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Review

Non-apoptotic functions of BCL-2 family proteins

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The BCL-2 family proteins are major regulators of the apoptosis process, but the mechanisms by which they regulate this process are only partially understood. It is now well documented that these proteins play additional non-apoptotic roles that are likely to be related to their apoptotic roles and to provide important clues to cracking their mechanisms of action. It seems that these non-apoptotic roles are largely related to the activation of cellular survival pathways designated to maintain or regain cellular survival, but, if unsuccessful, will switch over into a pro-apoptotic mode. These non-apoptotic roles span a wide range of processes that include the regulation of mitochondrial physiology (metabolism, electron transport chain, morphology, permeability transition), endoplasmic reticulum physiology (calcium homeostasis, unfolded protein response (UPR)), nuclear processes (cell cycle, DNA damage response (DDR)), whole-cell metabolism (glucose and lipid), and autophagy. Here we review all these different non-apoptotic roles, make an attempt to link them to the apoptotic roles, and present many open questions for future research directions in this fascinating field.
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Facts

- BCL-2 family members interact with each other and with resident mitochondrial proteins at the outer mitochondrial membrane to regulate the extrinsic and intrinsic pathways of apoptosis.
- BCL-2 family members are also distributed widely throughout the cell where they contribute to the regulation of numerous additional cellular functions.
- Several of the non-apoptotic functions of BCL-2 family members have a feed-back to apoptosis.
- Some of the non-apoptotic functions of BCL-2 family members seem to be completely distinct from their role in apoptosis.

Open Questions

- How are the non-apoptotic functions of distinct BCL-2 family proteins related to or exclusive from their apoptotic function? What determines when a BCL-2 family protein acts in one function *versus* another?
- Are there pathologic disease states dependent on non-apoptotic functions of BCL-2 family proteins?
- What are the protein domains and interacting proteins responsible for the non-apoptotic functions of BCL-2 family proteins?
- What are the appropriate cellular contexts for the non-apoptotic functions?
- How do BCL-2 family proteins regulate non-apoptotic functions of caspases?

Introduction: BCL-2 Proteins as Regulators of Mitochondrial Apoptosis

Programmed cell death, or apoptosis, is critical to both development and maintenance of tissues, and the BCL-2 family proteins are the major regulators of the apoptotic process.[1] The mechanisms by which BCL-2 family proteins regulate cell death are largely unknown, though it is thought that their function depends mostly on their ability to modulate the release of proteins from the intermembrane space of the mitochondria. The BCL-2 family includes both pro- (e.g., BAX) and anti- (e.g., BCL-2) apoptotic proteins that possess up to four conserved BCL-2 homology domains (BH1–4) with the BH3 functioning as a death domain (Figure 1). A subset of the pro-apoptotic proteins is the BH3-only group of proteins (e.g., BID).

The BH3-only members are pivotal sensors/mediators of cellular stress, and once activated are known to perform two tasks: (1) inactivate the anti-apoptotic BCL-2 family members (e.g., BCL-2), and (2) activate the pro-apoptotic BCL-2 family members BAX and BAK (Figure 2). These two events are thought to occur in parallel, eventually resulting in mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, caspase activation, and apoptosis. However, it remains a long-lasting debate whether both events, inhibition of anti-apoptotics and activation of pro-apoptotics, are absolutely required for the execution of apoptosis at the mitochondria.[2] Another important question that may shed light on the first question is whether BCL-2 family proteins play

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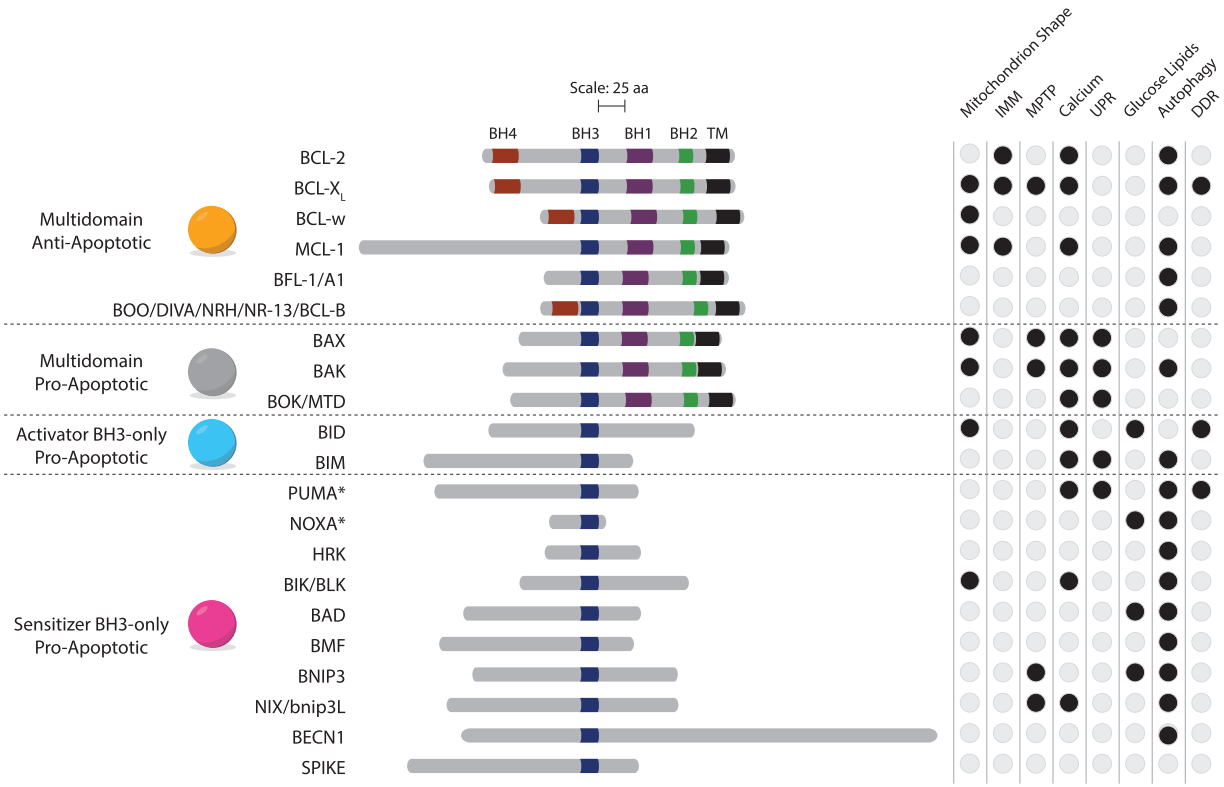

Figure 1 Classification of BCL-2 family members. BCL-2 family members are grouped by their ability to inhibit or activate apoptosis. Shared, conserved BCL-2 homology (BH) domains and transmembrane (TM) regions are depicted. *Indicates recent evidence for BH3-only proteins that might also act as activator BH3-only pro-apoptotic proteins. Darkened circles indicate where there is evidence to support an alternative function for the BCL-2 family member

additional roles related to mitochondrial function and whether these roles are related to their apoptotic role.

Regulation of Mitochondrial Morphology

Many of the BCL-2 family proteins reside at, or dynamically move on and off, the outer mitochondrial membrane (OMM).[1] It is well established that under apoptotic conditions, the BCL-2 proteins regulate MOMP, and this event is always accompanied by mitochondrial fission. Moreover, upon induction of apoptosis, DRP1, a dynamin-related protein that mediates OMM fission, translocates to the mitochondria to induce fission, and its inhibition blocks apoptosis.[3] In addition, during the initial stages of apoptosis, BAX colocalizes with DRP1 at mitochondria scission sites, and mitochondrial fusion is blocked independently of caspase activation.[4] Taken together, these pioneering studies from the Youle group[4] led to an exciting working hypothesis that mitochondrial fusion/fission (dynamics) is involved in MOMP, and that BCL-2 family members may also regulate mitochondrial dynamics under apoptotic (and perhaps also under non-apoptotic) conditions (Figure 3, middle panel).

In 2006, Karbowski *et al.*[5] elegantly demonstrated that in healthy cells BAX or BAK is required for normal fusion of mitochondria into elongated tubules, and that BAX seems to induce fusion via activation of MFN2, another dynamin family protein that mediates OMM fusion. Another study has shown that BAK regulates mitochondrial fragmentation during apoptosis via interaction with MFN1 and MFN2.[6] Moreover, it was demonstrated in a cell-free system of mitochondrial fusion that recombinant BAX positively stimulates fusion through MFN2.[7] In this respect, it is interesting to note that increasing the mitochondrial levels of BH3-only BID (by loss of its phosphorylation by the ataxia-telangiectasia mutated (ATM) kinase) or knocking out MTCH2, the mitochondrial receptor for BH3-only BID,[8] results in increased mitochondrial size/volume, and is associated with hematopoietic stem cell (HSC) mobilization and entry into active cell cycle.[9,10] This suggests that MTCH2 is possibly involved in regulating mitochondrial dynamics, and that the pro-apoptotic BCL-2 family members can possibly act via MTCH2 to regulate the structure of mitochondria.

Regulation of mitochondrial dynamics is not exclusively mediated by the pro-apoptotic BCL-2 family members, as it was demonstrated that anti-apoptotic BCL-X$_L$ also regulates mitochondrial dynamics via DRP1.[11,12] In this respect, an interesting chimera between BCL-X$_L$ and BAX (BCL-X$_L$/BAX-helix 5) induces substantial mitochondrial fragmentation in healthy cells via binding to mitofusins.[13] It was also shown that CED-9, the worm BCL-2 relative, promotes mitochondrial fusion via FZO-1/MFN1,2 and EAT-3/OPA1 in both mammalian cells and in *C. elegans*.[14,15] In addition, in *Drosophila* it has been demonstrated that Buffy and DEBCL, the BCL-2 fly relatives, affect mitochondrial dynamics, whereas there is no clear evidence for their involvement in apoptosis.[16]

In summary, the findings described above demonstrate that BCL-2 family members – in mammals, insects, and roundworms – are involved in the regulation of mitochondrial dynamics in both healthy and apoptotic cells. However, the

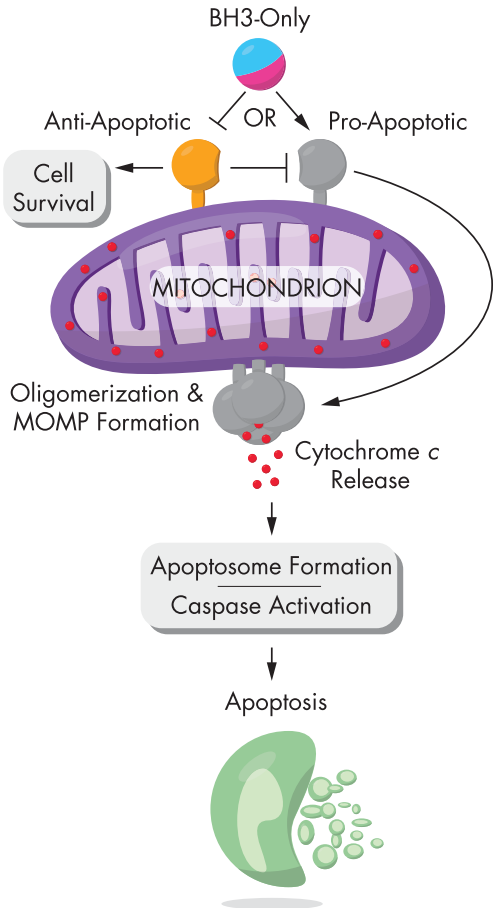

Figure 2 The intrinsic pathway of apoptosis. In response to stress signals, interactions among the different classes of BCL-2 family members at the outer mitochondrial membrane determine whether the cell is to survive or whether multidomain pro-apoptotic family members oligomerize and lead to mitochondrial outer membrane permeabilization (MOMP) with the release of several factors, including cytochrome *c*, thereby committing the cell to apoptosis

exact molecular mechanisms underlying how BCL-2 family members activate/inactivate the mitochondria dynamics machineries, and how these non-apoptotic functions may affect mitochondrial fragmentation during apoptosis, remain to be elucidated.

Regulation of the Mitochondrial Permeability Transition Pore

The mitochondrial permeability transition pore (MPTP) is a poorly characterized protein complex that mediates a regulated form of necrotic cell death.[17] Distinct from the BAX/BAK oligomers that lead to MOMP, the MPTP perforates both the inner and outer mitochondrial membrane leading to a loss of inner membrane potential, dissolution of ATP synthesis, massive swelling due to an influx of water into the solute-rich matrix, and eventual organelle rupture and cell death. Early evidence that BAX and BAK played a role in MPTP distinct from MOMP came from their interaction with the presumed components adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC)[18,19](Figure 3, left panel). However, the MPTP components remain elusive as the complete deletion of both ANT1 and 2 as well as all three VDAC genes show that neither protein is directly required for MPTP formation.[20,21] Recently, however, it has been demonstrated that the F-ATP synthase forms the MPTP.[17] Nonetheless, BAX and BAK still appear to be required for mitochondrial pore-dependent necrotic death as their loss inhibits mitochondrial swelling and rupture.[22,23] Moreover, this does not appear to be dependent on their ability to oligomerize and undergo MOMP as an oligomerization-deficient BAX mutant still permits MPTP-dependent mitochondrial swelling and necrotic cell death.[22,23] The mechanism by which BAX and BAK promote MPTP is still under investigation, with some data supporting a role for BAX-driven mitochondrial fusion as a means to lower the threshold for MPTP opening[22] and other data suggesting that BAX and BAK act as outer membrane permeability factors in their monomeric states.[23] Understanding the particular mechanism by which BAX and BAK influence MPTP is important in the development of targeting molecules to regulate this process.

Regulation of Metabolism at the Inner Mitochondrial Membrane

Several key pieces of data support distinct roles for BCL-2, BCL-X_L, and MCL-1 at the inner mitochondrial membranes (IMMs) (Figure 3, right panel). BCL-2 was originally biochemically purified as an IMM protein where it was hypothesized to function through various mitochondrial metabolic functions.[24] Identification of a carboxy-terminal anchor sequence shifted focus to functions at the OMM.[25] However, subsequent work confirmed that BCL-2 localizes to the IMM,[26] and a few reports suggest that it regulates mitochondrial respiration and binds to cytochrome *c* oxidase (COX) Va and cyclophilin D.[27,28] Further exploration will be necessary to determine the physiological contexts under which BCL-2 utilizes these additional functions and how that interplays with its role in cell death at the OMM.

BCL-X_L also localizes to the IMM where it interacts with the F_1F_0 ATP synthase and is important for the maintenance of the mitochondrial membrane potential.[29] Overexpression of BCL-X_L in resting neurons leads to a twofold increase in cytoplasmic ATP levels, whereas loss of BCL-X_L by genetic or pharmacologic inhibition increases mitochondrial ion leak and decreases ATP synthase function. Thus, BCL-X_L prevents the non-ATP producing proton leak into the matrix by directly modulating the F_1F_0 ATP synthase and helps enhance ATP production under conditions of increased energy demand that improves cellular survival in a BAX/BAK-independent but F_1F_0 ATP synthase-dependent manner.[29] In addition to its role in cellular survival, BCL-X_L increases synaptic transmission in neurons by increasing the numbers and size of synapses and the localization of mitochondria to synapses and synaptic vesicles through direct interaction with DRP1.[12] Precedent for a role in synaptic function was seen earlier by BAK that was surprisingly found to have an anti-apoptotic role protecting mice from kainite-induced seizures via synaptic activity regulation.[30] Taken together, BCL-X_L functions at the IMM by binding to the F_1F_0 ATP synthase in order to regulate pathways both directly involved in cell survival and distinct from it.

MCL-1 has also recently been demonstrated to be part of the group of anti-apoptotic BCL-2 family members that operate

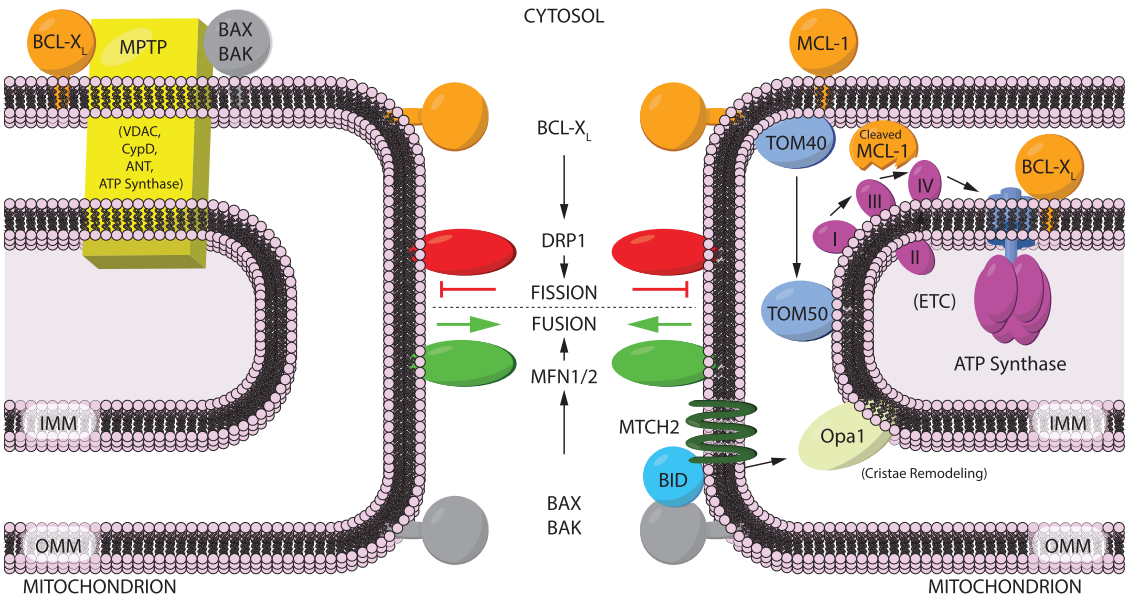

Figure 3 Alternative functions for BCL-2 family members at the mitochondria. BCL-2 family members have functions in regulation of the mitochondrial permeability transition pore (MPTP) (left), the process of mitochondrial fusion and fission (middle), and in regulation of oxidative phosphorylation through the electron transport chain (ETC) in the inner mitochondrial membrane (right), as discussed in the text

at the IMM.[31] IMM import for MCL-1 requires two proteolytic cleavage sites, a mitochondrial targeting sequence, and translocases of the OMM (TOM40) and IMM (TIM50).[31] *Mcl-1* deletion results in a number of mitochondrial defects, including (1) disorganized mitochondrial cristae, (2) loss of the tubular mitochondrial network, (3) decreased enzymatic activity of complex I (NADH dehydrogenase), complex II (succinate dehydrogenase) and complex III (cytochrome *c* oxidase), (4) decreased formation of respiratory supercomplexes and F_1F_0 ATP synthase higher-order oligomers, and (5) decreased ATP levels and oxygen consumption rates.[31] The close interdependence of these various features of mitochondrial structure, function, and dynamics makes it difficult to precisely determine the role of MCL-1 within this system. However, an MCL-1 isoform that only localizes to the IMM rescues the mitochondrial abnormalities, but does not provide anti-apoptotic activity in response to staurosporine or etoposide, whereas the opposite is observed with an MCL-1 isoform that only localizes to the OMM.[31] It will be of great interest to determine the relative contributions of the distinct functions of MCL-1 in apoptosis and mitochondrial function to its various physiological roles in development as well as in oncogenesis. Interestingly, loss of MCL-1 leads to a rapidly lethal cardiomyopathy that appears to be dependent on its apoptotic role as loss of BAX and BAK completely rescues the cardiac function despite the continued presence of mitochondrial ultrastructural and functional abnormalities.[32]

Regulation of Calcium Homeostasis at the Endoplasmic Reticulum

Multiple BCL-2 family members have been shown to regulate endoplasmic reticulum (ER) calcium by binding to the various inositol 1,4,5-trisphosphate receptors (IP3R1–3)[33] (Figure 4, right panel). The IP3Rs are ubiquitous, ER-resident, calcium release channels that are essential for numerous calcium signaling pathways. BCL-2, BCL-X_L, and MCL-1 each bind to the sixth transmembrane domain of the IP3R.[34,35] This interaction enhances calcium leak from the ER because of an allosteric shift that sensitizes the IP3R to very low IP3 levels present in resting cells.[34,35] In addition, BCL-2 also binds the middle of the IP3R regulatory region through its BH4 domain whereby it inhibits large, stress-induced release of calcium.[36] NRZ, a zebrafish anti-apoptotic homolog, also inhibits IP3-induced calcium release, but does so by competitive inhibition at the IP3 binding domain.[37] Beyond the IP3R, BCL-2 binds and inactivates the main ER calcium importer, the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump,[38] induces a decline in SERCA2b levels,[39] and even functions in increasing calcium extrusion from the cell through the calcium pumps in the plasma membrane.[40] The ability of BCL-2 to regulate cellular calcium levels at multiple levels may be important in order to establish an integrated handling of calcium throughout the cell.

Pro-apoptotic BCL-2 family members have also been implicated in IP3R-mediated calcium regulation.[41,42] The loss of BAX and BAK results in an increase in IP3R1 calcium leak leading to a reduced calcium resting concentration in the ER and a decreased uptake of calcium in the mitochondria.[42,43] Although BAX and BAK do not directly bind to the IP3Rs, BAX/BAK interaction with the anti-apoptotic proteins may compete away anti-apoptotic-IP3R binding. In the absence of BAX and BAK, there is increased BCL-2 and IP3R1 binding and IP3R1 hyperphosphorylation, making it sensitive to basal IP3 levels.[43] Similarly, tBID antagonizes BCL-X_L enhancement of IP3R-calcium release by blocking its interaction with the IP3R.[35] BIK, NIX, and PUMA also contribute to ER calcium depletion in a BAX/BAK-dependent manner.[44–46] In contrast,

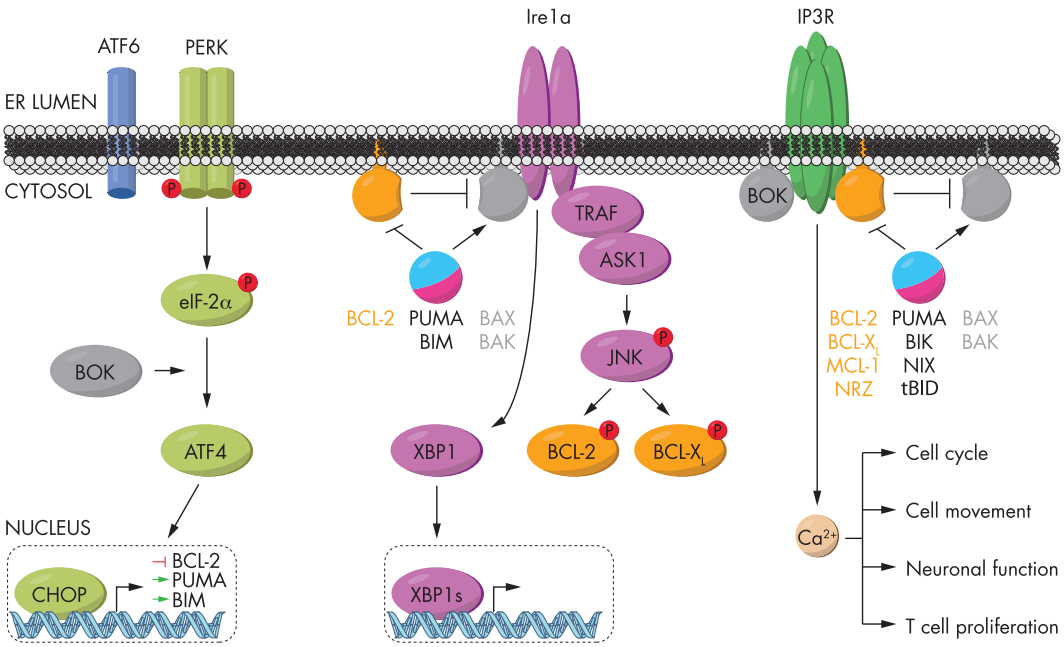

Figure 4 Alternative functions for BCL-2 family members at the endoplasmic reticulum. BCL-2 family members have functions in regulation of the unfolded protein response for PERK signaling (left) and IRE-1α signaling (middle) as well as in regulation of calcium release by the IP3R (right)

BOK does bind to the IP3R,[41] but might not bind to anti-apoptotic BCL-2 family members.[47] The binding of BOK BH4 domain to IP3R regulatory domain results in reciprocal protection from degradation, by caspase 3 for IP3Rs[41] and by the proteasome for BOK.[48] Surprisingly, initial experiments did not reveal a major effect of BOK on the calcium mobilizing function of IP3Rs in response to IP3 in permeabilized MEFs.[41] However, *Bok*$^{-/-}$ primary cortical neurons did reveal a decreased, but prolonged rise in cytoplasmic calcium levels in response to NMDA excitotoxicity.[49] Further elucidation of BOK regulation of IP3R calcium dynamics and its relationship to apoptosis is an important area of inquiry.

BCL-2 family members are able to affect numerous cellular pathways beyond cell death through their regulation of calcium, a well-known second messenger system. BCL-2 and BCL-X$_L$ promote G1 arrest through elevation of the calcium-regulated, cyclin-dependent kinase inhibitor p27, whereas BAX promotes S-phase entry through p27 depression.[50,51] Mutation of BCL-2 BH4 domain affects IP3-induced calcium release and BCL-2 regulation of the cell cycle, but not its anti-apoptotic function.[52] Cell movement is regulated in early vertebrate development by NRZ control of calcium and cytoskeletal dynamics[37] as well as in cancer cell invasion by BCL-2.[53] T-cell proliferation is dependent on BAX, BAK, and BIM, where loss of BAX and BAK from T cells leads to ER calcium store reductions and defects in T cell receptor-inducible calcium fluxes necessary for T-cell proliferation.[54] Similarly, loss of BIM from T cells leads to increased BCL-2-IP3R complexes, lower ER calcium release, and decreased NFAT activity.[55] Finally, overexpression of BCL-2 in CNS neurons promotes axonal growth, whereas deletion reduces axonal elongation.[56] This function requires BCL-2 to localize to the ER and is mediated by ERK and CREB that are activated by cytosolic calcium.[57] In fact, a single-nucleotide polymorphism (SNP) associated with bipolar disorder leads to decreased BCL-2 expression and abnormal IP3R release of calcium.[58] Taken together, calcium regulation by BCL-2 family members has a broad reach in regulating cellular processes like cell cycle, invasion, activation, and growth.

Regulation of the Unfolded Protein Response

The second major pathway in the ER affected by BCL-2 family members is the unfolded protein response (UPR). In this signaling pathway, three ER transmembrane proteins, IRE1α (inositol requiring enzyme 1), PERK (protein kinase like ER kinase), and ATF6 (activating transcription factor 6), are activated by the accumulation of misfolded proteins because of a variety of cellular stresses.[59] Altogether, the three arms of the UPR activate a cellular survival pathway designed to regain cellular homeostasis, but, if unsuccessful, will switch over into a pro-apoptotic pathway. The exact mechanism by which the UPR switches from an adaptive response to a pro-death pathway is still unclear.

IRE1α contains both an endoribonuclease domain and a kinase domain. Through the kinase domain, IRE1α communicates ER stress ultimately to JNK that phosphorylates and inhibits the anti-apoptotic functions of BCL-2 and BCL-X$_L$[60,61] (Figure 4, middle panel). In contrast, BAX and BAK bind IRE1α to modulate the amplitude of its signaling, such that *Bax*$^{-/-}$*Bak*$^{-/-}$ cells exhibit decreased endoribonuclease activity under ER stress.[62] The RNAse activity of IRE1α participates both in general RNA degradation to reduce protein synthesis[63] and direct splicing of a few target genes, including the transcription factor XBP-1 (X-box binding protein 1) that activates several genes that help restore ER folding

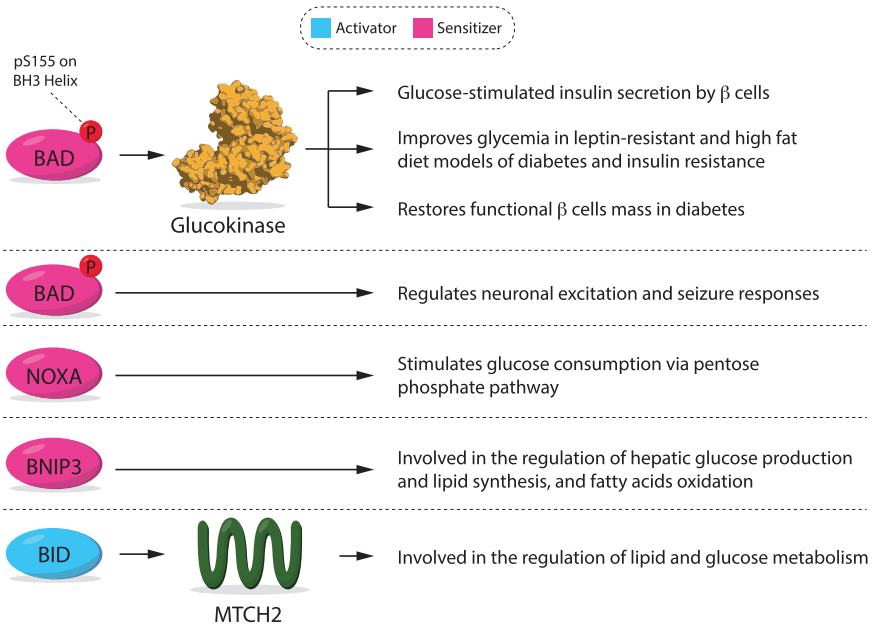

Figure 5 BCL-2 family members that regulate glucose and lipid metabolism. Both activator and sensitizer BH3-only BCL-2 family members participate in glucose metabolism. Whereas BAD and NOXA stimulate glucose consumption (although through different pathways), BNIP3 supports increased glucose production. BNIP3 and BID (potentially via MTCH2) also regulate lipid metabolism

capacity.[64,65] BCL-2 also enhances XBP-1 splicing, although this does not appear to be through direct binding of BCL-2 to IRE1*α*.[66] Finally, BIM and PUMA also bind IRE1*α* and regulate the splicing of XBP-1,[67] although these findings have not been universally identified.[68] Similar to their function as a rheostat for apoptosis, BCL-2 family members may act as a stress rheostat to modulate the kinetics and magnitude of adaptive responses through the UPR.

PERK dimerization and activation leads to the phosphorylation of elongation initiation factor 2*α* (eIF2*α*) that turns off global translation of new proteins, but also activates a select few proteins including activating transcription factor 4 (ATF4)[69] (Figure 4, left panel). ATF4 activates the expression of several genes, including the transcription factor C/EBP homologous protein (CHOP).[69] CHOP promotes apoptosis through repression of BCL-2 and activation of BIM and PUMA.[70,71] Loss of BAX and BAK attenuates ATF4 and CHOP activation, either through the PERK arm or by a crossover effect from the IRE1*α* arm.[66] Two different groups have also identified a role for BOK in the PERK pathway, although with some distinction in its activity.[47,72] Carpio *et al.*[72] showed that BOK-null cells exhibit a diminished UPR and apoptotic response to several ER stress agents, including bortezomib (a proteasome inhibitor) and thapsigargin (a SERCA inhibitor). Although PERK and eIF2*α* were activated in the BOK-null cells, ATF4 and CHOP expression were greatly diminished. Two different *Bok*$^{-/-}$ mouse models did not show resistance to the ER stress agent thapsigargin,[47,73] but one of the two studies showed that enforced BOK expression killed cells under proteasome inhibition.[47] Moreover, PERK inhibition resulted in BOK-dependent cell death in response to the ER stress agent Tunicamycin (an inhibitor of N-glycosylation).[47] In this setting, it was demonstrated that PERK inhibition resulted in loss of ERAD degradation of BOK that was then available to kill in response to the ER stress agent. Thus, two studies have implicated that under ER stress, the combination of BOK loss and PERK pathway inhibition is protective.[47,72] Taken together, two of the three arms of the UPR (IRE1 and PERK/CHOP) appear to be modulated by BCL-2 family members before irreversible cell damage, with the apparent aim of an adaptive response.

Regulation of Glucose and Lipid Metabolism

Mitochondria are the cellular hubs for metabolism and thus it was tempting to speculate that BCL-2 family proteins – regulators of life and death decisions - also possess non-apoptotic roles related to regulation of metabolism. The Danial group has clearly spearheaded this research direction by initially demonstrating (together with Stan Korsmeyer) that BH3-only BAD resides in a glucokinase-containing complex that regulates glucose-driven mitochondrial respiration.[74] Next, using a BAD BH3 peptide that targets glucokinase, it was demonstrated that BAD possesses a physiologic role in glucose-stimulated insulin secretion by *β* cells[75] (Figure 5). Moreover, BAD phosphomimic variants improve glycemia in leptin-resistant and high-fat diet models of diabetes and insulin resistant,[76] and restore functional *β* cell mass in diabetes.[77] The metabolic role of BAD is also relevant to the nervous system and is critical for regulating neuronal excitation and seizure responses.[78]

Thus, BAD is clearly the most investigated BCL-2 family member involved in glucose metabolism;[79] however, there were additional members that were studied in this context. BH3-only NOXA was demonstrated to stimulate glucose consumption and seems to enhance glucose turnover via

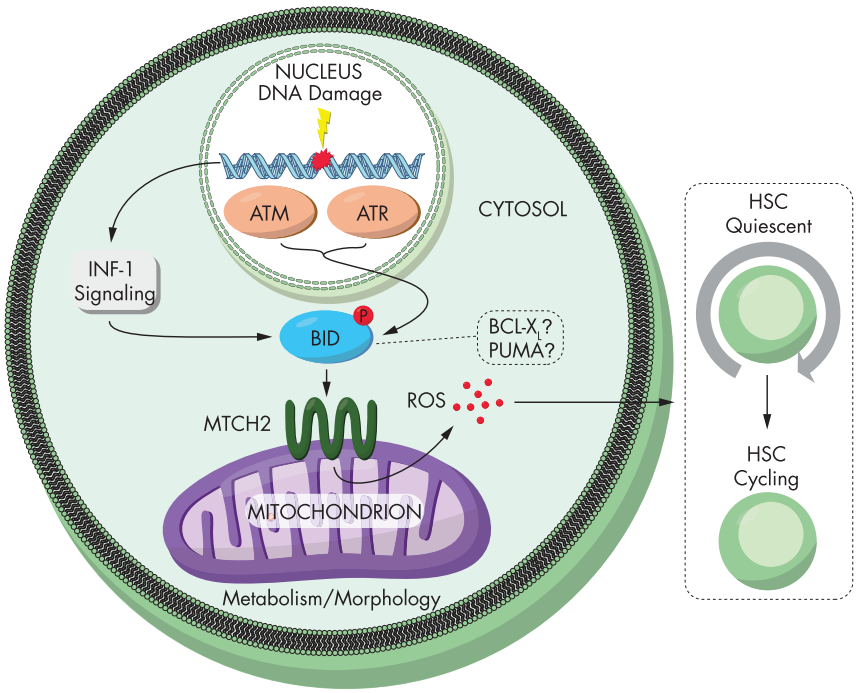

Figure 6 Regulation of hematopoietic stem cell (HSC) cycling by BCL-2 family members. BID, and potentially BCL-X_L and PUMA, shuttle from the nucleus to transmit the DNA damage response to regulate mitochondrial morphology and metabolism (including production of reactive oxygen species (ROS)) that regulate cycling of HSCs

the pentose phosphate pathway rather than through glycolysis.[80] On the other hand, BH3-only BNIP3, demonstrated to be involved in mitophagy,[81,82] was also shown to be involved in lipid metabolism. Knockout of BNIP3 results in increased hepatic lipid synthesis in the liver, reduced fatty acid oxidation (FAO), and failure to augment hepatic glucose production during fasting.[83] In addition, BH3-only BID was shown to be involved in lipid metabolism, demonstrating that truncated tBID inhibits FAO via inhibition of carnitine palmitoyltransferase-1 (CPT-1).[84] In this respect, it is interesting to mention that mice deficient for muscle MTCH2 (BID receptor) are protected from diet-induced obesity and hyperinsulinemia, and demonstrate increased energy expenditure.[9] Deletion of muscle MTCH2 also increases mitochondrial oxidative phosphorylation (OXPHOS) and mass, triggers conversion from glycolytic to oxidative fibers, increases capacity for endurance exercise, and increases heart function. Moreover, metabolic profiling of mice deficient for muscle MTCH2 reveals preference to carbohydrate utilization, and an increase in mitochondria TCA cycle activity and glycolytic flux in muscles.[9] On the other hand, overexpression of MTCH2 leads to the development of fatty livers and kidneys.[85] Thus, several BH3-only proteins affect lipid and glucose metabolism, but their exact mechanisms of action still need to be revealed.

Regulation of Macroautophagy and Mitophagy

Macroautophagy (henceforth autophagy) is a core metabolic process for recycling and biogenesis that initiates at the ER and OMM and ends with the release of the recycled macromolecules into the cytosol. Under conditions of nutrient starvation, a double-membrane structure forms at sites designated by the initiation complex that includes Beclin-1.[86] Beclin-1, the mammalian ortholog of yeast Atg6, has a functional BH3 domain.[87] Therefore, BCL-2, BCL-X_L, BCL-W, and MCL-1 all bind Beclin-1, prevent it from forming the initiation complex, and inhibit autophagy.[87,88] The Beclin-1/BCL-2 interaction is stabilized by NAF-1,[89] and phosphorylation of the Beclin-1 BH3 domain by Mst1,[90] but disrupted by phosphorylation of BCL-2 by JNK1.[91] In fact, mice with a BCL-2 that cannot be phosphorylated by JNK1 have increased affinity to Beclin-1 and deficient exercise- or starvation-induced autophagy with a decrease in endurance and altered glucose metabolism.[92] The presence of a metabolic defect in these BCL-2 mutant mice strongly supports BCL-2 alternative function in autophagy.

BH3-only proteins (e.g., BAD, BIK, BNIP3, and NOXA) and BH3 mimetics (e.g., ABT-737) affect autophagy by disrupting the anti-apoptotic-Beclin-1 complex.[87,93] The importance of BH3-only proteins in autophagy is conserved in *C. elegans* where gain-of-function EGL1 mutations increase autophagy and deletions of EGL1 impair autophagy.[87] In contrast to the pro-autophagic role ascribed to most BH3-only proteins in freeing Beclin-1 from the inhibitory anti-apoptotic complex, BIM inhibits autophagy by directly binding Beclin-1 and sequestering it to dynein light chains.[94] In response to nutrient starvation, JNK phosphorylates BIM and Beclin-1 is released to initiate autophagy. The difference between BIM and other BH3-only proteins in regulating autophagy through Beclin-1 may have implications on the manner by which the cell balances apoptosis with autophagy.

BCL-2 family members also function in the selective autophagic removal of dysfunctional mitochondria, known as

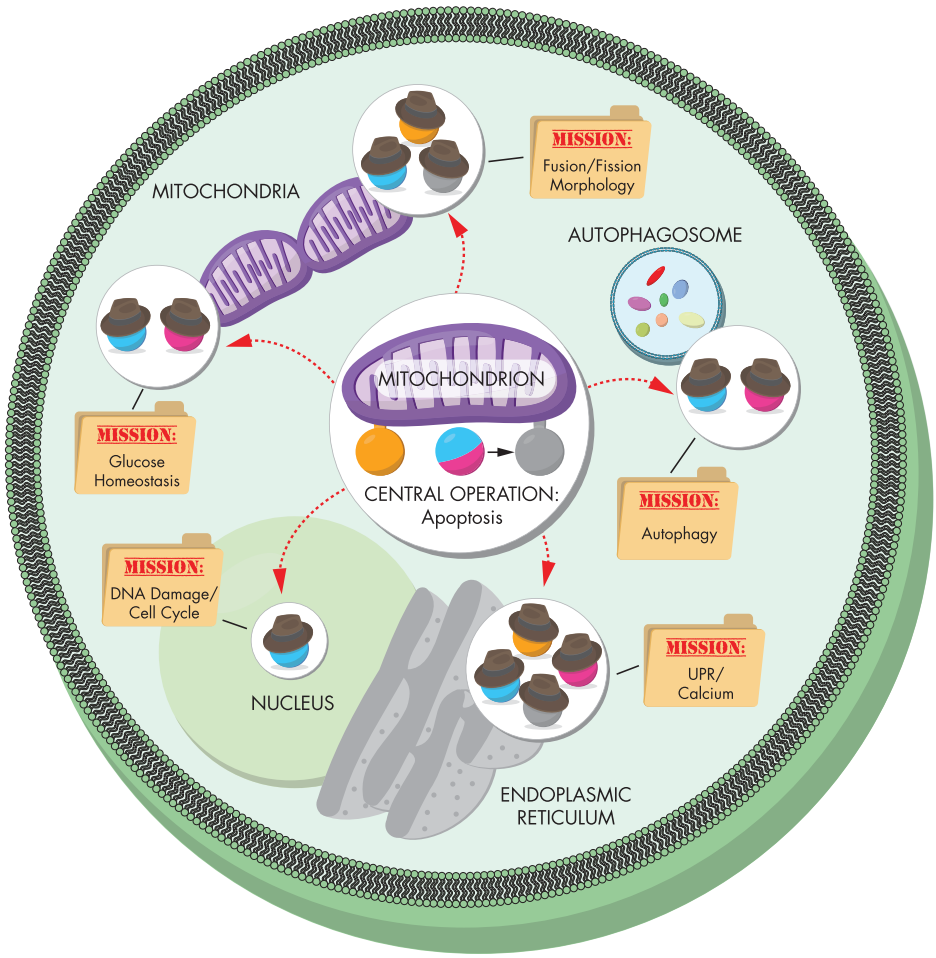

Figure 7 Conceptualization of BCL-2 family members as spies throughout the cell. Various BCL-2 family members have missions to participate in 'day jobs' throughout the cell and contribute to the cell's ability to function. How that mission proceeds determines if they need to report back and participate in their 'night job' function in the intrinsic pathway of apoptosis at the mitochondrion

mitophagy. In response to starvation and hypoxia, BNIP3 and NIX (BNIP3L) are transcriptionally activated by HIF-1α and mediate mitophagy.[95] BNIP3 promotes mitophagy (and ER-phagy) predominantly by acting as a receptor for LC3-II, a proteolipid component of the elongating autophagosome.[96] In addition, BNIP3 inhibits the cleavage and inactivation of PINK1, a mitochondrial kinase that recruits and activates PARKIN, an ubiquitin E3-ligase for multiple proteins on the mitochondria.[97] NIX has also been shown to promote mitophagy by acting as a receptor for LC3 proteins[98] and recruitment of PARKIN by regulating mitochondrial membrane potential.[99,100] Physiologically, NIX is required for mitochondrial clearance during erythroid maturation.[100] Mitophagy is inhibited by the anti-apoptotic BCL-2 proteins that bind and prevent the translocation of PARKIN.[101] This function can be neutralized by BH3-only proteins, BAD, BIM, NOXA, and PUMA, as well as a BH3-mimetic.[101] Taken together, pro-apoptotic signaling also stimulates mitophagy that might serve as a protective mechanism from loss of mitochondrial function and occur as an adaptive response before the need for apoptosis.

Regulation of the DNA Damage Response

In eukaryotes, each cell's genetic material is constantly subjected to DNA damage. Following DNA damage, the cell may activate a survival system that enables repair and continuation of its normal life cycle, or it may activate its apoptotic machinery in the face of extensive or irreparable damage.[102] One of the major responses associated with the cell survival network is the temporary arrest of cell cycle progression that reflects the activation of cell cycle checkpoints.[103,104] A prototype transducer of the DNA damage response (DDR) is the ATM kinase, a nuclear serine–threonine protein kinase.[105] Activated ATM phosphorylates a wide spectrum of substrates, and the Gross and Korsmeyer groups identified BID as one of these substrates.

DNA damage leads to rapid phosphorylation of BID by the ATM kinase on serines 61 and 78, and expression of a non-phosphorylatable BID mutant ($BID^{S61A/S78A}$ or BID^{AA}) results in a cell-cycle arrest defect and an increase in apoptosis.[106,107] These findings together with additional results demonstrating that BID shuttles between the nucleus and mitochondria[108] are consistent with the idea that BID

functions as a cellular sentinel of DNA damage (Figure 6). Moreover, the Gross group went on to show that *BID*^{AA} knock-in mice are hypersensitive to whole-body irradiation because of premature entry of HSCs into cycle and increased levels of HSC apoptosis.[109] In addition, loss of BID phosphorylation is associated with accumulation of BID at the mitochondria resulting in elevated levels of mitochondrial reactive oxygen species (ROS), suggesting that basal phosphorylation of BID is critical for balancing mitochondrial ROS thus regulating HSC quiescence.[110] More recently, it was demonstrated that MTCH2 (BID receptor[8]) is the regulator of mitochondrial ROS/metabolism in the ATM–BID pathway in HSCs.[10] Loss of MTCH2 increases mitochondrial OXPHOS, triggering HSC and progenitor entry into cycle. Elevated OXPHOS was accompanied by an increase in mitochondrial size, increase in ATP and ROS levels, and protection from irradiation-induced apoptosis. In contrast, BID^{AA} induced a similar increase in OXPHOS, but with higher ROS and reduced ATP levels, and was associated with hypersensitivity to irradiation.[10] Thus, MTCH2 is a negative regulator of mitochondrial OXPHOS downstream of BID, indispensable in maintaining HSC homeostasis.

A recent study further confirms the above findings, demonstrating that BID is a critical regulator of DNA damage-induced mitochondrial ROS in the interferon (IFN-1) signaling pathway in HSCs.[111] Importantly, in a series of elegant studies, the Zinkel group[112,113] demonstrated that BID mediates the DDR to replicative stress directed by the ATM and Rad3-related (ATR) kinase. In the initial studies it was found that BID is part of the DNA damage sensor complex (ATR/ATRIP/RPA) acting to amplify the ATR-directed cellular response to replicative DNA damage. Later, the relevance of these findings to the *in vivo* setting was demonstrated by showing that BID plays a critical role in protecting HSCs following chronic replicative stress[114] and delays T-cell leukemogenesis in *ATM*$^{-/-}$ mice.[115] All these studies together resolved an old controversy over whether BID plays a role in the DDR.[116]

Although BID is clearly the most investigated BCL-2 family member in the non-apoptotic arm of the DDR,[117] two additional members were studied in this context. The first study showed that ectopic expression of BCL-X_L in human cells enhances homology-directed repair (HDR) of DNA double-strand breaks (DSBs),[118] suggesting that BCL-X_L is actively involved in DNA repair. The second study showed that *Puma*$^{-/-}$ HSCs spared from radiation can be better maintained in a quiescent state, and can be induced to more efficient DNA repair,[119] suggesting that PUMA is possibly involved in triggering HSC exit from quiescence and inhibiting DNA repair.

Conclusions: BCL-2 Family Proteins as Regulators of Numerous Cellular Functions

It has taken over 30 years to elucidate the regulation of apoptosis by an elegant network of interacting BCL-2 family proteins. Our understanding of the complicated relative binding affinities and dynamic structural transformations has led to the development of small molecules that can selectively and specifically manipulate the primed state of a cell nudging it ever closer to death. However, during this first phase of our exploration we have seen multiple places where BCL-2 family members function beyond their canonical role in apoptosis. These alternative roles place BCL-2 family members throughout the cell, including the OMM, the IMM, the ER, the cytosol, and the nucleus. Many of these pathways (e.g., autophagy, electron transport chain, and gluconeogenesis) are essential for cell survival by regulating nutrient and energy metabolism, whereas others (e.g., UPR and DDR) provide critical respites that help a cell undergo repair before deciding whether the damage is beyond reproach and the cell needs to commit suicide. As such, one might view BCL-2 family members as 'sleeper agents' ready to inform on the state of the cell in their clandestine, night job as an agent of cell death (Figure 7). However, it is important to emphasize that the most effective sleeper agents integrate seamlessly into daily life and functions. Over time, their informant function that ties into apoptosis might become secondary to functions in cellular signaling. Deciphering how BCL-2 family members integrate into the broader cellular context over the next 30 years will likely yield further rich and sophisticated signaling networks that can be targeted within disease states.

Conflict of Interest

The authors declare no conflict of interest.

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