What is biological clock?

All eukaryotes and some prokaryotes display changes in gene activity, biochemistry, physiology, and behaviour that wax and wane through the cycle of days and nights – ***circadian rhythm*.**

Circadian rhythmicity is universally associated with the ability to perceive light, and the oscillators ("clocks") giving rise to these rhythms, which are feedback loops based on transcription and translation, are reset by light.

Examples:

- the level of the hormone melatonin that rises in our body during the night and falls during the day.
- fruit flies (***Drosophila***) hatch in greatest numbers just at dawn.
  Even when the organism is placed in constant conditions (e.g., continuous darkness), these rhythms persist. However, without environmental cues, they tend to be somewhat longer or somewhat shorter than 24 hours - giving rise to the name **circadian** rhythms (L. *circa* = about; *dies* = day).

Circadian Clock

- Endogenous cellular mechanism
- Temporal organization
- General models involve 3 basic elements:
  - input pathways
  - circadian oscillator
  - ouput pathways

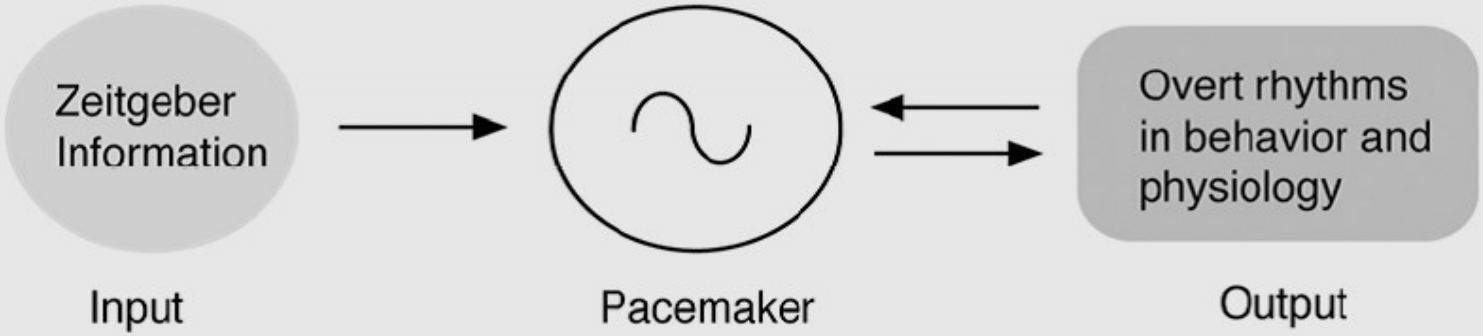
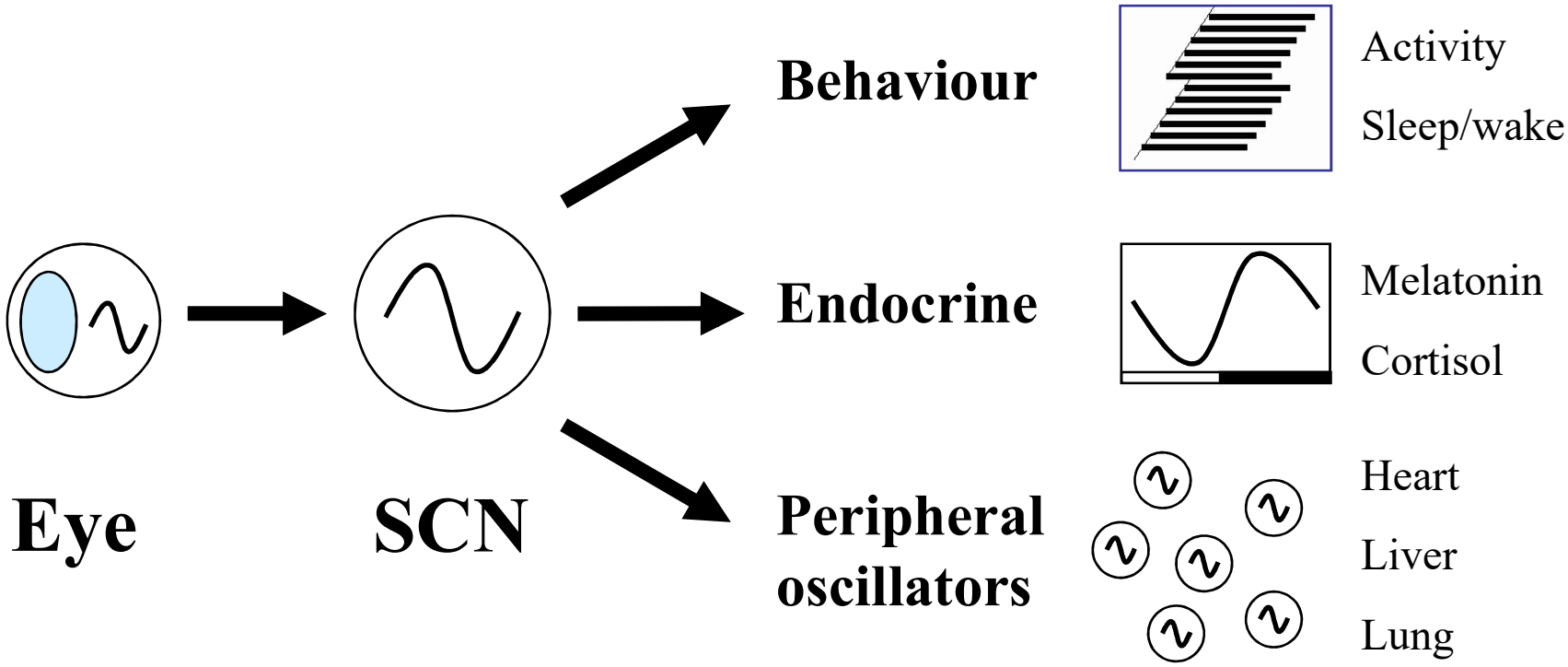


Figure 2:

Schematic diagram of the circadian system. It is composed of the input pathway, the pacemaker or clock and the output pathway. Note that the physiological state of the organism can influence the clock pacemaker to establish a crosstalk between clock pacemaker and output targets.

The Mammalian Circadian System



Even unicellular organisms show circadian behaviour.

The clock generating machinery need not be the product of cell:cell interactions.

What are clock molecules and how should they be identified?

Drosophila Mutants – Same Gene!

Activity	Eclosion	Mutation (*per*)
perN ~ 24	At Dawn	
perL ~ 28	> Dawn	Missense 243
perS ~ 19	< Dawn	Missense 589
per0	Arrhythmic	Nonsense 464 (truncated mRNA & no protein)

The *period* gene is located on the X chromosome as 7.2kb transcriptional unit that yields a 4.5kb mRNA and a 1218 amino acid protein.

Genes = Biological Clock

But what makes *Per* a clock gene?

How do you define where an input or output molecule stops and a clock molecule begins?

Criteria for Clock Molecule Identification

Do changes in the level or activity of the molecule alter clock driven behaviours/outputs?

- In the absence of the molecule do rhythms disappear?
- Will transient changes phase shift the rhythm?
- Will sustained changes alter period?

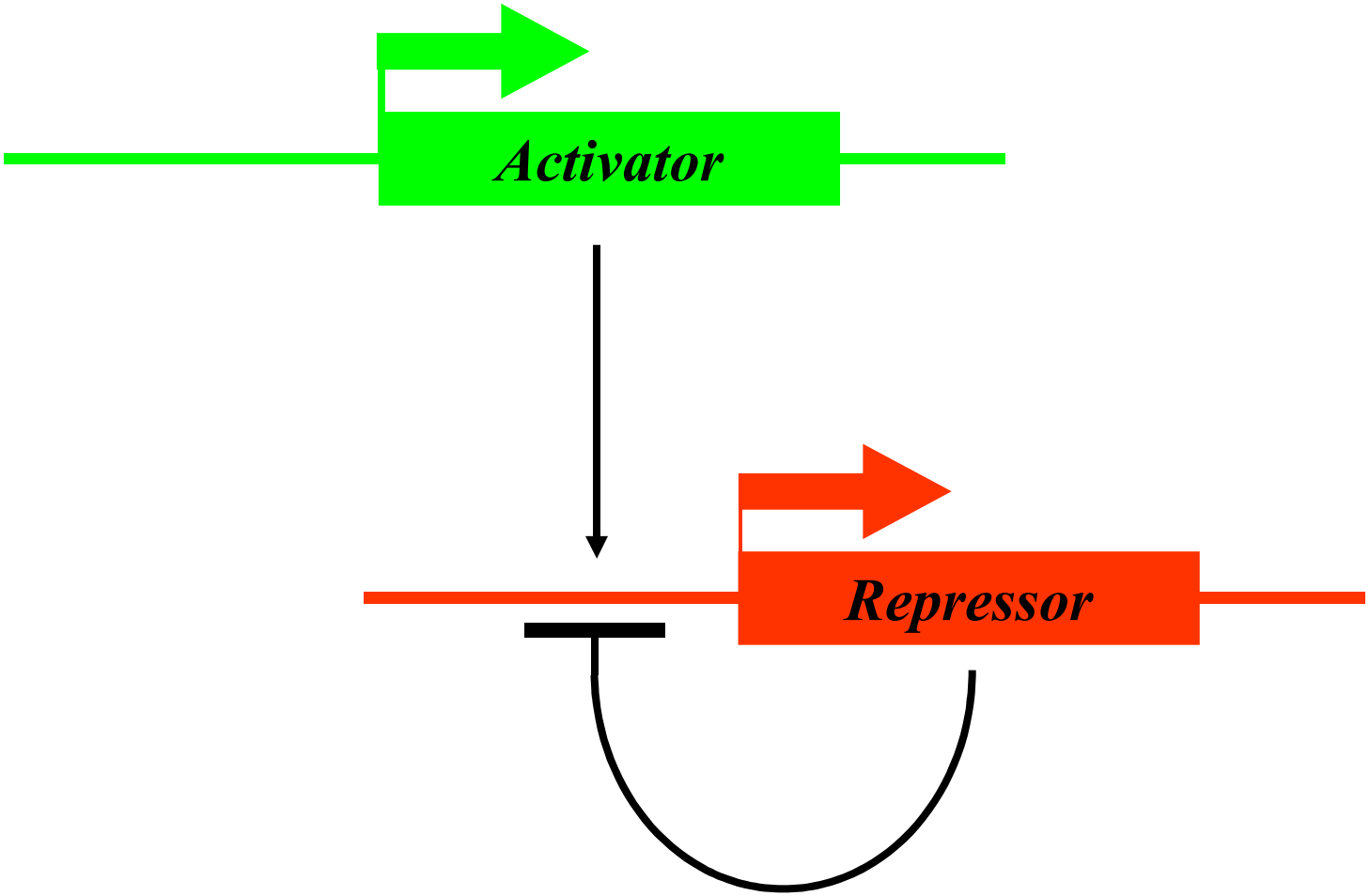
Do levels cycle with a circadian period under constant conditions?

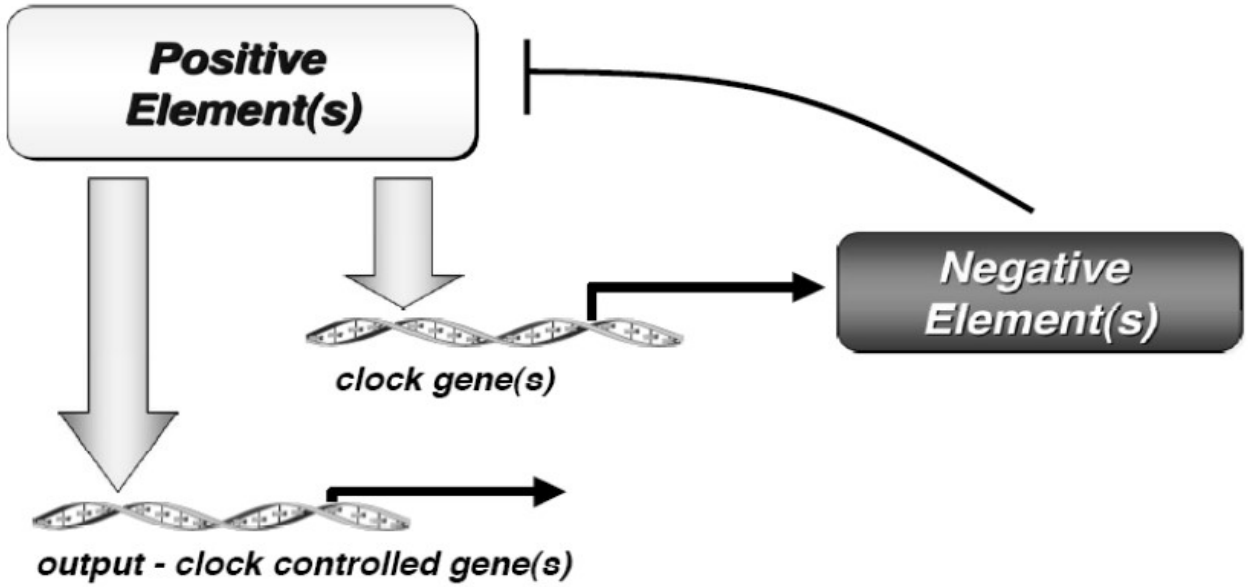
- Do protein levels cycle?
- Do mRNA levels cycle?
- Do they resemble regulatory molecules?

Cycles First

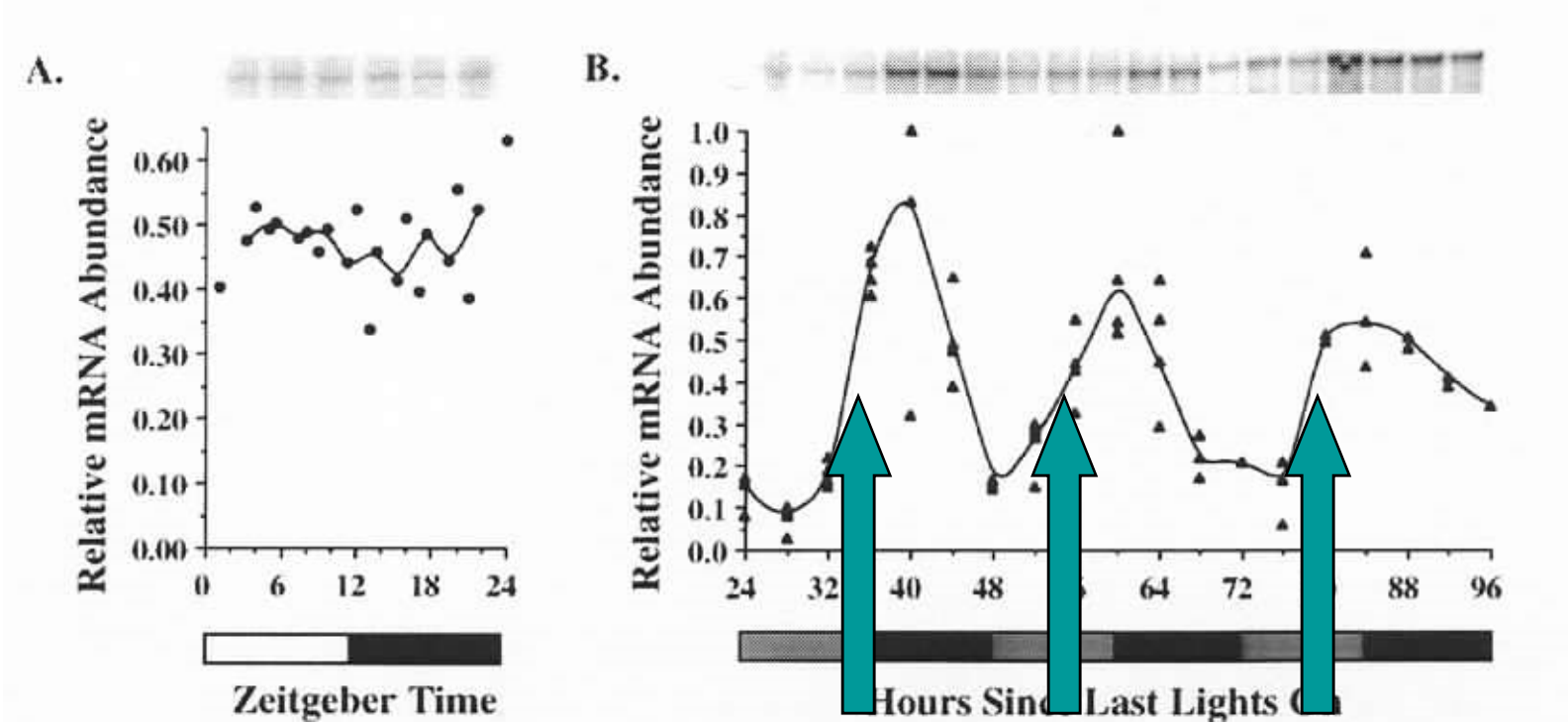
- Lots of different “clocks” in most organisms.
- Can have more or less central control (SCN - suprachiasmatic nuclei - in mammals; different organs have different clocks; different feedback loops in even unicellular organisms)

All known circadian oscillators consist of negative feedback loops of gene expression



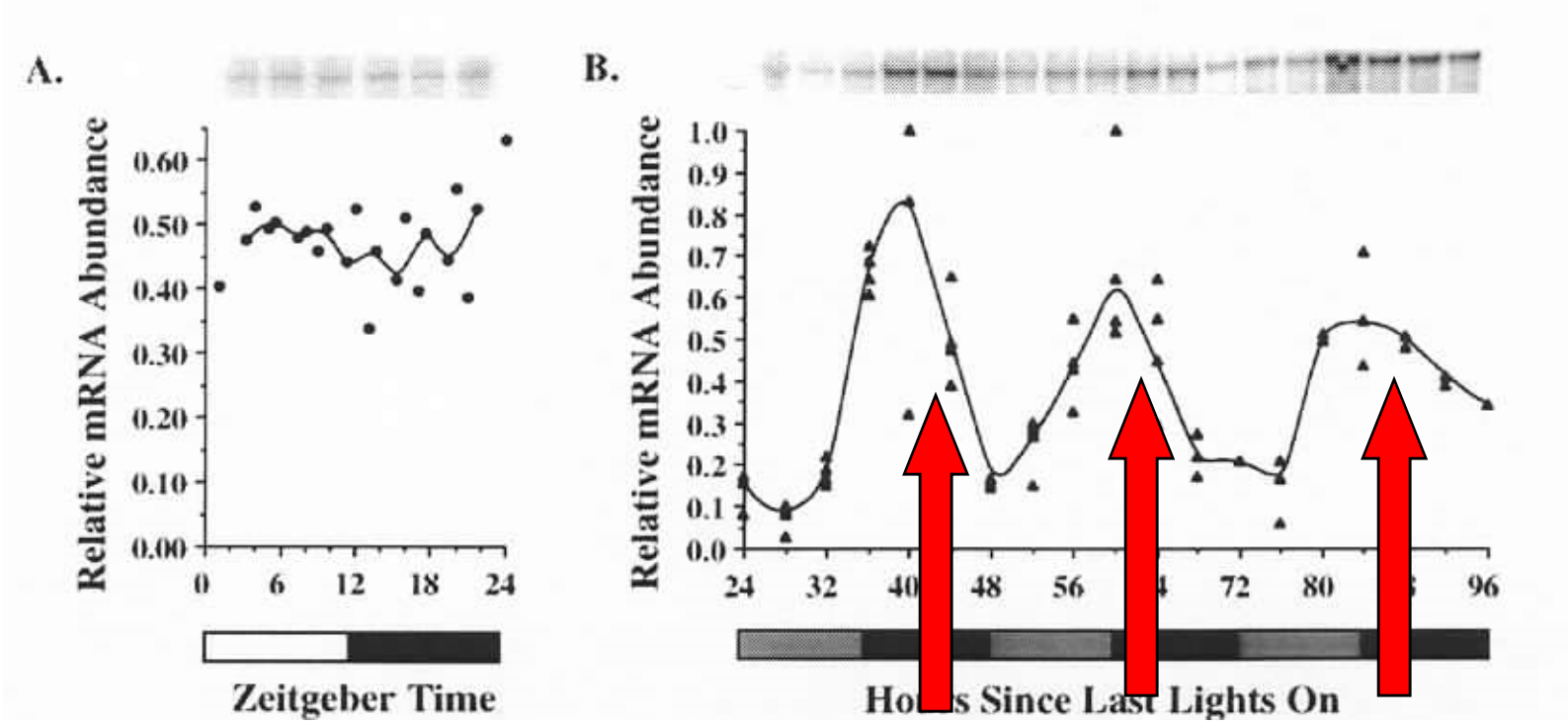

Positive Element(s)
Negative Element(s)
clock gene(s)
output - clock controlled gene(s)

Transcriptional Oscillations: Activation



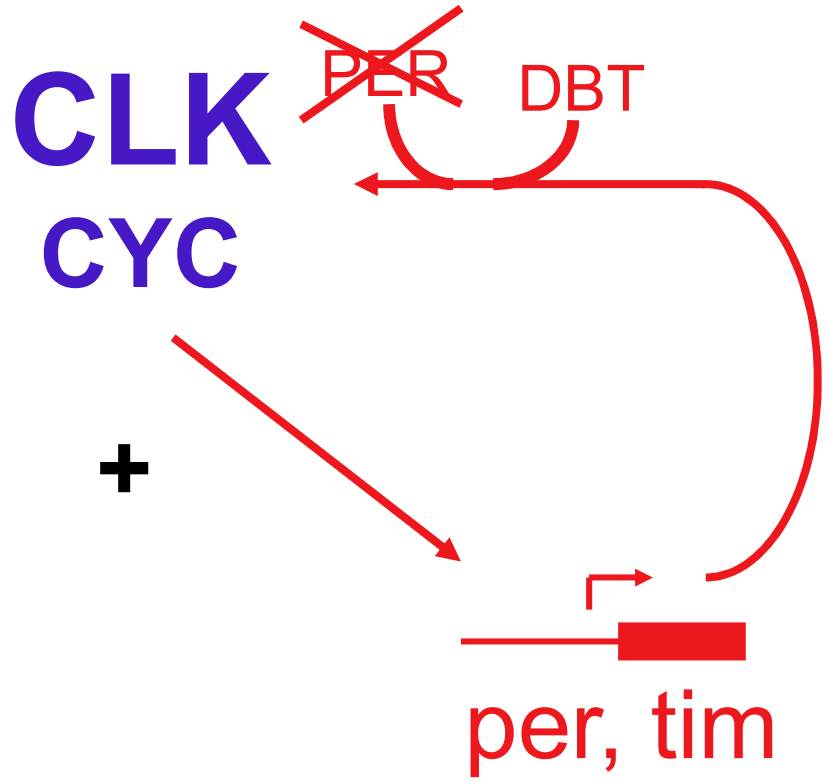
Hao et al., 1997

Transcriptional Oscillations: Repression

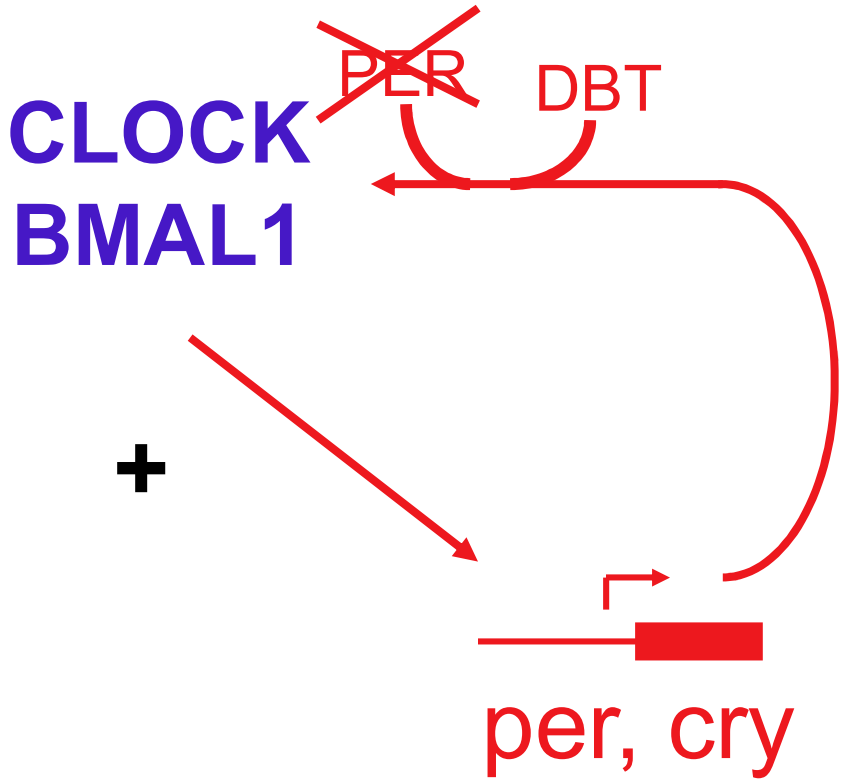


Hao et al., 1997

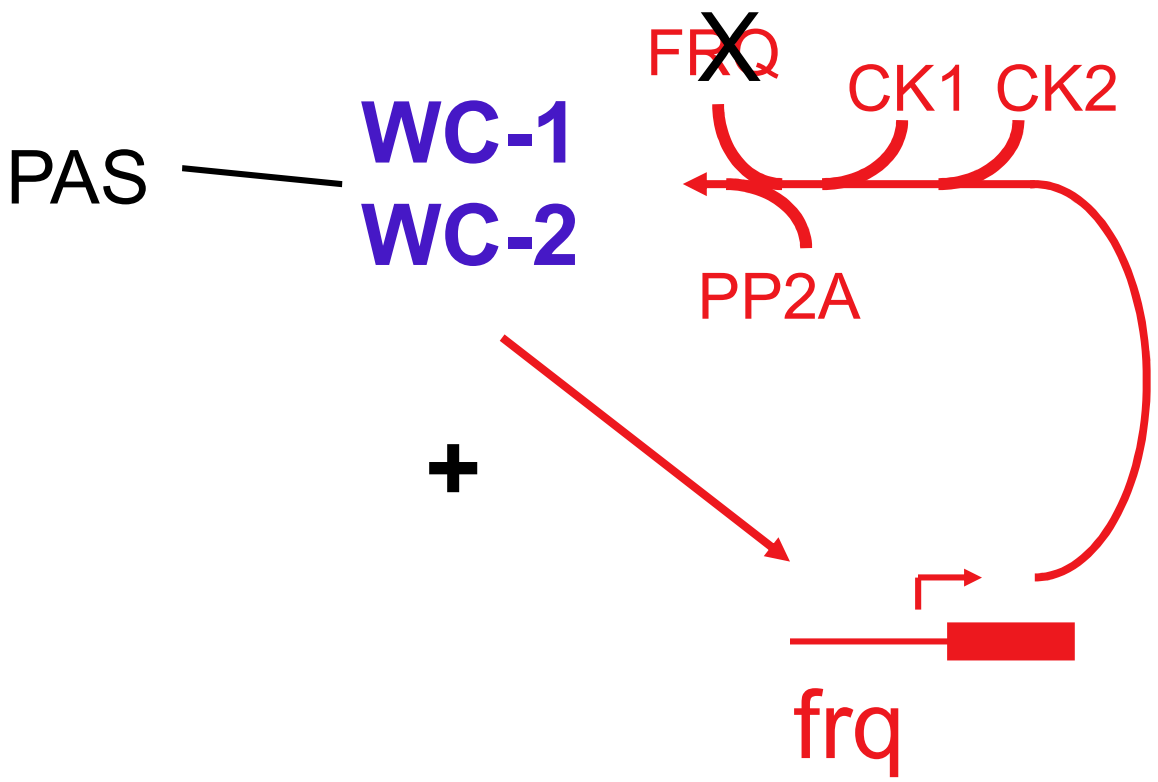
Transcriptional Feedback Loops

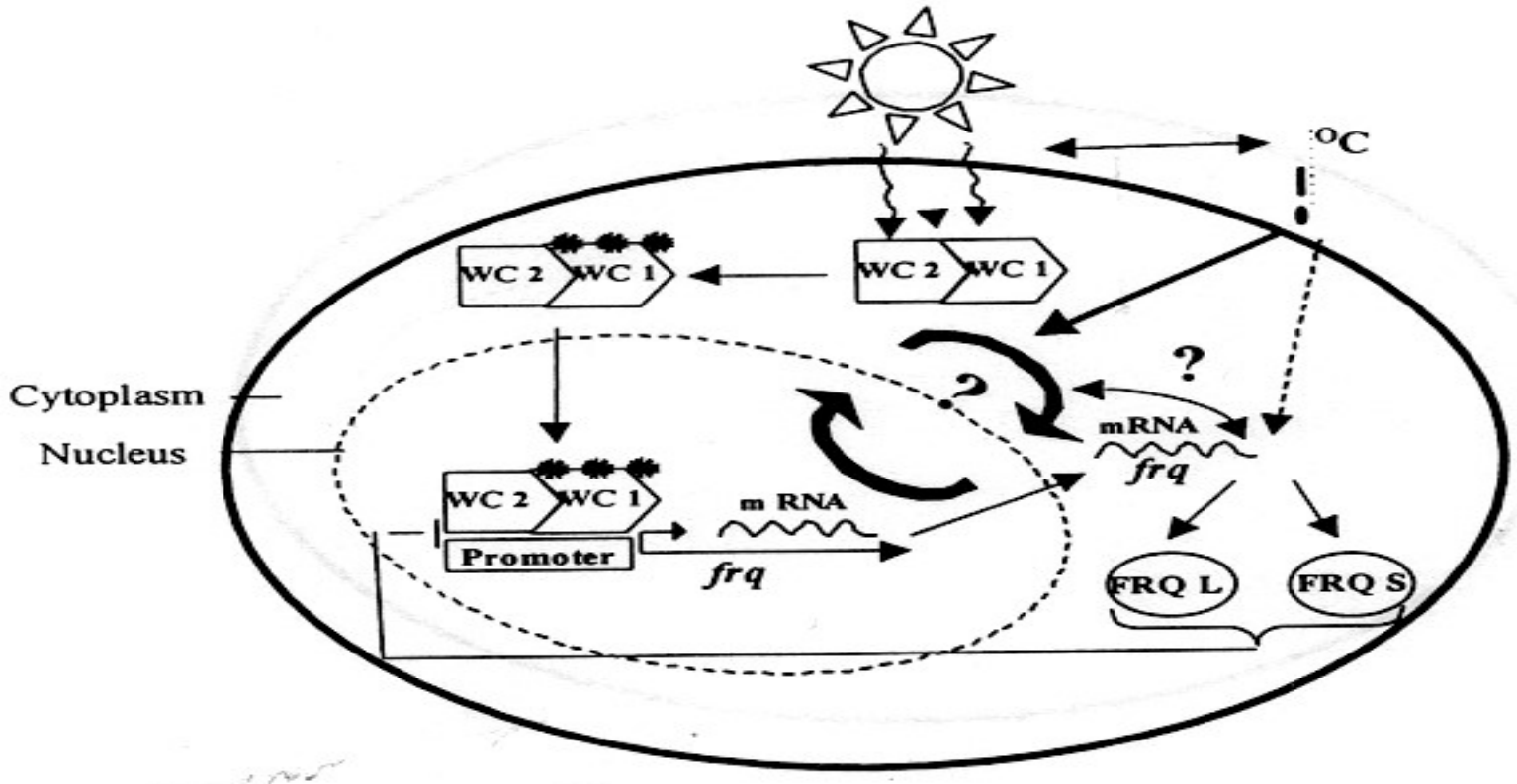


Transcriptional Feedback Loops



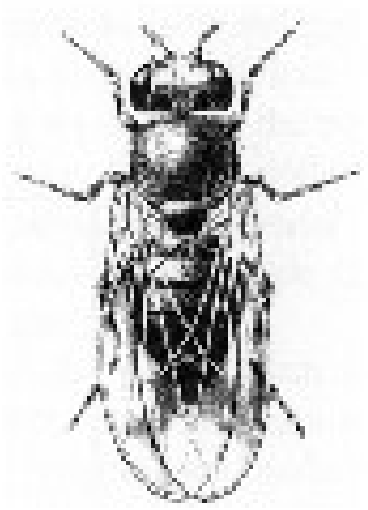
Neurospora Clock

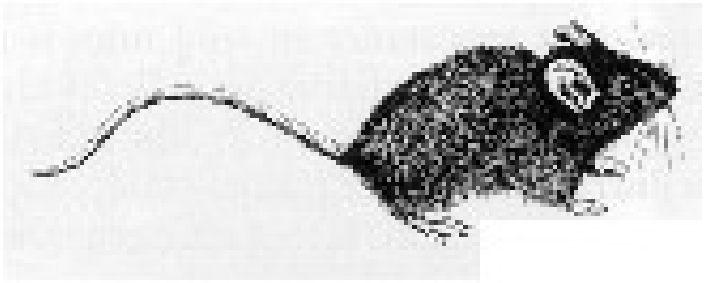




In Neurospora crassa, the core clock elements consists of *wcl, wc2* and_frq genes. The WCI and WC2 proteins dimerize and, following exposure to blue light, WCI is phosphorylated, dimer enters the nucleus and acts as a positive effector of the transcription of *frq.* In the cytoplasm frq MRNA can be translated from alternative start codons giving rise to 2 length variants (FRQ S and FRQ L). FRQ protein acts as an inhibitor of its own transcription. The temperature cycles can entrain in the absence of functional FRQ protein, suggesting a ':frq-less" oscillator (cycling arrows enclosing a question mark).

Are mammals just hairy flies?





The Circadian Clock in Mammals

The circadian clock in mammals resembles that in *Drosophila* in a number of ways, with many of the participating genes being homologous. However, there are some differences:

The transcription factors that turn **on** the light-induced promoters are dimers of the **CLOCK** protein and a protein designated **BMAL1**. These dimers turn **on**

- the ***per1 and per2*** genes;
- ***cry1 and cry2*** genes, the gene encoding cryptochrome
- **rev-ERB**α gene, whose product **inhibits** transcription of ***Bmal1***
- effector genes (such as the gene encoding vasopressin)

The **Per** and **Cry** mRNAs are exported to the cytoplasm where they are translated.

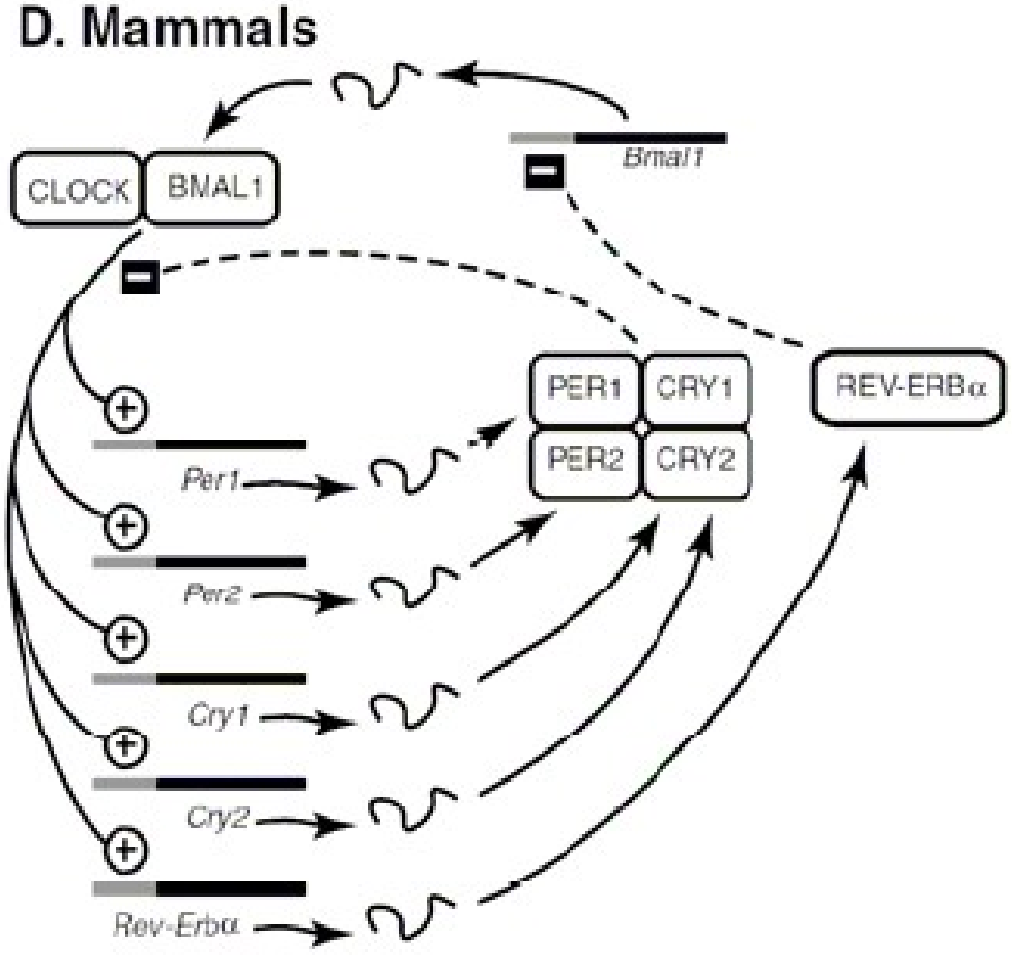
•The **PER** and **CRY** proteins then form dimers that enter the nucleus where They:

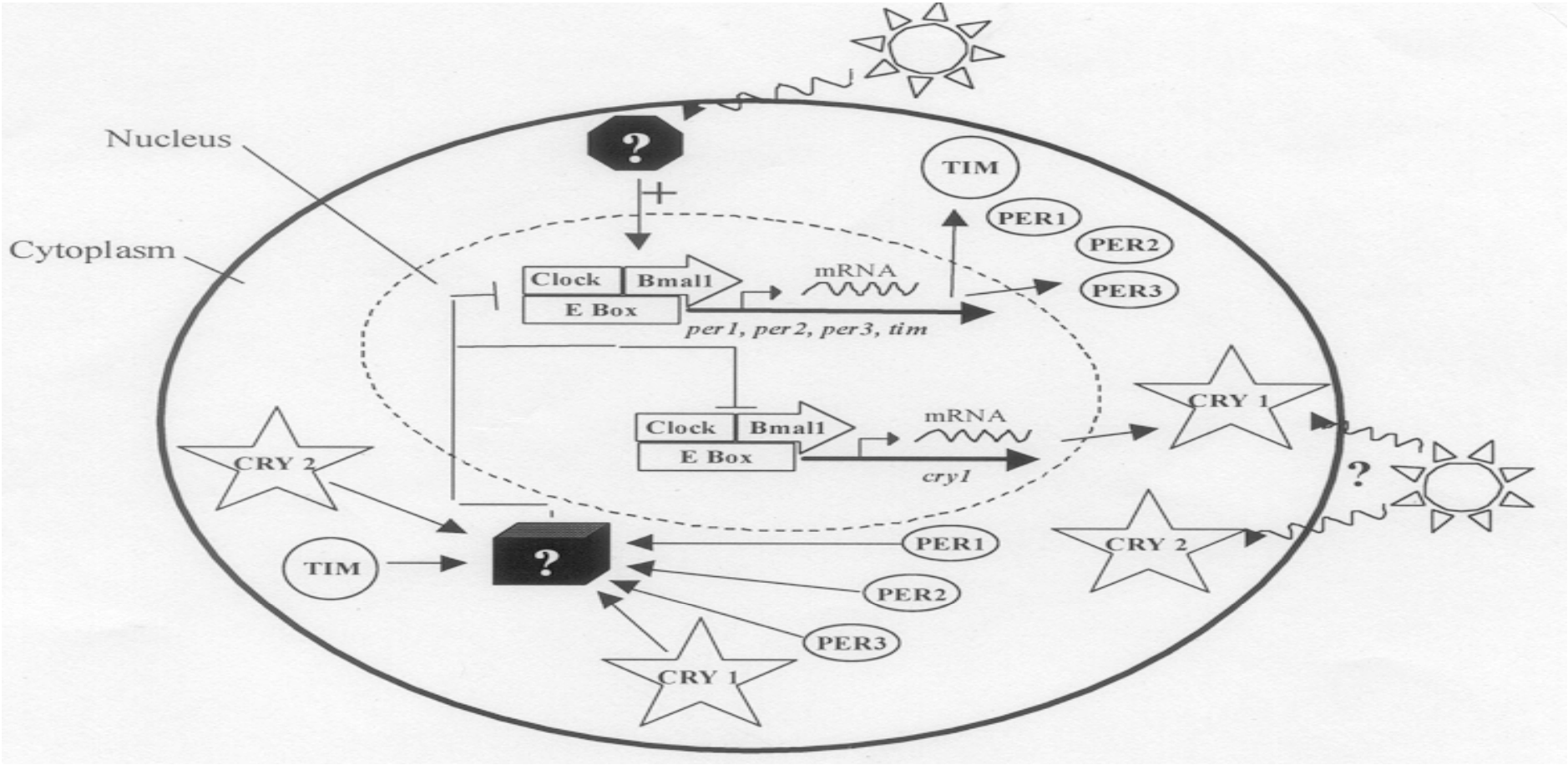
•turn OFF their own genes (as PER/TIM dimers do in Drosophila);

•turn OFF ***Rev-erb***α gene, thus removes the inhiibition on *Bmal1* transcription. So this double-negative effect causes the level of the BMAL1 protein to rise.

•These actions cause the levels of BMAL1 and PER/CRY to oscillate in opposite phases (as CLOCK and PER/TIM do in *Drosophila*).

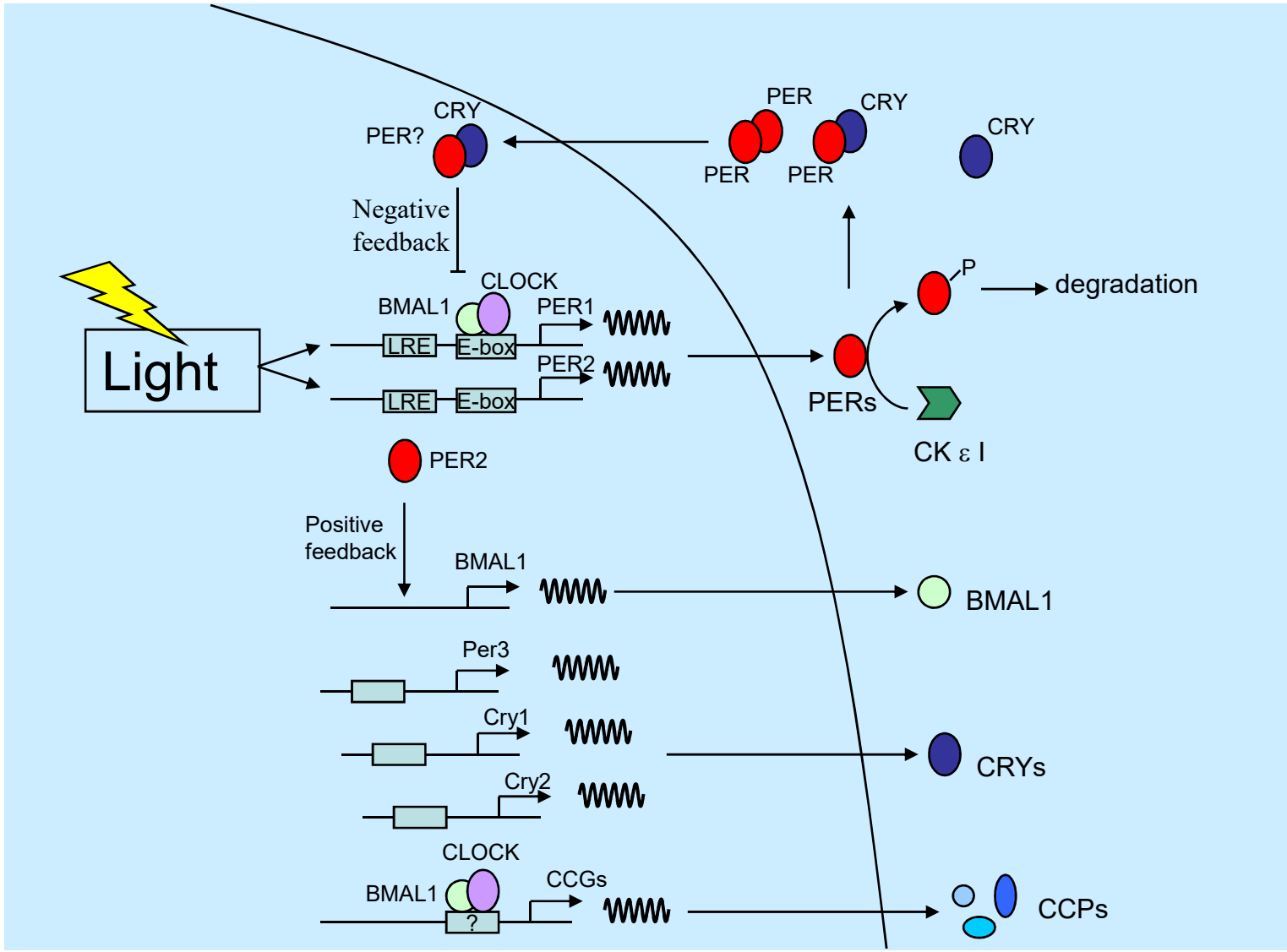
The Circadian Clock in Mammals



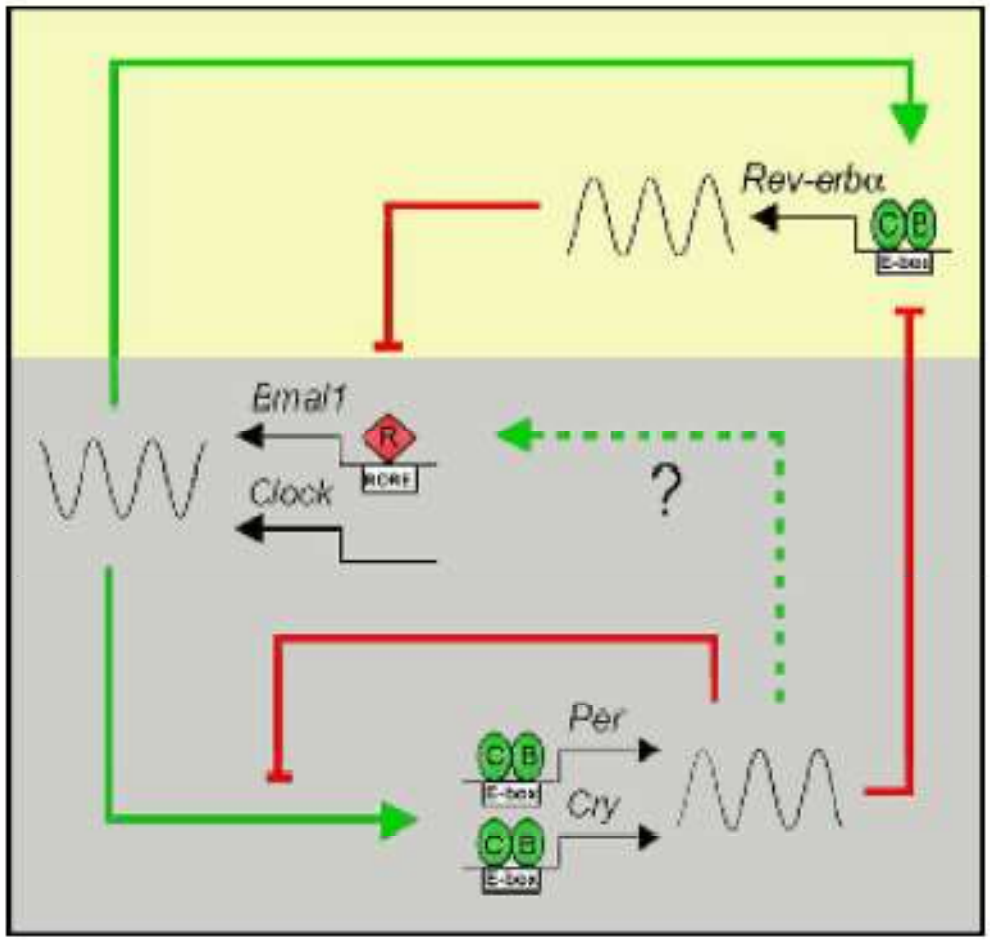


The circadian clock in mammals. Transcription of 3 *per genes,* tim and *cry 1* positively regulates by heterodimer formed by the protein products of the *clock and bmall* genes. For *per* genes, light plays a positive effector. The products of the 3 *per* genes, TIM, CRY I and CRY2 form complexes in various ways. However, it is unknown which complexes are actually formed *in vivo.* The actual role of CRYI and CRY2 in photoreception is uncertain. In the figure "plus" signs indicate positive effectors and lines terminating in perpendicular dashes indicate negative effectors.

Current Model of Clock Gene Interactions



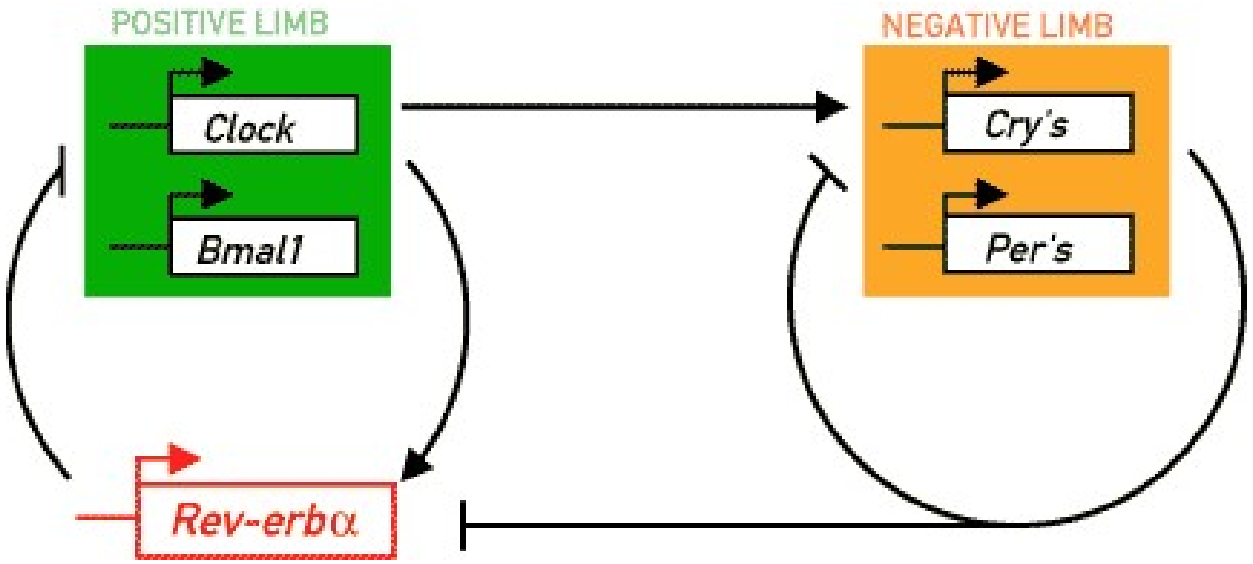
Nucleus **Cytoplasm**



Before the role of REV-ERBα was elucidated (grey), the CLOCK/BMAL1 heterodimer (C, B) was known to activate transcription of the *Per* and *Cry* genes by binding to E-box elements in their promoters. The oscillation of the *Per* and *Cry* gene products is thought to be generated by subsequent delayed inhibition of CLOCK/BMAL1 by the accumulating PER and CRY proteins. PER2 was proposed to rhythmically activate *Bmal1* transcription[3] through an unknown mechanism (dashed line). Preitner and colleagues[1], have now discovered (yellow) that, in fact, REV-ERBα regulates the rhythmic expression of *Bmal1*, so linking the positive and negative limbs of the feedback loop. *Rev-erbα* transcription is activated by CLOCK/BMAL1 and inhibited by PER and CRY proteins resulting in oscillation of the *Rev-erbα* gene product. REV-ERBα protein (R) binds to regulatory sequences (RORE) in the *Bmal1* promoter, rhythmically repressing *Bmal1* transcription and generating oscillations of *Bmal1* mRNA and protein. (Larger Version)

Kramer 2002

The nuclear orphan receptor REV-ERBα connects the two feedback loops by periodically repressing *Bmal1* and *Clock*



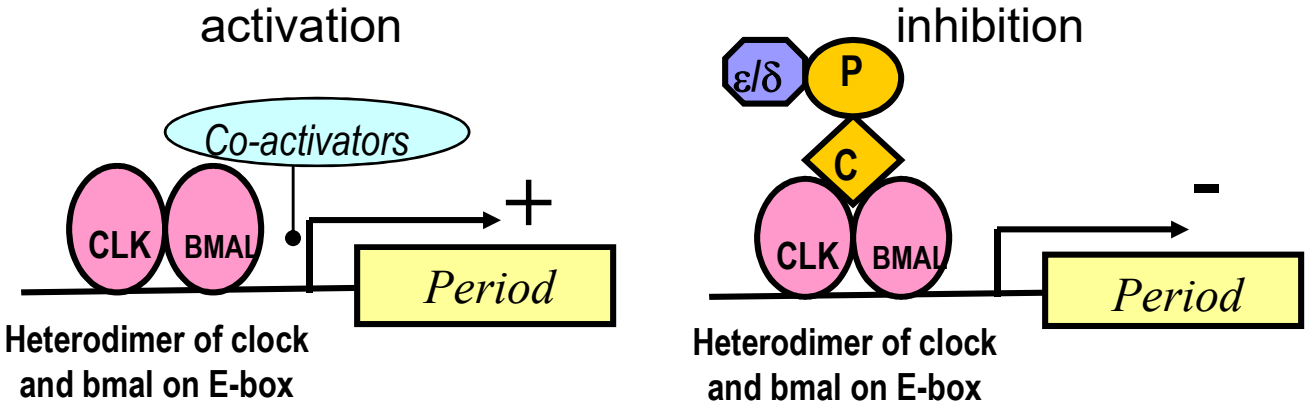
Simplified model of the circadian oscillator: The positive limb consists of the two PAS helix-loop-helix transcription factors CLOCK and BMAL1 that activate transcription of *Cry* and *Per*, which are members of the negative limb. CRY and PER proteins are translocated to the nucleus as multi-subunit protein complexes and, once these complexes have reached a critical concentration, they repress CLOCK:BMAL1-stimulated transcription. This feedback loop generates circadian rhythms of mRNA accumulation for members of the negative limb. In parallel, the same positive and negative elements periodically activate and repress, respectively, the transcription of the orphan receptor *Rev-erbα*. The circadian accumulation of the orphan receptor REV-ERBα then drives cyclic transcription of *Bmal1* and *Clock*

Simplified model of the circadian oscillator:

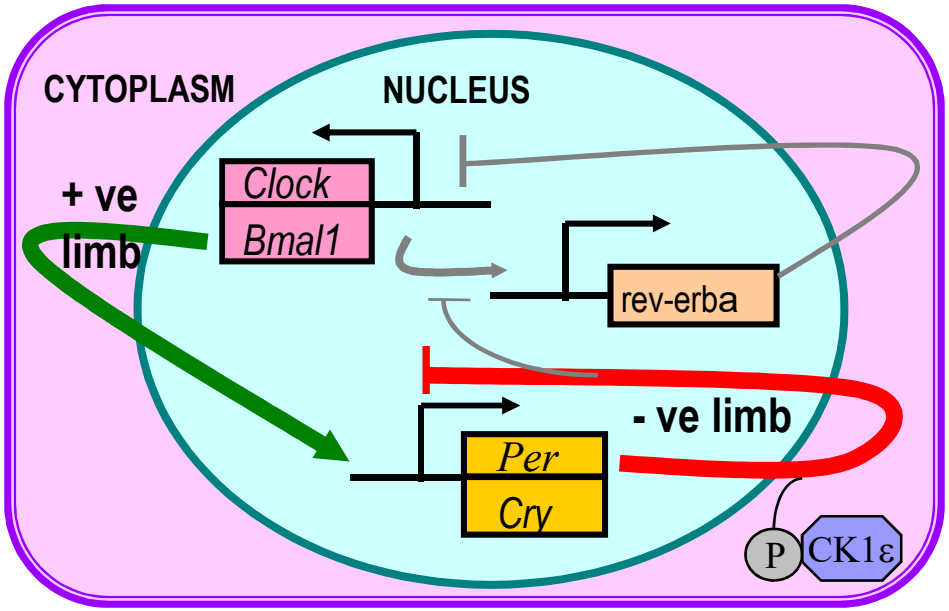
The positive limb consists of the two PAS helix-loop-helix transcription factors CLOCK and BMAL1 that activate transcription of *Cry* and *Per*, which are members of the negative limb. CRY and PER proteins are translocated to the nucleus as multi-subunit protein complexes and, once these complexes have reached a critical concentration, they repress CLOCK:BMAL1-stimulated transcription. This feedback loop generates circadian rhythms of mRNA accumulation for members of the negative limb. In parallel, the same positive and negative elements periodically activate and repress, respectively, the transcription of the orphan receptor *Rev-erbα*. The circadian accumulation of the orphan receptor REV-ERBα then drives cyclic transcription of *Bmal1* and *Clock*.

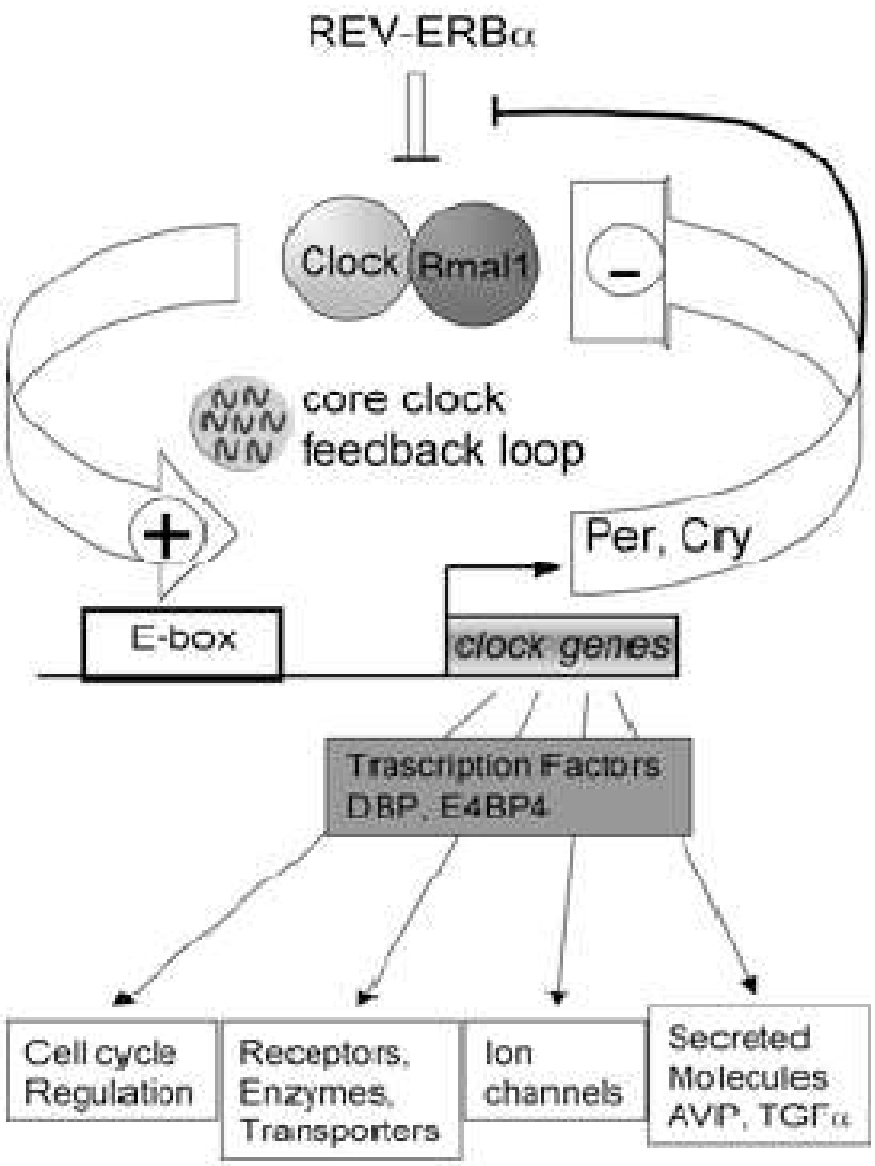
a) Transcriptional complex

Figure 2

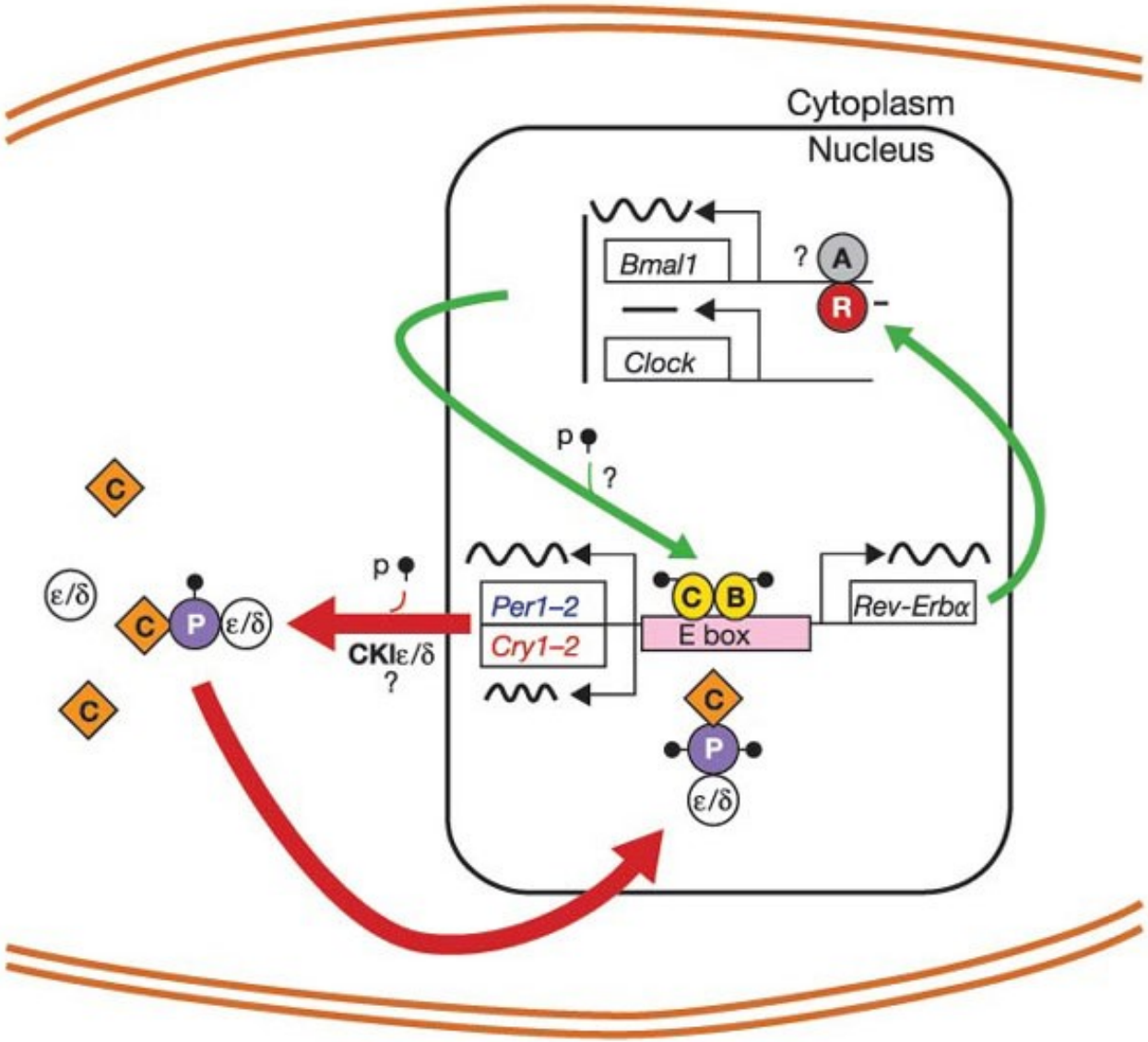


b) Molecular circuitry of the circadian clock





A basic-helix-loop-helix (bHLH) is a protein structural motif (a structural motif is a three-dimensional structural element or *fold* within the chain) that characterizes a family of transcription factors. The motif is characterized by two α helices connected by a loop. Transcription factors including this domain are typically dimeric, each with one helix containing basic amino acid residues that facilitate DNA binding. One helix is typically smaller and due to the flexibility of the loop, allows dimerization by folding and packing against another helix. The larger helix typically contains the DNA binding regions. bHLH proteins typically bind to a consensus sequence called an E-box (5'-CACGTG-3'). However some bHLH transcription factors bind to different sequences, which are often similar to the E-box.



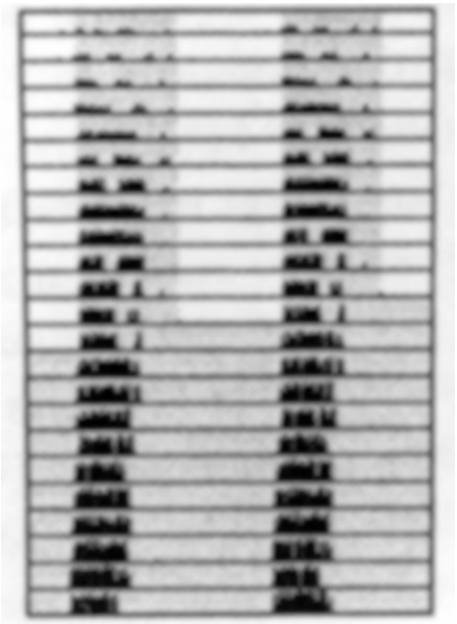
Interactive positive (green) and negative (red) feedback loops. CLOCK (C, oval) and BMAL1 (B, oval) form heterodimers and activate transcription of the Per, Cry and Rev-Erb genes through E-box enhancers. As the levels of PER proteins increase (P, blue circle), they complex with CRY proteins (C, diamond) and CKIe /CKId (e /d , circle), and are phosphorylated (p). In the nucleus, the CRY-PER-CKIe /CKId complexes associate with CLOCK-BMAL1 heterodimers to shut down transcription while the heterodimer remains bound to DNA, forming the negative feedback loop. For the positive feedback loop, increasing REV-ERB levels (R, circle) act through Rev-Erb/ROR response elements in the Bmal1 promoter to repress (-) Bmal1 transcription. CRY-mediated inhibition of CLOCK-BMAL1-mediated transcription de-represses (activates) Bmal1 transcription, because REV-ERB -mediated repression is inhibited. An activator (A, circle) may positively regulate Bmal1 transcription (?) alone or by interacting with mPER2. There are probably kinases (?) other than CKI and CKI that participate in phosphorylation of clock proteins.

Mammalian Cryptochromes

Cry 1 and Cry2
Rhythmic Expression
4-6h delay between mRNA & Protein via E-box?
Second transcriptional/Translational feedback loop!

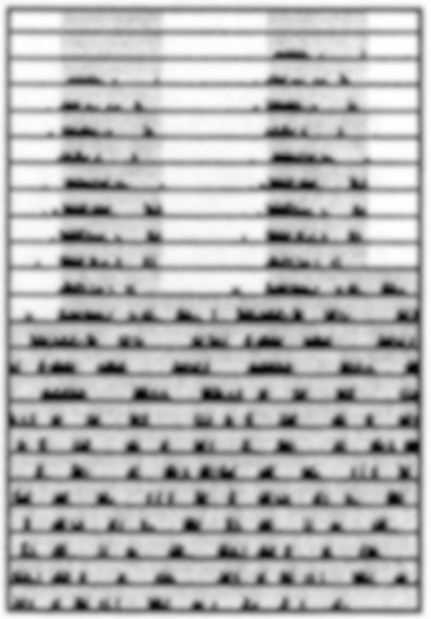
Cry KO mice
Cry 1 – short *tau*
Cry 2 – long *tau*

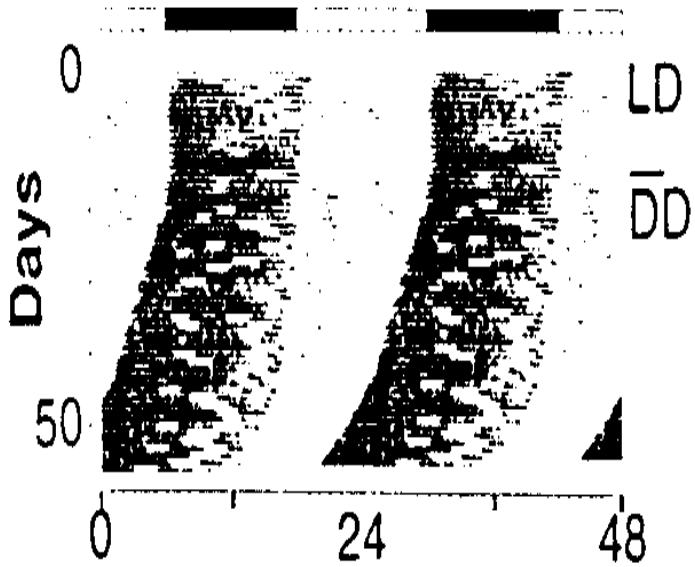
Wild type



Cry1 & Cry 2 KO mice are arrhythmic!

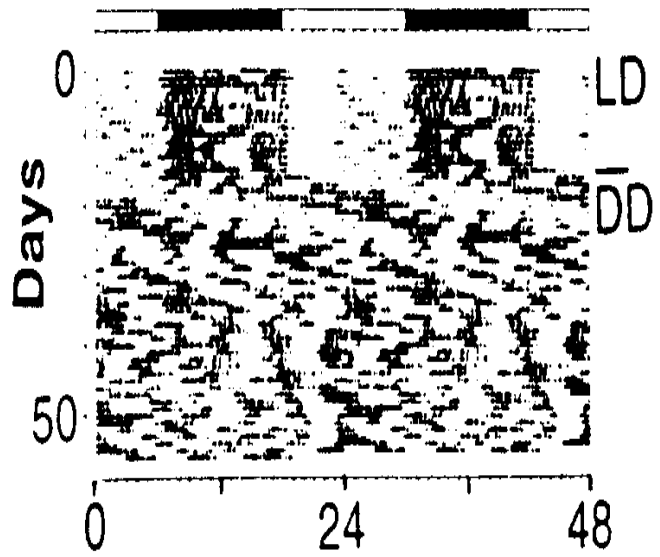
cry-less mice

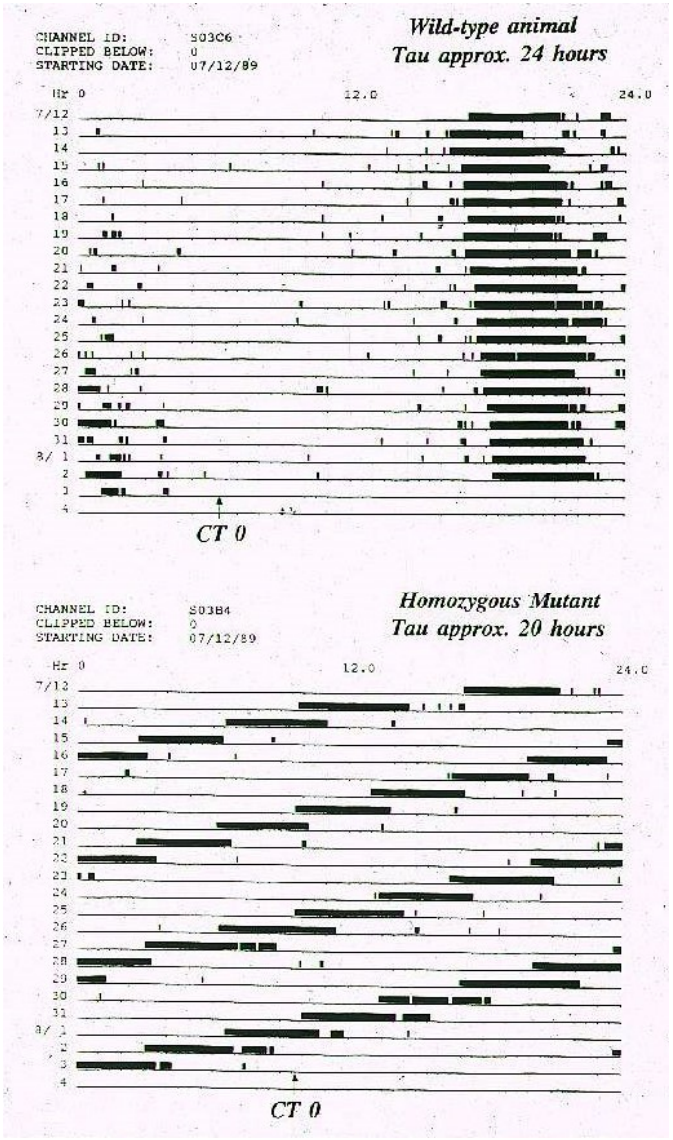




Circadian Locomotor Output Kaput *CLOCK*

Homolog identified in *Drosophila*, will bind to Bmal1 via a PAS domain to E-box elements.





CK1ε (casein-kinase 1ε) Homolog of Drosophila DBT

Probably phosphorylates PER

The Circadian Clock in Mammals - Setting The Clock

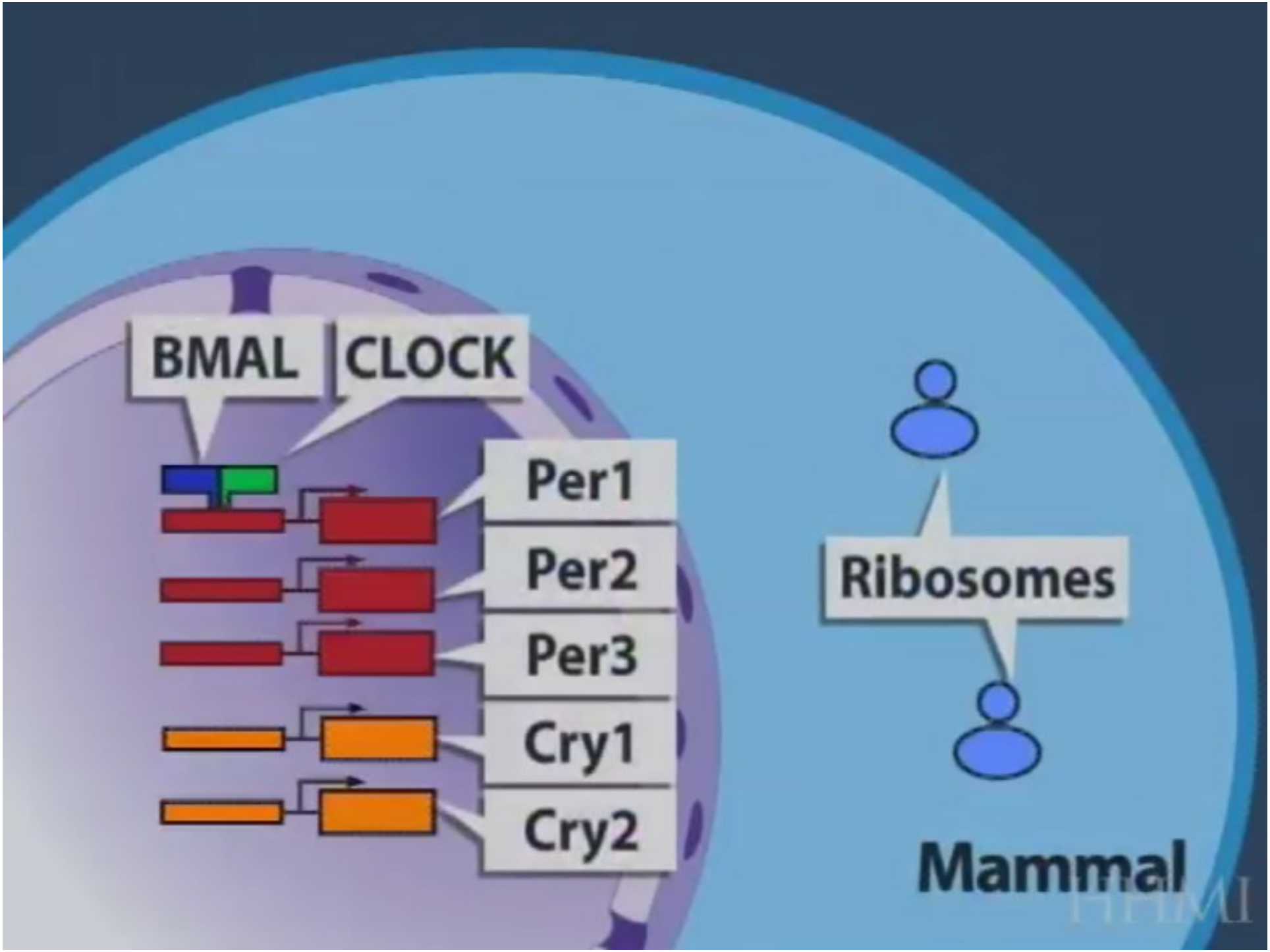
In mice, light acts through the retina and direct neural pathways to the suprachiasmatic nuclei (SCN) to stimulate per gene expression.

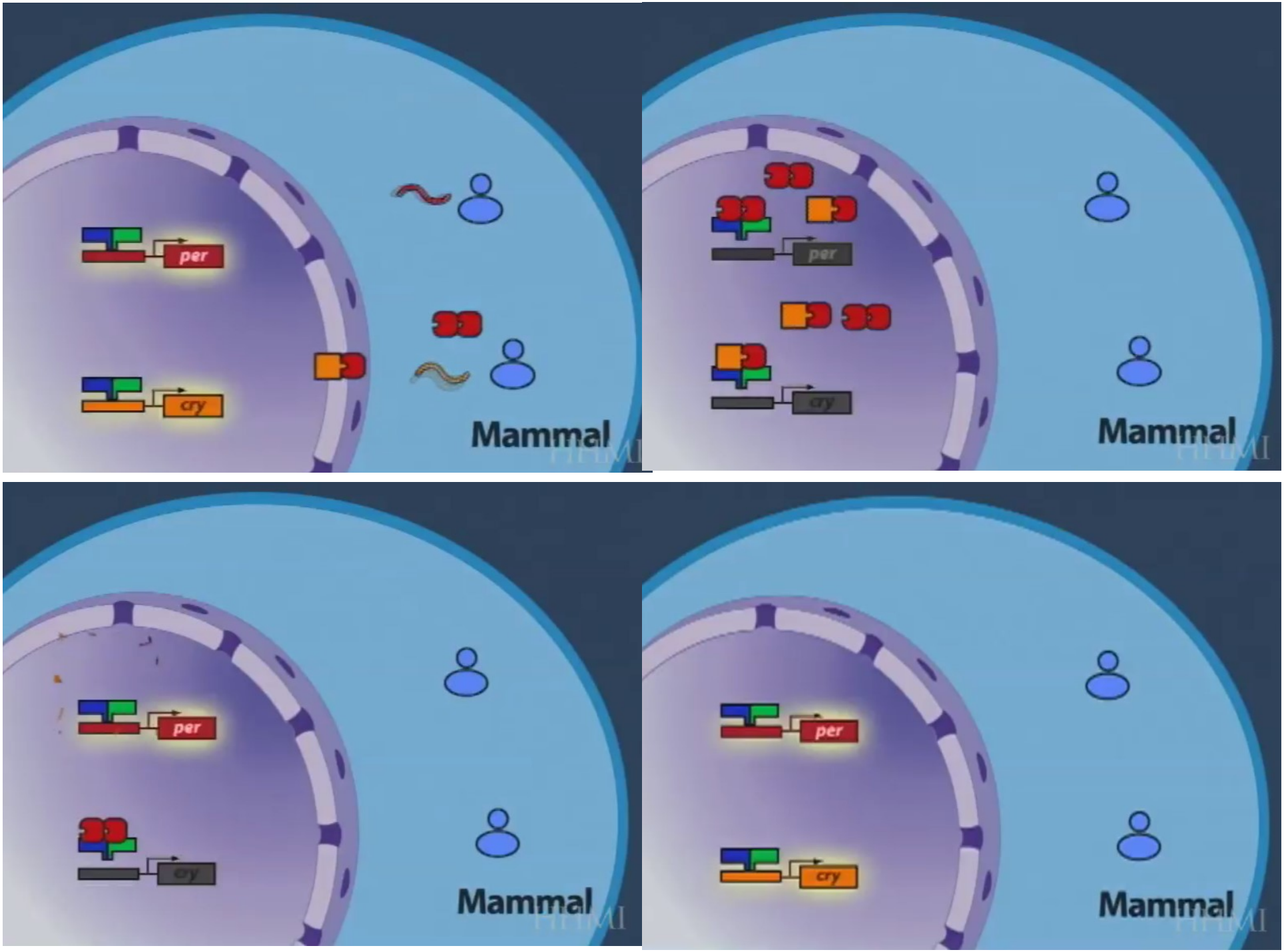
Mice who are totally blind (lacking both rods and cones) have no trouble keeping their circadian clock on time.

They are able to do this because:

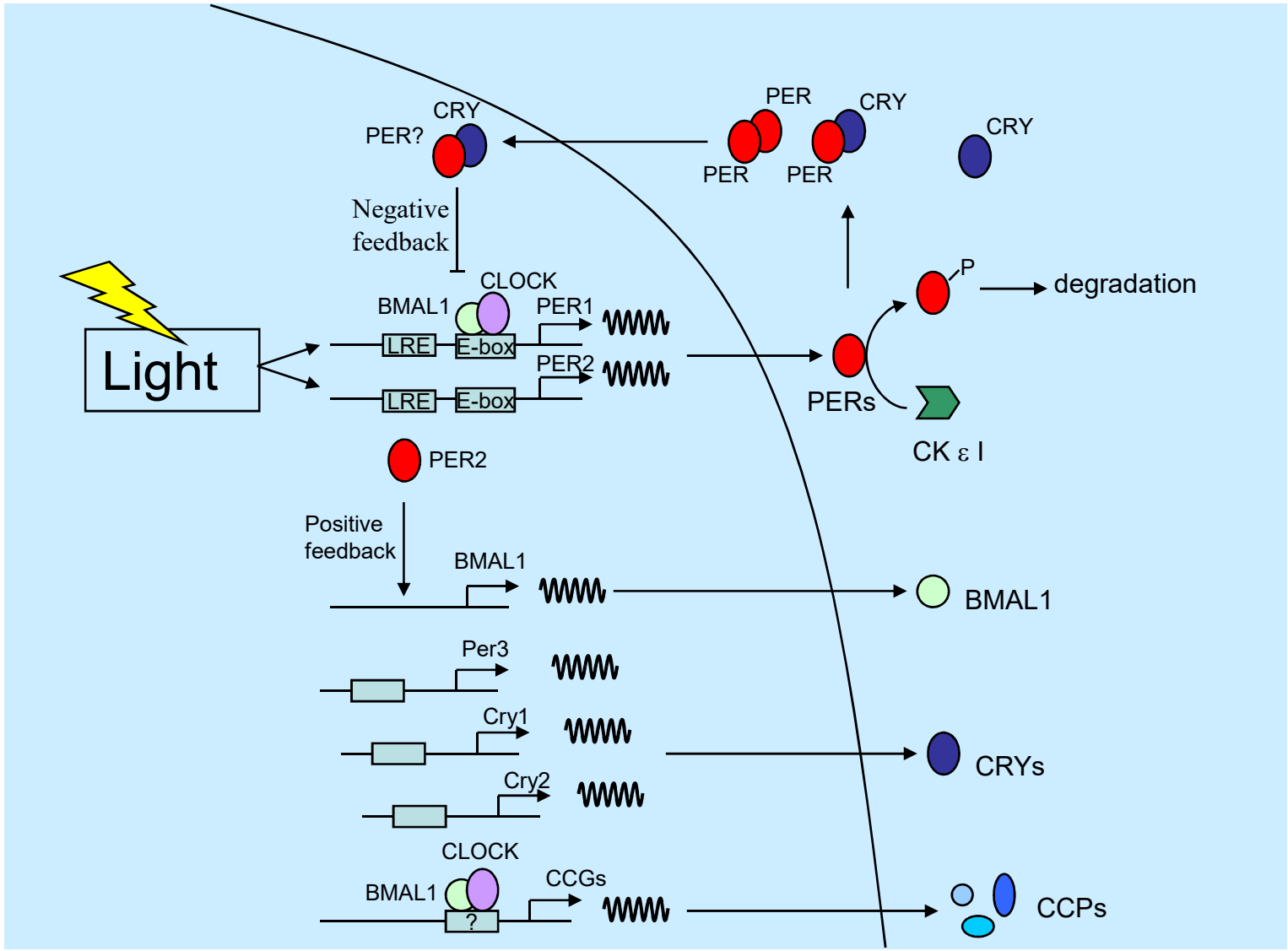
-- Some 1 - 2% of the ganglion cells in their retina - instead of depending on signals arriving from rods and/or cones - detect light directly.

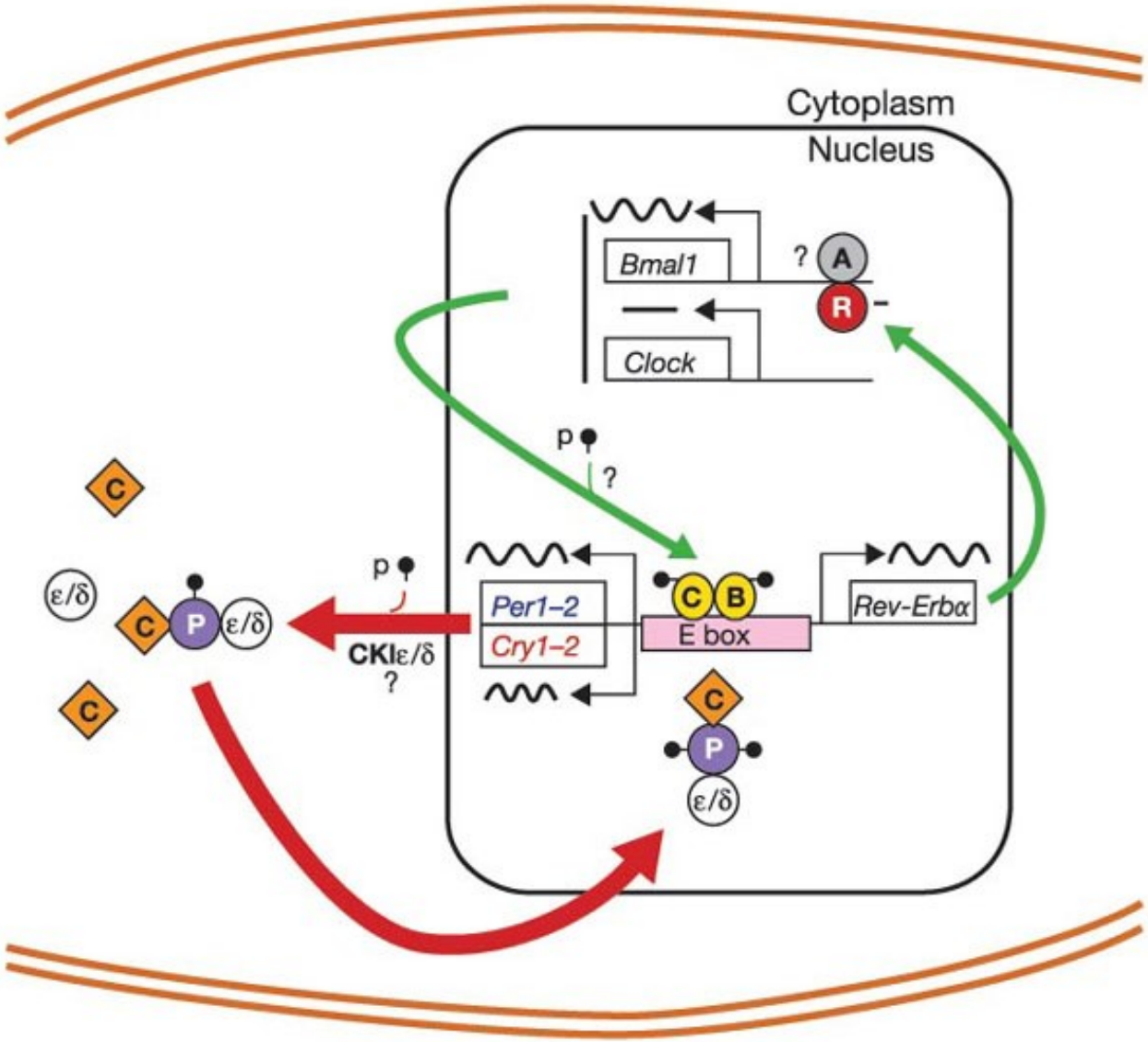
-- These ganglion cells have an extensive network of dendrites that contain the pigment **melanopsin**. When exposed to light (diffuse light is fine), these ganglion cells become depolarized and send their signals back to the SCN.


BMAL
CLOCK
Per1
Per2
Per3
Cry1
Cry2
Ribosomes
Mammal


per
cry
Mammal
per
cry
Mammal
per
cry
Mammal
per
cry
Mammal

Current Model of Clock Gene Interactions





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Circadian genes

- Most studied (sleep disorder implications, etc)
- Cyanobacteria 3 genes – kai A, B, & C
- *WC, frq* in *Neurospora*
- *clk, bmal, per, cry* in mammals
- *per, tim, cyc, clk* in *Drosophila*
- These genes are similar, and form similar feedback loops. Several of them are related.

External cues can reset the clock

- Light induced changes in protein conformation (that allow or block other molecules binding)
- Light induced degradation of molecules
- Most effective at dawn/dusk usually
- Other factors that can reset different oscillators: temperature, nutrient concentration, length of day

What do these clocks regulate?

- Gene expression!
- Metabolism!
- Behavior!

Table 1. Functions of Neurospora *ccgs*

Functional Category	*No. of ccgs*
Cell division	1
Signaling/communication	16
Cell structure/cytoskeleton	8
Cell defense	4
Development	11
Gene regulation	5
Metabolism	42
Protein processing	10
Protein synthesis	33
Unclassified	50

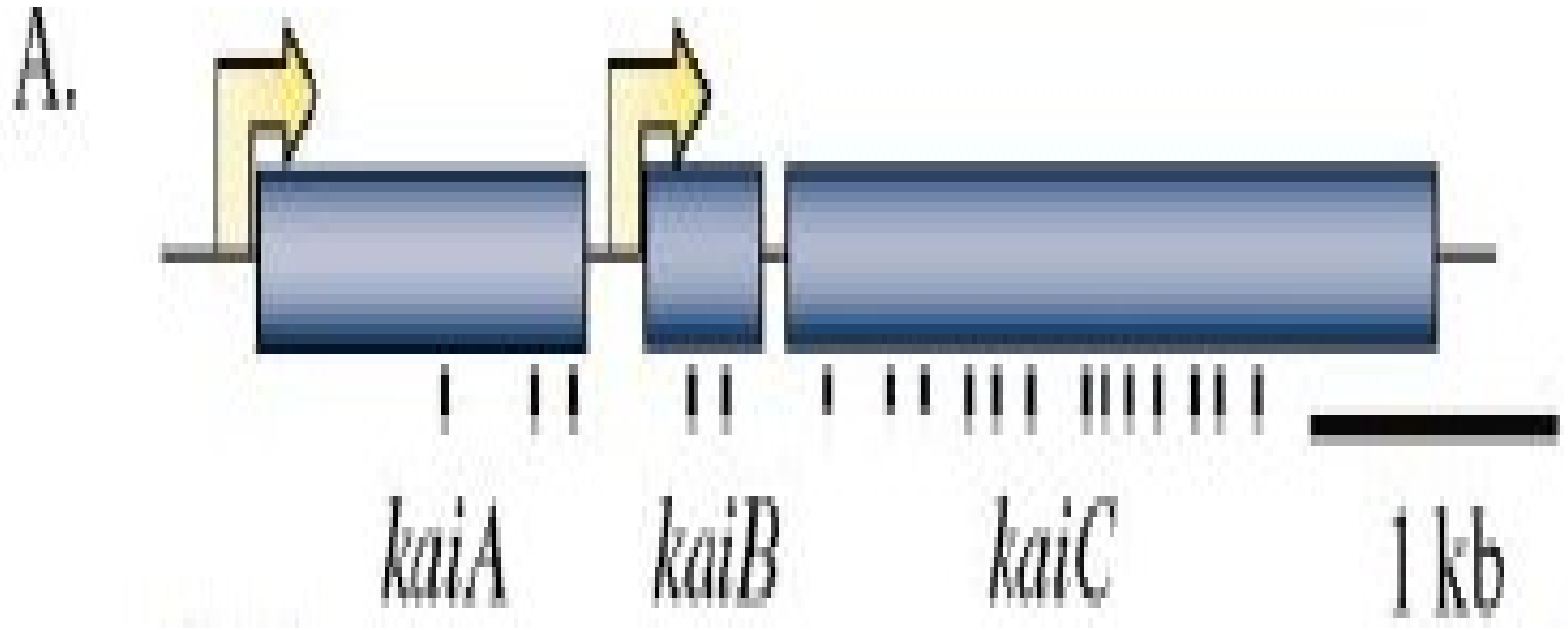
NOTE: ccg = clock-controlled gene. Data expanded from Correa et al. (2003) and Nowrousian et al. (2003).

Similar mechanisms operate on smaller scales to regulate smooth muscle contraction, neural firing, liver activity, and other processes.

Cyanobacteria

- Photoautotrophic prokaryotes
- Genetic lineage among oldest on Earth
- Oxygenic photosynthesis originated here
- Remarkable genetic diversity
- Morphological diversity
- Resides in nearly every habitat sunlight penetrates
- Wide range of growth rates and metabolic activities
- Participation in symbiotic associations

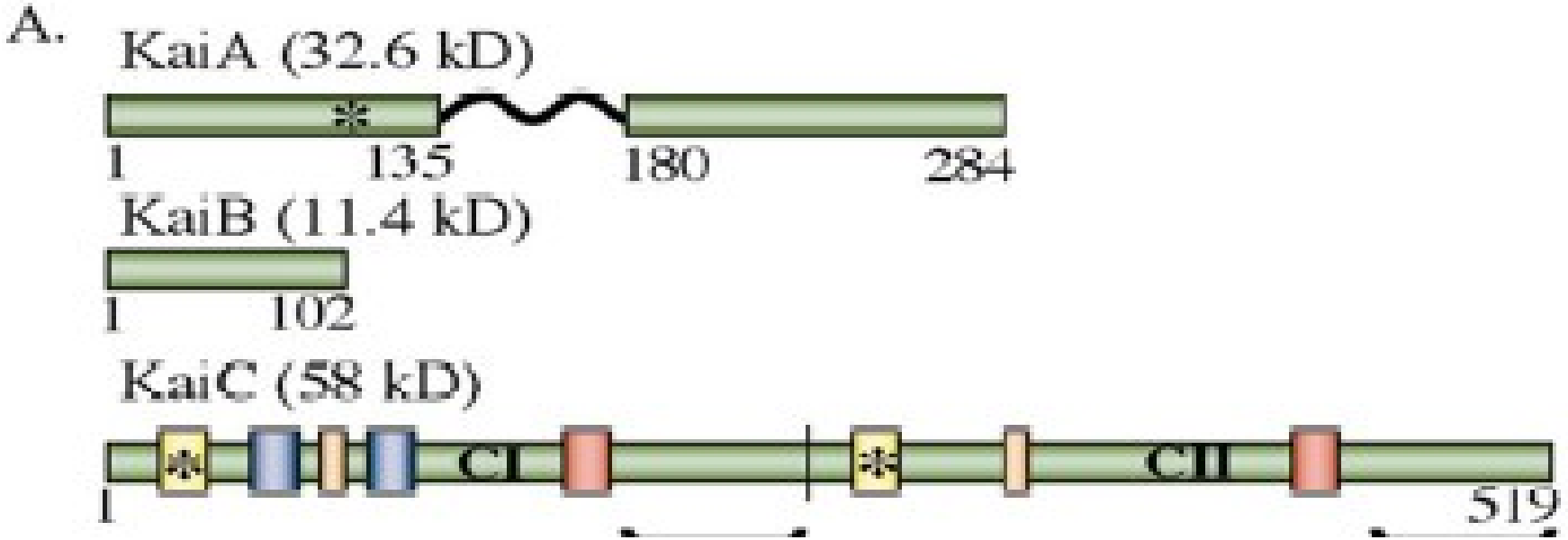
Clock comprised of at least 3 genes – kai A, B, and C



- At least 800 of 2700 genes expressed rhythmically
- Gene expression patterns can be categorized b/c differences are promoter specific
- Class I (most pervasive form) – peak near subjective dusk
- Class II – antiphase (peak at subjective dawn)

kai A, B, and C

- Kai A – 855bp, encodes 32.6 kD protein
- Kai B – 309bp, encodes 11.4 kD protein
- Kai C is 1560 bp, encodes 58 kD protein

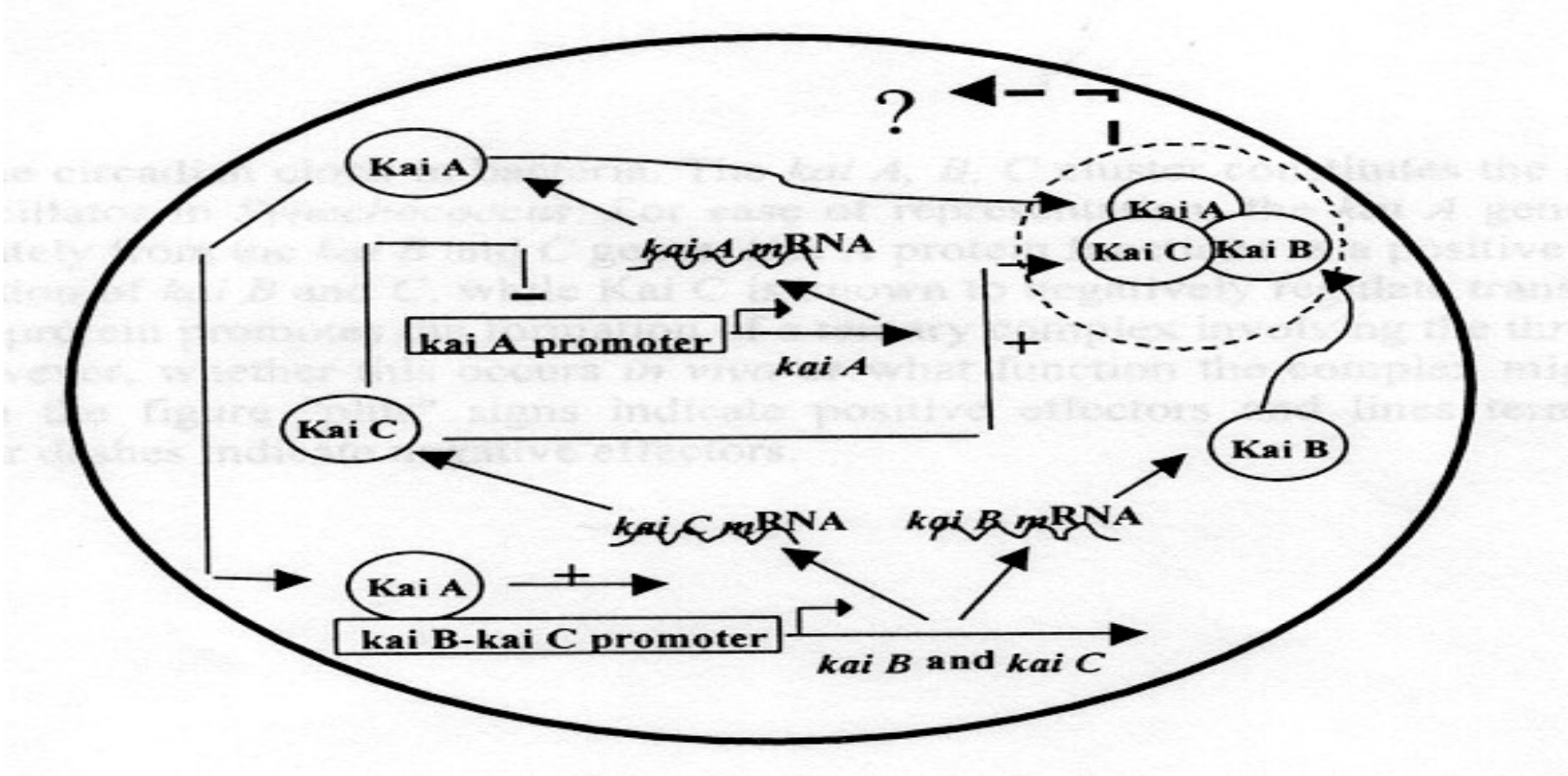


Transcriptional Control

- Negative feedback loops essential for regulation
- In eukaryotic systems, have positive elements and negative elements
- Kai C protein required for wt levels of expression in kaiBC promoter
- Kai A a positive activator
- Cooperative effort between Kai A and C?

- Kai genes confirmed to be essential components
- Genomes sequenced and all had at least one kai gene
- Kai A plays essential role in rhythm generation but N-terminal RR absent in many species
  - Not surprising due to evolutionary vastness

- Main requirement is ability to receive environmental stimuli and dock the Kai complex, inducing conformational change
- Evidence suggests that *kaiBC* operon is nearly 2 billion years old
- Seems likely that common ancestor between cyanobacteria and plastids had kai genes
- But where are the kai gene homologs in plastid lineage?



The circadian clock in bacteria. The *kai A, B, C* cluster constitutes the core of the circadian oscillator in *Synechococcus*. For representation, the *kai A* gene is drawn separately from *kai B* and C genes. Kai A protein functions as a positive effector of the transcription of *kai B* and C, while Kai C negatively regulates transcription of *kai A*. Kai C protein promotes formation of a ternary complex involving 3 *proteins in vitro*. In figure "plus", signs indicate +ve effectors and lines terminating in perpendicular dashes indicate negative effectors.

The Circadian Clock in *Drosophila*

A number of genes in Drosophila are turned on when the animal is exposed to light:

- **effector genes** whose products mediate the animal's responses (e.g. hatching or molting)
- **clock genes** whose products regulate the circadian clock.

Three key members of this group are:

- ***period*** (***per***)
- ***timeless*** (***tim***)
- ***vrille (vri)***

Activation of all of these genes requires that their promoters are bound by the protein transcription factors.

- **CLOCK** encoded by the gene ***clock*** (***clk***) and
- **CYCLE** encoded by the gene ***cycle*** (***cyc***)

The Circadian Clock in *Drosophila* – The Mechanism

The CLK/CYC proteins are transcription factors for ***per***, ***tim, vri*** and other effector genes.

The **PER** and **TIM** proteins (synthesized on ribosomes in the cytoplasm) form dimers.

•When the concentration of these gets high enough (early evening), they are transported into the nucleus.

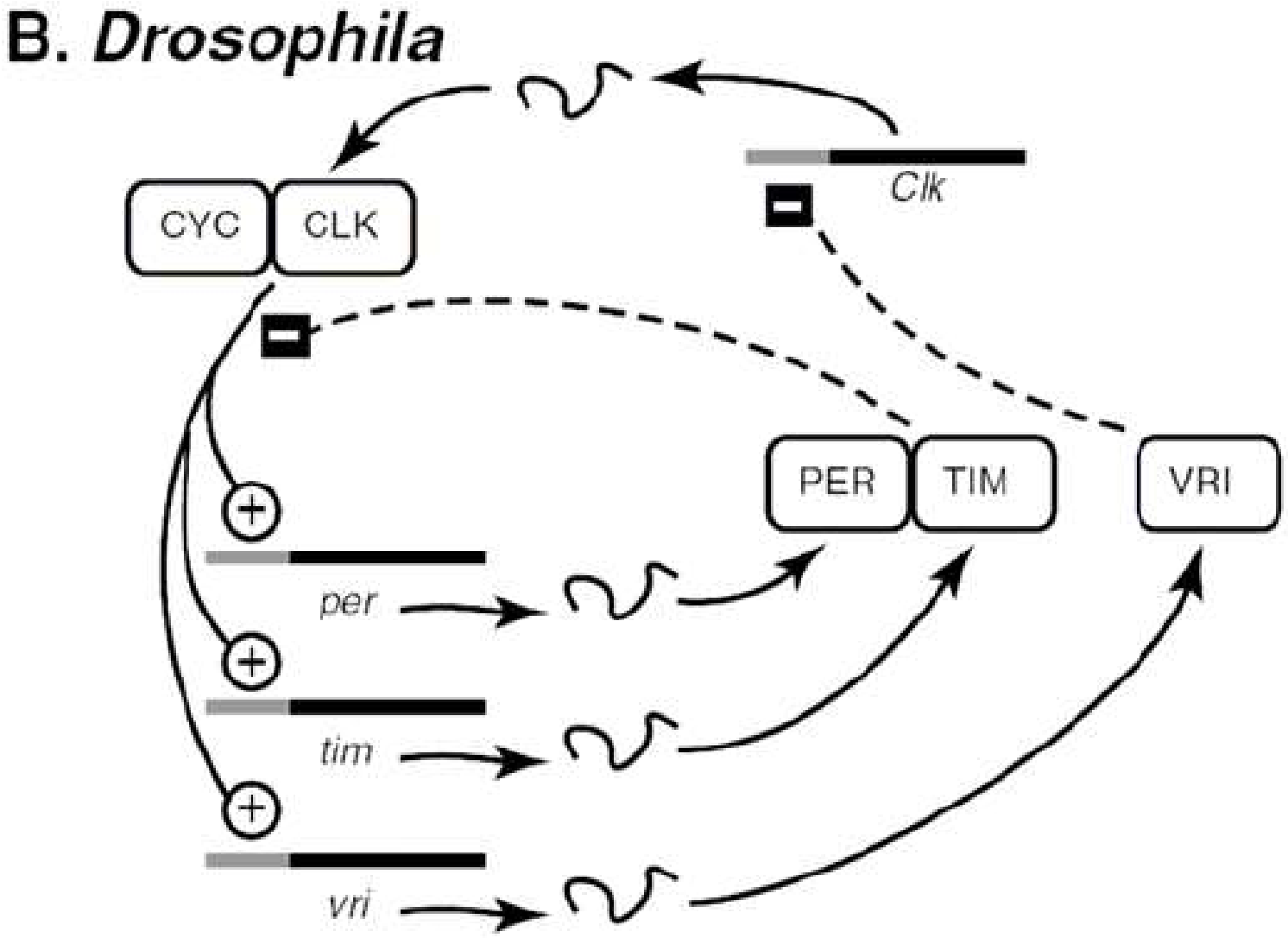
•The VRI protein also accumulates in the cytoplasm and goes into the nucleus, represses Clk gene transcription.

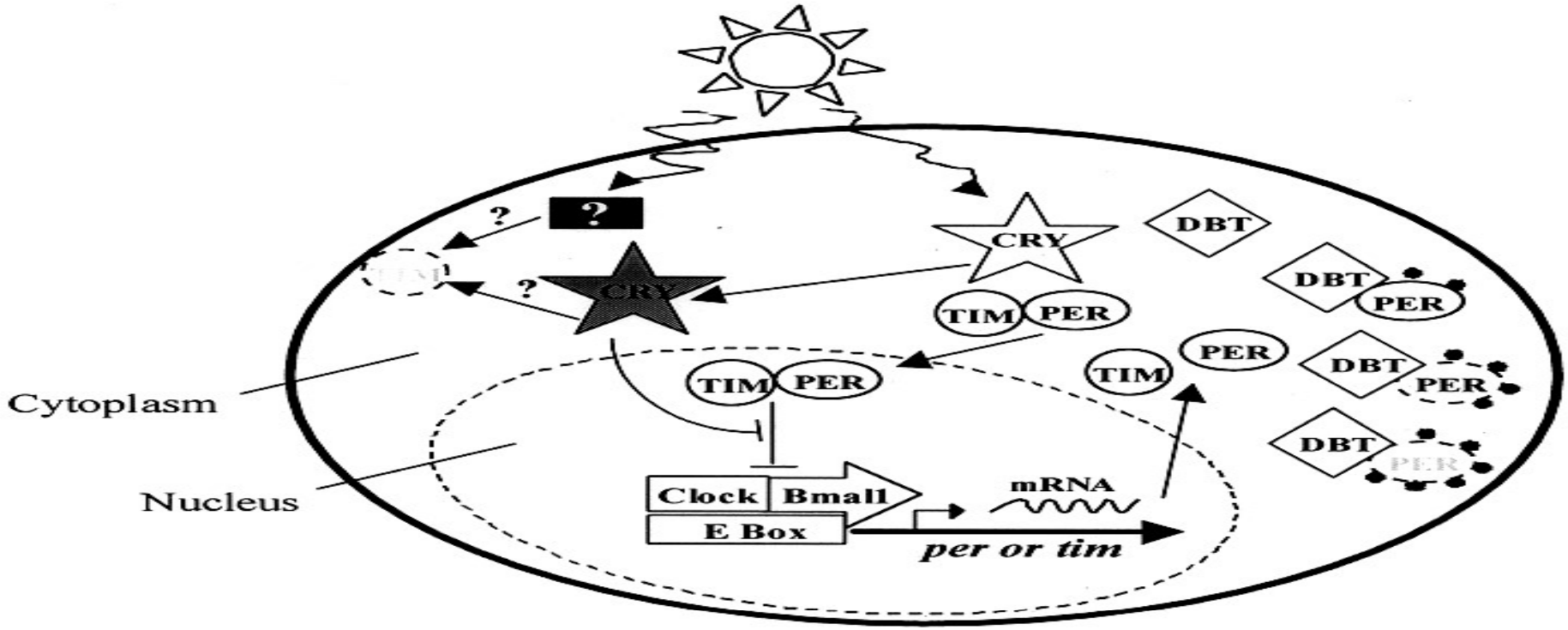
In the nucleus, PER/TIM

1) binds to the **CLK/CYC** transcription factors, removing them from the promoters of the genes they activate; thus shutting off transcription. Because these genes include ***per*** and ***tim***, the result is a negative feedback loop; that is, the products of the ***per*** and ***tim*** genes inhibit their own synthesis.

2) Shuts off ***vri*** transcription, thus remove inhibition on ***clk*** gene transcription.

3) As the level then falls, this inhibition is lifted and PER/TIM activity begins a new.

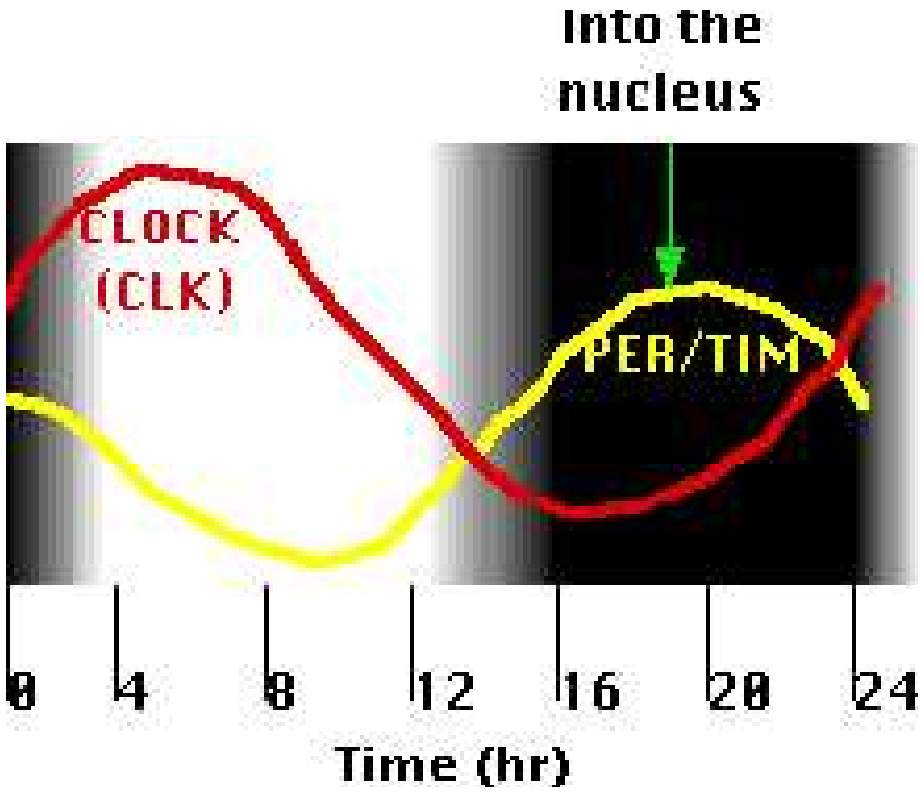

B. Drosophila
CYC
CLK
Clk
PER
TIM
VRI
per
tim
vri



In *Drosophila melanogaster* transcription of the clock genes *per and tim,* is positively regulated by the heterodimer formed by the protein products of the *clock and bmall (*d*cycle)* genes. PER and TIM form heterodimers which can then translocate into the nucleus, thereby negatively regulating their own transcription by interfering with the positively acting Clock-Bmall dimers. Moreover, the inhibiting action of the PER-TIM dimers is contrasted by the light-activated product (gray star) of the *cry* gene. The action of a constitutive homologue of human casein kinase type 1, encoded by the *dbt* gene, delays the formation of TIM-PER dimers by subtracting PER monomers, which are degraded via the ubiquitin pathway, following targeting via DBT-dependent phosphorylation (depicted from top to bottom along the right hand side of the cell). Clock resetting by light is effected by light-dependent degradation of TIM protein, although the precise mechanism by which light leads to this degradation has still to be described. In the figure "plus" signs indicate positive effectors and lines terminating in perpendicular dashes indicate negative effectors.

The Circadian Clock in *Drosophila* – The Mechanism

•The time required for the different effects results in the levels of **PER/TIM** and **CLOCK** oscillating in opposite phases with a circadian (~24 hr) rhythm.



The Circadian Clock in *Drosophila* – Setting The Clock

Even without any external cues (e.g., alternating light and dark), the cycles persist, although they tend to drift away from environmental time.

•Under natural conditions, the clocks are precise.

•This is because they are "set" (synchronized) by environmental cues, of which light is one of the most important.

In Drosophila, it works like this.

•Light (blue) is absorbed by the protein cryptochrome (**CRY**).

•This causes an allosteric change in its conformation enabling it to bind to **TIM**.

•This causes TIM to break down ending its inhibition on CYC/CLK.

•If this happens when **PER/TIM levels are rising** (late in the "day"), it sets the clock back.

•If it happens when **PER/TIM levels are declining** (late in the "night"), it sets the clock ahead.

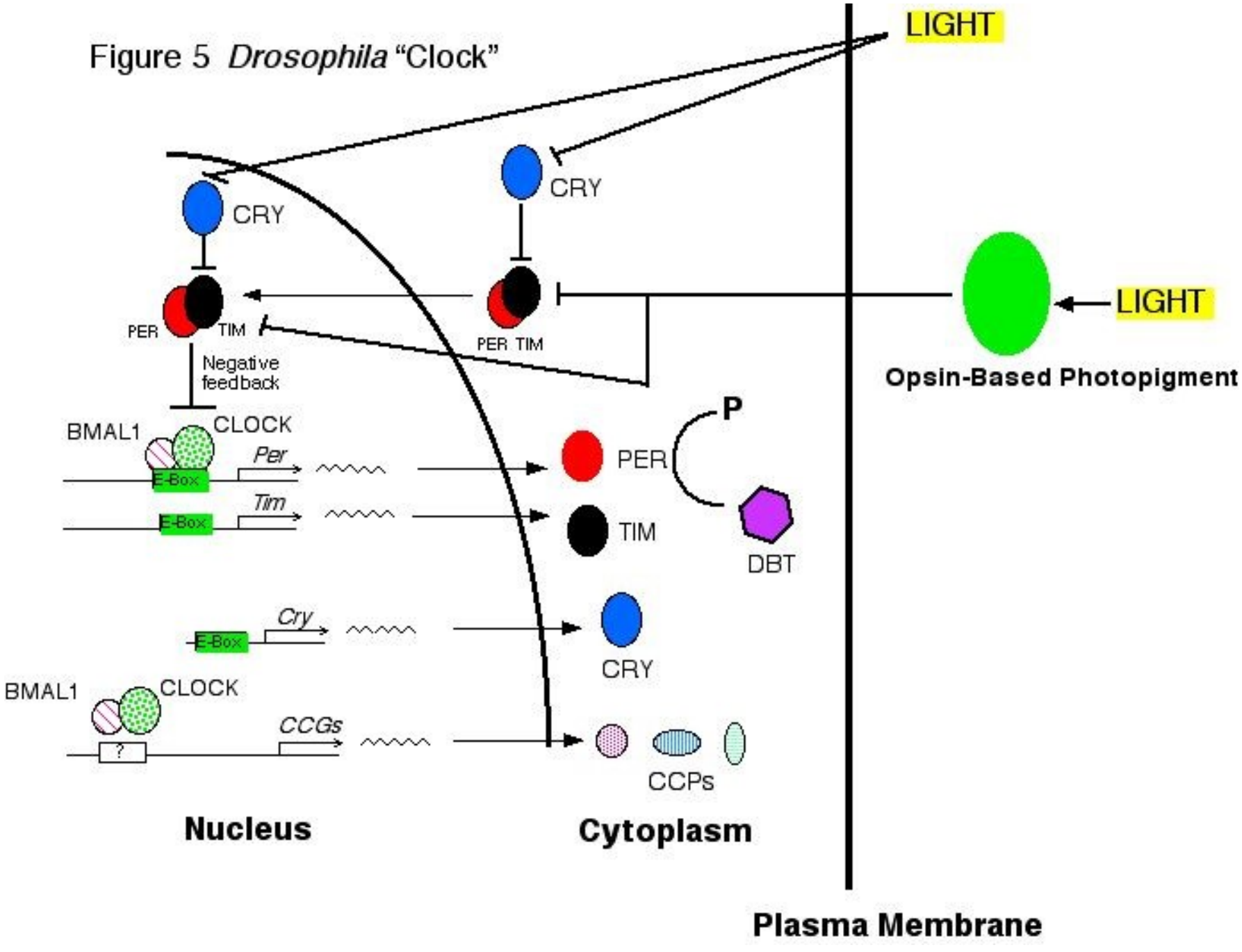
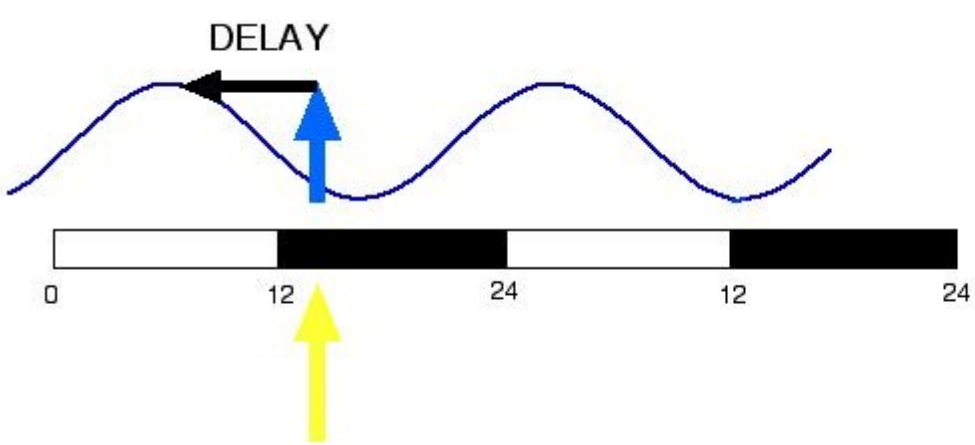
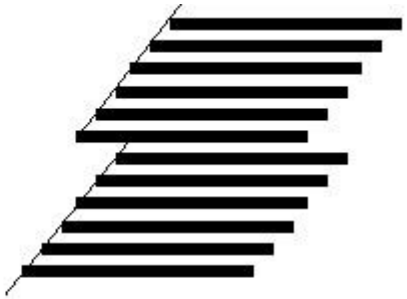
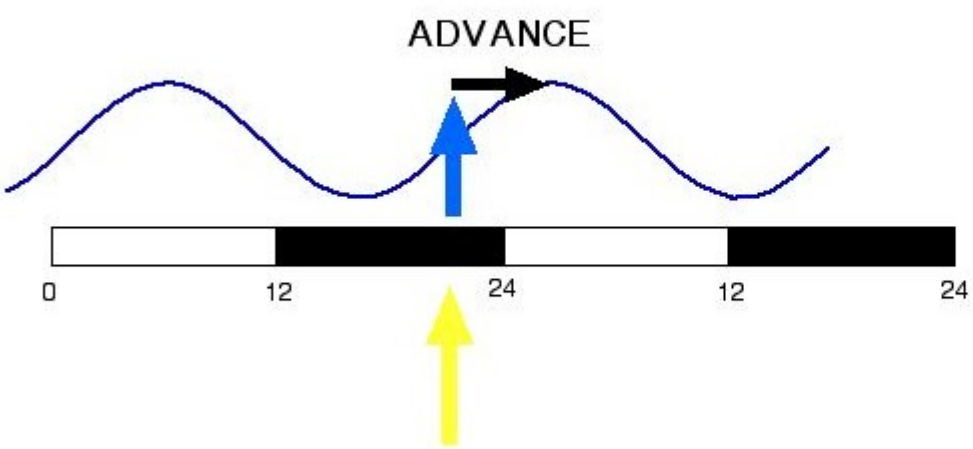


Figure 5 *Drosophila* "Clock"
LIGHT
CRY
CRY
PER
TIM
PER TIM
LIGHT
Opsin-Based Photopigment
Negative feedback
BMAL1
CLOCK
Per
E-Box
Tim
E-Box
PER
P
TIM
DBT
Cry
E-Box
CRY
BMAL1
CLOCK
CCGs
?
CCPs
Nucleus
Cytoplasm
Plasma Membrane

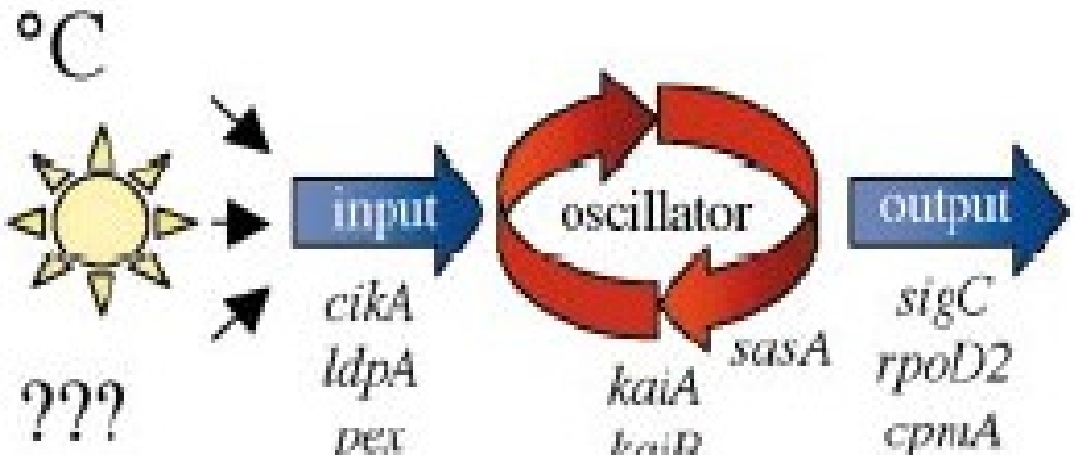
Molecular Entrainment







A.



Circadian Gene Expression:

Class 1 (peak at dusk)

1a { *kaiA*, *psbAI*

1b *kaiBC*

Class 2 (peak at dawn)

purF

opcA