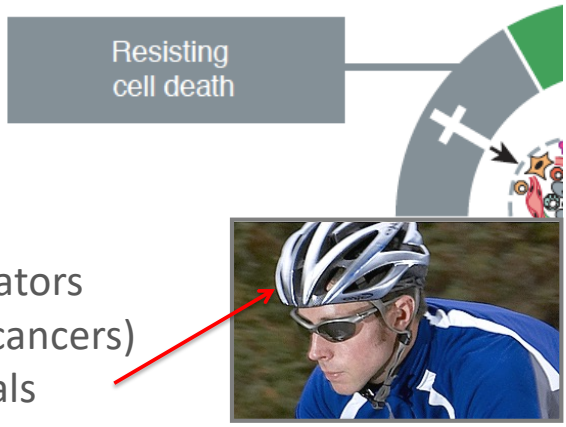
Hallmark: Resisting cell death

Specific barrier: Outlaw cells normally are eliminated by one or another form of 'programmed' cell death (most commonly apoptosis)



Acquired capability:

- inactivation of proapoptotic regulators (e.g. p53 is mutated in 25% of all cancers)
- activation of specific survival signals

Outline

1. Apoptosis as one form of Regulated Cell Death (RCD)
   - Distinctive features of apoptosis, and the role of caspases
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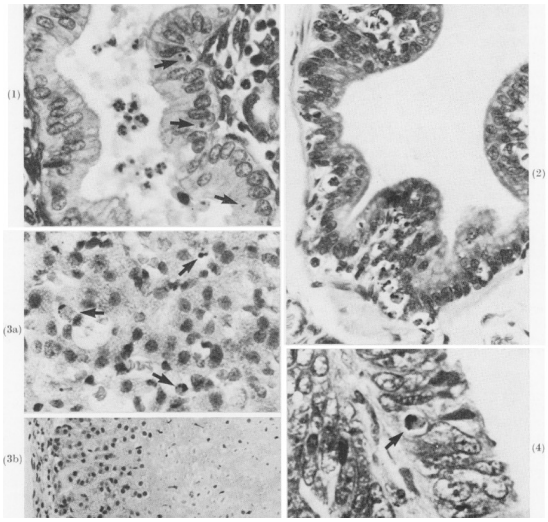
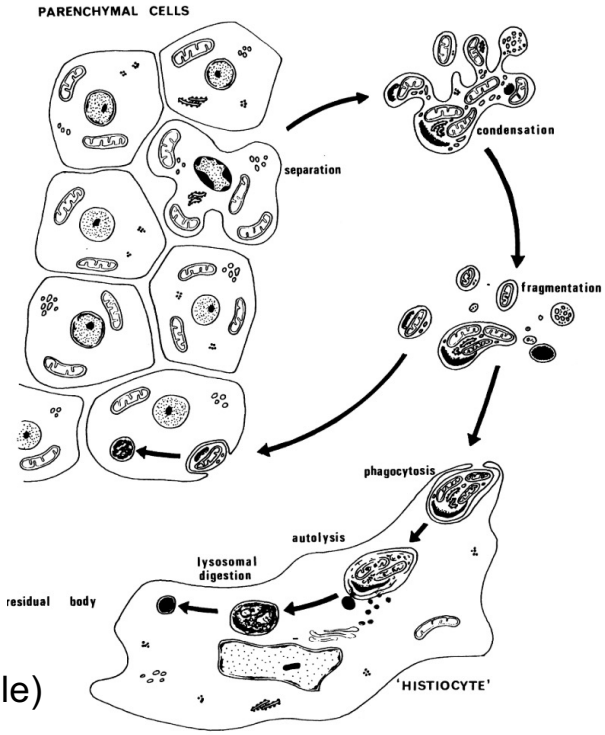
   - Discovery of mTOR and Rapamycin-like inhibitors (Rapalogs)
   - Remaining hurdles for 2nd and 3rd generation mTOR inhibitors

Br. J. Cancer (1972) **26**, 239

APOPTOSIS: A BASIC BIOLOGICAL PHENOMENON WITH WIDE-RANGING IMPLICATIONS IN TISSUE KINETICS

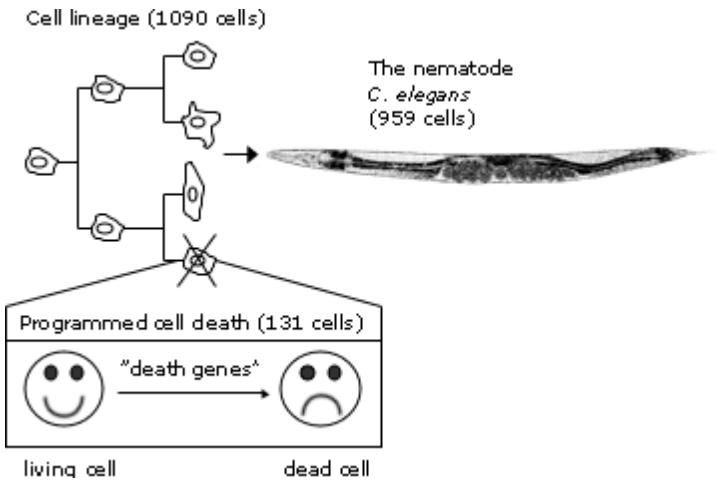
J. F. R. KERR*, A. H. WYLLIE AND A. R. CURRIE†

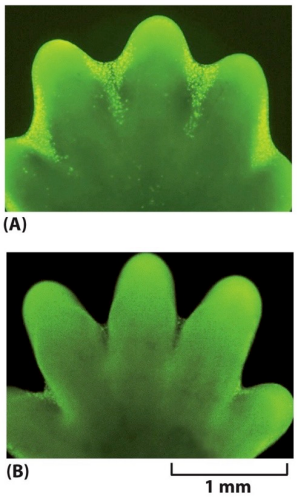




- Hormonally regulated
- Programmed (developmental schedule)
- Contributes to tumor shrinkage by therapies

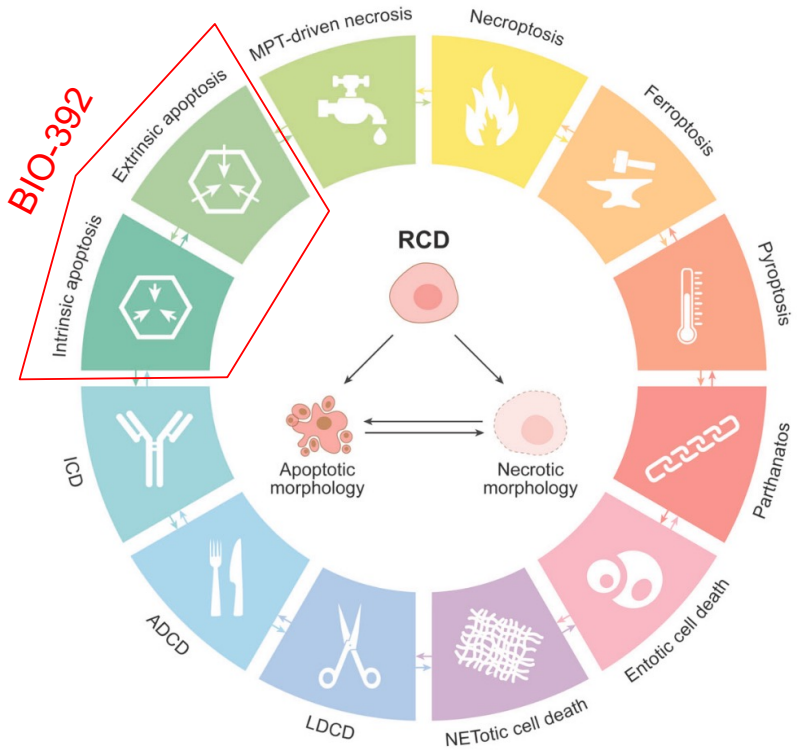
'Programmed' cell death during development





- Elimination of supernumerary cells
- Sculpting tissues&organs (digits, metamorphosis, hollow structures...)
- Homeostasis (e.g. negative selection of >95% of all T- & B-lymphocytes)
- Quality control (✝of stressed cells after DNA damage, infection...)

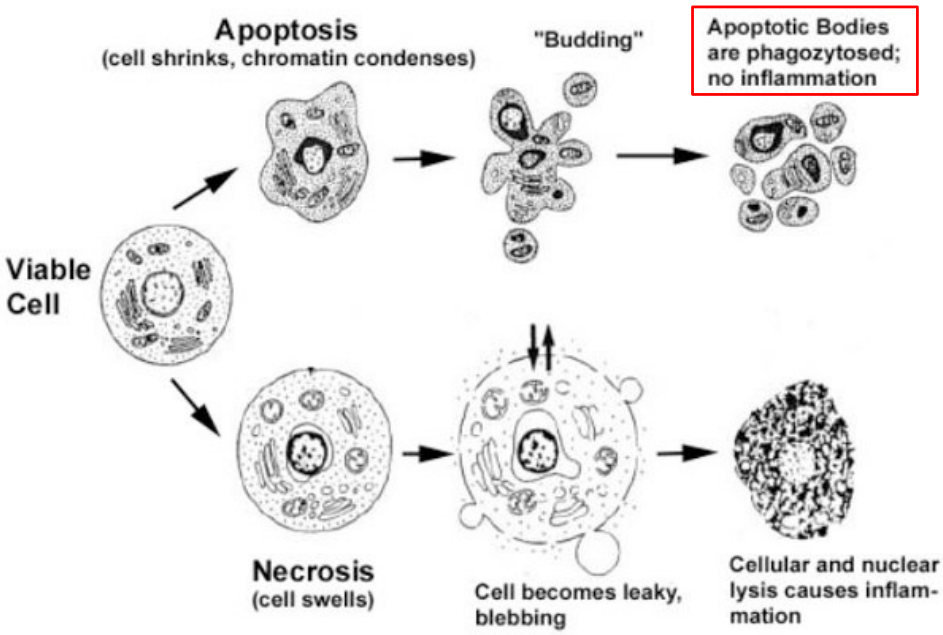
Disclaimer: There is *not* only apoptosis



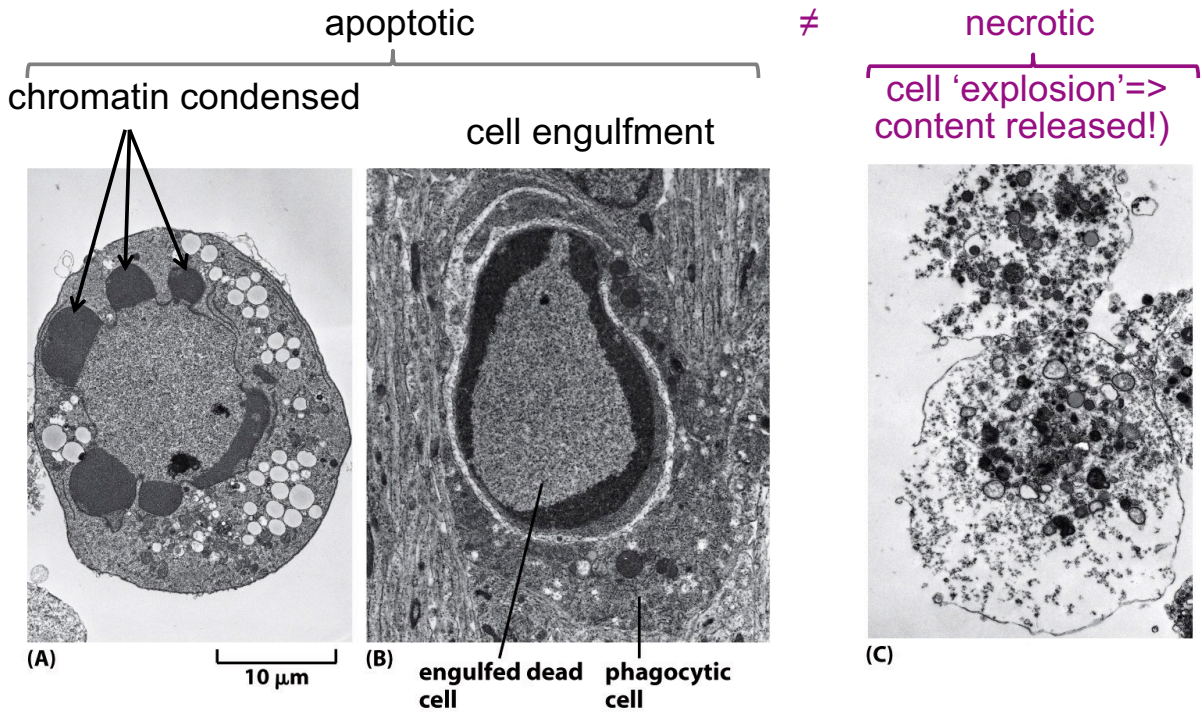
..but also other forms of **<u>regulated</u>** cell death (RCD)

Galluzzi et al. 2018, Cell Death and Differentiation 25:486-541

Distinctive features of apoptosis

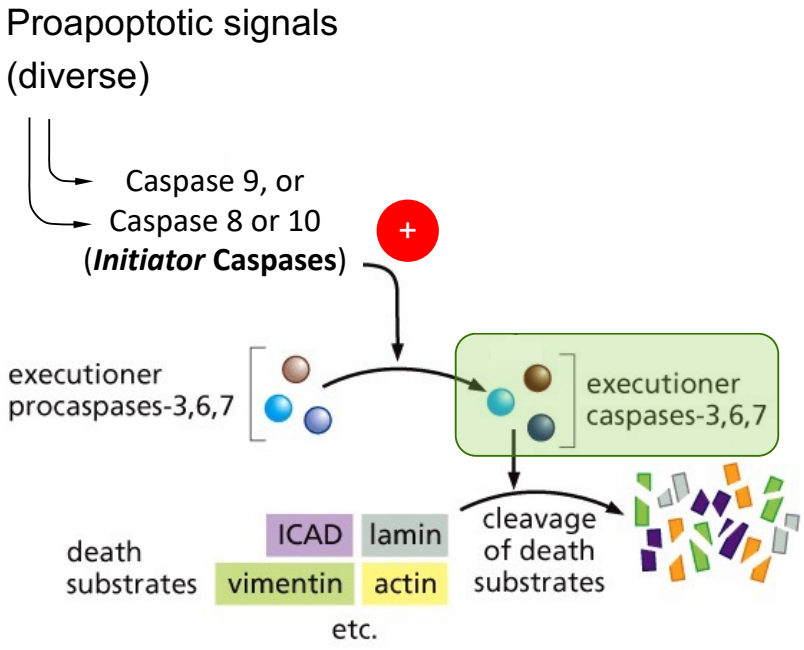


Morphological characteristics of apoptosis



Executioner caspases and their regulation

(cysteine-**asp**artic prote**ases)**



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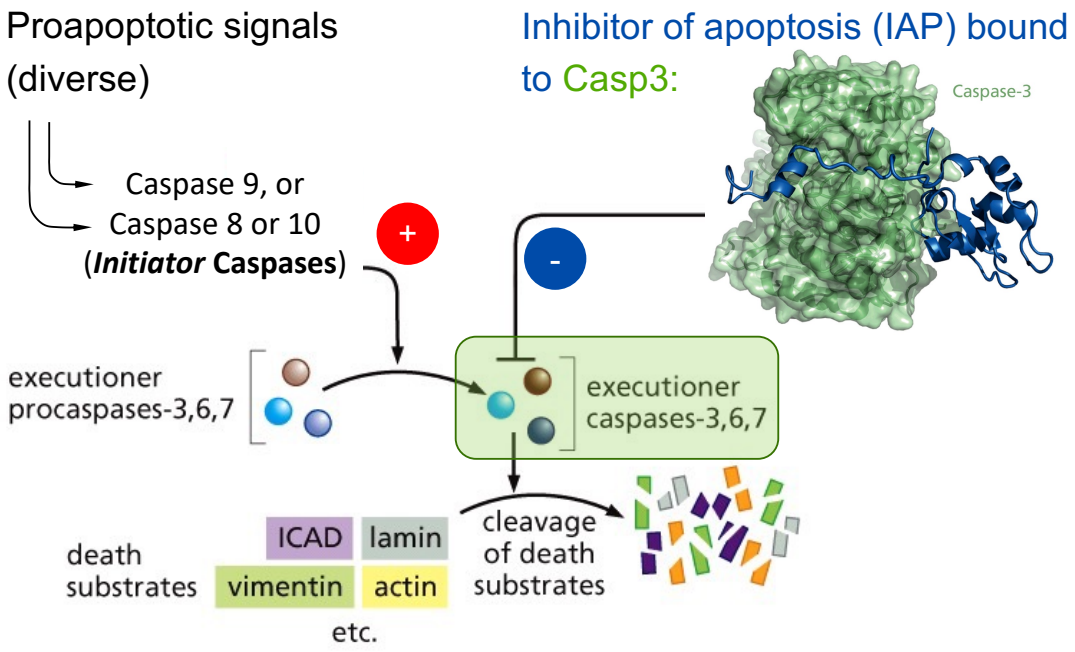

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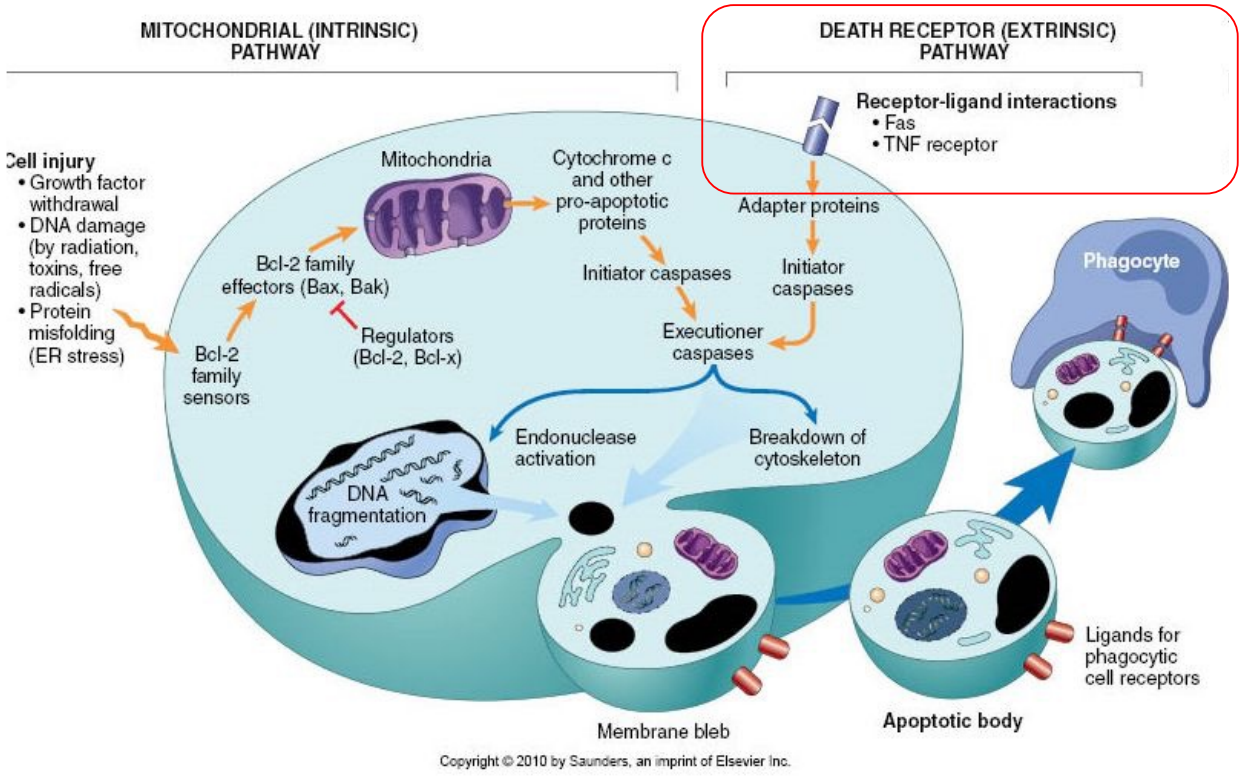
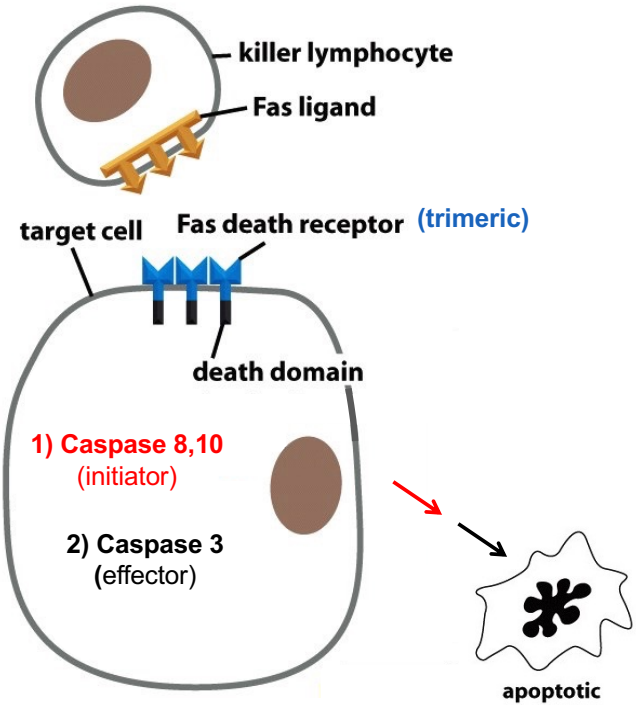
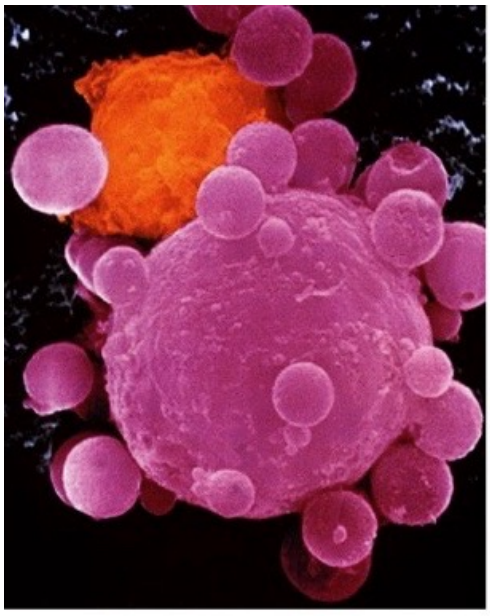
   - Remaining hurdles for 2nd and 3rd generation mTOR inhibitors

Two classes of apoptotic stimuli (intrinsic & extrinsic)



Cytotoxic T lymphocytes (CTL) can activate a *death receptor* in infected or cancerous cells





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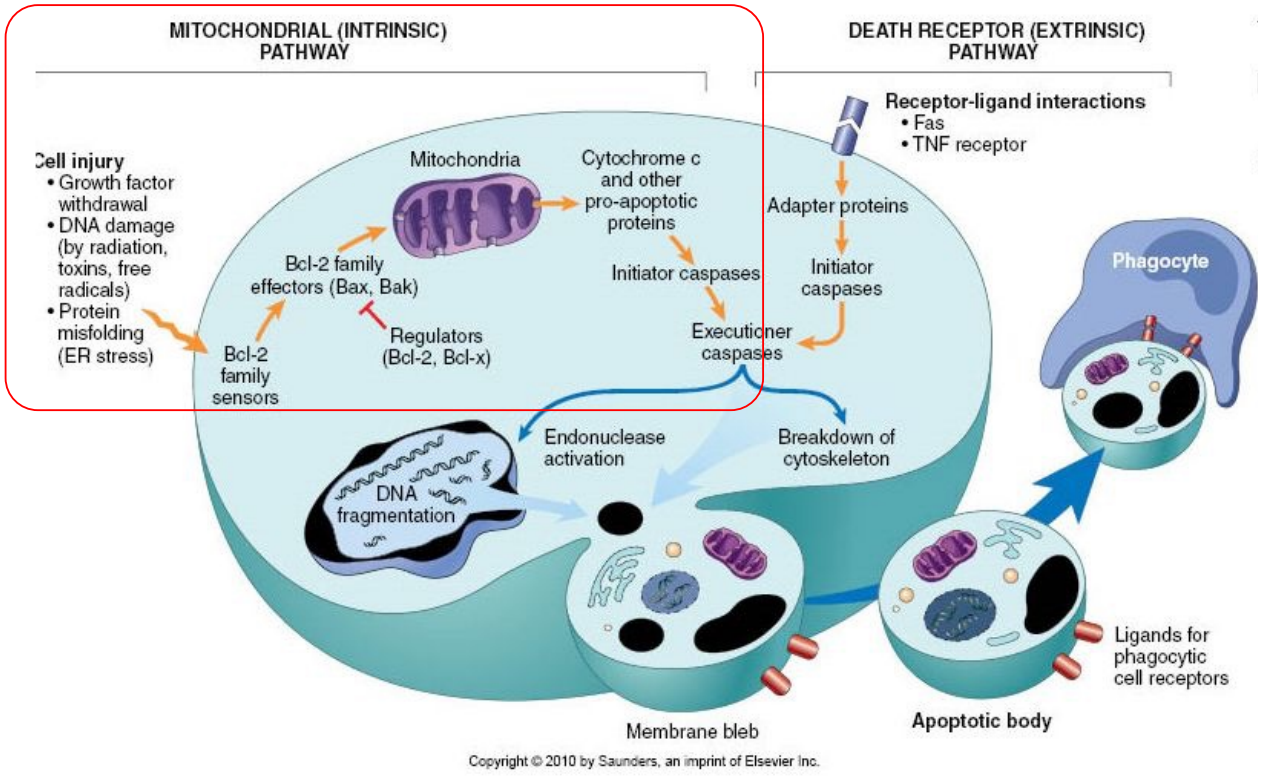
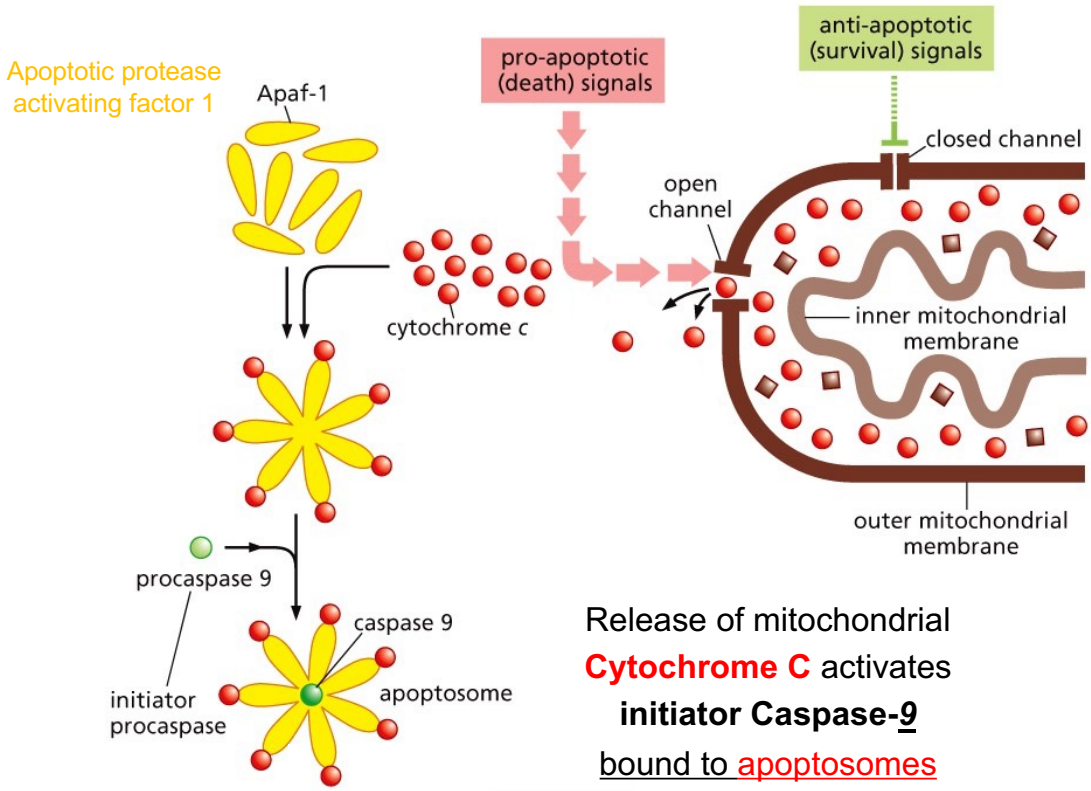


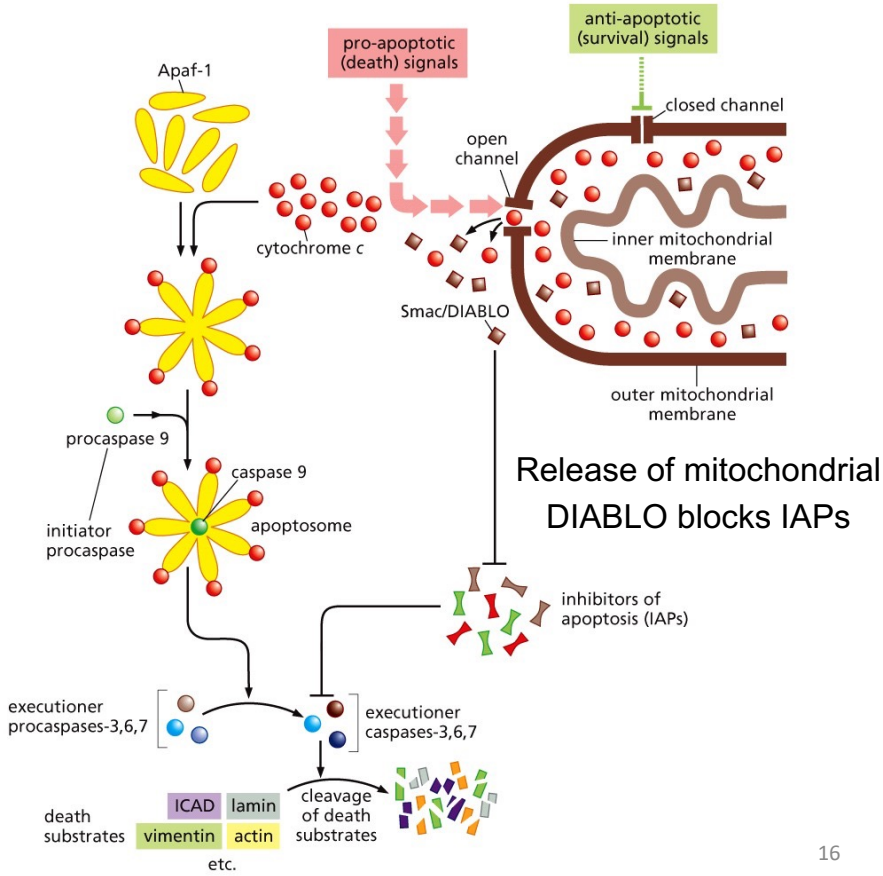
Mitochondrial (intrinsic) pathway: Caspase-9



Release of mitochondrial **Cytochrome C** activates **initiator Caspase-*9*** bound to apoptosomes

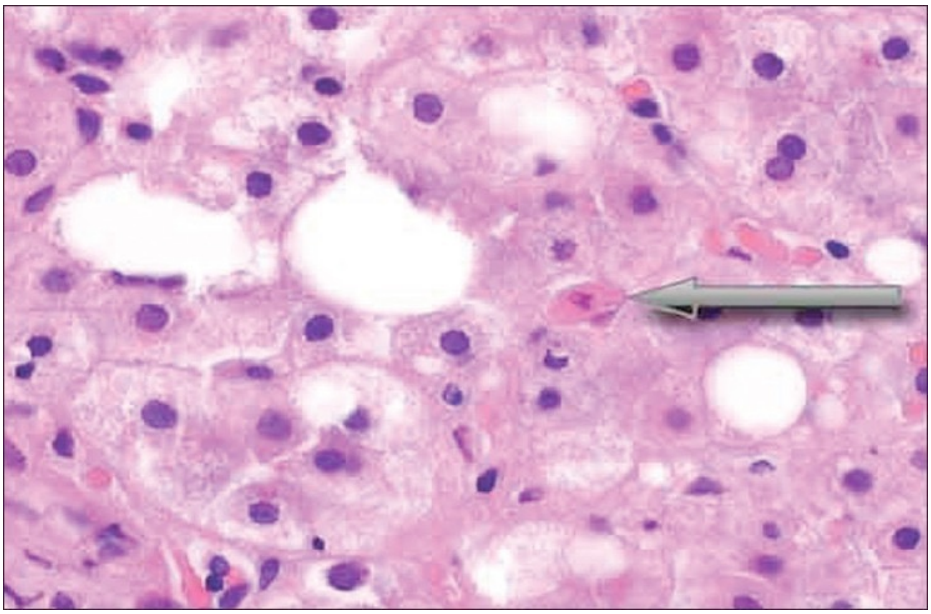
The intrinsic pathway also inhibits IAPs

Release of mitochondrial **Cytochrome C** activates **initiator Caspase-*9*** bound to apoptosomes:



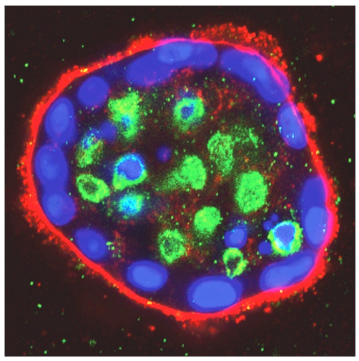
Release of mitochondrial DIABLO blocks IAPs

Detection of apoptosis: How?



Arrow: Example of an apoptotic hepatocyte

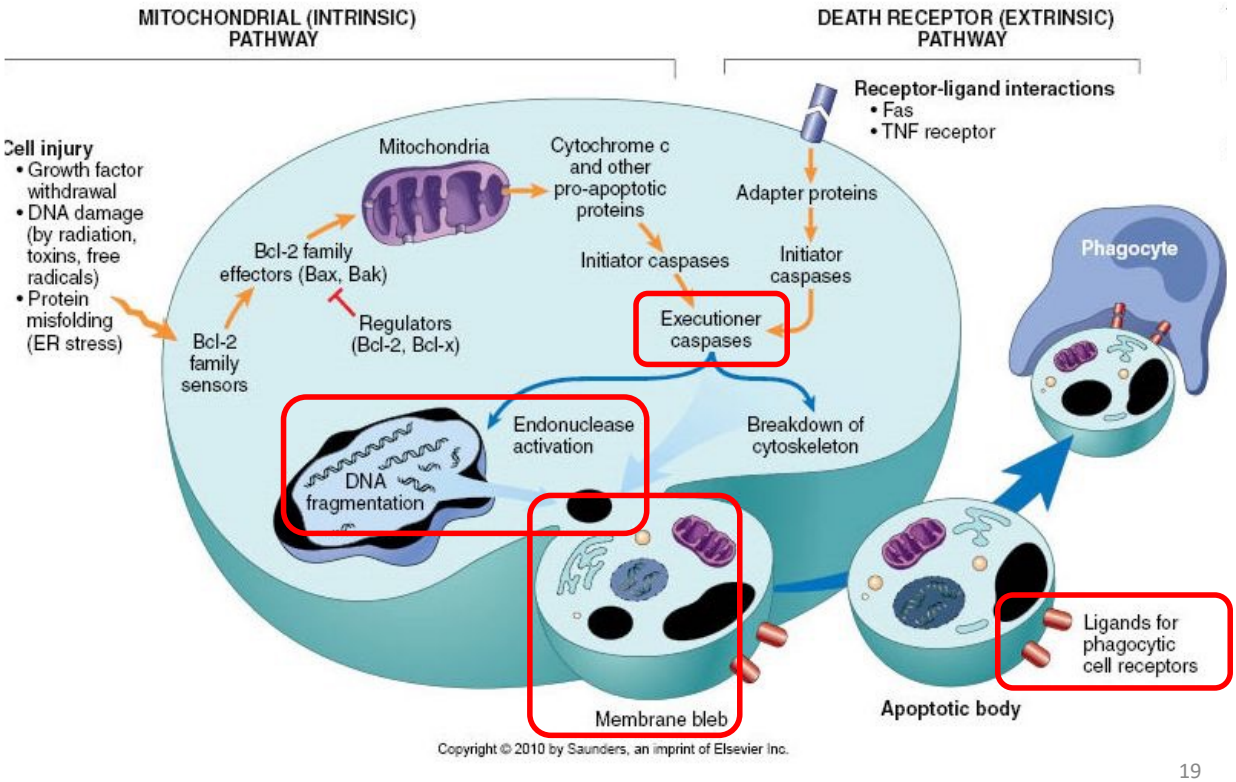
Immunostaing of activated Casp3 marks apoptotic cells



- Normal human mammary epithelial cells (MECs) grown in suspension as spheres form a basal lamina (red: laminin 5)
- Cells that fail to adhere to the basal lamina undergo **apoptosis** (marked by cleaved Caspase-3 staining, green)

Figure 9.22 *The Biology of Cancer* (© Garland Science 2014)

Changes in apoptotic cells induced by Executioner Caspases



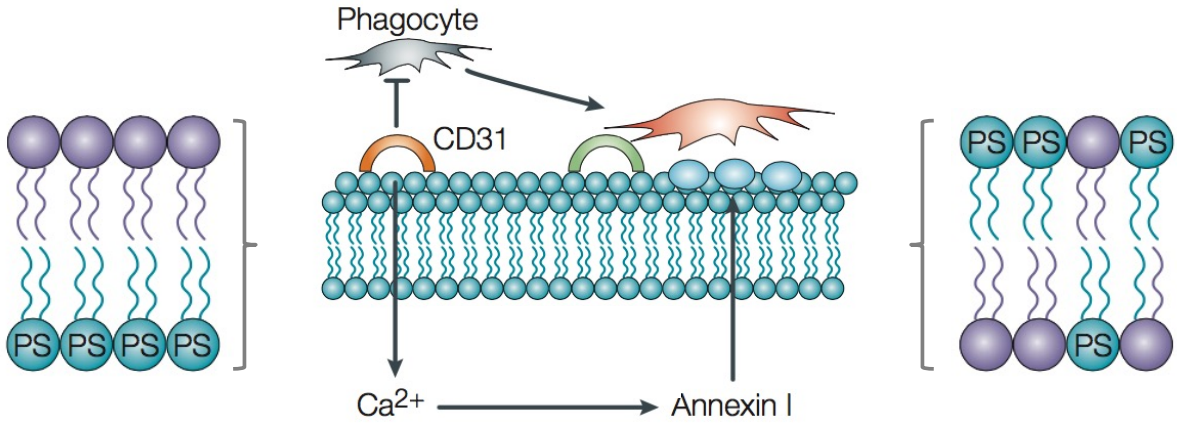
Phagocytosis of apoptotic bodies is induced by specific 'eat-me' signals

Healthy cells:

- translocate all phosphatidyl-serine to the inner leaflet of the plasma membrane
- display 'Don't eat-me' signals, e.g. CD31

Apoptotic cells:

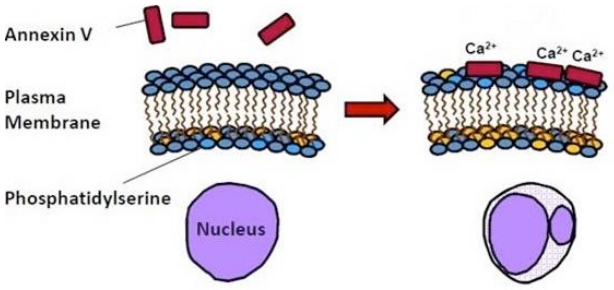
- externalize PS
- complexes of PS with Annexin I, and other factors act as 'Eat-me' signals

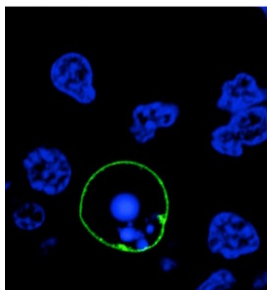


Orrenius et al. 2003 Nat Rev Mol Cell Biol

Four widely used methods to detect apoptosis

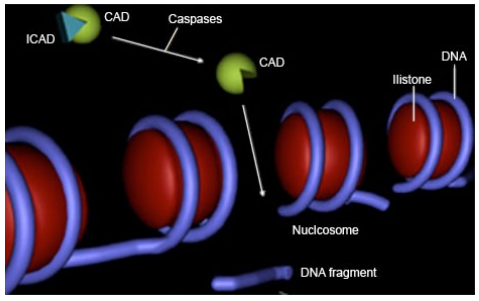
1. Immunostaining of activated caspase-3
2. Annexin V labelling of externalized phosphatidylserine:



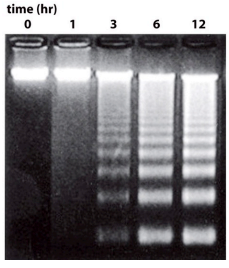


3. Gel electrophoresis of the genomic DNA

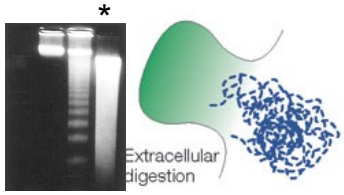
Characteristic fragmentation by Caspase-dependent nuclease (CAD)



180 bp 'ladder':

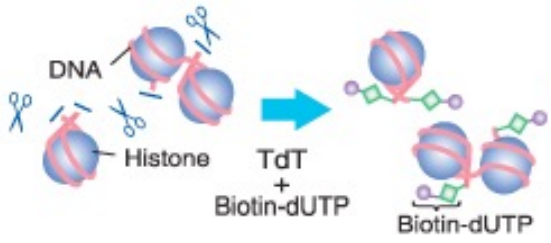


Contrasts the DNA smear of necrotic cells (*):

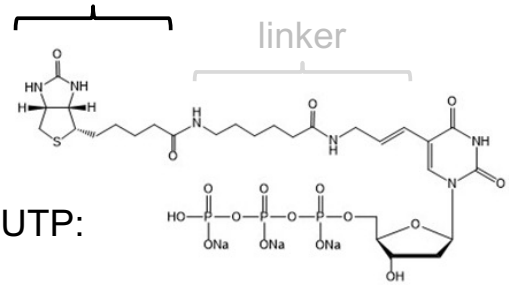


4. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP-mediated Nick End Labelling

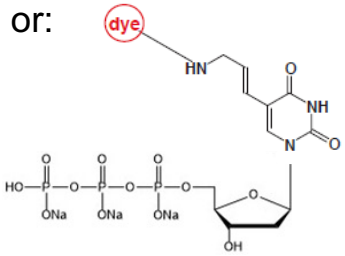


biotin (vitamin B7):



dUTP:

or:



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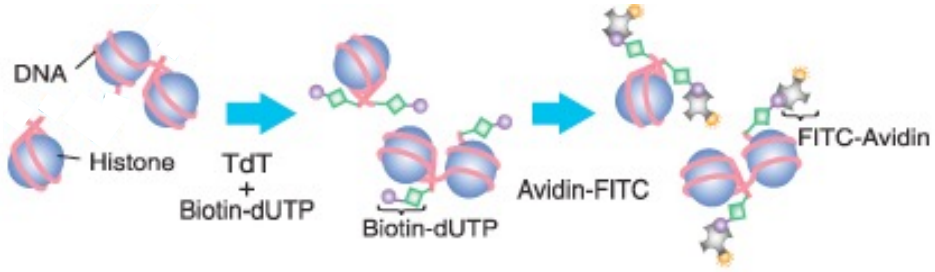
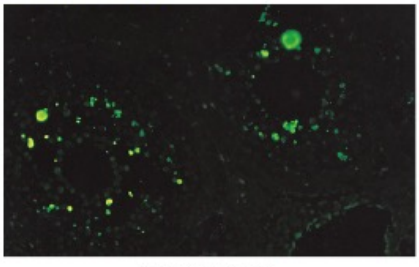
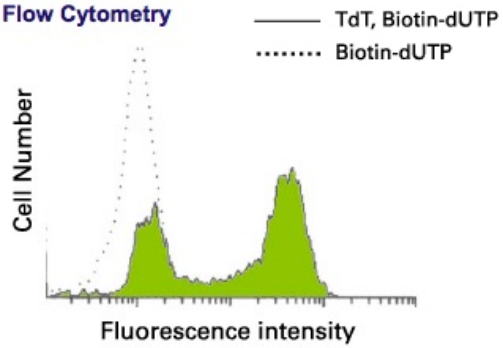


Histochemistry



Mouse ovary

Flow Cytometry



Distinct apoptotic and necrotic features

Feature	Necrosis	Apoptosis
Phagocytosis	Debris of lysed cells ingested by macrophages	Apoptotic bodies engulfed by variety of cells
Inflammatory response	significant	no
Occurrence	pathologic	pathologic and physiologic
Cell size	swelling	shrinkage
Plasma membrane	breaking up	blebbing, but largely intact
Organelles	lysosomal leakage	retained in apoptotic bodies
Chromatin	released	condensed
DNA fragmentation	random	180 bp ladder

Apoptosis – Concepts to remember

- Extrinsic (Fas) or Intrinsic (mitochondrial proteins) stimuli activate distinct 'initiator Caspases'
- 'Executioner Caspases' act downstream by cleaving specific cytoskeletal and other proteins, incl. ICAD
- Inactivation of ICAD by Casp3 activates the DNA nuclease CAD to triggers DNA fragmentation (180 bp ladder)
- Extrusion of phosphatidylserine and other specific "Eat-me" signals trigger phagocytosis of 'apoptotic bodies' *without inflammation*

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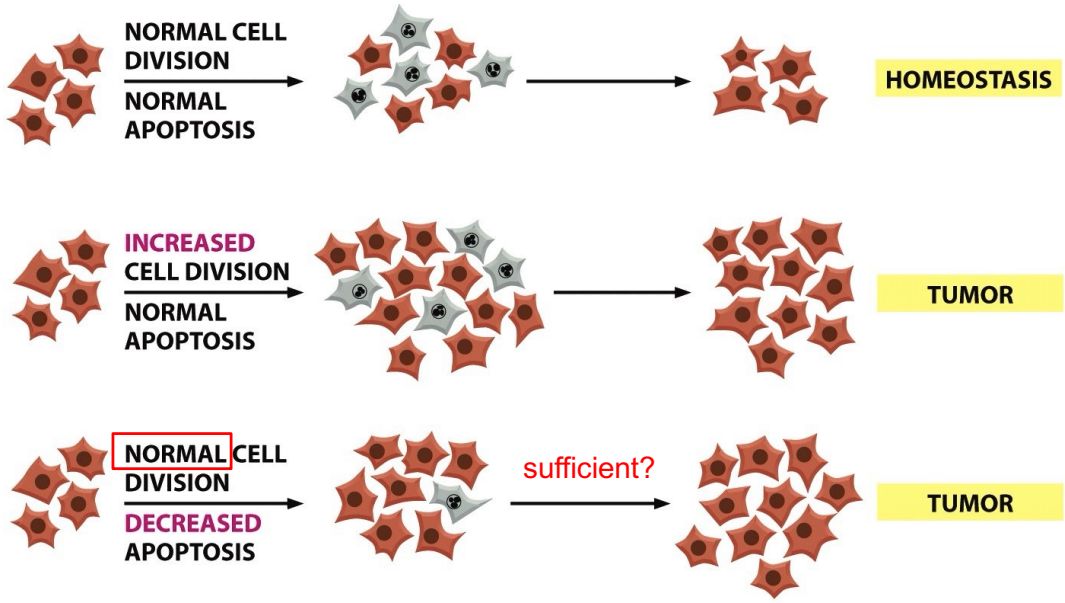
Impact of cell death on tumor volume

In normal adult tissue homeostasis (e.g. liver), unknown mechanisms balance cell death and proliferation:



Impact of cell death on tumor volume

Knockout mice lacking *individual* caspases are not cancer-prone

Nevertheless, caspases are frequently lost or downregulated in human cancers:

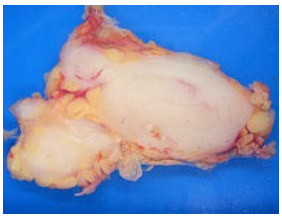
Table 1 Mutations and imbalances of caspase expression reported in human tumors and cell lines

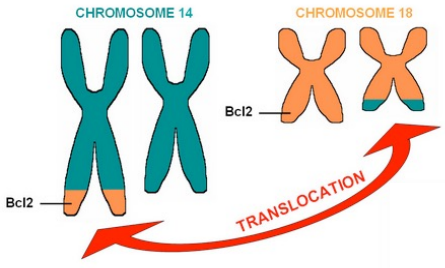
Caspase	Mutation	Protein expression	Cancer	Frequency	Reference
CASP8	Promoter methylation	Low/absent	Childhood neuroblastomas	11/42	Teitz *et al.*[32]
CASP8	Nonsense Missense Frameshift Deletion	NA	Advanced gastric	13/122	Soung *et al.*[37]
CASP8	Nonsense Missense Frameshift	NA	Invasive colorectal	5/98	Kim *et al.*[36]
CASP8	Missense	NA	Head and neck	1/1	Mandruzzato *et al.*[35]
CASP8	Frameshift	NA	Hepatocellular	9/69	Soung *et al.*[37]
Caspase-8	NA	Low/absent	Lung and breast tumor cell lines	6/55	Kischkel *et al.*[48]
Caspase-10	NA	Low/absent	Lung and breast tumor cell lines	31/55	Kischkel *et al.*[48]
Caspase-2	NA	Low/absent	Gastric	78/120	Yoo *et al.*[58]
Caspase-9	NA	Low/absent	Colorectal	12/26	Palmerini[82]
Caspase-6	NA	Low/absent	Gastric	57/120	Yoo *et al.*[58]
CASP7	Nonsense	NA	Colorectal	2/98	Soung *et al.*[81]
CASP7	Missense	NA	Esophageal	1/50	Soung *et al.*[81]
CASP7	Missense	NA	Head and neck	1/33	Soung *et al.*[81]
Caspase-7	NA	Low/absent	Colorectal	22/26	Palmerini[82]
Caspase-7	NA	Low/absent	Gastric	81/120	Yoo *et al.*[58]
Caspase-3	NA	Low/absent	Breast	23/31	Devarajan *et al.*[69]

Abbreviation: NA, not analyzed

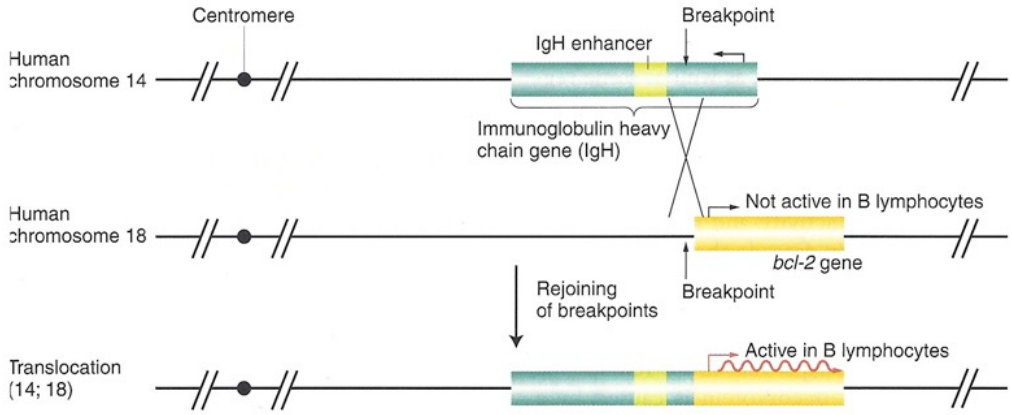
Olsson & Zhivotovsky, 2011, Cell Death and Differentiation 18:1441–1449

Direct evidence for the tumor-promoting role of anti-death signals:
B cell lymphoma 2 (Bcl-2) drives follicular lymphoma formation

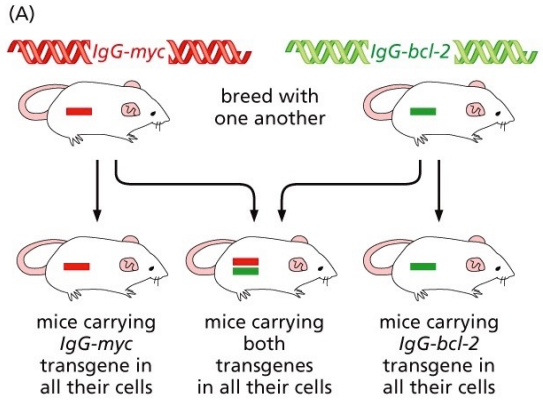


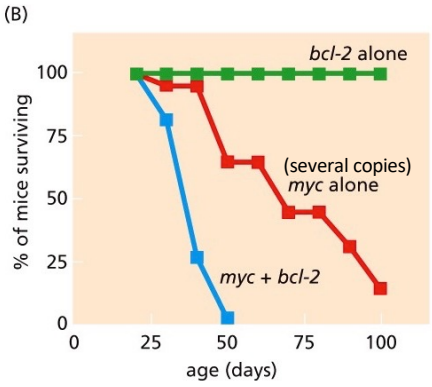


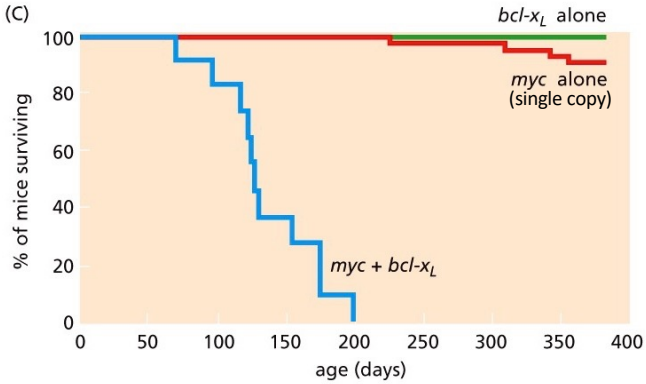
B-cell tumors in lymph nodes arising from *Bcl-2* translocations to IgH gene (=> strong expression in B-cells)



Blockade of apoptosis induces lymphoma (only) if…





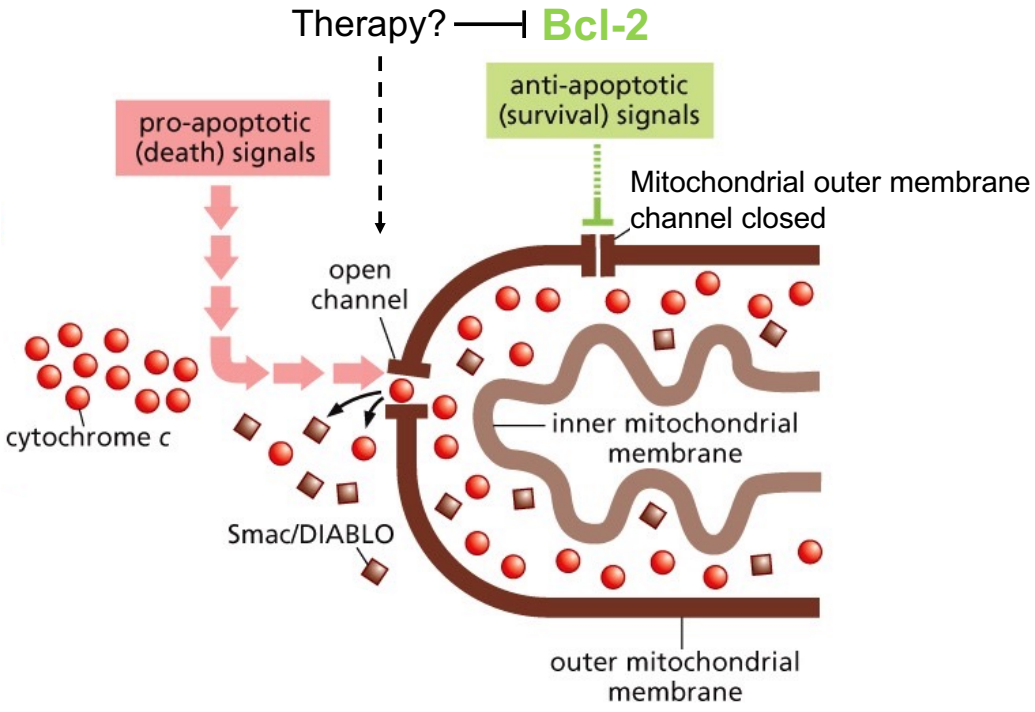


Bcl-2 or Bcl-X_L ***in combination with sustained proliferation signals:***

- e.g. with c-myc upregulation (additional hallmark capability!)

Bcl-2 alone prolongs ***cellular lifespan*** but without increasing cell proliferation

Role of Bcl-2 in sustaining mitochondrial membrane impermeability



Distinct classes of BH domain proteins inhibit or promote apoptotis, respectively

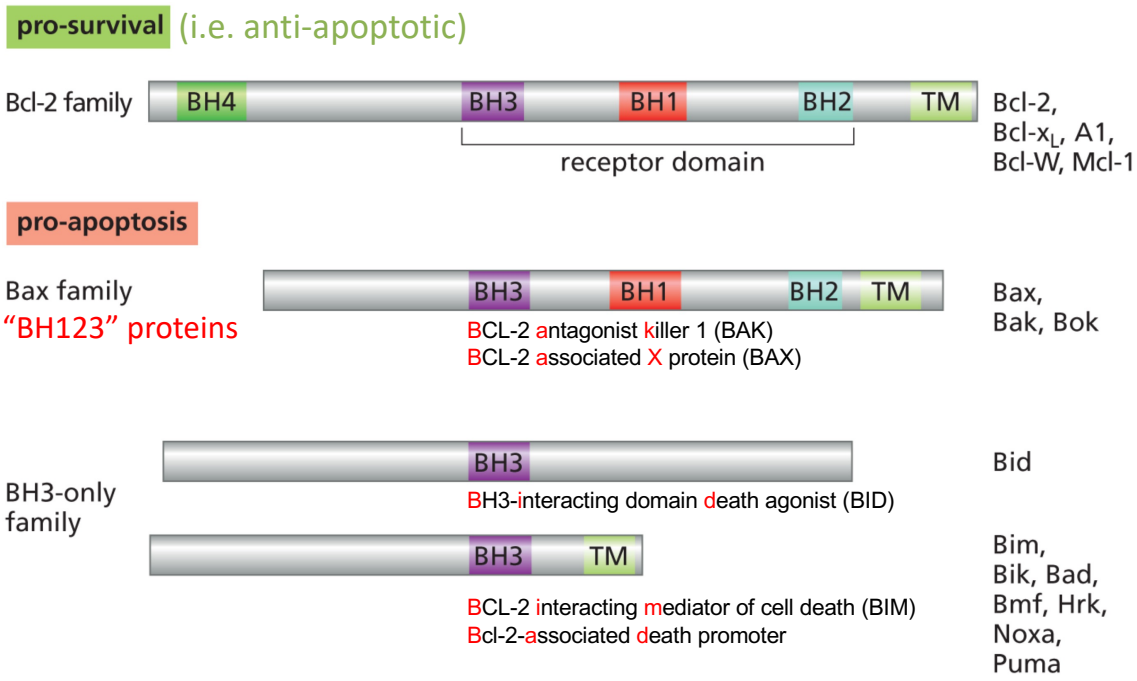
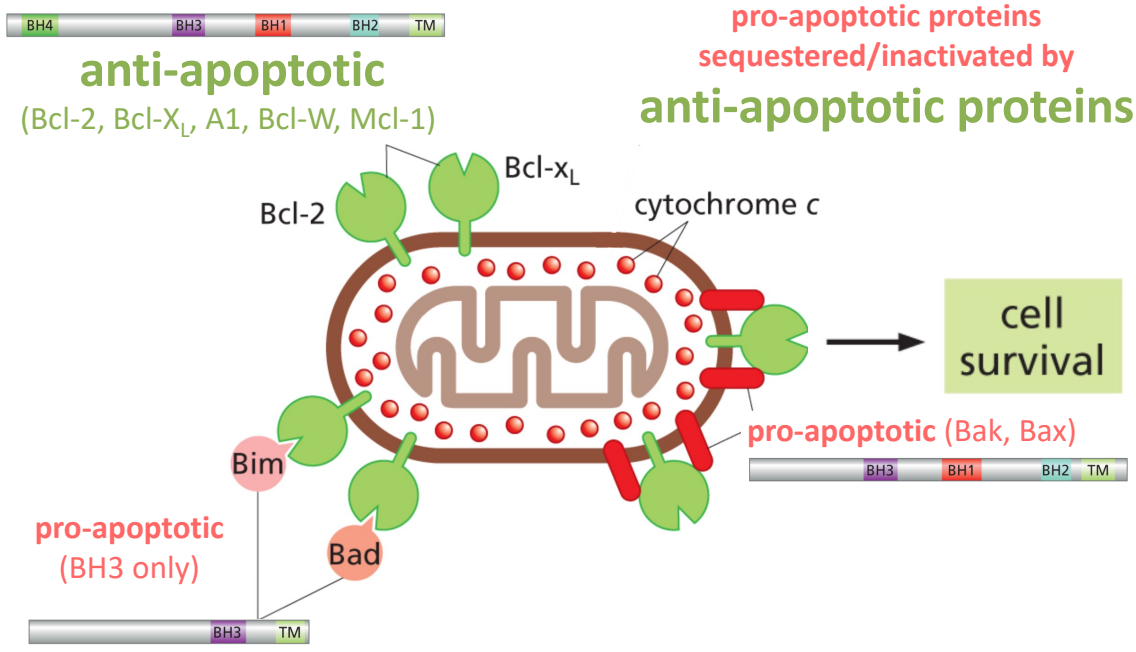


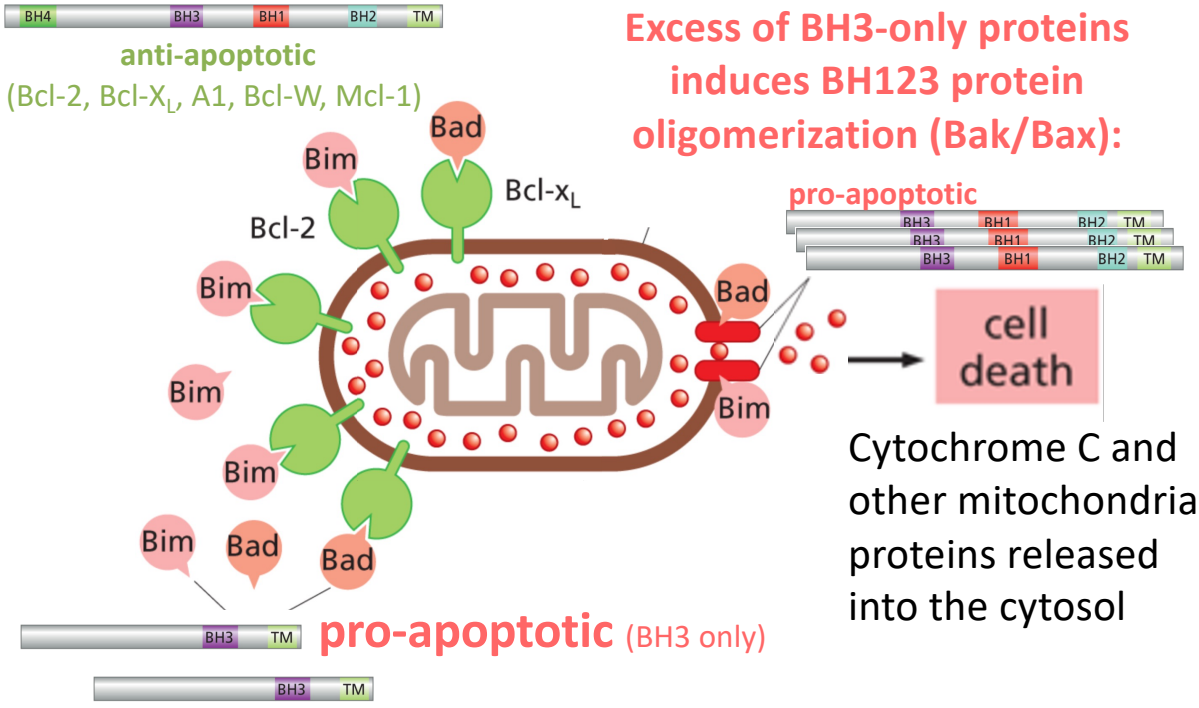
Figure 9.23 *The Biology of Cancer*

Intrinsic apoptosis is controlled by the ratio between pro- & anti-apoptotic BH domain proteins



after Fig. 9.23 *The Biology of Cancer*

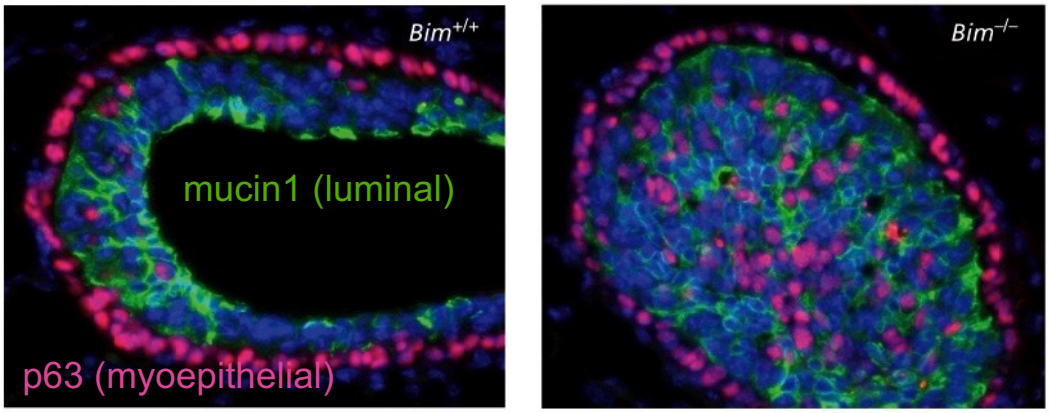
Under stress conditions, pro-apoptotic BH3-only proteins overwhelm anti-apoptotic factors



Cytochrome C and other mitochondrial proteins released into the cytosol

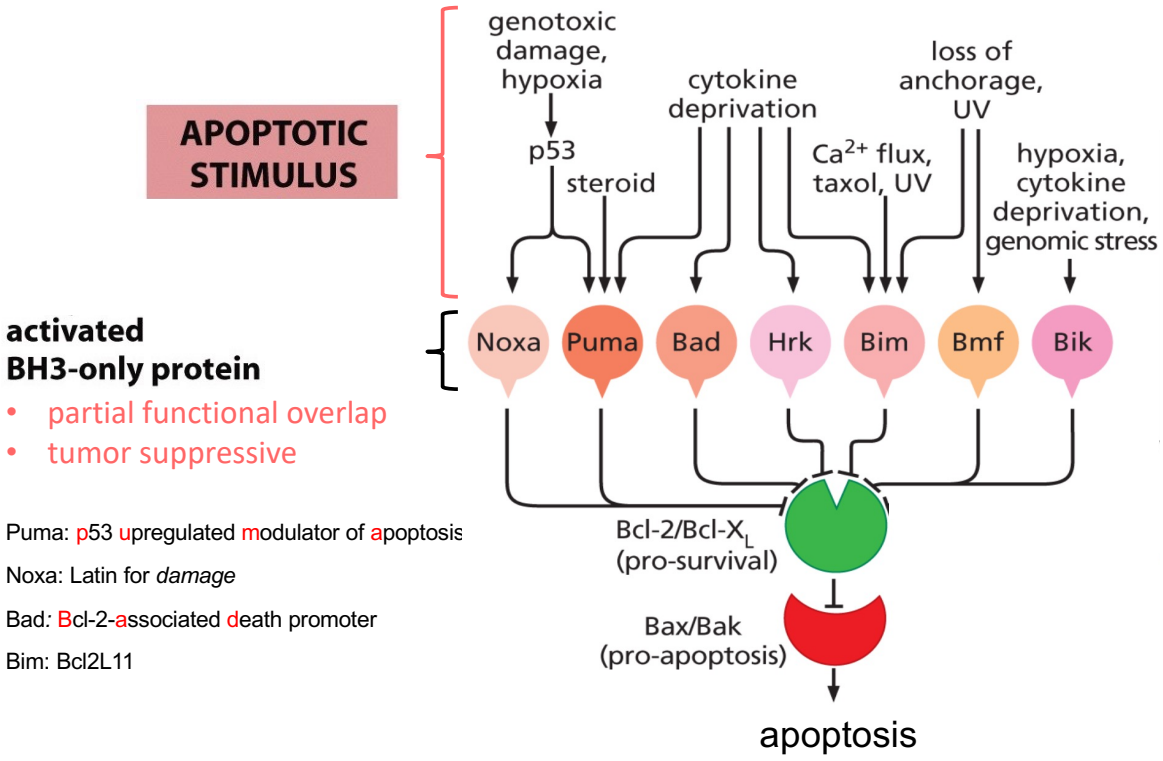
after Fig. 9.23 *The Biology of Cancer*

Testing the role of a BH3-only protein



- Bim mediates anoikis
  (= apoptosis induced by loss of ECM attachment of integrins)
- Example:
  Lumen formation by mouse mammary epithelial cells

BH3-only proteins sense diverse stress signals



- partial functional overlap
- tumor suppressive

Puma: p53 upregulated modulator of apoptosis

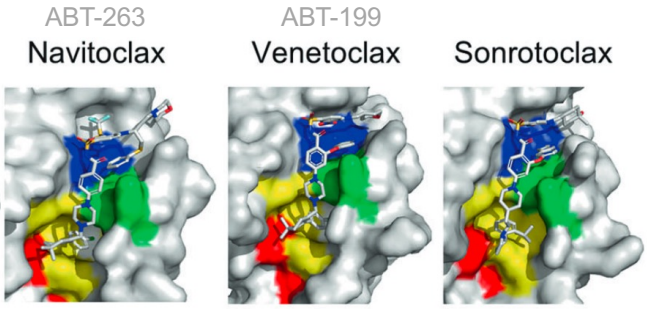
Noxa: Latin for *damage*

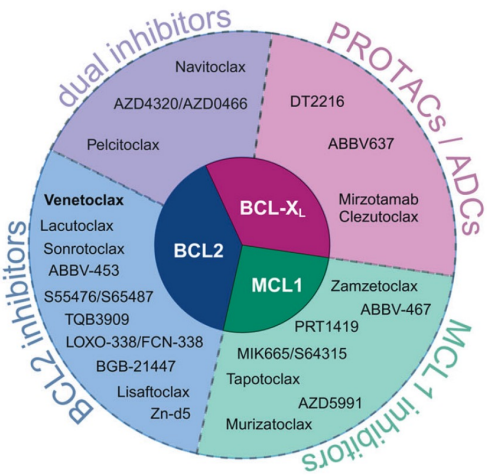
Bad: Bcl-2-associated death promoter

Bim: Bcl2L11

Figure 9.25A *The Biology of Cancer*

Induction of apoptosis by BH3-only mimics





- **BH3-mimics** that bind BCL2-like proteins:
  → Among the first small molecules used to successfully target a protein-protein interface
- **Navitoclax** (blocks BCL-2 and $-X_L$): Unacceptable on-target toxicity
- **Venetoclax** (selective for BCL-2): FDA-approved
  in chronic lymphocytic leukemia (CLL) & acute myeloid leukemia (AML)
- **Sonrotoclax** (higher affinity): Clinical trials ongoing

Vogler et al. 2025, Sig Transduct Target Ther 10:1-31

Development of BH3-only mimics

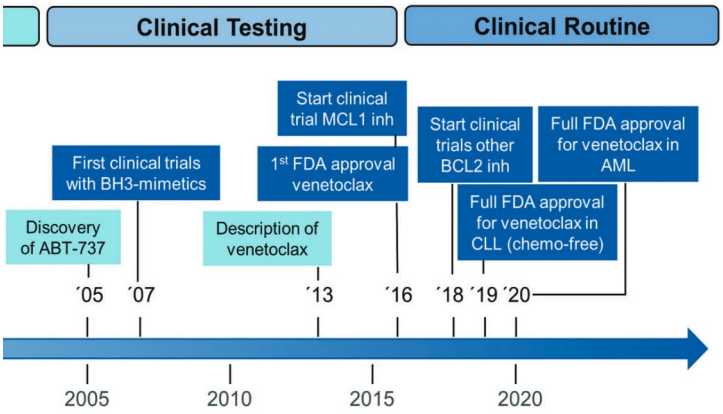


Table 1. BH3-mimetics in clinical development

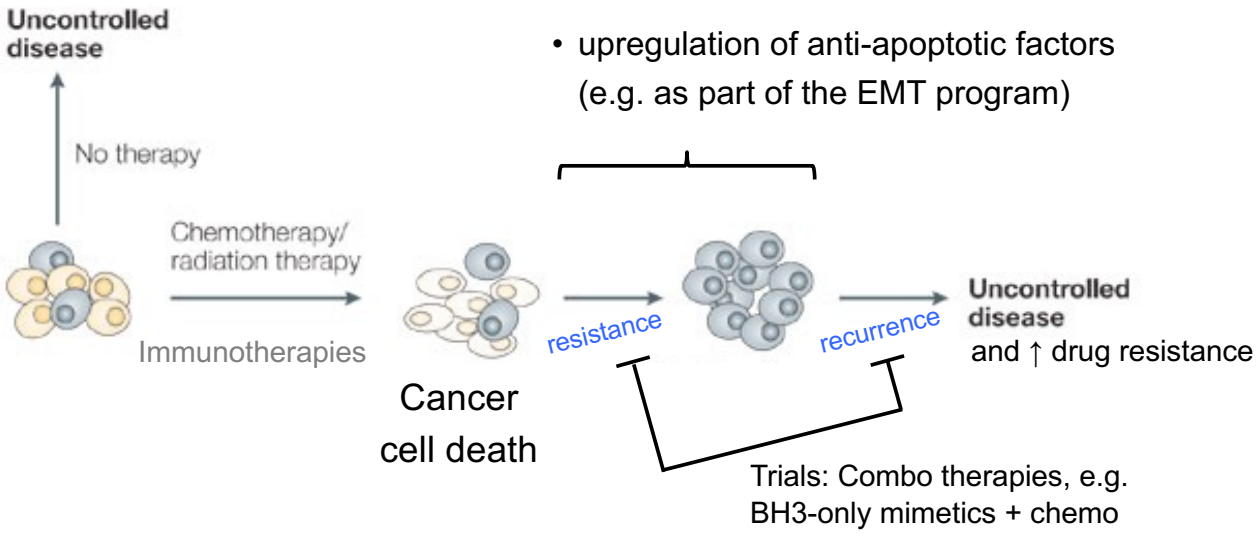
Name	Target	Status	Original Ref
Venetoclax / ABT-199	BCL2	FDA approved	32
Sonrotoclax / BGB11417	BCL2	Phase III	338
Lisaftoclax / APG-2575	BCL2	Phase III	527
ABBV-453	BCL2	Phase I	unpublished
ABBV-623	BCL2	Terminated	unpublished
S55746 / BCL201	BCL2	Phase I completed	336
S65487 / VOB560	BCL2	Phase I (halted)	337
FCN-338 / LOXO-338	BCL2	Phase I	346
BGB21447	BCL2	Phase I	unpublished
ZN-d5	BCL2	Phase I/II	344
Lacutoclax / LP-108	BCL2	Phase I/II	350
TQB3909	BCL2	Phase I/II	unpublished
Navitoclax / ABT-263	BCL2/ BCL-X_L	Phase III	27
AZD4320 and AZD0466	BCL2/ BCL-X_L	Terminated	438
Pelcitoclax / APG-1252	BCL2/ BCL-X_L	Phase I	365
Mirzotamab Clezutoclax / ABBV-155 (ADC)	BCL-X_L	Phase I (halted)	441,442
ABBV-637 (ADC)	BCL-X_L	Phase I (halted)	443
MIK665 / S64315	MCL1	Phase I (halted)	370
AZD5991	MCL1	Phase I	539
Tapotoclax / AMG176	MCL1	Phase I (halted)	373
Murizatoclax / AMG397	MCL1	Terminated	375
Zamzetoclax/ GS9716	MCL1	Phase I	378
ABBV-467	MCL1	Terminated	371
PRT1419	MCL1	Terminated	377

Vogler et al. 2025, Sig Transduct Target Ther 10:1-31

Defective apoptosis promotes tumor recurrence and drug resistance

Selection of cells:

- loss of pro-apoptotic factors, or
- upregulation of anti-apoptotic factors (e.g. as part of the EMT program)



Outline

1. Apoptosis as one form of Regulated Cell Death (RCD)
   - ✓ Distinctive features of apoptosis, and the role of caspases
   - ✓ Extrinsic versus intrinsic (mitochondrial) apoptosis
   - ✓ Techniques to detect apoptosis
2. Tumor suppressive role of apoptosis
   - ✓ Dual regulation of intrinsic apoptosis by the Bcl-2 family
   - ✓ BH3 mimetics as novel anti-cancer drugs
3. Anti-apoptotic cell survival signaling
   - Inactivation of p53 and/or its target genes
   - Phosphoralytion by Akt inactivates *multiple* pro-apoptotic signals
   - Examples: Upstream regulators of Bcl-2 and p53
4. Cell survival signaling by mTOR complexes
   - Discovery of mTOR and Rapamycin-like inhibitors (Rapalogs)
   - Remaining hurdles for 2nd and 3rd generation mTOR inhibitors

Loss of p53 impairs the expression of *multiple* pro-apoptotic genes

- ✓ cytostatic (p21, FoxO)
- proapoptotic (Fas, Bax, Puma...)
- anti-survival (IGF binding proteins)

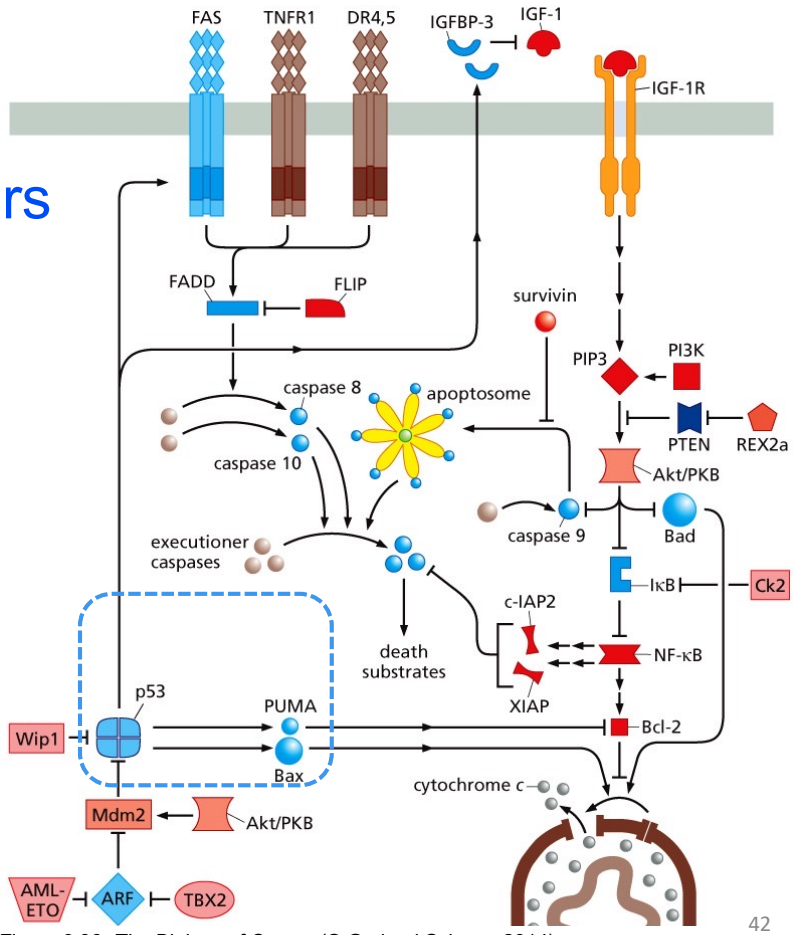


Figure 9.36 *The Biology of Cancer* (© Garland Science 2014)

Ways how cancer cells increase survival signals:

- upregulate Bcl-2 or IAPs
- or hyperactivate the transcription factor NF-kB or Akt acting upstream

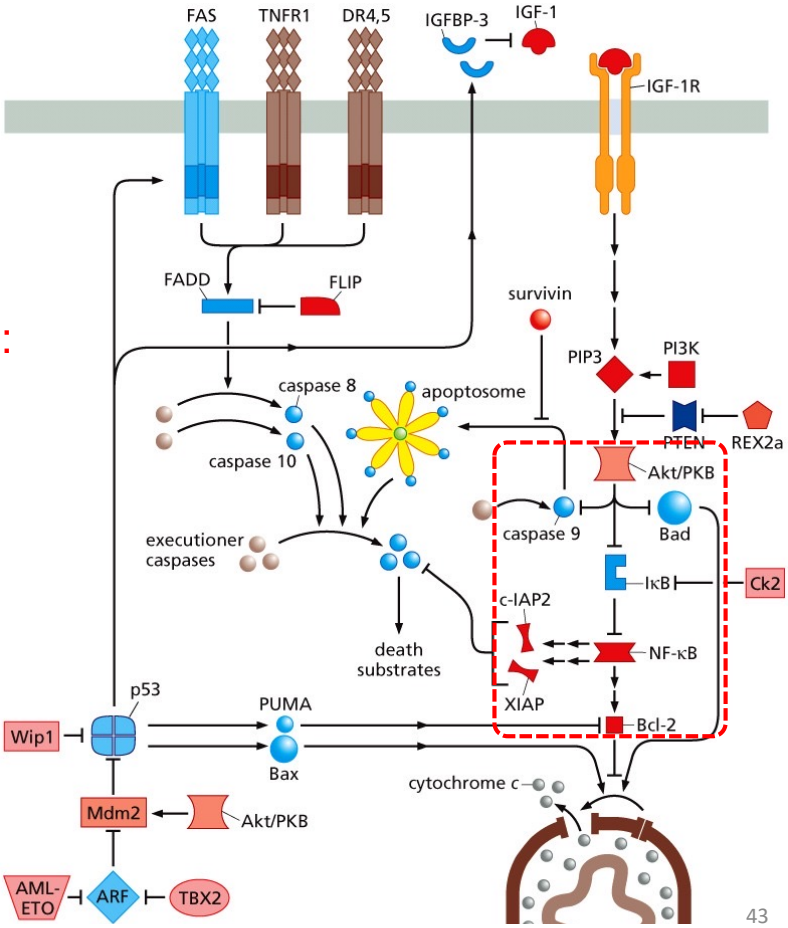


Figure 9.36 *The Biology of Cancer* (© Garland Science 2014)

Centrality of Akt for survival

- Tumors evade death directly by mutating p53, or through downregulation of pro-apoptotic factors (blue)
- or by inducing *Bcl-2* or inhibitors of apoptosis (IAPs), e.g. through the transcription factor NF-kB.
- Both p53 *and* NFkB are **regulated by Akt** (indirectly):

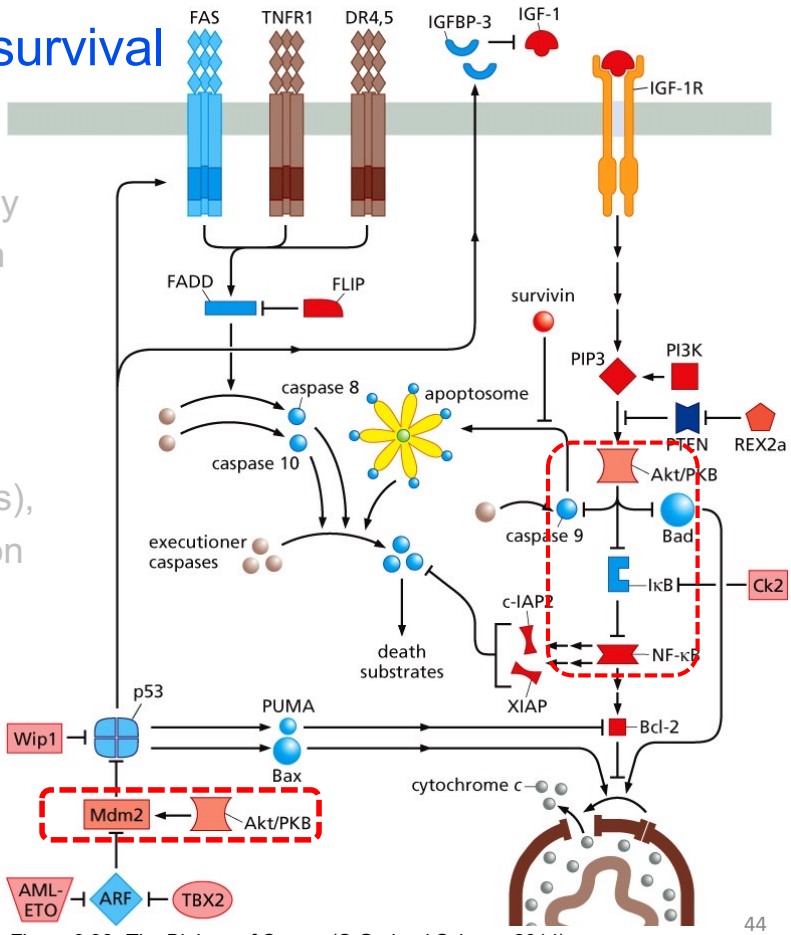
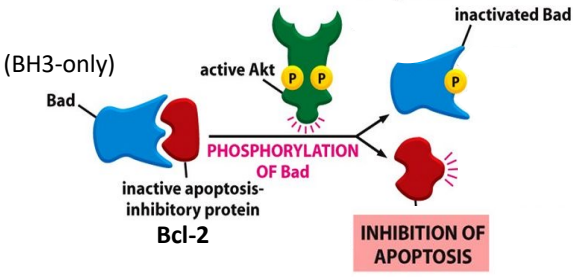
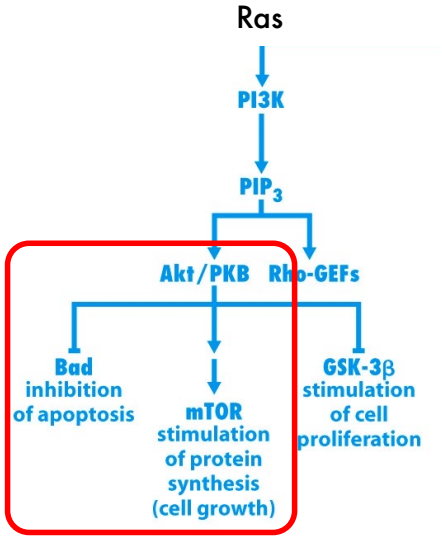


Figure 9.36 *The Biology of Cancer* (© Garland Science 2014)

Centrality of Akt (and downstream effectors) for survival

- Akt directly phosphorylates Bad to release the survival factor Bcl-2:





- Akt indirectly stimulates mTOR (and thus mRNA translation)

Outline

1. Apoptosis as one form of Regulated Cell Death (RCD)
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   - ✓ Examples: Upstream regulators of Bcl-2 and p53
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   - Discovery of mTOR and Rapamycin-like inhibitors (Rapalogs)
   - Remaining hurdles for 2nd and 3rd generation mTOR inhibitors

A metabolic switch: Role of Akt & mTOR

Energy demand of cancer cells is met by increased glucose uptake, **glycolysis** and lactate fermentation in the cytosol (Warburg effect)

Increased glycolysis also provides citrate for increased **fatty acid synthesis** (➔ cell size)

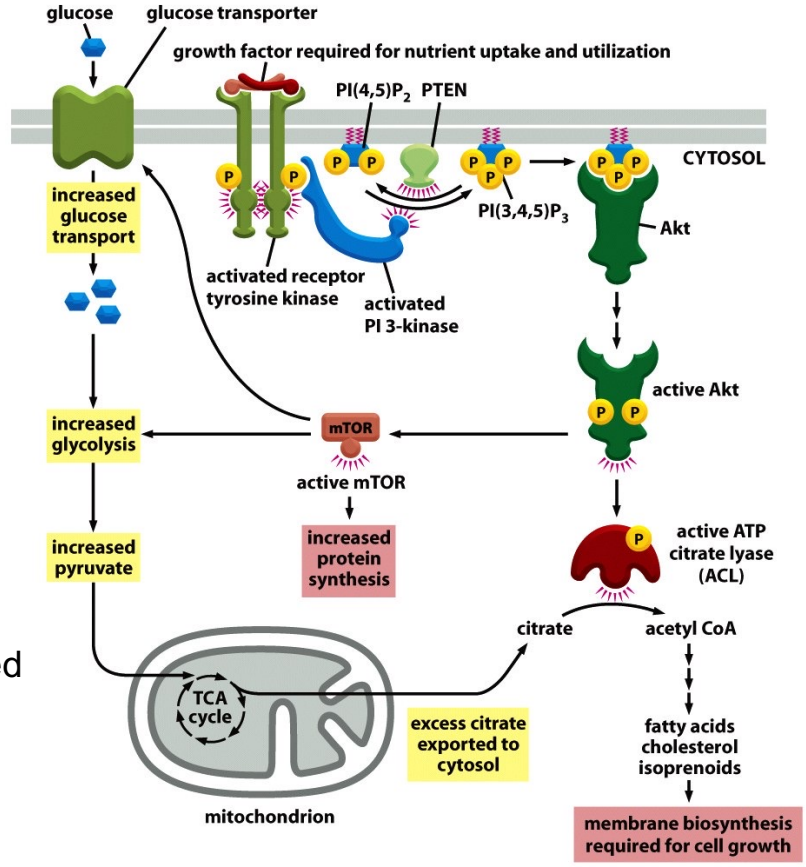
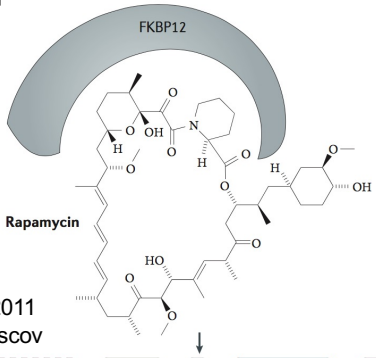


Figure 20-39b *Molecular Biology of the Cell* (© Garland Science 2008)

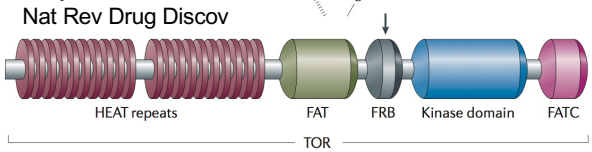
(mammalian) Target Of Rapamycin (m)TOR

FK506-binding protein:



mTOR:

Benjamin et al. 2011
Nat Rev Drug Discov

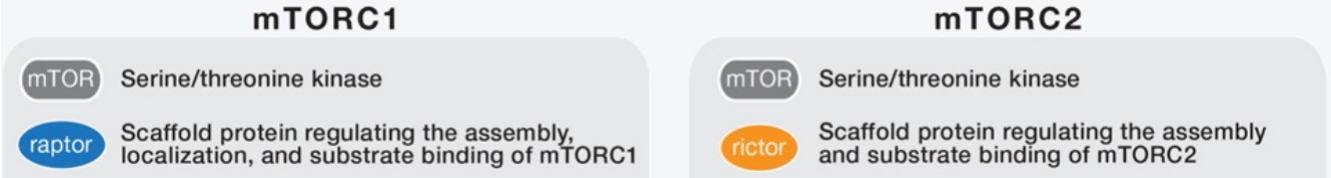


Not only the origin of "Kopfsteinpflaster" 😉

- 1975: Rapamycin isolated as an antifungal antibiotic of *Streptomyces* in soil from Rapa Nui (Easter Island)
- 1988: FK506 (Tacrolimus) isolated from another *Streptomyces* strain
- 1991: Recruitment of FKBP12 by Rapamycin allosterically inhibits TOR

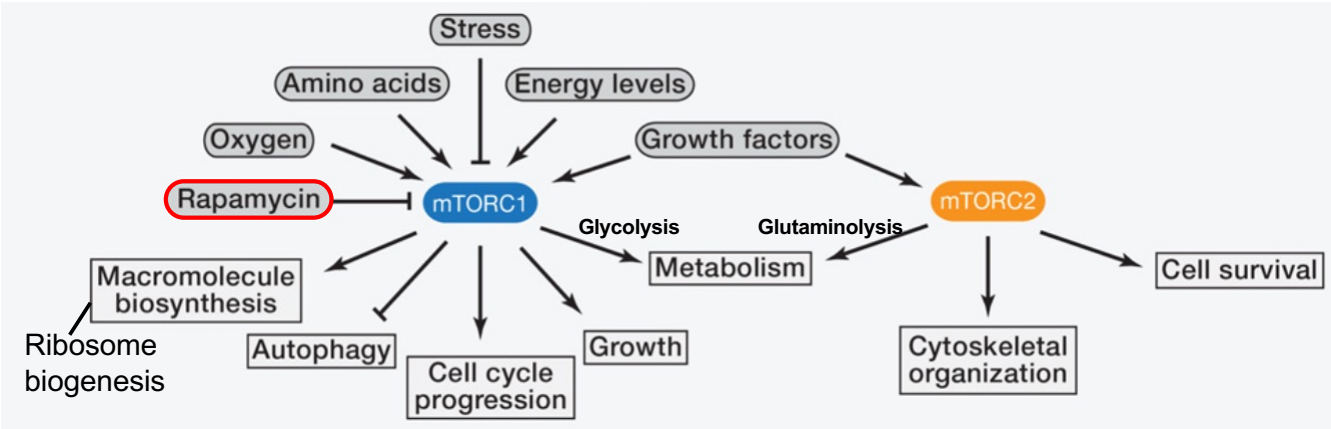
>1999: Rapamycin for organ transplant recipients (immune suppression)

Survival signaling by three mTOR complexes

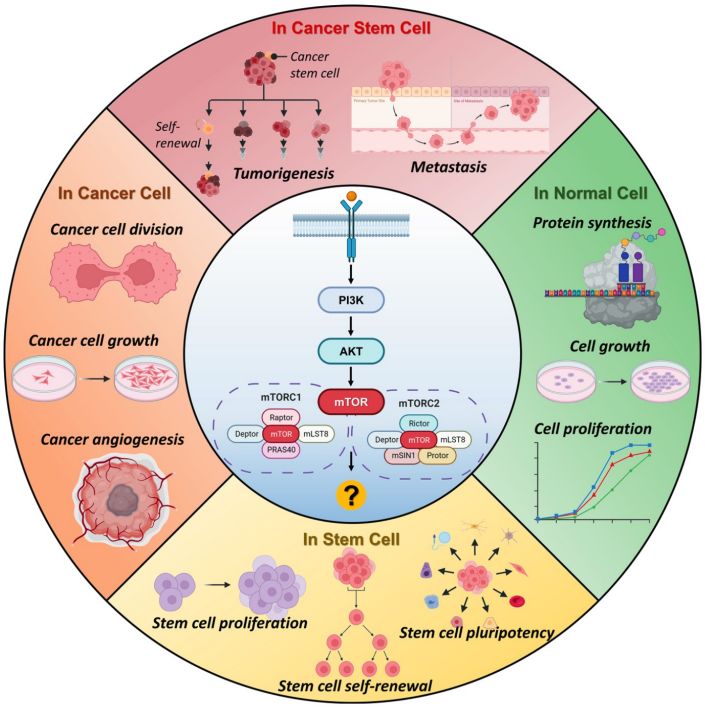


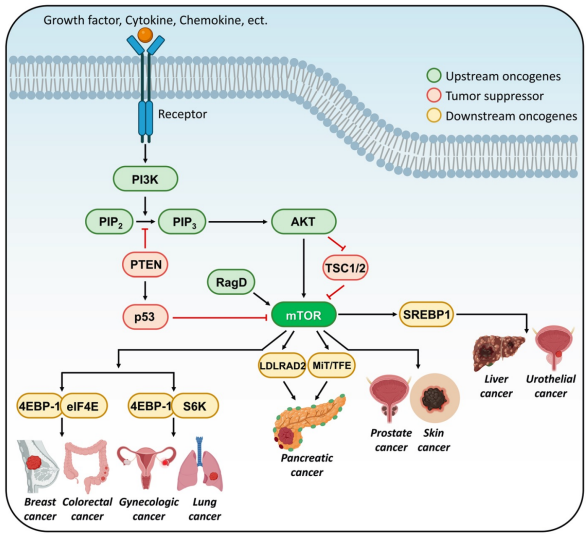
Raptor:
Regulatory-associated protein of mTOR

Rictor:
rapamycin-insensitive companion of mTOR



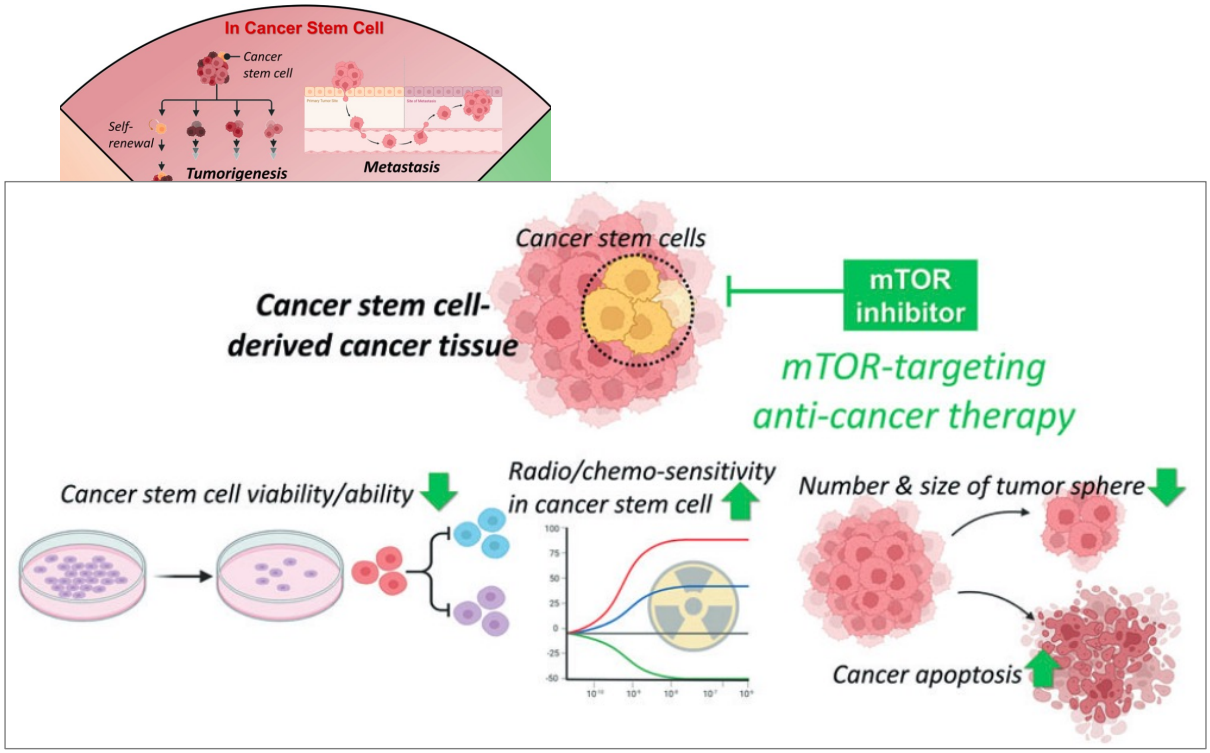
mTOR promotes cancer in multiple tissues





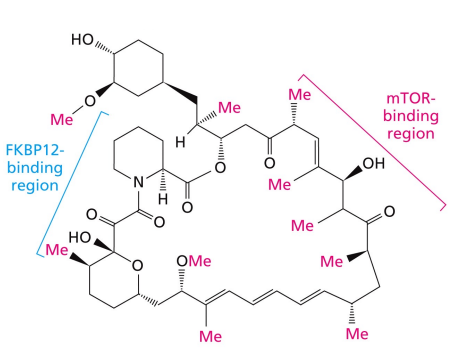
Son et al. 2024, Cell Death & Disease 9:1-18

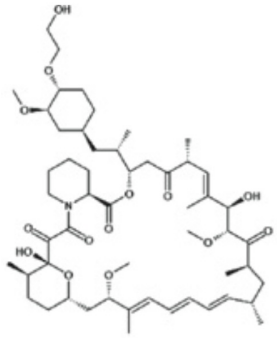
mTOR inhibitors to reduce cancer *recurrence*?

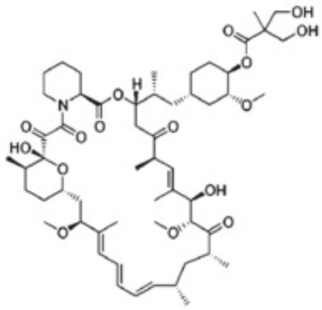


Son et al. 2024, Cell Death & Disease 9:1-18

1st generation mTORC1 inhibitors (rapalogs)







Sirolimus (=rapamycin)

Everolimus **Temsirolimus**

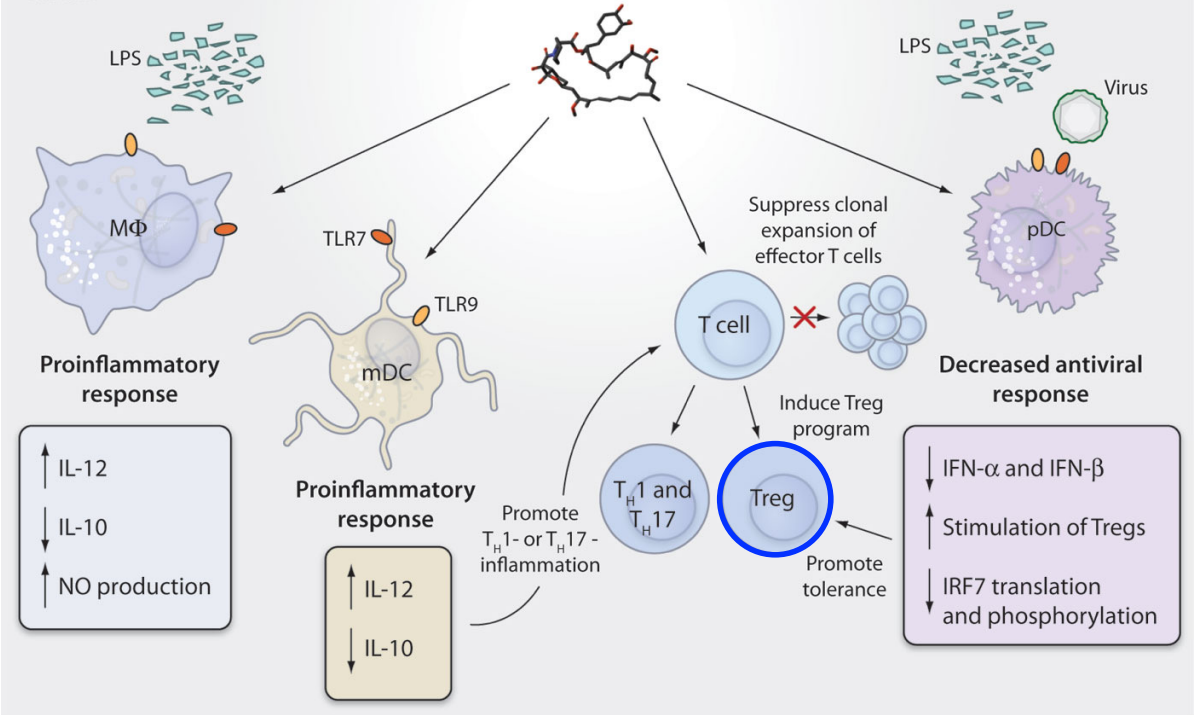
Improved bioavailability and pharmacodynamics

Table 1. Rapalogs and approved indications from the FDA and EMEA

Compound	Approved indication	Agency	References
Sirolimus	Prophylaxis of organ rejection in renal transplant patients	FDA/EMEA	39
Everolimus	Refractory advanced renal cell carcinoma	FDA/EMEA	54
Temsirolimus	Poor-prognosis untreated advanced renal cell carcinoma	FDA/EMEA	53
	Refractory mantle-cell lymphoma	EMEA	55

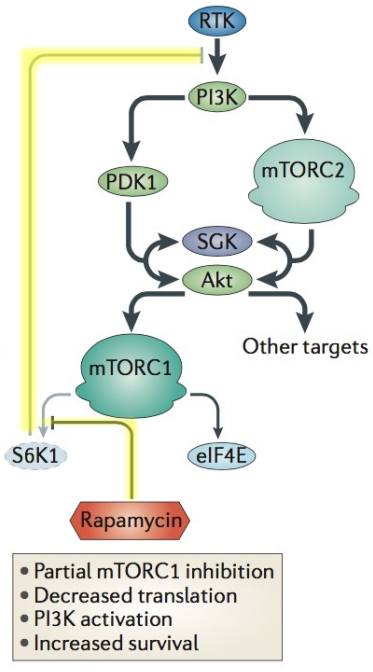
Vilar et al. 2011 Mol Canc Ther

Rapalogs induce immune tolerance by promoting immunesuppressive Treg cells



Janes & Fruman 2009, Sci Signal 67:pe25

Rapalogs showed only modest efficacy as anti-cancer drugs: Why?



Rapamycin and its analogs:

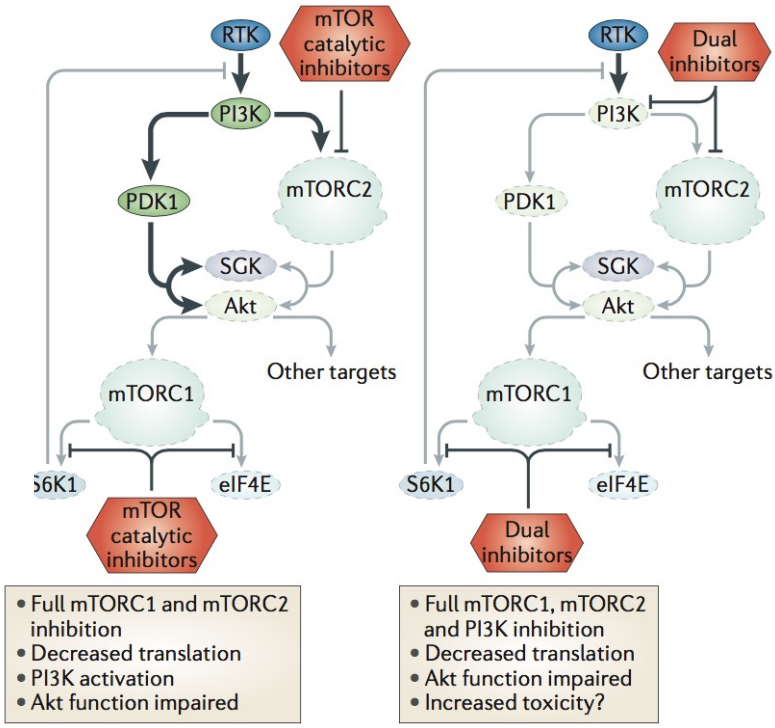
- have pro- and anti-inflammatory effects which overall promote immune tolerance
- they poorly inhibit mTORC2
- they only incompletely inhibit even mTORC1: eIF4 remains active!
- Paradox: Rapalogs hyperactivate PI3K/mTORC2/Akt signaling due to loss of of feedback inhibition by ribosomal protein S6 kinase (S6K)

Zoncu et al. 2011 Nat Rev Mol Cell Biol

Strategies to improve mTOR inhibition

2nd generation inhibitors:

- mTOR catalytic inhibitors
- Dual specificity inhibitors
- Issues:
  - increased toxicity
  - mTOR mutations that can confer resistance

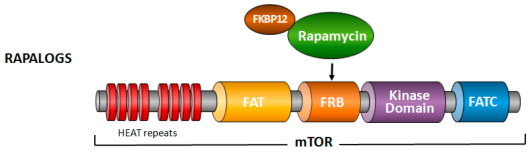


Zoncu et al. 2011 Nat Rev Mol Cell Biol

Overcoming mTOR resistance mutations with 3rd-generation inhibitors

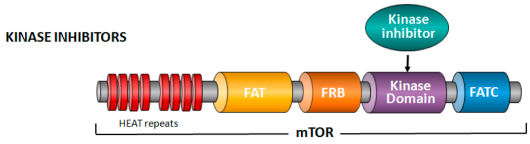
1st generation

Rapalogs (allosteric inhibitors)



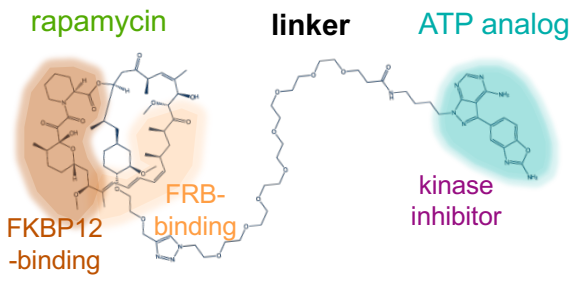
2nd generation

ATP analogs (kinase inhibitors)

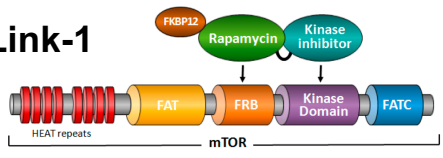


3rd generation

Rodrik-Outmezguine et al. 2016, Nature 534:272-276



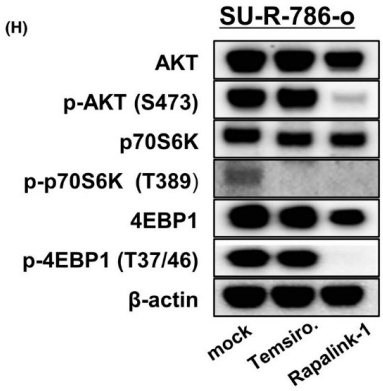
RapaLink-1



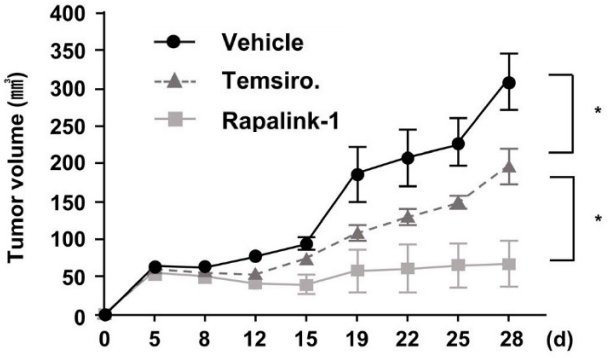
Still problematic: Effects on anti-tumor immunity?

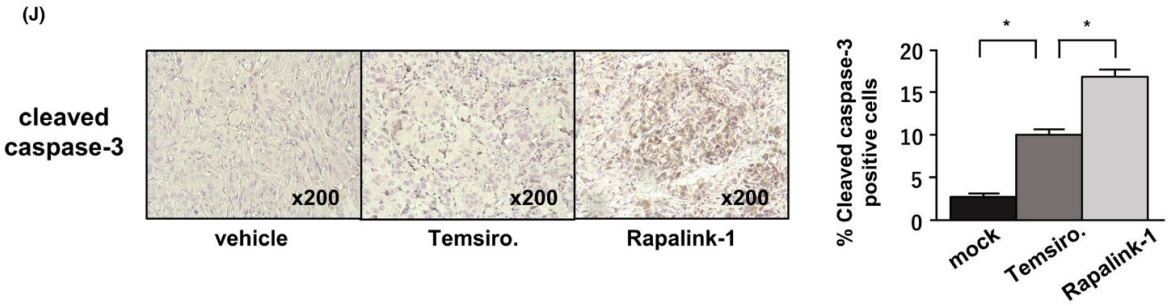
RapaLink-1 efficacy in preclinical models

in vitro



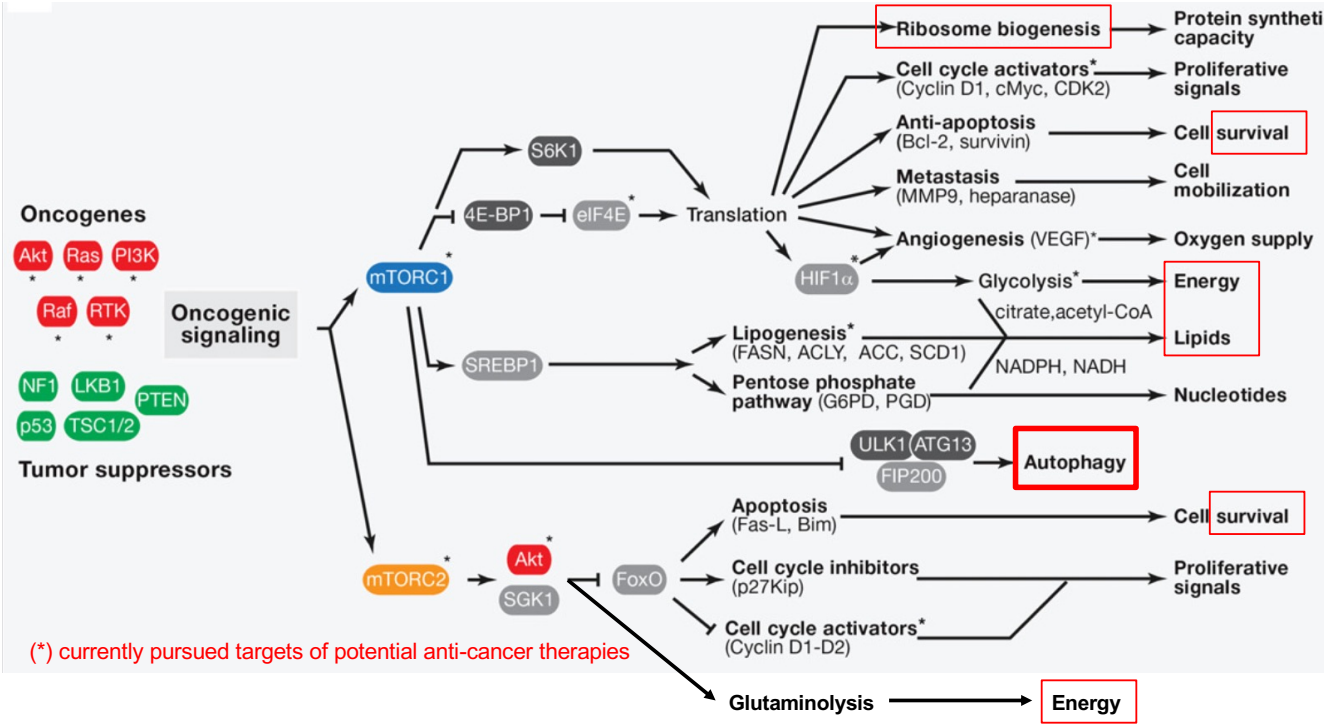
in vivo (xenografts)
(immunedeficient mice!)





Kuroshima et al. 2020, Cancer Science 111:1607-1618

mTORC1 regulation of autophagy



=> mTOR regulates aging, metabolic diseases (diabetes, obesity) and cancer

Laplante & Sabatini 2012 Cell 149:274-293

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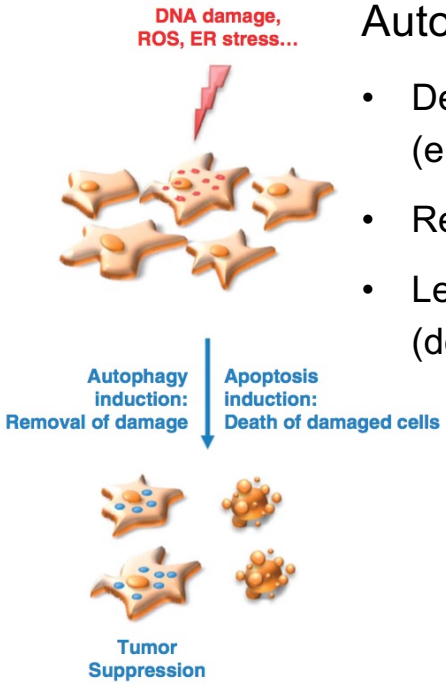
   - • Remaining hurdles for 2nd and 3rd generation mTOR inhibitors

Autophagy instead of apoptosis: Distinct outcomes

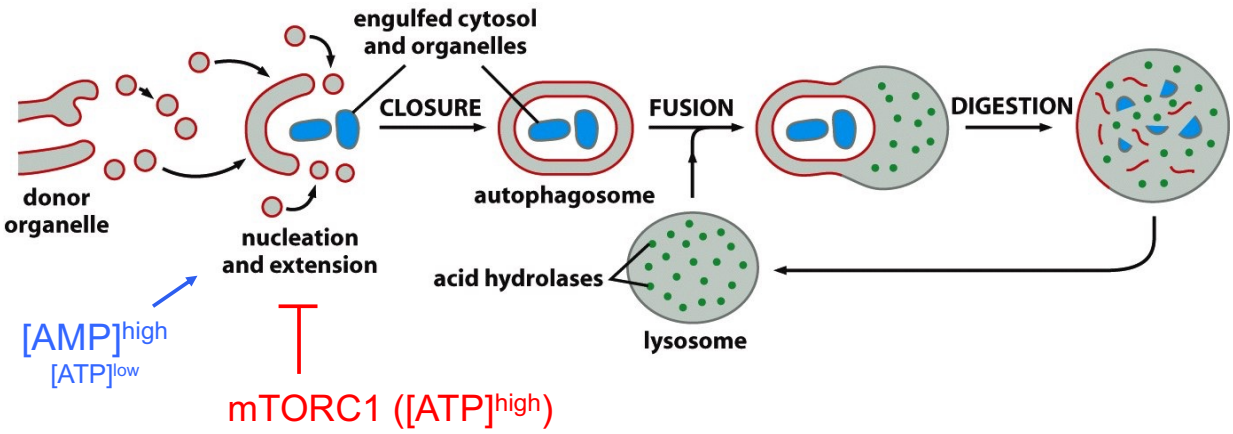


Autophagy is an alternative stress response:

- Degradation & recycling of defective organelles (e.g. mitochondria)
- Response to nutrient starvation
- Leads primarily to cell atrophy (death only follows after *prolonged* starvation)

Fimia et al. 2013 Oncogene

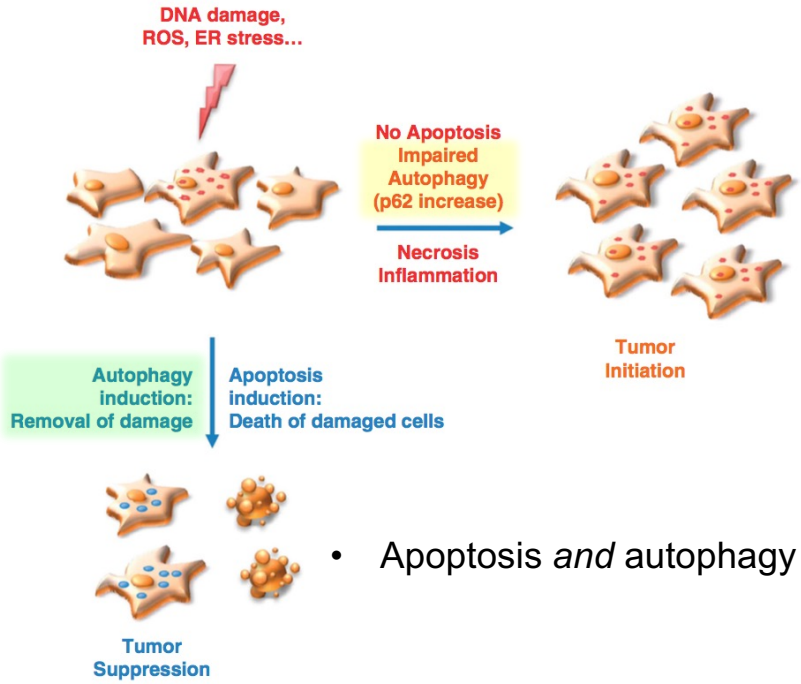
Autophagosome formation



- Membrane engulfment of entire organelles
- Degradation of cargo (e.g. p62) requires fusion with lysosomes
- Autophagy is induced by stress (e.g. starvation), but suppressed by Akt/mTORC1 signaling

Figure 13-43 *Molecular Biology of the Cell* (© Garland Science 2014)

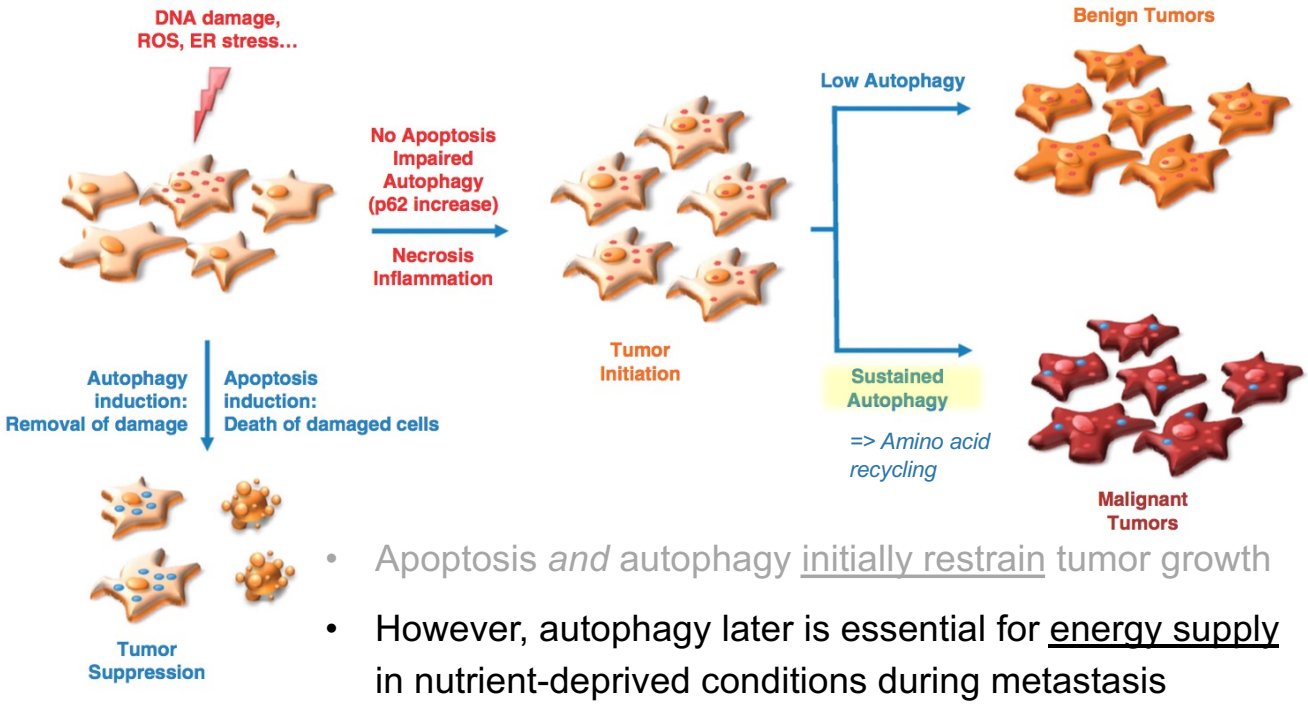
Dual role of autophagy in cancer



- Apoptosis *and* autophagy initially restrain tumor growth

Fimia et al. 2013 Oncogene

Dual role of autophagy in cancer



- Apoptosis *and* autophagy initially restrain tumor growth
- However, autophagy later is essential for energy supply in nutrient-deprived conditions during metastasis

=> *Implications for therapeutic strategies that target mTOR?*

Key concepts about apoptosis

- What is apoptosis, how can it be discerned, and what is its physiological role?
- How is it induced: Stimuli? Types of caspases and their roles?
- Roles of death receptors, Bcl-2 family members, mitochondria
- *How* do p53 and Akt regulate apoptosis? (at least a basic notion of mechanisms)
- mTOR inhibitors (classes & what they can achieve)
- Reasons why mTOR targeting proved to be more complicated than expected (feedback regulation, incomplete inhibition, three different mTOR complexes, autophagy activation...)