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Functional assessment of Bcl-2 family proteins in mitochondrial metabolism and beyond

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I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree.

Prague, 2024, MSc Dana Sovilj

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Contents

Abstract (EN)

Since their initial discovery in *C. elegans* and human cells more than 30 years ago, proteins from the Bcl-2 family were intrinsically linked with the induction, regulation and suppression of the mitochondrial branch of apoptotic signaling in mammalian cells but they also appear to modulate non-apoptotic signaling pathways. In this study we aimed, through comprehensive analysis, to enhance current knowledge of non-apoptotic roles of the main pro-apoptotic Bcl-2 family proteins BAX and BAK, with a specific focus on their role in cellular metabolism. We employed the CRISPR/Cas9 technique to generate Bax/Bak-deficient cancer cells of diverse tissue origins and assed impact of combined Bax and Bak deficiency on mitochondrial respiration and cellular glycolysis. While the ablation of Bax and Bak expression had no discernible effect on glycolysis across all tested cell lines, it modulated mitochondrial respiration in cell-type-specific manner. Elimination of Bax and Bak expression in HCT-116 colorectal cancer cells had no impact on mitochondrial respiration, but it largely affected mitochondrial respiration in Bax/Bak-deficient glioblastoma (U87) and lymphoma (HBL-2, UPF1H, UPF1G) cells. Bax/Bak-deficient U87 cells notably upregulated mitochondrial respiration as well as accelerated their proliferation and tumor growth in NSG mice. In contrast, Bax/Bak-deficient HBL-2 cells showed attenuated respiration and slower growth both in vitro and in vivo. The alterations in respiration were in Bax/Bak-deficient U87 cells accompanied by changes in metabolic pattern, such as an increase in NAD+/NADH ratio, increased mitochondrial membrane potential (MMP), and higher production of ATP. Subsequent analyses revealed upregulation/downregulation of mitochondria-encoded subunits of the respiratory chain complexes and stabilization/destabilization of the mitochondrial transcription elongation factor TEFM in Bax/Bak-deficient U87 and HBL-2 cells, respectively. Downregulation of TEFM expression using shRNAs resulted in attenuated mitochondrial

respiration not only in Bax/Bak-deficient U87 cells but also in parental HBL-2 cells. Our findings revealed that (post)translational regulation of TEFM protein levels in Bax/Bak-deficient cells modulates the expression levels of mitochondrial respiratory complex subunits, thereby influencing cellular respiration, metabolism, and proliferation dynamics. In collaborative projects, we participated in the assessment of the role Bcl-2 family proteins and mitochondrial metabolism in hematopoietic malignancies uncovering their response and resistances to therapeutically relevant BH3 mimetics.

Key words

BAX, BAK, Bcl-2 family, apoptosis, BH3 mimetics, metabolism, mitochondrial respiration, TEFM.

Abstract (CZ)

Od jejich prvotní identifikace v háďátku C. elegans a také v lidských buňkách před více než 30 lety jsou proteiny z rodiny Bcl-2 spojovány s indukcí, regulací a potlačením mitochondriální apoptotické signalizace, ale také se mohou uplatňovat při modulaci neapoptotických signálních dráh. V této studii jsme si stanovili za hlavní cíl rozšířit stávající znalosti o neapoptotických rolích hlavních proapoptotických proteinů z Bcl-2 rodiny, BAX a BAK, zejména pak na jejich roli v buněčném metabolismu. Pomocí genové editace využitím CRISPR/Cas9 jsme eliminovali expresi těchto proteinů v lidských nádorových buňkách různého tkáňového původu a v těchto Bax/Bak-deficitních buňkách jsme primárně analyzovali mitochondriální respiraci a buněčnou glykolýzu.

Zatímco eliminace exprese Bax a Bak neměla žádný patrný vliv na glykolýzu ve všech testovaných buněčných liniích, v závislosti na buněčném typu modulovala mitochondriální respiraci. Eliminace exprese Bax a Bak v buňkách rakoviny tlustého střeva HCT-116 neměla vliv na mitochondriální respiraci, ale zjevně ovlivnila mitochondriální respiraci v Bax/Bak-deficitních buňkách odvozených od glioblastomu (U87) a lymfomů (HBL-2, UPF1H, UPF1G). Bax/Bak -/- buňky U87 významně upregulovaly mitochondriální respiraci a akcelerovaly svou proliferaci a také nádorový růst nádoru u NSG myších. Naopak Bax/Bak -/- HBL-2 buňky vykazovaly sníženou respiraci a také pomalejší růst jak in vitro, tak in vivo. Změny v respiraci byly u Bax/Bak-deficitních U87 buněk doprovázeny změnami v buněčném metabolismu, jako nárůstem poměru NAD+/NADH, zvýšením mitochondriálního membránového potenciálu (MMP) a vyšší produkcí ATP. Následné analýzy odhalily zvýšení (U87) či snížení (HBL-2) proteinové exprese mitochondriálně kódovaných podjednotek respiračních řetězců a stabilizaci (U87) či destabilizaci (HBL-2) mitochondriálního faktoru TEFM v Bax/Bak-deficitních buňkách. Potlačení exprese TEFM pomocí shRNA vedlo k utlumení mitochondriální respirace nejen u buněk Bax/Bak -/- U87 buněk, ale i u parentálních HBL-2 buněk. Z našich výsledků plyne, že (post)translační regulace hladin proteinu TEFM v Bax/Bak-deficitních buňkách moduluje hladiny exprese mitochondriálních podjednotek respiračních komplexů, čímž ovlivňuje buněčnou respiraci, metabolismus a dynamiku proliferace. V rámci vědecké spolupráce jsme se podíleli na posouzení role proteinů z Bcl-2 rodiny a mitochondriálního metabolismu v odpovědi hematopoietických nádorových buněk na terapeuticky relevantní BH3 mimetika.

List of Abbreviations

AML - Acute Myeloid Leukemia

ALL - Acute Lymphoblastic Leukemia

ANT - Adenine Nucleotide Translocator

ATP - Adenosine Triphosphate

BAX - Bcl-2-Associated X protein

BAK - Bcl-2 Homologous Antagonist/Killer protein

BCL-W - B-Cell Lymphoma-W

BCL-XL - B-Cell Lymphoma-eXtra Large

BCL-2 - B-Cell Lymphoma 2 protein

BNE - Blue Native Electrophoresis

BOK - Bcl-2 Related Ovarian Killer

CDK - Cyclin-Dependent Kinase

COX - Cytochrome c Oxidase

DHODH - Dihydroorotate Dehydrogenase

DLBCL - Diffuse Large B-Cell Lymphoma

DRP1 - Dynamin-Related Protein 1

ETC - Electron Transport Chain

ER - Endoplasmic Reticulum

FAO - Fatty Acid Oxidation

GTP - Guanosine Triphosphate

HSC - Hematopoietic Stem Cell

IMM - Inner Mitochondrial Membrane

IMS - InterMembrane Space

IP3R - Inositol TrisPhosphate Receptor

MCL - Mantle Cell Lymphoma

MCL-1 - Myeloid Cell Leukemia sequence 1

MFN - Mitofusin

MMP - Mitochondrial Membrane Potential

MOMP - Mitochondrial Outer Membrane Permeabilization

MtDNA - Mitochondrial DNA

MPC - Mitochondrial Pyruvate Carrier

MPTP - Mitochondrial Permeability Transition Pore

NAD+ - Nicotinamide Adenine Dinucleotide

NADH - Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)

ND - NADH Dehydrogenase

NSG - NOD Scid Gamma

OMM - Outer Mitochondrial Membrane

OXPHOS - Oxidative Phosphorylation

PDX - Patient-Derived Xenografts

PKA - Protein Kinase A

POLRMT - RNA Polymerase Mitochondrial

PTP - Permeability Transition Pore

ROS - Reactive Oxygen Species

RCD - Regulated Cell Death

RC - Respiratory Complex

SDH - Succinate Dehydrogenase

SOD - Superoxide Dismutases

TCA - Tricarboxylic Acid Cycle

TEFM - Mitochondrial Transcription Elongation Factor

TFAM - Transcription Factor A, Mitochondrial

TOM - Translocase of the Outer Mitochondrial Membrane

TP53 - Tumor Protein 53

UPR - Unfolded Protein Response

VDAC - Voltage-Dependent Anion Channel

Introduction

Regulated cell death, apoptosis

Regulated cell death (RCD) is a fundamental process occurring in all cellular life forms, from single cell organisms as yeasts and protists to multicellular ones from both plant and animal kingdoms (2-5). By the definition RCD represents regulated. i.e. non-accidental cell death modalities, which lead to lytic or non-lytic cell demise. In mammalian cells among these modalities belong apoptosis, necroptosis, pyroptosis, ferroptosis and several others. The most known among them and in the last 50 years extensively studied is apoptosis (3, 6). The term 'apoptosis' was invented in 1972 by John F. R. Kerr and his colleagues for the definition of ordered cellular destruction in both adult organisms and during embryogenesis. The term apoptosis originates from the Greek words 'apo,' meaning 'away from,' and 'ptosis,' meaning ‘falling’ (6). Apoptosis is evolutionary conserved process that is essential for both the development and physiological homeostasis of organisms, and also plays important role under pathological conditions (7). Apoptosis is characterized by distinct features such as caspase activation, cellular shrinkage, membrane blebbing, and DNA fragmentation and represents in contrast to most of RCD modalities non-lytic, i.e. non-inflammatory mode of cell death (8). The apoptotic signalling can be initiated either via the extrinsic pathway, which largely involves the death receptors such as Fas/CD95, TRAIL receptors DR4/DR5 and TNFR1 and/or by the intrinsic, mitochondrial signalling with the essential participation of mitochondria (9). The intrinsic pathway is activated in response to cellular stresses such as DNA damage, oncogene activation, viral infection, or cellular exposure to radiation (9, 10). Both extrinsic and intrinsic apoptotic signalling pathways rely on the activation and activity of unique family of cysteine proteases named caspases. These enzymes can be divided into two groups: a/initiator caspases (e.g., caspase-8 and caspase-9) and

b/executioner caspases (e.g., caspase-3, caspase-6, and caspase-7), which cleave many specific

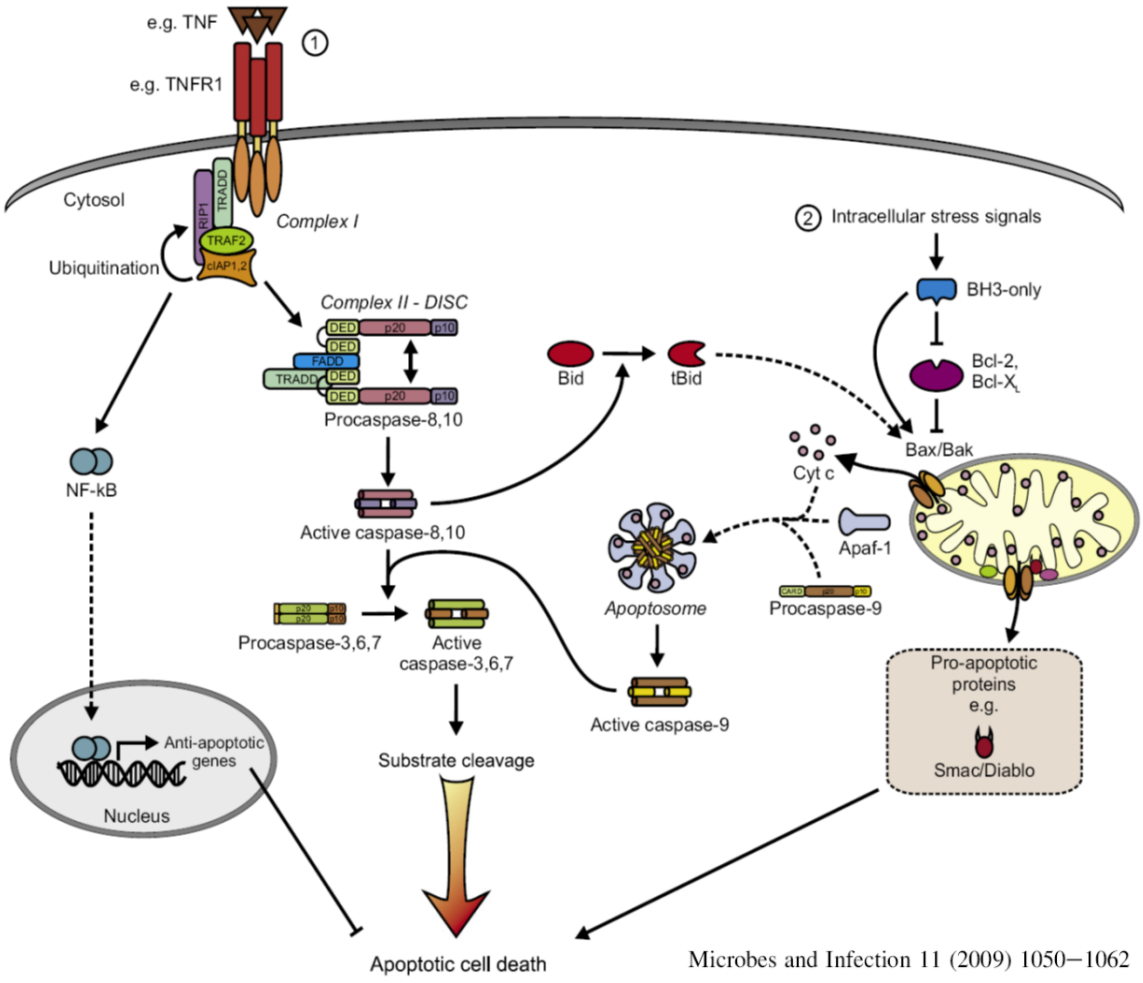

Figure 1. The basic signaling pathways of extrinsic (1) and intrinsic (2) apoptosis. Adapted from (1).

cellular targets and ensure cell demise (11, 12). The extrinsic and intrinsic apoptotic signalling is illustrated in Figure 1.

Proteins of the BCL-2 family

The activation and regulation of intrinsic apoptotic signalling is dependent on distinct group of proteins – members of the Bcl-2 family. Its first member, BCL-2 itself, was in 1984 discovered as

a protein overexpressed in follicular B-cell lymphoma (13). Its overexpression resulted from the translocation of Bcl2 gene from chromosome 18 to chromosome 14 next to the immunoglobulin heavy chain locus (6). Overexpressed BCL-2 enhanced the survival of tumor cells, marking it as a proto-oncogene (14). The intrinsic apoptotic pathway, which is largely regulated by Bcl-2 family proteins, relies on the pores/channels-mediated disruption of the outer mitochondrial membrane (OMM) and subsequent release of several pro-apoptotic proteins from the mitochondrial

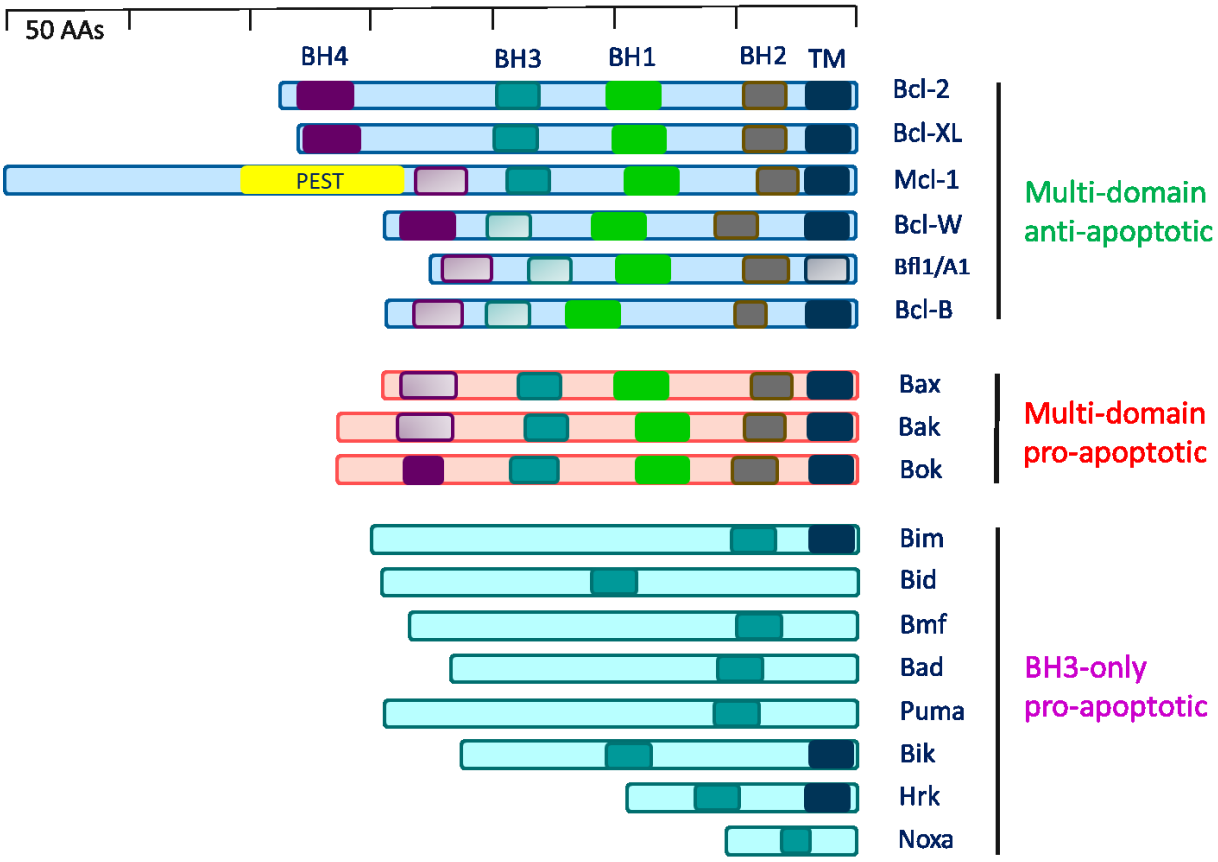

Figure 2. Overview of multi-domain anti-apoptotic, multi-domain pro-apoptotic and BH3-only members of human Bcl-2 family.

intermembrane space into the cytoplasm (15-19). One of these factors is cytochrome c, which in cytoplasm initiates formation pro-apoptotic complex named apoptosome. This complex also contains Apaf-1 adaptor protein and the initiator pro-caspase 9 and serves as a platform for autoprocessing-mediated activation of the initiator caspase-9. Among other proteins released from

mitochondria belong Smac/Diablo (binds to and neutralizes apoptosis inhibitory proteins (IAPs) in the cytoplasm), then nucleases Endonuclease G and AIF (both participate in cleavage of chromosomal DNA). Proteins from the Bcl-2 family contain 1-4 Bcl-2 Homology (BH) domains, and can be further sub-divided into three distinct subgroups: a/ multidomain anti-apoptotic (BCL-2, BCL-XL, MCL-1, BCL-W), b/multidomain pro-apoptotic (BAX, BAK, BOK), and c/BH3-only (such as BIM, BID, PUMA, BAD and others) proteins (20, 21). The pro-apoptotic BAX, BAK, and BOK can form large pores in OMM and the formation of these pro-apoptotic pores is prevented by their sequestering and dimerization with anti-apoptotic Bcl-2 proteins (20, 22, 23). Pro-apoptotic signalling triggers expression/release of BH3-only members of Bcl-2 family, which by competitive binding to the anti-apoptotic Bcl-2 proteins can release the pro-apoptotic ones. Released BAX and BAK then can form pores in the OMM leading both to attenuation of mitochondrial membrane potential and to release of cytochrome c and other proteins. Some BH3-only proteins such as truncated BID (tBID), BIM or PUMA can in addition also enhance pore formation capabilities of BAX and BAK proteins. Overview of the human Bcl-2 family members is shown in Figure 2. The dimerization of Bcl-2-family proteins occurs through protein-protein interactions dependent on their BH3 domain, which largely affects their ability to regulate apoptosis and form pores in OMM (24-29). The abundance and ratio between anti-apoptotic and pro-apoptotic Bcl-2 family proteins thus determine cellular sensitivity/resistance to apoptotic stimuli (27). Cells of different origin can have same sets of Bcl-2 family proteins, but various levels and posttranslational modifications of these proteins resulting in their different response to apoptotic stimuli (23). According to one model, apoptosis is triggered, when activated or induced BH3-only proteins neutralize all expressed anti-apoptotic proteins (30). This event leads to the release of BAX or BAK, which were initially bound and kept inactive by pro-apoptotic proteins

in healthy cells. The released BAX or BAK can then oligomerize, form pores and permeabilize OMM. Not all Bcl-2 proteins have the same affinities for dimerization with each other. PUMA, tBID and BIM exhibit comparable affinities for binding to all pro-survival Bcl-2 proteins, while BAD and BMF exclusively bind to BCL-2, BCL-XL, and BCL-W. In contrast, NOXA specifically binds to BFL1/A1 and MCL-1 (31). As mentioned above, three BH3-only proteins, namely BIM, tBID, and PUMA, can in addition to binding to anti-apoptotic Bcl-2 family proteins also enhance BAX/BAK-mediated pore formation via their direct binding to BAX or BAK (21, 28, 30, 31). These proteins are also referred to as 'direct activators.' On the other hand, BH3-only proteins such as NOXA, HRK, and BIK, classified as 'repressors,' carry out their apoptotic functions by just binding to anti-apoptotic Bcl-2 proteins and inhibiting their function. In non-apoptotic cells, the pro-apoptotic protein BAX largely resides in the cytosol (22, 32-35). Upon certain pro-apoptotic stimuli such as oxidation of its Cys62, BAX undergoes conformational changes, facilitating its translocation to the OMM. Once at the OMM, BAX inserts its transmembrane (TM) domain helix α9 into the membrane, oligomerizes with other BAX molecules, and forms pores. It is important to note that BAX is not exclusively a transmembrane protein, as it has both cytosolic and transmembrane regions (36). BAK, another pro-apoptotic protein, is primarily a transmembrane protein and is constitutively associated with the OMM (36). Pro-apoptotic stimuli exemplified by BIM binding to BAK lead to destabilization of helix α1, enabling BAK oligomerization with other BAK molecules (37). Structural analyses indicate that the BH3 peptide, an amphipathic α-helix spanning approximately 16–20 amino acids, binds to a hydrophobic pocket on anti-apoptotic Bcl-2-family proteins. This elucidates the structural foundation underlying the antagonistic interaction between pro- and anti-apoptotic family members, paving the way for drug discovery strategies focused on mimicking BH3 peptides with chemical compounds that target the same pocket (29,

38). In addition to their presence in the cytosol and mitochondrial membranes, Bcl-2 proteins can be also localized in the endoplasmic reticulum, nucleus, nuclear envelope, and Golgi apparatus (Figure 3) (39). This diverse localization confers additional functions to these proteins within the cell. The expression of Bcl-2 family proteins varies across distinct tissue types, and is meticulously regulated at the transcriptional, posttranscriptional, and translational levels. For instance, BCL-XL is vital for neuronal survival, and its absence leads to embryonic death (40). MCL-1 is expressed in diverse cell types, including hematopoietic cells, hepatocytes, cardiomyocytes, and neuronal cells and it has an essential role during embryonic development (41). BCL-2 and BCL-W exhibits a broad expression pattern across various tissues, including the testes, where BCL-W plays a crucial role in spermatogenesis (42, 43). The expression of BH3-only proteins PUMA and NOXA is in response to DNA damage upregulated at the transcriptional level by the tumor supresor and transcriptional factor TP53 (44, 45). Similar to BH3-only proteins, the pro-apoptotic proteins BAX and BAK also exhibit different affinities for the anti-apoptotic members of the Bcl-2 family. While BAX binds to all of them, BAK preferably interacts with BCL-XL and MCL-1 only (46). The expression and activity of the third pro-apoptotic Bcl-2 family member, BOK is regulated at the translational and posttranslational (proteosomal degradation) levels (47).

BCL-2 family proteins and cancer

In the context of carcinogenesis, various proteins from the Bcl-2 family can be categorized as either proto-oncogenes or tumor suppressors. Anti-apoptotic proteins, such as BCL-2, are recognized as proto-oncogenes with BCL-2 being overexpressed in small cell lung cancer, non-Hodgkin lymphoma and other malignancies (48-50). Moreover, the overexpression of other anti-apoptotic members of the Bcl-2 family is prevalent in many human solid and hematopoietic

cancers, thus contributing to tumor progression and poor prognosis (51-53). Conversely, BAX and BAK are recognized as tumor suppressors mainly due to their ability to induce apoptosis. Deletion and loss of function of BAX was found in several types of cancer, including colon adenocarcinoma, gastric and endometrial carcinomas and its loss promotes tumorigenesis and is correlated with a worse prognosis in patients (54-57). Recent report also documents that mutations in the BAX gene represent a novel mechanism of resistance to venetoclax (BH3 mimetic, BCL-2 antagonist) treatment in AML patients (58). Furthermore, in experimental settings, such mutations were shown to confer resistance to other BH3 mimetics as well. It is important to note that BAX and BAK are functionally interchangeable in initiating apoptosis. Rare loss of both BAX and BAK expression, resulting in resistance to intrinsic apoptosis, was documented in human AML patients (42). Indirectly, mutations in the TP53 transcription factor, which are prevalent in human cancers, can lead to altered expression of certain Bcl-2 family proteins, such as BAX, BID, PUMA, and NOXA (44, 59-61). Overexpressed, proto-oncogenic anti-apoptotic proteins from Bcl-2 family can be targeted by specific compounds known as BH3 mimetics (62-64). These compounds mimic BH3 domain and can specifically target anti-apoptotic proteins such as BCL-2, MCL-1, or BCL-XL. They sequester BAX, BAK and BH3-only proteins from these anti-apoptotic proteins, which are then free to initiate apoptosis in cancer cells. Venetoclax, selective inhibitor of human BCL-2, is the first therapeutic in this class, approved by the FDA for the treatment of AML and CLL and several other BH3 mimetics are currently in clinical trials (63, 64). In addition to the indirect approach of initiating apoptosis by targeting anti-apoptotic proteins, there are also compounds designed to directly target and activate BAX and BAK proteins (65-67).

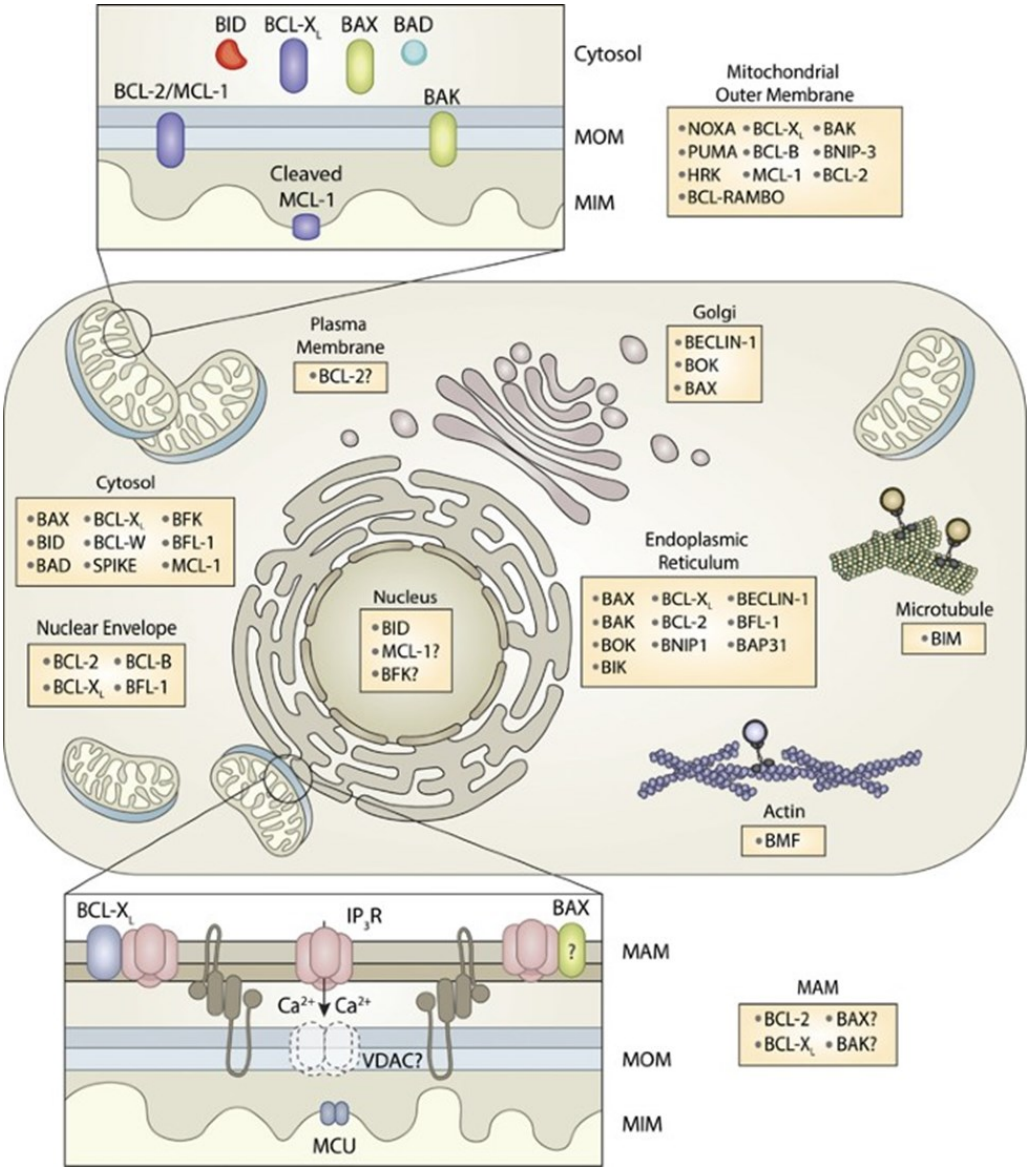

Figure 3. Cellular expression/localization of Bcl-2 family proteins. Adapted from (39).

Non-apoptotic functions of BCL-2 family proteins

In addition to their well-established role in apoptosis, Bcl-2 family proteins also participate in the regulation of various non-apoptotic cellular processes such as maintaining calcium homeostasis, shaping mitochondrial morphology, regulating mitochondrial permeability transition, facilitating

mitophagy, and participating in the DNA damage response (40, 41). This multifaceted involvement underscores the diverse and pivotal regulatory roles these proteins play in cellular physiology (68, 69). The anti-apoptotic BCL-2, BCL-XL, and MCL-1 proteins are also involved in the regulation of calcium homeostasis in the endoplasmic reticulum (ER) via their interaction with sixth transmembrane (TM) domain of the inositol trisphosphate (IP3) receptor (70, 71). BCL-2 can also interact with primary calcium exchanger on ER, the SERCA pump, and inhibit calcium release into the cytoplasm (72). BAX and BAK also participate in the regulation of calcium dynamics and their loss is accompanied by an increase in calcium leak through IP3R, resulting in decreased mitochondrial intake (73). Mitochondrial BCL-XL and BCL-2 can also enhance calcium uptake by mitochondria and its mitochondrial accumulation (74, 75). Another connection of Bcl-2 proteins with the ER involves their potential role in the regulation of the Unfolded Protein Response (UPR) process (76). IRE1-alpha, a crucial protein in UPR signaling responsible for the regulation of transcription of genes important for the recovery phase of UPR, is associated with and apparently activated by BAX and BAK (76). IRE1, through its kinase domain, also participates in activation of JNKs, which then can phosphorylate BCL-2 and BCL-XL proteins, thereby suppressing their anti-apoptotic function (77, 78). Proteins from the Bcl-2 family might also participate in the regulation of mitochondrial fusion and fission, processes, which are crucial for maintaining mitochondrial function, structure, and cellular homeostasis. During apoptosis, mitochondrial fission regulating dynamin-related protein 1 (DRP-1) apparently enhances oligomerization of BAX and pore formation, and BAK interaction with mitofusins MFN1 and MFN2 enhanced mitochondrial fragmentation during apoptosis (79, 80). In contrast, in non-apoptotic cells co-localization/interaction of BAX with DRP-1 apparently enhanced mitochondrial fusion at the fusion site (81). Recombinant soluble BAX also interact with MFN2 and promotes

mitochondrial fusion (82). BAX overexpression leads to more elongated mitochondria in DU145 cells, while elimination of BAX expression in HCT-116 cells caused mitochondrial fragmentation (81). These results demonstrate that BAX and BAK play pivotal roles not only in mitochondrial dynamics and morphology in dying cells but also in healthy cells. The anti-apoptotic BCL-XL protein also apparently regulates both mitochondrial fusion and fission in neurons, possibly via its interactions with DRP1 (83, 84). Recently, other reports indicate that Bcl-2 family proteins play a role in modulating mitochondrial metabolism and reactive oxygen species (ROS) production, exerting both positive and negative influences on cancer invasion and metastasis (68, 85). The anti-apoptotic BCL-2, through its interaction with COX5A in human lymphoblasts and Cyclophilin D in HEK239T cells, enhances mitochondrial respiration and when overexpressed, it exhibits pro-oxidant activity likely via its interaction with COX5b subunit of cytochrome c oxidase in the respiratory complex IV (86-88). Interestingly, knockdown of BCL-2 expression in primary AML cells led to attenuation of mitochondrial respiration (89). However, BCL-2 also showed antioxidant activity in neural cells and was reported to modulate the respiration of heart cells during ischemia by inhibiting ATP synthase (90, 91). Similarly to BCL-2, BCL-XL was reported to enhance cellular metabolism most probably through its direct interaction with the beta subunit of F1F0 ATP synthase in neurons, and by stabilizing MMP allowing better cell adjustment to changes in metabolic demands (92-94). Recently, Lucantoni et al reported that BCL-XL overexpression in breast carcinoma cells leads to an increase of mitochondrial respiration and higher levels of ATP resulting in improved resistance to metabolic stress (95). MCL-1, which is often overexpressed in tumors, increases mitochondrial respiration and consequently increases ROS production in breast cancer and AML cells (96, 97). Additionally, MCL-1 was shown to be a master regulator of fatty acids (FA) oxidation as Mcl-1 knockout leads to switching from using

FA acids as fuel to glucose (98). MCL-1, precisely its truncated variant, can interact with IMM and increase mitochondrial respiration, while elimination of MCL-1 expression causes dysfunction of CI,CII, CIV respiratory complexes (99, 100). Mcl-1-deficient mouse cardiomyocytes have attenuated respiration compared with cardiomyocytes expressing MCL-1, even in the background of Bax and Bak deficiency (101). Out of BH3-only group, Bim-deficient mouse embryonic fibroblasts (MEF) show increased OXPHOS accompanied by MMP decrease, while Bax/Bak deficiency did not affect the respiration of these cells (102). A recent study also reflected a role of BIM in drug-induced liver cell death, emphasizing its regulation of mitochondrial morphology and metabolism (103). Bim-deficient cells displayed increased mitochondrial fragmentation and metabolic shift from aerobic respiration to glycolysis. Bim-deficient hepatocytes are also susceptible to mitochondrial damage and metabolic crisis. In normal hepatocytes, this crisis is characterized by decreased ATP production and increased ROS generation, ultimately contributing to necrotic cell death. BAD, another protein from BH3-only group apparently participates in regulation of glycolysis. Akt or PKA-mediated phosphorylation of BAD inhibits its pro-apoptotic function while enhancing interaction with hexokinase IV, which leads to increased glycolysis rate in liver mitochondria (104, 105). Interestingly, tBID independently inhibits fatty acids oxidation in hepatocytes by blocking of carnitine palmitoyl-transferase-1 (106). Another BH3-only protein PUMA might also participate in the metabolic switch from aerobic respiration to glycolysis, by inhibiting MPC which transports pyruvate from cytoplasm to mitochondria (107). In contrast to the BCL-2 anti-apoptotic proteins primarily associated with enhancing mitochondrial respiration, the multidomain pro-apoptotic proteins were linked to the attenuation of mitochondrial respiration. BAX was reported to decrease ROS production by inhibiting ND5 subunit of respiratory complex (RC) I attenuating PI3K–Akt–MMP-2 pathway and tumor cells invasion (107-109). Similarly, in

cells expressing low levels of BAK as colorectal carcinoma cells LoVo, BAK seems to have the same attenuating effect on ROS production as BAX, as knock-down of Bak expression increases ROS production (109). Thus these data argue for BCL-XL and BCL-W antagonism with BAX and/or BAK to balance ROS production, contributing to tumor cell plasticity in tumor progression (110). As BAX and BAK apparently directly interact with RCI, pro-apoptotic proteins are suggested to be direct regulators of OXPHOS regulation, while anti-apoptotic indirectly regulates it by binding to BAX and BAK (85). However, Bax -/- HCT-116 cells tend to have lower mitochondrial respiration, lower ATP production ability, and reduced citrate synthase activity (111). Under conditions of nutrient limitation, BCL-2 and BCL-XL demonstrated enhanced ATP production efficiency compared to the parental cells, thereby aiding tumors with upregulated BCL-2 and BCL-XL to better cope with cellular stress and exhibit increased resistance (95).

The BH3 domain can be also found in non-Bcl-2 family proteins, which participate in other cell processes such as autophagy (112, 113). BH3 domain-containing Beclin-1 plays an essential role in cellular macroautophagy and via its BH3 domain can interact with other proteins from the Bcl-2 family. Notably, BCL-2, BCL-XL, BCL-W, and BIM can form complexes with Beclin-1, and thus suppressing autophagy. Phosphorylation of BCL-2 by JNK disrupts its interaction with Beclin-1 allowing thus the activation of autophagy (114). Other BH3-only proteins, such as BAD can stimulate autophagy by disrupting the interaction between Beclin-1 and anti-apoptotic proteins from the Bcl-2 family (115). However, an impact of pro-apoptotic proteins on the regulation of autophagy is not fully elucidated. Studies involving Bax/Bak-deficient cells suggested that the absence of these proteins may lead to increased autophagy, implying an inhibitory role for BAX and BAK in its regulation (116). In contrast, Yee et al. showed that the expression of BAX in osteocarcinoma Saos-2 cells can actually stimulate autophagy (117). The pro-apoptotic protein

kinase MST1 can also impact autophagy by phosphorylating the BH3 domain of Beclin-1 (118). This phosphorylation facilitates Beclin-1 interaction with anti-apoptotic Bcl-2 family proteins, thereby modulating autophagy. Interestingly, BH3 mimetics like ABT-737, known for inducing apoptosis, can also stimulate autophagy via binding to anti-apoptotic Bcl-2 proteins and releasing Beclin-1 (119). Another non-apoptotic, cell death-related function of Bcl-2 involves the regulation of the mitochondrial permeability transition pore (MPTP). The formation of MPTP is linked to necrotic cell death, leading to the disruption of both mitochondrial membranes, resulting in swelling and organelle disruption (120). BAX and BAK can interact with proteins ANT and VDAC families, which are one of the constituents of MPTP (121, 122). In their non-apoptotic and inactive states, BAX and BAK promote the formation of MPTP. This promotion may occur through acting as permeability factors or by rendering cells more sensitive to MPTP, particularly during BAX-mediated fusion (123, 124). Another unforeseen participation of Bcl-2 family proteins involves DNA damage response (DDR). Upon DNA damage, BH3-only protein BID is phosphorylated by the ATM kinase, playing a crucial role in mediating DDR and subsequently leading to BID-mediated regulation of reactive oxygen species (ROS) (125, 126). This process is particularly relevant in hematopoietic stem cells (HSC), where it induces quiescence (127). Expression of BID mutants, which cannot be phosphorylated, result in elevated mitochondrial ROS levels in HSCs and their exit from quiescence and re-entry into the cell cycle. The Bcl-2 family proteins are also recognized for their regulatory role in cell cycle progression. BCL-2 and BCL-XL can attenuate CDK2 activity, thereby slowing down the transition of the cell cycle from the G1 to the S phase (128-130). Interestingly, another anti-apoptotic Bcl-2 family protein MCL-1 can apparently suppress cell cycle progression by its interaction with proliferating cell nuclear antigen (PCNA) (131). In contrast, BAX has been implicated in regulating the cell cycle in the

opposite manner by stimulating it via reduction of protein levels of the cell cycle inhibitor p27/Kip1 (132).

Mitochondria

Mitochondria are multifunctional cellular organelles playing an important role in cellular homeostasis (133). Mitochondria regulate intracellular signaling, intrinsic apoptotic pathway, Ca2+ homeostasis, ROS production, metabolism of amino acids, nucleotides, lipids (133-135). Their most prominent cellular functions are linked to crucial anabolic and energetic processes such as de novo pyrimidine synthesis mediated by essential IMS enzyme DHODH, fatty acid oxidation, the TCA cycle, and last but not least generation of ATP by oxidative phosphorylation (133, 134, 136, 137). What makes these organelles special compared to others is certainly their evolutionary origin. The most widely accepted theory of mitochondrial origin is the endosymbiotic one, where proteobacteria entered into symbiosis with eukaryotic cells (138). This theory explains why these organelles have a double membrane and remaining autonomous genome. The three most important discoveries of the mitochondrial genome era were the existence of the non-nuclear genome, the first sequencing of the human mitochondrial genome, and the identification of pathogenic mitochondrial genomes (139-141). Mitochondrial genome encodes 2 rRNAs and 22 tRNAs and 13 proteins, all of them are part of the electron transport chain (139). The remaining proteins constituting the electron transport chain encoded by the nuclear genome (142). While textbooks often represent mitochondria as rod-shaped organelles, they have remarkably dynamic properties. Their shape and number reflects the cell type and ongoing processes of fusion and fission (143). Mitochondria are characterized by a double-layered membrane system consisting of the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM), separated by the

intermembrane space (IMS). The IMM is intricately folded into structures known as cristae, which provide a large surface area for crucial processes such as mitochondrial respiration via the electron transport chain (ETC) complexes (144, 145). IMM is solute impermeable and requires specialized transporters for the import and export of molecules into the mitochondrial matrix (146). In contrast, the OMM is permeable to small ions, nucleotides, some proteins and metabolites (147). The OMM plays important role in the intrinsic pathway of apoptotic signaling – see above and (32).

The mitochondrial central region, mitochondrial matrix, is a site for mitochondrial DNA (mtDNA) replication, transcription, and translation as well as for many essential biochemical processes such as the tricarboxylic acid (TCA) cycle (148, 149).

Recent discoveries unveiled the intriguing phenomenon of horizontal mitochondrial transfer, wherein cells demonstrate the ability to both export and receive mitochondria (150). This phenomenon was first described in 2006, highlighting instances where cells lacking mitochondrial DNA (mtDNA) could receive mitochondria from neighbouring donor cells exemplified by cancer cells acquiring mitochondria from stromal cells (151, 152). Cellular content of mitochondria is determined by two processes: mitochondrial biogenesis and mitophagy (135). Mitochondrial biogenesis, which involves the generation of new mitochondria, is regulated by the c-Myc transcription factor, coupling it with cell cycle progression (153). Mitophagy is a critical process for cellular fitness involving the clearance of damaged mitochondria (135). A typical sign of mitochondrial dysfunction is mitochondrial membrane depolarization, which activates the Pink1/Parkin pathway, the main pathway triggering mitophagy (154). Two other processes that regulate mitochondrial number and shape are mitochondrial fission and fusion (135). Mitochondrial fission is a process where mitochondria split into two or more individual mitochondria. Mitochondrial fission is promoted by DRP1 protein, which in response to cell cycle

and stress, translocates to mitochondria, binds to its receptor, and via its GTPase activity mediates fission (155). In contrast, mitochondrial fusion is the merging of two or more mitochondria, and mixing their mitochondrial content (156). The process is regulated by two groups of proteins—mitofusins (MFN1 and MFN2) and Opa1 (157, 158). MFN1 and MFN2 are located on the OMM and facilitate fusion of OMMs by homo- and heterodimerizing, while Opa1 is located on the inner mitochondrial membrane (IMM) and facilitates the IMM fusion. These two processes are synchronized. Mitofusins are regulated by ubiquitination on the OMM, while Opa1 is regulated by cleavage at the IMM (159). The intricate communication between mitochondria and the endoplasmic reticulum (ER) is facilitated through specialized structures known as mitochondrial-associated membranes (MAMs), wherein calcium ions serve as crucial signaling mediators (160). Mitochondrial uptake of calcium ions intricately modulates mitochondrial metabolism by activating select enzymes within the tricarboxylic acid (TCA) cycle, thereby enhancing adenosine triphosphate (ATP) production (161, 162). Conversely, the release of calcium ions from mitochondria triggers a cascade of events culminating in calcium-mediated cell death, characterized by the opening of permeability transition pore (PTP) channels and the subsequent release of cytochrome c (163).

Cellular metabolism

Oxidative phosphorylation

The important function of mitochondria is the generation of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS), a biochemical process integral to cellular energy metabolism. The inner mitochondrial membrane serves as the primary site for the OXPHOS, housing multiprotein complexes known as respiratory complexes (RC) (134). The flow of

electrons through the electron transport chain (ETC) is orchestrated to proceed from higher to lower energy states, ultimately culminating in molecular oxygen (O2) serving as the terminal electron acceptor as presented in Figure 4 (164). Concurrently, protons are actively transported across the inner mitochondrial membrane into the intermembrane space (IMS), resulting in the establishment of an electrochemical gradient. This proton gradient, or proton motive force, drives the synthesis of adenosine triphosphate (ATP) through the process of chemiosmosis (134). The TCA cycle generates reducing equivalents in the form of NADH and FADH, which serve as sources of energy for the ETC (165). Within the ETC, complexes I, III, and IV act as proton pumps, facilitating the movement of protons across the membrane. In contrast, complex II does not pump electrons but reduces ubiquinone, which acts as a source of electrons for complex III (CIII) (166).

Complex I (NADH:ubiquinone oxidoreductase) is the largest among the five respiratory complexes, with a mass of approximately 1 MegaDalton (MDa) (167). It consists of 45 functional subunits and requires 20 assembly factors for a proper assembly of these subunits (168). Out of these, the seven membrane subunits (ND1 through ND6 and ND4L) are encoded by mitochondrial DNA, while the remaining 38 subunits are encoded by the nuclear genome (169). The catalytic site where NADH oxidation occurs is known as a hydrophilic arm and consists of seven core subunits, flavin mononucleotide as an electron acceptor, and eight Fe-S clusters (170). Electrons are further passed to ubiquinone (coenzyme Q).

Complex II (succinate dehydrogenase)

Unlike the Complex I, all subunits of the Complex II are encoded by nuclear genome (166). It comprises four subunits: SDHA and SDHB, which are matrix-oriented and connected to the IMM via in the IMM-embedded SDHC and SDHD subunits (171). Complex II utilizes FAD as its

primary electron acceptor and contains three Fe-S clusters that accept electrons from succinate, a product of the TCA cycle. Subsequently, these electrons are transferred to ubiquinone.

Complex III (ubiquinol-cytochrome c reductase)

Respiratory complex III consists of 11 subunits including cytochrome b (the only subunit encoded by mitochondrial DNA), cytochrome c, and UQCRFS1 (172). Cytochromes contains heme groups, along with Fe-S clusters found within cytochrome b and UQCRFS1, facilitate the transfer of electrons from ubiquinol to cytochrome c, thereby reducing it (173). Simultaneously, this homodimeric complex acts as a proton pump, extracting two protons from the mitochondrial matrix and transporting four protons into the intermembrane space (IMS), thus contributing to the establishment of the electrochemical gradient across the inner mitochondrial membrane.

Complex IV (cytochrome c oxidase)

Respiratory complex IV is typically isolated as a dimer, with each monomer containing 13 subunits, three of them (MTCO1-3) are encoded by mitochondrial DNA (174). The main function of complex IV, also known as cytochrome c oxidase, is to accept electrons from cytochrome c and to transfer them through the heme groups of CO1 and copper centers within the complex, ultimately reducing oxygen to water (175). Additionally, complex IV is the last of the complexes in the electron transport chain to transport protons to the IMS.

Complex V (ATP synthase)

Complex V plays a pivotal role in the last and crucial step of oxidative phosphorylation - it generates ATP by exploiting the proton gradient generated by the CI, III, and IV complexes (176). Two main structural parts of ATP synthase are F_0 domain with a C ring, which is responsible for proton translocation, and F_1 domain which is responsible for phosphorylating ADP to ATP (173). F_1 domain is composed of α and β subunits, while the central stalk, which keeps F_0 and F_1 together,

is composed of γ, δ, and ε subunits. ATP synthase can depending on circumstances work in the opposite direction, hydrolyzing ATP for pumping the protons in opposite direction, i.e. from the mitochondrial matrix into IMS and thus maintaining the mitochondrial membrane potential (177).

Respiratory Supercomplexes

Respiratory complexes CI, CIII and CIV can be for increased effectivity assembled into higher order respiratory complexes, 'supercomplexes' such as the first described supercomplex of composed of CI, CIII, and CIV complexes, also known as the respirasome (178-180). The mitochondrial supercomplexes can be preserved by solubilizing mitochondria with digitonin or other mild detergents. Additionally, cryo-EM structures of CI-CIII and CIII-CIV were also reported (181, 182). From a functional point of view supercomplexes likely enhance OXPHOS efficacy, and participate in regulation of ROS production and in substrate channeling (182-184).

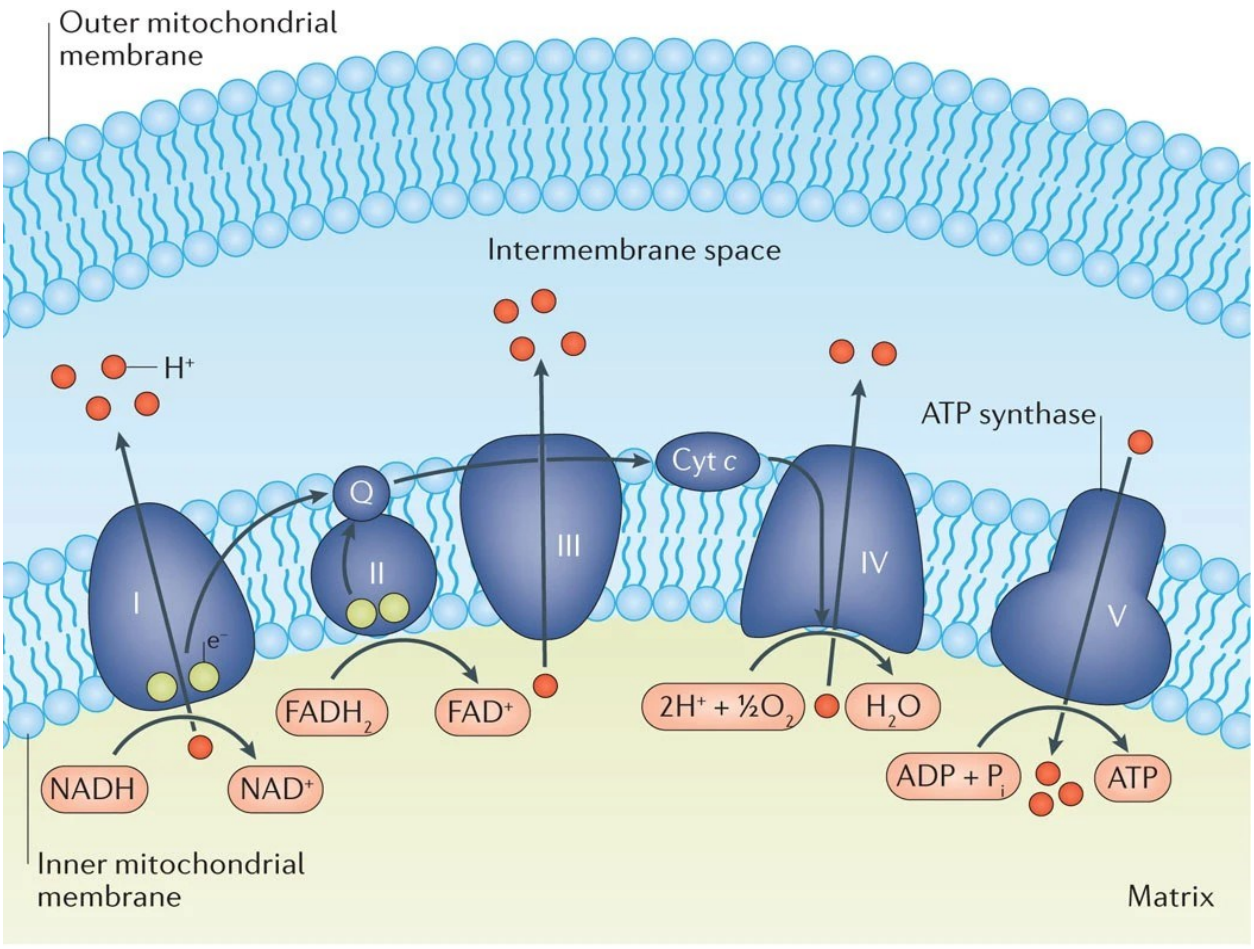

Figure 4. Organization of electron transport chain on Inner mitochondrial membrane. Adapted from (164) .

Reactive Oxygen Species (ROS)

Mitochondria, mainly through OXPHOS, are the main producers of ROS, which include superoxide, hydrogen peroxide, and hydroxyl free radicals and could be detrimental for mitochondrial proteins, lipids and nucleic acids (185). Therefore, mitochondria harbour efficient antioxidant systems to neutralize potentially harmful ROS, including superoxide dismutases

(SOD), glutathione peroxidase 1 (GPX1), thioredoxin reductase 2, and other (185). ROS are characterized by high reactivity and with an exception of hydrogen peroxide also a short half-life (145, 186). Mitochondrial superoxide, the primary ROS, is predominantly generated within the electron transport chain (ETC) at Complex I and Complex III due to electron leakage. Within mitochondria, superoxide is converted to hydrogen peroxide (H_2O_2) mainly via mitochondrial SODs. SOD1 localized in the IMS and cytosol, targets superoxide from Complex III, while SOD2, which is present in the mitochondrial matrix, converts superoxide from Complexes I and III. Following SOD-mediated conversion, peroxidases such as catalase, the thioredoxin system, and GSH peroxidase catalyze reduction of H_2O_2 to water (186).

Glycolysis

Glucose is a primary source of energy via a cascade of enzymatic steps called glycolysis. Glycolysis fulfils two major roles in cellular metabolism. Pyruvate, which is the final product of glycolysis enters further in TCA cycle and thus feeds OXPHOS. Additionally, the intermediates of glycolysis serve as direct precursors for synthesizing various cellular components (187, 188). During glycolysis, one glucose molecule undergoes a series of 10 reactions in the cytosol, ultimately producing two molecules of pyruvate. In the presence of oxygen, pyruvate is further oxidized to acetyl-CoA in the mitochondria. In hypoxic conditions with suppressed OXPHOS, glycolysis provides rapid though ineffective source of energy (ATP) for cellular functions and glucose is converted to lactate as a final product (187). Glycolysis generates a net gain of two ATP molecules per glucose molecule (in contrast to 30-32 molecules of ATP per glucose generated by OXPHOS), with an initial investment of two ATP molecules and a subsequent payoff of four ATP molecules (189).

TCA cycle

Glycolysis end-product pyruvate is translocated into mitochondria, where it is enzymatically oxidized to acetyl-CoA within the mitochondrial matrix (190). This process, catalyzed by the pyruvate dehydrogenase complex, involves the decarboxylation of pyruvate coupled with the reduction of NAD+ to NADH. The resultant acetyl-CoA serves as a pivotal substrate for the mitochondrial matrix-based TCA cycle. This cycle of linked enzymatic reactions serves as a pivotal hub in cellular metabolism due to its ability to integrate various substrates. It initiates with the condensation reaction of the two-carbon acetyl-CoA, derived from the oxidation of fatty acids, amino acids, or pyruvate, with the four-carbon oxaloacetate (OAA), resulting in the formation of the six-carbon citrate molecule (191). The entry of acetyl-CoA into the TCA cycle initiates a series of oxidative reactions, which includes eight different enzymes, leading to the generation of three NADH, one FADH2 and one ATP molecules per cycle (190). During its progression, metabolites from the TCA cycle are also exported to the cytosol, where they serve as precursors for other anabolic processes (192). Resulting NADH and FADH2 subsequently fuel complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) of the ETC, respectively (188). The interdependence between the TCA cycle and oxidative phosphorylation is essential, as the oxidation of NADH and FADH2 by complexes I and II sustains the functionality of the TCA cycle. Maintaining cellular stability relies on the intricate regulation of the TCA cycle, which involves numerous positive and negative allosteric regulators orchestrating metabolic flux and ensuring continuous feedback with oxidative phosphorylation (191).

Mitochondria in cancer

A role of mitochondria in cancer initiation and progression is rather complex and depends on genetic, environmental, and tissue-specific factors (135). Mitochondria can profoundly influence cancer development and progression through various mechanisms (Figure 5) (134, 193). Primarily, it has been recognized for decades that mitochondrial DNA (mtDNA) mutations are implicated in numerous diseases. Among these, mutations affecting the activities of subunits within the ETC are particularly prevalent and significant in pathological conditions (194). Oxidative stress, primarily caused by mitochondria-produced ROS, is another crucial factor in cancer initiation and

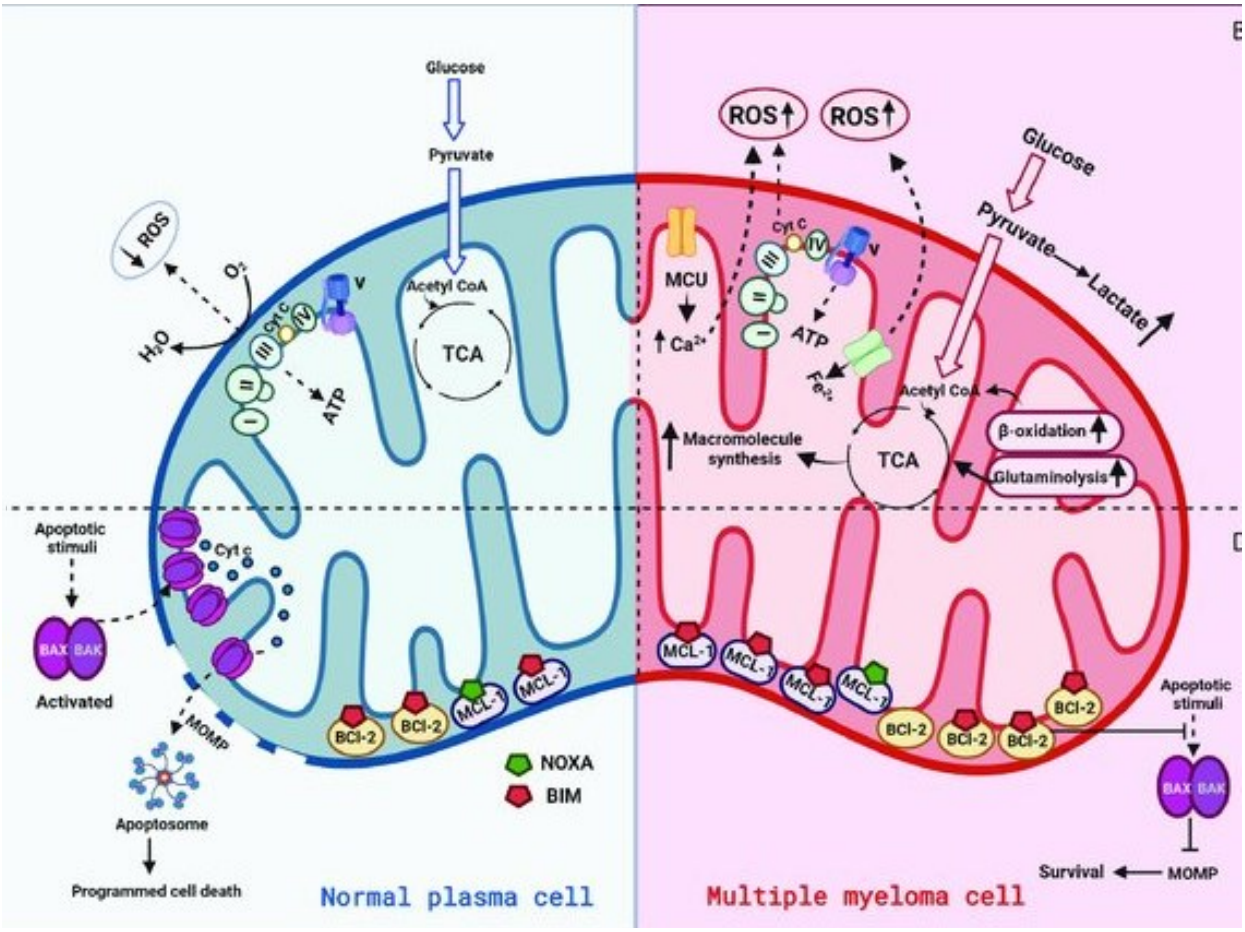

Figure 5. Mitochondrial functions in normal vs cancer cell. Adapted from (193).

progression (195, 196). These highly reactive molecules can damage cellular components but also

serve as signaling molecules in various metabolic pathways (197). Elevated ROS levels in cancer cells result from increased metabolic activity and compromised antioxidant defenses. Mitochondria also play an important role in controlling cell death processes such as apoptosis and necrosis (198). In apoptosis induction, proteins from the Bcl-2 family interact with mitochondria initiating and regulating MOMP and cytochrome c release. Anti-apoptotic Bcl-2 family proteins are potentially pro-oncogenic, while the pro-apoptotic proteins are mainly oncosupressive and their deregulation in cancers affects tumorigenesis and response to cancer therapy ((42), see also *Bcl-2 family proteins*). The equilibrium between pro- and anti-apoptotic proteins influences a cancer cell's sensitivity to apoptotic triggers and could serve as an indicator of the tumor's responsiveness to chemotherapy (199). Metabolic reprogramming, a hallmark of tumors, is also driven by mitochondria, which serve as central hubs for metabolic reactions facilitating macromolecule synthesis, fulfilling bioenergetic demands, and promoting cellular survival via various mechanisms (200). Metabolic reprogramming in cancer also involves pro-malignant mutations in genes encoding enzymes of the TCA cycle (201). Notably, certain TCA cycle intermediates such as fumarate, succinate, aspartate, and D-2-hydroxyglutarate (2HG) exhibit upon their accumulation significant pro-carcinogenic effects (202). TP53 tumour suppressor, frequently mutated tumors suppressor also plays a crucial role in modulating cellular metabolism by activating metabolic genes, inhibiting glycolysis, and promoting the transcription of genes essential for the ETC assembly and maintenance (203). Alongside mitophagy and mitochondrial biogenesis, which maintain a pool of functional mitochondria, cancer cells can also acquire functional mitochondria from non-malignant cells within the tumor microenvironment (204, 205). This transfer occurs through intercellular tunneling nanotubes (TNTs) and larger microtubes, facilitating the exchange of intact mitochondria (152).

Hypothesis and aims

Bcl-2 family proteins were among the earliest known regulators of apoptosis in the animal kingdom, with their involvement traced back to both apoptotic and non-apoptotic signaling pathways in the nematode *C. elegans*. In mammalian cells their predominant role is mainly though not exclusively linked to activation and regulation of mitochondrial intrinsic apoptotic signaling. Alongside their involvement in apoptosis, Bcl-2 family proteins were recently documented to be involved in the regulation of mitochondrial bioenergetics affecting both oxidative phosphorylation (OXPHOS) and glycolysis pathways. Recent findings in OXPHOS regulation indicate that anti-apoptotic Bcl-2 proteins exert a positive influence, enhancing respiration, ATP production, and the generation of reactive oxygen species (ROS). Conversely, there are controversial and inconclusive studies showing that pro-apoptotic proteins suppress or enhance these bioenergetic processes and mechanisms underlying these processes remain still poorly understood. To contribute to and enhance our knowledge of a role Bcl-2 family proteins in cellular bioenergetics, we eliminated expression of the pro-apoptotic BAX and BAK proteins using CRISPR/Cas9 approach in several well-established human cancer cell lines. We aimed to unravel the mechanisms governing their impact on mitochondrial respiration, shedding light on these processes that likely exert a significant impact on the fitness and proliferation of cancer cells.

Major aims:

1. Preparation of Bax/Bak-deficient cancer cells.
2. Bioenergetic profiling and in-depth characterization of Bax/Bak-deficient cancer cells.
3. Assessment of the role of Bcl-2 family proteins and mitochondrial metabolism in hematopoietic malignancies.

Aim 1.

Gene editing-mediated elimination of two main pro-apoptotic Bcl-2 family proteins expression in cancer lines of different origin. Selected phenotypically different human cancer cell lines originated from colon epithelial cancer (HCT-116), glioblastoma (U87) and B cell lymphoma (HBL-2) cells. CRISPR/Cas9 gene editing system was used to abolish expression of BAX and BAK in cells. BH3 mimetics targeting the major anti-apoptotic proteins BCL-2/BCL-XL/BCL-W inhibitor ABT-737, MCL-1 inhibitor S63846 and BCL-XL inhibitor A1155463 were used in a selection process, eliminating cells which were sensitive to BH3 mimetics-induced apoptosis (i.e. expressing BAX, BAK or both proteins), together with single-cell sorting. "Sleeping beauty" protein expression system using PSBbi plasmid was used to re-express BAX and BAK proteins.

Aim 2.

Bioenergetics and biochemical profiling of cell lines with eliminated expression of BAX and BAK proteins- analysis of respiration and glycolysis at functional and biochemical levels in Bax/Bak ablated clones. For the initial screening of basic energy-related processes in genetically modified cancer cell lines we employed high throughput assays in Seahorse XFe96 and Oroboros O2k instruments using non-permeabilised and permeabilised cells. Together with respiration/glycolysis analyses we aimed to determine other important metabolic parameters such as ATP, NAD+/NADH, MMP, cellular and mitochondrial ROS levels for more comprehensive information about energy-metabolic fitness of analyzed Bax/Bak-deficient cells. For the analysis of expression and composition of respiration complexes we used Blue Native Electrophoresis (BNE). For the analysis of changes in protein expression and levels of selected metabolites we employed proteomic and metabolomic approaches. We also determined growth of Bax/Bak-deficient cells in vitro and in immunodeficient mice.

Aim 3.

Our collaborative research aimed to assess the role of mainly anti-apoptotic Bcl-2 family proteins in hematopoietic malignancies, with a primary focus on their therapeutic targeting and overcoming resistance to treatment. We exploited BH3 mimetics for dual-targeting of anti-apoptotic Bcl-2 family proteins in B cell lymphomas and thus overcoming their acquired resistance to FDA-approved BCL-2 inhibitor venetoclax. We also analyzed alterations in mitochondrial metabolism in these cells.

Materials and Methods

All methods are thoroughly described in the publications listed in the results section. The most important techniques relevant to the Thesis and to the first-author publication are outlined and briefly explained below:

Cell lines

U87- Glioblastoma cell line – ATCC, https://www.atcc.org/products/htb-14

HCT-116- Colorectal carcinoma cell line – obtained from dr V. Korinek, IMG CAS, originally from ATCC, https://www.atcc.org/products/ccl-247

HBL-2, UPF1H, UPF1G – Mantle B-cell lymphoma cell lines – gift from Prof. P. Klener, 1st Faculty of Medicine, Charles University in Prague

SDS electrophoresis and western blotting

Both procedures were performed according to standard laboratory protocols.

Primary antibodies: anti-BAX (Cell Signaling Technology, #2772)

anti-BAK (Cell Signaling Technology, #12105)

anti-TEFM (ThermoFisher, PA5-46120)

anti-TOM20 (Cell Signaling Technology, #13929)

anti-VDAC1 (Cell Signaling Technology, #4661)

anti-α-Tubulin (Cell Signaling Technology, #3873)

anti-β-Actin (Cell Signaling Technology, #12620)

anti- mtND5 (Abcam, ab138136)

Genetic elimination of Bax and Bak, and their re-expression

Adherent cell lines were transfected using Lipofectamine CRISPRMAX (Thermo Fisher Scientific) and B-cell Lymphoma cell lines were transfected by electroporation ((Neon, Thermo Fisher Scientific).

TrueCut Cas9 Protein v2 (Thermo Fisher Scientific) and a mix of sgRNAs targeting both Bax and Bak (CRISPR Gene Knockout Kit v2 for human Bax and Bak, Synthego) were transfected/electroporated into target cells and Bax/Bak-deficient cells were selected with 5 μM mix of BH3 mimetics either as a mixed culture or cell-sorted for the preparation of clonal cultures.

Re-expression of BAX and BAK: Bax-IRES-Bak module was cloned into pSBbi-Pur (Addgene) and together with the Sleeping beauty recombinase expressing plasmid pCMV(CAT)T7-SB100 (Addgene) was transfected into Bax/Bak-deficient cells.

Cell proliferation

Kinetic profiles of cell proliferation were determined using the IncuCyte SX1 (Sartorius & Essen BioScience).

Analysis of mitochondrial respiration using the Oxygraph 2k respirometer

Respiration (routine, and CI- and CII-dependent) was assessed using the Oroboros O2k respirometer (Oroboros Instruments) using standard protocol as described in (206).

Analysis of mitochondrial respiration and cellular glycolysis using the Seahorse XFe96 analyser

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) evaluation were performed using the Seahorse XFe 96 analyser (Agilent Technologies) and standard protocols. For OCR we used the Agilent Seahorse XF Cell Mito Stress protocol: https://www.agilent.com/cs/library/usermanuals/public/XF_Cell_Mito_Stress_Test_Kit_User_Guide.pdf and for glycolysis the Agilent Seahorse XF Glycolysis Stress protocol: https://www.agilent.com/cs/library/usermanuals/public/XF_Glycolysis_Stress_Test_Kit_User_Guide.pdf .

RCI- and RCII-dependent respiration was assessed using the Agilent Seahorse permeabilization protocol, permeabilizing cells with PMP (Plasma Membrane Permeabilizator, Agilent): https://www.agilent.com/cs/library/technicaloverviews/public/XF-PMP-Limited-Tech-Brief-WEB.pdf

Results were normalized based on cell number counted using BioTek Cytation 5 instrument (Agilent) and analysed using Seahorse Wave softer (Agilent).

Analysis of mitochondrial membrane potential and formation of reactive oxygen species

For evaluation of inner mitochondrial membrane potential ($\Delta\Psi_m$), cells were stained with tetramethylrhodamine ethyl ester (TMRE) (TMRE kit, Abcam) and assessed using the LSR Fortessa SORP flow cytometer (Beckton Dickenson).

For generation/content of mitochondrial reactive oxygen species (ROS) levels, cells were stained with MitoSox (Mitochondrial indicator; Thermo Fisher Scientific) and analyzed using the LSR Fortessa SORP flow cytometer.

Determination of NAD^+/NADH ratio and ATP level

The oxidized and reduced forms of the coenzyme were quantified using the NAD^+/NADH Glo assay kit (Promega) and bioluminescence intensity was assessed using a Tecan Infinite 200 reader (TECAN).

Blue native gel electrophoresis and in-gel activity

For BNE and In-gel activity assay were used isolated and solubilized mitochondria and standard laboratory protocol as described in (207).

Primary antibodies used for BNE: -anti-NDUFA9 (Abcam, ab14713)

-anti-NDUFB8 (Abcam, ab192878)

-anti-SDHA (Abcam, ab14715)

-anti-SDHB (Abcam, ab14714)

-anti-UQCR22 (Abcam, ab14745)

-anti-COX5A (Abcam, ab110262)

-anti-ATP5B (Abcam, ab14748)

In-gel activity was assessed incubating native gels with specific CI and CII buffers and recording enzymatic activity by Azure c600 imaging system.

Assessment of mitochondrial DNA by qPCR

Genomic and mtDNA were isolated using the Wizard DNA purification Kit (Promega) and expression of B2M and mtCO1 was measured by CFX96 Touch Real-time detecting system (BioRad).

Formation of tumours in mice

Cells were injected subcutaneously (s.c.) into NSG mice (5 mil. cells per mouse) and growing tumours were measured using calliper.

Metabolomics and proteomics analyses

Metabolomics and proteomics analyses were performed at OMICS Proteomics and Metabolomics Core Services at BIOCEV - https://www.biocev.eu/en/services/omics-proteomics-and-metabolomics.6

The samples for targeted metabolomics were analyzed by a Dionex Ultimate 3000RS liquid chromatography system coupled to a TSQ Quantiva mass spectrometer (ThermoScientific).

Calcium imaging measurements

Calcium imaging microscopy was performed at the Laboratory of Cellular Neurophysiology of the Institute of Experimental Medicine, Czech Academy of Sciences - https://www.iem.cas.cz/en/department/department-of-cellular-neurophysiology/ .

Transmission electron microscopy

Mitochondrial shape and cristae structure were visualized using transmission electron microscopy (TEM) at the electron microscopy core unit of the Institute of Molecular Genetics, Czech Academy of Sciences - https://www.img.cas.cz/group/electron-microscopy/ .

Quantitative real-time PCR

Quantitative PCR was performed on CFX96 Touch Real-time detecting system (BioRad) using HOT FIREPol EvaGreen qPCR Supermix (Solis BioDyne OÜ)

Statistical analyses

The acquired data were analysed with the GraphPad Prism 7 using two-way ANOVA analysis. The results were analysed based on three independent experiments, presented as means ± standard errors. $P < 0.05$ was considered to indicate statistical significance (*$p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.005$).

Results

List of publications

1. **Sovilj D,** Kelemen CD, Dvorakova S, Zobalova R, Raabova H, Kriska J, Hermanova Z, Knotek T, Anderova M, Klener P, Filimonenko V, Neuzil J, Andera L. Cell-specific modulation of mitochondrial respiration and metabolism by the pro-apoptotic Bcl-2 family members Bax and Bak. *Apoptosis* 424–438 (2024). https://doi.org/10.1007/s10495-023-01917-2

2. Klanova M, Kazantsev D, Pokorna E, Zikmund T, Karolova J, Behounek M, Renesova N, **Sovilj D**, Kelemen CD, Helman K, Jaksa R, Havranek O, Andera L, Trneny M, Klener P; Anti-apoptotic MCL1 Protein Represents Critical Survival Molecule for Most Burkitt Lymphomas and BCL2-negative Diffuse Large B-cell Lymphomas. *Mol Cancer Ther* (2022). https://doi.org/10.1158/1535-7163.MCT-21-0511

3. Klener P, **Sovilj D**, Renesova N, Andera L. BH3 Mimetics in Hematologic Malignancies. *Int. J. Mol. Sci.* (2021) https://doi.org/10.3390/ijms221810157

4. Stemberkova-Hubackova S., Zobalova R., Dubisova M., Smigova J., Dvorakova S., Korinkova K., Ezrova Z., Endaya B., Blazkova K., Vlcak E., Brisudova P., Le, D.-D.T., Juhas, S., Rosel, D., Daniela Kelemen, C., **Sovilj, D**., Vacurova, E., Cajka, T., Filimonenko, V., Dong, L., Andera, L., Hozak, P., Brabek, J., Bielcikova, Z., Stursa, J., Werner, L. and Neuzil, J. (2022), Simultaneous targeting of mitochondrial metabolism and

immune checkpoints as a new strategy for renal cancer therapy. *Clin. Transl. Med*., 12: e645. https://doi.org/10.1002/ctm2.645

5. Malarikova, D., Jorda, R., Kupcova, K., Senavova, J., Dolnikova, A., Pokorna, E., Kazantsev, D., Nozickova, K., **Sovilj, D**., Bellanger, C., Chiron, D., Andera, L., Krystof, V., Strnad, M., Helman, K., Klanova, M., Trneny, M., Havranek, O., & Klener, P. . Cyclin dependent kinase 4/6 inhibitor palbociclib synergizes with BCL2 inhibitor venetoclax in experimental models of mantle cell lymphoma without RB1 deletion. *Exp Hematol Oncol.* 2024 Mar 25;13(1):34. doi: 10.1186/s40164-024-00499-2

6. Dolníková, A., Anděra, L., Kazantsev, D., Zikmundová, M., Pokorná, E., **Sovilj, D**., Kelemen, C. D., Tušková, L., Mráz, M., Hoferková, E., Helman, K., Čuřík, N., Poláková-Machová, K., Trněný, M., & Klener, P. Blockage of BCL-XL overcomes venetoclax resistance across BCL2-positive lymphoid malignancies irrespective of BIM status. *Blood Advances (2024)* (manuscript under the revision)

Results of thesis-related research articles

The foundation stone of the Thesis is the first author publication in the Apoptosis journal (2023 IF=7.2) supported by three other co-authored contributions (two already published publications and one manuscript under the revision). Each publication/manuscript is briefly summarized below, highlighting its relevance to the Thesis and the respective author's contributions:

1. **Sovilj D,** Kelemen CD, Dvorakova S, Zobalova R, Raabova H, Kriska J, Hermanova Z, Knotek T, Anderova M, Klener P, Filimonenko V, Neuzil J, Andera L. Cell-specific modulation of mitochondrial respiration and metabolism by the pro-apoptotic Bcl-2 family members Bax and Bak. *Apoptosis* 2024 29(3-4):424-438. doi: 10.1007/s10495-023-01917-2.

Although cancer cells often express reduced levels of the pro-apoptotic Bcl-2 family proteins BAX and BAK, their roles beyond apoptosis in cancer, particularly their impact on cancer metabolism, is not yet fully understood. In order to unravel the non-apoptotic, mitochondria-linked role of two these two pro-apoptotic proteins we employed CRISPR/Cas9-meditaed gene editing for concurrent elimination of their expression in human cancer cell lines of epithelial, glial, and hematopoietic origin. These Bax/Bak-deficient clonal and mixed (just U87 cells) cell cultures were then assessed for their mitochondrial respiration and glycolysis using Seahorse XFe 96 analyzer and O2k Oxygraph. Surprisingly we uncovered that Bax/Bak-deficient cancer cells of glial, epithelial, and hematopoietic origins differed in their mitochondrial respiration not only from the parental cells but also among themself. Specifically, while respiration significantly increased in Bax/Bak-deficient glioblastoma U87 cells, it was slightly suppressed in Bax/Bak-deficient B lymphoma cells and apparently unchanged in epithelial HCT-116 cells. Moreover, Bax/Bak-deficient U87 cells exhibited accelerated proliferation rates in culture, rapid tumor formation and growth in immunodeficient mice. They also displayed notable changes of cellular levels of mitochondria-linked metabolites, including a substantial increase in the NAD+/NADH ratio. In contrast, Bax/Bak-deficient HBL-2 lymphoma cells showed attenuated in vitro proliferation and slower tumor formation in immunodeficient mice. Subsequent proteomic and western blotting analyses of the parental vs Bax/Bak-deficient U87 and HBL-2 cells revealed substantial alterations in the

expression of mitochondria-encoded subunits of respiratory complexes and of the mitochondrial transcription elongation factor TEFM. We uncovered that the protein levels of TEFM in Bax/Bak-deficient cells are likely post-transcriptionally modulated (increased in U87 and decreased in HBL-2 cells) and directly influence the expression of mtDNA-encoded subunits of the respiratory complexes. ShRNA-mediated downregulation of TEFM expression in Bax/Bak-deficient U87 cells attenuated their mitochondrial respiration thus confirming its essential role in this fundamental cellular process. These findings led us to propose a mechanism whereby (post)translational regulation of TEFM levels in Bax/Bak-deficient cells orchestrates the expression of mitochondrial respiratory complex subunits, influencing mitochondrial respiration, cellular metabolism, and proliferation of these cells.

Author's contribution to the publication: I made substantial contributions to the study design and actively participated in various aspects, including planning and running the experiments, data analysis, drafting and revision of the manuscript. I participated in the experimental part of the project by:

- preparing clones with modulated expression of BAX/BAK and TEFM which includes, cloning processes, transfections, transductions, clonal selection and verification of genetically modified clones;

-performing in vitro measurements of proliferation;

-performing all bioenergetics analyses including Seahorse assay, Oxygraph measurements and other biochemical assays;

-performing BNE and In-gel activity analyses;

-performing PCR and Western Blotting experiments.

Furthermore, beyond the findings presented in this article, we uncovered that the elimination of BAX and BAK expression in two additional B lymphoma cell lines - UPF1G and UPF1H led to similar attenuation of their mitochondrial respiration as in Bax/Bak-deficient HBL-2 cells (see below – Figure 6).

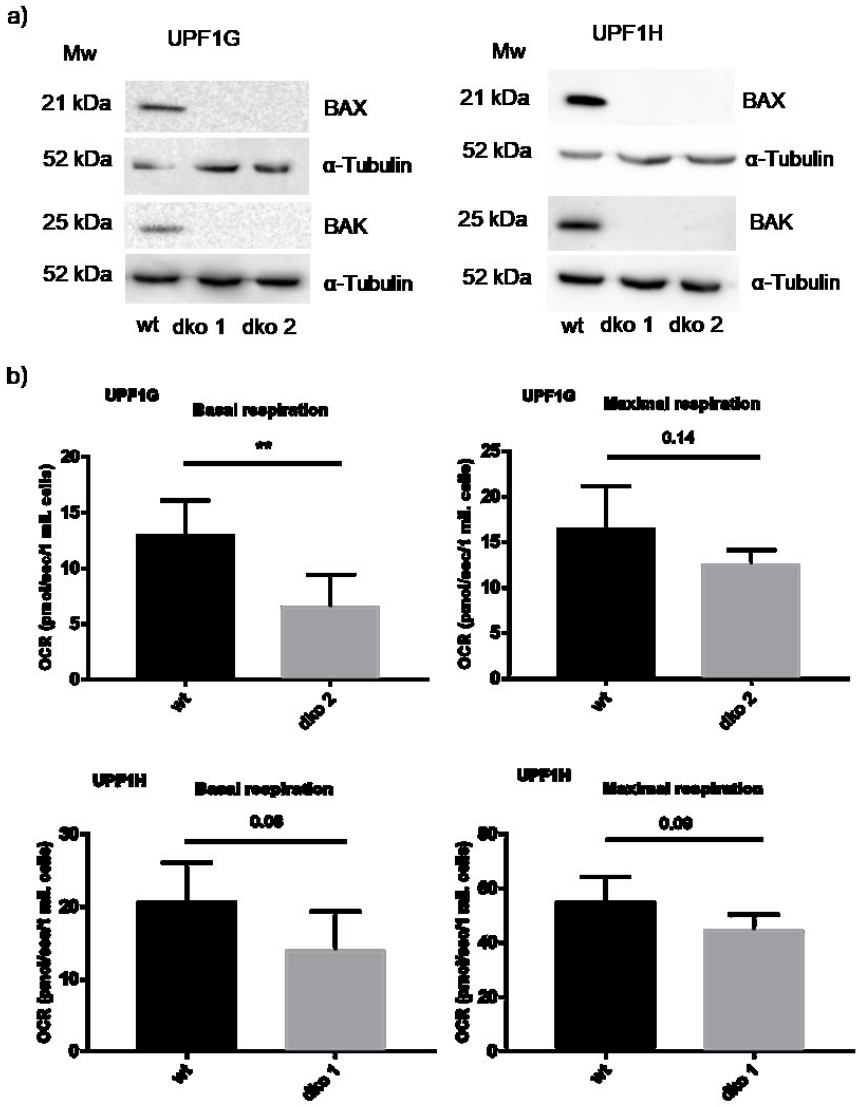

Figure 6. BAX/BAK deficiency in UPF1G and UPF1H lymphoma has a negative effect on their mitochondrial respiration.

a) Western blots of the parental (wt) and Bax/Bak-deficient UPF1G and UPF1H clones. b) Basal and maximal cellular respiration was determined using Oxygraph-2K instrument in UPF1G and UPF1H parental (wt) and Bax/Bak-deficient cells (dko 1). Data represent mean values from three independent experiments ± SD. *$P < 0.05$.

2. Klanova M, Kazantsev D, Pokorna E, Zikmund T, Karolova J, Behounek M, Renesova N, **Sovilj D**, Kelemen CD, Helman K, Jaksa R, Havranek O, Andera L, Trneny M, Klener P; Anti-apoptotic MCL1 Protein Represents Critical Survival Molecule for Most Burkitt Lymphomas and BCL2-negative Diffuse Large B-cell Lymphomas. *Mol Cancer Ther.* 2022 21(1):89-99. doi: 10.1158/1535-7163. (2023 IF = 5.7)

The overexpression of pro-survival protein MCL-1 commonly occurs in many human cancers, including non–Hodgkin B-cell lymphomas (B-NHL). S63845, a highly specific BH3 mimetic targeting MCL-1, which modified second-generation S64315 (MIK665) is being tested in clinical trials with various hematological malignancies. We also employed S63845 for the treatment of diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma cells. We found out that while majority of Burkitt lymphomas were sensitive to in vitro treatment with S63845, part of DLBCL was resistant and this resistance was primarily associated with high levels of BCL-2 protein. BCL-2 acted as a buffer for pro-apoptotic proteins released from MCL-1 upon exposure to S63845. Furthermore, the expression level and occupancy status of MCL-1 were identified as crucial factors influencing sensitivity to S63845. MCL-1-BIM/BAK complexes primed lymphoma cells for S63845-induced apoptosis, whereas MCL-1-NOXA complexes were linked to S63845 resistance. The expression levels and occupancy status of both BCL-2 and MCL-1 were thus the key determinants of sensitivity/resistance these cells to S63845. The combined treatment of S63845 resistant cells with selective BCL-2 inhibitor venetoclax and S63845 led to their increased apoptosis in vitro and to efficient growth suppression of B-NHL patient-derived xenografts in immunodeficient mice. Co-treatment of B-NHL with venetoclax can overcome BCL-2-mediated resistance to S63845, which might have possible therapeutic implications in aggressive B-NHL with BCL-2 positivity.

Relevance to the Thesis and author's contribution to the publication: Common narrative being focus on Bcl-2 family of proteins. Participated by preparation of B cell lymphomas with overexpressed Bcl-2 and Mcl-1 and clonal cultures of Bak-deficient B cell lymphomas.

3. Malarikova, D., Jorda, R., Kupcova, K., Senavova, J., Dolnikova, A., Pokorna, E., Kazantsev, D., Nozickova, K., **Sovilj, D**., Bellanger, C., Chiron, D., Andera, L., Krystof, V., Strnad, M., Helman, K., Klanova, M., Trneny, M., Havranek, O., & Klener, P. . Cyclin dependent kinase 4/6 inhibitor palbociclib synergizes with BCL2 inhibitor venetoclax in experimental models of mantle cell lymphoma without RB1 deletion. *Exp Hematol Oncol.* 2024 Mar 25;13(1):34. doi: 10.1186/s40164-024-00499-2. (2022 IF = 8.65)

Mantle cell lymphoma (MCL) is a recurring B-cell lymphoma characterized by disrupted cell cycle regulation. This study examines effectiveness, mechanisms, and predictive markers of response to palbociclib, a CDK 4/6 inhibitor, alone and in combination with venetoclax, a BCL-2 inhibitor in a panel of nine MCL cell lines and four patient-derived xenografts (PDX) from MCL patients resistant to chemotherapy and ibrutinib. Combining palbociclib and venetoclax showed significant synergy in killing MCL cells both in vitro and in vivo. Mechanistically, palbociclib reduced the levels of anti-apoptotic protein MCL-1, increased levels of pro-apoptotic protein BIM, and enhanced the sensitivity of MCL cells to apoptosis through metabolic and mitochondrial stress pathways. Resistance to palbociclib was observed in cells lacking the RB1 gene, while alterations in CDKN2A, CDK4, and MYC genes did not affect sensitivity. Thus these data support further exploration of the palbociclib and venetoclax combination as a novel treatment approach for post-ibrutinib MCL patients without RB1 deletion.

Relevance to the Thesis and Author's contribution to the publication: Not direct relevance just a methodical one – Seahorse XFe 96-mediated analysis of mitochondrial respiration and glycolysis, and measurements of ROS levels in parental and genetically manipulated MCL cells.

4. Dolníková, A., Anděra, L., Kazantsev, D., Zikmundová, M., Pokorná, E., **Sovilj, D**., Kelemen, C. D., Tušková, L., Mráz, M., Hoferková, E., Helman, K., Čuřík, N., Poláková-Machová, K., Trněný, M., & Klener, P. Blockage of BCL-XL overcomes venetoclax resistance across BCL2-positive lymphoid malignancies irrespective of BIM status. *Blood Advances (2024)* (manuscript under the revision, 2023 IF = 7.5)

In this study, venetoclax, known for its effectiveness against mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL), and diffuse large B-cell lymphoma (DLBCL) tumors, showed enhanced anti-tumor activity when combined with BCL-XL inhibitor A1155463. Employing a battery of lymphoma and leukemia cell lines as well as patient-derived xenografts (PDX), we found a significant synergy between venetoclax and A1155463. Immunoprecipitation assays and genetic manipulation of BCL-XL expression, highlighted the crucial role of BCL-XL in both inherent and acquired resistance to venetoclax. Notably, even in Bim-deficient, usually venetoclax-resistant cells, venetoclax and A1155463 combination demonstrated synthetic lethality. Immunoprecipitation experiments further pinpointed BAX as the primary driver of apoptosis in BIM-deficient malignant cells. The efficacy of this combined venetoclax + A1155463 treatment was validated in vivo using lymphomas PDX models. To mitigate thrombocytopenia, a common side effect of prolonged BCL-XL inhibition, in this study a treatment regimen of four days on and three days off was successfully tested, maintaining the desired anti-tumor synergy while managing platelet toxicity.

Relevance to the Thesis and Author's contribution to the publication: This manuscript is similarly as the Thesis focused on proteins from the Bcl-2 family and I/we contributed by preparation of Bcl-XL-deficient HBL-2 and Maver-1 cells.

Discussion

Analyses of the genome of the nematode *Caenorhabditis elegans* offered early insights into the intricate process of apoptosis regulated by Bcl-2 family proteins (208). Since the discovery of the first member of the Bcl-2 protein family over 30 years ago, their role in apoptotic signaling has been firmly established. In recent years, the multifaceted role of Bcl-2 proteins in mammalian cells has become increasingly recognized. In addition to their participation in the regulation of calcium signaling, cell cycle progression, and mitochondrial dynamics, Bcl-2 family proteins can affect also cellular metabolism, particularly mitochondrial respiration. Anti-apoptotic members such as BCL-2, BCL-XL, and MCL-1 have been recognized as positive regulators of mitochondrial metabolism. However, there is limited knowledge linking BAX and BAK with non-apoptotic signaling, particularly with mitochondrial metabolism. Among these reports, two studies pointed to BAX-mediated enhancement of respiration in HCT-116 cells, while in other communication BAX was involved in suppression of respiration in lung cancer cells (110, 111, 209). The conflicting findings from these studies were among the factors motivating us to investigate the potential involvement of BAX and BAK in mitochondrial respiration across human cancer cells of various origin, thus enhancing our knowledge on their role in cellular bioenergetics. The expression of Bcl-2 family proteins is dysregulated in various cancer types, disrupting apoptotic signaling, potentially affecting non-apoptotic pathways, and influencing the effectiveness of

cancer therapies. Therefore, in our collaborative projects, our aim was to expand our understanding not only of the pro-apoptotic group but also of the anti-apoptotic group and their roles in response to various cancer therapeutics targeting intrinsic apoptotic signaling.

To investigate possibly cell type-specific effects of BAX and BAK on cellular metabolism, we selected cell lines derived from three distinct tissue types: HCT-116 colorectal carcinoma (of epithelial origin), U87 glioblastoma (of glial origin), and B (mantle) cell lymphoma HBL-2 (of hematopoietic origin). Simultaneous elimination of both Bax and Bak expression in these cells using CRISPR/Cas9 gene editing and initial characterization of these cells led us to rather surprising discovery. Clonal and mixed cultures of Bax/Bak-deficient glioblastoma U87 cells respired better that the parental cells, while the mitochondrial respiration was attenuated in Bax/Bak-deficient lymphoma HBL-2 cells and also in two additional tested lymphoma UPF1G and UPF1H cells. In order to exclude potential clonal biases, in addition to at least three tested clones, we also generated mixed population of Bax/Bak-deficient U87 cells, which exhibited increased respiration similar to U87 clonal cultures. The clonal cultures of Bax/Bak-deficient HCT-116 cells prepared in our study consistently showed mitochondrial respiration like the parental cells. However, Bax/Bak-deficient HCT-116 cells obtained from Dr. Richard Youle at the National Institute of Health, consistently with previously published data, respired markedly less than the parental HCT-116 cells (111, 210). This discrepancy may be attributed to clonal variations within either our HCT-116 cells (with a conformity level of 75% to ATCC STR profiling) or the obtained HCT-116 Bax/Bak -/- cells (with conformity level of 81% to ATCC STR profiling) derived from Bax-deficient HCT-116 cells. Multiple Bcl-2 family proteins, primary from the BH3-only group, were also shown to modulate glycolysis (68, 104, 105). However, the Glycolytic Stress Test assay run on the Seahorse XFe 96 did not, in contrast to noticeable changes in mitochondrial

respiration, revealed any modulation of cellular glycolysis in Bax/Bak-deficient cells. The Permeabilization Seahorse protocol and in-gel activity assay provided further insights into the notable alterations in respiration observed in U87 cells. While the activity of complex II remained in Bax/Bak-deficient U87 cells unchanged, there was a significant increase in the complex I activity. Similarly, in permeabilized Bax/Bak-deficient U87 cells, the complex I respiration increased while complex II respiration was unaffected. Interestingly, the modulation of mitochondrial respiration in Bax/Bak-deficient U87 and HBL-2 cells was also reflected in their in vitro proliferation and in vivo tumor growth of cells engrafted into immunodeficient mice. Bax/Bak-deficient U87 cells exhibited enhanced proliferation compared to parental cells and faster tumor growth in NSG mice. In contrast, less respiring Bax/Bak-deficient HBL-2 clonal cultures attenuated their in vitro proliferation and tumor growth as well. Faster in vitro proliferation and tumor growth of Bax/Bak-deficient U87 cells could be also related to their acquired resistance to the intrinsic/mitochondrial apoptotic signaling. However, suppressed proliferation and growth of less respiring HBL-2 Bax/Bak -/- cells/tumours argues against presumed involvement of apoptotic signaling, though it might also indicate a pro-senescence phenotype, which was recently reported for Bax/Bak-deficient mouse embryonic fibroblasts and HCT-116 cells (211). However, possible pro-senescence phenotype cannot be extrapolated to fast-proliferating Bax/Bak-deficient U87 cells. While we successfully restored parental cell respiration in HBL-2 cells via the re-expression of BAX and BAK, we encountered difficulty in achieving the same outcome in U87 cells. This failure might be attributed to rapid and persistent cell death observed in U87 Bax/Bak -/- cells with re-expressed pro-apoptotic proteins. The surviving cells expressing low levels of BAX and BAK proteins did not attenuated their increased respiration. One possible explanation could be the long-term stability of respiratory complexes observed in cells of neural and cardiac origin, which may

also account for the persistence of increased respiration in Bax/Bak-deficient U87 glioblastoma cells with re-expressed minute levels of these pro-apoptotic proteins (212). The differences in mitochondrial respiration between Bax/Bak-deficient U87 and HBL-2 cells prompted us to conduct a more detailed analysis of their mitochondria and relevant, linked signaling pathways. The enhanced respiration of Bax/Bak-deficient U87 cells could not be relayed to increased mitochondrial content as both mitochondrial protein and mtDNA levels were comparable between the parental and Bax/Bak-deficient U87 cell. However, denser mitochondrial cristae network in Bax/Bak-deficient U87 cells could contribute to increased respiration as the cristae network content and mitochondrial respiration are directly linked – respiration-deficient cells lacking mitochondrial TFAM factor possess severely disorganized and even absent IMM cristae (213). Metabolic profiling further supported higher respiration rate in Bax/Bak-deficient U87 cells, indicated by a significantly reduced NAD+/NADH ratio and increased mitochondrial membrane potential. Decreased levels of NAD+ and increased levels of NADH in Bax/Bak-deficient U87 cells could be attributed to the higher efficiency of RCI, resulting in an elevated demand for NADH production. This increased production of NADH is essential for driving the ETC and subsequently leads to higher production of ATP and indeed we confirmed that ATP levels in Bax/Bak-deficient U87 were increased. Conversely, in the less respiring Bax/Bak-deficient HBL-2 cells, as anticipated, there was an increase in the NAD+/NADH ratio. Given the significant impact of BAX and BAK deficiency on mitochondrial respiration, particularly in CI-linked respiration, we sought to investigate how the absence of both pro-apoptotic proteins influences the mitochondrial proteome. High-throughput proteomic analysis revealed elevated levels of numerous proteins in Bax/Bak-deficient U87 cells. Specifically, proteins encoded by mitochondrial DNA (e.g., MT-ND5, MT-ND4, cytochrome b) showed increased expression levels. Additionally, expression

levels of proteins associated with mitochondrial respiration encoded by the nuclear genome were in these cells also elevated. Furthermore, using Blue Native Electrophoresis, we noted an increased level of the respirasome in these Bax/Bak-deficient U87 cells, suggesting a potential link to the observed changes in mitochondrial function. Data from proteomics analysis also revealed that among other proteins related to mitochondria, the protein level of TEFM (Transcriptional Elongation Factor-Mitochondrial) was significantly increased and western blot analysis confirmed this finding. Interestingly, while TEFM protein levels increased in Bax/Bak-deficient U87 cells, they were suppressed in Bax/Bak-deficient HBL-2 cells. These data thus raised a question: Is alternation of TEFM expression responsible for the opposite effects on respiration and proliferation in Bax/Bak-deficient U87 and lymphoma cells? This prompted us to explore its role in mitochondrial respiration. A little over a decade ago, TEFM, alongside TFAM, was discovered as an important player in the regulation of mitochondrial genome transcription. (Figure 7) (214, 215). RNA interference-mediated downregulation of TEFM expression lead to respiratory incompetence due to reduced levels of mitochondrial transcripts distal to the H- and L-strand promoters (214). TEFM via its binding to mtDNA forms a downstream "sliding clamp" that enhances processivity of the elongation complex (216). TEFM thus effectively prevents transcription termination events at a G-quadruplex region near the replication origin and promotes longer RNA transcripts (216, 217). Recently it has been reported that TEFM is crucial for mouse embryonic development, and its conditional knockout in heart resulted in a significant decrease in promoter-distal mitochondrial transcripts and a notable increase in promoter-proximal transcripts (218). Deep sequencing analysis revealed an accumulation of unprocessed transcripts in TEFM knockouts, indicating that TEFM plays a critical role in transcription elongation, replication primer formation, and RNA processing in mammalian mitochondria. TEFM's significant involvement in

regulating the expression of mitochondrial genes carries pathophysiological implications. Elevated TEFM expression is associated with tumor progression and metastasis, predicting unfavourable prognosis and survival in patients with hepatocellular carcinomas and gliomas (218-220). Additionally, mutations in TEFM are linked to early onset neurological disorders, including mitochondrial encephalomyopathy, epilepsy, among others (221). Our findings align with published studies, demonstrating that cells with elevated TEFM protein levels, such as Bax/Bak-deficient U87 cells, show significantly increased mitochondrial respiration and proliferation, whereas cells with reduced TEFM expression, as Bax/Bak-deficient HBL-2 cells do have downregulated respiration and attenuated proliferation. Furthermore, consistent with Minczuk et al. (214), we observed that reducing TEFM expression in Bax/Bak-deficient U87 cells attenuated their boosted mitochondrial respiration, although not to the levels as in parental U87 cells. Similarly, TEFM downregulation in HBL-2 cells resulted in suppression of mitochondrial respiration to levels comparable to the respiration in Bax/Bak-deficient cells. Hence, we propose that BAX and BAK influence TEFM protein levels in cancer cells, independently of their roles in apoptosis, thereby modulating the respiratory and proliferative activities of cancer cells in a cell type-specific manner. In the above-mentioned studies, TEFM and other factors were found to be upregulated at the transcriptional level. However, in our model cells, the elimination of BAX and BAK modulates TEFM at the protein level, as there is no change in its mRNA expression in Bax/Bak-deficient U87 and HBL-2 cells. Excluding regulation of TEFM at the transcriptional level, it is possible that BAX/BAK could potentially influence TEFM expression through several other mechanisms, including post-transcriptional regulation, translational regulation, post-translational modification, protein degradation, protein localization, and transport. Considering that TEFM is encoded by nuclear DNA and then transported to mitochondria and that BAK and

BAX are predominantly localized in the outer mitochondrial membrane and cytosol, we presume that either TEFM degradation (possible interaction of BAX/BAK with MPP-Mitochondrial processing peptidase) or protein localization and transport into mitochondria (possible interaction of BAX/BAK with TOM family proteins or others involved in transport of nuclear-encoded protein into mitochondria) might be in cell-specific manner BAX and BAK-mediated modulation of TEFM protein levels (Figure 8).

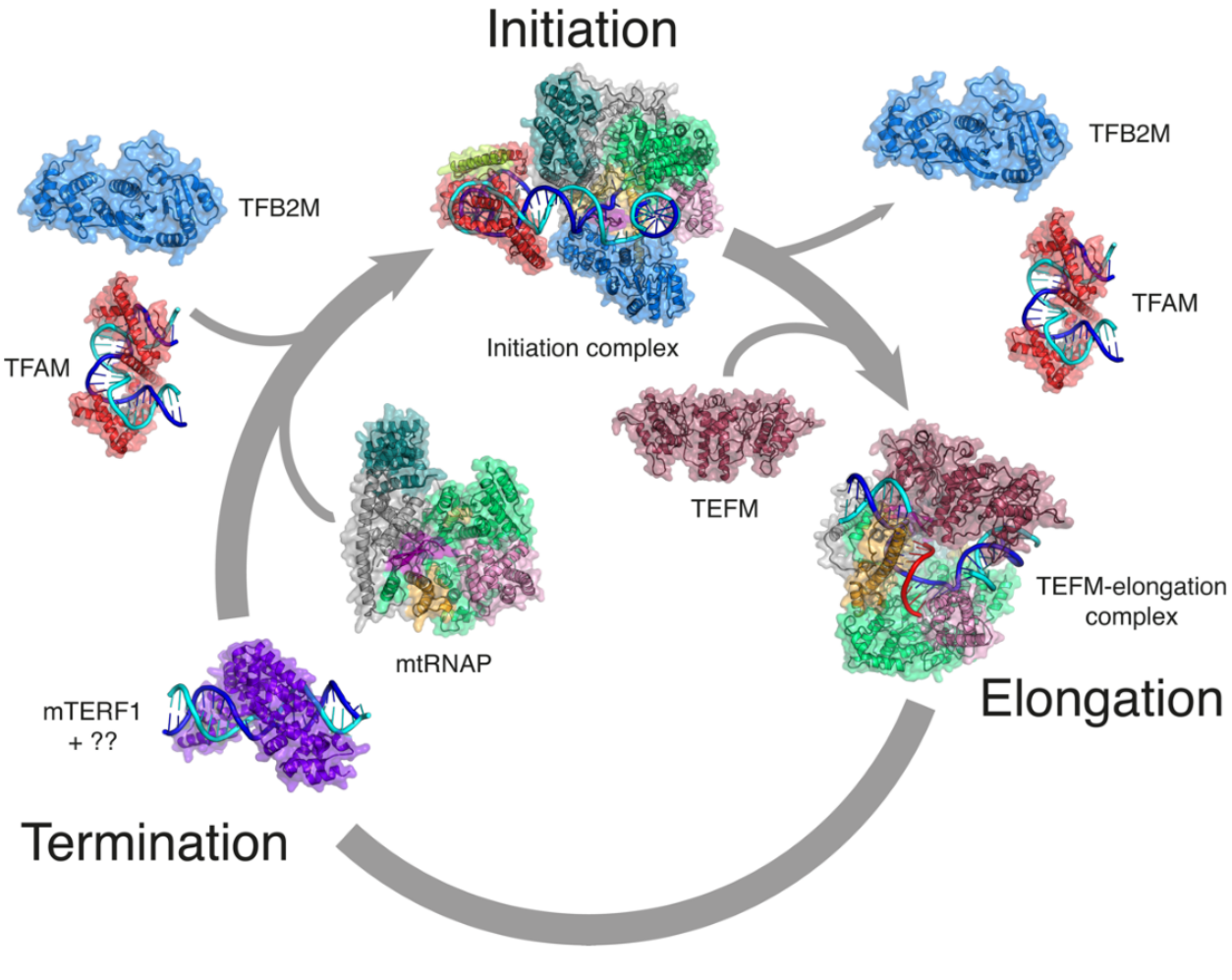

Figure 7. Structural view on the human mitochondrial transcription cycle. Adapted from (215).

Hence, we propose that BAX and BAK regulate the levels of the TEFM protein cancer cells, likely in cell type specific-manner and independently from their apoptosis-related function, which results in either the enhancement or reduction of both respiration and proliferation of these cancer cells.

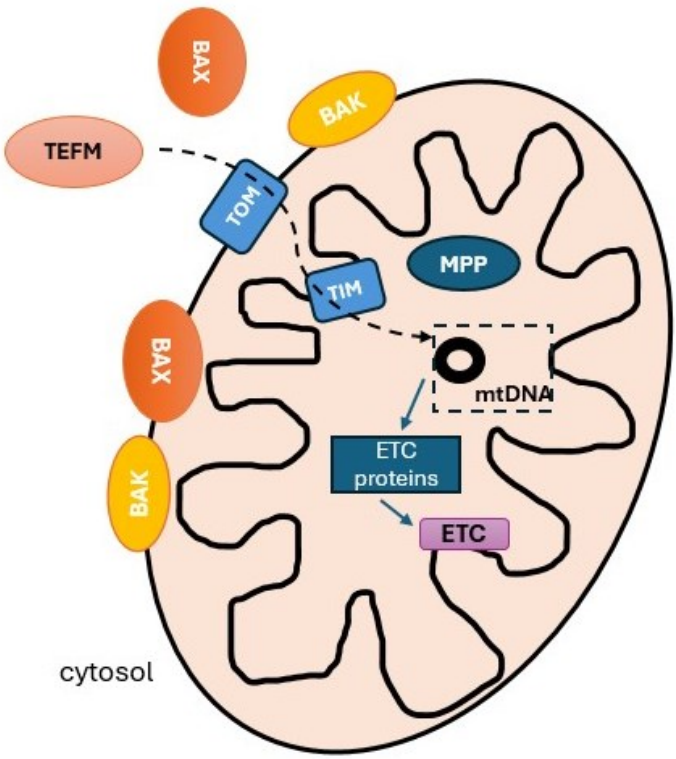

Figure 8. Cellular localization of potential players in BAK/BAK-mediated regulation of mitochondrial respiration.

Anti-apoptotic proteins from the Bcl-2 family as BCL-2, BCL-XL, and MCL-1 might enhance mitochondrial respiration and these proto-oncogenic proteins also play an important role in tumorigenesis. Resistance of cancer cells to apoptosis/RCD has been recognized as one of the hallmarks of cancer (222). This resistance is partly linked to the anti-apoptotic Bcl-2 family proteins and their targeting with BH3-mimetics represents a promising and novel approach in cancer therapy. Thus, our collaborative research with prof. Pavel Klener laboratory was mainly focus on the assessment of the role of anti-apoptotic Bcl-2 family proteins in human hematopoietic malignancies, their therapeutic targeting and overcoming resistances to targeted therapy.

Our study, which was focused on targeting MCL-1 protein in lymphoma treatment revealed that while some DLBCL and most Burkitt lymphoma cell lines were sensitive to the MCL-1-specific BH3 mimetic S63845, the acquired resistance to S63845 was primarily correlated with high levels of BCL-2 protein. BCL-2 acted as a buffer against pro-apoptotic proteins released by MCL-1 upon exposure to S63845. Sensitivity to S63845 was influenced by the expression level and occupancy status of MCL-1, with MCL-1-BIM/BAK complexes priming cells for apoptosis and MCL-1-NOXA complexes associated with resistance. Both BCL-2 and MCL-1 expression levels and occupancy status were found to be critical in determining sensitivity/resistance to S63845. Co-treatment with venetoclax (BCL-2 inhibitor) showed promise in overcoming BCL-2-mediated resistance to S63845, potentially enhancing the efficacy of MCL-1 inhibitors in aggressive B-NHL (B-Cell Non-Hodgkin Lymphoma) cases with BCL-2 positivity. Similarly, co-targeting MCL-1 and BCL-2 in acute myeloid leukemias and mantle cell lymphomas greatly enhanced their pro-apoptotic and growth suppressive potential (96, 223). We also addressed role of another anti-apoptotic protein, BCL-XL in human B cell lymphomas. Employing immunoprecipitation assays and genetic elimination or enhancement of Bcl-XL expression, emphasized its pivotal role in both

inherent and acquired resistance to venetoclax. Remarkably, even in venetoclax-resistant cells lacking the pro-apoptotic protein BIM the combination treatment of these cells with venetoclax and BCL-XL inhibitor A1155463 was synthetically lethal. Immunoprecipitation experiments identified BAX as the principal driver of apoptosis in BIM-deficient lymphoma cells. Moreover, asynchronous co-treatment of human lymphoma derived PDXs with venetoclax and A1155463 showed remarkable efficacy in growth suppression of these malignancies engrafted in NSG mice. Asynchronous treatment regime i.e. A1155463 application every fourth day suppressed thrombocytopenia, a common side effect of prolonged BCL-XL inhibition. In recent reports was also documented that-targeting BCL-XL and BCL-2 with a novel BH3 mimetic PROTAC 753B efficiently suppressed growth of acute myeloid leukemia PDXs (224) and interestingly co-treatment of solid and hematopoietic malignancies BCL-XL/BCL-2-targeting novitoclax and orally bioavailable BAX activator, BTSA1.2 effectively suppressed growth of these tumors (225). In addition to dual targeting of two anti-apoptotic proteins with BH3-mimetics we also explored the efficacy and mechanisms of combining palbociclib, a CDK 4/6 inhibitor, with venetoclax, in the treatment of mantle cell lymphomas. The combination showed significant synergy in killing MCL cells both in vitro and in vivo by reducing MCL-1 levels, increasing BIM levels, and sensitizing cells to apoptosis. Resistance to palbociclib was observed in cells lacking the RB1 gene (226). These findings suggest a potential chemotherapy-free treatment option for post-ibrutinib MCL patients without RB1 deletion.

Conclusion

The mitochondrial/intrinsic apoptosis in mammalian cells relies on the pro-apoptotic Bcl-2 family proteins BAX and BAK, but their apoptosis-unrelated cellular function is still poorly understood. Our study uncovered yet unknown role of these pro-apoptotic proteins in indirect, cell-type specific regulation of mitochondrial respiration. In Bax/Bak-deficient U87 glioblastoma cells, mitochondrial respiration was greatly enhanced, leading to accelerated cell proliferation and tumor formation in mice, and it was accompanied by significant metabolic changes such as increased NAD+/NADH ratio. Conversely, Bax/Bak-deficient HBL-2 B lymphoma cells showed slightly suppressed respiration and proliferation, in vitro and in vivo. The altered respiration of these Bax/Bak-deficient cells is linked to the modulation of TEFM protein levels in these cells and consequently to the enhanced (in U87 cells) or attenuated (in HBL-2 cells) expression of mitochondria-encoded subunits of respiratory complexes, and thus to increased or suppressed mitochondrial respiration. Targeted downregulation of TEFM attenuated mitochondrial respiration in both cell types, suggesting a crucial role for TEFM in modulating mitochondrial function and cellular metabolism in Bax/Bak-deficient cells. The association between BAX and BAK deficiency and the translational/posttranslational regulation of TEFM remains to be comprehensively investigated. The Bcl-2 family proteins also play a crucial role in tumorigenesis and in response of cancer cells to therapy. Thus, studying alterations in their expression levels and functions upon treatment with BH3 mimetics is of vital importance in understanding their therapeutic potential. Our collaborative articles have highlighted potential treatment combinations involving venetoclax, as well as elucidated the roles of specific Bcl-2 family members in the development of resistance to BH3 mimetics.

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