Bid, one of the first family members described with a BH3 domain only, is a cytosolic protein and lacks a membrane targeting sequence. Caspase-8 cleaves Bid to yield a truncated COOH-terminal 15 kD protein (tBid), which readily moves to mitochondrial membranes. All BH3-only proteins described to date lack C-terminal membrane spanning sequences and have non-mitochondrial localizations, with mitochondrial translocation taking place downstream of specific apoptotic signaling pathways. As for Bax, changes in protein conformation may be required for membrane targeting.
As Bid contains a buried BH3 domain, Bid can be classified as a latent pro-apoptotic protein. The specific activation mechanism for Bid, caspase-mediated cleavage, is predicted to lead to exposure of the BH3 domain. Similarly, caspase proteolysis at the NH$_2$-terminus of Bcl-2 and Bcl-x$_L$ generates pro-apoptotic versions of these proteins. The NH$_2$-terminal α-helix forms an undercarriage for the BH3 helix, thus removing this portion of the protein may untether the BH3 domains.

Lipid interactions may also be involved in membrane targeting of BH3-only proteins. Membrane insertion of tBid is favored in lipid bilayers containing >20% cardiolipin, a mitochondria-specific phospholipid normally restricted to the inner mitochondrial membrane. Post-translational lipid modification of tBid takes place by myristoylation at the NH$_2$-terminal glycine generated following caspase-mediated cleavage.

The pro-apoptotic Bad protein also lacks a COOH-terminal signal/anchor sequence but has two consensus 14-3-3 binding sites. Phosphorylation at serines 112 and 136 within the 14-3-3 binding site results in sequestration of Bad bound to cytosolic 14-3-3 proteins. Bad phosphorylation occurs downstream of growth factor signals and has been attributed to several kinases: protein kinase B/Akt, mitochondrial-anchored protein kinase A, p70S6 kinase and PAK1 kinase.

Other BH3 proteins interact with distinct extra-mitochondrial targets. Bim is localized to the microtubule dynein motor complex by an interaction with the dynein light chain, DLC 1 and Bmf associates with dynein light chain 2 (DLC2) in the myosin V actin motor complex. Several apoptotic stimuli, including paclitaxel (Bim) and detachment from solid supports (Bmf), result in translocation of these BH3 proteins to mitochondria.
Why is there such apparent redundancy for both pro- and anti-apoptotic BH proteins? In the case of the pro-apoptotic proteins, it has been suggested that the large number of pro-death family members is indicative of specialization. The emerging pattern for pro-apoptotic BH proteins is one of latent function requiring re-targeting, post-translational modifications and/or conformation changes for activation. The unique localization, protein associations and mechanism of activation found for the individual pro-apoptotic members Bax, Bad, Bid, Bim and Bmf support a hypothesis that each acts as a “sentry” for distinct damage signals, thereby increasing the range of inputs for endogenous death pathways.

Apoptotic pathways

In order to deal with the plethora of cues for apoptosis, several discrete signaling pathways are available. For the most part, the components of these circuits are pre-formed and do not require new gene expression. Since these stimulus-specific responses funnel into a common circuit involving mitochondrial disruption and/or activation of terminal caspases, altered expression or mutation of core apoptotic machinery affects apoptotic susceptibility to a wide-range of inducers. Conversely, since the proximal stimulus-specific pathways are reasonably linear, it is possible to ablate a stimulus-specific response (e.g. mutant p53, Fas/FasL). Core pathways have non-linear, feed forward features, as well as high levels of redundancy, such that complete cellular resistance to apoptosis has not been observed.
Death receptor signaling.

In opposition to growth factor receptors, death receptors are expressed on many cell types, where they function as a social restraint on cell viability (particularly for the immune system). The intracellular responses to death receptor engagement appear to be more limited than for growth factors, and the cytoplasmic sequences of members of the death receptor superfamily all contain an 80-residue death domain (DD). Once clustering of receptors by ligand binding occurs, the DD serves to nucleate formation of a caspase activation “machine” for initiator caspases (caspases 8,10) that bear distinct protein interaction motifs in their long pro-domains. Analogous to the apoptosome, this multi-protein complex has been designated as a death-inducing signaling complex (DISC).

Death receptors and proximal caspases do not bind each other directly, but through adaptors containing docking sites for each factor. Analogous to scaffolds in other signal transduction pathways, adaptor proteins amplify the initial receptor signal and approximate key factors, as well as provide opportunities for flexibility and regulation of signaling circuits.

There are eight mammalian death receptors (TNF-R1, Fas, TRAMP, DR4, DR5, DR6, NGF-R, EDA-R). The extracellular domains contain several cysteine-rich domains forming an extended structure stabilized by disulfide bonds. Death receptor ligands share a TNF homology domain and bind as trimers to cysteine-rich domains of the corresponding receptors. All known ligands are expressed as type II transmembrane proteins and are subject to limited proteolysis generating soluble forms. In most cases, soluble ligands are inferior to membrane bound forms for receptor activation. Thus, cell-cell contacts are necessary for death-receptor signaling, justifying the characterization of these apoptotic deaths as “fratricides”.

In the simplest example, binding of Fas ligand to the CD95/Fas receptor triggers receptor multimerization. An adaptor protein, FADD, binds at the Fas cytoplasmic domain using homotypic DD associations. Similarly, procaspase-8 is bound to FADD by homotypic death effector domain (DED) interactions. Unprocessed procaspase-8
enzymes assemble as dimers in the DISC, and acquire new proteolytic activity directed specifically against adjacent dimers. Receptor-activated assembly of the DISC has several complementary effects – stabilizing pro-caspase dimers, functional activation of dimers, and presentation of dimers for proteolytic processing – all of which require bound caspase-9. A N-proximal processing separates the caspase-8 prodomain from the catalytic subunits, allowing untethering of active caspase-8 from the DISC. Processed caspase-9 exists as a stable dimer capable of procaspase-3 processing, but can no longer process pro-caspase-9 dimers.

Cells with vigorous DISC assembly bypass Bcl-2 interdiction (Type I cells), while lower levels of DISC output are amplified by mitochondrial pathways that are regulated by Bcl-2 proteins.
Superimposed on this 3-component model are additional factors that can replace one of the core components. FLIP (FLICE/caspase-8 inhibitory protein) is homologous to caspase-8 but devoid of protease activity (the active site cysteine is replaced).

Different splice forms of FLIP retain the DED motif and either compete with caspase-8 for binding to FADD or prevent release of caspase-8 from the DISC. Thus, FLIP interrupts communication between Fas receptor and effector caspases, blocking apoptosis. Incorporation of FLIP in the DISC, in addition, leads to the recruitment of additional factors (Rip, Trafs) that connect to signal transduction pathways involving NF-κB and ERK.
Two arenas where death receptors act physiologically involve lymphocytes. Activation-induced cell death curtails T lymphocyte immune responses via Fas receptor signaling. Fas ligand and Fas are induced during T cell activation downstream of lck and NF-kB. Engagement of Fas on one cell by Fas ligand on a second cell triggers apoptosis. Thus, the Fas-FasL system provides an upper limit on the density of activated T cells at sites of inflammation.

Lymphocyte cell death is also directed by FasL expression on dissimilar cells. Fas expression in germinal center B lymphocytes appears to play a role in eliminating cells bearing self-reactive surface immunoglobulin, as mice expressing Fas only on T lymphocytes acquire high levels of auto-antibodies. In this case, FasL expression on T cells delivers the fatal blow. T lymphocytes can also be eliminated by FasL expressed on non-lymphoid cell types. Immune-privileged zones such as the eye and testis can be transplanted with allogeneic tissue due to the lack of immune surveillance at these sites, enabling corneal transplants from unrelated donors without need for immunosuppression. Gld-mice deficient in FasL expression do not manifest site-specific restrictions to immune responses, and develop vigorous inflammatory responses to viral infections and allogeneic cells. In place of a physical “barrier” to lymphocyte trafficking to immune-privileged sites, constitutive expression of FasL by interstitial and support cells in these locations effectively deletes trespassing cells.
Fas expression is constitutively expressed in some non-lymphoid tissues (hepatocytes, cardiac muscle, kidney epithelium) and in others, is induced during acute stress responses (UV and gamma-radiation). FasL-Fas interactions take place during cytotoxic T lymphocyte killing and in some circumstances Fas has proven necessary for target cell killing.

Targeted deletions of components of the Fas signaling pathway indicate the importance of this pathway in development. Mouse knockouts for caspase-8, FADD, as well as FLIP, die during embryogenesis with severe cardiac malformations. Although lack of normal developmental death can lead to abnormal morphogenesis, the ability of death receptors to communicate with other signal transduction pathways may have important physiologic consequences. Fas signaling in resting lymphocytes co-stimulated with antigen has co-mitogenic effects, indicating that entry to alternative signaling pathways dependents on cell activation state and receptor density.

**DNA damage**

Among other types of cellular damage, alterations in DNA structure as a result of oxidation, alkylation, single or double-strand breaks (including stalled replication forks) are notorious for triggering apoptotic pathways. Apoptosis is recognized as part of a larger DNA damage response, involving cell cycle checkpoint (control mechanisms ensuring dependency in the cell cycle) and repair pathways.
The transition from attempts at damage repair to throwing in the towel is usually thought to involve some quantitative aspect of DNA damage, perhaps the ability to repair DNA below a certain threshold before apoptotic mechanisms kick in. Some insight into the biochemical mechanism has come from understanding of p53 protein degradation pathways. In healthy cells, p53 protein has a half-life of 5-20 min, with ubiquitin-mediated degradation governed by its interactions with Mdm2. Mdm2 acts as a ubiquitin E3 ligase for p53. p53 functions as a tetrameric, sequence-specific transcription factor, thus transcriptional activity has an exponential dependence on p53 concentration in the nucleus. Activating signals for p53 result in increased stability of p53 protein and decreased Mdm2-mediated degradation. At early times following p53 activation, negative feedback control of p53 expression involves p53-dependent transcription of Mdm2, resulting in increased p53 degradation. However, continued stabilization of p53 activates another p53 target gene, the lipid phosphatase PTEN. PTEN inactivates the second messenger PI(3,4,5)P3, ultimately resulting in nuclear exclusion of Mdm2 and stabilization of p53. Pten is required for p53-mediated apoptosis in murine embryonic fibroblasts.

Several types of changes to the DNA template trigger activation of chromatin-associated kinases over long distances, probably through changes in higher order chromatin structure. In response to DNA strand breaks, the PI(3)K-related kinase ATM phosphorylates multiple substrates including p53, Mdm2, and the checkpoint kinase,
Chk2 (Cds1). Post-translational modification of p53 at several sites leads to inhibition of Mdm2 association and transcriptional activation. Transcriptional targets of p53 include several pro-apoptotic Bcl-2-related genes (Bax, Noxa, Puma), death receptors (Fas, DR5), oxidation-reduction enzymes (PIG3), and APAF-1. There are also examples of p53-dependent apoptosis that are independent of transcriptional activity; associations of p53 with mitochondria and a cytosolic E3 ligase (Parkin) have been described. Direct targeting of p53 to mitochondria triggers mitochondrial pathways of apoptotic cell death. Mitochondrial localization involves interactions with BCL-X₁ and BCL-2 proteins. (Mol Cell 11: 577-90, 2003).

**Protein stress (ER-IRE1-TRAF2-ASK1,caspase-12)**

Protein stress responses are a recent addition to apoptotic pathways. These highly conserved mechanisms provide feedback fidelity control of protein folding, and glycosylation and secretory pathways in the endoplasmic reticulum. Multiple inputs (amino acid deficiency, glucose deprivation, calcium dysregulation, proteasomal activity) trigger this pathway via their effects on ER protein folding.

In yeast models, IRE1, a unique ER transmembrane protein with both serine/threonine kinase and endoribonuclease activities, functions as a sensor for misfolded or unfolded ER proteins. IRE1 kinase activity is normally suppressed by binding to the ER chaperone protein GRP78/BiP. Unfolded proteins accumulating within the ER lumen recruit GRP78/Bip away from IRE1, allowing IRE1 oligomerization and auto-phosphorylation of IRE1. IRE1 Phosphorylation stimulates endoribonuclease activity for a specific target, HAC1 mRNA encoding a basic leucine zipper transcription factor. The HAC1 transcript is constitutively expressed, but contains a non-classical intron that inhibits translation. IRE1 removes this intron by two site-specific cleavages, and a third factor, Rlg1p, splices the remaining exons. With efficient translation of the spliced mRNA, HAC1 activates transcription of several ER
chaperones.

The mammalian version of the UPR incorporates two additional features: general suppression of translation, and a link to apoptotic pathways. Inhibition of translation is accomplished using an ER transmembrane kinase, PERK, related to the dsRNA-dependent kinase, PKR. PERK phosphorylates the eukaryotic initiation factor EIF2α, inhibiting assembly of pre-initiation complexes at ribosomes. Apoptosis is triggered via an ER-specific caspase, caspase-12. Caspase-12 binds to IRE1 and is processed in response to ER stress.

Oncogene-induced apoptosis

Hyperactivity of mitogenic oncogenes such as Myc, adenovirus E1A, and Ras triggers a common pathway of p53 accumulation, via induction of the ARF tumor suppressor gene. P14ARF (or P19ARF in mice) is encoded by an alternative reading frame in
the p16INK4a locus. ARF inhibits Mdm2, the p53 E3 ubiquitin ligase, and transports Mdm2 to the nucleolus, where additional ARF functions are evident. Processing of precursor rRNAs to 28S, 18S, and 5.8S rRNAs is inhibited by ARF, independently of p53 or Mdm2.

The proximal signals for oncogene-dependent induction of ARF are poorly understood at this time.

Survival signaling pathways

Social models of apoptosis postulate cell death as a default pathway for single cells, with a survival requirement for signals from neighboring cells (embryonic blastomeres are an exception). The prototypical example of intercellular survival signaling is the insulin-like growth factor-1 (IGF-1)- PI(3)kinase – Akt kinase pathway. Note that survival pathways are engaged downstream of most, if not all, growth factors.

The IGF-1 receptor tyrosine kinase is auto-phosphorylated in trans following ligand binding. An adaptor protein (Insulin Receptor Substrate = IRS) binds to the cytoplasmic domain of the receptor via a phosphotyrosine binding domain. The IRS adaptor is phosphorylated on tyrosines in turn, enabling the p85 regulatory subunit of PI(3) kinase to bind, and relieving inhibition of the catalytic PI(3) kinase. Phosphorylation of phosphatidylinositol in the plasma membrane yields PI3,4,5P3, PI3,4P2 and PI3P; these lipid signaling molecules function by recruiting signaling proteins containing Pleckstrin homology (PH) domains to the plasma membrane. One, the serine/threonine kinase PDK1, activates a second, Akt/PKB by phosphorylation in its active loop.
Several substrates for Akt/PKB serine/threonine kinase are involved in apoptosis. Bad and pro-caspase-9 are inhibited by phosphorylation. Another set of factors with pro-apoptotic effects are the Forkhead transcription factors. Phosphorylated forkhead proteins (FOXO) bind to 14-3-3 proteins and are exported from the nucleus and degraded in the cytosol. Transcriptional targets of FOXO relevant to apoptosis include FasL and Bim. A broader role for FOXO factors includes adaptation to stress and aging (mutants of the homolog DAF-16 in Caenorhabditis elegans have shortened lifespans).

**Metabolic regulation and apoptosis**

While the mitochondrial localization of multiple apoptotic regulators (BCL-2 family, cytochrome c, SMAC, etc.) may be happenstance, it is tempting to speculate that the metabolic functions of mitochondria are somehow linked with apoptosis regulation. Since the “apoptotic” cytochrome c, AIF, HtrA2/Omi proteins have physiologic, functions related to energy metabolism, it may turn out that we are looking at other apoptotic factors, such as BCL-2, IAPs and caspases, from a limited vantage point and broader aspects of their functions will become evident with time. Several observations may be pertinent in this regard:

The presence of BCL-2 survival proteins on the outer mitochondrial membrane maintains outer membrane permeability to adenine nucleotides following growth factor deprivation (Cell Death Differ 7: 1182-91, 2000). This appears to allow prolonged cell survival under glucose starvation conditions by facilitating oxidative metabolism of endogenous substrates (J Biol Chem 276: 12041-8, 2001).

Binding of hexokinase, which controls the first committed step in glycolysis, to the cytoplasmic surface of mitochondria inhibits activation of BAX and BAK following growth factor withdrawal (Mol Cell Biol 24: 730-40, 2004).

The BH3-only protein BAD associates with glucokinase (hexokinase IV) and regulates glucokinase activity in pancreas and liver, such that Bad-deficient mice display glucose intolerance (Nature 424, 952-956, 2003).
Several substrates for the Akt/PKB serine/threonine kinase are involved in apoptosis. Bad and caspase-9 are inhibited by phosphorylation. Another set of factors with pro-apoptotic effects are the Forkhead transcription factors. Phosphorylated forkhead proteins (FOXO) bind to 14-3-3 proteins and are exported from the nucleus and degraded in the cytosol. Transcriptional targets of FOXO relevant to apoptosis include FasL and Bim. A broader role for FOXO factors includes adaptation to stress and aging (mutants of the ortholog DAF-16 in Caenorhabditis elegans have shortened lifespans).

Akt-dependent survival (and by implication any growth factor receptors that utilize PI3K-Akt signaling for survival) differs from Bcl-2-dependent survival in the effect of glucose as a glycolytic substrate. Whereas Bcl-2 maintains cell viability in the absence of glucose, constitutive Akt activation only works if glucose is available. This is an important distinction, as it changes the focus from life vs. death to the different ways in which cells are viable.

In the case of Akt, anti-apoptotic effects depend on its ability to promote binding of hexokinase (isoforms I and II) to the mitochondrial outer membrane (Robey and Hay, Oncogene, 2006). Hexokinase binds to a protein channel, VDAC (voltage-dependent anion channel) involved in exchange of cytosolic ADP for ATP, although the details of how Akt regulates this step are not well understood. One effect of mitochondrial localization of hexokinase is believed to be the channeling of ATP synthesized by the mitochondria into the first step of glycolysis, glucose-6-phosphorylation. This, in conjunction with the higher intrinsic activity of mitochondrial compared with cytosolic hexokinase, may increase the "pull" on mitochondrial oxidative phosphorylation by increasing the concentration of ADP available for ATP synthesis.
Mitochondrial binding of hexokinase inhibits activation of Bax and Bak, and conversely, the BH3-only Bid protein can displace hexokinase from mitochondria. While these results suggest that hexokinase promotes survival by opposing Bax and Bak, some models of apoptosis that occur in Bax/Bak-/- cells are also inhibited by Akt and mitochondrial hexokinase (Majewski et al., Mol Cell, 2004).

There is also evidence that Bcl-xL and Bcl-2 have similar effects as hexokinase on mitochondrial transport of adenine nucleotides, although without the requirement for glucose. Withdrawal of the growth factor IL-3 from FL5.12 cells leads to accumulation of mitochondrial phosphocreatine in the intermembrane space, suggesting that phosphocreatine transport across the OMM is impaired (Vander Heiden et al., PNAS 2000). Bcl-2 and Bcl-xL prevent this accumulation, while Bcl-xL maintains the open state of the voltage-dependent anion channel in situ (and supports adenine nucleotide exchange across the outer mitochondrial membrane (Vander Heiden et al., J Biol Chem 2001).

Another link between metabolism and Bcl-2 family proteins is provided by the BH3-only protein Bad. The phosphorylated (and apoptosis-inactive) form of Bad associates with glucokinase (hexokinase IV) and regulates glucokinase activity in pancreas and liver, such that Bad-deficient mice display glucose intolerance (Nature 424: 952-956, 2003).

Autophagy (from N. Danial)

In response to the metabolic stress of nutrient starvation, cells activate a homeostatic pathway known as autophagy [from Greek meaning to eat “phagy” oneself “auto”], to degrade long-lived proteins and damaged organelles. An evolutionarily conserved set of proteins orchestrates the recruitment of protein/organelle cargo to vesicles that, in turn, deliver their contents to lysosomes (Levine and Klionsky, Dev Cell 2004). The recycling of organelles and proteins in autophagosomes produces amino acids that feed into the mitochondrial tricarboxylic acid (TCA) cycle and sustain the production of reducing equivalents (FADH$_2$ and NADH), ensuring that the flow of electrons through the mitochondrial respiratory chain complexes and oxidative phosphorylation remains uninterrupted. Autophagy serves multiple organismal functions, including tissue remodeling during development, in addition to survival in face of nutrient starvation or other environmental stresses.

Survival signaling pathways and autophagy responses are hardwired in opposite directions in response to the bioenergetic state of the cell. Survival signaling inhibits autophagy. Downstream of Akt, the mTOR (target of rapamycin) serine/threonine kinase promotes protein synthesis and represses autophagy. mTOR serves as a nutrient sensor activated by high levels of ATP, glucose and amino acids. During nutrient starvation, the activity of mTOR is inhibited by AMPK, another nutrient sensor kinase that is activated when the ratio of ATP to AMP decreases during metabolic stress. Through inactivation of mTOR, AMPK releases the brake on autophagy.
Interestingly, autophagy may have both pro- and anti-survival effects. A morphologic subset of cell deaths with prominent autophagic vacuoles (described as type II or lysosomal cell death) has common features with apoptosis, including the externalization of phosphatidylserine in the outer leaflet of the plasma membrane and lack of associated inflammation, but lacks caspase activation. Whether autophagy is primarily a mechanism of cell death or a means of cellular survival is the subject of intense investigation. Current findings support the notion that autophagy is primarily a self-limited survival response that can promote cell death under a limited set of conditions. Cells deficient in autophagy pathways have normal cell death responses to a variety of stimuli (Yue, PNAS 2003). Autophagy appears to have a greater role in cell death in circumstances when apoptosis is prevented by genetic alterations or pharmacologic inhibitors. In nutrient-poor conditions (or equivalently inhibited mTOR signaling), autophagy maintains survival (Lum JJ et al., Cell 2005). In contrast, under nutrient-rich conditions, autophagy appears to promote an non-apoptotic cell death in response to other types of stress (Shimizu et al., Nat Cell Biol 2004).

These seemingly conflicting observations can be explained by regarding apoptosis and autophagy as complementary responses to metabolic stress. During nutrient limitation, autophagy ensures that cellular ATP levels are maintained, and fulfills a critical survival function beyond suppression of apoptosis pathways. Inactivation of autophagy under these settings leads to necrosis. Persistent or deregulated autophagy beyond this physiologic context may deplete bioenergetic reserves and lead to futile cycles of macromolecular synthesis and degradation.

The interrelationship between apoptosis, autophagy and necrosis carries significant relevance in tumor settings where defects in apoptotic pathways (e.g. over expression of Bcl-2 or Bax/Bak deficiency) and abnormal proliferation (e.g. constitutive activation of PI3 kinase/Akt pathway) are common (Degenhardt et al., Cancer Cell 2006).