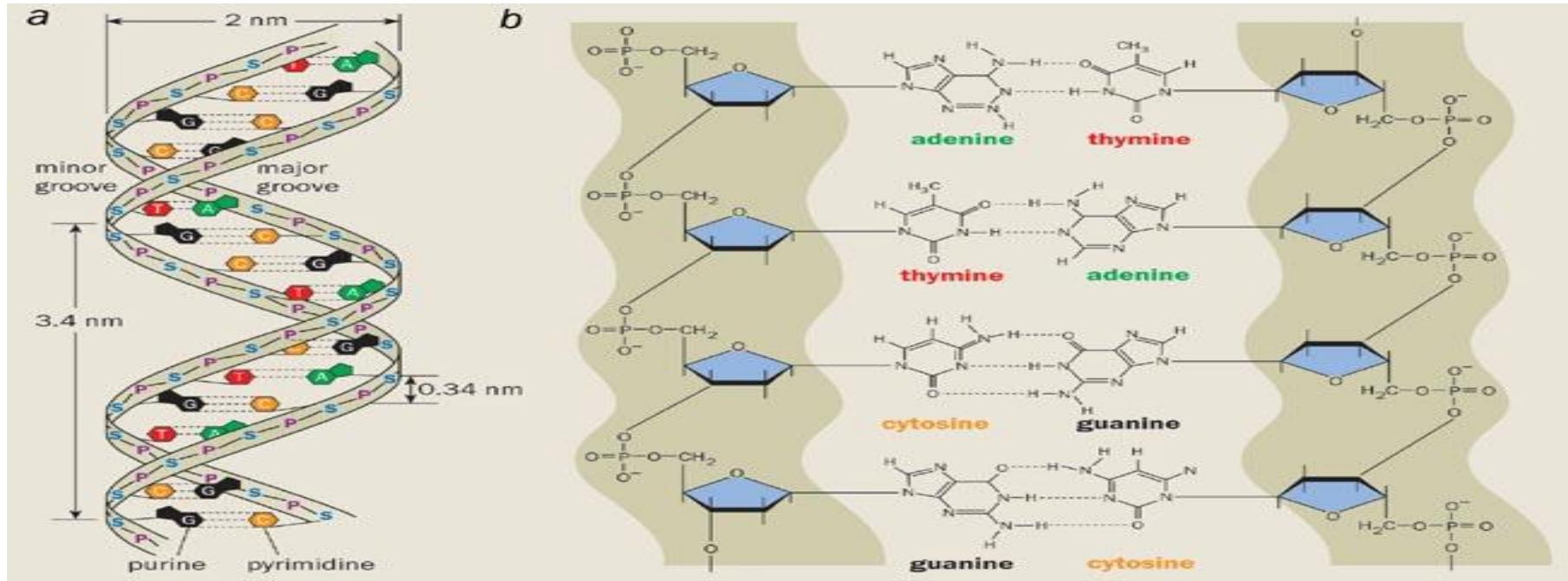


Molecular Biology Techniques and Mechanisms of Cell Death

Feyruz Rassool, Ph.D.

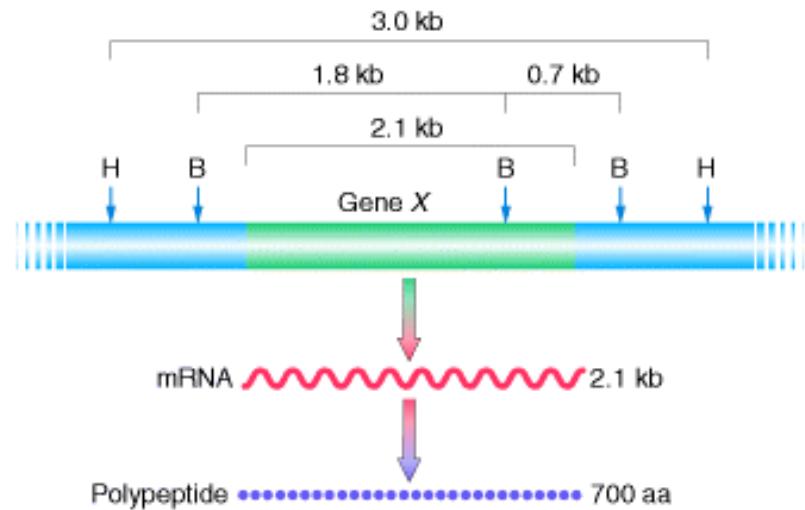
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DNA is the Code of Life



Central Dogma about DNA

DNA → mRNA → protein



Endonucleases: restriction enzymes

- enzymes that cleave specific recognition sequences within DNA.

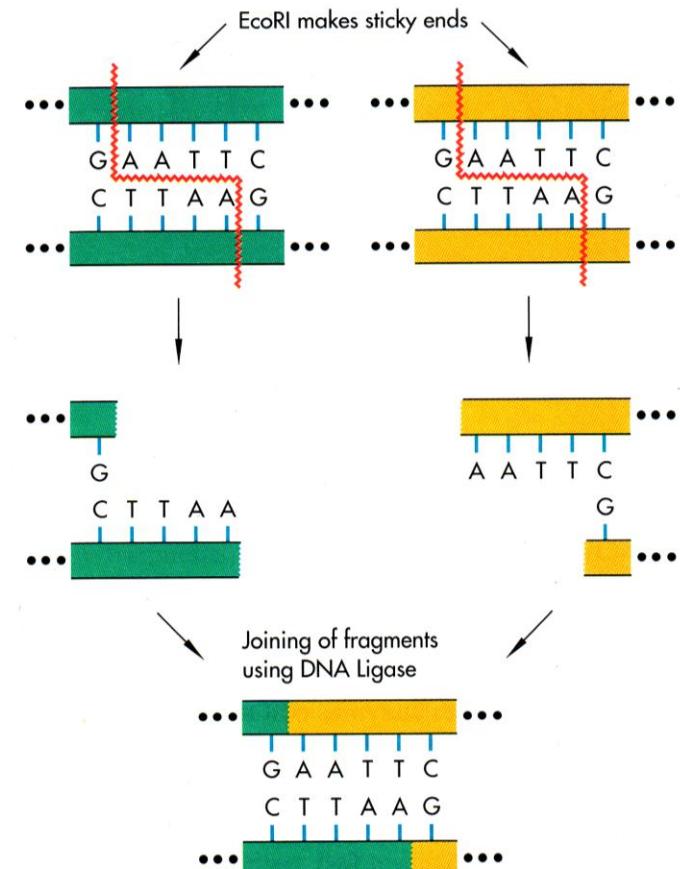
TABLE 5-1

Some Restriction Enzymes and Their Cleavage Sequences

MICROORGANISM	ENZYME ABBREVIATION	SEQUENCE	NOTES*
<i>Haemophilus aegyptius</i>	<i>HaeIII</i>	5' ... G G C C ... 3' 3' ... C C G G ... 5'	1
<i>Thermus aquaticus</i>	<i>TaqI</i>	5' ... T C G A ... 3' 3' ... A G C T ... 5'	2
<i>Haemophilus haemolyticus</i>	<i>HbaI</i>	5' ... G C G C ... 3' 3' ... C G C G ... 5'	3
<i>Desulfovibrio desulfuricans</i>	<i>DdeI</i>	5' ... C T N A G ... 3' 3' ... G A N T C ... 5'	4
<i>Moraxella bovis</i>	<i>MboII</i>	5' ... G A A G A (N) ₈ ... 3' 3' ... C T T C T (N) ₇ ... 5'	5
<i>Escherichia coli</i>	<i>EcoRV</i>	5' ... G A T A T C ... 3' 3' ... C T A T A G ... 5'	1
	<i>EcoRI</i>	5' ... G A A T T C ... 3' 3' ... C T T A A G ... 5'	2
<i>Providencia stuartii</i>	<i>PstI</i>	5' ... C T G C A G ... 3' 3' ... G A C G T C ... 5'	3
<i>Microcoleus</i>	<i>MspI</i>	5' ... C C T N A G G ... 3' 3' ... G G A N T C C ... 5'	4
<i>Nocardia otitidis-caviae</i>	<i>NoI</i>	5' ... G C G G C C G C ... 3' 3' ... C G C C G G C G ... 5'	6

* Notes:

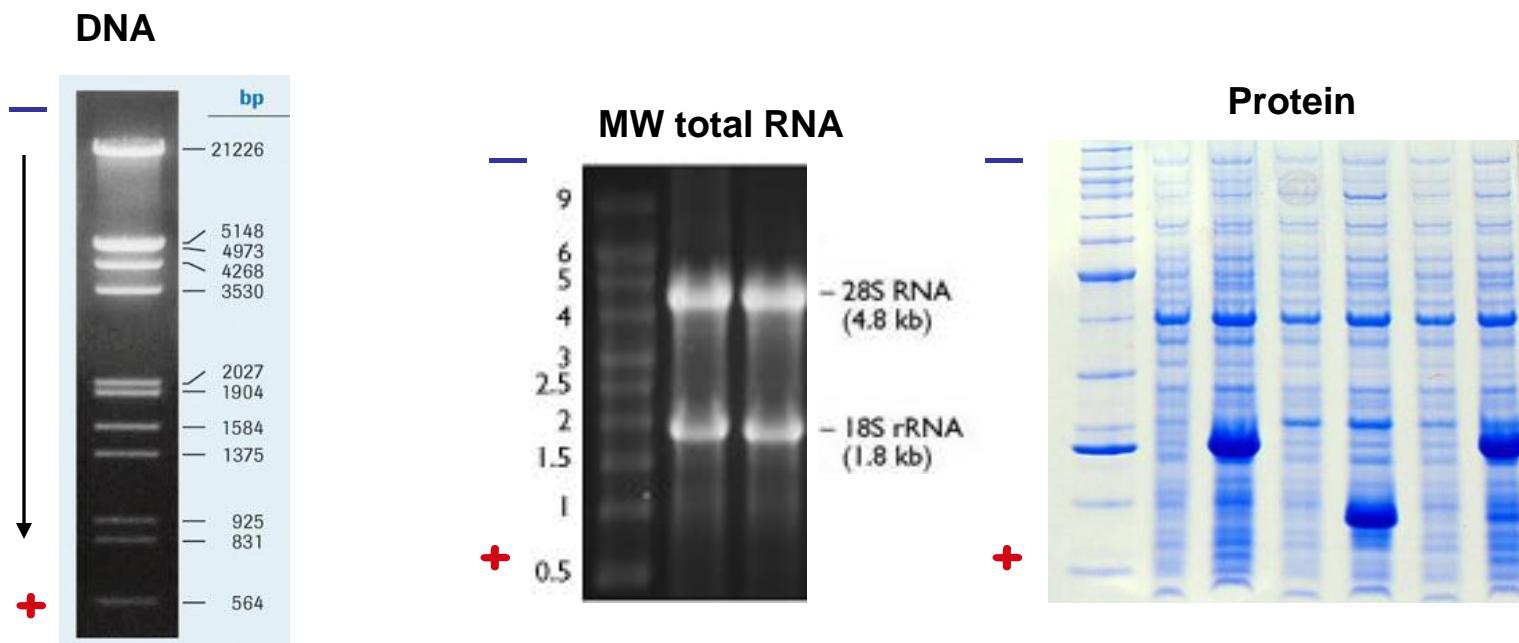
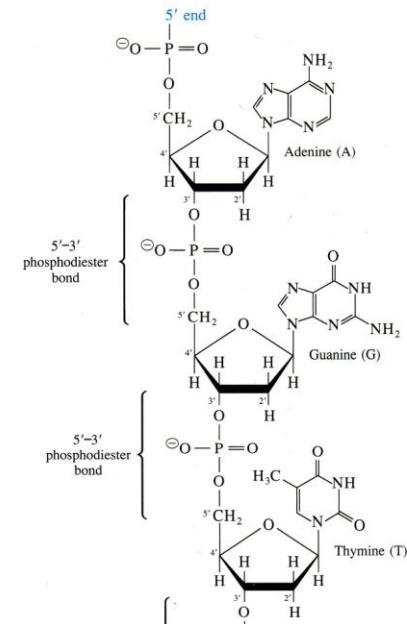
1. Enzyme produces blunt ends.
2. The single strand is the 5' strand.
3. The single strand is the 3' strand.
4. The base pair *N* can be any purine or pyrimidine pair.
5. The enzyme does not cut within the recognition sequence, but at whatever sequence lies eight nucleotides 3' to the recognition site.
6. *NoI* has an eight-base recognition sequence and cuts mammalian DNA very infrequently.



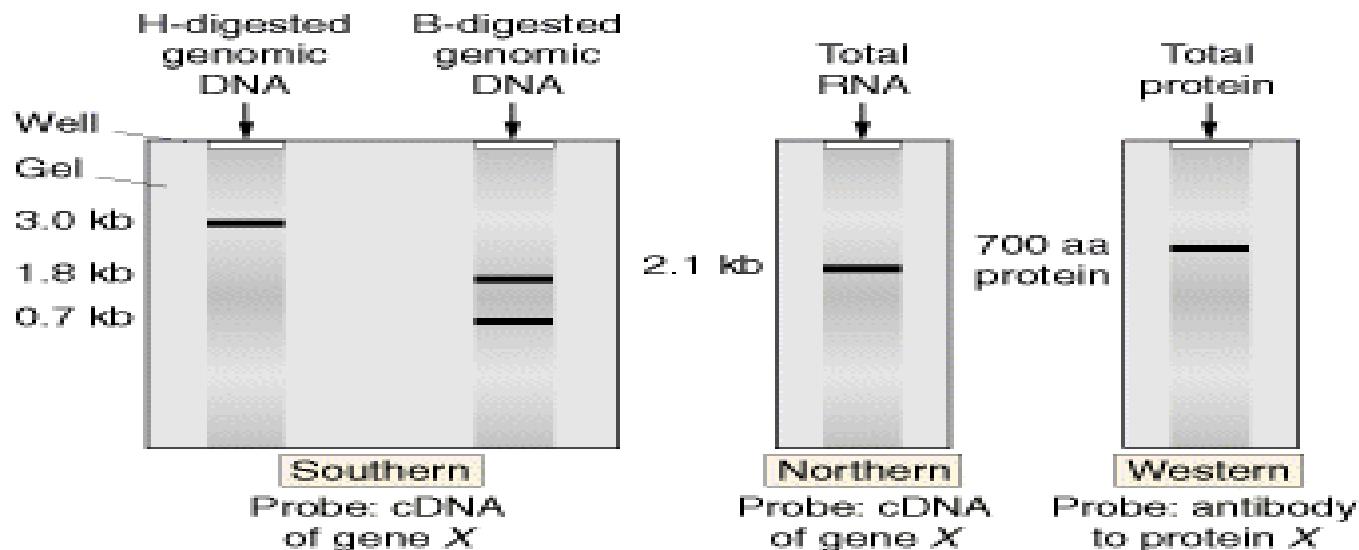
Visualization and detection of DNA, RNA, and protein

Gel electrophoresis:

- agarose or polyacrylamide
- separates by size



Southern, Northern, and Western blot analysis



Southern blot: DNA detection

DNA isolated, separated by gel electrophoresis and transferred to membrane.

Detection: annealing of single stranded DNA, cDNA, or RNA probe to complementary sequence on blot.

Northern blot: mRNA Detection

mRNA

Detection: annealing of single cDNA, or RNA probe

Western blot: Protein Detection

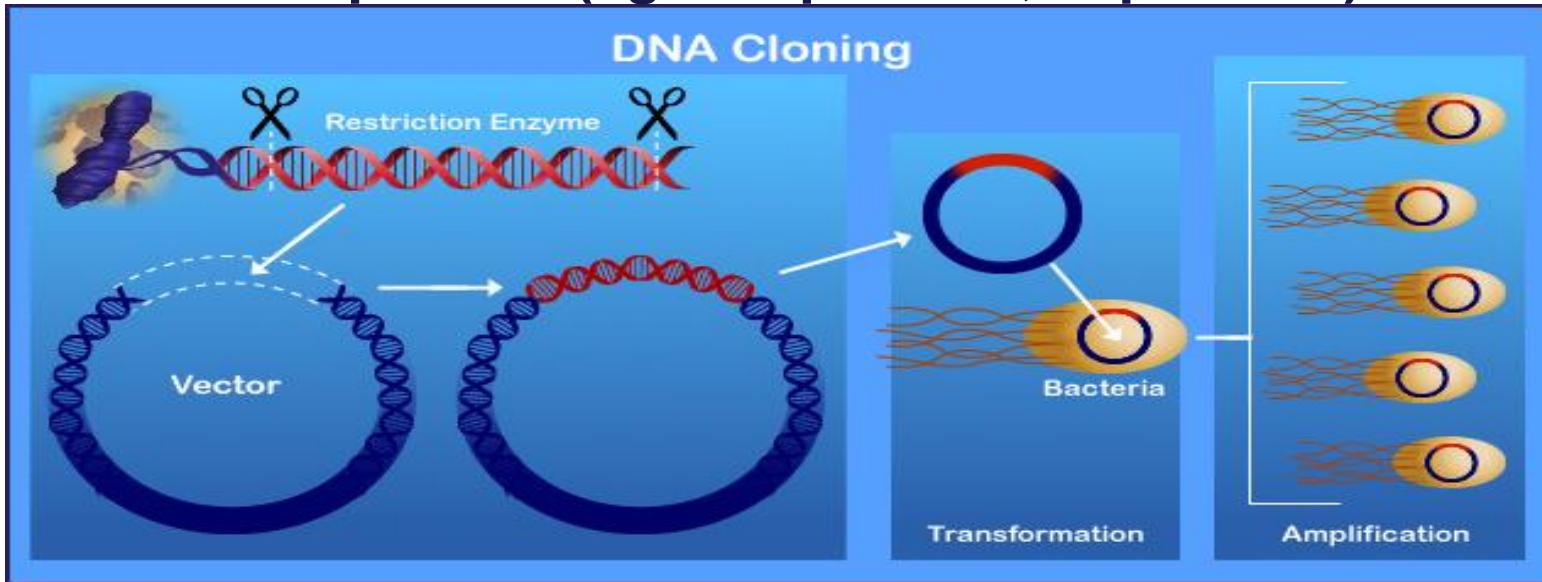
Protein in Cell extract separated by gel electrophoresis and transferred to membrane.

Detection: Use an antibody to detect protein of interest.

Modern Genetic Analysis

Griffiths, A., W.H. Freeman & Co., c1999

Gene Cloning: This process allows for the generation of multiple copies of a single gene. Once isolated, the gene can be manipulated (eg.: sequenced, expressed).



Vectors: DNA molecules into which foreign DNA has been inserted which is capable of replicating in a host organism.

Plasmids: Can accommodate up to approximately 15,000 bases (15 Kb).

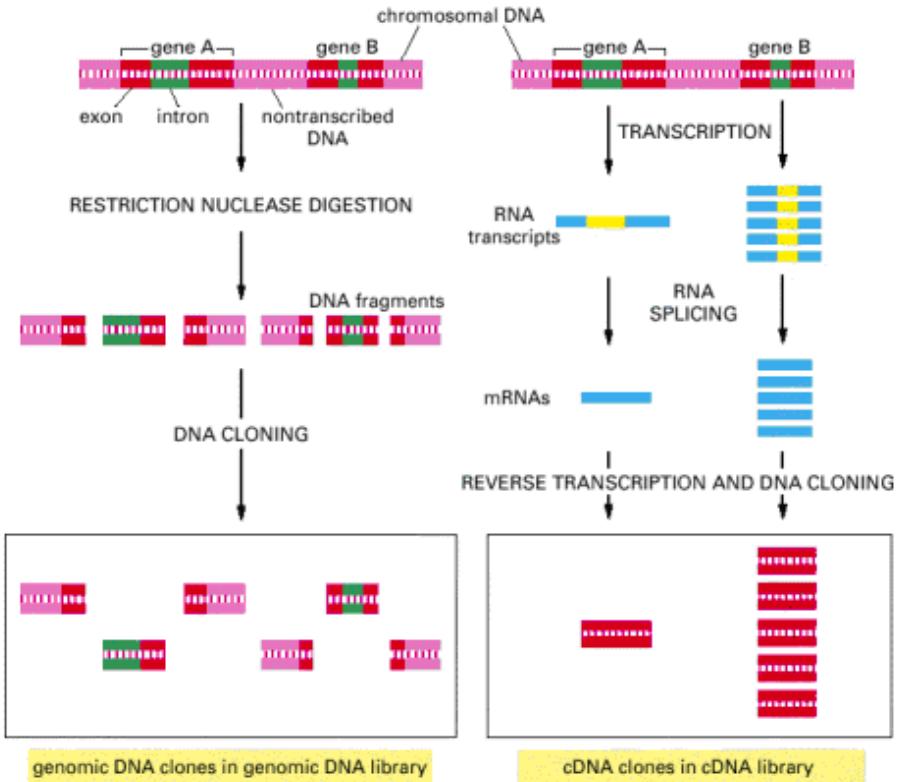
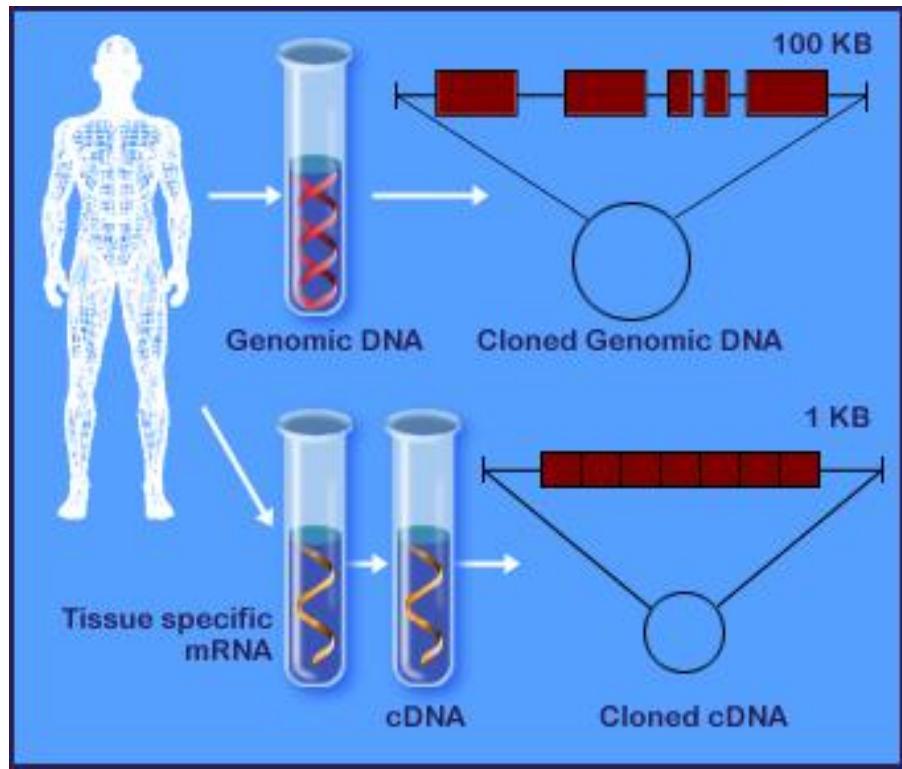
Bacteriophages: Bacterial viruses that accept inserts of up to 20,000 bases.

Cosmids: Recombinant/hybrid plasmids with bacteriophage components that accept inserts up to 45 Kb.

Bacterial Artificial Chromosomes (BACs): Can accept up to 150 Kb.

Yeast Artificial Chromosomes (YACs): Up to 1000 Kb of insert.

Libraries: Genomic and cDNA

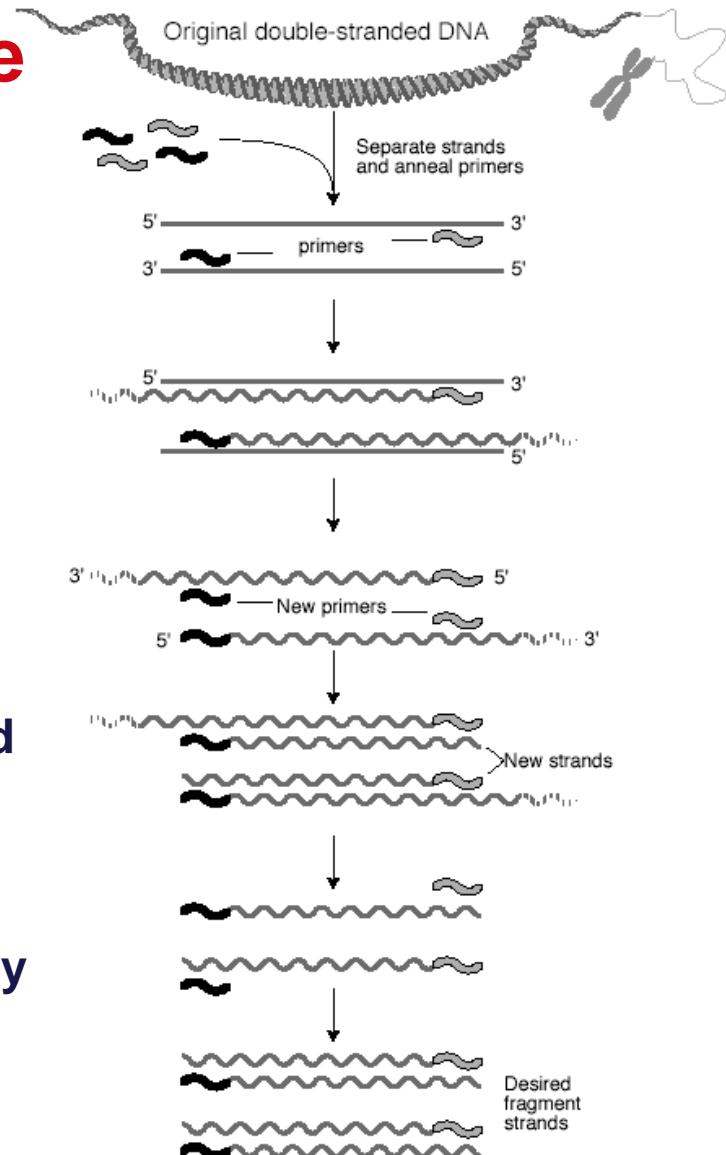


Collections of clones sufficient in number to include all of the genes (genomic library) of an organism or all of the expressed genes (complementary DNA/cDNA) of a particular cell type.

DNA Amplification: Polymerase Chain Reaction

How it works:

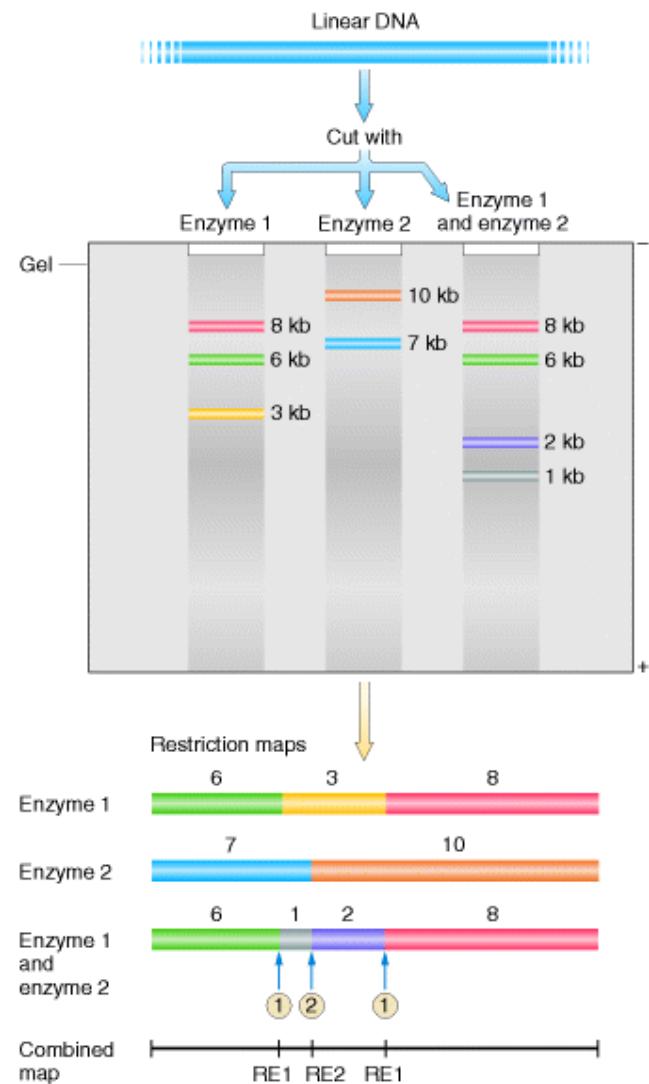
1. Separate the two strands with low heat
2. Reaction components: dNTPs, primers, and DNA Polymerase
 - Creates double stranded DNA from a single strand.
 - Polymerase uses primers, ssDNA template and dNTPs for synthesis of double stranded DNA.
3. Now you have two copies.
Repeat. Amount of DNA grows Exponentially
 $(1 \rightarrow 2 \rightarrow 4 \rightarrow 8 \rightarrow 16 \rightarrow 32 \rightarrow 64 \rightarrow 128 \rightarrow 256 \dots)$



Restriction Mapping

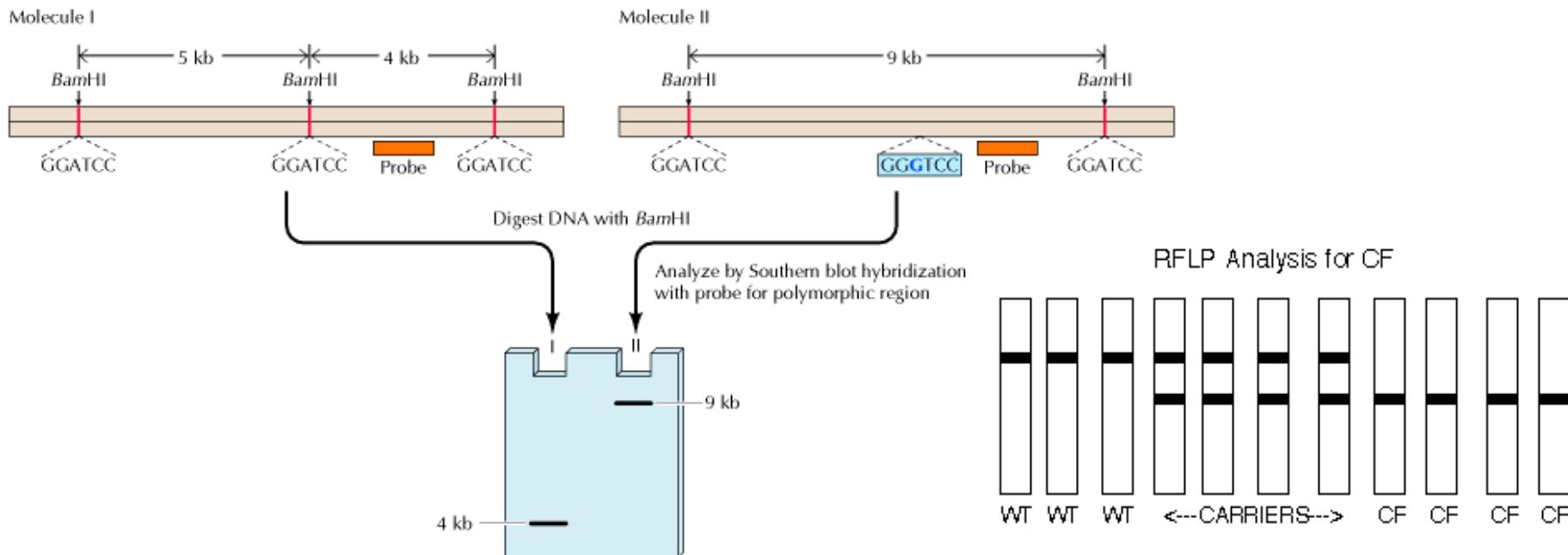
Once a gene is cloned, a restriction map can be made using two or more restriction enzymes.

Can also be done with genomic DNA, but you must do Southern blot analysis using a probe covering the whole gene.



Restriction Fragment Length Polymorphism: RFLP

- DNA from an individual specimen is extracted and purified and may be amplified by PCR. The DNA is then cut into *restriction fragments* using restriction endonucleases, which only cut the DNA molecule where there are specific recognition sequences. The restriction fragments are then separated according to length by agarose gel electrophoresis. The resulting gel may be analyzed by Southern blotting.



- RFLPs are inherited in a Mendelian manner and can be used for genetic linkage analysis with a diseased gene.

The Uses of Microarrays: High throughput approach

1. Determine gene expression at the mRNA or protein level.
2. Identify mutations and genotype at the genomic DNA level.
3. Detect and locate chromosomal changes (*CGH* = comparative genomic hybridization).

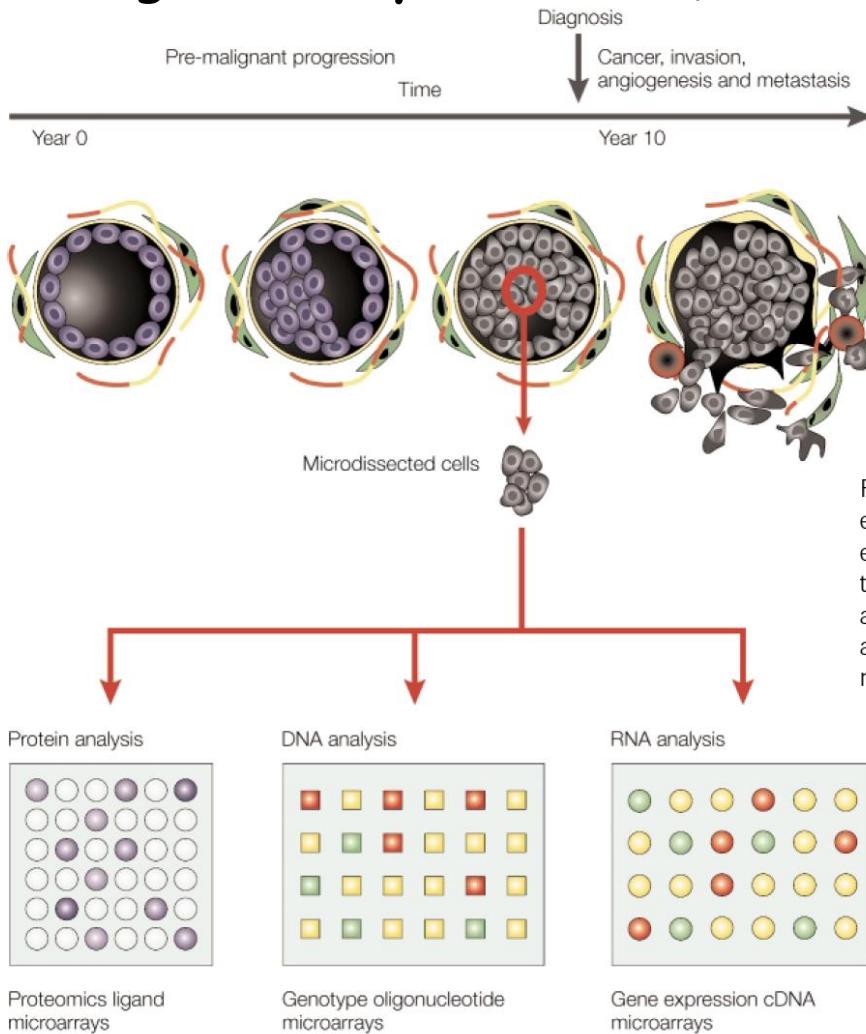
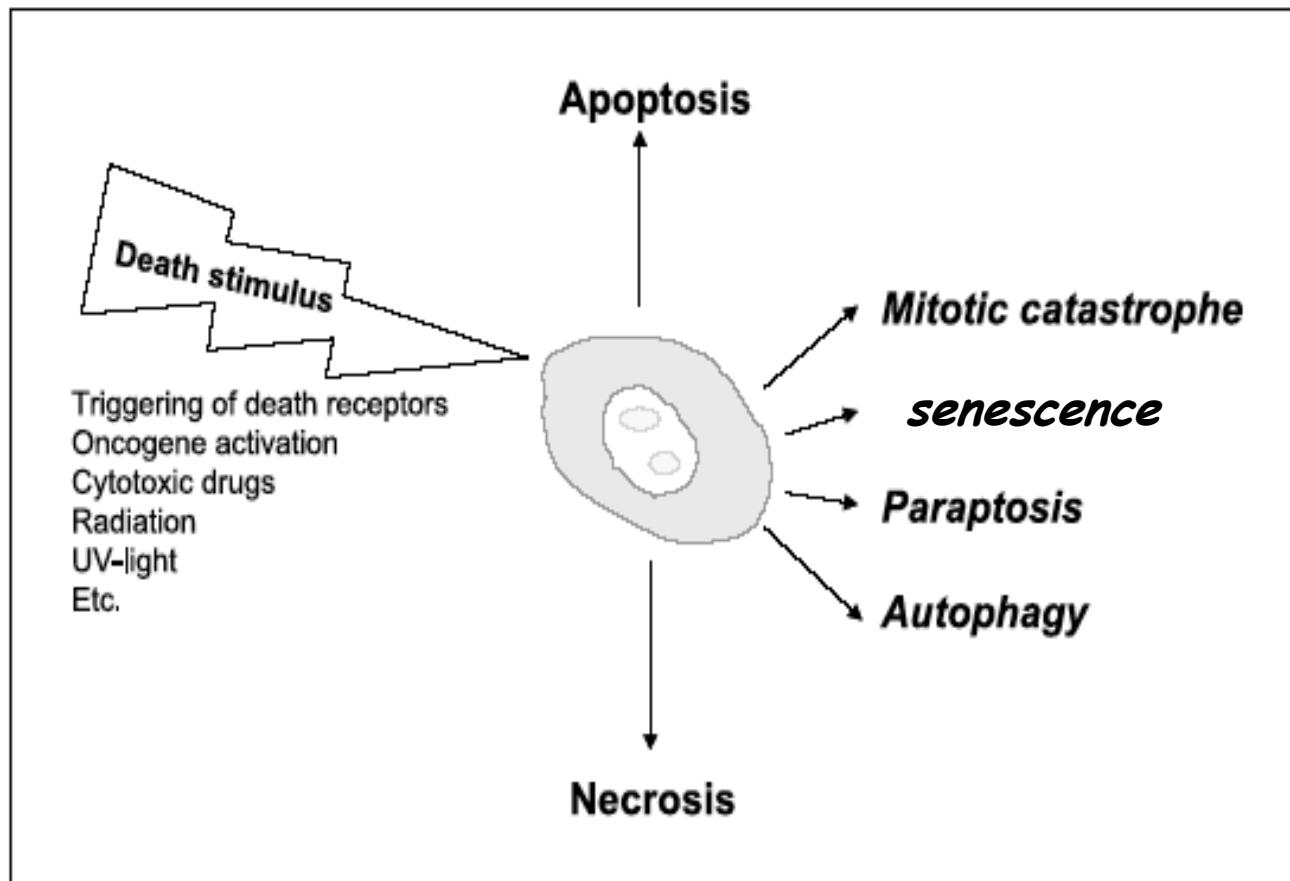


Figure 2 | **Molecular profiling of pre-malignant progression.** Invasive metastatic cancer emerges after a long pre-malignant phase that occurs in microscopic regions of the tissue epithelium. Microdissection makes it possible to profile the molecular patterns that track all the way through from normal epithelium to metastatic cancer, in the same patient^{16,18}. DNA analysis indicates the genes that have been mutated, deleted or amplified, whereas RNA and protein arrays reveal differences in transcription, protein synthesis and post-translational modification of proteins.

DNA damage and its Consequences

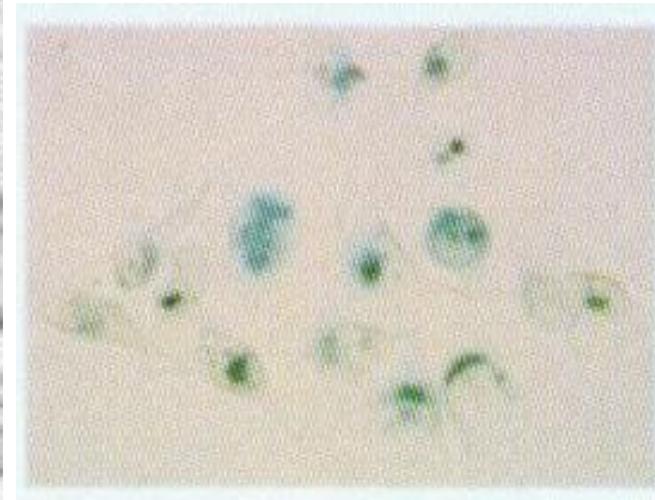
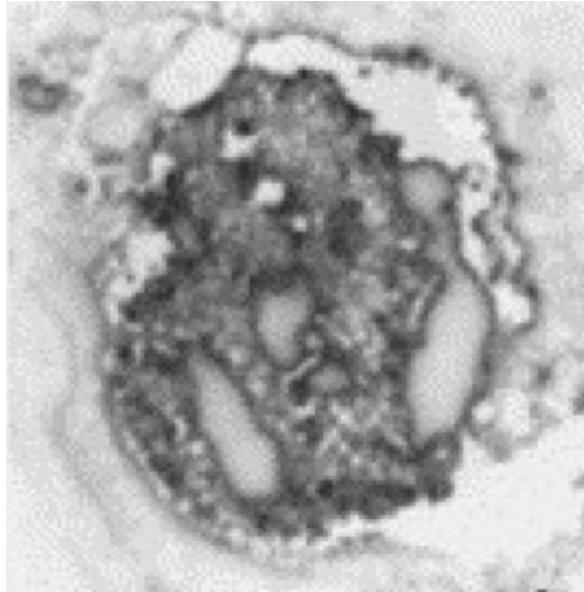
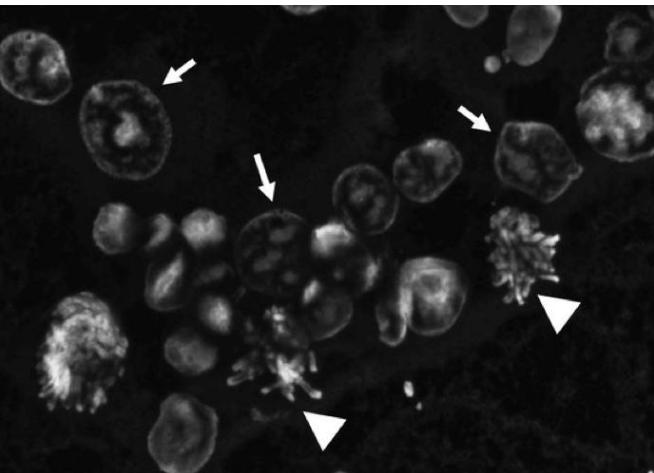


Importance of Cell Death

- 1. Radiation oncologists are primarily interested in loss (death) of cells following RT because this determines the outcome of cancer treatment - therapeutic ratio**
- 2. Death pathways contribute to intrinsic radiosensitivity**
- 3. Mode of death offers therapeutic opportunities**
- 4. Mutations in death pathways contribute to carcinogenesis**

Types of REPRODUCTIVE Cell Death

- 1. Mitotic catastrophe**
- 2. Programmed cell death / apoptosis**
- 3. Terminal growth arrest / senescence**



Mitotic Catastrophe

After irradiation, MOST reproductive cells die by a mitotic death, also known as mitotic catastrophe

- Sometimes occurs after several cell divisions
- Number of divisions depends on dose
- Traverse of multiple cell divisions increases the probability that some clonogenic progeny can survive, even if the majority die

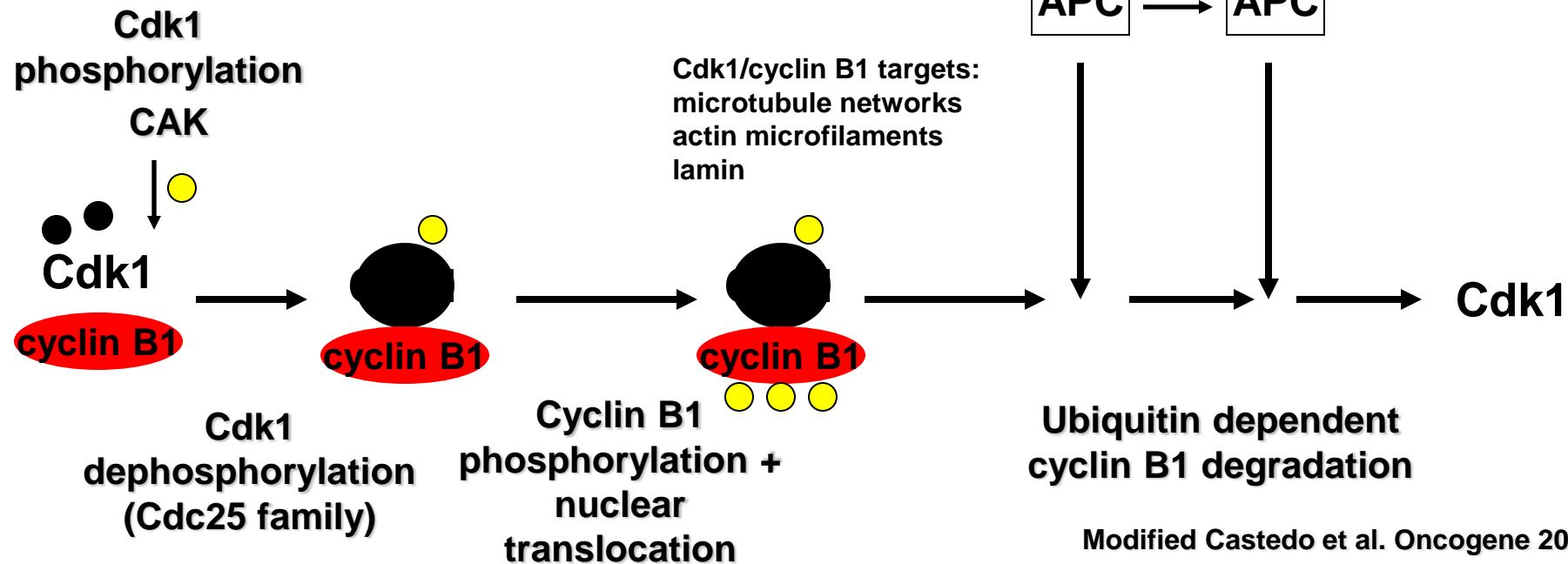
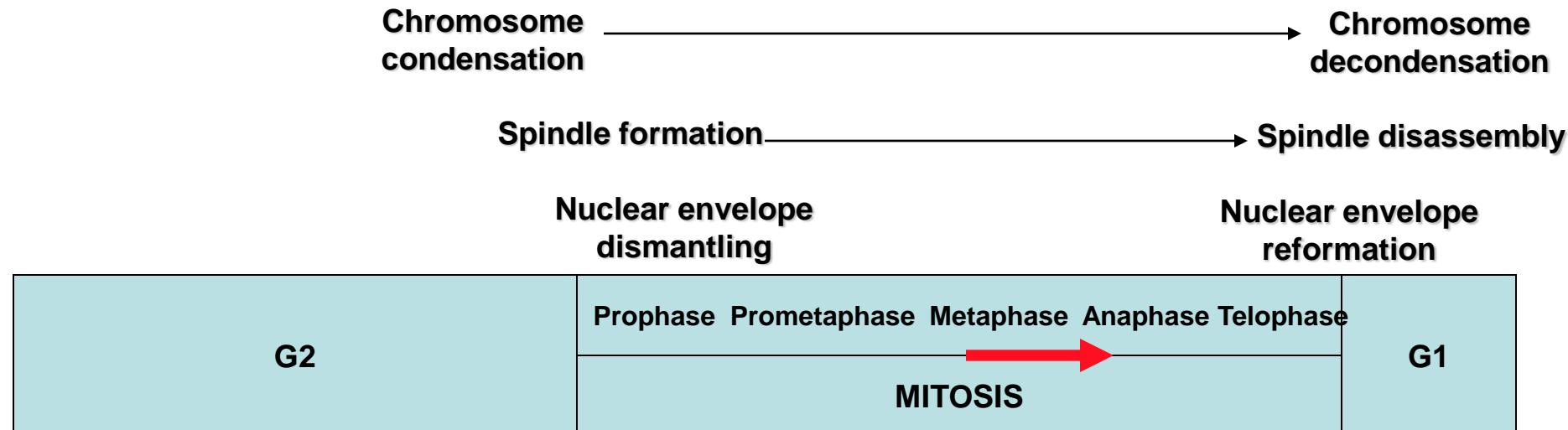
Mitotic Catastrophe

Mitotic catastrophe: failure to undergo complete mitosis (mitotic failure) after DNA damage (coupled to defective checkpoints) → tetraploidy/ endoploidy

Cell death occurs during mitosis as a result of DNA damage or deranged spindle formation + debilitation of different checkpoint mechanisms (e.g. DNA structure, spindle assembly)

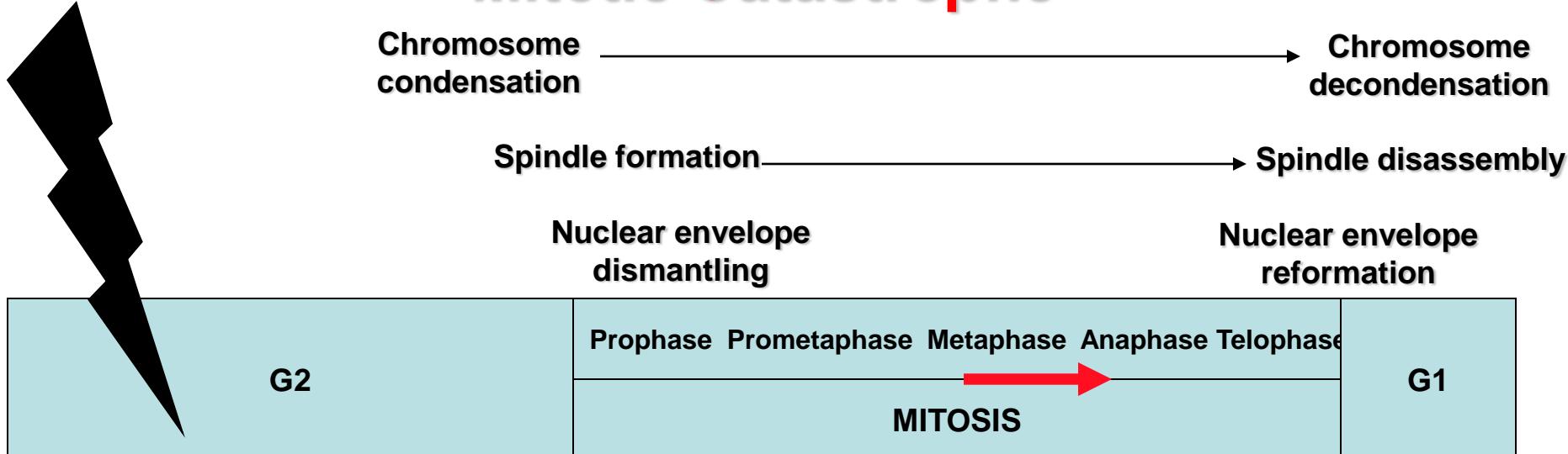
Involvement of Cdk1/cyclin B

Mitotic Catastrophe

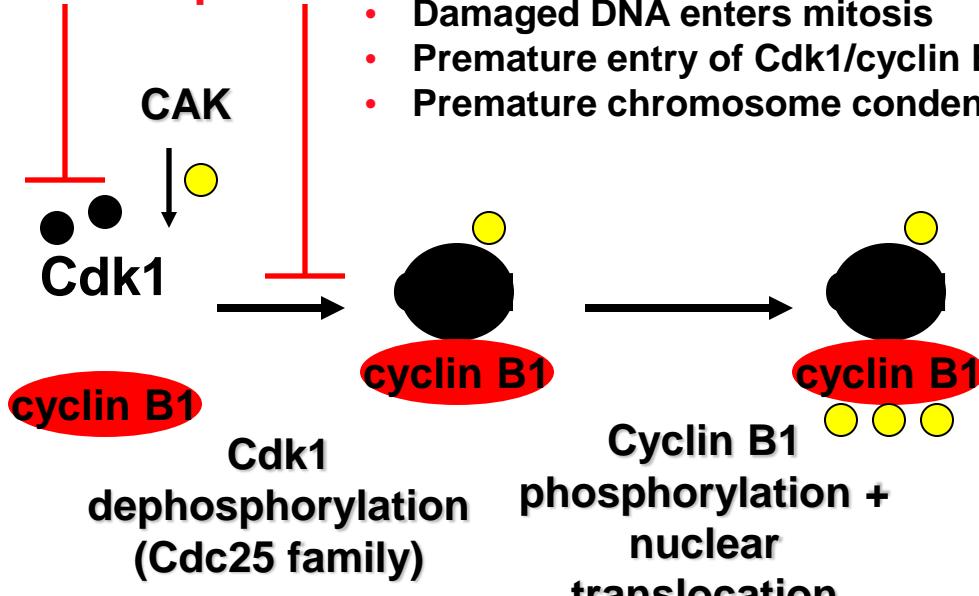


Modified Castedo et al. Oncogene 2004

Mitotic Catastrophe



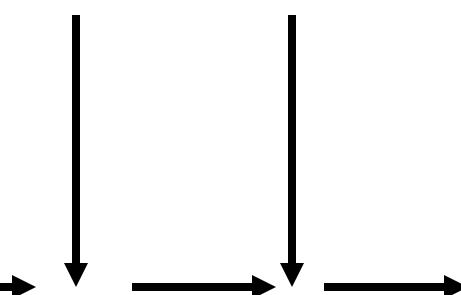
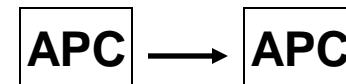
DNA structure checkpoint



Aberrant mitotic entry:

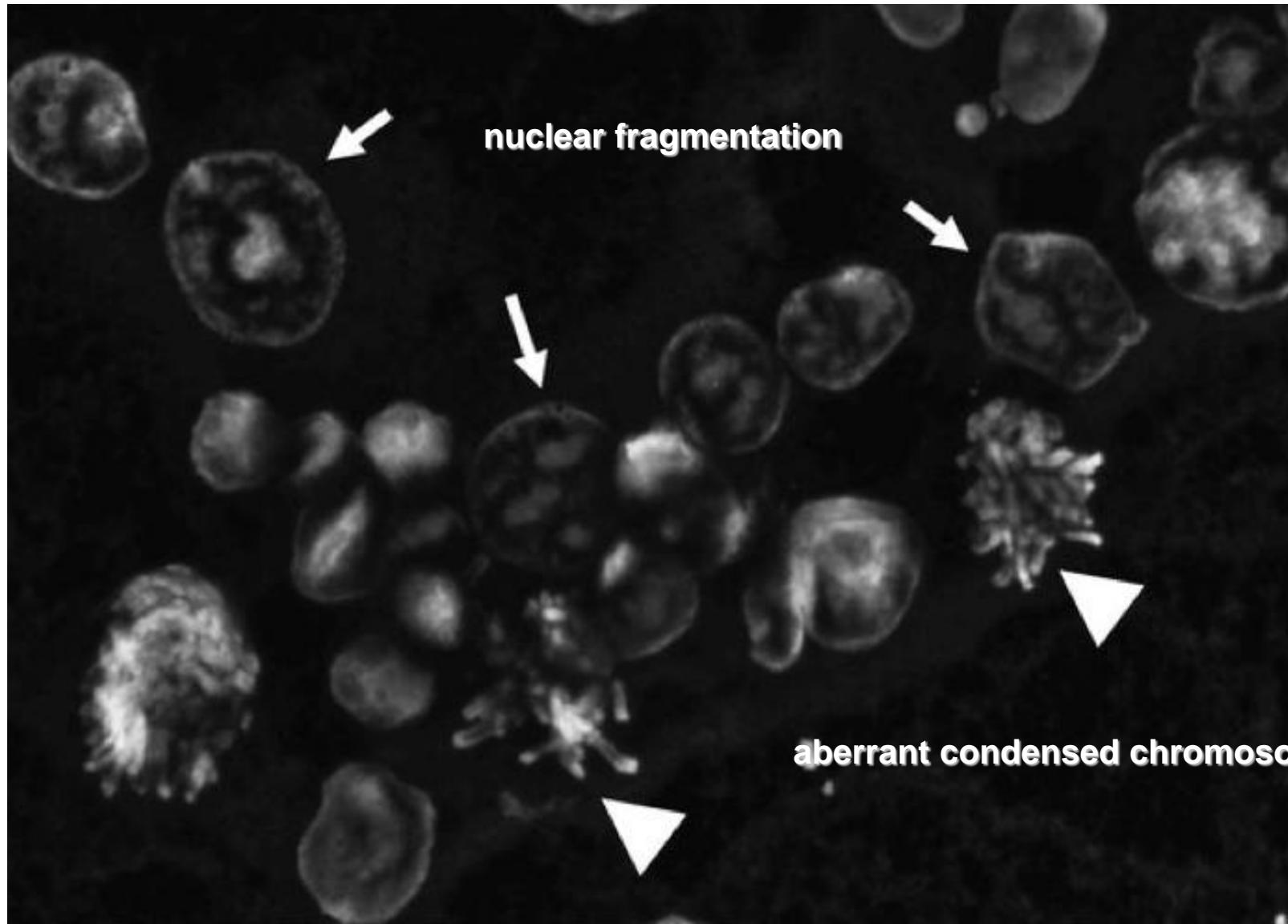
Compromise in DNA structure checkpoint:

- Damaged DNA enters mitosis
- Premature entry of Cdk1/cyclin B1
- Premature chromosome condensation



Cdk1

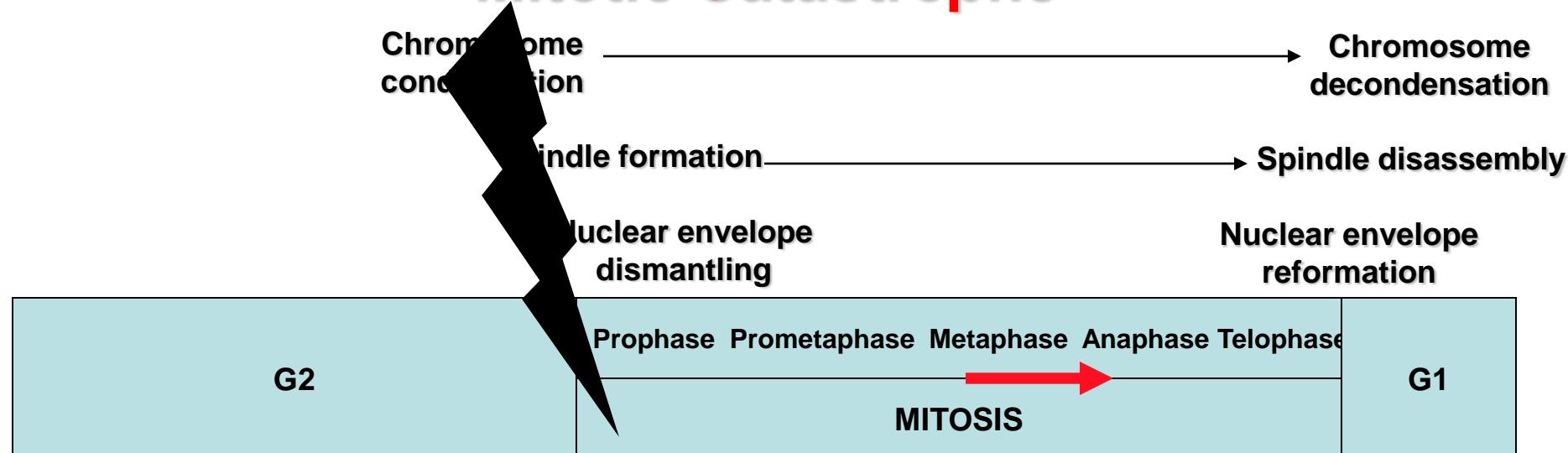
Ubiquitin dependent
cyclin B1 degradation



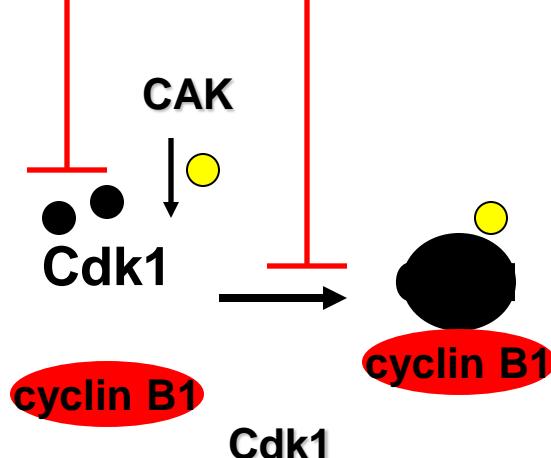
nuclear fragmentation

aberrant condensed chromosomes

Mitotic Catastrophe



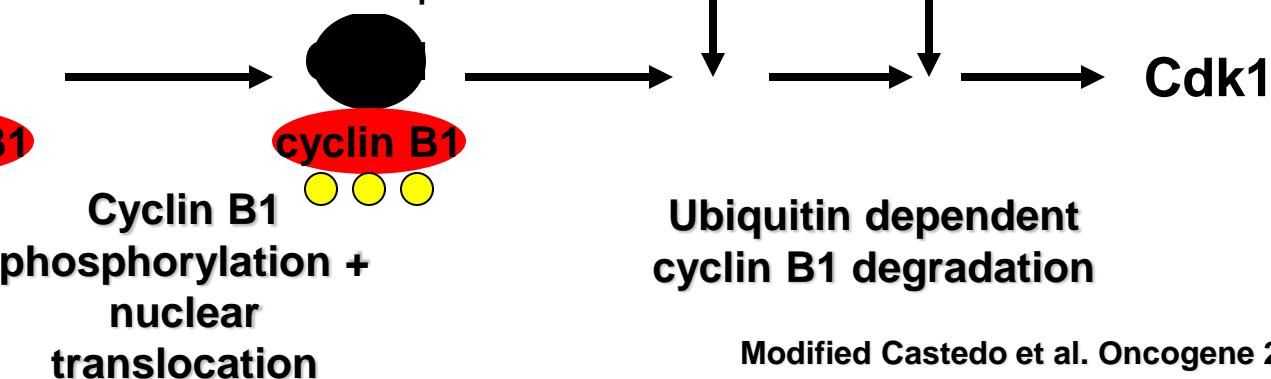
DNA structure checkpoint



Spindle assembly checkpoint

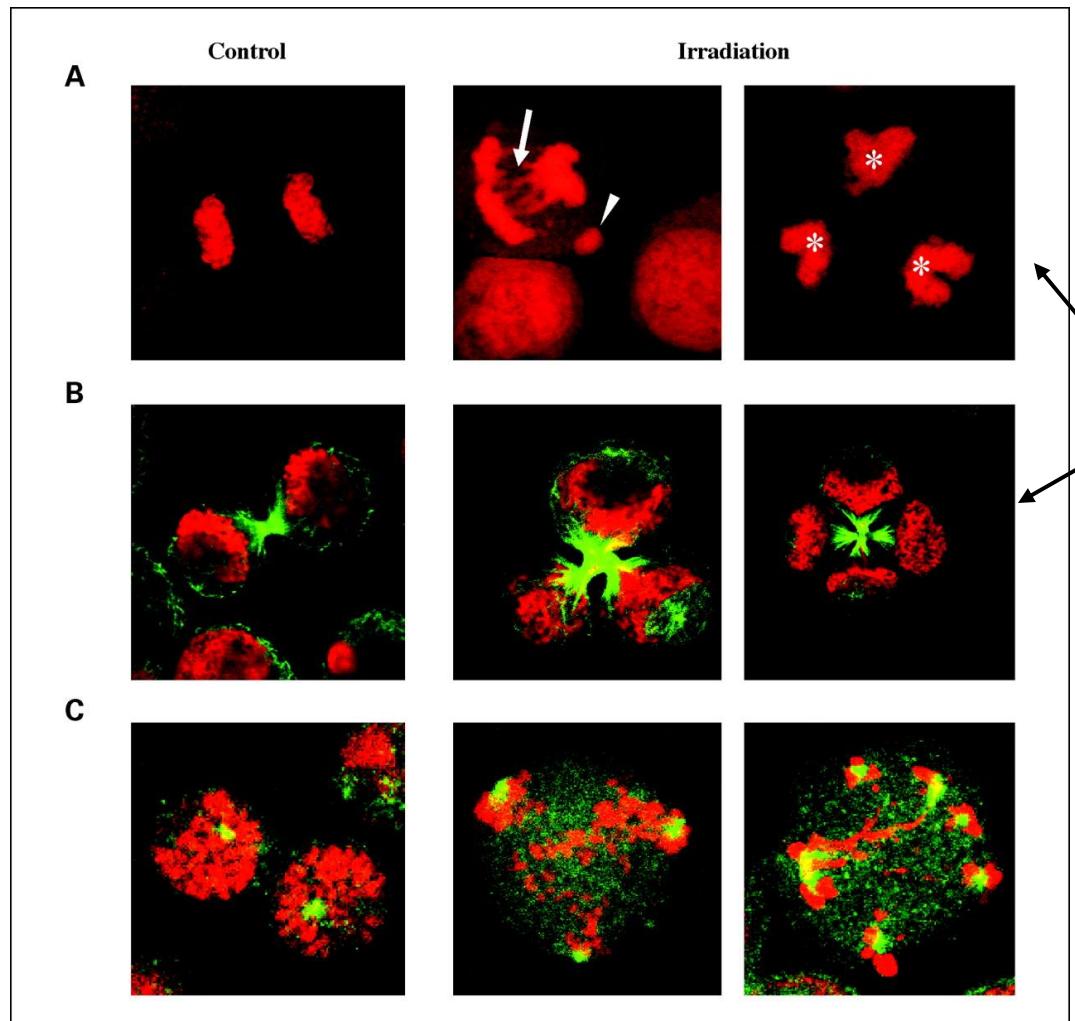
Aberrant mitotic entry:
Prolonged activation of spindle assembly checkpoint:

- Prevents assembly of bipolar spindle
- Centrosome overduplication



Ubiquitin dependent
cyclin B1 degradation

Mitotic Catastrophe



Eriksson, D. et al. Clin Cancer Res 2007;13:5501s-5508s

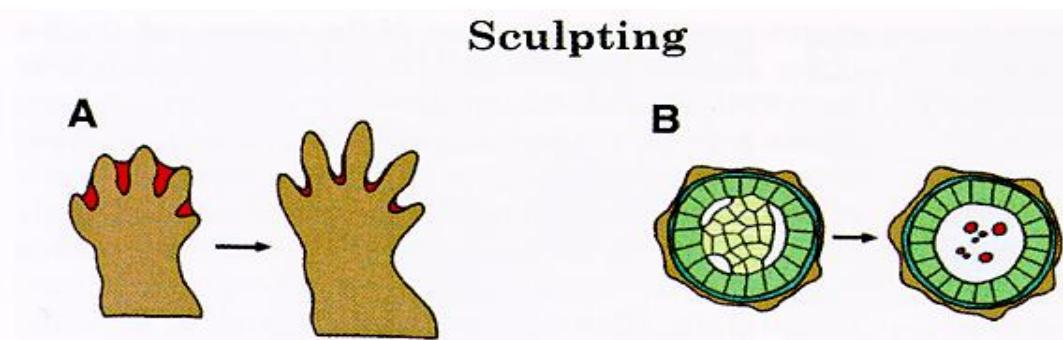
Interphase Death

In contrast to mitotic catastrophe, some cells die by interphase / programmed cell death without dividing (e.g. apoptosis)

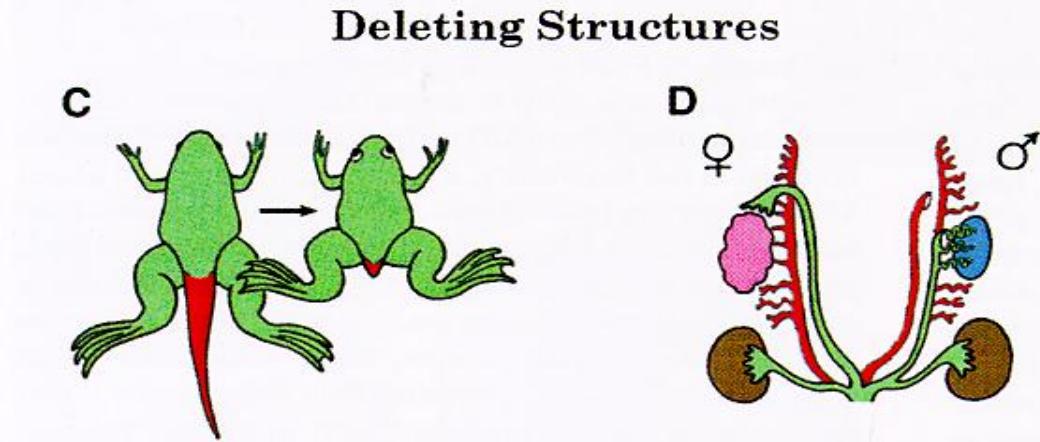
- **Normal process that is part of tissue homeostasis and development**
(note: approximately 10 billion cells die per human per day)
- **Genetically regulated (work in C. elegans); dysregulation associated with many diseases, including autoimmune disorders and cancer**

Programmed Cell Death in Development

Sculpting

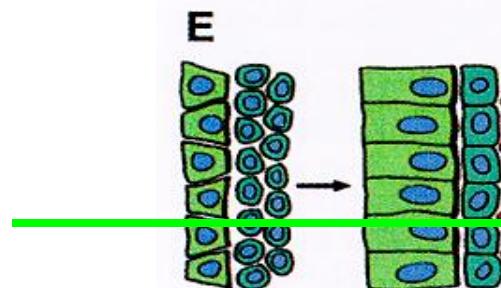


Deletion of unwanted structures



Controlling cell numbers

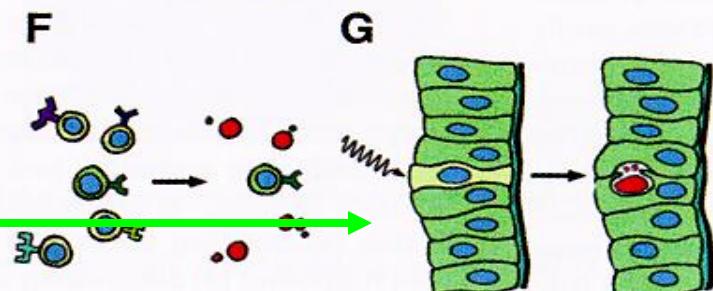
Adjusting Cell Numbers



Quality control

damaged

Eliminating Dangerous or Injured Cells



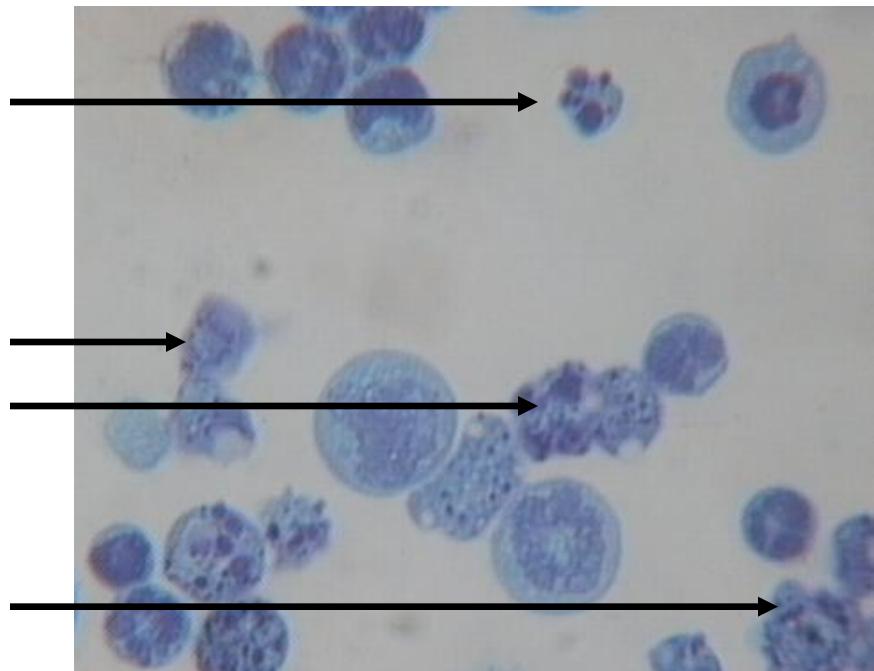
Programmed Cell Death

Apoptosis (type I programmed cell death)

A tightly regulated “active” cell death process, named from the Greek term for “falling” that describes the natural process of leaves falling from the tree.

Associated with:

- **cellular and nuclear shrinkage**
- **nuclear fragmentation with formation of apoptotic bodies**
- **blebbing of cell membrane, but no early loss of membrane integrity**
- **deletion of single cells in isolation**
- **lack of an inflammatory response and phagocytosis by local cells (a silent death)**



Apoptosis - Caspases

Definition: The morphological and biochemical hallmarks of apoptosis are, in general, the endpoint of a complex signaling cascade that culminates in the activation of intracellular proteases, “caspases”

- **15 caspases identified: caspase-2, -3, -6, -7, -8, -9, -10, and -12 key in regulation of apoptotic signal transduction**

2 groups:

- **Initiator caspases (caspase-2, -8, -9, -10, -12): activated by death signals to activate:**
- **Effector caspases (caspase-3, -6, -7): act on multiple substrates → apoptosis**

Apoptosis - Pathways

It is the relative abundance (balance) of pro- versus anti-apoptotic molecules in a cell at a given time that will determine its fate.

Intrinsic pathways:

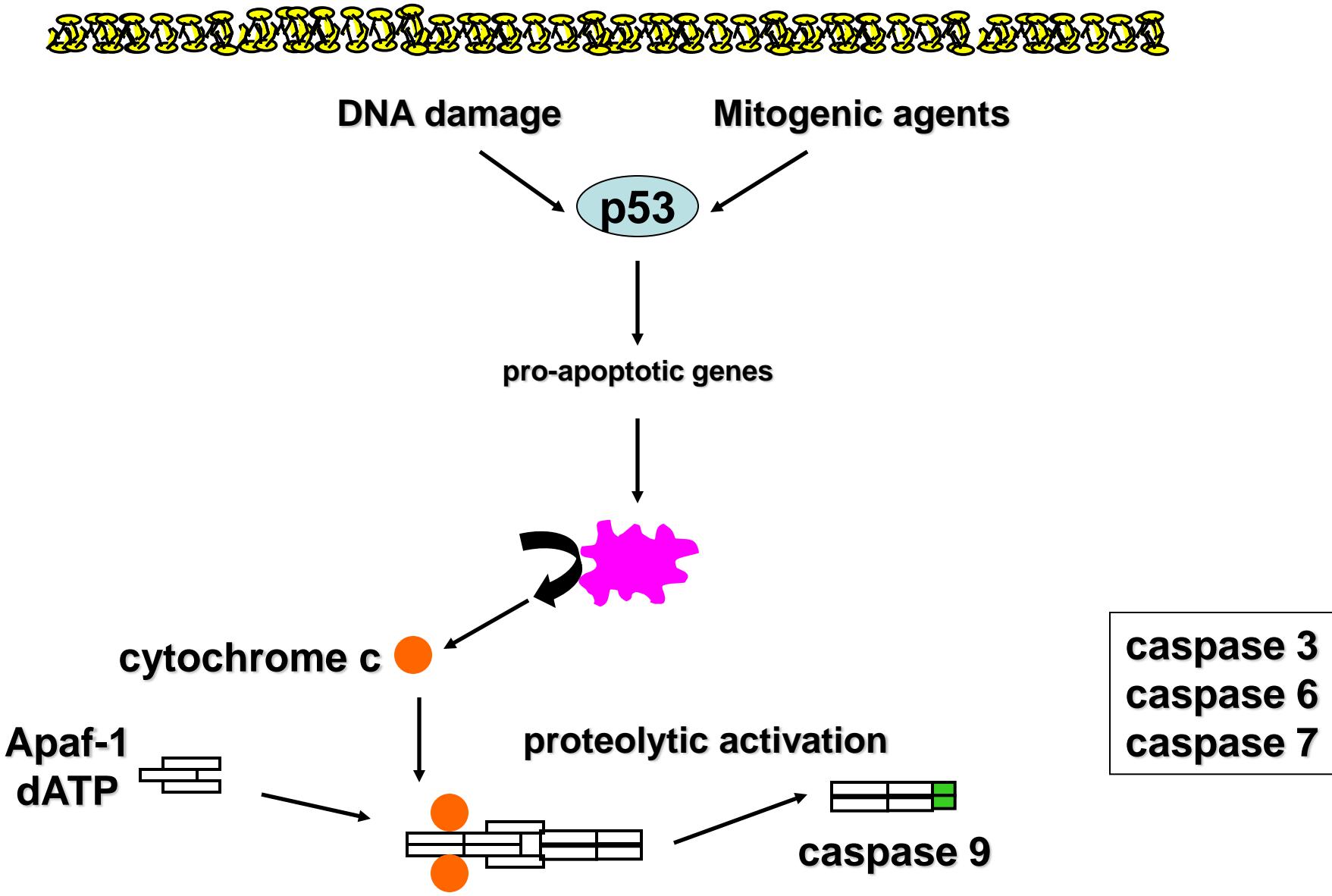
- 1. Mitochondrial pathway (caspase 9)** - cellular stress response to e.g. DNA damage, mitogenic
- 2. Endoplasmic reticular pathway (caspase-12)**

Extrinsic pathway:

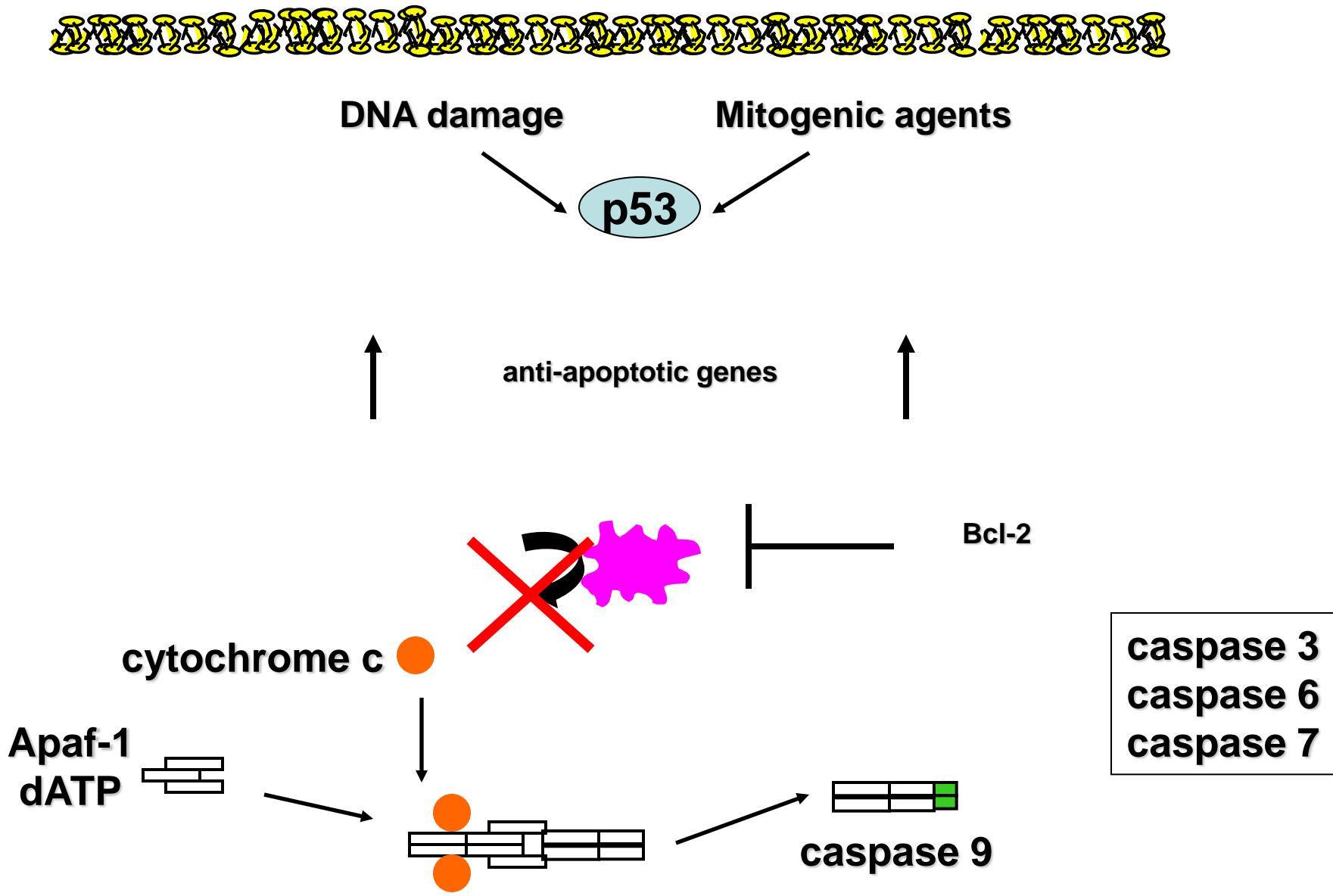
- 3. Death receptor pathway (caspase 8)** - wide variety of signals, including cytokines, hormones, oxidized lipids, etc.

Extrinsic-initiated apoptosis tends to be more acute and massive

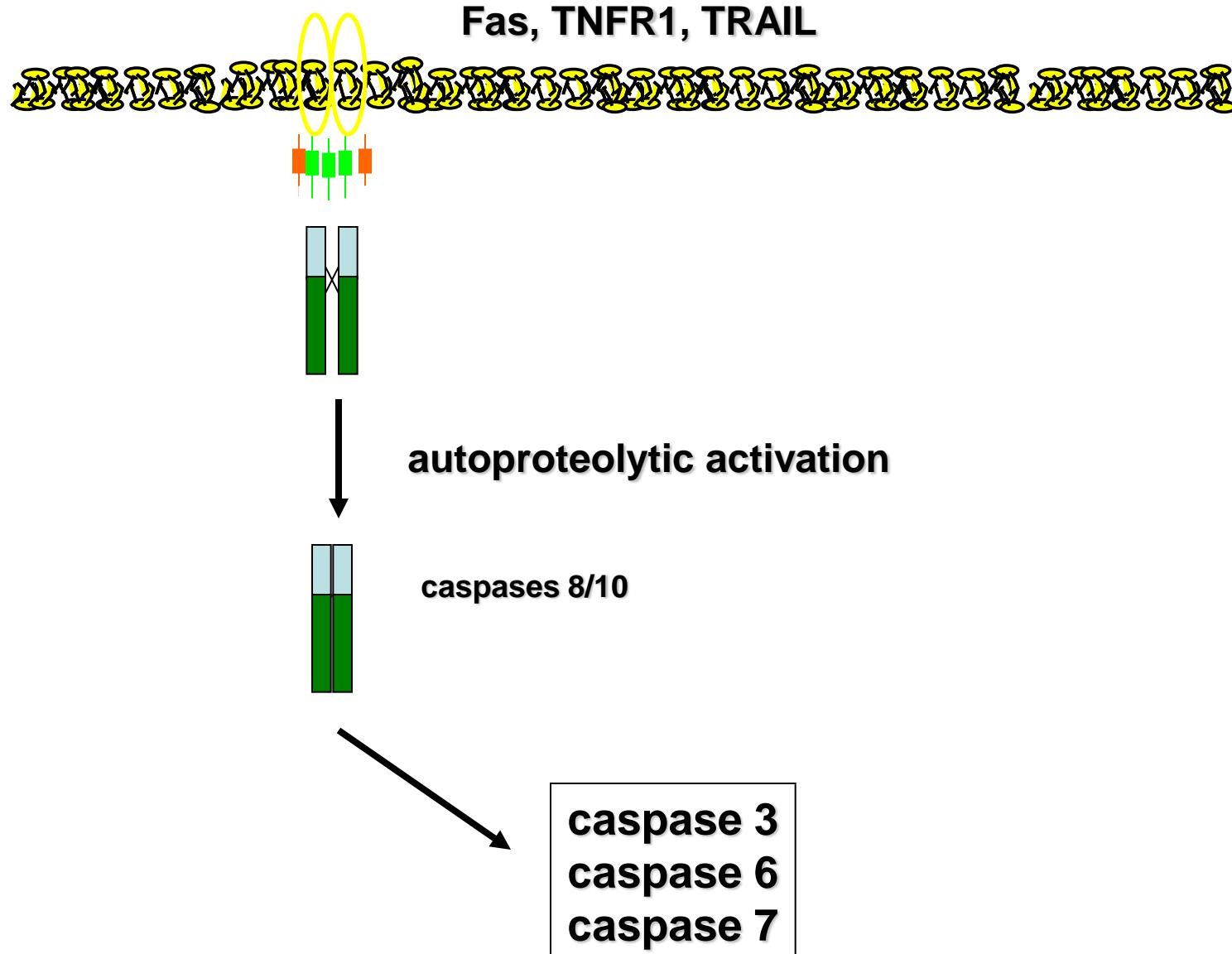
Intrinsic (Mitochondrial) Pathway



Intrinsic (Mitochondrial) Pathway



Extrinsic (Death Receptor) Pathway



Apoptosis - Caspases

Existence of multiple caspases suggests each may have specific role in apoptosis:

- **caspase 3 -/- and caspase 9 -/- mice develop ectopic masses in brain**
Extra cells escaped apoptosis during brain development
- **caspase 2 -/- mice show defective cytolysis of lymphoblasts; female mice have excess ovarian germ cells -- oocytes are resistant to chemotherapy-induced apoptosis**
- **caspase 7 -/- and caspase 8 -/- mice have development defects and die prenatally**

Lack of a dramatic phenotype in caspase 6 -/- mice suggests some functional redundancy

Radiation-Induced Apoptosis

Intrinsic pathway:

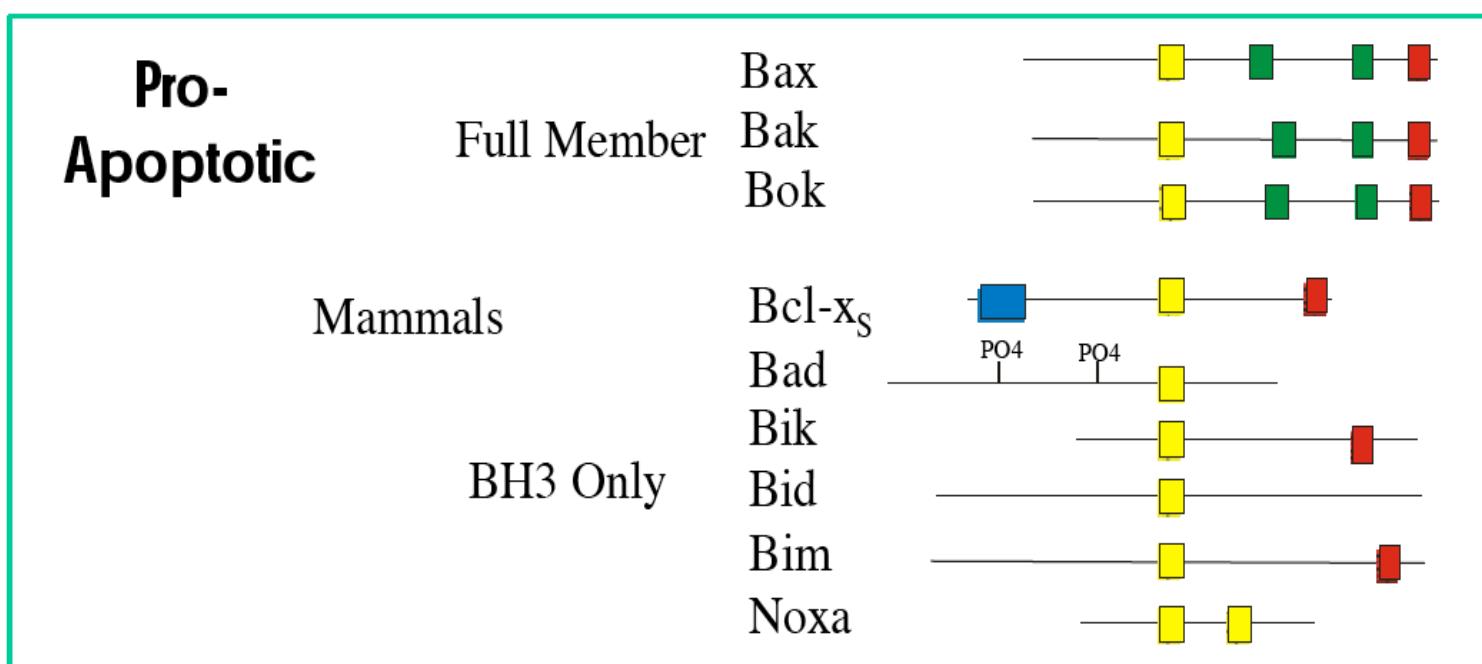
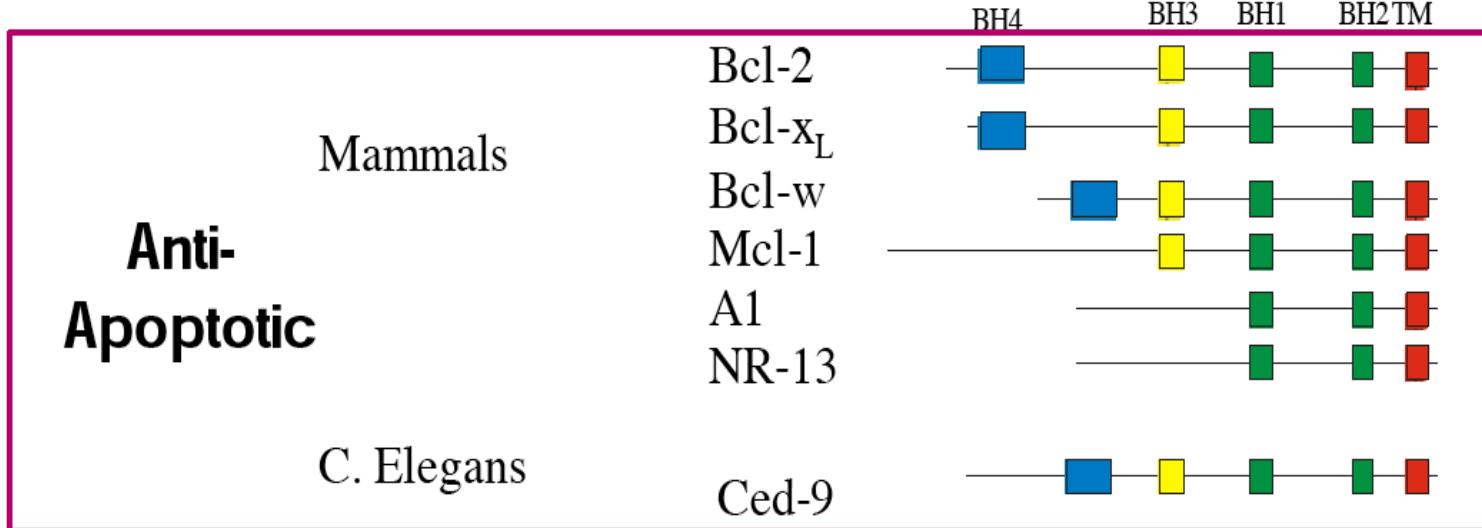
- activation of p53, which produces bax, causing mitochondrial leakage
- generation of the lipid, ceramide
- direct action on mitochondria

Extrinsic pathway:

- induces TNF α and TNFR family members
- generation of the lipid, ceramide

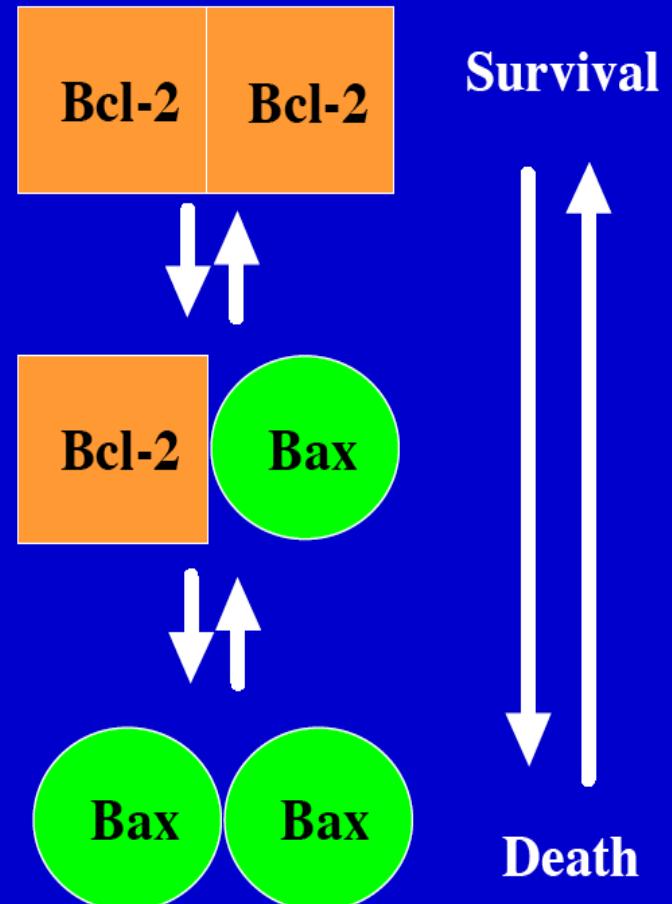
The decision to commit apoptosis is determined by an internal apoptotic “rheostat” within the cell i.e. cells have a pro-apoptotic or anti-apoptotic phenotype. Radiation increases the AI, but does not change a cell from anti-apoptotic to pro-apoptotic

Background: Overview of Bcl-2 Family Members



Bcl-2 Homologue discovered

- 1993-Bcl-2 IP identified Binding Partner-Bax
- Bax Homologous to Bcl-2
- Had the opposite activity when overexpressed.



Apoptosis and Cancer

- **Tumorigenesis associated with acquired mutations, including dysregulation in apoptosis**

Gene	Role in Cancer
P53	The most commonly mutated gene
RB	Mutated or nonfunctional in various cancers. Loss of Rb triggers p53 dependent and independent apoptosis
PTEN	Mutated or altered expression in cancer. Regulates Akt activation
BAK	Mutated or decreased expression in some tumors
BAX	Mutated or decreased expression in some tumors
APAF-1	Mutated or transcriptionally silenced in melanoma
CASPASE-8	Mutated or transcriptionally silenced in neuroblastoma
FAS	Mutated or down-regulated in lymphoid and solid tumors
Trail receptors	Mutated in metastatic breast cancers
DR4 and DR5	
BCL-2	Frequently overexpressed in cancer
IAPs	Frequently overexpressed in cancer
MDM2	Overexpressed in some tumors
AKT	Frequently amplified in solid tumors
FLIP	Overexpressed in some cancers

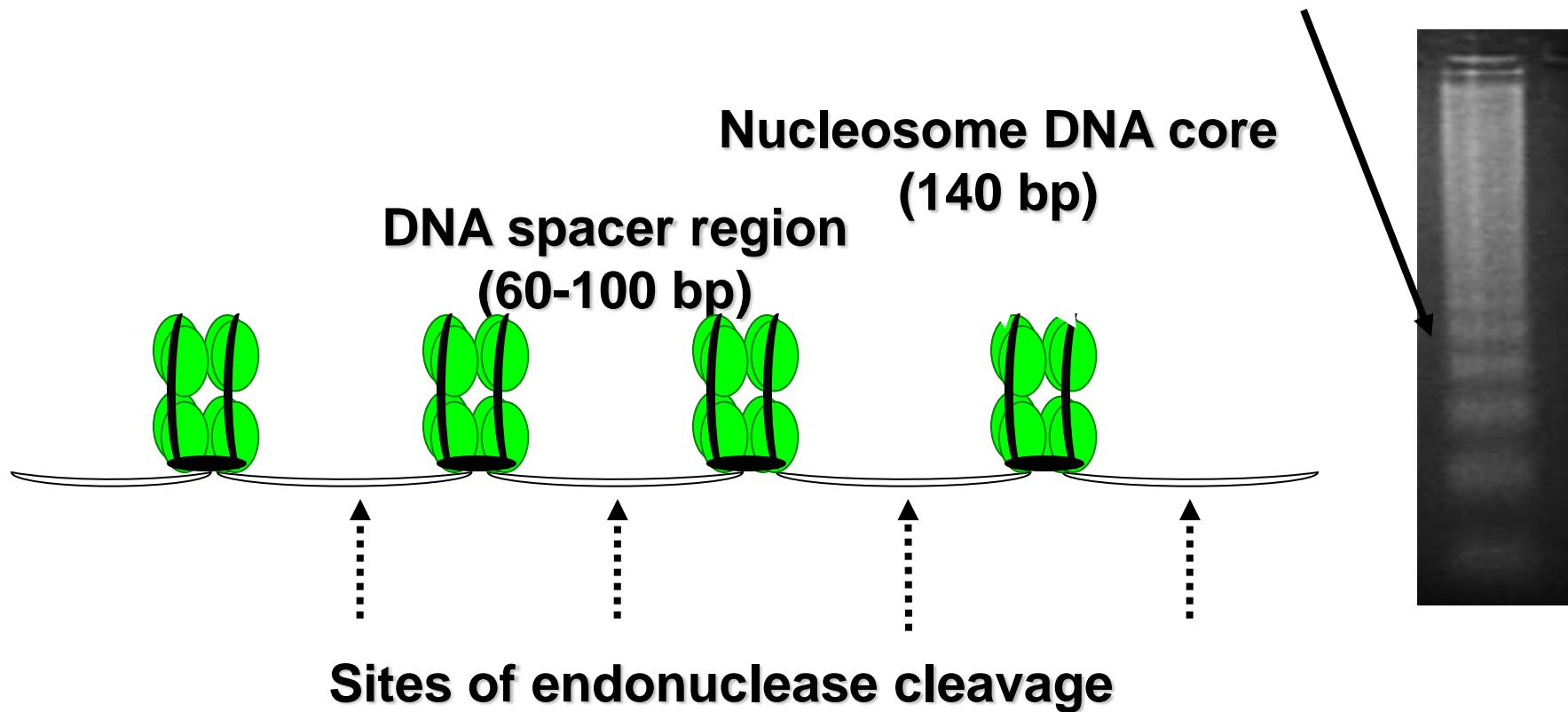
Apoptosis and Cancer

- Tumorigenesis associated with acquired mutations, including dysregulation in apoptosis
- Clinical RT response is superior in tumors with a pro-apoptotic tendency, but relationships between AI (or BAX/Bcl-2) and local tumor control/survival after RT are controversial
- In general, RT increases the AI only in cells with a pro-apoptotic phenotype
- Enhancing apoptosis in a proportion of cells does not necessarily affect the shape of the clonogenic survival curves following radiation - this depends on the response of the non-apoptosing cells
- Apoptosis may affect the clinical response of normal tissues to RT, e.g. serous cells - “dry mouth”

Detection of Apoptosis

During apoptosis, endonucleases are induced that cleave between nucleosomes.

On agarose gel electrophoresis, the DNA separates into fragments with sizes that are multiples of 180-200 bp. This forms a “ladder.”



Detection of Apoptosis

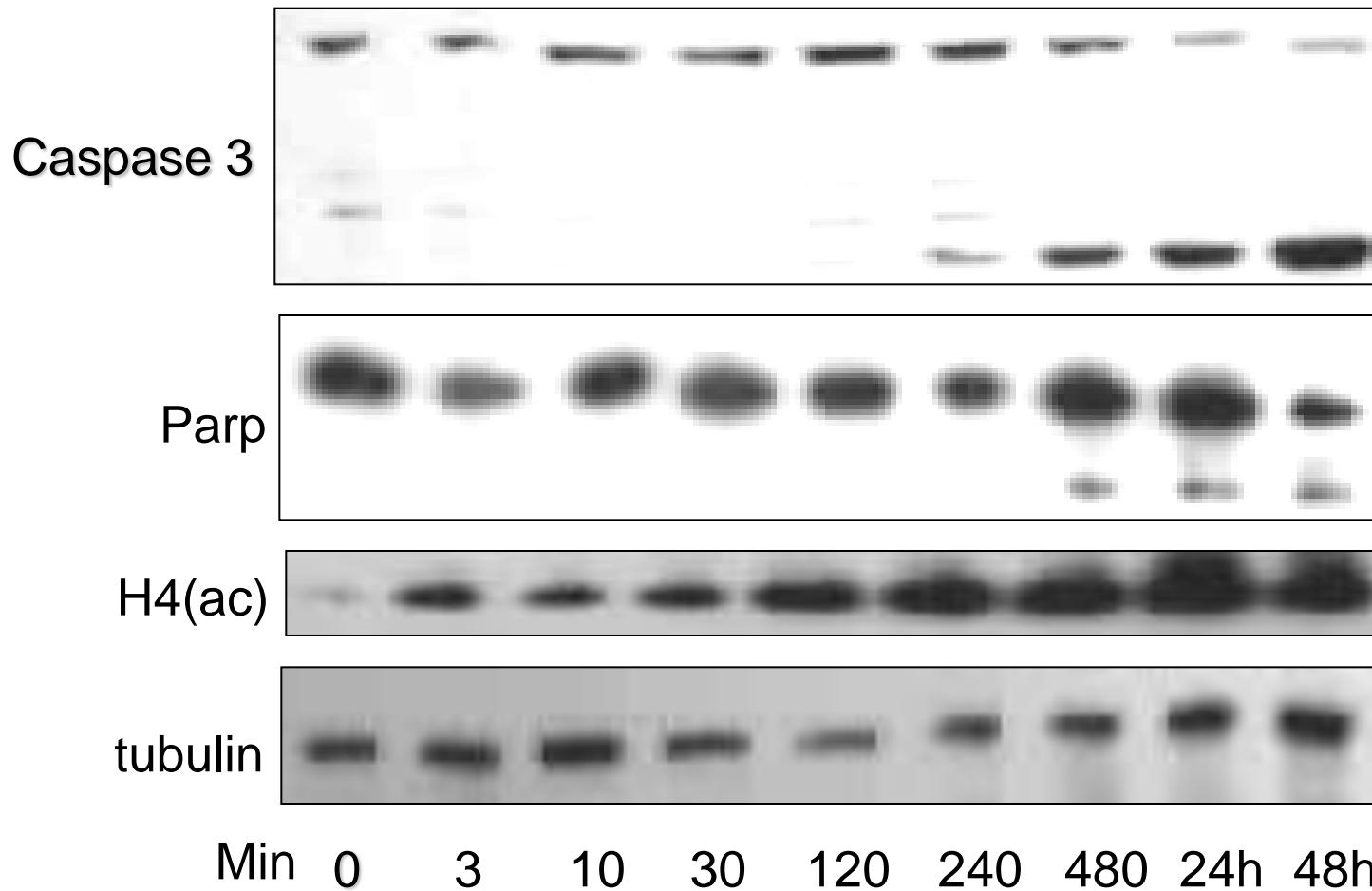
During apoptosis, endonucleases are induced that cleave between nucleosomes.

Apoptosis can be visualized using TdT (terminal deoxynucleotidyl transferase) to catalyze the addition of fluorescein-labeled nucleotides (dUTP) onto 3'-OH ends of double- or single-strand DNA breaks

TUNEL -- TdT-mediated dUTP nick end-labeling

**It is particularly useful for detecting DNA damage in tissue sections.
When combined with caspase staining, e.g. caspase 3: apoptotic index (AI)**

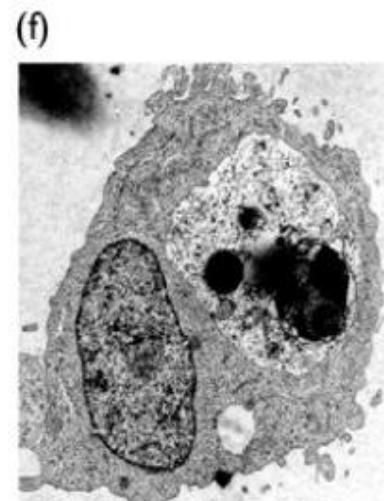
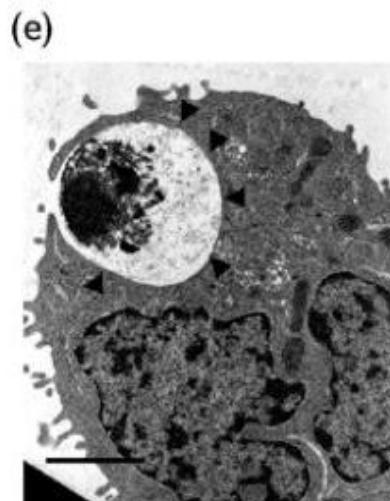
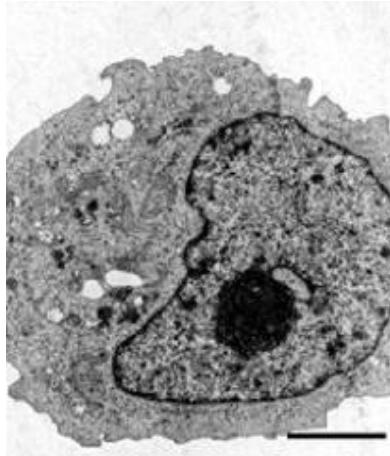
Apoptosis following treatment with HDAC Inhibitor in leukemia cell line HL60



Stress-induced programmed cell death

Autophagy (type II programmed cell death)

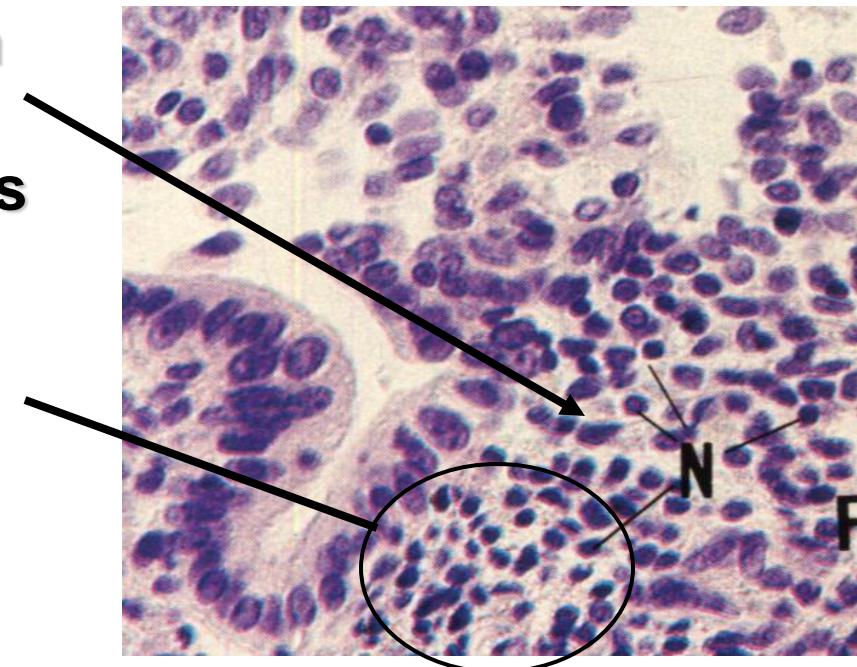
- A tightly regulated, ordered cell death process that is a response to stress, e.g. nutrient deprivation, cytokine damage, but is also seen in remodelling, etc.
- Organelles and other cell components are sequestered in autophagosomes that fuse with lysosomes (self-digestion)
- Increased endocytosis, vacuolation, membrane blebbing, nuclear condensation
- Appears to be a defensive reaction that leads to cell death



Necrosis

Necrosis serves to remove damaged cells from a organism. It is generally a passive, non-physiological process associated with:

- Loss of plasma membrane integrity and deregulated ion homeostasis
- Swelling and bursting of cells as water enters
- Groups of cells, rather than single cells, are affected
- There is no pattern to its fragmentation: DNA forms a “smear” on agarose gel.
- Necrosis generates inflammation and immune response



Apoptosis

Membrane blebbing, no loss of integrity

Shrinking of cytoplasm

Alteration of membrane asymmetry

Condensation of nucleus

Mono- and oligonucleosomal length fragmentation of nuclear DNA

Ends with fragmentation of cell into smaller bodies

Energy (ATP)-dependent

Activation of caspases

Involves at least two different pathways

No inflammatory response

Necrosis

Loss of membrane integrity

Swelling of cytoplasm and mitochondria

Membrane asymmetry preserved

Random digestion of DNA (smear of DNA after agarose gel electrophoresis)

Ends with total cell lysis

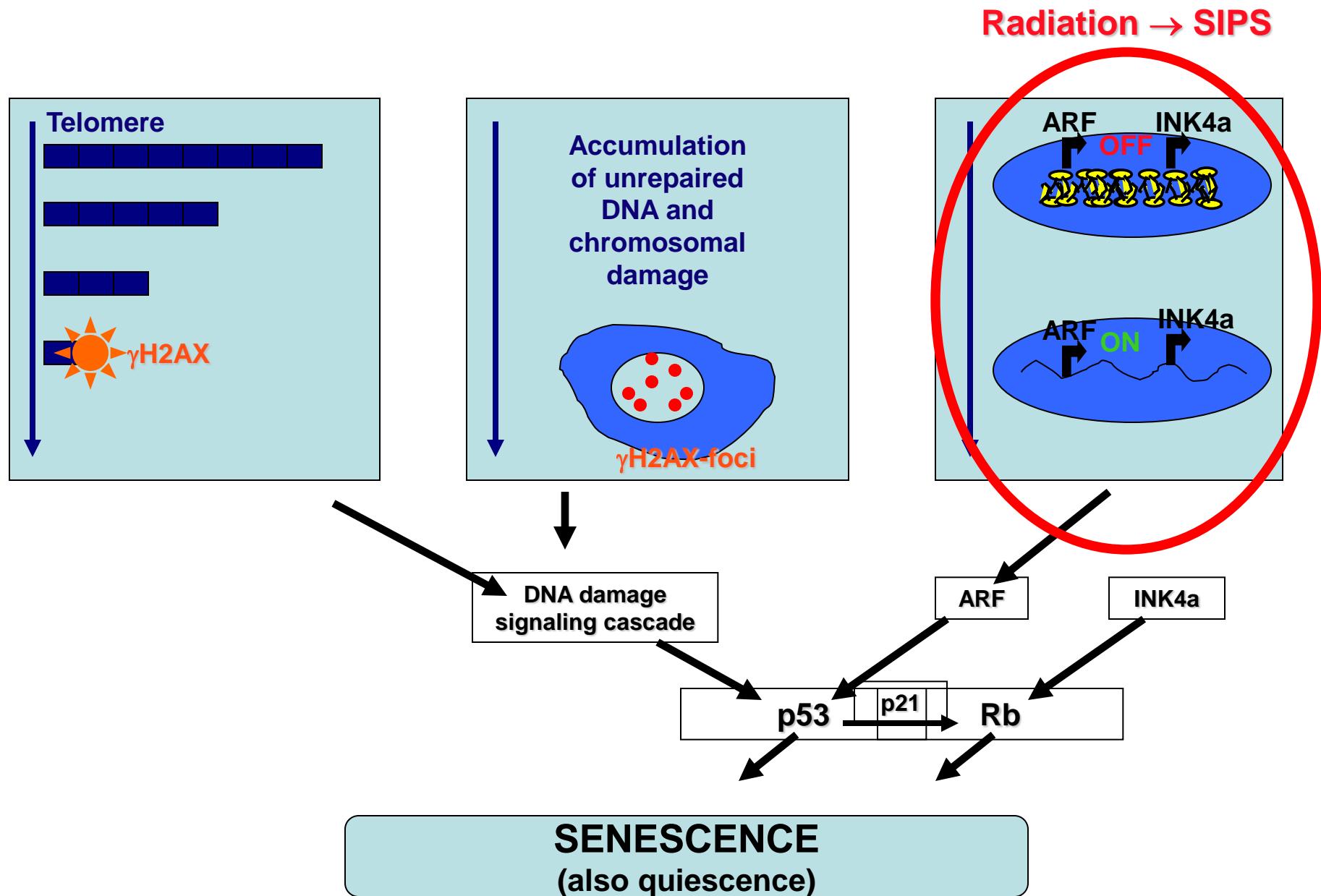
Energy independent

Inflammatory response

Replicative Senescence

- Hayflick and Moorhead (1961): irreversible growth arrest of normal versus tumor cells *in vitro*
- Hypothesis: “stopwatch” → aging
- Characterized by growth arrest, SA β -galactosidase
- Result of fixed number of cell divisions / stress
- *In vitro*: senescent cells may live indefinitely
- *In vivo*: senescent melanocytes (years) / senescent tumor cells in liver carcinomas (removed rapidly)
- Response to stress -- defense mechanism

“Hayflick Factors” in Senescence

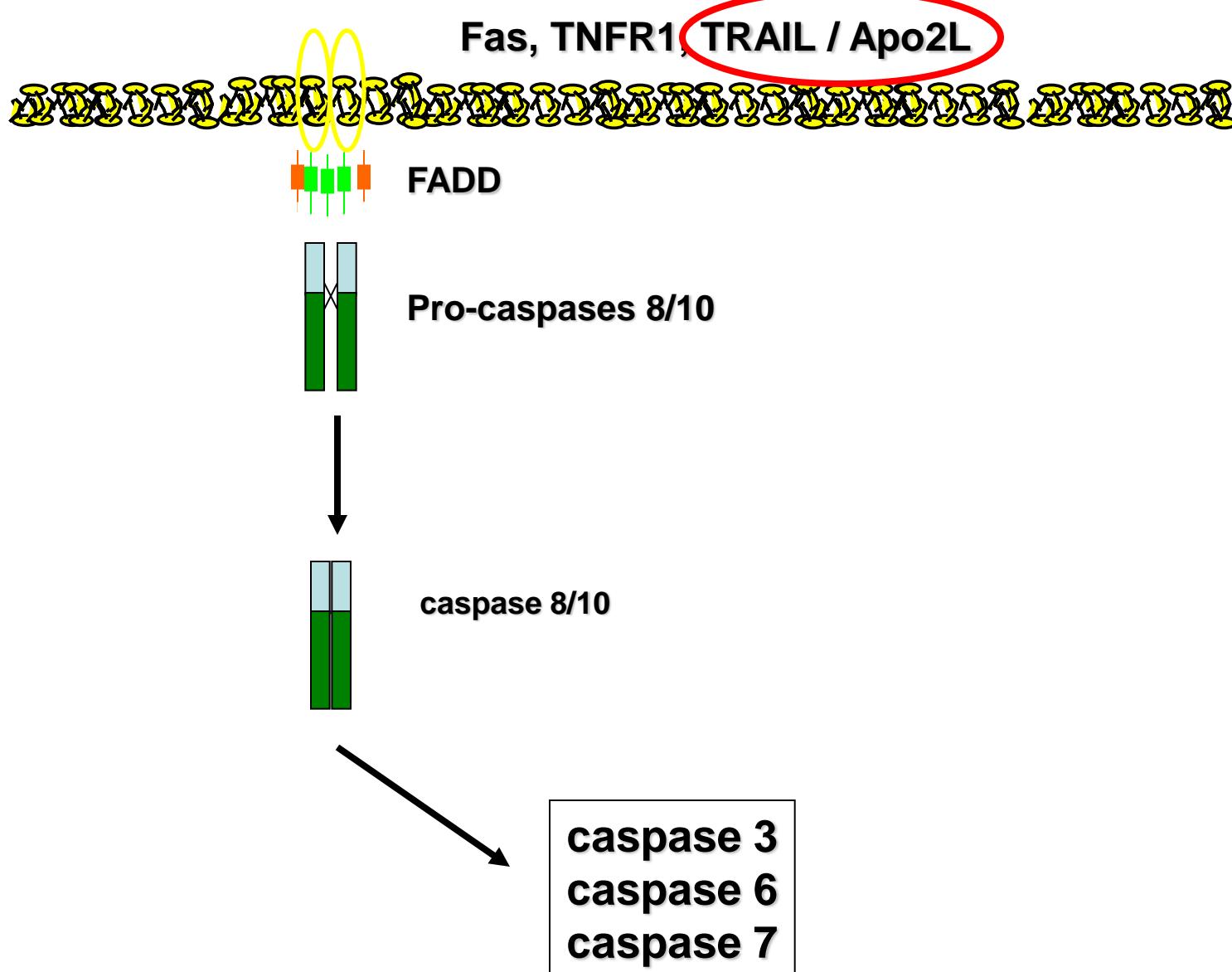


Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway:**
 - ❖ TRAIL (Apo-2L)

Extrinsic (Death Receptor) Pathway

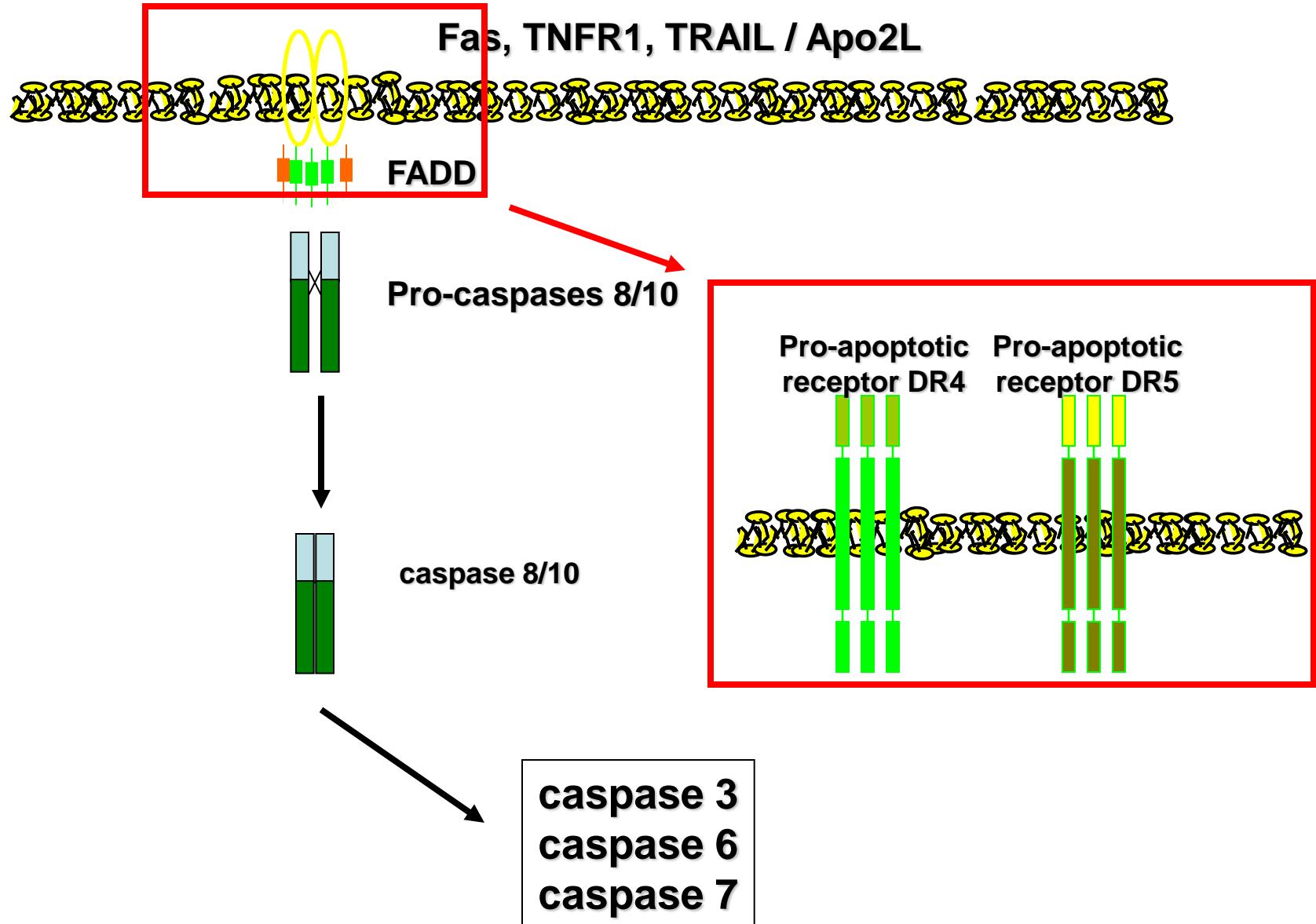


Therapies Targeted at Cell Death

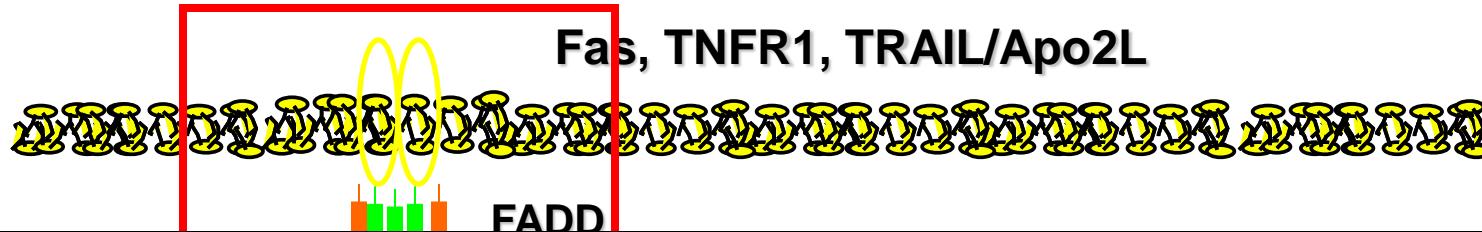
Apooptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway:**
 - ❖ **TRAIL (Apo-2L) - cloned**
in vitro: induced apoptosis in tumor cell lines vs. normal
preclinical: acts synergistically with other modalities
 - ❖ **Other pro-apoptotic receptors**

Extrinsic (Death Receptor) Pathway



Extrinsic (Death Receptor) Pathway



Agent/identifier	Mechanism/ pharmacology	Development stage
rhApo2L/TRAIL	rhApo2L/TRAIL targeting DR4 and DR5	Phase II (NHL, NSCLC)
Mapatumumab	Human mAb targeting DR4	Phase II (NHL, NSCLC)
Lexatumumab	Human mAb targeting DR5	Phase I
Apomab	Human mAb targeting DR5	Phase II (NHL, NSCLC)
AMG-655	Human mAb targeting DR5	Phase II (NSCLC)
LBY135	Chimeric mAb targeting DR5	Phase I/II
TRA-8/CS-1008	Murine DR5-targeting antibody	Phase I
AD5-TRAIL	Recombinant adenovirus encoding human Apo2L/TRAIL	Phase I

Ashkenazi et al. J Clin Invest, 2008.

Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway: direct cell kill - shift in dose response**
- **Intrinsic pathway:**
 - ❖ **Bcl-2 family**

Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway**
- **Intrinsic pathway:**
 - ❖ **Bcl-2 family - antisense oblimersen (phase II/III)**
 - ❖ **Other Bcl-2 targeting agents: ABT-263 (Bcl-2, Bcl-X_L, Bcl-W inhibitor); GX-15-070/obatoclax (Bcl-2, Bcl-X_L, Bcl-W, MCL-1, Bak inhibitor)**

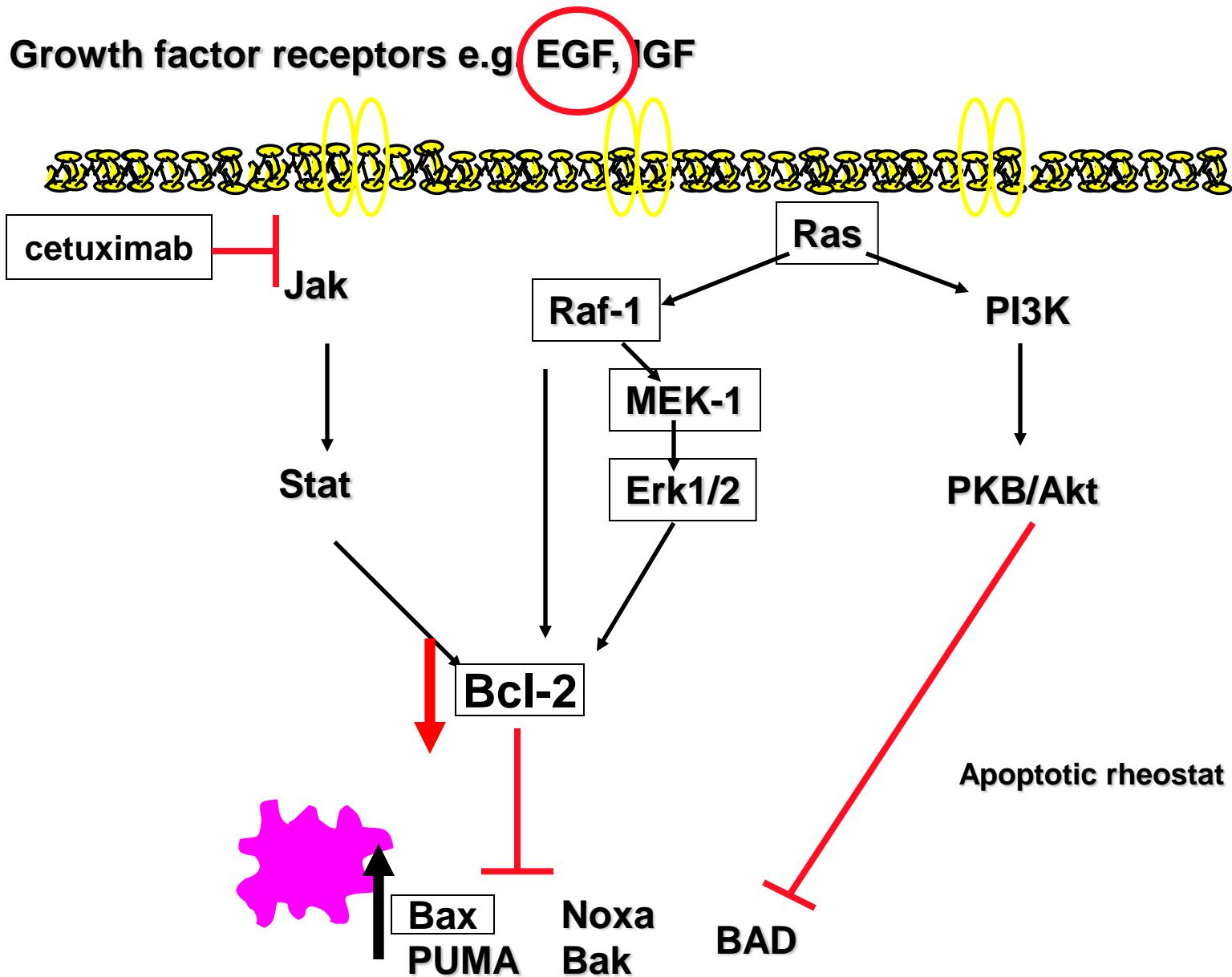
Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway**
- **Intrinsic pathway**
- **Survival pathways:**
 - ❖ **Epidermal growth factor receptor (EGFR)**

Survival Pathways

Growth factor receptors e.g. EGF, IGF



Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway**
- **Intrinsic pathway**
- **Survival pathways:**
 - ❖ **Epidermal growth factor receptor (EGFR) inhibition:** cetuximab (Erbitux) - combination therapy with RT improves locoregional control and overall survival in recurrent/metastatic H&N
 - ❖ **EGFR tyrosine kinase domain:** gefitinib (Iressa) and erlotinib (Tarceva) in NSCLC

Therapies Targeted at Cell Death

Apoptotic pathways most studied:- greatest dysregulation in tumors vs. normal tissues. Cancer cells have acquired blocks to apoptosis, although are driven towards apoptosis due to genomic and other aberrations

- **Extrinsic pathway**
- **Intrinsic pathway**
- **Survival pathways**
- **Radiation protection**
 - ❖ **Suppression of apoptosis: activation of NF κ B**

Conclusions

- The pathways that govern cell death/survival also govern radioresistance and radiosensitivity
- Manipulation of apoptotic pathways genetically or with drugs can affect clonogenic cell survival only when non-apoptosing cells are affected
- Other modes of cell death should not be ignored as targets for therapy
- Survival pathways form appropriate targets for tumor radiosensitization
 - ❖ EGF inhibitors - Iressa, Tarceva, C225, FTIs
 - ❖ COX-2 inhibitors
- Survival pathways form appropriate targets for normal tissue radioprotection
 - ❖ NF κ B agonists
 - ❖ Keratinocyte growth factor (KGF) in bone marrow transplant patients