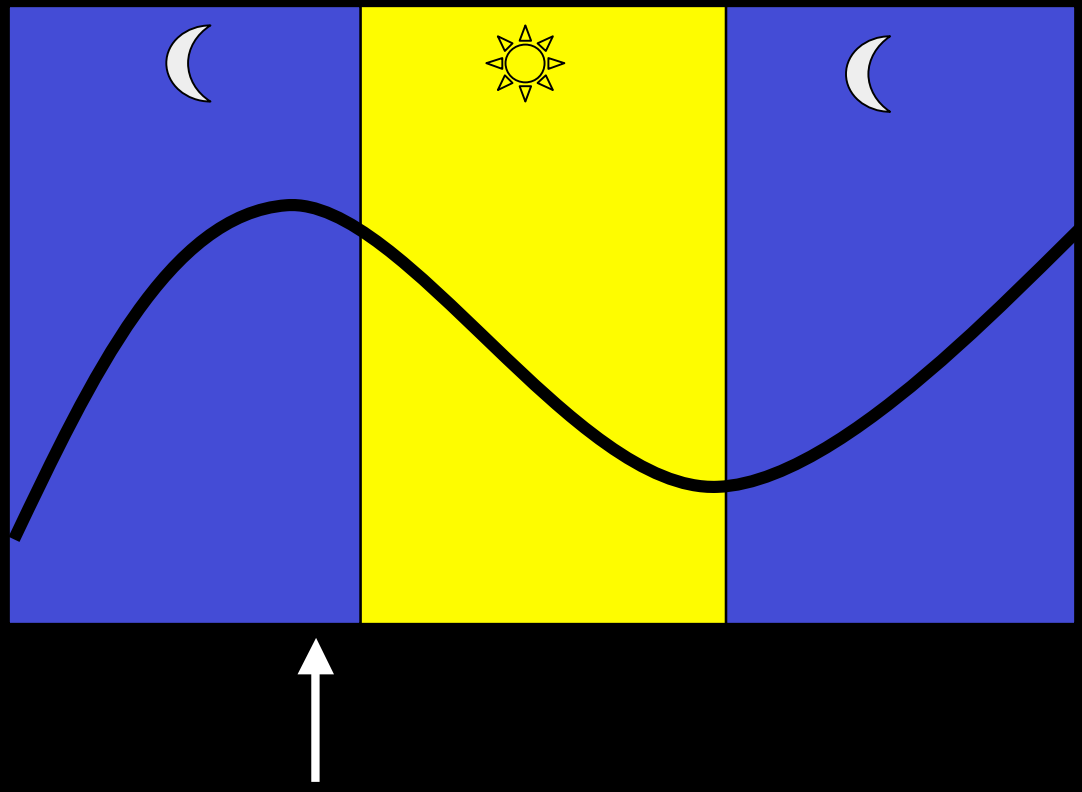
Complexity in the *Neurospora* circadian clock

1. Overview of FWO
2. FLO's
3. Output
4. Frequency Demultiplication

The "pacemaker" involves the interactions of <u>multiple feedback loops</u> that can program phase-dependent oscillations.

Endogenous Circadian Clocks Allow Anticipation of Environmental Cycles



Make protective proteins BEFORE sunrise

Neurospora crassa

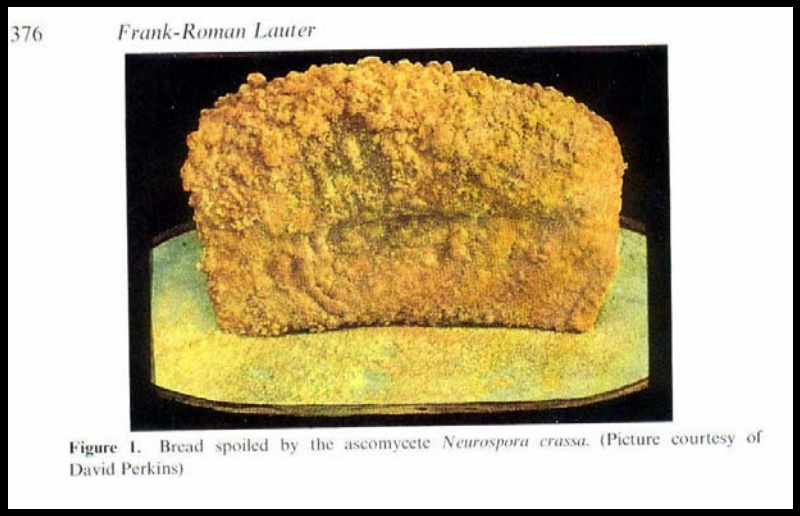
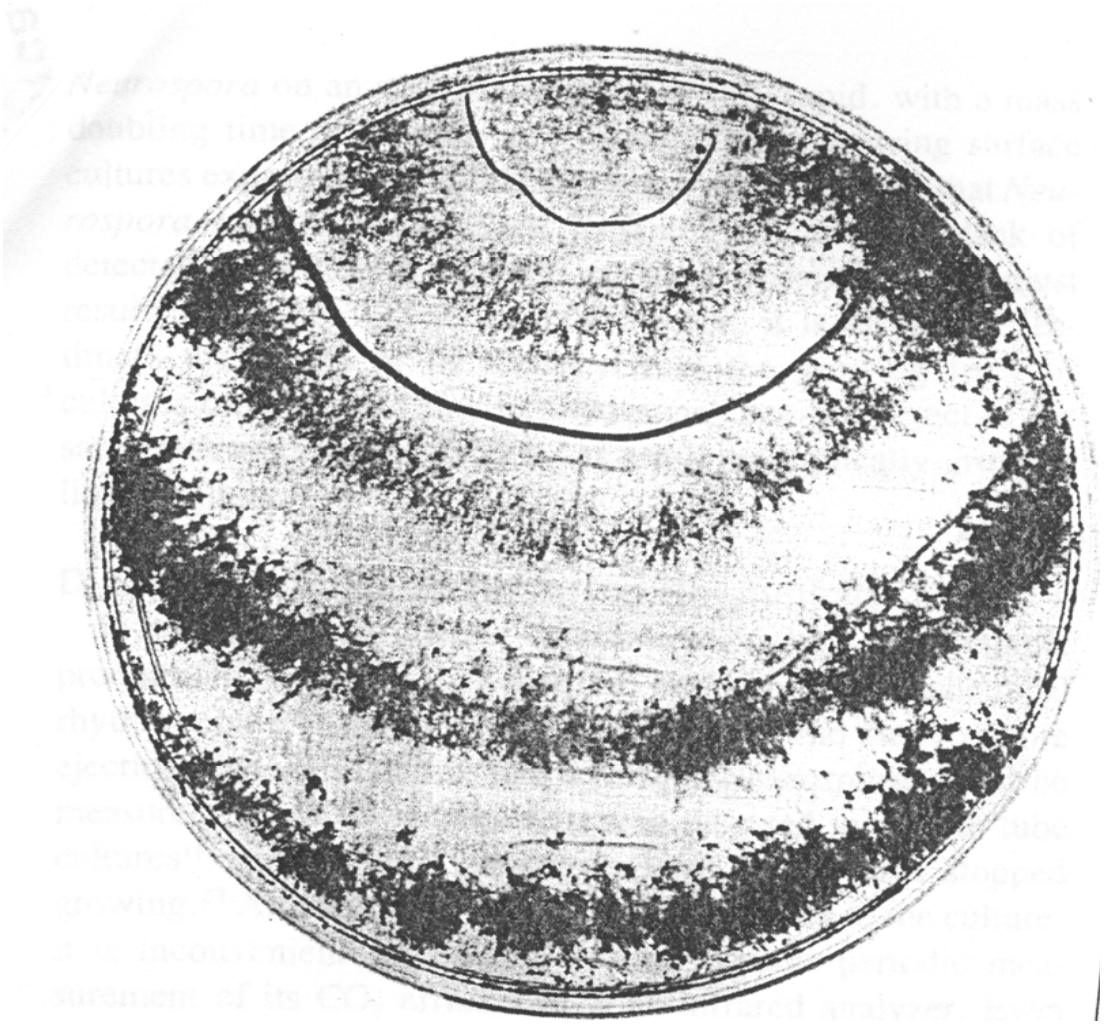

376 *Frank-Roman Lauter*

Figure 1. Bread spoiled by the ascomycete *Neurospora crassa.* (Picture courtesy of David Perkins)

- Haploid genome with 7 chromosomes, sequenced.
- Reproduces both asexually and sexually.
- Easy to manipulate both genetically and biochemically.
- Easy to monitor the circadian rhythm of asexual spore development.

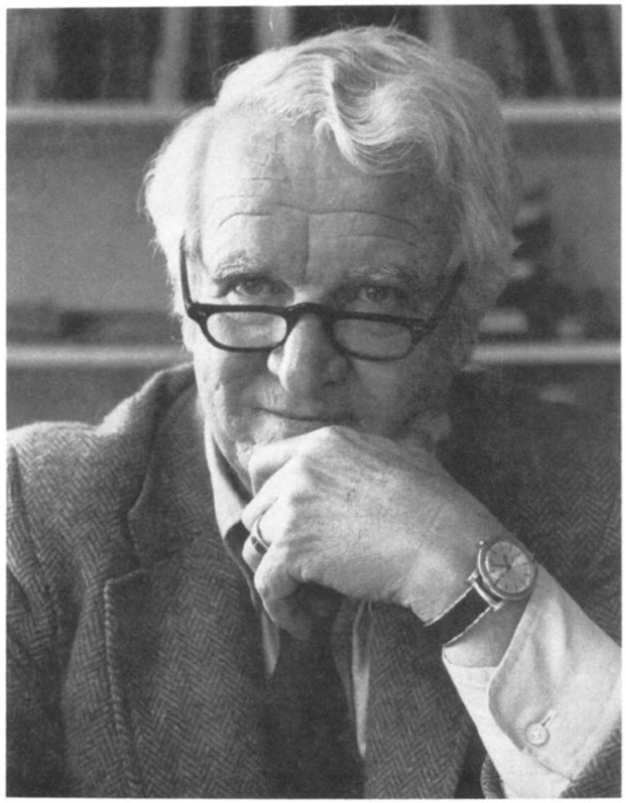
William Brandt (1953) *Mycologia*: Patch mutant (proline auxotroph), first report of a rhythm in *N. crassa*.



Pittendrigh CS, Bruce BVG, Rosenzweig NS, Rubin ML. 1959. A biological clock in *Neurospora*. Nature 184: 169-170.

Showed that the developmental rhythm is circadian (temp compensated, entrainable by light/dark cycles)

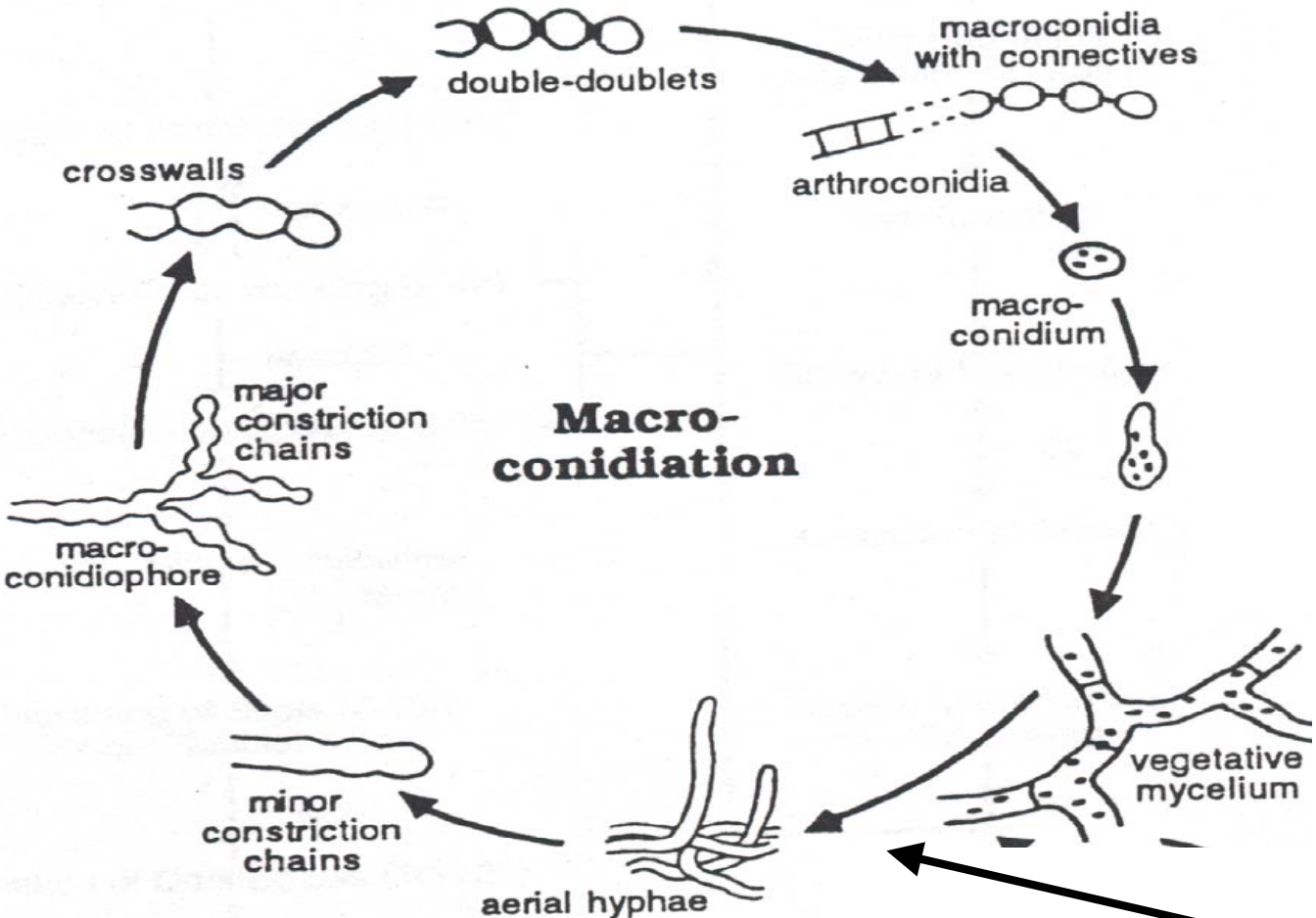
"the Neurospora system appears potentially valuable for an attack on the basic problem of the nature of oscillations that constitute the circadian clock"



Colin S. Pittendrigh

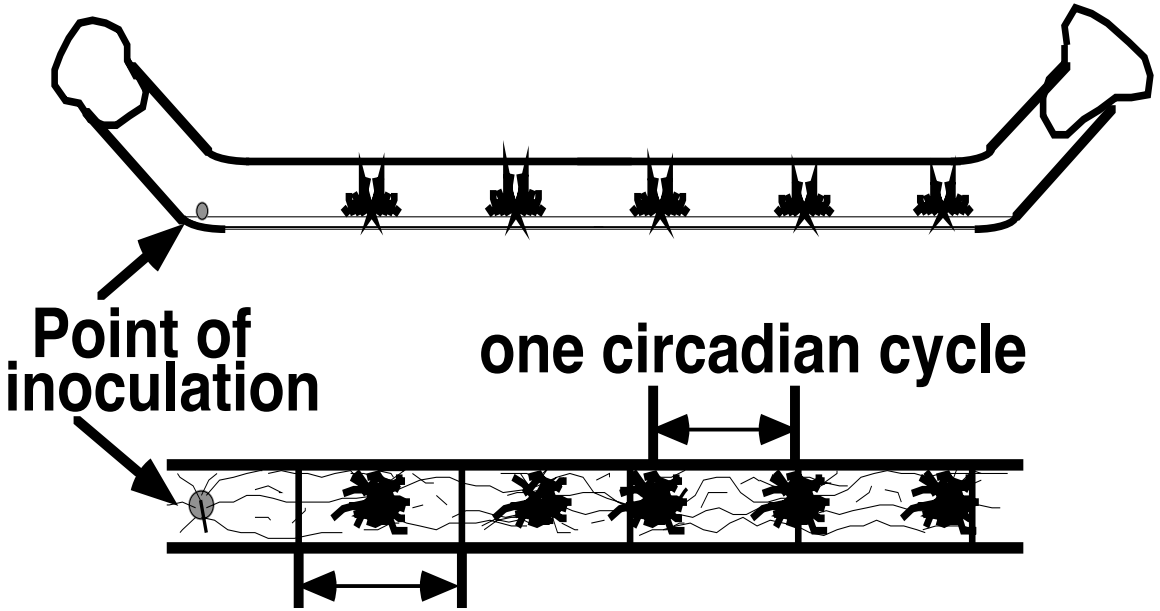
Malcolm Sargent identified the **band (bd)** mutation (1972) and developed conditions, using **race tubes**, to promote the best expression of the rhythm. He also showed that the rhythm can be reset by light and temperature pulses.

Asexual Development Cycle



Light
Nutrient Deprivation
Dessication
Endogenous Clock

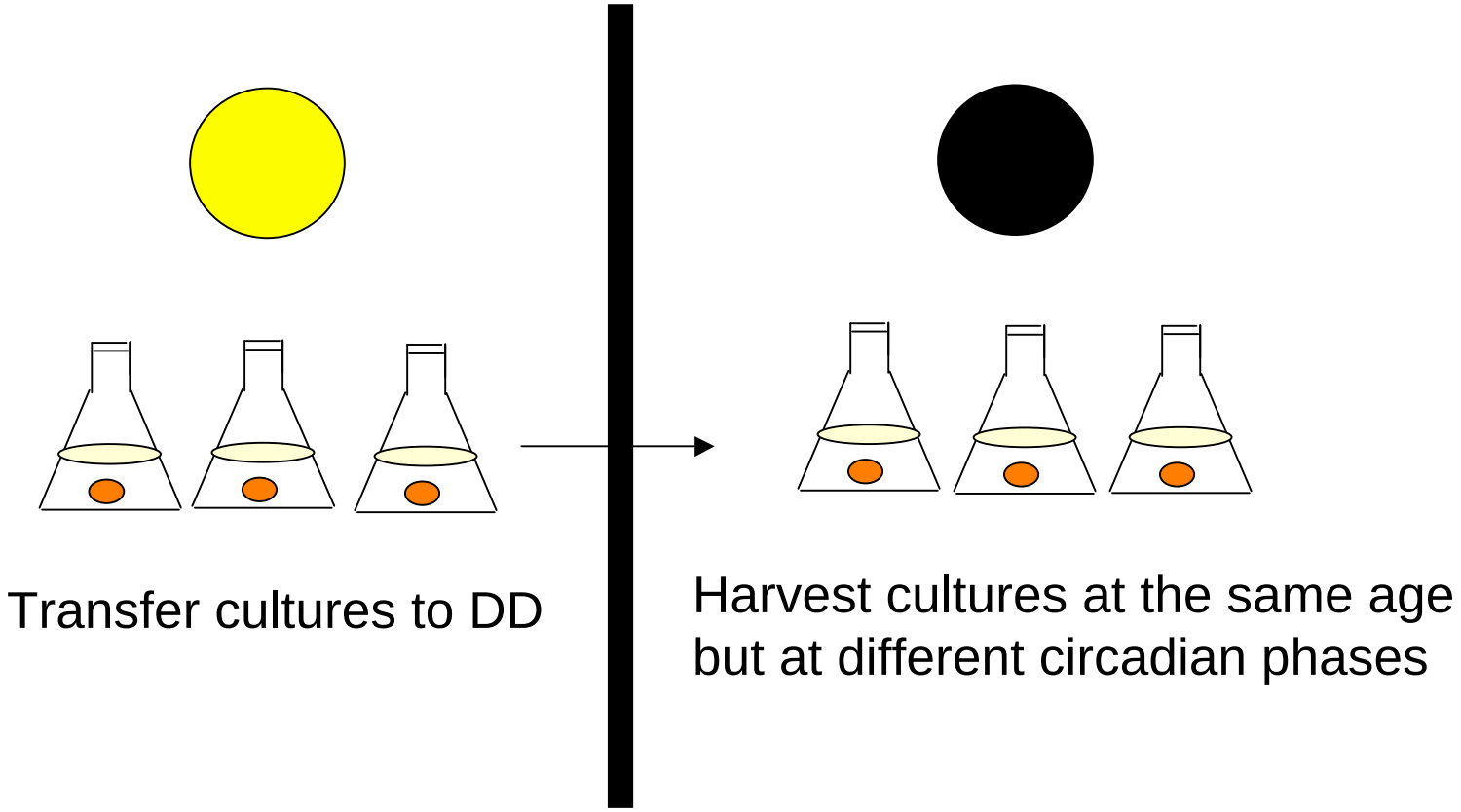
Diagram of a race tube



band (bd) strain

Time lapse video of Neurospora Circadian Conidiation Rhythms by Van Gooch and Jason Thoen.

Development of **liquid culture** methods (Nakashima) to monitor cycling mRNAs and proteins.



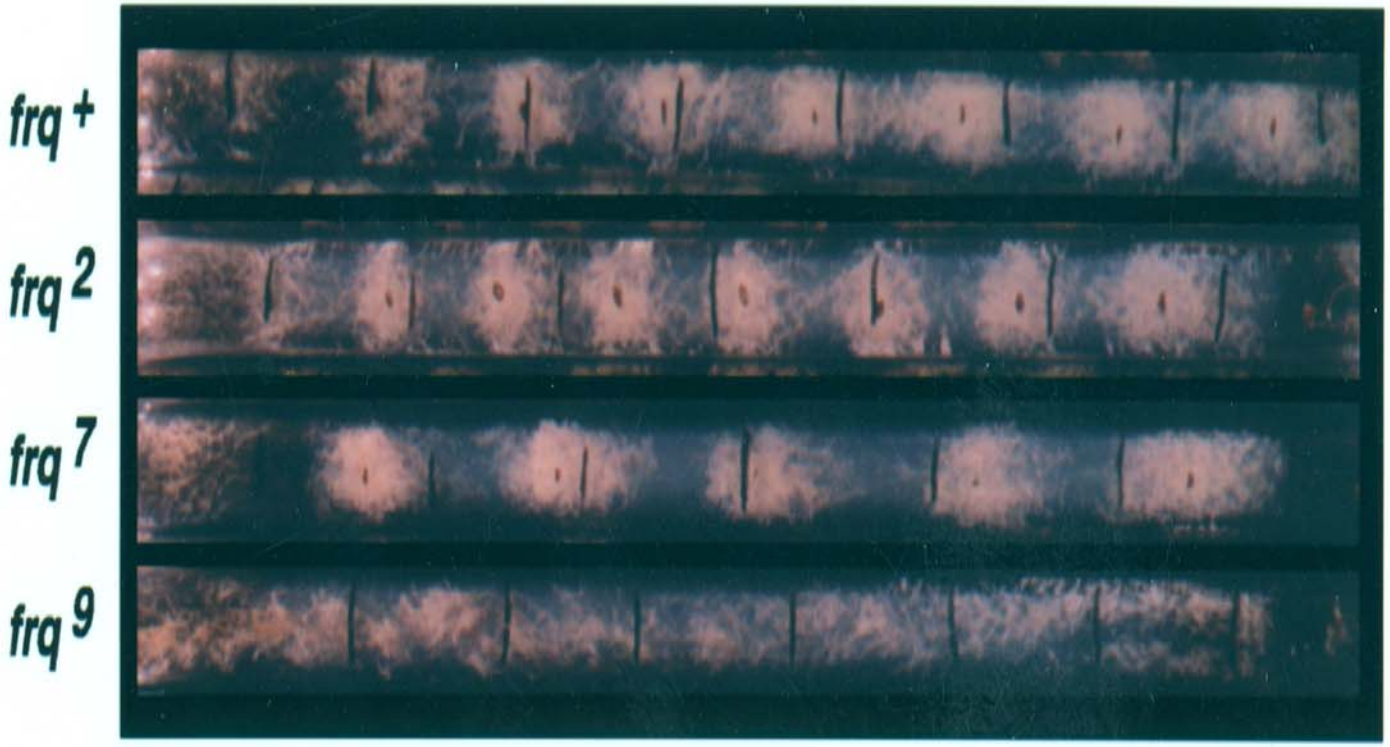
*** Luciferase and gfp variants**

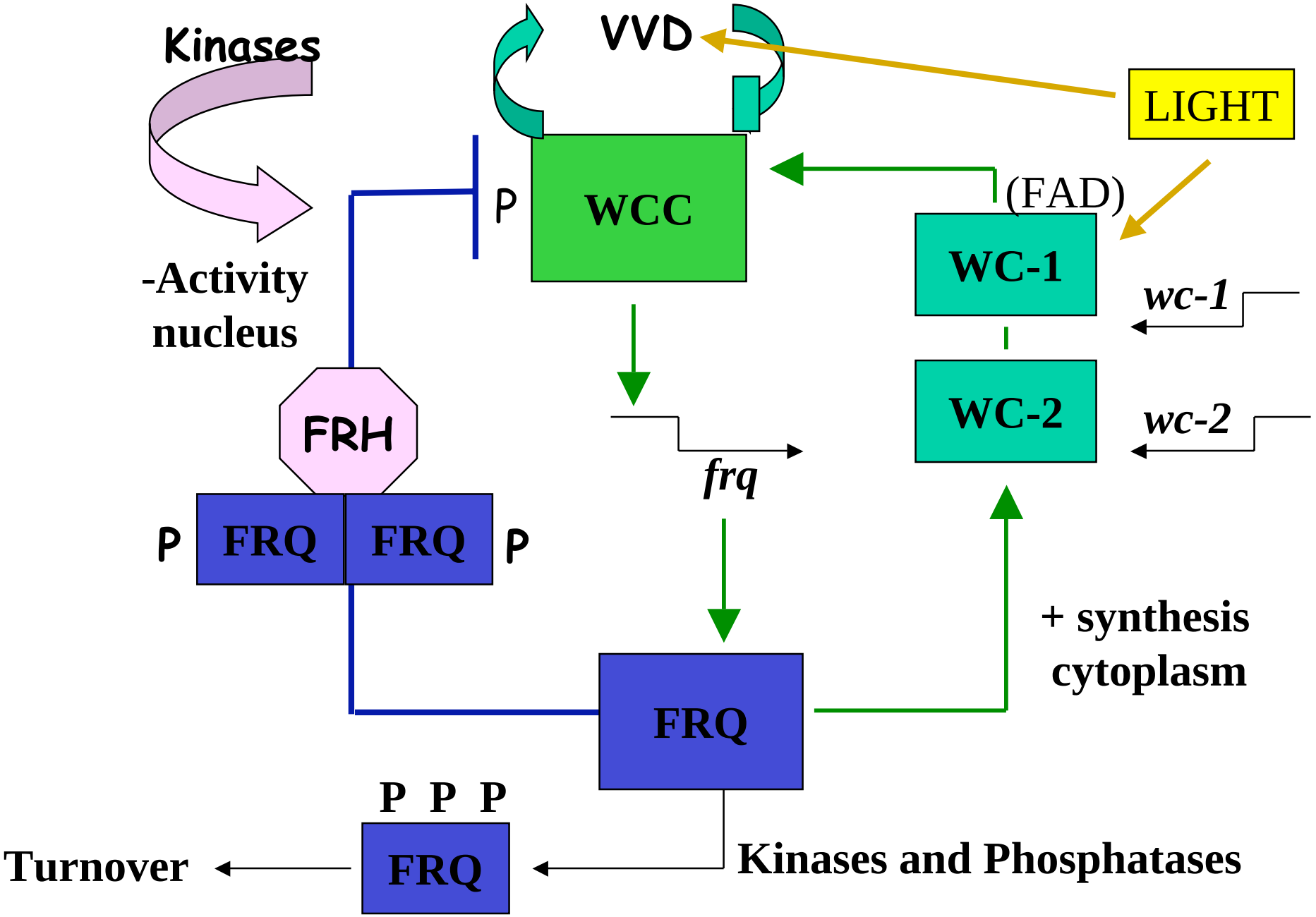
Jerry Feldman (1960s-1970s) was the first to identify mutations that alter the circadian rhythm of development in *Neurospora*.

Jay Dunlap and **Jennifer Loros** (1989) cloned the first clock gene (*frequency)* in *Neurospora* (McClung et al, 1989).

First global screen for ccgs (Loros et al, 1989)

The beginnings of molecular/genetic analysis of the *N. crassa* clock

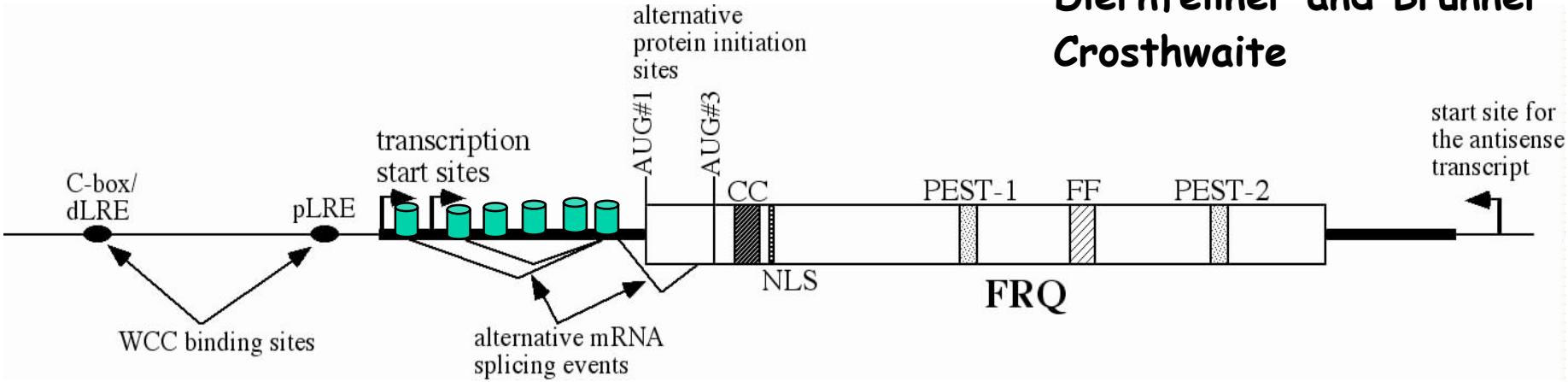



Kinases
VVD
LIGHT
P
WCC
(FAD)
WC-1
-Activity
nucleus
wc-1
WC-2
wc-2
FRH
frq
P
FRQ
FRQ
P
+ synthesis
cytoplasm
FRQ
P P P
Turnover
FRQ
Kinases and Phosphatases

WC-2
frq
lFRQ & sFRQ
WC-1
WC-1 &
WC-2
Circadian Time
0
4
8
12
16
20
24

The effects of temperature on the FWO:

Liu, Merrow, Loros, Dunlap
Colot, Loros, Dunlap
Diernfellner and Brunner
Crosthwaite



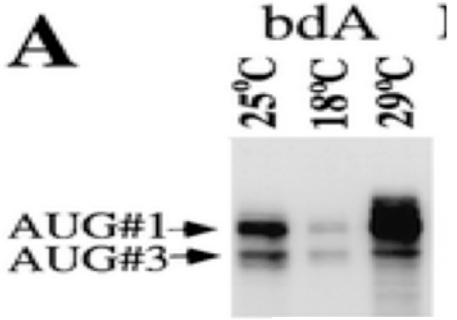
Temperature increase - *frq* mRNA levels go up, more FRQ protein made.
Transcription initiation more often at the downstream site of transcription.
The increase in FRQ protein levels depends on the 5' UTR and 6 uORFs. Initiation at uORFs is more efficient at low temp.

The ratio of l and sFRQ are temperature-dependent as well. See more lFRQ as the temp increases. Strains that can only express lFRQ are arrhythmic at high temps, whereas those that can only express sFRQ loose rhythmicity at low temp. Intron 6 contains the translation initiation site of lFRQ - very little splicing at high temp.

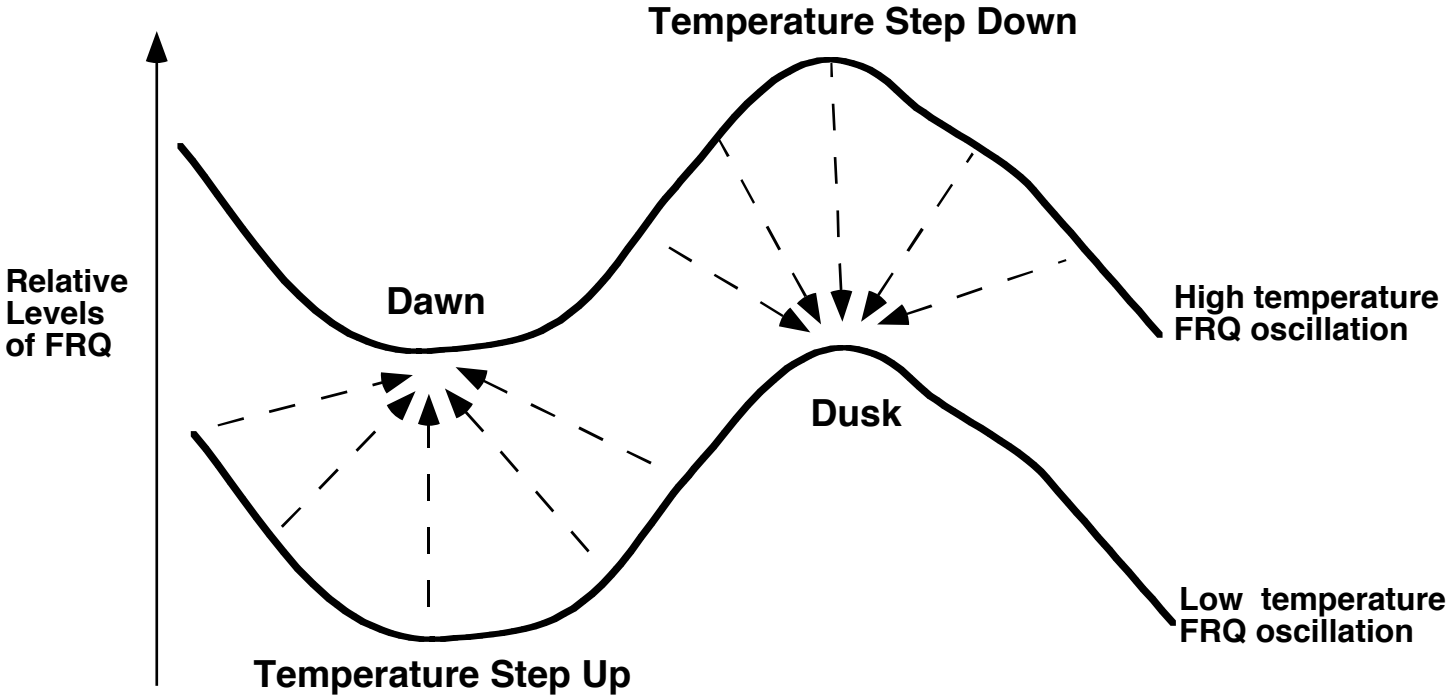
Helps to explain temperature entrainment, not compensation

Temperature resetting of the *Neurospora* clock

Posttranscriptional mechanism



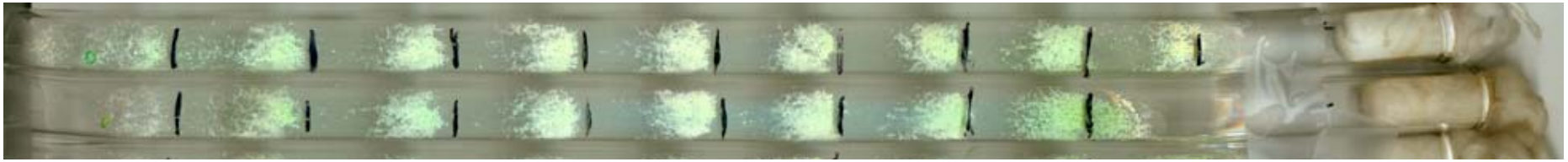
Liu Y, Garceau NY, Loros JJ, Dunlap JC. Cell. 1997 May 2;89(3):477-86



The effects of light on the FRQ/WCC oscillator

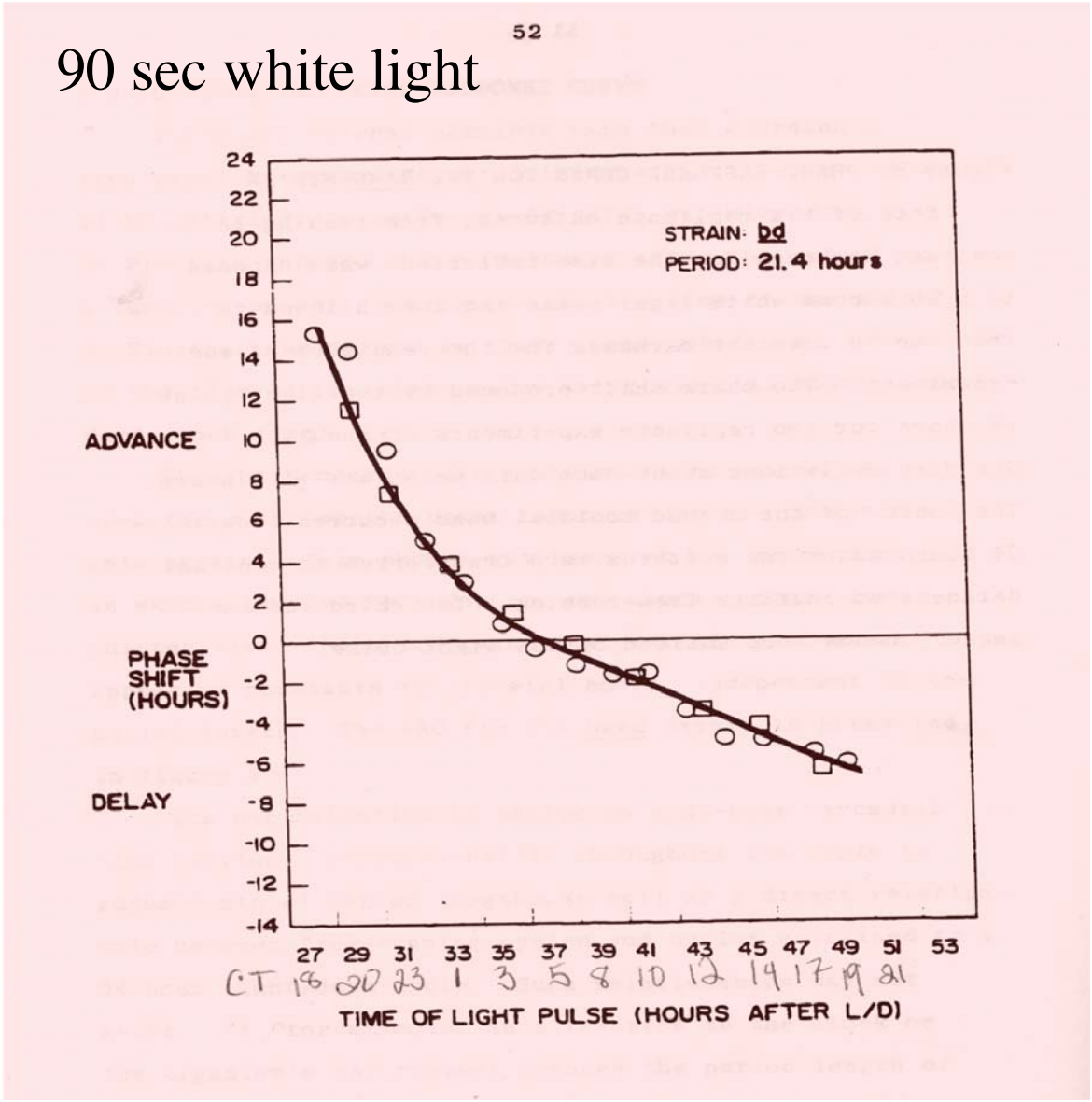
DD

LD 12:12



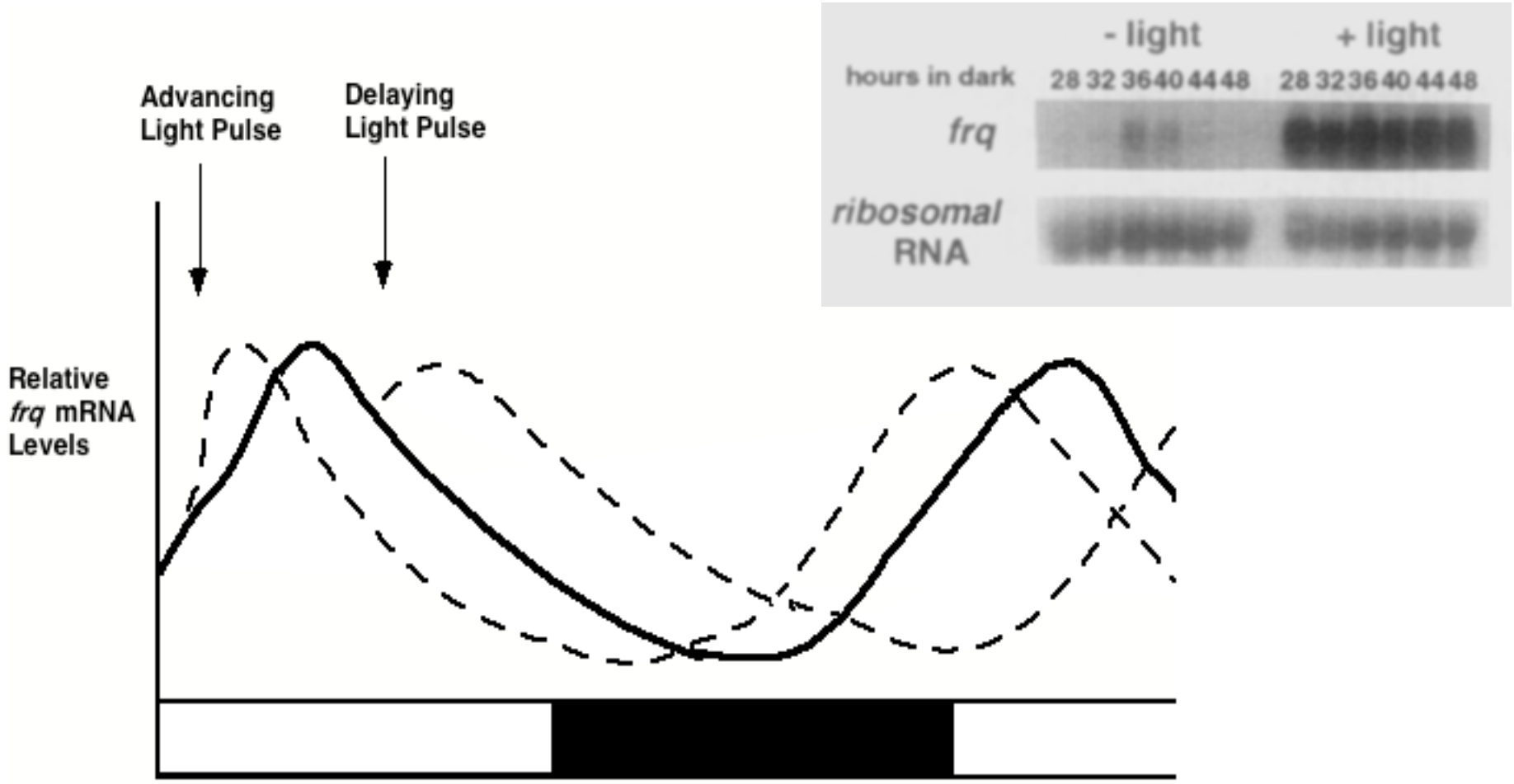
Constant light

90 sec white light



Dharmanda and Feldman

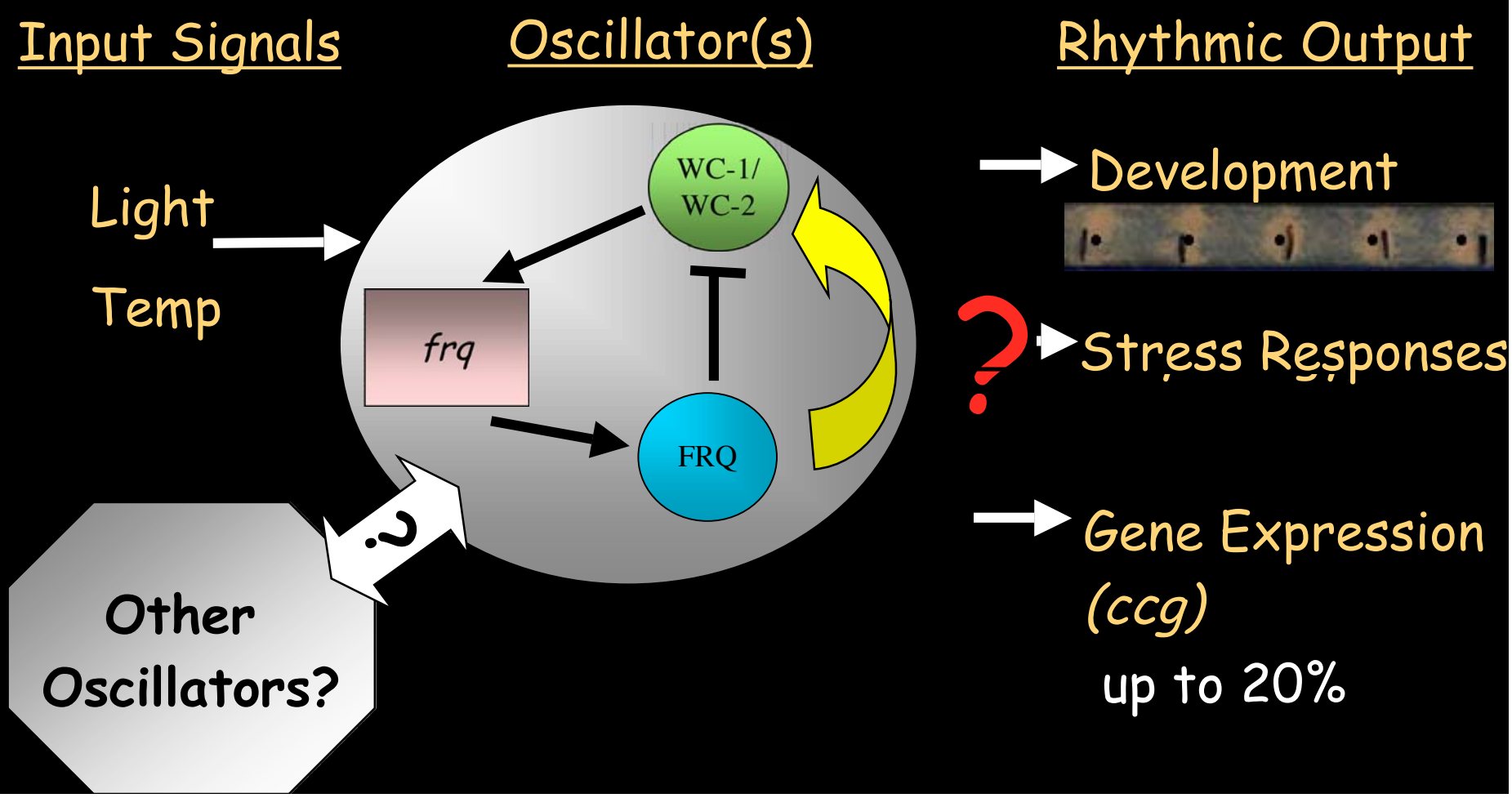
How Light Resets the Clock



Shows how the same signal can elicit a delay and an advance of the clock

Crosthwaite SK, Loros JJ, Dunlap JC Cell. 1995 Jun 30;81(7):1003-12.

The *Neurospora* Circadian Clock System

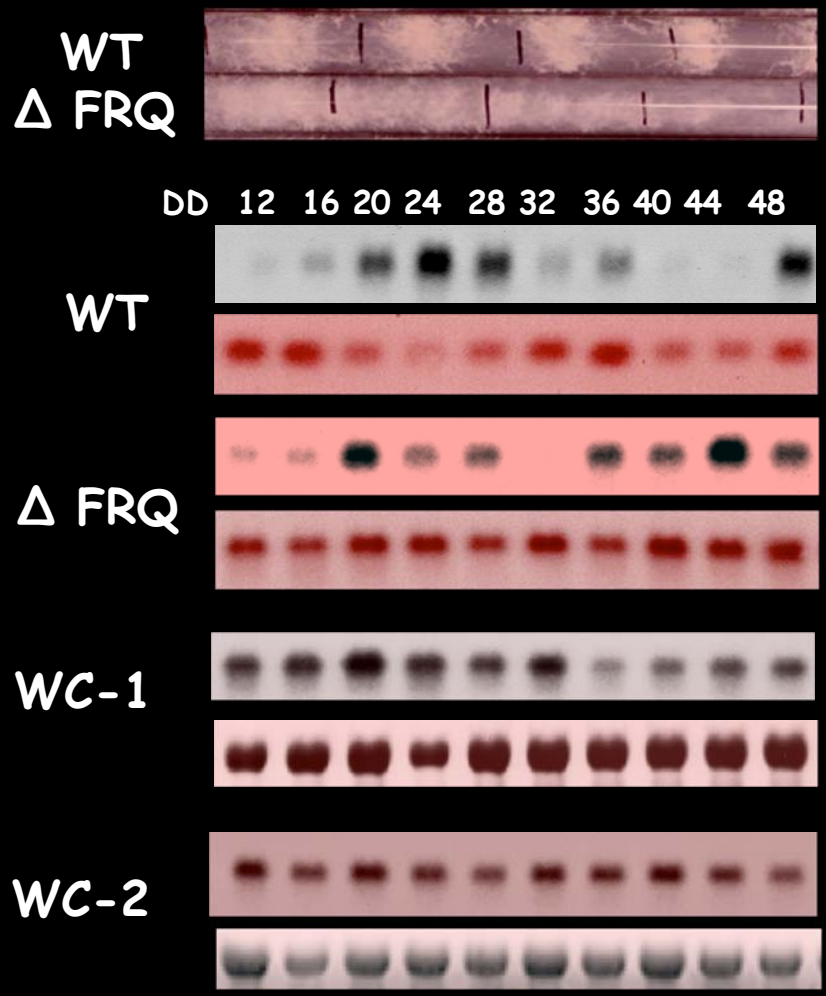


Evidence for multiple oscillators within Neurospora cells

- Genes/proteins that are rhythmic in the absence of a functional FRQ/WCC oscillator. Peter Ruoff - nitrate reductase

- Developmental rhythms can be observed in strains that do not have a functional FRQ/WCC oscillator under certain growth conditions and in certain mutant backgrounds. Jennifer Loros, Stu Brody, Pat Lakin-Thomas, Martha Merrow, Till Roenneberg, Yi Liu

The evening-specific *ccg-16* gene is regulated by a FRQ-less Oscillator (FLO)

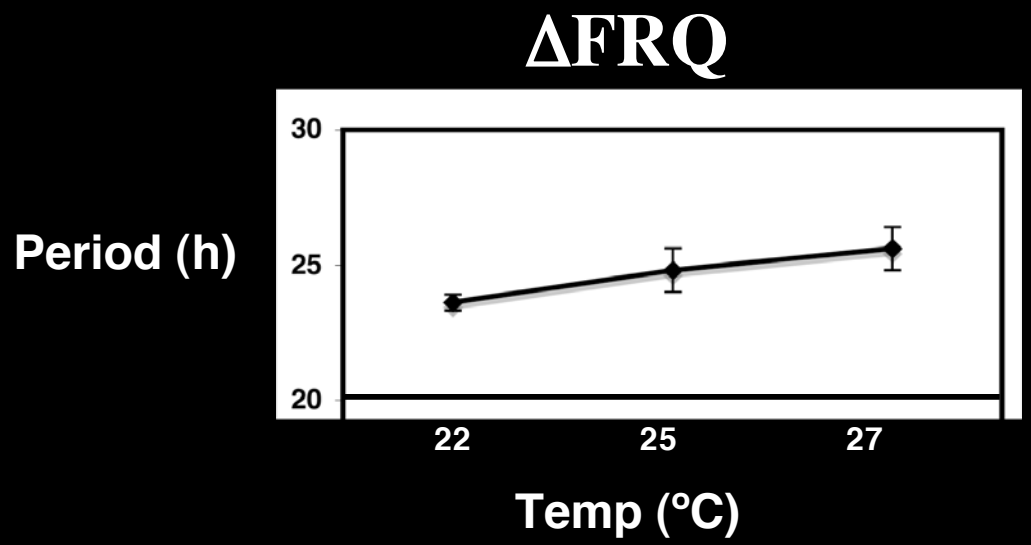


* WT = *bd*

Phase

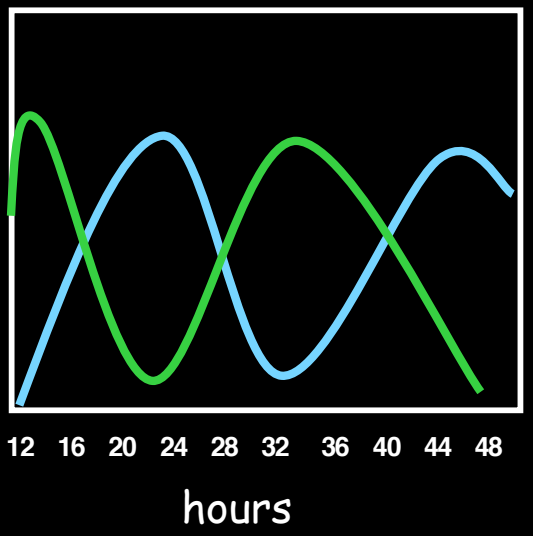
De Paula R, Lewis, Z et al 2006 JBR

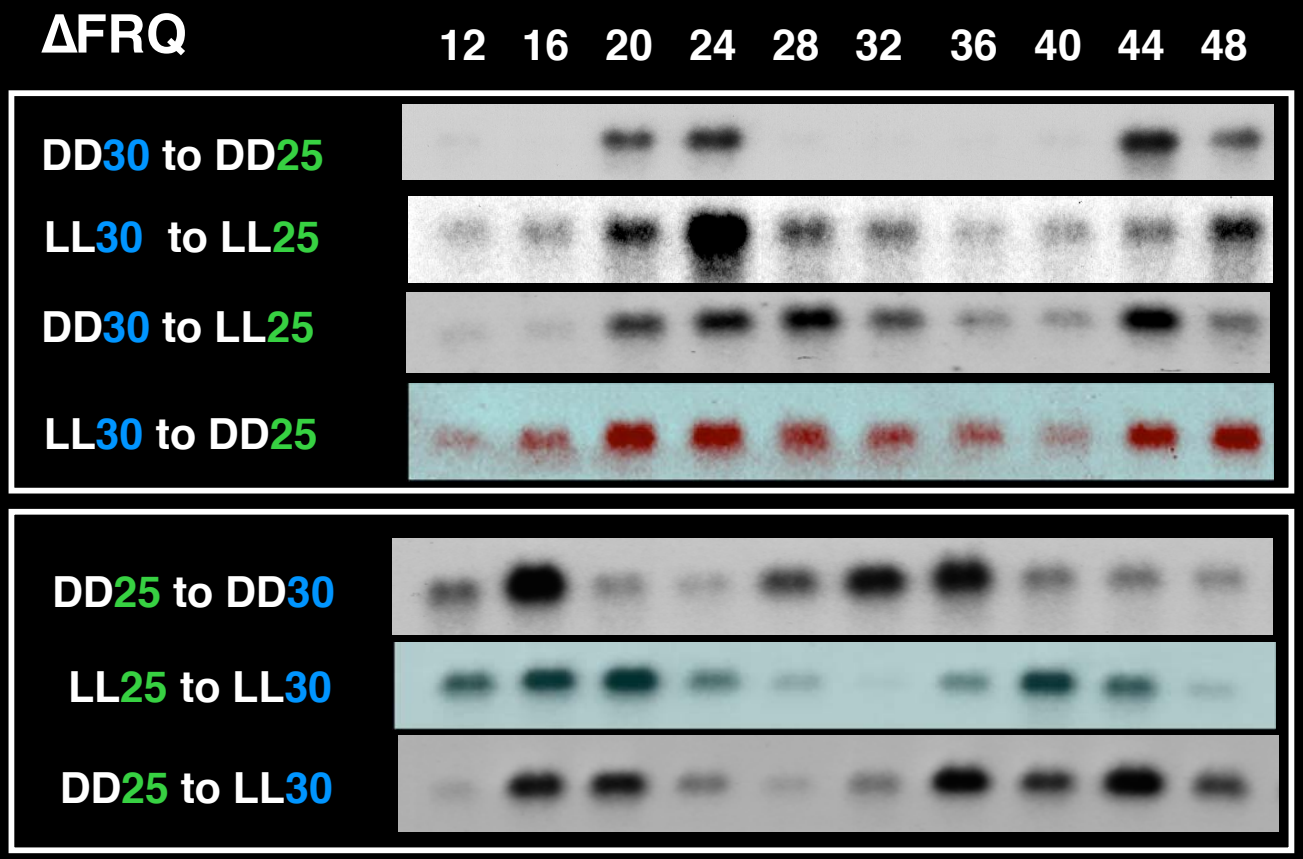
The *ccg-16* mRNA rhythm is temperature compensated



Q_{10} = 0.9

The FLO is synchronized by temperature shifts





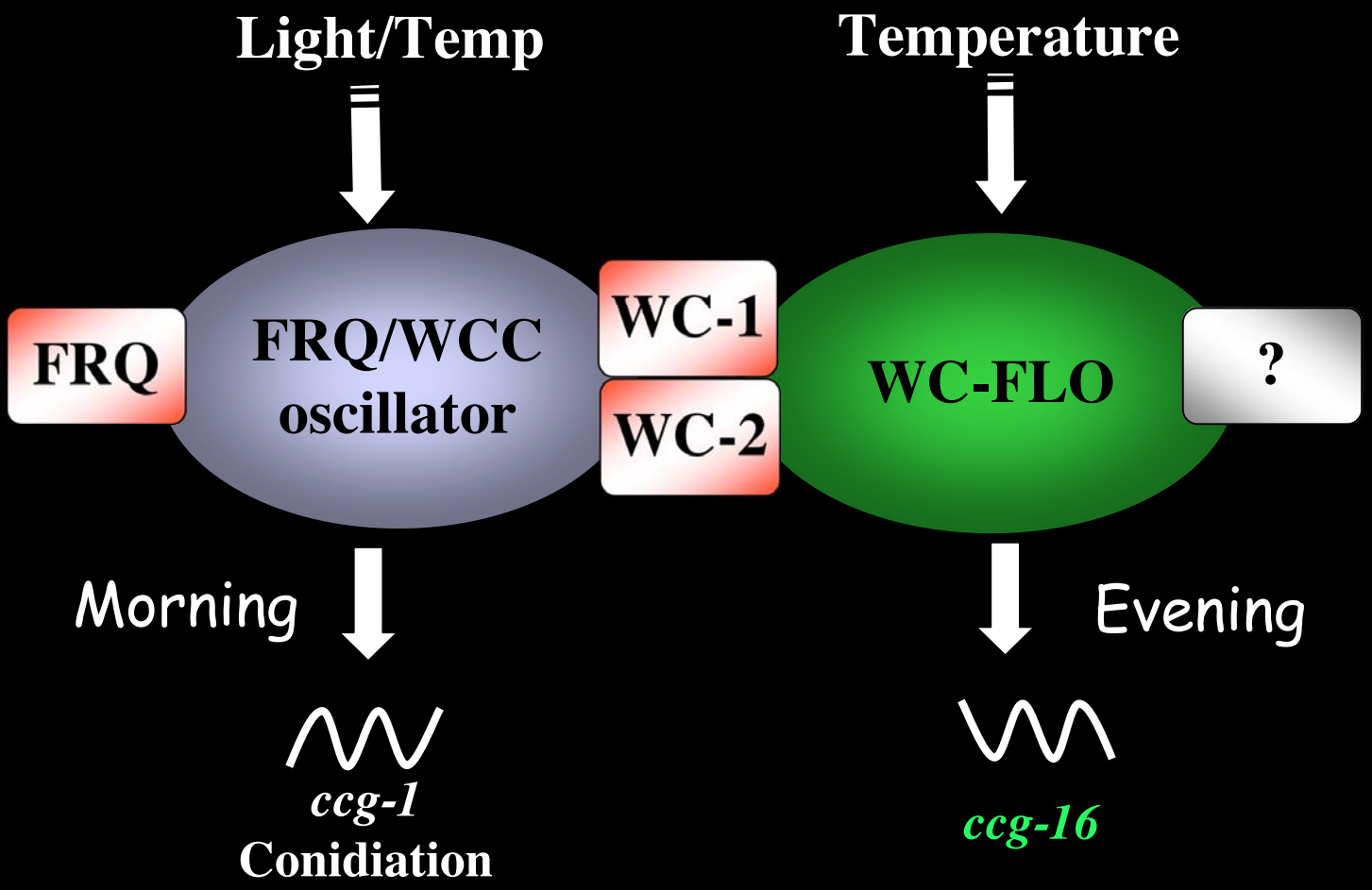
A circadian WC-FLO:

a. The WC-FLO programs a free-running rhythm in the absence of a functional FRQ oscillator.

b. The WC-FLO is temperature compensated.

c. The WC-FLO is responsive to temperature shifts.

Evidence of a 2nd Circadian Oscillator



Pitt E&M oscillators

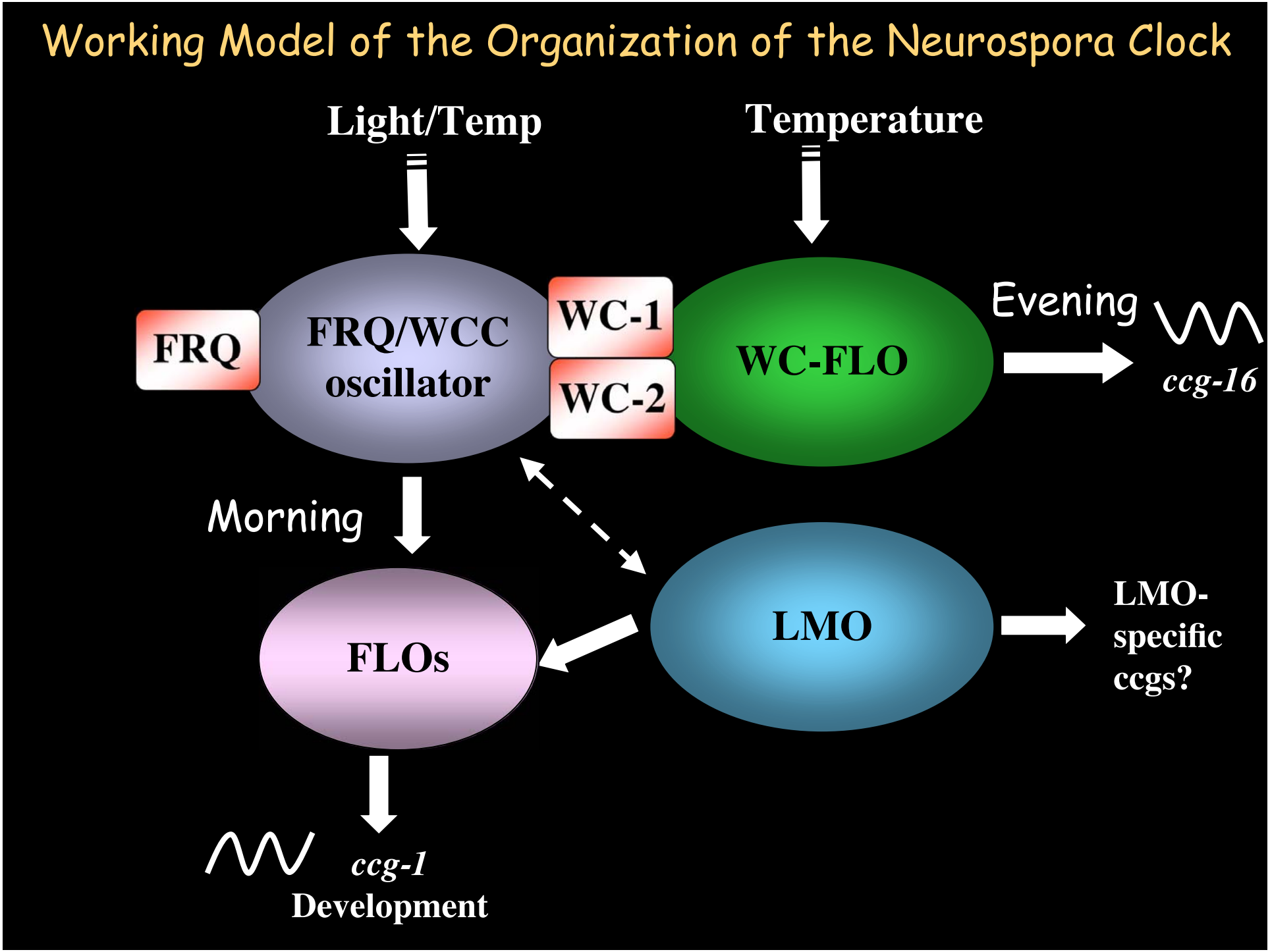
Evidence for a 3rd oscillator

Continuous bright light (1200 Lux)

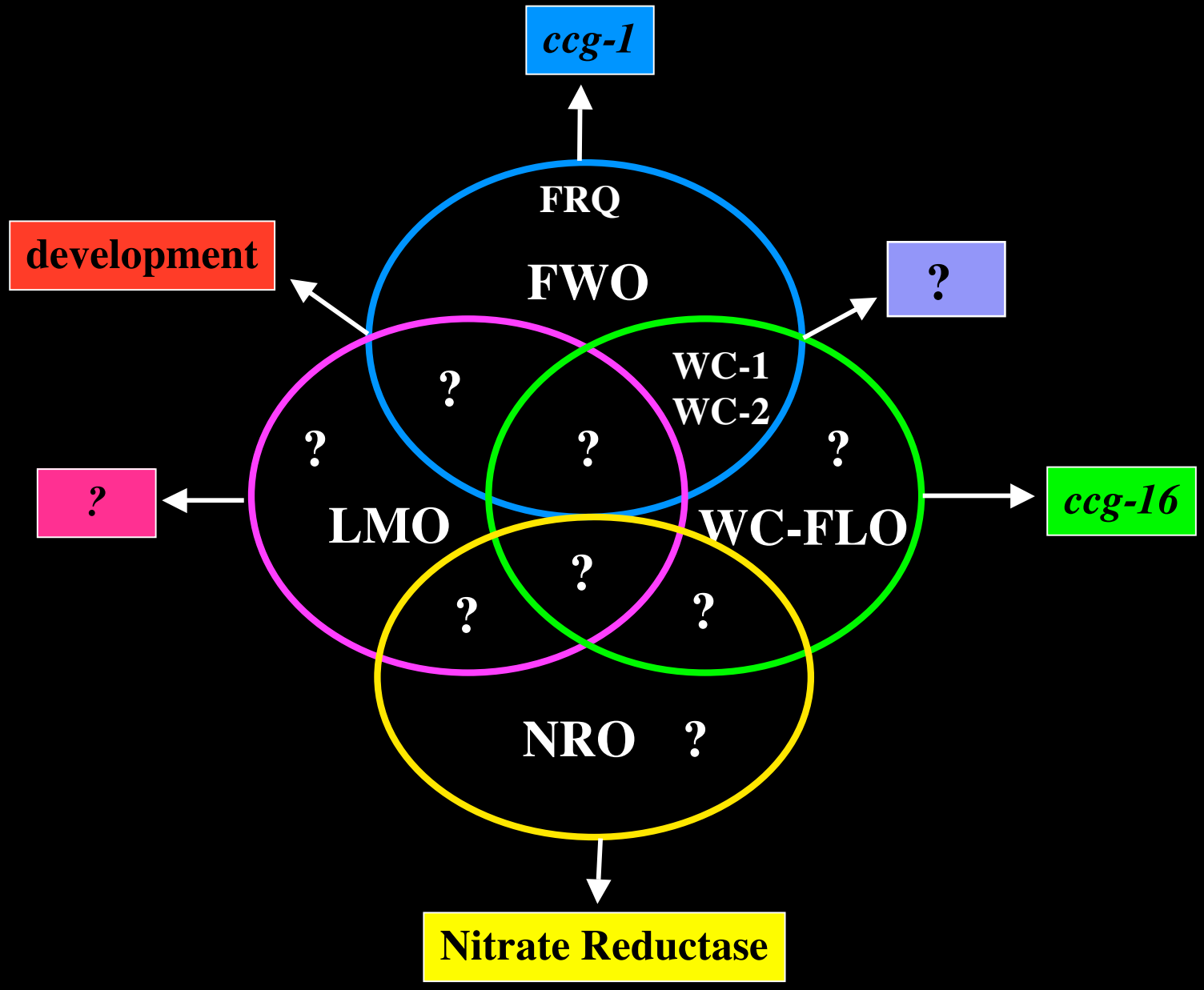
Strain		Period 22°C
WT		
LM1		18 h ± 1.2
ΔWC-1, LM1		23 h ± 1.8
ΔFRQ, LM1		23 h ± 1.6

Huang, H., Seo, K., unpublished

Working Model of the Organization of the Neurospora Clock



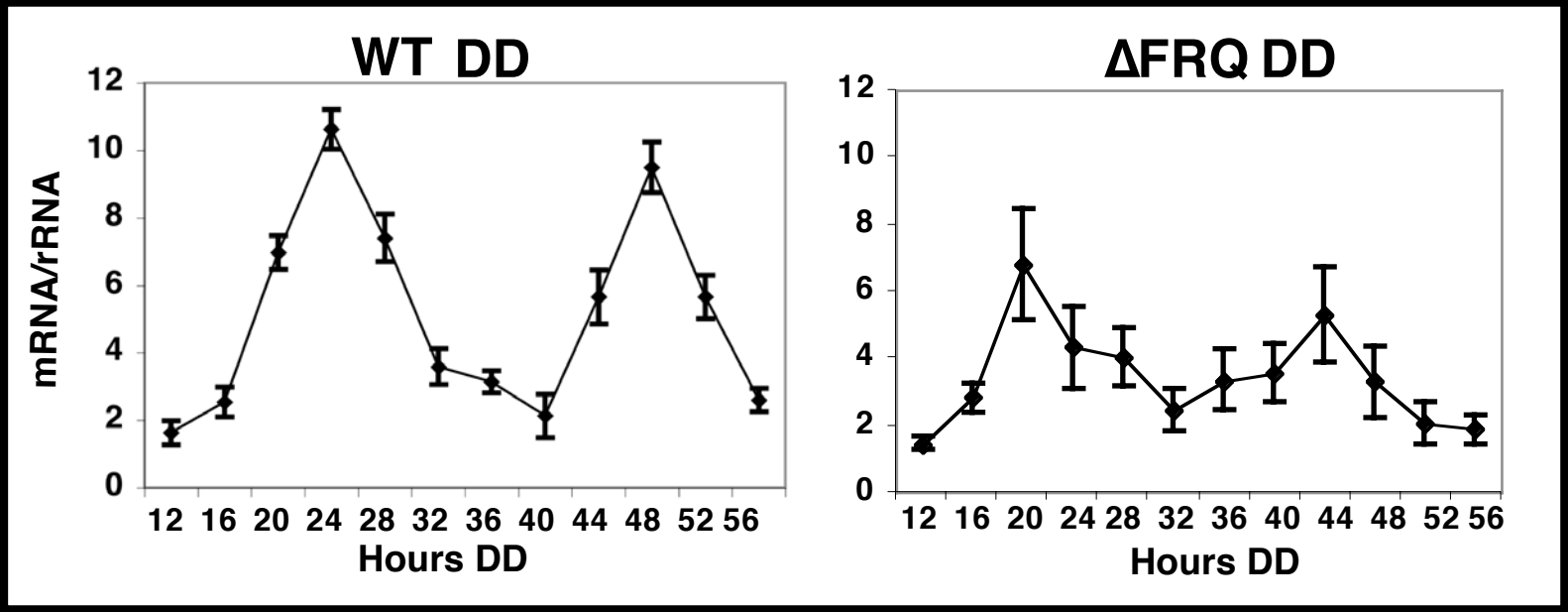
Multilayered Organization of a Circadian Clock



Why multiple oscillatory loops?

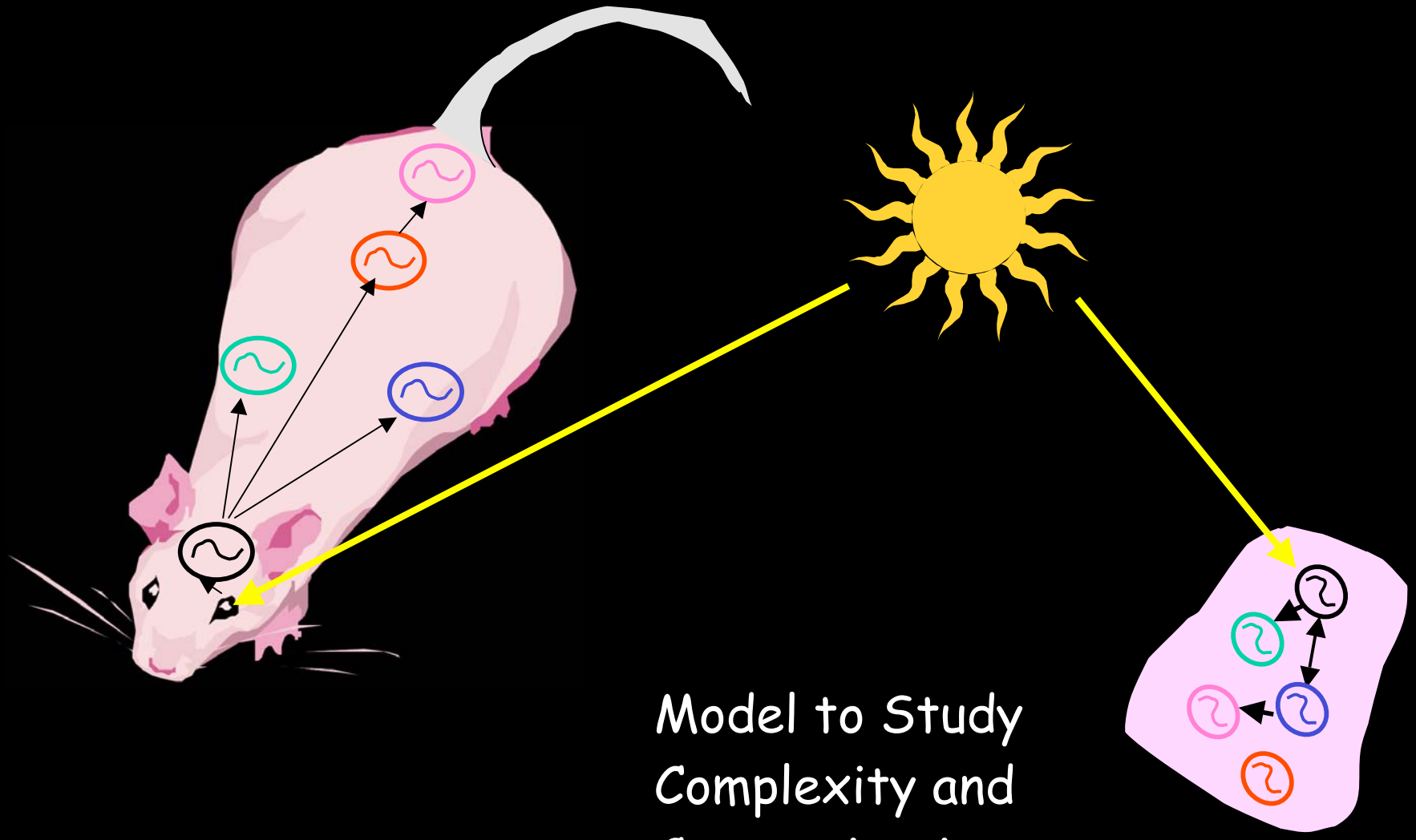
- Different inputs/outputs
- Provide precision?

The FRQ/WCC oscillator affects the phase and amplitude of *ccg-16* rhythms



N ≥ 5

Clock organization and oscillator complexity



Model to Study
Complexity and
Communication

Output pathways

How do oscillators signal through output pathways to control overt rhythmicity?

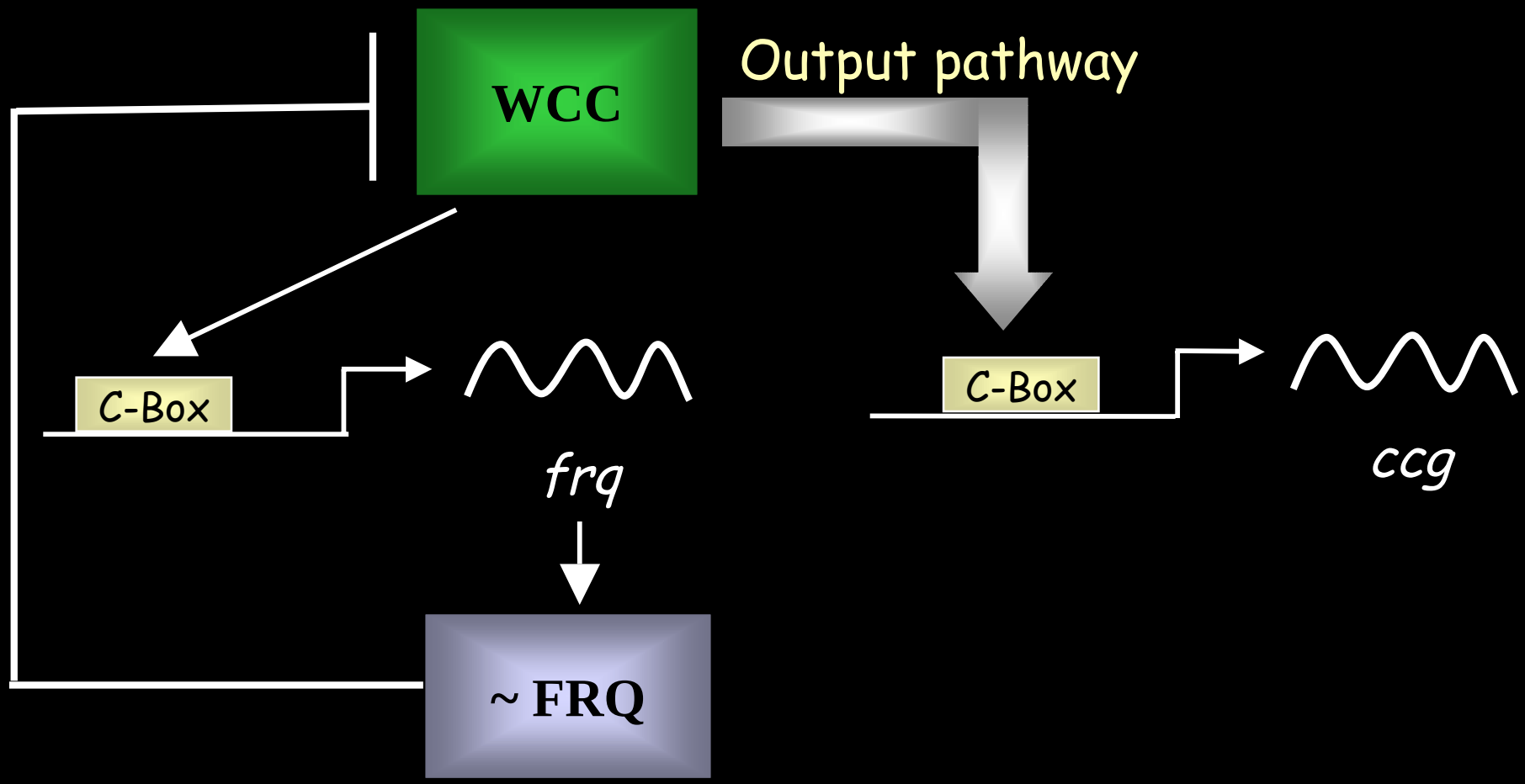
Focus on the FRQ/WCC oscillator

182 clock-controlled genes (ccgs)/~1400 that peak in expression at different times of day

Category	#
Cell Division	2
Cell Signaling/Communication	17
Cell Structure/Cytoskeleton	10
Cell Defense	6
Development	10
Gene Expression	5
Metabolism	32
Protein Synthesis	33
Protein Processing	10
Unclassified	57
Total	182

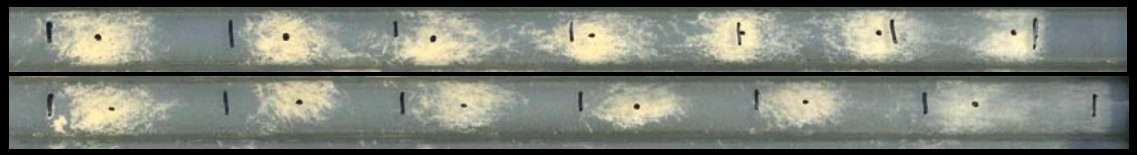
Full genome arrays in progress

18 ccgs contain a Clock (C) - box in their promoter



Knockout of candidate genes

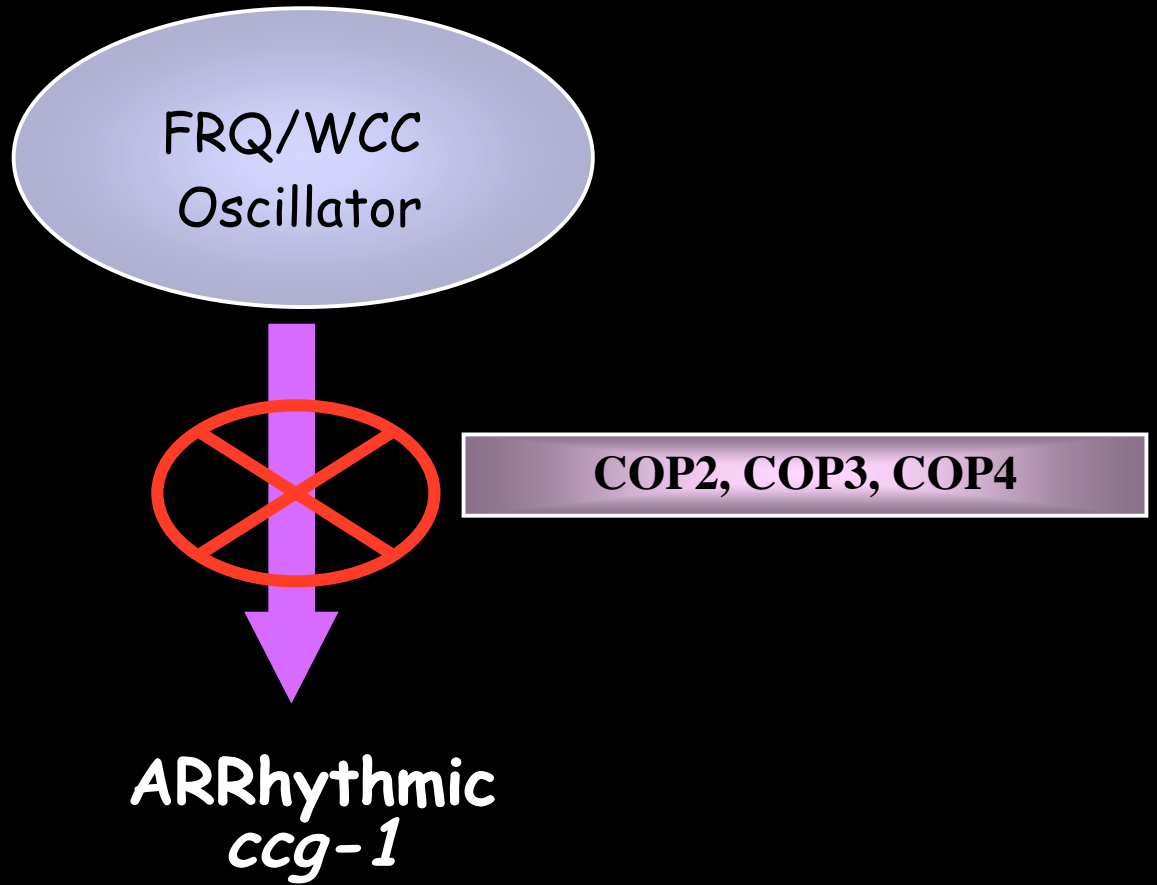
SHK-1 kinase binding protein (*skb-1*)



	Period
WT	22 h
Δskb-1	24 h

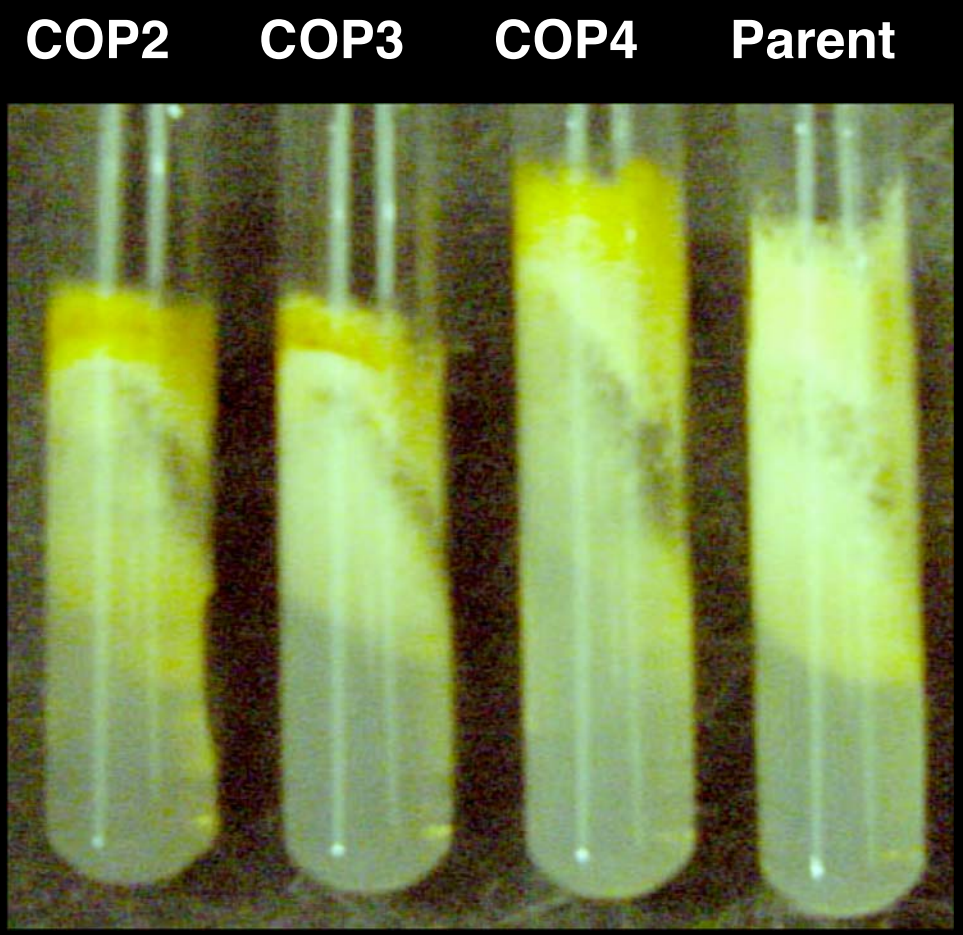
microarrays

Genetic selection for mutations in the output pathways regulating rhythmic *ccg-1* expression

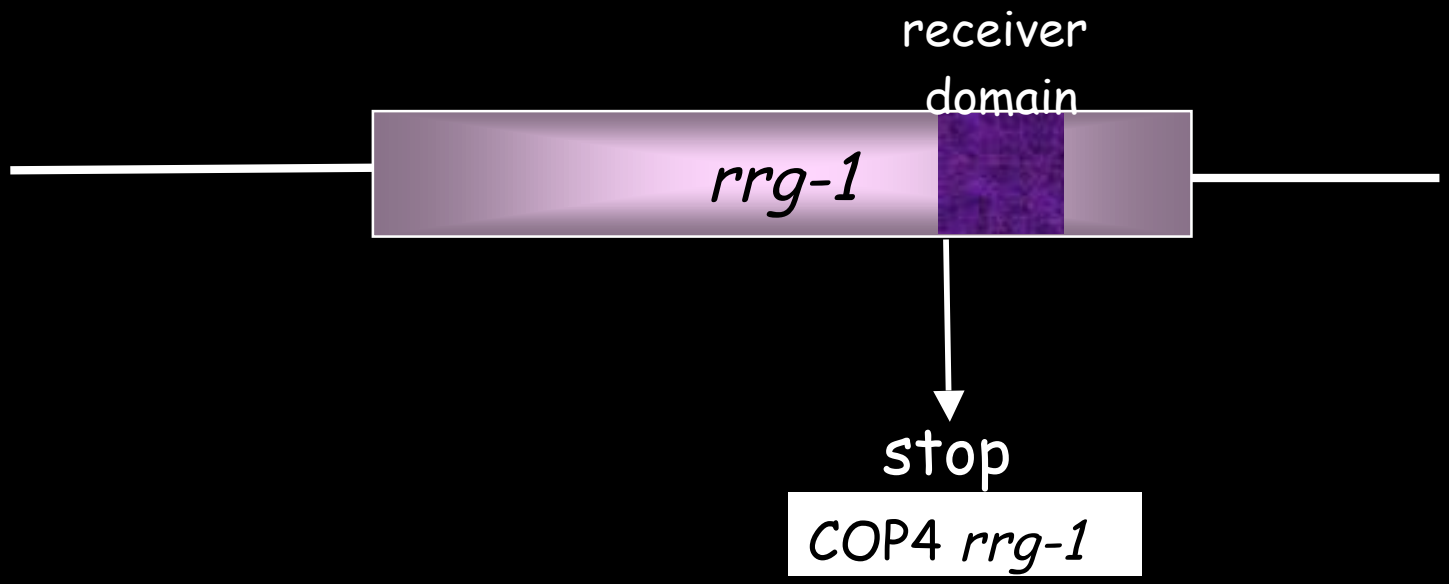


Vitalini et al, 2004 Genetics; Vitalini et al, PNAS submitted

The COP mutants have a phenotype

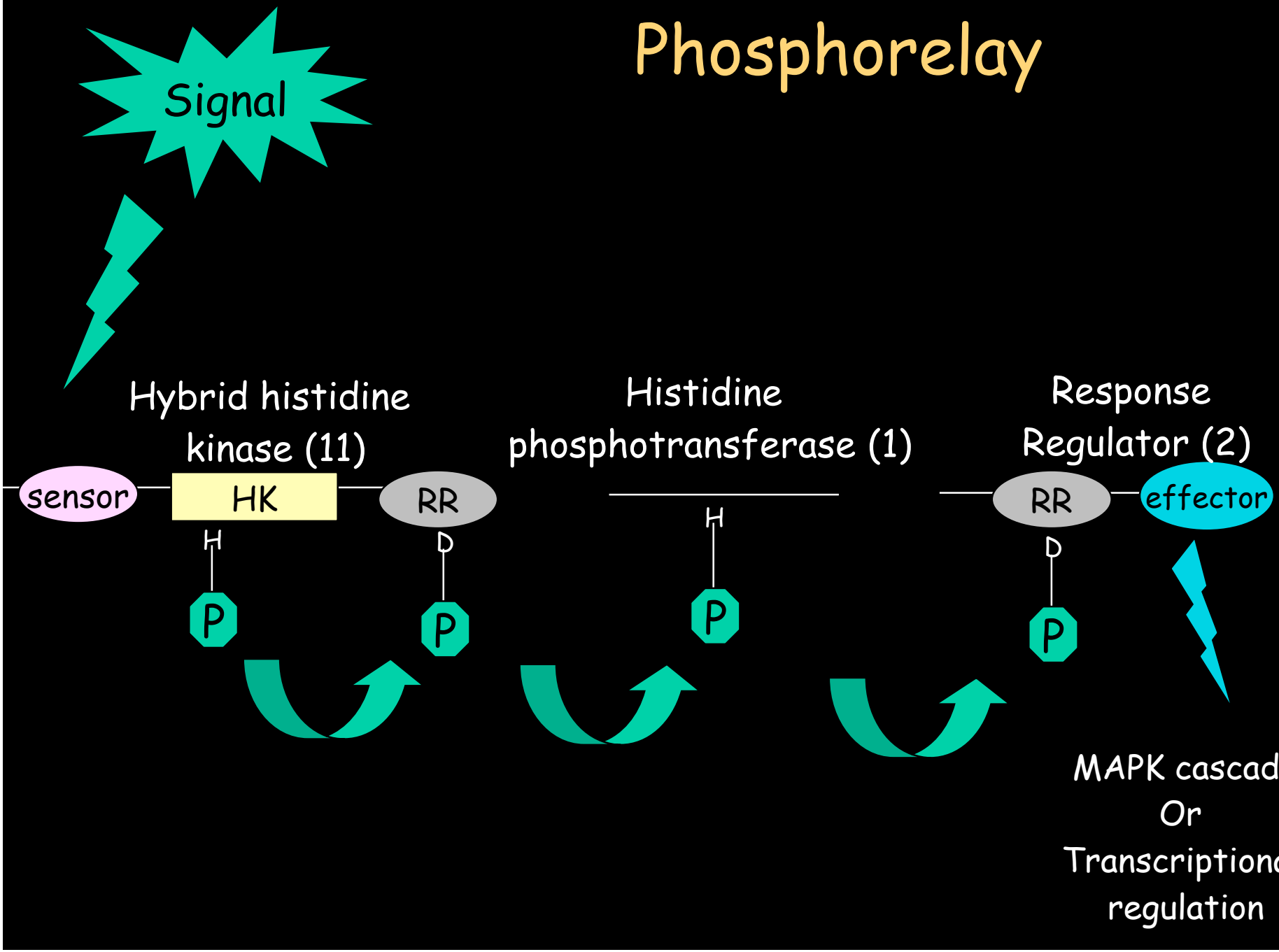


Genetic mapping localized COP4 to the *rrg-1* gene that encodes a response regulator

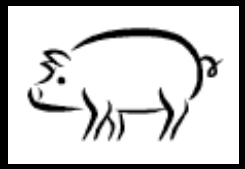


Jones, CA., Greer-Phillips, S., and Borkovich, KA. 2007 MBC in press

Phosphorelay



Phosphorelay



Signal

Sensor Histidine Kinase — OS-1

Histidine Phosphotransferase — HPT-1

Response Regulator — RRG-1 — COP4, COP3

MAPKKK — OS-4

MAPKK — OS-5

MAPK — OS-2 — COP2

Osmotic stress resistance, Conidial integrity, Sexual Dev't

Rhythmic gene expression

rrg-1 is not required for FRQ/WCC oscillator function

OS-1

HPT-1

RRG-1

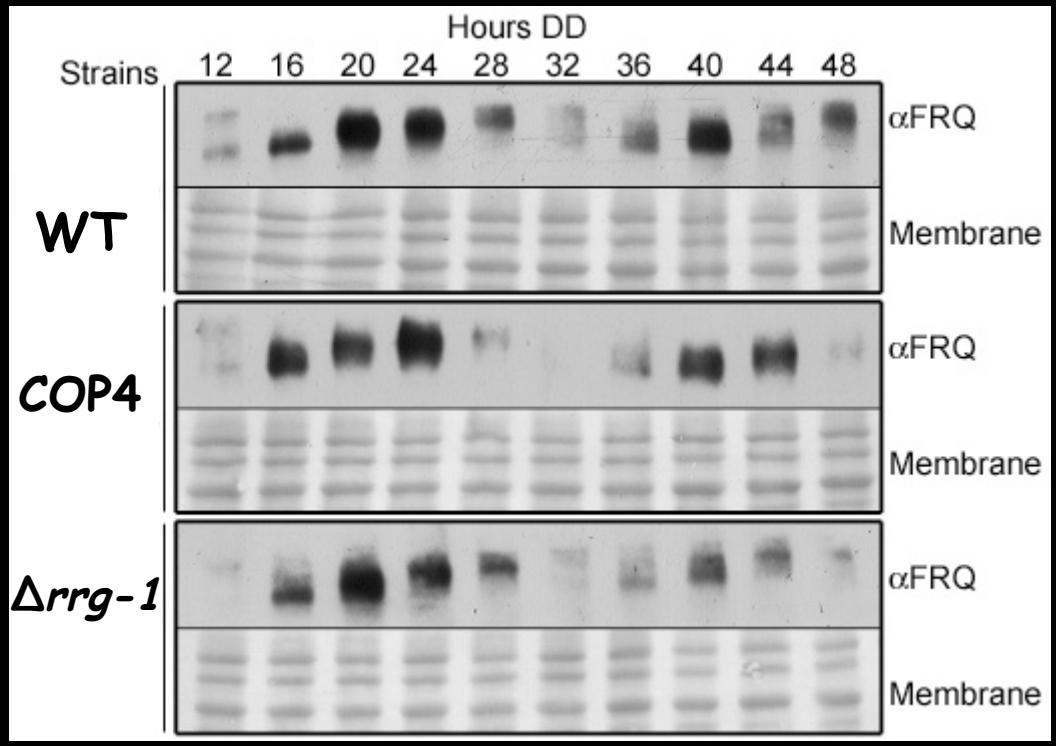
OS-4

OS-5

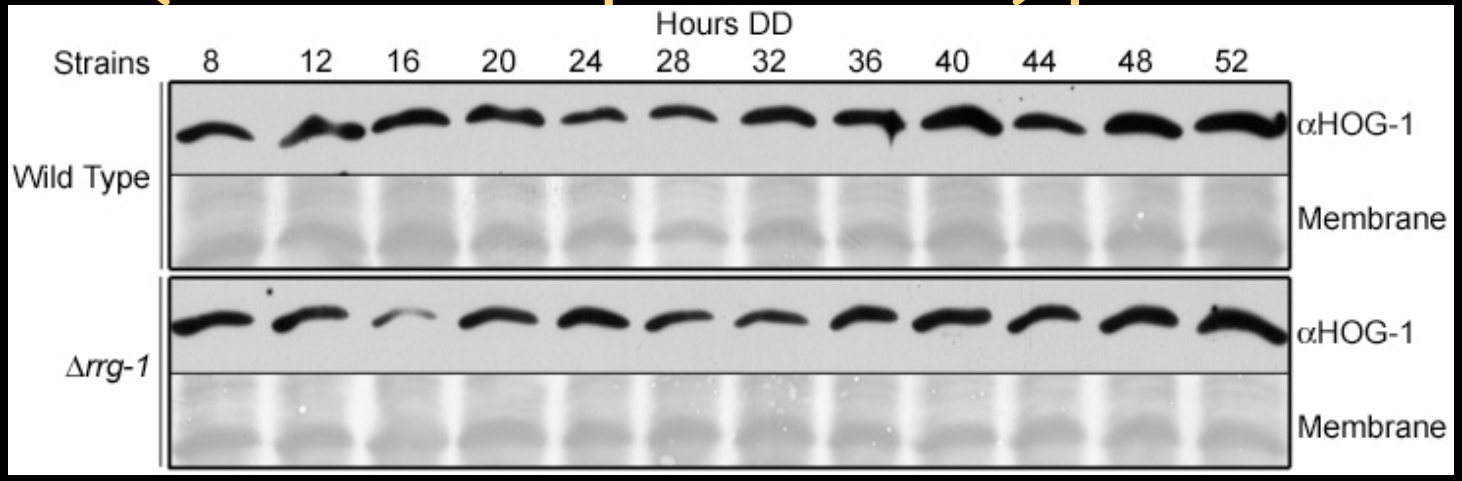
OS-2

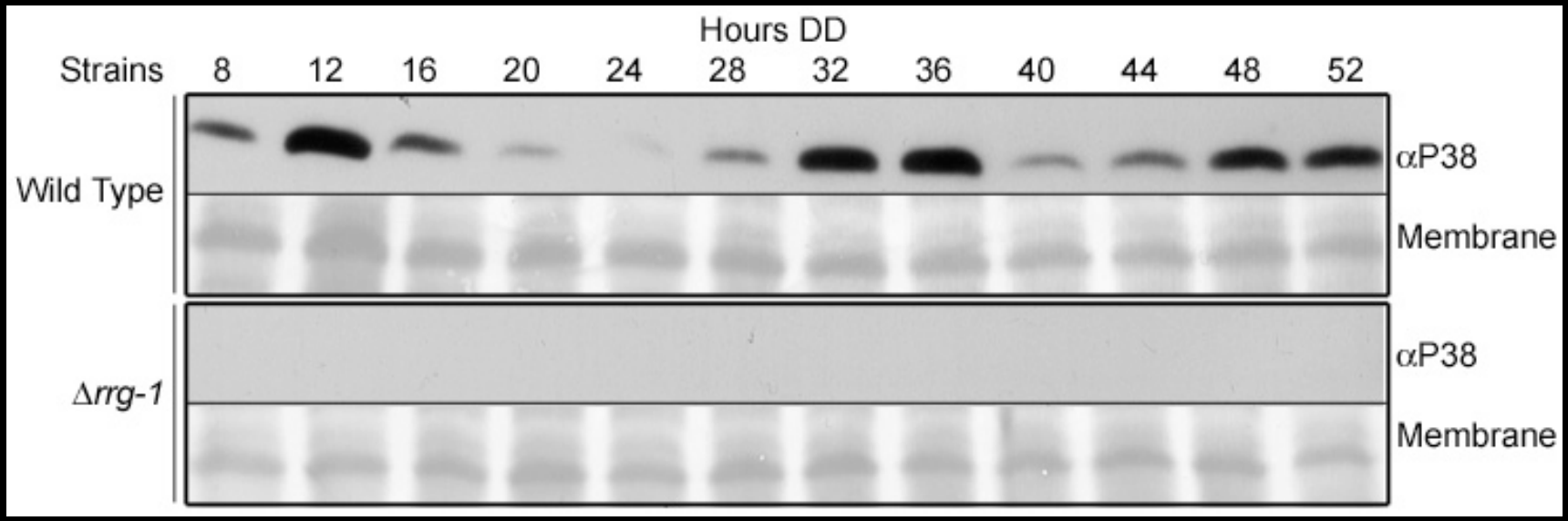
Rhythmic *ccg-1*

?

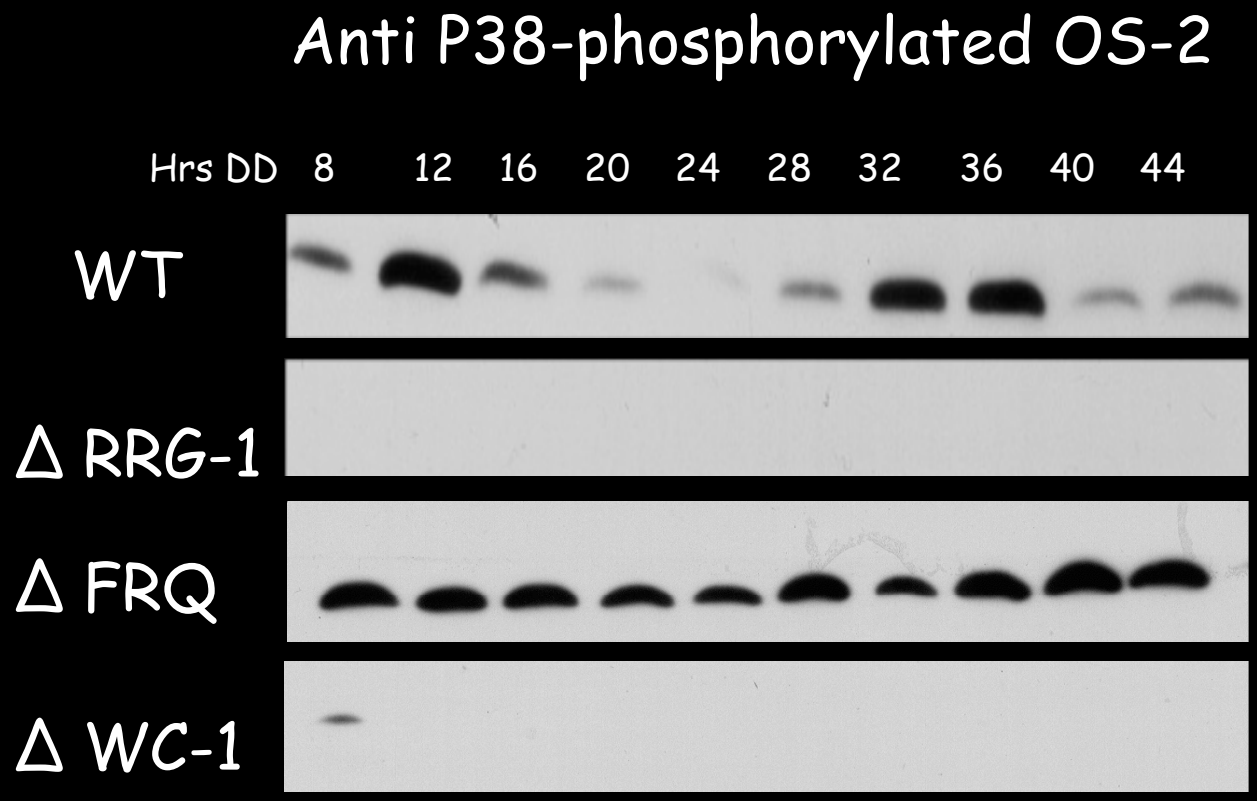


Circadian rhythm in phosphorylated OS-2 (conserved p38 MAPK) protein

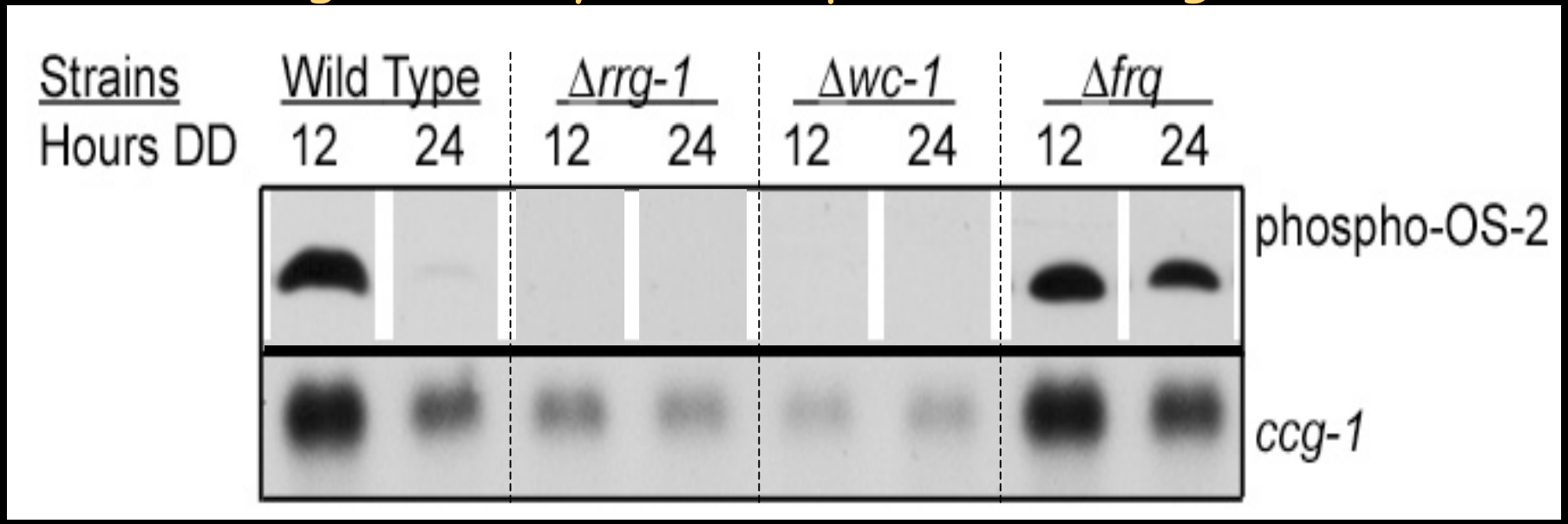


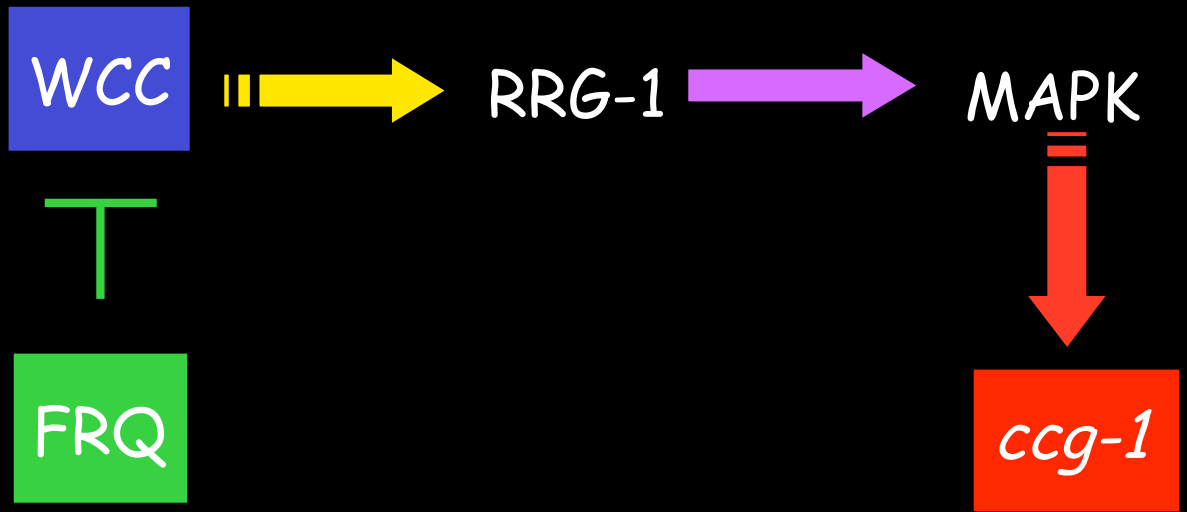


The activity of the Neurospora HOG pathway is regulated by the clock

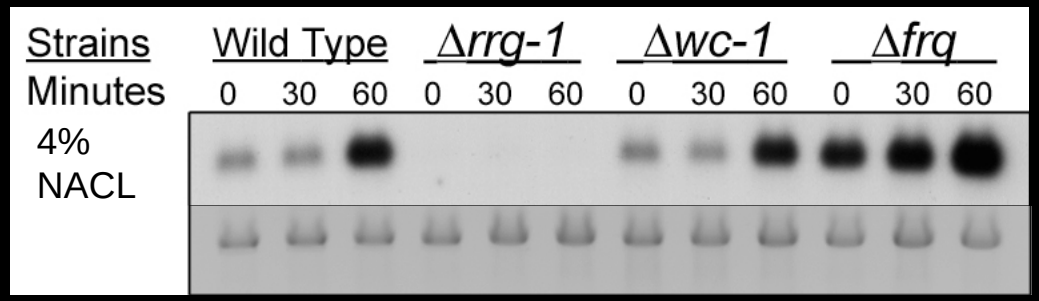


The HOG pathway is an output of the FRQ/WCC that regulates rhythmic expression of *ccg-1*





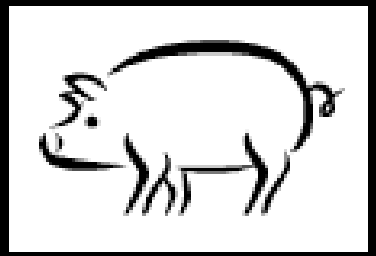
The FRQ/WCC oscillator is not necessary for the osmotic stress response



Osmotic Stress

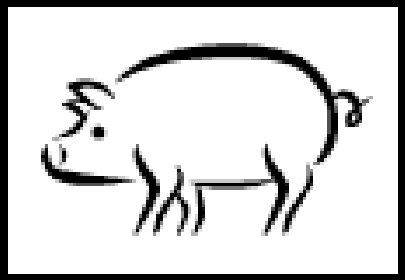
FRQ/WCC oscillator

~



Gene Expression (*ccg-1*)

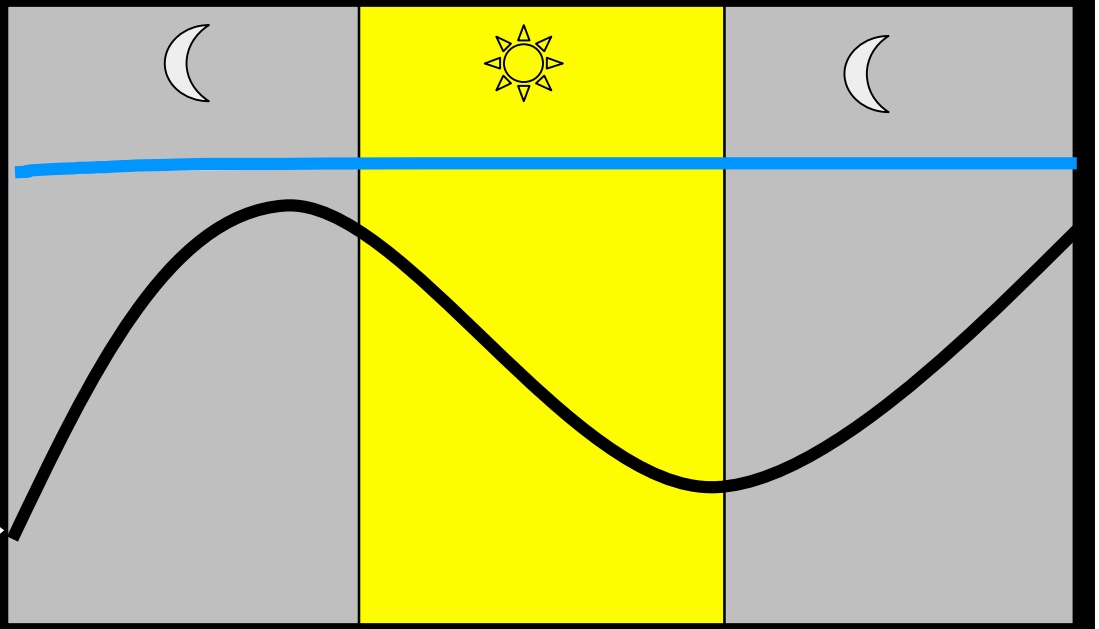
Osmotic Stress



Transcription factors

Gene Expression

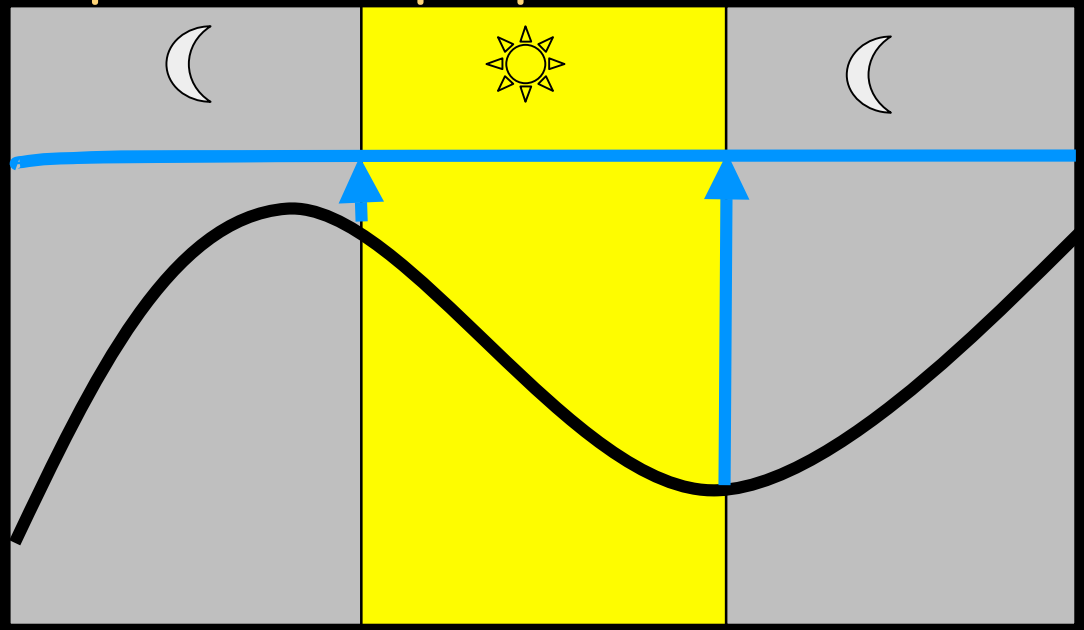
Control of the HOG pathway by the clock suggests that the rhythmic activity of the pathway allows anticipation and preparation for osmotic stress.

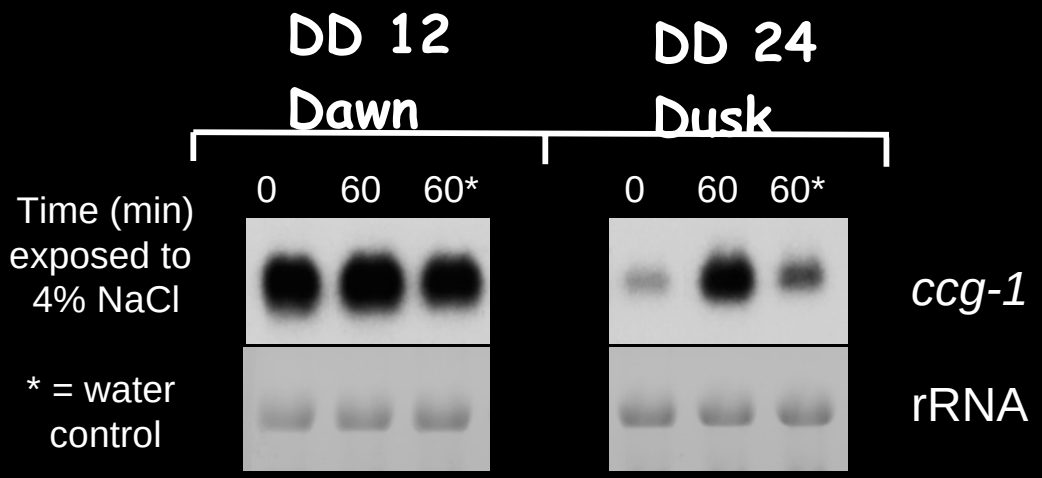


DD 12
Dawn

DD 24
Dusk

Control of the HOG pathway by the clock allows anticipation and preparation for osmotic stress





Summary:

Multiple (overlapping) oscillator loops exist within a single cell and function to regulate distinct biological rhythms, and may add to the precision of the clock.

- Important model for dissecting a multi-oscillator clock

Output pathways - The conserved HOG/P38MAPK pathway is an output pathway for the circadian clock.

- Circadian oscillators can hijack established signaling pathways
- P38 kinases mediate a variety of cellular behaviors including apoptosis, cell cycle, differentiation, inflammation, and tumorigenesis

Frequency Demultiplication

A work in progress….

Louis Morgan

*Oscillators can only be entrained within a given range of periods (limits of entrainment). When the period of the entraining cycle is close to half (third, fourth, etc.) of the oscillators endogenous period, the oscillator may entrain to every second entraining cycle (skipping a beat - known as frequency demultiplication).

In *N. crassa*, temperature, but not light cycles produce frequency demultiplied rhythms, suggesting the possibility that different mechanisms are used for entrainment.

i.e. does temp entrainment involve both the FWO and a FLO, whereas light involves only the FWO?

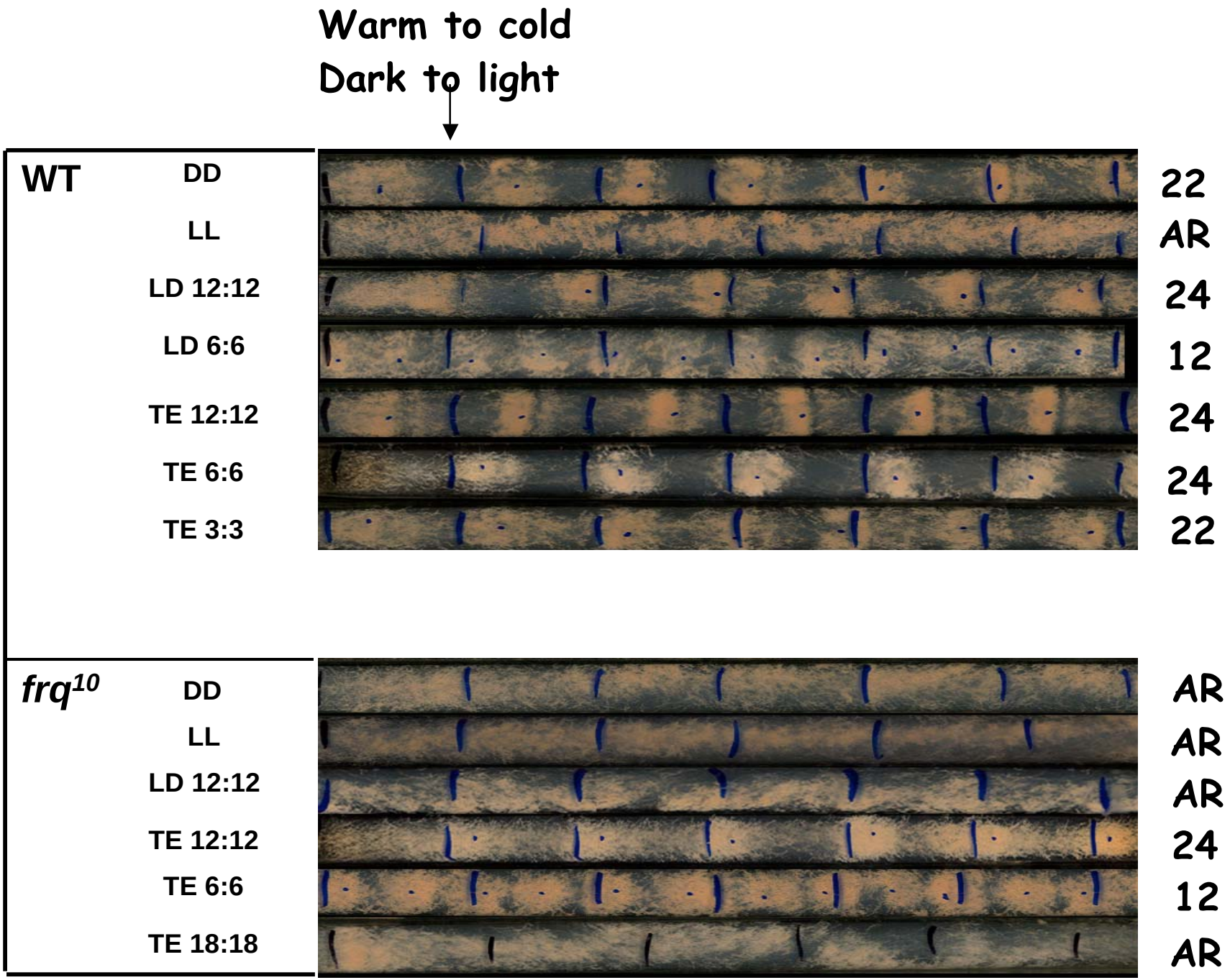
Or does light drive (mask) the rhythm

Frequency demultiplication was first discovered by Van der Pol in simulations of heart rhythms by electrical circuits, suggesting:

common features

between electrical and biological oscillators.

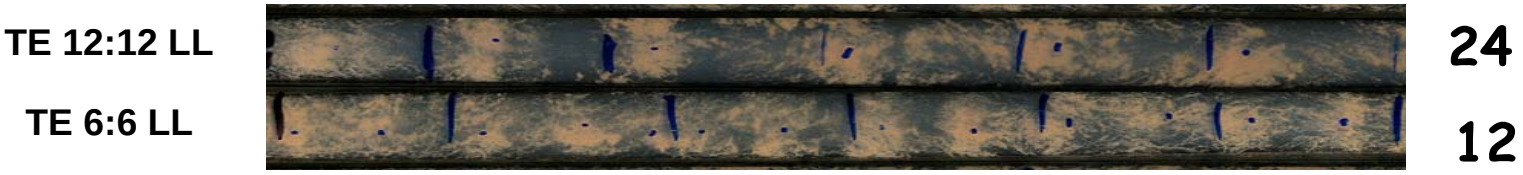
van der Pol, B. and van der Mark, J., "Frequency demultiplication", *Nature*, 120, 363-364, (1927)


Warm to cold
Dark to light
WT
DD
LL
LD 12:12
LD 6:6
TE 12:12
TE 6:6
TE 3:3
22
AR
24
12
24
24
22
frq10
DD
LL
LD 12:12
TE 12:12
TE 6:6
TE 18:18
AR
AR
AR
24
12
AR

Is an intact FRQ/WCC oscillator (and not just FRQ) required for FD?

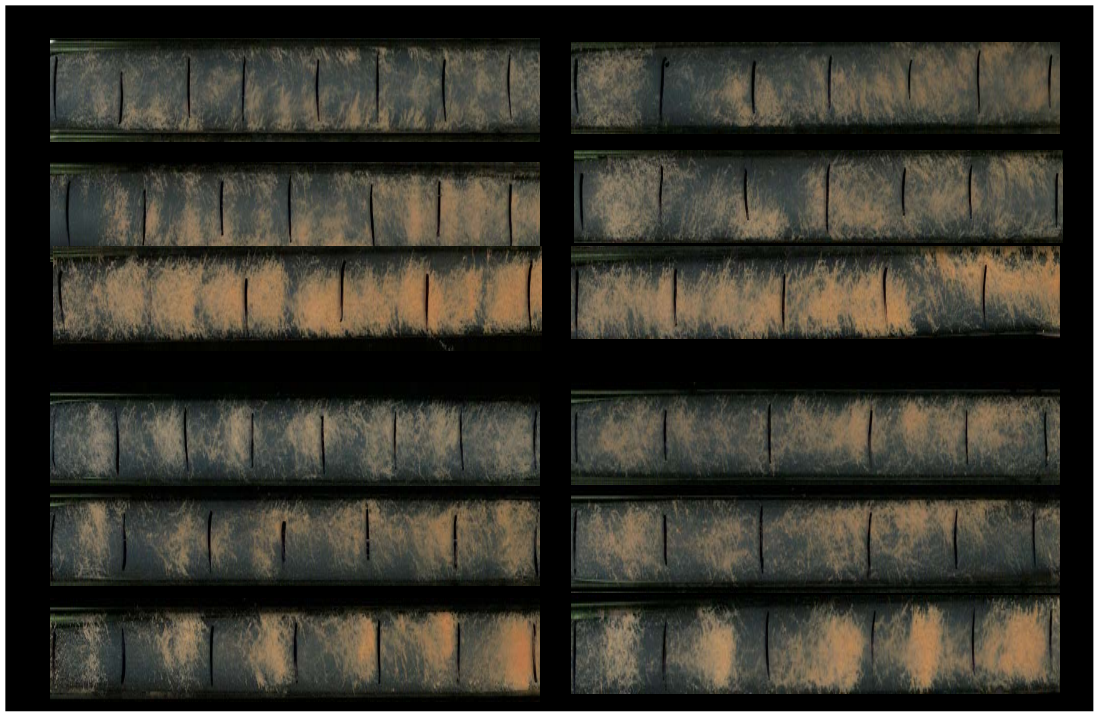
wc-1 and *wc-2* null strains also do not undergo FD

In LL, *frq* mRNA is constantly elevated (10X the DD peak)

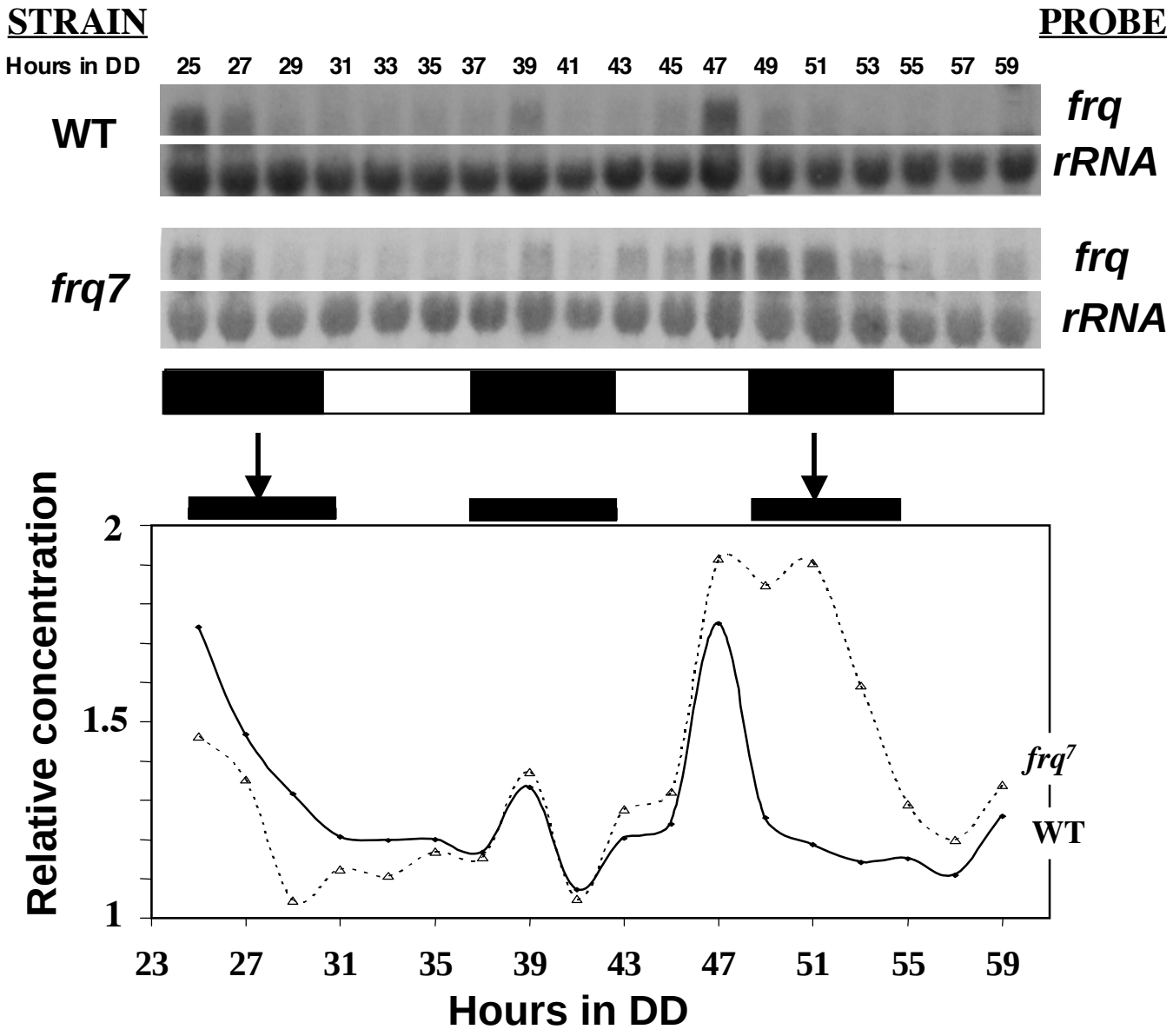


Suggests that saturation of the FRQ/WCC oscillator by LL blocks frequency demultiplication, but does not block the effects of temperature cycles on the conidiation rhythm generated by the FLO.

frq mRNA rhythms are required for overt rhythmicity
Are *frq* mRNA rhythms required for FD?



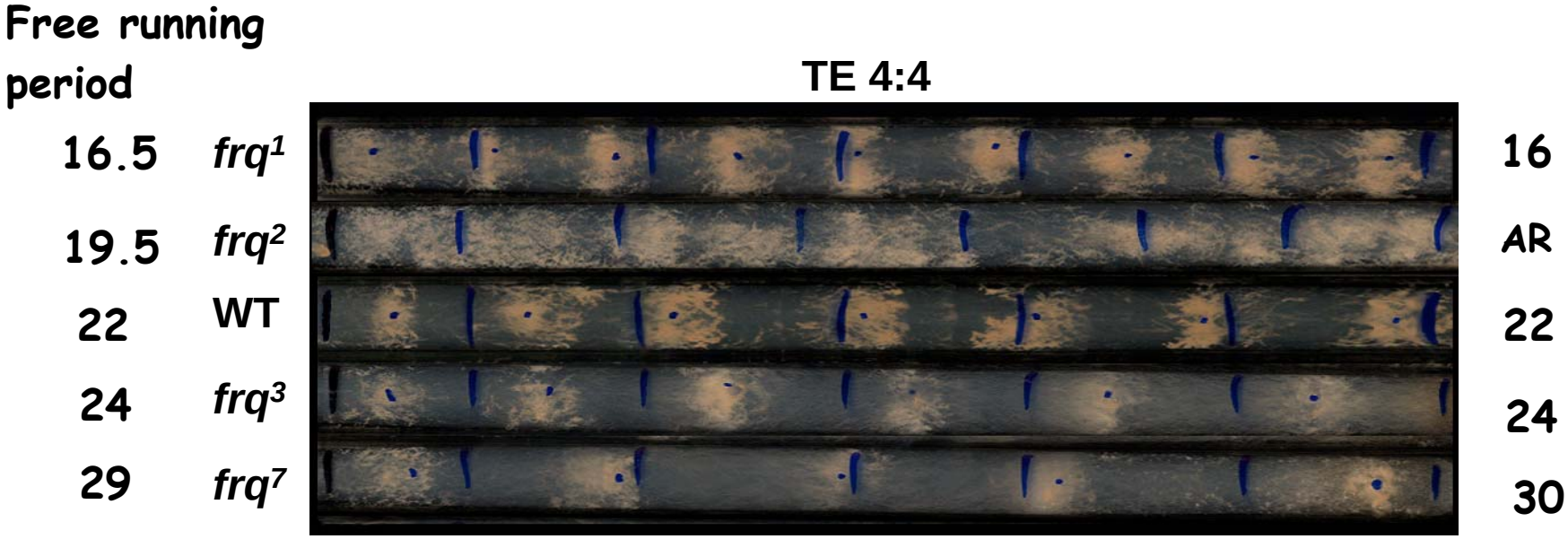
Lower threshold of inducer needed to cause FD vs. generate a free running rhythm in *qa:wc-1* suggests that the FLO can amplify the signal coming from the FRQ/WCC oscillator



Induction of *frq* by temperature is phase-dependent

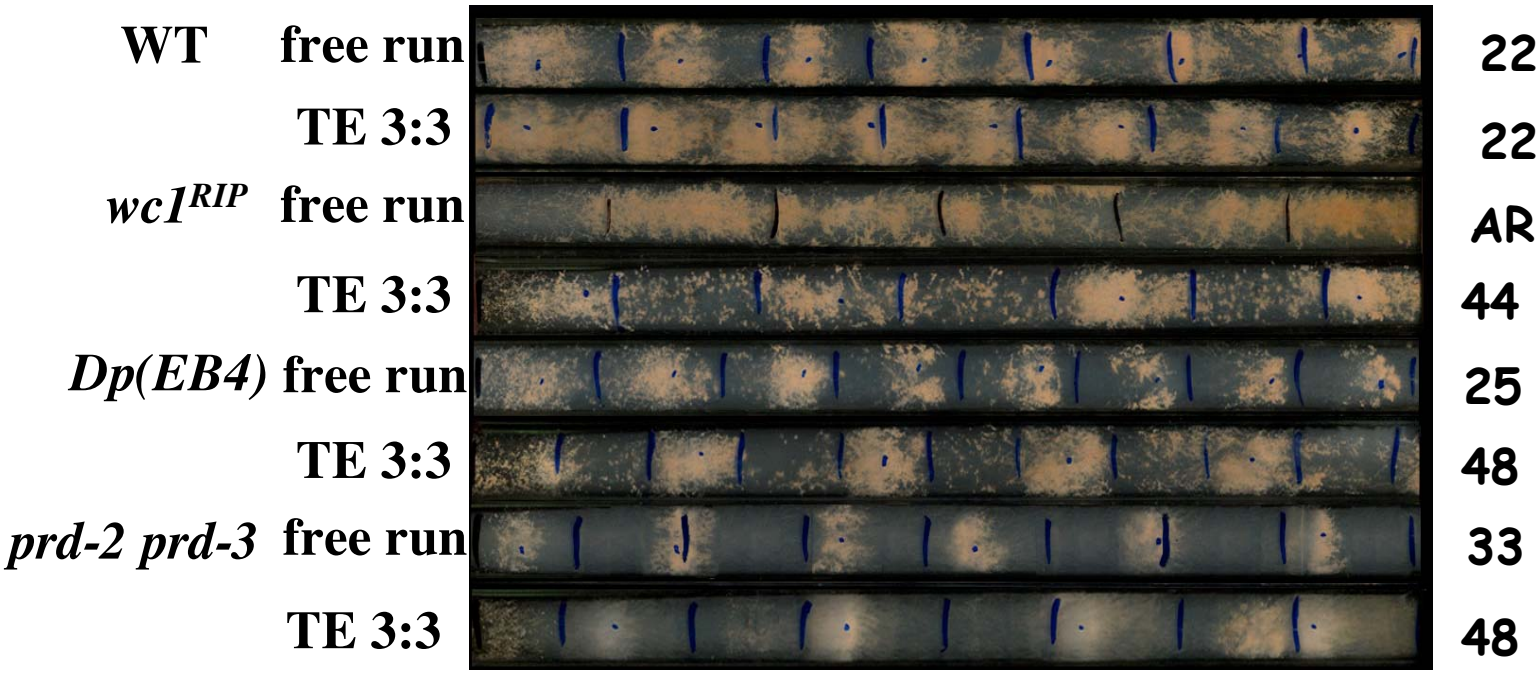
The period of the FRQ/WCC oscillator determines the period in temperature entrainment cycles

No defects in 12:12 LD or 12:12 TE cycles

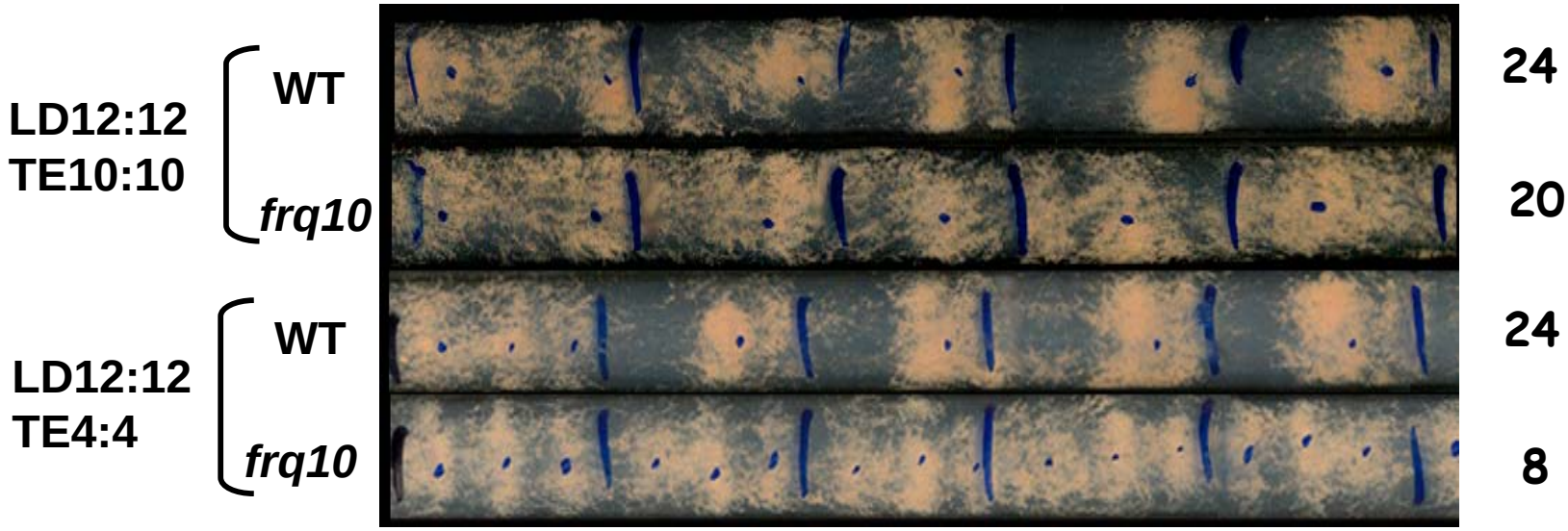


If the strains free-running period is close to 24 h (20-28) an ~ 24h rhythm was observed in TE 6:6 cycles. A 12 h rhythm if outside of this range. A rhythm close to the FRP was observed in shorter cycles, such as 4:4. But, some TE cycles caused arrhythmic behavior (frq2) or over de-multiplication!

Some strains "over-demultiply" in short TE cycles and adopt a rhythm longer than their free-running rhythm.



Light/dark cycles set the period of the FRQ/WCC Oscillator



In 4:4 TE cycles alone, WT is 22 h. Thus, the period of the demultiplied rhythm is controlled by the LD cycle period.

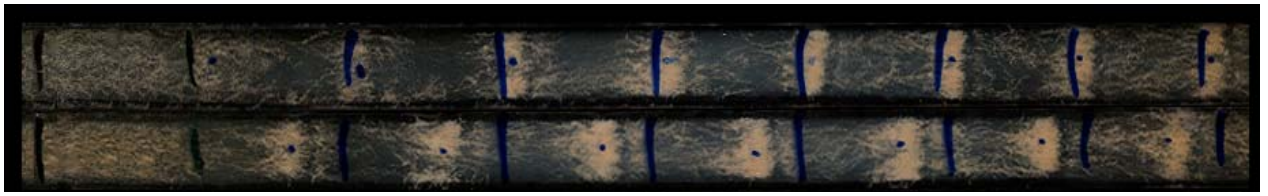
Time delay - synchronization of the oscillators is needed for FD?

Phase-locking dynamics

The adopted phase is dependent on initial conditions. WT strain grown in 6:6 TE cycles was transferred from LL 22°C to either the cold phase of the cycle (top) or warm phase of the cycle (bottom).

WT cold

WT warm



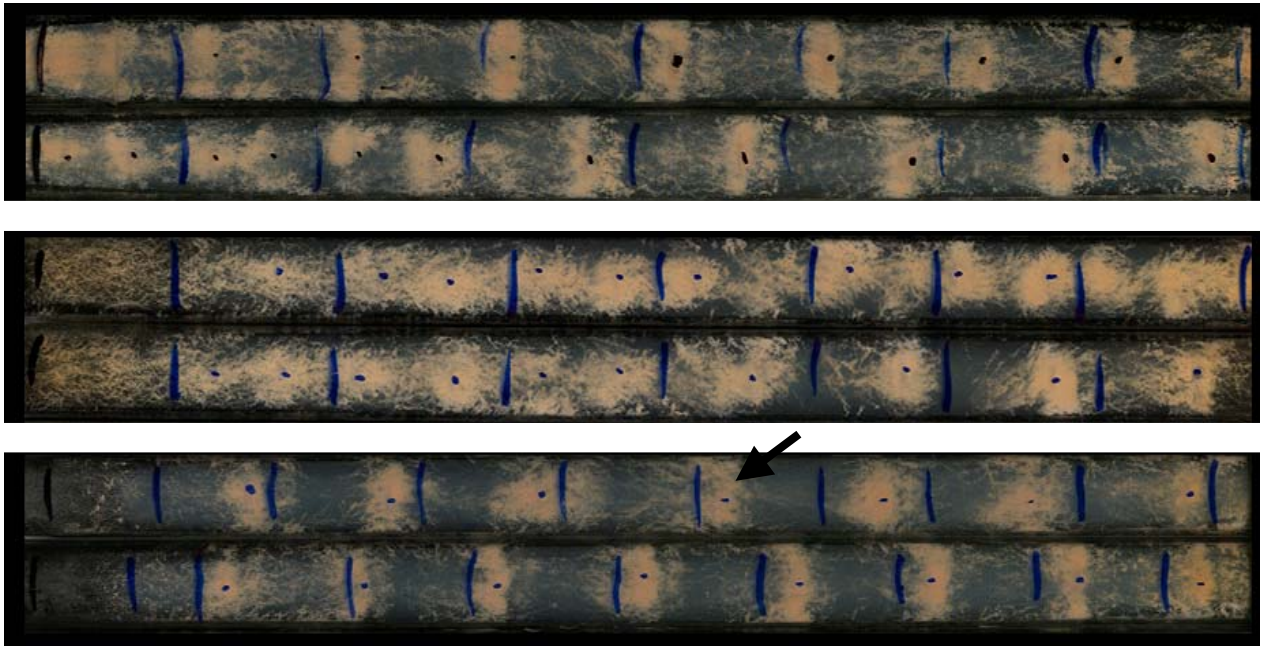
Delayed period doubling is shown between replicate cultures of the WT and *frq*^{2} strains grown in 6:6 TE cycles.)

WT

***frq*^{2}**

***frq*^{7}**

WT



The *frq*^{7} strain appears to temporarily slip out of a phase-locked condition before returning to the 24-hr entrained rhythm (black arrow); a WT strain in the same assay is shown for comparison.

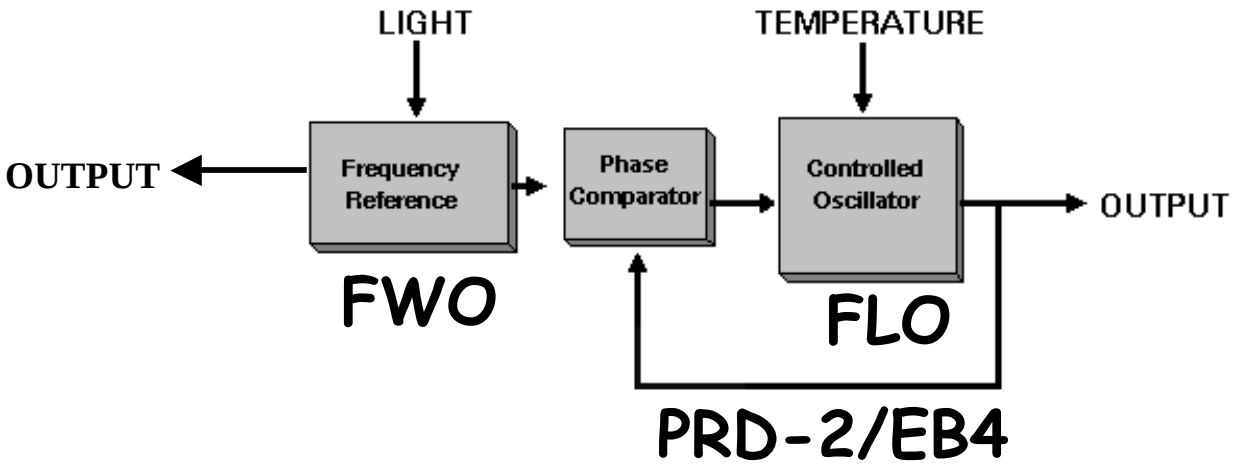
Summary:

1. Loss of oscillation of *frq* mRNA results in loss of FD, but not 1:1 entrainment in temperature cycles - consistent with presence of a temperature responsive FLO. Implies that the FRQ/WCC oscillator controls the FLO when it is intact.

2. The time delay before the onset of FD suggests that the phase-locking of the FRQ/WCC oscillator and the FLO occurs after a temperature induced oscillation is manifest in the FLO - suggests a mechanisms is present which controls the coupling between the FRQ/WCC oscillator and the FLO.

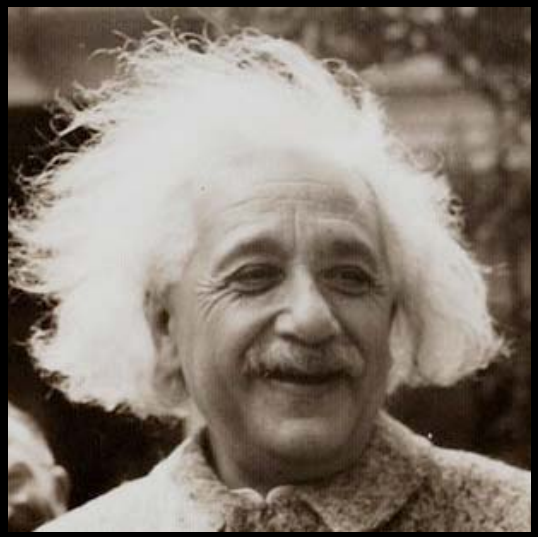
3. Over-demultiplication seen in *prd-2* and EB4 mutations suggest that these have defects in the oscillator coupling mechanism.

4. Demultiplied oscillations tend to be attracted towards the natural frequency of the system (their FRP).



“The only reason for time is so that everything doesn't happen at once.”

Albert Einstein



Acknowledgements

Output

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ccg-16

Renato de Paula

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Zachary Lewis

Andrew Greene

LM mutations

Kyung Seo

Howard Huang

Xiaoguang Liu

Kaitlyn Beasley

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UC Riverside

FRQ is a central component of the core Neurospora feedback loop

(Aronson et al, 1994)

- Loss of function mutations of *frq* result in loss of normal free running rhythms in conidiation.
- Partial function mutants alter period (16-29 h) and temperature compensation.
- FRQ protein and *frq* mRNA accumulation are rhythmic in DD and rhythmic expression is essential for conidiation rhythms.
- Overexpression of *frq* at an ectopic locus represses transcription from the endogenous locus - feedback loop

WC-1 and WC-2; regulate all known blue light responses in Neurospora (G. Macino) and are central oscillator components

(Crosthwaite et al, 1997).

Role in DD - null mutants are arrhythmic in DD

WC-1: A blue-light photoreceptor, binds the flavin chromophore FAD, protein accumulation is rhythmic.

WC-2: mRNA and protein are abundant and not rhythmic.

WC-1/WC-2 heterodimerize through their PAS domains to form a complex (WCC) which can activate *frq* transcription - WCC binds to the *frq* promoter at 2 sites.

How does the negative feedback loop keep from winding down?

Interlocked loops - observed in all eukaryotic circadian oscillators

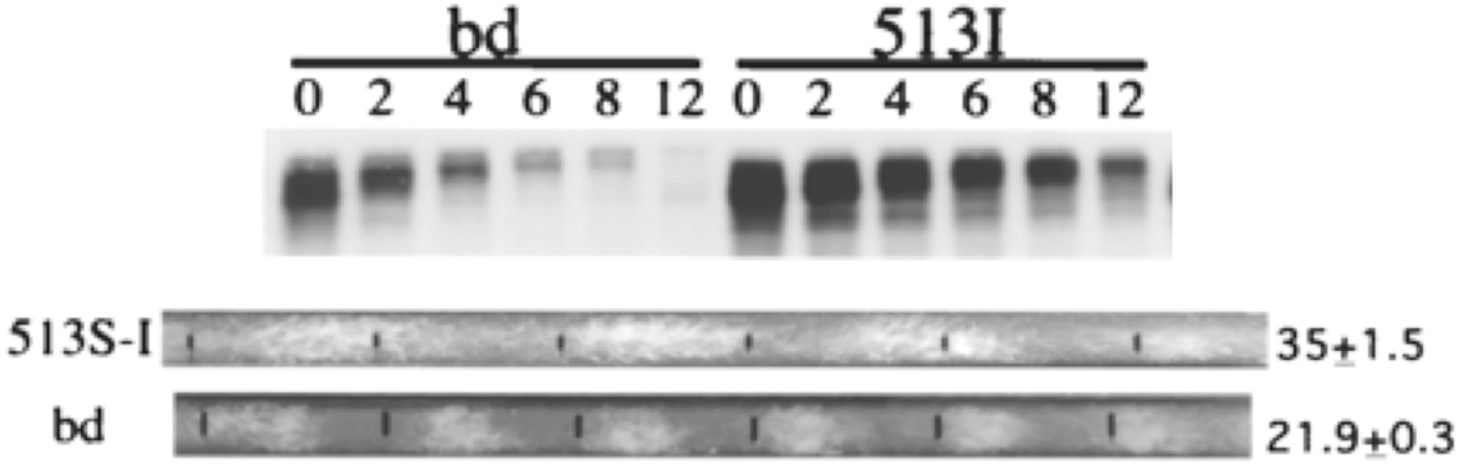
Lee K, Loros JJ, Dunlap JC.
Interconnected feedback loops in the Neurospora circadian system.
Science. 2000 Jul 7;289(5476):107-10.

FRQ turnover

Yi Liu

Several kinases can phosphorylate FRQ and mutation of the kinases affects the period and stability of FRQ.

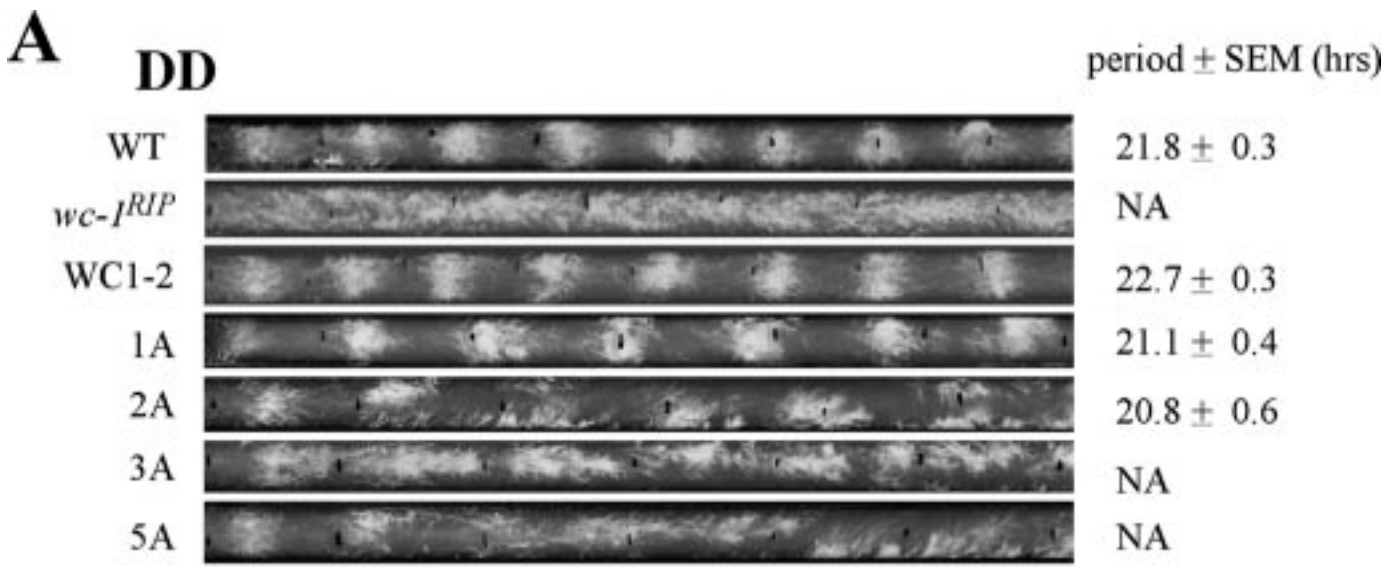
Ser 513 critical for FRQ function - a single substitution of isoleucine stabilizes FRQ and lengthens the period from 22 to 35 h.



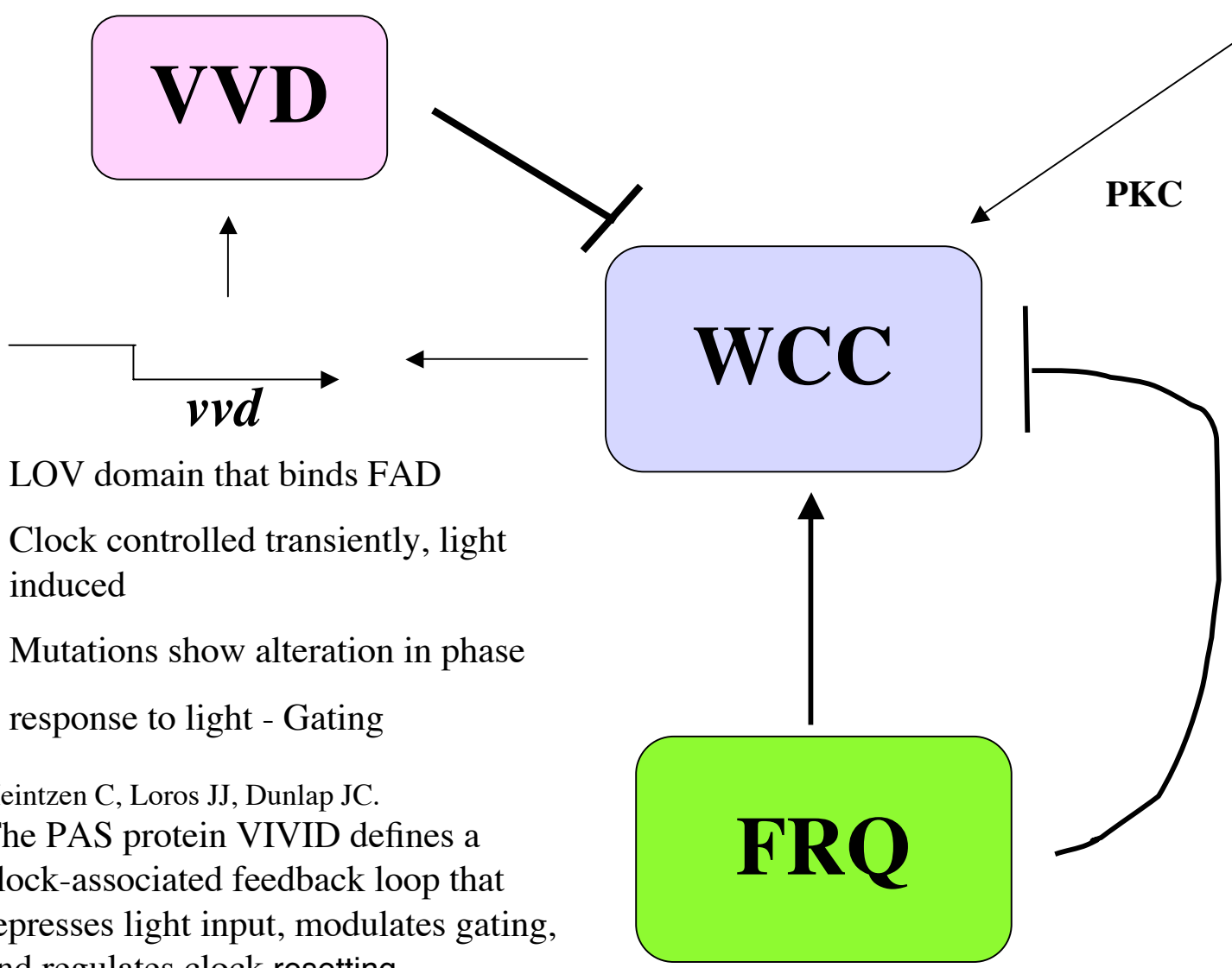
He Q, Shu H, Cheng P, Chen S, Wang L, Liu Y.

Light-independent phosphorylation of WHITE COLLAR-1 regulates its function in the Neurospora circadian negative feedback loop.
J Biol Chem. 2005 Apr 29;280(17):17526-32.

Identified 5 P sites on WC-1 and found that mutation show short period, low amplitude, or arrhythmic conidiation rhythms in DD.



VIVID



LOV domain that binds FAD

Clock controlled transiently, light induced

Mutations show alteration in phase

response to light - Gating

Heintzen C, Loros JJ, Dunlap JC.
The PAS protein VIVID defines a clock-associated feedback loop that represses light input, modulates gating, and regulates clock resetting
Cell. 2001 Feb 9;104(3):453-64.