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Bcl-2 family in inter-organelle modulation of calcium signaling; roles in bioenergetics and cell survival

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Abstract

Bcl-2 family proteins, known for their apoptosis functioning at the mitochondria, have been shown to localize to other cellular compartments to mediate calcium (Ca^{2+}) signals. Since the proper supply of Ca^{2+} in cells serves as an important mechanism for cellular survival and bioenergetics, we propose an integrating role for Bcl-2 family proteins in modulating Ca^{2+} signaling. The endoplasmic reticulum (ER) is the main Ca^{2+} storage for the cell and Bcl-2 family proteins competitively regulate its Ca^{2+} concentration. Bcl-2 family proteins also regulate the flux of Ca^{2+} from the ER by physically interacting with inositol 1,4,5-trisphosphate receptors (IP_3Rs) to mediate their opening. Type 1 IP_3Rs reside at the bulk ER to coordinate cytosolic Ca^{2+} signals, while type 3 IP_3Rs reside at mitochondria-associated ER membrane (MAM) to facilitate mitochondrial Ca^{2+} uptake. In healthy cells, mitochondrial Ca^{2+} drives pyruvate into the citric acid (TCA) cycle to facilitate ATP production, while a continuous accumulation of Ca^{2+} can trigger the release of cytochrome c, thus initiating apoptosis. Since multiple organelles and Bcl-2 family proteins are involved in Ca^{2+} signaling, we aim to clarify the role that Bcl-2 family proteins play in facilitating Ca^{2+} signaling and how mitochondrial Ca^{2+} is relevant in both bioenergetics and apoptosis. We also explore how these insights could be useful in controlling bioenergetics in apoptosis-resistant cell lines.

Keywords

Calcium signaling; Apoptosis; Bcl-2; Mitochondria-associated; ER membrane (MAM); Bioenergetics; ER; Mitochondria; Inositol; 1,4,5-trisphosphate receptor (IP_3R)

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Introduction

The growing Bcl-2 family (Fig. 1) consists of both anti- and pro-apoptotic members that share up to four conserved regions known as Bcl-2 homology domains (BH1-4). Anti-apoptotic family members typically contain all four BH domains and include Bcl-2 and Bcl-x_L in which the BH4 domain is relevant for anti-apoptotic activity. Other anti-apoptotic members; however, such as Mcl-1, merely possess strong sequence homology in the BH1-3 domains. Additionally, HAX-1 has anti-apoptotic properties although it shares sequence homology within BH1 and BH2 only. Pro-apoptotic proteins are subdivided into multi-domain Bcl-2 effector molecules such as Bax and Bak, which contain BH1-3, and BH3-only proteins. BH3-only proteins are further characterized as *activators* like Bid, Bim, and Puma that directly activate Bax and Bak, or *sensitizers* including Bad, Bik, and Nix/BNIP3 that interact with anti-apoptotic proteins to induce the release of activated multi-domain pro-apoptotic proteins (Brunelle and Letai 2009; Gallenne et al. 2009). Interactions between family members mediated by BH domains are vital for many aspects of their functioning. One of the unique features of anti-apoptotic Bcl-2 family proteins is a hydrophobic pocket defined by the BH1-3 domains, which allows for heterodimerization with pro-apoptotic proteins through insertion of their BH3 domains (Burlacu 2003; Petch and Al-Rubeai 2004; Gélinas and White 2005).

Bcl-2 family proteins are traditionally believed to reside or translocate to mitochondria and function as apoptotic regulators by controlling mitochondrial membrane permeability. Ultimately, the ratio between anti- and pro-apoptotic Bcl-2 proteins determines whether cells live or die (Hanson et al. 2008). As a result, Bcl-2 family proteins have been used to genetically engineer recombinant mammalian cells such as Chinese hamster ovary (CHO) and human embryonic kidney (HEK) 293 cell lines used to produce many biotherapeutics (Itoh et al. 1995; Chiang and Sisk 2005; Majors et al. 2007).

The Bcl-2 family; however, has additionally been detected at other subcellular locations in healthy cells including the endoplasmic reticulum (ER), nuclear membrane and within the cytosol and have also been linked to non-apoptotic functions including calcium (Ca^{2+}) homeostasis, inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated Ca^{2+} signaling and mitochondrial bioenergetics (Vander Heiden and Thompson 1999; Pinton et al. 2002; Majors et al. 2007). One can speculate that localization of these proteins may in some way have an impact on their function inasmuch as apoptosis and bioenergetics are dependent on the proper supply of Ca^{2+} by organelle channeling. Indeed, that Ca^{2+} signaling regulation might serve as an integrating role in which the activity of Bcl-2 family members relates bioenergetics with apoptosis. A better understanding of how Bcl-2 family members promote bioenergetics would be useful to further advances in our understanding of cell physiology and metabolic engineering. Thus, we aim to clarify the multi-organelle localization and functions of Bcl-2 members in mediating Ca^{2+} signals and investigate the implications of Ca^{2+} as it relates to bioenergetics and apoptosis.

Intracellular Ca^{2+} dynamics

Cytosolic Ca^{2+} homeostasis

The plasma membrane consists of several transporters and channels to modulate intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) including store-operated Ca^{2+} channels (SOCs) and Ca^{2+}-ATPases (PMCAs) to regulate Ca^{2+} entry and exit, respectively. Within the cytosol, Ca^{2+} chelators act as either buffers or sensors to regulate Ca^{2+} homeostasis, and include proteins from the annexin and EF-hand (a Ca^{2+}-binding motif containing an E-helix-loop-F-helix) families. Calmodulin and proteins from the S100 family are typical examples of Ca^{2+} sensors that undergo conformational changes upon binding Ca^{2+} to interact with specific targets. Ca^{2+} buffers, on the other hand, maintain $[Ca^{2+}]_i$ in the range of 20–100 nM under steady-state conditions. Cytosolic Ca^{2+} stores; however, are transient and serve to replenish ER and mitochondria Ca^{2+} stores and stimulate Ca^{2+} extrusion when cells are overloaded with Ca^{2+}.

ER Ca^{2+} homeostasis

The ER functions as the predominant Ca^{2+} storage facility within cells. Housed within the ER are Ca^{2+} binding-proteins that can be categorized as buffers or chaperones that are responsible for mediating ER Ca^{2+} homeostasis. Buffers, such as calreticulin, have a large Ca^{2+}-binding capacity and are responsible for maintaining ER Ca^{2+} concentration, $[Ca^{2+}]_{ER}$, within the physiological range of 0.2–1 mM. This allows the ER to generate Ca^{2+} signals directed into the cytosol or mitochondria that are central to a broad range of cellular and physiological functions. Additionally, Ca^{2+} can bind to the multiple Ca^{2+}-binding sites found on chaperones such as binding immunoglobulin protein (BiP/GRP78), calnexin or ERp57 to regulate their activity (Görlach et al. 2006).

Ca^{2+} accumulation in the ER lumen is mediated by sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases (SERCAs; Fig. 2a). Local rises in cytosolic Ca^{2+} concentrations are able to stimulate increases in SERCA activity. However, SERCA-mediated ER Ca^{2+} uptake is almost exclusively regulated within the ER lumen. $[Ca^{2+}]_{ER}$ plays an important role in controlling SERCA-dependent Ca^{2+} uptake, whereby high $[Ca^{2+}]_{ER}$ inhibits SERCA activity and low $[Ca^{2+}]_{ER}$ increases ER Ca^{2+} uptake by SERCA. Several studies have also shown that ER-resident proteins including, calnexin and calreticulin inhibit ER Ca^{2+} uptake by regulating SERCA activity (John et al. 1998; Roderick et al. 2000; Arnaudeau et al. 2002; Görlach et al. 2006).

In the event of ER Ca^{2+} depletion, capacitative Ca^{2+} entry (CCE) is employed through SOCs on the plasma membrane. Stromal interaction molecule 1 (STIM1), an intraluminal ER Ca^{2+} sensor, plays an essential role in activation of CCE by communicating $[Ca^{2+}]_{ER}$ to SOCs. Knockdown of STIM1 reduces store-operated currents in Jurkat T cells and store-operated Ca^{2+} entry in HEK293 cells and SH-SY5Y neuroblastoma cells (Putney 2005). Researchers later found that STIM1 aggregates in regions close to the plasma membrane to form a ternary complex with SOC subunits Orai1 and TRPC1 (canocial transient receptor potential 1) following ER Ca^{2+} depletion (Salido et al. 2009).

IP_3R-mediated ER Ca^{2+} efflux

Stimulated release of Ca^{2+} from the ER occurs when ex-tracellular stimuli bind G-coupled protein receptors, activators of phospholipase C (PLC), to produce inositol 1,4,5-trisphosphate (IP_3). The binding of IP_3 provokes the opening of inositol 1,4,5-trisphosphate receptors (IP_3Rs) and the release of Ca^{2+} (Fig. 2a). Three isoforms of IP_3Rs (type 1, 2, and 3) have been identified with varying degrees of IP_3 binding affinity and Ca^{2+} oscillations (Zhang et al. 2011). Knockdown studies of type 1 and type 3 IP_3Rs in CHO cells showed that cytosolic Ca^{2+} was reduced when type 1 knockdown occurred and mitochondrial Ca^{2+} was reduced with type 3 knockdown. Indeed, Type 1 IP_3Rs localize to the bulk ER to mediate Ca^{2+} efflux into the cytosol, whereas type 3 IP_3Rs reside at the direct ER-mitochondrion contact termed MAM (**m**itochondria-**a**ssociated ER **m**embrane) and have been shown to preferentially facilitate the flux of Ca^{2+} into the mitochondria (Mendes et al. 2005).

MAM: direct ER to mitochondria Ca^{2+} signaling

Mitochondria-**a**ssociated ER **m**embrane (MAM) is a specialized area of the ER in direct contact with mitochondria that enables ER proteins to associate directly with proteins and lipids on the outer mitochondrial membrane (OMM). This physical interaction between the ER and mitochondria was determined based on co-sedimentation and electron microscopic observations (Shore and Tata 1977; Mannella et al. 1998) and it was later shown that up to 12 % of the ER is in direct contact with the mitochondria (Hayashi et al. 2009). Although the ER and mitochondria are both anchored to the cytoskeleton, the MAM is predominately held together by tethering proteins such as S100B and mitofusion 2, which maintain a minimum distance between the MAM and OMM at 10–25 nm (Csordás et al. 2006; Hayashi et al. 2009).

The functional role of MAM in lipid metabolism has been well established in mammalian cells, where phosphatidylethanolamine (PE) synthesis depends on the transport of phosphatidylserine (PS) transport from the ER to mitochondria. Studies have shown that the enzymes involved in this process, including PS synthase and PE N-methyltransferase-2, are enriched at the MAM (Vance 2003). Likewise, key calcium signaling machinery has been observed to accumulate at the MAM; facilitating the efflux of Ca^{2+} directly from the ER into the mitochondria (Fig. 2b).

Furthermore, sigma-1 receptors (Sig-1Rs) in conjunction with the ER-resident chaperone, BiP, form a complex that stabilizes type 3 IP_3Rs at the MAM. Upon IP_3R activation by IP_3, Sig-1Rs dissociate from BiP and directly bind type 3 IP_3Rs to prevent degradation and prolong Ca^{2+} signaling (Hayashi and Su 2007). Ca^{2+} signaling at the MAM is additionally facilitated by the formation of "Ca^{2+} tunnels." "Ca^{2+} tunnels" are multi-molecular complexes comprised of IP_3Rs at the ER membrane linked to voltage-dependent anion channels (VDACs) on the OMM by glucose-regulated protein 75 (grp75). VDAC on the OMM functions to tightly control Ca^{2+} permeation during type 3 IP_3R-mediated Ca^{2+} signaling. The IP_3R3-VDAC complex shown in Fig. 2b ensures that Ca^{2+} released through IP_3R3 generates high Ca^{2+} microdomains at ER-mitochondria contact sites (Szabadkai et al. 2006).

Ca^{2+} transport into mitochondria: VDAC and uniporter

Ca^{2+} permeation through the mitochondria is controlled by two major components: VDAC on the OMM and Ca^{2+} uniporters on the inner mitochondrial membrane (IMM; Fig. 2b). The OMM was thought to freely-transmit Ca^{2+}; however, new evidence shows that VDAC may be tightly controlling Ca^{2+} permeation during short-lasting IP_3R-mediated Ca^{2+} signaling (Spät et al. 2008). This was demonstrated using truncated Bid (tBid) to increase the permeability of the OMM, while keeping the IMM intact. It was observed that the exposure of tBid enhanced IP_3R-mediated Ca^{2+} signaling, but failed to affect Ca^{2+} permeation into the mitochondria (Hajnóczky et al. 2002). Interestingly, overexpression of VDAC in HeLa cells stimulated with histamine, to generate IP_3, caused an increase in mitochondrial Ca^{2+} uptake (Rapizzi et al. 2002). Cytosolic components such as NADH and metabolic enzymes; however, are also able to alter the gating properties of VDAC (Rizzuto et al. 2009). Once Ca^{2+} transverses the OMM, highly selective Ca^{2+} uniporters on the IMM are engaged to transmit Ca^{2+} into the mitochondrial matrix (Hajnóczky et al. 2002; Spät et al. 2008). A recently discovered gene, *mitochondrial calcium uniporter* (*MCU*), was determined to encode for the pore-forming subunit of the uniporter and has been determined to be critical for mitochondrial Ca^{2+} uptake. Indeed, when *MCU* is silenced mitochondrial Ca^{2+} uptake is diminished (Baughman et al. 2011; Chaudhuri et al. 2013). The Ca^{2+} concentration within the mitochondrial matrix plays a crucial role in mediating oxidative phosphorylation and ATP production through regulation of the TCA cycle (Hayashi and Su 2007; Rizzuto et al. 2009), while also controlling the opening of permeability transition pore (PTP), which plays a role in apoptosis (White et al. 2005; Walter and Hajnóczky 2005; Gunter and Sheu 2009).

Subcellular localization and redistribution of the Bcl-2 family

Distribution of anti-apoptotic Bcl-2 family proteins

Bcl-2 and Bcl-x_L are tail-anchored proteins containing a C- terminal transmembrane (TM) domain consisting of a hydrophobic α-helix which functions as a membrane insertion device. The TM domain of Bcl-x_L, in particular, possesses an X-TMB sequence that is flanked by two basic amino acids and specifically targets it to the outer mitochondrial membrane (OMM), which accounts for its predominant localization at the mitochondria. Bcl-2, on the other hand, contains an X/2-TMB sequence within its TM domain that is far less basic and has no sequence homology when compared with the X-TMB sequence of Bcl-x_L (Kaufmann et al. 2003). As a result, Bcl-2 does not specifically target the mitochondria and can be found largely at the ER. In fact, Bcl-2 relies on the mitochondrial chaperone protein FKBP38, an atypical member of the FK506-binding immunophilin protein family, to shuttle it to the mitochondrial membrane (Portier and Taglialatela 2006).

The predominate ER localization of Bcl-2 can be attributed to its interaction with a reticulum (RTN) family protein, RTN- x_s. Cell fractionation of D98/AH2 cells stably expressing Bcl-2 showed a shift in subcellular localization from the mitochondrial fraction to the microsomal fraction when transiently transfected with RTN-x_s (Tagami et al. 2000). Interestingly, it has also been demonstrated that Bcl-2 is enriched at MAM (Meunier and

Hayashi 2010). A small fraction of Bcl-x_L has also been detected on the ER membrane due to interactions with RTN family members RTN-x_s and NSP-C (Tagami et al. 2000).

Mcl-1 is found largely on the OMM, but curiously lacks a mitochondrial targeting sequence in its TM domain. Thus, two mechanisms have been proposed as to how Mcl-1 targets to the mitochondria. Firstly, Mcl-1 mitochondrial targeting has been linked to its association with translocase of the outer mitochondrial membrane 70 (Tomm70). The internal domain of Mcl-1 contains an EELD sequence that binds Tomm70 and works in conjunction with its TM domain to facilitate membrane insertion (Chou et al. 2006). Mitochondrial targeting is also achieved by the first 79 amino acids on the NH_2-terminus of Mcl-1, which contains a PEST domain and several phosphorylation sites that promotes its association with the mitochondria. Deletion of the NH_2-terminal domain of Mcl-1 diminishes mitochondrial targeting and the anti-apoptotic functioning of the protein (Germain and Duronio 2007).

The first 43 residues on the NH_2-terminus of HAX-1 contain a predicated mitochondrial-targeting motif and acid box, which are essential for mitochondrial targeting (Chou et al. 2006), although a small fraction of HAX-1 has also been detected on the ER membrane. HAX-1 contains a binding region for the ER protein phospholamban (PLN) in its trans-membrane domain (aa 203–245), and when bound to PLN, HAX-1 distributes to the ER membrane. Indeed, when the transmembrane domain of HAX-1 is deleted, truncated HAX-1 (HAX-1 aa 1–181) only distributes to the mitochondria. Additionally, when compared to full length HAX-1 cotransfected with PLN, truncated HAX-1 cotransfected with PLN did not show distribution to the ER membrane (Kaufmann et al. 2003; Yap et al. 2010).

Although anti-apoptotic proteins reside mainly at the OMM and/or ER membranes, it is important to note that they have also been detected at other cellular locations. Bcl-2 and HAX-1, for instance, have been observed at outer nuclear membranes, while Mcl-1 and Bcl-x_L exists partially within the cytosol. Cytosolic Bcl-x_L, in particular, exists as a homodimer with its TM domain inserted into the hydrophobic pocket of the reciprocal dimer partner (Jeong et al. 2004).

Distribution of pro-apoptotic Bcl-2 family proteins

Bak mainly localizes to the OMM and integrates via its C- terminal TM domain. Similar to the TM domain of Bcl-x_L, the TM domain of Bak is flanked by basic amino acids that specifically target the mitochondria (Lindsay et al. 2011). Its pro-apoptotic activity; however, is restrained by association with Bcl-x_L, Mcl-1, or VDAC2 (Willis et al. 2005). In contrast, Bax in its inactive form is mainly found in the cytosol by hiding its C-terminal α helix containing a TM domain in its own binding pocket, while a fraction of Bax is also tethered to the OMM (George et al. 2010; Edlich et al. 2011). Subcellular fractionation studies have also shown that approximately 10–15 % of Bak and Bax reside at the ER (Zong et al. 2003).

Bik contains a C-terminal TM domain that exclusively targets it to the ER membrane (Mathai et al. 2002; Zhao et al. 2008). The hydrophobic C-terminal TM domain of Puma predominately targets it to the mitochondria, but is expressed at very low levels in cells until activation by p53 or alterations in intracellular Ca^{2+} (Nakano and Vousden 2001; Reimertz

et al. 2003). Similarly, the C-terminal TM domain of Nix/BNIP3 also targets it to the mitochondria, although a small amount is also detected at the ER (Zhang et al. 2009; Chen et al. 2010). Most other inactive forms of BH3-only proteins including Bid, Bad, and Bim; however, are found within the cytosol. Bid remains cytosolic since it lacks a membrane targeting sequence. Bad is regulated by phosphor-ylation of three serine sites—S112, S136, and S155. The three serines are phosphorylated by a number of kinases including protein kinase A, Akt/protein kinase B, and p21-activated kinases. When S112 and S136 are phosphorylated, Bad is cytosolically retained through interaction with 14-3-3 scaffold proteins (Masters et al. 2001). The two major isoforms of Bim, Bim_{EL} and Bim_{L}, are sequestered to the microtubule- associated dynein motor complex by binding to the LC8 dynein light chain (Puthalakath et al. 1999).

Translocation of Bcl-2 family proteins

BH3-only pro-apoptotic proteins mainly found in the cytosol serve to detect apoptotic stimuli in cells and are characterized as *activators* or *sensitizers*. Activators, such as Bid, directly activate multi-domain pro-apoptotic proteins. Upon stimulation, Bid is proteolytically cleaved by caspase-8 and undergoes a posttranslational modification to form truncated Bid (tBid), which then targets the mitochondria and activates Bax and Bak. Activated Bax dimerizes and undergoes a conformational change that exposes its transmembrane domain followed by translocation to mitochondrial and ER membranes. Subsequently, tBid induces a conformational change in Bak that breaks its association with Bcl-x_L, Mcl-1 or VDAC2 at the OMM (Korsmeyer et al. 2000). Alternatively, when activated, the BH3-only sensitizers like Bim and Bad translocate and then bind anti-apoptotic proteins to neutralize their activity. For example, apoptotic stimuli activate the release of Bim from its association with the dynein motor complex, and Bad undergoes dephosphorylation to dissociate from 14-3-3. The sensitizers are then able to bind Bcl-2 or Bcl-x_L to disrupt their complexes with multi-domain pro-apoptotic proteins; thereby activating the apoptotic cascade (Puthalakath et al. 1999; Masters et al. 2001). Bad has also been shown to hinder the activity of Bcl-x_L by inhibiting dimer formation. Interestingly; however, this also triggers the translocation of cytosolic Bcl-x_L to mitochondrial membranes by displacing homodimers of Bcl-x_L to expose Bcl-x_L TM domains (Jeong et al. 2004).

Although the mechanism is unknown, cytosolic Mcl-1 is also known to translocate to the OMM upon apoptotic stimulation. Interestingly, Mcl-1 degradation within the cytosol and at the OMM also facilitates cytosolic Bcl-x_L and Bax translocation to the mitochondria (Nijhawan et al. 2003). Once in the OMM, Bcl-x_L heterodimerizes with multi-domain and pro-apoptotic proteins to deter apoptosis (Cheng et al. 2001; Yi et al. 2003; Ruffolo and Shore 2003; Shore and Nguyen 2008). In particular, Bcl-x_L is able to retrotranslocate Bax from the mitochondria to the cytosol depending on its concentration at the OMM (Edlich et al. 2011).

Multi-organelle coordination of Ca^{2+} signaling by the Bcl-2 family

Bcl-2 family at the ER

Bcl-2 mediates ER Ca^{2+} stores—There is conflicting evidence as to the roles anti-apoptotic Bcl-2 family proteins play in ER Ca^{2+} homeostasis. Some studies show that ER Ca^{2+} is preserved when anti-apoptotic Bcl-2 proteins are overexpressed, while others report that Bcl-2 overexpression results in diminished Ca^{2+} stores. These different findings demonstrate the complexity of Ca^{2+} signaling and may be the result of unique differences between cell types or even clonal isolates. The data supporting the preservation of ER Ca^{2+} will be considered first. Under extracellular Ca^{2+}-free conditions, it was observed that Bcl-2 overexpression preserves ER luminal Ca^{2+} during apoptosis induced by inter-leukin (IL-3) withdrawal in IL-3-dependent hematopoetic cells. Indeed, direct measurement of ER luminal Ca^{2+} by Fura-2FF AM showed no difference between Bcl-2 positive and negative clones (Baffy et al. 1993). It was further demonstrated for W.Hb12 cells stably overexpressing Bcl-2 that ER Ca^{2+} was preserved due to enhanced ER Ca^{2+} uptake (He et al. 1997). A similar finding was observed in MCF10A cells stably expressing Bcl-2. It was additionally determined that Bcl-2 overexpressing MCF10A cells have upregulated SERCA2 mRNA and protein levels. Therefore, increased ER Ca^{2+} uptake was attributed to increased SERCA activity, and it was speculated that Bcl-2 might interact with SERCA pumps at the ER (Chen et al. 2004).

Evidence supporting reduced ER luminal Ca^{2+} in cells overexpressing anti-apoptotic proteins has been presented by a number of researchers using ER-targeted sensors including cameleons, Mag-fura 2-AM, and AEQ chimeras. There are three proposed mechanisms to explain how reduced ER luminal Ca^{2+} may occur. Some researchers believe that Bcl-2 overexpression decreases SERCA activity, thereby reducing the amount of Ca^{2+} that can enter the ER. This was demonstrated in a study showing that Bcl-2 overexpression decreases SERCA2b and calreticulin expression in LNCaP cells (Vanden Abeele et al. 2002). Furthermore, it was reported that anti-apoptotic HAX-1, although residing predominately at the mitochondria, can translocate to the ER to bind with and significantly downregulate SERCA2 thereby causing reduced ER Ca^{2+} loading (Vafiadaki et al. 2009). Another proposed mechanism for reduced ER luminal Ca^{2+} is that anti-apoptotic Bcl-2 family members increase Ca^{2+} leakage across the ER in order to reduce steady-state Ca^{2+} levels. Researchers observed a decrease in ER luminal Ca^{2+} in DT40 cells expressing Bcl-2 or Mcl-1; however, in the presence of IP_3R inhibitor heparin that blocks the exit of Ca^{2+} from the ER, ER luminal Ca^{2+} was preserved. Furthermore, Bcl-2 and Mcl-1 expression had no effect on ER Ca^{2+} stores in DT40 cells with all three IP_3R isoforms knocked-out (Eckenrode et al. 2010). An alternative scenario under consideration is that Bcl-2 indirectly decreases capacitative Ca^{2+} entry by downregulating SOC activity at the plasma membrane to limit the amount of Ca^{2+} stored in the ER (Li et al. 2002).

Pro-apoptotic Bcl-2 members regulate ER Ca^{2+} stores—Pro-apoptotic Bcl-2 family proteins have also be shown to regulate luminal ER Ca^{2+}. Experimentation with Bax^{-}/Bak^{-}double-knockout (DKO) cells (mouse embryo fibroblast cells deficient in Bax and Bak) showed a decrease in ER Ca^{2+} stores, which resulted in reduced flux of Ca^{2+} from

the ER into the cytosol and mitochondria compared to wild-type cells under thapsigargin (Tg) stimulation. Expression of recombinant Bax in DKO cells restored ER Ca^{2+} to nearly wild-type levels; however, expression of mitochondria-targeted Bax in DKO cells had no effect on ER Ca^{2+} stores. Thus, the expression of ER-targeted Bax/Bak may function to increase the ER luminal Ca^{2+} concentration (Scorrano et al. 2003; Oakes et al. 2005).

BH3-only pro-apoptotic proteins Bik and Nix/BNIP3 have been observed to increase ER Ca^{2+} leakage. In Hep3B cells with Bik overexpression, there was an observed increase in cytosolic Ca^{2+} over a 72 h time frame after depletion of ER Ca^{2+} (Zhao et al. 2008). Another study using kidney epithelial cells derived from wild-type or Bax^{-}/Bak^{-} DKO mice demonstrated that Bik overexpression results in the release of ER Ca^{2+} in wild-type cells, although Ca^{2+} release was not observed in DKO cells. Hence, Bik appears to function through a Bax/Bak-dependent mechanism to release ER Ca^{2+} stores (Mathai et al. 2005). Researchers have also observed greater increases in cytosolic Ca^{2+} in Nix-overexpressing mice compared to Nix-knockout mice and controls following caffeine stimulation (Diwan et al. 2009). Transiently overexpressing BNIP3 in Mes 23.5 cells was additionally determined to increase ER Ca^{2+} leakage, resulting in increased mitochondrial Ca^{2+} accumulation (Zhang et al. 2009).

Puma regulates ER Ca^{2+} depletion-induced apoptosis—Following ER Ca^{2+} depletion stimulated by Tg, researchers observed transcriptional upregulation of Puma, a BH3-only pro-apoptotic protein, in HCT116 human colon cancer cells. Increased Puma mRNA and protein levels resulted in activation of caspase-3, 8, and 9 and Bid as well as the release of cytochrome c into the cytosol (Luo et al. 2005). Likewise, increases in Puma mRNA and protein levels were detected in SH-SY5Y neuroblastoma cells after 4 and 12 h treatment, respectively, with tunicamycin (an ER stress inducer that increases intracellular Ca^{2+} levels). Corresponding increases in activated caspase-3 and 9 as well as cytochrome c release was observed. Morphological changes characteristic of apoptosis and substantial increases in cell death were also observed in GFP-positive SH-SY5Y cells transiently co-transfected with hemagglutinin-tagged Puma compared to control cells (Reimertz et al. 2003).

Researchers have found that Bax-KO cells provided some resistance to Tg-induced apoptosis. Increased Puma upregulation was also observed in these cells compared to controls, indicating an attempt by Puma to compensate for Bax deficiency. Thus, Puma appears to facilitate ER-stress-induced apoptosis through a Bax-dependent pathway. Additional evidence shows that Puma is vital for initiation of ER stress-induced apoptosis. BiP, an unfolded protein response target gene that aids in restoring normal ER functioning, was upregulated in response to prolonged Tg treatment in HCT116 control and Puma-knockout (KO) cells. Consistent with previous results Tg also triggered the upregulation of Puma in control cells. However, Puma-KO cells had substantially less apoptosis after exposure to Tg compared to controls (Reimertz et al. 2003; Luo et al. 2005).

Bcl-2, Bcl-x_L, and Mcl-1 bind IP_3Rs—Several studies confirm that Bcl-2, Bcl-x_L, and Mcl-1 bind to all three isoforms of IP_3Rs (Eckenrode et al. 2010). Researchers also observed enhanced Bcl-2 and type 1 IP_3R binding in Bax^{-}/Bak^{-} double-knockout (DKO) MEF cells

by immunoprecipation of Bcl-2 from ER fractions when compared to wild-type cells (Oakes et al. 2005). Furthermore, an increase in Bcl-2 and type 3 IP_3R interactions was shown by immunoprecipation of type 3 IP_3Rs or Bcl-2 in T47D breast cancer cells after apoptosis induction using gefitinib, a tyro-sine kinase inhibitor (Zannetti et al. 2008). Most studies; however, focus on determining the binding sites for Bcl-2 family protein interacting with type 1 IP_3Rs.

Pull-down studies using glutathione-*S*-transferase (GST) fusion constructs followed by immunoblotting showed that Bcl-2 binds to domain 3 (amino acids, aa, 293–1581) in the regulatory and coupling domain of type 1 IP_3Rs, and less significantly with domain 6 (aa 2590–2749) at the C-terminus. By dividing the binding region into smaller fragments and it was determined that Bcl-2 binds most strongly with amino acids 1347–1426 of domain 3 (Rong et al. 2009). In a similar study, Bcl-x_L was shown to interact with domain 6 at the C-terminus of type 1 IP_3R; interaction with domain 3 was not investigated (White et al. 2005). Furthermore, Flag-tagged Bcl-2, Bcl-x_L, and Mcl-1 were pulled-down by domain 6 (GST-IP_3R-TM6 + C; aa 2570–2749) of all three types of IP_3Rs (Eckenrode et al. 2010). Researchers have also been able to determine that the BH4 domain of Bcl-2 is necessary for binding with type 1 IP_3Rs. This was demonstrated using GST-IP_3R-domain 3 to pull down full-length Bcl-2, whereas ΔBH4Bcl-2 was not pulled down by either GST-IP_3R-domain 3 or 6 (Rong et al. 2009).

Bcl-2, Bcl-x_L, and Mcl-1 regulate IP_3R-mediated Ca^{2+} signaling—By binding IP_3Rs, Bcl-2 family proteins function to modulate ER Ca^{2+} efflux. Using a single type 1 IP_3R channel incorporated into an artificial planar lipid bilayer, Bcl-2 overexpression was shown to markedly reduce IP_3R channel opening (Rong et al. 2009) and decrease the binding affinity of IP_3Rs to IP_3. During single cell imaging with Fura- 2-loaded DT40 cells the initial amplitude of released Ca^{2+} was reduced in Bcl-2 overexpressing cells. However, Bcl-2 and Mcl-1 overexpression contributed to more sustained and extended Ca^{2+} oscillations. Separately, experimentation with siRNA against Bcl-2 showed an increase in the number of cells that responded to ATP stimulation as well as an increase in the amplitude of Ca^{2+} signals when compared to control cells (Chen et al. 2004).

Experiments with a single type 1 IP_3R channel showed that Bcl-x_L increased channel activity by increasing the binding affinity of IP_3Rs to IP_3 (White et al. 2005; Li et al. 2007). Interestingly, however; Bcl-x_L was also shown to diminish IP_3R expression. In fact, total RNA purified from FL5.12 cells stably transfected with Bcl-x_L, showed a marked decrease in the expression of type 1 IP_3R genes. Western blotting data showed lower levels of type 1 and 3 IP_3R expression in Bcl-x_L overexpressing FL5.12 and 2B4.11 cells when compared to control cells. Thus, Bcl-x_L overexpression resulted in an over-all decrease in the percentage of ER Ca^{2+} release as observed by researchers in which they showed that Bcl-x_L overexpressing vesicles release less than 25 % of total Ca^{2+}, compared to control vesicles that released approximately 40 % of total Ca^{2+} (Li et al. 2002).

Bcl-2 family at the mitochondria

Bcl-2 family proteins mediate VDAC opening on the OMM—VDAC is a highly abundant protein on the OMM and functions to facilitate the entry and exit of ATP, Ca^{2+}, cytochrome c, and other metabolites between the mitochondria and other cellular compartments. As previously discussed, VDAC is also known to form "Ca^{2+} tunnels" with IP_3R3 at the MAM via linkage with grp75 to tightly control ER Ca^{2+} signals into the mitochondria. There is some controversy; however, as to whether the open or closed conformation of VDAC assists in bioenergetics or induces cell death.

One theory is that VDAC closure sensitizes cells to apoptotic signals, while VDAC opening facilitates cellular metabolism (Vander Heiden et al. 2000; Tan and Colombini 2007). In its closed conformation, VDAC was observed to be highly permeable to Ca^{2+}, but has reduced permeability to metabolites and ATP. Alternatively, the open conformation of VDAC allows for metabolite flux, has a low permeability to Ca^{2+} and prevents cytochrome c release. In fact, recombinant Bcl-x_L was shown to increase the conductance of VDAC (Vander Heiden et al. 2001), while tBid induces VDAC channel closure (Rostovtseva et al. 2004).

Other researchers hypothesized that, in healthy cells, VDAC is continuously opening and closing to promote cellular metabolism. However, when pro-apoptotic proteins bind VDAC it causes the channel to remain in its open conformation, which is permeable to cytochrome c and leads to apoptosis. Anti-apoptotic proteins are able to counteract the effects of pro-apoptotic proteins by closing VDAC channels, but have no effect of VDAC conformation in healthy cells (Tsujimoto and Shimizu 2000). This was demonstrated by electrophysiological studies in which Bax caused a 4-fold increase in VDAC conductance, while Bcl-x_L almost completely closed the channel (Shimizu et al. 2000a). Of note, it was demonstrated that the BH4 domains of Bcl-2 and Bcl- x_L are required to inhibit VDAC opening, while the BH1 domain of Bcl-x_L is also required (Shimizu et al. 2000b).

Mcl-1 and BNIP3 regulate mitochondrial Ca^{2+} uptake—Although the mechanism is unknown, Mcl-1 has been shown to modulate mitochondrial Ca^{2+} uptake. Rhod-2 measurements of mitochondrial Ca^{2+} using confocal microscopy were used to compare Mz-ChA-1 cells transfected with Mcl-1, siRNA against Mcl-1, or were not transfected. Those cells transfected with siRNA against Mcl-1 or non-transfected cells showed an increase in mitochondrial Ca^{2+} when stimulated with ATP, while cells transfected with Mcl-1 showed lower mitochondrial Ca^{2+} increases. Researchers also observed an increase in mitochondrial Ca^{2+} in Mcl-1 knockdown cells, while mitochondrial Ca^{2+} remained constant in Mcl-1 overexpressing cells when stimulated with staurosporine to induce apoptosis (Minagawa et al. 2005).

Conversely, Mes 23.5 cells transiently overexpressing BNIP3 were observed to stimulate mitochondrial Ca^{2+} uptake. Cells were loaded with Fluo-4 and Rhod-2 Ca^{2+} dyes to monitor cytosolic and mitochondrial Ca^{2+}, respectively, after Tg-induced ER Ca^{2+} depletion. When compared to control, BNIP3 increased the ratio of mitochondrial-to-cytosolic Ca^{2+} over an 8 h period. This increase in mitochondrial Ca^{2+} was hindered when cells were first incubated with Ru360, a mito-chondrial uniporter inhibitor. Furthermore, transiently transfecting Bcl-2 in addition to BNIP3 also reduced mitochondrial Ca^{2+} uptake (Zhang et al. 2009).

Bcl-2 family at other subcellular localizations

Cytosolic Ca^{2+} increases modulate apoptosis—Increases in intracellular Ca^{2+} have been found to change the phosphorylation state of Bad through calcineurin, a serine-threonine phosphatase that is stimulated by Ca^{2+}. Immunoprecipitation studies have determined that phosphorylated Bad reside in the cytosol in complex with 14-3-3 scaffold proteins and calcineurin. After cytosolic Ca^{2+} concentration is increased by stimulating cells with Tg, ionomycin, A23187, or glutamate, Bad becomes dephosphorylated and dissociates from calcineurin and 14-3-3. Dephosporylated Bad then trans- locates to the mitochondria to associate with Bcl-x_L and results in increases in the activated form of caspase-3 (Wang 1999; Springer et al. 2000).

Ca^{2+} has also been found to activate a family of cytosolic cysteine proteinases called calpains (Khorchid and Ikura 2002). Active calpains cleave a number of target proteins in order to regulate apoptosis including members of the Bcl-2 family and caspases. Indeed, calpains are able to cleave Bid into its truncated form (tBid), resulting in cytochrome c release. Calpains can also activate Bax by cleaving its N-terminal region, which is responsible for heterodimerization with Bcl-2 and Bcl-x_L. Bcl-2 and Bcl-x_L can also be direct targets of calpains. Researchers determined that calpain- truncated Bcl-2/Bcl-x_L undergo conformational changes that make them unable to homo- or heterodimerize. Of note, calpain-truncated Bcl-2, but not calpain-truncated Bcl-x_L, was able to induce cytochrome c release. Taken together, the above data support the notion that calpain cleavage of Bcl-2 family proteins facilitates apoptosis. However, it is largely tissue-dependent whether calpain-cleaved caspases promotes death or cell survival (Gil-Parrado et al. 2002; Łopatniuk and Witkowski 2011).

Nuclear envelope-associated Bcl-2 is pro-apoptotic—The nuclear envelope acts as a barrier responsible for housing the genetic material of the cell within the nucleus. Continuous with the ER membrane, the cisternal space of the nuclear envelope also acts as a Ca^{2+} store mediated by SERCA pumps and IP_3Rs. The nuclear pore complex (NPC) is a large protein channel that spans both lipid layers of the nuclear envelope and function to exclusively regulate the transport of macro-molecular molecules into and out of the nucleus. The NPC is composed of over 100 proteins, including 16–25 copies of glycoprotein 210 that contains several Ca^{2+}-binding domains that extend into the cisternae of the nuclear envelope. Thus, cisternae Ca^{2+} stores were determined to regulate NPC structure (Gerace et al. 1982; Greber and Gerace 1995). Particularly, cisternal Ca^{2+} depletion causes the NPC to undergo conformational changes that increase NPC diameter, alter transport, and impede selectivity; all of which are detrimental to the cell (Erickson et al. 2006; Strasser et al. 2012).

Since NPC structure is mediated by cisternae Ca^{2+} concentration, it was hypothesized that nuclear envelope-associated Bcl-2 may function to mediate nuclear envelope permeability. In fact, it was determined that Bcl-2 overexpression lowers steady-state Ca^{2+} concentrations in the nucleus and increases the size-exclusion limit of the NPC when compared to control cells. Furthermore, only ER- and nuclear envelope-target Bcl-2, and not mitochondrial localized Bcl-2, increased nuclear envelope permeability. Since increased nuclear envelope permeability can lead to apoptosis, Bcl-2 has a pro-apoptotic role when localized at the

nuclear envelope (Portier and Taglialatela 2006; Strasser et al. 2012) and further supports the notion that subcellular localization is important to Bcl-2 family functioning.

Bcl-2 mediates Ca^{2+} entry at the plasma membrane—Bcl-2 has been shown to modulate Ca^{2+} entry into the cell, although conflicting evidence may be the result of unique differences between cell types. Some studies observed a decrease in Ca^{2+} entry in Bcl-2 expressing cells. For example, measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) using AEQ chimeras in HeLa cells transiently overexpressing Bcl-2 resulted in a lower increase in $[Ca^{2+}]_c$ compared to controls after CCE was activated (Pinton 2000). Similar results were also seen in LNCaP cells stably overexpressing Bcl-2. These cells were treated with fura-2 to measure $[Ca^{2+}]_c$, and researchers observed a longer latency period and slower rate of Ca^{2+} influx through SOCs. Further experimentation suggested that Bcl-2 expression decreases the number of functional SOCs, resulting in the observed downregulation of capacitative Ca^{2+} entry (Vanden Abeele et al. 2002). Conversely, experiments with HL60 and PW cell lines resulted in enhanced CCE in Bcl-2 overexpressing cell lines (Williams et al. 2000).

The role of Ca^{2+} in bioenergetics and apoptosis

The principle input for cellular carbon metabolism in mammalian cells is glucose. During glycosis, one glucose molecule is converted into two pyruvate molecules, and produces energy in the form of ATP. Pyruvate can then be transported into the mitochondria to enter the tricarboxylic acid (TCA) cycle for the production of NADH and $FADH_2$, or remains in the cytosol where it is converted into lactate. NADH and $FADH_2$ are then used in the electron transport chain to drive ATP production by means of oxidative phosphorylation. Ca^{2+} in the mitochondria assist in the regulation of cellular bioenerget-ics by stimulating Ca^{2+}-dependent enzymes in the TCA cycle (pyruvate dehydrogenase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase), as well as ATP synthase, adenosine nucleotide translocase (ANT; the ATP translocator), and sites on the electron transport chain (Fig. 3).

The electron transport chain is also responsible for maintaining the inner mitochondrial transmembrane potential ($\Delta\psi_m$) through regulation of the permeability transition pore (PTP). For many years the PTP was thought to consist of ANT found on the IMM and VDAC found on the OMM. However, PTP opening has been observed in the absence of these molecules (Kokoszka et al. 2004; Baines et al. 2007). In fact, new evidence indicates that the PTP is composed of ATP synthase dimers that bind cyclophilin D, a known regulator of the PTP. Although the mechanism is unknown, it is speculated that Ca^{2+} accumulated in the mitochondrial matrix binds ATP synthase causing conformational changes that induce PTP formation, with cyclophilin D increasing the Ca^{2+} binding affinity to the PTP (Giorgio et al. 2013).

Two open conformations of the PTP have been observed; a low-conductance state and a high-conductance state. At a low-conductive state, the PTP is permeable to small ions such as Ca^{2+} and K^+ and contributes to mitochondrial Ca^{2+} homeo-stasis by controlling mitochondrial Ca^{2+}-induced Ca^{2+} release (CICR). During CICR, Ca^{2+} is released quickly

during transient PTP opening without causing pathological increases in $\Delta\psi_m$. The high-conductive conformation of the PTP; however, is permeable to pro-apoptotic molecules like cytochrome c and irreversibly dissipates $\Delta\psi_m$ leading to apoptosis (Jouaville et al. 1998; Huang et al. 2000). Once in the cytosol, cytochrome c interacts with apoptotic-protease-activating factor-1 (Apaf-1) to form the apoptosome. The apoptosome will then activate initiator caspases to trigger a caspase cascade, thus resulting in apoptosis. Cytochrome c can also bind IP_3Rs to boost the influx of Ca^{2+} into the mitochondria and enhance apoptotic signaling (Boehning et al. 2003; Jeong and Seol 2008). The switch from a metabolic to apoptotic functioning of the PTP is dependent on matrix pH, which is dictated by the rate of mitochondrial Ca^{2+} influx and not the absolute amount (Jouaville et al. 1998). Thus, rapid or transient increases in mitochondrial Ca^{2+} will result in low-conductive opening of the PTP, while prolonged Ca^{2+} signals or mitochondrial Ca^{2+} overload causes high-conductive PTP opening.

There is some controversy as to whether the PTP regulates necrosis or apoptosis (Fig. 4). Bcl-2 overexpression was determined to diminish Ca^{2+} activation of the PTP, and block the dissipation of $\Delta\psi_m$ by ER-targeted BNIP3 (Murphy et al. 2001; Zhang et al. 2009); thus supporting an apoptotic role of the PTP. However, a study using cyclophilin D-deficient mice indicates that PTP opening is vital for necrosis (Nakagawa et al. 2005). This is further supported by the fact that ER localization of Nix also plays a role in PTP-dependent necrosis (Chen et al. 2010). There is additional evidence supporting the release of cytochrome c through the mitochondrial apoptosis-induced channel (MAC) formed on the OMM during early apoptosis. Although there is not a clear link between Ca^{2+} signaling and MAC, this channel is highly regulated by Bcl-2 family proteins (Guo et al. 2004; Martinez-Caballero et al. 2004, 2005; Dejean et al. 2005, 2006). There is also speculation that the PTP and MAC work together to facilitate cytochrome c release during mitochondrial mediated apoptosis. For instance, Ca^{2+} overload in the mitochondria matrix would cause highly-conductive PTP opening and dissipation of $\Delta\psi_m$ resulting in elevated cytosolic Ca^{2+} concentration. Increased cytosolic Ca^{2+} in return would lead to activation of pro-apoptotic proteins such as tBid and Bad, and induce MAC formation; therefore leading to OMM permeability and cyto-chrome c release (Wang 1999; Springer et al. 2000; Chen et al. 2002; Hajnóczky et al. 2009).

The Bcl-2 family: linking apoptosis and bioenergetics through Ca^{2+}

The relative amounts of anti- and pro-apoptotic proteins determine whether a cell remains viable or enters into apoptosis. In healthy cells, anti-apoptotic Bcl-2 family proteins dominate and function at the ER, mitochondria, nuclear envelope, and plasma membrane to mediate Ca^{2+} homeostasis, IP_3R-mediate Ca^{2+} signaling and mitochondrial Ca^{2+} uptake in order to maintain concentrations within physiological levels (Table 1). Sustained and complete release of Ca^{2+} into the mitochondria, alterations in the spatiotemporal pattern, or changes at the level of effectors activated by Ca^{2+} can cause Ca^{2+} signals to switch from physiological functioning to apoptosis initiation (Pinton et al. 2002), leading Bcl-2 family proteins to translocate to the mitochondrial membrane in order to regulate $\Delta\psi_m$ (Fig. 4). If death signals prevail, the PTP switches from a low-conductive state to a high-conductive state which dissipates $\Delta\psi_m$ (Jouaville et al. 1998).

Genetic engineering of mammalian cells has evolved to combat apoptosis through overexpression of anti-apoptotic proteins and the knockdown or silencing of pro-apoptotic proteins. A number of studies have centered on overexpression of Bcl-2, in which these cell lines had higher viabilities and improved productivity compared to control cells when exposed to apoptotic triggers (Mastrangelo et al. 1999, 2000). Overexpression of other anti-apoptotic Bcl-2 proteins, such as Bcl-x_L and Mcl-1, or combinations of anti-apoptotic proteins have also resulted in increases in viability and titer (Chiang and Sisk 2005; Nivitchanyong et al. 2007; Majors et al. 2009; Dorai et al. 2009). Likewise, Bax^{-}/Bak^{-} double-knockouts in DHFR knockout CHO suspension cells resulted in increased levels of recombinant protein production when compared to wild-type cells (Cost et al. 2010).

Since Bcl-2 family proteins also regulate bioenergetics, changes in cellular metabolism were observed in some apoptosis-resistant cell lines. For example, Bcl-2 expressing Myc-transformed Rat1 cells were shown to have an altered glucose metabolism with a lower molar ratio of lactate production to glucose consumption, indicating a more efficient shuttling of pyruvate into the TCA cycle (Papas et al. 1999). Apoptosis-resistant CHO cells were also shown to consume lactate at a higher rate when compared to control cells (Dorai et al. 2009), and lactate consuming cells were found to be more energy efficient based on metabolic flux analysis (Martínez et al. 2013). Thus, it was hypothesized that apoptosis-resistant cell lines could thrive in high glucose conditions without producing toxic levels of lactate. Indeed, anti-apoptotic cell lines could be cultured effectively in a high glucose medium, where viability, peak viable cell density, and antibody titer all significantly increased compared to controls (Dorai et al. 2009).

It has become clear that subcellular localization of Bcl-2 family proteins also plays an important role in mediating Ca^{2+} signals important in both bioenergetics and apoptosis. Thus, in the future, it may be useful to target Bcl-2 family proteins to specific subcellular locations to control bioenergetics, along with apoptosis. For example, ER-targeted Bcl-2 was shown to protect cells from apoptosis by Bax overexpression, while wild-type and mitochondrial-targeted Bcl-2 did not (Wang et al. 2001). Additionally co-expression of Bcl-2 and FKBP38, a mitochondrial chaperone protein that shuttles Bcl-2 to the mitochondrial membrane, reduced pro-apoptotic nuclear envelope-associated Bcl-2 and increased cell survival (Portier and Taglialatela 2006). More studies are needed; however, to determine if subcellular targeting of Bcl-2 family proteins can be effective in controlling bioenergetics or other aspects of cellular physiology beyond the cell death cascade.

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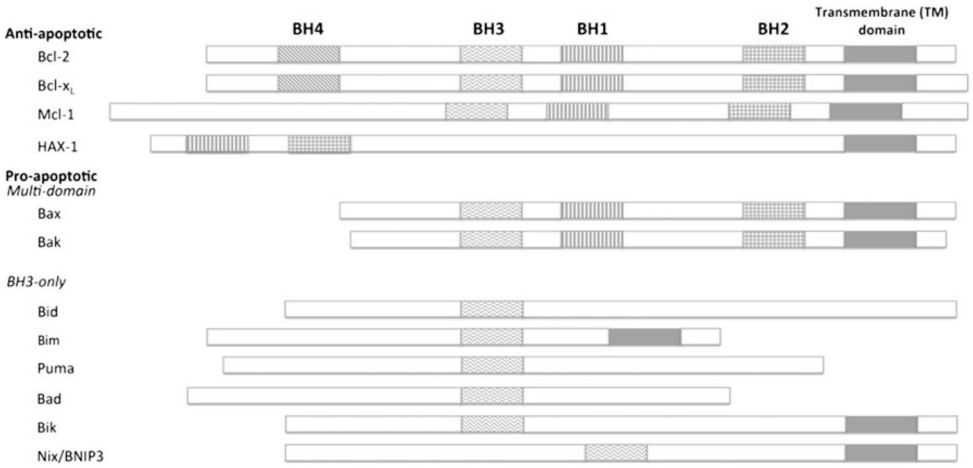

Fig. 1.

Schematic representation of key members of the Bcl-2 family of proteins. Anti-apoptotic members are typically comprised of all four BH domains and a transmembrane domain for membrane anchoring. Pro-apoptotic members contain multi-domain members that include BH1-3, or BH3-only members. Not all pro-apoptotic Bcl-2 family members; however, have a transmembrane domain

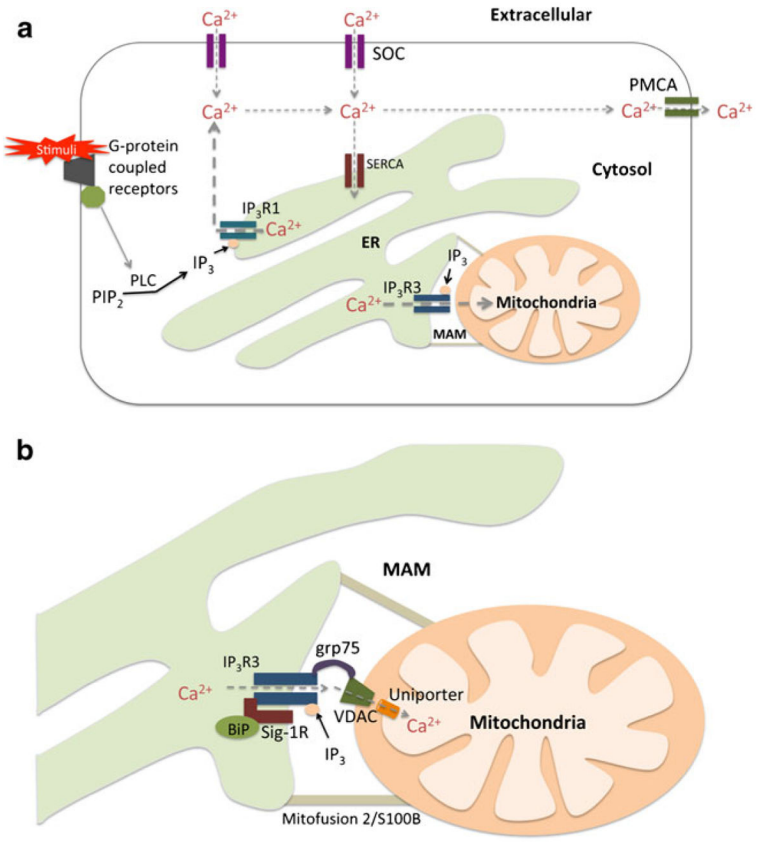

Fig. 2.

Schematic representation of intracellular Ca^{2+} dynamics. **a** Ca^{2+} entry and exit are regulated by SOC and PMCA channels, respectively, on the plasma membrane. Ca^{2+} is transported into the ER lumen by SERCA and released through IP_3R. Type 1 IP_3Rs (IP_3R1) mediate cytosolic Ca^{2+} efflux and type 3 IP_3Rs (IP_3R3) mediate mitochondrial Ca^{2+} efflux. **b** IP_3R3s enriched at MAM are found in a complex with Sig-1R and BiP, and are additionally linked to VDAC on the OMM by grp75. VDAC tightly controls Ca^{2+} permeation by IP_3R3-mediated Ca^{2+} signals. Once Ca^{2+} transverses the OMM, Ca^{2+} uniporters transmit Ca^{2+} into the mitochondrial matrix

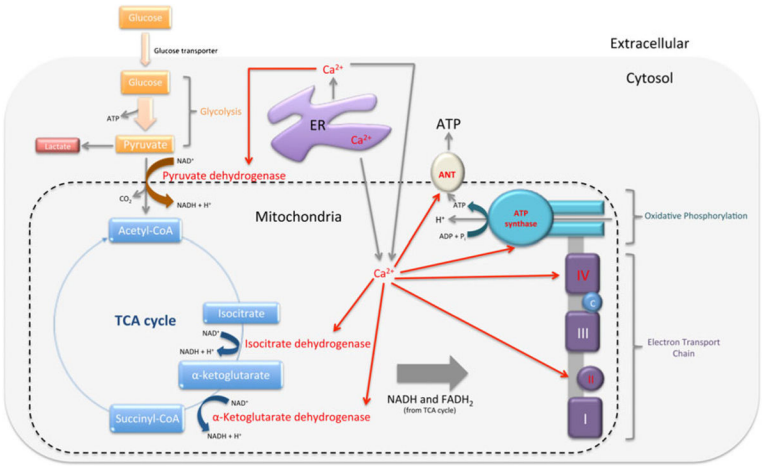

Fig. 3.

Ca^{2+}-dependent enzymes in bioenergetics are facilitated by Ca^{2+} signals from the ER into the cytosol and mitochondria. Pyruvate, produced during glycolysis, can remain in the cytosol where it is converted into lactate, or it is transported into the mitochondria by Ca^{2+}-dependent pyruvate dehydrogenase where it enters the TCA cycle. The TCA cycle consists of two other Ca^{2+}-dependent enzymes—isocitrate, and α-ketoglutarate dehydrogenases—and produces NADH and $FADH_2$. NADH and $FADH_2$ are used by the electron transport chain to drive oxidative phosphorylation and ATP production. Complex II and IVof the electron transport chain are Ca^{2+}-dependent as well as ATP synthase and ANT

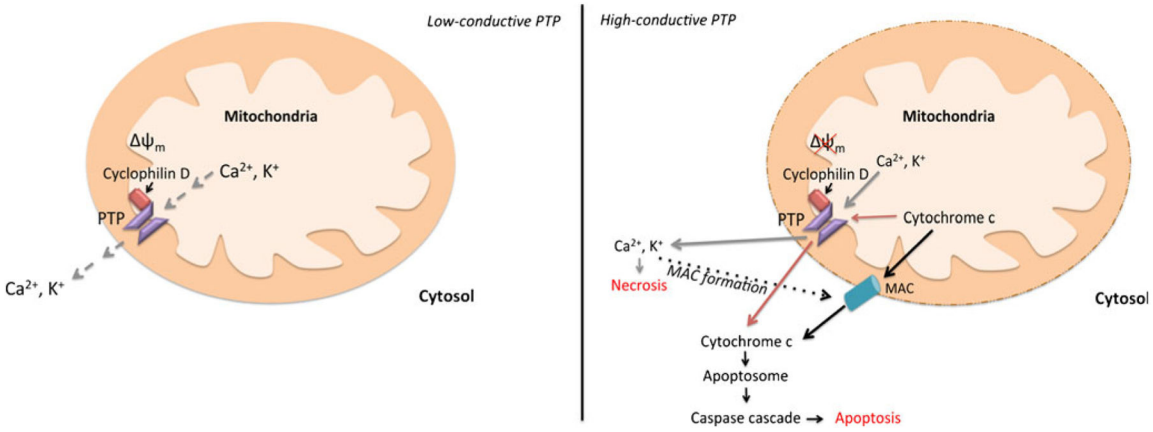

Fig. 4.

PTP opening is controlled by the rate of mitochondrial Ca^{2+} influx. Rapid or transient increases in mitochondrial Ca^{2+} influx results in low-conductive PTP opening permeable to small ions and controls transient Ca^{2+} release. Prolonged Ca^{2+} signals or mitochondrial Ca^{2+} overload causes high-conductive PTP opening, which increases cyctosolic Ca^{2+}. The highly-conductive PTP was observed to directly facilitate necrosis (*gray lines*) and apoptosis (*red lines*). There is additional evidence that the PTP works in conjunction with MAC during apoptosis initiation (*black lines*). High-conductive opening of the PTP increases cytosolic Ca^{2+} concentration, which results in activation of pro-apoptotic proteins, inhibition of anti-apoptotic proteins, and MAC assembly. MAC then functions to permeabilize the OMM and release cyto-chrome c, which leads to apoptosis

Table 1

Overview of Bcl-2 family functioning in intracellular Ca^{2+} homeostasis and IP_3R-mediated Ca^{2+} signaling at various subcellular localizations

Bcl-2 family protein	Localization	Functioning
Anti-apoptotic		
Bcl-2	ER	• Mediates ER Ca^{2+} stores • Binds IP_3R (type 1, 2, and 3) • Reduces IP_3R channel activity • Enhances IP_3R-mediated Ca^{2+} oscillations
	Mitochondria	• Mediates VDAC opening • Regulates PTP opening
	Plasma membrane	• Mediates CCE through SOC
	Nuclear envelope	• Pro-apoptotic functioning • Increases nuclear envelope permeability • Increases size-exclusion limit of NPC
Bcl-x_L	ER	• Binds IP_3R (type 1, 2, and 3) • Diminishes IP_3R expression • Increases IP_3R channel activity • Causes an overall decrease in the percentage of ER Ca^{2+} released
	Mitochondria	• Mediates VDAC opening
	Cytosol	• N/A
Mcl-1	ER	• Reduces ER luminal Ca^{2+} • Binds IP_3R (type 1, 2, and 3) • Enhances IP_3R-mediated Ca^{2+} oscillations
	Mitochondria	• Inhibits Ca^{2+} uptake
	Cytosol	• N/A
HAX-1	ER	• Downregulates SERCA2 expression • Reduces ER luminal Ca^{2+} concentration
	Mitochondria	• N/A
	Nuclear envelope	• N/A
Pro-apoptotic		
Bax/Bak	ER	• Increases ER luminal Ca^{2+} concentration • Binds IP_3R (type 1)

Bcl-2 family protein	Localization	Functioning
	Mitochondria	• Mediates VDAC opening
Bid/tBid	Mitochondria	• Mediates VDAC opening
Puma	Mitochondria	• Induces cytochrome c release
	Cytosol	• Upregulation by ER Ca^{2+} depletion • Expression is necessary for ER stress-induced apoptosis • Activates caspases-3, 8 and 9
Bad	Mitochondria	• Activates caspase-3
	Cytosol	• N/A
Bik	ER	• Enhances ER Ca^{2+} efflux (Bax/Bak-dependent)
BNIP3	ER	• Enhances ER Ca^{2+} efflux
	Mitochondria	• Increases Ca^{2+} uptake • Dissipates membrane potential
Nix	ER	• Enhances ER Ca^{2+} efflux • Mediates PTP opening
	Mitochondria	• N/A