while those of *comG* and *comS* are expressed anti-phase to one another. The promoter activity of these three elements show an approximately 24h oscillation under certain conditions[60]. While the cross-regulation between these components determines their relative change in abundance, one could speculate that a time-keeping system might underlie the periodicity of their expression.

Another example of *ca*. 24 h changes occurs during differentiation towards matrix production of *B. subtilis* under constant conditions that normally do not induce biofilm formation. Functionally, biofilm makes *B. subtilis* resilient in adverse conditions and also facilitates attachment to the root rhizosphere[59]. Norman *et al.*[61] monitored the expression of motility genes, by coupling the promoter of the *hag* gene encoding the flagellin subunit of the peritrichous flagellum to the red fluorescent mKATE2 reporter. They also monitored expression of matrix genes in *B. subtilis* at a single cell level, by placing the blue fluorescent protein-coding gene *cfp* under the control of the *tapA* promoter and using a microfluidic device[61]. This setup allowed the observation of a single cell for several days, and the activation of anti-phased expression of motility and matrix production pathways. The activation of matrix production and coupled chain formation occurred for only 7-8 generations of a cell in the absence of biofilm inducing signals. Intriguingly, the activation of matrix gene expression pulsed with an approximately 24 h period, suggesting that even under homogeneous

conditions the stochastic triggering of biofilm formation follows an internal cycle of this bacterium. Detailed analysis of this random initiation of matrix gene expression revealed that noisy antagonism within the complex regulatory pathway determine the chance of activation.

Yet another process that appears rhythmic in *B. subtilis* is spore formation, a well-documented clock regulated process in *N. crassa*[3]. The formation of highly resistant spores in *B. subtilis* depends on cell physiology (i.e. starvation) and environment[56]. The initiation of the spore development depends on the global regulator Spo0A, the activity of which is influenced by a phosphorelay. During sporulation, the forespore encompasses the whole bacterial chromosome and is subsequently decorated by a spore coat that enables the packing of genetic material[62]. The timing of this process was dissected, showing coordination of *spo0A* expression with DNA replication[63,64]. On the one hand, timed expression of feedback from the phosphorelay activating kinase (KinA) towards Spo0A phosphorylation ensures the cyclic, but continuous increase of Spo0A with each cell cycle[63]. On the other hand, the genomic arrangement of the genes involved in the phosphorelay leads to a transient gene dosage imbalance during chromosome replication that contributes to the coordinated pulsatile expression of Spo0A phosphorylation[64].

Cyclic changes in the Spo0A pathway also occur in a cell cycle-independent manner. Single-cell tracking of *B. subtilis* during the early progression to spore formation identified bursts of promoter activity of several genes with a 20 h period. These genes encode the receptor aspartyl phosphatase A (RapA, a negative effector of the phosphorelay) and the phosphotranferase Spo0F. These are key components of the phosphorelay signal transduction pathway implicated in cell fate decisions between sporulation and growth of the bacterium[65,66]. While the sum of these observations does not define a circadian oscillator in *B. subtilis*, these observations do show the possibility of daily regulation of gene expression in pathways involved in sensing and integrating environmental changes in bacteria.

Our survey of the literature suggests that non-photosynthetic prokaryotes are capable of generating rhythmic gene expression with periods that are considerably longer than the typical cell division times in most laboratory conditions. The data are also remarkable for what seems to be missing; stability of period length, self-sustainment of the free-running rhythm, systematic entrainment to T cycles (apparently not investigated) and, with one exception, evidence of temperature compensation. Does the evidence suggest that non-photosynthetic bacteria have circadian clocks that adhere to historical definitions[67]? Or are these examples of less sophisticated time-keeping mechanisms, described by Johnson as proto-circadian

oscillators[68]? If the former, perhaps laboratory conditions provide the equivalent to *B. subtilis* of constant dim light to a mouse: conditions that are incompatible with self-sustained, robust circadian rhythms. In the laboratory, the growth environment is most often reduced to simplicity. For example, a monoculture is typically grown in one of several standard, defined media in contrast to a noisy natural environment with complex signals derived from growth substrates and other microorganisms. Could the circadian properties be better observed under different conditions, for example using media in which cell division occurs less frequently, or in conditions where nutrients are not exhausted quickly; for example, in fermenters? If the latter, why would bacteria – organisms that are constantly bombarded with changing environmental conditions stemming from day/night cycles and capable of rapid genetic evolution – fail to have a demonstrable, robust circadian system? The analysis of microbiomes has been a revolution in microbiology. It is important to consider that microbes are virtually never found in isolation in nature, but rather in tremendously complex communities including bacteria, fungi and viruses. This may be an important observation, because the description and rhythmic activities of microbiomes as a whole could be the key to circadian rhythms in non-photosynthetic bacteria.

Microbiomes and daily rhythms

Intestinal microbiomes are remarkable for their complexity, reaching about 1,000 bacterial species in humans. 15 to 20 % of the taxonomic units in the intestinal microbiome shift in amount relative to one another each day[69–71]. This suggests an impact of the host on the gut bacterial content. By logical reasoning, this could be mediated either by behavior (ingestion of food at a certain time which would act via its food value for the bacteria), by the clock-regulated intestinal epithelium or by endogenous core body temperature cycles. Restricted and controlled feeding in clock mutant mice supports the former mechanism but the other factors are not ruled out.

There are many examples of rhythmic outputs from the gut microbiome impacting the host circadian clock. Amplitude of clock gene expression in the intestinal epithelium is altered in antibiotic treated mice. RevErbα, Per1 and Per2 transcript abundance increases whilst that of Bmal1, Cry1 and RORα decreases[72]. By changing the microbiome in a number of ways (diet, germ-free conditions), it has been demonstrated that the intestinal microbiome controls at least part of the rhythmic liver transcriptome[73–75]. In a remarkable experiment, disruption of the gut microbiome led to the retention of some daily rhythms in the gut and liver, abolished other rhythms in those tissues and resulted in *de novo* expression of yet another cohort of rhythmic

genes[76]. At least part of this set of regulated genes shows sexual dimorphism[77]. Furthermore, there are high amplitude changes to the numbers of bacteria associated with the intestinal wall according to time of day[76]. This could be mediated by either by the host (rhythmic chemokines) or by the microbial population (rhythmic responses to chemokines).

We therefore propose a model for interaction of local clocks in the intestine that encompasses numerous observations (Fig. 3A). The intestinal epithelial cells have rhythmic expression of clock genes with an attendant set of clock-controlled outputs that will modify the local environment over the course of the 24h day. They also are the source of intestinal melatonin[78]. Although we do not know whether the melatonin that they produce is rhythmic, there are rhythms in its direct precursor, serotonin[79]. Among other factors, melatonin will be utilized by at least some of the bacteria, possibly as a zeitgeber. The microbiome, in turn, will secrete metabolites reflecting daily rhythms in feeding of the host, inputs from the epithelium - and possibly endogenous bacterial timing programs, though this is not an essential feature of the model. The metabolites can be used in a number of ways: they might feed back to the intestinal epithelium; they could be utilized among the bacterial population, signaling within to the community; they clearly can impact peripheral liver clock-regulated genes. An important connection to make will be if the presence of a circadian rhythm in the gut (and its microbiome) impacts the clock in the brain. The gut – brain axis is clearly established, though how the clock might regulate this remains unknown. Clock-controlled outputs from the gut microbiome to the brain may form a feedback loop if they influence the timing of feeding behaviour.

If gut microbial communities serve a function within a circadian system, then other well-characterized microbiome/host arrangements might be expected to share some of the same principles. To that end, interactions between the root microbiome and circadian rhythms in plants have been reported. Clock regulation has a pervasive influence upon the physiology and development of plants, regulating processes ranging from the season of flowering to daily cycles of photosynthesis, stomatal opening, energy supply during the night, and fitness[80–84]. Cultivation of Arabidopsis short- or long period clock mutants on soil led to alterations in the microbiome composition compared with the composition of the microbiome surrounding wild type plant roots[85]. Conversely, wild type plants had decreased leaf rosette diameter and delayed germination when cultivated on soil that was previously used to grow circadian clock mutants. One potential explanation is that mutants with an altered free-running period associate an altered microbiome and this particular microbial community provides fewer benefits to the plant (here, assessed as from ‘conditioned’ soil)[85].

As with the mouse gut microbiome, the plant microbiome can alter the host circadian rhythms. Application of filtered soil samples that were either untreated or manipulated to disrupt the microbiome (sterilized or filter-sterilized) to tissue-cultured Arabidopsis seedlings led to differential effects upon their free-running period[85]. In another experimental protocol, the circadian period of *CCA1:LUC* in Arabidopsis is shortened by application of the bacterial flagellar peptide flg22[86]. Although this example relates to plant defense mechanisms, it provides a proof of concept that plant surface microbes can alter plant circadian rhythms. Thus, as with the mouse intestinal microbiome, the plant/soil microbiome shows bidirectional interactions. Although some differences will surely be employed by the plant and animal microbial communities, similar features are apparent between the two systems. The intersection of circadian clock regulation, microbiome and host may follow a set of common principles.

The microbiome as an organism and temporal structuring of mutualism

One of the most exciting developments in chronobiology research in the last decade is the characterization of circadian clocks as complex systems. On the one hand, within a single cell there exists a complex, multi-feedback loop network that comprises the clock, stretching across all levels from transcription to metabolism. On the other hand, these cells function not so much as individuals but together as organs which then must coordinate daily timing between each other. The clock is an evolved system that necessarily involves coordination between partners on many scales[7,87]. This concept may help in dissecting circadian clock properties in the microbiome.

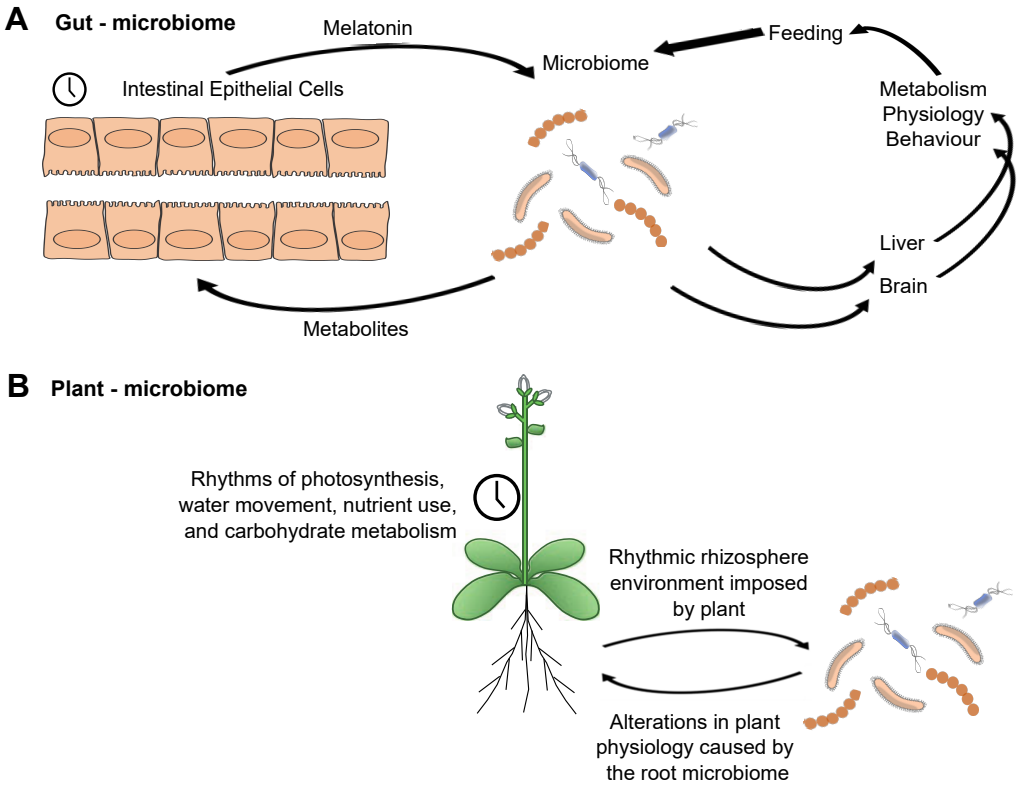


Figure 3. Interactions between circadian clocks and microbiome communities are established. (A). The clock in the host (here shown as the intestinal epithelial cells) shapes the gut microbiome community and the microbiome in turn impacts many clock regulated outputs. (B). Similar results have started to appear relative to plant microbial communities. It is as yet unclear how much endogenous clock-like function the microbes may possess. We propose that the microbial community may utilize mutualism with a temporal structure in such a way that is consistent with the observations published thus far.

How is organization of the gut microbiome like a higher-level organism? Mice, for example, take synchronizing signals at the behavioral level from the environment (zeitgeber cycles). The gut microbiome must take zeitgeber signals from its environment. These signals are coming from the host-temperature cycles, behaviorally-regulated feeding, the intestinal epithelium, and also from the surrounding microbial neighborhood. As microbes respond to timed signals such as periodic food supplies or higher or lower temperatures or endogenous hormone levels, they will adjust their physiology (Fig. 3). If zeitgebers are highly predictable, the organism will evolve a system to prepare for the regular input, the first step to an endogenous temporal structure.

Furthermore, the microbiome shows organized localization of communities of a given microbe according over time. At some times of day, certain species are intimate with the gut wall[76]. At other times, they are more internal to the lumen. This suggests that there is a coordination at the species level over time. Therefore, the gut-microbiome interactions occur across the three dimensions of space as well as the fourth dimension, time.

What we have described is essentially a variation of the holobiont concept[88,89], namely that individual organisms deliver emergent properties when observed within their endogenous community. We suggest that daily time, with its pervasive, extensive structure of cues and signals, is an essential element for microbe-rich holobionts. A simple mechanism whereby this may be implemented is cross-feeding. Cross-feeding is emerging as a major aspect of microbial biology that is thought to explain the inability to isolate certain species as individuals *in vitro*[90]. The organism benefits from coordinating resources with neighbors, as it can effectively shut off costly metabolic pathways. The ideal neighborhood would feature a collection of complementary producers. Several metabolites that are cross-fed by bacteria are circadian clock-regulated in their presence as well as in their regulatory proteins in eukaryotic clock systems (e.g. lactate in mice).

Bringing these ideas together, we propose the novel concept of temporal mutualism, defined as rhythmically structured metabolic exchanges between microbes for mutual benefit within a community. Temporal mutualism may therefore underlie rhythmic emergent behaviour within the community and have acted to select for timing systems in non-photosynthetic bacteria.

Why do we care?

The potential for circadian rhythms in the Eubacteria is a big deal. Bacteria play a crucial role in medical, agricultural and industrial processes. Knowledge of potential microbial circadian rhythms would allow us to capitalise on the rhythms to tune how we interact with bacteria in all of these areas.

The presence of a circadian clock in microbes could impact health problems stemming from or associated with microbes. One of the crises in medicine at this time is the prevalence of antibiotic resistant bacteria (MRSA). This reflects an antibiotic overuse, creating an evolutionary pressure on the bacteria to evolve escape mechanisms, as well as a deficit of new antibiotics. Application of new antibiotics would simply delay the problem until new escape tricks are evolved. The combination of invoking bacterial circadian clocks such that they slow down metabolism or replication paired with time-of-day specific administration of antibiotic may be a more powerful form of treatment, potentially delaying development of resistance. The escape drive would have to focus on multiple targets, including the timing system. Concerning the biology of infection itself, the humoral immune system, experimentally characterised by how an organism responds to lipopolysaccharide, a component of bacterial cell wall, has a high amplitude daily rhythm[91]. Administration of 20mg/kg to mice at ZT 6 (night-time for a mouse) leads to death of 50% of the animals within 24h. Almost all mice treated at ZT18 (in the middle of the active period) are alive after 3 days. Control of exposure times may lead to more or less successful infections.

There is an indication that viral infection and response to infection is time of day specific. Entry of viral particles of flaviviruses (RNA viruses including Deng and Zika) into cells as well as the replication of their genome is regulated by clock genes[92]. Infection of mice with Herpesvirus (a DNA virus) at the beginning of the light phase leads to a higher viral load relative to infection at the end of the light phase[93]. This outcome is dependent on a functional BMAL1 protein. We are not suggesting that virus will have an active clock they may borrow or plug into host temporal structures for optimal function.

The strongest case for the impact of a microbial circadian clock on health is emerging from the gut microbiome. Although we still do not know if the gut microbiota will possess an endogenous circadian clock, the interaction of host clock with microbe, the association of clock dysfunction with disease states, and the association of particular microbial populations with

disease makes it tempting to speculate on how microbial circadian clocks may be involved in maintaining health or transition to disease. If microbes are passive players, simply responding to host synchronisation, then attending mainly to supporting the host clock should restore healthy microbiome structure. If the microbiome has a clock of its own, then it will follow the rules of entrainment. The amplitude of rhythms, for instance, would be expected to decrease in phase shifting protocols[94], in contrast to a clock-less, driven system.

In business and industry, bacteria are used for qualitative and quantitative production. Invoking the circadian clock – working with the rhythmic physiology of the producers – could increase production as well as improve quality of the product[95]. Perhaps the most common application is fermentation which traditionally was performed in loosely controlled conditions (in the cellar or in a cabinet in a kitchen). Modern factory production strives for tightly controlled conditions which may inadvertently be a source of either decreased productivity or alteration in flavours. We made sauerkraut, beer and rice wine under strictly controlled temperature conditions as well as under appropriate 24h temperature cycles (data not shown). The results were taste tested in a single blind experiment. In all cases, the subjects could distinguish the foods from the two protocols. Preference was mixed. The deliberate imposition of controlled zeitgeber (e.g. temperature) cycles on fermentation processes should adjust the metabolic pathways thus leading to the accumulation of distinct metabolites, hence leading to unique flavours[95].

Agriculture has been employing microbes to optimize crop production for some time. Root-colonising bacteria (rhizobacteria) are beneficial for plant growth enhancement and agricultural disease suppression[96]. For example, *B. subtilis* forms associations with plant roots, contributing to the growth and health of crops. The nature of the interaction between plants and *Bacillus* spp. is complex and involves signalling between the plant and bacteria. The bacteria are initially attracted towards roots by the detection of root exudates such as L-malic acid, in a process involving chemotaxis[97]. Subsequently, the bacteria form adhesive biofilms on the root surface[98]. Recognition of plant cell wall polysaccharides activates bacterial matrix genes, causing the bacterial synthesis of extracellular polymers that form a matrix between bacterial cells[99]. After colonisation, the bacteria can utilize plant root exudates whilst benefiting the plant through growth enhancement and disease suppression. Examples of crop growth enhancement by *B. subtilis* include an increase in tomato root length and plant height by inoculation with *B. subtilis* PTS-394, increases in root elongation of chickpea seedlings of 70-74% after inoculation with *B. subtilis* strains CM1 and CM3, and increased leaf length, flowers per corm and stigma biomass after inoculation of saffron (*Crocus sativus*) with *B. subtilis*

FZB24[100–102]. There are also many examples of enhanced crop protection from pathogens by inoculation with *B. subtilis*, such as antifungal activity against peach brown rot (*Monilinia* spp.)[103] protection against grey mould (*Botrytis cinerea*) of apple post-harvest, and against damping-off caused by *Pythium ultimum* in bean seedlings[104]. In addition to fungal pathogens, *B. subtilis* colonization of *Arabidopsis thaliana* confers protection against the major agricultural bacterial pathogen *P. syringae* by secretion of the antimicrobial lipopeptide surfactin and the induction of systemic resistance[105].

Given the considerable agricultural and economic importance of rhizobacteria, understanding the mechanisms that underpin plant-bacteria interaction and growth enhancement is important for identifying new ways to sustainably enhance agricultural production. It is possible that circadian-regulated processes in plants or bacteria might contribute to these interactions. For example, plant circadian clocks respond to metabolites, phytohormones and nutrient conditions[106–108], all of which may be altered by microbial colonization. On the other hand, plant circadian regulation of processes such as photosynthesis and carbohydrate metabolism[82,83] might create a rhythmic host environment for the colonising bacteria. Importantly, the presence of circadian rhythms within roots[109,110] provides considerable potential for circadian programs within the plant to impact attraction and colonisation by beneficial bacteria. We also speculate that there might be circadian rhythms in root membrane transport processes such as proton pumping that impose a fluctuating environment upon root-colonising bacteria, although this is as yet uncharacterized. These bidirectional interactions between plant and bacterial circadian rhythms may be analogous to host-bacteria interactions in the gut microbiome[70,71]. It is possible to envisage that circadian programs in root-colonising bacteria might allow the bacterial colonies to co-ordinate their metabolism with rhythms derived from the host plant in root exudate secretion, in order to maximise the benefit of the bidirectional interaction.

A Perspective

The glimpses of circadian rhythms in non-photosynthetic Eubacteria are tantalizing. We suspect that the time-keeping abilities of such organisms are significant but that the witnessing of those abilities might require systems in which these microbes are in more complex environments than they are typically cultured. The interaction between the timekeepers in these complex environments will be important to understand for basic scientific reasons, as well as for medical and agricultural reasons.

Acknowledgments

The work of M.M. is supported by the Volkswagen Foundation, the German Science Foundation (DFG), the Friederich-Bauer Stiftung of LMU Munich, WiFoMed of LMU Munich and the Center for Advanced Studies of LMU Munich. The laboratory of Á.T.K. is supported by the Danish National Research Foundation (DNRF137) for the Center for Microbial Secondary Metabolites. The laboratory of A.N.D. is supported by the UK Biotechnology and Biological Sciences Research Council (BBSRC), the Leverhulme Trust, the Daiwa Anglo-Japanese Foundation and the Bristol Centre for Agricultural Innovation.

Author Contributions

B.A., J.B., Z.E.-C., F.S., L.E.H., A.N.D., Á.T.K., and M.M. wrote the paper.

Literature references

1. Abraham, D. *et al.* Restoration of Circadian Rhythmicity in Circadian Clock – Deficient Mice in Constant Light. *J. Biol. Rhythms* 21, 169–176 (2006).
2. Pittendrigh, C. S. & Caldarola, P. C. General homeostasis of the frequency of circadian oscillations. *Proc. Natl. Acad. Sci. U. S. A.* 70, 2697–2701 (1973).
3. Gardner, G. F. & Feldman, J. F. Temperature Compensation of Circadian Period Length in Clock Mutants of Neurospora crassa. *Plant Physiol.* 68, 1244–1248 (1981).
4. Barrett, R. K. & Takahashi, J. S. Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. *J. Neurosci.* 15, 5681–5692 (1995).
5. Zimmerman, W. F., Pittendrigh, C. S. & Pavlidis, T. Temperature compensation of the circadian oscillation in drosophila pseudoobscura and its entrainment by temperature cycles. *J. Insect Physiol.* 14, 669–684 (1968).
6. Pittendrigh, C. S. On Temperature Independence in the Clock System Controlling Emergence Time in Drosophila. *Proc. Natl. Acad. Sci.* (1954) doi:10.1073/pnas.40.10.1018.
7. Merrow, M., Spoelstra, K. & Roenneberg, T. The circadian cycle: daily rhythms from behaviour to genes. *EMBO Rep.* 6, 930–5 (2005).
8. Mrosovsky, N. Masking: History, definitions, and measurement. *Chronobiology International* (1999) doi:10.3109/07420529908998717.
9. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, (2010).
10. Aschoff, J. & Pohl, H. Phase relations between a circadian rhythm and its zeitgeber within the range of entrainment. *Naturwissenschaften* 65, 80–84 (1978).
11. Tan, Y., Dragovic, Z., Roenneberg, T. & Merrow, M. Entrainment dissociates transcription and translation of a circadian clock gene in Neurospora. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.02.035.
12. Rémi, J., Merrow, M. & Roenneberg, T. A circadian surface of entrainment: Varying T, τ, and Photoperiod in Neurospora crassa. *J. Biol. Rhythms* 25, (2010).
13. Kondo, T. *et al.* Circadian clock mutants of cyanobacteria. *Science (80-.).* (1994) doi:10.1126/science.7973706.
14. Liu, Y. *et al.* Circadian orchestration of gene expression in cyanobacteria. *Genes Dev.* 9, 1469–1478 (1995).
15. Nakajima, M. *et al.* Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science (80-.).* 308, 414–415 (2005).

16. Chen, A. H., Lubkowicz, D., Yeong, V., Chang, R. L. & Silver, P. a. Transplantability of a circadian clock to a noncircadian organism. *Sci. Adv.* 1, 1–6 (2015).
17. Aoki, S., Kondo, T., Wada, H. & Ishiura, M. Circadian rhythm of the cyanobacterium Synechocystis sp. strain PCC 6803 in the dark. *J. Bacteriol.* (1997) doi:10.1128/jb.179.18.5751-5755.1997.
18. Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Hirschie, C. Resonating circadian clocks enh ; ince fitness in cyanobacteria. *Evolution (N. Y).* (2009) doi:10.1073/pnas.95.15.8660.
19. Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K. & Johnson, C. H. The adaptive value of circadian clocks: An experimental assessment in cyanobacteria. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.08.023.
20. Welkie, D. G. *et al.* Genome-wide fitness assessment during diurnal growth reveals an expanded role of the cyanobacterial circadian clock protein KaiA. *Proc. Natl. Acad. Sci.* (2018) doi:10.1073/pnas.1802940115.
21. Van Praag, E., Agosti, R. D. & Bachofen, R. Rhythmic activity of uptake hydrogenase in the prokaryote Rhodospirillum rubrum. *J. Biol. Rhythms* (2000) doi:10.1177/074873040001500303.
22. Min, H., Guo, H. & Xiong, J. Rhythmic gene expression in a purple photosynthetic bacterium, Rhodobacter sphaeroides. *FEBS Lett.* (2005) doi:10.1016/j.febslet.2005.01.003.
23. Ehret, C. F. & Wille, J. J. The Photobiology of Circadian Rhythms in Protozoa and other Eukaryotic Microorganisms. in *Photobiology of Microorganisms* 369–416 (1970).
24. Kondo, T. *et al.* Circadian rhythms in rapidly dividing cyanobacteria. *Science (80-.).* (1997) doi:10.1126/science.275.5297.224.
25. Norman, T. M., Lord, N. D., Paulsson, J. & Losick, R. Stochastic Switching of Cell Fate in Microbes. *Annu. Rev. Microbiol.* (2015) doi:10.1146/annurev-micro-091213-112852.
26. Rogers, L. A. & Greenbank, G. R. The intermitent growth of bacterial cultures. *J Bacteriol.* 16, 181–190 (1930).
27. Halberg, F. & Conner, R. L. Circadian organization and microbiology: Variance spectra and a periodogram on behavior of Escherichia coli growing in fluid culture. *Proc Minn Acad Sci* 29, 227–239 (1961).
28. Sturtevant, R. Circadian patterns in linear growth of Escherichia coli. *Anat. Rec.* 175, 453 (1973).
29. Bibb, L. B. Long Tube Method of Cultivating Microorganisms with Observations on Mobile Colonies in Liquid Medium. *J. Bacteriol.* 10, 561 (1925).

30. Sturtevant, R. P. Circadian variability in Klebsiella demonstrated by cosinor analysis. *Int. J. Chronobiol.* (1973).
31. Paulose, J. K., Wright, J. M., Patel, A. G. & Cassone, V. M. Human gut bacteria are sensitive to melatonin and express endogenous circadian rhythmicity. *PLoS One* 11, (2016).
32. Soriano, M. I., Roibás, B., García, A. B. & Espinosa-Urgel, M. Evidence of circadian rhythms in non-photosynthetic bacteria? *J. Circadian Rhythms* 8, (2010).
33. Dvornyk, V., Vinogradova, O. & Nevo, E. Origin and evolution of circadian clock genes in prokaryotes. *Proc. Natl. Acad. Sci.* (2003) doi:10.1073/pnas.0130099100.
34. Loza-Correa, M., Gomez-Valero, L. & Buchrieser, C. Circadian clock proteins in prokaryotes: Hidden rhythms? *Front. Microbiol.* (2010) doi:10.3389/fmicb.2010.00130.
35. Cleyet-Marel, J. C., Rome, S., Salducci, X. & Wery, J. Importance des composés phénoliques dans les interactions entre plantes et microorganismes: Exemple des relations Rhizobium/légumineuses. *Acta Bot. Gall.* (1996) doi:10.1080/12538078.1996.10515349.
36. Rome, S., Fernandez, M. P., Brunel, B., Normand, P. & Cleyet-Marel, J.-C. Sinorhizobium medicae sp. nov., Isolated from Annual Medicago spp. *Int. J. Syst. Bacteriol.* (1996) doi:10.1099/00207713-46-4-972.
37. Bernal, P., Allsopp, L. P., Filloux, A. & Llamas, M. A. The Pseudomonas putida T6SS is a plant warden against phytopathogens. *ISME J.* (2017) doi:10.1038/ismej.2016.169.
38. Nambu, J. R., Lewis, J. O., Wharton, K. A. & Crews, S. T. The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* (1991) doi:10.1016/0092-8674(91)90292-7.
39. Crews, S. T., Thomas, J. B. & Goodman, C. S. The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* (1988) doi:10.1016/0092-8674(88)90538-7.
40. Hoffman, E. C. *et al.* Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science (80-.).* (1991) doi:10.1126/science.1852076.
41. Lindebro, M. C., Poellinger, L. & Whitelaw, M. L. Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J.* (1995).
42. Huang, Z. J., Edery, I. & Rosbash, M. PAS is a dimerization domain common to Drosophila period and several transcription factors. *Nature* (1993) doi:10.1038/364259a0.
43. Taylor, B. L. & Zhulin, I. B. PAS Domains: Internal Sensors of Oxygen, Redox

Potential, and Light. *Microbiol. Mol. Biol. Rev.* 63, 479–506 (1999).

44. Rockwell, N. C., Su, Y.-S. & Lagarias, J. C. PHYTOCHOME STRUCTURE AND SIGNALING MECHANISMS. *Annu Rev Plant Biol* 57, 837–858 (2006).
45. Davis, S. J., Vener, a V & Vierstra, R. D. Bacteriophytochromes: phytochrome-like photoreceptors from nonphotosynthetic eubacteria. *Science* 286, 2517–2520 (1999).
46. Bhoo, S. H., Davis, S. J., Walker, J., Karniol, B. & Vierstra, R. D. Bacteriophytochromes are photochromic histidine kinases using a biliverdin chromophore. *Nature* 414, 776–779 (2001).
47. Rottwinkel, G., Oberpichler, I. & Lamparter, T. Bathy phytochromes in rhizobial soil bacteria. *J. Bacteriol.* (2010) doi:10.1128/JB.00672-10.
48. Lee, H. J. *et al.* Stem-piped light activates phytochrome B to trigger light responses in arabidopsis thaliana roots. *Sci. Signal.* (2016) doi:10.1126/scisignal.aaf6530.
49. Nimmo, H. G. Entrainment of Arabidopsis roots to the light:dark cycle by light piping. *Plant Cell Environ.* (2018) doi:10.1111/pce.13137.
50. Losi, A., Polverini, E., Quest, B. & Gärtner, W. First evidence for phototropin-related blue-light receptors in prokaryotes. *Biophys. J.* (2002) doi:10.1016/S0006-3495(02)75604-X.
51. Gomelsky, M. & Klug, G. BLUF: A novel FAD-binding domain involved in sensory transduction in microorganisms. *Trends Biochem. Sci.* (2002) doi:10.1016/S0968-0004(02)02181-3.
52. Tschowri, N., Busse, S. & Hengge, R. The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of Escherichia coli. *Genes Dev.* (2009) doi:10.1101/gad.499409.
53. Memmi, S. *et al.* Photoactive yellow protein from the halophilic bacterium Salinibacter ruber. *Biochemistry* (2008) doi:10.1021/bi701486n.
54. Kumauchi, M., Hara, M. T., Stalcup, P., Xie, A. & Hoff, W. D. Identification of six new photoactive yellow proteins-diversity and structure-function relationships in a bacterial blue light photoreceptor. in *Photochemistry and Photobiology* (2008). doi:10.1111/j.1751-1097.2008.00335.x.
55. Bonomi, H. R. *et al.* Light regulates attachment, exopolysaccharide production, and nodulation in *Rhizobium leguminosarum* through a LOV-histidine kinase photoreceptor. *Proc. Natl. Acad. Sci.* (2012) doi:10.1073/pnas.1121292109.
56. Lopez, D., Vlamakis, H. & Kolter, R. Generation of multiple cell types in Bacillus subtilis. *FEMS Microbiology Reviews* vol. 33 152–163 (2009).
57. Kearns, D. B. A field guide to bacterial swarming motility. *Nature Reviews Microbiology* (2010) doi:10.1038/nrmicro2405.

58. Hölscher, T. & Kovács, Á. T. Sliding on the surface: bacterial spreading without an active motor. *Environmental Microbiology* (2017) doi:10.1111/1462-2920.13741.
59. Vlamakis, H., Chai, Y., Beauregard, P., Losick, R. & Kolter, R. Sticking together: Building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology* (2013) doi:10.1038/nrmicro2960.
60. Süel, G. M., Garcia-Ojalvo, J., Liberman, L. M. & Elowitz, M. B. An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* (2006) doi:10.1038/nature04588.
61. Norman, T. M., Lord, N. D., Paulsson, J. & Losick, R. Memory and modularity in cell-fate decision making. *Nature* 503, 481–486 (2013).
62. Higgins, D. & Dworkin, J. Recent progress in Bacillus subtilis sporulation. *FEMS Microbiology Reviews* (2012) doi:10.1111/j.1574-6976.2011.00310.x.
63. Levine, J. H., Fontes, M. E., Dworkin, J. & Elowitz, M. B. Pulsed feedback defers cellular differentiation. *PLoS Biol.* (2012) doi:10.1371/journal.pbio.1001252.
64. Narula, J. *et al.* Chromosomal Arrangement of Phosphorelay Genes Couples Sporulation and DNA Replication. *Cell* (2015) doi:10.1016/j.cell.2015.06.012.
65. Bischofs, I. B., Hug, J. A., Liu, A. W., Wolf, D. M. & Arkin, A. P. Complexity in bacterial cell-cell communication: Quorum signal integration and subpopulation signaling in the Bacillus subtilis phosphorelay. *Proc. Natl. Acad. Sci.* 106, 6459–6464 (2009).
66. Kuchina, A. *et al.* Temporal competition between differentiation programs determines cell fate choice. *Mol. Syst. Biol.* 7, 557 (2011).
67. PITTENDRIGH, C. S. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/SQB.1960.025.01.015.
68. Ma, P., Mori, T., Zhao, C., Thiel, T. & Johnson, C. H. Evolution of KaiC-Dependent Timekeepers: A Proto-circadian Timing Mechanism Confers Adaptive Fitness in the Purple Bacterium Rhodopseudomonas palustris. *PLoS Genet.* (2016) doi:10.1371/journal.pgen.1005922.
69. Zarrinpar, A., Chaix, A., Yooseph, S. & Panda, S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. *Cell Metab.* 20, 1006–1017 (2014).
70. Liang, X., Bushman, F. D. & FitzGerald, G. a. Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. *Proc. Natl. Acad. Sci.* 112, 10479–10484 (2015).
71. Thaiss, C. A. *et al.* Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell* 159, 514–529 (2014).
72. Mukherji, A., Kobiita, A., Ye, T. & Chambon, P. Homeostasis in intestinal epithelium is

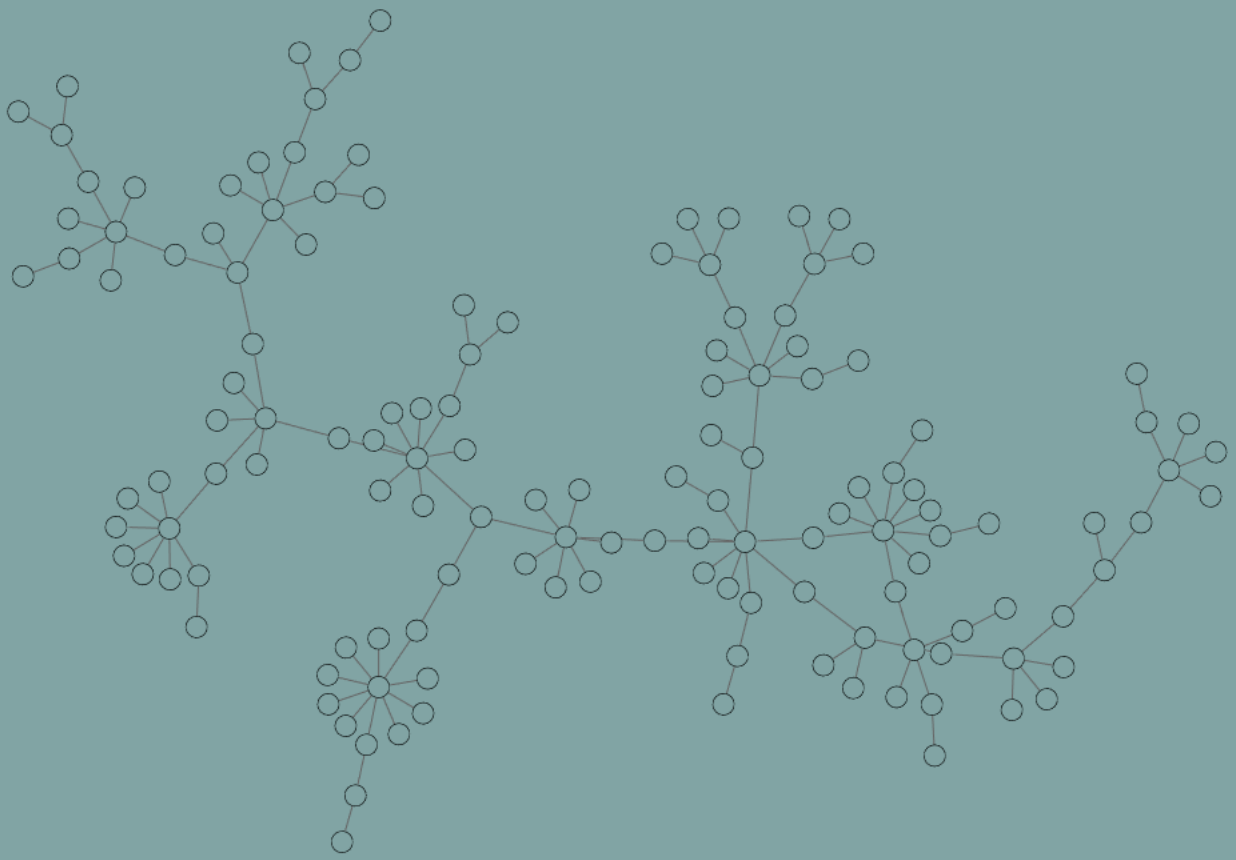
orchestrated by the circadian clock and microbiota cues transduced by TLRs. *Cell* (2013) doi:10.1016/j.cell.2013.04.020.

73. Montagner, A. *et al.* Hepatic circadian clock oscillators and nuclear receptors integrate microbiome-derived signals. *Sci. Rep.* (2016) doi:10.1038/srep20127.
74. Leone, V. *et al.* Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. *Cell Host Microbe* (2015) doi:10.1016/j.chom.2015.03.006.
75. Murakami, M. *et al.* Gut microbiota directs PPARγ-driven reprogramming of the liver circadian clock by nutritional challenge. *EMBO Rep.* (2016) doi:10.15252/embr.201642463.
76. Thaiss, C. A., Nobs, S. P. & Elinav, E. NFIL-trating the Host Circadian Rhythm—Microbes Fine-Tune the Epithelial Clock. *Cell Metab.* 26, 699–700 (2017).
77. Weger, B. D. *et al.* The Mouse Microbiome Is Required for Sex-Specific Diurnal Rhythms of Gene Expression and Metabolism. *Cell Metab.* (2018) doi:10.1016/j.cmet.2018.09.023.
78. Konturek, S. J. *et al.* Localization and biological activities of melatonin in intact and diseased Gastrointestinal Tract (GIT). *Journal of Physiology and Pharmacology* (2007) doi:10.1080/02692170701474751.
79. Thaiss, C. A. *et al.* Microbiota Diurnal Rhythmicity Programs Host Transcriptome Oscillations. *Cell* 167, 1495-1510.e12 (2016).
80. Hsu, P. Y. & Harmer, S. L. Wheels within wheels: The plant circadian system. *Trends in Plant Science* (2014) doi:10.1016/j.tplants.2013.11.007.
81. Hennessey, T. L. & Field, C. B. Circadian Rhythms in Photosynthesis : Oscillations in Carbon Assimilation and Stomatal Conductance under Constant Conditions. *Plant Physiol.* (1991).
82. Dodd, A. N. *et al.* Plant Circadian Clocks Increase Photosynthesis , Growth , Survival. *Science (80-.).* 630, 630–3 (2007).
83. Graf, A., Schlereth, A., Stitt, M. & Smith, A. M. Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. *Proc. Natl. Acad. Sci.* (2010) doi:10.1073/pnas.0914299107.
84. Green, R. M., Tingay, S., Wang, Z.-Y. & Tobin, E. M. Circadian {Rhythms} {Confer} a {Higher} {Level} of {Fitness} to {Arabidopsis} {Plants}. *Plant Physiol.* 129, 576–584 (2002).
85. Hubbard, C. J. *et al.* The plant circadian clock influences rhizosphere community structure and function. *ISME J.* (2018) doi:10.1038/ismej.2017.172.
86. Zhang, C. *et al.* Crosstalk between the Circadian Clock and Innate Immunity in

Arabidopsis. *PLoS Pathog.* (2013) doi:10.1371/journal.ppat.1003370.

87. Roenneberg, T. & Merrow, M. The circadian clock and human health. *Curr. Biol.* 26, (2016).
88. Margulis, L. & Fester, R. *Symbiosis as a Source of Evolutionary Innovation*. (1991).
89. Simon, J. C., Marchesi, J. R., Mougel, C. & Selosse, M. A. Host-microbiota interactions: from holobiont theory to analysis. *Microbiome* 7, 5 (2019).
90. D'Souza, G. *et al.* Ecology and evolution of metabolic cross-feeding interactions in bacteria. *Natural Product Reports* vol. 35 455–488 (2018).
91. Spengler, M. L. *et al.* Core circadian protein CLOCK is a positive regulator of NF- B-mediated transcription. *Proc. Natl. Acad. Sci.* (2012) doi:10.1073/pnas.1206274109.
92. Zhuang, X. *et al.* The circadian clock components BMAL1 and REV-ERBα regulate flavivirus replication. *Nat. Commun.* (2019) doi:10.1038/s41467-019-08299-7.
93. Edgar, R. S. *et al.* Cell autonomous regulation of herpes and influenza virus infection by the circadian clock. *Proc. Natl. Acad. Sci.* (2016) doi:10.1073/pnas.1601895113.
94. Dijk, D. J. *et al.* Amplitude reduction and phase shifts of Melatonin, cortisol and other circadian rhythms after a gradual advance of sleep and light exposure in humans. *PLoS One* 7, (2012).
95. Merrow, M. W. Process for the production of a compound or a composition employing a culture of microorganisms under circadian cultivation conditions. (2009).
96. Vejan, P., Abdullah, R., Khadiran, T., Ismail, S. & Nasrulhaq Boyce, A. Role of plant growth promoting rhizobacteria in agricultural sustainability-A review. *Molecules* (2016) doi:10.3390/molecules21050573.
97. Rudrappa, T., Czymmek, K. J., Pare, P. W. & Bais, H. P. Root-Secreted Malic Acid Recruits Beneficial Soil Bacteria. *PLANT Physiol.* (2008) doi:10.1104/pp.108.127613.
98. Allard-Massicotte, R. *et al.* Bacillus subtilis early colonization of Arabidopsis thaliana roots involves multiple chemotaxis receptors. *MBio* (2016) doi:10.1128/mBio.01664-16.
99. Beauregard, P. B., Chai, Y., Vlamakis, H., Losick, R. & Kolter, R. Bacillus subtilis biofilm induction by plant polysaccharides. *Proc. Natl. Acad. Sci.* 110, E1621–E1630 (2013).
100. Qiao, J. *et al.* Addition of plant-growth-promoting Bacillus subtilis PTS-394 on tomato rhizosphere has no durable impact on composition of root microbiome. *BMC Microbiol.* (2017) doi:10.1186/s12866-017-1039-x.
101. Swain, M. R. & Ray, R. C. Biocontrol and other beneficial activities of Bacillus subtilis isolated from cowdung microflora. *Microbiol. Res.* (2009) doi:10.1016/j.micres.2006.10.009.

102. Sharaf-Eldin, M. *et al.* Bacillus subtilis FZB24® affects flower quantity and quality of saffron (Crocus sativus). *Planta Med.* (2008) doi:10.1055/s-2008-1081293.
103. Hong, C., Holtz, B. A., Morgan, D. P. & Michailides, T. J. Significance of thinned fruit as a source of the secondary inoculum of Monilinia fructicola in California nectarine orchards. *Plant Dis* 81, 519–524 (1997).
104. Ongena, M. *et al.* Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of Bacillus subtilis. *Appl. Microbiol. Biotechnol.* (2005) doi:10.1007/s00253-005-1940-3.
105. Bais, H. P., Fall, R. & Vivanco, J. M. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiol.* 134, 307–319 (2004).
106. Hanano, S., Domagalska, M. A., Nagy, F. & Davis, S. J. Multiple phytohormones influence distinct parameters of the plant circadian clock. *Genes to Cells* 11, 1381–1392 (2006).
107. Haydon, M. J., Mielczarek, O., Robertson, F. C., Hubbard, K. E. & Webb, A. A. R. Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. *Nature* 502, 689–692 (2013).
108. Frank, A. *et al.* Circadian Entrainment in Arabidopsis by the Sugar-Responsive Transcription Factor bZIP63. *Curr. Biol.* 28, 2597-2606.e6 (2018).
109. Thain, S., Murtas, G. & Lynn, J. The circadian clock that controls gene expression in Arabidopsis is tissue specific. *Plant …* 130, 102–110 (2002).
110. James, A. B. *et al.* The circadian clock in Arabidopsis roots is a simplified slave version of the clock in shoots. *Science (80-.).* (2008) doi:10.1126/science.1161403.



Chapter 7

A Circadian Clock in a Non-photosynthetic Prokaryote

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Published: Science Advances 2021

Introduction

Circadian clocks contribute to the fitness of an organism[1,2]. The absence of characterized circadian rhythms in the first group of cellular organisms to populate the Earth is thus striking and unexpected. In order to challenge this, we selected the non-photosynthetic, gram-positive bacterium *B. subtilis* as an experimental model. We chose this organism because of circumstantial observations of rhythms approaching 24h, although these were not made under the controlled conditions normally used to study circadian clocks. For example, in single cells of *B. subtilis* in a microfluidic device there is pulsed activation of expression of a matrix gene approximately every 24h, indicating that under homogeneous conditions the stochastic triggering of biofilm formation may follow an internal daily cycle[3]. There are cyclic (every ~20 h) changes in the activity of the promoters of *rapA* and *spo0F*, which are important for cell fate decisions between sporulation and growth of *B. subtilis*[4,5] . These results suggest there might be daily changes in the expression of genes involved in sensing environmental changes in *B. subtilis*. Furthermore, *B. subtilis* is light-sensitive, harbouring blue- and red-light photoreceptors[6] that could potentially entrain a circadian clock to the 24h day. Together, these reports led us to hypothesize that this Eubacterium might entrain to its environment using light and/or temperature signals like other circadian systems.

We developed luciferase reporter strains to conduct high-throughput and non-invasive measurement of gene promoter activity. This approach is used widely to study circadian rhythms in other kingdoms of life[7]. The *B. subtilis* genome lacks homologs of the core clock proteins (KaiA, KaiB, KaiC) present in cyanobacteria. However, many bacteria including *B. subtilis* harbor genes encoding PAS domains, which are structural motifs present in all defined circadian clocks of eukaryotes[8]. We reasoned that such genes might encode circadian clock-associated proteins. Of 16 predicted PAS domain-encoding genes in *B. subtilis*, we first selected the promoter of *ytvA* to create a bioluminescent reporter strain. *ytvA* encodes a blue light photoreceptor[9] with a PAS domain accompanied by a PAC domain, which is a common pairing in circadian and sensory/signaling proteins[10]. Blue light photoreception is an integral part of circadian systems in all experimental models examined to date[11].

Synchronisation to 24h light or temperature cycles and free-running rhythms

We identified free-running rhythms of *ytvA* promoter activity in *B. subtilis*. Cultures were exposed to 24h zeitgeber cycles. Zeitgebers are predictable, recurring environmental signals that biological rhythms use for entrainment or synchronization. In these culture conditions, zeitgeber-sensitive rhythmic processes could be either initiated or synchronized between

bacterial cells, and therefore detected. We first determined whether light can act as a zeitgeber for *B. subtilis.* 24h light/dark (LD) cycles (12h L / 12h D) were applied to cultures. P_{ytvA}*::lux* gene expression increased during the dark phase and decreased during the light phase (Fig. 1A; Fig. S1A). The pattern appears to combine two common features observed in the process of entrainment by a circadian clock. First, the abrupt reversal of expression at the zeitgeber transitions resembles masking[12] (Fig. 1A; Fig. S1A). Second, the interaction of two oscillators (the circadian clock and the zeitgeber cycle) each with their own momentum and robustness is suggested by irregular expression of P_{ytvA}*::lux* luciferase from day to day, with a stable pattern appearing only after several days suggesting that the biological oscillator has reached a stable relationship with the 24h cycle (Fig. 1A; Fig. S1A). Importantly, a free-running rhythm in *ytvA* promoter activity occurred when the cultures were released to constant darkness (Fig. 1B, C; Fig. S1A). This occurred only in strains cultured in glucose-containing media (Fig. 1B, C; Fig. S2B). The period calculated over a 48h window following release to constant conditions was 28.66 ± 1.77 h. Over 5 days, the period increased in length and damped thereafter. In glucose-free media, damping occurred rapidly on release to constant darkness, precluding the determination of period length in this condition (Fig. S2B).

We validated our findings with an additional luciferase reporter strain that uses a promoter from another PAS-domain protein coding gene, *kinC*. KinC is a histidine kinase involved in the regulation of differentiation processes such as biofilm development and sporulation[13]. Sporulation is a clock-regulated output in the fungal species *Neurospora crassa*[14,15] and *Aspergillus*[16]. Following entrainment in 24h LD cycles (Fig. 1D, Fig. S1B), P_{kinC}::*lux* expression has circadian rhythms (Fig. 1E, F, Fig. S1B). As for the P_{ytvA}*::lux* strain, daily rhythms were detected in cultures containing glucose, but not in those deficient in glucose (Fig. S2D). The temporal expression pattern (phase and amplitude) of this reporter was similar to that of P_{ytvA}::*lux* (Fig. S3). This is not surprising based on published observations: the preponderance of gene expression in cyanobacteria at the end of the subjective night relative to other times of day[17] and furthermore, the congruent expression of these two genes in media containing different carbon sources[18] could suggest shared regulatory pathways. It is possible that as more clock-regulated genes are identified in *B. subtilis*, more phases of expression will be identified.

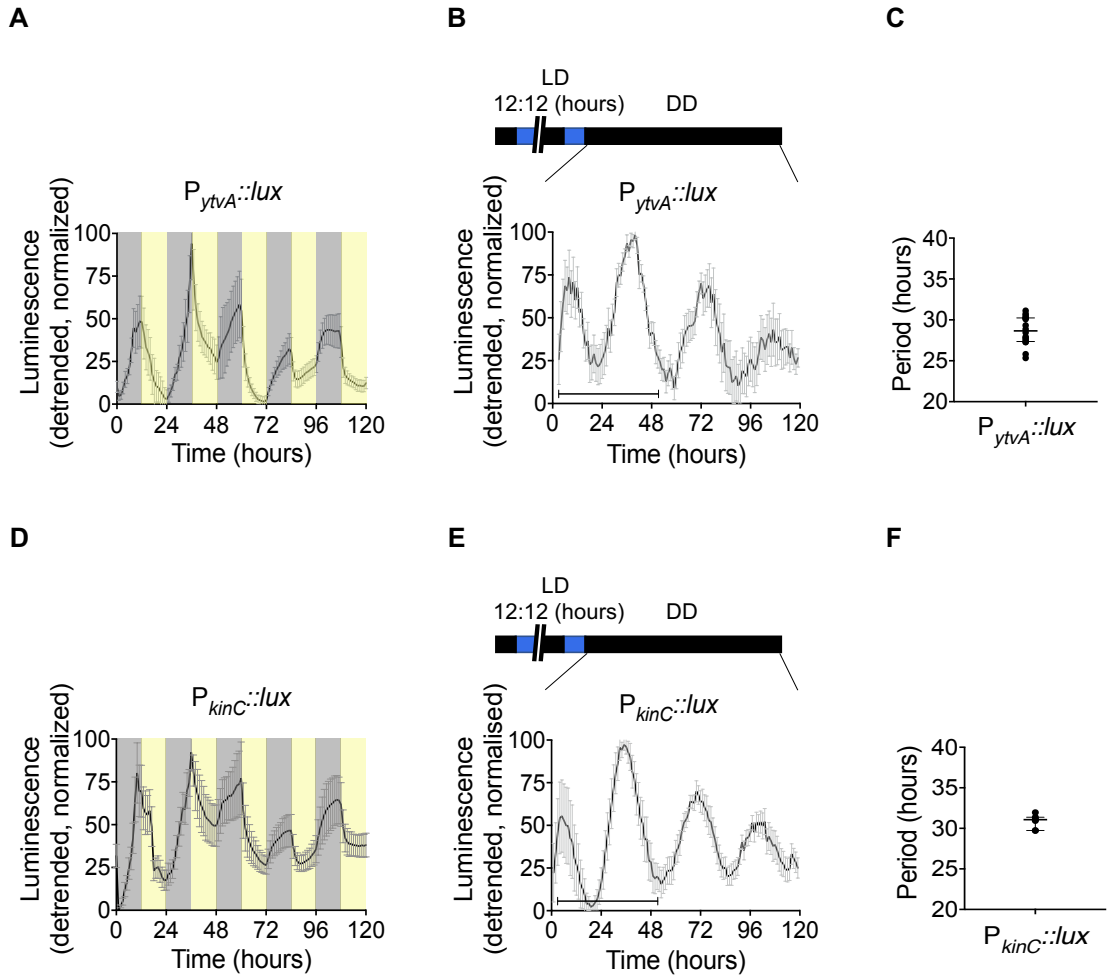


Figure 1. Entrainment by light and a free-running rhythm in *B. subtilis*. Bioluminescence of PytvA::lux (A) and PkinC::lux (D) under 5 days of entrainment with cycles of darkness and blue light (12h D /12h L) and after release to constant darkness conditions (DD; B, E) for 5 days. The temperature was kept constant at 25.5 °C. The de-trended data are presented as mean ± standard deviation. The shading in (A, D) shows the timing of the LD cycle (yellow, light phase; grey, dark phase) relative to the bioluminescence. The horizontal bar in (B, E, lower left) shows the time window of 48h selected for the analysis of period length. The calculated period length is plotted in (C, F); individual data points (C, N = 16; F, N = 7) are shown along with the median and interquartile range. See also Suppl. Table 1.

Self-sustained, free-running rhythms in promoter activity of *ytvA* also occurred following entrainment to temperature cycles (12h at 25.5 °C / 12h at 28.5 °C). Daily temperature fluctuations are reliable indicators of the time of day in nature, often serving as zeitgebers (recurring cues from the environment that are used by biological rhythms for their synchronization or entrainment) for a circadian clock. Temperature entrained cultures had daily oscillations in *ytvA* promoter activity (Fig. S4). In contrast to cultures entrained using cycles of blue light and darkness, promoter activity was generally higher during the warm phase (correspondent to daytime), which is the converse of its behavior during entrainment to light (compare Fig. 1 and Fig. S4). Further, persistent temperature-entrained free-running rhythms were detected only in media lacking glucose (with a period of 23.94 ± 1.64; Fig. 2A

and B). In contrast to the LD-entrained cultures, rhythms were suppressed by media containing glucose as a carbon source (Fig. 2D). Rhythms were also suppressed in the presence of glycerol (Fig. 2C) and in a variety of other media often used to culture *B. subtilis* (Fig. S5). Together with the longer free-running period following light compared to temperature entrainment, this sensitivity to nutritional composition suggests that the circadian clock of *B. subtilis* responds to the myriad of environmental conditions under which bacteria subsist. Carbon source and availability also impact the free-running rhythm in plants and fungi[19,20]. Further, nutritional composition and environmental conditions such as light and temperature determine how populations grow and differentiate[13,21]. 100% of the cultures exhibiting free-running, circadian rhythms formed a floating biofilm, whose presence was assessed qualitatively by visual observation of a pellicle forming at the air-liquid interface in the well.

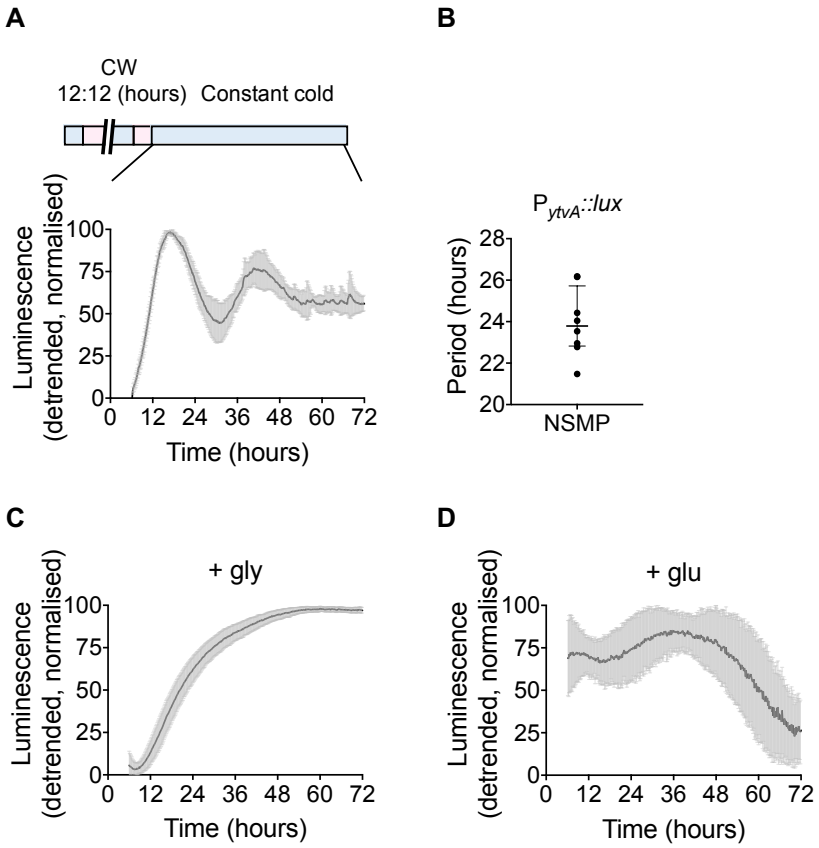


Figure 2. Free-running rhythms in B. subtilis following entrainment in temperature cycles. Bioluminescence of PytvA::lux in constant darkness at 25.5 °C following 5 days of entrainment in temperature cycles (12h at 25.5 °C / 12h at 28.5 °C) is shown. A free-running rhythm is observed in NSMP medium lacking glucose (A). The de-trended data are plotted as mean ± standard deviation. The calculated period length of PytvA::lux expression shown in (A) is plotted in (B), where individual data points (N = 8) are shown along with the median and interquartile range. No free-running rhythm is observed in NSMP medium containing glycerol (N = 40) (C) or glucose (N = 15) (D) as a carbon source. See also Suppl. Table 1.

A third hallmark of circadian clocks is temperature compensation. The period of the free-running circadian rhythm typically has a Q_{10} close to 1, meaning that it remains relatively stable across a physiologically relevant 10 °C temperature range. We entrained *B. subtilis* cultures to three different temperature cycles for 5 days, after which the cultures were released into constant conditions at the lower temperature of each cycle (Fig. 3A-C). The free-running periods measured in constant temperatures spanning a 6 °C range were not significantly different ($P > 0.05$; Fig. 3D). Q_{10} was calculated as 1.03, a moderate under-compensation. At temperatures outside this 6 °C range, free-running rhythms were not detected. Interestingly, the amplitude of the oscillation in gene expression was significantly greater at the intermediate temperature relative to both lower and higher temperatures (22.5 °C vs. 25.5 °C, p = 0.0013; 28.5 °C vs. 25.5 °C, p = 0.0311; Fig. 3E). Taken together with the damping of rhythms outside of this temperature range, we conclude that there is a narrow, optimal temperature range in which the *Bacillus* cultures can maintain free-running rhythms in our laboratory conditions.

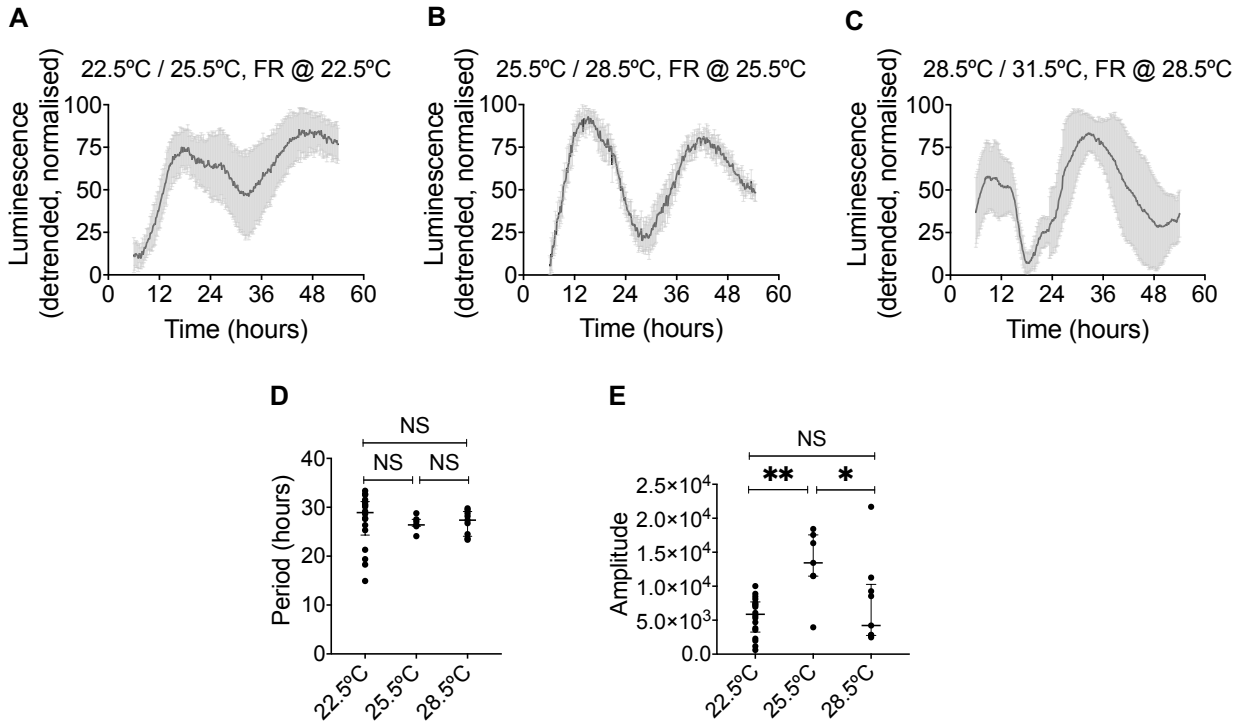


Figure 3. Circadian rhythms in B. subtilis are temperature compensated. Bioluminescence of PytvA::lux under constant conditions (22.5 °C, N = 25, A; 25.5 °C, N = 7, B; 28.5 °C, N = 9, C) following 5 days of entrainment with various temperature cycles (A: 12h at 22.5 °C / 12h at 25.5 °C; B: 12h at 25.5 °C / 12h at 28.5 °C; C: 12h at 28.5 °C / 12h at 31.5 °C). The de-trended data are presented as mean ± standard deviation. Period (D) and amplitude (E) of the bioluminescent signal of the data from panels A through C are shown as single data points, median and interquartile range. Data were analysed using Ordinary one-way ANOVA. NS, not significant (P values > 0.05), ** P = 0.0013, * P = 0.0311. See also Suppl. Table 1.

Phase relationship between the circadian rhythm and the temperature cycle

Our data identifies free-running rhythms and their temperature compensation. Perhaps the most important hallmark of circadian rhythms, entrainment, is implied by the synchronisation of cultures to 24h zeitgeber cycles as shown in figures 1, S1-S4. We further observed that the free-running rhythm started 180° out of phase in cultures that were entrained in antiphase (Fig. S6). We sought to test for explicit features of entrainment, namely the establishment of a distinct phase of entrainment – meaning when the biological rhythm reliably occurs within each day - according to the zeitgeber cycle. As it is the 24h temporal structures of zeitgebers that drove evolution of circadian clocks, systematic entrainment should be a built-in feature of the system. Adaptive entrainment is essential to accommodate circadian entrainment in a constantly changing photoperiodic environment.

To understand entrainment in *B. subtilis*, we tested the effect of varying the zeitgeber strength - the magnitude of the entrainment signal - on the phase of entrainment, since the two are related in circadian systems in other kingdoms of life[22–25]. Most humans, for instance, will entrain earlier in a zeitgeber cycle of higher amplitude. In our experiments with *B. subtilis*, we used temperature as a zeitgeber. Physical temperature perceived by living organisms is contextual; for example, a 3 °C amplitude temperature cycle is perceived as a different amplitude depending on the absolute or ambient temperature. Therefore, our cultures assayed for temperature compensation experienced different zeitgeber strengths at each set of entrainment temperatures. The phase of the first measured oscillation of bioluminescence varied, according to whether the temperature cycled around a lower or higher mean temperature (Fig. 3A-C). The phase of this first cycle during free-run was later at lower temperatures, and earlier at higher temperatures. This suggests that circadian phase of *B. subtilis* depends upon the zeitgeber strength and is not simply driven by the environmental transitions.

We next used “T cycles” (entraining cycles of different length) to distinguish non-circadian, environment-driven synchronization (masking) from circadian entrainment. “T” is defined as the duration of the entire entraining cycle, *e.g.* on Earth, T is about 24 h. A general feature of circadian rhythms is a relationship between the period of the rhythm and that of the zeitgeber cycle. A conspecific with a longer free-running period generally entrains later in a 24h cycle than will an individual with a shorter period. It follows that a given individual will entrain to a later phase in a shorter cycle and an earlier one in a longer cycle (so-called T cycles) [23, 26–28]. In contrast, a “masking” signal synchronises to the same phase irrespective of the structure of the zeitgeber cycle (*e.g.* if it is longer or shorter). Entrainment often contains elements of

adaptive phase angles that change according to the zeitgeber as well as evidence of masking. We found that *B. subtilis* entrained systematically later as T cycles became shorter (Fig. 4) despite evidence of masking (Fig. S7). The phase angles of the oscillation shifted significantly from a peak of P_{ytvA}*::lux* before the cold to warm transition (T = 24h; Fig. 4) to occur at the midpoint of the warm phase of the temperature cycle as the T cycle became shorter (T = 20h; Fig. 4). This provides evidence for a robust circadian system in *B. subtilis* that interprets the zeitgeber cycle as an oscillator, rather than simply responding to changes in the environment as a switch.

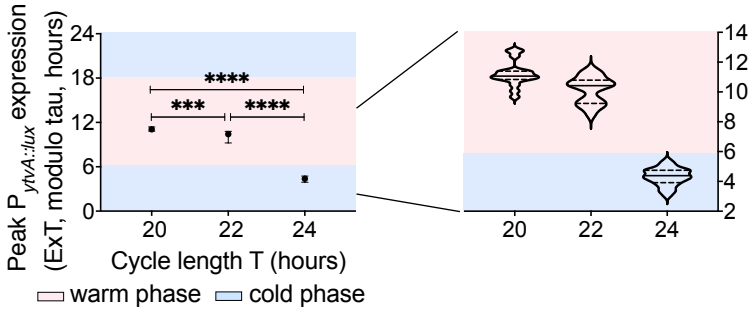


Figure 4. Phase angle of entrainment in T cycles. PytvA::lux was cultured under symmetrical temperature cycles (alternations between 25.5 °C (50% of cycle) and 28.5 °C (50% of cycle)) using different cycle lengths (T) (T20 (a 20h zeitgeber cycle): N = 17 , T22 (a 22h zeitgeber cycle): N = 16, T24 (a 24h zeitgeber cycle): N = 26). The phase (peak of luciferase expression; expressed as External Time (ExT), where midnight is 0) shifted to a later phase with shorter temperature cycles. The blue shaded areas indicate the cold phase and the pink shaded areas indicate the warm phase. The graph on the left shows median period with the interquartile range. The graph on the right is a violin plot of the same data. Phases observed in the different T cycles were compared using ordinary one-way ANOVA. All comparisons were significantly different from each other (**** P <0.0001, *** P =0.0005).

Discussion

Circadian clocks remain largely unknown in the non-photosynthetic bacteria, despite bacteria representing about 15% of the living matter on Earth[29]. We have identified circadian rhythms in a non-photosynthetic bacterium. Our experiments using promoters from two PAS-domain containing genes revealed free-running circadian rhythms, systematic entrainment to zeitgeber cycles, and temperature compensation of the circadian period. We conclude that the free-living bacteria *B. subtilis* has a circadian clock. Why have circadian clocks remained elusive in the bacteria? Data from the purple photosynthetic bacterium *Rhodospirillum rubrum* suggest that rhythmic processes occur (*e.g.* enzymatic activity[30]), but these rhythms have not yet been shown to function as a circadian clock. The purple bacteria *Rhodobacter sphaeroides*, which possesses KaiB and KaiC orthologs, can also show rhythmic behavior depending on environmental conditions. However, neither of these systems have been tested systematically for the hallmarks of circadian regulation. Recently, *Klebsiella aerogenes* has been shown to possess temperature-entrainable gene expression that can show *circa* 24h rhythms on release to constant conditions[31]. In this isolate from the gut microbiome of a patient, rhythms generally occur only in the presence of melatonin, suggesting that these bacteria might not generate free-running rhythms independently from host cues. 24h light cycles modified mediators of pattern formation in *Pseudomonas aeruginosa*, but no circadian rhythms were observed[32].

Our experiments indicate that robust or detectable circadian rhythms depend upon environmental characteristics such as nutrient supply and ambient temperature. Furthermore, in our conditions, only cultures that form biofilms will show circadian rhythms. This is an interesting observation because biofilms represent a distinct developmental state relative to planktonic cultures. Many microbes will produce biofilms under certain conditions and they have been associated with pathology. Effectively, biofilms arise when a microbial community shifts programs and produces a sticky matrix, thus creating a mechanism to form a differentiated population. This conditionality of the rhythms might be important for adaptive functions of the clock in bacteria, and perhaps the life history of *B. subtilis*. Conditionality of circadian regulation is common. For example, constant light conditions suppress the circadian clock[33] in almost every case other than photosynthetic organisms, whilst in plants many rhythms cease or change their period in constant darkness[34,35]. In *Drosophila*, a proportion of insects are arrhythmic, with this number depending on the strain[36]. Furthermore, in the model fungus *N. crassa,* rhythms in non-mutant wild type strains are highly dependent on media composition[37]. Together, this indicates that the conditionality of circadian rhythms due to

genotype, metabolism and environmental conditions is common across life. The wealth of information concerning the environmental regulation of metabolism in the Eubacteria make these organisms an excellent system in which to understand the functions of conditional rhythmicity.

Whilst the rhythms that we report might be regulated by a transcription-translation feedback system, there remain other possibilities. For instance, we cannot exclude the possibility that the rhythms are linked to metabolic cycles because this has been shown in a variety of organisms *(e.g. [20])*. It has been speculated that the ultradian rhythms in yeast that are tied to metabolic state (and also broadly integrated with transcriptional regulation) might be related to circadian clocks[38]. It is also possible that the presence of rhythms only in population harboring biofilms could indicate some role for the biofilm matrix in maintaining the robustness of the rhythm. It will be informative to investigate whether temperature and light are inputs to one master pacemaker, or whether *B. subtilis* might possess multiple oscillators, as described for a variety of unicellular and multicellular organisms[39,40]. It is also possible that *B. subtilis* might possess either a master oscillator and one or more downstream oscillators that are coupled to and entrained by a main pacemaker[41].

We suggest that the incorporation of temporal structures into industrial, biomedical and agricultural applications for bacteria might provide important translational opportunities. Our discovery of circadian rhythms in the Eubacteria should motivate future insights into the mechanisms and evolution of circadian rhythms across life.

Materials and Methods

Candidate gene selection

PAS-domain containing genes were identified using the Simple Modular Architecture Research Tool (SMART), a database containing annotated proteins and their domain architectures. Sixteen *Bacillus subtilis* genes encoding a PAS-domain were retrieved. From initially, 7 genes (ytvA, kinC, ydfH, resE, phoR, yycG, ykoW) promoter-luciferase reporter constructs were made. Five of these are histidine kinases, a type of enzyme known be involved in the circadian clocks of *i.e. Ceanobacteria*, YtvA is a blue-light photoreceptor, showing similarities with the WCC-complex in *N.crassa*. After an initial screen, using a variety of circadian culturing conditions, ytvA and KinC were selected based on their gene expression profiles as response to the different zeitgabers.

Strains and strain construction

All *B. subtilis* strains used in this study derived from stock 168 (Jena), a domesticated but biofilm-proficient isolate[42]. The promoter regions of *ytvA* and *kinC* genes were amplified using oligos ytvA_SacI_FW (5'- AGATCTGAGCTCCTTCATCATCACCTTCCTAAAG-3') – ytvA_SalI_REV (5'- CTCGAGGTCGACTTAGGCCGTCAGCTTGCTATG-3') and kinC_SacI_FW (5'- AGATCTGAGCTCTTTGTTTAATGACTGGAGAAATC-3') – kinC_SalI_REV (5'- CTCGAGGTCGACTGCCGCTTGTGTTTCTCTAC-3'), respectively. PCR products were digested with *Sac*I and *Sal*I enzymes (Thermo Scientific) and cloned into the corresponding sites of pAH321 harboring the promoter-less *luxABCDE* genes[43]. The vectors were verified by sequencing the cloned fragment and were subsequently transformed into *B. subtilis* 168 using natural competence[44]. Integration of the reporter cassettes into *amyE* locus was verified by the lack of amylase activity on 1% (w/v) starch containing Lysogeny Broth (LB) plates[45] and the presence of luminescence in the transformed strains.

Growth conditions

B. subtilis that had not been previously exposed to entrainment conditions was inoculated for overnight culture in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl). Strains were subsequently grown as on a variety of media, as described in the Results. Nutrient sporulation medium (NSMP) [46] (8 g l^{-1} Nutrient Broth (Difco), 1 µM $FeCl_3$, 700 µM $CaCl_2$, 50 µM $MnCl_2$, 1 mM $MgCl_2$, and 100 mM potassium phosphate) was used without carbon source or supplemented either with 2.56% (v/v) glycerol or 0.1% (w/v) glucose (Fig. 1, Fig. S1, Fig. 2, Fig. S2, Fig. S3, Fig. 3, Fig. 4, Fig. S5). The following media were used in Fig. S4: modified MSgg medium (5 mM potassium phosphate, 100 mM MOPS, 2 mM $MgCl_2$, 700 µM $CaCl_2$, 50

μM $MnCl_2$, 100 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine HCl, 2.56% (v/v) glycerol, 0.5% (w/v) monosodium glutamate and 50 μM L-tryptophan); LB supplemented with 1 mM $MnCl_2$; 2xSG medium (16 g l^{-1} Nutrient Broth (Difco), 2 g l^{-1} KCl, 0.5 g l^{-1}·$7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$·$4H_2O$, and 1 μM $FeSO_{4)}$ either with or without 0.1% (w/v) glucose; 10% (v/v) NSMP supplemented with 5 g l^{-1} NaCl; chemically defined medium (CDM35) as described in Ponomarova *et al.*, 2017[47]. The NSMP, MSgg and CDM35 media were made fresh from stock solutions on the day of the experiment, and the stock solution for iron was freshly prepared every two weeks.

For all luminometry experiments, white 96-well plates (Nunclon Delta, Thermo Scientific) were used, with each well inoculated with approximately 5 x 10^5 cells. Plates were sealed with a transparent, evaporation-free cover (Optical Adhesive Covers, Applied Biosystems®, Life Technologies™). For experiments with temperature entrainment, cultures were exposed to temperature cycles for 5 days, after which the cultures were released to conditions corresponding to the cooler temperature. We measured bioluminescence (Berthold Centro LB960 XS^3) for 1 second every 10-minutes. All experiments were carried out in temperature-controlled incubators (Panasonic MIR-154). For experiments with blue light entrainment, cultures were grown in NSMP medium without or with 0.1% (w/v) glucose and were exposed to a 12h darkness / 12h blue light cycle for 5 days, followed by release into constant darkness. The temperature was kept constant at 25.5 °C during these experiments. Bioluminescence was measured for 1 second each hour. The plate was ejected from the machine between readings, for exposure to blue light (LEDs with peak emission at 450 nm; Barthelme, Nürnberg, Germany) at a photon flux density of 35 μE m^{-2} s^{-1}.

Cell growth under entrained and free-running conditions

The presence of a biofilm was assessed qualitatively, by eye, as a pellicle forming at the air-liquid interface in the well. In order to start to understand the state of our rhythmic, biofilm forming cultures, we determined cell number from day 4 (one day before the end of the entraining cycle) and into day 7 (the second day in constant conditions). Cultures grown in 96-well plates (as for luminometry experiment, Fig. 2.) were exposed to a temperature cycle (12h 25.5 °C / 12 h 28.5 °C) for 5 days, after which the cultures were released to constant temperature of 25.5 °C. Cells were harvested every 12h, starting 30h before release to constant conditions until 42 hours after release. Samples were sonicated mildly (Diagenode Bioruptor, USA) at low power (130 watt) for 12 seconds, for 2 cycles, with a 5 second pause between cycles according to a protocol modified from Dragoš *et al*.[48]. Sonicated cells were examined by light microscopy (Leica, Germany) to confirm disruption of biofilm and cell viability. The sonicated cells were plated on LB agar and grown overnight at 37 °C. The number of colony-forming units was counted on the following day. Figure S8 shows that the cell growth was stable before release to constant conditions, whereupon it increased approximately 3-fold. In the 42h of constant conditions, the cell number gradually decreased about 50%.

Data analysis

Graphing

Bioluminescence traces were baseline detrended using the open-access web tool BioDare2 (https://biodare2.ed.ac.uk)[49] and values were normalised between 0% and 100%. GraphPad Prism 8.1 (GraphPad Software, La Jolla, CA) was used to plot all graphs.

Calculation of free-running period using non-linear modeling

For the analysis of the free-running period using continuous luminometry measurements, the period was calculated by analysis in the R programming language[50]. To describe and parameterize the data, a non-linear model was constructed which performs a decay trend correction and fits a cosine-based function to the signal by using a Nonlinear Least Squares (nls) method[51,52]. The model assumes an exponentially decaying baseline signal as well as an exponentially decaying oscillating (cosine) signal:

$$f(t) = a_0 \cdot e^{-k_0 \cdot t} + a_1 \cdot e^{-k_1 \cdot t} \cdot \cos\left(\frac{2\pi \cdot (t-\theta)}{T}\right)$$

with t = time (in h) from the start of the experiment. Here, a_0 is the amplitude (maximum) of the baseline signal and k_0 the decay rate of the baseline signal (in h^{-1}). The shape of the baseline is consistent with *e.g.* a first order decay of the *B. subtilis* population during the experiment, or a depletion of an essential nutrient. Furthermore, a_1 is the amplitude of the oscillation, k_1 is the decay rate (in h^{-1}) of the oscillation, T the period of the oscillation (in h) and θ the phase of the signal (in h) at the start of the experiment. The advantage of such a physical-biological model is that all model parameters have a correspondent biological reference. Under most experimental conditions, the decay rates are positive and the period is about 24 h. Some data was detrended (baseline detrending) using BioDare2[53] prior to this calculation of free-running period.

The nonlinear least squares (nls) method in R requires sufficiently well-chosen starting values of all six model parameters a_0, k_0, a_1, k_1, T and θ. For most experiments, the oscillatory part of the signal is much weaker than the baseline signal, hence $a_0 \gg a_1$. Therefore, the amplitude a_0 was set at the maximum value of the raw signal $y(t)$, so that $\hat{a}_0 = \max(y)$. Assuming that $a_1 \ll a_0$, the raw signal is approximately an exponentially decaying signal, $y(t) \approx a_0 \cdot \exp(-k_0 \cdot t)$. Hence, $\ln(y/\hat{a}_0) \approx -k_0 \cdot t$, therefore the decay rate k_0 can be estimated as the negative of the slope of $\ln(y/\hat{a}_0)$ for t (via linear regression without an intercept using the R function lm [52]). In order to have a crude estimate of the remaining parameters a_1, k_1, T and θ, we calculated a baseline-corrected signal $y_{\mathrm{corr}}(t) = y(t) - \hat{a}_0 \cdot \exp(-\hat{k}_0 \cdot t)$ that shows

damped oscillations around zero. The oscillation amplitude was estimated as the maximum of the absolute value of y_{corr} within the first 24 hours, therefore $\hat{a}_1 = \max(|y_{\mathrm{corr}}|,\ t < 24)$. The phase-shift θ was estimated as the time at which the maximum value of y_{corr} within the first 24 hours occurs, therefore $\hat{\theta} = \max_t(y_{\mathrm{corr}},\ t < 24)$. The oscillation period T was estimated initially as the difference in time between the maximum value of y within the first 24 hours and the maximum value of y within the second 24 hours such that $\hat{T} = \Delta(\max_t(y,\ t < 24), \max_t(y,\ t > 24)$. Finally, since $\ln(|y_{\mathrm{corr}}/\hat{a}_1|) = -k_1 \cdot t + |\cos(2\pi(t-\theta)/T)| \approx -k_1 \cdot t$, the decay rate of the oscillating signal $\hat{k}_1$ was roughly estimated as the negative of the slope of $\ln(|y_{\mathrm{corr}}/\hat{a}_1|)$ versus t (linear regression without intercept using the R function lm). Applying the nls function on the full nonlinear model using the set of starting values resulted in a set of least squares estimates of the parameters a_0, k_0, a_1, k_1, T and θ, as well as standard errors and p-values for each parameter.

As a measure of "goodness of fit", Akaike's An Information Criterion (AIC)[54] was used, by subtracting the baseline AIC from the final model AIC. Bonferroni multiple testing correction was applied on calculated p-values.

Phase angle determination of the T-cycle series

Once stable entrainment was observed, three entraining cycles were used for analysis of entrained phase. Each individual signal was trend corrected by subtraction of a second order polynomial fit of the raw data (Fig. S9A, B). As the masking peak is the most dominant feature in the signal, a fitted curve would mainly be a reflection of the zeitgeber. Therefore, in order to find the circadian component in the overall signal we applied a fitting procedure, a combination of a sine ($\sin(2\pi \cdot \frac{t}{24})$) and cosine ($\cos(2\pi \cdot \frac{t}{24})$) using the least square error method (Python numpy.linalg.lstsq) excluding data from the warm phase that shows extreme masking (Fig. S9C) from the fitting process (Fig. S9D). The resulting sine curve is a thus a representation of the underlying circadian component (Fig. S9E).

Literature references

1. Woelfle, M. A., Ouyang, Y., Phanvijhitsiri, K. & Johnson, C. H. The adaptive value of circadian clocks: An experimental assessment in cyanobacteria. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.08.023.
2. Dodd, A. N. *et al.* Plant Circadian Clocks Increase Photosynthesis , Growth , Survival. *Science (80-.).* 630, 630–3 (2007).
3. Norman, T. M., Lord, N. D., Paulsson, J. & Losick, R. Memory and modularity in cell-fate decision making. *Nature* 503, 481–486 (2013).
4. Bischofs, I. B., Hug, J. A., Liu, A. W., Wolf, D. M. & Arkin, A. P. Complexity in bacterial cell-cell communication: Quorum signal integration and subpopulation signaling in the Bacillus subtilis phosphorelay. *Proc. Natl. Acad. Sci.* 106, 6459–6464 (2009).
5. Kuchina, A. *et al.* Temporal competition between differentiation programs determines cell fate choice. *Mol. Syst. Biol.* 7, 557 (2011).
6. Van Der Steen, J. B. & Hellingwerf, K. J. Activation of the General Stress Response of Bacillus subtilis by Visible Light. in *Photochemistry and Photobiology* vol. 91 1032–1045 (2015).
7. Millar, A. J., Carré, I. A., Strayer, C. A., Chua, N. H. & Kay, S. A. Circadian clock mutants in Arabidopsis identified by luciferase imaging. *Science (80-.).* (1995) doi:10.1126/science.7855595.
8. Ponting, C. P. & Aravind, L. PAS: a multifunctional domain family comes to light. *Current biology : CB* (1997) doi:10.1016/s0960-9822(06)00352-6.
9. Ávila-Pérez, M., Hellingwerf, K. J. & Kort, R. Blue light activates the σB-dependent stress response of Bacillus subtilis via YtvA. *J. Bacteriol.* (2006) doi:10.1128/JB.00716-06.
10. Kay, S. A. PAS, present, and future: Clues to the origins of circadian clocks. *Science* (1997) doi:10.1126/science.276.5313.753.
11. Devlin, P. F. Signs of the time: environmental input to the circadian clock. *J. Exp. Bot.* (2002) doi:10.1093/jxb/erf024.
12. Mrosovsky, N. Masking: History, definitions, and measurement. *Chronobiology International* (1999) doi:10.3109/07420529908998717.
13. Lopez, D., Vlamakis, H. & Kolter, R. Generation of multiple cell types in Bacillus subtilis. *FEMS Microbiology Reviews* vol. 33 152–163 (2009).
14. Merrow, M. W. & Dunlap, J. C. Intergeneric complementation of a circadian rhythmicity defect: Phylogenetic conservation of structure and function of the clock gene frequency. *EMBO J.* 13, (1994).

15. Liu, Y., Merrow, M., Loros, J. J. & Dunlap, J. C. How temperature changes reset a circadian oscillator. *Science (80-.).* (1998) doi:10.1126/science.281.5378.825.
16. Greene, A. V., Keller, N., Haas, H. & Bell-Pedersen, D. A circadian oscillator in Aspergillus spp. regulates daily development and gene expression. *Eukaryot. Cell* (2003) doi:10.1128/EC.2.2.231-237.2003.
17. Liu, Y. *et al.* Circadian orchestration of gene expression in cyanobacteria. *Genes Dev.* 9, 1469–1478 (1995).
18. Zhu, B. & Stülke, J. SubtiWiki in 2018: From genes and proteins to functional network annotation of the model organism Bacillus subtilis. *Nucleic Acids Res.* (2018) doi:10.1093/nar/gkx908.
19. Loros, J. J., Feldman, J. F. & Loros, J. J. Loss of Temperature Compensation of Circadian Period Length in the frq-9 Mutant of Neurospora crassa. *J. Biol. Rhythms* (1986) doi:10.1177/074873048600100302.
20. Haydon, M. J., Mielczarek, O., Robertson, F. C., Hubbard, K. E. & Webb, A. A. R. Photosynthetic entrainment of the Arabidopsis thaliana circadian clock. *Nature* 502, 689–692 (2013).
21. Sonenshein, A. L. Control of key metabolic intersections in Bacillus subtilis. *Nature Reviews Microbiology* (2007) doi:10.1038/nrmicro1772.
22. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2043–2047 (2010).
23. Roenneberg, T. & Merrow, M. The circadian clock and human health. *Current Biology* (2016) doi:10.1016/j.cub.2016.04.011.
24. Pittendrigh, C. S. & Daan, S. A functional analysis of circadian pacemakers in nocturnal rodents - IV. Entrainment: Pacemaker as clock. *J. Comp. Physiol.* ■ *A* (1976) doi:10.1007/BF01417859.
25. Stothard, E. R. *et al.* Circadian Entrainment to the Natural Light-Dark Cycle across Seasons and the Weekend. *Curr. Biol.* (2017) doi:10.1016/j.cub.2016.12.041.
26. Bruce, V. Environmental entrainment of circadian rhythms. in *Cold Spring Harbor Symp. Quant. Biol.* 29–48 (1960).
27. Hoffmann, K. Zur beziehung zwischen phasenlage und spontanfrequenz bei der endogenen Tagesperiodik. *Zeitschrift fur Naturforsch. - Sect. B J. Chem. Sci.* (1963) doi:10.1515/znb-1963-0211.
28. Merrow, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurospora clock gene frequency. *Nature* 399, 584–586 (1999).
29. Bar-On, Y. M., Phillips, R. & Milo, R. The biomass distribution on Earth. *Proc. Natl. Acad. Sci.* 115, 6506–6511 (2018).

30. Van Praag, E., Agosti, R. D. & Bachofen, R. Rhythmic activity of uptake hydrogenase in the prokaryote Rhodospirillum rubrum. *J. Biol. Rhythms* (2000) doi:10.1177/074873040001500303.
31. Paulose, J. K., Cassone, C. V., Graniczkowska, K. B. & Cassone, V. M. Entrainment of the Circadian Clock of the Enteric Bacterium Klebsiella aerogenes by Temperature Cycles. *iScience* (2019) doi:10.1016/j.isci.2019.09.007.
32. Kahl, L. J., Price-Whelan, A. & Dietrich, L. E. P. Light-mediated decreases in cyclic di-GMP levels inhibit structure formation in Pseudomonas aeruginosa biofilms . *J. Bacteriol.* (2020) doi:10.1128/jb.00117-20.
33. Abraham, D. *et al.* Restoration of Circadian Rhythmicity in Circadian Clock – Deficient Mice in Constant Light. *J. Biol. Rhythms* 21, 169–176 (2006).
34. Millar, A. J., Straume, M., Chory, J., Chua, N. H. & Kay, S. A. Phytochrome and blue responsive photoreceptors regulate circadian period in Arabidopsis thaliana. *Science (80-.).* (1995) doi:10.1126/science.7855596.
35. Wang, Z. Y. & Tobin, E. M. Constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93, 1207–1217 (1998).
36. Beauchamp, M. *et al.* Closely Related Fruit Fly Species Living at Different Latitudes Diverge in Their Circadian Clock Anatomy and Rhythmic Behavior. *J. Biol. Rhythms* (2018) doi:10.1177/0748730418798096.
37. Kim, T.-S., Logsdon, B. A., Park, S., Mezey, J. G. & Lee, K. Quantitative trait loci for the circadian clock in Neurospora crassa. *Genetics* 177, 2335–2347 (2007).
38. Causton, H. C., Feeney, K. A., Ziegler, C. A. & O'Neill, J. S. Metabolic cycles in yeast share features conserved among circadian rhythms. *Curr. Biol.* 25, (2015).
39. Bell-Pedersen, D. *et al.* Circadian rhythms from multiple oscillators: Lessons from diverse organisms. *Nature Reviews Genetics* (2005) doi:10.1038/nrg1633.
40. Bell-Pedersen, D., Crosthwaite, S. K., Lakin-Thomas, P. L., Merrow, M. & Økland, M. The Neurospora circadian clock: Simple or complex? in *Philosophical Transactions of the Royal Society B: Biological Sciences* (2001). doi:10.1098/rstb.2001.0968.
41. Pittendrigh, C., Bruce, V. & Kaus, P. ON THE SIGNIFICANCE OF TRANSIENTS IN DAILY RHYTHMS. *Proc. Natl. Acad. Sci.* (1958) doi:10.1073/pnas.44.9.965.
42. Gallegos-Monterrosa, R., Mhatre, E. & Kovács, Á. T. Specific Bacillus subtilis 168 variants form biofilms on nutrient-rich medium. *Microbiol. (United Kingdom)* 162, 1922–1932 (2016).
43. Schmalisch, M. *et al.* Small genes under sporulation control in the Bacillus subtilis genome. *J. Bacteriol.* 192, 5402–5412 (2010).

44. Kunst, F. & Rapoport, G. Salt stress is an environmental signal affecting degradative enzyme synthesis in Bacillus subtilis. *J. Bacteriol.* (1995) doi:10.1128/jb.177.9.2403-2407.1995.
45. Harwood, C. R. & Cutting, S. M. *Molecular biological methods for Bacillus*. (Wiley & Sons Ltd., 1990).
46. Fortnagel, P. & Freese, E. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. *J. Bacteriol.* (1968) doi:10.1128/jb.95.4.1431-1438.1968.
47. Ponomarova, O. *et al.* Yeast Creates a Niche for Symbiotic Lactic Acid Bacteria through Nitrogen Overflow. *Cell Syst.* (2017) doi:10.1016/j.cels.2017.09.002.
48. Dragoš, A. *et al.* Division of Labor during Biofilm Matrix Production. *Curr. Biol.* 28, 1903-1913.e5 (2018).
49. Moore, A., Zielinski, T. & Millar, A. J. Online period estimation and determination of rhythmicity in circadian data, using the BioDare data infrastructure. *Methods Mol. Biol.* (2014) doi:10.1007/978-1-4939-0700-7_2.
50. core Team, R. R: A Language and Environment for Statistical Computing. *R Found. Stat. Comput. Vienna, Austria* (2018).
51. Bates, D. M. & Watts, D. G. *Nonlinear Regression Analysis and Its Applications*. (Wiley, 1988).
52. Bates, D. M. & Chambers, J. M. Nonlinear models. in *Statistical Models in S* (eds. Chambers, J. M. & Hastie, T. J.) (Wadsworth & Brooks/Cole, 1992).
53. Zielinski, T., Moore, A. M., Troup, E., Halliday, K. J. & Millar, A. J. Strengths and limitations of period estimation methods for circadian data. *PLoS One* (2014) doi:10.1371/journal.pone.0096462.
54. Sakamoto, Y., Ishiguro, M. & G., K. *Akaike Information Criterion Statistics*. (D. Reidel Publishing Company, 1986).

Acknowledgements

The authors thank Prof. Ben Aronson for critical discussions on this work, and Prof. Oscar P. Kuipers for comments and support during the early stages of the project. Work in the lab of MM is supported by the Volkswagen Foundation (Life? Funding Program: “The Fourth Dimension”) and the Friedrich Bauer Stiftung and the Verein zur Förderung von Wissenschaft und Forschung of the LMU Munich. AD is grateful to UK BBSRC for funding (Institute Strategic Programme GEN BB/P013511/1), ATK was supported by Danish National Research Foundation (DNRF137) for the Center for Microbial Secondary Metabolites. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Competing Interests

The authors declare that they have no competing interests.

Contributions

MM and JB initiated the project. All authors (MM, JB, AD, ZE-C, FS, AK) discussed experimental protocols. ZE-C, FS, JB, and AK performed experiments. ZE-C, FS and JB analysed data. All authors (MM, JB, AD, ZE-C, FS, AK) interpreted the data and wrote the paper.

Data and Materials Availability

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Link to all raw data: Eelderink-Chen, Bosman et al., 2020, raw data

Supporting information materials

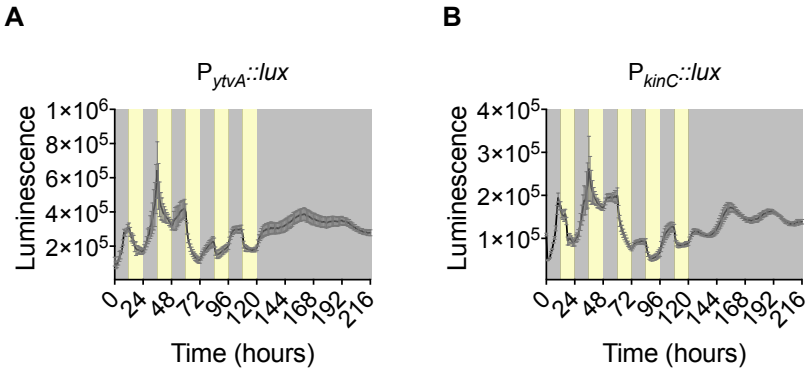


Figure S1. Raw bioluminescence traces. Unprocessed bioluminescence traces of PytvA::lux (N = 16) (A) and PkinC::lux (N = 7) (B) over 5 days of 24h blue light entrainment cycles (12h D / 12h L; represented as grey and yellow, respectively) and in constant darkness in NSMP medium containing glucose. The traces correspond to data shown in Fig. 1. The units simply correspond to the signal as measured by the luminometer (see Methods).

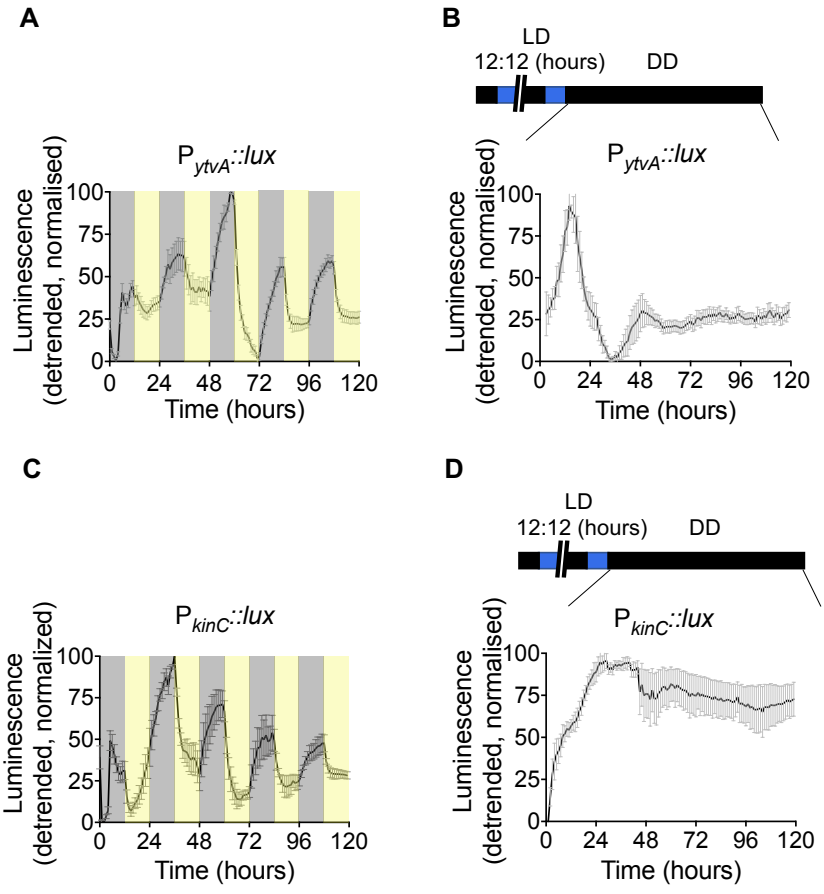


Figure S2. Blue light-mediated entrainment of free-running circadian rhythms by *B. subtilis* depends upon nutrient conditions. Bioluminescence traces of PytvA::lux in 5 days of 24h blue light entrainment cycles (12h L /12h D; A) and the following 5 days in constant darkness (B) (N = 9). C, D: Bioluminescence traces of PkinC::lux under equivalent entrainment and release conditions (N = 8). Bacteria were grown in NSMP medium lacking glucose at a constant temperature of 25.5 °C. The detrended data are shown as mean ± standard deviation. See also Suppl. Table 1.

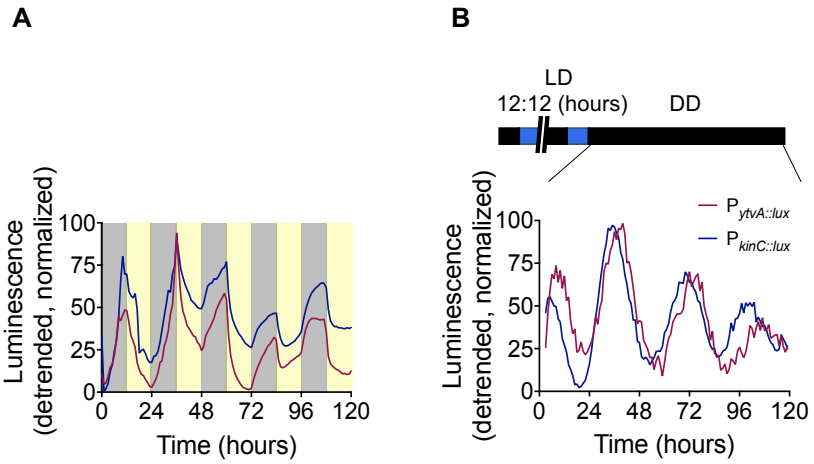


Figure S3. Temporal expression via ytvA and kinC promoters is similar. Bioluminescence traces of PytvA::lux (N = 16) and PkinC::lux (N = 7) during 5 days of 24h blue light entrainment cycles (12h D / 12h L; represented as grey and yellow, respectively) (A) and following release to constant conditions (B) in NSMP medium containing glucose. The traces correspond to data shown in Fig. 1. The de-trended data are presented as a mean value of rhythmic wells.

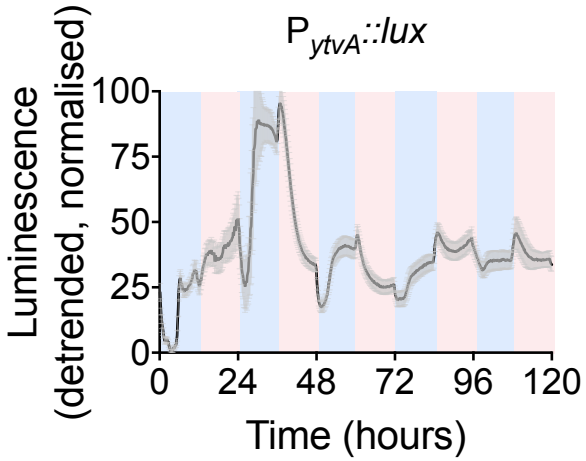


Figure S4. Entrainment of *B. subtilis* in temperature cycles. Bioluminescence of PytvA::lux during 5 days of entrainment to temperature cycles (12h at 25.5 °C / 12h at 28.5 °C) in NSMP medium. The de-trended data are presented as mean ± standard deviation (N = 20). The blue shaded areas indicate the cold phase and the pink shaded areas indicate the warm phase.

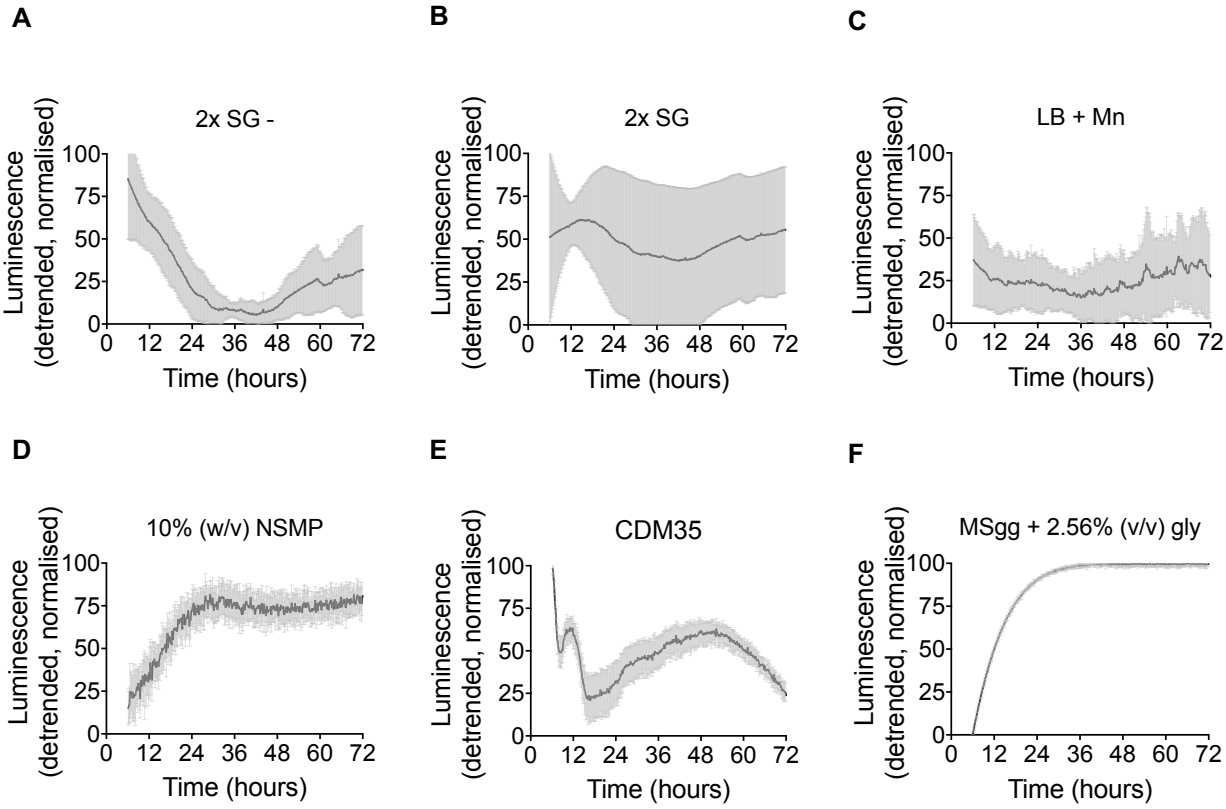


Figure S5. Bioluminescence recording of PytvA::lux in different media. Bioluminescence of PytvA::lux under constant conditions at 25.5 °C following 5 days of entrainment in temperature cycles (12h at 25.5 °C / 12h at 28.5 °C). Cultures were grown in 2xSG - glucose (N = 6) (A), 2x SG + glucose (N = 10) (B), LB + Mn (N = 12) (C), 10% NSMP (N = 16) (D), CDM35 (N = 10) (E), or MSgg + 2.56% glycerol (N = 29) (F). The de-trended data are shown as mean ± standard deviation. See also Suppl. Table 1.

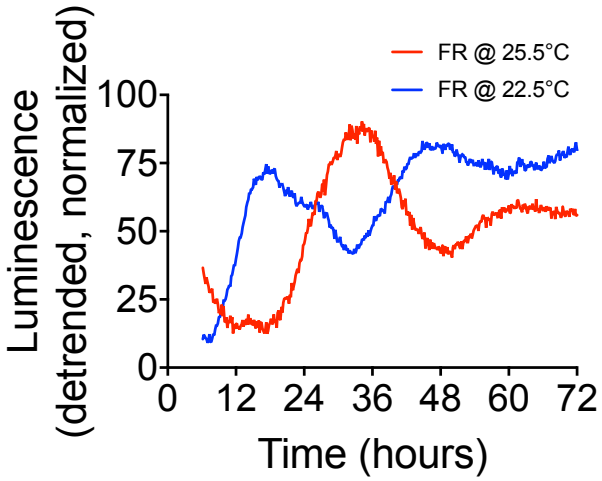


Figure S6. Free-running rhythms in *B. subtilis* following entrainment to antiphase temperature cycles. Bioluminescence of PytvA::lux under constant conditions (22.5 °C, N = 25, Blue line; 25.5 °C, N = 12, Red line) following 5 days of entrainment with antiphased temperature cycles (Blue line: 12h at 22.5 °C / 12h at 25.5 °C; Red line: 12h at 25.5 °C / 12h at 22.5 °C) is shown. The de-trended data are plotted as the mean. See also Suppl. Table 1.

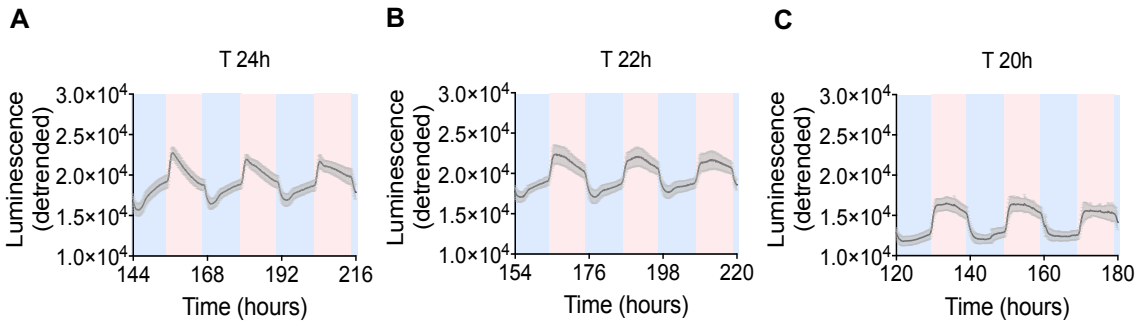


Figure S8. Cell growth in cultures with biofilm during temperature entrainment and in free run. Cultures were exposed to temperature cycles (12 h 25.5 °C / 12 h 28.5 °C) for 5 days, after which the cultures were released to constant temperature of 25.5 °C. The graph shows the cell numbers (quantified in cells per milliliter) at the end of the entrainment period and for two days after release into constant conditions. Data represent averages of results from 3 biological replicates ± standard deviation (SD). The blue shaded areas indicate the cold phase and the pink shaded areas indicate the warm phase.

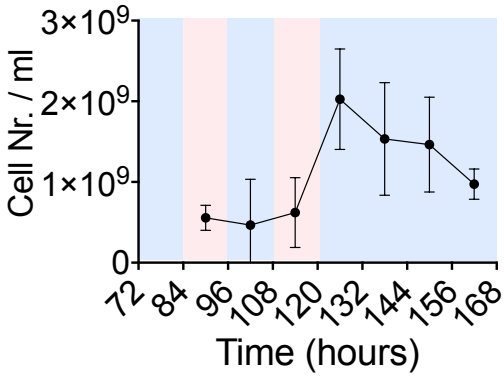


Figure S7. Bioluminescence traces of PytvA::lux in T cycles. PytvA::lux was cultured in temperature cycles (25.5 °C / 28.5 °C) of different cycle lengths (T). The de-trended bioluminescence data are presented as mean ± standard deviation (T24: N = 26, T22: N = 16, T20: N = 17). The blue shaded areas indicate the cold phase and the pink shaded areas indicate the warm phase.

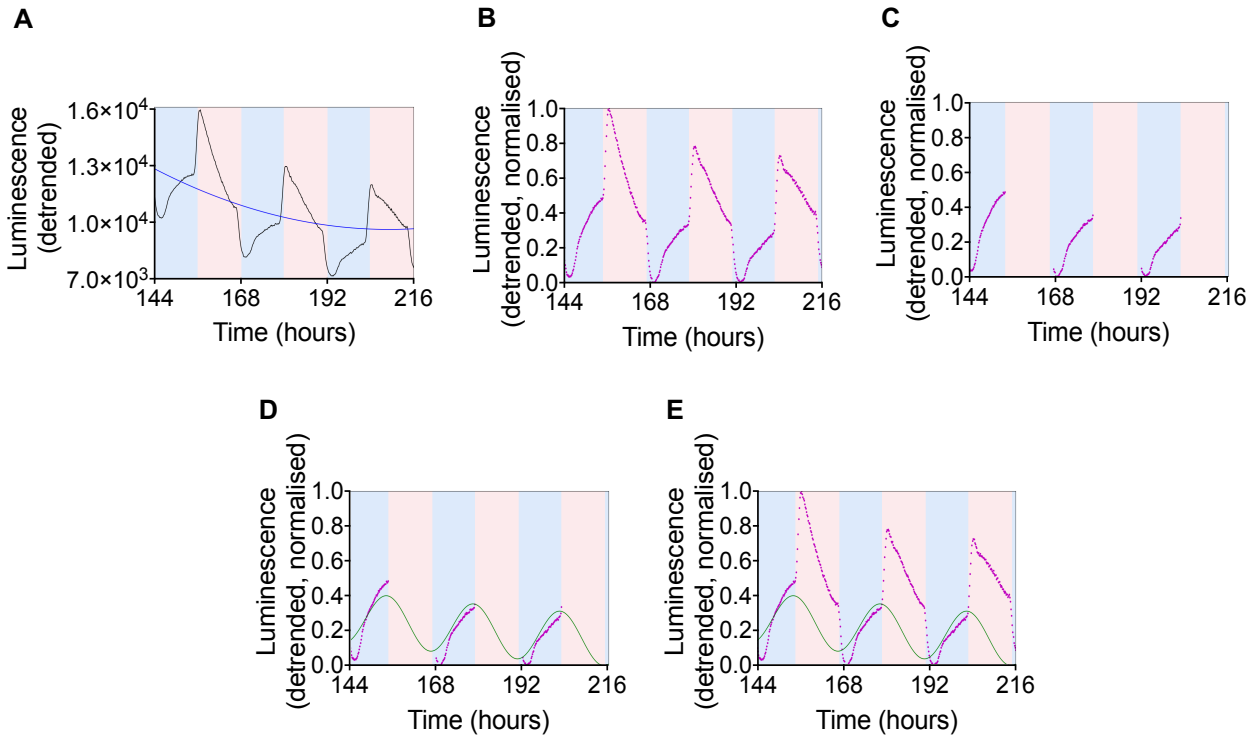
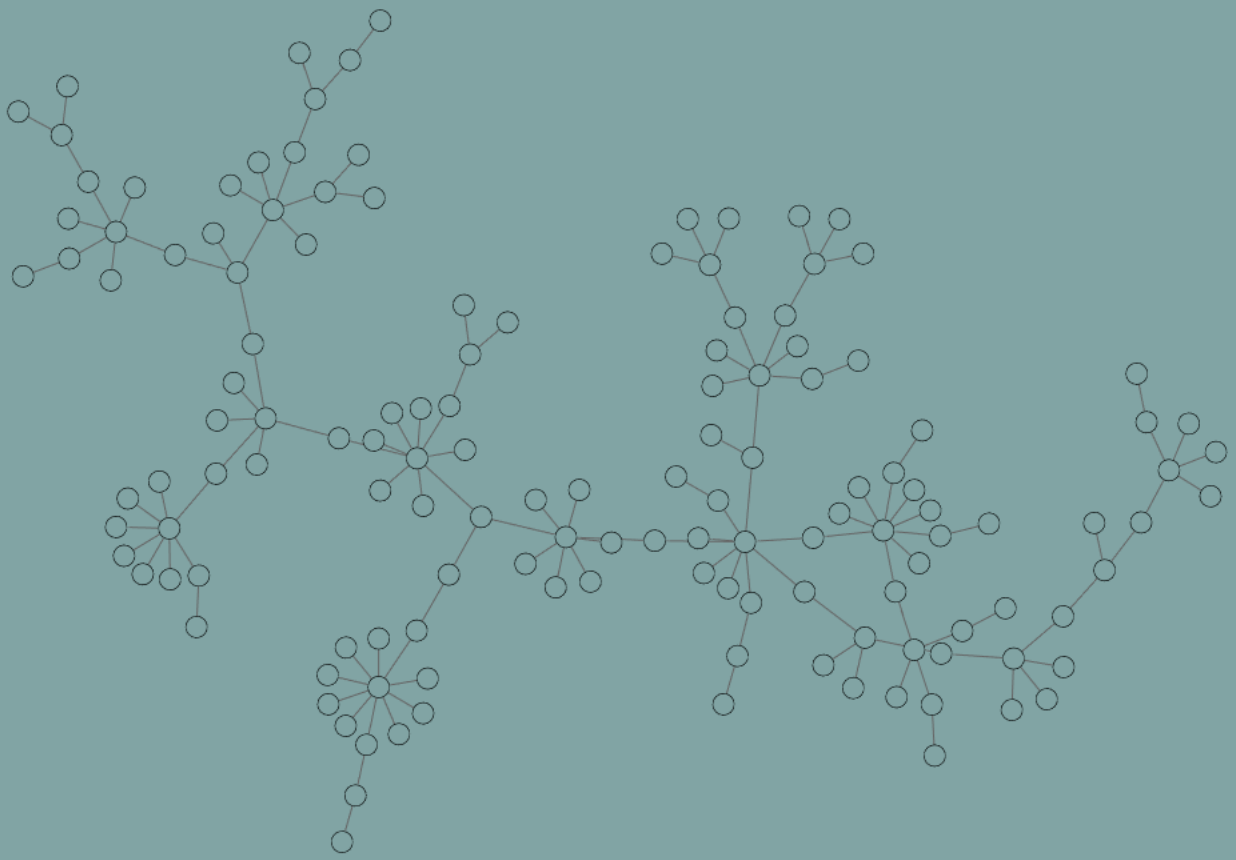


Figure S9. De-masking T cycle data. PytvA::lux was cultured under symmetrical temperature cycles using different cycle lengths (T). The blue shaded areas indicate the cold phase (25.5 °C) and the pink shaded areas indicate the warm phase (28.5 °C). Signals (Black solid line) were detrended (black solid line) using a 2nd order polynomial (blue solid line) (A). The resulting trend-corrected signal was rescaled between 0 and 1 (pink dotted line) (B). In order to avoid the pronounced effect of masking at ZT transition on phase estimation, only the data collected during the subjective cold phase were used for sine wave fitting (C). The best fitting sinewave (green solid line) was estimated using least squares regression (D) and imposed on the signal (E).

Table S1. Summary of entrainment conditions and sample sizes. Number of samples (N Rhythmic = numbers of samples exhibiting circadian rhythmicity under free-running conditions, Total N = total number of samples per condition; 1 sample = 1 well of a 96-well plate), and period length.

Reporter strain	*Entrainment condition**	*Free-running condition*	*N Rhythmic*	*Total N*	*Mean period* ± SD*	*Period* median with interquartile range*	*Reference to figure in main text*
P_{ytva}::lux	Dark:light 12:12 h in NSMP w glucose	25.5°C, DD	16	16	28.66 ± 1.77	28.66 (27.37 – 30.25)	Fig. 1B, 1C, S1A, S3B
P_{kinC}::lux	Dark:light 12:12 h in NSMP w glucose	25.5°C, DD	7	8	30.85 ± 0.83	31.07 (29.75 – 31.38)	Fig. 1E, 1F, S1B, S3B
P_{ytva}::lux	Dark:light 12:12 h in NSMP w/o glucose	25.5°C, DD	-	9	-	-	Fig. S2B
P_{kinC}::lux	Dark:light 12:12 h in NSMP w/o glucose	25.5°C, DD	-	8	-	-	Fig. S2D
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in NSMP w/o glucose	25.5°C, DD	8	8	23.94 ± 1.64	23.79 (22.82 - 25.72)	Fig. 2A, 2B
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in NSMP w glycerol	25.5°C, DD	-	42	-	-	Fig. 2C
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in NSMP w glucose	25.5°C, DD	-	16	-	-	Fig. 2D
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in 2 x SG w/o glucose	25.5°C, DD	-	12	-	-	Fig. S5A
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in 2 x SG w glucose	25.5°C, DD	-	12	-	-	Fig. S5B
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in LB + Mn	25.5°C, DD	-	12	-	-	Fig. S5C
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in 10% (w/v) NSMP w/o glucose	25.5°C, DD	-	16	-	-	Fig. S5D
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in CDM35	25.5°C, DD	-	16	-	-	Fig. S5E
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in MSgg w 2.56% (v/v) glycerol	25.5°C, DD	-	32	-	-	Fig. S5F
P_{ytva}::lux	22.5°C:25:5°C 12:12 h in NSMP w/o glucose	22.5°C, DD	25	32	27.28 ± 5.41	28.92 (24.32 - 31.17)	Fig. 3A, 3D, 3E
P_{ytva}::lux	25.5°C:28:5°C 12:12 h in NSMP w/o glucose	25.5°C, DD	7	8	26.62 ± 1.44	26.40 (26.13 - 27.53)	Fig. 3B, 3D, 3E
P_{ytva}::lux	28.5°C:31:5°C 12:12 h in NSMP w/o glucose	28.5°C, DD	9	18	26.87 ± 2.48	27.36 (24.06 - 29.13)	Fig. 3C, 3D, 3E
P_{ytva}::lux	22.5°C:25:5°C 12:12 h in NSMP w/o glucose	22.5°C, DD	25	32	27.28 ± 5.41	28.92 (24.32 - 31.17)	Fig. S6
P_{ytva}::lux	22.5°C:25:5°C 12:12 h in NSMP w/o glucose	25.5°C, DD	12	16	31.99 ± 3.55	32.32 (31.57 - 33.23)	Fig. S6

§ All cultures were entrained under a darkness–blue light (LD) or temperature (°C) cycle for 5 days and then released for 5 days under constant darkness and temperature conditions.
* Period was calculated over a period of 48 h as described in Material and Methods.



Chapter 8

General discussion and perspectives

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Epilogue

We believe that all organisms, even single celled, free living microbes have circadian clocks – due to expected evolution, selection via fitness advantages. During this thesis I development novel approaches to identify molecular circadian mechanisms for *S. cerevisiae* and *B. subtilis*. We used yeast in chemostats in conjunction with dedicated clock protocols, bacillus and reporter genes with similar protocols and bioinformatic *in-silico* approaches to drive hypothesis driven research. This type of multi-level analysis is needed to capture the diversity of mechanisms controlled by the circadian clock. In this thesis I discuss this entire package.

Microbes are subjected to environmental signals which serve as zeitgebers for characteriased clock systems. Light and temperature are in this group. Exposing microbes to cycling conditions *i.e.* light/dark or warm/cold, with a period of 24 hours entrains its circadian clock. After a period of entrainment, synchronized populations of microbes are released to a constant condition allowing discovery of a freerunning circadian rhythm, during which the molecular mechanism of the circadian clock maintains the microbes its rhythm.

Circadian mechanism impacted and predicted genes show a cycling gene expression profile with a 24-hour period under constant conditions. Performing a whole genome gene-expression patterns analysis from microbes sampled during a freerun, should reveal components which are either part of or under control of the (transcriptional) circadian mechanism. However, the clock impacts numerous processes and functions of a cell and therefore many genes show this characteristic oscillating pattern in their expression. Reducing these candidate genes to the most essential components / functions and processes is one of the major difficulties faced in this thesis.

An easy method for candidate reduction is lowering the significance threshold (p-value). However, true positives can be missed. On the other hand, higher p-values may cause false positive inclusion. I've tried to find a solution to this problem by developing the PREMONition method, a strategy to construct a fully connected protein-protein-interaction network, based on a small set of highly significant candidate genes. Missing connections are reconstructed based on published data and computational data curated in the STRING protein interaction database. The reconstructed network only contains the most probable interacting proteins and can be analysed for function using gene ontology enrichment analysis. Providing insights into the most essential processes and functions of the clock mechanism.

Circadian PREMONition networks reveal conserved processes and functions, across a variety of organisms regulated by the circadian clock. PREMONition networks can be constructed for any organism of which genetic or protein (or other molecular) interactions are curated. Comparing significantly enriched properties from reconstructed molecular-interaction networks, based on genes that exhibit daily oscillation at the transcript level, allowed prediction of the most propable processes impaired by the circadian clock.

Combining information collected from different experiments and analysis performed throughout this thesis, with respect to *S. cerevisiae*, results in a hypothetical model. Using the tools developed and experimental insights gained during my research project, a set of recurring genes is identified. Here, I present a hypothetical model, based on gene function, involved processes and pathways as a reference for future research to circadian mechanisms in *S. cerevisiae*.

Chapter summaries

In **chapter 2**, we showed that the mRNA expression of ammonium permease *MEP2* and the amino acid permease *GAP1* is phase advanced relative to the pH oscillation in 1 L fermontor cultures. We hypothesise the pH oscillations as follows: The periodic transport of ammonium and amino acids into the cytoplasm would increase cytoplasmic pH. The plasma membrane H^+-ATPase, Pma1p, maintains intracellular pH by controlling extracellular proton flux leading to secretion of excess protons and creating the observed oscillations. The observed oscillations reveal a circadian timing mechanism in yeast and discovered circadian clock regulated rhythms in the gene expression levels of enzymes involved in key metabolic pathways.

In **chapter 3**, the PREMONition method was developed. This tool reconstructs fully connected Protein-Protein-Interactions (PPI) networks based on an input set of candidate genes (essentially all rhythmic genes in a given condition). To validate this new method, known, rhythmically expressed genes were used to reconstruct PPI-networks for *H. sapiens*, *D. melanogaster* and *N. crassa*. Next, a PREMONition network for *S. cerevisiae* was constructed, based on a micro-array dataset collected from a temperature entrained 1 L fermentor culture (chapter 2). Comparing these networks using gene ontologies and orthologs, I identified 13 genes showing cross-organism relationships. The 13 genes can be cathegorized in 3 processes. First, nitrogen compound transport (*CDC5*, *ACT1*, *RPD3*, *RPN11*, *HRR25*, *HOG1*, *EFB1*, *CDC19* and *TKL1*) a process known process regulated by the circadian clock in cyanobacterium *Synechococcus.* Second, vacuole organization (*YPT7*, *ACT1* and *HRR25*) of which *HRR25* encodes a Casein kinase I, a key player in the mammalian circadian clock. And third, proteins showing kinase activity (*CDC5*, *HRR25*, *HOG1*, *CDC19*) known to be of importance in molecular circadian clock mechanisms.

In **chapter 4**, using the PREMONition method, I constructed circadian Protein-Protein-Interaction networks from all organisms annotated in the Circadian Gene DataBase (CGDB) database. These networks were analysed for common biological properties according to enrichment for GO-terms (biological process, molecular function and cellular component), KEGG pathways and SMART protein domains. Two different approaches were taken to identify genes involved in molecular clock mechanism of *S. cerevisiae*. First, after determining the most common biological properties within the network all yeast genes annotated in the STRING database were mapped on these properties. Resulting in 294 genes, of which 22 show a circadian pattern on the transcript level. The second approach - mapping highly

significant rhythmically expressed genes - delivered 9 genes. Among the set of 29 unique genes identified in these two approaches are the serine/threonine protein-kinases *SAK1*, *KCC4*, *PSK1*, *FUS3* and *IME2* the transcription factors *ACA1*, *YAP7*, *ARR1*. More importantly, as the analysis of the *M. musculus* PPI-network showed, some biological processes that are highly conserved in circadian PPI-networks. These processes include nitrogen compound metabolic process, oxidation-reduction process, glycolysis/ gluconeogenesis and fatty acid metabolism. These processes are known to be involved in the circadian mechanisms in different organisms including cyanobacteria, fungus and mammals[1–10].

In **chapter 5**, I applied the PREMONition method to a non-circadian network problem, namely regulation of gene expression in yeast be light. I characterised the physical and underlying molecular processes involved in the growth response of yeast to light. First, DNA microarrays were used to identify genes that are either up- or down- regulated (differentially expressed), when exposed to blue light. In addition, post-transcriptional genes from previous studies were included in the light sensitivity growth assay. Yielding 28 light-regulated genes derived from the DNA microarrays and 14 post-transcription genes show a light-induced growth phenotype. Construction a PREMONition PPI network using the 42 candidate genes, resulted in a network consisting of 73 nodes (31 NPC) connected by 58 edges. Functional network analysis revealed a set of biological enriched process including, ATP hydrolysis coupled transmembrane transport and ion homeostasis, age-dependent response to oxidative stress involved in chronological cell aging, positive regulation of TORC1 signaling and vacuolar acidification. The genes involved show a high degree of relatedness to genes in other organism being part of the circadian clock mechanism.

In **chapter 6**, a literature study towards circadian clocks in non-photosynthetic bacteria was undertaken. Giving the conservative nature of circadian mechanisms and the effects environmental changes have of the microbes it's likely a timekeeping mechanism also evolved in non-photosynthetic bacteria. The presence of a circadian mechanisms in these bacteria, playing a crucial role in medical, agricultural and industrial processes, could be of a great importance and enables a deeper understanding of circadian clocks and temporal programs, and provide valuable insights. Here, we suggest, based on experimental and bioinformatical evidence, that circadian rhythms can exist in non-photosynthetic Eubacteria.

In **chapter 7**, the non-photosynthetic Eubacteria *Bacillus subtilis* has been subject to a series of circadian protocols, exposing the cultures to light and temperature cycles with a period of 24h. The collected data shows the ability of *B. subtilis* to synchronise with a 24h light or temperature cycles and that biofilm populations can support a free-running period close to 24h upon release to constant dark and temperature conditions. This behavior has been observed for genes *KinC* and *YtvA*. *YtvA* encodes a blue light photoreceptor with a PAS domain accompanied by a PAC domain, which is a common pairing in circadian and sensory/signaling proteins. *KinC* is a histidine kinase involved in the regulation of differentiation processes, including biofilm development and sporulation. Sporulation is a clock-regulated output in the fungal species *Neurospora crassa* and *Aspergillus.*

Towards an integrated model

Environment sensing pathways

As with many models it starts with input parameters. Here the input parameters are the ever-changing cycling environmental conditions. These conditions vary *e.g.* in temperature, light, nutrition, humidity, salt- and nitrogen concentration. In yeast, these signals are conveyed by the MAPK-signaling pathways (Fig. 1). High temperature sensing is mediated via the Heat Shock Response (HSR) pathway a highly conserved anticipating mechanism aiming to prevent damage, rather than to promote recovery from existing damage[11]. Other harmful environmental stress factors are mediated by the Cell Wall Integrity (CWI) and Environmental Stress Response (ESR) pathways[12]. Following this logic, I expect an underlaying circadian mechanism as functional element in the anticipation of environmental change (an input pathway or a zeitgeber input pathway).

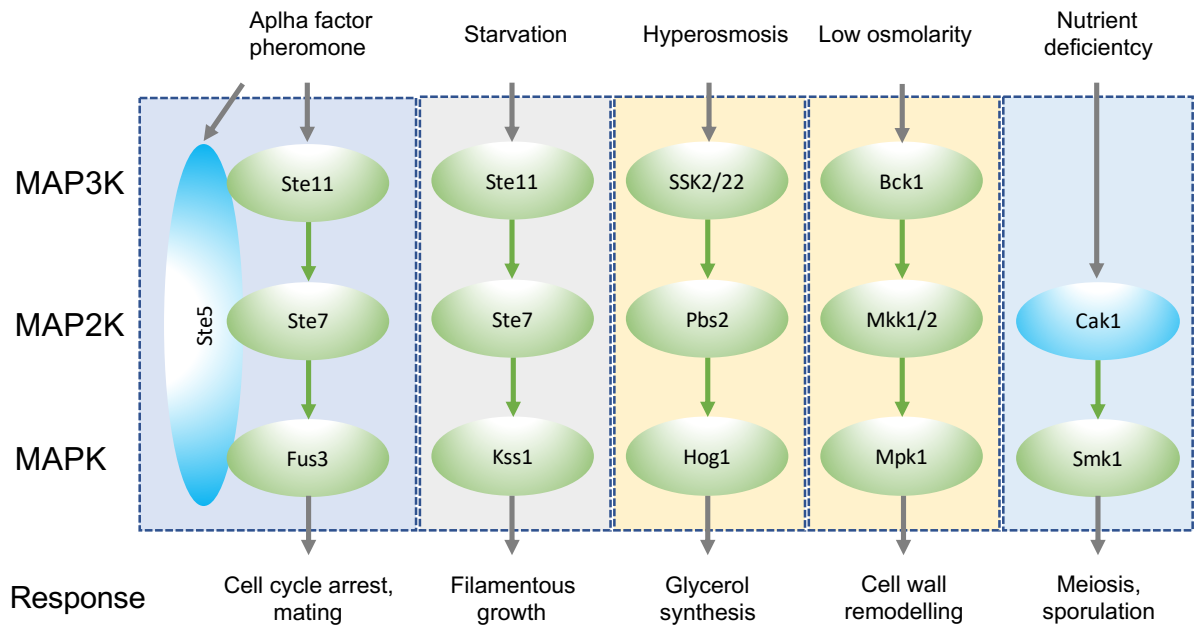


Figure 1. Schematic overview of the MAPK-signaling pathway in *S. cerevisiae*. Five modules mating, filamentation, hyperosmosis, cell wall integrity, sporulation pathways, are regulated independently and react to different environmental changes. The classical proteins are represented by green ellipses and the non-classical by blue ellipses. Figure redraw from Wikipedia under the Creative Commons Attribution-Share Alike 3.0 Unported license.

HSR can be activated in different temperature ranges. Commonly used temperatures are from mild (37°C), severe (42°C), to sublethal stress (46°C)[13]. During our yeast 1L thermostat entrainment experiments a physiological temperature range of 21/28 °C were applied. I expect that the HSP-pathway is not activated via a heat shock, however it could still be clock-regulated as has been observed in other eukaryotic organisms. Clock regulation may already

have been identified in yeast but as 'cellular memory'[14], known in yeast as the hyperosmotic stress response[15]. After a first exposure, yeast react faster to a second exposure. The High Osmolarity Glycerol (HOG) pathway allows yeast to physiologically adapt to hyperosmotic stress within 15–30 minutes[16]. Activation of Hog1p leads to the induction of nuclear adaptive responses. Nuclear responses include control of gene expression and the modulation of cell-cycle progression. Cytoplasmic responses include glycerol transport, metabolic enzymes, the control of ionic fluxes and protein translation[17]. The protein kinase HOG1 interacts with the promoter region of *MSN2/4* regulating its activity[18]. The activity of Msn2/4p is also impacted by the Target of Rapamysin (TOR) pathway (nutrition), Protein Kinase A (intracellular acidification), Snf1p (glucose sensor) and by activators including genes encoding for protein kinases (*MCK1*, *RIM15*, *YAK1* and *HOG1*) and phosphatases (*PSR1*, *PSR2*, and *GLC7*)[19].

Msn2/4p is a key regulator in of General STress Response Element (STRE) (Fig. 2). One of the genes regulated by Msn2/4p is PRX1, mitochondrial peroxiredoxin with thioredoxin peroxidase activity, a highly conserved element involved in circadian rhythms[20]. Prx1 is required for cellular homeostasis and Reactive Oxygen Species (ROS) neutralization and is induced during respiratory growth and oxidative stress. ROS species are mainly formed in mitochondria during cellular respiration[21], but also when exposed to visible light[22]. Oxidative stress is signaled via the HOG-pathway via phosphorylation of Sko1 by Hog1[23].

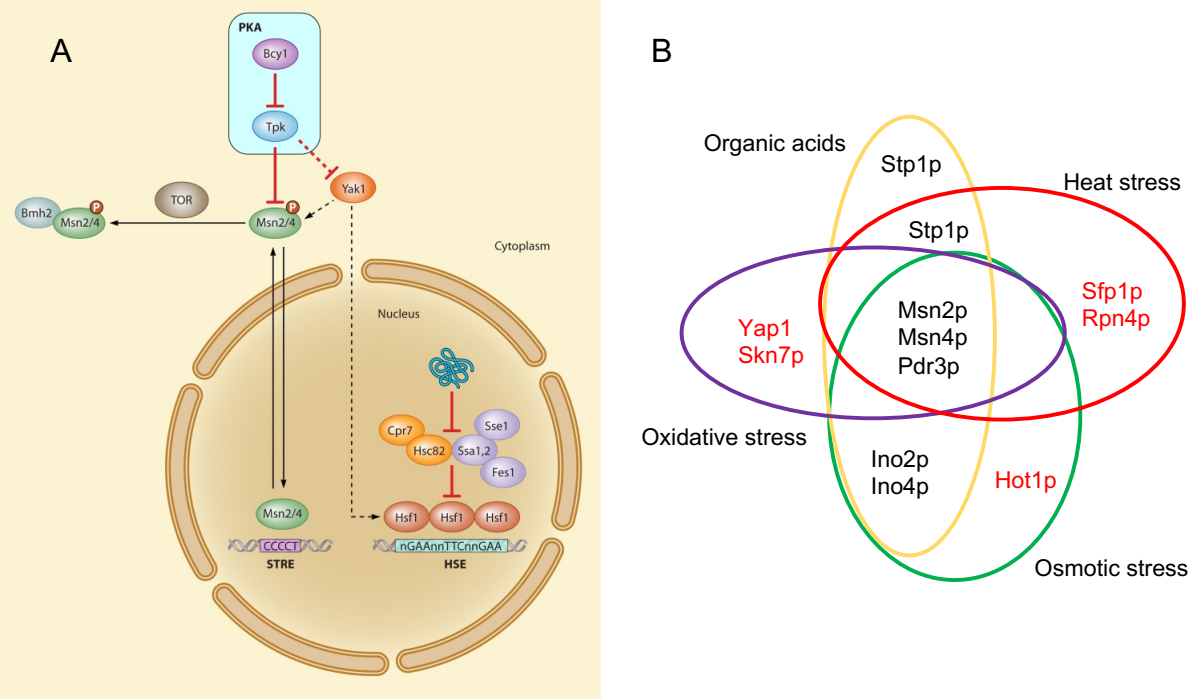


Figure 2. Transcription factor Msn2/4p is key regulator of cellular responses in *S. cerevisiae*. Msn2/4p is a key regulator of the general STress Response Element (STRE) and Heat shock Response (HSE). Dashed lines represent assumed interactions of the Yak1 kinase and red lines regulatory interactions of protein kinase A. Figure borrowed with permission from Verghese *et al*.[11] (A). Venn-diagram from "transcription factors specific for a particular type of stress or common transcription factors mediating the response to a heat, oxidative, and osmotic stresses and the presence of organic acids". Figure redrawn from Agata Święciło[12].

The main metabolic regulator in the activation of several of these pathways is glucose. High glucose concentration activates the HOG-pathway, induces cellular acidification activating the RAt Sarcoma (RAS) pathway and Snf1 becomes activated under low glucose. Glucose is normally fermented even in the presence of oxygen later the produced ethanol can be respirated. This switch from fermentation to respiration requires massive reprogramming of gene expression and is regulated by the transcriptional activator/ repressor Ethanol Regulated Transcription factor 1 (*ERT1*)[24]. Ert1 is one of the four per-ARNT-sim (PAS) domain containing proteins in *S. cerevisiae*. The other proteins are: Rds2, Psk1, Psk2. PAS-domains, is a sensory module that can be found in all kingdoms of life[25] and are commonly found in proteins at the heart of circadian clocks in different organisms. Interestingly Snf1 controls the expression of RDS2[26] which is part of the zinc cluster proteins Cat8, Sip4, and Rds2 and Adr1, required to mediate cellular gene expression reprogramming required for the metabolic fermentation to respiration switch, mediated by Ert1. Sbf1 also phosphorylates Psk1 and phosphorylates/ activates Pbp1 inhibiting torc1[27]. These parallels between glucose metabolism-involved genes, showing relationships between the circadian clock mechanisms in other organisms, are not surprising. It has been shown that glucose metabolism is under

circadian control in humans, rats and mice[28–30] and can function as a circadian entrainment cue[31]. In lower organisms, carbon source and availability also impact the free running rhythm in plants and fungi[32].

PREMONition network summaries

In this thesis I used temperature entrainment and a light sensitivity assays to identify candidate clock-involved genes in *S. cerevisiae*. This yielded 27 and 42 genes respectively. Starting with a PREMONition network constructed from candidates derived from temperature entrainment and a release to constant conditions (**chapter 2**), I observed that the sensory MAPK-pathway is significantly enriched in this set with the genes *MKK1* (Low osmolarity), *FUS3* (alpha-factor pheromone) and *IME2* (downstream of alpha-factor pheromone). Ime2 is a Serine/threonine protein kinase as are Psk1 (PAS domain containing), Kcc4 and Sak1. Although the topology of a PREMONition network is not related to functional categories, clusters of functionalities can be identified. These include glycolysis, alanine, methionine, threonine, amino- and sugar metabolism. In addition, 3 transcription factors Arr1, Yap1 and Yap7, mainly involved in oxidative stress responses, were identified in this experiment.

Inspecting the PREMONition network constructed using the light responsive genes (**chapter 3**) shows one “highly” connected hub around *SNF1*, apparently a key node. Snf1 is involved in the switch from metabolic fermentation to respiration. Snf1 is also 1 of the 10 tyrosine kinases contained within the network, which are involved in functions such as TOR signalling cascades (Ksp1, Sch9), cell wall remodelling (PKC1), cell proliferation (Pho85, Rim15), MAPK signalling cascades (Hog1), meiosis (Ime2), homeostatic adaptation (Ire2) and nutrient-responsive protein (Hal5). A second noticeable feature is the VMA’s cluster, involved in vacuolar acidification.

Constructing PREMONition networks from genes identified using two circadian clock-related protocols results in a network containing 101 nodes connected by 110 edges and 39 connectors (Fig. 3). Enrichment analysis shows, again, the MAPK and TOR pathways being significantly enriched. In addition, processes known to be circadian regulated in other organisms? such as cytochrome P450[33], the mitochondrial translational elongation[34] and sporulation[35,36]. The case of cytochrome P450 is interesting, since the cytochromes of the electron transport chain are photo-oxidized by light, and light also affects mitochondrial morphology. If mitochondrial disfunction is induced by light, yeasts can switch from respiration to fermentation utilizing enormous amounts of glucose, resulting in ROS formation[37] explaining

oxidative stress, cell wall integrity responses and vacuolar acidification[38]. These processes are mainly based on candidate genes from the light-regulation conditions, whereas those from the temperature-entrained, free running candidate genes are mainly involved in biosynthesis of metabolites.

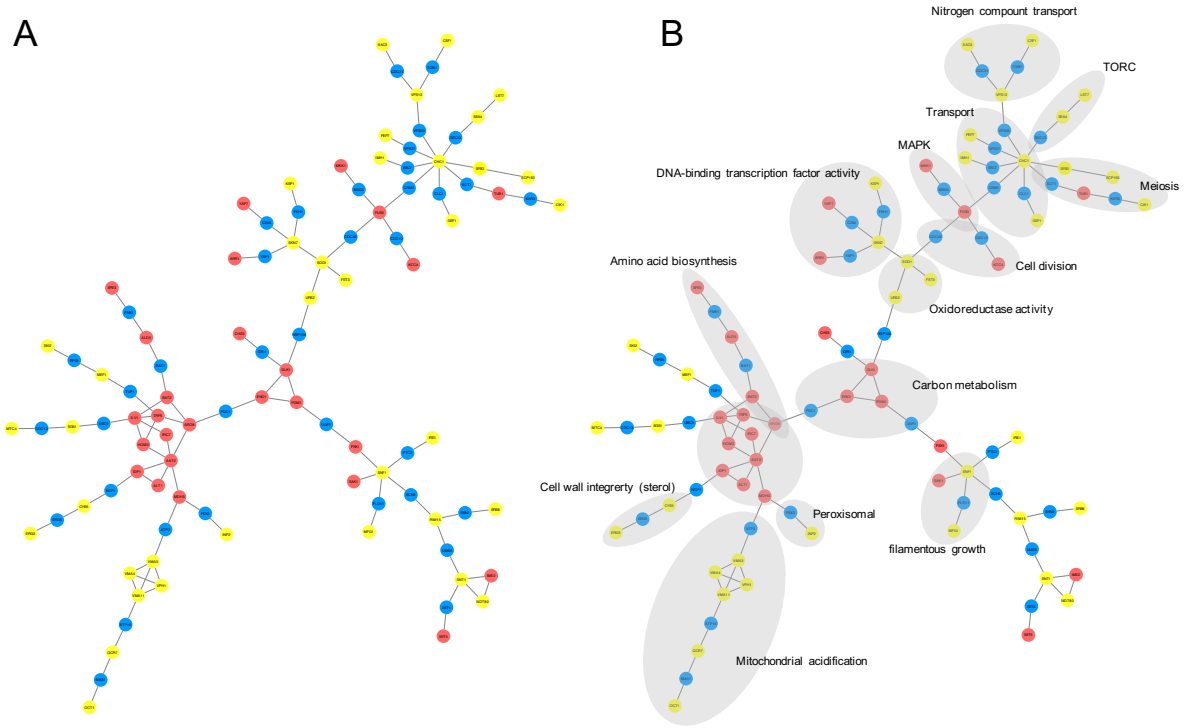


Figure 3. PREMONition network based on genes identified using temperature entrainment (chapter 2) (red spheres) and broad-spectrum light exposure (chapter 3) (yellow spheres). Blue spheres are genes, inserted by the algorithm to construct a fully connected network (A). Some areas of the network are functionally enriched (gray ellipses) (B)

Integrated model proposition

The interplay between different environmental factors, metabolic pathways, genetic regulation and cellular processes is not trivial. Extracellular nutrient combinations activate different metabolic pathways and cellular processes, intertwined with a high degree of genetic regulation. Here I propose a general molecular regulatory model in which some candidate genes and proteins, with a focus on PAS domain containing proteins, for a circadian clock mechanism in yeast are integrated (Fig. 4). Yeast has several environmental sensing mechanisms *e.g.* the aforementioned MAPK and TOR pathways. The PAS domain containing proteins are also involved in nutrient and glucose sensing. Glucose concentrations regulate for example Ert1 activity, a protein enabling the switch from fermentation to respiration and vice versa[24]. Interestingly, whilst Psk2 affects glycogen storage, it shows decreased activity

under respiratory conditions, suggesting the regulation and activation of this PAS kinase under respiratory conditions is primarily mediated via Psk1[39]or by the AMPK ortholog in yeast *SNF1*[27]. Snf1 is a central regulator of cellular energy homeostasis, which, in response to a fall in intracellular ATP levels, activates energy-producing pathways and inhibits energy-consuming processes[40]. Since respiration generates much more ATP, in comparison to fermentation, its importance is key under starvation conditions, during which the electron transport chain becomes essential. However, the cytochromes are heavily impacted by light[22,41].

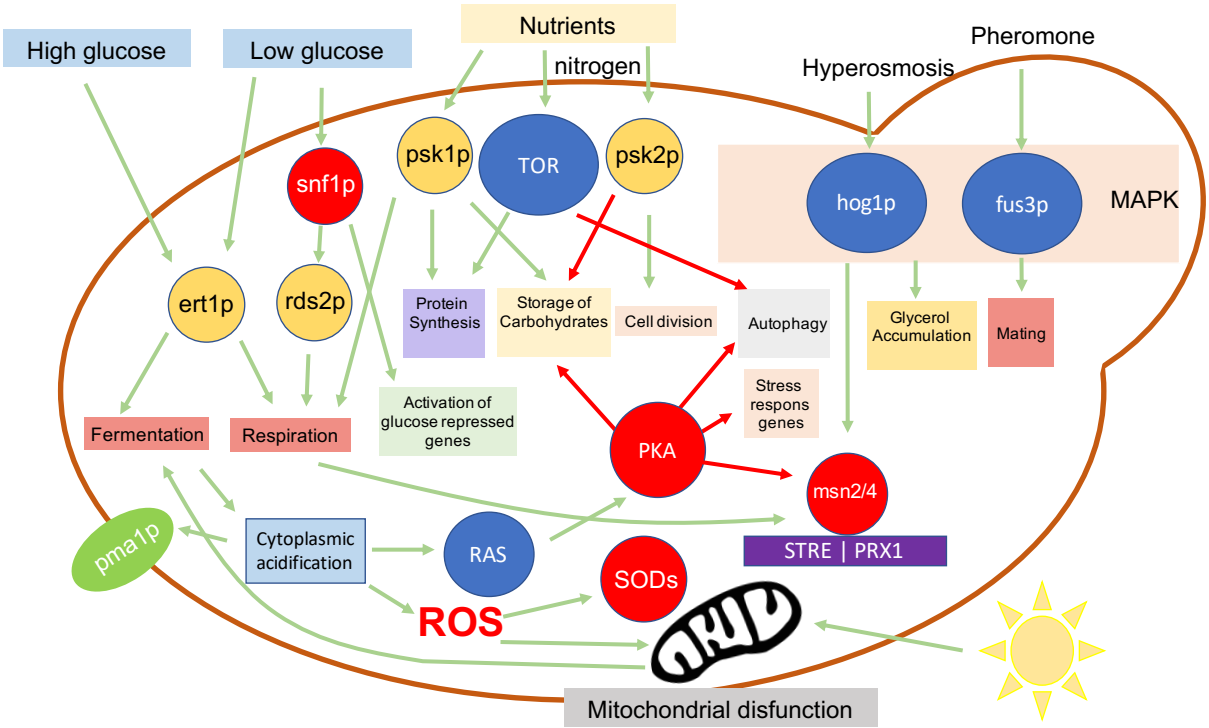


Figure 4. Proposed regulatory model, showing the interconnection of input pathways, environmental signals and internal organization, based on primarily the PAS-domain containing proteins in *S. cerevisiae*. Green arrows indicate some type of activation, stimulation or induction. Red arrows indicate deactivation, inhibition or repression. Yellow spheres represent the PAS-domain containing proteins, blue ellipses are the different environmental signaling pathways and red-spheres are the other key regulating proteins. See text for detailed description.

In **chapter 5**, I identified *SNF1*, *SOD1* and several VMA's and *CSF1*. The latter enables fermentation at low temperatures, suggesting that during our light exposure experiments yeast cells switch to fermentation. VMA's are subunits of the vacuolar ATP proton-translocating ATPases (V-ATPases) and are essential for compartment acidification. Fermentation causes acidification of the cytoplasm Molecular mechanisms of *Saccharomyces cerevisiae* favor adaptation and programmed cell death in response to acetic acid[42] which leads to ROS formation, activating SODs, Protein Kinase A and interestingly Pma1p. Pma1 is a plasma

membrane H^+-ATPase, pumping protons out of the cell. Eelderink-Chen *et al*[43] showed pH rhythms in 1 L fermentor cultures exposed to a temperature cycle. Sampling from these same cultures and analyzing intracellular metabolite concentrations using Nuclear Magnetic Resonance, rhythms in glucose concentration levels were detected (unpublished data). This suggests rhythms in glucose uptake and metabolism. One could assume that low glucose metabolism occurs during respiration. This process activates Msn2/4, which is also activated by a variety of extracellular conditions, such as nitrogen or carbon starvation, osmotic stress (HOG-pathway), DNA-damaging agents, ethanol, and heat stress[44], Msn2/4 regulates expression of glycolytic enzymes[45] and when inhibited by snf1p, PKA and Tor1p (high glucose) regulates the expression of *PRX1*[46]. Prx1 is a mitochondrial peroxiredoxin and is a conserved marker of a circadian clock[47]. Finally, the HOG-pathway can be activated by limitation of environmental nitrogen[48] which in its turn also regulates Msn2/4 activity (Fig. 3). Nitrogen metabolism is also known to be under circadian regulation in *Cyanobacteria*[4–6].

Conclusions

Throughout this thesis I tried to identify molecular components of a circadian mechanism in *B. subtilis* and *S. cerevisiae*. In order to do so, I've applied structured zeitgebers, *i.e.* light and temperature cycles, on yeast cultures. These two different stimuli seem to activate and regulate different aspects of cellular processes. In yeasts, temperature seems to regulate mainly metabolic processes and in terms of living environment this has some logic. Light seems to work more indirectly via photo-oxidation of mitochondrial cytochromes. Yeast has no known photoreceptors as *Bacillus* does, harbouring blue- and red-light photoreceptors. In chapter 4, we showed free-running rhythms in the gene-expression profiles of *ytvA* and *kinC,* obeying the circadian clock characteristics such as self-sustained rhythms and their temperature compensation. I've identified several potential candidate genes for being involved in a circadian clock mechanism in which energy metabolism seems to be an important theme. Other processes showing relevance in the molecular circadian clock mechanism in yeast are nitrogen compound metabolic process, oxidation-reduction process and fatty acid metabolism. All could serve as a starting point for further research to the circadian clock in yeast.

Future research perspectives

Many questions remained to be answered, but unicellular organisms including prokaryotes do contain circadian clock mechanisms, as we showed in *B. subtilis*, and it's evident that *S. cerevisiae* is also under the control of a molecular circadian clock mechanism.
Basically 3 types of experiments can be performed to gain more insights into this clock mechanism. i, micro-fluidics, trapping the mother cells and eluting their daughters ensuring entrainment of cell for a longer period. ii, luminometry, more promoter-luciferase constructs from candidate genes can be constructed, allowing real-time analysis of gene expression over a longer period of time. iii, more quantitative knockout-strains, can easily be achieved by spreading a higher dilution of yeast cells across a bigger surface area. This ensures growth of colonies originating from a single cell. Simple colony forming units can be determined as well as colony size. Two measures for simple characterization of light effects in yeast. All 3 types of experiments can be used to expose the cells to circadian protocols (24h cycles with 2 alternating period of 12h of stimulation, followed by a longer period of a constant condition). A variety of stimulations *i.e.* temperature, light and food can be used as zeitgabers to entrain the cells. All these types of experiments can be subjected to standard analytics such as RNA-sequencing (single cell). However, more complex analytics can be performed, like methylation profiling (epigenetics) or LC-MSMS to identify phosphorylation status of proteins. A more bioinformatics-heavy approach could be identification of Non-coding RNAs and their targets. These are known to ensure a cellular fast response to environmental changes.

Literature references

1. Baek, M. *et al.* Circadian clock regulation of the glycogen synthase (gsn) gene by WCC is critical for rhythmic glycogen metabolism in Neurospora crassa. *Proc. Natl. Acad. Sci. U. S. A.* (2019) doi:10.1073/pnas.1815360116.
2. Doi, R., Oishi, K. & Ishida, N. CLOCK regulates circadian rhythms of hepatic glycogen synthesis through transcriptional activation of Gys2. *J. Biol. Chem.* (2010) doi:10.1074/jbc.M110.110361.
3. Pattanayak, G. K., Phong, C. & Rust, M. J. Rhythms in energy storage control the ability of the cyanobacterial circadian clock to reset. *Curr. Biol.* (2014) doi:10.1016/j.cub.2014.07.022.
4. Chen, T. H., Huang, T. C. & Chow, T. J. Calcium requirement in nitrogen fixation in the cyanobacterium Synechococcus RF-1. *Planta* (1988) doi:10.1007/BF00403017.
5. Mitsui, A. *et al.* Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* (1986) doi:10.1038/323720a0.
6. Stal, L. J. & Krumbein, W. E. Temporal separation of nitrogen fixation and photosynthesis in the filamentous, non-heterocystous cyanobacterium Oscillatoria sp. *Arch. Microbiol.* (1987) doi:10.1007/BF00423140.
7. Poggiogalle, E., Jamshed, H. & Peterson, C. M. Circadian regulation of glucose, lipid, and energy metabolism in humans. *Metabolism.* (2018) doi:10.1016/j.metabol.2017.11.017.
8. Gooley, J. J. Circadian regulation of lipid metabolism. in *Proceedings of the Nutrition Society* (2016). doi:10.1017/S0029665116000288.
9. Zhou, M. *et al.* Redox rhythm reinforces the circadian clock to gate immune response. *Nature* (2015) doi:10.1038/nature14449.
10. Hoyle, N. P. & O'Neill, J. S. Oxidation-reduction cycles of peroxiredoxin proteins and nontranscriptional aspects of timekeeping. *Biochemistry* (2015) doi:10.1021/bi5008386.
11. Verghese, J., Abrams, J., Wang, Y. & Morano, K. A. Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (Saccharomyces cerevisiae) as a Model System. *Microbiol. Mol. Biol. Rev.* (2012) doi:10.1128/mmbr.05018-11.
12. Święciło, A. Cross-stress resistance in Saccharomyces cerevisiae yeast—new insight into an old phenomenon. *Cell Stress Chaperones* (2016) doi:10.1007/s12192-016-0667-7.
13. Mühlhofer, M. *et al.* The Heat Shock Response in Yeast Maintains Protein Homeostasis by Chaperoning and Replenishing Proteins. *Cell Rep.* (2019)

doi:10.1016/j.celrep.2019.11.109.

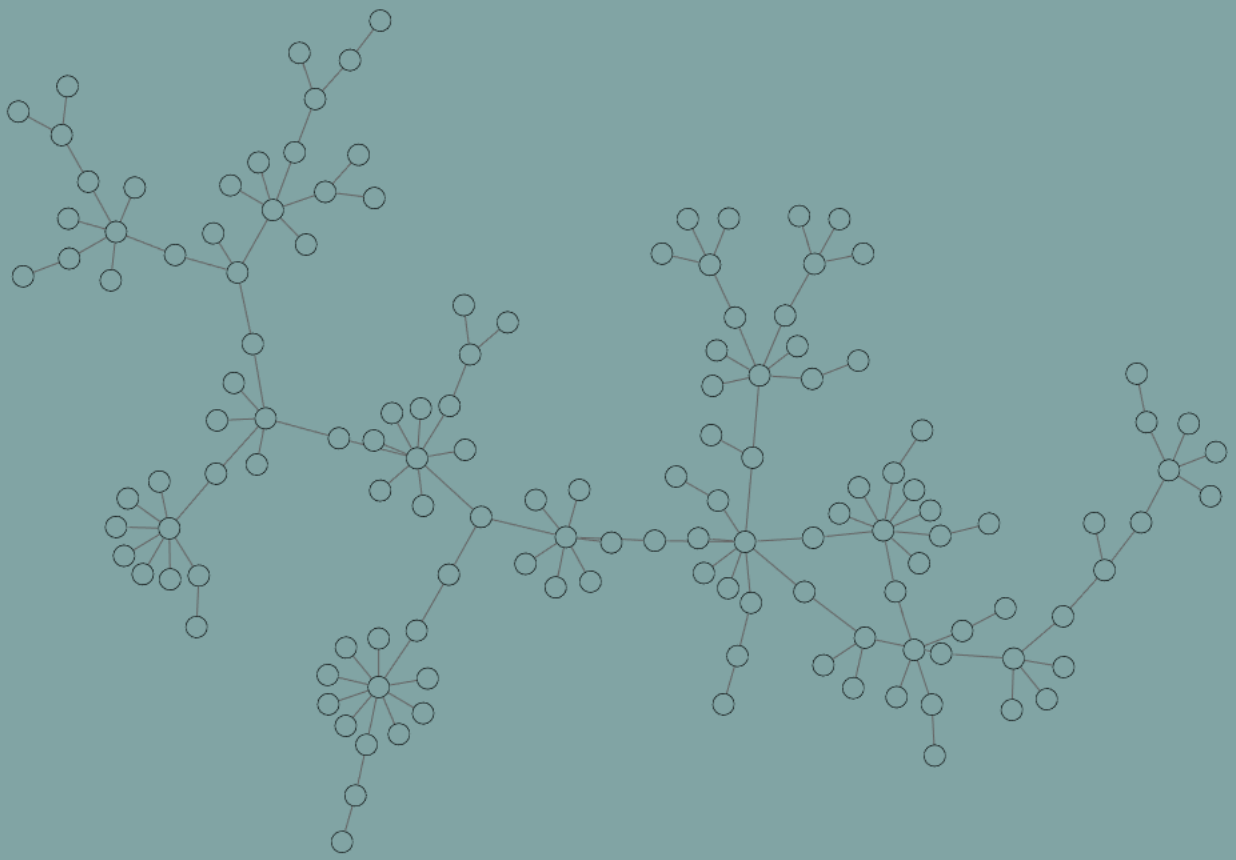
14. D'Urso, A. & Brickner, J. H. Epigenetic transcriptional memory. *Current Genetics* (2017) doi:10.1007/s00294-016-0661-8.
15. Ben Meriem, Z., Khalil, Y., Hersen, P. & Fabre, E. Hyperosmotic Stress Response Memory is Modulated by Gene Positioning in Yeast. *Cells* (2019) doi:10.3390/cells8060582.
16. Miermont, A., Uhlendorf, J., McClean, M. & Hersen, P. The Dynamical Systems Properties of the HOG Signaling Cascade. *J. Signal Transduct.* (2011) doi:10.1155/2011/930940.
17. Saito, H. & Posas, F. Response to hyperosmotic stress. *Genetics* (2012) doi:10.1534/genetics.112.140863.
18. Capaldi, A. P. *et al.* Structure and function of a transcriptional network activated by the MAPK Hog1. *Nat. Genet.* (2008) doi:10.1038/ng.235.
19. Sadeh, A., Movshovich, N., Volokh, M., Gheber, L. & Aharoni, A. Fine-tuning of the Msn2/4-mediated yeast stress responses as revealed by systematic deletion of Msn2/4 partners. *Mol. Biol. Cell* (2011) doi:10.1091/mbc.E10-12-1007.
20. Edgar, R. S. *et al.* Peroxiredoxins are conserved markers of circadian rhythms. *Nature* 485, (2012).
21. Farrugia, G. & Balzan, R. Oxidative stress and programmed cell death in yeast. *Frontiers in Oncology* (2012) doi:10.3389/fonc.2012.00064.
22. Woodward, J. R., Cirillo, V. P. & Edmunds, L. N. Light effects in yeast: Inhibition by visible light of growth and transport in Saccharomyces cerevisiae grown at low temperatures. *J. Bacteriol.* (1978) doi:10.1128/jb.133.2.692-698.1978.
23. Ikner, A. & Shiozaki, K. Yeast signaling pathways in the oxidative stress response. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis* (2005) doi:10.1016/j.mrfmmm.2004.09.006.
24. Gasmi, N. *et al.* The switch from fermentation to respiration in Saccharomyces cerevisiae is regulated by the Ert1 transcriptional activator/repressor. *Genetics* (2014) doi:10.1534/genetics.114.168609.
25. Nambu, J. R., Lewis, J. O., Wharton, K. A. & Crews, S. T. The Drosophila single-minded gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* (1991) doi:10.1016/0092-8674(91)90292-7.
26. Prielhofer, R. *et al.* Pichia pastoris regulates its gene-specific response to different carbon sources at the transcriptional, rather than the translational, level. *BMC Genomics* (2015) doi:10.1186/s12864-015-1393-8.
27. DeMille, D. *et al.* PAS kinase is activated by direct SNF1-dependent phosphorylation

and mediates inhibition of TORC1 through the phosphorylation and activation of Pbp1. *Mol. Biol. Cell* (2015) doi:10.1091/mbc.E14-06-1088.

28. Qian, J. & Scheer, F. A. J. L. Circadian System and Glucose Metabolism: Implications for Physiology and Disease. *Trends in Endocrinology and Metabolism* (2016) doi:10.1016/j.tem.2016.03.005.
29. La Fleur, S. E., Kalsbeek, A., Wortel, J., Fekkes, M. L. & Buijs, R. M. A daily rhythm in glucose tolerance: A role for the suprachiasmatic nucleus. *Diabetes* (2001) doi:10.2337/diabetes.50.6.1237.
30. Zhong, L. X. *et al.* Circadian misalignment alters insulin sensitivity during the light phase and shifts glucose tolerance rhythms in female mice. *PLoS One* (2019) doi:10.1371/journal.pone.0225813.
31. Pitts, S. N., Perone, E. & Silver, R. Food-entrained circadian rhythms are sustained in arrhythmic Clk/Clk mutant mice. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* (2003) doi:10.1152/ajpregu.00023.2003.
32. Zhu, B. & Stülke, J. SubtiWiki in 2018: From genes and proteins to functional network annotation of the model organism Bacillus subtilis. *Nucleic Acids Res.* (2018) doi:10.1093/nar/gkx908.
33. Lin, Y. *et al.* Bmal1 regulates circadian expression of cytochrome P450 3a11 and drug metabolism in mice. *Commun. Biol.* (2019) doi:10.1038/s42003-019-0607-z.
34. Xu, L. *et al.* Circadian gene Clock regulates mitochondrial morphology and functions by posttranscriptional way. *bioRxiv* (2018) doi:10.1101/365452.
35. Merrow, M. W. & Dunlap, J. C. Intergeneric complementation of a circadian rhythmicity defect: Phylogenetic conservation of structure and function of the clock gene frequency. *EMBO J.* 13, (1994).
36. Liu, Y., Merrow, M., Loros, J. J. & Dunlap, J. C. How temperature changes reset a circadian oscillator. *Science (80-.).* (1998) doi:10.1126/science.281.5378.825.
37. Pérez-Gallardo, R. V. *et al.* Reactive oxygen species production induced by ethanol in Saccharomyces cerevisiae increases because of a dysfunctional mitochondrial iron-sulfur cluster assembly system. *FEMS Yeast Res.* (2013) doi:10.1111/1567-1364.12090.
38. Charoenbhakdi, S., Dokpikul, T., Burphan, T., Techo, T. & Auesukaree, C. Vacuolar H+-ATPase protects Saccharomyces cerevisiae cells against ethanolinduced oxidative and cell wall stresses. *Appl. Environ. Microbiol.* (2016) doi:10.1128/AEM.00376-16.
39. Grose, J. H., Sundwall, E. & Rutter, J. Regulation and function of yeast PAS kinase: A role in the maintenance of cellular integrity. *Cell Cycle* (2009)

doi:10.4161/cc.8.12.8799.

40. Ulery, T. L., Jang, S. H. & Jaehning, J. A. Glucose repression of yeast mitochondrial transcription: kinetics of derepression and role of nuclear genes. *Mol. Cell. Biol.* (1994) doi:10.1128/mcb.14.2.1160.
41. Ułaszewski, S. *et al.* Light effects in yeast: Evidence for participation of cytochromes in photoinhibition of growth and transport in Saccharomyces cerevisiae cultured at low temperatures. *J. Bacteriol.* 138, 523–529 (1979).
42. Giannattasio, S., Guaragnella, N., Ždralević, M. & Marra, E. Molecular mechanisms of Saccharomyces cerevisiae stress adaptation and programmed cell death in response to acetic acid. *Frontiers in Microbiology* (2013) doi:10.3389/fmicb.2013.00033.
43. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2043–2047 (2010).
44. Gasch, A. P. *et al.* Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* (2000) doi:10.1091/mbc.11.12.4241.
45. Kuang, Z., Pinglay, S., Ji, H. & Boeke, J. D. Msn2/4 regulate expression of glycolytic enzymes and control transition from quiescence to growth. *Elife* (2017) doi:10.7554/eLife.29938.
46. Monteiro, G. & Netto, L. E. S. Glucose repression of PRX1 expression is mediated by Tor1p and Ras2p through inhibition of Msn2/4p in Saccharomyces cerevisiae. *FEMS Microbiol. Lett.* 241, 221–228 (2004).
47. Edgar, R. S. *et al.* Peroxiredoxins are conserved markers of circadian rhythms. *Nature* (2012) doi:10.1038/nature11088.
48. Adhikari, H. & Cullen, P. J. Metabolic Respiration Induces AMPK- and Ire1p-Dependent Activation of the p38-Type HOG MAPK Pathway. *PLoS Genet.* (2014) doi:10.1371/journal.pgen.1004734.



Chapter 9

English summary

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English summary

For millions of years, the rotation of the Earth has created the day and night cycle of this planet. As a result, the Earth's environmental conditions such as light, temperature, and humidity change in a predictable way over the course of the day over a 24-hour period. In order for life to flourish, many organisms developed mechanisms to exploit these rhythmically changing conditions. These mechanisms are known as circadian clocks, biological programs that provide a time structure for organisms to anticipate predictable changes in the environment. Such a mechanism, which regulates aspects of molecular biology, physiology and social interactions, can have many advantages. For this reason, circadian clocks (from the Latin "circa diem", about a day) are found in a large number of organisms from all phyla.

We believe that almost all organisms have developed a circadian clock mechanism. In this thesis I describe the analysis techniques we developed and the newly discovered molecular clock mechanisms in *Saccharomyces cerevisiae* and *Bacillus subtilis*. We used yeast in chemostats in combination with special circadian growth protocols, as well as bacillus cultures containing cloned reporter constructs under the influence of similar protocols. All this in conjunction with bioinformatic *in-silico* approaches.

The first indications and evidence for a circadian clock in *S. cerevisiae* have been found using fermentor cultures, subjected to temperature cycling over a 24 hour period, to create a rhythmic environment whose pH and dissolved oxygen (dO_2) were monitored. Gene expression analysis of yeast cells from the cultures showed that the mRNA expression of ammonium permease MEP2 and the amino acid permease GAP1 are phase shifted from the pH oscillation in 1 L of fermontor cultures. Our explanation for this is that the periodic transport of ammonium and amino acids to the cytoplasm increases the pH of the cytoplasm. To compensate for this effect, the membrane bound H^+-ATPase, Pma1p pumps the excess intracellular protons out of the cell. This leads to the observed pH oscillations that reveal circadian timing mechanism in yeast.

Large-scale gene expression analyzes lead to many candidate genes. These can be reduced in number by lowering the significance level (p-value). However, this can result in a functionally and relational incomplete set of genes. To solve this problem we have developed the PREMONition, PREdicting MOlecular Networks method. This tool is able to reconstruct a fully linked Protein-Protein-Interactions (PPI) network based on an input set of candidate genes. To validate this new method, genes known to have circadian rhythms in their gene expression

pattern were used to reconstruct PPI networks. This was done for *H. sapiens*, *D. melanogaster* and *N. crassa*. In addition, a PPI for *S. cerevisiae* has been constructed on the basis of a microarray dataset collected from a fermentor culture under the influence of a temperature cycling with a period of 24 hours. By comparing the obtained networks, 13 genes in yeast have been identified, showing overlapping functionalities with the human, fly and fungal genes. These 13 genes can be categorized into 3 processes. The first, the transport of nitrogen compounds (CDC5, ACT1, RPD3, RPN11, HRR25, HOG1, EFB1, CDC19 and TKL1), a process known to be regulated by the circadian clock in cyanobacterium *Synechococcus*. The second, vacuole organization (YPT7, ACT1 and HRR25) of which HRR25 encodes a casein kinase I, a major player in the mammalian circadian clock. And the third, proteins that exhibit kinase activity (CDC5, HRR25, HOG1, CDC19) that are known to be important in molecular circadian clock mechanisms.

Due to these promising results, we have applied PREMONition to all organisms annotated in the Circadian Gene DataBase (CGDB) database. These networks were then analyzed for common biological properties such as GO terms (biological process, molecular function and cellular component), KEGG pathways and SMART protein domains. We then applied two different strategies to identify circadian clock genes in yeast. The first, it is mapping all yeast genes annotated in the STRING database with similar traits. This resulted in 294 genes, 22 of which show a circadian pattern at the transcription level. The second approach, is to first construct a PPI network based on genes whose gene expression pattern shows a 24 hour rhythm. We then compare the networked yeast genes with the set of biological properties. A set of 9 genes was obtained through this approach. In the total set of 29 unique genes, the serine / threonine protein kinases SAK1, KCC4, PSK1, FUS3 and IME2, the transcription factors ACA1, YAP7, ARR1 have been identified.

More importantly, some biological processes are highly conserved in circadian PPI networks. These processes include nitrogen metabolic processes, the oxidation-reduction process, glycolysis / gluconeogenesis and fatty acid metabolism. These processes are known to be involved in the circadian mechanisms in various organisms, including cyanobacteria, fungi and mammals.

A general understanding has now been obtained of the genes that show a circadian gene expression pattern under a 24 hour temperature cycle. The next step is to study which genes change their expression pattern under the influence of light. To characterize the physical and underlying molecular processes involved in the growth response of yeast under the influence

of light. It is hypothesized that a day-night cycle (i.e. light-dark) is also a crucial factor in mediating the circadian rhythm in yeast. Therefore, we examined the growth of yeast under light and dark conditions and the effect of specific gene knockouts on its growth. A selection of potentially interesting genes has been compiled from two different sources. The first, DNA microarrays of cells exposed to blue light. From this, the genes were selected that are up- or down-regulated (differential gene expression) under the influence of blue light. And the second, post-transcriptional genes from previous studies. This photosensitivity growth assay yielded 28 light-regulated genes from the DNA microarrays and 14 post-transcription genes. Functional network analysis revealed a series of biologically enriched processes, including ATP hydrolysis coupled transmembrane transport and ion homeostasis, age-dependent response to oxidative stress involved in chronological cell aging, positive regulation of TORC1 signaling and vacuolar acidification. The genes involved show a high degree of relationship to genes in other organisms that are part of the circadian clock mechanism.

The above provides evidence for the existence of a circadian clock in *S. cerevisiae*. And so we now ask ourselves: "Are there any more Eubacteria in which a circadian clock has not been described before?" Given the conservative nature of circadian mechanisms and the effects of environmental changes of the microbes, it is plausible that a time recording mechanism has also evolved in non-photosynthetic bacteria. The presence of circadian mechanisms in these bacteria, which play a crucial role in agricultural and industrial processes, can be of great importance. Disturbances in the clock mechanism have been associated with clinical pictures. Our literature review, based on experimental and bioinformatic evidence, suggests that non-photosynthetic prokaryotes can generate rhythmic gene expression and we propose *Bacillus subtilis* as a potential model to investigate circadian mechanisms in non-photosynthetic bacteria. In addition, based on experimental and bioinformatic evidence, we suggest that circadian rhythms may occur in non-photosynthetic Eubacteria.

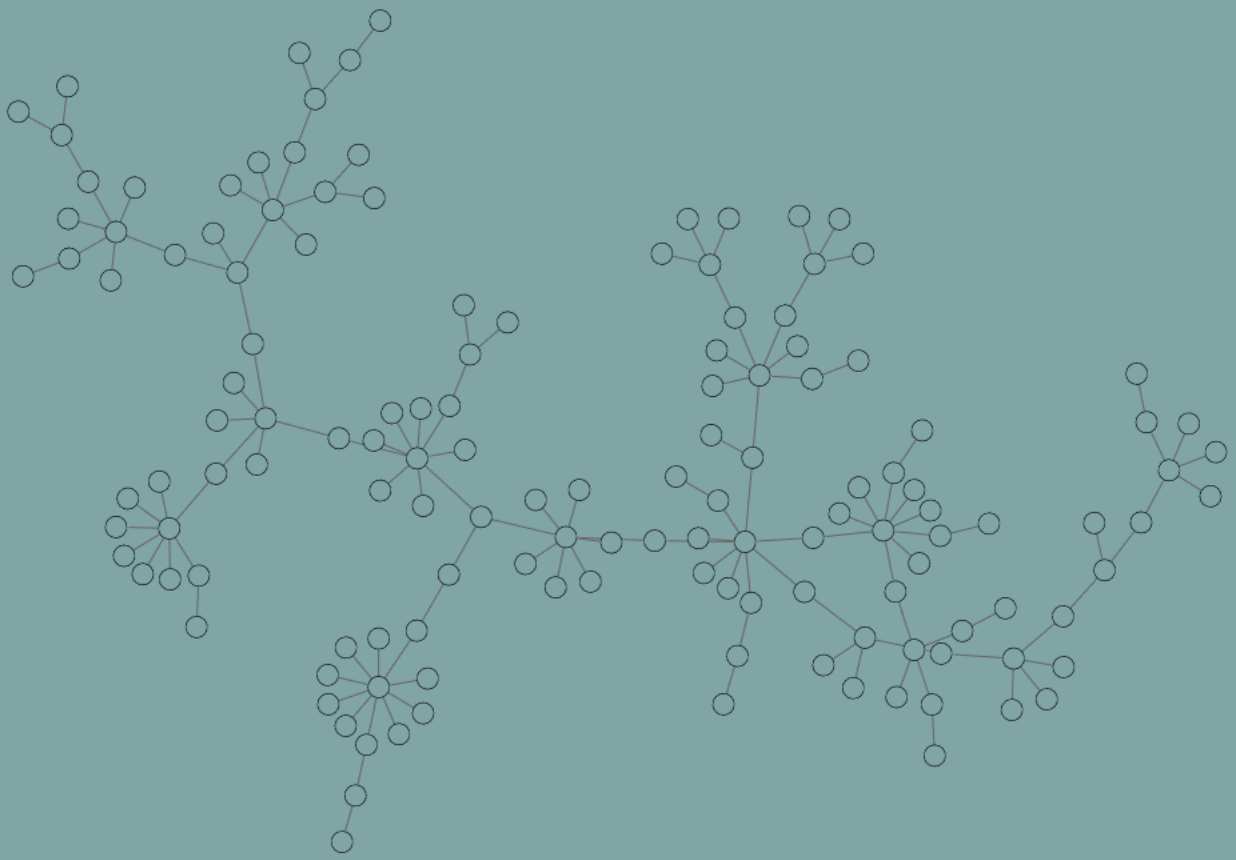
To follow up our own proposal, *Bacillus subtilis* as a potential model to investigate circadian mechanisms in non-photosynthetic bacteria. We subjected *Bacillus subtilis* cultures to a series of circadian protocols, in which the cultures were exposed to light and temperature cycling over a 24 hour period. To gain insight into how this organism reacts to these systematic changes, we selected two genes, the protein of which contains a PAS domain (YtvA and kinC), and constructed bioluminescent reporter strains from them. The YtvA promoter encodes a blue light photoreceptor with a PAS domain accompanied by a PAC domain. KinC is a histidine kinase involved in the regulation of differentiation processes, including biofilm development

and sporulation. We found that bacterial biofilm populations have a free-running rhythm of ytvA and KinC activity of almost 24 hours after release under constant dark and temperature conditions in *B. subtilis*. The free-running oscillations are temperature-compensated and have phase-specific characteristics of entrainment. These are the main features of a circadian clock mechanism, making it very likely that such a system exists in *B. subtilis*.

Finally, I present a hypothetical model for an integrated circadian clock mechanism in unicellular microbes with an emphasis on *S. cerevisiae*. This mechanism involves several metabolic pathways and the main regulator is the stress sensitive transcriptional activator Msn2p. Msn2p is regulated by Snf1p (Sucrose NonFermenting), Tor1p (Target Of Rapamycin (TOR) pathway) and Hog1p (High Osmolarity Glycerol (HOG) response) and Msn2p itself regulates the Peroxiredoxin Prx1. Peroxiredoxins are highly conserved and exhibit circadian oscillations in their oxidation state in human, murine and marine algae cells. This mechanism also allows feedback of glucose concentrations via the TOR pathway and via protein kinase A. The latter is associated with the *Neurospora crassa* clock. In addition to protein kinase A, serine / threonine protein kinases also play an important role and are highly conserved and critical components in known clock mechanisms.

In this thesis I tried to identify molecular components of a circadian mechanism in *B. subtilis* and *S. cerevisiae*. To do this, I applied structured zeitgebers, i.e. light and temperature cycles, to yeast cultures. These two different stimuli appear to activate and regulate different aspects of cellular processes. In yeasts the temperature seems to regulate mainly metabolic processes and in terms of living environment this seems logical. Light appears to act more indirectly via photo-oxidation of mitochondrial cytochromes. Yeast has no known photoreceptors like *Bacillus*, which contains blue and red light photoreceptors. We showed free-running rhythms in ytvA and kinC gene expression profiles, adhering to circadian clock features such as self-sustaining rhythms and temperature compensation.

I have identified several potential candidate genes to be involved in a circadian clock mechanism in which energy metabolism appears to be a major theme. Other processes relevant in the molecular circadian clock mechanism in yeast are: metabolic process of nitrogen compounds, oxidation-reduction process and fatty acid metabolism. All could serve as a starting point for further research on the circadian clock in yeast.



Chapter 10

Nederlandse samenvatting

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Nederlandse samenvatting

Miljoenen jarenlang heeft de rotatie van de aarde de dag- en nachtcyclus van deze planeet gecreëerd. Als gevolg hiervan veranderen de omgevingscondities op aarde zoals licht, temperatuur en vochtigheid op een voorspelbare manier, met het verloop van de dag gedurende een periode van 24 uur. Om het leven tot bloei te laten komen, ontwikkelden veel organismen mechanismen om deze ritmisch veranderende omstandigheden te exploiteren. Deze mechanismen staan bekend als circadiaanse klokken, biologische programma's die een tijds-structuur bieden aan organismen waardoor ze in staat zijn te anticiperen op voorspelbare veranderingen in het milieu. Een dergelijk mechanisme, dat aspecten van moleculaire biologie, fysiologie en sociale interacties reguleert, kan veel voordelen hebben. Om deze reden worden circadiaanse klokken (van het Latijnse "*circa diem*", ongeveer een dag) aangetroffen in een groot aantal organismen uit alle phyla.

Wij zijn van mening dat bijna alle organismen een circadiaans klokmechanisme hebben ontwikkeld. In dit proefschrift beschrijf ik de door ons ontwikkelde analysetechnieken en de hiermee nieuw ontdekte moleculaire klok mechanismen in *Saccharomyces cerevisiae* en *Bacillus subtilis*. We gebruikten gist in chemostaten in combinatie met speciale circadiaanse groeiprotocollen, daarnaast bacillus cultures met daarin gekloneerde reporter constructen onder invloed van vergelijkbare protocollen. Dit alles in samenspel met bioinformatische *in-silico*-benaderingen.

De eerste aanwijzingen en bewijzen voor een circadiaanse klok in *S. cerevisiae* zijn gevonden doormiddel van fermentor cultures, onderworpen aan temperatuurcycli met een periode van 24 uur, om een ritmische omgeving te creëren waarvan de pH en opgeloste zuurstof (dO_2) werden gemonitord. Genexpressie analyse van gistcellen uit de cultures, toonden aan dat de mRNA-expressie van ammoniumpermease MEP2 en de aminozuurpermease GAP1 in fase zijn verschoven ten opzichte van de pH-oscillatie in 1 L fermontorkweken. Onze verklaring hiervoor is, dat het periodieke transport van ammonium en aminozuren naar het cytoplasma de pH van het cytoplasma verhogen. Om dit effect dit compenseren pompt de membraam gebonden H^+-ATPase, Pma1p de overtollige intracellulaire protonen weer de cel uit. Dit leidt tot de geobserveerde pH oscillaties die circadiaans timingmechanisme in gist onthullen.

Grootschalige genexpressie analyses leiden tot veel kandidaat genen. Deze kunnen in aantal worden gereduceerd door het significantieniveau (p-waarde) te verlagen. Echter hierdoor kan er een functioneel en relationeel onvolledige set van genen ontstaan. Om dit probleem op te

lossen hebben we de PREMONition[2], PREdicting MOlecular Networks methode ontwikkeld. Deze tool is in staat om, op basis van een inputset van kandidaatgenen een volledig verbonden Protein-Protein-Interactions (PPI) -netwerk te reconstrueren. Om deze nieuwe methode te valideren werden genen, waarvan bekend is dat hun expressie patroon een circadiaanse ritmiek vertoond, gebruikt om PPI-netwerken te reconstrueren. Dit voor *H. sapiens*, *D. melanogaster* en *N. crassa*. Daarnaast is er een PPI voor *S. cerevisiae* geconstrueerd op basis van een microarray dataset verzameld uit een fermentorkweek onder invloed van een temperatuurcycli met een periode van 24 uur. Door de verkregen netwerken met elkaar te vergelijken, zijn er 13 genen in gist geïdentificeerd, die overlappende functionaliteiten ten opzichte van de menselijke, vlieg en schimmel genen laten zien. Deze 13 genen kunnen in 3 processen worden gecategoriseerd. De eerste, het transport van stikstofverbindingen (CDC5, ACT1, RPD3, RPN11, HRR25, HOG1, EFB1, CDC19 en TKL1), een proces waarvan bekend is dat het proces wordt gereguleerd door de circadiane klok in cyanobacterium *Synechococcus*. De tweede, vacuole-organisatie (YPT7, ACT1 en HRR25) waarvan HRR25 codeert voor een Caseïnekinase I, een belangrijke speler in de circadiane klok van zoogdieren. En de derde, eiwitten die kinase-activiteit vertonen (CDC5, HRR25, HOG1, CDC19) waarvan bekend is dat ze van belang zijn in moleculaire circadiane klokmechanismen.

Door deze veel belovende resultaten, hebben we PREMONition toegepast op alle organismen geannoteerd in de Circadian Gene DataBase (CGDB) database. Deze netwerken zijn vervolgens geanalyseerd op gemeenschappelijke biologische eigenschappen zoals GO-termen (biologisch proces, moleculaire functie en cellulaire component), KEGG-routes en SMART-eiwitdomeinen. Vervolgens hebben we twee verschillende strategieën toegepast om circadiaanse klok genen te identificeren in gist. De eerste, het is kaart brengen van alle gistgenen geannoteerd in de STRING-database met vergelijkbare eigenschappen. Dit resulteerde in 294 genen, waarvan er 22 een circadiaans patroon vertonen op transcriptieniveau. De tweede benadering, is het eerst construeren van een PPI netwerk op basis van genen waarvan het gen expressiepatroon een 24 uur ritme vertoont. Vervolgens vergelijken we de genetwerkte gistgenen met de verzameling biologische eigenschappen. Via deze aanpak is een set van 9 genen verkregen. In de totale set van 29 unieke genen, zijn o.a. de serine/ threonine-eiwitkinases SAK1, KCC4, PSK1, FUS3 en IME2, de transcriptiefactoren ACA1, YAP7, ARR1 geïdentificeerd.

Wat nog belangrijker is dat sommige biologische processen sterk geconserveerd zijn in circadiane PPI-netwerken. Deze processen omvatten stikstof metabolische processen, het oxidatie-reductieproces, glycolyse / gluconeogenese en vetzuurmetabolisme. Het is bekend dat deze processen betrokken zijn bij de circadiane mechanismen in verschillende organismen, waaronder *cyanobacteriën*, schimmels en zoogdieren.

Er is nu beeld verkregen van de genen die onder een 24 uurs temperatuur cyclus ook een circadiaans genexpressie patroon vertonen. De volgende stap is het bestuderen welke genen hun expressie patroon veranderen onder de invloed van licht. Om zo de fysische en onderliggende moleculaire processen te karakteriseeren die betrokken zijn bij de groeirespons van gist onder de invloed van licht. De hypothese is dat een dag-nachtcyclus (d.w.z. licht-donker) ook een cruciale factor is bij het bewerkstellen van het circadiane ritme in gist. Daarom onderzochten we de groei van gist onder lichte en donkere omstandigheden en het effect van specifieke gen-knock-outs op de groei ervan. Een selectie van potentieel interessante genen is samengesteld uit twee verschillende bronnen. De eerste, DNA-microarrays van cellen die zijn blootgesteld aan blauw licht. Hieruit zijn de genen geselecteerd die up- of down-gereguleerd worden (differentiële genexpressie) onder invloed van blauwlicht. En de tweede, post-transcriptionele genen uit eerdere studies. Dit lichtgevoeligheidsgroei-assay leverde 28 lichtgereguleerde genen afkomstig van de DNA-microarrays en 14 post-transcriptiegenen op. Functionele netwerkanalyse onthulde een reeks biologisch verrijkte processen, waaronder ATP-hydrolyse gekoppeld transmembraantransport en ionhomeostase, leeftijdsafhankelijke respons op oxidatieve stress wat betrokken is bij chronologische celveroudering, positieve regulering van TORC1-signalering en vacuolaire verzuring. De betrokken genen vertonen een hoge mate van verwantschap met genen in andere organismen die deel uitmaken van het circadiane klokmechanisme.

Het bovenstaande geeft bewijs voor het bestaan van een circadiaanse klok in *S. cerevisiae*. En daarom vragen wij ons nu af: “Zijn er nog meer *Eubacteria* waarin nog niet eerder een circadiaanse klok is beschreven?”. Gezien de conservatieve aard van circadiane mechanismen en de effecten van veranderingen in het milieu van de microben, is het aannemelijk dat een tijdregistratiemechanisme ook is geëvolueerd in niet-fotosynthetische bacteriën. De aanwezigheid van circadiane mechanismen in deze bacteriën, die een cruciale rol spelen in landbouw- en industriële processen, kan van groot belang zijn. Verstoringen in het klokmechanisme zijn in verband gebracht met ziektebeelden. Ons literatuuronderzoek, gebaseerd op experimenteel en bioinformatisch bewijs suggereert dat niet-fotosynthetische prokaryoten ritmische genexpressie kunnen genereren en we stellen *Bacillus subtilis* voor als

een potentieel model om circadiane mechanismen in niet-fotosynthetische bacteriën te onderzoeken. Daarnaast suggereren we, gebaseerd op experimenteel en bioinformatisch bewijs, dat circadiane ritmes kunnen voorkomen in niet-fotosynthetische Eubacteria.

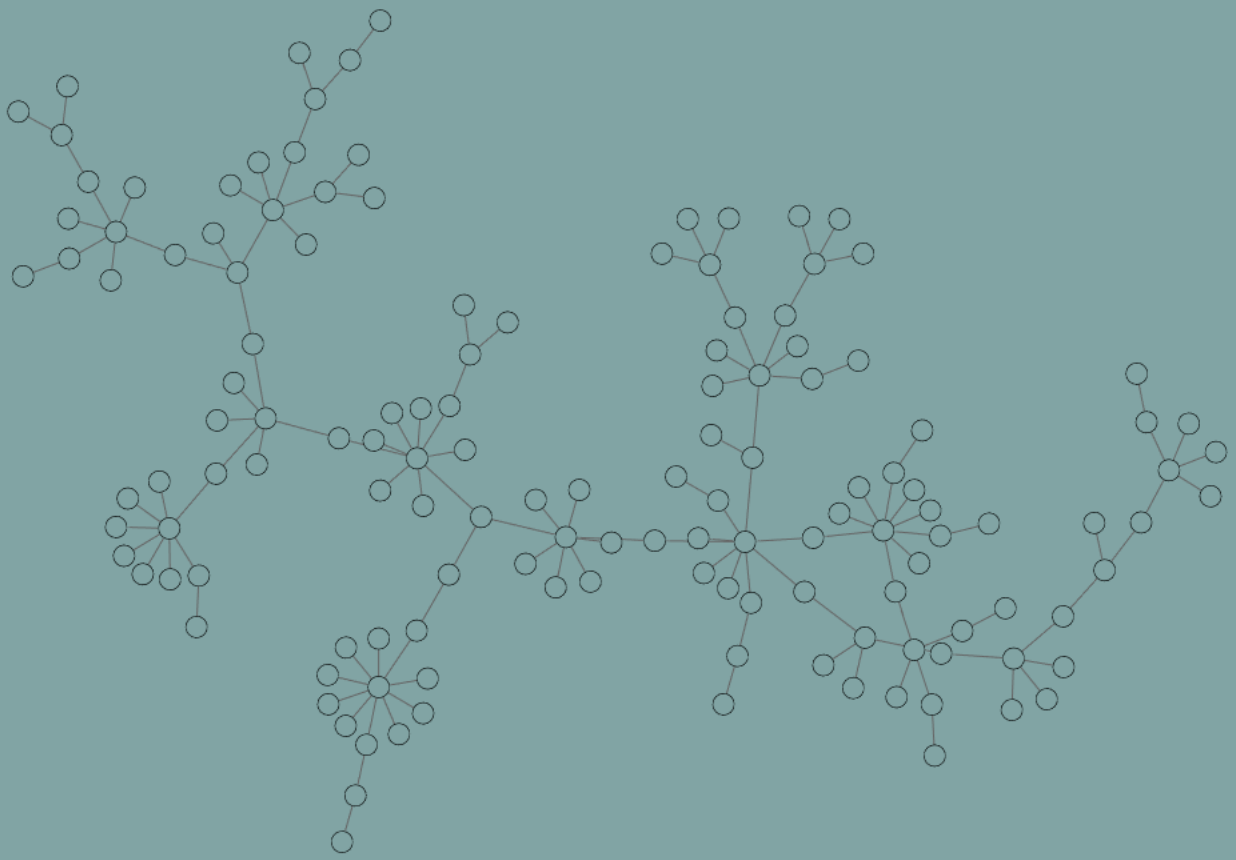
Om ons eigen voorstel, *Bacillus subtilis* als potentieel model om circadiane mechanismen in niet-fotosynthetische bacteriën te onderzoeken, op te volgen. Hebben we *Bacillus subtilis*-culturen onderworpen aan een reeks circadiaanse protocollen, waarbij de culturen zijn blootgesteld aan licht- en temperatuurcycli met een periode van 24 uur. Om inzicht te krijgen hoe dit organisme reageert om deze systematische veranderingen hebben we twee genen, waarvan het eiwit een PAS domein bevat (YtvA en kinC) geselecteerd en daaruit bioluminescente reporterstammen geconstrueerd. De promotor YtvA codeert voor een blauwlichtfotoreceptor met een PAS-domein vergezeld van een PAC-domein. KinC is een histidinekinase dat betrokken is bij de regulering van differentiatieprocessen, waaronder biofilmontwikkeling en sporulatie. We ontdekten dat bacteriële biofilmpopulaties een vrijlopend ritme van ytvA- en KinC-activiteit hebben van bijna 24 uur na vrijlating onder constante donkere en temperatuuromstandigheden in *B. subtilis*. De vrijlopende oscillaties zijn temperatuurgecompenseerd en hebben fasespecifieke karakteristieke meesleuringen. Dit zijn de belangrijkste kenmerken van een circadiaans klokmechanisme, waardoor het zeer waarschijnlijk is dat een dergelijk systeem bestaat in *B. subtilis*.

Tenslotte presenteer ik een hypothetisch model voor een geïntegreerd circadiaans klokmechanisme in eencellige microben met de nadruk op *S. cerevisiae* voor. Dit mechanisme omvat verschillende metaboleroutes en de belangrijkste regulator is de stressgevoelige transcriptionele activator Msn2p. Msn2p wordt gereguleerd door Snf1p (Sucrose NonFermenting), Tor1p (Target Of Rapamycin (TOR) -route) en Hog1p (High Osmolarity Glycerol (HOG) -respons) en Msn2p zelf reguleert de Peroxiredoxine Prx1. Peroxiredoxines zijn sterk geconserveerd en vertonen circadiane oscillaties in hun oxidatietoestand in cellen van mensen, muizen en zeealgen. Dit mechanisme maakt ook feedback van glucoseconcentraties mogelijk via de TOR-route en via proteïnekinase A. Dit laatste is geassocieerd met de *Neurospora crassa* klok. Naast proteïne kinase A spelen ook serine / threonine proteïne-kinasen een belangrijke rol en zijn sterk geconserveerde en kritische componenten in bekende klokmechanismen.

In dit proefschrift heb ik geprobeerd moleculaire componenten van een circadiaans mechanisme in *B. subtilis* en *S. cerevisiae* te identificeren. Om dit te kunnen doen, heb ik gestructureerde zeitgebers, d.w.z. licht- en temperatuurcycli, toegepast op gistculturen. Deze

twee verschillende stimuli lijken verschillende aspecten van cellulaire processen te activeren en te reguleren. In gisten lijkt de temperatuur voornamelijk metabolische processen te reguleren en in termen van leefomgeving lijkt dit logisch. Licht lijkt meer indirect te werken via foto-oxidatie van mitochondriale cytochromen. Gist heeft geen bekende fotoreceptoren zoals *Bacillus*, die blauw- en roodlicht-fotoreceptoren bevat. We hebben vrijlopende ritmes laten zien in de genexpressieprofielen van ytvA en kinC, daarbij gehoorzamende ze aan de circadiaanse klokkenmerken zoals zelf ondersteunende ritmes en temperatuurcompensatie.

Ik heb verschillende potentiële kandidaat-genen geïdentificeerd om betrokken te zijn bij een circadiaans klokmechanisme waarin energiemetabolisme een belangrijk thema lijkt te zijn. Andere processen die relevant zijn in het moleculaire circadiaanse klokmechanisme in gist zijn: metabolisch proces van stikstofverbindingen, oxidatie-reductieproces en vetzuur-metabolisme. Allen zou als uitgangspunt kunnen dienen voor verder onderzoek naar de circadiaanse klok in gist.



Chapter 11

Acknowledgements

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Acknowledgements

At this point I realise how many people intertwined with my life, for shorter or longer periods of time. From each interaction one learns, become inspired, activated or motivated. Thank you.

At first, I would like to thank **Martha Merrow** for giving me the opportunity for this PhD adventure, teaching me a numerous writing and laboratory skills and, of course, allowing me to participate in the yeast genetics course at Cold Spring Harbour Laboratory. My PhD project lasted a tiny bit longer than the conventional 4 years, still Martha kept supporting me. Together, we had numerous discussions resulting in new ideas and insights.

Next, the Merrow molecular chronobiology group. **Zheng Chen** (Yaoyao) for training me extensively in yeast fermentor cultures enabling us to keep them running for months at a time. Also, for performing several yeast and Bacillus experiments in München. **Margien Raven**, together we had so much fun in the lab and I would like to thank you for filling my head with all kinds of pubquiz facts and music of which I had never heard before. **Margriet van der Pol**, thank you for all your life lessons such as: “a clean house is a sign of a wasted life” and “When you are able to peel an apple while keeping the peel in one piece, then you’re ready for marriage”. Besides the wise words, you performed hundreds of minipreps for me in attempts to the construct yeast luciferase strains. **Gerard Overkamp** for building the light panel I used for my yeast light exposure experiments.

I like to thank all co-authors: **Emma Laing** for helping to improve the PREMONition publication, especially the homology analysis. **Tsjerk Wassenaar**, your statistical knowledge was a great resource for the analysis of the effects of light on the growth of yeast. Thank you for helping with the linear models and principal component analysis. **Ben Aronson**, **Francesca Sartor**, **Lauren Hibbert**, **Antony Dodd**, **Ákos Kovács** without your extensive knowledge on *Bacillus subtills* and insights in relationship to the circadian clock, the scientific impact of our two papers would never have been so high.

Arjen Krikken, you taught me the basics of confocal microscopy. Thank you for allowing me to use your (and **Ida van der Klei**) fantastic piece of equipment and for keeping up with my many questions concerning molecular techniques in *S. cerevisiae*.

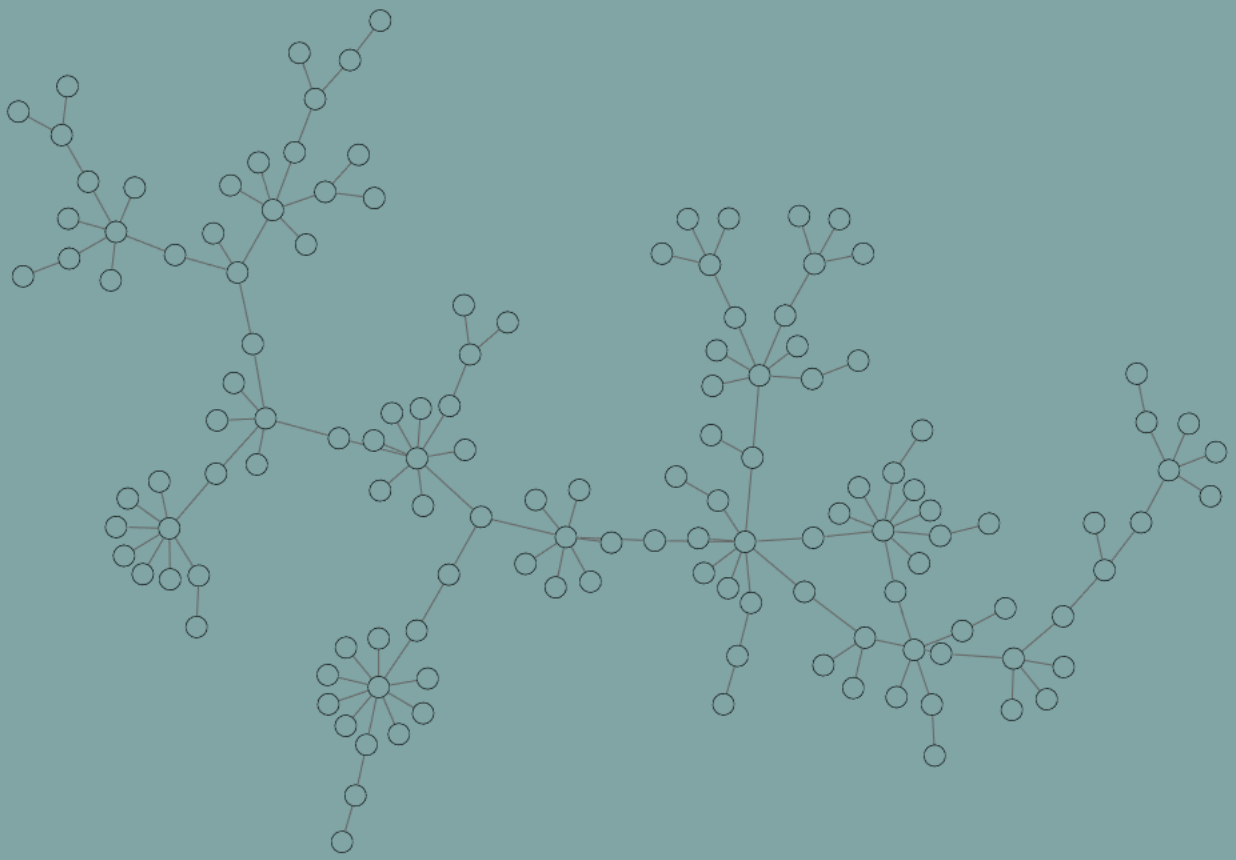
Jean-Christophe Billeter, I think we started around the same time. You as an assistant professor and I as a PhD student. Although our model organisms are quite different, we hypostasised on an interaction between the two. The odours of yeast attract fruit flies, so what if… Thank you for all our nice discussions, your friendly character and for helping me in the past with your HPLC (among many things) and now to complete the final steps of my PhD project.

As some of you know, I always have ideas for new experiments. One of these involved capturing yeast cells in a micro-fluidic device, enabling single cell analysis over time. **Patty Mulder,** you helped tremendously with putting this new idea to the test. You made all kind of micro-fluidic devices for me and helped setting up the experiment setup. With respect to this topic, I thank **Sabeth Verpoorte** and **Ingrid Molema** for showing me the fascinating world of micro-fluidics and HUVEC cells. A special word of gratitude goes to **Emile Apol**. Emile, your help was essential for modelling and anaysing the *Bacillus subtilis* gene expression profiles. Thank you very much for your patience and great advice. Next, I like to thank **Ida van der Veen** (Hanze University of Applied Science), who gave me opportunity and time to work on my thesis for 2 months without the many interruptions a teacher normally has. This time gave me the final push to finish my Thesis.

Marissa Dubbelaar, I want to thank you for many different things. Among these, all the nice projects you came up with for my students and the great supervision of them. But also, for answering all my questions related to finishing my thesis.

Besides all scientific contributors enabling my thesis, there are three other people I would like to thank. First, **my parents**. Who gave me the opportunity to get a proper education and stimulated be to be curious and ask questions.

Finally, the most important person in my life: my wife **Hadewig Oosterbeek.** I still remember the day we discussed the possibility for starting this PhD project, during a walk. How ignorant we were. So much has happened the last couple of years. We were blessed with two wonderful sons; Wout en Cas, bought two houses, changed jobs and much more. As all fellow scientists know, we spend many hours in the lab, analysing data etc. But as a circadian biologist I also had to spend many nights in the lab. I'm sorry I was not always around while I should have been. Thank you for all your support throughout the years, your trust, wise and motivational words and for believing in me.



Appendix A

Using Circadian Entrainment to Find Cryptic Clocks

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Published: Methods in Enzymology, Volume 55. 2015 Elsevier Inc.

Introduction

Colin Pittendrigh characterized circadian clocks according to a list of 16 "generalizations"[1]. We commonly use a distilled version of this list, describing them as having a ca. 24 h period in constant conditions, showing temperature compensation of that period and possessing a mechanism by which the free-running circadian rhythm is entrained to exactly 24 h by zeitgebers. A survey of the chronobiology literature quickly indicates that most experimental protocols in our research field rely on constant conditions, or at least an attempt to achieve them. The main reason to do this is that it simplifies experiments substantially. Scientific experimentation is all about standardizing and controlling conditions, usually keeping them unchanging. By adding entraining conditions (zeitgeber cycles) into the mix, we potentially introduce noise into our phenotype that stems from the physical stimulus (**Systematic name** masking), not from the biological clock. A near universal response to zeitgebers is masking[2,3], which is sometimes difficult to discern from unmasked entrainment. Activity in mice is an example of negative masking because it is suppressed by light[3]. frq RNA expression in Neurospora shows positive masking because it is induced with light exposure at any time of day[4], whereas the timing of FRQ protein expression and of clock-regulated conidiation (asexual spore formation) is an integration of photoperiod, cycle length (T), and free- running period[5]. FRQ accumulation depends on the structure of the zeitgeber (*i.e.* photoperiod and/or scotoperiod[6].

Despite many reasons to use free-running period as a clock output, we note that the clock exists in nature —and thus it evolved— in the entrained state. One might therefore expect that entrainment of circadian systems is a more robust property of circadian clocks. We also propose that a fuller understanding of the principles of entrainment —from cells to societies— will give important insights into other clock properties. We start by reviewing some entrainment protocols and what they can reveal about the circadian clock. We then describe two examples of how we used entrainment to show clock properties in the absence of a robust, free- running rhythm. Finally, we discuss additional implications of entrainment and constant condition protocols that are not actually constant.

1.1 Entrainment protocols

What are some of the entrainment methods that can be used to characterize circadian clocks? They all use the trick of changing zeitgeber structure to show a highly systematic set of responses. A very early conceptual breakthrough in circadian biology was the observation that the biological clock behaved like a physical oscillator in that it synchronizes or entrains differently if characteristics of the entraining oscillator change[7]. This is clearly an evolved response to, for example, seasons and other predictable features of the light/dark (LD) cycle.

Just as entraining cycles vary from day to day, free-running, circadian clocks in different individuals have different inherent properties (*e.g.* amplitude or period). This creates differences in entrainment between individuals in a given zeitgeber cycle, yielding a distribution of chronotypes[8,9]. Chronotype or phase of entrainment is systematic and depends on many aspects of the zeitgeber, as described below.

1.1.1 Photoperiod — Longer or shorter days, shorter or longer nights

One of the most basic and natural ways to characterize entrainment is to mimic the changing of the seasons by simply changing the photoperiod within a 24 h cycle. The relevance of this protocol lies in the profound, seasonal reproduction observed in some animals, in plants, and also in some fungi[10–12]. A masked response would follow the zeitgeber transition (either lights-on or lights-off), whereas a clock-regulated one will often change phase relationships, sometimes entraining relative to midnight or midday, regardless of photo- and scotoperiod. An example of these phenomena is found in Neurospora, where frequency (frq) mRNA transcription is induced every time the lights are turned on (masking), whereas FRQ protein is produced rapidly following lights-on in a short photoperiod (after a long night) or after as much as 8 h after lights-on in a long photoperiod entraining cycle (after a short night[6]). When spore formation in *Neurospora* was investigated, the entrained phase was highly systematic and could follow either a LD transition or midnight/midday, depending on for instance endogenous versus exogenous period lengths[5]. When mice are given light pulses of different length and are then analyzed for their phase response curves (suggesting entrainment characteristics), the midpoint of the light “pulse” —not the onset and not the offset—corresponds best to the resulting phase shift[13]. Interestingly, a large-scale study of the timing of human sleep behavior shows that people sleep at different times in summer and winter, indicating that we also show alternative entrainment as photoperiod/ scotoperiod changes[14].

1.1.2 Zeitgeber strength

It is clear that photoperiod and scotoperiod are both important for entrainment. One of the most dramatic experiments to show this compared activity in *Drosophila* held in LD cycles compared to those entrained in the more realistic light/moonlight cycles[15]. Flies exposed to light at night showed more overall activity with the two peaks of activity shifting earlier and later (they are generally biphasic, almost crepuscular) and the siesta becoming longer. The presence of light at night thus leads to different timing of and amount of activity/behavior. The implications for modern life, where we choose between sleeping with open curtains (with exposure to artificial lights) and closed curtains (shielding us from moonlight), are numerous. Interestingly, exposure to light at night has been suggested to be associated with various pathologies based on studies of shift workers[16,17]. Obviously, this form of light at night may be entirely different than what we are exposed to on a night with a full moon, presumably a condition that we have experienced over evolutionary time. Light at night may alter entrained phase by changing zeitgeber strength. The numerical difference between no light and a number of nanoeinsteins of light as occurs with a full moon is minimal but if this is expressed as a ratio or relative amount, the difference can be substantial. Part of the mechanism by which light at night may change entrainment could be by interfering with adaptation characteristics of the light input system. Exposure to light at night should change the responsivity of the system to light during the photoperiod. A quantitative demonstration of this principle can be seen in the type II phase response curves of Aschoff[18]. In these experiments, zeitgeber pulses were delivered in the first 24 h of constant conditions, expressly because the magnitude of the phase shift was much less in a light-adapted circadian system than if it was held in constant conditions for longer.

Zeitgeber strength can also be investigated experimentally by changing characteristics of the photoperiod —both amount and duration of light— on a dark background. The relevance to human behavior is evident, as exposure to high amplitude zeitgeber cycles (more daylight hours spent outside) results in an earlier chronotype[19].

1.1.3 Dawn and dusk transitions

The sudden nature of the lights/on and lights/off of a typical laboratory LD cycle ignores the potential complexity of signals in sunrise and sunset. In addition to a gradual change in the amount of total light, which would lead to different adaptation characteristics, the spectral quality of light changes drastically throughout dusk and dawn[20]. Experiments comparing dusk

and dawn transitions (quantitatively not qualitatively defined) in the lab relative to square-wave lighting conditions suggest that the former condition amounts to a stronger zeitgeber[21,22].

1.1.4 T cycles and phase angles

An important protocol in the circadian repertoire has been T cycles. “T” represents the exogenous cycle length. A given individual entrains later in a shorter cycle and earlier in a longer one[23–25]. This observation has led to a number of important extensions. First, a noncircadian, driven rhythm —one that is simply masking or responding directly to the zeitgeber transitions— will show no such phase angles and will synchronize to the same time relative to the zeitgeber, independent of cycle length. Second, the protocol can be turned around to understand what happens with entrainment of individuals with different free-running periods. In theory, individuals with shorter free-running periods should entrain earlier and those with longer ones should entrain later. This is generally correct[24,26] although there are other possibilities besides period (*e.g.* amplitude) that could explain an alternative entrained phase[27]. Finally, we also suggest that entrainment behavior in T cycles reveals the inherent clock property of robustness. A weak system would show drivenness, whereas only a robust one would change phase angles in concert with small changes in endogenous period. Eventually, lack of entrainment results as the range of entrainment is exceeded[23].

2. METHODS

What follows are two examples of using entrainment to deduce a circadian clock in organisms where a free-running rhythm has been difficult to discern. In both cases, protocols showing circadian entrainment were first optimized. Experiments to show free-running rhythms followed.

2.1 Saccharomyces cerevisiae

A haploid yeast strain was grown in 1 L chemostat cultures in YPD[28]. This is in contrast to most laboratory experiments where diploid *Saccharomyces cerevisiae* strains are grown continuously in Satroutdinov medium. In these other protocols, pH is strictly controlled[29–33] and dissolved oxygen (dO_2) in the media is the measured "output." Under these conditions, ultradian rhythms were observed. Rather, we primarily used extracellular pH to monitor the state of the yeast cultures, as *Gonyaulax* cultures showed a circadian oscillation in pH[34]. We determined optimal conditions in an iterative procedure. Chemostat cultures were subjected to two different temperature cycle conditions (18/25 ºC and 21/28 ºC) and to a variety of dilution rates. The changing phase angle between the pH rhythm and zeitgeber cycles was taken as an indication of a yeast circadian clock.

2.1.1 Identification of the optimal dilution rate

The temperature cycles consisted of an 11 h cold phase (18 ºC or 21 ºC) followed by a 60-min temperature transition to an 11 h warm phase (25 ºC or 28 ºC), which was followed by a 60 min temperature transition to the cold phase. The entire zeitgeber cycle was thus 24 h. This zeitgeber structure was implemented to avoid a "square wave" temperature cycle. Dilution rates describe the rate at which fresh media is pumped into the culture, while simultaneously an equivalent volume of conditioned or depleted media with cells is pumped out of the culture. Dilution rates of between 0.025 and 0.125 h 1 were tested (dilution rates refer to liters of media per hour.) In the cycles at the lower temperature, a great variety of waveforms were observed (Fig. 1). At 0.078 h 1, for instance, two peaks in the pH oscillation occurred. At higher dilution rates, the pH oscillation shifted earlier although the peak in hydrogen ion concentration still occurred in the warm phase.

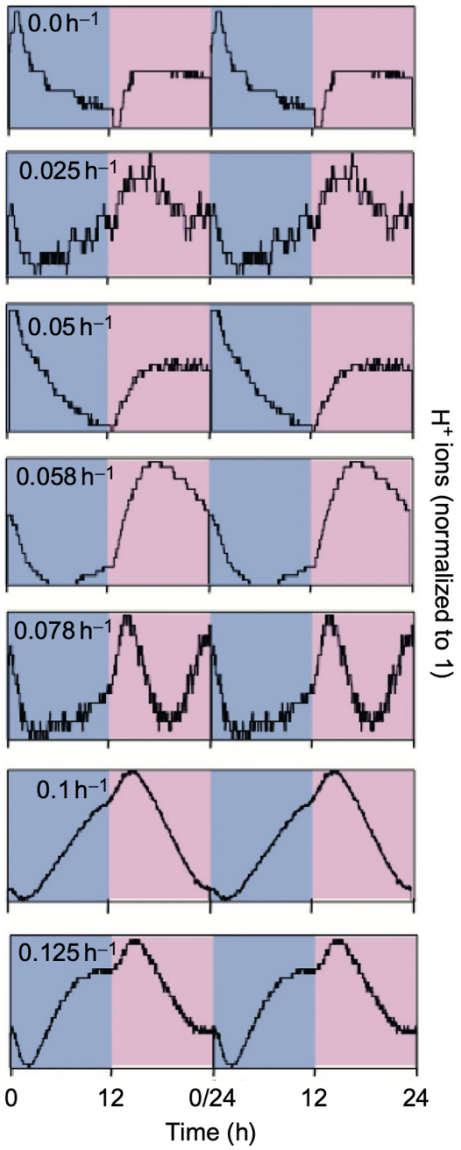

Figure 1. Yeast chemostat cultures in temperature cycles (18–25 °C). Oscillations in H^+ concentrations at different dilution rates. Gray and white areas indicate cool and warm phase (18 or 25 °C). The cycle was structured with 11 h at the cool temperature, a 60 min transition to the warm temperature, 11 h at the warm temperature and then a 60 min transition to the cool temperature. Here, the pH is converted to proton concentration. The H^+ oscillations were calculated without smoothing and trend correction. The dilution rate represents L/h (*e.g.* 0.078 h^{-1} means 0.078 L/h were circulated through the 1 L culture each hour). The data are double plotted.

Experiments at higher temperatures showed larger changes in phase angles (Fig. 2). Starting with the dilution rate of 0.054 h^{-1}, the phase of the pH oscillation became earlier with each increase in dilution rate. At the two highest dilution rates, the peak of the ion concentration switched to the cool phase. The experiments were additionally analyzed for robustness by comparing amplitude of the H^+ oscillation (Tables 1 and 2). The dilution rates that showed the most robust pH rhythm as defined by the r-value and the amplitude were 0.058 and 0.1 h^{-1} in the 18/25 ºC cycles and 0.1 h^{-1} in the 21/28 ºC cycle.

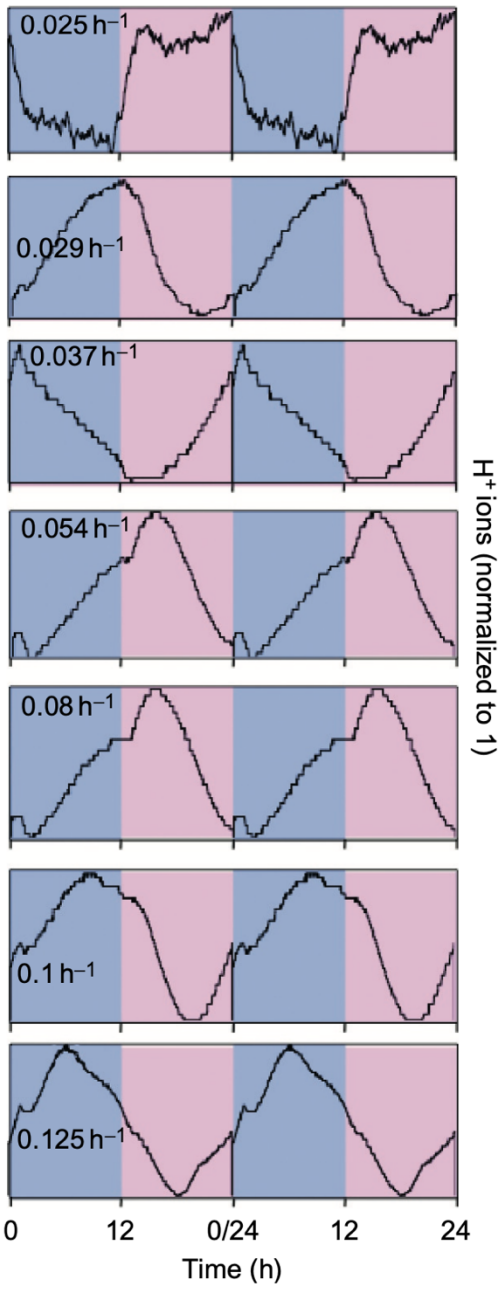

Figure 2. Yeast chemostat cultures in temperature cycles (21–28 °C). Oscillations in H^+ concentrations at different dilution rates in cycles at high temperature. See Fig. 1 for description of graphs except that here the temperatures used for the zeitgeber cycle are 21 and 28 °C. The data are double plotted.

Table 1. H^+ oscillations in a 12/12 h temperature cycle (18–25 ^oC) with various dilution rates

Dilution rate (h^{-1})	Amplitude (H^+ ions, M **X** 10^{-6})	r
0.025	9.71	0.95
0.050	18.16	0.92
0.058	31.15	0.99
0.078	5.02	0.78
0.078	34.63	1.00
0.125	9.08	0.97

Table 2. H^+ oscillations in a 12/12 h temperature cycle (21–28 ^oC) with various dilution rates

Dilution rate (h^{-1})	Amplitude (H^+ ions, M **X** 10^{-6})	r
0.025	7.23	0.89
0.029	18.08	0.99
0.037	114.26	0.99
0.054	26.24	0.99
0.080	26.13	0.99
0.100	27.32	0.99
0.125	26.40	0.99

2.1.2 T cycles

We investigated whether yeast synchronizes to symmetrical T cycles with systematically changing phase angles. In temperature cycles of 18/25 ºC with a dilution rate of 0.058 h^{-1}, fermenter cultures were subjected to T cycles with a period of 26, 24, 16, and 14 h (Fig. 3). The oscillation in ion concentration shifted later as the T cycles transitioned from long to short. The peak of the H^+ oscillation moves from the beginning of the warm phase in the 26 h cycle to the warm to cold transition in cycles of 14 h.

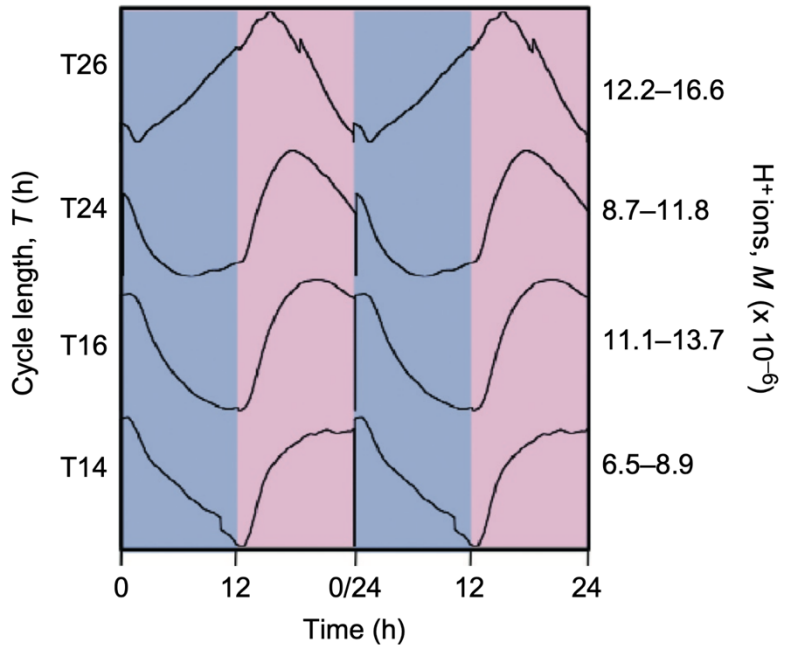

Figure 3. T cycles. Yeast chemostat cultures were held in temperature cycles of 18–25 °C with a dilution rate of 0.058 h^{-1}. Gray and white areas indicate cold and warm phase. The cycle lengths are indicated on the left and the concentrations of H^+ in each cycle are indicated on the right. The units on the x-axis represent each cycle, independent of its actual length, divided into 24 h of equal length. The phase of the H^+ oscillations changes systematically with cycle length. The waveform of the proton concentrations obviously changes in different cycle lengths. The data are double plotted.

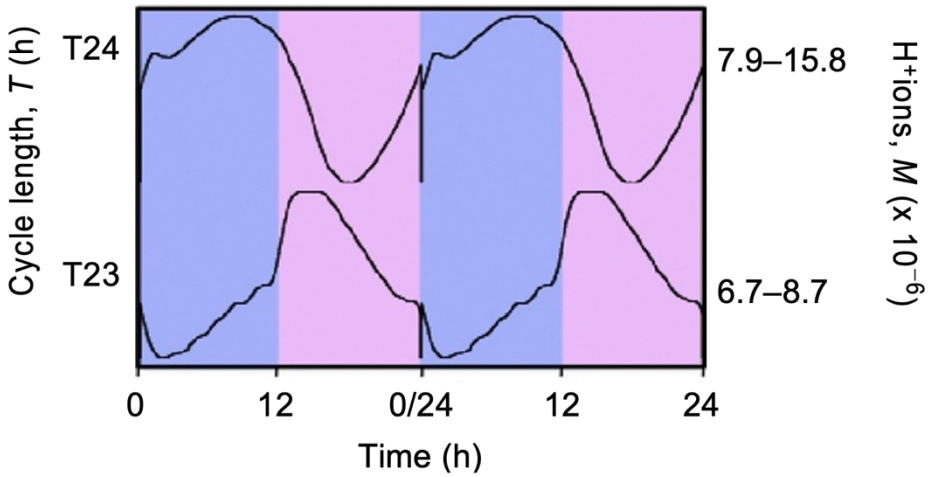

Figure 4. T cycles. Yeast chemostat cultures were held in temperature cycles of 21–28 °C with a dilution rate of 0.1 h^{-1}. Gray and white areas indicate cold and warm phase. The cycle lengths are indicated on the left and the concentrations of H^+ in each cycle are indicated on the right. The phase of the proton concentration oscillations varied with cycle length: the shorter cycle (T = 23 h) leads to a delayed phase. The data are double plotted.

We compared a 24 h cycling yeast culture with one in a 23 h cycle at the higher temperature conditions (21/28 ºC, dilution rate of 0.1 h^{-1}; Fig. 4). In this case, the phase of the oscillation shifted by much more than the 1^{-h} change in T. The peak of H^+ ions occurred in either the cold or the warm phase in the longer or shorter cycles, respectively. The troughs also switched position. In summary, in both conditions (18/25 ºC with a dilution rate of 0.058 h^{-1} and 21/28 ºC with a dilution rate of 0.1 h^{-1}), the phase of the pH rhythm changed with shortening of the T cycle by 1 h. Such an observation is assumed to indicate that there is an underlying oscillator —in yeast— that has a period that resonates with the entraining cycle. This oscillator must also possess sufficient robustness to deliver these very different phase angles in different T cycles. A very weak oscillator would give very small ones. In addition, for the entraining cycle to be "read" by the yeast, we assume that yeast possesses a zeitgeber sensory system, in this case, obviously for temperature.

2.1.3 Zeitgeber strength and entrainment of yeast

Zeitgeber strength was altered in two ways: (1) as referred to above, simply shifting the temperature cycle to lower mean level (18/25 ºC vs. 21/28 ºC) and (2) changing amplitude of temperature cycles (16/27, 18/25, 19/23, and 20/22 ºC; Fig. 5). The 20/22 ºC cycle failed to yield a rhythmic pH oscillation, suggesting that this zeitgeber condition is too weak to entrain a yeast circadian clock (data not shown). In the entraining temperature cycles with increasing amplitude, the phase of the pH rhythm becomes later. At lower temperatures, the phase of the H+ oscillation also moves later than the phase in higher temperatures. This suggests that, at least within the conditions tested, lower temperature (18/25 ºC) is perceived as a stronger zeitgeber than the higher temperature condition (21/28 ºC). That is, entrainment at the lower temperature is more like the higher amplitude temperature cycles.

2.1.4 Constant conditions: Free-running rhythm?

We interpreted the systematic entrainment response of the H^+ oscillations in the chemostat cultures as evidence of an underlying circadian clock. We further hypothesized that by entraining the yeast in the chemostat in temperature cycles, we might be synchronizing the system —within cells, organizing their metabolism and gene expression relative to the zeitgeber cycle, and between cells, bringing them to the same phase throughout the culture. In this case, one might expect to see free-running rhythms on release to constant conditions. The cultures were entrained for at least 1 week in 24 h temperature cycles until characteristic phase angles had been established. Once again, the pH was monitored. When cultures were released to the lower constant temperature (18 ºC), the pH rhythm damped after a single peak in H^+ (Fig. 6A). When the cultures were released to constant high temperature (25 ºC), they

showed one ca. 24 h oscillation before damping (Fig. 6B). This may mean that yeast has no machinery that supports a free-running rhythm, that the potential of the system to free-run under these conditions is limited, or that individual cells remain rhythmic but a free-running rhythm in the bulk culture rapidly damps. The latter condition would lead to an apparent arrhythmicity in constant conditions, as has been clearly documented in tissue culture cells[35].

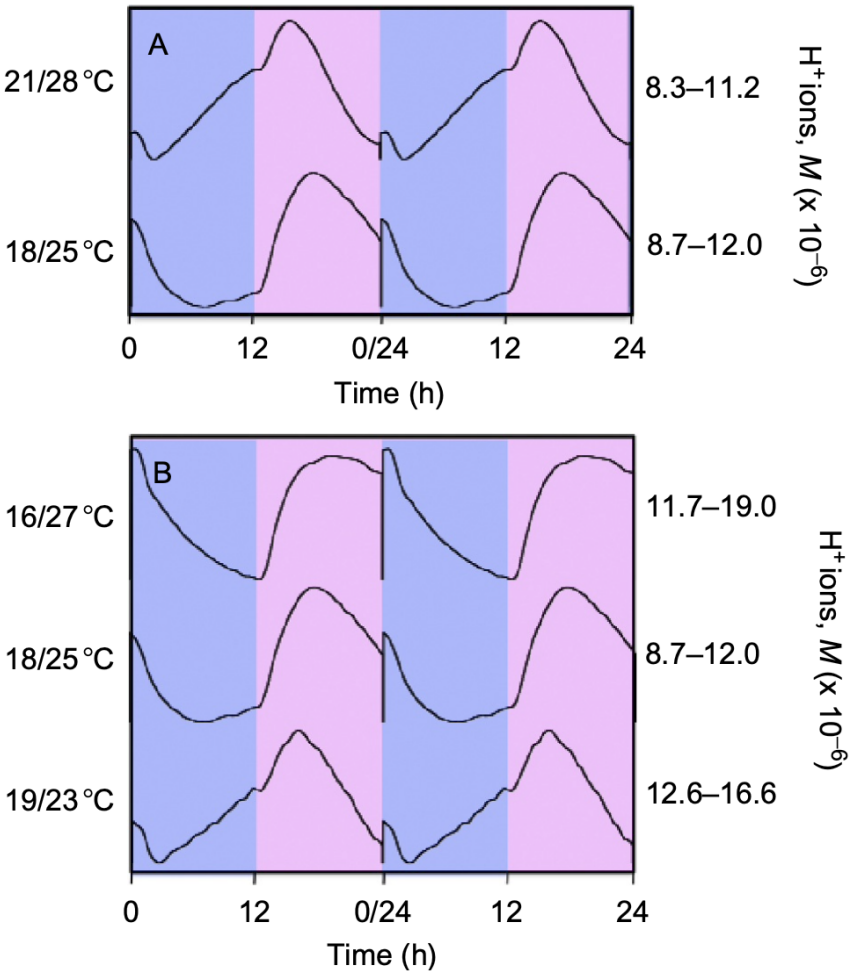

Figure 5. Phase relationships change with zeitgeber strength. Gray panels indicate cool phase; white panels indicate warm phase. (A) The phase of the H^+ oscillation in temperature cycles with the same amplitude but different absolute values. In lower temperature cycles (18–25 °C; lower panel), the peak of the H^+ oscillation shifted to the later position than the peak in warmer cycles (21–28 °C; upper panel). (B) The phase of the H^+ oscillation in temperature cycles with different amplitudes. The peak of the H^+ oscillation shifted to a later phase in the high-amplitude cycles. The data are double plotted.

2.2. *Caenorhabditis elegans*

Although many groups have published studies suggestive of a circadian clock in this nematode [36–41], quantitative protocols —which would be necessary to fully exploit this model system with genetic methods— are still lacking. As an approach to the study of circadian rhythms in *C. elegans*, we measured the daily regulation of olfaction. The circadian clock regulates sensory input pathways at many levels. Olfaction is under circadian control in insects and mammals [42,43]. The nematode *C. elegans* shows attractive and repulsive responses to a wide range of chemicals, and these are typically robust and lend themselves to quantitative assessment [44]. The olfactory response to a repellant (1-octanol) was measured in temperature cycles (12/12 h, 13/16 ºC) at hourly intervals on the sixth day of entrainment. At this time, the nematodes are young adults and have a completely developed nervous system. Under these conditions, there is a more pronounced response to 1-octanol during the warm phase of the cycle (Fig. 7A). The temperature transition from cold to warm coincided with increased olfactory responses. The stronger response in warm conditions might be a direct reaction to the temperature increase, be it due to a higher volatility of the chemical, a higher locomotor activity of the nematodes or other to factors that are not directly related to the olfactory sensitivity of the animal.

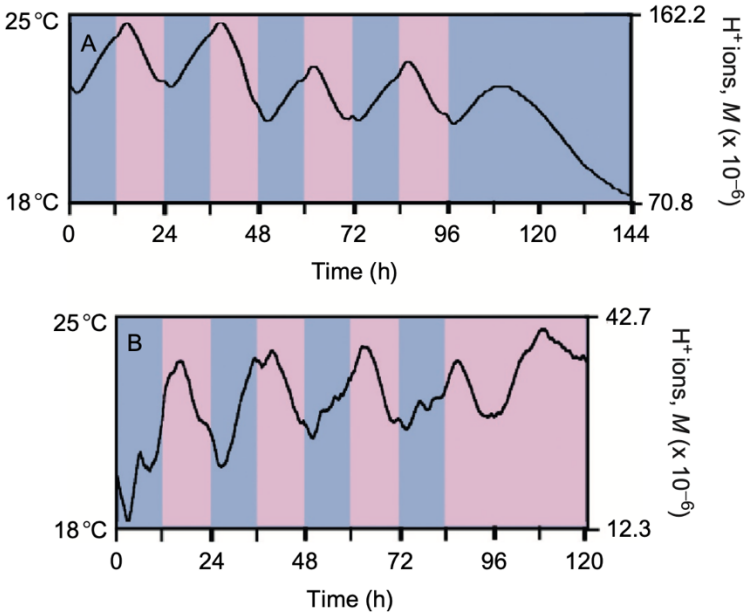

Figure 6. Entrained yeast cultures released to constant temperature. Chemostat cultures were entrained in 24 h temperature cycles of 18–25 °C with dilution rate of 0.1 h^{-1}. Gray and white areas indicate cold and warm phase. (A) A culture released to constant low temperature (18 °C). (B) A culture as released to constant high temperature (25 °C).

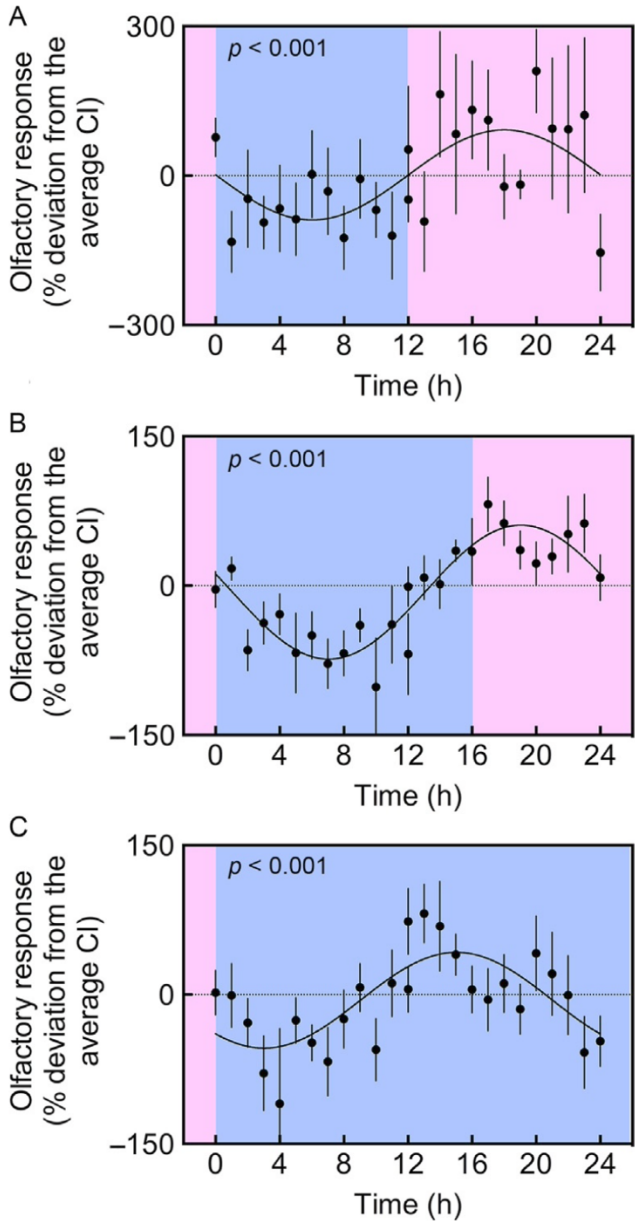

Figure 7. Olfaction in *C. elegans* in response to 1-octanol. Gray and white panels represent cold and warm phases of a temperature cycle (13/16 °C), respectively. The response to 1-octanol is measured as percentage deviation from the average chemotaxis index of the experiment. The timing of harvest (x-axis) is expressed as time from the end of the warm to cold transition at the beginning of the 6th cycle. A sinusoidal curve was fitted to the data (P<0.001) using Circwave. (A) Response to 1-octanol over 24 h in a 12/12 temperature cycle. (B) Response to 1-octanol over 24 h in a 16/8 cool/warm temperature cycle. (C) Response to 1-octanol over 24 h in constant conditions (14.5 °C).

We thus changed the structure of the zeitgeber cycle to 16/8 h (still at 13/16 ºC). A higher response at high temperature was again observed; however, importantly, the increase in the olfactory response starts during the cold period rather than with the transition to warm (Fig. 7B). This response pattern is typical for the quality called “anticipation” that is often referred to in circadian biology. Using the entrainment protocols to synchronize the system, we looked for

sustained rhythms upon release to a free run. The measurement of the response during the first day in constant conditions after five cycles of entrainment (16/8 h at 13/16 ºC) reveals a free-running rhythm with an earlier peak, suggesting a short free-running period and also the contribution of temperature-induced masking to the entrained phenotype (Fig. 7C).

Chapter 3

In this chapter, we discuss how and why entrainment can be used as a quantitative measure for circadian behavior, even in the absence of other overt clock properties. The clock evolved under highly predictable zeitgeber conditions and —as a phenotype— it may be more robust than free-running rhythms. The circadian system is tuned to accommodate and interpret changing seasons and environmental conditions using an active entrainment mechanism. Entrainment is furthermore the property that leads to chronotypes[14] or the differences in the timing of human behavior, and also jetlag conditions. Without entrainment, adjustment to shift work would be trivial and presumably less dangerous for health!

Competition experiments clearly demonstrate the power of zeitgeber conditions to drive selection for traits that concern temporal organization[45]. It is thus reasonable to hypothesize that all organisms continually exposed to 24-h zeitgebers will possess a system — a biological clock — for coping with daily exogenous structures. A battery of entrainment protocols exist that are useful for demonstrating systematic circadian entrainment — different states or phases for different conditions. Our key assumption in applying entrainment protocols to discover circadian clocks is the expectation that the entrained phase changes with each alteration in zeitgeber structure or amount. In the absence of the systematic changes, we would assume that we are not using conditions that support circadian entrainment. Conversely, when we do see phase angles, we hypothesize that we are synchronizing a circadian clock.

Here, we describe applying entrainment protocols to valuable genetic model systems (*C. elegans* and *S. cerevisiae*) that are not yet in the circadian toolkit. Anecdotally, over the years many attempts have been made to show free-running, circadian rhythms in these organisms, only yielding limited success. In the case of the nematodes, subtle rhythms were apparent [36,37,39–41] but these were generally not robust and robustness of phenotype is a requirement for full exploitation of genetics methods. A rhythm in growth rate was observed in yeast when it was cultured at very low temperatures (12 ºC) in bulk cultures in an LD cycle[46]. The rhythm persisted when constant conditions were initiated. Unfortunately, these experiments were

never repeated. Over the years, we have experimented with many methods for culturing *S. cerevisiae* that would allow visualization of circadian or clock-regulated properties. Just to list a few of these, we tried to see morphological differences in yeast cultures that were induced to grow as pseudohyphae by stressing nutritional state[47]. Changing nutrition had revealed a new set of clock-regulated behaviors in *Gonyaulax*[48]. We monitored pH of bulk cultures of unicellular yeast in entraining cycles and in constant conditions, most successfully growing *Neurospora* instead of our yeast. We tried a molecular approach, reasoning (in the 1990s) that the heat and cold shock system would be involved in normal circadian entrainment with temperature but failed to see systematic regulation of these genes in our cultures (all unpublished data). Without having any good clock gene candidates in yeast, the development of good protocols also lacked a genetic approach.

When two publications showed ultradian rhythms in yeast (with periods of 40 min and 4–5 h)[31,33,49] we hypothesized that these might be related to circadian oscillations. Using the same culturing conditions as Murray et al., we were able to show ultradian rhythms in budding yeast. The conditions used by Tu *et al.*[33]. require air pressure levels that are incompatible with our equipment. Interestingly, by increasing pressure within equipment specifications, we successfully extended the 40 min cycle of Murray *et al*[31]. into the hours range. Additional adjustment of the culturing conditions failed to extend the ultradian rhythms to circadian ones. Hence, we turned to ca. 24 h entrainment protocols.

In these protocols, yeast showed circadian entrainment but failed to show robust, self-sustained circadian rhythms. Why is this? Formally speaking, even systems that show systematic entrainment need not have a self-sustained free-running rhythm. Anticipation of a zeitgeber could be programmed based on the previous zeitgeber exposure. There are other explanations that may be more likely. For instance, the growth conditions for most model organisms in the lab are drastically different than those in nature. We rarely consider the ecology of the organism when designing our artificial growth media in the lab. Rather, considerations include short generation time and cost control. It is worth noting that in well-studied circadian model systems, the free-running rhythm is dependent on the particular set of constant conditions. In constant darkness (DD) the period is different than in dim LL, and in bright LL almost all nonphotosynthetic organisms become arrhythmic[50,51]. Changing nutritional conditions can lead to changes in the period, despite metabolic compensation[52]. Similarly, changes in temperature lead to changes in period despite temperature compensation[53–55].

It may be time to reconsider how or why we use a free-running rhythm. If there is no single free-running rhythm (as elaborated above), then what is the value therein? For modeling purposes, why is the period in DD used rather than in dim LL? After all, in the entrained state, there is some darkness and some light. Therefore, which is relevant? Furthermore, many protocols that claim constant conditions cannot deliver this. Consider the case of mice. We know much about the genetics of free-running rhythms due to the robustness of the behavioral clock phenotype in this tiny workhorse of a model organism. The catalog of free-running rhythms includes that of body temperature. Thus, even in animals in DD, all tissues will receive ca. 24 h temperature cycles. Thus the liver, heart, lung, etc., are harvested at “circadian” timepoints from these animals will be experiencing a physiologically relevant entraining temperature cycle, not constant conditions in any sense. Further- more, the internal zeitgeber strength will be altered on the days when the system is released from an LD cycle to DD, leading to new internal phase relationships. It may be that most of these experiments are harvested at a time when phases are volatile — in transition — and much less stable than if they had been maintained in a standardized LD cycle. We predict that this state would lead to a loss of information through underrepresentation of rhythmic genes as they shift their entrained state from one set of zeitgeber cycles and amplitudes to another. Genes that might be robustly rhythmic may be judged nonrhythmic simply due to lack of statistical power in a system that has more noise due to a less stable, transition state.

Given the importance of entrainment of the circadian system for the health and wellbeing of living organisms, it is important to look critically at our experimental paradigms and understand what they are delivering to us. We suggest that standardized entrainment protocols should replace free-running conditions, a practice that will deliver at once more relevant information in protocols that actually are what they are supposed to be.

Acknowledgments

We thank Till Roenneberg, Serge Daan, and Gabrielle Mazzotta for many helpful discussions. Our work is supported by the Dutch Science Foundation (NWO), the Rosalind Franklin Fellowship Program of the University of Groningen, and the Ludwig- Maximilians-Univeristat in Munich.

Literature references

1. PITTENDRIGH, C. S. Circadian rhythms and the circadian organization of living systems. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/SQB.1960.025.01.015.
2. Mrosovsky, N., Lucas, R. J. & Foster, R. G. Persistence of masking responses to light in mice lacking rods and cones. *Journal of Biological Rhythms* (2001) doi:10.1177/074873001129002277.
3. Mrosovsky, N. Masking: History, definitions, and measurement. *Chronobiology International* (1999) doi:10.3109/07420529908998717.
4. Crosthwaite, S. K., Loros, J. J. & Dunlap, J. C. Light-induced resetting of a circadian clock is mediated by a rapid increase in frequency transcript. *Cell* (1995) doi:10.1016/S0092-8674(05)80005-4.
5. Rémi, J., Merrow, M. & Roenneberg, T. A circadian surface of entrainment: Varying T, τ, and Photoperiod in Neurospora crassa. *J. Biol. Rhythms* 25, (2010).
6. Tan, Y., Dragovic, Z., Roenneberg, T. & Merrow, M. Entrainment dissociates transcription and translation of a circadian clock gene in Neurospora. *Curr. Biol.* (2004) doi:10.1016/j.cub.2004.02.035.
7. Bruce, V. Environmental entrainment of circadian rhythms. in *Cold Spring Harbor Symp. Quant. Biol.* 29–48 (1960).
8. Dominoni, D. M., Helm, B., Lehmann, M., Dowse, H. B. & Partecke, J. Clocks for the city: Circadian differences between forest and city songbirds. *Proc. R. Soc. B Biol. Sci.* (2013) doi:10.1098/rspb.2013.0593.
9. Roenneberg, T., Wirz-Justice, A. & Merrow, M. Life between clocks: Daily temporal patterns of human chronotypes. *J. Biol. Rhythms* (2003) doi:10.1177/0748730402239679.
10. Bunning, E. Opening Address: Biological Clocks. *Cold Spring Harb. Symp. Quant. Biol.* (1960) doi:10.1101/sqb.1960.025.01.003.
11. Elliott, J. A. & Goldman, B. D. Seasonal Reproduction. in *Neuroendocrinology of Reproduction: Physiology and Behavior* (ed. Adler, N. T.) 377–423 (Springer US, 1981). doi:10.1007/978-1-4684-3875-8_12.
12. Roenneberg, T. & Merrow, M. Seasonality and photoperiodism in fungi. *Journal of Biological Rhythms* (2001) doi:10.1177/074873001129001999.
13. Comas, M., Beersma, D. G. M., Spoelstra, K. & Daan, S. Phase and period responses of the circadian system of mice (Mus musculus) to light stimuli of different duration. *J. Biol. Rhythms* (2006) doi:10.1177/0748730406292446.

14. Roenneberg, T., Kumar, C. J. & Merrow, M. The human circadian clock entrains to sun time. *Current Biology* (2007) doi:10.1016/j.cub.2006.12.011.
15. Bachleitner, W., Kempinger, L., Wülbeck, C., Rieger, D. & Helfrich-Förster, C. Moonlight shifts the endogenous clock of Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* (2007) doi:10.1073/pnas.0606870104.
16. Haus, E. L. & Smolensky, M. H. Shift work and cancer risk: Potential mechanistic roles of circadian disruption, light at night, and sleep deprivation. *Sleep Medicine Reviews* (2013) doi:10.1016/j.smrv.2012.08.003.
17. Schernhammer, E. S. & Thompson, C. A. Light at night and health: The perils of rotating shift work. *Occupational and Environmental Medicine* (2011) doi:10.1136/oem.2010.058222.
18. School, F. S., Aschoff, J., Organization., N. A. T. & Division., S. A. Circadian clocks : proceedings of the Feldafing Summer School, 7-18 September 1964. in (North-Holland Pub. Co., 1965).
19. Roenneberg, T. & Merrow, M. Entrainment of the human circadian clock. in *Cold Spring Harbor Symposia on Quantitative Biology* (2007). doi:10.1101/sqb.2007.72.043.
20. Hailman, J. P. Ecology of Vision The Ecology of Vision J. N. Lythgoe. *Bioscience* (1981) doi:10.2307/1308439.
21. Boulos, Z., Macchi, M. M. & Terman, M. Twilights widen the range of photic entrainment in hamsters. *J. Biol. Rhythms* (2002) doi:10.1177/074873002129002654.
22. Comas, M. & Hut, R. A. Twilight and photoperiod affect behavioral entrainment in the house mouse (Mus musculus). *J. Biol. Rhythms* (2009) doi:10.1177/0748730409343873.
23. Aschoff, J. Circadian Rhythms Within and Outside Their Ranges of Entrainment. in (1978). doi:10.1007/978-3-642-66981-1_28.
24. Merrow, M., Brunner, M. & Roenneberg, T. Assignment of circadian function for the Neurospora clock gene frequency. *Nature* (1999) doi:10.1038/21190.
25. Hoffmann, K. Overt circadian frequencies and circadian rule. *J. Aschoff (ed.), Circadian clocks Amsterdam North-holl. Publ. Co* 87–94 (1965).
26. Duffy, J. F., Dijk, D. J., Hall, E. F. & Czeisler, C. A. Relationship of endogenous circadian melatonin and temperature rhythms to self-reported preference for morning or evening activity in young and older people. *J. Investig. Med.* (1999).
27. Granada, A. E., Bordyugov, G., Kramer, A. & Herzel, H. Human Chronotypes from a Theoretical Perspective. *PLoS One* (2013) doi:10.1371/journal.pone.0059464.
28. Eelderink-Chen, Z. *et al.* A circadian clock in Saccharomyces cerevisiae. *Proc. Natl.*

Acad. Sci. U. S. A. 107, 2043–2047 (2010).

29. Keulers, M., Suzuki, T., Satroutdinov, A. D. & Kuriyama, H. Autonomous metabolic oscillation in continuous culture of Saccharomyces cerevisiae grown on ethanol. *FEMS Microbiol. Lett.* (1996) doi:10.1016/0378-1097(96)00277-7.
30. Lloyd, D., Lemar, K. M., Salgado, L. E. J., Gould, T. M. & Murray, D. B. Respiratory oscillations in yeast: Mitochondrial reactive oxygen species, apoptosis and time; a hypothesis. *FEMS Yeast Res.* (2003) doi:10.1016/S1567-1356(03)00071-0.
31. Murray, D. B., Engelen, F. A. A., Keulers, M., Kuriyama, H. & Lloyd, D. NO+, but not NO., inhibits respiratory oscillations in ethanol-grown chemostat cultures of Saccharomyces cerevisiae. *FEBS Lett.* (1998) doi:10.1016/S0014-5793(98)00777-7.
32. Satroutdinov, A. D., Kuriyama, H. & Kobayashi, H. Oscillatory metabolism of Saccharomyces cerevisiae in continuous culture. *FEMS Microbiol. Lett.* (1992) doi:10.1111/j.1574-6968.1992.tb05525.x.
33. Tu, B. P., Kudlicki, A., Rowicka, M. & McKnight, S. L. Cell biology: Logic of the yeast metabolic cycle: Temporal compartmentalization of cellular processes. *Science (80-.).* (2005) doi:10.1126/science.1120499.
34. Eisensamer, B. & Roenneberg, T. Extracellular pH Is under Circadian Control in Gonyaulax polyedra and Forms a Metabolic Feedback Loop. *Chronobiol. Int.* (2004) doi:10.1081/CBI-120027979.
35. Leise, T. L., Wang, C. W., Gitis, P. J. & Welsh, D. K. Persistent cell-autonomous circadian oscillations in fibroblasts revealed by six-week single-cell imaging of PER2::LUC bioluminescence. *PLoS One* (2012) doi:10.1371/journal.pone.0033334.
36. Kippert, F., Saunders, D. S. & Blaxter, M. L. Caenorhabditis elegans has a circadian clock [2]. *Current Biology* (2002) doi:10.1016/S0960-9822(01)00670-4.
37. Migliori, M. L. *et al.* Daily variation in melatonin synthesis and arylalkylamine N-acetyltransferase activity in the nematode Caenorhabditis Elegans. *J. Pineal Res.* (2012) doi:10.1111/j.1600-079X.2011.00969.x.
38. Migliori, M. L., Simonetta, S. H., Romanowski, A. & Golombek, D. A. Circadian rhythms in metabolic variables in Caenorhabditis elegans. *Physiol. Behav.* (2011) doi:10.1016/j.physbeh.2011.01.026.
39. Saigusa, T. *et al.* Circadian behavioural rhythm in Caenorhabditis elegans [1]. *Current Biology* (2002) doi:10.1016/S0960-9822(01)00669-8.
40. Simonetta, S. H., Romanowski, A., Minniti, A. N., Inestrosa, N. C. & Golombek, D. A. Circadian stress tolerance in adult Caenorhabditis elegans. *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* (2008) doi:10.1007/s00359-008-0353-z.
41. Sengupta, P. *et al.* Genome-wide analysis of light- and temperature-entrained

circadian transcripts in Caenorhabditis elegans. *PLoS Biol.* (2010) doi:10.1371/journal.pbio.1000503.

42. Granados-Fuentes, D., Tseng, A. & Herzog, E. D. A circadian clock in the olfactory bulb controls olfactory responsivity. *J. Neurosci.* (2006) doi:10.1523/JNEUROSCI.3445-06.2006.
43. Krishnan, B., Dryer, S. E. & Hardin, P. E. Circadian rhythms in olfactory responses of Drosophila melanogaster. *Nature* (1999) doi:10.1038/22566.
44. Bargmann, C. I., Hartwieg, E. & Horvitz, H. R. Odorant-selective genes and neurons mediate olfaction in C. elegans. *Cell* (1993) doi:10.1016/0092-8674(93)80053-H.
45. Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Johnson, C. H. Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* (1998) doi:10.1073/pnas.95.15.8660.
46. Edmunds, L. N., Apter, R. I., Rosenthal, P. J., Shen, W. -K & Woodward, J. R. LIGHT EFFECTS IN YEAST: PERSISTING OSCILLATIONS IN CELL DIVISION ACTIVITY AND AMINO ACID TRANSPORT IN CULTURES OF SACCHAROMYCES CEREYISIAE ENTRAINED BY LIGHT-DARK CYCLES. *Photochem. Photobiol.* (1979) doi:10.1111/j.1751-1097.1979.tb07186.x.
47. Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: Regulation by starvation and RAS. *Cell* (1992) doi:10.1016/0092-8674(92)90079-R.
48. Rehman, J., Lindgren, K., & Roenneberg, T. No TitleLight and nitrate: Interactions of two zeitgebers in the marine unicellular alga Gonyaulax. *Pap. Present. Meet. Soc. Res. Biol. Rhythm. Amelia Island, FL, USA* (1996).
49. Murray, D. B., Klevecz, R. R. & Lloyd, D. Generation and maintenance of synchrony in Saccharomyces cerevisiae continuous culture. *Exp. Cell Res.* (2003) doi:10.1016/S0014-4827(03)00068-5.
50. ROENNEBERG, T. & HASTINGS, J. W. ARE THE EFFECTS OF LIGHT ON PHASE AND PERIOD OF THE Gonyaulax CLOCK MEDIATED BY DIFFERENT PATHWAYS? *Photochem. Photobiol.* (1991) doi:10.1111/j.1751-1097.1991.tb03665.x.
51. Somers, D. E., Devlin, P. F. & Kay, S. A. Phytochromes and cryptochromes in the entrainment of the Arabidopsis circadian clock. *Science (80-.).* (1998) doi:10.1126/science.282.5393.1488.
52. Dragovic, Z., Tan, Y., Görl, M., Roenneberg, T. & Merrow, M. Light reception and circadian behavior in ‘blind’ and ‘clock-less’ mutants of Neurospora crassa. *EMBO J.* (2002) doi:10.1093/emboj/cdf377.

53. Barrett, R. K. & Takahashi, J. S. Temperature compensation and temperature entrainment of the chick pineal cell circadian clock. *J. Neurosci.* 15, 5681–5692 (1995).
54. Dibner, C. *et al.* Circadian gene expression is resilient to large fluctuations in overall transcription rates. *EMBO J.* (2009) doi:10.1038/emboj.2008.262.
55. Gardner, G. F. & Feldman, J. F. Temperature Compensation of Circadian Period Length in Clock Mutants of Neurospora crassa. *Plant Physiol.* 68, 1244–1248 (1981).