

## Review

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# Bcl-2 family proteins: master regulators of cell survival

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**Abstract:** The most prominent function of proteins of the Bcl-2 family is regulation of the initiation of intrinsic (mitochondrial) pathways of apoptosis. However, recent research has revealed that in addition to regulation of mitochondrial apoptosis, proteins of the Bcl-2 family play important roles in regulating other cellular pathways with a strong impact on cell survival like autophagy, endoplasmic reticulum (ER) stress response, intracellular calcium dynamics, cell cycle progression, mitochondrial dynamics and energy metabolism. This review summarizes the recent knowledge about functions of Bcl-2 family proteins that are related to cell survival.

**Keywords:** Bcl-2 family proteins; calcium; cell survival; endoplasmic reticulum; mitochondria.

## Introduction

The most prominent function of proteins of the B-cell lymphoma 2 (Bcl-2) family is regulating the initiation of intrinsic (mitochondrial) pathways of apoptosis (1, 2). This is referred to as a canonical function of Bcl-2 family proteins. However, recent research has revealed that proteins of the Bcl-2 family also play important roles in the regulation of other intracellular pathways with a strong impact on cell survival like autophagy (3, 4), endoplasmic reticulum (ER) stress response (5–7), intracellular calcium dynamics (8–11), mitochondrial dynamics and energy metabolism (12), and cell cycle progression (3, 13).

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The first protein of the Bcl-2 family, Bcl-2, was identified almost 30 years ago as a product of the previously unknown gene *BCL2*, which was cloned from the breakpoint region of t(14;18) chromosomal translocation associated with human follicular lymphoma (14). To date, 25 members of the Bcl-2 family have been identified that are classified into three subfamilies based on the presence of conserved Bcl-2 homology (BH) domains and functions playing in mitochondrial apoptosis (1, 2) despite their involvement in other intracellular processes.

The anti-apoptotic subfamily includes Bcl-2, B-cell lymphoma-extra large (Bcl-XL), Bcl-W, myeloid cell leukemia 1 (Mcl-1), Burkholderia lethal factor 1 (BLF1)/A-1 and Bcl-B proteins that suppress apoptosis and contain four BH domains, designated as 1–4. Each anti-apoptotic Bcl-2 protein has a similar overall helical fold localized on a core hydrophobic helix with the BH1-3 domains arranged to expose a hydrophobic groove that is required for both their pro-survival activity and the binding of their pro-apoptotic partners.

Pro-apoptotic proteins, such as Bcl-2-like protein 4 (BAX), Bcl-2 homologous antagonist/killer (BAK), and Bcl-2 related ovarian killer (BOK), contain BH 1–3 domains and are termed ‘multi-domain proteins’, whereas other pro-apoptotic proteins, such as Bcl-2-like protein 11 (BIM), Bcl-2-associated death promoter (BAD), BH3 interacting domain death agonist (BID), p53 upregulated modulator of apoptosis (PUMA), Bcl-2 modifying factor (BMF), Harkiri, Bcl-2 interacting protein (HRK) and NADPH oxidase activator 1 (Noxa), are termed ‘BH3-only’ proteins as they contain only BH3 domain, which is the most important mediator of the interaction with anti-apoptotic proteins. In some reviews, Bcl-2 19-kDa interacting protein 3 (BNIP3) (14, 15) and BNIP3 like protein (BNIP3L) (14) are also involved among the members of BH3-only proteins based on limited sequence homology to the BH3 domain.

The proteins of the Bcl-2 family are located in cytoplasm and outer mitochondrial membrane (OMM) (16) while Bcl-2 interacting killer (BIK) is localized predominantly in ER membrane (17). The intracellular localization of Bcl-2 family proteins exhibits significant dynamics.

In viable cells, BAK resides on the mitochondria while BAX is primarily cytosolic, and different types of cytotoxic signals promote the accumulation of BAX on the mitochondria. Recent evidence indicates that BAX regularly cycles to the OMM in viable cells but is then translocated back, possibly by interactions with pro-survival proteins such as Bcl-XL. The mitochondrial enrichment of BAX during apoptosis may reflect either the activation of BAX in the cytosol, probably by a BH3-only protein, or an inhibition of retro-translocation of BAX from the mitochondria (18). In the cells of rat hippocampus, BAX has been localized mainly in mitochondria, and apoptosis initiation was supposed to be a result of translocation of BH3-only protein BAD to mitochondria (19). In fact, the BH3-only proteins appear to play an essential role in the initiation of mitochondrial pathway of apoptosis. The precise mechanism by which BH3-only proteins initiate mitochondrial apoptosis is not completely clear but experimental results are consistent with three mutually non-exclusive models (2). In the direct activation model, ‘activator’ BH3-only proteins, the truncated form of BID (tBID), BIM and perhaps PUMA, directly activate BAX and BAK. In the displacement model, the ‘sensitizer’ BH3-only proteins (BAD, BIK, BMF, HRK and Noxa) bind by the BH3 domain into the hydrophobic groove of anti-apoptotic proteins inhibiting their anti-apoptotic function that leads to the indirect activation of BAX and/or BAK. The unified model assumes that the anti-apoptotic proteins sequester not only BH3-only proteins but also activated BAX and BAK. These interactions are reversible, and disruption of this complex equilibrium may result in induction of apoptosis or by contrast in regression of apoptosis (15). These previously accepted hierarchical models of canonical initiation of mitochondrial apoptosis (20) were recently upgraded by an interconnected hierarchical model (21). Activation of BAX and/or BAK and consequent formation of the pore in OMM are followed by the release of cytochrome c and other pro-apoptotic proteins, including apoptosis inducing factor (AIF), Smac/DIABLO, EndoG and Omi/HtrA2, from intermembrane space of mitochondria to cytosol (2). The next step involves apoptosome formation associated with activation of initiator caspase 9 leading to activation of executor caspase 3 and consequent apoptosis execution (1, 2). Release of AIF, EndoG and Omi/HtrA2 can initiate mitochondrial apoptosis that is independent from caspase 3. In addition to canonical initiation of pore formation, the non-canonical initiation of OMM permeability involves stabilization of either tumor suppressor p53 (22, 23) or BOK (24) and their consequent interaction with Bcl-2 family proteins. Finally, recent study has documented BOK-dependent release of

cytochrome c and consequent apoptosis initiation in the absence of BAX and BAK (25).

In this review, we will shortly discuss non-canonical functions of Bcl-2 family proteins in the regulation of intracellular processes with a strong impact on cell survival.

## Autophagy

In mammalian cells, autophagy was initially proposed to be a mechanism promoting cell survival in conditions of starvation, but it was later considered also as a mechanism by which cells can commit suicide (26). The roles for Bcl-2 family proteins in the regulation of autophagy have been less well-characterized and are still a matter of debate (4). Previous studies have indicated that members of Bcl-2 family can also regulate autophagy (28–31). The anti-apoptotic Bcl-2 proteins including Bcl-2 (32), Bcl-XL (33), Bcl-W (34), and Mcl-1 (35, 36) have been proposed to inhibit autophagy owing to their interaction with the autophagy regulator Beclin 1. Structural and mutational studies showed that Beclin 1 contains a functional BH3 motif that is involved in binding to anti-apoptotic proteins (37, 38). The finding that the BH3 motif of Beclin 1 can be inserted into the hydrophobic groove on Bcl-XL in a manner similar to other BH3 domains of pro-apoptotic proteins (37–39) raises the possibility of the involvement of other BH3-containing proteins in autophagy regulation. In fact, BH3-only proteins including BAD and BIK have been showed to induce autophagy (33, 40), and the involvement of BH3-only proteins in autophagy appears to be evolutionarily conserved (37). In addition, the small molecule BH3-mimetic ABT-737 that binds to Bcl-2 or Bcl-XL can stimulate autophagy (33). As Bcl-2 and other family members are mainly involved in mitochondrial apoptosis regulation, it has been suggested that only ER-localized Bcl-2, and not mitochondrial Bcl-2, negatively regulates Beclin 1-dependent autophagy (32). The early step of Bcl-2-dependent autophagy inhibition involves disruption of Beclin 1 interaction with Vps34, class III phosphatidylinositol 3-kinase, as generation of phosphatidylinositol 3-phosphate by the Beclin 1/Vps34 complex is thought to be important in mediating the localization of other autophagy proteins to pre-autophagosomal membranes (32). In accord with this model, the ER-localized protein nutrient-deprivation autophagy factor-1 enhances the interaction of Bcl-2 with Beclin 1 to suppress autophagy and further promotes this cell-survival process by binding and inhibiting BH3-only ER-localized protein BIK (40). In contrary, the Bcl-2 localized in mitochondria was also able

to inhibit autophagy by interacting with and sequestering the Beclin 1-binding, positive regulator of autophagy, Ambra1 (41). The recent model for the role of the Bcl-2 family in autophagy suggested that the anti-apoptotic proteins of the Bcl-2 family do not bind directly to Beclin 1 but instead regulate autophagy by inhibiting BAX/BAK mediated apoptosis, a process that activates autophagy by an unknown mechanism (4).

There are controversial findings about the involvement of pore forming BAX and BAK in autophagy. The increased rate of autophagy in the cells deficient in both BAX and BAK suggest that BAX/BAK directly or indirectly inhibits autophagy (42), while other study found that BAX expression induces mitochondrial autophagy (43). In healthy cells, BAX promotes mitochondrial fusion, which could slow mitophagy, but overexpressed BAX induces mitochondrial fission in apoptotic cells that produce small mitochondria destined for destruction (44). The opposite effect of BAX/BAK could be attributed to later downstream caspase cleavage of autophagy proteins (45, 46). Finally, the results of a recent study suggest that pro-apoptotic protein kinase Mst1 coordinately regulates autophagy and apoptosis by phosphorylating Beclin 1 and consequently modulating a three-way interaction among Bcl-2 proteins, Beclin 1 and BAX (47). Thus, further research will be needed to determine whether these two processes are mutually exclusive, can occur in tandem, or whether they are specific to conditions.

## ER stress response

In addition to mitochondria that are considered as the primary target of Bcl-2 family proteins, some pro- and anti-apoptotic family members exert effects on ER mainly through the processes associated with ER stress response. The ER stress response constitutes a cellular process that is triggered by a variety of conditions that disturb synthesis and the folding of proteins in the ER (6). Eukaryotic cells have developed an evolutionarily conserved adaptive mechanism, the unfolded protein response (UPR), which aims to clear aberrant proteins and restore ER homeostasis. Similarly to autophagy, ER stress that cannot be reversed culminates in cell death via different mechanisms (48, 49).

The relationship between ER stress response and Bcl-2 family proteins is mutual as some components of ER stress response affect expression and function of some proteins of the Bcl-2 family and vice versa; some proteins of the Bcl-2 family are involved in regulating the ER stress response (7). In this review, we will focus on the impact of Bcl-2 family proteins on the ER stress response. Several reports

have indicated that different Bcl-2 family proteins regulate the activity of inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) which is the most conserved ER stress sensor (50). It has been documented that BAX/BAK-dependent activation of IRE1 $\alpha$  is mediated by an interaction between the BH1 and BH3 domains of BAX/BAK and the cytosolic domains of IRE1 $\alpha$ , possibly stabilizing its active form (51). The results were derived from the study of BAX/BAK double knockout cells that displayed a specific deficiency in the autophosphorylation and oligomerization of IRE1 $\alpha$ , leading to a stable association with BiP as well as decreased expression of IRE1 $\alpha$ -downstream signals including c-Jun N-terminal kinase (JNK) phosphorylation and x-box binding protein 1s (XBP-1s) expression under experimental ER stress conditions (51). Under prolonged ER stress, IRE1 $\alpha$  activity is turned off whereas still active protein kinase RNA-like endoplasmic reticulum kinase (PERK) possibly sensitizes critically damaged cells to apoptosis (52–54). Although BAX/BAK expression specifically affects IRE1 $\alpha$  activation, PERK-dependent signaling of the ER stress response is not altered (51, 55). In line with this, it has been reported that the ER located anti-apoptotic protein, transmembrane BAX inhibitor motif containing (TMBIM)-6, also known as BAX inhibitor-1, regulates IRE1 $\alpha$  activity (56–58). The inactivation of IRE1 $\alpha$  during ER stress possibly requires a direct binding of TMBIM and displacement of BAX/BAK from the UPRosome complex (55, 58). The involvement of other Bcl-2 family proteins in regulation of IRE1 $\alpha$ -dependent UPR remains unclear. Specific expression of the BH3-only proteins BIM and PUMA at the ER membrane led to the activation of IRE1 $\alpha$ /JNK pathway in a BAK-dependent manner (59). As these results were obtained in the absence of any ER stressor when the BIM and PUMA were overexpressed and targeted specifically to the ER, it has been suggested that these BH3-only proteins are potent indirect activators of the IRE1 $\alpha$  UPR branch but the exact mechanism is not completely clear. The finding that PUMA and BIM could bind under ER stress to IRE1 $\alpha$  in a BH3 domain-dependent manner and that pro-survival Bcl-2 is required for this interaction (60) were questioned with subsequent experiments (61).

In addition to the impact of Bcl-2 proteins on UPR, the effect of BAK, possibly involving Bcl-XL, a calcium transport via ryanodine receptor, on structure of ER was also documented (62).

## Intracellular calcium dynamics

Although Ca<sup>2+</sup> is the important second messenger affecting several intracellular functions, dysregulation of

intracellular  $\text{Ca}^{2+}$  dynamics is associated with either apoptotic or necrotic cell death (63). The proteins of the Bcl-2 family affect intracellular  $\text{Ca}^{2+}$  dynamics mainly at the level of ER  $\text{Ca}^{2+}$  transport with some minor impact on  $\text{Ca}^{2+}$  transport through plasma and mitochondrial membranes.

To date, there are conflicting findings as to the roles anti-apoptotic Bcl-2 family proteins play in ER  $\text{Ca}^{2+}$  homeostasis. Studies showed that overexpression of anti-apoptotic Bcl-2 proteins results either in preserved ER  $\text{Ca}^{2+}$  or in diminished  $\text{Ca}^{2+}$  stores. These controversial findings demonstrate the complexity of  $\text{Ca}^{2+}$  signaling and may be attributed to unique differences between cell types or even clonal isolates. The early study showed that overexpression of Bcl-2 can affect both  $\text{Ca}^{2+}$  signaling and redistribution of  $\text{Ca}^{2+}$  from ER to mitochondria (64). It was further demonstrated for cells overexpressing Bcl-2 that ER  $\text{Ca}^{2+}$  was preserved due to enhanced ER  $\text{Ca}^{2+}$  uptake (65). Later, it was documented that both Bcl-2 (66–68) and Bcl-XL (69) reduce basal ER  $\text{Ca}^{2+}$  levels specifically through increasing  $\text{Ca}^{2+}$  leak from ER. The original hypothesis that Bcl-2 proteins affect ER  $\text{Ca}^{2+}$  levels through their channel-forming ability was shown to be elusive, as it was documented that the effect of Bcl-2 does not depend on their putative pore-forming properties (70). Subsequent studies showed that regulation of ER  $\text{Ca}^{2+}$  levels by Bcl-2 family proteins occurs through direct or indirect modulation of ER  $\text{Ca}^{2+}$  transport proteins. Bcl-2 was found to decrease calcium pump, sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase 2b (SERCA2b), levels following overexpression (71) and to bind and inhibit the calcium pump, SERCA (72). This inhibition can be counteracted by heat-shock proteins like HSP70 (73). Another proposed mechanism for reduced ER luminal  $\text{Ca}^{2+}$  was to involve the Bcl-2 family proteins in regulation of inositol 1, 4, 5-trisphosphate receptor ( $\text{IP}_3\text{R}$ ) function, although the mechanisms involved are unclear (74). Several studies confirmed that Bcl-2, Bcl-XL, and Mcl-1 bind to all three isoforms of  $\text{IP}_3\text{Rs}$  (75) to modulate ER  $\text{Ca}^{2+}$  efflux and  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  signaling. Studies focused on determining the binding sites for Bcl-2 family proteins on type 1  $\text{IP}_3\text{Rs}$  have shown that Bcl-2 binds to domain 3 (amino acids, aa, 293–1581) in the regulatory and coupling domain of type 1  $\text{IP}_3\text{Rs}$ , and less significantly with domain 6 (aa 2590–2749) at the C terminus (76), while Bcl-XL was shown to interact with domain 6 of type 1  $\text{IP}_3\text{R}$  (69). Furthermore, Bcl-2, Bcl-XL, and Mcl-1 were shown to bind at domain 6 all three types of  $\text{IP}_3\text{Rs}$  (75). It was determined that the BH4 domain of Bcl-2 is necessary for binding with type 1  $\text{IP}_3\text{Rs}$  (76). Bcl-2 overexpression was shown to markedly reduce the  $\text{IP}_3\text{R}$  channel opening and decrease the binding affinity of  $\text{IP}_3\text{Rs}$  to  $\text{IP}_3$  (77). In contrary, the overexpression of Bcl-XL increased

$\text{IP}_3\text{R}$  channel activity by increasing the binding affinity of  $\text{IP}_3\text{Rs}$  to  $\text{IP}_3$  (69, 78); however, overexpression of Bcl-XL was also shown to diminish  $\text{IP}_3\text{R}$  expression and resulted in an overall decrease in the percentage of ER  $\text{Ca}^{2+}$  release (79). At least for Bcl-2, ER calcium regulation appears to be modulated by phosphorylation as phosphorylated Bcl-2, which resides primarily at the ER and cannot reduce basal ER  $\text{Ca}^{2+}$  levels (80, 81). With respect to the pro-apoptotic Bcl-2 family proteins, it has been documented that cells from BAX and BAK double knockout mice also have lower resting ER  $\text{Ca}^{2+}$  levels and secondarily decreased mitochondrial  $\text{Ca}^{2+}$  uptake (82, 83). These findings led to a proposal that in contrast to Bcl-2 and Bcl-XL, BAX and BAK decrease ER  $\text{Ca}^{2+}$  concentration without alteration of calcium pump SERCA2 or  $\text{IP}_3\text{R}$  levels (84). BAX and BAK have been shown to regulate  $\text{IP}_3\text{R1}$  and  $\text{Ca}^{2+}$  leak, but no direct interaction between BAX/BAK and  $\text{IP}_3\text{R}$  has been observed and these effects may be mediated through modulation of Bcl-2/ $\text{IP}_3\text{R1}$  interaction and the regulation of the PKA-dependent phosphorylation state of  $\text{IP}_3\text{R1}$  (84). The regulation of interaction of Bcl-2-family proteins with the  $\text{IP}_3\text{R}$  at the level of  $\text{IP}_3\text{R}$  phosphorylation may even be more complex than can be mediated through dopamine- and cAMP-regulated phosphoprotein 32 (DARPP-32) (85) and the phosphatase calcineurin and protein phosphatase 1 (86) on the  $\text{IP}_3$ -binding site. The binding of pro-apoptotic BOK to  $\text{IP}_3\text{Rs}$  protects them from proteolytic degradation (87). Finally, recent studies have documented interaction of either Bcl-2 (88) or Bcl-XL (89) with ryanodine receptor that is also intracellular  $\text{Ca}^{2+}$  channel localized on ER membrane. It has also been shown that ER-localized BIK might be required for BAX/BAK-dependent ER  $\text{Ca}^{2+}$  release in response to genotoxic stress (90).

With respect to mitochondrial  $\text{Ca}^{2+}$  transport, some proteins of the Bcl-2 family have impacts on mitochondrial  $\text{Ca}^{2+}$  uptake. Both Mcl-1 and BNIP3 have been shown to modulate mitochondrial  $\text{Ca}^{2+}$  uptake via an unknown mechanism. Downregulation of Mcl-1 was shown to increase in mitochondrial  $\text{Ca}^{2+}$  when stimulated with adenosine triphosphate (ATP), while cells overexpressing Mcl-1 showed lower mitochondrial  $\text{Ca}^{2+}$  increases (91). Localization of BNIP3 to the ER membrane facilitated release of  $\text{Ca}^{2+}$  from ER and subsequently increased uptake of  $\text{Ca}^{2+}$  into mitochondria (92).

There are some indications of the involvement of Bcl-2 family proteins in regulation of mitochondrial  $\text{Ca}^{2+}$  through voltage-dependent anion channel (VDAC) that is highly expressed on the OMM facilitating the entry and exit of ATP,  $\text{Ca}^{2+}$ , cytochrome c, and other metabolites between the mitochondria and other cellular compartments (93). It is also known to tightly control ER  $\text{Ca}^{2+}$  signals into the

mitochondria via formation of 'Ca<sup>2+</sup> tunnels' with IP<sub>3</sub>R3 and GRP75 at the mitochondria-associated ER membranes (MAM), region of the ER that is reversibly tethered to mitochondria. Anti-apoptotic Bcl-XL was shown to counteract the effect of pro-apoptotic BAX on electrophysiological properties of VDAC (94), but the effect of Bcl-2 family proteins on leak of Ca<sup>2+</sup> from mitochondria through VDAC is far from to be clear (10). BH4 domain of Bcl-XL but not related Bcl-2 was shown to be important for the interaction of Bcl-XL with VDAC (95). Finally, the recent study has documented that the association of Bcl-XL with IP<sub>3</sub>R3 at MAM enhances transient mitochondrial Ca<sup>2+</sup> levels upon ER Ca<sup>2+</sup> depletion induced by short-term, non-apoptotic cell treatment with ER stressor thapsigargin (96).

Bcl-2 has also been shown to modulate Ca<sup>2+</sup> entry into the cell with the conflicting evidence that may be the result of differences between cell types. Initial study observed a decrease in Ca<sup>2+</sup> entry in Bcl-2 expressing cells after capacitative Ca<sup>2+</sup> entry (CCE) was activated (97). Further experiments suggested that Bcl-2 expression decreases the number of functional store operating Ca<sup>2+</sup> channels, resulting in the observed downregulation of CCE (71). In contrary, other experiments resulted in enhanced CCE in Bcl-2 overexpressing cell lines (98).

## Mitochondrial dynamics and energy metabolism

Mitochondrial dynamics and energy metabolism are interconnected processes that have an important impact on cell survival (99) with a particular importance for large polar cells like neurones (100). Proteins of the Bcl-2 family can influence both mitochondrial dynamics and energy metabolism.

The involvement of Bcl-2 family proteins in changes of mitochondrial morphology and localization has emerged in recent years (101–103). Similarly to other non-canonical functions, Bcl-2 family proteins also appear to play opposite roles in the control of mitochondrial morphology, but with a twist. The impact of Bcl-2 proteins on mitochondrial energy (93) could also indirectly influence mitochondrial morphology. In addition, many studies suggested interactions between both anti- and pro-apoptotic Bcl-2 family proteins and both fission and fusion factors including the dynamin-like GTPases Drp1 and Mfn1/2 that are the main regulators of OMM fission and fusion, respectively (104, 105), but the connections between specific proteins and cell death/survival outcomes are not fully clear (106–110). Nevertheless, these mechanisms may be

conserved across species even when canonical functions of Bcl-2 family proteins appear not to be conserved (101). The involvement of Bcl-2 family proteins in mitochondrial dynamics was first indicated when Drp1 was shown to promote BAX-induced mitochondrial fission and cell death (111, 112). In contrast to dying cells, BAX promotes mitochondrial fusion in healthy cells (113). BAX-induced fusion can also be connected to the mitochondrial permeability transition pore opening, leading to necrosis (114). Bcl-XL can also bind and induce Drp1-dependent mitochondrial fission, but this seems to be not a cell death function (115). It is still not clear if the opposite effects of individual Bcl-2 family proteins on changes of mitochondrial morphology require any shared biochemical mechanisms. Recent *in vitro* studies indicated that Bcl-2 family proteins may play an exact role in membrane fusion reactions. Drp1 that induces a hemifusion state in OMM (116, 117) creates a local lipid topology promoting BAX oligomerization. These results are consistent with co-localization of Drp1 and BAX in spots found at sites of mitochondrial fission (113, 116). This function of Drp1 does not require its GTPase activity, which is required for mitochondrial fission. If BAX oligomerises at stalled fission junctions, it is conceivable that OMM permeability is the result of a defective, excessive, or incomplete fission process (116).

On the day-job side, development of a powerful *in vitro* fusion assay has served to show a role for BAX in mitochondrial fusion *in vitro*. The studies using mitochondria isolated from different cells revealed that either BAX or Bcl-XL can promote Mfn2-dependent fusion of isolated mitochondria *in vitro*, fitting with their functions in healthy mitochondria (118, 119).

In addition to the effects on mitochondrial structure exerted at the OMM, recent studies indicated that Bcl-2 proteins can also affect mitochondrial morphology and functions by acting in mitochondria (12). Several studies have shown that anti-apoptotic Bcl-2 proteins can be imported into mitochondria and likely associate with the inner mitochondrial membrane in which they are suggested to carry out non-canonical functions in regulation of mitochondrial respiration and ATP production (120–122).

First indications about the effect of Bcl-2 family proteins on energy metabolism are coming from the studies showing that the overexpression of Bcl-2 (123) and Bcl-XL (124) in osteosarcoma cells stimulates mitochondrial respiration and increases the mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). Further evidence suggesting that pro-survival Bcl-2 proteins promote mitochondrial energy metabolism was based on the study of Bcl-2-induced increase in cytochrome c oxidase activity, and mitochondrial respiration (125). Moreover, the overexpression of

Bcl-W or Bcl-XL in lung cancer cells increased complex-I activity,  $\Delta\Psi_m$ , and cellular ATP levels (126). With respect to non-malignant cells, the ability of Bcl-XL to increase mitochondrial energy and ATP levels was demonstrated analyzing Bcl-XL-overexpressing (120) or Bcl-XL-knockout neurons (121). An amino-terminally truncated isoform of Mcl-1 possesses a bona-fide mitochondrial pre-sequence that mediates  $\Delta\Psi$ -dependent import of Mcl-1 into the mitochondrial matrix. Matrix-localized Mcl-1 is necessary to facilitate normal mitochondrial fusion, ATP production, membrane potential, respiration, cristae ultrastructure and maintenance of oligomeric ATP synthase (122). These findings support the hypothesis that the anti-apoptotic proteins of the Bcl-2 family increase mitochondrial energy metabolism.

In contrast, pro-apoptotic proteins of the Bcl-2 family decrease mitochondrial energy metabolism. Addition of recombinant BAX protein reduced  $\Delta\Psi_m$  and mitochondrial respiration in permeabilized astrocytes (127), cardiomyocytes and in isolated rat heart mitochondria (128). The ability of BAX to reduce  $\Delta\Psi_m$  production has also been shown in oligomycin-treated mouse sympathetic neurons (129). Although in lung cancer cells the knockdown of BAX and BAK increased complex-I activity,  $\Psi_m$ , and ATP levels (126), BAX deletion did not improve neuronal energy and only slightly reduced basal cytosolic ATP levels in mouse cortical neurones (130). It seems that, at least in some cell types, anti-apoptotic and pro-apoptotic Bcl-2 proteins antagonistically regulate mitochondrial energy metabolism. In accord, BAX and Bcl-2 also influence the function of ATP/adenosine diphosphate (ADP)-translocator in opposing manners (131). Bcl-2 maintains the translocase activity at high levels, whereas BAX inhibits the translocase function of adenine nucleotide translocase (ANT).

## Cell cycle progression

Progression of the cells through the cell cycle is an essential process with respect to cell proliferation but it can also significantly affect cell survival as cell cycle arrest can further culminate in cell death (132).

Both Bcl-2 and Bcl-XL were recognized as negative regulators of the cell cycle, through several mechanisms. It has been shown that Bcl-2 prolonged G1 phase (133) and delayed the G1-S transition by inhibition of cyclin-dependent kinase-2 (CDK2) (134), upregulation of CDKN1 (135) and/or interfering with the transcriptional activity of E2F (135, 136). Similar mechanisms of cell cycle inhibition have been ascribed to Bcl-XL (137, 138), and the cell cycle effects of both Bcl-2 and Bcl-XL were shown to be reversed

by pro-apoptotic BH3-only protein BAD (137). Moreover, it has been suggested that two distinct functions of Bcl-2 (anti-apoptosis and cell cycle inhibition) are differentially regulated by post-translational mechanisms such as phosphorylation involving different protein kinases and pathways like CDC2K (139) and CDK1 (140) or apoptosis signal-regulating kinase (ASK)/Jun kinase pathway (141). Mcl-1 also significantly inhibited the cell cycle progression through the S-phase owing to interaction of Mcl-1 with the cell cycle regulator, proliferating cell nuclear antigen (142). In contrary, the pro-apoptotic Bcl-2 family proteins such as BAX and BAD stimulated cell cycle progression (134, 143) via different mechanisms like a BAX-dependent decrease of the levels of p27/Kip1 (144). It seems that the cell cycle regulatory functions of anti-apoptotic Bcl-2 family proteins are evolutionarily conserved (138, 145) and mediated by a BH4 protein domain that is not involved in the regulation of apoptosis (146). In accord with the stimulatory effect of pro-apoptotic proteins of the Bcl-2 family on cell cycle progression, G1-S transition was showed to be blocked in BID-deficient hepatocytes following DNA damage (147). BID-dependent DNA damage response has been attributed to ataxia telangiectasia mutated (ATM) kinase-induced BID phosphorylation (148), but these results have been questioned by subsequent study (149). The results of later study suggested a direct role for BID acting at the level of the damage sensor complex to amplify the ataxia telangiectasia mutated and Rad3-related protein (ATR) kinase-directed cellular response to replicative stress (150). Finally, recent study has demonstrated that expression of Bcl-2 reduces intracellular deoxynucleotides (dNTPs) by inhibiting ribonucleotide reductase activity (151). This retards DNA replication fork progression with increased fork asymmetry, leading to DNA replication stress that can be further associated with cell cycle arrest.

## Expert opinion and outlook

As briefly summarized in this review, Bcl-2 family proteins are not only involved in the regulation of intrinsic pathways of apoptosis, but also involved in other intracellular pathways associated with cell survival. There are still several open questions about whether and in which manner are particular proteins of Bcl-2 family involved in regulating processes distinct from apoptosis as were also indicated in this review. Unlike canonical functions, some non-canonical functions of Bcl-2 family proteins exhibit significant diversity depending on the cell type or particular cell architecture based on endogenous or exogenous

conditions. In addition, the majority of knowledge about non-canonical functions of the Bcl-2 family proteins were derived from experiments based on up- or downregulation of the expression of a particular protein, and little attention has been paid to the post-translational modifications of Bcl-2 family proteins or their targets with a focus on the functional consequences of such modifications. As Bcl-2 family proteins are involved in the mechanisms of neurodegeneration as well as in the initiation and progress of malignant diseases, several therapeutical strategies were developed on the basis of their functions in apoptosis to cope with the mentioned diseases. Thus, the studies of non-canonical functions of Bcl-2 family proteins and their mutual interactions with canonical functions as well as involvement of post-translational modifications in these interactions and their impact on Bcl-2 family protein functions represent great challenges for further research.

## Highlights

- Bcl-2 family proteins are not only involved in regulating intrinsic pathways of apoptosis, but are also involved in other intracellular pathways associated with cell survival.
  - The anti-apoptotic Bcl-2 proteins have been proposed to inhibit autophagy owing to their interaction with the autophagy regulator Beclin 1, whereas involvement pro-apoptotic pore forming BAX and BAK in autophagy regulation is not clear.
  - Several reports have indicated that different Bcl-2 family proteins regulate ER stress response mainly via regulation of activity of IRE1 $\alpha$  that is the most conserved ER stress sensor.
  - There are controversial findings about the involvement of Bcl-2 family proteins in regulating intracellular Ca<sup>2+</sup> dynamics but the interaction of some Bcl-2 family proteins with intracellular Ca<sup>2+</sup> channels, Ca<sup>2+</sup> pumps, was described.
  - Bcl-2 family proteins interact with both fission and fusion factors including Drp1 and Mfn1/2 that are the main regulators of OMM fission and fusion, respectively, but the connections between specific proteins and cell death/survival outcomes are not fully clear.
  - Anti-apoptotic and pro-apoptotic Bcl-2 proteins antagonistically regulate mitochondrial energy metabolism at least in some cell types.
  - Anti-apoptotic Bcl-2 family proteins inhibit cell cycle progression while the pro-apoptotic Bcl-2 family proteins stimulate cell cycle progression.
- There are still several open questions whether and in which manner are particular proteins of Bcl-2 involved in regulation of processes distinct from apoptosis.

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## List of abbreviations

|                   |   |
|-------------------|---|
| ADP               | adenosine diphosphate                                   |
| AIF               | apoptosis inducing factor                               |
| ANT               | adenine nucleotide translocase                          |
| ASK               | apoptosis signal-regulating kinase                      |
| ATM               | ataxia telangiectasia mutated                           |
| ATP               | adenosine triphosphate                                  |
| ATR               | ataxia telangiectasia mutated and Rad3-related protein  |
| BAD               | Bcl-2-associated death promoter                         |
| BAK               | Bcl-2 homologous antagonist/killer                      |
| BAX               | Bcl-2-like protein 4                                    |
| Bcl-2             | B-cell lymphoma 2                                       |
| Bcl-XL            | B-cell lymphoma-extra large                             |
| BID               | BH3 interacting domain death agonist                    |
| BIK               | Bcl-2 interacting killer                                |
| BIM               | Bcl-2-like protein 11                                   |
| BLF1              | Burkholderia lethal factor 1                            |
| BMF               | Bcl-2 modifying factor                                  |
| BNIP3             | Bcl-2/adenovirus E1B 19-kDa interacting protein 3       |
| BOK               | Bcl-2 related ovarian killer                            |
| CCE               | capacitative Ca <sup>2+</sup> entry                     |
| CDK2              | cyclin-dependent kinase-2                               |
| DARPP-32          | dopamine- and cAMP-regulated phosphoprotein 32          |
| dNTPs             | deoxynucleotides  |
| HRK               | Harakiri, Bcl-2 interacting protein                     |
| IP <sub>3</sub> R | inositol 1, 4, 5-trisphosphate receptor type            |
| IRE1 $\alpha$     | inositol-requiring enzyme 1 $\alpha$                    |
| JNK               | c-Jun N-terminal kinase                                 |
| MAM               | mitochondria-associated ER membranes                    |
| $\Delta\Psi_m$    | mitochondrial transmembrane potential                   |
| Mcl-1             | myeloid cell leukemia 1                                 |
| Noxa              | NADPH oxidase activator 1                               |
| OMM               | outer mitochondrial membrane                            |
| PERK              | protein kinase RNA-like endoplasmic reticulum kinase    |
| PUMA              | p53 upregulated modulator of apoptosis                  |
| SERCA2b           | sarco/endoplasmic reticulum Ca <sup>2+</sup> -ATPase 2b |
| TMBIM             | transmembrane BAX inhibitor motif containing            |
| UPR               | unfolded protein response                               |
| VDAC              | voltage-dependent anion channel.                        |
| XBP-1s            | x-box binding protein 1s                                |

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