

Apoptosis



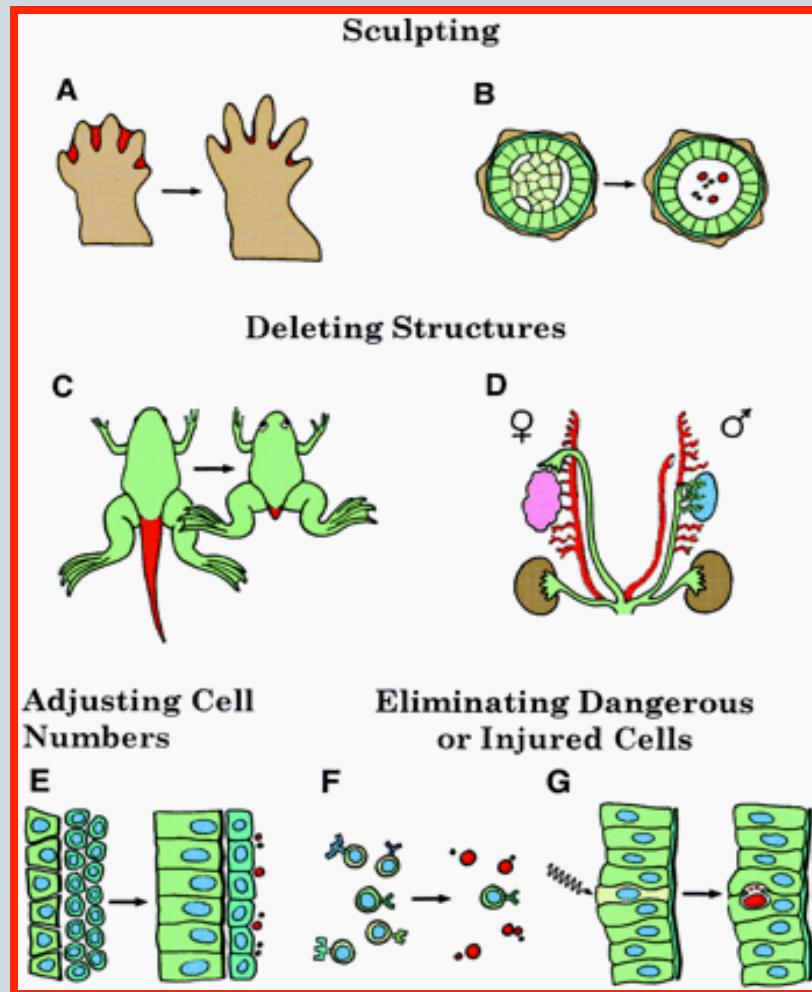
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Programmed Cell Death



- Apoptosis is programmed cell death
- Helps the organism in eliminating unwanted cells with minimal damage to surrounding cells
- An organized process in which various cellular components are broken down systematically

Apoptosis in Development and Maintenance



- A. Form digits
- B. Form lumina
- C. Vestigeal structure
- D. Müllerian/Wolffian
- E. Cull extras (neurons)
- F. Cull immune system
- G. Cull damaged cells

- Human body contains about 10^{14} cells (100 trillion)
• (10^6 million; 10^9 billion; 10^{12} trillion)
- Between 50 billion and 70 billion cells die each day due to apoptosis in the average human adult.
- For an average child between the ages of 8 and 14, approximately 20 billion to 30 billion cells die a day.
- In a year, this amounts to the proliferation and subsequent destruction of a mass of cells equal to an individual's body weight.
- <http://en.wikipedia.org/wiki/Apoptosis>

Apoptosis in Cancer



- **Every cell in a multicellular organism has the potential to die by apoptosis**
- **Tumor cells have faulty apoptotic pathways**
- **These defects not only increase tumor mass, but also render the tumor resistant to therapy**

Nobel Prize in Physiology and Medicine 2002



Sydney Brenner (b 1927), Berkeley, CA, USA, established ***C. elegans* as a novel experimental model organism**. Brenner's discoveries were carried out in Cambridge, UK

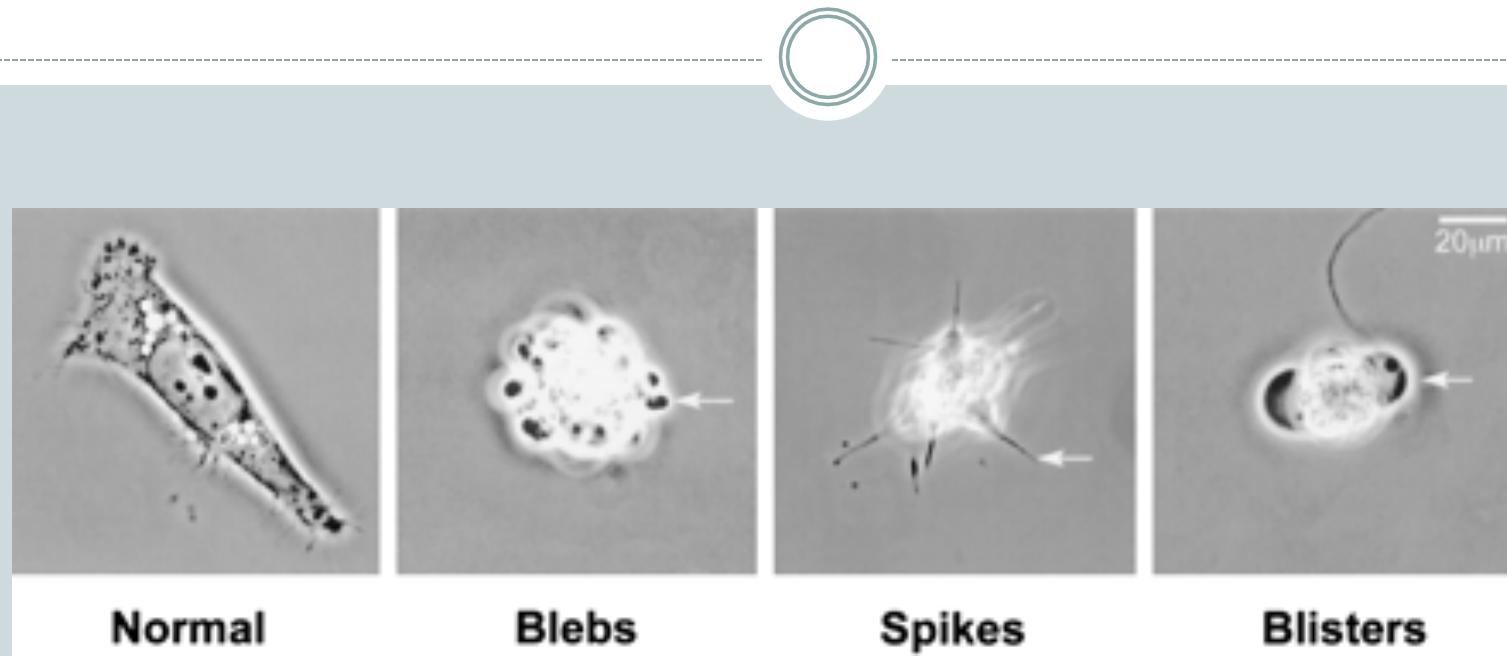


Robert Horvitz (b 1947), Cambridge, MA, USA, has discovered and characterized key genes controlling **cell death** in *C. elegans*. He has shown how these genes interact with each other and that corresponding genes **exist in humans**.



John Sulston (b 1942), Cambridge, England. He showed that specific cells undergo **programmed cell death** as an integral part of the normal differentiation process, and he identified the **first mutation of a gene participating in the cell death process**.

AN APOPTOTIC CELL IN CULTURE



Collins JA, et al. 1997

Diseases Associated with Deregulated Apoptosis



Increased Apoptosis

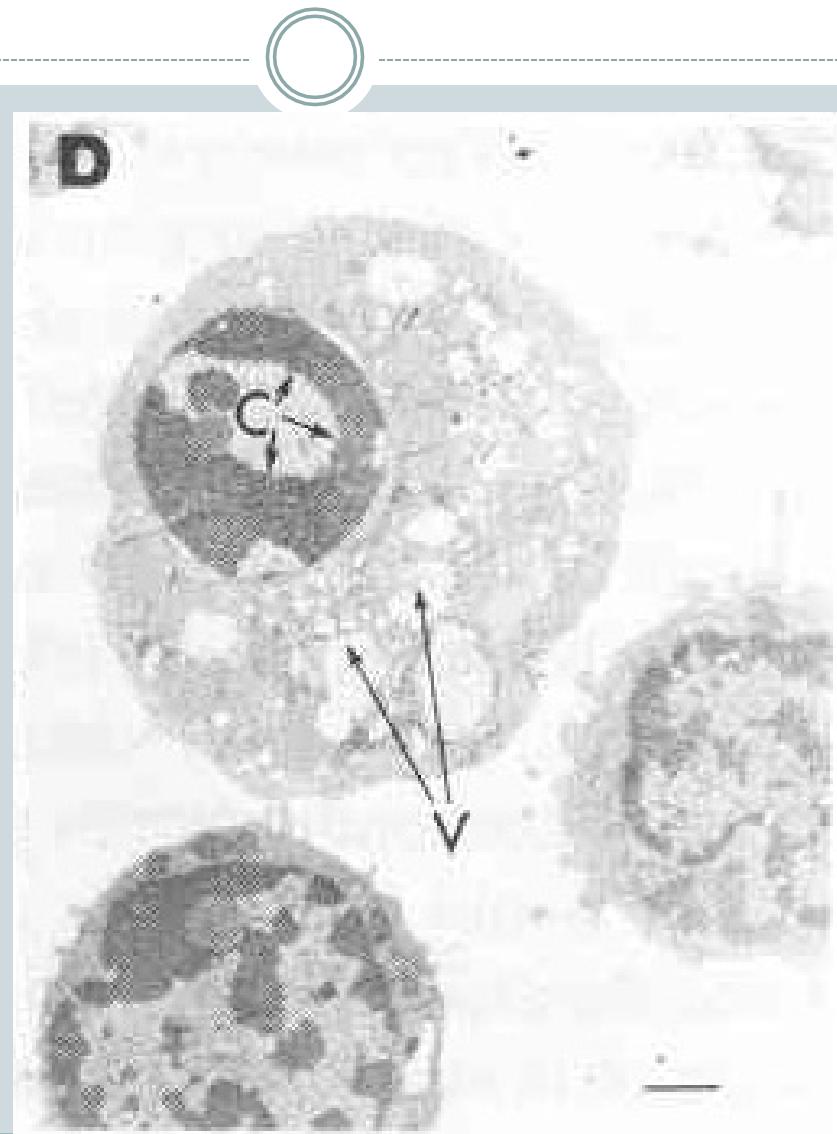
- AIDS
- Neurodegenerative disorders
 - Alzheimer's disease,
 - Parkinson's disease,
 - Amyotrophic lateral sclerosis
 - Retinitis pigmentosa
- Myelodysplastic syndromes
 - Aplastic anaemia
- Ischaemic Injury
 - Myocardial infarction,
 - Stroke,
 - Reperfusion injury
- Toxin-Induced liver disease
 - Alcohol

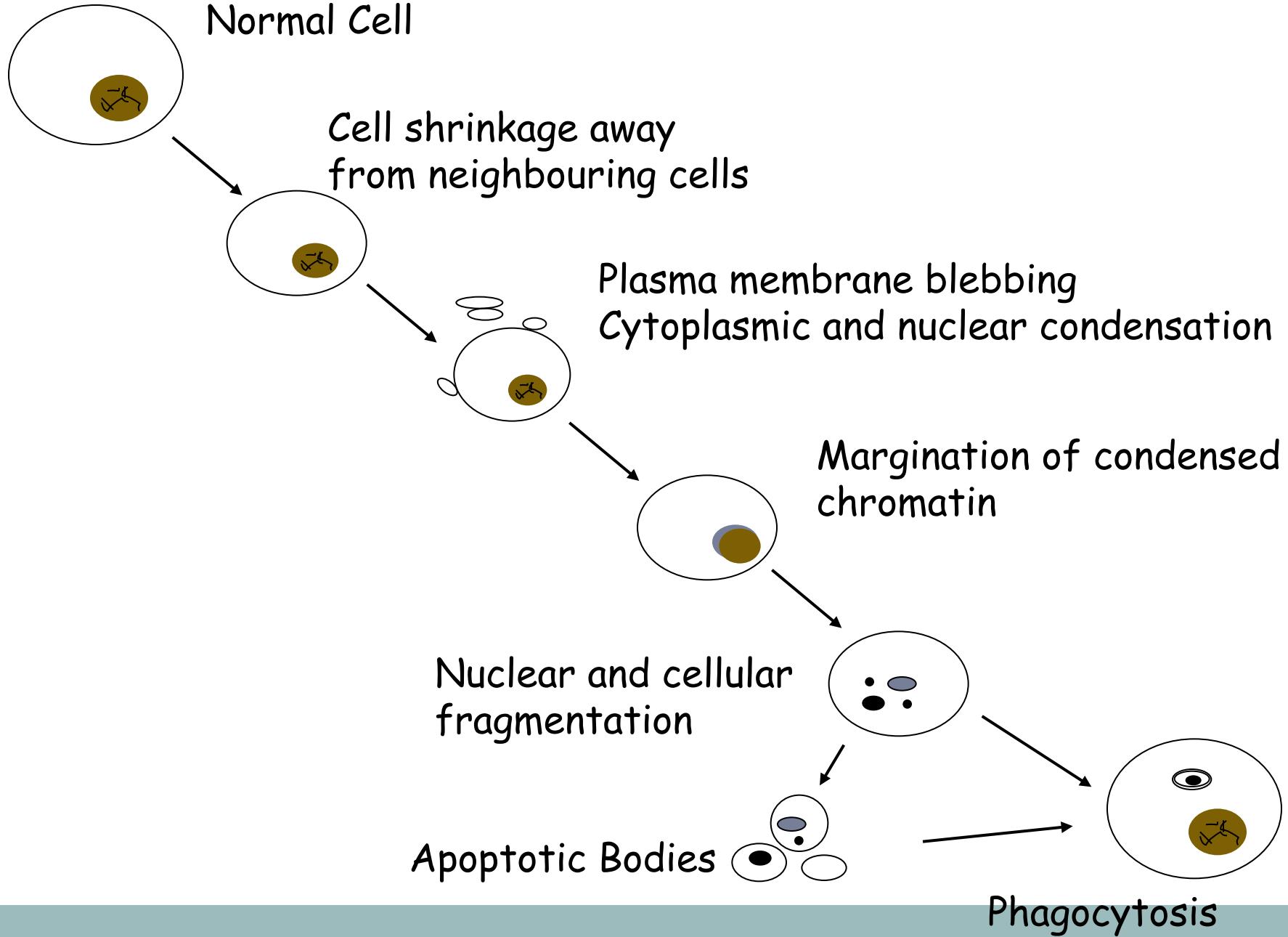
Inhibition of Apoptosis

- Cancer
 - Follicular lymphomas
 - carcinomas with p53 mutations
 - hormone dependent tumours:
 - breast cancer, prostate cancer,
 - ovarian cancer
- Autoimmune Disorders
 - Systemic lupus erythematosus
 - Immune-mediated glomerulonephritis
- Viral Infections
 - Herpesvirus, poxvirus, adenovirus

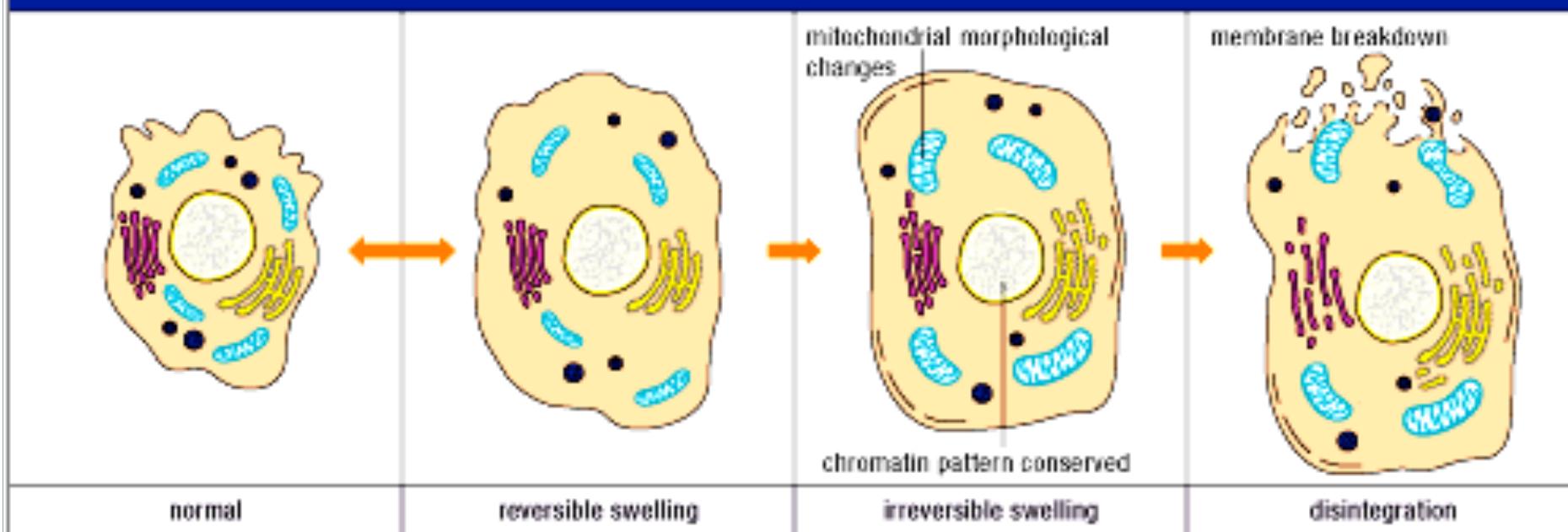
Morphology and Biochemistry of Apoptosis

- Chromatin Condensed
- DNA Fragmentation
- PS externalization
- PARP cleavage
- Cytosol Shrinks
- Vacuolation
- “Apoptotic Bodies”

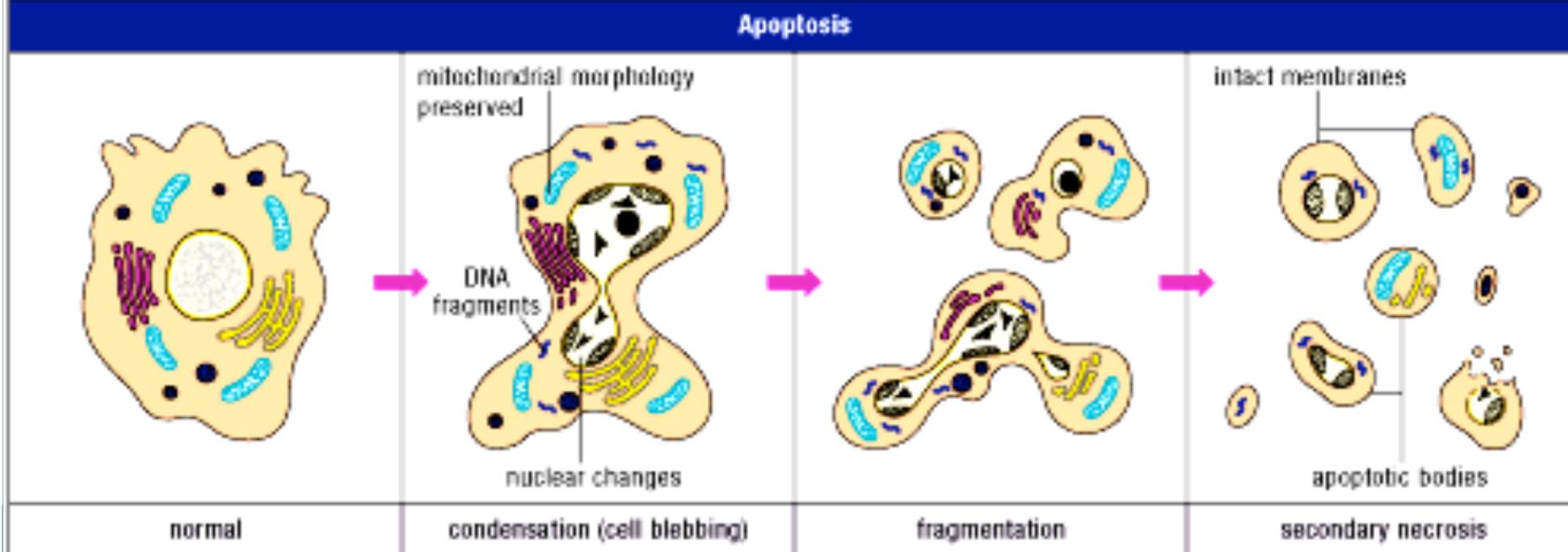




Necrosis



Apoptosis



Morphological Differences between Apoptosis and Necrosis



Apoptosis vs Necrosis

Nuclei	Dense Condensation of Chromatin	Irregular Chromatin Clumping
Cytoplasmic Organelles	Morphologically Intact	Disrupted
Cell Membrane	Apoptotic Bodies, Blebbing	Blebbing and Loss of integrity
Cell Volume	Cells shrink	Cells swell
In Tissues	Single Cells Affected	Groups of Cells Affected
Tissue Response	None	Inflammation

Caspases



- Cysteine proteases cleave Asp in P1 position of tetrameric recognition sequence
- Synthesised as inactive zymogens
- Activated by Asp cleavage
 - removes N-terminal prodomain
 - active tetramer formed
- **14 mammalian caspases identified to date**
 - caspase 1, 11, 4, and 5 → non-apoptotic function
 - remaining 10 caspases all implicated in apoptosis
- Activated by 3 mechanisms:
 - autoactivation (caspase 8)
 - trans-activation (caspase 3, 6 and 7)
 - conformational change (caspase 9)

Caspase Subfamilies



- ICE family which function as cytokine processors
 - caspases 1, 4, 5, and 11
- ICH/Nedd-2 family of apoptotic initiators
 - caspases 2, 8, 9, and 10
- Ced3 family of apoptotic executioners
 - caspases 3, 6, and 7
- Does redundancy exist amongst the caspases?

Knockout

- Caspase 1

- Caspase 3

Phenotypes Observed

- Develop normally
- Thymocytes show partial resistance to
 - Fas-induced apoptosis
- Die 1-3 weeks of age (brain development defect)
- Thymocytes show normal sensitivity to apoptosis

Caspase Substrates



- cleaved by executor caspases only (caspase 3, 6 and 7)

Group

Signalling Proteins

Substrates

RasGAD, D4-GDI, PKCd, FAK,
PAK2, RIP

Structural Proteins

Lamin A and B, G-actin, fodrin,
gelsolin

Cell cycle/DNA repair

PARP, pRb, Mdm2, DNA-PK

Anti-Apoptotic Proteins

Bcl-2, Bcl-xL

Pro-apoptotic Proteins

Bid

Nucleases

CAD/ICAD complex

Inducers of Apoptosis



Physiological Activators

- TNF family
 - Fas Ligand
 - TNF
- TGFb
- Neurotransmitters
 - Glutamate
 - Dopamine
 - N-methyl-D-aspartate
- Growth Factor withdrawal
- Loss of matrix attachment
- Calcium
- Glucocorticoids

Damage-Related Activators

- Heat Shock
- Viral Infection
- Bacterial Toxins
- Oncogenes
 - myc, rel, E1A
- tumour suppressors
 - p53
- Cytolytic T cells
- Oxidants
- Free Radicals
- Nutrient Deprivation
 - anti-metabolites

Therapy-associated Agents

- Chemotherapeutic drugs
 - cisplatin, doxorubicin,
 - bleomycin, cytosine
 - arabinoside, nitrogen
 - mustard, methotrexate,
 - vincristine
- Gamma-radiation
- UV-radiation

Two Main Pathways of Caspase Activation

Signals

TNF-related proteins

Death Receptors

Caspase Activator

Initiator Caspase

Effector Caspase

Survival factor withdrawal
Chemotherapeutic drugs
Developmental signals
Mitochondrial pore opening

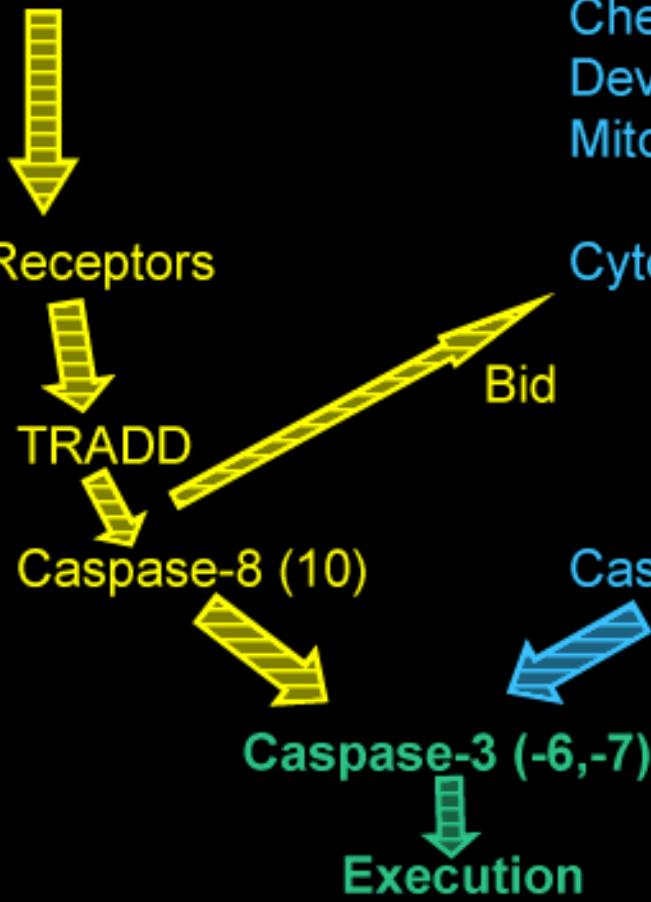
Cytochrome c Release

Apaf-1

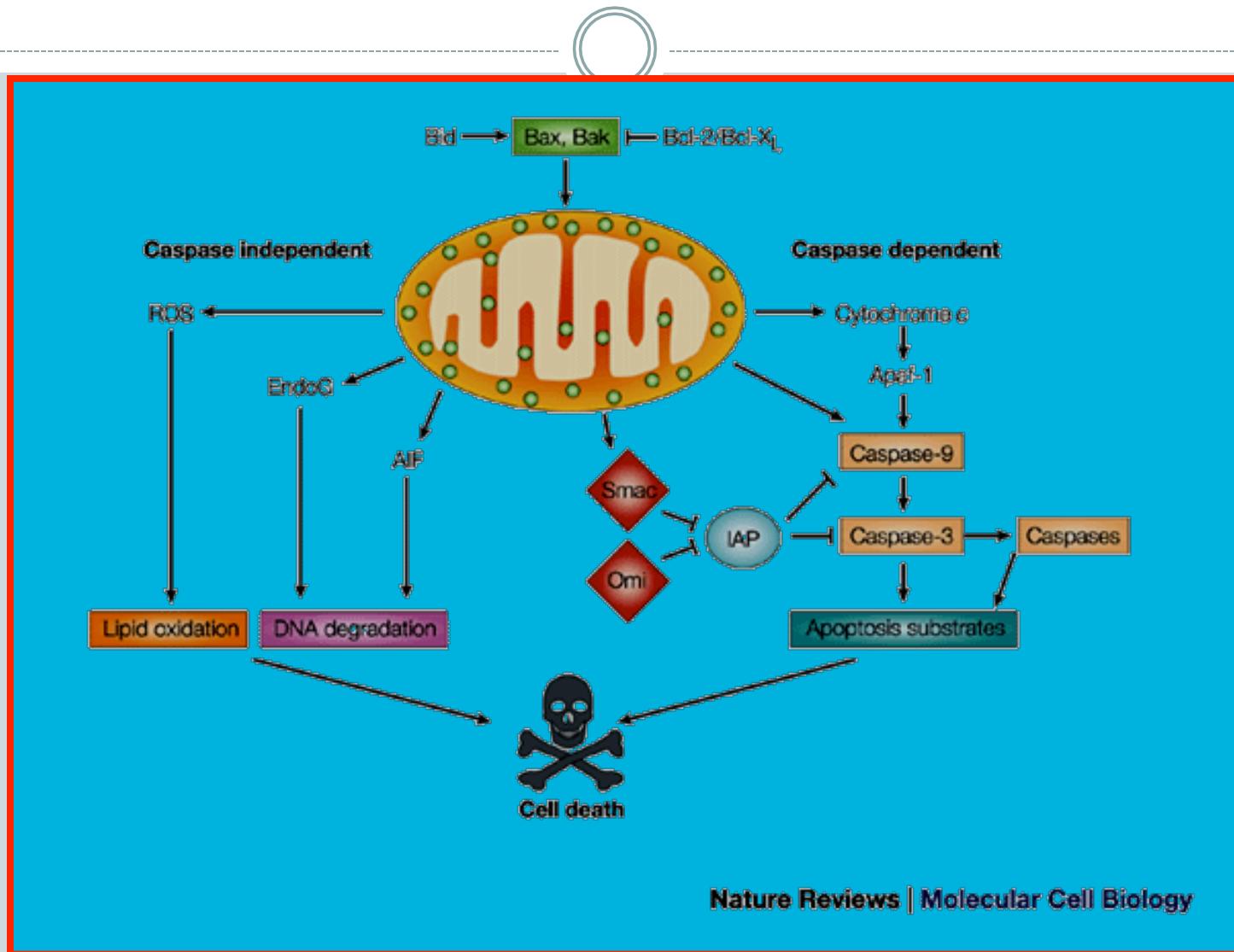
Caspase-9

Caspase-3 (-6,-7)

Execution



Caspase dependent or independent?





Proapoptotic Factors

Caspases, Cytochrome c, Smac, Bax, Bid, AIF, CAD, DFF-45

Antiapoptotic Factors

NF-Kappa B, Bcl-2, Bcl-XL, IAP, HSPs

- There exists a balance between pro and anti apoptotic factors
- Anti cancer agents shift this balance towards apoptosis
- Earlier, we shifted this balance experimentally by transfection of cells with NF- κ B (antiapoptotic) and tested the efficiency of curcumin to induce apoptosis

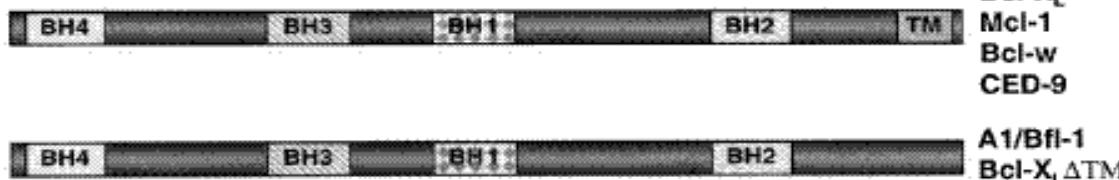
Bcl-2 Family Proteins



- Bcl-2 initially identified as a translocation breakpoint t(14;18) in tumour cells of ~80% patients with Follicular B-cell lymphoma
- Functional homologue of *C. elegans* ced9
- Bcl-2 translocation suppressed lymphocyte apoptosis
- Bcl-2 KO mice
 - normal at birth
 - later develop number of symptoms due to lack of apoptosis
 - (polycystic kidney disease, distortion of small intestine, postnatal degeneration of motor neurons, immune depletion of B and T cells)
- Normal development of Bcl-2 -/- mice suggests compensation for loss of Bcl-2 by other proteins

Bcl-2 Family Proteins

Anti-Apoptotic



Pro-Apoptotic



Modulating Apoptotic Response as a Therapy



- Achilles heel of the cancer cell is that many primary tumours are sensitive to anti-cancer agents
- Apoptosis sensitisation gained from oncogene deregulation
- This therapy window is short and further mutations erode this apoptosis sensitisation
- Reactivating p53 in the tumour cell to abolish chemosensitivity
- Bcl-2 anti-sense therapy to enhance killing in tumour cells
- Caspase Inhibitors as therapy to reduce aberrant cell death

p53 as a Cancer Therapy



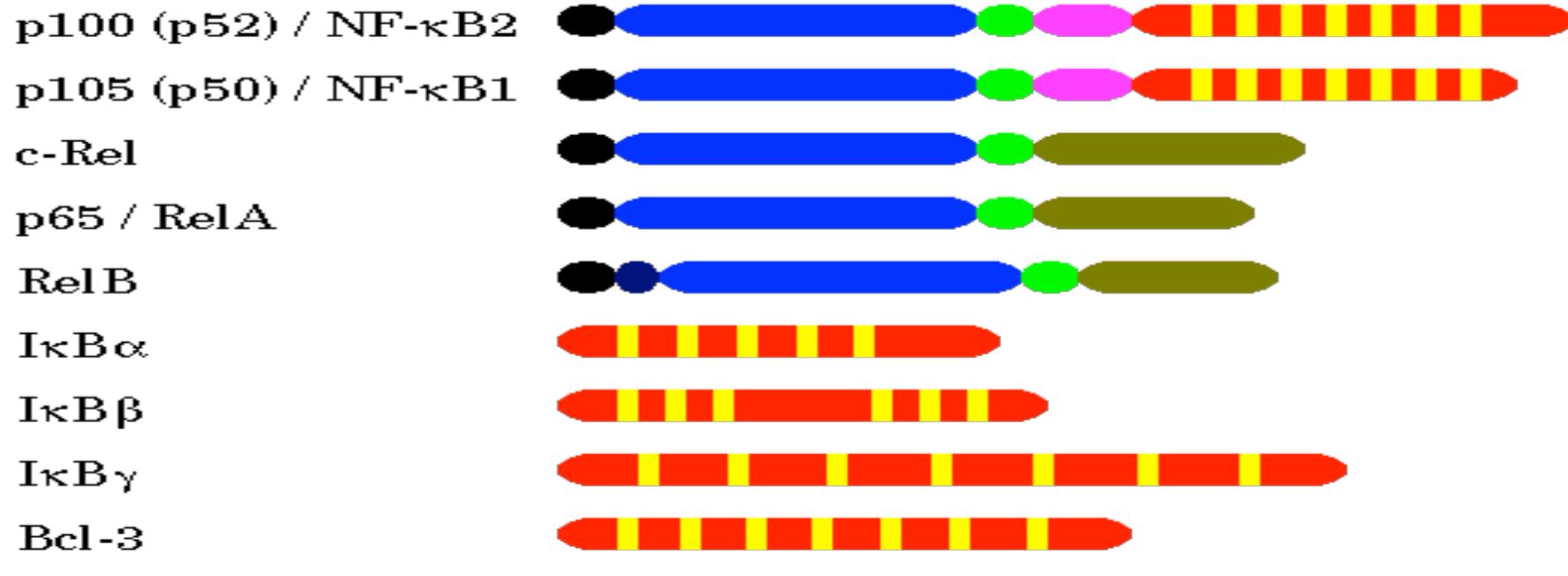
- p53 mutations present in more than 50% sporadic human cancers
- Most chemotherapeutics induce DNA damage, triggering p53 and activating apoptosis
- Strategies to reintroduce p53 include:
 - gene transfer of p53 (delivery problem?)
 - using synthetic peptides to restore wt p53 function
 - using monoclonal antibodies to increase transcriptional activation function
 - Stabilising DNA binding domain using low MW compound –
 - this rescues transcriptional activation function. Positive results in mouse model
 - block p53 turnover by inhibiting p53-Mdm2 interaction
- All of these strategies, although promising have the major obstacle of efficient tumour specific drug delivery

Anti-Sense Bcl-2 Therapy



- Bcl-2 levels elevated in a broad range of human tumours
 -
- Genta G-3139 anti-sense oligonucleotide in clinical trials on melanoma and non-Hodgkin's lymphoma
- G-3139
 - targets the first 18bp of Bcl-2 mRNA
 - functions by binding Bcl-2 mRNA, inhibits translation and targets the mRNA for degradation
 - alters balance in favour of pro-apoptotic Bcl-2 proteins
 - delivered subcutaneously, minimal side effects
(mild hyperglycaemia and mild thrombocytopenia)
 - animal models showed reduction in tumour size
 - had little effect on cell lines not expressing high levels of Bcl-2
apoptosis was enhanced when co-administered with standard chemotherapeutics

NF-κB Family



- NH₂-terminal domain
- Rel homology domain
- Nuclear localization signal (NLS)
- Glycine-rich domain
- Ankyrin repeats
- Trans-activation domain
- Leucine zipper

NF-κB Prevents Apoptosis

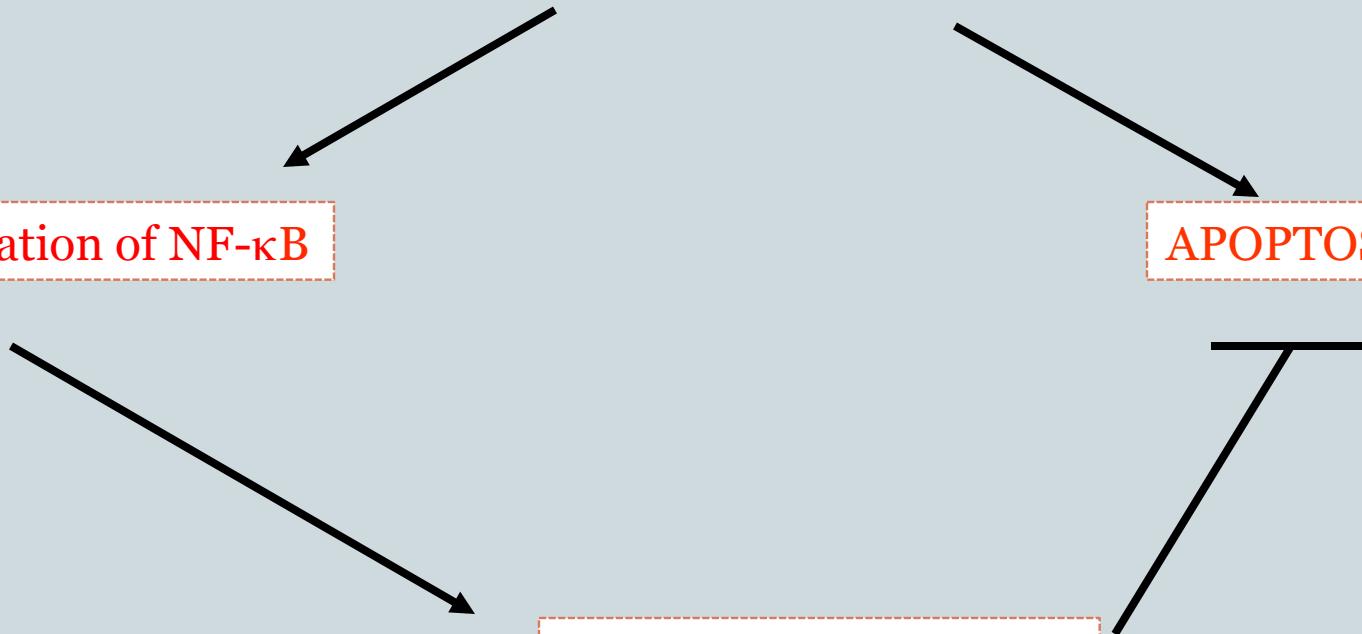


**Tumor Necrosis Factor
Radiation
Chemotherapeutic compounds**

Activation of NF-κB

APOPTOSIS

NF-κB-induced proteins



Heat Shock Proteins

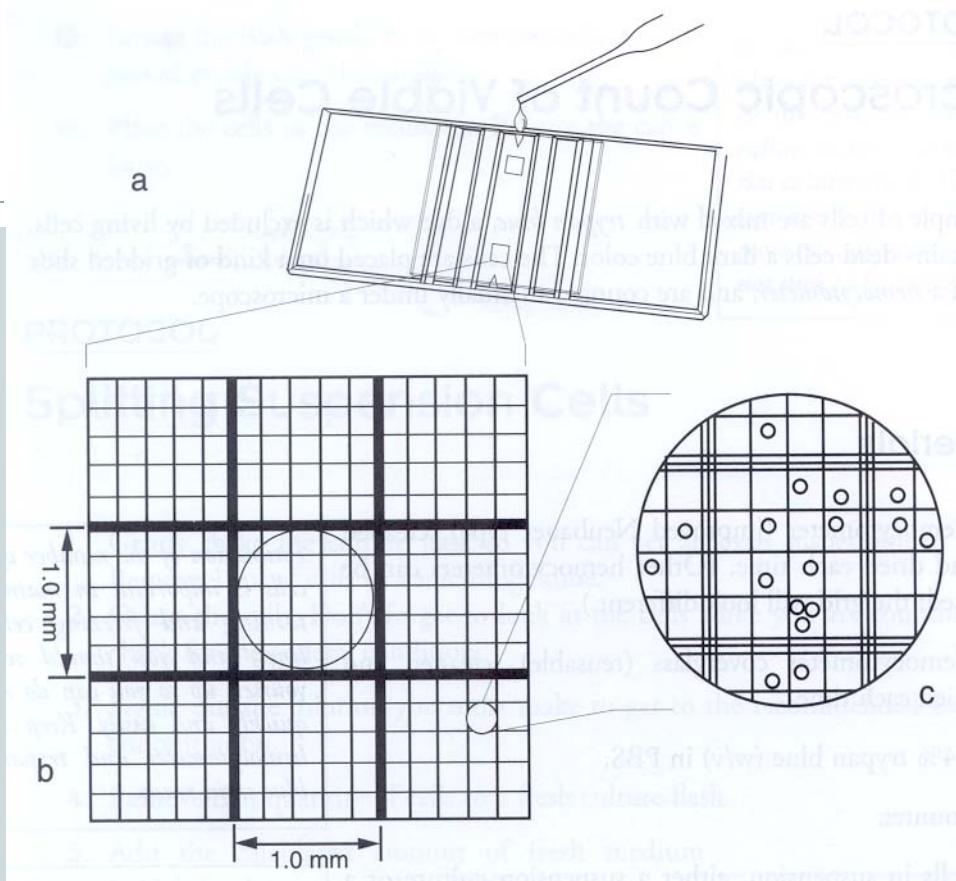


Hsp90, Hsp 70, Hsp 60 and Hsp 27

- Family of proteins induced by physiological or environmental stresses
- Most conserved proteins known
- Molecular chaperones
- Some of them function as Antiapoptotic proteins
- Expression correlates with oncogenic status
- High expression associated with metastasis, poor prognosis, resistance to chemo and radio-therapy

Apoptosis Assays





Count cells in 1 mm²

Example:

You have counts of 113, 99, and 118 (with an average of 110).

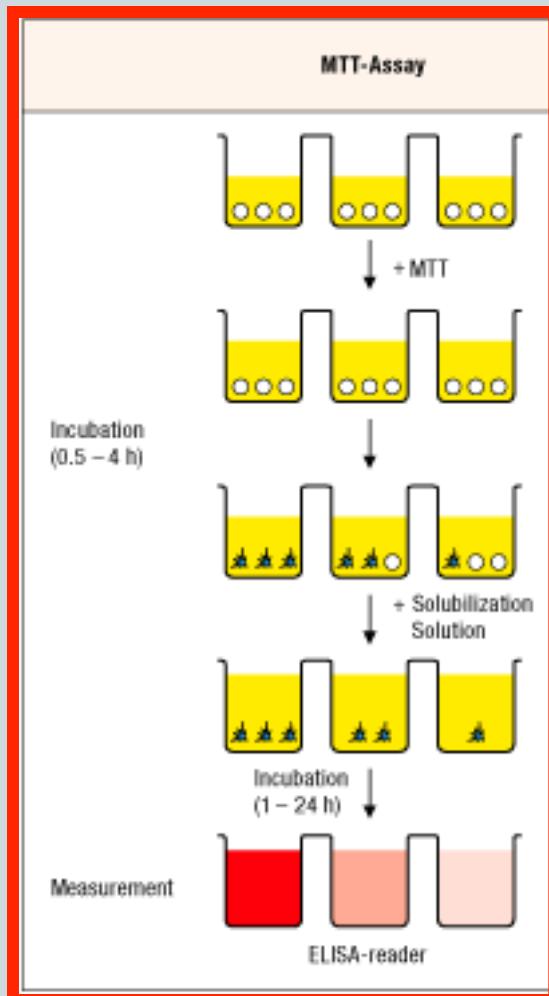
$$110 \times 10,000 = 1.1 \times 10^6 \text{ cells/ml}$$

Since you mixed 50 µl of cells with 50 µl of trypan blue, your dilution factor is 2.

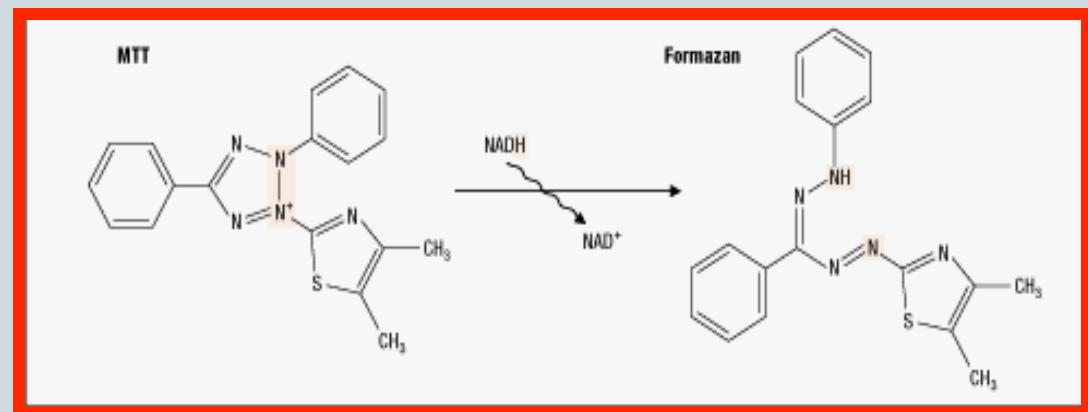
$$2 \times 1.1 = 2.2 \times 10^6$$

2.2×10^6 cells/ml is the number of cells in the original culture.

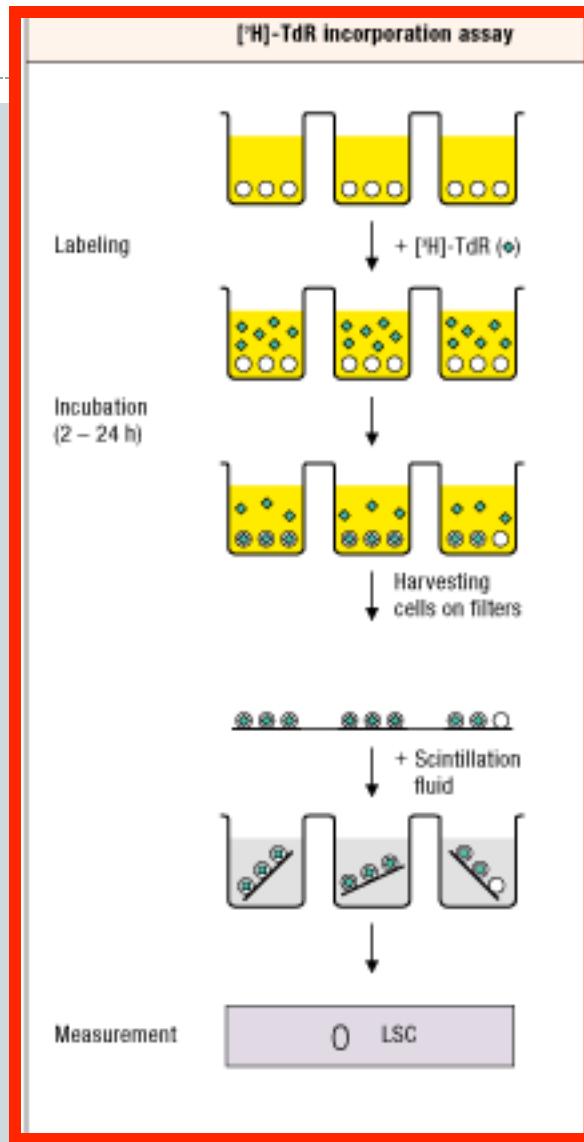
Cell Viability Assay using MTT



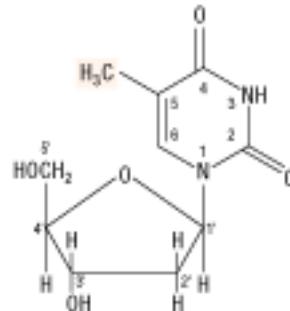
Chemical structure of MTT



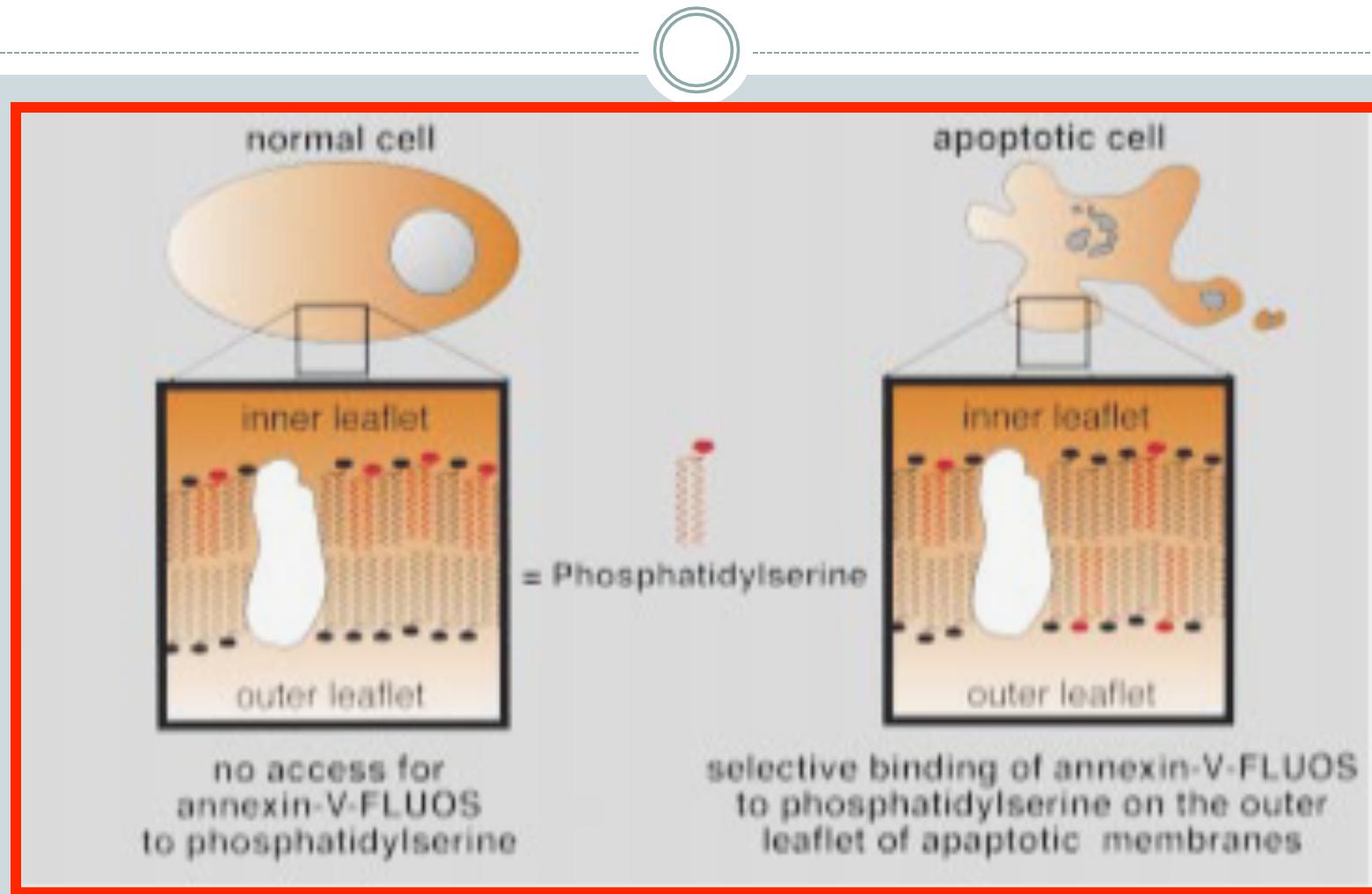
Determination of Levels of DNA Synthesis



Thymidine
(5-methyluracil-2'-deoxyribose)



Phosphatidyl serine flip-flops in apoptotic cells



Detected by Annexin V binding & PI staining

Dapi Staining

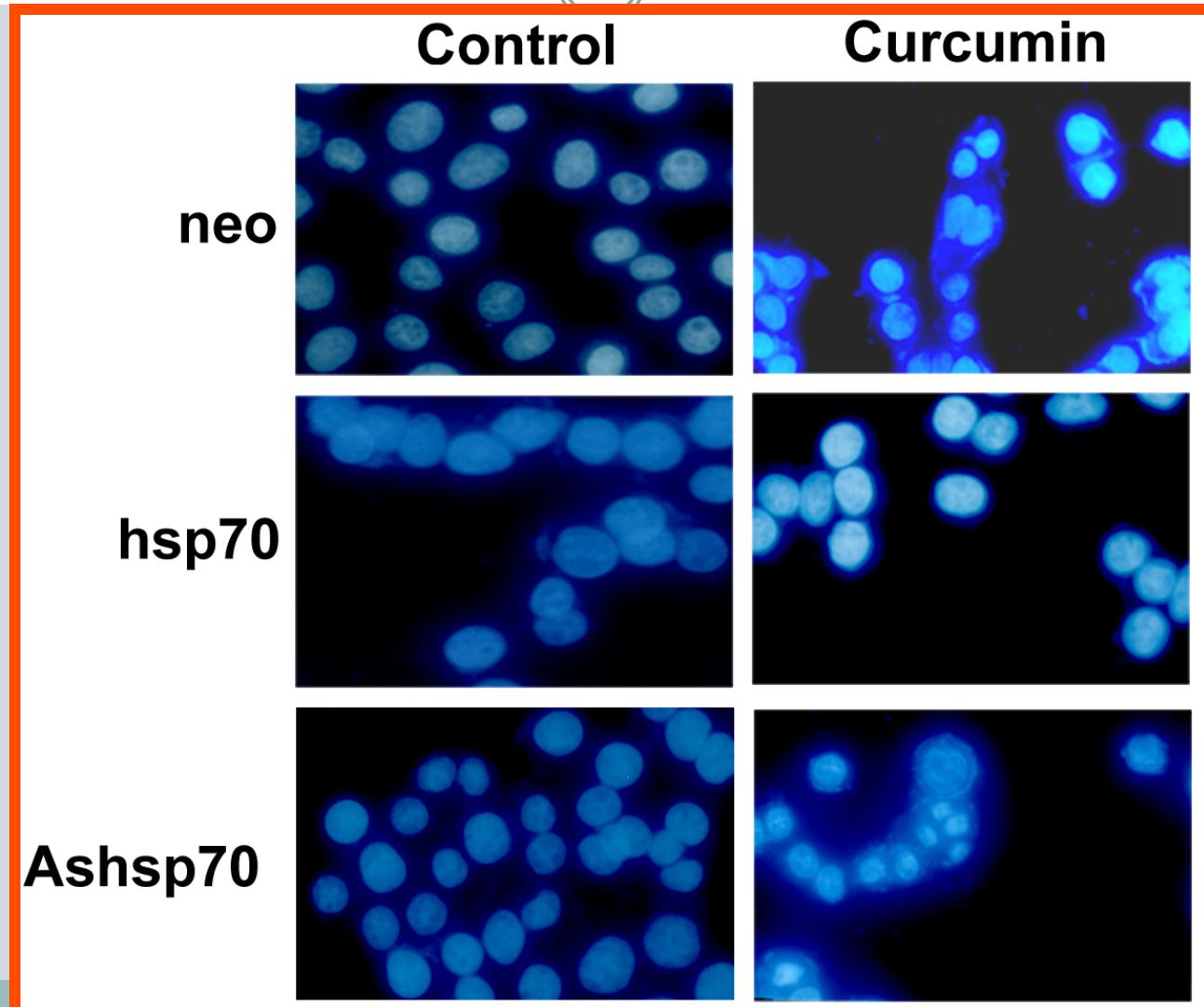
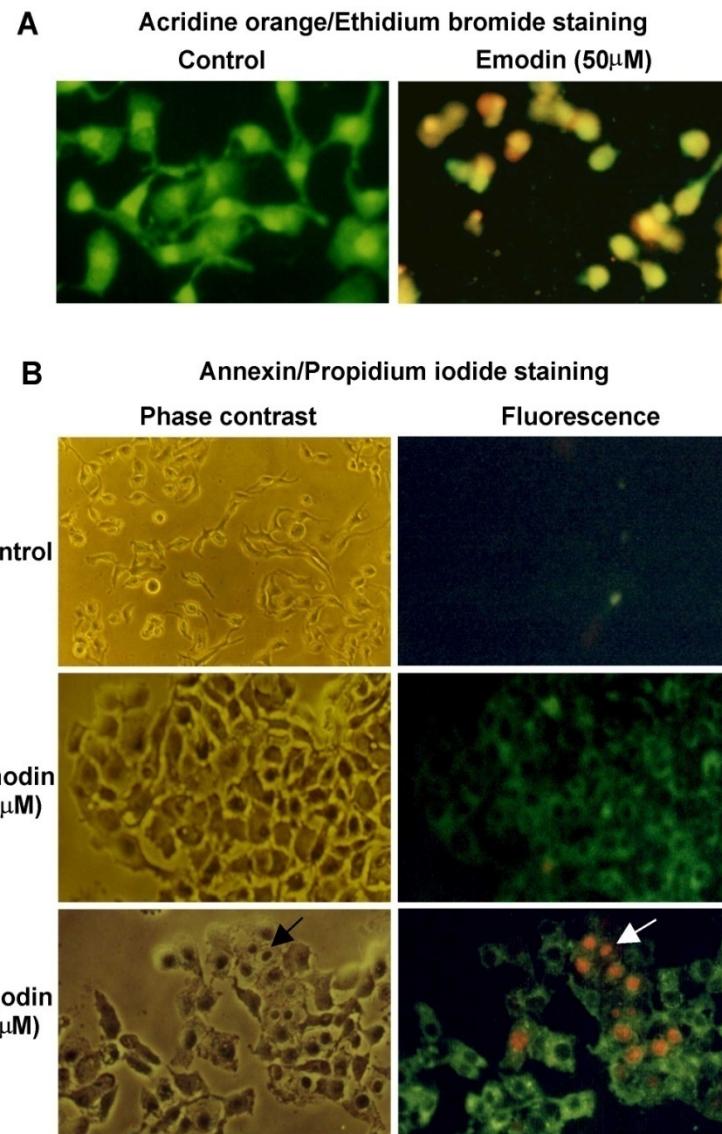
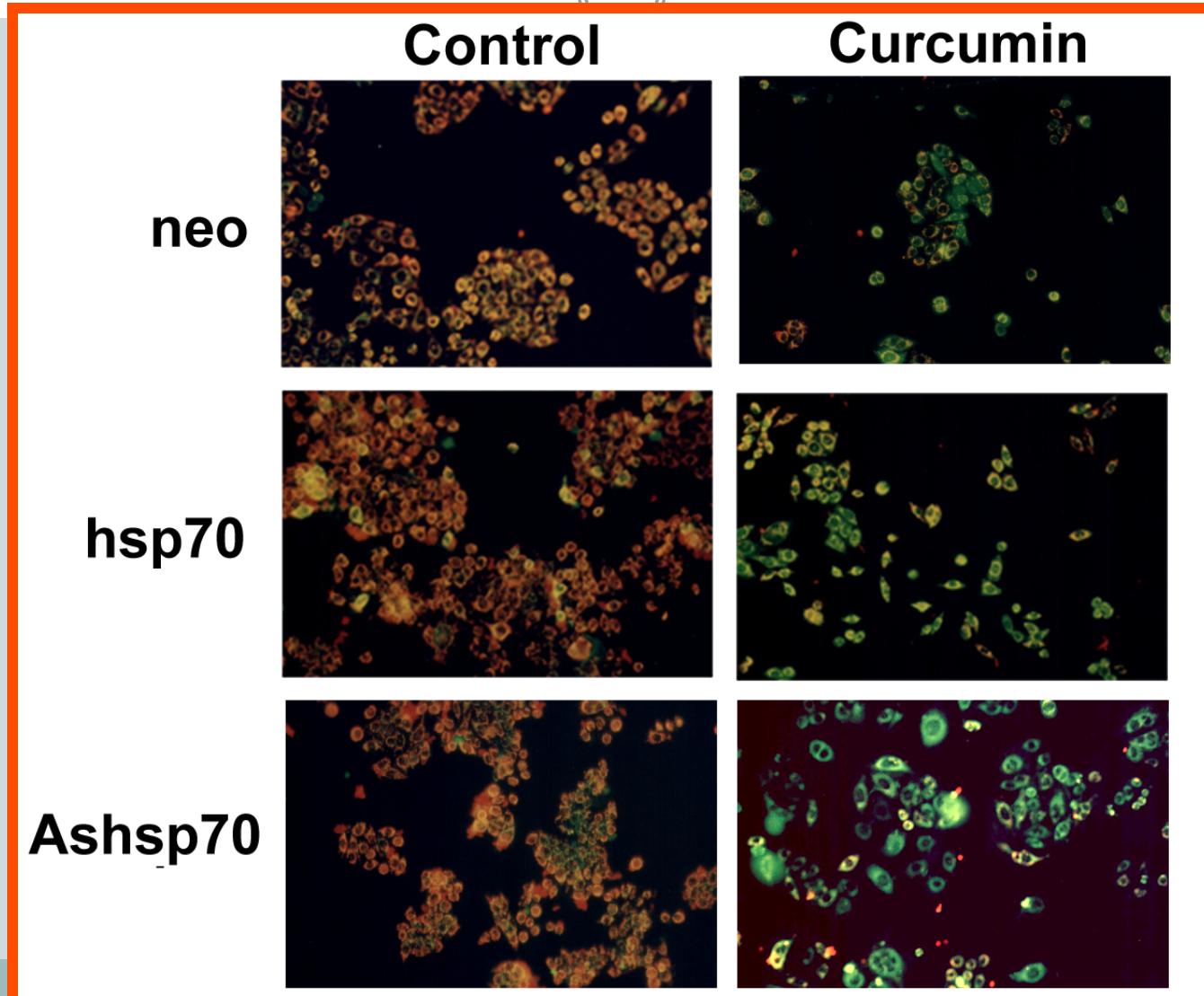


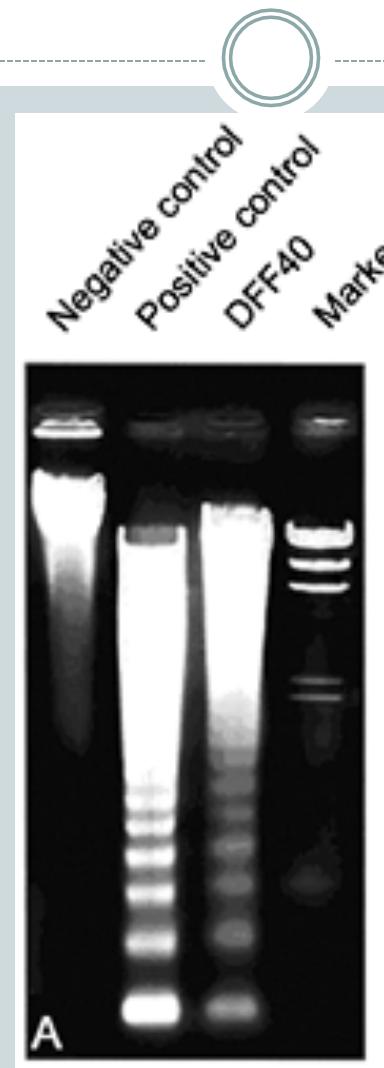
FIGURE 3



Mitochondrial Membrane Potential Detection



DNA Fragmentation



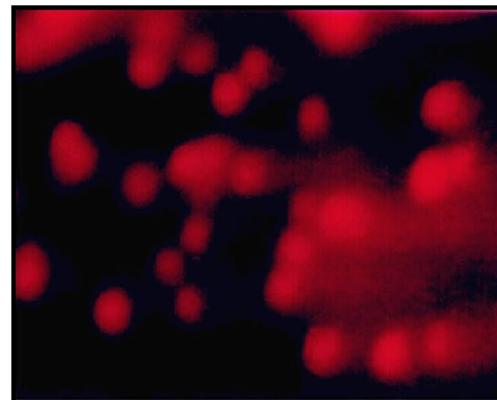
Ldff, the large molecular weight DNA fragmentation factor, is responsible for the large molecular weight DNA degradation during apoptosis in *Xenopus* egg extracts; Zhi Gang LU, Chuan Mao ZHANG and Zhong He ZHAI; *Nature Cell Research*

Comet Assay

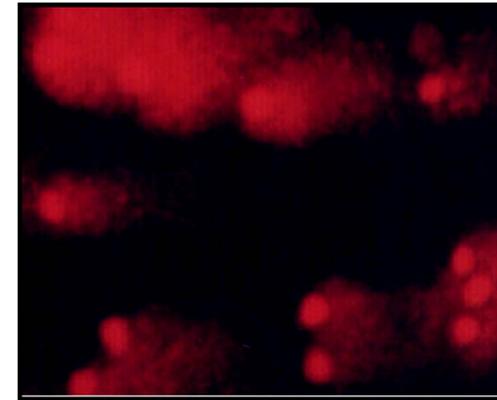


A431- Neo

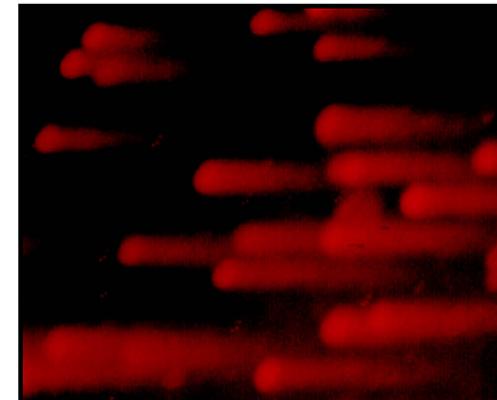
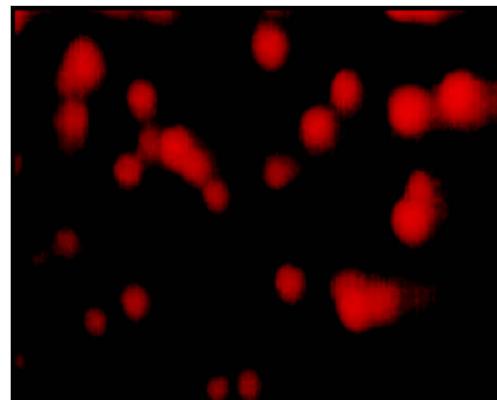
Control



EGF (5ng/ml)



A431- I_KB- α



PARP cleavage

