Molecular Biology of Apoptosis

The pathways that regulate cellular suicide, also known as programmed cell death - the price of being a metazoan
Apoptosis is characterized by characteristic morphological changes that occur in a stereotyped pattern.  
1972, seminal paper from Kerr, Wyllie and Currie  

Greek Derivation  

Apo-ptosis = “dropping off” or “falling off” as leaves from a tree - can be thought of as cells dying and dropping off their normal tissue  

Pronounciation:  
ancient Greek: “ay-Poh-toh-sys”  
modern Greek: “ay-pop-tuh-sys”
Programmed Cell Death

Required for the proper maintenance of adult tissues…

• 500 billion blood cells are eliminated by apoptosis every day to balance continual production

• A mechanism for the removal of damaged cells
  - cancerous or precancerous cells
  - radiation damaged cells
  - virally-infected cells
PCD in Development

...required to sculpt tissue during embryonic development

sexual differentiation

digit formation

metamorphosis
Apoptosis in *Drosophila* head segmentation

- **A**: Normal segment boundary
- **B**: Reaper mutant
- **C**: No apoptosis

Labels:
- ci
- olp
- P
- md
- mx
- lb
Apoptosis in the developing nervous system

- Neurons are produced in excess
- Up to 50% of developing neurons die
- Those that live do so because they receive survival signals from target cells
Cellular morphological changes during apoptosis

- DNA fragments and nuclei condense
- Dying cells shrink, condense and then fragment into apoptotic bodies
- Intracellular contents are not released
TEM and SEM of apoptotic membrane blebbing

DNA fragmentation to ~180 bp fragments
Apoptosis versus cellular necrosis

Apoptotic bodies engulfed by phagocytes
No spillage of cell contents
No inflammatory response

Cell and nuclear swelling
Rupture and spillage of cell contents
Inflammatory response!
Programmed cell death in *C. elegans*

- Of the 1090 cells of the nematode, 131 are removed by apoptosis during development (Sulston and colleagues)
- Genetic screens by Hedgecock and Horvitz for apoptotic genes identified *cell death* or *ced* mutants
- *ced* mutants identified apoptotic pathway components - in *ced-3* and *ced-4* mutants, cells live that normally suicide, in *ced-9* mutants, most cells go through apoptosis
Nobel prize for apoptosis, and C. elegans biology

Sydney Brenner NL

Ed Hedgecock IPD

John Sulston NLNS

Bob Horvitz NL
In 1992, Hengartner and Horvitz discovered that worm CED proteins had mammalian homologs
an example of the "principle of biological universality"

• The *ced-3* gene encodes a caspase: a protease that catalyzes cell death
• The *ced-4* gene encodes an apoptotic adaptor protein (mammal version, Apaf-1) that binds to caspases and promotes their activation
• The *ced-9* gene encodes a Bcl-2 class protein that inhibits *ced-4* and thus caspase activation
• the *egl-1* gene (apoptotic promoter) encodes a BH3-only domain protein, and inhibits *ced-9*
Overview of evolutionarily conserved apoptosis pathway

Egl-1

BH3 class

C. elegans

Vertebrates

Regulator

Adapter

Effector

Ced-9

Ced-4

Ced-3

Death

Bcl-2

Apaf-1

Casp9

Casp3

Death

C. elegans

Vertebrates

Regulator (Ced-9)

Ced-4

Adaptor (Ced-4)

Caspase (Ced-3)

Autocleavage

Active caspase (Ced-3)

Cell death

Mammals

Regulator (Bcl-2 family members)

Cytochrome c

Adaptor (Apaf-1)

Caspase-9

Autocleavage

Active caspase-9

Downstream caspases

Cell death
Molecular tests for apoptosis (versus simple cellular toxicity)

1. Loss of mitochondrial membrane potential, called delta psi m (TMRE, MitoTracker, DiOC$_6$) very early marker of apoptosis
2. Activation of caspases (Antibodies for cleaved forms of caspases)
3. Phosphatidylserine detection (Annexin V)
4. Loss of plasma membrane integrity (acridine orange, propidium iodide)
5. Cleavage of nuclear DNA (DNA laddering and TUNEL)
AnnexinV staining for phosphatidylserine

- phosphatidylserine is normally found on the inner leaflet of the plasma membrane
- in apoptotic cells it flips out so that it resides on the outer leaflet of membrane
- PS may serve as an “eat me” signal for phagocytes
- AnnexinV has high binding specificity for PS and other negatively charged phospholipids when calcium ions are present

red - annexinV stain
Acridine orange stains of *Drosophila* embryos reveals patterns of developmental apoptosis
TUNEL staining detects dying apoptotic nuclei

TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick End Label (requires double strand DNA with exposed 3’OH)

rat brain before chemically induced "stroke"

rat brain after "stroke"
TUNEL stain- brown
Effector and initiator caspases in the linear apoptosis pathway

C. elegans: Egl-1 → Ced-9 → Ced-4 → Ced-3 → Death

Vertebrates: BH3 class → Bcl-2 → Apaf-1 → Casp9 → Casp3 → Death

C. elegans:
- Regulator (Ced-9)
- Adaptor (Ced-4)
- Caspase (Ced-3)
- Active caspase (Ced-3)
- Cell death

Vertebrates:
- Regulator (Bcl-2 family members)
- Cytochrome c
- Adaptor (Apaf-1)
- Caspase-9
- Active caspase-9
- Downstream caspases
- Cell death
Caspases

- **Cysteine ASpartate ProteASES** (active site cysteine, cleave after aspartic acid residues) aka peptidase C-14 family - Caspase 8 prefers Leu/Val-Glu-X-Asp

- three domain proteins made as inactive proenzymes

- usually activated by caspase cleavage

- One class are initiator caspases e.g. caspase 8, that cleave and activate effector caspases (amplifiers)

- Second class are effector caspases (ced-3, caspase-3) that cleave many different cellular targets that results in blebbing, DNA fragmentation, cellular fragmentation
<table>
<thead>
<tr>
<th>Caspases</th>
<th>mammalsian</th>
<th>Worms: 1 - CED-3</th>
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<tbody>
<tr>
<td>caspase 1</td>
<td>ICE (interleukin-1β converting enzyme), pro-inflammatory</td>
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<tr>
<td>caspase 2</td>
<td>NEDD2/Ich-1L</td>
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<tr>
<td>caspase 3</td>
<td>CPP32/YAMA/Apopain</td>
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<td>ICE_{relII}/Tx/Ich-2</td>
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<td>caspase 6</td>
<td>Mch2</td>
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<td>caspase 7</td>
<td>Mch3/CMH-1/ICE-LAP3</td>
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<td>caspase 8</td>
<td>Mch5/FLICE/MACH</td>
<td>mouse mutant- heart defects</td>
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<td>caspase 9</td>
<td>Mch6/ICE-LAP6</td>
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<td>caspase 10</td>
<td>Mch4/FLICE-2</td>
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<td>caspase 14</td>
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Effector Caspase targets

substrates that lead to apoptosis:

- gelsolin (depolymerize F-actin; membrane blebbing)
- ICAD (Inhibitor of Caspase Activated DNase)
- Lamin (disassembly of nuclear membrane)
- Bcl-2 (block antiapoptotic signaling)
- FAK (cell shrinkage and detachment)
- Akt/PKB (block antiapoptotic signaling)
- hnRNPs (shut down of RNA synthesis)

Caspases also cleave procytokines in non-apoptotic pathways
Normal Lamin staining, with a few apoptotic cells
Effector caspases consist of cleaved homodimers formed of two p20 and two p10 subdomains.

Initiator caspses are homodimers that autoactivate by intimate proximity, requires apoptosome, possess Death Effector Domains (DED) or CASpase Recruitment Domains (CARD).
Adaptors in the linear apoptosis pathway

Egl-1

C. elegans

BH3 class

Vertebrates

C. elegans

Regulator (Ced-9)

Ced-4

Adapter (Ced-4)

Caspase (Ced-3)

Auto cleavage

Cell death

Vertebrates

Bcl-2

Apaf-1

Casp9

Casp3

Death

Mammals

Regulator (Bcl-2 family members)

Cyt c

Caspase-9

Auto cleavage

Active caspase-9

Downstream caspases

Cell death
Apaf-1(CED-4) is the scaffolding protein of the apoptosome

- Apaf-1 is homologous to worm CED-4
- Contains N-terminal **CAspase Recruitment Domain** domain (CARD domain), which binds the CARD domain of procaspase 9
- also binds dATP
- C-terminal WD-40 domain repeats of Apaf-1 bind Cytochrome c
The Apoptosome

- 1.4 Mda complex that includes Apaf-1 (Apoptotic protease activating factor), procaspase 9 & cytochrome c
- components purified from 100L of apoptotic HeLa cells (Xiaodong Wang, ‘96–’97)
- facilitate caspase-9 activation by bringing procaspase 9 molecules in intimate contact, triggering cross-cleavage
cytochrome c release from the mitochondrion triggers the formation of the apoptosome
Bcl-2 class regulators in the linear apoptosis pathway

Egl-1

BH3 class

C. elegans

Vertebrates

Ced-9

Ced-4

Ced-3

Death

Bcl-2

Apaf-1

Casp9

Casp3

Death
Regulators of Apoptosis, the most important for the intrinsic (mitochondrial) pathway is the Bcl-2 protein (CED-9 homolog)

- In mammals, Bcl-2 was discovered as an "oncogene" at a translocation breakpoint in B-cell lymphomas. The translocation results in the overexpression of Bcl-2, which blocks death
- There are many Bcl-2-like proteins, some of which have anti-apoptotic functions, some of which are pro-apoptotic
The Bcl-2 (Egl-1) Family

anti-apoptotic
mammalian
Bcl2, BclXL, Bclw, Mcl1, A1/Bfl1, Boo/Diva, Nr13, CED-9

C. elegans

pro-apoptotic
'multi-domain'
mammalian
Bax, Bak, Bok/Mtd

'BH3 domain-only'
Bld, Bad, Bik/Nbk, Bik, Hrk, Bim/Bod, Bnip3, Nix, Noxa, EGL-1

C. elegans

Pro-survival
Bcl-2 Subfamily

Regulation
Dimerization (receptor) domain
Membrane anchor

Pro-apoptosis
Bax Subfamily

Ligand domain
The mitochondrion and apoptosis

- Known that apoptosis resulted in disruption of electron transport, loss of mitochondrial membrane potential.
- Shown that in Xenopus egg extracts, suppression of "apoptosis" by recombinant Bcl-2 required an organelle fraction that was rich in mitochondria (Don Newmeyer & John Reed, 1994)
• Bcl-2 binds BH3 domains in BH3 killer proteins
• One way to overcome Bcl-2 inhibition is to transcriptionally activate genes for Bad, Bid, or NoxA, which overwhelm Bcl-2, and results in activation of Bax or Bak
• Bax or Bak multimerize, insert into mitochondrial outer membrane lead to loss of membrane potential, opening of permeability transition pore, release of cytochrome c, multimerization of Apaf-1

promoters of PCD: Bak, Bax
inhibitors of PCD: Bcl-2, Bcl-\(X_L\)
BH3 killers:  Bad, Bid, NoxA
Many intrinsic and extrinsic pathways result in activation of BH3 domain killers

- BH3 proteins are potent killers.
- **Intrinsic transcriptional regulation:** Noxa is p53 dependent
- **Extrinsic promotion of BH3 killers:** Bid is cleaved by caspase-8 to truncated Bid (t-Bid)
- **Regulation by sequestration:** Bad is phosphorylated, bound to 14-3-3, Bim is bound to dynein light chain, all can bind to Bcl-2

25 aa Bad peptide complexed with Bcl-x<sub>L</sub>
p53 regulation of apoptosis

Hypoxia

DNA damage

p53

Noxa, Puma (BH3)
Regulation from the "side" of the central apoptotic pathway (buffer regulation?)

RHG proteins

\[ \downarrow \]

IAP proteins

\[ \downarrow \downarrow \]

Egl-1 \[ \leftarrow C. elegans \]

Ced-9 \[ \rightarrow\] Ced-4 \[ \leftrightarrow\] Ced-3 \[ \rightarrow\] Death

BH3 class \[ \leftarrow \text{Vertebrates} \]

Bcl-2 \[ \rightarrow\] Apaf-1 \[ \rightarrow\] Casp9 \[ \rightarrow\] Casp3 \[ \rightarrow\] Death
Inhibitor of Apoptosis Proteins (IAPs)

- bind Apaf-1, procaspases and activated caspases and inhibit their activity
- examples include cIAP1, xIAP, cIAP2, DIAP (Drosophila), p35 protein (baculovirus)
- a death signal must remove this inhibition
- this occurs through binding of Reaper, Head involution defective (Hid), or Grim- the RHG proteins, mammal analogs are SMAC, Diablo

**IAP domain structure**

- BIR1: binds RHG proteins
- BIR2: binds pro-Dronc (fly Apaf-1)
- RING: required for ubiquitination of DIAP1

**BIR - Baculovirus IAP Repeats**

- bind Apaf-1, procaspases and activated caspases and inhibit their activity
- examples include cIAP1, xIAP, cIAP2, DIAP (Drosophila), p35 protein (baculovirus)
- a death signal must remove this inhibition
- this occurs through binding of Reaper, Head involution defective (Hid), or Grim- the RHG proteins, mammal analogs are SMAC, Diablo
Regulation of Apoptosis - lessons from *Drosophila*

*rpr*, *grim* and *hid* in one genomic region, deleted in Df(H99)

RHG proteins bind DIAP1, which then releases procaspase (Dronc)

RHG proteins induce auto-ubiquitination and degradation of DIAP1
A fly Hox protein, Deformed (DFD), activates *reaper* and apoptosis at a segment border.

*reaper* transcripts - red
DFD protein - green
ectopic *reaper* leads to ectopic apoptosis in fly embryos

acridine orange staining - green

**wild type embryo**

**embryo with ectopic patches of reaper in abdomen**
Extrinsic Death Signals

- **FAS/CD95** (Fas ligand)
- **Tumor Necrosis Factor Receptor** (TNFR (TNF ligand))
- **TNF Related Apoptosis-Inducing Ligand** (TRAIL receptors (TR1-4))

All of these receptors have a cytoplasmic **Death Domain** motif (DD)

Extrinsic activation of Caspase 8 via **Death Domain (DD)** and **Death Effector Domain (DED)** adaptor, e.g. FADD (**Fas Associated Death Domain**)
Extrinsic signals for survival (involvement of Akt/PKB)

Martin Raff proposed in 1992 that the default state for all metazoan cells is apoptosis, they must swim in a sea of survival signals to avoid death.