The Neurospora Circadian System - some new tools and new insights

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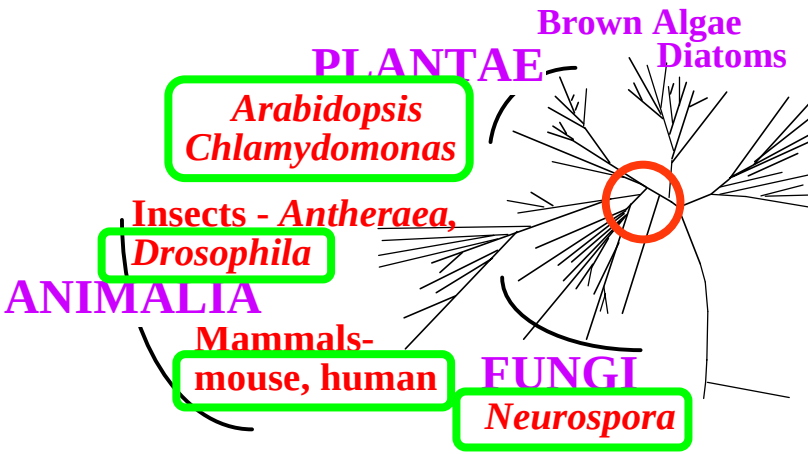
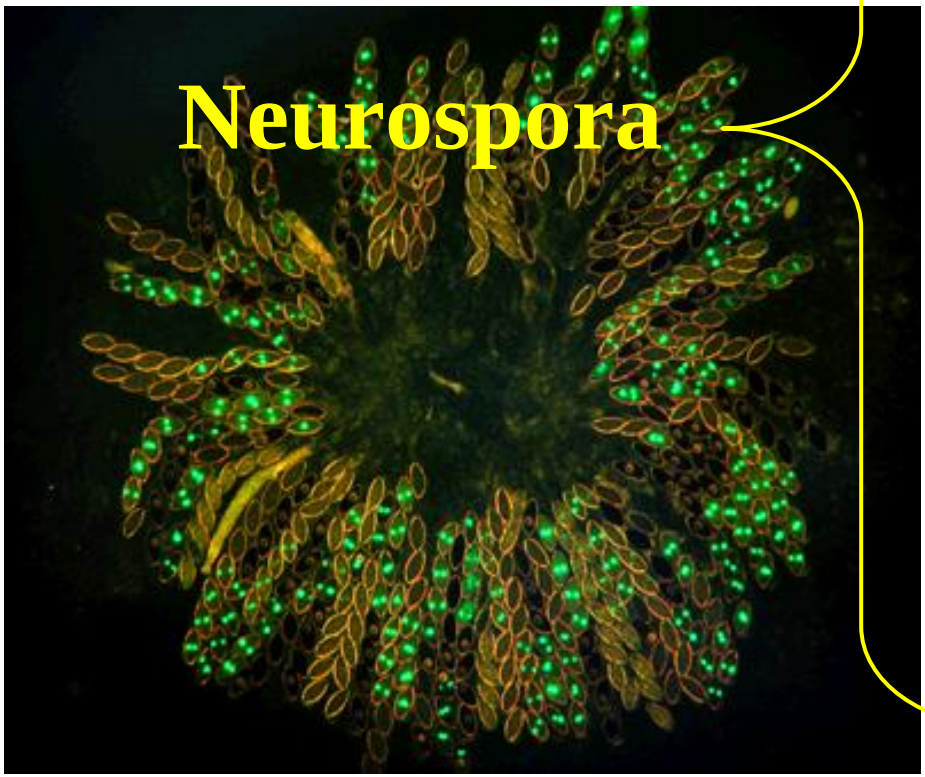
UC Santa Barbara
July, 2007

Circadian Systems in the Universal Tree of Life





Excellent genetics

Tractable molecular genetics

- **genome of 43 Mb fully sequenced**
  ~10,000 genes annotated
- **ongoing curation**
- **numerous regulatable promoters**
- **targeted replacements @98% efficiency**
  ~2500 genes knocked out + ~200/month
- **whole genome microarrays**

Typical eukaryotic gene structure

- **multiple introns**
- **combinatorial gene regulation**

28 cell types

Real world biology

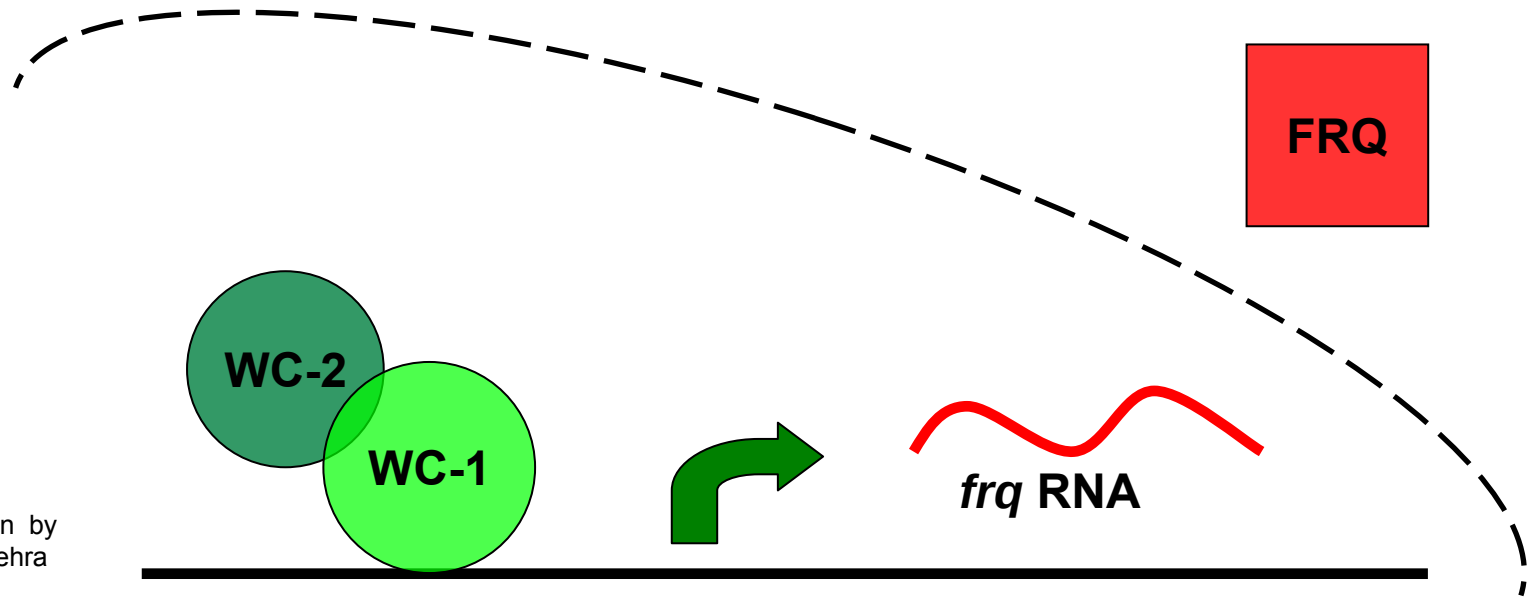
- **photobiology**
- **development**
- **cell/environmental interaction**
- **circadian rhythms**

Dunlap, Cell, 1999


FRQ/
WCC
kinas
CK-1,
CAMK-1,

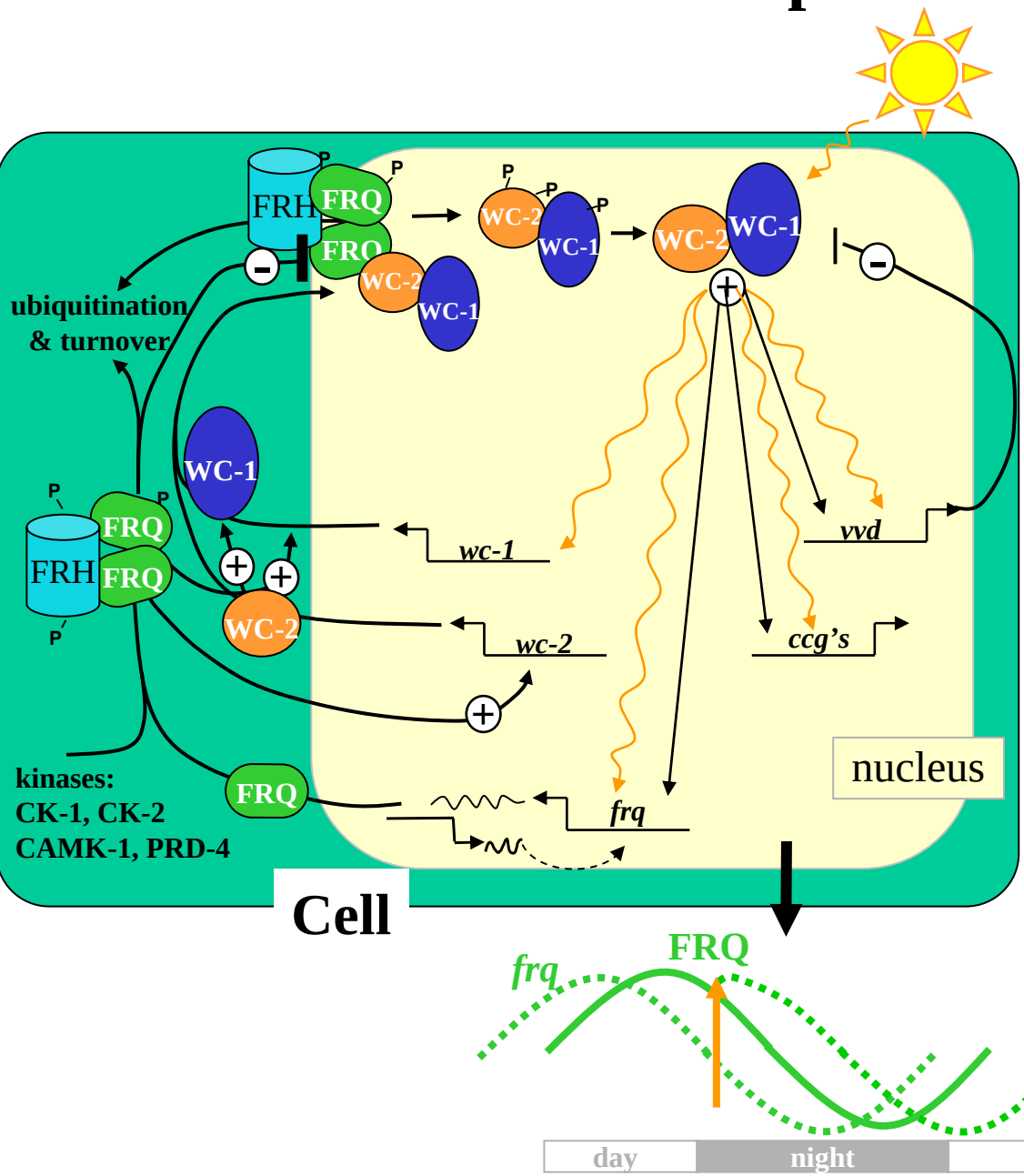
Simplified elements and dynamics of the *N. crassa* clock

And repeat every 22.5 h …



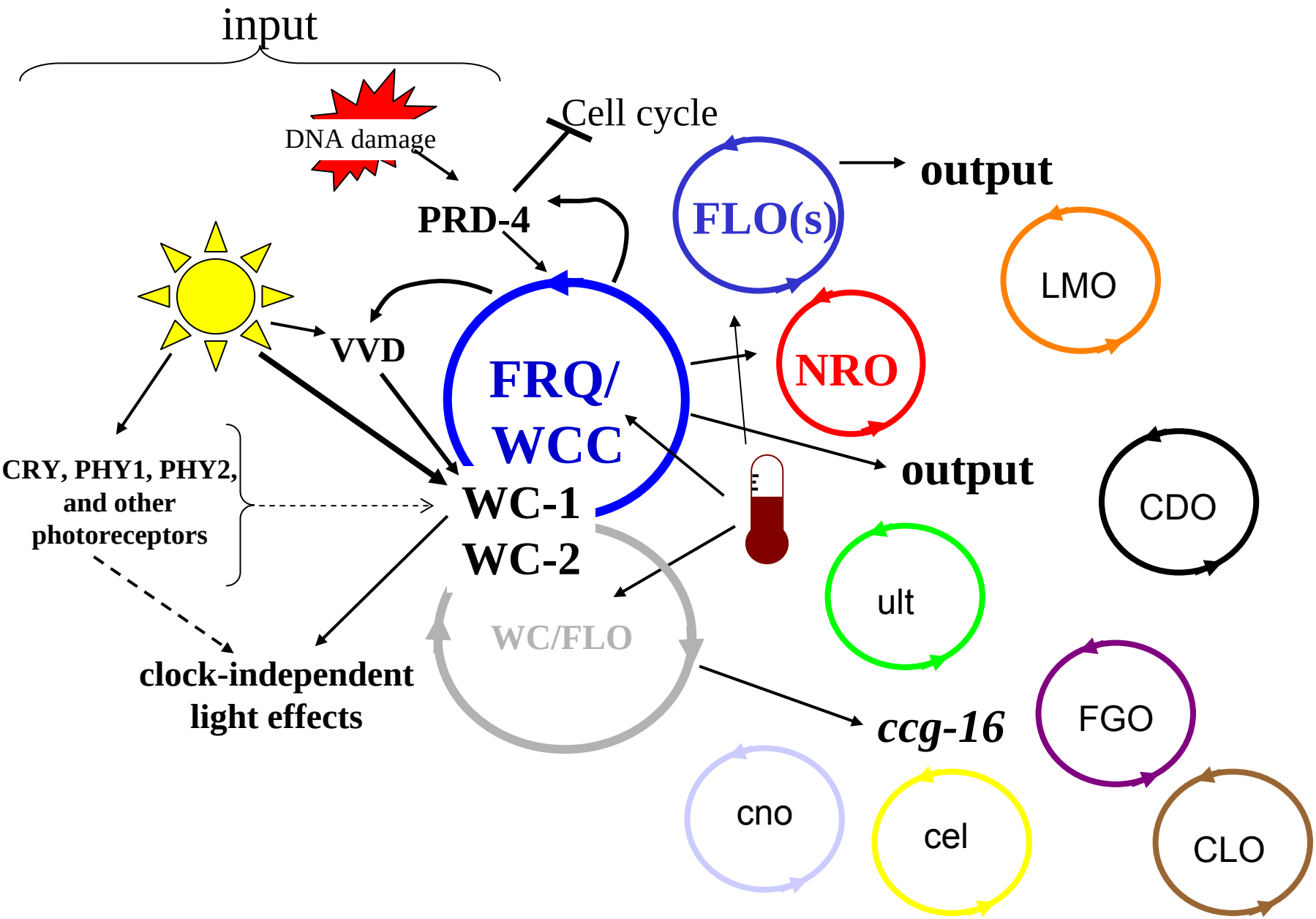
animation by
Arun Mehra

Known Molecular Components of the Neurospora clock



- WC-1 and WC-2 act positively on the production of *frq* transcript.
- FRQ makes a trimer with itself and the FRH helicase, acting negatively on the production of its own transcript. (negative feedback loop)
- FRQ acts positively on the production of *wc-2* transcript and WC-1. (positive feedback loops)
- FRQ promotes phosphorylation of WC-1 and WC-2, inactivating them.
- FRQ becomes phosphorylated which leads to its turnover, releasing WC-1 and WC-2.
- These interactions lead to oscillations in levels of *frq*/FRQ that are essential for all true circadian rhythms in Neurospora, including nested loops affecting input.
- Changes in *frq*/FRQ levels are directly translated into changes in circadian rhythms in Neurospora.

The Cellular Circadian System in Neurospora



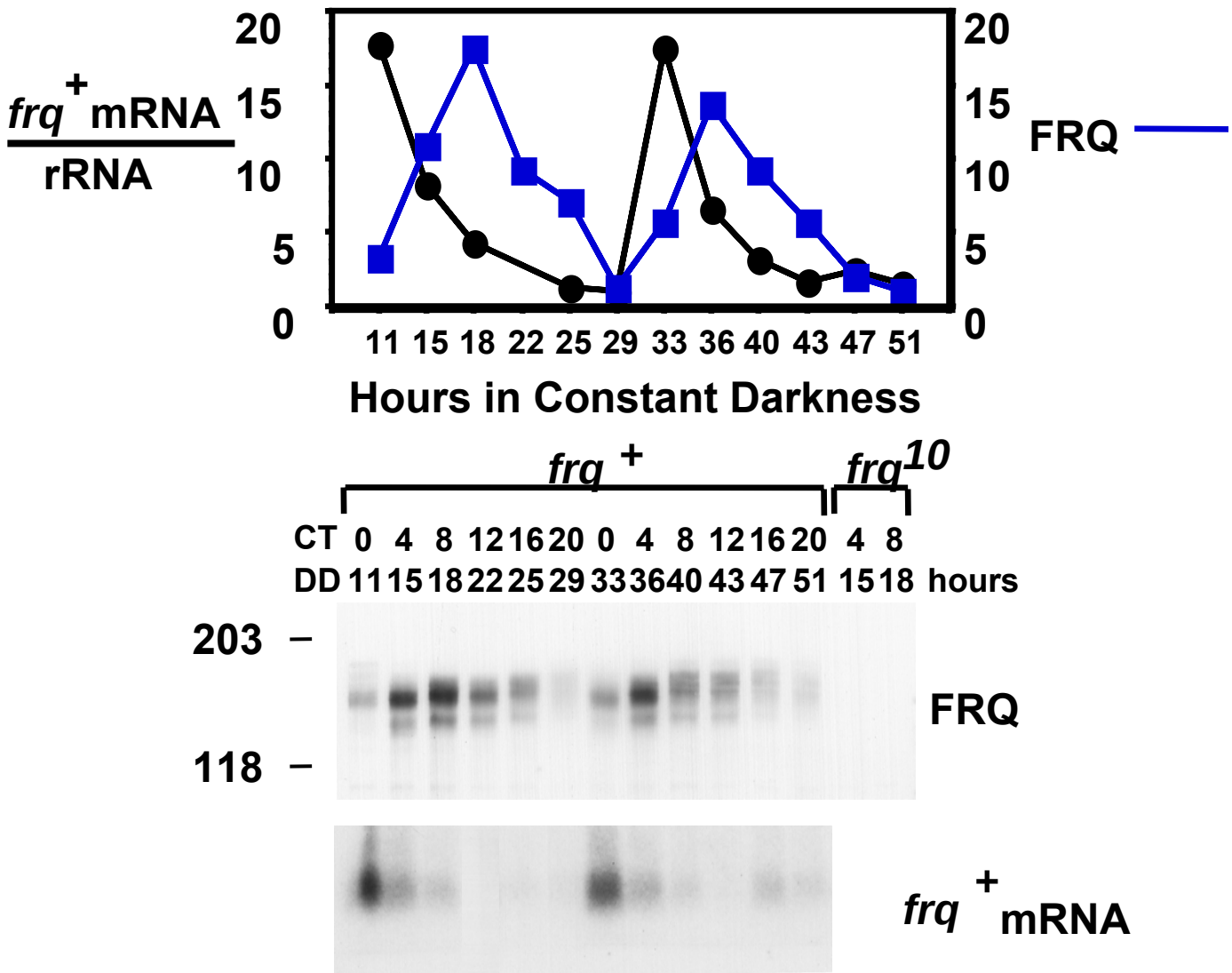
Outline - I'm going to cover (or attempt to cover) three topics that are linked by the fact that their resolution was made possible by technical improvements in the system.

1. A role for chromatin remodeling in the FRQ-WCC circadian feedback loop.

2. A role for casein kinase 2 in circadian temperature compensation.

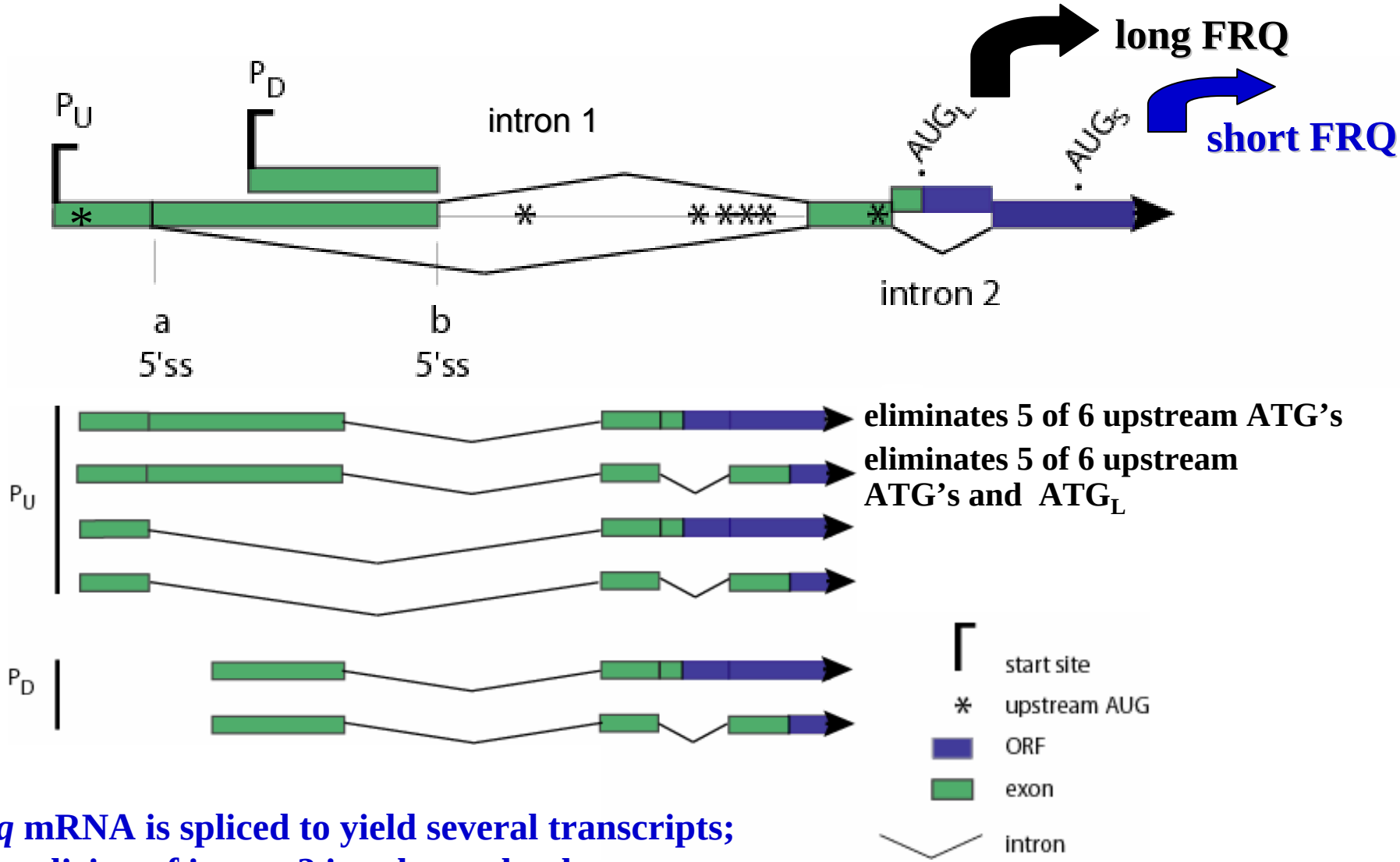
3. A method for simultaneous analysis of multiple oscillators in a single cell, and its use in establishing regulatory relationships between and among separate oscillators within the circadian system.

Rhythms in *frq* mRNA and FRQ protein



from Garceau et al, Cell, 1997

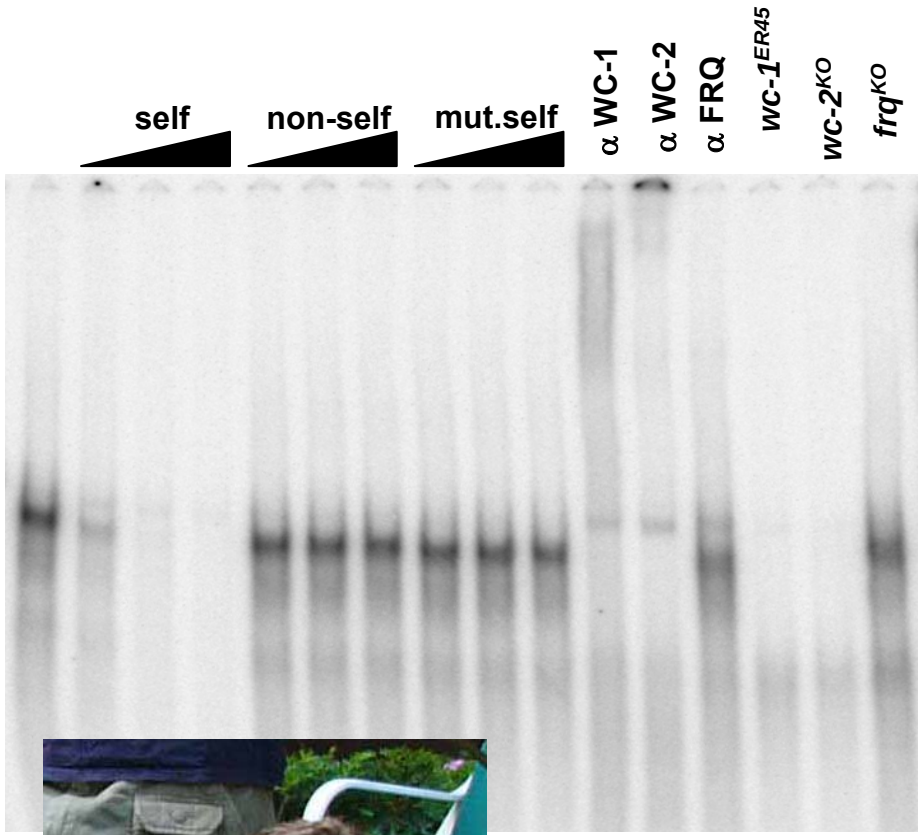
Expression of *frq* is surprisingly complex



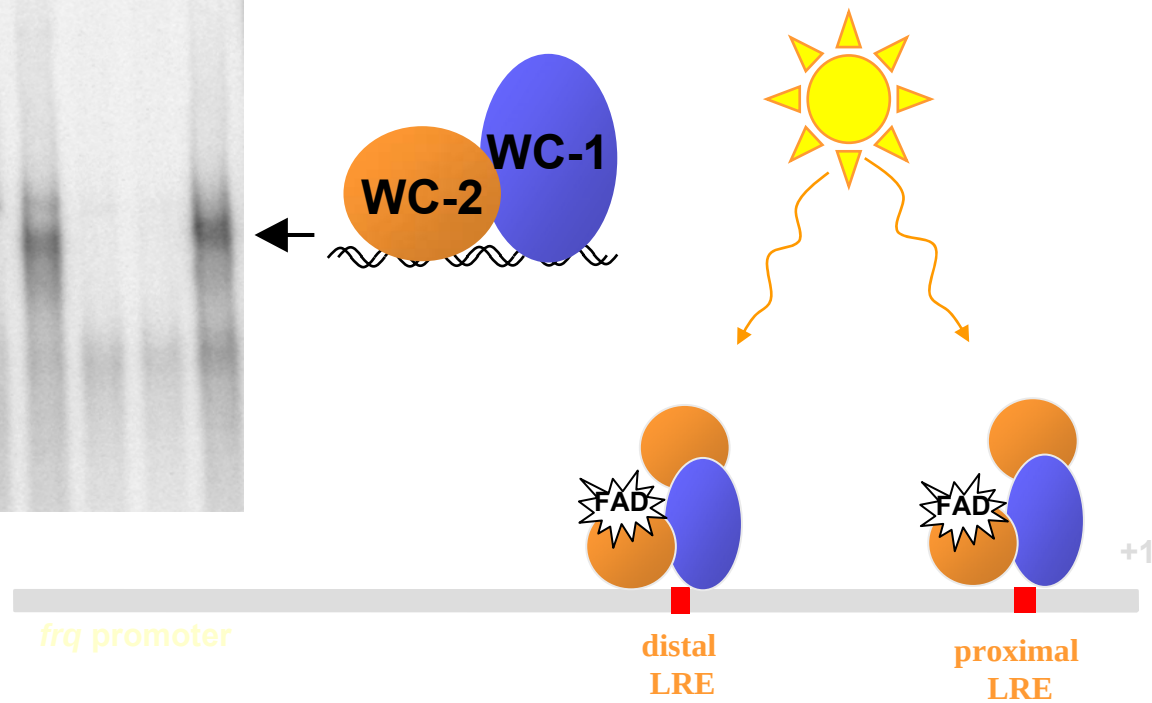
***frq* mRNA is spliced to yield several transcripts;**
splicing of intron 2 is enhanced at low temperatures;
uORFs reduce FRQ expression at lower temperatures by occupying ribosomes.

Colot et al. 2005, MBoC

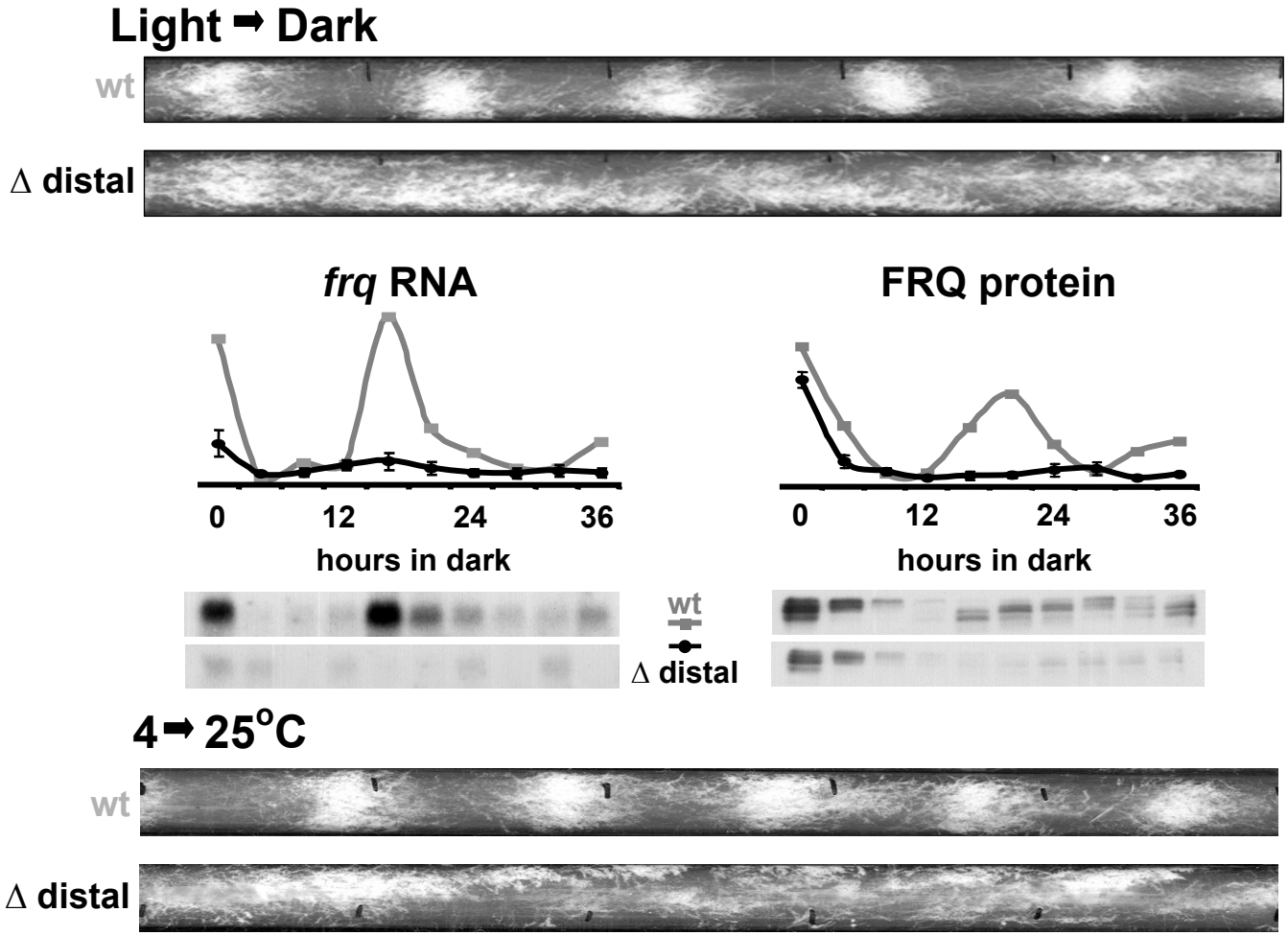
Two sites in the *frq* promoter are bound by WC-1/WC-2



Allan Froehlich

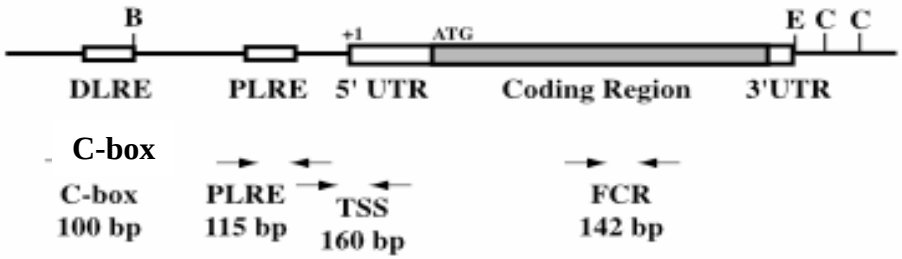


A distal WC binding site is necessary for rhythmicity

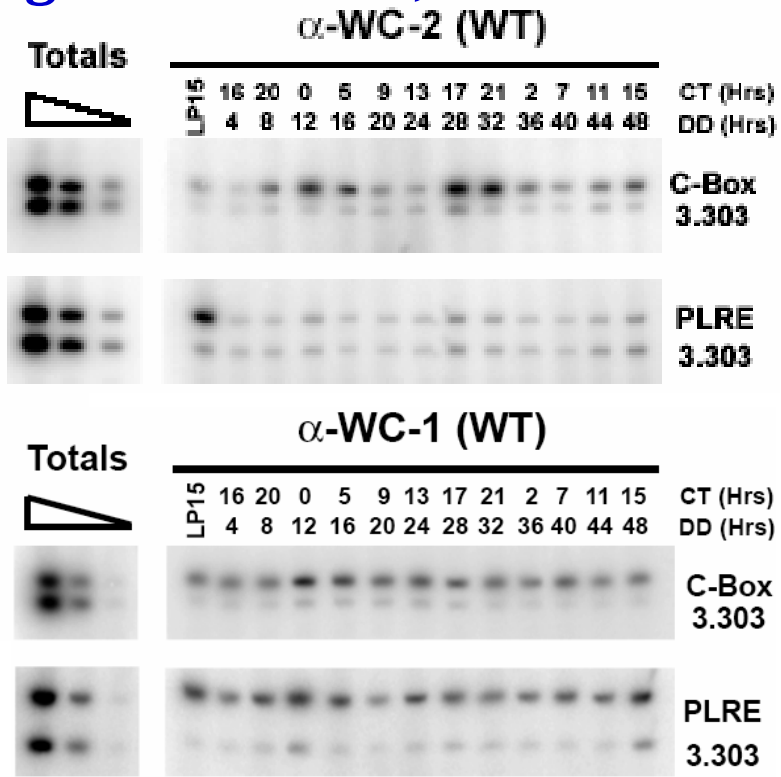


Therefore, important regulatory factors bind to that DNA, and it is now called the Clock-Box or C-Box.

Chromatin Immunoprecipitation assays show rhythmic association of WC-2 with the Clock-Box but no rhythmic changes in binding at the PLRE, TSS or FCR.



C-box: Clock Box (Distal Light Regulated Element)
PLRE: Proximal Light Regulated Element
TSS: Transcriptional Start Site
FCR: *frq* Coding Region
3.303: loading control of nontranscribed DNA

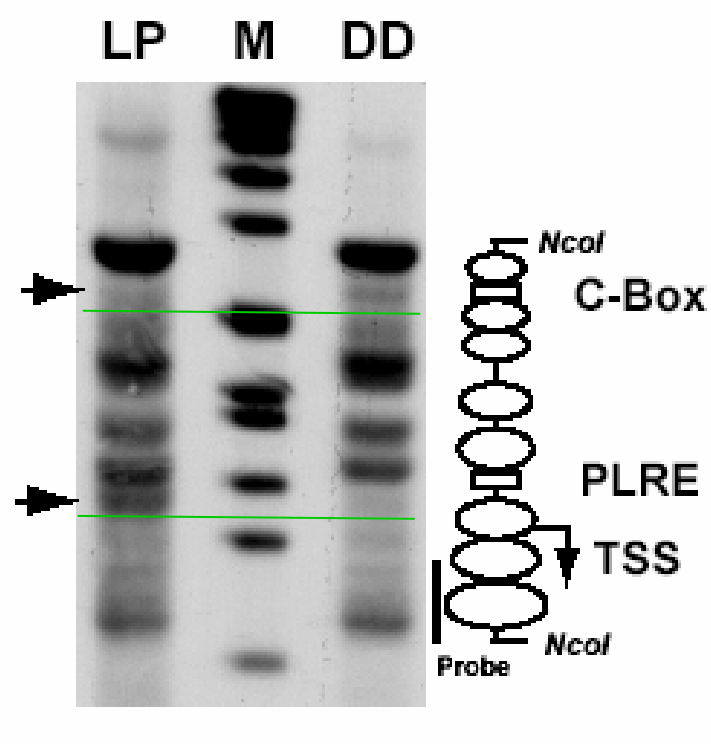


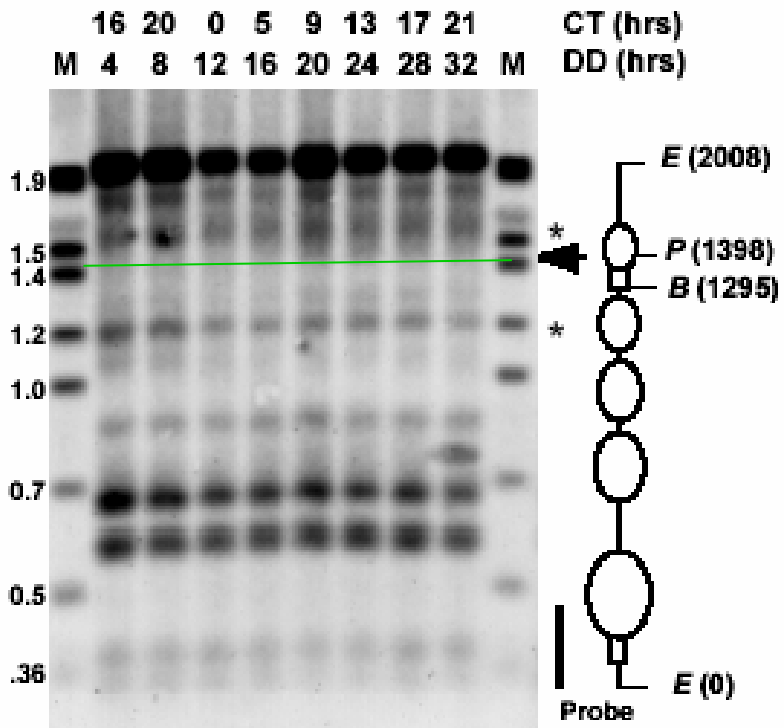
Bill Belden

Additional data suggested that chromatin remodeling was happening at *frq*.

(Chromatin-immunoprecipitation assay - proteins normally bound to DNA are chemically cross-linked to the DNA, which is then isolated, sheared to a smaller size, and immunoprecipitated with antisera to the specific transcription factors bound to the DNA. The cross links are reversed and the IP'd DNA is used in PCR to see what regions are enriched, that is, where the transcription factors were binding)

Limited digestion with micrococcal nuclease confirms light- and clock-associated changes in chromatin structure.

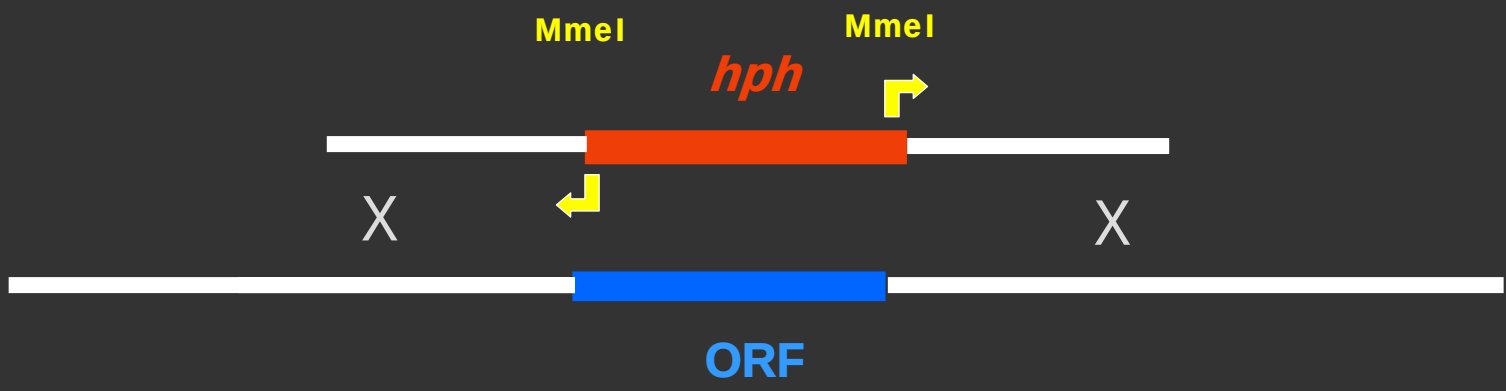




Light causes chromatin to close or be inaccessible at the Clock Box and to open up at the PLRE.

The Clock Box is generally bound during periods of clock-associated *frq* transcription and opens up as *frq* expression declines.

An enzyme is doing this - But there are 19 SWI/SNF-like homologs to chromatin remodeling enzymes in Neurospora. Which one(s) are important?



- The selectable marker *hph* is used to replace the ORF by homologous recombination
- Mme I sites are inserted at the ends of *hph* to provide molecular barcoding capabilities

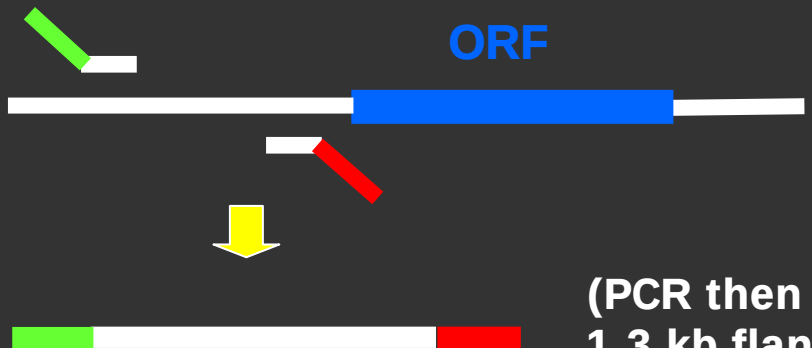
- **The knockout procedure overcomes three traditional roadblocks to knockout creation in Neurospora:**
  - Techniques for making deletion cassettes were cumbersome.
  - Long stretches of homology (2-3 kb) were required for reasonable efficiency of homologous recombination.
  - Ectopic integration was frequent.

Knockout cassettes were assembled using recombination cloning in yeast

Synthesize 4 primers for each gene

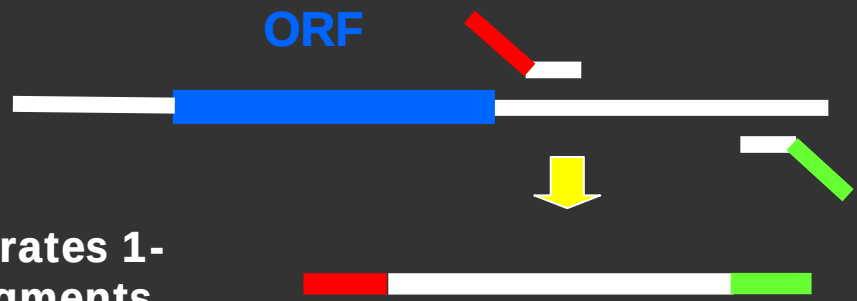
(An algorithm analyzes each ORF and picks a gene-specific 20 nt sequence as the 3' end of each primer. It then adds one of four generic 29 nt sequences that has homology to the vector or *hph*, as appropriate.)

PCR flank fragments from genomic DNA



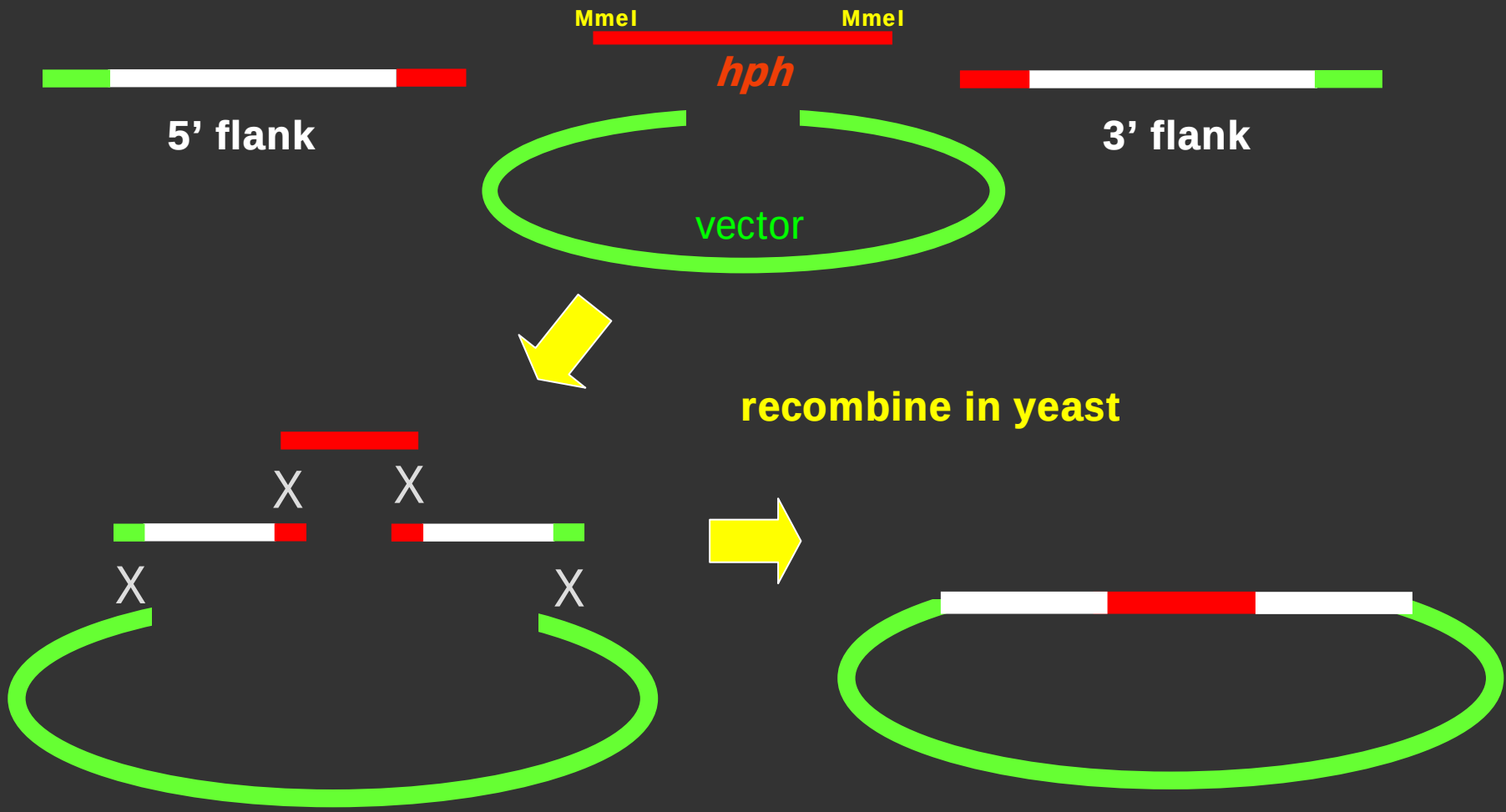
5' flank

(PCR then generates 1-1.3 kb flank fragments within the 1500 bases on 5' or 3' side of ORF.)

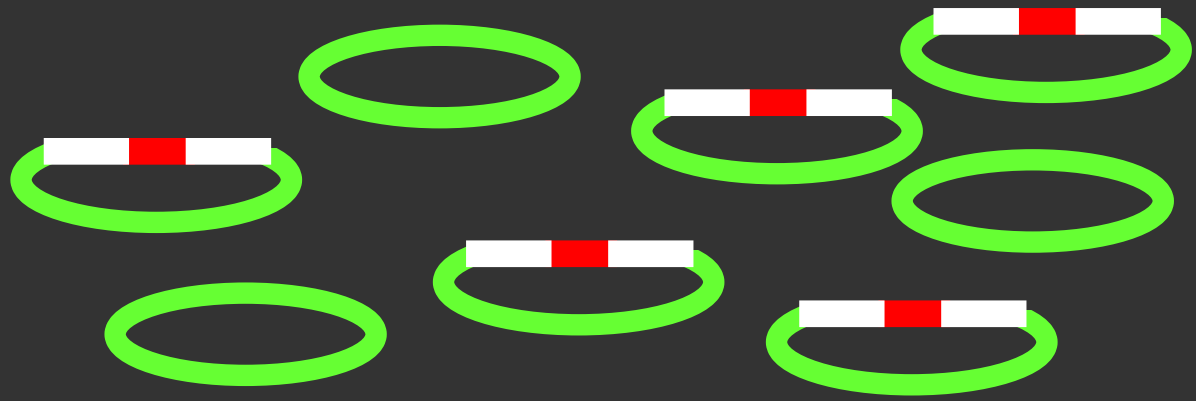


3' flank

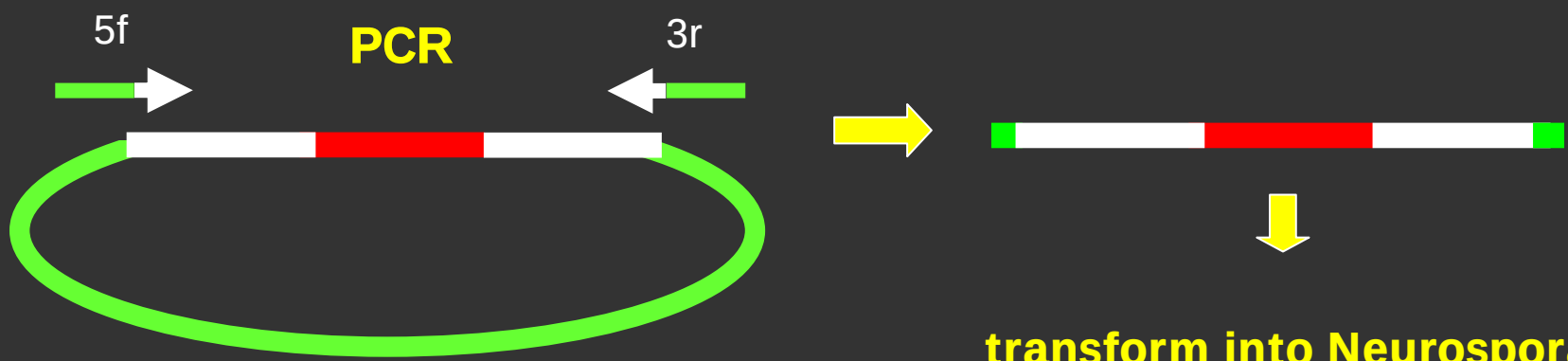
The two flanks from the PCR are mixed with the generic selectable marker (hph) and with the vector, and the 4 components cotransformed into yeast.



Yeast DNA is prepared from the mixed pool of transformants.



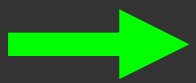
PCR directly from the mixed pool of DN
using the outside primers generates the
knockout cassette which can be used
without purification.



transform into Neurospora

Southern blot analysis of primary transformants

	KO construct transformed	Transformations yielding viable colonies	Transformants with Southern results	Homologous integration events	HR with ectopics
number	104	103	623	614	4
% of total	100	99	100	98.6	0.6

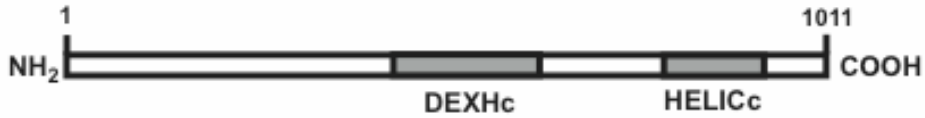


97.9% of transformants yield clean gene replacements

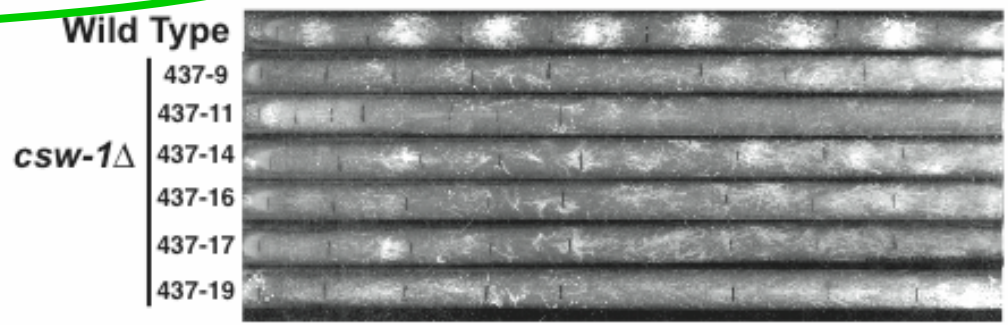
CSW-1, an ATP-dependent chromatin remodeling enzyme, is needed for rhythmic *frq* expression

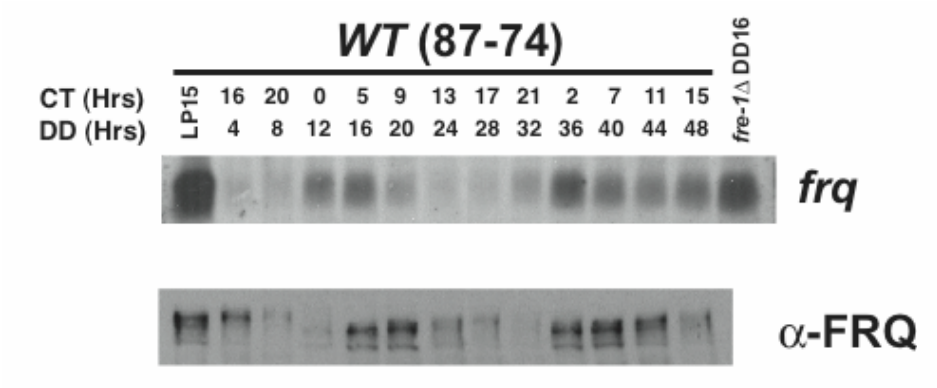
ATP-Dependent Chromatin Remodeling Enzymes

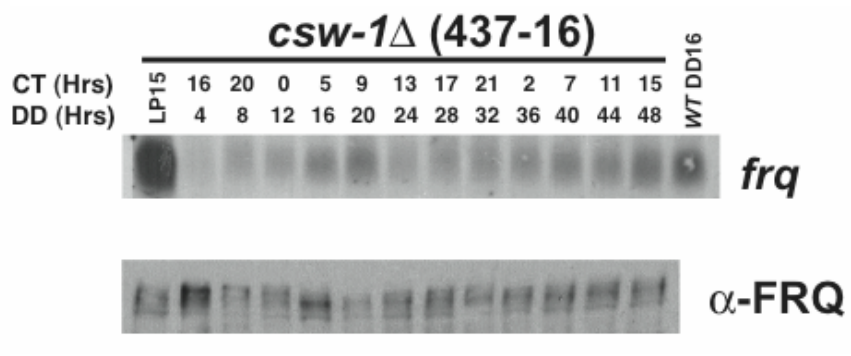
Neurospora orf	Protein	Phenotype
3875.1	*ScSth1 (RSC)/ISWI*	Not Viable
6488.1	*ScSwi2/Snf2*	Vialble
3060.1	*Sc Chd1*	Will not cross
1406.1	*Hs Mi-2*	Vialble
7556.1	*Hs TAF172*	Vialble
9106.1	*ScFun30/HsETL*	No circadian Banding
7837.1		Viable
4424.1		Viable
4445.1		Viable
5246.1		Viable
6306.1	*HsLSH*	Viable
7975.1		Viable
164.1		Viable
4786.1		Viable
631.1	*ScRis1*	Viable
2684.1		Viable
2910.1		Viable
7358.1		Viable
8919.1	*ScIno80*	Viable
9993.1	*ScSwr1*	Viable

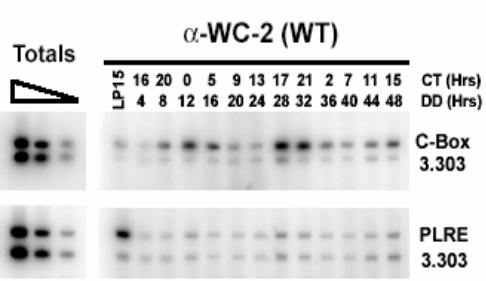


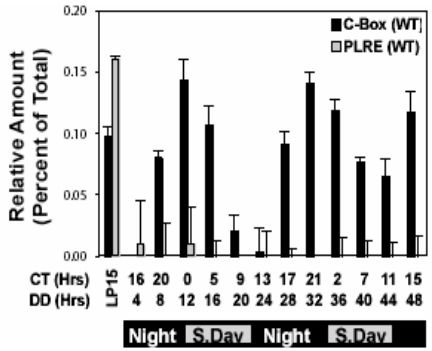
clock switch-1 {csw-1 }

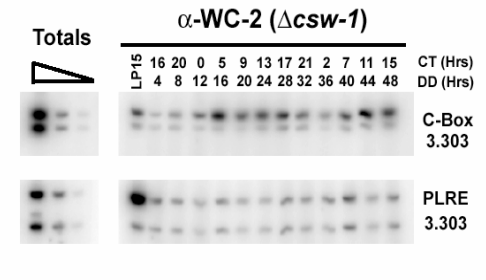


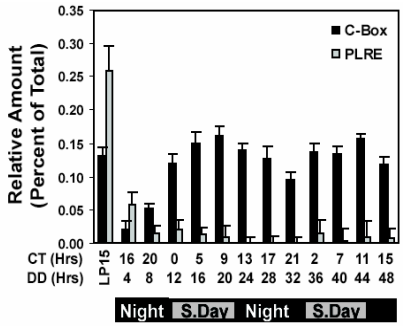


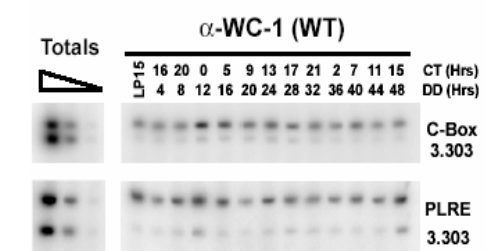


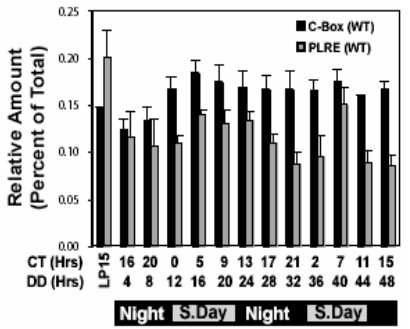


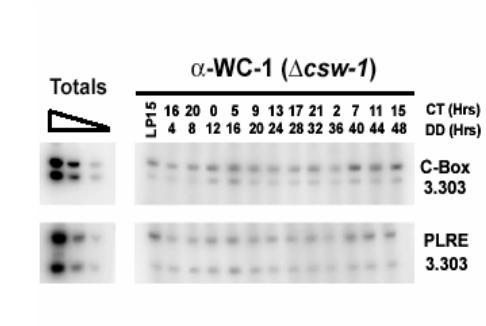


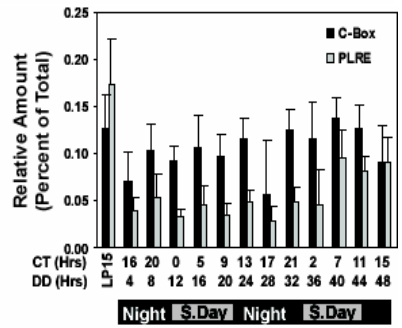










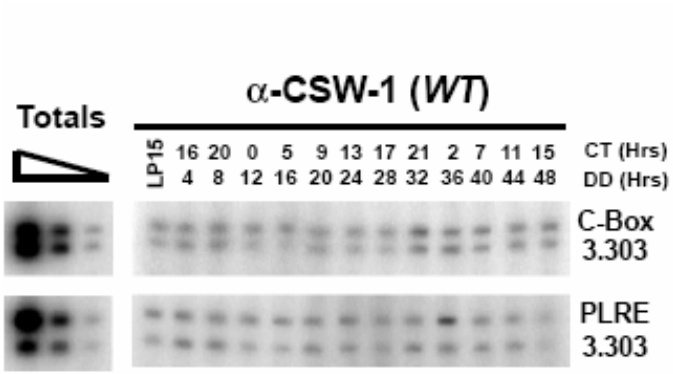


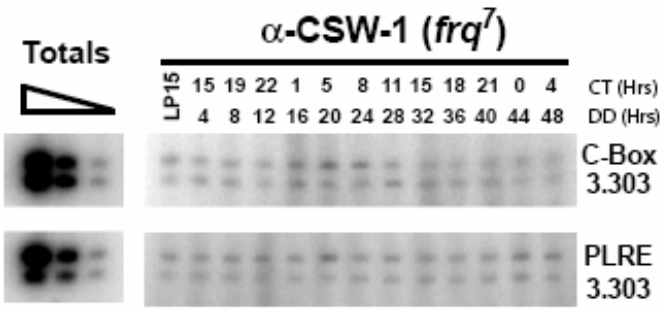
Loss of CSW-1 severely compromises rhythmicity in binding of WC-2 by preventing a return of the C-box to the unbound state,

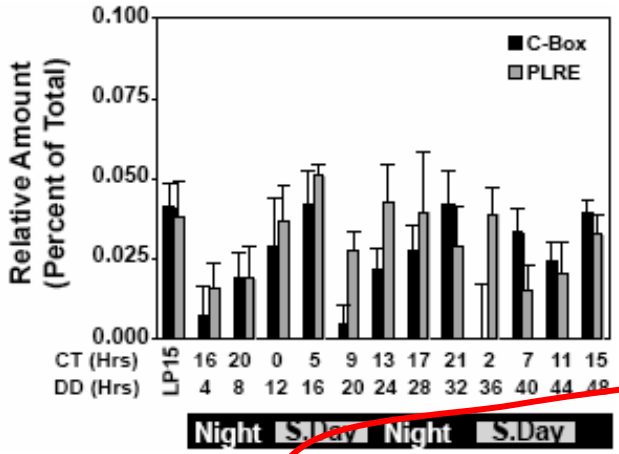
and reduces the magnitude of WC-1 binding.

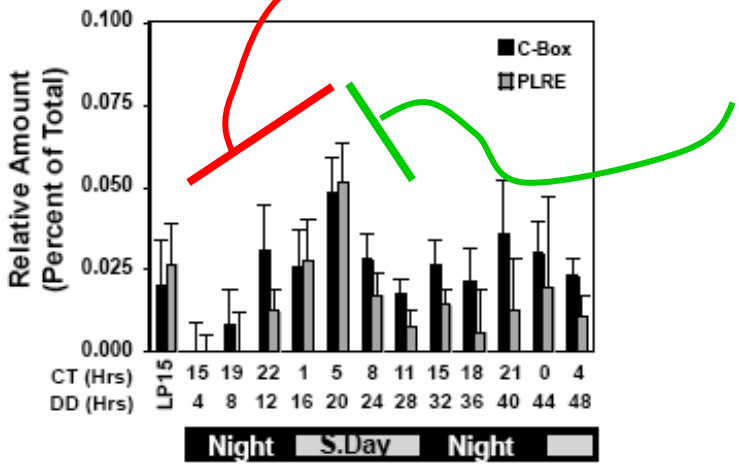
This suggests a model where CSW-1 is needed to help to eject WC-2 from the Clock-box to bring about the negative limb of the circadian feedback loop.

Consistent with this, CSW-1 binding to the C-box is lowest in the late night when *frq* transcription starts and steadily increases, peaking when the rate of transcription begins to decline







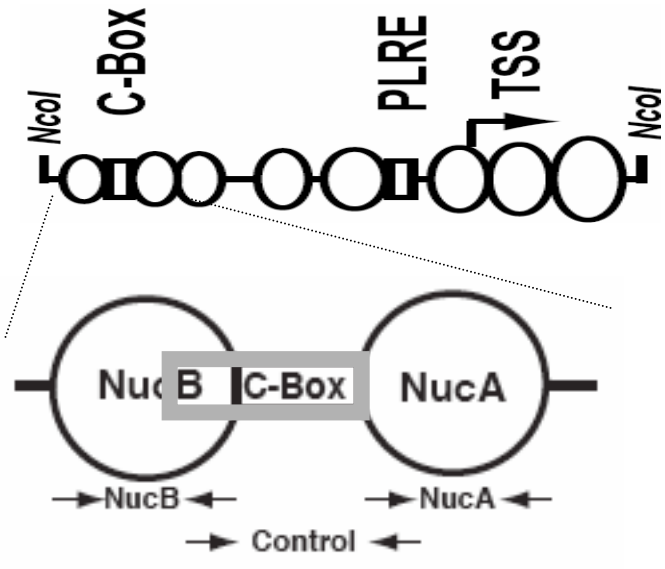


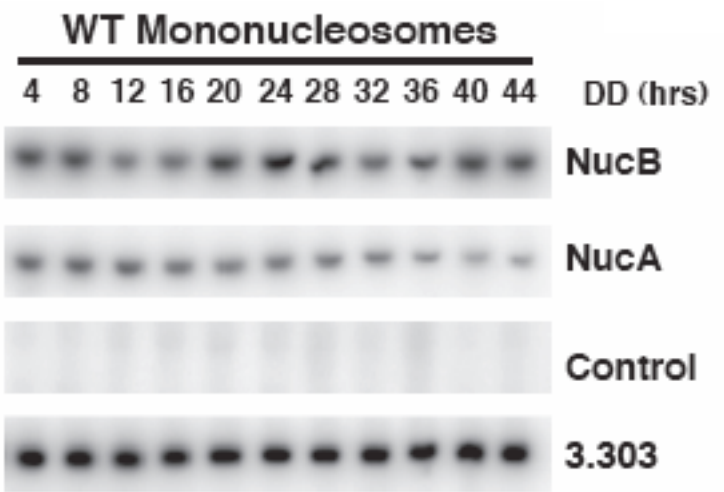
frq transcription increasing

frq transcription decreasing

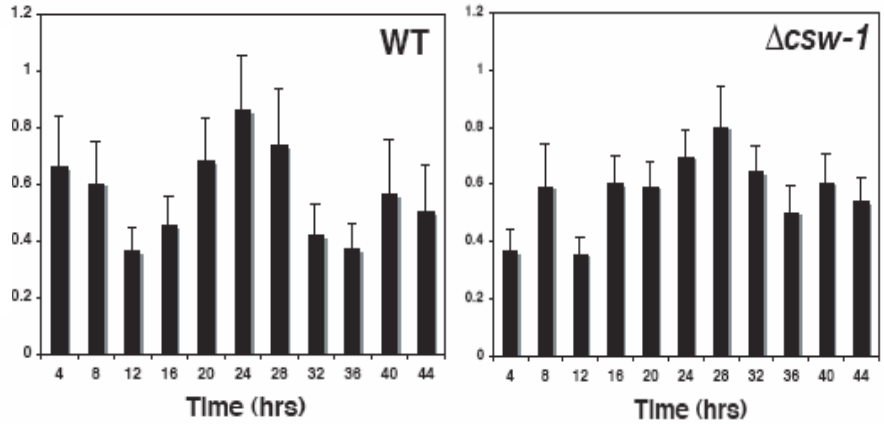
If this model is valid there should be rhythmic changes in nucleosome structure.

Limit nucleosome digestions show cyclical protection of the nucleosome next to the C-box





Relative amount of nucleosome B



Circadian remodeling of "nucleosome B" is consistent with cyclical opening of the C-box assisted by CSW-1

(limit nucleosome digestions: Isolate chromatin and digest to completion with micrococcal nuclease; only DNA tightly wrapped around the nucleosome core is protected.)

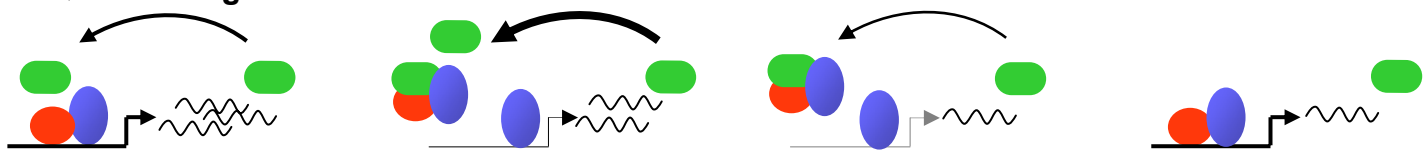
Molecular Events in the Neurospora Clock
(in constant darkness)

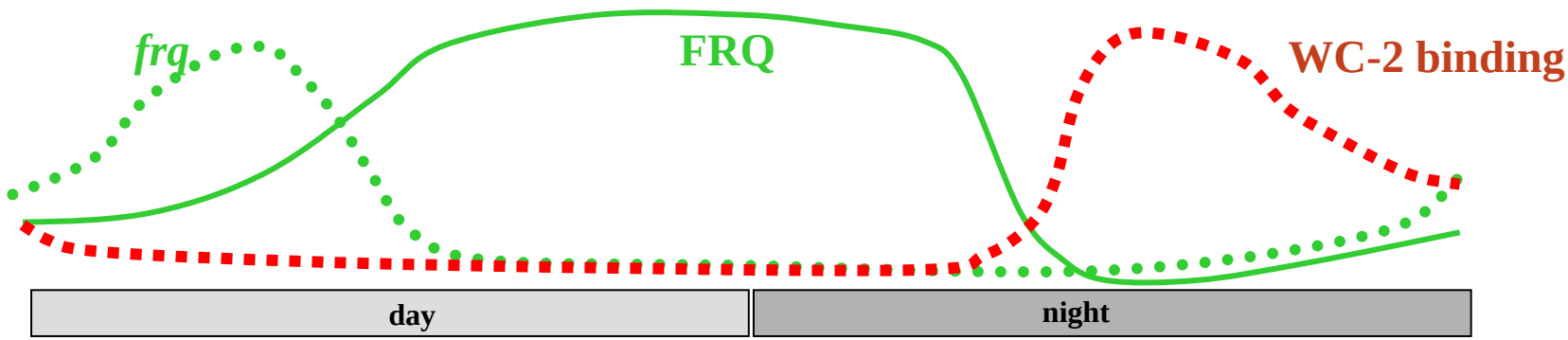
Midday ~CT6-10
- FRQ increasing to maximum
- WC-2 binding decreasing to minimum
- chromatin bound or in a closed state
- *frq* mRNA decreasing

Midnight ~CT18-22
- FRQ precipitously turns over and is low
- New WC-2 joins dephosphorylated WCC; binding to C-box rapidly increasing
- CSW-1 binding increasing; initiation of chromatin remodeling
- *frq* mRNA low, but increasing

Dawn ~CT0/24
- *frq* transcription high
- WC-1 and WC-2 bound as WCC
- WCC becoming phosphorylated causing activity to decrease
- FRQ increasing

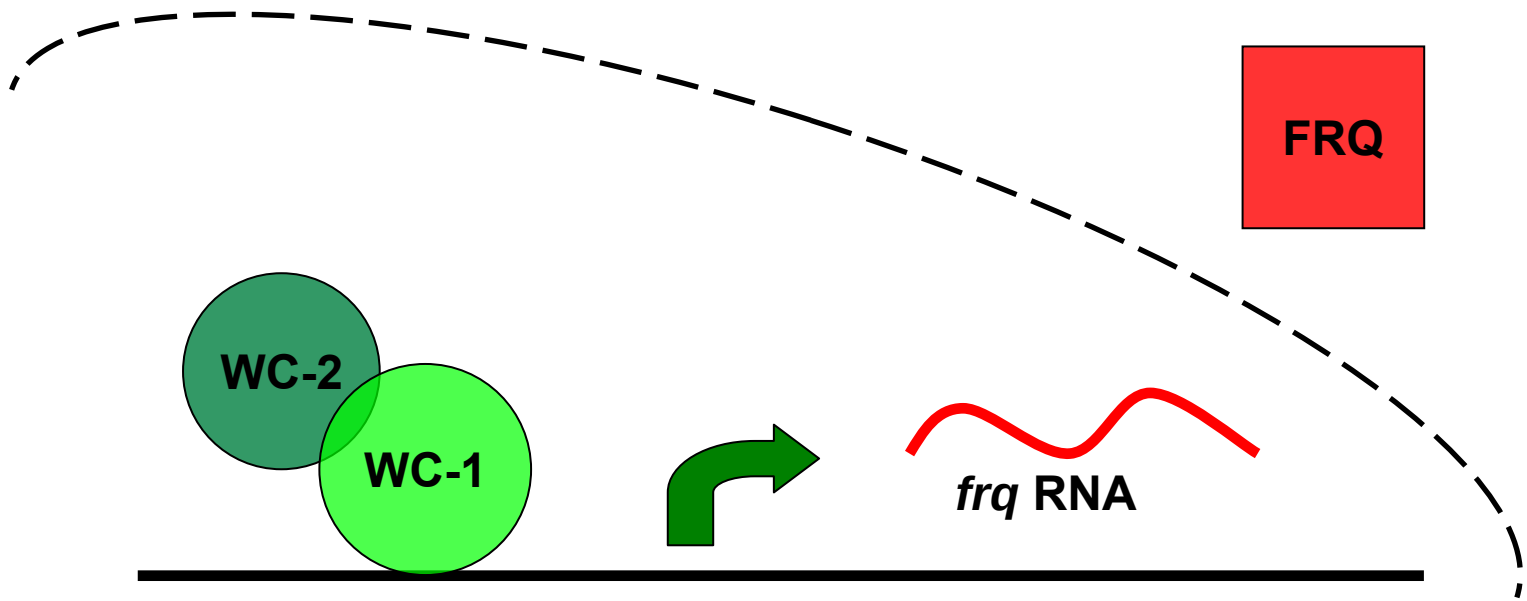
Dusk ~CT12
- *frq* mRNA low
- WC-2 binding low
- FRQ becoming phosphorylated and unstable





Simplified elements and dynamics of the *N. crassa* clock

And repeat every 22.5 h …



Circadian effects of temperature

Resetting and entrainment resulting from steps and pulses between temperatures within the physiological range (i.e. not heat shock)

- modeled as a result of temperature-dependent increases in [FRQ] (Liu et al, Science, 1998)

Temperature limits that are permissive for rhythmicity, within the physiological range of growth

-modeled as the result of temperature-dependent synthesis of two different isoforms of FRQ, a long and short form (Liu et al. Cell, 1997; Diernfellner et al Genes & Dev., 2005)

Temperature compensation of period length (perhaps an aspect of general compensation or homeostasis)

Proc Natl Acad Sci U S A. 1957 September 15; 43(9): 804–811. **ON THE MECHANISM OF TEMPERATURE INDEPENDENCE IN A BIOLOGICAL CLOCK*** J. Woodland Hastings and Beatrice M. Sweeney

"The experiments reported here describe the effect of temperature upon the luminescent rhythm. The results suggest that temperature independence is achieved by means of a compensation mechanism."

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

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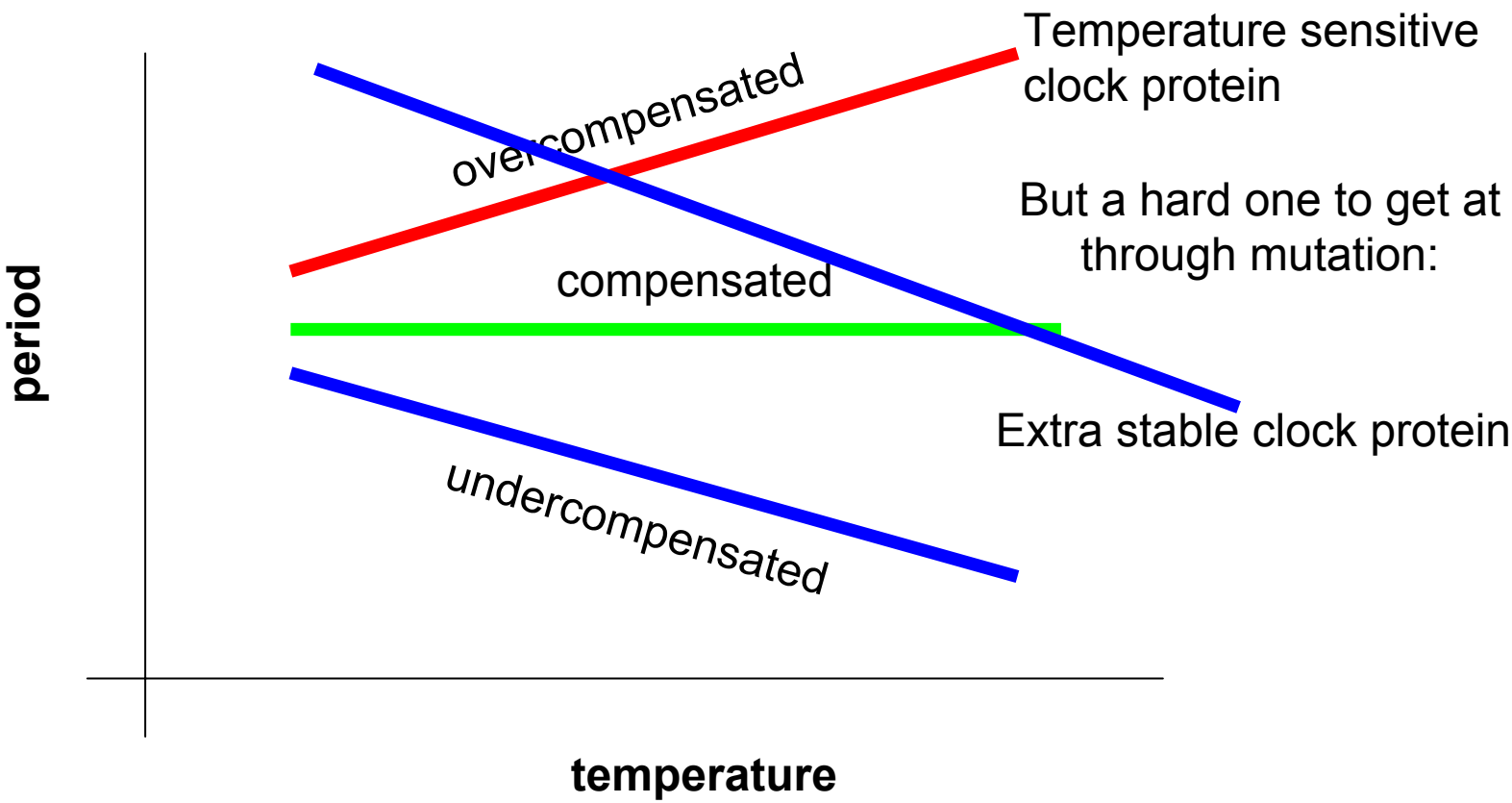
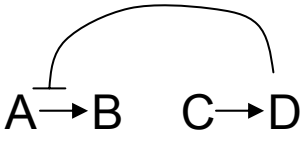
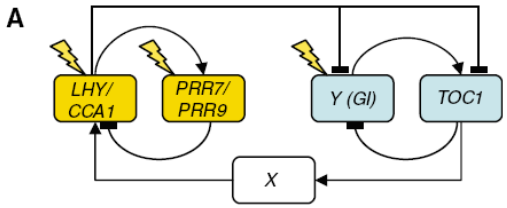
Even clocks in vertebrate cells in culture (NIH 3T3 cells, adipocytes, chick pinealocytes) and tissues (whole Xenopus or mouse retina) are compensated.

Temperature compensation (TC) is a defining clock property

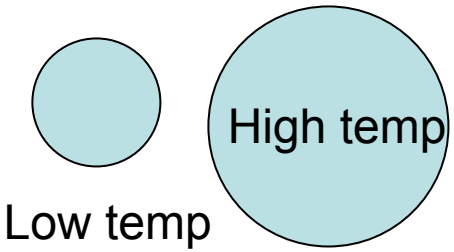


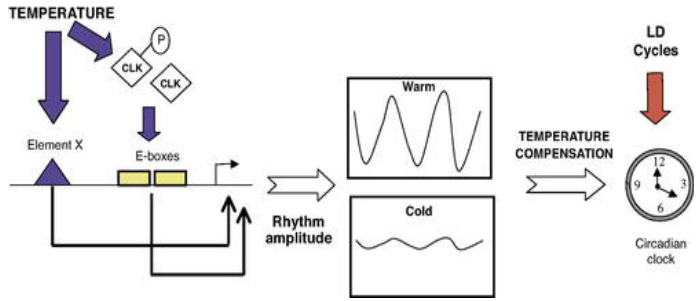
A variety of models and approaches have been used to understand compensation



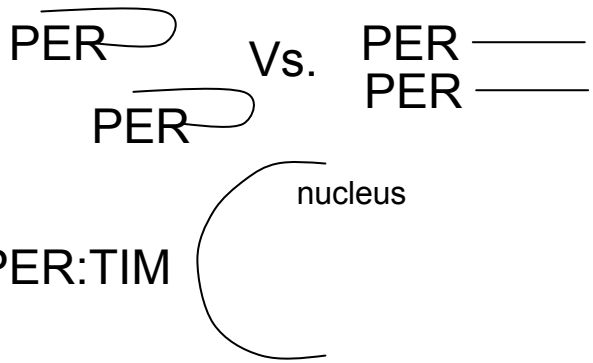


Feedback between loops in the *Arabidopsis* oscillator.(Locke *et al.*, Mol Sys Biol, 2006; Gould et al. 2006)





In zebrafish, increased temperature leads to increased amplitude of *per4* oscillation (From Lahiri *et al.*, PLoS Biology, 2005.)



Competing intramolecular vs intermolecular interactions. (Huang et al., 1995; Price, 1997)
(TG repeats - Sawyer et al. 1997)

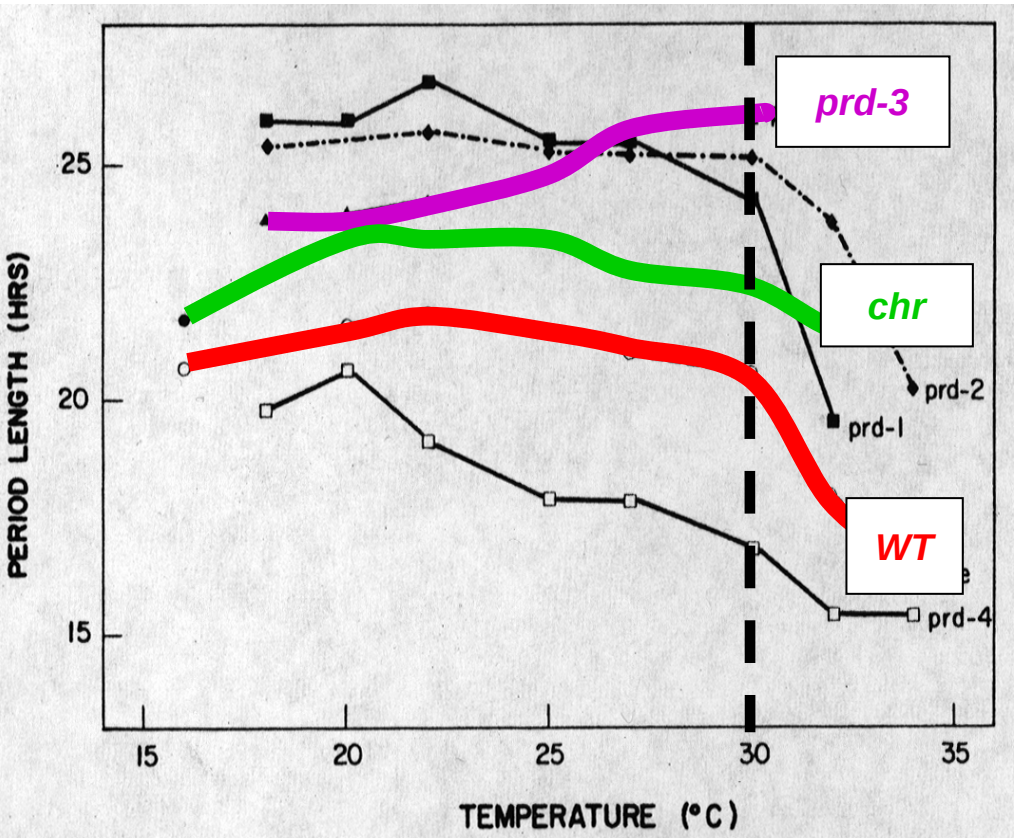
Opposing effects of dimerization (stable steady state) and nuclear entry (stable limit cycle). (Hong and Tyson, 1997; Hong, Conrad & Tyson, 2007)

$$\frac{d\ln P}{dT} = \frac{1}{RT^2}\sum_{i=1}^{N} C_i^P \cdot E_i = 0$$

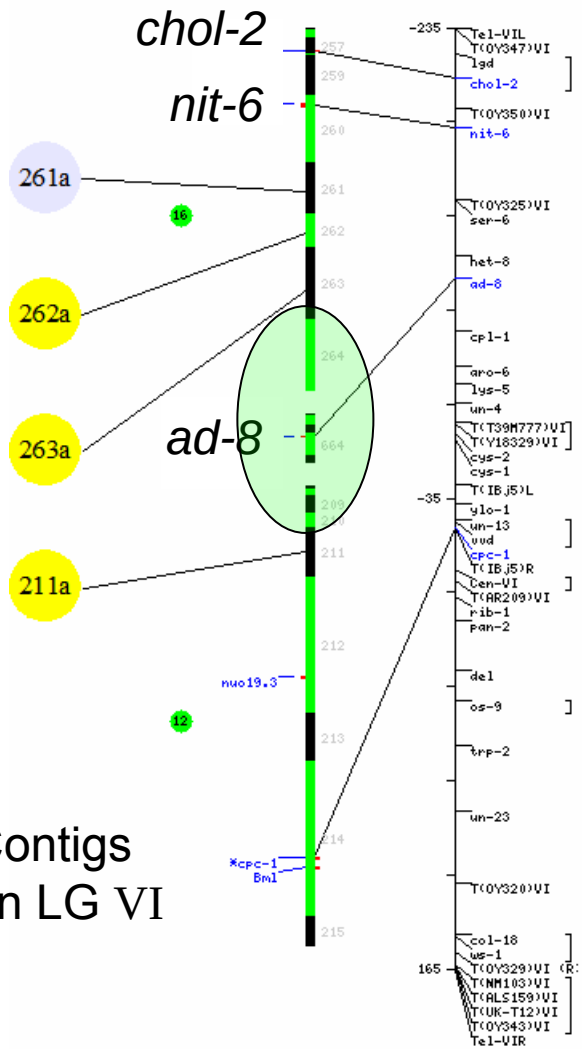
The cycle will the the summation of many reactions, and in the aggregate their control coefficients multiplied by their activation energies must sum to 0. (Ruoff et al., 2005)

Mutations exist in which compensation works better than in wild type, or in which there is even overcompensation.

So we decided to clone these to see if we could get at a mechanism for compensation



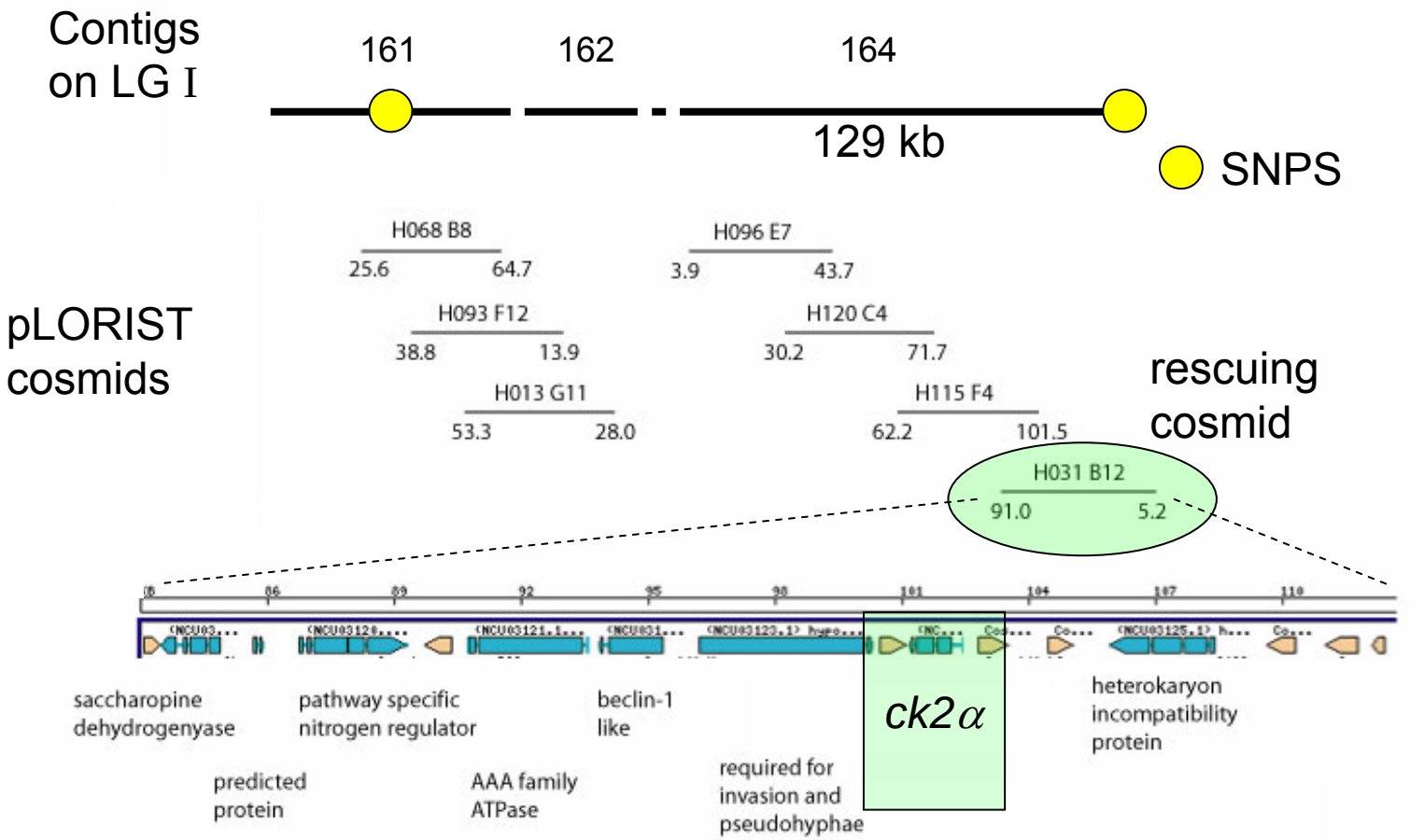
The β1 subunit of casein kinase 2 (*ckb-1*) was a candidate for ***chr***



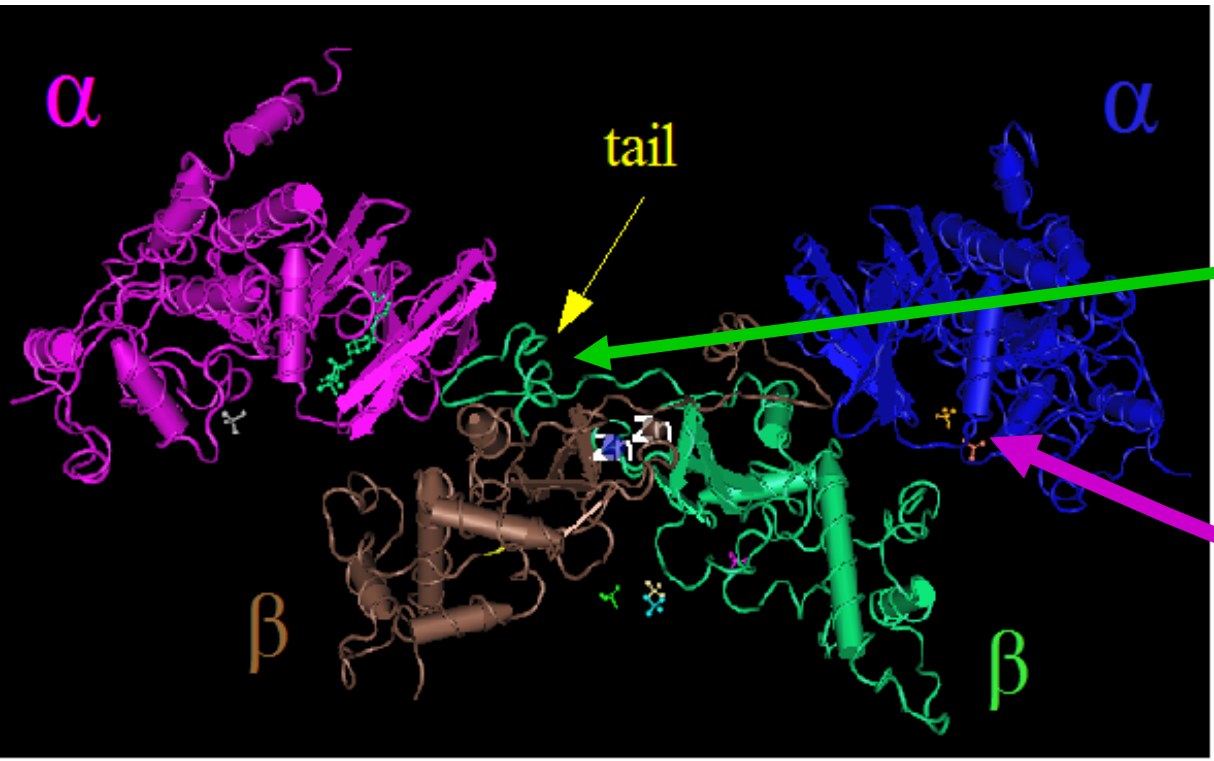
Contigs on LG VI

SNPS

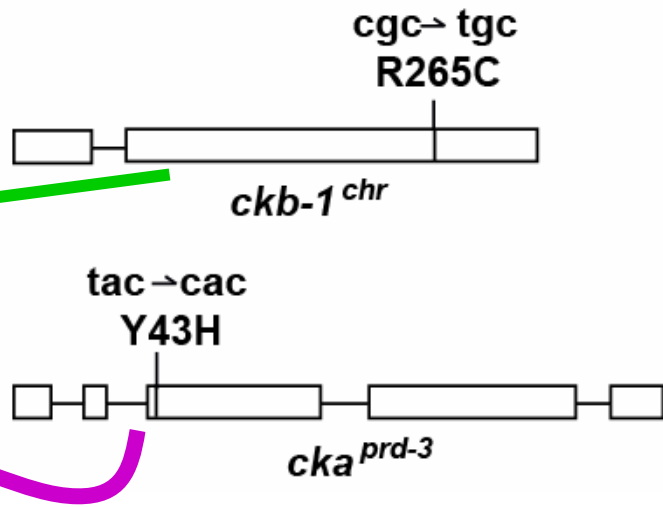
ck2α (*cka*) was identified as a candidate locus for ***prd-3***

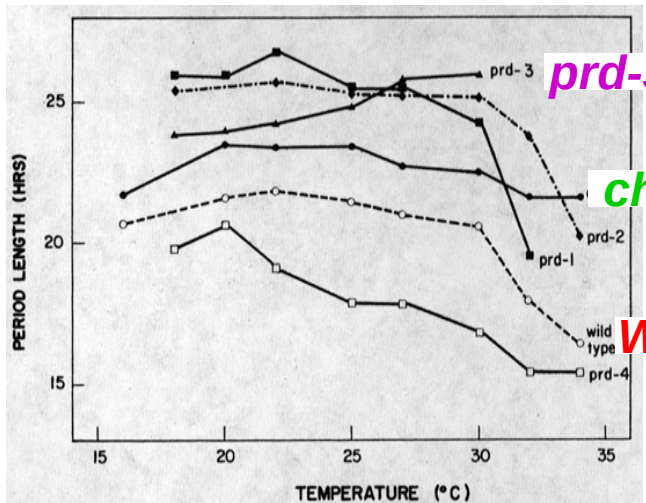


Casein kinase 2 (CK2) is a multifunctional heterotetramer



Tetramer of human casein kinase II.

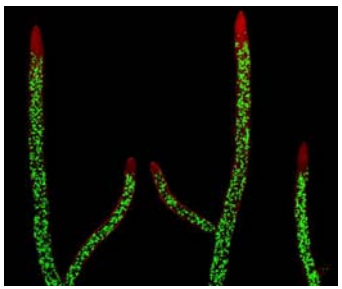




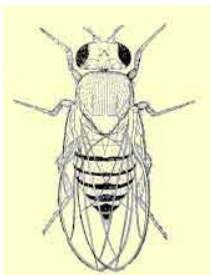
Independent mutations showing enhanced compensation identified distinct subunits of the same enzyme.

CK2 is well established as a clock affecting kinase

CKB3 associates with (in two-hybrid) and phosphorylates CCA1, overexpression of CKB3 shortens clock timing (E Tobin lab, 1999).

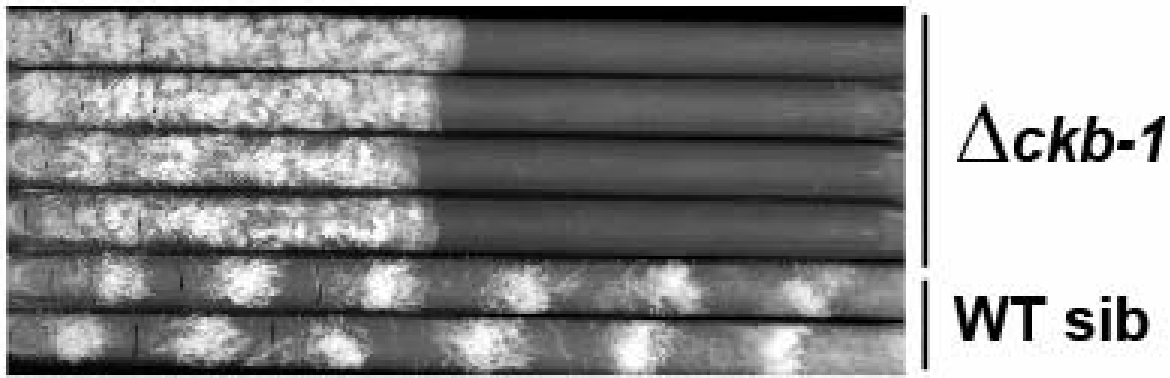


CK2 contributes to *in vitro* phosphorylation of FRQ; *ckb-1* repeat-induced mutation shows slowed FRQ rhythmicity (Y. Liu lab, 2002).



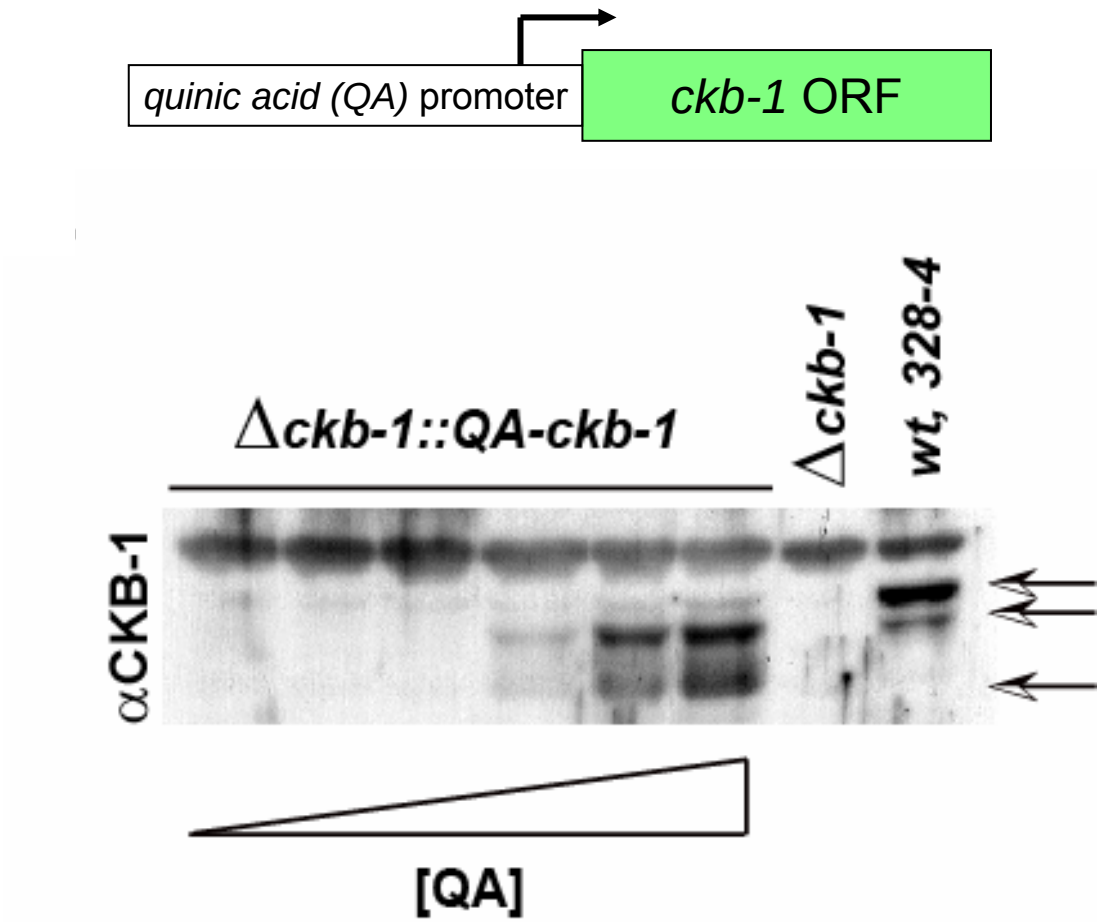
Two long period mutants, *Andante* and *Timekeeper* define CK2β and CK2α respectively; CK2α phosphorylates PER *in vitro*; PER nuclear entry is delayed in *Tik* (FR Jackson & R Allada labs, 2002-3).

Δ*ckb-1* is arrhythmic, while Δ*cka* spores failed to pass through meiosis

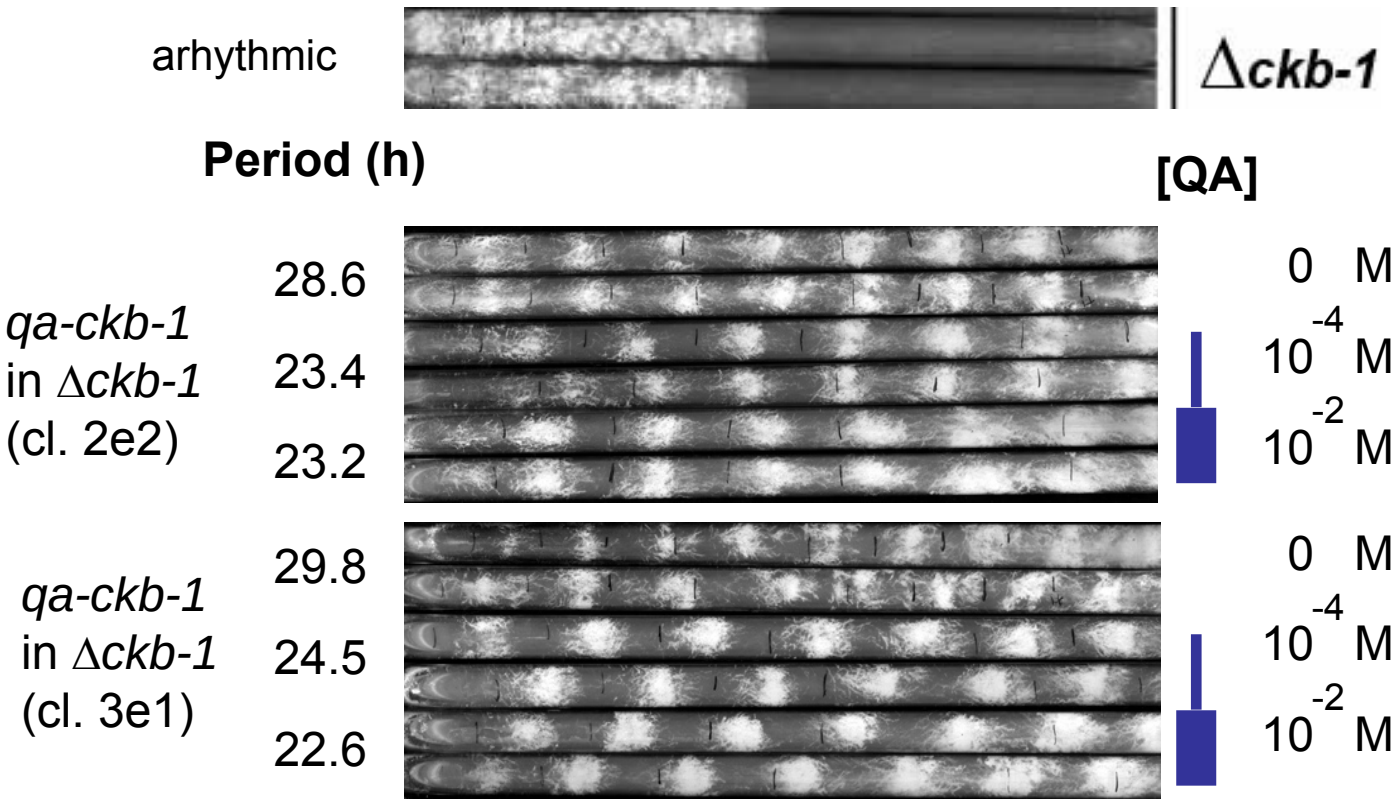


So this means we can look at the effect of **ckb-1** (=CK2) gene dosage on temperature compensation.

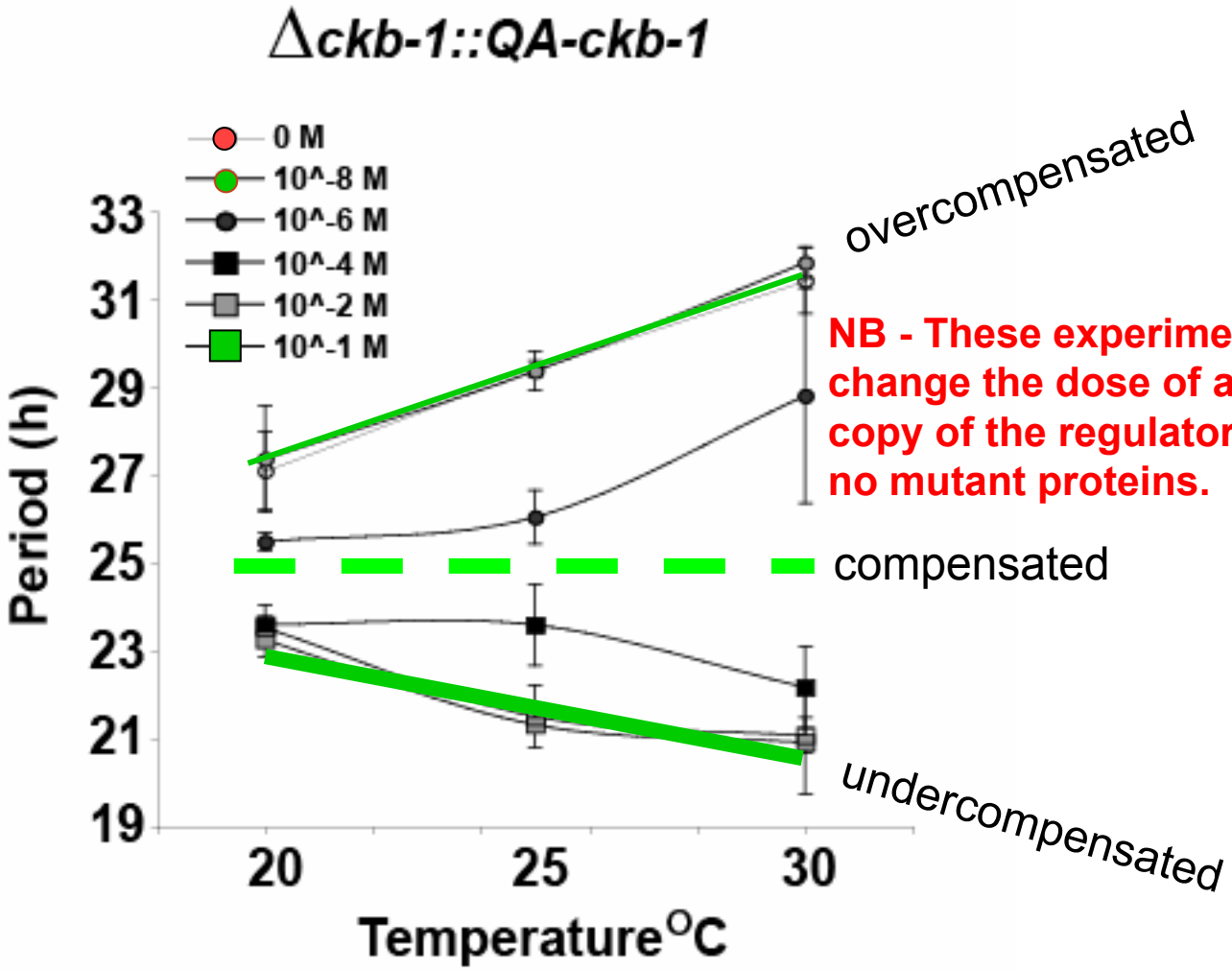
Dose dependent **CKB-1** production



In a Δ*ckb-1* background, increasing [**CKB-1**] first restores the rhythm and then reduces the period



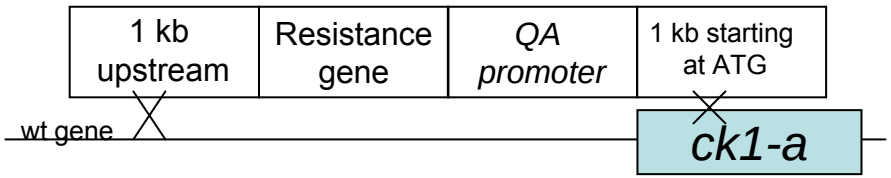
CKB-1 dose determines TC mode



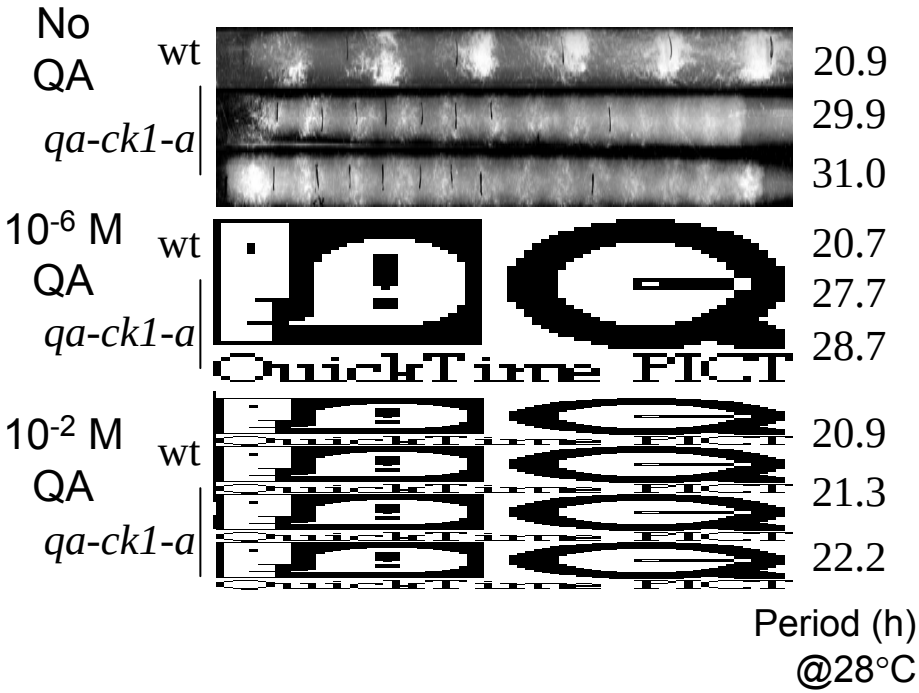
NB - These experiments only change the dose of a wild type copy of the regulatory subunit - no mutant proteins.

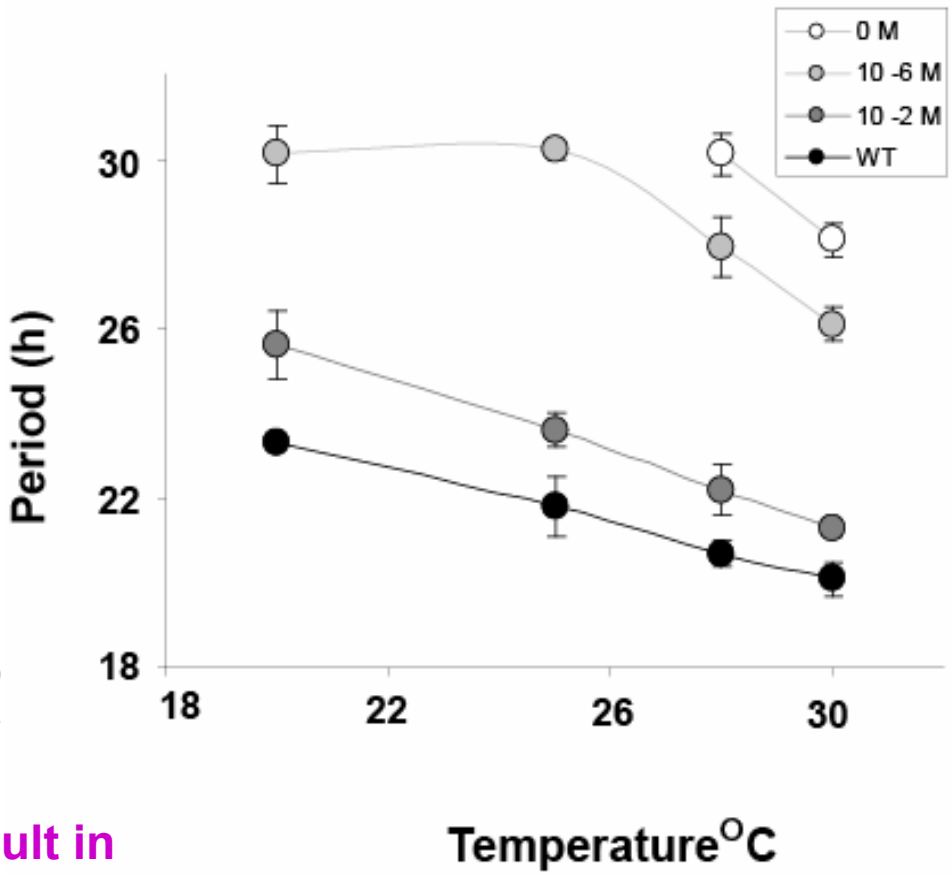
Well, perhaps anything that changes period length will affect compensation.

The dose of Casein Kinase 1 *(ck1-a)* affects period but not compensation



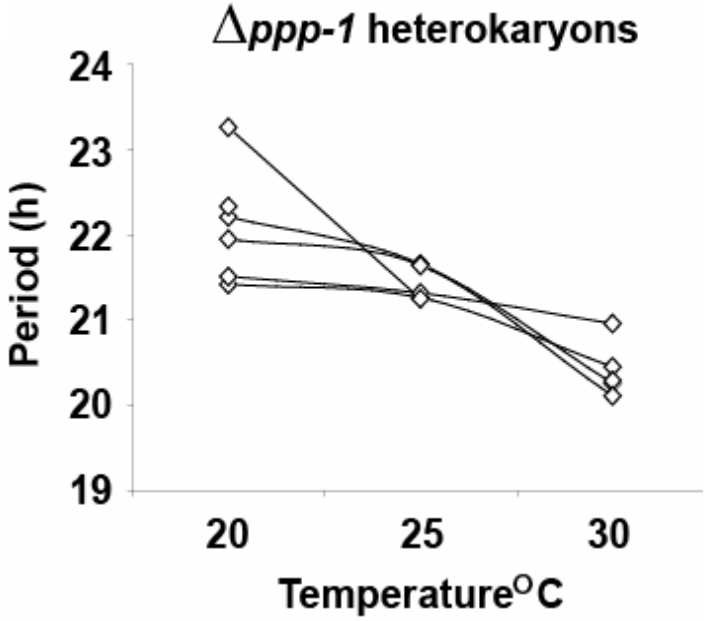
CK1-a interacts with FRQ, but no null mutants exist in the kinase. Since KOs in these kinases are likely lethal, Arun used a knock-in strategy - knocked in the QA promoter in front of CK1-a.





Low *ck1-a* dose results in normal undercompensation and does not result in anticompensation in the manner of low *ckb-1.*

Reduced dosage of two different phosphatases, PP1 and PP2A, does not affect compensation

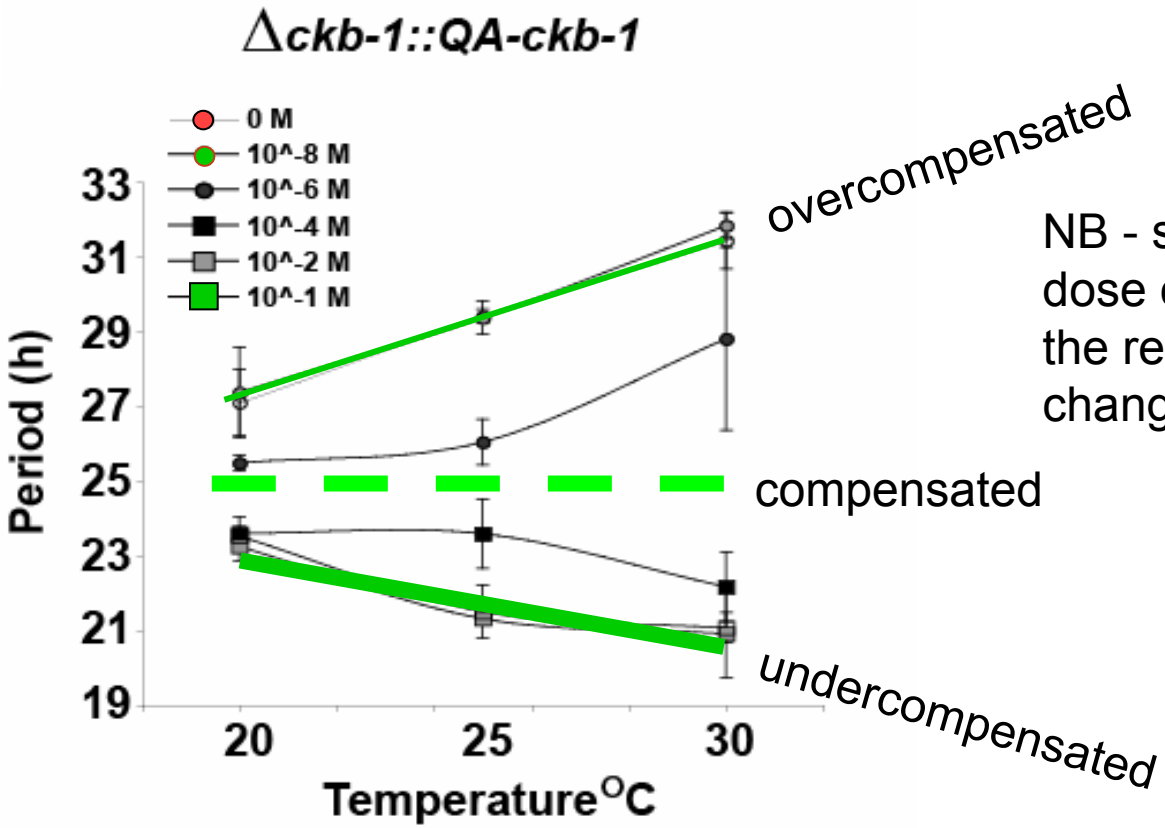


Deletion of

S/T protein phosphatase PP1 (NCU00043.2, *ppp-1*)- Δppp-1) (data on left)
and
S/T protein phosphatase PP2A catalytic subunit (NCU06630.2, *pph-1*) (not shown) showed no compensation phenotype when assessed as heterokaryons.

So it's not the case that changes in the dosage or activity of any modifying enzyme will affect compensation.

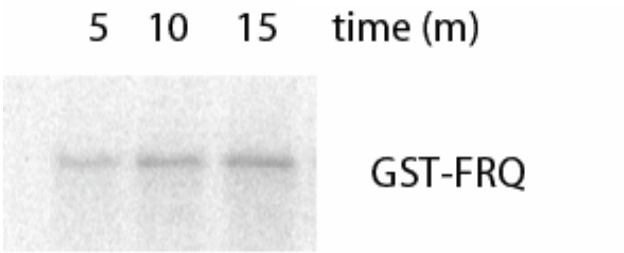
CKB-1 dose determines TC mode - i.e. casein kinase 2 is special



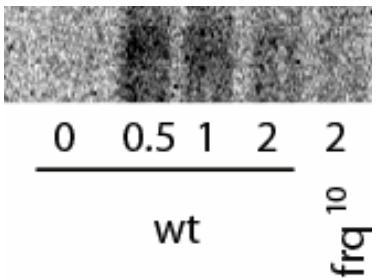
NB - simply changing the dose of a wild type copy of the regulatory subunit changes compensation.

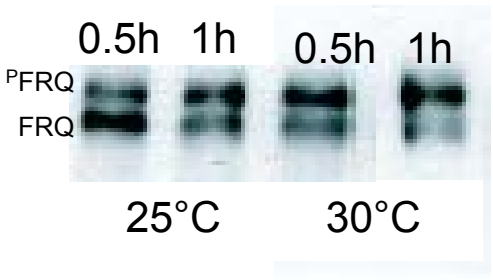
So - we've begun to focus more closely on CK2 activity as a function of dose and temperature.

CK2 directly phosphorylates FRQ

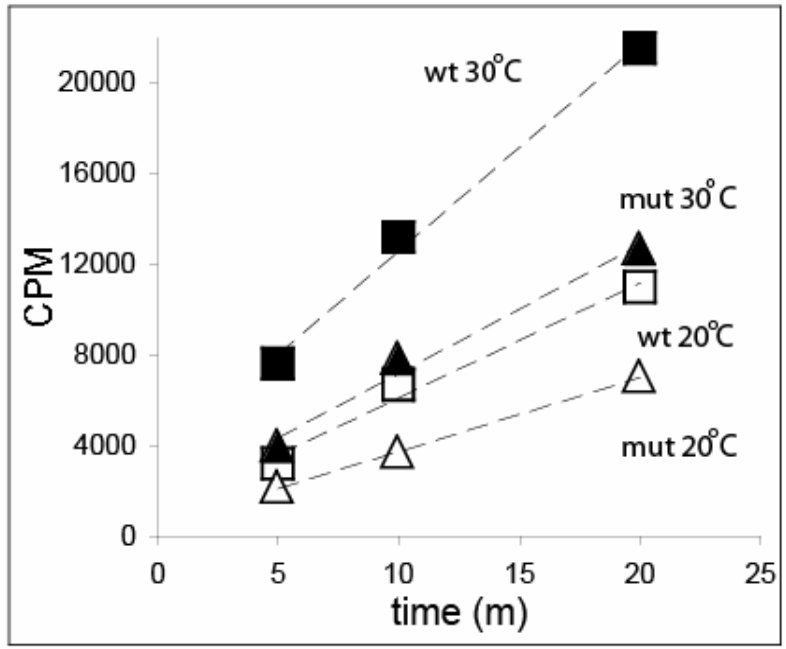


FRQ in extracts detected by FRH pull-down





CK2-specific phosphorylation of FRQ in whole cell extracts (by Western)

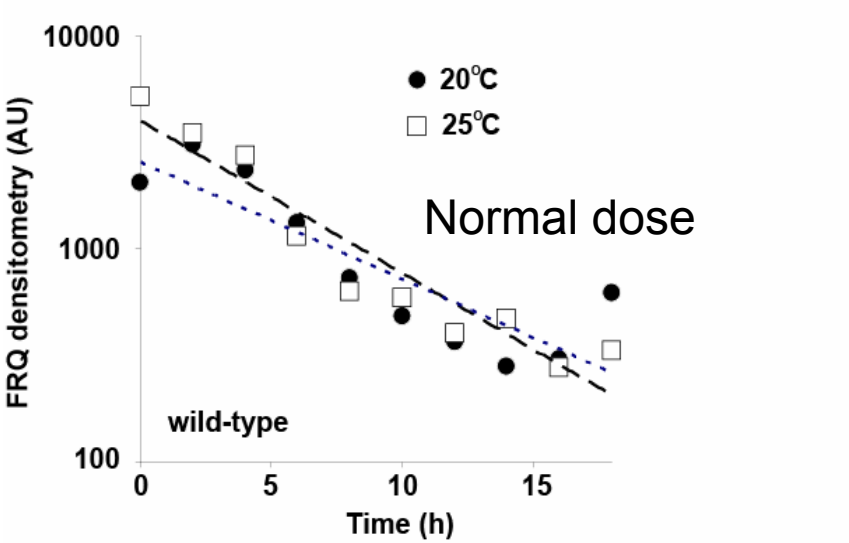


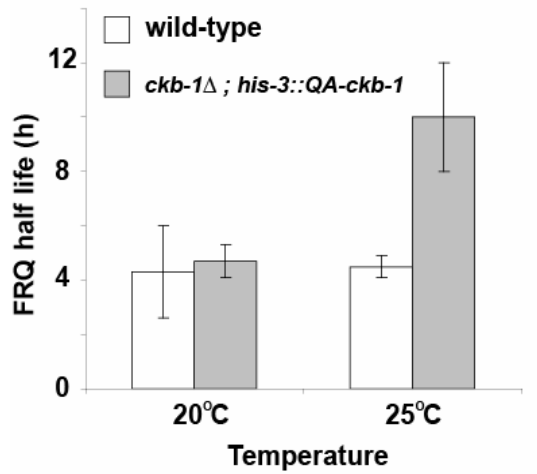
CK2 activity as assessed by an artificial peptide: Peptide phosphorylation;
mut = *his-3::QA-ckb-1, Δckb-1* with no QA

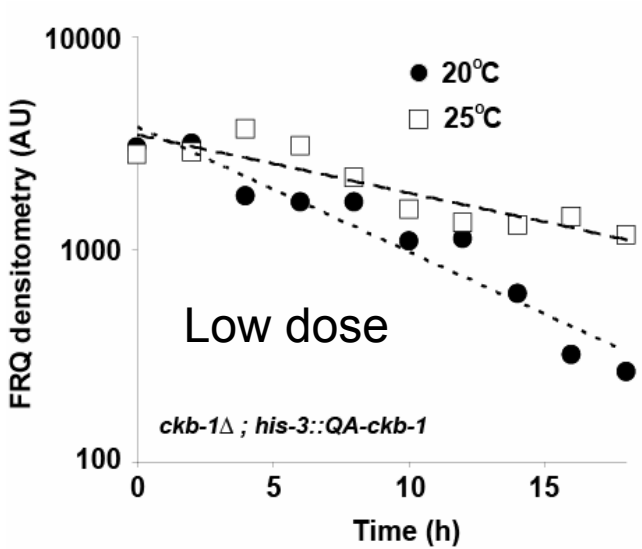
AND, even at low dosage, CK2 displays a normal temperature-activity profile;
i.e. CK2 phosphorylates FRQ more at higher temps.

But, even though CK2 activity increases at higher temperatures, at low [CKB-1],

FRQ degradation **decreases** *as a function of temperature*







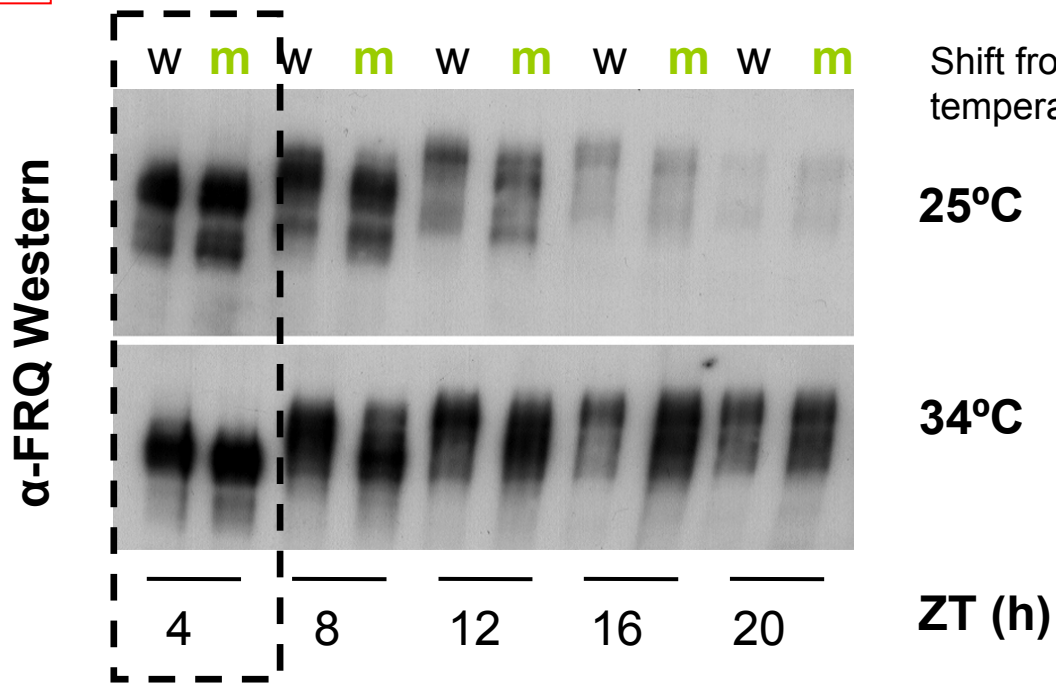
Since the kinetics of FRQ turnover are a major determinant of period length, these data are consistent with the observation of increasing period as a function of temperature (i.e. overcompensation) at low [CK2].

And they recapitulate the *chr* phenotype.

chr mutation in CK2 inefficiently phosphorylates FRQ thereby slowing degradation- and increasing period

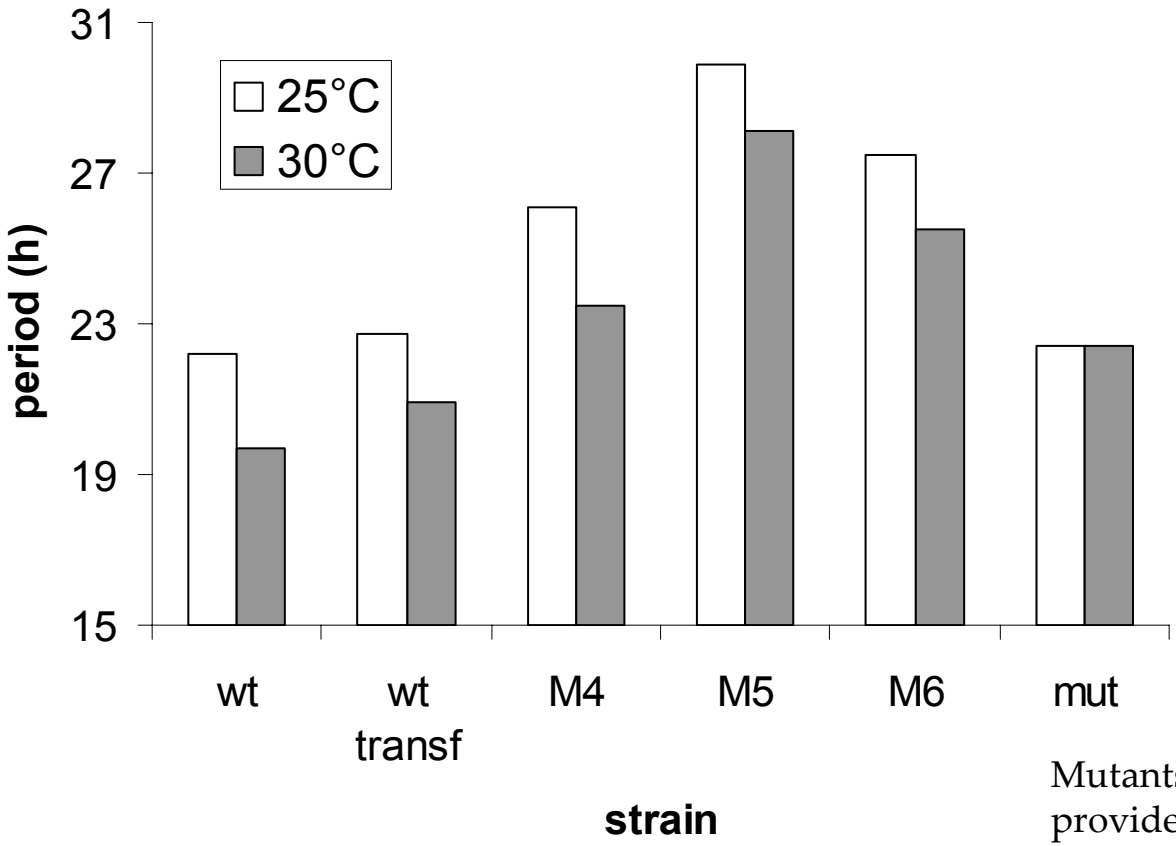
w = wild type
m = *chr*

This effect is exacerbated with increasing temperature.



This implies the existence of more than one class of CK2 phosphorylation sites on FRQ, one of which might specifically affect turnover at higher temperatures.

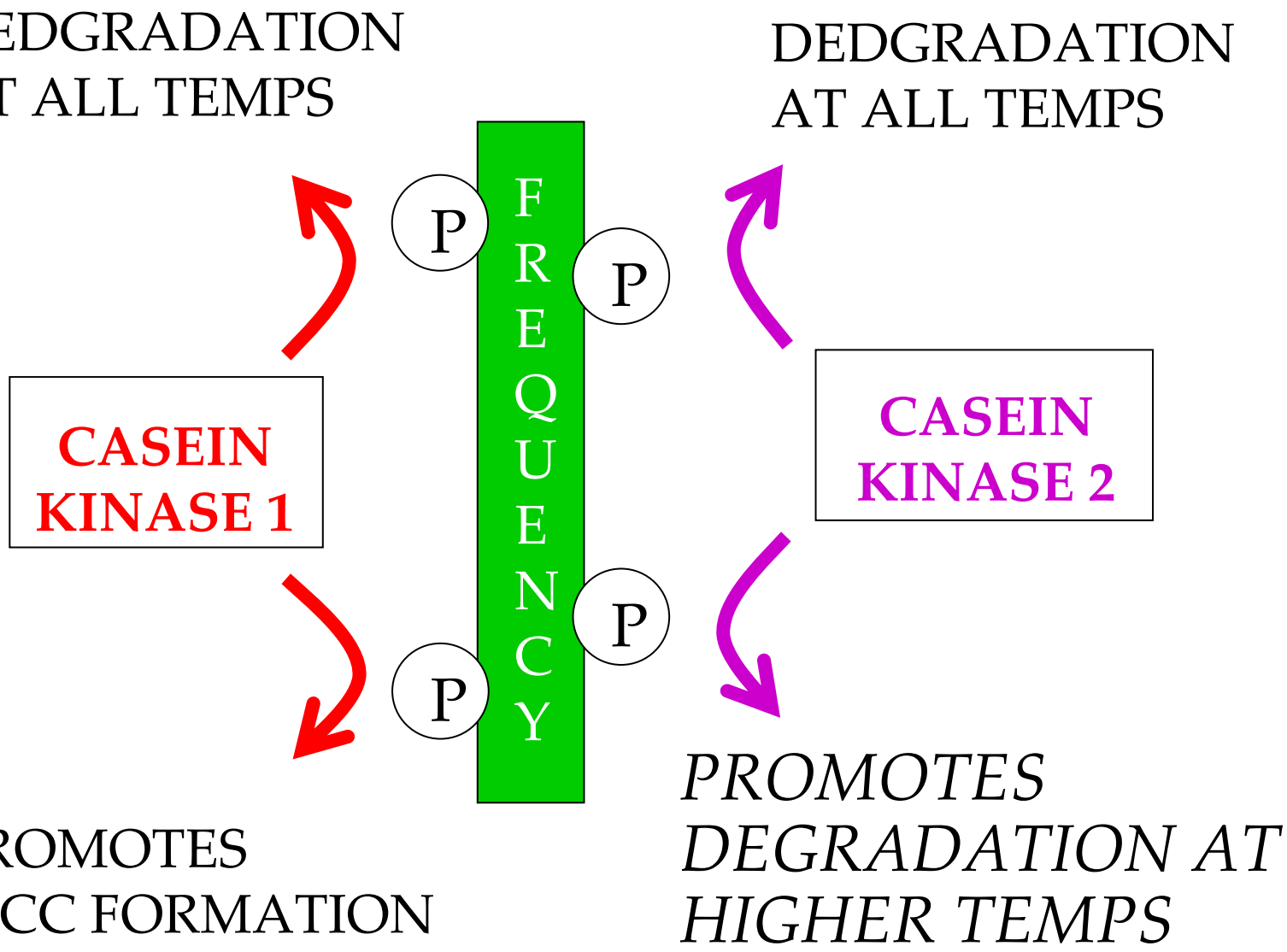
So, we searched among existing and novel (i.e. newly engineered) phosphorylation site mutants in FRQ, and found that mutation of a putative CK2 phosphosite in FRQ partially phenocopies *chr*.



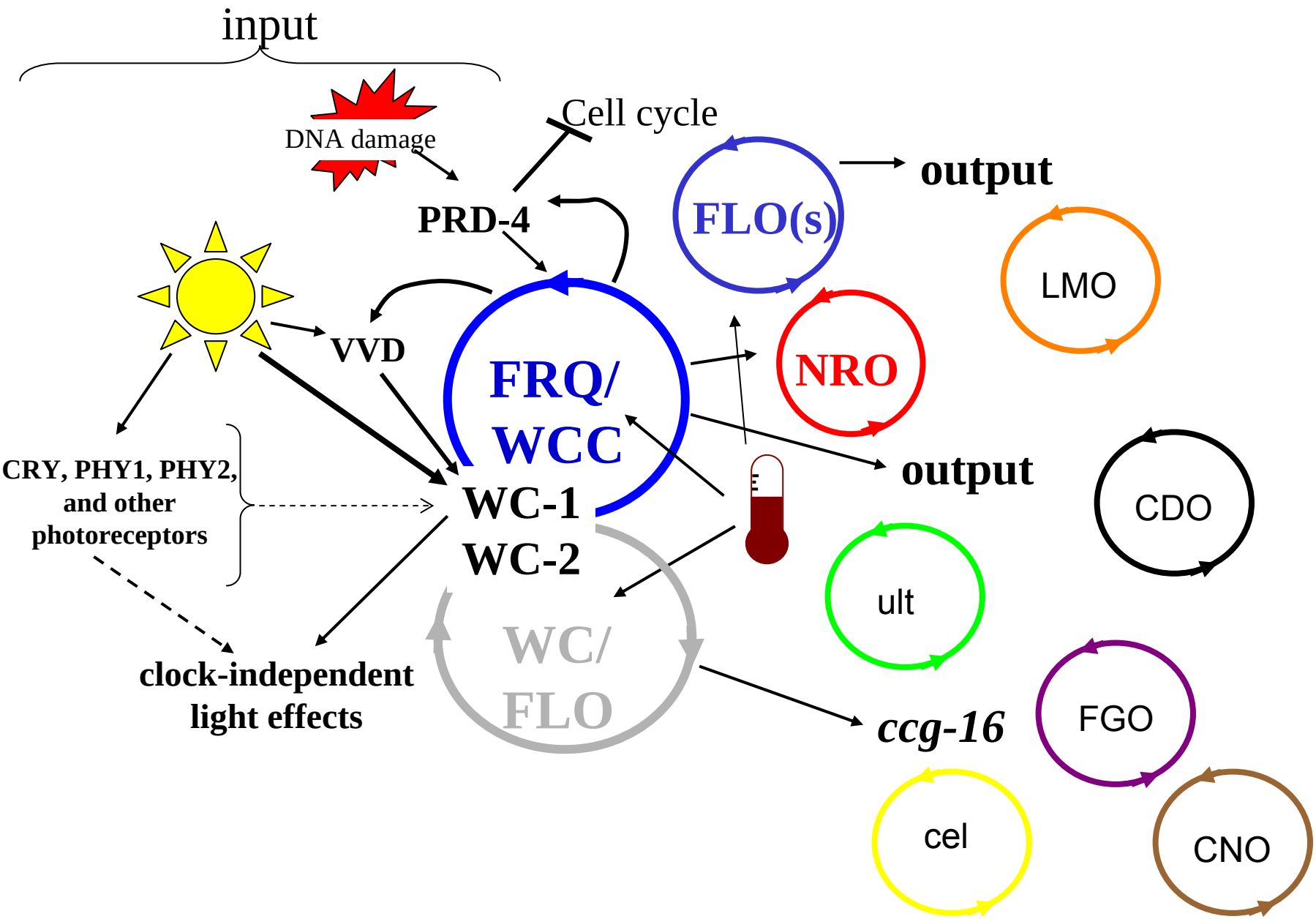
Mutants M4-M6 kindly provided by Y. Liu.

Working Model

FRQ has a number of phosphorylation sites, and phosphorylation of different sites can lead to or promote distinct consequences: *e.g.* degradation, WCC formation and degradation in a temperature-dependent manner.



The Cellular Circadian System in Neurospora



FLO
NRO
WC/FLO
cel
CDO
LMO
CNO
FGO
ult

-all have, or with manipulation can be made to have, period lengths within the circadian range

- all are distinguishable based on period, medium conditions, or other conditions

- only the NRO and WC/FLO are known to be connected to the FRQ/WC circadian feedback loop

- only the NRO and WC/FLO can be followed by any means other than race tubes

A cellular circadian clock controls an overt rhythm in developmental potential in Neurospora.

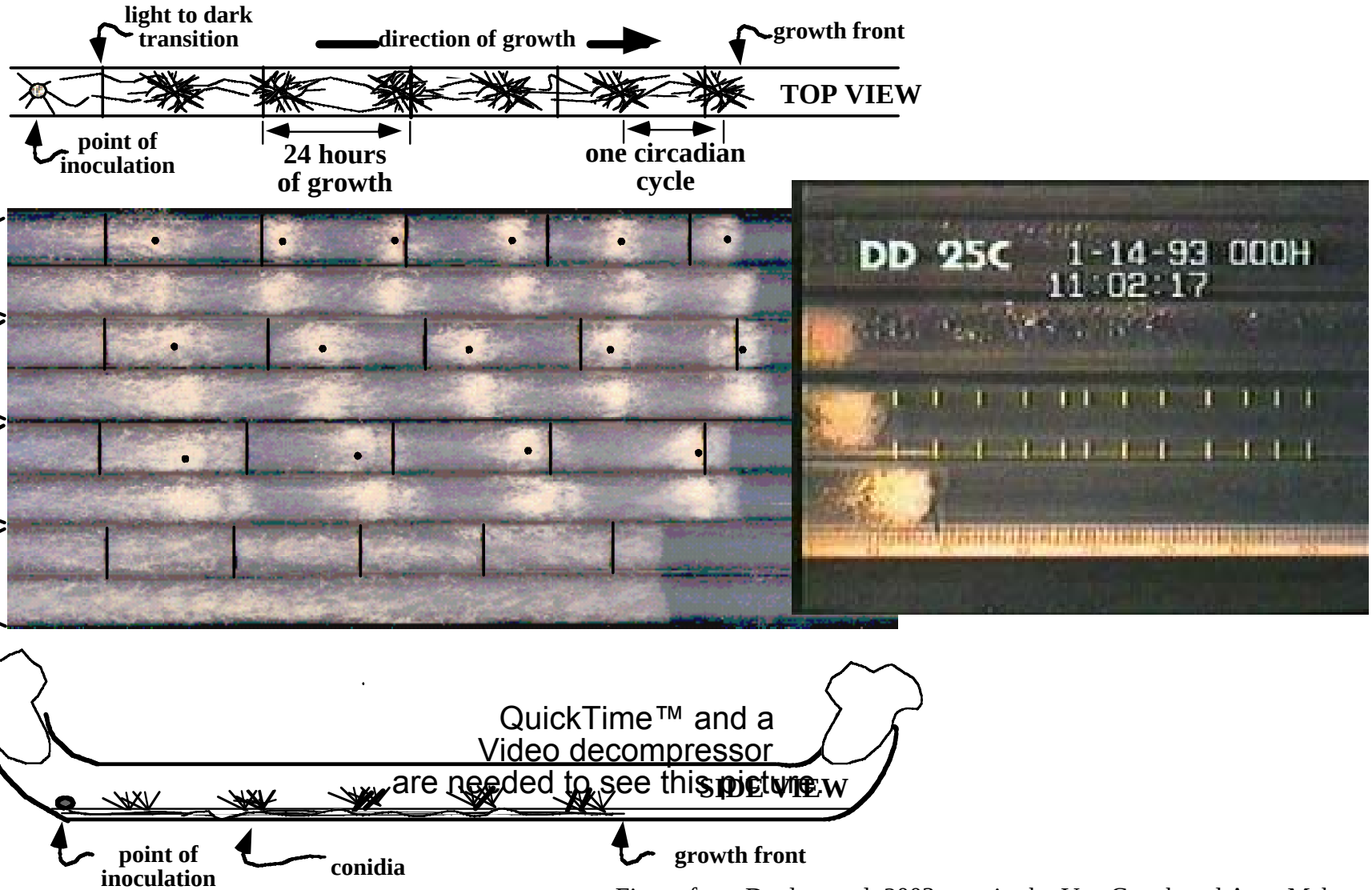
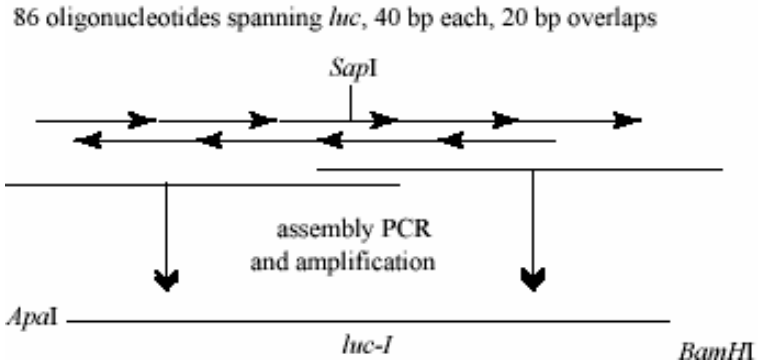
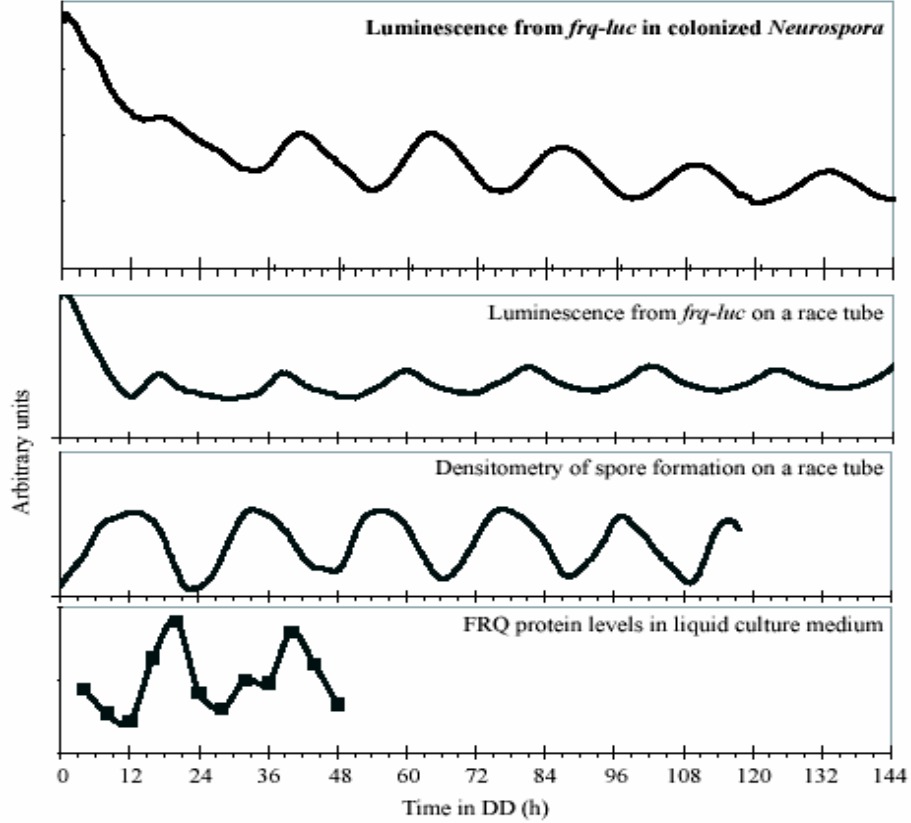
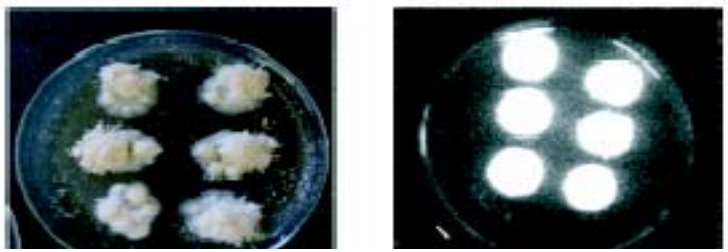


Figure from Dunlap et al, 2003; movies by Van Gooch and Arun Mehra

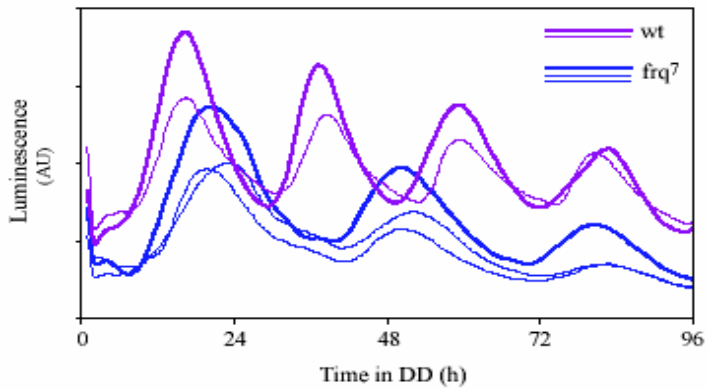






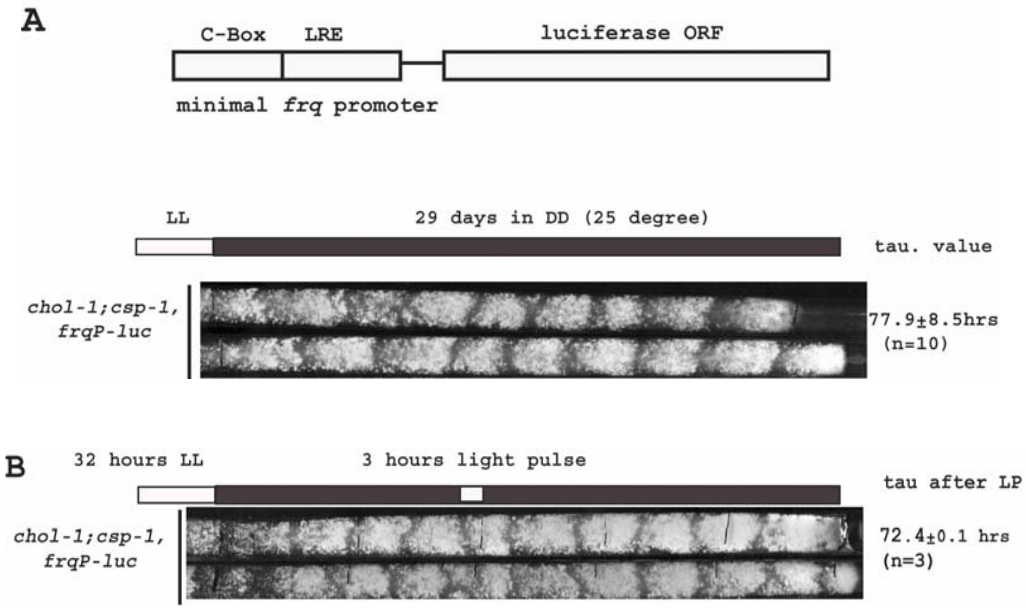
Strain Heterokaryon Homokaryon

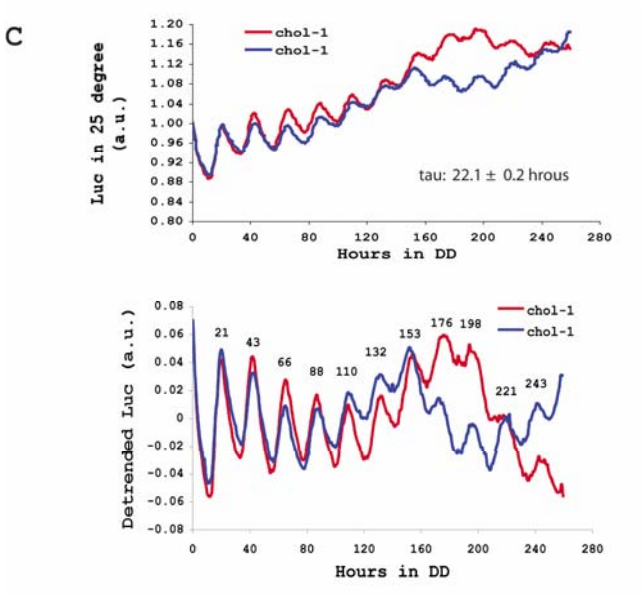
eas (*ccg-2*)-driven, partially optimized (36)[1]	0.007, 0.001	ND
eas (*ccg-2*)-*luc-I*[2]	53, 37	137, 38
eas (*ccg-2*)-*luc*[3]	87	63
frq-luc-I[4]	0.6, 0.26	1.0, 0.58

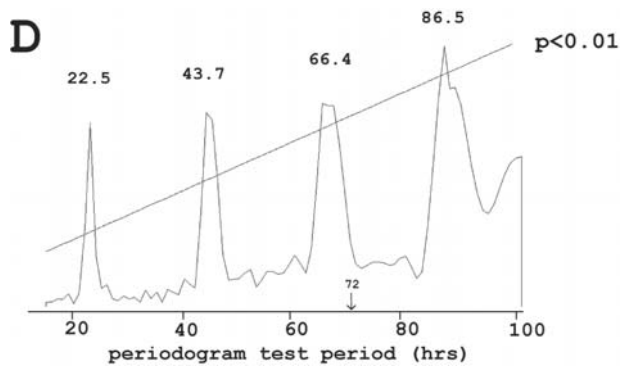


QuickTime™ and a
Sorenson Video decompressor
are needed to see this picture.

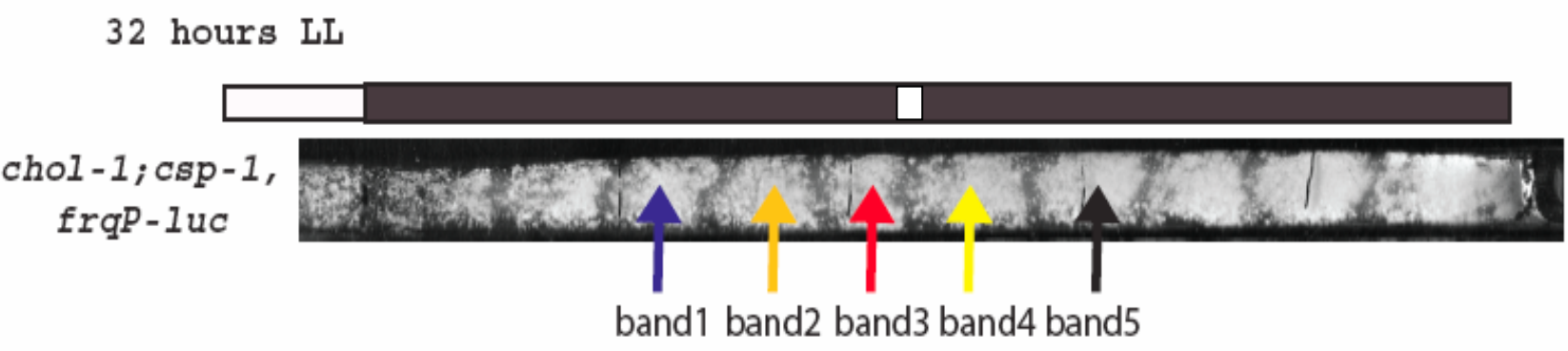
Circadian rhythms in luciferase from a strain exhibiting the long period choline-starvation rhythm





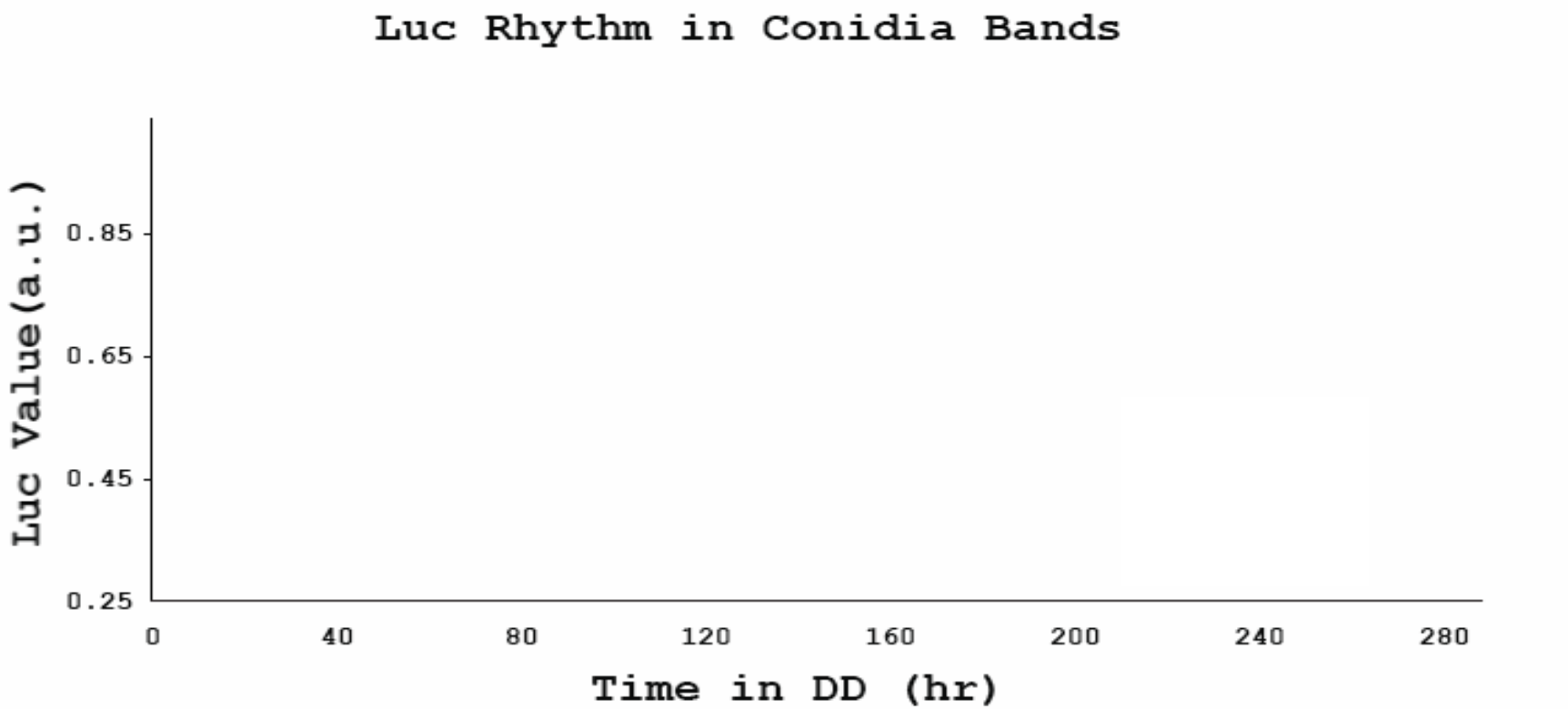


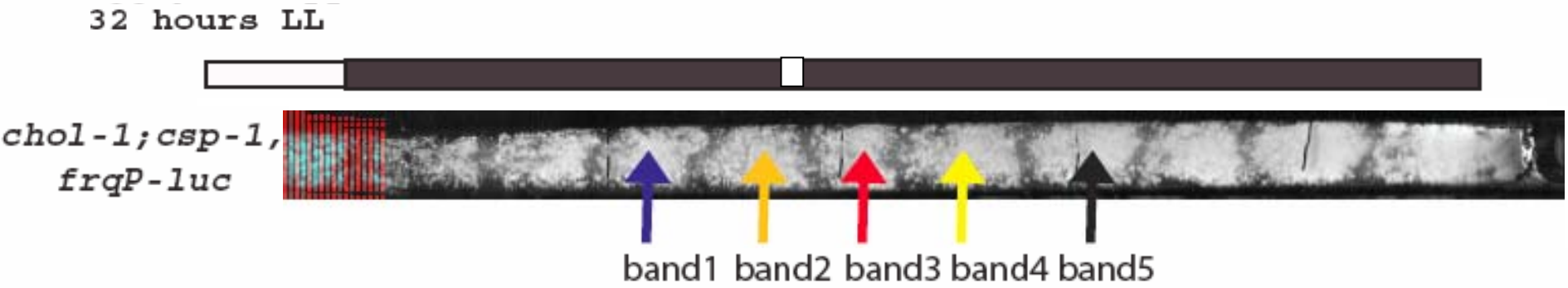
Luciferase construct under control of the *frq* promoter (panel A) was introduced into a *chol-1* strain. The strain showed a CDO rhythm. A light pulse was given in a race tube culture to synchronize the rhythms (panel B), and luciferase from the whole race tube was recorded for 260 hours (panel C). The top chart is the original data and the bottom one is detrended. The periodogram analysis of the luc rhythm only showed a period at 22.5 hours.



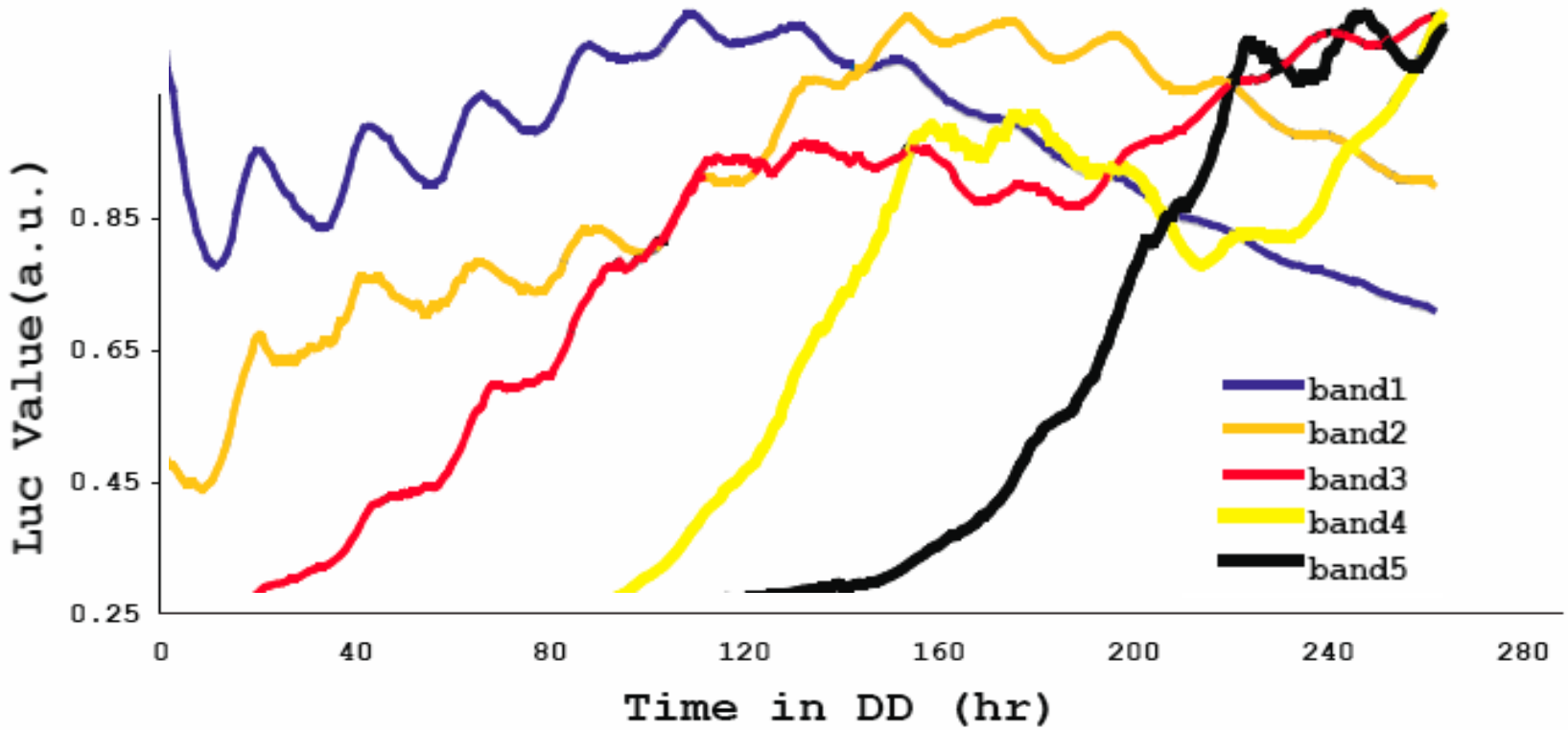
Period of conidiation rhythm = 72.9 ± 7 hrs

Give light after 292 hours, and then follow bioluminescence from each location.

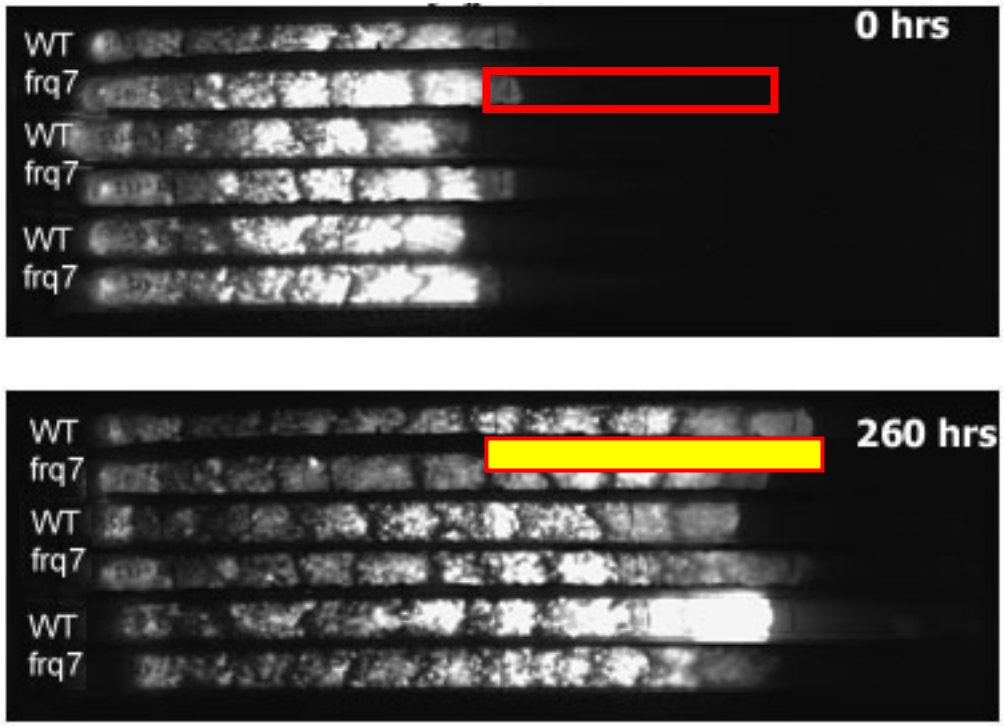




Give light after 292 hours, and then follow bioluminescence from each location.



his-3::frq p-luc, chol-1

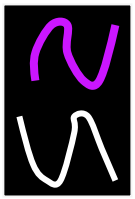


his-3*; *frq^{7}::frq p-luc, chol-1

As the culture grows down the rest of the race tube expressing the ~72 hr banding rhythm, we're going to follow *frq* expression by luciferase.

Simultaneous expression of the conidial banding rhythm and the FRQ/WC oscillator in real time

Conidial banding



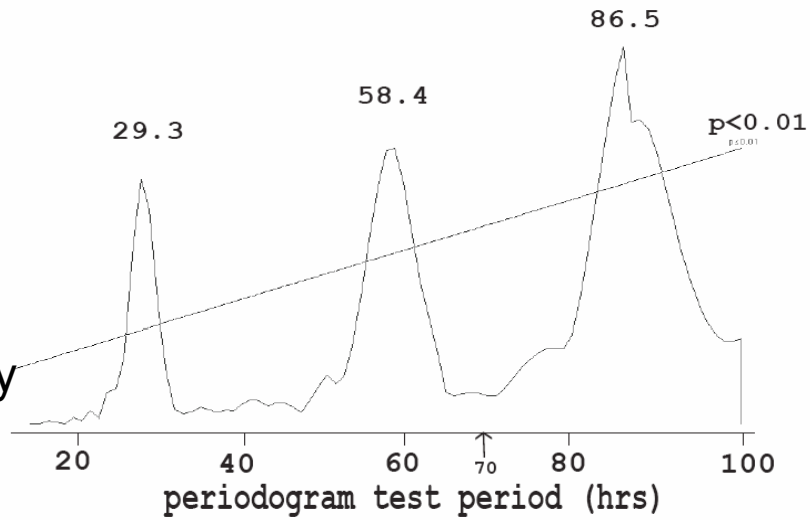
Luciferase

QuickTime™ and a
decompressor
are needed to see this picture.

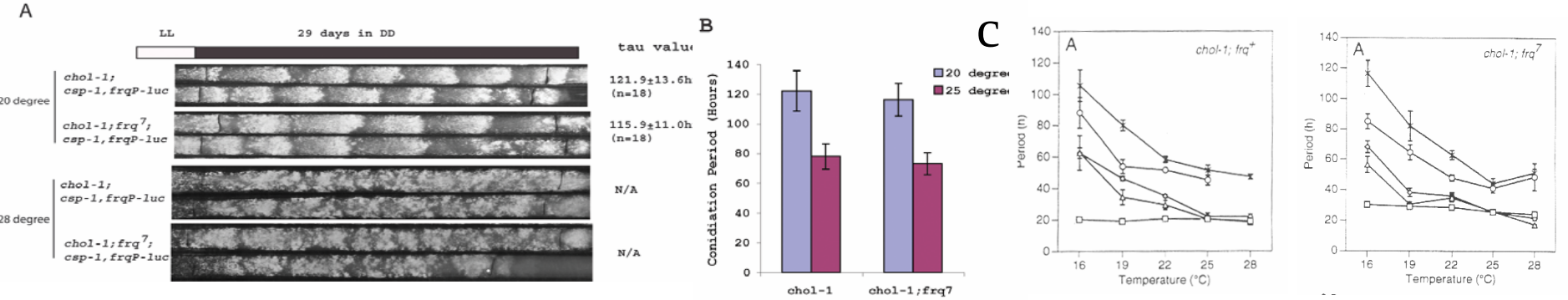
The two rhythms can be seen to run along independently of one another

his-3*; *frq*^{7}*::frq p-luc, chol-1

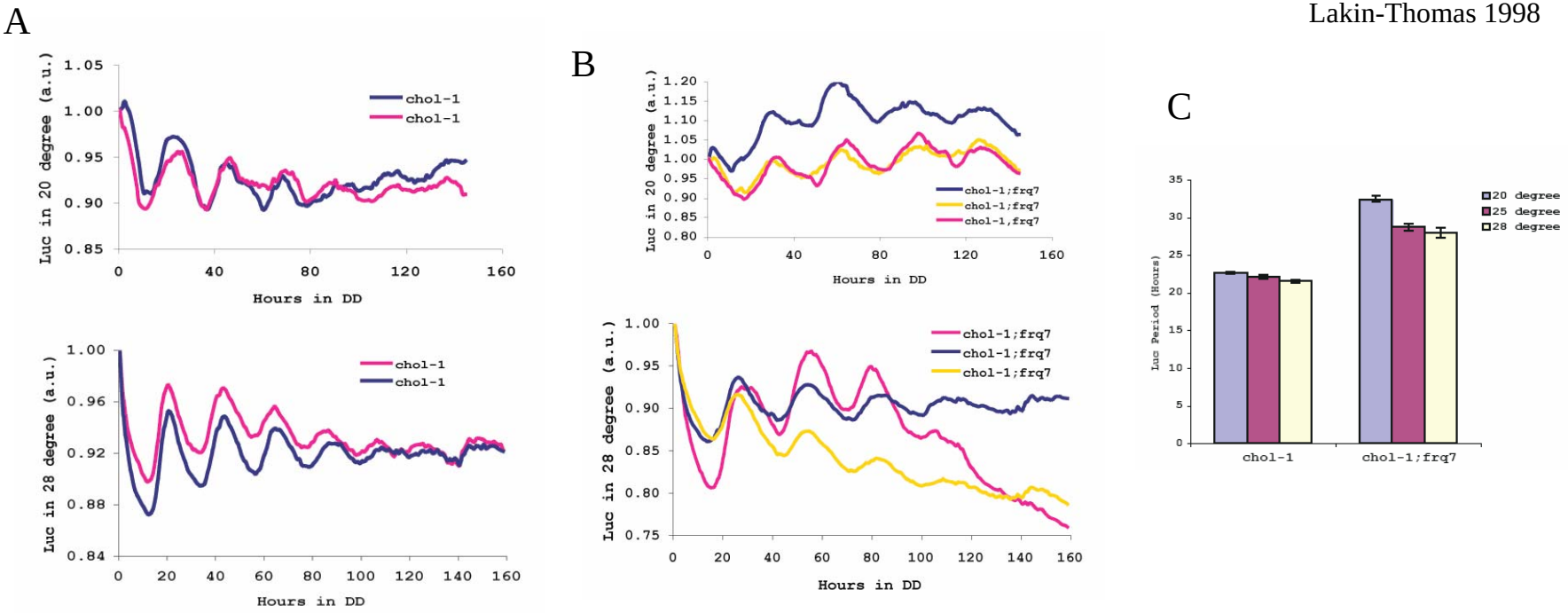
And this is consistent with periodogram analysis of the luciferase data, which shows no apparent contribution from the 72 hour conidiation rhythm elicited by choline starvation.



CDO is not temperature compensated whereas the *frq*-luc rhythm is compensated.

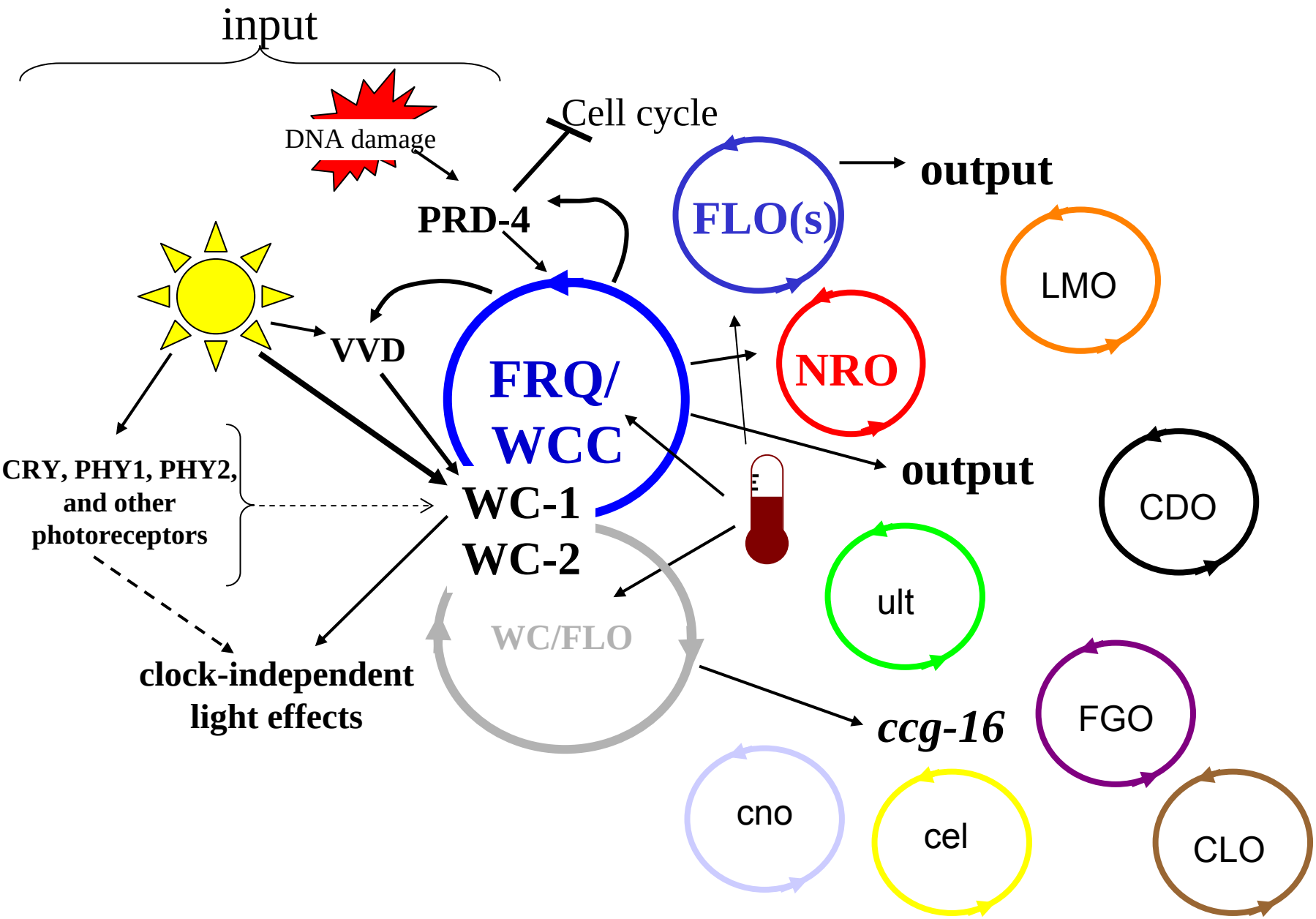


Lakin-Thomas 1998



Luciferase analysis was performed in both low and high temperatures. In contrast with the conidiation rhythm controlled by the CDO, the luc rhythm showed a typical temperature compenstation profile in *frq*$^{+}$ and a temperature under-compensation in *frq*^{7} strain.

The Cellular Circadian System in Neurospora



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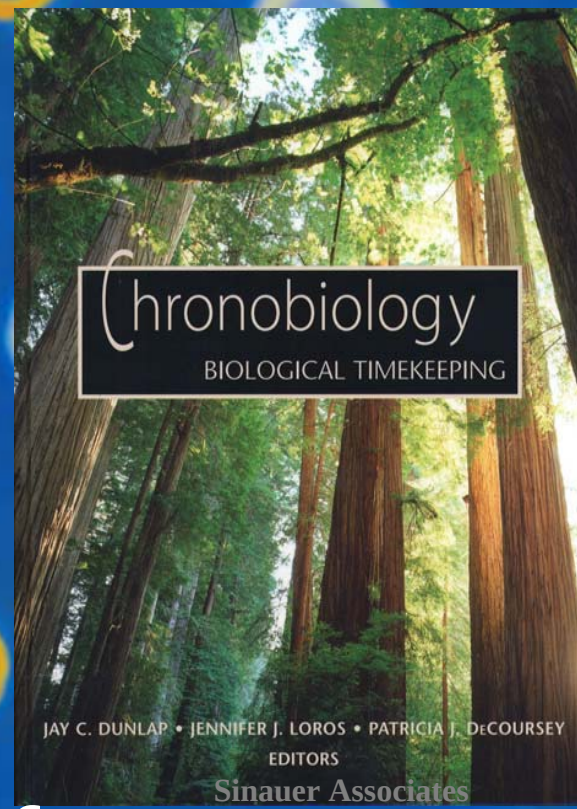
Bill Belden

Arun Mehra

Mi Shi

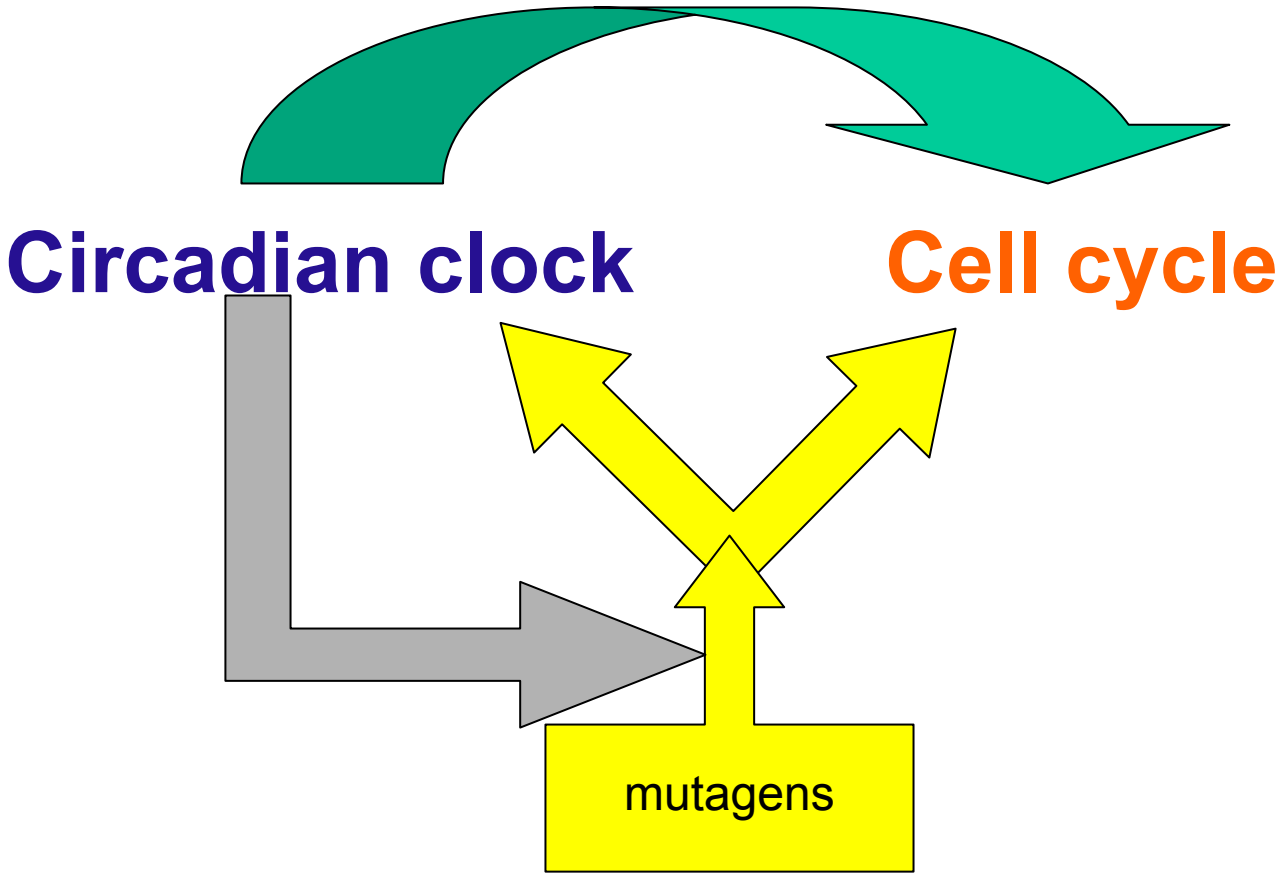
Collaborators: Peter Ruoff, Van Gooch



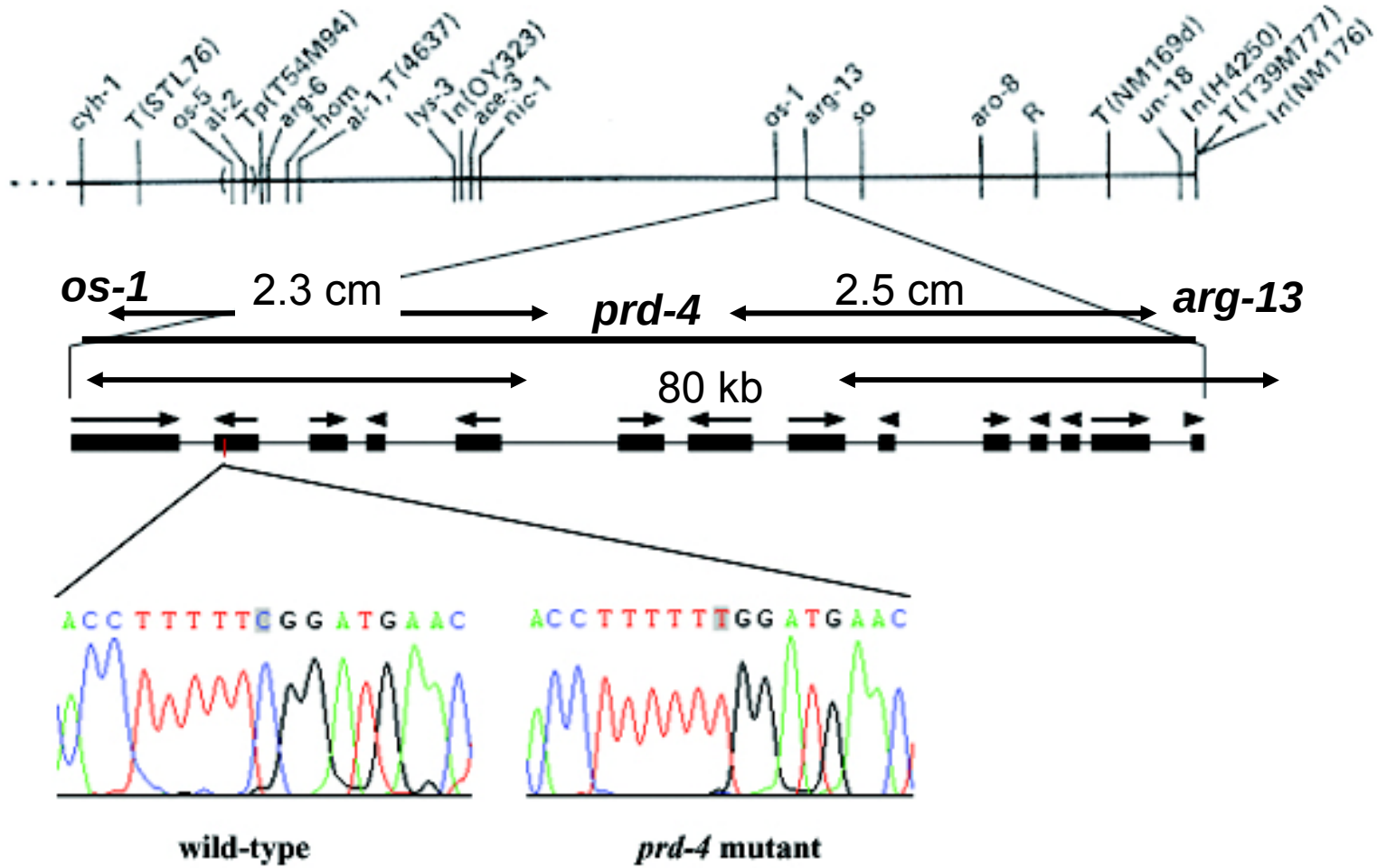
Chris Hong

Luis Larrondo

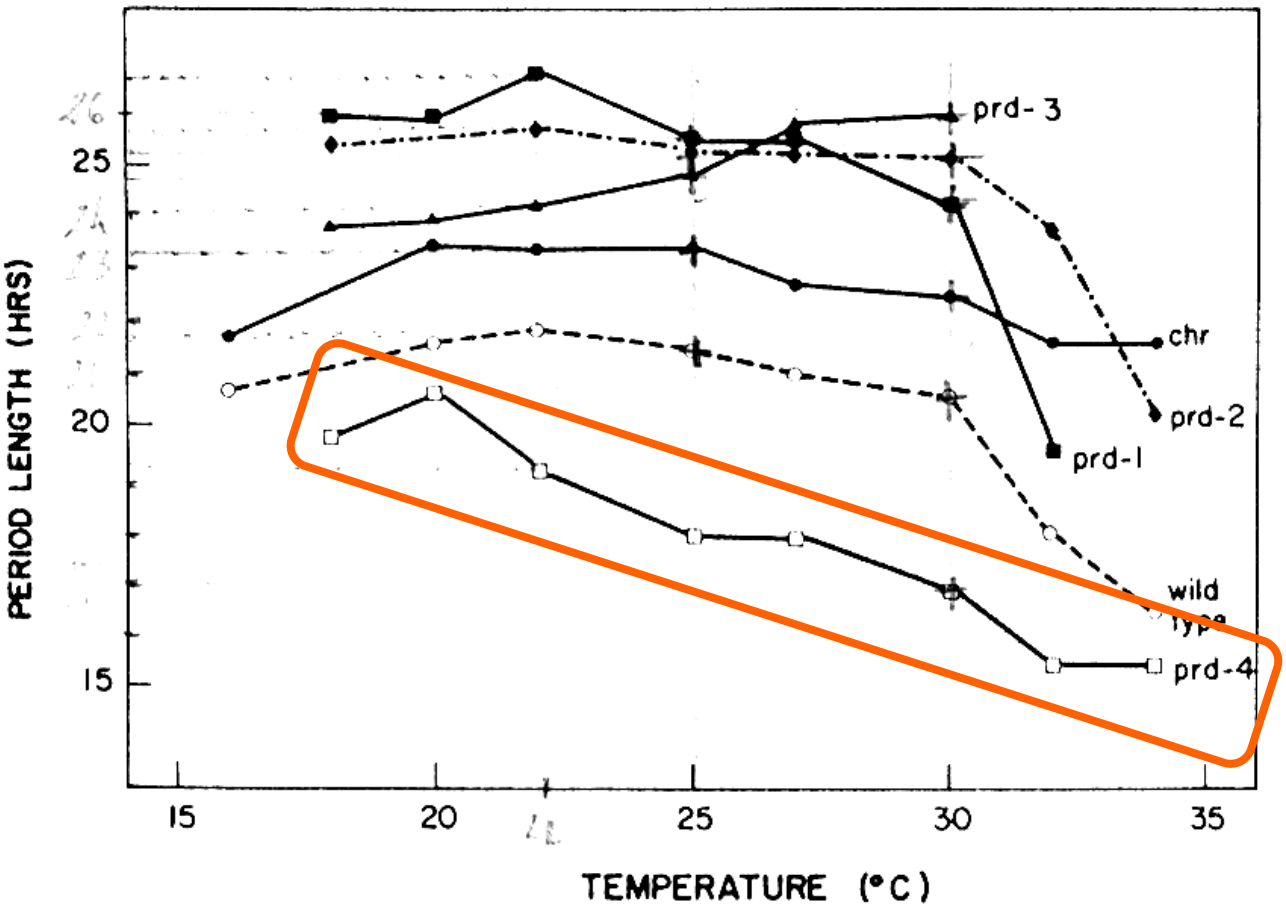
Jennifer Loros


Circadian clock
Cell cycle
mutagens

Identification of the *prd-4* gene



Characteristics of the *prd-4* mutant



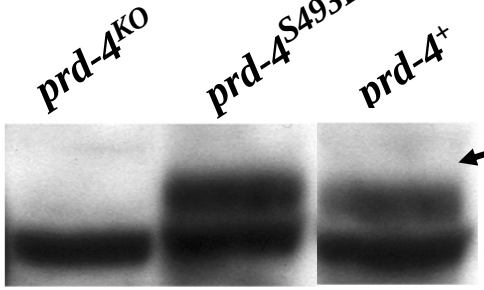
- **short period**
- **partial loss of temperature compensation**
- **semi-dominant mutation**

FIG. 2. Period lengths of wild-type and clock mutants not at the *frq* locus at different temperatures. The average SD for each strain was as follows: Wild-type, 0.5 h; *prd-1*, 1.1 h; *prd-2*, 0.4 h; *prd-3*, 0.5 h; *prd-4*, 0.3 h; *chr*, 0.5 h.

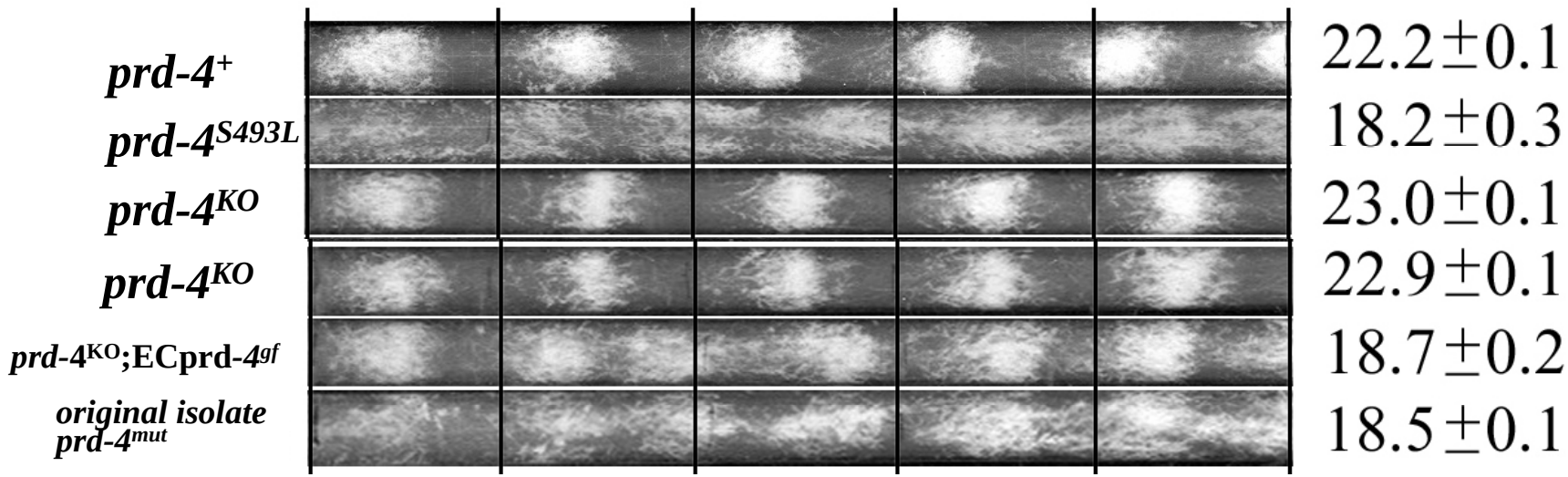
Gardner and Feldman, 1981

Identification of the *prd-4* gene

S493L



prd-4 mutant is semidominant and expresses protein so its probably a gain -of-function

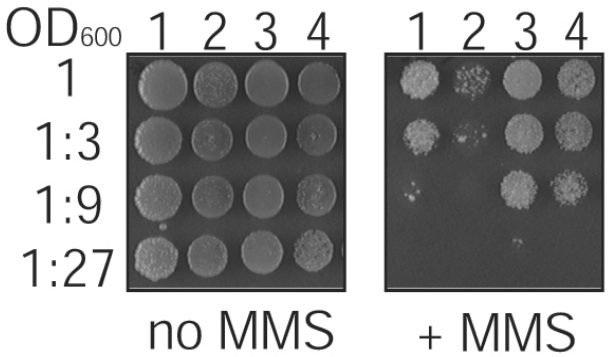


PRD-4 is not essential for clock function, but can modify the function of the circadian system.

What is PRD-4?

Protein	Accession no.	Species	E score
protein kinase Chk2	NP_446129	*Rattus norvegicus*	6e-64
RAD53 homolog (S.cerevisiae); Protein kinase Chk2; Cds1 homolog (S. pombe)	NP_057890	*Mus musculus*	5e-64
CHK2 checkpoint homolog (S. pombe); RAD53 homolog (S.cerevisiae)	NP_009125	*Homo sapiens*	3e-67
protein kinase Cds1	AAG59884	*Xenopus laevis*	4e-65
protein kinase Chk2	AAK52419	*Danio rerio*	1e-51
CeCHK2	BAB15803	*Caenorhabditis elegans*	Not found
PROTEIN KINASE CDS1 (CHECKPOINT KINASE CDS1)	Q09170	*Schizosaccharomyces pombe*	3e-46

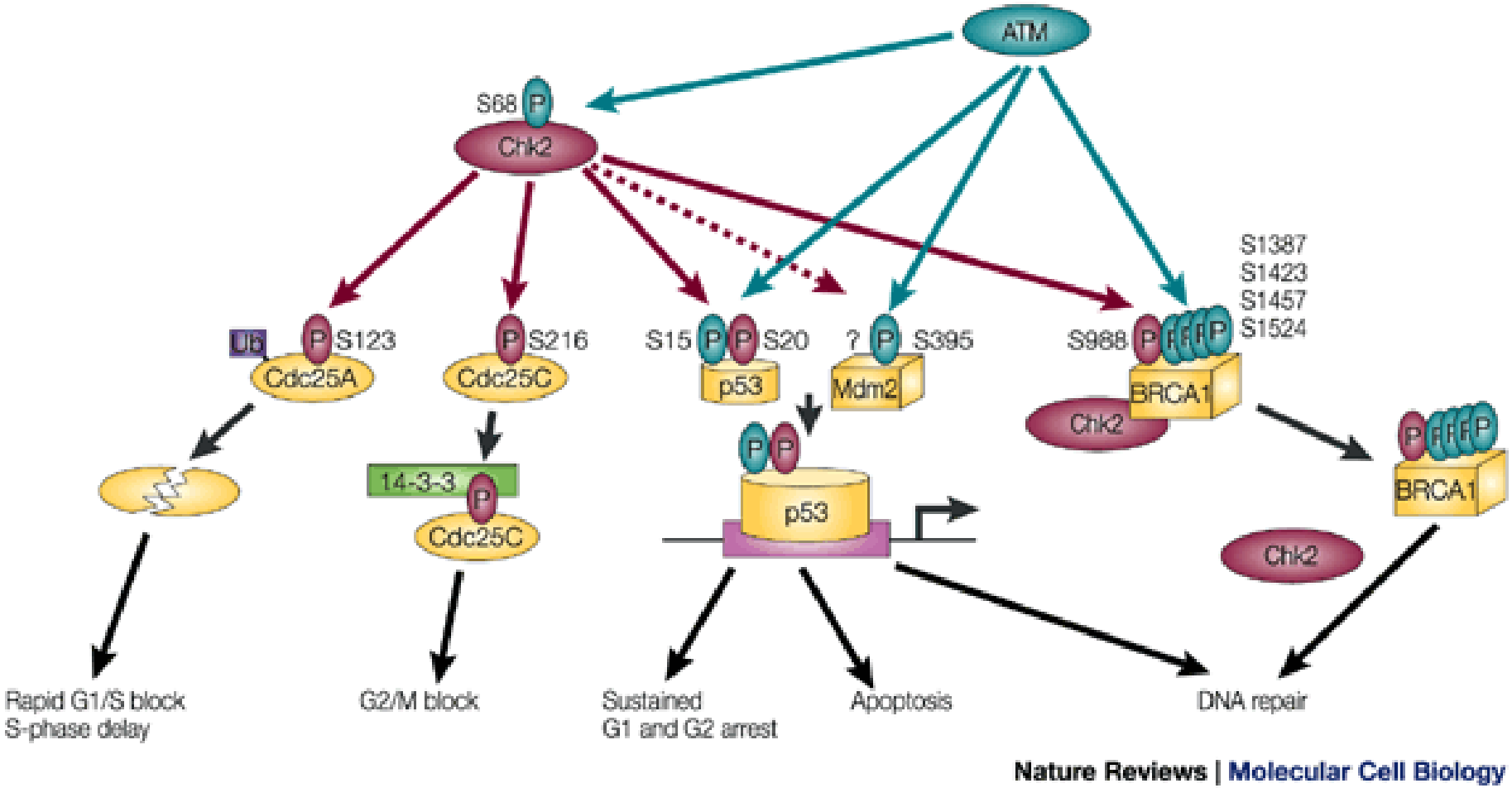
PRD-4 is checkpoint kinase 2.



1 pMH267 (mChk2)
2 pBJ245 (vector)
3 pBJ245::RAD53
4 pBJ245::NcPRD-4

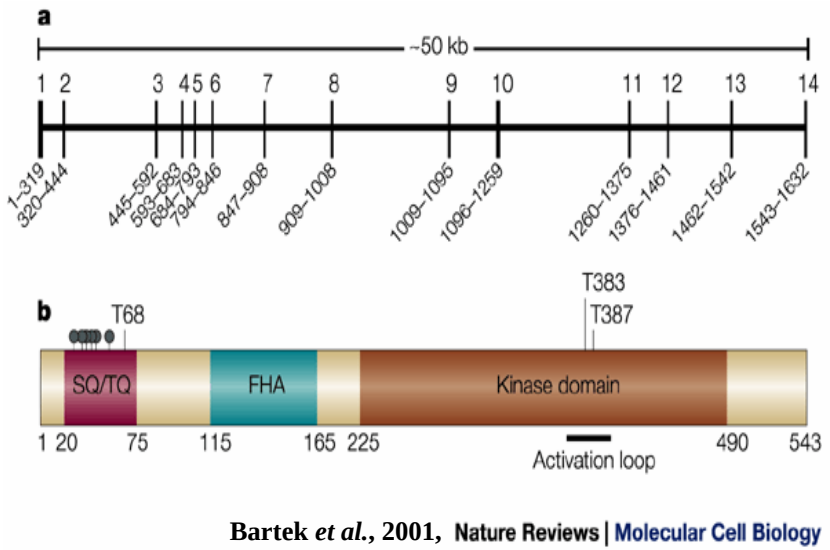
PRD-4 complements yeast loss-of-function knockouts in RAD53 in a manner comparable to mChk2.

Roles of *checkpoint kinase 2* (Chk2)

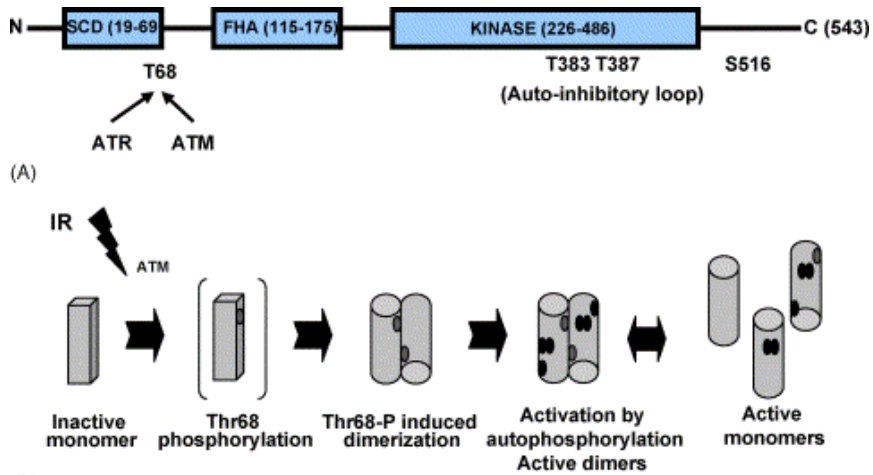


Bartek *et al.*, 2001

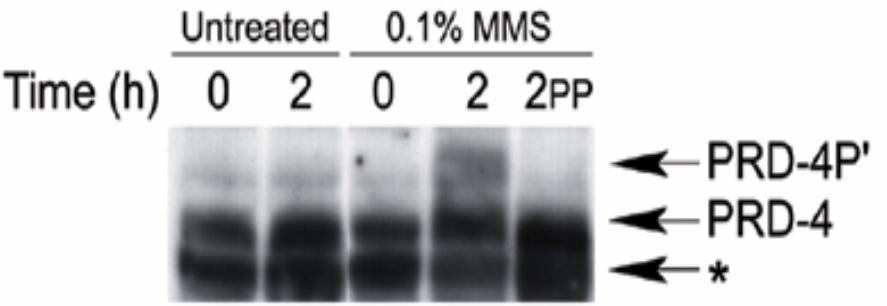
How Chk2 works

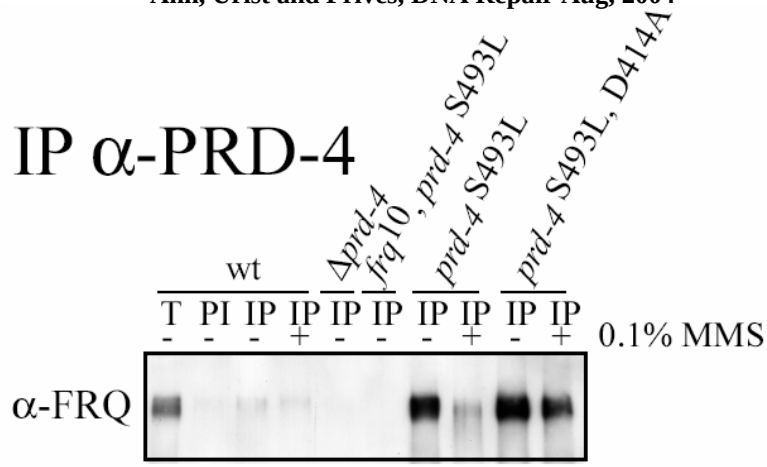


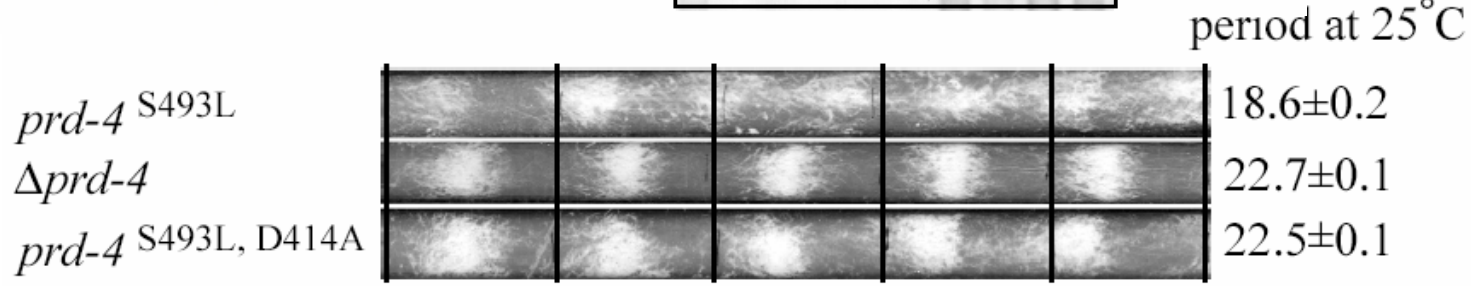
Bartek *et al.*, 2001, Nature Reviews | Molecular Cell Biology

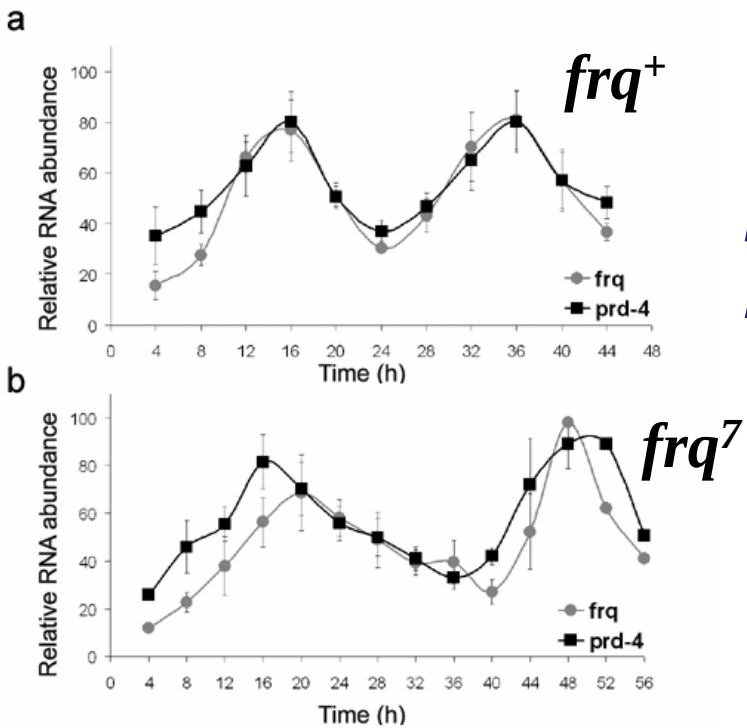


Ahn, Urist and Prives, DNA Repair Aug, 2004

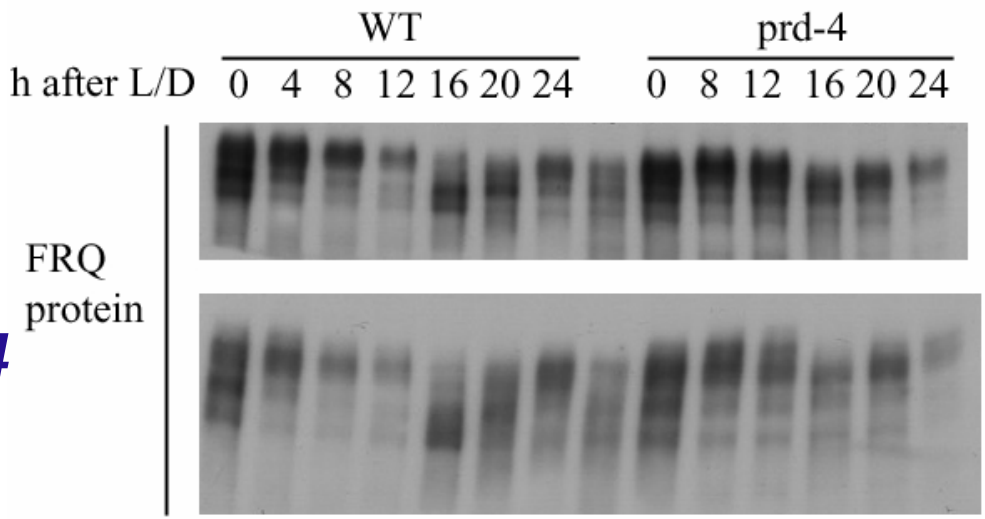








Expression of prd-4 is regulated by the circadian clock.

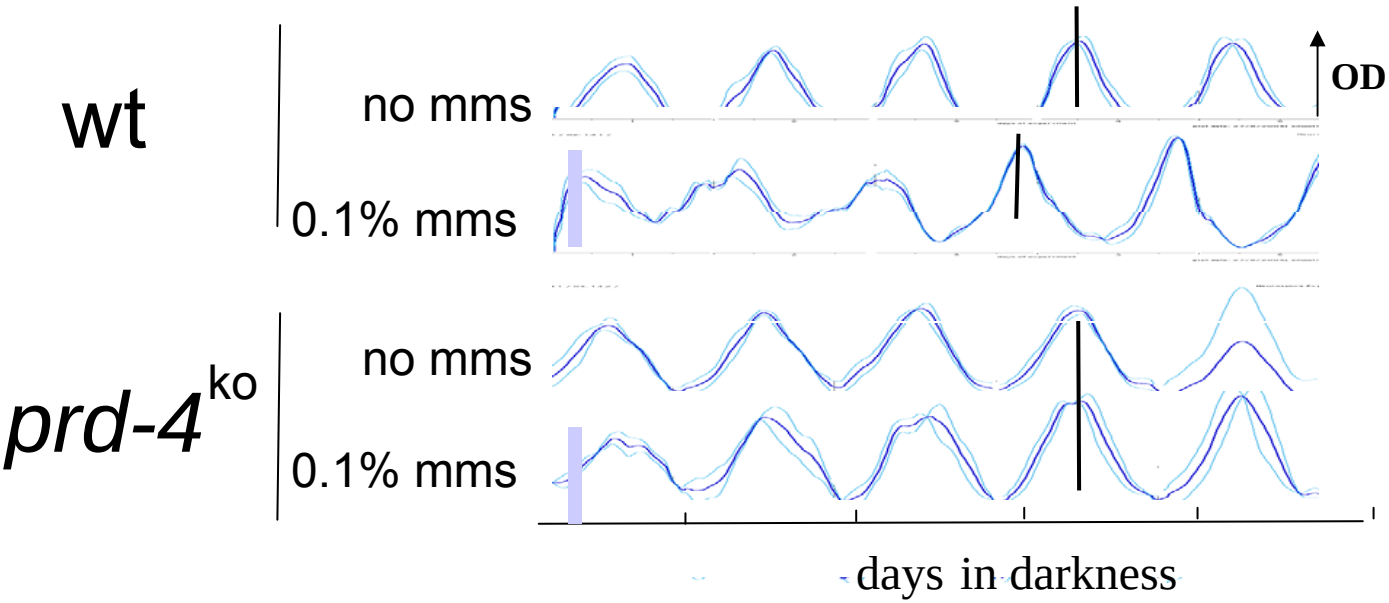


The S493L mutation of prd-4 results in premature phosphorylation of FRQ.

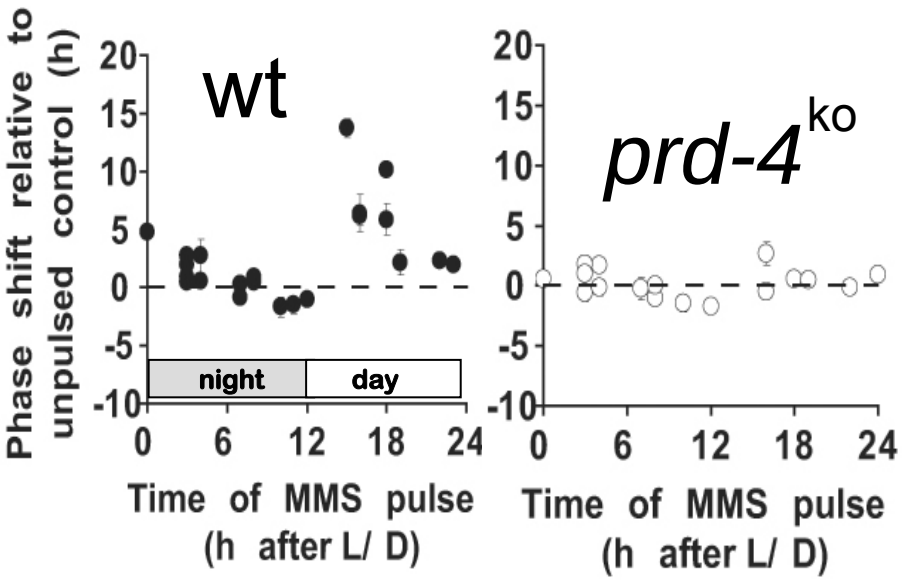
OK, so PRD-4 is clock-controlled and its mutation probably affects FRQ, but what's the evidence that wild type PRD-4 has any normal function related to the clock?

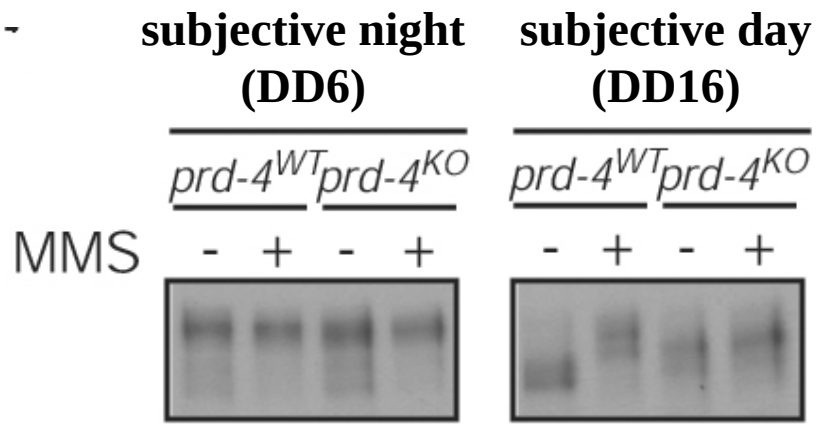
Since CHK2 is activated by DNA damage, we looked at clock effects of DNA damage.

Clock resetting by MMS requires PRD-4

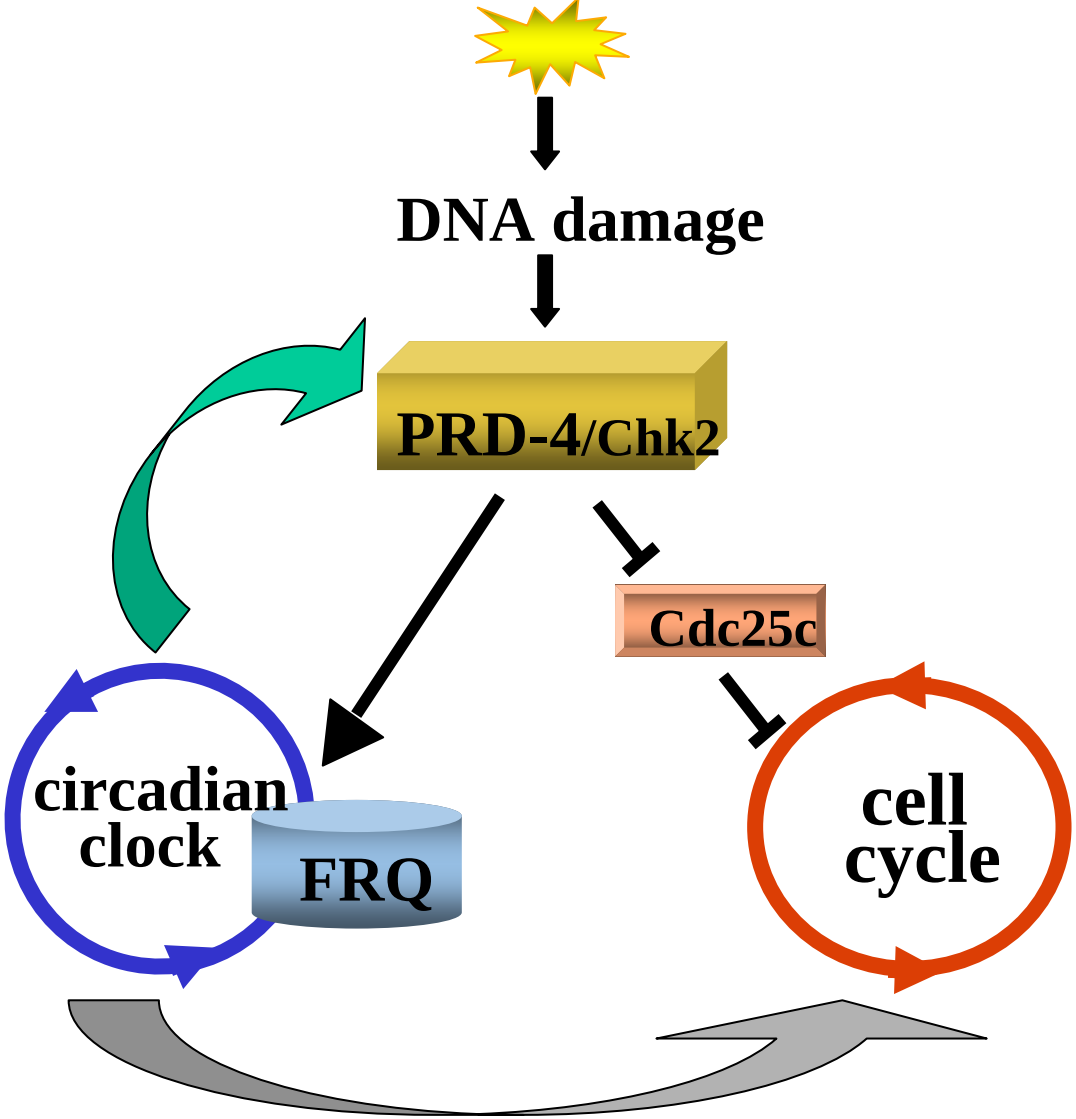


a 2 hr long treatment with 0.1% MMS resets the clock in WT but not in *prd-4^ko^*





clock resetting correlates with FRQ phosphorylation



prd-4^{mut} is a semidominant clock mutation characterized by a short period and partial loss of temperature compensation.

PRD-4 = Neurospora CHK2 (checkpoint kinase-2)

prd-4 expression is clock-regulated.

PRD-4 function is required for clock-resetting effects of the radiomimetic drug MMS.

Identification of *prd-4* has brought to light an additional feedback loop that closes around the clock, conditionally connecting output with input.

Long and short FRQ isoforms help to expand the physiological range permissive for rhythmicity, but they do not play a role in establishing temperature compensation of period length.

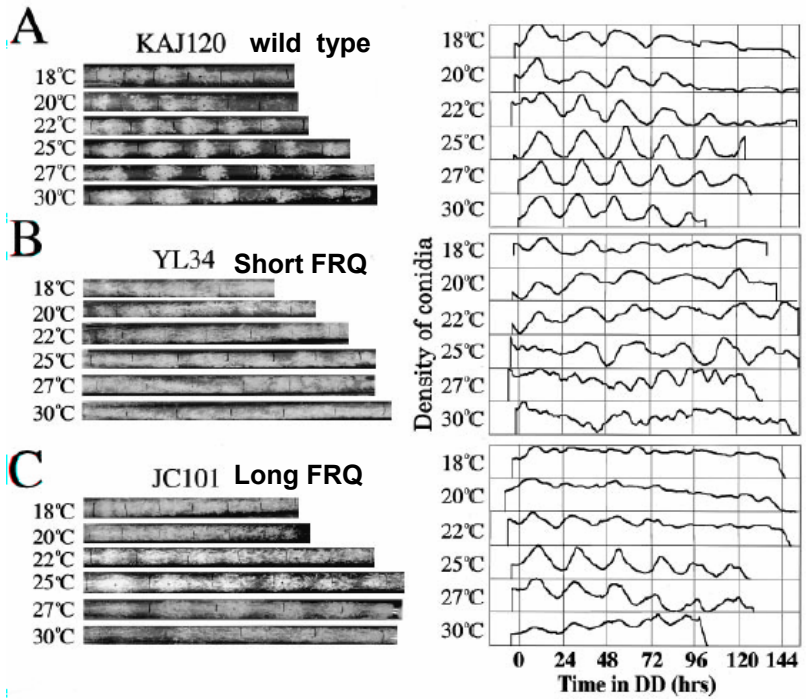


Figure 2. Mutation of Initiation Codons within the FRQ ORF Reduces the Temperature Range Permissive for Rhythmicity

Race tube data are shown on the left, and the densitometric analysis of the images is shown on the right (see Experimental Procedures).

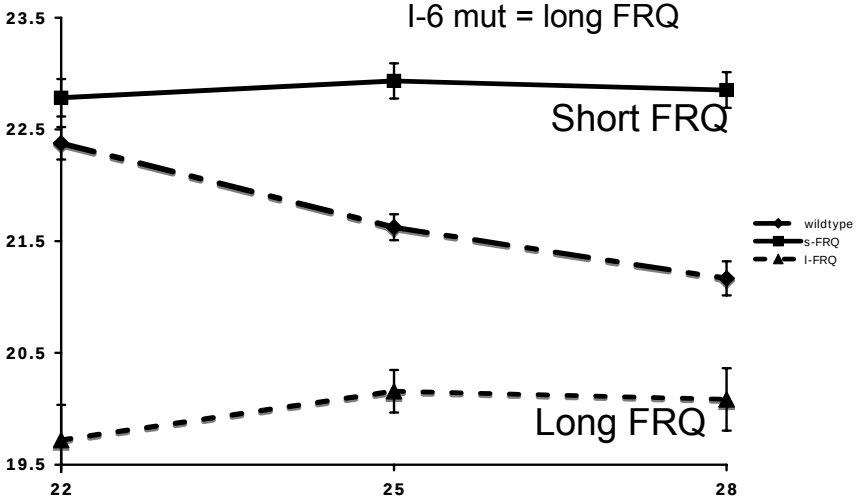
(A) Transformants bearing the intact *frq* locus (KAJ120) exhibit normal rhythmicity at all temperatures across the physiological range.

(B) Deletion of AUG#1 in YL34-S eliminates overt rhythmicity at temperatures near the high end of the physiological temperature range.

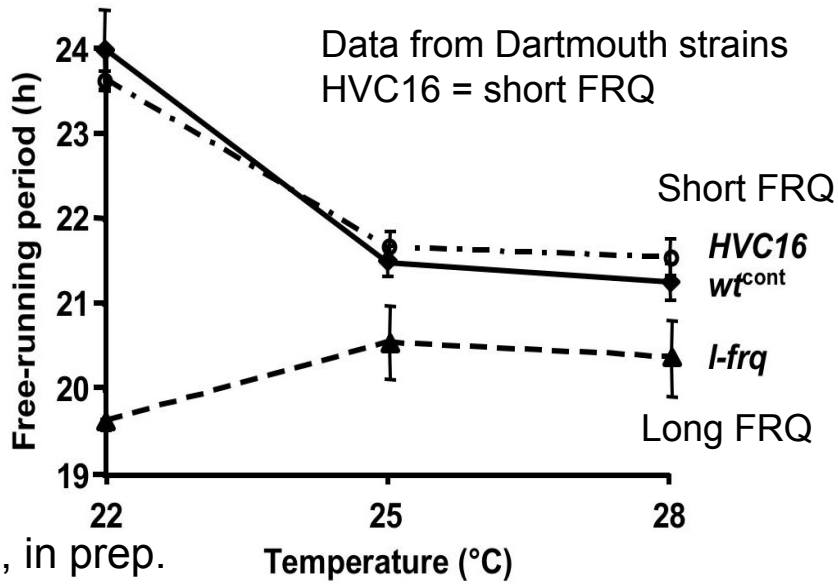
(C) Mutation of AUG#3 in JC101-L transformants selectively eliminates overt rhythmicity at temperatures near the low end of the physiological temperature range.

Liu et al. Cell, 1997

Data from Heidelberg strains
I-6opt = short FRQ
I-6 mut = long FRQ



Data from Dartmouth strains
HVC16 = short FRQ



Diernfellner et al, in prep.