

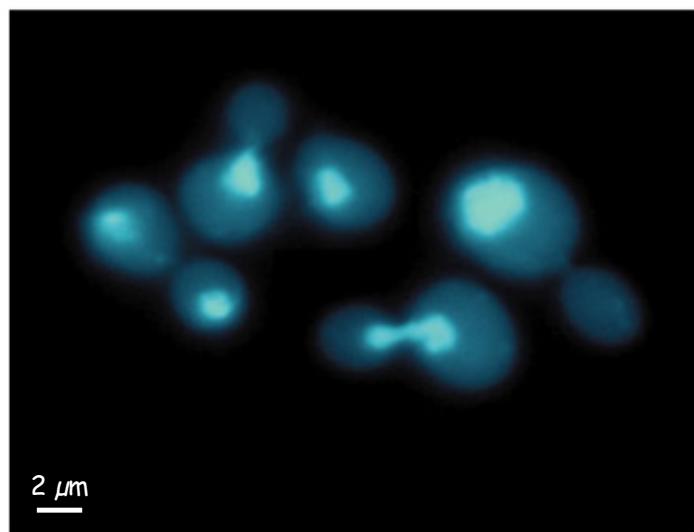
Module 1
The Eukaryotic Cell Cycle

Michaelmas Term 2025
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Lecture 1

The language of GENETICS & Yeast as a model

A genetic model: the budding yeast
Saccharomyces cerevisiae



Please review the Yeast Genetics Primer (Pg 5)

Dissecting the cAMP pathway in yeast using Genetics

I) Mutants dependent on cAMP for growth

1) Isolate mutants that cannot grow in the absence of external cAMP:

one essential locus identified: *CYR1* -

a *cyr1* mutant stops division before bud emergence in the absence of external cAMP

~same response as absence of nutrients~

2) Isolate SECOND SITE SUPPRESSORS allowing a *cyr1* cell to grow without cAMP

one non-essential locus identified: *BCY1*.

In the absence of nutrients, a *bcy1* mutant continues to divide and dies.

II) Find mutants from unrelated screens with similar phenotypes

cdc35 was ALLELIC to *cyr1*

cdc25 can be rescued by exogenous cAMP but it is not allelic to *cyr1*

and with Molecular Biology-based tools.....

- Screen a library for clones COMPLEMENTING a *cdc35^{ts}*: sequence showed "similarity" to adenylyl cyclase. *cdc35^{ts}* at RESTRICTIVE TEMPERATURE had low levels of cAMP. *CDC25* was also cloned by complementation of *cdc25^{ts}* and mapped to a separate locus.

- Based on the clones, the endogenous genes were DISRUPTED: both essential. Lethality could be rescued by exogenous cAMP or by a *bcy1* mutation.

- *CDC35* encodes adenylyl cyclase while *CDC25* may encode an activator

III) Finding more genetic interactors

- Screen for MULTICOPY SUPPRESSORS of a *cdc25^{ts}*->3 loci: *TPK1*, *2*, and *3*.

- These loci encode the catalytic subunit of cAMP-dependent protein kinase

- Triple deletion is lethal. Cannot be rescued by adding cAMP or a *bcy1* mutation

- Screen for SECOND SITE SUPPRESSORS of a *cdc25^{ts}*: isolated 4 additional loci- *PDE1*, *2* and *IRAI* and *2*.

- The sequence of the *PDE* genes suggested similarity to phosphodiesterases (enzymes that convert cAMP into 5'AMP)

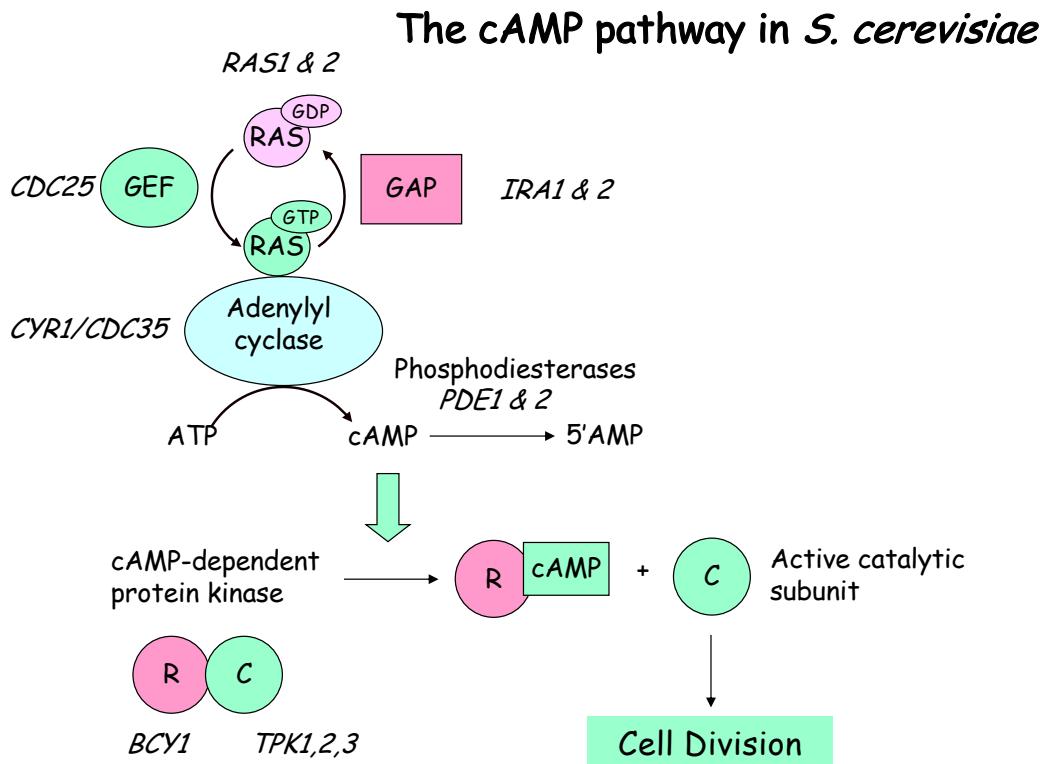
IV) The missing link: identification of yeast **homologues** of human **RAS**

- Using the cDNA of the protooncogene **Harvey-ras** as a probe, two loci were identified in yeast by Southern blot analysis.
- The genes were cloned by colony hybridisation using the same probe: **RAS1 & 2**.
- Double disruptant is dead but can be rescued by cAMP or a *bcy1* mutation
- Lethality was also rescued by **Harvey-ras** expressed in yeast!!!
- A mutation analogous to the oncogenic "activated" H-ras^{Val12} was introduced in RAS2: **RAS2^{Val19}**. Activated RAS2^{Val19} was oncogenic in mammalian cells!!!!
- A **RAS2^{Val19}** strain contains high cAMP levels and fails to stop dividing in the absence of nutrients (like *bcy1* mutants)
- **RAS2^{Val19}** suppresses a *cdc25^{ts}* BUT NOT a *cdc35^{ts}* strain
- The sequence of the **IRAI** and **2** genes predicted products related to human GAP (GTPase activating protein). Ira1 and Ira2 were shown to act as GAPs for yeast RAS.

Based on these data,

can you suggest a model for

the cAMP pathway?



Homework: complete the table & answer the questions - submit for feedback

Mutant	Relevant phenotypes		
	Division in response to nutrients	intracellular cAMP levels	Suppression by exogenous cAMP
<i>ira1 ira2</i>	cannot arrest	high	n/a
<i>RAS2^{val19}</i>	cannot arrest	high	n/a
<i>bcy1</i>	cannot arrest	low	n/a
<i>cdc25</i>	cannot bud	very low	yes
<i>ras1 ras2</i>	cannot bud	very low	yes
<i>cdc35</i>	cannot bud	undetectable	yes
<i>tpk1,2,3</i>	cannot bud	high	no
<i>cdc25 RAS2^{val19}</i>	cannot arrest	high	n/a
<i>cdc35 RAS2^{val19}</i>	cannot bud	undetectable	yes
<i>cdc25 ira1 ira2</i>	?	?	?
<i>cdc35 ira1 ira2</i>	?	?	?
<i>ira1 ira2 ras1 ras2</i>	?	?	?
<i>ira1 ira2 ras1 ras2 bcy1</i>	?	?	?
<i>ira1 ira2 tpk1,2,3</i>	?	?	?

With the help of the diagram for the cAMP pathway and the Appendix on *S. cerevisiae* genetics in the next pages, answer these questions:

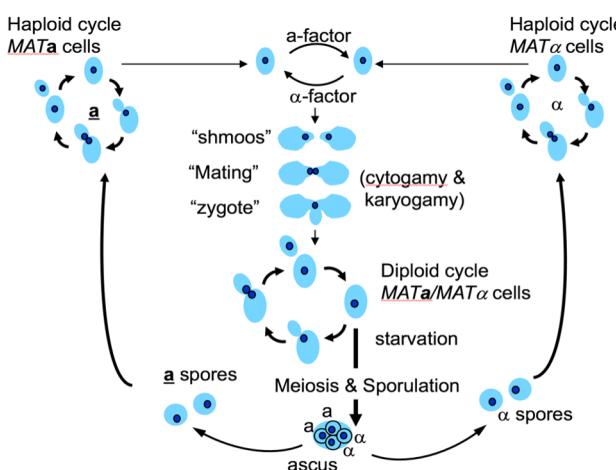
A dominant allele *TPK1^W* was isolated as a second site suppressor of a *cdc35ts*.

- a) What might be the mechanism of rescue by this mutant?
 - b) Suggest a simple experiment involving plasmid-based manipulations to confirm your hypothesis.
- A dominant allele *BCY1^C* was isolated as second site suppressor of *RAS2^{val19}* lethality upon starvation.
- c) What might be the mechanism of rescue?
 - d) Would your hypothesis be consistent with *BCY1^C* being suppressed by *TPK1^W*?

----- Appendix -----
S. cerevisiae - a genetic model

A primer to yeast genetics tools has been added to this handout to facilitate reading and understanding primary literature later. Be ready to discuss any emerging issues in supervisions. Taught in 1a Biology of Cells and 1b Cell and Developmental Biology.

Saccharomyces cerevisiae life cycle



S. cerevisiae exists as distinct cell types during the life cycle: *a* and *α* haploid cells or *a/α* diploid cells. Those can proliferate by mitotic cell division. *a* haploid cells can mate with *α* cells, a process triggered by reciprocal signals (*a* and *α* factor), followed by cell and nuclear fusion to give rise to *a/α* diploid cells.

a/α diploids cannot mate but can undergo meiosis upon nutritional starvation. The **four progeny spores of a single meiosis** are wrapped up together in an **ascus**. In general, laboratory strains have been manipulated to be "heterothallic" for ease of genetic analysis as these strains exist as stable *a* or *α* haploid populations that give rise to diploids solely when mixed together.

a and *α* factor (aka mating pheromones) cause **cell cycle arrest** of *a* and *a* cells, respectively **in late G₁** in preparation for mating. **Synthetic α-factor** can be used to **synchronise a cell cultures**. After arresting in *G₁*, the pheromone is washed off giving rise to a population that proceeds synchronously - very useful in cell cycle studies. Other drugs can be used to block cells in S phase or mitosis.

Yeast Genetic Nomenclature

Usually hints at function or acronym from initial phenotypic characterisation

TUB1

locus name

In classic genetic analysis: number designates a single "complementation group"

The gene product of *TUB1* (the protein) is designated Tub1 or Tub1p

Wild type gene, uppercase, italics: e.g. *TUB1* is the locus encoding alpha tubulin (Tub1)

Recessive alleles, lowercase, italics: e.g. *tub1-203*

allele number

Dominant alleles, uppercase, italics: e.g. *RAS2^{Val19}* (superscript denotes a substitution)

Also:

Deletions are designated by a Δ, e.g. *tub1Δ*

Temperature-sensitive alleles are designated by a ts superscript, e.g. *tub1-5^{ts}*

PLEASE NOTE
and use correct nomenclature
in your essays

Yeast shuttle vectors (simplified)

Designed for manipulation by standard DNA recombinant technology and propagation in bacteria. In addition, they carry genetic elements for *selection* and *maintenance* in yeast:

- A bacterial-plasmid backbone: bacterial replication origin, bacterial selectable marker (AmpR, TetR)
- A yeast selectable marker (see below)

- In autonomous plasmids (as opposed to integrative), a yeast origin of replication:

a) **Low copy plasmids:** a minimal CEN sequence (centromere) and ARS (yeast origin of replication)

b) **Multicopy plasmids:** a fragment of the yeast 2 μ m plasmid including the origin of replication and copy number control elements

- Expression vectors: to express a specific ORF (e.g. for inducible expression &/or to achieve over-expression - *GAL1-10* promoter (OFF in dextrose, ON in galactose) *MET3* promoter (OFF if methionine added, ON in the absence of methionine))

Yeast selectable markers (examples)

Auxotrophic markers

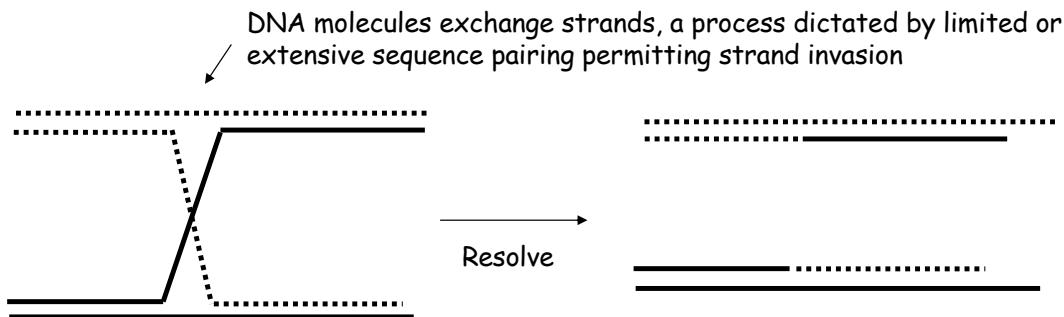
ura3-52 Loss-of-function, recessive allele of *URA3*. Mutant cannot grow in medium lacking uracil
A plasmid carrying the wild type *URA3* gene COMPLEMENTS the mutation. Transformants with the plasmid can be SELECTED by growing the cells in the absence of uracil

Drug-resistance markers

KAN^R Yeast is naturally sensitive to the drug G418

A plasmid carrying *KAN^R* (a bacterial gene) confers RESISTANCE to the drug in yeast. Transformants can be SELECTED by growing the cells in medium containing G418.

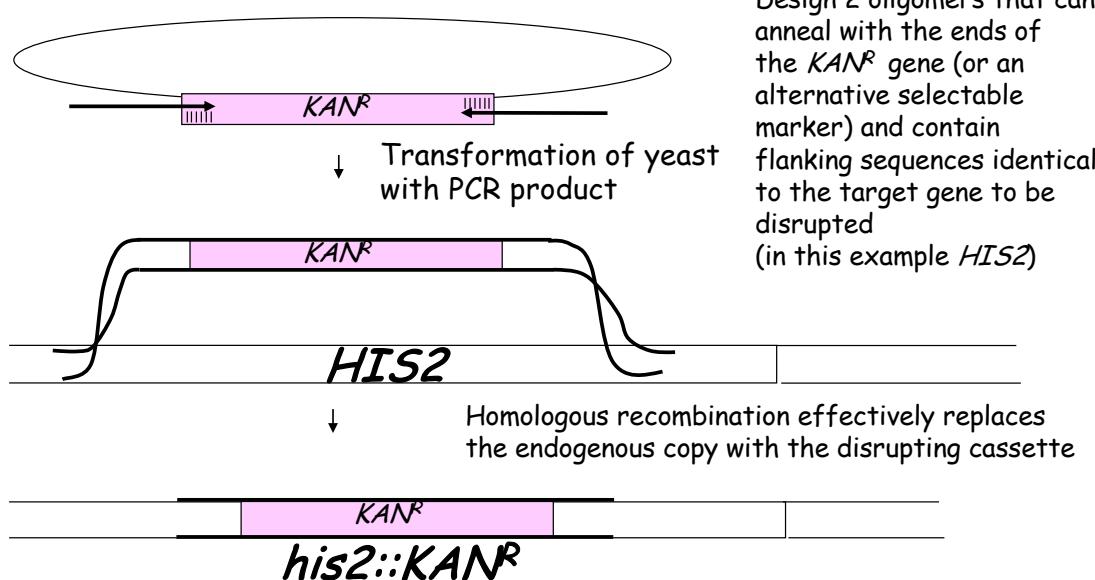
Homologous recombination



A highly efficient process in *S. cerevisiae*
It provides a basis for manipulating endogenous loci at will.

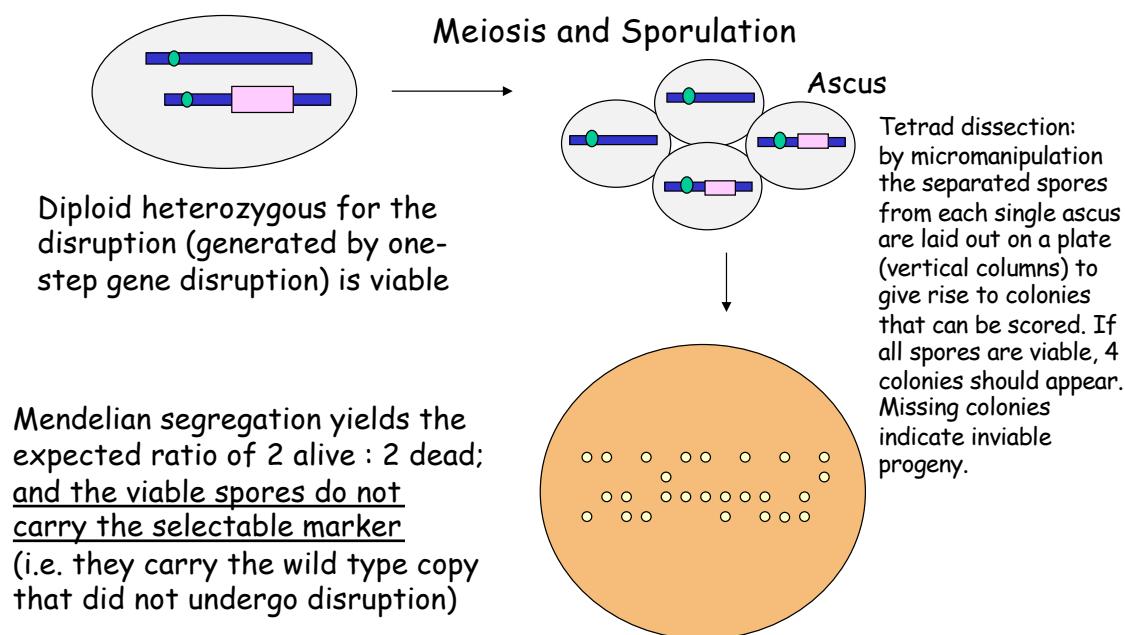
Alleles, deletions, tagging constructs, etc. are designed such that when introduced into yeast by transformation, they will undergo targeted integration at the intended locus by *homologous recombination*. The result is a precise replacement of the endogenous locus by the construct.

Creating deletion mutants: PCR-based one-step gene disruption



Transformants will be resistant to G418 (as the bacterial *KAN^R* gene confers resistance to the drug G418 aka geneticin) and unable to grow in the absence of histidine

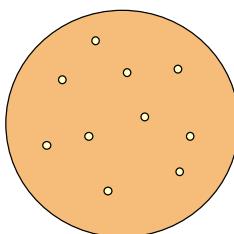
One-step gene disruption of an *ESSENTIAL* gene



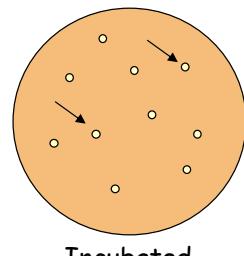
→ the disruption of an essential gene is LETHAL

Conditional mutants: screening for temperature-sensitive mutations

Plate a mutagenised population of cells (e.g. UV-, MMS-treated, etc.) to obtain individual colonies and incubate at 25°C



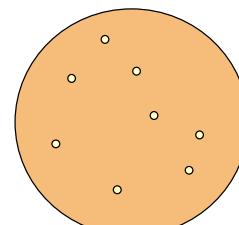
Replica plating
→



Incubated at 25°C

Colonies missing at high temperature most likely correspond to *ts* mutants for an essential gene

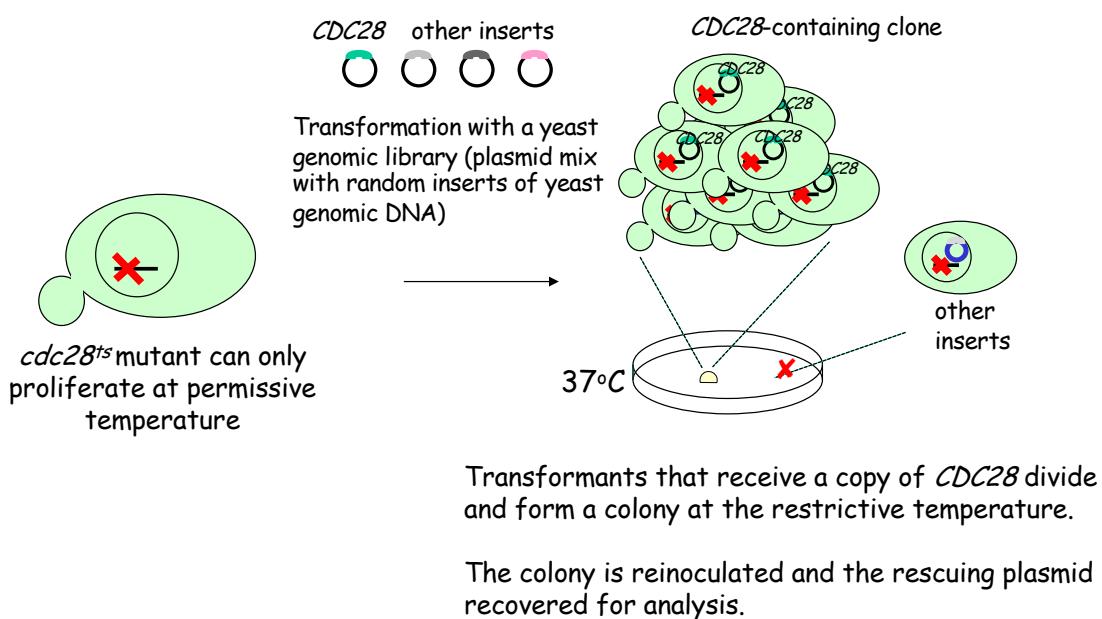
i.e. *ts* mutants for an essential gene are
CONDITIONAL LETHAL mutants



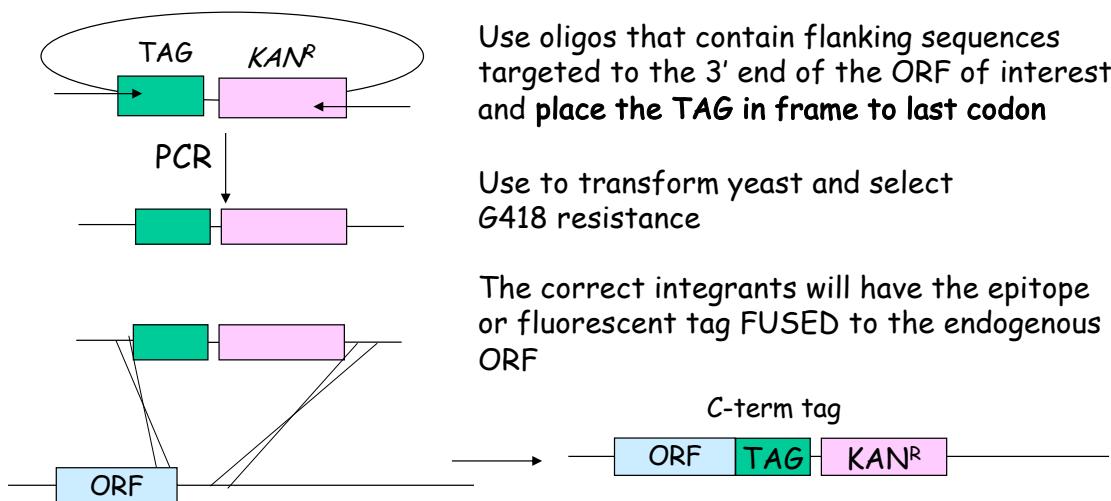
Incubated at 37°C

Such an approach was used for isolating a collection of *cdc* (cell division cycle) mutants

Isolation of a wild type gene by complementation of a *ts* mutant



PCR-based tagging of yeast sequences

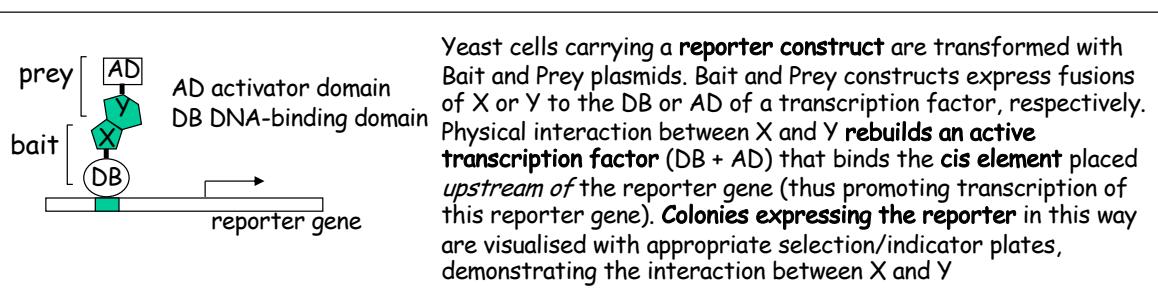


Popular tags for protein analysis: HA₃, Myc₆, FLAG₃, His₆, GST, TAP, MBP, etc.

Popular tags for live fluorescence microscopy: GFP, CFP, YFP, DsRed, mCherry

Other yeast-as-test tube-based approaches

- Identification of structural and functional homologues using yeast mutants (by screening heterologous expression libraries; e.g. we will learn how this approach led to identification of the human mitotic CDK, Cdc2^{Hs} and human Cyc D and E)
- Dissecting the mechanism of function or conserved regulatory pathways of heterologous proteins studied in the context of yeast (e.g. we'll learn how this approach was used to figure control of human cyclin E proteolysis)
- Two-hybrid screens: a method to probe protein-protein interactions



**The sequence of *S. cerevisiae*
(1st eukaryotic genome to be sequenced)**
can be accessed at <http://www.yeastgenome.org/>

Outlook for Genetics, M1 & beyond?

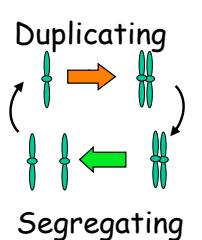
- The Yeast Genome Project has been a model for other projects to follow
- Genome data informs genetic manipulation, genetic analysis and screens
- Overall genome organisation in physical terms
- Comparison of complete genomes (genetic variation & evolution)
- Global/systematic/genome wide analysis/reverse genetics (deletions, tagging, etc.)
- Live imaging, transcriptomics, proteomics, epigenomics, metabolomics, interactome
- Single-cell & population analysis
- Systems and synthetic biology

Lecture 2

Cell cycle control in eukaryotic cells (I)

Basic principles & master regulators

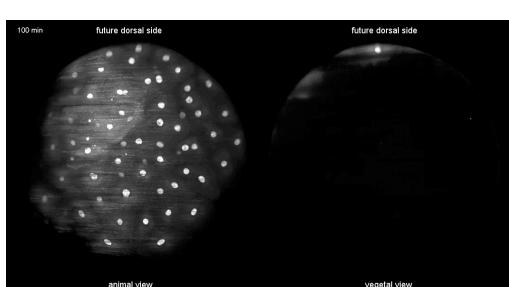
The simplest cell cycle:
cells specialized for rapid proliferation



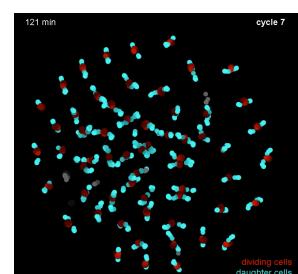
- Embryonic cleavage:
- . cell cycle *without* cell growth
 - . synchronous



From synchronous to asynchronous divisions in the embryo

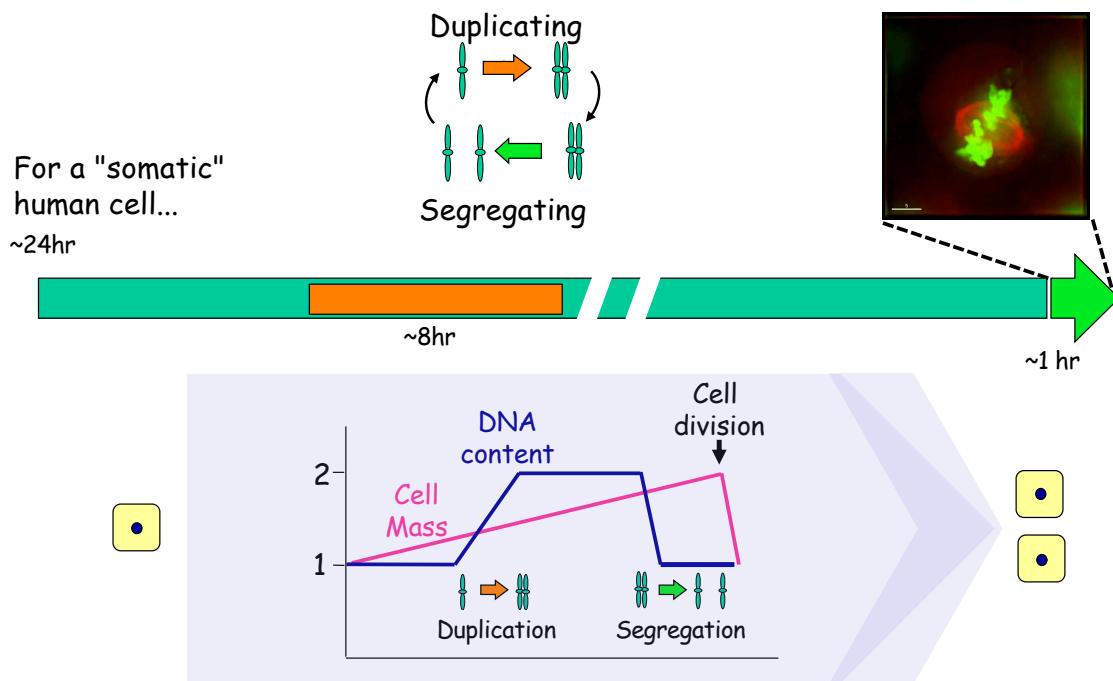


Zebrafish embryo

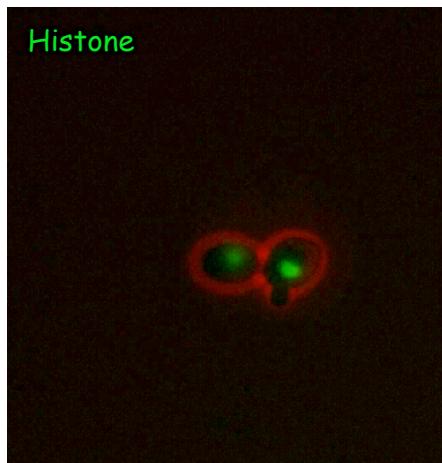


Keller et al. (2008) *Science* 322: 1065

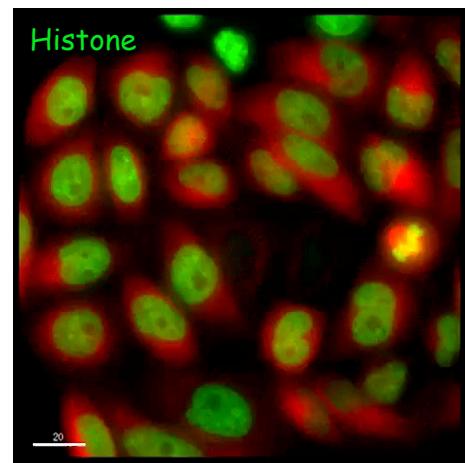
Cell division and cell growth



The somatic cell cycle: cell division and cell growth

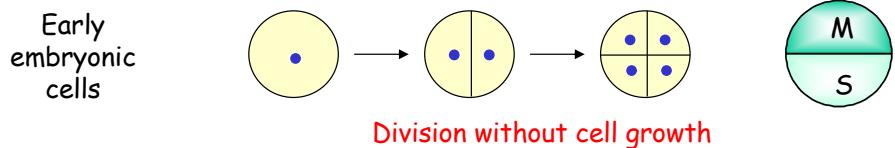


Saccharomyces cerevisiae



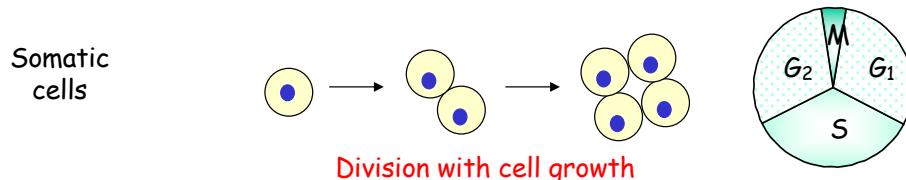
HeLa cells

Embryonic vs Somatic cell cycle



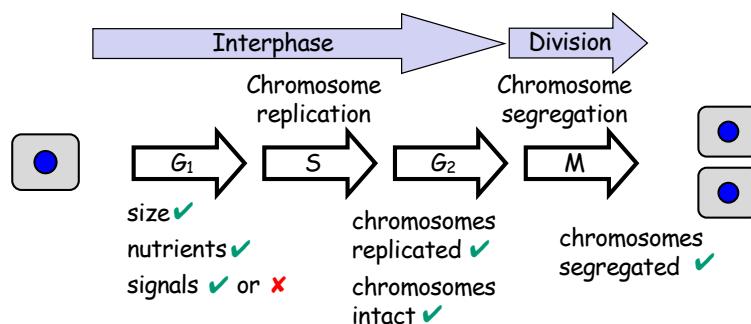
Fertilised eggs are exceptionally set for proliferation thanks to massive maternal stockpiles of cellular transcripts and factors promoting fast cell divisions (cleaving embryo):

- Cells specialised for rapid and synchronous cell division without cell growth \Rightarrow no G₀
- Cell division produces progressively smaller cells in successive cell cycles
- Popular models: frogs, marine invertebrates, also fruit fly syncytial embryo



- Most other cells dividing mitotically. Also, fission and budding yeast.
- Canonical cell cycle: G₁, S, G₂, M; prominent control points; can be synchronised by drugs
- Cells coordinate cell division and cell growth, thus ensuring cell size homeostasis
- In both fission and budding yeast, morphology may be correlated with cell cycle position
- Budding yeast: bud emergence is an important landmark
- For animal cells, new markers developed in recent years distinguish G₁, S, G₂ and M

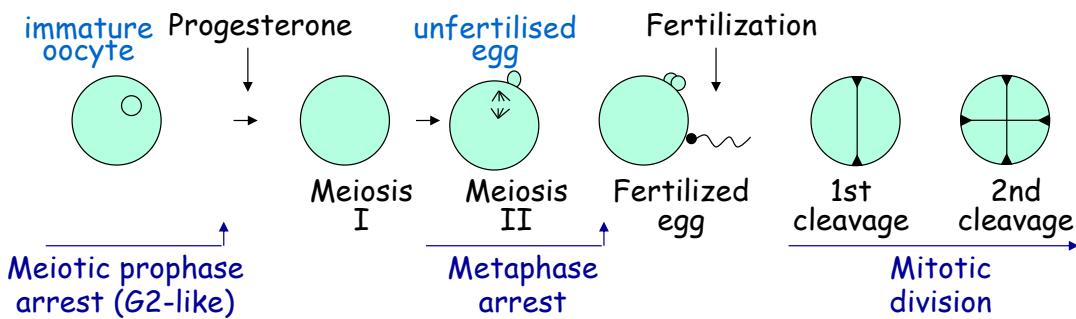
Significance of cell cycle *control*



- Order: accuracy and fidelity — genome and cell integrity
- Staging cycling dynamics according to developmental context
- Govern a fundamental decision — *to cycle or not to cycle*
 - subject to nutrients, stresses & internal cues
 - mitogenic and anti-mitogenic signals
 - cell fate —exit indefinitely or not

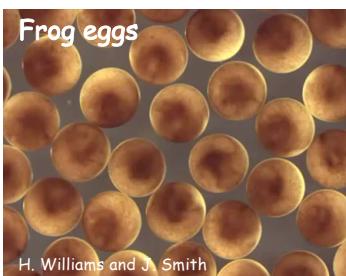
Maturation Promoting Factor, a bit of history

- Early in development, a pool of oocyte precursors is set aside and remains arrested as "immature oocytes" in a "G2-like state in early meiosis
- Progesterone triggers "maturation" i.e. completion of the first meiotic division and arrest at metaphase of meiosis II. After fertilization, the egg completes meiosis followed by the first mitotic divisions in the cleaving embryo. The entire process has been studied in multiple models including Xenopus, clams & sea urchins

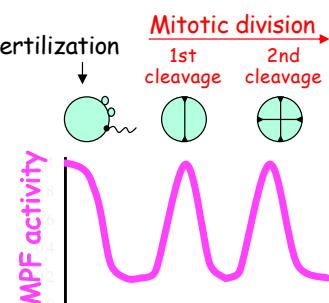


Cytoplasmic transfer from oocytes that underwent maturation triggered by progesterone, drove maturation of immature oocytes *bypassing progesterone use*. A factor that induces such maturation was postulated and called **maturation promoting factor (MPF)**. MPF activity was found to oscillate with the period of cleaving divisions and ultimately recognized as an **M-phase inducer**.

An activity that correlates with M phase



- An M-phase inducer was identified as "Maturation-promoting factor, **MPF**"
- Oocyte fertilization triggers synchronous mitotic divisions in the "cleaving" embryo
- MPF activity oscillates with the period of those cleaving divisions



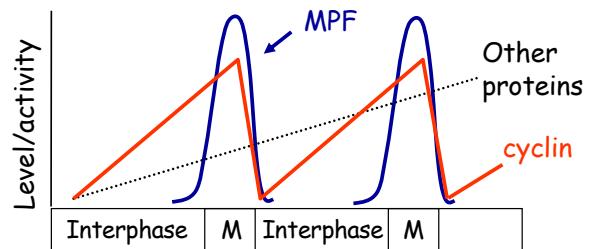
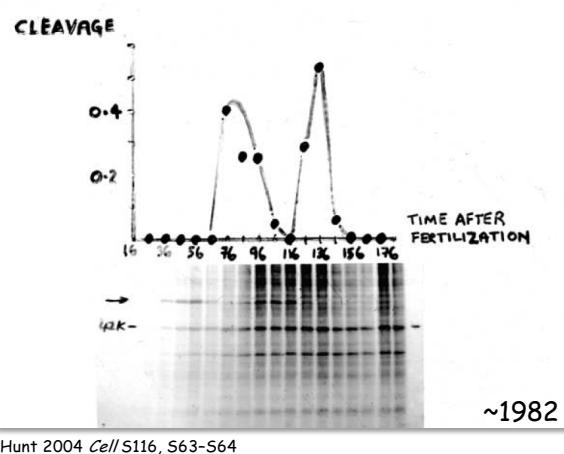
An activity that correlates with M phase

Cyclin B accumulation mirrors MPF activation

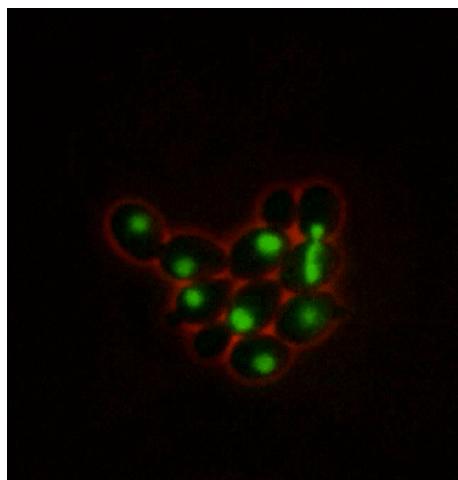
- Peaks coincident with M phase
- Disappears at the end of mitosis

Cyclin B is a component of MPF

- Purified MPF contains two polypeptides:
 - ~ 46 kDa, cyclin B
 - ~ 32 kDa ???



The genetic quest for a master regulator of the cell cycle



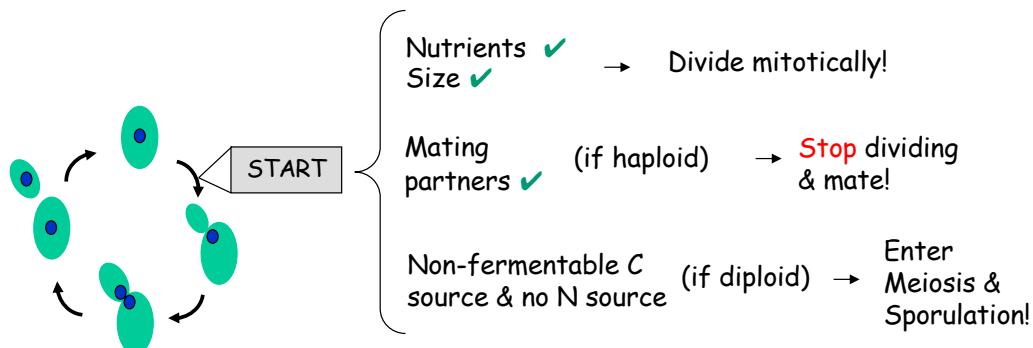
...Saccharomyces cerevisiae!
Behold the awesome power of yeast genetics SGD

Budding yeast

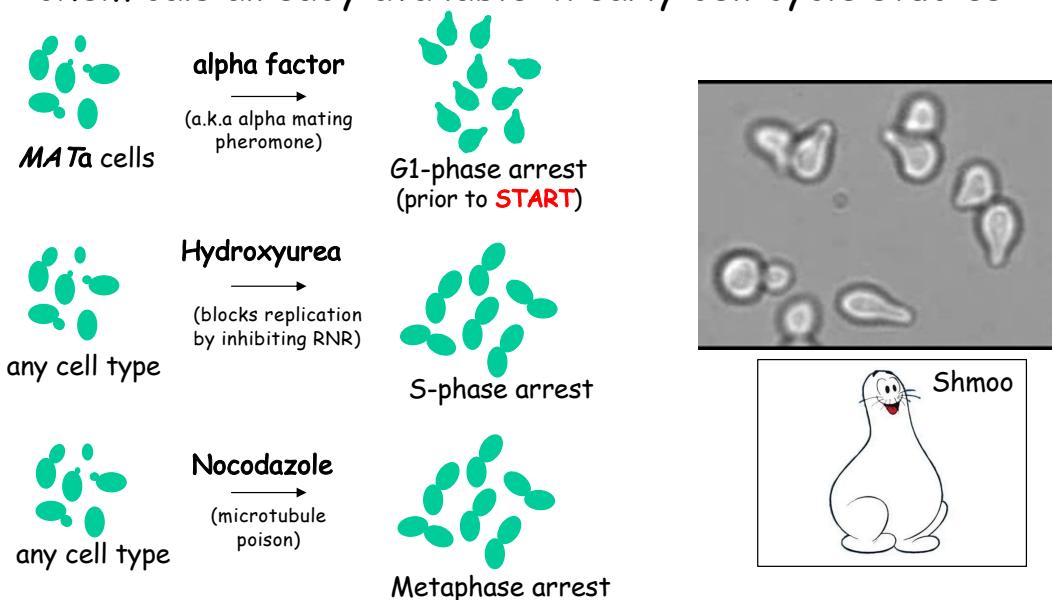
All aspects of somatic cell cycle control

Morphology is diagnostic of cell cycle position

START, a commitment point in the yeast cell cycle

Hartwell et al. (1974) *Science* 183:46-51

Yeast cells could be synchronised by blocks induced by chemicals already available in early cell cycle studies:



Compounds that specifically block a given cell cycle event caused a yeast culture to arrest with a uniform cell morphology (often indicative of the position of the block in the cell cycle) **without otherwise impeding cell growth**. Thus, in principle, *cdc* mutations that disable progression across cell cycle control points could be expected to result similarly in cells arresting with uniform morphology, i.e. synchronised at the point of the block.

Isolation of mutants defective in cell cycle control

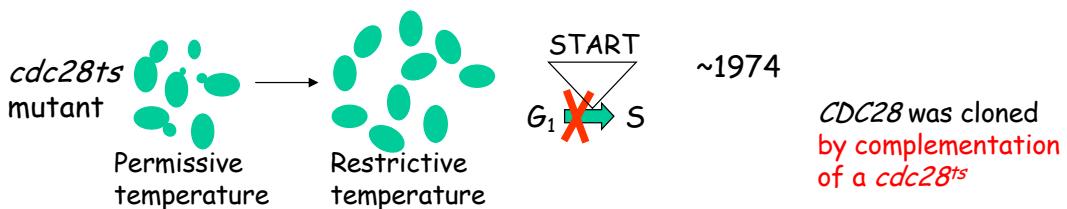
- Conditional *ts* mutants
- Cannot proceed along the cell cycle at the restrictive temperature
- Arrest with uniform morphology (terminal phenotype)
- A major landmark in cell cycle research



A collection of *Cell Division Cycle* mutants was isolated and characterised independently first in *S. cerevisiae* and later *S. pombe*

Among the mutants isolated, budding yeast *cdc28ts* cells failed to proceed past START at the restrictive temperature

CDC28 encodes a conserved protein kinase



- *CDC28* encodes a ~34kd protein with similarity to protein kinases
- *CDC28* and *S. pombe cdc2⁺* encode functional homologues (*CDC28* can replace *cdc2⁺* in *S. pombe* and vice versa)
- By screening a human cDNA expression library in a *S. pombe cdc2^{ts}* mutant, the corresponding human homologue was identified (~1987)

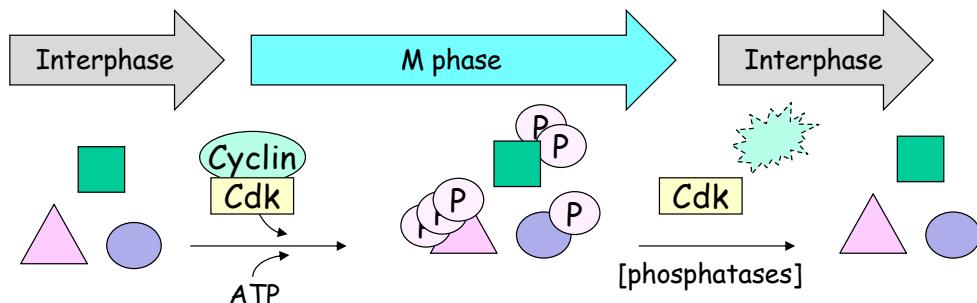
p34^{cdc2/CDC28} is a key cell cycle regulator
conserved throughout evolution

Antibodies raised against Cdc2 cross-react with the 32kDa subunit of MPF

MPF is the prototype of an evolutionary conserved family of Ser/Thr protein kinases:

Cyclin-dependent Kinase (CDK)

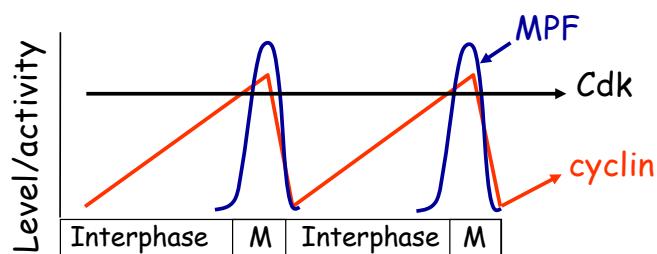
Cyclin: regulatory subunit
Cdk: catalytic subunit



2001 Nobel Prize in Physiology or Medicine

"for their discoveries of key regulators of the cell cycle"
to Lee Hartwell, Tim Hunt and Paul Nurse

MPF activity correlates with the mitotic state



Is the "master regulator" one and the same?

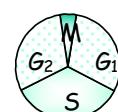
Early embryonic cells

 Division without cell growth



Somatic cells

 Division with cell growth



The somatic cell cycle: more genetic screens, more regulators

High copy number suppressors* of *cdc28-4^{ts}* (arrests prior to START):

- . *CLN1*, *CLN2* and *CLN3* encoding "G1 cyclins"
- . *P_{GAL1}:CLN2** *cln1Δ cln2Δ cln3Δ*** strain shifted from galactose to glucose-containing medium*** arrests prior to START.

High copy number suppressors of *cdc28-1N^{ts}* (arrests prior to M):

- . *CLB2*, encoding a "B-type cyclin"
- . Also *CLB1, 3 & 4*, based on sequence similarity

High copy number suppressors of *cln1cln2cln3Δ*:

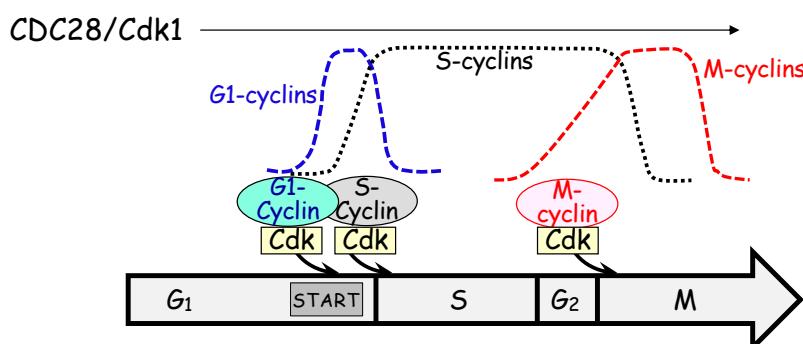
- . *CLB5 & CLB6* encoding "S phase cyclins"

* A dosage interactor: multiple copies of a wild type gene (in a high copy number plasmid i.e. 2μm-based) can compensate or bypass (suppress) the defect caused by *cdc28-4^{ts}*. In this case, inability to proceed past START at the restrictive temperature.

***GAL1* promoter (*P_{GAL1}*) is used to control expression of an ORF of interest. In this case, *CLN2* is expressed in the presence of galactose but repressed when cells are transferred to glucose thus allowing for handling the strain and exploring phenotypes resulting from inactivation of all three *CLN* genes.

***At least one *CLN* gene is sufficient for viability suggesting functional redundancy

Cyclin/Cdk complexes drive the cell cycle



Stage-specific control based on multiple oscillatory cyclins (both transcription and protein accumulation "oscillate" – more later)

Ongoing debate: is order imposed by stage-specific CDK complexes as a result of qualitative differences or by virtue of quantitative differences i.e. overall activity levels? (we will return to this later)

Are S-phase & M-phase promoting factors universal?

Is START universal?

A Restriction Point for Control of Normal Animal Cell Proliferation

Pardee (1974) Proc Natl Acad Sci USA 71:1286-1290

- “...normal animal cells possess a unique regulatory mechanism to shift them between **proliferative** and **quiescent** states.”
- Cells stop proliferation under diverse suboptimal nutritional conditions, but resume from the same point in G1 when nutrition is restored.

The specific time in the cell cycle for this critical decision-making event was called the **Restriction Point (R)**

- Malignant cells are proposed to have lost their restriction point control.

Are START and R one and the same?

The somatic cell cycle in humans: learning from yeast

a) Screen for human cDNA clones that can rescue *cln1Δ/cln2Δ/cln3Δ* lethality:

Isolated cyclin D and cyclin E → Higher eukaryotes like us have G1 cyclins!!!!

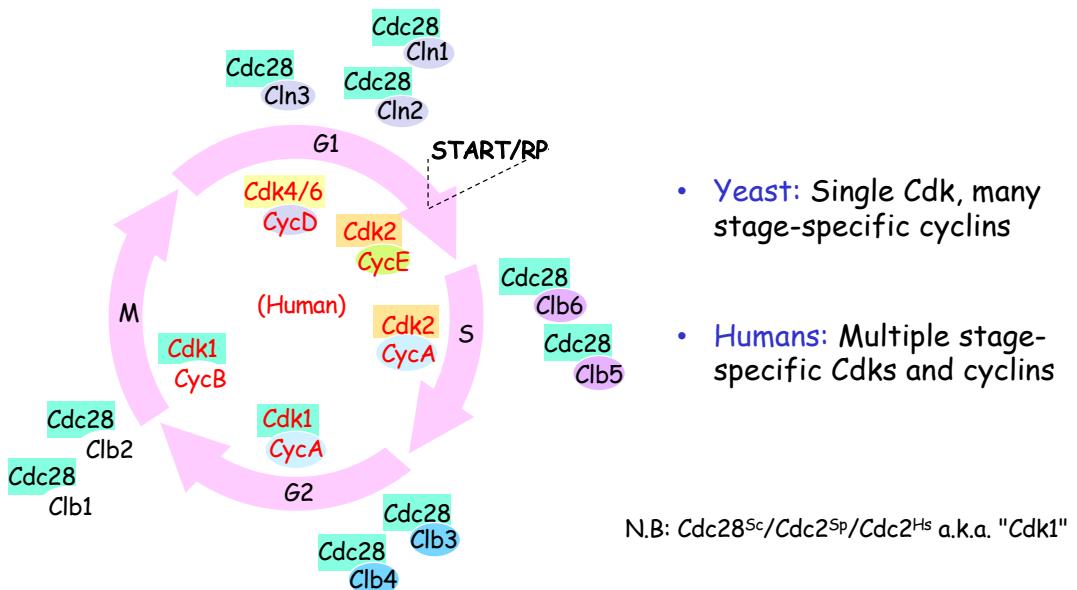
b) Screen for Cdk-related genes based on sequence similarity to *cdc2+*:

various CDK-related sequences were isolated, in addition to the precise counterpart of *cdc2+*



Metazoans have several stage-specific Cdks!

From yeast to humans: stage-specific CDK complexes drive the cell cycle (simplified)



How does CDK set a switch-like transition?

Next lecture... Spotlight on G1/S

To keep in mind during this lecture series!!!!

Our goal is to understand the **general themes** in cell cycle control.

The good news: the key regulators and principles are *exquisitely conserved* throughout evolution.

The bad news: research in different model systems proceeds independently so we will struggle to cross-reference players (oh yes, same component, many names....) emerging from diverse research.

One more point: the precise details and complexity of the circuitry of the cell cycle from yeast to humans may vary while the key principles hold throughout....

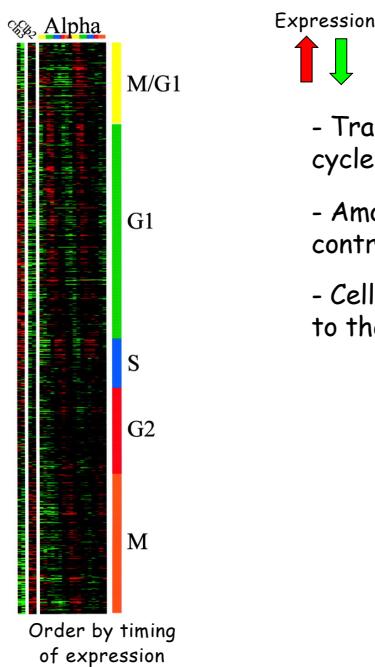
WE WILL FOCUS ON THESE PRINCIPLES!!!!

Lecture 3

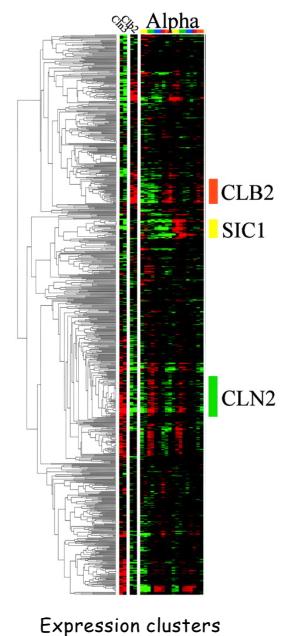
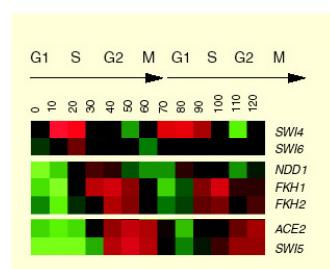
Cell cycle control in eukaryotic cells (II)

The G1/S transition lessons from yeast

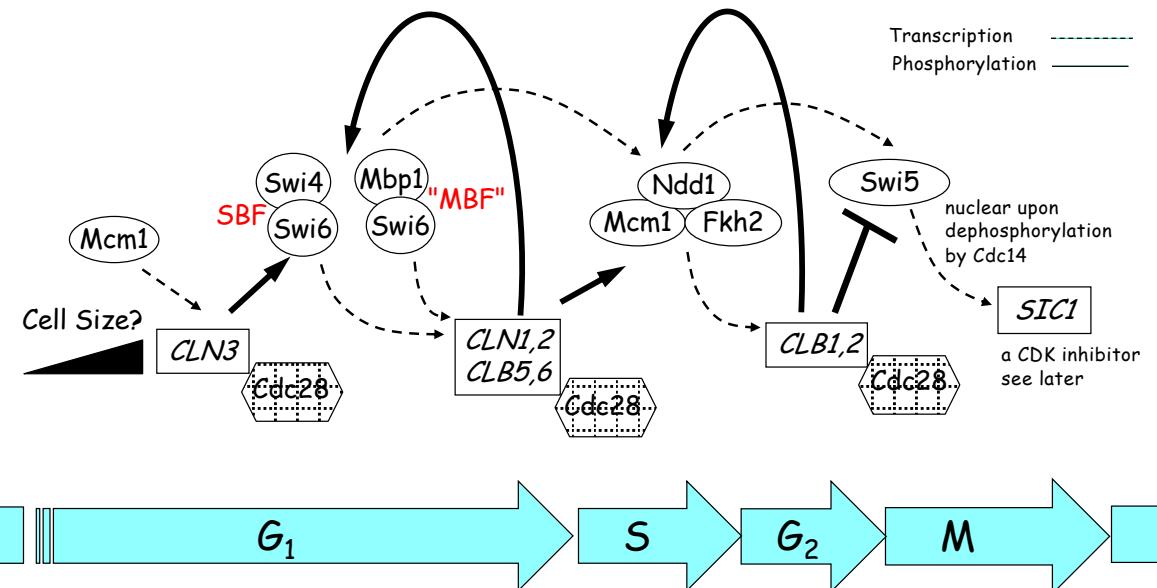
The transcription of cyclin genes is cell cycle-regulated



- Transcription of ~ 600 genes is cell cycle-regulated, including cyclin genes
- Among them also, the TFs that control those expression waves
- Cell cycle-regulated TFs peak prior to their targets, e.g.,

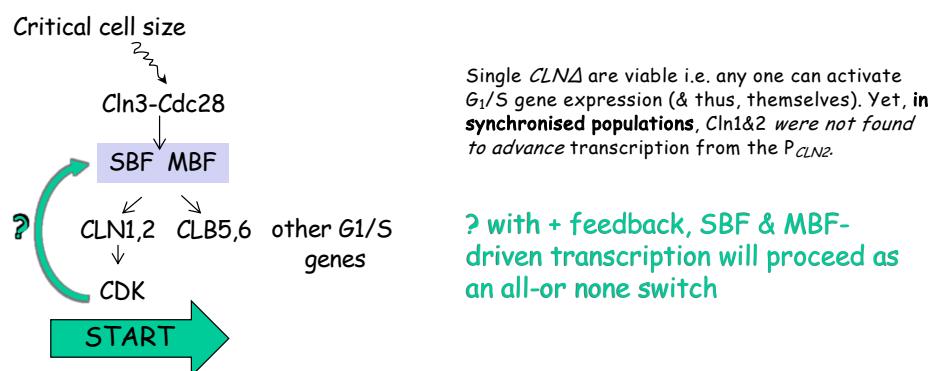


A “transcriptional cycle” based on interdependent waves (simplified)



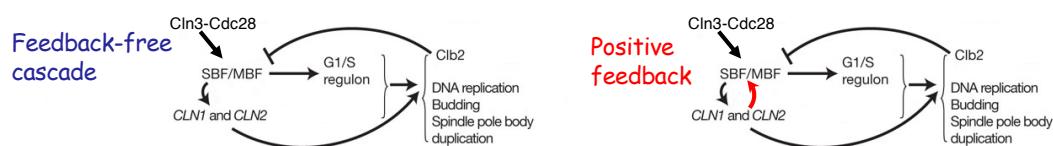
- Key transcription factors peak with each transcriptional wave
- Cyclins are among transcriptional targets
- CDK phosphorylation modulates transcription factor activity and is therefore crucial for transcriptional oscillations

Positive feedback of G₁ cyclins ensures coherent cell cycle entry



Revisit this issue by single-cell analysis to distinguish between:

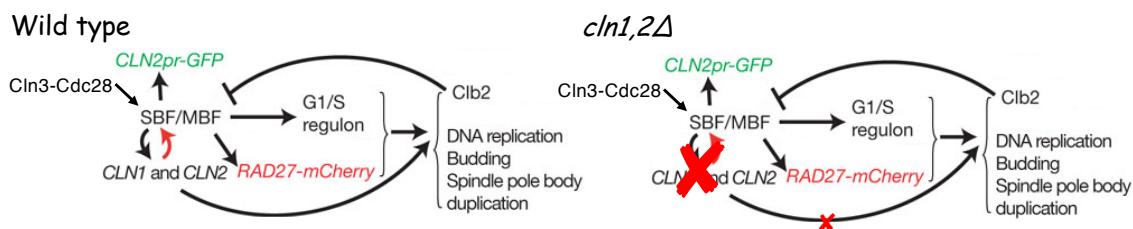
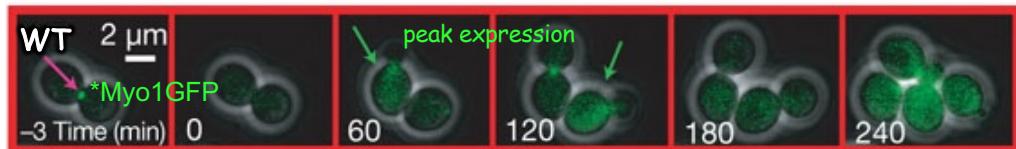
Skotheim et al. (2008) *Nature* 454, 291-297



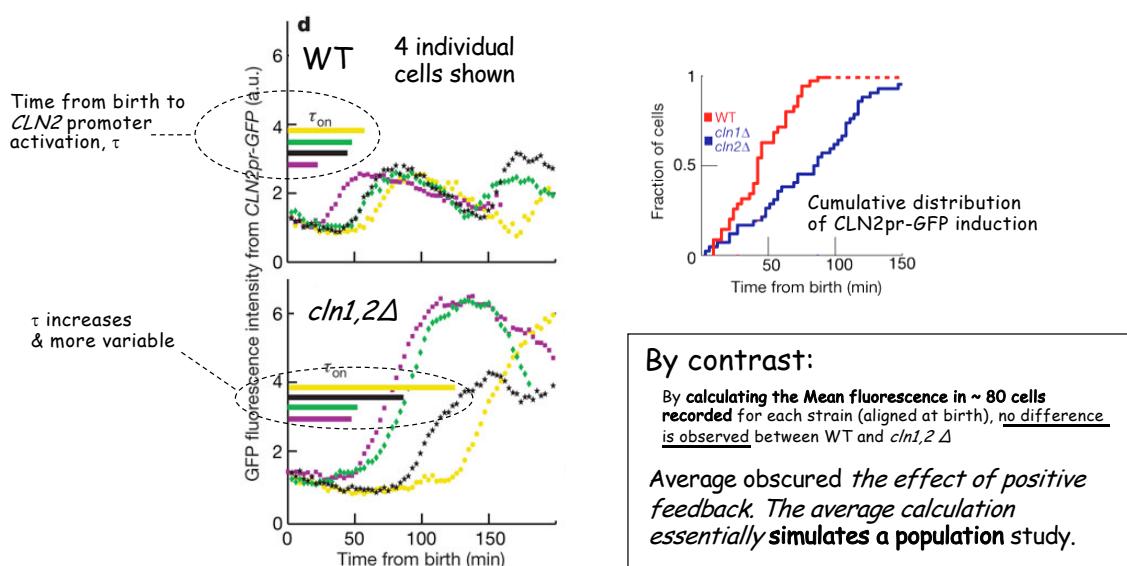
Live imaging analysis of *CLN2prom*-dependent transcription

Setup:

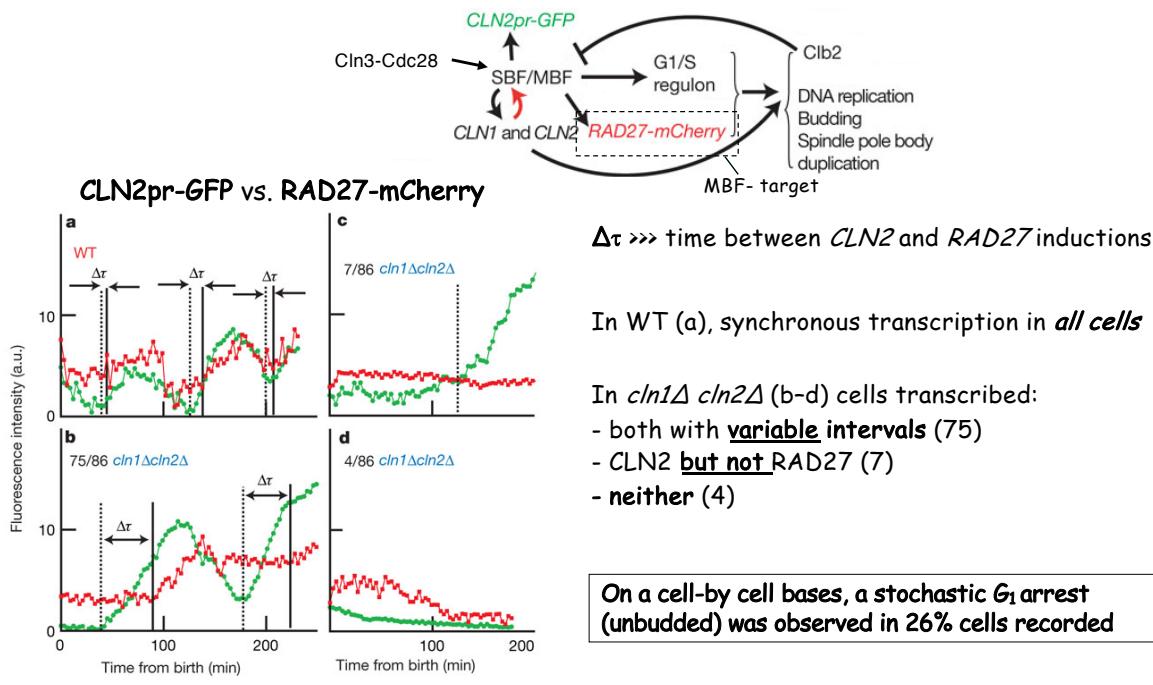
- *Myo1-GFP: reference for T_0 (cytokinesis)
- *CLN2prom*-GFP: a *CLN2* expression reporter
- RAD27-mCherry: representative MBF-dependent transcription reporter



Cln1 and Cln2-dependent feedback contributes to *CLN2* expression

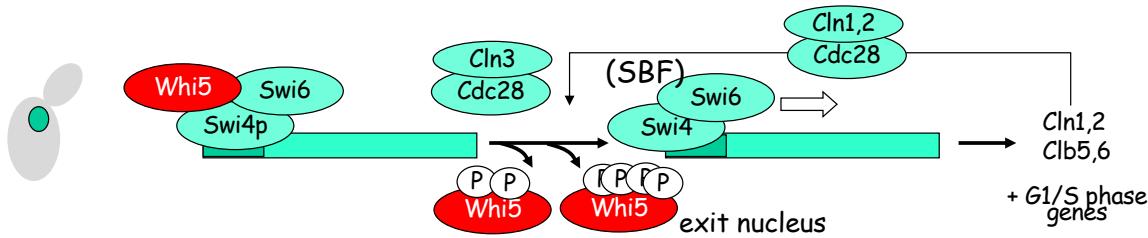


Given the variable induction of *CLN2* promoter in *cln1Δ/cln2Δ*, is the programme of G₁/S transcription disrupted without positive feedback?

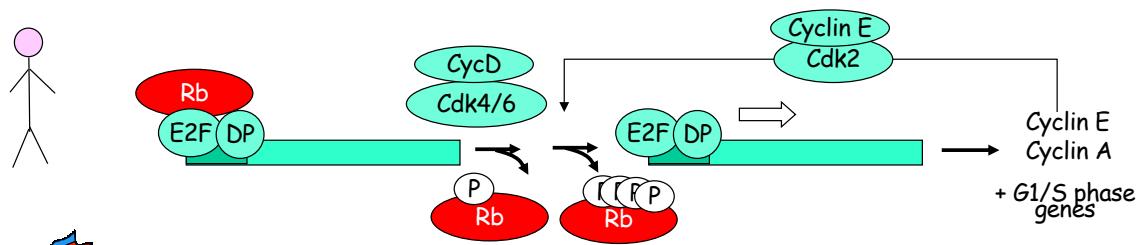


⇒ *Cln1 & Cln2* drive coherent expression of the SBF/MBF-dependent targets

Positive feedback centres on the inactivation of a repressor of the G₁/S regulon

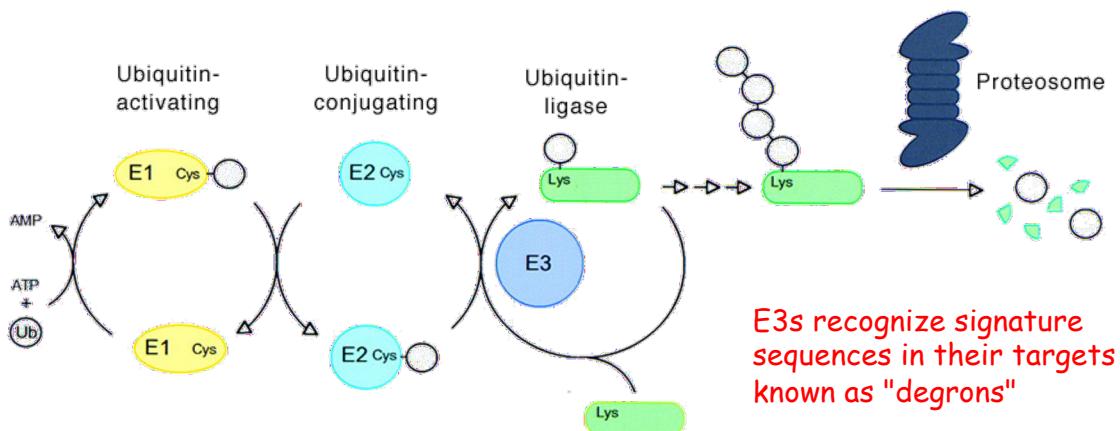


The specific contribution of each G₁/S-CDK to phosphorylation is still work in progress both in yeast and humans...



Rb, a critical negative regulator of the cell cycle, a prototypic tumour suppressor

Ubiquitin-dependent proteolysis



2004 Nobel Prize in Chemistry

"for the discovery of ubiquitin-mediated protein degradation"
to Aaron Ciechanover, Avram Hershko and Irwin Rose

Two evolutionary conserved multisubunit cullin-RING E3s
target critical cell cycle regulators

SCF: Skp1, Cdc53, F-box protein

[CUL1]

Substrate receptor: e.g. Cdc4, Grr1

- SCF targeting requires "phospho-degrons"
- Temporal control by degron phosphorylation
- First identified as the E3 that targets G1 cyclins and CDK inhibitors in yeast, its role in cell cycle regulation is conserved

(This lecture)

APC/C: Anaphase Promoting Complex / Cyclosome

(Next lecture)

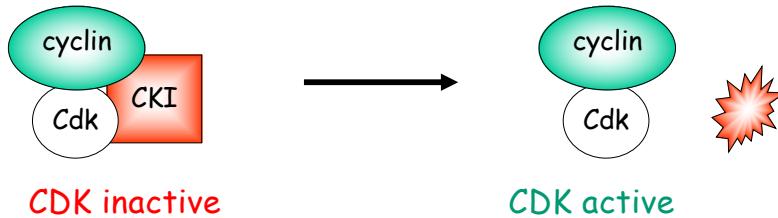
two alternative
(mutually exclusive)
activators

Cdh1
Cdc20

- degrons: destruction box and "KEN" box
- APC/C is controlled by phosphorylation
- its role and regulation are conserved

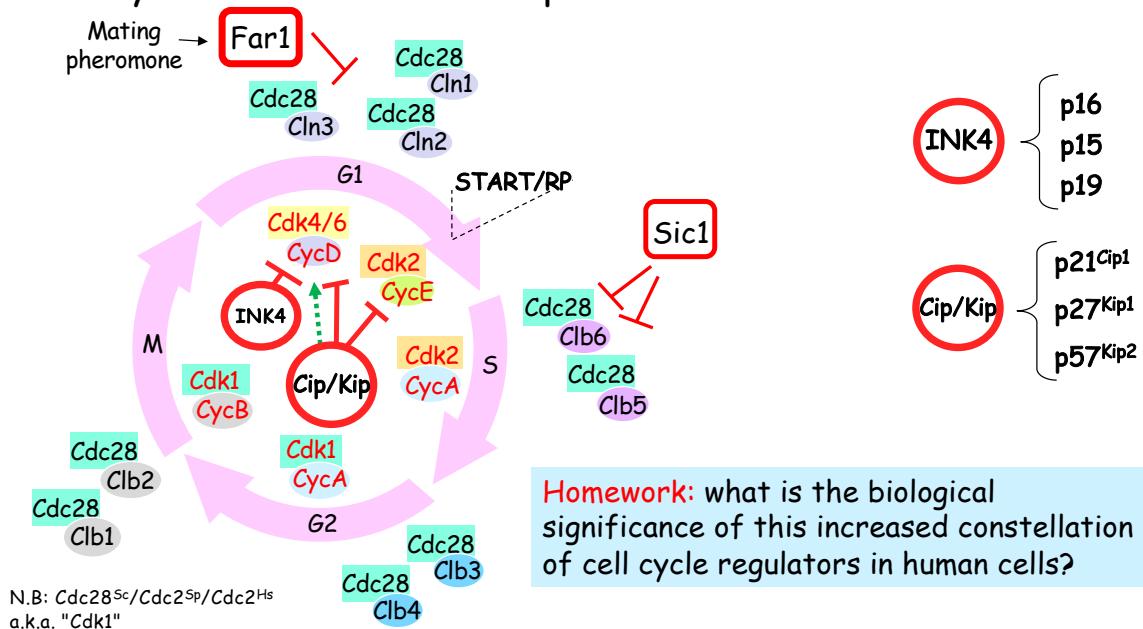
Cullin-RING ligases (CRLs) are the largest family of E3s. They consist of a cullin protein, a RING protein (that recruits an E2), an adaptor protein and one of many possible substrate-receptor proteins recruiting the target. SCF and APC/C are prototypical multisubunit E3 members of the CRL superfamily.

CDK Inhibitors (CKIs) contribute to switch-like cell cycle transitions



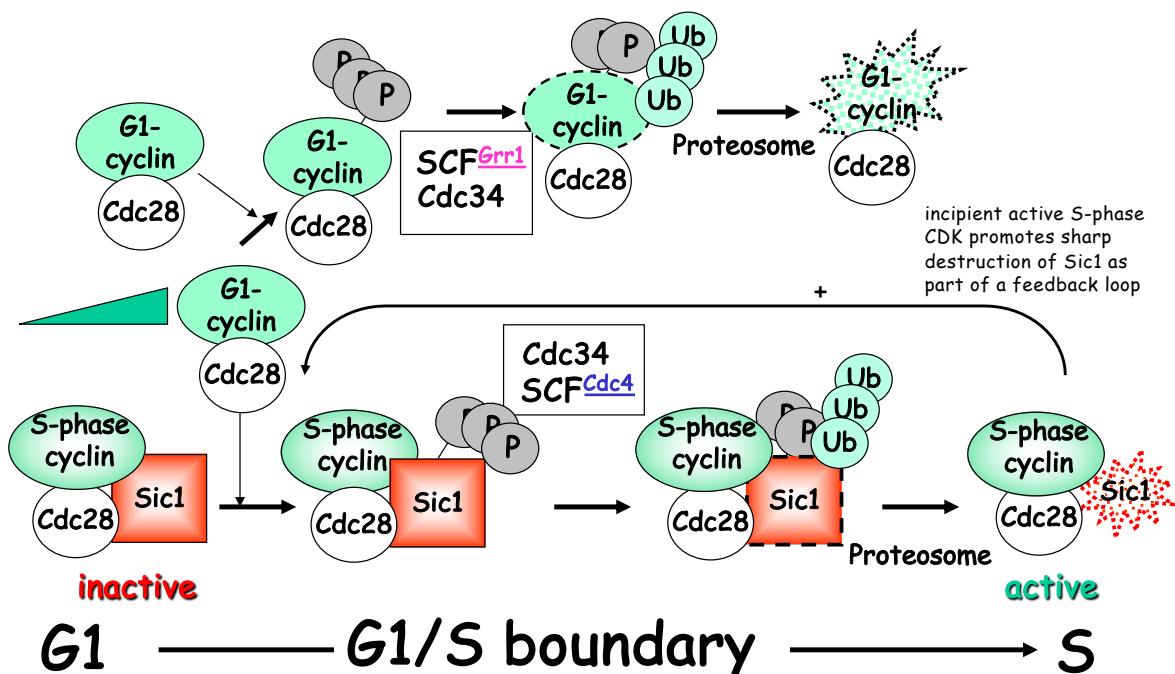
- Isolated by yeast two-hybrid screens using CDKs as bait, and independently, by biochemical purification.
- SCF-dependent destruction of the CKI causes the switch-like activation of a preformed CDK complex
- Here we will discuss **Sic1**, a CKI of yeast Clb-CDKs. Human cells contain two families of CKIs with multiple members, adding combinatorial control to the G1/S transition

From yeast to humans: multiple CDKs and CKIs in humans



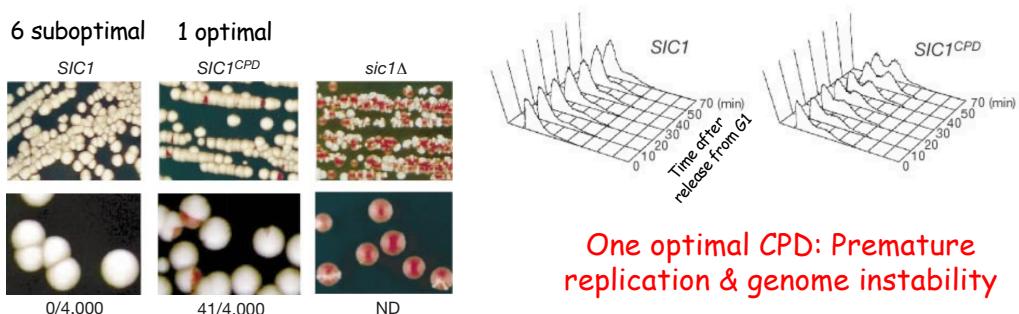
- * Ink4 inhibits exclusively Cdk4 and Cdk6 by binding the monomeric Cdk and preventing cyclin binding
- * Cip/Kip assists assembly of Cdk4/6-CyclinD complexes. Inhibition may be terminated by phosphorylation and proteolysis
- * Cip/Kip holds Cdk2-cyclin complexes inactive. Inhibition may be terminated by titration and/or phosphorylation & proteolysis

Cln1, Cln2 and the CKI Sic1 are targeted for ubiquitin-dependent proteolysis by Cdc34(E2) and SCF(E3)

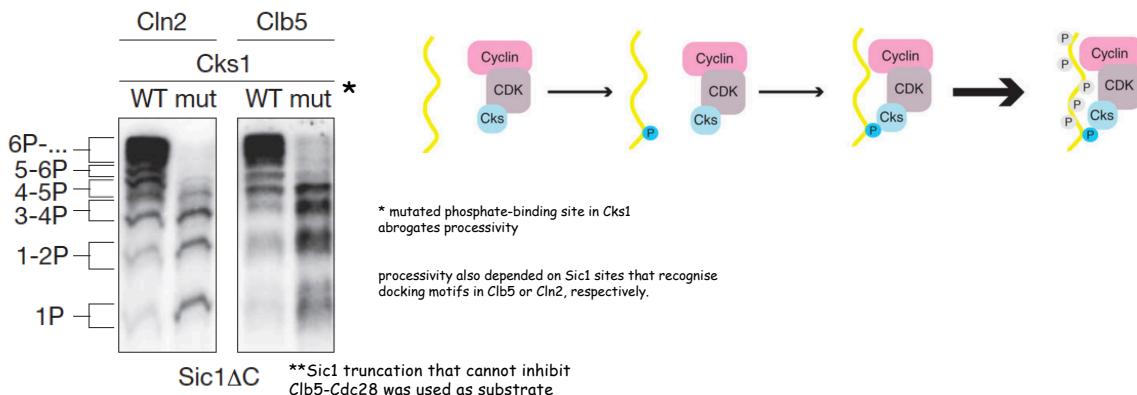


Multisite phosphorylation of Sic1 preserves G1 phase integrity

- 9 suboptimal *Cdc4* phospho-degron (CPD) motifs
- at least 6 required for *Cdc4* recognition
- an "optimal CPD" was identified (in vitro binding assay with peptide arrays)
- a *Sic1* version containing a single optimal CPD was constructed and expressed in yeast



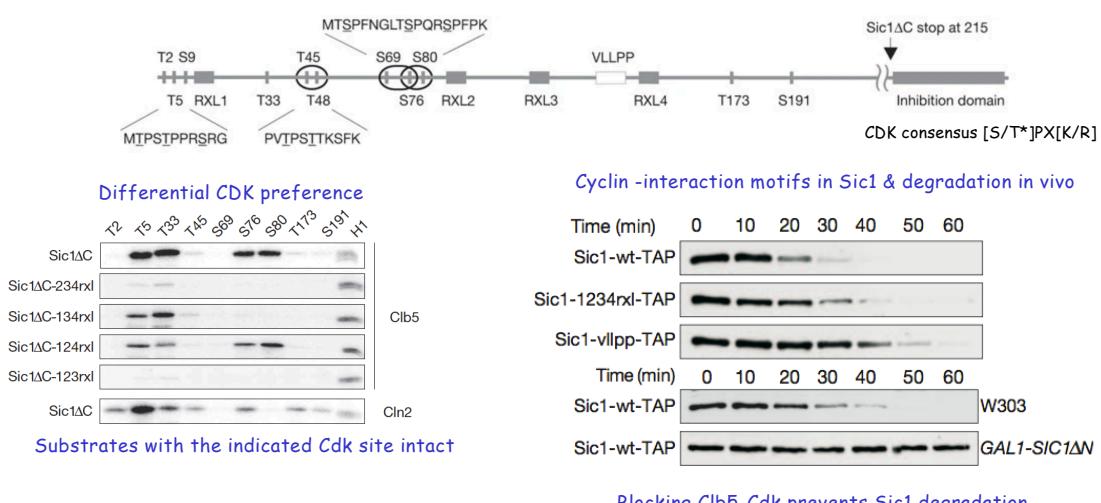
Cks1 phospho-dependent docking promotes processive phosphorylation of Sic1 by Cln2-Cdc28 & Clb5-Cdc28

Kõivomägi et al. (2011) *Nature* 480, 128-132

Note: *In vitro* kinase assay with full-length Sic1



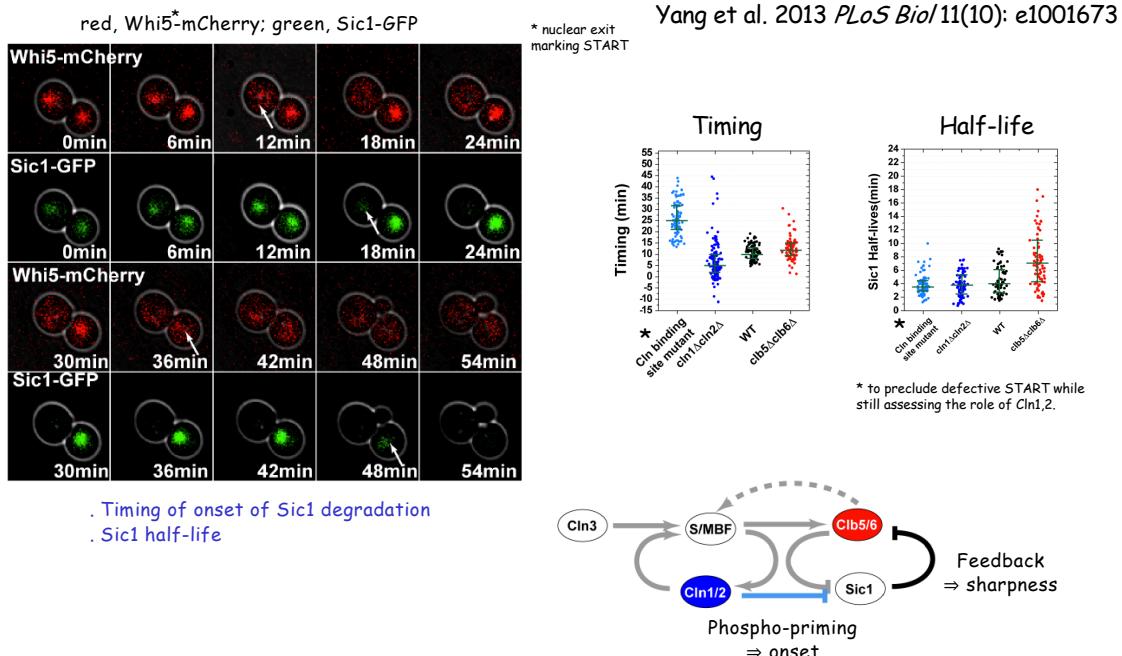
Cyclin docking motifs and phospho-priming ensure orderly phosphorylation at suboptimal phosphodegron motifs

Kõivomägi et al. 2011 *Nature* 480, 128-132***

- Cyclin-docking motifs and phospho-priming dictate the kinetics of Sic1 phosphorylation and ensuing degradation
- Interplay between Cln-CDK & Clb5-CDK sharpens Sic1 destruction into an all-or-none switch

Control of Sic1 destruction by Cdk *in vivo*

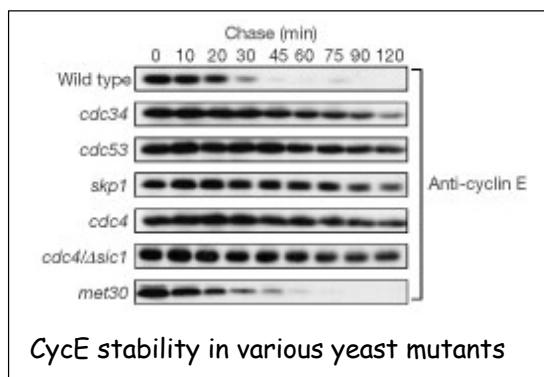
Cln1/2-Cdc28 sets the proper *timing* and Clb5/6-Cdc28 the *rate* of Sic1 destruction



More lessons from yeast: SCF-dependent proteolysis of cyclin E

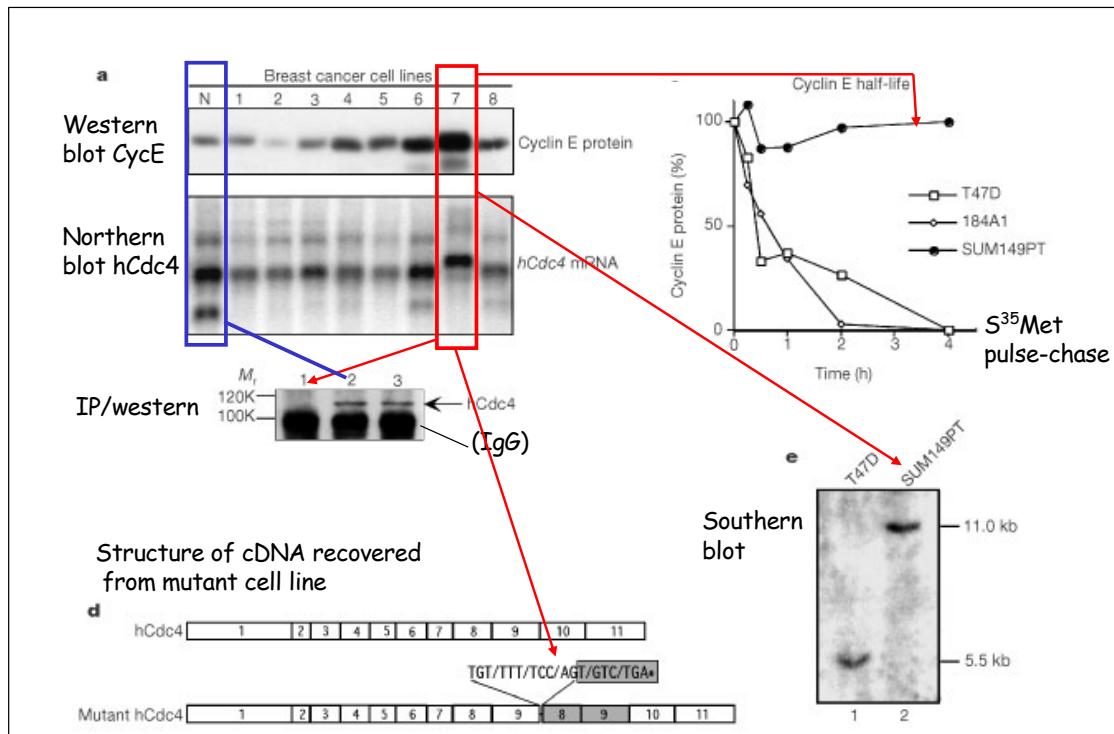
Strohmaier et al. (2001) *Nature* 413:316-322

- Human cyclin E expressed in yeast undergoes SCF^{Cdc4}-dependent turnover



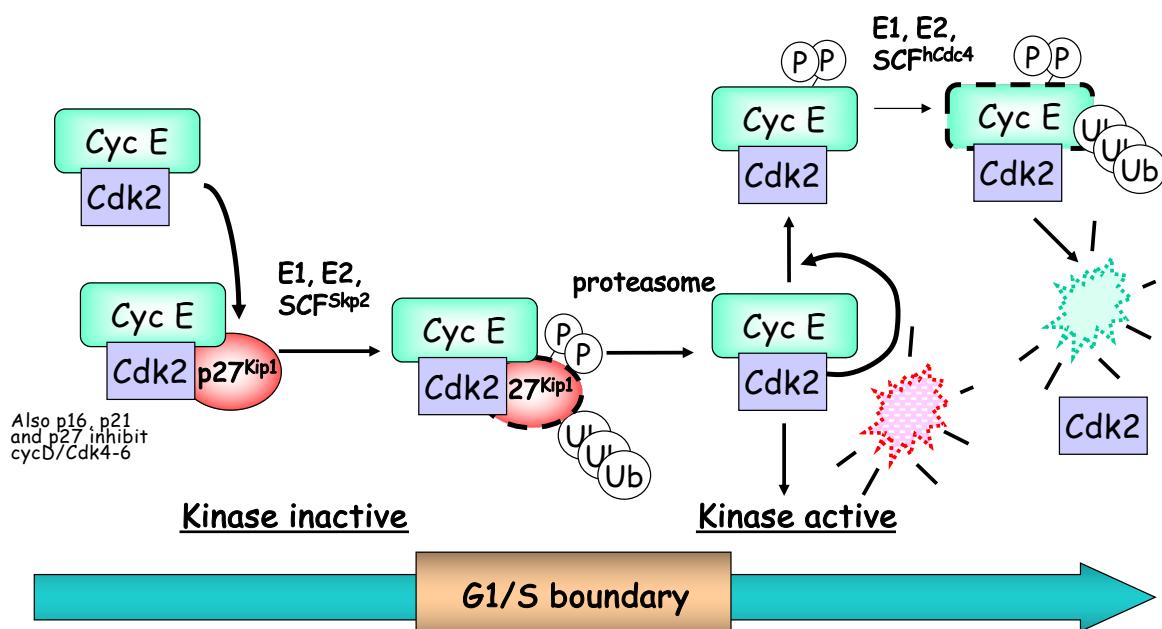
- A search of the Human Genome Database using the sequence of the "F box" of yeast Cdc4 as query pointed to a human counterpart of Cdc4
- It encoded THE F-box protein targeting cyclin E to degradation

- cycE protein can be upregulated without gene amplification or excess transcription



- human Cdc4 acts as a tumour suppressor

CKI and cyclin E are critical **SCF targets** in G1/S progression in human cells (simplified)



Homework: consider this diagram along with the role of E2F/RB in transcriptional control and feedback. Compare with the yeast paradigm.

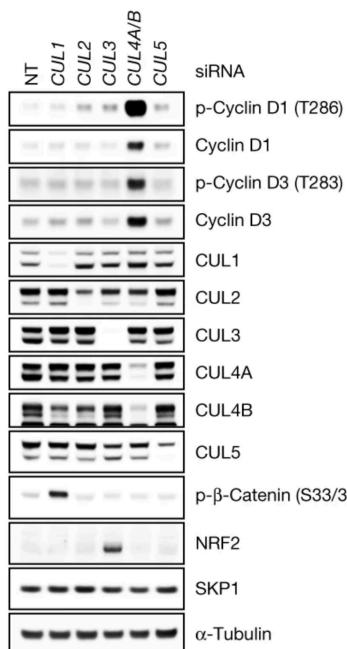
The E3 CRL4^{AMBRA1} (**NOT SCF**) targets CycD for destruction

Simoneschi et al. 2021 *Nature* 592:789; Maiani et al. (2021) *Nature* 592:799; Chaikovsky et al. (2021) 592:794

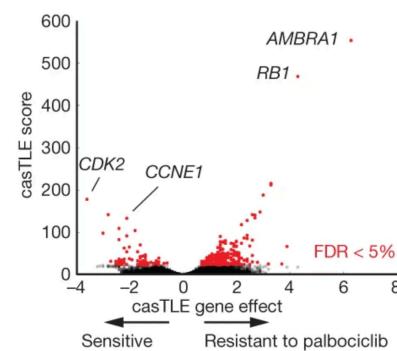
- CycD1 phosphorylation at T²⁸⁶ controls ubiquitin-dependent degradation by the proteasome (the identity of the kinase remains controversial).
- CycD1^{T286A} is stabilised. T²⁸⁶ is found mutated in uterine and endometrial cancers. CycD1^{T286A} expression drives spontaneous cancers in mice.

What E3 targets CycD for proteolysis?

- siRNA candidate screen with all human cullins: CRL4 silencing stabilized CycD
- Substrate receptor?>>>AMBRA1
 - . siRNA screen for all predicted CRL4-associated factors
 - . Whole-genome CRISPR-Cas9 screen for regulators of CycD1
 - . Proteomic screen for interactors with CycD1 but not CycD1^{T286A} (a stable phospho site mutant)
- A knock-out mouse model also demonstrated AMBRA1 role in CycD control.
- CRISPR-Cas9 screen in U2OS human cells for insensitivity to the Cdk4/6 inhibitor palbociclib identified AMBRA1.



Homework: interpret the following data on the characterisation of AMBRA1 taken from the studies outlined above. Consider the significance of these findings



Volcano plot of a CRISPR-Cas9 screen for genes that regulate the response to palbociclib in U937 cells, analysed using the Cas9 high-throughput maximum likelihood estimator (casTLE). FDR, false-discovery rate (From Chaikovsky et al. 2021)

HCT-116 cells were transfected with a non-targeting (NT) siRNA or siRNAs against *CUL1*, *CUL2*, *CUL3*, *CUL4A* and *CUL4B* (*CUL4A/B*) or *CUL5*, and cell lysates were blotted with the indicated antibodies for proteins and phosphorylated (p-) proteins (From Simoneschi et al. 2021)

The E3 CRL4^{AMBRA1} targets CycD for destruction

— significance in cancer —

Simoneschi et al. 2021 *Nature* 592:789; Maiani et al. (2021) *Nature* 592:799; Chaikovsky et al. (2021) 592:794

- AMBRA1 might act as a tumour-suppressor controlling CycD levels.
- The *AMBRA1* gene is mutated in human cancers (check depmap (<https://depmap.org/>)).
- Levels of *AMBRA1* and CycD found to be inversely correlated in human tumours (with low levels of *AMBRA1* linked to poor prognosis).
- In *AMBRA1*-depleted cells, CycDs found paired with Cdk2 — these complexes are insensitive to CDK4/6 inhibitors (palbociclib, ribociclib & abemaciclib, CDK4/6 inhibitors approved for the treatment of advanced breast cancers).
- Loss of *AMBRA1*, triggers DNA damage and replication stress. *AMBRA1*-depleted cancer cells were hypersensitive to inhibitors of the checkpoint kinase CHK1 — potential therapeutic target for *AMBRA1*-deficient tumours.

Circuitry at the G1/S transition is fundamentally conserved

- Transcriptional circuitry: CDK thresholds for inactivation of Whi5/Rb are central to START or the RP. Note: Rb is the first tumour suppressor identified.
- CKIs avert premature S phase entry and associated genetic instability
- In human cells, CKIs are key to combinatorial control by mitogenic vs. antimitogenic signals modulating the switch between proliferation and quiescence. Regarded as tumour suppressors.
- Ubiquitin-dependent proteolysis of G1/S cyclins and CKIs are key to dynamics of G1/S regulators. Various factors that target cycD and E are proven tumour suppressors.

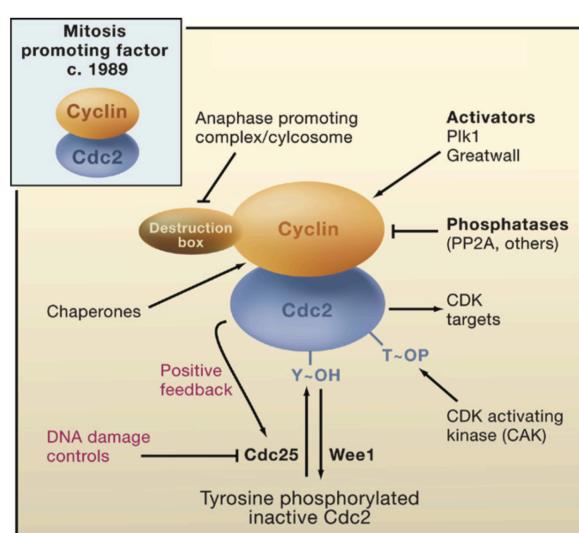
Next... In & out of Mitosis

Lecture 4

Cell cycle control in eukaryotic cells (III)

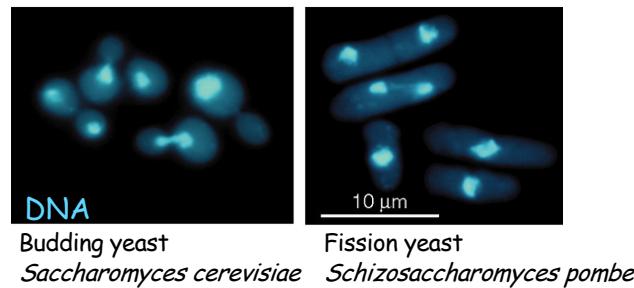
In and out of mitosis & the perfect oscillator

Overview of MPF interactors & post-translational modifications

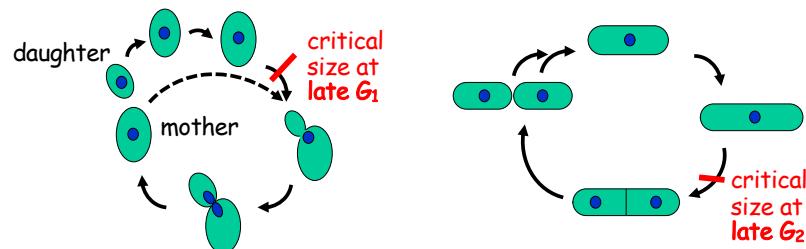


Post-translation modifications turn MPF into an all-or-none switch, and.... yes, feedback loops!!!!

Yeast: models for somatic cell cycle control

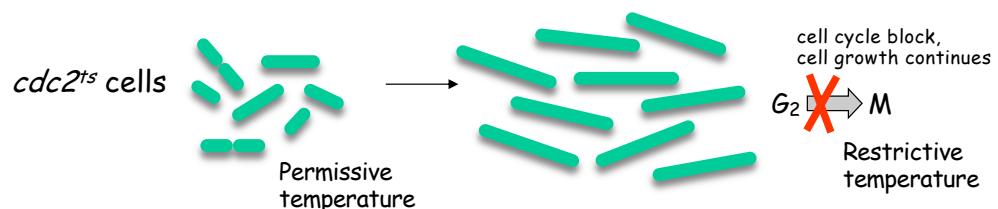
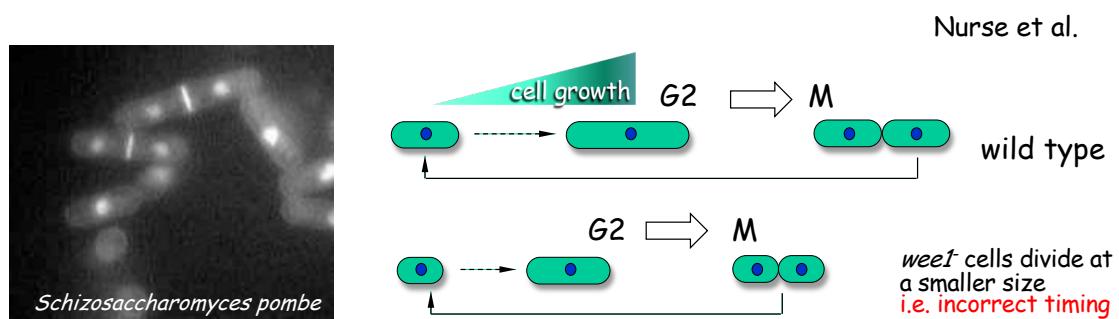


GAP phases are key to coordination of cell growth with proliferation: a critical size is a prerequisite for progression



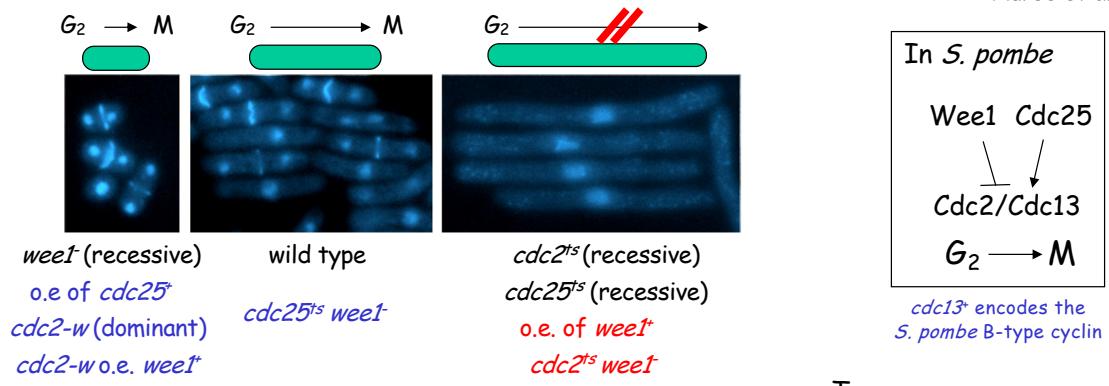
A critical size in *S. pombe* is a precondition for entering M phase. In *S. cerevisiae* daughter cells must reach a critical size prior to bud emergence.

Fission yeast: cell length correlates with cell cycle position

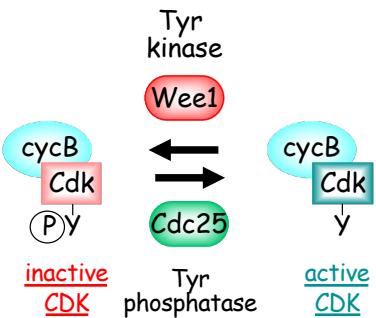


Genetic analysis in *S. pombe* uncovered a conserved mitotic trigger

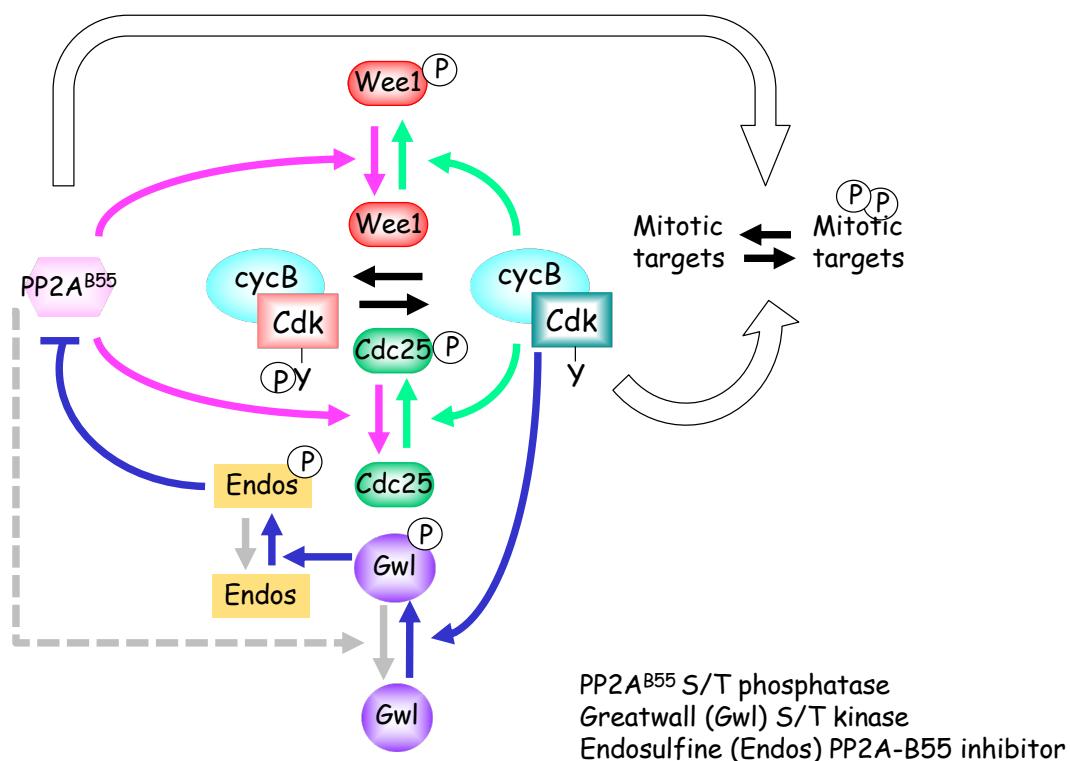
Nurse et al.



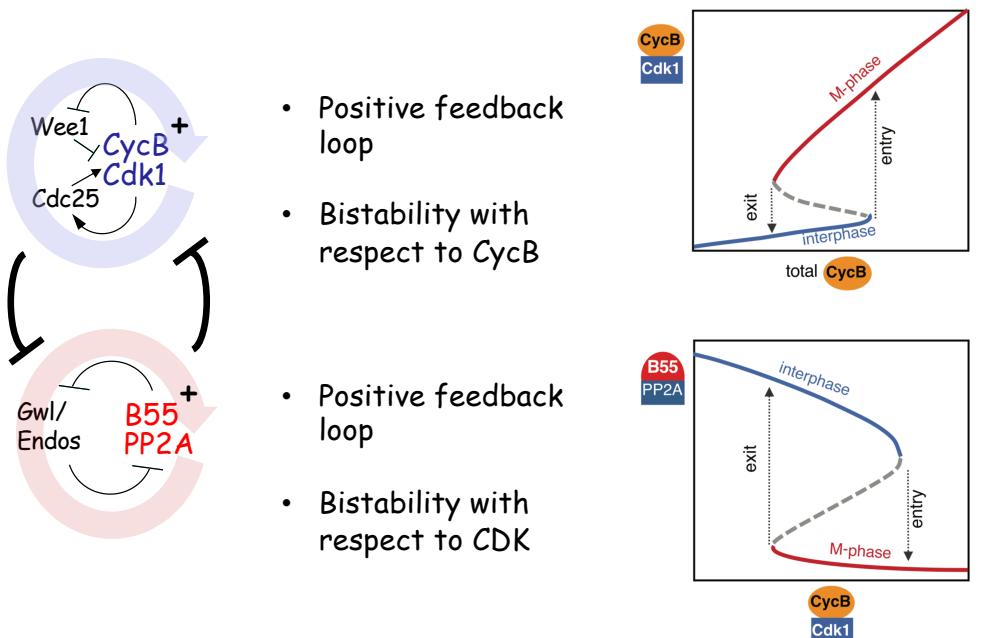
- *wee1^r* encodes a protein kinase that acts as a dosage inhibitor of M
- *cdc25^r* encodes a protein phosphatase that acts as a dosage activator of M



A mitotic switch centred on Tyr phosphorylation/dephosphorylation of CDK

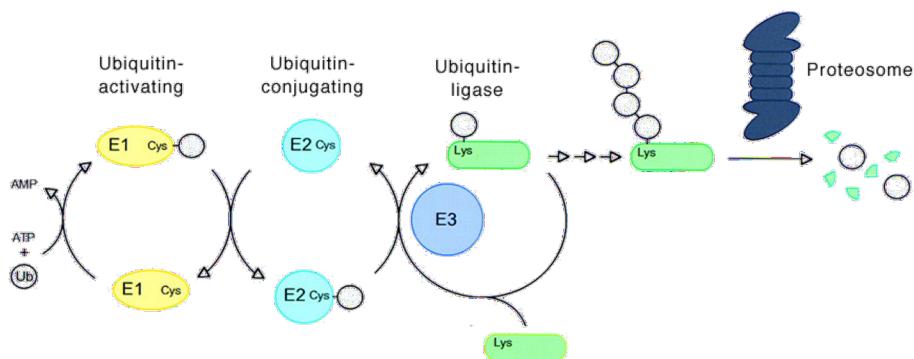


A mitotic switch: distinct thresholds for mitotic entry and exit



Rata et al., 2018 *Curr Biol* 28, 3824-3832

Ubiquitin-dependent proteolysis & mitosis



- The first cell cycle regulator found to undergo ubiquitin-dependent proteolysis is Cyclin B. A critical motif essential for targeted proteolysis was identified: the destruction box.
- The quest for the machinery that targets mitotic cyclins uncovered a fundamental regulatory mechanism that ensures the fidelity of chromosomal segregation

The destruction box* targets yeast B-type cyclins for proteolysis

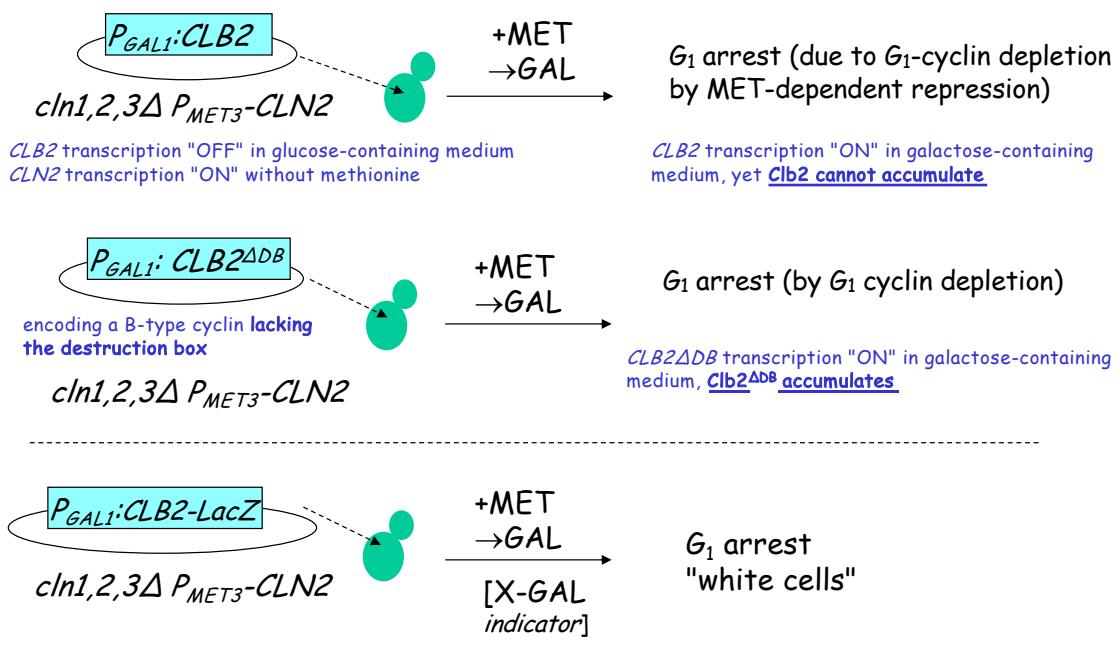
- a) A $Clb2^{\Delta db}$ is stable and blocks mitotic exit.
- b) $Clb2$ begins to accumulate during late S/G2 (transcriptional control). It becomes unstable in late mitosis and cannot accumulate during G1 (proteolysis is active during G1 and transcription is normally off).
- c) $CLB2^{\Delta db}$ BUT NOT $CLB2$ expression under the $GAL1$ promoter in G₁ leads to cyclin accumulation.
- d) A genetic screen was therefore designed to identify elements participating in B-type cyclin degradation (i.e. targeted by the destruction box).

*APC/C-mediated ubiquitination depends on either one of two poorly defined motifs in the substrate, the destruction box (D-box; first described in mitotic cyclins) and the KEN box (first described in Cdc20). Yeast $Clb1$ - $Clb5$ contain destruction boxes. Clbs mutant versions lacking this motif are stable (as originally demonstrated for Cyclin B).

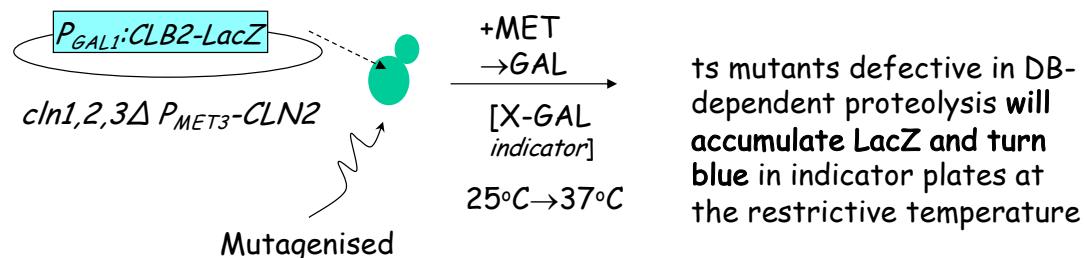
Destruction box-containing proteins cannot accumulate in G₁

How to screen for the components of the system targeting cyclins for degradation?

Consider the following:



d) A genetic screen was therefore designed to identify elements participating in cyclin degradation



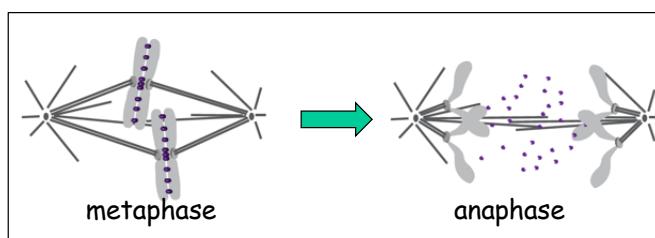
Among others, it identified several previously known genes: *CDC23*, *CDC16*

If in doubt - read on: Irniger et al. (1995) *Cell* 81:269-278

Cdc23 and *Cdc16* are part of a complex controlling the metaphase/anaphase transition

- *cdc23^{ts}* & *cdc16^{ts}* cells arrest at metaphase following shift to the restrictive temperature
- Yet, Clb2 is normally degraded in late M. *Clb2^{Δdb}* blocks mitotic exit.
- There must be another target of ubiquitin-dependent proteolysis acting as an anaphase inhibitor (not degraded upon shift of *cdc23^{ts}* or *cdc16^{ts}* cells)
- *Cdc23* and *Cdc16* are components of the Anaphase Promoting Complex/cyclosome: APC/C
- The APC/C is activated by *Cdc20* or *Cdh1* in a mutually exclusive manner (see later)

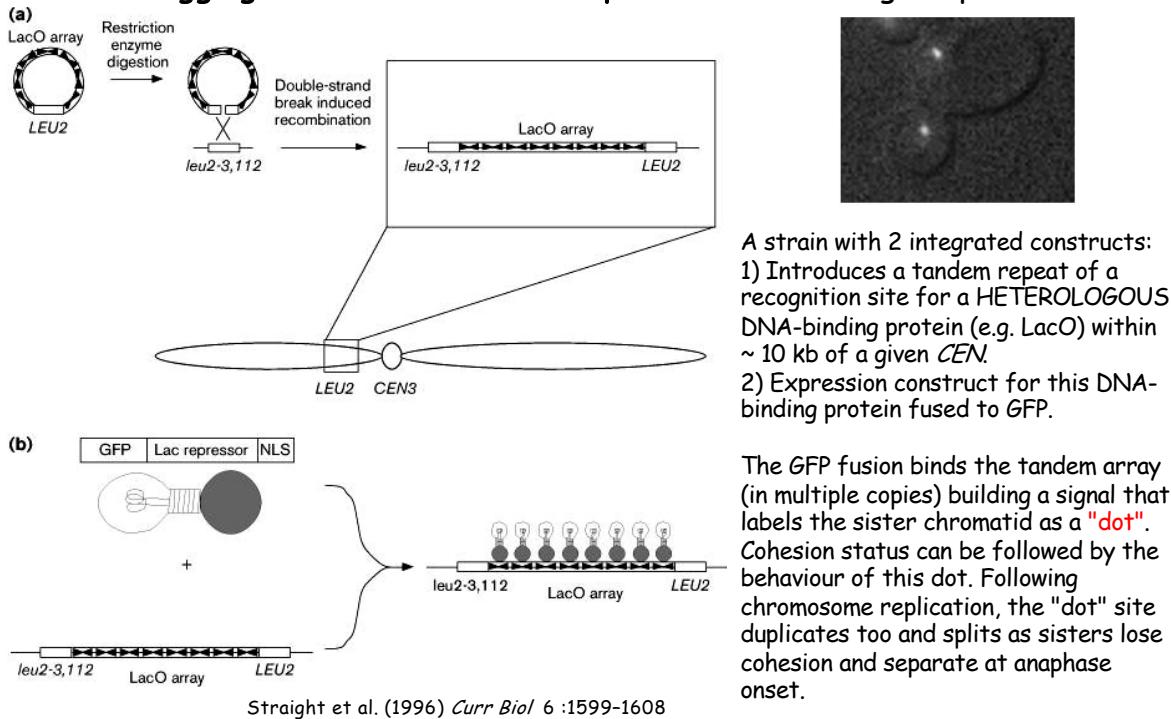
Both the "anaphase inhibitor" & B-type cyclins are targets of the APC/C



Homework: Several cell cycle controls converge onto this step, as you learn about them consider their biological significance

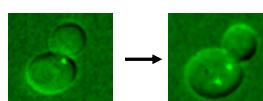
Monitoring loss of cohesion with limited chromosomal condensation

GFP-tagging of subcentromeric sequences: monitoring anaphase onset



A strain with 2 integrated constructs:
 1) Introduces a tandem repeat of a HETEROLOGOUS DNA-binding protein (e.g. LacO) within ~ 10 kb of a given *CEN*.
 2) Expression construct for this DNA-binding protein fused to GFP.

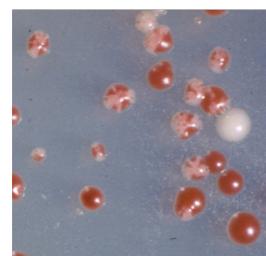
The GFP fusion binds the tandem array (in multiple copies) building a signal that labels the sister chromatid as a "dot". Cohesion status can be followed by the behaviour of this dot. Following chromosome replication, the "dot" site duplicates too and splits as sisters lose cohesion and separate at anaphase onset.



Screening for defective anaphase onset

a) Mutations increasing chromosomal loss (sectoring assay) and bypassing LOC defect of a *cdc16^{ts}* (extra notes below).

Identified *SCC1*: *Scc1* does not have a destruction box. May be the "glue protein". An *scc1^{ts}* strain undergoes premature LOC (prior to G_2).

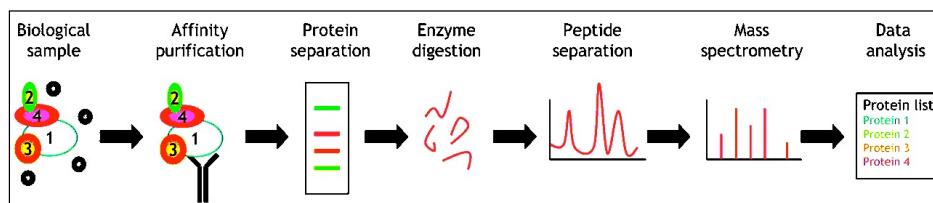


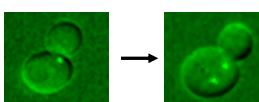
b) Mutants exhibiting LOC in the presence of nocodazole.

Identified *PDS1*: *Pds1* contains a destruction box and is degraded at MET/ANA transition. Expression of *PDS1^{Δdb}* blocks MET>ANA progression. The protein is stable in cells blocked by nocodazole. *Pds1* role in maintaining cohesion is **INDIRECT**.

c) *Pds1* coIPs with a 180kDa protein: *Esp1*.

An *esp1^{ts}* mutation prevents loss of cohesion at the restrictive temperature.

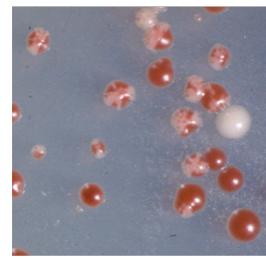




Screening for defective anaphase onset (expanded notes)

a) Mutations increasing chromosomal loss (sectoring assay) and bypassing LOC defect of a *cdc16^{ts}*.

Defective sister chromatid cohesion is expected to cause mis-segregation and chromosomal instability. Using a setup in which the loss of a "minichromosome" changes the colour of a colony, chromosomal instability may be scored and candidate mutants selected. Those are filtered by a secondary screen to identify those specifically impaired for cohesion.



Chromosomal instability assessed by a sectoring assay

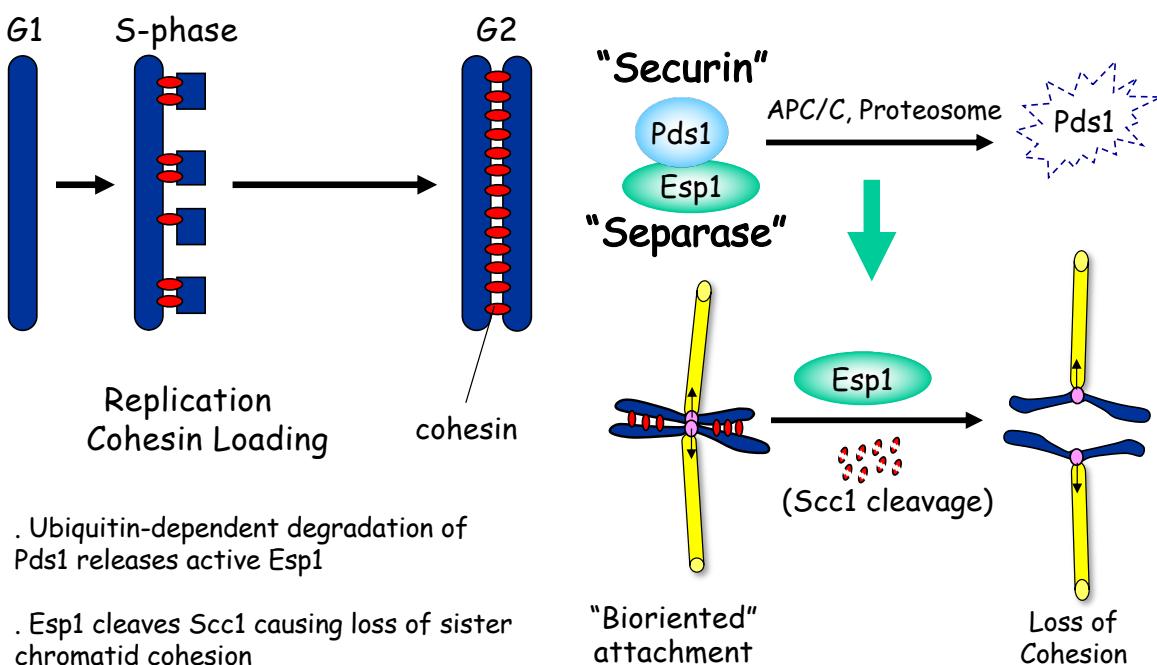
i) Screen for "x" mutants displaying excess chromosomal loss

- wild type yeast >> ~ white colonies
- ade2*- mutant accumulates a red pigment >> red colonies
- maintaining a minichromosome carrying *ADE2* (wild type gene) in an *ade2* yeast mutant gives rise to white colonies
- defective maintenance of a minichromosome carrying *ADE2* in an *ade2*- mutant strain gives rise to red sectors in a lighter colony background (the sectors are due to progeny of cells within the growing colony that "lost" the plasmid due to mis-segregation *per se* or other factors)

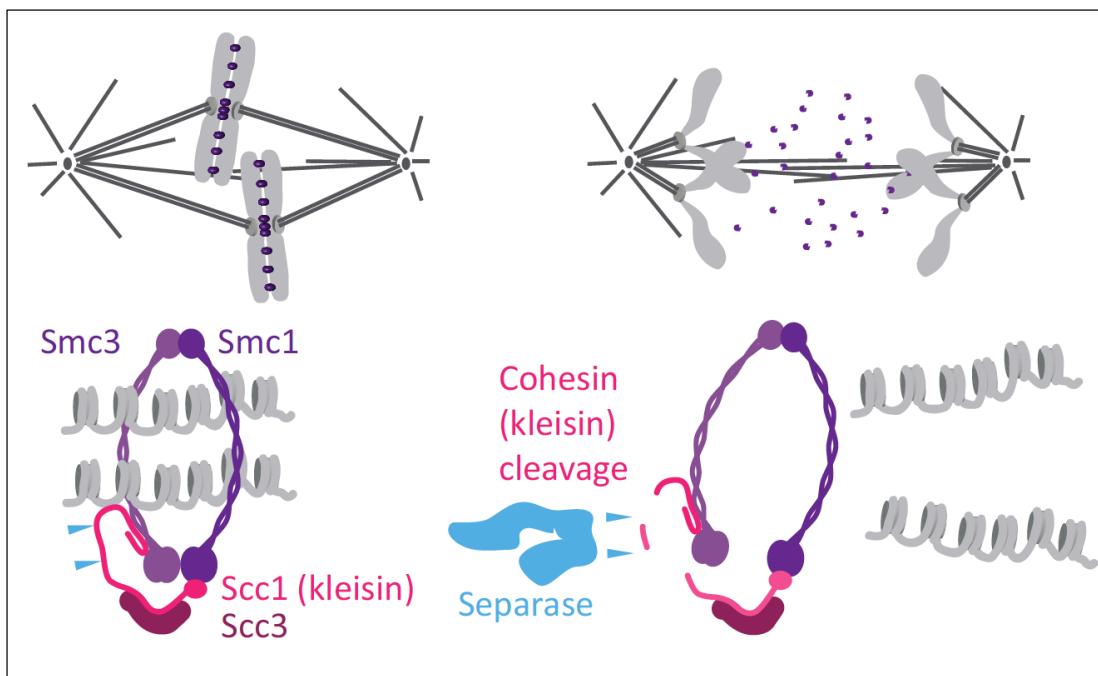
ii) Secondary screen of x- mutants from i) to select those in which cohesion is disabled

- cdc16^{ts}*: arrest at metaphase with sister chromatids held together by cohesion
- cdc16^{ts}* x-: cohesion lost >> 2 "dots"

Anaphase is triggered by Ubiquitin-dependent proteolysis of Pds1

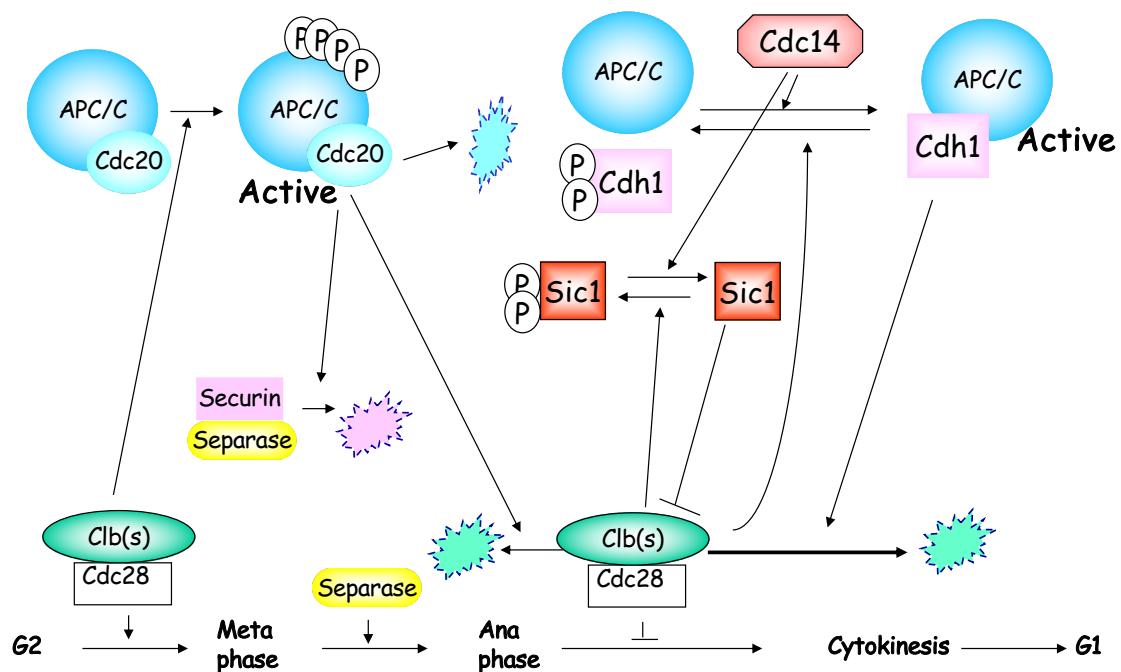


A conserved mechanism: cleavage of Kleisin by separase triggers chromosome separation



Kamenz and Hauf (2017) *Trends Cell Biol* 27:42-54

APC/C^{Cdc20} handover to APC/C^{Cdh1}: from anaphase onset to mitotic exit



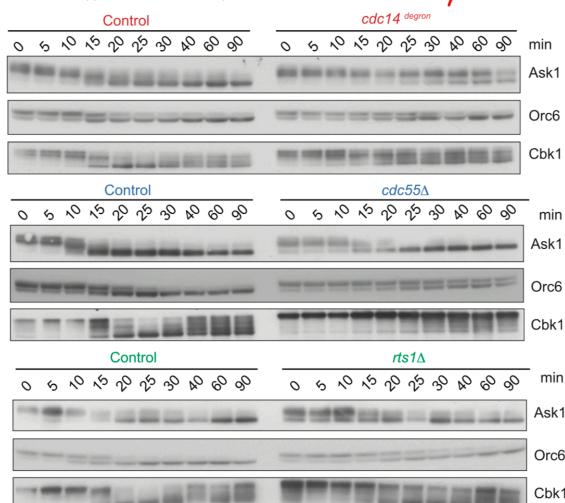
- The APC/C is a key effector of checkpoint pathways (next lecture)
- Cdc14 is the critical trigger for mitotic exit (more, in next 2 lectures)

Specific & redundant contributions of Cdc14 and PP2A phosphatases toward mitotic exit

P_{GAL1}-CDC20: time course from META block/release until next G1 (ending by α-factor)

Touati et al., 2019, *Cell Rep* 29, 2105-2119

Time course & western blot analysis



Time course & phospho-proteomics

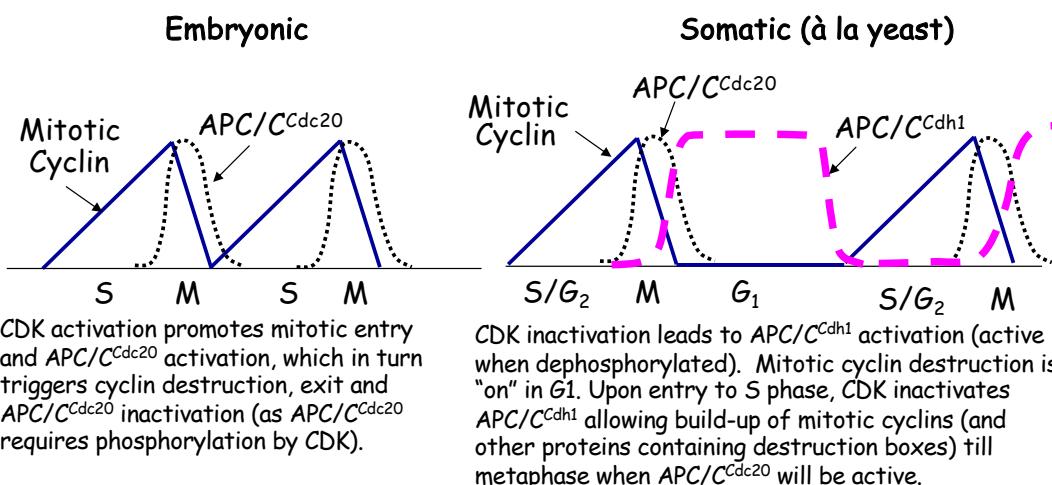
- Phosphatases influence each other's activation dynamics. They also modulate mitotic kinase waves
- Time-resolved dephosphorylation patterns
- Ordered dephosphorylation hinges on Cdc14 preference for pS over pT on CDK sites
- Overlapping preference of PP2ACdc55 and PP2A^{Rts1} for pT on CDK sites. Also non-CDK sites.
- PP2A^{Rts1} and PP2ACdc55 may play non-overlapping roles in control of late mitotic kinases (AurK and NDRK)

Differential impact on dephosphorylation dynamics upon depletion of Cdc14 (down to ~20% using a conditional degron) or deletion of the genes encoding PP2A regulatory subunits

In human cells, PP2A counteracts CDK phosphorylation at mitotic exit while the involvement of Cdc14 paralogs is unclear

Early embryonic vs. somatic cell cycles (revisited)

Homework: consider the message from the schematics below and the lectures so far to create a "wire diagram" (components and connectivity) of cell cycle regulators bringing together the role of CDK, SCF and APC/C in enforcing GAP phases in budding yeast. Use budding yeast protein names.

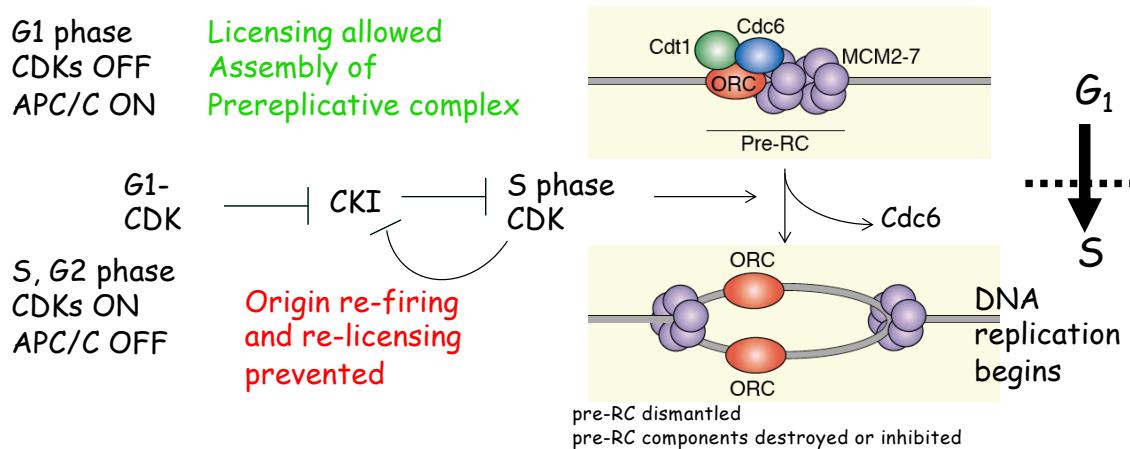


The APC/C-based double oscillator enforces GAP phases

"Downstream events"

respond to the
cell cycle oscillator

Control of initiation of DNA replication by CDK
replication origins are licensed and fired only once per cell cycle



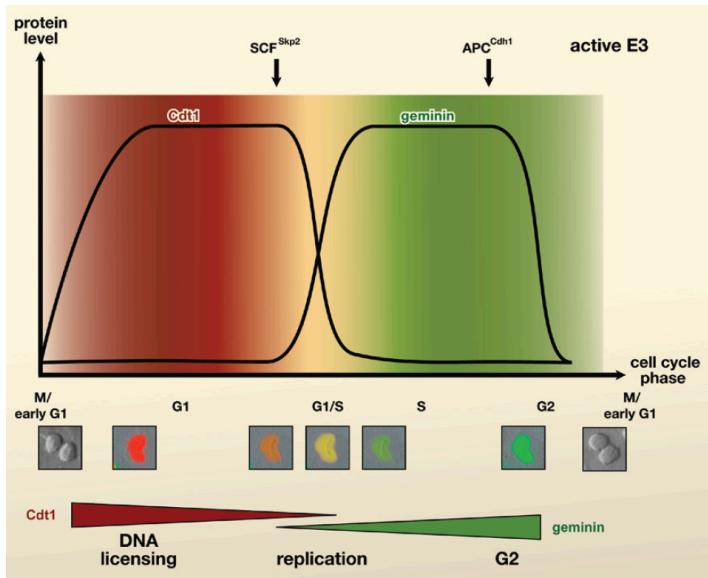
ORC is bound to chromosomes throughout the cell cycle. The ordered assembly of the preRC (Cdt1, Cdc6 and MCM proteins) occurs during early G₁ (low Cdk activity). Cdk-Clbs in S trigger initiation of replication.

Following "origin firing", formation of new preRCs is inhibited by high Cdk-Clb activity until exit from mitosis. This ensures that any given replication origin can only fire once per cell cycle.

In higher eukaryotes, there is further interplay between positive and negative regulators subject to cyclic proteolysis (Cdt1 and Geminin).

BONUS: visualizing cell cycle phases live!

Fluorescent Ubiquitination-based Cell Cycle Indicator



Red and Green fluorescent proteins fused to Cdt1 (G_1) and Geminin (S/G_2), respectively.

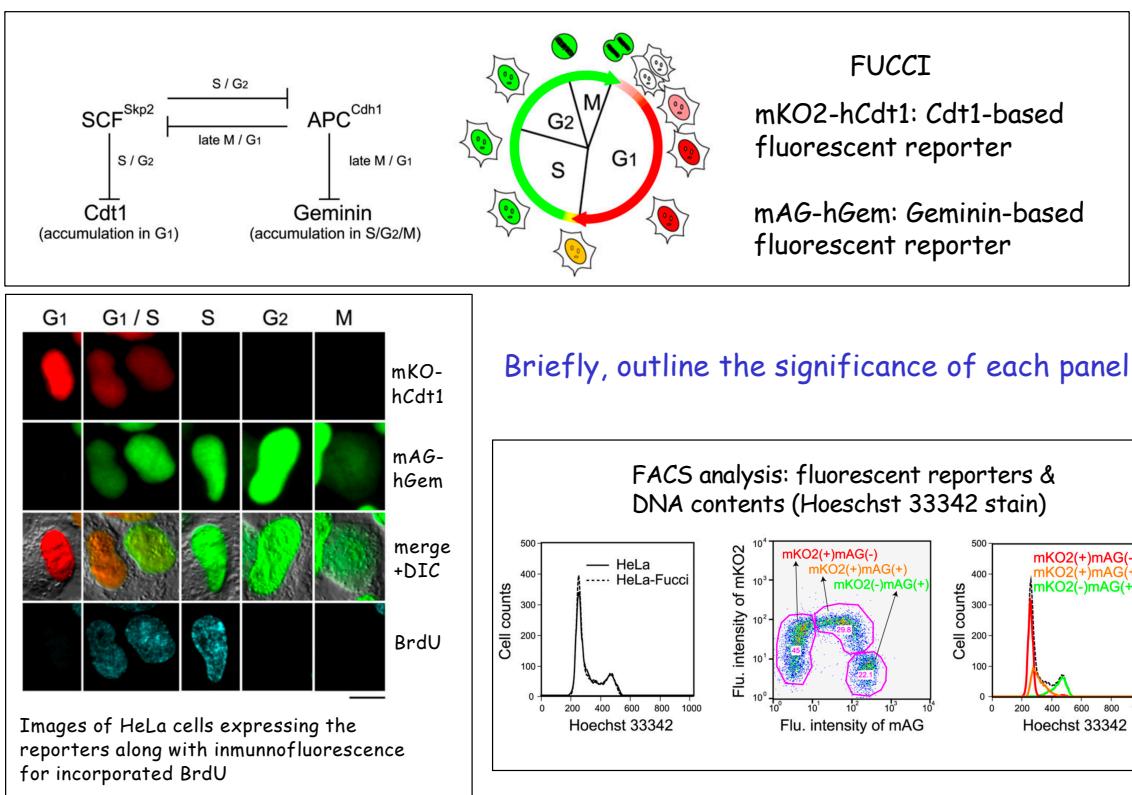
The constructs are cleverly designed to display cell cycle control of proteolysis without otherwise interfering with endogenous cell cycle controls, thus acting strictly as reporters.

By digital overlays, nuclei in G_1 are red, yellow in early S and green in late S and on.

Newer versions with 4 colours!!!!

Méchali & Lutzmann 2008 *Cell* 132: 341-3
Sakae-Sawano et al. 2008 *Cell* 132: 478-98

Homework: interpret the figures attached. Data from *Cell* 2008 132:487-498.

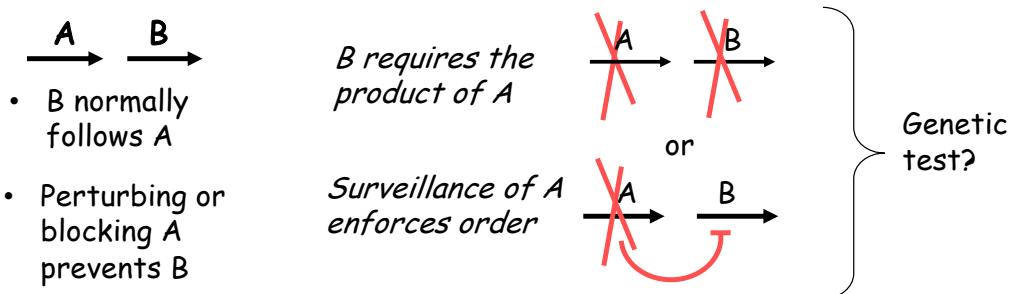


Lecture 5

Cell cycle control in eukaryotic cells (IV)

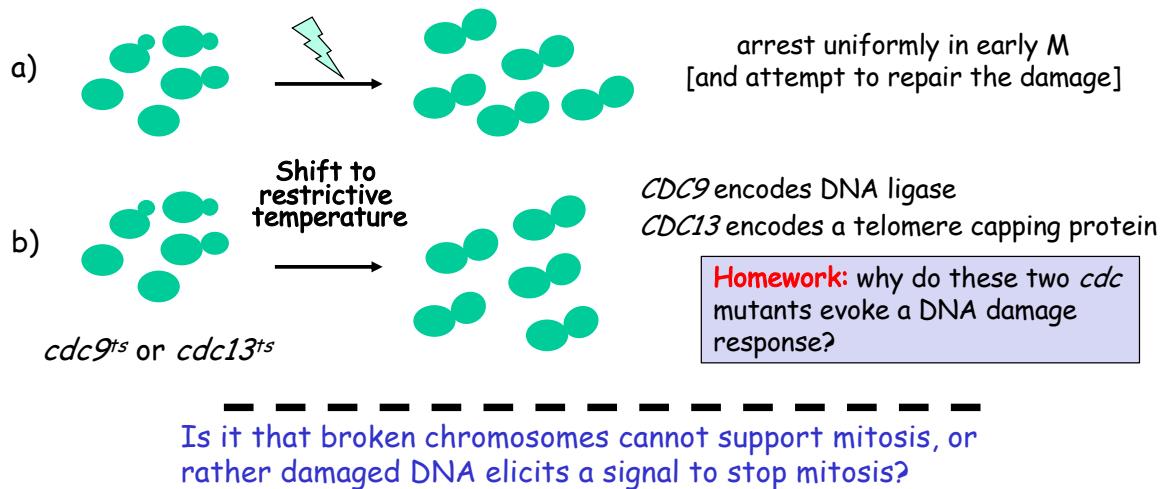
Checkpoints, order & genome integrity

Is order in the cell cycle enforced by checkpoint mechanisms?



If B depends on completion of A due to a surveillance mechanism, it should be possible to isolate **LOSS OF FUNCTION** mutations that relieve the dependence of B on A

DNA damage response in budding yeast



Screening for DNA damage *checkpoint* mutants:



RAD9 is critical for the response to DNA damage

Weinert & Hartwell

- *rad9* cells do not delay the cell cycle and die upon induction of DNA damage.
 Repair-defective mutants (e.g. *rad52*) still display a delay (i.e. they are checkpoint proficient) but die unable to repair.

- *cdc9ts rad9* or *cdc13ts rad9* mutants do not display a *cdc* phenotype and die at the restrictive temperature

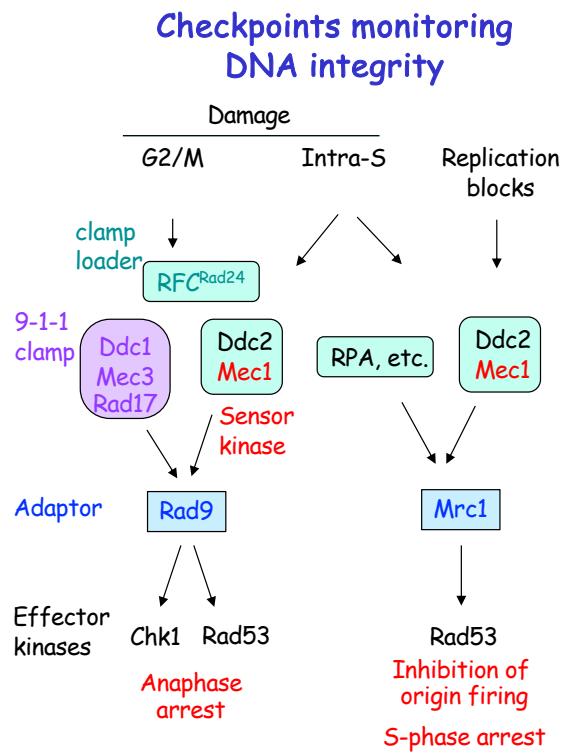
- *RAD9* is not an essential gene and *rad9* mutants show no obvious defect during a normal, unperturbed cell cycle

rad9 is the prototypic “checkpoint” mutant

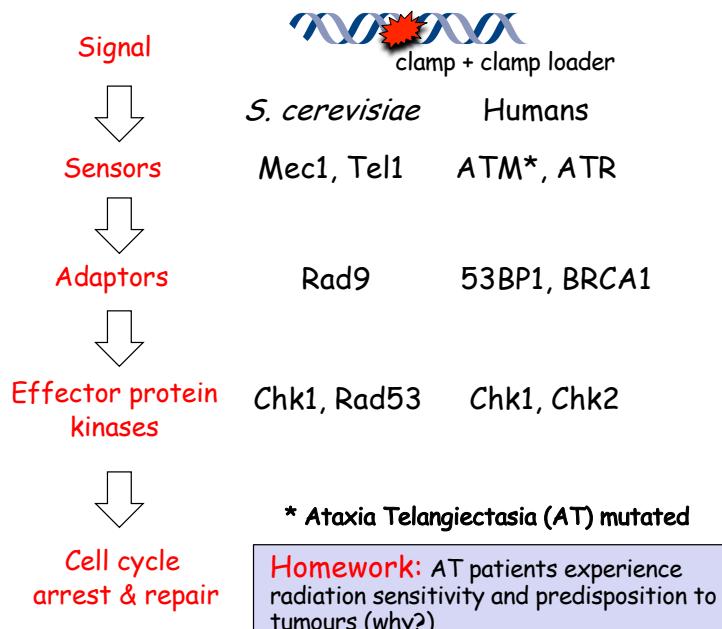
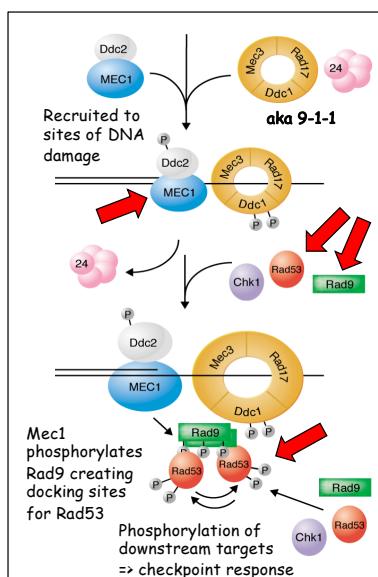
The DNA damage vs. DNA replication checkpoint

Are replication forks under surveillance?

- Mutants that fail to arrest upon treatment with HU:
 - mec1* (mitosis entry checkpoint)
 - rad53*
- These two mutants also proved defective in the DNA damage checkpoint.
- Yet, *rad9* is only defective in the DNA damage checkpoint.



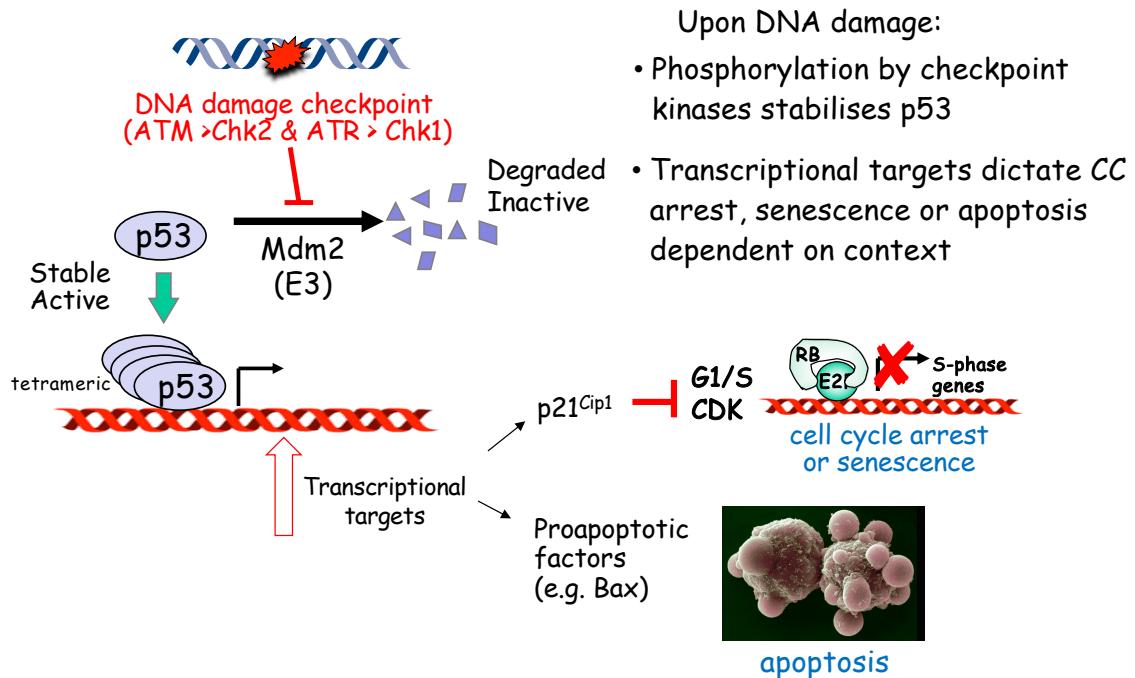
Checkpoints monitoring DNA integrity are conserved between yeast and humans



The significance of these checkpoint pathways is illustrated by predisposition to cancer arising from inactivation of *ATM* or *BRCA1*

p53 is a critical target of the DNA damage checkpoint

Its inactivation is a major hallmark in tumourigenesis

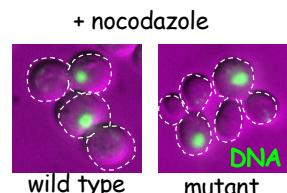


More checkpoint genes...

Can we genetically probe the mechanisms coupling cell cycle progression with the integrity of the mitotic spindle and the success of chromosomal segregation?

→ Mutations preventing cell cycle arrest in response to microtubule poisons

Screen for mutants that rebud (and die) upon treatment with benomyl or nocodazole:



bub1 bub2 bub3 Budding Uninhibited by Benzimidazole*

mad1 mad2 Mitotic Arrest Defective

* benzimidazole-based microtubule-depolymerising drugs: benomyl and nocodazole

Those screens uncovered elements of 2 separate checkpoint pathways

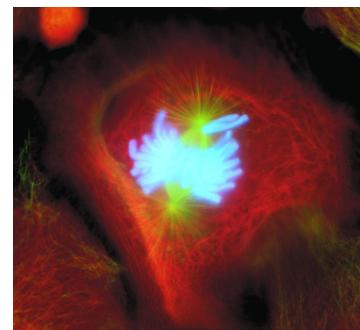
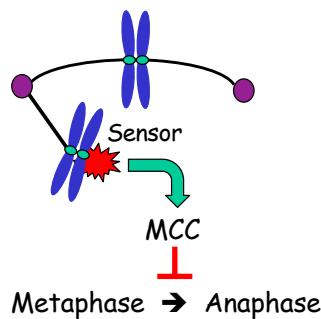
Two checkpoints ensure correct chromosomal segregation in yeast:

Spindle Assembly Checkpoint: restrains onset of anaphase until the spindle is correctly assembled with bi-oriented sister chromatids attached (held together by cohesins).

Spindle Position Checkpoint: restrains mitotic exit to couple completion of chromosomal segregation and cytokinesis. It is the prime surveillance mechanism for spatial coordination between the axis of chromosomal segregation (i.e. the spindle axis) and the cell division plane by targeting the Mitotic Exit Network (MEN).

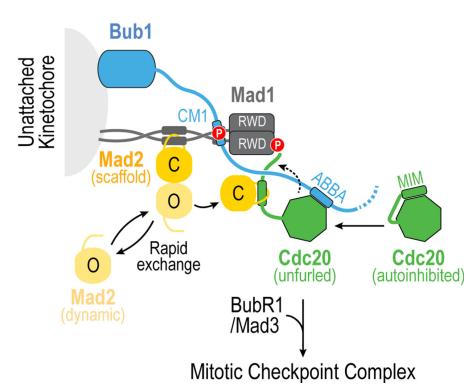
Components of these signalling systems are also conserved throughout evolution

Spindle assembly checkpoint (SAC)



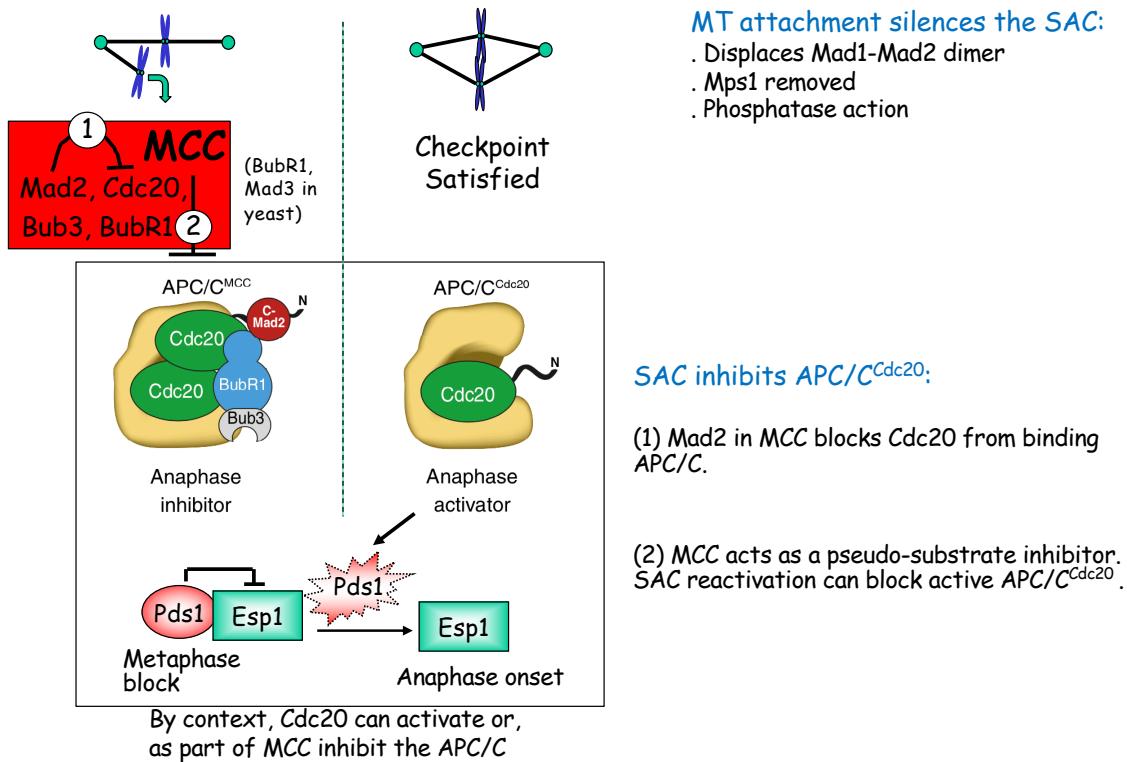
A model for Mitotic Checkpoint Complex (MCC) formation:

- the unattached kinetochore docking of Bub1-Bub3 and Mad1-Mad2 is controlled by the protein kinase Mps1
- "Mad2 template model" based on 2 conformations:
 - closed (C) bound to Mad1 or to its target Cdc20
 - open (O) when unbound
- Cdc20 is recruited at the "ABBA" motif
- Mad2-Cdc20 binding is established at the kinetochore through a (C)(O) dimer intermediate (bound to Mad1)
- BubR1/Mad3 is added to yield MCC

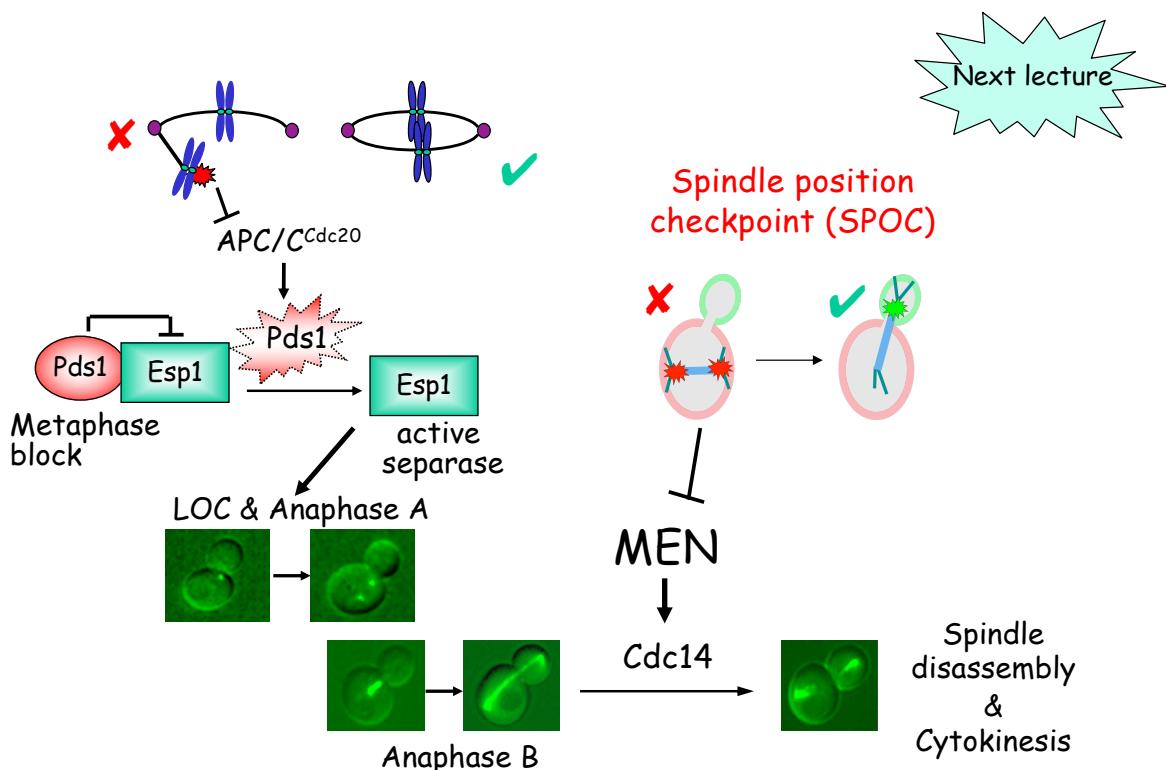


Lara-Gonzalez et al. (2021) *Semin Cell Dev Biol* 117 (2021) 86-98

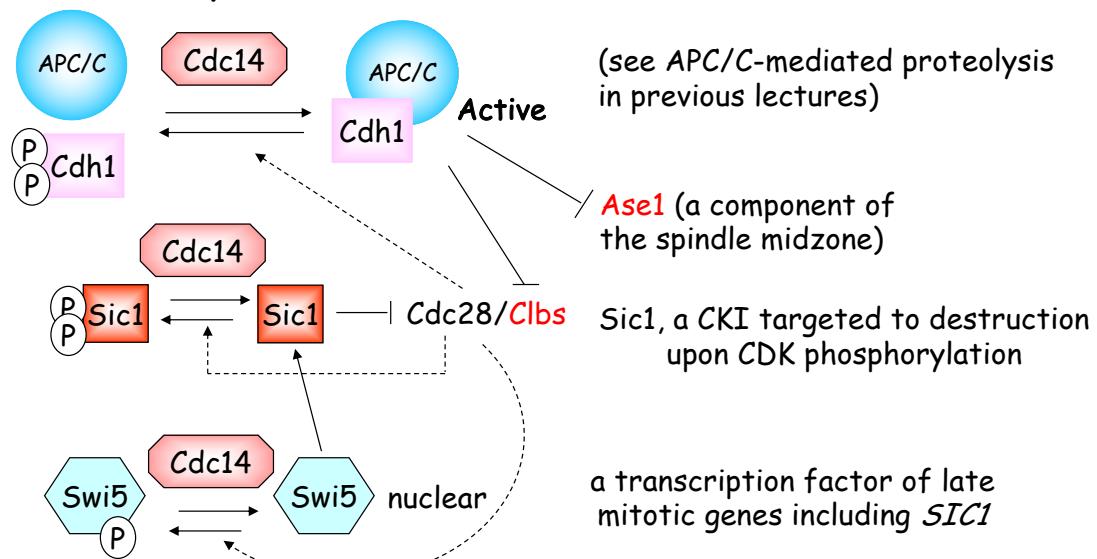
SAC-dependent inhibition of APC/C^{Cdc20}



Checkpoints surveilling spindle integrity & function



Cdc14 reverses CDK phosphorylation of key targets to promote mitotic exit (sneak preview)

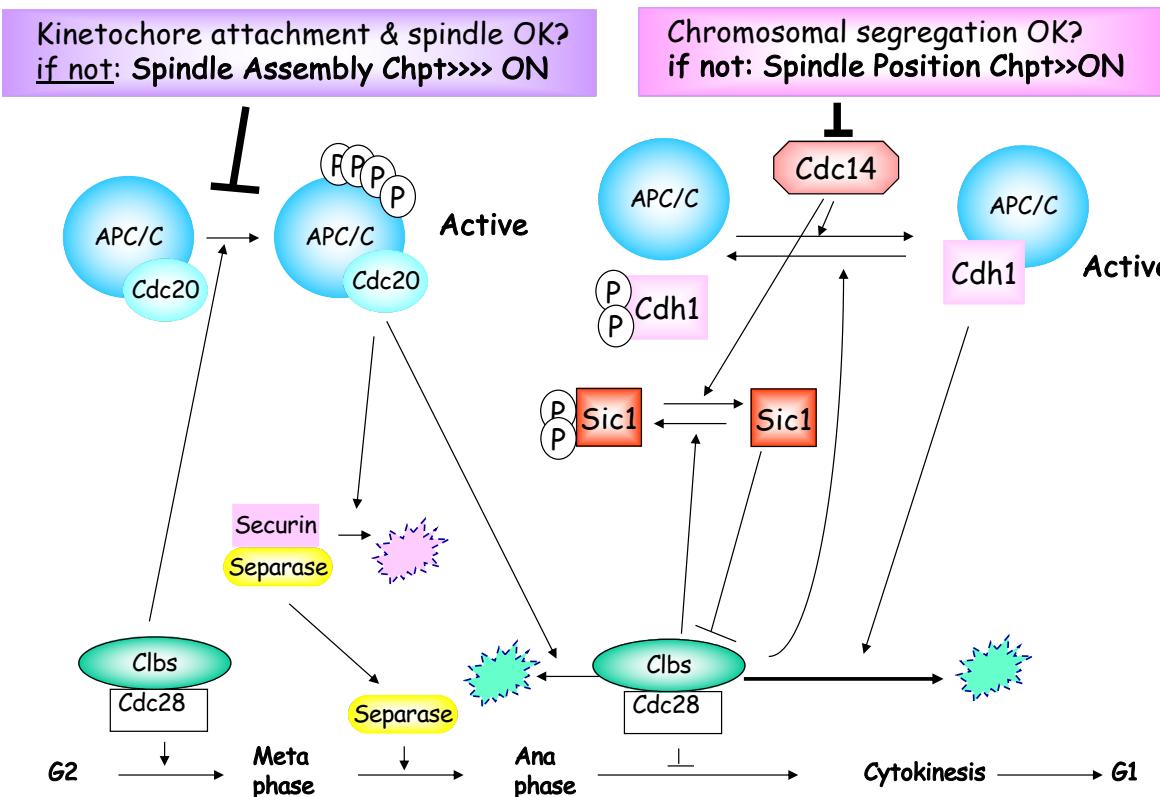


Cdc14 reverses CDK phosphorylation prompting **irreversible** inactivation of Clb-CDKs via

- targeted cyclin destruction by APC/C^{Cdh1}
- accumulation of active Sic1

Homework: What would be the terminal phenotype of *cdc14ts* cells?

APC/C, an effector of mitotic checkpoints



Understanding the mechanisms that make cells tick as well as the pathways enforcing order of cell cycle events is of utmost importance because.....

**Genes encoding Cell cycle and
Checkpoint Proteins
are Frequently Mutated and/or
Dysregulated in Cancer**

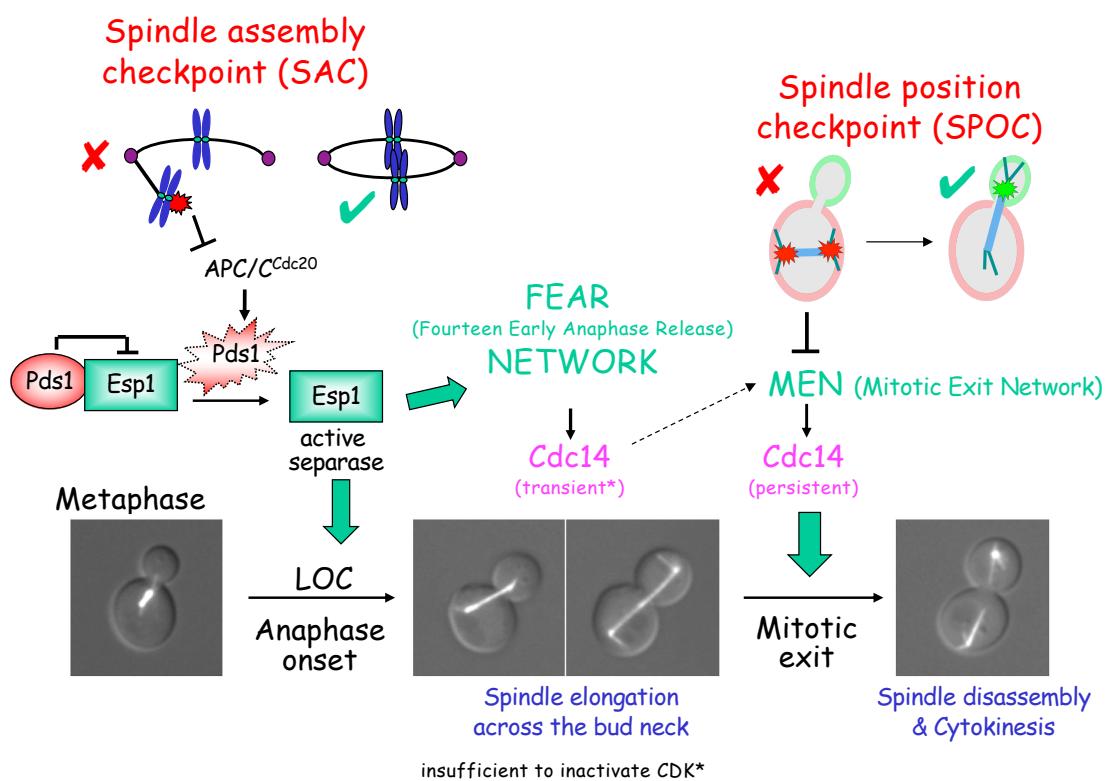
Homework: explore your favourite cell cycle players in depmap (<https://depmap.org/>) and cBioPortal (<https://www.cbioportal.org/>). Submit a summary of your findings.

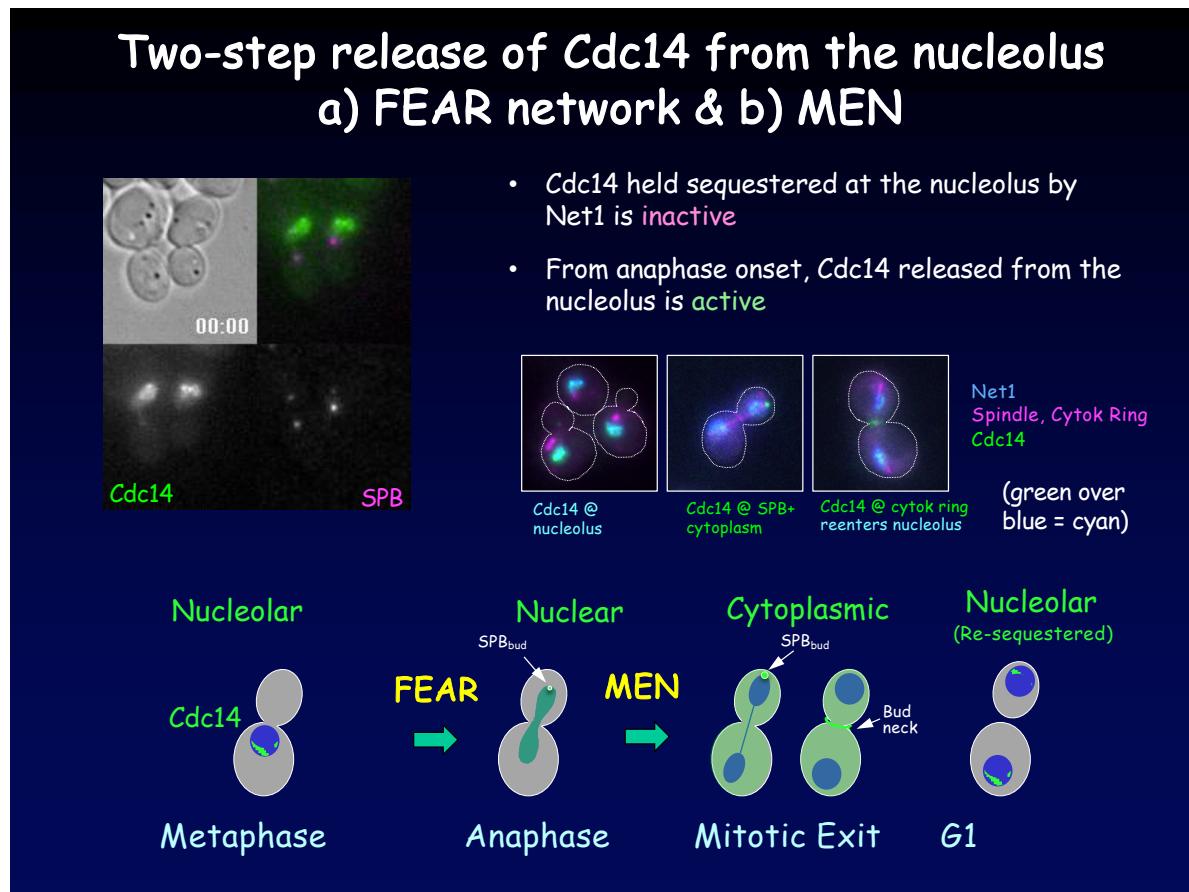
Lecture 6

Cell cycle control in eukaryotic cells (V)

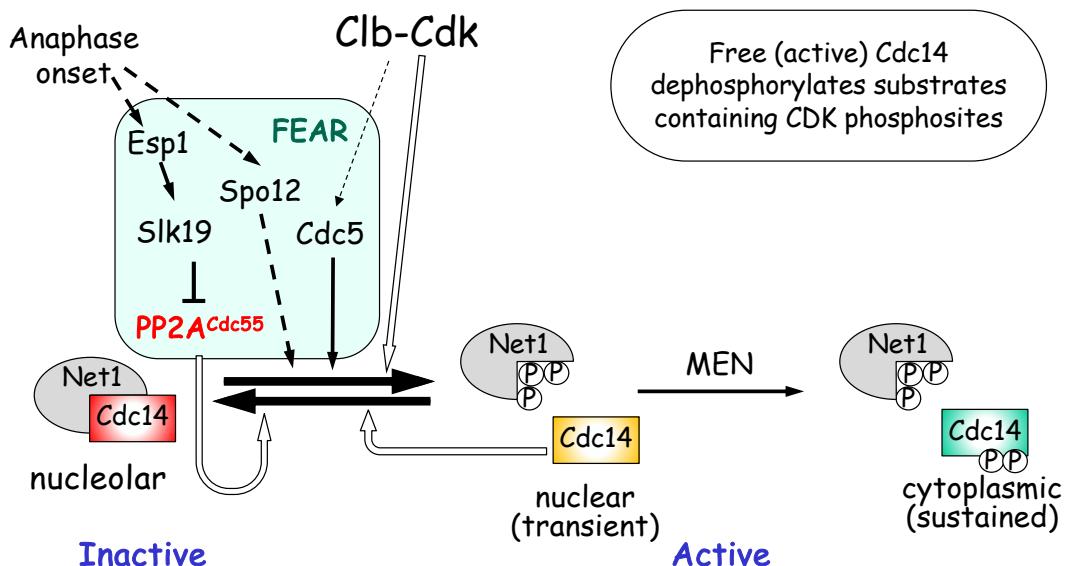
The Spindle Position Checkpoint:
the end of mitosis justifies the means

Control of *Cdc14* activation following anaphase onset





(1) The FEAR network

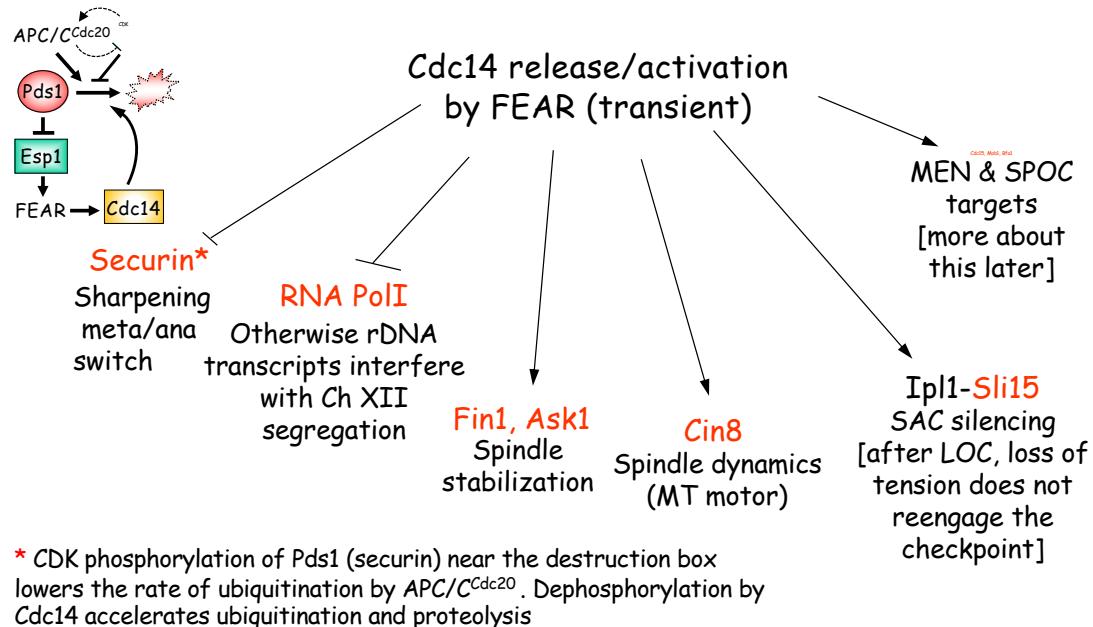


- Cdc14 release by the FEAR network is **TRANSIENT**
- Unless MEN activation follows:
 - Cdc14 is re-sequestered in the nucleolus
 - Cells do not exit mitosis

*Cdc5 = yeast polo kinase

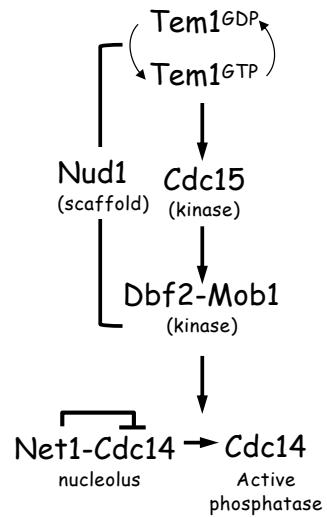
Significance of the FEAR network

Cdc14 coordinates and promotes anaphase dynamics through multiple targets in addition to supporting crosstalk between FEAR, SPOC and MEN



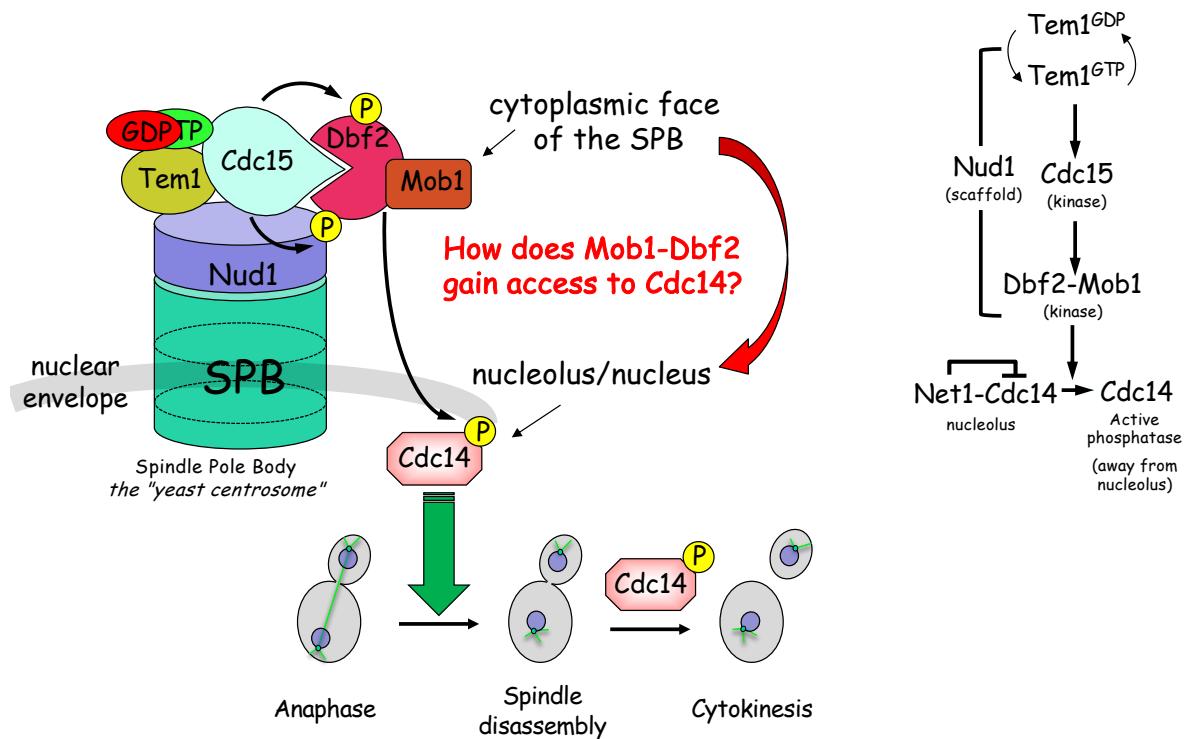
(2) The Mitotic Exit Network

- An **essential** signalling pathway controlled by the small GTPase Tem1
- Active Tem1 drives the recruitment of Cdc15 to the cytoplasmic face of the SPB entering the bud
- Cdc15 phosphorylates the SPB component Nud1 (acting as a scaffold), creating a docking site for the protein kinase complex Dbf2-Mob1
- After docking at the SPB, Dbf2-Mob1 is activated by Cdc15 phosphorylation
- Active Dbf2-Mob1 enforces Cdc14 complete release over the cell by phosphorylation

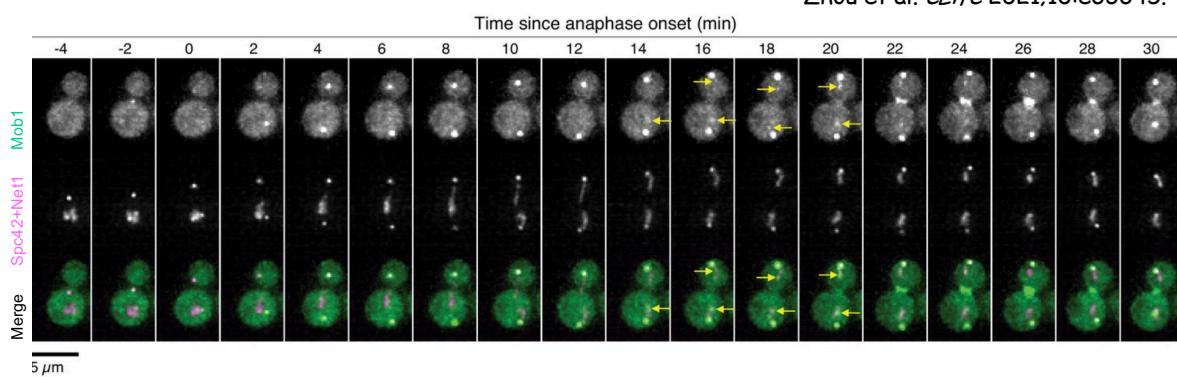


Homework: Why did *cdc14^{ts}* and *cdc15^{ts}* mutants meet the premise of the *CDC* screen. What might be the phenotype of *tem1^{ts}* or *dbf2^{ts}* mutants? Should those have been *CDC* genes? Comment.

(2) The Mitotic Exit Network

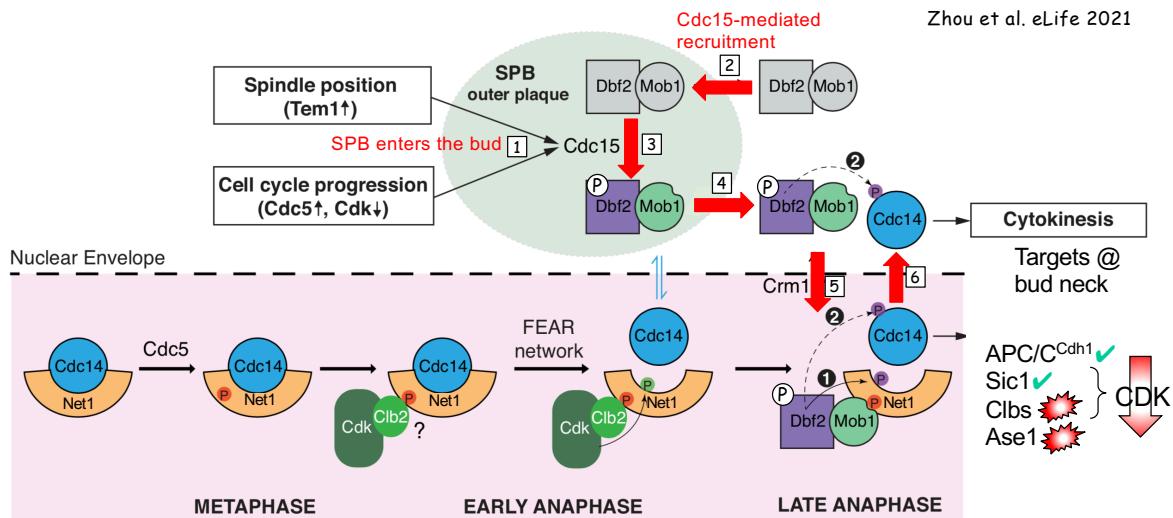


Cross-compartment signal propagation in the mitotic exit network

Zhou et al. *eLife* 2021;10:e63645.

- Nuclear import of Mob1-Dbf2 depends on Cdc15 activity (suppress NES)
- Cdc5 (polo kinase) phosphorylates Net1, creating a docking site for Mob1-Dbf2 in the nucleolus
- Mob1-Dbf2 phosphorylates Net1 (on different sites than CDK or Cdc5) and Cdc14 to promote Cdc14 persistent release

A model for cross-compartment signalling in the MEN

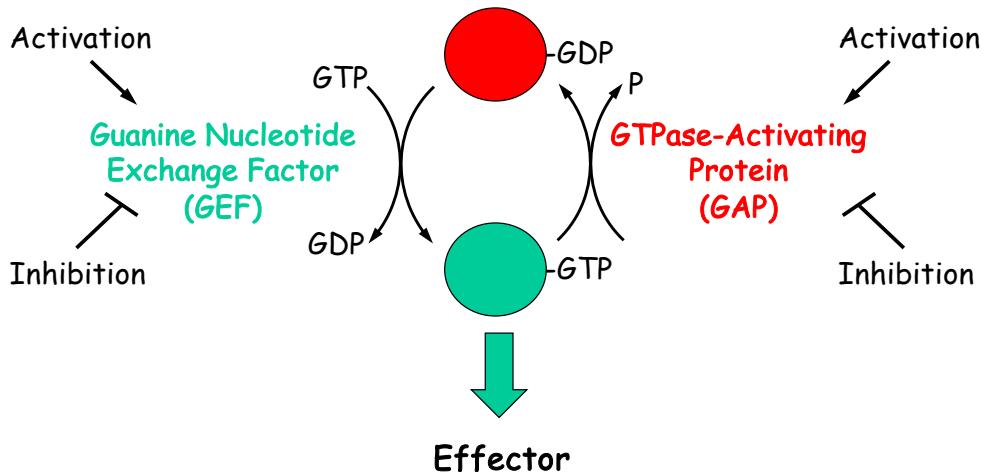


- Spatial and temporal cues instruct Tem1 activation (and thus the MEN).
- For our delight, multiple layers of cell cycle control are superimposed on the deceptively linear pathway leading to Cdc14 release
- The FEAR/MEN/SPOC system is a showcase for the complex architecture of cell cycle control mechanisms that continues to amaze

Homework: Phospho-proteomic analysis mapped various phospho-sites on Net1 responding to the consensus for Cdc5, CDK or Mob1. Knowing that S/T to A substitutions on a substrate impede phosphorylation and S/T to D mimic phosphorylation, outline a strategy combining *NET1* phospho-site mutants and the use of conditional alleles of *CDC5* or MEN kinases to test aspects of this model.

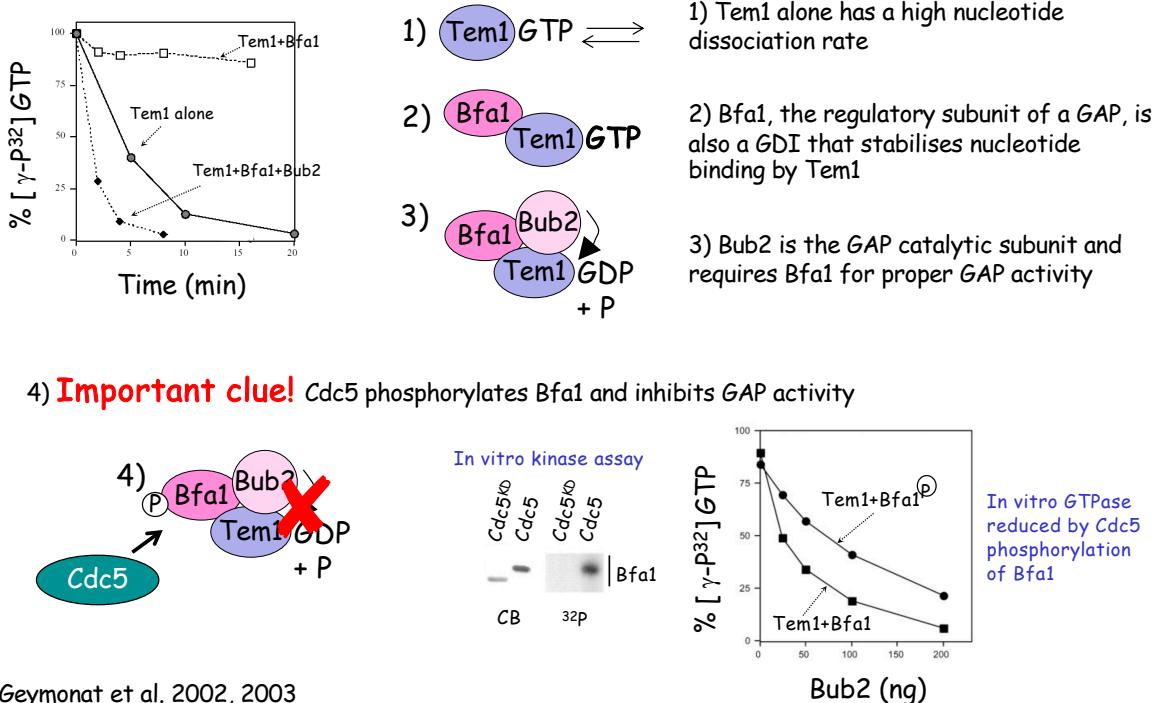
(3) Spotlight on the small GTPase Tem1, as target of the SPOC

Canonical small GTPase cycle



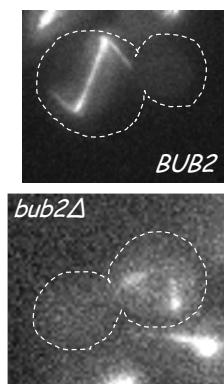
How is the Tem1 cycle regulated?

Bfa1-Bub2, a very special two-component GAP that downregulates Tem1



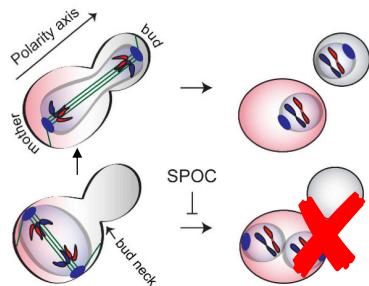
Geymonat et al. 2002, 2003

Inactivation of the GAP by *bub2Δ* or *bfa1Δ* abrogates the SPOC



SPOC-proficient cells misorienting the spindle restrain mitotic exit until one SPB has entered the bud (i.e. one set of chromosomes has been delivered to the daughter cell)

Incorrect mitotic exit due to *bub2Δ* (i.e. no GAP): the mispositioned spindle disassembles within the mother cell



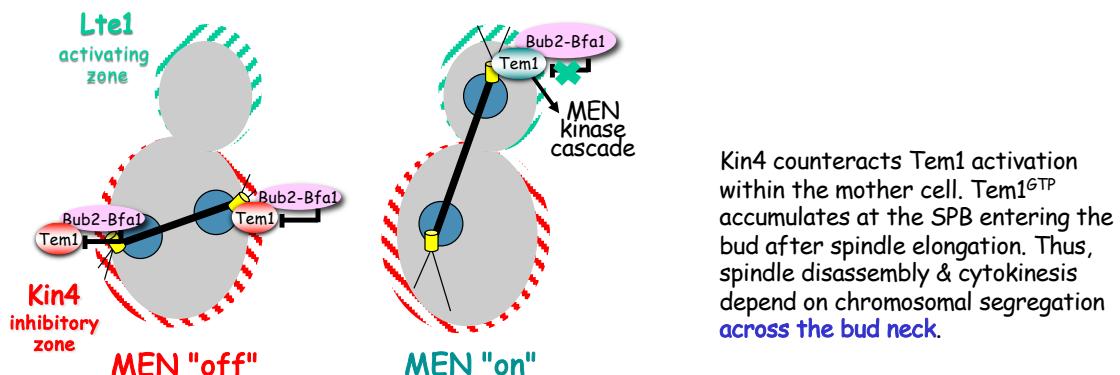
The cell monitors the translocation of one SPB in the bud compartment

How?

SPOC "two-zone model"

Proposed by Angelika Amon
Many labs have contributed

- Antagonistic components occupy opposite cell compartments effectively controlling MEN activity in response to the position of the anaphase spindle
- Kin4 marks the MEN-inhibitory domain and Lte1 the MEN-activating domain in the mother cell or bud, respectively. Cortical compartmentalisation is strict.
- The Tem1 module at the SPB acts as a **spatial sensor** also regulated by **cell cycle cues**

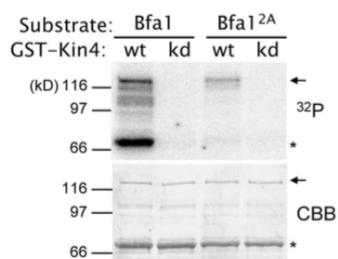


How does the protein kinase Kin4 inhibit MEN activation?

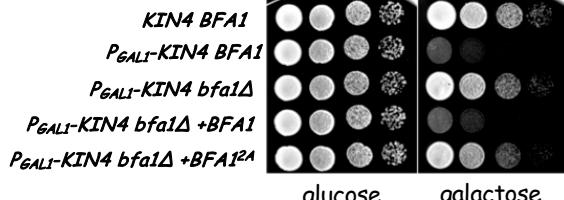
Kin4 protects Bfa1 from the inhibitory phosphorylation by Cdc5

. Bfa1 but not Cdc5 is a substrate of Kin4 *in vitro*

. Two main phospho-sites identified

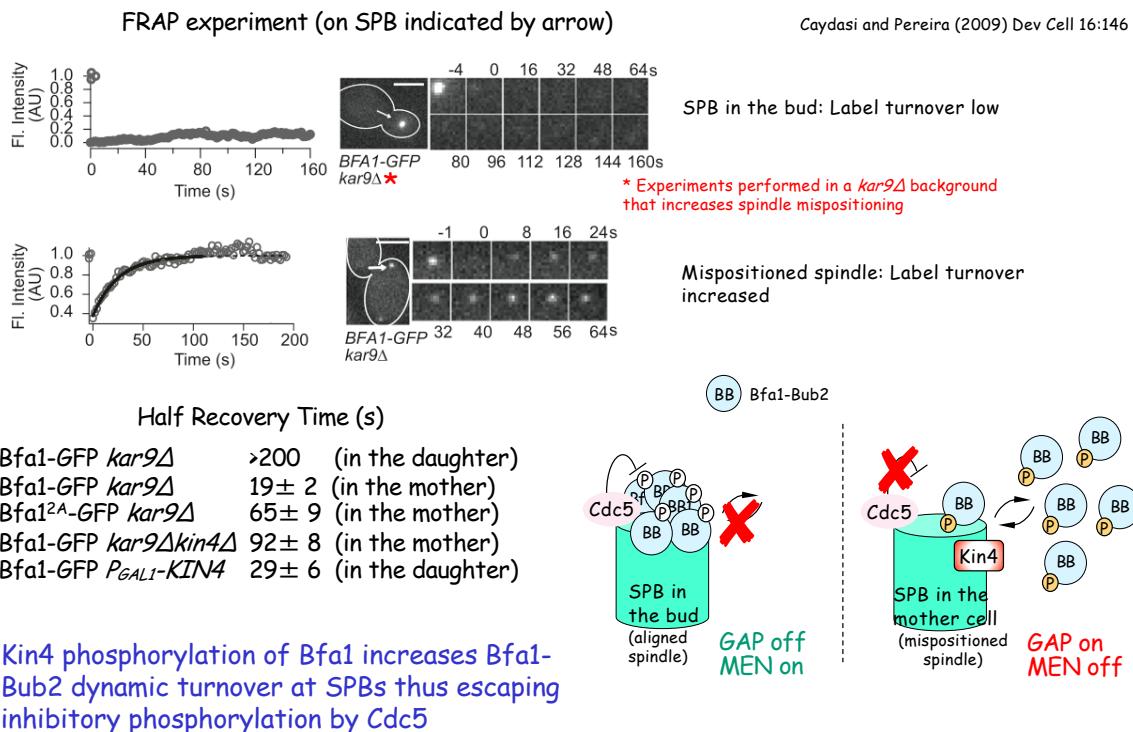


CBB = Coomassie brilliant blue
(a protein stain)



Lethality is prevented by substitutions that cancel the Kin4-dependent phospho-sites on Bfa1.

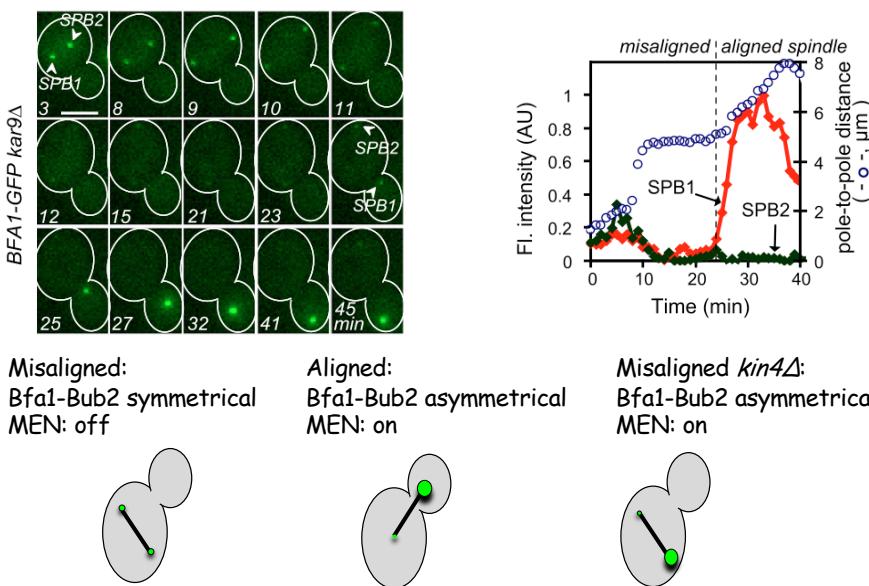
Kin4 increases Bfa1-Bub2 turnover at the SPB protecting the GAP from inactivation by Cdc5 during SPOC arrest



Localisation of Bfa1-Bub2, linked to mobility is cell cycle regulated and symmetric while the SPOC is on

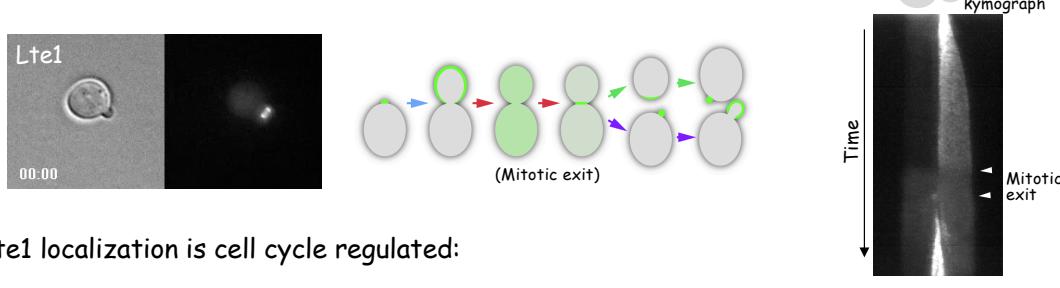
Caydası and Pereira (2009) Dev Cell 16:146

During spindle re-orientation, symmetric → asymmetric as the SPOC is satisfied



Lte1 a GEF-like protein like no other...

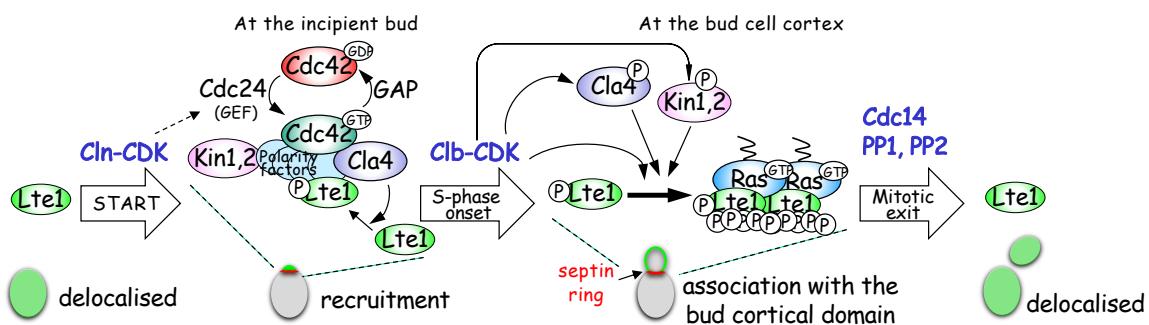
- A positive regulator of mitotic exit, its overexpression abrogates the SPOC
- It contains signature domains of GEFs, yet, experiments failed to prove that it is the GEF for Tem1



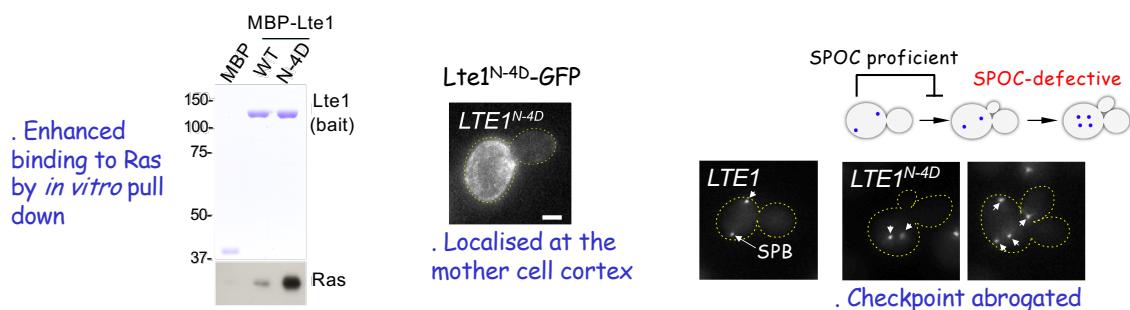
- Lte1 localization is cell cycle regulated:
 - Cdc42 triggers Lte1 recruitment to the incipient bud **after START**
 - Multisite phosphorylation by CDK, the p21-activated kinase (PAK) Cla4 and the yeast PAR-1 homologs Kin1 and Kin2 promotes Lte1 anchoring to Ras-GTP
 - The involvement of **polarised kinases restricts** the association between Lte1 and Ras* **to the bud**. The septin ring forming at the bud neck during bud emergence creates a diffusion barrier between mother and bud cortex precluding Lte1 access to the mother cell membrane domain.

* The conserved Master Regulator of cell polarity, note also a *CDC* product
 * Ras is evenly distributed over the entire plasma membrane

Lte1 integrates cell polarity and cell cycle cues for strict compartmentalization to the bud

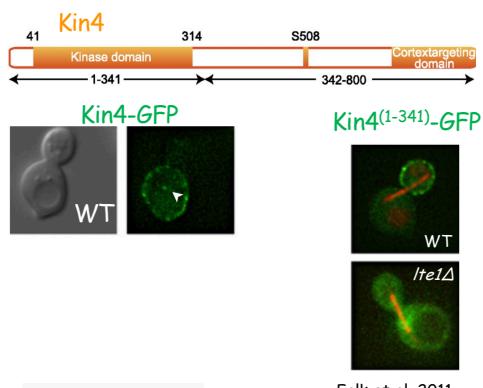


- Phospho-mimetic substitutions constitutively enhancing Lte1 binding to Ras, allow Lte1 recruitment to the mother cell cortex before bud emergence and abrogate the SPOC

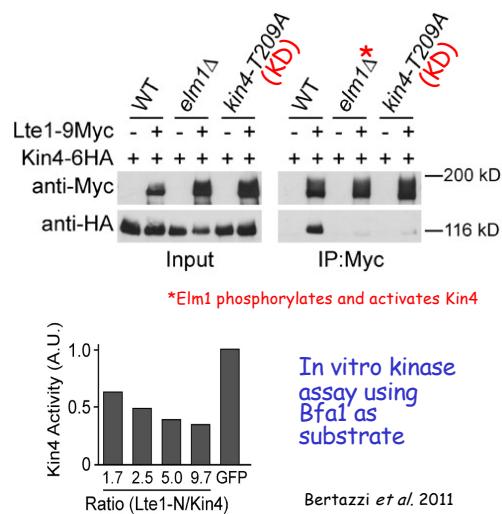


Lte1 binds and inhibits Kin4

- *In vivo* interaction requires active Kin4
- Lte1 inhibits Kin4 activity *in vitro*
- The interaction with Lte1 is mediated by the kinase domain of Kin4



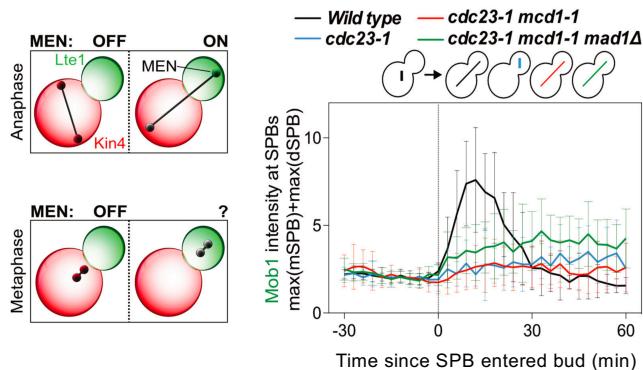
Falk et al. 2011



Work in progress

However, Lte1 also promotes mitotic exit independent of Kin4. The molecular mechanism is unknown. Is it directly activating Tem1?????

What confines MEN activation to late anaphase?



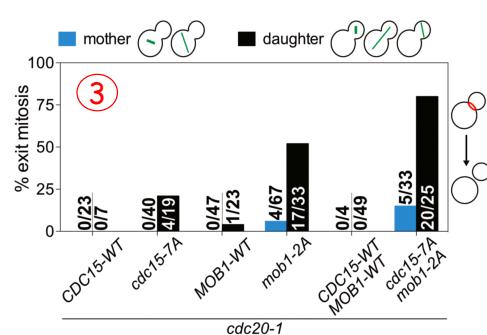
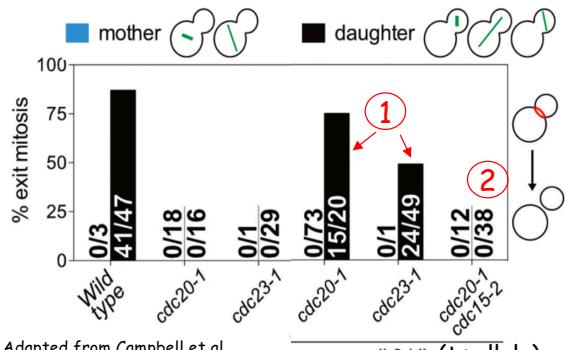
Notes:

- *MCD1* = *SCC1* (cohesin)
- *cclin23-1* and *mcd1-1* are *ts* alleles
- *Mob1-GFP* marks the SPB if MEN active (see before)
- strain colour-coded above the plot depicts the extent of cell cycle progression (line represents the spindle)

- SPB entry into the bud or spindle elongation is insufficient to activate the MEN without APC/C^{Cdc20} activation—why?

A wild type strain over-expressing *Cib2Δdb* under *P_GAL1* control enters anaphase (i.e. APC/C^{Cdc20} activated) yet, if fails to localise *Mob1-GFP* to the SPB \Rightarrow mitotic CDK antagonises the MEN

MEN integrates cell cycle and spatial cues to confine activation to late anaphase



- Spindle position & septin ring loss (a proxy for mitotic exit) were scored at restrictive temperature:

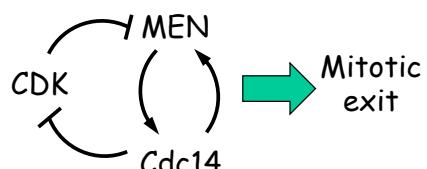
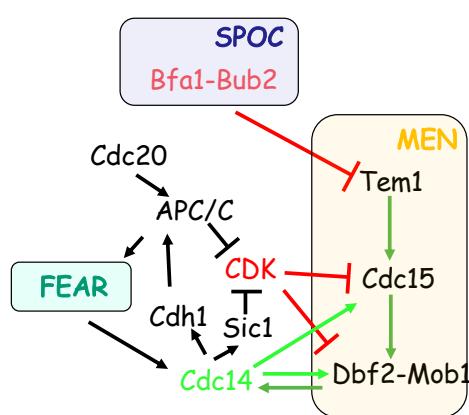
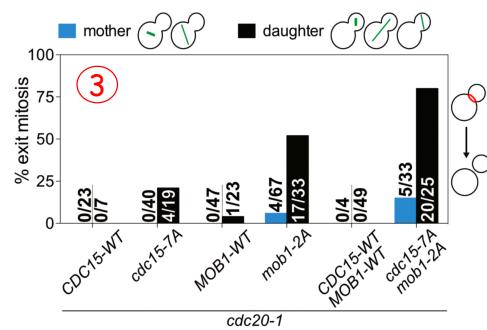
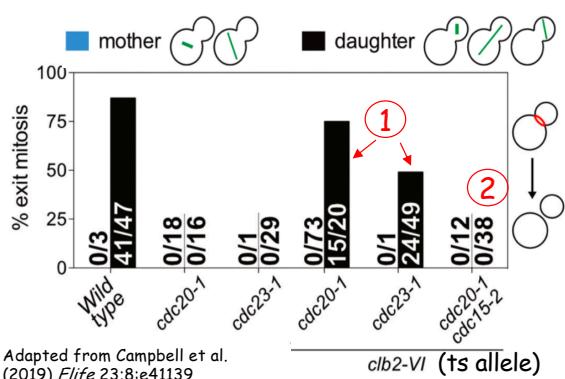
1) Clb2-Cdk activity allows mitotic exit of metaphase cells (APC/C-blocked) provided a spindle pole has entered the bud

2) Clb2-Cdk cannot bypass the MEN

3) Mob1 and Cdc15 are CDK substrates. S/T to A substitutions preventing phosphorylation enable APC/C-blocked cells to exit mitosis without CDK downregulation provided a spindle pole has entered the bud

→ CDK at metaphase inhibits the MEN by targeting Cdc15 and Mob1

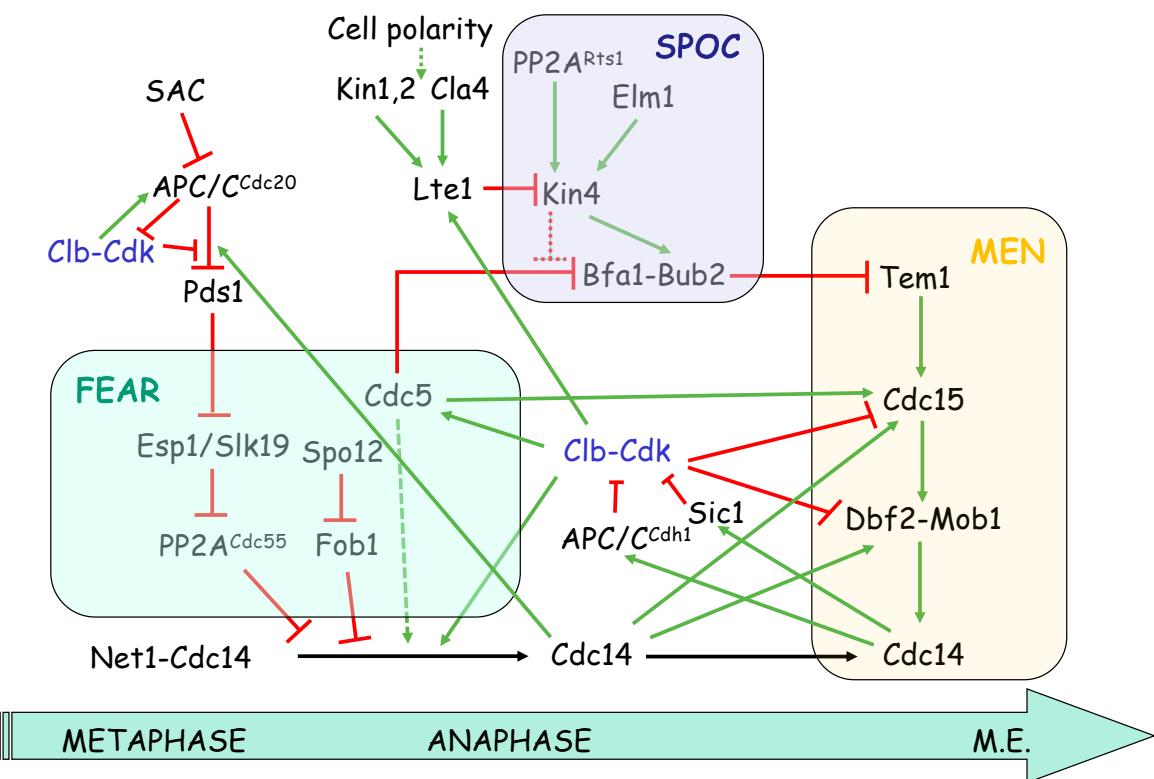
MEN integrates cell cycle and spatial cues to confine activation to late anaphase



⇒ Logical "AND gate" insulates MEN against spindle movement acting as a trigger prior to anaphase

Summary: spatial and temporal control of Cdc14 activation

--logical "AND" gate, network interplay--



Bonus: The MEN core kinase module is conserved in the Hippo pathway

Upstream signals include cell polarity and mechanical cues, cell density and stress

