



Review

# Bcl-2 Family of Proteins in the Control of Mitochondrial Calcium Signalling: An Old Chap with New Roles

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**Abstract:** Bcl-2 family proteins are considered as one of the major regulators of apoptosis. Indeed, this family is known to control the mitochondrial outer membrane permeabilization (MOMP): a central step in the mitochondrial pathway of apoptosis. However, in recent years Bcl-2 family members began to emerge as a new class of intracellular calcium (Ca<sup>2+</sup>) regulators. At mitochondria-ER contacts (MERCs) these proteins are able to interact with major Ca<sup>2+</sup> transporters, thus controlling mitochondrial Ca<sup>2+</sup> homeostasis and downstream Ca<sup>2+</sup> signalling pathways. Beyond the regulation of cell survival, this Bcl-2-dependent control over the mitochondrial Ca<sup>2+</sup> dynamics has far-reaching consequences on the physiology of the cell. Here, we review how the Bcl-2 family of proteins mechanistically regulate mitochondrial Ca<sup>2+</sup> homeostasis and how this regulation orchestrates cell death/survival decisions as well as the non-apoptotic process of cell migration.

**Keywords:** Bcl-2 proteins; mitochondrial calcium homeostasis; VDAC; IP<sub>3</sub>R; apoptosis; cell migration



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## 1. Introduction

Apoptosis is a form of regulated cell death by which complex multicellular organisms orchestrate the regulated removal of unwanted or damaged cells. It is well established that apoptosis plays critical roles in development, tissue homeostasis and the response to cellular stress [1]. Aberrations in the apoptotic program contribute to the aetiology of a broad range of human pathologies including cancer and neurodegenerative diseases [2,3].

Mitochondria play a central role in apoptosis execution. Actually, these genuine intracellular powerhouses contain in their intermembrane space (IMS) several cytotoxic proteins including Omi, SMAC/Diablo, and cytochrome c [4–8]. Following cellular stress and apoptosis induction, the outer mitochondrial membrane (OMM) is permeabilized, leading to their release into the cytosol and subsequent activation of cysteine-aspartic proteases, called caspases [4].

This mitochondrial outer membrane permeabilization (MOMP) is under the tight control of the Bcl-2 family of proteins [9]. Initially discovered within the chromosomal translocations of follicular lymphomas, the Bcl-2 proteins (an acronym for B-cell lymphoma 2 gene) are considered as one of the main MOMP regulators [10–14]. These intracellular proteins possess one or up to four conserved sequences called Bcl-2 homology (BH) domains or motifs [14,15]. As MOMP regulators, they are divided into three groups: multidomain pro-apoptotic Bax-like, which have pore-forming activity and induce MOMP, multidomain anti-apoptotic Bcl-2-like, which bind to Bax-like thus repressing MOMP, and pro-apoptotic BH3-only proteins. Structurally, Bax-like and Bcl-2-like family members are related as they possess four BH motifs (BH1 to 4). The sequence spanning between BH1 to BH3 organizes into the canonical BH3-binding groove where a BH3 motif can bind. In

this regard, BH3-only proteins are considered pro-apoptotic as interaction between their BH3 motifs and the BH3-binding groove results in activation of Bax-like or repression of Bcl-2-like proteins, shifting the balance towards MOMP [14,16].

*Bcl2*-related genes are found only in multicellular animals and thus they are referred to as markers of multicellularity, evolutionarily selected to regulate apoptotic cell removal in development and sustain tissue homeostasis in metazoans [17,18]. This was first demonstrated in *C. elegans* in which the Bcl-2 homolog CED-9 was mutated. Loss-of-function of the *ced9* gene resulted in widespread death of embryonic cells [19,20]. Subsequent observations in knockout (KO) mice for *bcl2* homologs solidified their critical role in apoptosis regulation [21–23]. However, more recent experiments have demonstrated that Bcl-2 family members are actually multifunctional proteins involved in non-MOMP related processes [24–28]. Indeed, many Bcl-2-related proteins have a C-terminal hydrophobic transmembrane (TM) motif allowing them to be anchored not only to mitochondria but also to the endoplasmic reticulum (ER) [29–33]. At the level of these internal membranes, Bcl-2 family members dynamically control the exchange of  $\text{Ca}^{2+}$  ions [34–37].

$\text{Ca}^{2+}$  ions are important secondary messengers participating in many cellular functions [38].  $\text{Ca}^{2+}$  is able to enhance mitochondrial bioenergetics by promoting the activities of pyruvate dehydrogenase, isocitrate dehydrogenase and  $\alpha$ -ketoglutarate dehydrogenase [39]. In contrast, mitochondrial  $\text{Ca}^{2+}$  is also required for the efficient execution of apoptosis, while  $\text{Ca}^{2+}$  overload induces the opening of the elusive mitochondrial permeability transition pore (mPTP), leading to necrotic cell death [40,41]. The role of  $\text{Ca}^{2+}$  in the balance between life and death underlies the need for tight regulation of mitochondrial  $\text{Ca}^{2+}$  pools. Bcl-2 family of proteins participates in this process through direct interactions with various intracellular  $\text{Ca}^{2+}$  transporters or channels, which have profound consequences for mitochondrial  $\text{Ca}^{2+}$  homeostasis and downstream  $\text{Ca}^{2+}$  signalling pathways. Here, we review the role of Bcl-2 proteins in mitochondrial  $\text{Ca}^{2+}$  homeostasis and how this regulation orchestrates not only survival/death decisions but also non-apoptotic processes like cell migration.

## 2. Mitochondria-ER Contacts (MERCs): A Signalling Platform for Mitochondrial $\text{Ca}^{2+}$ Homeostasis

Mitochondria are dynamic intracellular organelles that can store and exchange with the surrounding environment substantial amounts of  $\text{Ca}^{2+}$  ions [42]. As mitochondria are encompassed by a double membrane,  $\text{Ca}^{2+}$  is required to cross both layers in order to reach the matrix. The inner mitochondrial membrane (IMM) mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU) has a low affinity for  $\text{Ca}^{2+}$ , with a  $K_d$  of 10  $\mu\text{M}$  [43]. This therefore means that mitochondria are unable to uptake  $\text{Ca}^{2+}$  directly from the cytosol, but rather require the direct transfer of  $\text{Ca}^{2+}$  from other stores through membrane contact sites (MCS), in order to be able to maintain their  $\text{Ca}^{2+}$  pools [44]. While lysosomes [45] can directly transfer  $\text{Ca}^{2+}$  to mitochondria, the most understood pathway is the transfer of  $\text{Ca}^{2+}$  from the ER to mitochondria at mitochondria-ER contacts (MERCs) [46]. Mitochondria engage in MCS with the ER forming specialized structures known as MERCs or mitochondria-associated ER membranes (MAMs). Around 20% of the mitochondrial surface is involved in ER contacts, with average inter-organelle distances ranging around 10 to 50 nm [47,48]. MERCs are formed and stabilized by tethering proteins, such as Mitofusin-2 (MFN2) [49] or PDZ domain-containing 8 (PDZD8) [50] in mammals regulating the optimal distance between both organelles. This mitochondria-ER interface is essential for several other processes including the synthesis and exchange of lipids, autophagosome formation and mitochondrial dynamics, thus providing a signalling platform to coordinate cell fate [51–53].

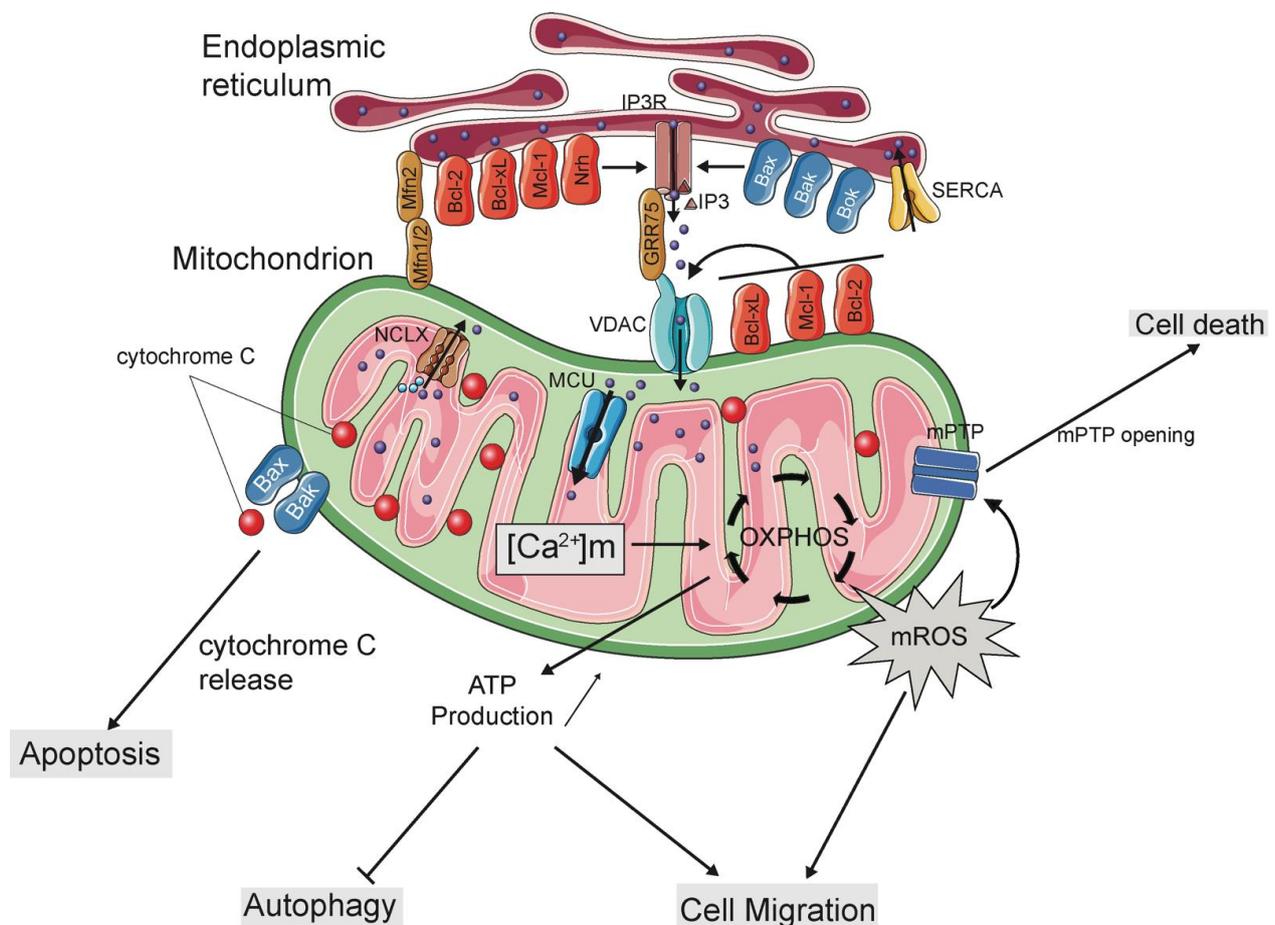
At the MERCs, a specialized subdomain exists to enable the efficient ER to mitochondria  $\text{Ca}^{2+}$  transfer. The ER-localized inositol 1,4,5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ) and OMM-localized voltage-dependent anion channel (VDAC) are bridged by the glucose regulated protein-75 (GRP75), forming a tethering complex between both organelles [54,55]. Upon stimulation, the natural ligand  $\text{IP}_3$  binds to  $\text{IP}_3\text{R}$  leading to opening of the channel

and subsequent  $\text{Ca}^{2+}$  release into cytosol and mitochondria, through VDAC [54] (Figure 1).  $\text{Ca}^{2+}$  transfer at the MERCs generates a high local concentration of  $\text{Ca}^{2+}$ , called  $\text{Ca}^{2+}$  microdomains, enabling the MCU complex to uptake  $\text{Ca}^{2+}$  into the mitochondrial matrix [43]. The formation of this functional  $\text{Ca}^{2+}$  signalling platform at MERCs organizes all of the appropriate machinery required to efficiently transfer  $\text{Ca}^{2+}$  from the ER to mitochondria. The high local concentration of  $\text{Ca}^{2+}$  generated at this interface enables the IMM- and MERCs-localized MCU [56] to allow the entry of  $\text{Ca}^{2+}$  into the matrix. The spatial organization and coordination of the ER-, OMM- and IMM-localized  $\text{Ca}^{2+}$  channels/receptors are therefore crucial in order to efficiently uptake  $\text{Ca}^{2+}$ .

Interestingly, biochemical subcellular fractionation studies have shown the presence of the anti-apoptotic proteins Bcl-2 and Bcl-xL at MERCs at steady state [57], with the recruitment of Bcl-2 to this specific interface, mediated by TOM20, being enhanced upon apoptotic stimulations [58]. In addition, microscopy analyses revealed that the apoptosis accelerator Bax is recruited to MERCs during tBid-induced apoptosis [59]. Upon mild stress induced by thapsigargin in Chinese hamster ovary (CHO) cells, Bcl-xL can also translocate specifically to MERCs promoting the increase of mitochondrial  $\text{Ca}^{2+}$  by regulating  $\text{IP}_3\text{R}$ -induced ER- $\text{Ca}^{2+}$  release and cellular bioenergetics [60].

While the subcellular localization of Bcl-2 proteins at this ER-mitochondria interface allows direct interaction with components of the  $\text{Ca}^{2+}$  homeostasis machinery, there is little evidence showing that Bcl-2 proteins can directly regulate ER and mitochondria membranes apposition. Recently, it has been shown that the pro-apoptotic member Bok was localized at MERCs where it controls the optimal distance between the two membranes for an efficient ER to mitochondria  $\text{Ca}^{2+}$  transfer to control cell death [61]. These results are in accordance with recent evidence indicating that overexpression of both Mcl-1 and Bok TMs leads to an increase of MERCs number and cell death [32]. An interaction between Bcl-2 and Bcl-xL with GRP75 was also identified, and it may be plausible that this interaction could regulate MERCs by controlling the  $\text{IP}_3\text{R}$ -GRP75-VDAC tethering complex [62]. Together, these data suggest that Bcl-2 proteins are not only localized to MERCs but could directly regulate them to sustain efficient ER to mitochondria  $\text{Ca}^{2+}$  transfer (Figure 1).

In the next sections, we will describe how Bcl-2 proteins regulate  $\text{Ca}^{2+}$  transients at the ER and mitochondrial interface to promote the uptake of mitochondrial  $\text{Ca}^{2+}$  required for cell death and the complex process of cell migration.



**Figure 1.** Schematic representation of ER to mitochondria  $\text{Ca}^{2+}$  regulation by Bcl-2 proteins. Members of the Bcl-2 family including pro- and anti-apoptotic proteins are found at the mitochondria-endoplasmic reticulum contacts (MERCs). At this interface, they control mitochondrial  $\text{Ca}^{2+}$  trafficking via the interaction with ER- and mitochondria-localized  $\text{Ca}^{2+}$  channels and transporters, which has an important implication in mitochondrial  $\text{Ca}^{2+}$ -dependent processes. Through mitochondrial  $\text{Ca}^{2+}$  pools regulation, Bcl-2 proteins control bioenergetics, ATP production and reactive oxygen species (ROS), thus influencing cell fate decisions including apoptosis, cell survival and cell migration. At the ER, the anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1 and Nrh interact with IP<sub>3</sub>R to decrease ER- $\text{Ca}^{2+}$  release into mitochondria to sustain mitochondrial bioenergetics and to protect from  $\text{Ca}^{2+}$ -induced cell death. At the mitochondria, Bcl-2, Bcl-xL and Mcl-1 interact with VDACs to promote or inhibit mitochondrial  $\text{Ca}^{2+}$  uptake, depending on cell types and the cellular metabolic state. In contrast, the pro-apoptotic members Bax and Bax can also localize to the ER where they promote ER- $\text{Ca}^{2+}$  release and cell death. Recently, ER-localized Bok has been shown to directly regulate MERCs number and to interact with IP<sub>3</sub>R promoting ER- $\text{Ca}^{2+}$  release and mitochondrial  $\text{Ca}^{2+}$  uptake required for cell death.

### 3. Regulation of Mitochondrial $\text{Ca}^{2+}$ Uptake Machinery by Bcl-2 Family Proteins

The OMM is highly permeable to ions and low molecular weight molecules, due to the presence of VDACs, whereas the IMM-localized MCU complex enables  $\text{Ca}^{2+}$  uptake into the matrix [63–65]. Three VDAC isoforms are found in vertebrates (VDAC1–3), representing the most abundant proteins of the OMM [66]. They can adopt two conformational stages: an open state, observed at low membrane potential (–10 mV to +10 mV), which is permeable for cations and small anionic metabolites, and a closed state at high mitochondrial membrane potential exhibiting only cation permeability [67]. All three VDAC isoforms are able to transfer  $\text{Ca}^{2+}$  ions through the OMM, however, functional implications differ. For instance, VDAC1 allows the passage of the low-amplitude apoptotic  $\text{Ca}^{2+}$  signals following IP<sub>3</sub>R stimulation [68], whereas VDAC2 is involved in transfer of  $\text{Ca}^{2+}$  from sarcoplasmic reticulum (SR) and in the rhythmicity of cardiomyocytes [69].

Bcl-2 family members are mainly OMM-resident proteins, so they exert a control over mitochondrial  $\text{Ca}^{2+}$  uptake mainly through the control of VDACs permeability (Figure 1), however this regulation is still a matter of debate. The first evidence for the implication of a Bcl-2 family member in VDAC permeability came from Craig Thompson's lab in the late 90s. Vander Heiden and collaborators demonstrated that following growth factor deprivation, cells overexpressing Bcl-xL survive by sustaining ATP/ADP exchanges in the mitochondria, suggesting that Bcl-xL maintains VDAC in an open state [36,70]. Supporting this model, the dephosphorylation of the BH3-only protein Bad, which causes its translocation to the OMM, disrupts the interaction between Bcl-xL and VDAC leading to mitochondrial  $\text{Ca}^{2+}$  overload [71]. However, using liposomes embedded with purified VDAC proteins, Shimizu and colleagues demonstrated that Bcl-xL binds to and inhibits VDAC opening [72]. Interestingly, in this latter experimental system pro-apoptotic Bax and Bak have the opposite effect and lead to VDAC opening [72].

More recently, the team of Chi Li demonstrated that *bclx* KO mouse embryonic fibroblasts (MEFs) uptake less  $\text{Ca}^{2+}$  into the mitochondria compared to control cells [73]. Notably, mitochondrial  $\text{Ca}^{2+}$  uptake was restored when KO MEFs were complemented with exogenous Bcl-xL targeted to the mitochondria but not to the ER. It has then been proposed that Bcl-xL promotes mitochondrial  $\text{Ca}^{2+}$  entry via direct interaction with the VDAC1 and VDAC3 channels [74], and that its N-terminal BH4 motif was required for this interaction and therefore  $\text{Ca}^{2+}$  regulation [57]. In this respect, a peptide corresponding to the BH4 motif of Bcl-xL reduces agonist-induced mitochondrial  $\text{Ca}^{2+}$  uptake and protects cells from apoptosis [57,75].

Actually, within the Bcl-2 family, VDACs participate in functional interactions with Bcl-2 and Mcl-1 as well. Several studies have shown that Bcl-2 interacts with VDAC1 N-terminal  $\alpha$ -helix, thereby leading to a reduction in mitochondrial  $\text{Ca}^{2+}$  uptake [76]. This probably also requires the BH4 motif of Bcl-2 because peptides corresponding to this region close VDAC and suppress pro-apoptotic stimuli [77]. In contrast, overexpression of Bcl-2 in neurons and myotubes has opposite effects, leading to an increase of mitochondrial  $\text{Ca}^{2+}$  [78,79]. Finally, Mcl-1 has also been shown to directly interact with VDACs to promote mitochondrial  $\text{Ca}^{2+}$  uptake and bioenergetics in a non-small cell carcinoma cell line [80].

The discrepancies regarding mitochondrial  $\text{Ca}^{2+}$  trafficking highlight the complex interactions between anti-apoptotic Bcl-2 proteins and VDACs. Due to their role in cell survival and death, it can be hypothesized that under physiological conditions, anti-apoptotic Bcl-2 members enhance mitochondrial  $\text{Ca}^{2+}$  uptake to regulate mitochondrial metabolism and bioenergetics, whereas upon apoptotic stimulations they protect mitochondria from deleterious massive  $\text{Ca}^{2+}$  overload by interacting with VDACs. This hypothesis is notably supported by findings in the heart of transgenic mice describing that Bcl-2 decreases mitochondrial  $\text{Ca}^{2+}$  efflux via the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger, NCLX, to maintain mitochondrial ATP production [81].

#### 4. Remote Control of Mitochondrial $\text{Ca}^{2+}$ Signalling by ER-Based Bcl-2 Proteins

The ER is the major storage organelle for cellular  $\text{Ca}^{2+}$ . ER-dependent  $\text{Ca}^{2+}$  release controls basal cytosolic  $\text{Ca}^{2+}$  levels and mitochondrial  $\text{Ca}^{2+}$  uptake through the direct transfer of  $\text{Ca}^{2+}$  ions at MERCs [42,48,82]. At the level of the ER, this occurs via the release of  $\text{Ca}^{2+}$  through ER- $\text{Ca}^{2+}$  channels.  $\text{IP}_3$ Rs and ryanodine receptors (RyRs) are the two major families of ER  $\text{Ca}^{2+}$  channels [83]. In vertebrates, there are three  $\text{IP}_3$ R isoforms ( $\text{IP}_3$ R1-3), which are often co-expressed in most mammalian cell types. The three isoforms differ in their affinity for the  $\text{IP}_3$  ligand;  $\text{IP}_3$ R2 exhibiting the highest sensitivity while  $\text{IP}_3$ R3 has the weakest [84]. Interestingly,  $\text{IP}_3$ R2 isoform is the most effective in delivering  $\text{Ca}^{2+}$  to the mitochondria [55].  $\text{IP}_3$ , the natural ligand for  $\text{IP}_3$ R, is produced upon G-protein coupled receptor (GPCR) activation by ligands such as histamine or ATP at the plasma membrane. GPCR activation leads to hydrolysis of phosphatidyl inositol-4,5-bisphosphate ( $\text{PIP}_2$ ) by phospholipase C resulting in the production of  $\text{IP}_3$ .  $\text{IP}_3$  then diffuses through the cell and

binds to the IP<sub>3</sub>-binding domain of IP<sub>3</sub>R oligomers resulting in the opening of the Ca<sup>2+</sup> channel, which subsequently allows Ca<sup>2+</sup> flux into the cytosol and mitochondria [85,86].

Actually, many anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-xL, Mcl-1 and Nrh, possess dual mitochondrial and ER localizations and are able to interact with IP<sub>3</sub>R [34,35,87] (Figure 1). Although these interactions regulate IP<sub>3</sub>R-dependent Ca<sup>2+</sup> release, the binding sites and the functional consequences of this regulation are different. For instance, Bcl-2 is able to bind to the central modulatory and transducing domain II (MTD II) of IP<sub>3</sub>R, which requires the N-terminal BH4 motif of Bcl-2 [34,88]. This interaction lowers Ca<sup>2+</sup> release from the ER and inhibits the transfer of toxic Ca<sup>2+</sup> insults to the mitochondria. Conversely, Bcl-xL interacts with IP<sub>3</sub>R through its BH3-binding groove [89]. Indeed, Yang and colleagues identified two new BH3-like helices in the IP<sub>3</sub>R C-terminus that are able to bind to Bcl-xL with high affinity. This interaction leads to IP<sub>3</sub>R opening and subsequent ER-Ca<sup>2+</sup> release. Interestingly, the mode of action of Bcl-xL appears to be concentration-dependent because increasing Bcl-xL levels lead to a secondary IP<sub>3</sub>R inhibition, which occurs through the binding of Bcl-xL at the Bcl-2 interaction site in the MTDII domain [89]. Thus, the regulation of IP<sub>3</sub>R by Bcl-xL seems to be biphasic. At low levels, ER-based Bcl-xL favors the release of Ca<sup>2+</sup> ions from IP<sub>3</sub>R and transfer to the mitochondria thus enhancing mitochondrial bioenergetics by activating the Ca<sup>2+</sup>-dependent dehydrogenases of the Krebs cycle. In contrast, at high protein concentration levels, Bcl-xL inhibits IP<sub>3</sub>R-dependent Ca<sup>2+</sup> release and subsequent apoptosis initiation [89,90]. Of note, as described previously, the BH4 motif of Bcl-xL does not interact with IP<sub>3</sub>R but preferentially binds to VDAC1, controlling its permeability [57]. The difference between Bcl-2 and Bcl-xL BH4 motifs can be explained by subtle differences in their respective amino acid compositions. Indeed, in the BH4 motif of Bcl-2 a lysine residue at position 17 (Lys17) is critical for its interaction with IP<sub>3</sub>R. The corresponding residue in Bcl-xL is an aspartate at position 11 (Asp11). Mutating Lys17 into Asp in Bcl-2 leads to complete loss of IP<sub>3</sub>R binding capacity, whereas changing of Asp11 into Lys in BH4 of Bcl-xL converts Bcl-xL into an IP<sub>3</sub>R binder and inhibitor [57].

Mcl-1 is another IP<sub>3</sub>R interactor shown to control mitochondrial Ca<sup>2+</sup> uptake. Actually, Mcl-1 and Bcl-xL seem to behave in a similar manner. Both proteins bind with comparable affinities to the C-terminus of all three IP<sub>3</sub>R isoforms suggesting that Mcl-1, like Bcl-xL, requires its BH3-binding groove to interact with IP<sub>3</sub>R channels [91]. In addition, the BH4 motif of Mcl-1 has a pronounced tropism for the OMM, where it inhibits mitochondrial Ca<sup>2+</sup> signalling [92].

An outsider of this Bcl-2-IP<sub>3</sub>R interaction group is the Bcl-2 homolog Nrh (also referred to as Bcl-B or BCL2L10). In breast cancer (BC) cells, Nrh is exclusively found at the ER where it is able to interact with the N-terminal IP<sub>3</sub> binding domain of the IP<sub>3</sub>R1 via its BH4 motif [87]. This interaction prevents IP<sub>3</sub>R1 opening, which in turn dampens the unfolded protein response (UPR). Actually, the UPR is an adaptive reaction that prevents the accumulation of misfolded proteins in the ER lumen to maintain cell viability. If stressful conditions persist, the UPR can prime cells for cell death through the activation of the BH3-only protein Bim [93,94]. The UPR is often suppressed in tumor cells in order to promote protein synthesis and cell survival. In this regard, Nrh expression in BC cells inhibits the UPR and induces drug resistance, whereas Nrh silencing makes BC cells more sensitive to drugs currently used in chemotherapy [87]. Interestingly, at MERCs, Nrh and IRBIT, another IP<sub>3</sub> binding domain protein, exert an additive inhibitory effect over IP<sub>3</sub>R at resting states [95]. However, upon apoptotic stress, IRBIT is dephosphorylated, thus inhibiting Nrh and leading to Ca<sup>2+</sup> accumulation in the mitochondria and subsequent apoptosis [95].

Finally, Bcl-2 proteins have also been proposed to interact with other ER-Ca<sup>2+</sup> channels [96]. Both Bcl-2 and Bcl-xL can interact with the ryanodine receptor (Ryr) via their BH4 domains and decrease their activity [97,98]. Indeed, overexpression of Bcl-xL inhibits caffeine-induced Ryr-dependent Ca<sup>2+</sup> release into the mitochondria [98]. Together, by direct interaction with ER-Ca<sup>2+</sup> channels, Bcl-2 proteins tightly control ER to mitochondria Ca<sup>2+</sup> transfer required for cell fate decisions.

## 5. Bridging the Gap between Mitochondria and ER during Cell Death and Survival

Mitochondrial  $\text{Ca}^{2+}$  plays a pivotal role in the balance between cell survival and cell death events [99]. While a minimal amount of  $\text{Ca}^{2+}$  is required to maintain mitochondrial bioenergetics and metabolism, larger and toxic mitochondrial  $\text{Ca}^{2+}$  levels have been proposed to facilitate apoptosis [100] and to trigger mPTP opening [41]. As already described, the anti-apoptotic Bcl-2 proteins are key executioners regarding the control of mitochondrial  $\text{Ca}^{2+}$  homeostasis as well as the cell death and survival balance. During apoptosis, the number of mitochondria-ER contact sites increases [59,101], fostering mitochondrial  $\text{Ca}^{2+}$  uptake [59,95], which has been associated with IMM remodelling and OPA1-dependent cristae reorganization, thus facilitating cytochrome c release and apoptosis [59]. Mitochondrial  $\text{Ca}^{2+}$  overload may also lead to IMM cardiolipin oxidation, increasing ROS production and mPTP opening [102]. Due to their roles in ER to mitochondria  $\text{Ca}^{2+}$  fluxes described above, anti-apoptotic Bcl-2 proteins have been widely shown to inhibit apoptosis by decreasing ER-induced  $\text{Ca}^{2+}$  release or decreasing VDAC1-dependent  $\text{Ca}^{2+}$  uptake [103] (Figure 1). In recent years, different peptides derived from their BH4 domain have been developed and their effects have been characterized in different cancer cell models [104]. Such peptides are able to disrupt the interactions between  $\text{IP}_3\text{R}$  and several Bcl-2 proteins and impact on the apoptotic  $\text{Ca}^{2+}$  signals transfer to the mitochondria. For instance, a BH4-domain-targeting peptide of Bcl-2, called Bcl-2/ $\text{IP}_3$  receptor disrupter-2 (BIRD-2), has been shown to have cell death-inducing effects in different cancer cell lines [34,105–108]. Interestingly, such cell death has been shown to depend on ER-induced mitochondrial  $\text{Ca}^{2+}$  overload and caspase activation [109].

While the role of the pro-apoptotic proteins in basal mitochondrial  $\text{Ca}^{2+}$  homeostasis has been less described, there is evidence supporting their contribution to the  $\text{Ca}^{2+}$ -dependent apoptotic process (Figure 1). Bok is the only multidomain pro-apoptotic member which has been shown to interact with the  $\text{IP}_3\text{R}$  coupling domain of both  $\text{IP}_3\text{R1}$  and  $\text{IP}_3\text{R2}$  via its BH4 domain [110,111]. This interaction has been initially reported to protect both  $\text{IP}_3\text{Rs}$  and unbound Bok from proteolysis and proteasomal-dependent degradation, respectively, and to control mitochondrial morphology [112]; however, no ER or mitochondrial  $\text{Ca}^{2+}$  defects were observed in these KO cell lines. Interestingly, a study has recently shown that KO of Bok resulted in a deregulation of intracellular  $\text{Ca}^{2+}$  signalling [61]. Indeed, these Bok KO MEFs harbored a reduction of  $\text{Ca}^{2+}$  transfer from ER to mitochondria and of apoptosis [61]. This study also showed that Bok-KO induces a decrease of MERCs number observed by microscopy, and a mislocalization and decrease of MERCs-resident proteins [61], suggesting that Bok can directly control MERCs to maintain mitochondrial  $\text{Ca}^{2+}$  pools and sustain cell viability. Rescue experiments with a Bok mutant unable to interact with  $\text{IP}_3\text{R}$  was shown to rescue the MERCs defect but not the mitochondrial  $\text{Ca}^{2+}$  phenotype [61]. Interestingly, restoring MERCs by an artificial tether, was insufficient to rescue the  $\text{Ca}^{2+}$  defects induced by Bok loss [61]. These data suggest a specific and mutually exclusive role of Bok in controlling  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  release and MERCs number.

Although no direct interaction with ER-localized  $\text{Ca}^{2+}$  channels/receptors have been reported, Bax and Bak can also localize to the ER where they control  $\text{Ca}^{2+}$ -dependent apoptosis [113–115]. Indeed, overexpression of Bax and Bak leads to an increase of ER- $\text{Ca}^{2+}$  release and mitochondrial  $\text{Ca}^{2+}$  levels accompanied by cytochrome c release and cell death [113], suggesting that Bax/Bak at the ER can control ER to mitochondria  $\text{Ca}^{2+}$  fluxes. In addition, Bax/Bak DKO MEFs have reduced ER- $\text{Ca}^{2+}$  content, leading to decreased mitochondrial  $\text{Ca}^{2+}$  uptake and apoptosis upon ER- $\text{Ca}^{2+}$  stimulation [114]. Importantly, re-expression of SERCA or ER-targeted Bax/Bak was able to restore ER- $\text{Ca}^{2+}$  content and efficient apoptosis, indicating that Bax and Bak directly control ER  $\text{Ca}^{2+}$  concentration [114]. Mechanistically, it has been proposed that this increased ER  $\text{Ca}^{2+}$  leak was associated to an increase of Bcl-2- $\text{IP}_3\text{R1}$  interaction and protein kinase A-dependent  $\text{IP}_3\text{R1}$  phosphorylation in Bax and Bak DKO cells [116]. Other studies have confirmed the contribution of Bax and Bak regarding ER-induced  $\text{Ca}^{2+}$  release and cell death regulation following different cellular stresses [115,117]. Alternatively, reports have shown that Bax and Bak are able

to permeabilize the ER membrane leading to the release of the ER lumen contents to the cytosol [118,119]. Indeed, the oligomerization of Bax and Bak on the ER membrane could lead to the formation of pores, similar to mitochondrial [120,121] and peroxisomal [122] pore formations, which could potentially allow the passage of  $\text{Ca}^{2+}$  in the cytosol during apoptosis. Finally, BH3 only proteins [123,124], including Bik [125], can also control Bax/Bak-dependent ER- $\text{Ca}^{2+}$  release to enhance mitochondrial  $\text{Ca}^{2+}$  uptake and cell death. In hyperplastic cells, not only Bik disrupts the Bcl-2-IP<sub>3</sub>R complex to promote ER- $\text{Ca}^{2+}$  release, but it can also activate and translocate Bak to the ER to form a complex with DAPK1 leading to an increase of MERCs and mitochondrial  $\text{Ca}^{2+}$  uptake, subsequently leading to cell death [126].

The complex regulation of  $\text{Ca}^{2+}$  by Bcl-2 proteins reflects the critical and opposing functions of  $\text{Ca}^{2+}$  about life and death decisions. Therefore, several modes of regulation must exist to tightly control mitochondrial  $\text{Ca}^{2+}$  levels, depending on environmental conditions.

## 6. Role of Bcl-2 Family Proteins in $\text{Ca}^{2+}$ -Dependent Cell Migration

Intracellular  $\text{Ca}^{2+}$  dynamics regulates many cellular processes including cytoskeleton remodelling and cell migration [37]. Most of these regulations occur by modifying the cytosolic  $\text{Ca}^{2+}$  signals, which has been reviewed extensively elsewhere [127,128]. The significance of Bcl-2 family proteins in cell migration and invasion during embryonic development and cancer progression, however, has only recently emerged.

Actually, the first evidence came from experiments conducted in the zebrafish model. In this vertebrate, a highly divergent Bcl-2 homolog, called Bcl-wav (acronym for Bcl-2 homolog found in water-living anamniote vertebrates) was identified [28]. Bcl-wav is a mitochondrial resident pro-apoptotic Bcl-2 homolog, the knockdown of which affects convergence and extension (C&E) movements during zebrafish embryogenesis [28]. C&E movements are critical for the establishment of the anterior-posterior and dorsoventral embryonic axes. Bcl-wav orchestrates these morphogenic movements through the control of intracellular  $\text{Ca}^{2+}$  trafficking. Indeed, *bclwav* knockdown was correlated with a decrease in mitochondrial  $\text{Ca}^{2+}$  levels and concomitant increase of cytosolic  $\text{Ca}^{2+}$  levels [28]. At the level of the mitochondria, Bcl-wav interacts with VDAC1 via its BH4 motif and enhances mitochondrial  $\text{Ca}^{2+}$  uptake thus controlling the kinetics of actin polymerization and blastomeres migration. Interestingly, C&E movements seem to be strongly depended on mitochondrial  $\text{Ca}^{2+}$  uptake since knockdown of *mcu* resulted in a similar phenotype [28].

The importance of the MCU-dependent  $\text{Ca}^{2+}$  transport was further emphasized in the motility of cancer cells [129,130]. Indeed, *mcu*-silencing in highly invasive triple-negative breast cancer (TNBC) cell lines resulted in altered F-actin cytoskeleton dynamics, cell polarization loss and impairment of the focal adhesion proteins dynamics [129]. These processes are mediated by the reduction of  $\text{Ca}^{2+}$ -dependent Calpain and Rho-GTPases activities [129]. In addition, Tosatto and collaborators showed that the knockdown of *mcu* also resulted in decreased cell motility and invasiveness as well as reduction of tumor growth [130]. However, they linked this phenotype to mitochondrial ROS (mtROS) production and downregulation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) [130]. This suggests that mitochondrial  $\text{Ca}^{2+}$  uptake could probably control multiple downstream signalling pathways. High mtROS production is detrimental for cell survival, however, in cancer cells sub-lethal mtROS levels promote cell proliferation, migration and invasion [131,132]. In this respect, several studies have demonstrated that Bcl-2 family members control cancer cell motility via mtROS production, independently of their role in apoptosis [80,133,134]. For instance, Mcl-1 was proposed to promote migration in non-small cell lung carcinoma through its interaction with VDAC1 and 3 and its capacity to control mitochondrial  $\text{Ca}^{2+}$  homeostasis [80]. Indeed, *mcl1*-silencing or treatment with peptides that suppress VDAC-based  $\text{Ca}^{2+}$  uptake led to reduced mtROS generation. Bcl-xL and Bcl-2 were also shown to act as accelerators of cell motility, invasiveness and metastasis spreading. As it is the case for Mcl-1, mitochondrion-localized Bcl-xL, but not ER-based Bcl-xL, contributes to cell migration

through the generation of reactive mtROS [133]. At the level of the mitochondria, Bcl-xL binds to VDAC1 via its BH4 motif thus promoting  $\text{Ca}^{2+}$  entry and mtROS production. Interestingly, one study linked this regulation with the effect of metalloprotease-processed CD95L (cl-CD95L) on TNBC accelerated metastatic dissemination and poor patient prognosis [134]. Actually, CD95-mediates  $\text{Ca}^{2+}$  release from the ER to mitochondria at MERCs. In this particular case, mitochondria-targeted Bcl-xL and ER-targeted Bcl-2 were proposed to increase  $\text{Ca}^{2+}$  transfer between the ER and the mitochondria, thus accelerating ATP production and mtROS generation [134]. Interestingly in this case, the use of BH3-mimetics was sufficient to decrease cell migration suggesting that these molecules may be useful not only to kill tumor cells but also to prevent metastatic dissemination [134].

## 7. Conclusions

The role of Bcl-2 family of proteins in the initiation of apoptosis has been well studied, which has led to our current understanding of how cells integrate stress signals at the level of the mitochondria, leading to initiation of the death program. The role of  $\text{Ca}^{2+}$  in mediating cell death decisions has also been emphasized, but recent evidence support additional functions for mitochondrial  $\text{Ca}^{2+}$  on top of mitochondrial bioenergetics and cell death. With their capacity to be localized at the mitochondria-ER interface and to interact with key channels or receptors on both ER and mitochondrial membranes, Bcl-2 proteins have emerged as key regulators of intracellular and mitochondrial  $\text{Ca}^{2+}$  homeostasis, and subsequently to several other processes such as cell migration. Due to this connection, numerous studies are currently directly targeting Bcl-2-IP<sub>3</sub>R or Bcl-2-VDAC interactions to modulate  $\text{Ca}^{2+}$  signalling and to control cell fate in different types of cancer cell models. Together, future studies identifying precisely how mitochondrial  $\text{Ca}^{2+}$  is regulated by Bcl-2 proteins may identify new strategies for therapeutic intervention.

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