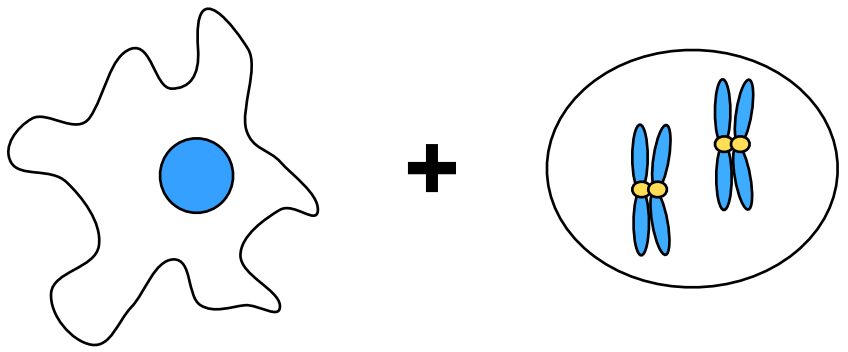
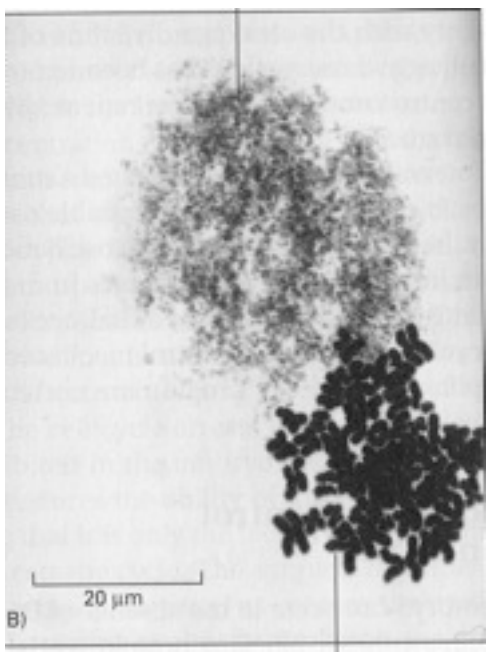
Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

A. What happened to the S-phase nucleus' chromosomes (they are the upper ones)? Why?



S **M**



S + M

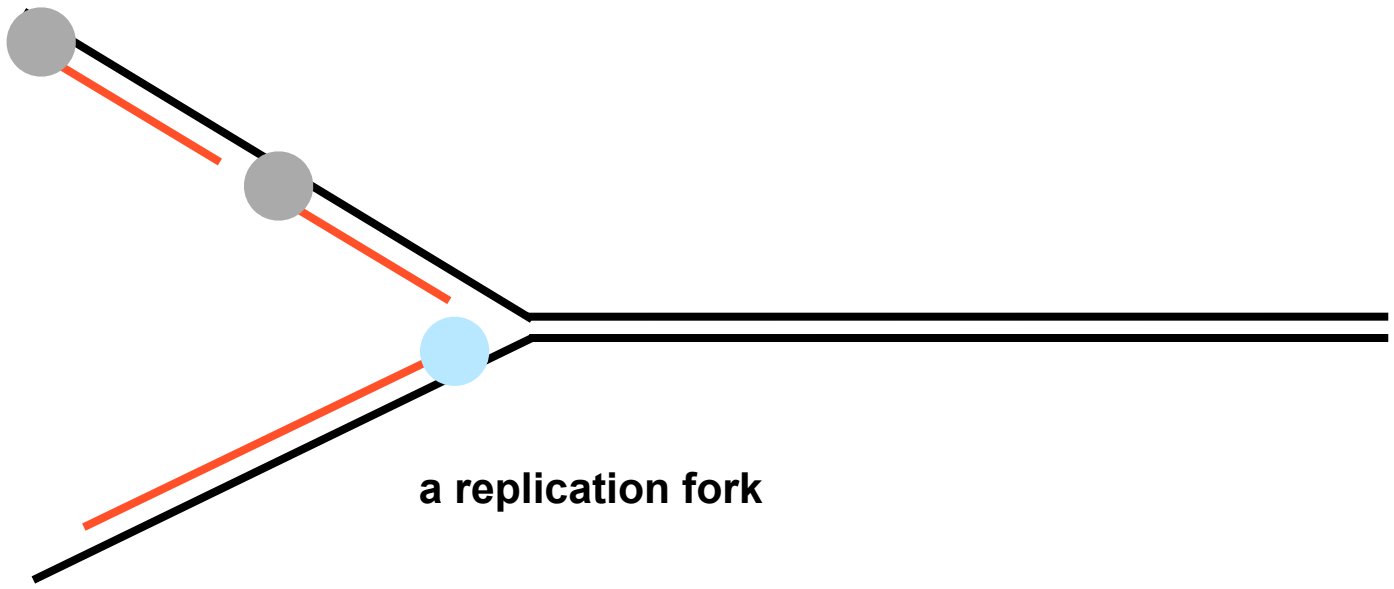
Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

A. What happened to the S-phase nucleus' chromosomes (they are the upper ones)? Why?

- the chromosomes are fragmented. Presumably this fragmentation happened as the S-phase chromosomes attempted to condense.

- Condensing S-phase chromosomes could cause fragmentation because of all the single stranded breaks caused from unligated replicated DNA (especially the Okazaki fragments), from lower nucleosome density during S-phase interfering with normal chromosome condensation, or a combination of both.



a replication fork

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

B. Propose a model for how an S-phase cell prevents mitosis from happening.

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

B. Propose a model for how an S-phase cell prevents mitosis from happening.

possible models could be:

1. a signal from the replication fork blocking MPF activation. When replication is complete, the inhibitory signal decays

2. A protein required for mitosis is also used for DNA replication and it only becomes available once replication is complete.

3. replicated DNA sends a positive signal to promote mitotic entry.

4. An S-phase promoting factor is a mitosis inhibiting factor.

5. A timing mechanism - once S-phase starts mitosis will happen after a certain period of time which is longer than it takes to complete S-phase.

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

C. What would happen to a cell that has two rounds of S-phase with no intervening mitosis?

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

C. What would happen to a cell that has two rounds of S-phase with no intervening mitosis?

the cell would increase its ploidy. However centrosome duplication usually happens early in the cell cycle, so it may also increase its centrosome number. Would this be a problem? How could cells deal with this?

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

D. What would happen to a cell with two mitosis with no intervening S-phase?

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

D. What would happen to a cell with two mitosis with no intervening S-phase?

the cell would decrease its ploidy.

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

E. What would happen to cells that don't undergo cytokinesis, but still alternate S and M phases?

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

E. What would happen to cells that don't undergo cytokinesis, but still alternate S and M phases?

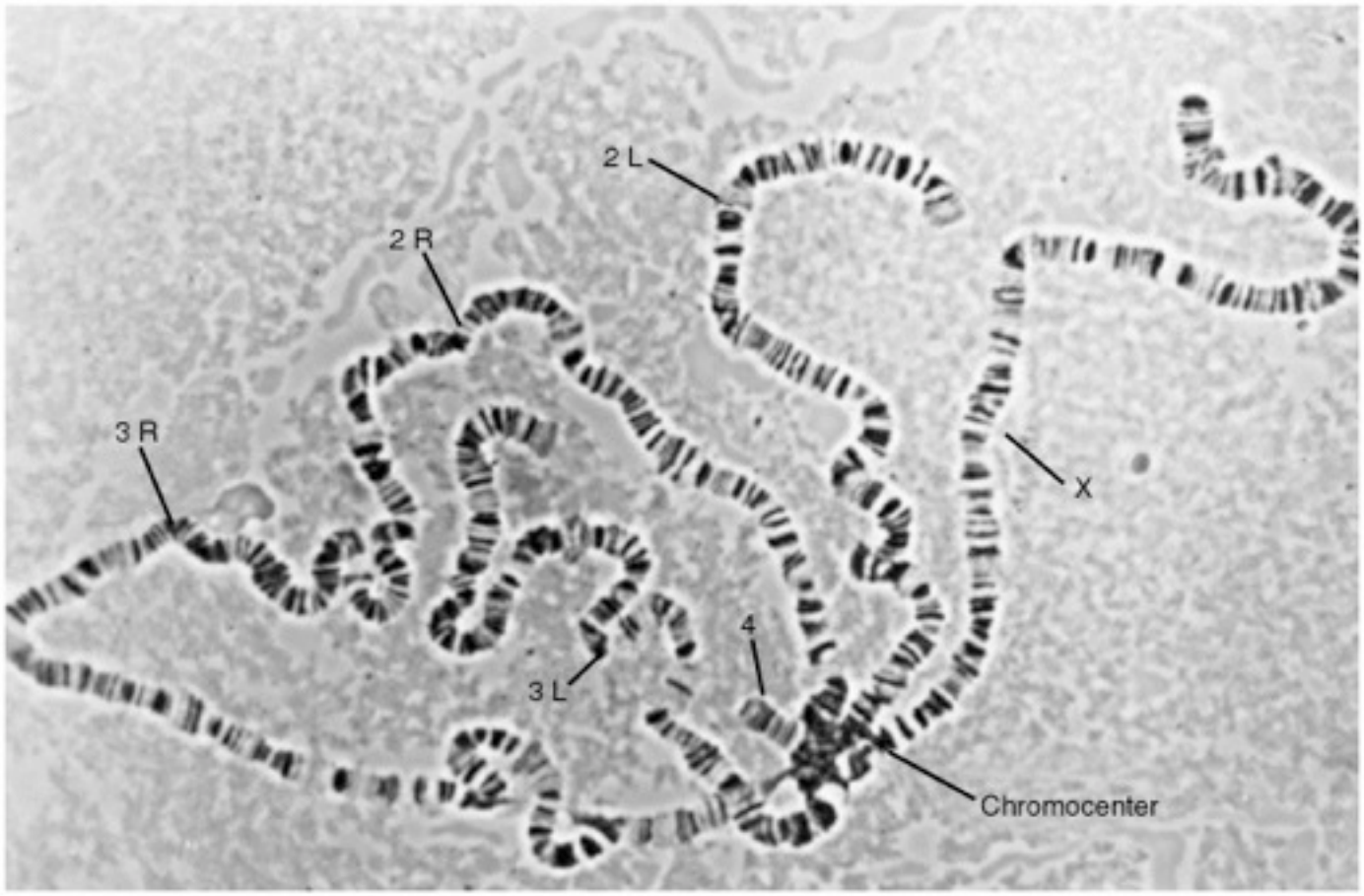
the cell would become multi-nucleate. If the nuclei fused it could increase its ploidy.

Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

F. Extra credit - can you think of examples of normal cells that behave as in C, D and E?

C. Polytene chromosomes in Drosophila salivary glands are caused by endoreduplication.



Answers to the questions.

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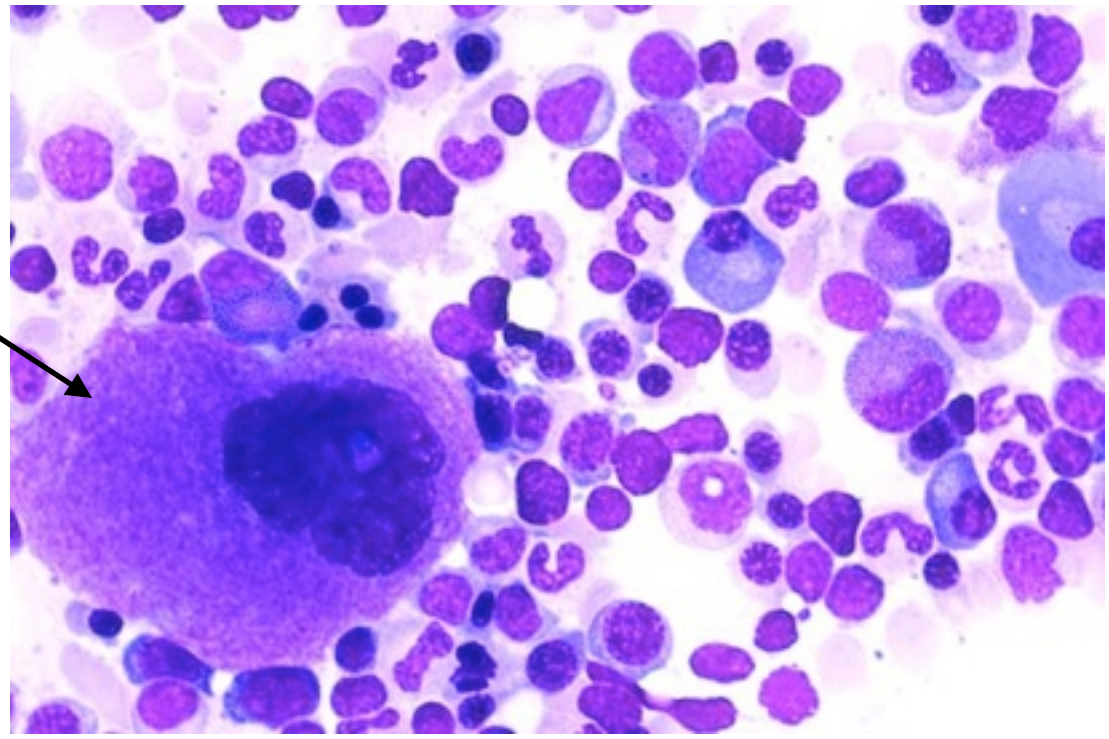
F. Extra credit - can you think of examples of normal cells that behave as in C, D and E?

C. Megakaryocytes are polyploid as are many bred plants.

triploid: apple, banana, citrus, ginger, watermelon
tetraploid: apple, durum wheat, potato, cabbage, peanuts, tobacco
hexploid: wheat, oat, kiwi
octaploid: strawberry, sugar cane, dahlia

megakaryocyte

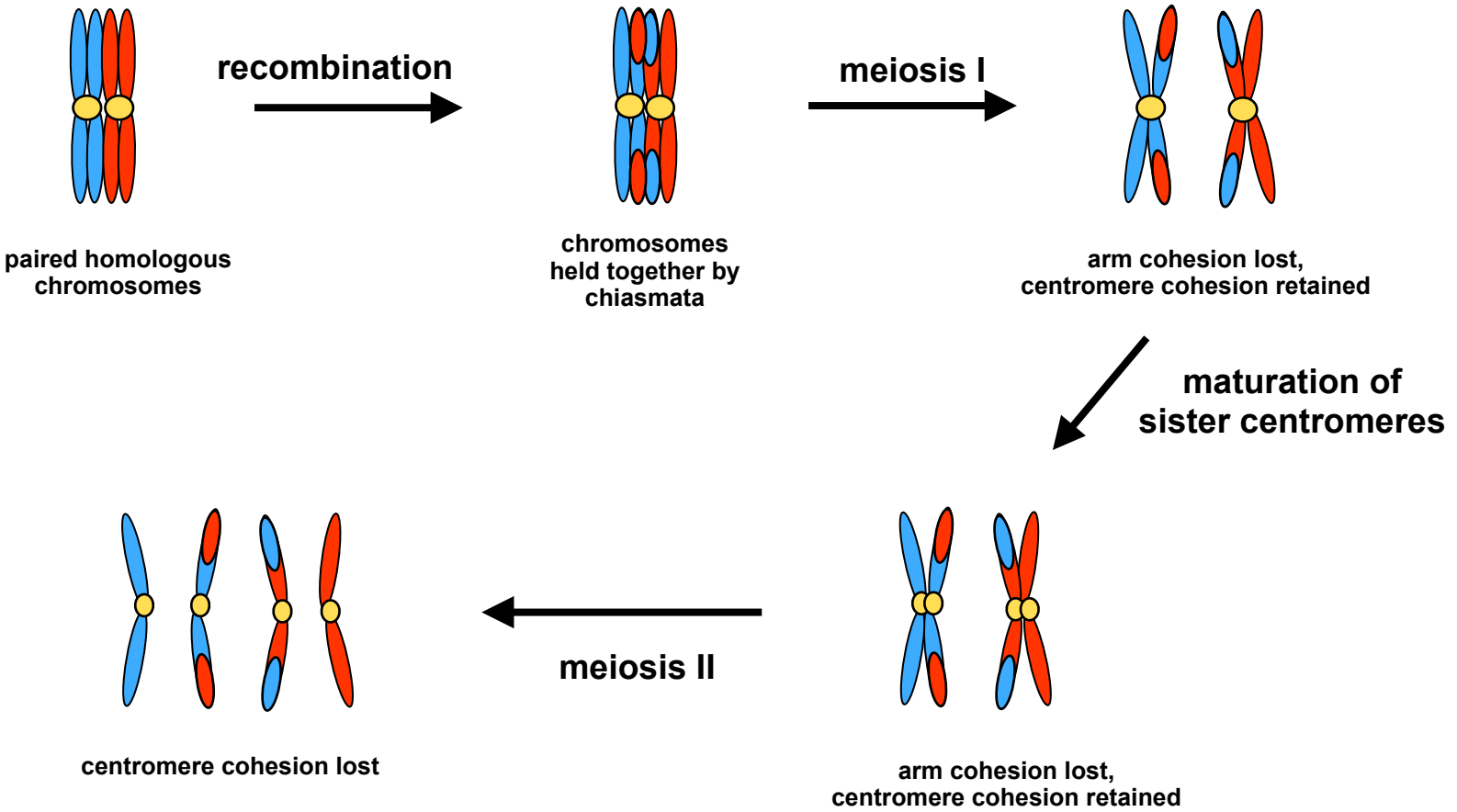


Answers to the questions.

Rao and Johnson fused S and M phase cells together and saw that the M phase nucleus drove the S phase nucleus into mitosis. The photo below shows what the chromosomes from the fused cell looked like.

F. Extra credit - can you think of examples of normal cells that behave as in C, D and E?

D. All organisms that have a meiotic cycle go through two two rounds of mitosis (meiosis I and II) with no intervening S-phase.



Answers to the questions.

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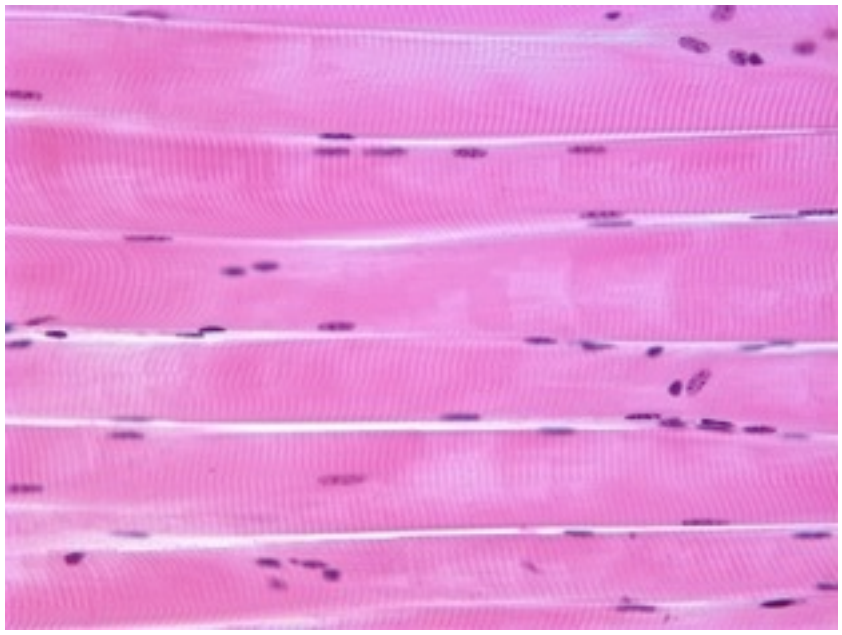
F. Extra credit - can you think of examples of normal cells that behave as in C, D and E?

E. Muscle cells are multinucleated and are the result of a block to cytokinesis. Hyphal fungi are as well.



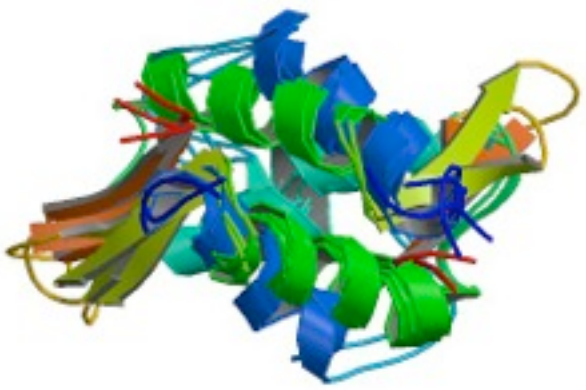
muscle cells

a hyphal fungi

Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

A. If histone H1 were a true substrate of MPF, what process do you think it might have regulated? Propose what MPF phosphorylation might do to histone H1 and chromatin structure.



histone H1

Answers to the questions.

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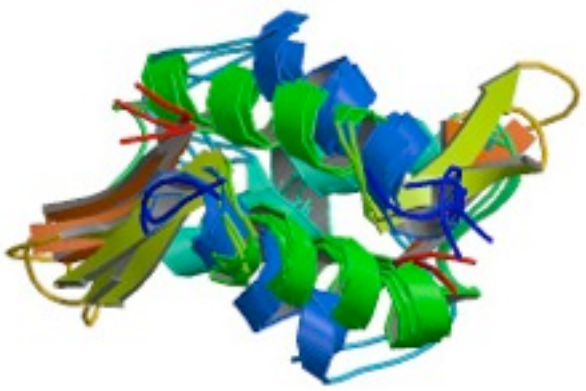
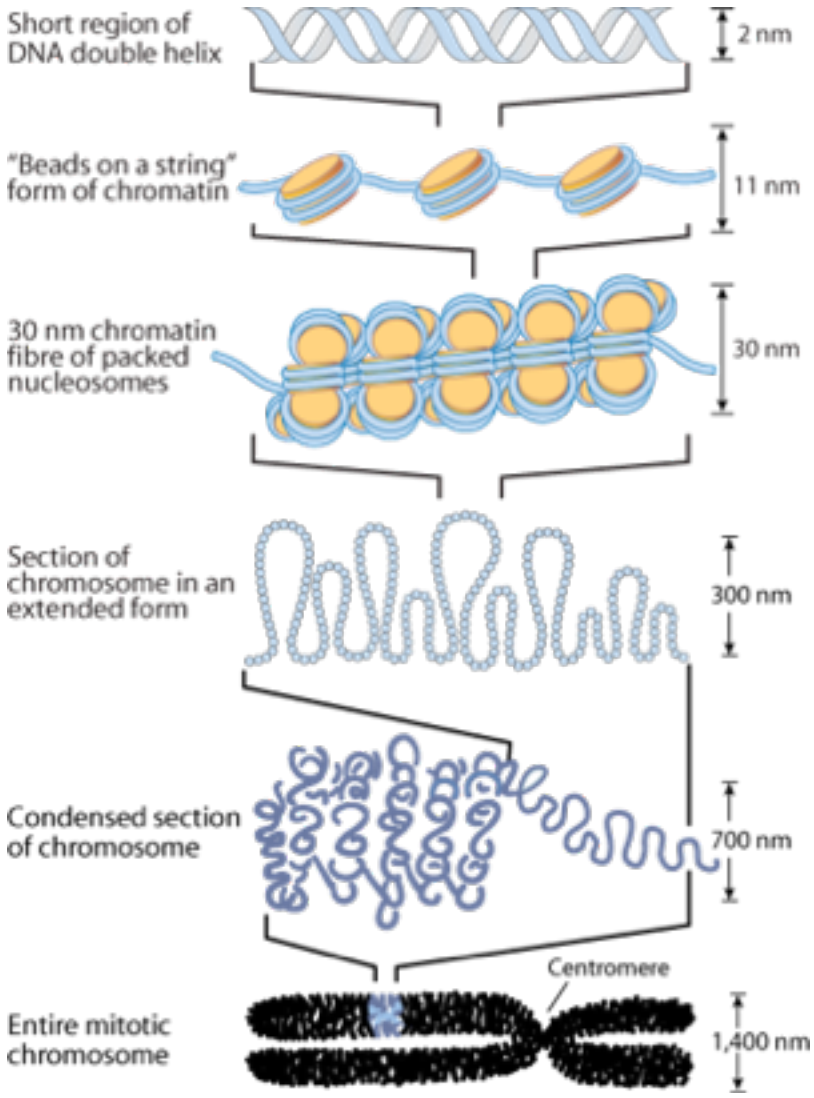
A. If histone H1 were a true substrate of MPF, what process do you think it might have regulated? Propose what MPF phosphorylation might do to histone H1 and chromatin structure.

perhaps it would regulate chromosome condensation. Histone H1 binds to the linker region on nucleosomal DNA, and phosphorylation might help nucleosomal DNA compact from the 11nm fiber to 30nm or beyond.



histone H1



Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

B. An eager graduate student, Marion, maps 12 sites of phosphorylation (which are all serine and threonine residues) on histone H1 and mutates them to alanine. Why? What phenotype do you think she expects to see when she expresses this mutant version of histone H1?

Answers to the questions.

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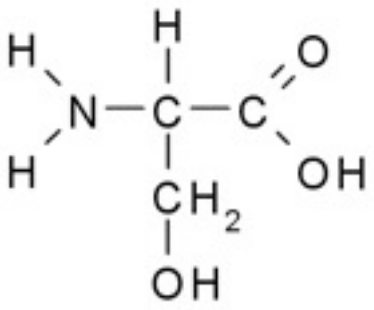
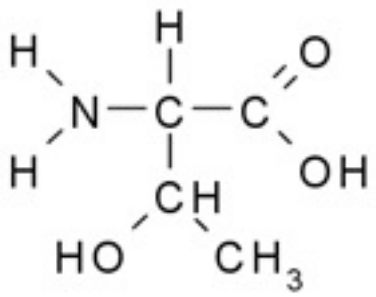
B. An eager graduate student, Marion, maps 12 sites of phosphorylation (which are all serine and threonine residues) on histone H1 and mutates them to alanine. Why? What phenotype do you think she expects to see when she expresses this mutant version of histone H1?

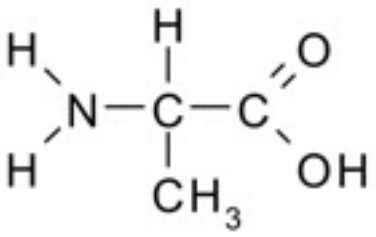
Alanine is the amino acid that looks the most like serine and threonine, but can't be phosphorylated. If phosphorylation of histone H1 contributes to chromosome condensation then expressing the mutant form in cells might slow or prevent normal condensation.



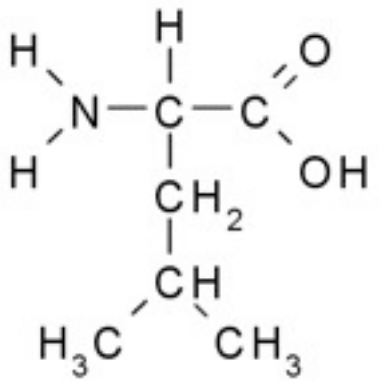
serine



threonine



alanine



valine

Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

C. Despite all her hard work, she sees no phenotype in her cells. Propose one reason why.

Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

C. Despite all her hard work, she sees no phenotype in her cells. Propose one reason why.

Some possible reasons:

1. Phosphorylation of histone H1 is unimportant. Perhaps she should have checked that it was phsophorylated on these sites in vivo?

2. Phosphorylation may be important, but there are redundant mechanisms that regulate condensation, and the other mechanisms can substitute for phosphorylation of histone H1.

3. She was only expressing her mutant histone H1 in cells, so the wild type version is still being expressed. Does her mutant version function at all? If not, it may have no effect because it can't interact with nucleosomes - essentially a non-result.

4. Maybe she can't express it to high enough levels to see a phenotype. Can she replace her mutant version for the wild type?

Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

D. Do you think histone H1 could be the only target of MPF phosphorylation? Explain.

Answers to the questions.

Early on researchers realized that MPF activity was associated with high levels of histone H1 kinase activity, and this was exploited as an easy assay when researchers were studying MPF. Histone H1 is the linker histone, which binds to the DNA between nucleosomes.

D. Do you think histone H1 could be the only target of MPF phosphorylation? Explain.

Seems unlikely. There are so many things MPF appears to do: regulate condensation, nuclear envelope breakdown, spindle assembly, anaphase initiation, as well as other things (to be discussed). It is very hard to imagine histone H1 could regulate all these things, so there are probably multiple different substrates.

Answers to the questions.

a short primer on reading papers

1. Conclusions are restated multiple times. Your goal is to figure out the one or two main conclusions.

2. Abstract. Often confusing.

3. Introduction. Contains all the background information you need to know to appreciate (and understand) the conclusions.

4. Results. Can be confusing, and requires frequent consulting of the figures/methods. If well written won't require much information beyond what is in the Introduction.

5. Figures/Tables. Virtually all the critical information should be in the figures. Spend most of your time making sure you understand how the experiments presented were done, if they are well controlled and what the author concludes from them. Do you conclude the same thing? Good figures will be understandable without reading every word of the figure legend.

6. Discussion. Restates the conclusions, but often confusing. Your interpretations are usually more important than the authors. If well written, the discussion will make you think about the data in a way you didn't before.

7. Methods. Can be minimal, and unhelpful, but often can contain critical information.

8. References. Useful to follow-up on a question about past work. Unless it is your field, hard to appreciate if all the right papers have been cited.

Good luck reading the assigned paper. Hopefully this short primer will help you focus on what is most important. The questions I have posted on the website are things you should be able to answer, as well as questions that are similar to them.

Russell et al., 1986

1. Most of the figures are unimportant!

2. Don’t worry too much about the details of cloning *cdc25*, RNA levels, fusions, and how they made the disruption (figures 1, 2, 3, 4, 6 and 7).

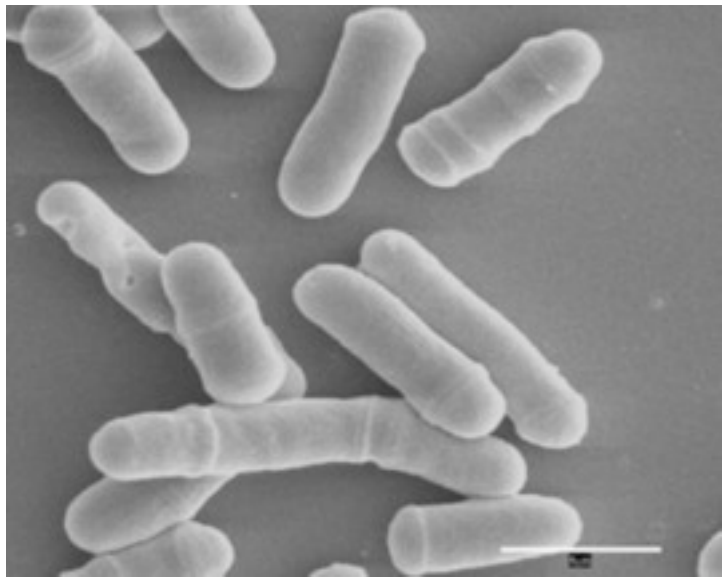
3. More important is the reasoning behind the double mutant combinations they make and the data in Table 1, figures 5, 8 and 9. The text has the most important things to pay attention to.

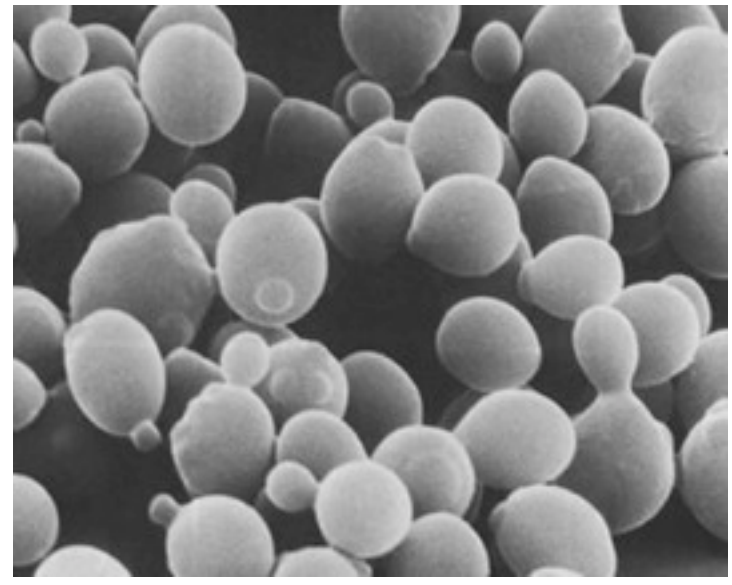
4. Not a great paper in its layout, but it makes some critical observations from simple and powerful genetic experiments.

5. Read carefully!

Things to look out for in the paper for Tuesday.

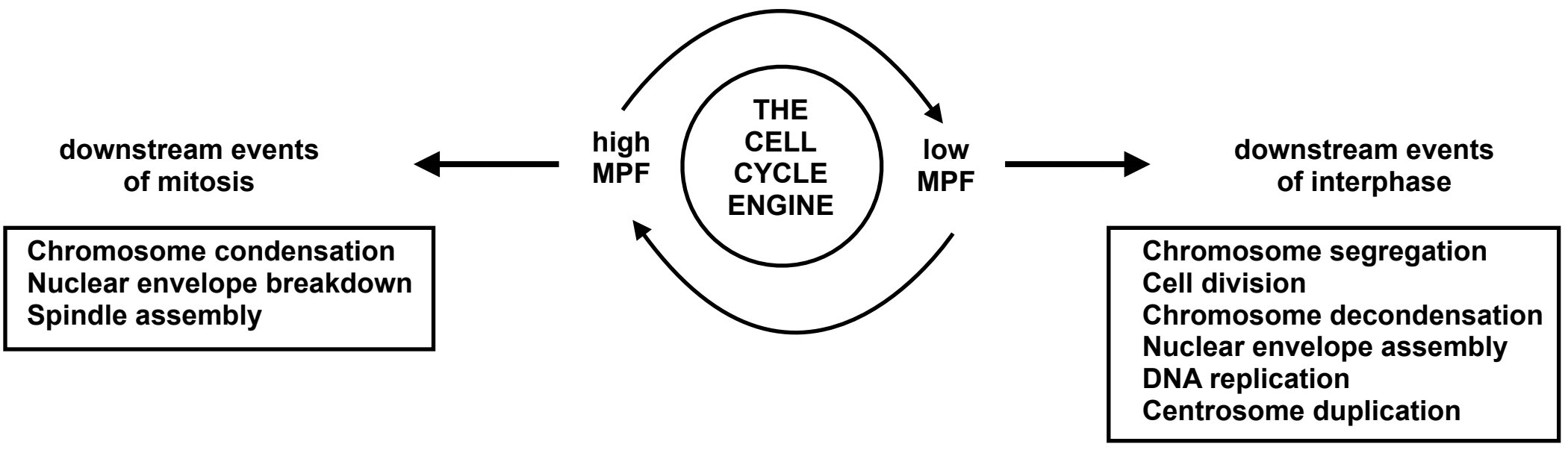
Lecture 2
Conservation of the cell cycle engine





We'll begin to explore genetic experiments performed in fission and budding yeast in this lecture.

A simple model for how changes in MPF drive key cell cycle events



The idea of MPF led to the following model that hypothesized oscillations in MPF activity is the main driver of key cell cycle events. This model obviously focused on the events of mitosis and didn't really have an explanation for how the absence of MPF would trigger all the non-mitotic events.

Today

the identity of MPF

Outline of Today's Lecture

1. Biochemistry vs. Genetics.

2. *cdc* mutants in budding and fission yeast.

3. *cdc* mutants helped determine dependencies of events in the cell cycle

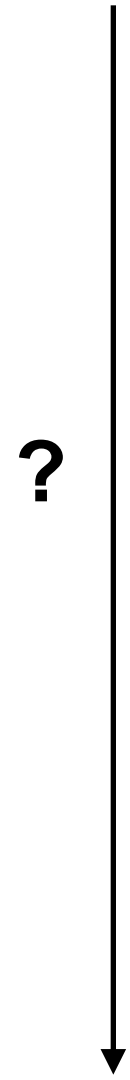
4. How to order genes in pathway using epistasis.

5. Conservation of Cdc2/Cdc28

Outline of this lecture. Part of the lecture will cover the science - following up on MPF and talking about some key mutants in budding and fission yeasts. Part will take you through some important genetic methodology - how the cdc mutants were isolated, ordering mutants by looking at dependencies, and ordering by epistasis.

How to figure out the mechanism of a biological process?

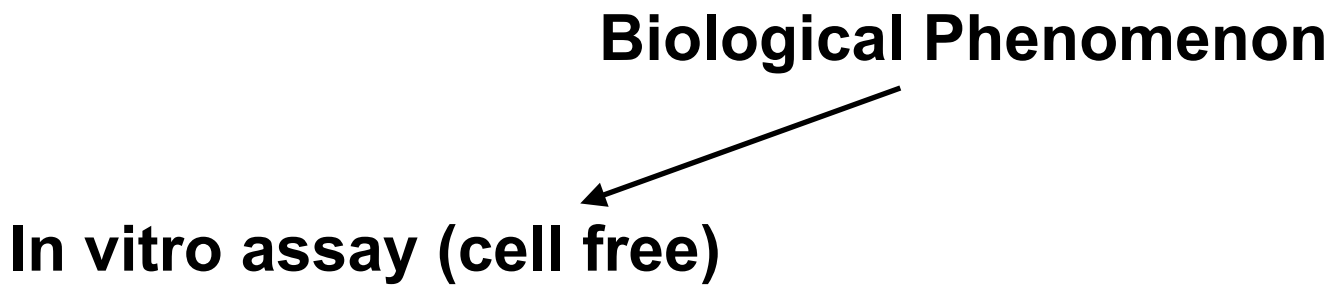
Biological Phenomenon



MECHANISM

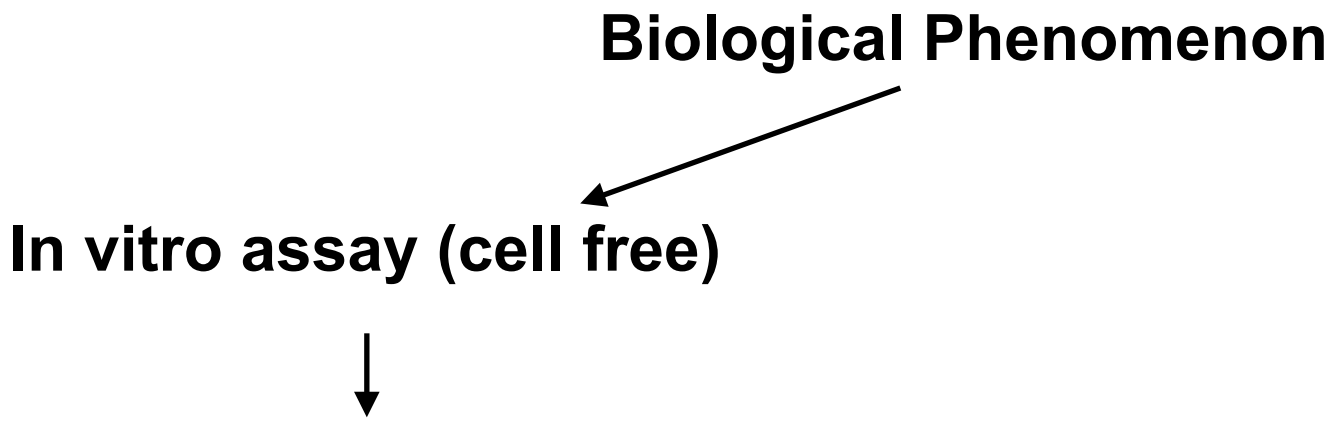
Before we get into the details of what MPF is, I wanted to talk briefly about how researchers go from biological phenomenon to mechanism, which is the goal of cell biological research.

Biochemical Approach:



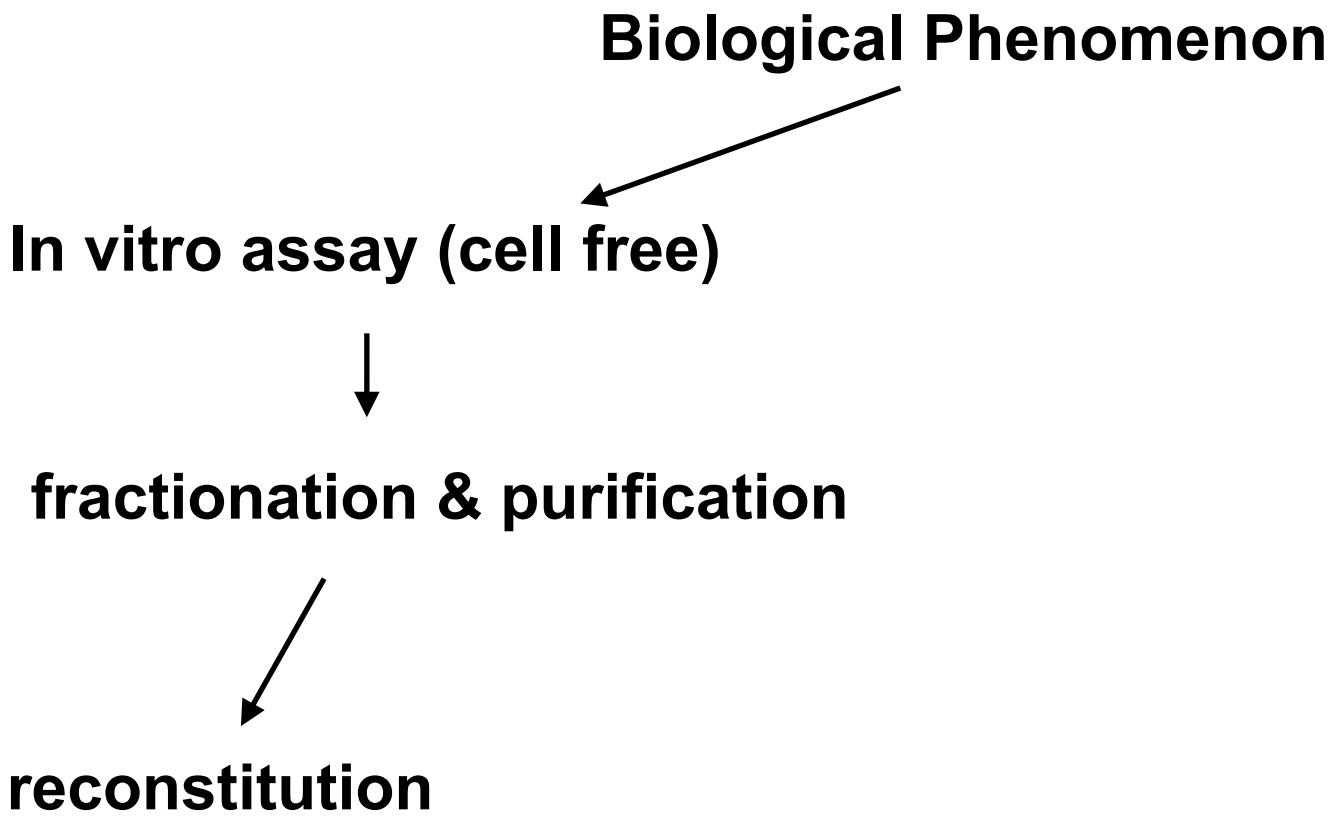
The biochemical approach begins by defining an in vitro assay, which is usually cell free and performed in a test tube, but in the case of MPF, is done on immature oocytes dissected from female frogs. In some respects the immature oocytes are acting like little tubes for this assay. The goal of any in vitro assay is to recapitulate some aspect of a biological process, and be set up in a way that allows for it to be dissected.

Biochemical Approach:



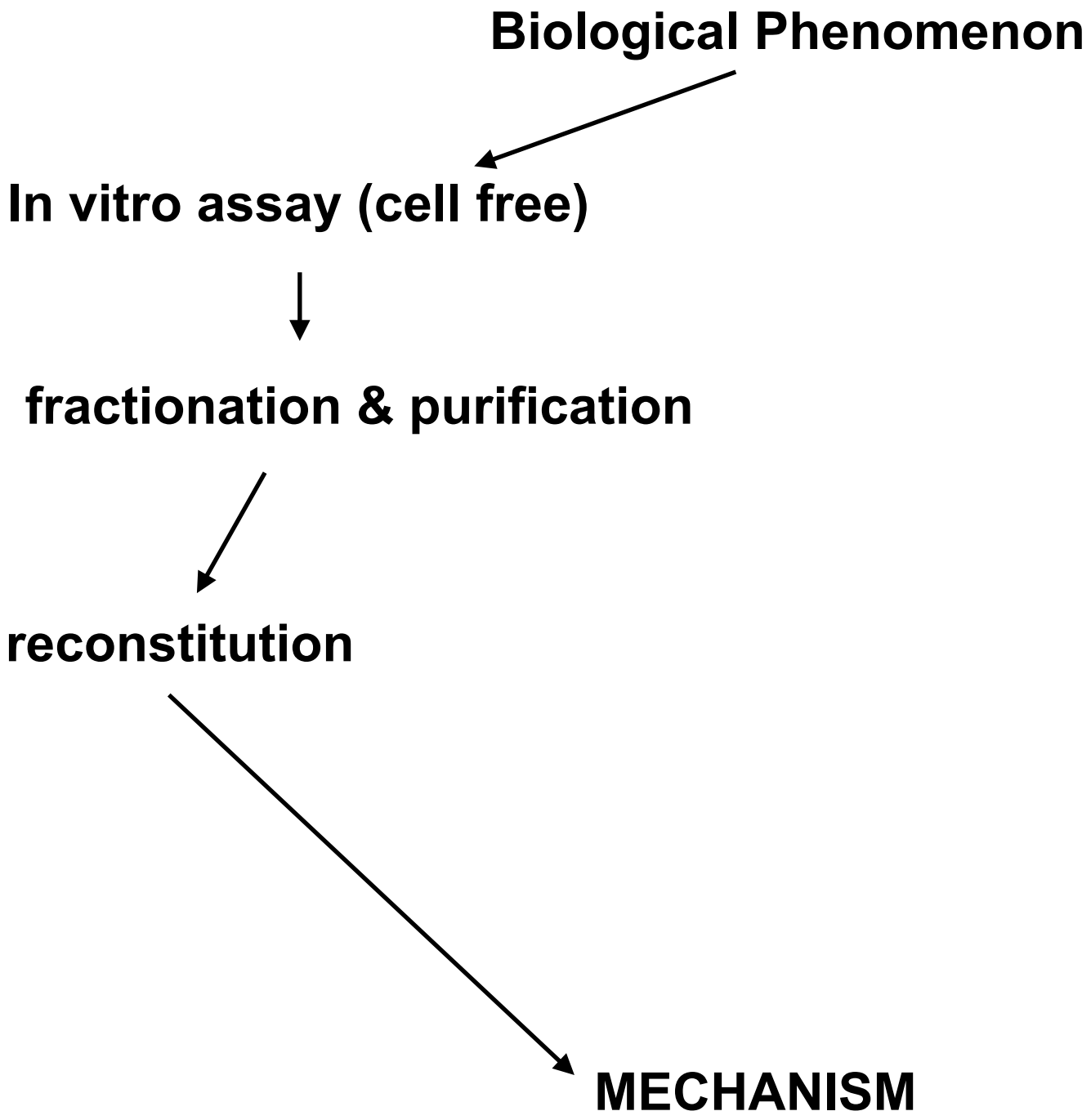
An assay is dissected by fractionation and then purification. Does the assay depend on one protein or factor, or many? Can that protein be purified to homogeneity and then identified? For any fractionation/purification you need an activity that you measure in an assay. For example for MPF, the activity was oocyte maturation and the assay was injecting cytoplasm or fractionated cytoplasm into immature oocytes. Early on people recognized that MPF was associated with histone H1 kinase activity, so people thought MPF was a protein kinase, so they often also measured H1 kinase activity (which is much easier than measuring oocyte maturation).

Biochemical Approach:



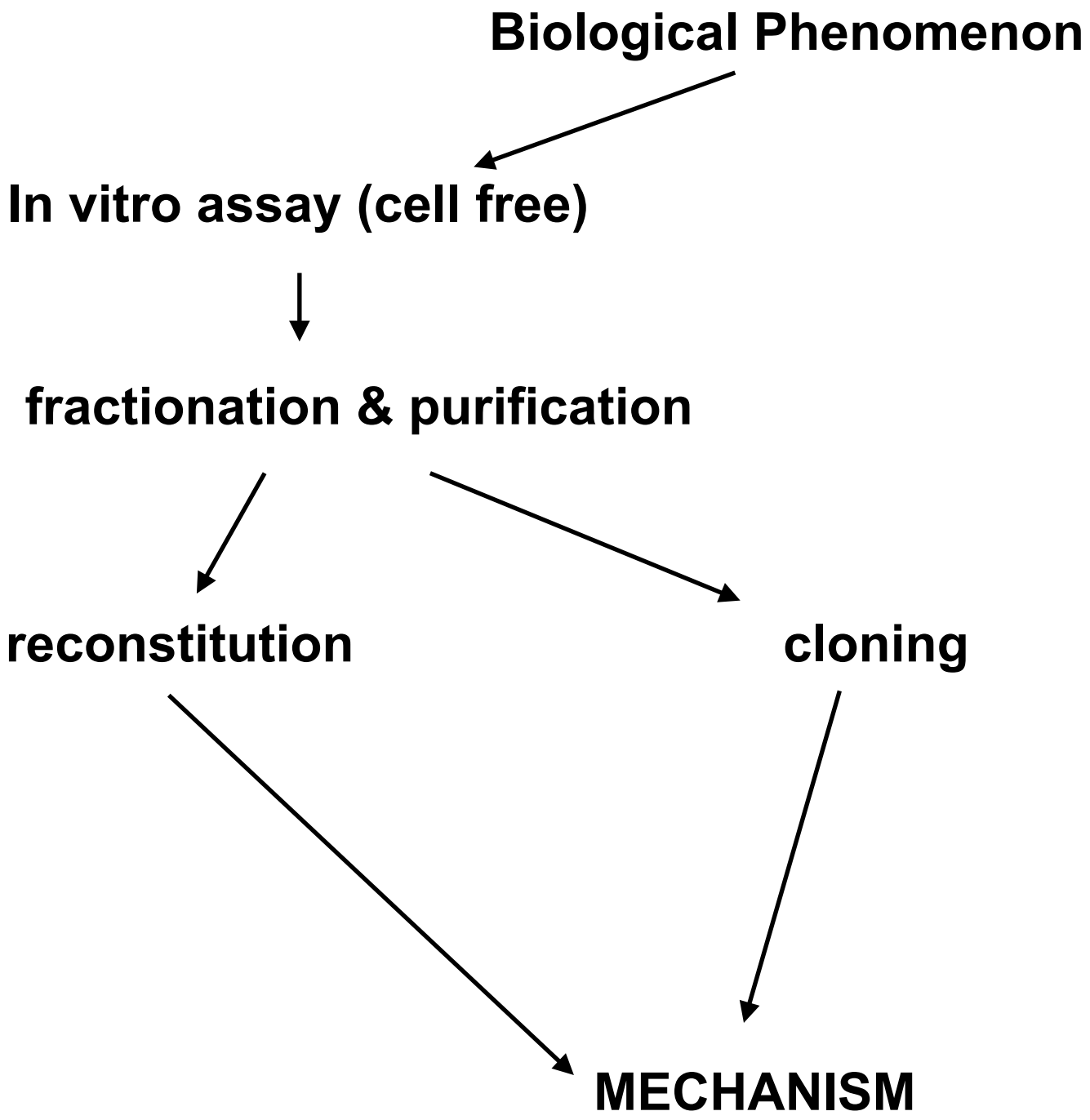
Once your factors are purified, the high road of biochemistry is to reconstitute your activity with pure components. This confirms that all you need to make a reaction go is the components you add. The classic example of this is Arthur Kornberg's reconstitution of DNA replication. Reconstitution tells you a lot about what factors are sufficient for a process, but doesn't necessarily tell you that particular proteins are necessary for the process in vivo.

Biochemical Approach:



The end goal of a biochemical approach is to tell you something about the molecular mechanism of a particular process. In the example of MPF, people wondered what sort of protein or proteins triggered oocyte maturation, and if the same proteins also triggered entry into mitosis. Knowing the activity of the proteins that composed MPF would open up the field of cell cycle research because it was assumed knowing what MPF was would lead to finding all the downstream targets that were needed for each cell cycle transition.

Biochemical Approach:



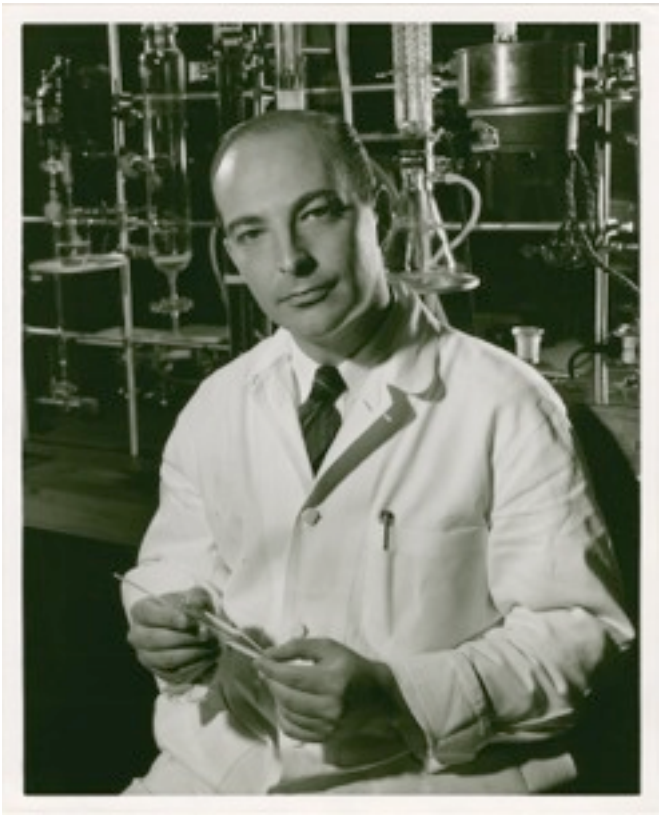
Fractionation and purification can also lead to the cloning of the gene or genes that encode proteins involved in a particular process. Its important to remember that sometimes the activities measured are not always proteins, so there isn't always a gene to clone! With current methods (in particular mass spectrometry) cloning of genes encoding candidate proteins happens quickly, and allows reconstitution and fractionation to proceed more rapidly.

Biological Phenomenon studied with Biochemistry

1. Metabolic enzymes.
How is glucose converted to glucose-6-phosphate?

2. DNA replication

You may be more used to Biochemistry helping to discover things like the metabolic enzymes. Things like hexokinase which converts glucose to glucose-6-phosphate. Researchers likely set up a simple assay for this process and purified the enzyme responsible. Purifying MPF uses biochemistry to understand a complicated cell biological process - oocycte maturation which probably requires 100 of proteins, but the maturation assay allowed researchers to look for key regulators of the process. The hope was knowing the identity of MPF would then lead to finding the proteins downstream that induce all the major changes during maturation.

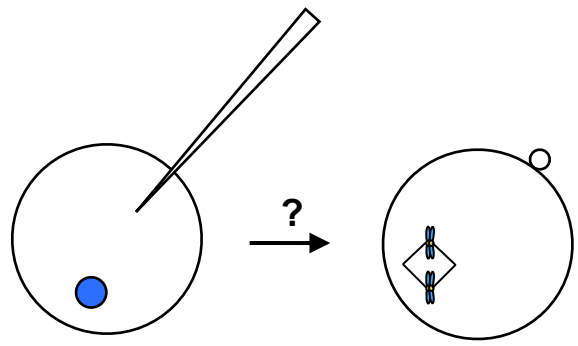


“Implicit in the devotion to purifying enzymes is the faith of a dedicated biochemist of being able to reconstitute in a test tube anything a cell can do.” Arthur Kornberg

Arthur Kornberg popularized this approach. He was the first person to try and reconstitute a very complicated cell biological process - DNA replication.

What is MPF?

In vitro assay (“cell free”):

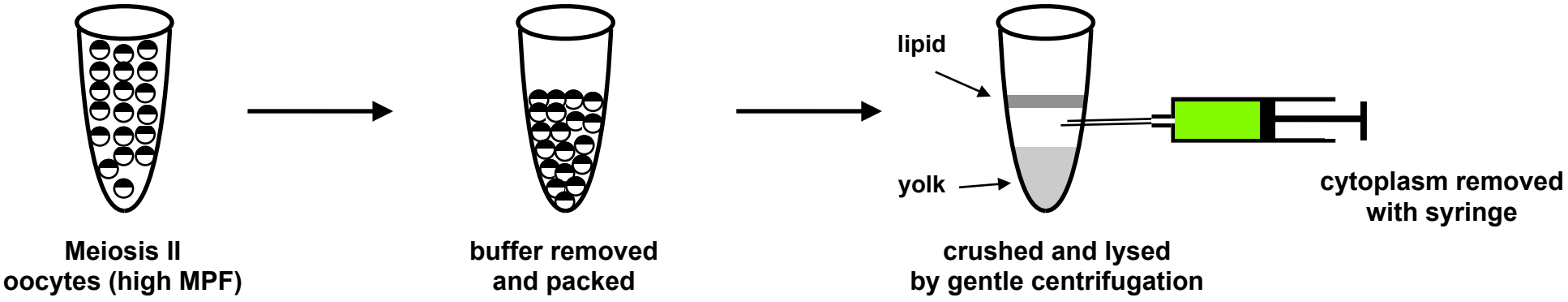


oocyte maturation

MPF was first defined as an activity, so biochemical methods seemed most appropriate. Extracts of eggs could be made, fractionated, and the fractions tested in the oocycte maturation assay. On paper a good idea, but it was exceedingly difficult because the maturation assay is so time consuming. Other methods (to be discussed) discovered the molecular components of MPF.

What is MPF?

material to fractionate:

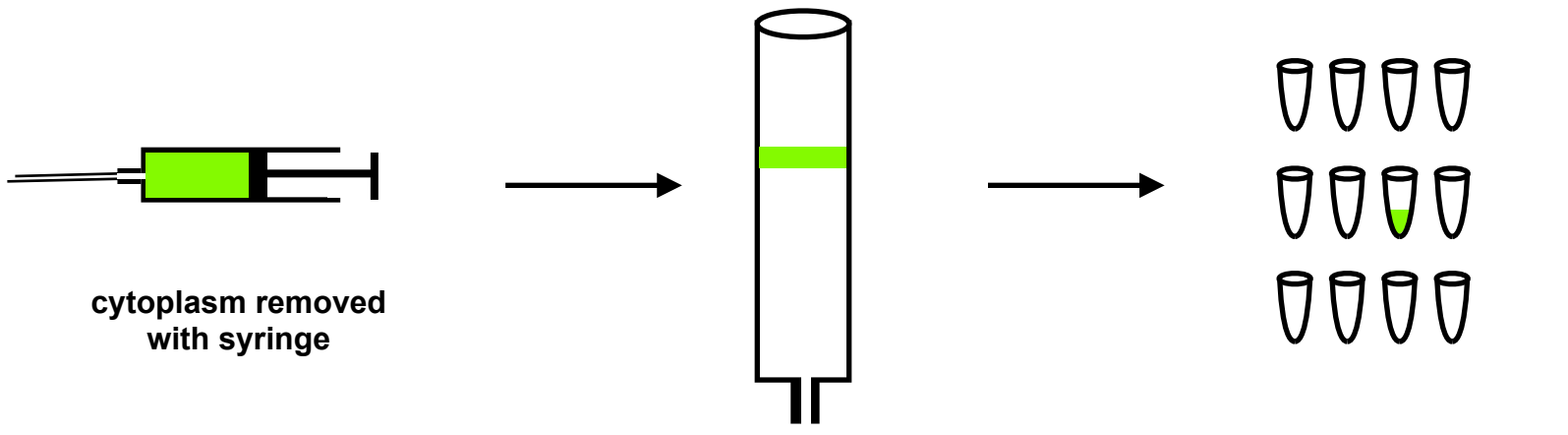


frog egg cytoplasm

The material used to fractionate was extracts of mature oocytes that are arrested in metaphase of meiosis II. People knew these had high MPF activity. The oocytes were collected, packed in a tube and then crushed by high speed centrifugation. The contents separate into three phases: lipid, cytoplasm and yolk. MPF was present in the cytoplasm which is a good thing because it is tricky to fractionate lipids and yolk!

What is MPF?

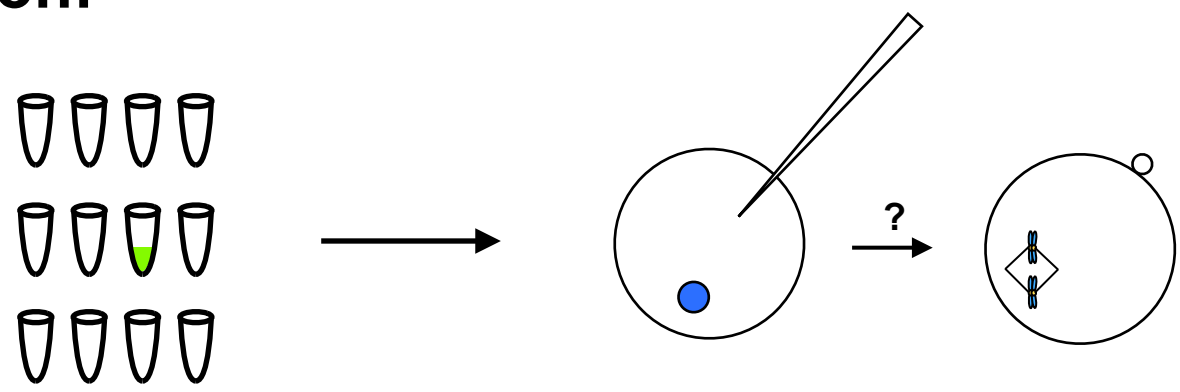
fractionation:



The cytoplasm was then fractionated primarily by column chromatography. I'm showing one column in this cartoon, but the actual purification involved many columns. Chromatography separates the components of the cytoplasm into different fractions based on intrinsic properties of proteins - charge, size, hydrophobicity.

What is MPF?

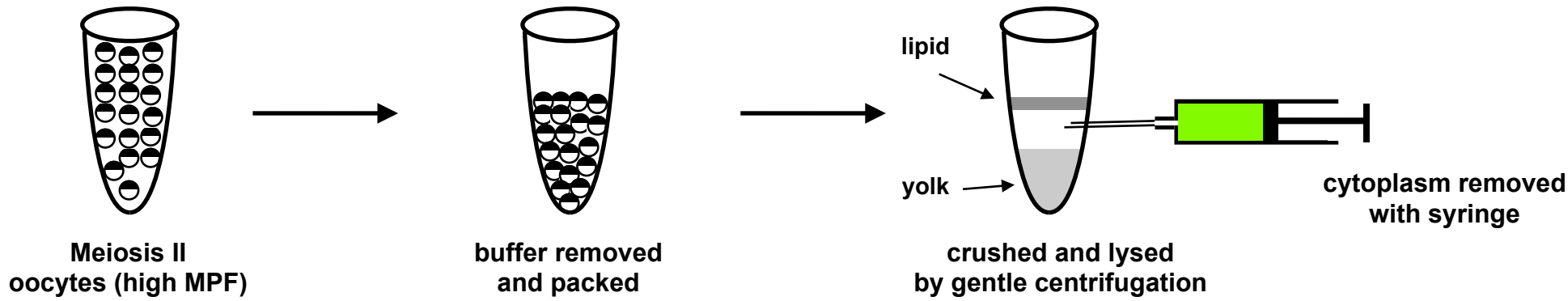
reconstitution:



fractionated extract **oocyte maturation**

Each fraction was then tested in the maturation assay. The hope was to eventually have a fraction that contained only MPF that could support oocyte maturation.

What is MPF?



Meiosis II
oocytes (high MPF)

buffer removed
and packed

crushed and lysed
by gentle centrifugation

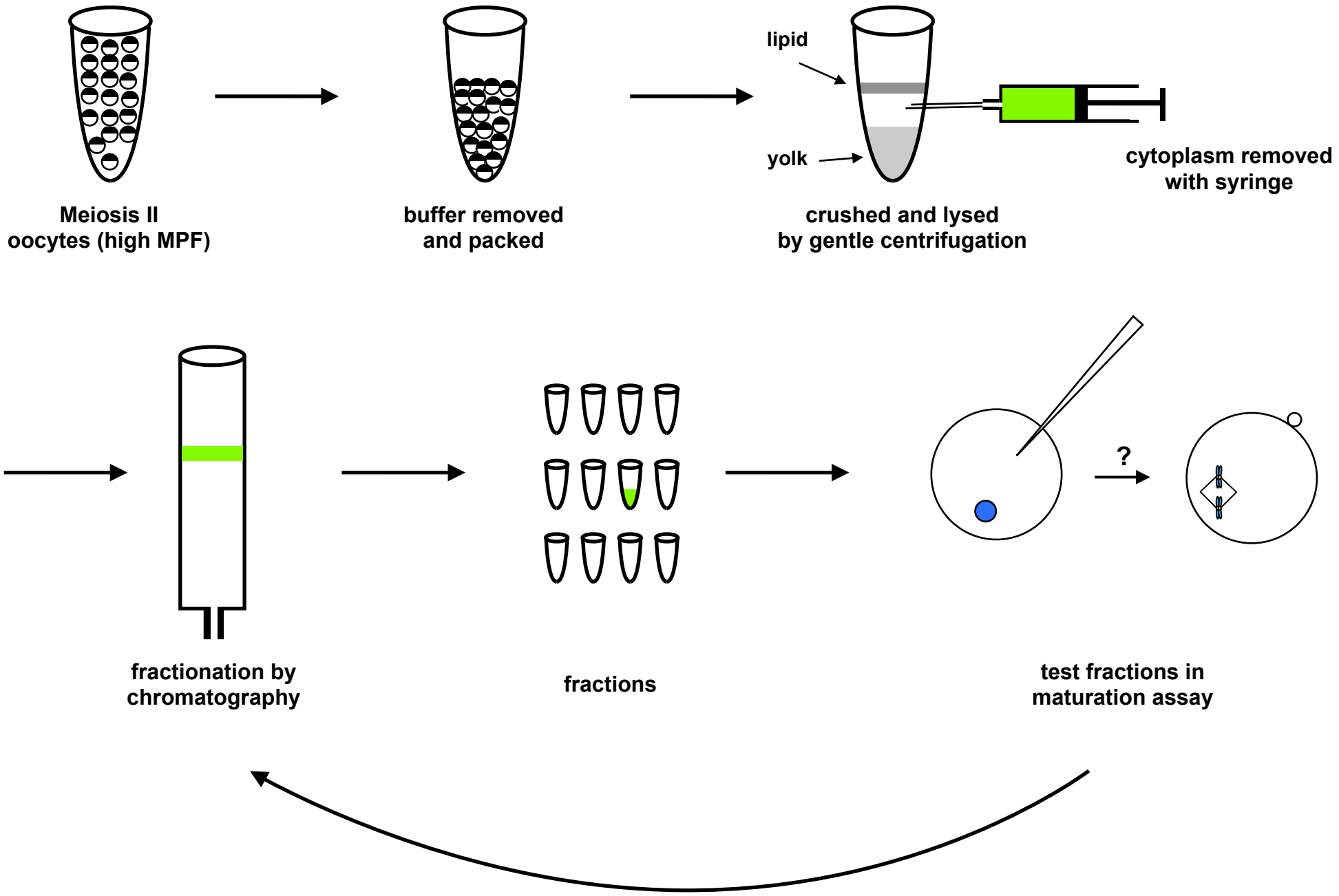
fractionation by
chromatography

fractions

test fractions in
maturation assay

Here is the whole work flow.

What is MPF?



The fraction that contained MPF was then fractionated further on additional columns.

An example of the best MPF purification scheme:
(though it was published a year after the identity of MPF was already determined!)

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

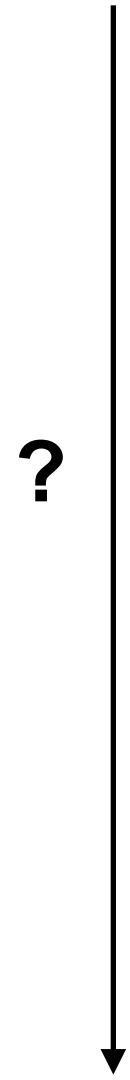
One unit of H1 kinase activity corresponds to 1 μ1mol of ^{32}P transferred per min at 25°C. One unit of MPF corresponds to the amount of MPF required in 50 nl of a microinjected sample for 50% of the recipient Xenopus oocytes to undergo GVBD (Wu and Gerhart, 1980).

Labbe et. al., Cell 1989

This is a table showing the best purification of MPF that was performed. After a high speed spin and six columns MPF was purer, but not pure enough to identify the components of MPF. Note that H1 kinase activity was measured in parallel with oocyte maturation activity. At the time researchers were pretty sure they were the same thing, but not yet positive.

How to figure out the mechanism of a biological process?

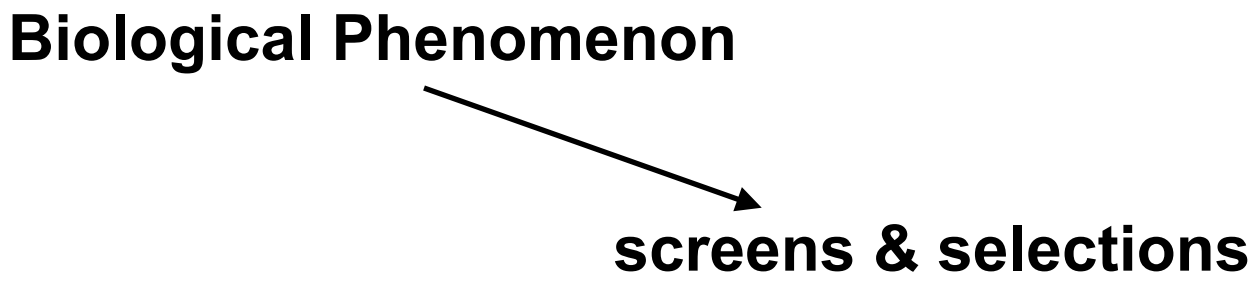
Biological Phenomenon



MECHANISM

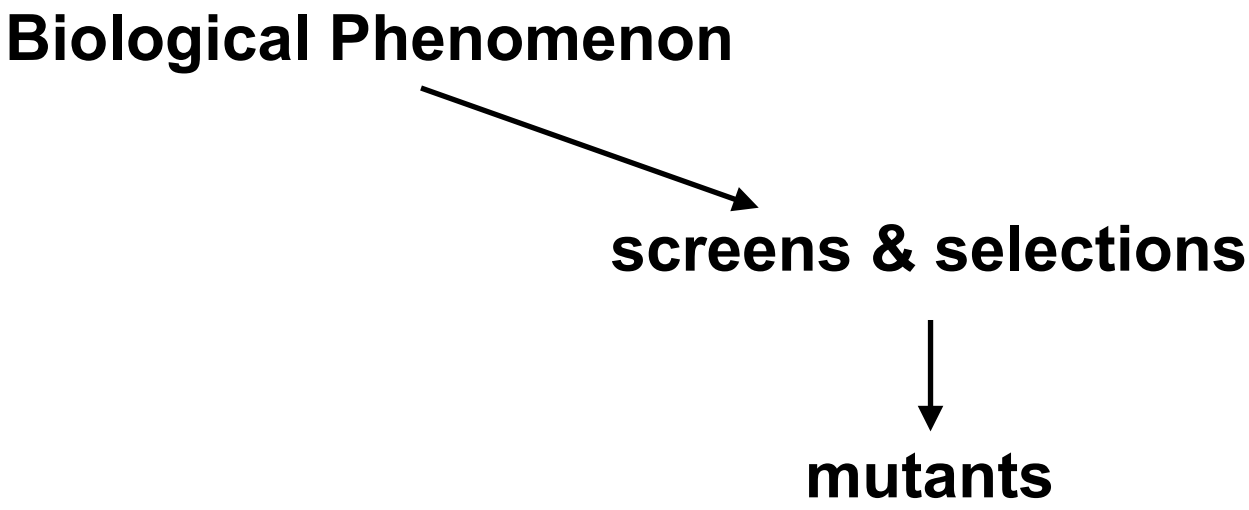
Before we get into the details of what MPF is, I wanted to talk briefly about how researchers go from biological phenomenon to mechanism, which is the goal of cell biological research.

Genetic Approach:



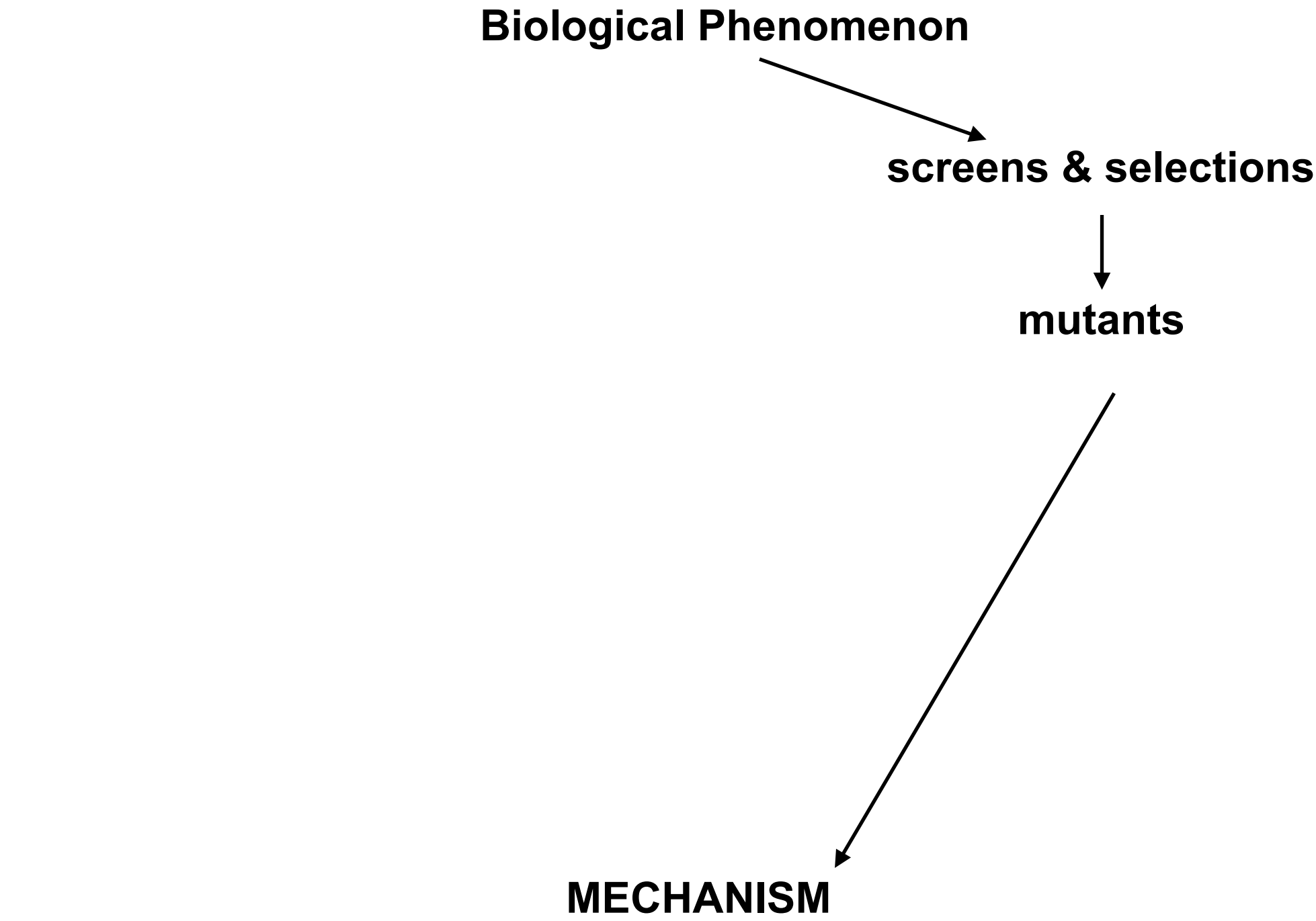
An alternative approach that was used with great success to figure out the core principles of the cell cycle was a genetic approach. In this approach a phenomenon, in this case the ability of a cell to duplicate its chromosomes and divide, is studied by finding mutants defective in these processes. These were mainly done by screens – where a particular phenotype (here, cell cycle arrest) is screened for. Selections are usually more powerful, but rely on a mutant to provide a growth advantage so that it can be selected from the starting strain. Arresting the cell cycle – the phenotype screened for – clearly would cause a serious growth disadvantage, so a screen was the only option.

Genetic Approach:



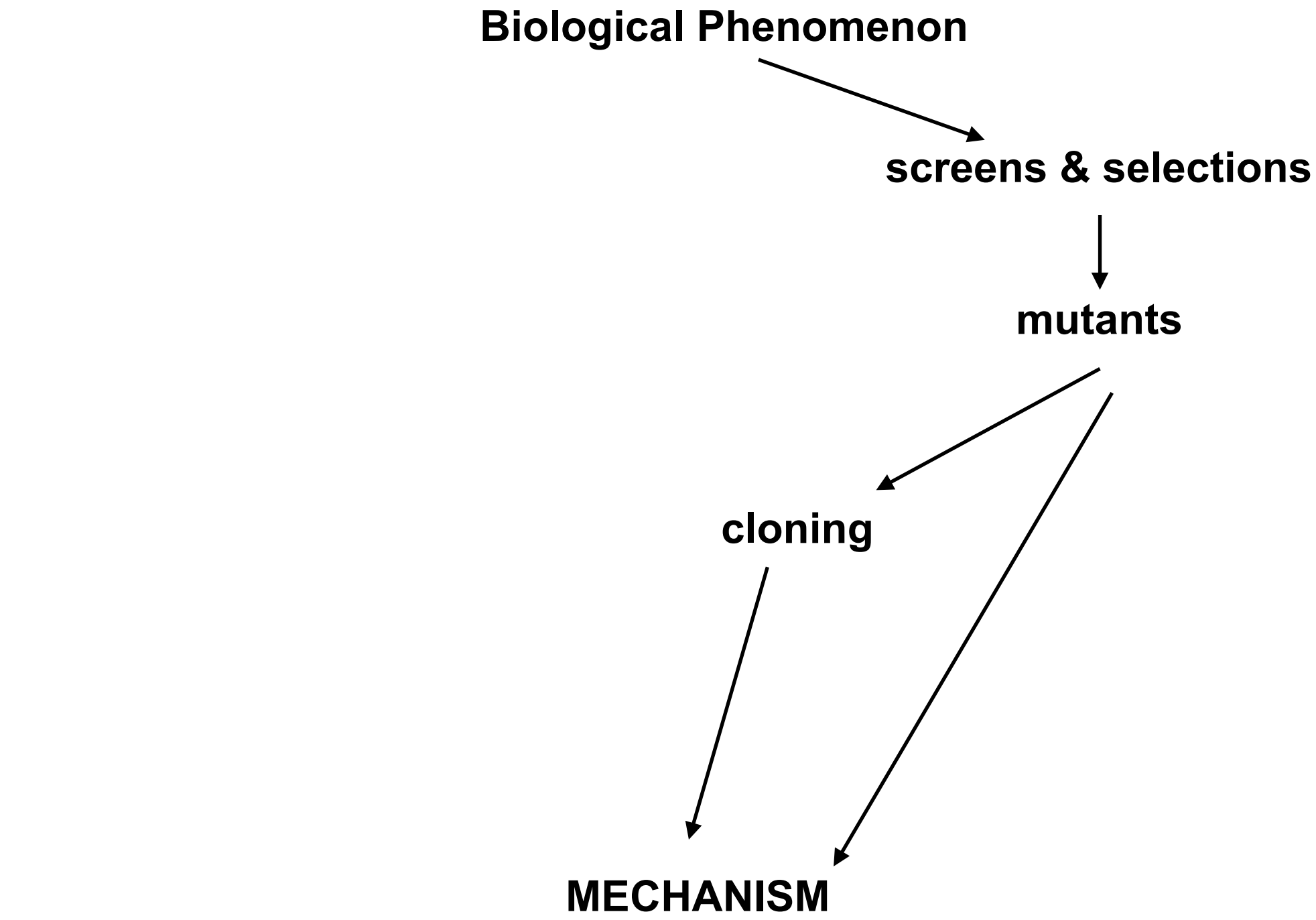
The screens and selections lead to the isolation of mutants defective in a given phenomenon. The existence of certain mutants will sometimes provide proof that a particular process actually exists. For example, when mutants defective in the cell cycle were first isolated, people did not know that such mutants could exist and that they would arrest at particular stages of the cell cycle. The fact that they existed meant that there were important transitions that were regulated by the activity of proteins.

Genetic Approach:



Mutants are also useful because using them you can order a pathway and begin to understand the mechanism of a process.

Genetic Approach:



They also allow researchers to clone the gene that is mutated, which often reveals a lot about the mechanism of a process.

Biological Phenomenon studied with Genetics

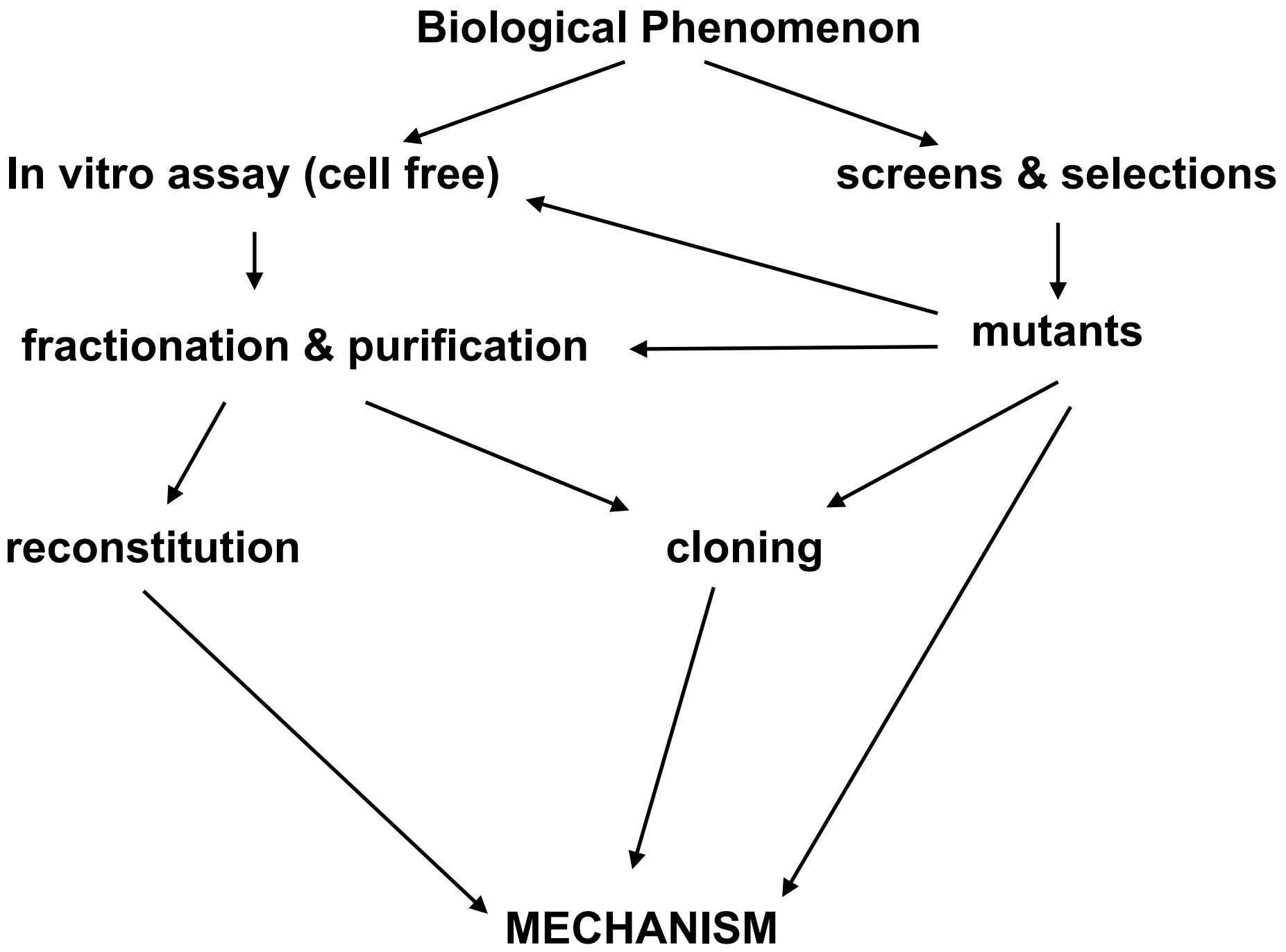
1. Metabolic processes.
How is uracil synthesized?

2. Morphological processes
Why are Drosophila wings in the right place?
What determines Drosophila eye color?
What makes a pea seed smooth/wrinkled?

3. Cell Biological processes.
How do cells traverse from one point in the cell cycle to another?

As with the biochemical approach, you may be more used to genetics being used to study metabolic processes. A lot of early microbial genetics was used for this. For example, if I was interested in how uracil is synthesized in yeast, I could look for mutants that could no longer survive without adding exogenous uracil into the media (wild yeast strains can make uracil for simpler constituents). I could isolate such ura mutants, and they would genetically define the steps needed to make uracil. I'll talk later about how you would order such steps, but the key concept is that if such mutants were to exist, it shows that a defined biochemical pathway exists that can be disrupted with a mutation in a single gene. For a metabolic process this might seem obvious, but for cell biological processes its not always clear that certain pathways even exist! Genetics has also been used to study larger scale morphological pathways - drosophila eye color and Mendel's peas are good examples. The peas could be smooth or wrinkled, and a single mutation could trigger that change (even though hundreds of genes are probably needed to cause such a morphological change. The genetics done to understand the cell cycle were of a different variety - researchers looked for mutations that would block specific steps in a cell biological process - specifically would block transitions between different phases of the cell cycle. People didn't know how these steps were regulated and if such mutants actually existed. They do exist and these mutants have been essential for understanding how the cell cycle functions.

Usually a combination:



In reality the biochemical and genetic approaches are used in combination - where mutants are used to help establish in vitro assays and perform fractionation/purification. And the combined evidence from in vivo analysis of mutants, and in vitro analysis of the proteins encoded by the mutated genes gives the fullest picture of the molecular mechanism of a given process. This happened with the cell cycle where the discovery of MPF happened at the same time as the isolation of cdc mutants (more below).

Biochemistry vs. Genetics

The Demise of Bill

by Douglas R. Kellogg

The Sullivan Laboratory
Molecular, Cell and Developmental Biology
University of California, Santa Cruz

The Salvation of Doug
A Tale of Two Retired Scientists and Some Rope

by William T. Sullivan

"I use this story in my introductory genetics classes to explain the rationale behind mutational analysis and to show younger students the basic differences between genetics and biochemistry."

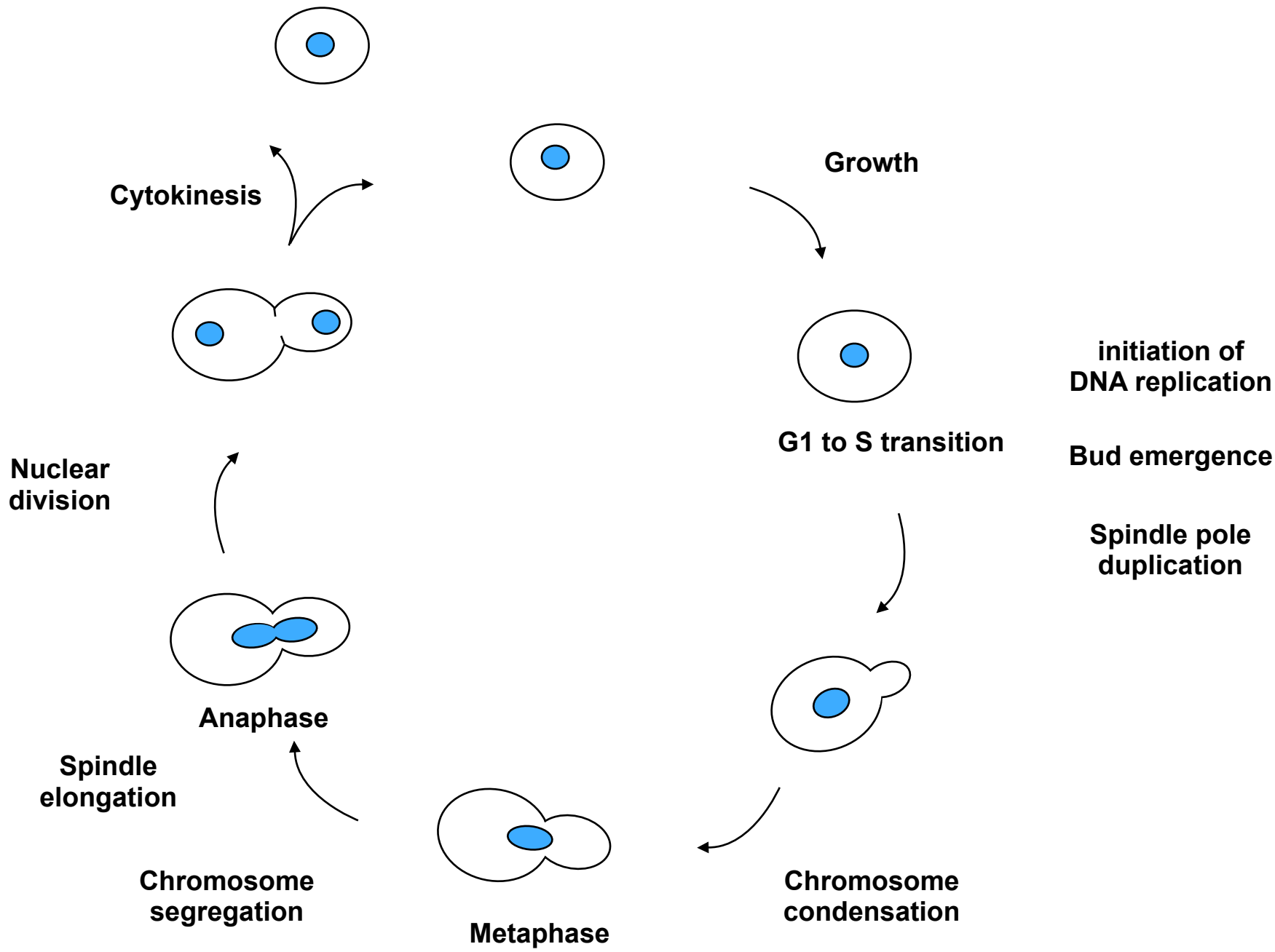
- Bill Sullivan

-

I've posted on the web site two short pieces about the benefits and drawbacks of biochemical and genetic techniques written by two professors at UC, Santa Cruz. They use car manufacturing as an example of a process they want to understand – but a car is not so different from the cell cycle – many small machines tied together into a larger device, run by a central engine.

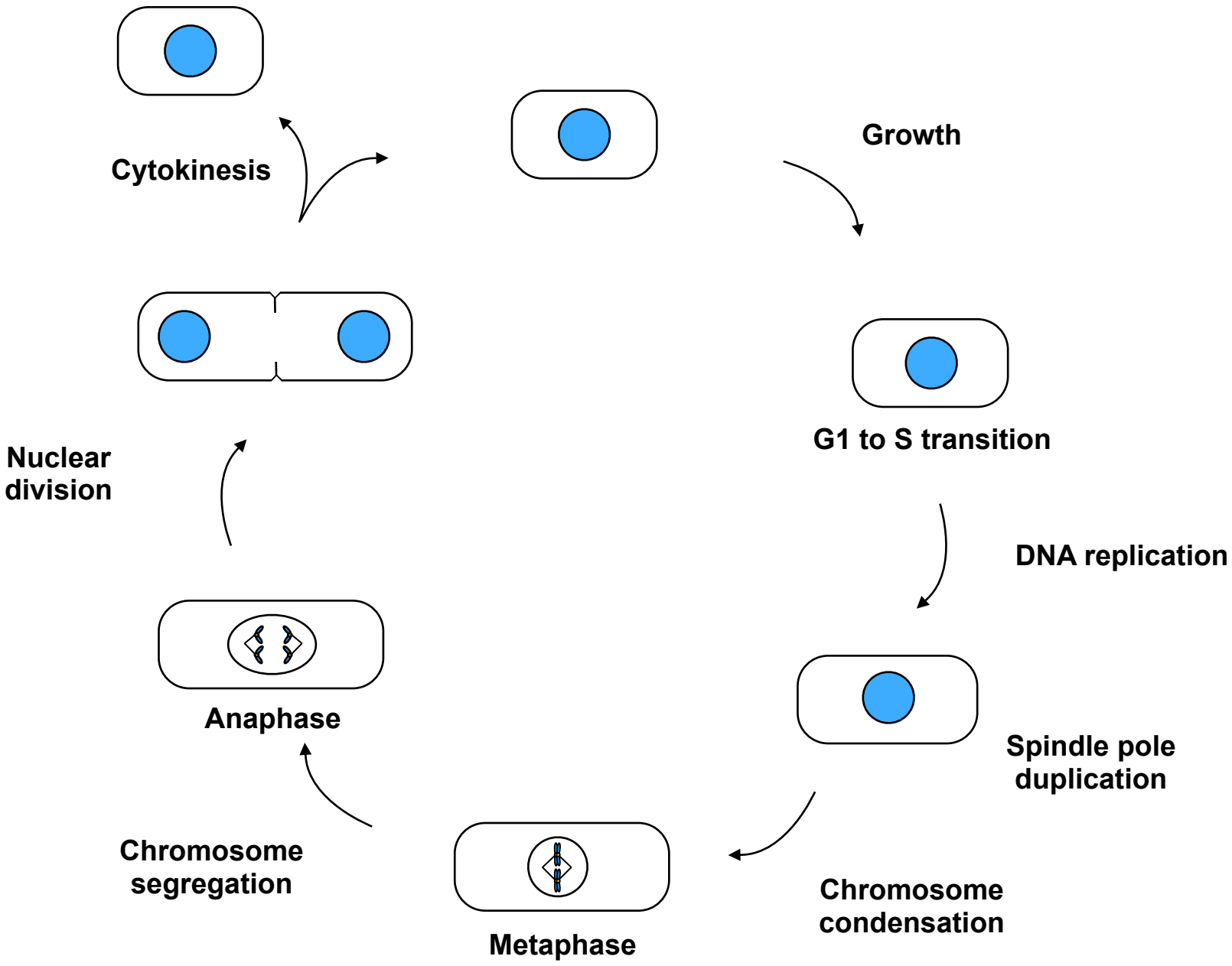
A movie illustrating how budding yeast grow and divide.

budding yeast cell cycle - size of bud determines where cells are in the cell cycle



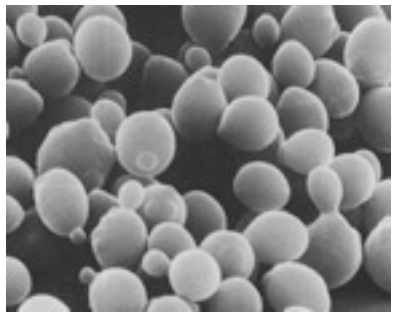
The cell cycle in the budding yeast, Saccharomyces cerevisiae. Budding yeast cells in G1 are round cells, but after the G1 to S transition initiate three events: DNA replication, bud emergence and spindle pole duplication. After budding, most growth occurs in the bud. A consequence of budding is that the placement of the cytokinetic furrow is determined early in the cell cycle. Unlike fission yeast, chromosomes are not visible during mitosis in budding yeast. Despite this the cell cycle position of budding yeast can be determined by the size of the bud - unbudded cells are in G1, small budded cells in S phase, and large budded cells in mitosis. In this picture I've "stained" the DNA in blue - it lights up the whole nucleus.

fission yeast cell cycle - length of the cell determines where cells are in the cell cycle

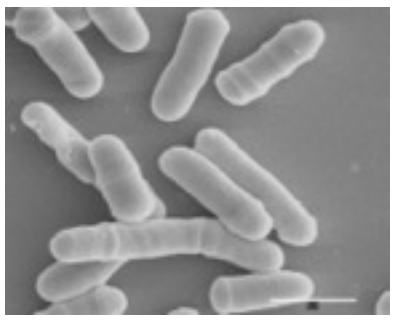


The cell cycle in the fission yeast, Schizzosaccharomyces cerevisiae. This yeast is rod shaped, grows from its two ends, and divides by fission, hence its name. After anaphase it lays down a septum at the midpoint of the mother cell. Fission yeast chromosomes are visible during mitosis, but it undergoes a “closed” mitosis - the nuclear envelope doesn’t breakdown as it does in vertebrate cells. The G1 to S transition in fission yeast and budding yeast is called START, and is the point at which the cell commits to going through the entire cell cycle. One of the critical determinants for passing START is that the cells reach a critical size. A similar point, called the “restriction point” exists in vertebrate cells. The restriction point was defined as the point after which serum could be removed from culture media, and the tissue culture cells would still traverse on cell cycle. Like START, the restriction point, provides a point to halt cell cycle progression when nutrients are unavailable. After START DNA replication initiates.

budding yeast

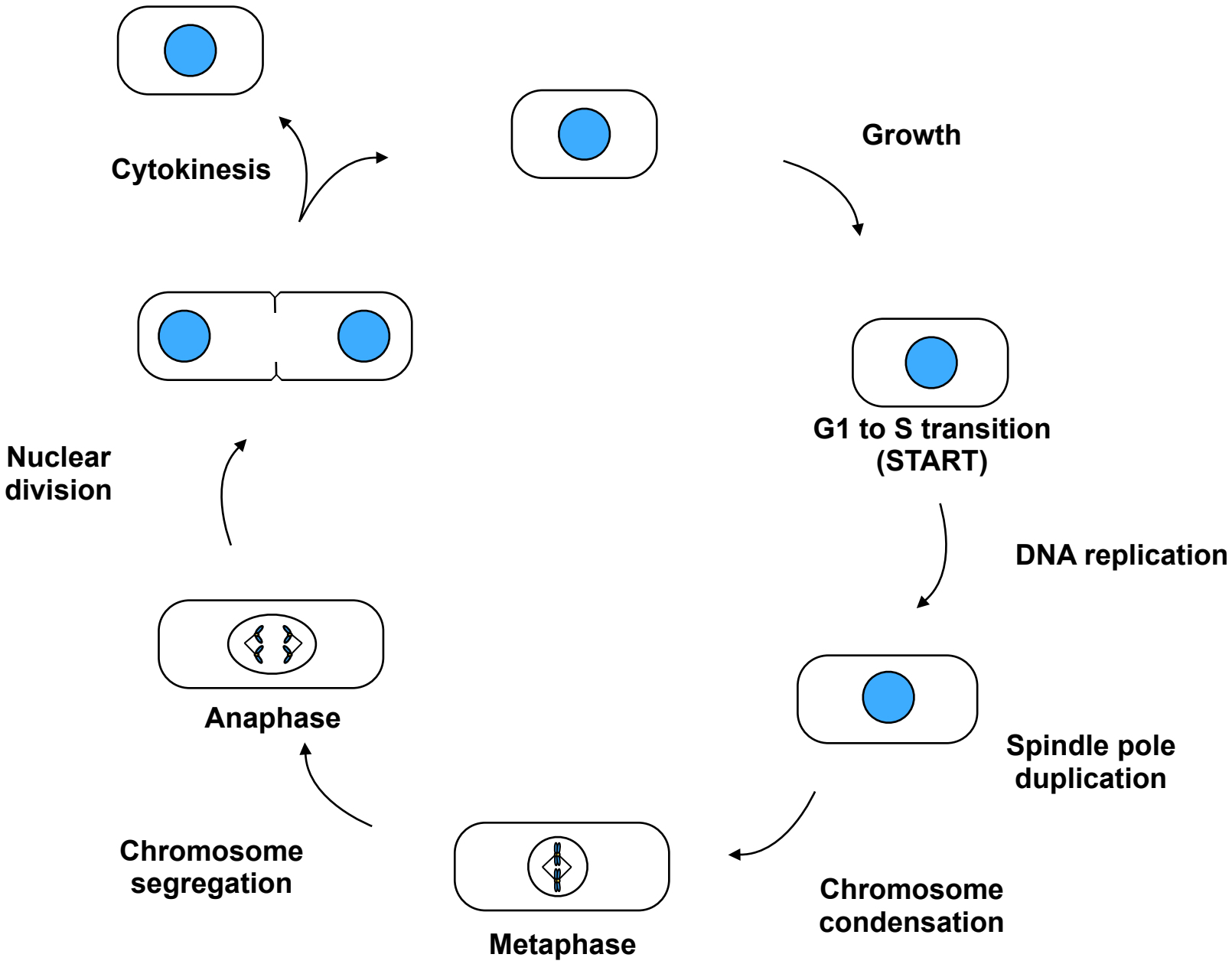


fission yeast

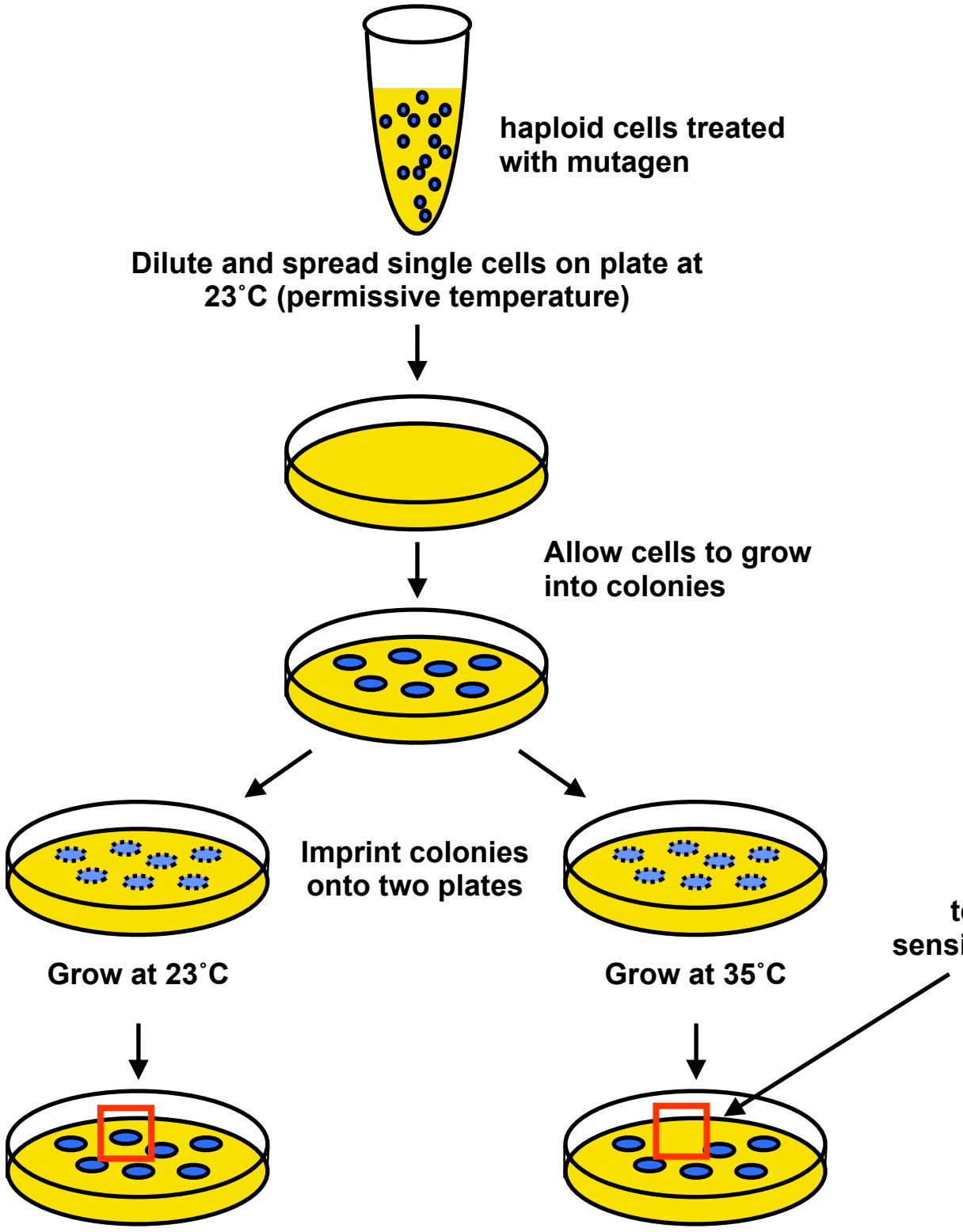


The organization of the fission and budding yeast cell cycle. As discussed in the last lecture, the fission yeast cell cycle has a very short (or non-existent) G1. As a consequence, fission yeast cells have passed the threshold size for passing START before they have completed mitosis. Budding yeast spend a long time in G1, and have virtually no G2 phase. The difference between the two cell cycles may be connected to the fact that fission yeast exist in the wild as haploids and budding yeast exist as diploids (or tetra- or octa-ploids). Haploids are more sensitive to the effects of DNA damage in G1 because they don't have a homologous chromosome to repair off of. Therefore, a short G1 is advantageous to fission yeast. Budding yeast will usually have homologues to repair from, so a long G1 is not a problem. Both budding and fission yeast are studied as haploids in the lab.

do mutants exist that block specific cell cycle transitions?



The strategy for studying the cell cycle in fission and budding yeast was to look for mutants that block different transitions in the cell cycle.



Since mutants that block cell cycle transitions will be lethal, researchers isolated “conditional” mutants. Conditional mutations render a gene inactive in one condition (the restrictive condition) and active in a second condition (the permissive condition). In these experiments researchers isolated temperature sensitive mutants. At the high temperature ts mutants die. The yeast are mutated with a mutagen (often EMS or MMS, two chemicals that covalently modify DNA and cause specific base changes). Cells recover at the permissive temperature, and then are “replica plated” onto two plates - one placed at the permissive temperature and the other at the restrictive temperature. Colonies that grow at the permissive temperature and not the restrictive temperature identify ts mutants. in some cases a ts mutant may contain multiple mutations. It is standard practice to “back cross” the mutant to a wild type strain in order to find out if a single gene is mutated, or if several are. It is also important to note that any mutants found don’t tell you anything about the gene mutated, or the protein it encodes. ts mutants are only one type of conditional mutant that can be screened for - researchers can also look for cold sensitive mutants, chemically sensitive mutants, light sensitive etc. In general ts mutants are loss-of-function recessive mutations. At the restrictive temperature the mutated gene is not expressed at all (ie loss-of-function).

Researchers looked for temperature sensitive (*ts*) mutants

1. *ts* mutants are conditional - alive at one temperature (the <u>permissive</u> condition - usually a low temperature) and dead at another (the <u>restrictive</u> condition - usually a high temperature).

Properties of ts mutants that are important to know.

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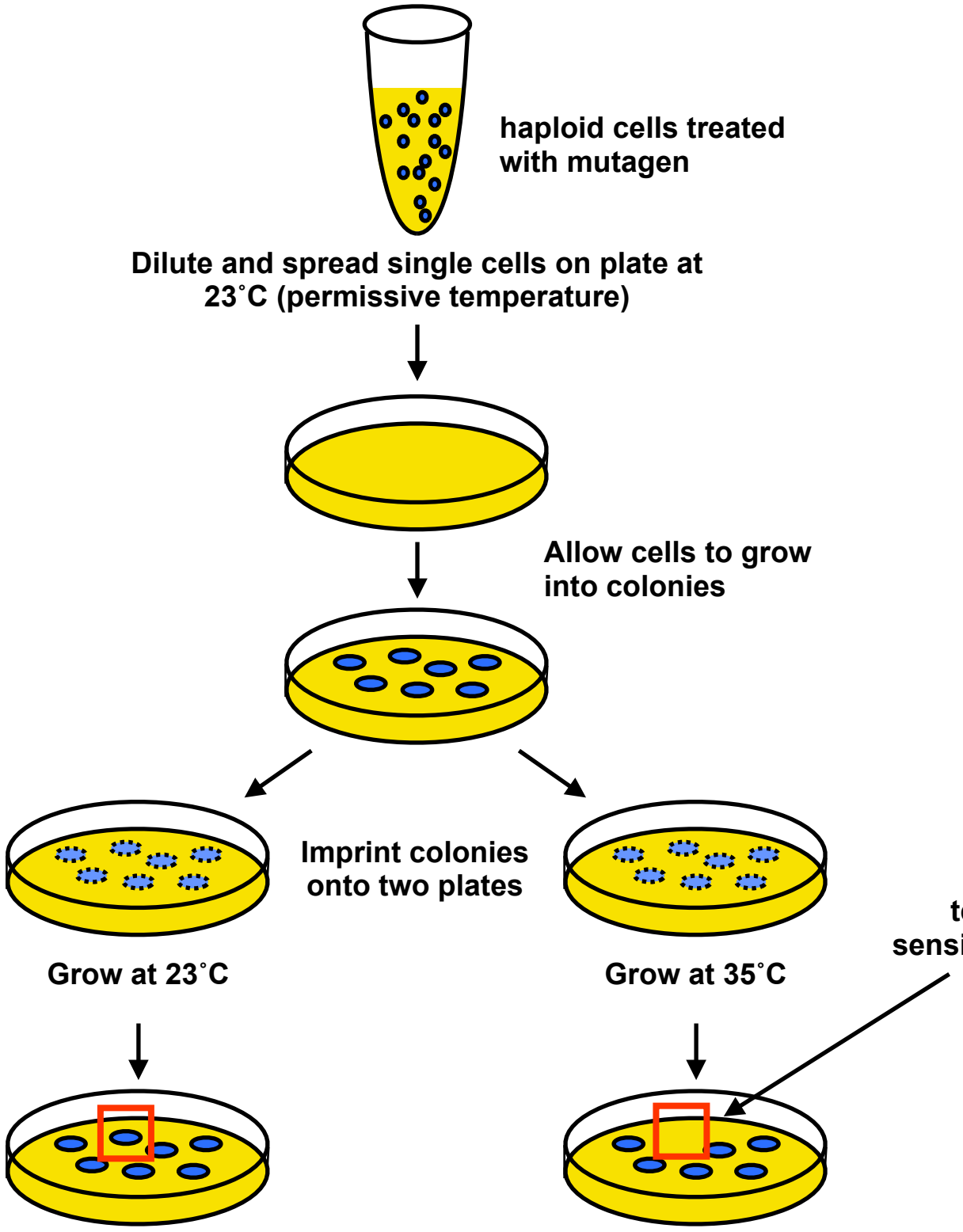
7. *ts* mutants are rare because they often require specific substitutions of amino acids at specific positions.

Properties of ts mutants that are important to know.



23 degrees is the permissive condition, and 35 degrees is the restrictive condition. At 35 degrees the ts mutant dies, and is therefore a mutant in an essential gene. This doesn't tell you that the mutant is blocked in the cell cycle, only that its function is required to grow at high temperature.

Nomenclature

fission yeast

wild type gene: *cdc2*$^{+}$
mutant gene: *cdc2*^{ts} or *cdc2-3w*^{D}
protein: Cdc2 or Cdc2-3w^{D}

budding yeast

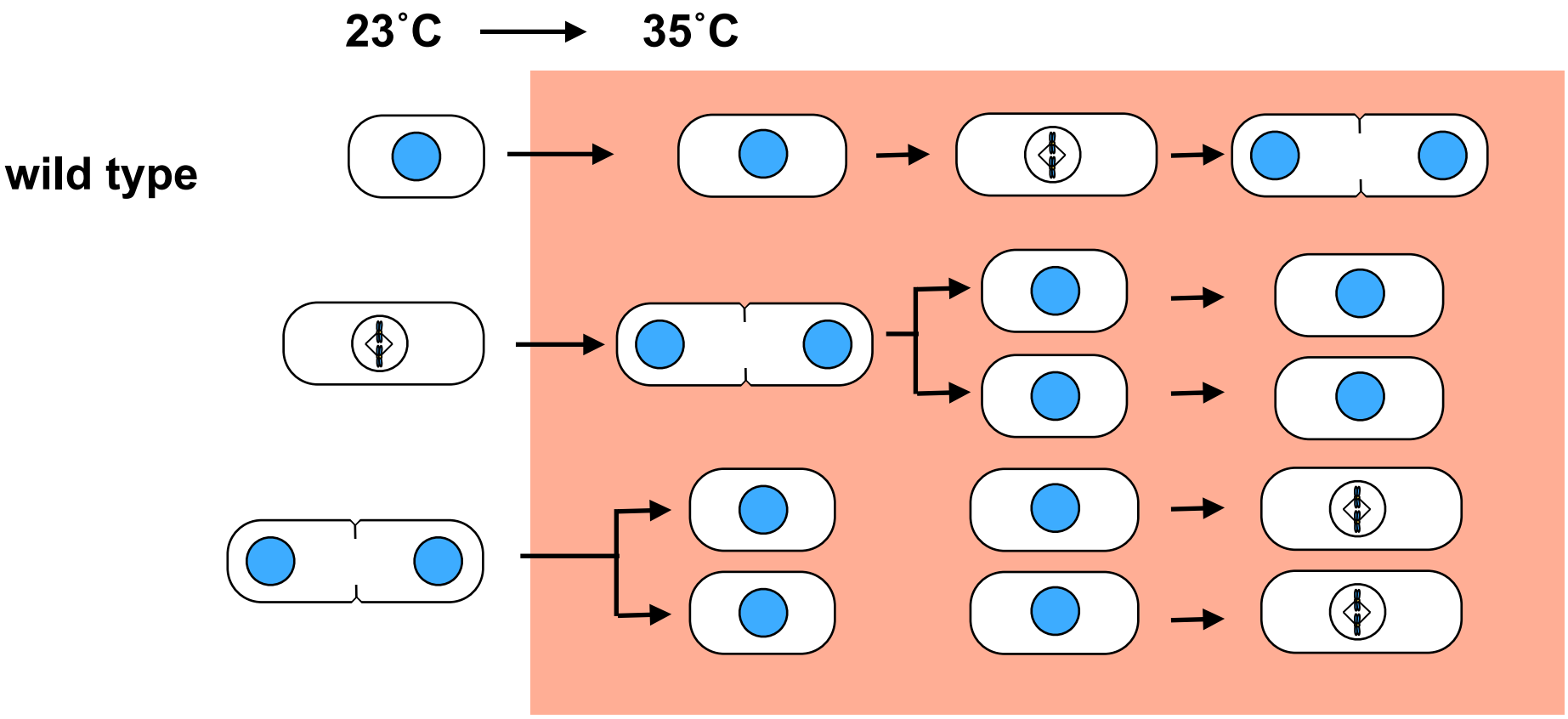
wild type gene: *CDC2*
mutant gene: *cdc2*^{ts} or *cdc2-1* or *cdc2*^{D}
protein: Cdc2 or Cdc2-1

Genes of the same name are not always homologous!

***cdc2*$^{+}$ is <u>not</u> *CDC2* (for example)**

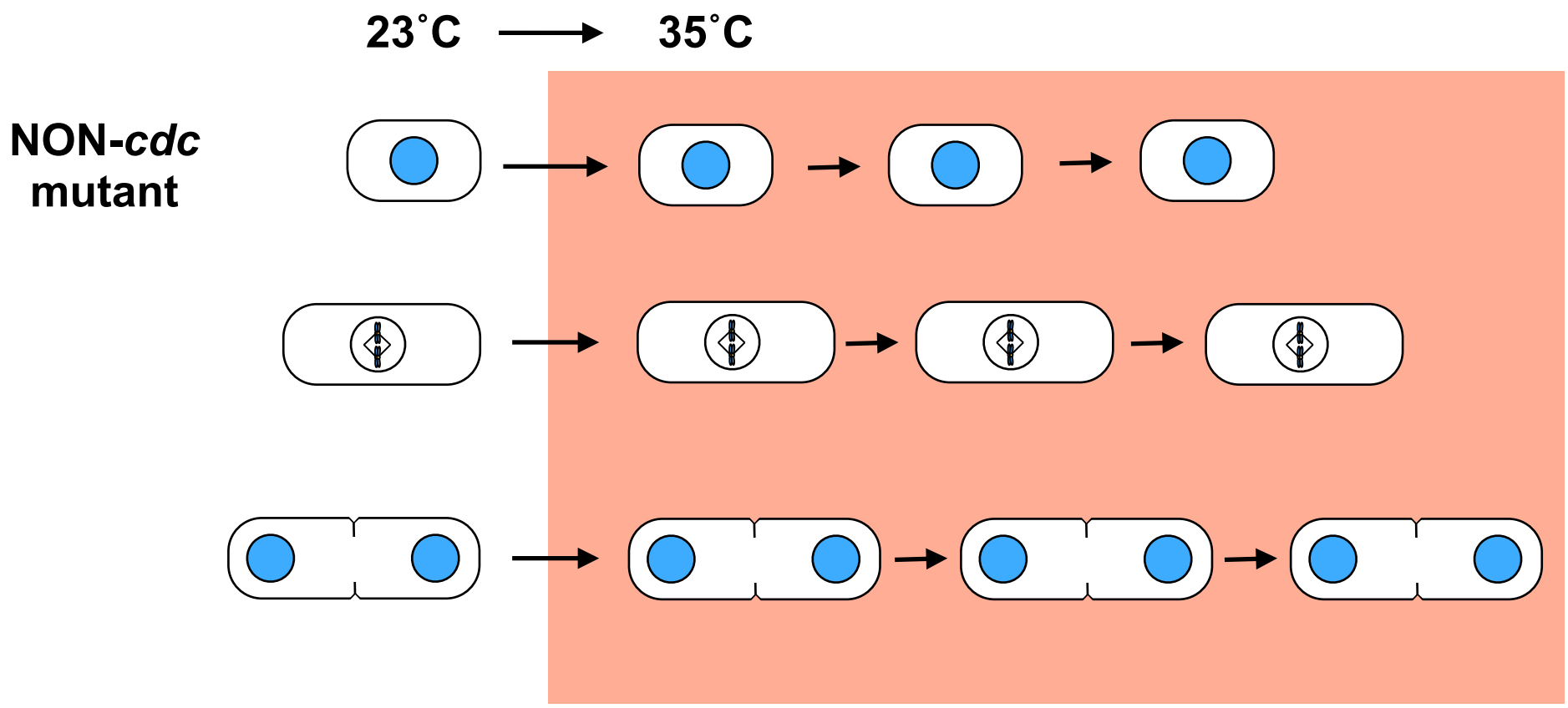
Some nomenclature issues that will be helpful for future lectures. Italics are used when referring to a gene, normal text for a protein. Budding and fission yeast differ on the use of capitals/lowercase. Most important is to remember that the same name can be a different gene in different organisms.

Isolating temperature sensitive cell division cycle (*cdc)* mutants in fission yeast



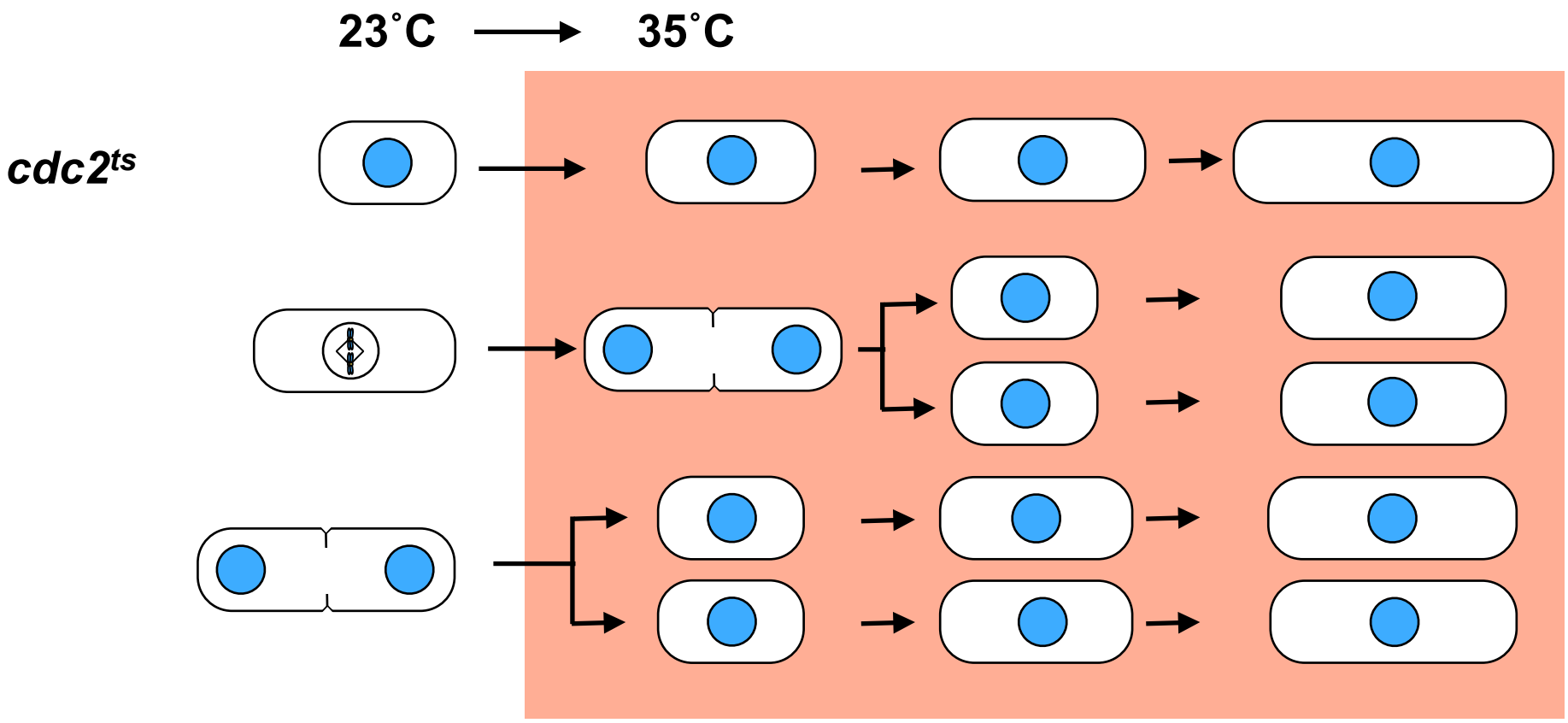
These mutants were called cdc (for cell division cycle) mutants. When wild type cells are shifted to the restrictive temperature (35˚C in this screen) they continue to divide.

Isolating temperature sensitive cell division cycle (*cdc)* mutants in fission yeast



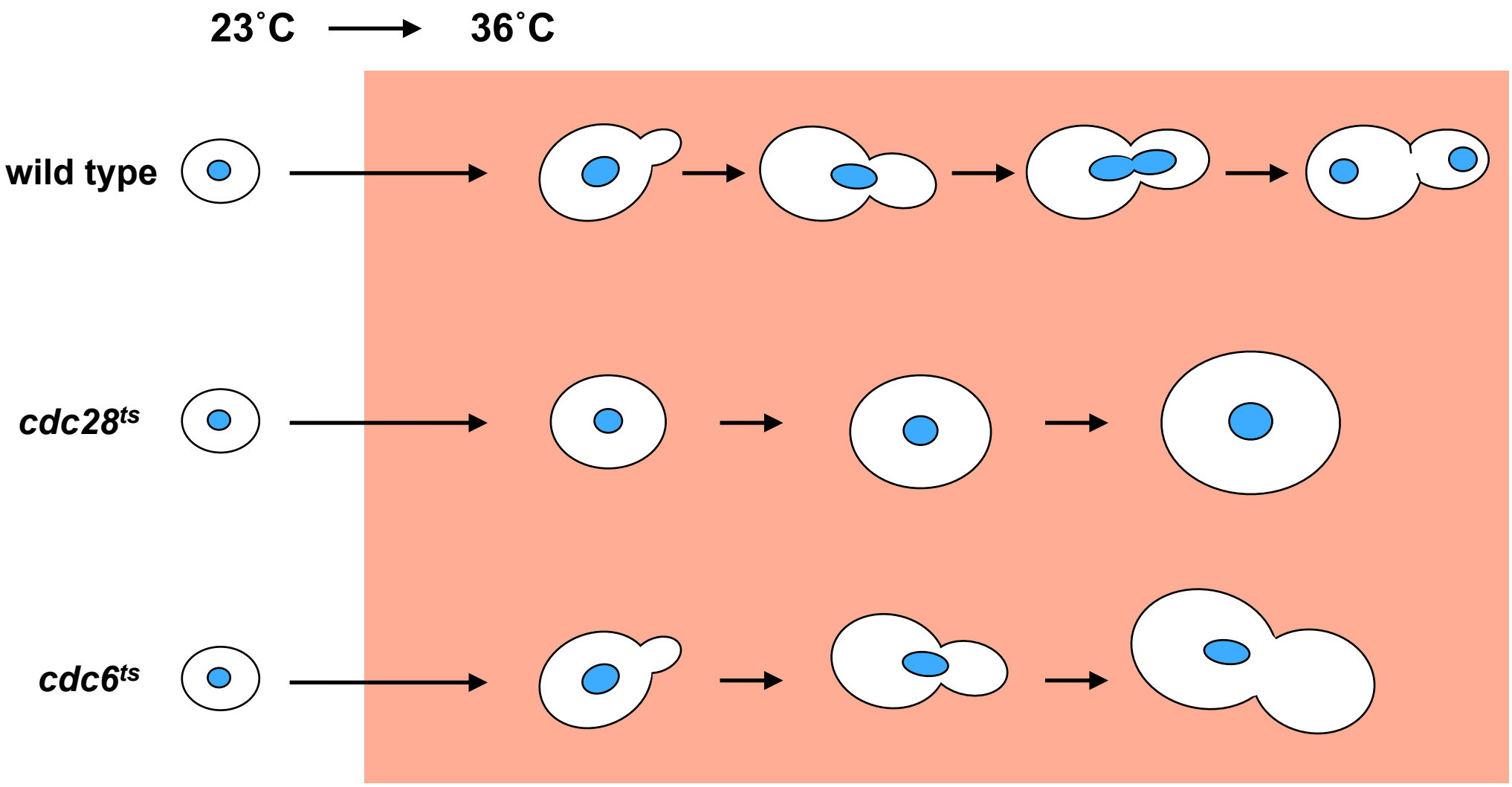
Cdc mutants are special. Most temperature sensitive mutants stop cell growth and division wherever they are in the cell cycle. For example mutations in genes involved in respiration will stop production of ATP and completely stop all cell processes.

Isolating temperature sensitive cell division cycle (*cdc)* mutants in fission yeast



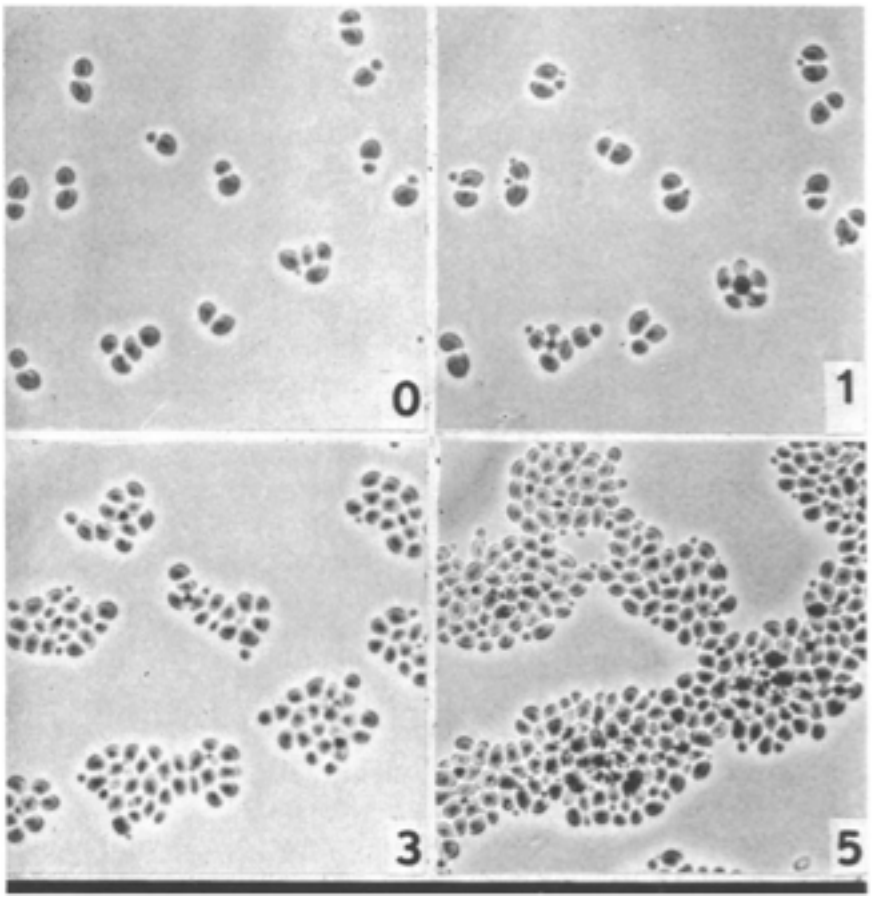
One mutant that was found is cdc2-ts. At the high temperature cdc2-ts arrest in the G2 phase of the cell cycle. Cells will continue to grow until they reach G2 and then arrest. For example, if the cells are in mitosis when shifted to 35˚C, they will complete mitosis, divide into two cells, both will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

Cell division cycle (*cdc)* mutants in budding yeast

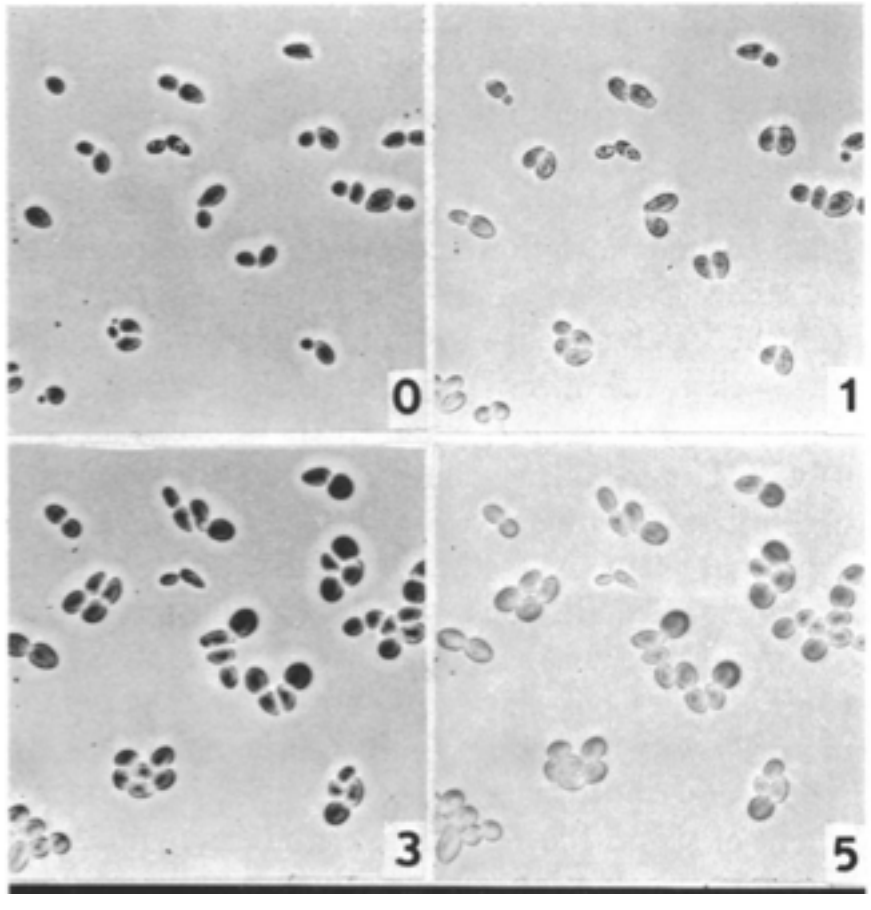


Temperature sensitive cdc mutants in budding yeast were isolated in much the same way as in fission yeast. They arrested primarily in two ways – as unbudded cells, like cdc28-ts, or as large budded cells, like cdc6-ts. Initially researchers believed the unbudded cells were arresting in G1 and the large budded cells were arresting in mitosis.

one of the original *cdc* mutants



wild type



cdc2^{ts}

Hartwell, et al., 1970

One of the original cdc mutants which arrests as a large budded cell after growth at high temperature for a few hours. Note that the same field of cells are imaged at each time. Wild type cells grow a lot during this time.

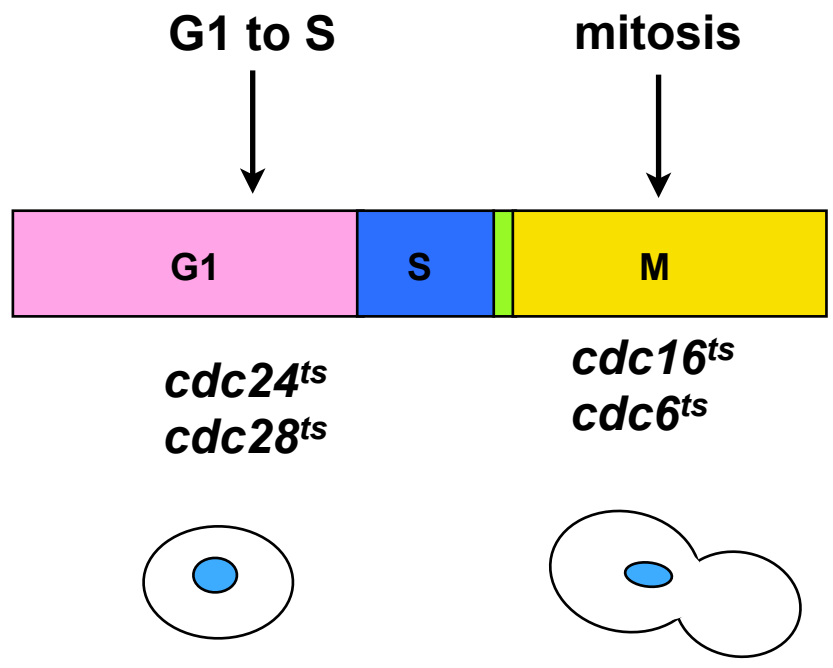
Each mutant was analyzed in a few ways

1. Termination or arrest point

2. Ability to perform events in the cell cycle that are easily measured

3. Execution point

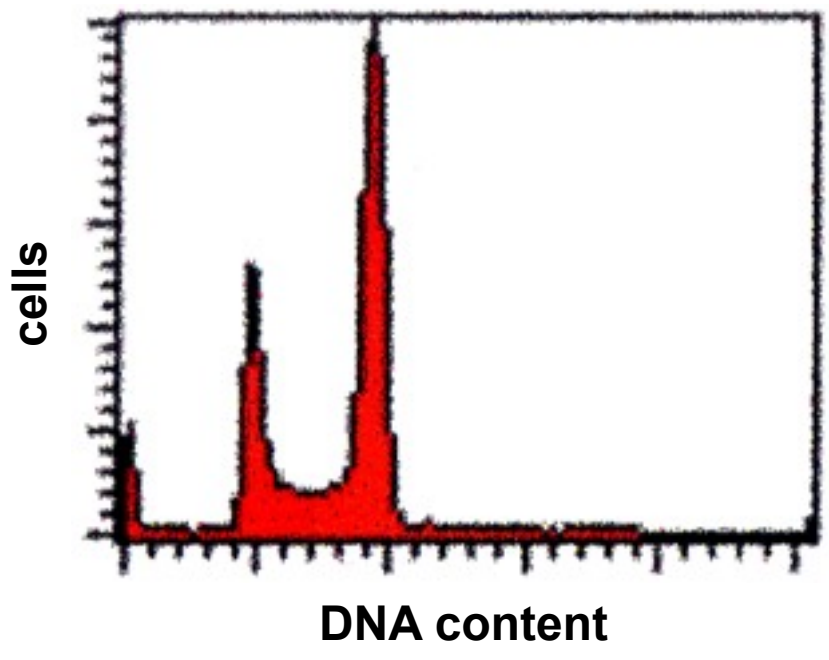
Arrest point of a mutant is its terminal arrest point. When simply by looking at the morphology of cells, there are two basic arrest points



Clearly the mutants that arrested as unbudded cells had a good likelihood of regulating START, but what about all the mutants that arrested as large budded cells? Could they also regulate START? A clue that some might came from an analysis of the "execution point" of the mutants. Before I discuss the execution point, we need to define the "arrest point" or "termination point" - this is where the mutants arrest. As I showed before some mutants arrest point was apparently at START (cdc24-ts and cdc28-ts), while others arrested as large budded cells, apparently in mitosis (cdc6-ts, cdc31-ts and cdc16-ts).

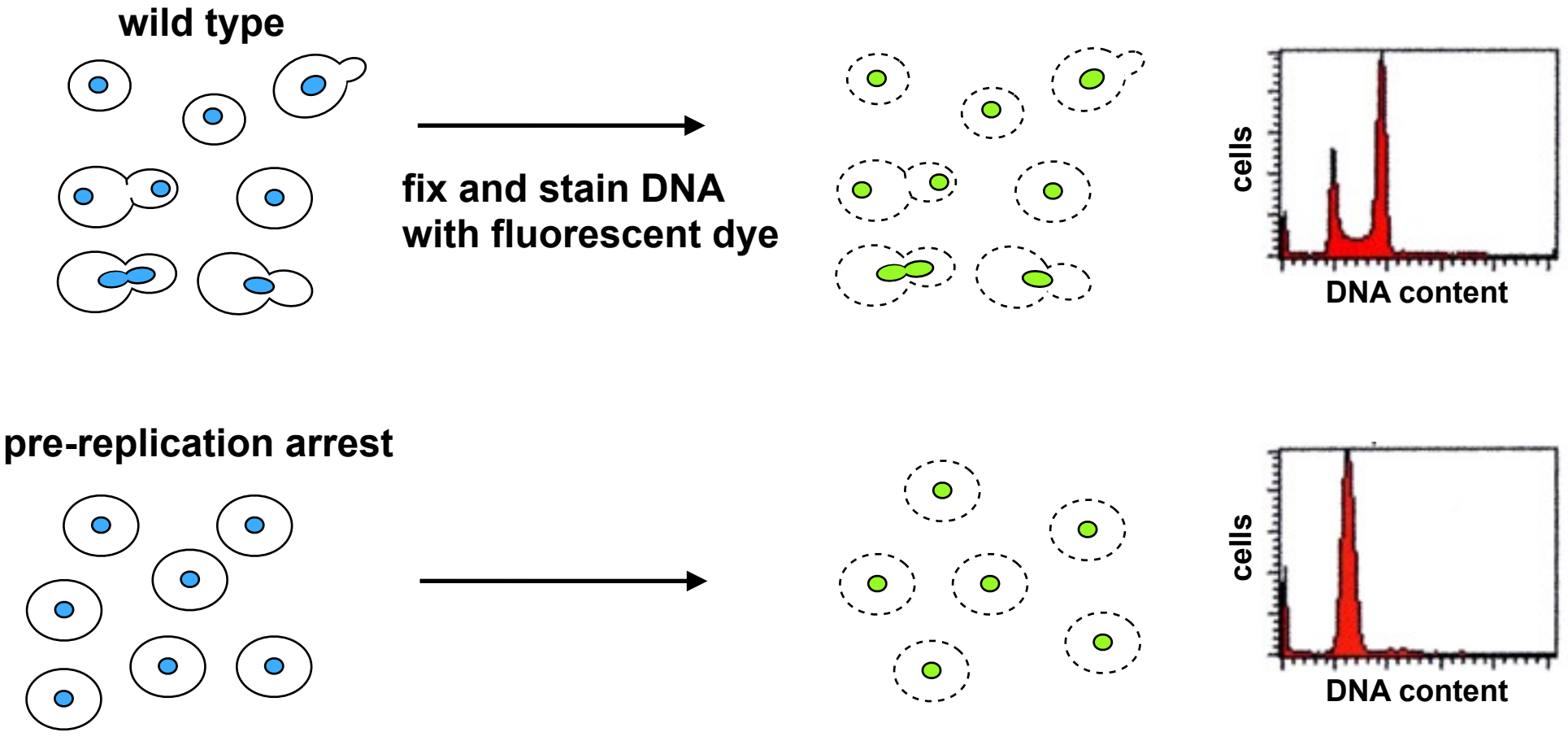
Other events in the cell cycle were determined using other assays

S-phase: BrdU labeling/FACS analysis



You are probably more familiar with FACS to analyze DNA content, but early experiments used other techniques that relied on incorporation of radioactive nucleotides or nucleotides of different masses (Heavy/Light labeling - similar to what was used for showing DNA replication is semi-conservative).

Fluorescence Activated Cell Sorting (FACS)

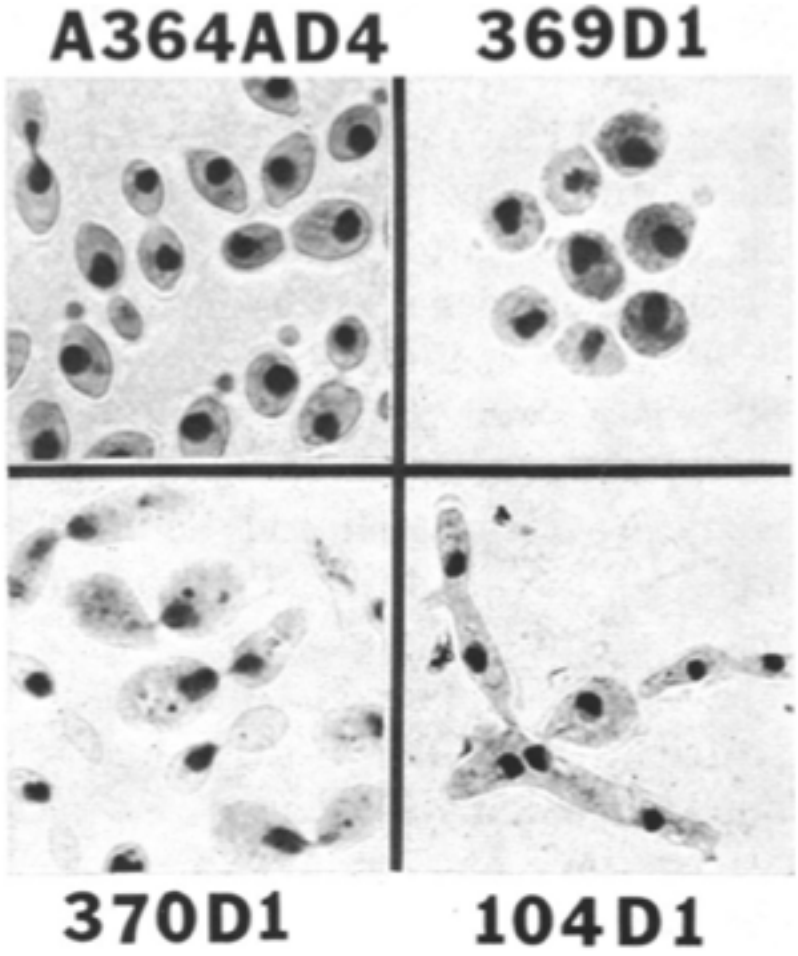


FACS (fluorescence activated cell sorting) can be used to measure DNA content in cells. Briefly, the cells are fixed and then the DNA is stained with a fluorescent dye. The FACS machine uses the amount of fluorescence as a measure of the DNA content. If the cells are growing asynchronously, the FACS plot will show cells in G1 (with a 1N DNA content – these are haploid cells), cells in mitosis (with a 2N content), and cells undergoing DNA replication (the cells between the two peaks). If cells are arrested before DNA replication they'll arrest uniformly with a 1N DNA content, if they are arrested after DNA replication, they arrest uniformly with a 2N DNA content.

Other events in the cell cycle were determined using other assays

Nuclear division: DNA stains and microscopy

wild type



cdc1^{ts}

cdc2^{ts}

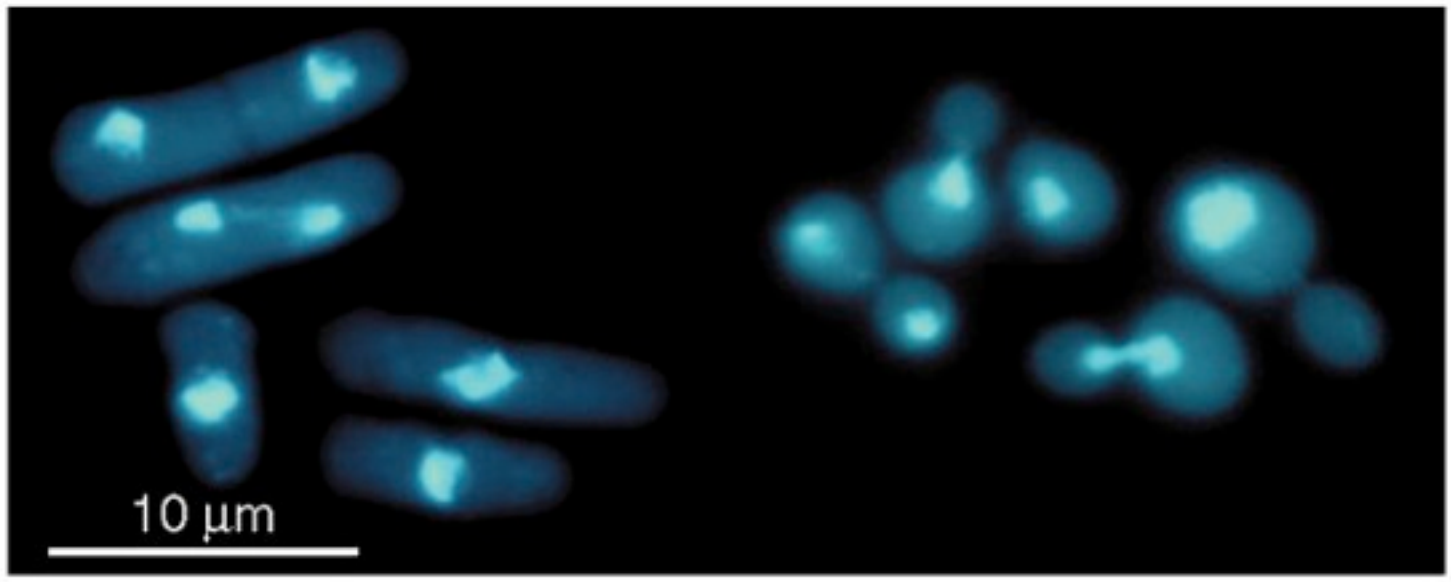
cdc3^{ts}

Hartwell, et al., 1970

DNA stains can be used with light microscopy – here Giemsa stain is used in the original cdc paper. Many subtleties of nuclear position/migration could be determined using these stains.

Other events in the cell cycle were determined using other assays

Nuclear division: DNA stains and microscopy



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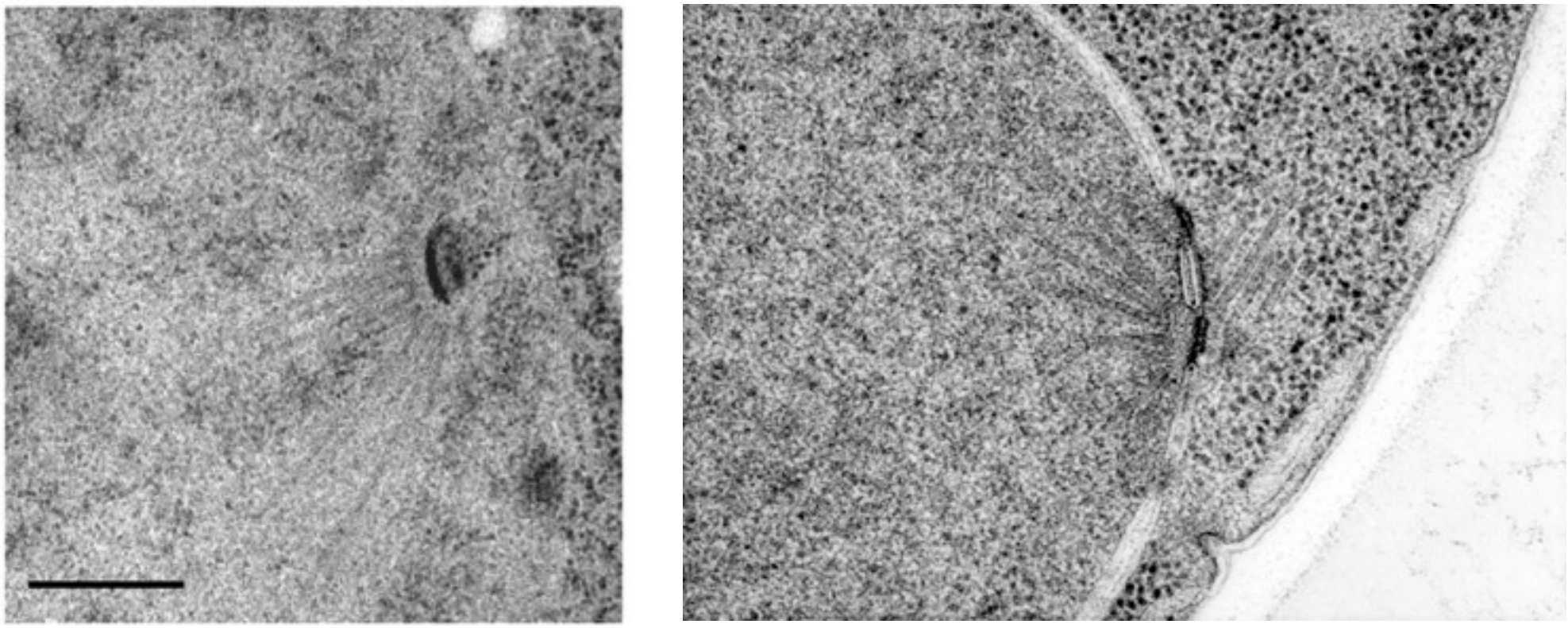
DAPI (4',6-diamidino-2-phenylindole) is the most common

Now people use DAPI and fluorescence microscopy. Here you see fission yeast on the left and budding yeast on the right.

Other events in the cell cycle were determined using other assays

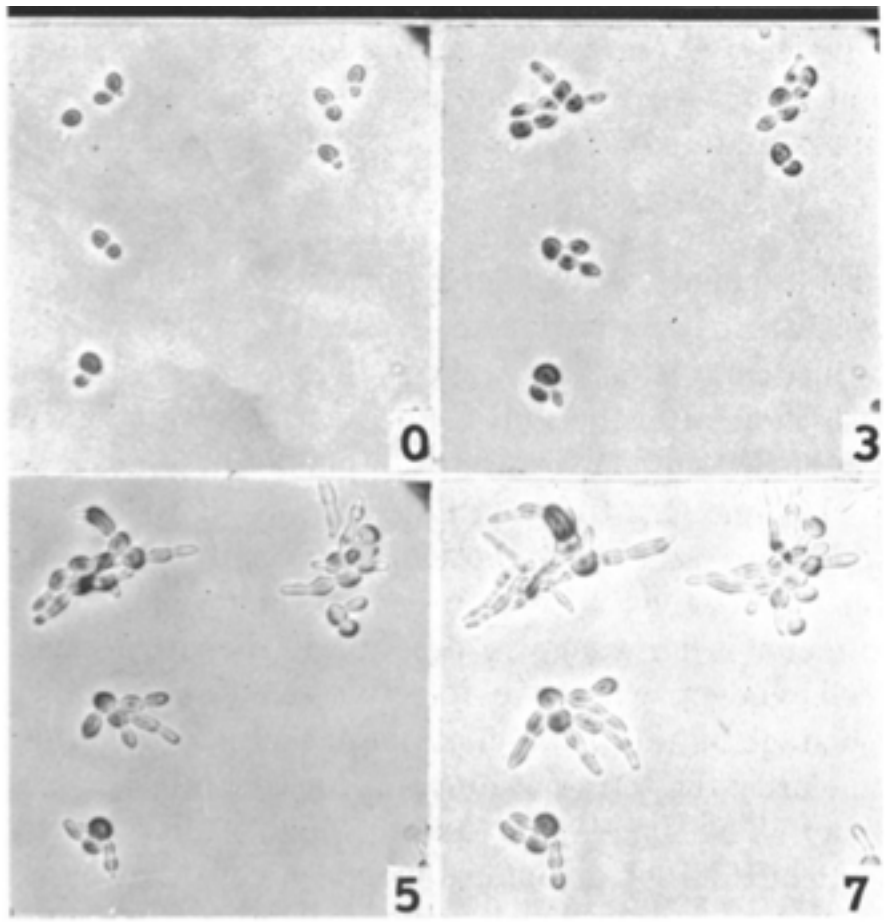
spindle pole duplication: electron microscopy



The yeast SPB or centrosome can be seen by EM. Its a tri-laminar structure with microtubules emanating from it. On the left is one SPB probably in a mitotic cell. On the right is a cell with duplicated but unseparated SPBs. The bottom SPB has both cytoplasmic and nuclear microtubules, and it is linked to the top SPB by the half-bridge. The top SPB is not yet functional.

Other events in the cell cycle were determined using other assays

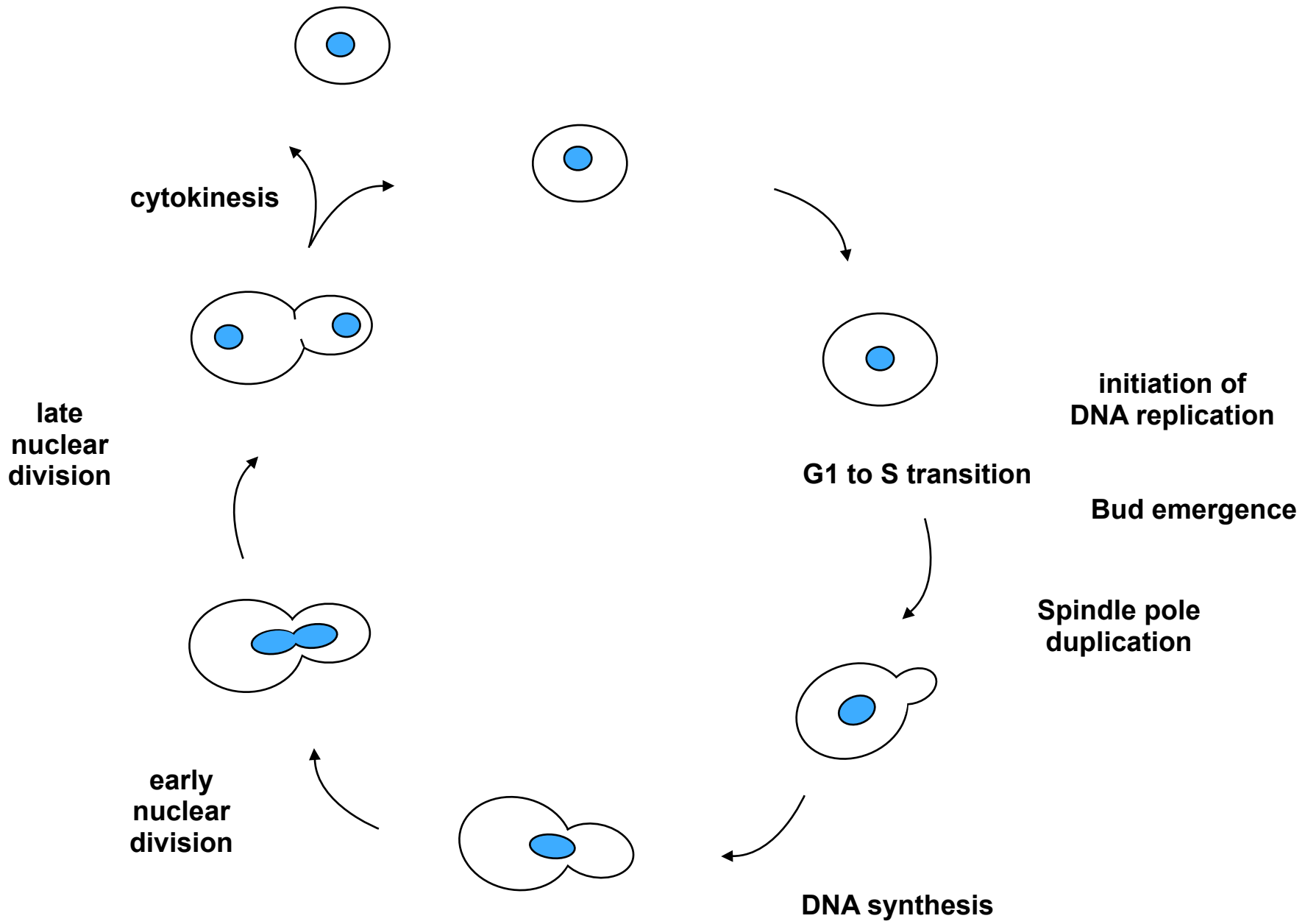
cytokinesis: simply looking



cdc3^{ts}

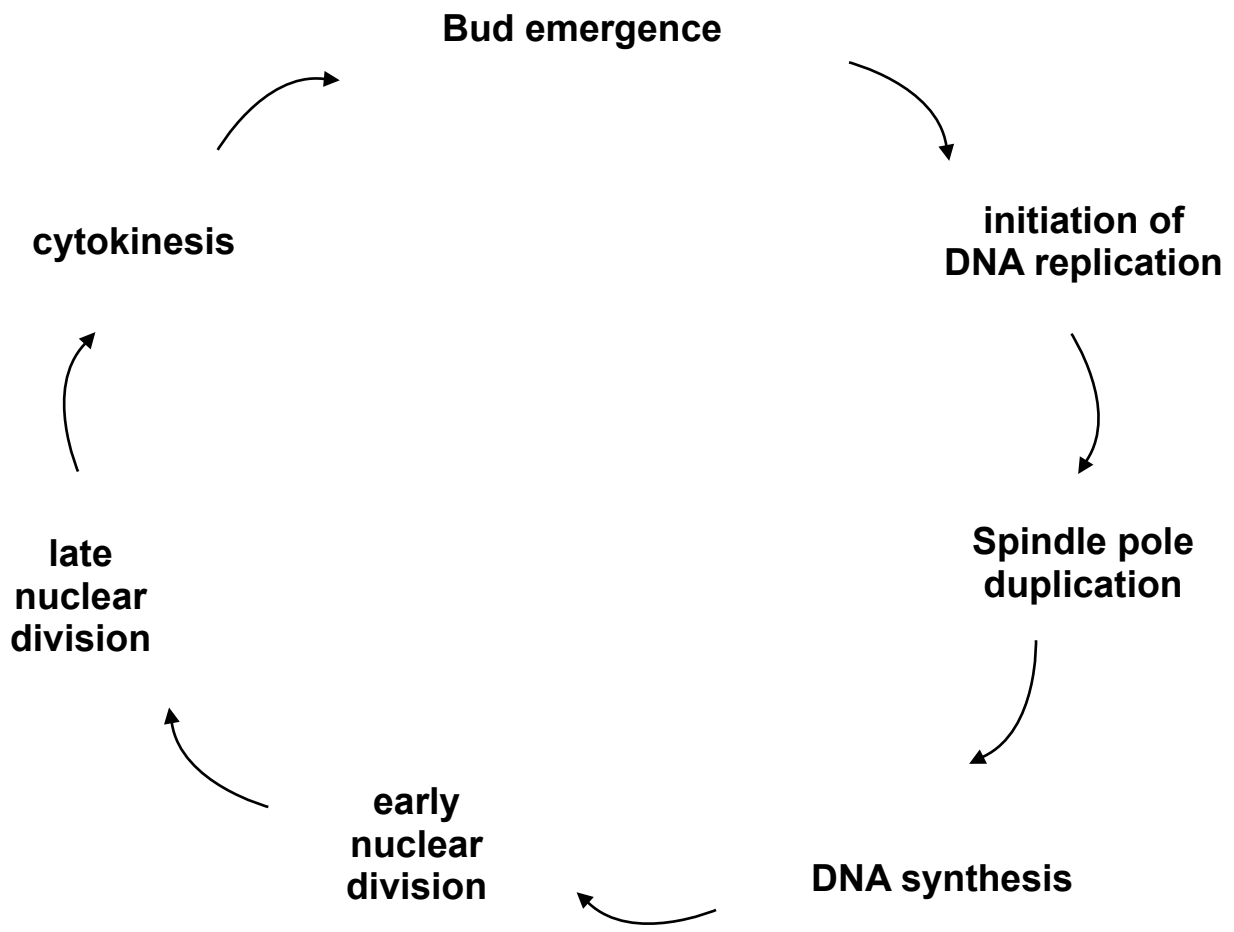
Mutants defective in cytokinesis form chains of cells that eventually lyse. They can't be separated from one another, and if their cell walls are degraded (with a glucanase) they still form chains. They are not strictly cdc because they still grow and divide, but they do die at the restrictive temperature and their morphology was so distinctive that they were included in the original collection of cdc mutants (cdc3, cdc10, cdc11 and cdc12).

Collection of *cdc* mutants analyzed for their ability to perform a subset of events

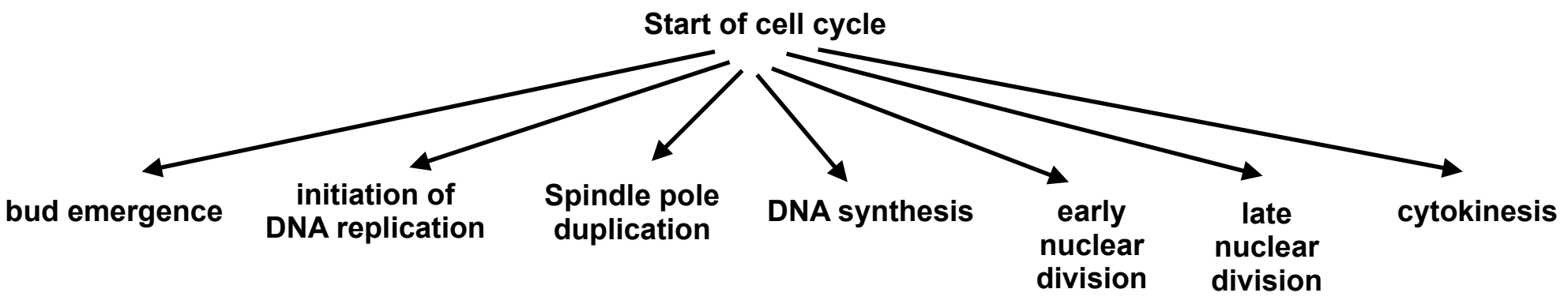


Many of the mutants arrested as unbudded cells, and researchers initially focused on trying understand how these mutants regulated the G1 to S transition, or START. As I mentioned earlier, three events happen at START in budding yeast: DNA replication, bud emergence and spindle pole duplication.

Are these events dependent on one another?



Or independent of one another?



A third version

dependent pathway model

A → B → C → D → E → F

independent pathways model

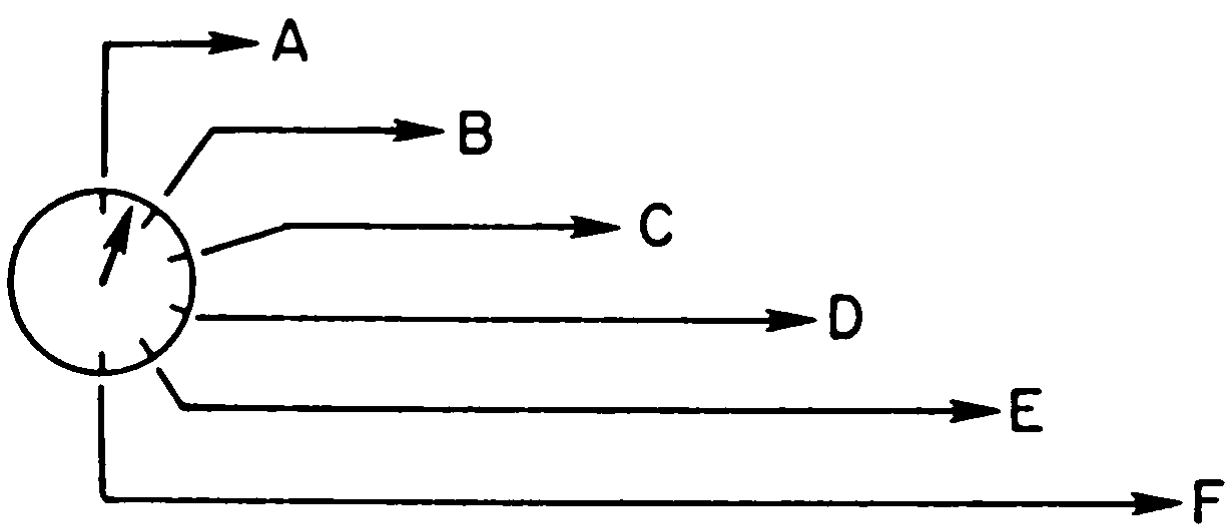


Fig. 2. Two models to account for the ordering of cell cycle events.

Hartwell, et al., 1974

These two options are very similar to the two depicted in this model.

Examples of mutants that are defective in each of these processes

bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	***cdc6^{ts}***	***cdc31^{ts}***	***cdc21^{ts}***	***cdc16^{ts}***	***cdc15^{ts}***	***cdc3^{ts}***

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bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	***cdc6^{ts}***	***cdc31^{ts}***	***cdc21^{ts}***	***cdc16^{ts}***	***cdc15^{ts}***	***cdc3^{ts}***

All events occur except cytokinesis

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bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	***cdc6^{ts}***	***cdc31^{ts}***	***cdc21^{ts}***	***cdc16^{ts}***	***cdc15^{ts}***	***cdc3^{ts}***
					↓	↓
					All events occur except cytokinesis, late nuclear division	**All events occur except cytokinesis**

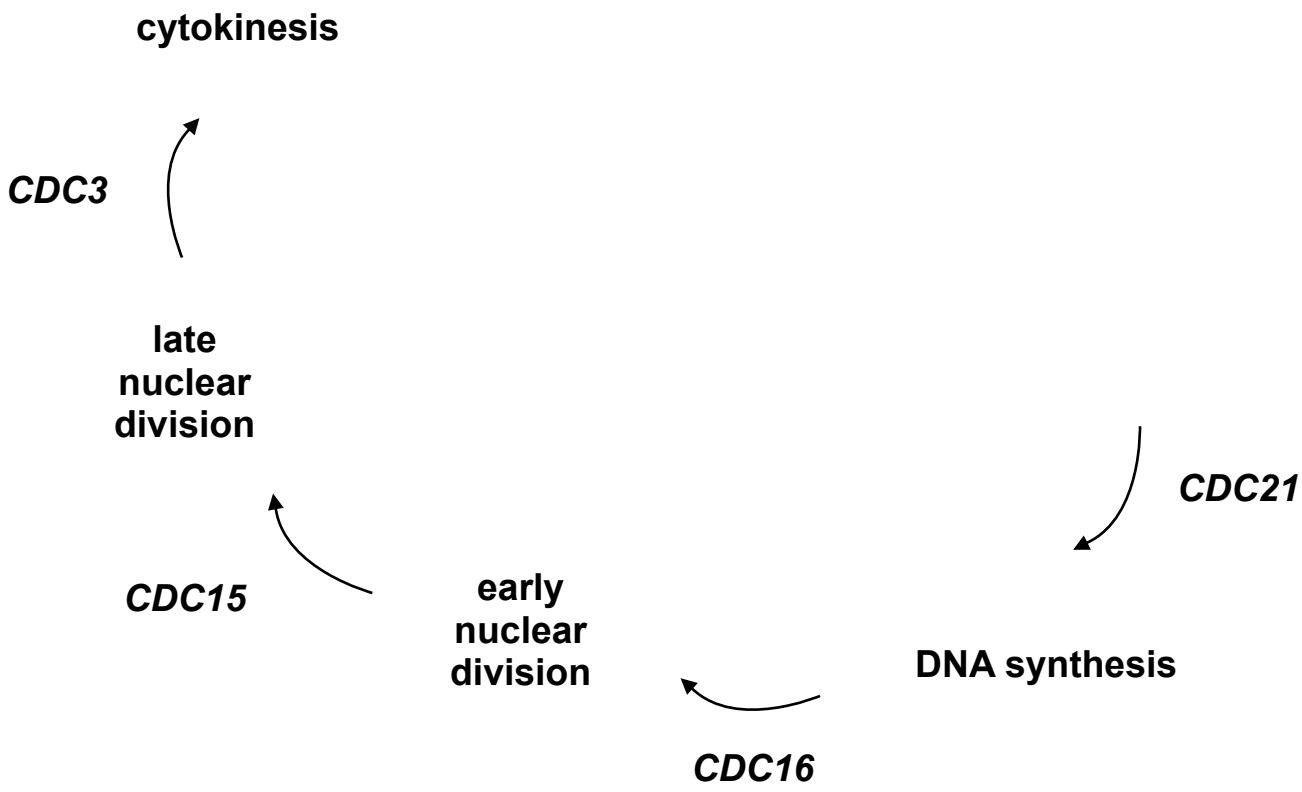
Examples of mutants that are defective in each of these processes

bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	*cdc6^{ts}*	*cdc31^{ts}*	*cdc21^{ts}*	*cdc16^{ts}*	*cdc15^{ts}*	*cdc3^{ts}*
				↓	↓	↓
				All events occur except cytokinesis, late nuclear division, early nuclear division	All events occur except cytokinesis, late nuclear division	All events occur except cytokinesis

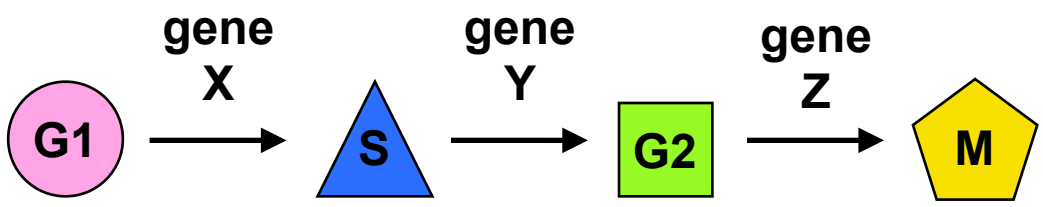
Examples of mutants that are defective in each of these processes

bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	*cdc6^{ts}*	*cdc31^{ts}*	*cdc21^{ts}*	*cdc16^{ts}*	*cdc15^{ts}*	*cdc3^{ts}*
			↓	↓	↓	↓
			All events occur except cytokinesis, late nuclear division, early nuclear division, DNA synthesis	All events occur except cytokinesis, late nuclear division, early nuclear division	All events occur except cytokinesis, late nuclear division	All events occur except cytokinesis

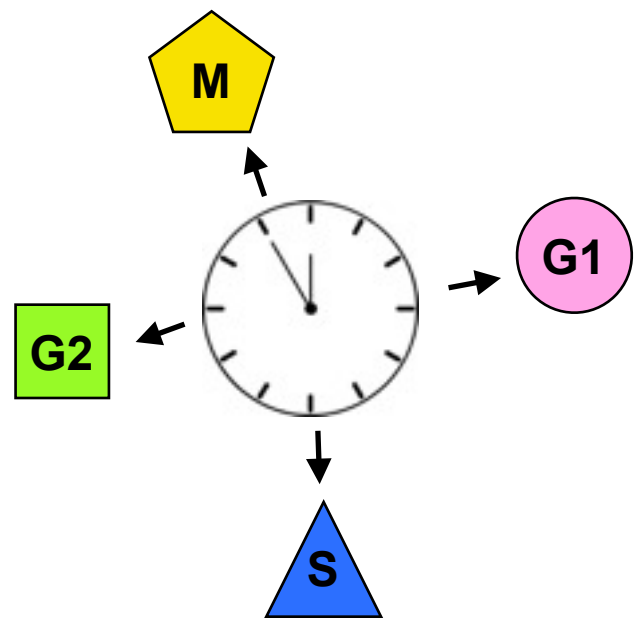
Completion of these events appear to depend on one another



domino theory



clock theory



Which model is supported by this data?

Examples of mutants that are defective in each of these processes

bud emergence	initiation of DNA replication	Spindle pole duplication	DNA synthesis	early nuclear division	late nuclear division	cytokinesis
cdc24^{ts}	*cdc6^{ts}*	*cdc31^{ts}*	*cdc21^{ts}*	*cdc16^{ts}*	*cdc15^{ts}*	*cdc3^{ts}*
			↓	↓	↓	↓
			All events occur except cytokinesis, late nuclear division, early nuclear division, DNA synthesis	All events occur except cytokinesis, late nuclear division, early nuclear division	All events occur except cytokinesis, late nuclear division	All events occur except cytokinesis

the earliest events, however are independent of each other.

These events are independent

bud emergence

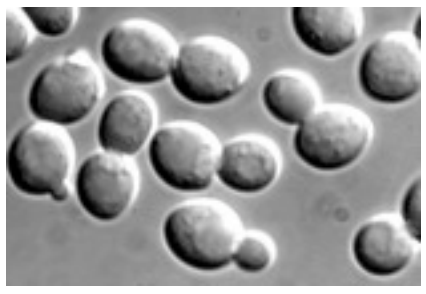
cdc24^{ts}

↓

DNA replication

NO budding

Spindle pole duplication



initiation of DNA replication

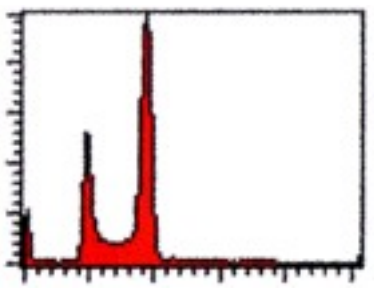
cdc6^{ts}

↓

NO DNA replication

Budding

Spindle pole duplication



Spindle pole duplication

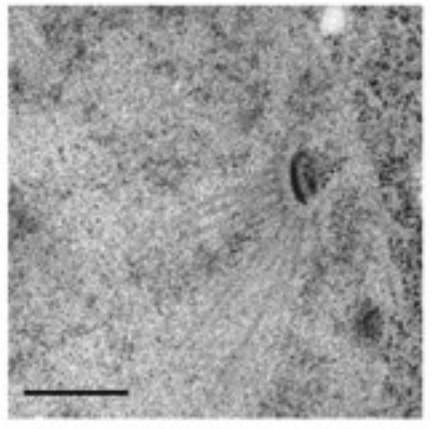
cdc31^{ts}

↓

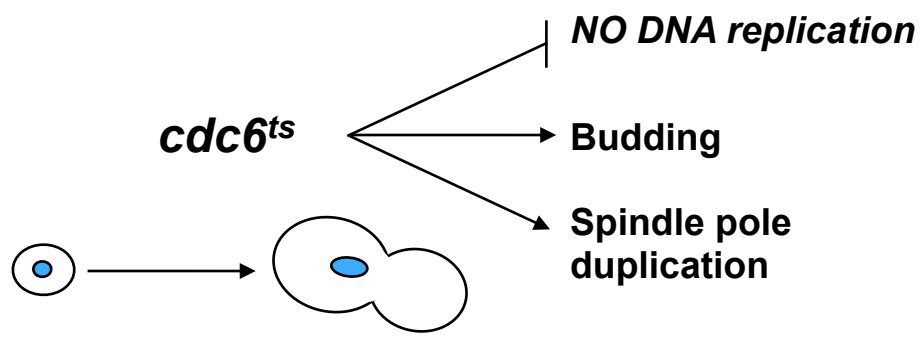
DNA replication

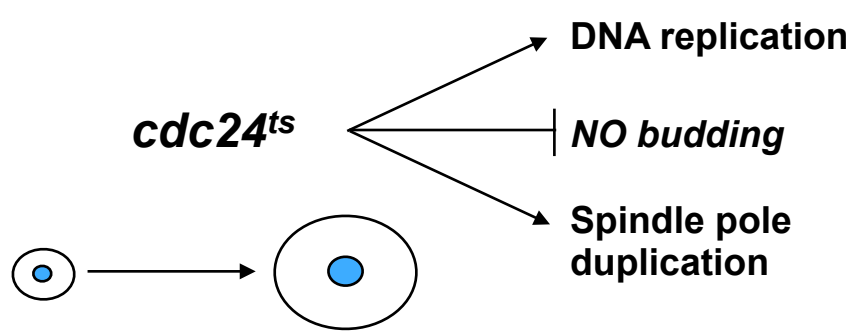
Budding

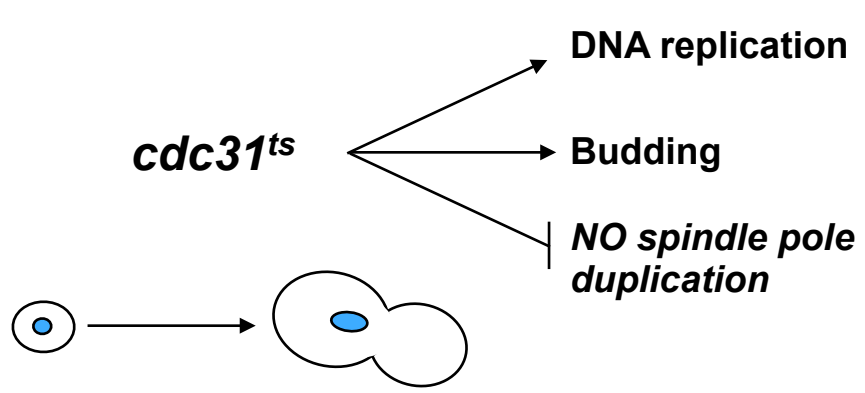
NO Spindle pole duplication



the earliest events, however are independent of each other.

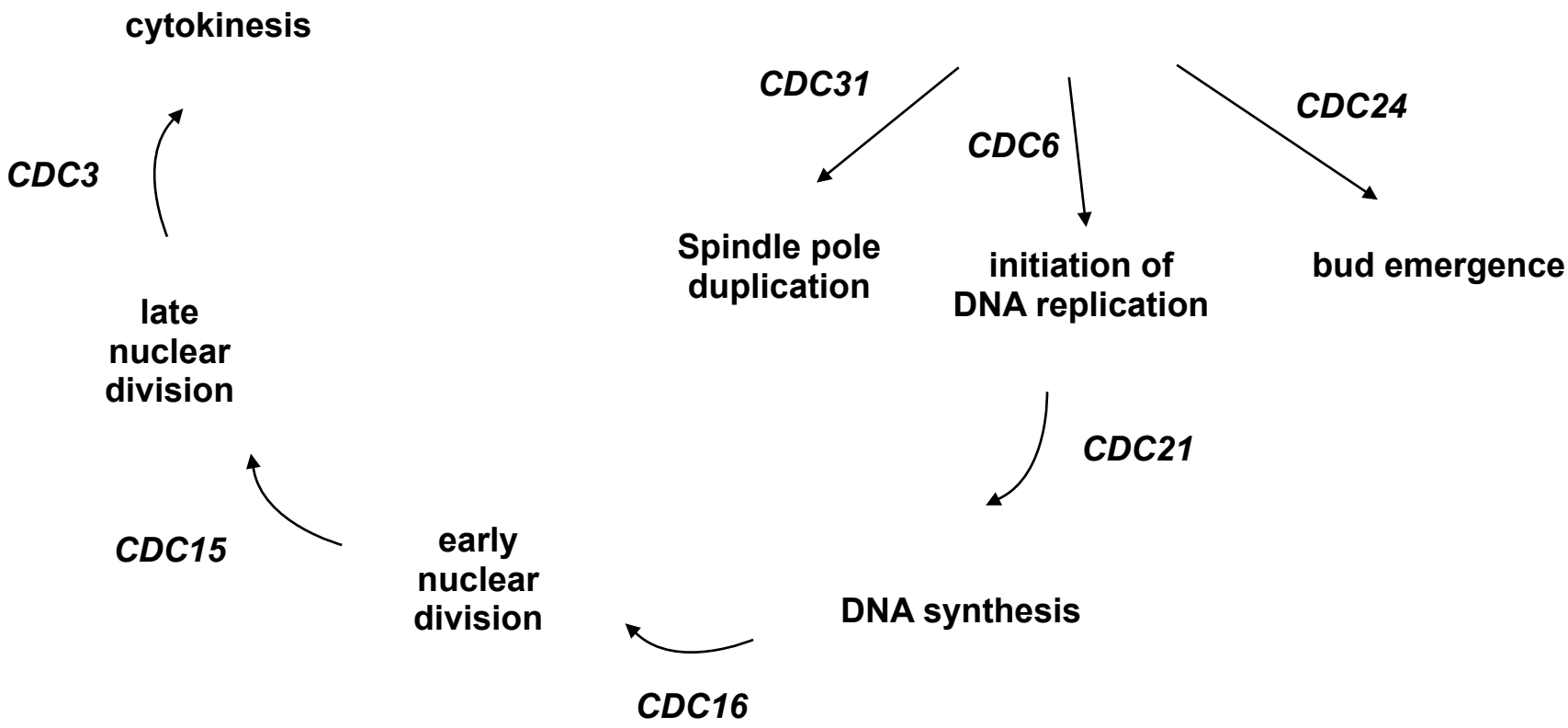






When three mutants with execution points in G1 (cdc6-ts, cdc24-ts and cdc31-ts) were analyzed, they each were defective in one of the three parameters. cdc6-ts does not undergo DNA replication, cdc24-ts does not bud, and cdc31-ts does not duplicate its spindle pole.

What happens at the start of the cell cycle?



cdc28^{ts} was a special mutant

cdc28^{ts}

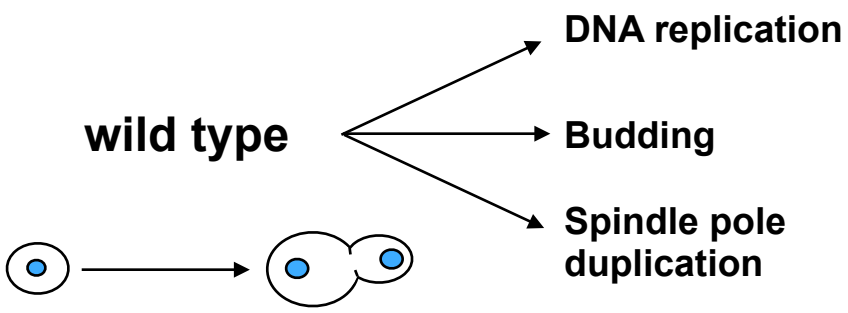
↓

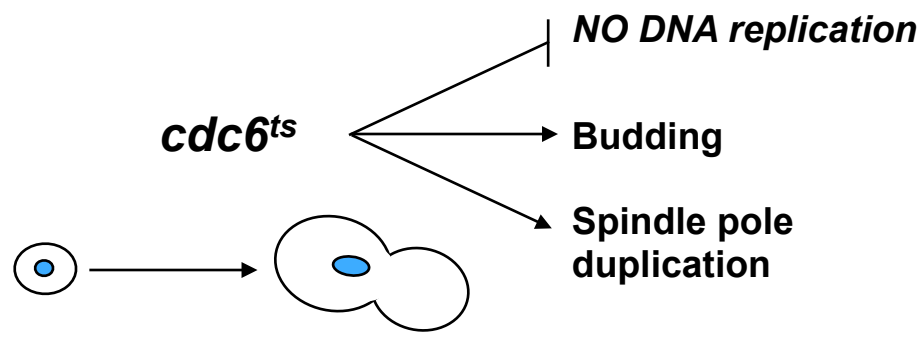
NO DNA replication

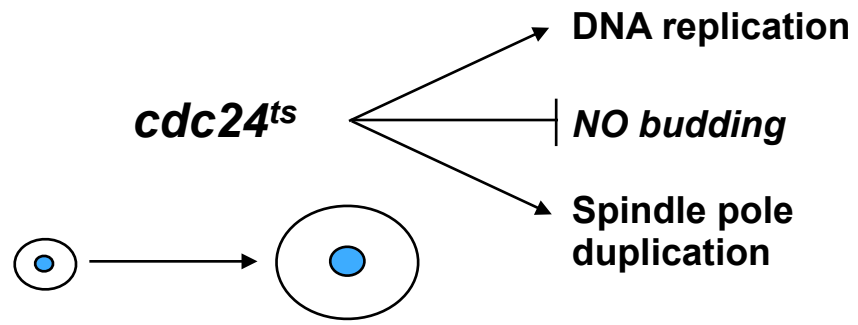
NO Budding

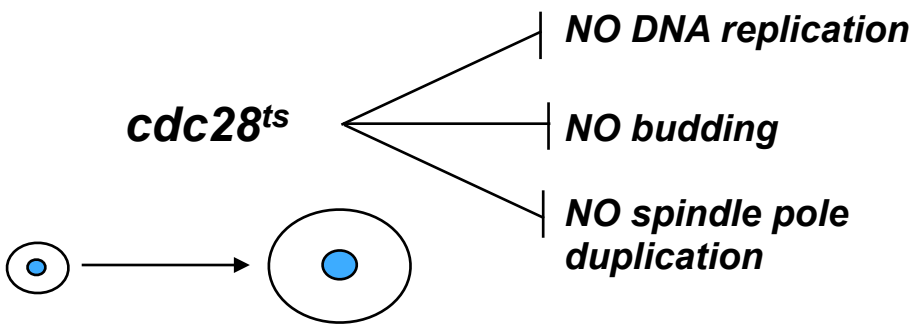
NO Spindle pole duplication

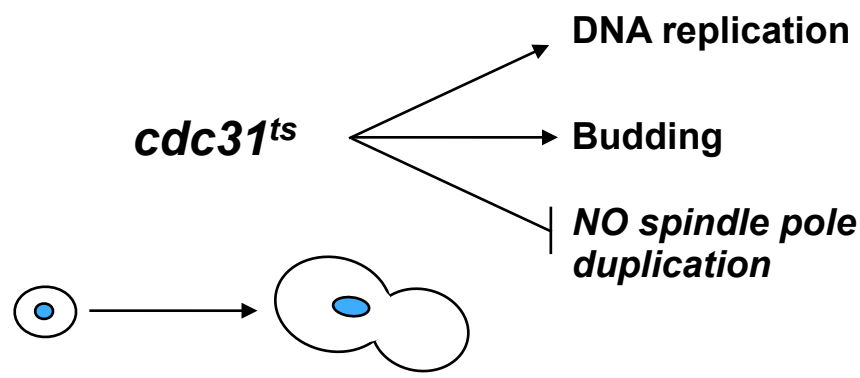
cdc28 was special because it blocked all three of these processes at the beginning of the cell cycle.


wild type
DNA replication
Budding
Spindle pole duplication

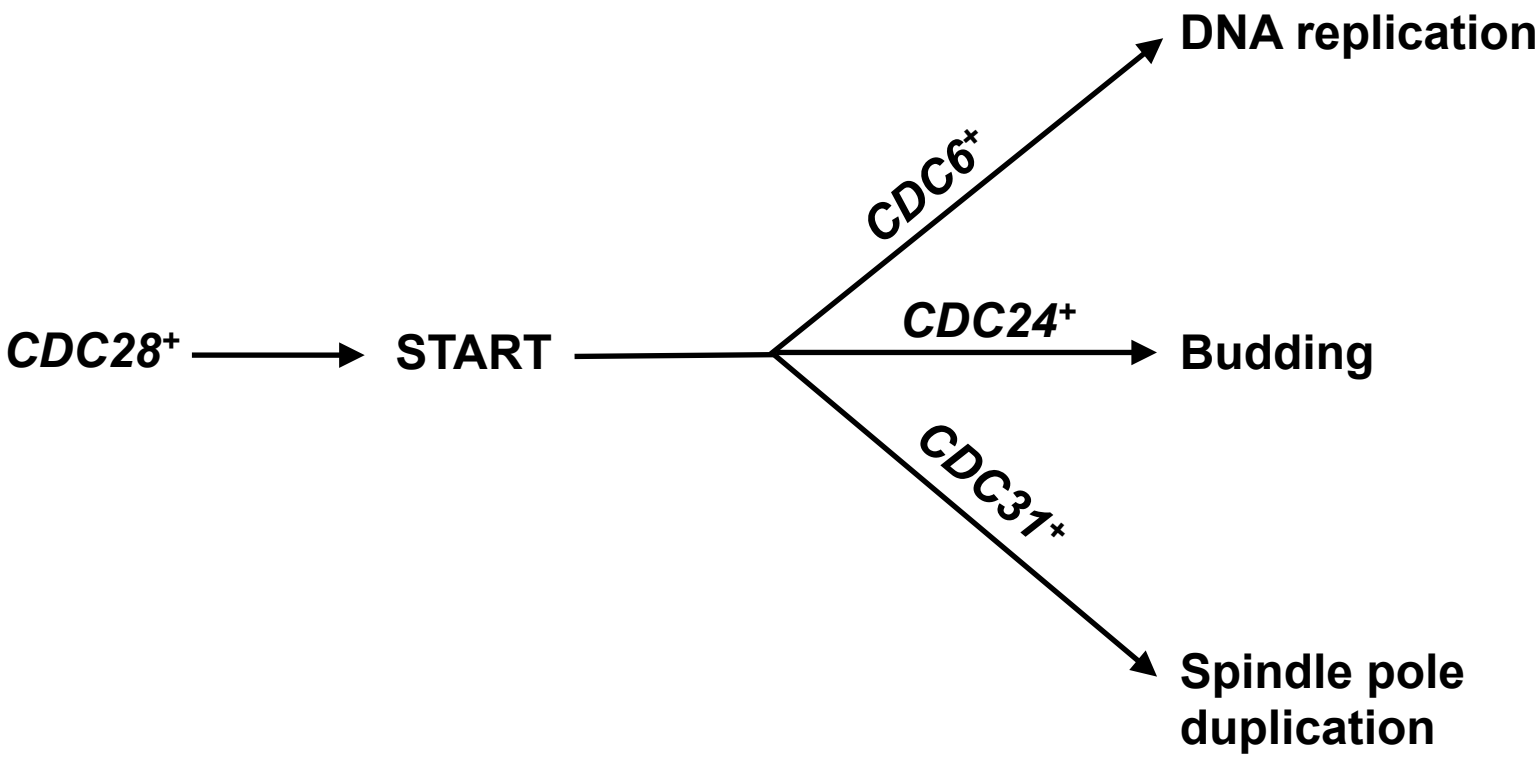

cdc6ts
NO DNA replication
Budding
Spindle pole duplication


cdc24ts
DNA replication
NO budding
Spindle pole duplication


cdc28ts
NO DNA replication
NO budding
NO spindle pole duplication


cdc31ts
DNA replication
Budding
NO spindle pole duplication

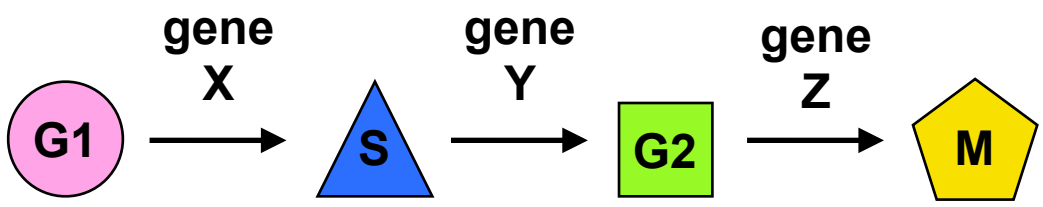
CDC28 defines START



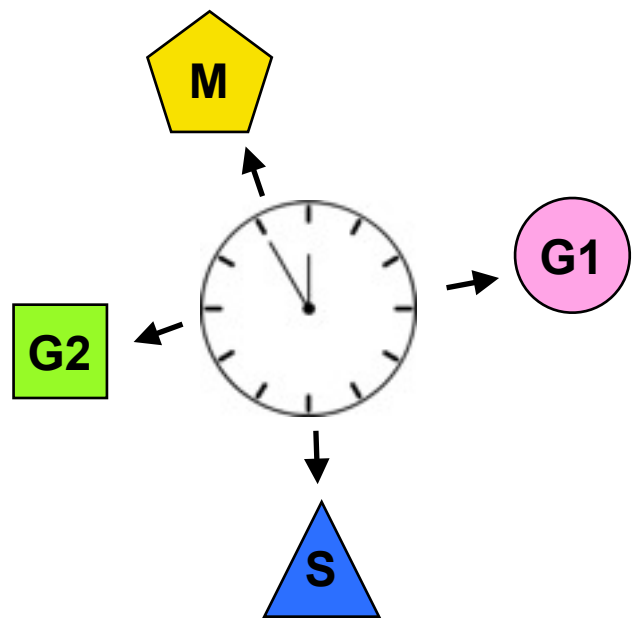
These experiments led to the model that CDC28 regulates START (and all of its downstream events), while CDC6, CDC24 and CDC31 independently regulate the three downstream events of START. CDC28 thus clearly has a special role as an important regulator of the G1 to S transition.

Note that in budding yeast the wild type gene name is indicated in capital letters (CDC28) while the mutant is indicated in lowercase letters (cdc28-ts). This is different from fission yeast where both wild type and mutant are indicated in lower case, but the wild type with a + (cdc2^{+}) and the mutant with an allele number (cdc2-ts or cdc2-1 or cdc2-3wD).

domino theory



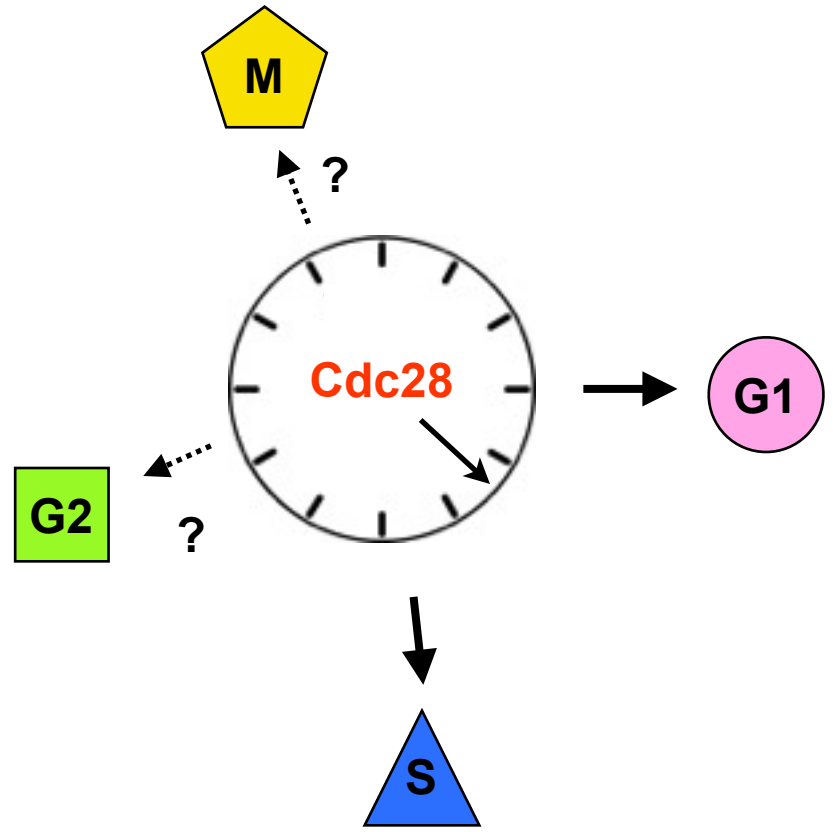
clock theory



Which model is supported by the cdc28 mutant?

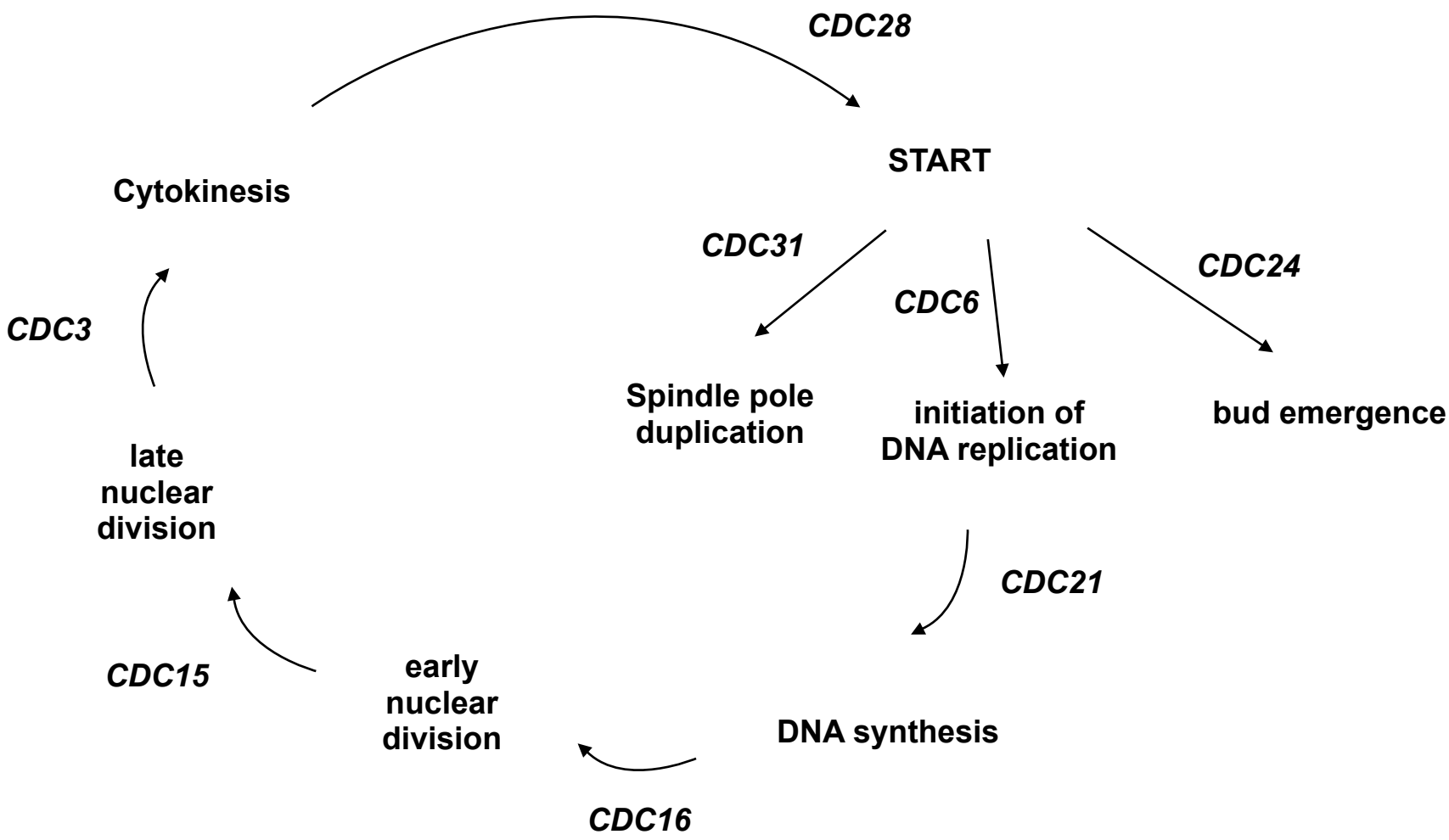
Cdc28 is required for triggering three events that occur at G1 to S

clock theory



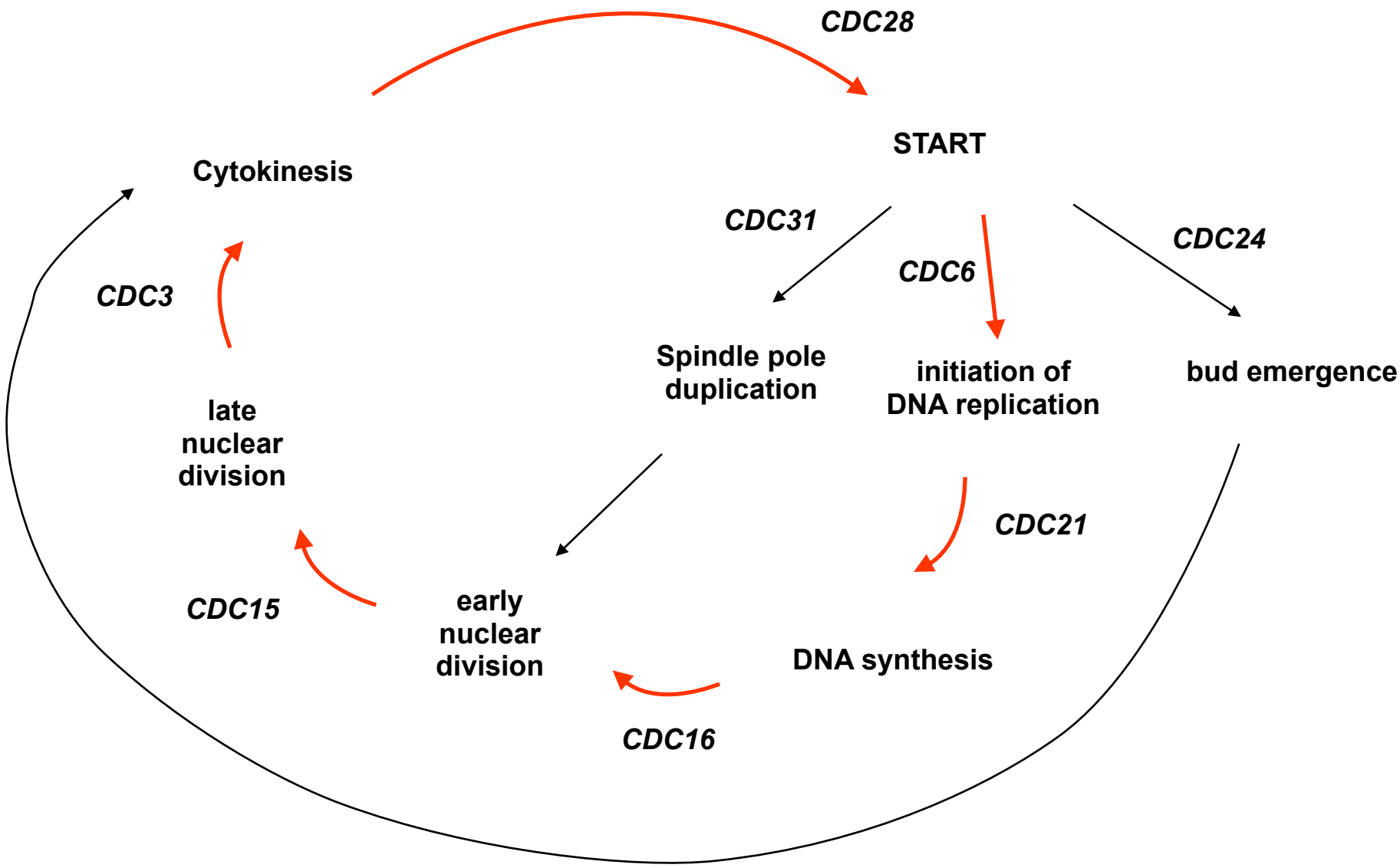
Cdc28, like Cdc2 appears to be a master regulator of cell cycle transitions, here the transition form G1 to S. But as with the analysis of Cdc2, does it only regulate one cell cycle transition?

CDC28 defines START

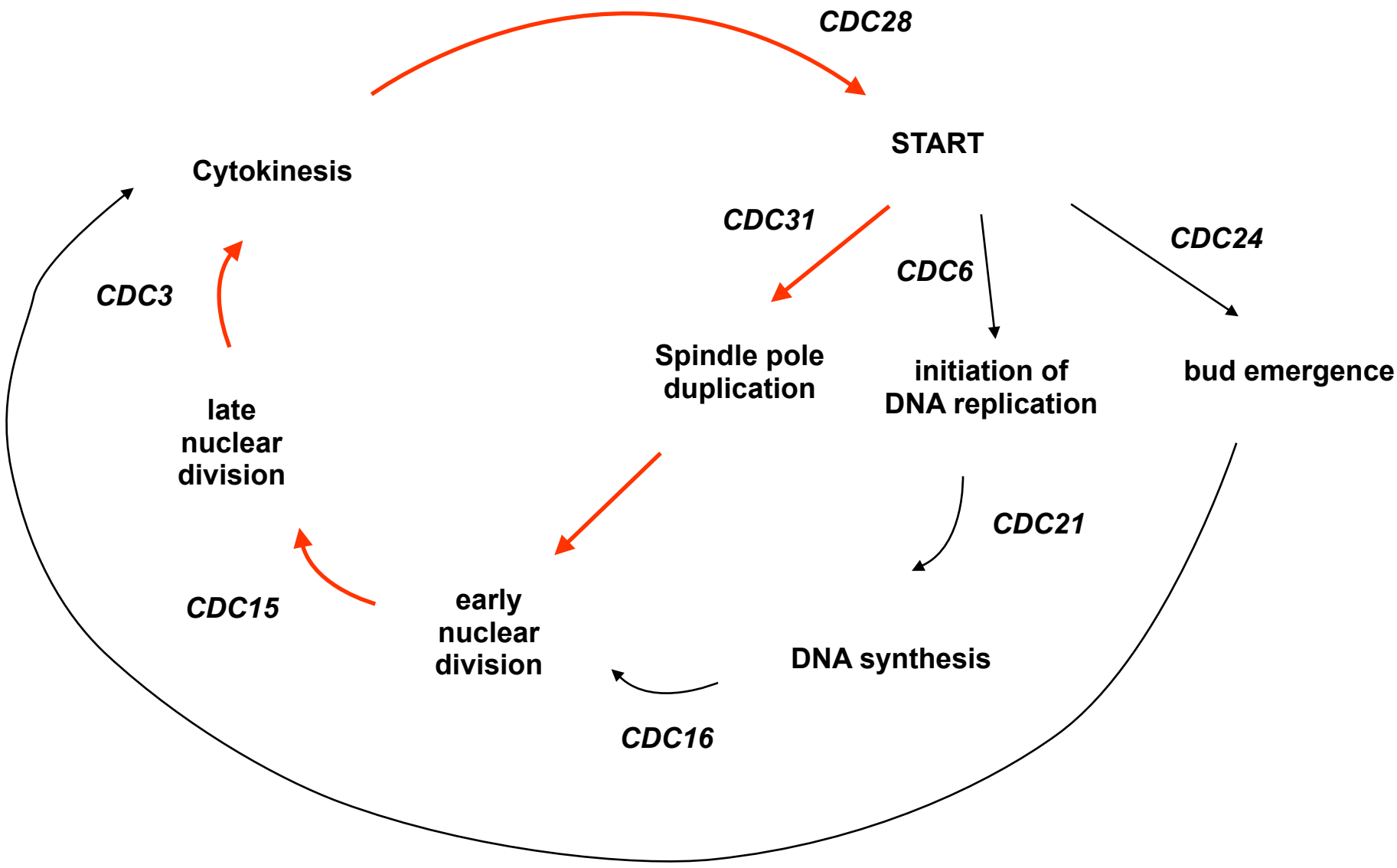


The cdc28 mutant was used to define the start of the cell cycle or START. START is similar to the mammalian restriction point. It is the time in the cell cycle where many decisions are made - to enter the cell cycle, to enter meiosis (for diploids), to mate (for haploids), to stop dividing if nutrients are scarce, to enter a different developmental program (call pseudo-hyphal growth). In mammalian cells it where decisions about differentiation are made.

Three inter-connected cycles in budding yeast
a DNA replication/mitosis cycle

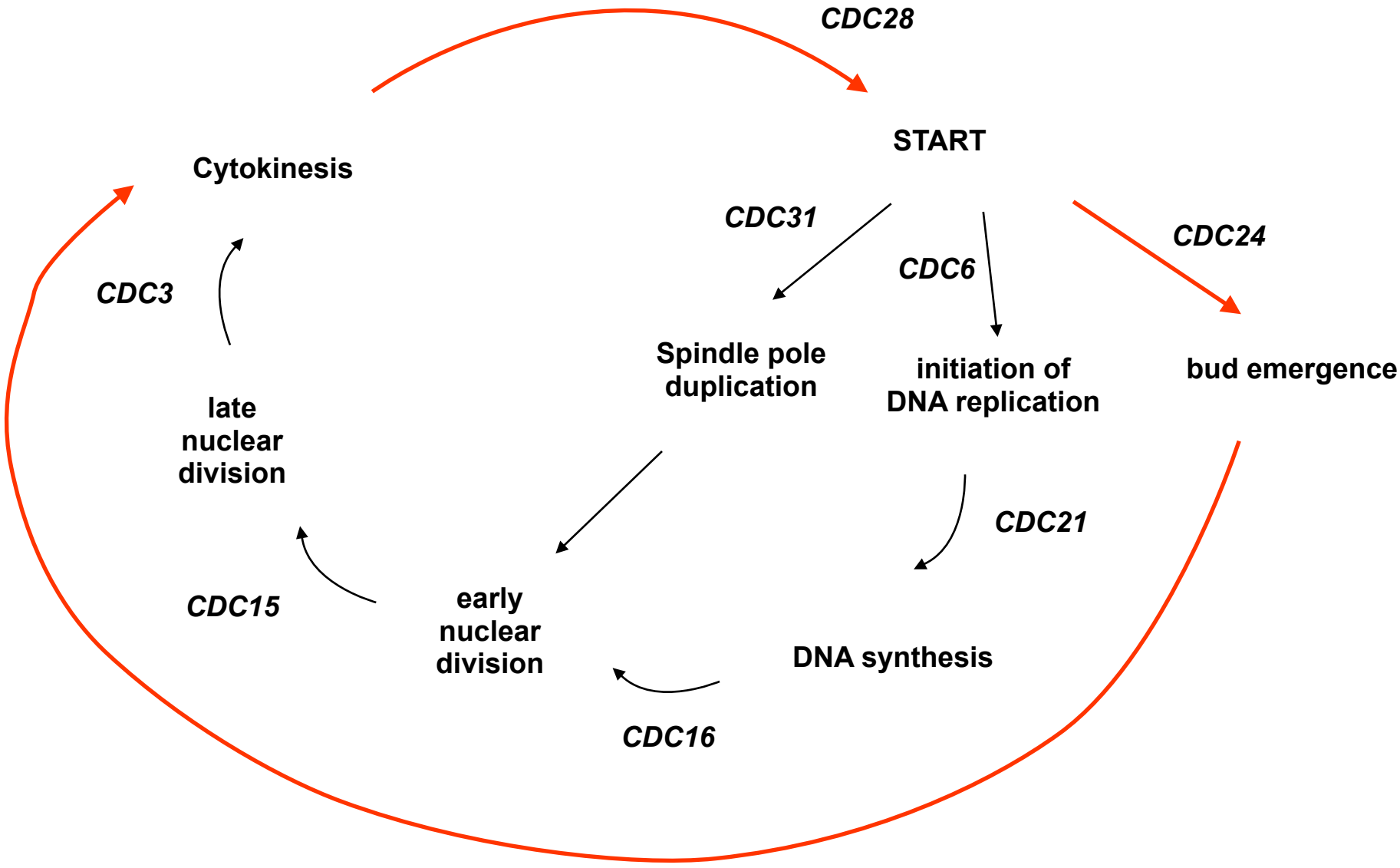


Three inter-connected cycles in budding yeast
a SPB duplication/mitosis cycle



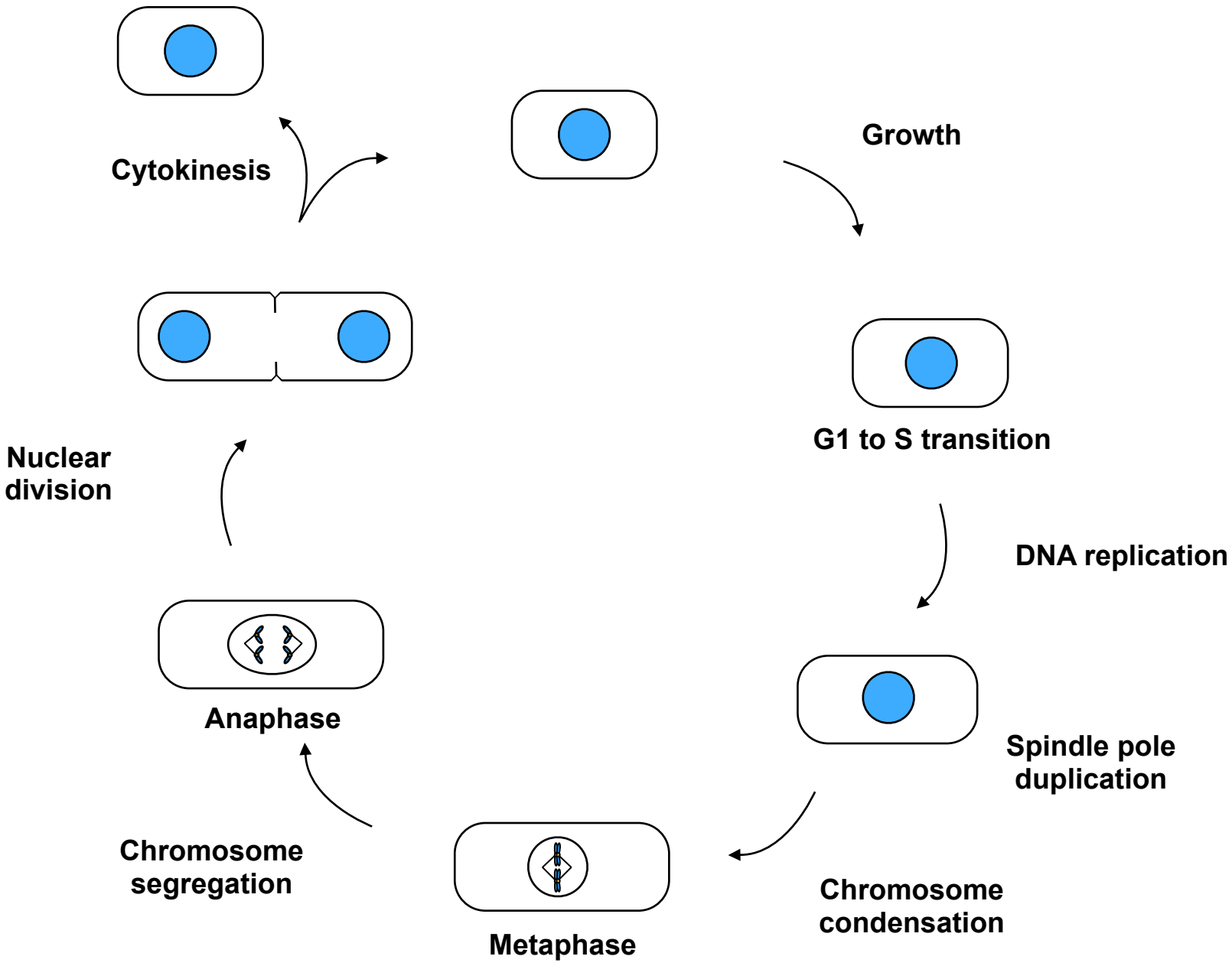
SPB duplication is obviously a pre-requisite for forming a mitotic spindle successfully.

Three inter-connected cycles in budding yeast
a budding/cytokinesis cycle



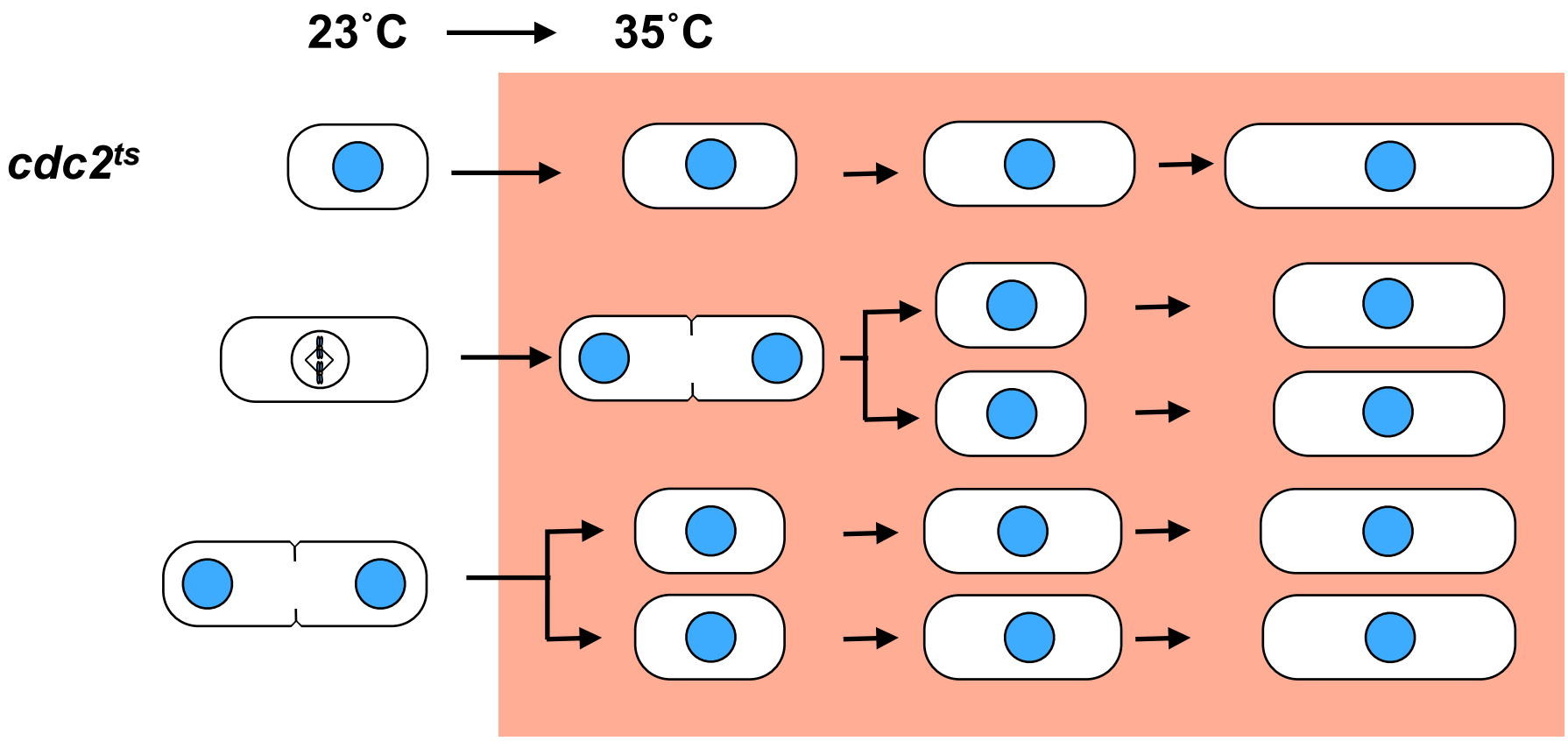
Bud emergence is obviously needed for proper cytokinesis in budding yeast.

back to the fission yeast cell cycle

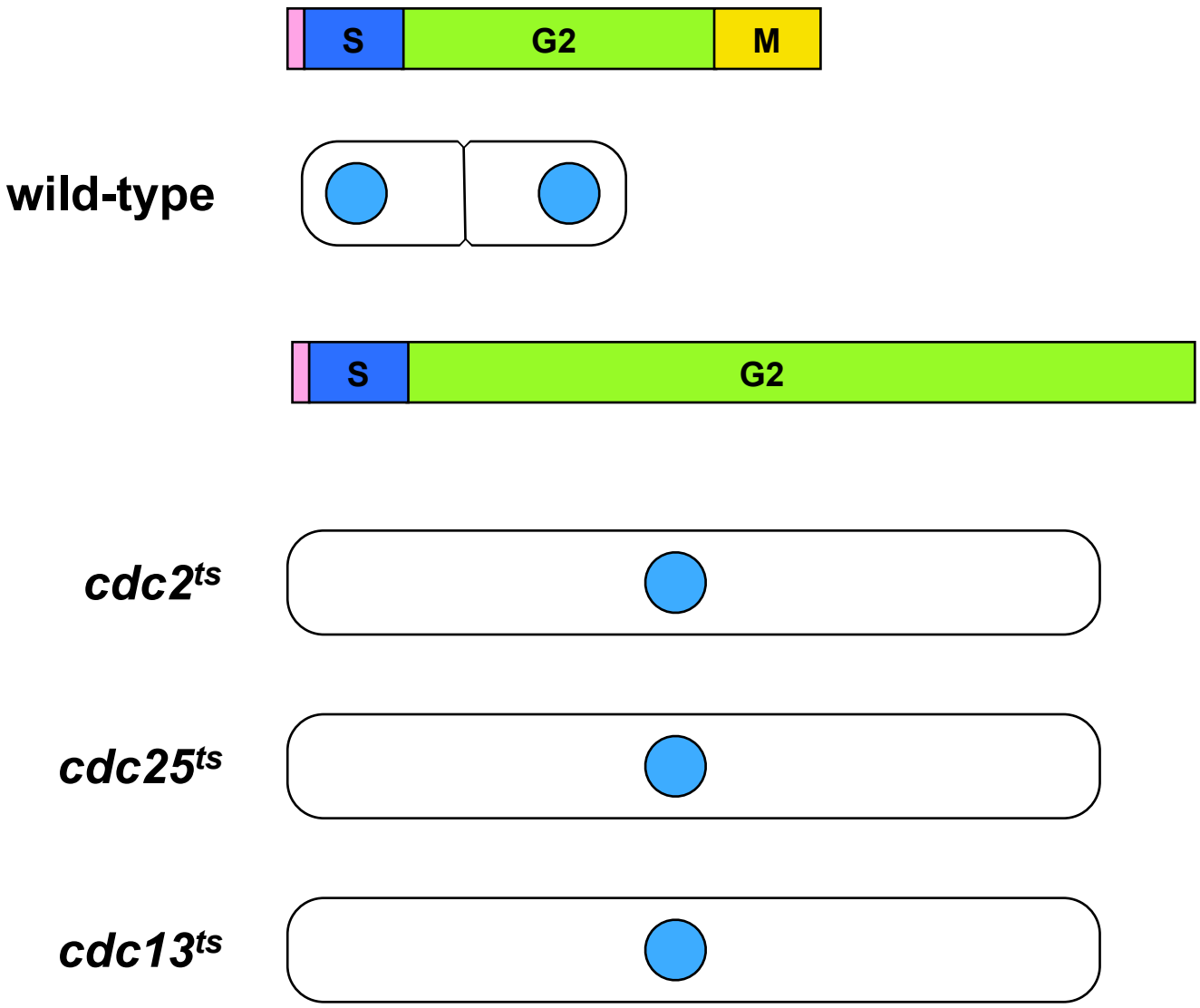


The cell cycle in the fission yeast, Schizzosaccharomyces cerevisiae. This yeast is rod shaped, grows from its two ends, and divides by fission, hence its name. After anaphase it lays down a septum at the midpoint of the mother cell. Fission yeast chromosomes are visible during mitosis, but it undergoes a “closed” mitosis - the nuclear envelope doesn’t breakdown as it does in vertebrate cells. The G1 to S transition in fission yeast and budding yeast is called START, and is the point at which the cell commits to going through the entire cell cycle. One of the critical determinants for passing START is that the cells reach a critical size. A similar point, called the “restriction point” exists in vertebrate cells. The restriction point was defined as the point after which serum could be removed from culture media, and the tissue culture cells would still traverse on cell cycle. Like START, the restriction point, provides a point to halt cell cycle progression when nutrients are unavailable. After START DNA replication initiates.

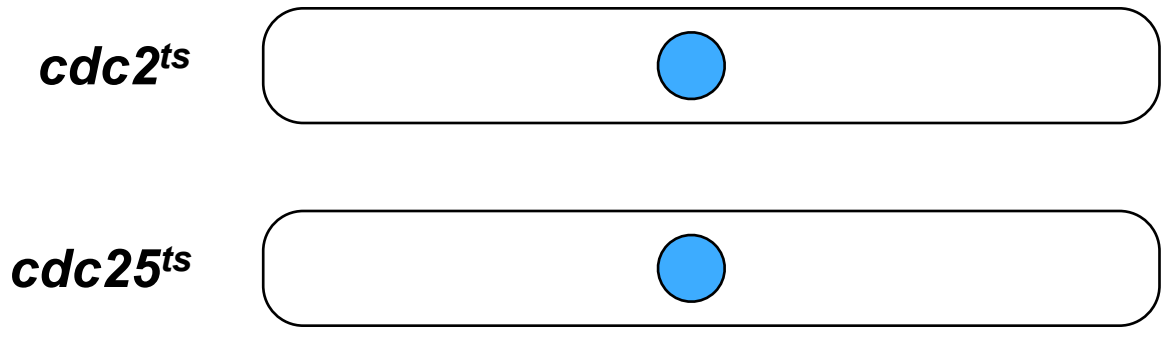
many *cdc* mutants arrested in G2



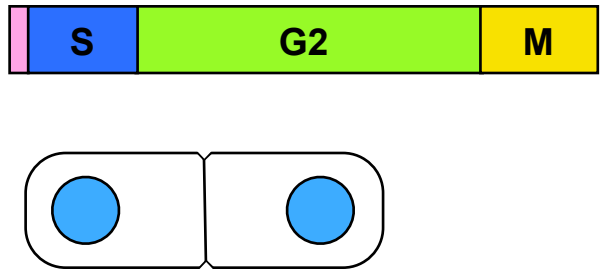
One mutant that was found is cdc2-ts. At the high temperature cdc2-ts arrest in the G2 phase of the cell cycle. Cells will continue to grow until they reach G2 and then arrest. For example, if the cells are in mitosis when shifted to 35˚C, they will complete mitosis, divide into two cells, both will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



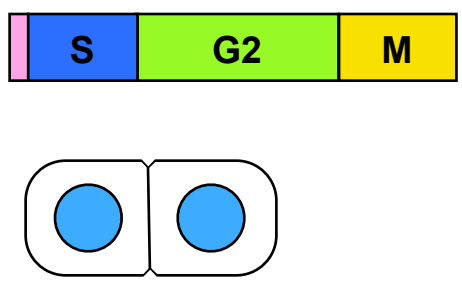
Many cdc mutants arrest in the G2 phase at the restrictive temperature. When fission yeast cells arrest in G2 they continue to grow from their ends and become very long. Three cdc mutants that arrest in G2 are cdc2-ts, cdc25-ts and cdc13-ts.



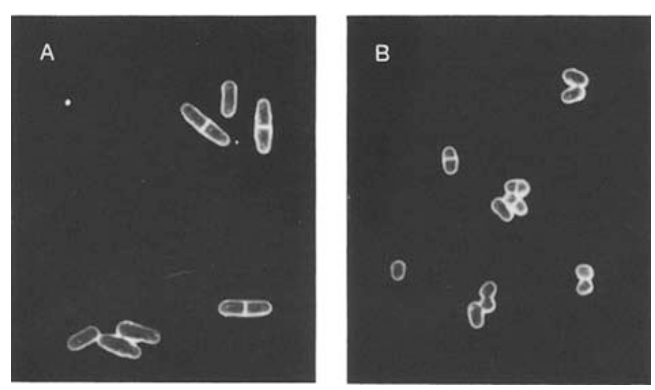
After finding these 5 mutants in four genes that all seem to affect the transition from G2 to M, Paul Nurse wondered if the mutated genes all function in a single pathway to regulate the transition from G2 to M.



wild-type



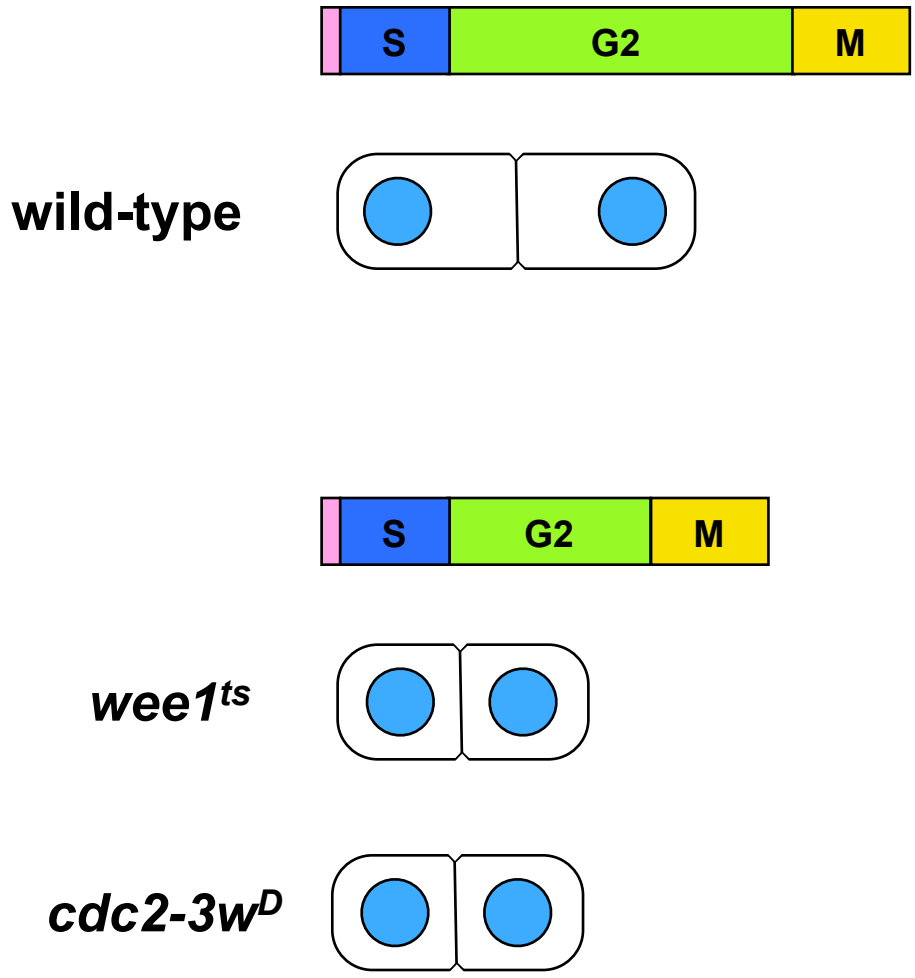
wee1^{ts}



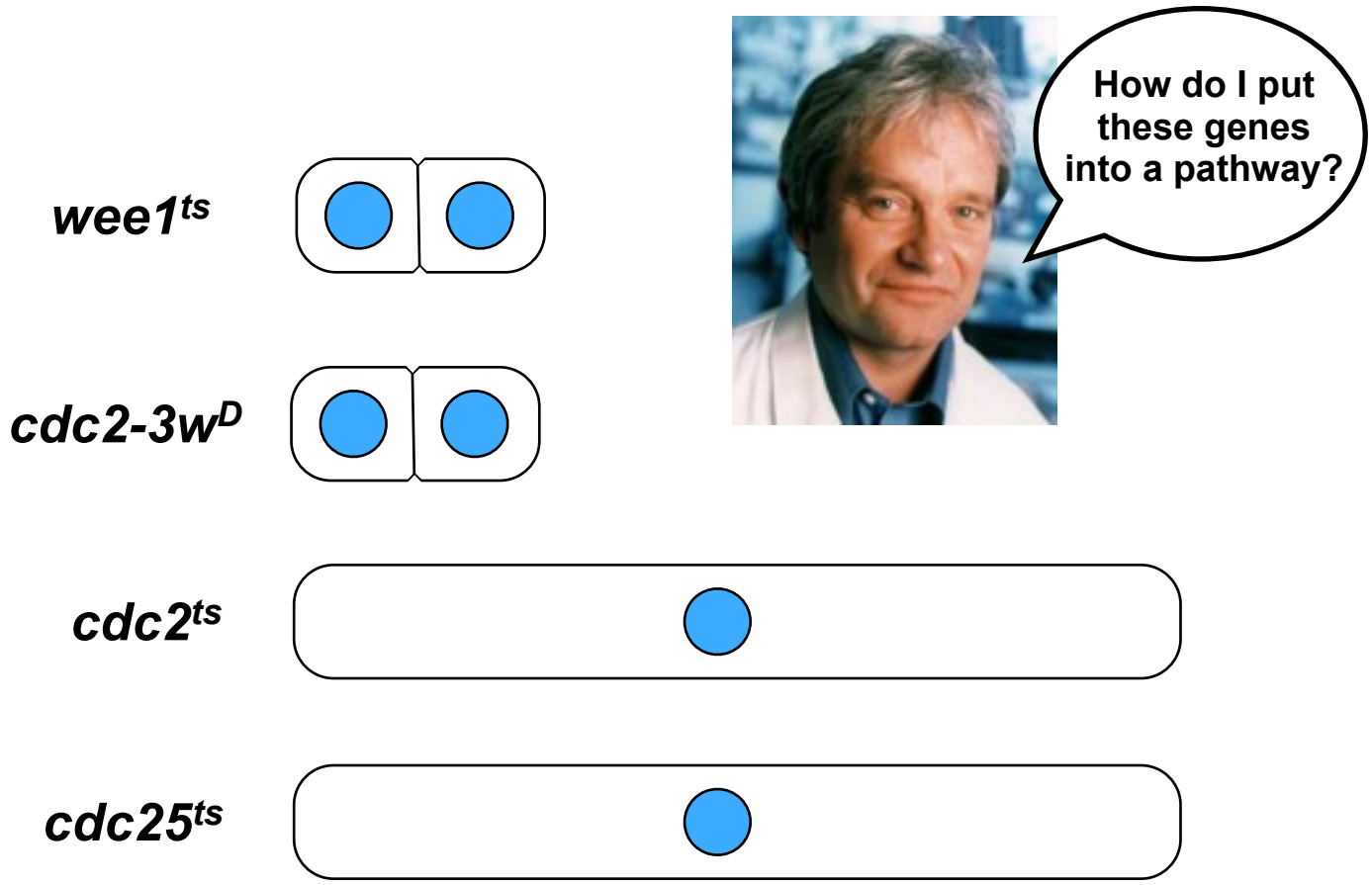
Thuriaux, Nurse and Carter, 1978

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.

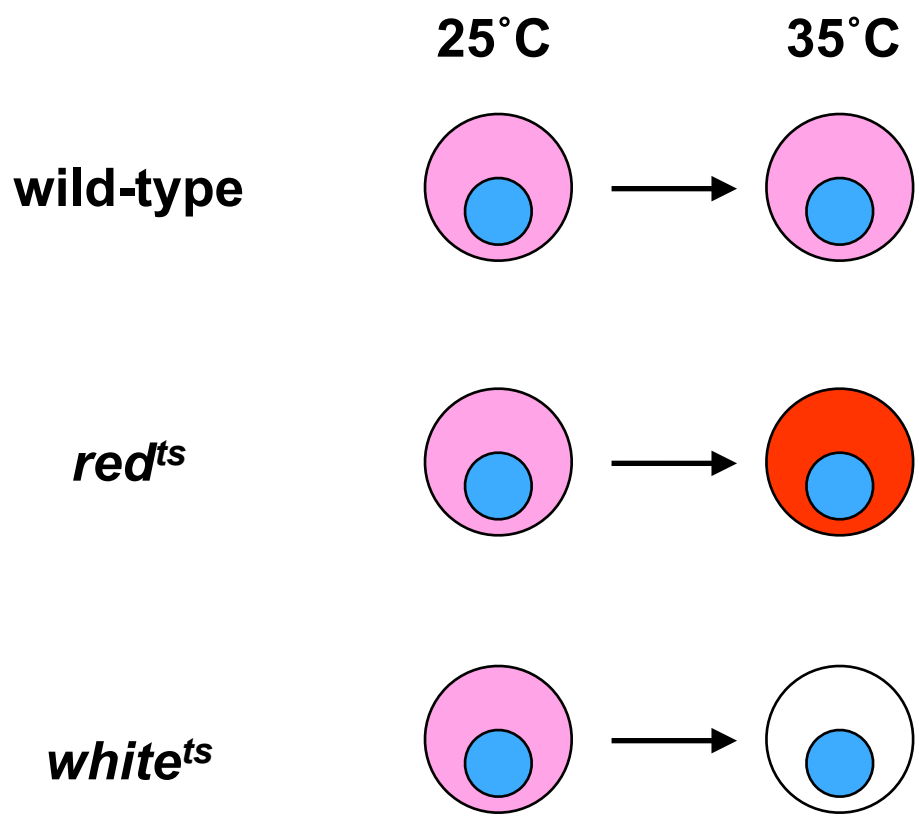


A second wee mutant was a mutant in cdc2, cdc2-3wD. cdc2-3wD is a dominant mutant that like wee1-ts, shortens the G2 phase. The fact that mutations in cdc2 can either cause a long G2 (or an arrest in G2 - cdc2-ts) or a short G2 (cdc2-3wD) clearly pointed to the fact that cdc2 was a critical regulator 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).



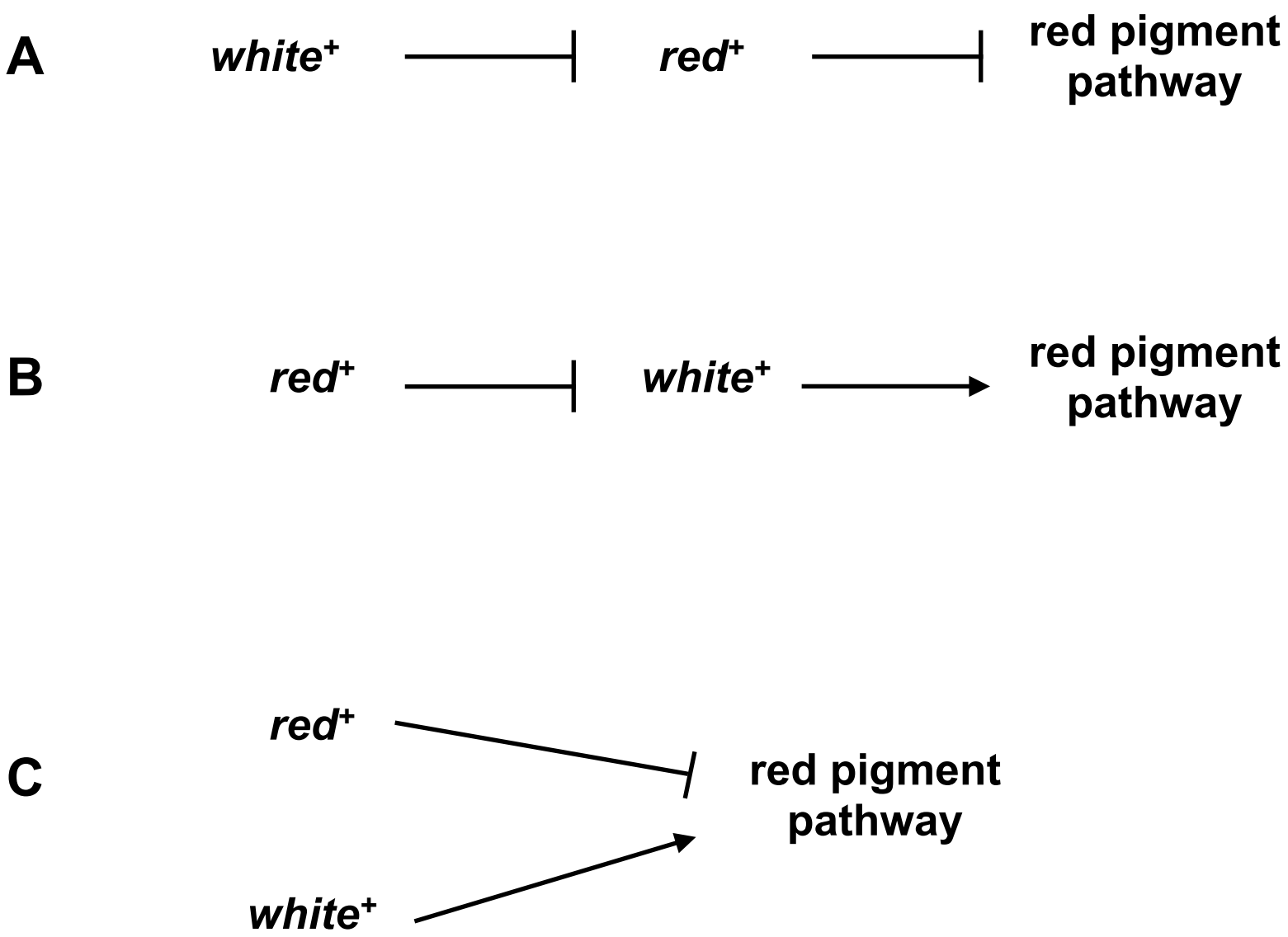
After finding these 5 mutants in four genes that all seem to affect the transition from G2 to M, Paul Nurse wondered if the mutated genes all function in a single pathway to regulate the transition from G2 to M.

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



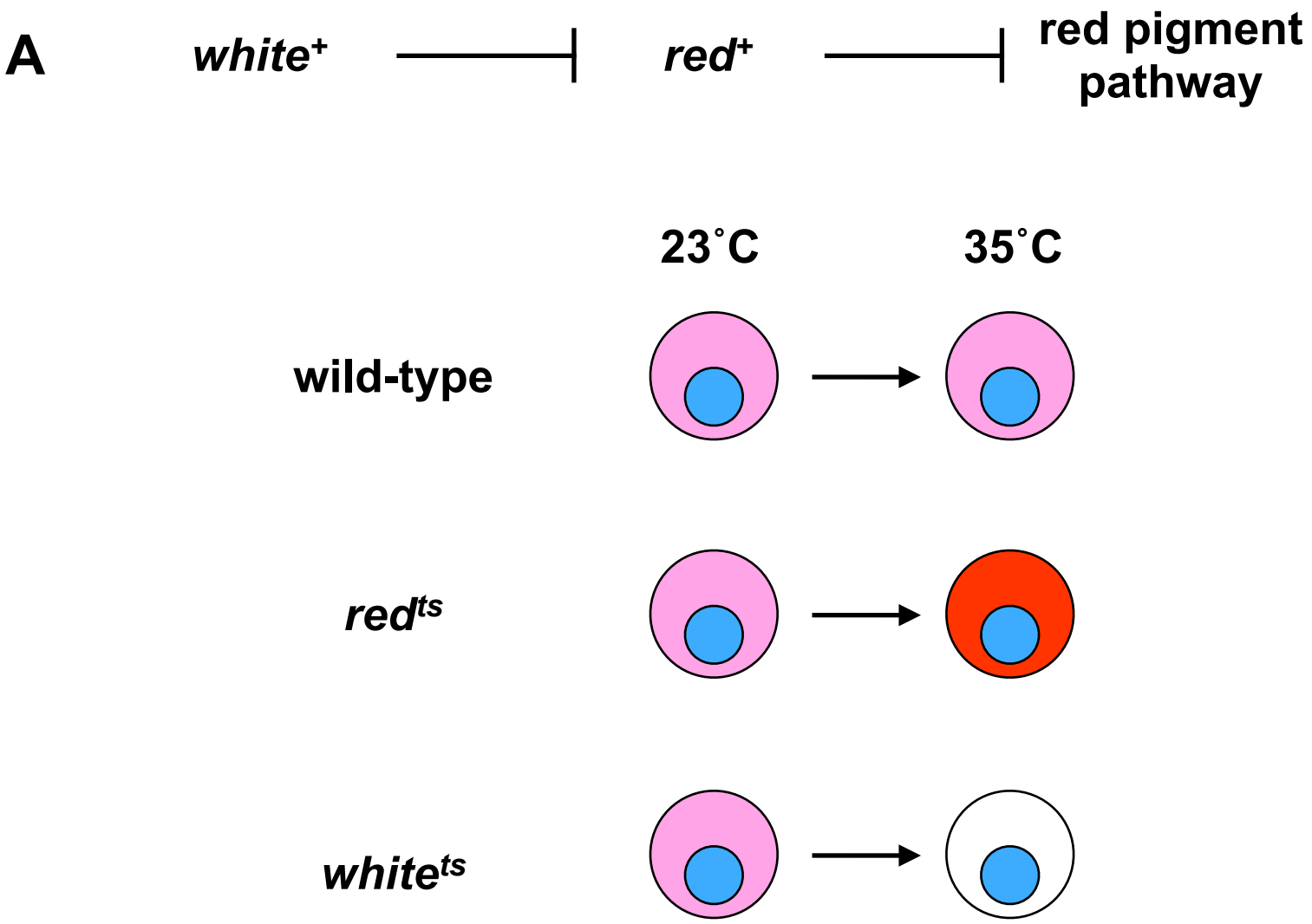
We can envision three models for how the red and white genes regulate cell color. We can assume that cell color is regulated by a red pigment pathway. In wild type cells the red pigment pathway makes enough pigment to make the cells pink. The mutants red-ts and white-ts somehow modulate this pathway.

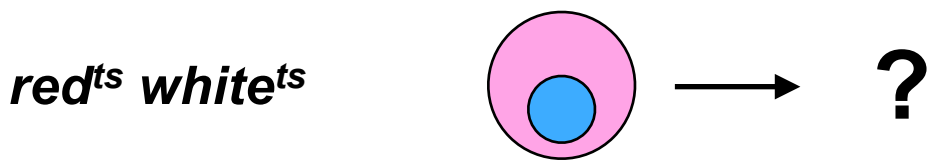
In Model A, the functional red gene inhibits the red pigment pathway, and the white gene inhibits the red gene. In the red-ts mutant, the inhibition of the red pigment pathway is removed so more red pigment is made, so the cells are red. In the white-ts mutant the inhibition of the red gene is removed, 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.

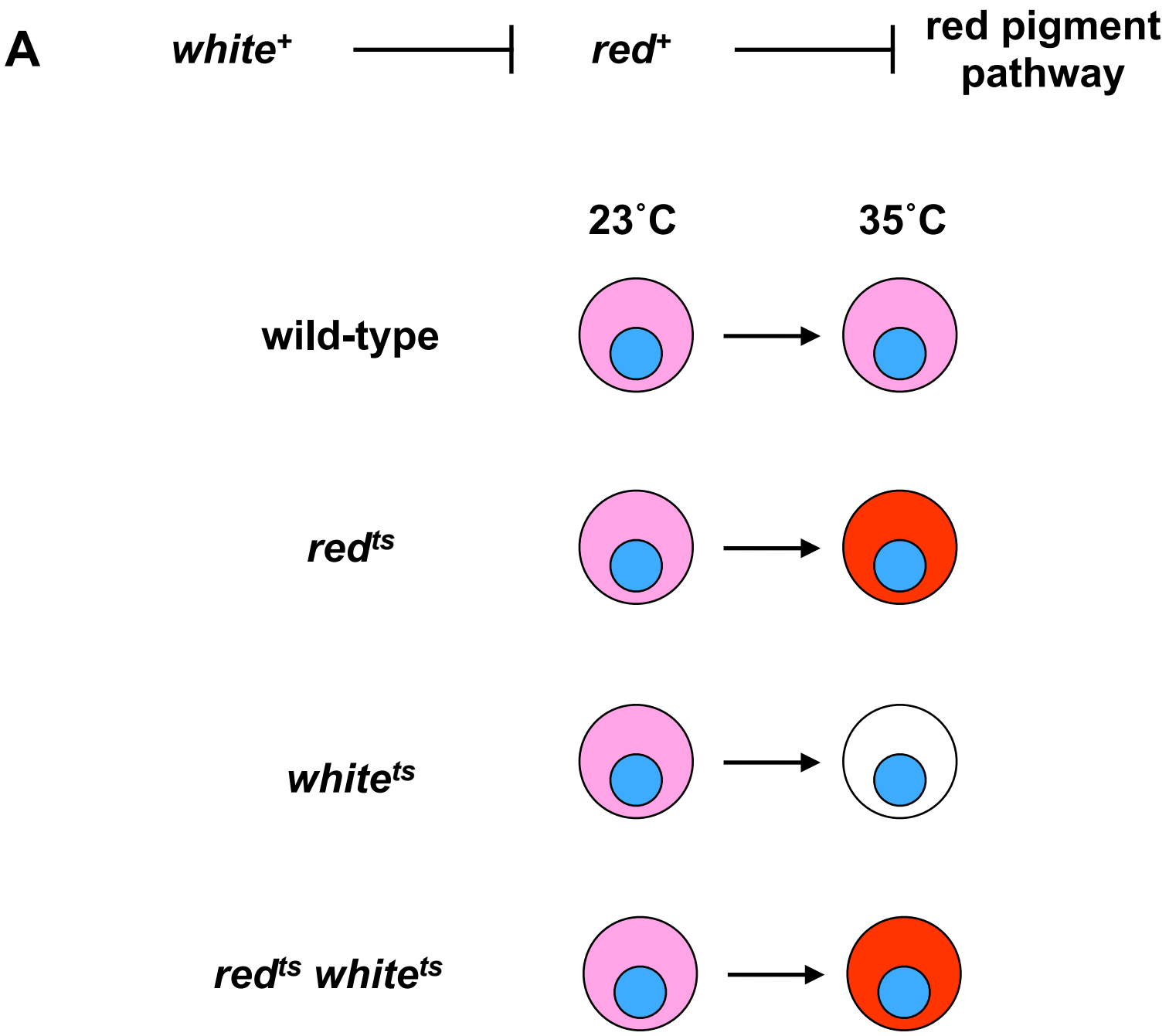
In Model C, both genes independently regulate the red pigment pathway. The red gene inhibits the pigment pathway and the white gene activates the pigment pathway. In a red-ts mutant, the inhibition of the pigment pathway is removed and the cells are red, and in the white-ts mutant the activation of the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



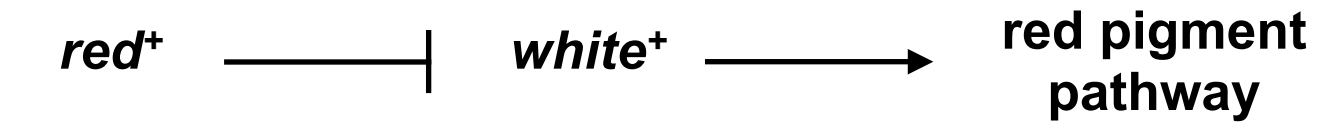


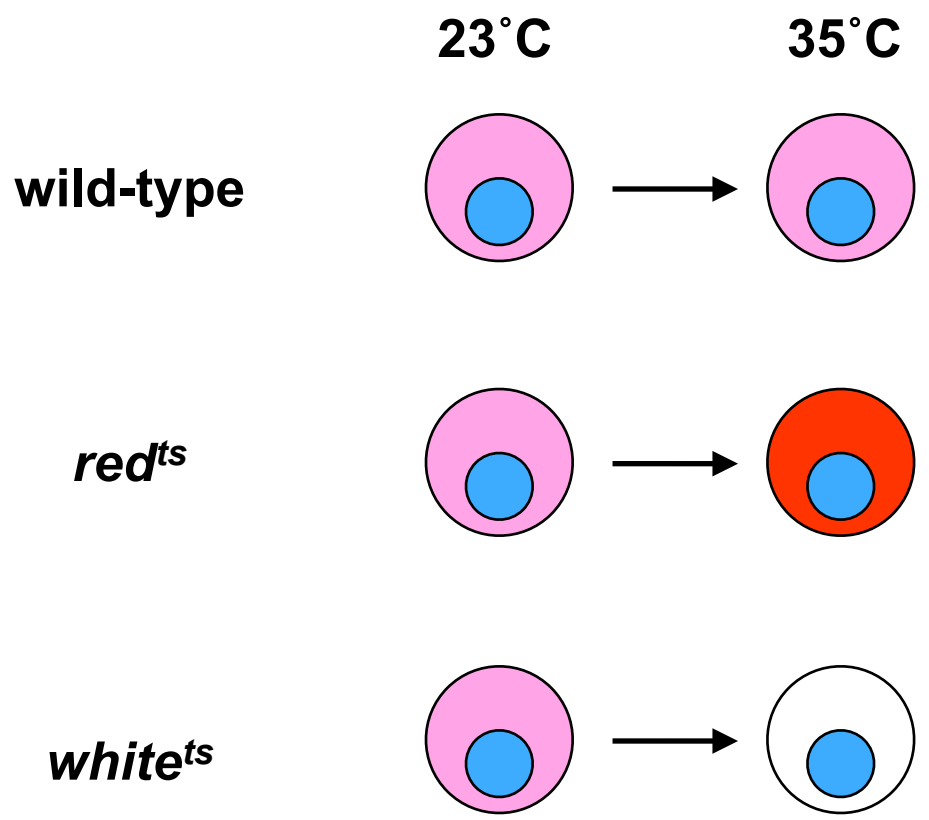
To test all three models we need to know the phenotype of the white-ts red-ts double mutant. Some organisms are not amenable to genetic analysis, like frogs, but others like yeasts, are. Constructing double mutants in yeast requires simple genetic crosses. If Model A is correct we expect that the red-ts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

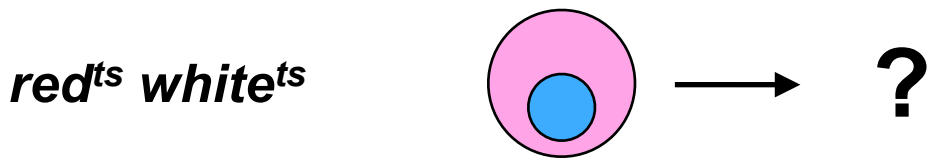


red. Mutating the white gene has no affect if the red gene is already mutated.

B

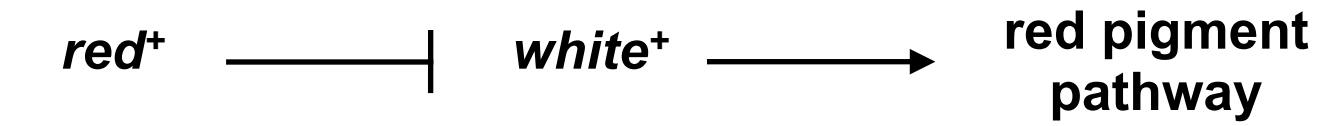


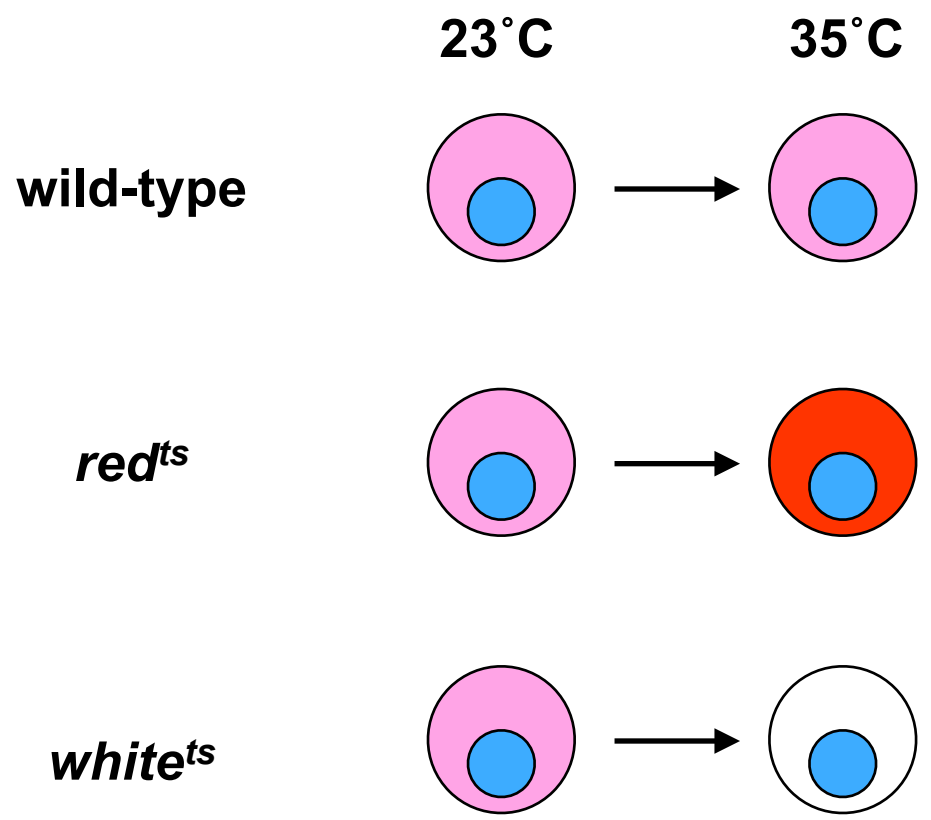


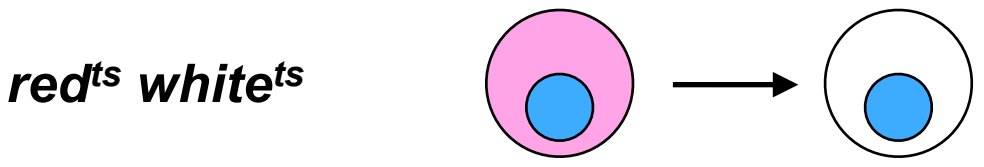


In Model B the red-ts white-ts double mutant will be.....

B

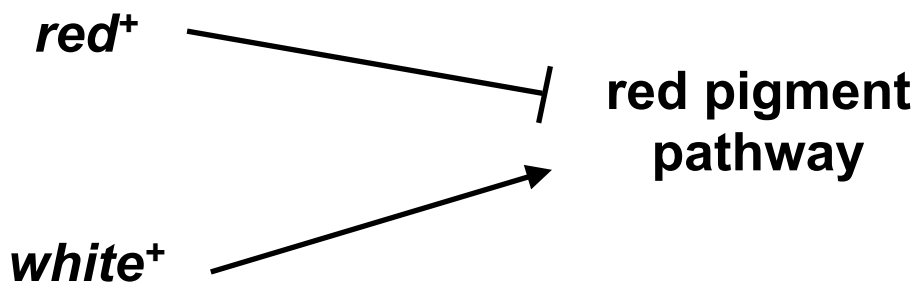


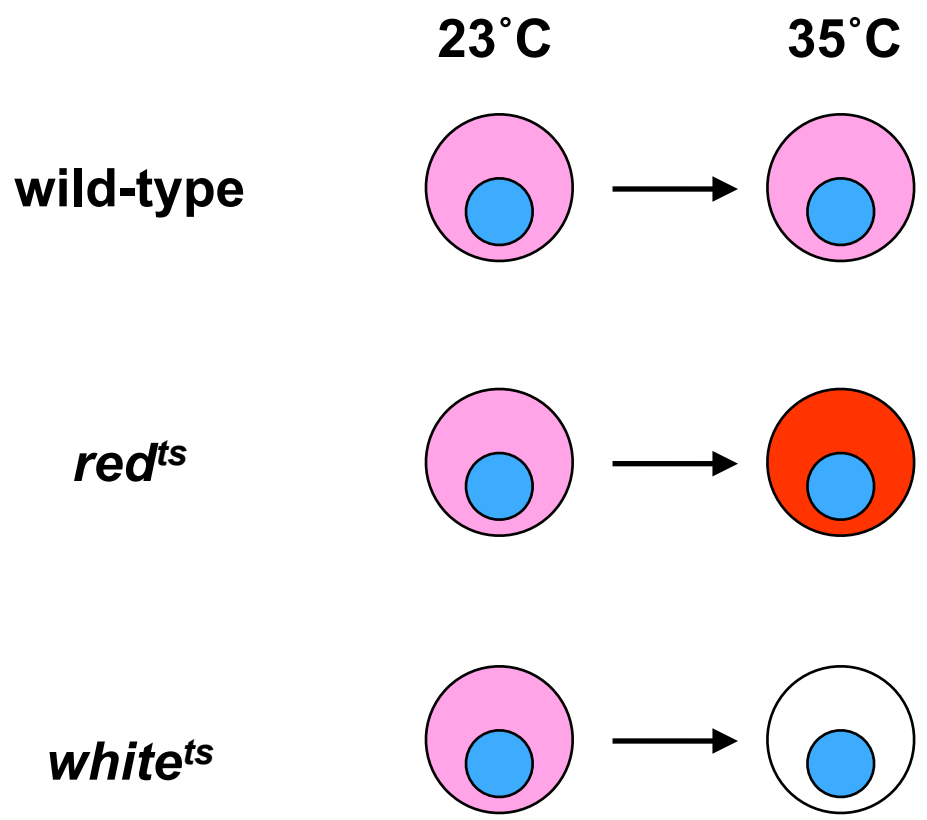




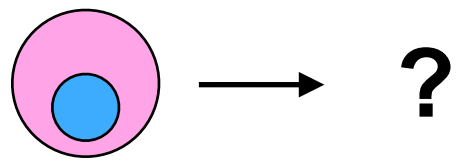
white. Because in this model mutating the red gene has no affect if this white gene is already mutated.

C

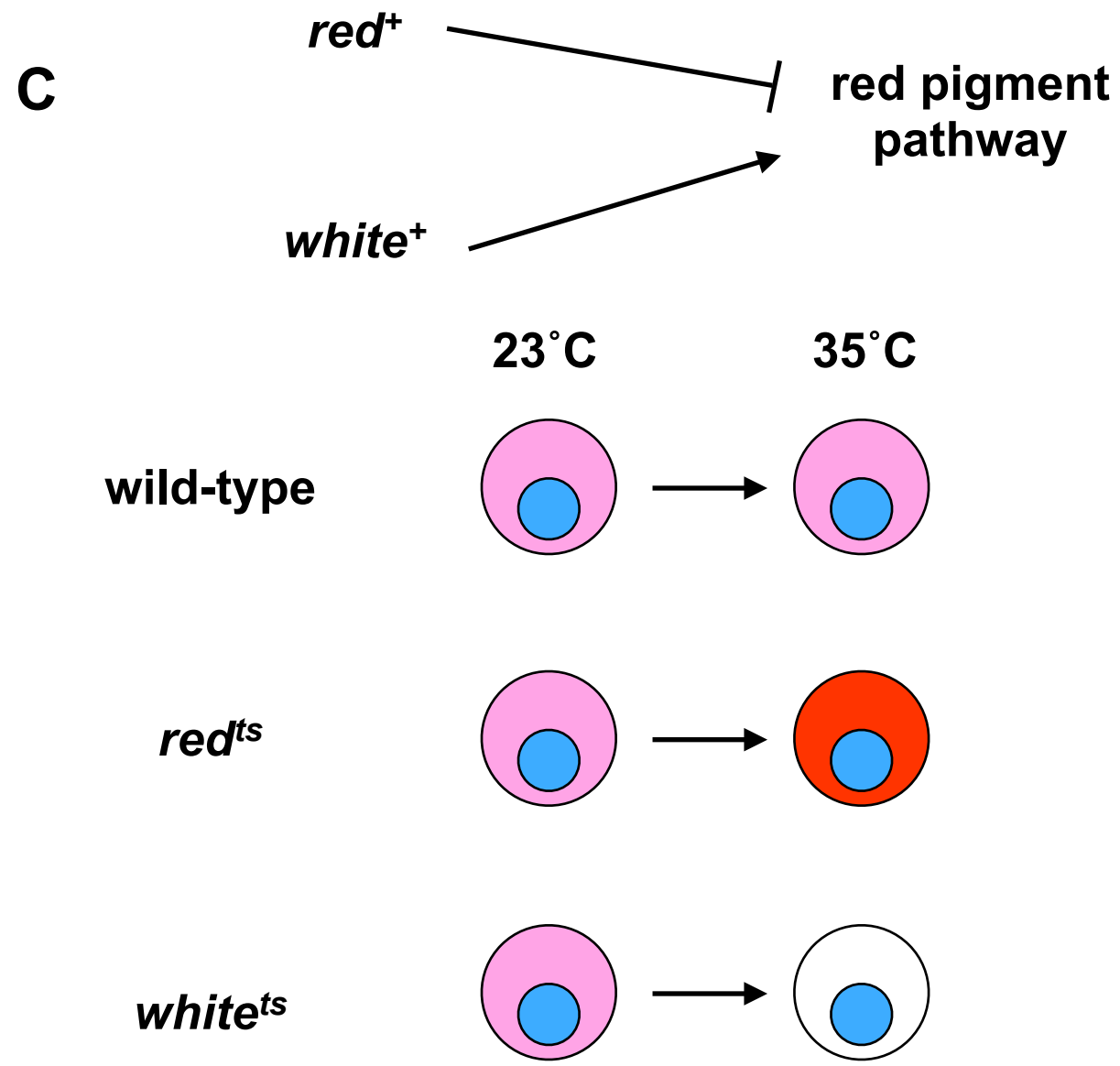


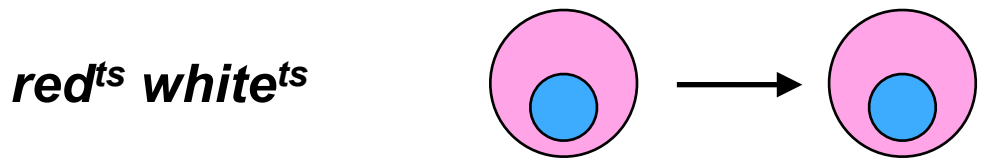


redts whitets

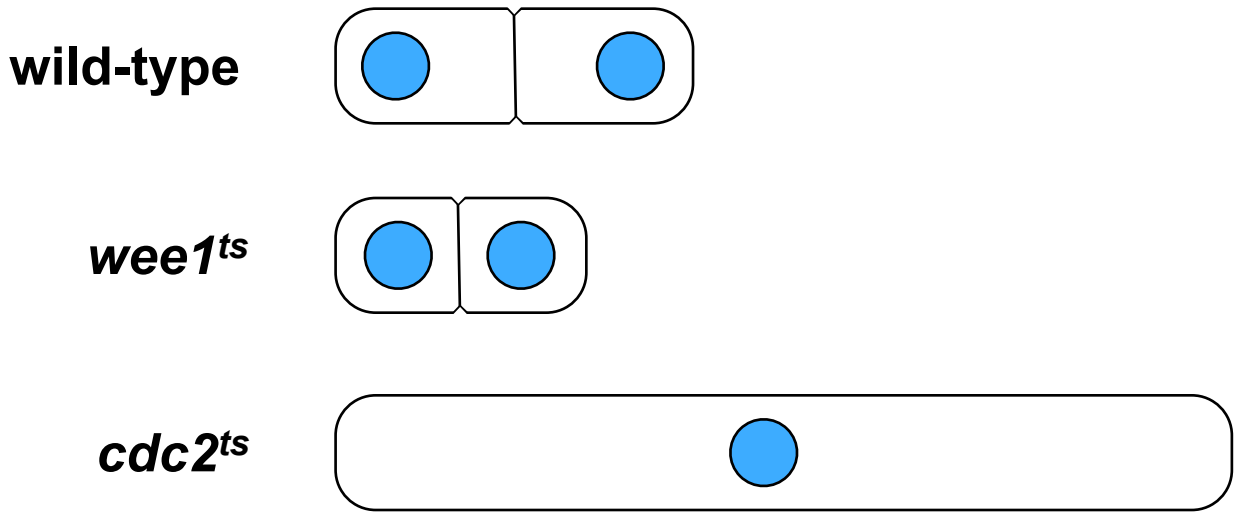


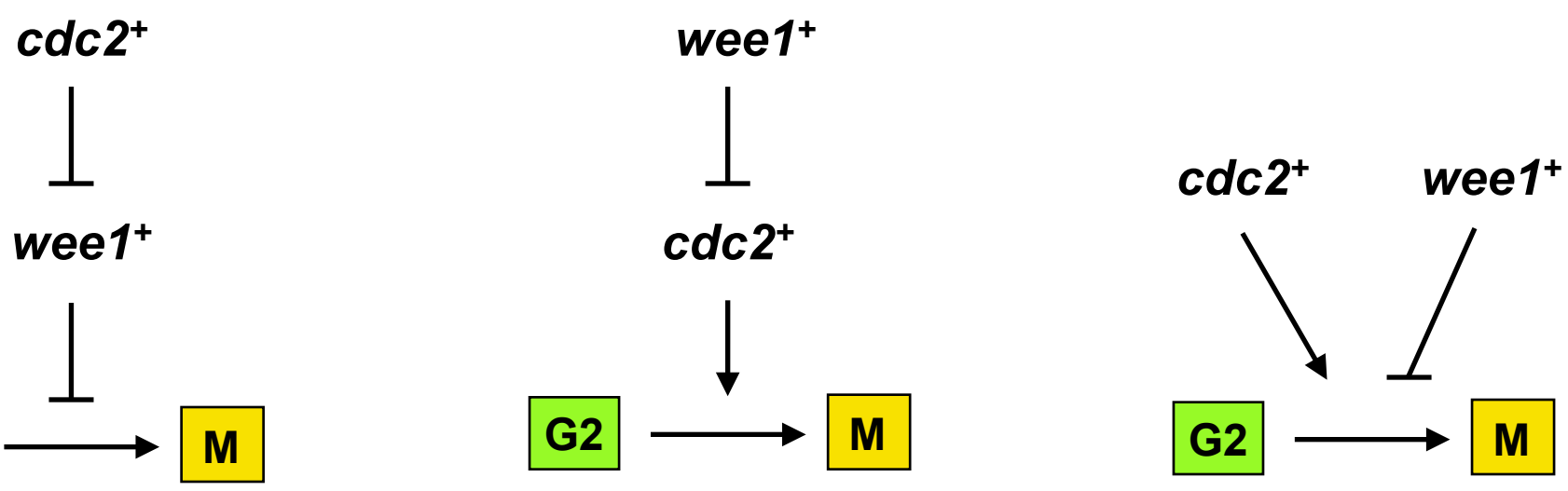
And finally in Model C the red-ts white-ts double mutant would be.....



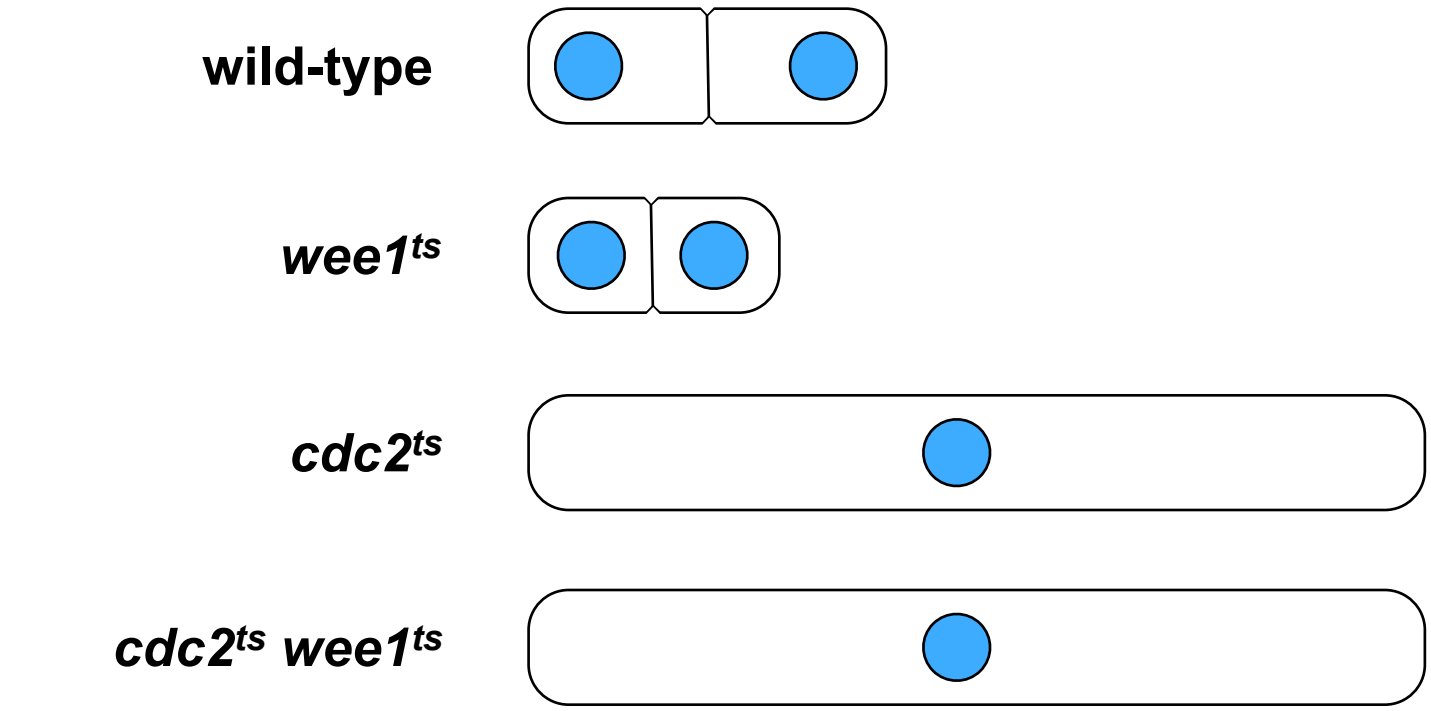


likely pink. This isn't a certainty, but it is the most likely outcome. This would occur if there is some basal level of activity of the pigment pathway. The white and red genes function to modulate that activity, but removing both still leaves the basal activity intact.

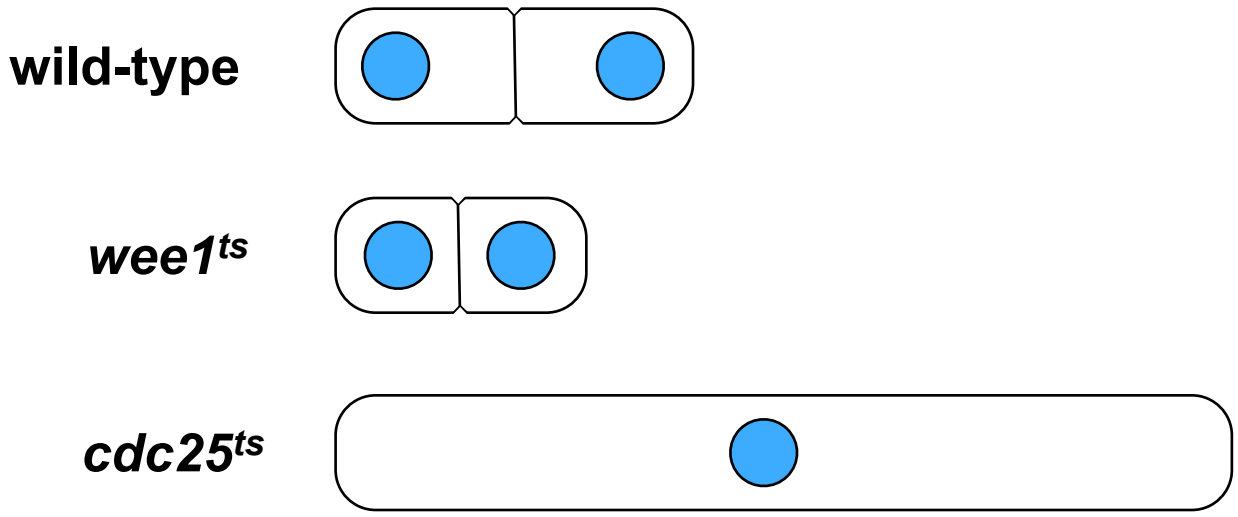


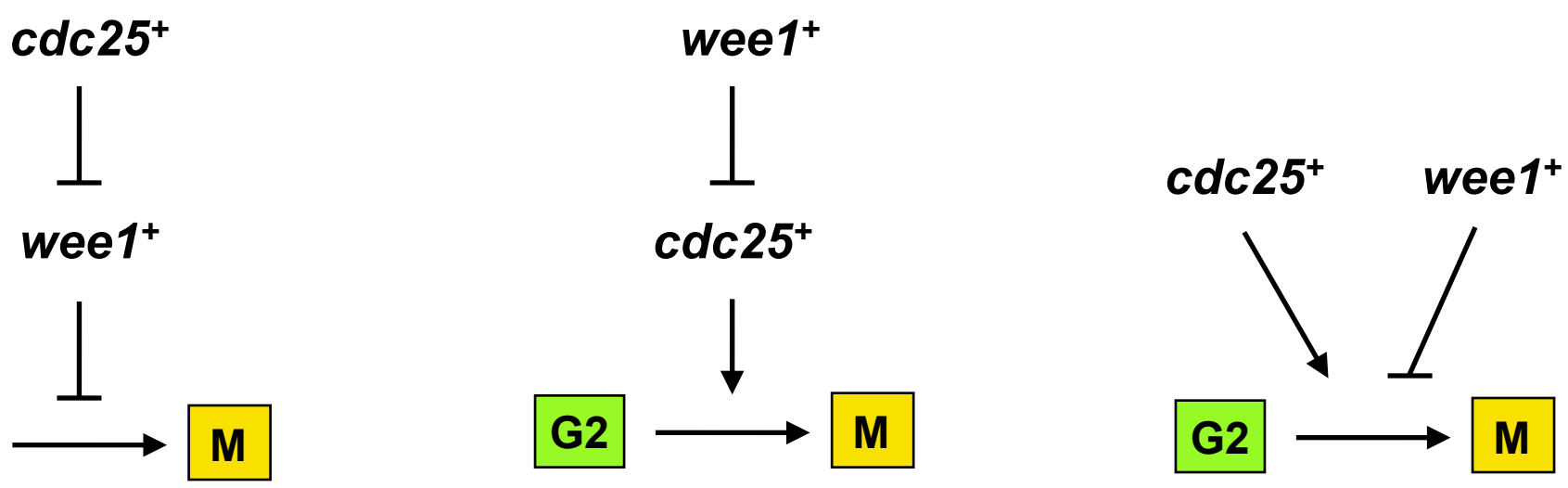


Paul Nurse wondered what the relationship was between wee1 and cdc2, and came up with three possible models (which are exactly analogous to the red/white/pink model). In one, cdc2 inhibits wee1 which inhibits the transition from G2 to M, in a second wee1 inhibits cdc2 which activates the G2 to M transition,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

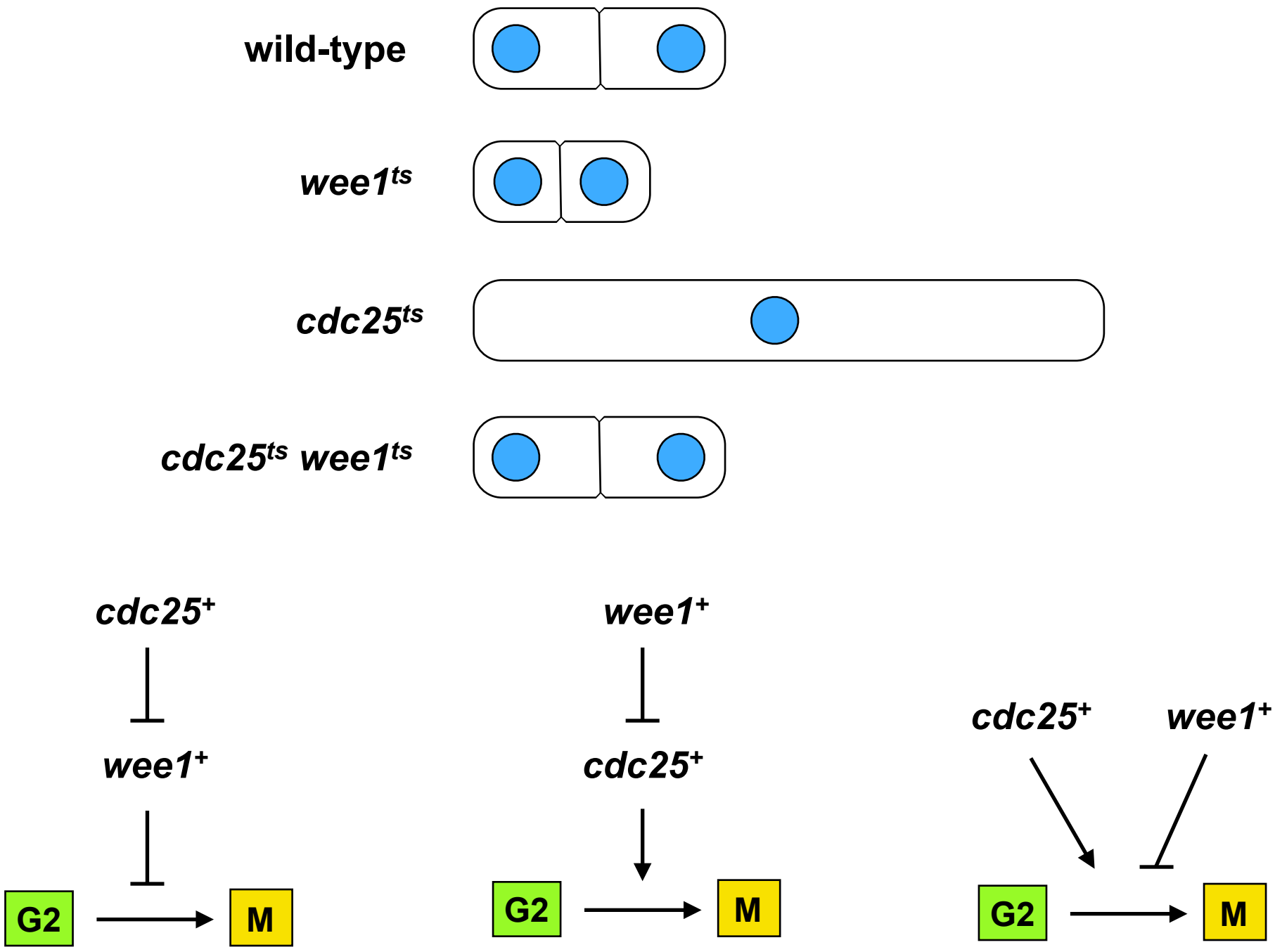


When he made the cdc2-ts wee1-ts double mutant he found that it was large (and identical) to the cdc2-ts mutant alone. This supports which model? The second, because wee1 function is upstream of cdc2, so its mutation has no affect when cdc2 is already mutated.

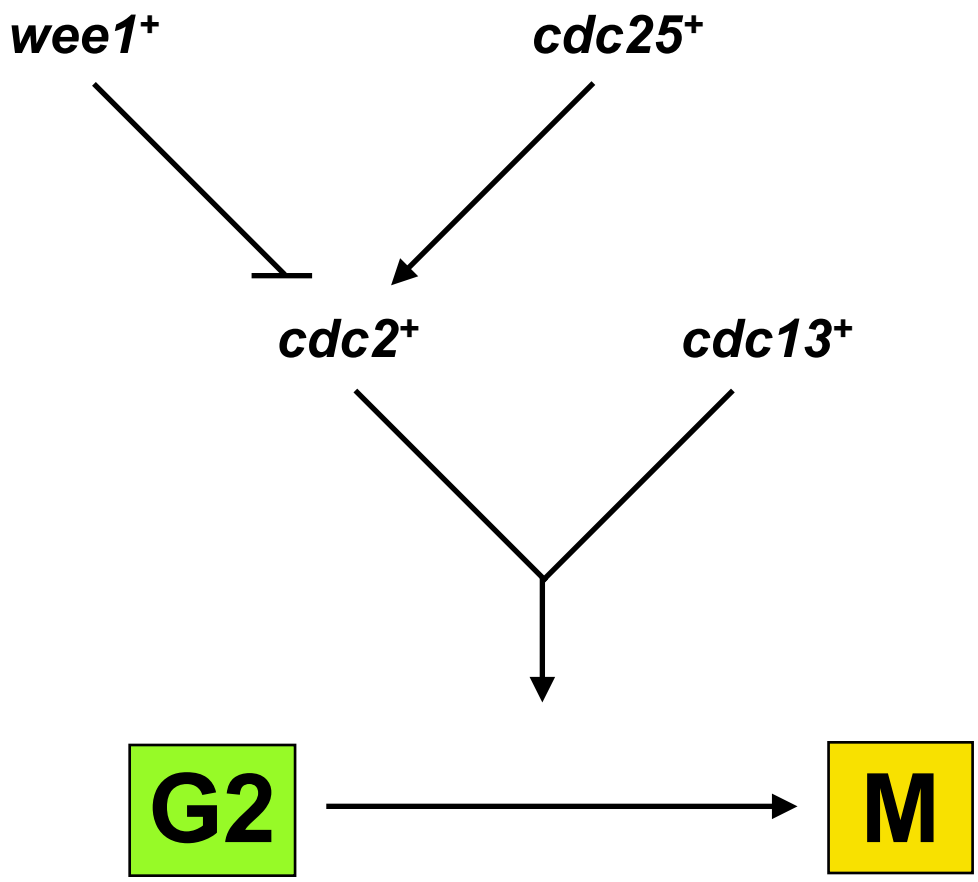




He imagined the same three possible models for how wee1 and cdc25 interact.

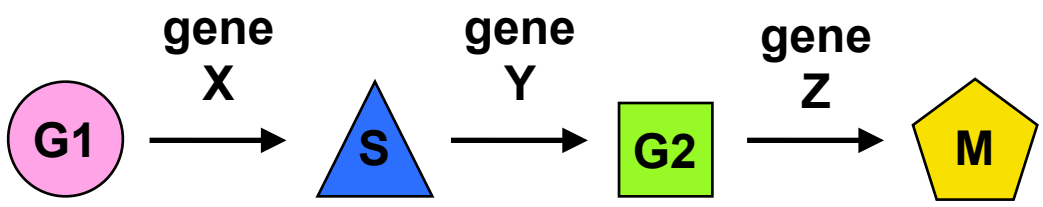


When he examined the cdc25-ts wee1-ts double mutant he found that it was the same size as wild type cells, which supports which model? The third model, that the two genes independently regulate the G2 to M transition.

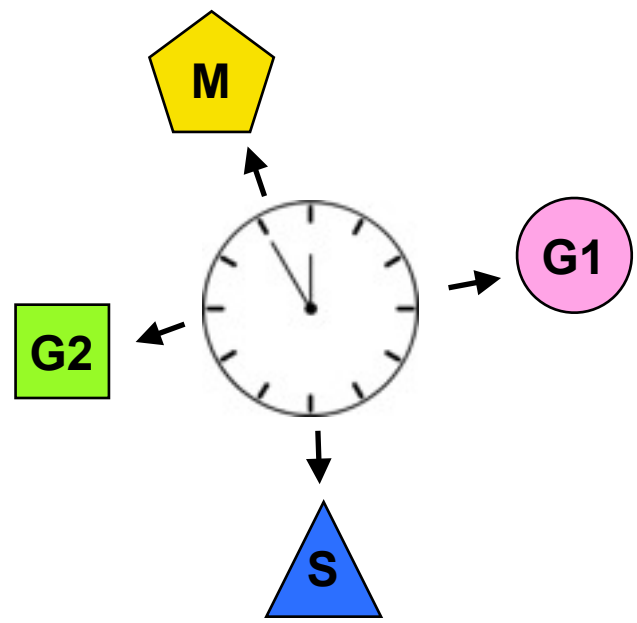


These two examples plus a few others I'm not showing led to the following model for how these four genes interact. Cdc2 and Cdc13 together activate the G2 to M transition, while Wee1 and Cdc25 modify the activity of Cdc2. These analyses were done before the identity of these proteins were known and simply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

domino theory



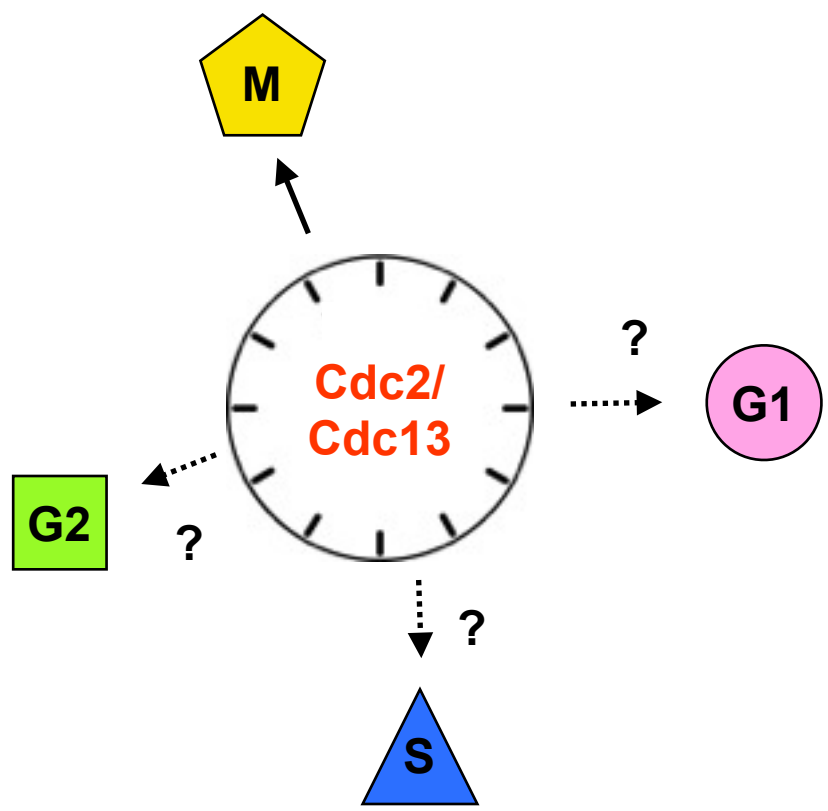
clock theory



Which model is supported by this data?

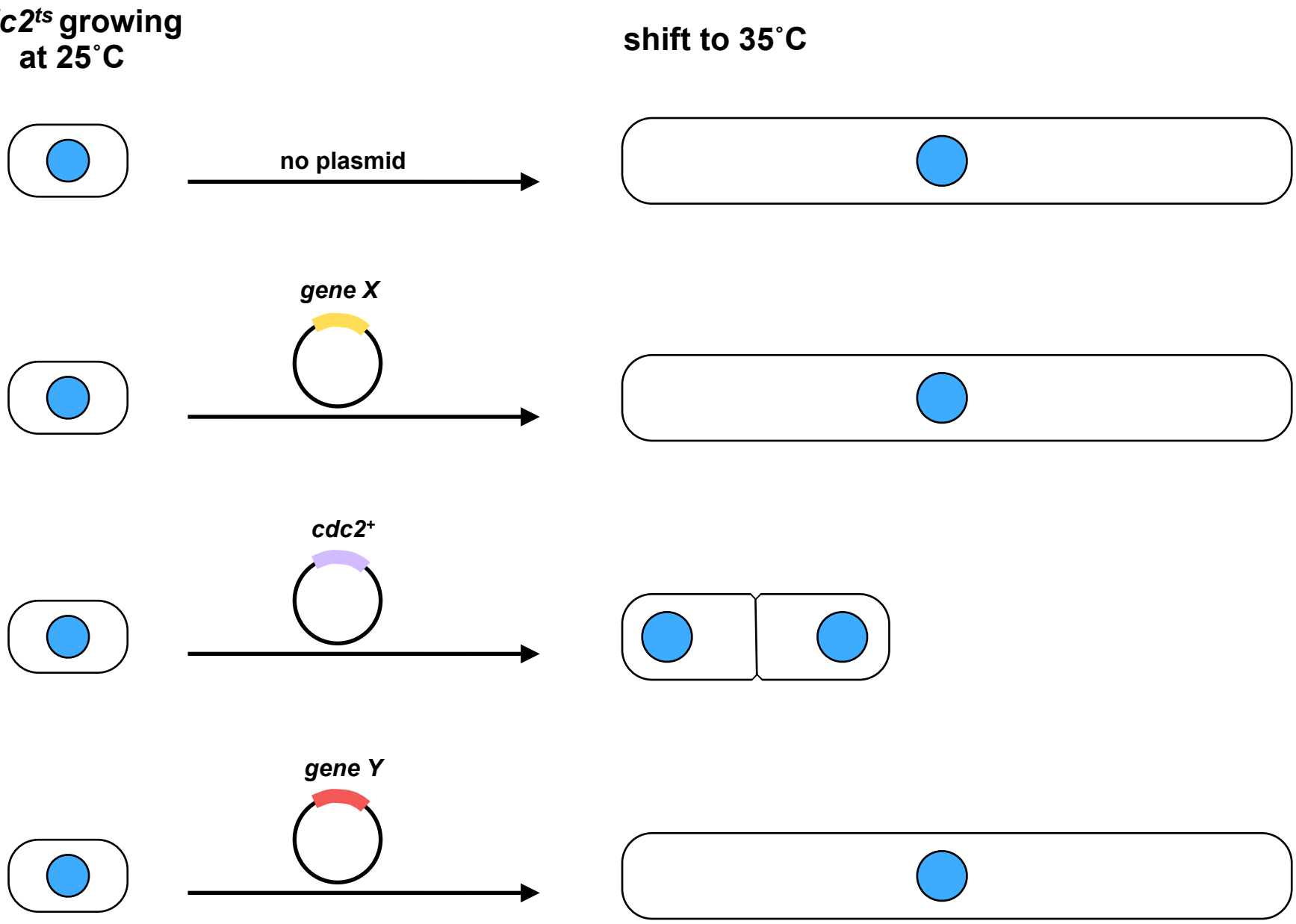
Cdc2, like MPF, is required for entry into mitosis

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

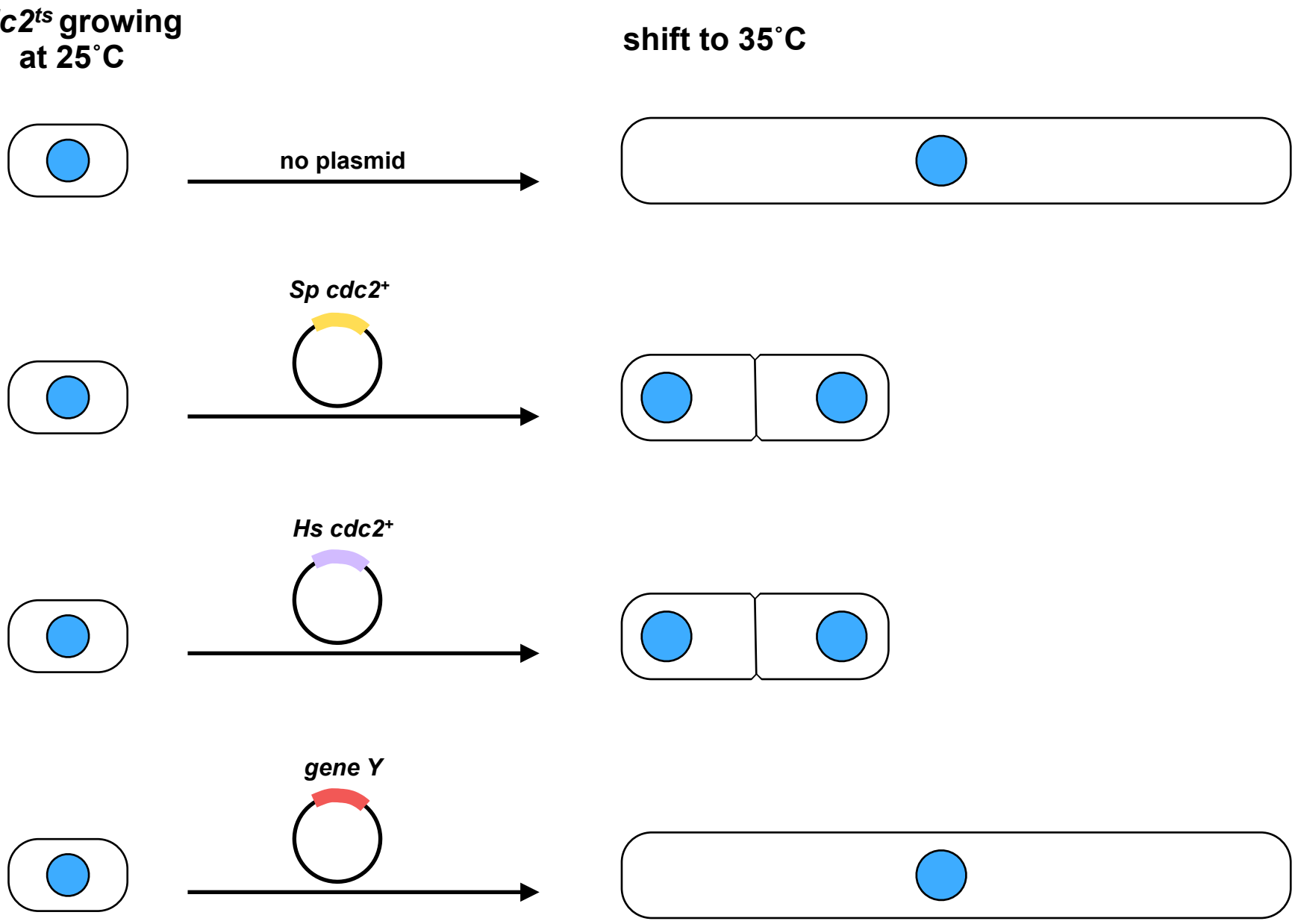
To clone these genes researchers used what is now a very standard approach. At the time, these were some of the first genes to be cloned by "complementation." The researchers made a "library" or collection of all fission yeast genes on a plasmid. Each different plasmid contains one fission yeast gene. 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.

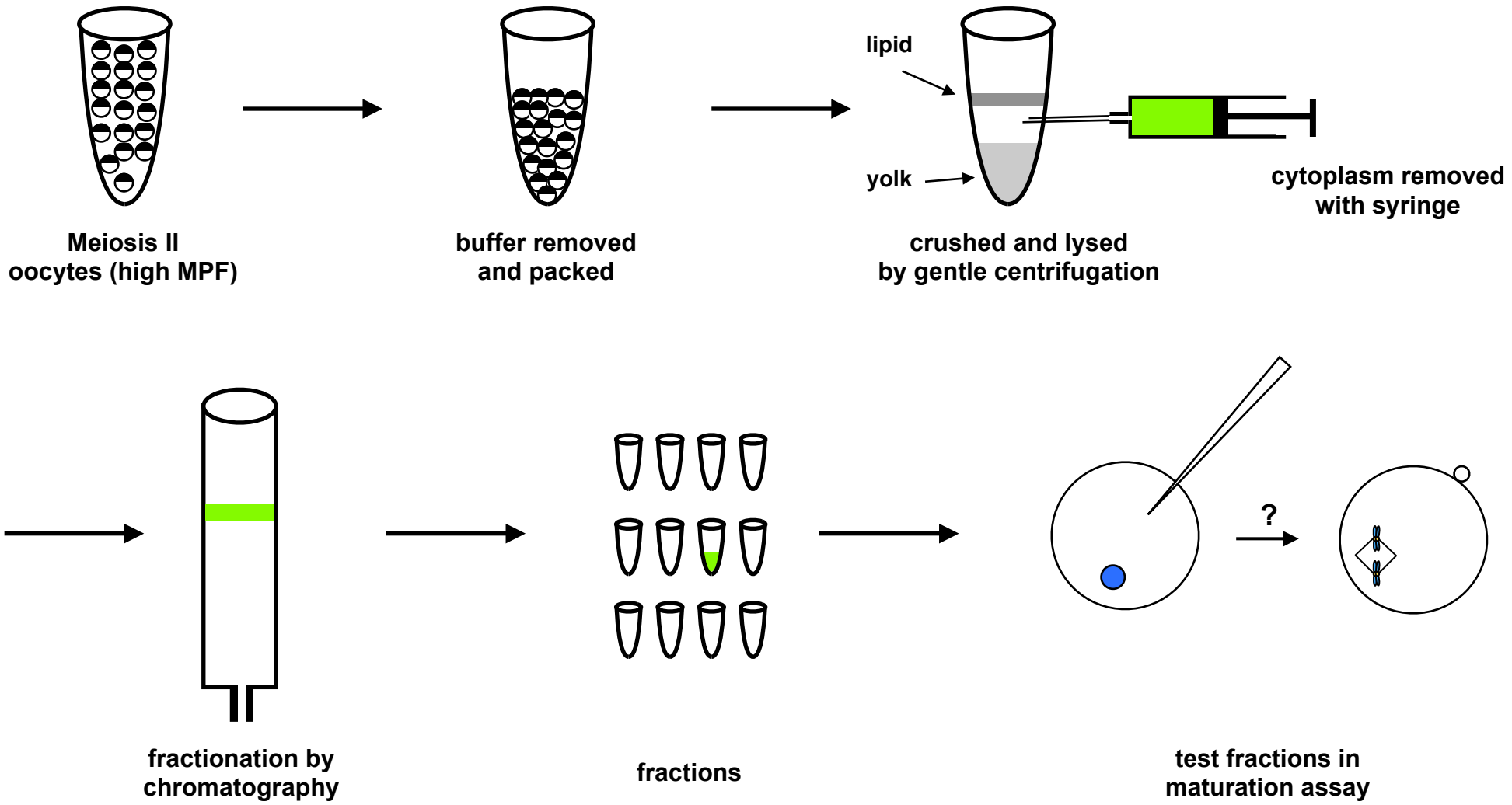
Cdc2 is universally conserved



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

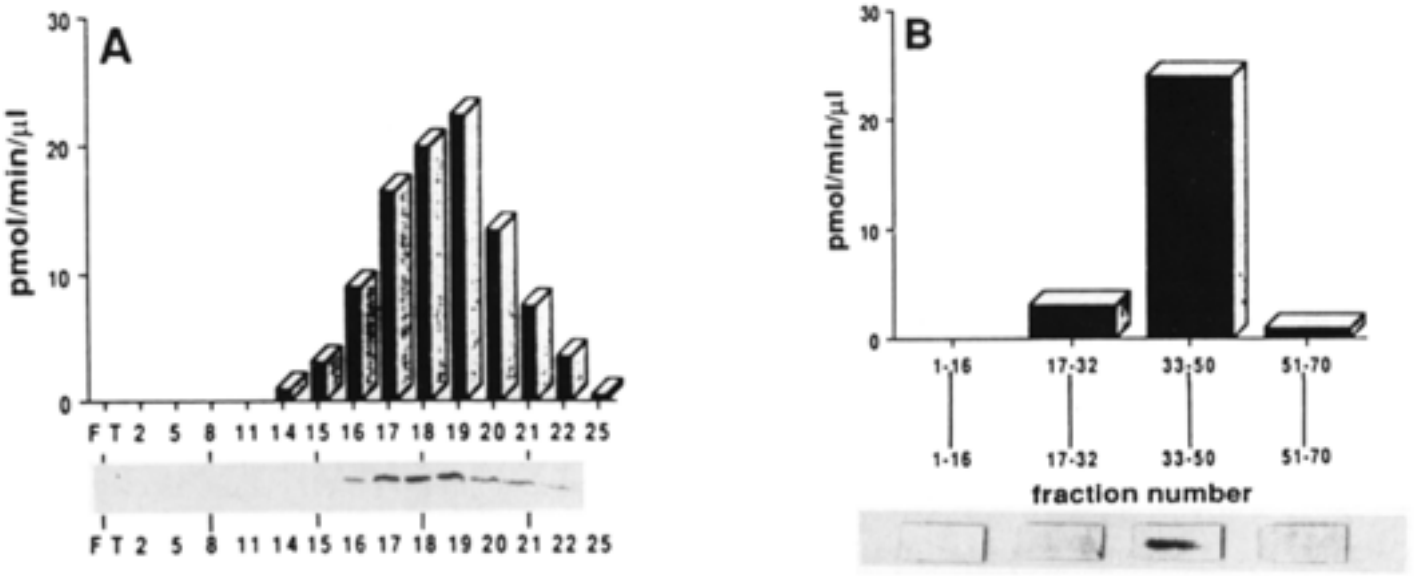
As I mentioned at the start of the lecture purification of MPF was not so successful. However, once it was recognized that Cdc2 and Cdc28 were conserved critical regulators of the cell cycle researchers took there partly purified MPF and checked to see if the frog homologue of Cdc2 was present. It was, 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

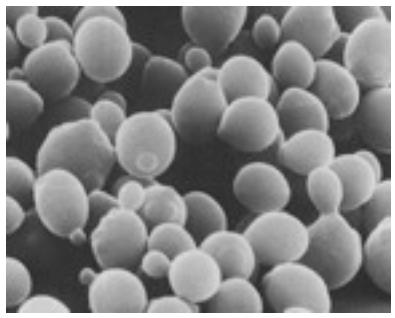
One unit of H1 kinase activity corresponds to 1 μ1mol of ^{32}P transferred per min at 25°C. One unit of MPF corresponds to the amount of MPF required in 50 nl of a microinjected sample for 50% of the recipient Xenopus oocytes to undergo GVBD (Wu and Gerhart, 1980).



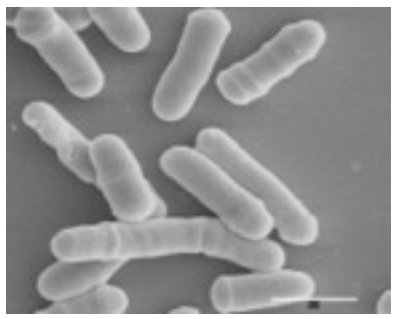
To show that MPF contained Cdc2 the researchers who had purified MPF used an early antibody against Cdc2 (called anti-PSTAIR - a motif found in all Cdc2 homologues) and showed that Cdc2 co-purified with histone H1 kianse activity (and a 34kD protein). The left figure are the fractions off the hydroxylapatite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

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

budding yeast

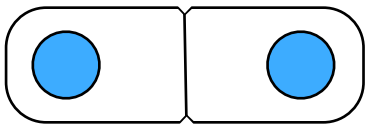


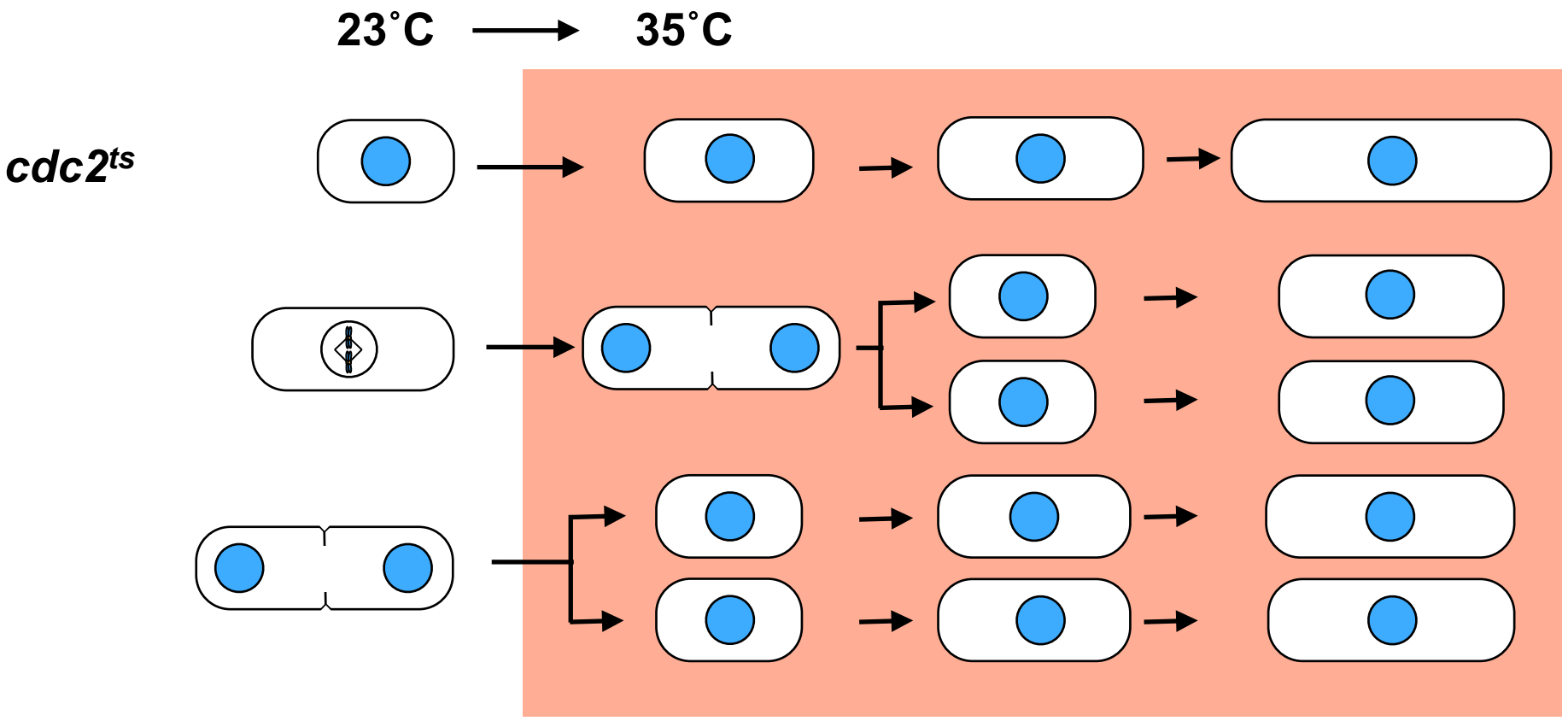
fission yeast



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.

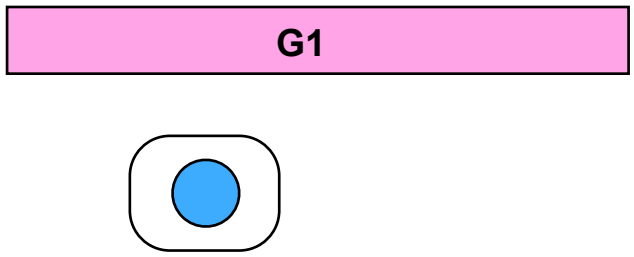
Asynchronous fission yeast contain virtually no cells in G1



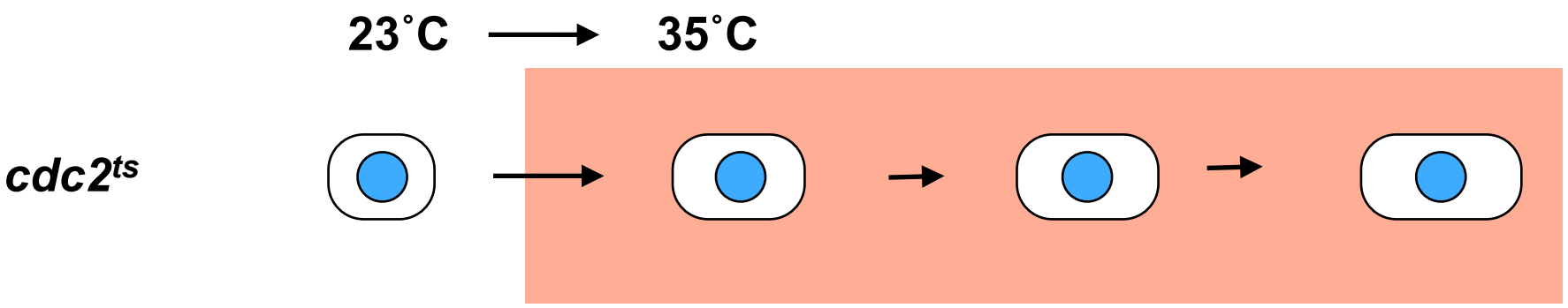


Although cdc2-ts arrests in G2, when a asynchronous population of fission yeast are shifted to 35 degrees its unlikely any of them would arrest in G1 because most cells in the population don't have a G1 phase.

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

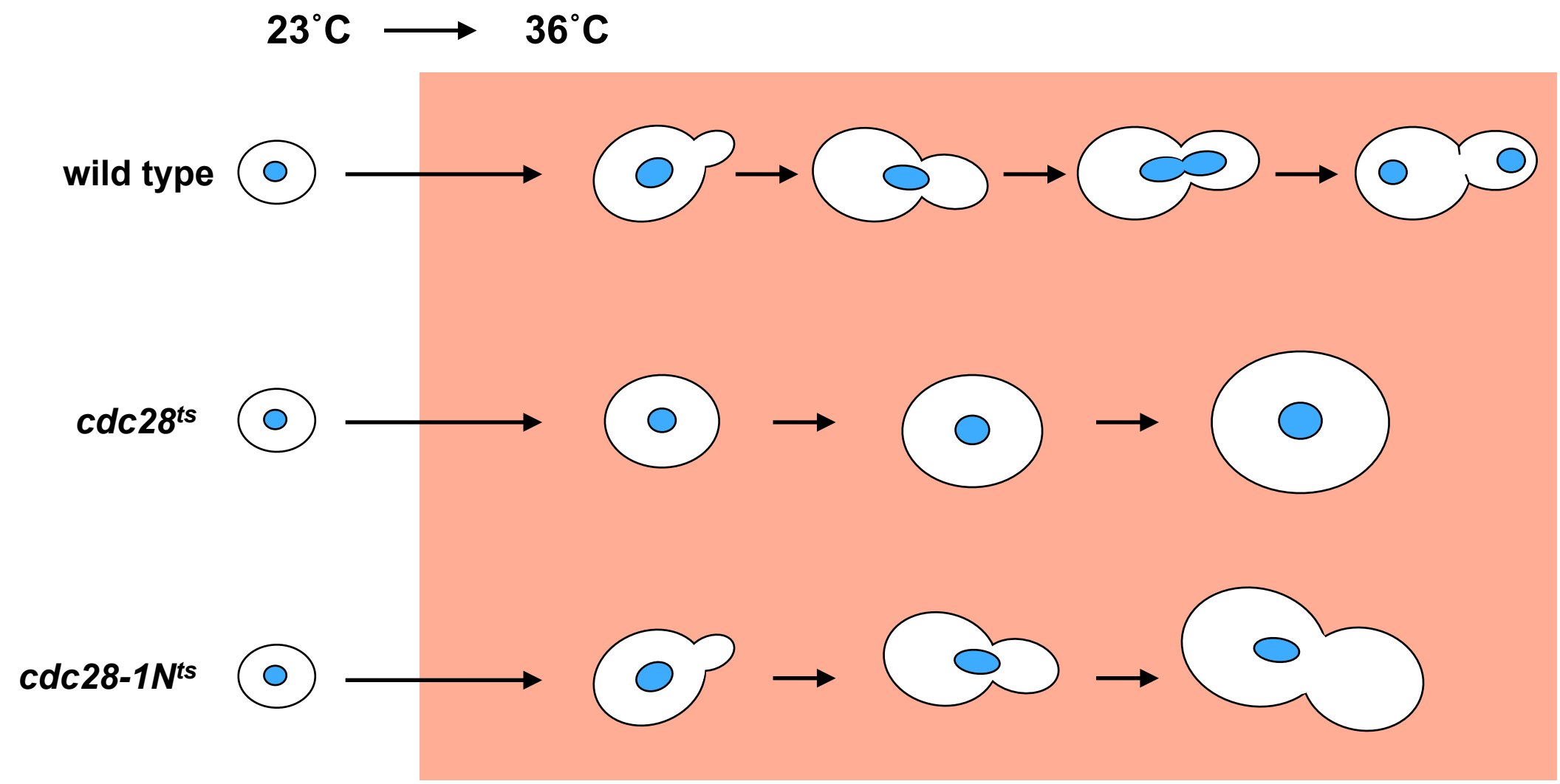


When released into fresh nutrients at the restrictive temperature, *cdc2* mutants arrest in G1 at START like *cdc28*^{ts} mutants in budding yeast



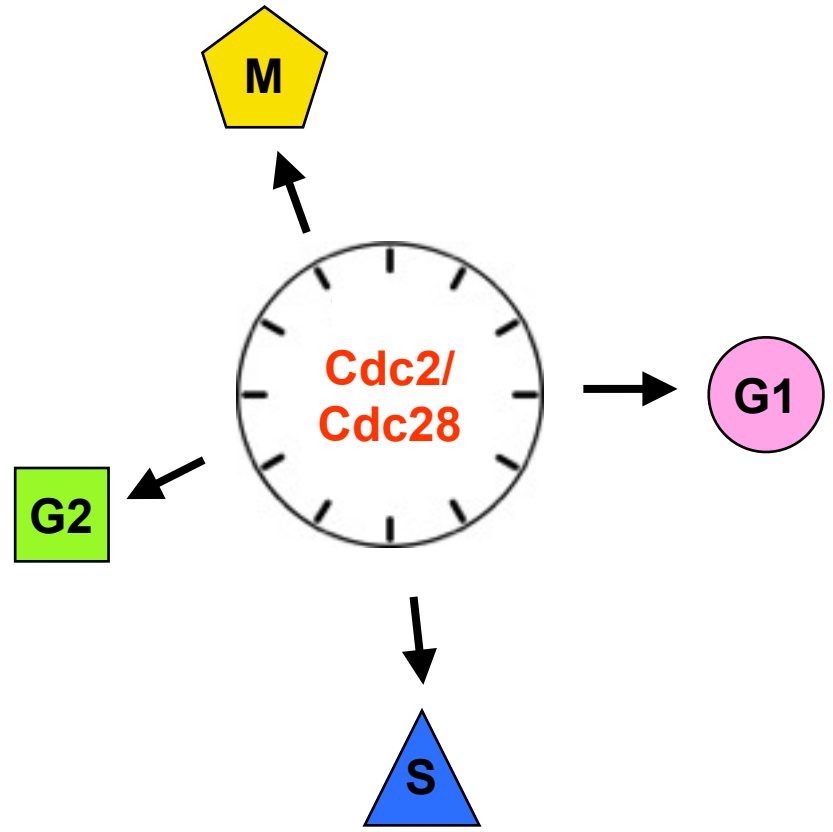
Nutrient starvation is a way to cause a prolonged G1-like arrest (actually cells arrest in G0), if cdc2-ts are released from this arrest at 35 degrees they arrest in G1, not G2, showing that Cdc2 in fission functions at two cell cycle stages, G1 and G2.

Alleles of *cdc28* can arrest at two points



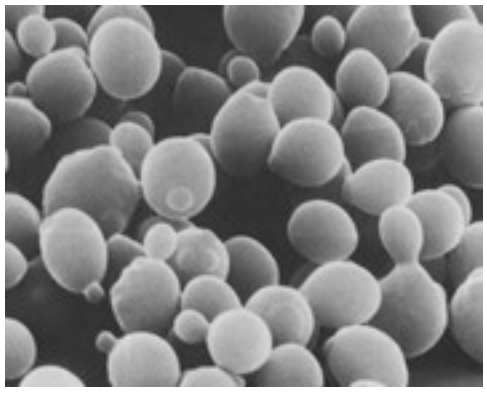
A special allele of CDC28 revealed that Cdc28 in budding yeast also regulates mitosis. cdc28-1N, a different allele of cdc28 arrested in mitosis and its execution point is in mitosis. It is thought that this allele was difficult to find because the requirement for Cdc28 protein is much lower in mitosis 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

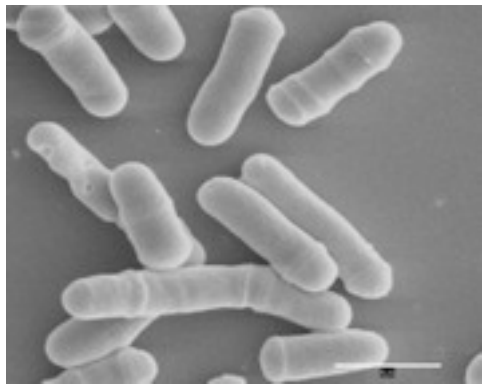


This suggested that Cdc28 in budding yeast and Cdc2 in fission yeast regulate multiple cell cycle transitions, and behaves as we might expect the clock or engine of the cell cycle to behave.

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

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

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Russell et al. cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell (1986) vol. 45 (1) pp. 145-53. How *cdc25* functions to regulate *cdc2*.

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*.

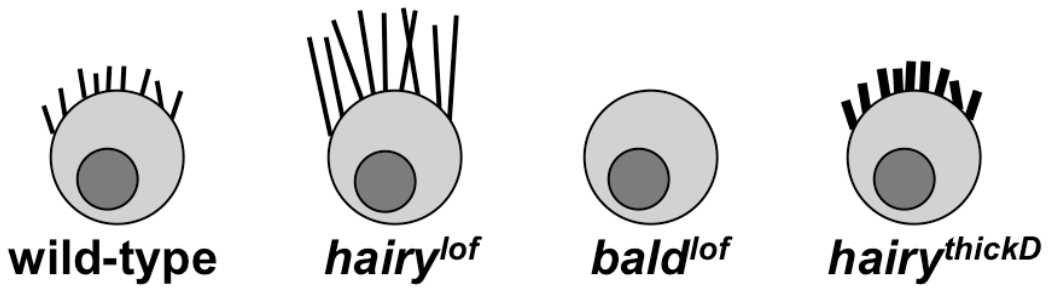
Wood, J.S. & Hartwell, L.H. A dependent pathway of gene functions leading to chromosome segregation in Saccharomyces cerevisiae. J Cell Biol 94, 718–726 (1982).

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).

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).

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?