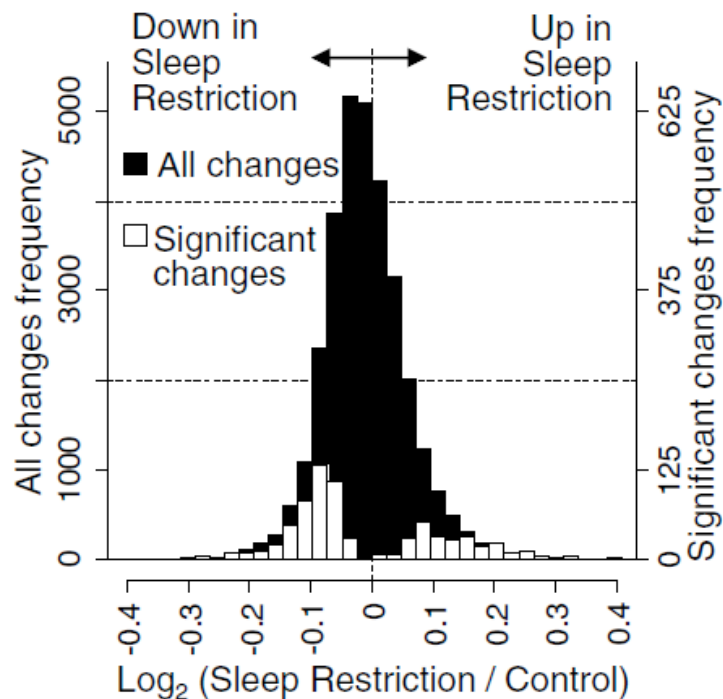


V4: Circadian rhythms (wrap up)

Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome

Carla S. Möller-Levet¹, Simon N. Archer¹, Giselda Bucca¹, Emma E. Laing, Ana Slak, Renata Kabiljo, June C. Y. Lo, Nayantara Santhi, Malcolm von Schantz, Colin P. Smith¹, and Derk-Jan Dijk^{1,2}



Frequency distribution of expression fold-changes after sleep restriction relative to control.

Filled area: Histogram of changes in all transcripts (n = 31,685 probes that target 22,862 genes)

Open area: changes in transcripts identified as having a statistically significant (multiplicity corrected P value < 0.05) main effect of Sleep Condition

(n = 744 transcript that target 711 genes).

Bonferroni correction for multiple testing

The **Bonferroni correction** is a method used to counteract the problem of multiple comparisons. It is considered the simplest and **most conservative** method to control the familywise error rate.

The Bonferroni correction is based on the idea that if an experimenter is testing m dependent or independent hypotheses on a set of data, then one way of maintaining the familywise error rate is to test each individual hypothesis at a statistical significance level of $1/m$ times what it would be if only one hypothesis were tested.



Carlo Emilio Bonferroni
(1892-1960)

www.wikipedia.org

False-discovery rate (FDR)

	Null hypothesis is true (H_0)	Alternative hypothesis is true (H_1)	total
Declared significant	V (# false positives) = „false discoveries“	S (# true positives)	R (# rejected null hypotheses) = „discoveries“
Declared non-significant	U (# true negatives)	T (# false negatives)	$m - R$
Total	m_0	$m - m_0$	m

In m hypothesis tests of which m_0 are true null hypotheses, R is an observable random variable, and S , T , U and V are unobservable random variables.

We can define Q as the ratio of false discoveries : $Q = V / R$

We set $V / R = 0$ if $R = 0$.

This is the false discovery rate:

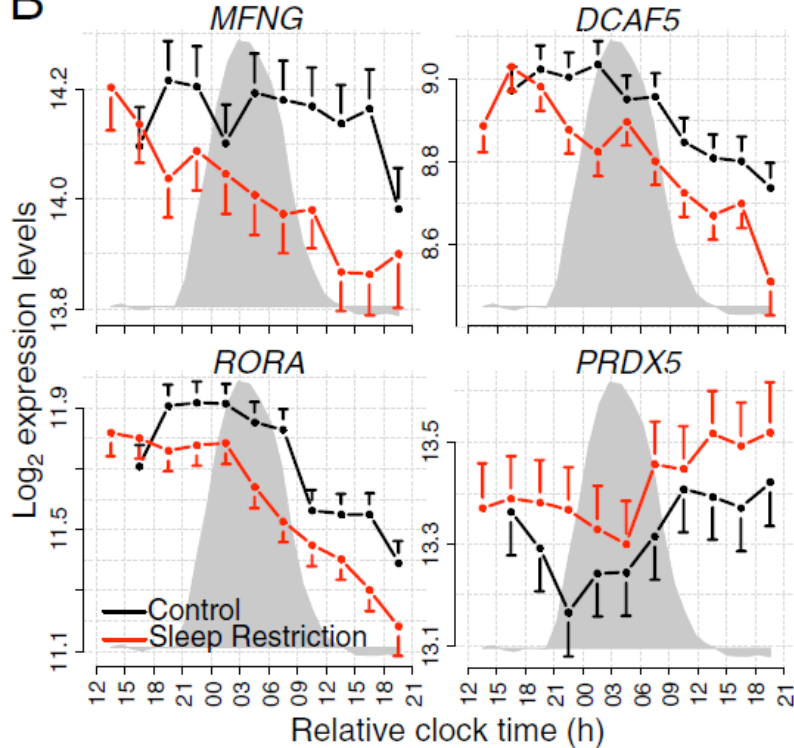
$$FDR = Q_e = E[Q] = E\left[\frac{V}{V + S}\right] = E\left[\frac{V}{R}\right],$$

The **Benjamini-Hochberg** method is one FDR-method.

www.wikipedia.org

Examples of genes with significant effect of Sleep Condition

B



$$P < 1 \times 10^{-6}$$

MFNG: O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase

DCAF5: is a protein-coding gene ...

RORA: retinoic acid receptor-related orphan receptor alpha is a nuclear hormone receptor – associated with circadian rhythms

PRDX5: peroxiredoxin 5

Greyed areas: melatonin profile averaged for the two conditions.

Individual data were aligned relative to the individual melatonin rhythm and sorted into discrete circadian phase bins.

Because of the shift in circadian phase after sleep restriction and to individual variation, the 10 melatonin samples covered 11 circadian phase bins after sleep restriction.

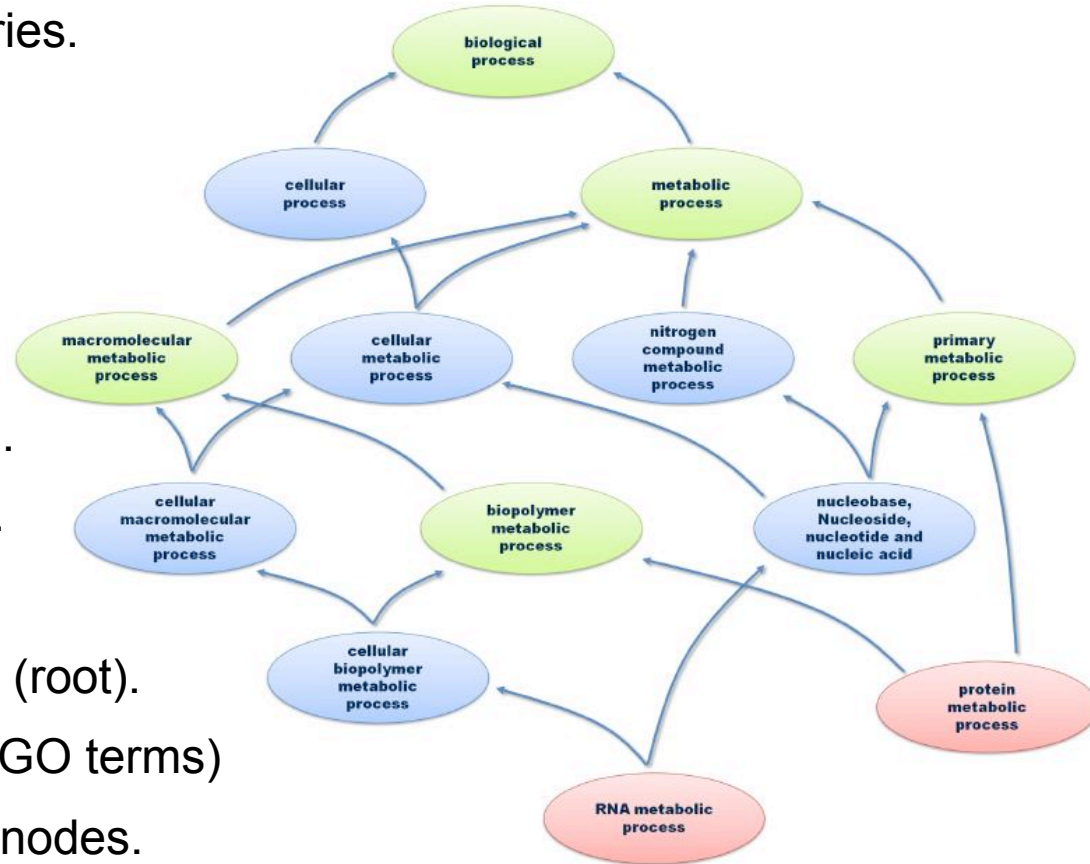
Gene Ontology (GO)

Ontologies are structured vocabularies.

The Gene Ontology has 3 tracks:

- biological process (BP)
- molecular function (MF)
- cellular component (lokalisation).

Shown here is a part of the BP tree.



At the top: most general expression (root).

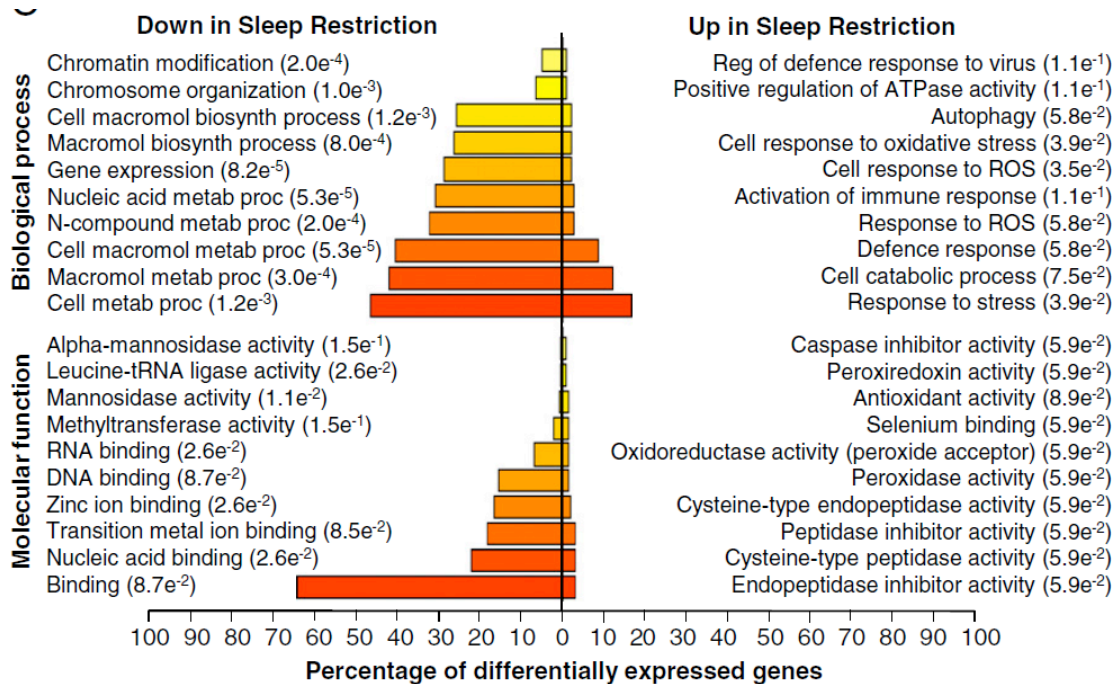
Red: leafs of the tree (very specific GO terms)

Green: common ancestors of 2 red nodes.

Blue: other nodes.

Lines: „Y is contained in X“- relationships

What sort of genes are differentially expressed?



The top 10 enriched GO biological processes and molecular functions within the statistically significant differentially expressed gene list as identified by WebGestalt when using the human genome as background. P-values corrected by Benjamini-Hochberg method.

Down-regulation: chromatin modification and organization, metabolism

Up-regulation: cellular response to oxidative stress and reactive oxygen

Over-representation analysis

4 services used for **over-representation analysis** of GO terms in gene sets:

2 with Agilent Probe IDs

(1) **DAVID** (NIH)

(2) **MetaCore™** (Thomson-Reuters)

- Features

Pathway analysis of 'omics' data for drug discovery

Knowledge mining of the database for hypothesis generation

Target and biomarker assessment and validation

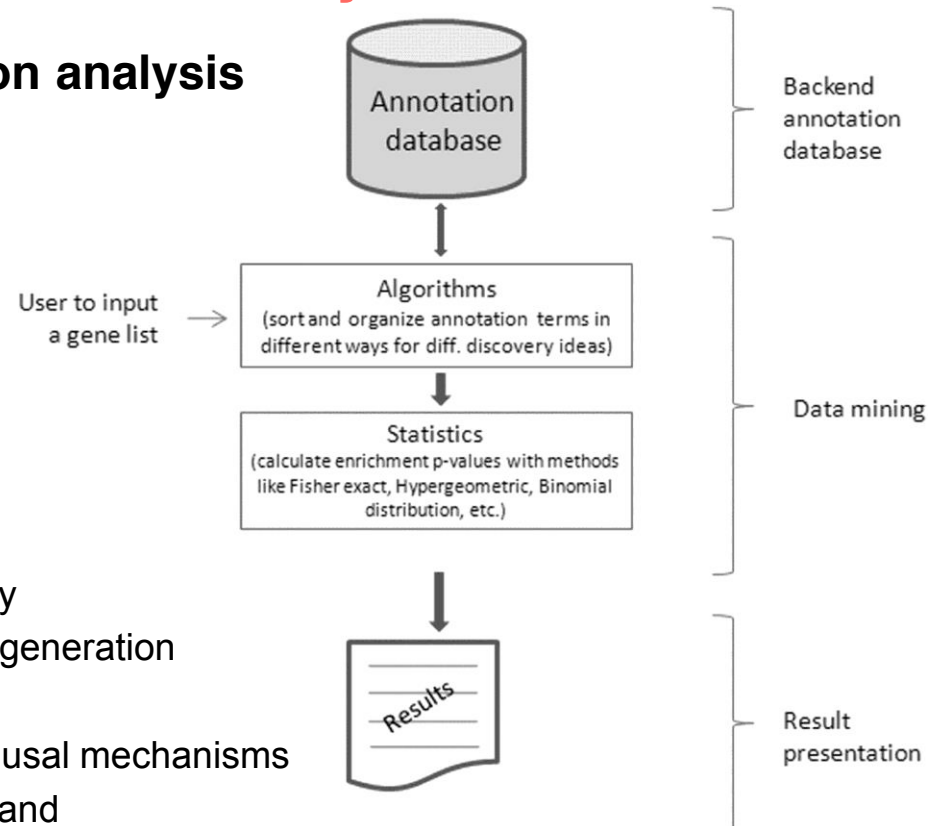
Disease pathway modeling and investigation of causal mechanisms

Patient stratification, comprehensive comparison, and
functional fingerprinting

2 with HUGO gene symbols

(3) **Toppcluster** uses the hypergeometric test, Bonferroni/FDR

(4) **Webgestalt** uses hypergeometric test or the Fisher's exact test



Huang, Sherman, Lempicki,
Nucl. Acids Res. (2009) 37: 1-13

Over-representation analysis (WebGestalt)

Suppose that we have n genes in the interesting gene set (A) and m genes in the reference gene set (B).

Suppose further that there are k genes in A and j genes in B that are in a given category (C) (e.g. a GO category, a KEGG pathway, a BioCarta pathway etc.).

Based on the reference gene set, the expected value of k would be

$$k_e = (n/m) \times j$$

If k exceeds the above expected value, category C is said to be **enriched**, with a **ratio of enrichment** (r) given by $r = k/k_e$.

Zhang, Kirov, Snoddy (2013)
Nucl Ac Res 33: W741-W748

Over-representation analysis (WebGestalt)

If B represents the population from which the genes in A are drawn, WebGestalt uses the **hypergeometric test** to evaluate the significance of enrichment for category C in gene set A,

$$P = \sum_{i=k}^n \frac{\binom{m-j}{n-i} \binom{j}{i}}{\binom{m}{n}}$$

Interpretation: draw $i = k$ genes for A that belong to category C from the j genes from B that belong to C.

→ The other $n - i$ genes in A do not belong to C. They are drawn from the $m - j$ genes in B that do not belong to C.

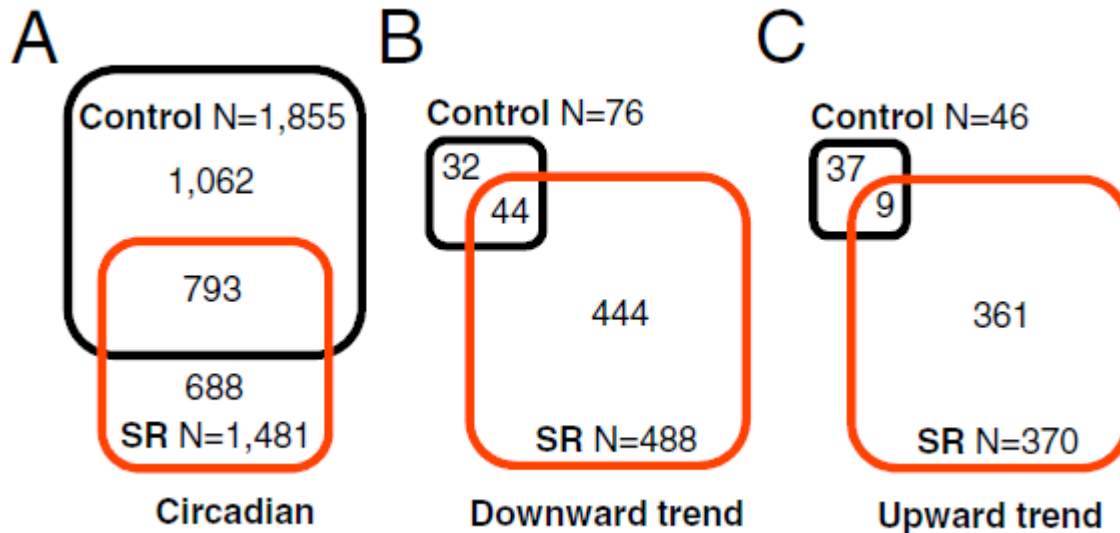
Normalization is done by total number of possibilities to draw n genes from m genes.

If A and B are two independent gene sets, WebGestalt uses **Fisher's exact test** instead,

$$P = \sum_{i=k}^n \frac{\binom{n}{i} \binom{m}{j+k-i}}{\binom{m+n}{j+k}}$$

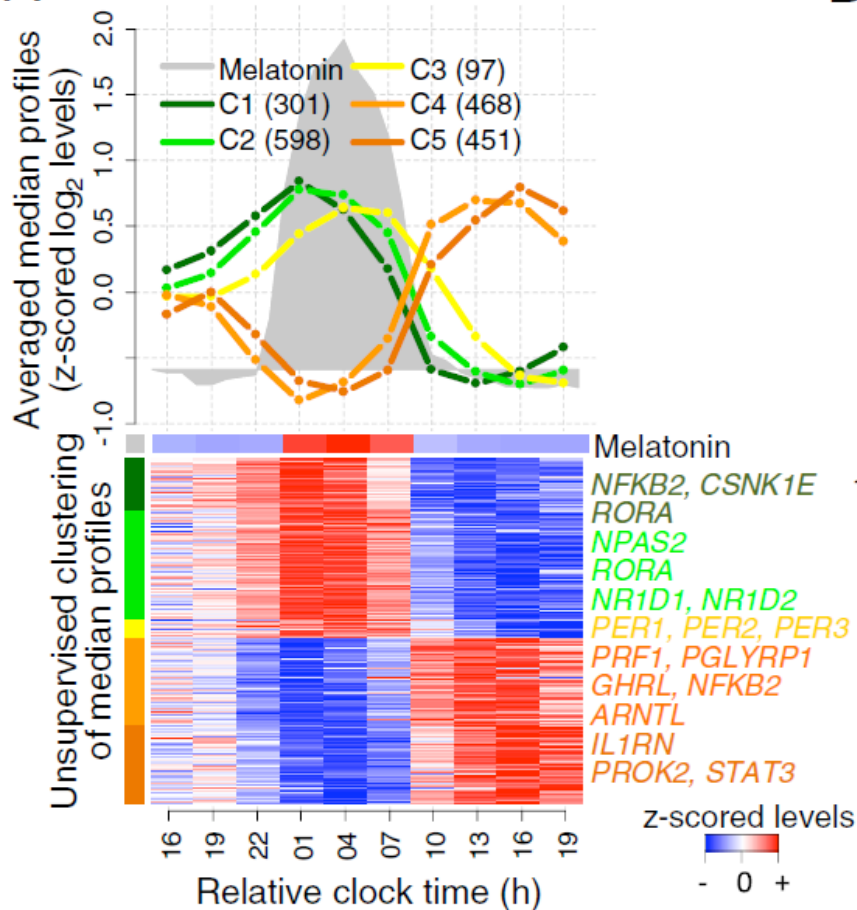
Zhang, Kirov, Snoddy (2013)
Nucl Ac Res 33: W741-W748

Overlap of gene sets



Intersection of genes identified as circadian and time-awake–dependent in control and sleep-restriction (SR) conditions. (A) Venn diagram of prevalent circadian genes. (B) Venn diagram of genes identified as having a prevalent time-awake upward trend. (C) Venn diagram of genes identified as having a prevalent time-awake downward trend.

Effect of sleep restriction on phases of circadian genes



Genes with a prevalent circadian variation during the constant routine/total sleep deprivation after the control condition (2,103 probes that target 1,855 genes, FDR <5%). Heatmap rows correspond to the median of the melatonin-aligned probe values across all participants in the control condition.

Rows are clustered based on a circular self-organizing map. Cluster means are plotted above as time-series.

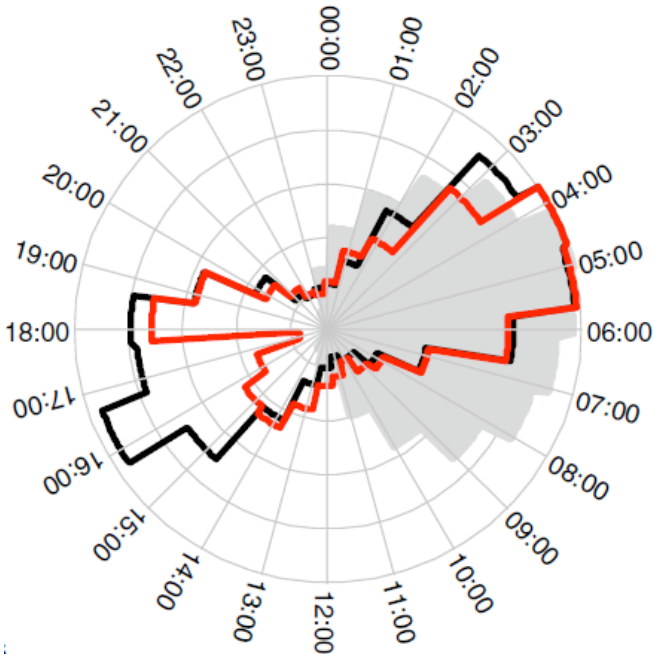
In parenthesis: number of genes per cluster (C1–C5).

Color codes left of heatmap match the colors of the clusters. Genes related to circadian rhythmicity and sleep (according to GO) are indicated in the heatmap (colors indicate cluster location).

5

— Control
— Sleep Restriction
— Melatonin

Shift in melatonin-aligned peak times



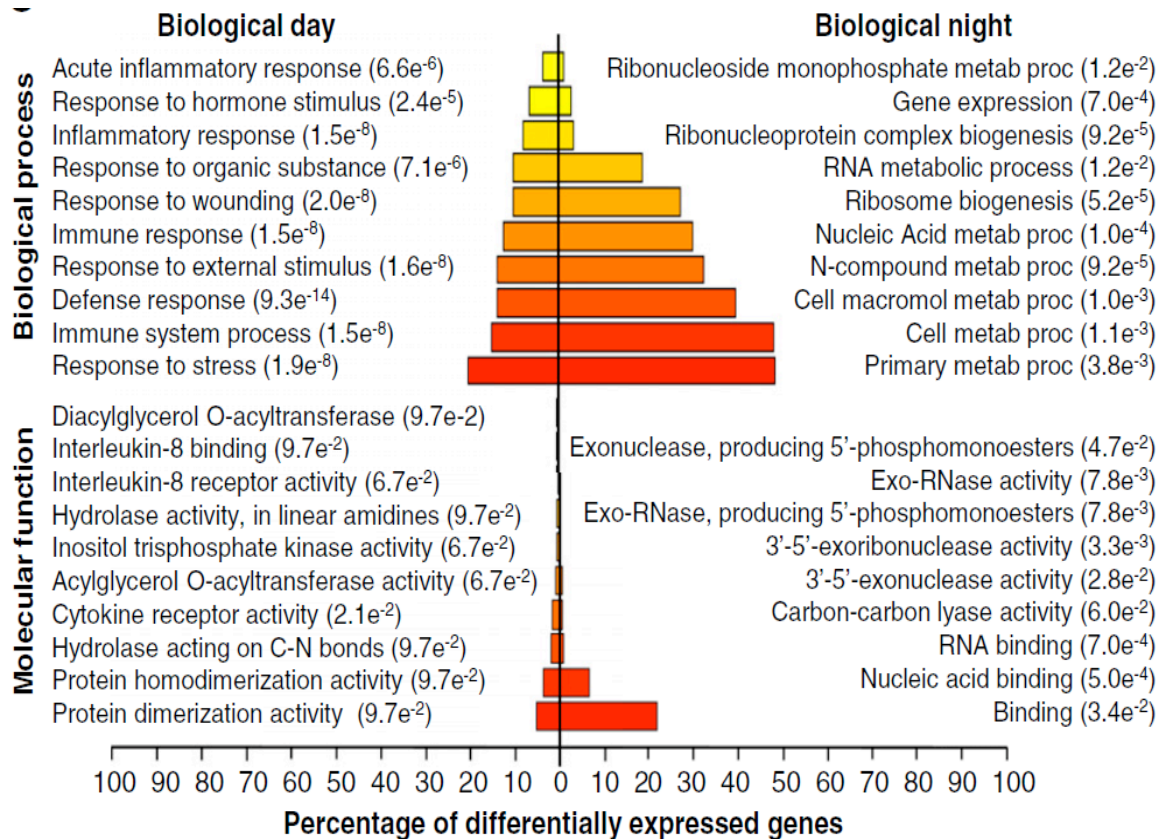
Genes with **night peaks** (control) are enriched in GO terms for: gene expression, RNA metabolic processes, cellular metabolic processes

Genes with day peaks (control): Response to hormone and stress, inflammatory, immune and defense response, interleukin and cytokine activity.

Phase histogram of melatonin-aligned peak times of prevalent circadian genes following and sleep restriction.

The distribution of the phases is significantly different between conditions.

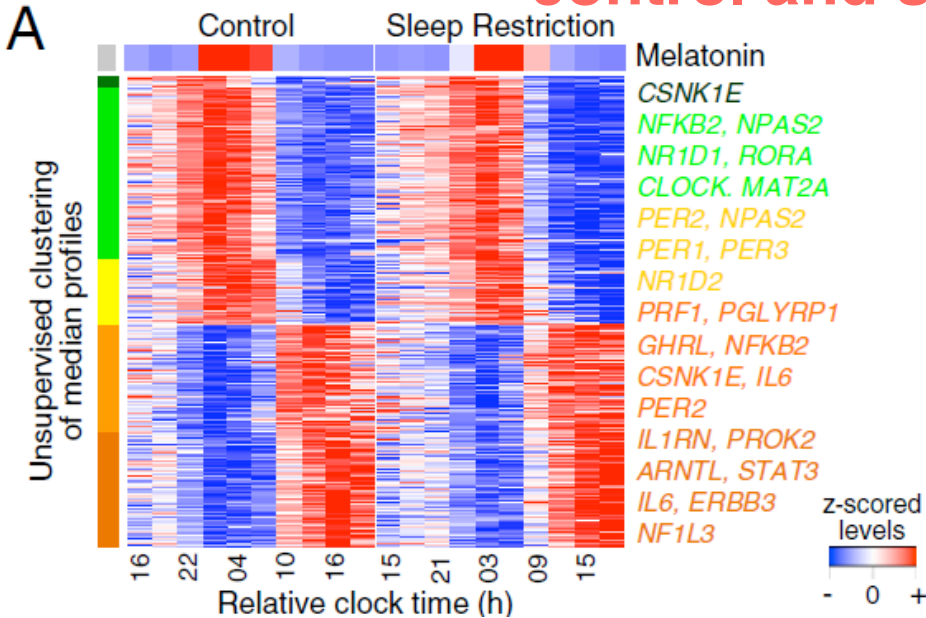
Gene functions of „normal“ circadian genes



Top 10 enriched GO BPs and MFs within the circadian gene list of the control condition using the human genome as a background.

Percentages are based on the number of unique gene symbols annotated as belonging to a specific biological process/ molecular function compared with the number of unique gene symbols within the entire gene list. P values corrected by Benjamini and Hochberg.

Circadian variations in the transcriptome following control and sleep restriction

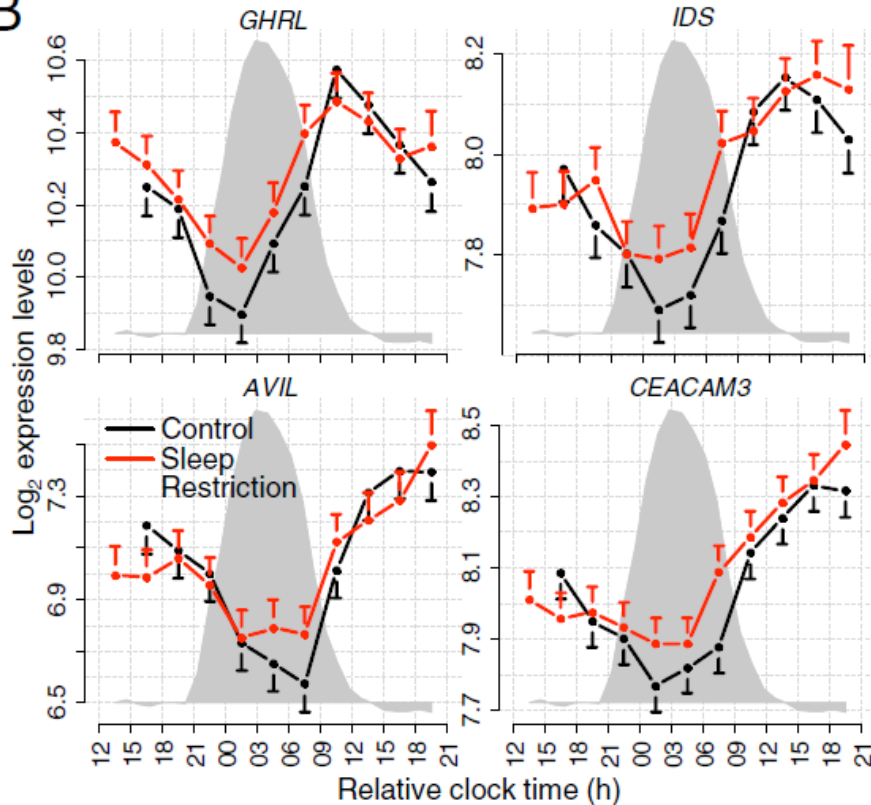


Genes with a prevalent circadian variation during the constant routine/total sleep deprivation after control and/or sleep restriction (n = 2,859 probes that target 2,510 genes).

Heatmap rows correspond to the median of the melatonin-aligned probe values across all participants per sleep condition. Rows are clustered based on a circular SOM. Color codes on the left side of the heat map identify the clusters. Relative clock times and melatonin profiles are average values across all participants per sleep condition. Genes related to circadian rhythmicity and sleep (according to Gene Ontology) are indicated in the heatmap (gene colors indicate cluster location).

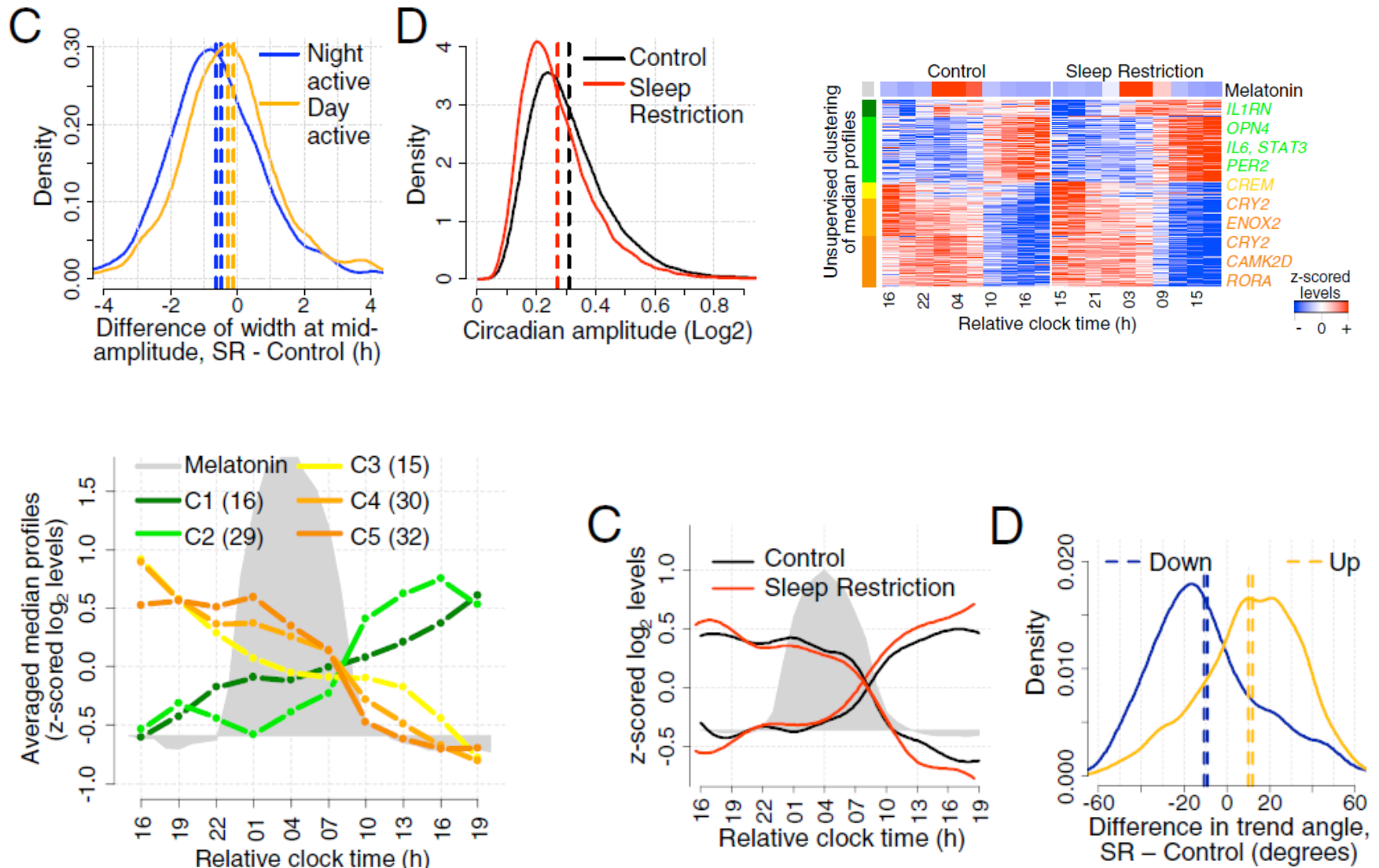
Genes with significant difference in circadian amplitude

B

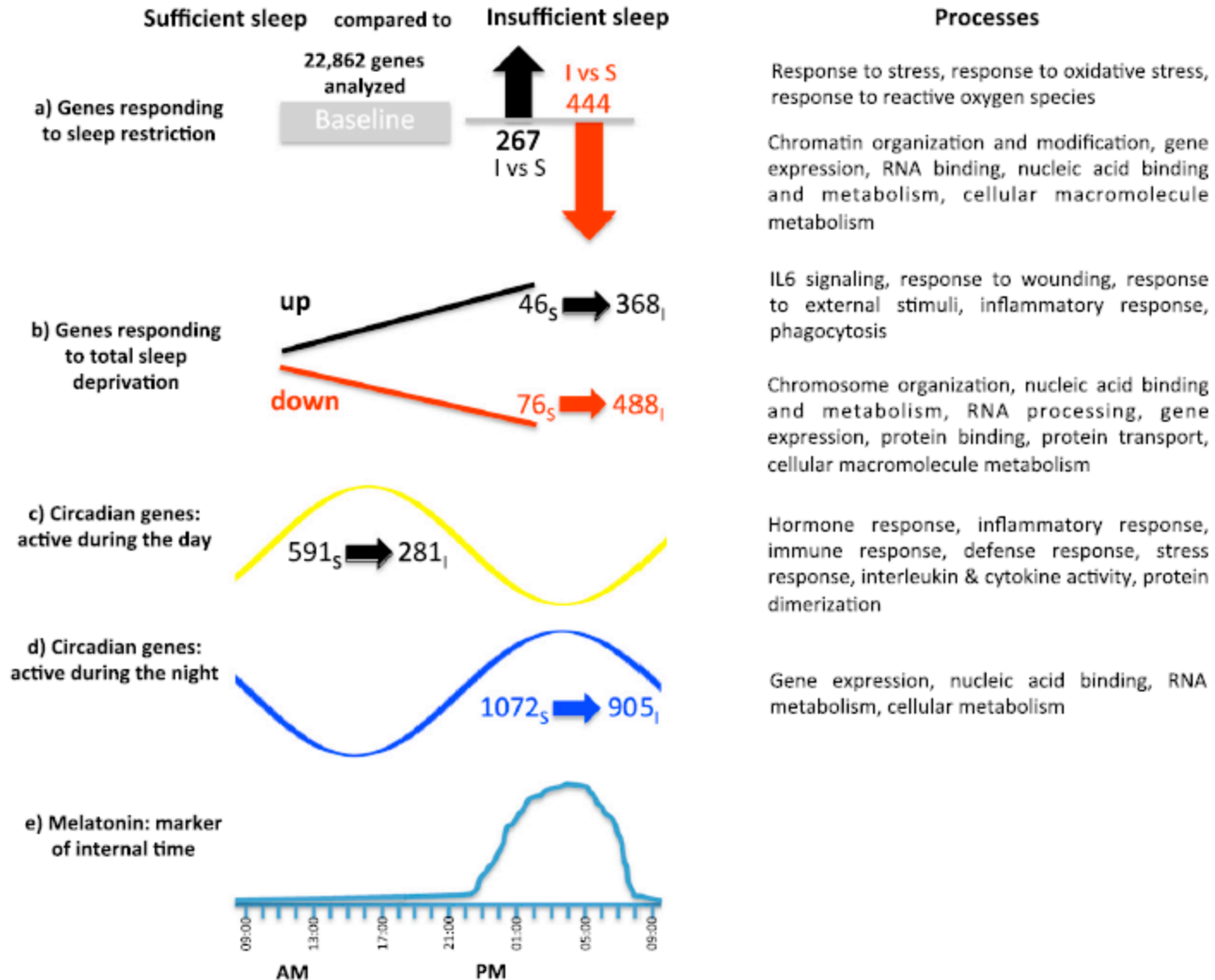


(B) Examples of genes with a significant difference in circadian amplitude:
GHRL,
IDS
AVIL, and
CEACAM3

Other results (not further discussed)



Summary of results



Circadian rhythms are coupled to metabolism

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

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

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

Review:

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

They drive the sleepwake cycle and coordinate subordinate 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 causes delay,
- 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) 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.

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 of amplitude, phase, and period.

Roles of cAMP?

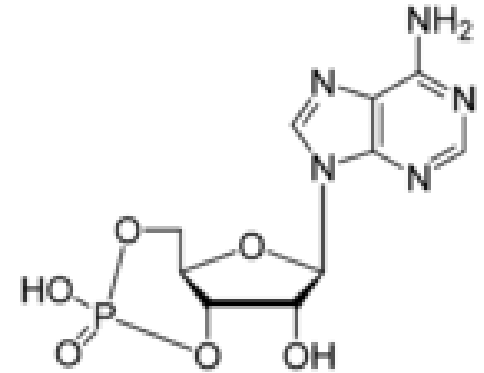
In molluscs, birds, and the mammalian SCN, cAMP is implicated in entrainment or maintenance of clocks, or both, or mediation of clock output. It was not considered as part of the core oscillator so far.

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

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.



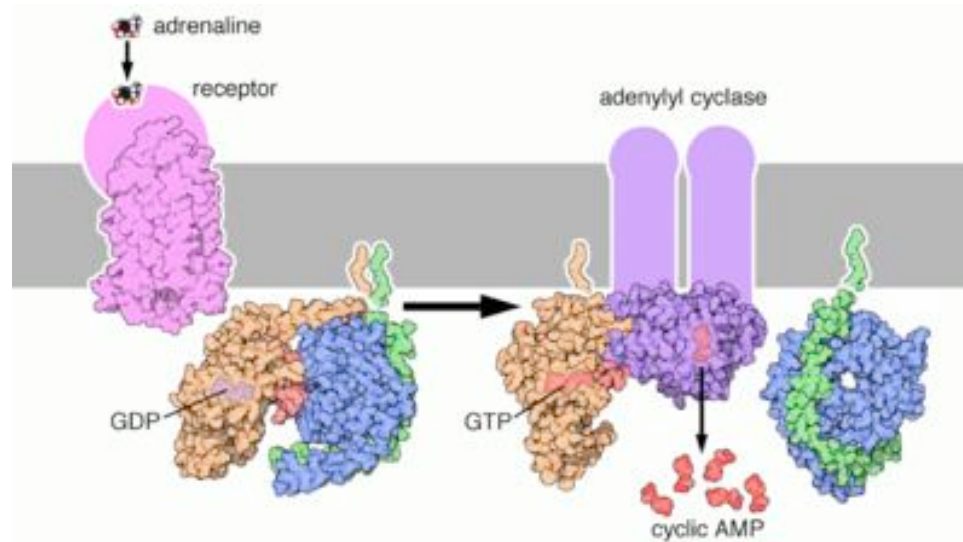
In humans, cyclic AMP works by activating **cAMP-dependent protein kinase (PKA)**.

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 and enables them to phosphorylate substrate proteins.

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 releasing hormone causes release growth hormone

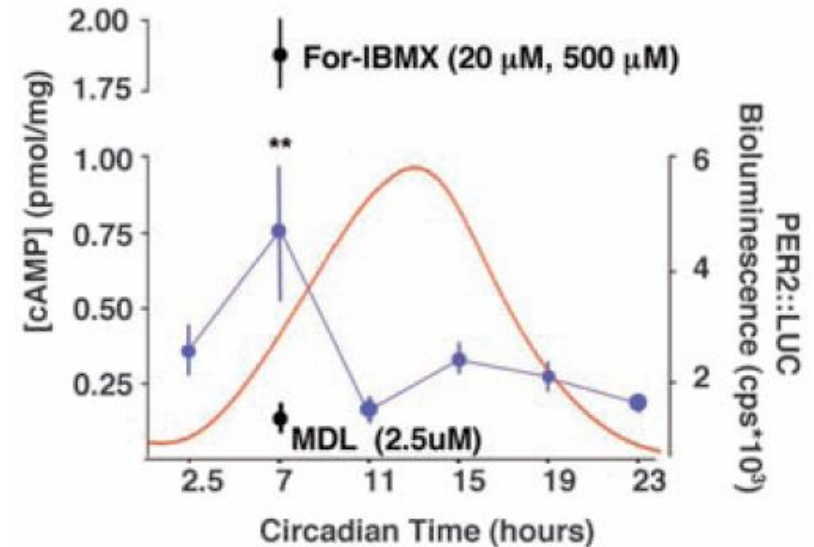
Picture: Epinephrine (adrenaline) binds its receptor, that associates with an heterotrimeric G protein. The G protein associates with adenylyl cyclase that converts ATP to cAMP, spreading the signal

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.

Rhythmic luciferase activity controlled by the Per1 promoter (Per1::luciferase) revealed circadian transcription, and a fusion protein of mPER2 and LUCIFERASE (mPER2::LUC) reported circadian protein synthesis rhythms.

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



Circadian oscillation of cAMP concentration (blue) and PER2::LUC bioluminescence (red), as well as cAMP concentration in SCN slices treated with MDL-12,330A (MDL) or with forskolin plus IBMX.

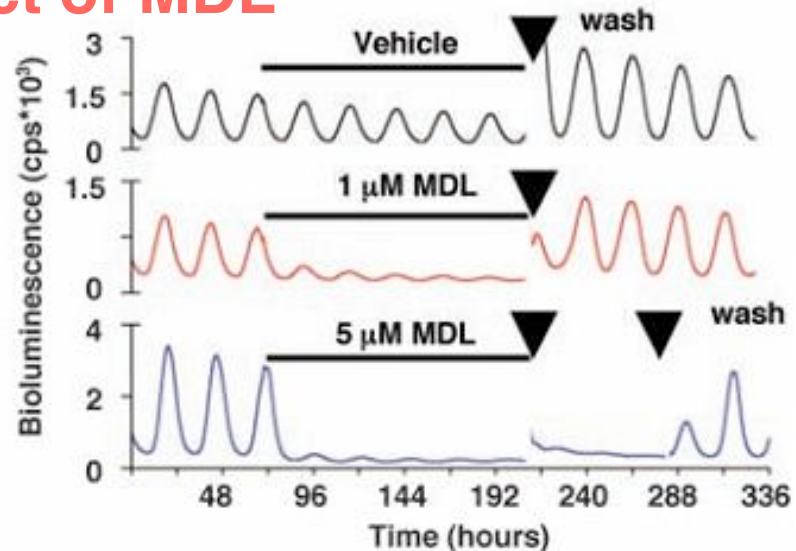
O'Neill et al.
Science, 320, 949 (2008)

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.



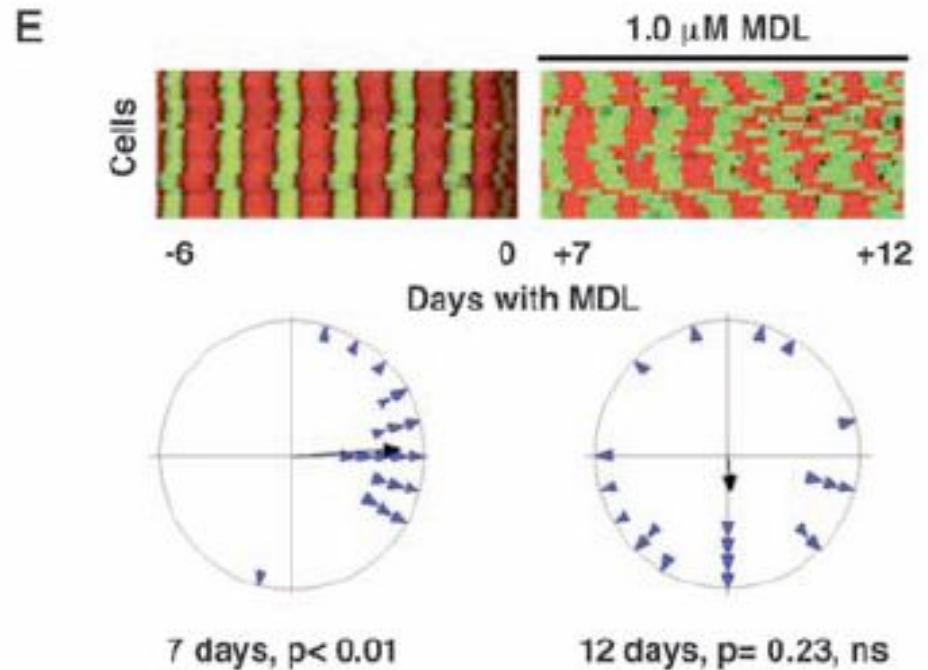
Interpretation: MDL rapidly suppressed circadian CRE::luciferase activity, presumably through loss of cAMP-dependent activation of CRE sequences.

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 ($1.0 \mu\text{M}$) suppressed and desynchronized the transcriptional cycles of SCN cells.



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

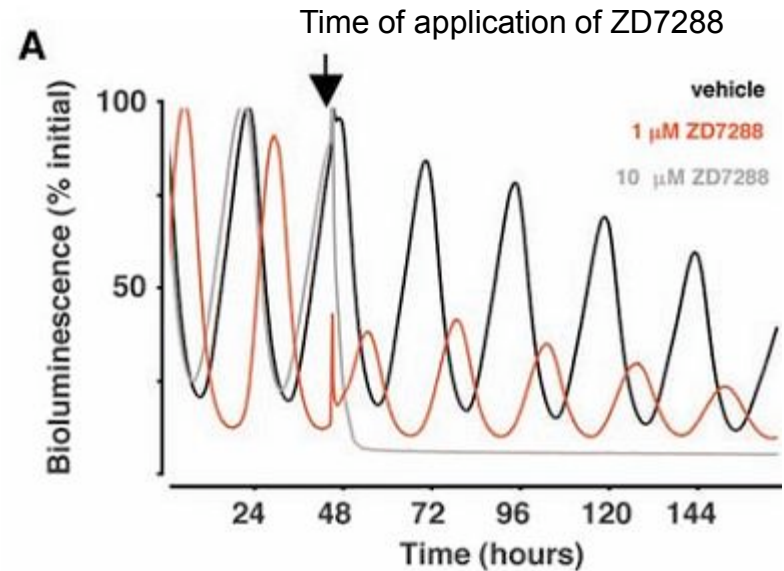
Can one block cAMP action?

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).

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



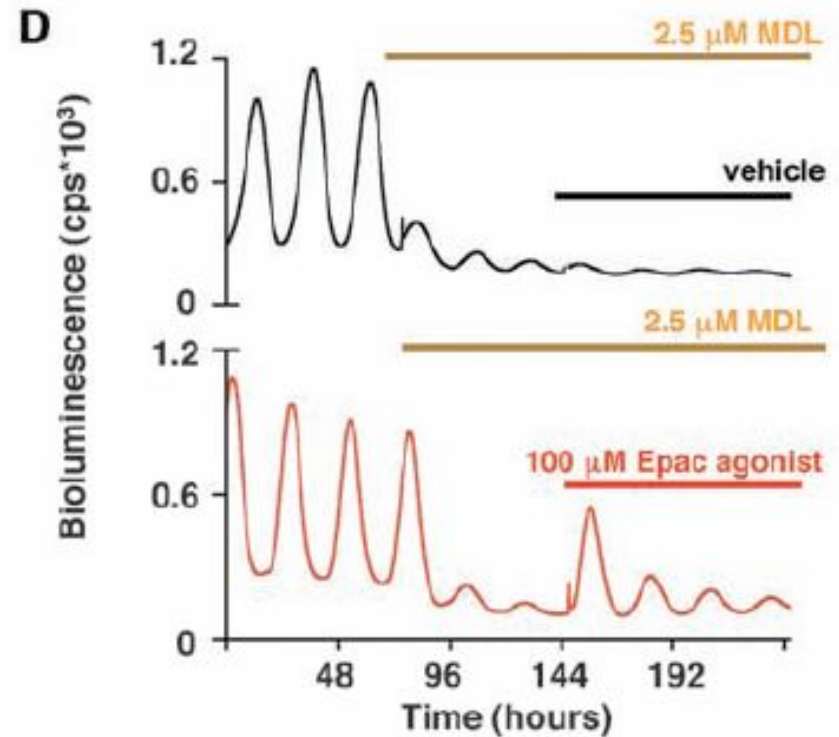
The irreversible HCN channel blocker ZD7288, which would be expected to hyperpolarize the neuronal membrane, dose-dependently damped circadian gene expression in the SCN.

This is consistent with disruption of transcriptional feedback rhythms.

Can cAMP stimulation be recovered?

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

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

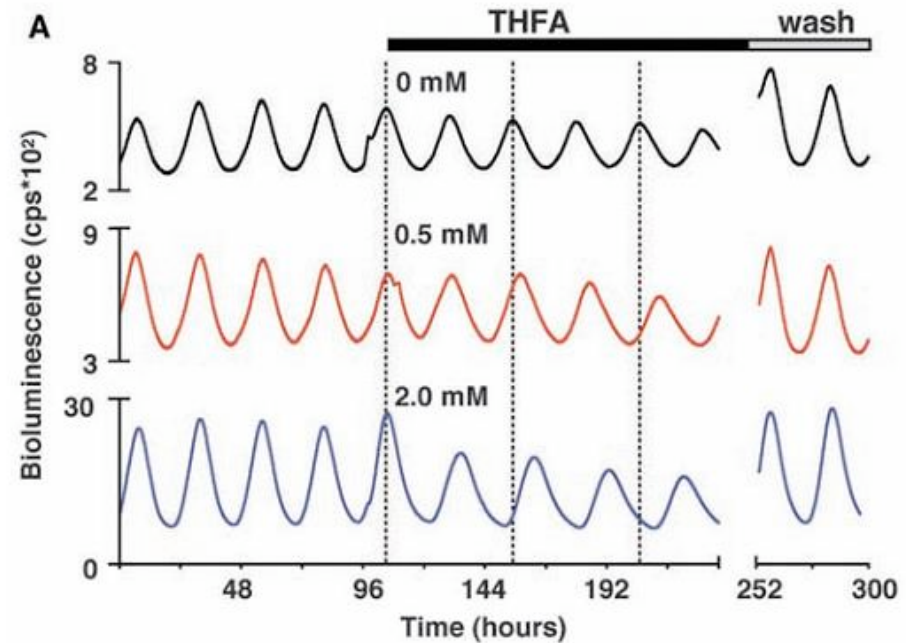


O'Neill et al.
Science, 320, 949 (2008)

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

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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)

Circadian regulation of epigenetic chromatin

Circadian Regulator CLOCK Is a Histone Acetyltransferase

Doi, Hirayama, Sassone-Corsi, Cell 125, 497 (2006)

Masao Doi,^{1,3} Jun Hirayama,^{1,2} and Paolo Sassone-Corsi^{1,2,*}

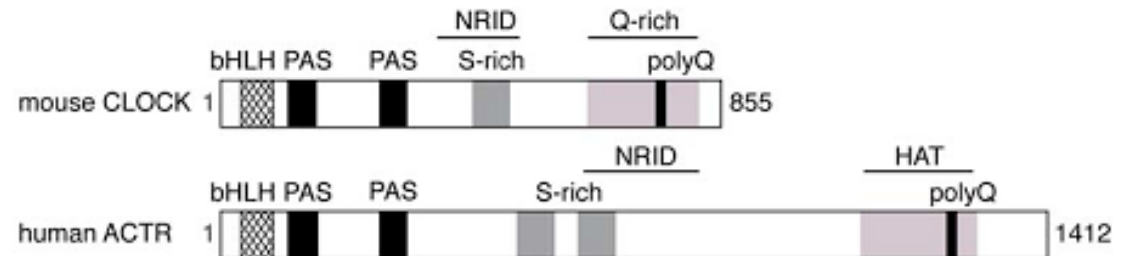
¹Institut de Génétique et de Biologie Moléculaire et Cellulaire, B.P. 10142, 67404 Illkirch, Strasbourg, France

²Present address: Department of Pharmacology, University of California, Irvine, 360D MedSurge II, Irvine, CA 92697, USA

³Present address: Division of Molecular Brain Science, Department of Brain Sciences, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan

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DOI 10.1016/j.cell.2006.03.033



Schematic representation of the primary structures of mouse CLOCK and human ACTR with common features; a basic helix-loop-helix (bHLH) motif (indicates binding to DNA), Per-Arnt-Sim (PAS) domains, serine-rich (S-rich) regions, a nuclear receptor interaction domain (NRID), a glutamine-rich (Q-rich) region containing a poly-glutamine (polyQ) stretch.

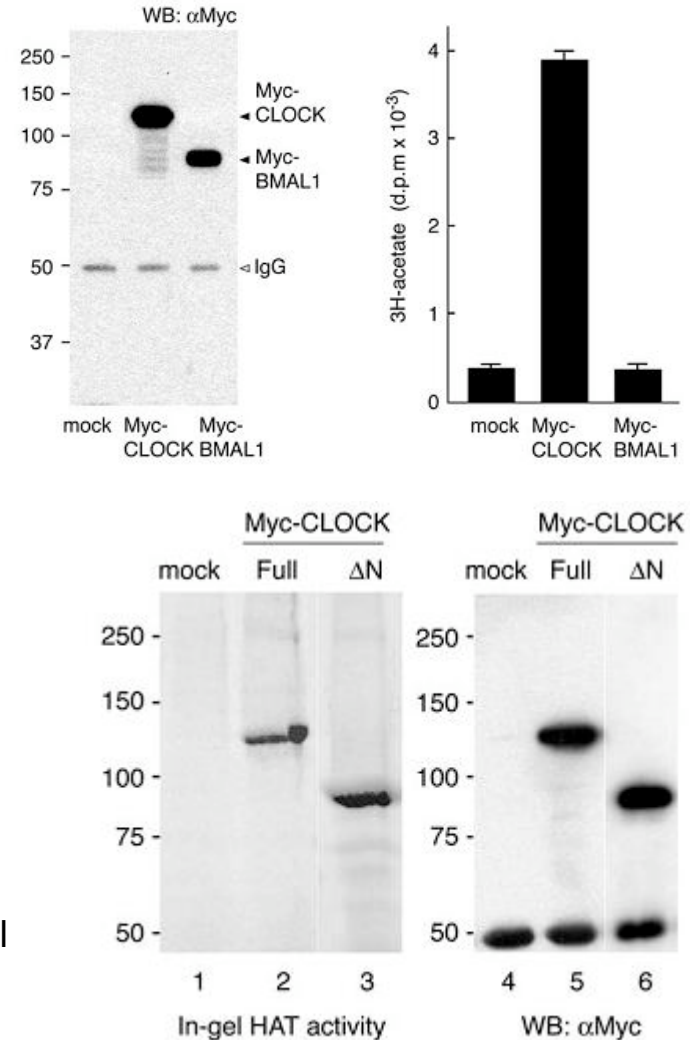
A horizontal line above hACTR indicates a region known to have HAT activity.

CLOCK is a histone acetyl transferase

(A) Myc-mCLOCK-specific immunoprecipitation of HAT activity. Myc-mCLOCK or Myc-mBMAL1 were transiently expressed in JEG3 cells and then immunoprecipitated with antiMyc 9E10 antibody. After extensive washing, the resulting immunoprecipitates were incubated with [3H] acetyl-CoA and a mixture of histone H3 and H4 amino-terminal tail peptides. The incorporated [3H] acetate was detected by filter binding assays. As a **control**, cells transfected with an empty vector (**mock**) were also subjected to the immunoprecipitation HAT assay.

Representative Western blot, illustrating the protein levels of the immunoprecipitated Myc-tagged proteins, is shown on the left.

(B) In-gel HAT activities of Myc-CLOCK. Either a full-length (Full) or an N-terminally truncated (DN) mCLOCK protein was expressed in JEG3 cells and immunoprecipitated as described in (A). The immunoprecipitates were resolved on a 7.5% SDS-PAGE gel containing core histones and processed to detect acetyltransferase activity (left). Identical immunoprecipitated samples were electrophoresed in a parallel SDS-PAGE gel and immunoblotted with antiMyc 9E10 antibody (right).

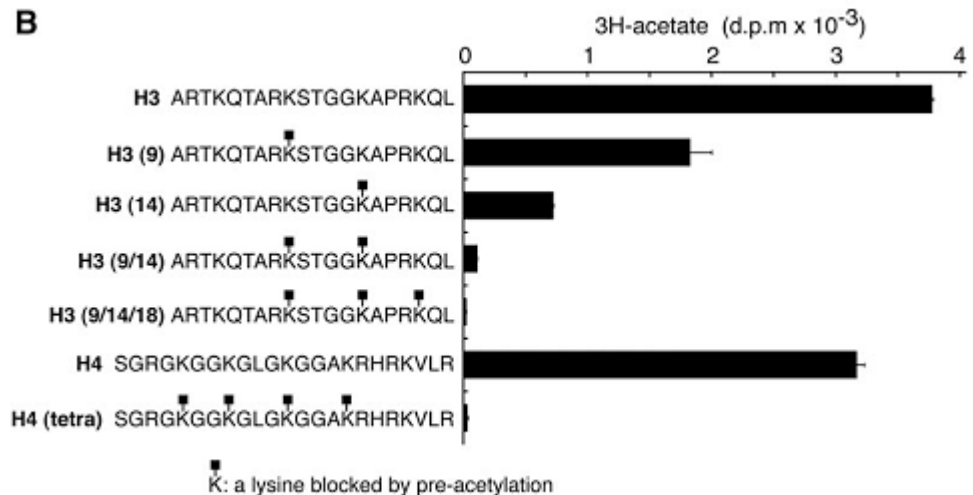
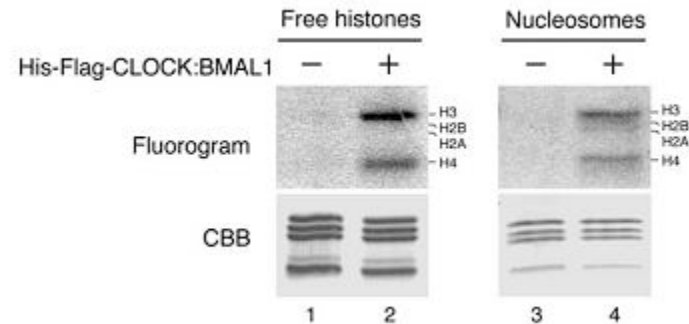


Doi, Hirayama, Sassone-Corsi, Cell 125, 497 (2006)

CLOCK is a histone acetyl transferase

TOP: HAT assays using either free core histones or mononucleosomes were performed and the reaction products analyzed on SDSPAGE. The mCLOCK protein acetylated primarily histones H3 and H4 on both free histone and mononucleosomes,

BOTTOM: Specificity of CLOCK enzymatic activity was then investigated by using H3 and H4 tails with preacetylated lysines. In this approach, putative HAT substrate sites are occupied, resulting in a block of potential de novo acetylation. Our results determined that histone H3 Lys-14, and in a lesser extent Lys-9, are the major sites acetylated by mCLOCK.



Doi, Hirayama, Sassone-Corsi, Cell 125, 497 (2006)

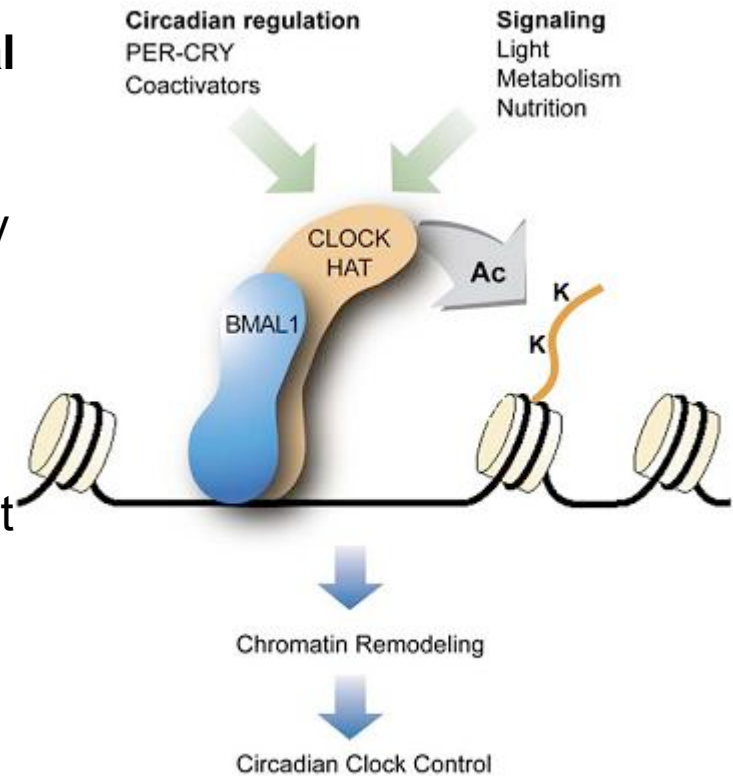
Schematic model

Schematic Model of CLOCK-Mediated Histone Acetylation and Its Role within the Physiological Pathways of Circadian Rhythmicity

The HAT function of CLOCK activity is enhanced by BMAL1, its natural heterodimerization partner, with which it binds to E box promoter elements within clock gene promoters (such as *per1*).

Acetylation by CLOCK, e.g. at H3 Lys-14, is thought to elicit chromatin remodeling by inducing a transcription-permissive state.

Metabolic, nutritional, and environmental circadian cues likely modulate the HAT function of CLOCK.



Doi, Hirayama, Sassone-Corsi,
Cell 125, 497 (2006)

Current understanding: clock – chromatin - metabolites

Circadian transcription is associated with **rhythmic changes in epigenetic marks** at circadian promoters, such as H3K4 trimethylation and H3K9 and H3K14 acetylation.

The histone methyltransferase mixed-lineage leukemia 1 (**MLL**) contributes to the **recruitment of CLOCK-BMAL1** to chromatin and thereby to the expression of clock-controlled genes.

Sirtuins are a class of NAD⁺-dependent deacetylases.

Circadian fluctuation of NAD⁺-levels induce rhythmicity in SIRT1 enzymatic activity.

NAD⁺-oscillation is dictated by CLOCK-BMAL1 which control the gene *Nampt*, encoding the nicotinamide phosphoribosyltransferase enzyme.

Aguila-Arnal et al. show that MLL1 is an acetylated protein and its enzymatic activity is controlled by SIRT1-dependent deacetylation.

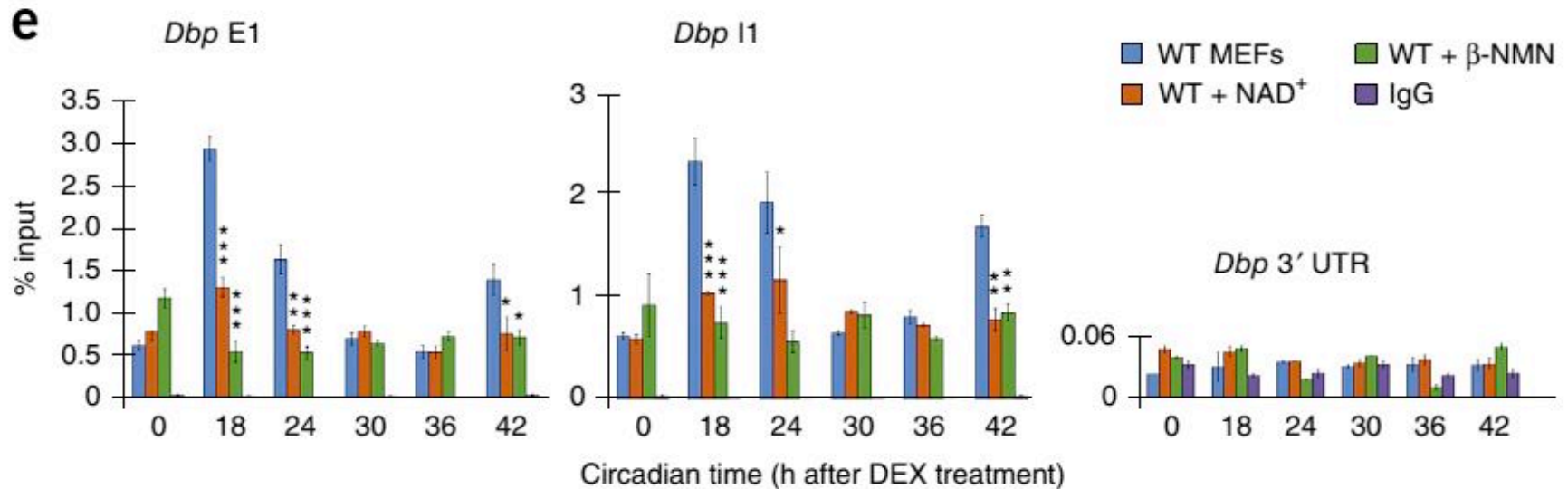
Aguila-Arnal et al.
Nature Struct Mol Biol
22, 312 (2015)

CLOCK is a histone acetyl transferase

NAD⁺-SIRT1 control of H3K4 trimethylation through circadian deacetylation of MLL1

Lorena Aguilar-Arnal¹, Sayako Katada^{1,2}, Ricardo Orozco-Solis¹ & Paolo Sassone-Corsi¹

Fig. (e) shows H3K4 ChIP-data for the promoter of the circadian gene *Dbp*.
→ H3K4-methylation levels are modified by changing the NAD⁺ concentration.

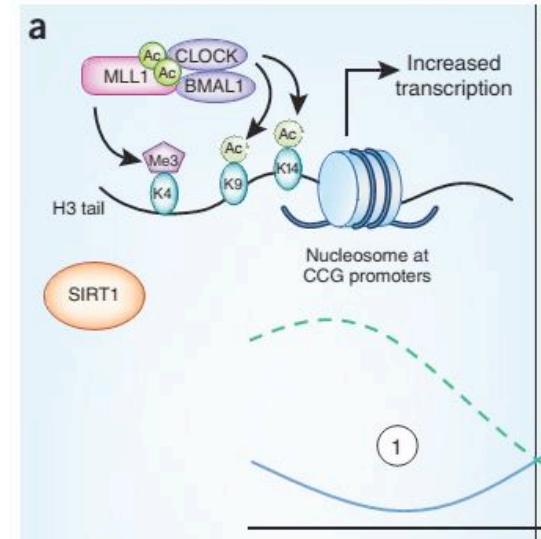


Aguila-Arnal et al.
Nature Struct Mol Biol
22, 312 (2015)

Interpretation: Circadian regulation of epigenetic chromatin

Dynamic cross-talk between cellular energy metabolism and chromatin regulation controls circadian rhythmicity of gene expression.

Circadian fluctuations in NAD^+ levels and SIRT1 activity drive oscillations of the transcriptionally activating H3K4 trimethyl mark at promoters of clock-controlled genes (CCGs).



(a) At circadian times with low NAD^+ levels (1), SIRT1 deacetylase activity is low, and MLL1 remains acetylated and active, increasing H3K4me3 levels at the promoters of CCGs.

Acetylated MLL1 also favors recruitment of the HAT complex, CLOCK–BMAL1, and acetylation of H3K9 and H3K14 at these promoters. Together, the activating methyl and acetyl histone marks promote transcription of CCGs.

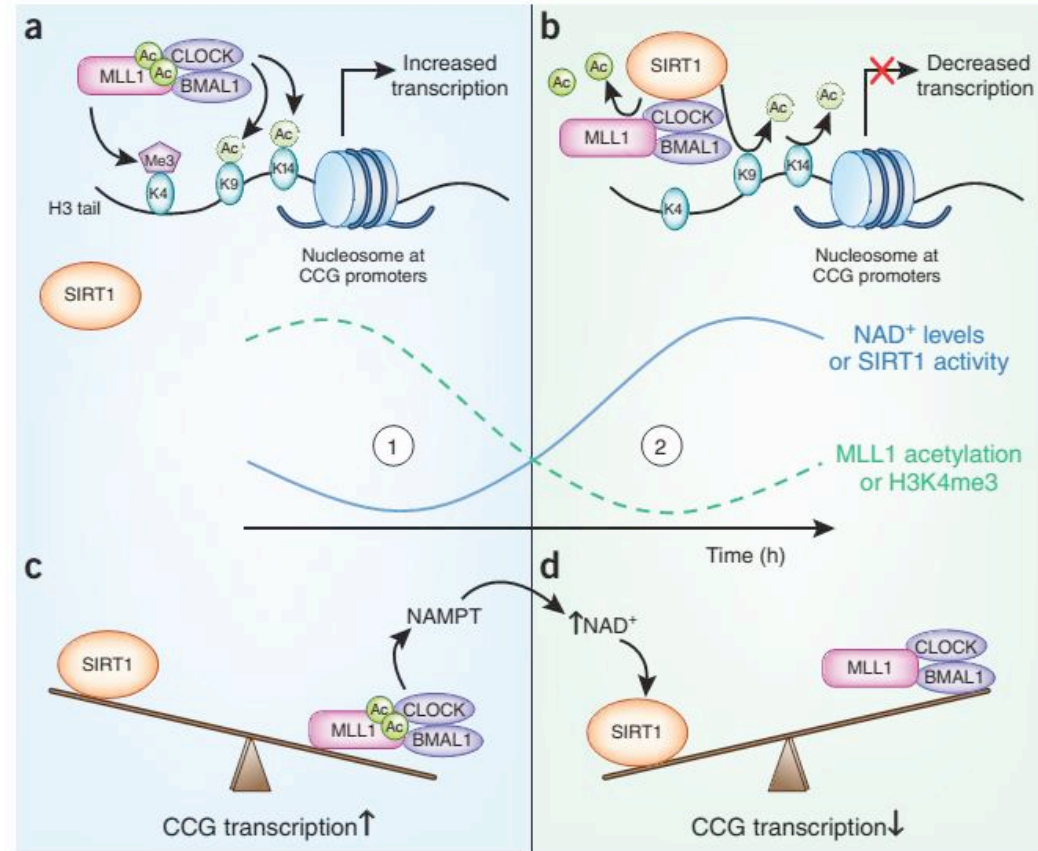
Tasselli & Chua,
Nat Struct Mol Biol 22, 275 (2015)

Interpretation: Circadian regulation of epigenetic chromatin

(b) As NAD^+ levels increase over time, SIRT1 is activated, and it deacetylates MLL1.

This reduces the methyltransferase activity of MLL1 and thus decreases H3K4me3 occupancy at CCG promoters.

This, together with SIRT1 deacetylation of H3K9 and H3K14, results in reduced transcription of CCGs.

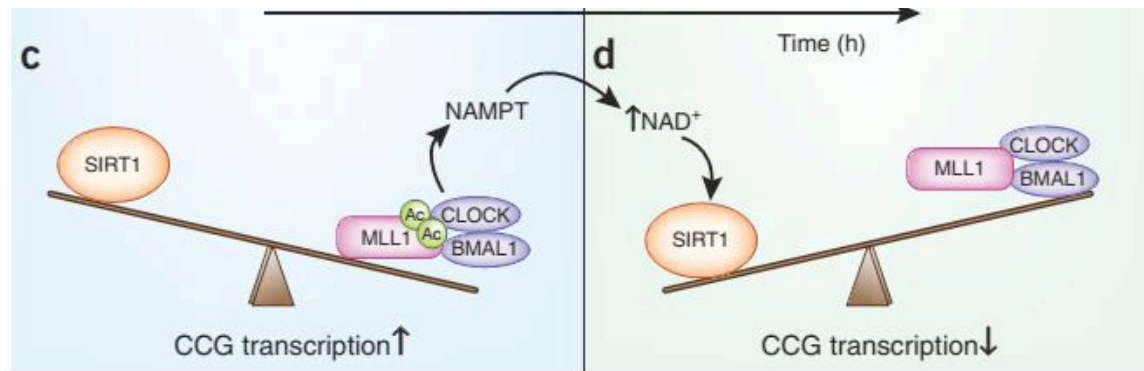


Tasselli & Chua,
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Interpretation: Circadian regulation of epigenetic chromatin

(c,d) Schematic illustrating the shifting balance between SIRT1 versus MLL1 activities over circadian time. The circadian oscillations in these activities are linked to each other and to the cellular bioenergetic state via feedback loops involving cyclic production of NAD^+ . In conditions of low cellular NAD^+ (c), the balance favors transcription dependent on MLL1 and CLOCK–BMAL1.

Among the CCGs is the *Nampt* gene, which encodes a key enzyme in NAD^+ biosynthesis. Over time, as NAD^+ synthesis continues, rising NAD^+ levels tilt the balance back toward SIRT1 activity and transcriptional repression (d).



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