Bioanalytical Chemistry Fall 2010

Schedule (Mondays 2:30-5:30 pm)

September 13	Lecture 1
September 20	Lecture 2
September 27	Lecture 3
October 4	Lecture 4
October 11	No classes – Reading Week
October 18	Lecture 5
October 25	Lecture 6
November 1	**Midterm exam (30%)**
November 8	Lecture 7
November 12	Last date to drop courses without receiving a grade
November 15	Lecture 8
November 22	Lecture 9
November 29	Lecture 10
December 6	**Project presentation (30%)**
December 12	**Exams start, Final exam (40%)**

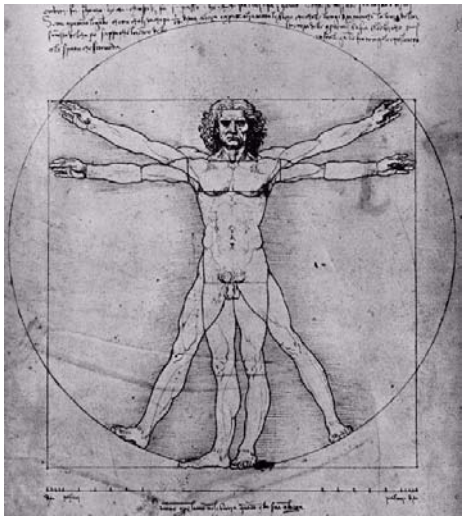
http://www.chem.yorku.ca/profs/krylov/

Teaching

Homeostasis of Multi-cellular Organisms

Homeostasis - definition

Homeostasis is the ability or tendency of an organism or cell to sustain internal equilibrium by adjusting its physiological processes (The American Heritage Dictionary of the English Language)



Vitruvian. Leonardo Da Vinci 1452-1529

Homeostasis in multi-cellular organisms: the number of cells, the shape and the size of the body are relatively constant.

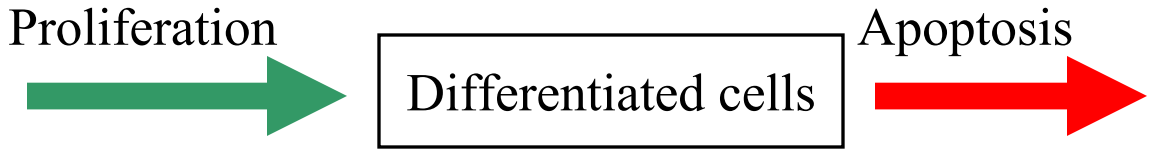
Homeostasis is maintained by precise balance of three key developmental processes:

1. Cell proliferation (cell division)
2. Cell differentiation (generation of specialized cells)
3. Apoptosis (programmed cell death)

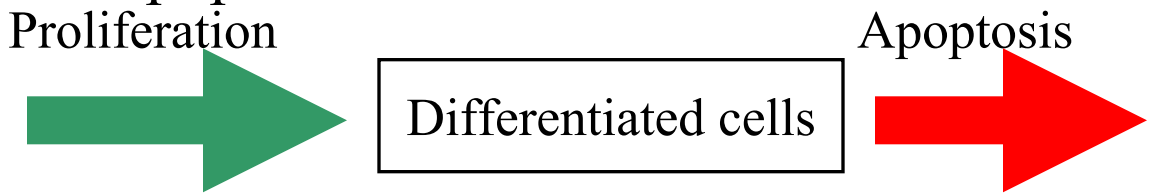
Venera, Capitoline Museum, Rome

Balance Between Proliferation and Apoptosis in a Healthy Organ(ism)

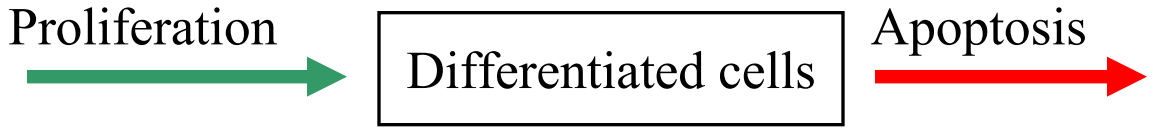
1. Normal rate of apoptosis



2. Increased apoptosis



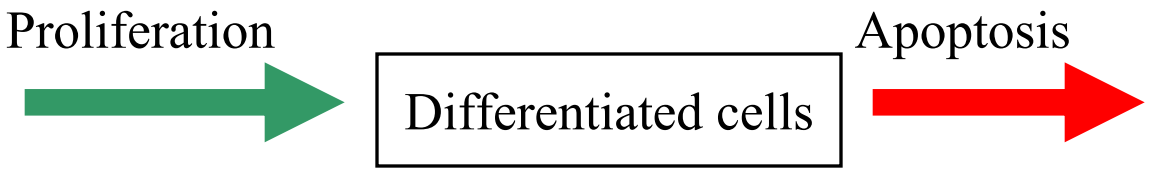
3. Increased survival



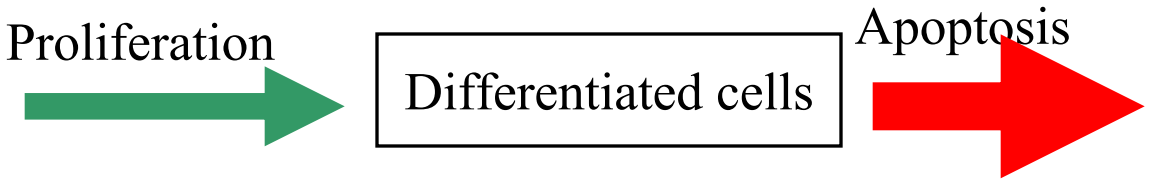
Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult. In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight

Imbalance Between Proliferation and Apoptosis

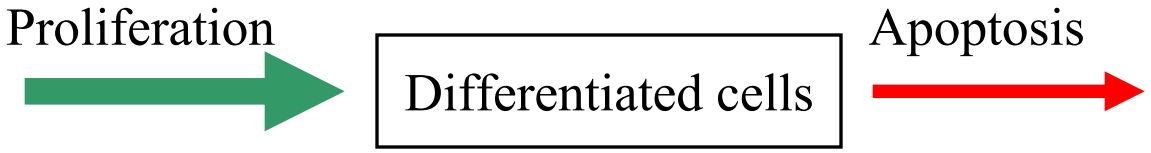
1. Homeostasis



2. Disorder of cell loss



3. Disorder of cell accumulation



Homeostasis Disorders

In adult organisms, if Proliferation Rate ≠ Apoptosis Rate then we deal with Homeostasis Disorders:

Cell loss disorders
apoptosis > *proliferation*
- AIDS
- Neurodegradation
- Ischemic injuries

Cell accumulation disorders
proliferation > *apoptosis*
- Autoimmunity
- Viral Infections
- Cancer

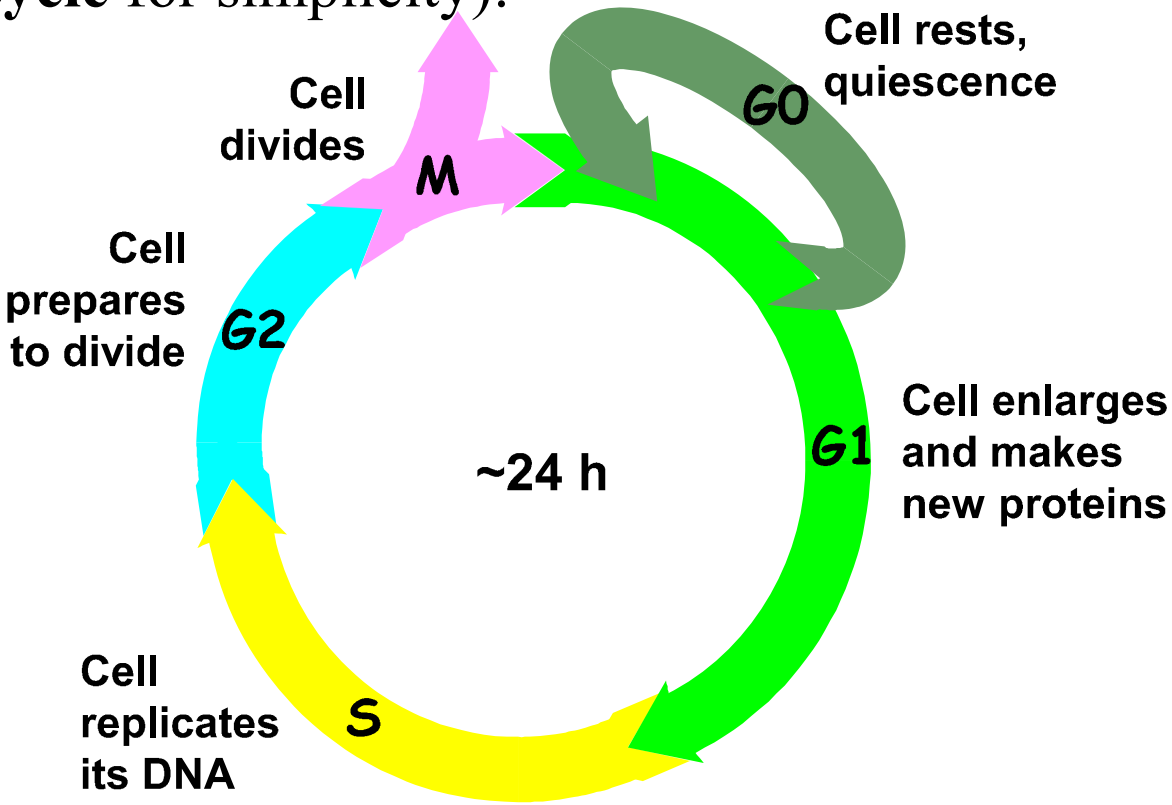
Being able to treat these disorders requires that:

1. The molecular mechanisms of cell *proliferation*, *cell differentiation* and *apoptosis* be well understood
2. Interplay of the three mechanisms be well understood (intuitively we feel that this interplay can be achieved through common molecules participating in the three processes)

Cell Proliferation

Most of cells in our organism do not and cannot proliferate. Such cells are called differentiated cells.

Three types of cells can proliferate: 1) germ cells, 2) stem cells and 3) tumor cells. Non-germ cells proliferate through a mitotic cycle (or **cell cycle** for simplicity):

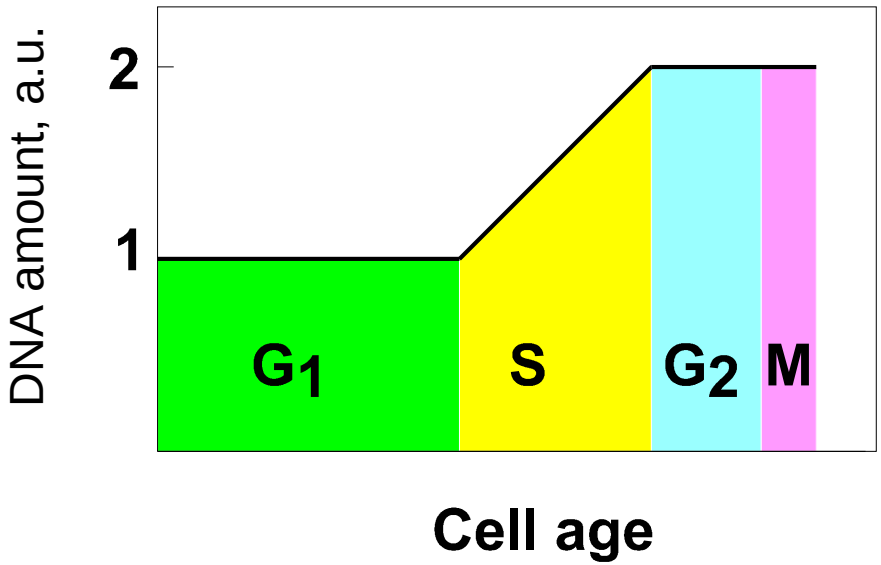


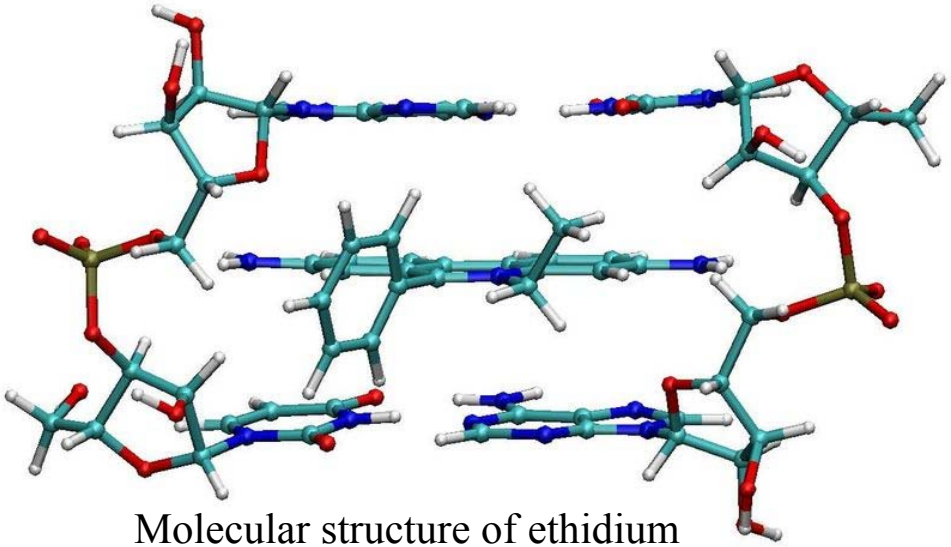
RNA and protein synthesis occurs continuously, DNA synthesis occurs only during the S phase

This feature can be used to determine cell's position in the cell cycle

Determining Cell Position in the Cell Cycle

By measuring the amount of DNA in a cell we can distinguish three positions in the cell cycle: G1, S, and G2/M.
The amount of DNA can be measured with DNA intercalating fluorescent dyes such as propidium iodide (stains only fixed cells), Hoechst (stains live cells, but requires UV excitation) or DRAQ5 (www.biostatus.com stains life cells, excited and fluoresces in visible range)





Molecular structure of ethidium bromide intercalated between two pairs of adenine-uracil base pairs.

Key Molecular Players in the Cell Cycle

(all of them are proteins)

Kinases are the main engines of the cell cycle machinery

Kinase - enzyme catalyzing phosphorylation (in contrast to phosphatase that catalyzes de-phosphorylation)

Cyclin Dependent Kinase (CDK) Machinery

CDK itself	catalytic subunit (***Engine***)
	serine/threonine protein kinase
	always present in the cells
Cyclin	regulatory subunit (***Gas pedal***)
	associates with and activates CDK
	accumulates during cell cycle (origin of the name)
	and destroyed during mitosis
CKI	Cyclin Dependent Kinase Inhibitor (***Brake***)

Cell Cycle Progression. Cascade of Events

G0 resting cell

Growth factor interact with the cell

G1 *Cyclin D* synthesis is triggered by the *growth factor*

CDK4 is activated by association with *Cyclin D*

Retino-blastoma protein (Rbp) is phosphorylated by Cyclin D-CDK complex

Transcription Factor *E2F* is released by phosphorylated Rbp

Expression of genes important for S phase (e.g. *Cyclin A*) is activated by E2F

CDK2 is activated by *Cyclin A*

S *Chromosome replication* is mediated by *Cyclin A-CDK2* (Role is not exactly known but cyclin A-CDK2 co-localizes with replication apparatus)

G2 *Cyclin B* is synthesized and activate *cdc2*

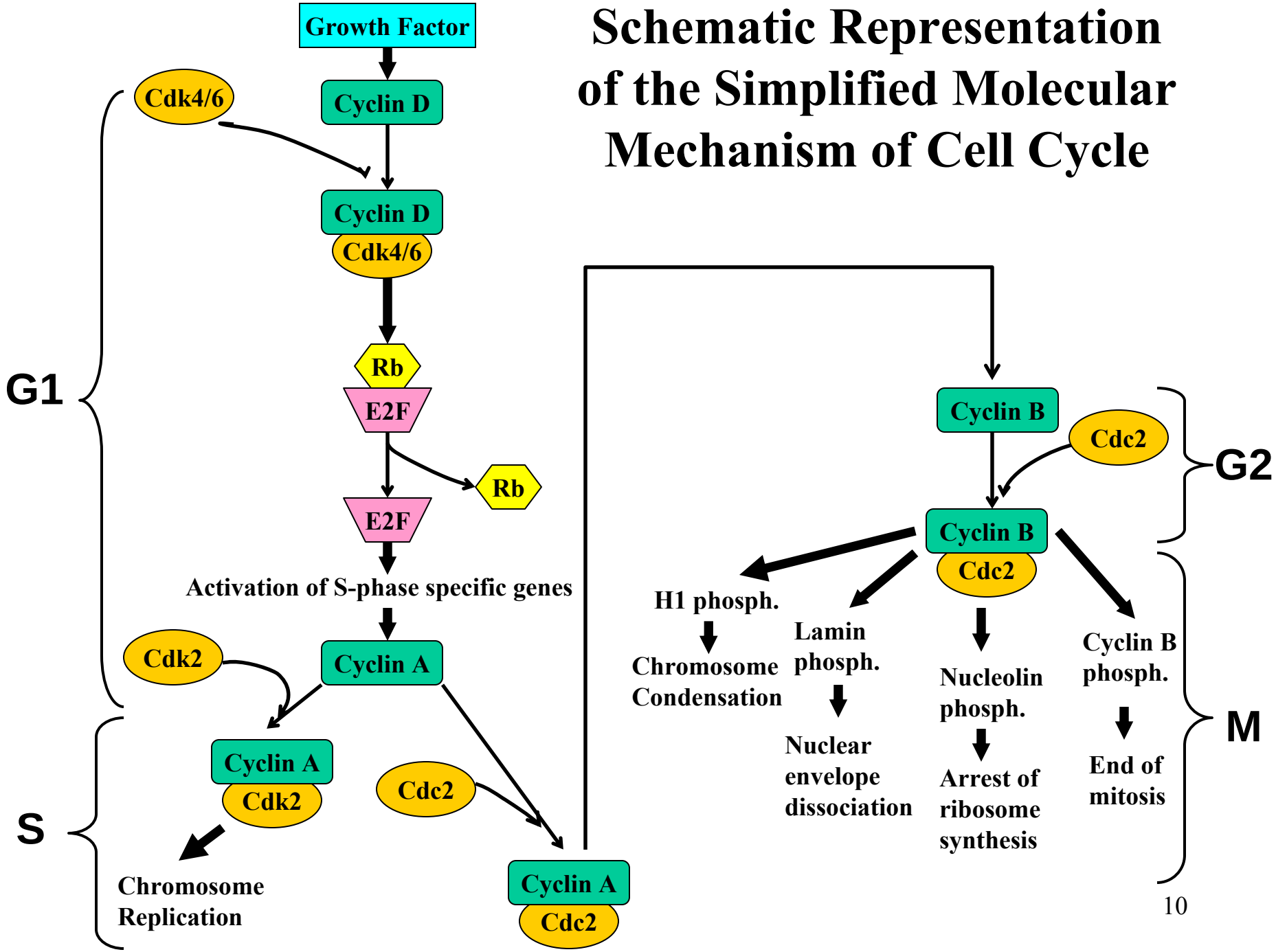
M *Histone 1 is phosphorylated* by *Cyclin B-cdc2* resulting in the condensation of the chromosomes

Lamins (proteins forming nuclar lamina) are phosphorylated by *Cyclin B-cdc2* resulting in dissociation of nuclear envelope

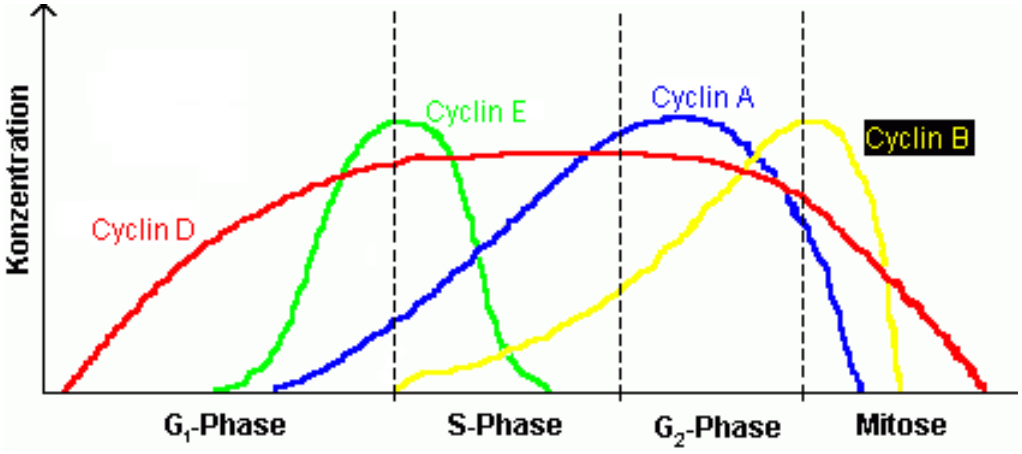
Nucleolin (involved in the synthesis of ribosomes) is phosphorylated by *Cyclin B-cdc2* resulting in the arrest of ribosome synthesis

Proteolytic destruction of Cyclin B is initiated by *Cyclin B-cdc2* itself results in the end of mitosis

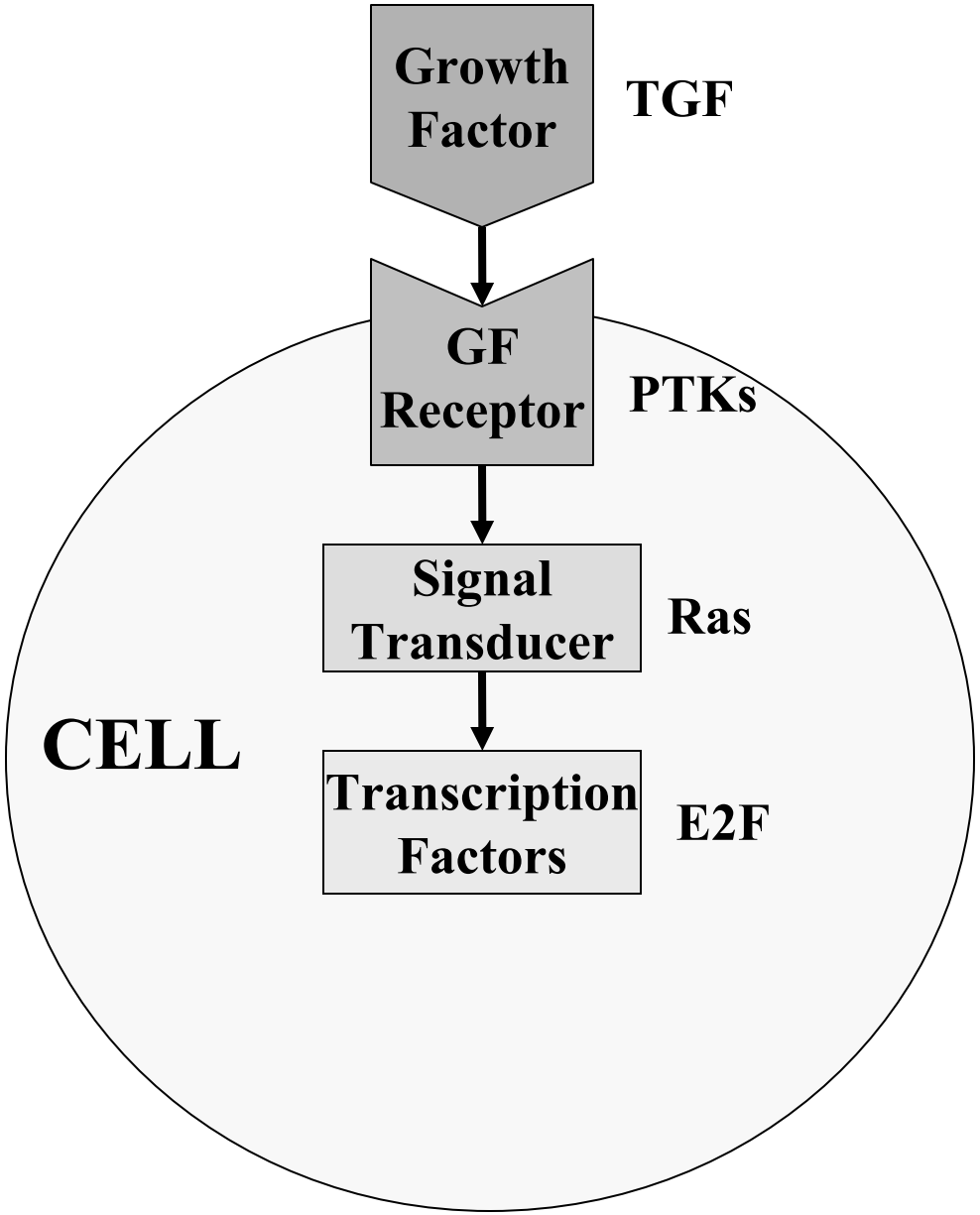
Schematic Representation of the Simplified Molecular Mechanism of Cell Cycle



Kinetics of cyclins during the cell cycle



External signal is required to initiate cell cycle in normal cells



Note:

1. All players in this pathway are proteins
2. Most these proteins are encoded by proto-oncogenes, that can become oncogenes upon mutations (retinoblastoma is a tumor suppressor gene as it blocks the E2F transcription factor)

Generalization: The Role of External Factors

1. External factors are proteins that are secreted by cells to induce a proper response in neighboring cell.
2. External factors (they are often called ligands) work through interaction with cellular receptors
3. Cellular receptors are membrane proteins
4. Upon interaction with external factors, cellular receptors start a cascade of reactions that lead to the expression of specific genes.

In general, the cells need external factors to start proliferation, differentiation or apoptosis

Control of Cell Cycle

Restriction point

Point of decision making - to enter the S phase or not to enter (to divide or not to divide)

After the decision to divide is made, the abortion is not allowed and if a serious problem is encountered, the cell should undergo apoptosis

Checkpoints

Control loops which make initiation of one event dependent on successful completion of an earlier event.

Examples of

Checkpoint working in specific phases:

1. Completion of previous mitosis before passing R point (G1)
2. Completion of DNA replication before entering G2 (S)

Checkpoint working in all phases (externally-induced damages)

1. DNA damage by radiation
2. Oncogene activation
3. DNA tumor viruses
4. Hypoxia

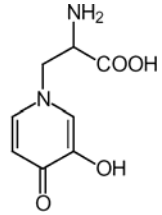
Manipulations with the Cell Cycle

are required to generate many cells in the same cell-cycle position for the analysis of chemical contents of cells in this position

Synchronization

Natural synchronization	***Stimulated synchronization***
Synchrony of embryo development	1. Arrest
Lasts for several cycle cycles	2. Release
Becomes asynchronous suddenly	Lasts for less than a cycle

Traditional Ways of Cell Cycle Arrest



Phase	***Method***
G0	Growth to confluence, Contact Inhibition
G1	L-mimosine (a rare plant amino acid, inhibitor of serine hydroxymethyltransferases)
S	Inhibitors of synthesis of deoxyribonucleotide triphosphate (Thymidine)
G2	DNA topoisomerase II inhibitors (Hoechst 33432)
M	Inhibitors of tubulin assembly (Calcimine)

Note: ***Physical arrest*** (contact inhibition) does not disturb normal biochemistry, ***Chemical arrest*** (inhibition of one normal cellular process which occurs only at a specific stage of cell cycle, checkpoint controls arrest the cycle at this point) disturbs normal cellular biochemistry, often results in cell death

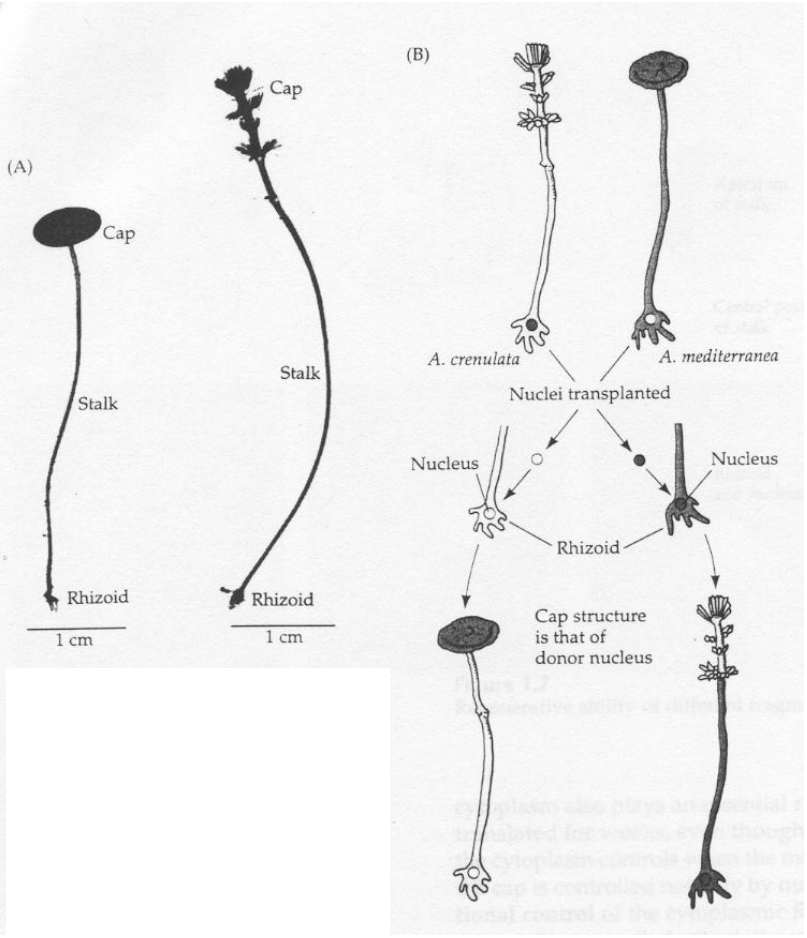
It is better to work with single cells so that synchronization and arrest are not needed ***but working with single cells requires advanced bioanalytical methods***

Cell Differentiation

Example: Differentiation in *Dictyostelium discoideum*

Dictyostelium discoideum,
mexamoebae (social amoebae [ə mēʹbə])
part-time multi-cellular organism

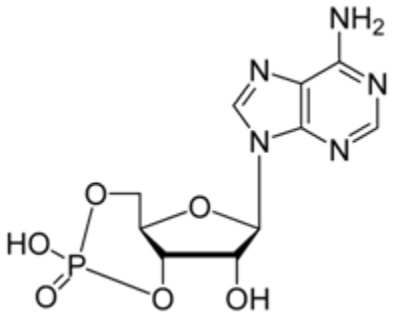
Lives on decaying logs
Eats bacteria



In shortage of food supply
Thousands of single amoebae aggregate
They form a multi-cellular organism
with cells performing different functions
It can move and find new food supply
Part of cells differentiate to form spores. Spores are disseminated
Every spore gives live to new mexamoebae

Mechanism and Some Clues and Conclusions

- Starving cells release cAMP
- Cells move against the gradient of cAMP
- ***Differentiation is initiated by external signal***



Cyclic-adenosine-monophosphate

- cAMP initiates expression of new proteins
- These proteins are responsible for:
  - cell adhesion
  - cell differentiation

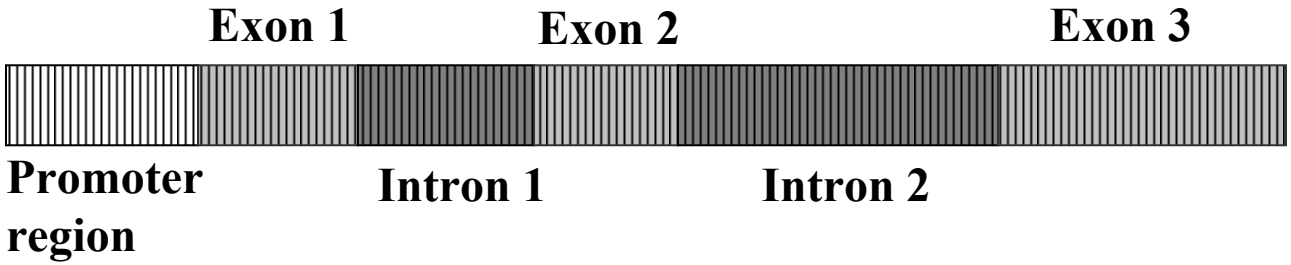
- ***Differentiation is the production of different proteins in different types of cells***

- Several types of cells differentiate from identical ancestor-cells
- ***All single mexamoebae cells have identical genes***
- ***Only parts of genes are expressed***
- ***Expression pattern can be changed in response to external signals – Differential Gene Expression***

Mechanism of Differential Gene Expression?

Gene

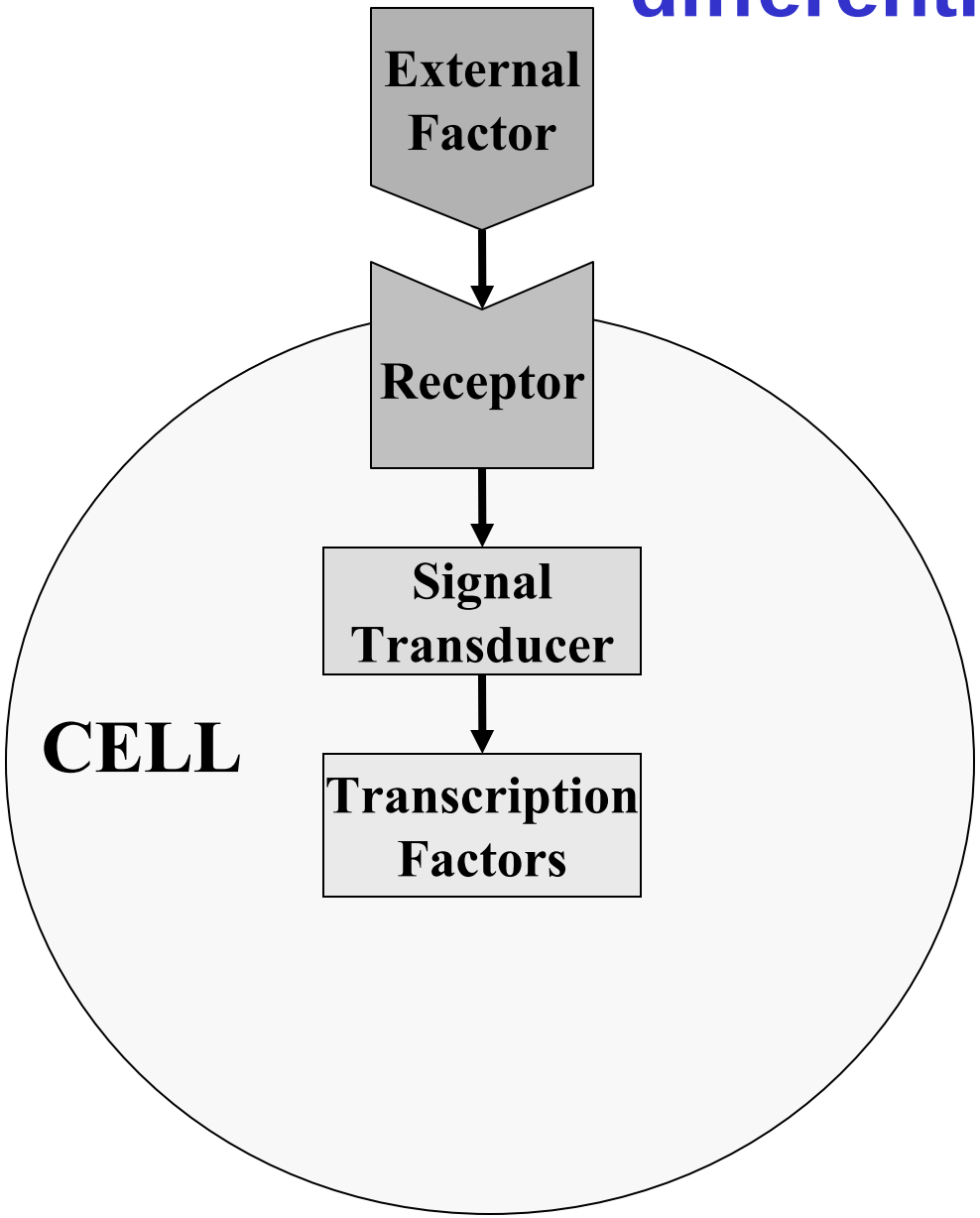
Gene is a fragment of DNA that encodes a protein.



It consists of:

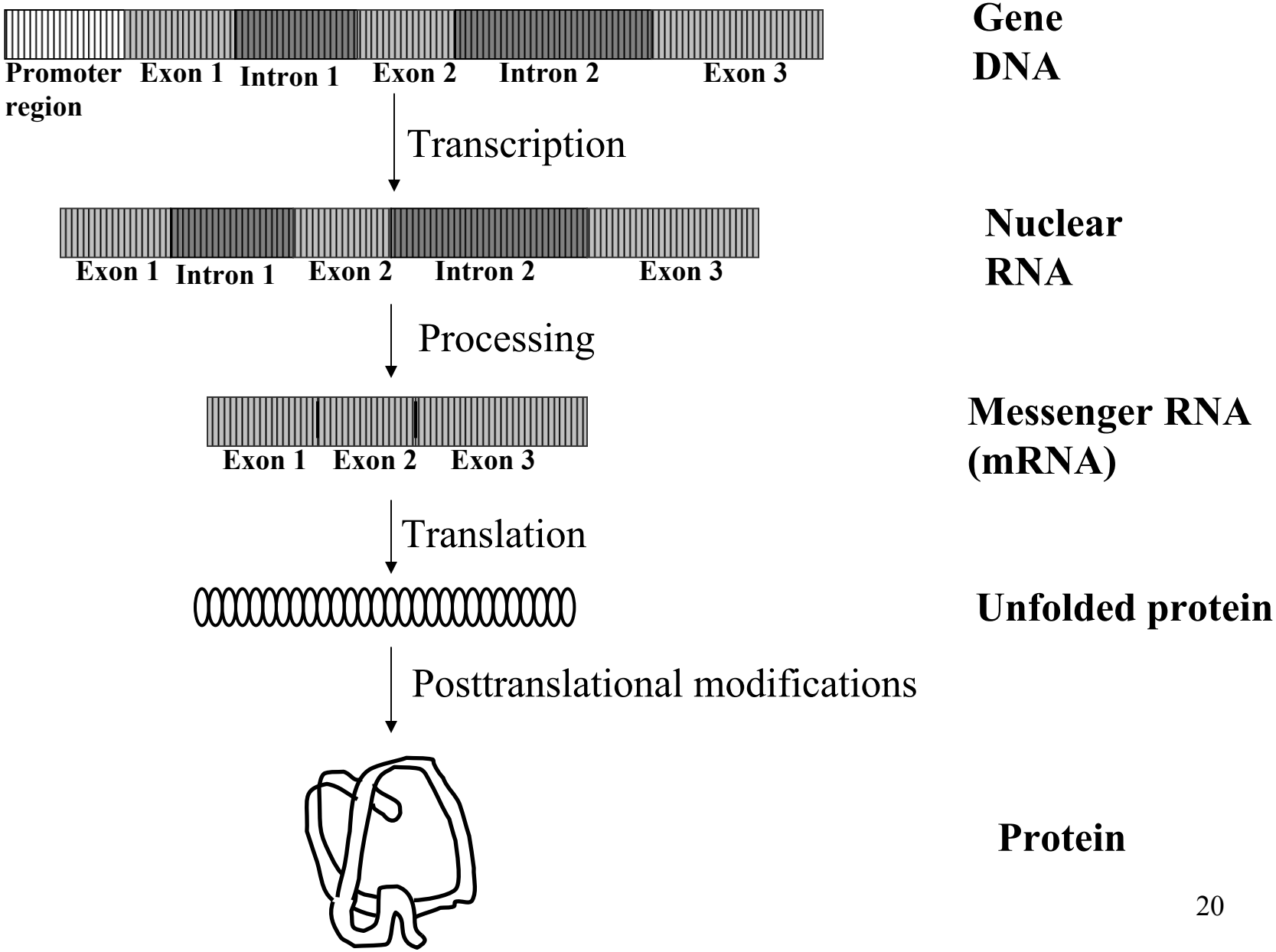
1. Promoter region, responsible for:
   - RNA polymerase binding
   - Initiation of transcription
2. Exons - protein-coding regions
   - Encode the amino-acid sequence of a protein
3. Introns - do not code the protein
   - Are often the enhancers

External signal is required to initiate cell differentiation



Note: The signal transduction pathway is in general similar to that of cell cycle initiation

Gene expression - Protein production



4 Steps of Gene Expression

1. Transcription
   - Synthesis of nuclear RNA from DNA matrix
   - Nuclear RNA contains both exons and introns
   - Transcription machinery - protein complex
   - Proceeds in nucleus
2. Processing
   - Synthesis of mRNA from nuclear RNA matrix
   - mRNA contains just exons
   - Proceeds in nucleus
   - mRNA is transported to the cytoplasm
3. Translation
   - Synthesis of a protein from mRNA matrix
   - Takes place in the cytoplasm
   - Ribosomes are involved in the translation machinery
4. Posttranslational modifications
   - Folding the protein
   - Glycosylation
   - Phosphorylation
   - Lipidation
   - Adding prosthetic groups (non-amino acid component of a protein)

Gene expression can be regulated on any of these 4 steps

Transcriptional regulation

1. Promoters and enhancers

- Regulatory DNA sequences
- Promoters bind RNA polymerase
- Enhancers activate the use of the promoters
- Enhancers regulate tissue-specific transcription

2. Transcription factors

- Regulatory proteins
- Bind to the enhancer and/or promoter regions
- Regulated by phosphorylation

3. DNA Methylation

- 5th base in DNA, 5-methylcytosine
- Differences between male and female pronuclei are due to differences in their DNA methylation patterns

Transcriptional regulation contd.

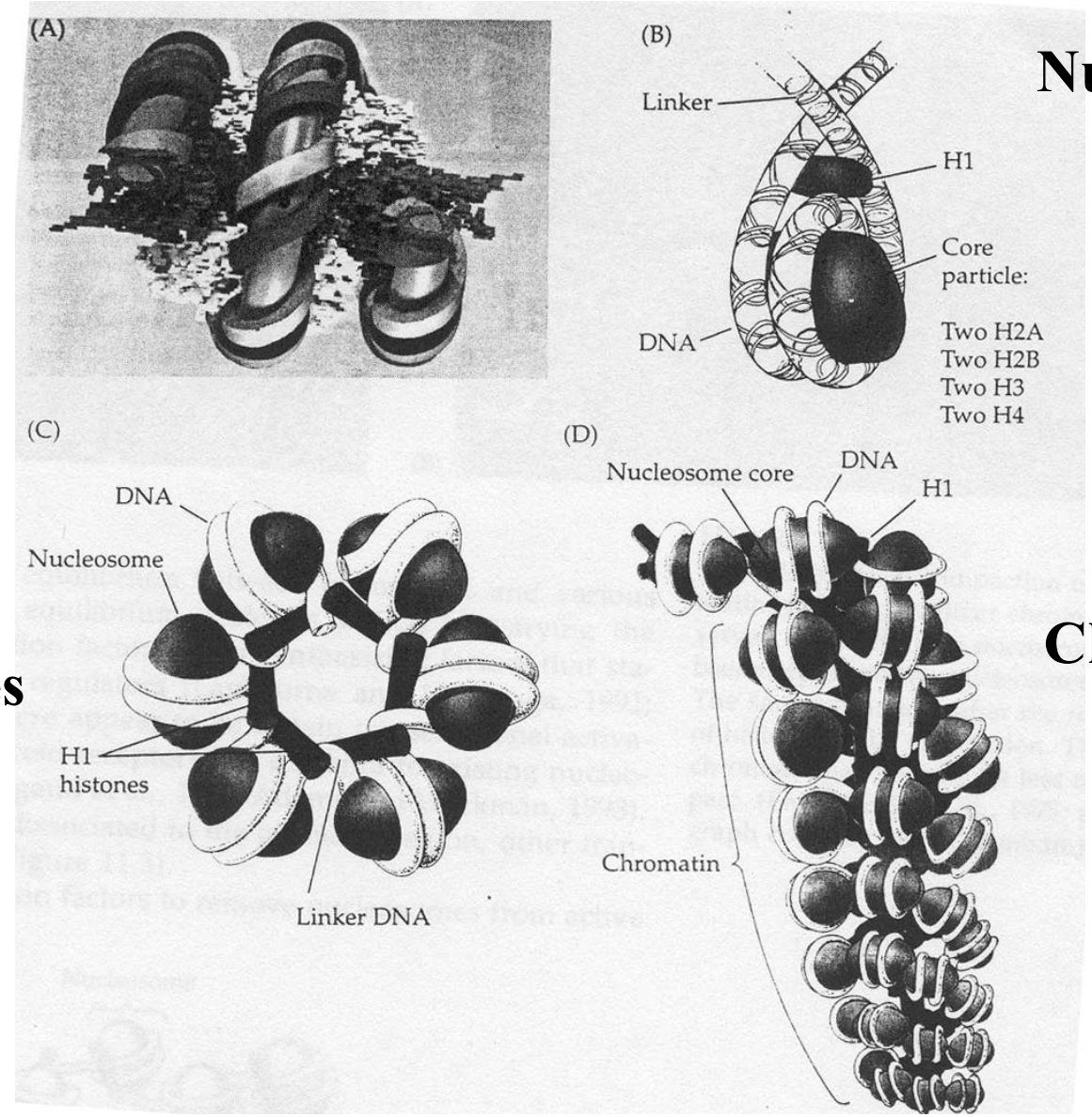
4. The activation of chromatin

- Chromatin is DNA-protein complex
- DNA is tightly and regularly packed in chromatin
- Chromatin consists of nucleosomes
- Nucleosome is composed of a histone core, H1 histone, and two loops of DNA (~140 bp)
- Nucleosome bids are linked with ~60 bp DNA
- DNA in chromatin is not accessible for transcriptional factors unless chromatin is "activated"

Hypotheses of chromatin "activation":

1. Transcription factors compete with histone H1 for specific DNA sequences
2. Transcriptional activators (proteins) disrupt nucleosome so that transcription factor can reach the promoter region.

Illustration: Nucleosome and Chromatine Structure



Nucleosome

Packing of nucleosomes

Chromatin

Regulation by RNA processing

1. Control of processing of nuclear RNA into mRNA
- Not all the genes transcribed to nuclear RNAs have corresponding mRNAs in the cytoplasm

2. Splicing different combination of potential exons leads to a variety of related proteins
- Thus, one gene may create a family of related proteins
- Spliceosomes (RNA-protein complexes) cut introns and splice exons. Differential regulation of spliceosomes can change the RNA processing pattern.

Note: There are only less then 25,000 human genes encoding proteins instead of ~160,000 thought a couple of decades ago. Alternative splicing is an important way to enrich the pool of proteins with this "small" genome. Analysis of splice isoforms of proteins is difficult due to their similarities in structures

Translational regulation

Fact: mRNA is present in the cytoplasm but may or may not be translated

1. Degradation or stabilization of mRNA

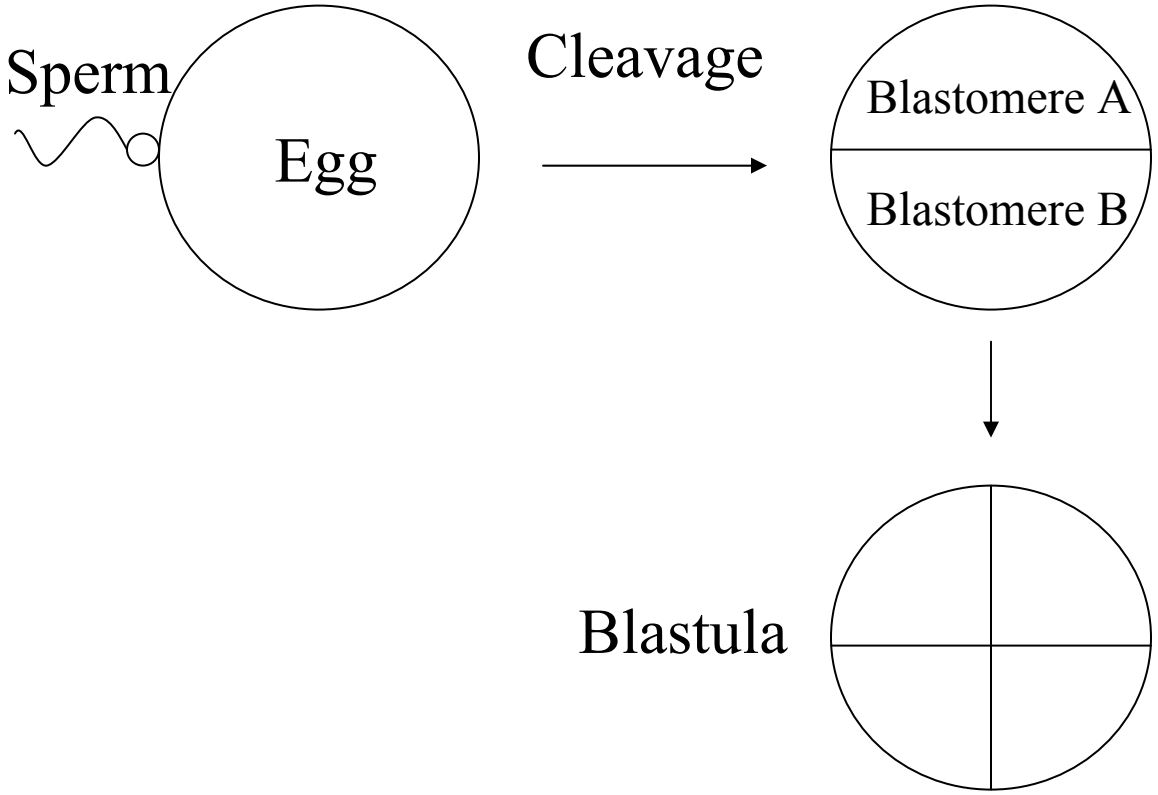
Example:
Lifetime of mRNA of β-globin is 17 h
Lifetime of mRNA of growth factors is <30 min

Some hormones influence mRNA stability rather than nuclear RNA production

2. Competitive inhibition of complex formation between mRNA and ribosomes

Differentiation in Embryo Development

Blastula development



Reproductive machinery of the egg

- Egg is a reproductive cell
- Relatively big
- Cleaves (divides) rapidly after fertilization
- Blastomers are the cells produced by cleavage
- Blastula is the embryo on the stage of cleavage
- *During cleavage blastula does not change size*

- At a certain stage the speed of cleavage reduces
- Further divisions proceed with increasing overall size of embryo

- The egg contains (2-5) × 10^4 of different mRNA ~2,000 copies of each. This is the greatest message complexity of any known cell type

- Reproductive machinery of the egg is ready for embryo development

Initial Asymmetry

For differentiation of blastomeres in the embryo, the asymmetry in the embryo has to appear at some stage

Asymmetry occurs at different stages for different species

1. Asymmetry of mRNA distribution in the egg's cytoplasm

Example: *Acrobeloides* (relative to *C. elegans*)

- Asymmetry inducer is an unknown component of mother's reproductive tract
- The side of the egg facing the vulva consistently develops the anterior
- Fertilization does not induce any developmentally important asymmetry

(Current Biology, 1998, 9, 157-160)

Initial Asymmetry (cont.)

2. Asymmetry introduced by the sperm cell

Example: *C. elegans*

- **Asymmetry inducer is the sperm cell**
- **P granules containing mRNA-protein complexes migrate to the site opposite to that of sperm entry**

(Development 1996, 122, 1467-74)

Note: the difference between two close relatives -> possible mistake

3. Asymmetry induced by a component of mother's uterus, which the blastula attaches to

Example: *Mammals*

- **Initial cleavages produce symmetrical blastomeres; exchanging the places of such blastomeres does not influence normal differentiation at later stages**
- **Manipulation with blastomeres after the differentiation started can result in defective embryo development**

Note: Recent observations showed asymmetry in antibody staining in a mammal blastula at the 2-cell stage

Anterior-posterior asymmetry

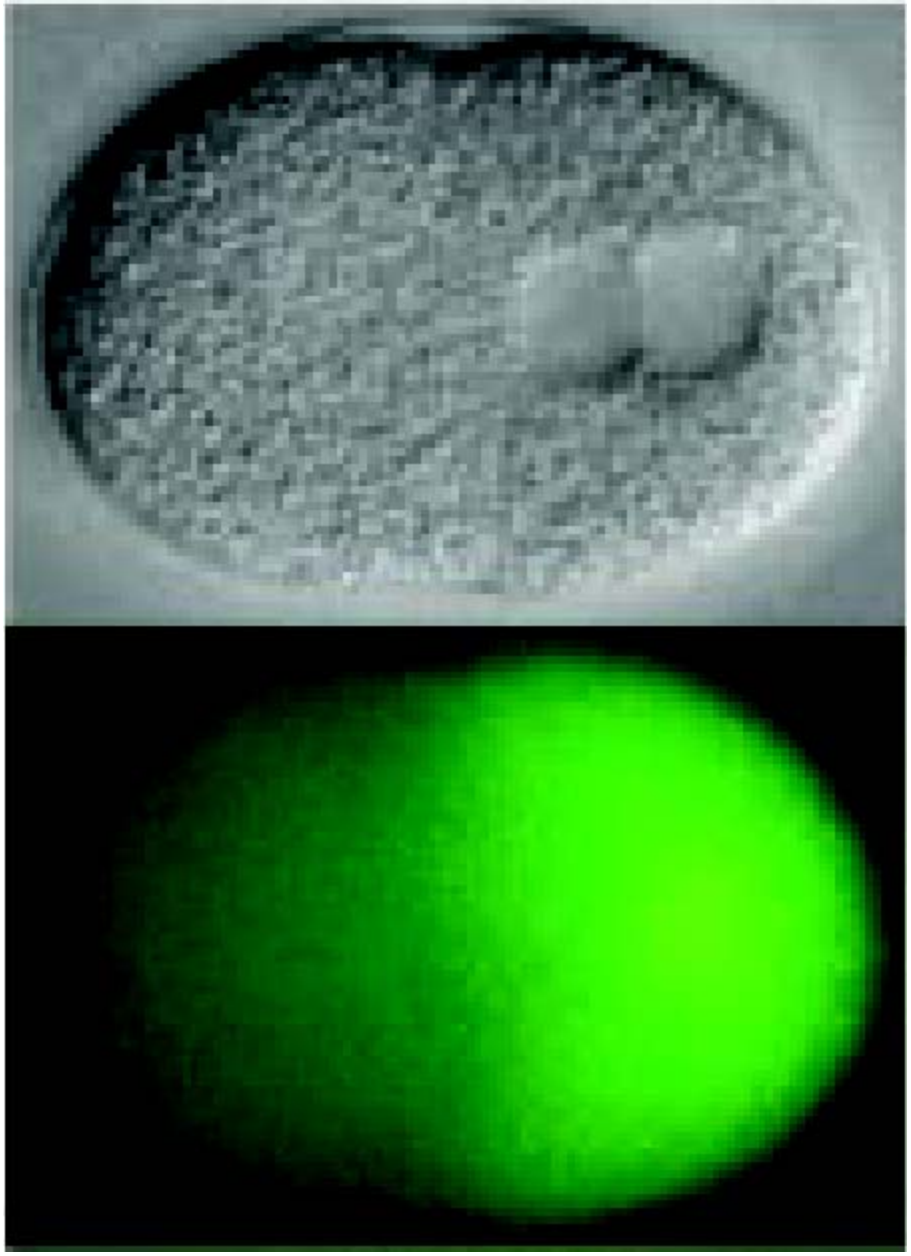
All three considered above scenarios deal with an anterior-posterior asymmetry or "single-dimensional asymmetry"

In *C. elegans* three genes are involved in anterior-posterior axis formation: *par, Wnt, lit-1*
(Nature, 1997, 390, 294-298)

- Anterior-posterior asymmetry can produce only axially-symmetric organisms with only asymmetry along the AP axis

- Mammals have two-dimensional asymmetry (one dimensional symmetry: right-left)

- One extra asymmetry initiator has to participate in differentiation



Science (2002), 298(5600), 1946-1948.

Intercellular interaction during differentiation

- The signal to differentiate is a chemical signal coming form outside the cell or the embryo

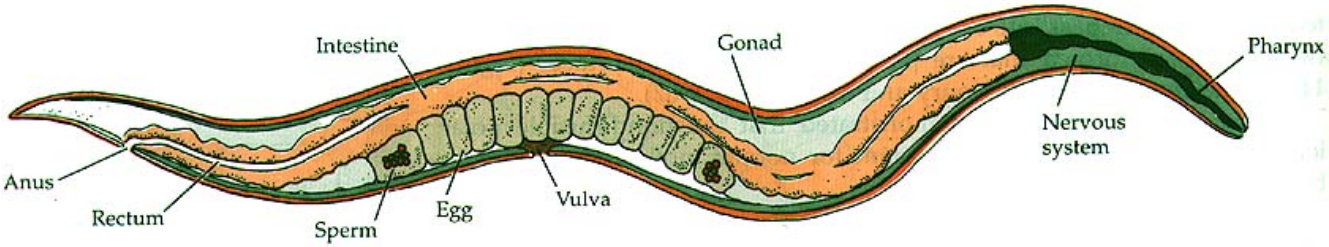
- While asymmetries are introduced, what does govern further differentiation?

Further differentiation is regulated by:

1. Memory about the initial asymmetry

2. Signals from neighboring cells

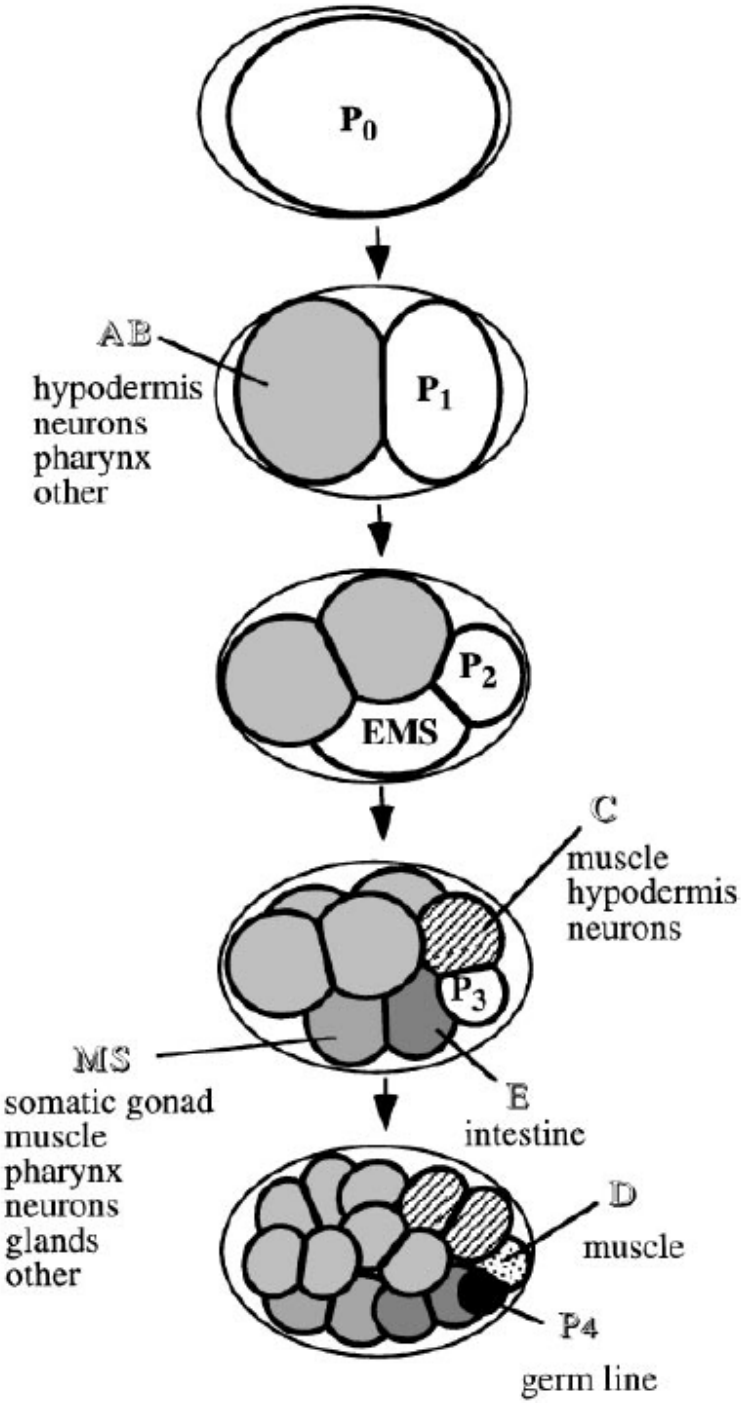
Example: *C. elegans*



- **All the gut cells are produced from a single precursor cell (E cell)**
- **Nothing else is produced from the E cell**
- **E cell can be produced only from the EMS progeny cell (memory factor)**
- **EMS cell appears to be symmetric**
- **Further differentiation of EMS to MS and E cells depends on the presence of the P2 cell (neighbor factor)**
- **The E cell is always neighboring the P2 cell**

Early embryogenesis in *C. Elegans*

Formation of founder cells. Anterior is to the left. The founder cell names and their contributions to the embryo are indicated. Clones of cells from each founder are indicated by shading. Asymmetrically dividing cells P_0, P_1, P_2, EMS, and P_3 are unshaded, with blastomere names inside the cell borders. The 16-cell embryo at the bottom is highly schematized for simplicity; it shows only approximate relative positions of blastomeres and does not reflect the fourth AB cleavage, which happens at roughly the same time as the P3 division.



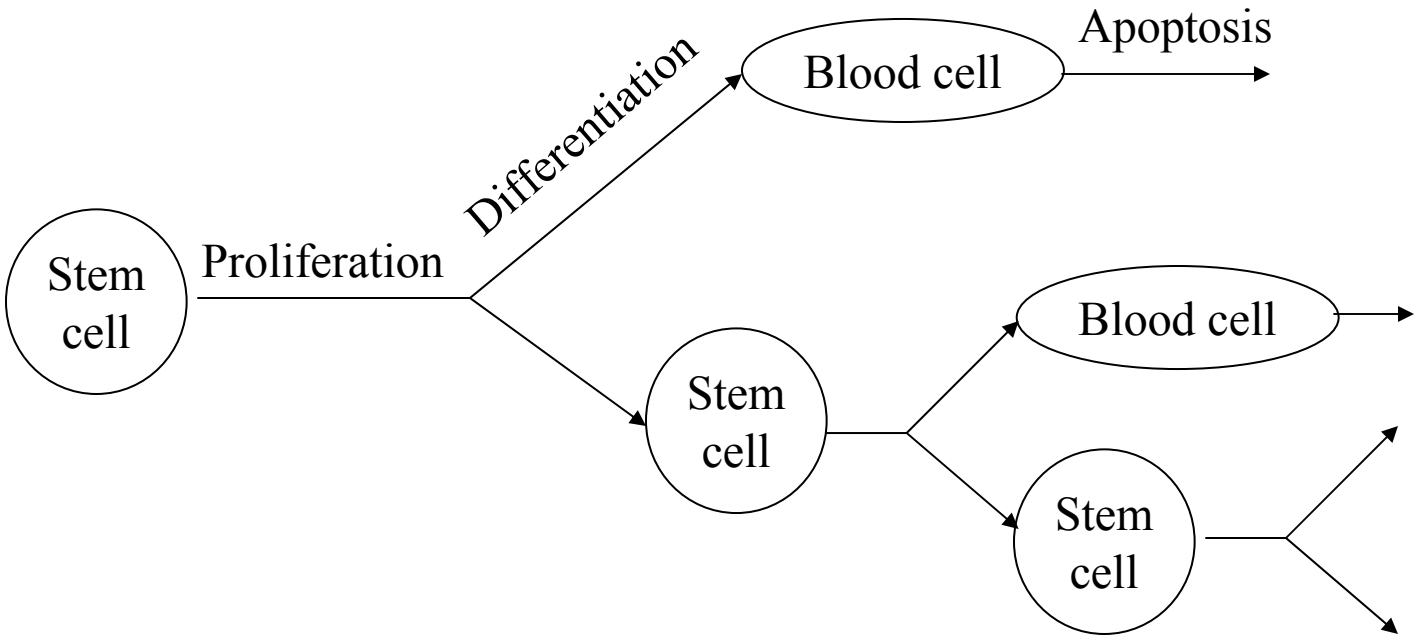
Differentiation in a mature organism

Differentiated cells do not proliferate

Most of somatic cells are differentiated

Stem cells do proliferate. There should be many stem cells in tissues undergoing continuous renewal (skin, intestine, blood)

Example: proliferation, differentiation and apoptosis of blood cells



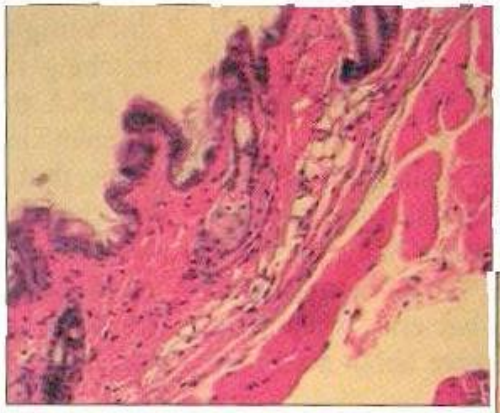
Science, September 5, 2003

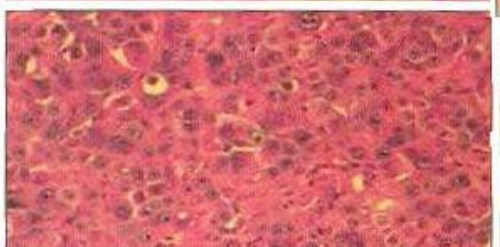
Mutant Stem Cells May Seed Cancer

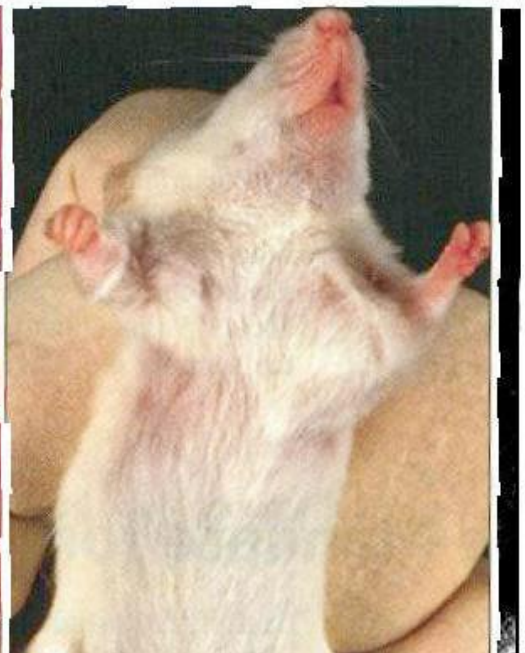
Stem cells have acquired a golden glow in the past few years as a possible tool for reversing the damage diseases wreak on organs. Many researchers predict that stem cell transplants—whether derived from embryonic tissue or from adult cells that retain the flexibility to develop into various tissues—will someday repair hearts crippled by heart attacks or brains under attack by Alzheimer's or Parkinson's disease. But the very qualities that make these cells so attractive for medicine—especially their capacity to replicate ad infinitum—also hint at a dark side. Recent evi dence suggests that the may be the source of th mutant cells that give rise t cancerous tumors and main tain their growth.

Researchers have identi fied what they call "cance stem cells" in blood cancers such as leukemias, and ir breast and brain cancers. The finding raises the possibility that the mutations that drive cancer development may have originated in the body's small supply of naturally occurring stem cells. Cancer stem cells resemble those

tions of the work as "absolutely huge … it has the chance of leading to new ways of thinking about cancer and new kinds of therapies." To cure cancer completely, Weissman and others say, it may be necessary to design therapies that target cancer stem cells, assuming that can be done without also wiping out the stem cells needed to maintain tissues such as the bone marrow and intestinal lining. In addition, the growing suspicion that stem cells may be the source of some cancers sounds a note of caution for efforts to use stem cells for organ repair.







of human hematopoietic stem cells, whi give rise to the various types of blood cell

The model starts with an extreme immunodeficient mouse strain called t NOD/SCID mouse. The researchers irradia the animals to destroy their bone marrow a then introduce human stem cells to see if th will produce a new complement of bloc cells. After showing that normal huma hematopoietic stem cells can do this, Dic and his team used the approach to study t cancer-causing power of acute myeloi leukemia (AML) cells freshly harvested fro human patients. By progressive ly diluting a known number c leukemia cells, they establishe that only the very rare AM cell—about one in a million— has the ability to reproduce th disease in the animals.

Because this was a muc smaller fraction of cells tha could form colonies in culture the result indicated that th simple ability to grow didn' equate with the ability to devel op into leukemias in living ani mals. Dick speculated that the leukemia-initiating cells had a greater developmental potential than the vast majority of clone-

Differentiation and cancer

Why does cancer kill?

- Cancer cells are undifferentiated
- They do not produce tissue-specific proteins
- They do not serve as the normal tissue cells
- They suppress survival of normal cells
- Undifferentiated cancer cells establish colonies in new tissues where normal differentiated cells would not survive

How does undifferentiation happen?

- Cancer destroys anti-mutation machinery
- Tissue-specific genes are also mutated
- Cancer cells up-regulate and down-regulate the expression of the intact genes
- Undifferentiation of cancer cells is often reversible
- Differentiation in cancer cells can be induced by hormones etc.

Example: colon cancer

Downregulation of:

fatty-acid binding proteins
cytokeratin
carbonic anhydrase
guanylin
uroguanylin

Upregulation of:

growth factors
enzymes involved in glycolysis

Science 1997, 276, 1268-1271

The role of Cell Differentiation in cancer diagnostic and therapy

- **Differentiation of cancer cells is an important prognostic factor**
- **More differentiated cancer cells are more susceptible to therapies and easier undergo apoptosis**

- **Pregnancy protects from breast cancer through the induction of a complete differentiation of the mammary gland**

- **Retinoic acid is known as differentiation inducer in cancer cells**
- **It is a potential anticancer drug**

APOPTOSIS

Two means of cell death

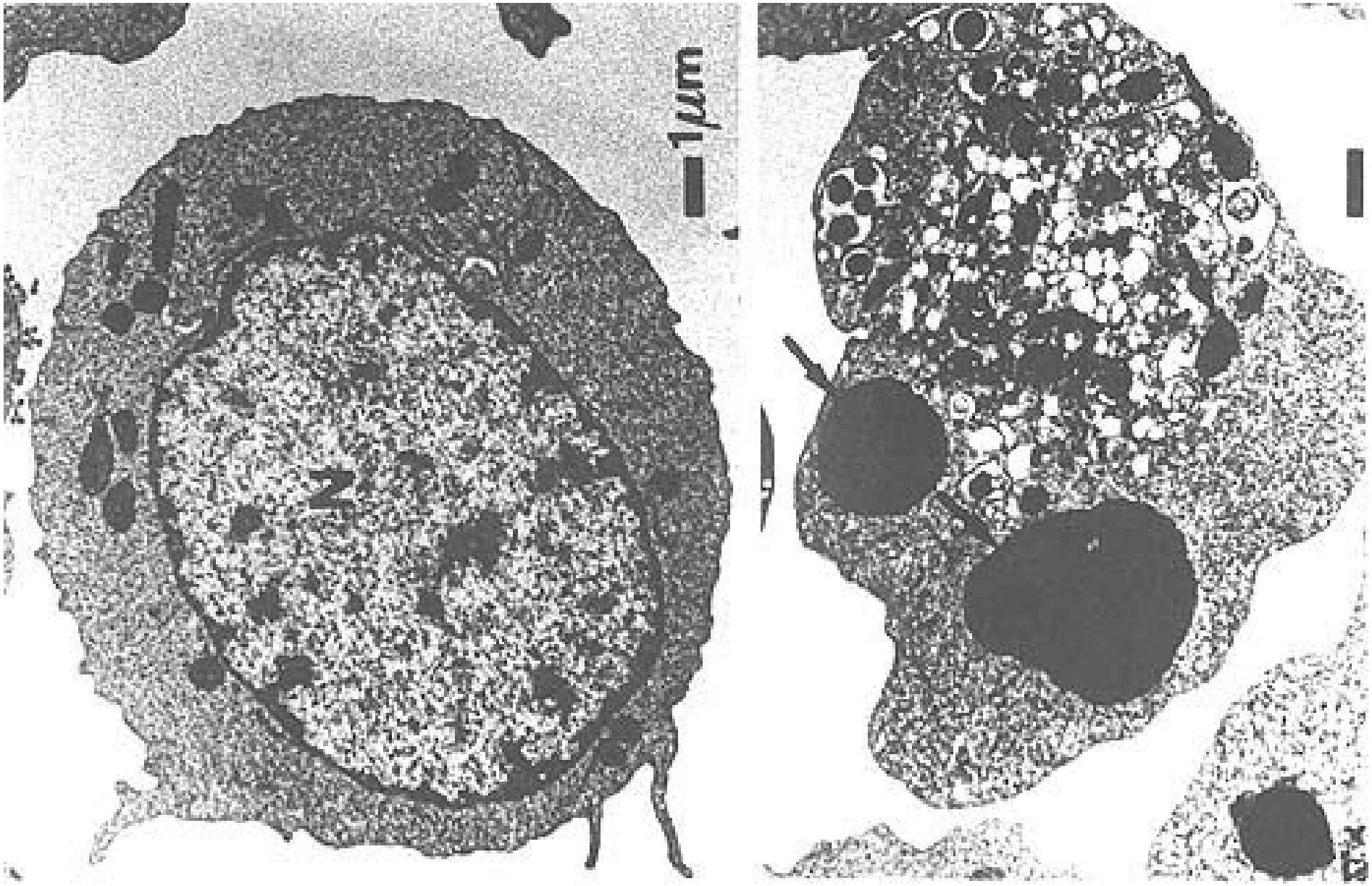
Necrosis **(Pathological cell death caused by acute cell injury)**

- Destruction of cellular membrane
- Leakage of cytoplasmic contents
- Inflammatory response

Apoptosis **(Programmed cell death through controlled autodigestion of the cell)**

- Integrity of cellular membrane is maintained
- Endogenous proteases digest important proteins
- Endogenous nucleases degrade DNA
- Disassembled cells brake down into apoptotic bodies
- Apoptotic bodies are phagocytosed by neighboring cells
- No inflammatory response

Morphologies of Normal and Apoptotic Cells



The role of cell suicide

1. **Developmental death during embryogenesis**

Example: ***C. elegans*** **consists of 959 cells. Extra 131 cells die through apoptosis during development.**

2. Normal tissue turnover of a mature organism

Example: We lose 10^7 blood cells every second; most of them die by apoptosis.

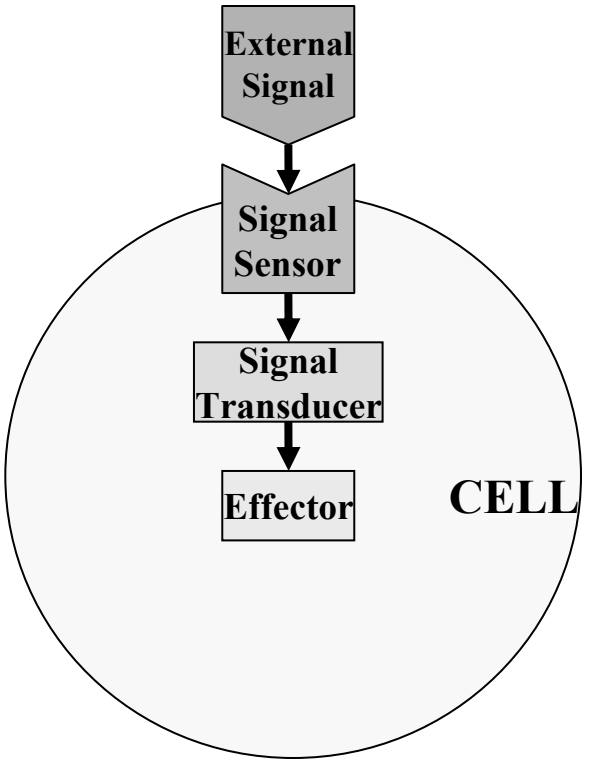
3. Elimination of diseased cells

Examples:

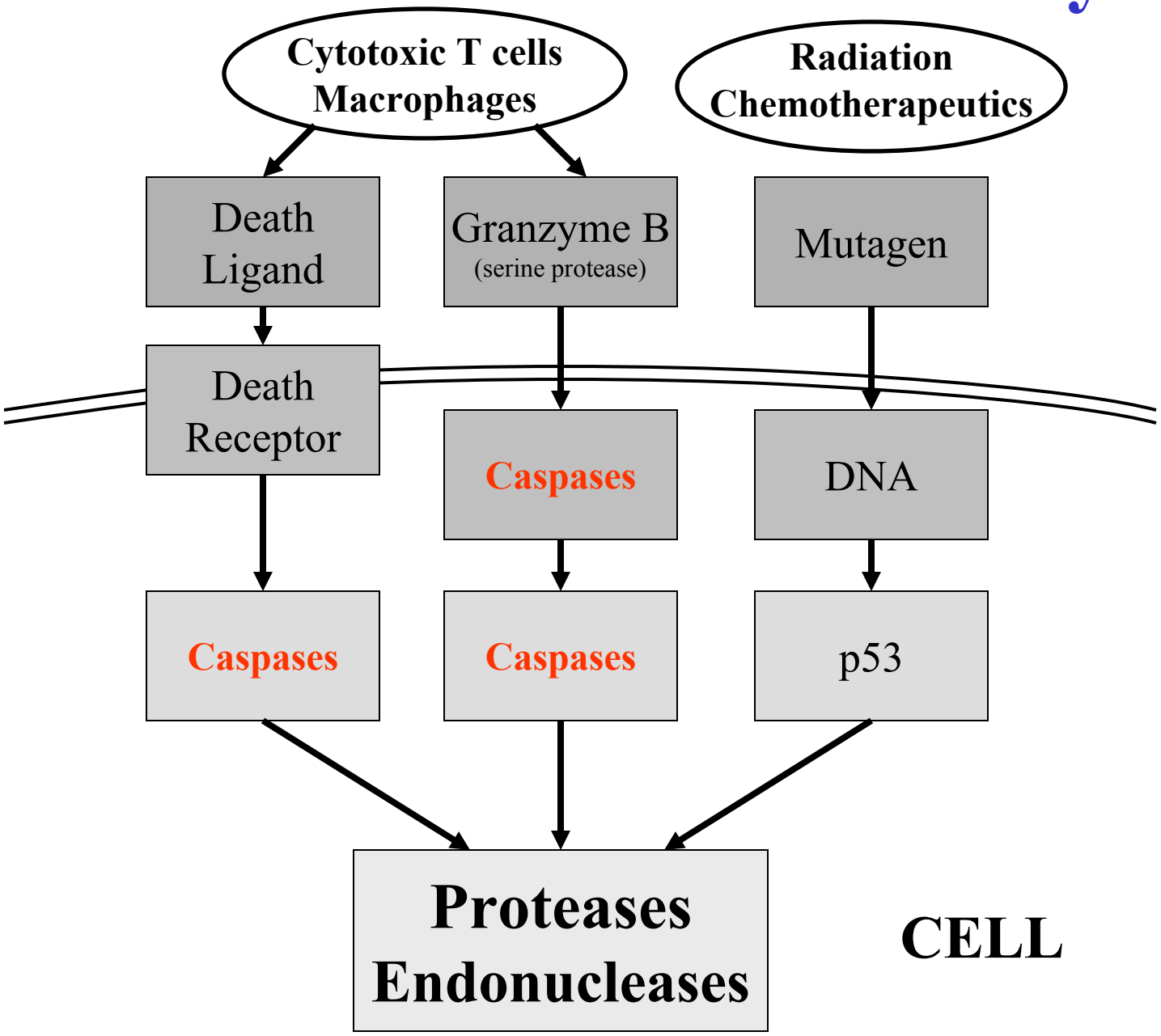
- Viral infection

- Mutagen-caused DNA damage

External signal is needed to initiate apoptosis



Suicide machinery



Caspases: Enemies Within

- ☆ **Cysteine proteases**
- 🕐 **Synthesized as inactive precursors**
- 🕑 **Precursors are activated by proteolysis**
- 🕒 **Highly specific**
- 🕓 **Highly efficient**
- 🕔 **Targets:**
    1. **Procaspases (caspase precursors)**
    2. **Inhibitors of caspases and endonucleases**
    3. **Lamina (which supports nuclei integrity)**
    4. **Proteins involved in cell-to-cell adhesion**

Suicide machinery is always present

Fact: No nuclei is required for apoptosis

↓

All the proteins involved in apoptosis are always expressed

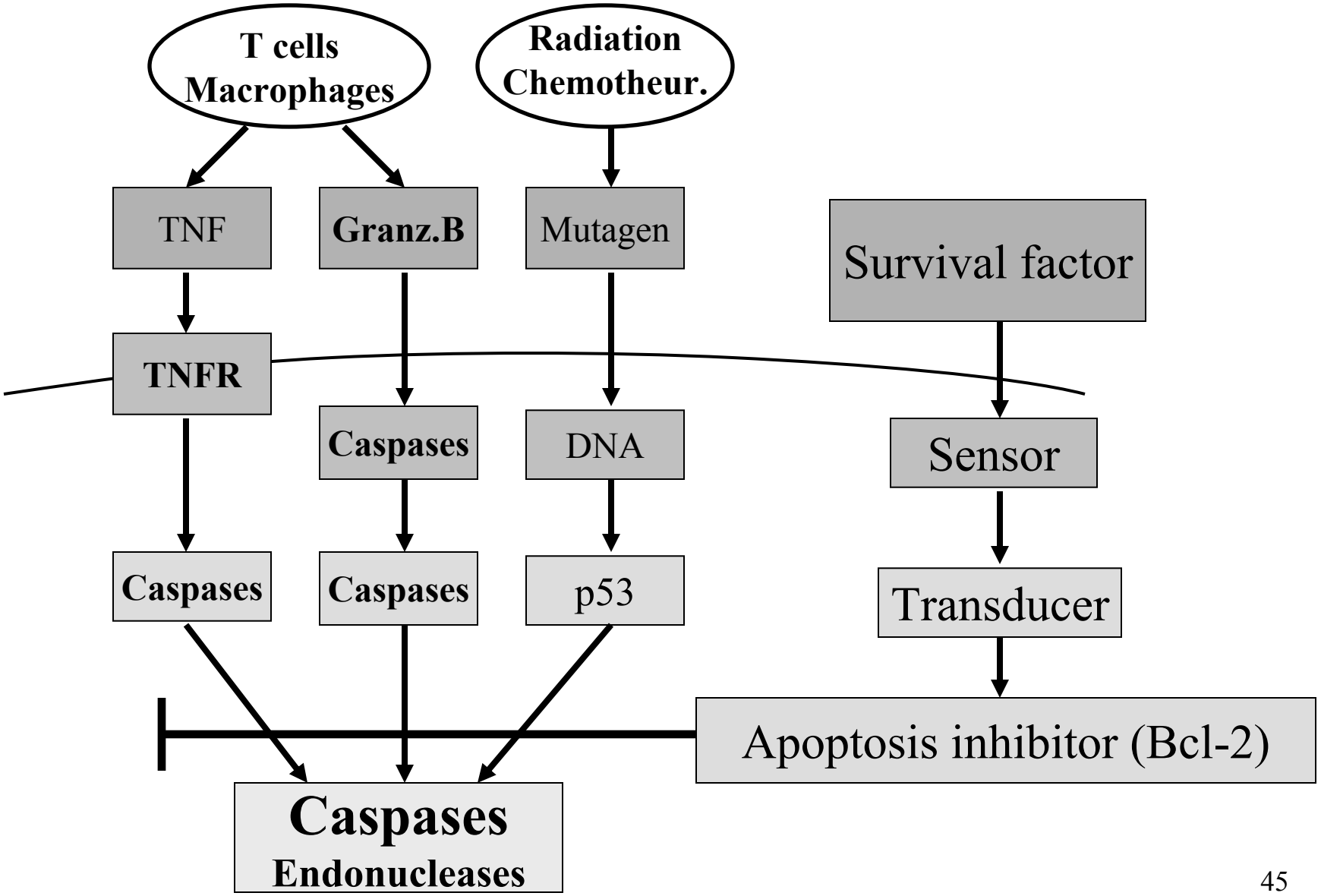
↓

How does the cell survive in the presence of suicide machinery ?

↓

There is apoptosis inhibitory mechanism

Inhibition of apoptosis



Survival Factors

Physiological

1. Growth factors
2. Extracellular matrix
3. Neutral amino acids
4. Zinc
5. Androgens

Pharmaceutical agents

1. Calpain Inhibitors
2. Cysteine protease inhibitors
3. Tumor promoters
   Phenobarbital
   α-Hexachlorocyclohexane

Viral Genes

1. Adenovirus *E1B*
2. Baculovirus *p35*
3. Cowpox virus *crmA*
4. Epstein-Barr virus *BHRF1*, *LMP-1*
5. Herpesvirus γ1 34.5

There should be a balance between apoptotic factors and survival factors!