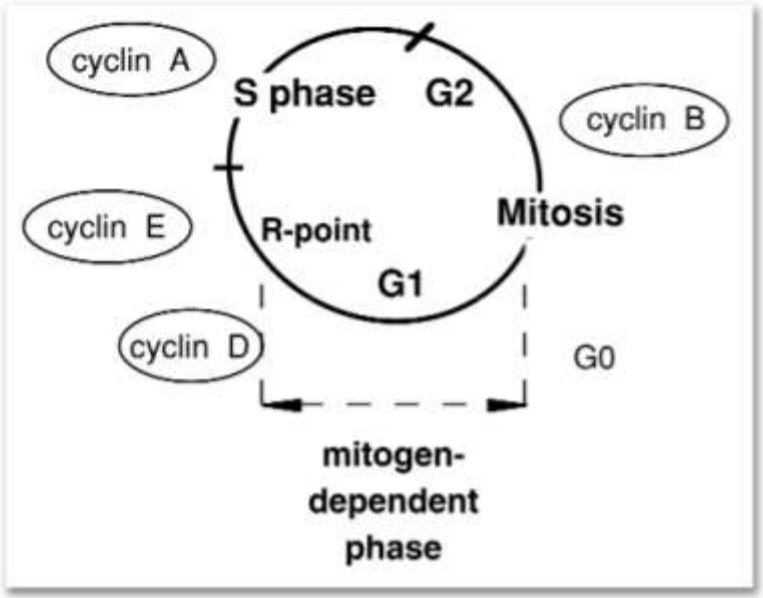
Cell cycle 4

(Graphics from internet)

Cell Cycle Control in Mammalian Cells

Cyclin D is essential for passing through restriction point



As cells proceed through the cycle, four major cyclins are produced sequentially (D, E, A, and B), and they activate CDKs. Cyclins D (D1, D2, D3) and E drive a cell into S-phase. During cell cycle progression, D cyclins start accumulating at mid-G1, whereas cyclin E appears later, prior to the G1/S transition. Progression through S phase requires cyclin A. B-type cyclins associate with p34cdc2 to trigger mitosis.

S. cerevisiae	Cln3 (Cdk1)	Cln 1,2 (Cdk1)	Clb 5,6 (Cdk1)	Clb 1,2,3,4 (Cdk 1)
H. sapiens	cyclin D 1,2,3 (Cdk4, Cdk6)	cyclin E (Cdk2)	cyclin A (Cdk2, Cdk1)	cyclin B (Cdk1)

The specific cyclin subtypes along with their corresponding CDK (in brackets) are:

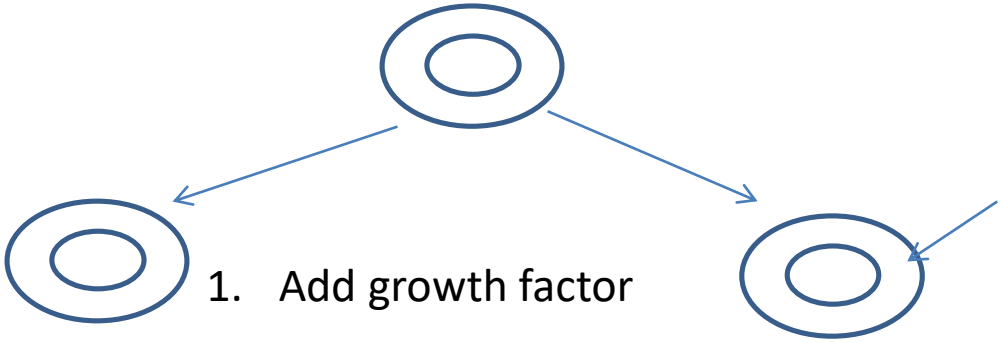
Species	G1	G1/S	S	M
S. cerevisiae	Cln3 (Cdk1)	Cln 1,2 (Cdk1)	Clb 5,6 (Cdk1)	Clb 1,2,3,4 (Cdk 1)
S. pombe	Puc1? (Cdc2)	Puc1, Cig1? (Cdc2)	Cig2, Cig1? (Cdc2)	Cdc13 (Cdc2)
D. melanogaster	cyclin D (Cdk4)	cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B, B3 (Cdk1)
X. laevis	either not known or not present	cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B, B3 (Cdk1)
H. sapiens	cyclin D 1,2,3 (Cdk4, Cdk6)	cyclin E (Cdk2)	cyclin A (Cdk2, Cdk1)	cyclin B (Cdk1)

The point **at G1 at which commitment occurs and the cell no longer** requires growth factors to complete the cell cycle has been termed the restriction (R) point. The R point has been temporally mapped at 2–3 hours prior to the onset of DNA synthesis.

What is the purpose of having a restriction point during the cell cycle?

Regulation of Cell Proliferation by the Restriction Point. The restriction point is a molecular “gate” that **regulates the expression of genes required for cell-cycle progression**. The gate is based on proteins that are related to the Rb susceptibility protein and a family of essential transcription factors known as E2F.

Cyclin D is essential for passing through restriction point



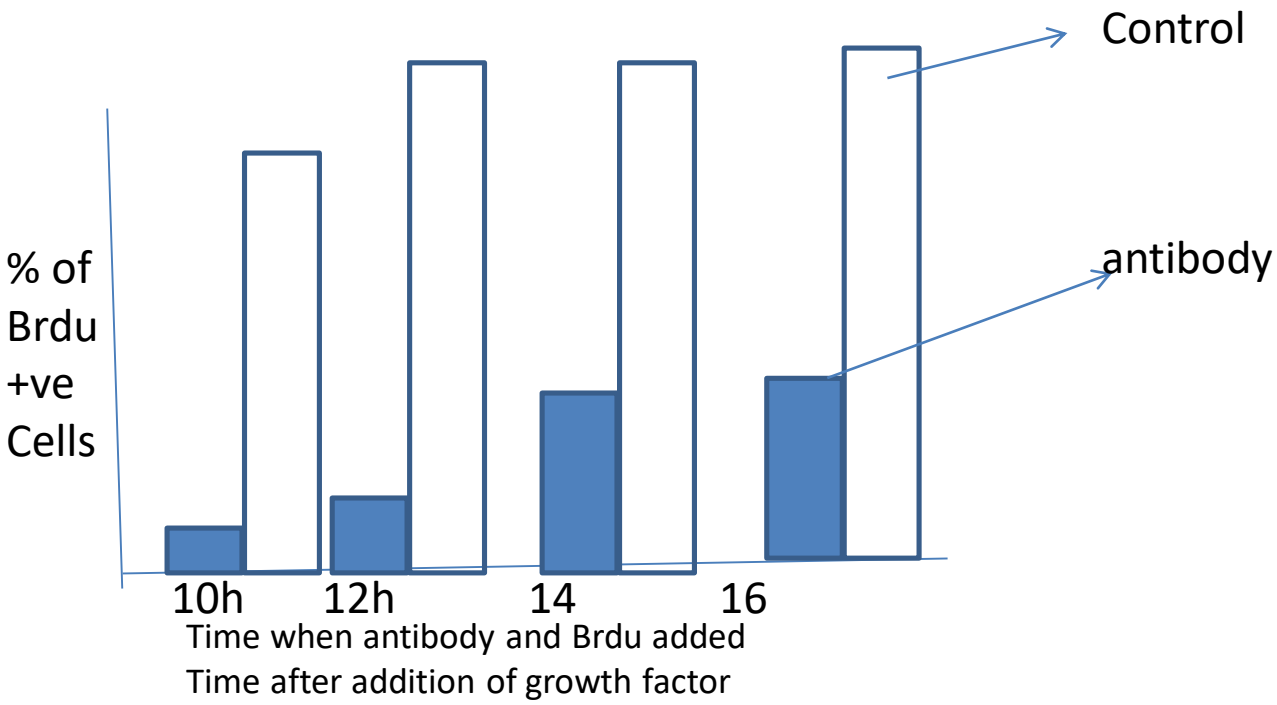
1. Add growth factor

Add antibody to Cyclin D

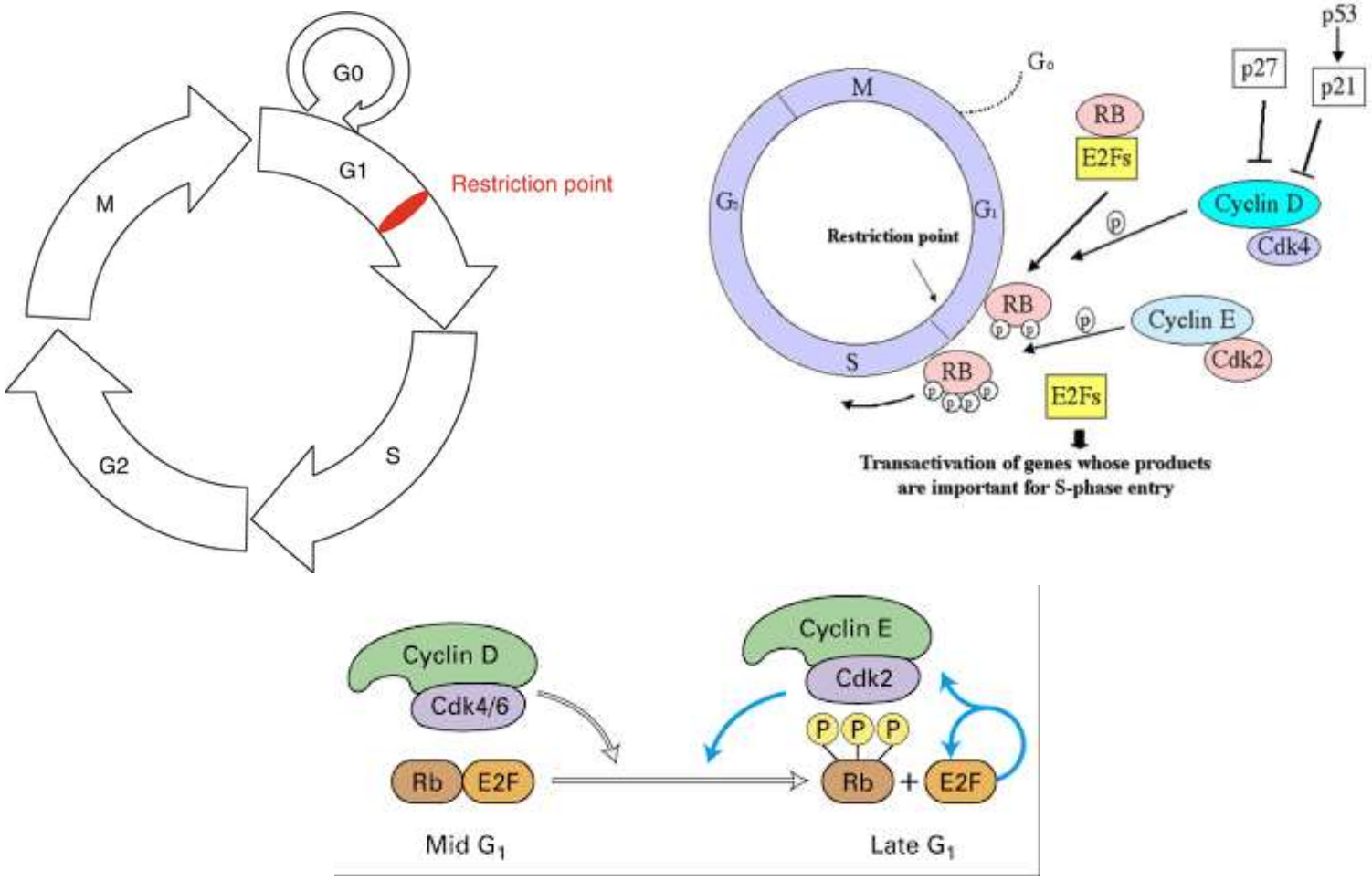
10-16h later

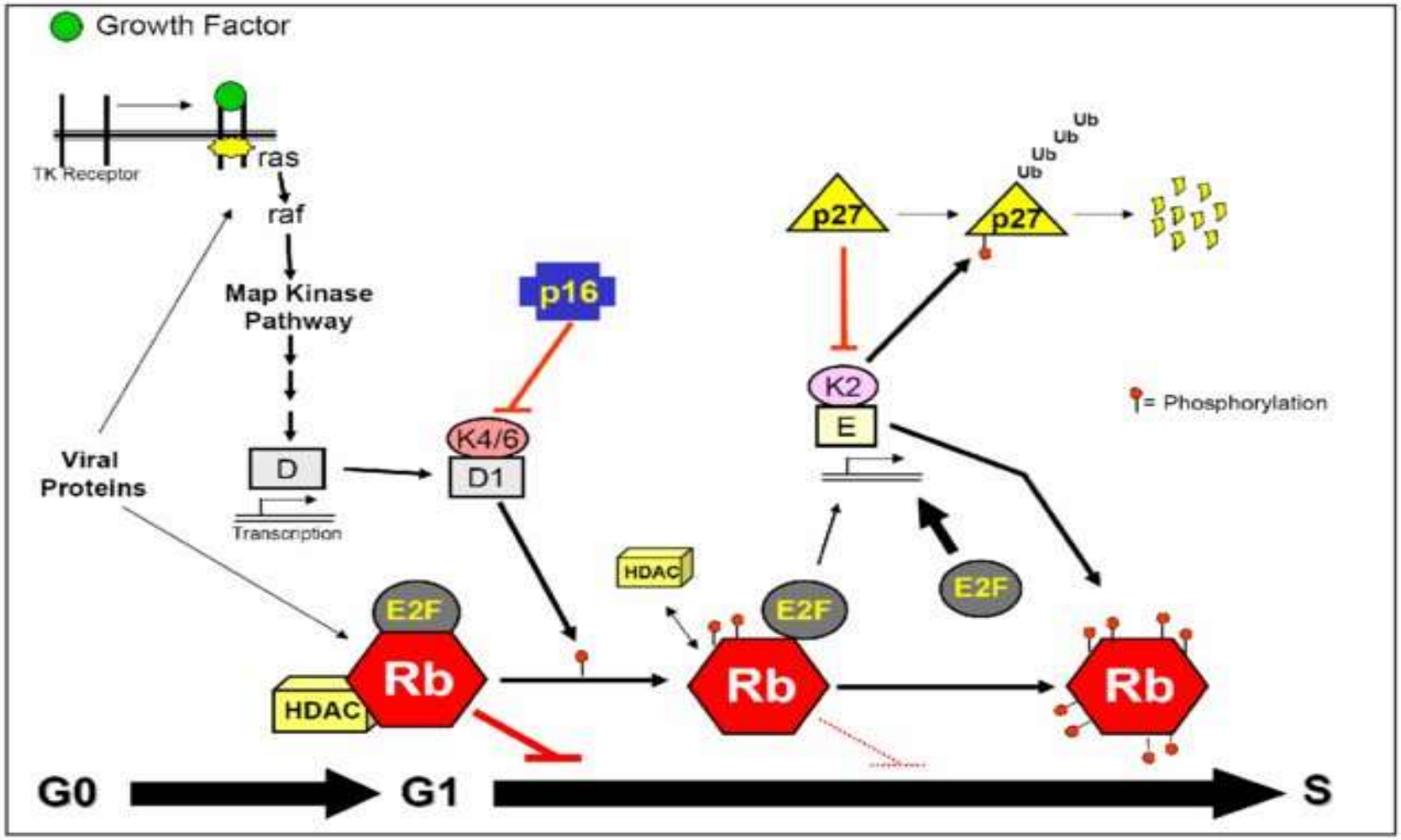
2 . Add Brdu

3. Determine Brdu +ve cells



The **restriction point** (R), also known as the Start or G_1/S checkpoint, is a cell cycle checkpoint in the G_1 phase of the animal cell cycle at which the cell becomes "committed" to the cell cycle, and after which extracellular signals are no longer required to stimulate proliferation.

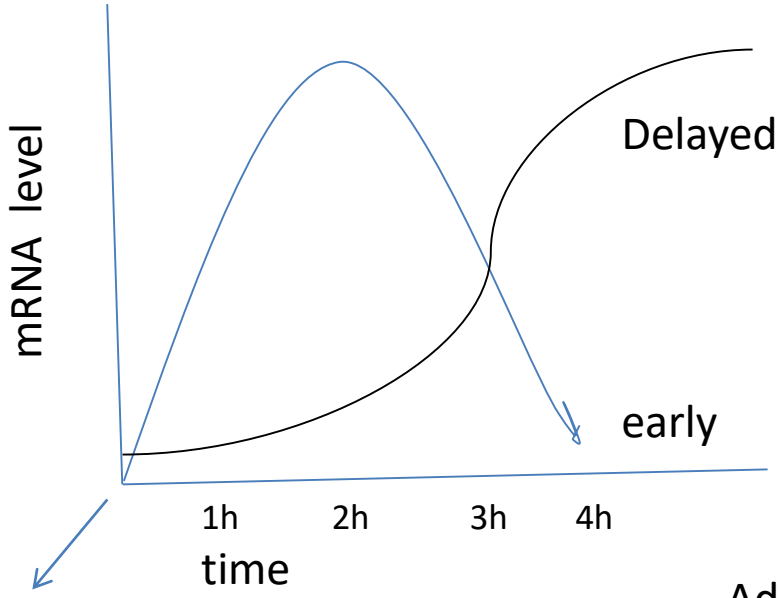


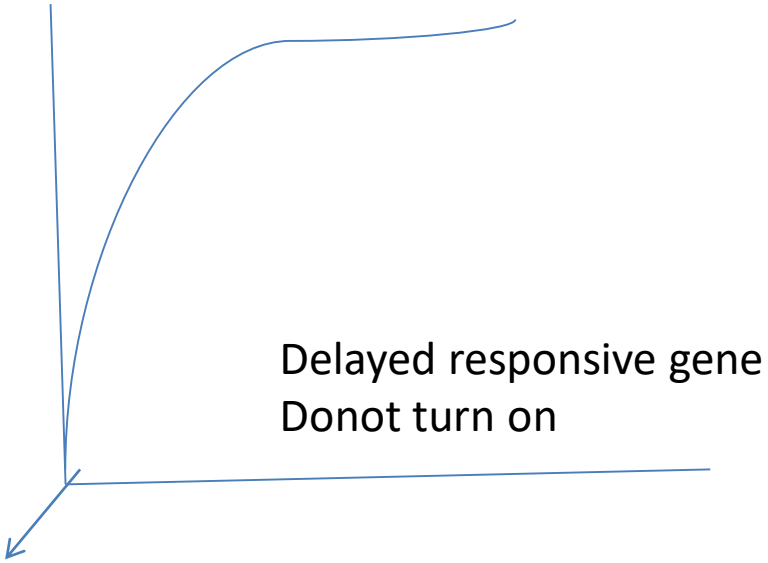


The G1 Restriction Point For cells to progress from G0 into the cell cycle they require the correct growth factor stimuli to up-regulate the expression of cyclin D, which can then bind to its partner CDK4. This results in the partial phosphorylation and inactivation of the retinoblastoma protein Rb, and subsequent release of E2F leading to up-regulation of the genes required for S phase progression.

Regulated expression of two classes of genes direct G0 into cell cycle

Early and delayed responsive gene

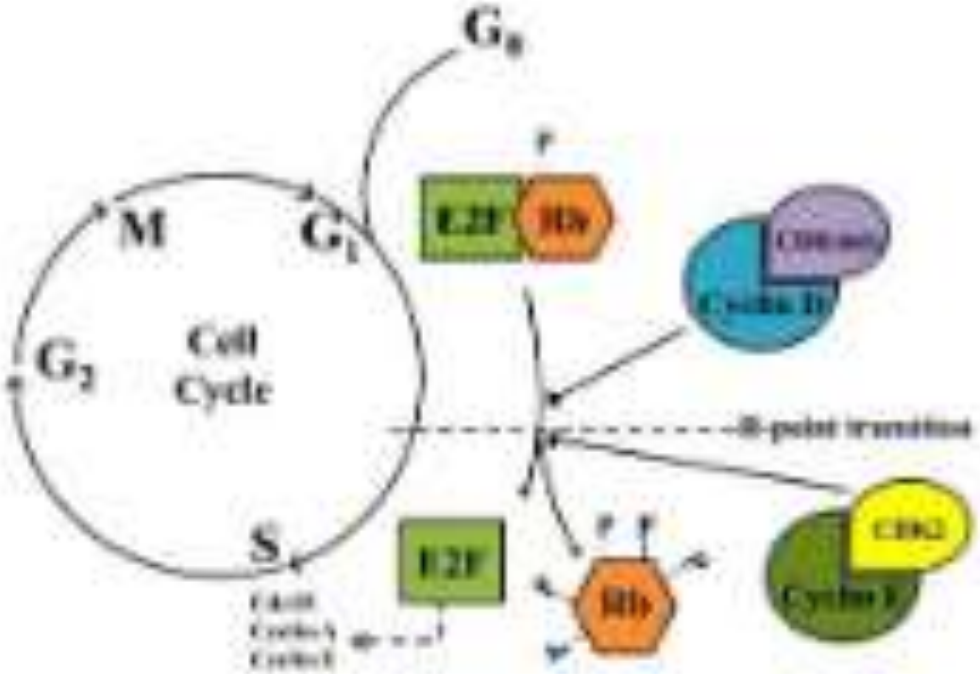




Early responsive gene : c-fos, c- jun

Induce expression delayed responsive genes
E,D type cclin, cdk 2,4,6, E2F etc

The Rb tumor suppressor protein (pRb) binds to the E2F1 transcription factor preventing it from interacting with the cell's transcription machinery. In the absence of pRb, E2F1 mediates the trans-activation of E2F1 target genes that facilitate the G1/S transition and S-phase. E2F targets genes that encode proteins involved in DNA replication (for example DNA polymerase, thymidine kinase, dihydrofolate reductase and cdc6), and chromosomal replication (replication origin-binding protein HsOrc1 and MCM5). When cells are not proliferating, E2F DNA binding sites contribute to transcriptional repression. In vivo footprinting experiments obtained on Cdc2 and B-myb promoters demonstrated E2F DNA binding site occupation during G0 and early G1, when E2F is in transcriptional repressive complexes with the pocket proteins.
pRb is one of the targets of the oncogenic human papilloma virus protein E7, and human adenovirus protein E1A. By binding to pRB, they stop the regulation of E2F transcription factors and drive the cell cycle to enable virus genome replication



The Rb:E2F pathway. Sequential phosphorylation by kinase complexes Cyclin D:Cdk 4/6 and Cyclin E:Cdk 2, respectively, causes conformational changes to the Rb structure and release of E2F. The release of E2F is necessary for the expression of S-phases genes.

Rb protein remain phosphorylated through out S, G2 and M by Cdk2 and cdk1 Cuclins complex. After the cell enter into G0 or G1 phase cyclin-cdk level fall And Rb is dephosphorylated by unopposed Phosphatase.

S. Prombe and S.cerevisea ➡ single cdk

But mammalian cells ➡ A small family of related cdk to regulate progression through cell Cycle (cdk1,2,4,6)

Also produce multiple cyclins

S,G2, early M ➡ Cyclin A,B

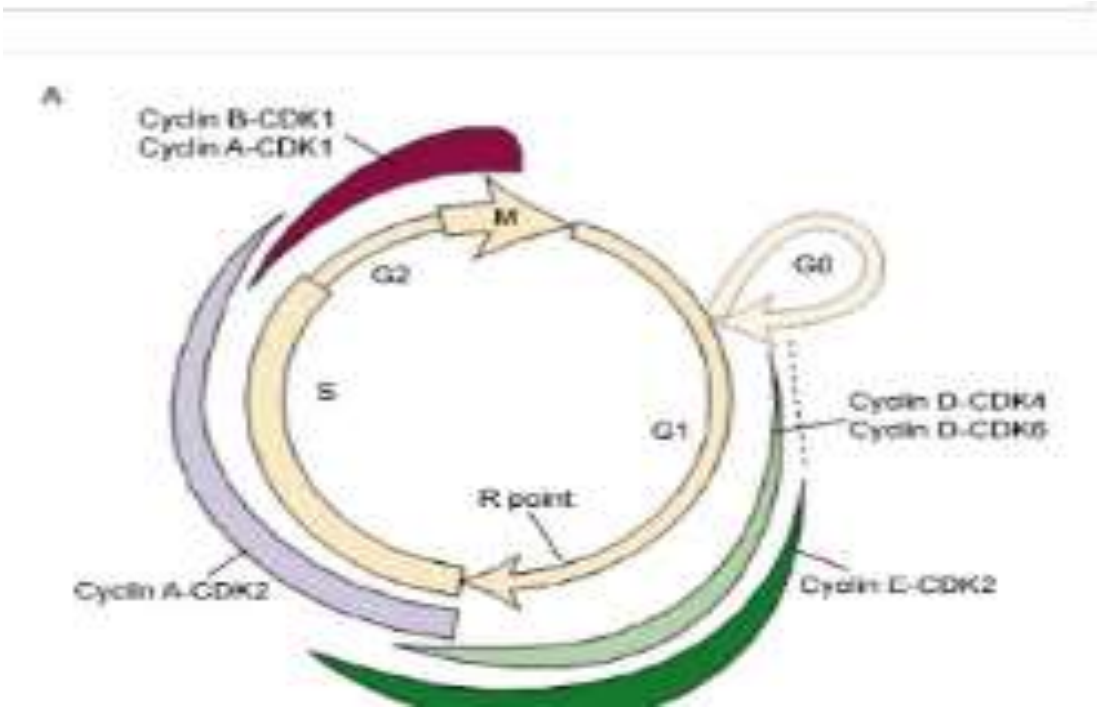
G1 ➡ Cyclin D,E (like Cln)

Cdks

G1 ➡ cdk2,4,6

S ➡ cdk2

M ➡ cdk1



Mammalian Cyclin kinase inhibitor ➡ cell cycle control

Several cyclin kinase inhibitors

CIP (Cdk inhibiting proteins

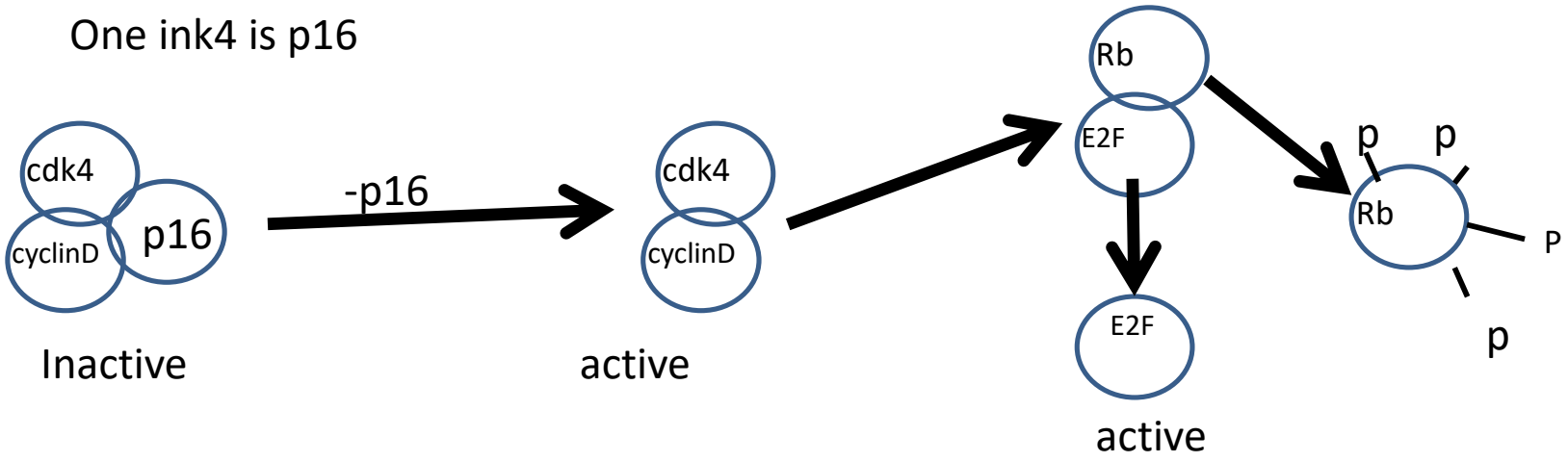
CKI

Ink4 (inhibitor of kinase 4) / ARF

CIP binds and inhibits cdk1,cdk2,cdk4 ,cdk6

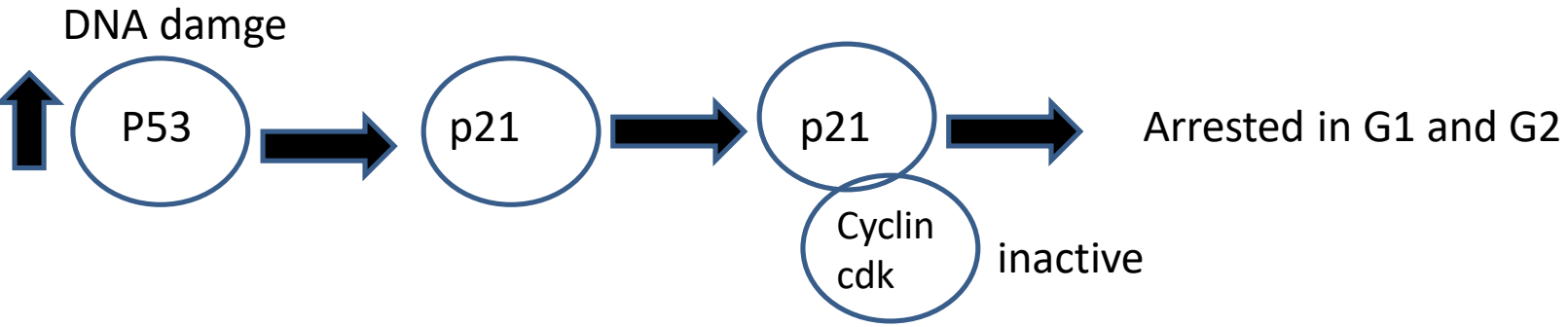
INK4 cdk4-cyclinD, cdk6-cyclinD

One ink4 is p16



cIP,s are p21, p27,p57

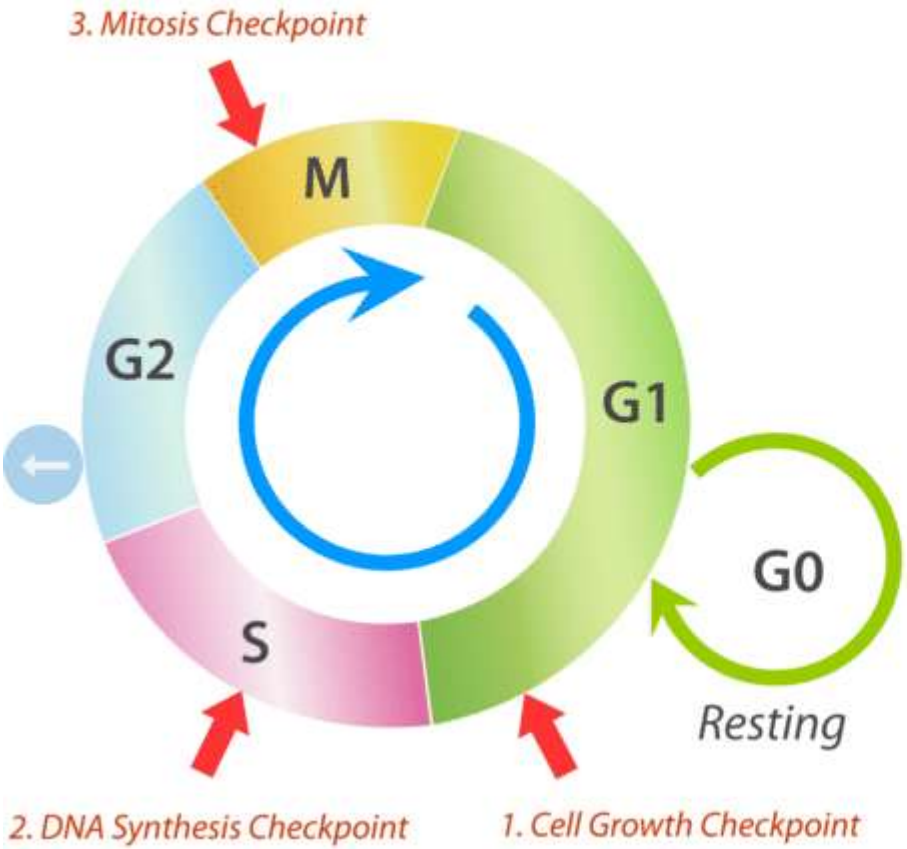
P21 expressed by p53 ----- another tumor supressor



P27 inhibits cdk2 hence E2F activation

P57 inhibits G1 cyclin, role in growth
Now it was found that p57 is also involved in the regulation of other cellular processes including apoptosis, differentiation, development, and migration in tumorigenesis

Cell cycle check point



1. Cell Growth Checkpoint

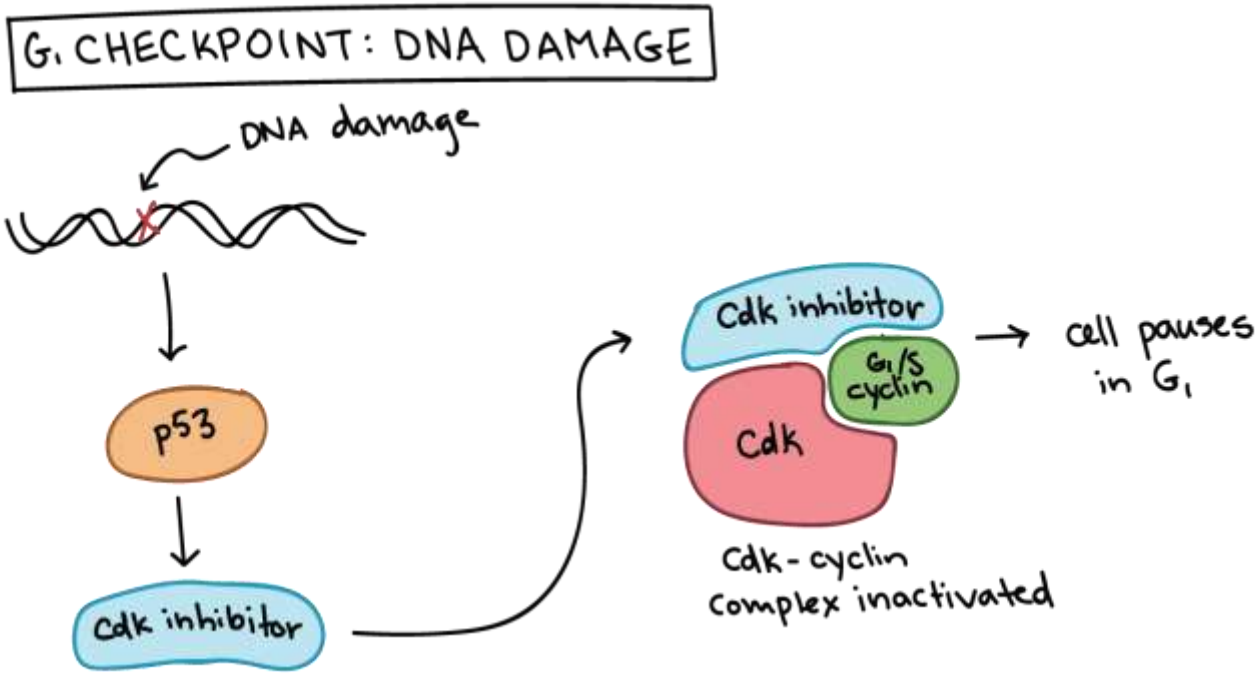
- Occurs toward the end of growth phase 1 (G1).
- Checks whether the cell is big enough and has made the proper proteins for the synthesis phase.
- If not, the cell goes through a resting period (G0) until it is ready to divide.

2. DNA Synthesis Checkpoint

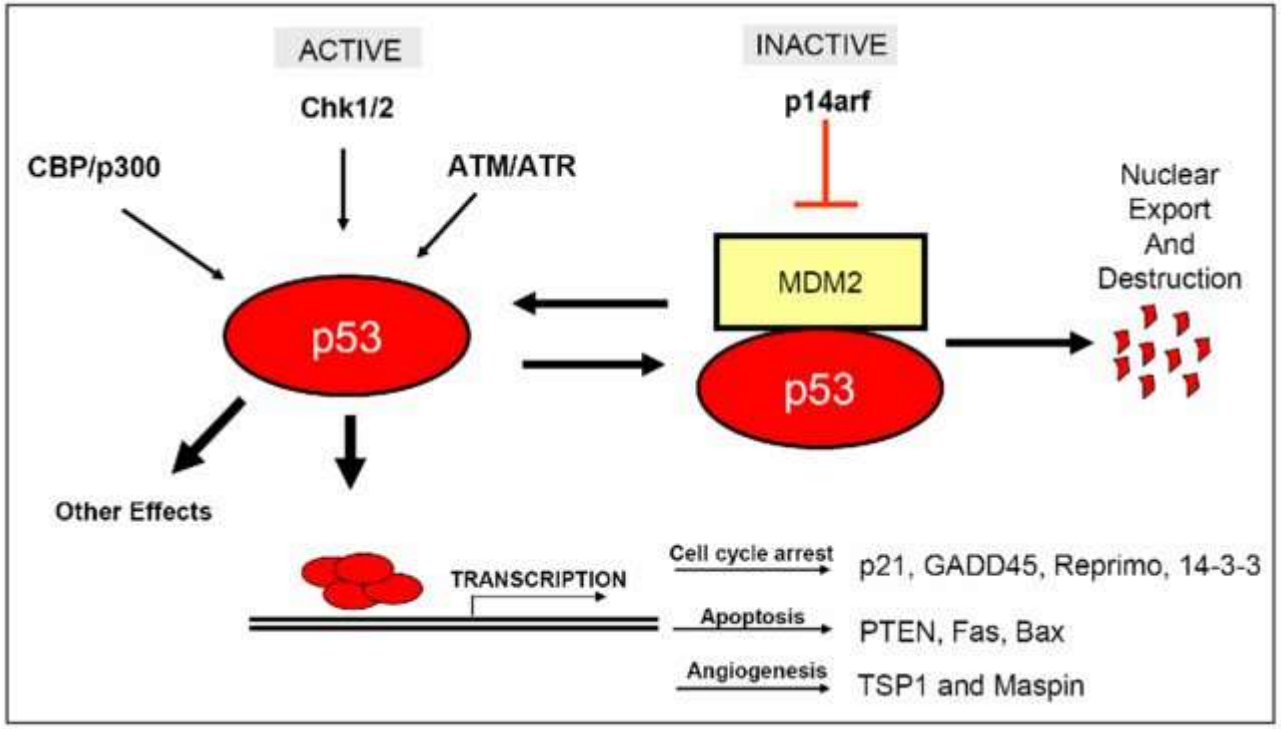
- Occurs during the synthesis phase (S).
- Checks whether DNA has been replicated correctly.
- If so, the cell continues on to mitosis (M).

3. Mitosis Checkpoint

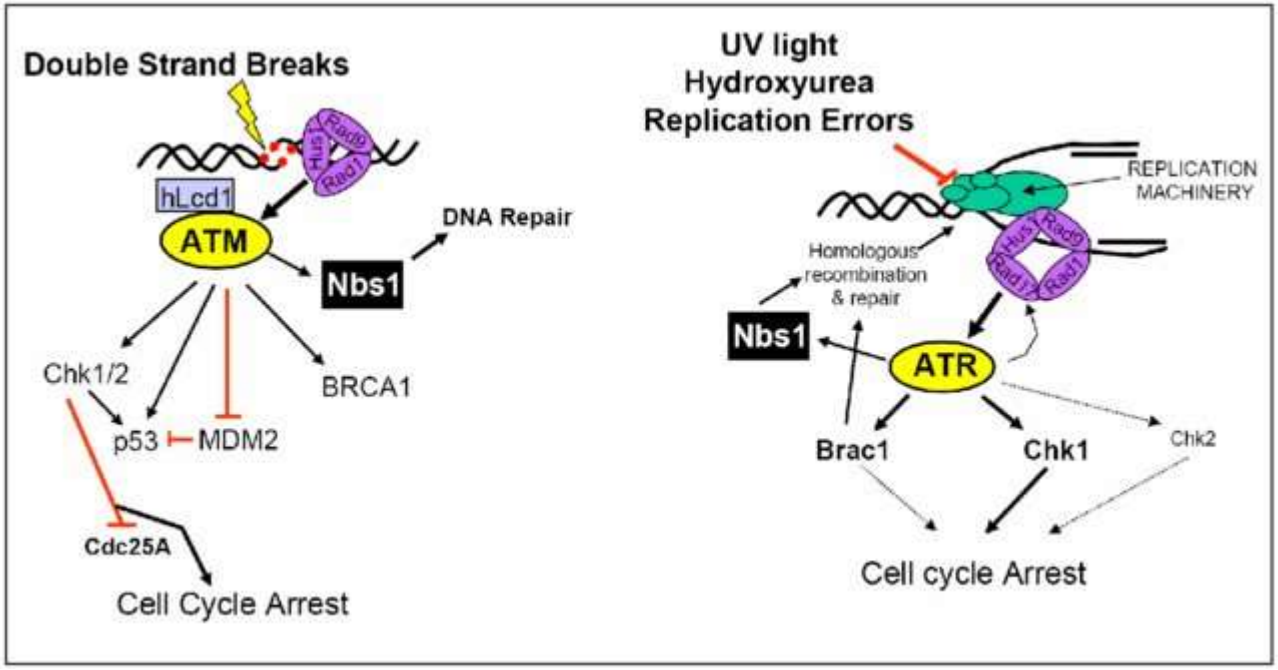
- Occurs during the mitosis phase (M).
- Checks whether mitosis is complete.
- If so, the cell divides, and the cycle repeats.



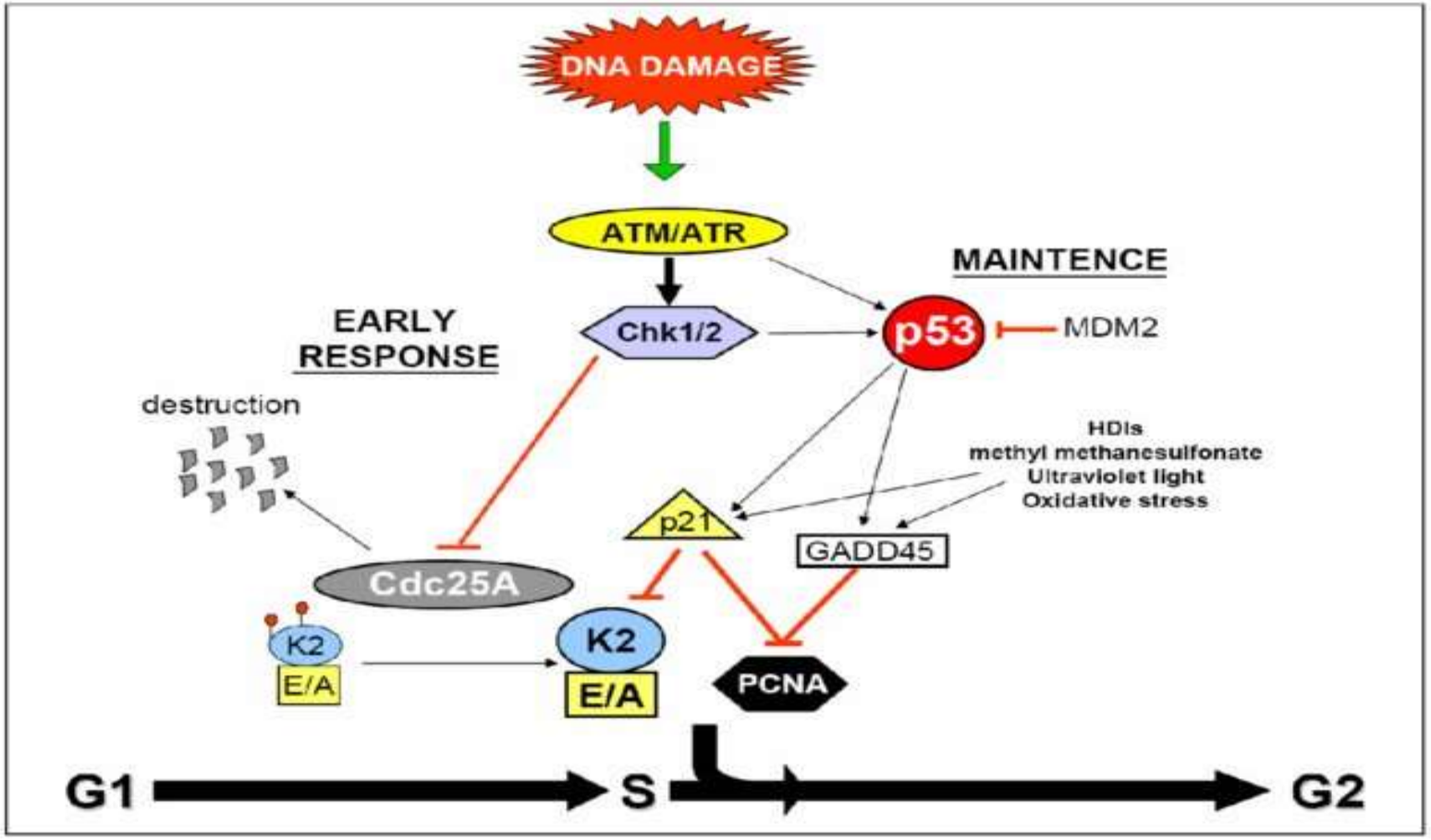
p53 works on multiple levels to ensure that cells do not pass on their damaged DNA through cell division. First, it stops the cell cycle at the G1 checkpoint by triggering production of **Cdk inhibitor** (**CKI**) proteins. The CKI proteins bind to Cdk-cyclin complexes and block their activity ,buying time for DNA repair. p53's second job is to activate DNA repair enzymes. If DNA damage is not fixable, p53 will play its third and final role: triggering programmed cell death so damaged DNA is not passed on.



General Regulation and Functions of the Tumour Suppressor Protein p53 Stimuli such as DNA damage or cellular stress and the subsequent signalling through ATM/ATR or the checkpoint kinases Chk1/2, results in the phosphorylation and stabilisation of p53 protein. This leads to a number of effects including the up-regulation of a number of p53 target genes.

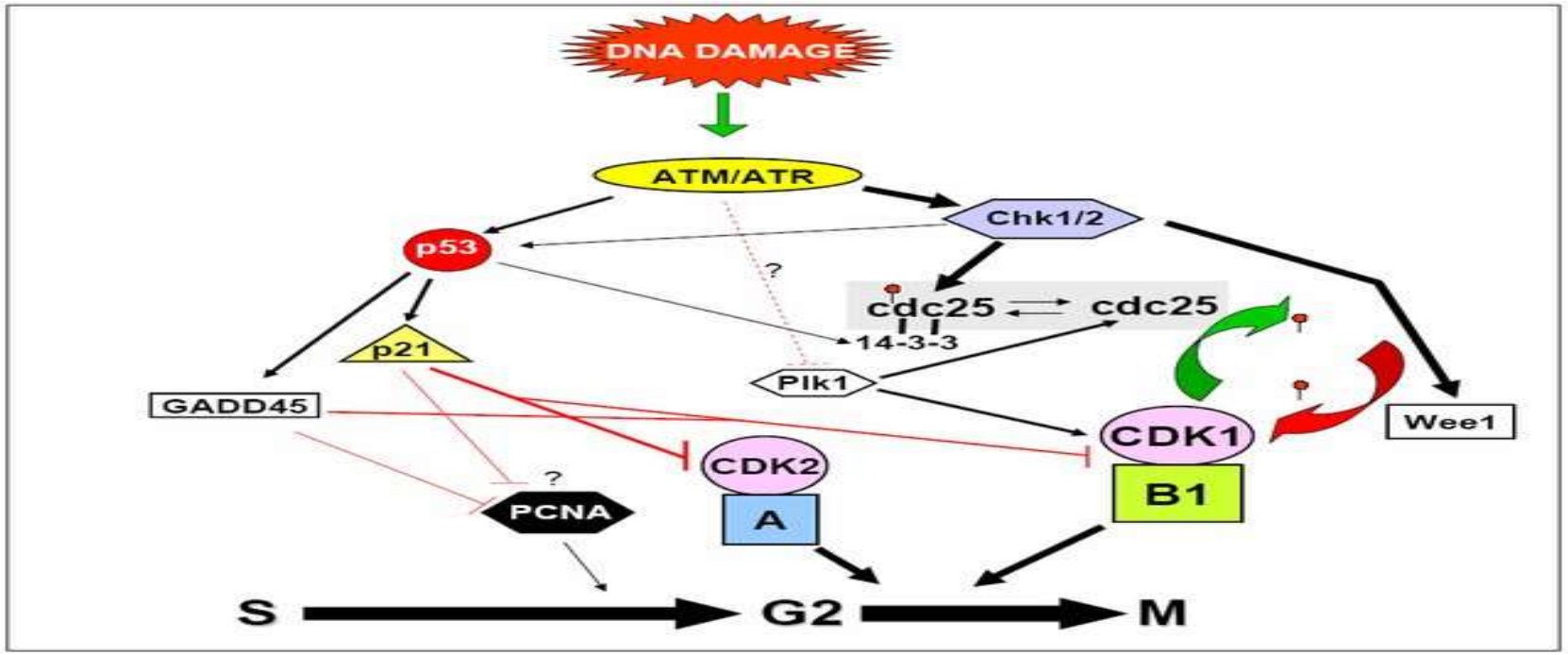


ATM/ATR Responses to DNA Damage DNA double strand breaks such as those induced by IR are associated with recruitment of ATM to the sites of damage. While those agents that induce single strand breaks or stalled replication forks, such as UV light and hydroxyurea appear to favour the recruitment of ATR.



G1 and S phase Checkpoint Responses DNA damage during G1 and S phase signals through ATM/ATR to initially block cdc25A thus preventing the removal of inhibitory phosphorylation on cyclin E/CDK2 and progression through G1 and S phase. A second slower p53 dependent pathway is also activated to help maintain the arrest. The slower mechanism involves the phosphorylation and stabilisation of p53.

G2/M DNA Damage Checkpoint

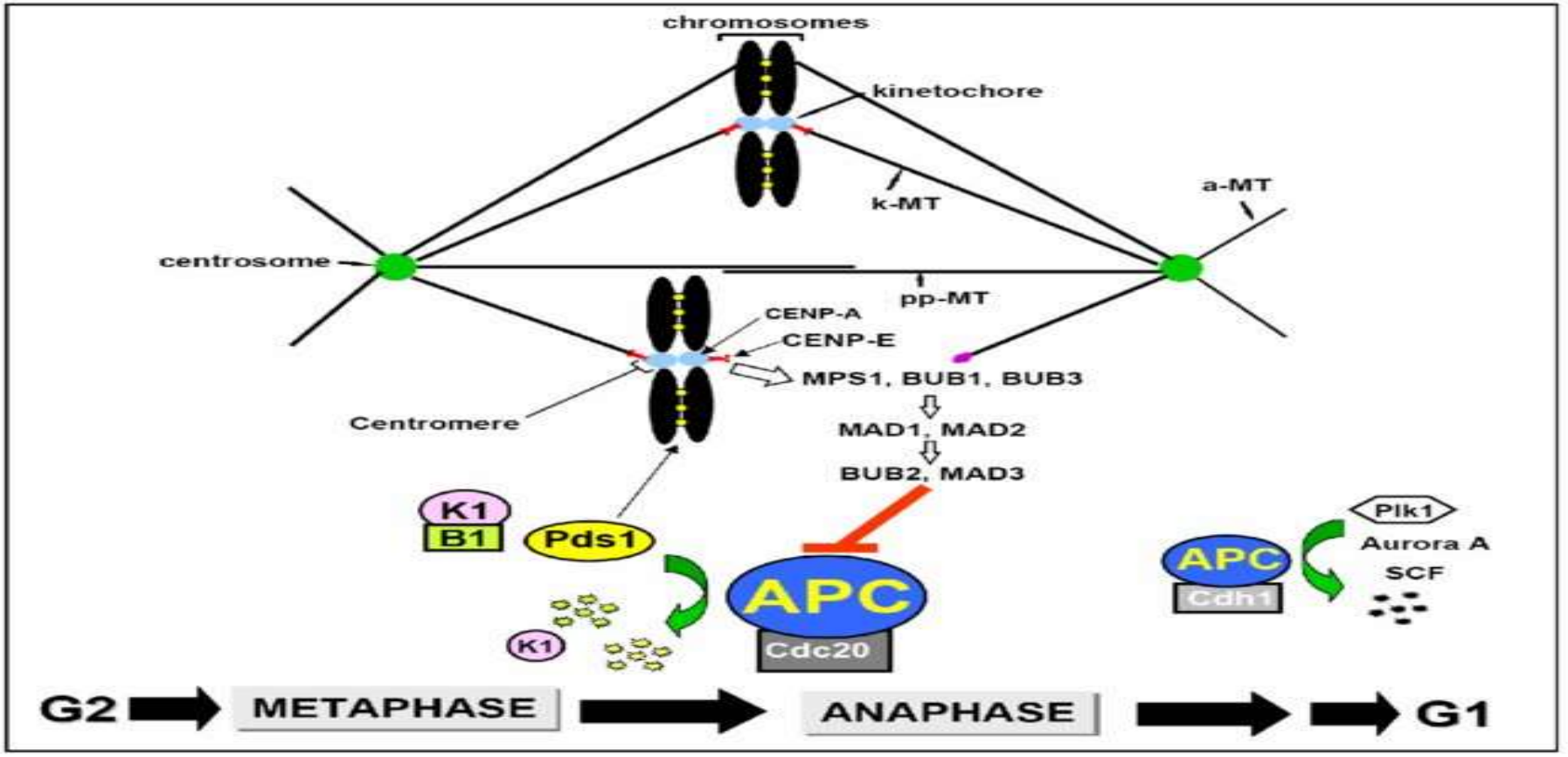


The G2/M DNA Damage Checkpoint DNA damage activates ATM/ATR which can then signal through either p53 dependent or independent pathways, both of which ultimately target and inhibit cyclin B1/CDK1 and cyclin A /CDK complexes in G2/M, resulting in a cell cycle arrest.

Gadd45 binds to the proliferating cell nuclear antigen and p21 proteins

14-3-3 protein, **regulate the cell cycle by binding to various cyclin-dependent kinases (CDKs) and Cdc25 phosphatases (Cdc25A, -B, and -C) at different phases of the cell cycle**

Spindle Assembly Checkpoint



The Spindle Assembly Checkpoint The spindle assembly checkpoint responds to unattached, misaligned or unequal tension across the kinetochores. These signal through the MAD and BUB checkpoint genes that inhibit the anaphase promoting complex (APC) **WAIT ANAPHASE,** which is responsible for the destruction of key substrates such as cyclin B and Pds1 (cohesion).

Thus : The cell cycle is based on three main checkpoints:

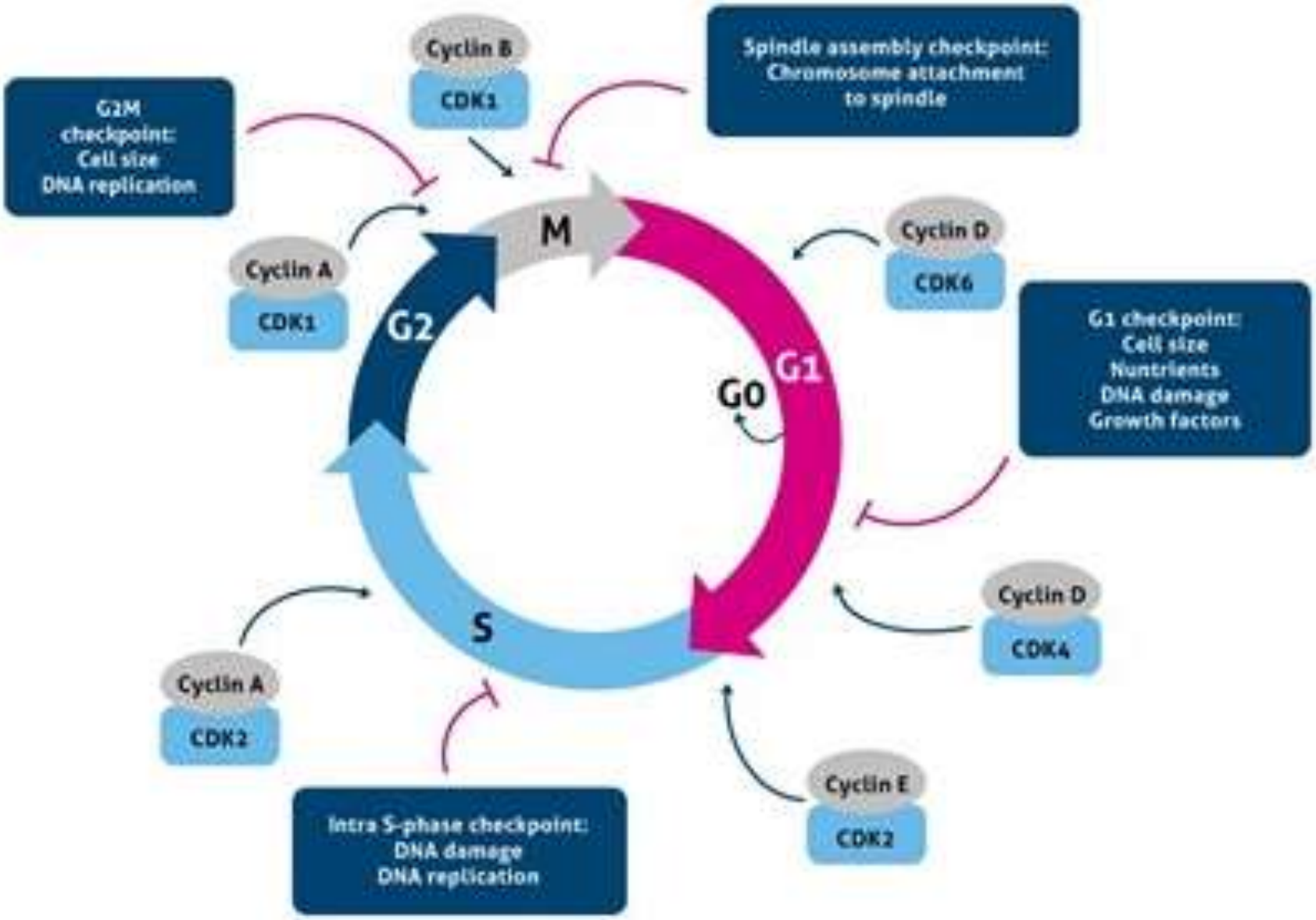
- Phase G1 – DNA integrity and cell size
- Phase G2 – DNA damage and chromosome duplication
- Phase M – Attachment of kinetochore and a spindle fiber

The key role of checkpoint proteins is to detect DNA damage and send a signal to delay cell cycle advance until the damaged chromosomes are repaired

What controls the cell cycle at key checkpoints?
There are two key classes of regulatory molecules:

- Cyclins – a group of proteins that control the progression of cells through the cell cycle by activating cyclin-dependent kinase (CDK) enzymes
- CDKs – a family of protein kinases that are involved in regulating transcription, mRNA processing, and the differentiation of nerve cells

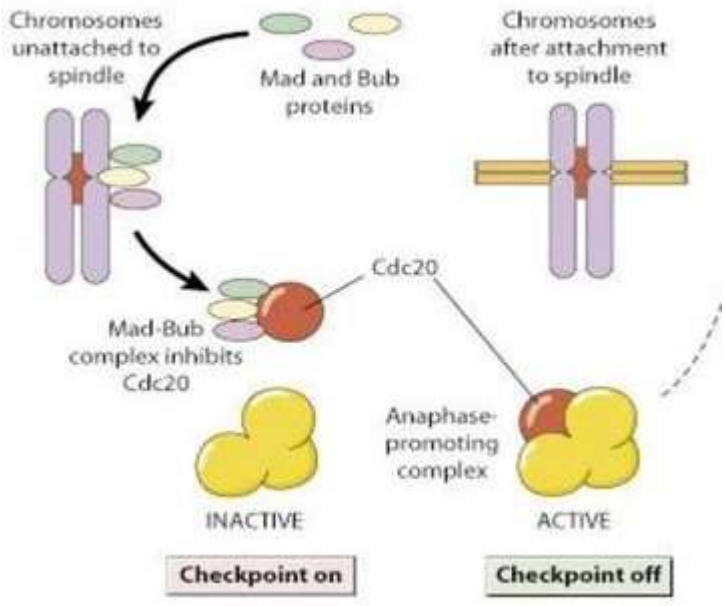
The cell cycle and its checkpoints



The normal function of MAD2 is to accumulate at kinetochores and generate a wait signal preventing the cell from progressing to anaphase of the cell cycle until the spindle microtubules have correctly aligned with the kinetochores on each chromosome.

Bub1 has two roles at the kinetochore: **detecting the absence of microtubules and ensuring that sister chromatids attach to opposite spindle poles**. In contrast, Mad3 aids another checkpoint component, Mad2, in turning off the APC.

MAD2 inhibits the anaphase-promoting complex when chromosomes are unattached to the mitotic spindle. **It acts as a tumor suppressor gene** because MAD2+/–cells enter anaphase early and display chromosome instability, leading to the formation of lung tumors in mice. Mad2 (mitotic arrest deficient 2) is **an essential spindle checkpoint protein**.



Control of the Cell Cycle

The length of the cell cycle is highly variable, even within the cells of a single organism. In humans, the frequency of cell turnover ranges from a few hours in early embryonic development, to an average of two to five days for epithelial cells, and to an entire human lifetime spent in G_0 by specialized cells, such as cortical neurons or cardiac muscle cells. There is also variation in the time that a cell spends in each phase of the cell cycle. When fast-dividing mammalian cells are grown in culture (outside the body under optimal growing conditions), **the length of the cycle is about 24 hours. In rapidly dividing human cells with a 24-hour cell cycle, the G_1 phase lasts approximately nine hours, the S phase lasts 10 hours, the G_2 phase lasts about four and one-half hours, and the M phase lasts approximately one-half hour.** In early embryos of fruit flies, the cell cycle is completed in about eight minutes. The timing of events in the cell cycle is controlled by mechanisms that are both internal and external to the cell.

progress.

Regulation of the Cell Cycle by External Events

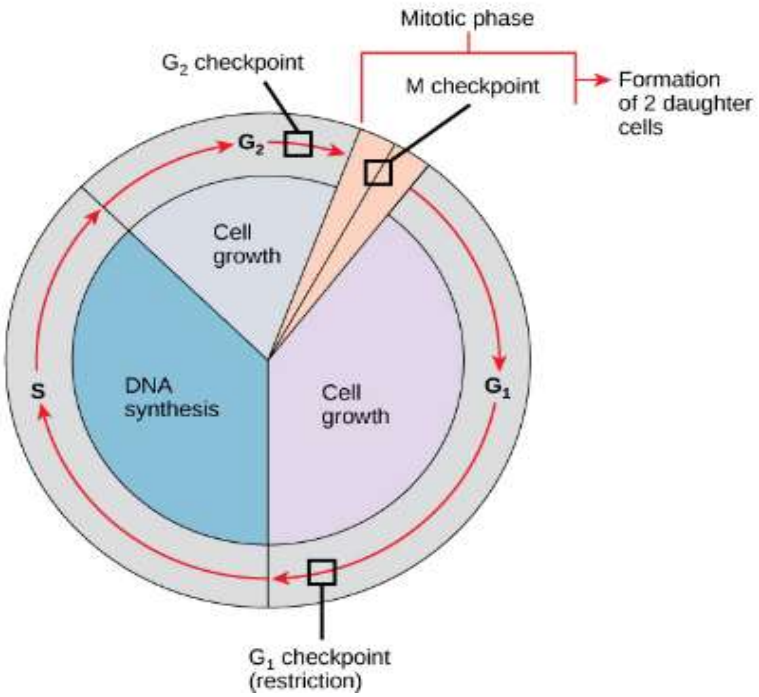
Both the initiation and inhibition of cell division are triggered by events external to the cell when it is about to begin the replication process. An event may be as simple as the death of a nearby cell or as sweeping as the release of growth-promoting hormones, such as human growth hormone (HGH). A lack of HGH can inhibit cell division, resulting in dwarfism, whereas too much HGH can result in gigantism. Crowding of cells can also inhibit cell division. Another factor that can initiate cell division is the size of the cell; as a cell grows, it becomes inefficient due to its decreasing surface-to-volume ratio. The solution to this problem is to divide.

Whatever the source of the message, the cell receives the signal, and a series of events within the cell allows it to proceed into mitosis. Moving forward from this initiation point, every parameter required during each cell cycle phase must be met or the cycle cannot

Regulation at Internal Checkpoints

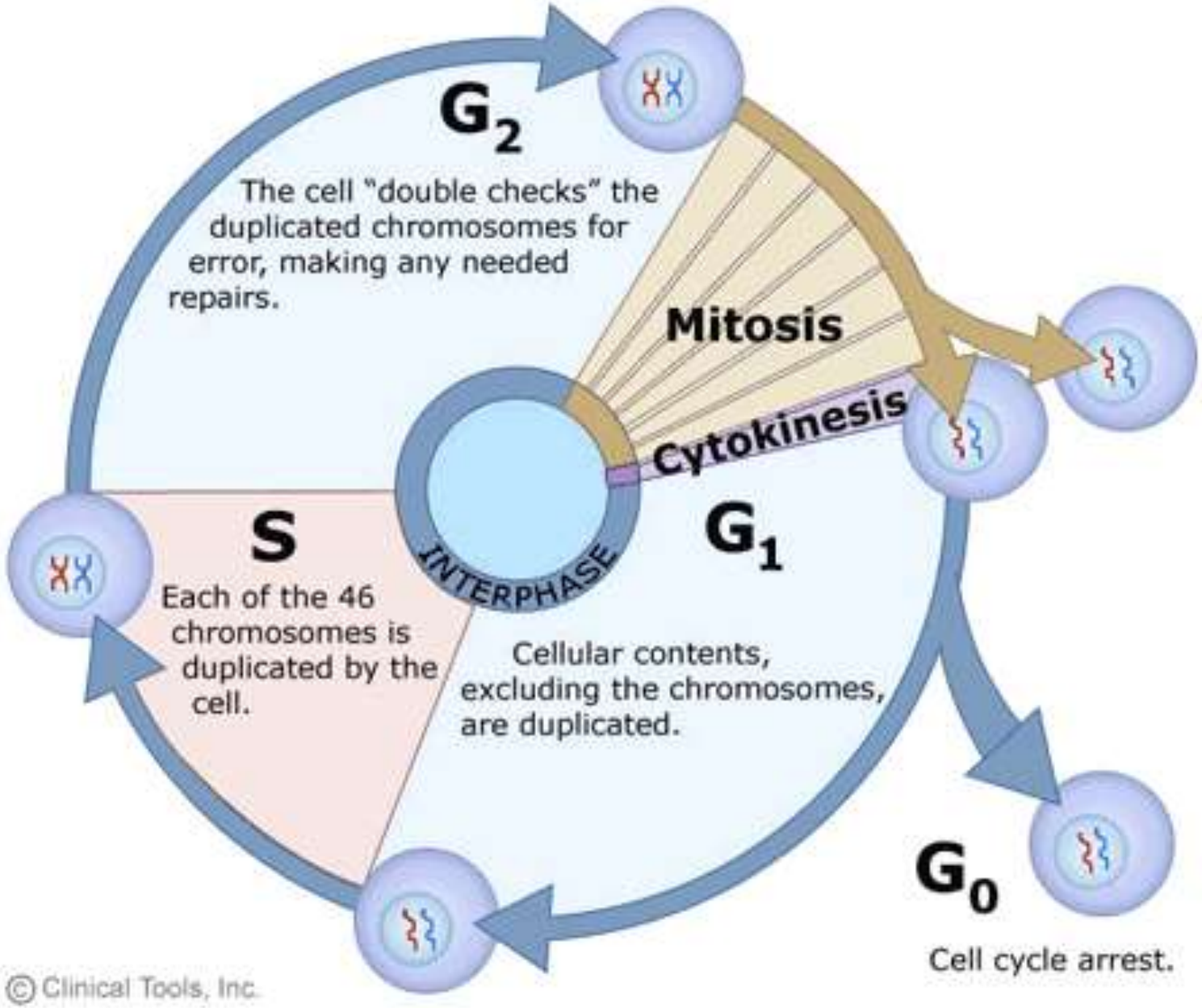
It is essential that the daughter cells produced be exact duplicates of the parent cell. Mistakes in the duplication or distribution of the chromosomes lead to mutations that may be passed forward to every new cell produced from an abnormal cell. To prevent a compromised cell from continuing to divide, there are internal control mechanisms that operate at three main cell cycle checkpoints.

A checkpoint is one of several points in the eukaryotic cell cycle at which the progression of a cell to the next stage in the cycle can be halted until conditions are favorable. These checkpoints occur near the end of G_1, at the G_2/M transition, and during metaphase



The cell cycle is controlled at three checkpoints. The integrity of the DNA is assessed at the G_1 checkpoint. Proper chromosome duplication is assessed at the G_2 checkpoint. Attachment of each kinetochore to a spindle fiber is assessed at the M checkpoint.

Cell cycle and its Regulation (graphics from internet)


G2
The cell "double checks" the duplicated chromosomes for error, making any needed repairs.
Mitosis
Cytokinesis
S
Each of the 46 chromosomes is duplicated by the cell.
INTERPHASE
G1
Cellular contents, excluding the chromosomes, are duplicated.
G0
Cell cycle arrest.
© Clinical Tools, Inc.

1. G1 (gap1) phase:

➢The first stage of interphase is called the G1 phase (first gap) because, from a microscopic aspect, little change is visible. However, during the G1 stage, the cell is quite active at the biochemical level.

➢It is characterized by a change in chromosome from condensed state to more extended state and series of metabolic events that leads to initiation of DNA replication. During G1 phase, chromatin fibres become slender, less coiled and fully extended and more active for transcription. The transcription results in synthesis of RNAs (tRNA, mRNA and rRNA) ad series of proteins molecules required for initiation of DNA replication.

➢The length of G1 phase varies from cell to cell and also the length of G1 phase is more than other three phase in cell cycle.

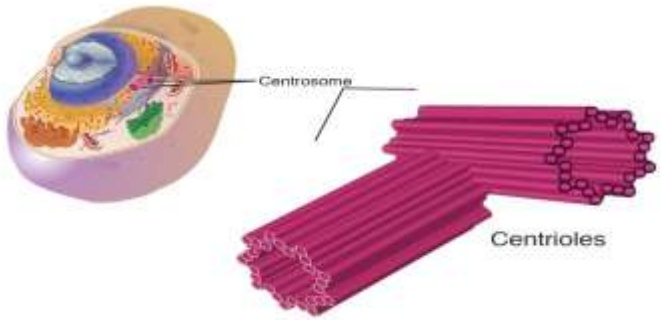
➢G1 phase represents 25-40% of generation time of a cell.

➢G1 phase is very significant phase of cell cycle as the cell grows and accumulates the building blocks of chromosomal DNA and the associated proteins as well as sufficient energy reserves to complete the task of replicating each chromosome.

➢Within G1 phase there is a definite check point at which DNA synthesis is initiated and once the biochemical events associated with that point have occurred cell proceeds towards division.

2. S (synthesis) phase:

- The synthesis phase of interphase is biochemically a phase of active DNA synthesis and histone synthesis.
- In the S phase, chromosome numbers doubles which is accomplished by DNA replication and associated proteins. Although some of the histone protein synthesis occurs in G1 phase, most of it is synthesized during S phase.
- DNA replication is semi conservative and discontinuous type which results in the formation of identical pairs of DNA molecules.
- After doubling of chromosome, sister chromatids are still firmly attached to the centromeric region.
- At the center of each animal cell, the centrosomes of animal cells are associated with a pair of rod-like objects, the centrioles, which are at right angles to each other. Centrioles help organize cell division. Centrioles are absent in plants and most fungi.
- The centrosome (centriole) is also duplicated during the S phase. The two centrosomes will give rise to the mitotic spindle, the apparatus that mediate the movement of chromosomes during mitosis.



3. Gap2 (gap2) phase:

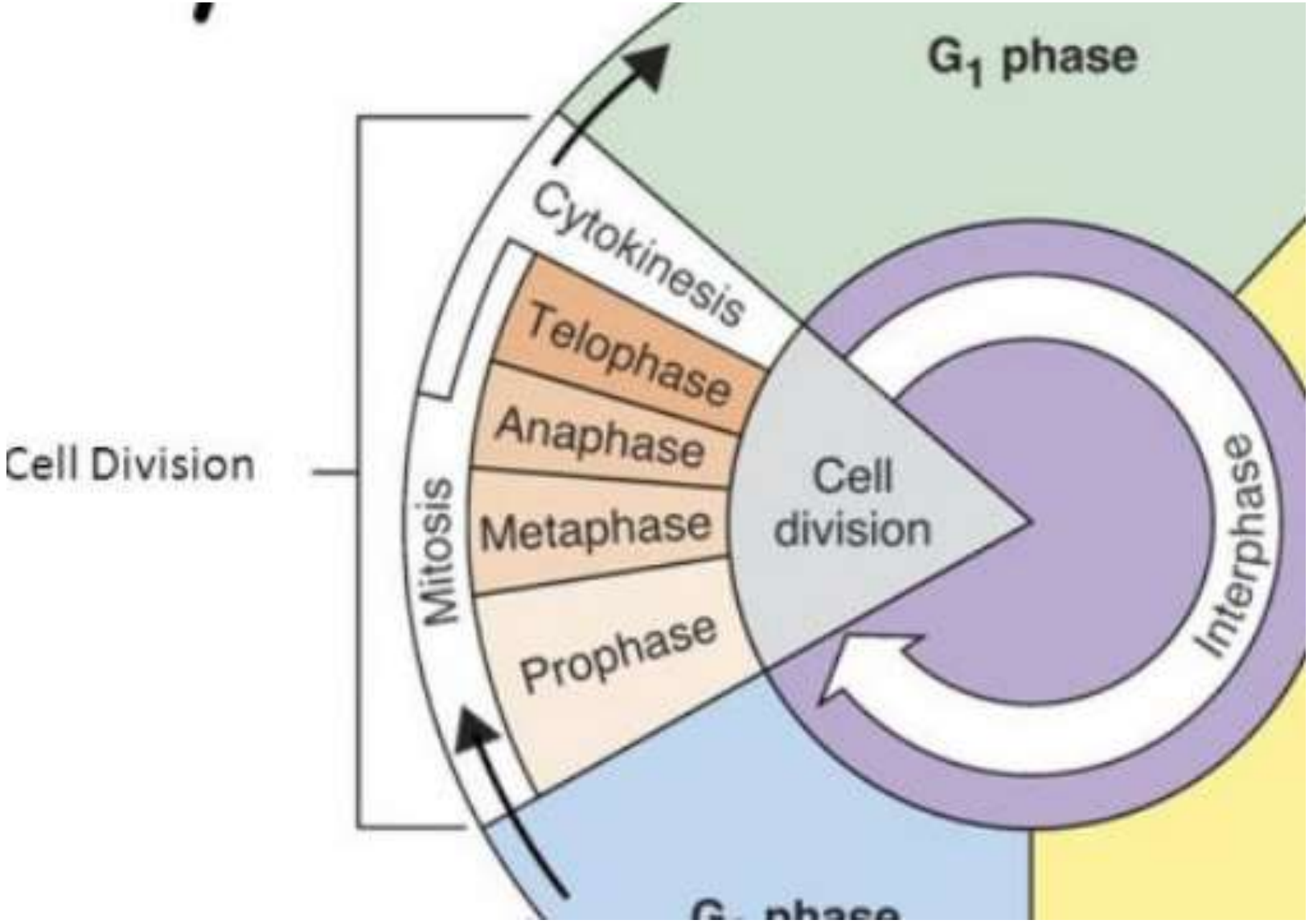
- G2 phase follows S phase. This phase represents 10-25% of generation time of cell.

- In G2 phase chromosome consists of two chromatids ie the cell has twice the amount of DNA content.

- In the G2 phase, the cell restore its energy stores and synthesizes proteins necessary for chromosome manipulation.

- Some cell organelles are duplicated, and the cytoskeleton is dismantled to provide resources for the mitotic phase.

- There may be additional cell growth during G2. The final preparations for the mitotic phase must be completed before the cell is able to enter the first stage of mitosis


Cell Division
G_1 phase
Cytokinesis
Telophase
Anaphase
Metaphase
Prophase
Mitosis
Cell division
Interphase

M (mitotic) phase:

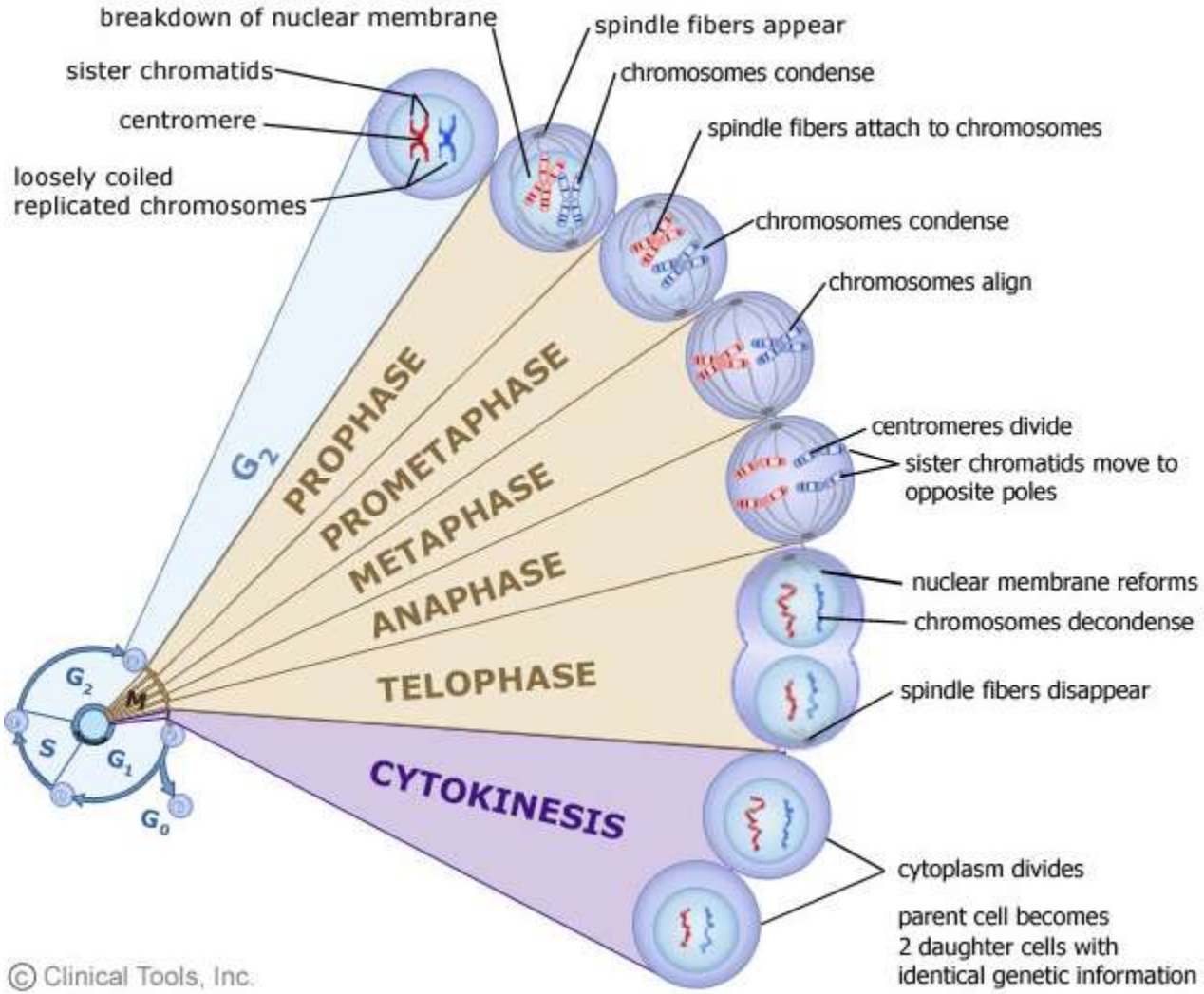
M phase follows G2 phase. During this phase cell divides into two daughter cell with equal distribution of chromosome among daughter cells. After M phase cell enter into G1 phase and next cell cycle is repeated. However, some cell after completion of mitosis do not enter into G1 phase, those cell are referred as G0 cells.

M phase consists of following sub –phases;

- **During prophase**, the nuclear membrane disappears, spindle fibers form, and DNA condenses into chromosomes (sister chromatids).
- **During metaphase**, the sister chromatids align along the equator of the cell by attaching their centromeres to the spindle fibers.
- **During anaphase**, sister chromatids are separated at the centromere and are pulled towards opposite poles of the cell by the mitotic spindle.
- **During telophase,** chromosomes arrive at opposite poles and unwind into thin strands of DNA, the spindle fibers disappear, and the nuclear membrane reappears.

Cytokinesis is the actual splitting of the cell membrane; animal cells pinch apart, while plant cells form a cell plate that becomes the new cell wall.

Cells enter the G_0 (inactive) phase after they exit the cell cycle when they are not actively preparing to divide; some cells remain in G_0 phase permanently


breakdown of nuclear membrane
sister chromatids
centromere
loosely coiled
replicated chromosomes
spindle fibers appear
chromosomes condense
spindle fibers attach to chromosomes
chromosomes condense
chromosomes align
centromeres divide
sister chromatids move to
opposite poles
nuclear membrane reforms
chromosomes decondense
spindle fibers disappear
cytoplasm divides
parent cell becomes
2 daughter cells with
identical genetic information
G2
PROPHASE
PROMETAPHASE
METAPHASE
ANAPHASE
TELOPHASE
CYTOKINESIS
G2
M
S
G1
G0
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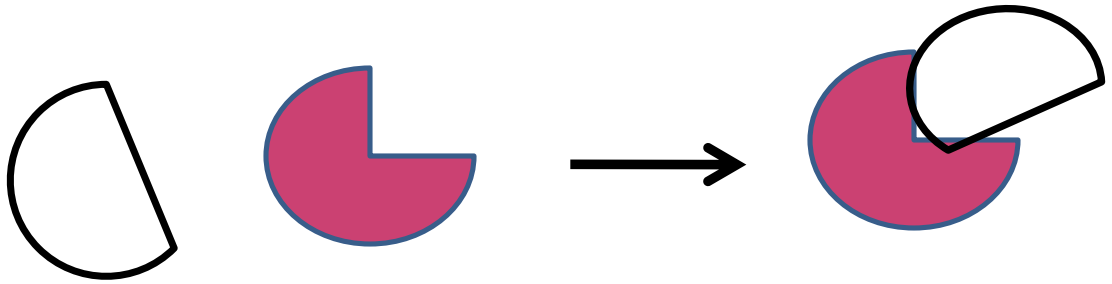
Control of cell cycle

- Regulated phosphorylation /activation
- Regulated degradation

Small number of heterodimeric protein kinase called cyclin.Their catalytic components cyclin dependent kinase is inactive in the absence of cyclins



Cyclin inactive CDK active CDK

Three classes of cyclin complexes

G1-CDK
S-CDK
G2-CDK

Their level rise and fall with the progression of cell cycle

G1-CDK ---- Express first

Induce expression of transcription factors for DNA synthesis and **S-CDK** complexes

Held in check by inhibitors

S-CDK -------Phosphorylation of pre-replication complexes which were assembled during G1-phase resulting initiation, also prevent reassembly of new pre-replication complex, so each chromosome is replicate once

M-CDK------- Synthesized in S and G2 but their activity is held in check

Activated M-CDK ------ chromosome condensation

breakdown of nuclear envelope

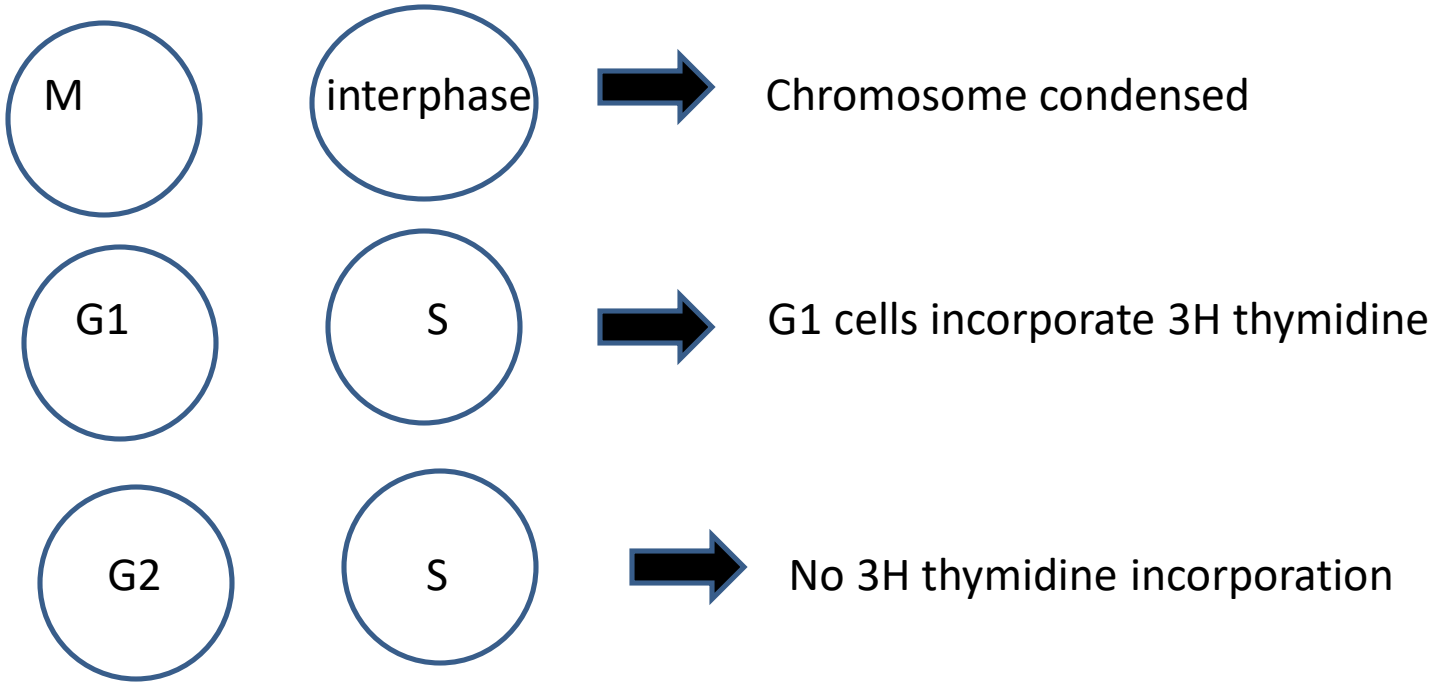
assembly of mitotic spindle

alingment of condensed chromosomes

activate **APC**

- Ubiquitation mediated degradation of anaphase inhibitor
- Inactivated protein complxes that connect sister chromatids
- Degradation of M-CDK (Late anaphase)
- Nuclear envelop reform arround daughter chromosome
- Cytokinesis

Cell fusion experiments



Thus the diffusible factors in S-phase cells induce DNA synthesis in G1 cells but not in G2

Regulation of cell cycle

Protein factors

Cyclin

CDK-cyclin-dependent kinase

Cyclin complex

- G_1 cyclin-CDK complexes
- S phase cyclin-CDK complex
- Mitotic cyclin-CDK complex

Cell cycle inhibitors

Cell cycle promoting factor - ubiquitin ligase lead to degradation of cell cycle inhibitors

- SCF and APC

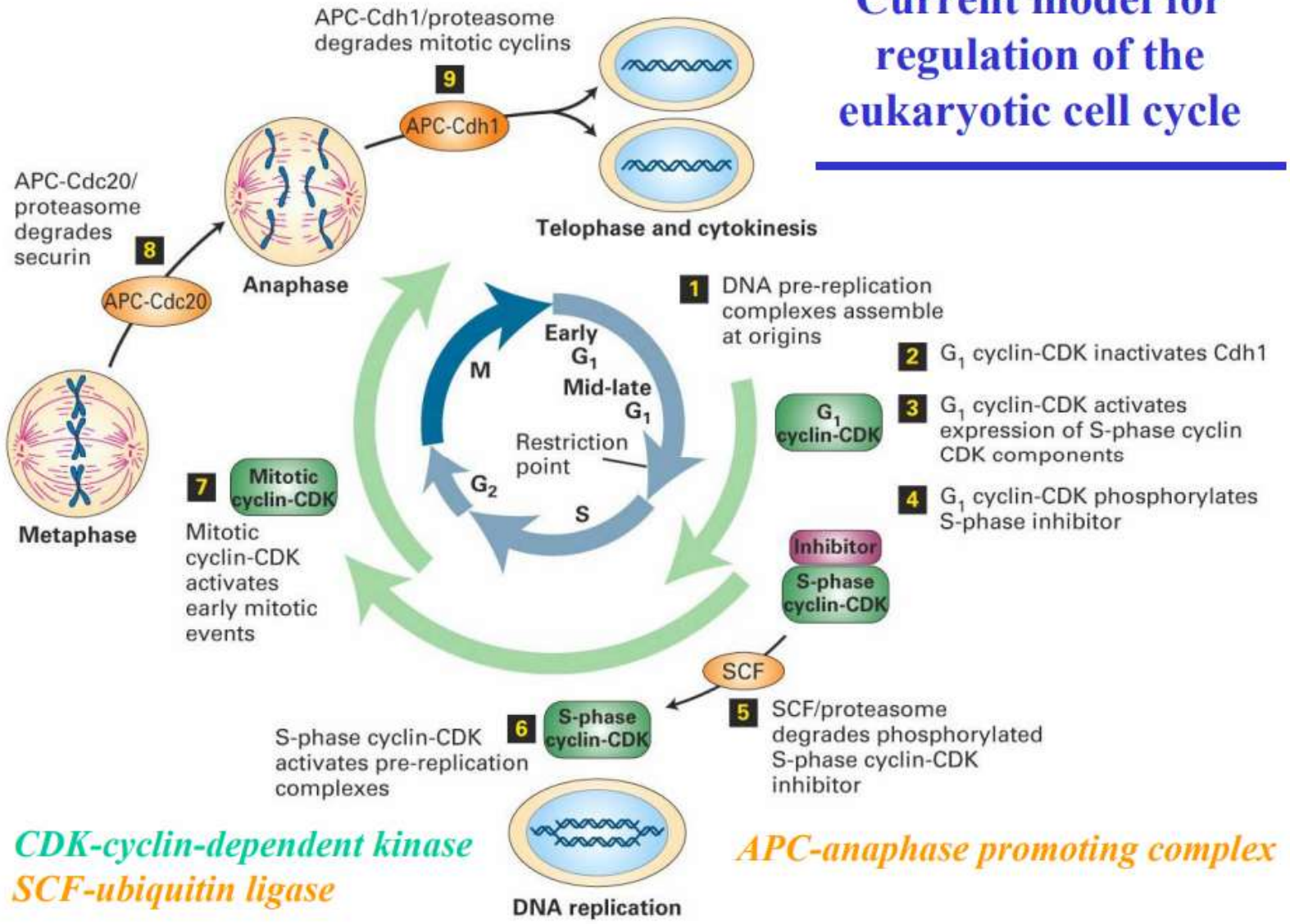
Mechanisms

Phosphorylation and dephosphorylation

Activation of gene transcription and translation

Proteasome-mediated degradation

Current model for regulation of the eukaryotic cell cycle

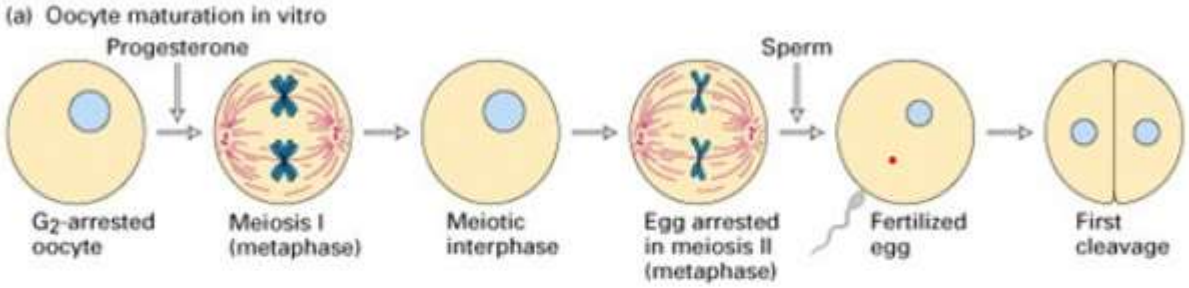


CDK-cyclin-dependent kinase

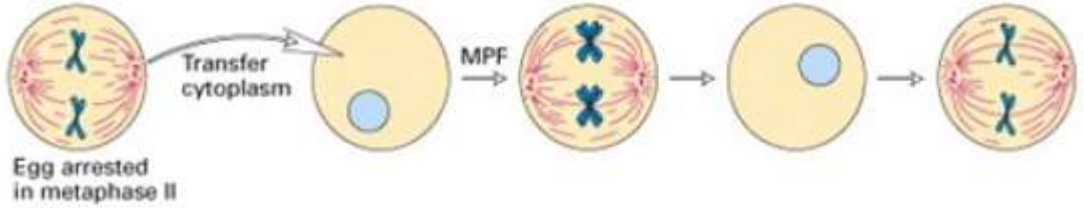
SCF-ubiquitin ligase

APC-anaphase promoting complex

The Maturation of Frog Eggs

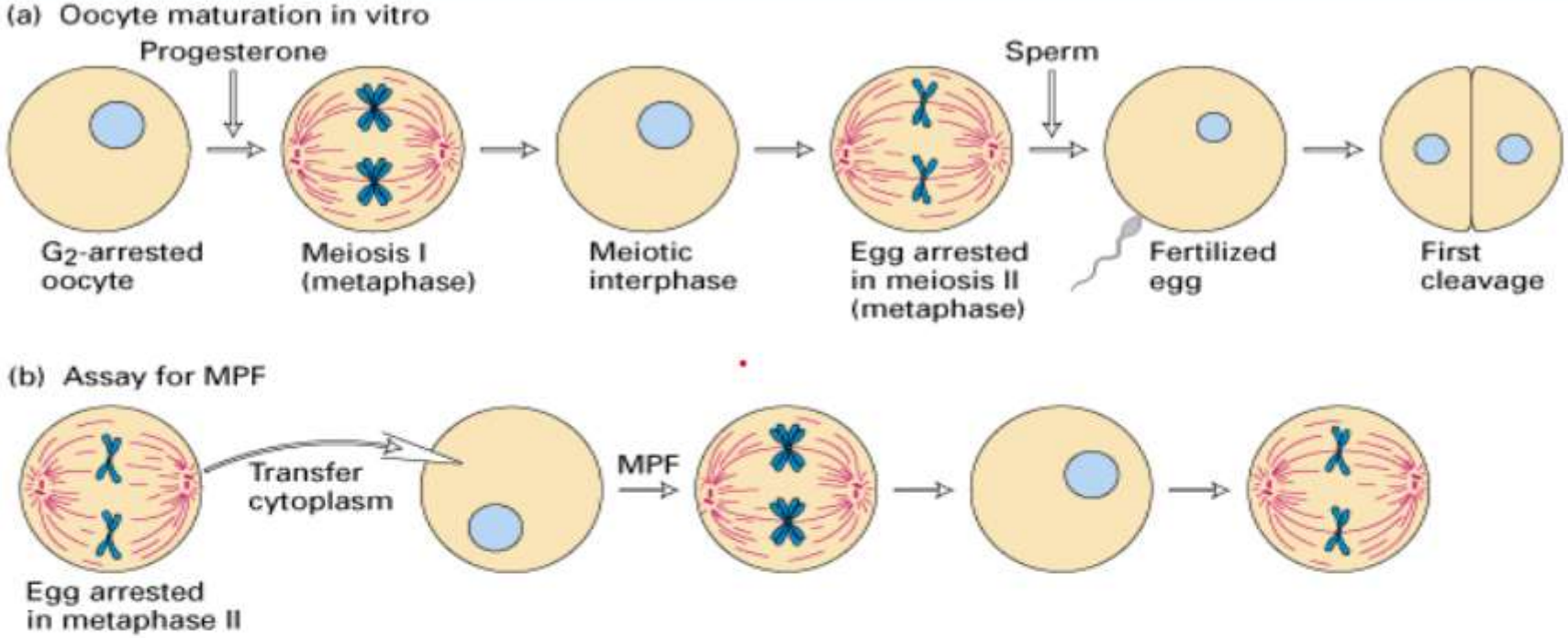


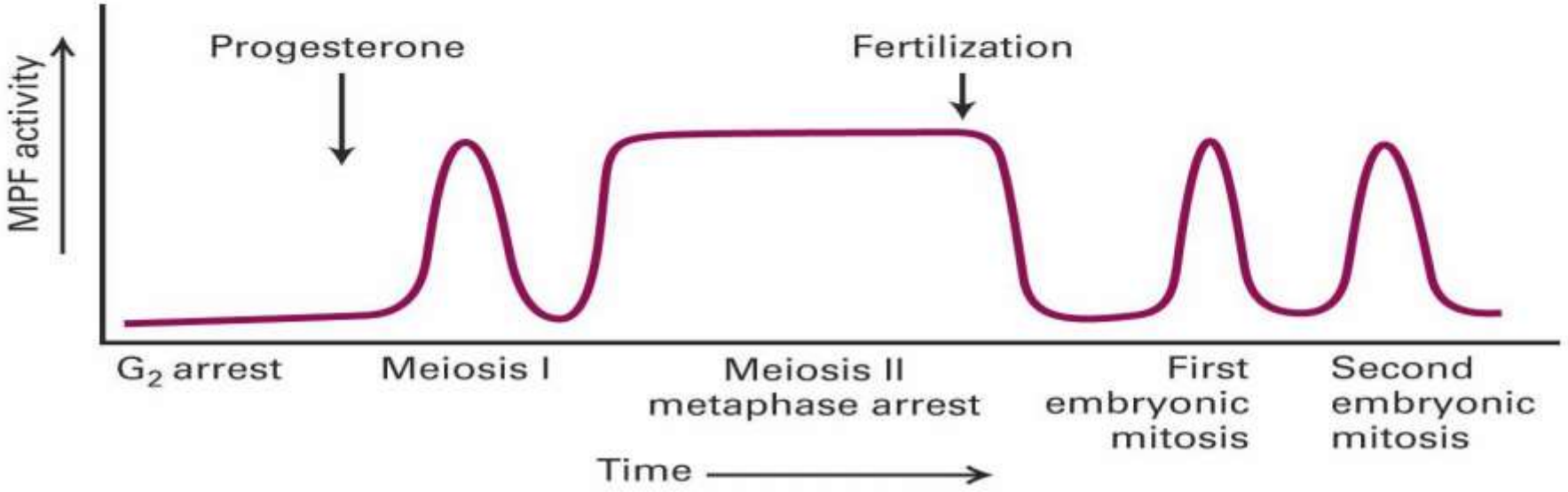
An Assay for Maturation Promoting Factor (MPF)



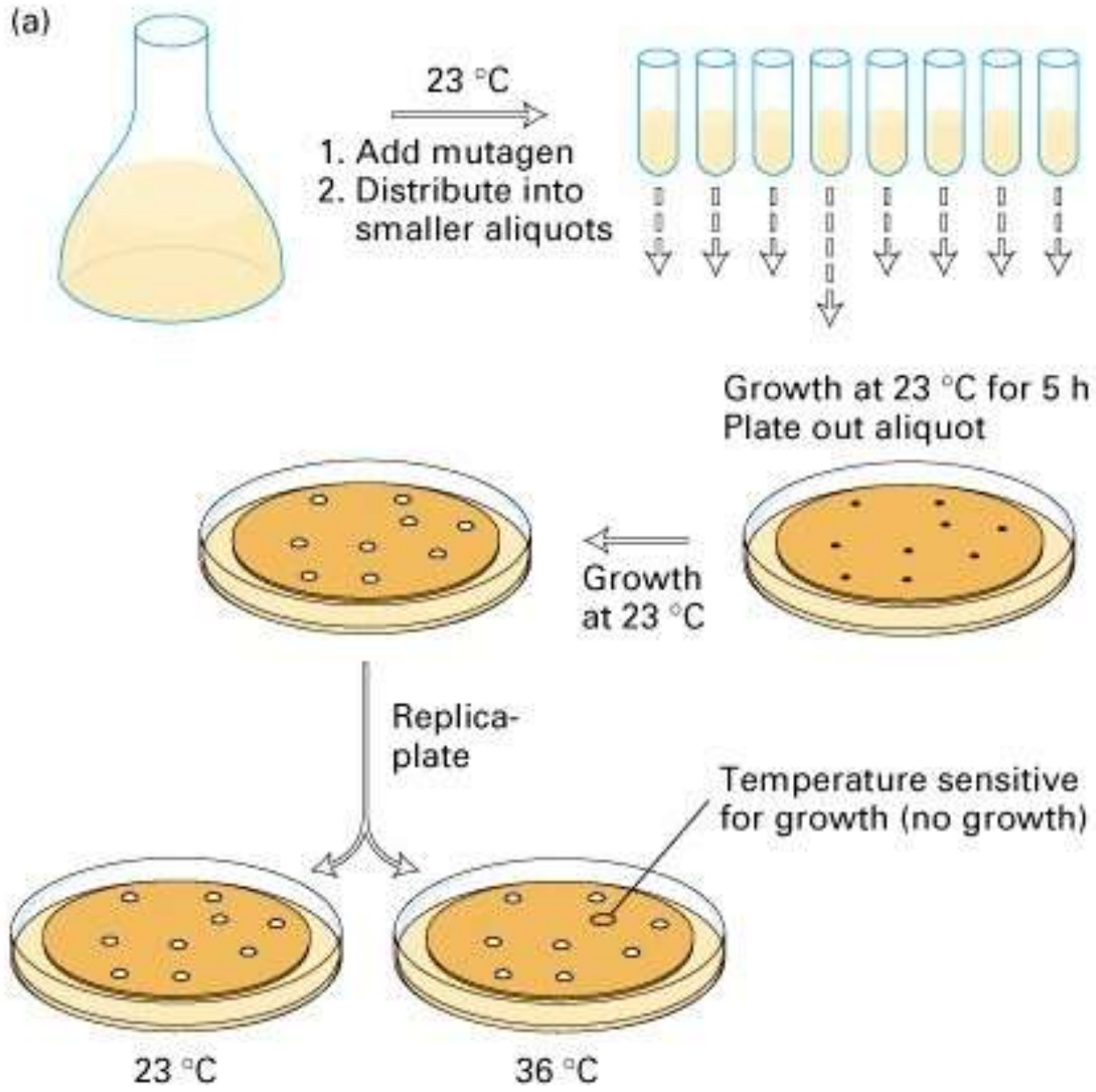
Yoshio Masui, 1971

A diffusible factor in arrested Xenopus eggs promotes meiotic maturation

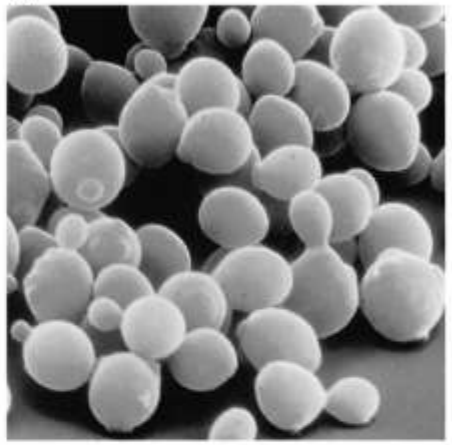




Temp Sensitive yeast mutant



Isolation of cell-division cycle (*CDC*) genes from a *S. cerevisiae* genomic library by functional complementation of *cdc* mutants



cdc28^{ts} cells grown at 25 °C

Transform with plasmid library of wild-type *S. cerevisiae* DNA

Gene *X*

Gene *Y*

cdc28

Transformed *cdc28*^{ts} cells grown at 35 °C

No colony formation

Isolate plasmid

cdc28

Cells in colony at various cell-cycle stages

S.Cerevasie

Cdc2 for prombe

Grow out of bud, whose size during the growth increase during cell cyle

Mutant cells arrested at budding process → cdc mutant

S. pombe

Increase in length and divide in middle cdc mutant divide

Without dividing and form enormously elongated cells at Non-permissive temperature

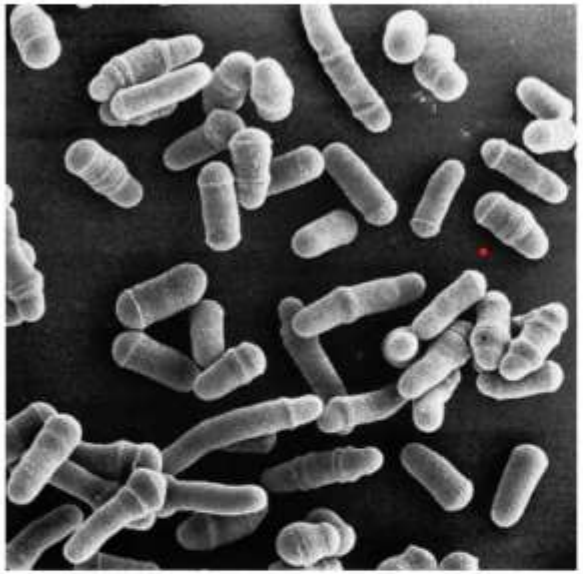
Also S.prombe mutant grow smaller in size → wee mutant

Cdc2 is a key regulator to entry into mitosis : protein kinase :

Another cdc gene cdc 13 also required for entry into mitosis : Cyclin B

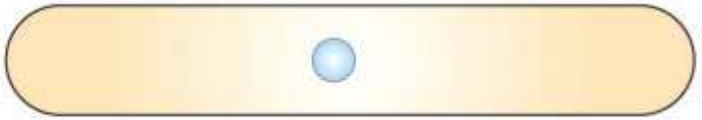
Using *S. pombe cdc* mutants to study the cell cycle regulation

(a)



(a)

Deficit of Cdc25
or
Excess of Wee1



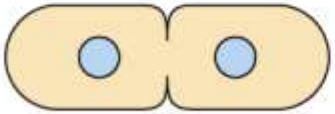
Elongated cells
(increased G_2)

Deficit of Wee1
or
Excess of Cdc25

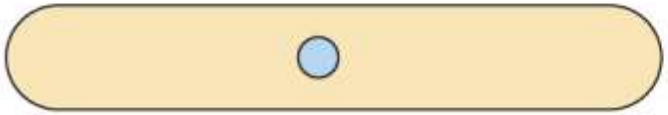
Small cells
(decreased G_2)

(b)

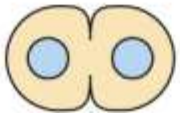
cdc2^{+}

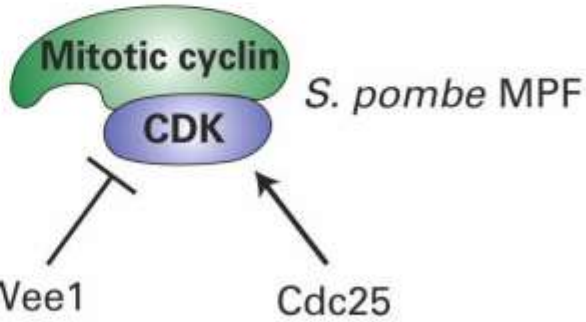


cdc2^{-}

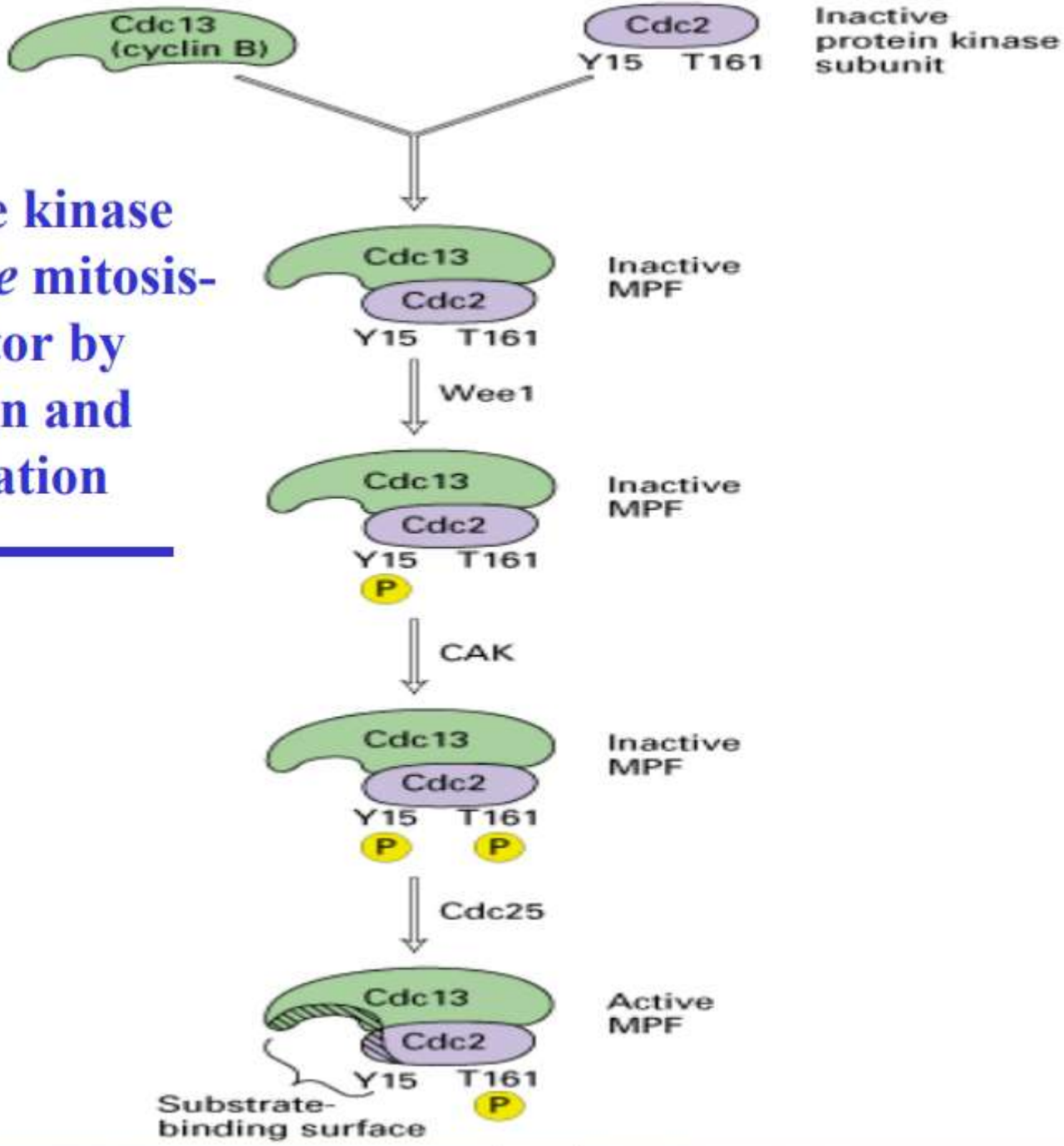


cdc^{D}



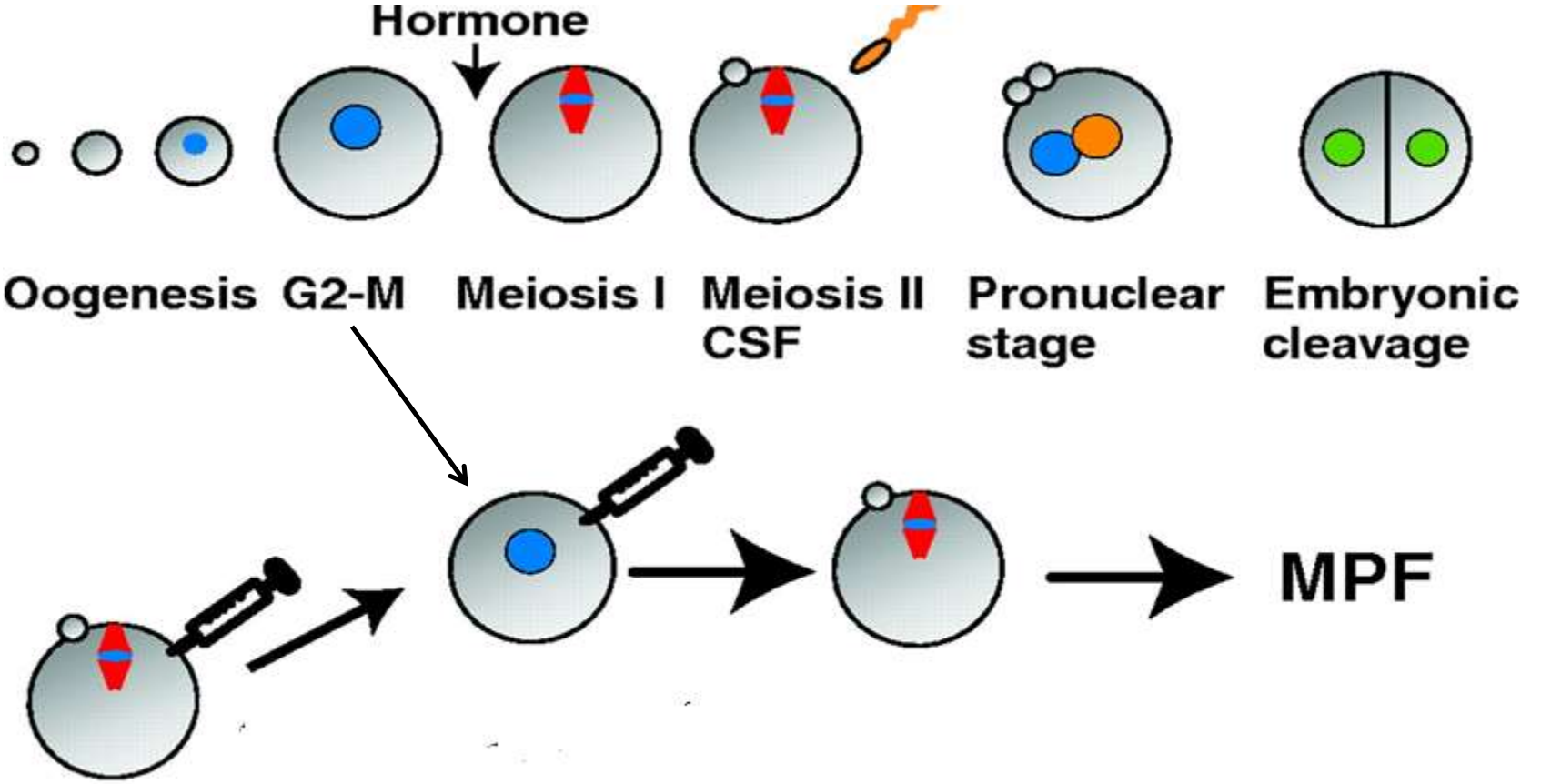


Regulation of the kinase activity of *S. pombe* mitosis-promoting factor by phosphorylation and dephosphorylation

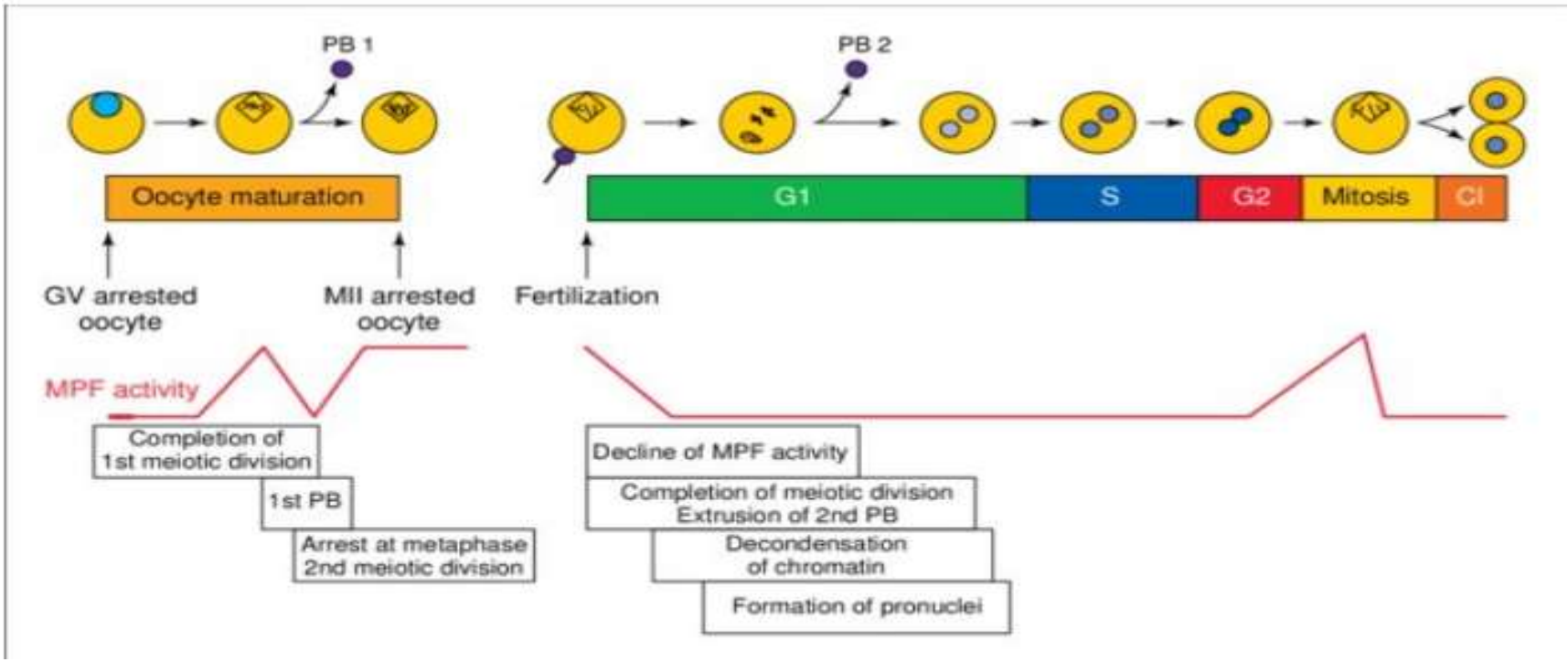


oocytes await fertilization while arrested at metaphase of meiosis II

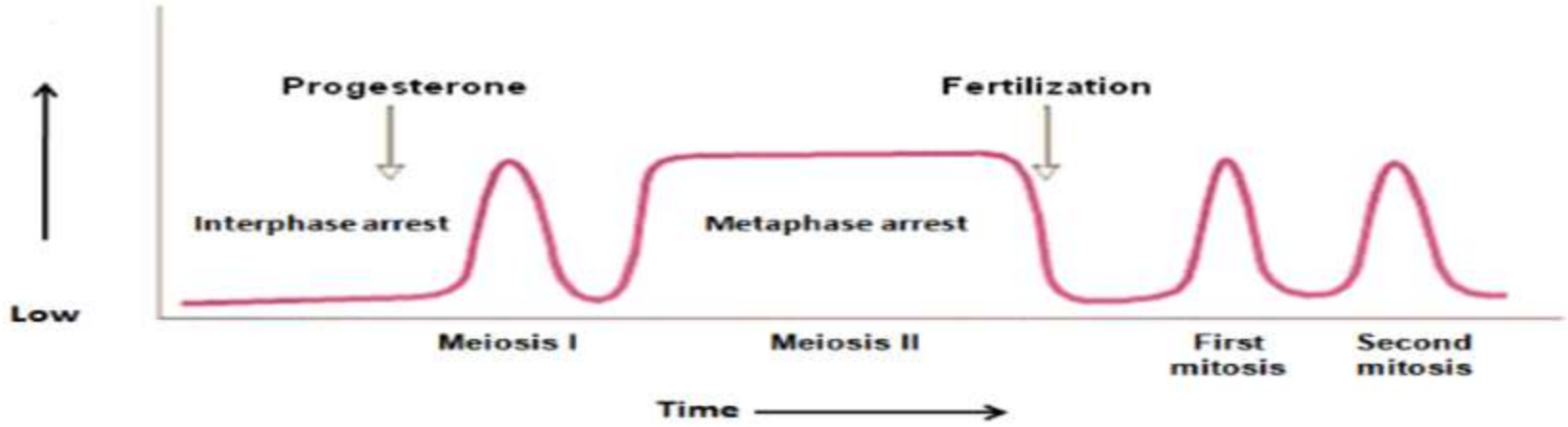
Progestorene



Transfer
cytoplasm



MPF activity: A diffusible factor that promotes the entry of cells into mitosis –**cyclin-cdk**



Maturation-promoting factor (abbreviated **MPF**, also called **mitosis-promoting factor** or **M-Phase-promoting factor**) is the cyclin-Cdk complex that was discovered first in frog eggs. It stimulates the mitotic and meiotic phases of the cell cycle. MPF promotes the entrance into mitosis (the M phase) from the G_2 phase by phosphorylating multiple proteins needed during mitosis. MPF is activated at the end of G_2 by a phosphatase, which removes an inhibitory phosphate group added earlier.
The MPF is also called **the M phase kinase** because of its ability to phosphorylate target proteins at a specific point in the cell cycle and thus control their ability to function.

MPF ⟹ Enter and exit from mitosis even when nucleus is removes
Thus cell cycle clock is operated in cytoplasm independent of nucleus

All required information for progression through cell cycle are stores in unfertilized egg.
specific mRNA must be produced at particular point in the cell cycle

Thus extract of Xenopus egg ⟹ all material for cell cycle enzymes, precurssor of DNA,RNA

⟹

Egg extract_ + sperm → Nucleus around sperm,

↓

DNA replicate

↓

Chromosome condensed

↓

Nuclear envelope breaks

↓

At this point all cyclin B degrades

↓

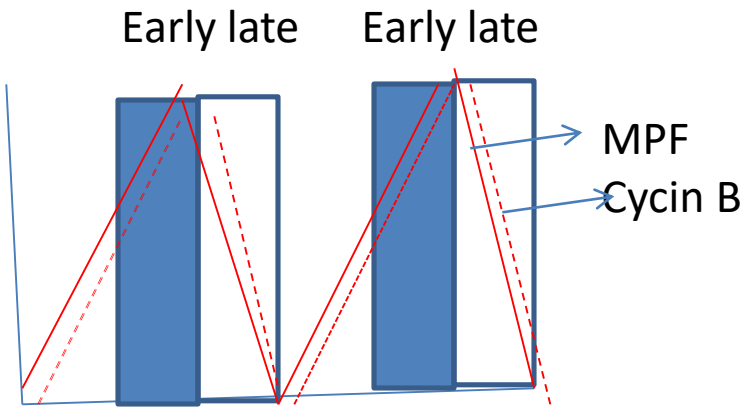
Then sperm chromosome decondensed

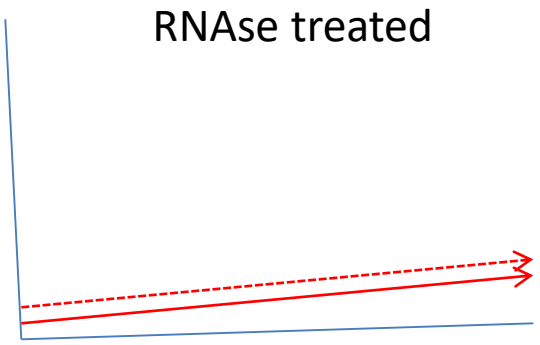
↓

Nuclear envelope reform

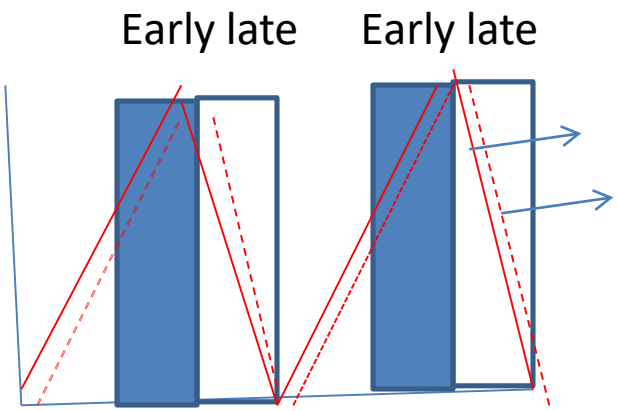
↓

After 20 min Cell cycle begins again

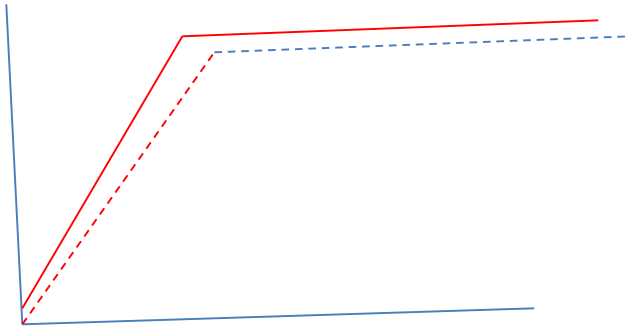




RNAse + Wt Cyclin B treated

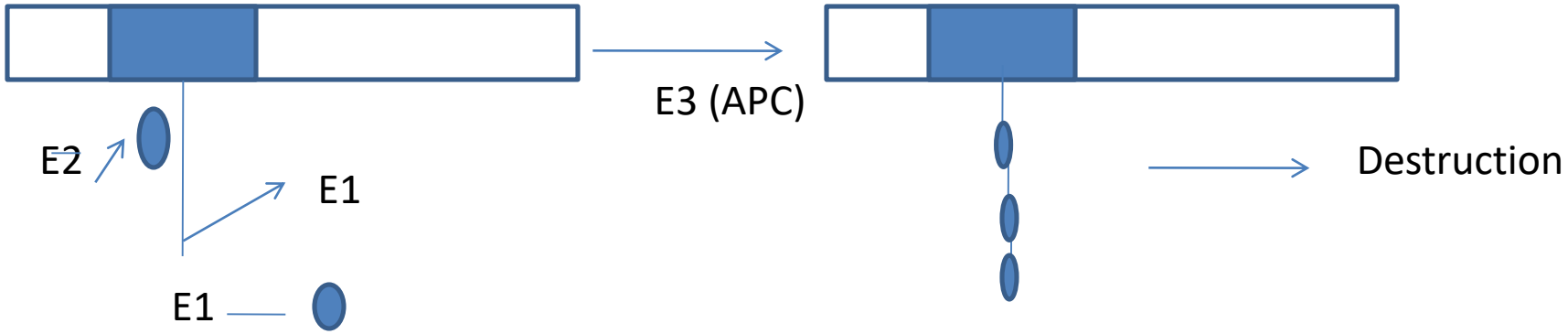


RNAse + non degradable Cyclin B treated



Thus ubiquitation mediated degradation of mitotic cyclin promotes exit from mitosis

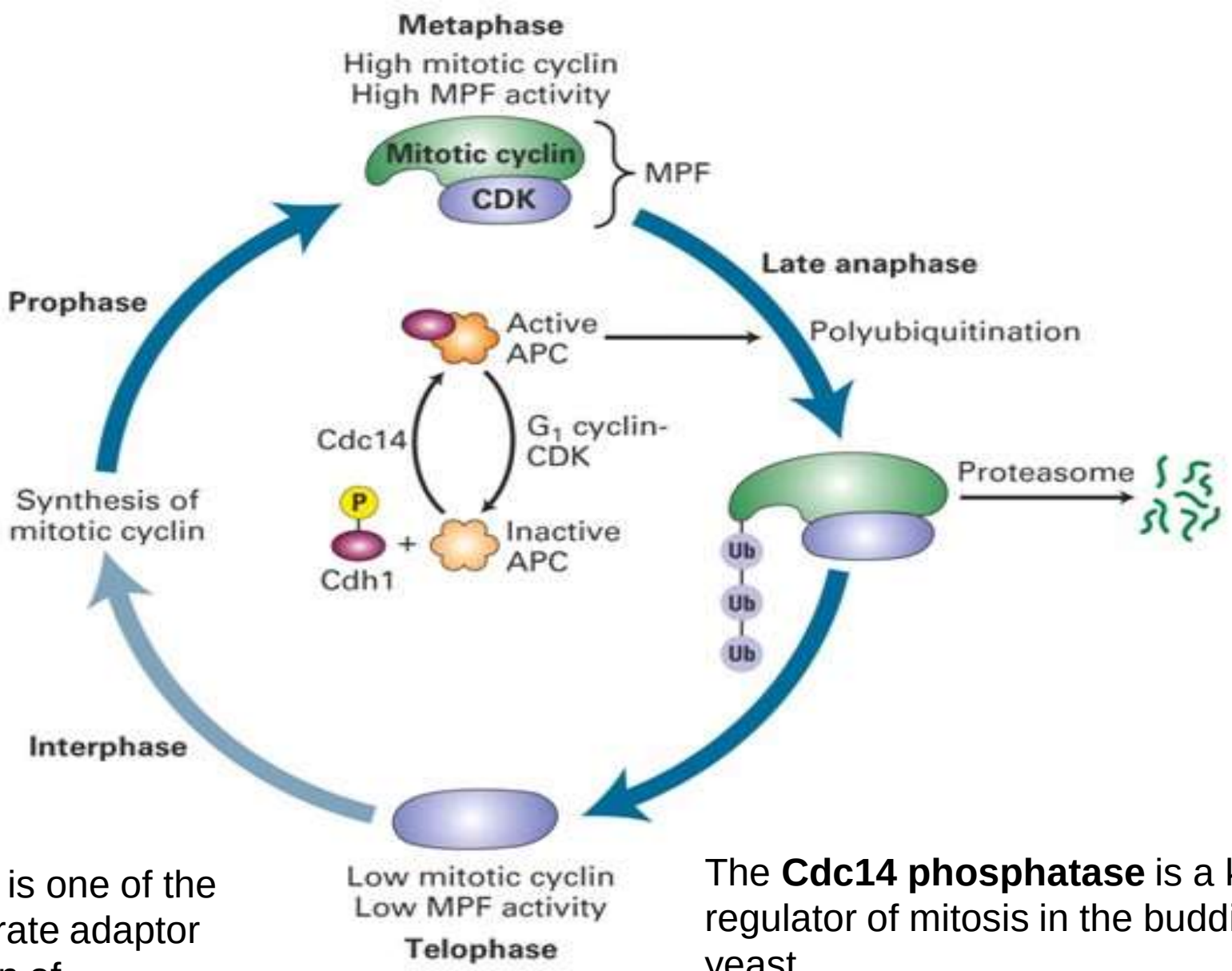
cDNA sequencing – N-terminal destruction box



Mutant protein that lacks destruction box is not degrades at all
E3 is APC : regulation of APC activity controls degradation of cyclin B
Low level of APC activity: Arrested in metaphase
High level of APC --- after metaphase is completed

Subunit of APC --- high activity when phosphorylated
removal of phosphate --- low activity

When MPF activity reaches maximum----it phosphorylates APC, which degrades cyclin B
APC is deactivated in late G1—Thus permitting rise in cyclin B which synthesize continuously during cell cycle

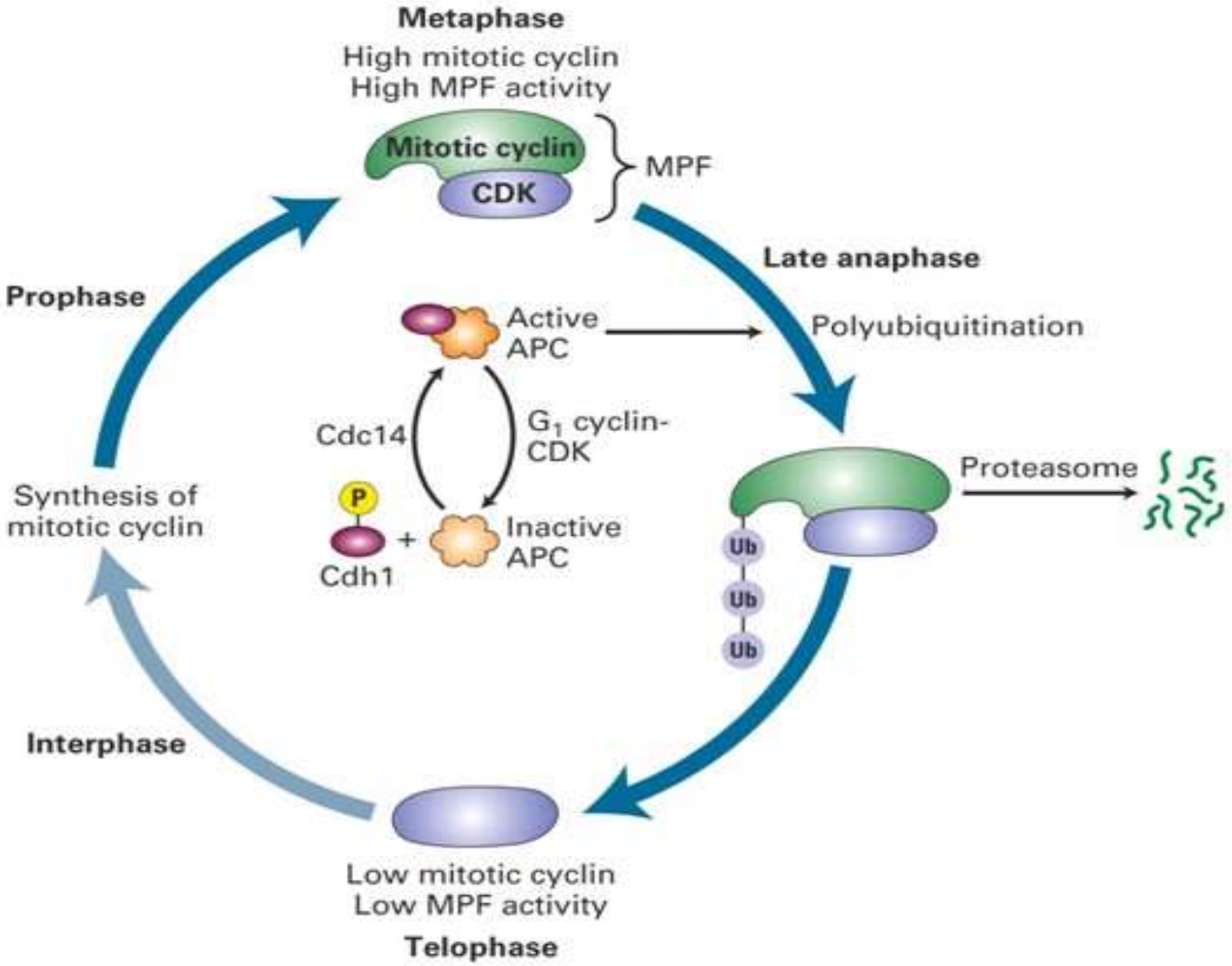


Cdh1 is one of the substrate adaptor protein of the anaphase-promoting complex (APC)

The **Cdc14 phosphatase** is a key regulator of mitosis in the budding yeast

Cell cycle -2

(Graphics are from internet)


Metaphase
High mitotic cyclin
High MPF activity
Mitotic cyclin
CDK
MPF
Late anaphase
Polyubiquitination
Proteasome
Ub
Ub
Ub
Active
APC
Cdc14
G1 cyclin-
CDK
P
+
Cdh1
Inactive
APC
Prophase
Synthesis of
mitotic cyclin
Interphase
Low mitotic cyclin
Low MPF activity
Telophase

The studies with ***Xenopus*** **egg extracts described clearly show that continuous synthesis of cyclin B followed by its periodic degradation at late anaphase is required for the rapid cycles of mitosis** observed in early animal embryos.

Identification of the **catalytic subunit of MPF and further insight into its regulation came from genetic analysis of the cell cycle in the fission yeast *S. pombe*.** This yeast grows as a rod-shaped cell that increases in length as it grows and then divides in the middle during mitosis to produce two daughter cells of equal size

➢Complementation and recombination analyses of *S. pombe* mutants have identified a number of different *cdc* and *wee* genes, designated with individual numbers.

➢Wild-type genes are indicated in italics with a superscript plus sign (e.g., $cdc2^{+}$);

➢ genes with a recessive mutation, in italics with a superscript minus sign (e.g., $cdc2^{-}$).

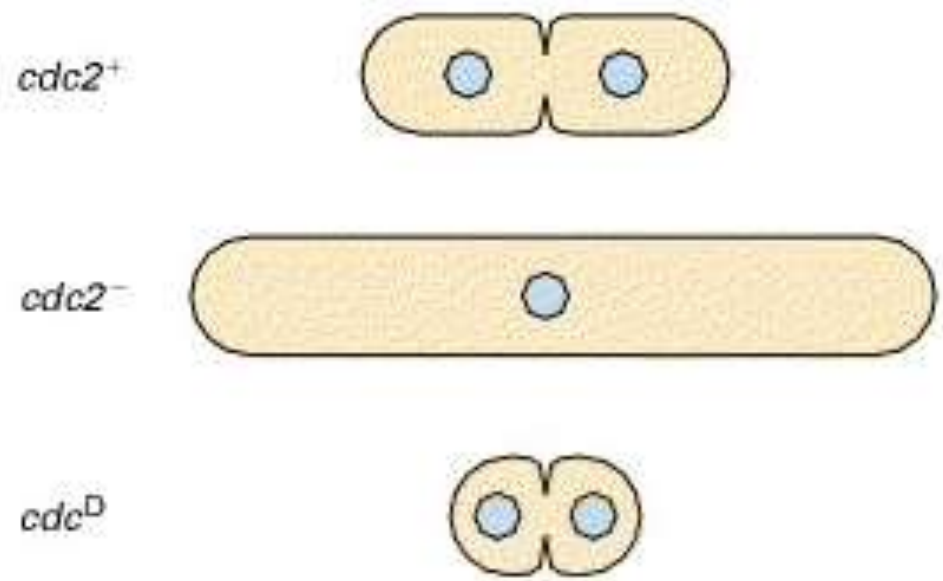
➢The protein encoded by a particular gene is designated by the gene symbol in Roman type with an initial capital letter (e.g., Cdc2).

➢Temperature-sensitive recessive mutations in several different *cdc* genes in *S. pombe* prevent cells from entering mitosis and thus they grow much longer than normal . Dominant mutations in one of these genes, designated *cdc2,* gives rise to the wee phenotype.

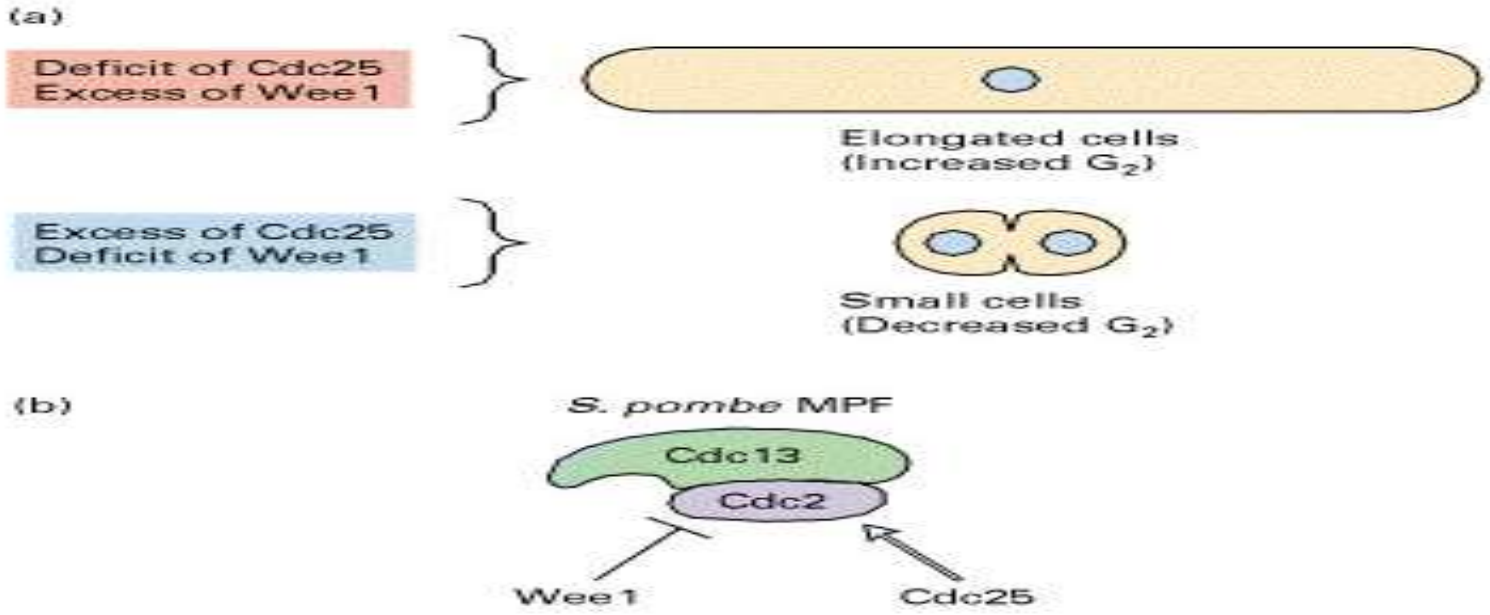
➢**Generally, recessive phenotypes result from the absence of wild-type protein function, whereas dominant phenotypes are due to increased protein function, either because of overproduction or lack of regulation**.

➢***Isolation of these mutants indicates that an absence of Cdc2 activity prevents entry into mitosis, while an excess of Cdc2 activity brings on mitosis earlier than normal.***

➢These findings **identified Cdc2 as a key regulator of entry into mitosis in *S. pombe*.** The wild-type $cdc2^{+}$ gene contained in a *S. pombe* plasmid library was identified and isolated by its ability to complement $cdc2^{-}$ mutants . Sequencing showed that *it encodes a 34-kDa protein with homology to eukaryotic protein kinases.*



Wild-type cell *(cdc2^{+})* is depicted just before cytokinesis with two normal-size daughter cells.
A recessive *cdc2*$^{-}$ mutant cannot enter mitosis at the nonpermissive temperature and appears as an elongated cell with a single nucleus, which contains duplicated chromosomes.
A dominant *cdc2*^{D} mutant enters mitosis prematurely before reaching normal size in G_2; thus, the two daughter cells resulting from cytokinesis are smaller than normal, that is, they have the wee phenotype.



(a) Cells that lack Cdc25 or Wee1 activity, as a result of recessive temperature- sensitive mutations in the corresponding genes, have the opposite phenotype. Likewise, cells with multiple copies of plasmids containing *cdc25*$^+$ or *wee1*$^+$, and which thus produce an excess of the encoded proteins, have opposite phenotypes.

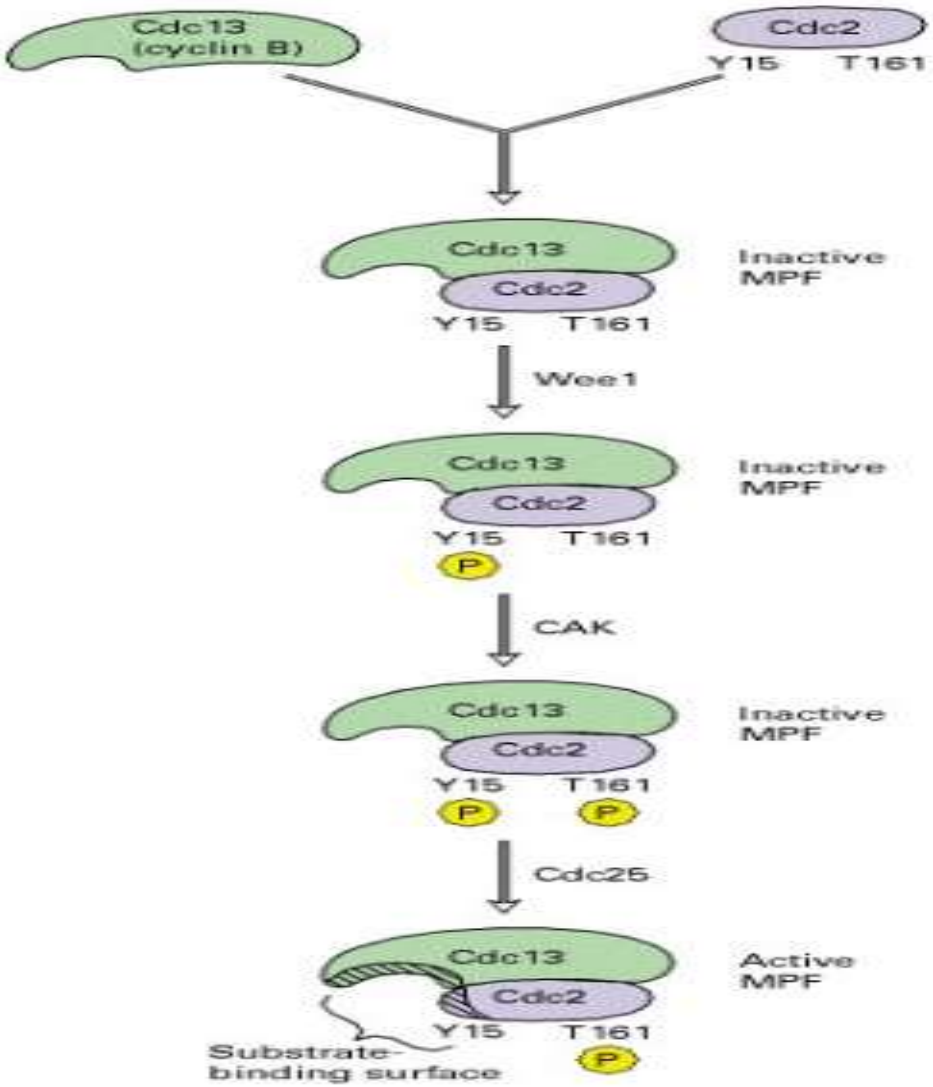
(b) These phenotypes imply that the Cdc2-Cdc13 complex is activated (→) by Cdc25 and inhibited by Wee1

- In *cdc25*$^{-}$ cells, the inhibitory activity is unopposed and MPF activity is inhibited, blocking entry into mitosis and resulting in elongated cells.

- When Cdc25 is produced at higher-than-normal levels, it offsets the inhibitory effect of Wee1, so MPF activity rises faster than in wild-type cells, causing premature entry into mitosis, which results in small (wee) cells.

- In *wee1*$^{-}$ mutants, the stimulatory effect of Cdc25 is unopposed, so MPF activity rises faster than normal, leading to premature entry into mitosis and small (wee) cells.

- Conversely, overproduction of Wee1 inhibits MPF activity more than normal, delaying entry into mitosis and producing elongated cells.



Wee1 and CAK
are protein kinases, and Cdc25 is **a protein phosphatase**. Once bound by Cdc13, the catalytic Cdc2 subunit can be phosphorylated at two regulatory sites, **tyrosine-15 (Y15) and threonine-161(T161**). Only when Cdc2 is monophosphorylated at T161 is MPF active. Cdc13 contributes to the specificity of substrate binding, probably by forming part of the substrate-binding surface (cross-hatch), which also includes the inhibitory Y15 residue.

Regulation of mitotic activity

Entry into mitosis : Regulated increase in MPF
:phosphorylation of specific proteins
:Chromosome condensation
Formation of spindle
breakdown of nuclear envelope

MPF activity is controled by

1. Regulation of the concentration of cyclin
2. Regulation of kinase activity

Other regulations like controlled activity of wee1, cdc25.
transcription of MPF components

Nuclear envelop

Disassembly of the **nuclear envelope**, which parallels a similar **breakdown** of the endoplasmic reticulum, involves changes in all three of its components:
The **nuclear** membranes are fragmented into vesicles, the **nuclear** pore complexes dissociate, and the **nuclear** lamina depolymerizes.

The **nuclear envelope** consists of two lipid bilayer membranes, an inner **nuclear membrane** & an outer **nuclear membrane**. The outer **nuclear membrane** is continuous with the endoplasmic reticulum **membrane**. The **nuclear envelope** has many **nuclear** pores that allow materials to move between the cytosol and the **nucleus**.

The **nuclear envelope** needs to be broken apart so that the chromosomes can be found, aligned in the middle of the cell, and then pulled apart.

➢The process of nuclear envelope breakdown is triggered by active maturation-promoting factor (MPF), which is thought to be a complex of cyclin B and cdc2/cdk1 kinase.

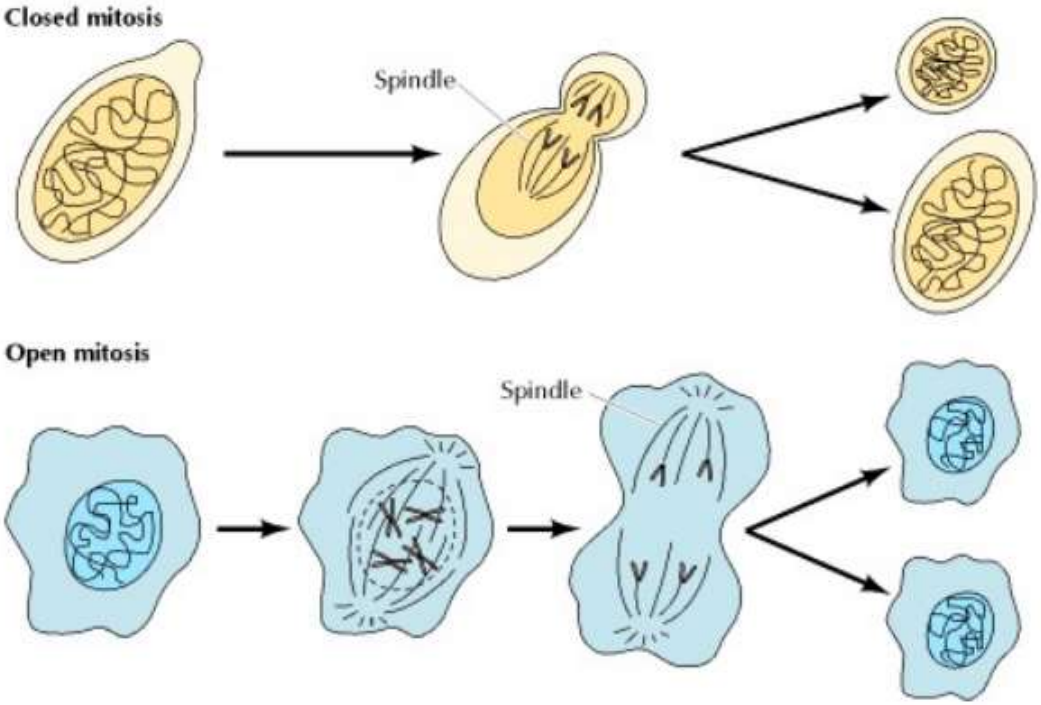
➢MPF moves into the nucleus where it directly phosphorylates or causes the phosphorylation of several targets .

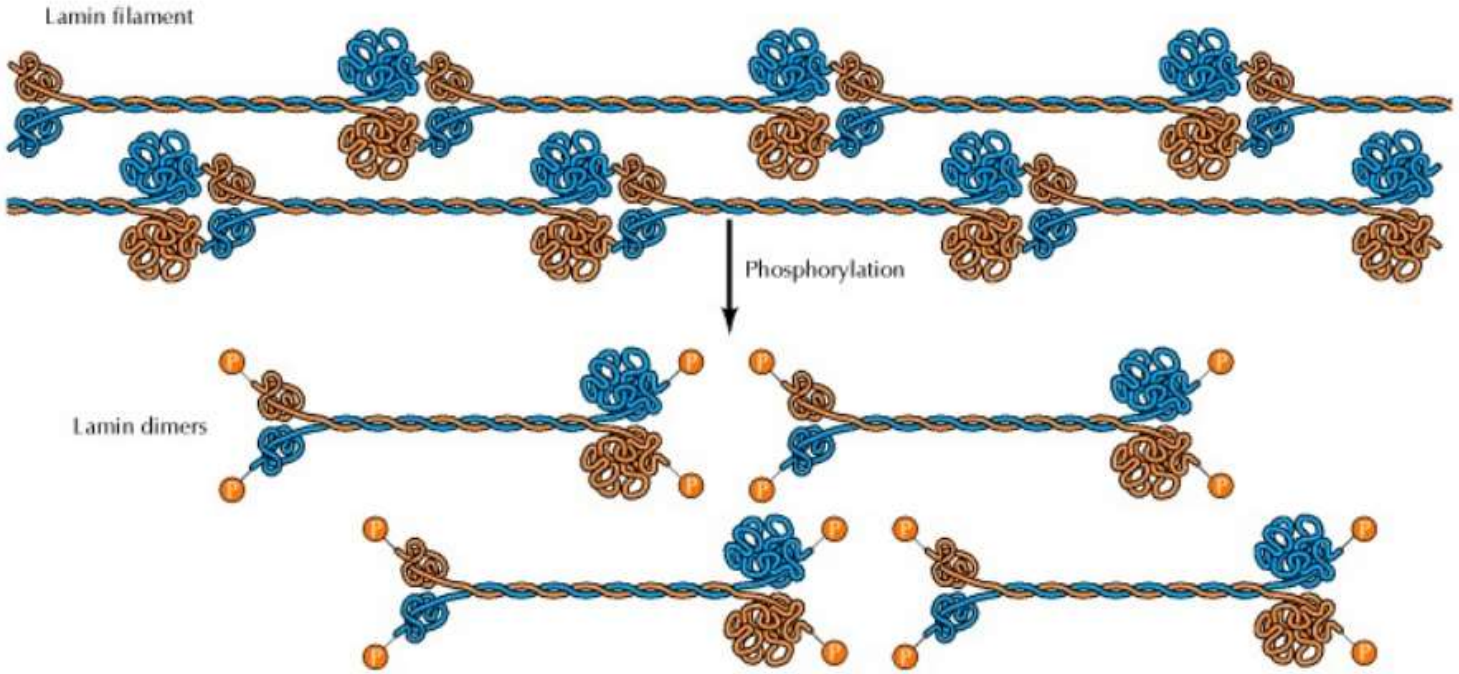
➢The lamins were the among the first and most clearly demonstrated target of MPF.

➢**Phosphorylation of polymerized lamins causes depolymerization in vitro**, and expression of mutant lamins lacking phosphorylation sites interferes with nuclear lamina disassembly in living cells .

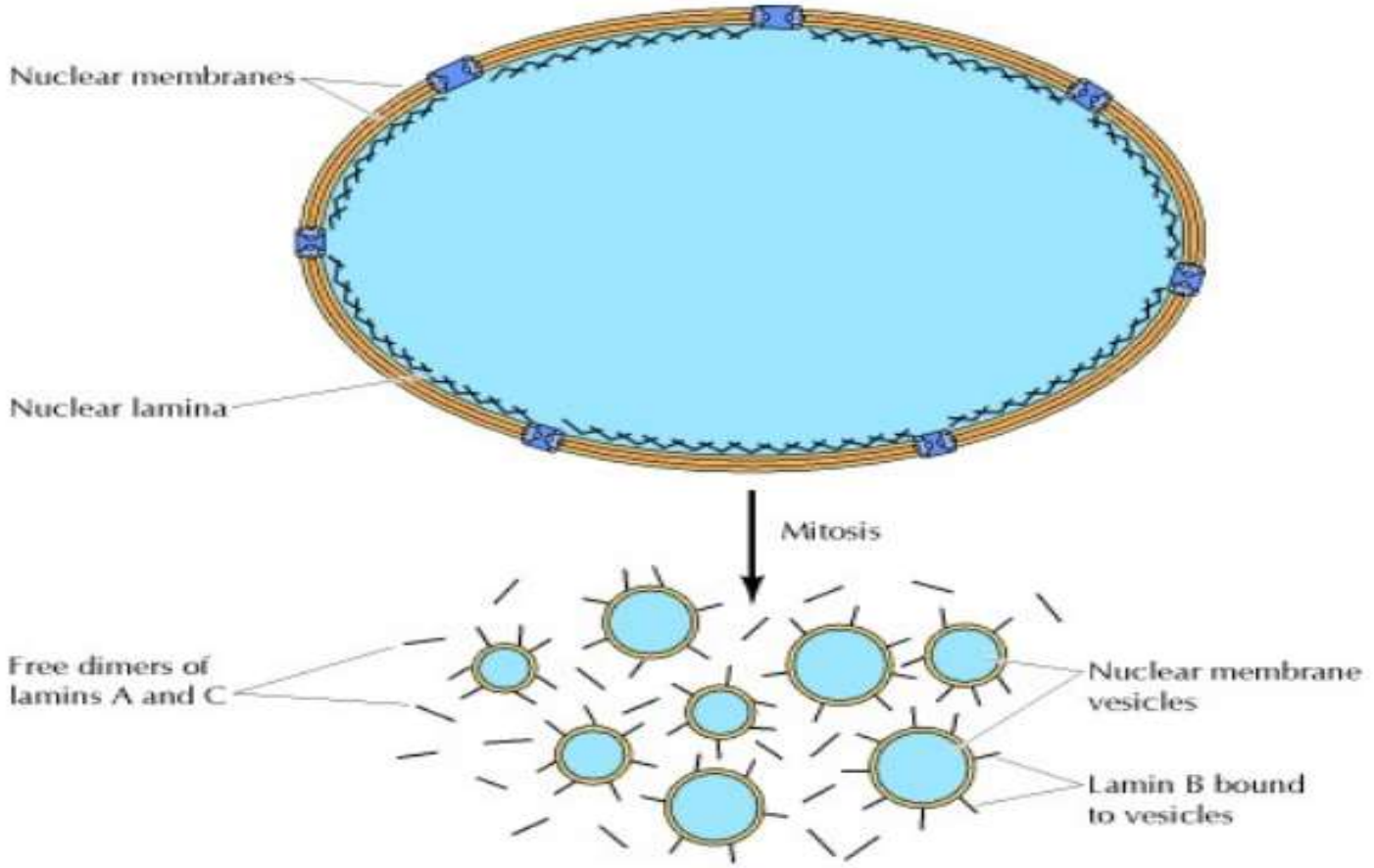
These experiments showed that phosphorylation is required for lamina disassembly and that lamina disassembly is required for normal mitosis.

However, this disassembly of the nucleus is not a universal feature of mitosis and does not occur in all cells. Some unicellular eukaryotes (e.g., yeasts) undergo so-called closed mitosis, in which the nuclear envelope remains intact (Figure). In closed mitosis, the daughter chromosomes migrate to opposite poles of the nucleus, which then divides in two. The cells of higher eukaryotes, however, usually undergo open mitosis, which is characterized by breakdown of the nuclear envelope.





The nuclear lamina consists of a meshwork of lamin filaments.
At mitosis, Cdc2 and other protein kinases phosphorylate the lamins, causing the filaments to dissociate into free lamin dimers.

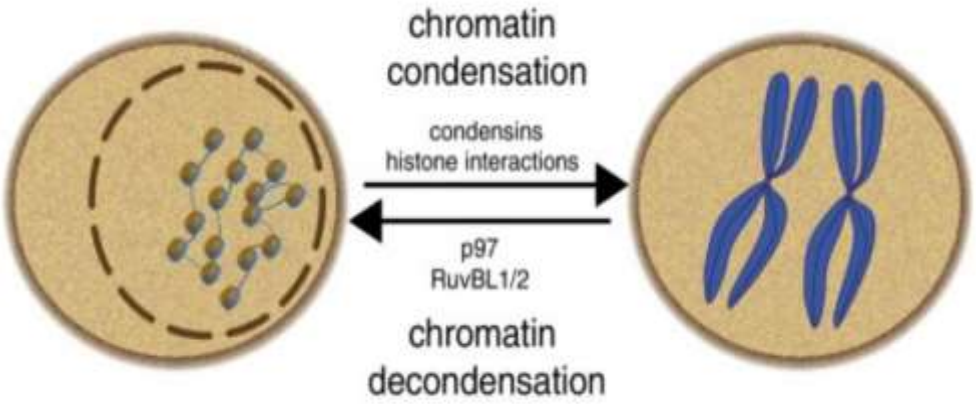


Breakdown of the nuclear membrane. As the nuclear lamina dissociates, the nuclear membrane fragments into vesicles. The B-type lamins remain bound to these vesicles (**through isoprenyl group)** , while lamins A and C are released as free dimers

➢The other major change in nuclear structure during mitosis is chromosome condensation. The interphase chromatin, which is already packaged into nucleosomes, condenses approximately a thousand fold further to form the compact chromosomes seen in mitotic cells .

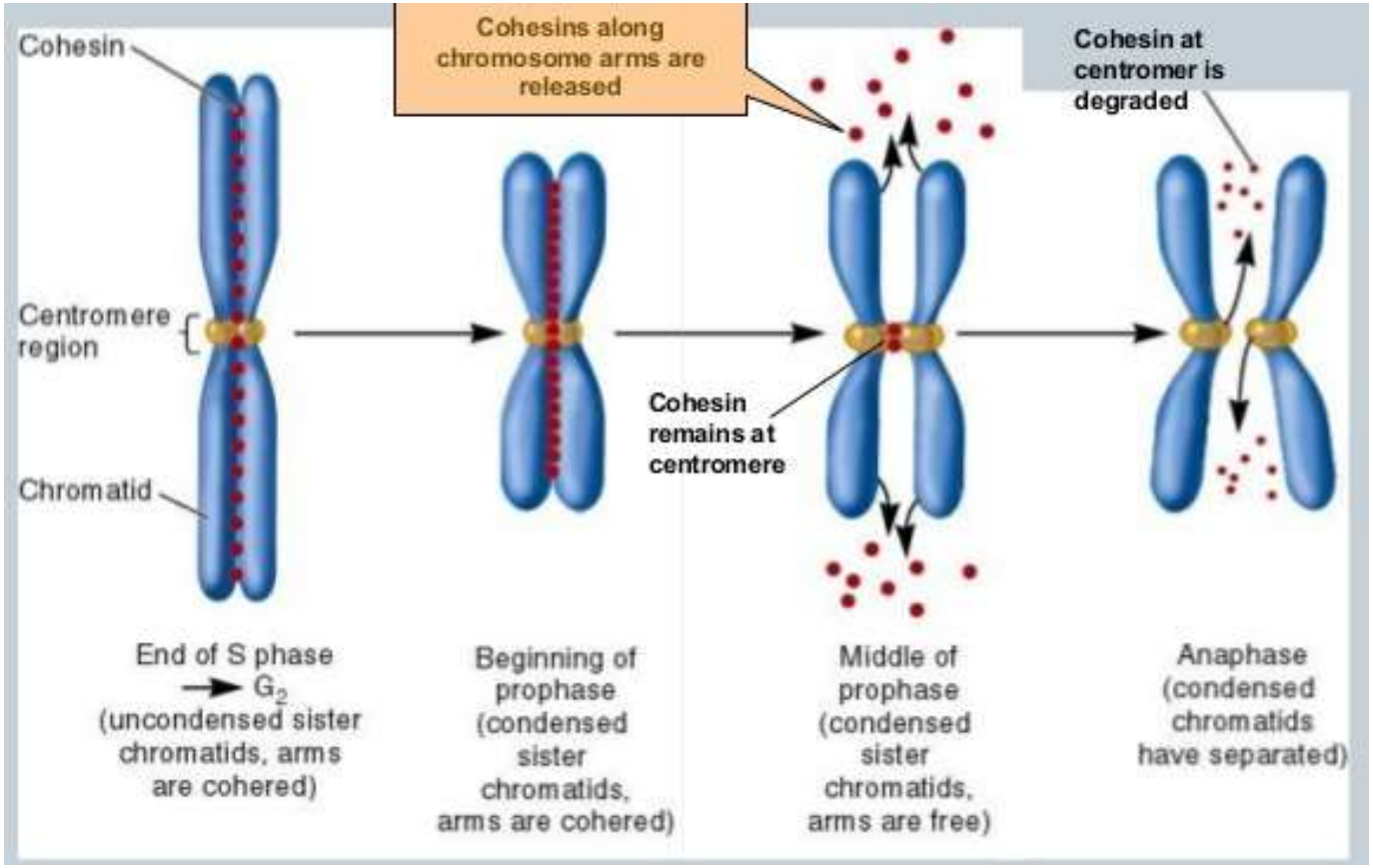
➢This condensation is needed to allow the chromosomes to move along the mitotic spindle without becoming tangled or broken during their distribution to daughter cells.

➢DNA in this highly condensed state can no longer be transcribed, so all RNA synthesis stops during mitosis. As the chromosomes condense and transcription ceases, the nucleolus also disappears.



During interphase (1), chromatin is in its least condensed state and appears loosely distributed throughout the nucleus.
Chromatin **condensation** begins during prophase (2) and **chromosomes** become visible. **Chromosomes** remain condensed throughout the various stages of mitosis .

Chromosome condensation, is a process by which long strands of DNA are wound tightly together with proteins, to make compact structures that we see as the clearly defined chromosomes in a dividing cell. This condensation process is carried out with the help of protein complexes called **condensins**. **Condensins are inactive until they are phosphorylated by MPF. Once they are phosphorylated, they begin the process of condensation.**



Cohesin glues replicated sister chromatids together until they split at anaphase, where as **condensin** reorganizes chromosomes into their highly compact mitotic structure.

*In experiments with purified condensin and DNA, phosphorylated condensin binds to DNA and winds it into **supercoils** in a reaction requiring the hydrolysis of ATP. These results have lead to the model that individual condensin complexes, activated by MPF or another protein kinase regulated by MPF, bind to DNA at intervals along the chromosome scaffold. Self-association of the bound complexes via their coiled-coil domains and supercoiling of the DNA segments between them is proposed to cause chromosome condensation*

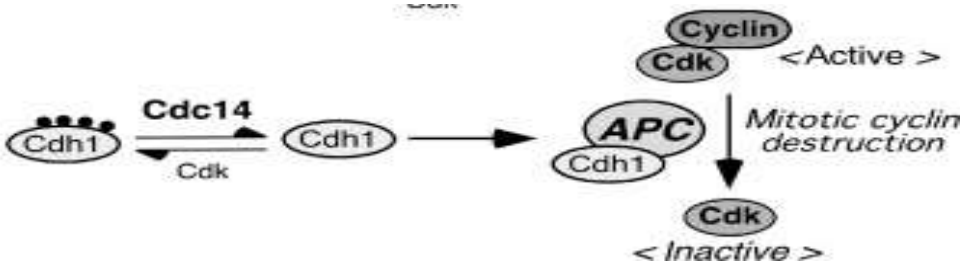
SMC complexes represent a large family of ATPases that participate in many aspects of higher-order chromosome organization and dynamics. SMC stands for **Structural Maintenance of Chromosomes**.

Eukaryotes have at least six SMC proteins in individual organisms, and they form three distinct heterodimers with specialized functions:

- A pair of SMC1 and SMC3 constitutes the core subunits of the **cohesin** complexes involved in **sister chromatid cohesion**.
- Likewise, a pair of SMC2 and SMC4 acts as the core of the **condensin** complexes implicated in **chromosome condensation**.
- A dimer composed of SMC5 and SMC6 functions as part of a yet-to-be-named complex implicated in DNA repair and checkpoint responses

APC-Dependent Unlinking of Sister Chromatids Initiates Anaphase

In the late anaphase and telophase stages of mitosis, APC-mediated polyubiquitination of cyclin B targets it for destruction **. Additional experiments with RNase-treated *Xenopus* egg extracts provided evidence that polyubiquitination and subsequent degradation of noncyclin proteins also is required to initiate anaphase.** In these studies, the mitotic spindle, which is formed from tubulin-containing microtubules, was visualized by including fluorescent-labeled tubulin in the reaction mixtures. When RNase-treated egg extracts and sperm chromatin were incubated in the presence of mRNA encoding wild-type cyclin B, the mitotic spindle apparatus and condensed sperm chromosomes aligned between the spindle poles were visible, similar to their appearance during metaphase in intact cells. After 15 minutes of incubation, the chromosomes were seen to move toward the spindle poles, just as they do during anaphase in intact cells. Cyclin B degradation and the resulting precipitous decrease in MPF activity began after this point, and over the next half hour the spindle depolymerized and the chromosomes decondensed



APC first target non cyclin protein

RNase-treated egg extracts and sperm chromatin were incubated in the presence of mRNA encoding wild-type cyclin B

The mitotic spindle apparatus and condensed sperm chromosomes aligned between the spindle poles were visible, similar to their appearance during metaphase in intact cells.

After 15 minutes of incubation, the chromosomes were seen to move toward the spindle poles, just as they do during anaphase in intact cells

Cyclin B degradation and the resulting precipitous decrease in MPF activity began after this point

RNase-treated egg extracts and sperm chromatin were incubated in the presence non degradable cyclin B

Ch. Segregation is there but decondensation never happen

RNase-treated egg extracts and sperm chromatin were incubated in the presence destruction box

No segregation

Cohesin function is regulated by the **anaphase inhibitor***,* a protein that is a target of APC-directed polyubiquitination. In yeast, anaphase inhibitor functions together with another protein to stimulate the proper association of cohesin with daughter chromosomes

As cells enter anaphase, anaphase inhibitor is polyubiquitinated by the APC and degraded by proteasomes . As a consequence, cohesin function is inactivated, allowing the poleward force exerted on kinetochores to move sister chromatids toward opposite spindle poles

After APC is activated, it initially acts on certain **target proteins such as the anaphase inhibitor**, but it does not act on cyclin B until late in anaphase . This is necessary in order to maintain MPF activity until late anaphase, keeping chromosomes in their condensed state until they have segregated to opposite spindle poles. Recent genetic studies in budding yeast indicate that this stage-dependent targeting of APC activity is due to the regulated activity of two APC-associated proteins.

CDC20 to bind the APC/C, specific APC/C subunits **must be phosphorylated by Cdk1** (among other Cdks). Therefore, when cdk activity is high in mitosis, and the cell must prepare to enter anaphase and exit mitosis, the APC/C^{Cdc20} complex is activated

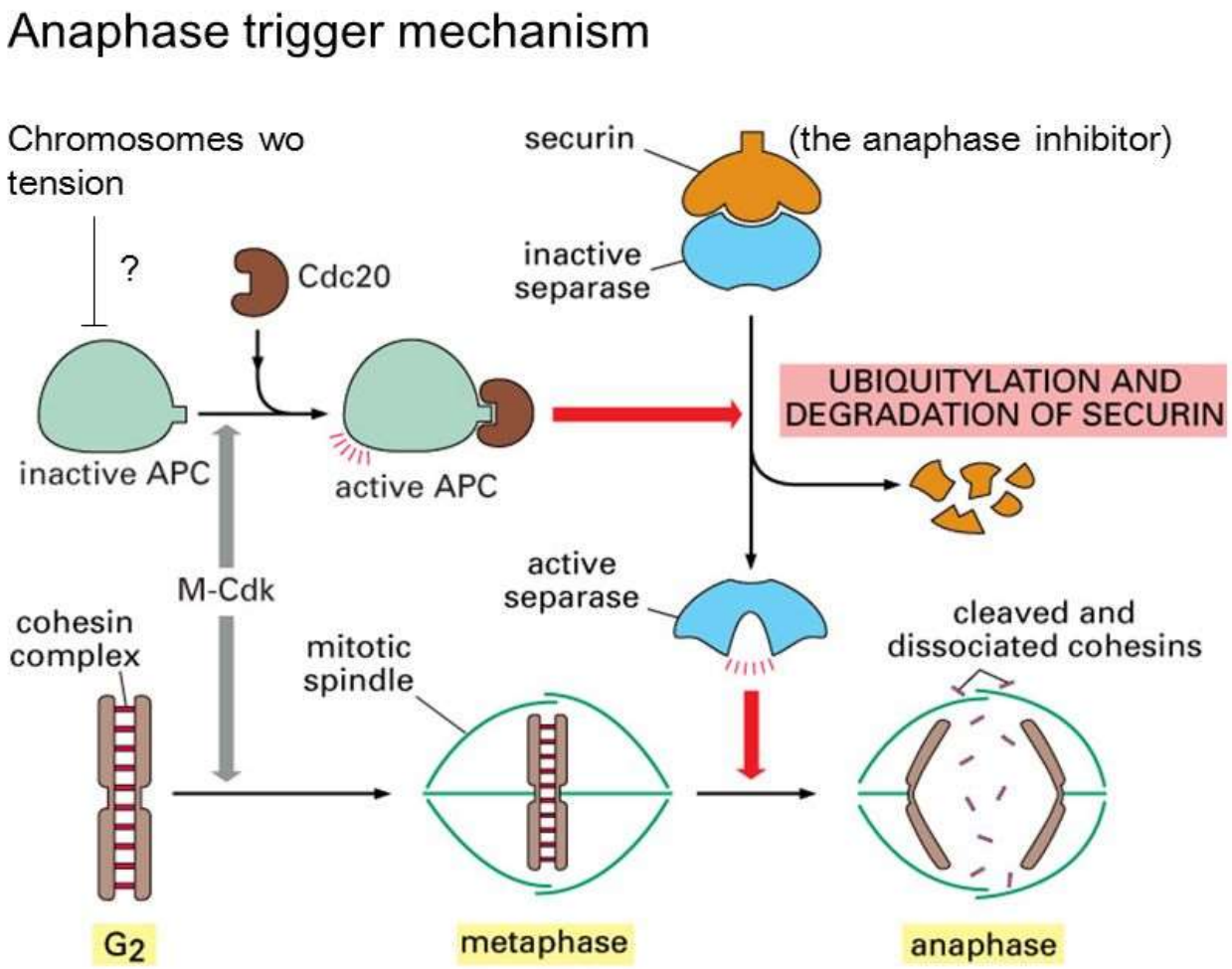
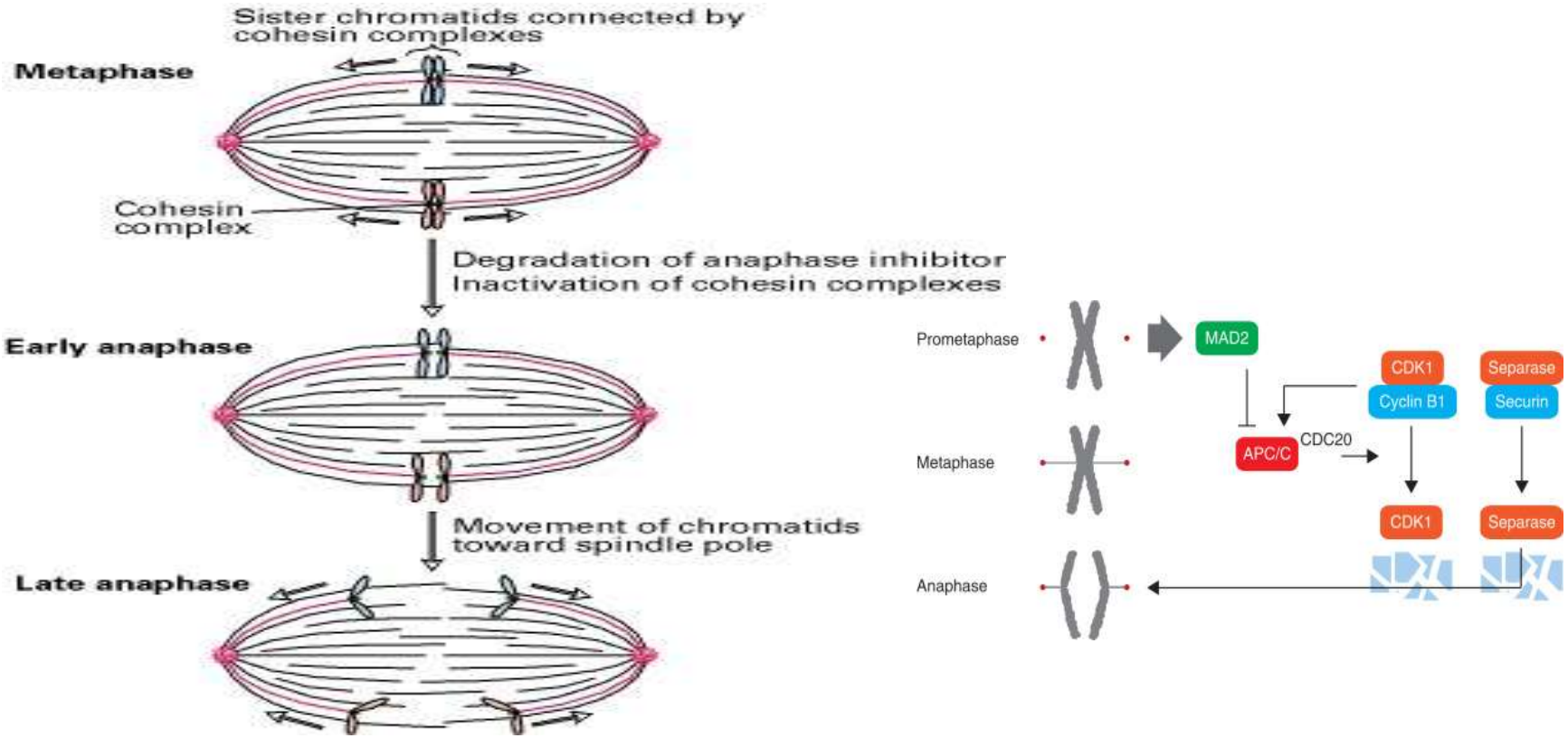


Figure 17–26. Molecular Biology of the Cell, 4th Edition.

Model for induction of anaphase by regulation of cohesin complexes

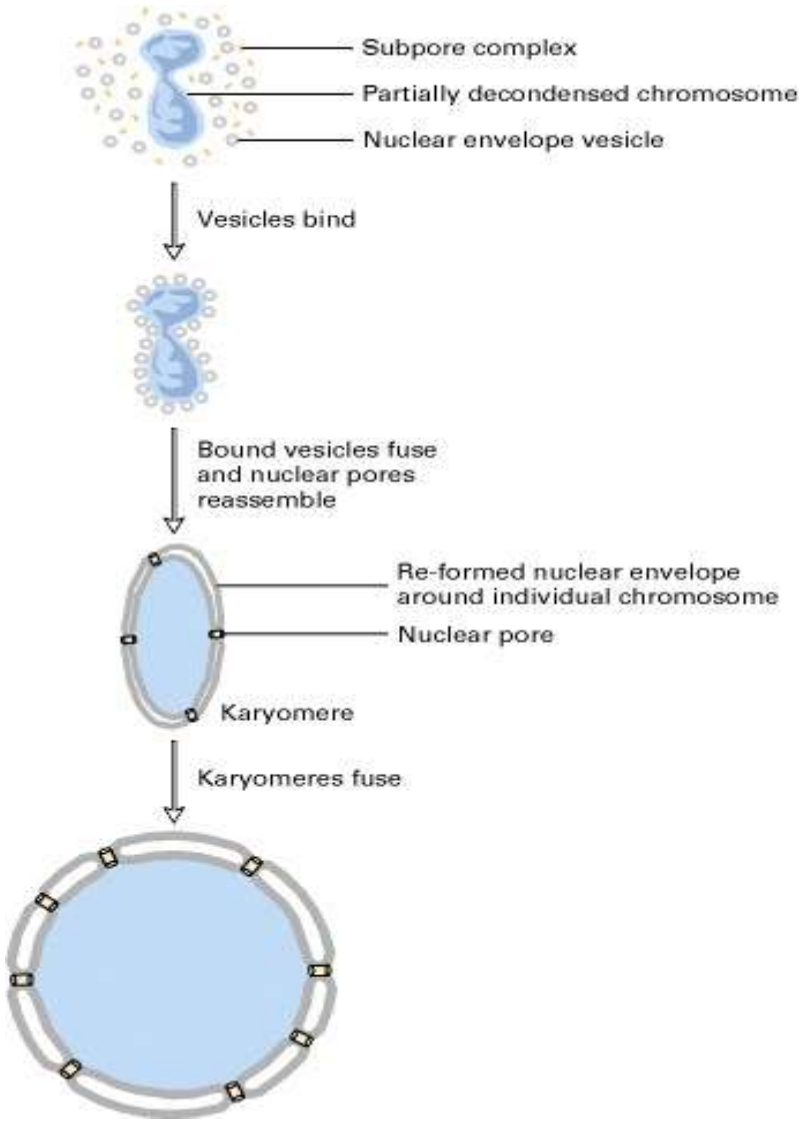


Arrows indicate direction of the forces acting on the kinetochores. Cohesin complexes are shown connecting centromeres, but they also occur along the arms of sister chromatids. Cohesin function is regulated by anaphase inhibitor, a protein that is polyubiquitinated by the APC during early anaphase. The subsequent degradation of anaphase inhibitor by proteasomes results in the inactivation of cohesins, permitting the polewise movement of the chromatids.

Phosphorylation of microtubule-associated proteins by MPF probably is required for the dramatic changes in microtubule dynamics that result in the formation of the mitotic spindle and asters . In addition, all vesicular traffic in the cell ceases during mitosis, and the endoplasmic reticulum and Golgi complex break down into small vesicles as the nuclear membrane does. Phosphorylation of proteins associated with these membranous organelles, by MPF or other protein kinases activated by MPF-catalyzed phosphorylation, likely is responsible for these mitotic events as well.

Reassembly of the Nuclear Envelope and Cytokinesis

Removal of these phosphates coincides with lamin repolymerization and re-formation of the nuclear lamina associated with the daughter-cell nuclei during telophase. Studies with *Xenopus* egg extracts and analyses of various organisms with temperature-sensitive mutations in protein phosphatases indicate that specific protein phosphatases indeed are required for reassembly of the nuclear lamina and the nuclear envelope. When MPF is inactivated by the degradation of cyclin B late in anaphase, the action of these phosphatases, which remove the lamin regulatory phosphates, is unopposed; consequently, the lamins are rapidly dephosphorylated.



Nuclear envelope vesicles, generated by the breakdown of the envelope during prophase, associate with decondensing chromosomes and then fuse. Subpore complexes reassemble into nuclear pores, forming individual mininuclei called karyomeres. The enclosed chromosome further decondenses, and subsequent fusion of the nuclear envelopes of all the karyomeres at each spindle pole forms a single nucleus containing a full set of chromosomes.

Reassembly of the nuclear lamina in the daughter nuclei probably is initiated on lamin B molecules, which remain associated with the nuclear-envelope vesicles throughout mitosis via the isoprenyl anchors covalently linked to the C-terminal region of lamin B.

- Although most substrates of MPF remain to be identified, nuclear lamins, subunits of condensin, and myosin light chain are three identified substrates.

- MPF-catalyzed phosphorylation of specific lamin serines early in mitosis causes depolymerization of lamin filaments, leading to breakdown of the nuclear envelope .
- In addition, phosphorylation of condensin complexes by MPF or a kinase regulated by MPF is thought to promote chromosome condensation.

- When MPF activity falls in late anaphase and telophase, protein phosphatases remove the regulatory phosphates from lamins A, B, and C, permitting reassembly of the nuclear lamina in the two daughter cell nuclei.

- MPF-catalyzed phosphorylation of the myosin light chain prevents cytokinesis. Since MPF activity does not fall until the completion of anaphase, cytokinesis is delayed until sister chromatids have been segregated to opposite poles of the spindle apparatus.

The APC-directed degradation of the anaphase inhibitor causes inactivation of the cohesin complexes that connect sister chromatids. This unlinking of sister chromatids heralds the onset of anaphase and allows sister chromatids to move apart . Later, the same APC targets cyclin B for destruction, causing the decrease in MPF activity that marks the onset of telophase.

Cell cycle 3

(Graphics are from internet)

S phase promoting complex

Three cyclins in S.cerevasae: Cln1, Cln2 and Cln 3; KO of all three is lethal

Deletion of Cln3 ⟹ extend G1 for several hours

Over production of any Cln decrease G starts prematurally

In the absence of any Cln ⟹ Cell arrested in G1

These Cln are G1 cyclin

The complex Cdc28 and G1 cyclin ⟹ SPC

Cln3 –expressed constant level through out cell cycle

Cln1 and Cln2 Express during 2nd half of G1 when they increase concentration

Rapidly ⇨ peaking at the onset of S phase and diminishing thereafter until they are eliminated by the time of mitosis

Cdc28-cln3 ⇨ phosp and activate transcription factor SBF and MBF------

these induce Cln1 and Cln2, DNApol, RPA,ligase, enzymes for DNA synthesis

Cdc-28-Cln1 and Cln2 ---- phosphorylate APC and inactivate

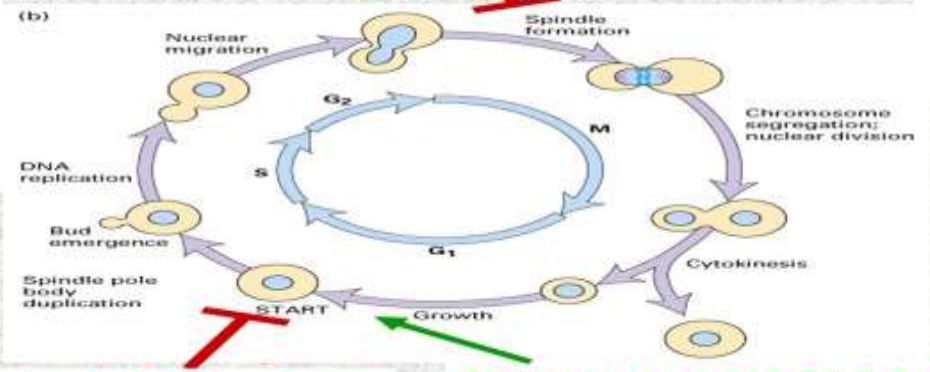
Yeast Cyclin Genes: CLNs and CLBs

Figure 13-22

Quadruple clb1,2,3,4 Mutant

G2 Cyclins
CLB1
CLB2
CLB3
CLB4

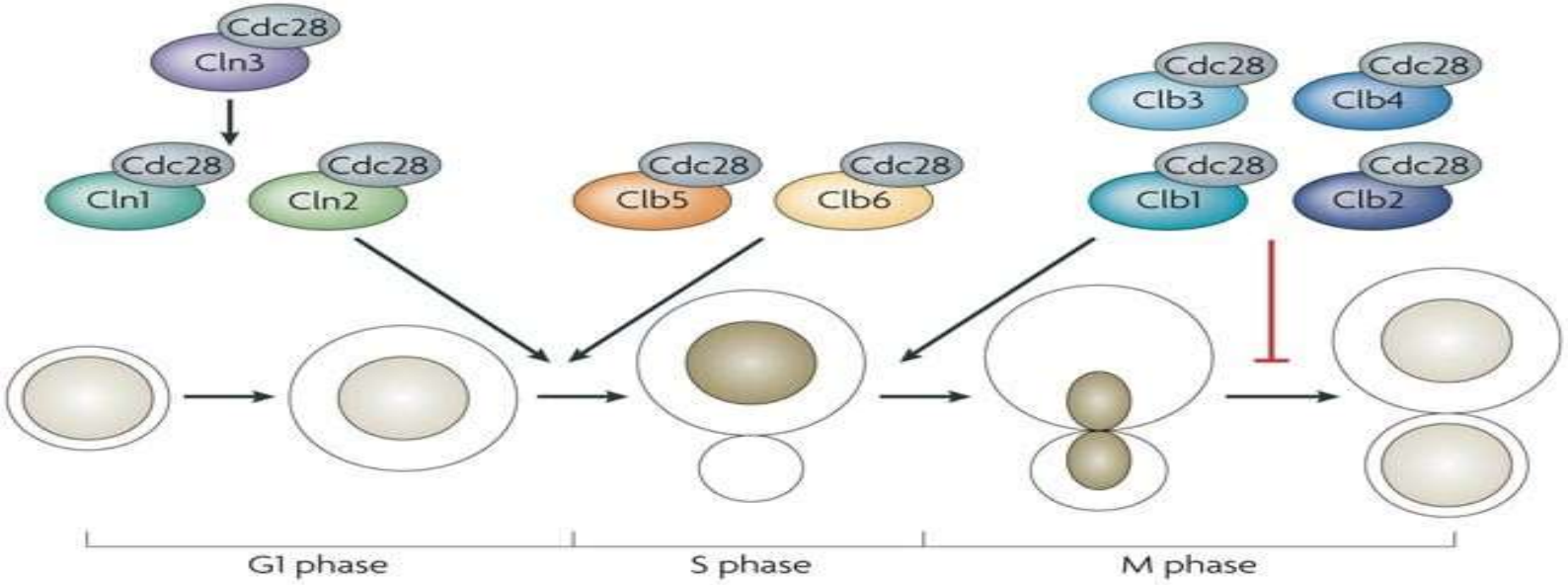
S phase Cyclins
CLB5, CLB6
NO PHENOTYPE
- double mutants
-gain of function
-????????????



G1 Cyclins:
CLN1
CLN2
CLN3

Triple cln1,2,3 mutant

Dominant CLN Mutations



Nature Reviews | Molecular Cell Biology

One of the important substrates of the Cdc28-Cln1 and Cdc28-Cln2 complexes is the yeast **anaphase-promoting-complex**, APC. Recall that the APC is activated during anaphase of the previous mitosis, probably by MPF phosphorylation. Activated APC then directs polyubiquitination and hence proteasome degradation of the anaphase inhibitor, yeast B-type cyclins, and components of the spindle apparatus . Phosphorylation of APC by Cdc28 - Cln1 or Cdc28 - Cln2 inactivates the complex in late G_1.

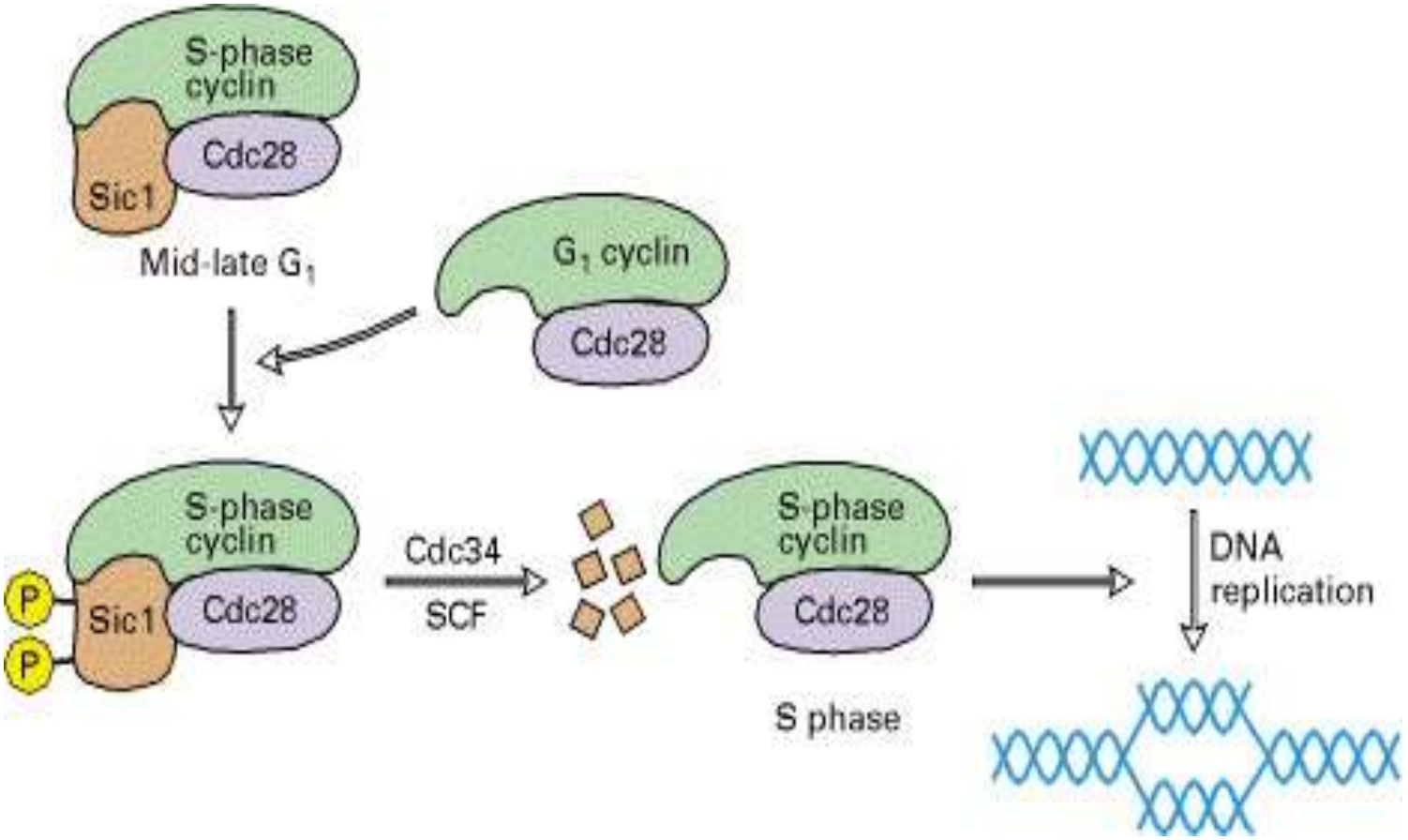
Two B-type cyclin genes, called *CLB5* and *CLB6*, also are regulated by MBF and transcribed beginning in late G_1. The corresponding proteins, Clb5 and Clb6, accumulate because of the inactivation of the APC, which would otherwise cause the degradation of these B-type cyclins.

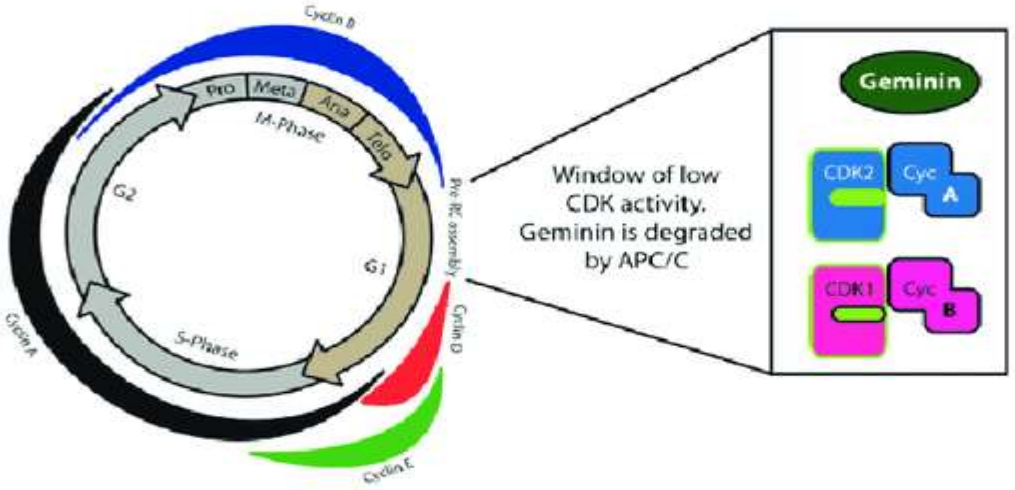
➢As Cdc28-Clb5 and Cdc28-Clb6 heterodimers accumulate in late G_1, they are immediately inactivated by binding of Sic1, which is expressed late in mitosis and early G_1.

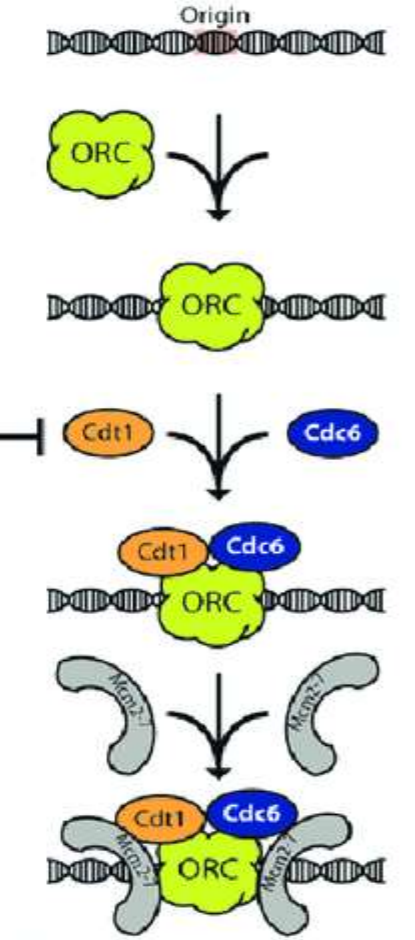
➢ Sic1 functions as an *S-phase inhibitor,* specifically **inhibiting Cdc28 – B-type cyclin complexes but having no effect on the Cdc28 – G_1 cyclin (Cln) complexes**.

➢Cells enter the S phase (i.e., DNA replication initiates at origins) when the Sic1 inhibitor is precipitously degraded following its polyubiquitination by a distinct **E2 ubiquitin-conjugating enzyme (Cdc34) associated with an E3 ubiquitin ligase called SCF.**

➢Once Sic1 is degraded, the Cdc28-Clb5 and Cdc28-Clb6 kinases induce DNA replication from origins by phosphorylating as-yet unidentified substrates. This mechanism for activating these Cdk-cyclin complexes — that is, inhibiting them as they are synthesized and then precipitously degrading the inhibitor — permits the sudden activation of large numbers of complexes, as opposed to the gradual increase in kinase activity that would result if no inhibitor were present during synthesis of the S-phase cyclins.


S-phase cyclin
Cdc28
Sic1
Mid-late G1
G1 cyclin
Cdc28
S-phase cyclin
P
P
Sic1
Cdc28
Cdc34
SCF
S-phase cyclin
Cdc28
S phase
DNA replication





Pre-replication complex

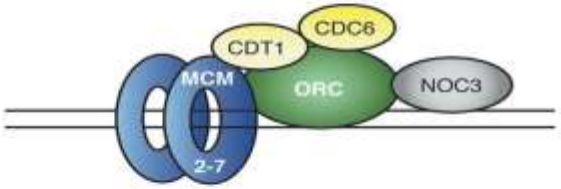
Origin assembly

Mitotic Cell Cycle

Initiation complexes assemble in late M and early G1

licensing

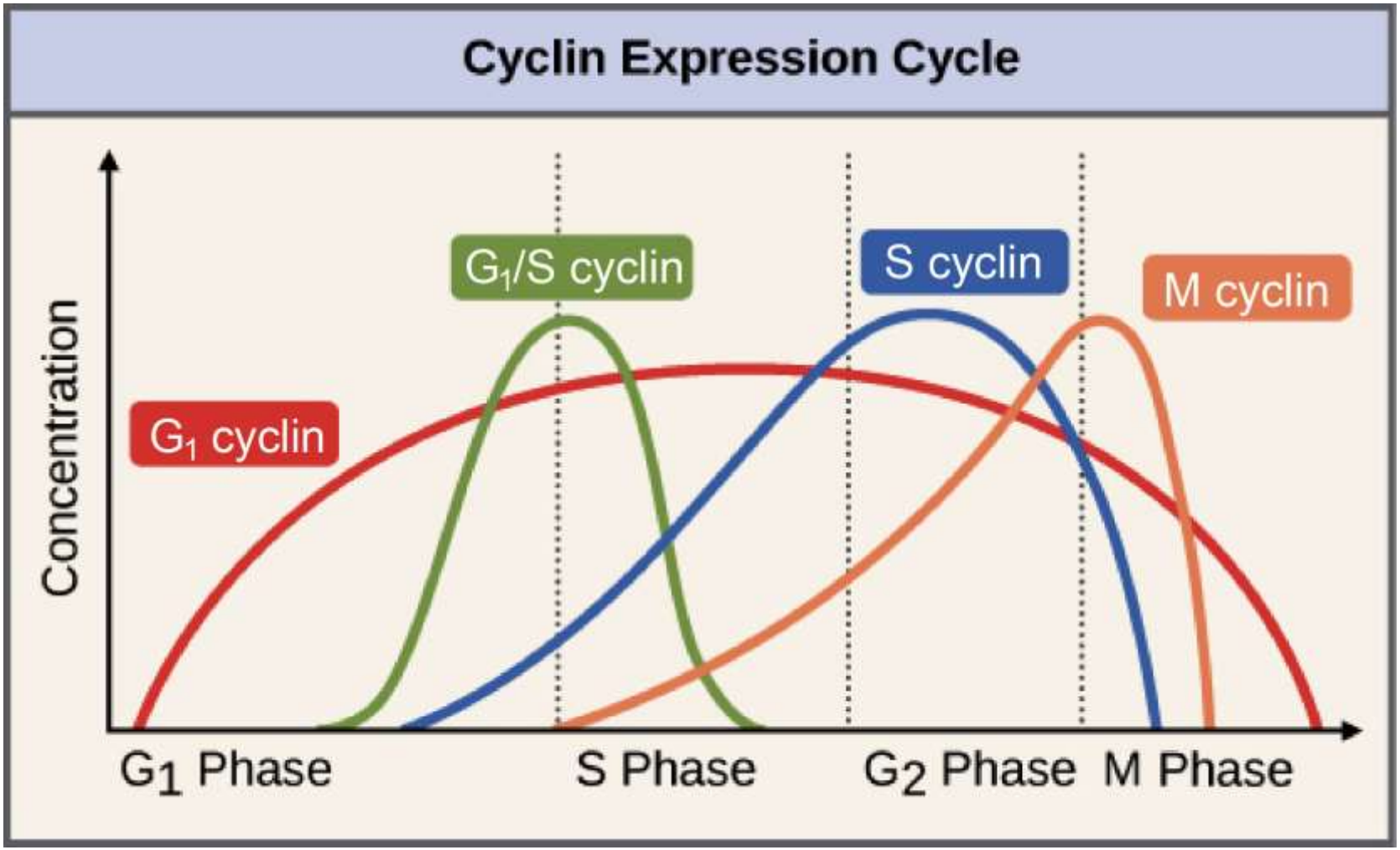
Pre-replication complex



ORC - origin recognition complex
MCM - replicative DNA helicase
CDT1/CDC6 - MCM loader

The specific cyclin subtypes along with their corresponding CDK (in brackets) are:

Species	G1	G1/S	S	M
S. cerevisiae	Cln3 (Cdk1)	Cln 1,2 (Cdk1)	Clb 5,6 (Cdk1)	Clb 1,2,3,4 (Cdk 1)
S. pombe	Puc1? (Cdc2)	Puc1, Cig1? (Cdc2)	Cig2, Cig1? (Cdc2)	Cdc13 (Cdc2)
D. melanogaster	cyclin D (Cdk4)	cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B, B3 (Cdk1)
X. laevis	either not known or not present	cyclin E (Cdk2)	cyclin E, A (Cdk2,1)	cyclin A, B, B3 (Cdk1)
H. sapiens	cyclin D 1,2,3 (Cdk4, Cdk6)	cyclin E (Cdk2)	cyclin A (Cdk2, Cdk1)	cyclin B (Cdk1)



Cdk levels remain relatively constant across the cell cycle, but Cdk activity and target proteins change as levels of the various cyclins rise and fall. In addition to needing a cyclin partner, Cdks must also be phosphorylated on a particular site in order to be active , and may also be negatively regulated by phosphorylation of other sites

➢We can now see that regulated proteolysis directed by two ubiquitinating complexes, Cdc34-SCF and APC, controls three major transitions in the cell cycle:

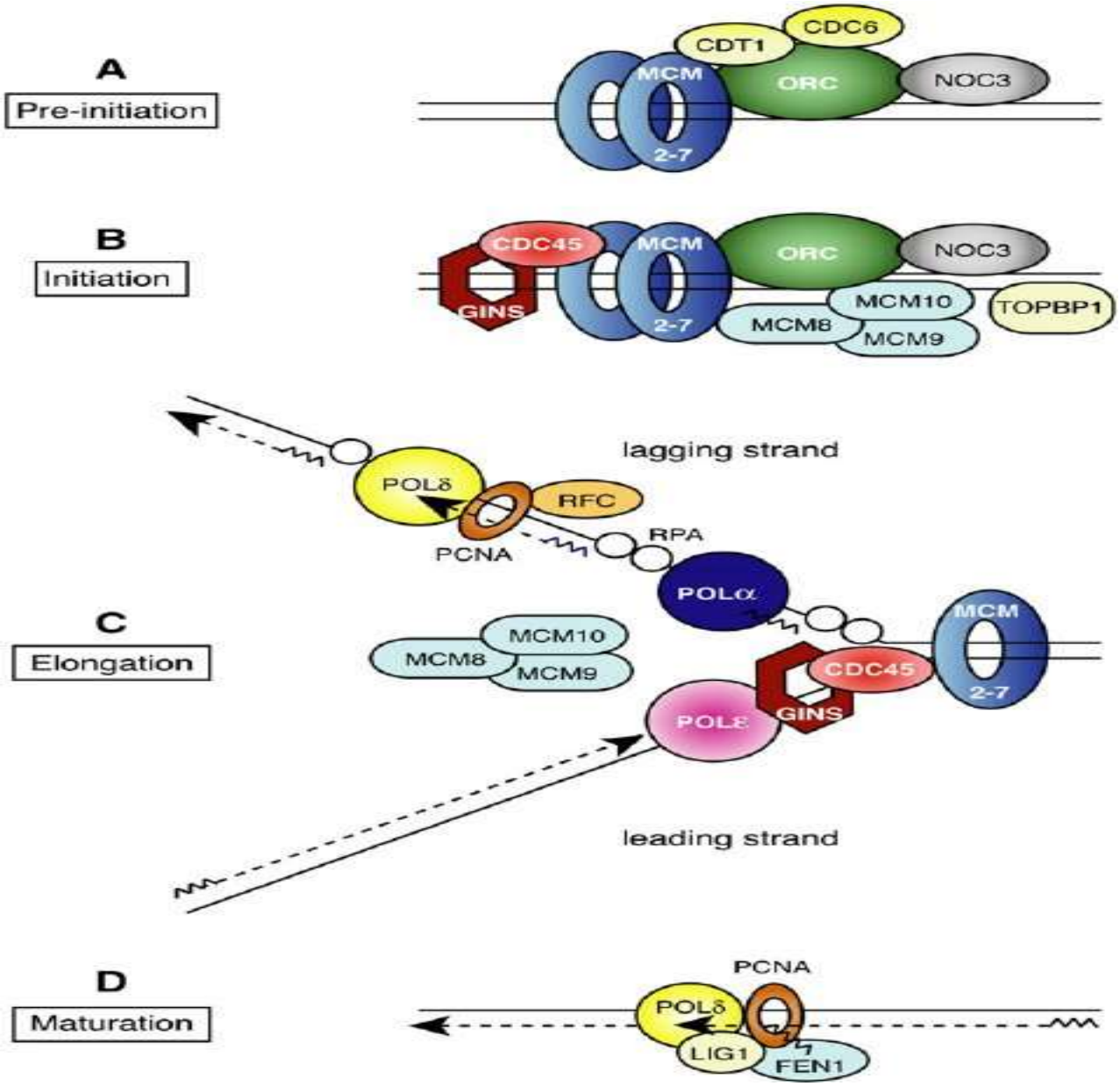
➢onset of the S phase, beginning of anaphase, and exit from mitosis. As discussed earlier, the APC must be activated before anaphase can proceed .

➢In contrast, the activity of Cdc34-SCF is not directly regulated. Rather, control is exerted by marking its substrate, Sic1, for polyubiquitination by phosphorylation by a Cdc28-G_1 cyclin .

➢This difference in strategy probably occurs because the APC has several substrates, including the anaphase inhibitor and B-type cyclins, which must be degraded at different times in the cycle. In contrast, entry into the S phase requires the degradation of only a single protein, Sic1.

➢ An obvious advantage of proteolysis for controlling passage through these critical points in the cell cycle is that protein degradation is an irreversible process, ensuring that cells proceed irreversibly through the cycle.

Skp, Cullin, F-box containing complex (or SCF complex) is a multi-protein E3 ubiquitin ligase complex


A
Pre-initiation
CDT1
CDC6
MCM
ORC
NOC3
2-7
B
Initiation
CDC45
GINS
MCM
ORC
NOC3
2-7
MCM10
TOPBP1
MCM8
MCM9
lagging strand
POLδ
RFC
PCNA
RPA
POLα
C
Elongation
MCM10
MCM8
MCM9
MCM
CDC45
2-7
POLε
GINS
leading strand
D
Maturation
PCNA
POLδ
LIG1
FEN1

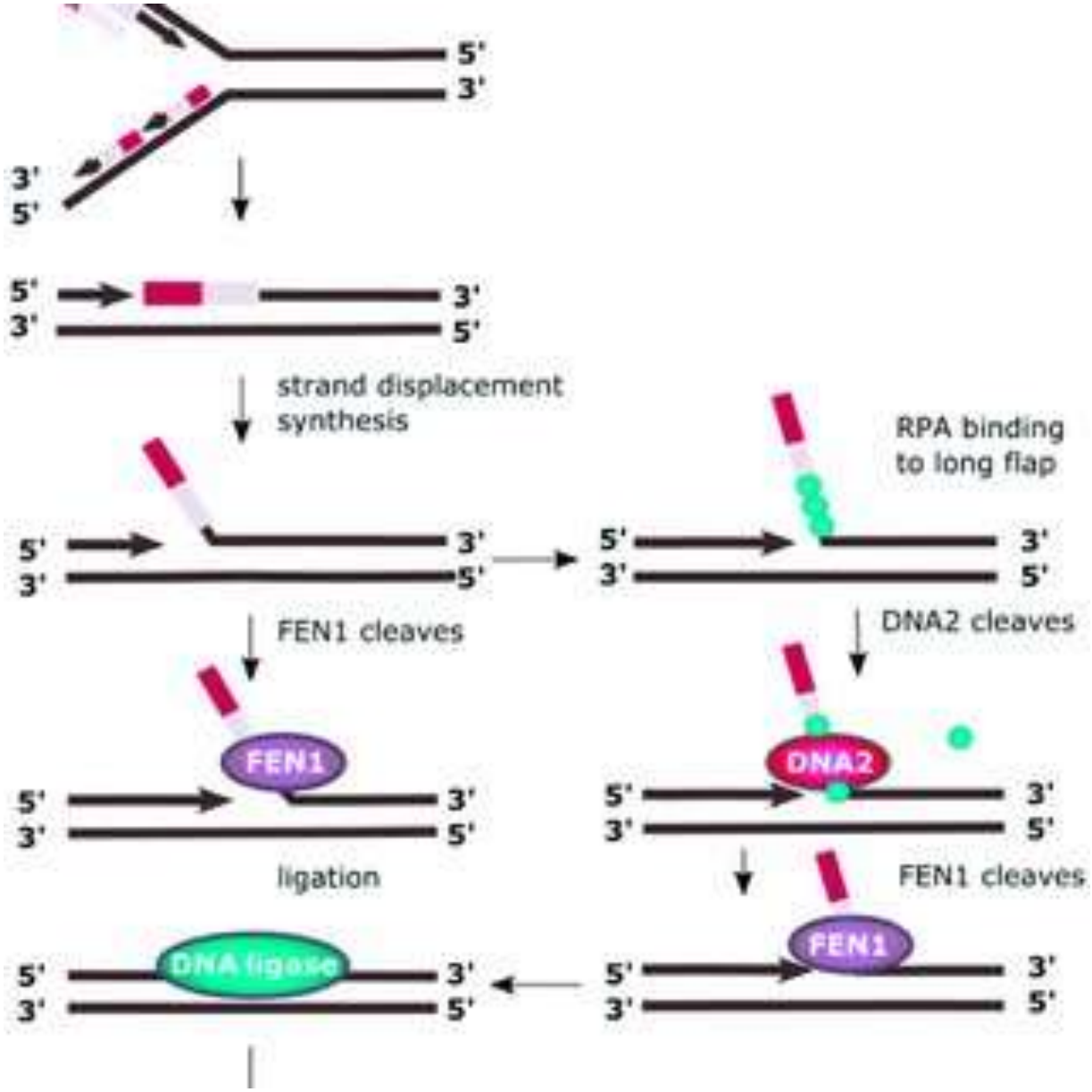
Model depicting the core eukaryotic DNA replication machinery from initiation through Okazaki fragment maturation.

A, Components of the preinitiation complex. DNA bound ORC recruits NOC3, CDC6, and CDT1 in early G1. Reiterative loading of 10 to 40 MCM complexes forms a licensed origin. After MCM loading is complete, CDC6 and CDT1 dissociate from the origin.

B, At the G1/S transition a subset of licensed origins transition to an initiation complex. The precise order of events is not clear and may vary between systems. CDC45, TOPBP1, and MCM8-10 contribute to GINS complex loading, DNA unwinding, and recruitment of the polymerases.

C, Components of the active DNA replication fork. MCM2-7, CDC45, and GINS unwind the duplex DNA. Leading strand synthesis is accomplished primarily by POLE. GINS increases the processivity of POLE. On the lagging strand, RPA stabilizes ssDNA, POLA lays down a short RNA/DNA primer and then is replaced by POLD, which completes the Okazaki fragment. RFC loads PCNA, which increases the processivity of POLD. The precise role of MCM8-10 in this process is not clear.

D, The dominant mechanism of Okazaki fragment maturation requires FEN1 to cleave the RNA/DNA flap, resulting in a nick that is sealed by LIG1.


5'
3'
3'
5'
5'
3'
3'
5'
strand displacement synthesis
5'
3'
3'
5'
FEN1 cleaves
FEN1
5'
3'
3'
5'
ligation
DNA ligase
5'
3'
3'
5'
RPA binding to long flap
5'
3'
3'
5'
DNA2 cleaves
DNA2
5'
3'
3'
5'
FEN1 cleaves
FEN1
5'
3'
3'
5'

Pre-replicative complex

Eukaryotic origins of replication control the formation of a number of protein complexes that lead to the assembly of two bidirectional DNA replication forks. These events are initiated by the formation of the pre-replication complex (pre-RC) at the origins of replication. This process takes place in the G_1 stage of the cell cycle.

The pre-RC formation involves the ordered assembly of many replication factors including the **origin recognition complex (ORC), Cdc6 protein, Cdt1 protein, and minichromosome maintenance proteins (Mcm2-7**).

Once the pre-RC is formed, activation of the complex is triggered by two kinases, cyclin-dependent kinase 2 (CDK) and Dbf4-dependent kinase (DDK) that help transition the pre-RC to the initiation complex prior to the initiation of DNA replication. (Dumbbell former 4 protein)

This transition involves the ordered assembly of additional replication factors to unwind the DNA and accumulate the multiple eukaryotic DNA polymerases around the unwound DNA.

Central to the question of how bidirectional replication forks are established at replication origins is the mechanism by which ORC recruits two head-to-head Mcm2-7 complexes to every replication origin to form the pre-replication complex.

Origin recognition complex

The first step in the assembly of the pre-replication complex (pre-RC) is the binding of the origin recognition complex (ORC) to the replication origin. In late mitosis, Cdc6 protein joins the bound ORC followed by the binding of the Cdt1-Mcm2-7 complex. **ORC, Cdc6, and Cdt1 are all required to load the six protein minichromosome maintenance (Mcm 2-7) complex onto the DNA**.

The ORC is a six-subunit, Orc1p-6, protein complex that selects the replicative origin sites on DNA for initiation of replication and ORC binding to chromatin is regulated through the cell cycle. Generally, the function and size of the ORC subunits are conserved throughout many eukaryotic genomes with the difference being their diverged DNA binding sites.

Cdc6 protein

- Binding of the cell division cycle 6 (Cdc6) protein to the origin recognition complex (ORC) is an essential step in the assembly of the pre-replication complex (pre-RC) at the origins of replication.
- Cdc6 binds to the ORC on DNA in an ATP-dependent manner, which induces a change in the pattern of origin binding that requires Orc1 ATPase.
- The ORC-Cdc6 complex forms a ring-shaped structure and is analogous to other ATP-dependent protein machines.
- The levels and activity of Cdc6 regulate the frequency with which the origins of replication are utilized during the cell cycle.

Cdt1 protein

The chromatin licensing and DNA replication factor 1 (Cdt1) protein is required for the licensing of chromatin for DNA replication. In *S. cerevisiae*, Cdt1 facilitates the loading of the Mcm2-7 complex one at a time onto the chromosome by stabilising the left-handed open-ring structure of the Mcm2-7 single hexamer.

Cdt1 has been shown to associate with the C terminus of Cdc6 to cooperatively promote the association of Mcm proteins to the chromatin. ***Cdt1 activity during the cell cycle is tightly regulated by its association with the protein geminin, which both inhibits Cdt1 activity during S phase in order to prevent re-replication of DNA and prevents it from ubiquitination and subsequent proteolysis.***

Minichromosome maintenance protein complex

Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7 form a hexameric complex that has an open-ring structure with a gap between Mcm2 and Mcm5.The assembly of the Mcm proteins onto chromatin requires the coordinated function of the origin recognition complex (ORC), Cdc6, and Cdt1. ***Once the Mcm proteins have been loaded onto the chromatin, ORC and Cdc6 can be removed from the chromatin without preventing subsequent DNA replication.***

Initiation complex

During the G_1 stage of the cell cycle, the replication initiation factors, origin recognition complex (ORC), Cdc6, Cdt1, and minichromosome maintenance (Mcm) protein complex, bind sequentially to DNA to form the pre-replication complex (pre-RC).

At the transition of the G_1 stage to the S phase of the cell cycle, S phase–specific cyclin-dependent protein kinase (CDK) and Cdc7/Dbf4 kinase (DDK) transform the pre-RC into an active replication fork.

During this transformation, **the pre-RC is disassembled with the loss of Cdc6**, creating the initiation complex.

In addition to the binding of the Mcm proteins, cell division cycle 45 (Cdc45) protein is also essential for initiating DNA replication. Studies have shown that Mcm is critical for the loading of Cdc45 onto chromatin and this complex containing both Mcm and Cdc45 is formed at the onset of the S phase of the cell cycle.

Cdc45 targets the Mcm protein complex, which has been loaded onto the chromatin, as a component of the pre-RC at the origin of replication during the G_1 stage of the cell cycle.

Cdc45 protein

Cell division cycle 45 (Cdc45) protein is a critical component for the conversion of the pre-replicative complex to the initiation complex.

The Cdc45 protein assembles at replication origins before initiation and is required for replication to begin in *Saccharomyces cerevisiae*, and has an essential role during elongation.

Thus, Cdc45 has central roles in both initiation and elongation phases of chromosomal DNA replication.

The loading of Cdc45 onto chromatin is critical for loading other various **replication proteins, including DNA polymerase α, DNA polymerase ε, replication protein A (RPA) and proliferating cell nuclear antigen (PCNA) onto chromatin**

GINS

The six minichromosome maintenance proteins and Cdc45 are essential during initiation and elongation for the movement of replication forks and for unwinding of the DNA. **GINS are essential for the interaction of Mcm and Cdc45 at the origins of replication** during initiation and then at DNA replication forks as the replisome progresses.

The GINS complex is composed of four small proteins Sld5 (Cdc105), Psf1 (Cdc101), Psf2 (Cdc102) and Psf3 (Cdc103), GINS represents 'go, ichi, ni, san' which means '5, 1, 2, 3' in Japanese.

Cdc45, Mcm2-7 and GINS together form the CMG helicase, the replicative helicase of the replisome. Although the Mcm2-7 complex alone has weak helicase activity Cdc45 and GINS are required for robust helicase activity

The CMG (Cdc45-MCM-GINS)

DDK and CDK kinases

At the onset of S phase, the pre-replicative complex must be activated by two S phase-specific kinases in order to form an initiation complex at an origin of replication. One kinase is the Cdc7-Dbf4 kinase called Dbf4-dependent kinase (DDK) and the other is cyclin-dependent kinase (CDK).

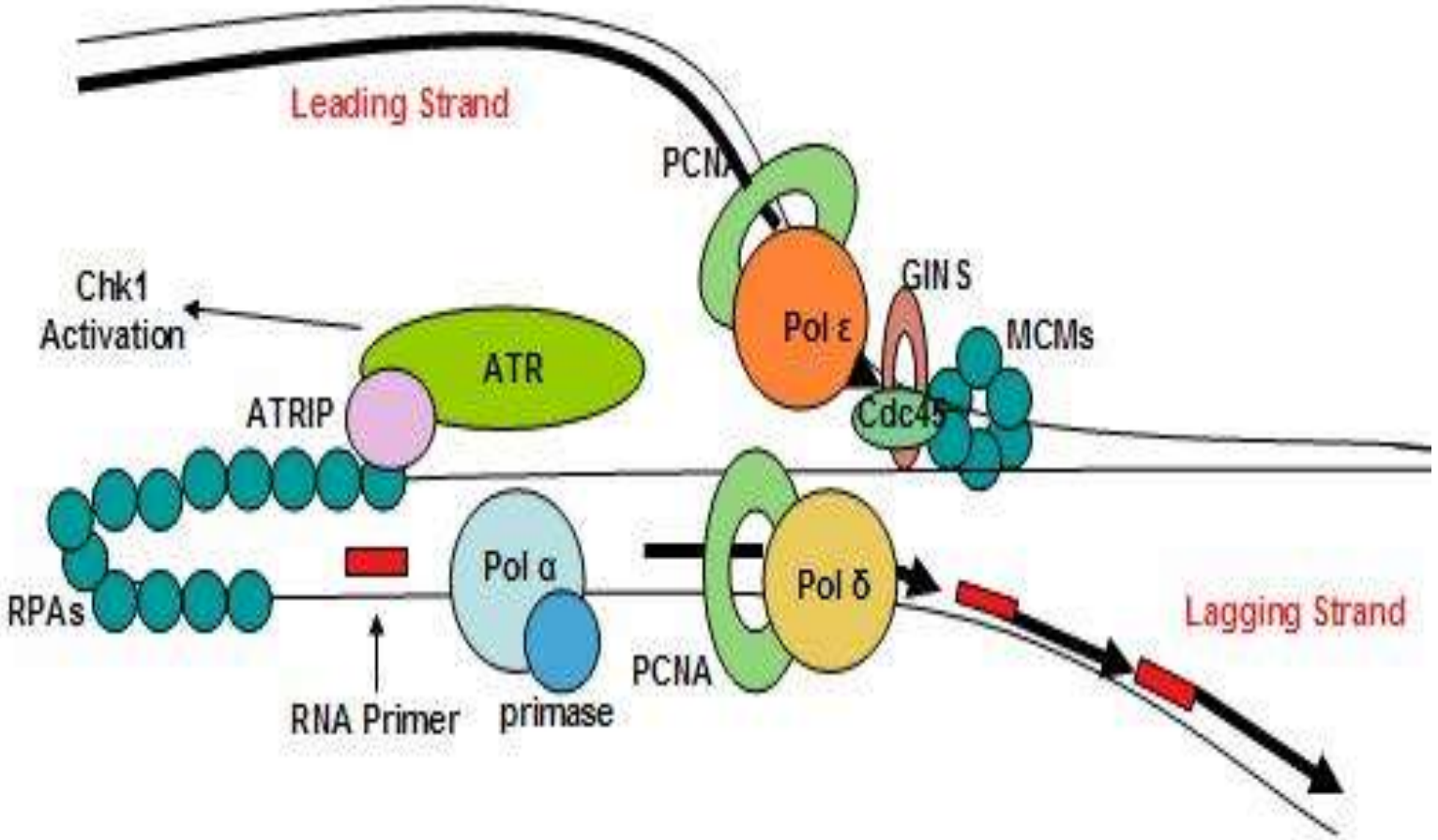
Chromatin-binding assays of Cdc45 in yeast and *Xenopus* have shown that a downstream event of CDK action is loading of Cdc45 onto chromatin.

Cdc6 has been speculated to be a target of CDK action and the CDK-dependent phosphorylation of Cdc6. The CDK-dependent phosphorylation of Cdc6 has been considered to be required for entry into the S phase.

Both the catalytic subunits of DDK, Cdc7, and the activator protein, Dbf4, are conserved in eukaryotes and are required for the onset of S phase of the cell cycle.

Both DDK and Cdc7 are required for the loading of Cdc45 onto chromatin origins of replication. The target for binding of the DDK kinase is the Mcm complex, possibly Mcm2. DDK targets the Mcm complex, and its phosphorylation leads to the possible activation of Mcm helicase activity

M phase
G1
S phase
Pre-replication complex (preRC) assembly
Initiation
Elongation
Cdt1
ORC
Cdc6
MCM
Cdc45
Sld2, 3
GINS
Dpb11
Polymerase
ε
Cdc7
CDK
P
Polymerase
α
δ
Ctf4, Mrc1


Leading Strand
PCNA
GINS
Chk1
Activation
Pol ε
MCMs
ATR
ATRIP
Cdc45
Pol α
Pol δ
RPAs
Lagging Strand
PCNA
RNA Primer
primase

Cell death

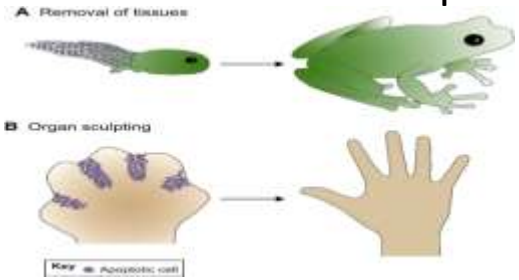
(Graphics are from internet)

Apoptosis

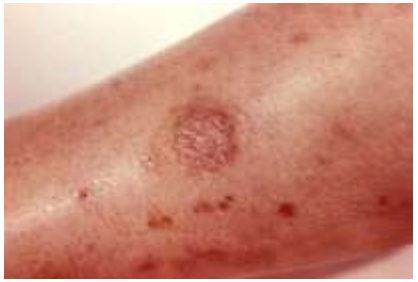
What is difference between necrosis and apoptosis?
The main **difference between apoptosis** and **necrosis** is that **apoptosis** is a predefined cell suicide, where the cell actively destroys itself, maintaining a smooth functioning **in the** body whereas **necrosis** is an accidental cell death occurring due to the uncontrolled external factors **in the** external environment of the cell

In **apoptosis**, the cell death is part of the normal developmental process; in **necrosis**, by contrast, the cell death is the result of something gone wrong.

Programmed cell death is as needed for proper development as mitosis is. **Examples**: The resorption of the tadpole tail at the time of its metamorphosis into a frog occurs by **apoptosis**. The formation of the fingers and toes of the fetus requires the removal, by **apoptosis**, of the tissue between them.



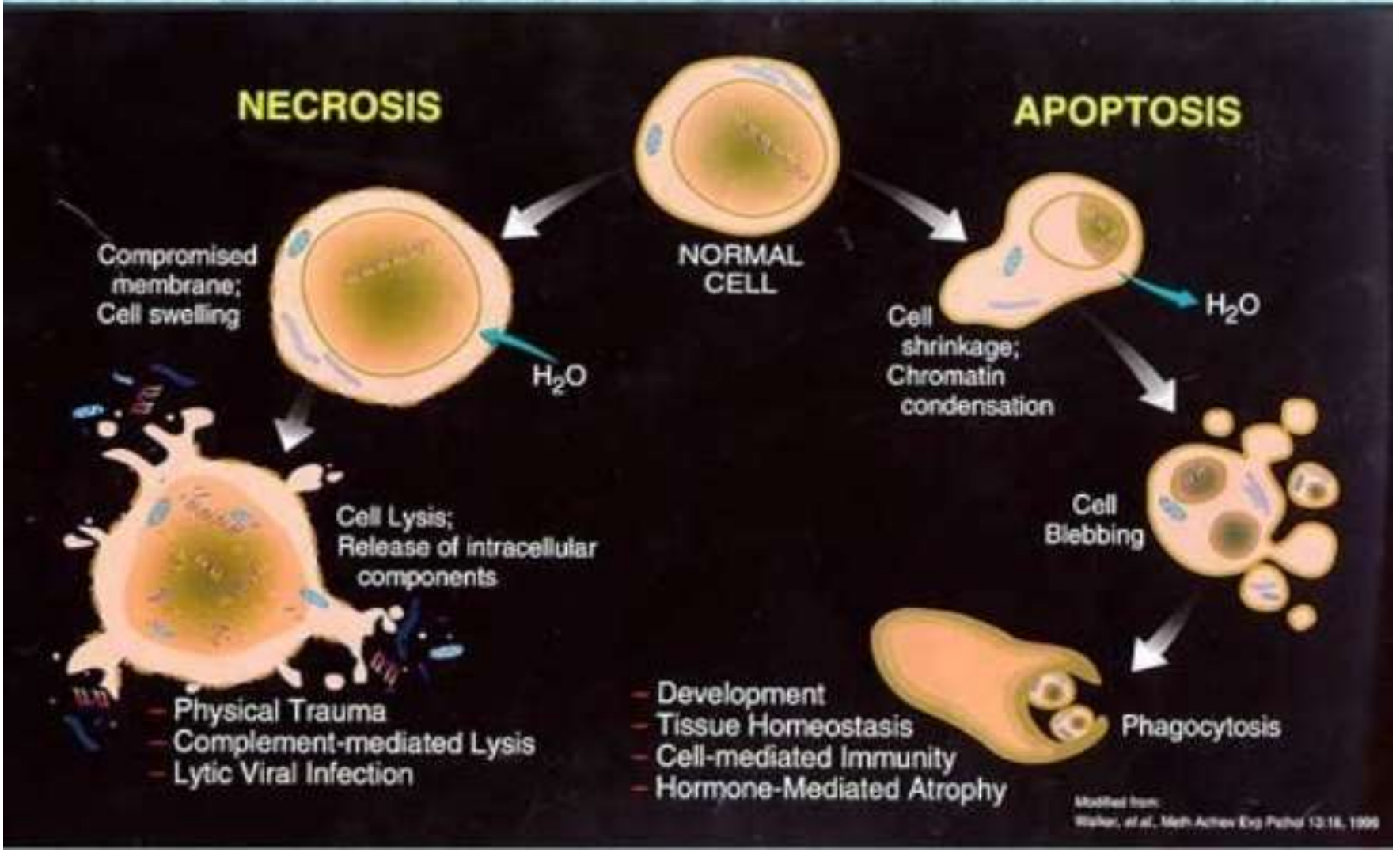
Necrosis
Human disease.
Tissue.Gangrene.Lung infarction.Ulcer.Lesion.Osteonecrosis.



Patient with localized tissue necrosis caused by a bite from a brown recluse spider.

APOPTOSIS (Physiological cell death)	NECROSIS (Pathological cell death)
Functional form of cell death	Accidental form of cell death
Occurs under physiological conditions	Seen under pathological conditions
Energy (ATP)- dependent	No energy requirement
Cell shrinks and pulls away from its neighbours	Cell swelling in a defining features
Nucleus ruptures	Entire cell balloons and ruptures
Induced by physiological stimuli(lack of growth factor, changes in harmonal environment	Induced by non-physiological disturbances lytic viruses, hypothermia,hypoxia, ischaemia,metabolic poisons
No inflammation follows apoptosis	Necrosis is followed by inflammation

NECROSIS Vs APOPTOSIS



Reasons of apoptosis

- **Withdrawal of positive signals**

  examples :
  - growth factors for neurons
  - Interleukin-2 (IL-2)

- **Receipt of negative signals**

  examples :
  - increased levels of oxidants within the cell
  - damage to DNA by oxidants
  - death activators :
    - Tumor necrosis factor alpha (TNF-α)
    - Lymphotoxin (TNF-β)
    - Fas ligand (FasL)

Inducers of Apoptosis

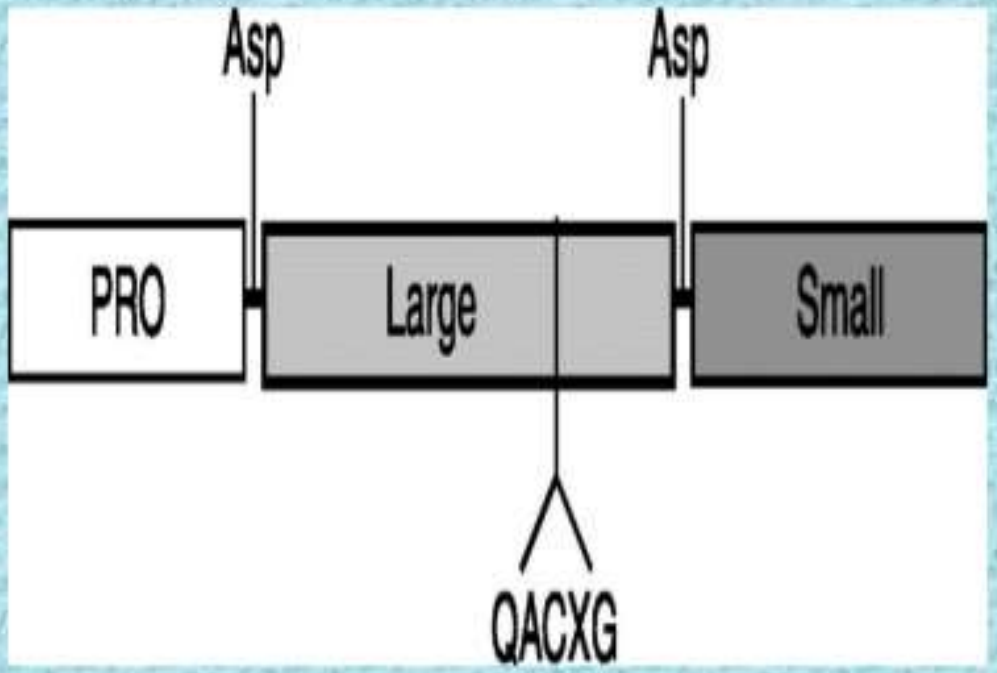
- TNF family
- Growth factor withdrawl
- Calcium
- Oncogenes
- Nutrient deprivation
- Toxins
- UV radiation
- Gamma radiation

- Important in normal physiology
  - **Development**: Immune systems maturation, Morphogenesis, Neural development
  - **Adult**: Immune privilege, DNA Damage and wound repair.
- Excess apoptosis
  - Neurodegenerative diseases
- Deficient apoptosis
  - Cancer
  - Autoimmunity

Caspases

- Caspases stands for **c**ysteine **asp**artate-specific prote**ase**.
- Caspases have the characteristics of high specificity for substrates containing Asp, and use a Cys for catalyzing peptide bond cleavage.
- Synthesized in the cell as precursors named procaspase.
- Caspases are the major executioners in apoptosis.

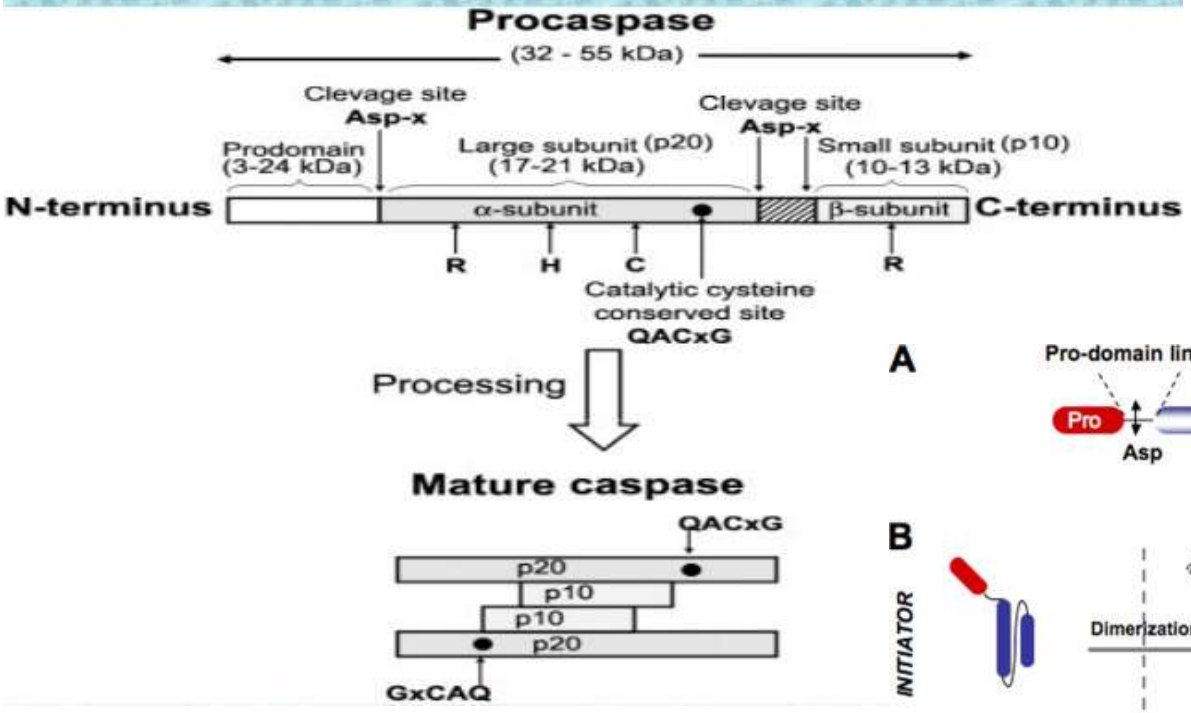
Caspase Structure

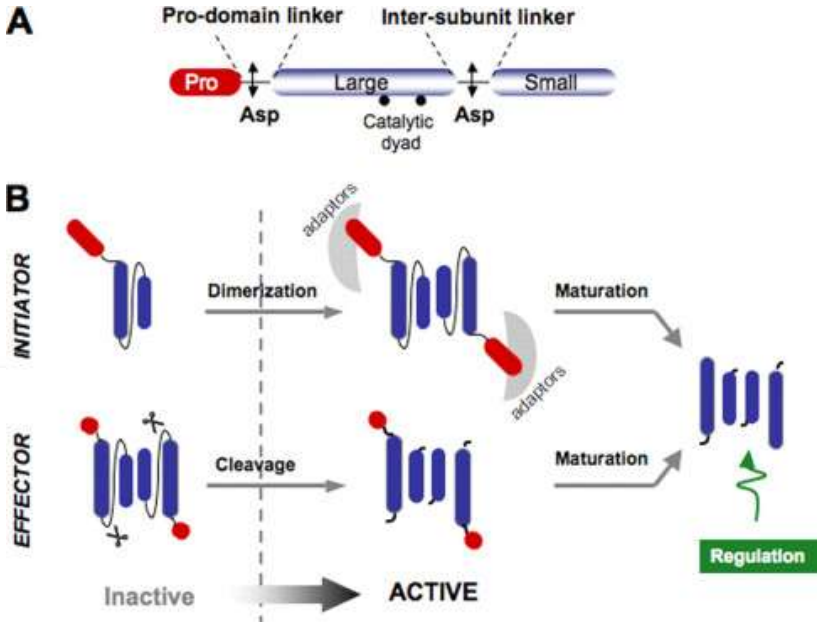


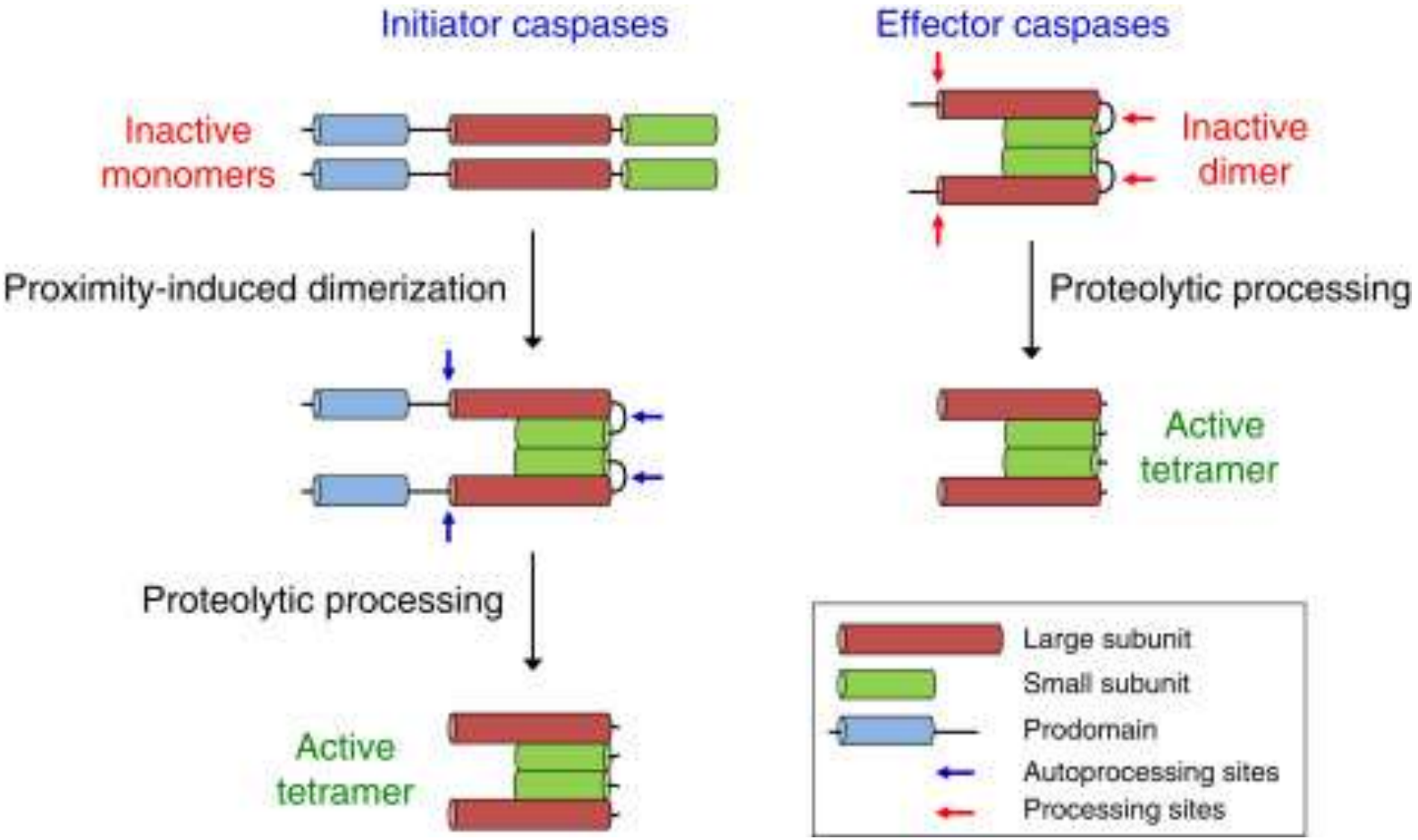
- NH_1-terminal domain
- Large subunit (~20kD)
- Small subunit (~10kD)

All caspases are synthesized in cells as catalytically inactive zymogens, and must undergo an activation process. The activation of an effector caspase, such as caspase-3 or -7, is performed by an initiator caspase, such as caspase-9, through an internal cleavage to separate the large and small subunits

Caspase Activation







Depending on the structure of their prodomains, caspases are activated by different mechanisms. Long prodomain-containing initiator caspases exist as inactive monomers and are activated by proximity-induced dimerization which can sometimes be facilitated by adaptor proteins. Upon dimerization, interchain autocatalytic cleavage generates a catalytically activate enzyme. Further processing leads to the removal of the prodomain and formation of a mature tetramer assembled by the homodimerzation of heterodimers. Effector caspases have short prodomains and exist as inactive dimers. They are activated by cleavage (mediated by activated initiator caspases) between the small and large subunits of the catalytic domain. As with initiator caspases, enzymatically active effector caspases are comprised of tetramers derived from two large and two small subunits.

Caspase Role in Apoptosis

- Cut off contact with surrounding cells
- Reorganize cytoskeleton
- Shut don DNA replication and repair
- Interrupt splicing
- Destroy DNA
- Disrupt nuclear structure
- Induce cell to display signals marking it for phagocytosis
- Disintegrate cells into apoptotic bodies

Types

Two types:

- those **involved in apoptosis:**

Initiators – activate downstream effector caspases to initate activation cascades:

Caspases2

Caspases9

Caspases8

Caspases10

Effectors - cleave target proteins resulting in morphological and biochemical markers of apoptosis:

Caspases3

Caspases6

Caspases7

Caspases14

Two distinct pathways converge on caspase acticvation

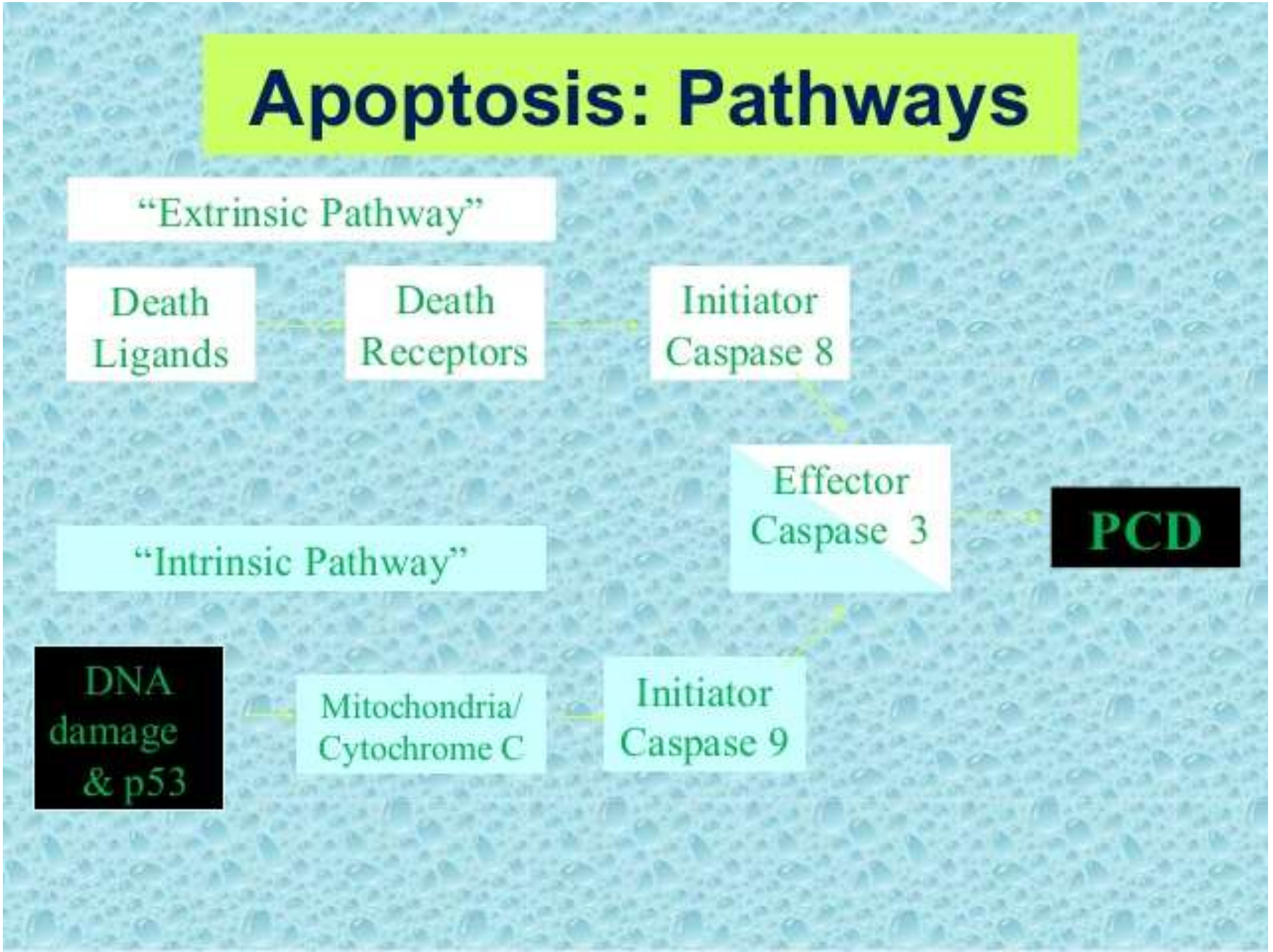
Mitochondrial pathway

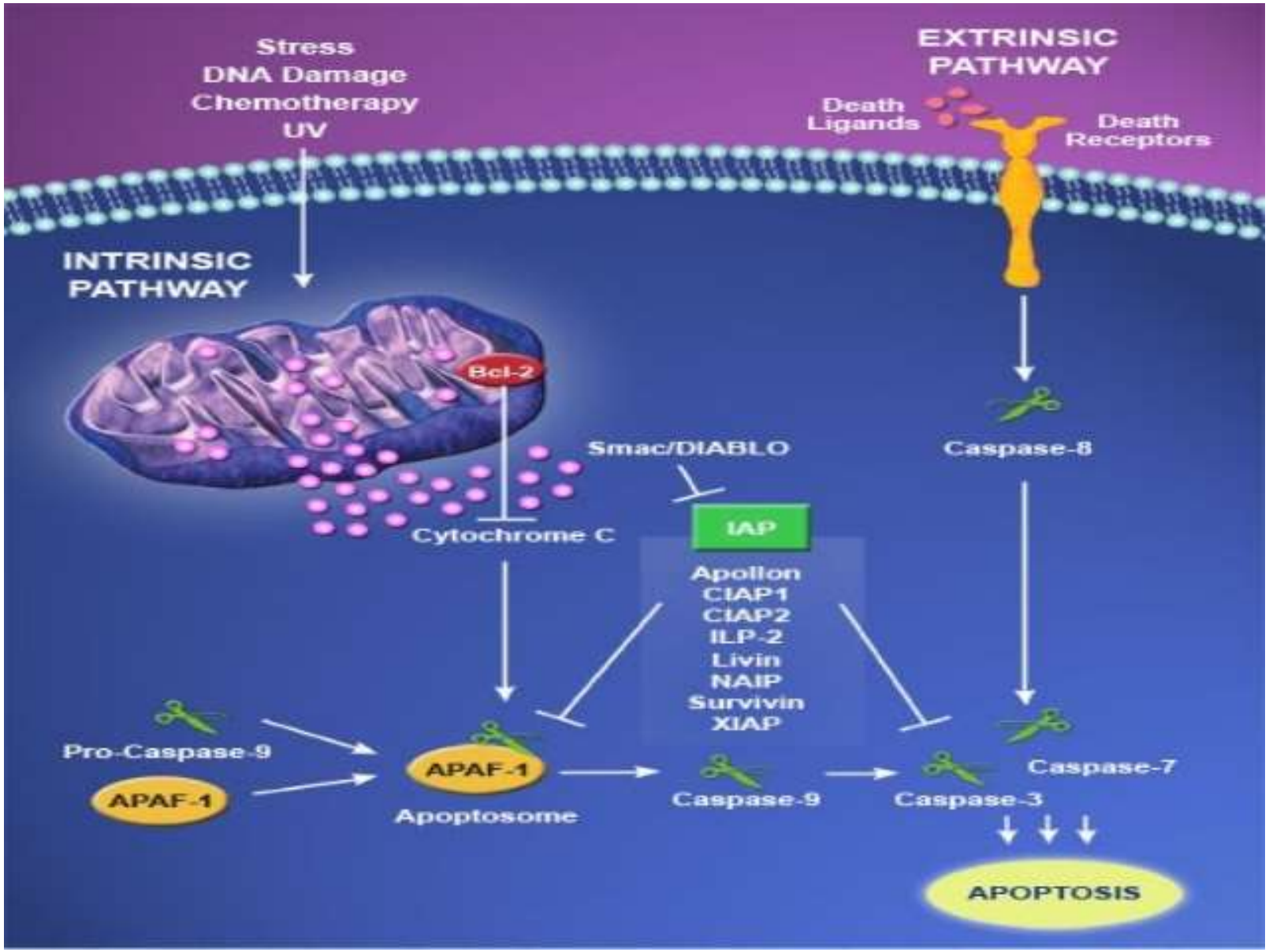
- Intrinsic pathway

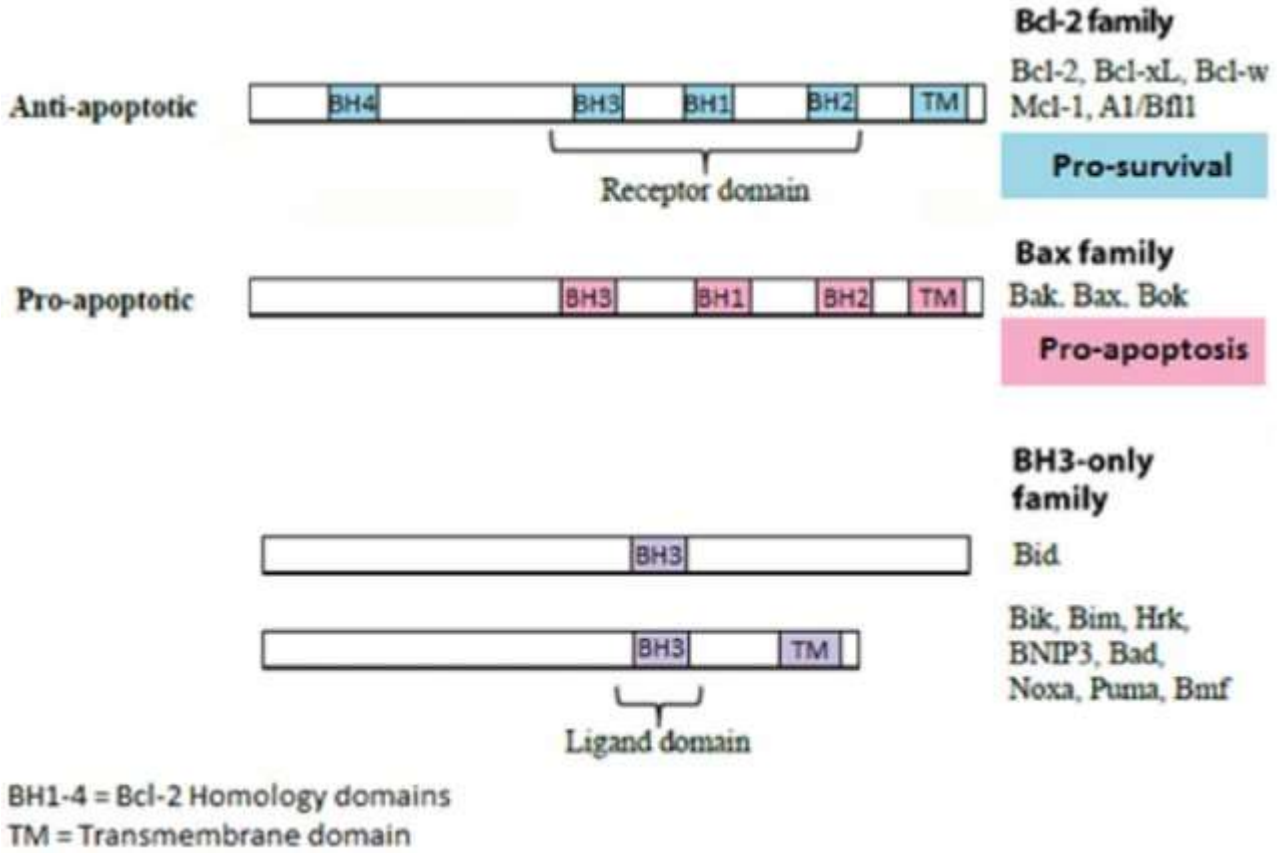
The death receptor pathway

- Extrinsic pathway

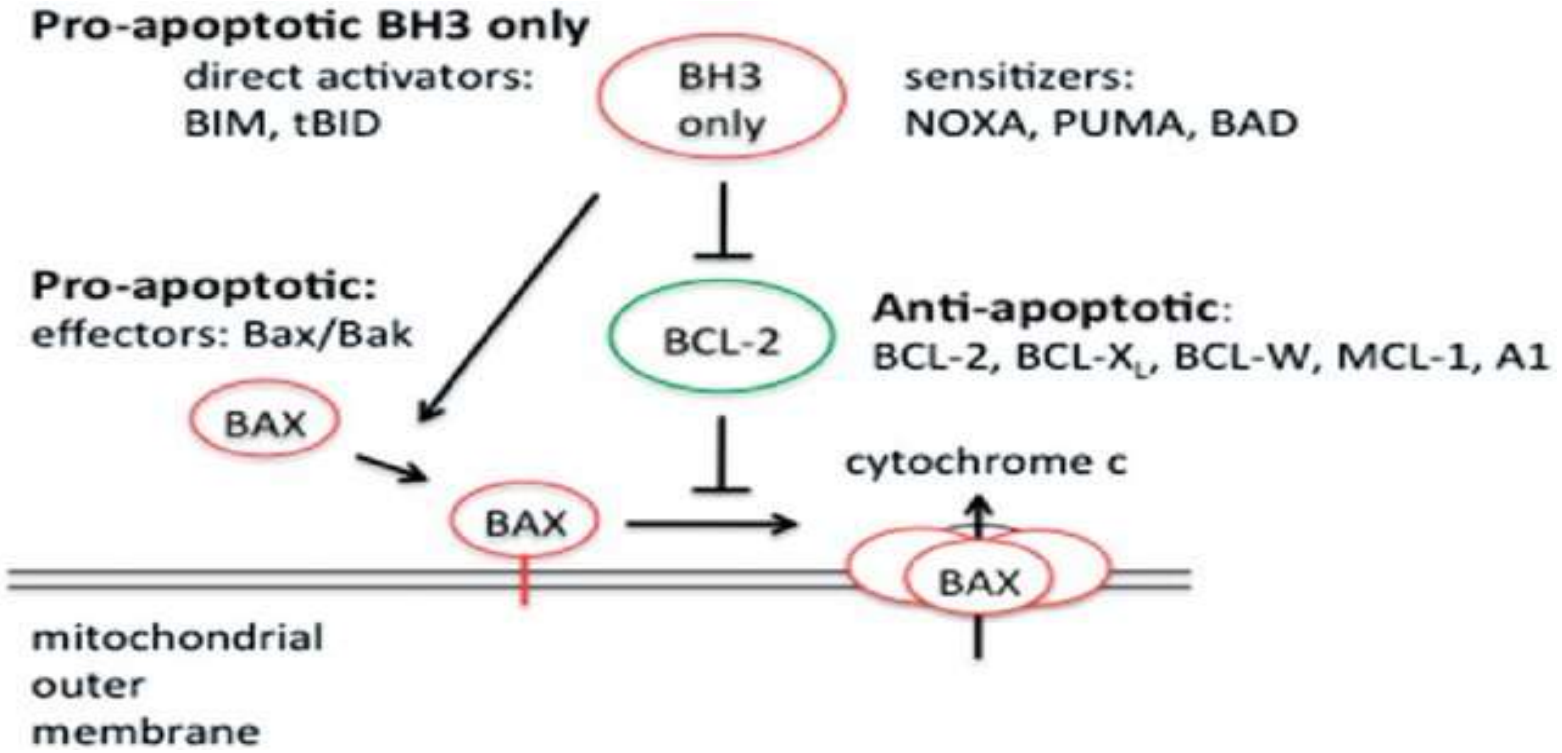
Apoptosis: Pathways



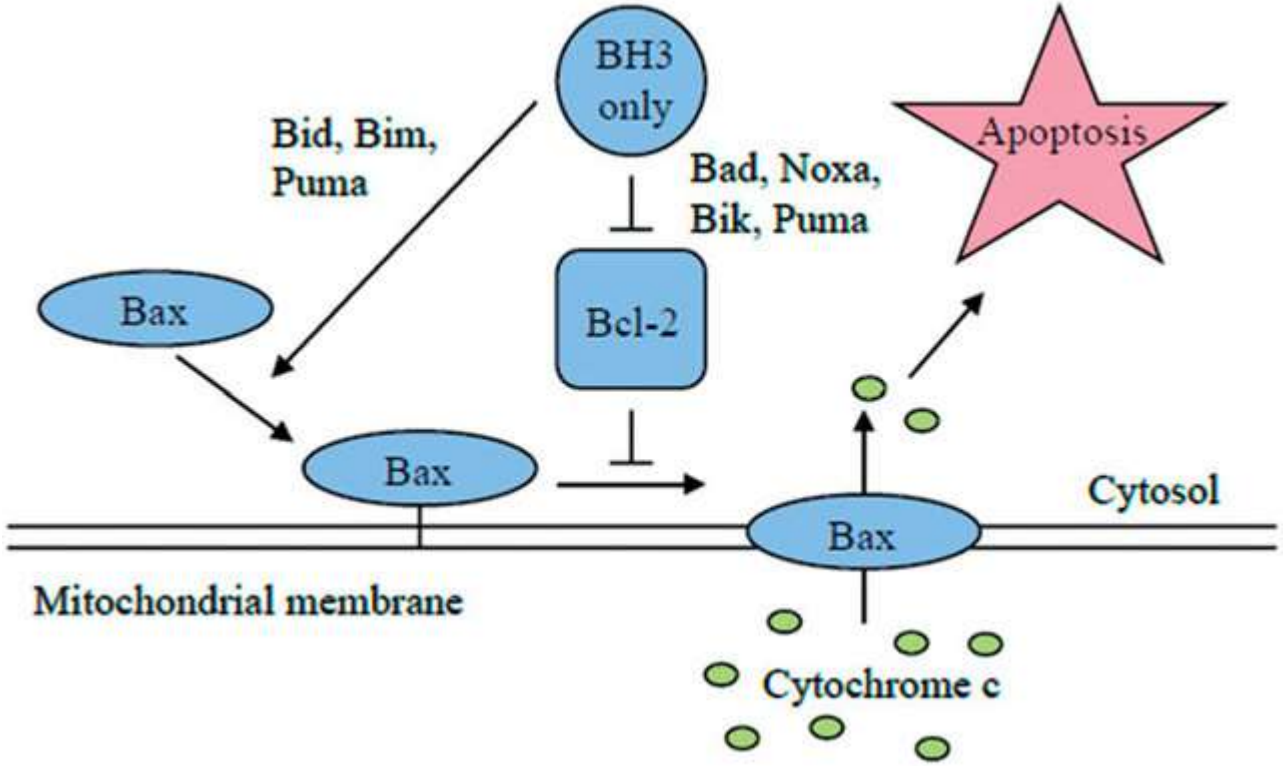

Stress
DNA Damage
Chemotherapy
UV
EXTRINSIC PATHWAY
Death Ligands
Death Receptors
INTRINSIC PATHWAY
Bcl-2
Smac/DIABLO
Caspase-8
Cytochrome C
IAP
Apollon
CIAP1
CIAP2
ILP-2
Livin
NAIP
Survivin
XIAP
Pro-Caspase-9
APAF-1
APAF-1
Apoptosome
Caspase-9
Caspase-3
Caspase-7
APOPTOSIS



Structure of the Bcl-2 protein family members. The BH1-3 domain forms a hydrophobic receptor domain able to sequester the BH3 domain-only proteins. BH1/2/3 domain of pro-survival Bcl-2 proteins acts as a receptor for the BH3 domain of other Bcl-2 family members.



Schematic model of apoptosis regulation by Bcl-2 members protein family. Apoptosis is initiated by the oligomerization of Bax (or Bak) in the mitochondrial outer membrane, which causes the formation of a channel for the release of cytochrome c and other apoptogenic factors. The anti-apoptotic Bcl-2 family members inhibit Bax and Bak oligomerization. The pro-apoptotic BH3-only members of the Bcl-2 family are the direct sensors of cellular stress. They trigger cell death by either inhibiting the antiapoptotic Bcl-2 members or in the case of BIM and tBID by directly activating BAX and BAK. The BH3-only proteins are regulated by a number of mechanism included increased gene expression or posttranslational modifications.

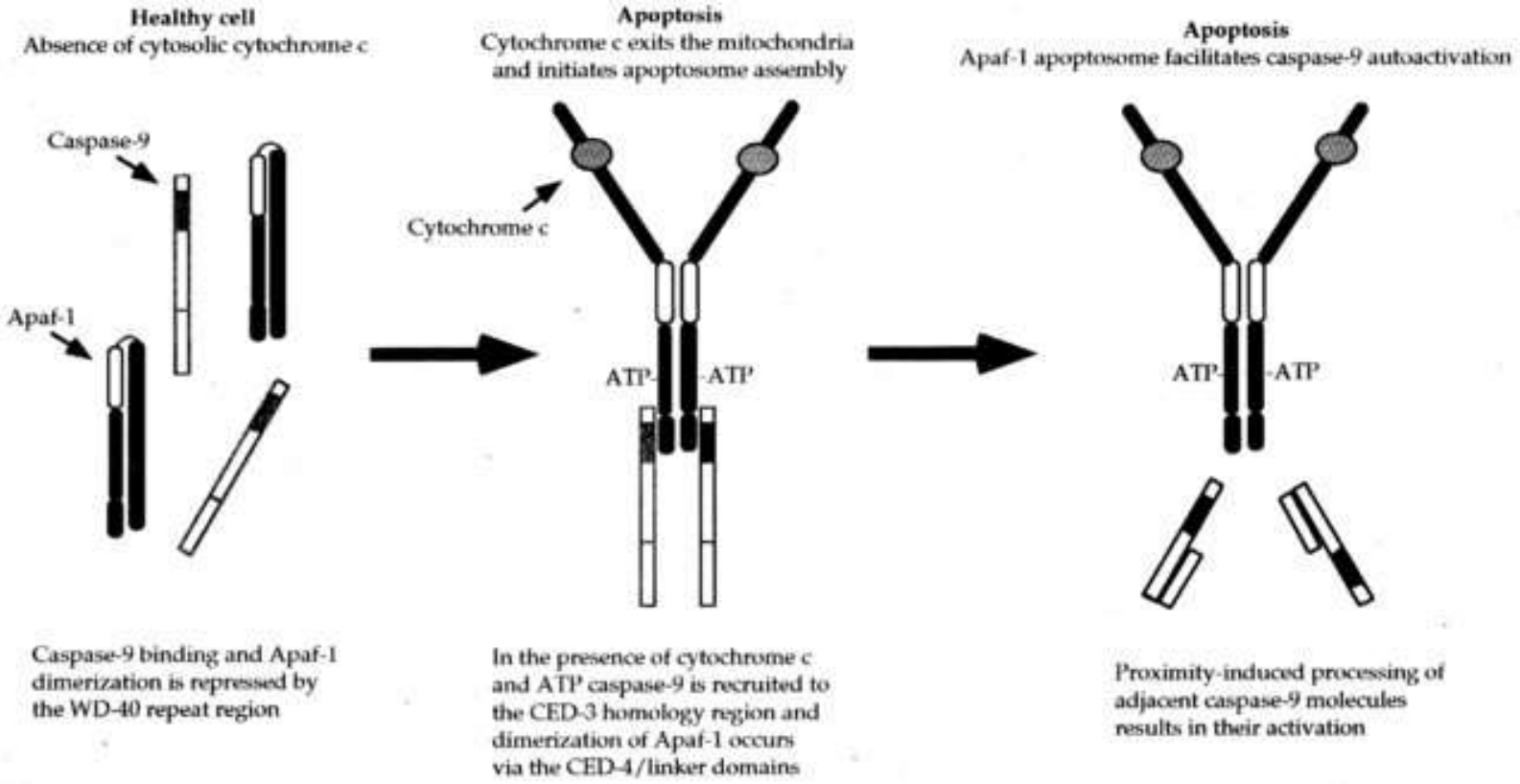


Comparison of the direct and indirect model of Bax and Bak activation. In the direct model, the BH3-only members (Bim, tBid and PUMA) act as activators and bind to Bax and Bak directly to induce pore formation in the OMM that permits the release of cytochrome c. On the other hand, the remaining BH3-only proteins act as sensitizers and bind to the Bcl-2 members, releasing bound Bim and tBid and allowing them to directly activate Bak and Bax. The indirect model shows that BH3-only proteins do not bind directly to Bax and Bak; however, they engage the anti-apoptotic proteins, causing the release of Bak and Bax. Some BH3-only proteins bind to specific anti-apoptotic Bcl-2 family proteins (selective) while others bind to all anti-apoptotic Bcl-2 proteins (promiscuous).

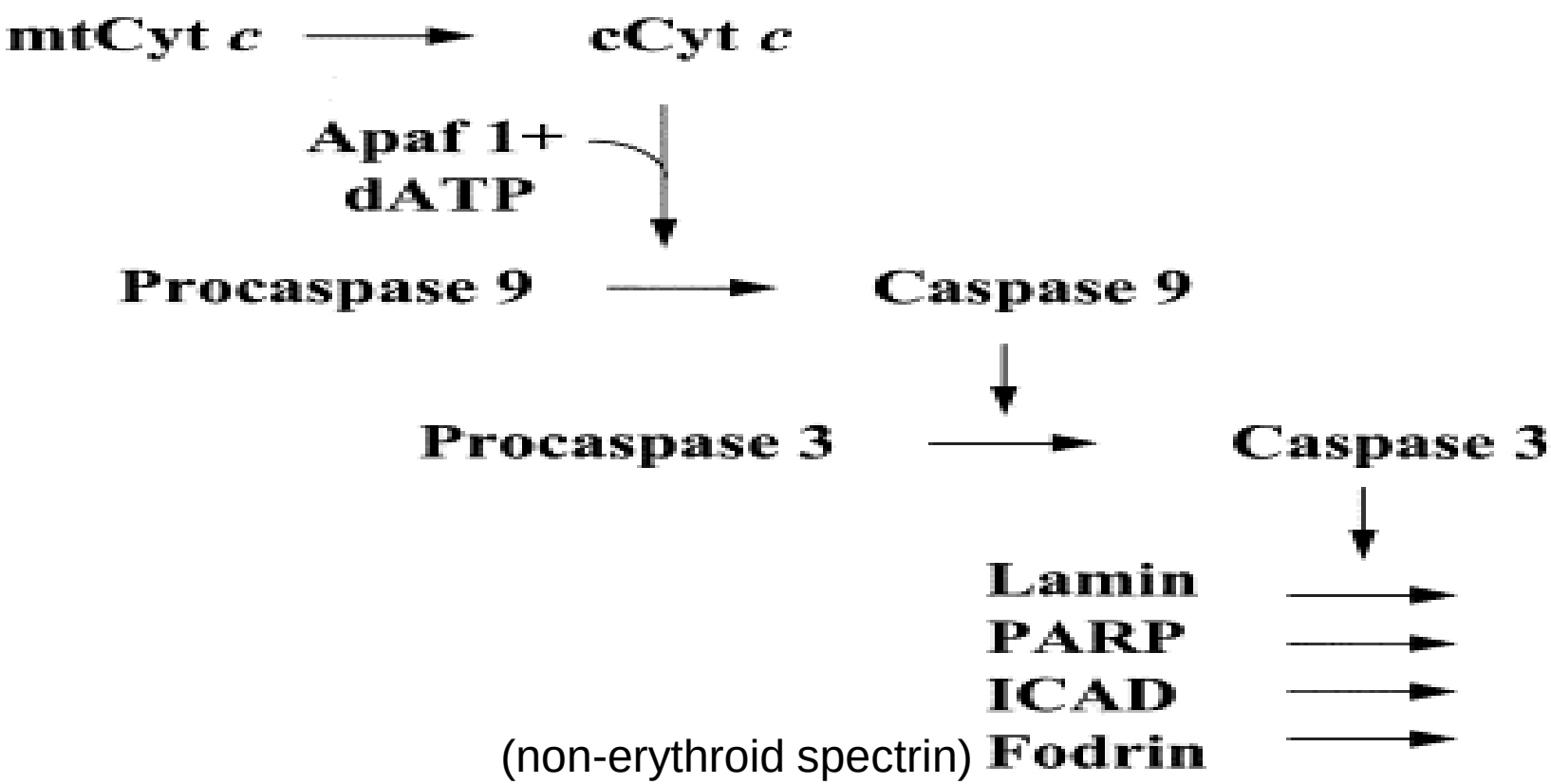
In the cytosol, Cyt c mediates **the allosteric activation of apoptosis-protease activating factor 1**, which is required for the proteolytic maturation of caspase-9 and caspase-3. Activated caspases ultimately lead to apoptotic cell dismantling

How does cytochrome c help in apoptosis?
Mitochondrial cytochrome c (cyt c) has been found to have dual functions in **controlling both cellular energetic metabolism and apoptosis**. Through interaction with apoptotic protease activating factors (Apaf), cyt c can initiate the activation cascade of caspases once it is released into the cytosol.

How does cytochrome c activate caspase-9?
A critical component of the intrinsic apoptotic pathway is caspase-9, which is activated by **Apaf-1 in the apoptosome, a large complex assembled in response to release of cytochrome c from mitochondria**. Caspase-9 cleaves and activates effector caspases, predominantly caspase-3, resulting in the demise of the cell.



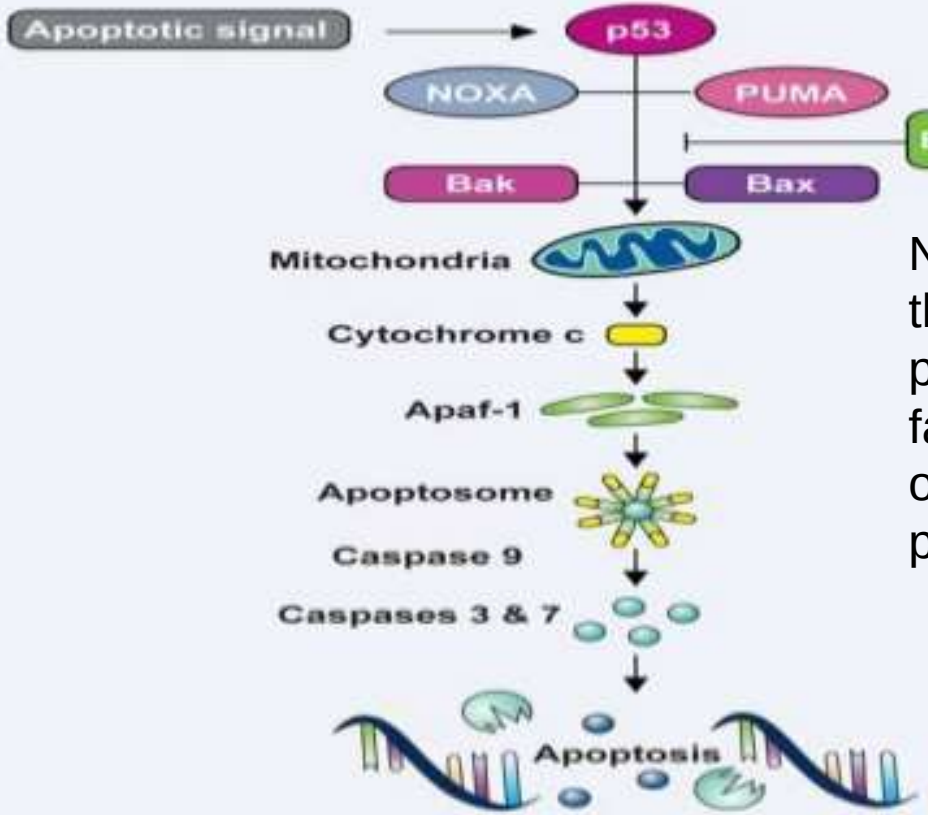
Apaf-1 promotes caspase-9 activation by oligomerization. Cytochrome c and ATP regulate the ability of Apaf-1 to recruit caspase-9 and form multimers



Activation cascade initiated by mitochondrial cytochrome *c* release. Release of mitochondrial cytochrome *c* (mtCyt *c*) from the intermembrane space into the cytoplasm (cCyt *c*) allows interaction with Apaf-1 which, in the presence of micromolar concentration of dATP or millimolar concentration of ATP, results in proteolytic activation of procaspase-9. Active caspase-9 activates procaspase-3 by proteolytic cleavage. Active caspase-3 subsequently cleaves a variety of substrates resulting in characteristic morphologic changes in the nucleus, DNA fragmentation, and appearance of the phagocytic marker phosphatidylserine on the cell surface.

THE INTRINSIC PATHWAY

Figure 3.1. Elements of the mitochondrial pathway

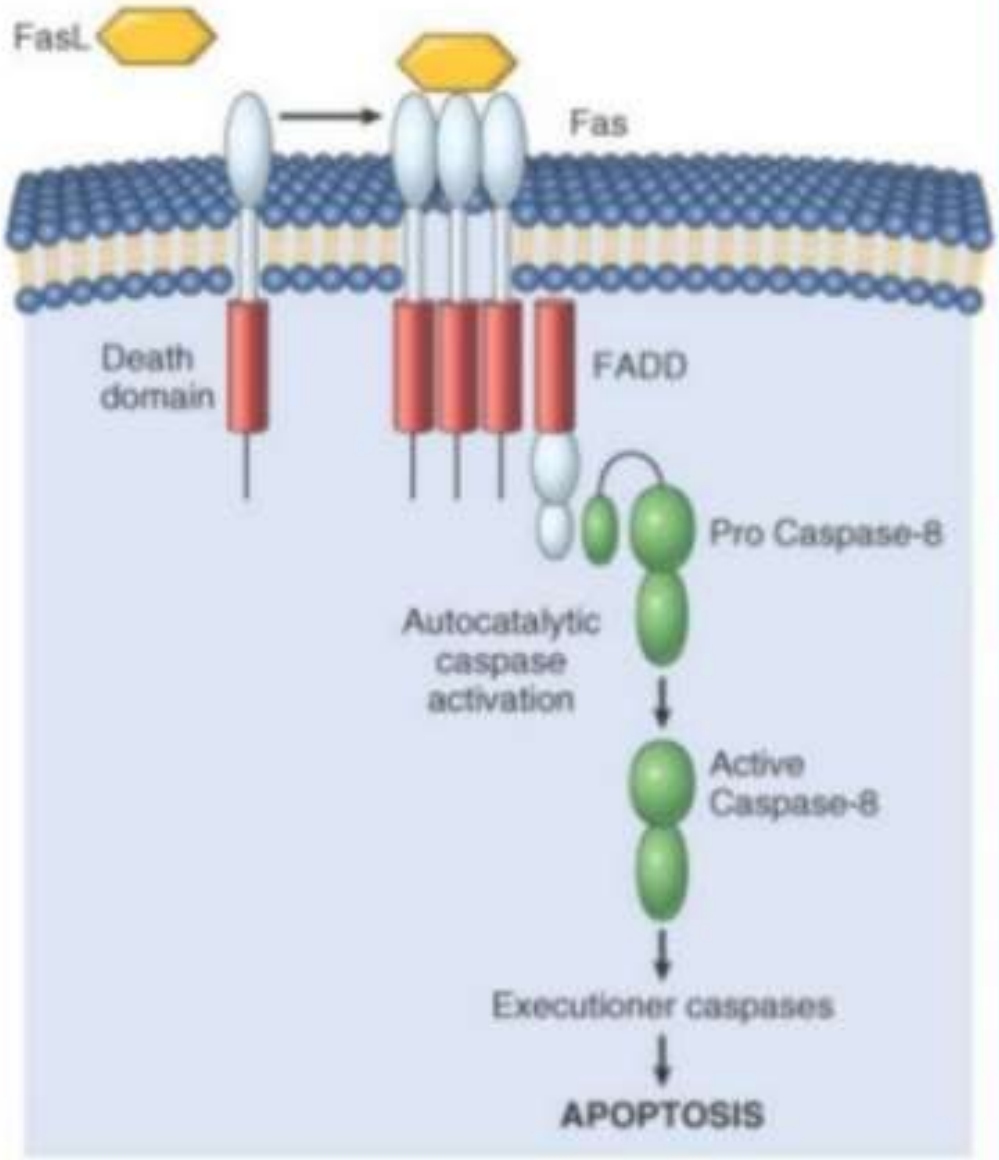


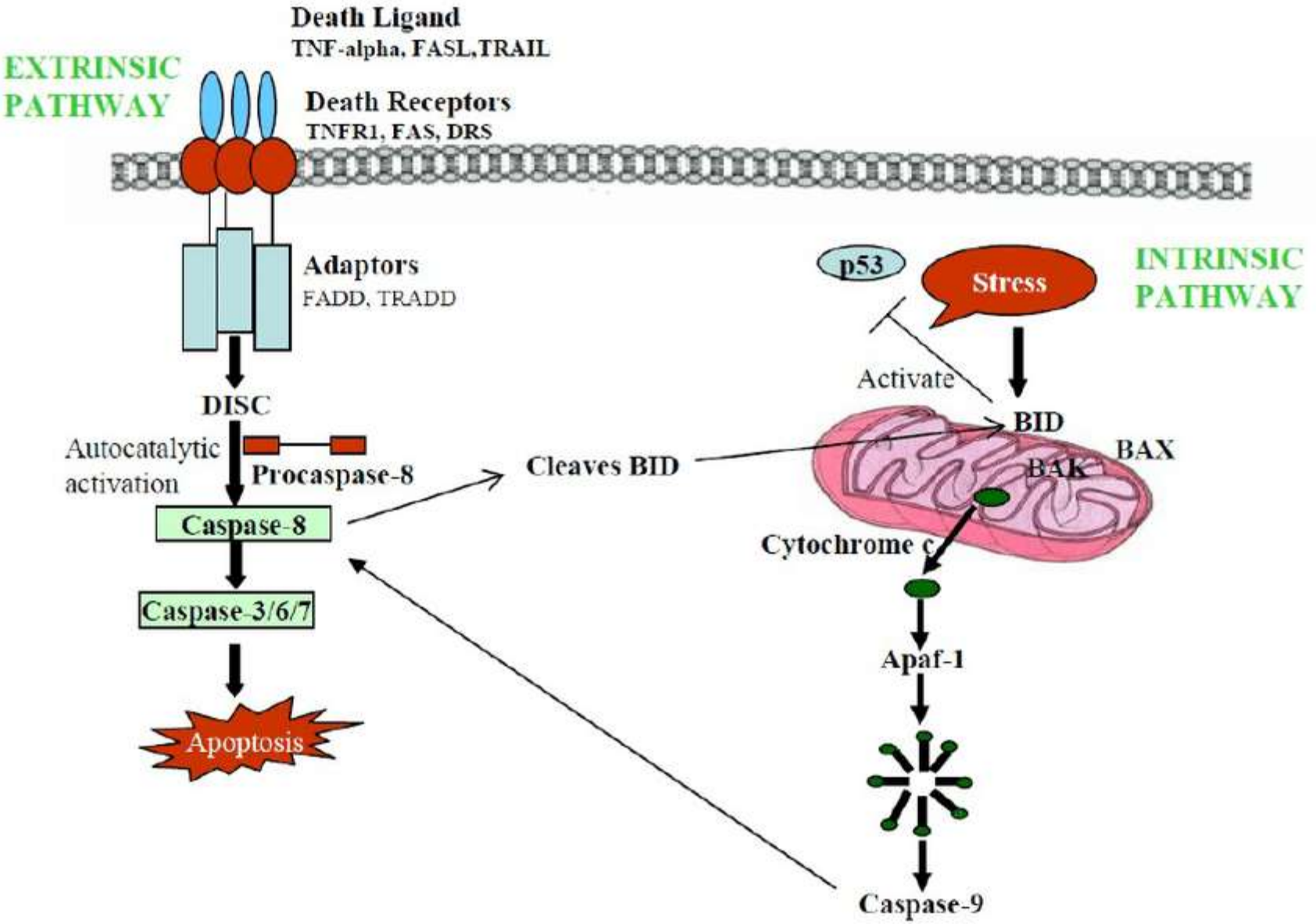
PUMA (p53 upregulated modulator of apoptosis) bind to Bcl-2, localize to the mitochondria to induce cytochrome c release

NOXA neutralizing the prosurvival Bcl2 proteins Mcl1/A1, facilitating activation of Bax and/or Bak proteins

- Extrinsic pathway

The death receptor pathway




Death Ligand
TNF-alpha, FASL,TRAIL
EXTRINSIC PATHWAY
Death Receptors
TNFR1, FAS, DRS
Adaptors
FADD, TRADD
DISC
Autocatalytic activation
Procaspase-8
Caspase-8
Caspase-3/6/7
Apoptosis
Cleaves BID
p53
Stress
INTRINSIC PATHWAY
Activate
BID
BAX
BAK
Cytochrome c
Apaf-1
Caspase-9

Extrinsic and Intrinsic pathway of apoptosis: In extrinsic pathway, the death ligands bind to transmembrane death receptors present on the cell destined to die.

The binding of ligand to the receptors on the target cell triggers receptor clustering on the cell surface.

This aggregation recruits the adaptor proteins on the cytoplasmic site of the receptors, forming death inducing signalling complex (DISC).

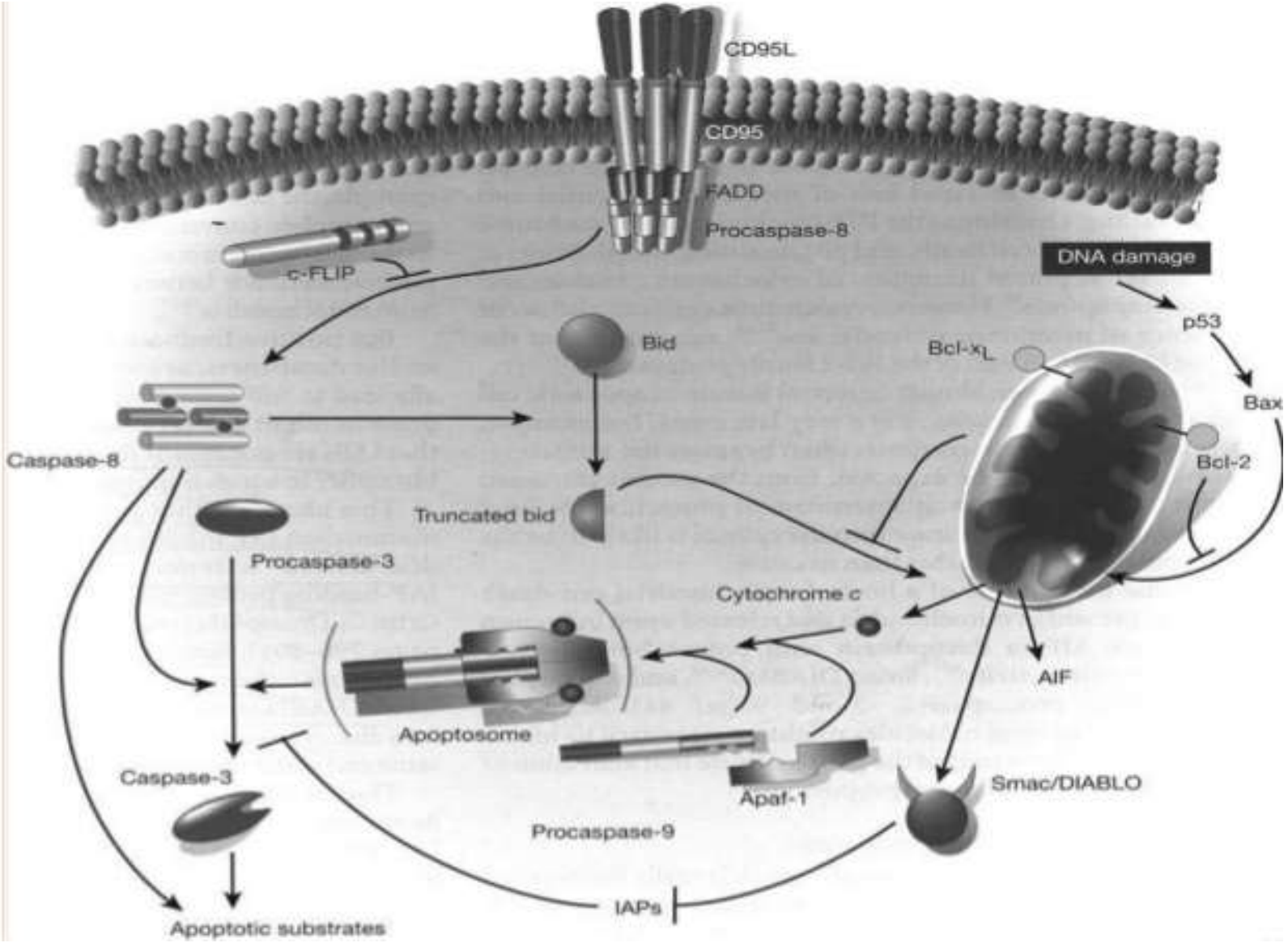
Formation of DISC brings procaspase molecules close to one another, which facilitates their autocatalytic activation and release into the cytoplasm where they activate caspase cascade.

Active caspase-8 also mediates the cleavage of proapoptotoc protein, BID which subsequently releases mitochondrial proapoptotic factors linking the two pathways.

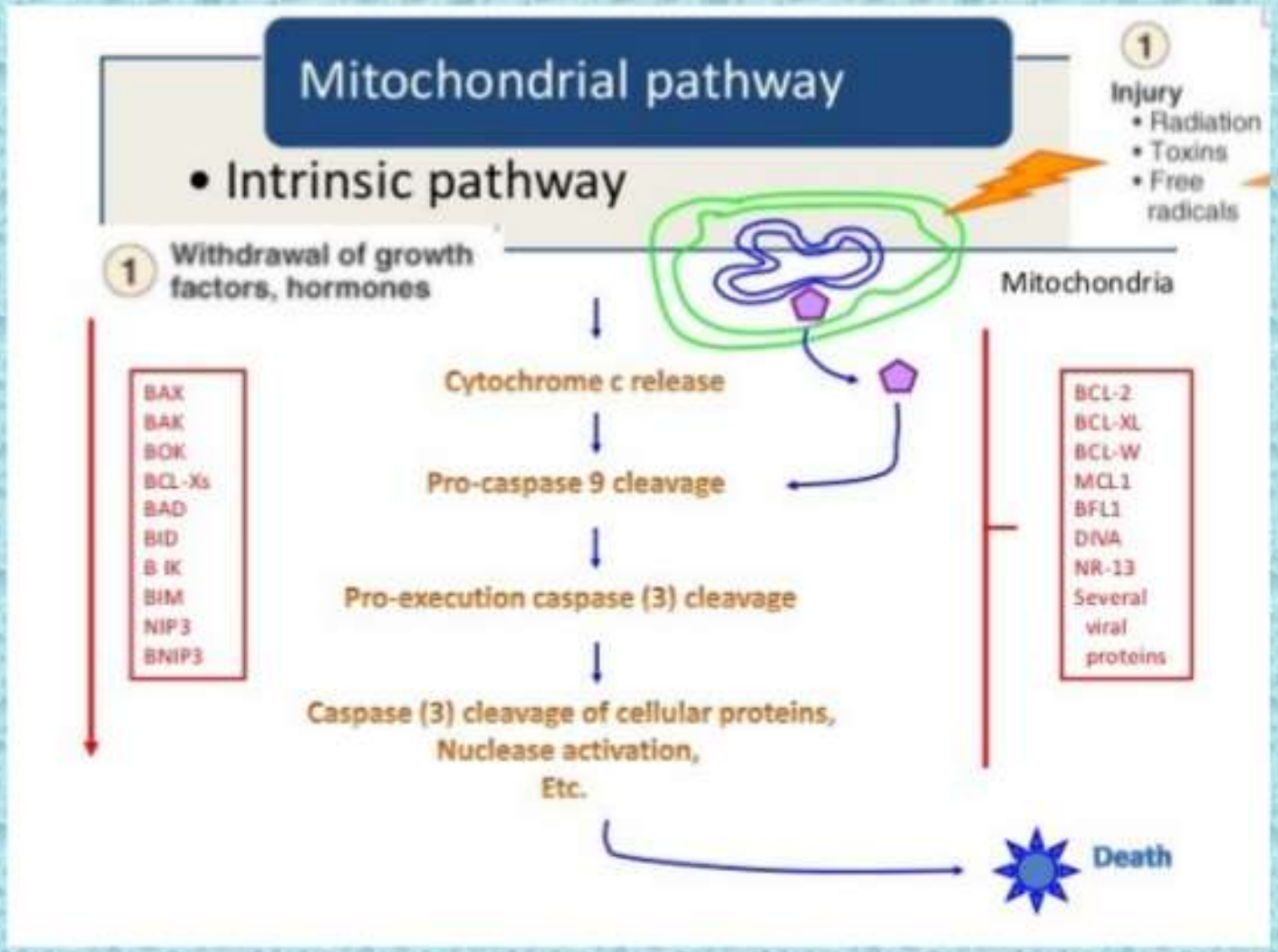
In case of intrinsic pathway, stress signal causes the binding of cytoplasmic proteins, BAX and BID to the outer membrane of mitochondria.

Another mitochondrial protein BAK interacts with BAX and BID causing release of cytochrome c into the cytosol. This binds to Apaf-1 which then forms apotosome that triggers the activation of procaspase-9. Activated caspase-9 further initiates the caspase cascade leading to apoptosis. Tumor suppressor p53 protein is a sensor of cellular stress and play a vital role in initiating apoptosis by transcriptionally activating proapoptotic proteins BID and BAX.

FLIP acts as an intracellular apoptosis-suppression protein, which can competitively bind to the Fas-associated death domain (FADD), the apoptosis-mediated protein in the death receptor apoptosis pathway, hence blocking the Fas–FasL mediated apoptosis pathway



The inhibitor of apoptosis proteins (IAPs) are a family of antiapoptotic proteins that **block cell death, in part, by inhibiting the downstream portion of the caspase activation pathways**.


Mitochondrial pathway
• Intrinsic pathway
1
Injury
• Radiation
• Toxins
• Free radicals
1
Withdrawal of growth factors, hormones
Mitochondria
BAX
BAK
BOK
BCL-Xs
BAD
BID
B IK
BIM
NIP3
BNIP3
Cytochrome c release
Pro-caspase 9 cleavage
Pro-execution caspase (3) cleavage
Caspase (3) cleavage of cellular proteins,
Nuclease activation,
Etc.
BCL-2
BCL-XL
BCL-W
MCL1
BFL1
DNA
NR-13
Several viral proteins
Death

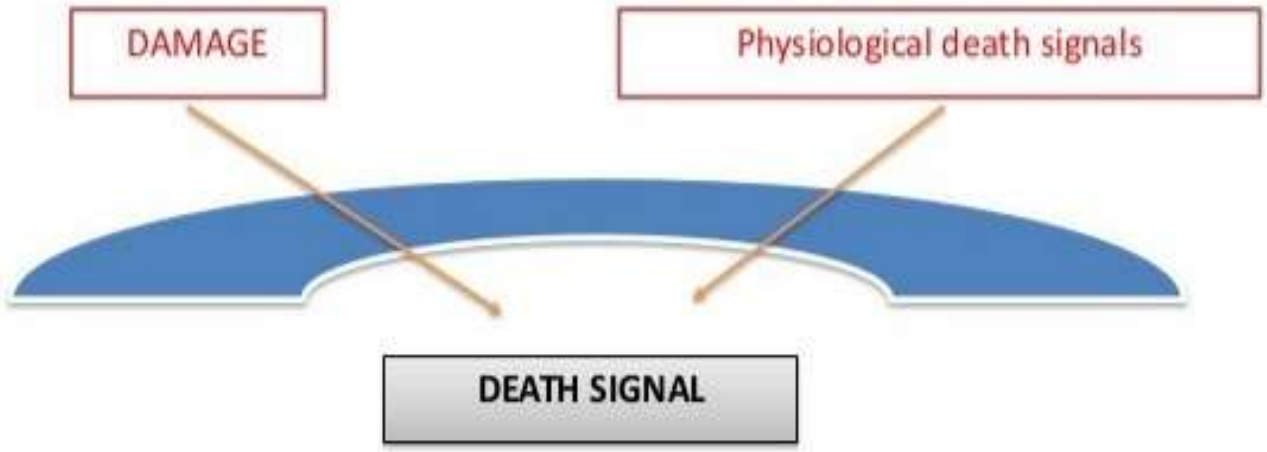
Intrinsic Pathway

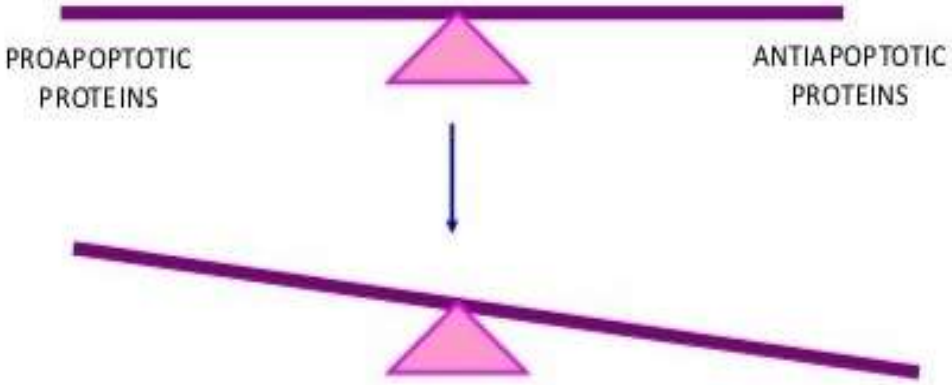
- Initiated from within the cell.
- Activated in response to signals such as DNA damage, loss of cell survival factors ,cell stress.
- Hinges on balance brtween pro and antiapoptotic signals of Bcl-2 family.
- Apaf-1,cytochrome-c,ATP(apoptosome) activate procaspase-9 complex.
- pro apoptotic proteins released which activate caspase proteases

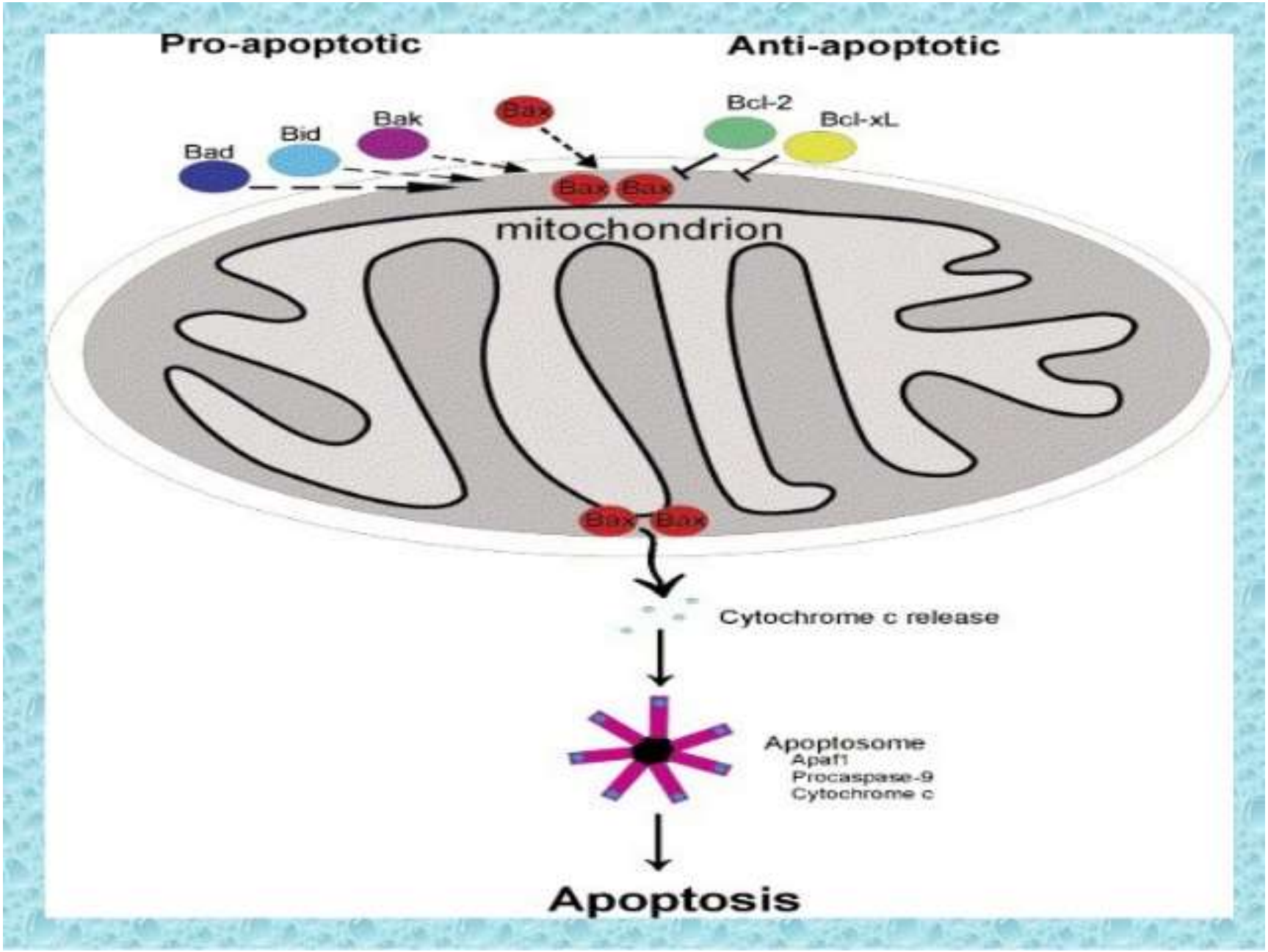
Extrinsic Pathway

- Begins outside the cells.
- Activation of death receptors (Fas-R,TNF-R ,DR-3,DRY/DR5) by death ligands (Fas-L,TNF-alpha,Apo3L,Apo2L)play major role.
- Death induced signalling compex (DISC) activated.
- On DISC activation same effctor pathway as intrinsic pathway is adopted

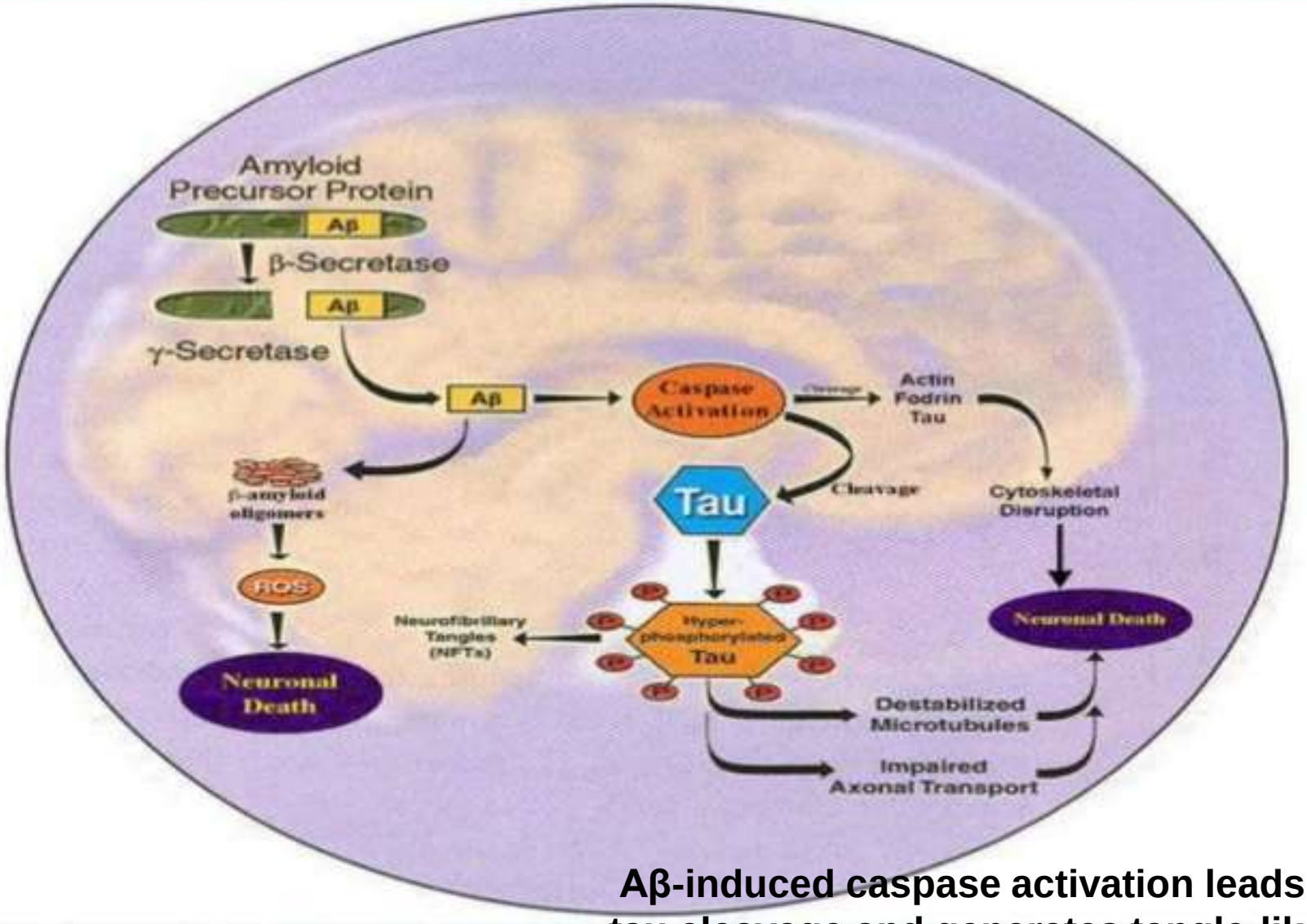
CELLS ARE BALANCED BETWEEN LIFE AND DEATH






Pro-apoptotic
Anti-apoptotic
Bad
Bid
Bak
Bax
Bcl-2
Bcl-xL
Bax Bax
mitochondrion
Bax Bax
Cytochrome c release
Apoptosome
Apaf1
Procaspase-9
Cytochrome c
Apoptosis

Role of Caspases in Alzheimer's Disease

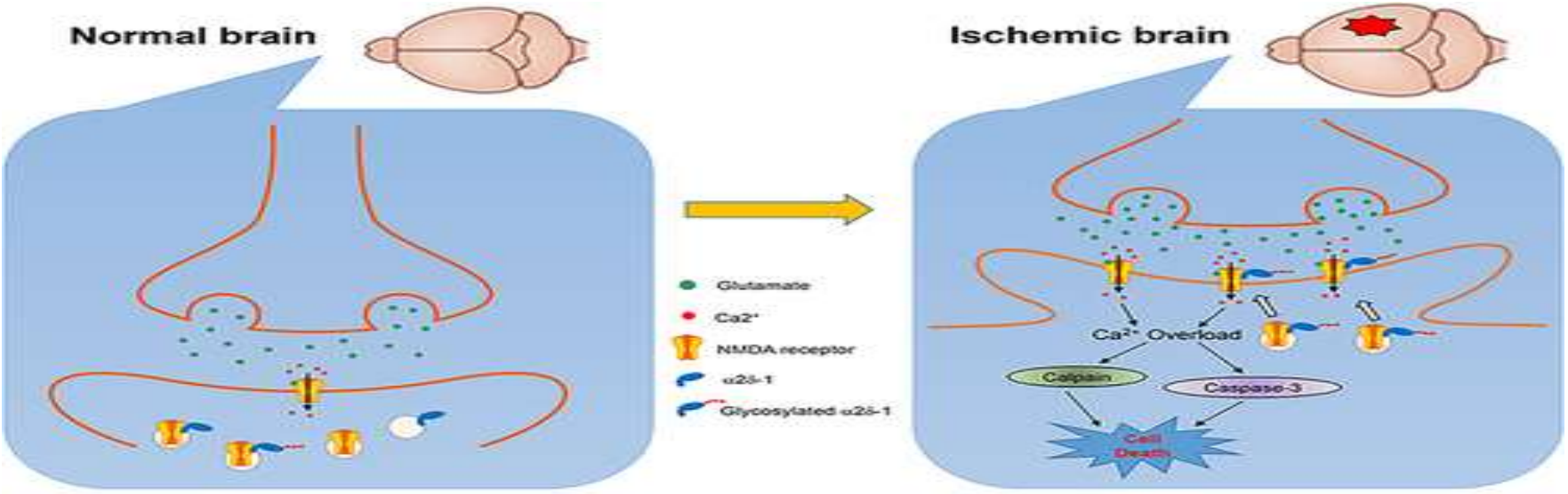


Aβ-induced caspase activation leads to tau cleavage and generates tangle-like morphology.

Table 2. Summary of caspases found to be activated in acute and chronic human neurological disease.

	CASPASE ACTIVATION
Parkinson's Disease	3?
Alzheimer's Disease	3
Huntington's Disease	1, 8
Amyotrphic Lateral Sclerosis	1, 3-like
Stroke	3
Brain/Spinal Cord Trauma	1, 3

Apoptosis may contribute to a significant proportion of neuron death following acute brain ischemia (ABI), but the underlying mechanisms are still not fully understood. **Brain ischemia may lead to stroke**, which is one of the main causes of long-term morbidity and mortality in both developed and developing countries.



Under normal conditions, the NMDA receptor is not bound to α2δ-1. Cerebral ischemia increases the glycosylation of α2δ-1 proteins and the α2δ-1–NMDA receptor interaction, which promote the synaptic trafficking of α2δ-1–bound NMDA receptor complexes and lead to neuronal NMDA receptor hyperactivity. NMDA receptor hyperactivity induces excessive calcium influx and subsequent activation of calpain and caspase-3, leading to cell death and brain infarction.

N-methyl-D-aspartate NMDA receptors are critically regulate a physiologic substrate for memory function in the brain

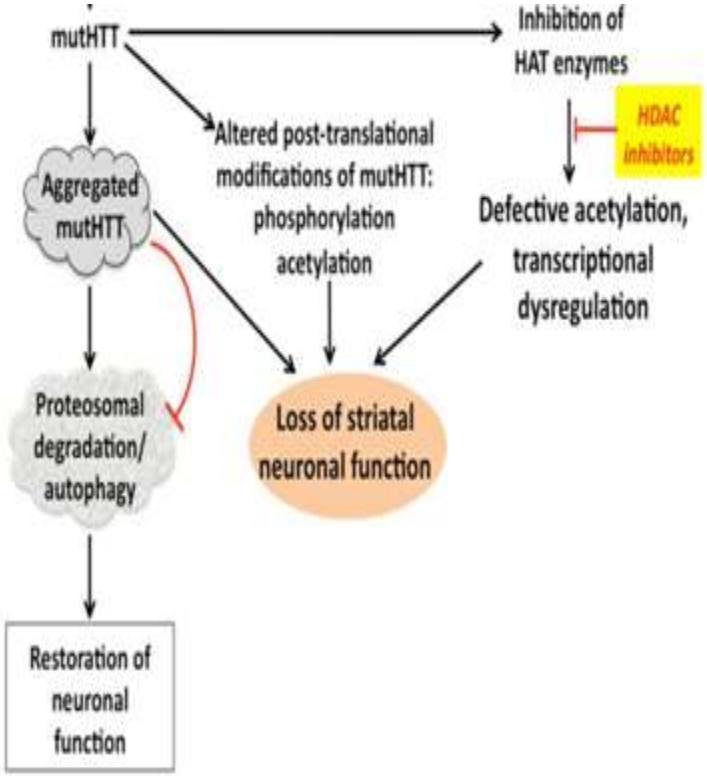
α2δ-1, commonly known as a voltage-activated Ca^{2+} channel subunit, is a binding site of gabapentinoids used to treat neuropathic pain and epilepsy

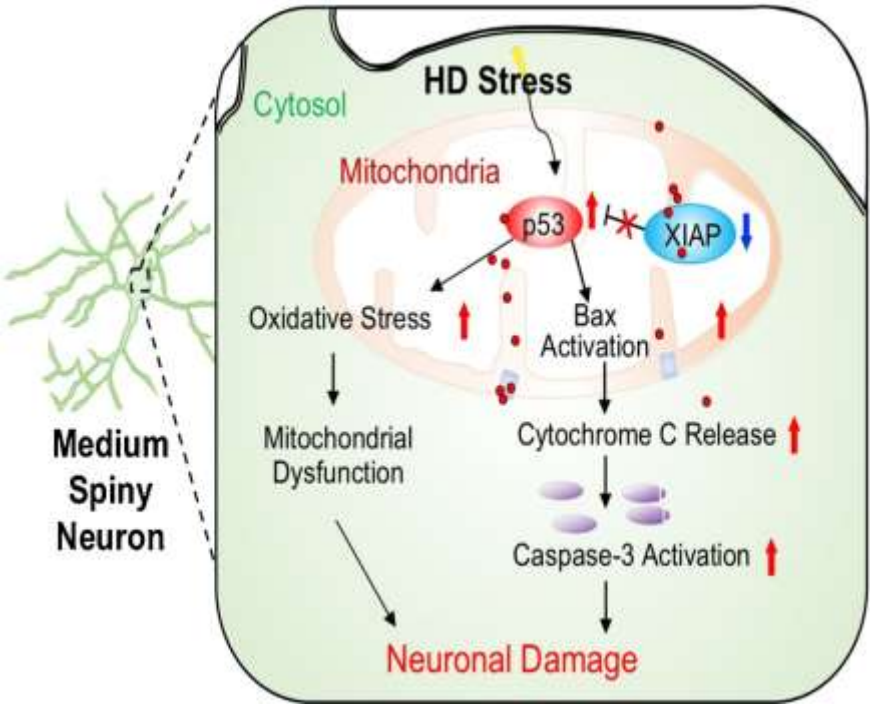
ALS affects the nerve cells that control voluntary muscle movements such as walking and talking (motor neurons). **ALS causes the motor neurons to gradually deteriorate, and then die**. Motor neurons extend from the brain to the spinal cord to muscles throughout the body. Early symptoms of ALS usually include **muscle weakness or stiffness**. Gradually all voluntary muscles are affected, and individuals lose their strength and the ability to speak, eat, move, and even breathe

there is increased expression or activation of caspases-1 and -3, and the dying motor neurones in human cases exhibit morphological features reminiscent of apoptosis.

Huntington's disease, which gets worse over time, attacks motor control regions of the brain (those involved with movement), as well as other areas. People with HD develop problems with behavior, emotion, thinking, and personality, along with uncontrollable dance-like movements (called chorea) and abnormal body posture

Mutations in the **HTT gene** cause Huntington disease. The HTT gene provides instructions for making a protein called huntingtin. Although the function of this protein is unclear, it appears to play an important role in nerve cells (neurons) in the brain





Apoptosis: Role in Disease

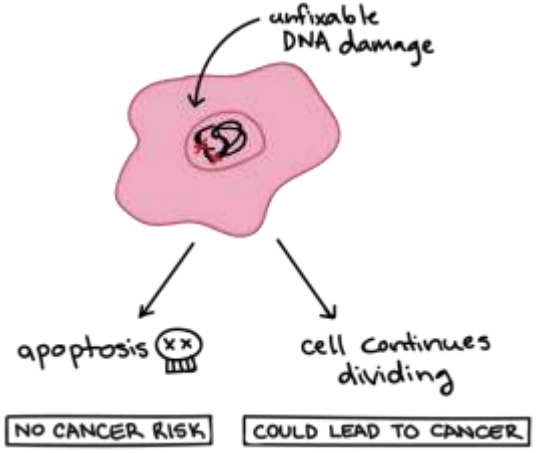
Cancer

- Apoptosis eliminates damaged cells (damage => mutations => cancer

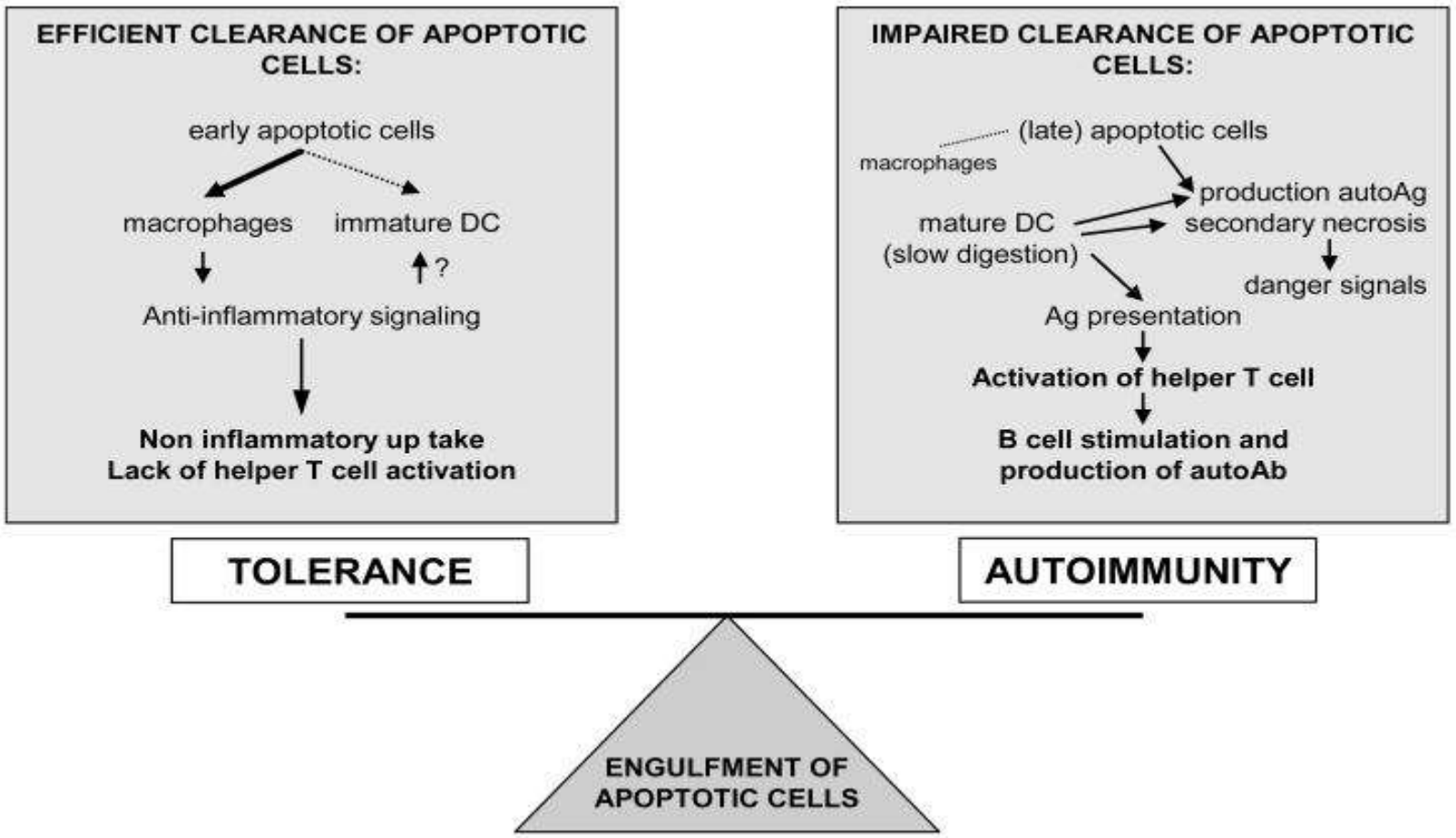
- Tumor suppressor p53 controls senescence and apoptosis responses to damage.

- Most cancer cells are defective in apoptotic response(damaged, mutant cells survive)

- High levels of anti-apoptotic proteins
  or
- Low levels of pro-apoptotic proteins ===> CANCER

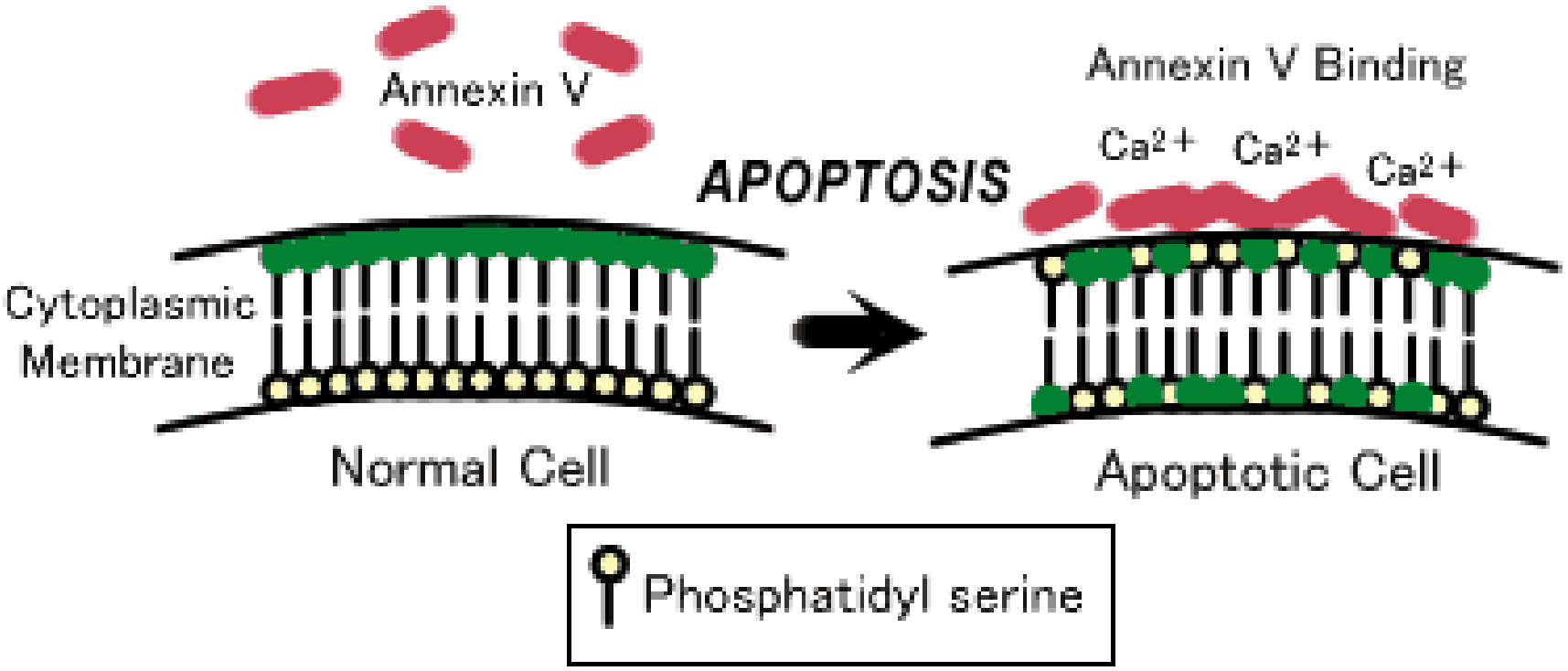


Apoptosis and autoimmunity



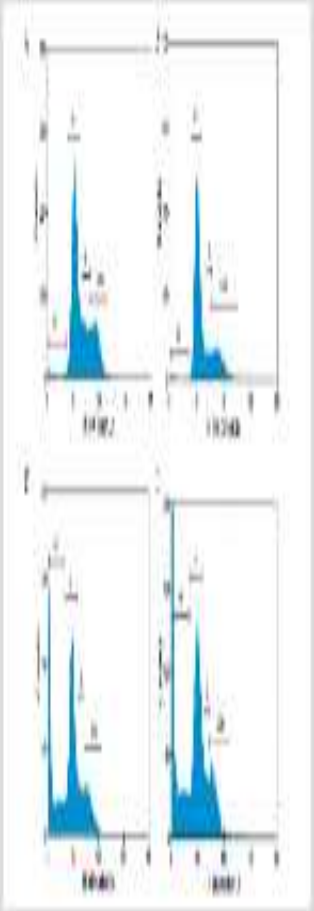
The uptake of dying cells is usually followed by an anti-inflammatory response and not associated with loss of tolerance. However, an ineffective removal of apoptotic cells is related to generation of “neo-antigensf that, presented to the T cells by mature DC can stimulate the production of autoantibodies.

Apoptosis assay:

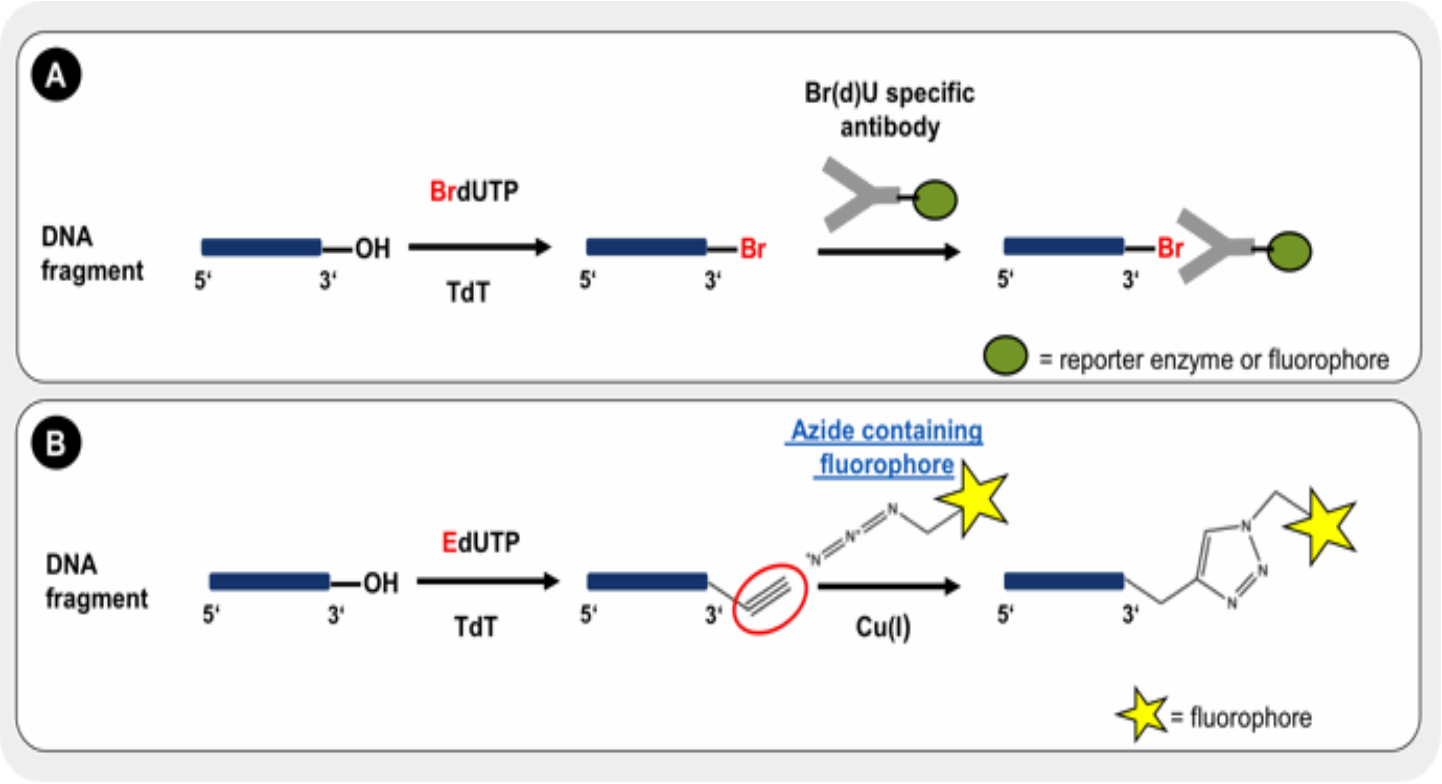


1 2 3

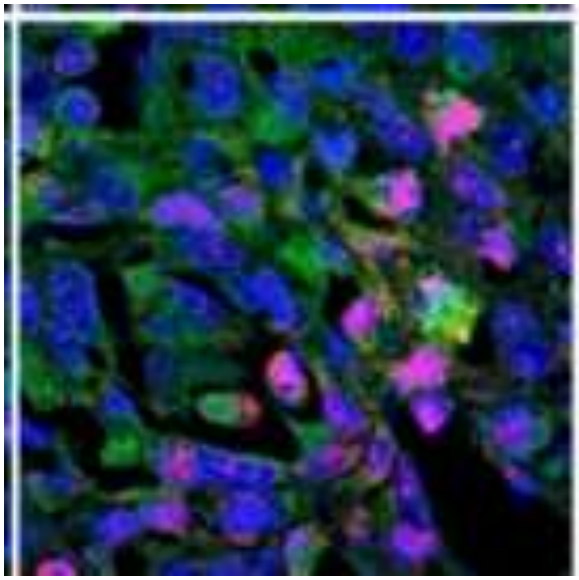
Quick Detection of Apoptotic DNA Ladder in Jurkat Cells. Apoptosis was induced in Jurkat cells with camptothecin (2 μM) for 0 hr (Lane 1), 6 hrs (Lane 2) and 12 hrs (Lane 3). Chromosomal DNA was prepared using the Quick Apoptotic DNA Ladder Detection Kit according to the kit instructions. 20 μl of each sample was electrophoresed on a 1.2% agarose/EtBr gel.



Cell cycle analysis of HepG2 cancer cells treated with oleanolic acid. The distribution of cells undergoing apoptosis and in various phases of the cell cycle was determined in HepG2 cells following treatment with oleanolic acid at various concentrations. (A) G0/G1-5%, S-30%, G2M-65%; (B) G0/G1-15%, S-25% G2M-60%; (C) G0/G1-38%, S-20%, G2M-42%; (D) G0/G1-55%, S-15%, G2M-30%; representing treatment with 0, 5, 25 and 50 µM, respectively. Values are presented as the mean ± standard error of mean of three determinations. PI, propidium iodide.



The principle of TUNEL assay relies on terminal deoxynucleotidyl transferase (TdT)-mediated addition of a modified dUTP (X-dUTP) to 3'-OH ends of DNA fragments that are generated as a result of apoptosis induction. To avoid the loss of fragmented DNA and to allow enzyme and nucleotide entrance, cells need to be fixed and subsequently permeabilized prior to the labeling reaction. Incorporated bromoylated dUTP (BrdUTP) is detected by specific antibody conjugates with a reporter enzyme or fluorescent dye (A). The incorporation of alkyne-containing dUTP (EdUTP) is visualized by Cu(I)-catalyzed alkyne-azide click chemistry (CuAAC) with an azide containing fluorphore.



Red: TUNEL signal

Green: pan cytokeratin

Blue: DAPI

Autophagy as type II cell death is the natural, conserved degradation of the cell that removes unnecessary or dysfunctional components through a lysosome-dependent regulated mechanism.

Although initially characterized as a primordial degradation pathway induced to protect against starvation, it has become increasingly clear that autophagy also plays a major role in the homeostasis of non-starved cells.

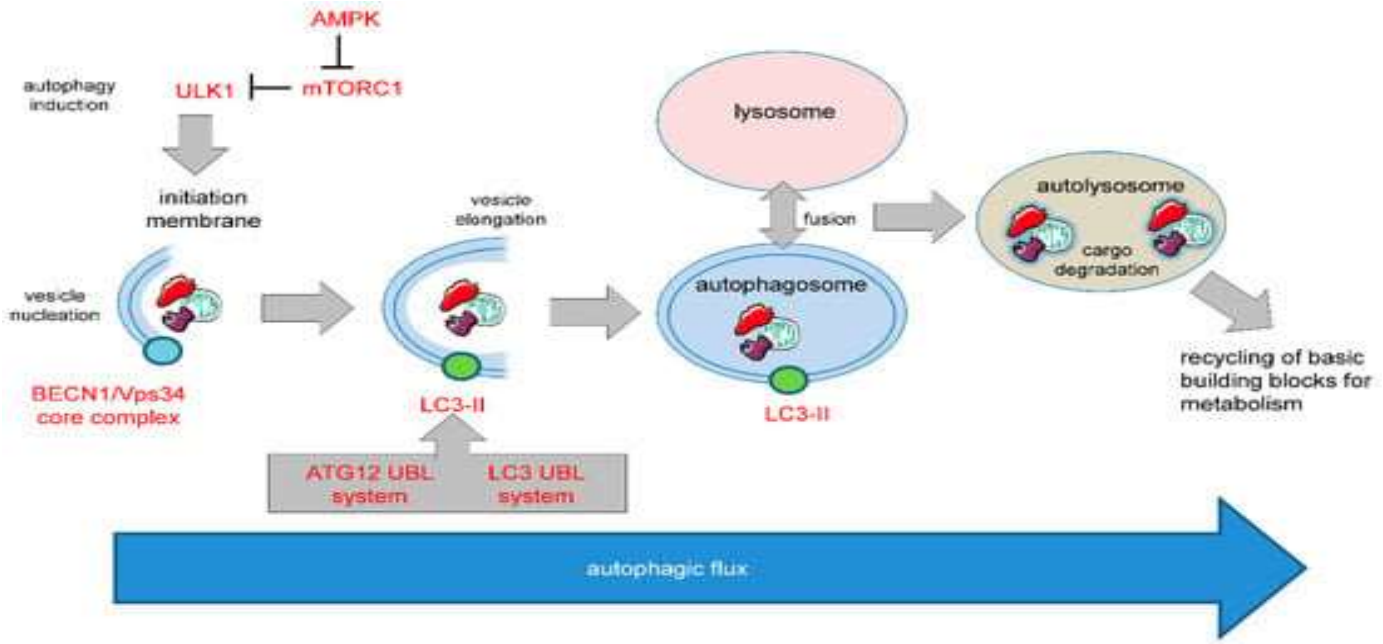
Defects in autophagy have been linked to various human diseases, including neurodegeneration and cancer, and interest in modulating autophagy as a potential treatment for these diseases has grown rapidly

Macroautophagy, is the main pathway, used primarily to eradicate damaged cell organelles or unused proteins.First the phagophore engulfs the material that needs to be degraded, which forms a double membrane known as an autophagosome, around the organelle marked for destruction.The autophagosome then travels through the cytoplasm of the cell to a lysosome in mammals, or vacuoles in yeast and plants,and the two organelles fuse.Within the lysosome/vacuole, the contents of the autophagosome are degraded via acidic lysosomal hydrolase.

Microautophagy, on the other hand, involves the direct engulfment of cytoplasmic material into the lysosome.This occurs by invagination, meaning the inward folding of the lysosomal membrane, or cellular protrusion

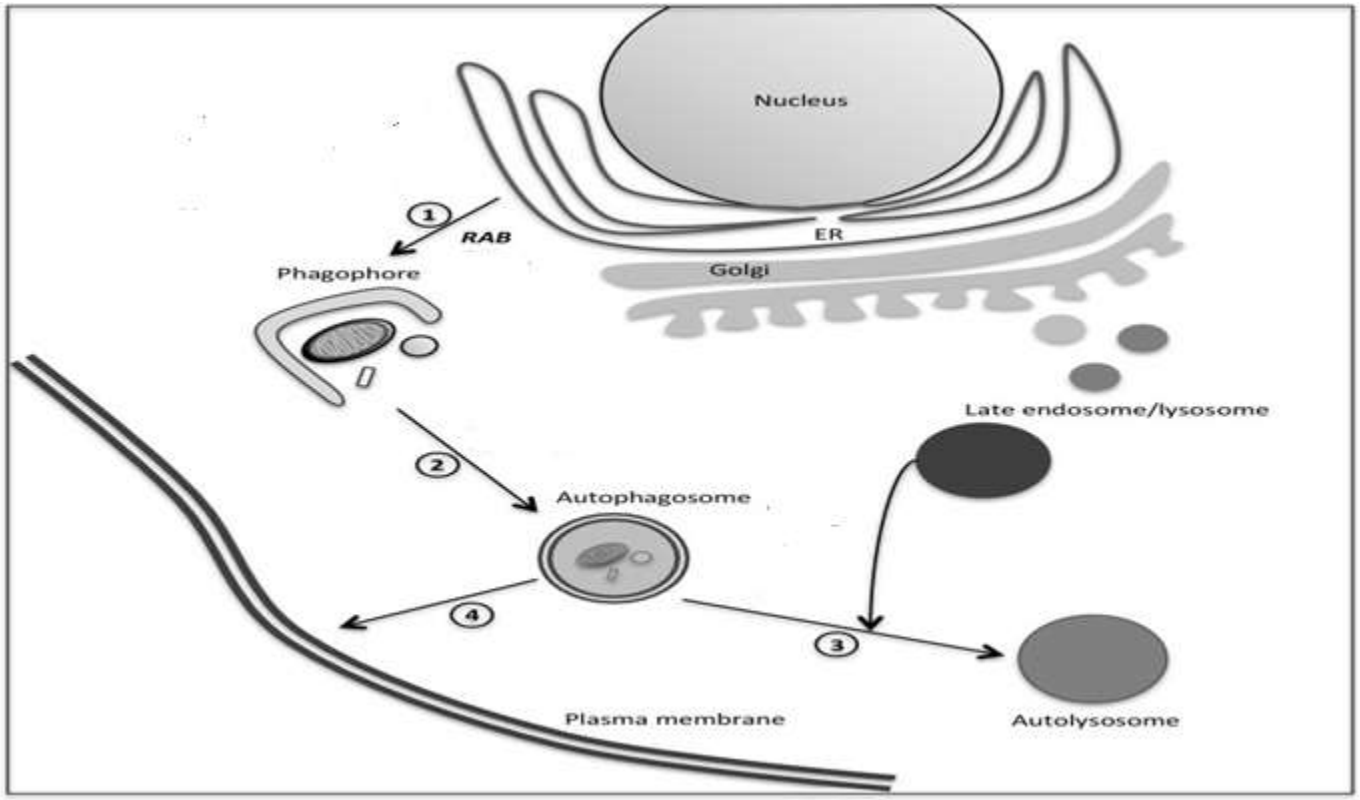
Chaperone-mediated autophagy is a very complex and specific pathway, which involves the recognition by the hsc70-containing complex. This complex then moves to the lysosomal membrane-bound protein that will recognise and bind with the CMA receptor. Upon recognition, the substrate protein gets unfolded and it is translocated across the lysosome membrane with the assistance of the lysosomal hsc70 chaperone.CMA is significantly different from other types of autophagy because it translocates protein material in a one by one manner, and it is extremely selective about what material crosses the lysosomal barrier.

Mitophagy is the selective degradation of mitochondria by autophagy. It often occurs to defective mitochondria following damage or stress. Mitophagy promotes the turnover of mitochondria and prevents the accumulation of dysfunctional mitochondria which can lead to cellular degeneration.



It requires the formation of double-membrane-containing autophagosomes that sequester proteins, lipids, organelles or invasive microbes and fuse with lysosomes for digestion of content by acidic hydrolases. ULK1, a protein kinase serving as the central initiator of autophagy, is inhibited by the mTORC1 complex that contains mTOR. AMPK serves as a nutrient sensor and negative regulator of mTORC1. Autophagosome biogenesis starts with the formation of an initiation membrane that is derived either from the endoplasmatic reticulum (ER) or from several other cellular membrane sources. Vesicle nucleation is promoted by the BECN1/Vps34 core complex containing the lipid kinase Vps34. Vesicle elongation is regulated by the two ubiquitin-like conjugation systems (UBLs) ATG12-UBL and LC3-UBL that cooperate to catalyze the conjugation of phosphatidylethanolamine (PE) to LC3 and facilitate the conversion of cytosolic LC3-I into a membrane-associated LC3-II that is translocated to the autophagosomal membrane. Following vesicle closure, mature autophagosomes fuse with lysosomes to generate autolysosomes that digest the autophagosomal content by lysosomal proteases for cellular recycling

Autophagic vacuoles (AVs) **arise when membranes of the ER sequester parts of the cytoplasm, forming a new, double-membraned vacuole, to which lysosomal enzymes are then delivered**.



(1) phagophore formation from specialized regions of the endoplasmic reticulum; (2) phagophore elongation and sealing of the isolated membrane to generate a doublemembrane compartment called autophagosome; and (3) autophagosome maturation through interactions with late endosomes and lysosomes. (4) Autophagosomes may also fuse with the plasma membrane in order to release their content to the extracellular space.

Cellular aging and Replicative senescence

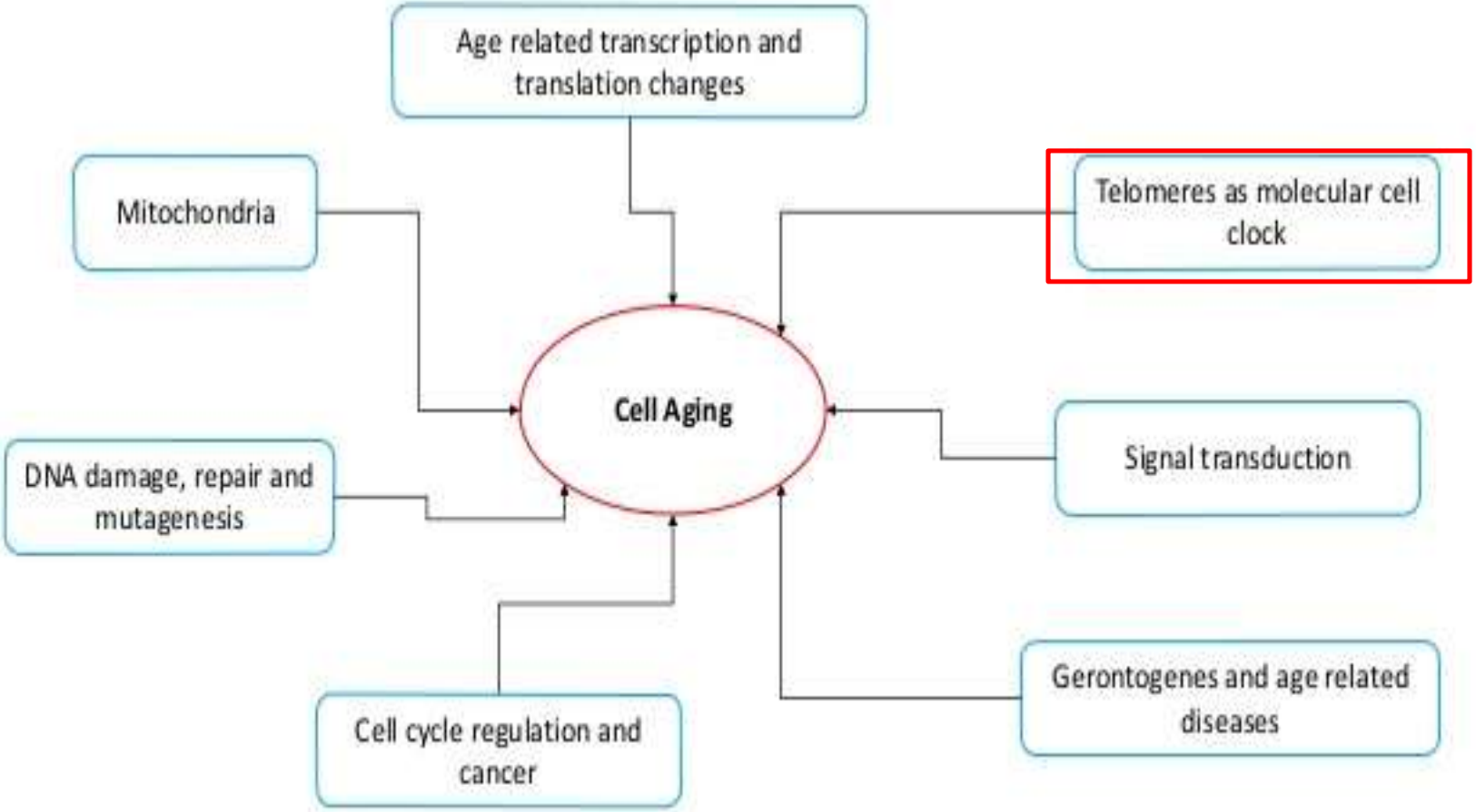
(Graphics are from internet)

Cellular ageing is generally defined as the progressive decline in the resistance to stress and other **cellular** damages, causing a gradual loss of **cellular** functions and resulting eventually in **cell** death. Replicative **ageing**, which refers to the limited number of divisions that a single **cell can** attain

Scientists now know that many factors – including physical exercise, sleep, depression, and certain gene mutations – are associated with reduced telomere length, and, by extension, can lead to premature biological **aging**

Introduction

- Aging is generally characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased risk of aging-associated diseases.
- Death is the ultimate consequence of aging.
- Differences in maximum life span between species correspond to different "rates of aging".
- A degenerative process, only.
- Has no positive features.



The main factors acting in aging process

Molecular Gene Theories

- **Codon restriction** - Fidelity/accuracy of mRNA translation is impaired due to inability to decode codons in mRNA.
- **Error catastrophe** - Fidelity of gene expression declines with age, resulting in increased fraction of abnormal proteins.
- **Somatic mutation** - Accumulation of molecular damage, primarily to DNA/genetic material.
- **Dys-differentiation** - Gradual accumulation of random molecular damage impairs regulation of gene expression.
  - **Gene regulation** - Aging caused by changes in gene expression regulating both aging and development. Gene expression protein folding and activity

Cellular Theories

- **Free radical** - Oxidative metabolism produces highly reactive free radicals that subsequently damage protein and DNA. Mitochondrial DNA Damage
- **Wear and tear** - Accumulation of normal injury – Glycooxidation Theory of Aging (products from glucose with proteins + oxidation; AGE (advanced glycation end products – Inflammation Theory of Aging
- **Apoptosis** - Programmed cell death resulting from intrinsic damage and genetically determined events or genome crisis.

- **Senescence** - Phenotypes of aging are caused by an increase in frequency of senescent cells. Senescence may be the result of telomere loss (replicative senescence) or cell stress (cellular senescence).

System Theories

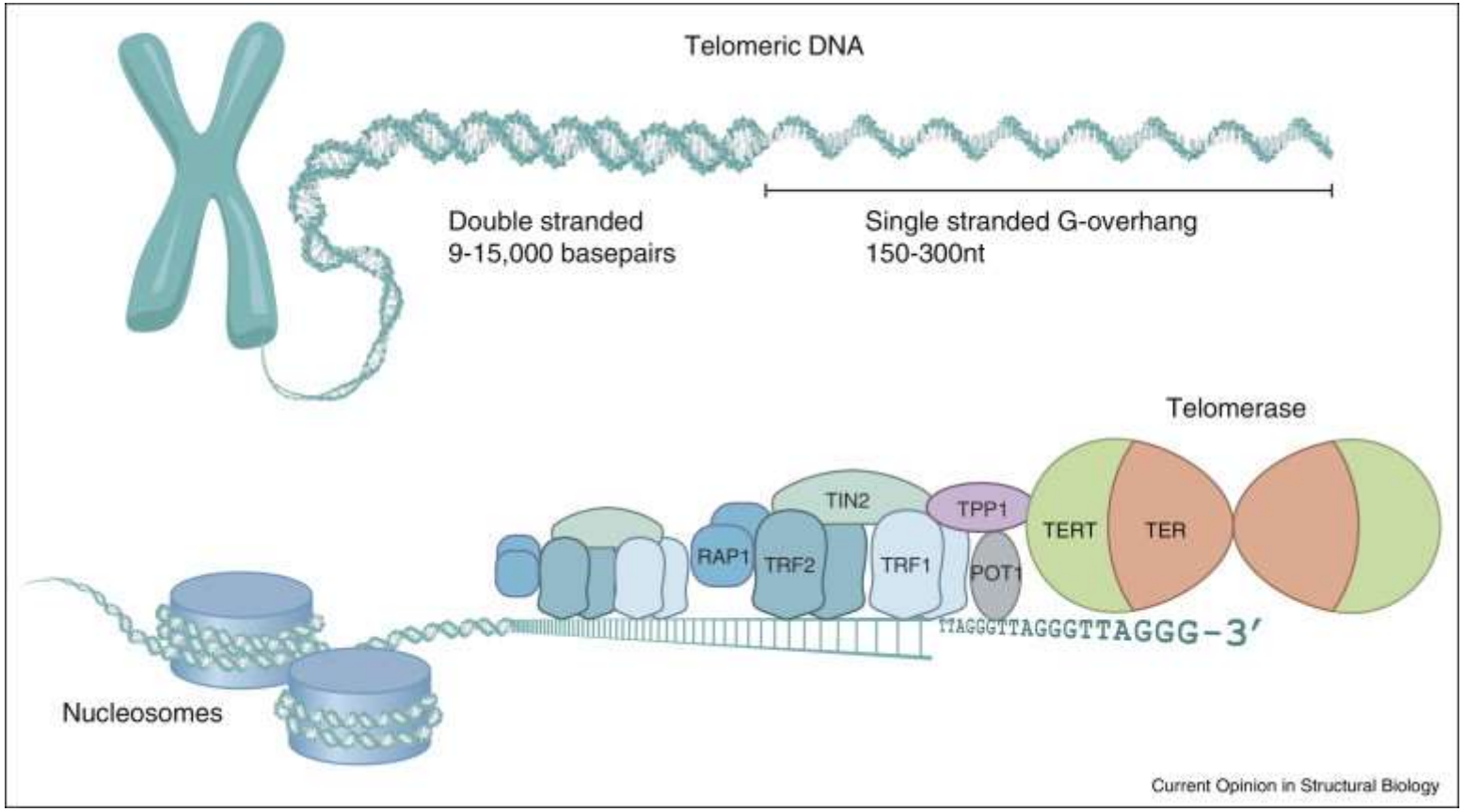
- **Rate-of-living** - Assumes a fixed amount of metabolic potential for every living organism (live fast, die young).
- **Neuroendocrine** - Alterations in neuroendocrine control of homeostasis results in age-related physiological changes also referred as Neuroendocrine Theories of Aging.
- **Immunologic** – decline of immune function with age results in increased incidence of disease also referred as Immunological Theory of Aging

Evolutionary Theories

- **Disposable Soma** - Somatic cells are maintained only to ensure continued reproductive success, following reproduction the soma is disposable. (life span theory)
- **Antagonistic Pleiotropy** - Genes that are beneficial at younger ages are deleterious at older ages.
- **Mutation Accumulation** - Mutations that affect health at older ages

DNA in eukaryotic cells is linear and contain mostly non coding region

The shelterin complex



Telomere shortening causes cell senescence

- Somatic cells usually lack telomerase activity, which means that telomeres shorten with each cell division.

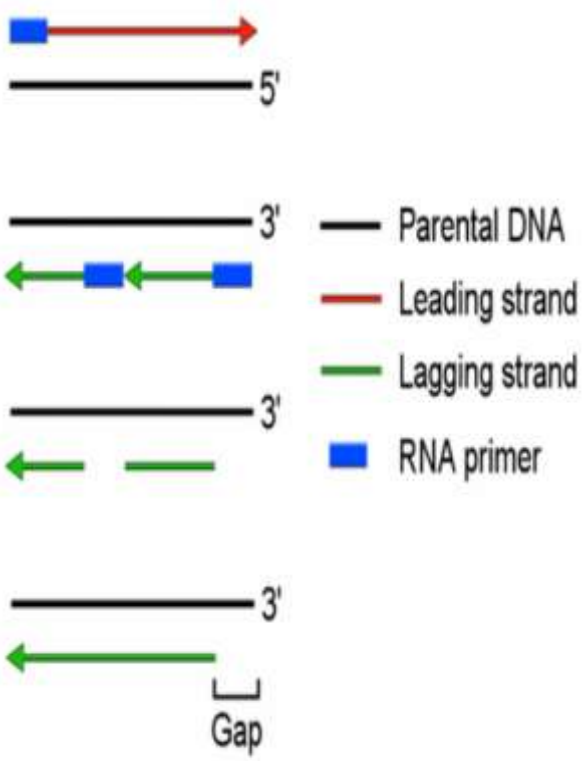
- Cultured cells may go into crisis as the result of reaching zero telomere length.

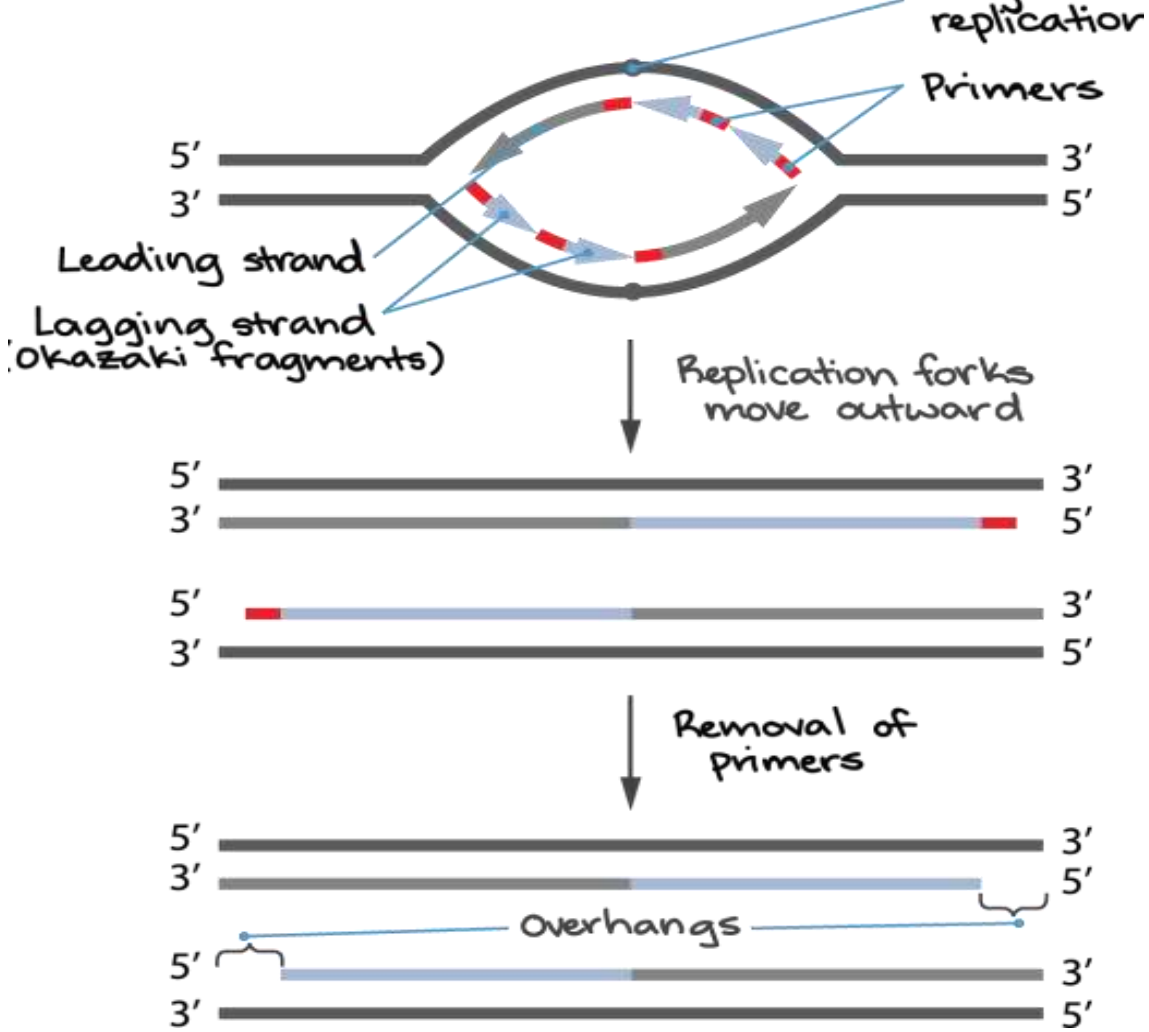
- Reactivation of telomerase enables cells to survive crisis and to become immortal.

Telomeres are sections of DNA found at the ends of each of our chromosomes[?]. They consist of the same **sequence** of bases repeated over and over. In humans the **telomere sequence** is TTAGGG. This **sequence** is usually repeated about 3,000 times and can reach up to 15,000 base pairs in length

End replication problem

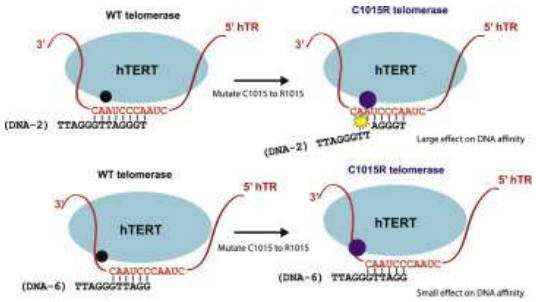
Unlike bacterial chromosomes, the chromosomes of eukaryotes are linear (rod-shaped), meaning that they have ends. These ends pose a problem for DNA replication. The DNA at the very end of the chromosome cannot be fully copied in each round of replication, resulting in a slow, gradual shortening of the chromosome. Why is this the case? When DNA is being copied, one of the two new strands of DNA at a replication fork is made continuously and is called the **leading strand**. The other strand is produced in many small pieces called Okazaki fragments, each of which begins with its own RNA primer, and is known as the **lagging strand**.



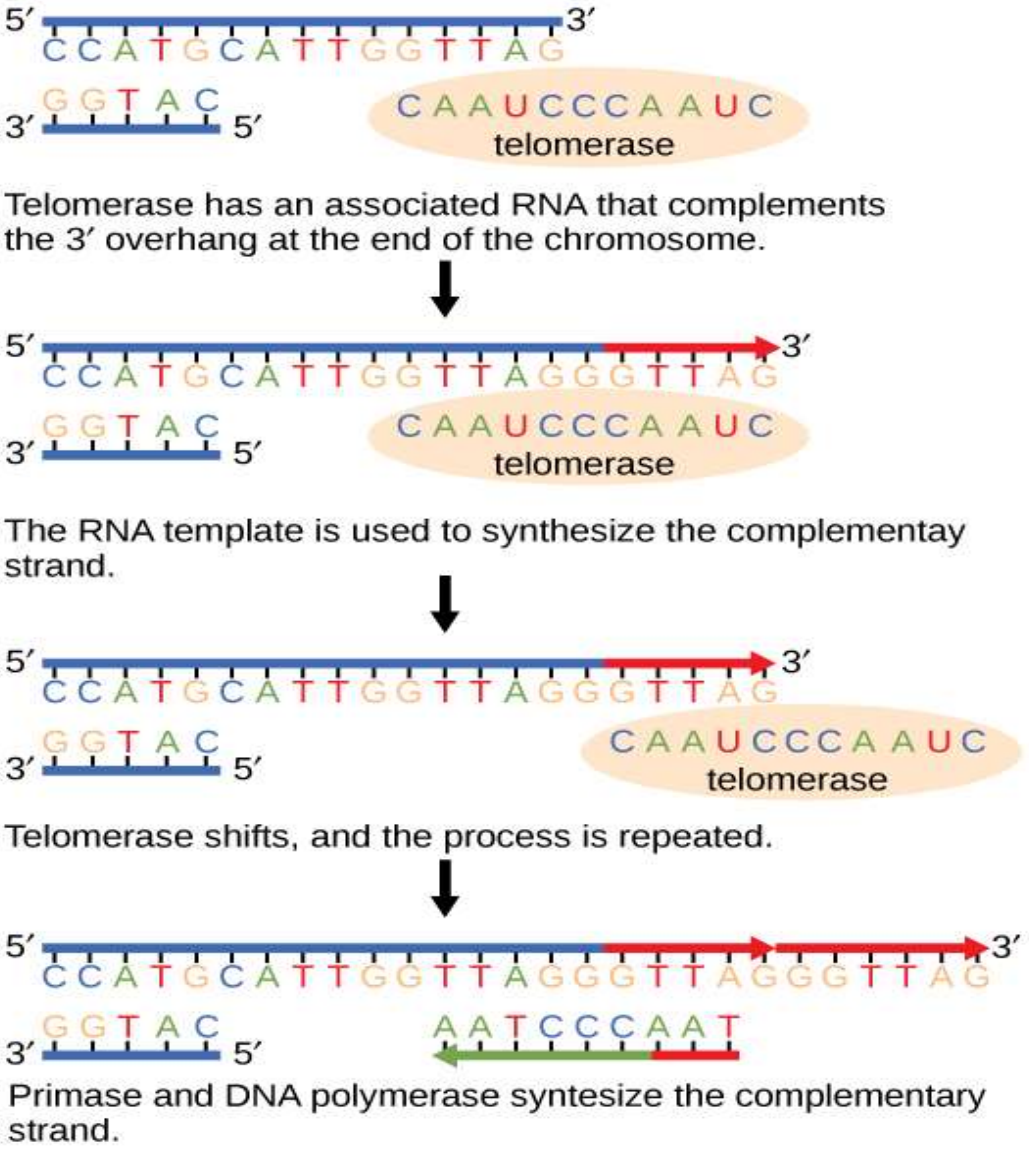


To prevent the loss of genes as chromosome ends wear down because of the end-replication problem, the tips of eukaryotic chromosomes have specialized DNA “caps” called telomeres. Telomeres consist of hundreds or thousands of repeats of the same short DNA sequence that protect the ends of chromosomes

Telomerase activity



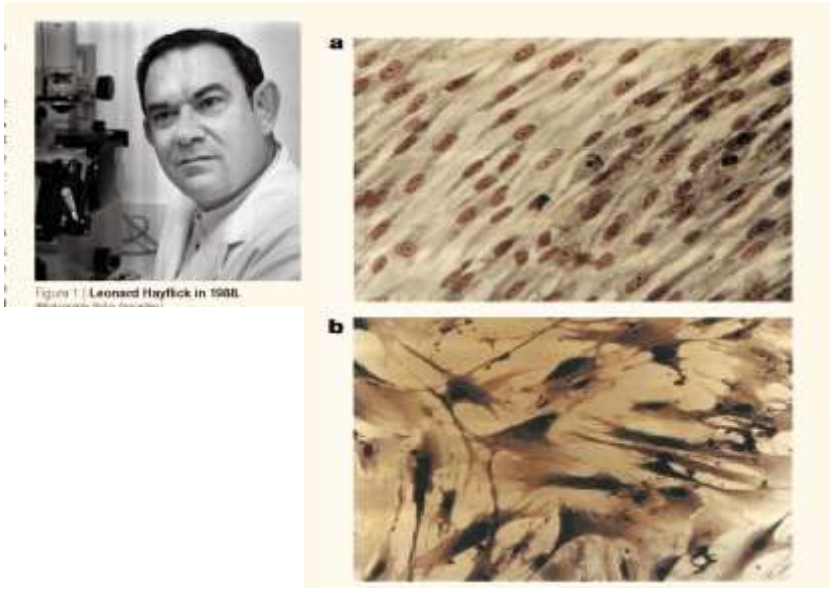
Telomerase is a <u>reverse transcriptase</u> enzyme that carries its own RNA molecule which is used as a template when it elongates telomeres. Telomerase is active in normal stem cells, in gametes and most cancer cells, but is normally absent from, or at very low levels in, most somatic cells.



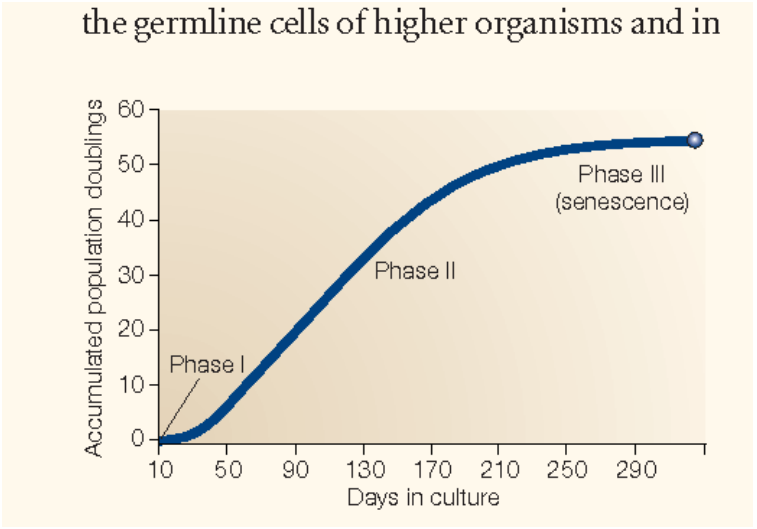
Telomerase is important for maintaining chromosome integrity: The ends of linear chromosomes are maintained by the action of the telomerase enzyme.

Hayflick limit and cellular ageing

Almost 40 years ago, Leonard Hayflick discovered that cultured normal human cells have limited capacity to divide, after which they become senescent — a phenomenon now known as the ‘Hayflick limit’. Hayflick's findings were strongly challenged at the time, and continue to be questioned in a few circles, but his achievements have enabled others to make considerable progress towards understanding and manipulating the molecular mechanisms of ageing

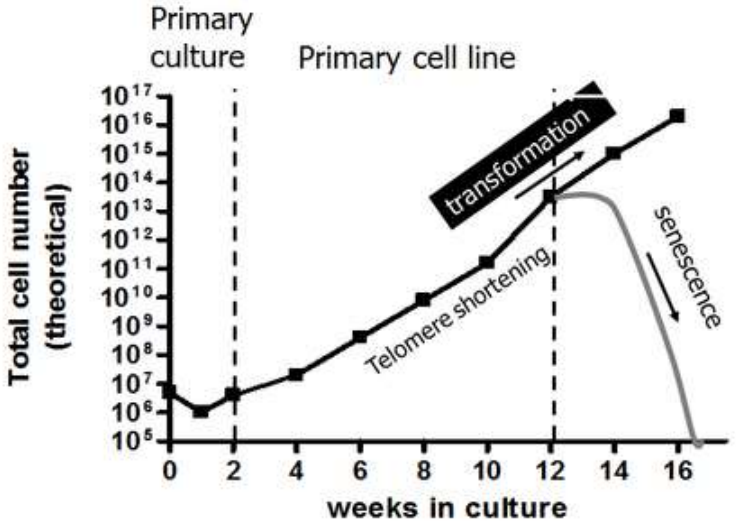


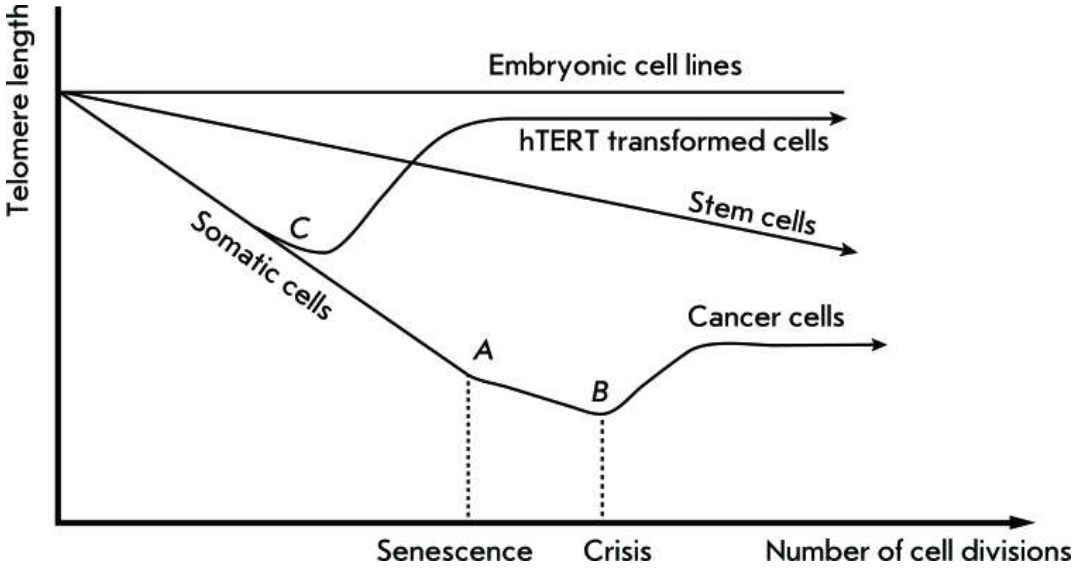
Young and old human diploid cells (strain WI-38). a | Young cells in phase II at population doubling 20. b | Old cells in phase III at population doubling 55



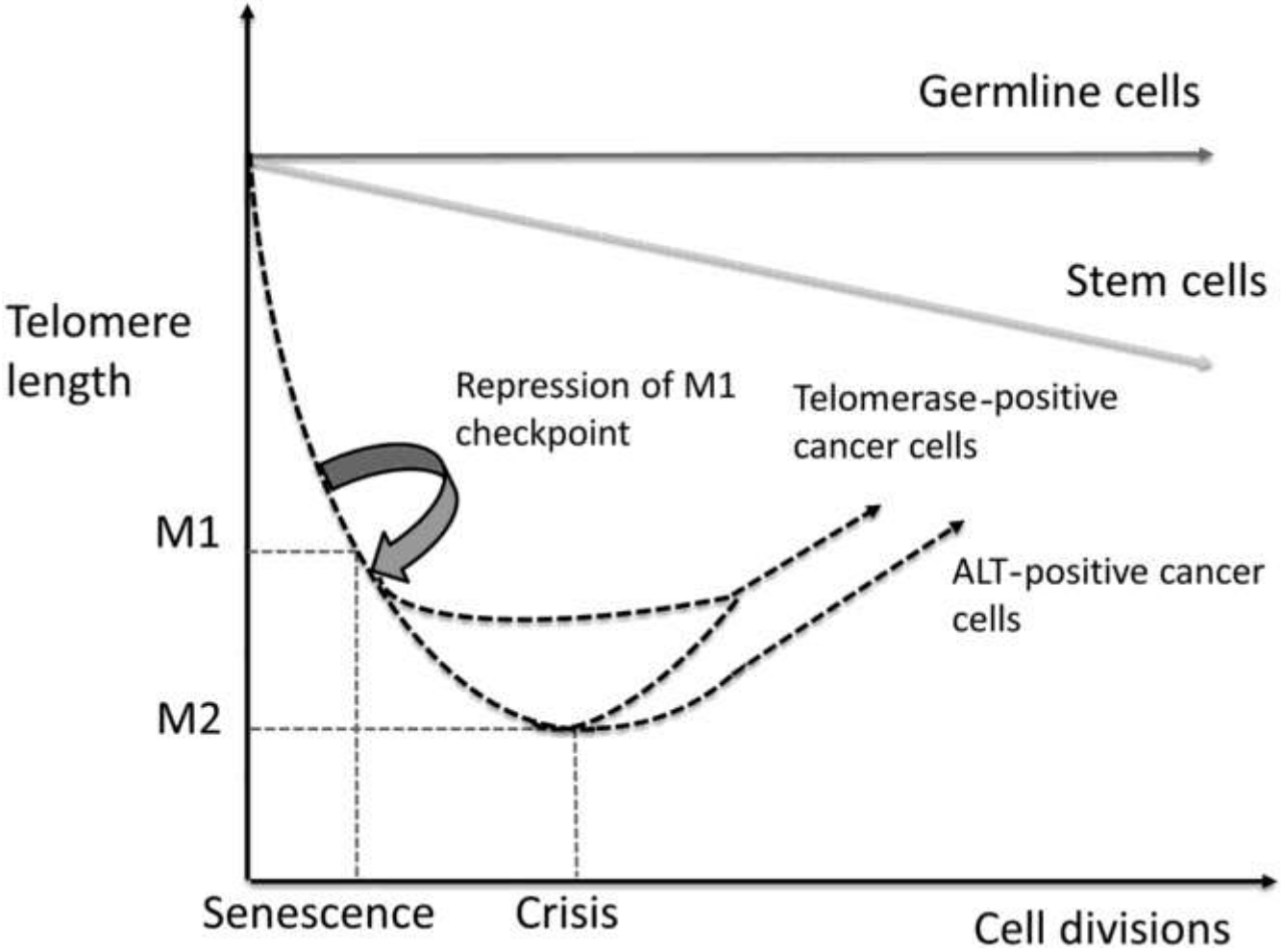
Hayflick’s three phases of cell culture. Phase I is the primary culture; phase II represents subcultivated cells during the period of exponential replication. Phase III represents the period when cell replication ceases but metabolism continues. Cells may remain in this state for at least one year before death occurs.

Hayflick limit- finite number of cell divisions





Alternative lengthening of telomeres (ALT) is a telomerase-independent but recombination-dependent process that extends telomeres.



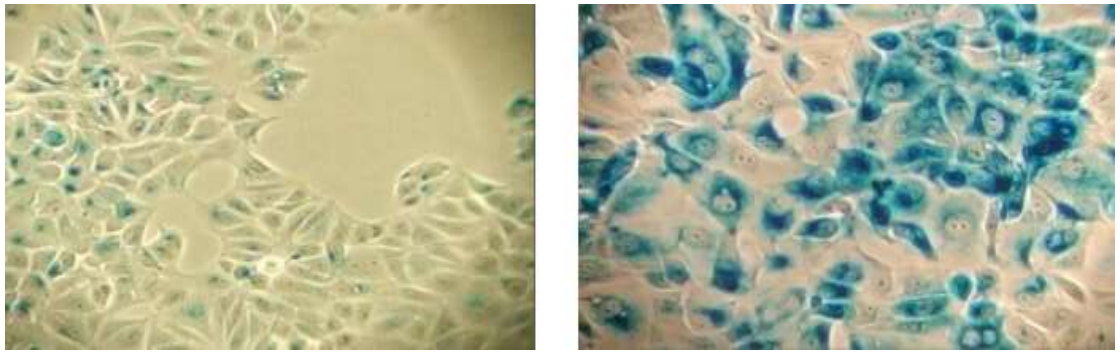
Cellular Senescence

What Is Senescence?

- **Cellular senescence refers to a state of stable cell cycle arrest in which proliferating cells become resistant to growth-promoting stimuli, typically in response to DNA damage.**
- **Senescence was first described by Leonard Hayflick upon the observation that human fetal fibroblasts eventually stopped dividing, but remained viable and metabolically active after prolonged time in culture.**
- **It is now generally accepted that only transformed malignant cells replicate indefinitely, while non-transformed cells do not, with the exception of cell types with stem-like properties. These include endogenous germline and somatic stem cells, in addition to embryonic or induced pluripotent stem cells developed under controlled in vitro conditions.**
- **Senescent cells are distinct from both quiescent cells which can reenter the cell cycle and from terminally differenti ated cells.**
- **Senescent cells are characterized by morphological and metabolic changes, chromatin reorganization, altered gene expression, and adoption of a pro-inflammatory phenotype known as the senescence-associated secretory phenotype (SASP).**

The biological role of senescence is complex as both protective and deleterious effects of senescent cells have been described, largely dependent upon physiological context. For example, while senescence has likely evolved as a mechanism to avoid malignant transformation of damaged cells, the onset of senescence may contribute to many age-associated pathologies, including cancer, tissue degeneration and inflammatory diseases.

The terms aging and cellular senescence cannot be used interchangeably. Aging is a progressive decline with time whereas senescence occurs throughout the lifespan, including during embryogenesis. The number of senescent cells increases with age, but senescence also plays an important role during development as well as during wound healing



β-Galactosidase staining detects expression of pH-dependent β-galactosidase activity (cells stained in blue), a known characteristic of senescent cells. Normal cells (left panel); Senescent cells (right panel).

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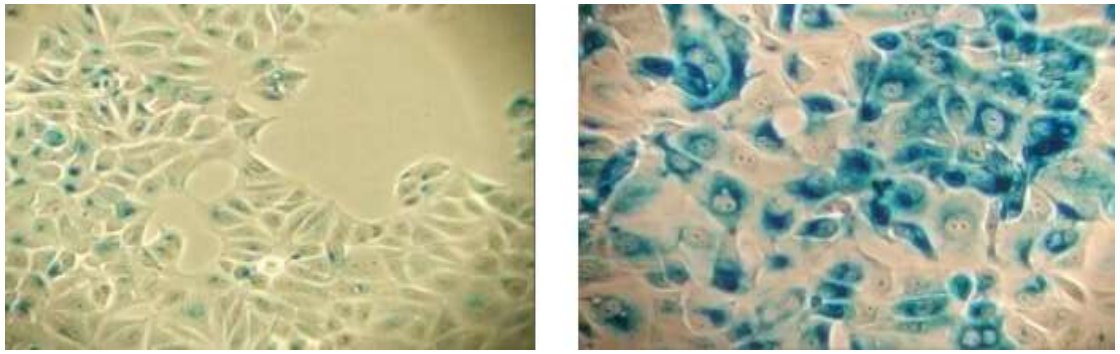


β-Galactosidase staining detects expression of pH-dependent β-galactosidase activity (cells stained in blue), a known characteristic of senescent cells. Normal cells (left panel); Senescent cells (right panel).

DNA Damage-Induced Senescence

DNA damage triggers the DNA repair machinery, apoptosis, or senescence depending on the extent of damage and physiological context. Senescent cells are characterized by a persistent DNA damage response (DDR), including chronic ATM (Ataxia Telangiectasia mutated) and ATR (Ataxia Telangiectasia and Rad3 related) kinase signaling which ultimately invokes cell cycle arrest and senescence through activation of the p53/p21 and p16/pRb pathways. Persistent DNA damage and subsequent senescence can also be induced by ionizing radiation, chemotherapeutics, genotoxic stress, and oxidative stress.

Oncogene-Induced Senescence

Cellular senescence is induced in response to oncogenic signaling as a potent cell autonomous anti-cancer mechanism. Senescence occurring in cells with oncogenic signaling is a response intended to prevent their transformation to malignant cells. Oncogene-induced senescence (OIS) results from the hyperactivation of oncogenes like H-Ras or the inactivation of tumor suppressors such as PTEN. For example, expression of H-RASV12, an oncogenic form of the GTPase H-RAS, triggers OIS by inducing chronic p38 mitogen-activated protein kinase (p38 MAPK) signaling. Strong mitogenic signaling can also induce DNA damage via replication stress which triggers the collapse of stalled replication forks.

Biomarkers of Senescence

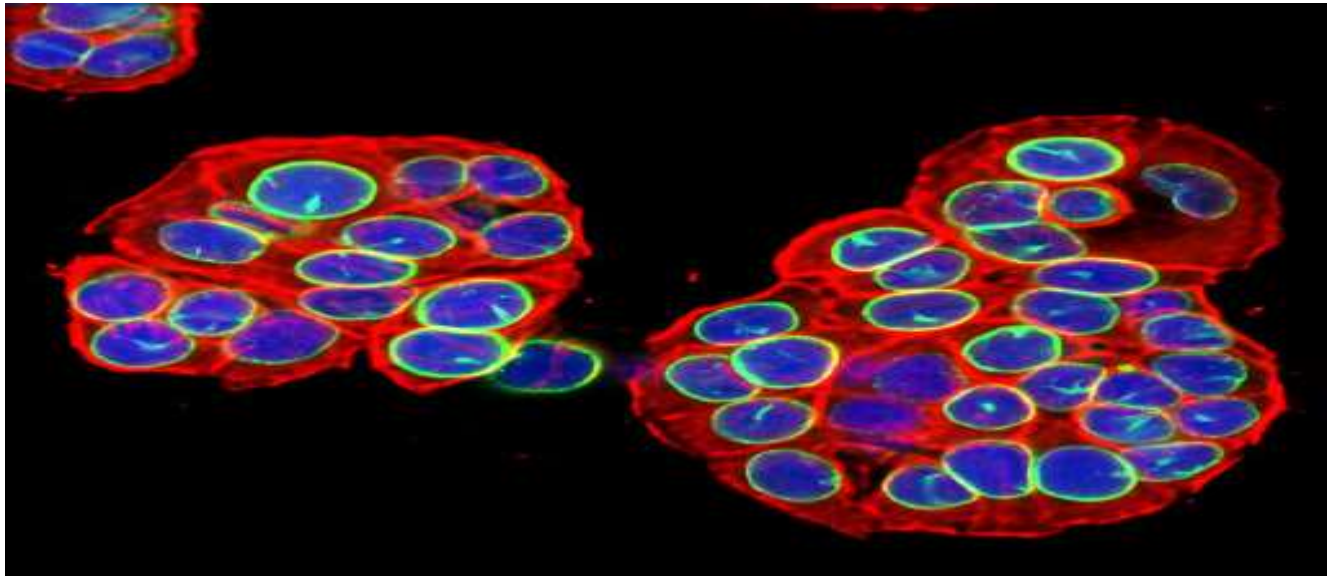
Senescent cells are characterized by stable cell cycle arrest as well as morphological and metabolic changes, chromatin reorganization, altered gene expression, and acquisition of the senescence-associated secretory phenotype (SASP). It is important to note that not all senescent cells display all biomarkers of senescence. In addition, senescence biomarkers are not necessarily specific to senescent cells, as some markers are observed in apoptotic cells or quiescent cells, for example. Therefore, the identification of senescent cells depends on the observation of several biomarkers, such as those described below:

Stable cell cycle arrest

Only cells with stable cell cycle arrest are considered senescent. Unlike a quiescent cell, a senescent cell will not reenter the cell cycle in response to any known physiological stimuli. Cell cycle arrest is mediated by the p53/$p21^{CIP1}$ and $p16^{INK4A/pRb}$ tumor suppressor pathways, described in more detail below. Expression of $p16^{INK4A}$ is frequently observed in senescent cells, serving as a useful biomarker. However, $p16^{INK4A}$ is also highly expressed in pRb-negative tumors and cell lines.`

Morphological and metabolic changes

Senescent cells typically have an enlarged size and flattened shape compared to their dividing cell counterparts. Senescent cells display extensive vacuolization and are sometimes multi-nucleated. In addition, disrupted nuclear envelope integrity is observed due to a loss of lamin B1 expression. Senescent cells accumulate dysfunctional mitochondria and display increased levels of reactive oxygen species (ROS). Increased lysosomal content and altered lysosomal activity is also observed, which is reflected by increased levels of β-galactosidase activity at pH 6.0, leading this to be widely adopted as a biomarker of cellular senescence



Loss of Lamin B1 (shown here in green) is a marker of cellular senescence.

The most widely used senescence marker is senescence-associated β-galactosidase (SA β-gal) activity. This enzymatic activity, which is found in many normal cells under physiological conditions (pH 4.0–4.5), is significantly amplified in senescent cells as a result of increased lysosomal content . Because of this, histochemical detection of β-gal activity at pH 6.0 (suboptimal for normal cells) allows specific identification of senescent cells . Since SA β-gal activity is detected in most senescent settings, both in vitro and in vivo, it is considered a de facto hallmark of senescence. However, cells deficient in *GLB1* (the gene encoding for lysosomal β-gal) do not exhibit impairments in the functional aspects of senescence.

Senescence-associated beta-galactosidase (**SA-β-gal** or **SABG**) is a hypothetical hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides only in senescent cells. Senescence-associated beta-galactosidase, along with $p16^{Ink4A}$, is regarded to be a biomarker of cellular senescence.

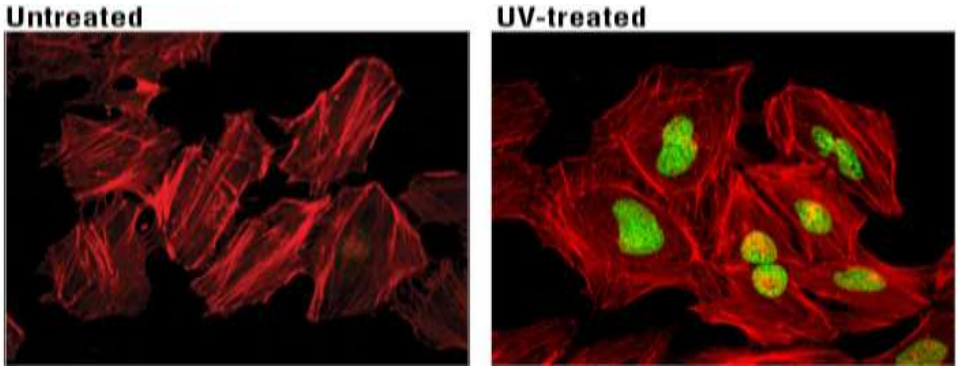
Its existence was proposed in 1995 by Dimri et al. following the observation that when beta-galactosidase assays were carried out at pH 6.0, only cells in senescence state develop staining. They proposed a cytochemical assay based on production of a blue-dyed precipitate that results from the cleavage of the chromogenic substrate X-Gal, which stains blue when cleaved by galactosidase. Since then, even more specific quantitative assays were developed for its detection at pH 6.0

Chromatin reorganization and altered gene expression

A hallmark feature of senescent cells is extensive chromatin reorganization, most notably the formation of senescence-associated heterochromatin foci (SAHF). These sites of facultative heterochromatin play a role in silencing genes that promote proliferation including E2F target genes like cyclin A. Senescent cells typically contain 30-50 SAHF which are characterized by bright DAPI staining and macroH2A, heterochromatin protein 1 (HP1), and lysine 9 di-or-tri-methylated histone H3 (H3K9Me2/3) immunoreactivity. Although SAHF is frequently observed during senescence, some cells undergo senescence without forming SAHF.

DNA damage and persistent DNA damage response (DDR)

DNA damage, such as DNA double strand breaks, is a prominent feature of senescence. Senescent cells display a persistent DNA damage response (DDR) which ultimately triggers cell cycle arrest. Senescent cells contain nuclear foci called DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), which associate with PML nuclear bodies and accumulate DDR proteins such as activated p53, ATR, and ATM. DNA-SCARS that occur at uncapped telomeres are called telomere dysfunction-induced foci (TIF). Another indicator of DNA damage is γ-H2A.X, which is the phosphorylated form of H2A.X, a variant histone required for checkpoint-mediated cell cycle arrest and DNA repair following double-stranded DNA breaks. DNA damage, caused by ionizing radiation, UV-light, or radiomimetic agents, results in rapid phosphorylation of H2A.X at Ser139.

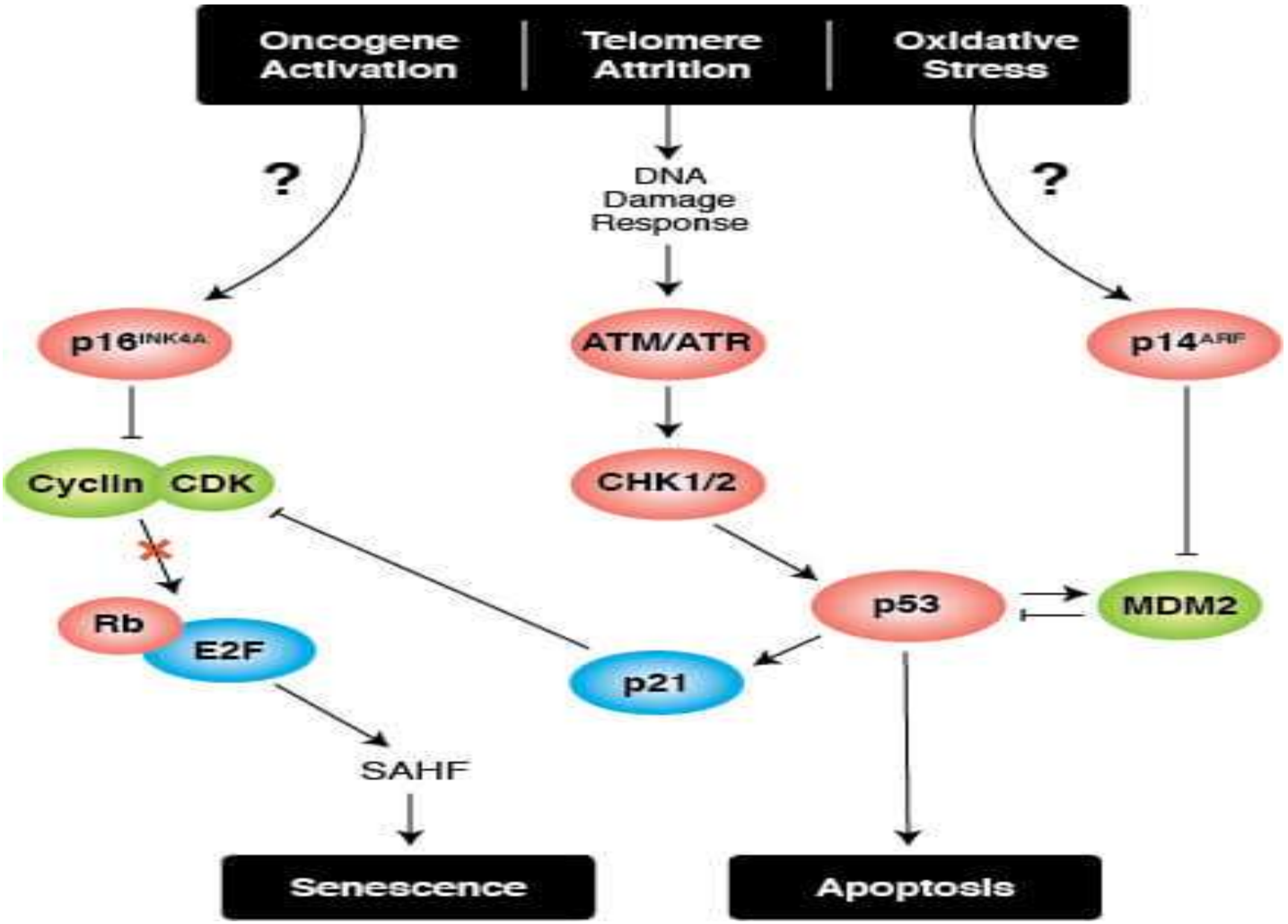


H2A.X is a commonly used marker of cell senescence. Confocal immunofluorescent analysis of HeLa cells, untreated (left), treated with UV (100 mJ/cm^2) using Phospho-Histone H2A.X (Ser139) Mouse mAb (green). Actin filaments were labeled with DyLight™ 554 Phalloidin (red

Senescence-associated secretory phenotype (SASP)

Many senescent cells acquire a pro-inflammatory senescence-associated secretory phenotype (SASP) that mediates non-cell autonomous effects of senescence, both beneficial and deleterious. The SASP is comprised of a highly complex mixture of secreted cytokines, chemokines, growth factors, and proteases, with the precise composition varying markedly by cell and tissue context and the senescence-inducing stimulus. These secreted factors facilitate communication with neighboring cells and the immune system, which ultimately influences the fate of the senescent cell. For example, the SASP recruits immune cells to senescent cells, thereby facilitating their elimination, which serves a tumor suppressor function. Paradoxically, however, the SASP has been shown to promote tumor cell progression through secretion of factors that promote angiogenesis, extracellular matrix remodeling, or epithelial-mesenchymal transition (EMT). Additionally, chronic senescence-induced inflammation can induce systemic immunosuppression, potentially leading to the onset of diseases including cancer. This chronic inflammation may also drive tissue damage and degeneration associated with aging.

Signaling Pathways for Senescence

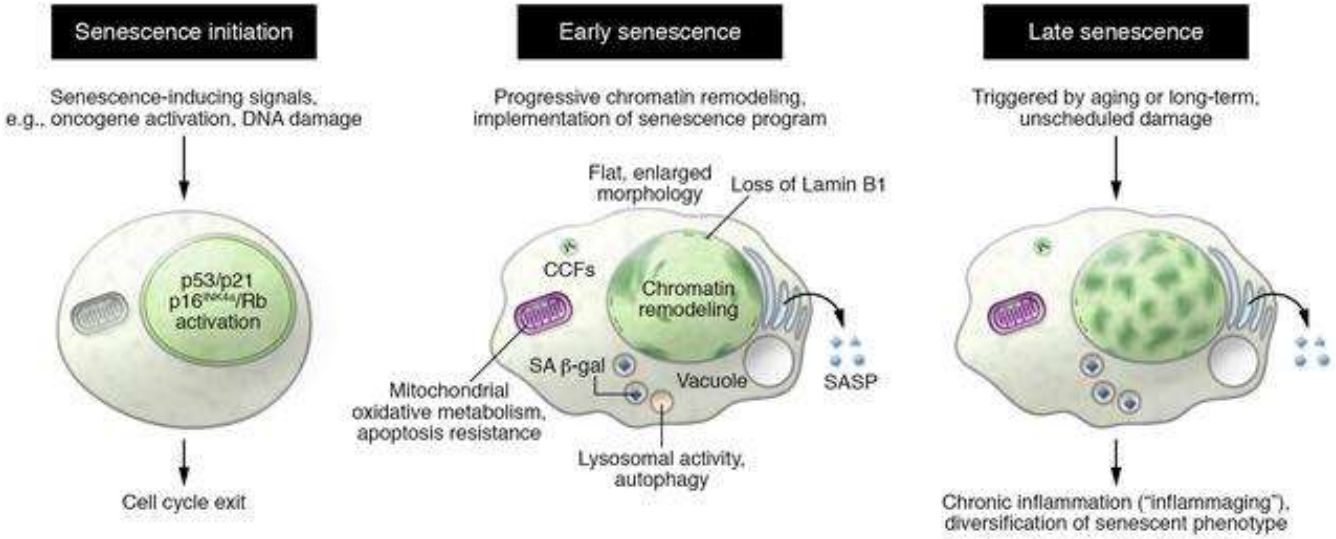


Transcriptional Regulation of Cellular Senescence

Most Common Proteins in Senescence

Protein or Marker	Role in Senescence
Senescence-associated β-galactosidase	Increased activity at pH 6.0 in senescent cells
p53	Activation can trigger cell cycle arrest
Rb (Retinoblastoma tumor suppressor protein)	Inhibition triggers cell cycle arrest
$p21^{CIP1}$	Inhibits cyclin dependent kinases; downstream of p53
$p16^{INK4A}$	Inhibits phosphorylation and inactivation of pRb
Bcl-2	Increased expression in senescent cells, inhibits apoptosis
macroH2A1.1	macroH2A1 isoform; marker of SAHF
macroH2A1.2	macroH2A1 isoform; marker of SAHF
H3K9Me2/3 (lysine 9 di-or-tri-methylated histone H3)	Marker of SAHF
HP1 (heterochromatin protein 1)	Marker of SAHF
Phospho-Histone H2A.X (Ser139)	Marker of DNA damage
Lamin B1	Expression reduced in senescent cells leading to disruption of nuclear envelope
HMGB1 (High mobility group protein B1)	SASP component
IL-6 (Interleukin 6)	SASP component
TNF-α (Tumor necrosis factor α)	SASP component
MMP3 (Matrix metalloproteinase-3)	SASP component

Phenotypic characteristics of senescent cells.



Cellular senescence was initially considered to be a cell-intrinsic program. Increasing evidence, however, has shown that senescent cells have the ability to signal and influence their surrounding environment. **Senescent cells produce a complex mixture of soluble and insoluble factors that are collectively termed senescence-associated secretory phenotype (SASP) or senescence-messaging secretome .**

SASP is the general term given to the combination of cytokines, chemokines, extracellular matrix proteases, growth factors, and other signaling molecules secreted by senescent cells. Importantly, its specific composition varies depending on the cell type and the senescence inducer. Likewise, the functions attributed to the SASP, or at least some of its members, are also very diverse and depend not only on the nature of the SASP, but on the surrounding environment and the genetic context of the cells being exposed to the senescent secretome. The SASP is the best-studied mechanism by which senescent cells influence their neighbors, but is not the only one. For example, senescent cells can signal and influence adjacent cells through juxtacrine NOTCH/JAG1 signaling or ROS production , or by cargo transfer, which occurs via formation of cytoplasmic bridges or release of exosomes .

Functions of the SASP. The SASP can have beneficial or detrimental effects . It is important to recognize, however, that the SASP's effects in specific contexts are pleiotropic.

- The SASP reinforces the senescence growth arrest in vitro by implementing an **autocrine positive-feedback loop**. This autocrine loop contributes to the tumor-suppressive function of senescence.
- The SASP can also induce nonmalignant proliferating neighbor cells to undergo senescence (termed paracrine senescence) . This suggests that senescent cells could also amplify the antitumoral response by limiting the proliferation of nearby cells exposed to similar stressors.

The senescent secretome can also promote tumorigenesis. In fact, the SASP has an important **proinflammatory nature, and inflammatory mediators** are powerful drivers of tumor progression.

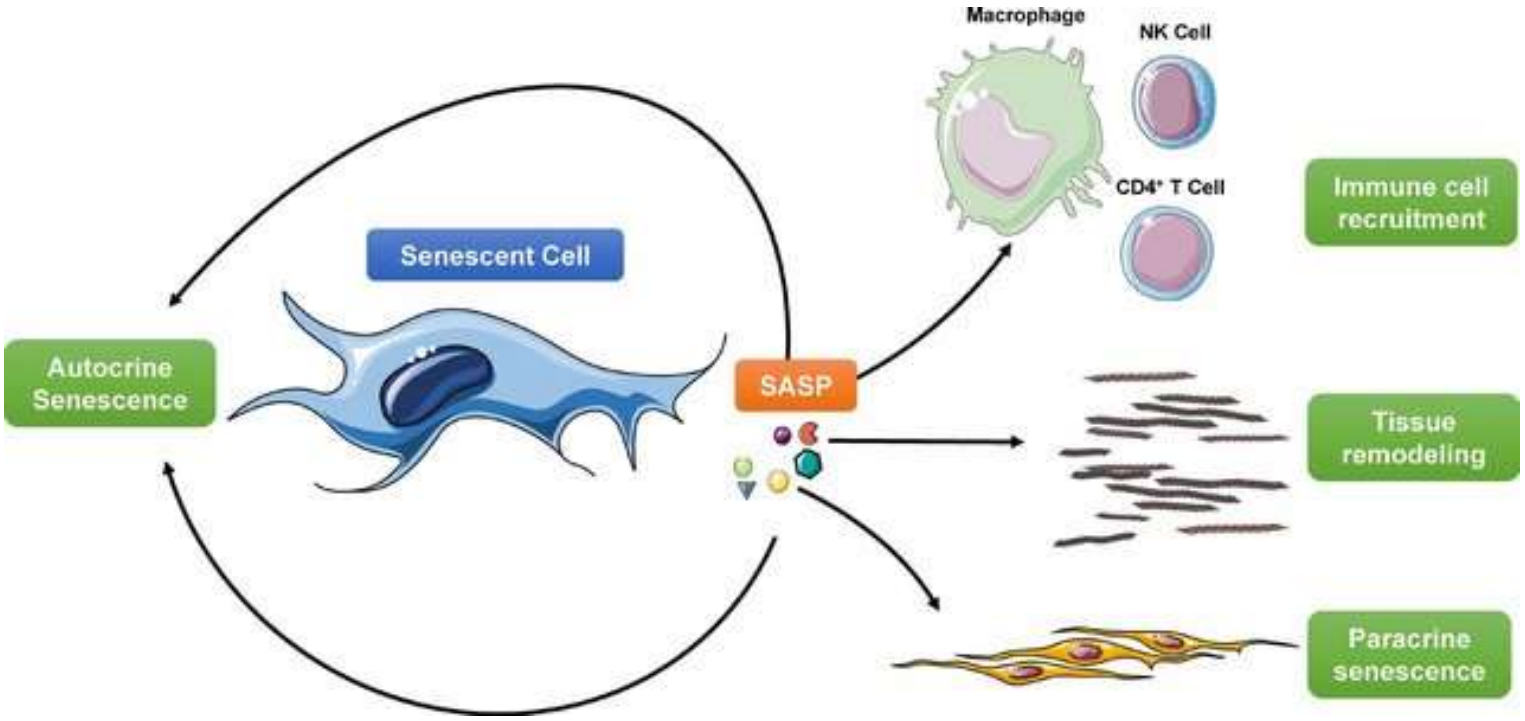
- The SASP of senescent fibroblasts can promote proliferation and metastatic features in premalignant epithelial cells or increase tumor vascularization in xenograft transplants.
- The SASP of senescent hepatic stellate cells (HSCs) promotes the proliferation and malignancy of the surrounding hepatocytes in obese mice treated with chemical carcinogens .

The interplay **between the SASP and the immune response** is also complex. On one hand, it is believed that the SASP might have initially evolved as a way to recruit the immune system to eliminate senescent cells. Indeed, during cancer initiation, SASP-dependent recruitment of Th1 cells, NK cells, and macrophages is essential to clear incipient preneoplastic cells and prevent the progression of hepatocellular carcinoma (HCC) .

On the other hand, the SASP can have immunosuppressive properties . For instance, Eggert and colleagues when premalignant senescent hepatocytes coexist with liver cancer cells, the SASP-dependent recruitment of immature myeloid cells may promote HCC progression by impairing the function of NK cells, suggesting a multifaceted interaction between the SASP, immune cells, and cancer.

The SASP has also been strongly linked to aging and age-related diseases.

Low-level chronic inflammation (also referred to as “sterile inflammation” or “inflammaging”; underlies many age-related pathologies. It seems that the SASP could explain, at least in part, this local inflammation within tissues. Indeed, the elimination of senescent cells reduces levels of proinflammatory cytokines such as IL-6, IL-1α, and TNF-α in fat, kidneys, and skeletal muscle of aged mice .Given the positive impact of eliminating these senescent cells, it is tempting to speculate that SASP suppression may underlie many of the beneficial effects of senolysis



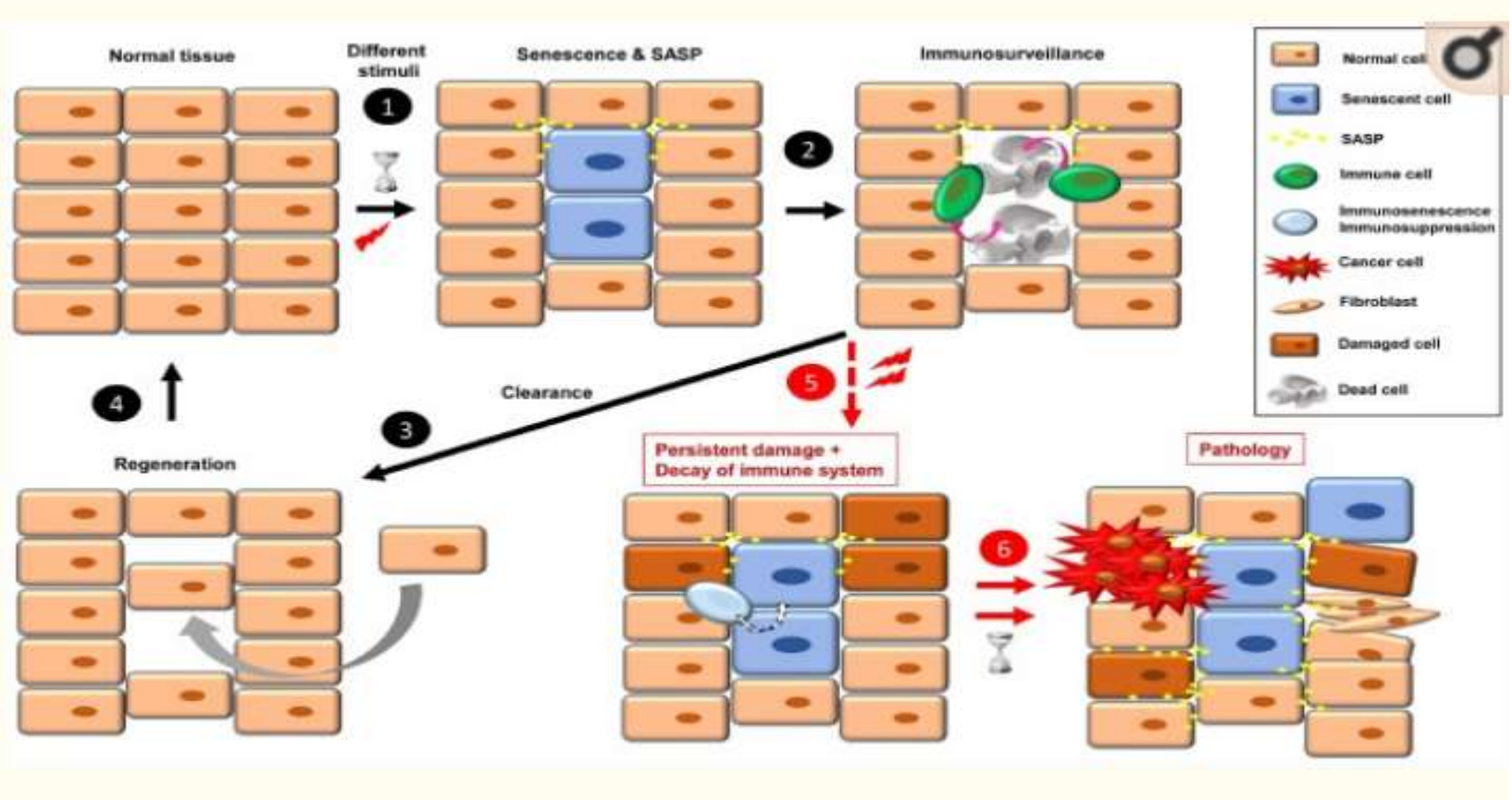
Functions of the SASP. The SASP mediates many of the cell-extrinsic functions of senescent cells. Among those it reinforces several aspects of senescence including growth arrest and the SASP itself via an autocrine loop. The SASP also recruits immune cells, such as macrophages, neutrophils, and natural killer (NK) cells to phagocytose and eliminate the senescent cell. Secretion of MMPs and factors such as VEGF can remodel the surrounding tissue, inducing angiogenesis and reducing fibrosis. Finally, secretion of molecules such as TGF-β can spread the senescence phenotype in a paracrine manner to surrounding cells.

Senescence is associated with large-scale chromatin rearrangements .

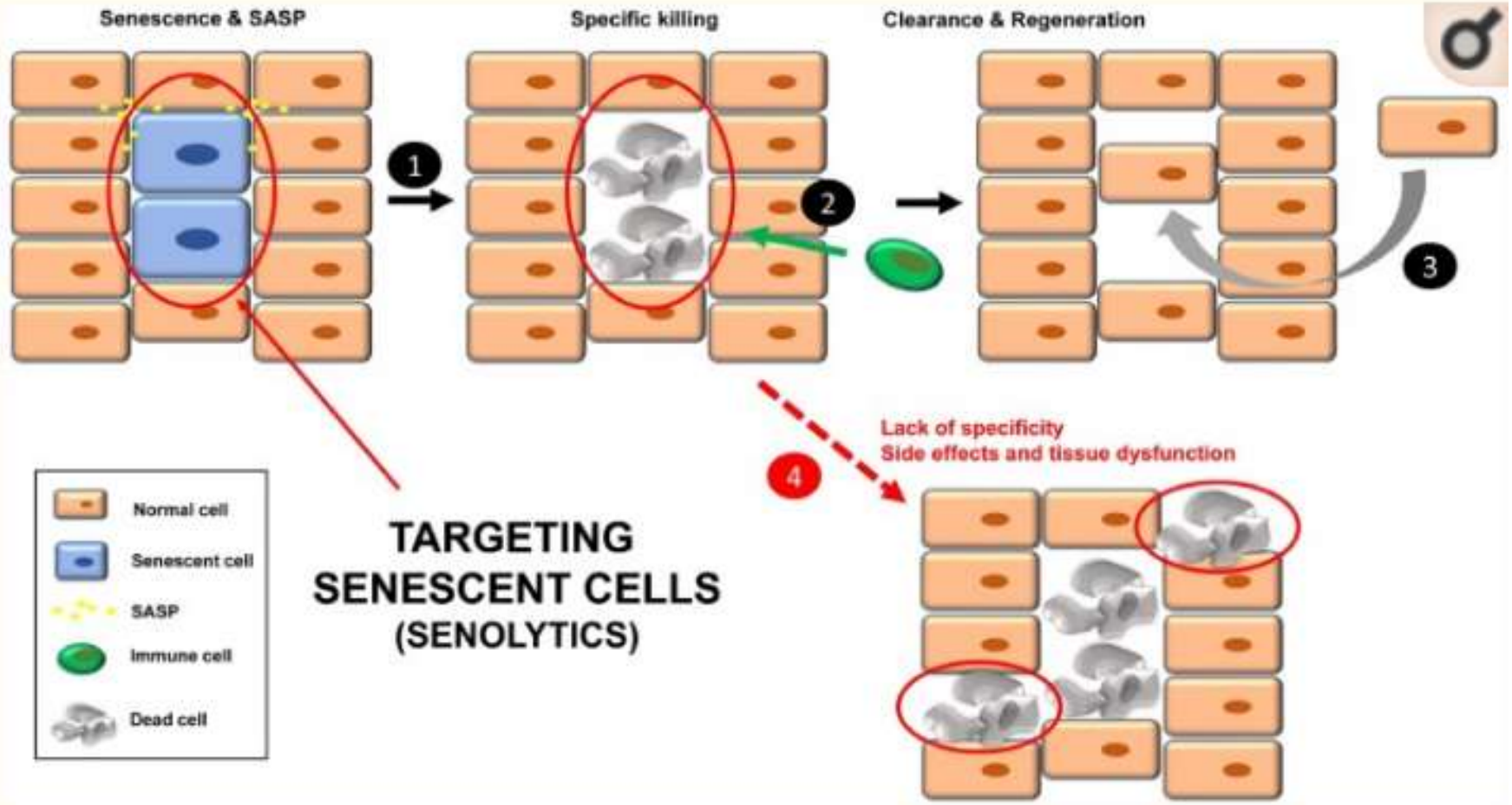
Besides the already described DDR and the formation of PML bodies (a type of matrix-associated nuclear domain) , the most striking chromatin change observed in senescent cells is the formation of **senescence-associated heterochromatic foci (SAHFs),** which are more prominent in human cells undergoing OIS . These foci can be identified by DAPI staining and are characterized by enrichment of repressive marks such trimethylated H3K9 and heterochromatic protein 1 (HP1), accumulation of high-mobility group HMGA proteins, and loss of linker histone H1 . Therefore, it was hypothesized that SAHFs may represent a senescence-specific heterochromatic compartment. The SAHFs are the result of the spatial repositioning of preexisting repressive marks rather than being caused by global changes in histone methylation . Interestingly, genomic regions contained in the SAHFs are found in lamina-associated domains (LADs) in proliferating cells . Upon induction of senescence and loss of lamin B1 (LMNB1), these LADs detach from the nuclear periphery and cluster within the nuclei.

In cell culture, senescence is normally accompanied by significant morphological changes. **Senescent cells become flat, enlarged, and vacuolized, and sometimes appear with multiple or enlarged nuclei**. Changes in shape rely on the status of the scaffolding protein caveolin 1 and the Rho GTPases Rac1 and CDC42 , and vacuolation has been associated with ER stress caused by the unfolded protein response . Senescent cells also form cytoplasmic bridges that allow them to signal to neighboring cells via direct intercellular protein transfer . Beyond these examples, the functional significance of most morphological changes associated with senescence is unclear. In vivo, senescent cells appear to preserve the morphology dictated by the architecture of the tissue. However, recent studies have discovered that SA β-gal^{+} cells in aged mice increase in size

A **senolytic** (from the words senescence and -lytic, "destroying") is among a class of small molecules under basic research to determine if they can selectively induce death of senescent cells and improve health in humans These cells accumulate in many tissues with aging and at sites of pathology in multiple chronic diseases.



The onset of cellular senescence in normal tissue takes place in response to different stimuli . Some SASP factors are involved in immune cell recruitment, which act in the clearance of the senescent cells . Then, to restore the normal tissue, a regeneration process is necessary . When a combination of persistent damage and immune system decay occurs, senescent cells accumulate, creating a pro-inflammatory and pro-tumorigenic environment and fibrotic tissue. Over time, this leads to disease, such as cancer progression, insulin resistance, osteoarthritis, atherosclerosis, and brain pathologies, among others .



Treatment with senolytics to specifically kill senescent cells . Over time, these apoptotic bodies will be cleared by the immune system . Finally, a regenerative process will lead to normal tissue functions . Normal cells could be affected by either the lack of specificity of the senolytics or chronic treatment, leading to tissue dysfunction