Geldner, N., Fernie, A.R., *et al*. (2012). AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr. Biol. *22*, 1207–1212.
12. Berthet, S., Demont-Caulet, N., Pollet, B., Bidzinski, P., Cezard, L., Le Bris, P., Borrega, N., Herve, J., Blondet, E., Balzergue, S., *et al*. (2011). Disruption of LACCASE4 and 17 results in tissue- specific alterations to lignification of *Arabidopsis thaliana* stems. Plant Cell *23*, 1124–1137.
13. Lu, S., Li, Q., Wei, H., Chang, M.-J., Tunlaya-Anukit, S., Kim, H., Liu, J., Song, J., Sun, Y.-H., Yuan, L., *et al*. (2013). Ptr-miR397a is a negative regulator of laccase genes affecting lignin content in *Populus trichocarpa*. Proc. Natl. Acad. Sci. USA *110*, 10848–10853.
14. Pesquet, E., Zhang, B., Gorzsás, A., Puhakainen, T., Serk, H., Escamez, S., Barbier, O., Gerber, L., Courtois-Moreau, C., Alatalo, E., *et al*. (2013). Non-cell-autonomous postmortem lignification of tracheary elements in Zinnia elegans. Plant Cell *25*, 1314–1328.

Division of Plant Sciences, College of Life Sciences, University of Dundee at the James Hutton Institute, Dundee DD2 5DA, UK.
E-mail: c.halpin@dundee.ac.uk

http://dx.doi.org/10.1016/j.cub.2013.10.033

Circadian Rhythms: Hijacking the Cyanobacterial Clock

Using basic research to advance a practical application, a recent study demonstrates that the circadian clock in cyanobacteria can be 'reprogrammed' to improve yields of heterologous protein production — a green future surely beckons.

Nathaniel P. Hoyle and John S. O'Neill*

The production of limitless carbon-free energy is a long-sought dream of scientists and politicians alike. One strategy for achieving this aim is the production of hydrogen by photosynthetic microorganisms — harnessing the effectively limitless power of the sun to power our cars, toasters and PCR machines. It may be tempting to think of host expression systems as miniature factories given over entirely to the production of our molecule of interest. However, the biological nature of the host must be taken into account if we are to maximize productivity. The circadian rhythm, a cell-intrinsic ~24-hour biological clock found in most organisms, is one such aspect that has received scant attention but is likely to be of particular importance to photosynthetic host systems. In this issue of *Current Biology* Xu *et al.* [1] describe how our knowledge of the *Synechococcus elongatus* circadian clock can be leveraged to improve the production of exogenous proteins, including those involved in the production of hydrogen.

Co-Opting the KaiABC Oscillator

S. elongatus has been a useful model for understanding circadian regulation of gene expression, independently of its potential utility in biotechnology [2]. The *S. elongatus* circadian clockwork is based upon rhythms of phosphorylation in the protein KaiC and its regulatory binding partners KaiA and KaiB (Figure 1A). In its simplest form the KaiABC complex will undergo circadian rhythms in autophosphorylation and dephosphorylation when its constituents are mixed *in vitro* in the presence of ATP [3]. *In vivo,* this basic biochemical oscillation is elaborated by autoregulation of the KaiABC gene cluster via accessory transcription factors [4]. This KaiABC oscillator also feeds into the genome-wide regulation of transcription to generate rhythmic outputs from the circadian clock [5,6]. Genes which accumulate during the day and peak at dusk are known as Class I genes. Those with an opposing peak at dawn are termed Class II. Overexpression of the KaiA protein enhances gene expression from the KaiBC promoter [4], giving the cells an apparently continuous dusk signal. Conversely, expression of KaiC has been claimed to globally repress gene expression [7]. With this in mind, the production of potentially useful gene products might be enhanced throughout the night by exogenous overexpression of KaiA — effectively holding the cells in a permanent dusk-like state (Figure 1B). The goal: to turn the circadian *S. elongatus* protein factory into a round-the-clock production line for heterologous protein.

In their efforts to stably enhance gene expression, Xu *et al.* found that overexpression of KaiA (KaiA-OX) in constant light conditions causes about 20% of genes to be up-regulated at the transcript level. Around 12% were down-regulated by KaiA-OX. Interestingly the over-expression of KaiC has the opposite effect on individual genes. Referencing Taoism, the authors describe this reciprocal regulation by KaiA and KaiC as a Yin–Yang model of regulation. Circadian rhythms of transcript abundance are produced by induction and repression of specific loci at opposing phases of the circadian cycle — presumably enhancing the amplitude of rhythmic gene expression in wild-type cells.

The native NAD/NADP-utilizing hydrogenase in *S. elongatus* displays a peak-at-dusk (Class I) profile and has enhanced expression during KaiA-OX. This raises the tantalizing possibility of increasing hydrogenase activity by pausing the circadian oscillator at subjective dusk when these genes are most prolifically transcribed.

When Is a 'Neutral Site' Not a Neutral Site?

Hydrogenases have evolved naturally in cyanobacteria to catalyze the reversible reaction $2H^+ + 2e^- \rightarrow H_2$ using NADH, NADPH and ferredoxin as electron donors. The direct production of H_2 circumvents the inefficient Calvin cycle and offers the prospect of highly efficient fuel production. Currently, yield is poor due to the oxygen sensitivity of hydrogenases and energetic favorability of H_2 uptake over production [8].

The RC41 *S. elongatus* strain was developed by Weyman *et al.* in an effort to optimize H_2 production from cyanobacteria [9]. The endogenous bidirectional hydrogenase HoxYH was deleted and the HynSL hydrogenase and accessory gene cluster from *Alteromonas macleodii* expressed

under the control of an inducible promoter at neutral site NS I. Unfortunately, RC41 has rather disappointing levels of hydrogenase activity owing to poor expression of hydrogenase and its accessory proteins [9].

An intriguing observation by Xu *et al.* revealed that several constitutive promoters from *Escherichia coli* behave in a circadian manner when expressed in *S. elongatus* from 'Neutral Sites' NS I and NS II. Neutral Sites are so called because their loci can be disrupted by incorporation of exogenous DNA with no aberrant phenotype in the host cell. As expression varies over the circadian cycle, it is perhaps confusing to label them neutral sites [10]. In particular, the *conIIp* constitutive promoter drives strong rhythmic expression of the luxAB reporter from NS I or NS II in *S. elongatus*, which has a peak-at-dusk expression profile similar to that observed in KaiA-OX-induced genes. KaiA-OX potentiates luxAB activity both in constant light and constant darkness, which suggests that NS I and NS II are circadian-regulated loci.

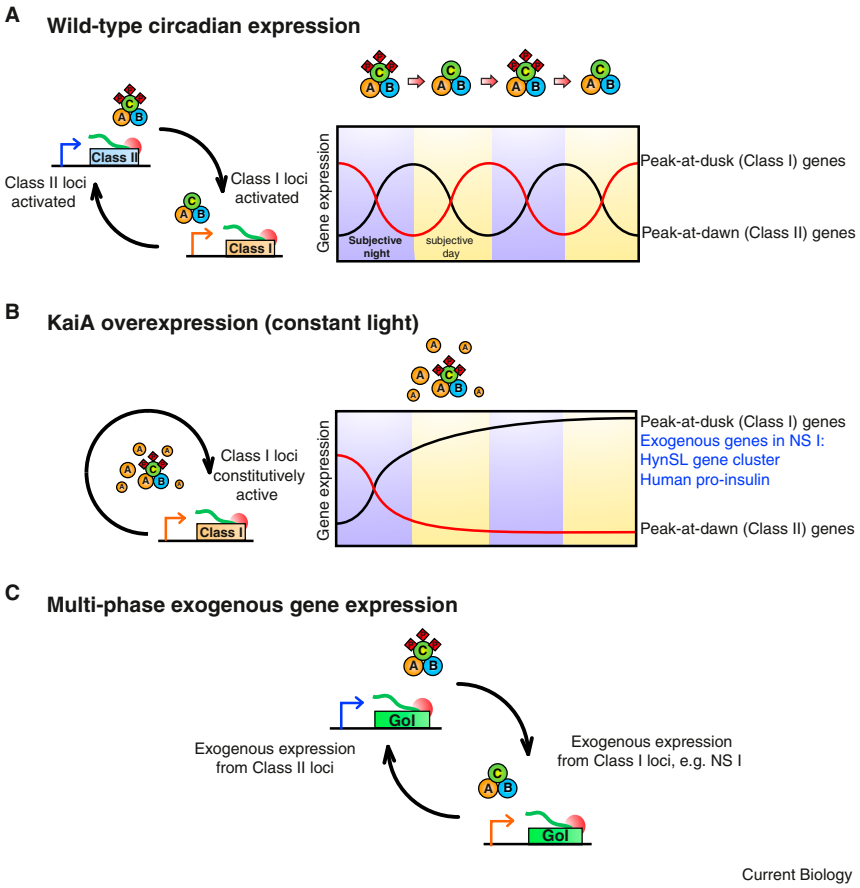

Figure 1. Exogenous gene expression in cyanobacteria can be increased by strategies that exploit the endogenous circadian clock.

(A) Circadian oscillations in gene expression are linked to the phosphorylation–dephosphorylation cycle of the KaiABC complex. Class I and Class II genes oscillate out of phase with one another, and maintain their rhythm during constant light. (B) When KaiA is overexpressed a subset of genes including Class I genes are expressed stably. This strategy was successfully employed to improve expression of exogenous HynSL hydrogenase and human pro-insulin. (C) Another potential strategy for round-the-clock expression of exogenous genes would be to incorporate genes at multiple sites with varying Class I and Class II character — enabling stable expression of any gene of interest (GoI) without the need to disrupt the circadian rhythm of the host by KaiA overexpression.

Towards Round-the-Clock Hydrogen Production

The observed increase in endogenous hydrogenase activity during KaiA overexpression in constant light conditions, coupled with the possibility of enhanced NS I activity during KaiA-OX prompted Xu *et al.* to optimize HynSL production in RC41.

Overexpression of KaiA in RC41 improved the expression of the HynL and increased H_2 production two-fold. This dramatic improvement in the production of H_2 is unlikely to spark an immediate clean energy revolution, however, as the hydrogenase activity was still lower than that observed in native *S. elongatus*. Clearly, there exist other limiting factors that need to be overcome before *S. elongatus* becomes a viable system for H_2 production.

Accordingly, when the KaiA-overexpressing *S. elongatus* was used to produce human pro-insulin fused to glutathione S-transferase, the results were more impressive — achieving up to a five-fold increase relative to cells not overexpressing KaiA. This demonstrates that pausing the circadian clock in a locus' most productive time window is a viable strategy for maximizing the production of exogenous proteins. Interestingly, the potentiation of gene expression by KaiA-OX was maximal during the dark phase of the light:dark cycle, which is unexpected given that NS I integrated genes normally behave like Class I genes. It may be that the general inactivity of *S. elongatus* gene expression machinery during the dark phase generates spare capacity for exogenous protein expression and thus allows it to occur more efficiently.

The attempt to leverage our knowledge of the circadian cycle to enhance exogenous gene expression has yielded interesting but perhaps unexpected results. The efficient production of human pro-insulin during the subjective night may hint that other heterologous proteins might be better suited to expression at this time. Perhaps HynSL production would be more efficiently produced from a KaiC-OX-induced locus or when present at multiple temporally divergent genetic loci — allowing an even greater production of protein throughout the circadian cycle without the need for the permanent 'on' switch of KaiA-OX (Figure 1C). Certainly, however, this study clearly demonstrates that there is immediate utility in considering the influence of a cell's endogenous clock upon the expression of exogenous gene products.

References

1. Xu, Y., Weyman, P.D., Umetani, M., Xiong, J., Qi, X., Xu, Q., Iwasaki, H., and Johnson, C.H. (2013). Circadian Yin-Yang regulation and its manipulation to globally reprogram gene expression. Curr. Biol. *23*, 2365–2374.
2. Johnson, C.H., Stewart, P.L., and Egli, M. (2011). The cyanobacterial circadian system: from biophysics to bioevolution. Annu. Rev. Biophys. *40*, 143–167.
3. Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T., and Kondo, T. (2005). Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. Science *308*, 414–415.
4. Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C.R., Tanabe, A., Golden, S.S., Johnson, C.H., and Kondo, T. (1998). Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science *281*, 1519–1523.
5. Liu, Y., Tsinoremas, N.F., Johnson, C.H., Lebedeva, N.V., Golden, S.S., Ishiura, M., and Kondo, T. (1995). Circadian orchestration of gene expression in cyanobacteria. Genes Dev. *9*, 1469–1478.
6. Nair, U., Ditty, J.L., Min, H., and Golden, S.S. (2002). Roles for sigma factors in global circadian regulation of the cyanobacterial genome. J. Bacteriol. *184*, 3530–3538.
7. Nakahira, Y., Katayama, M., Miyashita, H., Kutsuna, S., Iwasaki, H., Oyama, T., and Kondo, T. (2004). Global gene repression by KaiC as a master process of prokaryotic circadian system. Proc. Natl. Acad. Sci. USA *101*, 881–885.
8. Carrieri, D., Wawrousek, K., Eckert, C., Yu, J., and Maness, P.C. (2011). The role of the bidirectional hydrogenase in cyanobacteria. Bioresour. Technol. *102*, 8368–8377.
9. Weyman, P.D., Vargas, W.A., Tong, Y., Yu, J., Maness, P.C., Smith, H.O., and Xu, Q. (2011). Heterologous expression of Alteromonas macleodii and Thiocapsa roseopersicina [NiFe] hydrogenases in Synechococcus elongatus. PLoS ONE *6*, e20126.
10. Andersson, C.R., Tsinoremas, N.F., Shelton, J., Lebedeva, N.V., Yarrow, J., Min, H., and Golden, S.S. (2000). Application of bioluminescence to the study of circadian rhythms in cyanobacteria. Methods Enzymol. *305*, 527–542.

MRC Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge, CB2 0QH, UK.
*E-mail: oneillj@mrc-lmb.cam.ac.uk

http://dx.doi.org/10.1016/j.cub.2013.09.046

Mitochondrial Disease: mtDNA and Protein Segregation Mysteries in iPSCs

Mitochondrial diseases cause a range of clinical manifestations even in patients carrying the same mtDNA mutations. New work reveals that a common disease-associated mtDNA mutation is selectively segregated from wild-type mtDNA during the reprogramming of induced pluripotent stem cells and that high levels of this mutation in differentiated neurons upregulate Parkin-mediated mitophagy.

Alicia M. Pickrell and Richard J. Youle*

Multiple copies of mitochondrial DNA (mtDNA) reside in mitochondria and this DNA encodes the tRNA and rRNA machinery required to translate 13 components of all of the complexes of the respiratory chain, except complex II. Mutations in the mtDNA that affect oxidative phosphorylation function are common; it is estimated that 1 in 5,000 children and adults have mitochondrial diseases caused by mtDNA mutations [1]. MtDNA mutations reach varying levels of heteroplasmy — the ratio of wild-type to mutated mtDNA molecules — in different tissues and present a wide range of clinical symptoms, even between patients harboring the same type of mutation. The most common mtDNA mutation, m.3243A>G, disrupts the gene encoding tRNA Leucine$^{(UUR)}$ and causes two distinct mitochondrial diseases: maternally inherited diabetes and deafness (MIDD) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) [1]. To date, the most puzzling question is how mtDNA mutations affect different cell types to cause different phenotypes and how mutational load is determined in different tissues. Recent work by Hämäläinen *et al.* [2], published in *Proceedings of the National Academy of Sciences,* used an induced pluripotent stem cell (iPSC) model to provide mechanistic insight into how mtDNA mutations affect neurons differently from other cell types and how mtDNA mutations segregate in iPSCs to affect their differentiated progeny.

This study from Suomalainen's group shows that the m.3243A>G mutation causes a defect in respiratory chain complex I in differentiated neurons, but has no detrimental effect on oxidative phosphorylation activity in iPSCs [2]. Disrupting the proof-reading domain of the nuclear gene polymerase γ, which controls mtDNA replication, causes an accelerated accumulation of mtDNA mutations in a premature aging Mutator mouse model. These mtDNA mutations impact mitochondrial function with age, causing Mutator mice to suffer from weight loss, cardiomyopathies, age-related muscle wasting, fur graying, and other phenotypes that mimic human aging [3,4]. Prior work from the Suomalainen group showed that high mtDNA mutational loads in neural stem cells (NSCs) from Mutator mice do not result in a respiratory defect, but lead to oxidative phosphorylation dysfunction in adult neurons later in life [5]. MtDNA mutations in iPSCs or NSCs do not have the same adverse effects on oxidative phosphorylation activity as in other cell types, likely due to the heavy reliance of these stem cells on glycolysis for energy metabolism [6]. However, mtDNA mutations negatively affect the survival and proliferative abilities of stem cells, possibly due to alternative signaling pathways, such as the generation of reactive oxygen species [5]. It remains mysterious how a tRNA Leucine$^{(UUR)}$ mutation selectively impairs complex I in post-mitotic neurons when it is needed for the translation of all mitochondrial genes.

Neurons are complex specialized cell types categorized by location and by the type of neurotransmitters they release. Often, this view itself is simplistic; for example, different subtypes of dopaminergic neurons express different calcium-binding proteins and have distinct baseline neuronal firing oscillations. This is important because different disruptions in mtDNA integrity cause divergent neuroanatomical susceptibilities in the central nervous system [7]. Knocking out the function of complex III or complex IV in the same