Death signaling

Hao Wu

References

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- 3. Goyal, L. Cell death inhibition: keeping caspases in check. *Cell* 104, 805-808. (2001).
- 4. Green, D. R. Apoptotic pathways: the roads to ruin. *Cell* 94, 695-698. (1998).
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- 6. Hengartner, M. O. Apoptosis: corralling the corpses. *Cell* 104, 325-328. (2001).
- 7. Huang, D. C. & Strasser, A. BH3-Only proteins-essential initiators of apoptotic cell death. *Cell* 103, 839-842. (2000).
- 8. Johnstone, R. W., Ruefli, A. A. & Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 108, 153-164. (2002).
- 9. Shi, Y. A structural view of mitochondria-mediated apoptosis. *Nat Struct Biol* 8, 394-401. (2001).

- Color PDF file of handouts can be found at Wu lab web-page: http://venus.med.cornell.edu

Paper Discussion

- Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-13, 1997.
- Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. Cell 91: 479-89, 1997.

Apoptosis: an orderly process of cellular suicide

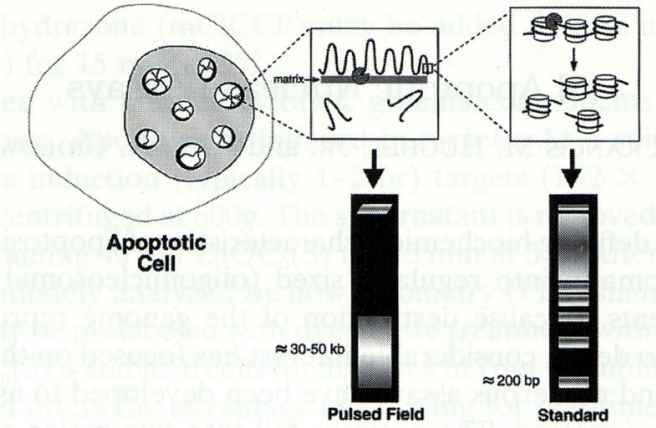
- Apoptosis refers to the shedding of leaves from trees in Greek. It was first observed by Carl Vogt in 1842. The word 'apoptosis' was introduced by Kerr, Wyllie and Currie in 1972 to describe the kind of cell death that is distinct from a necrotic cell death.
- It is associated with characteristic morphological changes:
  - Detachment from the surrounding tissue.
  - Shrinkage and condensation of cytoplasm and nucleus.
  - DNA fragmentation: ~180bp ladders, corresponding to inter-nucleosomal cleavages.
  - Plasma membrane blebbing and packaging of cell contents into enclosed apoptotic bodies. The cell surface undergoes changes (e.g. PS externalization) that signal the surroundings of their apoptotic state to assist phagocytosis and disposal.
- Rapid and contained, avoiding massive inflammatory responses often associated with tissue injury and necrotic cell death.

Apoptosis plays important roles in many biological processes

- Physiological conditions
  - An intrinsic and integral component of physiology, just like proliferation and differentiation.
  - Embryonic development: e.g. in *C. elegans,* 131 out of a total of 1090 somatic cells are programmed to undergo apoptosis at pre-defined stages.
  - Cellular homeostasis: e.g. lymphocytes
- Pathological conditions
  - Down-regulation of apoptosis: e.g. cancer, autoimmune disorders, persistent viral infections...
  - Up-regulation of apoptosis: e.g. many forms of degenerative disorders such as Alzheimer's disease, ischemic injury from stroke (heart disease), post-menopausal osteoporosis...

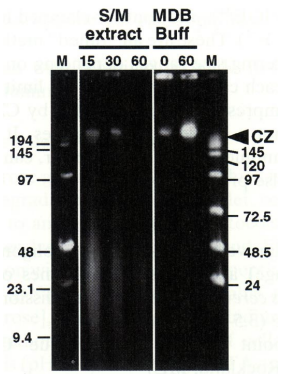
Detection of DNA fragmentation

Two types of DNA fragmentation

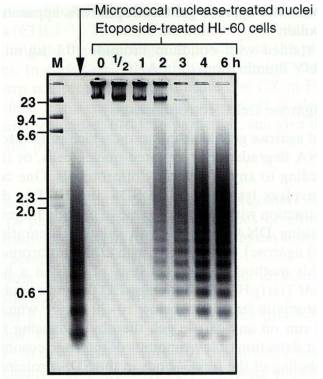


- **By gel electrophoresis**

50-500kbp domain sized fragments; mediated by AIF and other proteins



Internucleosomal DNA degradation, multiples of ~180bp; mediated by Caspase-Activated Deoxyribonuclease (CAD), also known as DNA Fragmentation Factor (DFF40).



Methods in Enzymology Vol 322, 2000.

TUNEL assay

Terminal Transferase-Mediated dUTP Nick End-Labeling Method (TUNEL). Enzymatic labeling of DNA double strand breaks induced by apoptotic stimuli, blunt, or with overhang.

Case I:

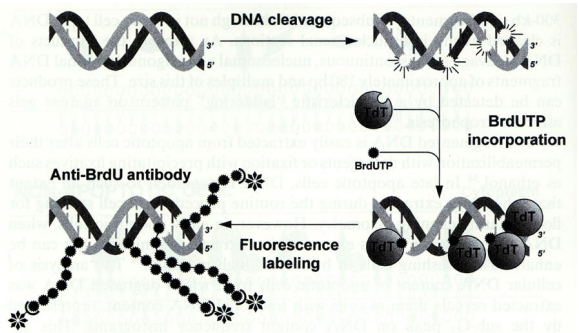


FIG. 2. Scheme illustrating the labeling of DNA strand breaks in apoptotic cells with BrdUTP, using exogenous terminal deoxynucleotidyltransferase (TdT) and anti-BrdU MAb.

TUNEL assay

Case II:

TdT, terminal deoxynucleotidyltransferase, can be used to add biotin-labeled uridylate to the free 3' ends of the DNA fragments. Biotin is then detected by peroxidase-coupled streptavidin.

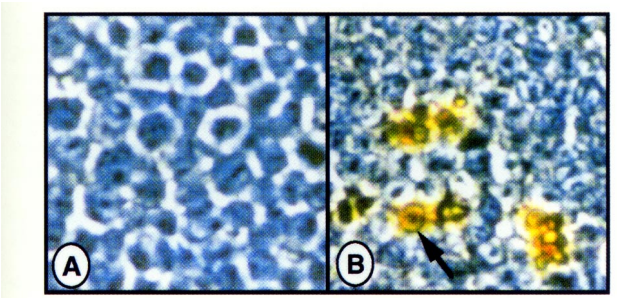


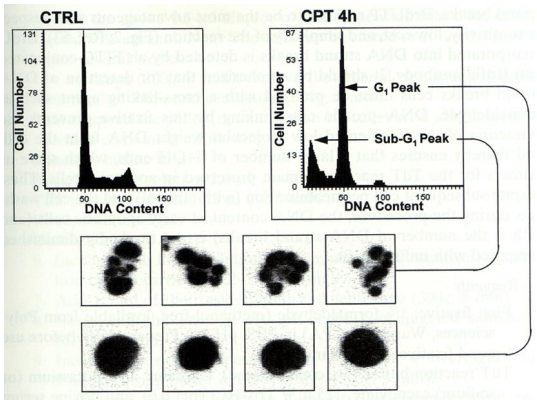
FIG. 3. Detection of DNA cleavage *in situ*, using the TUNEL technique. Sections of mouse thymus were deparaffinized in xylene, rehydrated in PBS, and incubated sequentially with biotinylated dUTP in the absence (A) or presence (B) of TdT, peroxidase-coupled streptavidin, AEC, and fast green FCF as described in text. Note that occasional cells in (B) (e.g., arrow) are stained brown, indicating incorporation of biotinylated nucleotide. In the absence of TdT, a signal is not detected (A).

PI staining

The low molecular weight fragments are readily extracted from ethanol-fixed cells by treatment with aqueous buffers, DNA-content in apoptotic cells is low compared to normal cells after staining with DNA-binding dyes such as propidium iodide (PI) and detected by flow cytometry.

Morphological detection

Hoechst 33342 dye staining for condensed chromatin

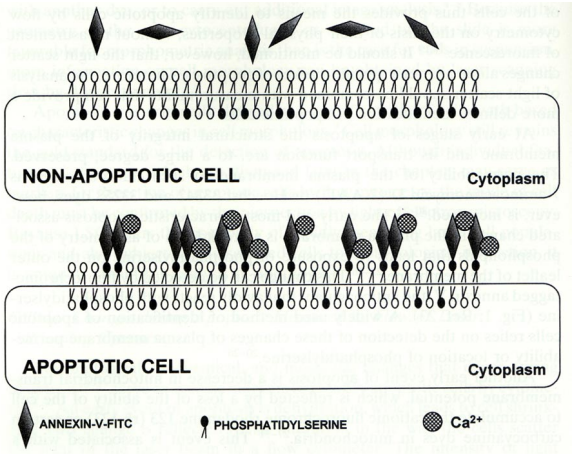


- Annexin V labeling of externalized PS

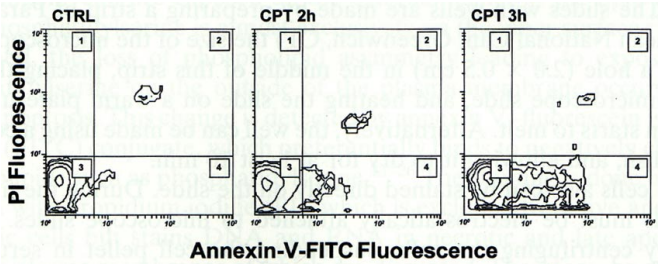
  Early detection of apoptosis;

  PS, phosphatidylserine, is normally confined to the inner leaflet of the plasma membrane. During apoptosis, PS translocates to the cell surface. This externalization of PS marks the apoptotic cells for phagocytosis and removal.

  Once on the cell surface, PS can be labeled by binding of fluorescein isothiocyanate (FITC)-labeled annexin V, followed by flow cytometry detection or fluorescent microscope observation.



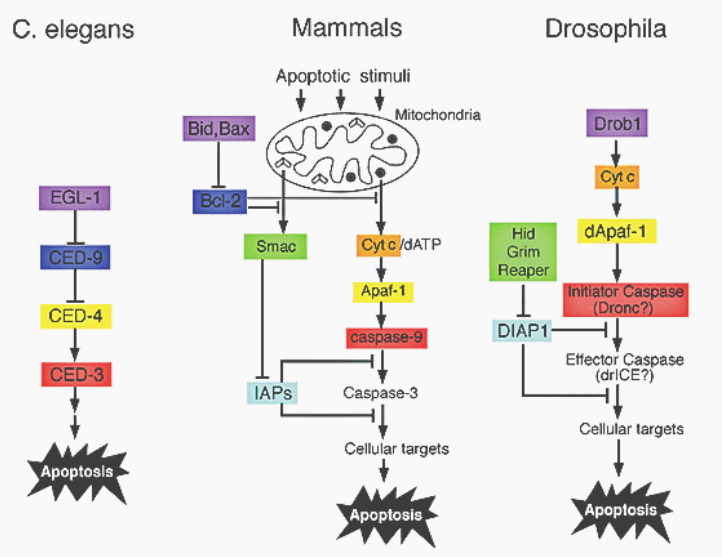
Flow cytometry of Annexin-V-stained apoptotic cells



Apoptosis-cellular suicide-programmed cell death

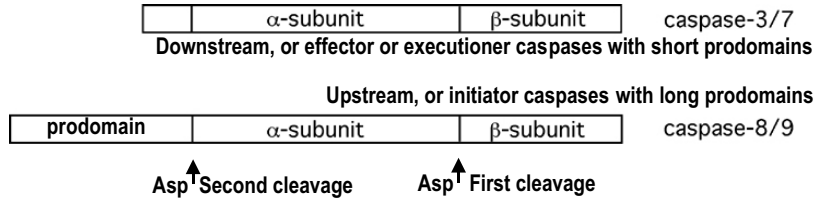
- 'active' (not passive) form of cell death by orchestrating its own silent demise.
  - Disables homeostatic and repair processes
  - Halts cell cycle progression
  - Induces structural disassembly and morphological changes
  - Marks the dying cells for engulfment and disposal
- Several phases of an apoptotic process:
  - Initiation, execution and disposal

Parallel paradigms of apoptosis in C. elegans, Drosophila and mammals: the importance of caspases



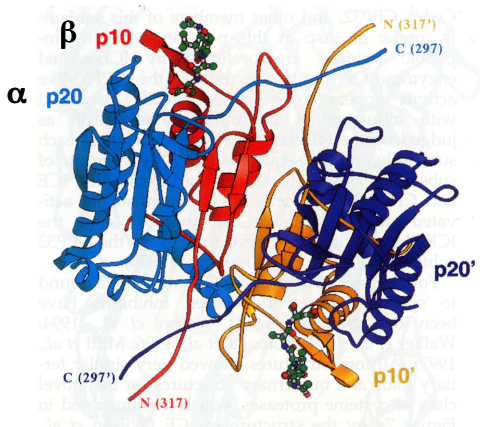
Apoptosis is executed by Caspases: Cysteinyl aspartate-specific proteinases: Death by a thousand cuts!

- **Constitutively present in most cells, residing in the cytosol as single chain zymogens.**

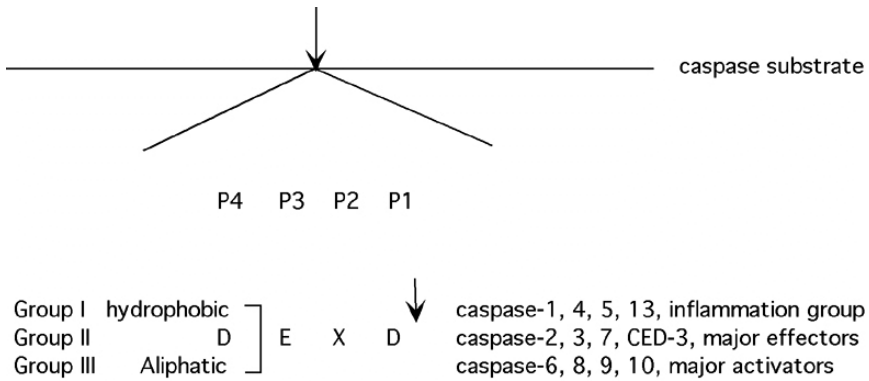


- **Procaspases may possess low but significant activity, e.g. procaspase-8 has ~1-2% of the activity of the mature caspase-8.**
- **Caspases are fully activated by a first proteolytic cleavage between the large and small subunits and a second cleavage to remove the prodomain.**

- **Mature caspases contain an $\alpha_2\beta_2$ arrangement**
  - **Mature caspase-1, or ICE, the first structure of a caspase**



Caspases recognize specific tetrapeptide motifs

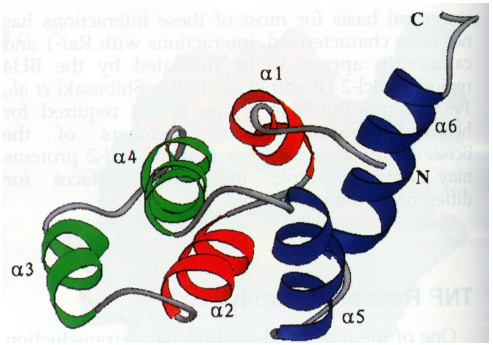


Several means of caspase activation

- By signaling cascades, which lead to oligomerization of upstream procaspases to allow auto- and trans-processing;
- By caspase cascades, in which upstream caspases cleave and activate downstream caspases to amplify caspase activation;
- By other proteases such as granzyme B, which is introduced into cells by cytotoxic lymphocytes.

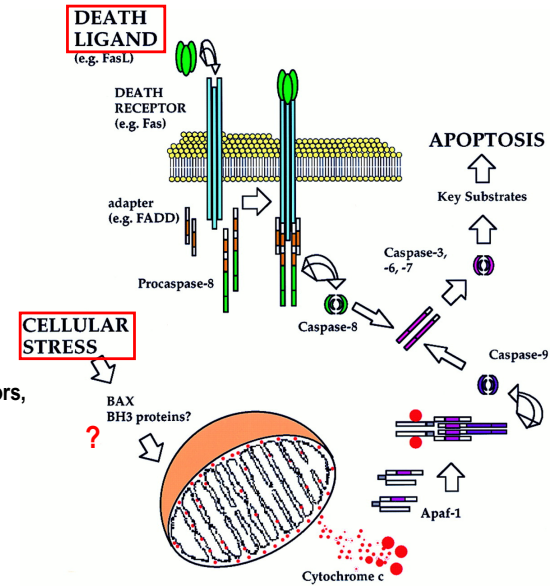
- Procaspase oligomerization is mediated by the binding of adapter molecules to caspase prodomains.
  - Caspase-8 and -10 each contain two tandem death effector domains (DEDs), which interact with adapter proteins such as FADD.
  - Caspase-9 (as well as -1, -2, -4 and -5) contains caspase recruitment domains (CARDs), which interacts with cytosolic protein Apaf-1.
- DEDs, CARDs, and death domains (DDs) all have a conserved structural arrangement with six closely packed, amphipathic antiparallel α helices.

Fas DD

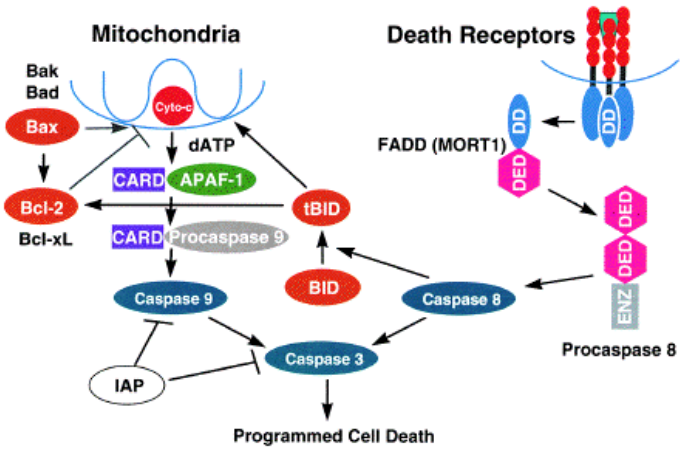


Intrinsic (mitochondria-mediated) and extrinsic (receptor-mediated) pathways in mammals

Serum starvation
Ultraviolet irradiation
DNA damage
Chemotherapeutic agents
Activation of tumor suppressors, such as p53
Developmental cues?



Caspases play differential roles in each cell death cascade



Caspase-knockout phenotypes

Caspases	Development	Apoptotic phenotype
caspase-1	normal	Fas? (thymocytes)
caspase-2	normal	germ cells
caspase-3	perinatal lethal	neuroepithelial progenitors; lack of or delayed morphological changes and DNA fragmentation
caspase-6	normal	N/D
caspase-7	embryonic lethal	N/D
caspase-8	embryonic lethal	death receptors (Fas, TNF, DR3) pathways
caspase-9	embryonic lethal	neuroepithelial progenitors; mitochondrial pathways (thymocytes)
caspase-11	normal	Fas? (thymocytes)

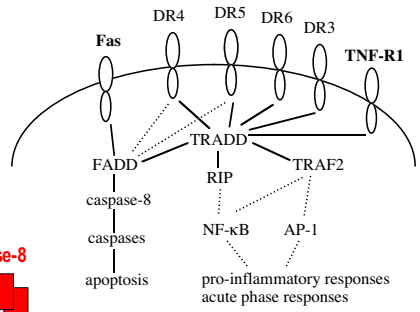
Relative importance of different caspases in different cells and under different conditions.

Zheng, T. S., Hunot, S., Kuida, K., and Flavell, R. A. (1999). Caspase knockouts: matters of life and death. Cell Death Differ *6*, 1043-53.

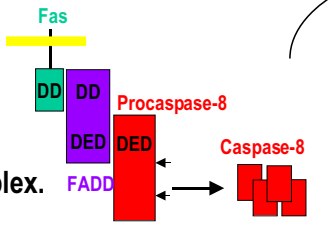
Death ligands and receptors: the TNF and TNFR superfamily

Receptors	Ligands	Functions	Signaling Proteins
TNFR1	TNFα/LTα/LTα$_2$β$_1$	apoptosis, growth, inflammation	TRADD, FADD, TRAF2, RIP
Fas	FasL	apoptosis, peripheral tolerance	FADD
P75 NGFR	Neurotrophins	neuron survival or death	TRAF6?
DR3	Apo3L	apoptosis, NF-κB activation	TRADD, FADD, TRAF2, RIP
DR4	Apo2L (TRAIL)	apoptosis, NF-κB activation?	FADD, TRADD?
DR5	Apo2L (TRAIL)	apoptosis, NF-κB activation?	FADD, TRADD?
DR6	?	apoptosis, NF-κB activation	TRADD, FADD, TRAF2, RIP

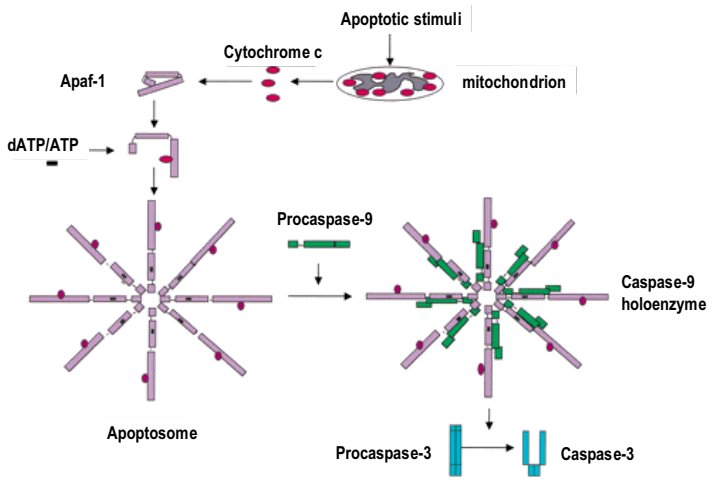
Two types of signaling cascades:
Fas-FADD-procaspase-8;
TNFR1-TRADD-FADD-procaspase-8;



Activated caspase-8 gets released From the signaling complex.

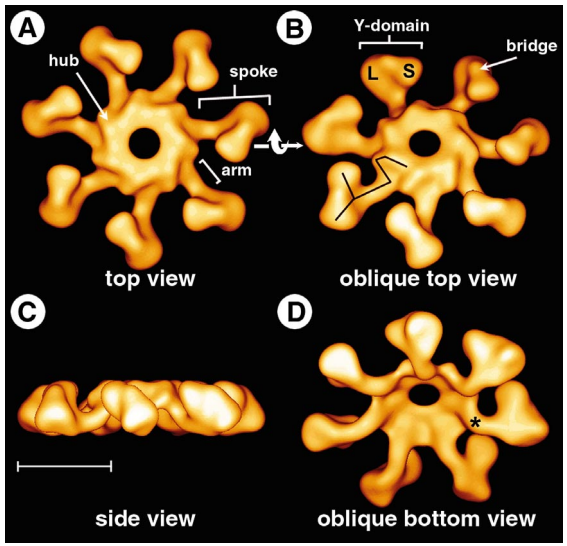


Apaf-1 cascade for caspase activation



- **In the absence of Apaf-1, procaspase-9 and mature caspase-9 possess similar catalytic activities.**
- **Activated caspase-9 remains bound with Apaf-1.**

EM structure of apoptosome (Apaf-1 + cytC)



Apaf-1 | CARD | CED-4 | WD | WD

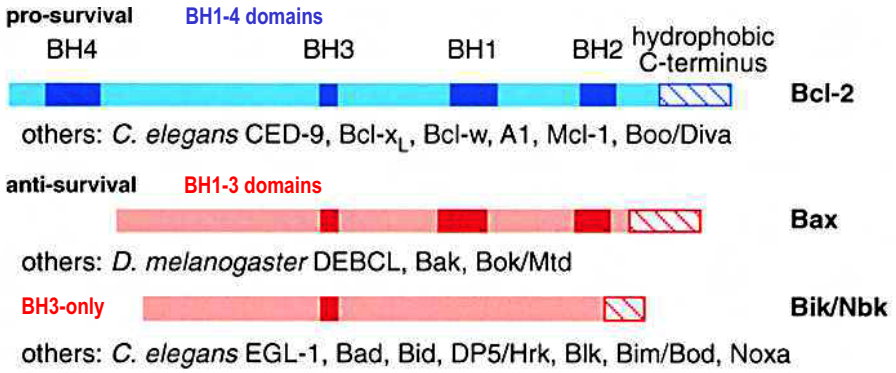
Targeted deletion of Apaf-1

- Defects are found in essentially all tissues whose development depends on cell death, including loss of interdigital webs, formation of the palate, control of neural cell number, development of the lens and the retina.
- However, some forms of apoptosis are partially or completely intact, e.g. cell death induced by glucocorticoids, staurosporine, and other agents, which appears to depend on the mitochondria, but not Apaf-1.
  - Are these Apaf-1 independent apoptotic processes achieved via death receptor signaling since cellular stress can induce expression of death ligands? Or other undiscovered Apaf-1 like molecules maintain apoptotic responses in thses cases? Or some caspase-independent and mitochondria-dependent processes exist?
- Caspase-9 knockout does not accurately mimic the Apaf-1 knockout. Additional apoptosomes? i. e. Additional caspases activated by Apaf-1?

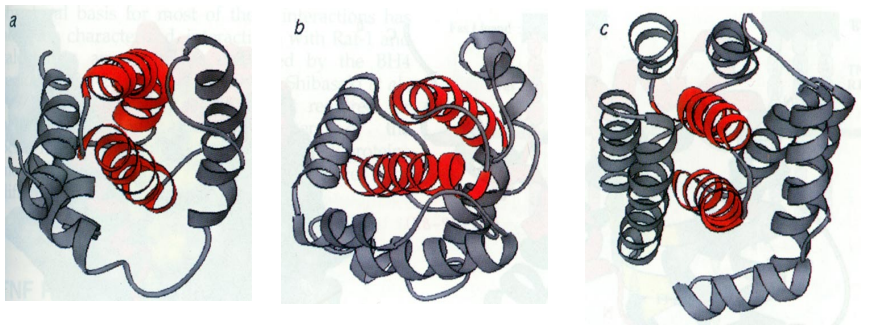
The involvement of mitochondria and cytC in Apaf-1 mediated apoptosis

- Dual functions of mitochondria: energy metabolism and apoptosis.
- Cytochrome c resides at the intermembrane space of the mitochondria. Only heme-bound cytochrome c, i.e. cytochrome c from mitochondria, is apoptogenic.
- What triggers cytochrome c release from the mitochondria?
  - The fundamental role of the mitochondria in apoptosis is established.
  - Mechanism of cytochrome c release is not fully established.
    - Loss of transmembrane potential
    - Permeability transition
    - Proapoptotic and anti-apoptotic Bcl-2 family members can cause and inhibit cytochrome c release, respectively.
    - Formation of channels for cytochrome c release?

Bcl-2 family

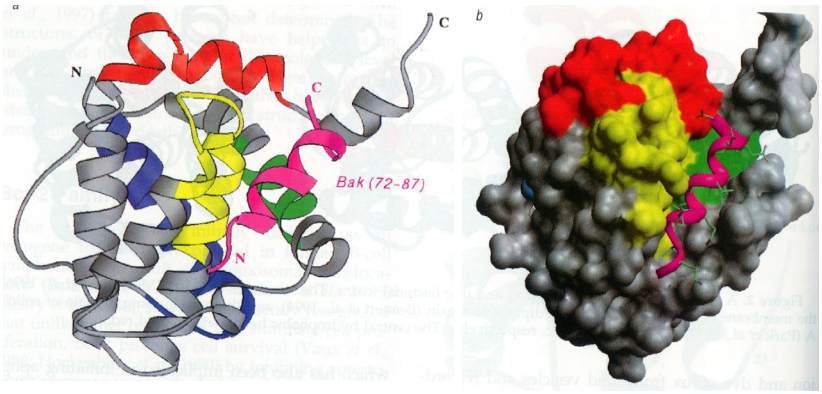


The structure of Bcl-xL is similar to pore-forming toxins



Bcl-x_L | diphtheria toxin (membrane-translocation domain) | colicin A (pore-forming domain)

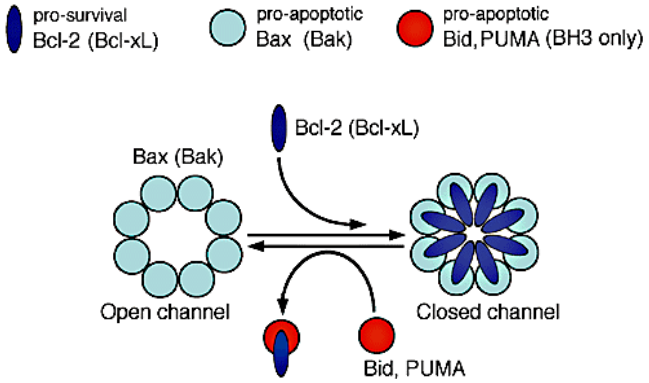
Bcl-2 family members can dimerize with each other.



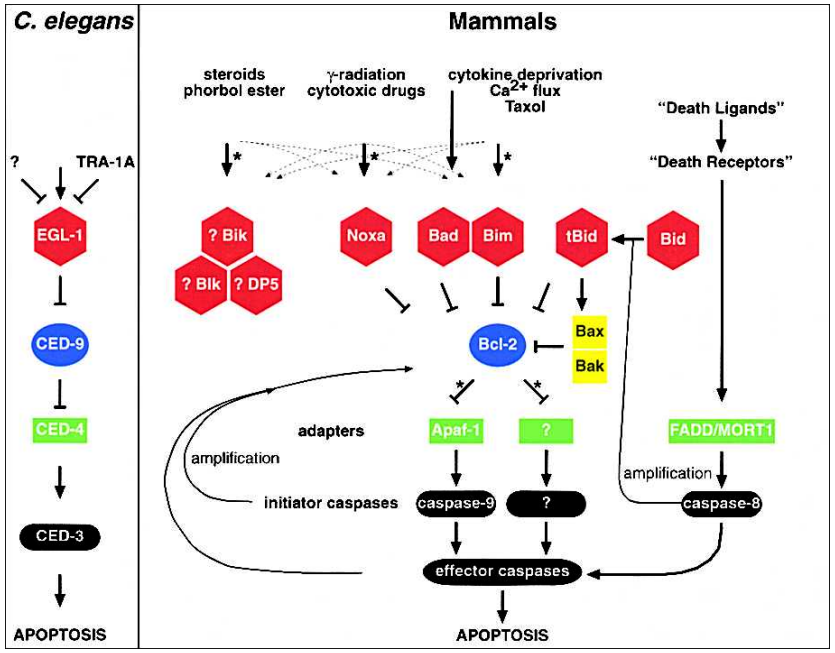
Bcl-xL/Bak peptide complex

A model for the function of Bcl-2 family proteins

- Pro-survival Bcl-2 and Bcl-xL contain all four BH domains.
- Pro-apoptotic Bax and Bak contain BH1, BH2 and BH3.
- Pro-apoptotic Bid and PUMA contain only BH3 domain.



Model for the activity of BH3-only proteins

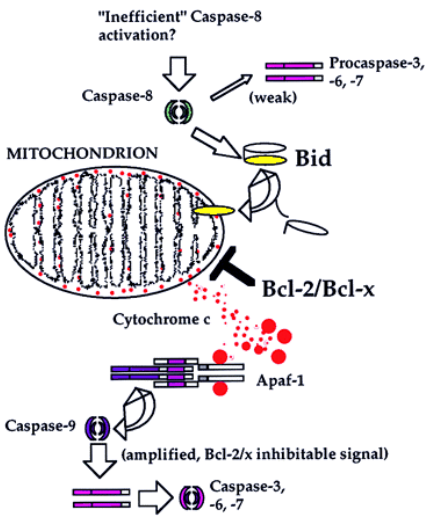


BH3-only proteins, essential initiators of apoptosis?

- Some of these proteins are regulated transcriptionally;
  - EGL-1: regulated by transcription; Noxa and PUMA, induced by p53 transcription.
  - DP5/Hrk, induced when neurons are deprived of growth factors or exposed to β-amyloid protein.
- Some of these proteins are regulated post-translationally;
  - Bad, phosphorylation of Bad leads to its sequestration by binding to 14-3-3 scaffold proteins.
  - Bim, is sequestered to microtubular complexes by interacting with dynein light chain LC8.
  - Growth factor and/or cytokine deprivation and certain other apoptotic stimuli result in the release of Bad and Bim, leading to the initiation of apoptosis.
- Some of these proteins may be used to amplify caspase activation;
  - Bid, becomes apoptogenic after cleavage by caspase-8.
- Thymocyte apoptosis induced by glucocorticoids and phorbol esters may be induced by yet unknown BH3-only proteins.

The role of mitochondria in receptor-mediated apoptosis: the cross-talk

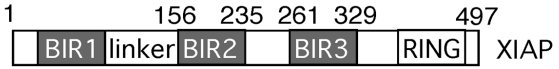
- Receptor-mediated death signaling pathway should be resistant to inhibition by Bcl-2 or Bcl-xL.
- However, in some cell types, when procaspase-8 activation is inefficient, Bcl-2 and Bcl-xL can interfere with Fas- and TNFR1-mediated cell death, because caspase activation in this case requires amplification by the mitochondria.
- Caspase-8 cleaves Bid to generate tBID, which triggers cytochrome c release and enlists Apaf-1 for caspase-3 activation.



- Bid may be a better substrate for caspase-8 than procaspase-3.

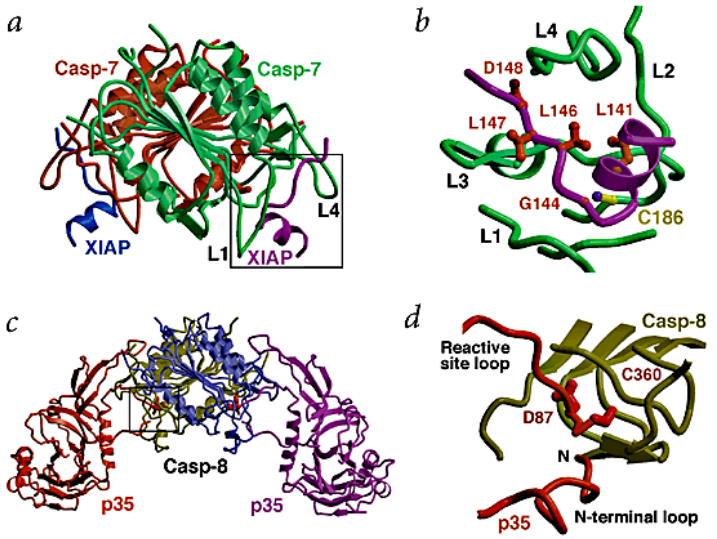
Protein caspase inhibitors

- Metazoan
  - Inhibitors-of-apoptosis (IAPs)
    XIAP, c-IAP1, c-IAP2, Op-IAP, Survivin, NAIP…
    XIAP is a protein with 'many talents':

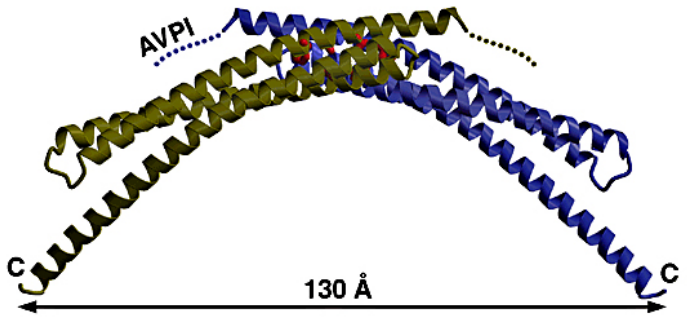


    linker: inhibits effector caspases, such as caspase-3 and caspase-7.
    BIR3: inhibits caspase-9, an initiator caspase.
    BIR2 and BIR3: interacts with Smac, a mitochondrial protein and an IAP antagonist.
    RING: may act as an E3 for ubiquitination and degradation.
- Viral
  - IAPs
  - p35 from baculoviruses
  - CrmA from Cowpox viruses: a serpin

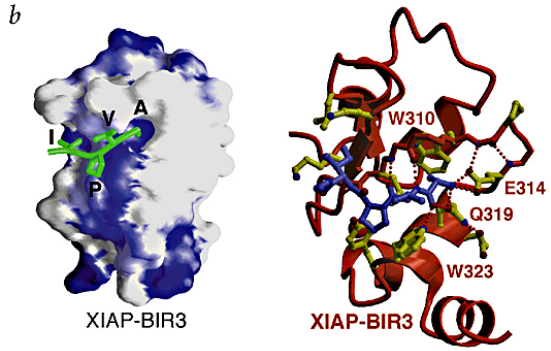
Molecular mechanisms of caspase inhibiton

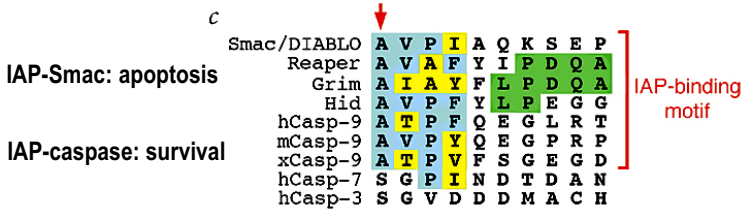


Structure of Smac



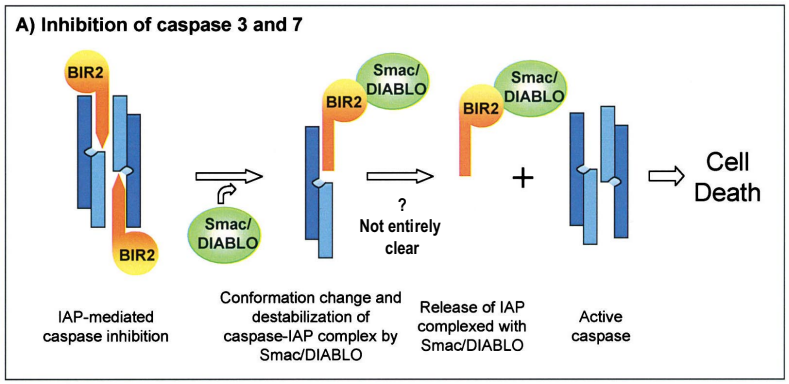
IAP-binding motif:
competition between IAP-caspase interaction and IAP-Smac interaction



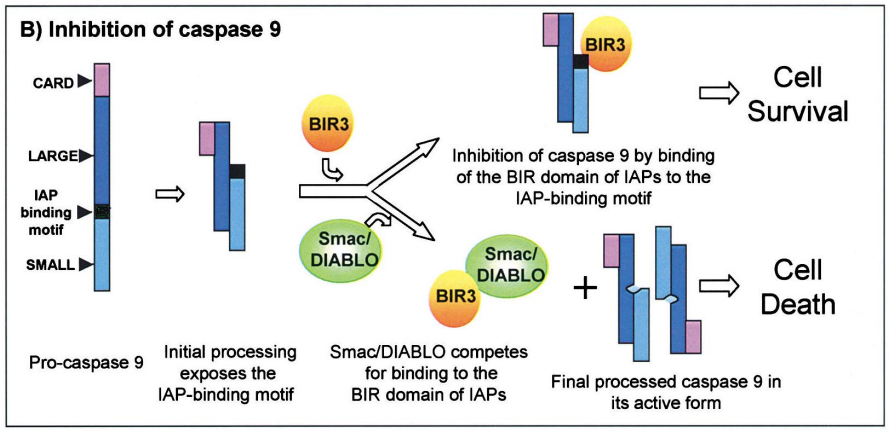


A model of XIAP and Smac in caspase regulation

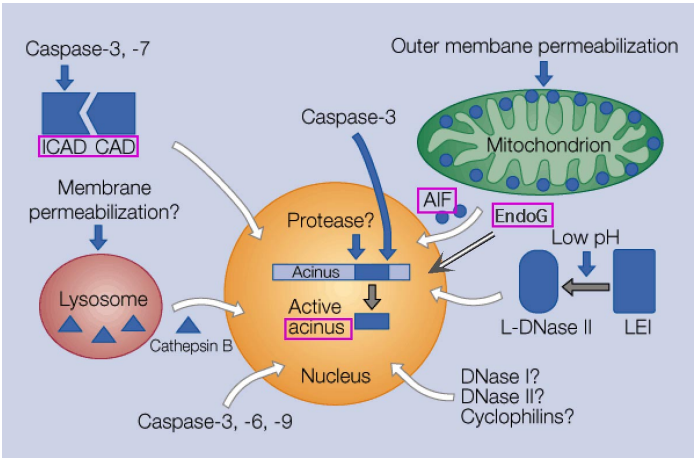
Effector caspases



Initiator caspases



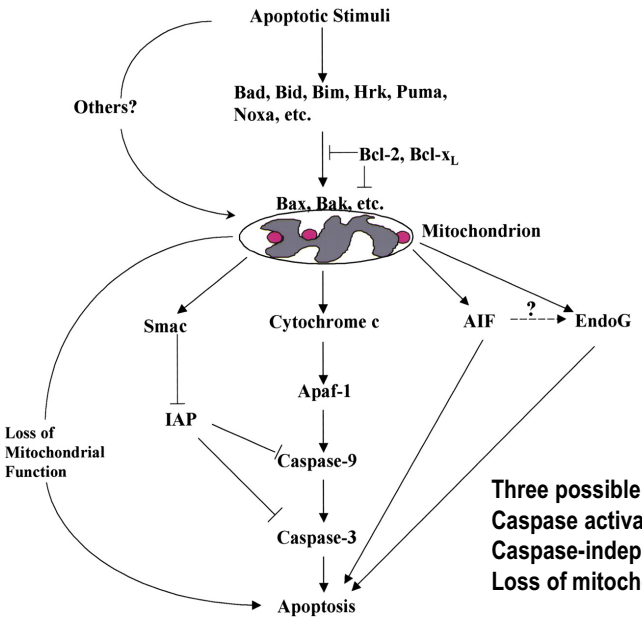
Protein factors (caspase-dependent and caspase-independent) in DNA condensation and fragmentation



AIF (mitochondria) → AIF (nucleus) → DNA condensation and large-fragment DNA cleavage ~50kbp

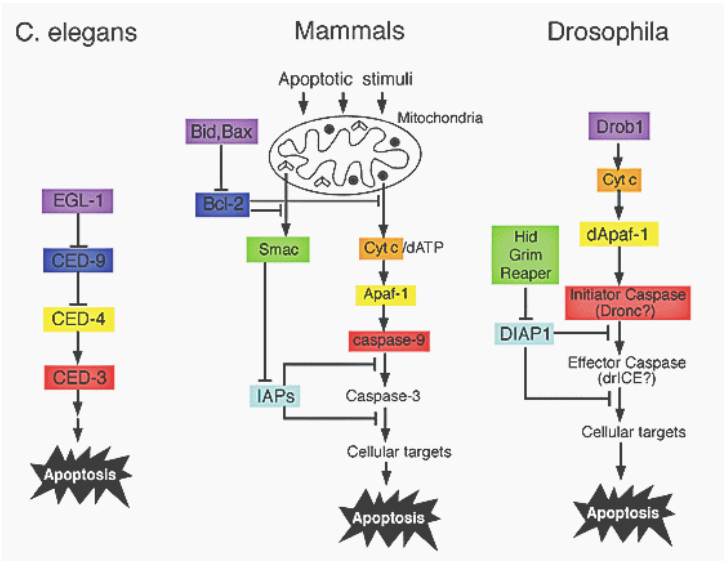
caspase
CAD/ICAD (cytosol) → CAD (nucleus) → oligonucleosomal DNA fragmentation, ~200bp

Summary of mitochondria-mediated cell death



Three possible mechanisms:
Caspase activation
Caspase-independent factors
Loss of mitochondrial function

Parallel paradigms in C. elegans, Drosophila and mammals

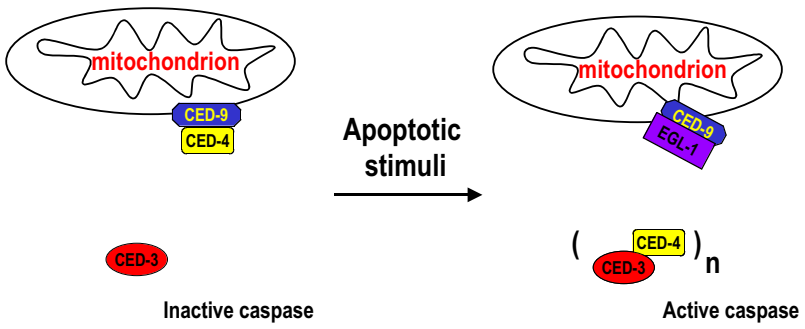


Cytochrome C; Smac-IAP; CED9/CED4 physical interaction

Apoptotic paradigm in C. elegans: differences with the mammalian system

- In mammals, cytochrome c release is involved in caspase activation, while in C. elegans, the involvement of mitochondria has not been demonstrated.
- The C. elegans CED-9/CED-4 interaction does not appear to be conserved in mammals as an Bcl-2/Apaf-1 interaction.

CED-3 (caspase); CED-4 (Apaf-1);
CED-9 (antiapoptotic Bcl-2 like);
EGL-1 (proapoptotic BH3-only Bcl-2 member)



Surviving cells

Apoptotic cells

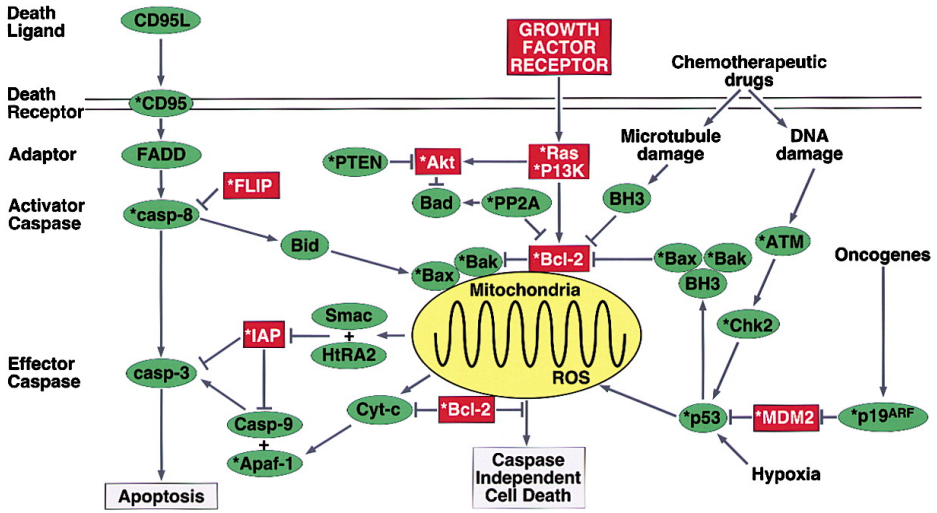
Where is the point of no return in apoptosis: mitochondrial damage?

- Triggers of apoptotic process presumably target mitochondria for cytochrome c release, prior to the involvement of Apaf-1, caspase-9 or caspase-3.
- Apaf-1-independent death in development (e.g. cells of the interdigital webs) occur a couple of days later.
- Cell death can sometimes proceed in the absence of caspases.
- Caspase inhibitors block the apoptotic phenotype, death in cell lines proceeds when induced by a variety of agents. An exception is cell death induced by ligation of death receptors; in this case the commitment is dependent on caspases and inhibitors therefore maintain cell viability.
- Mitochondrial damage leads to disruption of electron transport, generation of reactive oxygen species and so on-- do cells die due to disruption of mitochondrial function?

Apoptosis and cancer

- Since mitochondrial changes may be lethal, whether or not caspases are activated, tumor cells often express antiapoptotic proteins that act on the level of the mitochondria, such as Bcl-2 and Bcl-XL.
- On the other hand, tumor cells do not appear to select for cells with defects in caspase activation, because the lack of caspase activation may not provide a significant survival advantage.
- Thus, it is possible to use this intact apoptotic machinery to induce tumor cell apoptosis: e.g. activate caspases in tumor cells through inhibition of IAP.

The integrated apoptotic pathways and cancer: upstream regulators



Red components: inhibit apoptosis; Green components: promote apoptosis;
*: frequently mutated or aberrantly expressed in human cancers.

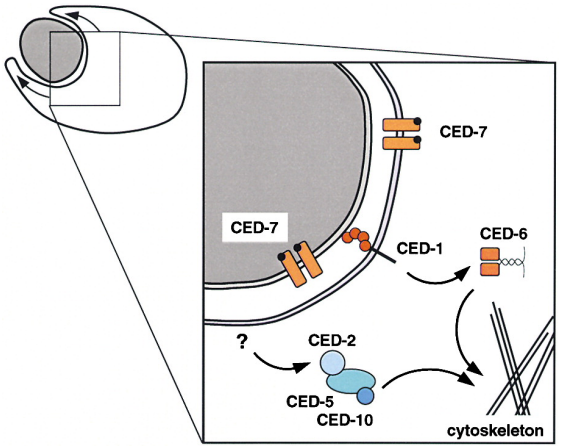
Recognizing death: how the organism disposes of its dying cells

- In mammals, a dozen heterogeneous candidate receptors have been described that promote recognition and/or internalization of apoptotic cells. These receptors belong to scavenger receptors, including SR-A, CD36, CD14.
- Genetic studies in Drosophila showed that croquemort (CD36 homologue), is required for the removal of apoptotic cells during development.
- What do these receptors recognize? One likely candidate is phosphatidylserine (PS), which is normally confined to the inner leaflet of the plasma membrane, but is present in the outer leaflet in apoptotic cells. It is not clear how the loss of phospholipid asymmetry is achieved. However, this PS exposure is a quite specific marker of apoptosis, which may act as an engulfment signal.
- There appears to be redundancy in this process. Blocking a particular candidate signal or receptor leads to partial block in the uptake of apoptotic cells.

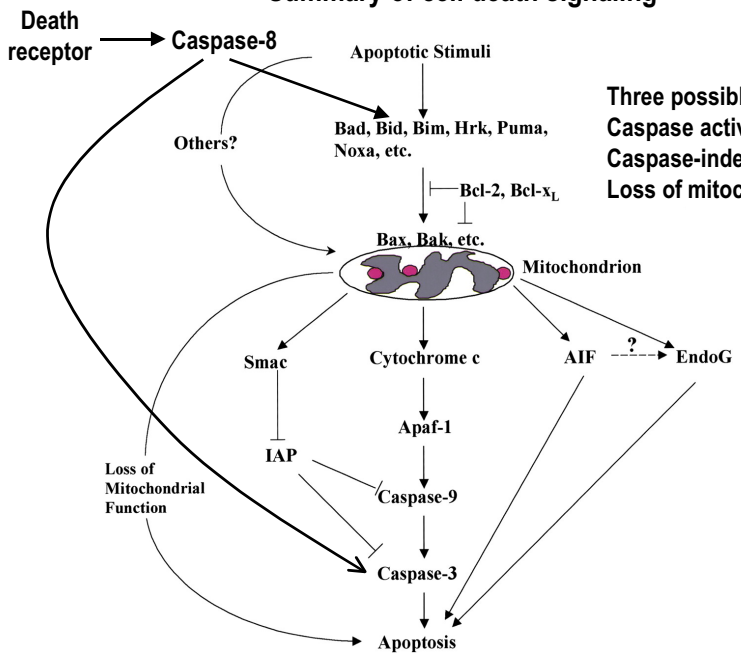
Genetic studies in worm reveal the engulfment machinery: essential genes/proteins for clearance of apoptotic cells

- The first group: may be essential for cell migration and engulfment.
  - CED-2/CED-5/CED-10 complex relays information from the cell surface to the cytoskeleton and induces the cytoskeleton rearrangement.
- The second group: may be involved in recognition of apoptotic cells.
  - CED-1: a scavenger receptor, highly expressed on large cells. It is clustered on membranes facing apoptotic cells and on internal membranes surrounding fully engulfed corpses. May recognize PS? Intracellular domain contains SH2 (YXXL) and PTB (NPXY) sites.
  - CED-7: required for CED-1 clustering around the apoptotic cell. Its mammalian homologue, ABC1 transporter, plays a role in cholesterol efflux. It is required on both apoptotic and engulfment cells.
  - CED-6: intracellular signaling molecule of CED-1? It contains a PTB domain, coiled-coil, and potential SH3 sites.

Model for the engulfment of apoptotic cells



Summary of cell death signaling



Three possible mechanisms:
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Pressing issues

- How do the wide range of apoptotic signals such as developmental cues, UV radiation, glucocorticoid treatement and other stress signals engage apoptotic pathways (i. e. mitochondria)?
- Where is the point of no return in these pathways?
- What does the apparent differences between the apoptotic paradigms in C. elegans and in mammals mean?
- Caspase-independent processes in apoptosis?
- How do these pathways may be best utilized for therapeutic means?