Noncanonical Cell Fate Regulation by Bcl-2 Proteins

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Stephen Jun Fei Chong[1], Saverio Marchi[2], Giulia Petroni[3], Guido Kroemer[4,5,6,7,8,9], Lorenzo Galluzzi[3,9,10,11,12,*] and Shazib Pervaiz[9,13,14*]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

[2]Department of Clinical and Molecular Sciences, Marche Polytechnic University, Ancona, Italy

[3]Department of Radiation Oncology, Weill Cornell Medical College, New York, NY, USA

[4]Equipe labellisée par la Ligue contre le cancer, INSERM U1138, Centre de Recherche des Cordeliers, Paris, France

[5]Metabolomics and Cell Biology Platforms, Gustave Roussy Comprehensive Cancer Institute, Villejuif, France; [6]Pôle de Biologie, Hôpital Européen Georges Pompidou, AP-HP, Paris, France

[7]Suzhou Institute for Systems Medicine, Chinese Academy of Sciences, Suzhou, China

[8]Department of Women's and Children's Health, Karolinska University Hospital, Stockholm, Sweden

[9]Université de Paris, Paris, France

[10]Sandra and Edward Meyer Cancer Center, New York, NY, USA

[11]Caryl and Israel Englander Institute for Precision Medicine, New York, NY, USA

[12]Department of Dermatology, Yale School of Medicine, New Haven, CT, USA

[13]Department of Physiology, YLL School of Medicine and NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore

[14]National University Cancer Institute, National University Health System, Singapore.

*Correspondence: Lorenzo Galluzzi (deadoc80@gmail.com) or Shazib Pervaiz (phssp@nus.edu.sg)

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Abstract

Bcl-2 proteins are widely known as key controllers of mitochondrial outer membrane permeabilization, arguably the most important step of intrinsic apoptosis. Accumulating evidence indicate that most, if not all, members of the Bcl-2 protein family also mediate a number of apoptosis-unrelated functions. Intriguingly, many of such functions ultimately impinge on cell fate decisions via apoptosis-dependent or -independent mechanisms, delineating a complex network through which Bcl-2 family members regulate cell survival and death. Here, we critically discuss the mechanisms through which Bcl-2 proteins influence cell fate as they regulate autophagy, cellular senescence, inflammation, bioenergetic metabolism, Ca^{2+} fluxes and redox homeostasis.

The Bcl-2 Protein Family

Originally discovered in the context of a chromosomal translocation expressed by a fraction of follicular lymphomas (t14:18) [1], BCL2 apoptosis regulator (BCL2, also known as Bcl-2) is the founder of a large family of proteins sharing one or more BCL2 homology (BH) domains [2]. Subsequent work determined the ability of BCL2 to mediate potent cytoprotective functions by preserving mitochondrial integrity in the course of intrinsic apoptosis [3] and revealed that most (but not all) other Bcl-2 family members also regulate mitochondrial outer membrane permeabilization (MOMP) [2]. Bcl-2 family members are classically categorized into three main groups based on the presence of specific BH domains that endow them with anti- or pro-apoptotic functions [2]. While multidomain anti- and pro-apoptotic Bcl-2 proteins share four BH domains as well as a transmembrane (TM) motif that enables constitutive or inducible localization to intracellular membranes, so-called "BH3-only proteins" – which promote apoptotic cell death upstream of their multidomain pro-apoptotic relatives – only share a BH3 domain and an optional TM motif [2]. Thus, the Bcl-2 family has long been known to occupy a central position in the biology of higher eukaryotes as a gate-keeper of mitochondrial integrity [2].

Over the past three decades, the realization that some neoplastic cells, especially of hematological origin, depend on anti-apoptotic Bcl-2 proteins for survival and proliferation prompted an intense wave of investigation aimed at the development of Bcl-2 inhibitors for use in cancer patients [4]. In this context, early strategies based on *BCL2*-targeting antisense oligonucleotides (ODNs) such as oblimersen (also known as G3139) provided promising preclinical results and entered clinical testing, but ultimately failed development most likely because of the pharmacodynamic and pharmacokinetic issues linked to the use of ODNs *in vivo* [5]. Alongside, multiple small chemical pharmacological inhibitors of one or more anti-apoptotic Bcl-2 family members were developed and demonstrated

superior activity in multiple preclinical tumor models, rapidly prompting clinical development [4]. The first among such "BH3 mimetics" demonstrating potent anticancer activity *in vivo* were oblatoclax (also known as GX15-070) [6], ABT-737 [7], and ABT-263 (best known as navitoclax) [8]. However, none of these agents completed clinical development owing to severe on-target toxicity reflecting the inhibition of BCL2 like 1 (BCL2L1, best known as BCL-X_L) in platelets and consequent thrombocytopenia [9]. To circumvent this issue, highly specific BH3 mimetics have been developed including ABT-199 (best known as venetoclax), which selectively targets BCL2 [4]. Ultimately corroborating the clinical value of targeting Bcl-2 proteins for cancer therapy, venetoclax is currently approved by the US Food and Drug Administration (FDA) for the treatment of chronic lymphocytic leukemia (CLL) [10] and demonstrated promising single-agent activity in patients with BCL2-overexpressing breast cancer [11].

Perhaps starting with the identification of a nuclear pool of BCL2 in actively proliferating cells [12, 13], a large amount of preclinical literature has accumulated in support of the notion that both pro- and anti-apoptotic Bcl-2 family members mediate numerous non-canonical functions [14], most of which ultimately impinge on cell fate decisions even though they do not directly influence apoptotic signaling (**Table 1**). Here, we discuss the ability of Bcl-2 proteins to influence cell fate by regulating autophagy, cellular senescence, inflammation, bioenergetic metabolism, Ca^{2+} fluxes and redox homeostasis.

Regulation of autophagy by Bcl-2 family members

Macroautophagy (herein referred to as 'autophagy') is a lysosome-dependent catabolic pathway through which virtually all eukaryotic cells dispose of superfluous or potentially dangerous cytosolic entities in support of cellular homeostasis [15]. Autophagy involves the formation of novel double-membraned organelles (*i.e.*, autophagosomes) that – upon sequestration of cytosolic substrates – fuse with lysosomes to initiate substrate degradation within so-called autolysosomes, a process that is regulated by a complex molecular machinery that interfaces with many other cellular functions [16, 17]. Multiple Bcl-2 proteins interact with such machinery, ultimately influencing the ability of cells to mount cytoprotective autophagic responses to stress (**Figure 1**).

One of the core regulators of autophagy in mammalian cells, *i.e.*, beclin 1 (BECN1), was identified as an antiviral BCL2-interacting protein as early as in 1998 [18]. Shortly thereafter, BECN1 was shown to promote autophagy [19] by promoting the catalytic activity of phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3, best known as VPS34) [20], and the depletion of BCL2 was reported to initiate autophagy in human leukemic HL60 cells [21], pointing to a mechanism for autophagy inhibition by anti-apoptotic Bcl-2 proteins. Consistent with the ability of BCL2 to inhibit BECN1, BCL2 mutants unable to bind BECN1 lose the capacity to inhibit autophagy in both yeast and mammalian cells, and transgene enforced overexpression of BCL2 in mouse cardiomyocytes limits autophagy in the heart [22]. Moreover, BECN1 mutants that cannot bind BCL2 as well as chemical BCL2 inhibitors enable unrestrained autophagic responses to stress that support, rather than limit, cell death [22, 23], and mice expressing a BECN1 mutant with decreased affinity for BCL2 at the whole body level exhibit increased autophagic responses at baseline linked to prolonged lifespan [24]. Intriguingly, at least in some settings including human breast cancer MCF7 cells, the anti-autophagic (but not the anti-apoptotic) function of BCL2 appears to be critical for the malignant phenotype [25].

Comforted by the notion that BECN1 and other components of the autophagy apparatus are lost or downregulated in a considerable fraction of human tumors, these findings document the important oncosuppressive roles of autophagy [26].

BCL-X_L resembles BCL2 in its ability to inhibit BECN1-dependent autophagic responses, a physical and functional interaction that depend on a *bona fide* BH3 domain in BECN1 [27, 28]. In line with this notion, both the transgene-enforced overexpression of the BH3-only protein BCL2 associated agonist of cell death (BAD) as well as exposure to ABT-737 potently induce autophagy by displacing BECN1 from inhibitory interactions with BCL-X_L [28]. The ability of BAD to interfere with BECN1 inhibition by BCL-X_L is shared by truncated BH3 interacting domain death agonist (BID) [29], but the functional consequences of these findings remain to be explored. Similarly, dynamin 1 like (DNM1L; best known as DRP1) a protein involved in mitochondrial autophagy (mitophagy), reportedly displaces BECN1 from BCL2 and BCL-X_L [30], but whether DRP1 drives mitophagy upon increased BECN1 activity remains unclear.

It has been proposed that the capacity of ABT-737 to drive autophagy would originate indirectly, downstream of the ABT-737-mediated release of BCL2 associated X, apoptosis regulator (BAX) and BCL2 antagonist/killer 1 (BAK1) from inhibitory interactions with anti-apoptotic Bcl-2 proteins [31]. However, both mouse embryonic fibroblasts (MEFs) and human colorectal carcinoma HCT 116 cells genetically engineered to prevent BAX and BAK1 expression undergo normal autophagic responses upon exposure to ABT-737, reinforcing the notion that BH3 mimetics can drive autophagy upon the direct displacement of BECN1 from BCL2-like proteins [32].

In physiological settings, the inhibitory interactions between BECN1 and BCL2 or BCL-X_L are destabilized upon phosphorylation of these BECN1-binding partners on specific serine or threonine residues, including T69, S70 and S87 on BCL2 and S62 on BCL-X_L [33, 34]. Stress-responsive kinases

that catalyze such phosphorylation events encompass (but may not be limited to) mitogen-activated protein kinase 8 (MAPK8, best known as JNK1) [33-35] as well as 5'-AMP-activated protein kinase (AMPK) [36], which also promotes autophagy by phosphorylating other BECN1 interactors [16].

Both BCL2L2 (also known as BCL-W) and MCL1 apoptosis regulator, BCL2 family member (MCL1), but not BAX, can also engage in physical interactions with BECN1, albeit with diverse affinity [29]. In line with the ability of MCL1 to inhibit autophagy downstream of BECN1, nutrient deprivation (a potent autophagy activator) causes rapid MCL1 degradation in human lung carcinoma H1299 cells while transgene-enforced MCL1 overexpression inhibits autophagy in the same experimental setting [37]. Moreover, conditional knockout of *Mcl1* in the mouse forebrain is sufficient to drive a robust autophagic response [37]. Intriguingly, BECN1 appears to compete with MCL1 for stabilization by the deubiquitinase ubiquitin specific peptidase 9 X-linked (USP9X), potentially adding another contact-independent mechanism whereby anti-apoptotic Bcl-2 proteins inhibit autophagic responses [38]. In line with this notion, MCL1 destabilization by the BH3-only protein phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1, best known as NOXA), reportedly drives autophagic responses in the context of BECN1 stabilization [38].

Taken together, these observations suggest that at least part of the oncogenic effects of anti-apoptotic Bcl-2 proteins may originate from the inhibition of autophagy, which generally limits malignant transformation [39]. Moreover, at least in some scenarios, anti-apoptotic Bcl-2 proteins may mediate cytoprotective effects by preventing disproportionate autophagic responses with catastrophic outcomes.

Bcl-2 family members and cellular senescence

The term 'cellular senescence' refers to the permanent proliferative inactivation of cells accumulating molecular damage in the absence of overt apoptotic signaling [40]. Senescent cells display a number of biochemical markers including an exacerbated β-galactosidase activity as well as the secretion of multiple bioactive mediators commonly referred to as "senescence-associated secretory phenotype" (SASP) [41]. Although in some instances (e.g., liver regeneration) cellular senescence mediate beneficial functions, the accumulation of senescent cells contributes to numerous aging-associated pathologies [42]. Accumulating evidence demonstrates that anti-apoptotic Bcl-2 proteins are essential for the establishment and long-term maintenance of senescence, at least in part as they elevate the ability of cells to cope with molecular damage, suggesting that BH3 mimetics may be employed as senolytic agents.

A number of reports linked endogenous or exogenous (transgene-enforced) elevations in BCL2 to the activation of senescence over apoptosis in multiple cellular models of stress, including (but not limited to): human fibroblasts deprived of serum [43] or forced to express oncogenic HRAS [44, 45]; rodent fibroblasts subjected to oncogene inactivation [46], serum deprivation [47], or DNA damage [47]; human cervical carcinoma HeLa cells treated with an inhibitor of DNA replication [48]; and mouse melanoma B16 cells maintained in hypoxic conditions [49]. Similar observations have been made for BCL-$X_{L,}$ MCL1 and/or BCL-W in human lung fibroblasts and MEFs subjected to DNA damage, replicative exhaustion or oncogene activation [50], irradiated human cholangiocytes [51, 52], human colorectal carcinoma HCT 116 cells exposed to topoisomerase inhibitors [53], as well as human triple negative breast cancer (TNBC) cells responding to BET inhibitors [54]. Importantly, in many of these settings, pharmacological or genetic inhibition of BCL2-like proteins prevented senescence establishment as it promoted cell death [49-51, 53, 54]. Consistent with this notion, TNBC cells

undergoing apoptosis upon exposure to BET inhibitors exhibited reduced BCL-X_L levels as compared to their senescing counterparts [54]. Moreover, induction of apoptosis over senescence by transgene-enforced overexpression of the cell cycle inhibitor cyclin dependent kinase inhibitor 2A (CDKN2A; best known as $p16^{INK4}$) in human non-small cell lung cancer (NSCLC) cells relied on BCL2 downregulation upon tumor protein p53 (TP53) activation [55]. Conversely, the androgen-mediated activation of retinoblastoma 1 (RB1), which blocks the cell cycle at the G_1-to-S transition and is involved in some variants of cellular senescence, reportedly limits *BCL2* transcription by E2F transcription factors in prostate cancer cells [56]. However, whether androgens establish *bona fide* senescence or a reversible cell cycle arrest (quiescence), which may explain this apparent discrepancy, remains to be clarified.

Apparently at odds with the protective role of MCL-1 in oncogene-induced senescence [45], HCT 116 cells overexpressing MCL1 are protected from chemotherapy-driven senescence as compared to their wild-type counterparts, while MCL1 downregulation mediates senescence-sensitizing effects that depend on the endogenous cell cycle inhibitor cyclin dependent kinase inhibitor 1A (CDKN1A; best known as $p21^{CIP1}$) [57]. Intriguingly, such anti-senescence effects of MCL1 cannot be mapped to its BH3-binding and TM domains, which are instead required for MCL1 to inhibit MOMP [57]. Conversely, chemotherapy-driven senescence regulation by MCL1 depends on four residues (*i.e.*, K194, K197, P198, G203) located outside of classical BH domains [58], and appears to reflect the ability of MCL1 to interfere with reactive oxygen species (ROS) production by NADPH oxidase 4 (NOX4) [59]. However, whether such pathway operates in cellular contexts other than colorectal carcinoma HCT 116 cells remains unverified.

Perhaps with the exception of MCL1, anti-apoptotic Bcl-2 proteins stand out as major regulators of cellular senescence and hence as potential targets for the development of novel senolytic approaches.

Preclinical data supporting the therapeutic potential of BH3 mimetics for human conditions involving the accumulation of senescent cells such as pulmonary fibrosis [60] or aging-associated hematopoietic dysfunction [61] have already begun to emerge. However, whether the anti-aging effects of BH3 mimetics only stem from their senolytic activity as opposed to a multitude of cellular activities including autophagy activation, which is known to postpone multiple manifestations of aging[24], remains unclear.

Immunomodulation by Bcl-2 family members

Several pro- and anti-apoptotic members of the Bcl-2 family are required for a variety of immune cells, including macrophages [62-64], B and T lymphocytes [65-67], and natural killer (NK) cells [68] to mature normally and mediate physiological effector functions. Moreover, numerous Bcl-2 proteins regulate inflammation by interacting with the molecular machinery for innate immune signaling (**Figure 2**).

In endothelial cells, both BCL2 and BCL-X_L suppress the core pro-inflammatory transcription factor NF-κB by preventing the degradation of NFKB inhibitor alpha (NFKBIA; best known as IκBα), in a process that relies on their BH2 and BH4 domains [69]. Although the precise mechanism underlying these observation remains to be elucidated, it is tempting to speculate that the ability of anti-apoptotic Bcl-2 proteins to prevent IκBα degradation relates to one or more of these observations: (1) limited inflammasome activation, knowing that IκBα contains a caspase 1 (CASP1)-like cleavage consensus site [69], and that anti-apoptotic Bcl-2 proteins prevent inflammasome activation by CASP8 downstream of MOMP [70, 71]; or (2) direct sequestration of heterodimeric NF-κB by BCL2 [72]. Irrespective of these unknowns, BCL2-overexpressing monocytes have been shown to limit inflammation and heart failure in a mouse model of cardiomyopathy correlating with decreased production of NF-κB-dependent cytokines [73], confirming the pathophysiological relevance of NF-κB or inflammasome inhibition by anti-apoptotic Bcl-2 proteins.

Importantly, MOMP regulation by Bcl-2 proteins influences inflammasome activation not only as a consequence of CASP8 activation downstream of the MOMP-dependent depletion of inhibitor of apoptosis proteins (IAPs) [70] or as a function of ROS overgeneration [74], but also linked to mitochondrial DNA (mtDNA) release into the cytosol [75]. Besides driving interleukin 1 beta (IL1B, best known as IL-1β) and interleukin 18 (IL18) maturation by the inflammasome [75], cytosolic

mtDNA also initiates type I interferon (IFN) secretion by cyclic GMP-AMP synthase (CGAS) and stimulator of interferon response cGAMP interactor 1 (STING1) [76, 77]. Notably, mtDNA is not fully released by mitochondria undergoing MOMP, but rather bulges into cytosol through BAX and BAK1 oligomers [78]. Moreover, inflammasome activation seems to initiate a feed-forward loop that exacerbates inflammatory signaling by mtDNA [79], although the contribution of Bcl-2 proteins to the latter process remains obscure.

In this context, CASP3 activation downstream of MOMP plays a major anti-inflammatory role, not only as it limits type I IFN-secretion upon CGAS activation [76, 77, 80], but also as it accelerates the terminal inactivation of stressed, type I IFN-secreting cells [81, 82], and it favors the release of anti-inflammatory mediators including prostaglandin E_2 (PGE_2) [83]. Of note, BID and BAX appears to participate in the inflammatory response that drives pulmonary fibrosis downstream of transforming growth factor beta 1 (TFGB1) signaling [84], largely as they stimulate the secretion of extracellular matrix-remodeling enzymes in the context of MOMP [84]. BAX has also been linked to prostaglandin-endoperoxide synthase 2 (PTGS2)-dependent inflammation in the absence of overt MOMP [85], but the precise mechanisms underlying this observation remain to be elucidated.

Irrespective of these incognita, while MOMP is intrinsically endowed with a potent immunostimulatory potential under regulation by Bcl-2 proteins, apoptotic caspases (at least in part) operate to limit inflammation in the context of apoptotic cell death, suggesting that BH3 mimetics coupled with caspase inhibitors may be employed to achieve superior immunostimulation where needed (*e.g.*, to boost anticancer immune responses). As multiple pro-inflammatory pathways impinge on the upregulation of anti-apoptotic Bcl-2 proteins (**Box 1**), however, negative feedback loops may be in place to support cell survival in the context of inflammation. Moreover, at least in some systems, BCL2 has been shown to promote (rather than limit) inflammation, as a consequence of mitochondrial ROS

overproduction and activation of pro-inflammatory transcriptional programs orchestrated by signal transducer and activator of transcription 3 (STAT3) or NF-κB [86, 87]. Taken together, these observations suggest that multiple factors influence the ability of Bcl-2 proteins to regulate inflammation, including (but not limited to) mitochondrial metabolism, as discussed here below.

Regulation of bioenergetic metabolism and Ca^{2+} fluxes by Bcl-2 family members

The ability of Bcl-2 proteins to regulate bioenergetic metabolism mainly reflects their capacity to interact with components of the electron transport chain (ETC) and hence influence oxidative phosphorylation (OXPHOS) [88] (**Figure 3**). Although some of these interactions may occur at the interface between the outer and inner mitochondrial membranes, others rely on the non-canonical localization of some Bcl-2 family members at the inner (rather than the outer) mitochondrial membrane or the mitochondrial matrix [89]. BCL2 itself was originally characterized as an inner mitochondrial membrane (IMM) protein [3], where it interacts with cytochrome c oxidase subunit 5A (COX5A) [90], a component of respiratory complex IV (CIV), as well as mitochondrial matrix proteins such as peptidylprolyl isomerase F (PPIF, best known as CYPD) [91].

Transgene-enforced BCL2 overexpression in human leukemic CEM cells boosts CIV activity along with increased oxygen consumption and ROS production [92], hence favoring proliferation. Conversely, in the presence of metabolic stress, BCL2 minimizes mitochondrial respiration by inhibiting CIV [92, 93], thus avoiding ROS to reach cytotoxic amounts (see below). In line with these observations, quiescent leukemia stem cells require the BCL2-dependent control of bioenergetics and redox state to survive [94], suggesting that the oncogenic function of anti-apoptotic Bcl-2 proteins also relates to the optimization of bioenergetic metabolism and redox homeostasis.

Both BCL-X_L and MCL-1 share with BCL2 the capacity to boost OXPHOS, although via different mechanisms [95]. Thus, while BCL-X_L physically interacts with the beta subunit of the F_1F_O-ATP synthase (CV) [96] from the IMM to boost CV activity via a mechanism controlled by a mitochondrial pool of cyclin B1 (CCNB1) and cyclin-dependent kinase 1 (CDK1) [97-99], a cleaved form of MCL1 facing the mitochondrial matrix appears to promote OXPHOS by favoring CV assembly [89]. In line with this notion, a MCL1 mutant that cannot enter the mitochondrial matrix negatively influences

respiratory capacity [100], and *MCL1* deletion results in ultrastructural mitochondrial defects accompanied by reduced transmembrane potential [89]. Moreover, mice lacking the BH3 protein BIM exhibit improved fatty acid oxidation (FAO) and superior ATP synthesis in multiple tissues [101], and BAD phosphorylation on S155 inhibits its pro-apoptotic functions as it supports metabolic functions in pancreatic beta cells and hepatocytes [102, 103]. Thus, the metabolic role of MCL1 within the mitochondrial matrix isoform is not restricted to OXPHOS regulation, but also involves lipid metabolism. This reflects the BH3-dependent interaction with the very long-chain acyl-CoA dehydrogenase (VLCAD), the first enzyme of FAO [104]. Such a dual control of bioenergetic metabolism by MCL1 emerges as a key downstream event of multiple pathways, including tumor suppression by prolyl hydroxylase 3 (PHD3) and neuroprotection by oncostatin M (OSM) [105, 106]. Along similar lines, CV control by BCL-X_L is crucial for proper cardiac functions [107]. Taken together, these observations delineate multiple metabolic functions for anti-apoptotic Bcl-2 proteins that ultimately impinge on cellular and organismal fitness.

Ca^{2+} fluxes are also regulated by both mitochondrial and extramitochondrial pools of Bcl-2 proteins (**Figure 3**). BCL2 localized at the endoplasmic reticulum (ER) and at mitochondria-associated ER membranes (MAMs) limit reticular Ca^{2+} content [108], by inducing an ER Ca^{2+} leak [109, 110] or increasing the sensitivity of inositol 1,4,5 trisphosphate receptors (IP_3Rs) to agonist-driven opening [111]. Since mitochondria can efficiently take up cytosolic Ca^{2+} coming from the ER because of their proximity to the ER membranes, these BCL2 functions facilitate the delivery of moderate Ca^{2+} amounts to the mitochondrial matrix, *de facto* supporting mitochondrial metabolism, while preventing Ca^{2+} overload-dependent cell death [109, 111, 112]. A similar activity has been attributed to both BCL-X_L [113-115] and MCL1 [111, 116]. Lowered ER Ca^{2+} concentrations at baseline have been also observed in cells lacking both BAX and BAK1 [117], most likely as a result of increased levels of unoccupied anti-apoptotic Bcl-2 proteins. Apparently at odds with these observations, BCL2 inhibits IP_3Rs in

lymphoma and leukemia cells [112, 118], and the peptide-mediated disruption of BCL2-IP_3R complexes in this setting results in cytosolic Ca^{2+} overload-driven cell death [118]. Whether this approach can ultimately translate in a novel therapeutic approach to leukemia remains unclear. Similarly, whether part of the therapeutic activity of venetoclax stems from primary cytosolic Ca^{2+} accumulation has not yet been investigated in patient samples, although data from human diffuse large B-cell lymphoma cell lines appear to negate this possibility [119]. Of note, the pro-apoptotic multidomain Bcl-2 protein BCL2 family apoptosis regulator BOK (BOK) also interacts with IP_3Rs, but fails to regulate Ca^{2+} fluxes [120]. Conversely, BOK-IP_3R complexes support mitochondrial morphology and bioenergetic metabolism [120], although the molecular mechanisms that underlie such a non-canonical function of BOK remain to be clarified.

The mitochondrial pools of BCL2, BCL-X_L and MCL1 interact with numerous members of the voltage-dependent anion channel (VDAC) protein family, a class of porins that regulate ionic fluxes across the OMM and participate in mitochondrial permeability transition (MPT)-driven regulated necrosis [121, 122]. Whether anti-apoptotic Bcl-2 proteins favor VDAC opening or closure to drive MPT has been the subject of an intense debate [123, 124], most likely reflecting the pro-survival effects of physiological VDAC activity supported by BCL2 and BCL-X_L *versus* the cytotoxic effects of excessive VDAC activity inhibited by BCL2 and BCL-X_L. Similarly, contrasting reports exist on the ability of anti-apoptotic Bcl-2 proteins to positively regulate VDAC functions in support of Ca^{2+} uptake and a metabolic boost that promotes migration [114, 116], or inhibit VDAC to prevent mitochondrial Ca^{2+} overload [115, 125]. Of note, the specific downregulation of OMM-localized MCL1, which is accompanied by the compensatory upregulation of a short MCL1 variant with pro-apoptotic functions, reportedly predisposes human cervical carcinoma HeLa cells to cell death resulting from increased Ca^{2+} uptake by mitochondria [126]. These latter findings suggest that specific splicing events may regulate the capacity of the Bcl-2 family to control Ca^{2+} fluxes. That said, it is likely that numerous

other factors influence the net effects of anti-apoptotic Bcl-2 protein on mitochondrial Ca^{2+} accumulation, including (but not limited to): (1) baseline ER Ca^{2+} levels, and (2) activity of the mitochondrial calcium uniporter (MCU) complex, which is ultimately responsible for the transport of Ca^{2+} ions across the IMM [127].

Importantly, the ability of multiple Bcl-2 family members to control Ca^{2+} fluxes at the ER appears to influence cell proliferation beyond its direct impact on metabolism and cell death. In particular, lowered ER Ca^{2+} concentrations have been shown to block cell cycle progression as a consequence of $p21^{CIP1}$ and cyclin dependent kinase inhibitor 1B (CDKN1B, best known as $p27^{KIP1}$) upregulation [128], which explains, at least in part, the proliferative defect of some *Bax*$^{-/-}$*Bak1*$^{-/-}$ cells [129]. Ultimately, the observations above delineate yet another mechanism whereby Bcl-2 family members influence cell fate, that is, the regulation of bioenergetic metabolism and Ca^{2+} fluxes.

Redox homeostasis and Bcl-2 family members

Numerous Bcl-2 family members regulate redox homeostasis, which is not surprising since (1) the mitochondrial ETC is a major site of ROS generation, especially at CI and CIII [130, 131], (2) Bcl-2 proteins directly regulate OXPHOS by interacting with CIV and CV [89, 90, 97], and (3) as part of its canonical function, the Bcl-2 family control mitochondrial integrity (which is necessary for OXPHOS to operate physiologically [2].

Early work performed in the context of the rheostat model initially proposed by the late Stanley J. Korsmeyer largely demonstrated that MOMP, as mediated by pro-apoptotic Bcl-2 proteins and prevented by their anti-apoptotic counterparts, is associated with ROS bursts that (at least to some degree) contribute to apoptotic cell death. Notably, BCL2 was shown to protect mouse pro-B lymphocytes and T cell hybridomas from cell death driven by menadione and hydrogen peroxide (H_2O_2), two strong pro-oxidants, in the absence of direct effects on ROS production by the stressed ETC [132]. Alongside, BCL2 has been shown to increase the mitochondrial pool of reduced glutathione (GSH), a potent anti-oxidant, in a BH3 domain-dependent manner [133]. Similar antioxidant properties have been attributed to MCL1, at least in cells undergoing senescence upon exposure to the DNA damaging agent doxorubicin [57, 59]. In this setting, MCL1 has been shown to limit both the expression and mitochondrial localization of NOX4 by a mechanism that involves MCL1 residue P198 [59]. More recently, ROS production by NOX4 has been shown to support apoptotic cell death by favoring MCL1 downregulation [134], suggesting the existence of a complex network whereby ROS levels and anti-apoptotic Bcl-2 proteins regulate each other. Further supporting the importance of mitochondrial integrity for the preservation of physiological ROS homeostasis, BAX has been demonstrated to support ROS production in both apoptotic and non-apoptotic cells, via a mechanism that involves lethal and sublethal CASP3 activation, respectively [135]. In this context,

NOX4-derived ROS have been linked to increased BAX phosphorylation at residue T167 (an activatory site) but not at S184 (an inhibitory site) [136], reinforcing the existence of a complex crosstalk between ROS and Bcl-2 proteins.

Apparently at odds with the observations above, transgene-enforced overexpression of BCL2 in *Escherichia coli* lacking the core antioxidant superoxide dismutase (SOD) resulted in increased mutational rate and upregulation of another antioxidant enzyme (catalase) [137], pointing to ROS overproduction. Supporting the possibility that BCL2 can mediates pro-oxidant effects, at least in some settings, CEM cells stably transfected to overexpress BCL2 exhibited increased ROS levels as compared to their control counterparts [92], which contributed to the cytoprotective effects of BCL2 (as demonstrated by pharmacological or genetic approaches to limit ROS levels in BCL2-overexpressing CEM cells) [138]. The pro-oxidant effects of BCL2 are not only due to increased OXPHOS linked to BCL2-dependent CIV hyperactivation (an activity that depends on both the BH2 domain and the C-terminal domain of BCL2) [90], but also to physical interactions between BCL2 and with Rac family small GTPase 1 (RAC1) and consequent ROS overgeneration by NADPH oxidases at both mitochondrial and extramitochondrial sites [139]. Of note, BCL2 overexpression in otherwise BCL2-negative human lymphoma Daudi cells resulted in baseline ROS overgeneration, but prevented further ROS elevations by ceramide or tumor necrosis factor (TNF), correlating with considerable cytoprotection [140]. Similar results have been obtained with CEM cells deprived of serum, glucose or oxygen [90, 92], identifying an hormetic, cytoprotective mechanism similar to the one operating under ischemic pre-conditioning [141]. Of note, MCL1 has also been shown to promote ROS overgeneration in human TNBC cells, in thus far cooperating with MYCN proto-oncogene, bHLH transcription factor (MYCN) in the establishment of chemoresistance [100]. In this case, however, the pro-oxidant effects of MCL1 appear to relate mostly to its capacity to boost mitochondrial respiration directly or via increased Ca^{2+} uptake (see above) [89, 116]. In turn, ROS overgeneration by TNBC cells exposed to

BH3 mimetics reportedly stabilizes MCL1 to support chemoresistance and disease progression [142]. Taken together, these observations delineate the ability of ROS and Bcl-2 proteins to cooperate in support of oncogenesis and tumor progression, reflecting the mitogenic activity of mild ROS elevations that can be prevented from favoring cell death by Bcl-2 overexpression.

In summary, redox regulation by Bcl-2 proteins is not homogenous across all physiological and pathological settings, largely reflecting the divergent impact of decreased OXPHOS (which limits ROS production) and MOMP (which exacerbates ROS production). Key factors that determine whether specific Bcl-2 family members operate as anti- or pro-oxidants include (but are not limited to): (1) bioenergetic metabolism at baseline, and notably OXPHOS *versus* glycolysis usage for ATP synthesis; (2) oxygen availability; and (3) physiological occupancy of anti-apoptotic Bcl-2 proteins by their pro-apoptotic counterparts (also known as mitochondrial priming) [143] (**Figure 4**). Moreover, specific post-translational modifications may be key to convert Bcl-2 family members from anti- to pro-oxidants. As an example, BLC2 phosphorylation at S70 has been shown to occur in the course of oxidative stress as a consequence of the ROS-dependent inactivation of protein phosphatase 2 regulatory subunit B'delta (PPP2R5D) [144, 145]. However, whether this applies to members of the Bcl-2 family other than BCL2 remains obscure.

Concluding Remarks

Although the Bcl-2 family regulates a variety of cellular processes beyond apoptosis, most (if not all) such processes ultimately influence cell fate via cell-intrinsic or -extrinsic pathways (Figure 5, Key Figure). Thus, Bcl-2 proteins appear to orchestrate very complex programs for the maintenance of cellular and organismal homeostasis that intersect with, but are not restricted to, apoptotic cell death [42], with a number of unresolved issues (see Outstanding Questions). First, it remains unclear which of the numerous functions ensured by Bcl-2 proteins in modern mammals evolved first (and hence which of the evolutionary pressures these functions address emerged first). It would be tempting to speculate that such function is fully unrelated to apoptotic regulation, based on the notion that the latter involves (at least in modern mammals) multiple members of the Bcl-2 family, which evolved by gene duplication and diversification events [146]. However, the yeast and plant genomes encode a single protein that contains a rudimentary BH3 domain, physically and functionally interacts with human Bcl-2 proteins including BAX and BCL-X_L and regulate MOMP in a variety of experimental models [147-150]. Interestingly, this protein (whose human homologue is commonly known as Bax inhibitor 1, BI1) is mostly localized to the ER and resembles mammalian Bcl-2 family members in its capacity to regulate autophagy [151], potentially pointing to the latter as to the pristine process regulated by Bcl-2 proteins.

Second, the full complexity of cell fate regulation by Bcl-2 proteins remains to be disentangled, especially with respect to the precise role of ROS. Indeed, ROS are involved in all processes under regulation by Bcl-2 family members, including apoptosis [152], autophagy [153], senescence [154], inflammation [155], energy metabolism [156, 157], and Ca^{2+} fluxes [158], potentially pointing to redox control as the key mechanism though which Bcl-2 proteins ultimately influence cell fate. Supporting this notion, some (apparently) ROS-independent pathways whereby Bcl-2 proteins regulate processes

other than apoptosis ultimately appear to impinge on redox status. As an example, the mtDNA-dependent activation of multiple inflammatory processes downstream of MOMP, including pathways initiated by CGAS, the inflammasome and Toll-like receptor 9 (TLR9), specifically involves oxidized mtDNA [75-77, 79, 159-162]. That said, the precise implication of ROS in other (apparently) ROS-independent pathways controlled by Bcl-2 proteins, such as the inhibition of BECN1 [22], remains to be investigated.

Third, it will be important to clarify the extent to which the pharmacological inhibition of anti-apoptotic Bcl-2 proteins with BH3 mimetics such as venetoclax mediates clinical effects by driving apoptosis directly (*i.e.*, by triggering MOMP) *versus* indirectly (*i.e.*, following the modulation of other cellular processes). Indeed, multiple BH3 mimetics including venetoclax, ABT-737 and the natural compound (-)-gossypol, have been shown to influence autophagy [28, 163], senescence [164], redox homeostasis [133], OXPHOS [165] and Ca^{2+} fluxes [166] in preclinical settings, but whether the same occurs in clinical samples and whether such modulation influence therapeutic responses has not yet been investigated. Preclinical models that enable the dissociation of apoptotic Bcl-2 functions from their non-apoptotic counterparts are urgently awaited in this sense.

Finally, it will be crucial to investigate whether the modulation of Bcl-2 proteins can be exploited as a therapeutic paradigm for human disorders other than cancer. Accumulating data suggest that various inflammatory and allergic conditions may benefit from the administration of BH3 mimetics as a function of the extraordinary sensitivity of specific cell populations (*e.g.*, mast cells) [167]. However, the implementation of similar strategies will have to take the possibility that systemic inhibition of anti-apoptotic Bcl-2 proteins could also support inflammatory reaction downstream of sublethal MOMP under attentive consideration.

In conclusion, Bcl-2 proteins operate at the core of cellular biology to regulate a variety of functions that directly or indirectly impinge on cell fate. We are convinced that the in-depth investigation of these functions from an integrated perspective ultimately will expand the therapeutic potential of current Bcl-2 modulators and favor the development of new molecules for clinical use.

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Box 1. Regulation of Bcl-2 proteins during inflammation

Numerous pro-inflammatory mediators including tumor necrosis factor (TNF), fms related receptor tyrosine kinase 3 ligand (FLT3LG), interleukin 6 (IL6), IL17 and trigger transcriptional programs that encompass the upregulation of anti-apoptotic Bcl-2 proteins [168]. The ability of TNF to drive the transactivation of *BCL2* and *BCL2L1* largely reflects derepression of NF-κB by the IκB kinase (IKK) complex as a consequence of TNF receptor superfamily member 1A (TNFRSF1A) proximal signaling [169, 170]. Ligand-bound fms related receptor tyrosine kinase 3 (FLT3) as well as the constitutively active oncogenic FLT3 variant FLT3-ITD can initiate signal transduction cascades that can favor *BCL2*, *BCL2L1* or *MCL1* transactivation as well as repression of *BAX* or *BCL2L11* (which encodes BH3-only protein known as BIM) [171-174], an effect that (at least in some settings) depends on the transcription factors forkhead box O3 (FOXO3) [173] or signal transducer and activator of transcription 5 (STAT5) [174]. Intriguingly, the capacity of STAT5 to upregulate MCL1 downstream of FLT3-ITD signaling appears to rely on phosphatidylinositol 3-kinase (PI3K)-dependent AKT serine/threonine kinase 1 (AKT1) activation, resulting in stabilization of MCL1 at the protein level [174]. STAT5 and other STAT family members like STAT3 are also intimately involved in the upregulation of anti-apoptotic Bcl-2 family members by IL3 [175], IL6 [176], IL15 [177], IL17 [178] and erythropoietin (EPO) [179]. Moreover, constitutively active variants of *STAT5* or Janus kinase 2 (*JAK2*), which operates immediately upstream of STAT5, have been shown to support malignant transformation in the hematopoietic system correlating with upregulation of anti-apoptotic proteins [180, 181]. Taken together, these observations corroborate the notion that inflammatory processes are generally accompanied by the upregulation of anti-apoptotic Bcl-2 proteins downstream of cytokine signaling, most likely as a mechanism for immune cells (which express cytokine receptors) to avoid the cytotoxic outcomes of inflammation while targeting other cellular compartments of the local

microenvironment (*e.g.*, epithelial cells, endothelial cells). In support of this notion, the cytotoxic activity of TNF plus interferon gamma (IFNG, best known as IFN-γ) on pancreatic β cells, which requires both TNFRSF1A and interferon gamma receptors (IFNGRs), relies on the STAT1-dependent downregulation of *BCL2* [182]. Thus, cytokine receptors and their signal transducers expressed in specific pattern dictate cell fate in the context of inflammation. As multiple tumors develop in the context of indolent inflammation [183, 184], these observations point to the upregulation of anti-apoptotic Bcl-2 proteins as to one of oncogenic mechanisms initiated by inflammation.

Figure 1. Autophagy regulation by Bcl-2 proteins. A. BCL2, BCL-X_L, BCL-W and MCL1 engage in inhibitory physical interactions with the core autophagy regulator beclin 1 (BECN1), hence limiting its ability to drive autophagic responses as part of a multiprotein complex with class III phosphatidylinositol 3-kinase (PI3K) activity. In line with this model, select BH3-only proteins including BID and BAD, chemical BH3 mimetics, as well as the mitophagy mediator dynamin 1 like (DNM1L; best known as DRP1), promote autophagy upon displacement of BECN1 from inhibitory interactions with Bcl-2 proteins. **B.** In addition, MCL1 has been shown to indirectly inhibit autophagy by virtue of its ability to compete with BECN1 for stabilization by the deubiquitinase ubiquitin specific peptidase 9 X-linked (USP9X). PI, phosphatidylinositol; PI3P, phosphatidylinositol 3-phosphate; VPS34 (official name: PIK3C3): phosphatidylinositol 3-kinase catalytic subunit type 3.

Figure 2. Control of inflammation by Bcl-2 family members. The Bcl-2 family is intimately involved in the control of inflammatory processes, largely reflecting the ability of anti-apoptotic Bcl-2 proteins including BCL2 and BCL-X_L to limit NF-κB, inflammasome, cyclic GMP-AMP synthase (CGAS) and prostaglandin E_2 (PGE_2) secretion as well as the release of extracellular matrix-remodeling enzymes by mitochondrial outer membrane permeabilization (MOMP)-dependent or MOMP-independent mechanisms. In addition, BCL2 has been reported to modulate inflammation downstream of reactive oxygen species (ROS) overgeneration, at least in some settings. CASP, caspase; IAP, inhibitor of apoptosis protein; IFN, interferon; IL, interleukin; PM, plasma membrane; PTGS2, prostaglandin-endoperoxide synthase 2; STAT3, signal transducer and activator of transcription 3; TGFB1, transforming growth factor beta 1.

Figure 3. Bioenergetic metabolism, Ca^{2+} fluxes and Bcl-2 proteins. BCL2, BCL-X_L and MCL1 generally reduce Ca^{2+} levels within the endoplasmic reticulum (ER) as they favor Ca^{2+} release by inositol 1,4,5 trisphosphate receptors (IP_3Rs). Cytosolic Ca^{2+} enters mitochondria via voltage-

dependent anion channels (VDACs) at the outer mitochondrial membrane (OMM) and mitochondrial calcium uniporter (MCU) at the inner mitochondrial membrane (IMM), ultimately boosting oxidative phosphorylation (OXPHOS), reactive oxygen species (ROS) production and ATP synthesis. The modulation of VDAC functions by Bcl-2 proteins most likely exhibits considerable degree of context dependency. Anti-apoptotic Bcl-2 proteins also boost oxidative phosphorylation (OXPHOS) by favoring respiratory complex IV (CIV) and CV activity or stability. Moreover, MCL1 supports fatty acid oxidation (FAO) in a BIM-regulated manner. Finally, BOK appears to support mitochondrial homeostasis by interacting by IP_3Rs, but in the absence of over alterations in Ca^{2+} fluxes. ETC, electron transport chain; MCU, mitochondrial calcium uniporter.

Figure 4. Bcl-2 family members in redox homeostasis. Specific Bcl-2 proteins can regulate redox homeostasis in diametrically opposed manners, at least in part as a function of baseline bioenergetic metabolism, oxygen availability, and mitochondrial priming. In particular, BCL2 and BCL-X_L appear to enable baseline reactive oxygen species (ROS) production to increase via oxidative phosphorylation (OXPHOS)-dependent and OXPHOS-independent mechanisms, thus favoring cell proliferation, and at the same time protecting cells from ROS overgeneration and consequent cell death. Both these redox functions of anti-apoptotic Bcl-2 proteins can be abolished by BH3 mimetics.

Figure 5. Key Figure. Integrated cell fate regulation by the Bcl-2 family. Bcl-2 family members control multiple cellular processes beyond mitochondrial outer membrane permeabilization (MOMP), including autophagy, senescence, inflammation, bioenergetic metabolism, Ca^{2+} fluxes and redox homeostasis, which can all impinge on cellular fate via cell-intrinsic or -extrinsic pathways.

Table 1. Non-canonical cell fate regulation by Bcl-2 proteins

Protein	Main localization*	Canonical function	Non-canonical process	Non-canonical effect	Depends on MOMP?	Interactor(s)	Ref.
BAD	Cytosol	BH3-only	Autophagy	Activation	N	BCL2 BCL-X_L MCL1	[28]
BAD	Cytosol	BH3-only	Metabolism	Activation	N	GCK	[102, 103]
BAK1	OMM ER	Multidomain pro-apoptotic	Ca^{2+} homeostasis	ER Ca^{2+} retention	N	BCL2 BCL-X_L MCL1	[117]
BAK1	OMM ER	Multidomain pro-apoptotic	Inflammation	Pro-inflammatory	Y	BAX	[70, 76-78]
BAX	Cytosol	Multidomain pro-apoptotic	Ca^{2+} homeostasis	ER Ca^{2+} retention	N	BCL2 BCL-X_L MCL1	[117]
BAX	Cytosol	Multidomain pro-apoptotic	Inflammation	Pro-inflammatory	Y/N	BAK1 PTGS2?	[70, 76-78, 84, 85]
BAX	Cytosol	Multidomain pro-apoptotic	Redox homeostasis	Pro-oxidant	Y/N	BCL2	[133, 135]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Autophagy	Inhibition	N	BECN1	[21, 22, 28, 29]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Ca^{2+} homeostasis	ER-to-mitochondria Ca^{2+} transfer	N	IP_3Rs VDAC	[109-112]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Inflammation	Anti-inflammatory	Y/N	BAK1 BAX IκBα NF-κB	[69, 70, 72, 75-77]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Metabolism	Activation	N	COX5A	[90]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Redox homeostasis	Anti-oxidant	Y/N	BAK1 BAX	[132, 133]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Redox homeostasis	Pro-oxidant	N	COX5A RAC1	[90, 139]
BCL2	OMM ER MAMs Nucleus	Multidomain anti-apoptotic	Senescence	Establishment and maintenance	Y/N	?	[43, 44, 46-49, 55]
BCL-W	OMM ER	Multidomain anti-apoptotic	Autophagy	Inhibition	N	BECN1	[29]
BCL-W	OMM ER	Multidomain anti-apoptotic	Senescence	Establishment and maintenance	?	?	[50]
BCL-X_L	OMM ER MAMs	Multidomain anti-apoptotic	Autophagy	Inhibition	N	BECN1	[27–29]

BCL-X_L	OMM ER MAMs	Multidomain anti-apoptotic	Ca^{2+} homeostasis	ER-to-mitochondria Ca^{2+} transfer	N	IP_3Rs	[113-115]
BCL-X_L	OMM ER MAMs	Multidomain anti-apoptotic	Inflammation	Anti-inflammatory	Y	BAK1 BAX IκBα	[69, 76]
BCL-X_L	OMM ER MAMs	Multidomain anti-apoptotic	Metabolism	Activation	N	CV	[97]
BCL-X_L	OMM ER MAMs	Multidomain anti-apoptotic	Senescence	Establishment and maintenance	Y/N	?	[50, 51, 53, 54]
BID	Cytosol	BH3-only	Autophagy	Activation	N	BCL2 BCL-X_L MCL1	[29]
BID	Cytosol	BH3-only	Inflammation	Pro-inflammatory	Y	BCL2 BCL-X_L	[84]
BIM	Cytoskeleton	BH3-only	Metabolism	Inhibition	N	MCL1?	[101]
BOK	OMM ER	Multidomain pro-apoptotic	Metabolism	Activation	N	IP_3Rs	[120]
MCL1	OMM ER	Multidomain anti-apoptotic	Autophagy	Inhibition	N	BECN1, USP9X	[28, 29, 38]
MCL1	OMM ER	Multidomain anti-apoptotic	Ca^{2+} homeostasis	ER-to-mitochondria Ca^{2+} transfer	N	IP_3Rs VDAC	[111, 116]
MCL1	OMM ER	Multidomain anti-apoptotic	Inflammation	Inhibition	Y	BAX BAK1	[70, 76, 78]
MCL1	OMM ER	Multidomain anti-apoptotic	Metabolism	Activation	N	CV VLCAD	[89, 104]
MCL1	OMM ER	Multidomain anti-apoptotic	Redox homeostasis	Anti-oxidant	N	NOX4	[59]
MCL1	OMM ER	Multidomain anti-apoptotic	Senescence	Establishment and maintenance	Y/N	BAX BAK1 NOX4?	[57]
NOXA	Cytosol	BH3-only	Autophagy	Activation	N	MCL1	[38]

Abbreviations: COX5A, cytochrome c oxidase subunit 5A; ER, endoplasmic reticulum; GCK, glucokinase; IP_3R, inositol 1,4,5 trisphosphate receptor; MAMs, mitochondria-associated ER membranes; MOMP, mitochondrial outer membrane permeabilization; NOX4, NADPH oxidase 4; OMM, outer mitochondrial membrane; RAC1, Rac family small GTPase 1; USP9X, ubiquitin specific peptidase 9 X-linked; VLCAD, very long-chain acyl-CoA dehydrogenase. *at baseline in physiological conditions.

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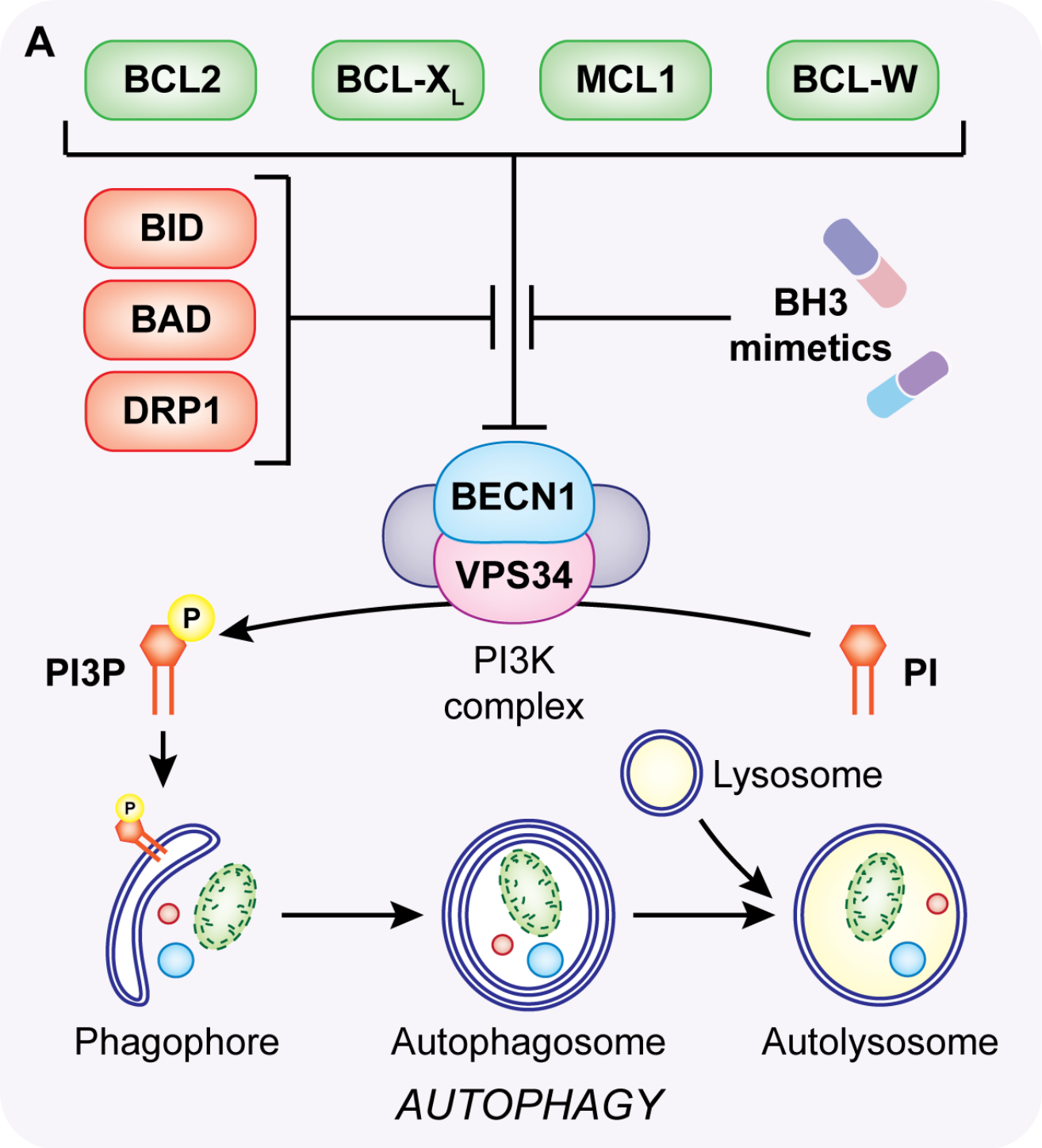
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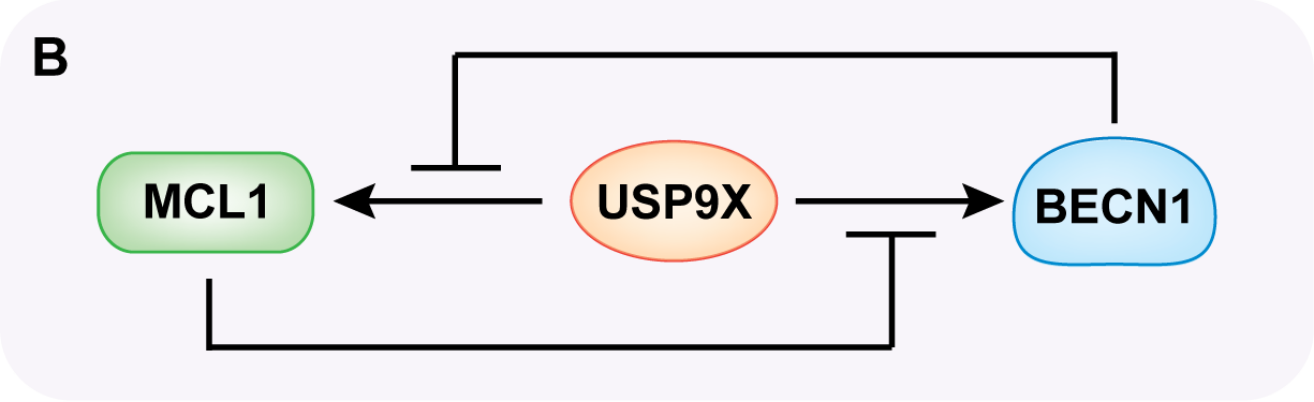
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Figure 1

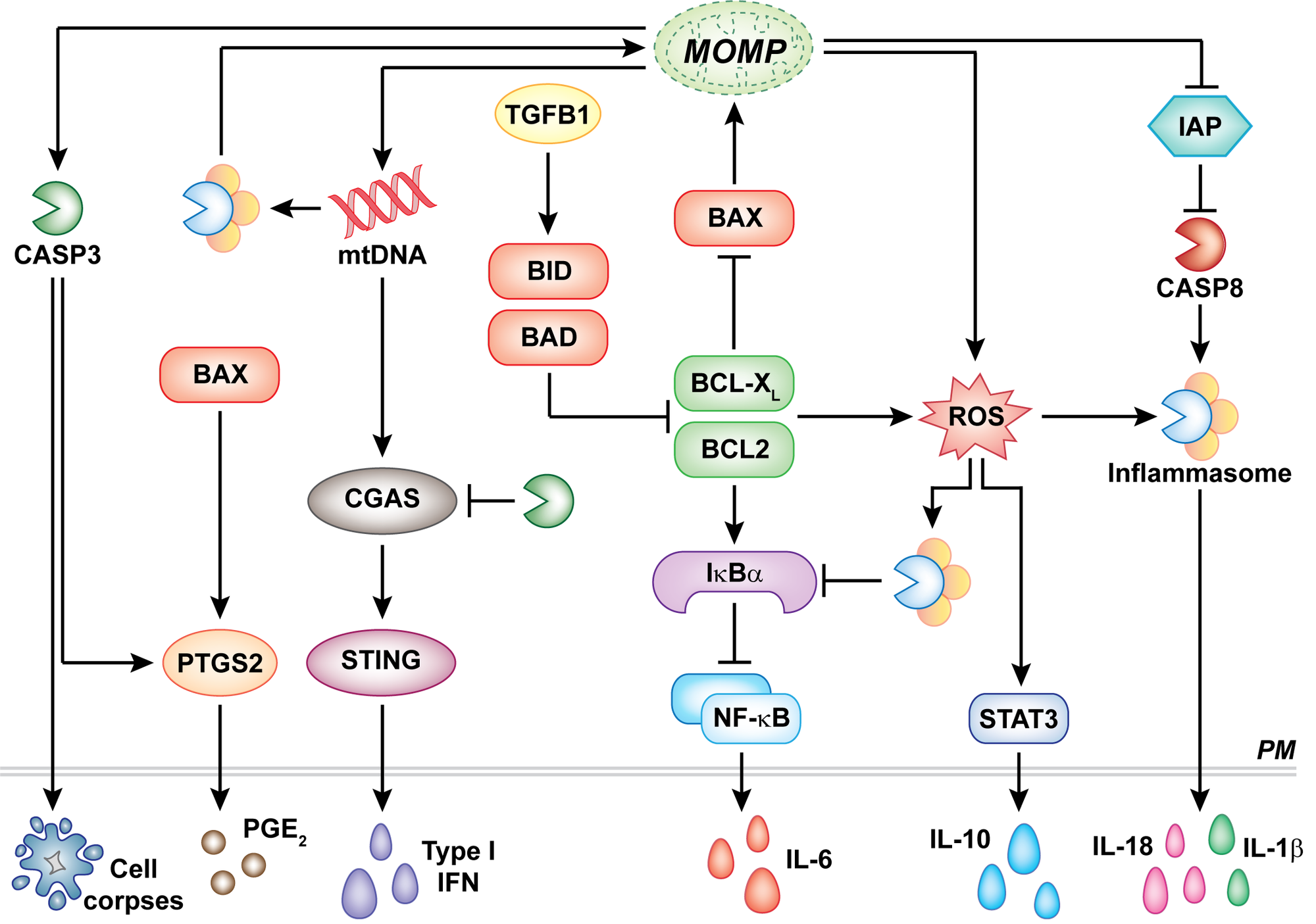
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Figure 2

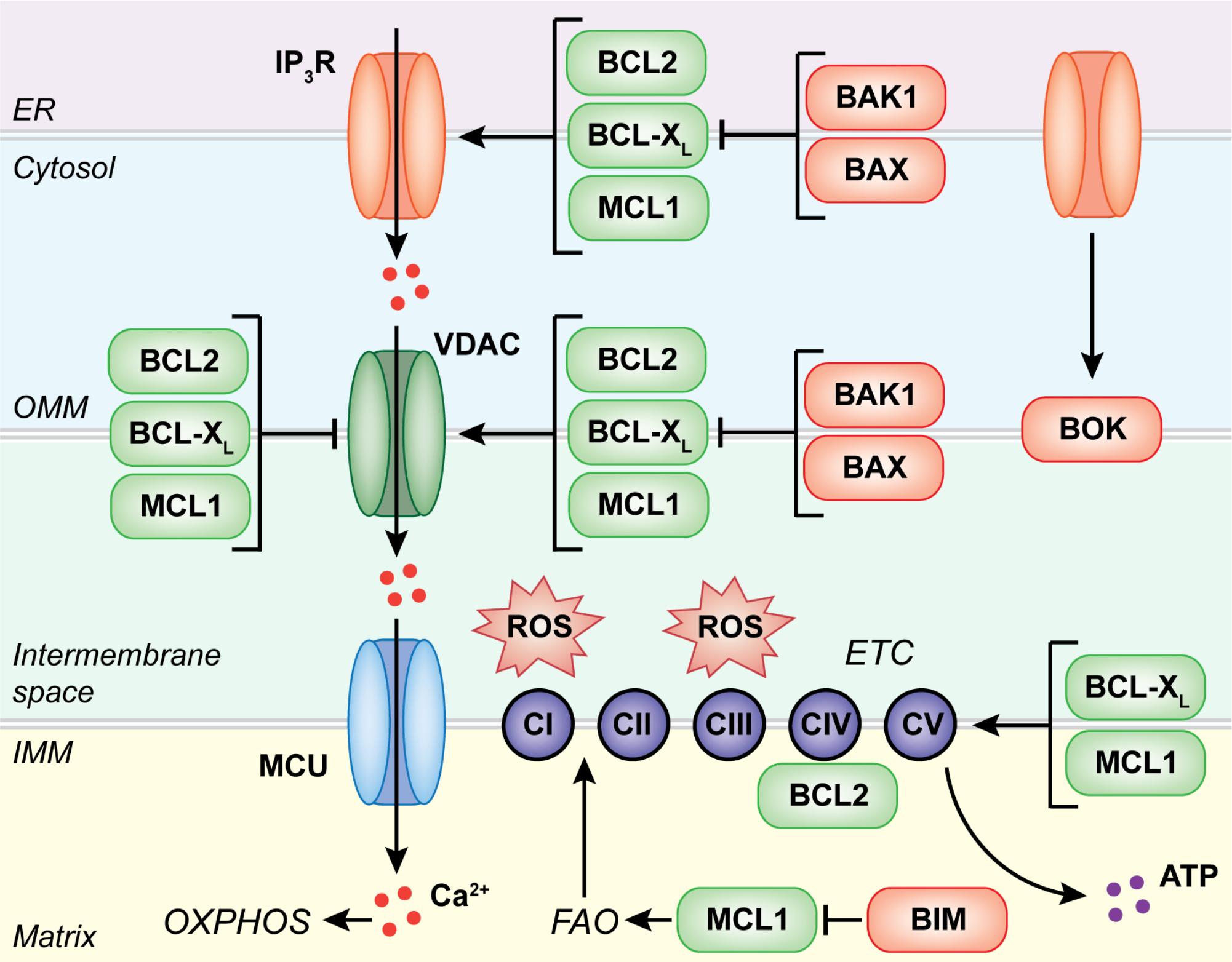
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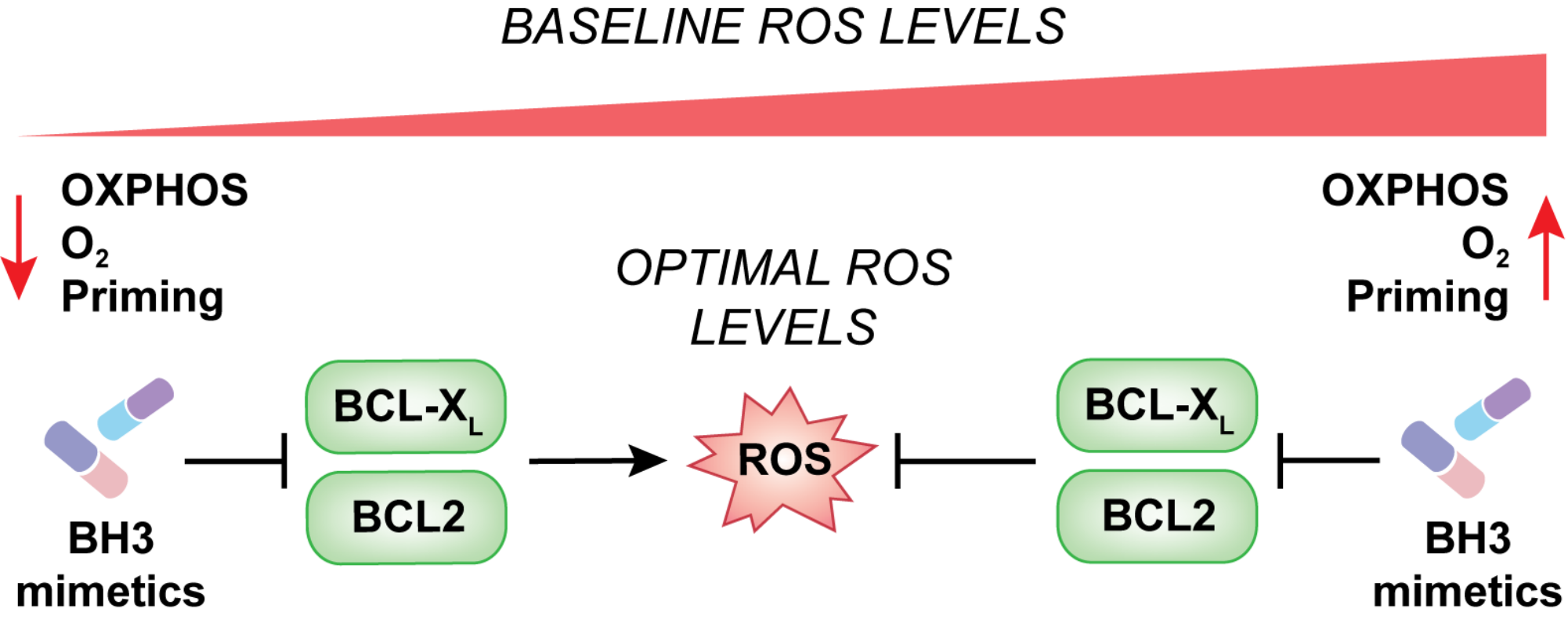
Figure 3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BH3 mimetics
BH3 mimetics

Figure 4