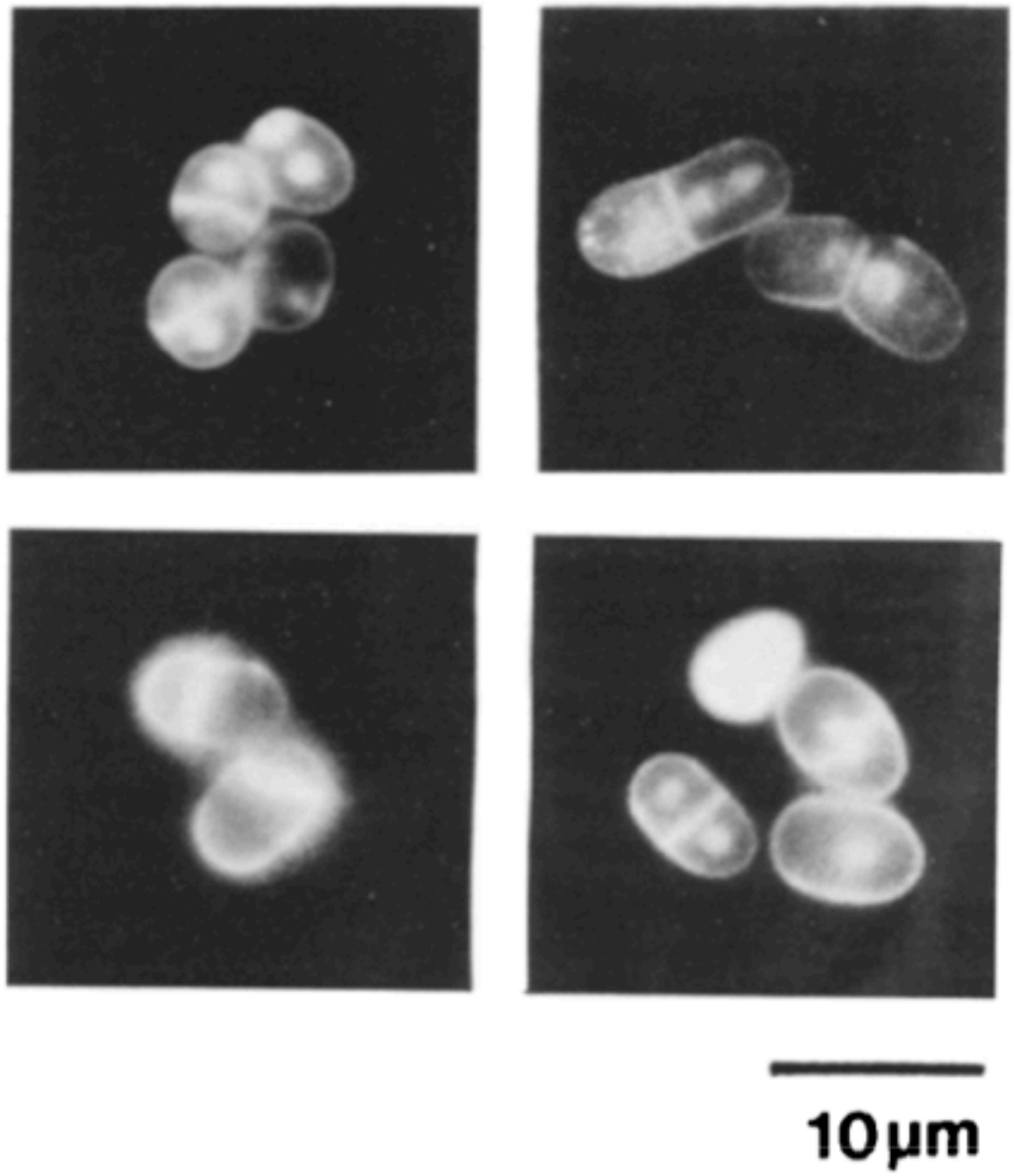
Lecture 3
more cell cycle genetics



Today

Outline of Today's Lecture

1. How to order genes in pathway using epistasis.

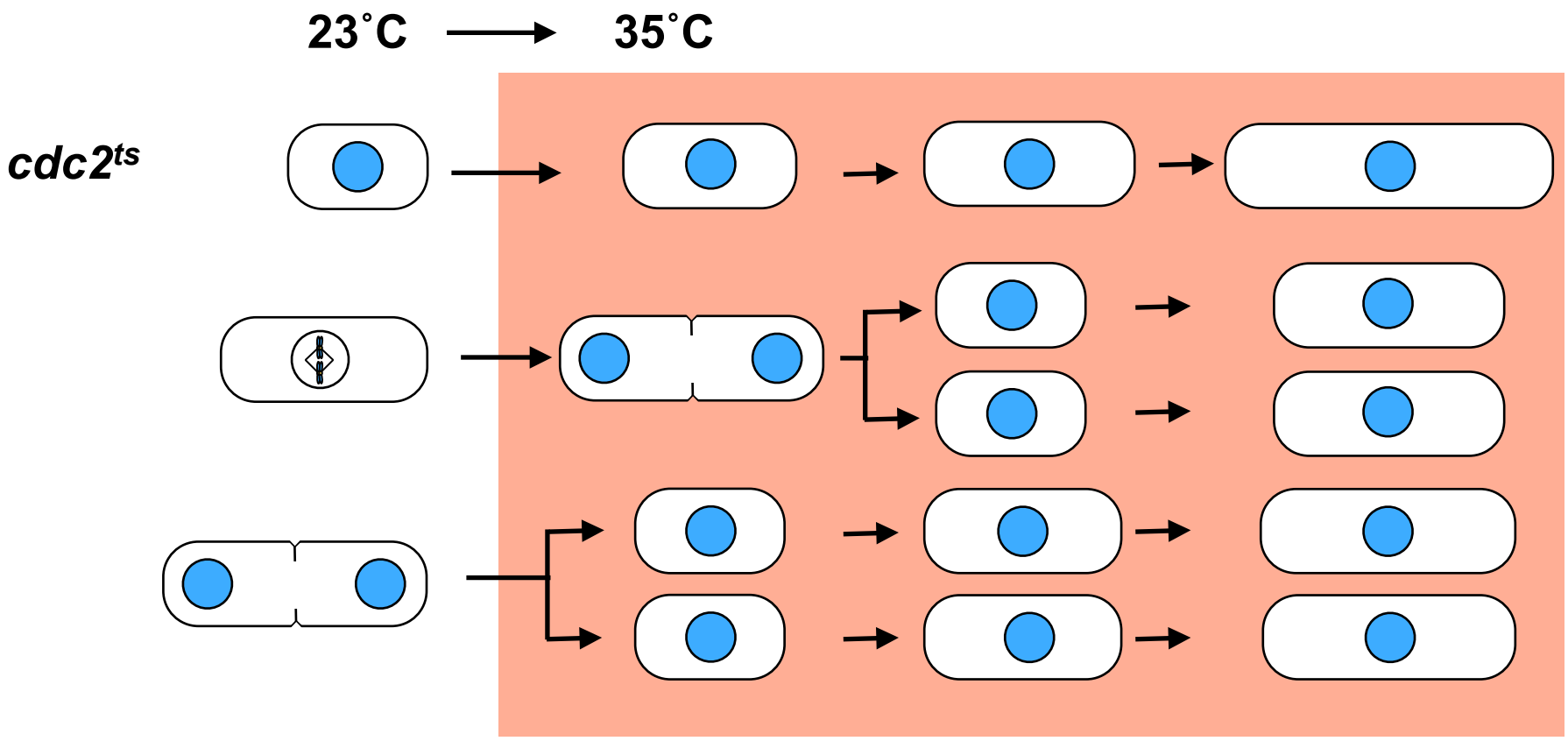
2. Conservation of Cdc2/Cdc28

3. Russell and Nurse, 1986 - how epistasis is actually done.

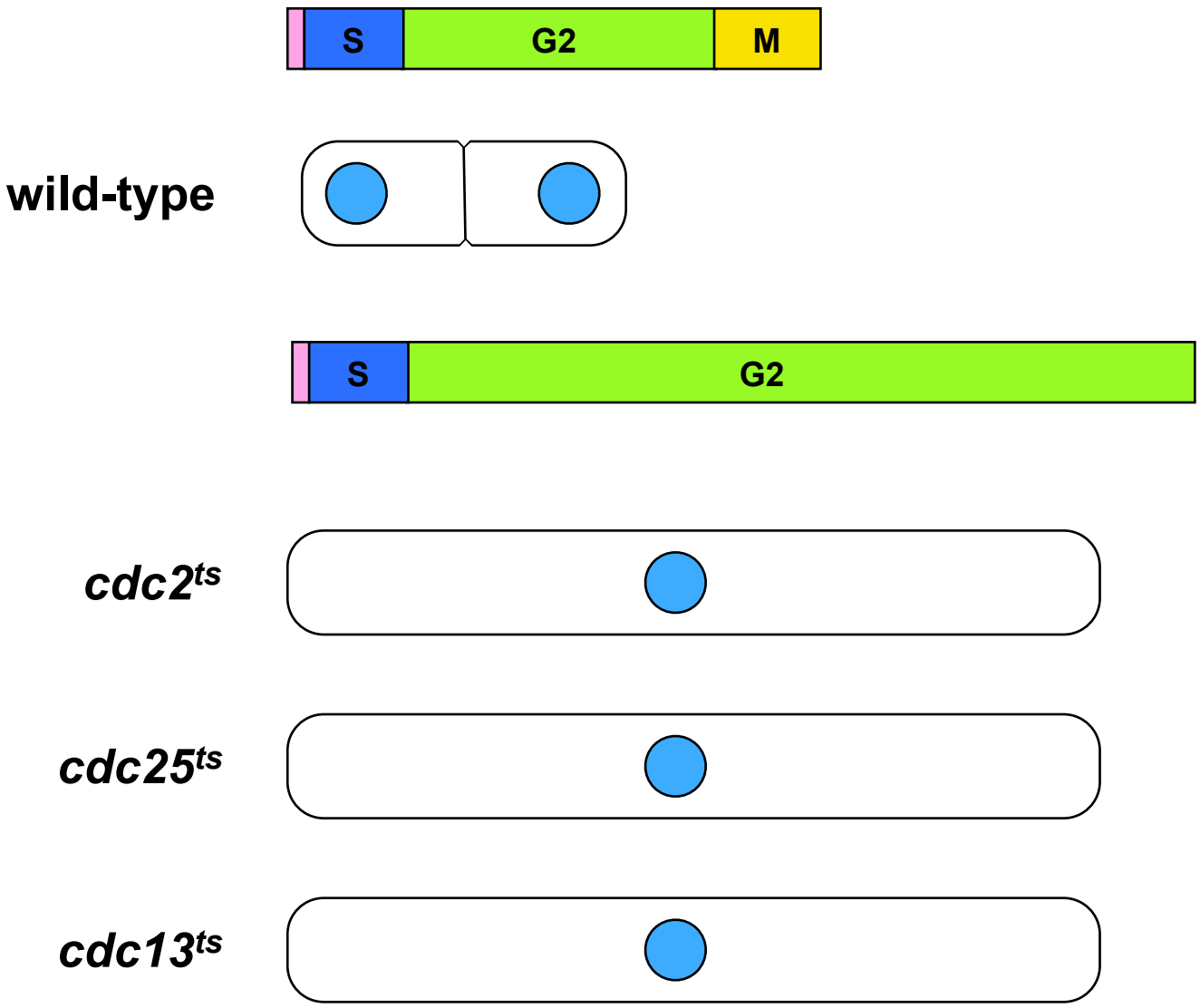
4. Execution points.

Outline of this lecture.

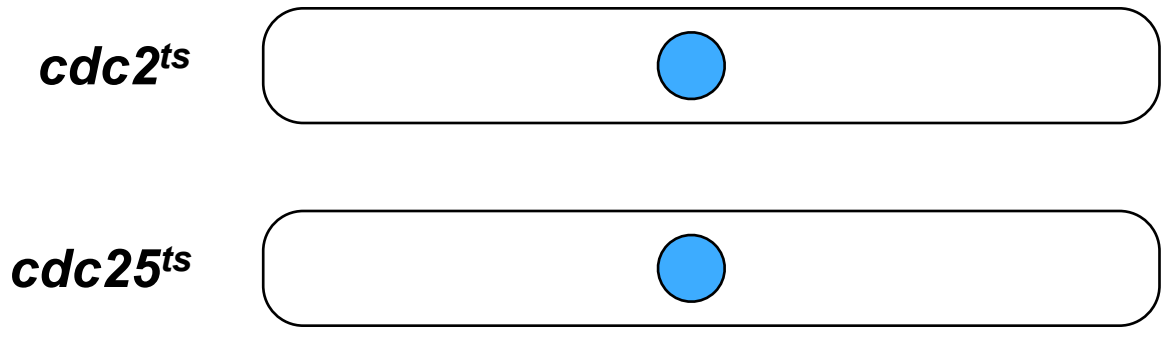
many *cdc* mutants arrested in G2



One mutant that was found is cdc2-ts. At the high temperature cdc2-ts arrest in the G2 phase of the cell cycle. Cells will continue to grow until they reach G2 and then arrest. For example, if the cells are in mitosis when shifted to 35˚C, they will complete mitosis, divide into two cells, both will go through S-phase and then arrest in G2. Most of the cdc mutants found in fission yeast arrested in the G2 phase.



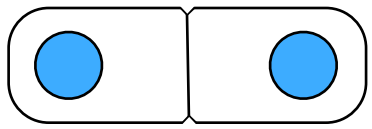
Many cdc mutants arrest in the G2 phase at the restrictive temperature. When fission yeast cells arrest in G2 they continue to grow from their ends and become very long. Three cdc mutants that arrest in G2 are cdc2-ts, cdc25-ts and cdc13-ts.



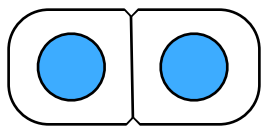
After finding these 3 mutants that all seem to affect the transition from G2 to M, Paul Nurse wondered if the mutated genes all function in a single pathway to regulate the transition from G2 to M.

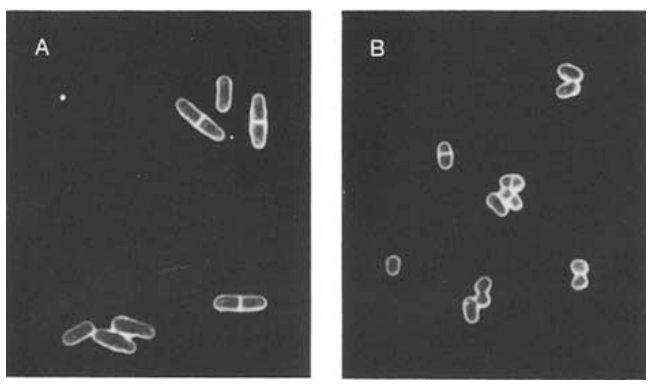
wee1 is an inhibitor of mitotic entry

wild-type



***wee1*^{ts}**

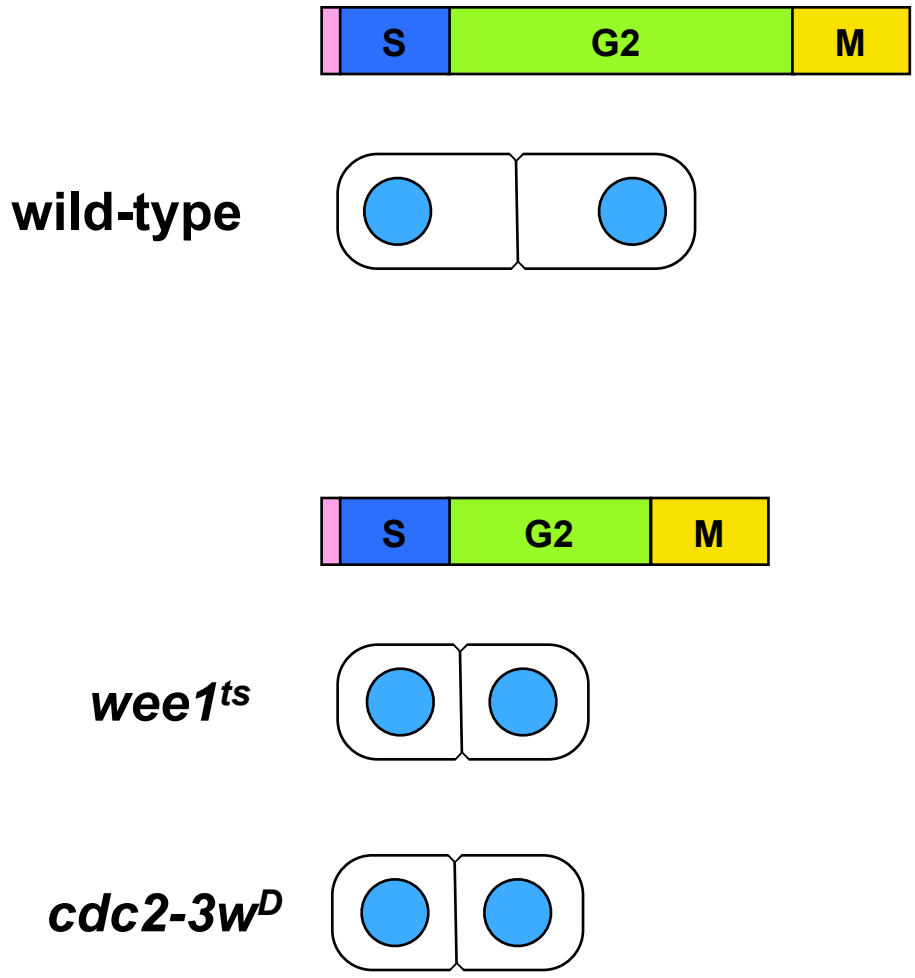




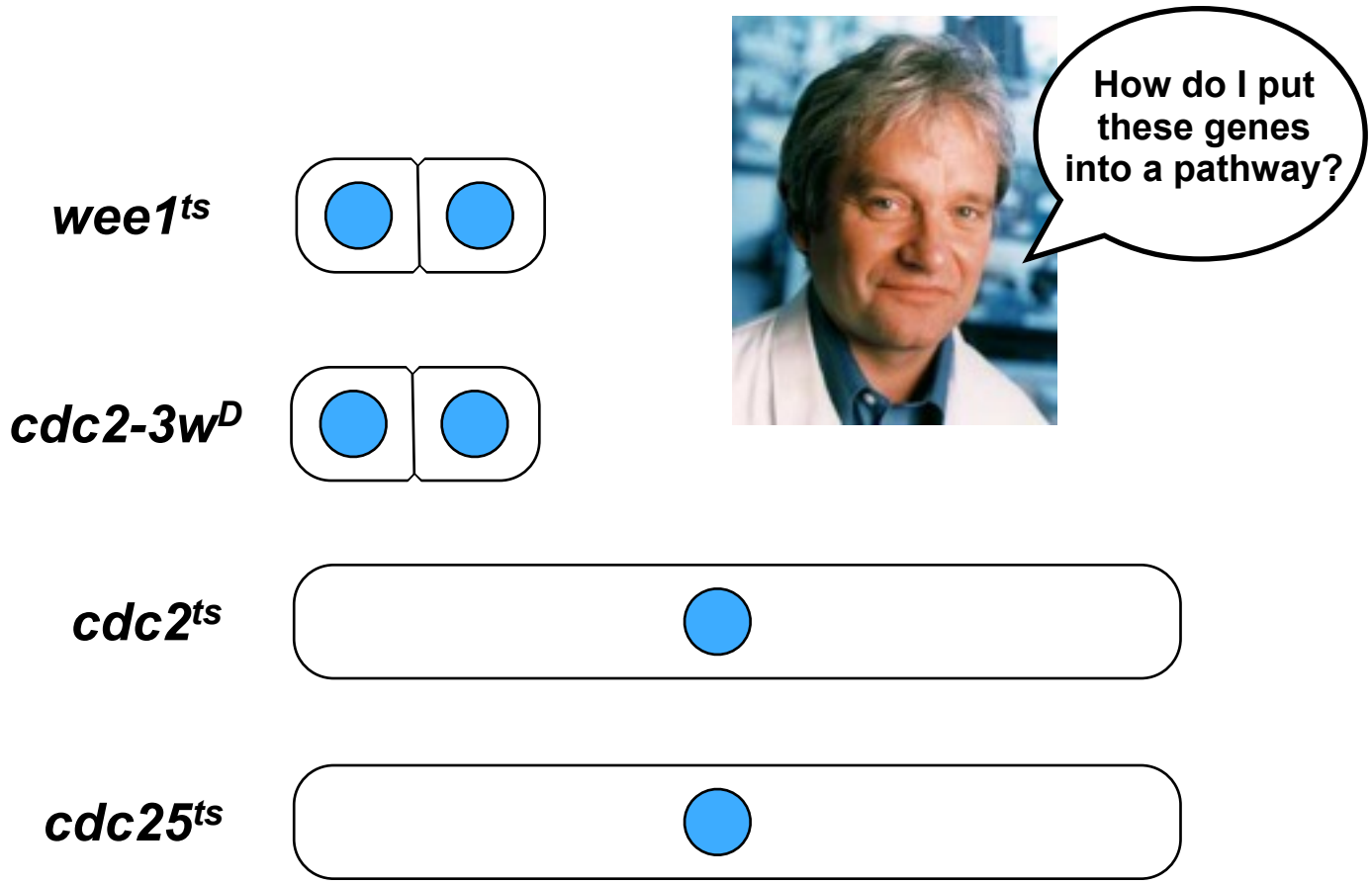
Thuriaux, Nurse and Carter, 1978

Fig. 1A and B. Photomicrographs of wild type and *wee* 1-50 cells. Cells grown at 35° C and photographed under dark field optics. Both plates at a final magnification of ×500. **A** 972h$^-$ wild type strain, **B** *wee* 1-50h$^-$

In a separate screen researchers looked for ts mutants that were small or “wee”. Wee1 was one of these mutants. When analyzed carefully they discovered that wee1-ts cells shorten their G2 phases and enter mitosis at a smaller cell size. Given that many of the cdc mutants arrested in G2, the wee1-ts mutant were critical in analyzing these mutants. It was assumed that Wee1 was an inhibitor of mitotic entry.

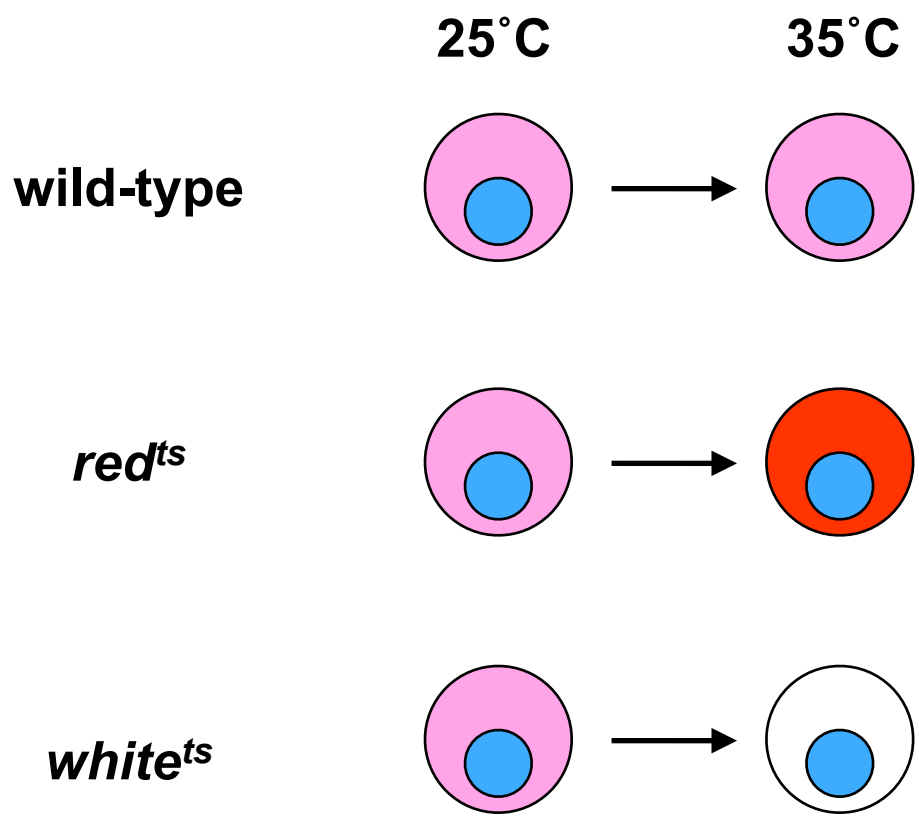


A second wee mutant was a mutant in cdc2, cdc2-3wD. cdc2-3wD is a dominant mutant that like wee1-ts, shortens the G2 phase. The fact that mutations in cdc2 can either cause a long G2 (or an arrest in G2 - cdc2-ts) or a short G2 (cdc2-3wD) clearly pointed to the fact that cdc2 was a critical regulator of the transition from G2 to M. Because cdc2-3wD is a dominant mutant it is likely a gain-of-function mutant, so that the Cdc2 gene product has either more activity than the wild type gene product, or it has some new function. Therefore it is not surprising that cdc2-ts (a loss-of-function mutant) has the opposite phenotype as cdc2-3wD (a gain-of-function mutant).



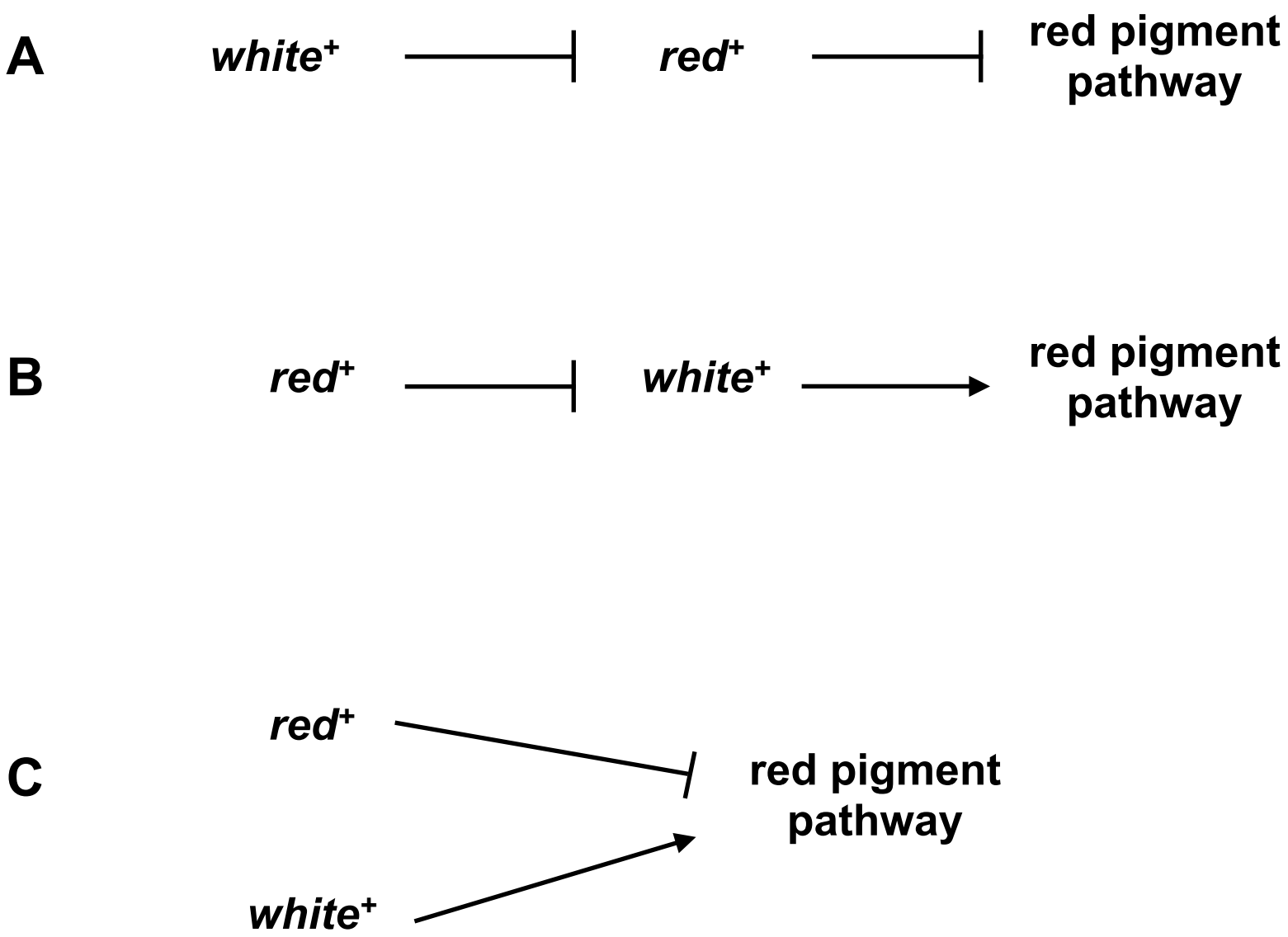
After finding these 5 mutants in four genes that all seem to affect the transition from G2 to M, Paul Nurse wondered if the mutated genes all function in a single pathway to regulate the transition from G2 to M.

Genetic epistasis or how to order a genetic pathway



Before I describe what was done in fission yeast, I wanted to go over how researchers can order genetic pathways using a hypothetical example. This ordering is called “epistasis analysis.” In this example we are studying cell color. This cell type is normally pink, at both the permissive and restrictive temperature. You isolate two mutants, red-ts which makes the cells red at the restrictive temperature and white-ts which makes the cells white at the restrictive temperature. Note that the temperature sensitivity is for color, not viability.

Three models



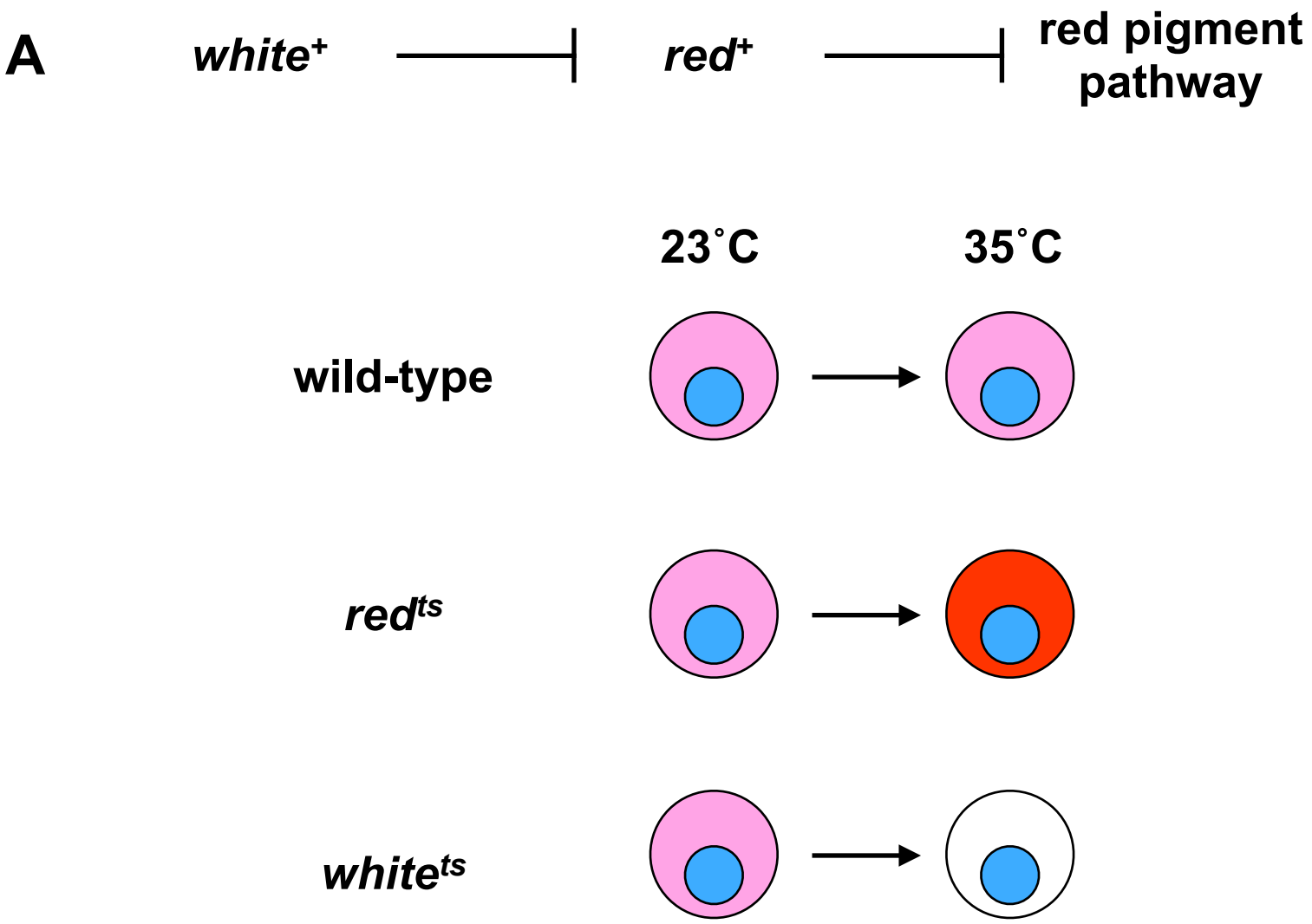
We can envision three models for how the red and white genes regulate cell color. We can assume that cell color is regulated by a red pigment pathway. In wild type cells the red pigment pathway makes enough pigment to make the cells pink. The mutants red-ts and white-ts somehow modulate this pathway.

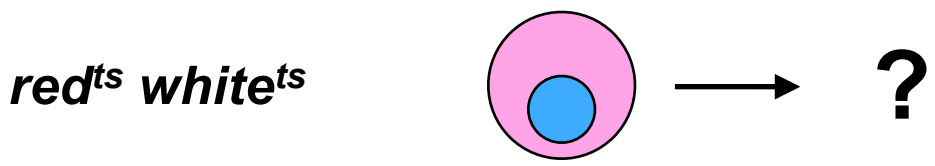
In Model A, the functional red gene inhibits the red pigment pathway, and the white gene inhibits the red gene. In the red-ts mutant, the inhibition of the red pigment pathway is removed so more red pigment is made, so the cells are red. In the white-ts mutant the inhibition of the red gene is removed, so the red gene inhibits the red pigment pathway at a greater level than normal, so less red pigment is made and the cells are white.

In Model B, the white gene activates the red pigment pathway and the red gene inhibits the white gene. In the white-ts mutant, the red pigment pathway is turned off, and the cells are white. In the red-ts mutant, the inhibition of the white gene is removed, and the white gene activates the pigment pathway more than in a wild type cell, therefore the cells are red.

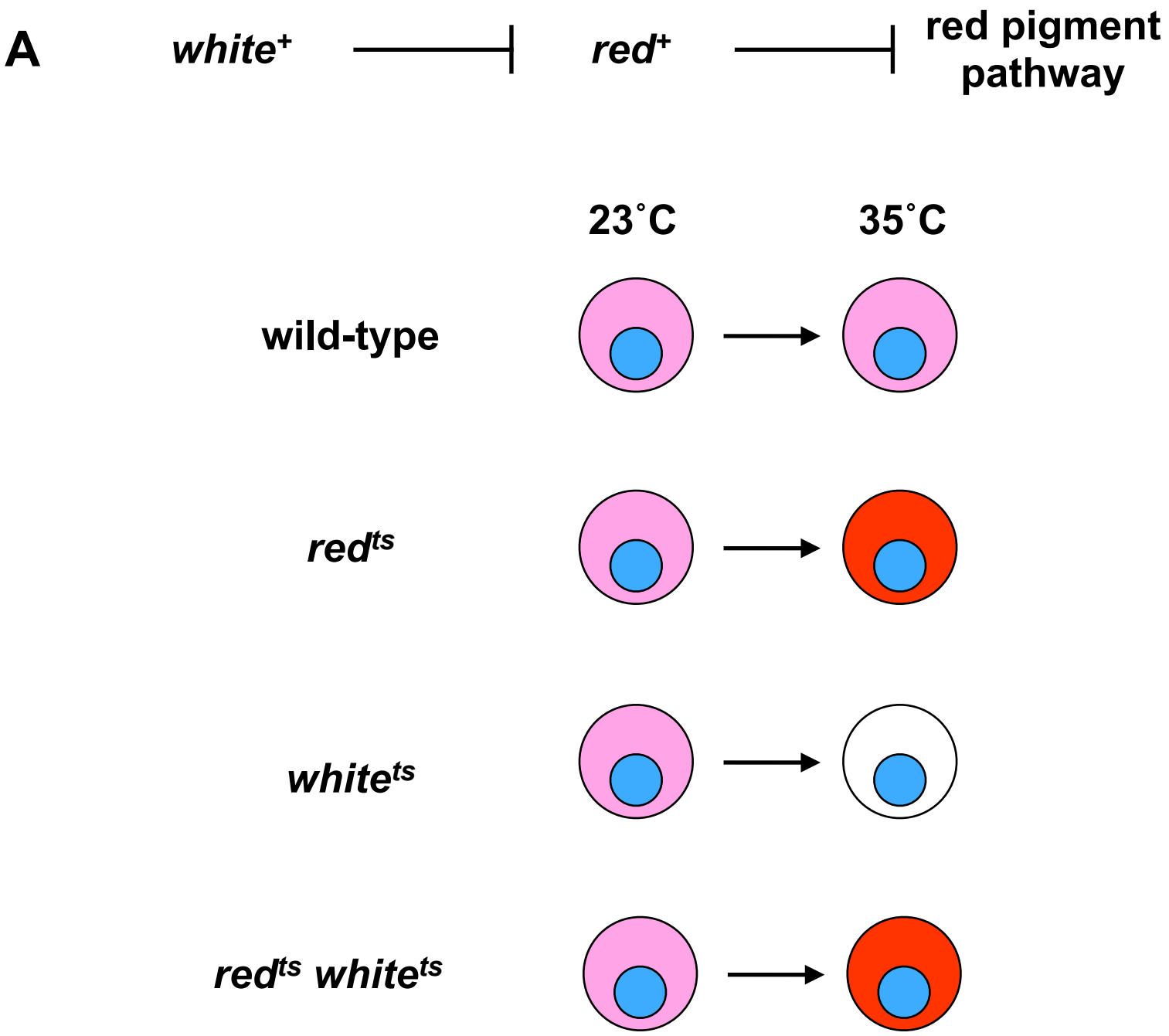
In Model C, both genes independently regulate the red pigment pathway. The red gene inhibits the pigment pathway and the white gene activates the pigment pathway. In a red-ts mutant, the inhibition of the pigment pathway is removed and the cells are red, and in the white-ts mutant the activation of the pigment pathway is removed, and the cells are white.

All three models are possible, based on the known phenotypes of the two mutants. Some interactions are not possible. For example the white gene cannot inhibit the pigment pathway directly, because removing the white gene would be predicted to make the cells red not white. When constructing a possible genetic model you first need to make sure the model is consistent with known data, and then you make predictions about what to expect with mutant combinations (see next slide).



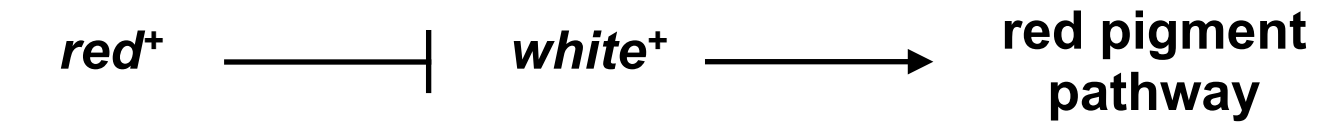


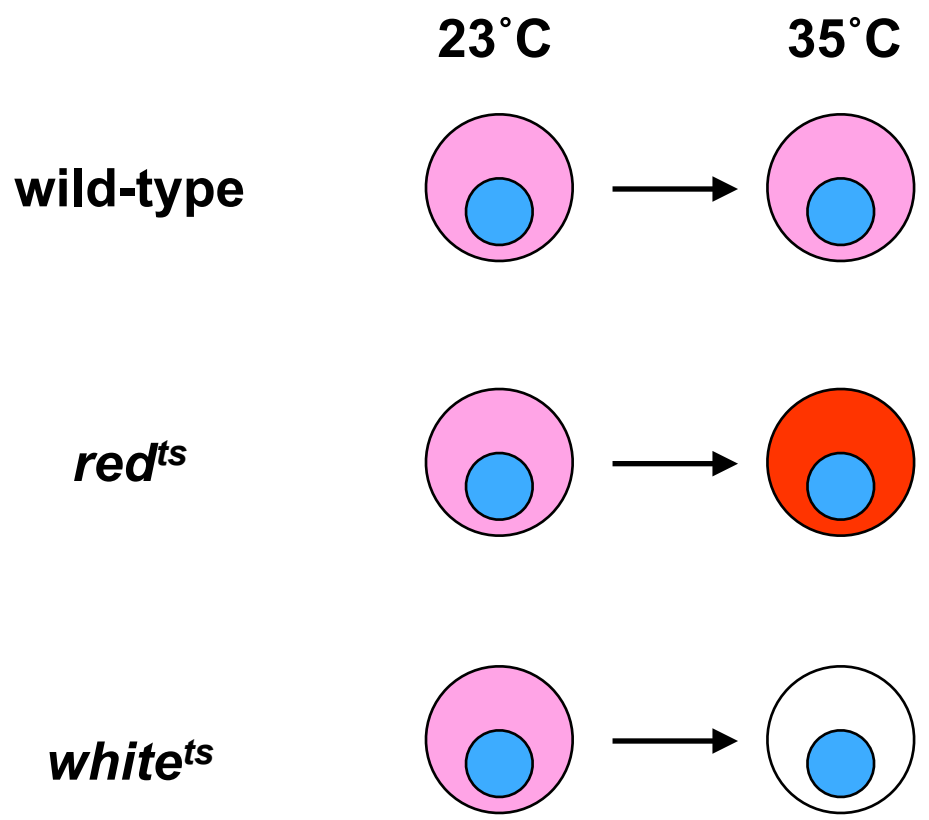
To test all three models we need to know the phenotype of the white-ts red-ts double mutant. Some organisms are not amenable to genetic analysis, like frogs, but others like yeasts, are. Constructing double mutants in yeast requires simple genetic crosses. If Model A is correct we expect that the red-ts white-ts double mutant will be.....

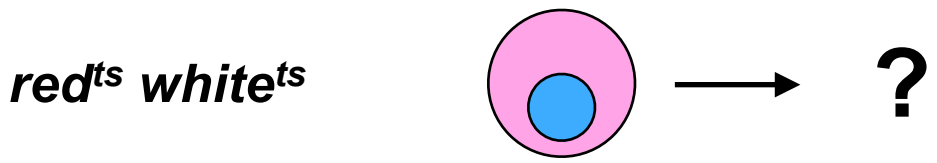


red. Mutating the white gene has no affect if the red gene is already mutated.

B

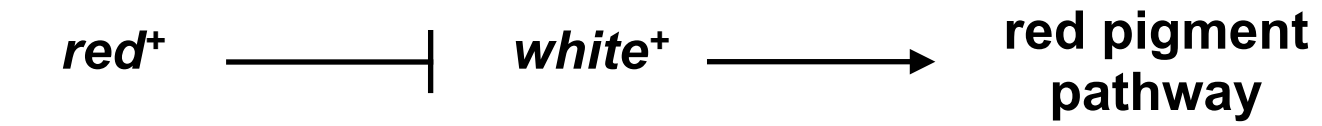


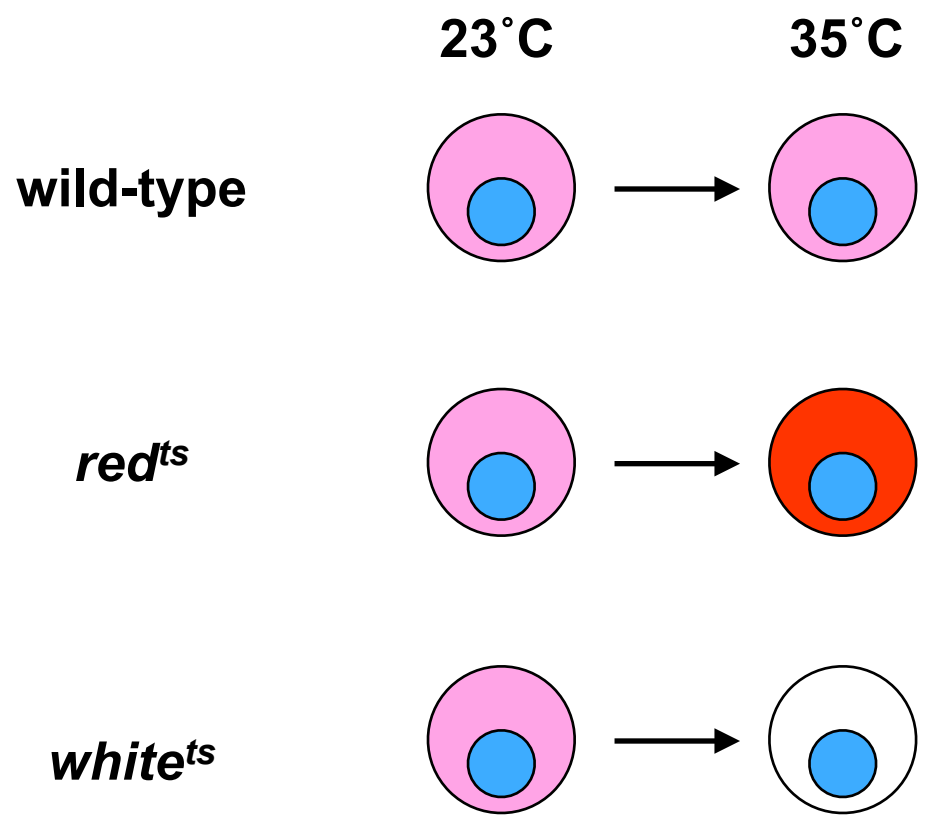


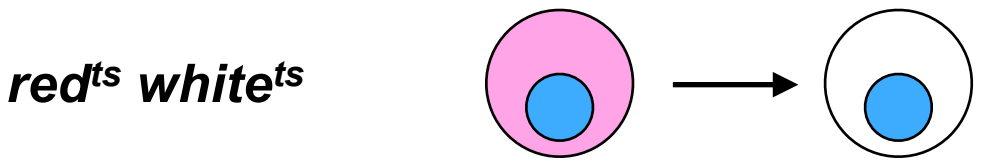


In Model B the red-ts white-ts double mutant will be.....

B

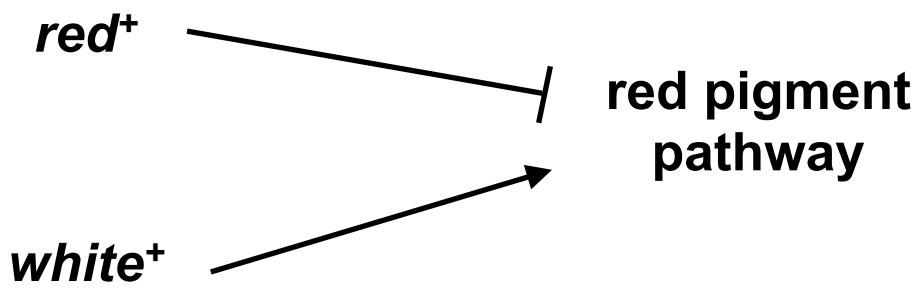


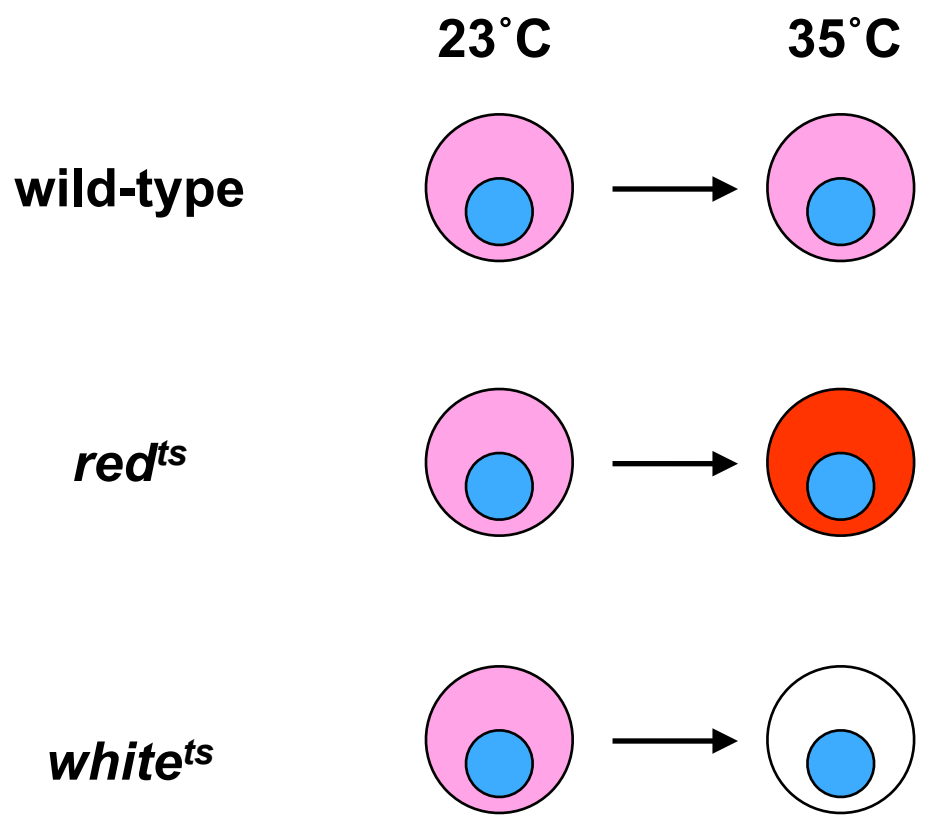




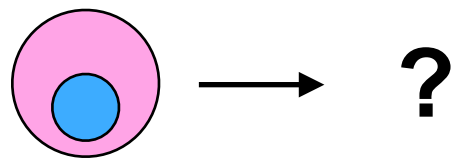
white. Because in this model mutating the red gene has no affect if this white gene is already mutated.

C

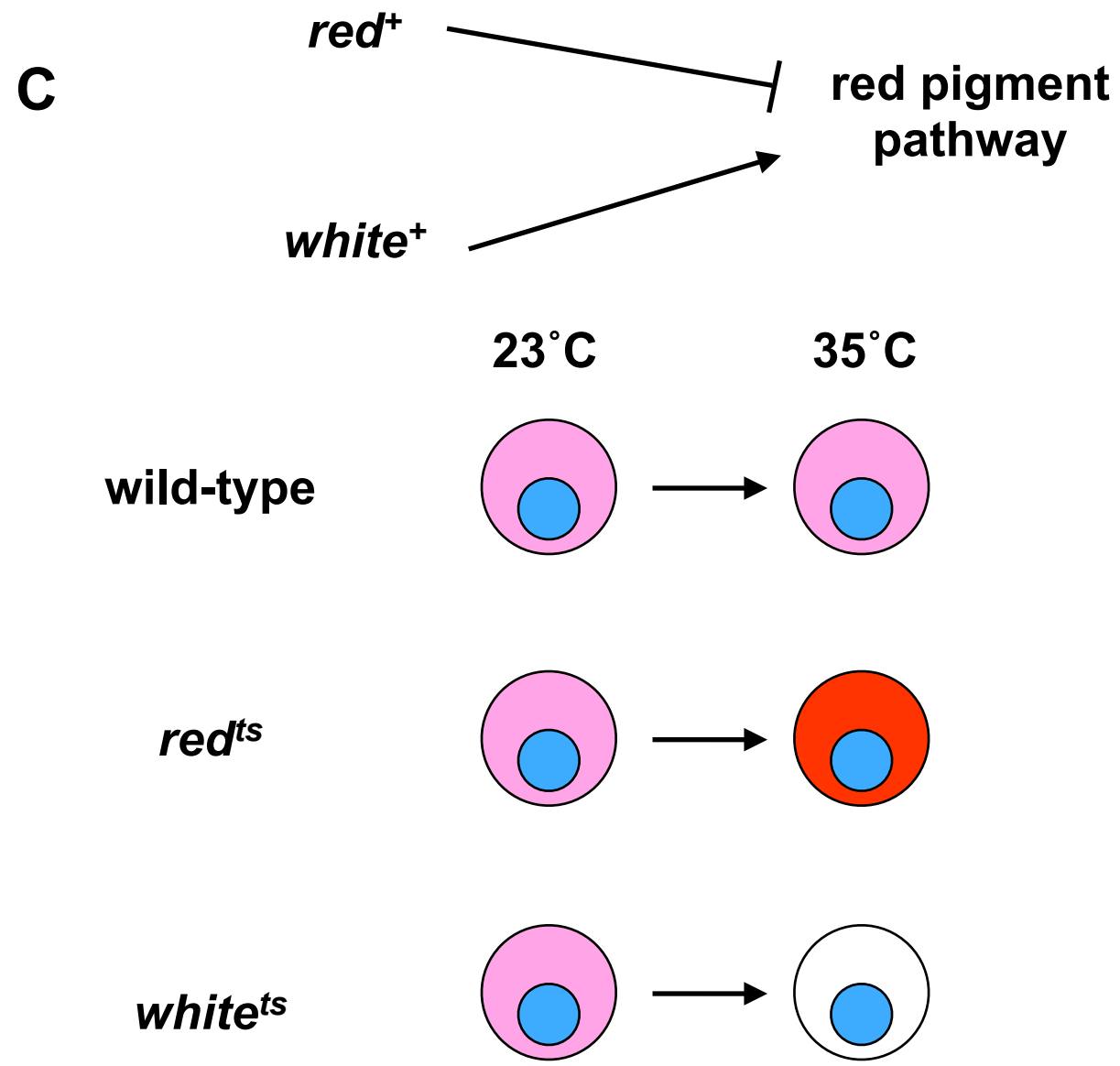


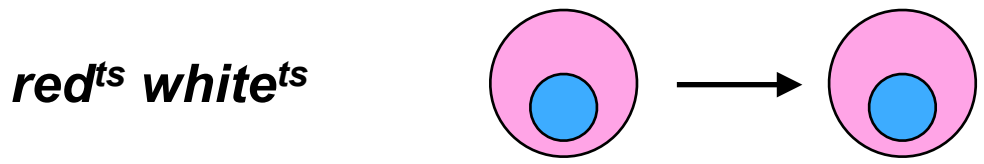


redts whitets

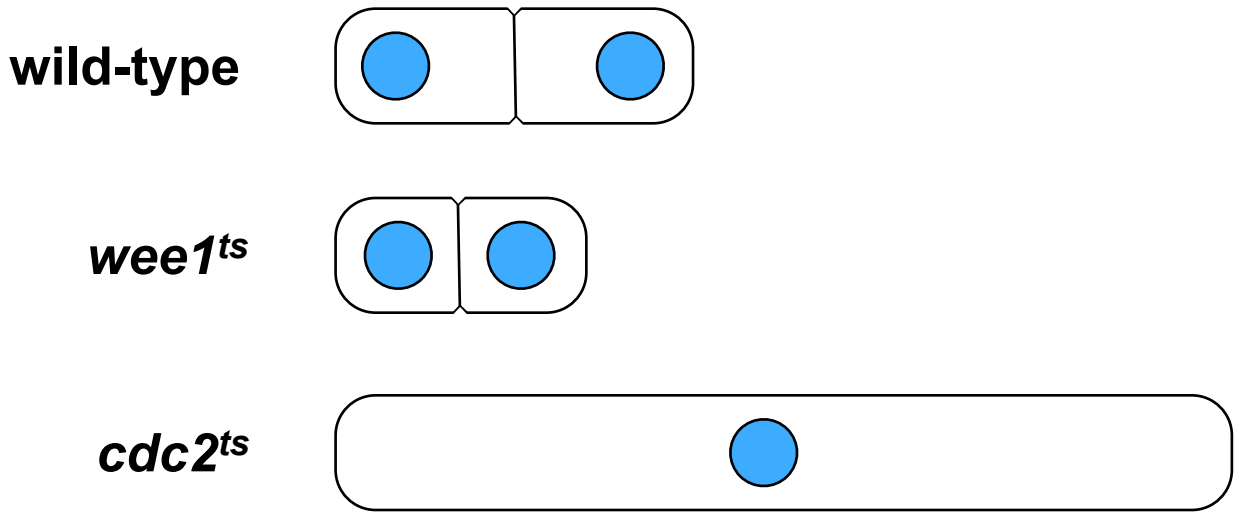


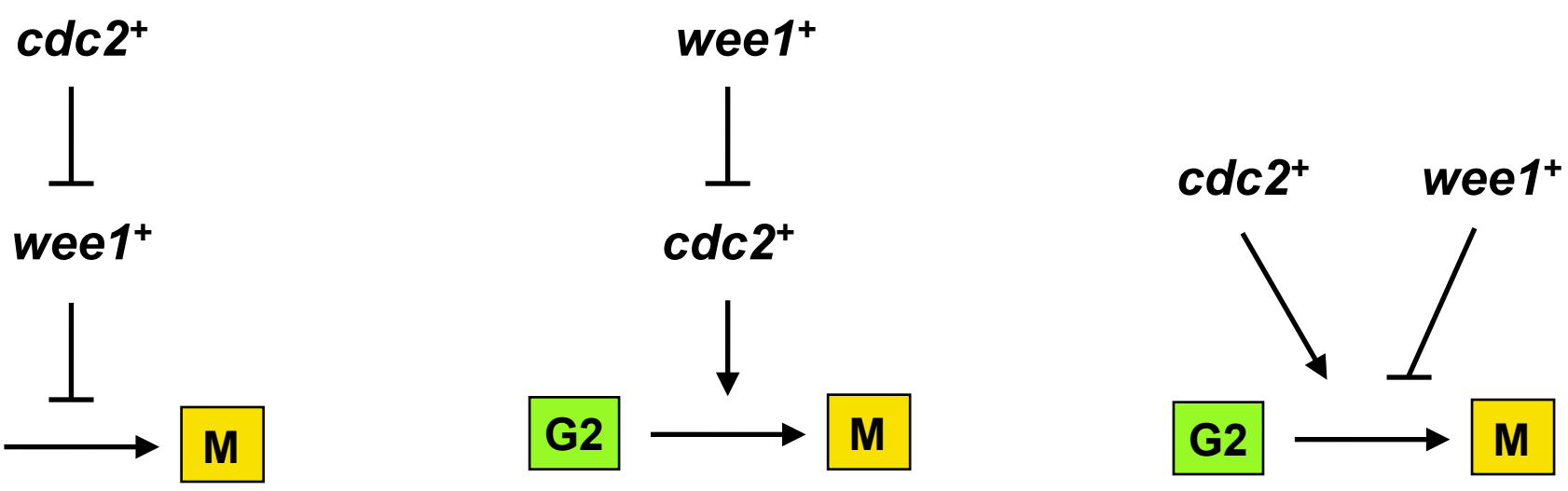
And finally in Model C the red-ts white-ts double mutant would be.....



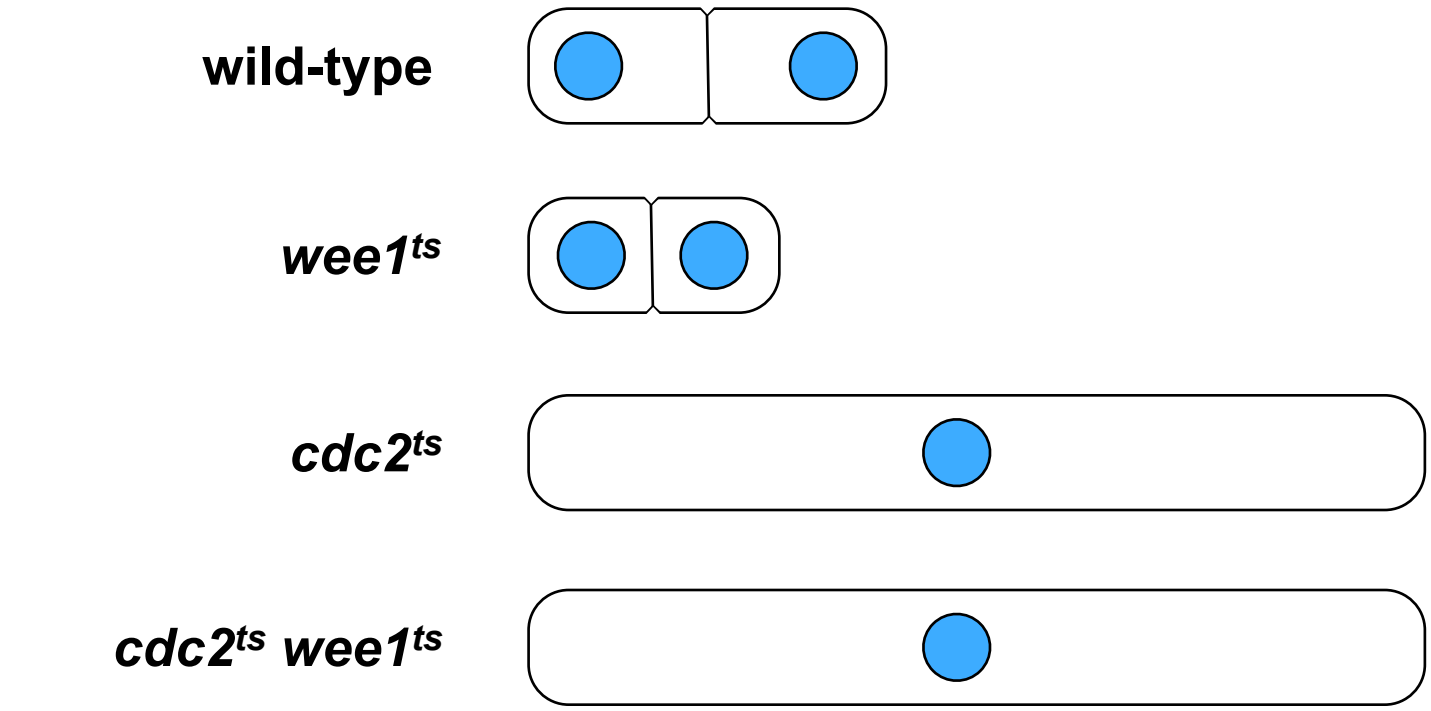


likely pink. This isn't a certainty, but it is the most likely outcome. This would occur if there is some basal level of activity of the pigment pathway. The white and red genes function to modulate that activity, but removing both still leaves the basal activity intact.

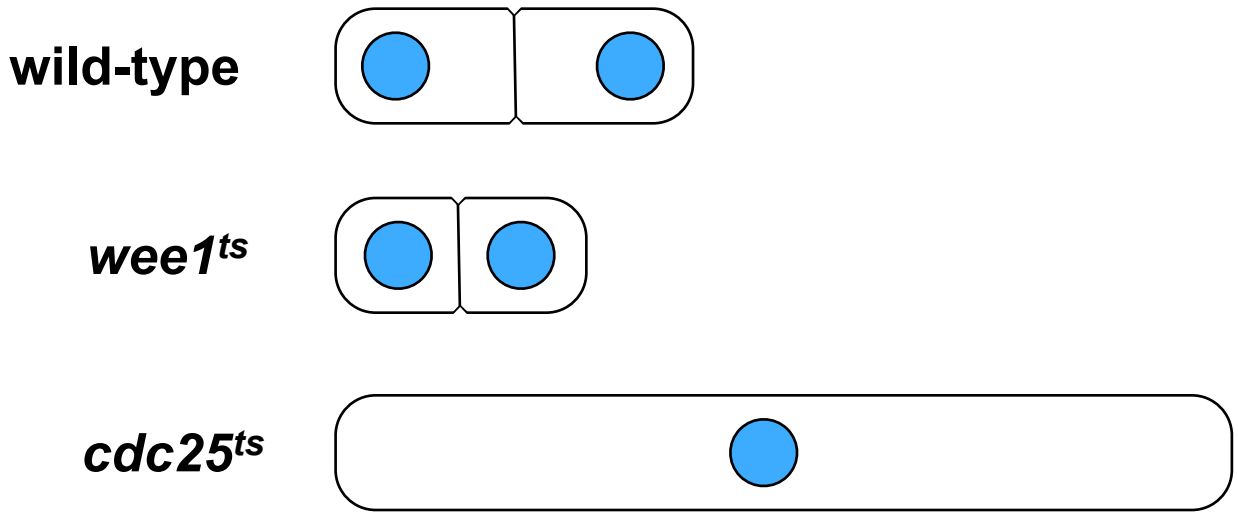


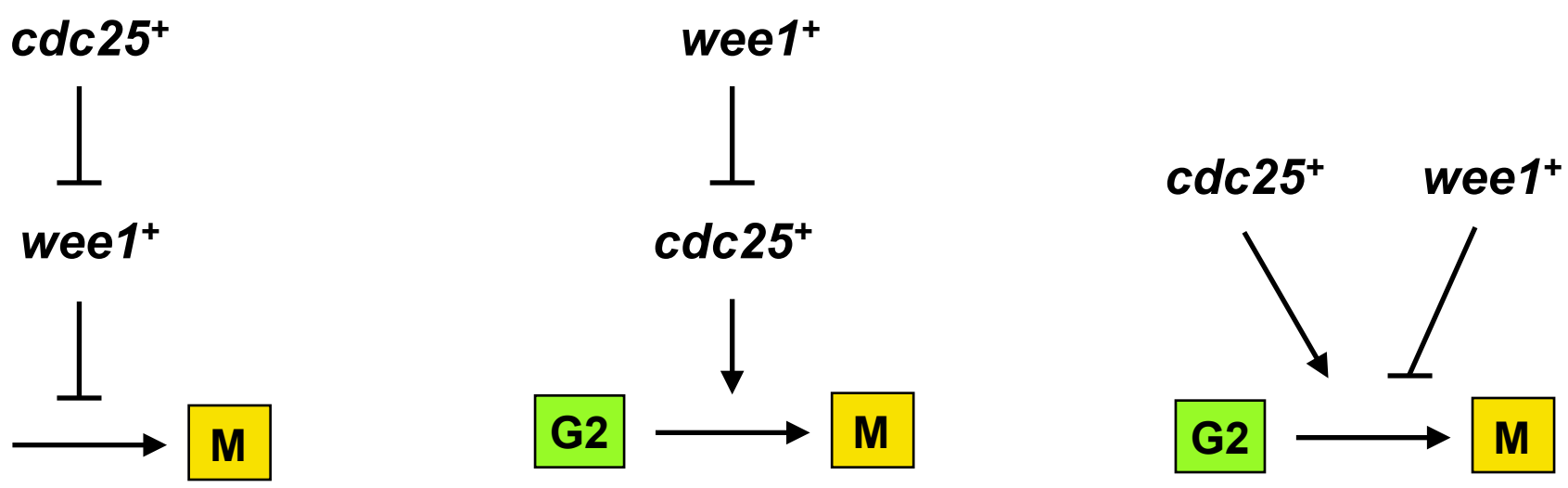


Paul Nurse wondered what the relationship was between wee1 and cdc2, and came up with three possible models (which are exactly analogous to the red/white/pink model). In one, cdc2 inhibits wee1 which inhibits the transition from G2 to M, in a second wee1 inhibits cdc2 which activates the G2 to M transition, and in the third they both independently regulate the G2 to M transition.

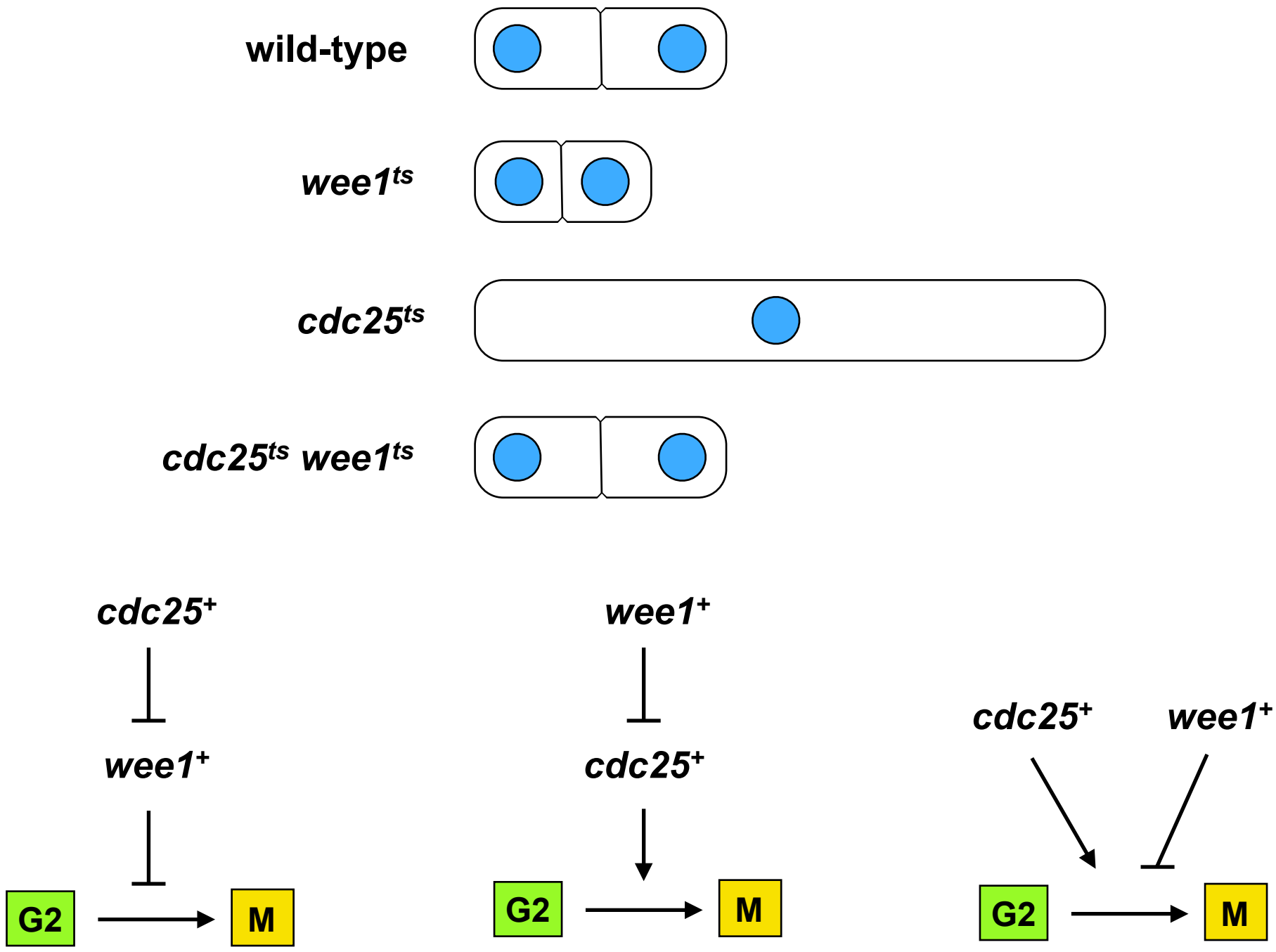


When he made the cdc2-ts wee1-ts double mutant he found that it was large (and identical) to the cdc2-ts mutant alone. This supports which model? The second, because wee1 function is upstream of cdc2, so its mutation has no affect when cdc2 is already mutated.

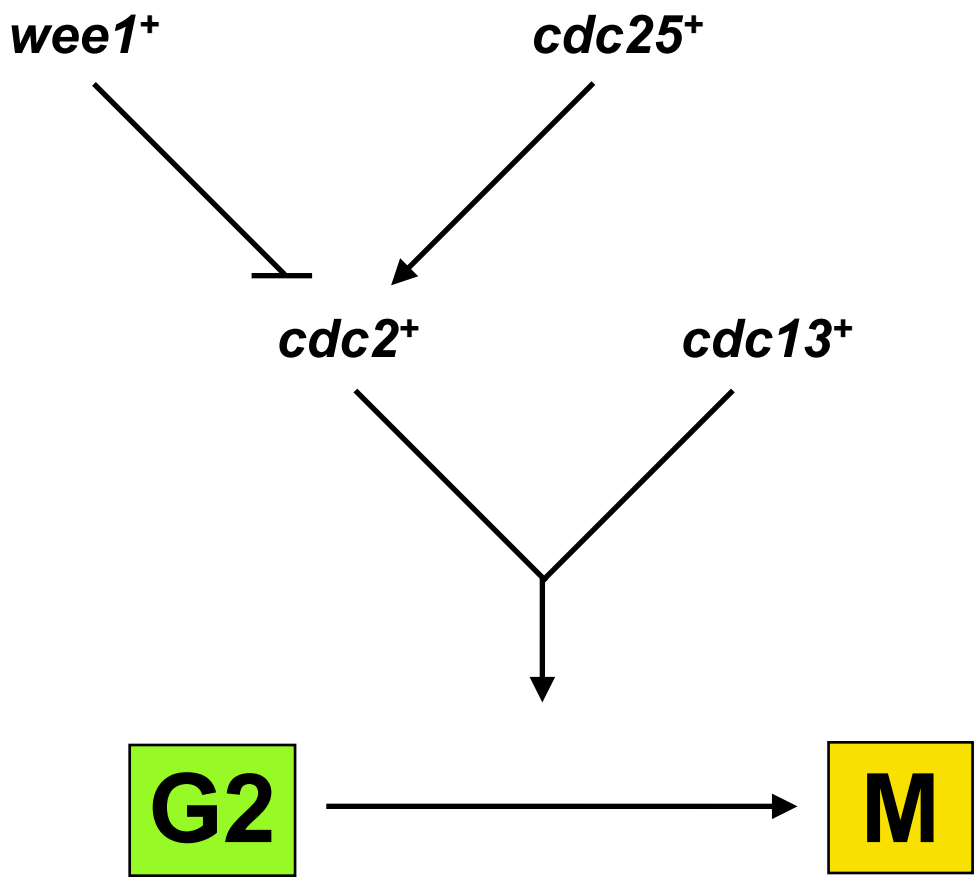




He imagined the same three possible models for how wee1 and cdc25 interact.

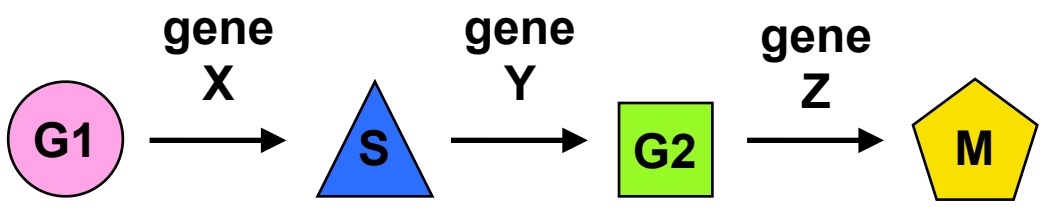


When he examined the cdc25-ts wee1-ts double mutant he found that it was the same size as wild type cells, which supports which model? The third model, that the two genes independently regulate the G2 to M transition. Later in the lecture you'll see that this isn't exactly what he saw, but hypothetically this result would make the analysis much easier. It was more complicated which led to a bunch of the experiments done in Russell and Nurse.

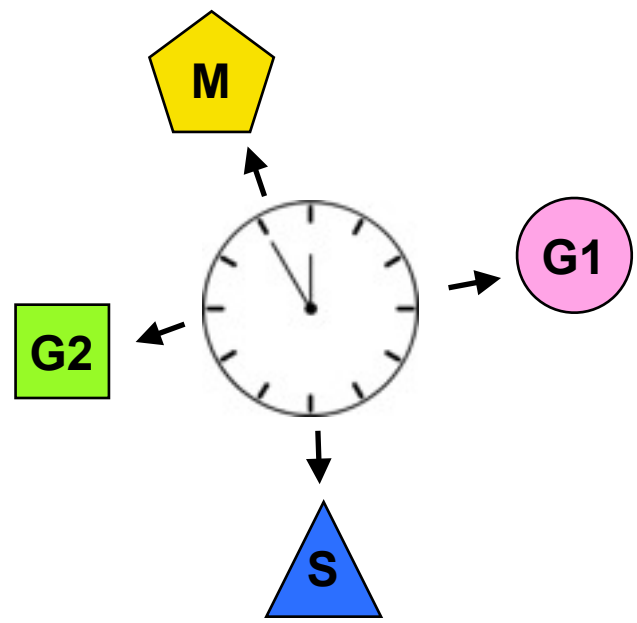


These two examples plus a few others I'm not showing led to the following model for how these four genes interact. Cdc2 and Cdc13 together activate the G2 to M transition, while Wee1 and Cdc25 modify the activity of Cdc2. These analyses were done before the identity of these proteins were known and simply were genetic relationships.

domino theory



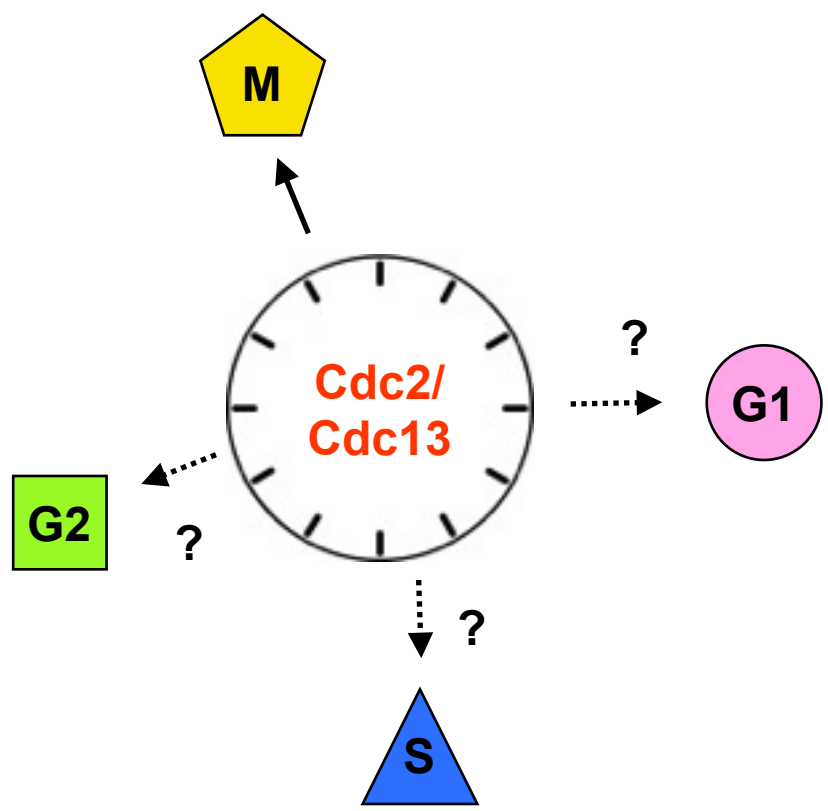
clock theory



Which model is supported by this data?

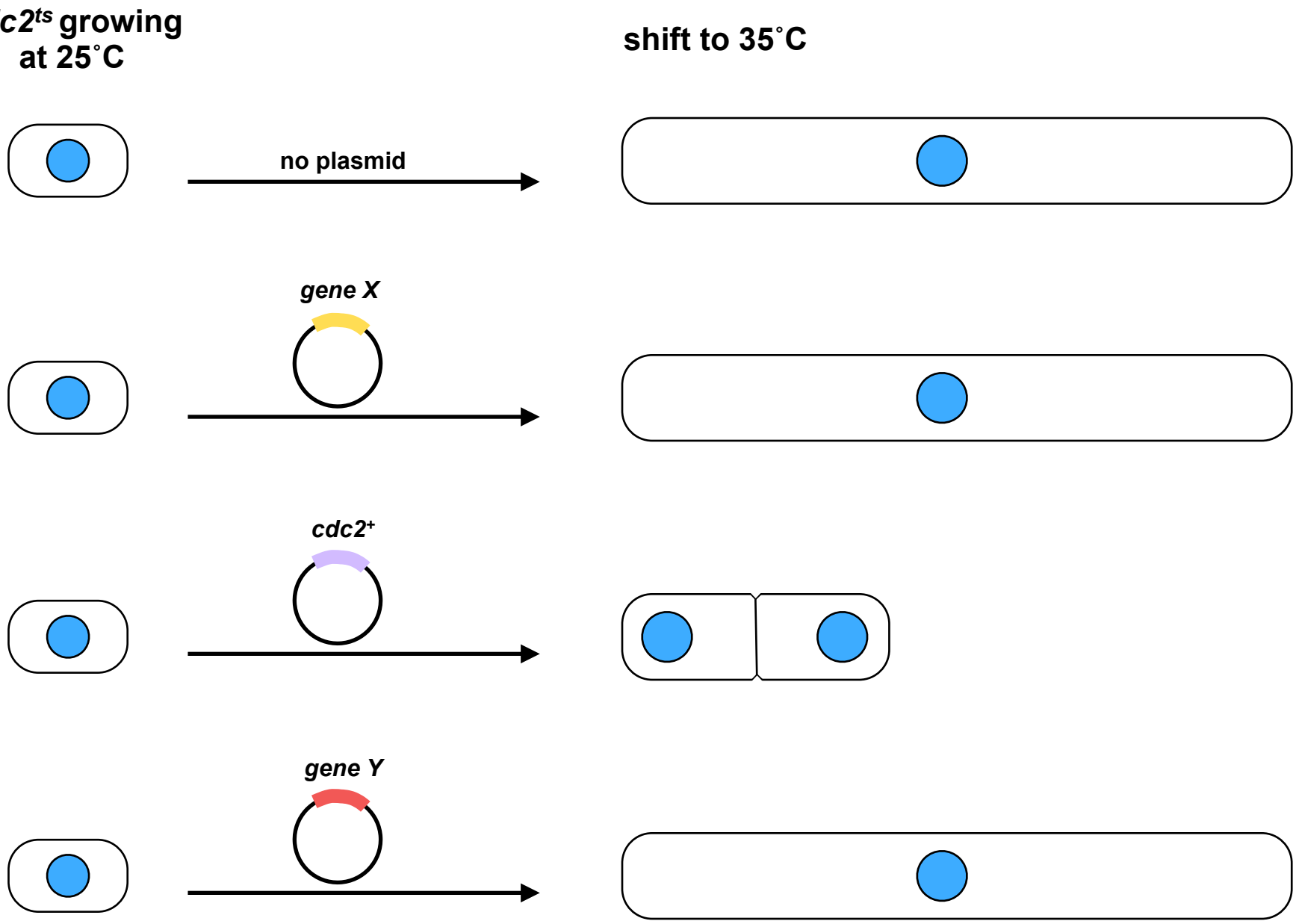
Cdc2, like MPF, is required for entry into mitosis

clock theory



Cdc2 and Cdc13, like MPF, are important regulators of mitosis, but do they support the clock theory? Currently we haven't discussed any experiments that show that Cdc2, Cdc13 and MPF regulate anything other than mitosis. The genetic experiments in fission yeast did support the clock theory because most cdc mutants arrested in one place, G2, suggesting that there weren't multiple dependencies that drove cells through different transitions within mitosis.

cloning of *cdc2* by complementation



Transfect with plasmid library

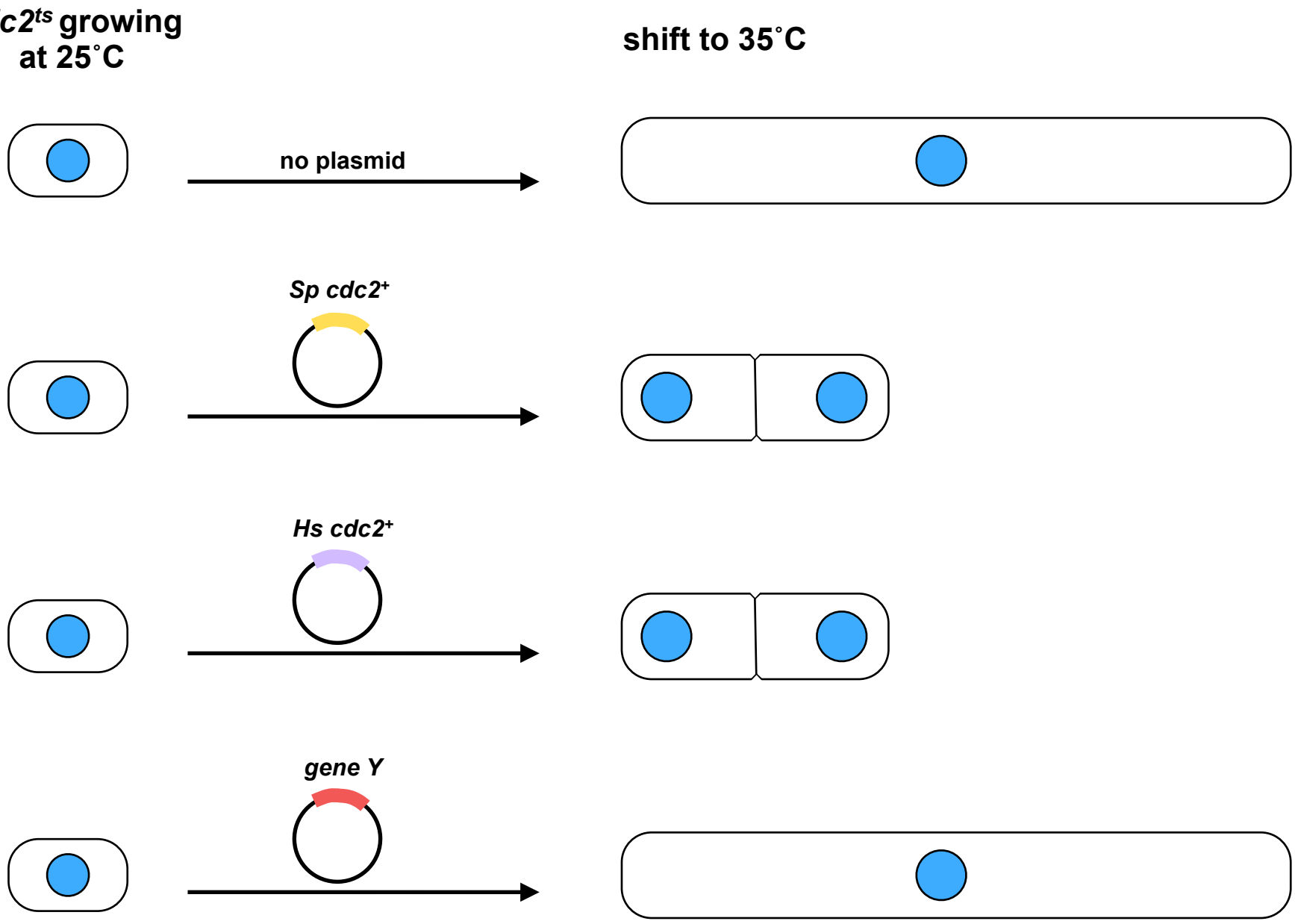
To clone these genes researchers used what is now a very standard approach. At the time, these were some of the first genes to be cloned by "complementation." The researchers made a "library" or collection of all fission yeast genes on a plasmid. Each different plasmid contains one fission yeast gene. The collection of plasmids were transfected into a cdc mutant, in this example cdc2-ts. After transfection the yeast containing plasmids are shifted to the restrictive temperature. In a cdc2-ts strain with no plasmid, the cdc2-ts mutant arrests in G2 and dies. In this example gene X and gene Y do not "complement" the cdc2-ts mutant, and when cdc2-ts contains these plasmids it arrests in G2 at the restrictive temperature and dies. If the plasmid contains the wild type cdc2 gene it "complements" and now cdc2-ts containing this plasmid grows normally at the restrictive temperature. Isolating the plasmid out of this strain (called the "rescuing" plasmid), and sequencing the gene on the plasmid allowed the identity of the mutated gene to be determined.

Cdc2 = Cdc28

a highly conserved 34 kD protein kinase

Cdc2 in fission yeast was a 34 kD protein kinase, and it turned out that Cdc28 was the homologous protein in budding yeast. The two genes have similar functions, but apparently at different cell cycle transitions.

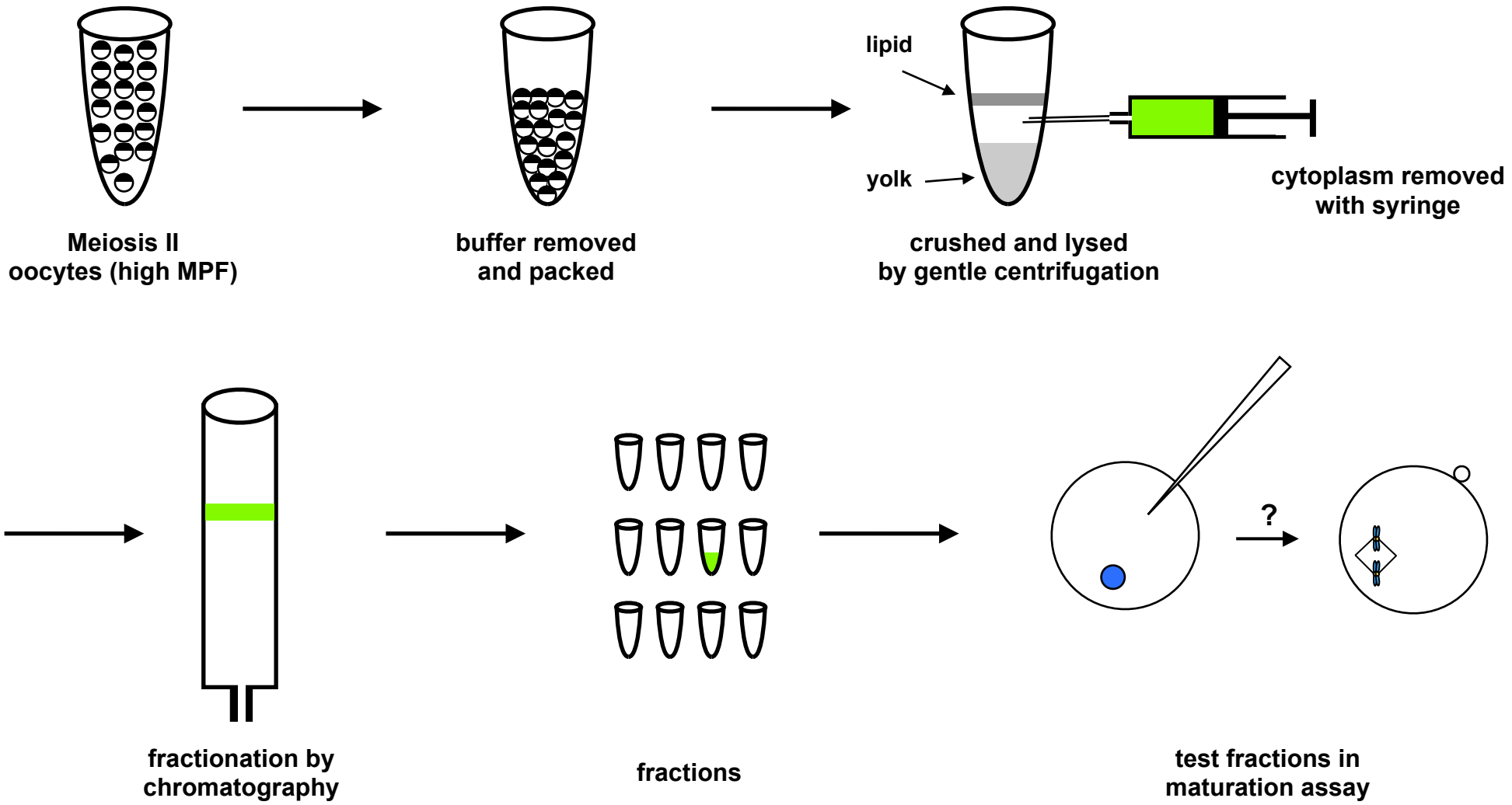
Cdc2 is universally conserved



Transfect with *human* cDNA library

Just how conserved is Cdc2 and Cdc28? The most incredible demonstration of conservation was the cloning of the human cdc2 gene (Hs cdc2). This was done in fission yeast, where one gene in a library of human cDNAs could complement the cdc2-ts mutation, and allow it to grow at the restrictive temperature. That one gene was the human homologue of cdc2. Fission yeast and humans are not very similar in most ways, and are separated by millions of years of evolution, but still the human gene can carry out all the functions needed for the survival of fission yeast.

Cdc2 is a component of MPF



Meiosis II oocytes (high MPF)

buffer removed and packed

crushed and lysed by gentle centrifugation

fractionation by chromatography

fractions

test fractions in maturation assay

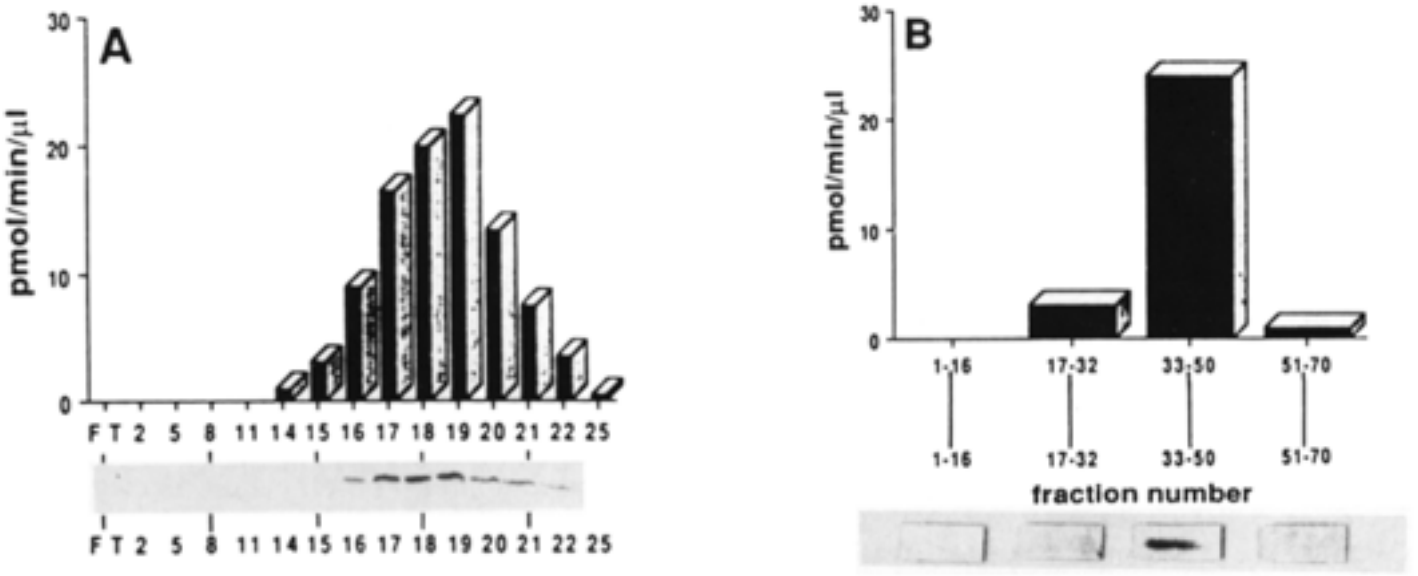
As I mentioned at the start of the lecture purification of MPF was not so successful. However, once it was recognized that Cdc2 and Cdc28 were conserved critical regulators of the cell cycle researchers took there partly purified MPF and checked to see if the frog homologue of Cdc2 was present. It was, and it became clear that Cdc2 is a component of MPF, and the histone H1 kinase activity that was followed in many purifications was the protein kinase activity associated with Cdc2. What is amazing about this story is that the genetics and biochemistry converged at precisely the same time, and disparate observations from many different organisms turned out to all point to Cdc28/Cdc2.

Cdc2 is a component of MPF

Table 1. Copurification of MPF and of the M Phase–Specific H1 Histones Kinase from Starfish Oocytes

Step	Total Protein (mg)	Total Activity		Specific Activity		Recovery (%)	
		H1 Kinase (Units × 10^3)	MPF (Units × 10^{-5})	H1 Kinase (Units × 10^3/mg)	MPF (Units × 10^{-3}/mg)	H1 Kinase	MPF
Supernatant (100,000 × g)	4800	2448	250	0.51	5	100	100
DEAE cellulose	1100	2398	210	2.2	19	98	85
Hydroxylapatite	254	1152	140	4.5	55	47	55
Phosphocellulose	100	1050	150	10.5	150	43	60
TSKG 3000 SWG	9	463	50	51	550	19	20
Mono Q	0.85	128	10	150	1750	5.2	4
Mono S	0.042	22	2	520	4800	0.9	0.8

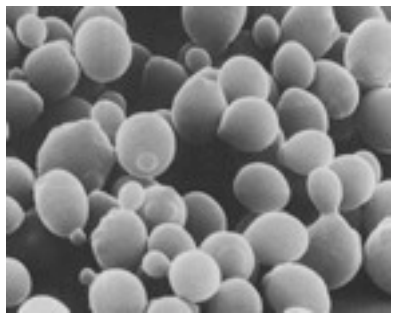
One unit of H1 kinase activity corresponds to 1 μ1mol of ^{32}P transferred per min at 25°C. One unit of MPF corresponds to the amount of MPF required in 50 nl of a microinjected sample for 50% of the recipient Xenopus oocytes to undergo GVBD (Wu and Gerhart, 1980).



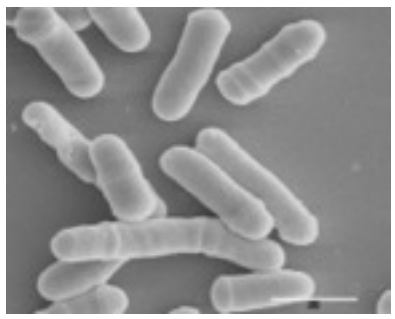
To show that MPF contained Cdc2 the researchers who had purified MPF used an early antibody against Cdc2 (called anti-PSTAIR - a motif found in all Cdc2 homologues) and showed that Cdc2 co-purified with histone H1 kianse activity (and a 34kD protein). The left figure are the fractions off the hydroxylapatite column, the right off the TSKG 3000 SWG column.

Why do *cdc2* and *cdc28* mutants behave so differently?

budding yeast

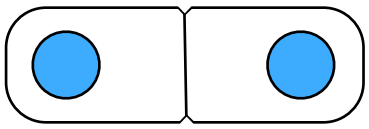


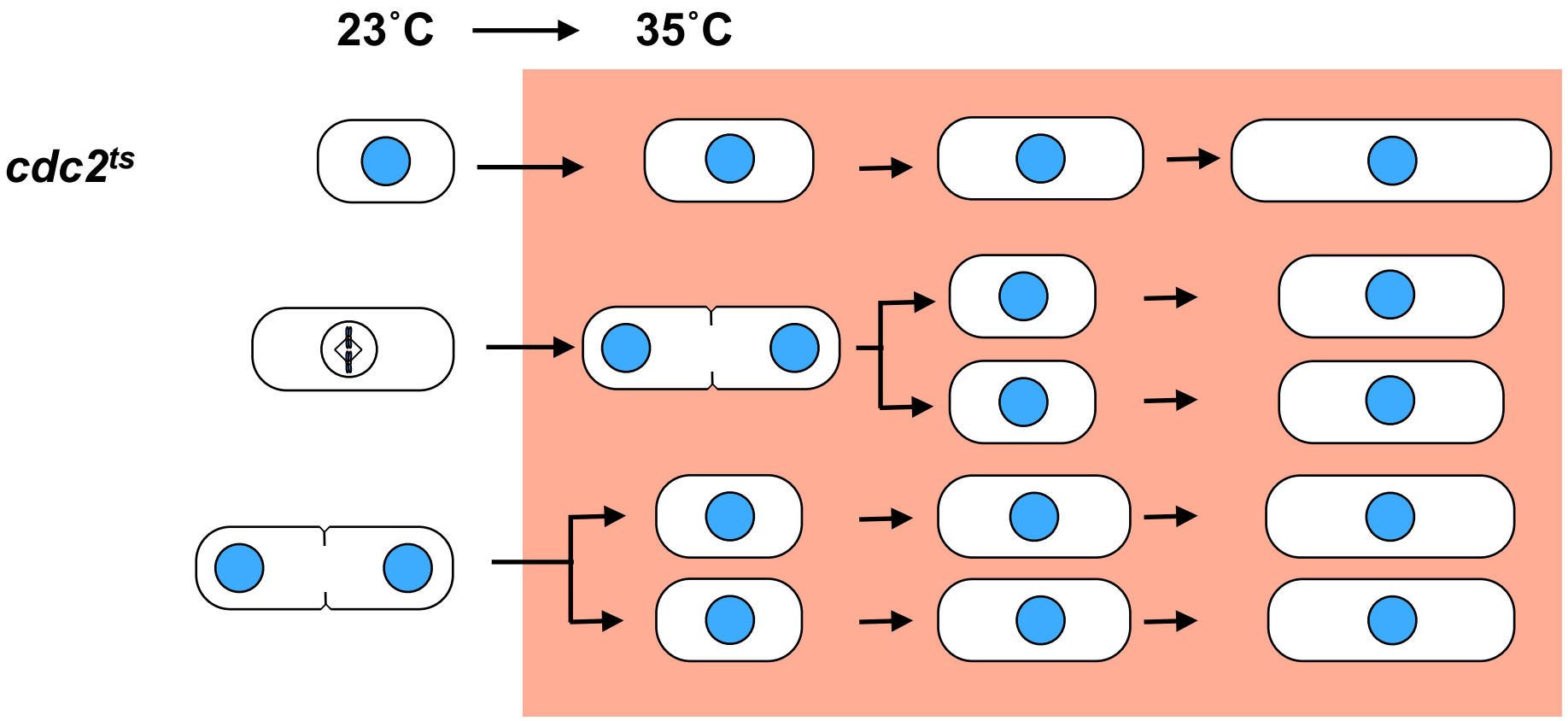
fission yeast



One clue as to why the two mutants are so different are the way their cell cycles are organized. If you remember, fission yeast barely has a G1, while budding yeast barely has a G2.

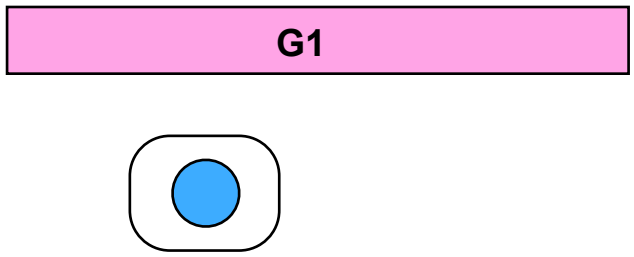
Asynchronous fission yeast contain virtually no cells in G1



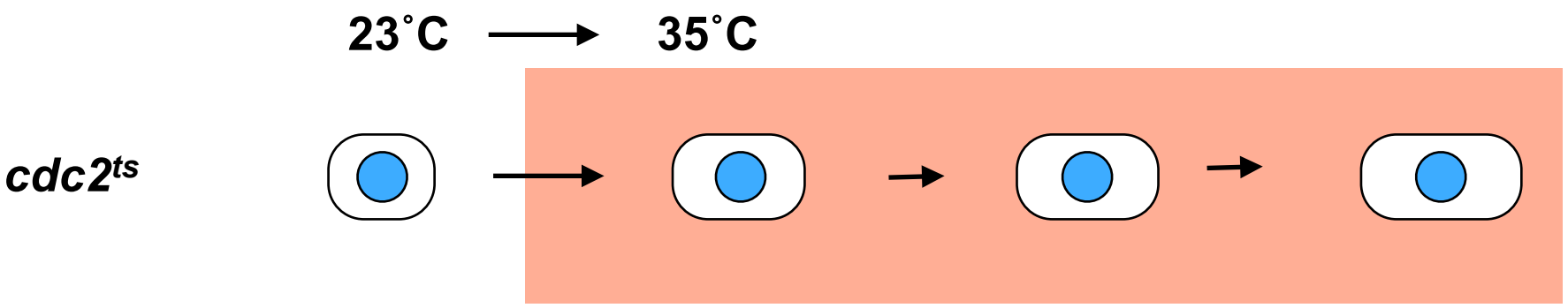


Although cdc2-ts arrests only in G2, when a asynchronous population of fission yeast are shifted to 35 degrees its unlikely any of them would arrest in G1 because most cells in the population don't have a G1 phase.

Nutrient starved fission yeast arrest in stationary phase which is G1-like

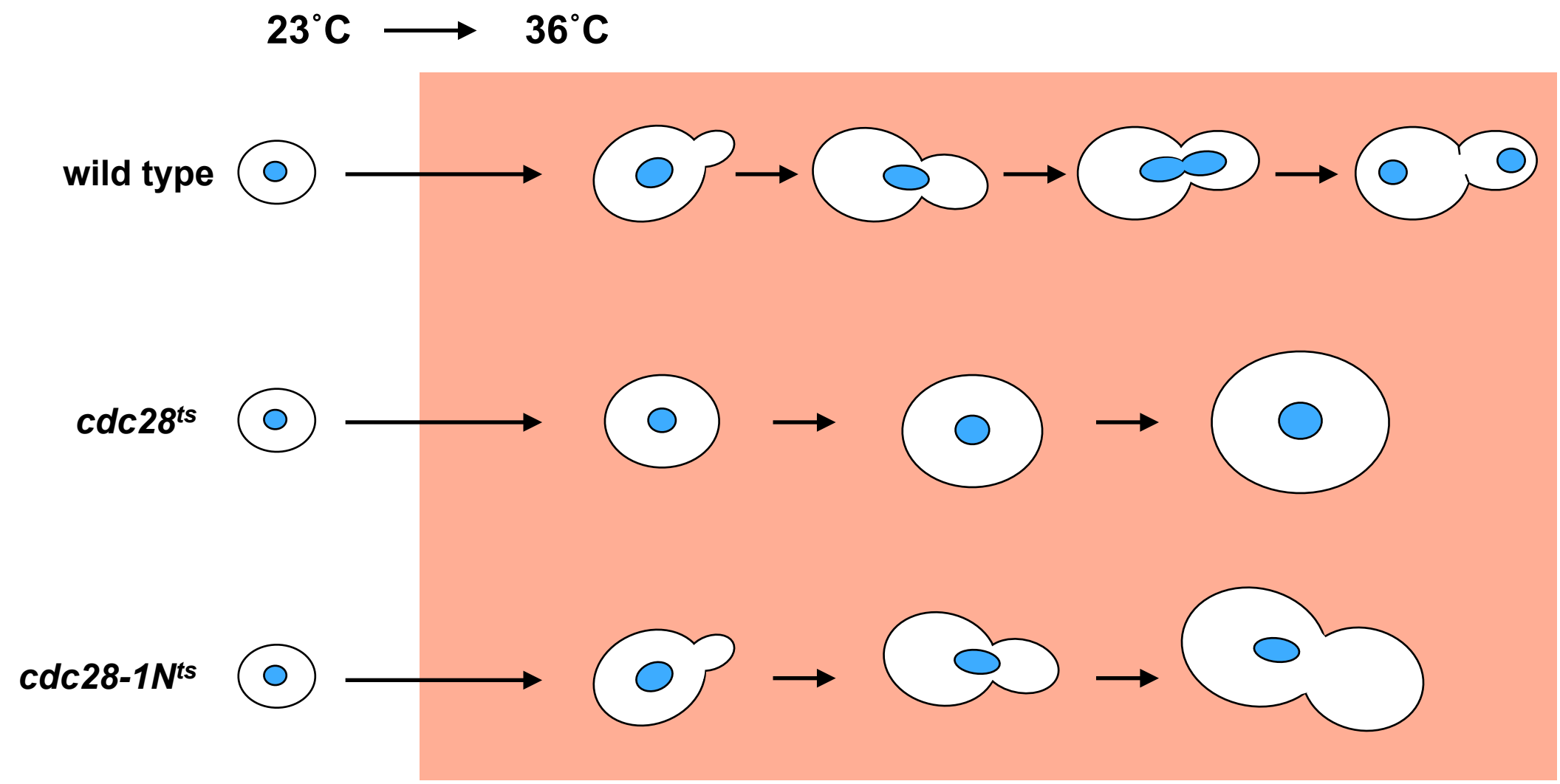


When released into fresh nutrients at the restrictive temperature, *cdc2* mutants arrest in G1 at START like *cdc28*^{ts} mutants in budding yeast



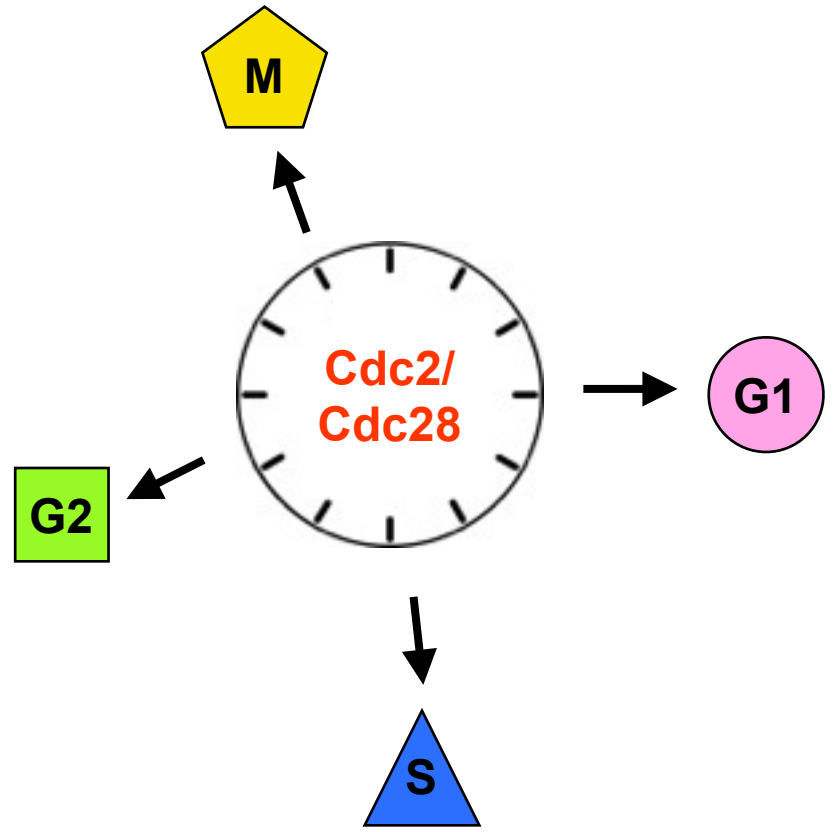
Nutrient starvation is a way to cause a prolonged G1-like arrest (actually cells arrest in G0), if cdc2-ts are released from this arrest at 35 degrees they arrest in G1, not G2, showing that Cdc2 in fission functions at two cell cycle stages, G1 and G2.

Alleles of *cdc28* can arrest at two points



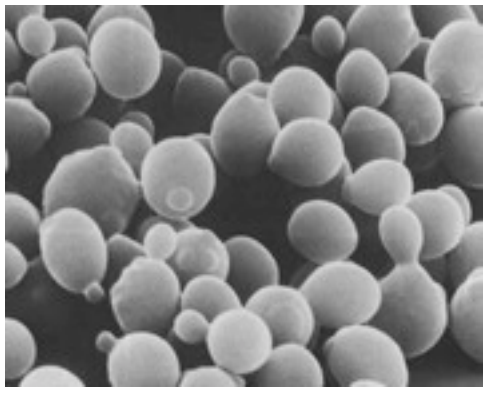
A special allele of CDC28 revealed that Cdc28 in budding yeast also regulates mitosis. cdc28-1N, a different allele of cdc28 arrested in mitosis and its execution point is in mitosis. It is thought that this allele was difficult to find because the requirement for Cdc28 protein is much lower in mitosis than in G1, so that alleles that arrest in G1 retain enough activity to pass through mitosis. Cdc28-1N has a special (still undetermined) defect that causes an arrest in mitosis.

Cdc2/Cdc28 drives the transitions through most cell cycle stages

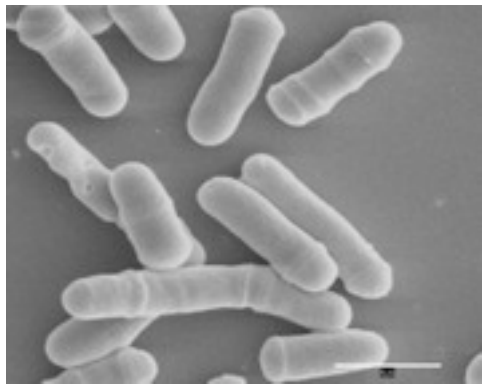


This suggested that Cdc28 in budding yeast and Cdc2 in fission yeast regulate multiple cell cycle transitions, and behaves as we might expect the clock or engine of the cell cycle to behave.

Cyclin-dependent kinases



Cdc28 = Cdk1



Cdc2 = Cdk1

Cdc2 = Cdk1
Cdk2
Cdk4
Cdk6
Cdk7

To sum up Cdc28 and Cdc2 encode homologous protein kinases, which have been renamed Cdk1 (cyclin-dependent kinase 1), and Cdk1 encodes a conserved master regulator of multiple cell cycle transitions. In vertebrates there are additional homologs of Cdk1, which I'll discuss briefly in the third lecture. In budding and fission yeast there is only one Cdk1, which regulates multiple cell cycle transitions. In the next lecture we'll discuss the cyclin-dependent part of Cdk.

What is cyclin?

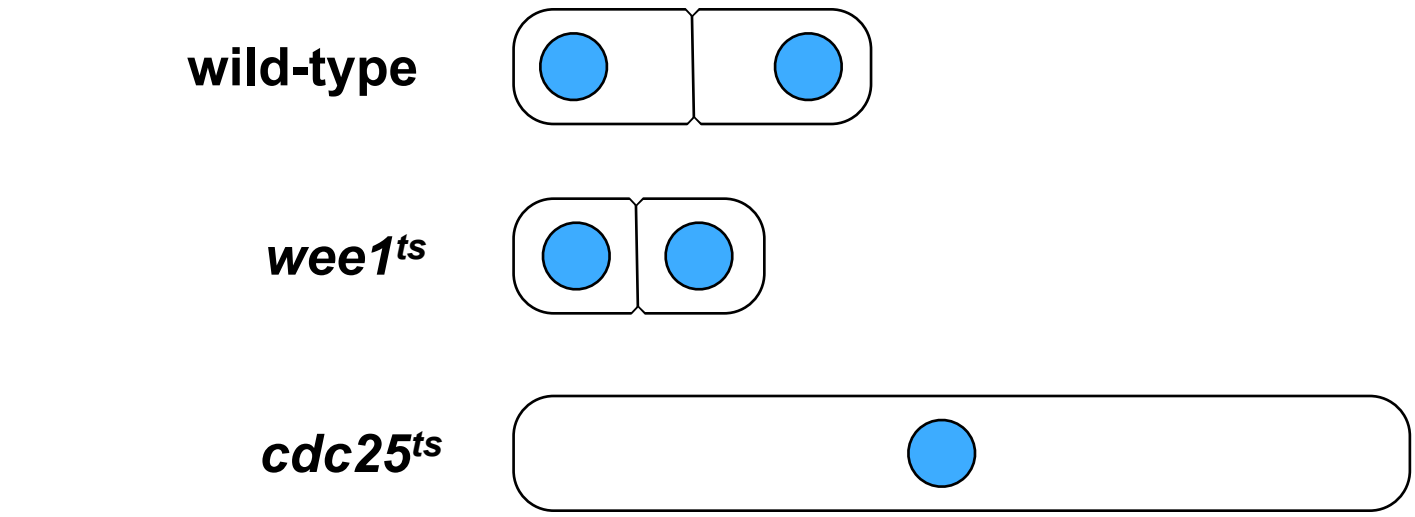
Cyclins are proteins that bind to Cdks

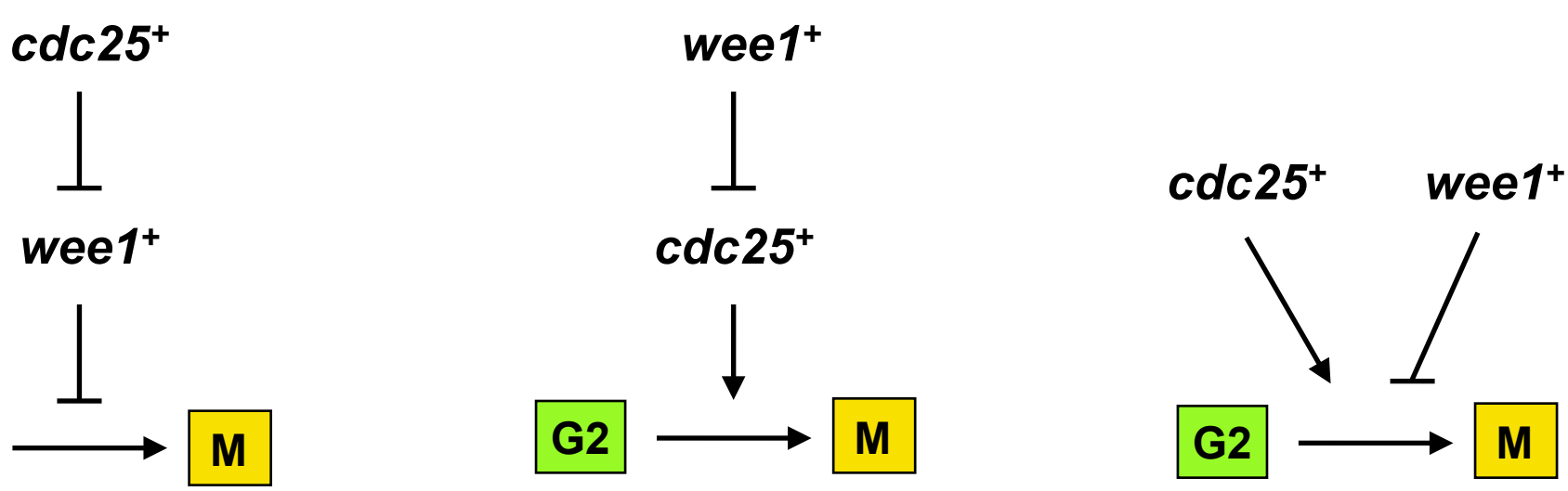
fission yeast *cdc13*$^{+}$ encodes a cyclin

As its name implies, cyclin protein levels cycle and play a key role in what makes the cell cycle cycle

We'll talk a lot more about cyclin

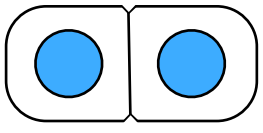
Russell and Nurse, 1986 determined the relationship between *cdc25* and *wee1*



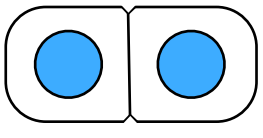


Russell and Nurse, 1986 illustrates the nuts and bolts of epistasis analysis and shows its often messier than showed in my hypothetical example. The data in the paper did get it right and sort out how these two genes interact. We'll go through the paper by answering the questions I gave you.

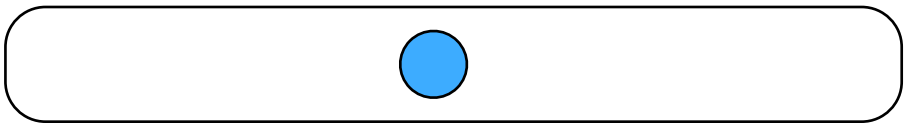
1. Based on information in the Introduction why do the authors focus on *wee1*, *cdc25* and *cdc2*? Why is *cdc2* so special?

wee1^{ts} 

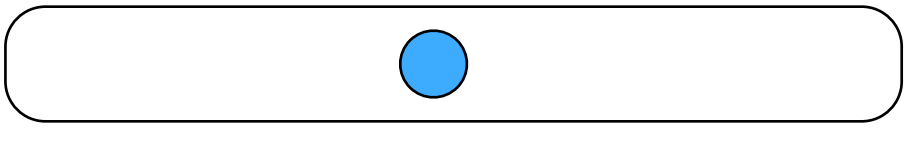
Speeds the transition from G2 to M

cdc2-3w^{D} 

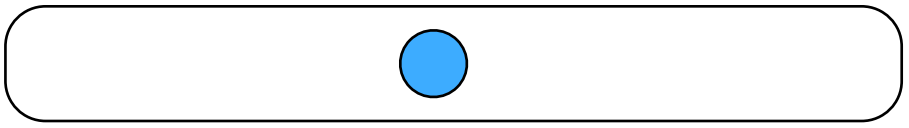
Speeds the transition from G2 to M

cdc2^{ts} 

Blocks the transition from G2 to M

cdc25^{ts} 

Blocks the transition from G2 to M

cdc13^{ts} 

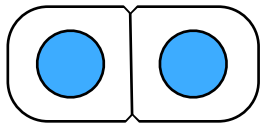
Blocks the transition from G2 to M

The two types of mutants clearly indicated that cdc2 was special.

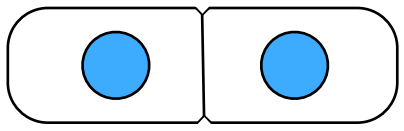
Three critical bits of data from the Introduction

1. *wee1*$^{-}$ cells speed transition from G2 to M. *wee1*$^{+}$/*wee1*$^{-}$ cells have intermediate phenotype suggesting that the dose of Wee1 regulates the speed of this transition

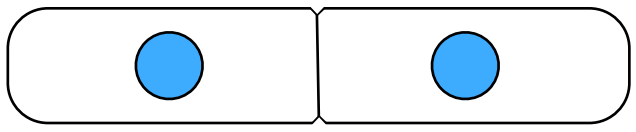
***wee1*$^{-}$/*wee1*$^{-}$**



***wee1*$^{+}$/*wee1*$^{-}$**



***wee1*$^{+}$/*wee1*$^{+}$**

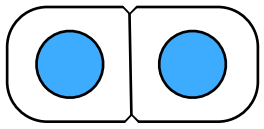


From the Introduction we get three critical bits of data. 1) Wee1 is dose dependent.

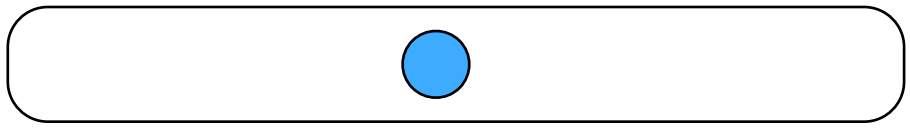
Three critical bits of data from the Introduction

2. There are two classes of *cdc2* mutants, those that arrest and those that are wee. The two classes suggest Cdc2 is an inducer of mitosis, and can be mutated to a gain-of-function allele that speeds this transition

cdc2-3w^{D}



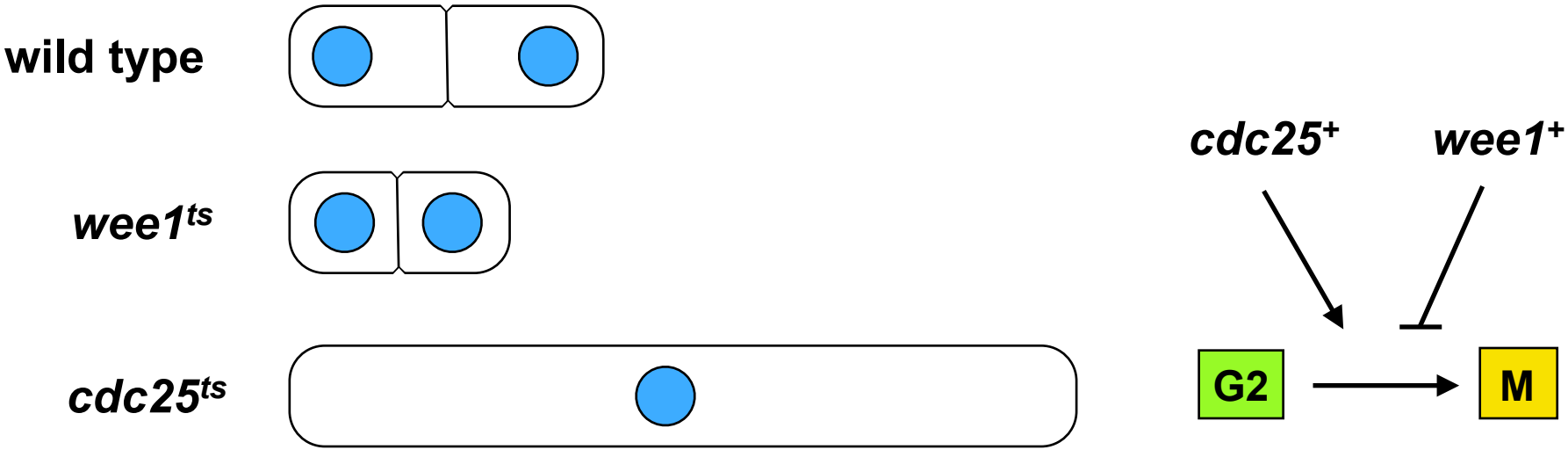
cdc2^{ts}



2) There are two types of cdc2 mutant.

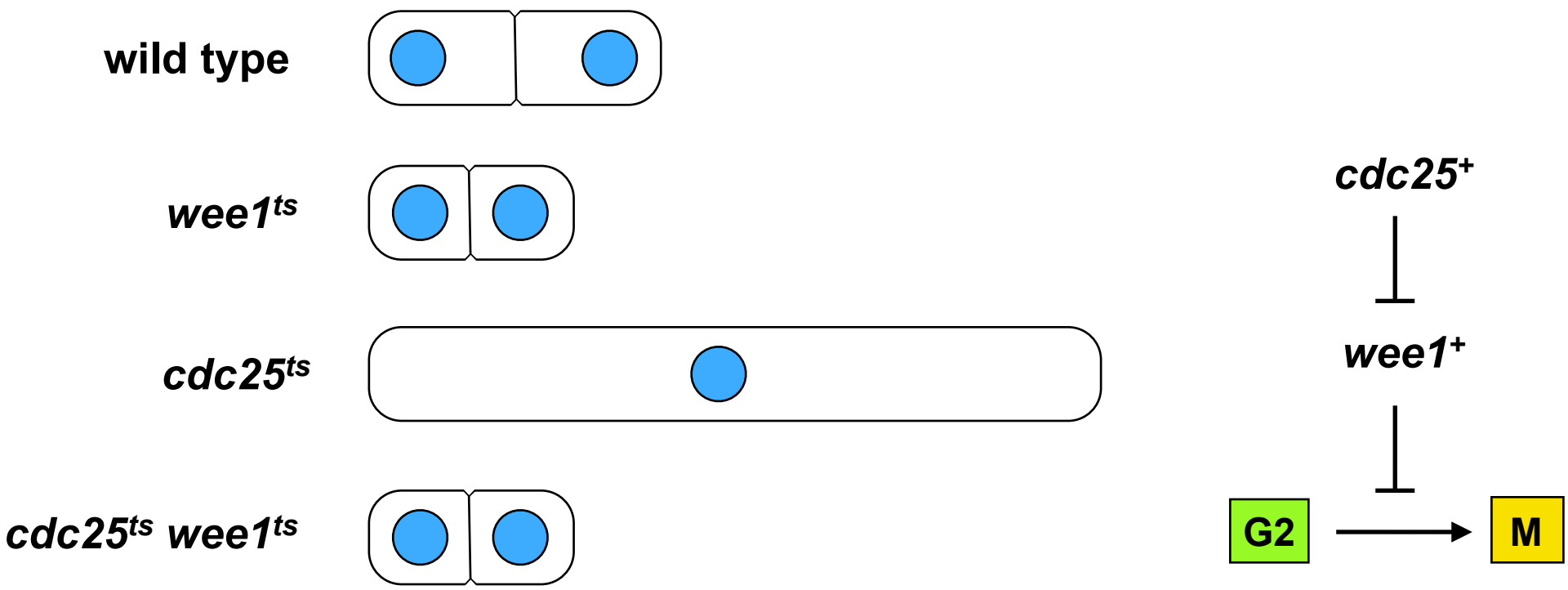
Three critical bits of data from the Introduction

3. *cdc25* mutants also block the transition into M, but this mutation can be suppressed by *wee1* mutants.



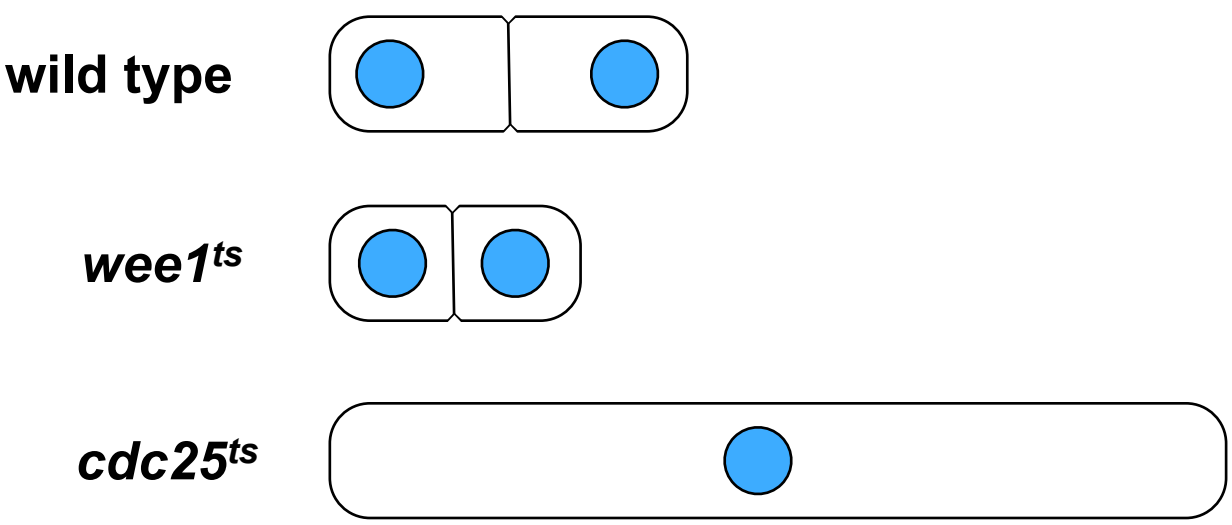
3) wee1 mutants suppress cdc25 mutants. I drew this as supporting model 3, where cdc25 and wee1 act independently – but this depends on two falsehood I told you – one is that the double is actually the same size as wild type – which it isn't,

3. *cdc25* mutants also block the transition into M, but this mutation can be suppressed by *wee1* mutants. And they are wee, not wt.



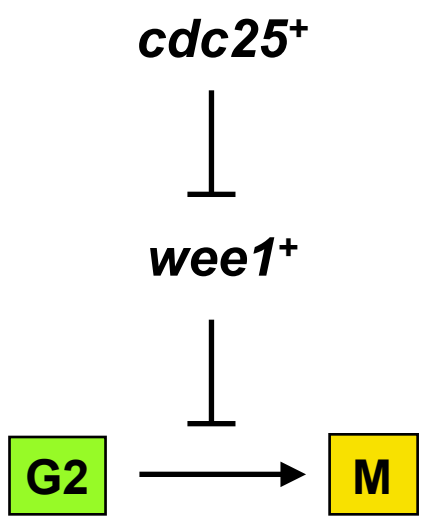
they are actually wee. This actually supports model 1, where wee1 is downstream. It also depends on the assumption that the cdc25 and wee1 alleles are complete nulls.

3. *cdc25* mutants also block the transition into M, but this mutation can be suppressed by *wee1* mutants. And they are wee, not wt. If *cdc25* mutants had residual activity then *wee1* could even be upstream of *cdc25.*

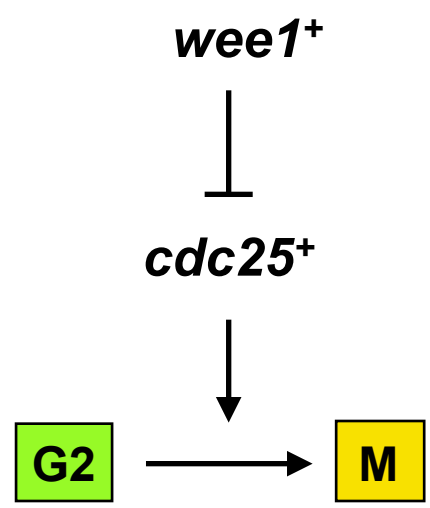


The authors thought wee1 mutants were null, but they thought there was residual Cdc25 activity in the cdc25 mutants. This then could support model 2 (or model 3)! So early on the authors weren't so sure how Cdc25, Wee1 and Cdc2 interacted.

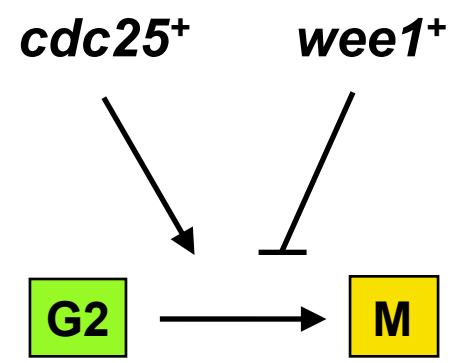
Russell and Nurse, 1986 determined the relationship between *cdc25* and *wee1*



if both *wee1-50* and *cdc25-22* are null mutants



if both *wee1-50* is null, but *cdc25-22* has residual activity

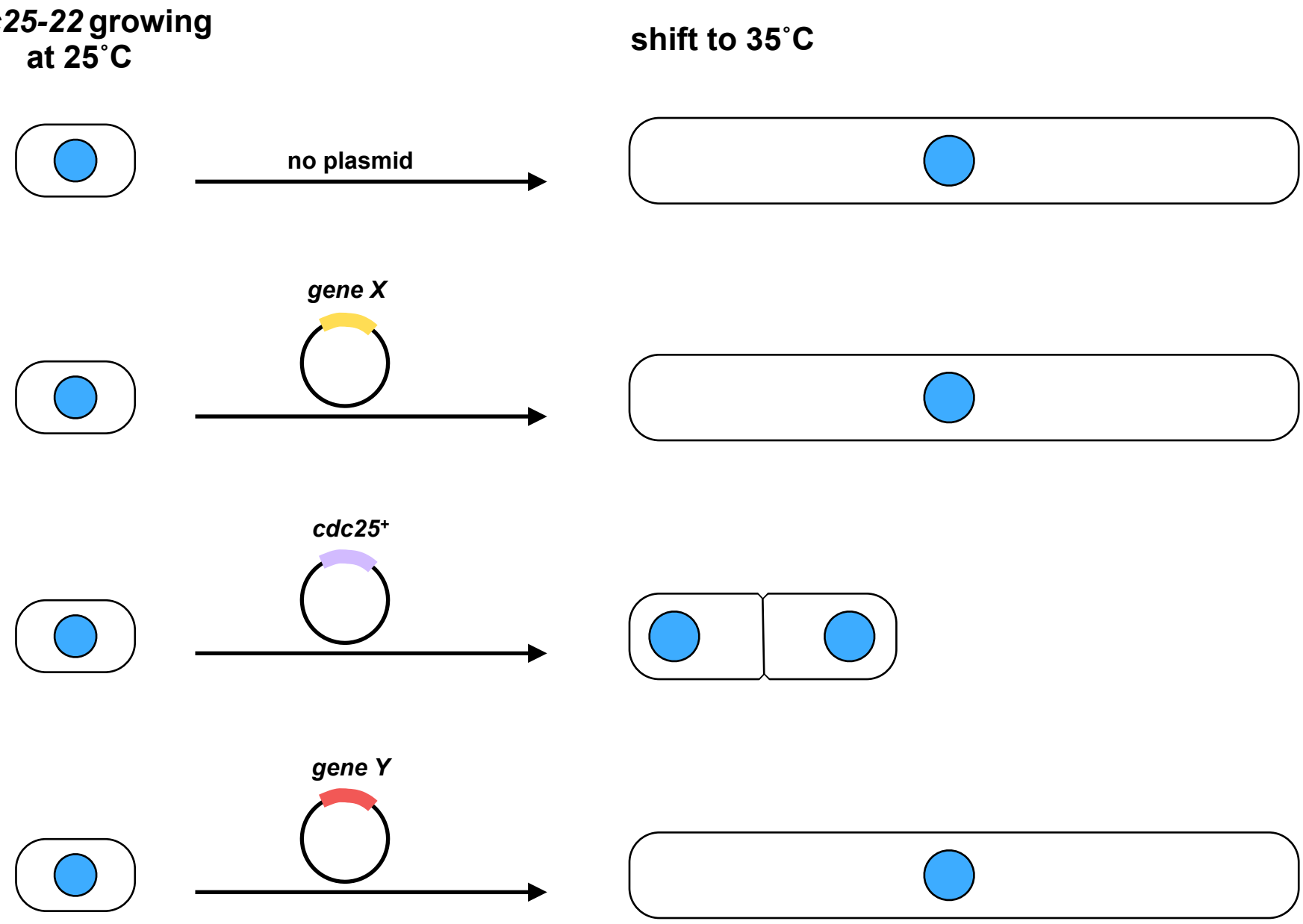


Given the information in the introduction any of these models may be correct.

What does Cdc25 encode?
And is it upstream of *wee1*?

This paper reports the cloning of Cdc25 and how having the sequence allowed them to do experiments to rule out model 1 and 2, and support model 3, that Wee1 and Cdc25 act independently.

cloning of *cdc25* by complementation



Transfect with plasmid library

Cdc25 was cloned by complementation: To clone these genes researchers used what is now a very standard approach. At the time, these were some of the first genes to be cloned by "complementation." The researchers made a "library" or collection of all fission yeast genes on a plasmid. Each different plasmid contains one fission yeast gene. The collection of plasmids were transfected into a cdc mutant, in this example cdc2-ts. After transfection the yeast containing plasmids are shifted to the restrictive temperature. In a cdc2-ts strain with no plasmid, the cdc2-ts mutant arrests in G2 and dies. In this example gene X and gene Y do not "complement" the cdc2-ts mutant, and when cdc2-ts contains these plasmids it arrests in G2 at the restrictive temperature and dies. If the plasmid contains the wild type cdc2 gene it "complements" and now cdc2-ts containing this plasmid grows normally at the restrictive temperature. Isolating the plasmid out of this strain (called the "rescuing" plasmid), and sequencing the gene on the plasmid allowed the identity of the mutated gene to be determined.

the complementing clones

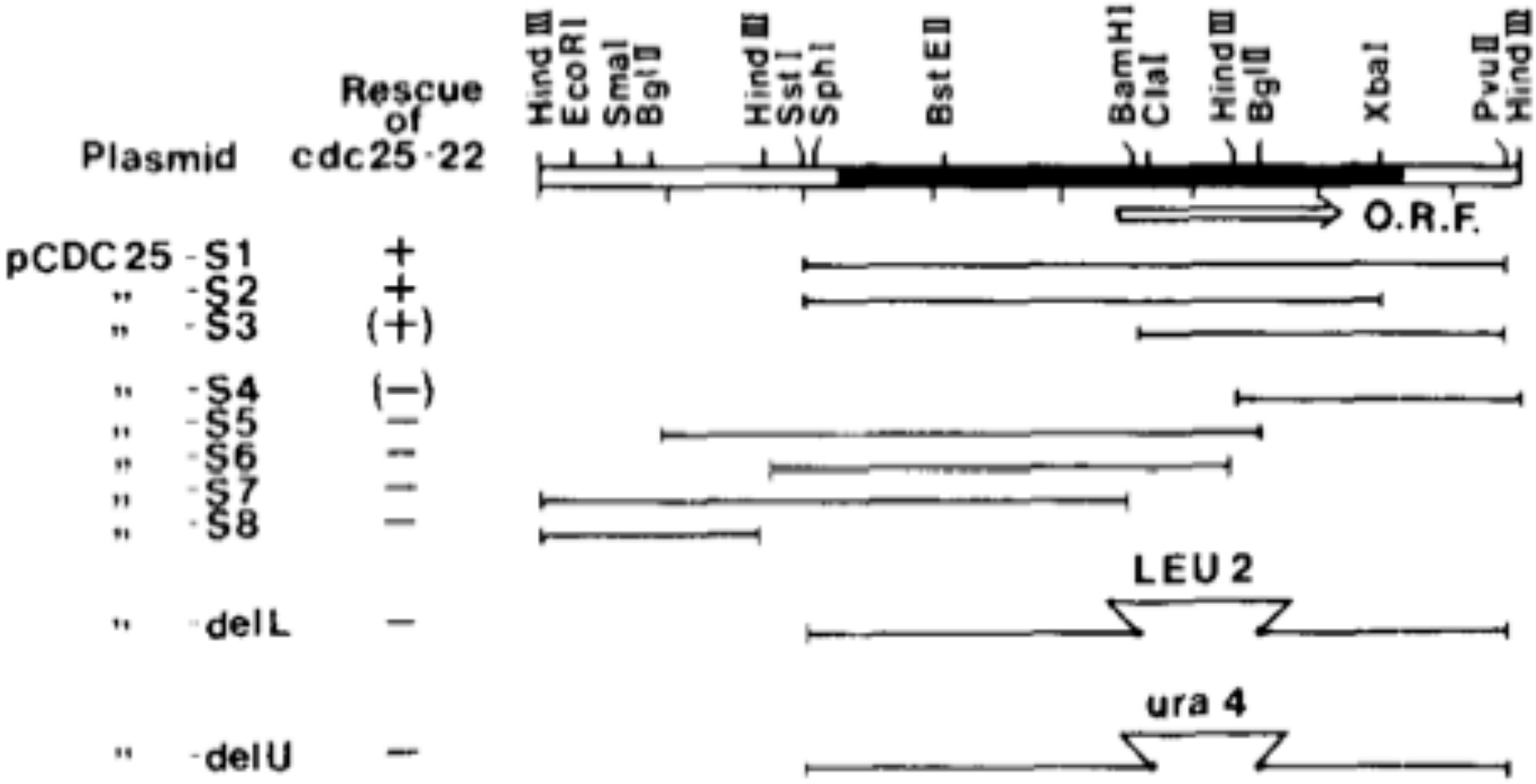


Figure 1. Subcloning and Mapping *cdc25*$^{+}$

The complementing clones. Very old school figure.

the sequence! Didn't look like anything....
(we'll get to what it encodes)

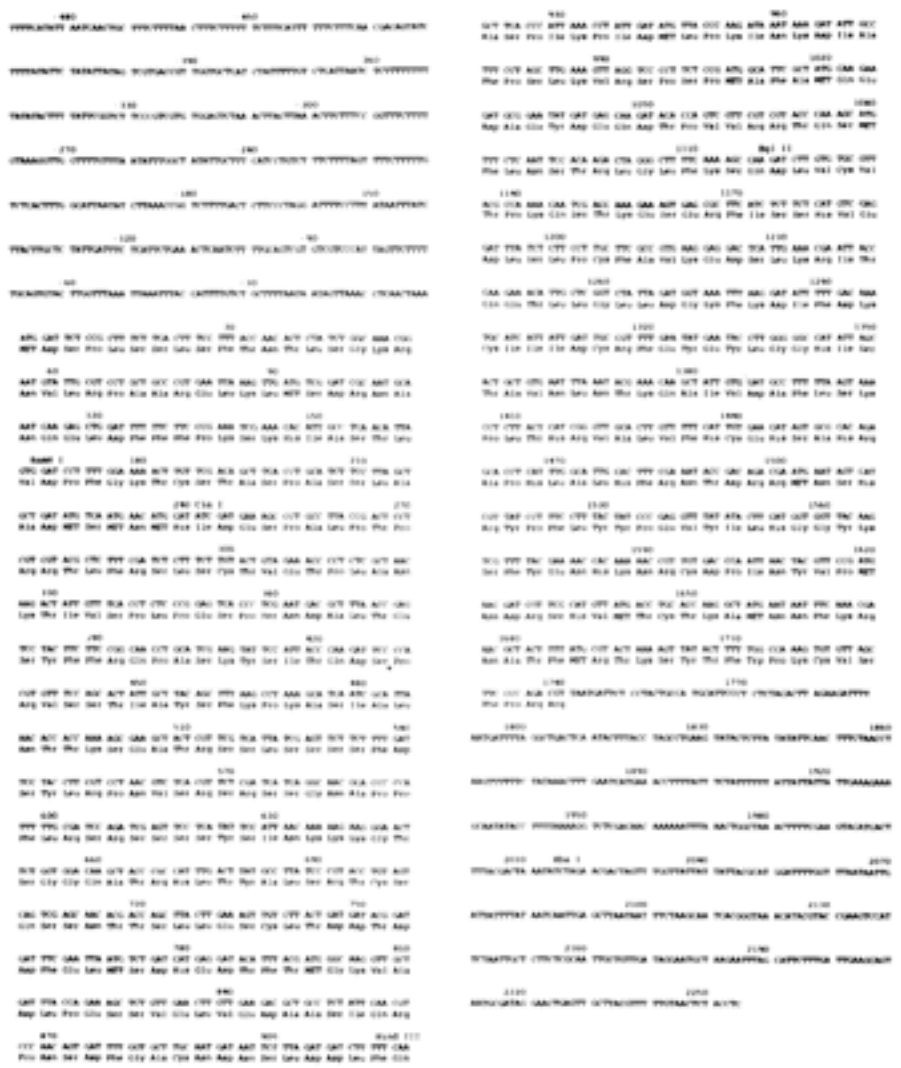


Figure 2. DNA Sequence and Predicted Amino Acid Sequence of cdc25⁺

The DNA sequence of a 2745 nucleotide region containing the 1.75 kb cdc25⁺ ORF is shown. The sites of restriction enzymes mentioned in the text are shown. Nucleotide numbering starts from the start of the ORF.

The sequence. A requirement, but it didn't tell them much at the time.

Once Cdc25 was cloned, what can they do with it?

1. Overexpress it.

2. Delete it.

What can they do with the sequence. Back then two techniques were just being developed - homologous recombination to delete genes, and tools for overexpression.

What happens when Cdc25 is overexpressed?

What happens when *cdc25* is expressed to very high levels?
What two things does this tell us about *cdc25*?

1. Integrate multiple copies of *cdc25* into a genomic locus

2. Drive Cdc25 expression from the *adh* promoter.

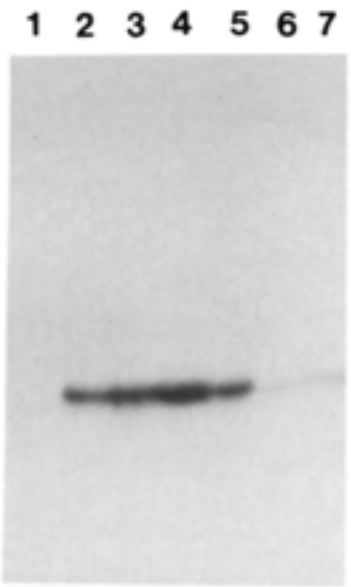


Figure 3. Quantitative Determination of *cdc25*⁺ RNA Levels in pCDC25-1 Tandem Integration Strains Using S1 Nuclease

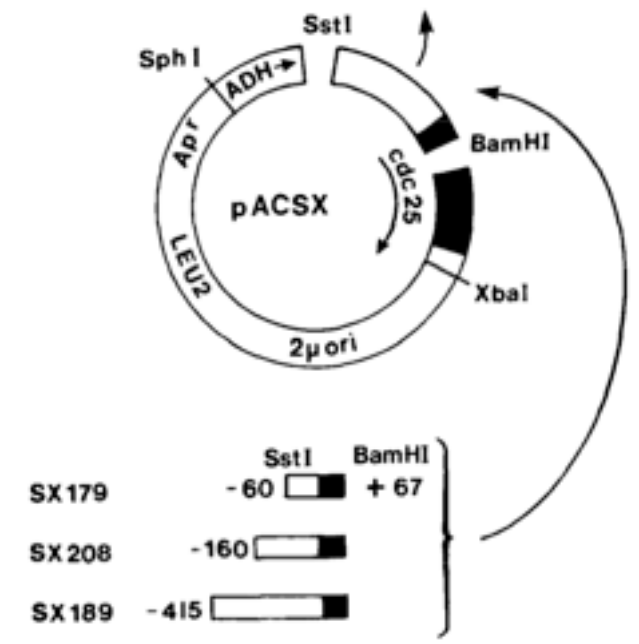


Figure 4. Fusions of *cdc25*⁺ to *adh* Promoter

To overexpress Cdc25 they either integrated many copies (confirmed by Northern analysis) or droves its expression from a constitutive promoter (adh).

Cells are smaller

Table 1. Cell Division Lengths and Generation Times

	Strain	Length at Cell Division (μm)	Generatio at 25°C (
wild type	972	14.3 (100%)	240
5X *cdc25+*	TI5-4	10.9 (76%)	260
adh/cdc25+	pSX-179 Integrant	7.8 (55%)	300
wee1-1	wee1-1	8.2 (57%)	300
control	pDB248 Integrant	14.5 (101%)	260

The key table. Overexpressing Cdc25 makes cells smaller! This is all done by measuring length at division.

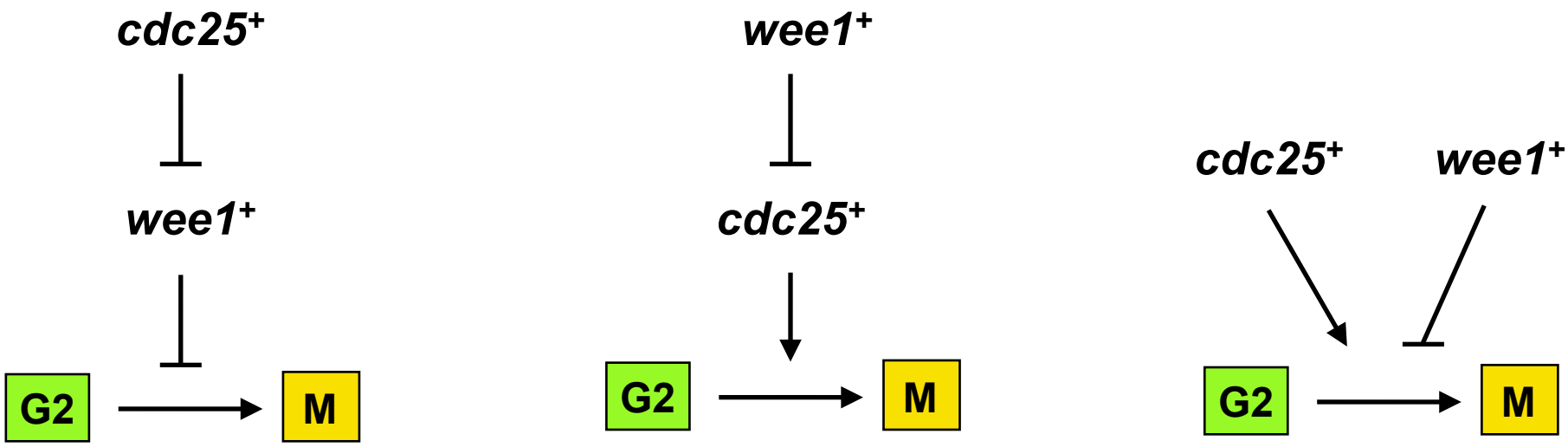
What happens when Cdc25 is overexpressed?

What happens when *cdc25* is expressed to very high levels?
What two things does this tell us about *cdc25*?

1. Cdc25 is a mitotic inducer (like Cdc2)

2. Cdc25 is dose dependent/rate-limiting (like Wee1).

3. This data doesn't really rule out one of these models, though it is hard to explain how both Cdc25 and Wee1 could be dose-dependent if they regulated each other.



The conclusions. They don't really point to one model.

What happens when *cdc25* is over-produced in *wee1-50* cells?
Why does the phenotype require a shift to 35˚C?

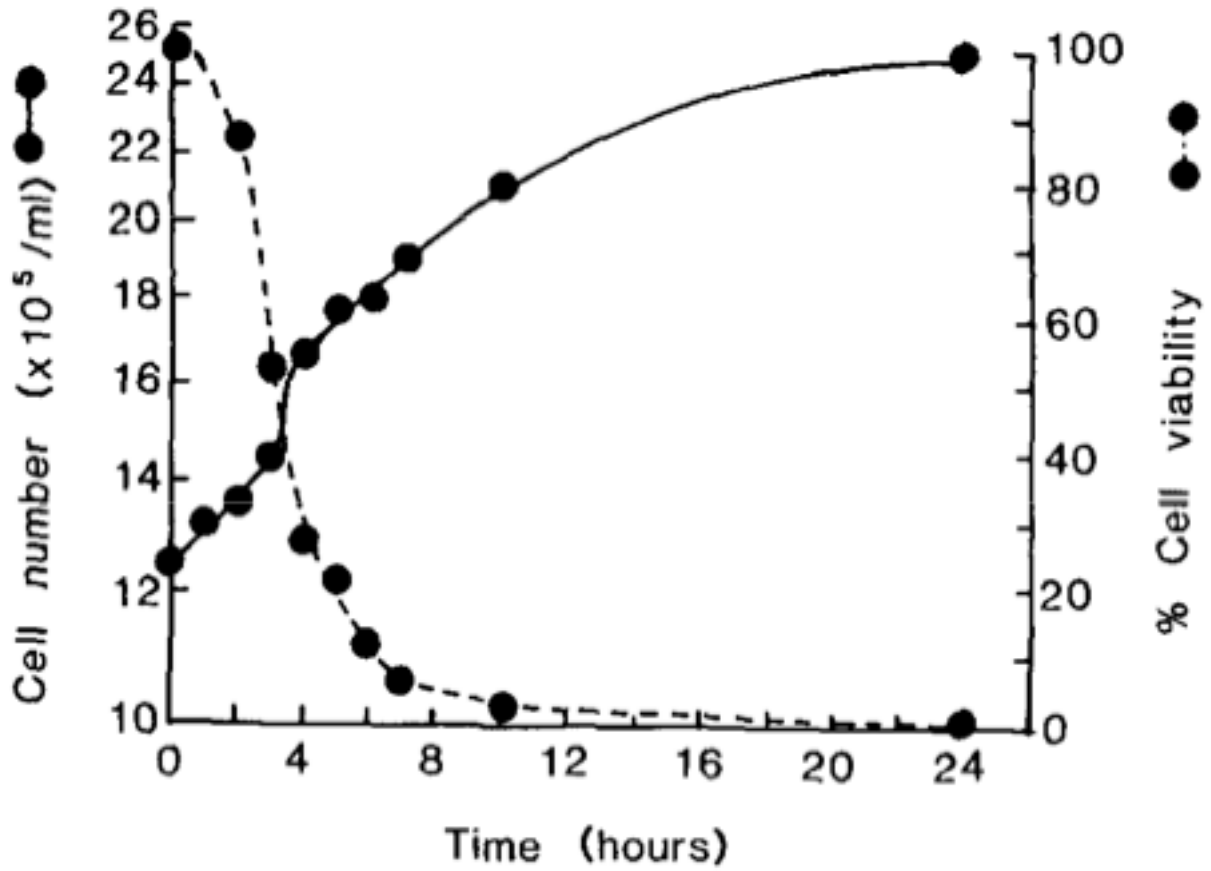


Figure 8. Viability Response of *wee1-50* pSX-179 Integrant Strain Shifted from 25°C to 35°C

However if Cdc25 is overexpressed in wee1 cells it kills them (only after the shift to 35˚C to inactivate Wee1).

What is wrong with the cells in Figure 9?

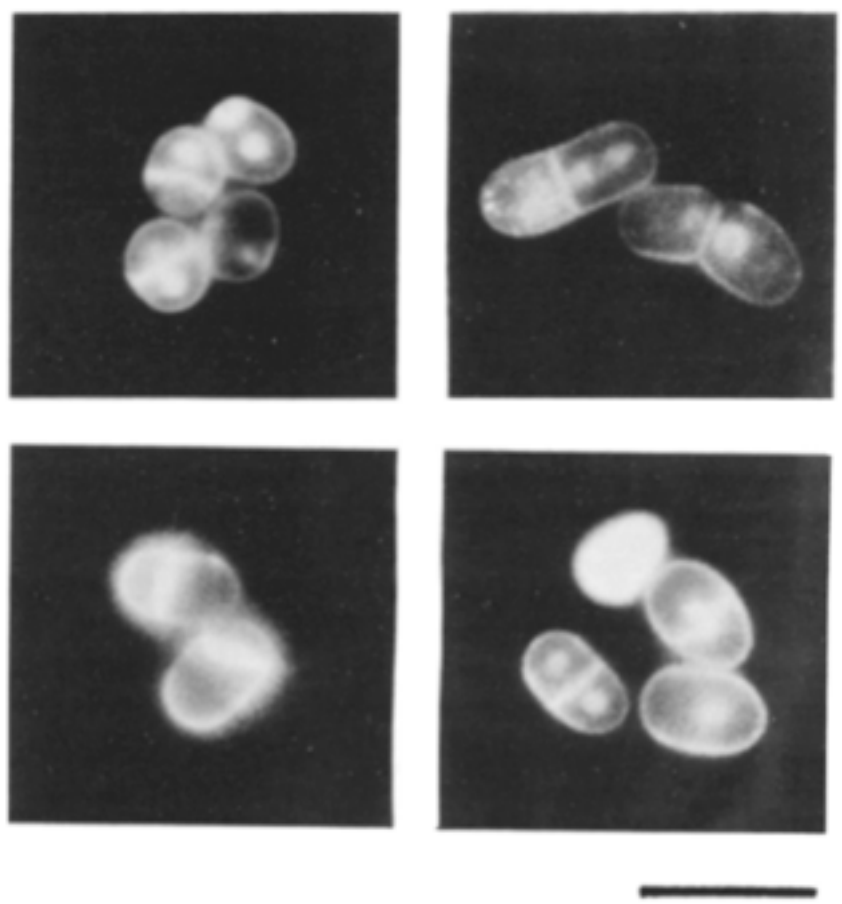


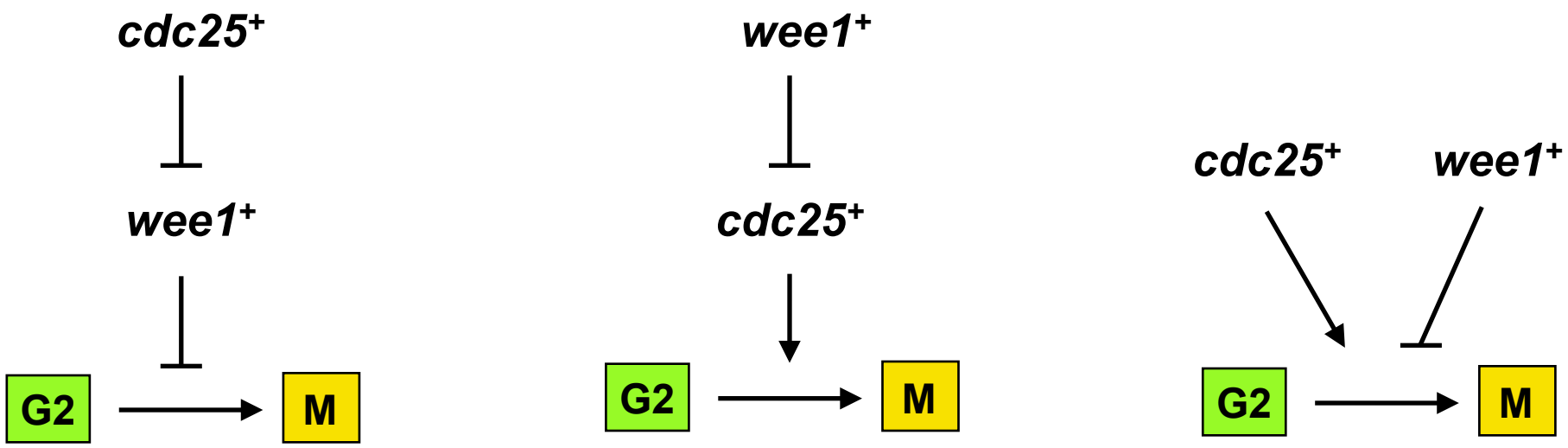
Figure 9. Abnormal Morphologies in *wee1-50* pSX-179 Integrant Strain Caused by Temperature Shift to 35°C

Here are some of these cells. They are super small. So small it kills the cells. You can see some septa cutting through the chromosomes in these pictures.

1. Overexpression of Cdc25 in *wee1-50* cells make the cells <u>very small</u> and kills them.

2. Argues against the first model, but is not conclusive. The authors cannot be sure if there is residual Wee1 activity in the *wee1-50* mutant. But they think there is not.

3. Can they rule out model 2 or 3 by deleting *cdc25*$^{+}$?

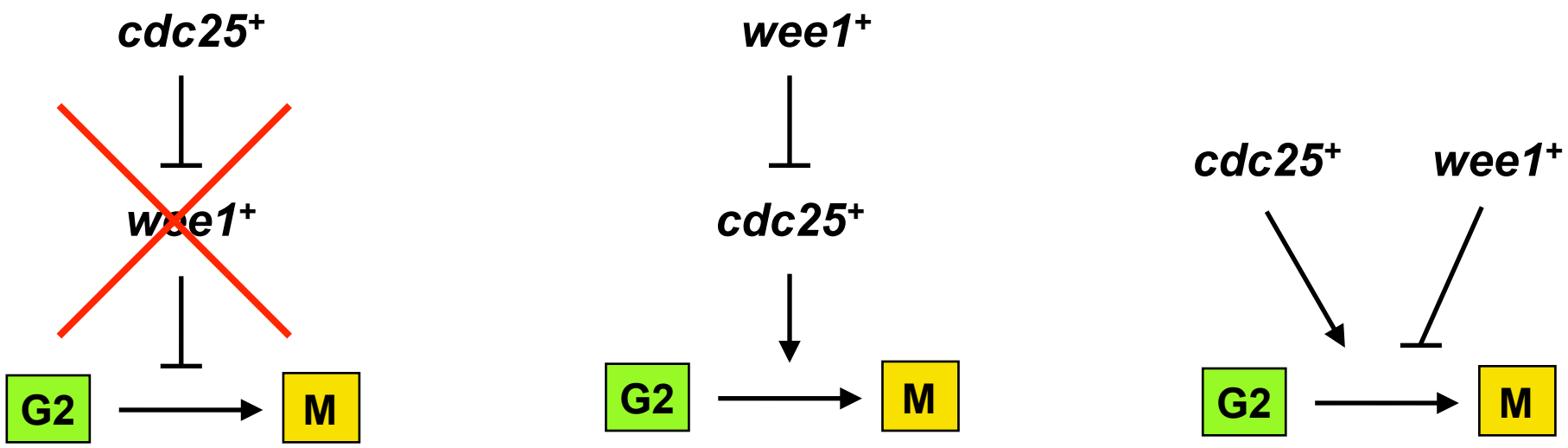


The conclusions of this experiment. Argues against first model if wee1-50 is a null. They think it is because although wee1-50 is ts, most wee1 alleles are not - and there is a nonsense suppressed allele (which means there is a premature STOP codon). They later learned that wee1 is not essential, so their assumption about wee1-50 is likely correct. Why is model 1 ruled out? Why not model 2 and 3?

1. Overexpression of Cdc25 in *wee1-50* cells make the cells very small and kills them.

2. Argues against the first model, but is not conclusive. The authors cannot be sure if there is residual Wee1 activity in the *wee1-50* mutant. But they think there is not.

3. Can they rule out model 2 or 3 by deleting *cdc25*$^+$?



Can they rule out 2 or 3 by deleting cdc25?

Why do the authors disrupt *cdc25* in a diploid?

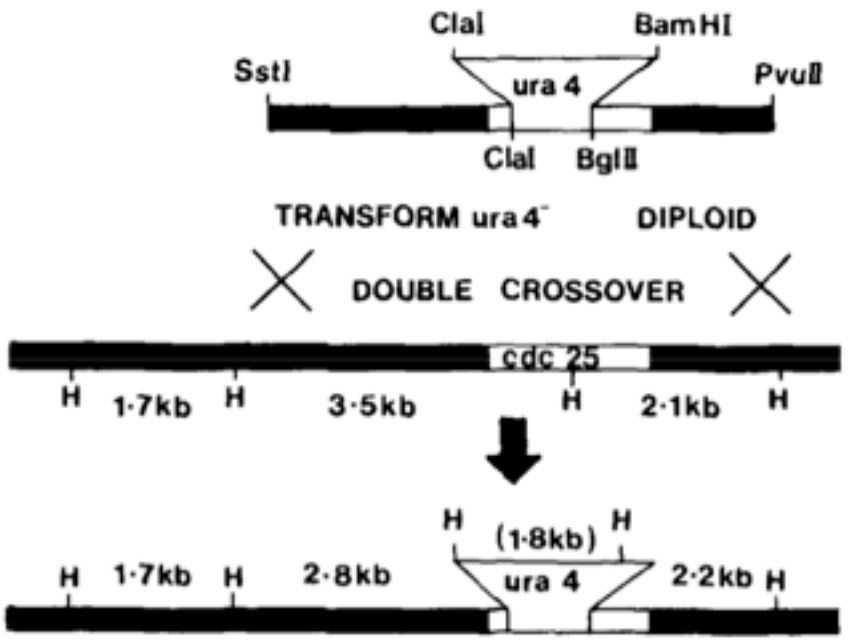


Figure 6. Gene Disruption of *cdc25*⁺

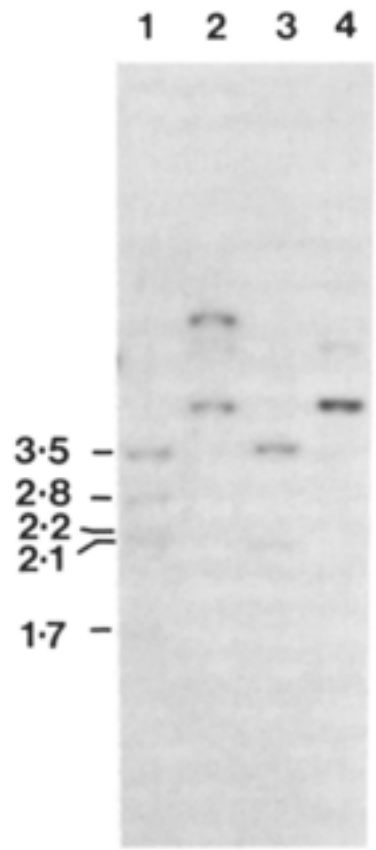
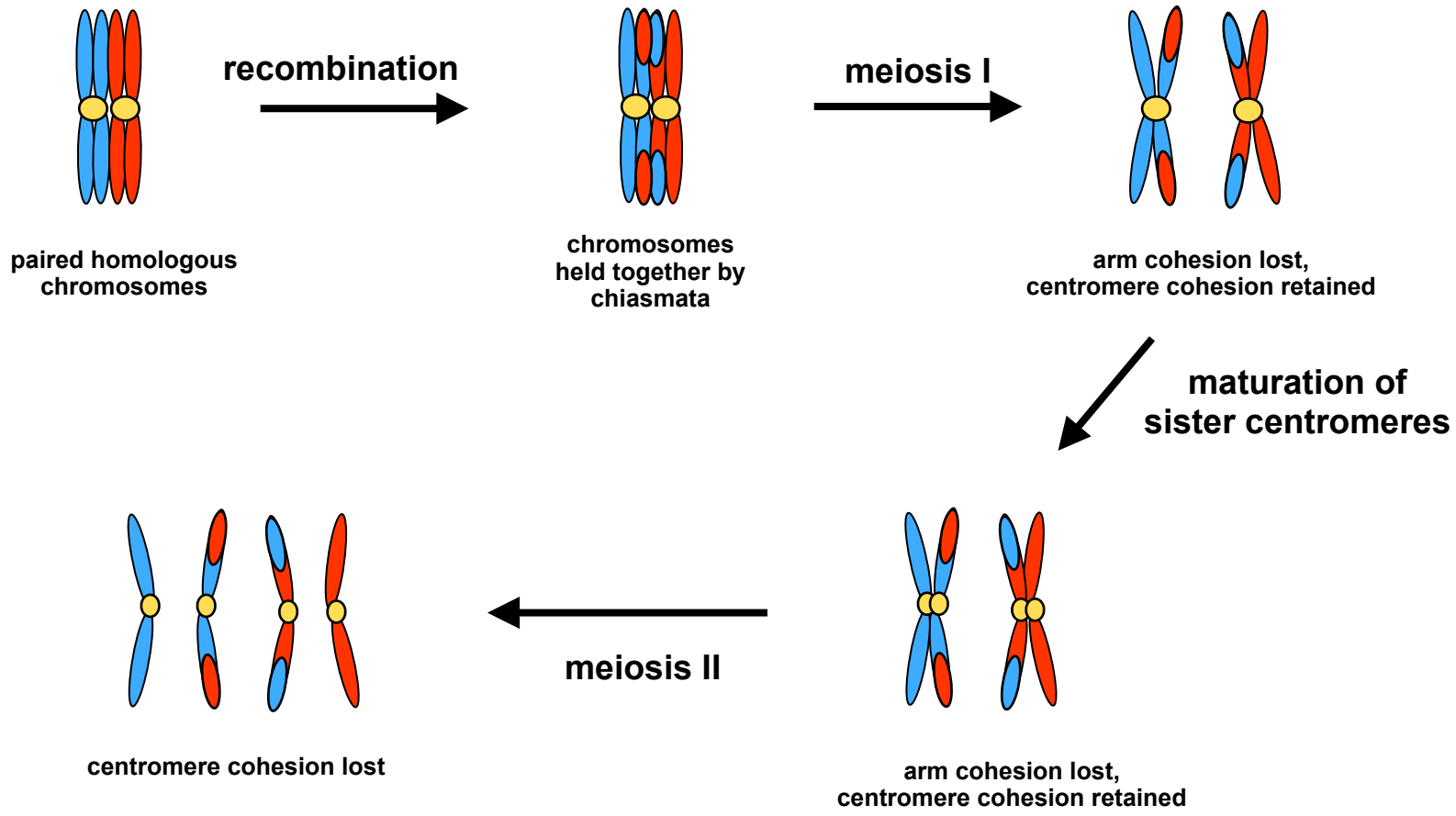


Figure 7. Southern Analysis of *cdc25*⁺ Gene Disruption Strain

The technique for deletion and confirmation by Southern blot. cdc25 is essential, so they had to delete it in a diploid.

***cdc25* is essential, so they delete it in a diploid and then put the diploid through meiosis. In fungi the four meiotic products form spores, that can be separated and germinated to form haploid colonies.**



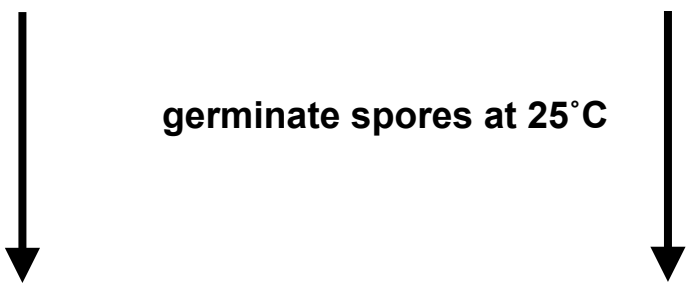
A reminder about meiosis.

1. *cdc25* mutants can be suppressed by *wee1* mutants.

2. can the *cdc25Δ::ura4* be suppressed by a *wee1* mutant?

***cdc25Δ::ura4/cdc25*$^{+}$**

***cdc25Δ::ura4/cdc25*$^{+}$**
***wee1-50/wee1*$^{+}$**



2 viable spores
&
2 dead spores

2 viable spores
&
2 dead spores

A reminder that cdc25 mutants are suppressed by wee1 mutants, so the experiment is to see if deletes could be isolated in a wee1-50 background. At permissive temperature 2 spores will be dead, as in the wee1+ situation.

1. *cdc25* mutants can be suppressed by *wee1* mutants.

2. can the *cdc25Δ::ura4* be suppressed by a *wee1* mutant?

cdc25Δ::ura4/cdc25⁺

germinate spores at 25˚C

2 viable spores
&
2 dead spores

cdc25Δ::ura4/cdc25⁺
wee1-50/wee1⁺

germinate spores at 25˚C

2 viable spores
&
2 dead spores

cdc25Δ::ura4/cdc25⁺
wee1-50/wee1⁺

germinate spores at 35˚C

authors were able
to isolate *ura⁺* spores
(that were also *wee1-50)*

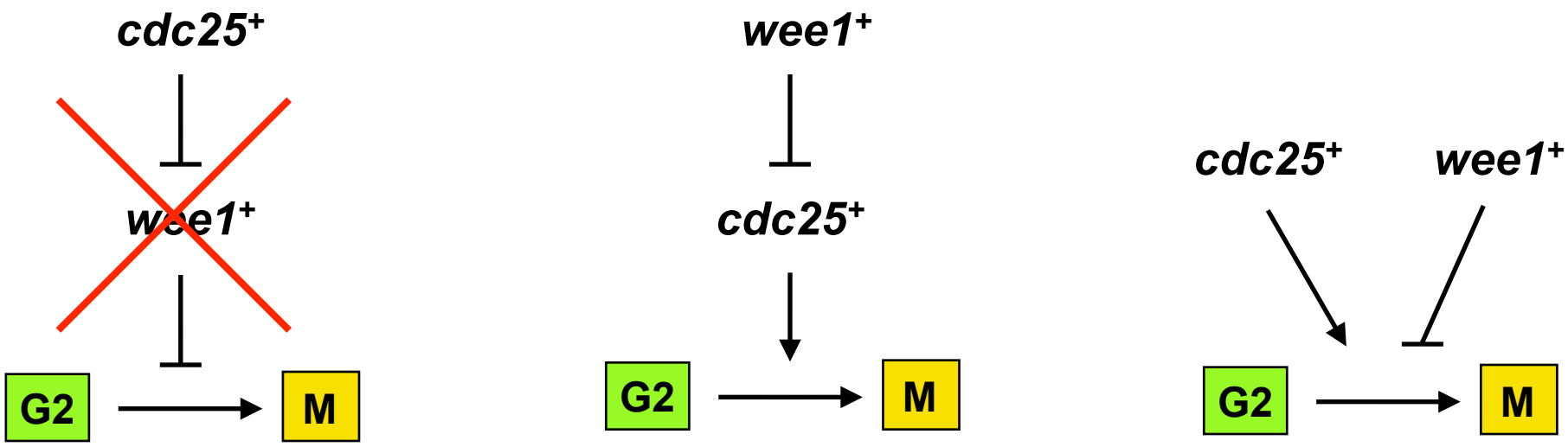
But at 35˚C, they were able to isolate ura+ spores, showing that inactivation of wee1 suppresses the cdc25Δ.

What important conclusion do the authors make about the relationship between *cdc25* and *wee1* from the viability of the *cdc25::ura4 wee1-50* cells at 35˚C? Why do these cells die at 25˚C? How do they die?

1. *wee1-50* suppresses *cdc25*Δ.

2. *cdc25*$^+$ is not required to induce mitosis.

3. Rules out middle model, leaving only the third model as the favored model.



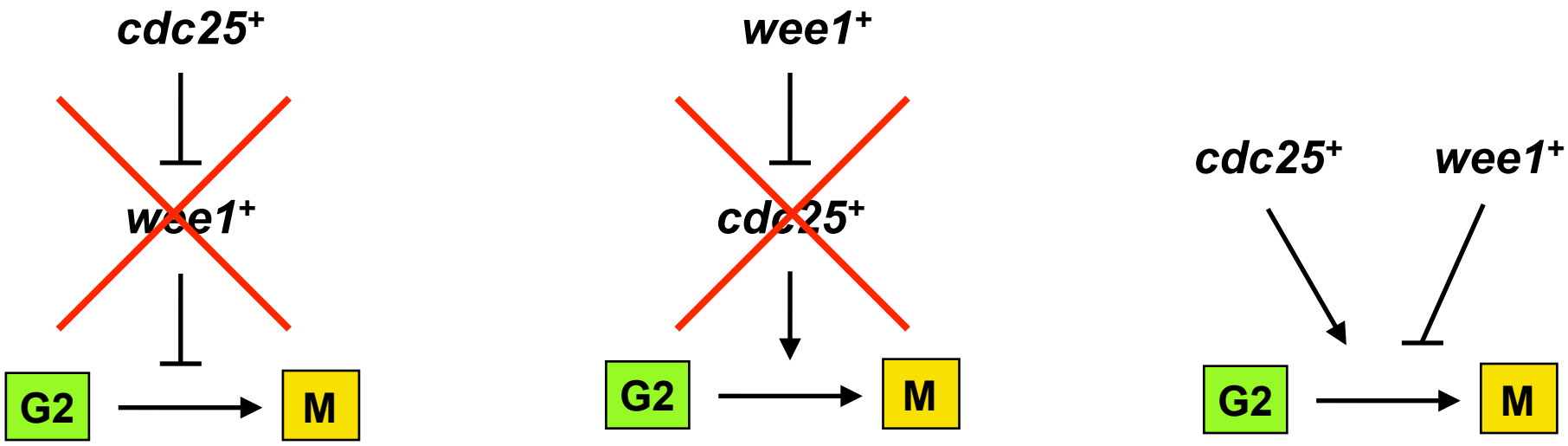
The conclusions of this experiment. wee1-50 suppresses the complete absence of cdc25. cdc25 therefore can't be absolutely required for mitosis. This

What important conclusion do the authors make about the relationship between *cdc25* and *wee1* from the viability of the *cdc25::ura4 wee1-50* cells at 35˚C? Why do these cells die at 25˚C? How do they die?

1. *wee1-50* suppresses *cdc25*Δ.

2. *cdc25*$^+$ is not required to induce mitosis.

3. Rules out middle model, leaving only the third model as the favored model.



rules out the middle model, leaving only the third model. The cells die at 25˚C because Wee1-50 is active at 25 degrees. This experiment was also done with cdc2Δ, and wee1 can't suppress that deletion, suggesting that cdc2 is essential for mitosis.

What important conclusion do the authors make about the relationship between *cdc25* and *wee1* from the viability of the *cdc25::ura4 wee1-50* cells at 35˚C? Why do these cells die at 25˚C? How do they die?

***wee1-50 cdc25Δ* at 25˚C**

wild type

adh/cdc25+

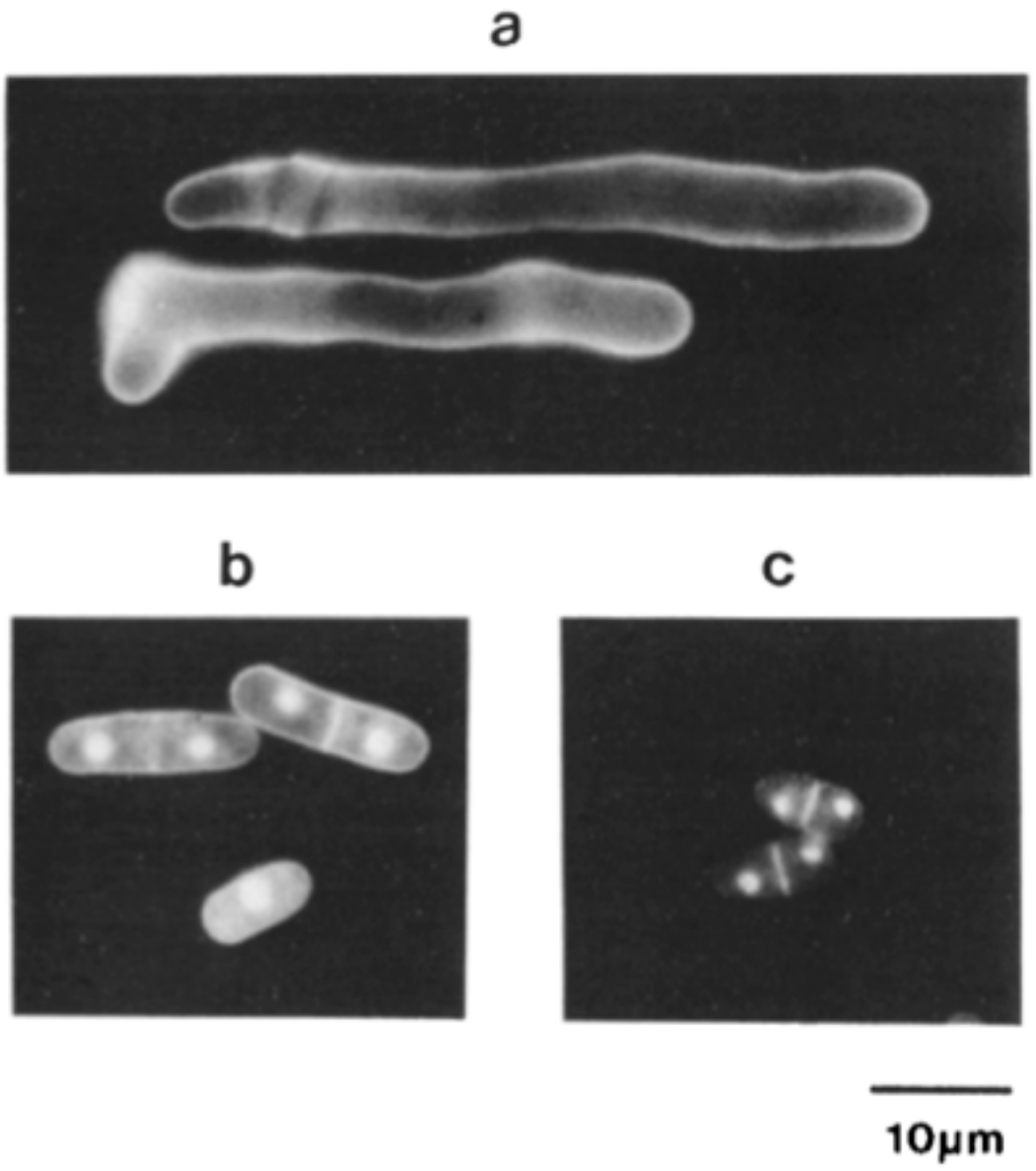


Figure 5. Cellular Morphologies

The wee1-50 cdc25Δ at 25˚C die by arresting permanently in G2 (like a cdc25 allele). They can never enter mitosis.

Conclusions

1. Cloned Cdc25.

2. Overexpression shows it is rate limiting for mitosis

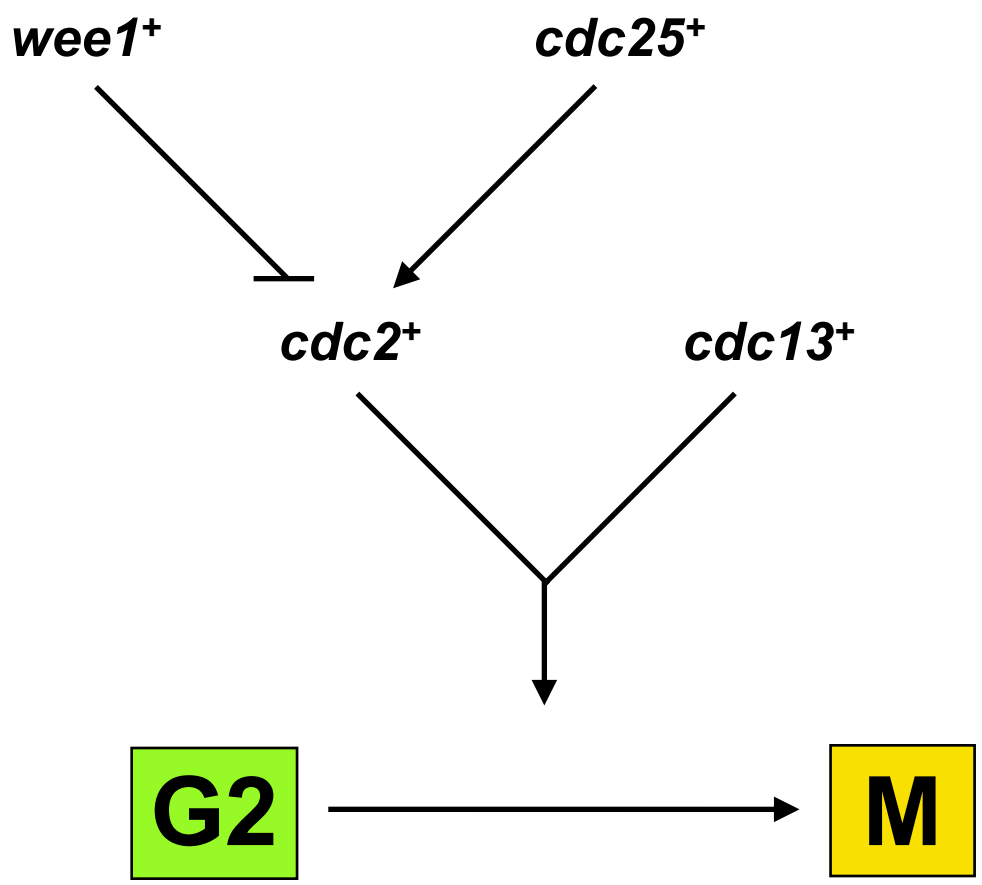
3. Overepxression in *wee1* mutant suggested that it was not upstream of *wee1*$^+$

4. Deletion showed that it doesn't act downstream of *wee1*$^+$

5. Data consistent with *cdc25*$^+$ and *wee1*$^+$ acting independently to regulate mitotic onset

The main conclusions.

the model



The model again. The genetics showed this, but

Future work

1. Cloned Wee1.

2. Cleared up that *wee1*+ isn't downstream of *cdc25*$^{+}$

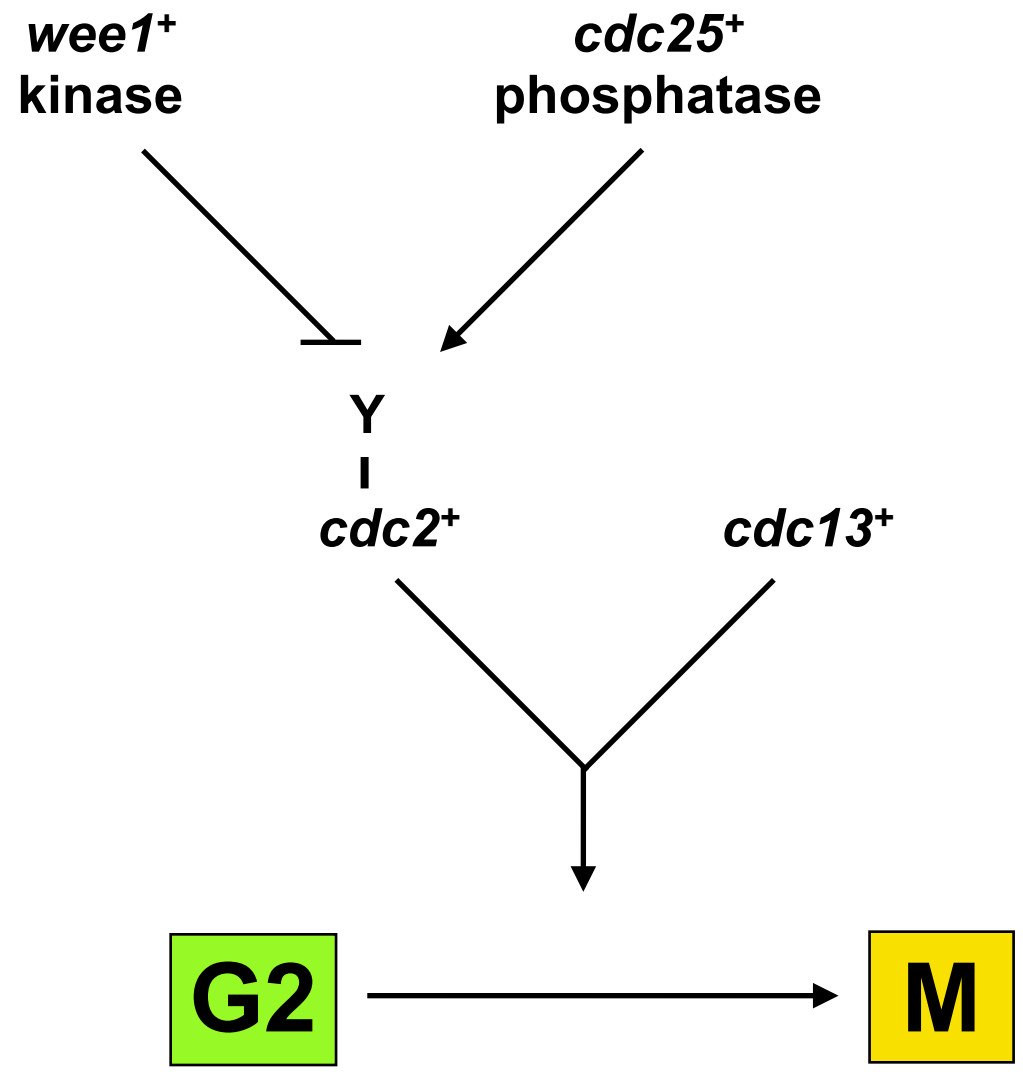
3. Showed that *wee1* mutants can't suppress *cdc2*Δ

4. Used *cdc2-w*^{D} mutants to show that *cdc2*$^{+}$ is downstream of both *cdc25*$^{+}$ and *wee1*$^{+}$.

5. Biochemical characterization confirmed the genetics.

Their future work showed....

Wee1 is a tyrosine kinase and Cdc25 is a tyrosine phosphatase. Both act on a conserved tyrosine residue on Cdc2 that when phosphorylated inhibits Cdc2 kinase activity.



biochemistry really nailed it. Wee1 and Cdc25 act on the same residue on Cdc2 – when its phosphorylated Cdc2 is inhibited and blocks entry into mitosis.

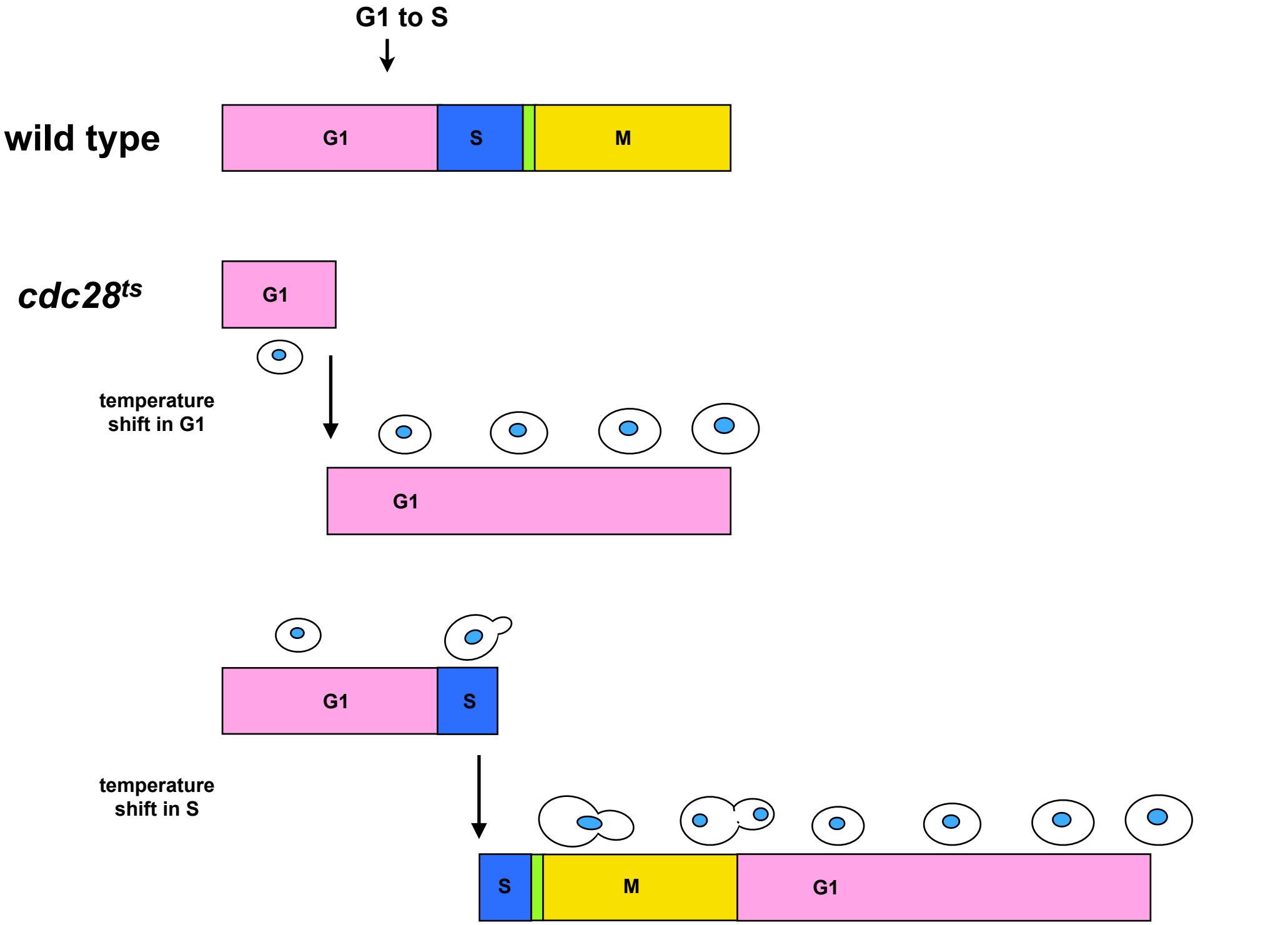
A bit more genetics

If there is time we'll get into two genetic techniques that were used to characterize many cdc mutants.

Execution points
when a gene is needed in the cell cycle

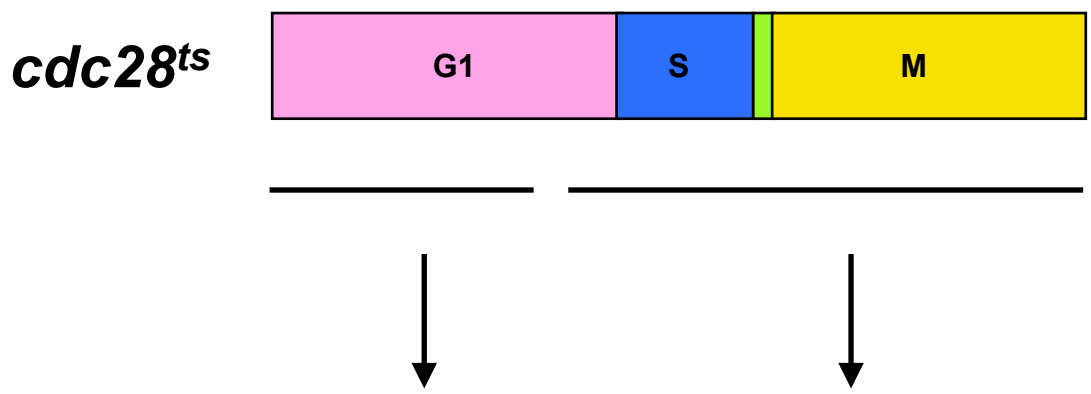
The execution point of a mutant can tell us when the gene is needed. The execution point of a mutant is the latest time at which an essential *cdc* gene product can act to allow passage to the next cell cycle. It therefore defines the point in the cell cycle at which the wild type gene product is needed

Execution points tell you when the function of a gene is needed. The dependency experiments I just describes makes assumptions about when specific cdc genes function, but execution point experiments locate the specific time that a gene is needed.



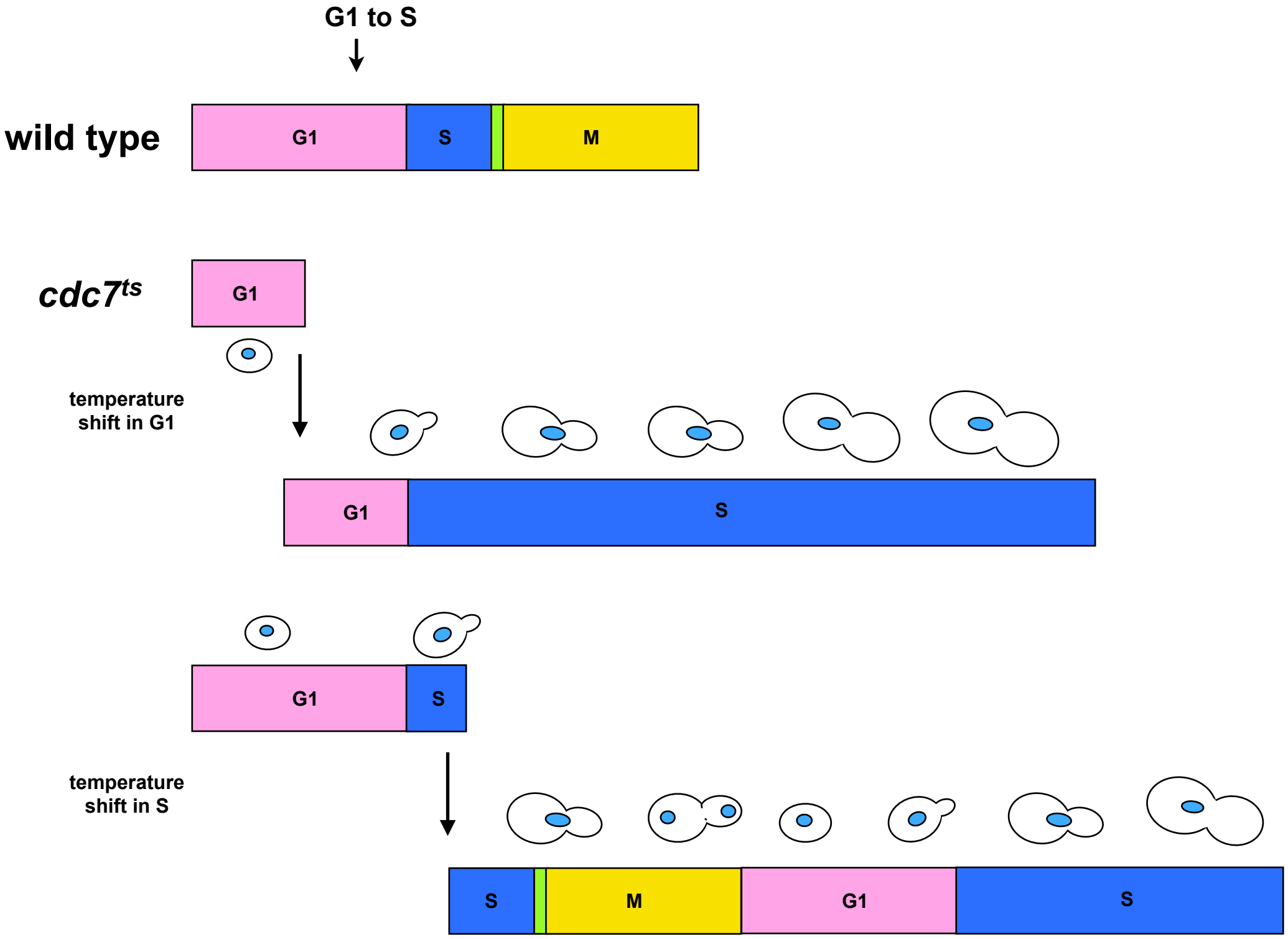
The execution point is the point in the cell cycle that a cdc mutant needs to pass at the restrictive temperature in order to display its cdc phenotype in the same cell cycle. An example is helpful. To determine the execution point of a mutant, here cdc28-ts, we'll take a population of synchronized cells (don't worry about how you get a synchronized population). All the cells start in G1 at the permissive temperature and are released into the cell cycle. At different times the cells are shifted to the restrictive temperature and we observe how the reach their arrest point. In the example with cdc28-ts, if the cells are shifted during G1, they arrest immediately and remain arrested in G1 during the course of the experiment. If they are shifted after they had budded, the cells need to traverse the whole cell cycle until they'll arrest in the subsequent G1 (a second cycle arrest). In this example the execution point for cdc28-ts is in G1, which is not so surprising because the arrest point is in G1.

when an asynchronous culture is shifted to the non-permissive condition



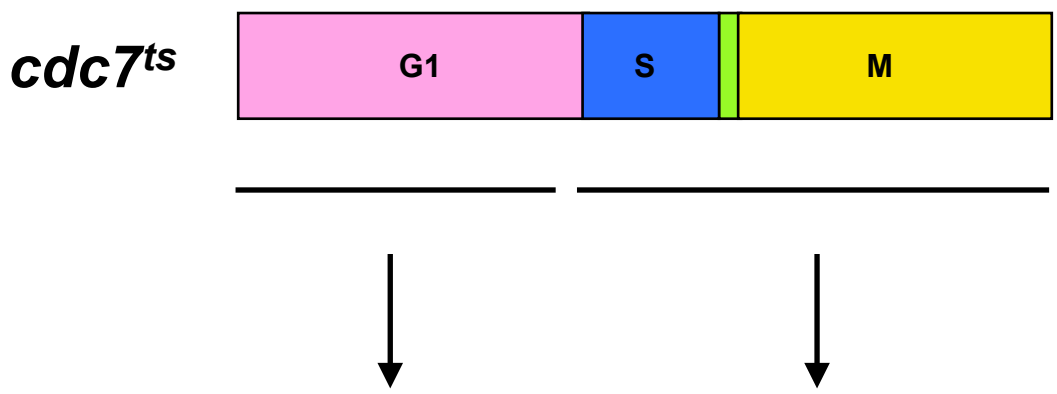
if the length of a cycle is 1, then the execution point for *cdc28ts* is roughly 0.25.

Another way to think about this is for cdc28-ts you can divide the cell cycle into two parts. The times when it will arrest in the first cycle, and the later times when it will take until the second cycle to arrest. If the whole cycle is defined as lasting 1 unit length of time, the execution point can be assigned a value, which for cdc28-ts would be around 0.25.



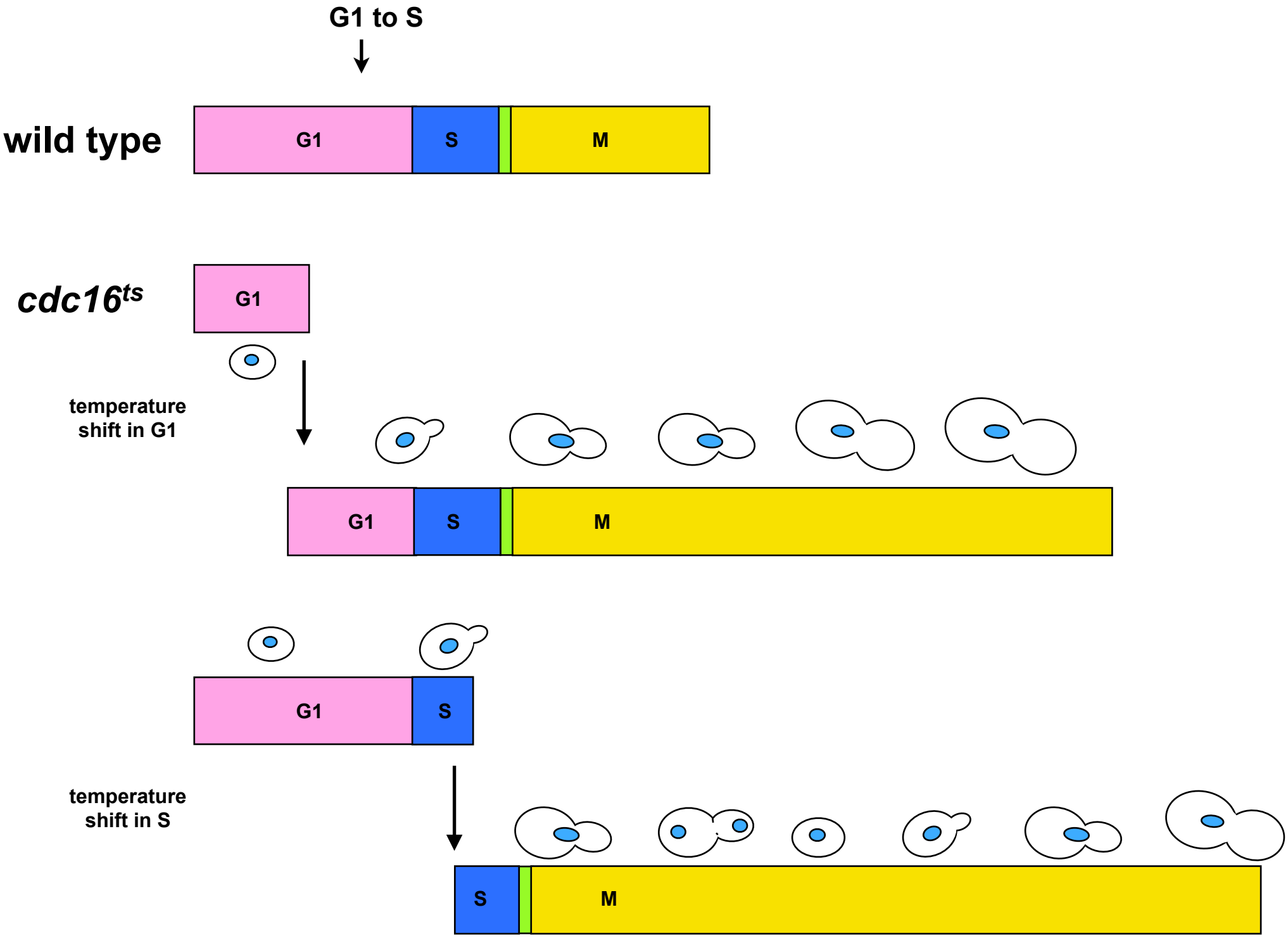
If the same experiment is performed on cdc7-ts we get a surprising result. If the cells are shifted in G1 they'll arrest in as large budded cells in the same cycle presumably in mitosis. If they are shifted after the cells bud and DNA replication has begun, the cells will traverse the entire cycle, go into the next cycle and then arrest as large budded cells in the second cycle. So although the arrest point is as a large budded cell (and apparently in mitosis), the execution point is in G1 like cdc28-ts. A number of the mutants that arrested as large budded cells had execution points in G1 - cdc7-ts and cdc31-ts being two examples. Later in the lecture it will be clear why these two mutants bud, yet have an early execution point.

when an asynchronous culture is shifted to the non-permissive condition

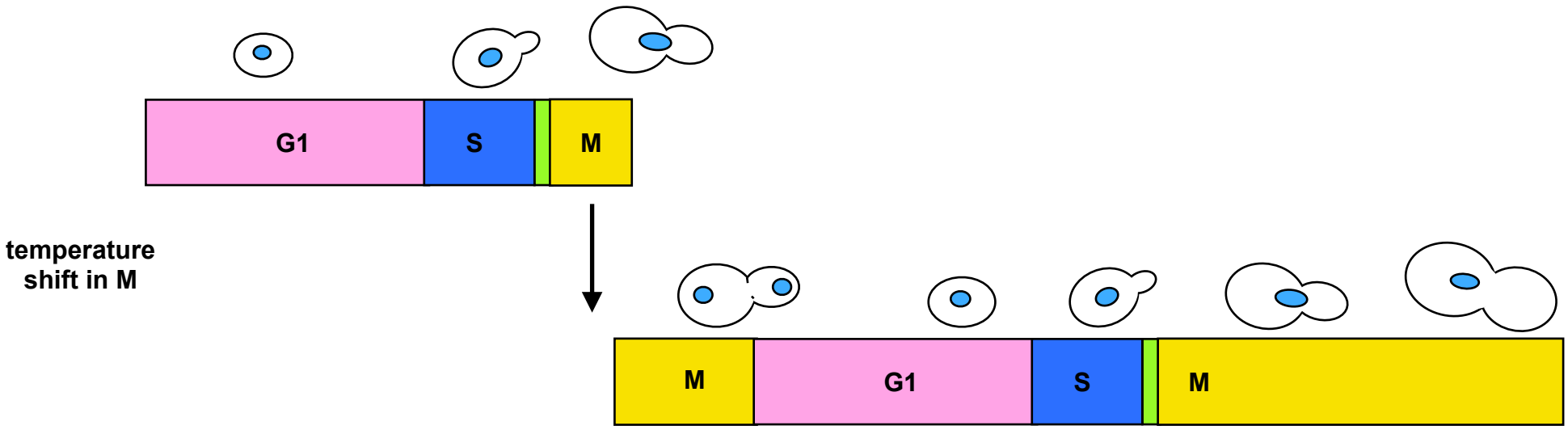


if the length of a cycle is 1, then the execution point for *cdc7^{ts}* is roughly 0.3.

For cdc7-ts, the execution point is roughly 0.4.

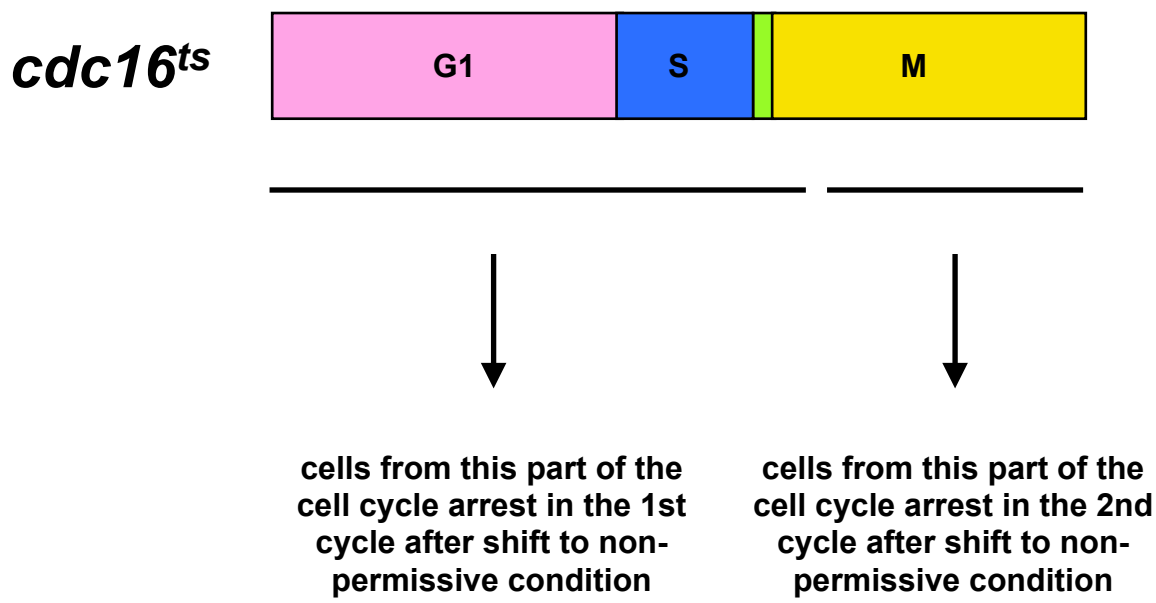


A third example is the ts mutant cdc16-ts If the cells are shifted in G1 they'll arrest in as large budded cells in the same cycle presumably in mitosis. If they are shifted after the cells bud and DNA replication has begun, they still arrest in the same cycle.



Only when cdc16-ts is shifted later in mitosis will it take an additional cell cycle to arrest.

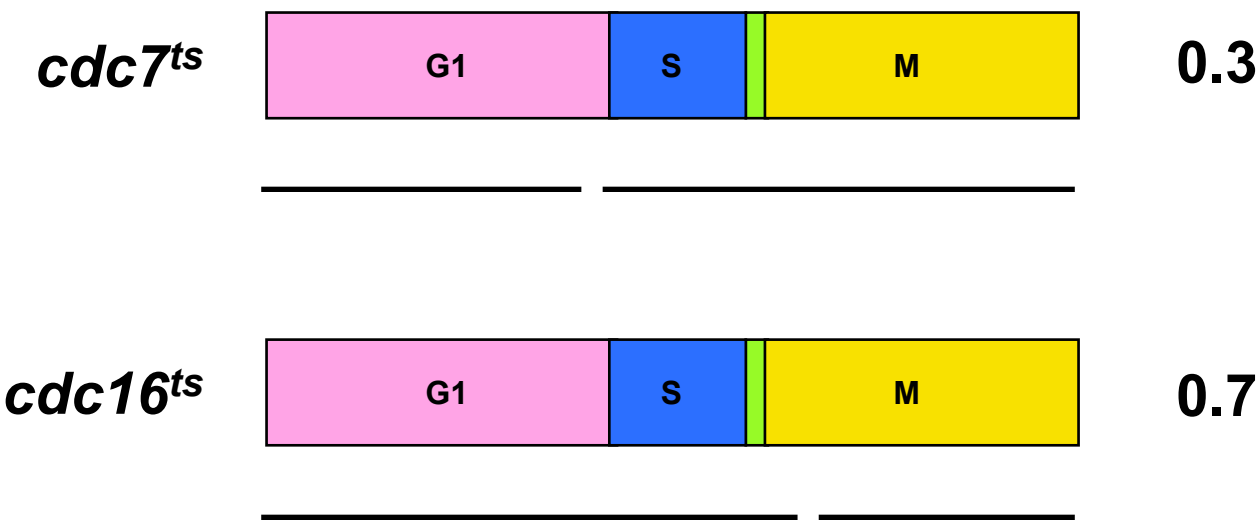
when an asynchronous culture is shifted to the non-permissive condition



if the length of a cycle is 1, then the execution point for *cdc16ts* is roughly 0.7.

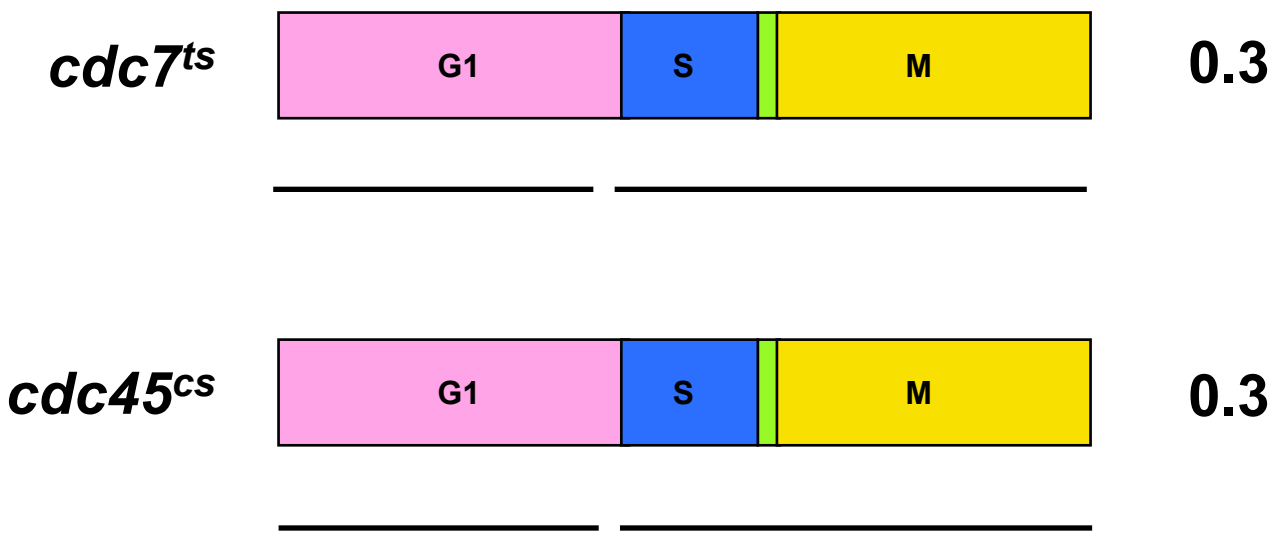
For cdc16-ts the execution point is roughly 0.7.

How can we know for sure that *CDC7* acts before *CDC16*?



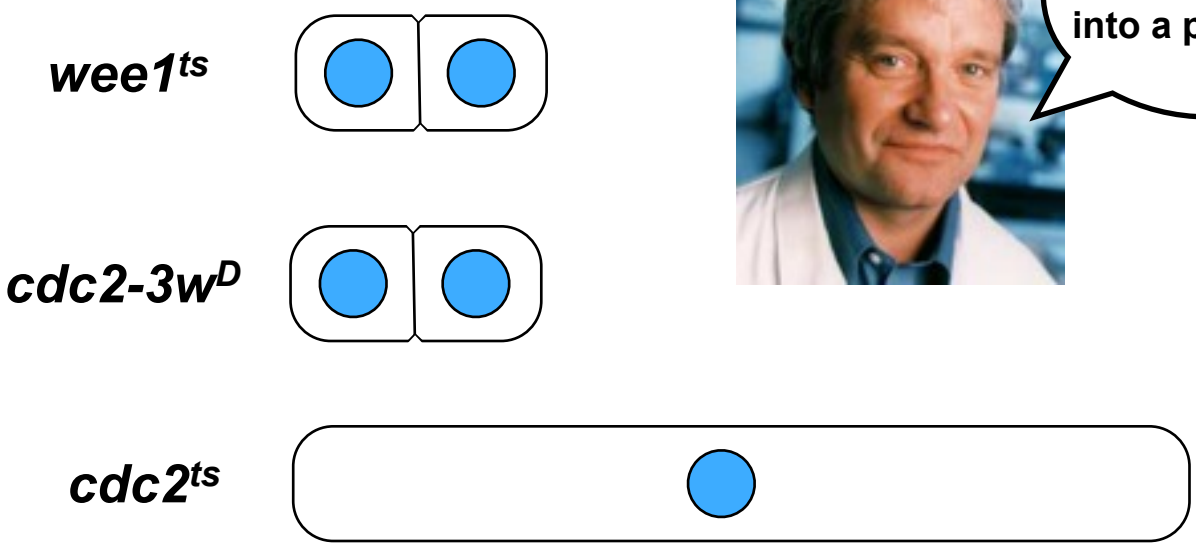
Although the execution points suggest that CDC7 acts before CDC16, we can't know this for sure. In some circumstances execution point analysis can be mis-leading, and ideally we could use a method that directly looks at the interaction between CDC7 and CDC16.

And which functions first, *CDC7* or *CDC45*? Or are they independent of or inter-dependent on one another?



This is an even bigger issue when two mutants have very similar execution points, as is the case for these two mutants, cdc7-ts and cdc45-cs. Both mutants arrest before initiating S-phase, so they appear to act during the same cell cycle transition. How do we know if the function of one depends on the other? Or if they are independent or inter-dependent?

Paul Nurse was able to order these mutants because he had two different phenotype to work with

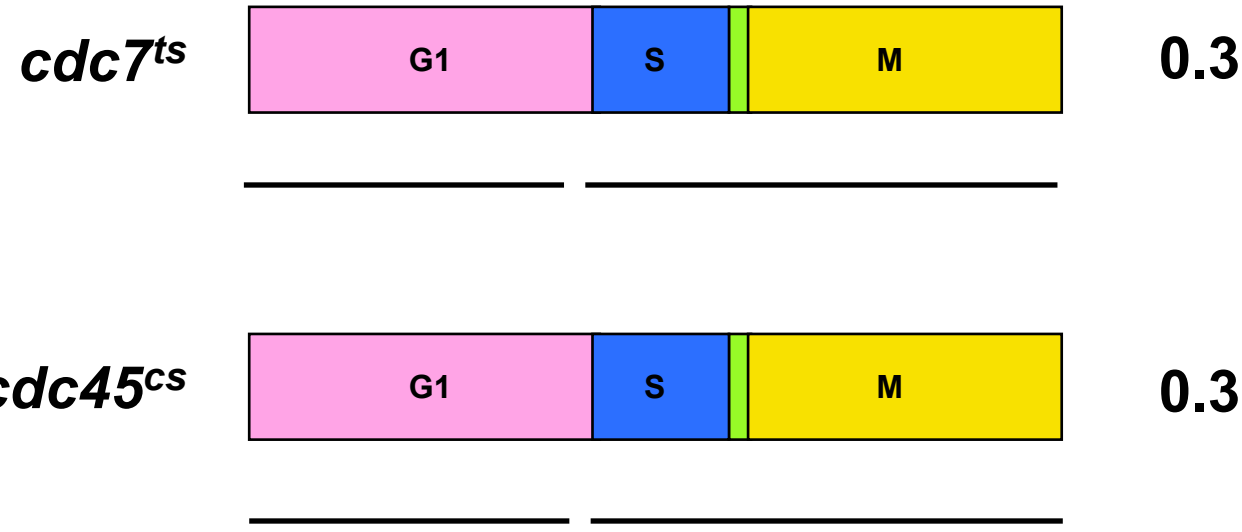


but what if you don’t have mutants with different phenotypes?

Reciprocal shift experiments

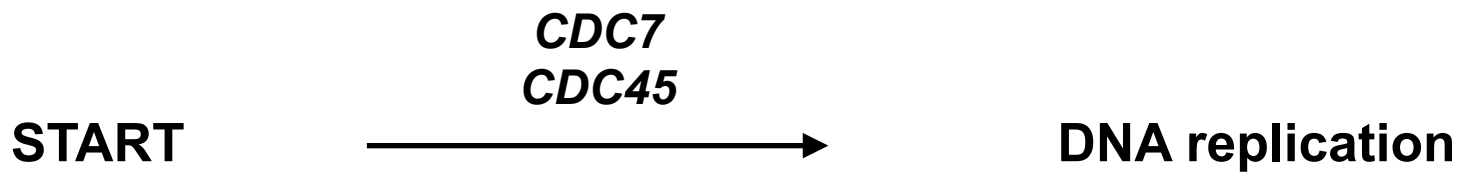
a way to test for dependencies between genes.

***CDC7* and *CDC45* are both required for S-phase and are needed at roughly the same time in the cell cycle.**



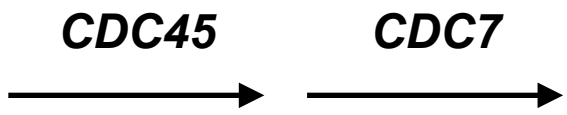
A method that can be used to order genes with similar phenotypes is called a reciprocal shift experiment, and is what was described in Wood and Hartwell (a paper on the website). It is a great way to test for dependencies between genes and has been used in many fields, not only the cell cycle.

What are the possible relationships between *CDC7* and *CDC45*?

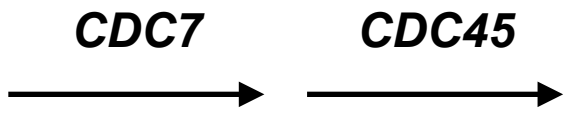


The first thing to consider are the four possible relationships between CDC7 and CDC45.

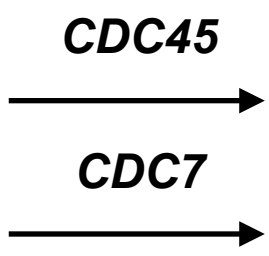
1. ***CDC45*** **acts before** ***CDC7***



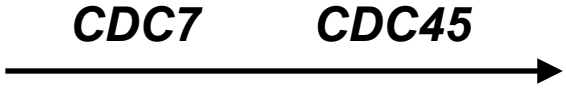
2. ***CDC7*** **acts before** ***CDC45***



3. ***CDC45*** **and** ***CDC7*** **are independent**



4. ***CDC45*** **and** ***CDC7*** **are inter-dependent**



There are four: 1) CDC45 functions before CDC7 (and its function needs to be completed before CDC7 can perform its function), 2) CDC7 acts before CDC45, 3) CDC45 and CDC7 act independently of one another and the order in which they function is unimportant, 4) CDC45 and CDC7 are inter-dependent and the function together (perhaps in a protein complex or as co-regulators of a process). Reciprocal shift experiments seek to determine which of these four relationships hold for a given pair of genes (or conditions, see below).

Reciprocal shift experiments require
two different ways to inactivate two different genes.

cdc7^{ts} **inactivates *CDC7* at 37˚C (ie. temperature sensitive)**

cdc45^{cs} **inactivates *CDC45* at 16˚C (ie. cold sensitive)**

Reciprocal shift experiments do require that there are two different ways to inactivate the two genes (or processes). In the CDC7/ CDC45 example cdc7-ts is inactivated at 37˚C, and cdc45-cs is inactivated at 16˚C (ie its cold sensitive).

Experimental design:

Arrest cells at one temperature block

↓

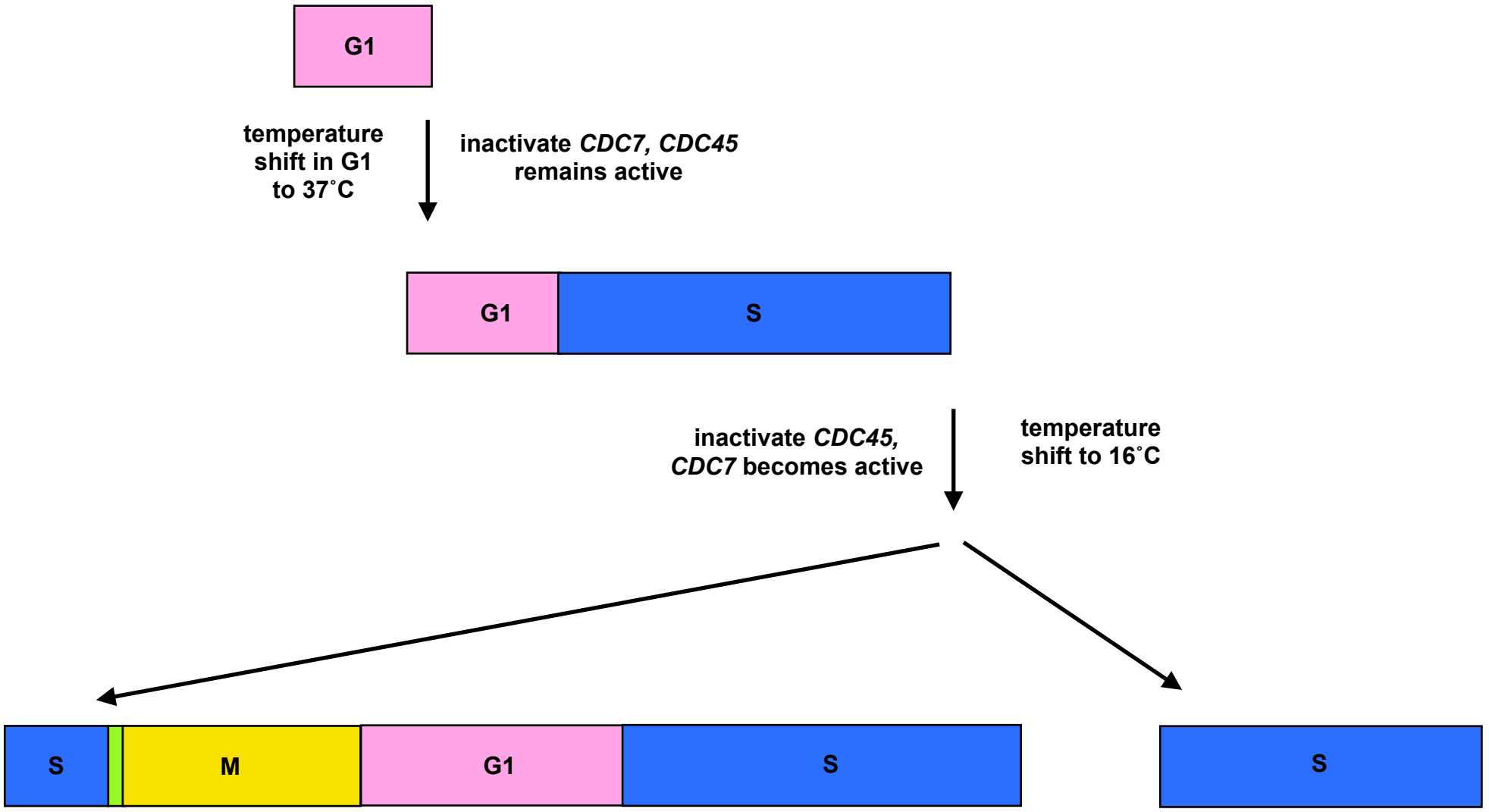
Shift from one temperature block to the second

↓

Do cells remain arrested or traverse one cell cycle?

The basic outline of one of these experiments is as follows: double mutants between the two genes cdc7-ts cdc45-cs are arrested at one temperature block, the cells are then shifted to the other temperature block and we examine if the cells remain arrested or if they traverse one cell cycle and then re-arrest.

cdc45^{cs} cdc7^{ts} (37˚C ⟶ 16˚C)

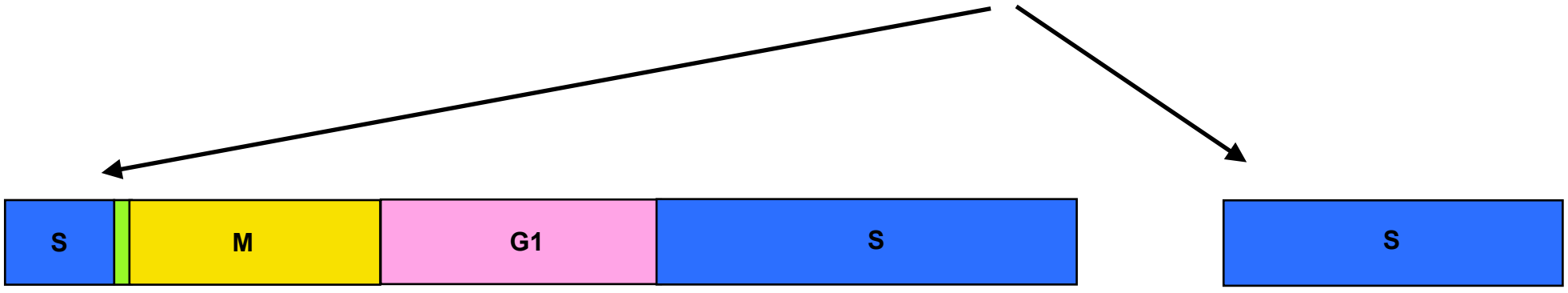


outcome 1: cells complete S-phase and the rest of the cell cycle, rearresting in the S-phase of the next cycle

outcome 2: cells remain arrested in S-phase

I've diagrammed one of the possible experiments in this slide. cdc45-cs cdc7-ts is arrested at 37˚C and the cells arrest at the start of S-phase. During this arrest cdc45-cs remains active. The cells are then quickly transferred to 16˚C to inactivate cdc45-cs, and to re-activate cdc7-ts. There are two possible outcomes of this second shift. On the left, the cells complete S-phase and the rest of the cell cycle and then arrest in the S-phase of the next cell cycle. On the right, the cells remain arrested in S-phase after the shift down to 16˚C. These two outcomes can be interpreted as follows....

cdc45^{cs} cdc7^{ts} **(37˚C ⟶ 16˚C)**



outcome 1: cells complete S-phase and the rest of the cell cycle, rearresting in the S-phase of the next cycle

outcome 2: cells remain arrested in S-phase

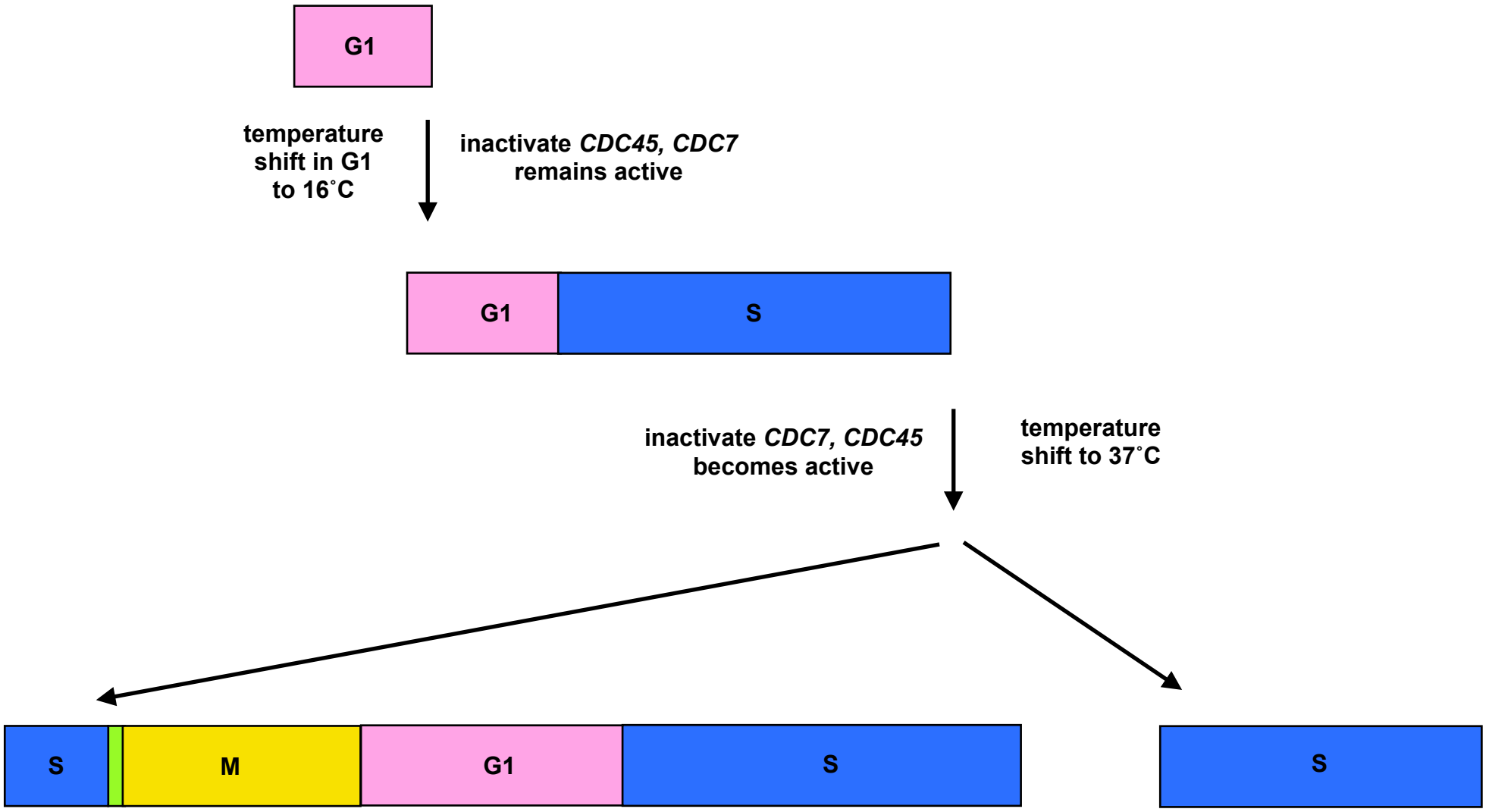
CDC45 acts before *CDC7*

CDC45 ⟶ ***CDC7*** ⟶

CDC7 acts before *CDC45*

CDC7 ⟶ ***CDC45*** ⟶

Outcome 1 suggests that CDC45 acts before CDC7, so that when the cells are shifted down to 16˚C cdc45-cs has already completed its function and cdc7-ts which has been reactivated can complete its function and the cells traverse one cell cycle before re-arresting. Outcome 2 suggests that CDC7 acts before CDC45 and when the cells are shifted to 16˚C the cells simply move from one pre-S-phase arrest (cdc7-ts) to a second pre-S-phase arrest (cdc45-cs). Doing only the shift from 37˚C to 16˚C doesn't tell us about inter-dependence orindependence, we need to do the other shift, from...

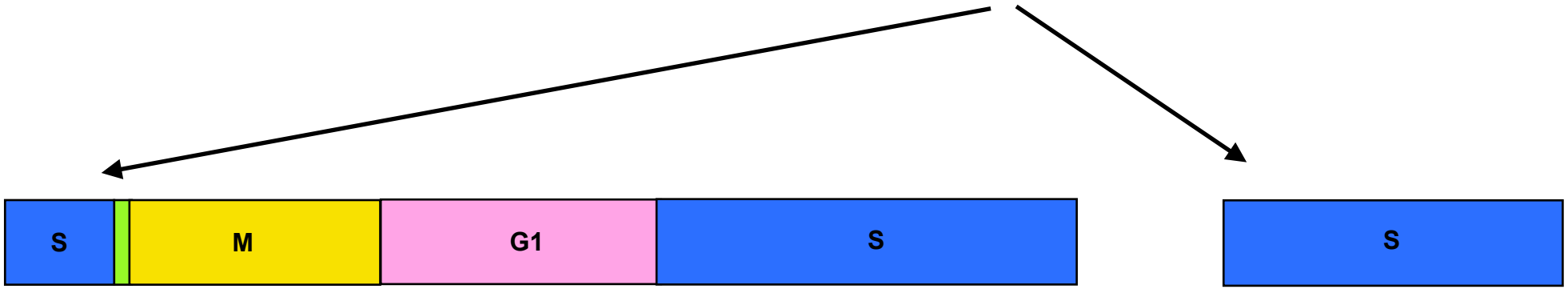


outcome 1: cells complete S-phase and the rest of the cell cycle, rearresting in the S-phase of the next cycle

outcome 2: cells remain arrested in S-phase

16˚C to 37˚C. The set-up is identical as previously but the temperatures are flipped. If the two genes are independent both shifts (37˚C to 16˚C and 16˚C to 37˚C) will have outcome 1, if the two genes are inter-dependent both experiments will have outcome 2. If one depends on the other, one experiment will have outcome 1 the other outcome 2.

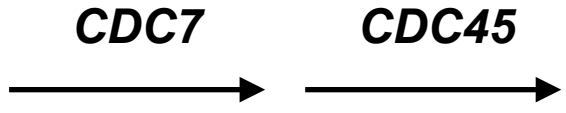
cdc45^{cs} cdc7^{ts} **(16˚C ⟶ 37˚C)**



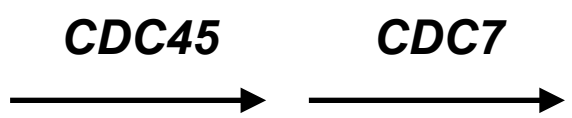
outcome 1: cells complete S-phase and the rest of the cell cycle, rearresting in the S-phase of the next cycle

outcome 2: cells remain arrested in S-phase

CDC7 acts before *CDC45*



CDC45 acts before *CDC7*



Outcome 1 suggests that CDC7 acts before CDC45, so that when the cells are shifted up to 37˚C cdc7-ts has already completed its function and cdc45-cs which has been reactivated can complete its function and the cells traverse one cell cycle before re-arresting. Outcome 2 suggests that CDC45 acts before CDC7 and when the cells are shifted to 37˚C the cells simply move from one pre-S-phase arrest (cdc45-cs) to a second pre-S-phase arrest (cdc7-ts). Combining this experiment with the prior experiment will tell us about inter-dependence and independence.

	(16˚C → 37˚C)	(37˚C → 16˚C)
1. *CDC45* acts before *CDC7*		
CDC45 → ***CDC7*** →	**outcome 1**	**outcome 2**
2. *CDC7* acts before *CDC45*		
CDC7 → ***CDC45*** →	**outcome 2**	**outcome 1**
3. *CDC45* and *CDC7* are independent		
CDC45 → ***CDC7*** →	**outcome 1**	**outcome 1**
4. *CDC45* and *CDC7* are inter-dependent		
CDC7 ***CDC45*** →	**outcome 2**	**outcome 2**

The results of these two experiments (the shift from 16 to 37, and 37 to 16) will determine the relationship between the two genes.

Reciprocal shift experiments can be done with other conditional treatments, like drug sensitivity

MBC (methyl-benzimidazole-3-ylcarbamate) causes the depolymerization of microtubules and a cell cycle arrest in mitosis. It is similar to the drugs nocodazole, benomyl and colchicine which do the same thing.

Wood and Hartwell ordered *cdc* mutants and MBC using reciprocal shift experiments

The example I gave is using a ts and a cs mutant, but these experiments can be done by blocking processes in other conditional ways - like using drugs. Wood and Hartwell used MBC which causes the rapid depolymerization of microtubules and arrests cells in mitosis. Wood and Hartwell ordered cdc mutants and the MBC arrest using reciprocal shift experiments. These experiments told them something about what genes might be involved in the response of cells to MBC, and helped order many of the mitotic cdc mutants by comparing their relationships to MBC.

Additional Reading:

Murray et al. Dominoes and clocks: the union of two views of the cell cycle. Science (1989) vol. 246 (4930) pp. 614-21

Russell et al. Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell (1987) vol. 49 (4) pp. 559-67. How *wee1* works antagonistically to *cdc25*.

Gould, K.L., and P. Nurse. 1989. Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature*. 342:39–45. doi:10.1038/342039a0.

Lee, M.G., and P. Nurse. 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature*. 327:31–35. doi:10.1038/327031a0.

Nurse, P. 1990. Universal control mechanism regulating onset of M-phase. *Nature*. 344:503–508. doi: 10.1038/344503a0.

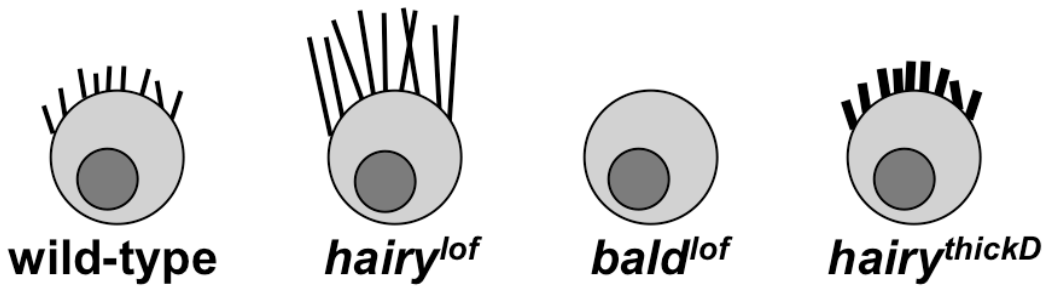
Labbé, J.C. et al. Purification of MPF from starfish: identification as the H1 histone kinase p34cdc2 and a possible mechanism for its periodic activation. Cell 57, 253–263 (1989).

Wood, J.S. & Hartwell, L.H. A dependent pathway of gene functions leading to chromosome segregation in Saccharomyces cerevisiae. J Cell Biol 94, 718–726 (1982).

Moir, D. & Botstein, D. Determination of the order of gene function in the yeast nuclear division pathway using cs and ts mutants. Genetics 100, 565–577 (1982).

Some references.

1. You are interested in understanding how hair growth on cells is regulated so you perform a genetic screen looking for regulators of hair growth. You initially find two mutants, hairy^{lof} in which the mutant cells are very hairy, and bald^{lof} in which the mutant cells have no hair. Both mutants are recessive loss-of –function mutants.



A. Draw three possible ways in which the wild type genes hairy^{+} and bald^{+} may regulate hair growth.

B. When you make the double mutant hairy^{lof} bald^{lof} you discover that the double mutant cells are hairy. Which of the pathways in A describes the relationship between hairy^{+} and bald^{+}?

C. You also find an interesting mutant that you name thick. Thick mutant cells have normal length hair, but it's thicker than normal. After careful analysis you learn that the thick mutant is in fact a dominant-gain-of function allele of hairy, so you rename it hairy^{thickD}. What do you predict the phenotype of the hairy^{thickD}bald^{lof} double mutant will be?

Both hydroxyurea (HU) and benomyl are drugs that arrest the cell cycle in budding yeast and their execution points are very similar to one another. You want to know if one acts before the other, if they act independently or if they act inter-dependently so you perform a reciprocal shift experiment to determine their dependency relationship. This involves the following two experiments:

1. Arrest the cells in media containing HU, wash away the HU and place the cells in media containing benomyl.

2. Arrest the cells in media containing benomyl, wash away the benomyl and place the cells in media containing HU.

If benomyl acts <u>after</u> HU, what happens after switching from one drug to the other in each of these two experiments? If benomyl and HU acted independently of each other, what would the outcomes be?