Meeting report

The rhythms of life

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A report on the symposium 'Clocks and Rhythms', Cold Spring Harbor, USA, 30 May-4 June 2007.

Many metabolic and physiological processes occur in a periodic fashion and, surprisingly enough, many of these rhythms rely on relatively simple biochemical reactions. A recent symposium on biological rhythms held at the Cold Spring Harbor Laboratory was a follow-up to a pioneering symposium held there in 1960, which many people in the field consider as the start of modern chronobiology. This year's symposium not only provided insights into the latest research of circadian rhythms in cyanobacteria, *Neurospora*, *Drosophila*, *Arabidopsis* and mammals, but also on metabolic cycles in yeast, the segmentation clock in mammals, regulation of life span, and many more. I will focus here only on some aspects of the meeting.

Periodicity in yeast and vertebrate development

More than 30 years ago, conditions for the culture of baker's yeast were established to allow homeostatic growth. The constant supply of oxygen and nutrients provokes multiple, highly synchronized metabolic cycles with a period length of about 40 minutes. These self-sustaining cycles can be separated into three phases: an oxidative, that is, oxygen-consuming, phase, a reductive building phase and a reductive charging phase. It appears that hydrogen sulfide secretion and clearance are major synchronizers of these processes. Robert Klevecz (Beckman Research Institute, Duarte, USA) provided evidence that each phase has its own pattern of gene transcription and that the replication of DNA (about 10% of the cells during a cycle) is gated to the reductive phase. Computer simulations indicate that stochastic noise is swept up and dampened during these cycles, which may be a common theme for other rhythmic processes. Benjamin Tu (University of Texas Southwestern Medical Center, Dallas, USA) has established a similar model system but with a period length of about 5 hours, allowing the entire culture to divide in a more synchronized fashion. Using mass spectrometry, he found about 60% of the metabolites in the yeast to be rhythmic. He presented detailed insights into NADP(H) and sulfur metabolism during the yeast metabolic cycle and described mutants that interfere with the cycle. An accompanying poster from Tu and colleagues described that in different cell-cycle mutants forced to replicate their DNA more and more in the oxidative phase, a concomitant increase in the mutation rate occurred. The protection of the integrity of the genome may be a recurring theme in rhythmic processes.

The segmental patterning of amniotes is based on axis elongation and somitogenesis - the generation of the embryonic blocks of tissue that give rise to the vertebrae and associated muscles. For example, in the development of the mouse, specific cells in the embryonic presomitic mesoderm proliferate and elongate the structure anteriorly, while blocks of cells left behind start to differentiate rhythmically into somites with a period length of about 2 hours. A segmentation clock determines the pace of this rhythmic differentiation process. Olivier Pourquié (Stowers Institute for Medical Research, Kansas City, USA) described a high-resolution transcriptome analysis of the developing presomitic mesoderm of the mouse. He found rhythmic expression of modulators of the Notch and fibroblast growth factor (FGF) signaling pathways in opposite phase to Wnt signaling. As a model, Pourquié proposed that the proliferating cells secrete FGF8, which will form a dynamic gradient because the differentiating cells left behind can no longer produce this protein. Dilution of the gradient yields a determination zone or wave front, where blocks of cells could start to differentiate into somites. The segmentation clock gates this process rhythmically.

Ryoichiro Kageyama (Kyoto University, Kyoto, Japan) has analyzed in detail the basic helix-loop-helix transcription factor Hes7, whose rhythmicity of expression is dependent on the segmentation clock. It was previously known that in Hes7 knockout mice or Hes7 transgenic mice, the somites

become fused, indicating that rhythmicity has been lost. Kageyama has now engineered a mouse strain with a more stable than normal Hes7 protein. Interestingly, during the development of these mice, five out of eight somites also fuse, indicating that the abundance and stability of this protein affects the resonance of the segmentation clock. Kageyama's analysis indicated that Hes7 expression relies on FGF8 signaling, whereas Notch signaling is important for the oscillation in the posterior presomitic mesoderm. Taken together, the results point strongly to a model in which a cell-autonomous oscillator rhythmically modulates a morphogenic gradient.

Circadian clocks

Circadian clocks display a free-running period length of about a day. They are based on cell-autonomous oscillators that govern the periodic changes in metabolism, physiology and gene expression. For instance, more than 70% of the transcriptome of the cyanobacterium *Synechococcus elongatus* is under circadian control. The circadian oscillator of these bacteria can be reconstituted *in vitro*: multiple circadian cycles of phosphorylation of the hexameric protein KaiC occur in a test tube filled with with the clock's subunits - KaiC, the regulatory subunits KaiA and KaiB, and ATP. To gain insight into this molecular oscillator, Takeo Kondo (Nagoya University, Nagoya, Japan) has analyzed in detail the progressive phosphorylation states of the hexameric KaiC protein, and the switch from its intrinsic ATPase activity to phosphatase activity. Although measured as extremely weak, the ATPase activity of KaiC is very stable and defines the circadian period. Carl Johnson (Vanderbilt University, Nashville, USA) pointed out that phase-specific exchanges of subunits between the KaiC hexamers must occur to allow for synchronization of the entire population of oscillators. Johnson's computer simulations also reveal that the transcription and translation feedback cycle, although not important *in vitro*, has a stabilizing and synchronizing effect on the cyanobacterial oscillator *in vivo*.

The *Neurospora* circadian oscillator is based on transcriptional and post-translational feedback loops composed of the White collar transcriptional activators WC-1 and WC-2 and the repressor Frequency (FRQ), which affects the phosphorylation and abundance of the two activators. Rhythmic changes in phosphorylation constitute an important feature of the feedback loop. Michael Brunner (University of Heidelberg Biochemical Center, Heidelberg, Germany) provided new evidence that FRQ, in complex with protein kinase 1a, phosphorylates and sequesters WC-2 in the cytoplasm, thereby blocking the transcription of the *frq* gene. Levels of WC-1 and WC-2 are also affected by another kinase, protein kinase A, according to Yi Lui (University of Texas Southwestern Medical Center, Dallas, USA). Although WC-1 and WC-2 are very unstable as nonphosphorylated proteins in an arrhythmic *pka* mutant background, the small amounts of them that remain are sufficient to drive high-level expression of the *frq* gene, because the non-phosphorylated forms have a very high affinity for DNA. Jay Dunlap (Dartmouth Medical School, Hanover, USA) examined a third class of kinase involved in the oscillator, exemplified by CK2. This kinase has an effect on the temperature compensation of the oscillator by promoting FRQ degradation at higher temperatures.

There is an increasing number of reports of oscillators in *Neurospora* that are independent of FRQ, some of which behave as simple 'slave oscillators' - that is they are driven solely by the rhythmicity of an organism and not directly by an oscillator, for example, the nitrate reductase cycle - and others as real self-sustaining oscillators. For example, Jennifer Loros (Dartmouth Medical School, Hanover, USA) has found that the gene *ccg-16* was still rhythmically expressed and temperature-compensated in an otherwise arrhythmic *frq* deletion strain. Sorting out the function and the relationships of all of these different oscillators will certainly be one of the tasks for the future.

The circadian oscillator of *Arabidopsis* is composed of at least three interconnected feedback loops. The central loop is composed of the reciprocal regulation of the two regulators TIMING OF CAB EXPRESSION 1 (TOC1) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) Joanne Chory (Salk Institute, La Jolla, USA) has carried out a microarray analysis of genes expressed during the day and found that 90% of the transcripts display a time-of-day-specific expression, including about 31% of circadian transcripts. Subsequent analysis revealed three classes of transcriptional response elements that drive expression in different circadian phases.

Steve Kay (Scripps Institute, La Jolla, USA) has developed a large-scale transcription factor/promoter element interaction assay, to identify additional regulators of *Cca1*. With this screen, for example, he identified *in vitro* the transcription factor TCP21 as a regulator of *Cca1*. Because of the redundancy of TCP21 with TCP7, this regulation had not been observed in negative TCP21-mutant plants. David Somers (Ohio State University, Columbus, USA) reported on the blue-light sensor Zeitlupe. He has found that this F-box containing E3-ubiquitin ligase is stabilized by direct interaction with the *Gigantea* protein, an effect that is greatly enhanced by blue light. Both proteins together gate the appearance of the Toc1 protein during the day/night cycle. This regulator is an example of the direct influence of light on the three-loop oscillator of *Arabidopsis*.

The *Drosophila* circadian oscillator is also composed of interconnected transcriptional and post-translational feedback loops. One loop consists of the Period (Per) and Timeless (Tim) proteins that, probably as heterodimeric complexes, counterbalance the activity of the transcriptional

activators Clock and Cycle to govern circadian rhythmicity. The main targets of these transcription factors are the genes for Per (*per*) and for cryptochrome (*cry*). Michael Young (Rockefeller University, New York, USA) has developed a fluorescence resonance energy transfer assay to detect interactions between these proteins in *Drosophila* S2 cells. He reported that Per and Tim associate in the cytoplasm but, surprisingly, enter the nucleus separately. Instead, he found that Per and the kinase Double-time enter the nucleus together.

Although a significant part of the transcriptome is expressed in a circadian fashion, only five genuine target genes have been identified for repression by the Per-Tim loop of the oscillator. Transcriptional repression of these targets seems to be important, however, and to directly affect the period length and the phase of the molecular oscillator, as demonstrated by Michael Rosbash (Brandeis University, Waltham, USA). He designed a genome-wide approach to identify new direct Clock targets and identified *clockwork orange* (*cwo*) as a protein that synergises with Per for transcriptional repression and, therefore, represents a new core-oscillator component. Surprisingly, although the circadian amplitudes of core-oscillator genes in *cwo*-mutant flies become slightly dampened, the flies are phenotypically arrhythmic. This may indicate that the transcriptional feedback-loops are important for the *Drosophila* oscillator.

Oxidative stress can interfere with the circadian oscillator, as investigated by Amita Seghal (University of Pennsylvania Medical School, Philadelphia, USA), who has found that this effect seems to be mediated specifically in the fat bodies by the transcriptional regulator Foxo, which is normally involved in insulin signaling. Astonishingly, young *Foxo*-null flies show a rapid degradation of their free-running rhythms, strongly resembling in this respect much older flies. Jeff Hall (Brandeis University, Waltham, USA) cautioned on the interpretation of the circadian phenotypes of many existing mutant flies, because at least some of these are based on genetic background and/or gain-of-function effects.

The mammalian circadian clock

The mammalian circadian oscillator resembles the *Drosophila* oscillator. The mammalian Per and Cry proteins counterbalance the activity of the BMAL1/MOP3 and Clock (or NPAS2) transcriptional activators to govern circadian rhythmicity. At the meeting, new insights into the molecular make-up of the mammalian oscillator were provided. At the meeting two talks reported the discovery of the F-box-containing E3-ubiquitin ligase Fblx3 as a new core oscillator component in mice. This protein targets specifically the Cry proteins with ubiquitin and thereby provokes proteosome-mediated degradation. As a consequence, in Fblx3 mutant mice, the Cry proteins become stabilized and repress for a prolonged time BMAL1/MOP3 and Clock governed transcription, resulting in a very long free-running period length of over 27 hours.

The half-lives and/or nuclear retention of Per proteins, on the other hand, can be regulated by phosphorylation. Louis Ptácek (University of California, San Francisco, USA) has transferred a mutation of *Per2* found in humans with familial advanced sleep phase syndrome (in which people feel sleepy in the early evening and wake up a couple of hours after midnight) to mice and obtained a similar phenotype. Further analysis of this experimental system confirmed the importance of phosphorylation in fine-tuning the half-life of the Per2 protein, which is shorter for the mutated protein. As a consequence, the free-running period of these mice (and of people with this mutation) is shorter than normal.

Other post-translational modifications are also involved in regulating the mammalian oscillator. Paolo Sassone-Corsi (University of California, Irvine, USA) recently identified an intrinsic acetyltransferase activity in the Clock protein, and he has now identified the BMAL1 protein as a direct target. Acetylated BMAL1 may be subsequently recognized by Cry1 to engage transcriptional repression.

The liver circadian oscillator has been the subject of very detailed analyses. Using microarray analysis with a 1 hour resolution as a base, John Hogenesch (Novartis Research Foundation, San Diego, USA) identified not only many new circadian transcripts, but also two new rhythms with 12 and 8 hour lengths. The 12 hour rhythms included, for example, a secretory network containing Sec22b and a regulatory network containing CDK2/HDAC1 and CDK2/HDAC2. Whether these rhythms are linked to the circadian oscillator remains to be seen. Rhythms with a period length of about a day in liver tissue without a functional oscillator were reported by other groups. As Ueli Schibler (University of Geneva, Geneva, Switzerland) outlined, these rhythms, including most surprisingly a robust cycling of the *Per2* gene, are driven from outside the tissue. Using a novel *in vitro* screen for circadian DNA-binding activities in liver nuclei, he identified the transcription factor HSF1 as a potential upstream regulator of *Per2*.

Where do the rhythms go? Hopefully not in circles! Research seems to have shifted a bit from the core oscillators to a plethora of new rhythmic phenomena and it will take some time to sort out the relationships between them. In the near future many new components of the mammalian oscillator will be identified, and we will gain much more insight into the relationship between circadian rhythms and diseases such as cancer and depression. We will also understand much better the circadian rhythmicity of humans and the problems associated with this. Hopefully we will not have to wait another 47 years for the next conference on 'Clocks and Rhythms' at Cold Spring Harbor.