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FACULTY NAME	**SCIENCE**
DEPARTMENT NAME	**BIOSCIENCES**
SUBJECT NAME	**CELL BIOLOGY**
COURSE	**M.Sc. BIOCHEMISTRY**
COURSE DURATION	**2 Years**
SUBTOPIC NAME	**Cell Cycle and its regulation**
CONTENT TYPE	**PDF**
SEARCH KEYWORDS	**cell cycle**

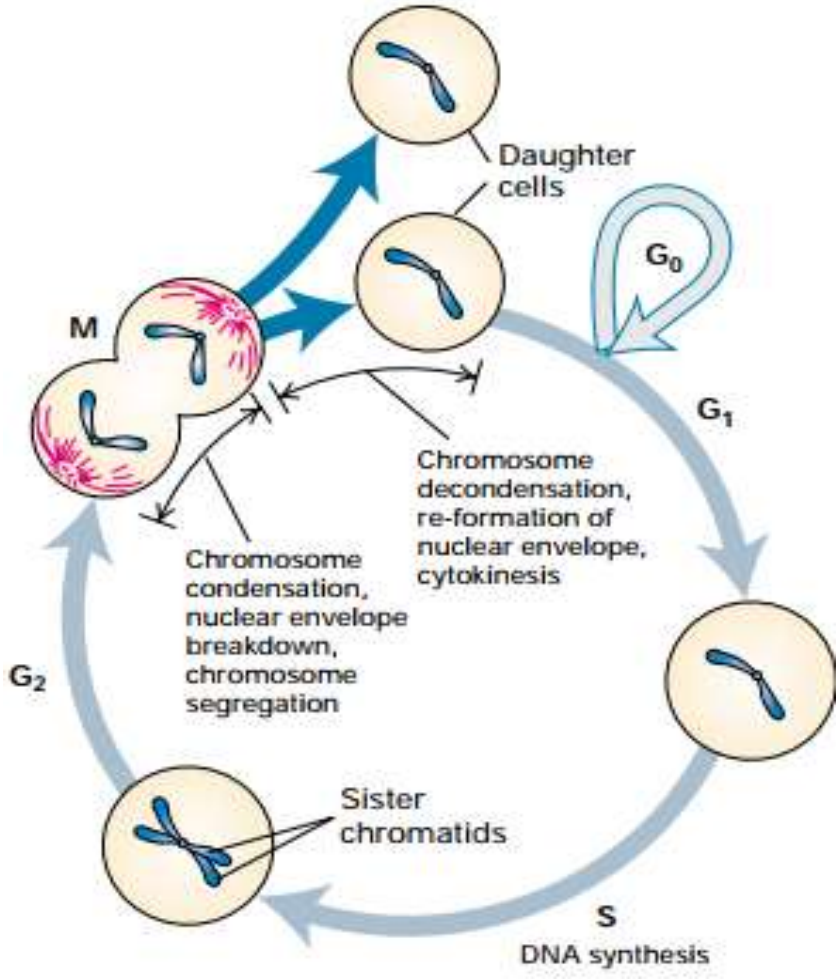
Cell Cycle and its regulation

Objectives:

To acquaint the students about:

1. Cell cycle and its different phases;
2. Critical points for regulation of cell cycle
3. Mechanism of cell cycle regulation by Cdk-cyclin complexes
4. Different regulatory proteins involved in cell cycle regulation

The division cycle of most cells consists of 4 coordinated processes: cell growth, DNA replication, distribution of duplicated chromosomes to daughter cells, and cell division. In bacteria, cell growth and DNA replication take place throughout the cell cycle, and duplicated chromosomes are distributed to daughter cells in association with PM. In eukaryotes, however, cell cycle is more complex and consists of 4 discrete phases.



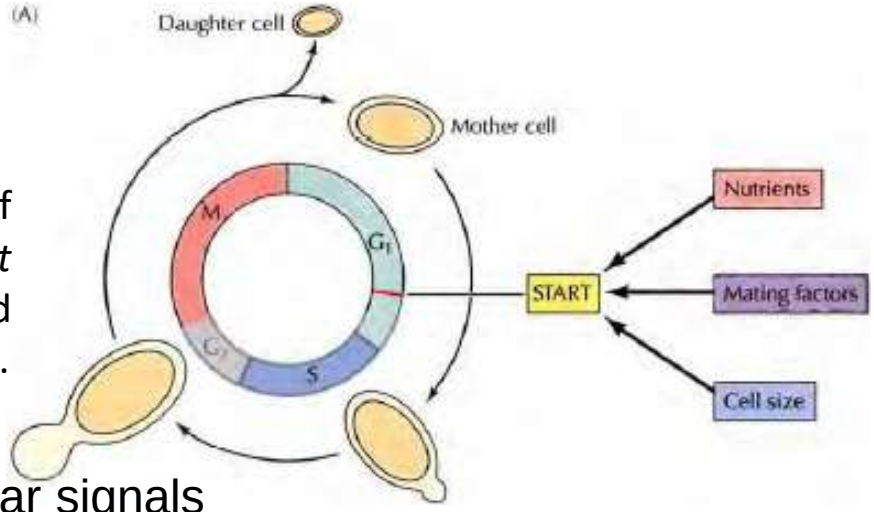
For a typical human cell with a total cycle time of 24 hours, the G1 phase might last ~ 11 h, S phase ~8 h, G2 ~4 h, and M ~1 h.

Regulation of cell cycle by cell growth and extracellular signals

Progression of cells through division cycle - regulated by internal and extracellular signals from environment as growth factors which monitor and co-ordinate various processes during cell cycle phases. Different cellular processes as cell growth, DNA replication and mitosis all must be coordinated during cell cycle progression. This is accomplished by control points which regulate progression through various processes which take place during different cell cycle phases. Cell cycle progression depends on discrete control points at which decision is taken on whether to proceed to another cycle.

A major cell cycle regulatory point in many cells is in late G1 and controls progression from G1 to S (first seen in budding yeast - *S. cerevisiae*) called as **START**.

-Once cells have passed START, they get committed to entering S phase and undergo one cell division cycle. But passage through START is highly regulated event in yeast cell cycle, controlled by availability of nutrients and cell size. eg if yeast cells have shortage of nutrients, they arrest cell cycle at start and enter resting state rather than proceeding to S-phase. Thus Start is the decision point at which cell determines if sufficient nutrients are available to support progression through rest of division cycle. Polypeptide factors that signal yeast mating also arrest cell cycle at START allowing haploid cells to fuse instead of progression to S phase.



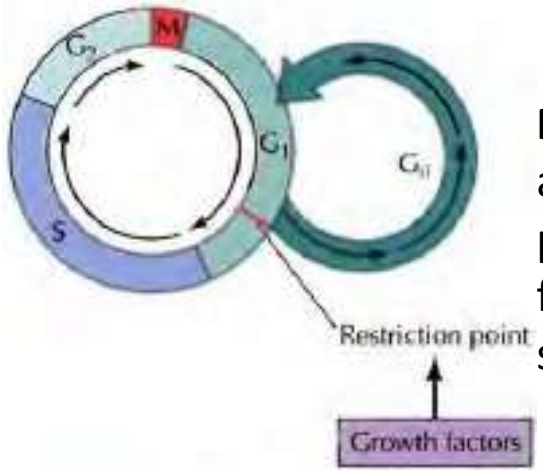
Regulation of the cell cycle of budding yeast Cell cycle of *Saccharomyces cerevisiae is regulated primarily at a point in late G1 called START.* Passage through START is controlled by the availability of nutrients, mating factors, and ceiJ size.

START- Decision point for monitoring extracellular signals

-Point at which cell growth coordinated with DNA replication and cell division.

This regulation - very important in yeast cells as division leads to large mother cell and small daughter cell. Thus, to maintain constant size, small daughter cell must grow more than large mother cell before next division. Thus, cell size must be monitored to co-ordinate cell growth with other cell cycle events this regulation accomplished by control mechanism which requires each cell to attain a minimum size before passing start their small daughter cell spends longer time in G1 phase and grows more than mother cell.

- In animal cells, decision point in late G1 phase cal is called **Restriction point**.
Passage of animal cells through cell cycle regulated by extracellular growth factors rather than cell size/nutrients. When appropriate growth factors - not available in G1, progression through cell cycle stops at restriction point. Such arrested cells enter **Quiscent phase Go** and may remain for long time without proliferating. Go cells are metabolically active, cease growth and have reduced protein synthesis. Many animal cells remain in Go unless stimulated by growth factors or extracellular signals for proliferation. e.g. skin fibroblasts arrested in Go phase until stimulated and cells divide as required to repair wound damage. During clotting blood platelets release platelet-derived growth factors signal, which signal fibroblast proliferation in vicinity of injured tissues.



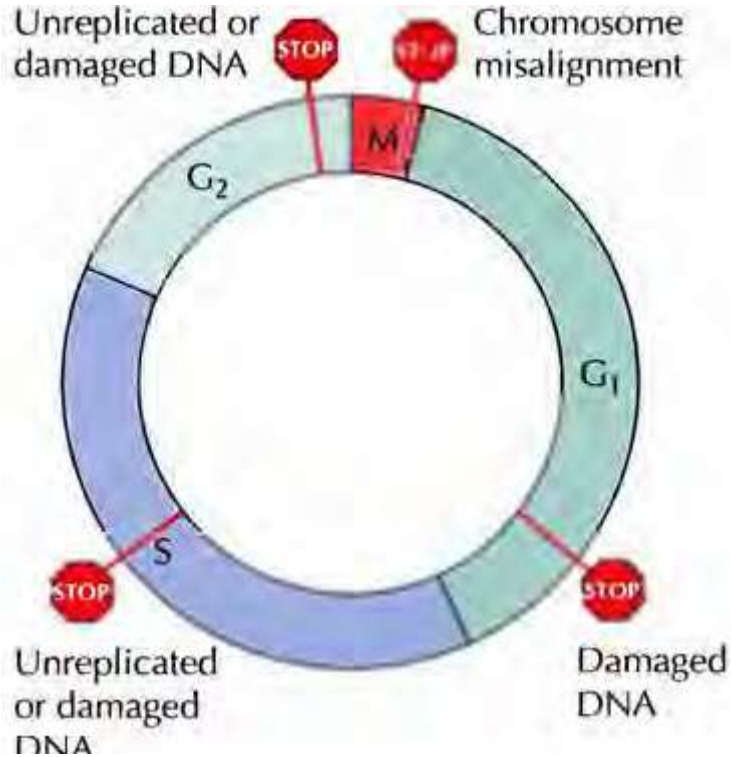
Regulation of animal cell cycles by growth factors. The availability of growth factors controls animal cell cycle at a point in late G1 called the restriction point. If growth factors are not available during G1, cells enter a quiescent stage of cycle called G0.

- Although proliferation of most cells - regulated in G1, some cell cycles controlled in G2. Commitment to mitotic division - at end of G2 phase. If cells do not divide at this point, it remains with twice the normal complement of chromosomes e.g. 1. fission yeast *S. pombe,* 2. Oocytes of animal vertebrates (remain arrested in G2 for decades till M phase is triggered by hormonal stimulation), 3. Some haploid mosses, 4 some insects (during embryogenesis nuclei divide and rest in tetraploid stage). In haploid world cells often rest in G2, affords protection against DNA damage, since 2 copies of DNA.

Cell cycle checkpoints

The orderly progress through cell cycle controlled by checkpoints which prevent one stage from proceeding unless necessary earlier stages have been completed.

For a cell with a division with a cycle determined through G1 control, START marks point at which cells decide do I divide? Beginning of S phase marked by point at which replication apparatus begins to synthesise DNA Start of mitosis identified by moment at which cells begin to reorganize for division Each of these event represents discrete moments at which a molecular change occurs in a regulatory molecule.



Checkpoints control the ability of cell to progress through cycle by determining whether earlier stages have been completed successfully

A check point works by acting directly on factors which control progression through cell cycle
They ensure - incomplete or damaged chromosomes are not replicated and passed on to daughter cells.

Checkpoints can arrest cell cycle at many points in response to intrinsic or extrinsic conditions

Arrest at	Responds to
G1	DNA damage or cell size or nutrients or growth factors
S	Incomplete replication
G2	DNA damage or cell size
M	Unattached kinetochore (chromosome attached to spindle)

Cell cycle checkpoints

Besides regulation of cell cycle in response to extracellular signals, cell size and growth factors, events in different stages of cell cycle must be coordinated with one another so that they occur in appropriate order. eg.it is important that cells not begin mitosis and till genome replication is completed otherwise catastrophic cell division occurs and daughter cell fail to inherit complete copies of genetic material.

Mostly - coordination between different phases of cell cycle is dependent on system of checkpoints and feedback controls which prevent entry into next phase of cell cycle untill preceding phase events are complete.

Several cell cycle checkpoints ensure incomplete or damaged chromosomes are not replicated and passed on to daughter cells

1. **G2 checkpoint** *prevents mitosis initiation untill DNA replication is completed*, senses unreplicated DNA, which generates a signal leading to cell cycle arrest. Therefore prevents M-phase initiation before S phase competition, so cells remain in G2 until genome has been completely replicated. Only then is the inhibition of G2 progression relieved, allowing the cell to initiate mitosis and distribute the completely replicated chromosomes to daughter cells.

-G2 checkpoint also senses DNA damage, such as from irradiation. If DNA is damaged, arrest at G2 checkpoint allows time for the damage to be repaired, rather passed on to daughter cells.

2. G1/S checkpoint

DNA damage not only arrests the cell cycle in G1 and S phase and allows damage repair to take place before the cell enters S phase, where damaged DNA would be replicated. The S-phase checkpoint provides continual monitoring of integrity of DNA to ensure that damaged DNA is repaired before it is replicated and also repair any errors introduced during DNA replication.

In mammalian cells, arrest at G1 checkpoint-mediated by action of p53 protein that is rapidly induced in response to damaged DNA.

-Gene encoding p53 – frequently mutated in human cancers.

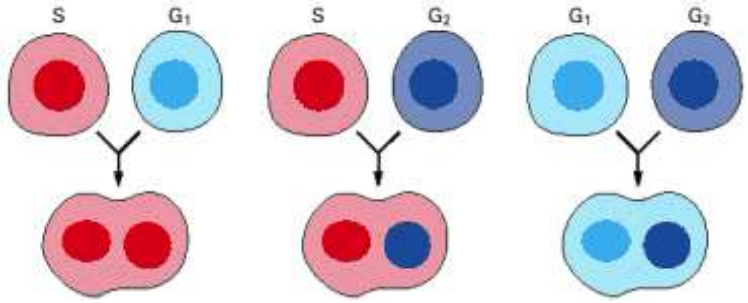
-- loss of p53 due to mutations, prevents G1 arrest in response to DNA damage, thus the damaged DNA id replicated and passed to daughter cells instead of being repaired.

-Inheritance of damaged DNA increases frequency of mutations and general instability of cellular genome, thereby contributing to cancer development.

3. M (spindle assembly) checkpoint maintains integrity of genome at end of mitosis. This checkpoint monitors alignment of chromosomes on the mitotic spindle, thus ensuring that a complete set of chromosomes is distributed accurately to the daughter cells. For example, the failure of one or more chromosomes to align properly on the spindle causes mitosis to arrest at metaphase, prior to the segregation of newly replicated chromosomes to daughter nuclei. As a result of the spindle assembly checkpoint, the chromosomes do not separate until a complete complement of chromosomes has been organized for distribution to each daughter cell.

Cell Fusion experiments identify cell cycle inducers

Each event in the cell cycle, represents discrete moments at which a molecular change occurs in a regulatory molecule. Presence of different regulator at different stages of cell cycle were observed by experiments which used cells in different stages of cell cycle. Fusion was done by mixing cultured mammalian cells in presence of chemicals (as PEG) or viral agents caused plasma membrane to fuse. The resulting hybrid cell had two nuclei in a common cytoplasm.



Cell-fusion experiments

Cell 1	Cell 2	Heterokaryon		Explanation
		Nucleus 1	Nucleus 2	
S phase	G1 phase	Replicates	Replicates	S phase cell cytoplasm has an activator of DNA replication. It was observed that the increase in ratio of S phase to G1 phase nuclei increases rate at which G1 nuclei enter replication. This regulator was called as ***S phase Activator***.
G2 phase	S phase	Waits (unable to initiate DNA synthesis even and presence of a cytoplasm	S phase nucleus replicates	Apparently DNA synthesis in G2 nucleus is prevented by a mechanism which blocked re-replication until after mitosis takes place. G2 nucleus had to pass through M phase and enter G1 before another round of DNA replication could be initiated.
M phase	Interphase	Mitotic	Enters pseudomitosis (divides)	M phase inducer present in dividing cells.

Regulators of Cell Cycle Progression

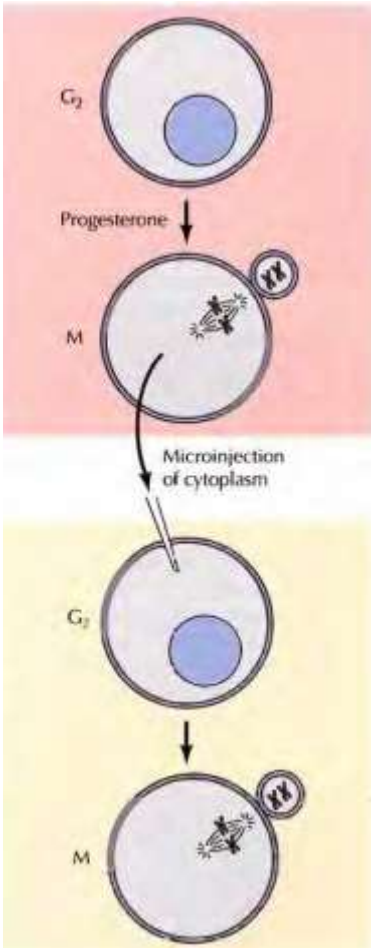
•-Cell cycle of all eukaryotes (yeasts, sea urchins, frogs, mammals) is controlled by a conserved set of protein kinases, responsible for triggering major cell cycle transitions.
•-Cell cycle control system is based on cyclically activated protein kinases, that exhibit increase or decrease in activity as cell progress through cell cycle.
•**MPF** (Maturation Promoting Factor, also called as Mitosis Promoting Factor) is a dimer of cdc2 and cyclin.

Protein Kinases and Cell Cycle Regulation

Three distinct experimental approaches contributed to identification of the key molecules responsible for cell cycle regulation.

<u>Ist approach based on studies on frog oocytes</u>

•Frog oocytes remain arrested in G2 phase of cell cycle until hormonal stimulation triggers entry into M phase of meiosis.
•When G2-arrested *Xenopus* oocytes are removed from a female frog and treated with progesterone, they undergo oocyte maturation from a G2-arrested oocyte to egg in meiosis.
•In 1971, two teams of researchers (Yoshio Masui and Clement Markert, as well as Dennis Smith and Robert Ecker) found that oocytes arrested in G2 could be induced to enter M phase (meiosis) and mature into eggs, by microinjection of cytoplasm from oocytes that had been hormonally stimulated. *The experiment by* demonstrated that a cytoplasmic factor called as *maturation promoting factor* (MPF) is sufficient to induce entry to M phase of meiosis, in absence of progesterone.
•Later, MPF turned out to be the key factor that regulates initiation of mitosis in all eukaryotic cells.

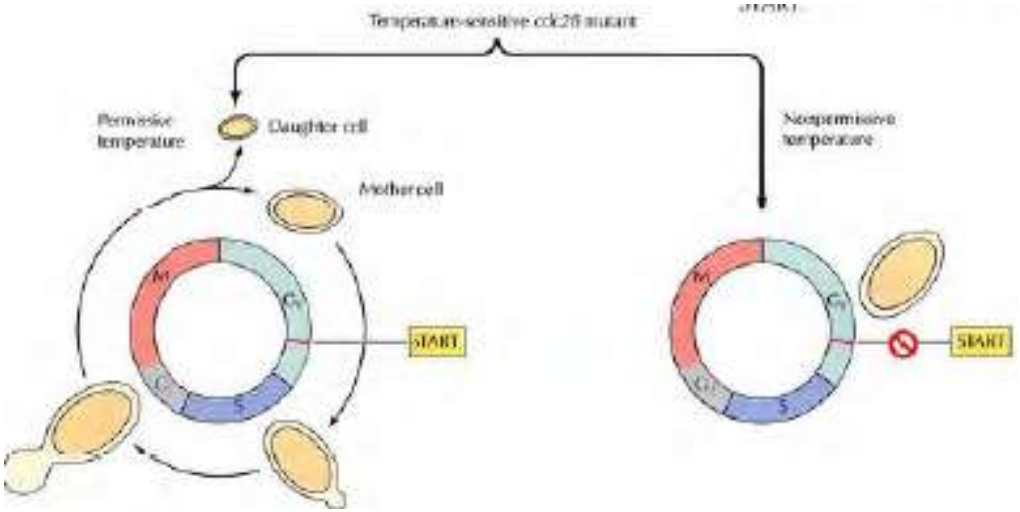


identification of MPF

IInd approach – based on genetic analysis of yeasts.

- Lee Hartwell et al (1970s) identified temperature-sensitive mutants in budding yeast *Saccharomyces cerevisiae,* that were defective in cell cycle progression (*cdc* for cell division cycle mutants) and underwent growth arrest at specific points in the cell cycle. For example, mutant *cdc28 caused cell cycle to arrest at START,* indicating that Cdc28 protein is required for passage through the regulatory point in G1 .
- *Paul Nurse et al showed - i*n fission yeast *Schizosaccharomyces pombe cdc2 mutants, arrests cell* cycle both in G1 and at G2 to M transition.
- Comparative analysis showed that *S. cerevisiae cdc28 and S. pombe cdc2 are functionally homologous genes, required for passage* through START and entry to mitosis in both yeasts.

- Molecular cloning and nucleotide sequencing demonstrated that *cdc2 and cdc28 encoded a* ***protein*** **kinase**-the first indication of the prominent role of protein phosphorylation in regulating cell cycle.
- Also, a human gene related to cdc2 was identified and shown to function in yeasts, providing a dramatic demonstration of conserved activity of cell cycle regulator.
- The protein kinase encoded by yeast *cdc2 and cdc28 genes has since been shown to be a conserved cell cycle* regulator in all eukaryotes, which is known as **Cdk1**.



Properties of *S. cerevisiae* cdc28 mutants
Temperature-sensitive *cdc28 mutant* replicate normally at the permissive temperature. At nonpermissive temperature, progression through the cell cycle is blocked at START.

IIIrd approach- based on studies of protein synthesis in early sea urchin embryos.

•Eventually converged with the identification of MPF and yeast genetics.

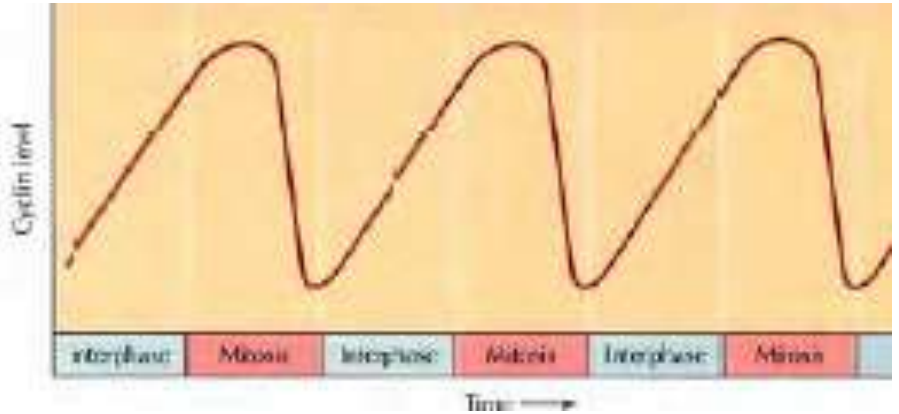
•Following fertilization, these embryos go through a series of rapid cell divisions.

•Studies with protein synthesis inhibitors showed that entry into M phase of embryonic cell cycles requires new protein synthesis.

•In 1983, Tim Hunt et al identified 2 proteins that display a periodic pattern of accumulation and degradation in sea urchin and clam embryos.

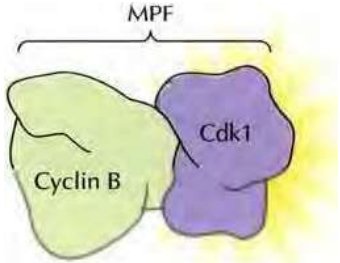
•These proteins accumulate throughout interphase and are then rapidly degraded toward the end of each mitosis. *Hunt* called these proteins **cyclins** (designated cyclin A and cyclin B) and suggested that their function as induction of mitosis, with their periodic accumulation and destruction controlling entry and exit from M phase.

•Direct support for such role of cyclins came in1986, when *Joan Ruderman et al* showed that microinjection of cyclin A into frog oocytes is sufficient to trigger the G2 to M transition.



Accumulation and degradation of cyclins in sea urchin embryos. The cyclins were identified as proteins that accumulate throughout interphase and are rapidly degraded toward the end of mitosis

In 1988 MPF was purified from frog eggs in laboratory of James Maller. Molecular characterization of MPF showed this regulator to be composed of 2 subunits: Cdkl and cyclin B.



Structure of MPF MPF is a dimer consisting of cyclin B and Cdkl protein kinase.

The Cell-Cycle Control System Is Based on Cyclically Activated Protein Kinases:

Families of Cyclins and Cyclin-Dependent Kinases

- There are 4 classes of cyclins, each defined by stage of the cell cycle at which they bind Cdks and function.

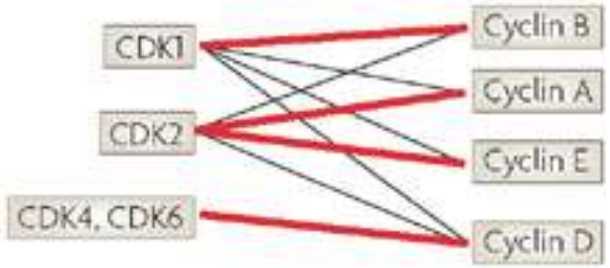
1. **G1/S-cyclins** bind Cdks at end of G1 and commit the cell to DNA replication.
2. **S-cyclins** bind Cdks in S phase and are required for initiation of DNA replication.
3. **M-cyclins** promote the events of mitosis.
4. **G1-cyclins** helps promote passage through Start or restriction point in late G1.

- In yeast cells, a single Cdk protein binds all classes of cyclins and drives all cell-cycle events by changing cyclin partners at different stages of cycle.
- In vertebrate cells, there are 4 Cdks. Two interact with G1-cyclins, one with G1/S- and S-cyclins, and one with M-cyclins.

CYCLIN-CDK COMPLEX	VERTEBRATES CYCLIN	VERTEBRATES CDK PARTNER	BUDDING YEAST CYCLIN	BUDDING YEAST CDK PARTNER
G_1-Cdk	cyclin D*	Cdk4, Cdk6	Cln3	Cdk1**
G_1/S-Cdk	cyclin E	Cdk2	Cln1, 2	Cdk1
S-Cdk	cyclin A	Cdk2	Clb5, 6	Cdk1
M-Cdk	cyclin B	Cdk1**	Clb1, 2, 3, 4	Cdk1

* There are three D cyclins in mammals (cyclins D1, D2, and D3).

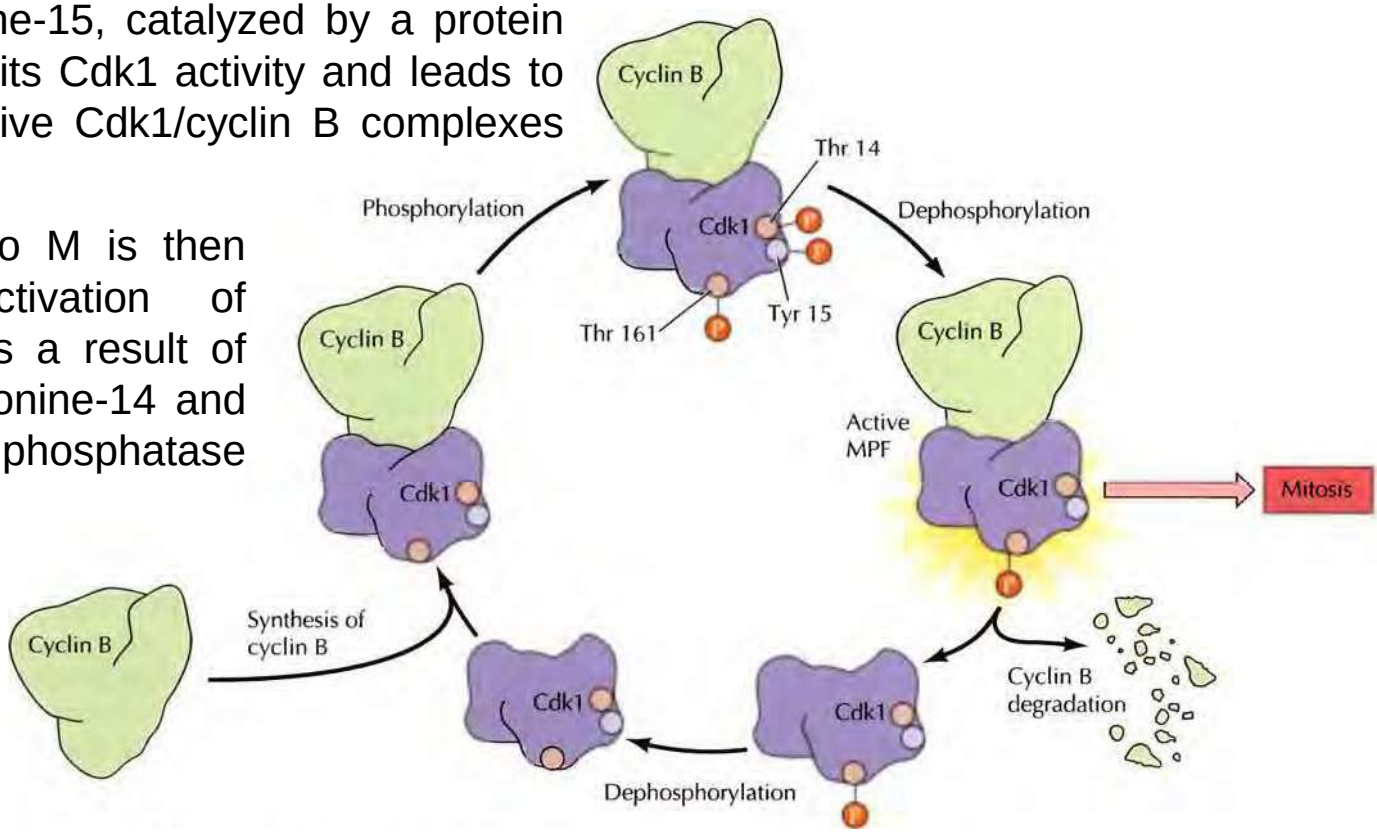
** The original name of Cdk1 was Cdc2 in both vertebrates and fission yeast, and Cdc28 in budding yeast.



CDK1 and CDK2 bind to multiple cyclins (cyclin types A, B, D and E), whereas CDK4 and CDK6 only partner D-type cyclins. Thick lines represent preferred pairing for each kinase.

- **Cyclin B** is regulatory subunit required for catalytic activity of Cdk1 protein kinase. MPF activity is controlled by by phosphorylation and dephosphorylation of Cdkl.
- In mammalian cells, cyclin B is synthesized and forms complexes with Cdk1 during G2 phase.
- As these complexes form, Cdk1 is phosphorylated at two critical regulatory positions. One of the phosphorylations occurs on threonine-161 and is required for Cdk1 kinase activity. The second is a phosphorylation of tyrosine-15 and of the adjacent threonine-14 in vertebrates.

- Phosphorylation of tyrosine-15, catalyzed by a protein kinase called Wee1, inhibits Cdk1 activity and leads to the accumulation of inactive Cdk1/cyclin B complexes throughout G2.
- The transition from G2 to M is then brought about by activation of Cdk1/cyclin B complex as a result of dephosphorylation of threonine-14 and tyrosine-15 by a protein phosphatase called Cdc25C.
- Once activated, Cdk1 phosphorylates a variety of target proteins that initiate events of M phase, and triggers cyclin B degradation that inactivates Cdk1, leading the cell to exit mitosis and return to interphase.



MPF regulation

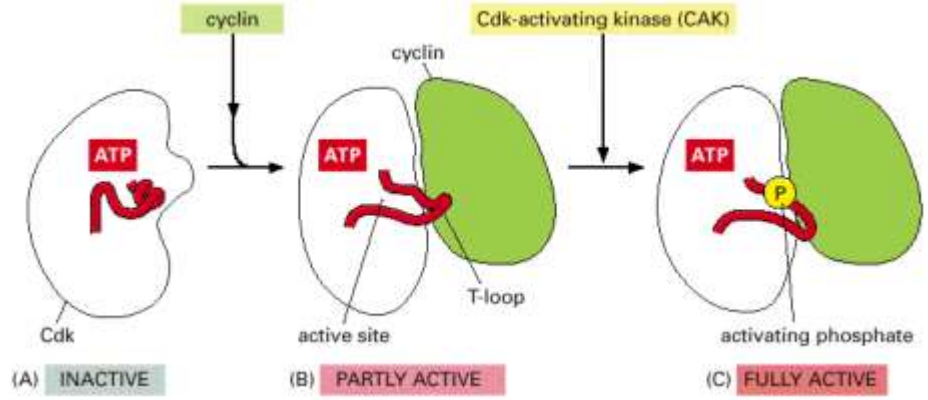
Cdk activation

How do different cyclin-Cdk complexes drive different cell-cycle events?

-Cyclin protein does not just activate its Cdk partner but also directs it to specific target proteins.

-Each cyclin-Cdk complex phosphorylates a different set of substrate proteins. The same cyclin-Cdk complex can also induce different effects at different times in cycle, because the accessibility of some Cdk substrates changes during the cell cycle. for example, certain proteins that function in M phase, may become available for phosphorylation only in G2.

3-dimensional structures of human Cdk2, as determined by x-ray crystallography showed that in absence of cyclin, Cdk active site is partly concealed by a slab of protein, like a stone blocking the entrance to a cave. Cyclin binding causes the slab to move away from active site, resulting in partial activation of the Cdk enzyme. Full activation of cyclin-Cdk complex then occurs when a **Cdk-activating kinase (CAK)**, phosphorylates an amino acid near Cdk active site. This causes a small conformational change that ncreases activity of Cdk, allowing kinase to phosphorylate its target proteins effectively and thus induce specific cell-cycle events.



The structural basis of Cdk activation. (A) In inactive state, without cyclin bound, active site is blocked by a region of protein called the T-loop. *(B) Cyclin binding* causes T-loop to move out of active site, resulting in partial activation of Cdk2. (C) Phosphorylation of Cdk2 (by CAK) at a threonine residue in T-loop further activates enzyme by changing shape of T-loop, improving ability of Cdk2 to bind its substrates.

Cdk Activity Can Be Suppressed Both by Inhibitory Phosphorylation and by Inhibitory Proteins

Four proteins regulate the protein kinase activity of the *S. pombe* CDK:

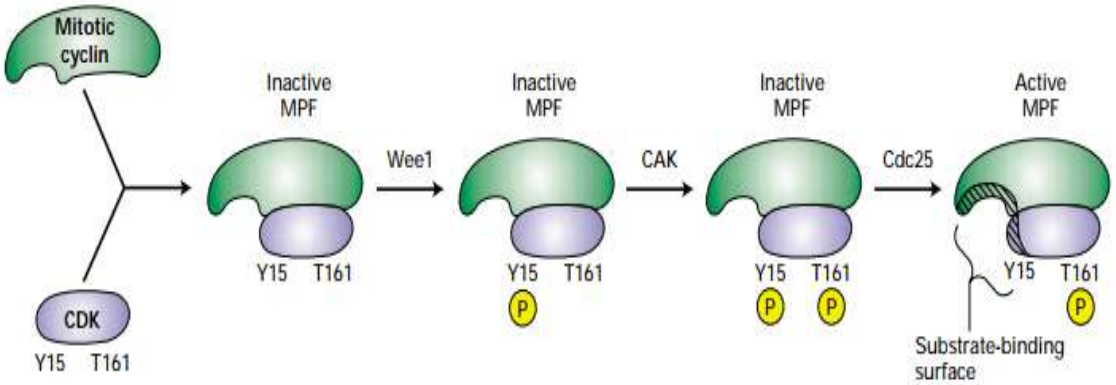
1st is Cdc13, mitotic cyclin of *S. pombe* (cyclin B), that associates with CDK to form MPF with extremely low activity.

2nd is **Wee1 protein-tyrosine kinase,** which phosphorylates an inhibitory tyrosine residue (Y15) in CDK subunit.

3rd is CDK-activating kinase (CAK), which phosphorylates an activating threonine residue (T161). When both residues are phosphorylated, MPF is inactive.

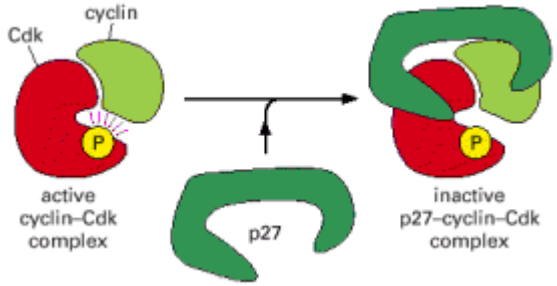
4th - Finally, *Cdc25 phosphatase* removes the phosphate from Y15, yielding highly active MPF.

Site-specific mutagenesis changed Y15 in *S. pombe* CDK to a phenylalanine, which cannot be phosphorylated, produced mutants with wee phenotype, similar to that of *wee1* mutants. Both mutations prevent inhibitory phosphorylation at Y15, resulting in inability to properly regulate MPF activity, and, consequently, premature entry into mitosis.



Regulation of the kinase activity of *S. pombe* mitosis-promoting factor (MPF).

Cyclin-Cdk complexes are also regulated by binding of **Cdk inhibitor proteins (CKIs)** that are primarily employed in control of G1 and S phase.



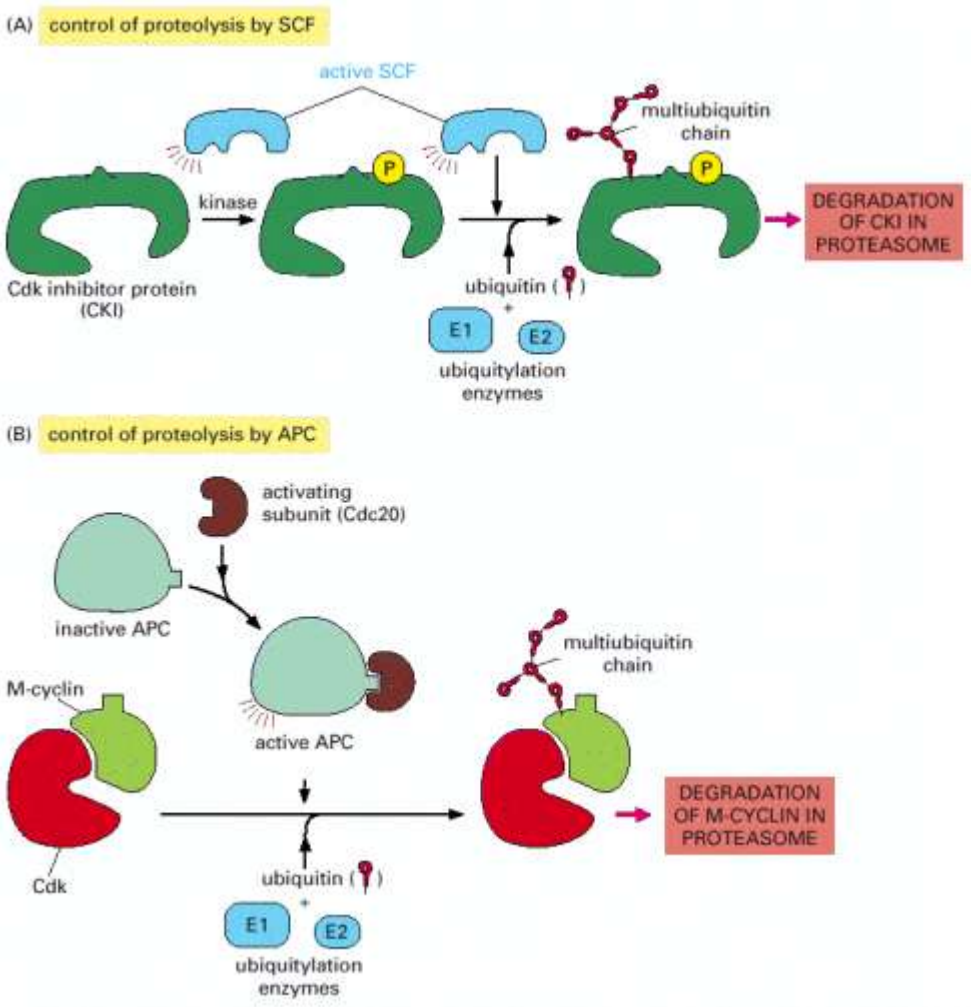
The inhibition of a cyclin-Cdk complex by a CKI. The 3D' structure of human cyclin A-Cdk2 complex bound to CKI p27, by x-ray crystallography shows that p27 binds to both the cyclin and Cdk in the complex, distorting the active site of Cdk. It also inserts into ATP-binding site, further inhibiting enzyme activity.

The Cell-Cycle Control System Depends on Cyclical Proteolysis

-Cell-cycle control depends crucially on at least 2 distinct enzyme complexes, acting at different times in cycle to cause proteolysis and inactivation of key proteins of cell-cycle control system by a ubiquitin-dependent mechanism.
E.g. Cyclin-Cdk complexes are inactivated by regulated proteolysis (destruction) of cyclins at certain cell-cycle stages.
-An activated enzyme complex recognizes specific amino-acid sequences on cyclin and attaches multiple copies of ubiquitin to it, marking it for complete destruction in proteasomes.
-Rate-limiting step in cyclin destruction is the final ubiquitin-transfer reaction catalyzed by enzymes known as ***ubiquitin ligases***. Two ubiquitin ligases are important in the destruction of cyclins and other cellcycle regulators.

-In G1 and S phase, an enzyme complex called *SCF (after its* three main protein subunits) is responsible for the ubiquitylation and destruction of G1/S-cyclins and certain CKI proteins that control S-phase initiation. In M phase, the *anaphase-promoting complex (APC) is responsible* for the ubiquitylation and proteolysis of M-cyclins and other regulators of mitosis.

These two large, multisubunit complexes contain some related components, but they are regulated in different ways. SCF activity is constant during the cell cycle. Ubiquitylation by SCF is controlled by changes in the phosphorylation state of its target proteins: only specifically phosphorylated proteins are recognized, ubiquitylated, and destroyed . APC activity, by contrast, changes at different stages of the cell cycle. APC is turned on mainly by the addition of activating subunits to complex.



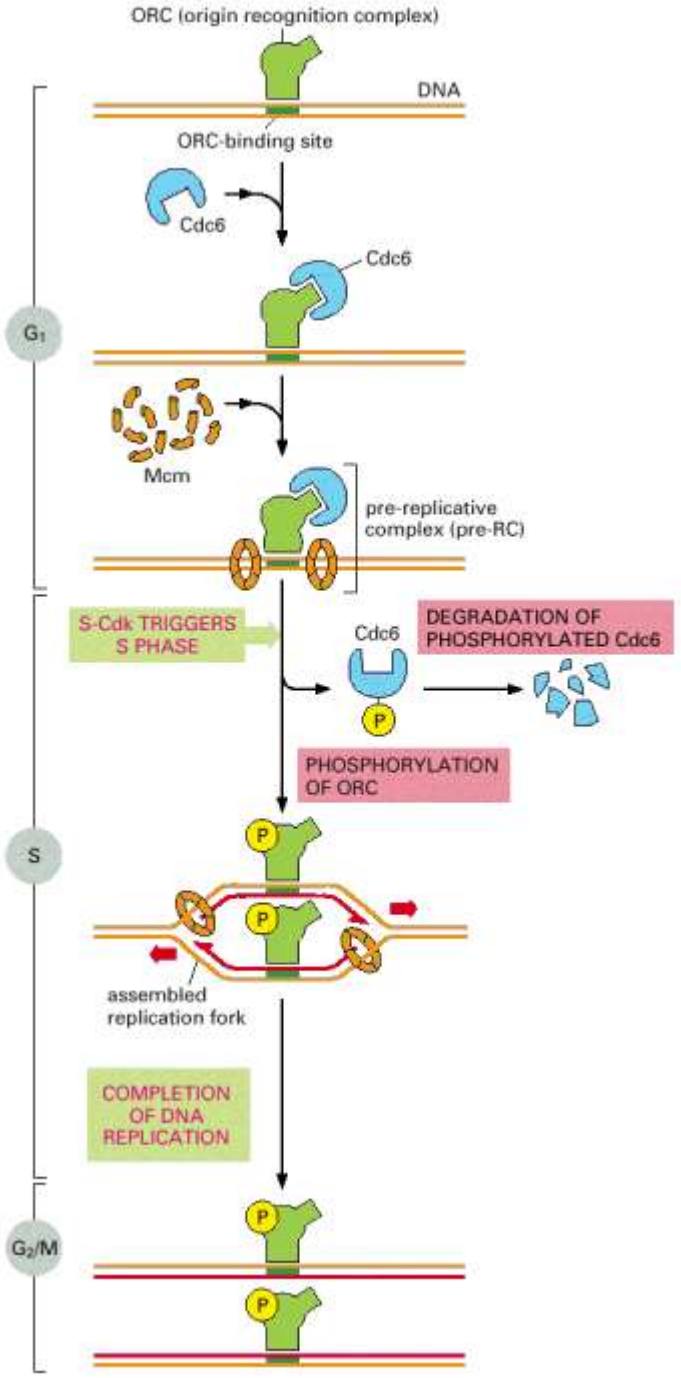
The control of proteolysis by SCF and APC during cell cycle. (A) The phosphorylation of a target protein, as CKI, allows protein to be recognized by constitutively active SCF. With help of 2 proteins: E1 and E2, SCF serves as a ubiquitin ligase that transfers multiple ubiquitin molecules onto CKI protein. The ubiquitylated CKI protein is then recognized and degraded in a proteasome. (B) M-cyclin ubiquitylation is performed by APC in late mitosis. Both SCF and APC have binding sites that recognize specific amino acid sequences of target protein.

Intracellular Control of Cell-Cycle Events

- Different cyclin-Cdk complexes serves as a molecular switch that triggers a specific cell-cycle event.
- We now consider how the switches initiate the events and how the cell-cycle control system ensures that switches fire in correct order and only once per cell cycle.
- Two central events of the cell cycle are:
  - -replication of DNA during S phase, and
  - -chromosome segregation and cell division of M phase.
- DNA replication must occur with 'extreme accuracy' to minimize the risk of mutations in the next cell generation, but every nucleotide in the genome must be copied once, and only once, to prevent the damaging effects of gene amplification.
- Thus, the cell-cycle control system initiates the replication process and, at the same time, prevents it from happening more than once per cycle.
- Cell fusion studies provided a clear hint that only G1 cells are competent to initiate DNA replication and that cells that have completed S phase (i.e. G2 cells) are not able to re-replicate their DNA, even when provided with S-Cdk activity. Passage through mitosis is required for the cell to regain the ability to undergo S phase.
- The molecular basis of these cell fusion experiments has been deciphered now.

- DNA replication begins at *origins of replication,* scattered in chromosome. Large, multiprotein complex known as **origin recognition complex (ORC) bind to replication origins in** budding yeast *S. cerevisiae* throughout cell cycle and serve as landing pads for several additional regulatory proteins. A regulatory protein **Cdc6**, is present at low levels during cell cycle but increases in early G1 and binds to ORC at replication origin, where it is required for binding of **Mcm proteins**. The resulting large protein complex formed at an origin is known as the **prereplicative complex, or pre-RC.**
- Once pre-RC is assembled in G1, replication origin is ready to fire. S-Cdk activation in late G1 pulls trigger and initiates DNA replication. S-Cdk also helps to prevent rereplication in several ways.
- First, it causes Cdc6 to dissociate from ORC after an origin has fired, resulting in disassembly of pre-RC, and preventing replication from occurring again at same origin.
- Second, it prevents Cdc6 and Mcm proteins from reassembling at any origin. By phosphorylating Cdc6, it triggers Cdc6 ubiquitylation. Thus, any Cdc6 protein not bound to origin is rapidly degraded in proteasomes. S-Cdk also phosphorylate Mcm proteins, and trigger their export from nucleus, ensuring - Mcm protein complex cannot bind to origin.
- S-Cdk activity remains high during G2 and early mitosis, preventing rereplication after S phase completion. M-Cdk also helps ensure that rereplication does not occur during mitosis by phosphorylating Cdc6 and Mcm proteins.
- The G1/S-Cdks also induce Mcm export from nucleus, ensuring that excess Mcm proteins not bound to origins in late G1 are taken out before replication begins.
- Thus, several cyclin-Cdk complexes restrain pre-RC assembly and prevent DNA rereplication after S phase.
- How, then, is the cell-cycle control system reset to allow replication to occur in the next cell cycle? At the end of mitosis, all Cdk activity in cell is reduced to zero. The resulting dephosphorylation of Cdc6 and Mcm proteins allows pre-RC assembly to occur once again, readying the chromosomes for a new round of replication.

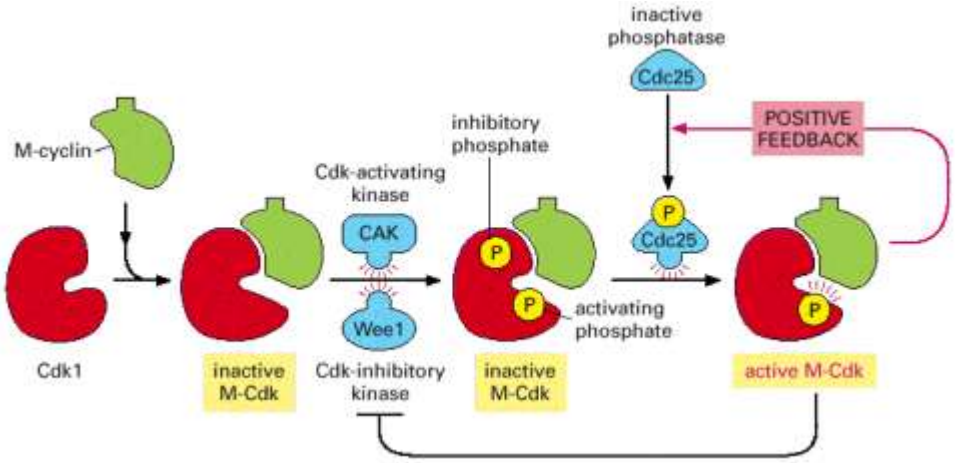
S-Phase Cyclin-Cdk Complexes (S-Cdks) Initiate DNA Replication Once Per Cycle



The initiation of DNA replication once per cell cycle. The ORC remains associated with a replication origin throughout the cell cycle. In early G1, Cdc6 associates with ORC. Aided by Cdc6, Mcm ring complexes then assemble on the adjacent DNA, resulting in the formation of the prereplicative complex. The S-Cdk then triggers origin firing, assembling DNA polymerase and other replication proteins and activating the Mcm protein rings to migrate along DNA strands as DNA helicases. The S-Cdk also blocks rereplication by causing dissociation of Cdc6 from origins, its degradation, and the export of all excess Mcm out of the nucleus. Cdc6 and Mcm cannot return to reset an ORC-containing origin for another round of DNA replication until M-Cdk has been inactivated at the end of mitosis

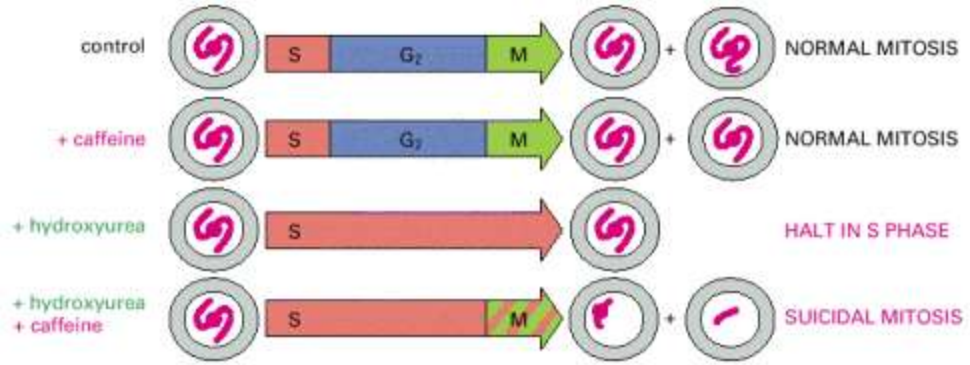
The Activation of M-Phase Cyclin-Cdk Complexes (MCdks) Triggers Entry into Mitosis

- In G2 phase- DNA is present in 2 copies of entire genome, with each replicated chromosome having 2 identical *sister chromatids glued along their length. Now cell* enters M phase, in which duplicated chromosomes and other cell contents are distributed equally to 2daughter cells. Events of mitosis are triggered by M-Cdk, which is activated after S phase is complete.
- **M-Cdk activation begins with the accumulation of M-cyclin (cyclin B)**
- In most cells, M cyclin synthesis increases in G2 & M, by increased *M-cyclin gene transcription,* leading to M-Cdk (Cdk1-M-cyclin complex) accumulation as cell approaches mitosis.
- Though Cdk -phosphorylated at an activating site by the enzyme CAK, it is held in inactive state by inhibitory phosphorylation at 2 neighboring sites by protein kinase Wee1. Thus, by the time the cell reaches end of G2, it has abundant stockpile of M-Cdk primed and ready to act, but M-Cdk activity is repressed by 2 phosphate groups that block its active site.
- What, then, triggers activation of M-Cdk stockpile? The crucial event is activation in late G2 of protein phosphatase Cdc25, that removes inhibitory phosphates restraining M-Cdk. At the same time, the activity of the inhibitory kinase Wee1 is also suppressed, further ensuring rapid increase in M-Cdk activity.
- 2 protein kinases activate Cdc25: i. Polo kinase, phosphorylates Cdc25 at a site and ii. M-Cdk itself phosphorylates a different site on Cdc25. M-Cdk also phosphorylates and inhibits Wee1. The ability of M-Cdk to activate its own activator (Cdc25) and inhibit its own inhibitor (Wee1) suggest - M-Cdk activation involves a positive feedback loop and quickly promote complete activation of all M-Cdk complexes in the cell.



Entry into Mitosis Is Blocked by Incomplete DNA Replication

- If a cell is driven into mitosis before it finishes replicating DNA, it will pass on broken or incomplete sets of chromosomes to its daughter cells. This disaster is avoided in most cells by a **DNA replication checkpoint** mechanism, which ensures that initiation of mitosis cannot occur until last nucleotide in genome has been copied.
- Sensor mechanisms detect unreplicated DNA (unfinished replication forks) and send a negative signal to cell-cycle control system, blocking M-Cdk activation.
- Thus, normal cells treated with chemical inhibitors of DNA synthesis, as hydroxyurea, do not progress to mitosis. If checkpoint mechanism is defective as in mammalian cells treated with high caffeine doses, cells plunge into suicidal mitosis despite incomplete DNA replication.
- Final targets of negative checkpoint signal are enzymes that control M-Cdk activation. The negative signal activates a protein kinase that inhibits Cdc25 protein phosphatase. As a result, MCdk remains phosphorylated and inactive until DNA replication is complete.



The DNA replication checkpoint. Mammalian cells in culture treated with caffeine and hydroxyurea, either alone or in combination. Hydroxyurea blocks DNA synthesis. This block activates a checkpoint mechanism that arrests cells in S phase, delaying mitosis. But if caffeine is added as well as hydroxyurea, checkpoint mechanism fails, and cells enter mitosis with incompletely replicated DNA. As a result, the cells die.

M-Cdk Prepares the Duplicated Chromosomes for Separation

•The protein kinase- M-Cdk – brings all the diverse and complex rearrangements occuring in early stages of mitosis.

•M-Cdk - induce the assembly of mitotic spindle
-ensure that replicated chromosomes attach to the spindle.
-triggers chromosome condensation, nuclear envelope breakdown, actin cytoskeleton rearrangement, and reorganization of Golgi apparatus and ER.

• Each of these events is thought to be triggered when M-Cdk phosphorylates specific structural or regulatory proteins involved in the event.

•The ***breakdown of nuclear envelope***, for example, requires disassembly of *nuclear lamina (underlying shell of polymerized lamin filaments,* giving nuclear envelope its structural rigidity). Direct phosphorylation of lamin proteins by M-Cdk results in their depolymerization, which is an essential first step in the dismantling of envelope.

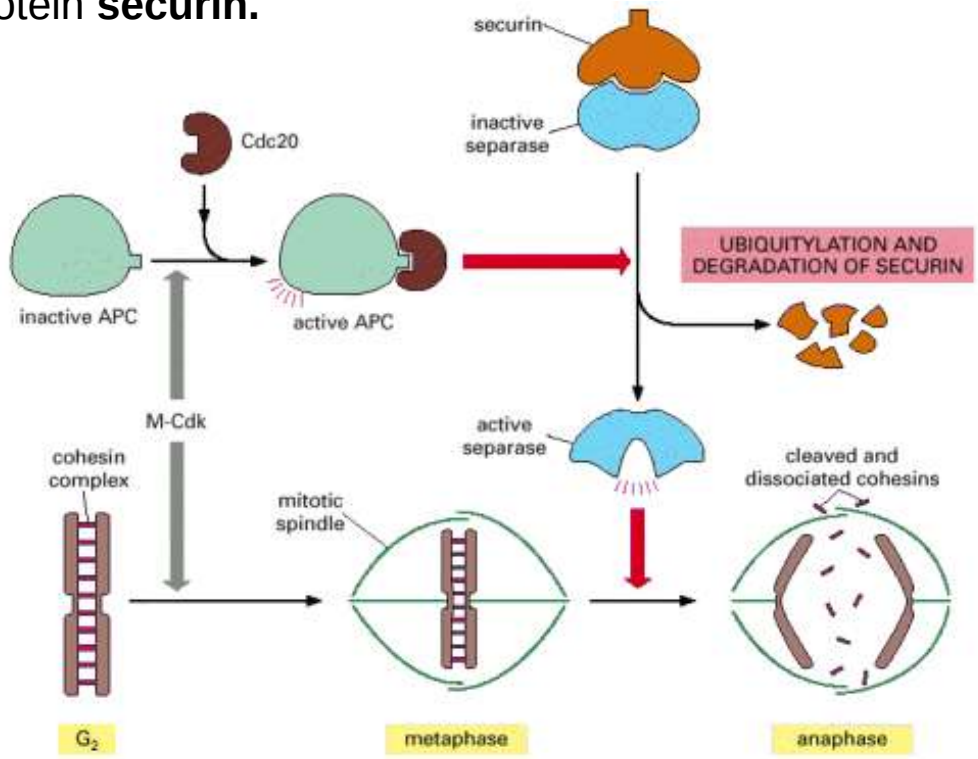
•Chromosome condensation is also a direct consequence of phosphorylation by M-Cdk.

•A complex of five proteins, known as the condensin complex, is required for ***chromosome condensation*** in *Xenopus* embryos. After M-Cdk has phosphorylated several subunits in the complex, two of the subunits are able to change the coiling of DNA molecules. It is thought that this coiling activity is important for chromosome condensation during mitosis.

•Phosphorylation by M-Cdk also triggers the complex microtubule rearrangements and other events that lead to the assembly of mitotic spindle. M-Cdk is known to phosphorylate a number of proteins that regulate microtubule behavior, causing the increase in microtubule instability that is ***required for spindle assembly***.

Sister Chromatid Separation Is Triggered by Proteolysis

- After M-Cdk has triggered rearrangements in early mitosis, cell cycle reaches metaphase-to-anaphase transition. Although M-Cdk activity sets the stage for this event, an **anaphase-promoting complex (APC)** throws the switch that initiates sister-chromatid separation. This ubiquitin ligase promotes destruction of several mitotic regulatory proteins.
- Sister chromatids are bound tightly together, both at centromeres and all along their arms. Sister-chromatid cohesion depends on cohesin complex, deposited along chromosomes as they duplicate in S phase. Anaphase begins with a sudden disruption of cohesion between sister chromatids, allowing them to separate and move to opposite poles of spindle. Sister-chromatid separation requires APC enzyme complex activation, suggesting that proteolysis is central to the process. The target of APC is protein **securin.**
- Before anaphase, securin binds to and inhibits activity of a protease called **separase.** Securin destruction at end of metaphase releases separase, which then cleaves subunits of cohesin complex. Instantly, the cohesin complex falls away from chromosomes, and sister chromatids separate. If APC triggers anaphase, what triggers APC? APC activation requires protein Cdc20, which binds and activate APC at mitosis. 2 processes regulate Cdc20 and its association with APC. 1st, Cdc20 synthesis increases as cell approaches mitosis, owing to an increase in its transcription. 2nd, APC phosphorylation helps Cdc20 bind to APC, thereby helping to create an active complex.



Triggering of sister-chromatid separation by APC

Unattached Chromosomes Block Sister-Chromatid Separation: The Spindle-Attachment Checkpoint

- A spindle-attachment checkpoint mechanism operates to ensure that all chromosomes are properly attached to the spindle before sister-chromatid separation occurs.
- The checkpoint depends on a ***sensor mechanism*** that *monitors the state of kinetochore, the* specialized region of chromosome that attaches to microtubules of the spindle.
- Any kinetochore that is not properly attached to spindle sends out a negative signal to cell-cycle control system, **blocking Cdc20-APC activation** and **sister-chromatid separation**.
- The nature of signal generated by an unattached kinetochore is not clear, although several proteins, including ***Mad2****, are recruited to unattached* kinetochores and are required for the spindle-attachment checkpoint to function. Even a single unattached kinetochore in the cell results in Mad2 binding and the inhibition of Cdc20-APC activity and Securin destruction.
- Thus, sister-chromatid separation cannot occur until the last kinetochore is attached.

Exit from Mitosis Requires the Inactivation of M-Cdk

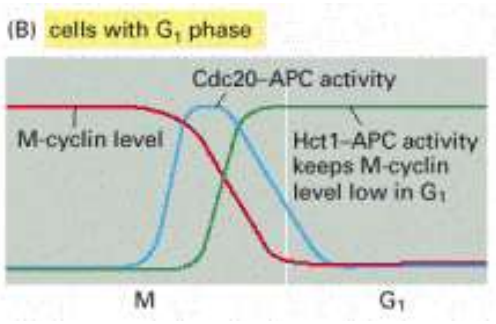
- After chromosomes have segregated to poles of spindle, cell must reverse the complex changes of early mitosis. The spindle must be disassembled, chromosomes decondensed, and nuclear envelope reformed.
- As early mitosis involves phosphorylation of various proteins, exit from mitosis requires dephosphorylation of these proteins. This is triggered by M-Cdk inactivation (by ubiquitin-dependent proteolysis of M-cyclins) and activation of phosphatases.
- Ubiquitylation of cyclin is usually triggered by the same Cdc20-APC complex that promotes the destruction of Securin at the metaphase-to anaphase transition.
- Thus, the activation of Cdc20-APC complex leads not only to anaphase, but also to M-Cdk inactivation which in turn leads to all of other events that take cell out of mitosis.

The G1 Phase Is a State of Stable Cdk Inactivity

In early animal embryos, M-Cdk inactivation in late mitosis is due to action of Cdc20-APC. However, M-Cdk stimulates Cdc20-APC activity. Thus, destruction of Mcyclin in late mitosis soon leads to inactivation of all APC activity in these cells.
Cdk reactivation is prevented after mitosis mainly by 2 mechanisms:

1st: by another APC-activating protein *Hct1*. Although both Hct1 and Cdc20 bind and activate APC, while Cdc20-APC complex is activated by M-Cdk, Hct1-APC complex is inhibited by M-Cdk, that directly phosphorylates Hct1. Thus Hct1-APC activity increases in late mitosis after Cdc20-APC complex has initiated destruction of M-cyclin. M-cyclin destruction therefore continues after mitosis: although Cdc20-APC activity declines, Hct1-APC activity is high.

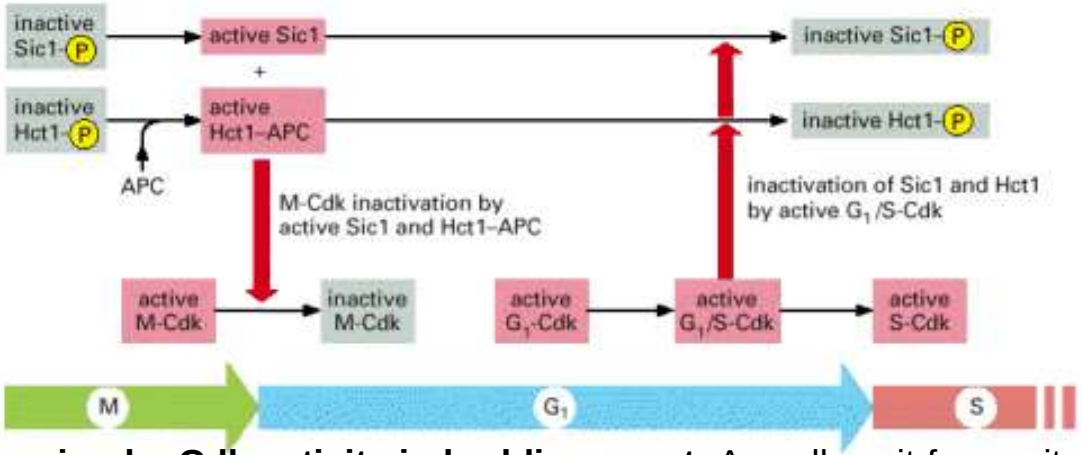
2nd depends on increased production of CKIs (Cdk inhibitory proteins). Budding yeast cells has a CKI protein - *Sic1, that binds to and inactivates M-Cdk in late mitosis* and G1. Like Hct1, Sic1 is inhibited by M-Cdk, which phosphorylates Sic1 during mitosis. M-Cdk also phosphorylates and inhibits a gene regulatory protein required for Sic1 synthesis, resulting in decreased Sic1 production. Thus, ***Sic1 and M-Cdk, like Hct1 and M-Cdk, mutually inhibit each other***. As a result, the decline in M-Cdk activity in late mitosis triggers rapid accumulation of Sic1 protein, ensuring stable inhibition of M-Cdk activity after mitosis.
Additionally, inactivation of M-Cdk by Hct1 and Sic1 leads to decreased M-cyclin gene transcription and thus decreased M-cyclin synthesis (feedback loop).



Creation of G1 phase by stable Cdk inhibition after mitosis.

In G1 phase cells, drop in M-Cdk activity in late mitosis leads to activation of Hct1-APC (and CKI proteins accumulation). This ensures a continued suppression of Cdk activity after mitosis

- In summary Hct1-APC activation, CKI accumulation, and decreased cyclin production act together to ensure that the early G1 phase is a time when all Cdk activity is suppressed.
- How do cells escape from this stable G1 state to initiate S phase? Escape usually occurs through accumulation of G1-cyclins. In budding yeast, these cyclins are not targeted for destruction by Hct1-APC and are not inhibited by Sic1. Thus, G1 cyclin accumulation lead to increased G1-Cdk activity. G1-Cdk activity triggers transcription of G1/S-cyclin genes, leading to increased synthesis of G1/S-cyclins and formation of G1/S-Cdk complexes, also resistant to Hct1-APC and Sic1.
- The increased G1/S-Cdk activity initiates the events that commit cell to enter S phase. It stimulates transcription of S-cyclin genes, leading to synthesis of S-cyclins and formation of S-Cdk complexes. These complexes are inhibited by Sic1, but G1/S-Cdk phosphorylates and inactivates Sic1, thereby causing S-Cdk activation. G1/S-Cdk and S-Cdk also phosphorylate and inactivate Hct1-APC.
- Thus, the same feedback loops that trigger rapid M-Cdk inactivation in late mitosis now work in reverse at the end of G1 to ensure the rapid and complete activation of S-Cdk activity.



Control of G1 progression by Cdk activity in budding yeast. As cells exit from mitosis and inactivate M-Cdk, the resulting increase in Hct1 and Sic1 activities results in stable Cdk inactivation during G1. When conditions are right for entering a new cell cycle, the increase in G1-Cdk and G1/S-Cdk activities leads to inhibition of Sic1 and Hct1 by phosphorylation, allowing S-Cdk activity to increase.

The Rb Protein Acts as a Brake in Mammalian G1 Cells

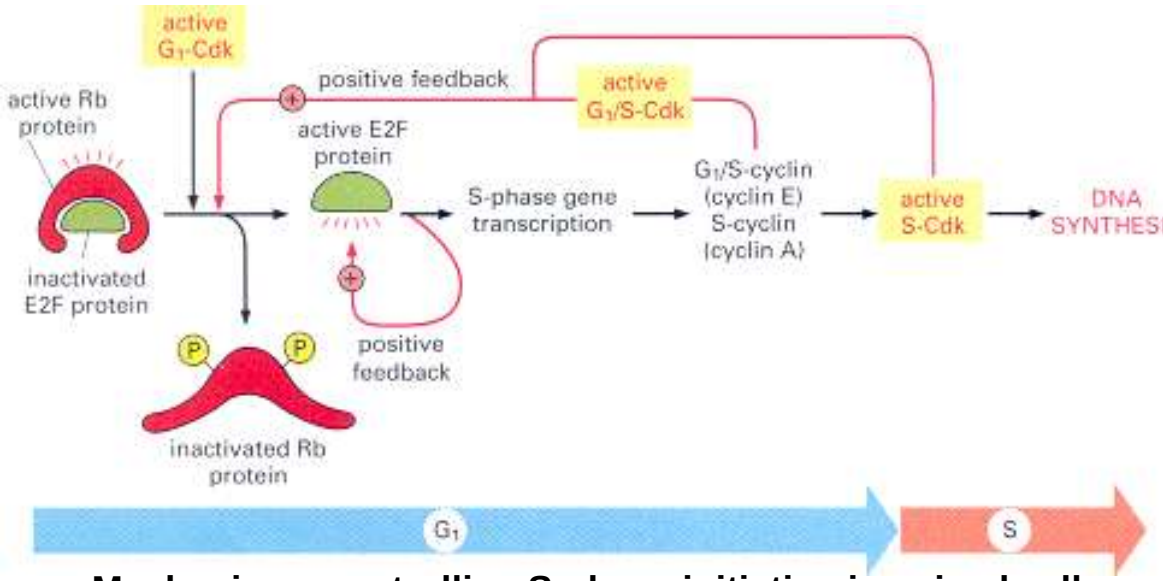
- Animal cells suppress Cdk activity in G1 by same 3 mechanisms: Hct1 activation, accumulation of a CKI protein (p27 in mammalian cells), & inhibition of cyclin gene transcription.
- As in yeasts, the activation of G1-Cdk reverses all 3 inhibitory mechanisms in late G1.
- The effects of G1-Cdk activity in animal cells are mediated by a gene regulatory protein - **E2F. It binds to specific DNA sequences in** promoters of genes that encode proteins required for S-phase entry, as G1/S-cyclins and S-cyclins. E2F function is controlled by interaction with the **retinoblastoma protein (Rb), an inhibitor of cell-cycle** progression.
- During G1, Rb binds E2F and blocks the transcription of Sphase genes. When cells are stimulated to divide by extracellular signals, active G1-Cdk accumulates and phosphorylates Rb, reducing its affinity for E2F. Rb then dissociates, allowing E2F to activate S-phase gene expression. This transcriptional control system has feedback loops sharpen G1/S transition:

i. The liberated E2F increases transcription of its own gene.

ii. E2F-dependent transcription of G1/S-cyclin and S-cyclin genes lead to increased G1/S-Cdk and S-Cdk activities, that increase Rb phosphorylation and promote further E2F release.

iii. Increase in G1/S-Cdk and S-Cdk activities enhance phosphorylation of Hct1 and p27, leading to their inactivation or destruction.

•Rb protein was identified from cells of eye cancer (*retinoblastoma*) in children. Loss of both copies of Rb gene lead to excessive cell proliferation in immature retina, suggesting that Rb protein is particularly important for restraining cell division rate in developing retina.

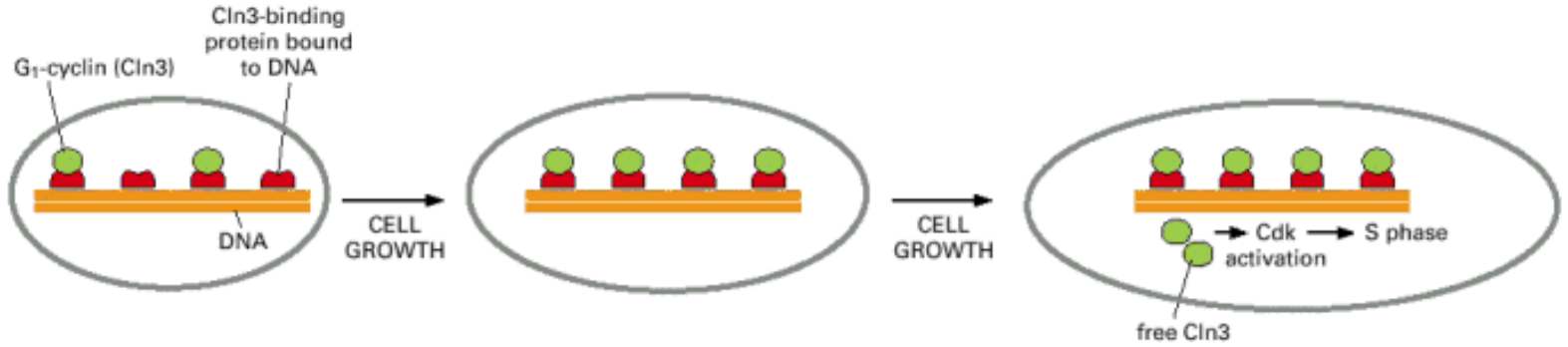


Mechanisms controlling S-phase initiation in animal cells.

Cell-Cycle Progression Is Somehow Coordinated With Cell Growth

For proliferating cells to maintain a relatively constant size, the length of cell cycle must match the time it takes the cell to double in size. If cycle time is shorter than this, cells will get smaller with each division; if it is longer, cells will get bigger with each division.

Budding yeasts coordinate their growth and cell-cycle progression by monitoring the total amount of a G1 cyclin called **Cln3**. Because Cln3 is synthesized in parallel with cell growth, its concentration remains constant while its total amount increases as cell grows. If amount of Cln3 is artificially increased, cells divide at a small size, whereas if it is artificially decreased, cells divide at a larger size than normal. Thus the cells commit themselves to division when the total amount of Cln3 reaches some threshold value. How, does cell monitor the total amount of Cln3? One possibility is that cells inherit a fixed amount of an inhibitor that can bind to Cln3 and block its activity. When amount of Cln3 exceeds the amount of inhibitor, the extra Cln3 triggers G1-Cdk activation and a new cell cycle. Since all cells receive a fixed and equal quantity of DNA, it is speculated that Cln3 inhibitor could be DNA itself, or some protein bound to DNA



A hypothetical model of how budding yeast cells may coordinate cell growth and cell-cycle progression.

Cell-Cycle Progression is Blocked by DNA Damage and p53: DNA Damage Checkpoints

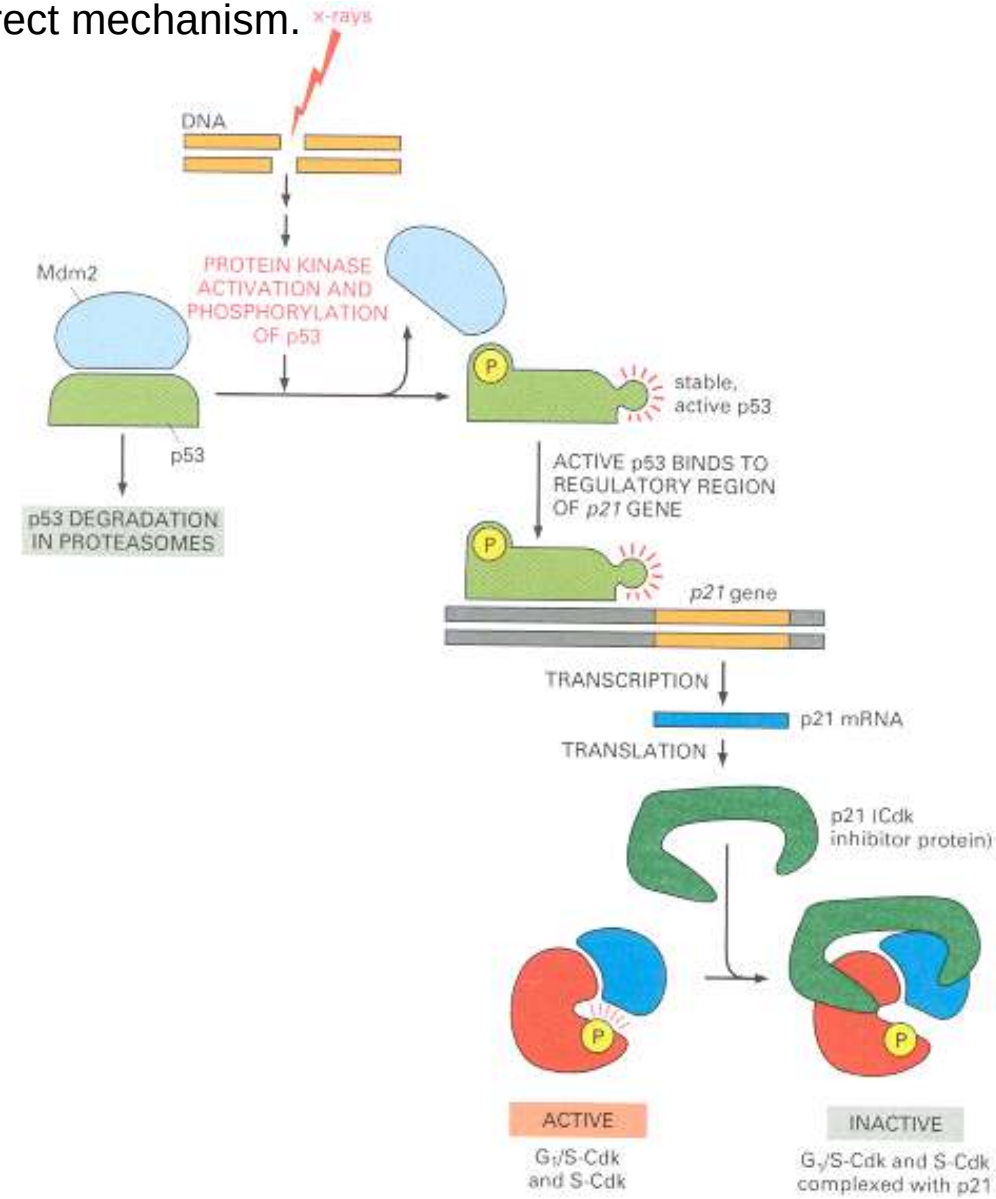
- The cell-cycle control system readily detects DNA damage and arrest the cycle at **DNA damage checkpoints**. Most cells have 2 such checkpoints:
  - in late G1, which prevents entry into S phase, and
  - in late G2, which prevents entry into mitosis.

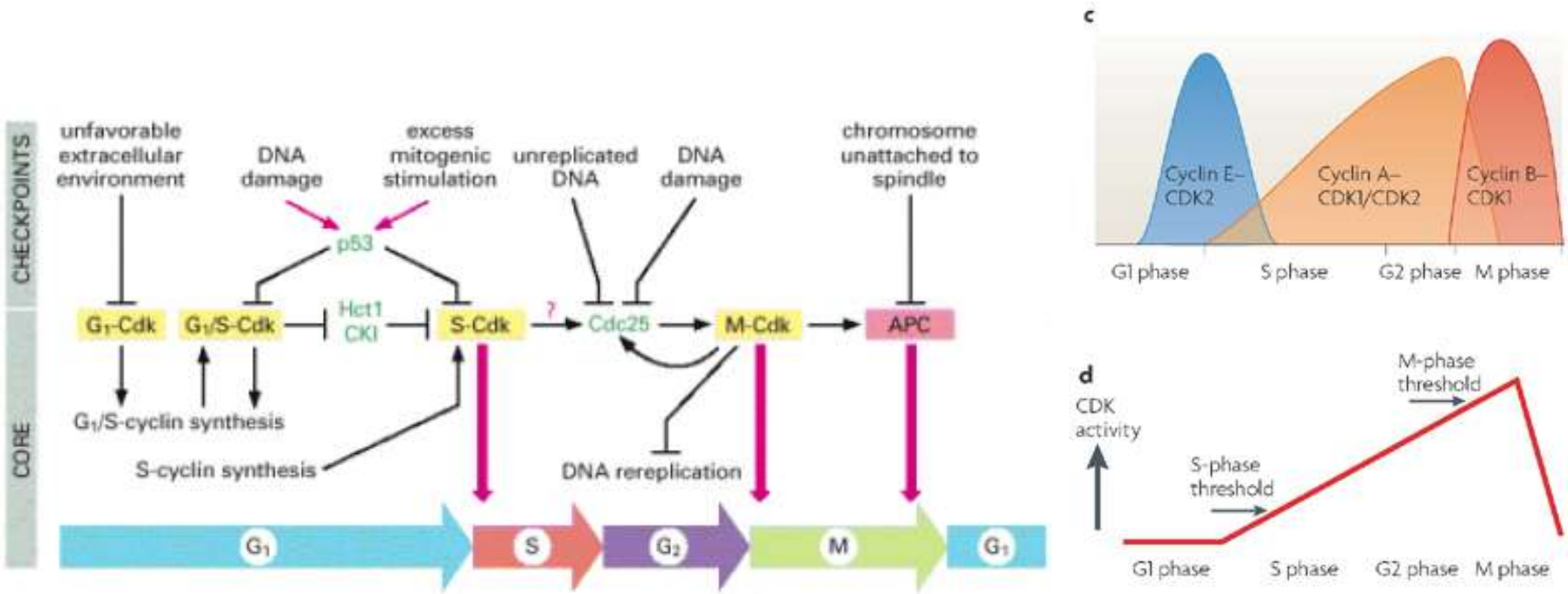
- The G2 checkpoint mechanism is similar to the mechanism that delays entry into mitosis in response to incomplete DNA replication.
- When cells in G2 are exposed to damaging radiation, for example, the damaged DNA sends a signal to a series of protein kinases that phosphorylate and inactivate the phosphatase Cdc25. This blocks the dephosphorylation and activation of M-Cdk, thereby blocking entry into mitosis.
- When the DNA damage is repaired, the inhibitory signal is turned off, and cell-cycle progression resumes.

The G1 checkpoint blocks progression into S phase by inhibiting the activation of G1/S-Cdk and S-Cdk complexes.

DNA damage arrests cell cycle in G1

- In mammalian cells, DNA damage leads to activation of gene regulatory protein p53, which stimulates transcription of several genes. One of these genes encodes a CKI protein - **p21**, which binds to G1/S-Cdk and S-Cdk and inhibits their activities, thereby blocking entry into S phase. DNA damage activates p53 by an indirect mechanism.
- In undamaged cells, p53 is highly unstable and is present at very low concentrations, as it interacts with a protein, **Mdm2, that acts as a ubiquitin ligase** and targets p53 for destruction by proteasomes. DNA damage activates protein kinases that phosphorylate p53 and thereby reduce its binding to Mdm2. This decreases p53 degradation, and results in marked increase in p53 concentration in cell. In addition, the decreased binding to Mdm2 enhances the ability of p53 to stimulate gene transcription.
- Like many other checkpoints, DNA damage checkpoints are not essential for normal cell division if environmental conditions are ideal. Conditions are rarely ideal, and low level of DNA damage occurs normally in cell, that accumulates in cell's progeny if the damage checkpoints are not functioning, leading to an increased frequency of cancer-promoting mutations. Indeed, mutations in p53 gene occur in at least half of all human cancers.





An overview of the cell-cycle control system. The core of the cell cycle control system consists of a series of cyclin-Cdk complexes. *The* activity of each complex is also influenced by various inhibitory checkpoint mechanisms, which provide information about the extracellular environment, cell damage, and incomplete cell-cycle events.

(Note: All the original contributors of the concept and findings published elsewhere are gratefully acknowledged while preparing the E-content for the purpose of student reading material in convenient form for biochemistry and allied discipline).

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