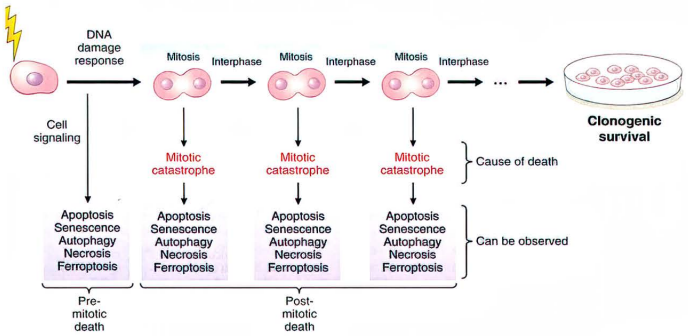
Mechanisms of Cell Death

A. In the old days of radiobiology, there was only one kind of cell death...and that was called **clonogenic death**, operationally defined as ***the inability of an irradiated cell to reproduce indefinitely***

1) today, we know that hidden in that definition of "clonogenic death" are several distinct death pathways, each of which has both unique, and (in some cases) common, molecular features



2) the types of cell death recognized today – not all of which are directly elicited by radiation exposure (but could be indirectly) – include:

Of these, the three most relevant to how ionizing radiation kills cells are:

Mitotic Catastrophe
Apoptosis
Senescence

Type of cell death	Morphological changes: Nucleus	Cell membrane	Cytoplasm	Biochemical features
Apoptosis	Chromatin condensation; nuclear fragmentation; DNA laddering	Blebbing	Fragmentation (formation of apoptotic bodies)	Caspase-dependent
Autophagy	Partial chromatin condensation; no DNA laddering	Blebbing	Increased number of autophagic vesicles	Caspase-independent; increased lysosomal activity
Mitotic catastrophe	Multiple micronuclei; nuclear fragmentation	–	–	Caspase-independent (at early stage) abnormal CDK1/cyclin B activation
Necrosis	Clumping and random degradation of nuclear DNA	Swelling; rupture	Increased vacuolation; organelle degeneration; mitochondrial swelling	–
Senescence	Distinct heterochromatic structure (senescence-associated heterochromatic foci)	–	Flattening and increased granularity	SA-β-gal activity

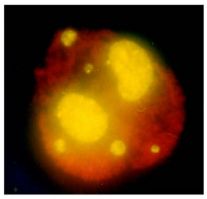
CDK1 cycline-dependent kinase 1, *MDC* monodansylcadaverine, *MPM2* mitotic phosphoprotein 2, *SA-β-gal* senescence-associated β-galactosidase, *RB* retinoblastoma protein

Zhou J (ed.), *Multidrug Resistance in Cancer*, 2010

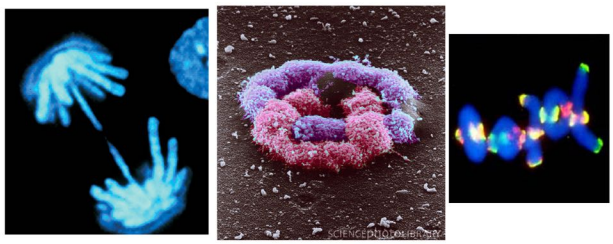
a) arguably, the closest thing to a marker for mitotic catastrophe (short of actually videoing a cell as it attempts mitosis) is the observation of binucelate cells and/or cells with multiple micronuclei, but even this isn't apparent until after the fact

1. historically, mitotic catastrophe has been both difficult to define and difficult to identify, because it really doesn't have a particular molecular marker or hallmark characteristic

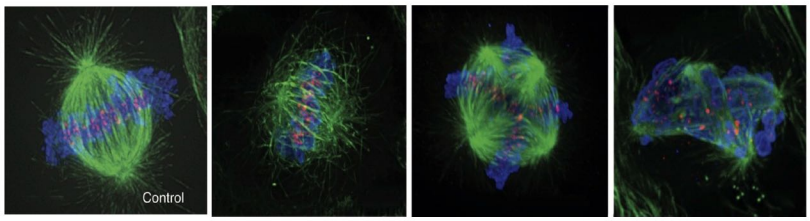
a) arguably, ***the closest thing to a marker for mitotic catastrophe is the appearance of binucleate cells and/or cells with multiple micronuclei following irradiation*** (being in mind that these won't be apparent until the cell next attempts to divide)



Binucleate, oversized cell with multipe micronuclei. If this had been caused by radiation exposure, the dose was probably in the 5–8 Gy range (X-rays)



Prelude to (mitotic) catastrophe: examples of types of chromosome aberrations that physically interfere with the process of cell division, and lead to prompt cell death



Sometimes, a mitotic spindle defect causes mitotic catastrophe, rather than a chromosome defect per se

Mitotic catastrophe is, by far, the most common mode of cell death associated with exposure to ionizing radiation; always has been, always will be. (This is not to say that there aren't notable exceptions, however...)

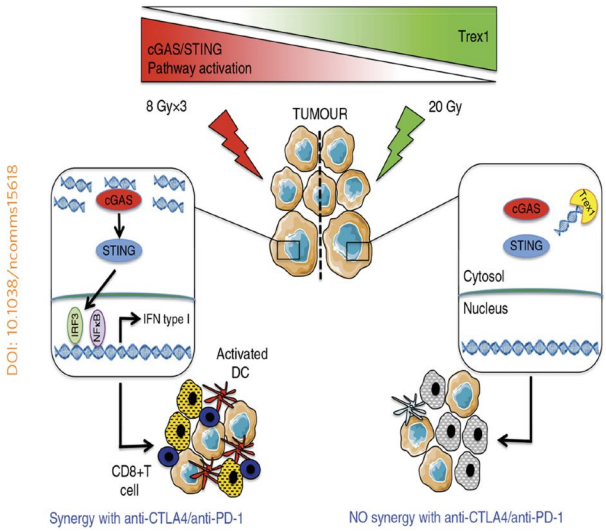
2. another interesting factoid about mitotic catastrophe:

> it doesn't always occur at the first attempted mitosis after irradiation – although this is more likely the higher the radiation dose – but can also do so "unexpectedly" after several subsequent rounds of cell division

3. **another consequence of a cell having micronuclei in its cytoplasm following mitotic catastrophe is that the presence of extranuclear double-stranded DNA triggers the cGAS/STING signaling pathway**

a) this is a component of the innate immune system designed to detect invading viruses, but can be fooled by the presence of micronuclei

b) once triggered, the pathway leads to the upregulation of interferons and other inflammatory cytokines



cGAS = cyclic GMP-AMP synthase
STING = stimulator of interferon genes

The Good News?

- Activating the cGAS-STING pathway stimulates an immune response and potentially, the killing of tumor cells.

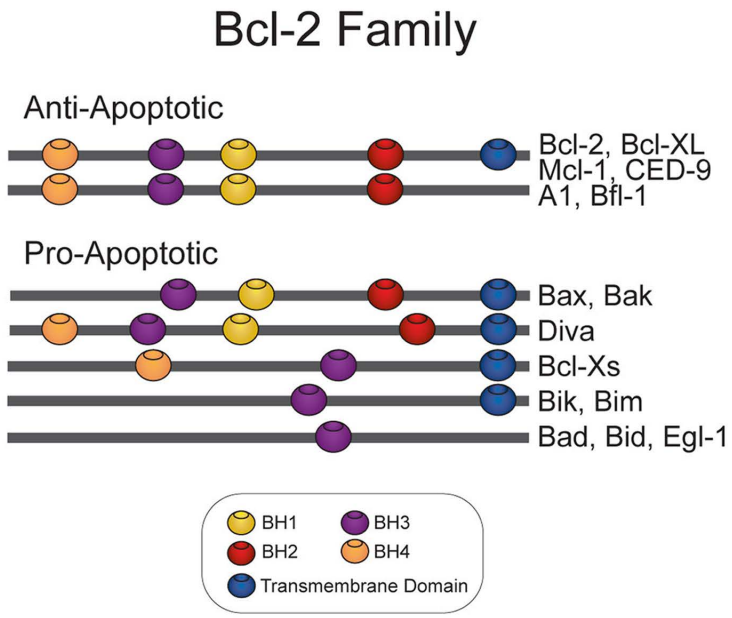
The Bad News?

- In the surrounding irradiated normal tissue, chronic inflammation can promote carcinogenesis, and increase the frequency and severity of late complications.

- However, higher radiation doses (think: SBRT) cause upregulation of an enzyme called **TREX1**, which degrades extranuclear dsDNA and therefore shuts off the cGAS-STING response, possibly muting any immune system (re-)activation.

Apoptosis *a genetically-regulated process characteristic of multicellular organisms for the deliberate removal of aged, damaged or unnecessary cells that is carefully controlled by an interplay between pro- and anti-apoptotic proteins of the Bcl-2 family*

a) thus, *the relative abundance of pro- and antiapoptotic factors determines whether a cell is "competent" to undergo apoptosis in response to environmental signals*



OK, so who's "pro" and who's "anti"?

Somewhat surprisingly, most of these proteins come from the same general family, main difference being the order, number and arrangement of so-called BH domains.

The best characterized anti-apoptotic proteins are Bcl-2, Bcl-XL and Mcl-1.

The best characterized pro-apoptotic proteins are Bax, Bak, Bad and Bid.

b) *the proapoptotic proteins are located on the mitochondrial membrane surface, and either promote or discourage "leakage" of cytochrome C; the release of cytochrome C into the cytoplasm then initiates a sequence of events that culminates in apoptosis*

2] why should cells have a built-in mechanism for committing suicide?

a) *apoptosis plays a critical role during embryological development*

b) after birth however, *multi-cellular organisms that contain specialized (i.e., differentiated) cells need to carefully—and actively—balance matters of life and death*

3] Which cell types undergo radiation-induced apoptosis?

Lymphocytes (thymus, spleen, lymph nodes)
Parenchymal cells of the salivary gland
Gut crypt cells of the GI tract (in some cases)
Glial cells in the (developing) nervous system
Germ cells (in some cases)
Vascular endothelial cells *(some conflicting evidence, but potentially quite important)*

4] *apoptosis and cancer - one of the hallmarks of most malignancies is the loss of the apoptotic pathway, in many cases because of a decrease or loss of proapoptotic proteins (like bax) and/or an upregulation of antiapoptotic proteins (like bcl-2)*

B. The Apoptotic Phenotype - a series of characteristic morphological and biochemical changes allow cells at various stages in the apoptotic process to be identified

1] morphological changes associated with apoptosis (in approximate order of appearance):

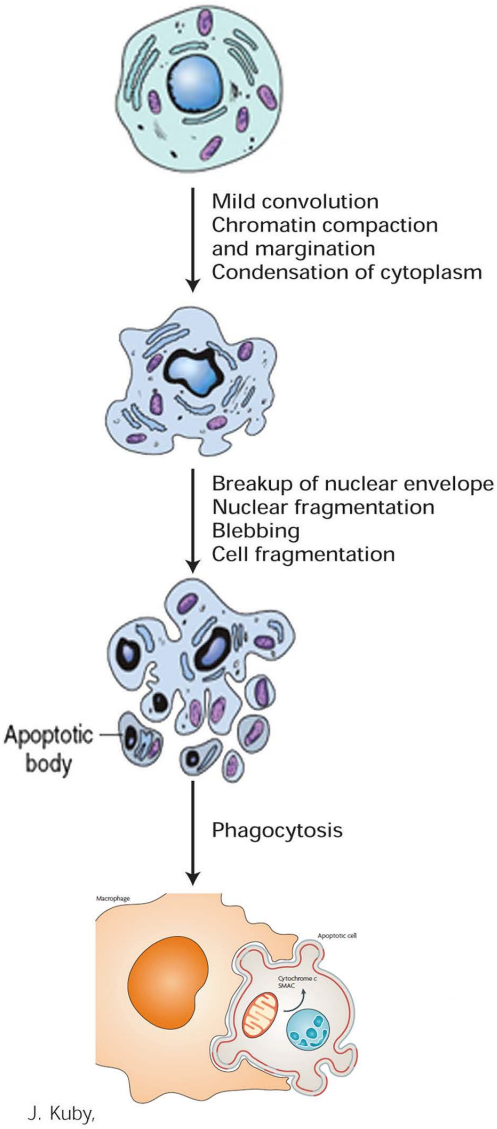
a) condensation and marginalization of the chromatin in the cell nucleus

b) some shrinkage of the total cytoplasmic volume, while at the same time, swelling of some of the organelles, especially the endoplasmic reticulum

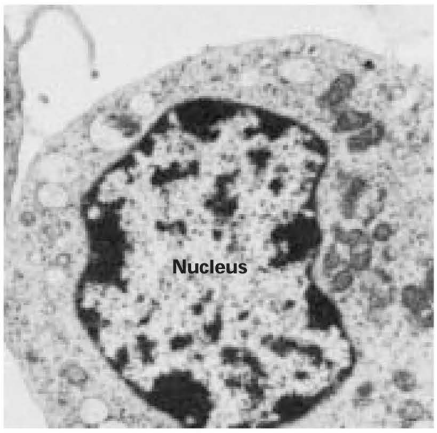
c) breakdown of the nuclear envelope, followed by fragmentation of the nuclear contents

d) "blebbing" of the outer cell membrane, followed by complete fragmentation of the cell into clumps called **apoptotic bodies**

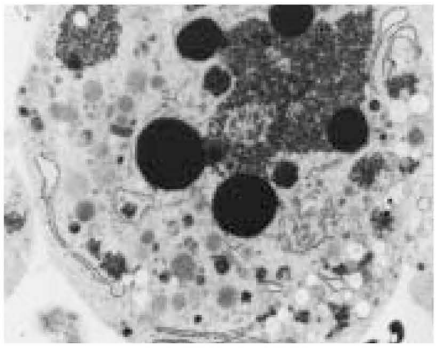
e) ***phagocytosis and destruction of the apoptotic bodies by surrounding cells, leaving no trace of the original cells, i.e., no spillage of cellular contents into the extracellular space and no localized inflammatory reaction or immune response***



J. Kuby, 1997, *Immunology*, 3d ed., W. H. Freeman & Co., p. 53.

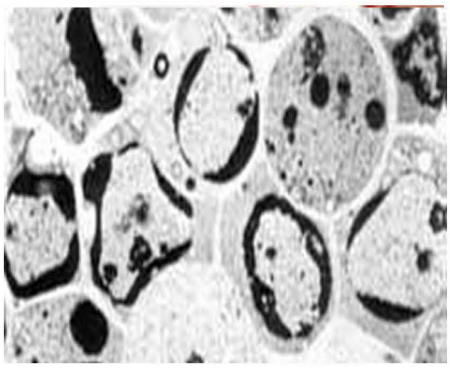


Normal cell



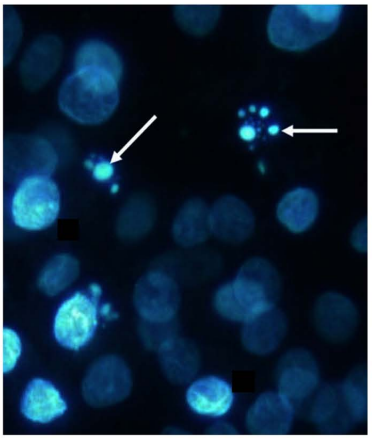
Apoptotic cell

1991, *Int'l. Rev. Exp. Pathol.* **32**:223



"Marginalized" Chromatin

Membrane Blebbing



Apoptotic Bodies

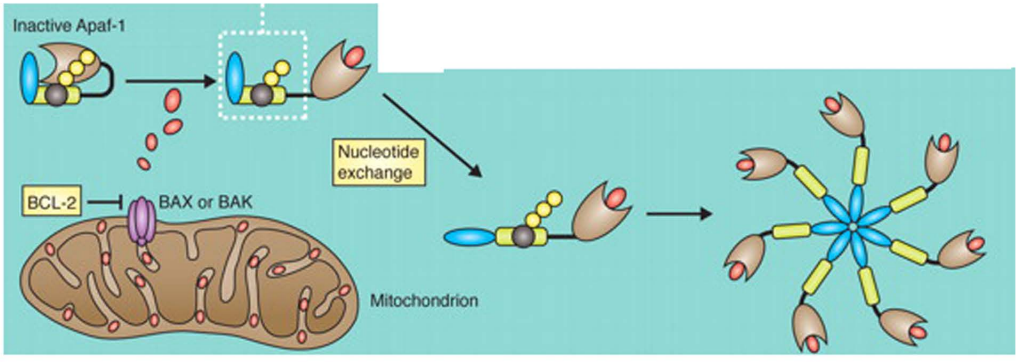
Molecular Biology and Biochemistry of Apoptosis

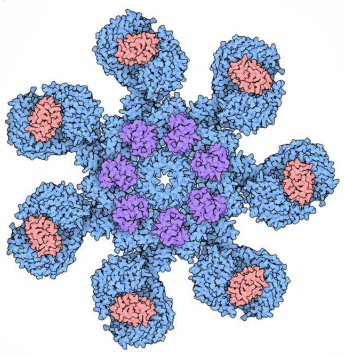
1. Stepwise biochemical changes and their consequences

a) **increase in cytosolic levels of Ca++, causing the leakage of cytochrome C** (and AIF, "apoptosis inducing factor") from mitochondria

1] *this is facilitated by proteins BAX, BAK and BID, and is antagonized by BCL-2*

2] the release of cytochrome C activates *APAF-1* ("apoptotic protease activating factor 1"), which binds to and triggers conformational changes and ultimately, assembly into a structure known as an **apoptosome**





3] **apoptosomes then bind pro-caspase 9**, converting it to its active form, caspase 9

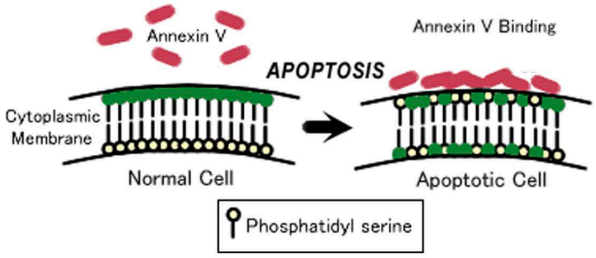
a. various proteins, collectively called *"inhibitors of apoptosis" or IAP*s – one in particular is called **survivin**, which is overexpressed in some cancers – antagonize the activation of caspase 9 in an attempt to prevent the apoptosis process from going forward

(1) IAPs in turn are antagonized by a mitochondrial-released protein called **SMAC/DIABLO** which has the net effect of allowing apoptosis to continue

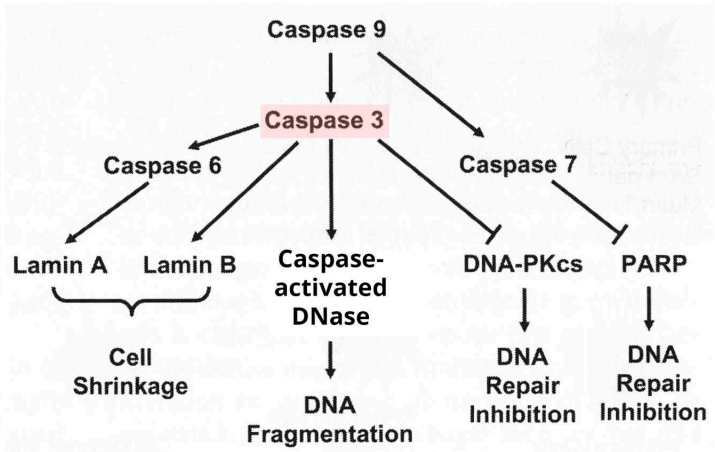
b) almost simultaneous with the release of cytochrome C from the mitochondria, there are also changes in the outer cell membrane, in particular, the **exteriorization of phosphatidyl serine residues**

1] this identifies the cell as being in the midst of apoptosis, which signals resident macrophages to be ready to clean up the debris once apoptosis is complete

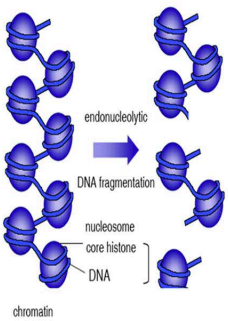
2] phosphatidyl serine also binds the protein *Annexin V*, which is used as a marker for the detection of cells in the early stages of apoptosis

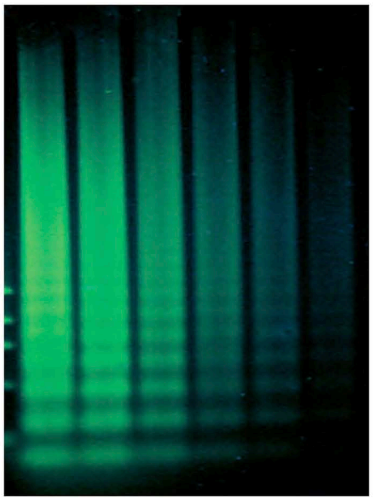


c) once caspase 9 is activated by the apoptosome (above), it then activates **caspase 3, termed the "executioner caspase"** and it initiates the destruction of the cell in a stepwise manner



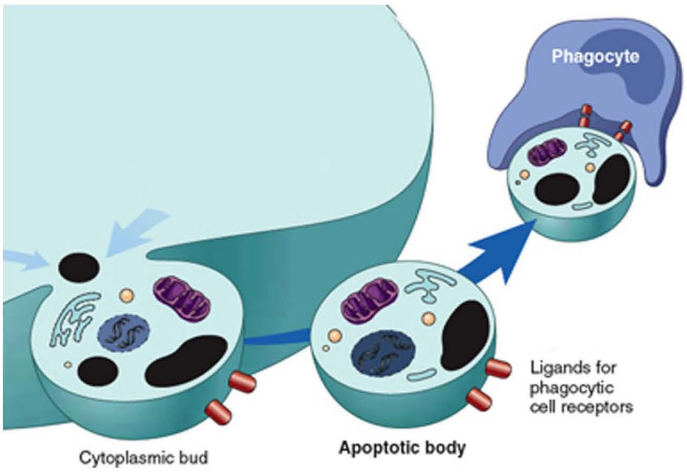
1] the next step is the condensation of the chromatin around the edges of the nucleus, and the DNA's fragmentation by endonucleases into chunks of uniform length, creating a **ladder pattern on a DNA gel**

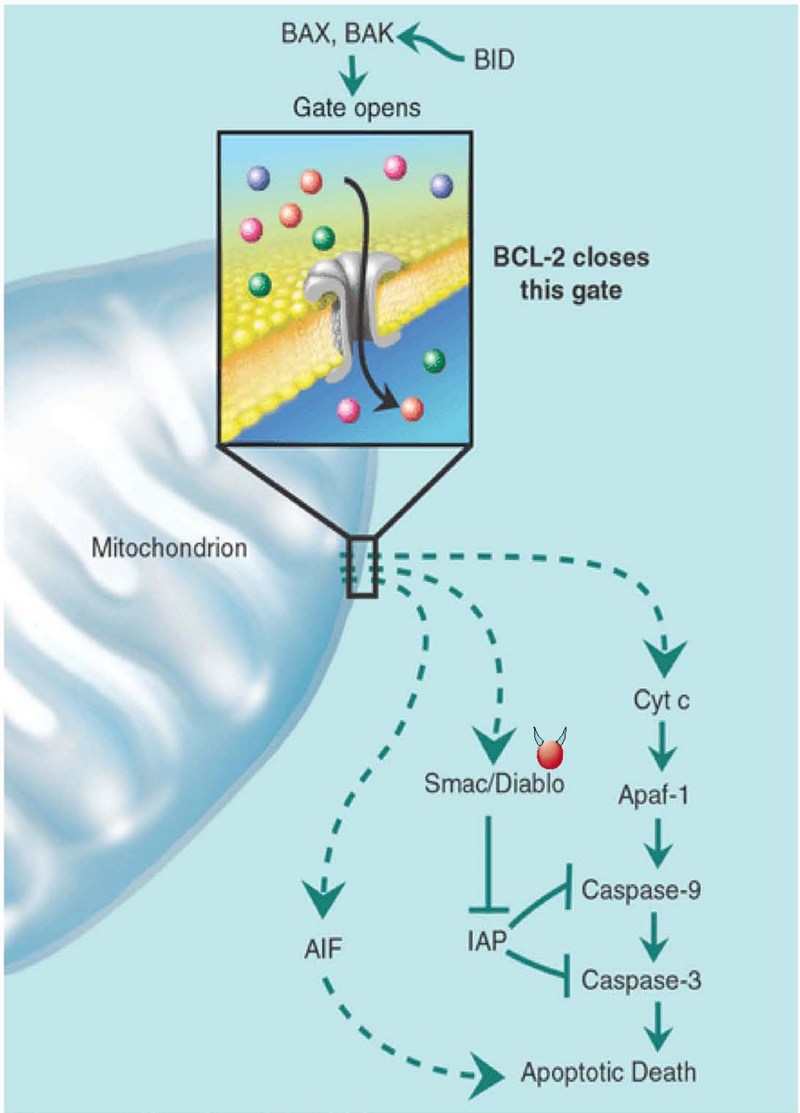




Note also that caspase 3 orchestrates the shut down of DNA damage sensing and repair, so the fragmentation goes to completion

d) finally, **caspase 6 activity causes the cell to start to shrink, its membrane bleb, and then it fragment into small apoptotic bodies that are consuned by neighboring phagocytes**





Science 292: 624-626, 2001

Summary graphic outlining the pro- and anti- molecular regulators of apoptosis

Clinical Correlate/Emerging Science!

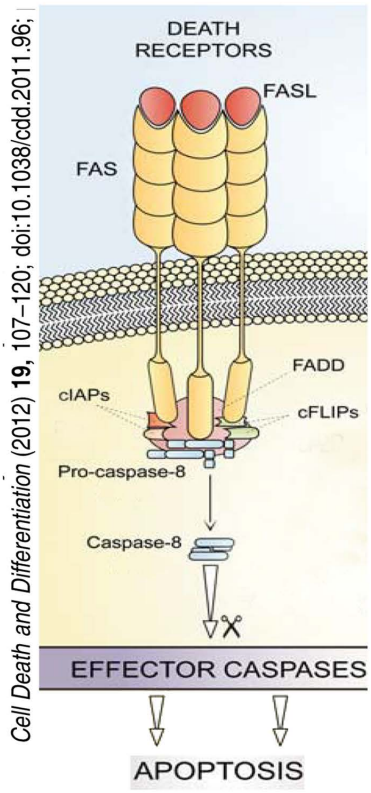
Debio 1143 is a novel compound currently in clinical trials as a possible radiosensitizer when given in combination with standard chemo-radiotherapy in patients with high-risk, locally advanced H&N squamous cell carcinoma (mostly heavy smokers with HPV- tumors). A recent Phase 2 trial by GORTEC showed improved locoregional tumor control 18 months after the completion of treatment in the Debio group, compared to the control group that received chemoradiotherapy only.

How does Debio 1143 work? It mimics the activity of SMAC/DIABLO, i.e., it inhibits the inhibitors of apoptosis, presumably upregulating apoptosis as a mode of cell death in tumor cells that typically have it downregulated.

Lancet Oncol. 2020 Aug 3:S1470-2045(20)30327-2

C. What triggers apoptosis in the first place?

1. positive induction of apoptosis occurs via the **fas signaling pathway**



Cell Death and Differentiation (2012) **19**, 107–120; doi:10.1038/cdd.2011.96;

a) **the fas complex is a transmembrane receptor that, upon binding of its specific extracellular ligand(s) – of which the cytokine tumor necrosis factor is one – signals the cell to commit suicide by apoptosis** (the fas signaling pathway is also involved in inflammatory responses)

1) the fas ligand pathway is commonly used by the immune system, and is also involved in inflammatory responses

2) this pathway also goes by the name "***extrinsic pathway to apoptosis***"

3) note that the initiator caspase for the extrinsic pathway is caspase 8, NOT caspase 9

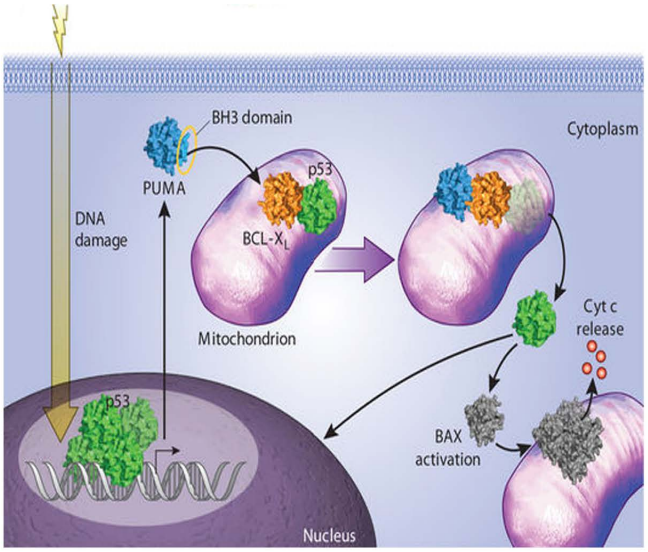
another example of positive induction of apoptosis: **the p53-mediated DNA damage response**

BOARDS!

a) p53's role in the DNA damage response:

1. p53 increases rapidly, but transiently, after DNA damage
2. its job is to coordinate the other cellular responses to the damage such as:
   - cell cycle arrest through upregulation of p21
   - positive induction of apoptosis if the damage is too extensive or severe:

     in response to DNA damage, p53 activates PUMA which inactivates Bcl-2, and also activates Bax which stimulates the release of cytochrome c from the mitochondria which triggers the start of apoptosis

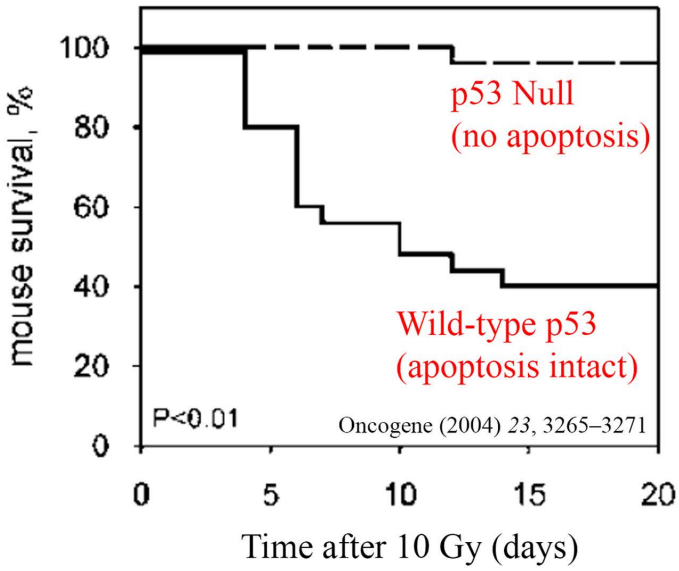


1. What happens if the p53-mediated apoptotic response to DNA damage is lost or deranged?

(a) ***for cells that either lack, or express a mutant form of p53 (characteristic of most tumor cells), the apoptotic response does not occur, meaning more cells survive than should be the case***, because they've gone ahead and propagated with residual DNA damage present; this leads to increased genomic instability, and often, a "worsening" phenotype

2. the loss of the p53-mediated apoptotic response after irradiation can partially explain one previously-unexplained observation: **that for two otherwise identical cell lines, one with the p53 pathway intact, and one with it mutated or absent, the mutant cell line often (BUT NOT ALWAYS) seems to be more radiation and/or chemotherapy resistant**

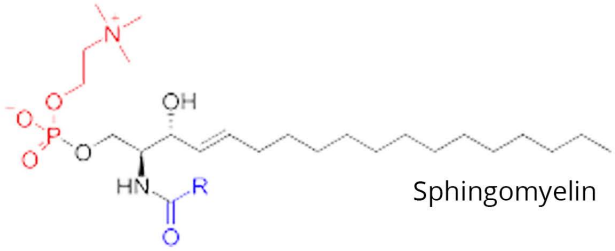
Mice that lack p53 are also resistant to radiation-induced apoptosis of lymphocytes and GI crypt epithelial cells. Loss of p53 **prevents death** from bone marrow failure (10 Gy whole body irradiation)

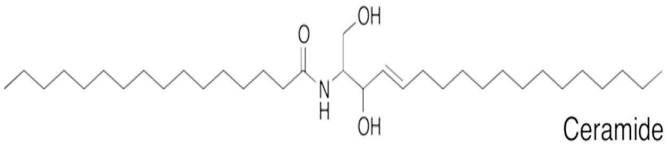


4] **Free radical/ceramide induction of apoptosis** - final example of positive induction of apoptosis; different from and independent of the fas ligand and p53 pathways

a) radiation-induced free radical damage to cell membranes causes activation of the enzyme acid sphingomyelinase (ASMase)

b) ASMase catalyzes the conversion of sphingomyelin (a phospholipid) to ceramide





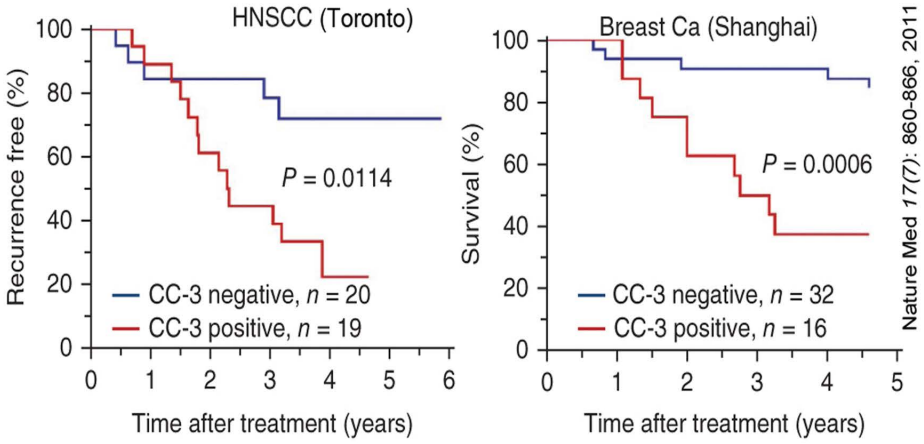
c) ***ceramide is a potent activator of the caspase cascade, which serves as a trigger for apotosis***

1. some speculate that the ability of radiation to (fairly quickly) impair vascular function – especialy in tumor vasculature – is secondary to ceramide-induced apoptosis of vascular endothelial cells

Clinical Correlate!

2] Does the presence (or upregulation) of one or more apoptosis-related proteins in tumors predict for better clinical outcomes, i.e., that a tumor that is still "pro-apoptotic" might be more sensitive to treatment?

a. *a few (small) clinical studies have attempted to relate an excess of the caspases (caspase 3 in particular) – which would indicate that apoptosis is occuring – with improved patient survival*

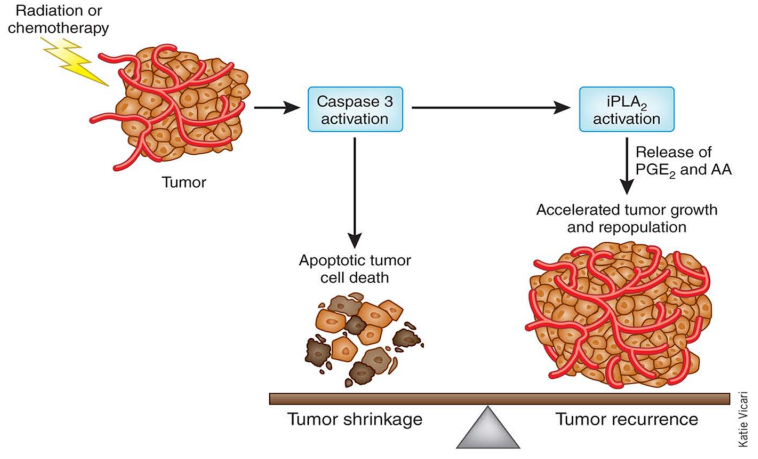


SURPRISE!!!!

The results indicated (for two different types of tumors in two different studies) that increased expression of caspase 3 that is typically associated with cells undergoing apoptosis was actually a ***negative*** prognostic indicator.

What's up with THAT?

1) **turns out that caspase 3 , the main "executioner" caspase** (i.e., the last link in the chain of caspases that actually begins the cellular self destruction) **has other functions besides initiating apoptosis..**



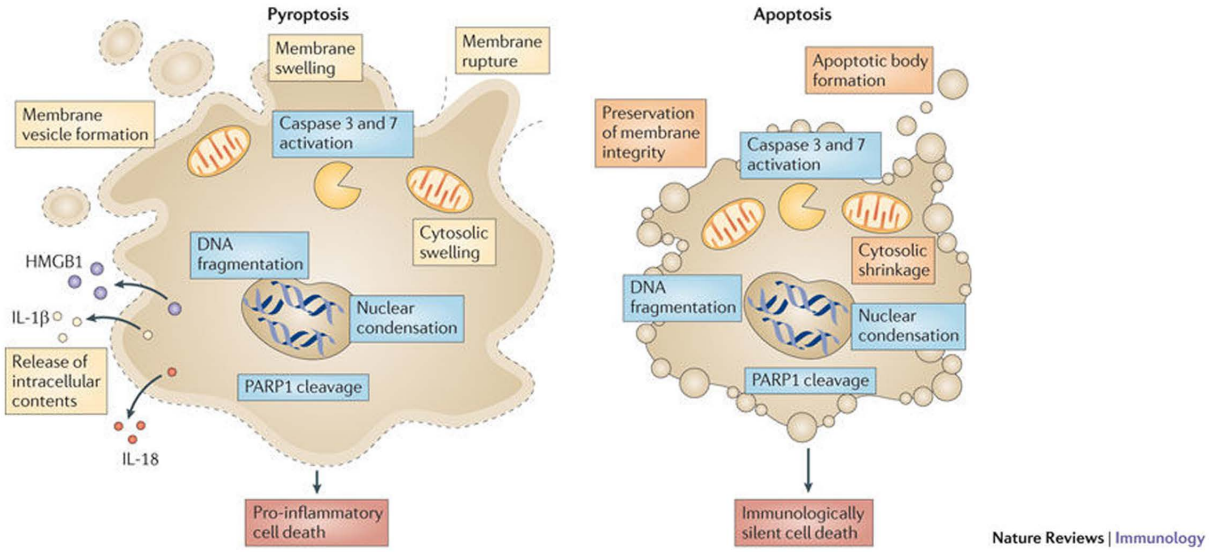
Huang *et al.* show that cytotoxic oncology therapies induce caspase 3 activation, which, in turn, can generate competing effects on apoptotic cell death and stimulation of tumor growth. Thus, the balance between these two competing processes determines the final outcome for the tumor: shrinkage or recurrence. AA, arachidonic acid; $iPLA_2$, calcium independent phospholipase A_2.

...INCLUDING STIMULATING SURVIVING TUMOR CELLS TO ACCELERATE THEIR REPOPULATION, which explains why an excess of caspase 3 may be a bad thing clinically

b) **Pyroptosis** - this is a variant of apoptosis, but with some important differences (and is not a radiation-related phenomenon *per se*)

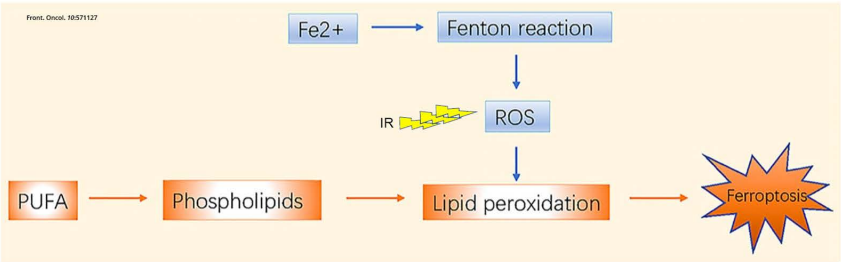
1. pyroptosis has best been characterized in macrophages, and occurs in response to microbial infection, and *shares in common with apoptosis the activation of certain caspases*

2. *unlike apoptosis however, pyroptosis is a highly inflammatory mode of cell death*, and culminates in the cell fragmenting and releasing its contents into the extracellular space



c) **Ferroptosis** - another mode of programmed cell death caused by excessive lipid peroxidation, which can occur under conditions of chronic oxidative stress, especially when natural antioxidants (e.g., glutathione) are in short supply or absent

1. ***ferroptosis gets its name from the fact that the chemical intermediate between the oxidative free radicals and the peroxidized lipids is iron***



a] worth mentioning is the fact that most tumors are under chronic oxidative stress anyway, such as from inflammation, metabolic reprogramming, intermittent hypoxia, etc., and that irradiation can further add to the free radical burden

1) *in theory then, this means that at least some tumor cells should be especially susceptible to ferroptosis, and that modulation of iron content could be harnessed for cancer treatment*

b) characteristics include: cell swelling and eventual rupture of the outer cell membrane, releasing "DAMPS" and leading to inflammation; mitochondria shrink and involute but the nucleus remains largely intact; iron accumulation; downregulation of glutathione; cascading amounts of lipid peroxidation

Senescence/"Permanent Growth Arrest"

Normal cells have:

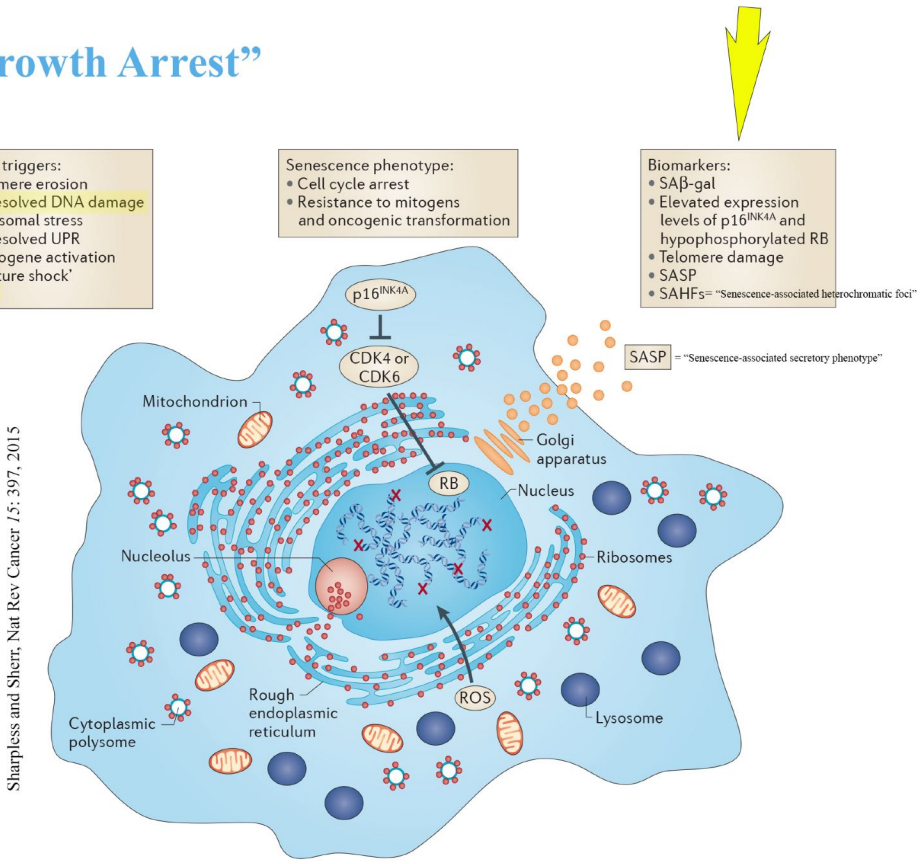
a finite reproductive lifespan, under the control of the telomere-shortening "clock"; once past this point, cells enter senescence... although they may not die in the literal sense at all

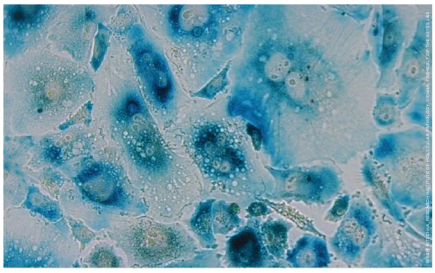
When this occurs as a pathological response to DNA damage, the phenomenon is called "permanent growth arrest". Senescent and growth arrested cells appear very similar, but their conditions may result from different type of genetic or epigenetic regulation.

In the case of radiation exposure, permanent growth arrest can occur in cells irreversibly stuck at cell cycle checkpoints, and in some of the cells that survive mitotic catastrope

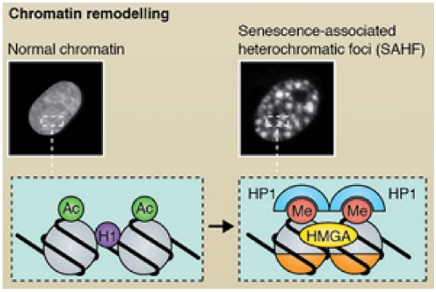
Of the senescence-associated biomarkers, the ones most likely to show up on the Boards are: the expression of SAβ-gal; and the persistent overexpression of p16.

The "closed" chromatin conformation of senescent cells (SAHFs) is also a dead giveaway.





Staining for SAβ-gal in cells entering senescence due to telomere erosion



Chromatin staining in senescent cells looks "spotty" due to its closed conformation and arrangement into distinctive foci, termed "SAHF"

Genes regulating senescence induction in cancer cells*

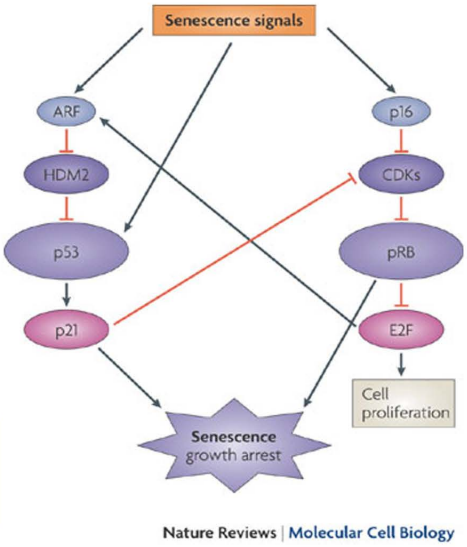
Mechanism and gene	Function	p53 Dependent†	p53 Independent†
Mitotic and stress signaling			
Raf1	Mitogenic/stress signaling	+	
MAP2K6/p38	Mitogenic/stress signaling	+	
Aurora kinase A	Mitogenic signaling		+
PTEN	Oncogenic stress signaling	+	
SKP2	Mitogenic/stress signaling		
Major tumor suppressors			
p53	Transcription factor		+
p63	Transcription factor		+
p73	Transcription factor		+
Rb	Transcription regulator		+
CDKIs			
p21Waf1/Cip1	Kinase inhibitor		+
p16Ink4a	Kinase inhibitor		+
p57Kip2	Kinase inhibitor		
p15Ink4b	Kinase inhibitor		
Mitochondrial integrity and function			
OPA1	Mitochondrial membrane structure	+	
Proinflammatory signaling			
IL-6/CXCR2	Cytokine/receptor	+	
IGFBP-rP1	Cytokine/IGF signaling, modulators		
IGFBP7		+	+

* CXCR2 = chemokine (C-X-C motif) receptor 2; IL-6 = interleukin 6; IGFBP-rP1= insulinlike growth factor–binding protein-related protein 1; IGFBP7 = insulinlike growth factor–binding protein 7; MAP2K6 = mitogen-activated protein kinase kinase 6; OPA1= optic atrophy 1; p38 = mitogen-activated protein kinase 14; $p21^{Waf1/Cip1}$ = CDKN1A/cyclin-dependent kinase inhibitor 1A; $p16^{Ink4a}$ = CDKN2A/cyclin-dependent kinase inhibitor 2a; $p57^{Kip2}$ = cyclin-dependent kinase inhibitor 1C; $p15^{Ink4b}$ = cyclin-dependent kinase inhibitor 2B; PTEN = phosphatase and tensin homolog; Raf1 = raf-1 murine leukemia viral oncogene homolog 1; Rb = retinoblastoma protein; Skp2 = S-phase kinase-associated protein 2.

† Senescence-inducing activity shown to be dependent or independent of intact p53 function, denoted by (+). Blank means undetermined.

JNCI Vol. 102, Issue 20 | October 20, 2010

Arguably though, the two master regulators of senescence are p16 and p21. Both are classic "gatekeeper" tumor suppressor genes that function as CDK inhibitors, rendering cells unable to pass cell cycle checkpoints...permanently



Nature Reviews | Molecular Cell Biology

To become immortalized, tumor cells have to find ways to outsmart one or both of these proteins, thereby evading senescence (and often, apoptosis too). Once this happens though, it is common to end up with chronically elevated p16 and p21 levels, as they valiantly keep trying to shut the cell down.

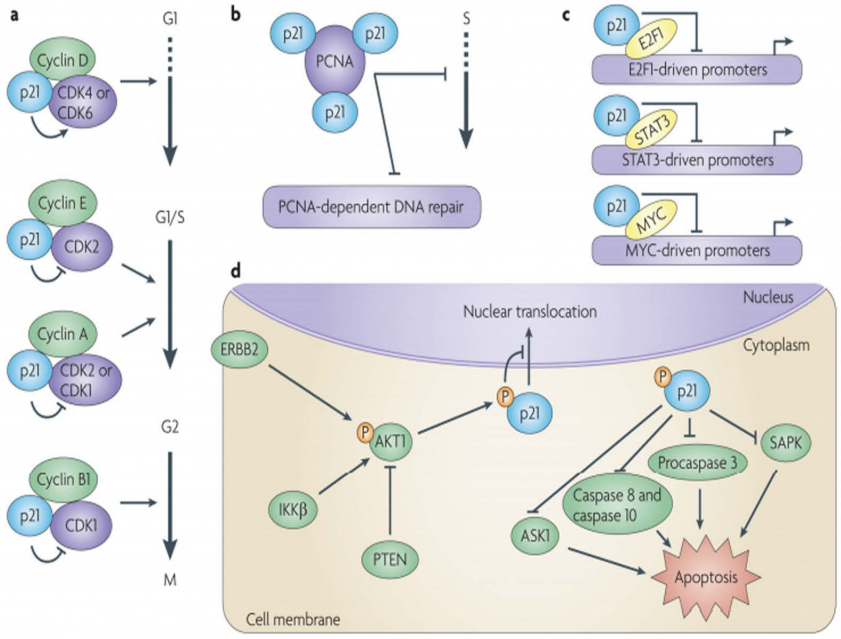
Clinical correlate: This is why HPV+ head and neck cancers overexpress p16...so much so that it can be used as a surrogate for HPV.

1. p21 is more than just a regulator of cell cycle arrest though; it's a master effector of several different tumor suppressor pathways beyond checkpoint control (panel a)...

• it also shuts down PCNA, and therefore, all the DNA repair pathways it participates in (panel b)

• it permanently shuts down the E2F, STAT3 and MYC transcription factors, all related to cell proliferation (panel c)

• *and* it migrates from the nucleus to the cytoplasm, where it inactivates several caspases, meaning that, seemingly paradoxically, it *shuts down apoptosis* (panel d)



The molecular basis of p21 function in cancer
The figure shows activities of p21 in the nucleus and cytoplasm.

(I guess p21 <u>really</u> must want to divert cells toward senescence instead of apoptosis.)

Inducing senescence as a clinical strategy?

1. turns out that there are quite a few cancer drugs that already do this..

Drugs that induce senescence in cancer cell lines and tumors*

Agent	p53 status†	Mechanism	In vitro‡	In vivo§
Aphidocolin	+	DNA polymerase inhibitor	+	
Bleomycin	+	DNA damage	+	
Camptothecin	+/−	DNA damage	+	
Carboplatin + docetaxel	+/−	DNA damage	+	Human lung tumors
Cisplatin	+/−	DNA damage	+	
Cyclophosphamide + doxorubicin + 5-Fluorouracil		DNA damage		Human breast tumors
Diaziquone/AZQ	+/−	DNA damage	+	Prostate xenograft tumors
Doxorubicin	+/−	DNA damage	+	
Epigallocatechin gallate	+	Telomerase inhibition	+	
Etoposide	+/−	DNA damage	+	
Gamma irradiation	+/−	DNA damage	+	+
Hydroxyurea	+	ROS	+	
K858	+/−	KIF11	+	Xenografts tumors
Lovastatin	−	HMG-CoA-reductase inhibitor	+	
Mitoxantrone	+/−	DNA damage	+	Human prostate tumors
MLN4924	−/+	Cul1 SCF subunit inhibitor	+	Prostate xenografts tumors
MLN8054	+	Aurora kinase A inhibitor	+	Colon xenograft tumors
Pyrithione	+/−	Zinc/calcium regulation, ROS	+	
Resveratrol	+	ROS	+	
Retinols	+	Differentiation	+	
TPA, PEP005, PEP008	+	PKC activating	+	
VO-OHpic	+	PTEN	+	Prostate xenograft tumors

* Cul1= cullin 1; HMG-Co-A reductase = 3-hydroxy-3-methylglutaryl-CoA-reductase; KIF11 = kinesin family member 11; PKC = protein kinase C; PTEN = phosphatase and tensin homolog; ROS = reactive oxygen species; SCF = Skp1/Cul1/F-box protein complex.

† p53 status of cells in which the drug induces senescence. (+) denotes active p53 in cancer cells in which the drug induces senescence, whereas (−) denotes senescence induction by the drug in cancer cells in which p53 is deleted or mutated.

‡ Senescence-inducing activity of drug in cancer cells in vitro; (+) denotes induction of senescence in vitro, whereas an empty cell denotes that results were not determined.

§ Senescence-inducing activity of drug in vivo in patients or tumor models. An empty cell denotes that results are not determined; (+) denotes senescence induction in various in vivo tumor models.

This must mean that some tumors still have intact senescence pathways, that we can better learn to manipulate.

In particular, it's DNA intercalating agents that produce the most senescence, radiation intermediate, and microtubule targeting agents the least.

Theoretically, a treatment that induces senescence might be useful for large tumors that have long since bypassed senescence. On the other hand, it might also be useful to help prevent the progression of premalignant lesions, or to treat small, well-differentiated tumors most likely to still have senescence pathways intact.

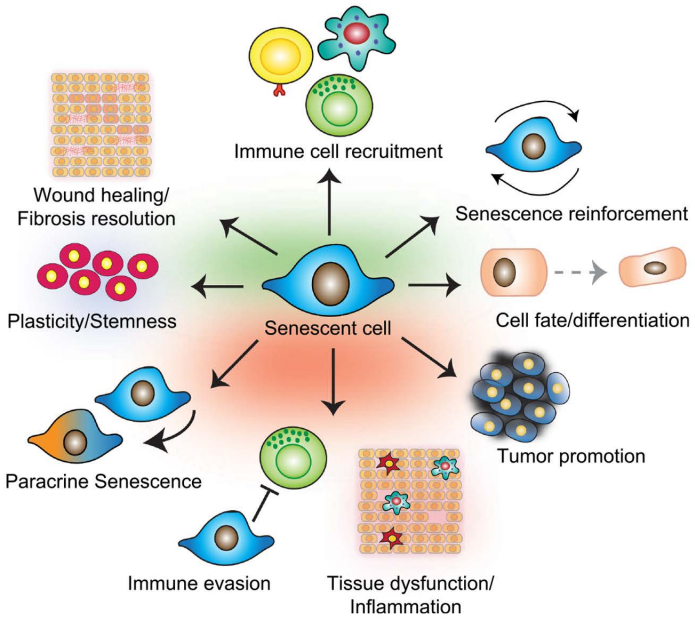
Lots of possibilities!

But wait...senesecence has a downside too

Even though senescence can serve a protective function in the short term by helping preserve tissue structure and preventing overly-damaged cells from proliferating and potentially becoming transformed, in the long term, *the SASP (senescence-associated secretory phenotype) can flood the tissue with inflammatory cytokines that can cause normal tissue dysfunction and further promote carcinogenesis*. And this would only be exacerbated in irradiated tissue that's already damaged.

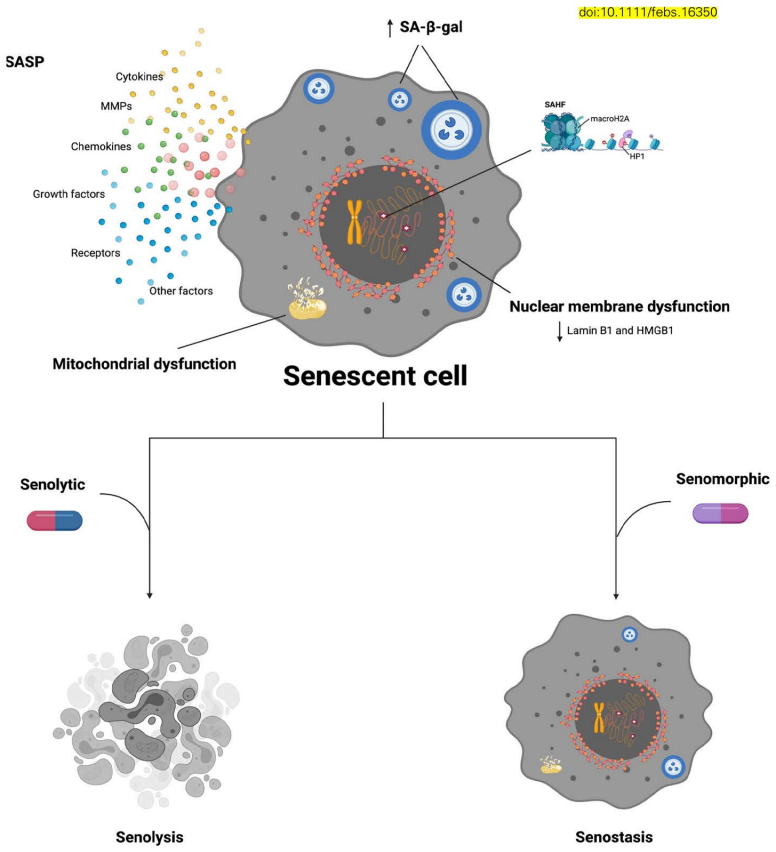
So from this perspective, a different clinical strategy might be to try to *prevent* the accumulation of senescent cells (in tumors or normal tissues).

This is why there's growing interest in the use of senotherapeutics, drugs that selectively kill senescent cells by reactivating apoptosis or another mode of cell death (senolytics), or drugs that shut off or neutralize the SASP but otherwise leave the senescent cells intact (senomorphics).



The pleiotropic functions of the SASP. Shown here is a summary of the effects exerted by senescent cells (in the *middle*) that are mediated by the SASP. The effects *above* the senescent cell (in green) represent those that are considered beneficial, whereas those at the *bottom* (in red) reflect some of the detrimental consequences of the SASP.

GENES & DEVELOPMENT 34:1565–1576, 2021



General hallmarks of cellular senescence and action mechanisms of senolytic versus senomorphic compounds.

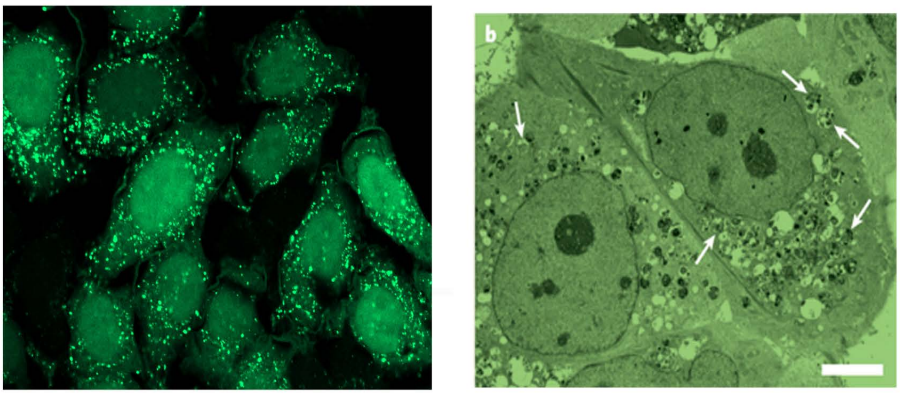
Autophagy - *(from the Greek, "auto" oneself, "phagy" to eat)* **is a tightly regulated catabolic process that involves the degradation of intracellular components via the lysosomes**

1. Autophagy occurs at low basal levels in virtually all cells to perform recurring functions such as the removal of long-lived or defective protein and organelles.

2. However, **under stress conditions – including starvation, growth factor withdrawal and high energy demand – autophagy is rapidly upregulated, such that the cell can recycle its own components for energy generation or a supply of nutrient building blocks**

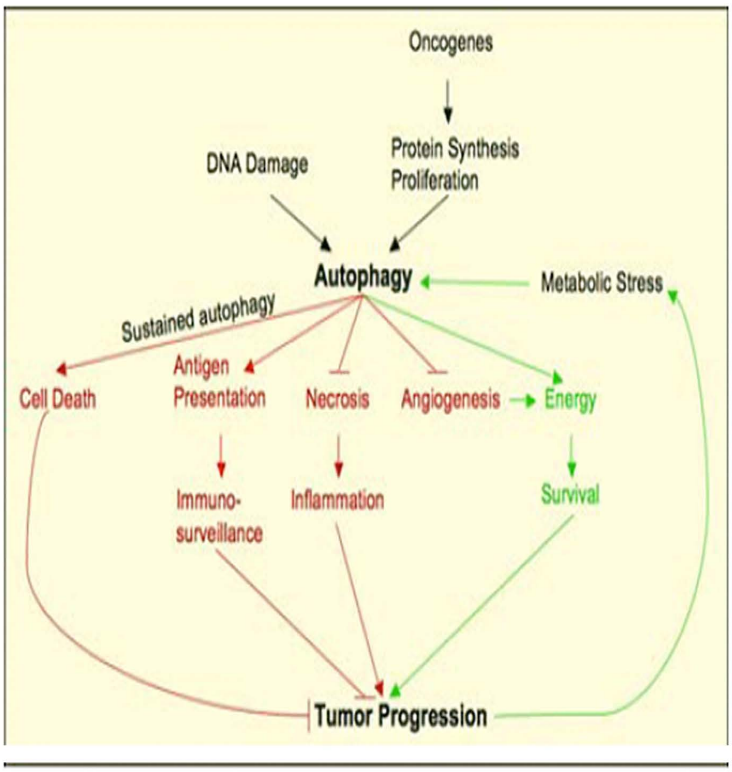
a. autophagy is also responsive to other environmental cues such as temperature, oxygen concentration (both hyperoxia and hypoxia can induce it), and cell density

Nat Chem Biol 3: 331-338, 2007



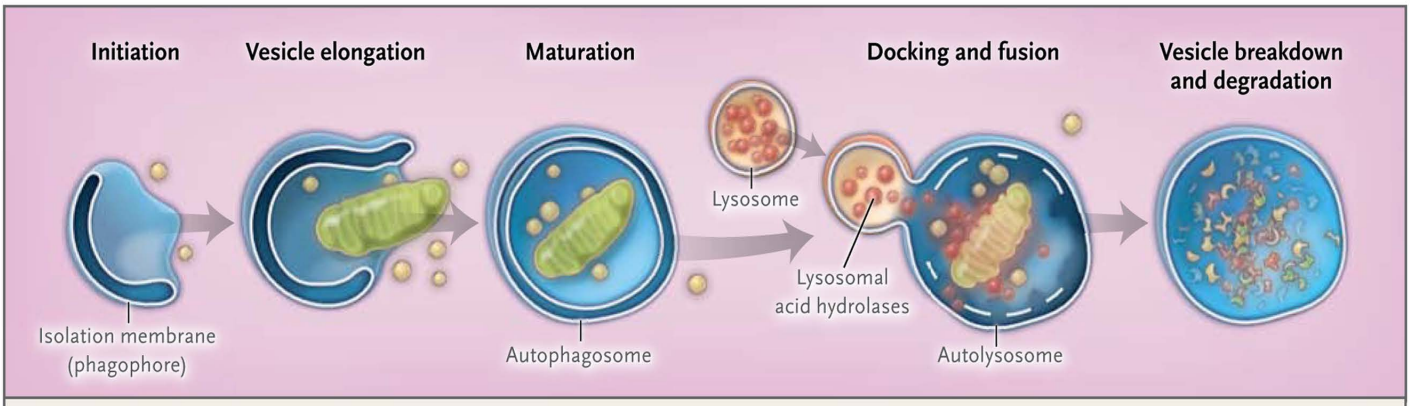
3. So...is autophagy a good thing or a bad thing?

Technically, it's both – it has both tumor-promoting and tumor-suppressing activity, depending on the exact context



4. How autophagy works:

approximately 15 proteins are involved in autophagy



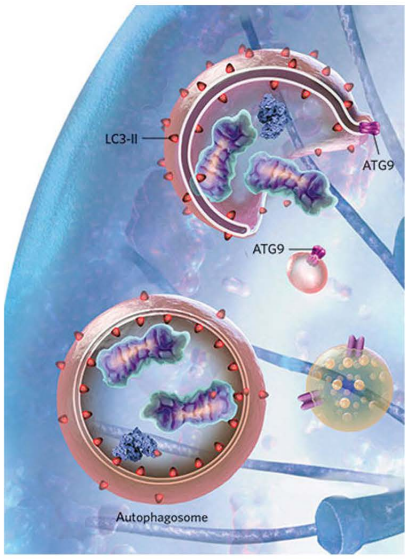
Phases of the Autophagic Pathway.

The autophagic pathway proceeds through several phases, including initiation (formation of a preautophagosomal structure leading to an isolation membrane, or phagophore), vesicle elongation, autophagosome maturation and cargo sequestration, and autophagosome–lysosome fusion. In the final stage, autophagosomal contents are degraded by lysosomal acid hydrolases and the contents of the autolysosome are released for metabolic recycling.

N ENGL J MED 368;7 NEJM.ORG FEBRUARY 14, 2013

- First, sequestration of the materials to be degraded begins with the formation of a **phagophore** that expands into a double-membrane **autophagosome**
- Second, the autophagosome fuses with a lysosome forming an **autolysosome** whose hydrolytic enzymes degrade the materials

- Finally, the autolysosome is degraded and recycled into fresh lysosomes



Two of the key proteins that help orchestrate autophagy are beclin and LC3, which are involved in the formation and maintenance of the autophagosome.

LC3 is being evaluated as a potential target for new drug development with the goal of trying to inhibit autophagy.

Clinical Strategies for Dealing with Autophagy – it's complicated and confusing!

A. Activating / Upregulating Autophagy

1. why you might want to do this: if allowed to continue unchecked, autophagy would *eventually* kill the cell and that, in turn, could lead to improved treatment outcomes

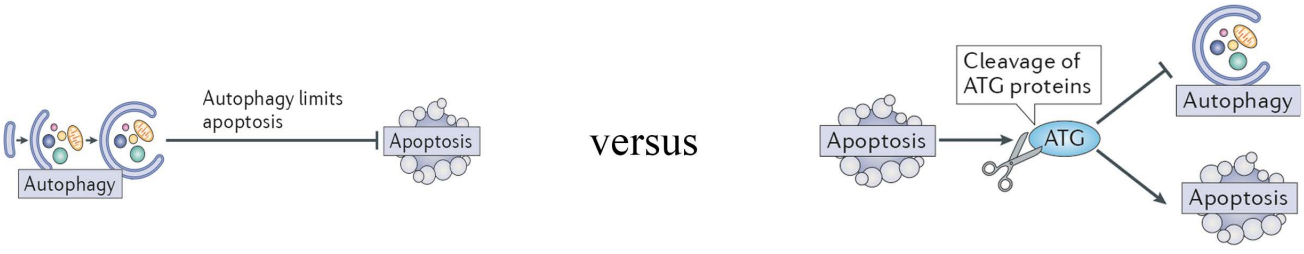
2. why you might not want to do this: it could further tumor progression by providing the energy and raw materials surviving tumor cells need to adapt to suboptimal growth conditions, and facilitate their proliferation

B. Inhibiting / Downregulating Autophagy

1. why you might want to do this:

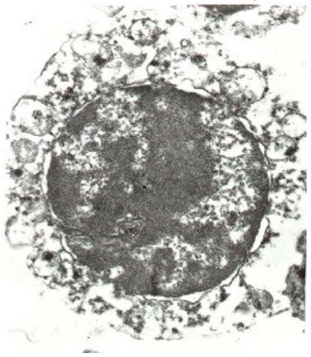
a) in many (but not all) studies using radiosensitization as an endpoint, cells capable of vigorous autophagy were more radioresistant, not more sensitive, meaning that *inhibiting* autophagy would make more sense

b) under some conditions, inhibition of autophagy can cause reactivation of apoptosis; this can happen because ***we now know that all the different modes of cell death "talk" to each other, so that the selective manipulation of one could be used to influence the others***



Necrosis - loss of reproductive integrity (and ultimately, frank cell death) secondary to localized nutrient or oxygen deprivation; cells that undergo necrosis, unlike the case of apoptosis, swell and rupture, spilling their contents into the intercellular space and often, eliciting an immune response

J. Submicrosc. Cytol. Pathol. *22:* 191, 1990



Swollen, "foamy" appearance of cells undergoing necrosis.

Outer cell membrane is also punctured, causing cellular contents to be dumped into the surrounding microenvironment.

a) *historically, necrosis was viewed as a relatively unregulated process* that occurred in response to severe conditions of nutrient or energy deprivation, and involved the following steps:

- oncosis - cell swelling
- membrane blebbing
- pyknosis - nuclear shrinkage
- karyolysis - nuclear degradation
- outer membrane rupture

...culminating in a brisk immune response and inflammation

b) **Necroptosis, aka "Programmed Necrosis"** - a (somewhat) apoptosis-like variant of necrosis

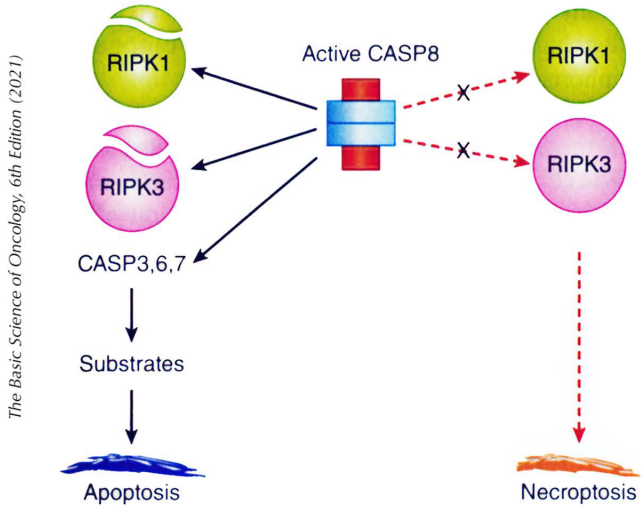
1. **necroptosis was first identified as a defense mechanism against viral infection, however it can also be triggered by a large excess of reactive oxygen species or DNA damage, or when PARP is over-activated**

Necroptosis requires the kinase activities of RIP1 and RIP3 ("receptor-interacting protein"), which leads to the disintegration of plasma, mitochondrial and lysosomal membranes.

Necroptosis sometimes fills in for apoptosis when the latter is inhibited, however unlike apoptosis, necroptosis is a highly inflammatory mode of cell death.

If caspase-8 is active, the RIP kinases are degraded, meaning apoptosis is available, but not necroptosis. If caspase-8 is *in*active, the RIP kinases are stabilized, and then necroptosis occurs, but not apoptosis.

More crosstalk between death pathways!

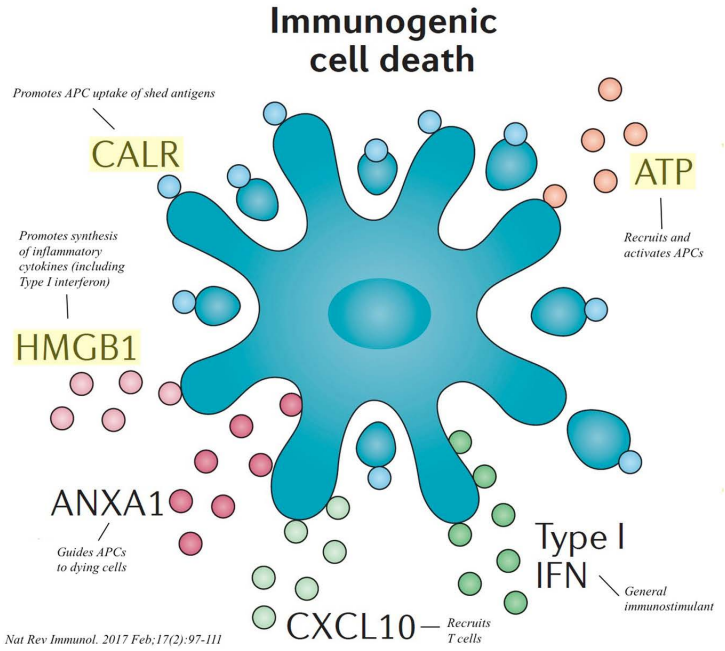


The Basic Science of Oncology, 6th Edition (2021)

Representation of the crosstalk between apoptosis and necroptosis.

Immunogenic Cell Death

a mode of cell death that can be induced by radiation exposure, that is highly inflammatory in nature, and shares some characteristics with both necrosis and apoptosis



1) ***immunogenic cell death involves the necrosis-like release of DAMPS ("danger-associated molecular patterns"), in particular, calreticulin, ATP and HMGB1, which prime antigen-presenting dendritic cells***

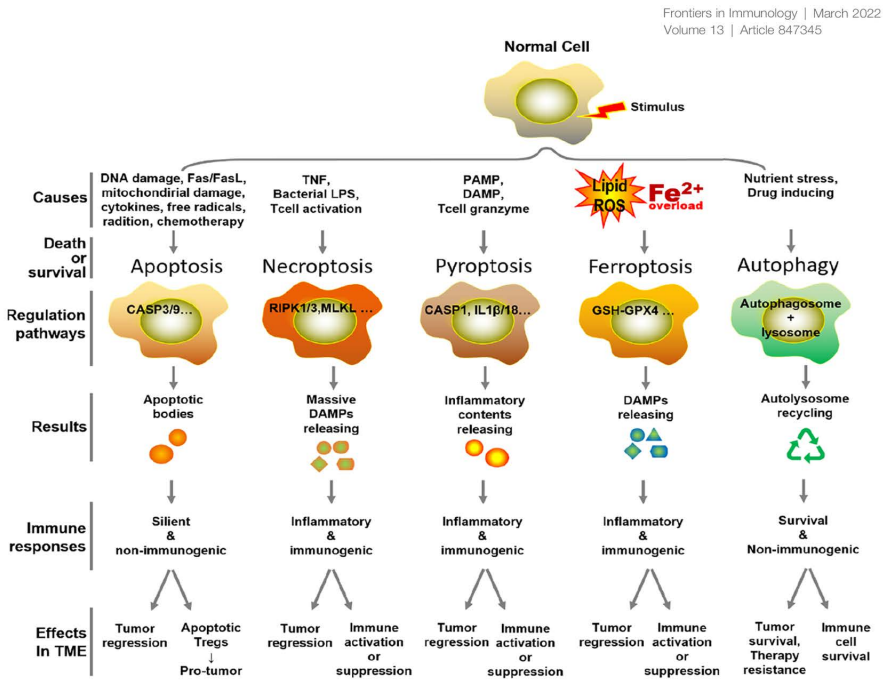
2) the dendritic cells then go on to prime cytotoxic T lymphocytes (CTLs) for an adaptive immune response

3) after the release of DAMPS, the dying cell then "implodes" more akin to apoptosis

Immunogenic cell death, along with the other pro-inflammatory death pathways, are of growing interest to radiation oncology, because they are more likely to occur when large doses are used (think SBRT), and if combined with immune checkpoint inhibitors, could lead to enhanced immunity and abscopal effects

Appendix Materials

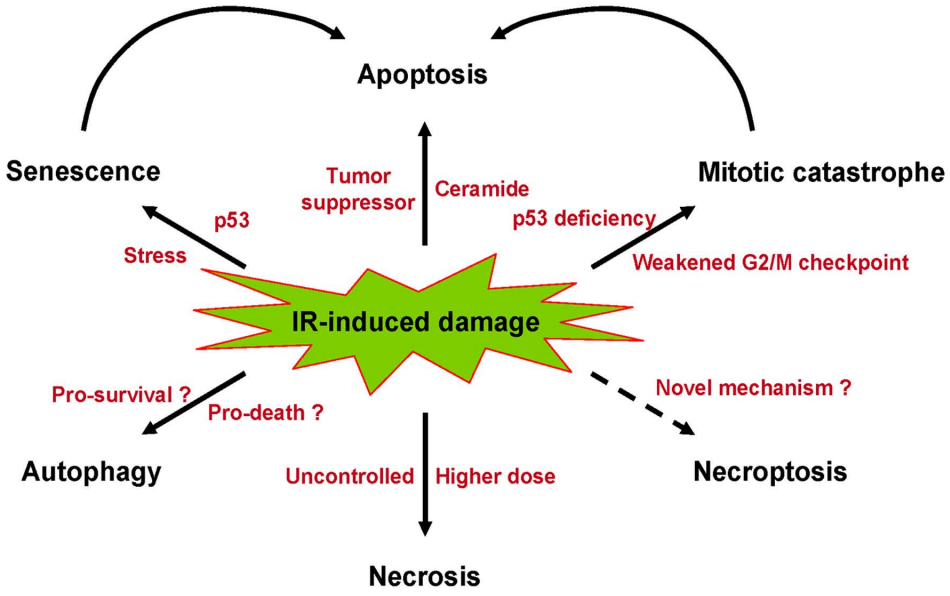
Modes of Cell Death Compared (graphical format)



Modes of Cell Death Compared (tabular format)

	Mode of Cell Death after Irradiation				
MODE OF CELL DEATH	**Mitotic Catastrophe**	**Apoptosis**	**Necrosis**	**Autophagy**	**Senescence**
HOW/WHY	Triggered by faulty mitoses caused by the presence of chromosome aberrations and/or spindle defects	Triggered by irreparable nuclear or mitochondrial DNA damage and/or an excess of ROS. Excess ROS can also activate acid sphingomyelinase (ASMase), that converts sphingomyelin to ceramide, which in turn activates caspases, thereby initiating apoptosis.	Triggered by extreme nutrient and/or oxygen deprivation	Triggered by nutrient (including O2) or growth factor withdrawal and/or high energy demand; causes degradation of intracellular components via the lysosomes…but **doesn't necessarily culminate in cell death unless persistent for extended periods**	Normal cell cycle-stopping process that occurs secondary to telomere shortening and followed by up-regulation of p16 and p21; **when induced by radiation or drugs, the preferred term is permanent growth arrest (i.e., not "normal")** and is triggered by unrepaired DNA, unfolded proteins, and/or excess ROS
SIGNIFICANCE VIS-A-VIS RADIOTHERAPY	**Main mode of cell death after irradiation for most types of cells**	**Main mode of cell death after irradiation for a small subset of normal cells, but usually absent in tumor cells**; loss of apoptosis renders some tumor cell types more radioresistant	Because cells swell (oncosis), explode and dump their contents into extracellular space, necrosis is highly inflammatory	Importance for radiotherapy not clear, however generally speaking, autophagy plays a protective role against cellular insults, and inhibiting it can lead to radio- or chemo-sensitization	More likely to be induced after higher radiation doses than lower ones; can also be induced by DNA intercalating agents
TIMESCALE	Hours at minimum, but could also be days or even weeks depending on the radiation dose (**higher dose = shorter time frame**)	24 hours or less in most cases	Hours to days	An ongoing, basal-level process, but that ramps up under stressful conditions within a day or two	Minutes to hours to trigger, however arrested cells could persist for days, weeks or longer
BIOMARKER(S)?	Flattened cells that are either multi-nucleated and/or contain micronuclei in the cytoplasm	**DNA laddering**, cell blebbing, **TUNEL assay, transport of phosphatidyl serine to outer cell membrane; caspase activiation**, etc.	Random DNA clumping and degradation; mitochondrial swelling and organelle degradation; cytoplasmic vacuoles; for necroptosis, **increased activity of RIP1 and RIP3 kinases.**	Autophagic vacuoles; assembly of the auto-phagosome facilitated by **beclin** and LC3	"Fried egg" cellular morphology; **SA-beta galactosidase expression**; release of inflammatory cytokines (**SASP**); increased glucose metabolism; closed chromatin conformation; chronically active DDR; **persistent over-expression of p16 and p21**
VARIATIONS		Anoikis: occurs secondary to loss of cell anchorage or down-regulation of EGFR; not immunogenic; helps facilitate survival of circulating tumor cells; Pyroptosis: highly inflammatory mode of cell death triggered by microbial infection or the presence of host dsDNA in the cytoplasm (AIM2 is the sensor)	Necroptosis: a programmed version of necrosis that occurs when there is an excess of DNA damage or ROS; and/or when PARP or ALKBH7 is hyper-activated; highly inflammatory Immunogenic Cell Death: highly inflammatory and elicits a strong immune response	Mitophagy: autophagy of mitochondria only Xenophagy: autophagy of infectious particles only	
INTER-RELATIONSHIPS	Has a few features in common with apoptosis, including the expression of a subset of caspases. **Presence of micronuclei in the cytoplasm (containing dsDNA) can trigger the cGAS-STING pathway,** upregulating the expression of inflammatory cytokines and eliciting an immune response	Apoptosis competes with senescence under some circumstances. Pyroptosis has some features in common with both apoptosis and necrosis.	Necroptosis can sometimes substitute for apoptosis when the latter is down-regulated, or when caspases are inhibited. Immunogenic cell death has some features in common with apoptosis and autophagy	Autophagy can stave off apoptosis or necrosis in certain circumstances. ATP produced during autophagy can be used to power other modes of cell death	Senescence sometimes competes with apoptosis by up-regulating anti-apoptosis proteins. **"Senolytic" drugs** aim to kill senescent cells by reactivating apoptosis and/or interfering with glucose metabolism
ULTIMATE FATE OF "DEAD" CELLS	Cleared (eventually) via phagocytosis or sometimes, apoptosis	Rapid phagocytosis of apoptotic bodies, however in the cases of pyroptosis,"DAMPS" are released into the microenvironment first	Phagocytosis of any larger cellular fragments by tissue macrophages and other immune cells	Persistent, extreme autophagy eventually does kill cells, typically by apoptosis or necrosis	Cleared (eventually) via phagocytosis by immune cells, or sometimes, apoptosis

Radiation's Proposed Roles in Different Modes of Cell Death: It's Complicated!



(Note: It is assumed here that the inactive, giant cells that result from senescence or mitotic catastrophe die (again!) by undergoing late apoptosis)

Apoptosis Assays

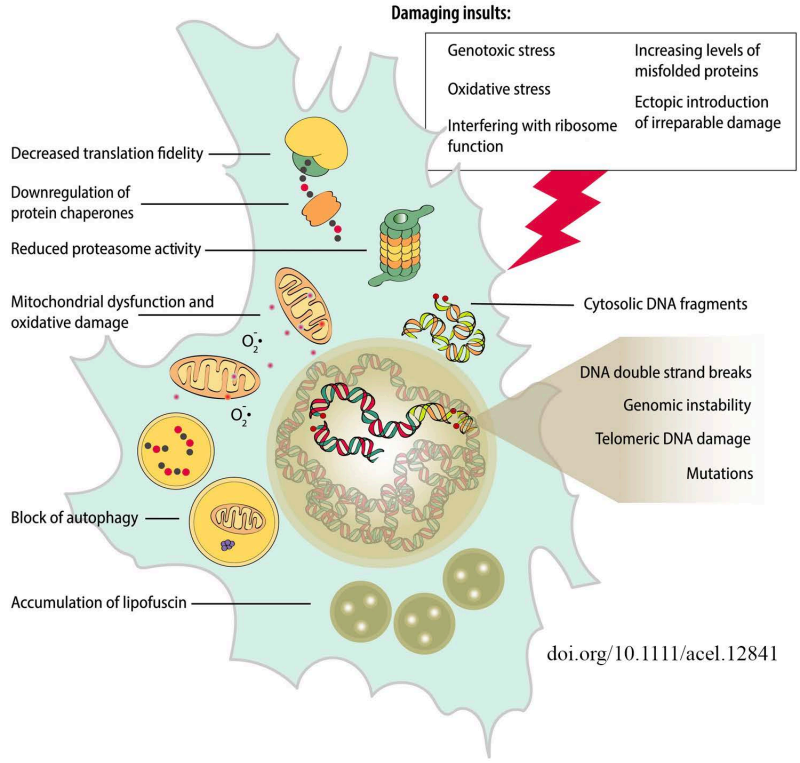
1. **DNA Laddering:** results from the action of specific endonucleases activated during apoptosis; the pattern of uniformly nicked (every 200 base pairs or so) genomic DNA looks like a ladder when using gel electrophoresis

2. **TUNEL Assay** or "Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling": an imaging technique that labels the DNA fragments with either a fluorescent or chromogenic probe that allows apoptotic cells to be visualized in tissue sections

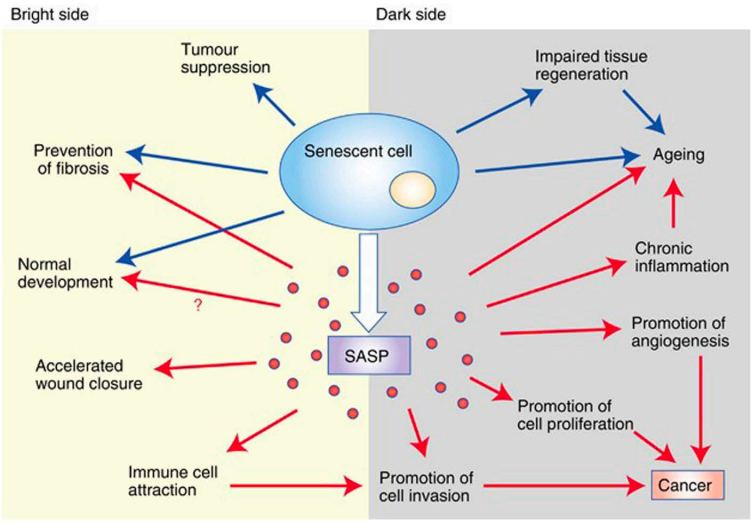
3. **Annexin V Binding**: during the early stages of apoptosis, phosphatidylserine (PS) becomes exposed on the outer cell membrane, and annexin V binds to it, allowing apoptotic cells to be visualized using immunological reagents

4. **Caspase Activity**: caspases are activated when cytochrome C is released from the mitochondria, and go on to disassemble the cell proteolytically; the activity of these enzymes can be assayed biochemically by monitoring the loss of one of their specific substrates, PARP

Senescence: A Deeper Dive

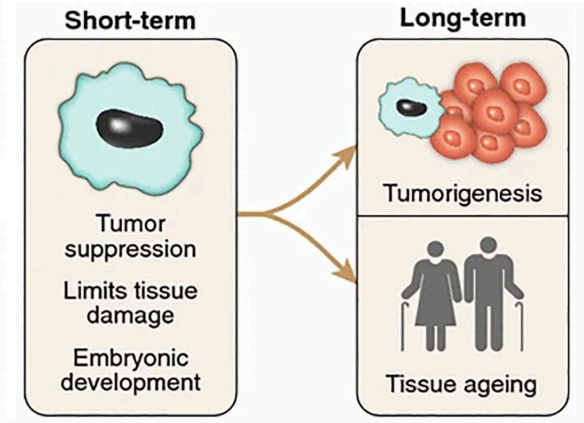


Different triggers for senescence and some of the cellular consequences (besides the permanent suspension of cell cycling).



Br J Cancer 114:1180-1184, 2016

The "good news/bad news" aspects of senescence



Short and long term biological consequences of cellular senescence