# V4: Circadian rhythms – summary

(1) Example from a previous mini test (lecture cellular programs – WS 2017)

(2) Schein conditions (V1)

(3) **Content of minitest #1:**

- Lecture V1 (slides 17-25),
- V2 (slides 13-16)
- V3 (slides 4, 6-8, 10-23)

- Specified content from Papers 1 to 3:
  methods, results and discussion section related to the indicated figures.

# Assignment 1 – will be discussed in tutorial on May 8, 2.15 pm

We have received solutions from the 33 students listed below.

**If your name is NOT included in the list, but you believe that you had submitted your solution by e-mail to us, please resend your solution to**
volkhard.helms@bioinformatik.uni-saarland.de until May 8, 10 am.

Andres, B.
Andres, S.
Asariardakani
Balaji Kuttae
Chavarria Rivera
Czaja
Dillmann
Ebby
Eimer
George
Golemi
Hasan
Havlik
Heggen
Joy
Kamada
Kamath
Laradji
Ludt
Manisha
Mekountchou
Moturu
Nobakht
Nobin
Paul
Sah
Senatorov
Singh, A.
Singh, N.
Solomon
Sudharshini
Sultan
Thomas

# Conditions for certification

(1) There will be 6 biweekly **assignments**. Students need to write short essays about topics covered in the lecture and in assigned research papers.

There are three possible grades: excellent, pass, failed. Students need to get a "pass" grade on at least *5* assignments or *3* "pass" and one "excellent" grade.

(2) There will be three 45-minutes **tests** on different parts of the lecture.

Students need to pass at least two out of the three tests.

Tests will cover the content of the lecture and of the assigned research papers.

(3) Students need to **present** at least once during the lecture on the content of an assigned research paper (**team work**, 20 min. powerpoint presentation and 10 min. discussion).

# (from V1) Circadian clocks in mammals and plants

Most organisms (animals, plants, fungi and cyanobacteria) enhance their fitness by coordinating their development with daily environmental changes through **molecular timekeepers (circadian clocks).**

**Mammals** display circadian rhythms in behavioral and physiological processes, such as

- sleep
- feeding
- blood pressure and
- metabolism

Roles in **plants** e.g.:

- opening of flowers in the morning and their closure at night

Circadian rhythms are guided by **external light–dark signals**
that are integrated through intrinsic central and peripheral molecular clocks

McClung Plant Cell 18, 792 (2006)

# Circadian rhythms

(1) Circadian rhythms are the subset of biological rhythms with period of 24 h.
The term circadian combines the Latin words “circa” (about) and “dies” (day).

(2) Circadian rhythms are **endogenously generated** and **self-sustaining**.

They persist under constant environmental conditions, typically constant light (or dark) and constant temperature.
Under these controlled conditions, the free-running period of **24 h** is observed.

(3) For all circadian rhythms, the **period** remains relatively **constant** over a range of ambient temperatures.

This is thought to be one property of a general mechanism that buffers the clock against changes in cellular metabolism.

**Chemical reactions are usually faster at higher temperatures.**

McClung Plant Cell 18, 792 (2006)

# Essential elements of biological clocks

Our biological clocks contain 3 essential elements:

(1) a **central oscillator** that keeps time;

(2) the ability to **sense time cues** in the environment and to **reset the clock** as the seasons change; and

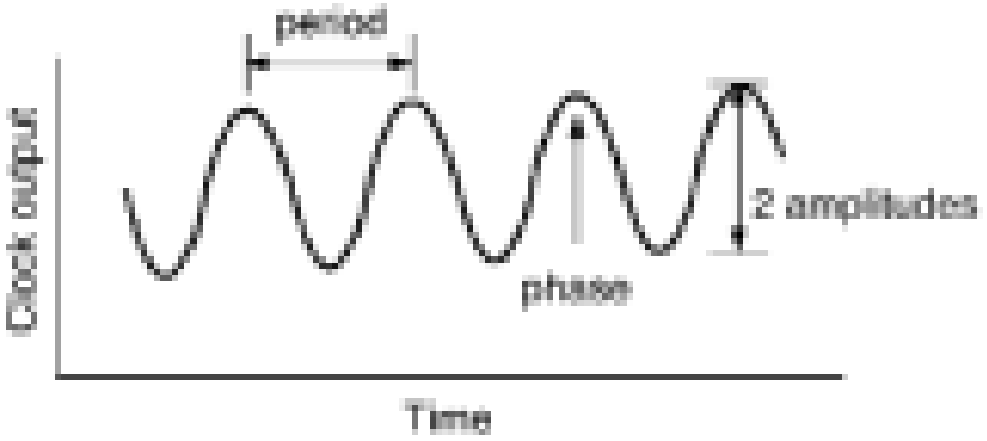
(3) a series of outputs tied to distinct phases of the oscillator
that regulate activity and physiology.

Gallego et al. Nat.Rev.Mol.Cell.Biol. 8, 140 (2007)

# Parameters of Circadian clocks

**Period** : time to complete one cycle.

**Amplitude** of the rhythm :
one-half the peak-to-trough distance.




**Phase** : time of day for any given event.
E.g. if the peak in a rhythm occurred at dawn,
the phase of the peak would be defined as 0 h.

Phase is often defined in **zeitgeber time (ZT).**

Zeitgeber is German for „time giver“, and any stimulus
that imparts time information to the clock is a zeitgeber.
The onset of light is a powerful zeitgeber, and dawn is defined as ZT0.

McClung Plant Cell 18, 792 (2006)

# Suprachiasmatic nucleus (SCN)

In mammals, the central clock resides in the suprachiasmatic nucleus (SCN), a small region of the brain that contains ca. 20,000 neurons.

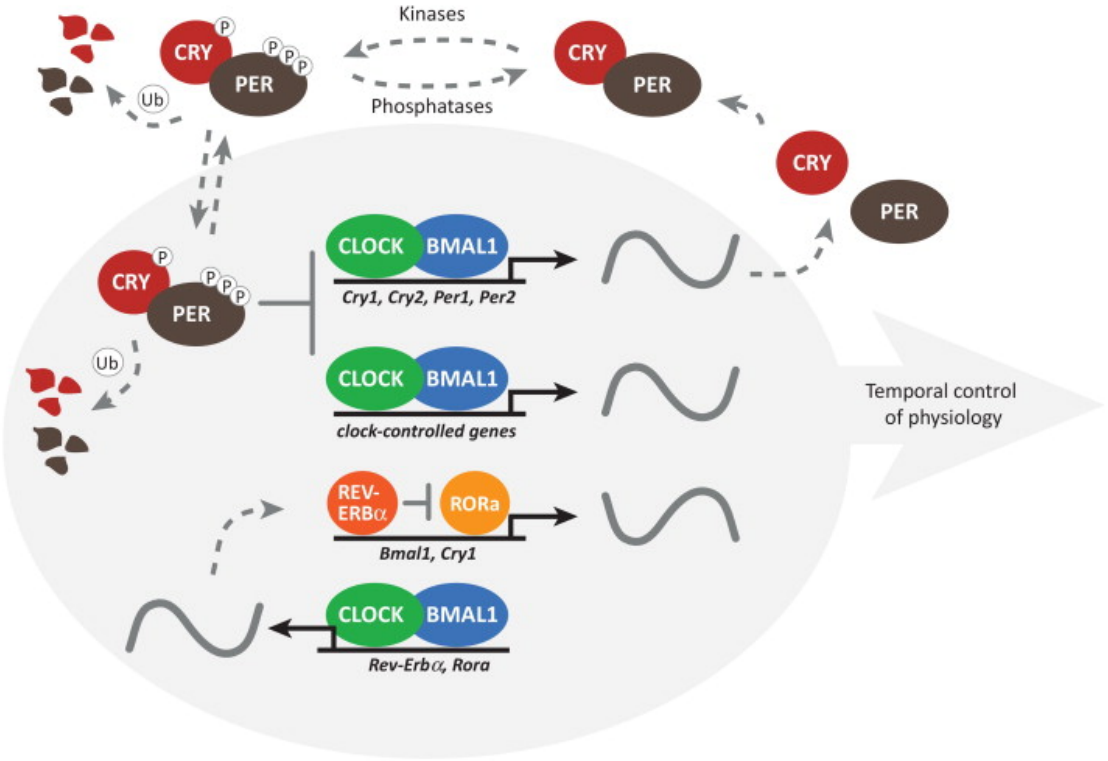
The SCN produces a **rhythmic output** that consists of a multitude of neural and hormonal signals that influence sleep and activity.

Most importantly, the SCN signals **set the peripheral clocks** present throughout the body.

The SCN clock is reset by external **light**, which is **sensed** by the ganglion cells of the **retina**.

Gallego et al. Nat.Rev.Mol.Cell.Biol. 8, 140 (2007)

# The molecular circadian clock in mammals




The cell-autonomous molecular clock in mammals is generated by 2 interlocking transcription/translation feedback loops (TTFL) that function together to produce robust 24 h rhythms of gene expression.

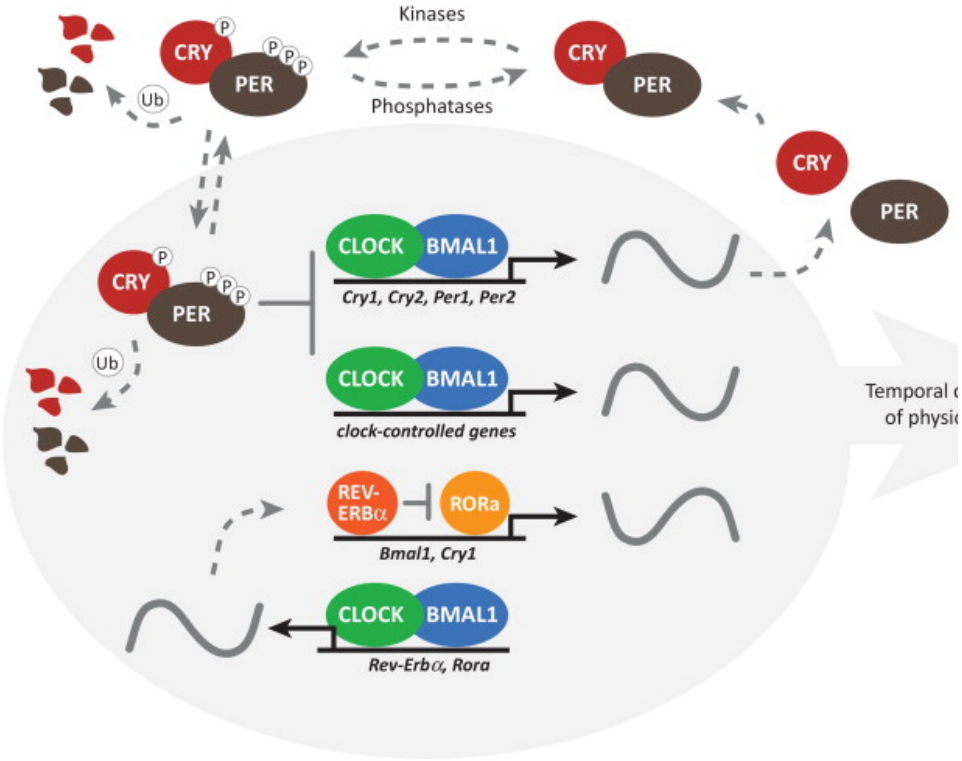
The core TTFL is driven by 4 integral clock proteins:

2 activators (CLOCK and BMAL1) and 2# repressors (PER and CRY), as well as by kinases and phosphatases that regulate the phosphorylation (P) and thereby localization and stability of these integral clock proteins.

BMAL1, brain and muscle ARNT-like 1
CLOCK, circadian locomotor output cycles kaput
CKI: casein kinases I CKIα, CKIδ, and CKIε;
CRY: cryptochrome
PER: period
PP: protein phosphatases PP1, PP5.

Partch et al. Trends Cell Biol 24, 90 (2014)

# The molecular circadian clock in mammals



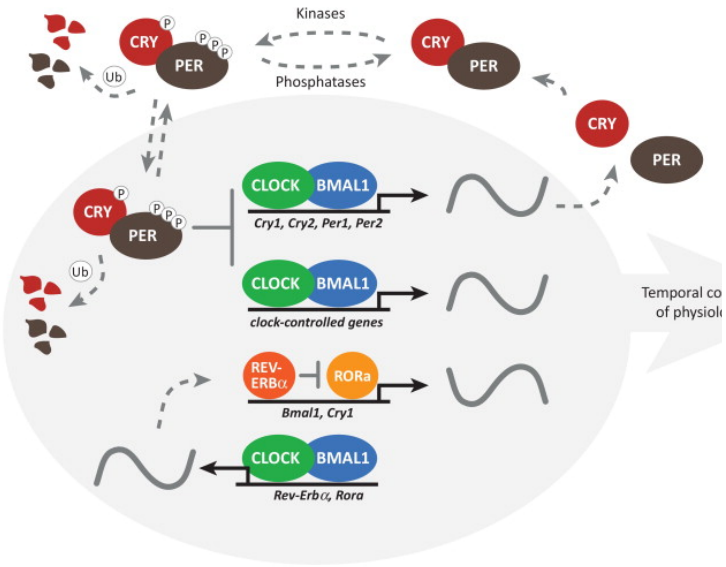

CLOCK and BMAL1 are subunits of the heterodimeric basic helix-loop-helix-PAS (PER-ARNT-SIM) transcription factor CLOCK:BMAL1, which activates transcription of the repressor *Per* and *Cry* genes, as well as other clock-controlled output genes.

PER and CRY proteins heterodimerize in the cytoplasm and translocate to the nucleus to interact with CLOCK:BMAL1, inhibiting further transcriptional activation.

As PER and CRY proteins are degraded through ubiquitin (Ub)-dependent pathways, repression on CLOCK:BMAL1 is relieved and the cycle begins again with ~24 h periodicity.

Partch et al. Trends Cell Biol 24, 90 (2014)

# The molecular circadian clock in mammals



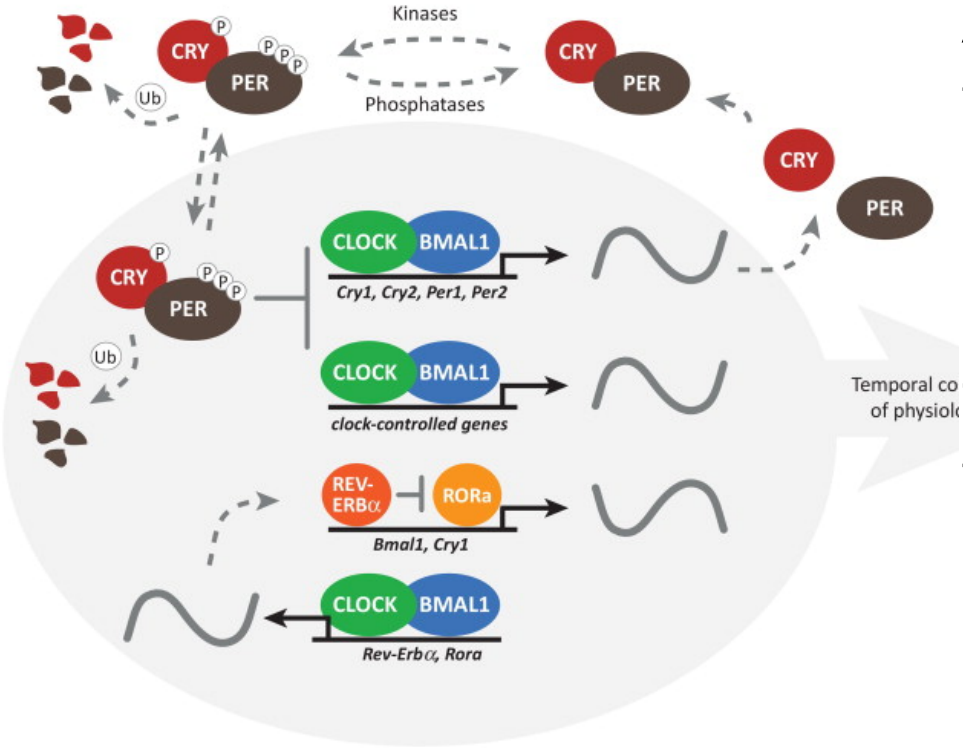

The casein kinases CKIδ and CKIε play an important role in determining the intrinsic period of the clock by controlling the rate at which the PER:CRY complexes are either degraded or enter the nucleus, and their activity is either counteracted or regulated by the phosphatases PP1 and PP5, respectively.

Notably, **familial mutations** resulting in the loss of a single phospho-acceptor site on PER2 (S662G) or a loss-of-function mutation in CKIδ (T44A) shorten the intrinsic period of the clock in mice and give rise to sleep phase disorders in humans.

A key role for the casein kinases in establishing period length has also been demonstrated pharmacologically via modulation of the kinases with **small-molecule inhibitors**, which dramatically lengthen the period by modulating PER localization and stability.

Partch et al. Trends Cell Biol 24, 90 (2014)

# The molecular circadian clock in mammals




A second TTFL is generated through transcriptional activation by the retinoid-related orphan receptors (RORa, b, c) and repression by REV-ERBα/REV-ERBβ.

This TTFL drives rhythmic changes in *Bmal1* transcription and introduces a **delay** in *Cry1* mRNA expression that offsets it from genes regulated strictly by CLOCK:BMAL1 and is crucial for proper circadian timing

The presence of cooperative, interlocking feedback loops provides **robustness** against noise and environmental perturbations to help maintain accurate circadian timing, and also helps to generate **phase delays** in circadian transcriptional output that optimally time gene expression for local physiology.

Partch et al. Trends Cell Biol 24, 90 (2014)

# (from V2) Core clock proteins interact with chromatin and chromatin-modifying complexes

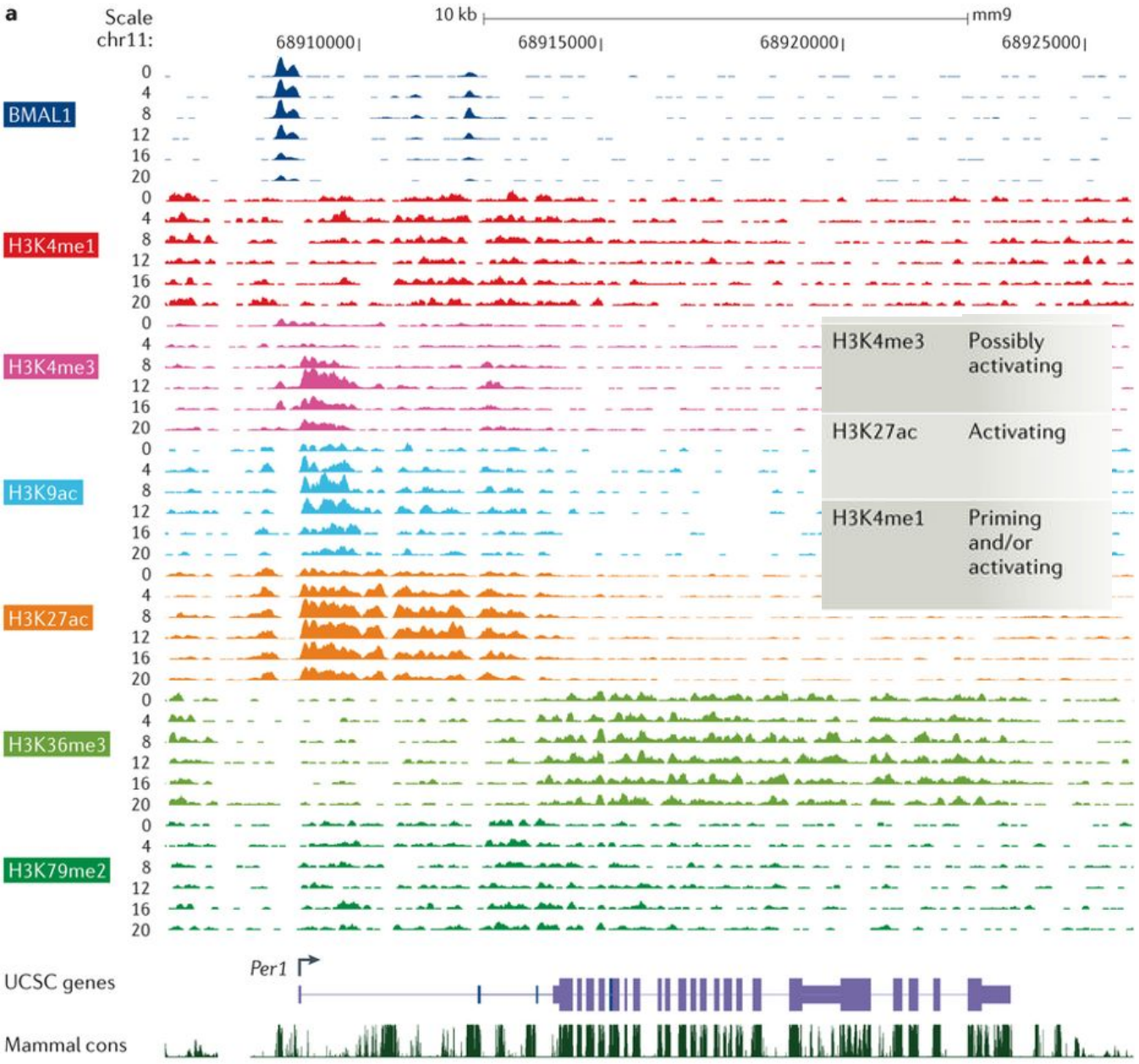
At the beginning of the transcription cycle, the activators CLOCK and BMAL1 interact with the **histone acetyltransferases** (HATs) p300 and CREB-binding protein (CBP), respectively, to acetylate histones and provide an accessible chromatin state for transcription.

**CLOCK** also has **intrinsic HAT activity** and acetylates histone H3 on Lys9 (H3K9) and Lys14 residues (H3K14).

The $NAD^+$-dependent histone deacetylase (HDAC) **sirtuin 1** (SIRT1) associates with CLOCK, BMAL1 and PER2, and a circadian rhythm in $NAD^+$ levels driven by the expression of the CLOCK–BMAL1 target gene *Nampt* in turn leads to a rhythm in SIRT1 activity that feeds back to inhibit the CLOCK–BMAL1 complex.

Takahashi
Nature Rev Genet
18, 164–179 (2017)

# Circadian chromatin states in the mouse liver

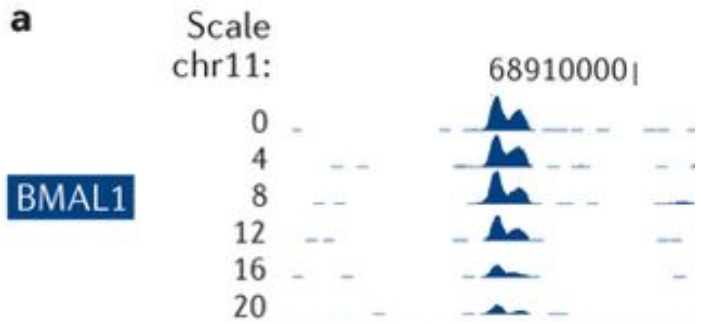



UCSC genome browser view of histone methylation and acetylation at the *Per1* gene at 6 circadian times (CTs) of the day (0, 4, 8, 12, 16 and 20 hours).

The colours of the wiggle plots of chromatin immunopreci-pitation followed by sequencing (ChIP–seq) signal indicate the following: **BMAL1 occupancy**, **monomethylation of Lys4 at histone H3 (H3K4me1), H3K4me3**, etc.

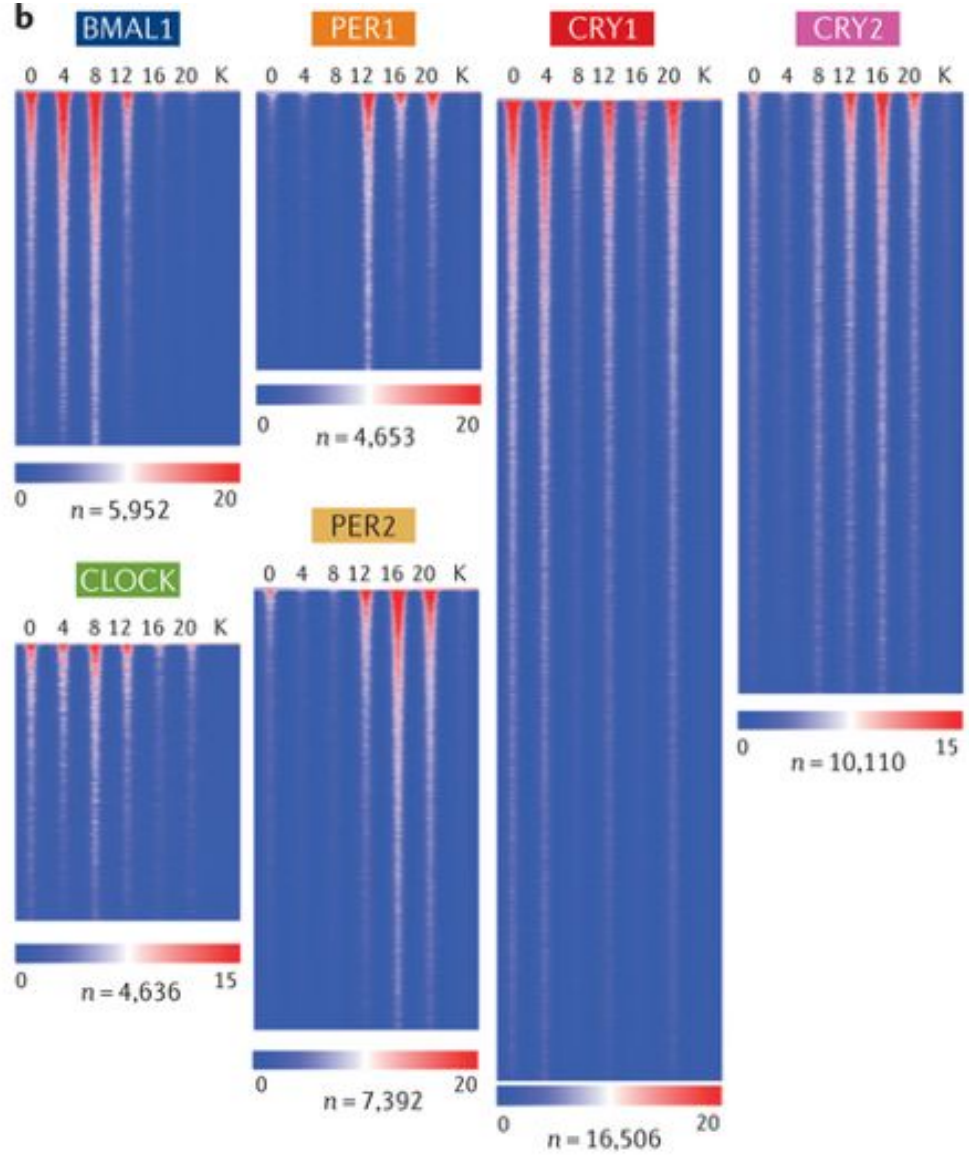
Takahashi
Nature Rev Genet
18, 164–179 (2017)

# BMAL1:CLOCK activity in the mouse liver



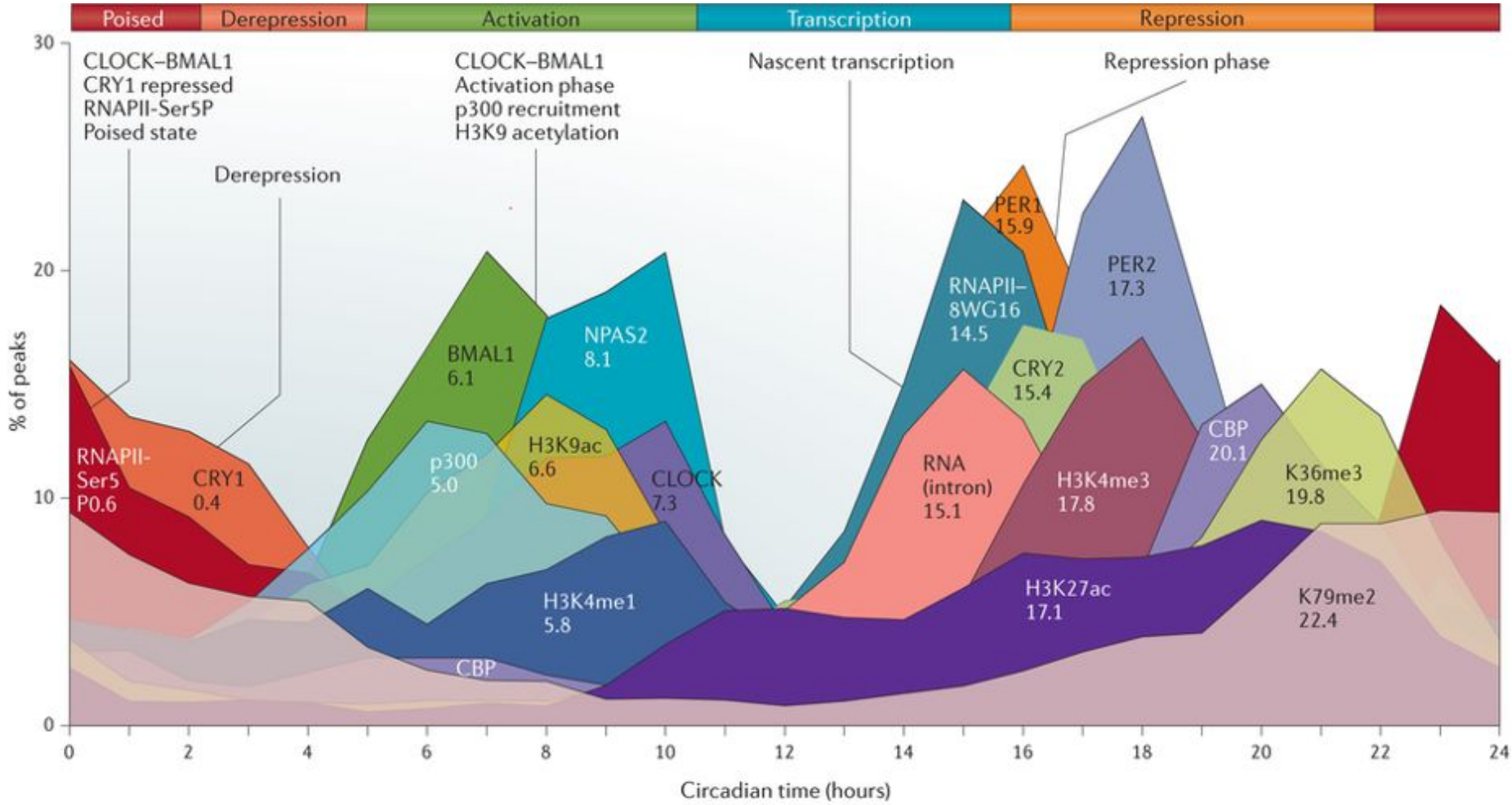

At Per1, the activators BMAl1 and CLOCK bind in a cyclic manner at the promoter between circadian time zero (CT0) and CT12, with maximal binding observed at CT8.

In genome-wide analyses, CLOCK and BMAL1 bind to more than 4,600 and 5,900 sites, respectively, corresponding to ca. 3000 unique genes in the liver.




Takahashi
Nature Rev Genet
18, 164–179 (2017)

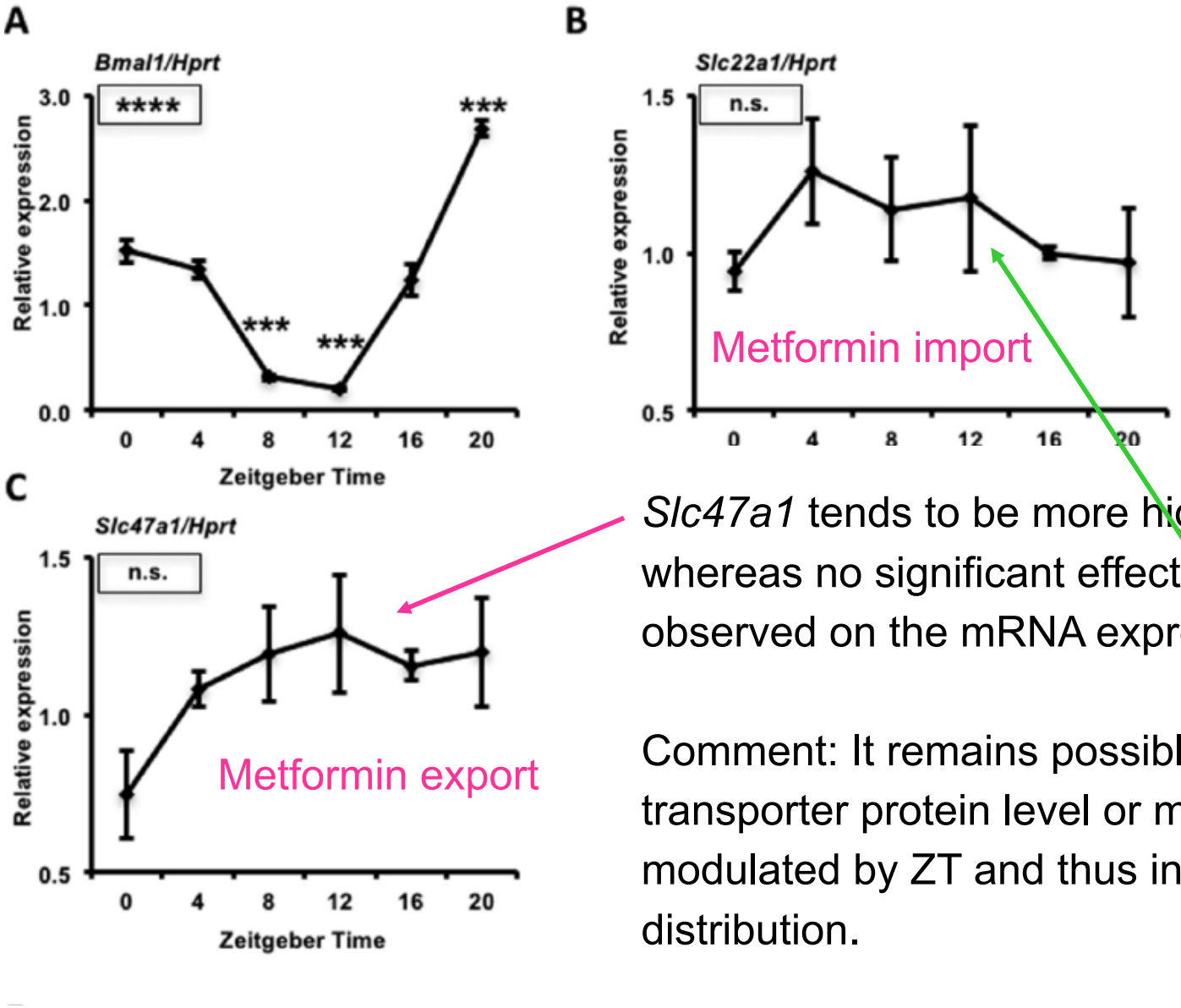
# Circadian cycle consists of 6 distinctive phases




Histograms showing the phase distributions of each factor as a function of time of day. ac, acetylation; CBP, CREB-binding protein; CRY, cryptochrome; me, methylation; NPAS2, paralogue of CLOCK; PER, period; RNAPII, RNA polymerase II; Ser5P, phosphorylation on Ser5.

Takahashi
Nature Rev Genet
18, 164–179 (2017)

# mRNA expression of Bmal1 and solute carriers




*Slc47a1* tends to be more highly expressed at night, whereas no significant effect of zeitgeber time was observed on the mRNA expression of *Slc22a1*.

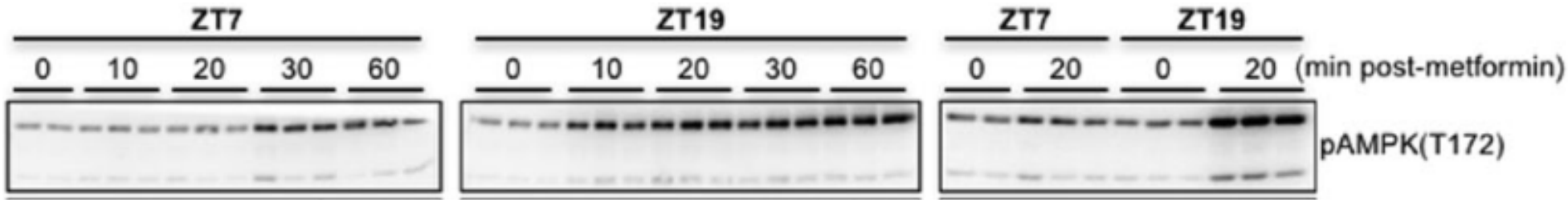
Comment: It remains possible that *Slc22a1* transporter protein level or membrane localization is modulated by ZT and thus influences drug distribution.

# Activation of AMPK (AMP-activated protein kinase)

What downstream consequences may result from the observed differences in metformin effects on blood glucose?

→ measure the kinetics of the **signal transduction response** to metformin at ZT7 and ZT19. These 2 time points exhibit similar basal blood glucose levels but markedly different reductions in blood glucose in response to metformin.

Consistent with the observed enhanced reduction of blood glucose during the night, the activating phosphorylation of AMPK on threonine 172 (T172) occurred more quickly after metformin treatment at ZT19 (10 min) compared with treatment at ZT7 (30 min).




Also the phosphorylation of the target of AMPK-directed posttranslational modification, RAPTOR was enhanced at ZT19

# (from V3) Circadian rhythms are coupled to metabolism

## cAMP-Dependent Signaling as a Core Component of the Mammalian Circadian Pacemaker

John S. O'Neill,[1]* Elizabeth S. Maywood,[1] Johanna E. Chesham,[1] Joseph S. Takahashi,[2] Michael H. Hastings[1]†

O'Neill et al.
Science, 320, 949 (2008)

Review:

The suprachiasmatic nuclei (SCN) of the hypothalamus are the principal **circadian pacemaker** in mammals,

They drive the sleepwake cycle and coordinate peripheral clocks in other tissues.

Current understanding:

The molecular clockwork within the SCN is being modeled as a combination of **transcriptional** and **posttranslational negative feedback loops**.

Protein products of *Period* and *Cryptochrome* genes periodically suppress their own expression.

# Control of circadian rhythms?

Open question: It is unclear how long-term, high-amplitude oscillations with a daily period are maintained.

In particular, transcriptional feedback loops are typically less precise than the oscillation of the circadian clock and oscillate at a higher frequency than one cycle per day.

Possible explanations:
- Phosphorylation (e.g. casein kinase) causes delay (see V1),
- secondary loops give stabilization.

O'Neill et al.
Science, 320, 949 (2008)

# Evidence for coupling of circadian clocks with metabolism

(1) Recombinant cyanobacterial proteins can **sustain circadian cycles** of autophosphorylation in vitro, in the absence of transcription,

(2) The intracellular signaling molecules cyclic adenosine diphosphate–ribose (**cADPR**) and $Ca^{2+}$ are essential **regulators** of circadian oscillation in *Arabidopsis* and *Drosophila*.

This indicates that transcriptional mechanisms may not be the sole, or principal, mediator of circadian pacemaking.

O'Neill et al.
Science, 320, 949 (2008)

# Example of a gene regulatory network

O'Neill and co-workers showed that the transcriptional feedback loops of the SCN are sustained by cytoplasmic cAMP signaling.

cAMP signaling determines their canonical properties (amplitude, phase, period).

Roles of cAMP?

In molluscs, birds, and the mammalian SCN, cAMP is implicated in entrainment or maintenance of clocks, or both, or mediation of clock output.

It was not considered as part of the core oscillator sofar.

These findings extend the concept of the mammalian pacemaker beyond transcriptional feedback to incorporate its integration with rhythmic cAMP-mediated cytoplasmic signaling.

O'Neill et al.
Science, 320, 949 (2008)

# What is cAMP

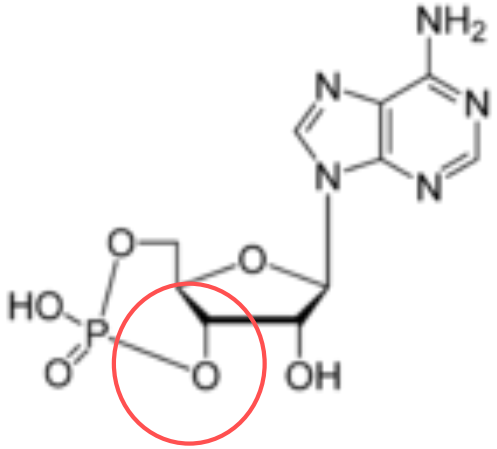
**Cyclic adenosine monophosphate** (**cAMP**) is a second messenger that is important in many biological processes.

cAMP is derived from ATP and used for intracellular signal transduction in many different organisms, conveying the cAMP dependent pathway.

In humans, cyclic AMP works by activating **cAMP-dependent protein kinase** (PKA).

Cyclic AMP binds to specific locations on the regulatory units of the protein kinase, and causes dissociation between the regulatory and catalytic subunits

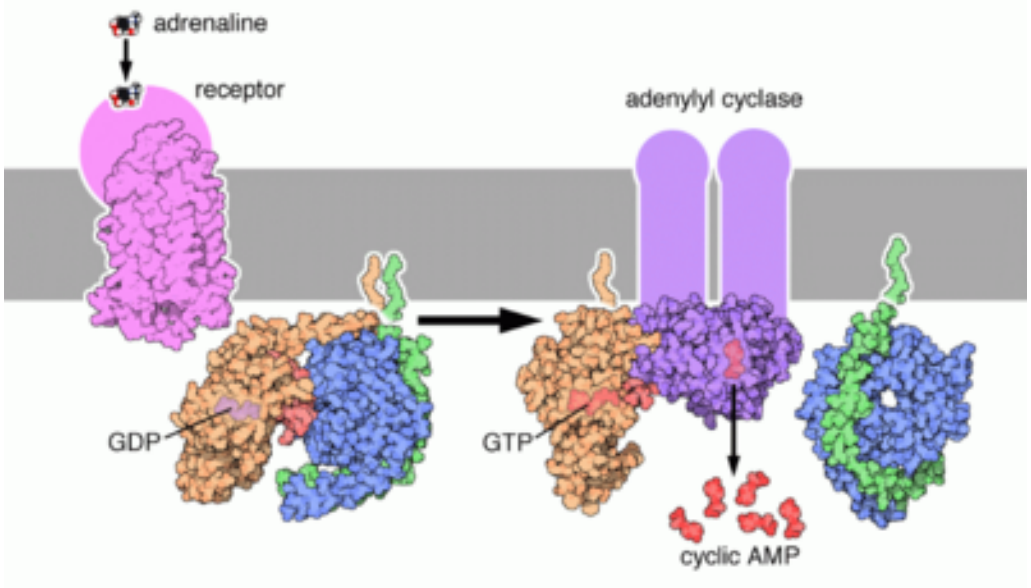
Thus it activates the catalytic units of PKA and enables them to phosphorylate substrate proteins.




„cyclic“

www.wikipedia.org

# Side functions of cAMP




There are some minor PKA-independent functions of cAMP, e.g. activation of calcium channels.
This provides a minor pathway by which growth hormone is released.

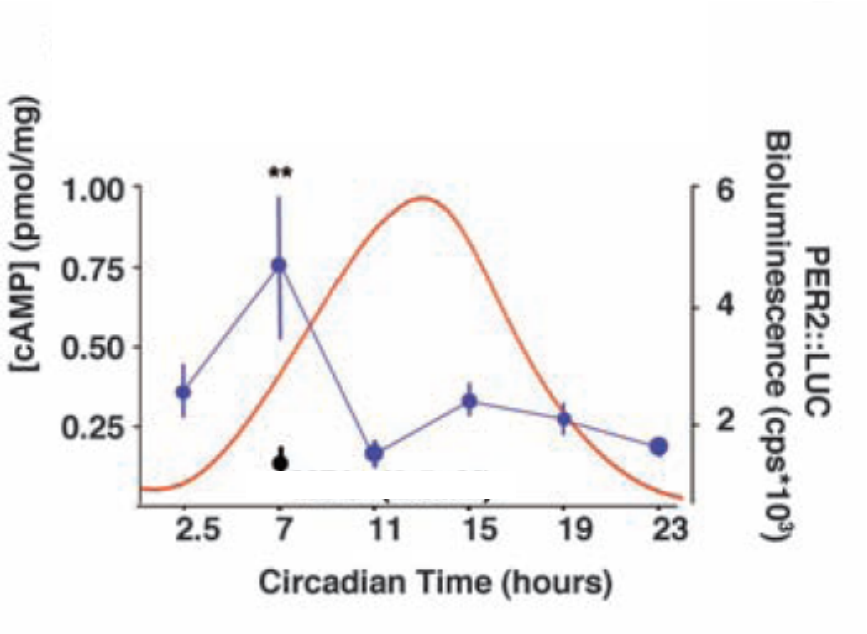
Picture: Epinephrine (adrenaline) binds its receptor, that associates with an heterotrimeric G protein. The G protein associates with adenylyl cyclase that converts ATP to cAMP, spreading the signal.

www.wikipedia.org

# Cyclic cAMP levels in mouse brain

The molecular oscillations of the SCN were tracked as circadian emission of bioluminescence by organo-typical slices from transgenic mouse brain.

Picture: a fusion protein of mPER2 and LUCIFERASE (mPER2::LUC) reported circadian protein synthesis rhythms.



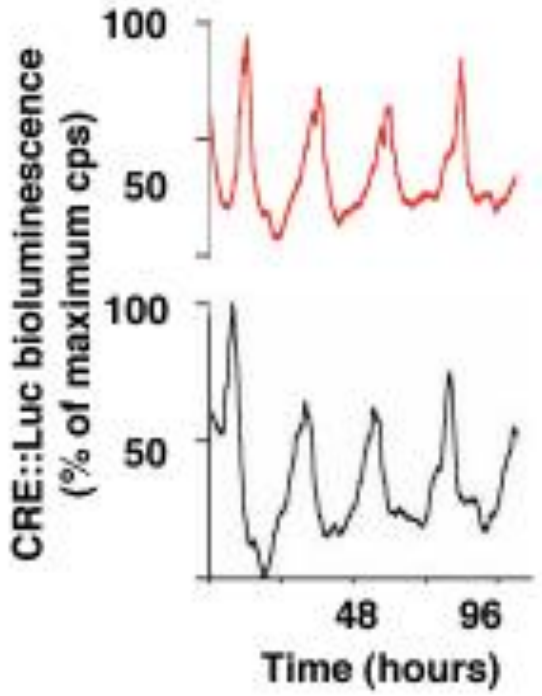

Circadian oscillation of cAMP concentration (blue) and PER2::LUC bioluminescence (red).

Interpretation: Under these conditions, the cAMP content of the SCN was circadian.

O'Neill et al.
Science, 320, 949 (2008)

# Cyclic cAMP levels in mouse brain

The circadian cAMP content of the SCN is accompanied by a circadian cycle in activity of cAMP response element sequences (CRE) reported by a *CRE::luciferase* adenovirus.




Circadian oscillation of CRE activity in two representative SCN slices (red and black) reported by *CRE:luciferase* adenovirus.

O'Neill et al.
Science, 320, 949 (2008)

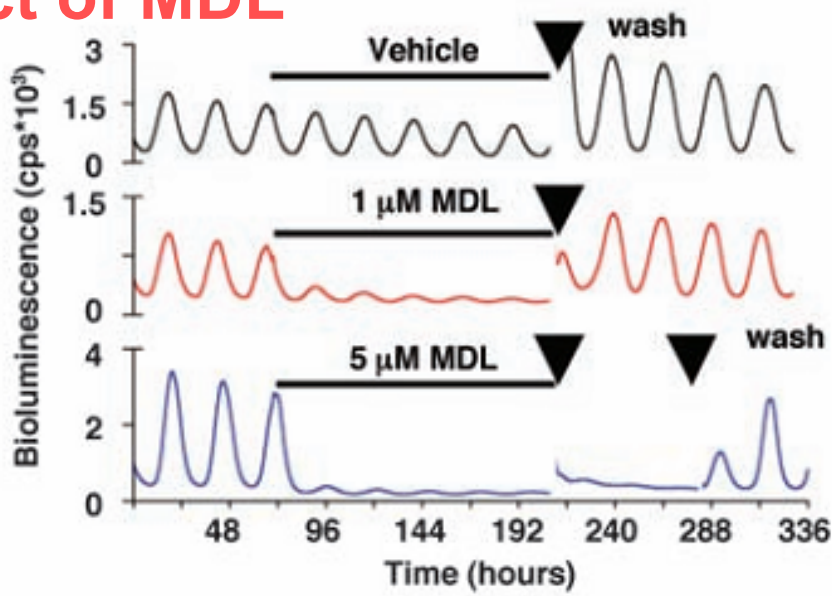
# Effect of MDL

**Idea**: can one show that cAMP is the reason for the oscillations?

**Realization**: need to suppress cAMP-production in the cell.

**Experiment**: treat SCN slices with MDL, a potent, irreversible inhibitor of the enzyme adenylyl cyclase (that synthesizes cAMP) to reduce concentrations of cAMP to basal levels.

"Vehicle" is a control experiment.



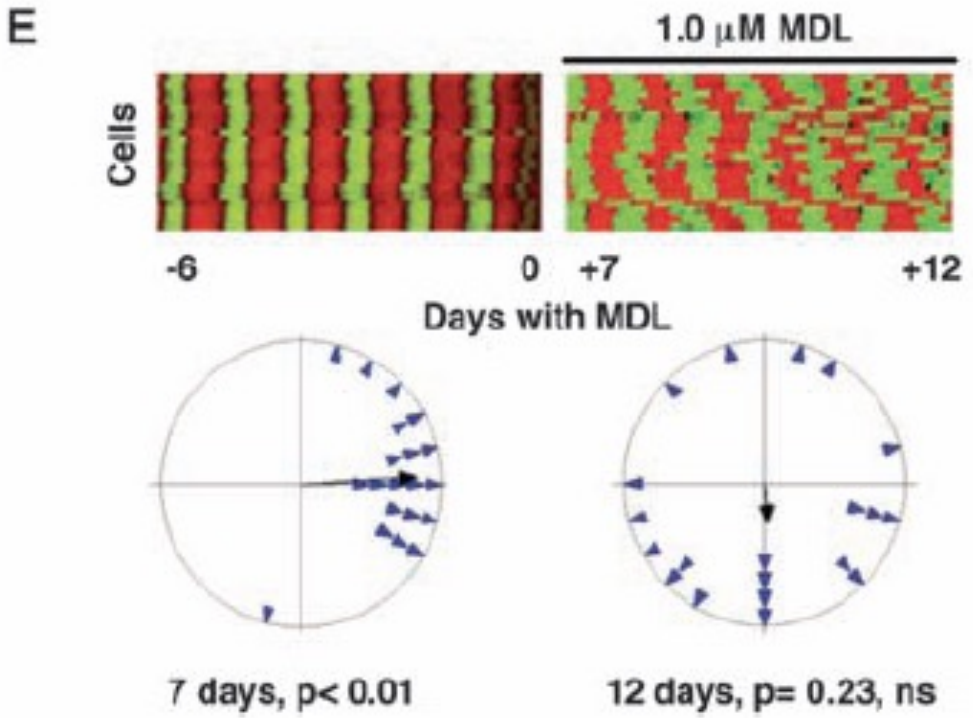

**Interpretation**: MDL rapidly suppressed circadian CRE:luciferase activity, presumably through loss of cAMP-dependent activation of CRE sequences.

This caused a dose-dependent **decrease** in the **amplitude** of cycles of circadian transcription and protein synthesis observed with mPer1::luciferase and mPER2::LUC.

O'Neill et al.
Science, 320, 949 (2008)

# MDL also affects the synchronization of the clock

Prolonged exposure to mild levels of MDL (1.0 μM) suppressed and desynchro-nized the transcriptional cycles of SCN cells.



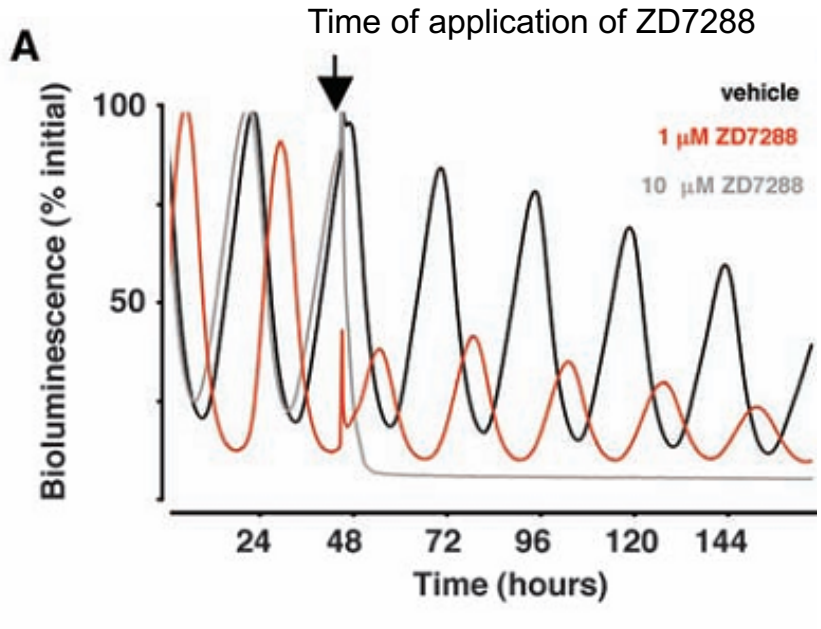

O'Neill et al.
Science, 320, 949 (2008)

# Can one block cAMP action?

**Idea**: If cAMP sustains the clock, interference with cAMP effectors should compromise pacemaking.

**PlanA**: treat brain slices with **inhibitors** of cAMP-dependent protein kinase.
This had no effect, however, on circadian gene expression in the SCN.

**PlanB**: But cAMP also acts through hyperpolarizing cyclic nucleotide–gated ion (HCN) channels and through the guanine nucleotide–exchange factors Epac1 and Epac2 (Epac: exchange protein directly activated by cAMP).




The irreversible HCN channel blocker ZD7288, which would be expected to hyperpolarize the neuronal membrane, dose-dependently damped circadian gene expression in the SCN.
This is consistent with disruption of trans-criptional feedback rhythms.

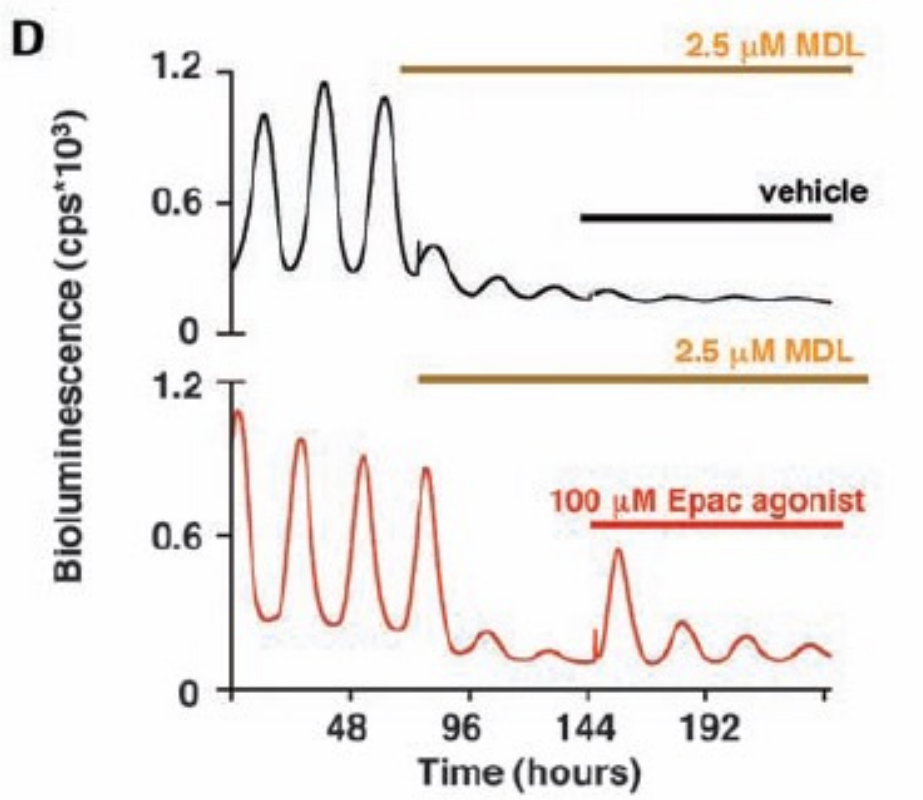
O'Neill et al.
Science, 320, 949 (2008)

# Can cAMP stimulation be recoved?

Experimentalists typically interrupt a cellular process and then restore it by a side-process.

Idea: **Direct activation** of the **effectors** might compensate for inactivation of adenylate cyclase by MDL.

Observation: A hydrolysis-resistant Epac agonist (bottom plot) transiently activated oscillations in transcriptional activity in SCN treated with MDL.

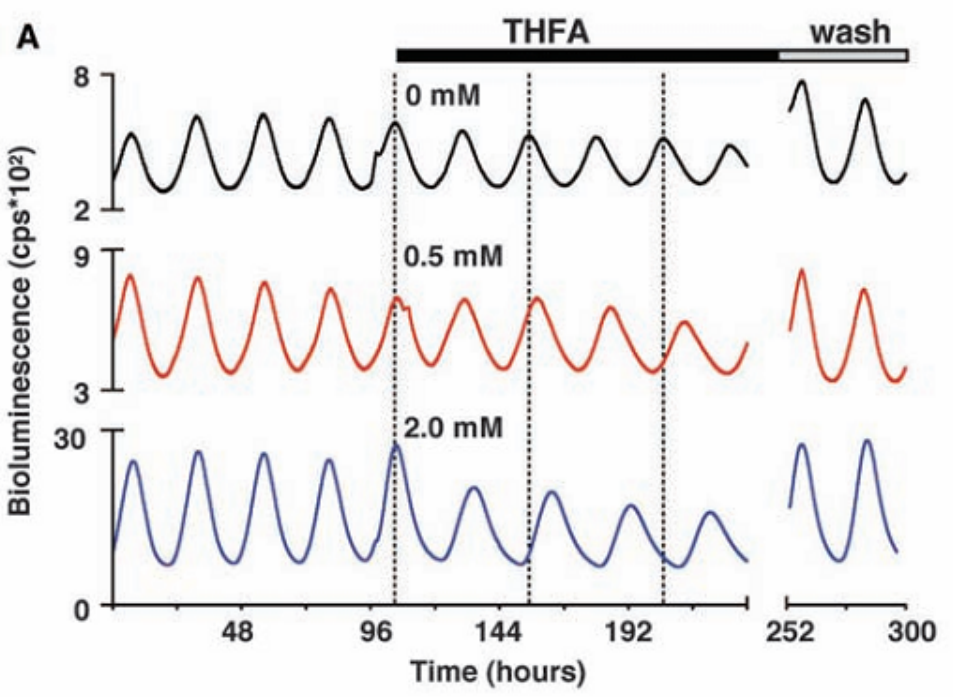



O'Neill et al.
Science, 320, 949 (2008)

# slowing cAMP synthesis

**Idea**: if cAMP signaling is an integral component of the SCN pacemaker, altering the rate of cAMP synthesis should affect circadian period.

**Experiment**: 9-(tetrahydro-2-furyl)-adenine (THFA) is a noncompetitive inhibitor of adenylate cyclase that slows the rate of $G_s$-stimulated cAMP synthesis, which attenuates peak concentrations.




**Interpretation**: THFA dose-dependently increased the period of circadian pacemaking in the SCN, from 24 to 31 hours, with rapid reversal upon washout

O'Neill et al.
Science, 320, 949 (2008)

# Conclusions on cAMP-coupling

Circadian pacemaking in mammals is **sustained**.

Its canonical properties of **amplitude**, **phase**, and **period** are determined by a reciprocal interplay in which transcriptional and posttranslational feedback loops drive rhythms of cAMP signaling.

Dynamic changes in cAMP signaling, in turn, regulate transcriptional cycles.

Thus, output from the current cycle constitutes an input into subsequent cycles.

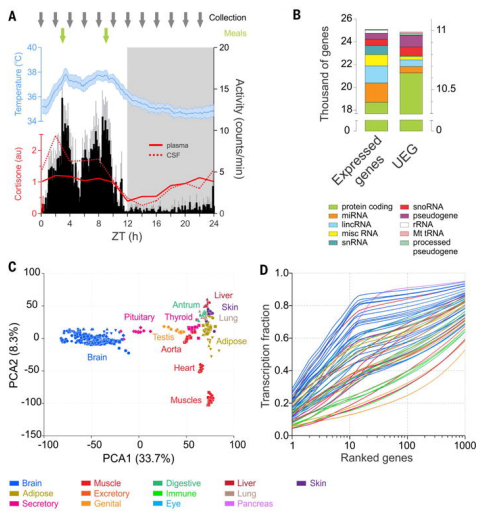
The interdependence between nuclear and cytoplasmic oscillator elements we describe for cAMP also occurs in the case of $Ca^{2+}$ and cADPR.

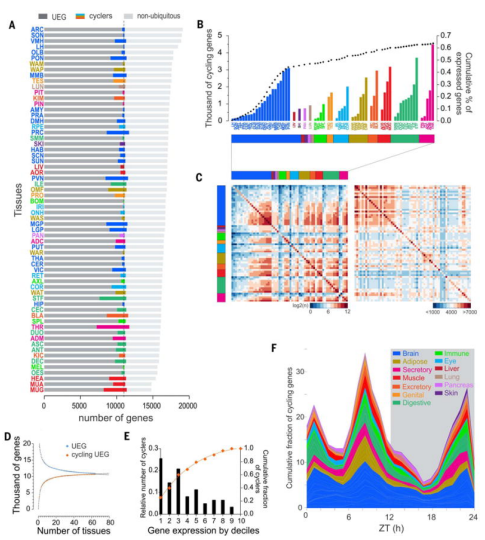
This highlights an important newly recognized common logic to circadian pacemaking in widely divergent taxa.

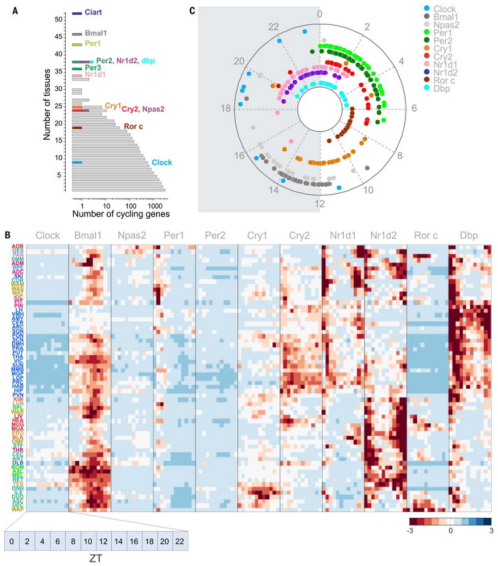
O'Neill et al.
Science, 320, 949 (2008)

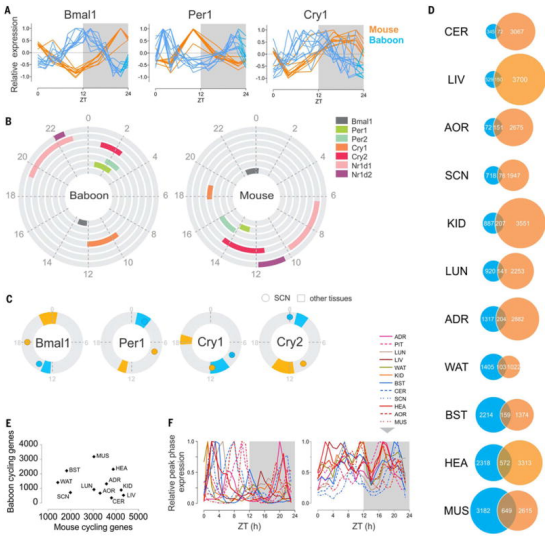
# Content from paper 1 that is relevant for mini test #1

ONLY: methods and results related to Figs 2, 3, 5, 7



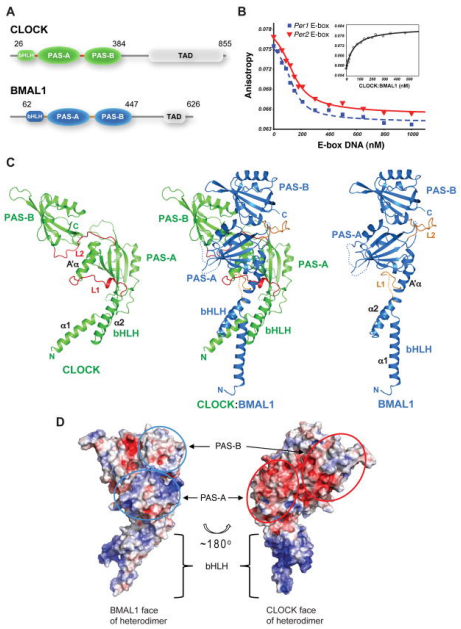



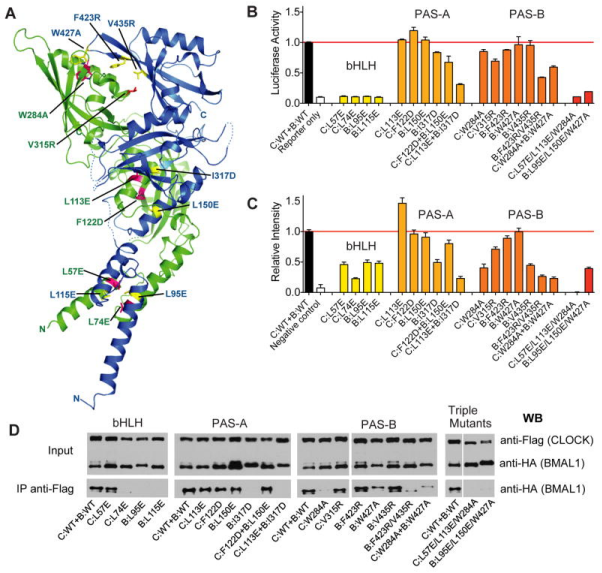



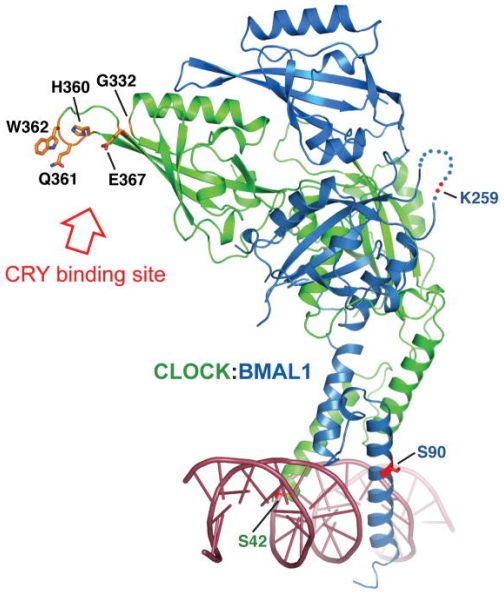



# Content from paper 2 that is relevant for mini test #1

ONLY: methods and results related to Figs 1, 4, 5



# Content from paper 3 that is relevant for mini test #1

ONLY: methods and results related to Figs 1, 2, 5, 7