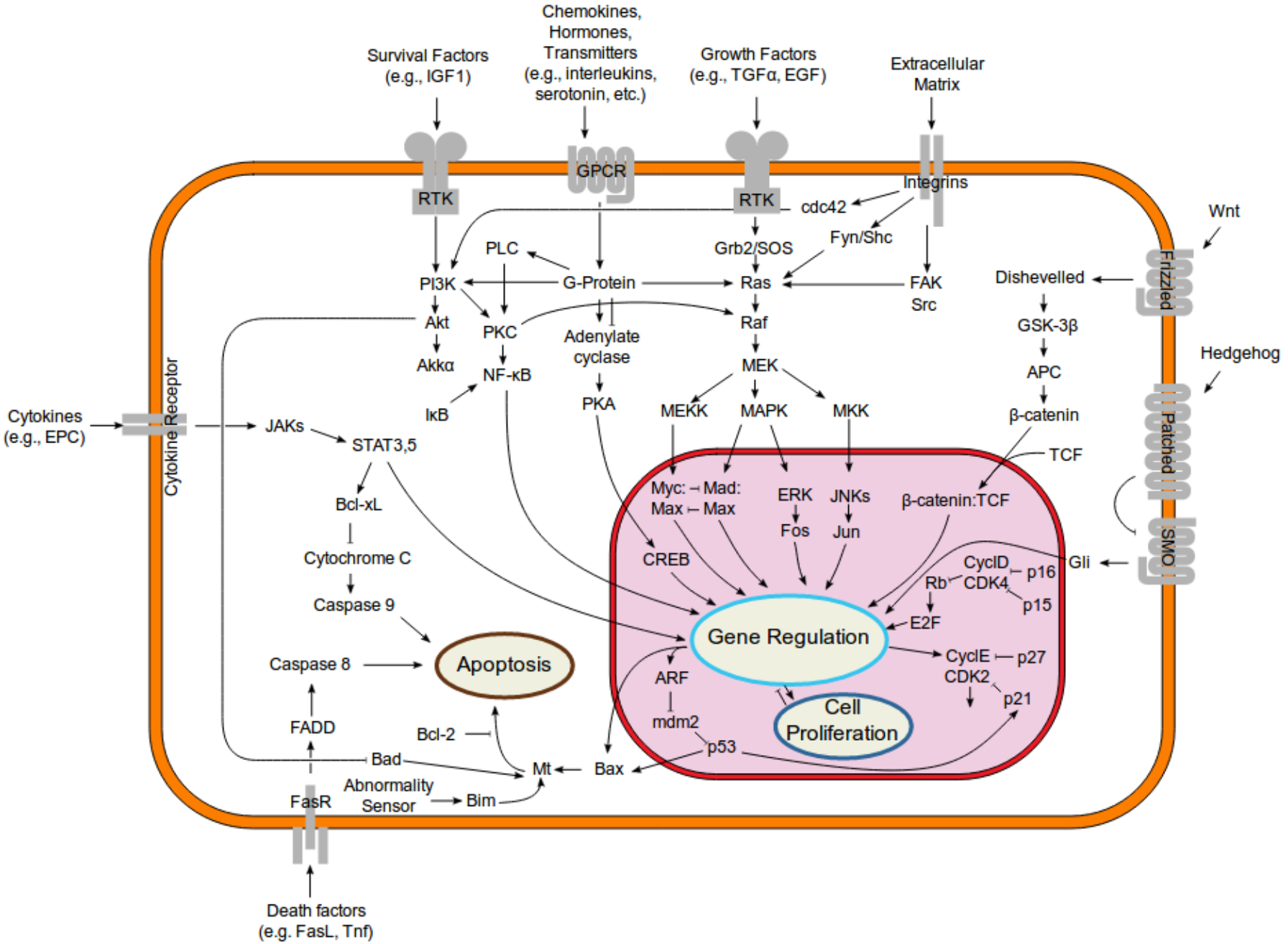
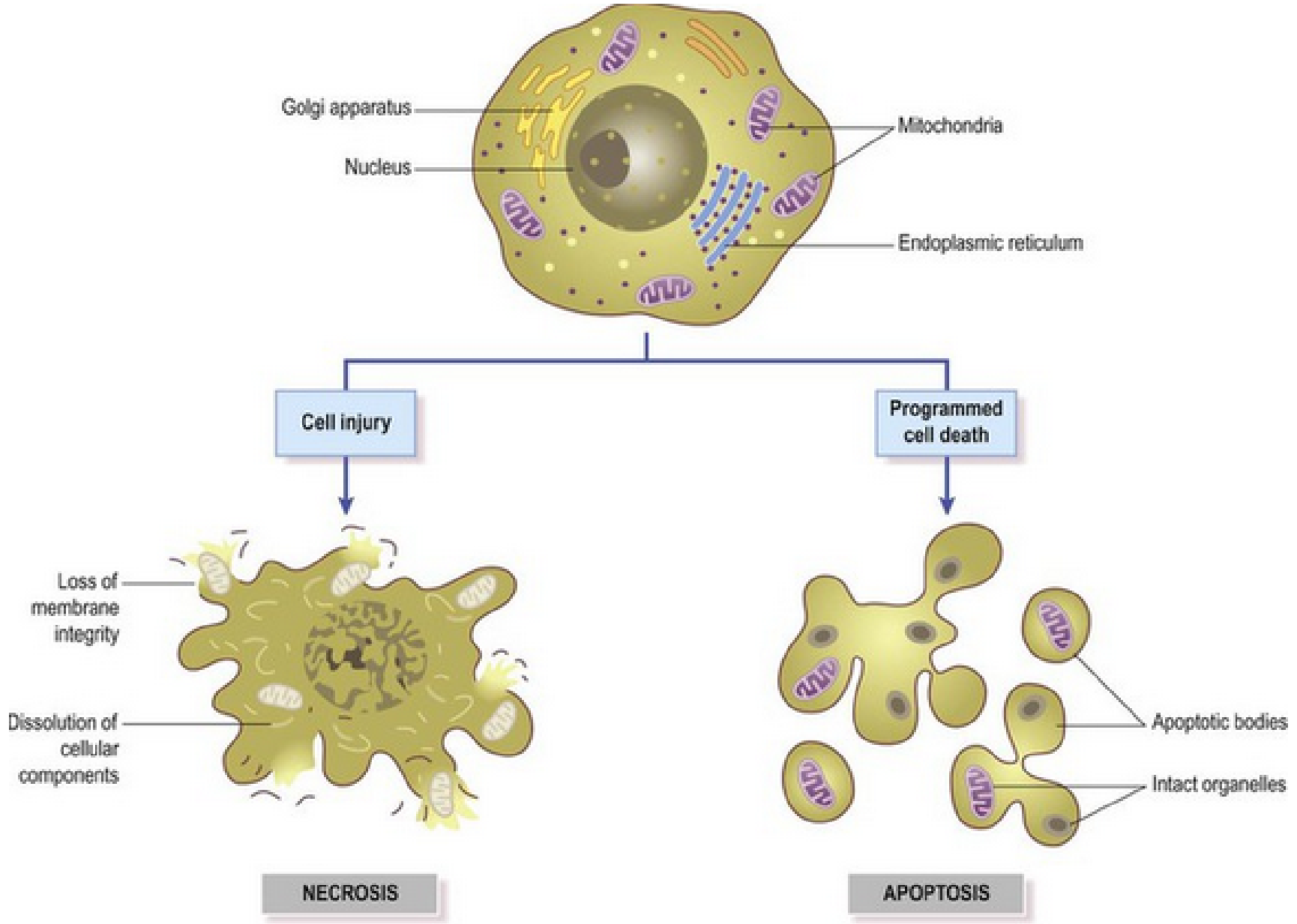
The complex process of programmed cell death (PCD)



Apoptosis vs necrosis



Apoptosis: main roles

Apoptosis occurs during all phases of development, body shaping and organogenesis

- involution of Miller's ducts (reproductive system)
- removal of interdigital membranes
- formation of the intestinal lumen
- formation of cavities in general

Apoptosis is required to maintain the activity of tissues that need a rapid turnover

- epithelial cell of the gut

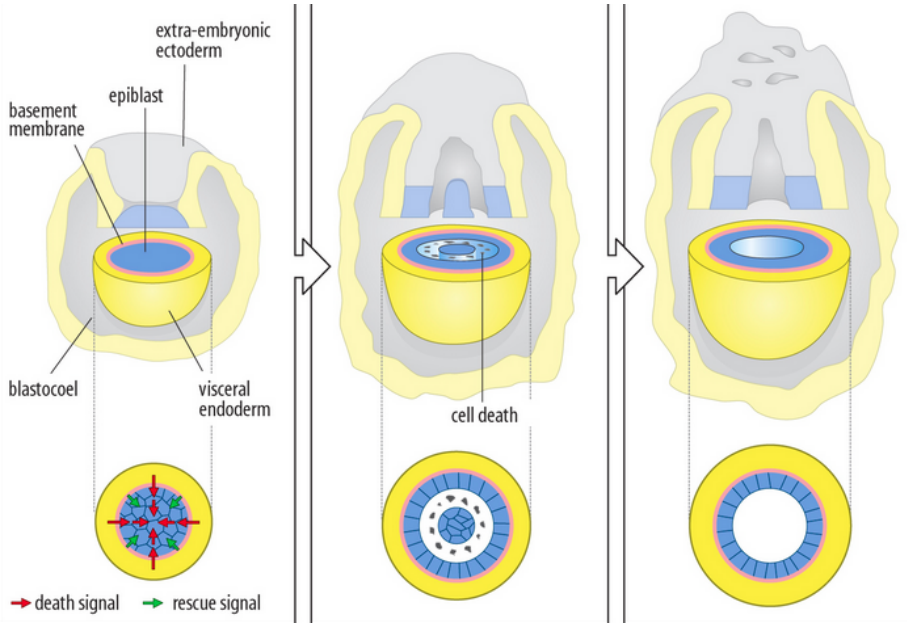
Apoptosis is activated to arrest the immunological response after stimulus

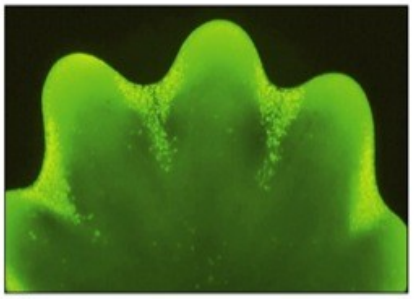
- by decreasing the levels of B- and T-cells.
- by removing netrophil granulocites after their invasion of the blood vessels, to stop inflammation

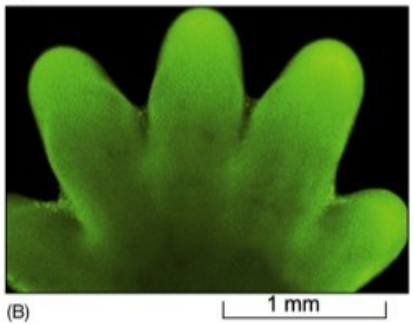
Apoptosis is fundamental to remove cells that, because of DNA damage by radiation or toxic agents, risk to become aberrant

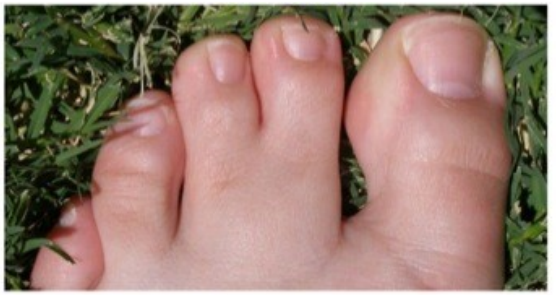
Apoptosis is involved in neurodegenerative diseases.

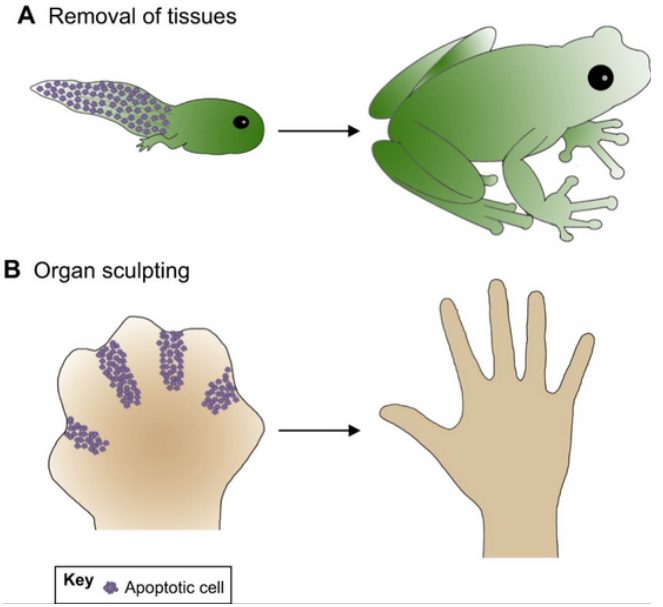
Apoptosis


extra-embryonic ectoderm
epiblast
basement membrane
blastocoel
visceral endoderm
cell death
death signal
rescue signal

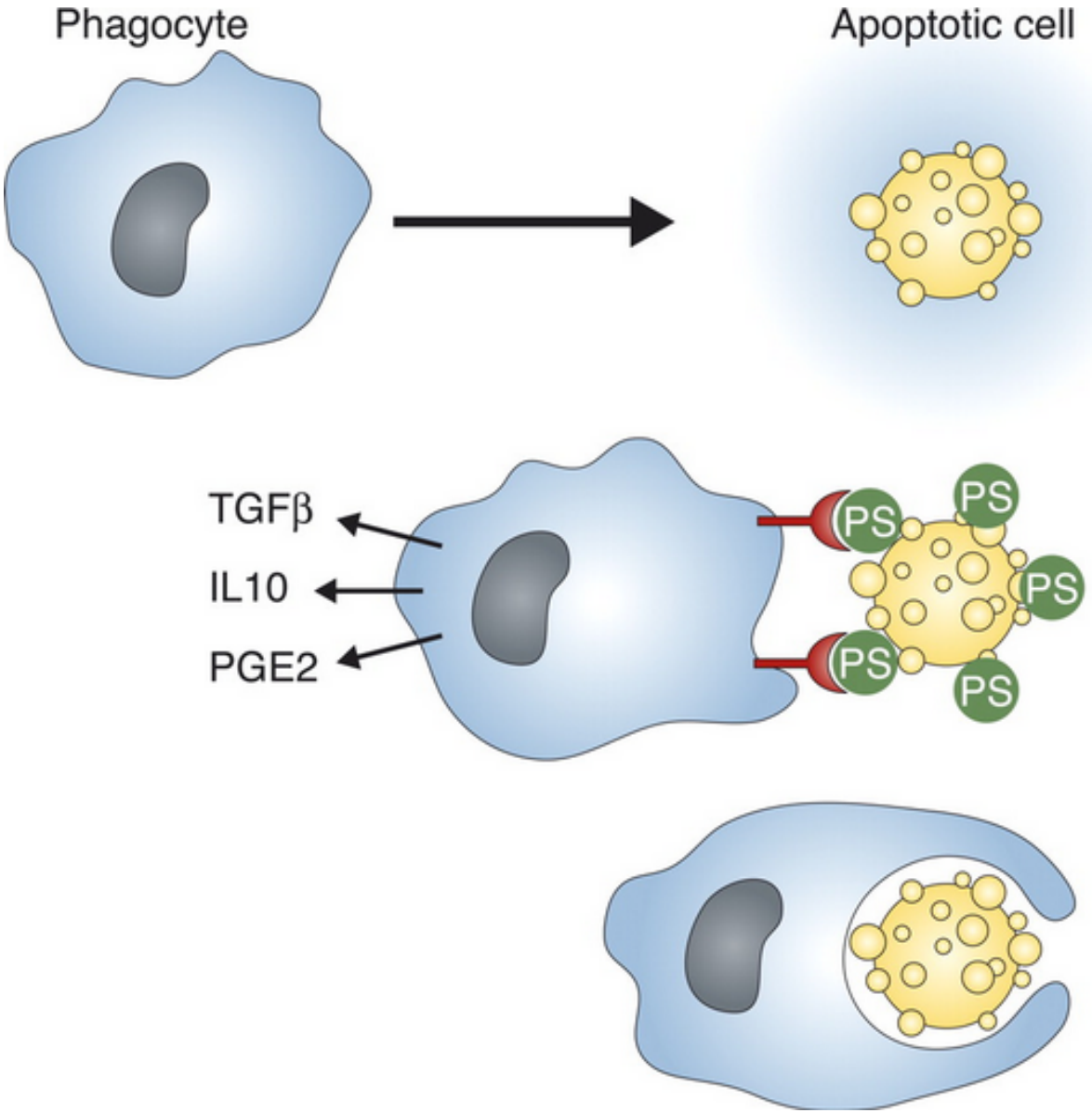

(A)


(B)
1 mm




A Removal of tissues
B Organ sculpting
Key
Apoptotic cell

Apoptosis and the “eat-me” signal



The flip of phosphatidylserine: the “eat-me” signal

Virtually all cells in the body restrict phosphatidylserine (PS) to the inner leaflet of the plasma membrane, keeping it asymmetrical.

Apoptotic cells rapidly randomize the asymmetric distribution, bringing PS to the surface.

This is a potent signal for inducing phagocytosis (not only in apoptosis, as frequently observed in the immune system or coagulation processes).

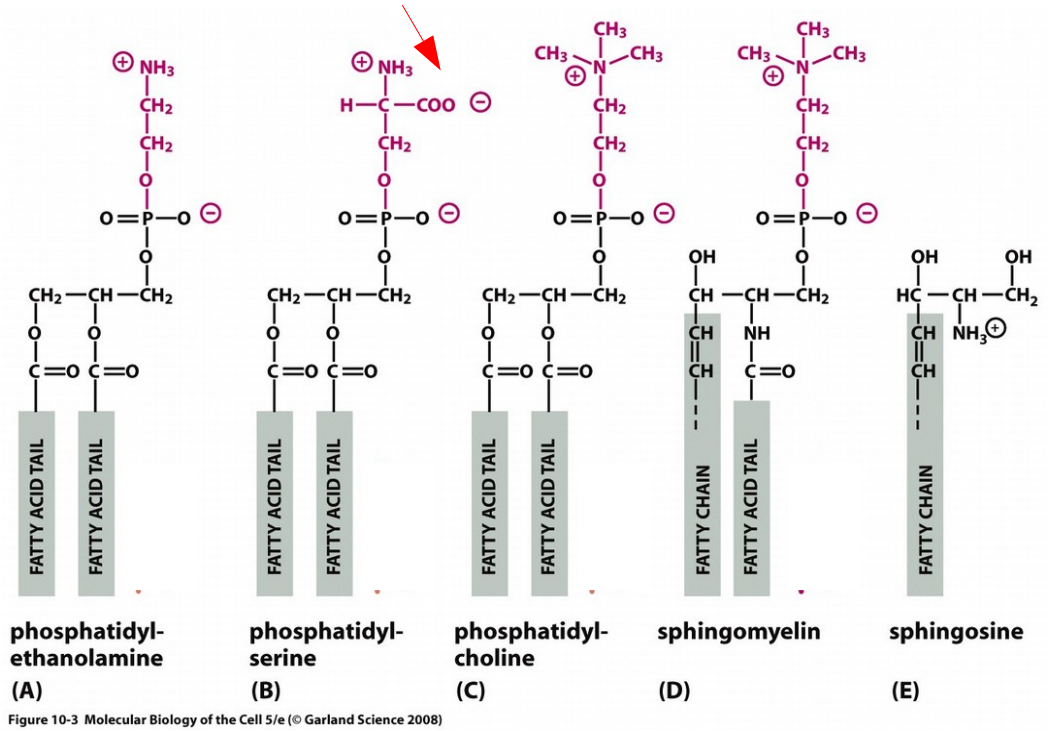
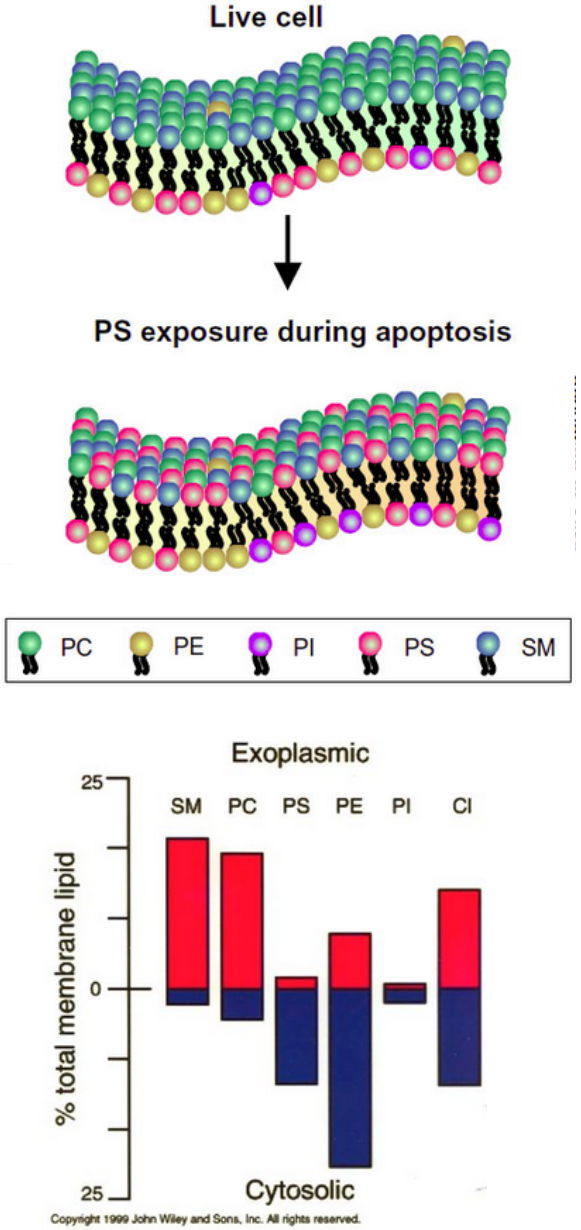


Figure 10-3 Molecular Biology of the Cell 5/e (© Garland Science 2008)



Rysavy et al. 2014 Bioarchitecture 4(4-5):127-37

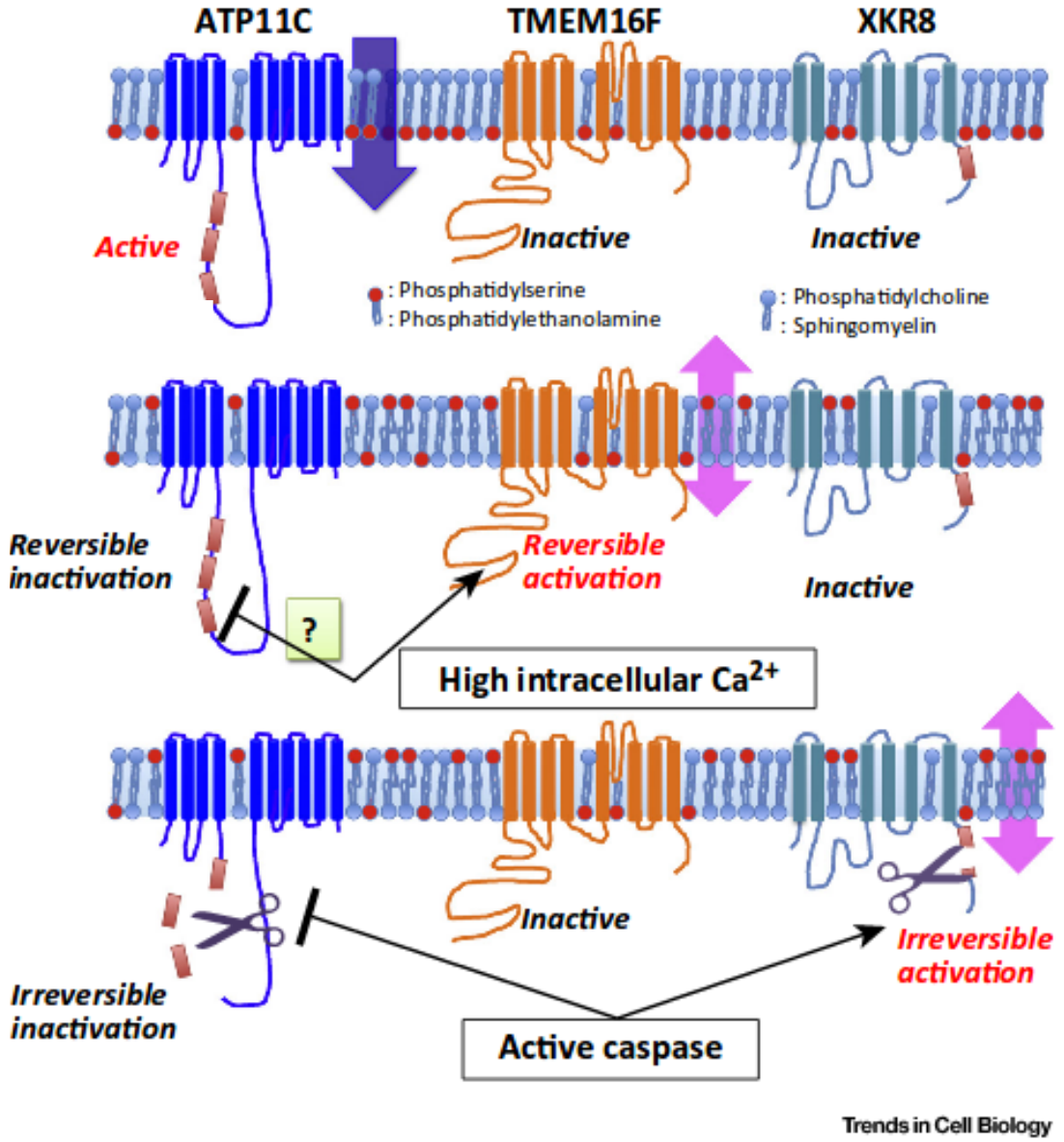
Homeostasis of cell membrane and PS asymmetry

In resting cells, ATP-dependent flippases (e.g. ATP11C) actively and continuously flip out→in PS in the plasma membrane. Scramblases (e.g. TMEM16F or Xkr8) are inactive. This maintain an asymmetric membrane.

In activated cells, a transient increase of Ca^{2+} activates TMEM16F and inhibits ATPC11, making the two sides of the membrane similar. Normally, intracellular Ca^{2+} goes back to normal levels and asymmetry is restored.

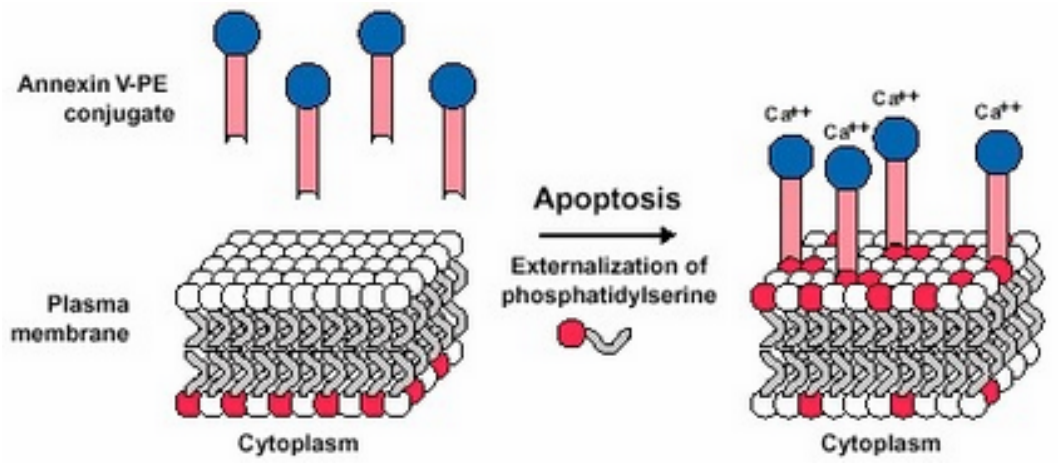
During apoptosis, TMEM16F and XKR8 are cleaved by caspases.

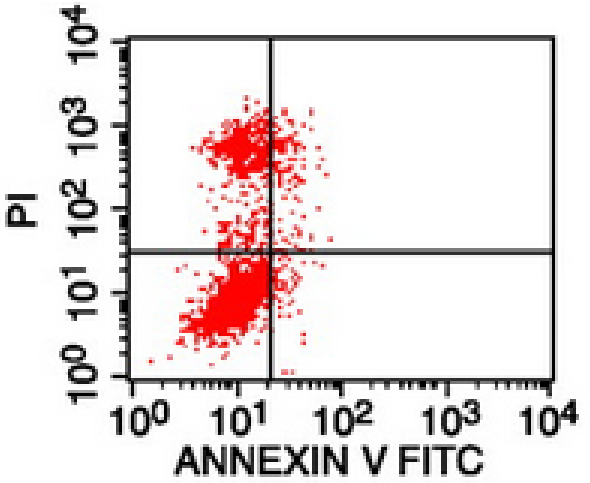
The TMEM16F is inactivated, XKR8 is activated and membrane asymmetry is definitely lost.

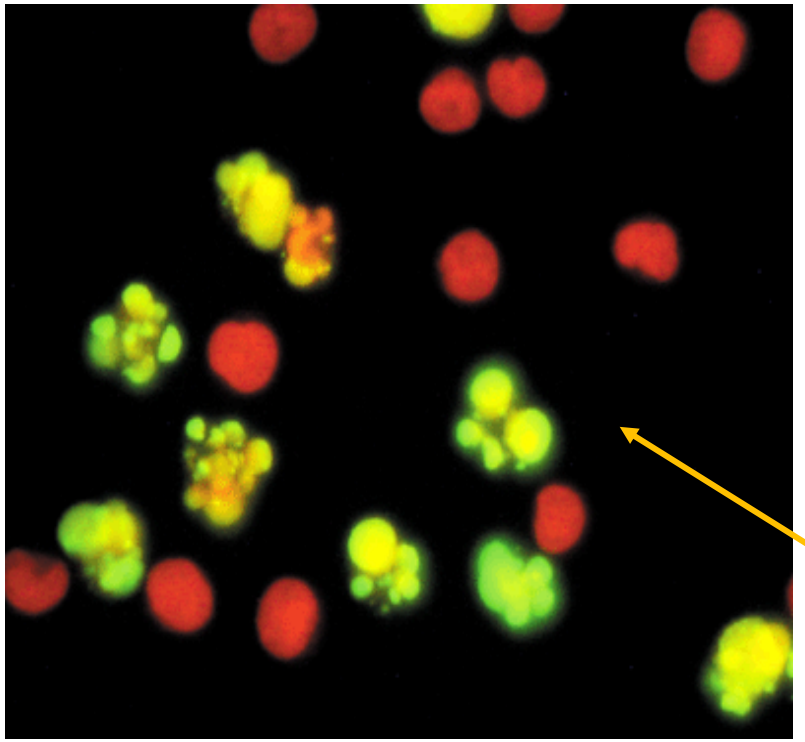


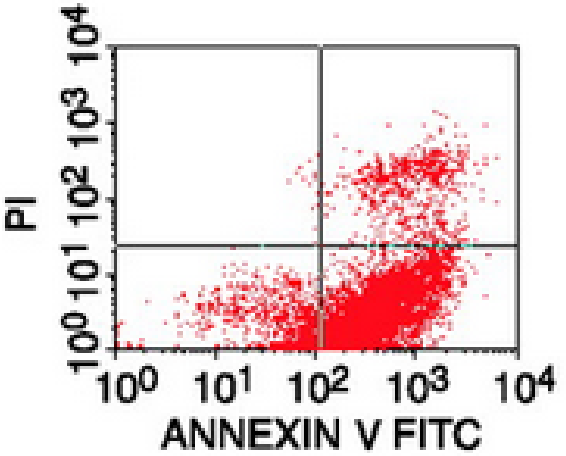
Nagata et al. 2016, Cell Death Differ 23(6):952-61
Segawa and Nagata 2015, Trends Cell Biol 25(11):639-650

Annexin V is a useful tool to detect flipped PS









Apoptotic bodies
Conjugated annexin V

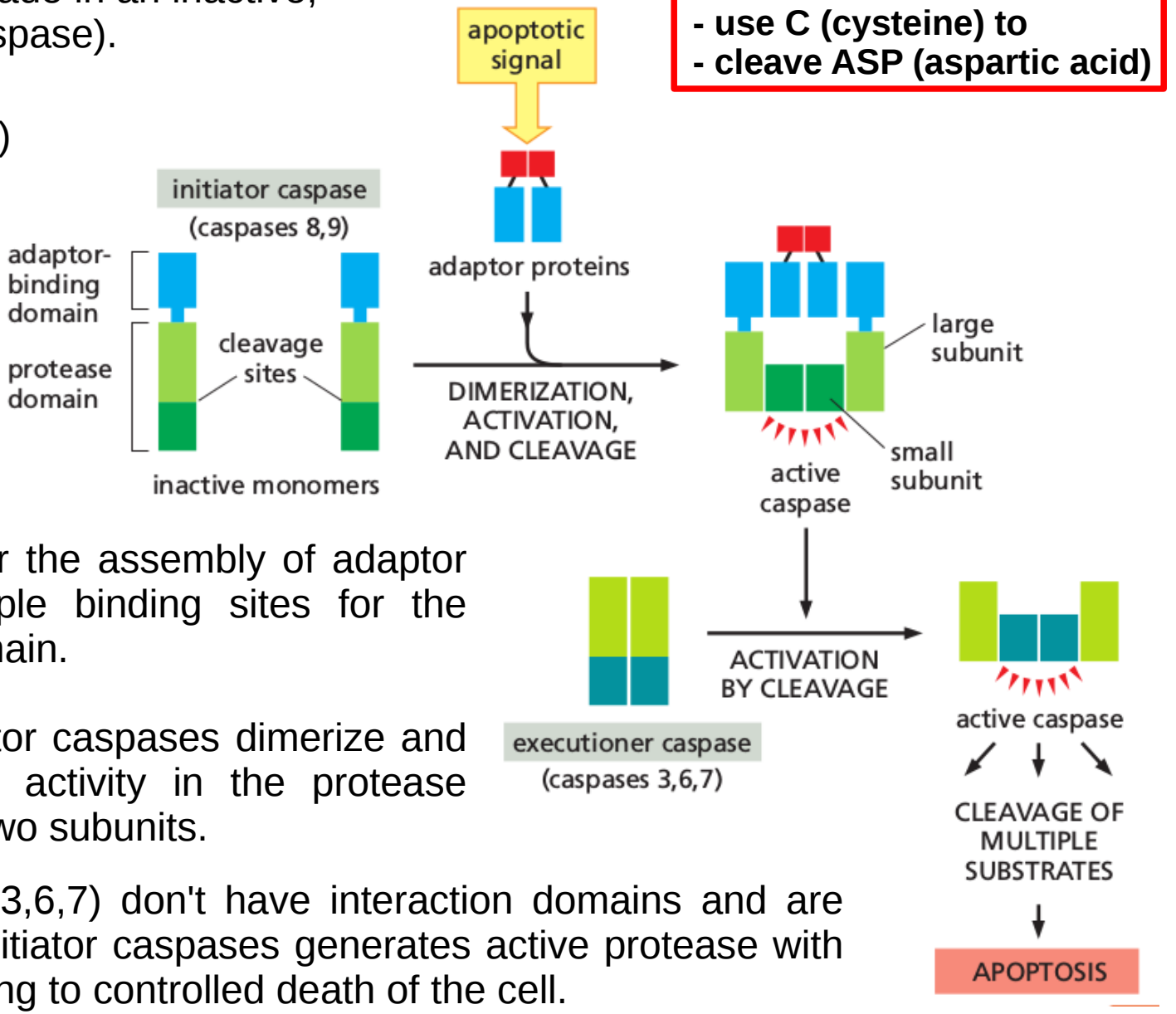
Apoptosis is triggered by caspases

Casapses are initially made in an inactive, monomeric form (procaspase).

C-ASP-ase
- use C (cysteine) to
- cleave ASP (aspartic acid)

An initiator caspase (8,9) contains

- a protease domain (c-term)
- an interaction domain (n-term)

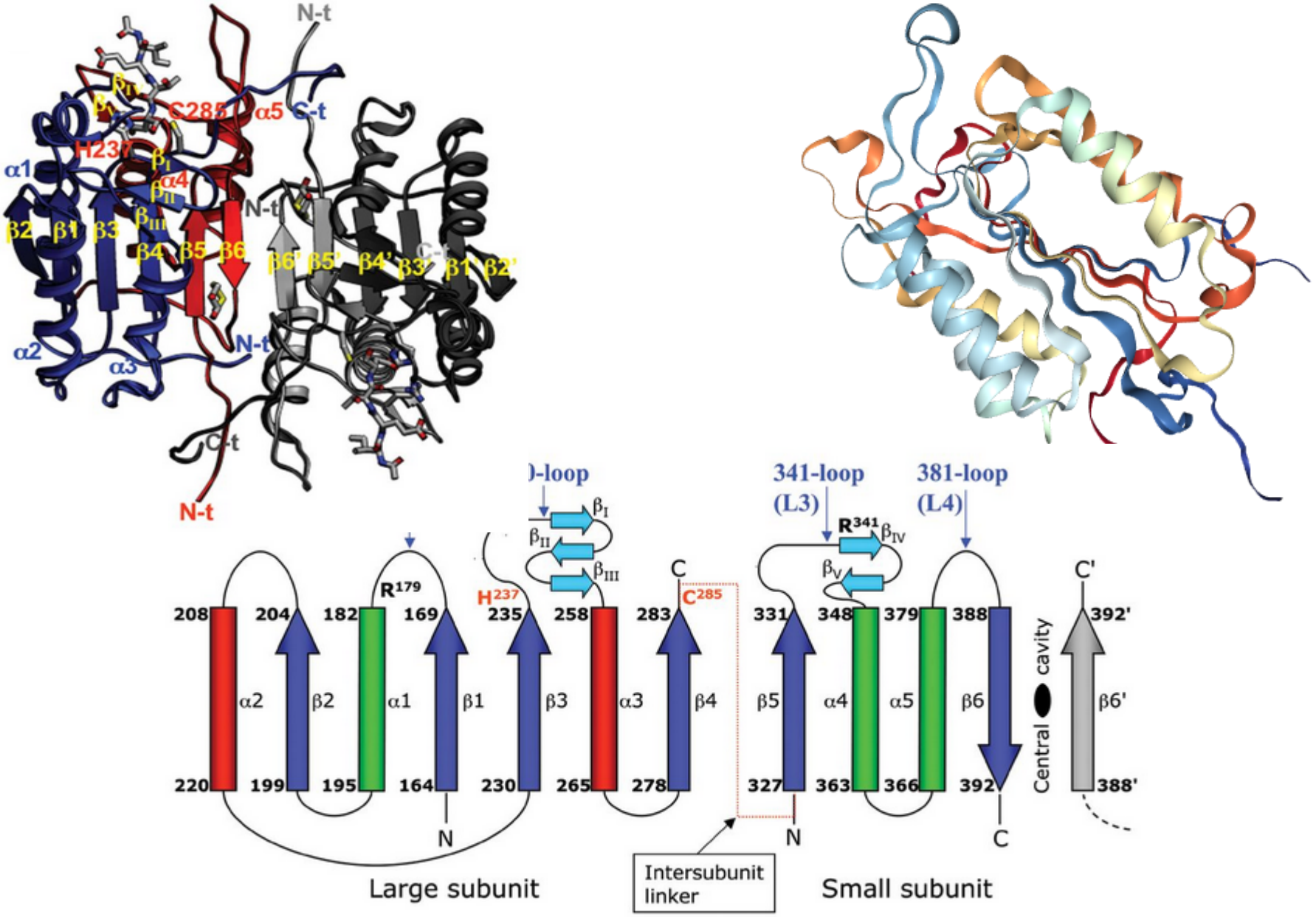


Apoptotic signals trigger the assembly of adaptor proteins carrying multiple binding sites for the caspase interaction domain.

Upon binding, the initiator caspases dimerize and activates a self-cutting activity in the protease domain, detaching the two subunits.

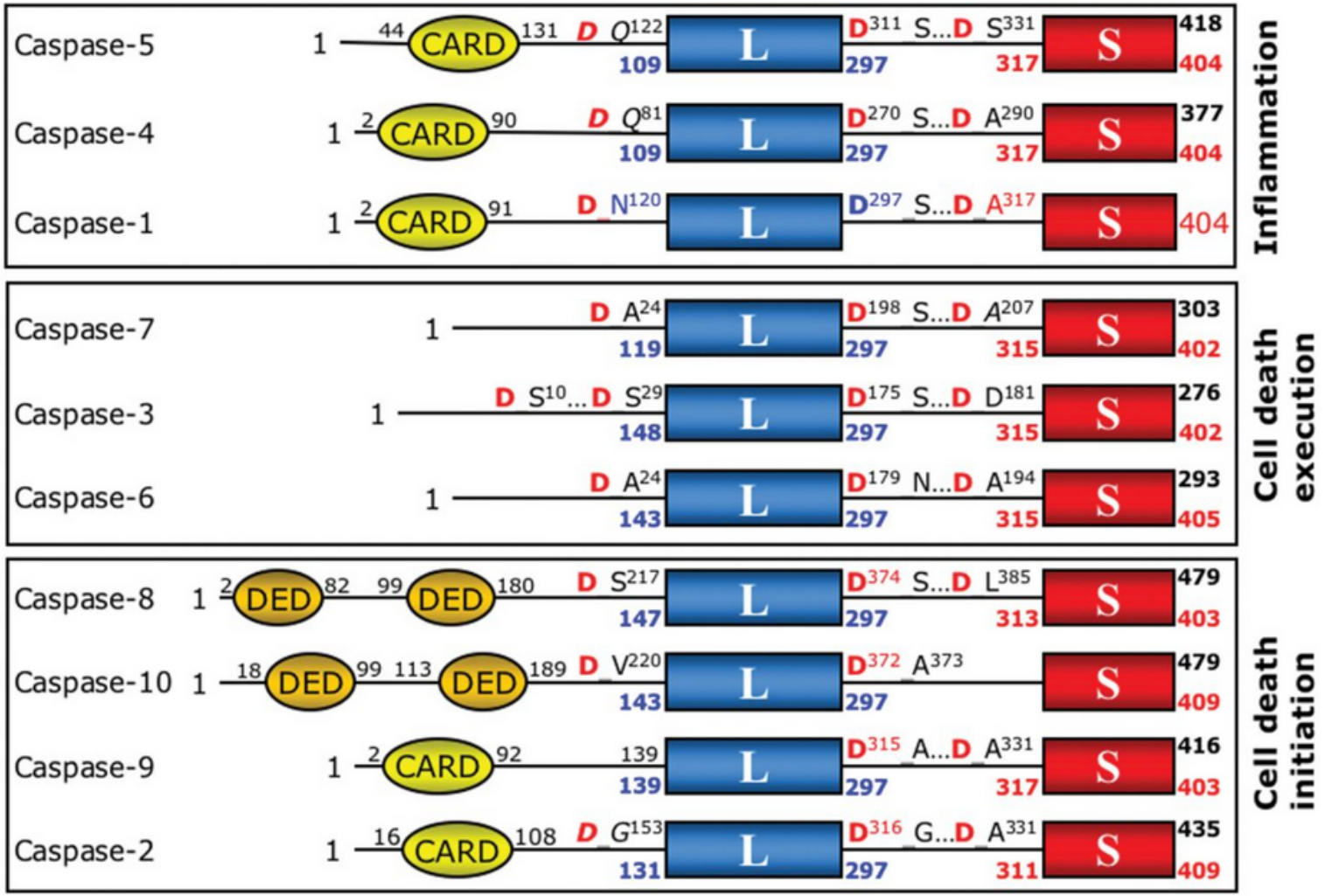
Executioner caspases (3,6,7) don't have interaction domains and are inactive. Cleavage by initiator caspases generates active protease with a variety of targets leading to controlled death of the cell.

Caspases have a compact two-domain fold



Fuentes-Prior and Salvesen 2004 Biochem J. 384(2):201-232

The caspase family

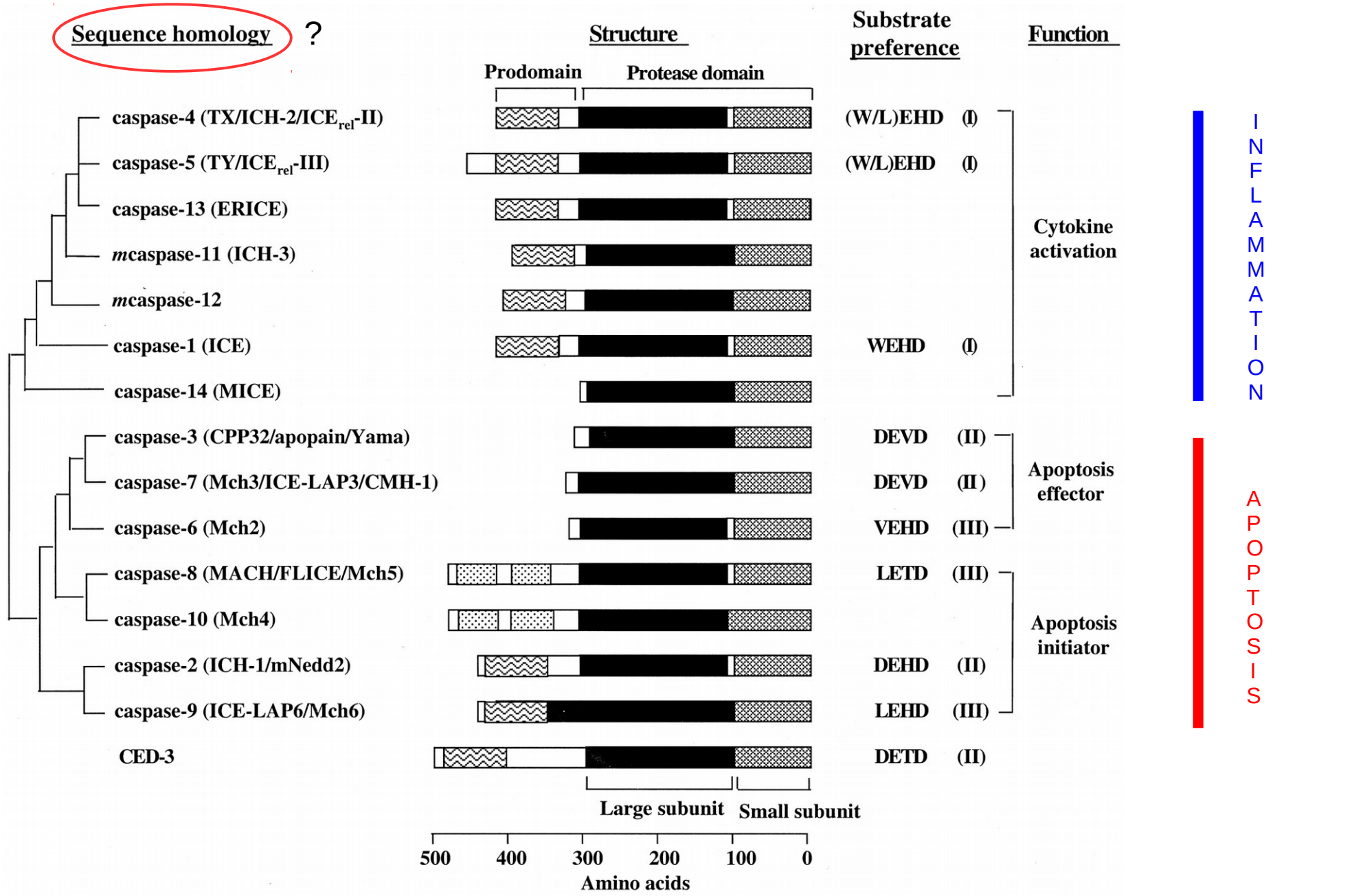


CARD — CAspase-Recruitment Domain

DED — Death Effector Domain

Fuentes-Prior and Salvesen 2004 Biochem J. 384(2):201-232

The caspase family



Caspases: domains and monomers

Caspase 8

www.uniprot.org/uniprot/P41790

Sites

Feature key	Position(s)	Description
Active site	121	By similarity
Active site	163	By similarity

Molecule processing

Feature key	Position(s)	Description
Propeptide (PRO_0000004628)	1 – 216	
Chain (PRO_0000004629)	217 – 374	Caspase-8 subunit p18
Propeptide (PRO_0000004630)	375 – 384	
Chain (PRO_0000004631)	385 – 479	Caspase-8 subunit p10

Amino acid modifications

Feature key	Position(s)	Description
Modified residue	188	Phosphoserine By simil
Modified residue	211	Phosphoserine By simil
Modified residue	224	N6-acetyllysine By simi
Modified residue	334	Phosphotyrosine Comb
Modified residue	387	Phosphoserine; by CDK1

Caspase 3

www.uniprot.org/uniprot/P42574

Sites

Feature key	Position(s)	Description
Active site	317	
Active site	360	

Molecule processing

Feature key	Position(s)	Description
Propeptide (PRO_0000004569)	1 – 9	
Propeptide (PRO_0000004570)	10 – 28	1 Publication
Chain (PRO_0000004571)	29 – 175	Caspase-3 subunit p17
Chain (PRO_0000004572)	176 – 277	Caspase-3 subunit p12

Amino acid modifications

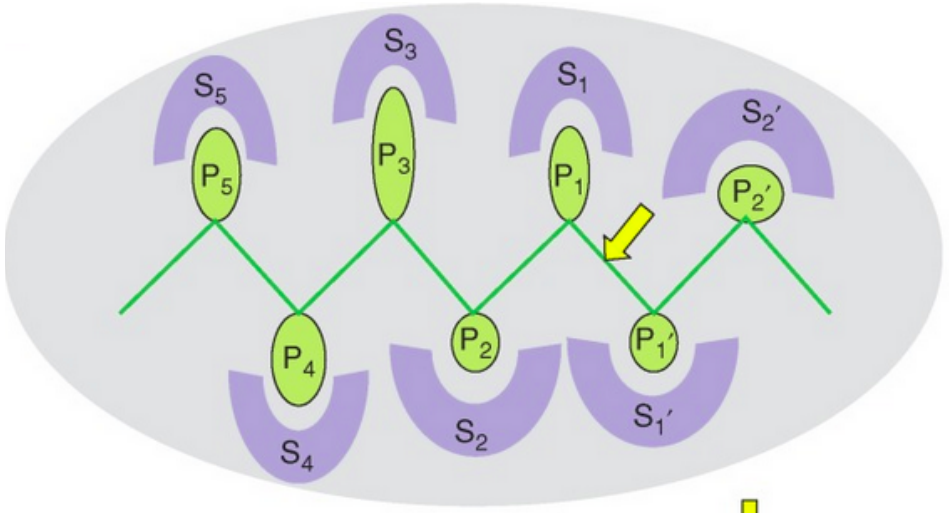
Feature key	Position(s)	Description
Modified residue	1	N-acetylmethionine Combined sou
Modified residue	11	N6-acetyllysine By similarity
Modified residue	26	Phosphoserine Combined sources
Modified residue	163	S-nitrosocysteine; in inhibited form

Caspases consensus sites

Casapses, as many other families of proteases, are characterized by consensus recognition sites. In particular, the different classes of proteases are slightly different in their consensus sites.

Proteins containing such sequences are potentially cleaved by caspases

=> caspase targets can be predicted!

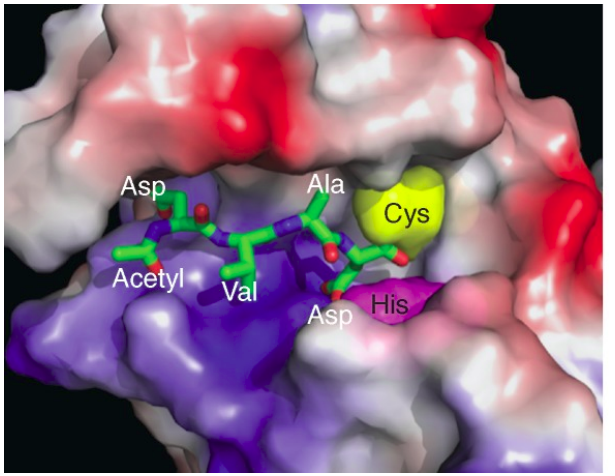


Caspase-	P_5	P_4	P_3	P_2	P_1	P_1'
1, 4, 5, 14		W/Y	E	X	D	ϕ
8, 9, 10		I/L	E	X	D	ϕ
3, 7		D	E	X	D	ϕ
6		V	E	X	D	ϕ
2	V/L	D	E	X	D	ϕ

CaspDB

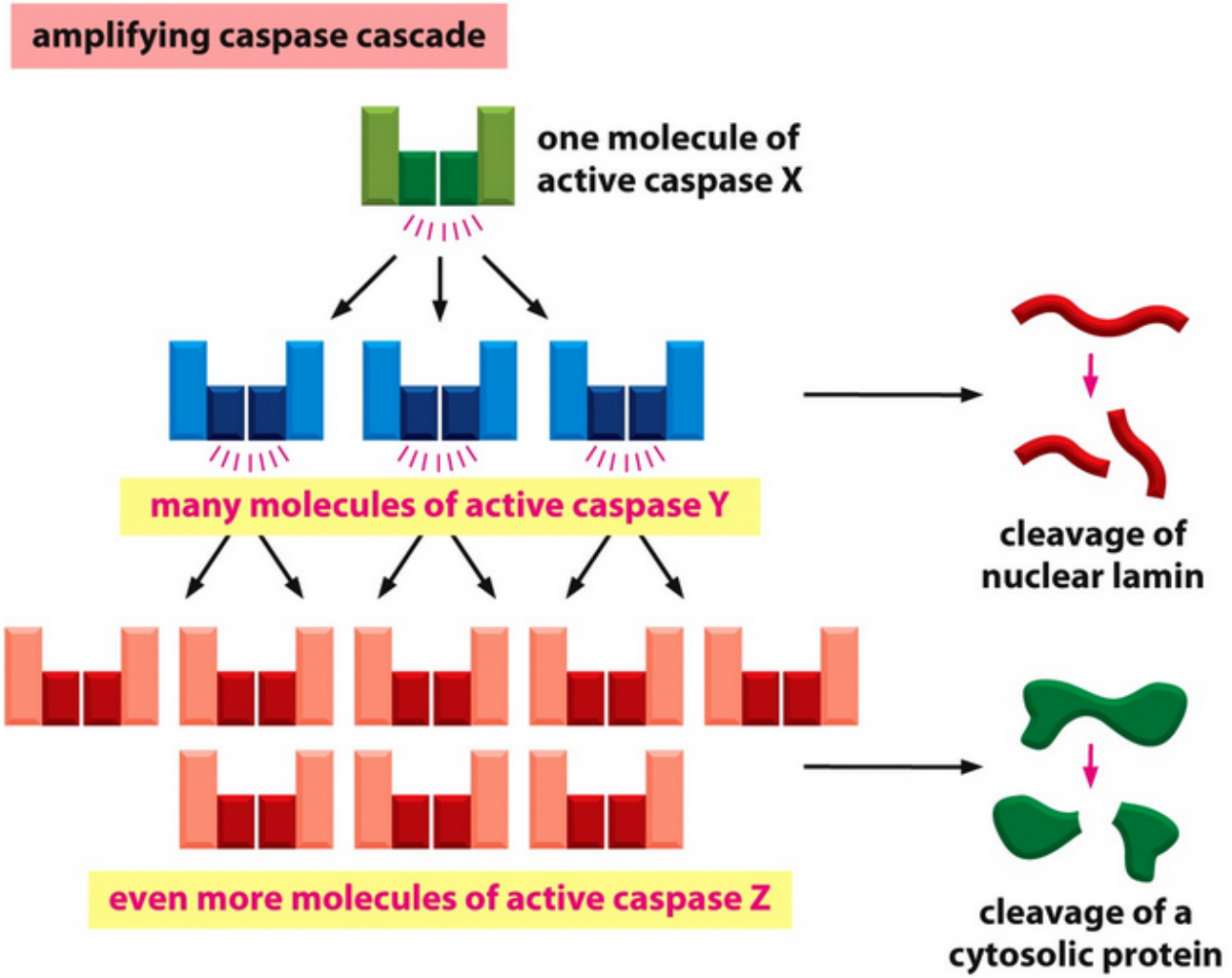
Database statistics	
Human uniprot reviewed protein :	20235
Secreted protein :	3368
Non secreted protein :	16867

http://caspdb.sanfordburnham.org/



Poreba et al. 2013 Perspect Biol. 5(8):a008680

Caspase activation is a cascade, a signal amplification



Nucleus disgregation

DNAse inhibitors
Cytoskeleton components
Adhesion proteins
Various apoptosis effectors

Changes in nucleus during apoptosis

Since its discovery in 1974, apoptosis is distinguished from necrosis based on the changes in the nuclear morphology. During apoptosis we observe:

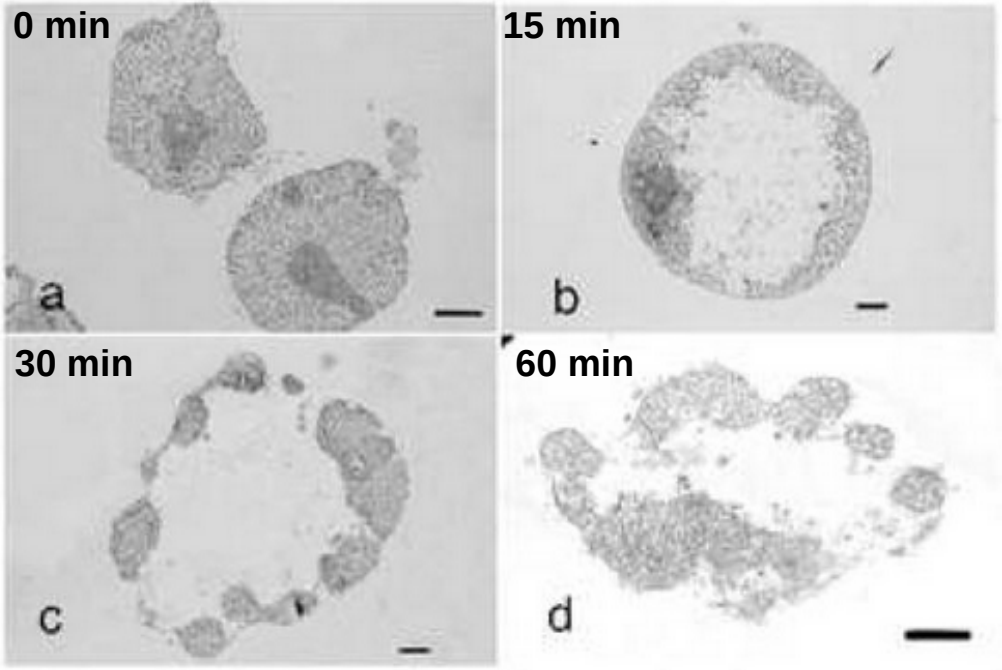
- condensation of chromatin at the nuclear periphery

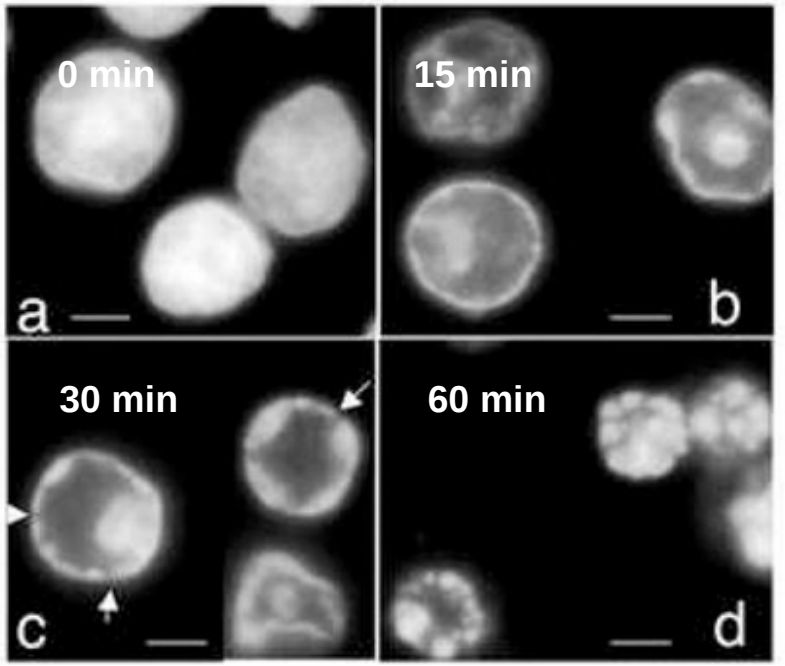
- disassembly of nuclear scaffold proteins

} **Stage 1**

- DNA fragmentation

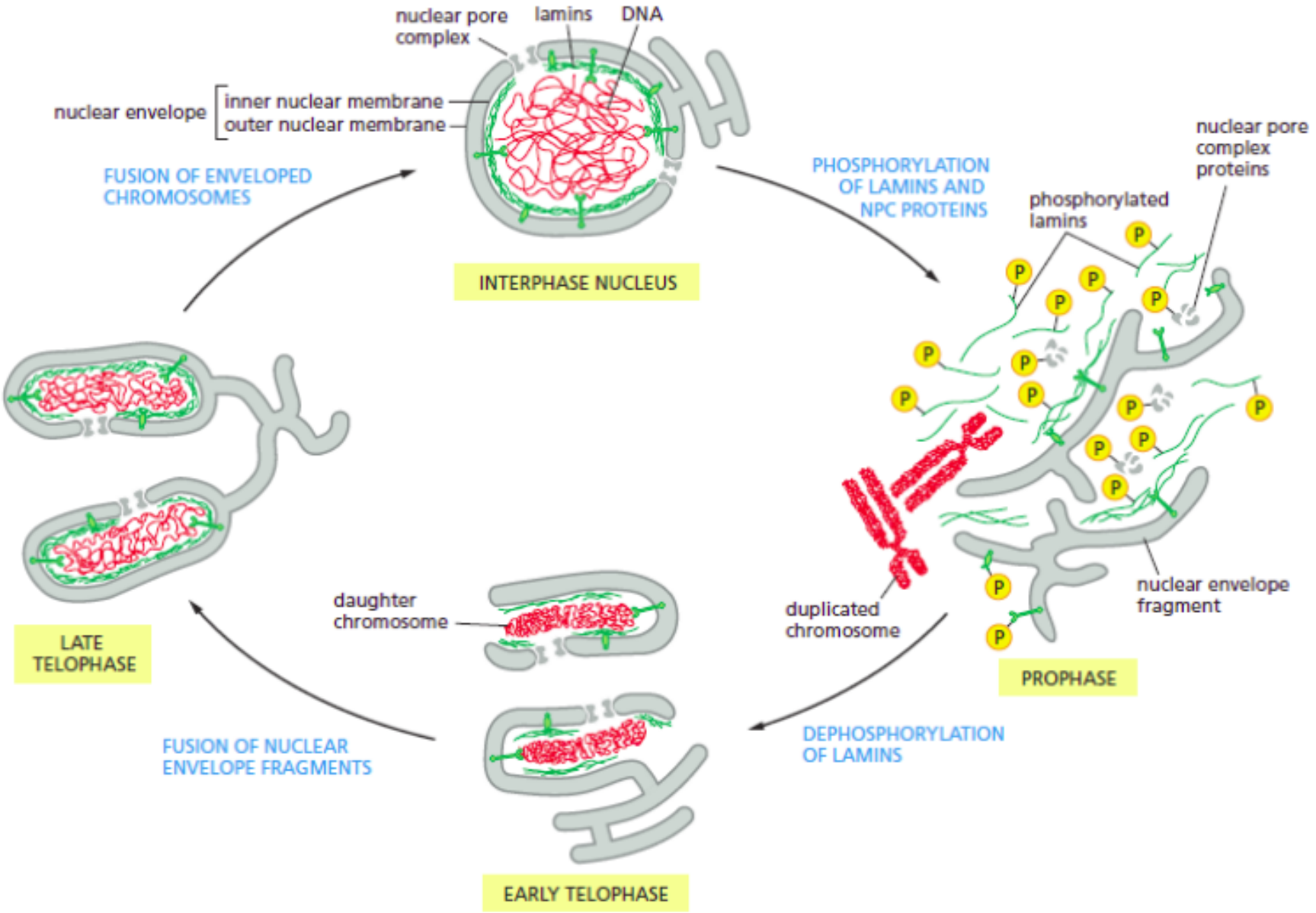
} **Stage 2**



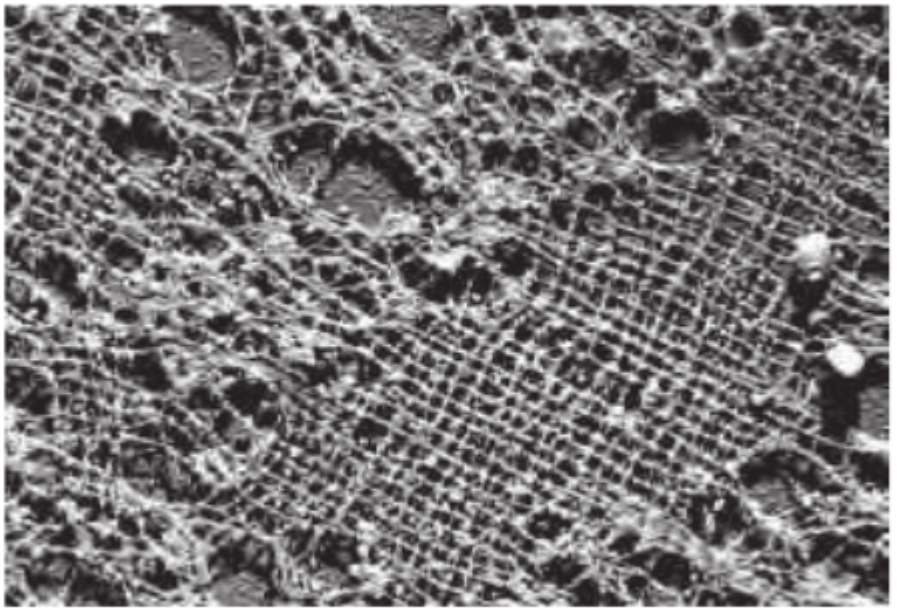


Prokhorova et al.2015 Cel Mol Life Sci 72(23):4593-4612
Tonè et al 2007 Exp Cell Res.313(16):3635-3644

Step back: lamin, nuclear envelope and cell division



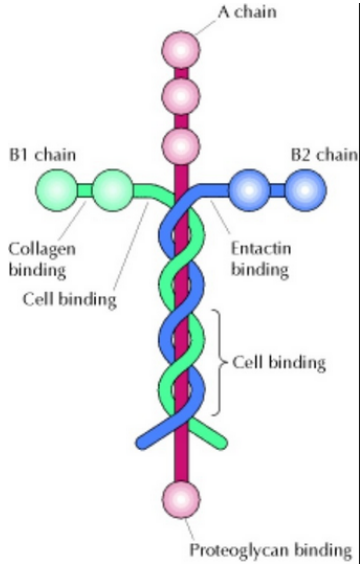
Stage 1: nuclear lamin is cleaved by caspases

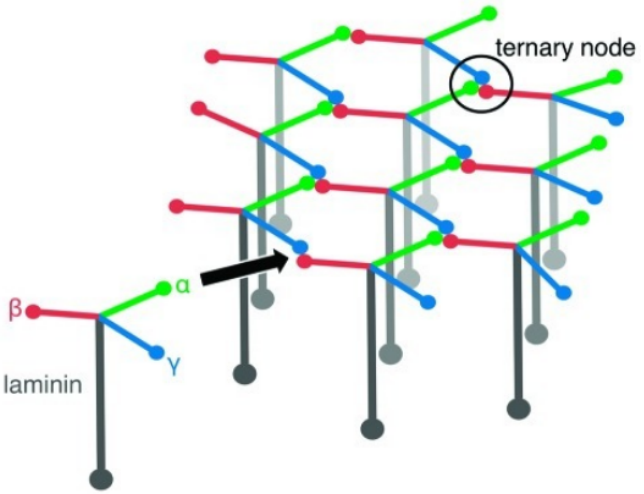


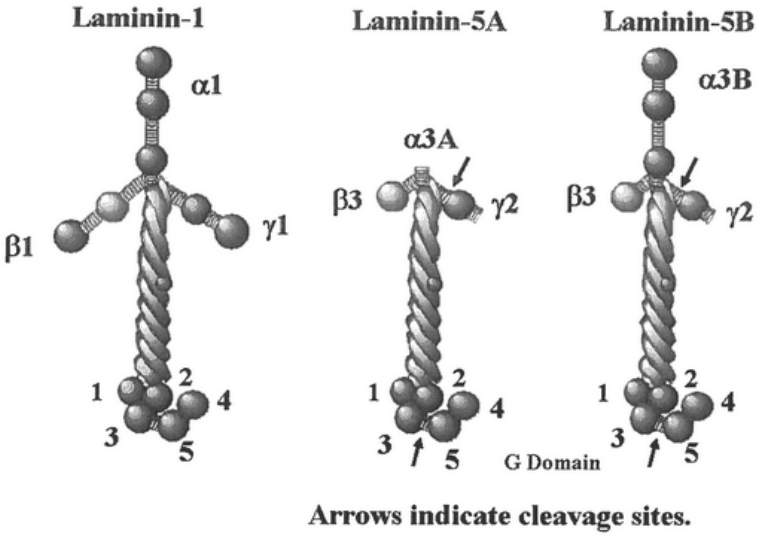
Laminins are crucial targets for apoptosis due to their close associations with chromatin and the nuclear envelope.

Caspases target nuclear lamin and cleave both A- and B-types lamins.

Chromatin separates from the nuclear lamina and condenses. Then, nucleus starts fragmenting and forms "blebs", a typical hallmark of apoptosis.







Step back: the nuclear protein traffic

The transport of protein from cytoplasm to the nucleus is mediated by the nuclear pore complex. The main actors of transport are:

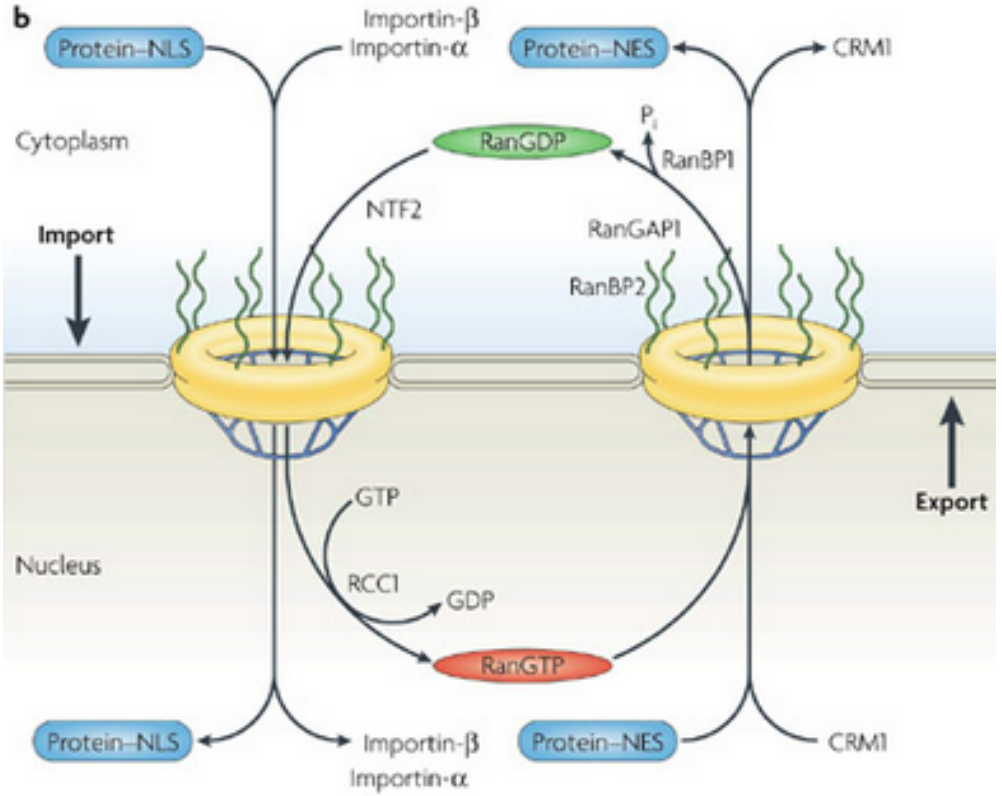
- **Importins (α and β)**

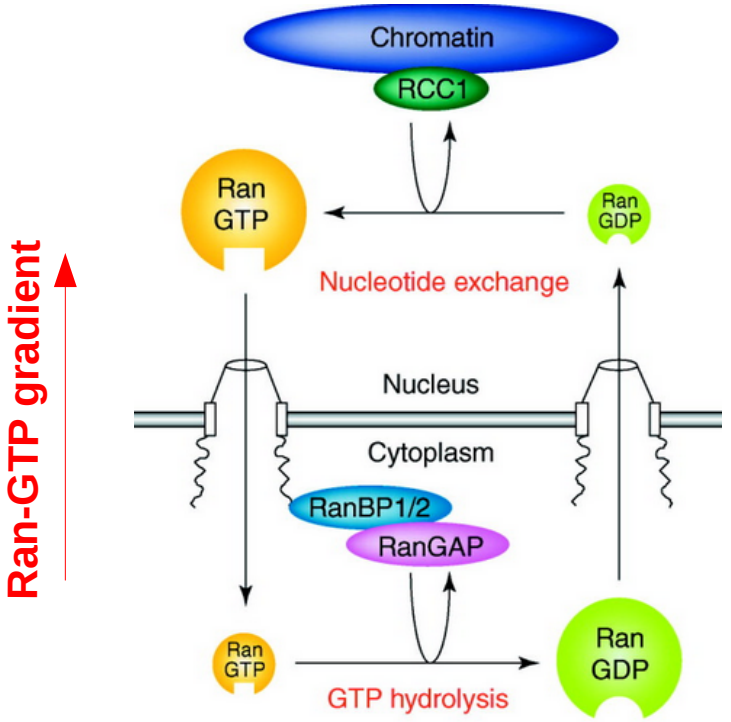
- **The small GTPase Ran**

- **RCC1, the Ran GTP/GDP exchange factor**

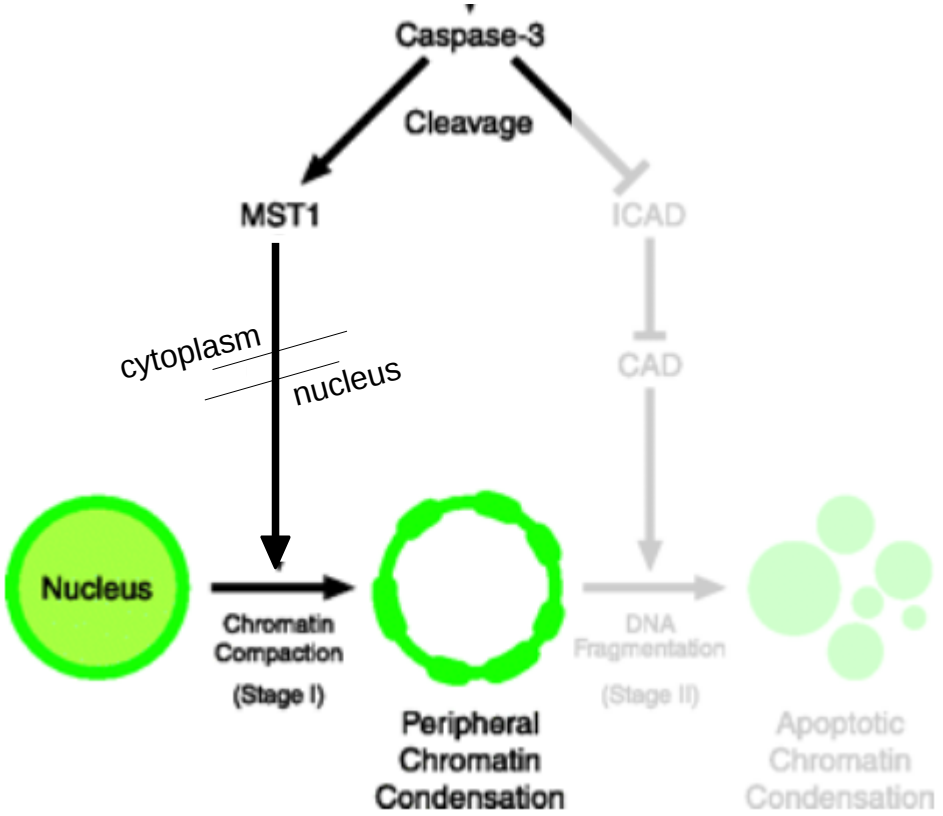
RCC1 is tightly associated to chromatin (histone H2A and **H2B**).

Histone 2B (H2B) phosphorylation status regulates RCC1 activity, and the RanGTP gradient as well.

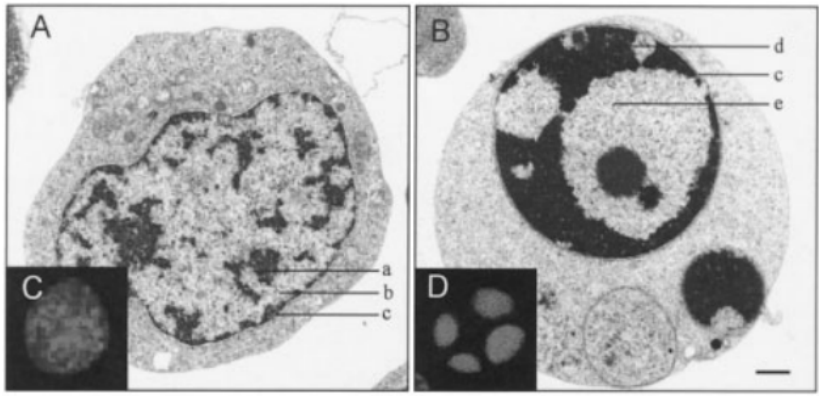




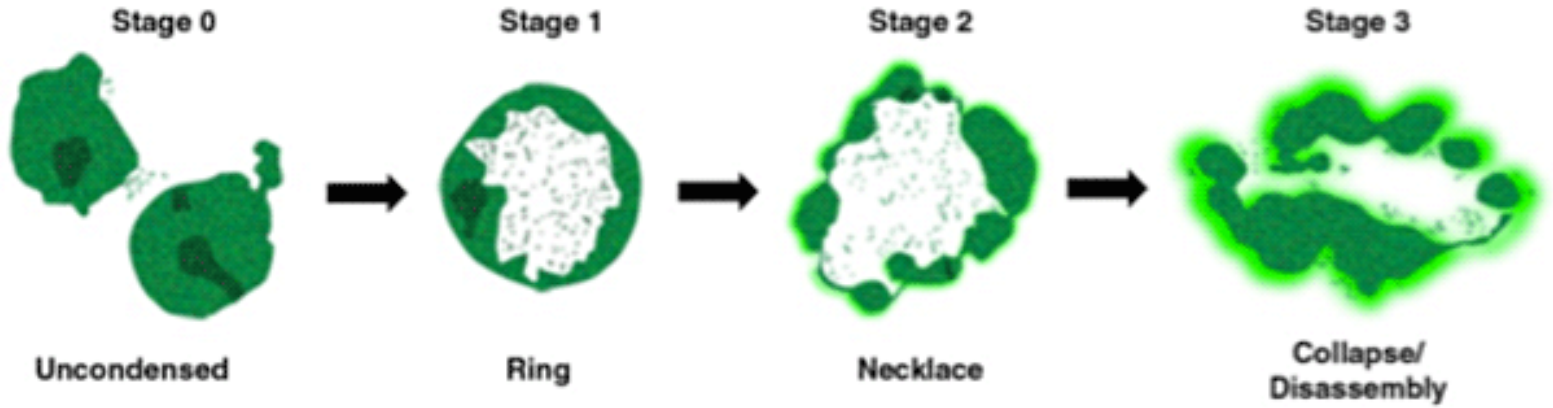
Stage 1: cleaved MST1 drives chromatin condensation



Healthy nucleus Apoptotic nucleus



Cleavage of MST1 by caspases drives its nuclear localization.
Once in the nucleus, MST1 phosphorylates **H2B** histone (Ser14). *p*-H2B in turn reduces the activity of RCC1, impairing the RanGTP gradient and the overall protein import. **Including NFκB**.

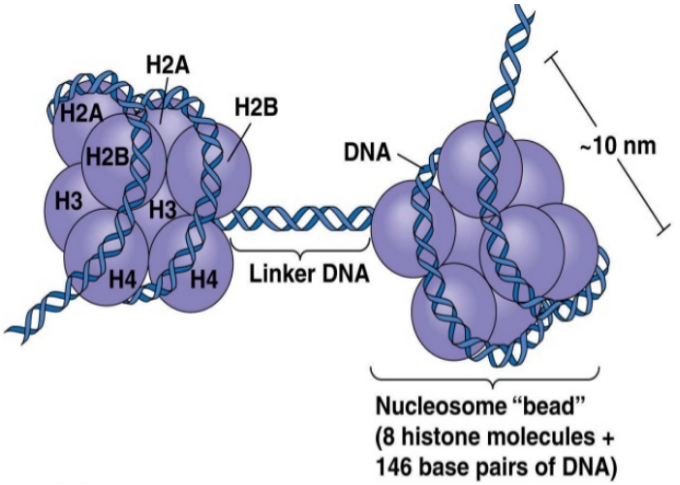


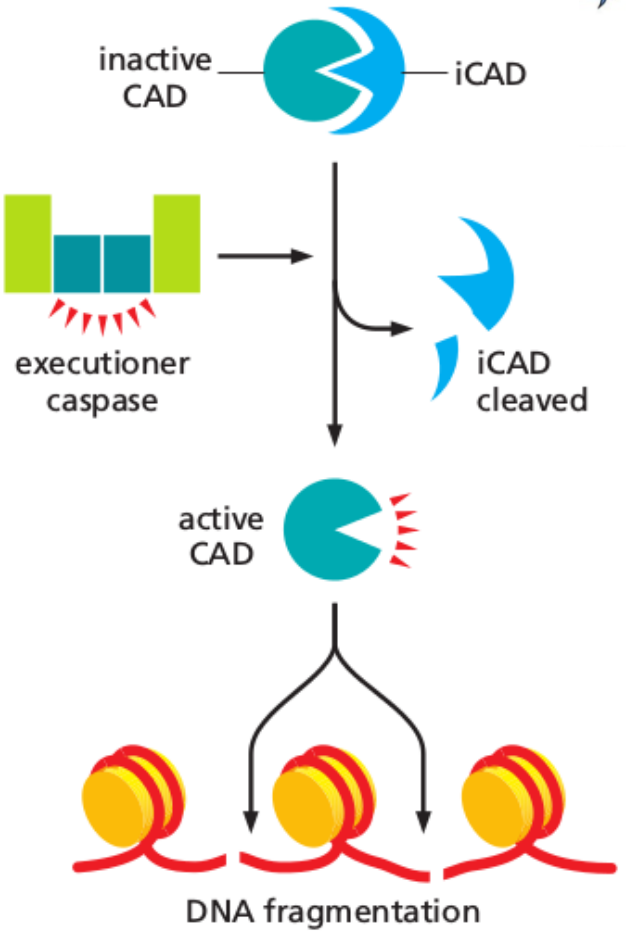
Stage 2: CAD mediates DNA fragmentation

- The endonuclease CAD (Caspase-Activated DNase) is normally inhibited by its partner iCAD.

- Executioner caspases (e.g. caspase 3) cut and inactivate iCAD, so that CAD is activated.

- CAD cuts DNA in histone free DNA (linker regions), producing fragments with quite regular sizes (nucleosome units).





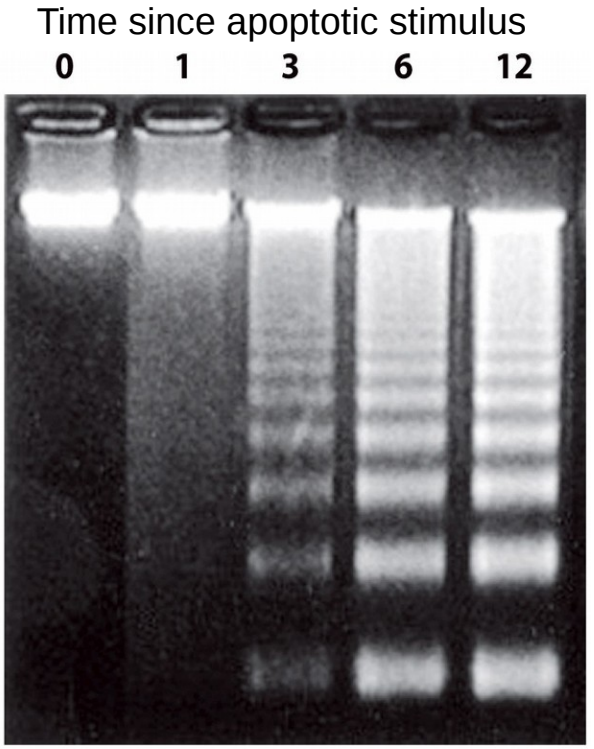


Figure 18-4a Molecular Biology of the Cell 5/e (© Garland Science 2008)

CAD is also known as DFFB (DNA fragmentation factor subunit beta) or DFF40.

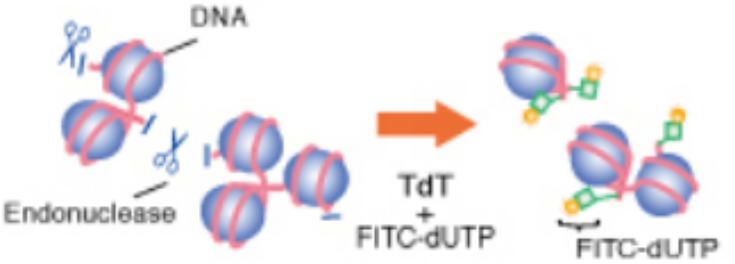
iCAD is also known as DFFA (DNA fragmentation factor subunit beta) or DFF45.

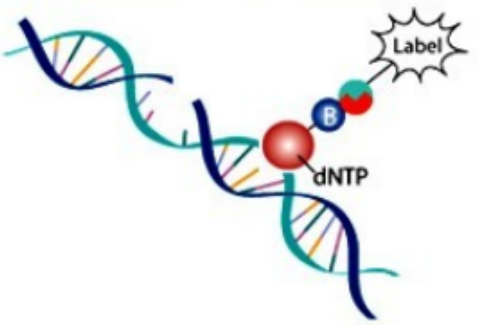
Measuring DNA fragmentation: the Tunel assay

DNA fragmentation in apoptosis produce nicked DNA.

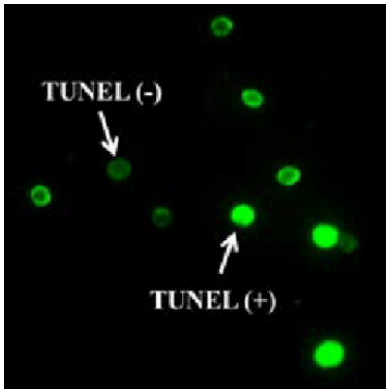
The enzyme Terminal deoxynucleotidyl Transferase (TdT) adds labeled deoxynucleotide (usually FITC-dUTP) to the 3′-OH ends of DNA fragments.

The presence of large numbers of DNA fragments therefore results in bright fluorescent dots in apoptotic cells.

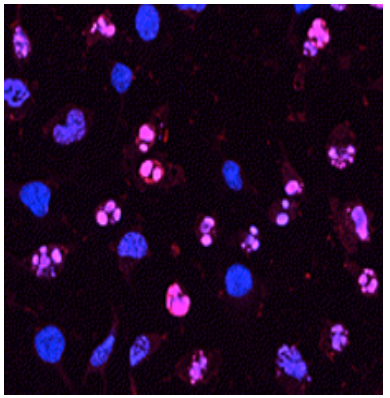




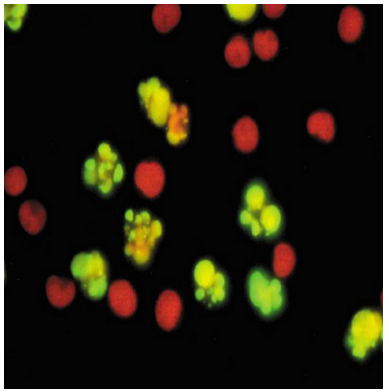
Single Tunel



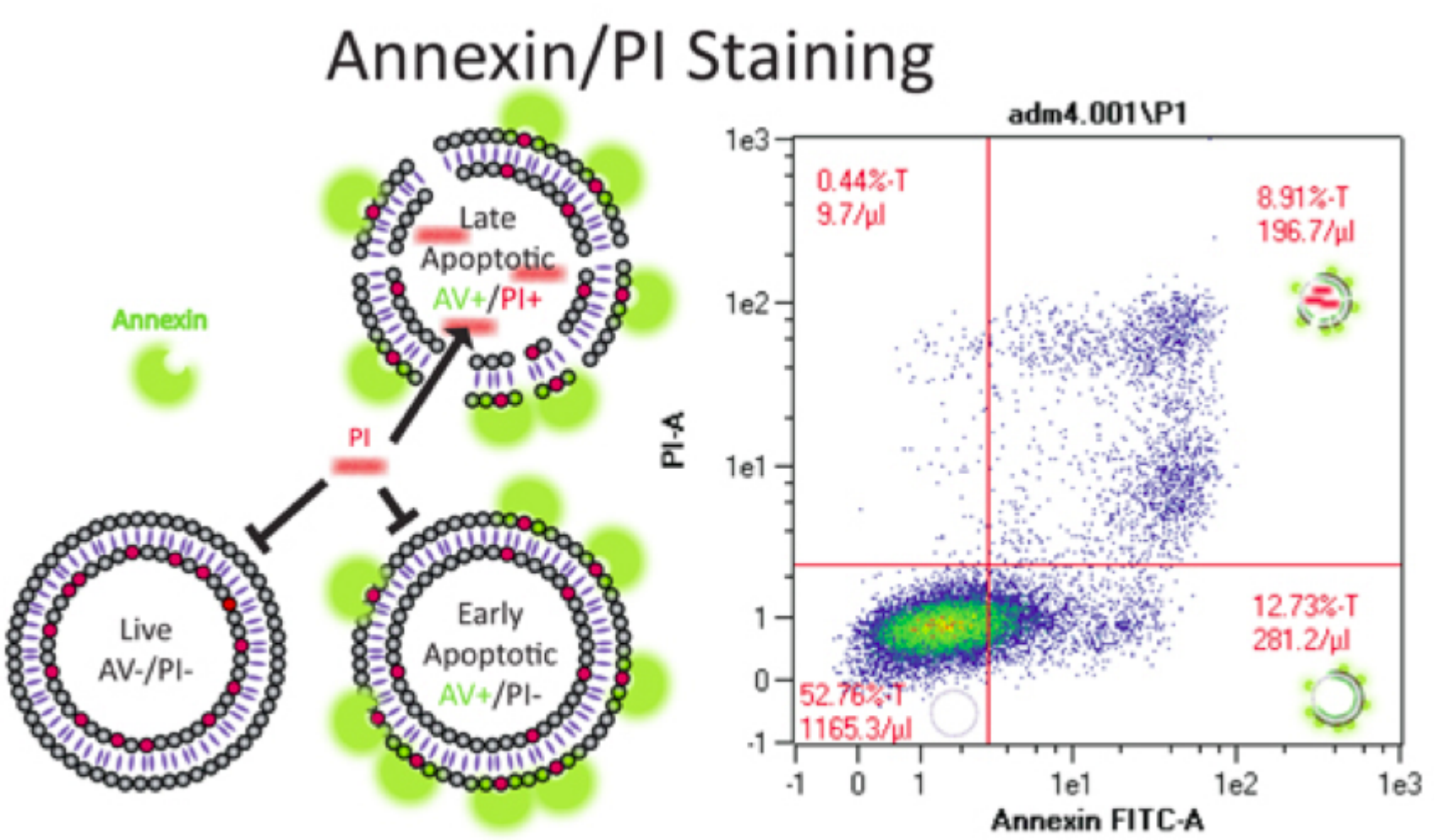
Co-stain with Hoechst 33342



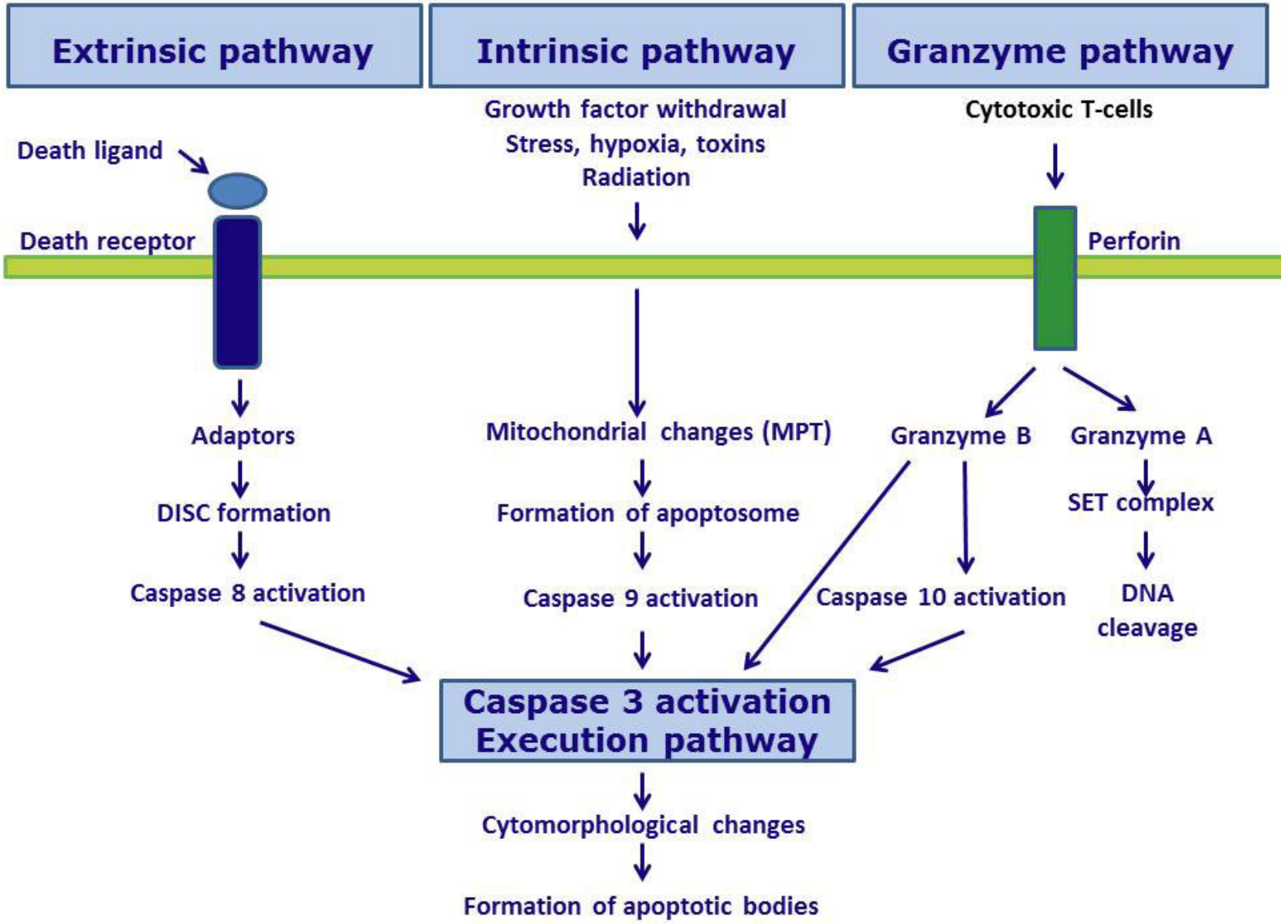
Co-stain with Propidium iodide



Annexin V / Propidium iodide in cytofluorimetry



Apoptosis can be triggered in different ways

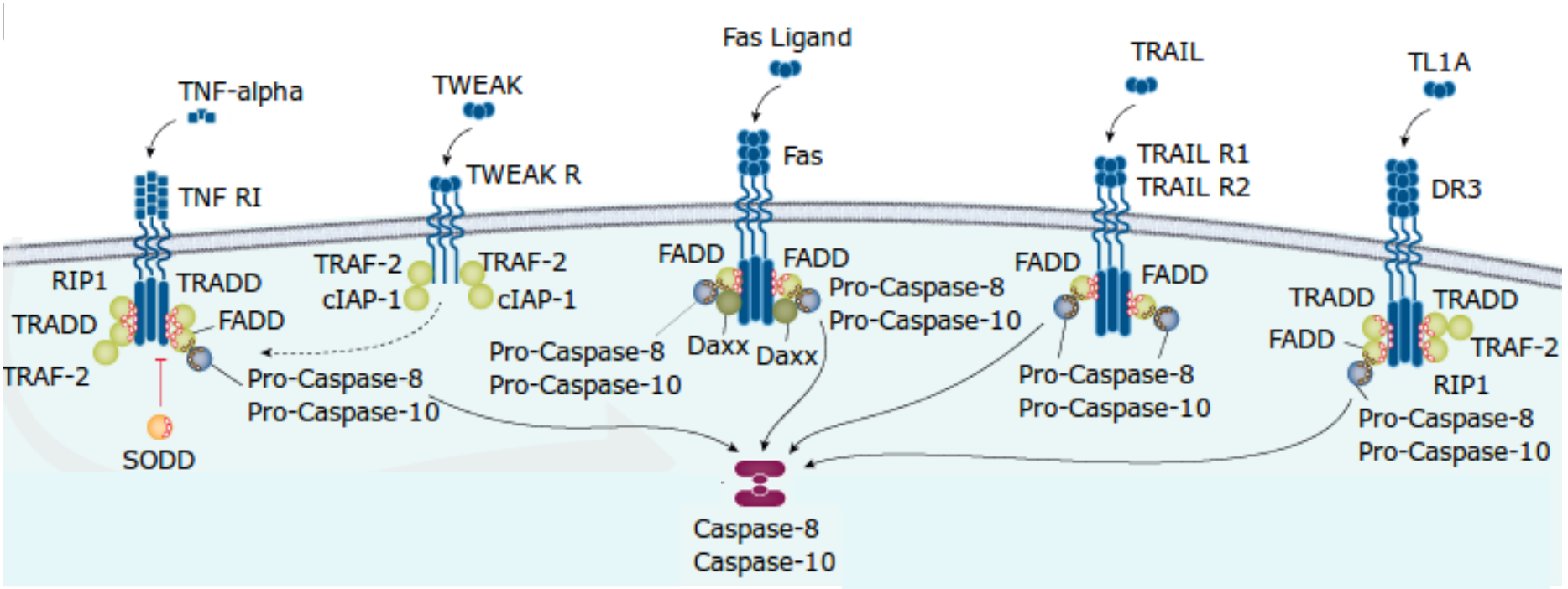


Extrinsic pathway: death receptors

Death receptors are trimers containing **2-4 cysteine-rich repeats** in their extracellular domain, required for ligand binding and an intracellular **death domain** capable of recruiting **specific adaptors** that define their downstream signals.

In all cases, the signals converge into the activation of Caspase 8 and/or 10.

Subsequently, effector caspases (e.g. caspase 3) are activated and this execution pathway is shared by all activation methods.



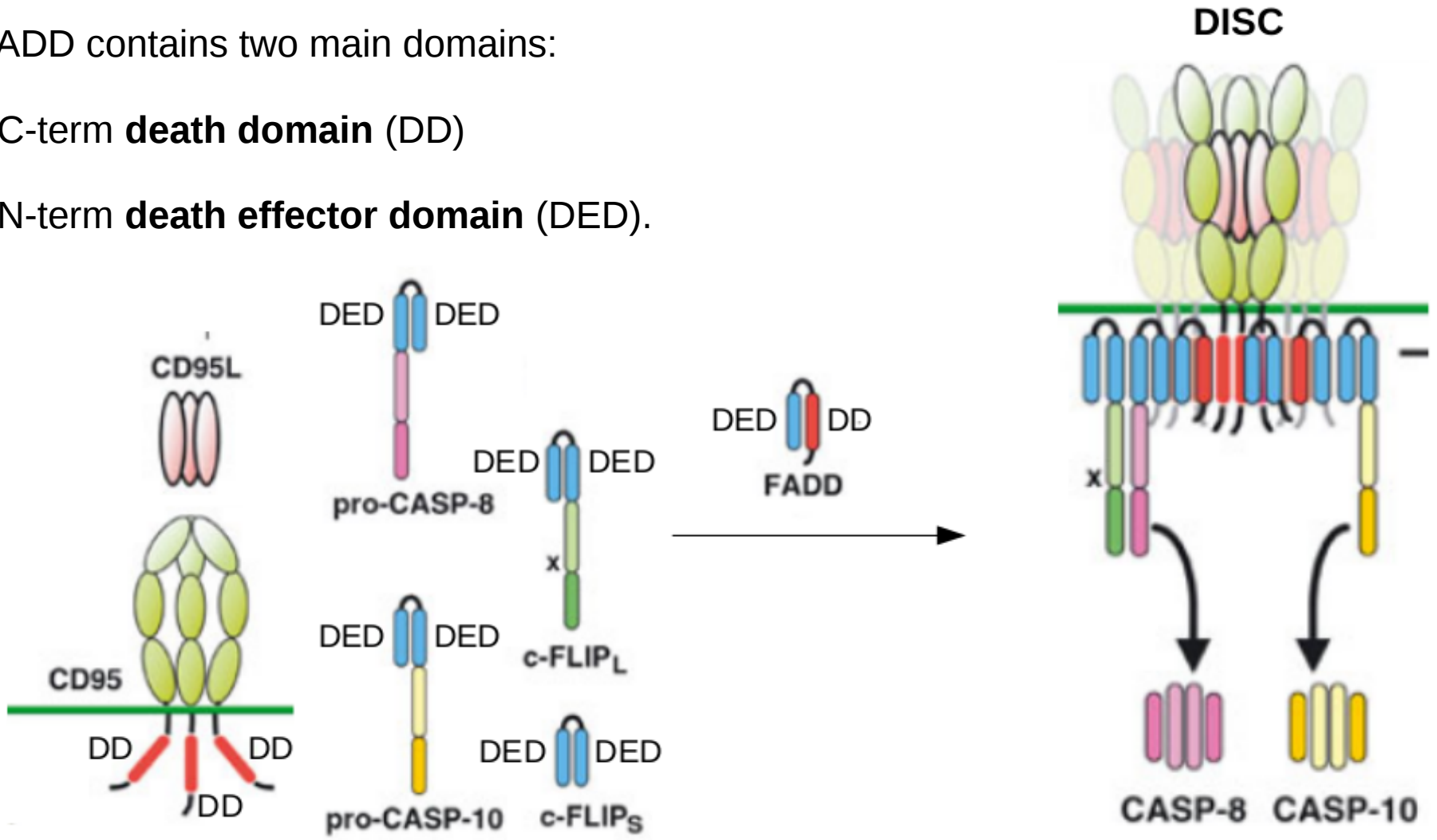
Bridging receptors and caspases: FADD and the DISC

Fas-Associated protein with Death Domain (FADD, a.k.a. MORT1) is an adaptor protein that bridges members of the tumor necrosis factor receptor superfamily, such as the Fas-receptor, to pro-caspases 8 and 10 to form the Death-Inducing Signaling Complex (DISC) during apoptosis.

FADD contains two main domains:

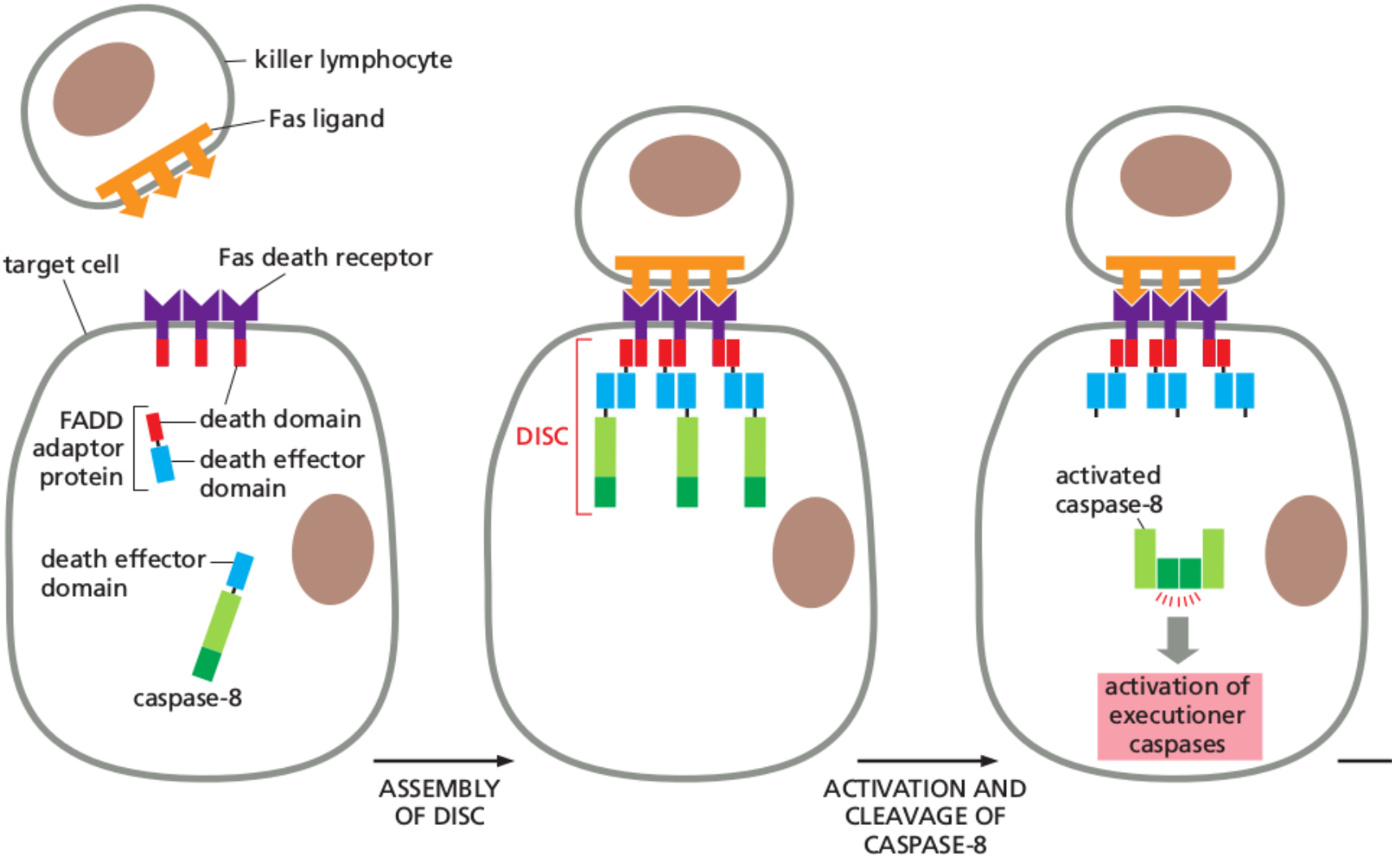
- C-term **death domain** (DD)

- N-term **death effector domain** (DED).



Extrinsic pathway 1: FAS ligand signaling

FAS ligand is expressed on the surface of killer limphocytes (CD8). Once a cell has been targeted for elimination, a cell-cell contact is established via FASL/FAS binding. This allows 1. the DISC to form, 2. the initiator caspases 8 to activate and in turn 3. downstream executioner caspases to activate.



Extrinsic pathway 2: TNF related signaling

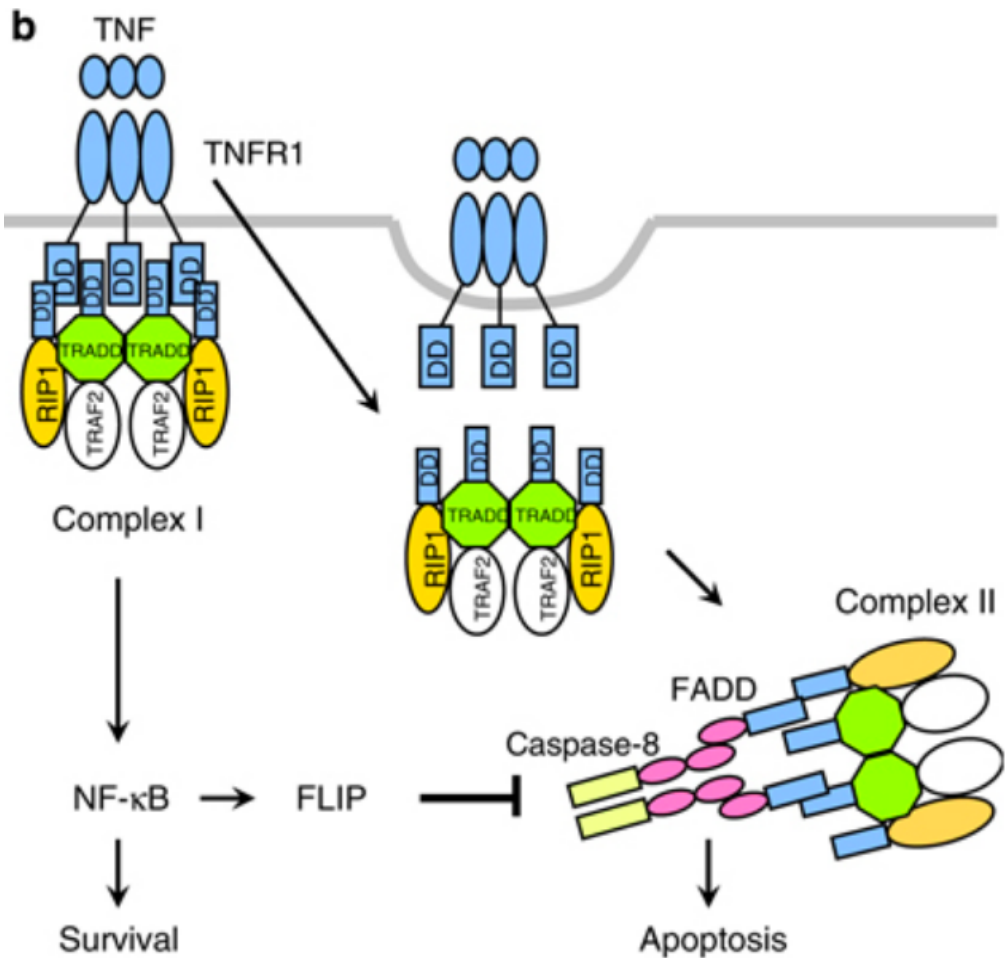
The apoptotic stimulus by TNFR1 is more complex because it is actually a balanced signalling.

The adaptor protein TRADD (only DD domains), bridges the association of TRAF2 and RIP (complex I). This complex rapidly activates NF-kB and enhance survival.

In a second stage, complex I dissociate from receptor and associate to FADD and caspase-8 (Complex II).

This could trigger apoptosis, but the FLIP protein (similar to CAS, but inactive) binds the complex and inhibit it.

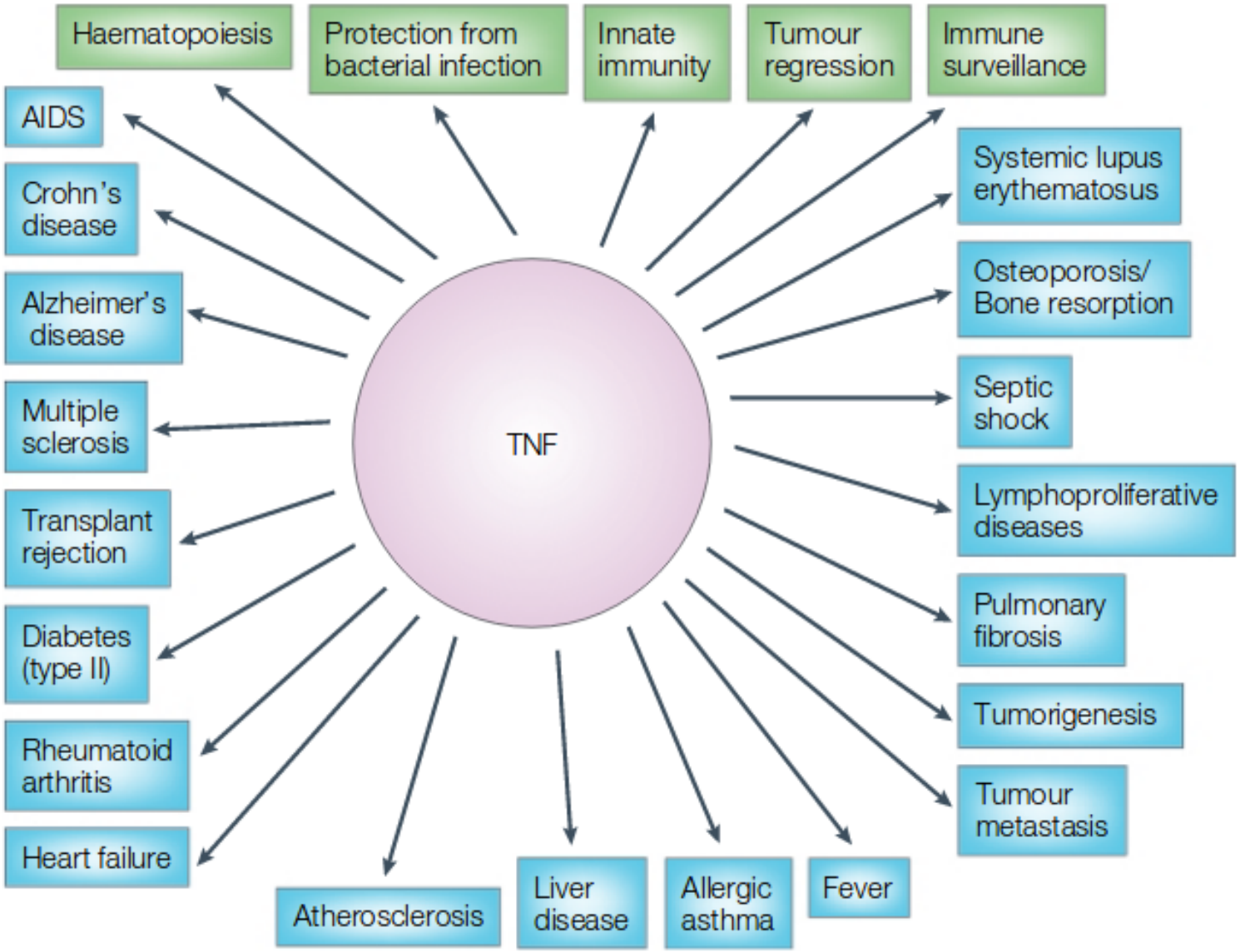
If survival signal is ineffective (**NF-kB** signaling), FLIP decreases and apoptosis can be stimulated.



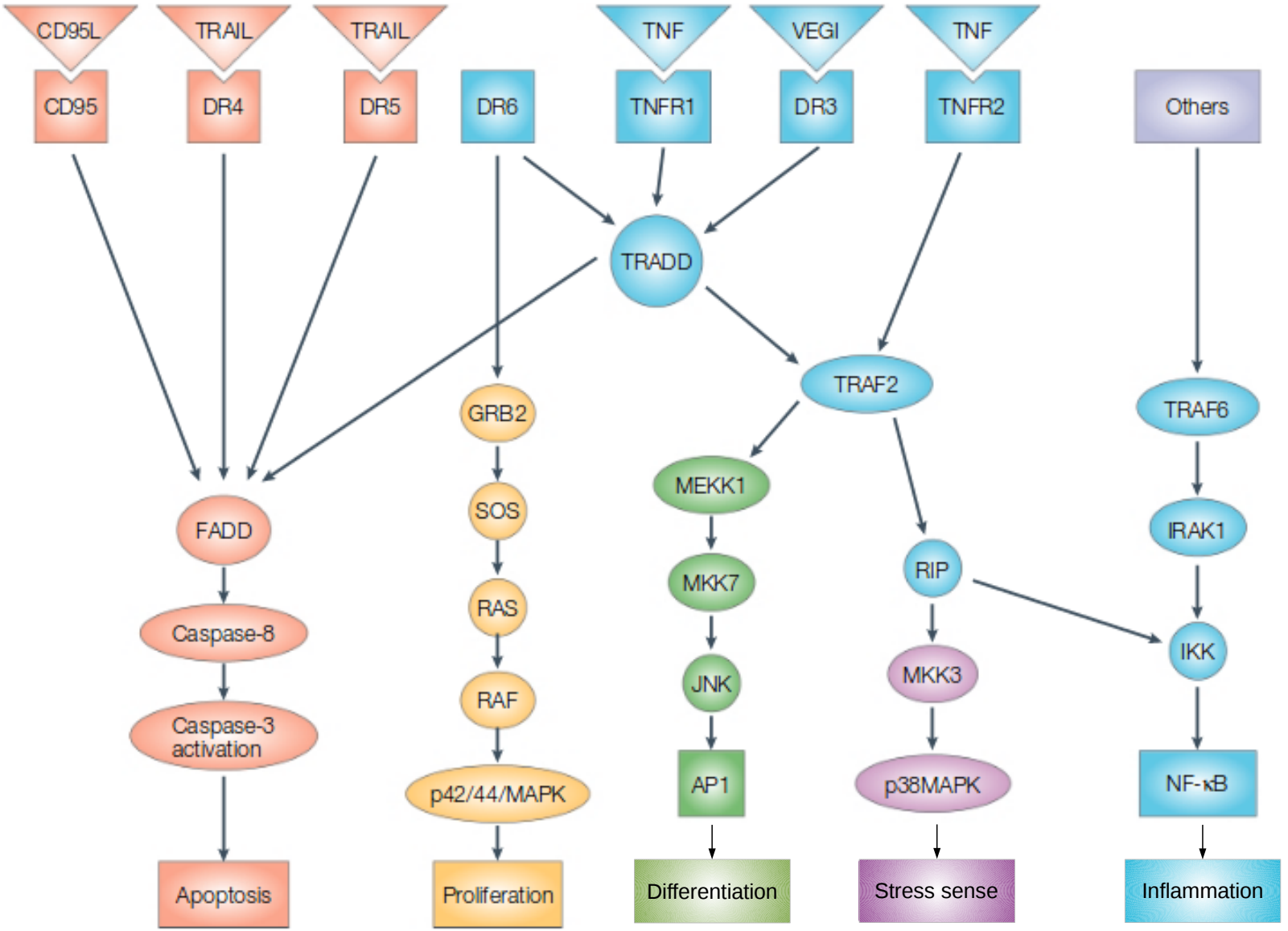
=> apoptosis is subjected to a survival-associated checkpoint

Sedgera and McDermott 2014 Cytokine Growth Factor Rev 25(4):453-472

Roles of Tumor Necrosis Factor (TNF) members



Pathways of TNF have several different fates



Aggarwal 2003 Nat Rev Immunol 3(9):745-756

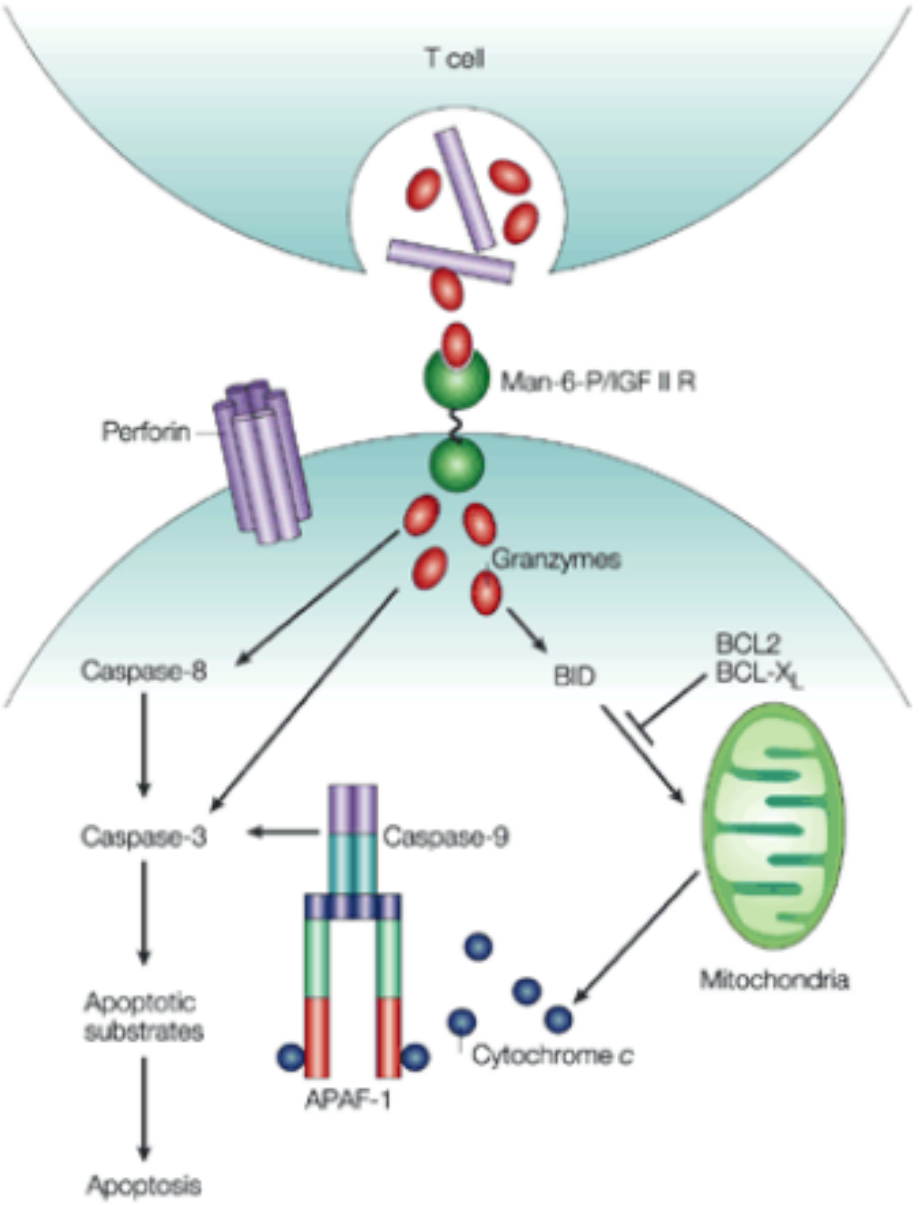
Extrinsic pathway 3: perforin/granzyme

This pathway arises from cytotoxic lymphocytes and natural killers to clear virus-infected or transformed cells.

Granzyme B (GrB) is the most abundant serine protease which is stored in secretory granules of CTLs and NK cells

Similar to caspases, GrB has a preference for cleaving peptide bonds immediately adjacent to Asp residues in several hundreds of potential substrates, among which:

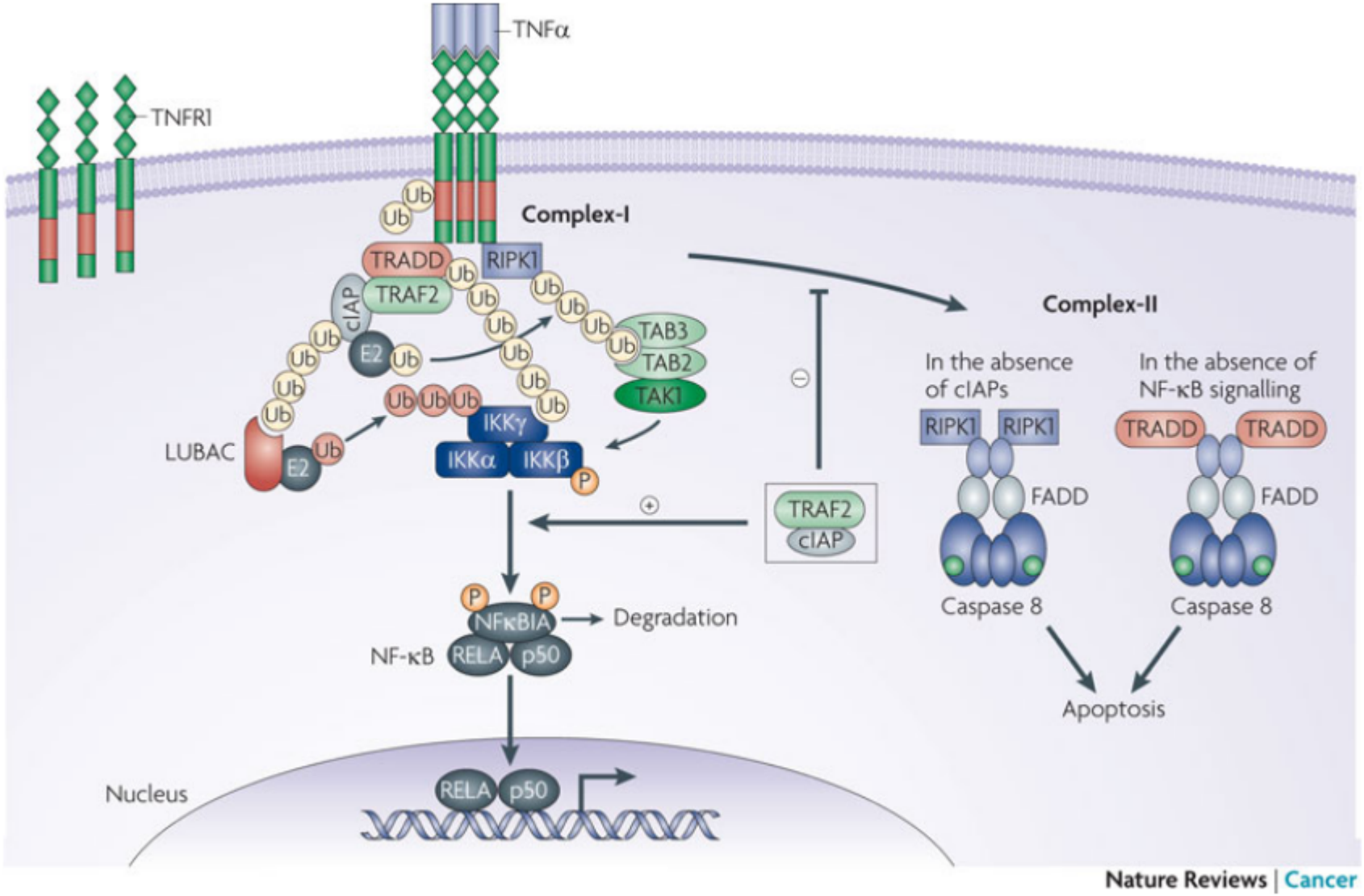
- Effector procaspase-3 and -7
- Bid, that promotes mitochondrial membrane permeabilization
- Lamin B, that scaffold nuclear membrane and impair nuclear membrane
- iCAD, after nuclear translocation, leading to DNA laddering



Rousalova and Krepela 2010 Int J Oncol.37(6):1361-78

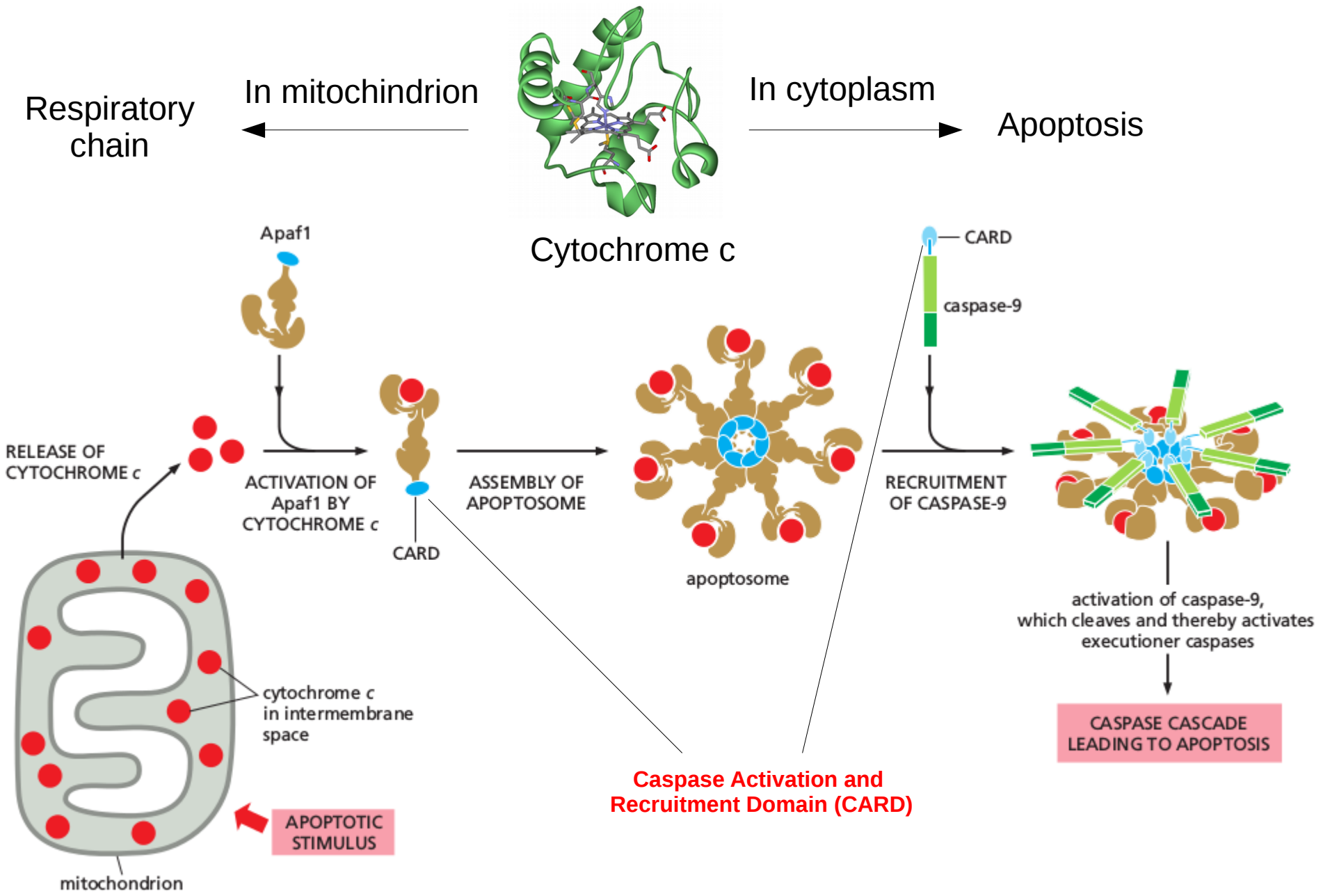
Inhibition of apoptosis

Since it is risky to start an apoptotic irreversible signal to the cells, the extrinsic pathways are provided with specific inhibitors termed IAPs



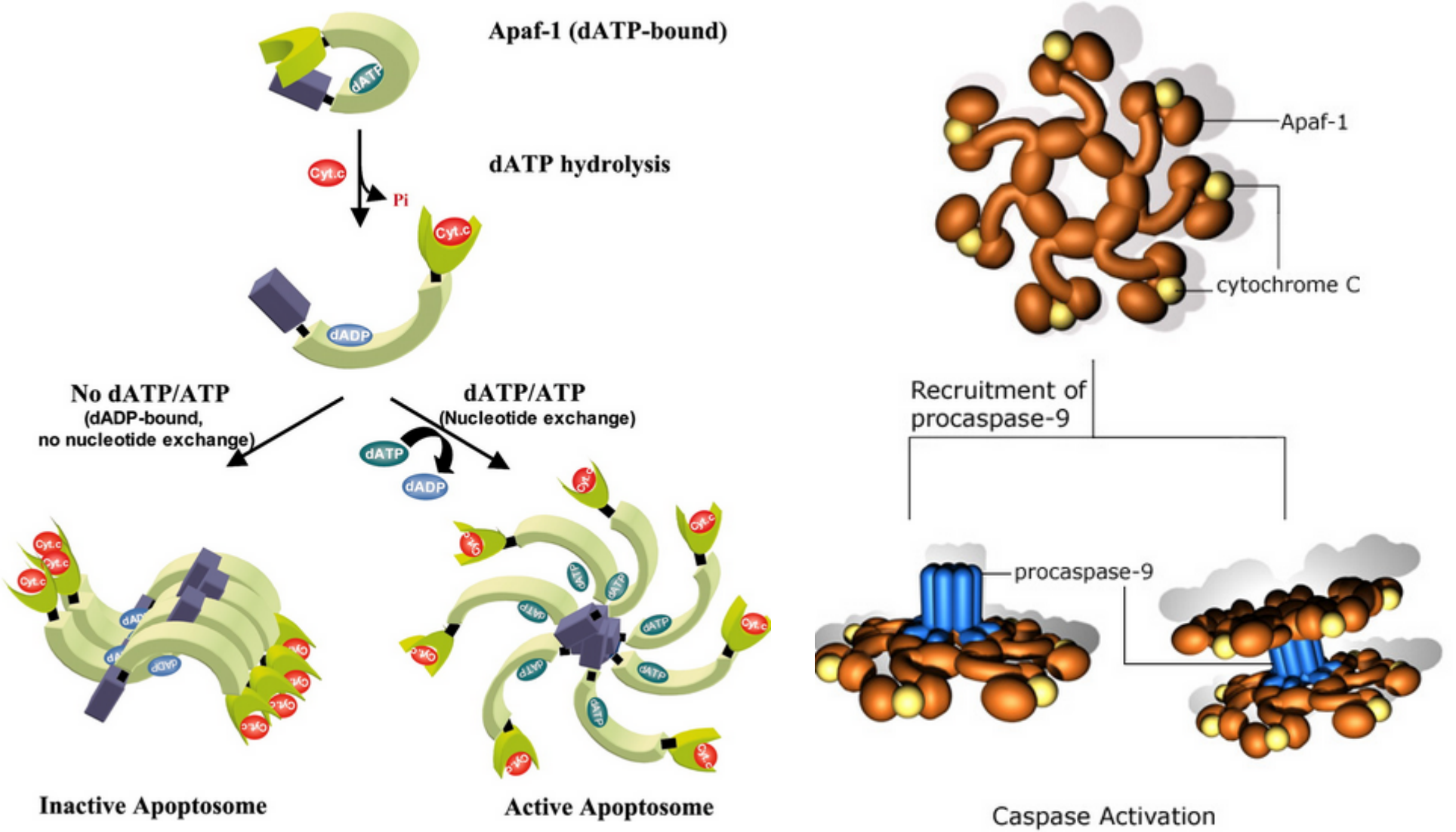
Gyrd-Hansen and Meier 2010 Nat Rev Cancer.10(8):561-74

Intrinsic pathway in vertebrates



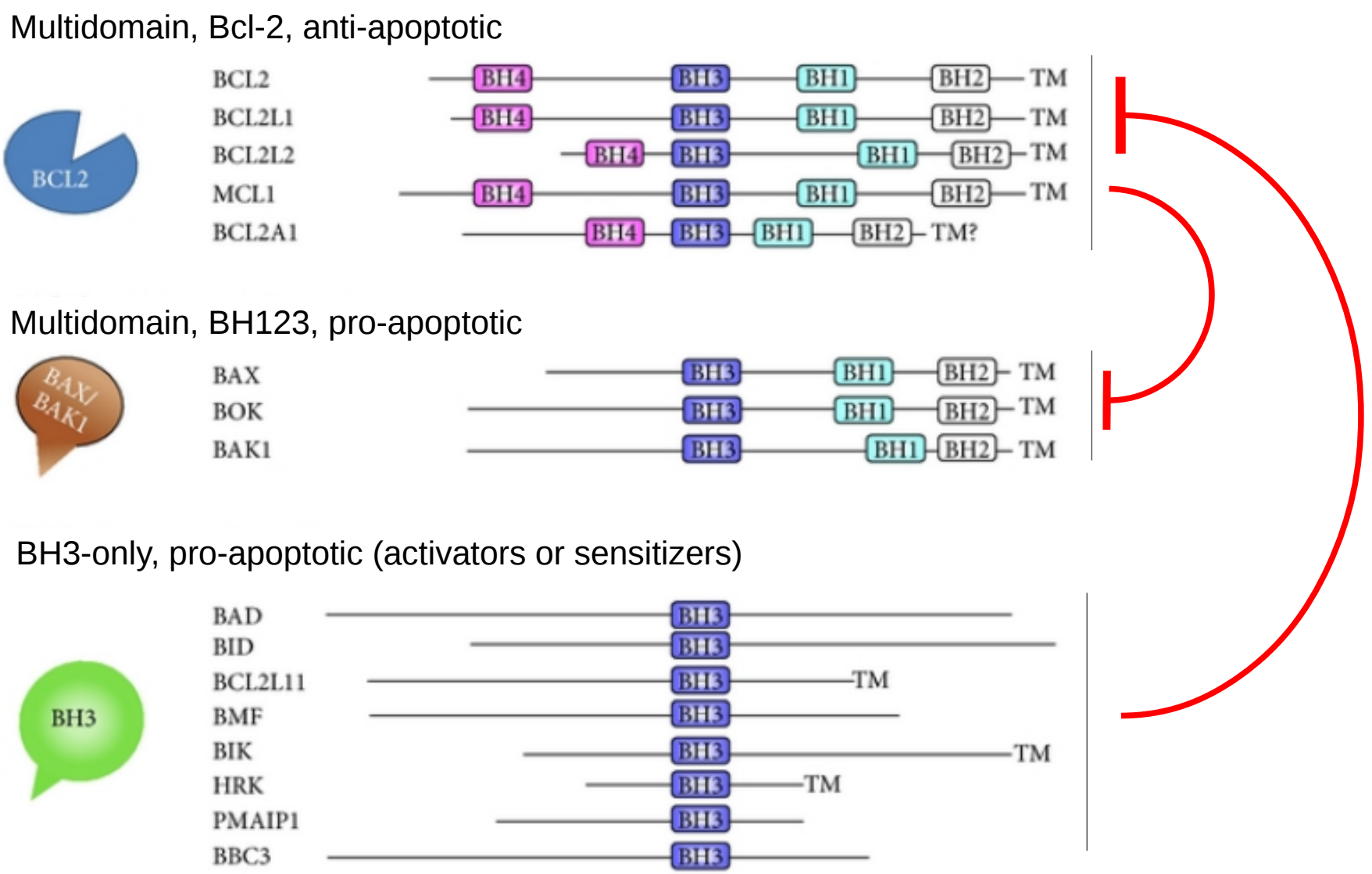
Intrinsic pathway: the apoptosome

Apaf-1 contains a domain that binds cytochrome c and a second domain that binds procaspase-9. Upon contact, procaspase-9 is cleaved by proximity with other procaspase-9, that trigger the caspase cascade.

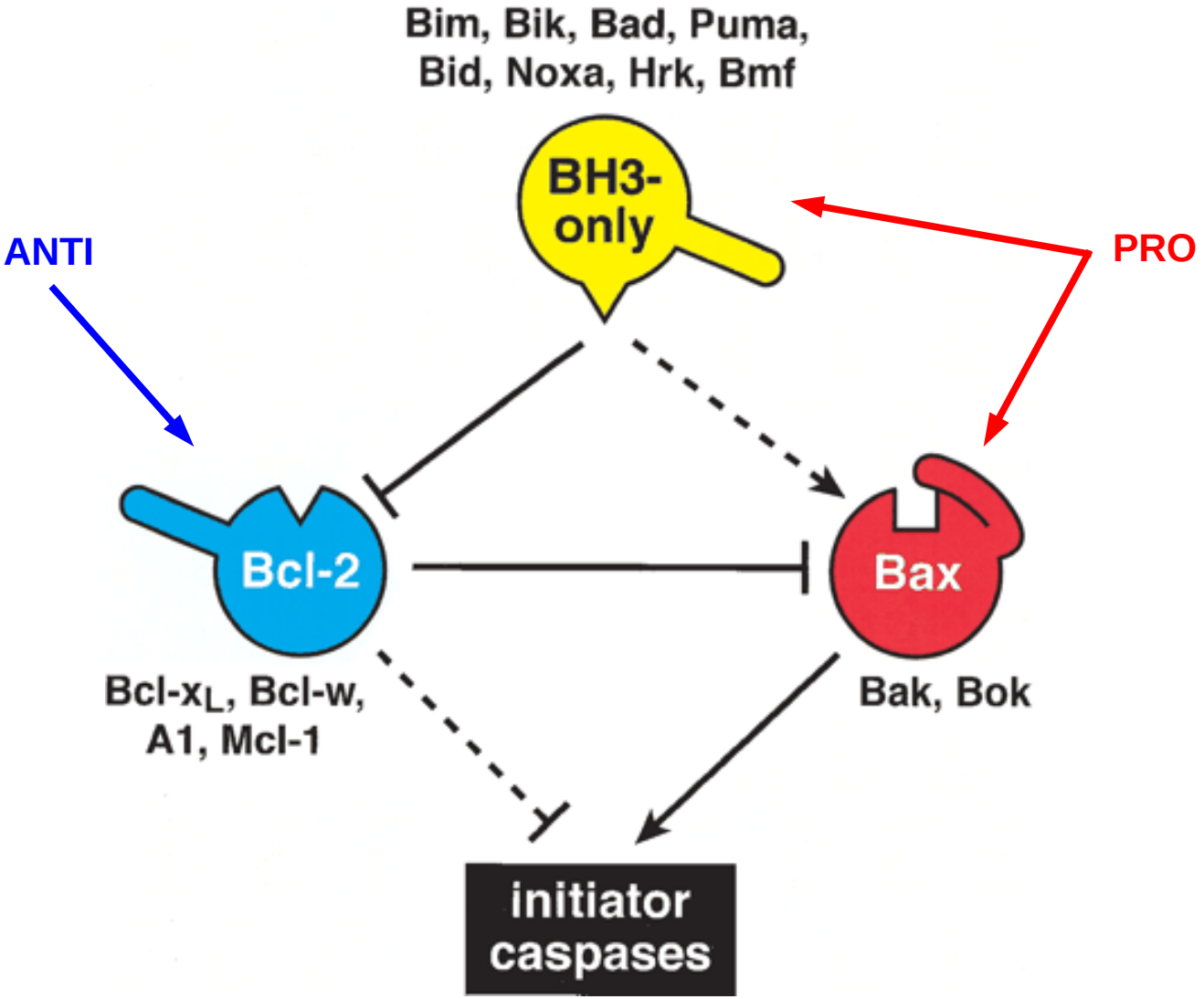


Bcl-2 family members: regulators of the intrinsic pathway

Proteins of the B-cell lymphoma-2 (Bcl-2) family share a variable number of Bcl-2 homology (BH) domains. Their functions depend on the nature and the number of the contained BH domains. In general, Bcl-2 inhibits BH123 and are inhibited by BH3-only.



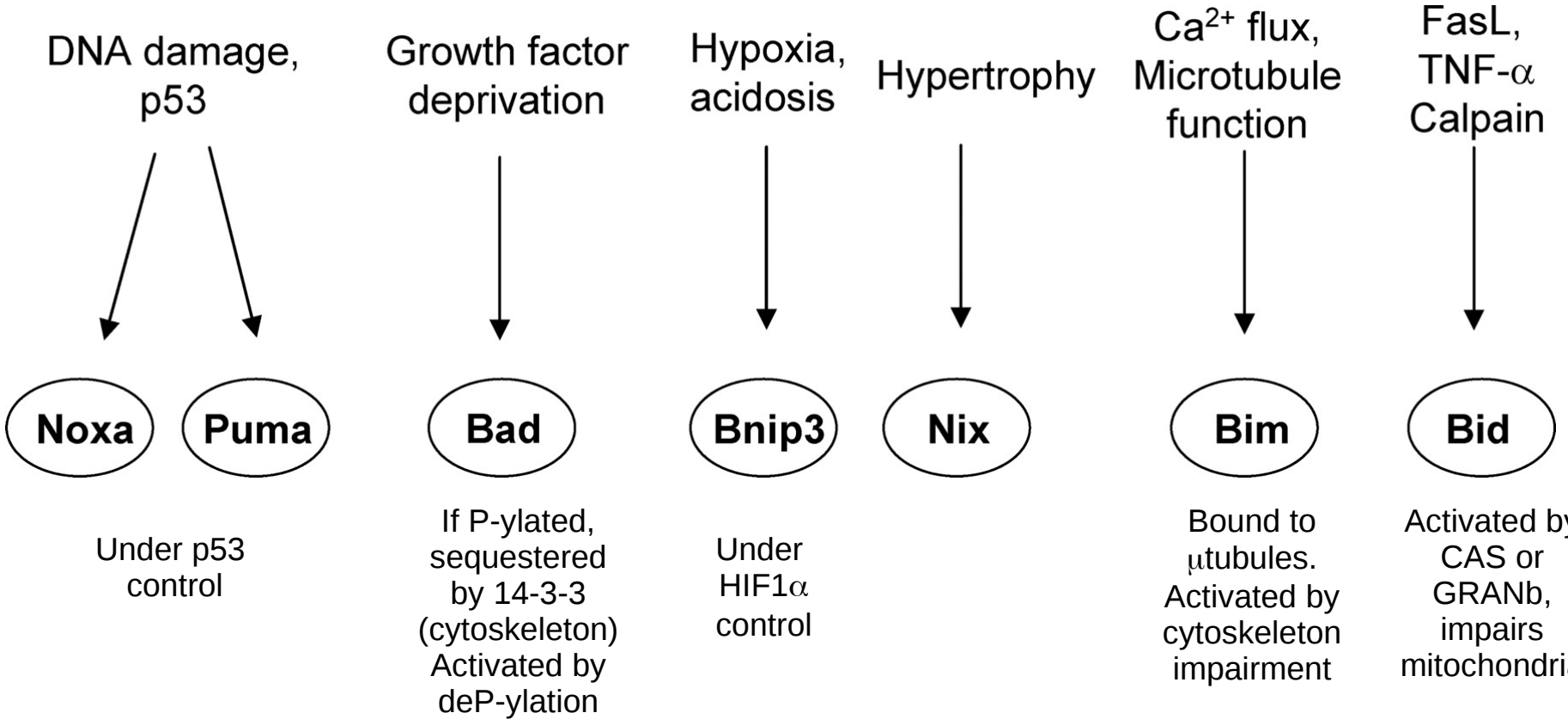
Regulation network among the Bcl-2 members



BH3-only are sensors for apoptotic stimuli

The BH3-only proteins function as death signal sensors in the cell and play a major role in transducing signals from the cytosol to the mitochondria. In mammals, ≥10 different BH3-only proteins, which differ in their expression pattern and mode of activation, have been identified.

Their pro-apoptotic activity is regulated by transcription and/or post-translational modification, and they selectively respond to specific death signals in the pathways they monitor



Gustafsson and Gottlieb 2007 Am J Physiol Cell Physiol. 292(1):C45-51

Activation of the intrinsic pathway

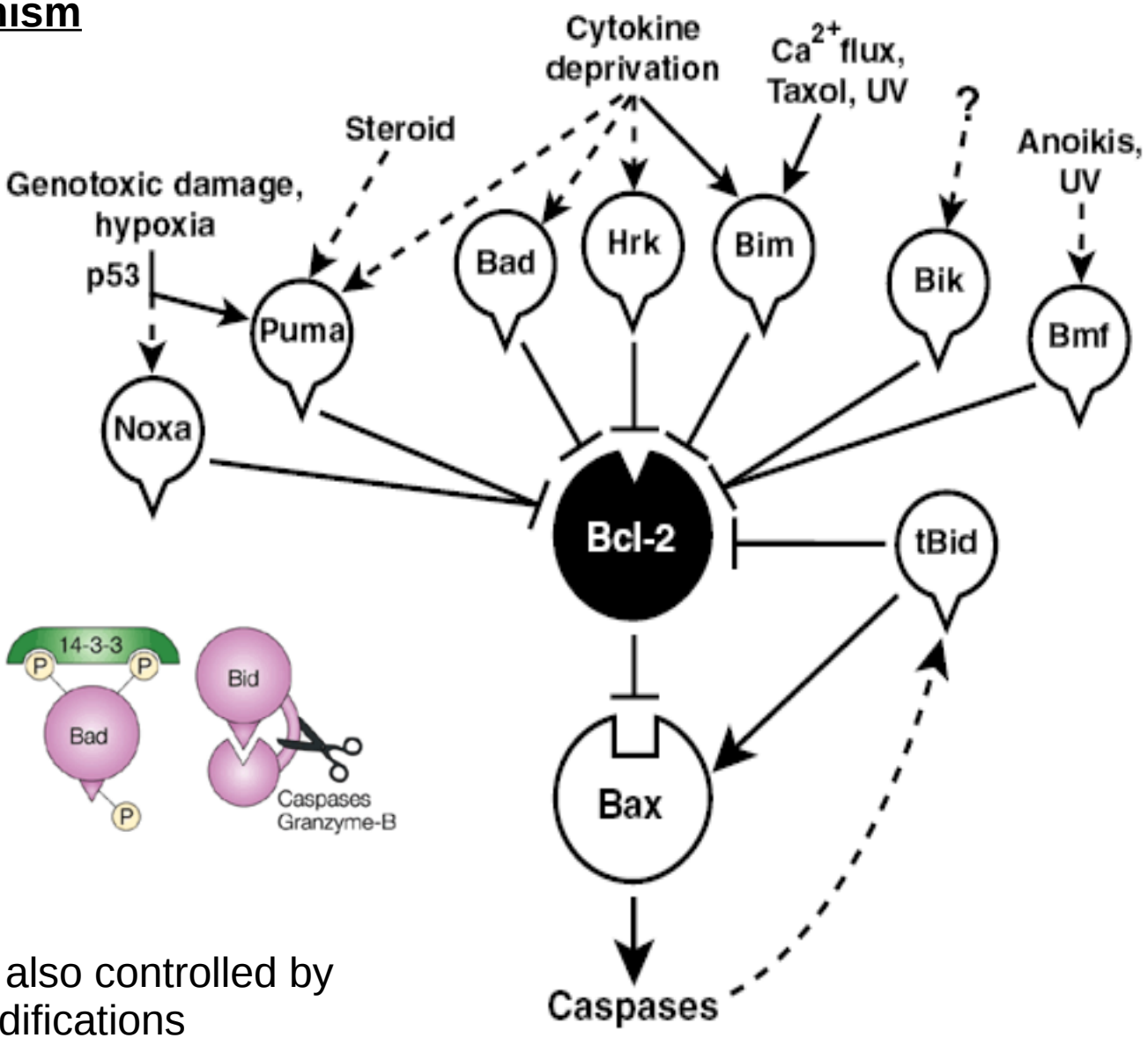
A double inhibition mechanism

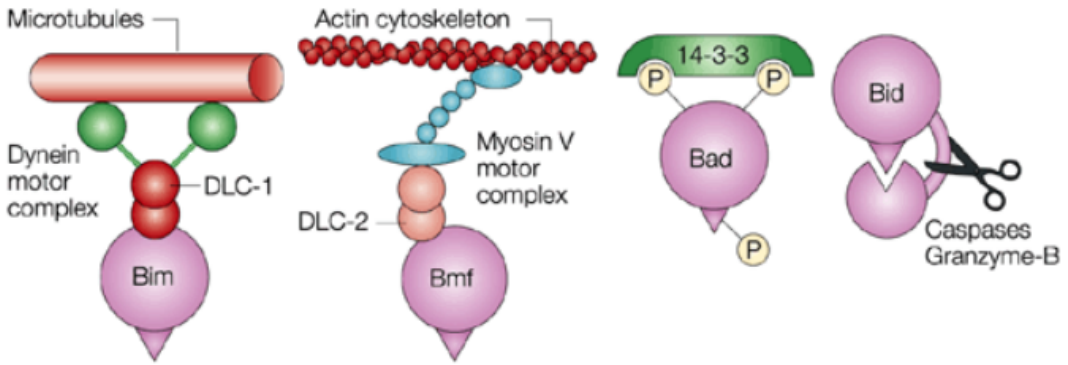
The control is:

- on controllers (BH3-only)

- of inhibitors (Bcl-2)

- of effectors (BH123)





Many BH3-only proteins are also controlled by post-translational modifications

Activation of the intrinsic pathway

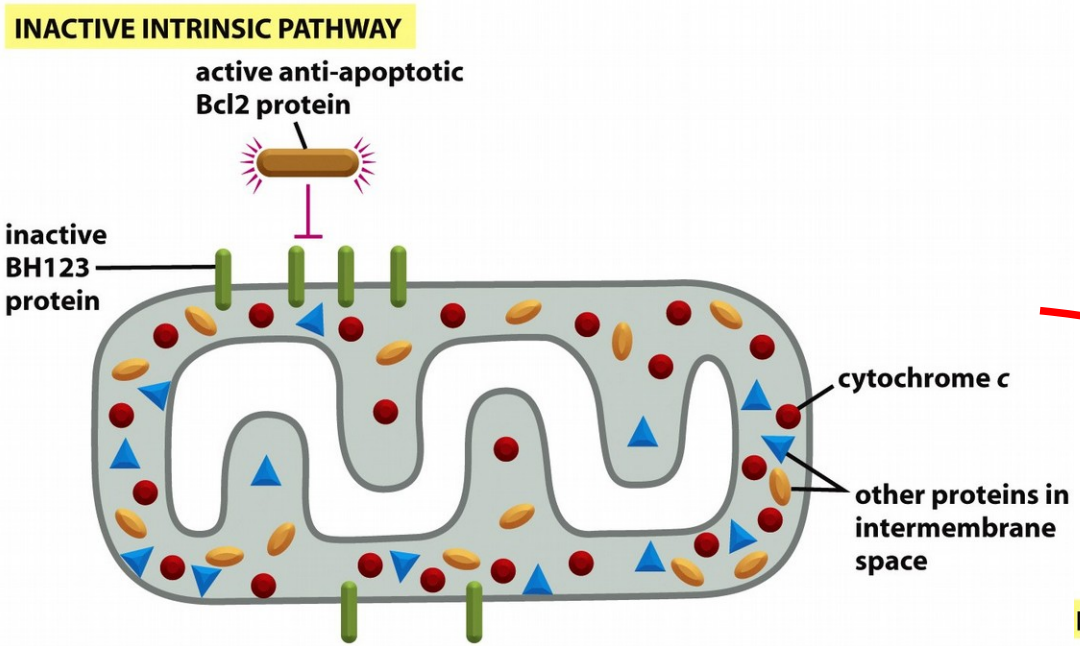


Figure 18-11a Molecular Biology of the Cell 5/e (© Garland Science 2008)

Activation by BH3-only proteins

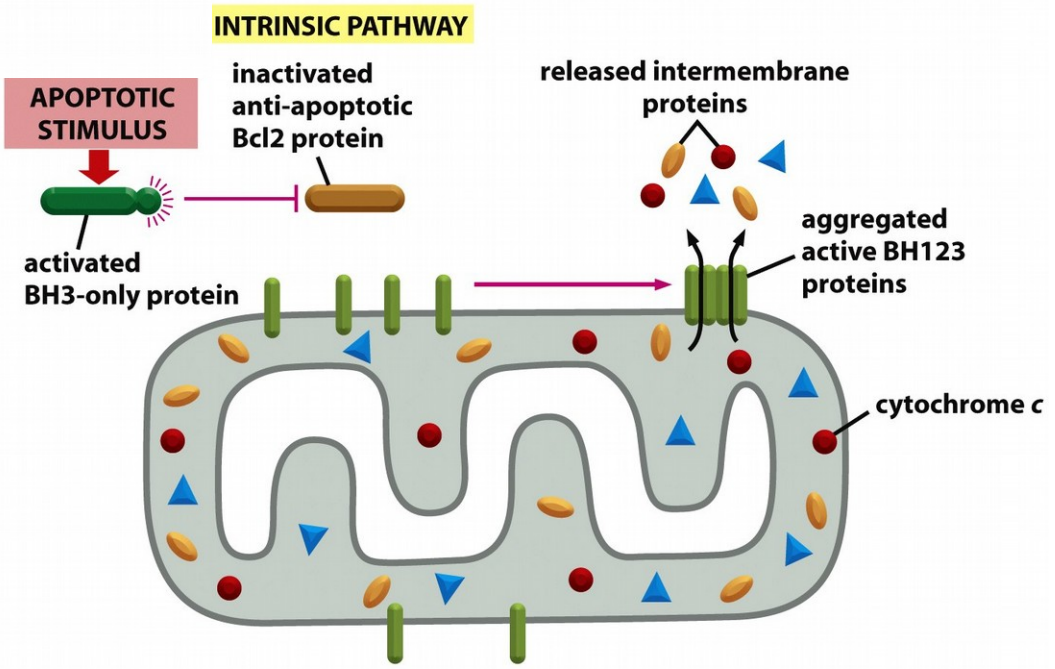
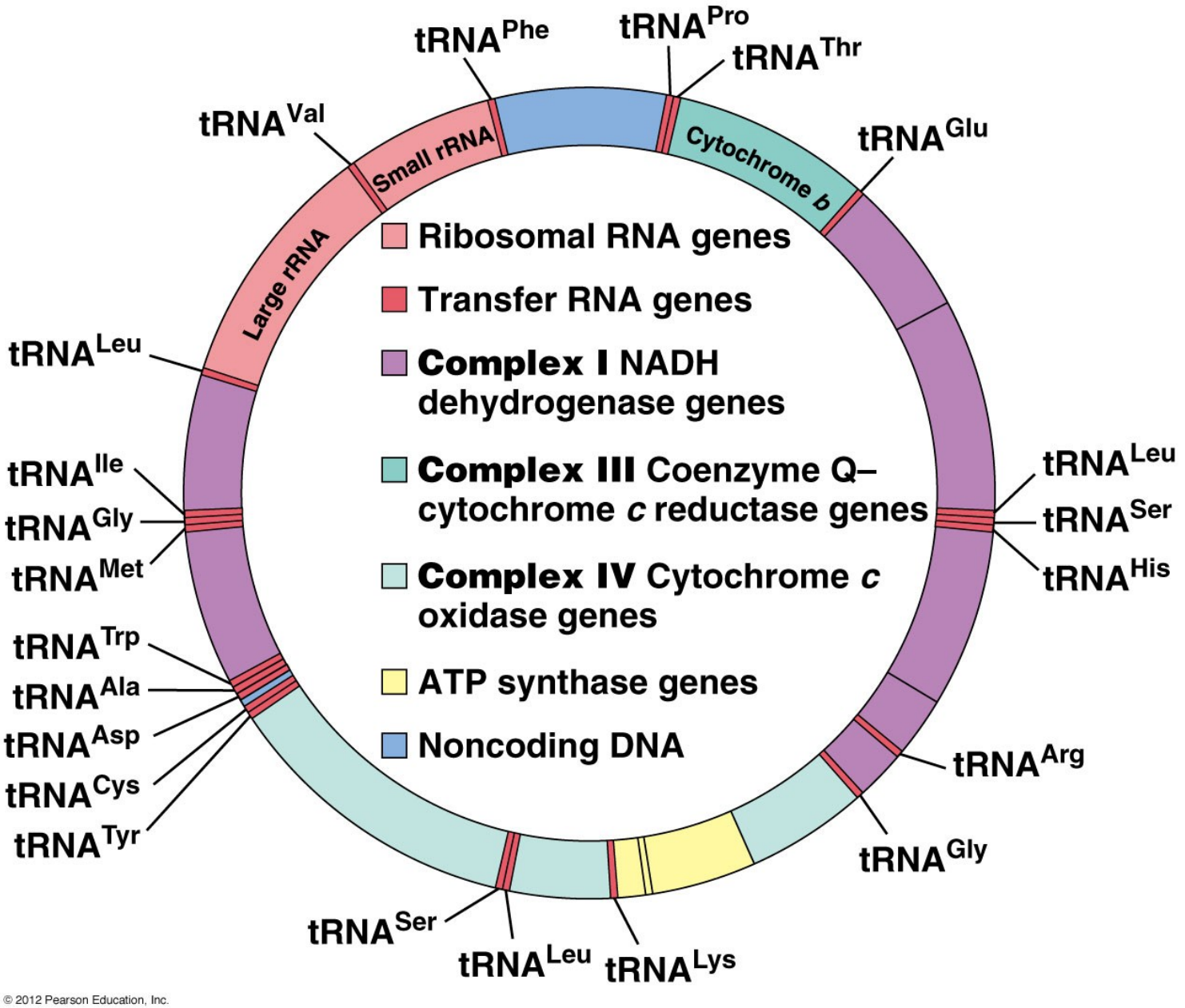


Figure 18-11b Molecular Biology of the Cell 5/e (© Garland Science 2008)

Step back: the mitochondrial genome



Step back: protein traffic to mitochondria

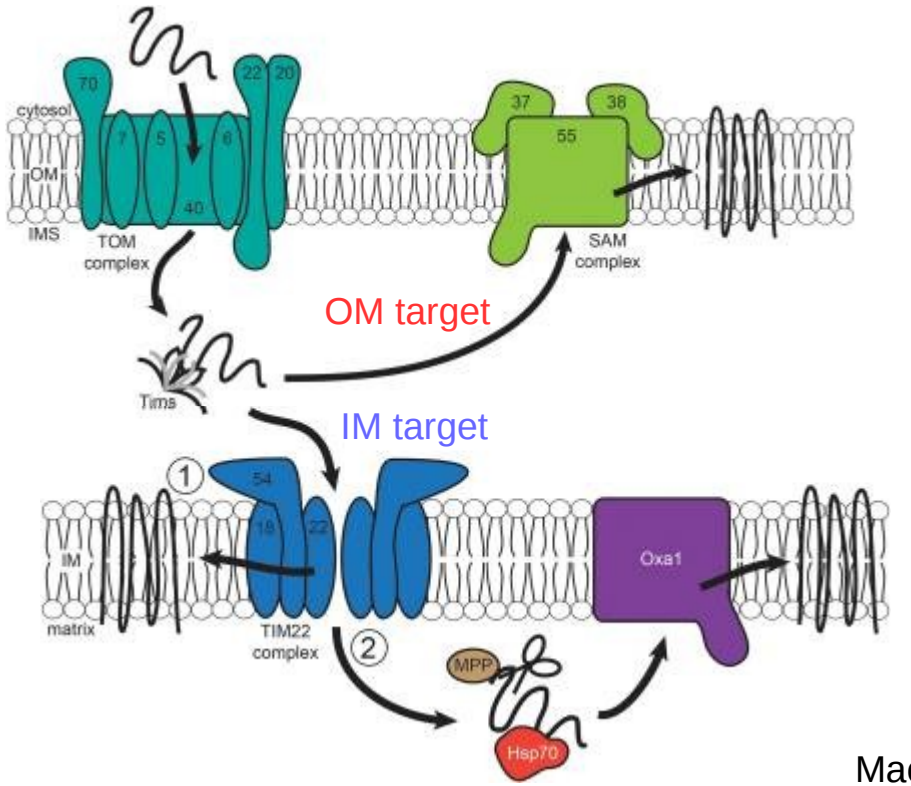
Most mitochondria proteins are encoded cytosolically (from the genome) and have signal sequences so that chaperones (Hsp70,Hsp90) drives them to TOM (translocase of the outer membrane) complex: depending on their final destination, further processing are needed, by:

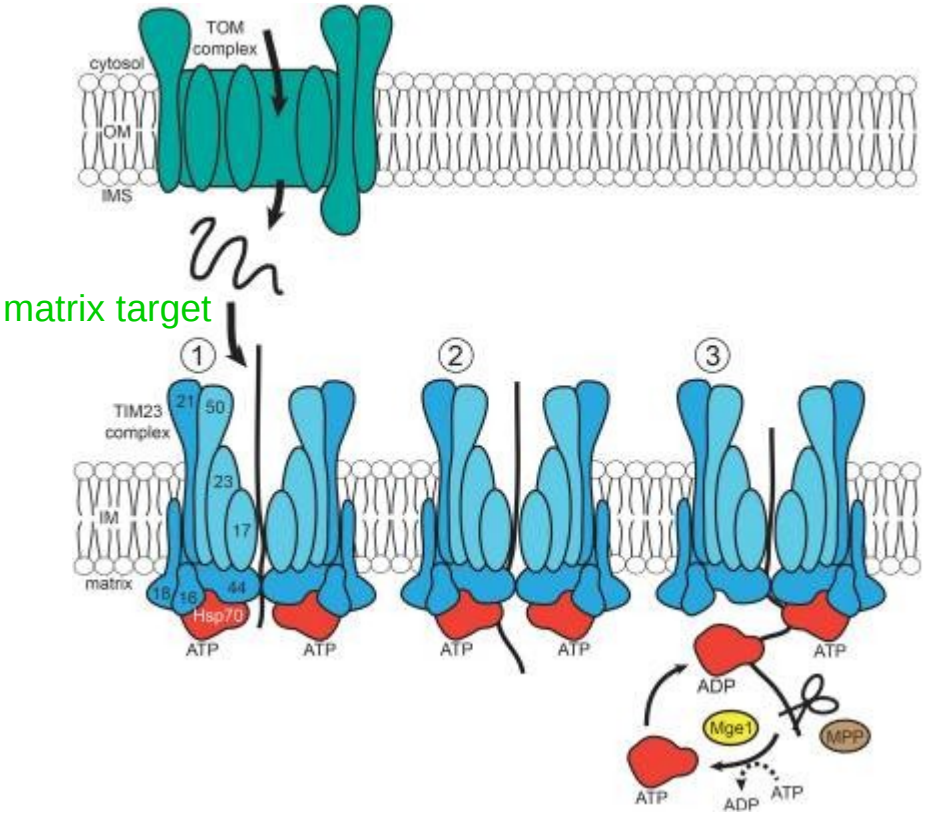
SAM: sorting and assembly machinery

TIM22: translocase of the inner membrane

Oxa1: cytochrome oxidase activity 1

TIM23: translocase of the inner membrane





MacPherson & Tokatlidis Biochem J. 2017 474(15): 2533–2545

Step back: protein traffic to mitochondria

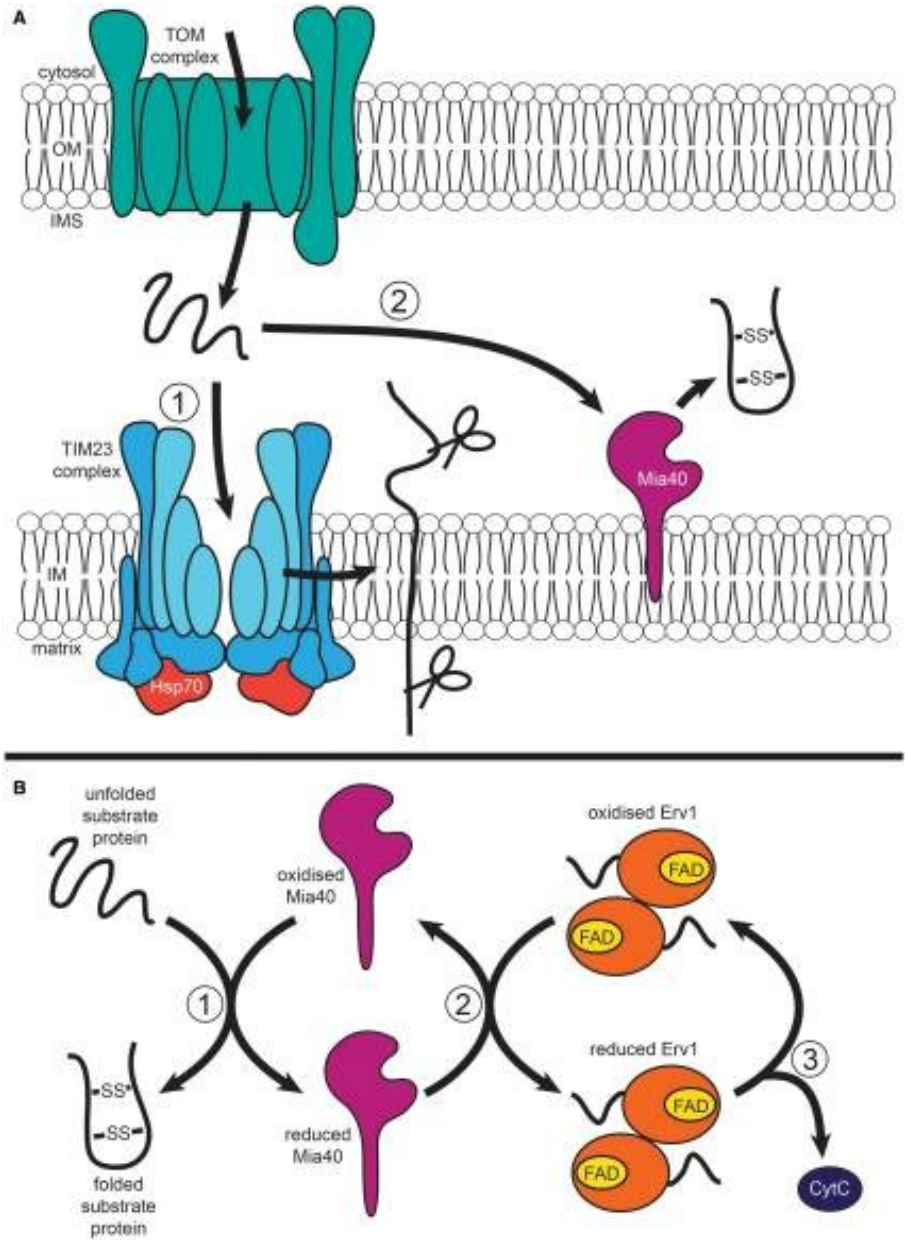
Proteins to be targeted to the intermembrane space (IMS) follows a more complex pathway:

- Import via TOM
- Retention in the inner membrane by TIM23
- Cleavage
- Oxidation and S-S formation by Mia40

Folded proteins cannot pass membranes anymore and are retained in the IMS.

Mia40 is then reoxidized by the homodimer Erv1, containing FAD as a cofactor.

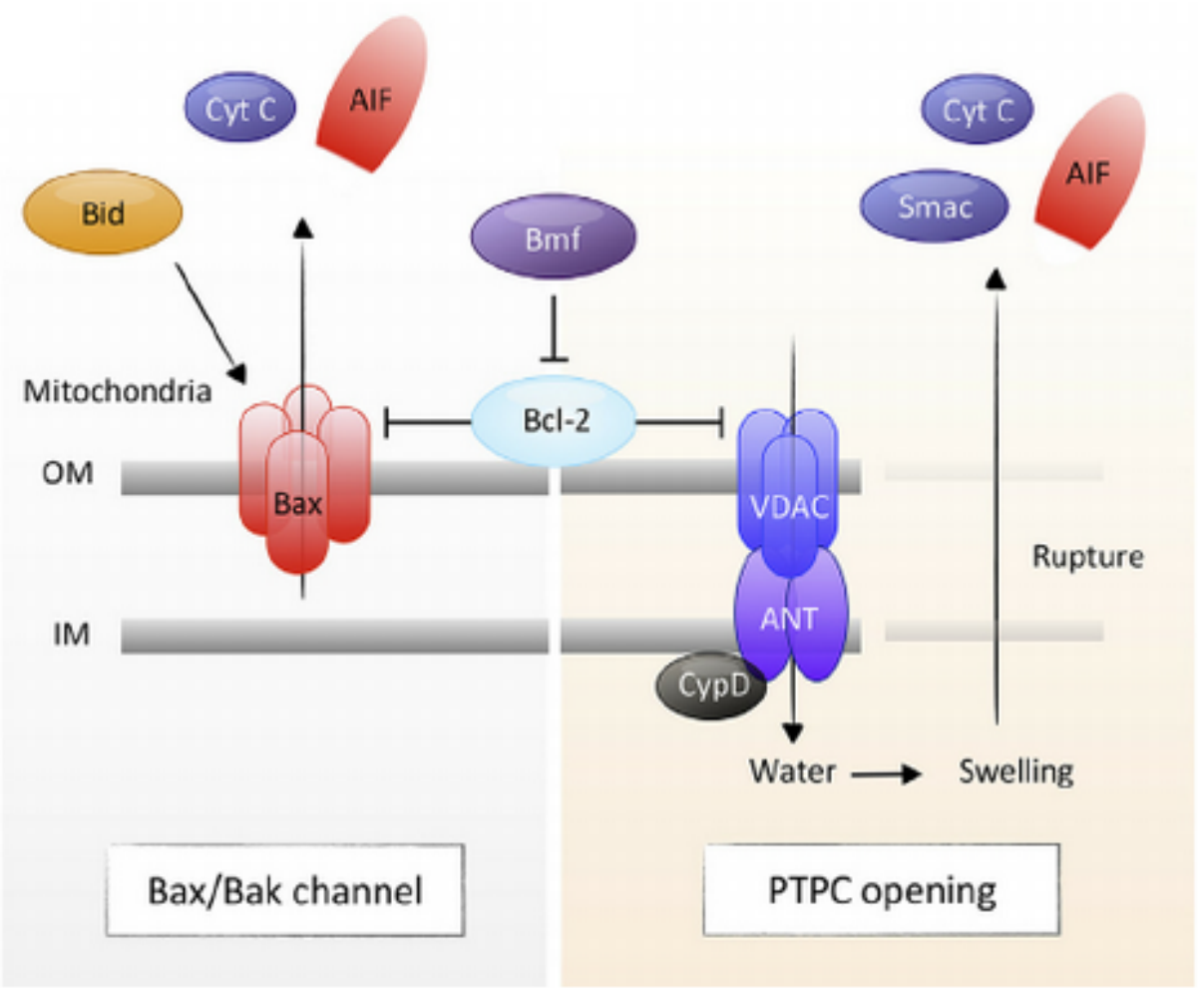
Reduced Erv1 is then reoxidized through the movement of electrons to the final electron acceptor cytochrome C-



MacPherson & Tokatlidis Biochem J. 2017 474(15): 2533–2545

Mitochondrial Outer Membrane Permeabilization (MOMP)

MOMP is a crucial event for apoptosis. The pores formed in the OM of the mitochondrion by Bax/Bak polymerization allows cytochrome C and other pro-apoptotic molecules to reach the cytosol. In the meanwhile, the permeability transition pore complex (PTPC) gets opened, imbalancing the permeability of the OM and dismantling a number of physiological processes.

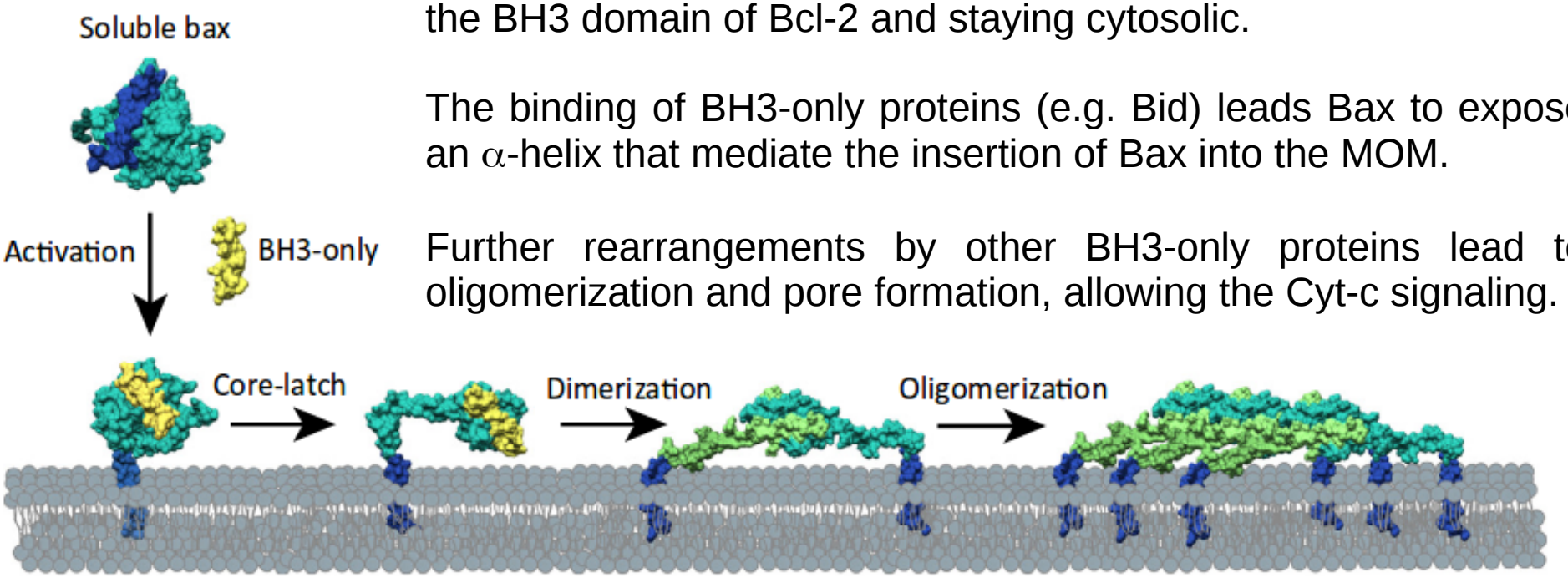


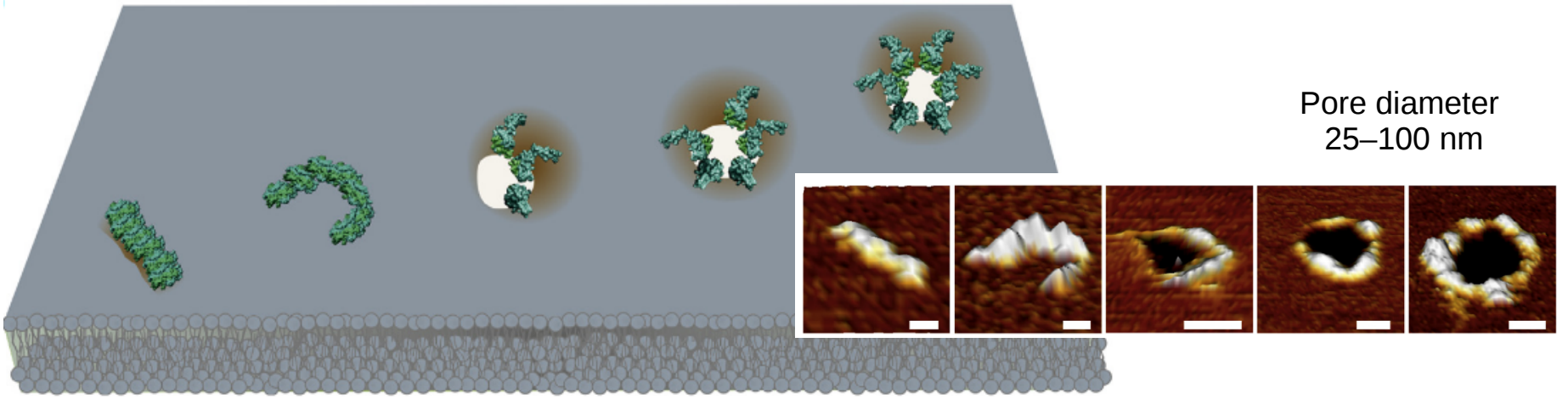
The opening of the Bax channels

The inactive form of Bax (BH123) is globular, capable of binding the BH3 domain of Bcl-2 and staying cytosolic.

The binding of BH3-only proteins (e.g. Bid) leads Bax to expose an α-helix that mediate the insertion of Bax into the MOM.

Further rearrangements by other BH3-only proteins lead to oligomerization and pore formation, allowing the Cyt-c signaling.



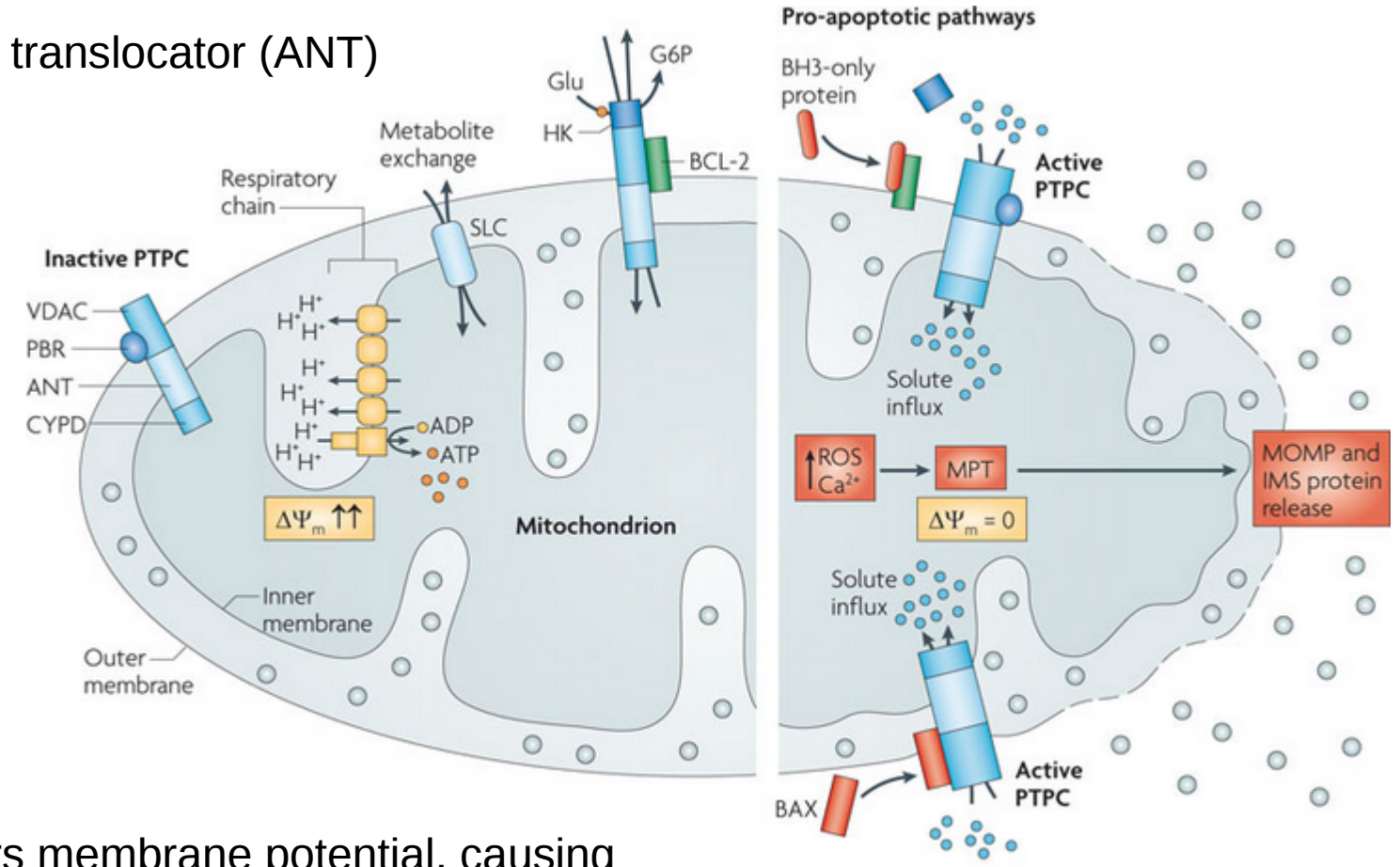


Cosentino and García-Sáez 2017 Trends Cell Biol.;27(4):266-275

The opening of the PTPC channels

The permeability transition pore complex (PTPC) is a mitochondrial complex aimed at controlling membrane permeability. Several members of the complex interact with Bcl-2 proteins. PTPC contains, among others:

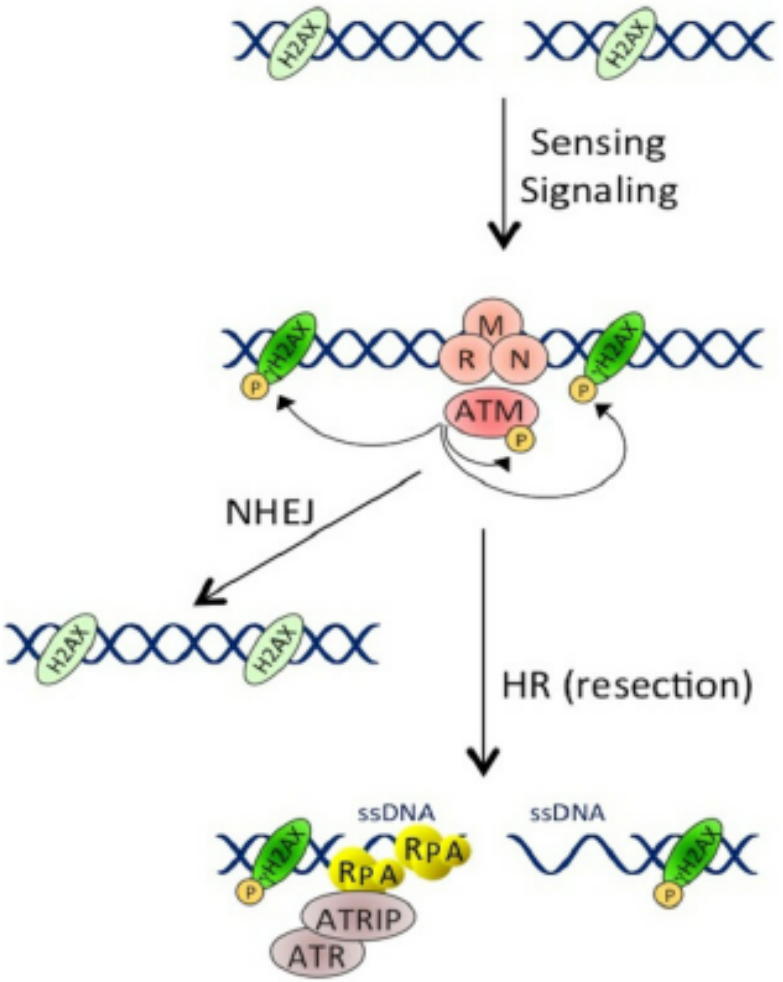
- voltage-dependent anion channel (VDAC)
- adenine nucleotide translocator (ANT)
- hexokinase II
- glycerol kinase
- creatine kinase
- cyclophilin D



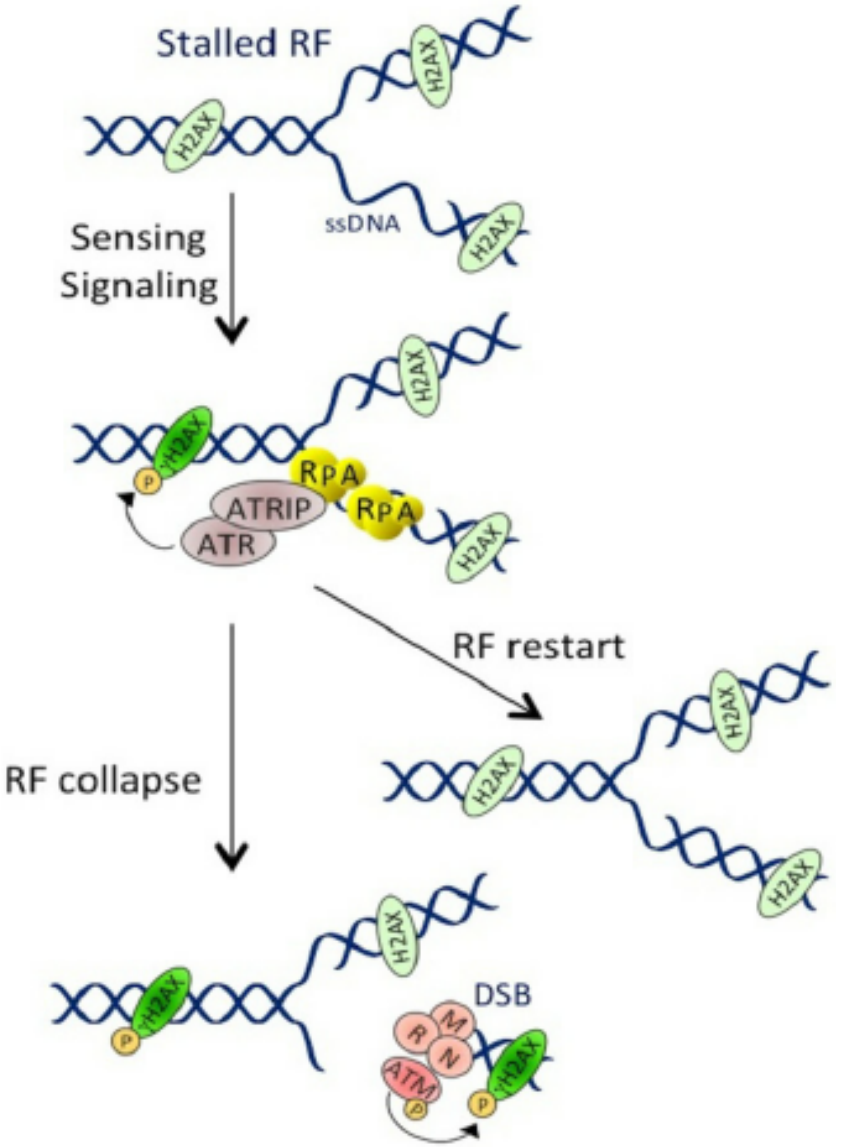
PTP opening impairs membrane potential, causing
a reduced OXPHOS, an increase of ROS and a potentiation of MOMP, leading to a further release of pro-apoptitic factors including SMAC (Cyt-c from Bax is insufficient)

DNA damage: ATM and ATR sensors

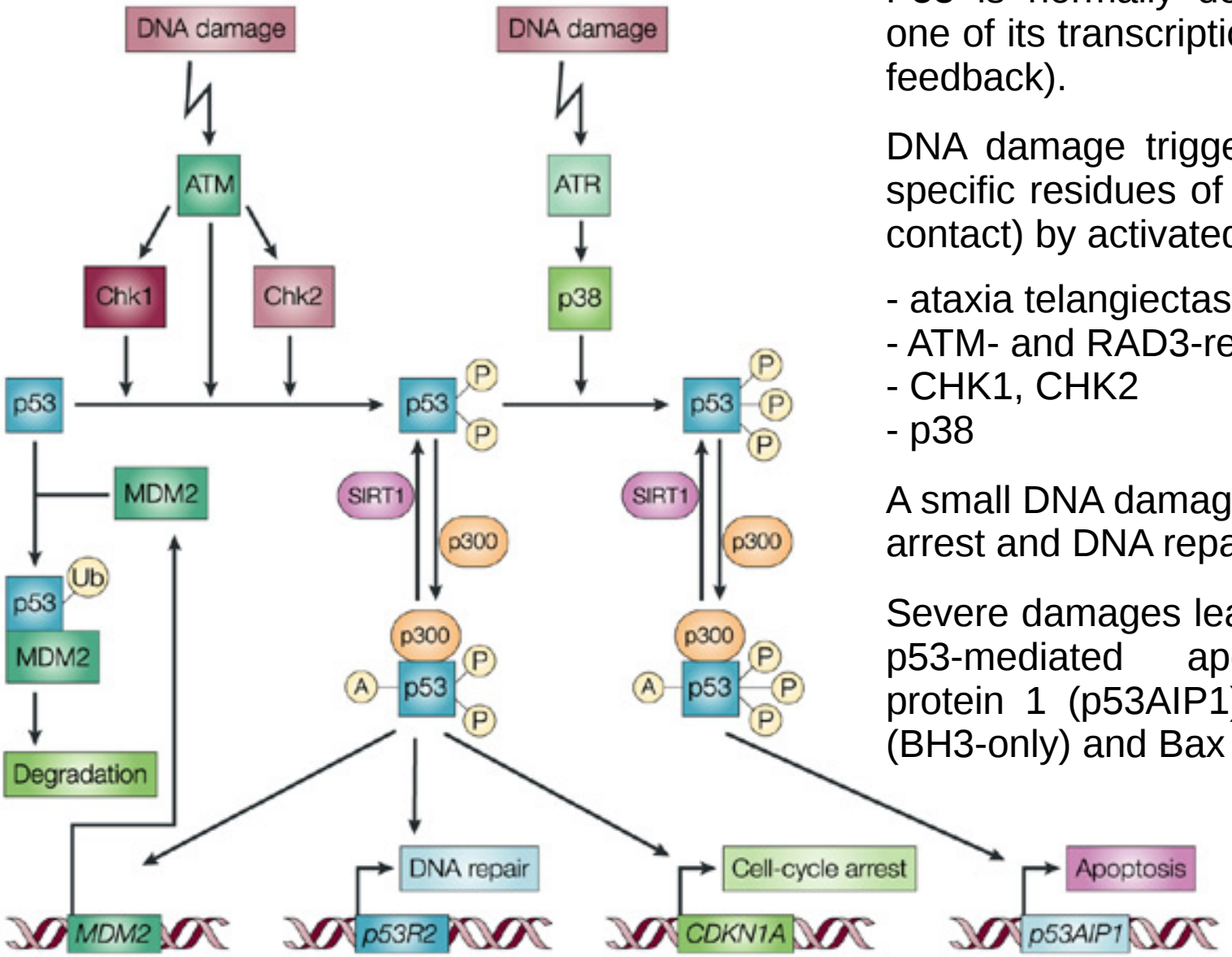
double-stranded DNA breaks



Various damages (UV, IR, Ox, EtOH)



DNA damage and p53-induced apoptosis



P53 is normally degraded by MDM2, one of its transcription targets (negative feedback).

DNA damage triggers the P-ylation of specific residues of p53 (loss of MDM2 contact) by activated kinases:

- ataxia telangiectasia mutated (ATM)
- ATM- and RAD3-related (ATR)
- CHK1, CHK2
- p38

A small DNA damage leads to cell-cycle arrest and DNA repair.

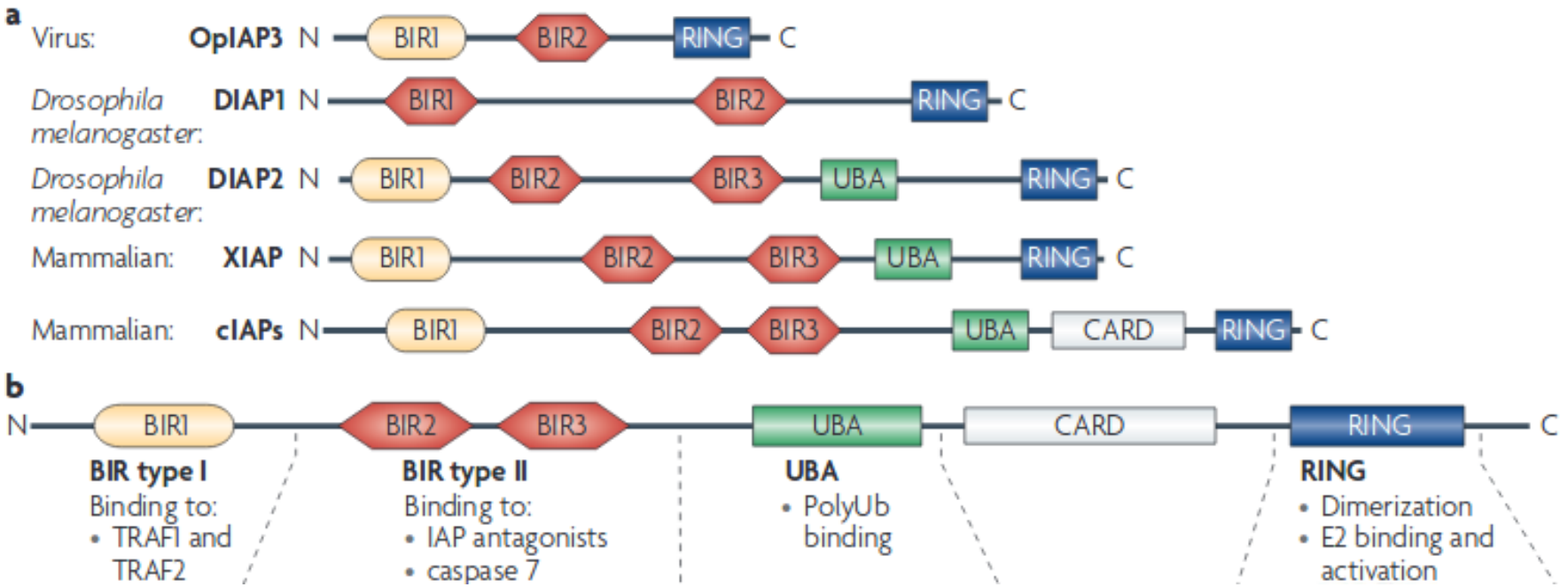
Severe damages leads to apoptosis via p53-mediated apoptosis, inducing protein 1 (p53AIP1), Puma and Noxa (BH3-only) and Bax (BH123).

Inhibitor of apoptosis (IAP) protein family

IAP proteins are characterized by the presence of the baculovirus IAP repeat (BIR) domain, a zinc binding fold that mediates protein-protein interactions. The virus prevented cell death with its IAPs. IAPs are constitutively expressed at low levels to increase the “activation threshold” of apoptosis.

IAPs are fundamental for the survival of certain types of cancer, but their inhibition is completely safe to normal cells. It may seem counterintuitive, but their inactivation is fundamental for certain type of cancer.

At least 8 IAPs have been identified in humans, carrying 1 to 3 BIR doimains.



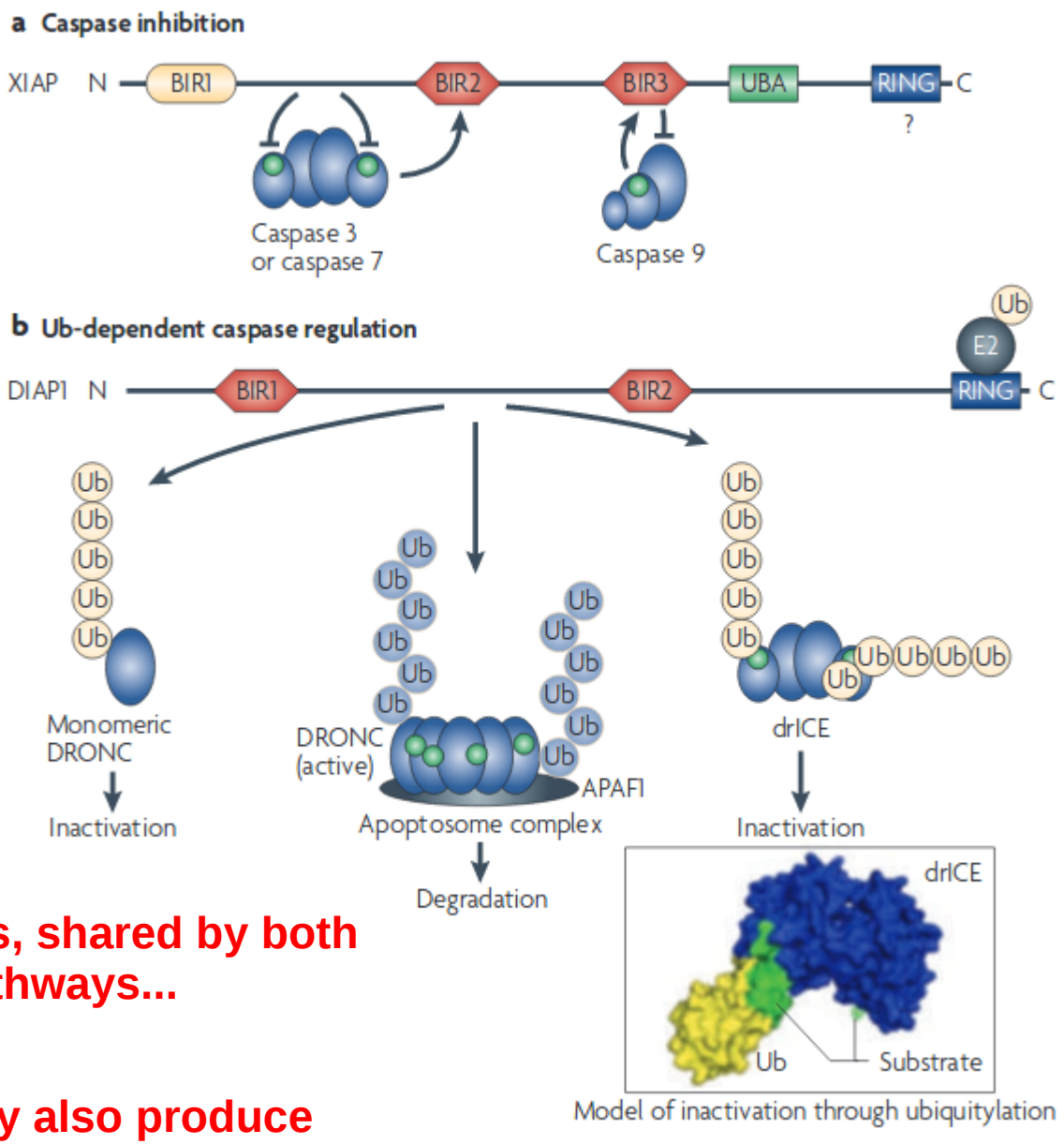
Gyrd-Hansen and Meier 2010 Nat Rev Cancer 10: 561-574

Inhibition strategies of IAPs

They exhibit at least two different modes of inhibition:

- **Direct caspase binding**, on:
  - caspase 9
    → via BIR3 domain
  - caspase 3 and 7
    → via BIR1-BIR2 linker

- **Ubiquitin-mediated**, on:
  - caspase 3 and 7
    → via monoubiquitylation
    → via polyubiquitinylation



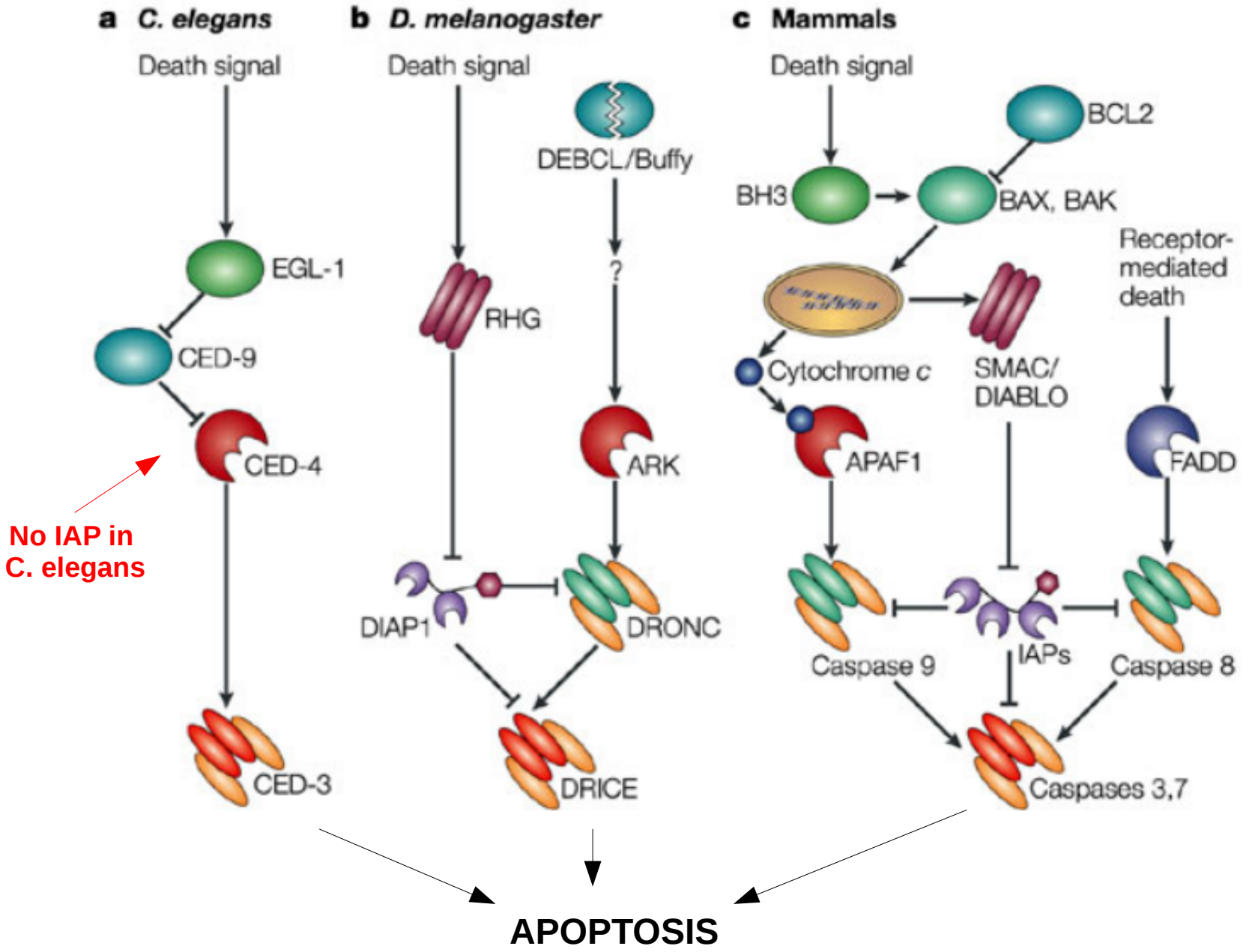
CAS3 and CAS7 are effector CASs, shared by both intrinsic and extrinsic pathways...

↓

...but intrinsic activation pathway also produce anti-IAPs (SMAC/DIABLO)

Gyrd-Hansen and Meier 2010 Nat Rev Cancer 10: 561-574

IAPs evolution in model organisms



Inhibition of apoptosis: the survival factors

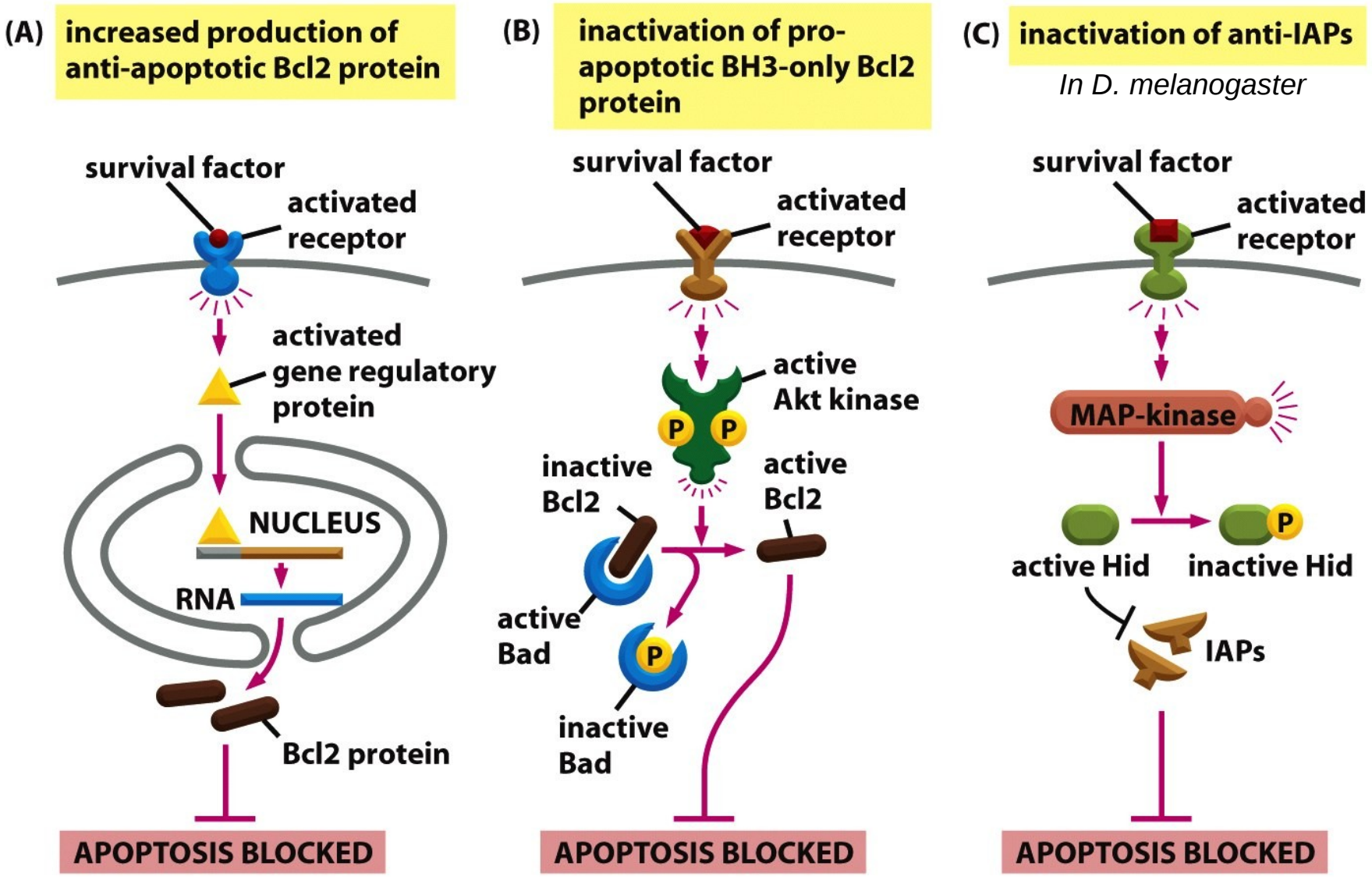


Figure 18-14 Molecular Biology of the Cell 5/e (© Garland Science 2008)