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Role of the Bcl-2 family of cell death regulators at the Endoplasmic Reticulum

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Role of the Bcl-2 family of cell death regulators at the Endoplasmic Reticulum

Rôle des protéines de la famille Bcl-2 au niveau du Réticulum Endoplasmique

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MANDATORY FEATURES

Titre Français

Rôle de la protéine anti-apoptotique Bcl-xL au niveau du Réticulum Endoplasmique

Mots-clés en Français

Protéines Bcl-2 – Bcl-xL – mitochondrie – réticulum endoplasmique – calcium – apoptose – cancer

Résumé en Français

Les protéines de la famille Bcl-2 sont des régulateurs majeurs de la voie mitochondriale de l'apoptose. Bcl-xL, un membre anti-apoptotique de cette famille, est surexprimé dans le cancer du sein et favoriserait le potentiel métastatique des cellules cancéreuses. Bcl-xL est localisé à la mitochondrie ainsi qu'au réticulum endoplasmique (RE). Cependant, les fonctions de Bcl-xL au niveau de RE sont encore mal connues.

Notre projet vise à évaluer les fonctions des pools mitochondriaux et réticulaires de Bcl-xL. Dans ce but, nous avons généré des souris génétiquement modifiées exprimant Bcl-xL exclusivement au niveau du RE (ER-xL) ou des mitochondries (Mt-xL). Des expériences de fractionnement subcellulaire et d'immunofluorescence sur les fibroblastes embryonnaires de souris (MEFs) ont validé la localisation exclusive de Bcl-xL chez ces souris.

De manière très intéressante, les MEFs exprimant la protéine Bcl-xL uniquement au niveau du RE sont plus résistantes au stress réticulaire par rapport aux MEFs de type sauvage (WT) et Mt-xL, du fait de l'inhibition du relargage de calcium de IP3R, un canal calcique localisé au RE. Les MEFs ER-xL ont montré moins d'initiation de la réponse aux protéines non-repliées ou Unfolded Protein Response (UPR) lors de l'induction du stress RE. En conclusion, nos résultats mettent en évidence un nouveau rôle de Bcl-xL localisé au niveau du RE en régulant négativement la libération de calcium médiée par IP3R et en protégeant les cellules contre le stress du RE (UPR).

Titre Anglais

Role of the anti-apoptotic protein Bcl-xL at the Endoplasmic Reticulum

Mots-clés en Anglais

Bcl-2 proteins – Bcl-xL– mitochondria – endoplasmic reticulum – calcium – apoptosis – cancer

Résumé en Anglais

Bcl-2 proteins are major regulators of the mitochondrial pathway of apoptosis. Bcl-xL, an anti-apoptotic Bcl-2 homolog, is overexpressed in breast cancer where it increases metastatic potential. This member is present at the mitochondria as well as the endoplasmic reticulum (ER). However, the subsequent function of its ER localization is poorly explored.

Our project aims at evaluating the respective functional contributions of the mitochondrial and reticular pools of Bcl-xL. To do so, we generated genetically modified mice expressing exclusively Bcl-xL at the ER referred to as ER-xL or mitochondria referred to as Mt-xL. Subcellular fractionation experiments on mice embryonic fibroblasts (MEFs) validated the exclusive Bcl-xL localization in these mice. By performing cell death assays, we showed that ER-xL MEFs have increased vulnerability to apoptotic stimuli compared to wild-type (WT) and Mt-xL MEFs but are more resistant to ER stress. Moreover, ER-xL MEFs displayed increased calcium sensibility at the ER but a reduced Inositol triphosphate receptor (IP3R)-mediated calcium release. Importantly, the ER-localization of Bcl-xL seems to confer resistance through the Unfolded Protein Response (UPR). We have shown for the first time that upon ER stress, Bcl-xL at the ER negatively regulates IP3R inhibiting ER calcium depletion necessary for UPR initiation and subsequently apoptosis. Overall, this work reveals a moonlighting function of Bcl-xL at the ER, apart from its cliché regulation of apoptosis.

Résumé substantiel en Français

L'apoptose est un type de mort cellulaire programmée bien étudié. Ce processus induit naturellement le renouvellement des cellules, maintient l'homéostasie tissulaire et favorise la morphogénèse lors du développement embryonnaire. Par conséquent, toute perturbation de sa régulation aura d'énormes conséquences en particulier sur les maladies liées à l'âge ou le cancer. Deux voies apoptotiques principales ont été décrites. La voie extrinsèque est activée lors d'interactions récepteurs de la mort – ligands qui déclenchent une cascade d'événements entraînant l'activation de certaines protéases appelées caspases responsables du clivage de nombreuses protéines entraînant la mort cellulaire. La voie intrinsèque, aussi appelée voie mitochondriale, est déclenchée par des stimuli non associés aux récepteurs, comme le stress intracellulaire et extracellulaire et le manque de facteurs de croissance. La famille des protéines Bcl-2 est connue pour être un régulateur de l'apoptose mitochondriale. Cette famille se compose de deux groupes fonctionnels principaux : les protéines anti-apoptotiques qui inhibent la mort cellulaire et les protéines pro-apoptotiques qui l'induisent. En fait, le deuxième sous-groupe est responsable de la perméabilisation de la membrane externe mitochondriale menant à la libération de protéines inter-membranaires comme le cytochrome c dans le cytosol. Ce dernier conduit à l'activation des caspases et à l'apoptose. Le groupe anti-apoptotique inhibe les pro-apoptotiques en les liant et les séquestrant. La surexpression des membres anti-apoptotiques est observée dans de nombreux types de cancers.

Il a été montré que la surexpression de Bcl-xL, un membre anti-apoptotique de la famille Bcl-2, observée dans de nombreux cancers comme le cancer du sein et le carcinome hépatocellulaire favoriserait le potentiel métastatique et l'invasion des cellules cancéreuses. Il est intéressant de noter que l'inhibition de l'expression de *bclx* induit chez la souris une mortalité embryonnaire suggérant un rôle majeur du Bcl-xL dans le développement. En effet, pendant l'ontogenèse neuronale, l'expression de Bcl-xL augmente et reste élevée jusqu'à ce qu'elle atteigne son apogée dans les cellules qui se différencient. De plus, Bcl-xL joue un rôle majeur dans le système hématopoïétique où il a été démontré qu'il favorise la survie des thymocytes et régule la différenciation des mégacaryocytes et érythroblastes. Il est important de noter que le ciblage thérapeutique dans les cancers de cette protéine par des thérapies

mimétiques BH3 a induit une thrombocytopénie sévère due à l'inhibition du Bcl-xL qui constitue un facteur essentiel pour la survie plaquettaire.

Cette protéine possède une double localisation subcellulaire, elle est localisée dans les mitochondries et au niveau du réticulum endoplasmique (RE). Son rôle dans les mitochondries est liée à l'inhibition de l'apoptose. Cependant, sa fonction au niveau RE reste mal connue. Au niveau de ce dernier organe, Bcl-xL interagit avec plusieurs canaux calciques comme le récepteur à l'inositol triphosphate (IP3R) où il le sensibilise à un faible taux de IP3. En dehors de la régulation de l'homéostasie calcique au niveau du RE, on sait peu de choses sur les autres fonctions non canoniques de la protéine. En conséquence, nous sommes intéressés au rôle fonctionnel du pool réticulaire de Bcl-xL. Pour ce faire, nous avons généré des souris génétiquement modifiées par le système de recombinaison Cre-LoxP et avons obtenu des souris exprimant Bcl-xL exclusivement au RE (ER-xL) ou aux mitochondries (Mt-xL). Tout d'abord, nous avons vérifié l'expression du Bcl-xL dans des cellules extraites de plusieurs organes de souris et dans des fibroblastes embryonnaires de souris (MEFs) de type sauvage (WT), ER-xL et Mt-xL extraits d'embryons de souris. Ensuite, nous avons validé la localisation de cette protéine dans ces MEFs par fractionnement subcellulaire, immunofluorescence et marquage ImmunoGold. Ensuite, nous avons effectué des tests de mort cellulaire sur les MEFs en les traitant avec des drogues cytotoxiques tels que la staurosporine. Les MEFs ER-xL sont plus vulnérables à la staurosporine mais plus résistantes aux inducteurs de stress ER comme la thapsigargine. De plus, ces MEFs ont montré moins d'initiation de la réponse aux protéines non-repliées ou Unfolded Protein Response (UPR) lors de l'induction du stress RE, comme le prouve le traitement cinétique suivi d'un test de détection par Western blot pour les marqueurs d'UPR. Nous avons émis l'hypothèse que la résistance observée pourrait impliquer une régulation du calcium au niveau du RE. En effet, nous avons pu montrer que Bcl-xL interagit avec IP3R (par Proximity Ligation Assay) et régule négativement la libération de calcium médiée par IP3R uniquement lorsqu'il est présent au RE (par microscopie confocale et dosage du calcium-45). Afin d'établir s'il existe un lien entre la résistance observée au stress du RE et la diminution induite par Bcl-xL sur l'activité d'IP3R, nous avons bloqué IP3R à l'aide d'inhibiteurs et traité les MEFs avec la thapsigargine. L'analyse par WB des marqueurs d'UPR montrent que les MEFs WT et Mt-xL sont plus résistantes au stress réticulaire. Ces données mettent en évidence un nouveau rôle de Bcl-xL au RE en régulant négativement la libération de calcium médiée par IP3R et en protégeant contre le stress RE.

En nous basant sur les fonctions non canoniques des protéines Bcl-2 et sur le nouveau rôle de Bcl-xL que nous avons mis en évidence, nous voulions savoir si ces fonctions étaient conservées au cours de l'évolution des métazoaires. En effet, après la découverte du gène Bcl-2, de nombreux membres de cette famille ont émergé chez l'homme et chez d'autres espèces comme l'hydre et les nématodes. Nous étions intéressés par une branche basale des métazoaires appelée placozoaire. Un des représentants de cet embranchement est le *Trichoplax adhaerens* (T. adhaerens.) C'est un petit organisme d'environ un millimètre de diamètre, plat, dépourvu de structures internes et d'organes avec seulement six types cellulaires. En effet, T. adhaerens a un génome plus petit que celui d'autres animaux, mais il présente un contenu et une structure génétique conservés par rapport aux génomes humains et autres métazoaires. Il a été démontré que T. adhaerens est capable d'induire une apoptose lorsque des inhibiteurs spécifiques de survie sont utilisés. Cela soulève la question de savoir si les mêmes mécanismes apoptotiques et non apoptotiques se produisent dans l'organisme métazoaire le plus primitif. Très peu de choses sont connues sur l'origine et l'évolution de ces fonctions. Cependant, le génome de T. adhaerens a été séquencé en 2008 et nous avons pu mettre en évidence que quatre gènes codant potentiellement pour des protéines ayant une similarité structurelle avec des protéines Bcl-2 multidomaines. Nous les avons nommés trBcl-2Like (trBcl-2L). Les analyses phylogénétiques ont démontré que cet animal possède deux homologues anti-apoptotiques (trBcl-2L1 et -2L2) et deux pro-apoptotiques (trBcl-2L3 et -2L4). Nous avons pu démontrer que trBcl-2L3 est une véritable protéine de type Bax capable d'induire une perméabilisation de la membrane externe mitochondriale. En revanche, trBcl-2L4 est un homologue de type Bak au niveau de la structure, mais agit comme sensibilisateur BH3-only, réprimant les activités anti-apoptotiques de trBcl-2L1 et trBcl-2L2. Enfin, nous avons démontré la double localisation subcellulaire des trBcl-2L1 et trBcl-2L2 présentes à la fois aux mitochondries et au RE. Dans ce dernier cas, il a été démontré que le trBcl-2L1 contrôle les flux calciques dépendants de l'IP3R. Dans l'ensemble, ces données suggèrent que la pléiotropie des protéines Bcl-2 serait conservée dans l'évolution métazoaire et que ces membres, en plus de réguler l'apoptose, sont impliqués dans d'autres processus cellulaires chez les animaux les plus simples et les plus complexes.

En conclusion, nos données mettent en lumière une nouvelle fonction de la protéine anti-apoptotique Bcl-xL au RE d'une part et suggèrent que les fonctions des membres de la famille Bcl-2 seraient conservées au cours de l'évolution des animaux multicellulaires d'autre part.

Publications

Article 1: **Jabbour L**, Popgeorgiev N, Gadet R, Bultynck G, Lohez O, Chalabi M, Mikaelian I, Rimokh R and Gillet G.

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Pleiotropy of Bcl-2 family proteins is an ancient trait in the metazoan evolution.

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Review article: Popgeorgiev N, **Jabbour L**, Gillet G.

Subcellular localization and Dynamics of the Bcl-2 family of proteins.

Frontiers in Cell and Developmental Biology. February 13th 2018. doi: [10.3389/fcell.2018.00013](https://doi.org/10.3389/fcell.2018.00013).

(Refer to ANNEX II)

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If I am writing this today, it is thanks to my family. I would not be here, nor have done any of this, if it were not for you Mom and Pap. You are my source of support and motivation. I only wish I can always make you proud. My beautiful sisters Reina, Perla and Joya, you are and always will be my stars, my guidance and my source of positive energy. You always believed in me and helped me overcome my fears. I love you.

PREFACE

There is a thin line between life and death. However, this line harbors complicated mechanisms regulating the switch between the two. Life and death govern ecosystems, organisms and cells that are constantly dying and constantly living. One of the most elaborated programmed cell death is apoptosis. This process preserves the balance of cell death/cell survival where it eliminates harmful cells and shapes the body during development. In contrast, 'successful and resistant' cancer cells are able to escape the apoptotic machinery and stimulate tumor initiation and progression, in other words, promoting their immortality. One of the main regulators of apoptosis is the Bcl-2 protein family. Throughout the years, numerous Bcl-2 members have been discovered and characterized. Several functions have been also elaborated after being long retained unmasked.

In this manuscript, we try to discern the complexity of this family and its involvement in numerous cellular processes. We also introduce a novel role of an anti-apoptotic member called Bcl-xL at a certain subcellular localization while questioning the conservation of known functions of the whole family during multicellular animal evolution.

On step closer towards the understanding and battling of cellular immortality because after all,

'Immortality is a fate worse than death.'

(Edgar A. Shoaff)

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INTRODUCTION

1. Apoptosis and Bcl-2 proteins: *“To kill or not to kill, that is the question.”*

1.1. What is Apoptosis?

“By the time I was born, more of me had died than survived.” By this, Lewis Thomas perfectly states the importance of programmed cell death (PCD) type 1, or Apoptosis (Eckardt, 2006). The latter originates from the Greek word “ἀούφωσις” meaning “falling-off” of petals or leaves from flowers or trees, respectively (Kerr et al., 1972). Life and death were and remain the most problematic issues. For the sake of continuity of life, death must occur. This natural phenomenon happens daily in the human body: one cell shall die, for another to live. When the cell is “programmed” to die, it will shrink, be fragmented forming apoptotic bodies that will then be phagocytized by neighboring cells mainly macrophages. The hallmark of apoptosis is the DNA fragmentation by selectively activated DNases (Saraste and Pulkki, 2000). This process naturally induces the turnover of the cells, maintains tissue homeostasis and promotes morphogenesis during embryonic development. Accordingly, any dysregulation of PCD will result in tremendous consequences involving age-related diseases (neurodegenerative diseases, immune system decline) (Tower, 2015) or cancer (Reed, 1999). In general, apoptosis is performed under three steps: Initiation, regulation and execution. During the first step, the cell receives stress signals *via* its sensor proteins. Next, Bcl-2 proteins join the game: the regulation step relies on this family whose actions change the equilibrium towards apoptosis execution (step 3) or cell survival. In the case of apoptosis execution, several Cystein-Aspartate proteases are activated. They cleave and neutralize cellular proteins leading to cell death.

1.1.1. Caspases: effectors of the job

As mentioned above, Cystein–Aspartate proteases called caspases, are basically the hallmark of apoptosis execution. These proteases are able to cleave target proteins – via their Cystein residue found in their active site – at their Aspartate residue.

They have a conserved structure: N–terminal pro–domain, central catalytic domain and a C–terminal conserved motif. Caspases are in an inactive precursor form. Upon cell death signals, they dimerize with the aid of helper proteins and might require a pro–caspase N–terminal cleavage to be active (Mcllwain et al., 2013). Caspases are divided into initiator and effector. Initiator caspases include caspases 2,8,9 and 10 and effector caspases include caspases 3, 6 and 7. Another type of caspases, called inflammation caspases, regroups caspases 1, 4, 5 and 12.

In fact, initiator caspases have a longer N–terminal domain. Caspases 2 and 9 are characterized by a Caspase Recruitment Domain (CARD) and Caspases 8 and 10 are known to have a DED (or a Death effector domain). Accordingly, these two domains are indispensable for the formation of complexes with adaptor proteins necessary for the induction of apoptosis (**Figure 1**) (Mcllwain et al., 2013).

Effector Caspases 3, 6 and 7 are considered the molecular scissors. Caspase 3 is the main effector caspase to date. These caspases do not possess special domains such as DED or CARD and are directly activated by initiator caspases proteolytic cleavage (Pop and Salvesen, 2009) (**Figure 1**).

Upon activation signals, initiator caspases are activated by dimerization and cleave effector caspases. Once active, effector caspases cleave numerous protein substrates through proteolytic cascade initiation resulting in apoptosis (Lüthi and Martin, 2007).

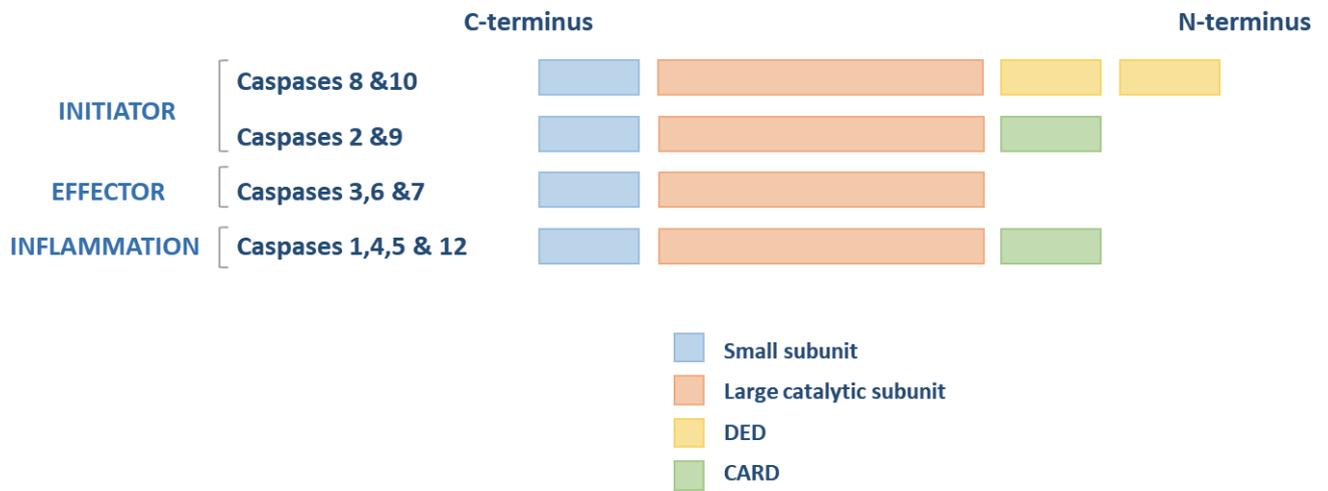


Figure 1: *Different domains of human caspases.*

Caspases are subdivided into 2 groups in the process of apoptosis execution: initiator and effector caspases. Another subtype is involved in inflammation. All caspases share a small subunit along with a large catalytic one. They might possess DED or CARD domains at their N-terminal necessary for protein-protein interactions and their subsequent activation.

1.2. Bcl-2 proteins: *discovery and evolution*

The Bcl-2 family of proteins is mostly known for its regulation of cell death precisely, apoptosis. Indeed, they decide the fate of the cell. Throughout the years, numerous members of this family were identified and characterized. Moreover, an increasing number of non-apoptotic functions of these proteins started to emerge modifying the cliché role of regulating apoptosis.

Discovery of Bcl-2 proteins

In 1984, Pegoraro and colleagues were the first to show that two chromosome rearrangements – t(14,18) and t(8,14) – contribute to oncogenic transformation in a B-cell lymphoma case (Pegoraro et al., 1984). In the t(14,18), an unknown oncogene placed on the chromosome 18 was identified and appointed Bcell lymphoma 2 (*bcl2*) which initiated the nomenclature of the *Bcl-2* oncogene. One year later, DNA probes that recognize the region of the double strand break promoting the translocation on the chromosome 18, allowed the cloning of the *bcl2* gene. The t(14,18) translocation places *bcl-2* under the control of Immunoglobulin heavy chain gene promoter which was responsible for its overexpression (Tsujiimoto et al., 1985).

Few years after its discovery, the function of the protein encoded by the *bcl2* gene started to show. It was demonstrated that this protein promoted the survival of B lymphocytes deprived of Interleukin 3 (IL-3), on the contrary to *c-myc*-encoded one that promotes cell proliferation (Vaux et al., 1988). Accordingly, a new class of oncogenes was identified promoting the survival of the cell under compromising conditions.

bcl2 and related genes are found only in metazoans and are currently considered as one of the hallmarks of multicellularity. Apoptosis was firstly characterized in metazoans as a way to eliminate damaged/abnormal cells without inducing an immune response (Engelberg-Kulka et al., 2006). It has been shown that there is a conservation of the molecular process of PCD from nematodes to mammals. In fact, the nematode *Caenorhabditis elegans* (*C. elegans*) was found to have several genes that regulate and execute PCD. *Ced9* (for cell death abnormal-9) negatively regulates the apoptotic pathway. It was the first Bcl-2 homolog discovered in non-vertebrate organisms and is structurally and functionally

related to pro-survival Bcl-2 protein (Hengartner and Horvitz, 1994). Further studies have identified two Bcl-2 homologs in drosophila: a pro-apoptotic one (Drop-1) and the other with anti-apoptotic function (Buffy) (Richardson and Kumar, 2002).

Evolution of Bcl-2 proteins

Knowing that at least one Bcl-2 homolog is found in *C. elegans*, two are found in drosophila and much more are discovered in humans, one might think that Bcl-2 related gene expression increased in a linear fashion during metazoan evolution. For instance, by checking basal branching metazoans such as hydra or sea urchins, highly divergent Bcl-2 conserved gene and protein structures are found indicating that *bcl2* genes might possibly have a common origin among species.

In fact, placozoans represent a basal group of metazoa (multicellular organisms). One of the very few representatives of this phylum is *Trichoplax adhaerens*. It is a small organism with a diameter of around one millimeter, flat, lacking internal structures and organs with only two cellular layers and four cell types. Indeed, *T. adhaerens* has a smaller genome compared to other animals, but it shows conserved gene content and structure in relation to human and other metazoan genomes (Srivastava et al., 2008a). It has been shown that *T. adhaerens* is capable of undergoing apoptosis whenever specific inhibitors of survival are used. This raised the question whether the same apoptotic and non-apoptotic mechanisms occur in the most primitive metazoan organism as they do in more derived organisms. Till now, no definitive indications on the actual apoptotic proteins present (von der Chevallerie et al., 2014). However, the genome of *T. adhaerens* was sequenced in 2008 and four genes were shown to potentially code for proteins with structural similarity to multidomain Bcl-2 proteins. These were called tr Bcl-2Like by our team (Popgeorgiev et al., 2019) (See Results).

1.3. Classification of the Bcl-2 proteins: *A family larger than yours*

Soon after Bcl-2 discovery, a huge interest was shown in the structure and function of this protein. Other studies have identified proteins with a high sequence homology with Bcl-2 in many species. Accordingly, Bcl-2 became the image of the whole family. On the structure level, to gain a membership in the Bcl-2 fraternity means to possess at least one Bcl-2 Homology (BH) domain consisting of a conserved short amino acid (aa) sequence along – or not – a transmembrane domain (TM) allowing the anchorage into intracellular membranes. Four BH domains exist (BH1–4) with BH3 being the only domain present in all Bcl-2 members. On the function level, this family is divided into pro-apoptotic (or anti-survival) and anti-apoptotic (or pro-survival) depending on their way of regulating apoptosis (Aouacheria et al., 2013). As a result, they are classified into three main groups: multidomain anti-apoptotic, multidomain pro-apoptotic and BH3-only pro-apoptotic proteins (**Figure 2**).

1.3.1. Multidomain anti-apoptotic proteins

Not long after the discovery of the *Bcl-2* gene in 1984 and the Bcl-2 protein coding region in 1986, other multidomain members were identified. In 1993, a gene called *bclx* was isolated and reported to function in the regulation of apoptosis in a Bcl-2-independent manner. In fact, alternative splicing of this gene results in completely distinct mRNAs, the larger one – the known multidomain anti-apoptotic – Bcl-xL (Bcl-2L1) consists of 233 aa. The other splice variant – called Bcl-xS – is pro-apoptotic and encodes for a shorter protein of 63 aa (Boise et al., 1993a). During the same year, Mcl-1 (Bcl-2L3) and Bfl-1 (Bcl-2A1 in mice) were discovered to have sequence homology with Bcl-2. They were also shown to promote viability and its concomitant maintenance enlarging thus the anti-apoptotic clan (Kozopas et al., 1993; Lin et al., 1993). Soon after, BCL2L10 (also called Nrh/Bcl-B in humans and Diva/Boo in mice) was identified in our team (Aouacheria et al., 2001; Gillet et al., 1995). After discovering quail NR-13 gene, Gillet's team was able to identify Nrh as an inhibitor of apoptosis in humans by preferentially associating with the anti-survival protein Bcl-xS. One year after NR-13 gene unmasking, Bcl-W (also called Bcl2L2) was the newest addition to the Bcl-2 family. It was considered as an attractive proto-oncogene candidate

based on its functional similarity with Bcl-2 in promoting cell survival in different cell lines (Gibson et al., 1996).

Apart from being anti-apoptotic, all of those proteins share the four BH domains. BH1 and BH2 domains were firstly shown to be required for the inhibition of apoptosis through the heterodimerization of Bcl-2 with the pro-apoptotic protein Bax. Mutations in the mentioned domains inhibit the interaction between the two proteins and compromise cell survival capacity of Bcl-2 (Yin et al., 1994). However, an important domain for the protective functions of the Bcl-2 multidomain class is BH4. Found in the native N-terminus of the multidomain proteins, BH4 comprises a stretch of 10 to 30 aa organized in an α -helical structure ($\alpha 1$) (Lee et al., 1996; Petros et al., 2001). This domain was identified by multiple sequence alignment of a subgroup of closely related Bcl-2, Bcl-xL and Bcl-W. On the other hand, Bfl-1, Mcl-1 and Bcl-2L10 seem to have a divergent BH4 motif, along with the other multidomain pro-apoptotic members. Consequently, the BH4 domain is the least conserved domain in the Bcl-2 clan (Aouacheria et al., 2005a; Huang et al., 1998; Reed et al., 1996).

All multidomain proteins possess a transmembrane domain responsible for their anchorage in intracellular membranes such as the mitochondrial outer membrane (MOM) and the nuclear membrane. As an exception, Bfl1 and Bpr have a less defined but a functional TM domain. Accordingly, any loss of the TM results in the accumulation of the protein in the cytosol. Sequences in the TM might decide the localization of the protein. However, other factors might affect the targeting to specific organelles such as intracellular protein-protein interactions. To date, the dynamics of the Bcl-2 proteins remain poorly understood; check (part 1.6.2.) (Popgeorgiev et al., 2018; Schinzel et al., 2004a).

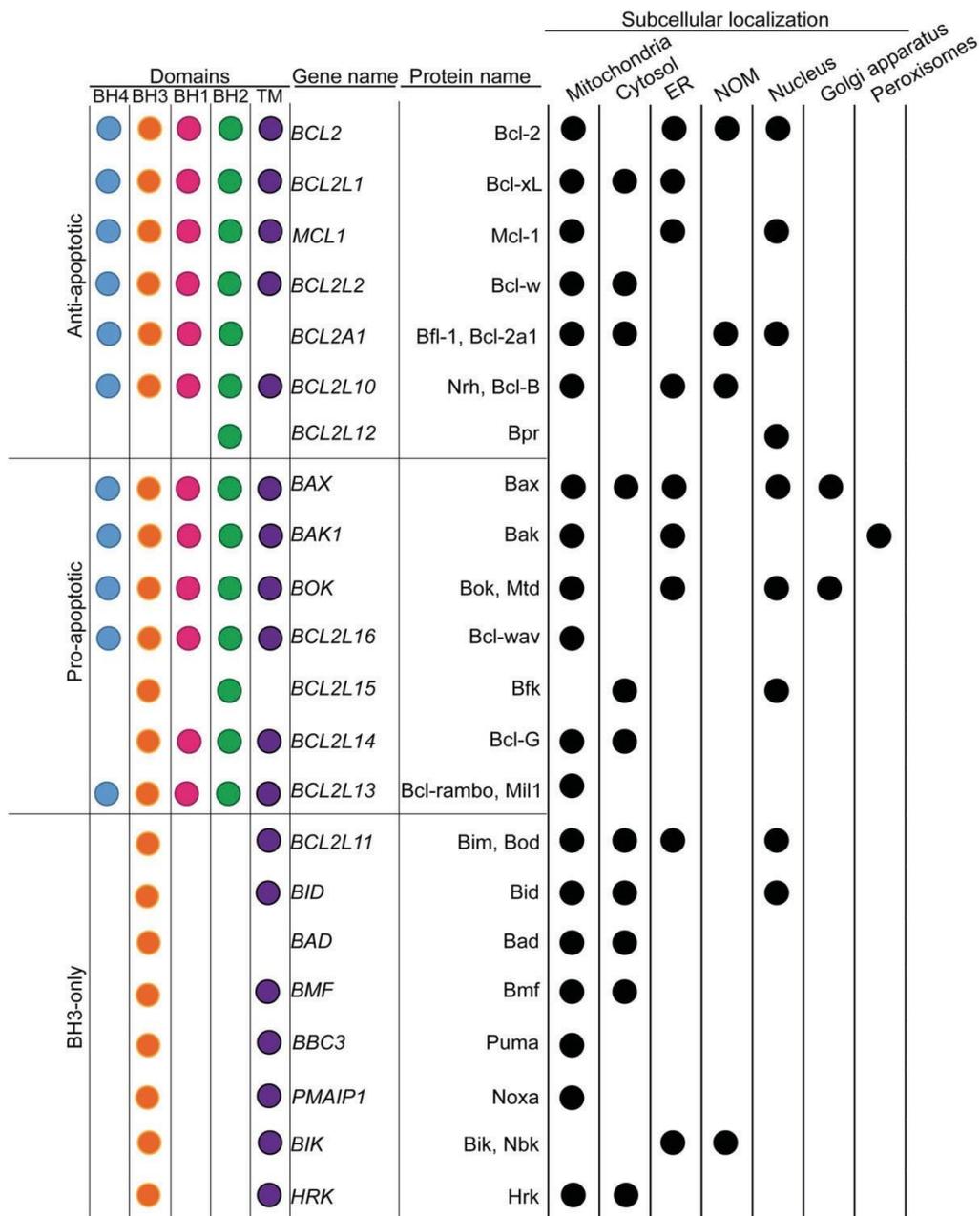


Figure 2: BH structure and subcellular localization of Bcl-2 proteins.

The classification of Bcl-2 members into 3 groups is based on their structure and function. Their subsequent subcellular localization is depicted. ER: endoplasmic reticulum. NOM: nuclear outer membrane (Popgeorgiev et al., 2018).

1.3.2. Multidomain pro-apoptotic proteins

The second sub-gang of the Bcl-2 family is composed of the “cell murderers” or the pro-apoptotic proteins. Even though they are less numerous than the other groups, their action could have detrimental effects on the cell. Cloning of the first pro-apoptotic protein Bax occurred in 1989. In fact, Korsmeyer’s team aimed to decipher the interactors with Bcl-2. They discovered a 21kDa partner (a) that has extensive aa homology with Bcl-2. Alternative RNA splicing gives rise to both membrane (a) and cytosolic (β and \wedge). Moreover, Bax was able to homodimerize or to heterodimerize with Bcl-2 repressing its pro-survival function. It appeared that the ratio of Bcl-2 to Bax determines the fate of the cell (Korsmeyer et al., 1993; Oltvai et al., 1993). One year later, the same group put in evidence the importance of BH1 and BH2 of Bcl-2 in its heterodimerization with Bax and subsequent repression of cell death (Yin et al., 1994). During the same year, *Bcl-2* and *Bax* were considered as p53 regulated genes since the tumor suppressor (TS) p53 downregulates Bcl-2 and upregulates Bax (Selvakumaran et al., 1994). In 1995, a new member was added to this group: Bak. It appeared that Bak binds the adenovirus protein E1B 19K along with the anti-apoptotic protein Bcl-xL and blocks their protective effect consequently accelerating apoptosis in sympathetic neurons deprived of nerve growth factor (Farrow et al., 1995). Also in serum-deprived fibroblasts, overexpression of Bak resulted in rapid and extensive apoptosis (Chittenden et al., 1995). It has been shown that the expression of Bak mRNA is widely distributed in the tissues and that the regulation of apoptosis is mainly controlled by the expression of proteins that inhibit it, in other terms, anti-apoptotic proteins (Kiefer et al., 1995). The multidomain pro-apoptotic group includes also Bok. Unlike Bak, the expression of Bok was firstly shown to be restricted to the reproductive tissues such as ovaries, uterus and testis (Hsu et al., 1997). Furthermore, Bok induces its anti-survival function with selective heterodimerization with anti-apoptotic members. In fact, Bok dimerizes with selective anti-apoptotic proteins such as Mcl-1 through its BH3 moiety. Any mutation in its BH3 domain leads to destabilization of this interaction without affecting its pro-apoptotic function (Hsu and Hsueh, 1998). Years later, the expression of Bok mRNA was indicated to be induced upon serum deprivation of HC11 mammary epithelial cells (Ha et al., 2001). Even though Bok is mainly expressed in the reproductive tissues, it was also shown to be present at readily detectable levels in the brain and myeloid cells (Ke et al., 2012a). Of note, Bok promotes the apoptotic response in ER stress situations independently from

Bax/Bak which is consisting with its predominant ER subcellular localization (Carpio et al., 2015a, 2016). However, these results were confronted with newer data showing that Bok might not be essential in promoting apoptosis faced to ER stress in primary and immortalized Bok^{-/-} hepatocytes (Fernandez-Marrero et al., 2016). Taken together, Bok seems to induce its function in a very selective and specific-tissue manner. The mostly known multidomain pro-apoptotic proteins Bax, Bak and Bok share BH1-4 domains along with a TM domain at their carboxy-terminal moiety (Kvansakul et al., 2008). Known to be the downstream effectors of apoptosis, these pro-apoptotic members are always kept on-check by anti-apoptotic proteins to control and prevent cell death.

Other Bcl-2 pro-apoptotic proteins were identified throughout the years: Bcl-G, containing BH2 and BH3, is present in the form of two proteins resulting from alternative splicing of the gene. Bcl-G(L) long or Bcl-G(S) short, when overexpressed, induces apoptosis in cells (Guo et al., 2001). Bcl-rambo possesses BH1-4 and is known to induce apoptosis by its unusual membrane-anchored c-terminal domain and not by any BH domain. It doesn't bind any Bcl-2 protein and is found strictly at the mitochondria (Kataoka et al., 2001). Bfk, also lacking BH1 and BH4 like Bcl-G, doesn't bind any Bcl-2 protein and weakly prompts apoptosis when overexpressed. Bfk appeared to have a role in mammary development and is mainly present in the nucleus (Coultas et al., 2003; Pujianto et al., 2007). Bcl-wav, found in fish and frogs, interacts with calcium (Ca²⁺) transporters and affects actin microfilaments dynamics guiding blastomere migration in zebrafish (Prudent et al., 2013a).

1.3.3. BH3-only pro-apoptotic proteins

The BH3-only group was and still is the hardest to define. As the name implies, the proteins belonging to this group possess only one BH3 domain. Accordingly, eight canonical members exist to date: Bad, Bik, Bid, Hrk, Bim, Noxa, Puma and Bmf.

In 1995, the first one – Bad – was shown to dimerize with Bcl-xL and Bcl-2 (with a higher affinity to Bcl-xL) thus, liberating Bax and prompting apoptosis induction (Yang et al., 1995). Then, Bik was shown to exert its pro-apoptotic activity by interacting with cellular, as well as, viral pro-survival proteins such as Epstein Barr virus-BHRF1 and adenovirus E1B-19 kDa (Boyd et al., 1995). Another death agonist with BH3-only domain was identified: Bid. The team of Korsmeyer has demonstrated that the BH3 domain

itself is a death domain and that Bid constitutes a death ligand for Bax. Moreover, Bid resides in an inactive form in the cytosol. Upon death stimuli, Bid is truncated (tBid) and translocated to the mitochondria in order to promote Bax/Bak dependent apoptosis. Apparently, the co-expression of Bcl-xL diminished the apoptotic changes following Bid cleavage (Gross et al., 1999; Li et al., 1998a; Wang et al., 1996). In 1997, a new gene Harakiri was cloned and characterized. The protein encoded by this gene, *hrk*, interacts with Bcl-xL and Bcl-2 and not with Bax and Bak. It was proven to have a high sequence homology with the BH3 domain and any mutation of this sequence resulted in a destabilization of its interaction with the pro-survival proteins, thus, provoking cell death (Inohara et al., 1997). Then, Bim was described as a potent death ligand that interacts and neutralizes certain anti-apoptotic proteins. Alternative splicing yields three Bim isoforms with the capacity to induce apoptosis (O'Connor et al., 1998). In the beginning of the twentieth century, Noxa and Puma were identified as BH3-only proteins which ectopic expression induces their translocation to the mitochondria where they prompt cell death execution (Nakano and Vousden, 2001; Oda et al., 2000). In fact, the latter proteins are transcriptionally regulated by p53. Upon DNA damage, p53-regulated genes are activated resulting in the localization of both proteins at the mitochondria and their interaction with resident anti-apoptotic proteins triggering thus cell death pathway. Hence, Noxa and Puma are considered to be critical mediators of apoptosis induced by p53 response (Schuler and Green, 2001; Villunger et al., 2003a). The last canonical BH3-only protein to be identified was Bmf that also performs its anti-survival action by binding to and inhibiting the pro-survival Bcl-2 members. In fact, Bmf is sequestered to actin filament network where it is associated with myosin V actin motor complex. Upon cytoskeleton disorganization, Bmf is unleashed and free to bind anti-apoptotic proteins in order to induce anoikis (or disruption of the interactions between normal epithelial cells and extracellular matrix-induced apoptosis) (Puthalakath et al., 2001).

This group was initially subdivided into sensitizers and direct activators of Bax and Bak. In other terms, they can indirectly or directly activate the multidomain pro-apoptotic Bax and Bak.

This model was under debate in 2016 based on studies by O'Neill and colleagues that have shown that the activation of Bax and Bak is only dependent on the inactivation of pro-survival Bcl-2 proteins (O'Neill et al., 2016). Furthermore, a recent study provided the "Membrane-mediated Permissive" model in which only the indirect activation of cell death effectors Bax and Bak was confirmed in cells lacking all eight pro-apoptotic BH3-only proteins. In fact, BH3-only members target primarily Bcl-xL and Mcl-1.

Nonetheless, neither Bim nor Bid were able to distinguish between Bax and Bak nor improve their apoptotic potential following the inactivation of Bcl-xL/Mcl-1. The whole model insinuates the indirect way of action of the BH3-proteins in the execution of apoptosis (Huang et al., 2019).

Here, we present the family tree timeline of the Bcl-2 proteins (**Figure 3**).

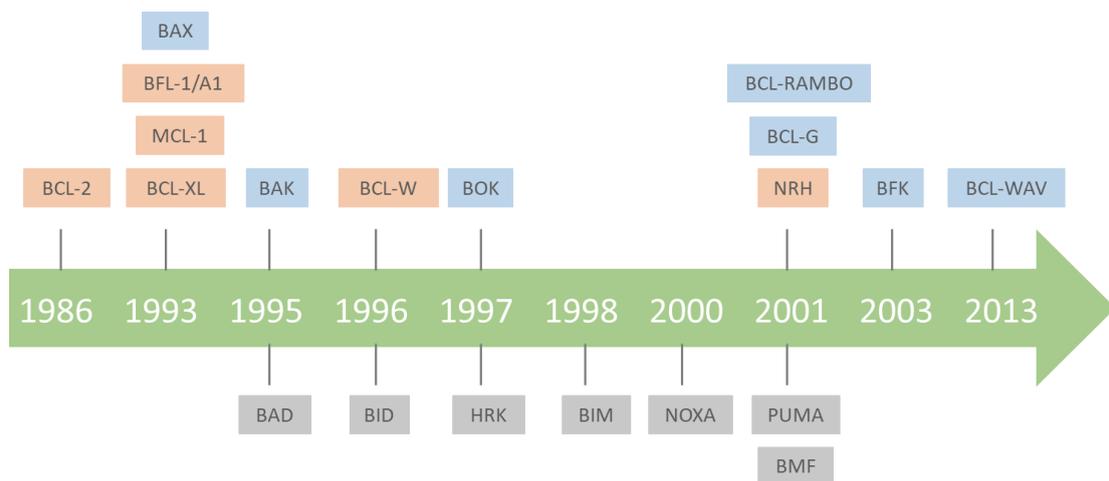


Figure 3: *Timeline of the cloning of Bcl-2 family members.*

From the cloning of human Bcl-2 in 1986 until the discovery of Bcl-wav in 2013, the cloning year of all Bcl-2 proteins is showed. Pink: Multidomain anti-apoptotic; Blue: Multidomain pro-apoptotic; Gray: BH3-only pro-apoptotic.

1.4. Bcl-2 members: Masterminds of apoptosis control

At the molecular level, two apoptotic pathways have been described: Extrinsic (death receptor-mediated pathway) and intrinsic (mitochondrial pathway) (Danial, 2007).

1.4.1. Extrinsic Regulation of apoptosis

The extrinsic pathway – also called Death Receptors pathway – is mainly activated when abnormal immune cells proliferate and might harm the organism either by inducing an inflammatory response or by over-proliferating causing lymphomas. The extrinsic pathway involves transmembrane receptor– ligand interactions. These receptors belong to TNF (Tumor necrosis factor) receptor gene superfamily and localize to the plasma membrane. The ligands include CD95 (or APO-1 or Fas) and TNF-Related Apoptosis Ligand (TRAIL). TNF receptors are composed of several domains: an extracellular Cysteine-rich (CRD) one, responsible for the linkage with the ligand, a transmembrane domain and a cytosolic one, often called Death Domain (DD) of around 80 aa which allows the trimerization of the receptor. This DD transmits the death signal from the outside to the inside of the cell. Many cytoplasmic adaptor proteins are recruited upon ligand binding to the receptor, and in their turn, bind the receptor. For example, the adaptor protein Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) is recruited, along with another adaptor: FAS-associated Death Domain (FADD) as TNF binds TNF receptor (Elmore, 2007). Then, the caspase cascade is involved: they are normally found as catalytically inactive zymogens (pro-caspases) and can only be activated following an internal cleavage. Accordingly, the recruited adaptor proteins have a DED allowing them to interact with initiator Caspases 2, 8 and 10. For instance, FADD recruits through its DED caspases 8/10 forming thus a complex – with TRAIL or CD95 receptors – called Death Inducing Signaling Complex (DISC). Once activated, these caspases induce the activation of effector Caspases 3 and 7, leading to apoptosis (**Figure 4**) (Shi, 2004).

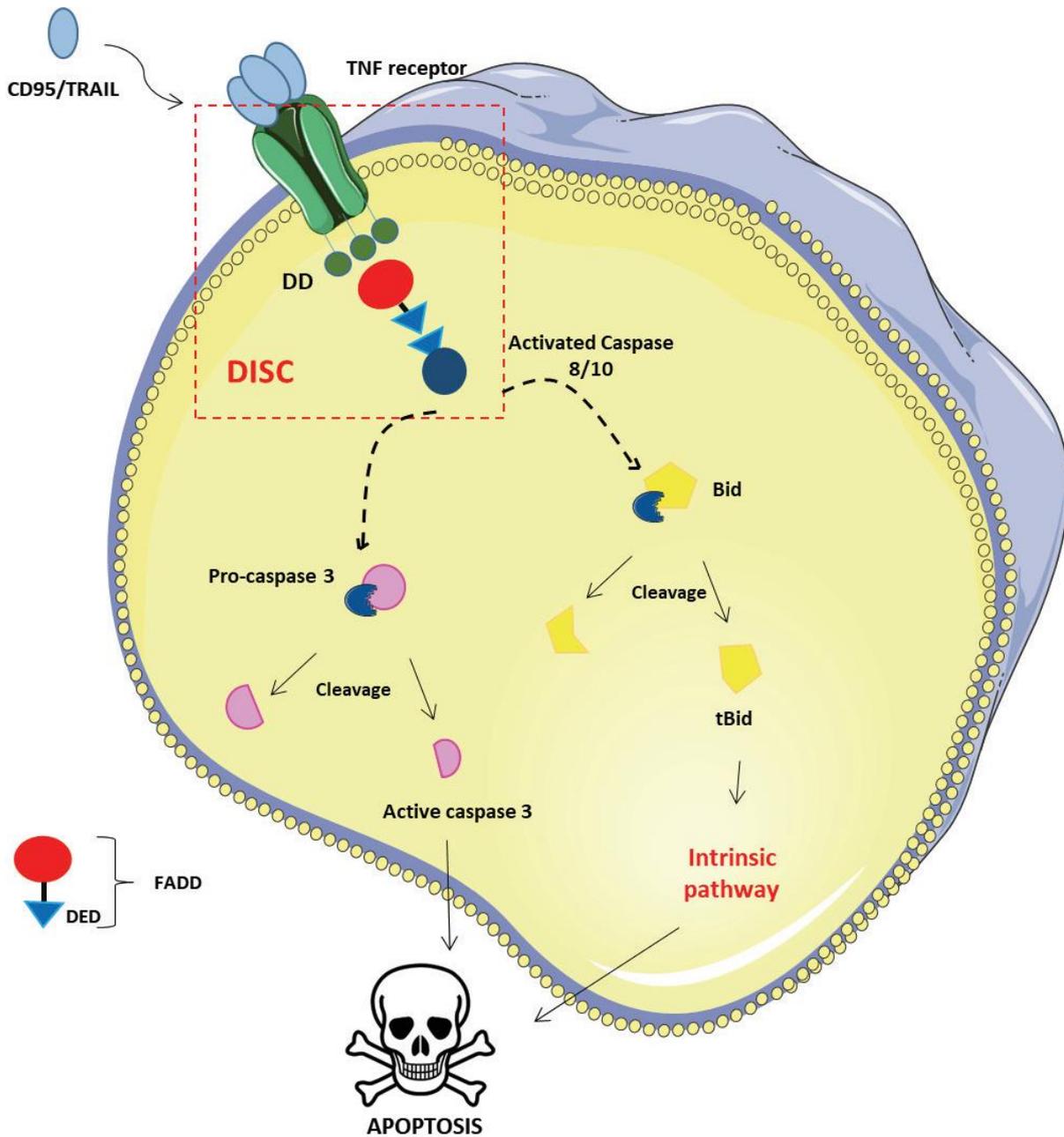


Figure 4: *The extrinsic pathway of apoptosis.*

Upon the ligand (CD95/TRAIL) binding to its subsequent Death Receptor, the latter trimerizes and recruits FADD via its DD. On its turn, FADD recruits and activates Caspase 8 or 10 via their DED. Accordingly, these active caspases can either directly activate, through cleavage, effector caspase 3 or can trigger the intrinsic-mitochondrial pathway through the BH3-only protein Bid cleavage. In both cases, the final result would be the death of the cell known as apoptosis.

1.4.2. Intrinsic Regulation of apoptosis

The intrinsic pathway is mainly triggered by non-receptor associated stimuli. These stimuli include stress (radiation, toxins, DNA damage), lack of growth factors, deprivation of cytokines and hormones and many others (Elmore, 2007). This pathway is also known as the “mitochondrial” pathway since this organelle represents a central hub for integrating various stress signals. Indeed, caspase activation one of the downstream events of the mitochondrial outer membrane permeabilization (MOMP) which is considered by many as the point of no-return in apoptosis execution. This permeabilization is regulated by many proteins of which the Bcl-2 family plays a major role. In general, active pro-apoptotic proteins called Bax and Bak create holes in the MOM inducing MOMP (Fulda and Debatin, 2006) (**Figure 5**).

Once the MOM is disrupted, most of the intermembrane space (IMS) proteins are released in the cytosol including Cytochrome c. The latter normally acts as an electron carrier in the respiratory chain (Bossy-Wetzel et al., 1998). However, the release of Cytochrome c in the cytosol leads to caspase activation. Indeed, cytosolic Cytochrome c binds Apoptosis Protease Activating Factor-1 (Apaf-1), an adaptor protein with a CARD. The CARD is homologous to that of Caspase 9, promoting Caspase 9-dependent activation of Caspase 3 while forming a complex called apoptosome. The whole process requires ATP hydrolysis (Pandey et al., 2000). In its turn, this ring-like structure prompts the activation of initiator pro-caspases which clip effector procaspase dimers (procaspase 3 and 7). The activated caspases then induce cellular proteolysis, and the death of the cell (Yuan and Akey, 2013). This process is regulated by the action of Bcl-2 proteins on the MOM.

Other proteins might leave the IMS. Second mitochondria-derived activator of caspase/Direct Inhibitor of Apoptosis-Binding protein with LOw pI (SMAC/Diablo) and Omi are two IMS-resident proteins. SMAC/Diablo function was long unexplored. However, recent studies have shown its involvement in lipid synthesis regulation that is important in cancer growth and development. Furthermore, this protein is overexpressed in cancers and is considered a potent favorable prognostic factor mainly in locally advanced breast cancer (LABC) (Paul et al., 2018; Pluta et al., 2018). As for Omi, also called HtrA2, it is a serine protease that leads to cell death either through caspase activation or in a caspase-independent way *via* its protease activity (Cilenti et al., 2003). Both mentioned IMS proteins interact and inhibit inhibitors of apoptosis (IAPs) once released in the cytosol. The IAP family is composed of 8 members in

mammalian cells and is known to interact and inhibit both initiator and effector caspases (Fulda and Vucic, 2012).

Two other IMS factors the Apoptosis Inducing Factor (AIF) and Endonuclease G (EndoG) are released upon MOMP. In fact, AIF is a protease residing in the mitochondria. It performs double actions: it can cause Caspase 3 activation when in the cytosol, or lead to DNA fragmentation when in the nucleus. Interestingly, EndoG is required for AIF nuclease activity (Susin et al., 1997; Wang et al., 2002). EndoG, acts independently of caspases and is linked to chromatin DNA cleavage into nucleosomal fractions (Li et al., 2001).

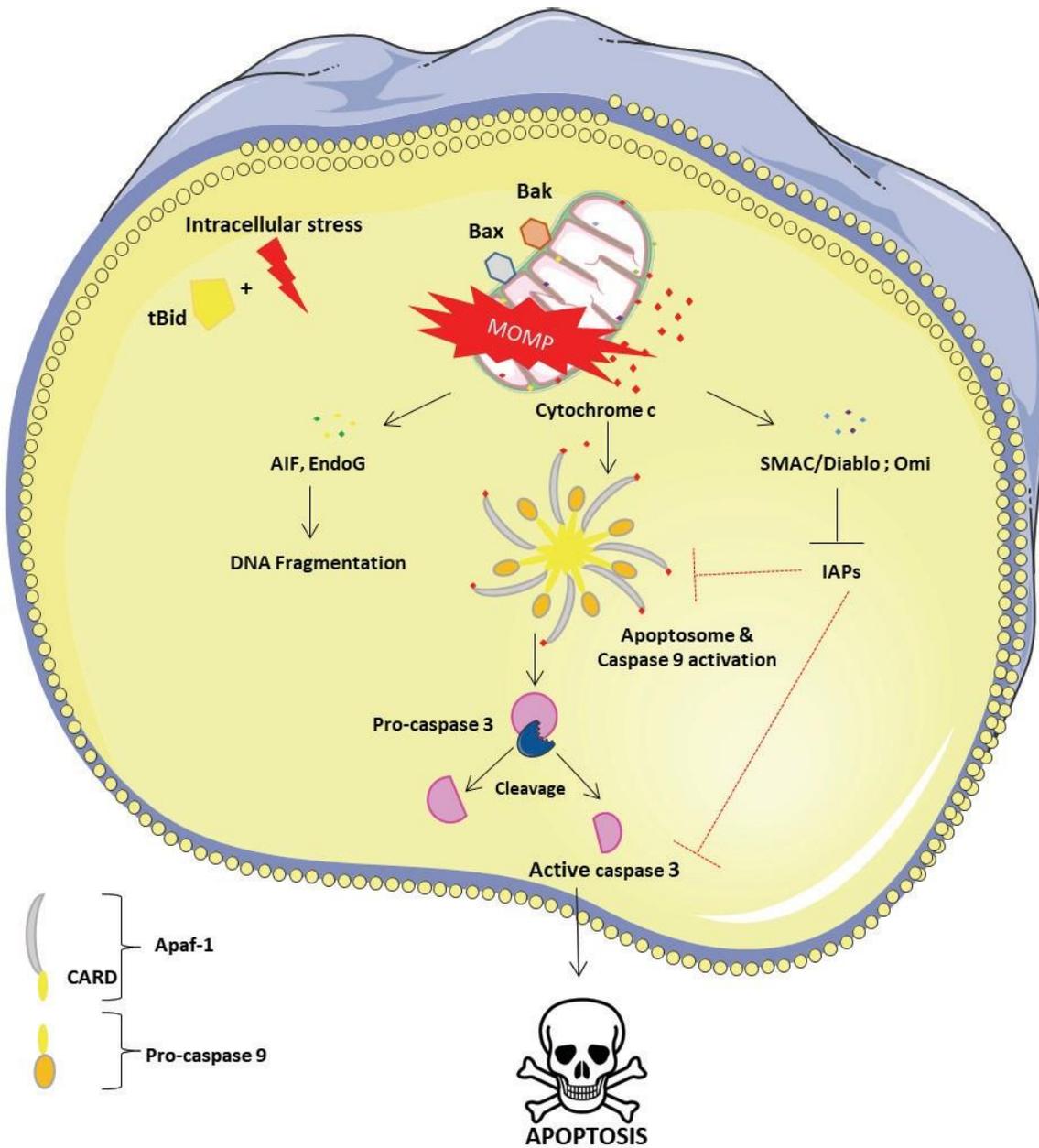


Figure 5: *The intrinsic pathway of apoptosis.*

Intracellular stresses including DNA damage or lack of growth factors or Bid cleavage trigger the mitochondrial pathway of apoptosis. Upon Bax/Bak activation and induction of MOMP, several pro-apoptotic factors are released in the cytosol. Of which, Cytochrome c prompts apoptosome assembly leading to the activation of Caspase 9. The latter activates executioner Caspase 3 and its consequent proteolysis leading to apoptosis. Both Caspases can be inhibited by IAPs, which are in their turn inhibited by SMAC/Diablo or Omi. Finally, other MOMP-related but caspase-independent factors are released in the cytosol upon MOMP including AIF and EndoG that are responsible for apoptotic DNA degradation.

Calcium-dependent apoptosis

Whenever cytosolic Ca^{2+} concentration increases, mitochondria take up Ca^{2+} which will then enhance the activity of several Ca^{2+} -dependent enzymes such as pyruvate and isocitrate dehydrogenases of the Transport Chain Activity (TCA) cycle. Accordingly, mitochondrial Ca^{2+} prompts ATP production and mitochondrial integrity. However, excess of mitochondrial Ca^{2+} is detrimental leading to mitochondrial swelling and eventual MOMP (Bonneau et al., 2013).

Accordingly, Bax and Bak are not the only factors in MOMP induction. These mitochondria can be permeabilized by the opening of the mitochondrial Permeability Transition Pores (mPTPs) found within the mitochondrial inner membrane (MIM). They consist of a protein complex that allows solutes to diffuse freely (Bernardi, 2013). The nature of the mPTP was and still is a matter of debate. It has been noted that the mPTP is composed of several proteins: Cyclophilin D, Adenine Nucleotide Transporter (ANT) both present on the MIM and VDAC on the MOM (Lemasters et al., 2009). A newer component of the complex was identified and consists of the F_0F_1 ATP synthase. It suggests that the dimer of F_0F_1 constitutes the mPTP (Giorgio et al., 2013). Also, Cyclophilin D was shown to modulate the activity of the F_0F_1 ATPase. To go further on the molecular level, the c subunit of this ATP synthase forms a voltage-sensitive channel. If this channel stays open, uncontrolled depolarization occurs on the MIM. Continuous high matrix Ca^{2+} concentration enlarges the c-subunit ring and unhooks it from Cyclophilin D binding site in the ATPase providing a mechanism for the opening of mPTP (Alavian et al., 2014). Moreover, opening of the mPTP induces mitochondrial matrix swelling by osmosis what leads to the remodeling of cristae that mainly contain cytochrome c stockpiles. Eventually, mitochondria swell and the MOM is permeabilized leading to the release of IMS proteins in the cytosol, activation of caspases and cell death (**Figure 6**) (Kroemer et al., 2007). Novel data suggest the existence of a Bax/Bak and cyclophilin (a major regulator of the mPTPs) – independent alternative intrinsic apoptotic pathway emerging from ER–mitochondria crosstalk (Zamorano et al., 2012).

Of note, both pathways are linked through Bid. Indeed, Bid is cleaved by Caspase 8 into tBid during death receptor apoptotic signaling. tBid then migrates to the mitochondria inducing Bax/Bak-dependent MOMP and thus, apoptosis (Billen et al., 2008) (**Figure 4-5**).

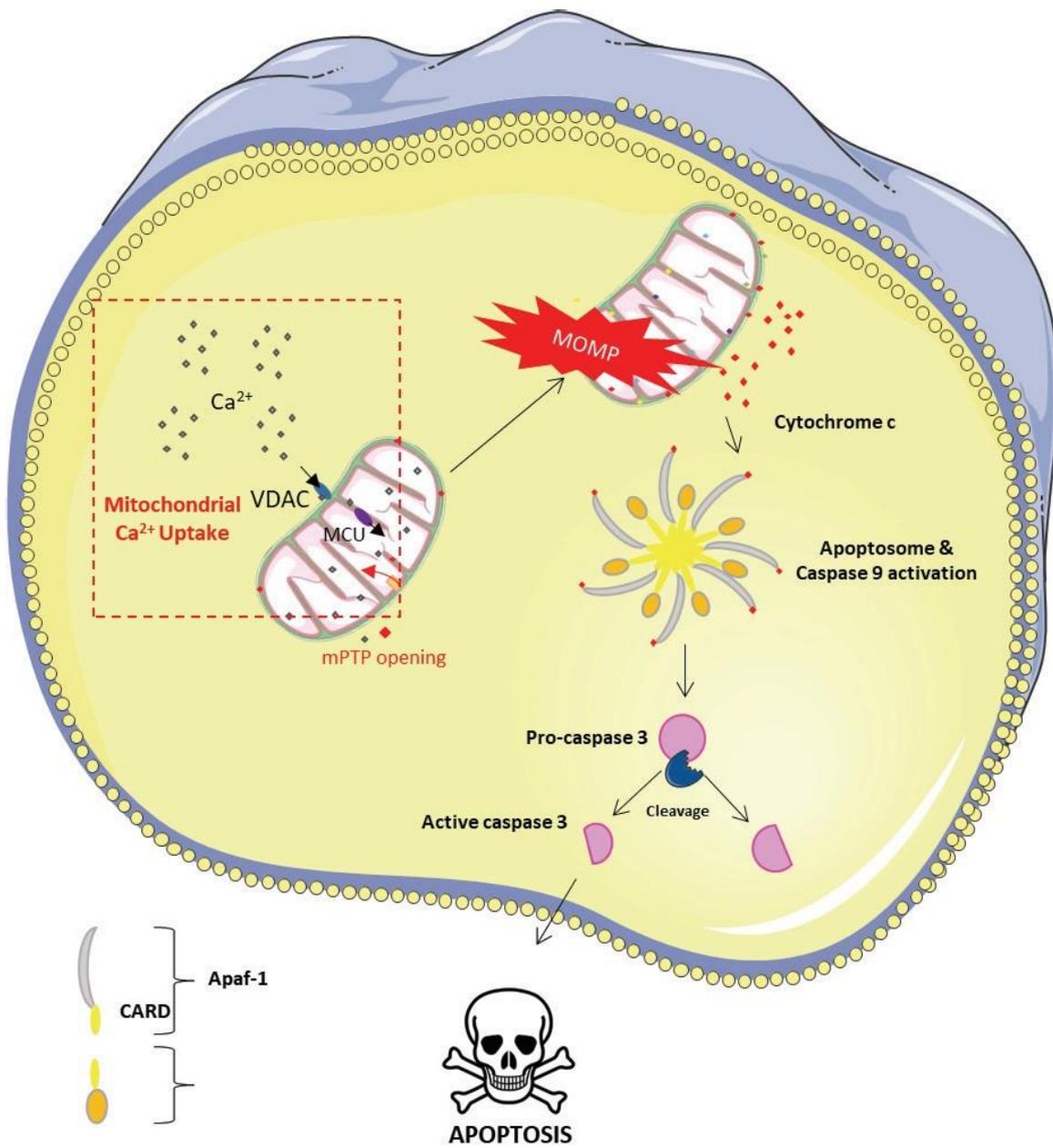


Figure 6: Calcium-mediated MOMP.

Excessive mitochondrial calcium uptake through VDAC and the mitochondrial calcium uniporter (MCU) channels induce the mPTP opening causing an osmotic shock. This triggers mitochondrial matrix swelling followed by MOMP. Consequently, IMS proteins are released in the cytosol and provoke the caspase cascade activation resulting in apoptosis.

To date, there is no evidence of the direct regulation of mPTP opening by the Bcl-2 family of proteins. However, the Bcl-2 gang cannot but interfere in approximately every cellular process: they indirectly affect mPTP-dependent MOMP through direct regulation of ER-Ca²⁺ release and mitochondrial Ca²⁺ influx as it will be described in (part 2).

1.4.2.1. Bax/Bak-mediated apoptosis

The BH1-3 hydrophobic groove of the anti-apoptotic proteins is indispensable for the interaction with the BH3 domain of the pro-apoptotic members (Sattler et al., 1997). Accordingly, the “cell killers” are unable to perform their duty in physiological situations. Upon apoptosis triggering, Bax and Bak are released from their unwelcomed partners and ready to finally pursue their sole life aim: MOMP. Several questions are to be answered:

How are Bax and Bak activated?

The key step for Bax/Bak activation is their translocation to the mitochondria. Even though Bak is majorly mitochondrial, both pro-apoptotic members are frequently translocated to the cytosol by anti-apoptotic proteins in healthy cells (Todt et al., 2015; Wolter et al., 1997). On the mitochondria, Bak must disengage from other anti-apoptotic members such as Mcl-1 and Bcl-xL (Willis et al., 2005).

‘Kiss-and-run’

A transient interaction of BH3-only proteins with the hydrophobic groove of Bax/Bak at the MOM is required for the first described model: *kiss-and-run*. Accordingly, Bax and Bak undergo conformational changes leading to dimer formation. These homodimers were considered as the “minimal unit” necessary for the assembly of higher-order oligomers responsible for MOMP (Pang et al., 2012; Shamas-Din et al., 2014; Subburaj et al., 2015).

Several models were proposed to enlighten the complex network of Bcl-2 proteins and their involvement in MOMP. Here, we briefly describe them.

The ‘**direct activation model**’ and the ‘**neutralization/displacement model**’ mainly divide BH3-only proteins into two main groups. The direct activators including tBid, Bim and Puma activate the

executioner proteins. As for the sensitizers (Bad, Noxa, Bik, Bmf, Hrk) – also called derepressors – they neutralize the anti-apoptotic proteins.

Not all BH3-only proteins bind and engage multidomain proteins in the same specificity (**Table 1**) (Chen et al., 2005; Letai et al., 2002; Shamas-Din et al., 2013a).

ANTI PRO	Bcl-2	Bcl-xL	Bcl-w	Mcl-1	Bfl-1	Nrh
Bax	x	x	x	x	x	x
Bak		x		x	x	
Bim	x	x	x	x	x	x
Bid	x	x	x	x	x	
Puma	x	x	x	x	x	
Bmf	x	x	x	x		
Bad	x	x	x			
Bik		x	x		x	x
Hrk		x	x		x	
Noxa				x	x	

Table 1: Summary of Bcl-2-regulated apoptotic interactions.

The pro-apoptotic proteins (shown in green) interact and inhibit (green checks) the listed anti-apoptotic ones (shown in red). As for the BH3-only members, they are divided into direct activators (shown in dark blue) and sensitizers (shown in light blue) and interact (blue checks) with the mentioned anti-apoptotic proteins. Inspired by (Rautureau et al., 2012).

The ‘**embedded together model**’ focuses on the anti-apoptotic proteins as they are the center of MOMP prevention. They can either sequester BH3-only proteins (MODE 1) or Bax/Bak (MODE 2). They can also sequester both (Leber et al., 2007).

The **'Unified model'** insists on the efficiency of MODE 2 in which anti-apoptotic proteins mainly sequester Bax/Bak. Upon stress induction, more effective inhibition of MOMP onset takes place in MODE 2, when prosurvival proteins directly sequester Bax/Bak. Ultimately, when both modes of survival are overcome, Bax/Bak oligomerization leads to MOMP (**Figure 7**) (Llambi et al., 2011). Another MODE 3 is starting to emerge and involves Bok. This model is independent of the anti-apoptotic proteins and their interactions and relies on the degradation the pro-apoptotic member Bok. Emerging data decipher the role of Bok in different cellular stresses including ER stress and this model needs further investigation (Llambi et al., 2016a).

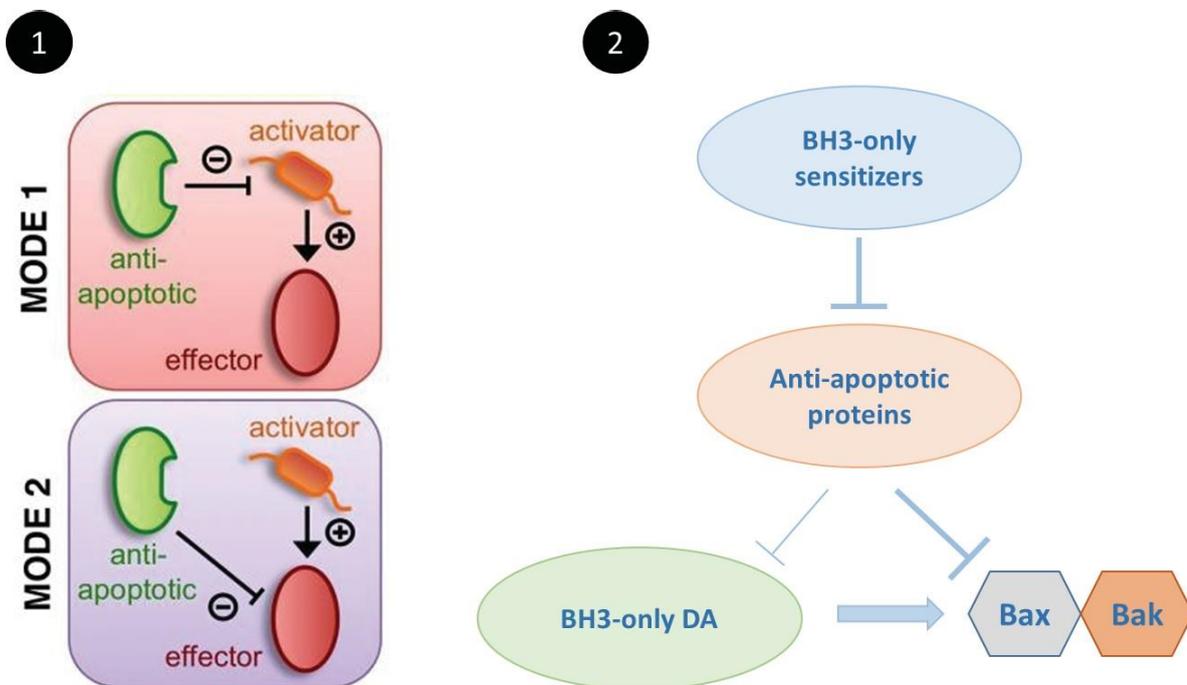


Figure 7: *Bax/Bak activation modes and unified model.*

(1) A schematic representation of MODES 1 and 2 of MOMP inhibition by anti-apoptotic Bcl-2 proteins. In MODE 1, anti-apoptotic proteins directly inhibit activator BH3-only members. In MODE 2, anti-apoptotic proteins directly inhibit Bax and Bak (Llambi et al., 2011). **(2)** A schematic summary of the Unified model of Bax/Bak activation. The sensitizer BH3-only proteins inhibit the anti-apoptotic proteins which in turn, inhibit more efficiently Bax/Bak than the BH3-only Direct Activators (DA).

In a very recent study, Huang and colleagues argue the dogma of the activation model of Bax/Bak and demonstrate that BH3-only proteins mainly target the pro-survival members Bcl-xL and Mcl-1. In this **“Membrane-mediated permissive”** model, activated BH3-only proteins bind to and neutralize the pro-survival Bcl-2 proteins on the MOM, which then provides a permissive environment for the spontaneous activation of Bax/Bak, culminating in their homo-oligomerization and the formation of Cytochrome c releasing pores (Huang et al., 2019) (**Figure 8**).

Choosing one path for the activation of pro-apoptotic members over the other is definitely difficult. However, two mechanisms are indispensable: 1- the localization of these members on the mitochondria and 2- the conformational changes leading to their oligomerization (discussed below). Both mechanisms are controlled by interactions with other Bcl-2 members.

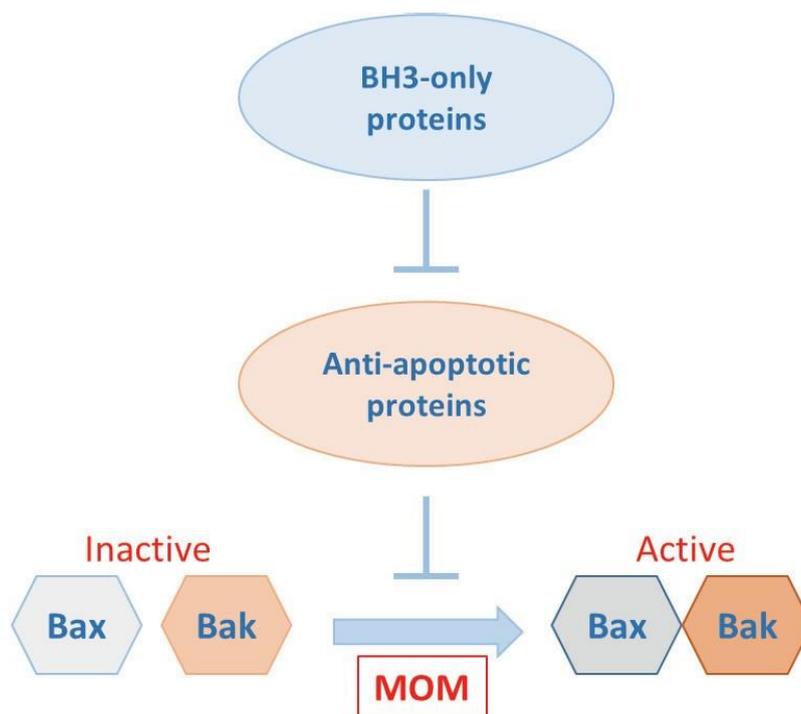


Figure 8: The ‘Membrane-mediated Permissive Model’ of Bax/Bak activation.

During apoptosis, activated BH3-only proteins interact with and neutralize the anti-apoptotic Bcl-2 proteins on the MOM. The latter grants a permissive environment for the subsequent activation and homo-oligomerization of Bax/Bak and eventually MOMP induction.

How do Bax and Bak form pores on the MOM?

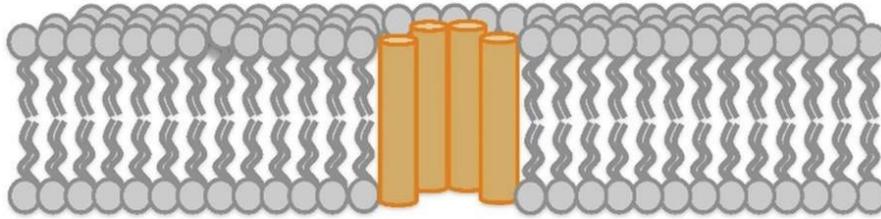
Once at the mitochondria, activated Bax and Bak perforate the MOM leading to the release of IMS proteins responsible for cell death. During Bax and Bak activation, they undergo different conformational changes allowing them to oligomerize at the MOM. This oligomerization is indispensable for MOMP induction as Bax/Bak deficient cells cannot undergo MOMP (Dewson et al., 2008; Westphal et al., 2011a).

In fact, to homodimerize, the BH3 domain interacts with the hydrophobic surface groove (α 4–5–helices of BH1) in a symmetric manner. Moreover, these dimers can oligomerize via the α 6–helices. Bax and Bak can also hetero-oligomerize in a BH3/groove manner similar to their homo-oligomerization (Dewson et al., 2012).

Bax/Bak oligomerization leads to the formation of pores in the MOM. Two theories exist on the nature of the MOM pores: are they proteinaceous or lipidic?

Proteinaceous pores are made of Bax/Bak oligomers forming stepwise growing channels (Zhang et al., 2017). Super-resolution microscopy data put in evidence the rings formed by Bax oligomers on the MOM (Große et al., 2016). However, considering the technical difficulties, a lipidic pore stays an option (**Figure 9**). Furthermore, increasing evidence demonstrate a lipidic nature of Bax/Bak-induced pores (called toroidal/proteolipidic pores) (Schafer et al., 2009). The underlying biophysical explanation is that a huge membrane tension occurs when proteins are integrated in an asymmetric manner into the lipid bilayer. This leads to membrane instability and pore opening. As the pore rim itself has a line of tension that provokes a force for pore closure, thus counteracting the membrane tension, the role for Bax/Bak in this toroidal model is mainly their integration into the pore edges, thereby decreasing the line tension and stabilizing the pore. The (proteo) lipidic pore model is most compatible with large and growing pores (García-Sáez, 2012; Gillies et al., 2015).

1- Proteinaceous pore



2- Toroidal pore

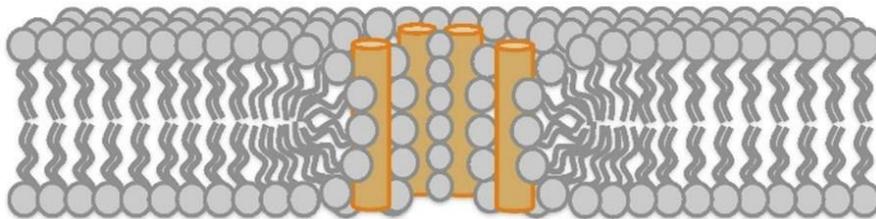


Figure 9: *Two mechanisms of pore formation by Bax and Bak.*

In the proteinaceous model, α -helical peptides (yellow) align to form a central cavity in the form of a barrel (1). In the toroidal model, α -helical peptides (yellow) cause membrane curvature forming a mixed cavity of peptides adjacent to lipid headgroups. From (Westphal et al., 2011b)

1.4.2.2. “MOMP: The point of no-return?”

MOMP is considered the point of non–return where it inevitably leads the cell to its grave. However, this scientific dogma has been challenged over the years: the release of Cytochrome c was proven to be a reversible event. In fact, the intracellular heterogeneity in the induction of MOMP has impacts on the survival and differentiation of the cell (Deshmukh and Johnson, 1998; Ichim and Tait, 2016; Martinou et al., 1999). To date, two different pathways of MOMP that do not commit the cell to suicide have been elaborated (**Figure 10**):

- 1– Incomplete MOMP called iMOMP that insinuates that not all mitochondria within a cell undergo MOMP.
- 2– Minority MOMP where few mitochondria undergo MOMP upon a sub–lethal stress.

For a cell to survive after iMOMP, caspase activity should be either inhibited or totally absent (Jia et al., 2001; Monian and Jiang, 2012; Tait et al., 2010). If caspase activity is inhibited, iMOMP occurs due to the unequal expression of anti–apoptotic members on the mitochondria. These proteins are able to repopulate the cell and provide its survival under apoptotic stimuli (Tait et al., 2010). On the other hand, minority MOMP triggers caspase activation but not at lethal levels. Consequently, DNA damage and genomic instability along with non–apoptotic caspase–mediated functions might provoke the cell’s oncogenic transformation (Ichim et al., 2015; Liu et al., 2015).

Both scenarios of partial MOMP open the field of possibilities for the cell to respond to anti–survival signals. They also insist on the importance of the ‘all or nothing’ decision of the cell when it encounters MOMP in order to determine its fate. Like MOMP, some data insinuate that caspase activity could also be reversed whenever the pro–apoptotic stress is abolished.

This new phenomenon was called in Greek ‘anastasis’ that actually means *rising to life* (Tang et al., 2015). Indeed, carcinogenesis could be a harmful side effect. However, potential benefits of anastasis might comprise the preservation of cells that are difficult to replace and stress–induced genetic diversity (Tang et al., 2012).

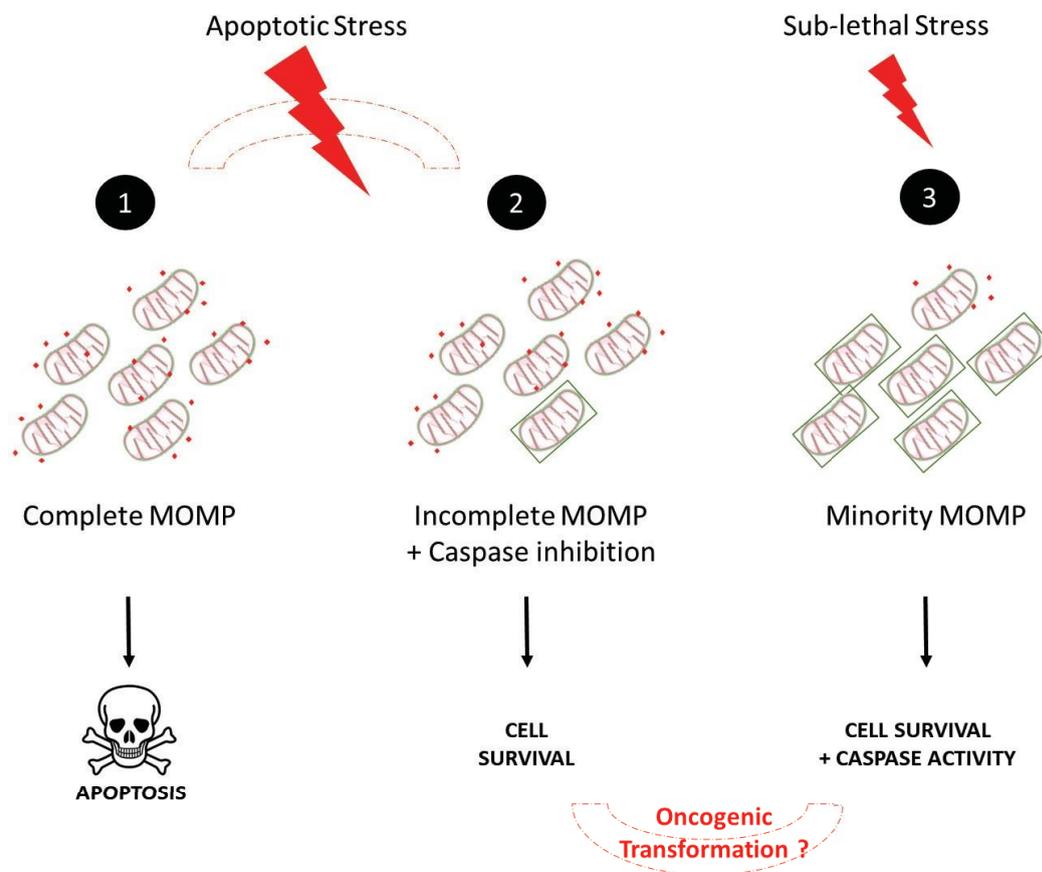


Figure 10: Overview of the different scenarios of MOMP.

Usually, apoptotic stress results in complete MOMP and eventually leads to apoptosis (1). However, in some cases, not all mitochondria in a cell undergo MOMP. If caspases are inhibited, the cell can survive with incomplete MOMP (2). Minority MOMP occurs when only a few mitochondria undergo MOMP upon a sub-lethal stress what maintains cell survival (3). Partial MOMP might cause DNA damage favoring malignant transformation. Green boxes: healthy mitochondria.

For the most part, despite the fact that apoptosis is capable of ending the survival of oncogenic transformed cells, partial MOMP might be fully capable of initiating cancer and promoting its progression (Czabotar et al., 2014). In cancer cells, the depletion of caspases is noted. If the threshold of complete MOMP activation is not crossed, it can prompt the survival of cancer cells facing apoptotic stresses and therapies. Accordingly, it can increase the risk of secondary malignancies such as leukemia after chemotherapy or even sarcomas after irradiation (Hars et al., 2006; Huang et al., 2011; Labi and Erlacher, 2015).

1.5. Bcl-2 members: *pretty much close and interactive*

Intra and interfamilial interactions are at the basis of the Bcl-2 activities. For example, Bcl-2 homodimerizes with itself involving a head-to-tail interaction between the BH4 domain (N-terminal domain) and the BH1-BH3 groove. However, the interaction of Bcl-2 with Bax constitutes a tail-to-tail heterodimerization requiring the BH1-3 groove of Bcl-2 and the BH3 domain of Bax. This exact domain of Bax is necessary for both, its homodimerization and its anti-survival action in yeast and mammalian cells (Reed et al., 1996). As for Bcl-xL, its crystal and solution structure were generated by X-ray crystallography. Two hydrophobic alpha-helices, surrounded by amphipathic helices constitute Bcl-xL. The hydrophobic cleft formed by BH1-3 embodies the binding site for other Bcl-2 proteins (Muchmore et al., 1996). Accordingly, the interaction of Bcl-xL/Bak peptide(72-87aa) was crystallized as an interaction between an amphipathic alpha helix with the hydrophobic groove of Bcl-xL (**Figure 11**). The Bcl-xL alpha-helices arrangement is reminiscent of the membrane translocation domain of bacterial diphtheria toxin and the colicins. The resemblance of helical structure of Bcl-2 proteins with that of bacterial ones shed new light on how Bcl-2 members, in particular pro-apoptotic ones, induce the mitochondrial permeabilization. The BH1-3 cleft is thus indispensable for the interaction with the BH3 domain of the pro-apoptotic killers (Sattler et al., 1997).

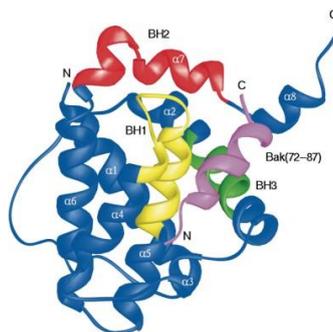


Figure 11: *Bcl-xL/Bak peptide structure.*

Depiction of the superimposed NMR-derived structures of Bcl-xL (shown in blue, red (BH2), green (BH3) and yellow (BH1)) complexed with Bak peptide of 16 aa (shown in purple) inserted in the hydrophobic cleft (Sattler et al., 1997).

1.6. Subcellular localization and dynamics of Bcl-2 members

[The following part is inspired by the review “Subcellular localization and dynamics of the Bcl-2 family of proteins” by Popgeorgiev N., Jabbour L., Gillet G. in *Frontiers in Cell and Developmental Biology*. doi: 10.3389/fcell.2018.00013. Refer to (Annex I)]

Intracellular compartmentalization has been considered a hallmark of eukaryotic cell and the proper subcellular localization of a given protein is critical for its action. Hence, Bcl-2 members are mainly dependent on their localization to prevent/promote the slaughter of the cell. Even though they are known to perform their actions at the level of the mitochondria, Bcl-2 proteins are “cell-omnipresent” in other terms, found in many cellular compartments. They can localize to the ER, Golgi apparatus, nucleus, nuclear outer membrane (NOM), peroxisomes and cytosol. Accordingly, numerous non- canonical functions, not involving apoptosis regulation, of this family have emerged throughout the years. Likewise, this gang can never be static: Bcl-2 proteins depend on their dynamic subcellular distribution to be able to interact with other proteins and perform their functions. Here, we elaborate what is currently known about the localization and the mobility of the Bcl-2 squad.

1.6.1. Subcellular localization

Although Bcl-2 proteins are majorly considered as regulators of mitochondrial outer membrane permeabilization, they could localize to many organelles. Bcl-2 protein was initially reported to be found at the level of the cytosolic leaflet of intracellular membranes. It wasn't, until 1990, proven to be located at the MIM (Hockenbery et al., 1990). Then, it was shown to be also located at the MOM with the help of its TM anchoring it to the membrane. As a consequence, Bcl-2 was able to regulate the actions of several MIM proteins (cytochrome c oxidase Va, Cyclophilin D) due to the fact that is mainly present at contact points between MIM and MOM (Nakai et al., 1993; Nguyen et al., 1993). The MIM seems to harbor many Bcl-2 members since Bcl-xL was also detected at the MIM of mitochondria isolated from hippocampal neurons. It actually binds the β -subunit of the F_1F_0 ATP synthase improving synaptic transmission (Alavian et al., 2011a; Jonas et al., 2003a). Moreover, Mcl-1 localizes to both mitochondrial

membranes where it fulfills different functions, accordingly. In fact, to be targeted to the mitochondrial matrix, Mcl-1 needs to undergo proteolytic cleavage of its N-terminal mitochondria-targeting sequence. Once there, Mcl-1 fosters mitochondrial bioenergetics. When at the MOM, this protein executes its anti-apoptotic function (Perciavalle et al., 2012a).

Apart from the mitochondrial localizations, several Bcl-2 members reside in the ER. Of which we cite multidomain anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1 and Bcl2L10) and pro-apoptotic proteins (Bax, Bak and Bok) as well as BH3 only proteins (Bim, Bik) (**Figure 2**). Accordingly, Bcl-2 proteins keep on controlling apoptosis in a remote fashion while performing additional non-canonical actions through their interactions with Ca²⁺ transporters and mediators of the Unfolded Protein Response (UPR).

Withal, Bcl-2 was also found in the nucleus. This translocation depends on the phosphorylation status of Thr56 where nuclear Bcl-2 appears to take part in a multiprotein complex comprising Cdk1, PP1 and Nucleolin (Barboule et al., 2005, 2009).

Finally, the Bcl-2 squad may also possess some “exotic” subcellular localizations, including Bax at the Golgi apparatus (Dumitru et al., 2012) and the lysosomes (Guan et al., 2015) or Bak shifting to the peroxisomes in the case of the loss of Voltage Dependent Anion Channel 2 (VDAC2) where VDAC2/Bax axis regulates peroxisomes’ membrane permeability (Hosoi et al., 2017). These different localizations are the reason for the orchestration of Bcl-2 members’ pleiotropic functions inside the cell.

1.6.2. Dynamic localizations

It is now clear that Bcl-2 proteins possess several subcellular localizations. As mentioned before, the COOH-terminal hydrophobic region is responsible for their anchorage to intracellular membranes. Nevertheless, a lot of these proteins were found soluble in the cytosol. Bax, for example, is an integral protein – bound to organelle membranes – and a cytosolic one. This multiple localization of proteins is in fact a proof of their dynamics. Indeed, Wolter and colleagues have shown that Bax translocates from the cytosol to the mitochondria upon apoptosis induction. They explored the localization of Bcl-2, Bcl-xL and Bax in living cells by green fluorescent protein (GFP) fusion to their N-termini. Deletion of the C-terminal hydrophobic domain from GFP-Bax prevented its redistribution during apoptosis and repressed the death-promoting activity of both Bax and GFP-Bax further proving the importance of this domain in at

least, Bax's subcellular localization (Wolter et al., 1997). Nevertheless, constitutive targeting of Bak or induced relocalization of Bax to the MOM does not imply that these proteins are active once the TM regions are MOM-integral. Actually, direct activation of Bak and Bax prompts diverse conformational changes such as $\alpha 1$ helix exposure, transient BH3 exposure, protection of the membrane-embedded Bcl-2 core and increased proximity of embedded monomers (George et al., 2007). In addition, it was also revealed that the $\alpha 5$ helix was both necessary and sufficient for Bax oligomerization and apoptosis induction (Chipuk et al., 2010; George et al., 2007).

In fact, the retrotranslocation of Bax from the mitochondria to the cytosol by the anti-apoptotic protein Bcl-xL was demonstrated in 2011 by the team of Edlich. They hindered conformational changes in the BH3 motif of Bax to check its involvement in Bax activity. Fluorescence Loss In Photobleaching (FLIP) measured an increase in mitochondrial Bax off rates by Bcl-xL with no competition between Bax and Bcl-xL for MOM binding proving that wildtype (WT) Bax retrotranslocates from the mitochondria to the cytosol. Bcl-xL seemed to localize predominantly to the mitochondria and re-locate to the cytosol at a slow rate in the absence of Bax. However, the overexpression of Bax accelerated this translocation suggesting that they interact on the mitochondria, retrotranslocate together and dissociate in the cytosol. Of note, the overexpression of Bcl-2 and Mcl-1 accelerated Bax retrotranslocation in a similar manner to that of Bcl-xL (Edlich et al., 2011). Thereafter, Bcl-xL C-terminal residues were proven important for Bax retrotranslocation after undergoing a conformational change exposing Bax BH3 domain (Martinou and Youle, 2011; Todt et al., 2013). Indeed, Bax retrotranslocation depends on two interactions: recognition of the Bax BH3 domain by the hydrophobic groove of Bcl-xL and binding of the Bcl-xL membrane anchor (MA) to Bax. (Todt et al., 2013). Interestingly, Bax shuttling can also implicate Bcl-xL-independent mechanisms. Adhesion survival signals such as Focal Adhesion Kinase (FAK) and Akt signaling regulate the subcellular distribution of Bax by affecting its dissociation from mitochondria to the cytosol. FAK was shown to be required for the detachment of mitochondrial Bax in MEC cell line (Schellenberg et al., 2013).

In 2015, Todt and colleagues studied the differential retrotranslocation of both major multidomain pro-apoptotic proteins. They found that Bak is present in the cytosol and that Bcl-xL is responsible for its

retrotranslocation from the mitochondria. Overexpression of Mcl-1, but not Bcl-2, accelerates this shuttling similar to Bax's. Actually, the same retrotranslocation process occurs for both Bax and Bak: interactions between the BH3 domain of Bax/Bak and the hydrophobic groove of Bcl-xL are mandatory for their translocations. Increased retrotranslocation is vital to protect cells from Bax activation and slow Bax redistribution commits cells to apoptosis. In this mechanism, not only Bax and Bak are being shuttled, but also membrane-integral Bcl-2 proteins such as MOM-integral Bcl-xL. Despite the fact that Bax and Bak are being transported by the same process from the mitochondria, cells are forced to increase Bax retrotranslocation rates in order to prevent commitment to cell death at low Bax shuttling rates when no apoptotic signals are present. Although being predominantly mitochondrial, Bak commits the cell to apoptosis only in the presence of apoptotic stimuli (Todt et al., 2015). On the other hand, Bax activation inhibits its shuttling to the cytosol thus active Bax is not translocated (Edlich et al., 2011). Therefore, under physiological conditions, cells block Bax-dependent apoptosis by increasing its transport into the cytosol further reducing the time spent on the MOM in a way to prevent Bax activation under non-apoptotic circumstances (Edlich, 2015; Todt et al., 2015).

Indeed, Bax and Bak are shuttled between the cytosol and the mitochondria, however, they also localize to other subcellular compartments. In fact, Bax is localized in a specific manner to the trans-Golgi compartment with a total absence within the Cis compartment. In addition, after DNA damage, Bax seemed to translocate from Golgi to the mitochondria in a p53-dependent manner in these cells (Dumitru et al., 2012). Another study in 2015 has shown that lysosomes can also be involved in Bax-mediated apoptosis. It revealed a novel crosstalk process between autophagy and apoptosis through DRAM1 (DNA damage-regulated autophagy modulator 1). Actually, Bax – at the lysosomes – was shown to affect their permeability and the release of cathepsins (Feldstein et al., 2006; Oberle et al., 2010). Upon treatment with the mitochondrial complex II inhibitor, 3-nitropropionic acid (3NP), DRAM1 and Bax expressions were upregulated contributing to a lysosomal Bax localization in a DRAM1-dependent manner causing the release of lysosomal cathepsin B and cleavage of Bid (Guan et al., 2015).

Actually, the presence of Bax at the lysosomes and Trans-Golgi compartments suggest that lysosomes could act as transport vesicles of Bax from trans-Golgi to the lysosomes but this needs further elaboration. As for the MOM-ER trafficking, Bax and Bak could cross the Mitochondria-Associated Membranes (MAMs) – the zone of close contact between the two organelles. As a matter of fact,

mitochondria and ER are continually moving along the cytoskeleton and their architectures change via processes of membrane fission, fusion, elongation, degradation and renewal. It has been shown that mitochondria readily import phospholipids from the ER but are not quite involved in protein interchange. However, the close proximity of these two suggests a sort of protein exchange even if it's not direct but with the aid of specific factors (Bravo-Sagua et al., 2013).

Here, we present a schematic representation of Bcl-2 proteins' dynamics inside the cell (**Figure 12**).

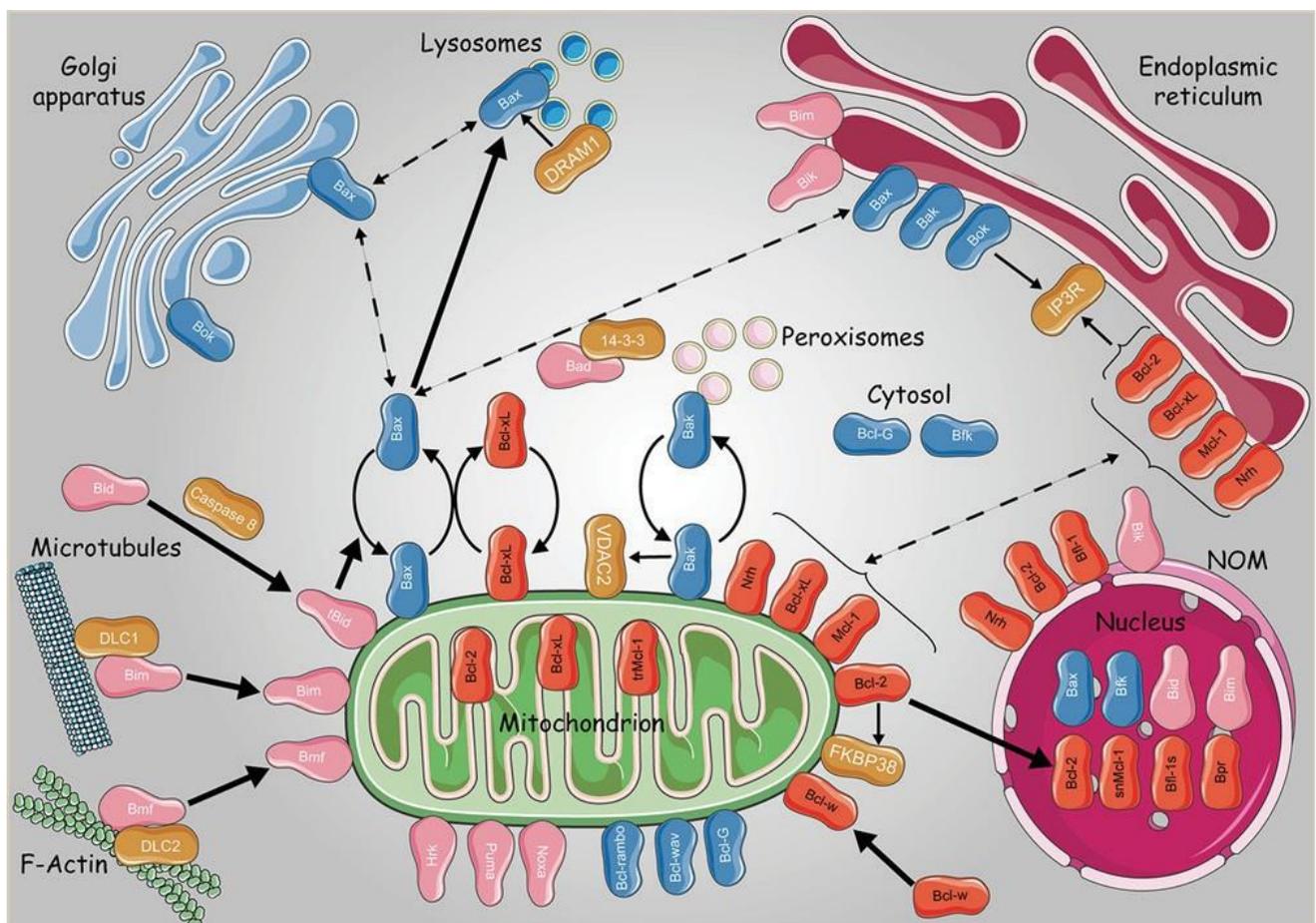


Figure 12: Subcellular dynamics of the Bcl-2 family of proteins.

The subcellular localization and dynamics of Bcl-2 anti-apoptotic proteins (shown in red), pro-apoptotic proteins (shown in blue) and BH3-only proteins (shown in pink) are presented. Other proteins are shown in brown. Continuous and bold lines represent protein translocation in physiological and stress conditions, respectively. Dashed lines represent hypothetical subcellular translocation (Popgeorgiev et al., 2018).

1.6.3. Moonlighting functions at extra-mitochondrial localizations

Indeed, Bcl-2 proteins localize and shuttle between different cellular compartments. However, their initial role is maintained at the level of the mitochondria for apoptosis regulation. Throughout the 35 years of research about this family, one can clearly say that Bcl-2 proteins participate in functions – other than apoptosis regulation – known as non-canonical (Bonneau et al., 2013). They appear to do so by modifying their subcellular localization.

At the level of the ER, Bcl-2 proteins regulate Ca^{2+} homeostasis. In fact, they do so by interacting with several calcium transporters; refer to (part 2.3.).

At the nuclear level, Bcl-2 proteins seem to be involved in the regulation of vital processes. A portion of the BH3-only protein Bid is localized to the nucleus (Kamer et al., 2005; Zinkel et al., 2005). As a matter of fact, Bid was shown to have a role in the DNA damage response. Following DNA damage, the DNA damage kinases ataxia telangiectasia mutated (ATM) and ATR are activated causing eventually cell cycle arrest allowing DNA repair or apoptosis. One of the targets of ATM is nuclear Bid that will be phosphorylated by this exact kinase and is needed for cell cycle arrest at the S phase or the induction of apoptosis (Gross, 2006). Zinkel and colleagues suggested that Bid is transported to the nucleus as part of a protein complex moving in response to DNA damage since it lacks an obvious nuclear localization signal. Accordingly, Bid might either help to stabilize the complex or facilitate subsequent enzymatic steps. Bid phosphorylation is important for maintaining genomic stability at the intra-S phase checkpoint. Consequently, Bid acts as a mediator between apoptosis and cell cycle regulation at the S phase (Kamer et al., 2005; Zinkel et al., 2005).

As for Bcl-2, many evidence proved its interaction – either direct or indirect – with transcription factors. In 1998, D. de Moissac and colleagues have demonstrated the regulation of I κ B α by Bcl-2. In fact, I κ B α is the cytoplasmic inhibitor of the ubiquitously expressed transcription factor Nuclear Factor κ B (NF κ B). Bcl-2 mediates the degradation of the inhibitor activating eventually NF κ B (de Moissac et al., 1998). Another study in 2001 presented direct evidence that NF κ B transcriptionally regulates the *bcl-2* gene and directly links the Tumor Necrosis Factor α (TNF- α)/ NF κ B signaling pathway with the expression of Bcl-2 in human prostate carcinoma cells (Catz and Johnson, 2001). A recent study delineated the

regulation of gene transcription by extra-mitochondrial Bcl-2 proteins, notably Bcl-2, Bcl-xL and Mcl-1. In fact, these proteins engage a BH domain sequence in the tumour suppressor and the antagonist of GLI DNA-binding proteins, SUFU (Suppressor of fused). By doing so, they allow its turnover, impede its interaction with GLI and allow the expression of GLI target genes (*Bcl-2*, *Bcl-xL* and *Mcl-1*) thus prompting the survival and growth of cancer cells (Wu et al., 2017).

Finally, members of the Bcl-2 family are also found at the Golgi apparatus. Bok, for example, was suggested to work upstream of Bax and Bak controlling the communication between the ER-Golgi and the mitochondria through apoptotic signals. In fact, Echeverry and colleagues saw that Bok, when overexpressed, contributed to the fragmentation of Golgi and the ER before the activation of caspases and when knocked out, Golgi disassembles under stress conditions thus boosting ER stress further activating BH3-only proteins needed for the execution of apoptosis (Echeverry et al., 2013).

1.7. Non-cliché roles of Bcl-2 proteins

Indeed, Bcl-2 proteins are famous for their control over the cell fate through their regulation of apoptosis. We have already mentioned that these little creatures are widespread in the cell; refer to (part 1.6.1.). So, it is no surprise that this family has broadened its action range and covered non-canonical functions, in a very discrete manner. Moreover, their localization at the ER allowed them to interfere with ER-resident Ca^{2+} channels and transporters. Thus, Bcl-2 proteins regulate either directly or indirectly intracellular Ca^{2+} homeostasis. Here, we briefly describe some of the non-cliché roles of this family.

1.7.1. Autophagy

Autophagy involves the re-organization of intracellular membranes in order to deliver cytoplasm and organelles to the lysosome where they will be sequestered, degraded and recycled under stress conditions like starvation. This phenomenon is regulated by a range of proteins encoded by autophagy related genes (Atg) that are evolutionarily conserved (Klionsky et al., 2003). The mammalian ortholog of Atg6 is Beclin 1. This protein is a part of a complex necessary for the formation of the autophagosome (Kihara et al., 2001). The latter is composed of double-membrane vesicles that engulf proteins and organelles to be sequestered.

Several outcomes might result from autophagy: cellular adaptation, cell survival or death. Furthermore, this phenomenon has interesting qualities in tumor suppression. Accordingly, any disruption of the process might prompt cellular transformation and tumor progression (Qu et al., 2003). One interesting fact about Beclin 1 is that it was originally isolated as a Bcl-2-binding partner by Liang team in 1998 demonstrating a functional crosstalk between apoptosis and autophagy (Liang et al., 1998). It was then demonstrated that Bcl-2 itself negatively regulates Beclin 1-mediated autophagy. Therefore, a new route was discovered for Bcl-2 proteins in which these members perform their oncogenic roles not only by inhibiting apoptosis, but also by blocking autophagy (Pattingre and Levine, 2006). In addition, Beclin1 was identified as a BH3-only protein because of the conserved BH3 domain that it contains. This

domain is necessary as well as sufficient to interact with the anti-apoptotic protein Bcl-xL as shown in **(Figure 13)** (Oberstein et al., 2007).

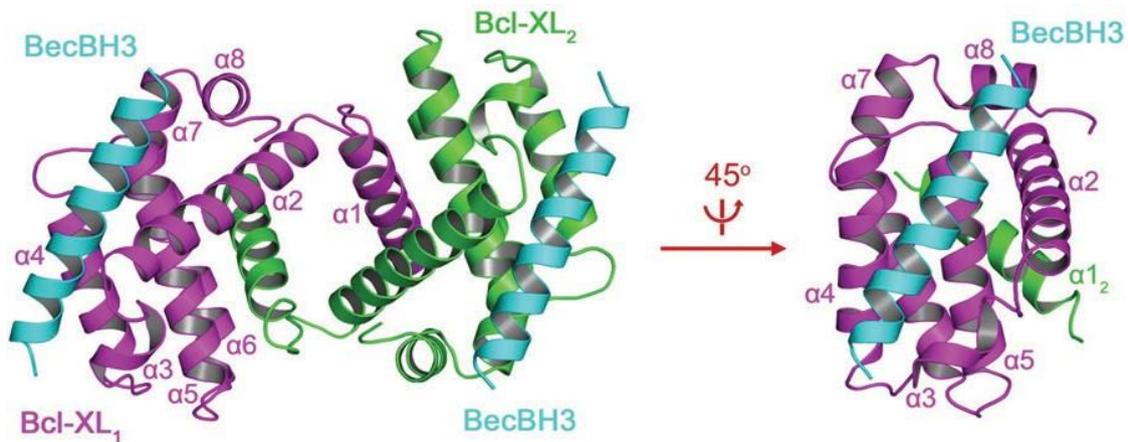


Figure 13: Structure of the BeclinBH3-Bcl-xL complex.

The interaction between Beclin 1 BH3 peptide and Bcl-xL is presented as a tetramer composed of two heterodimers. Crystallization of the tetramer show the intramolecular swapping of each Bcl-xL $\alpha 1$ helix with its neighbor one. Blue: Beclin 1 BH3 domain; Purple and Green: Bcl-xL (Oberstein et al., 2007).

The interaction between Bcl-2, Bcl-xL, Bcl-w and Mcl-1 with Beclin 1 resembles that of the Anti/Pro-apoptotic proteins: The BH3 domain of Beclin 1 interacts with the hydrophobic cleft of the pro-survival members. In general, the importance of this interaction relies on the prevention of the assembly of the pre-autophagosomal structure, thereby inhibiting autophagy (Erlich et al., 2007; Liang et al., 1998).

As for survival-mediated autophagy, Bcl-2 and Bcl-xL acquire novel roles in cell survival maintenance. In fact, in cells lacking Bax, Bcl-xL and Bcl-2 are required for the induction of the autophagic pathway in case of starvation. More precisely, Bcl-xL was shown to regulate in a tighter way autophagy compared to Bcl-2. Moreover, in autophagic cells, Bcl-xL re-localized to lighter membrane fractions which insists on

the importance of the subcellular localization in the execution of different functions. The activation of autophagy, in this case, is Beclin 1-independent (Priault et al., 2010).

Recent data showed the design of a novel BH3-target peptide in which L116 is substituted with M116 what might enhance the binding affinity with Bcl-xL (Fu et al., 2019). Furthermore, Beclin 1 was demonstrated to interact with pro-apoptotic Bax as well as active Caspase 3 in oocytes. The increase in Beclin 1 expression leads to an upregulation of the expression of pro-apoptotic proteins what could explain the activation of the apoptotic pathway during oocyte elimination (Escobar et al., 2019). Altogether, autophagy and apoptosis might appear like two separate mechanisms but are controlled by proteins from the same family what suggests a functional crosstalk between the two.

1.7.2. Cell cycle

Multiple observations have suggested a link between cell cycle and cell death. In general, cycling cells are more vulnerable to cell death than quiescent ones. Furthermore, cells that undergo apoptosis are known to activate cell cycle activators such as Cyclin-dependent kinase (Cdk) and inhibit cell cycle progression (Meikrantz et al., 1994). Surprisingly, Bcl-2 is not only an anti-apoptotic protein but also an anti-proliferative one. In 1988 was the first observation of Bcl-2 protein involvement in cell cycle: When Bcl-2 was overexpressed in Interleukin 3 (IL-3) – dependent FDC-P1 cells, these cells were smaller in the presence of IL-3 and were arrested in G0/G1 (Vaux et al., 1988). Afterwards, a series of studies ensued elaborating not only the effect of Bcl-2 on G0 arrest but also on cell cycle progression. In fact, overexpression of Bcl-2 in mouse T cells retarded the G0 to S transition. Bcl-2 impaired nuclear factor of activated T cells (NFAT) – mediated transcription of IL-2 necessary for cell cycle progression (Linette et al., 1996). Furthermore, Bcl-xL overexpression displayed the same consequences on cell cycle in T and B lymphocytes and quiescent fibroblasts. These data were another proof on the implications of the Bcl-2 family, especially the anti-apoptotic members, in normal cell turnover and tumor progression (O'Reilly et al., 1996). Expression of Bcl-w also retarded the progression to the S phase confirming the fact that the effect on cell cycle is neither Bcl-2 restricted nor cell type restricted (Print et al., 1998). On the contrary to previously cited anti-apoptotic proteins, Mcl-1 is involved in S phase and not G0/G1. The overexpression of this member significantly inhibited the progression into to the S phase. The latter

phase is regulated by the cell cycle regulator Proliferating Cell Nuclear Antigen (PCNA) that actually interacts with Mcl-1 (Fujise et al., 2000). It is interesting to note that tyrosine mutation in BH4 domain in Bcl-2, Bcl-xL and Bcl-w does not affect their capacity to bind several pro-survival members and prevent cell death but it actually abolishes their effect on cell cycle regulation (Huang et al., 1997).

Whereas the anti-apoptotic proteins are also anti-proliferative, the pro-apoptotic ones are proliferative. Overexpression of Bax in mature T cells accelerates the transition into the S phase in response to IL-2 stimulation (Brady et al., 1996). As for Bad, its overexpression inhibits the capacity of the cell to be arrested in G0 and continuously activates Cdk2 necessary for G1/S transition. However, this effect is completely dependent on Bad binding to Bcl-2 and Bcl-xL (Chattopadhyay et al., 2001).

Interestingly, Bcl-2 proteins require a post-translational modification, phosphorylation, to perform their diverse actions in the regulation of cell cycle. This section will be covered in (part 1.9.1).

1.7.3. Cellular and mitochondrial metabolism and dynamics

As mentioned above, mitochondria are dynamic organelles that constitute the cellular hubs for energy metabolism. These organelles also participate in the regulation of intracellular calcium fluxes.

The first proof of the implication of Bcl-2 proteins in metabolism was the involvement of Bad in glucose homeostasis. In fact, this BH3-only member interacts directly with glucokinase (GK, hexokinase IV). The latter is an essential factor of the glucose sensing machinery in mammals. It has principal roles in insulin secretion and glucose use and storage in hepatocytes. Moreover, this interaction depends on the phosphorylation status of Bad. In case of glucose deprivation, Bad is de-phosphorylated resulting in Bad-mediated cell death (Danial et al., 2003; Matschinsky, 2009). Actually, the activation of the GK depends on a conserved serine phosphorylation in the BH3 domain of Bad. Either Ser155 (mouse) or Ser118 (human), are phosphorylated in a cell-specific manner regulated by several signaling pathways. However, when the BH3 domain is not phosphorylated, Bad cannot bind to and activate GK but can bind and inhibit Bcl-2, Bcl-xL and Bcl-w so the induction of cell death is only indirect (Danial, 2008).

In the liver, Bad has an equilibrium role between hepatic glucose production, use and storage. These processes are mainly affected by mitochondrial functioning through Fatty Acid Oxidation (FAO), TCA cycle and electron transport chain (ETC) (Satapati et al., 2012). In the brain, Bad affects substrate metabolism important for neuronal excitability. Even though glucose is the main source of energy in the brain, neurons can switch to ketone bodies under glucose deprivation. Accordingly, Bad is phosphorylated at Ser155 in neuronal cells which stimulates glucose metabolism and inhibits the utilization of ketone bodies what creates a switch in the glucose-to-ketone response (Giménez-Cassina et al., 2012; Mergenthaler et al., 2013).

Another BH3-only member, Noxa, also controls glucose metabolism *via* its phosphorylation on Ser13. In fact, Cdk5, in a glucose-dependent way, is responsible for this phosphorylation upstream of the BH3 domain resulting in the nourishing of the pentose phosphate pathway (PPP) and not glycolysis. This process is important for T cell activation (Lowman et al., 2010; MacIver et al., 2013).

At the level of the mitochondria, some Bcl-2 proteins affect ETC and the oxidative phosphorylation (OXPHOS). Mcl-1, for example, is involved in ETC complex assembly. Deletion of this protein will result in tremendous effects on the tubular mitochondrial network as well as peculiarities in the morphology of mitochondrial cristae (Perciavalle et al., 2012b). The C-terminal TM domain of Mcl-1 targets this protein to the MOM. However, a cleavage signal at the N-terminus targets this same protein to the mitochondrial matrix where it normally couples mitochondrial fusion to respiration (Huang and Yang-Yen, 2010; Perciavalle et al., 2012b; Warr et al., 2011). Also, the N-terminus was demonstrated to be cleaved to produce a shorter protein that remains at the MOM if the mitochondrial membrane potential is intact. The MOM Mcl-1 product performs anti-apoptotic functions while the matrix one is involved in the assembly of large respiratory complexes comprising complexes I, III and IV which is highly dependent on the cristae ultrastructure integrity. In the absence of Mcl-1, changes in mitochondrial respiration occur along with morphological changes consistent with the ETC activity alterations (**Figure 14**) (Lapuente-Brun et al., 2013).

As for the Godfather of the gang, Bcl-2 triggers mitochondrial respiration in tumor cells *via* its interaction with the cytochrome c oxidase (COX) Va subunit of the respiratory complex IV. Upon activation, the latter

subunit recruits the COX Vb subunit and leads to the increase in mitochondrial respiration (**Figure 14**) (Chen and Pervaiz, 2007, 2010).

Bcl-xL in neurons was shown to interact with the β subunit of the F1FO ATP synthase and increases the coupling activity of mitochondria (**Figure 14**). This is done by a small fraction of Bcl-xL residing at the MIM (Alavian et al., 2011b; Chen et al., 2011b). Furthermore, Bcl-xL performs its pro-survival role also by affecting the OXPHOS. In fact, this protein lowers the levels of acetyl-CoA in Bcl-xL-overexpressed cells resulting in a reduction of N-alpha-acetylated proteins in the cell (Yi et al., 2011).

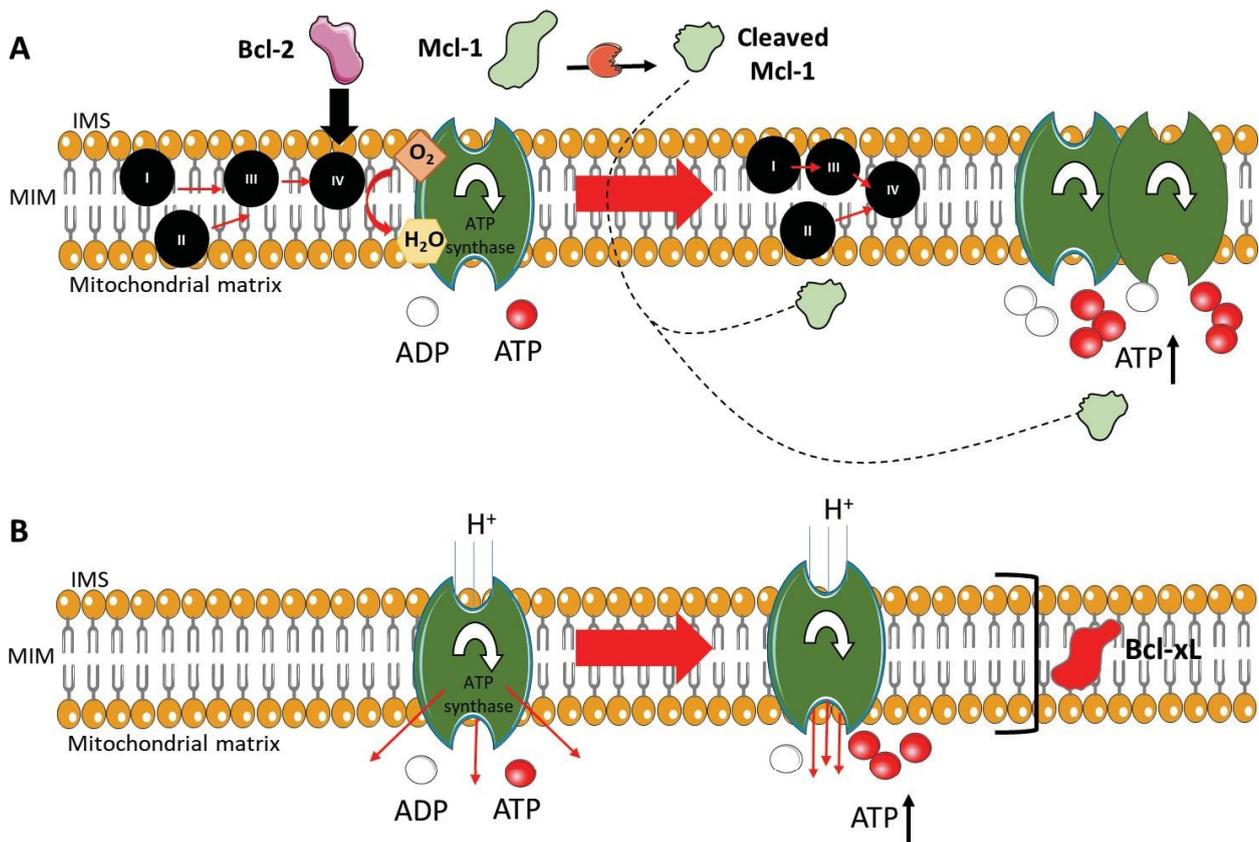


Figure 14: The implications of some Bcl-2 proteins in OXPHOS.

(A) Mcl-1 undergoes proteolytic cleavage at its N-terminus to produce a protein targeted to the mitochondrial matrix. There, it stabilizes the assembly of the respiratory complexes I, III and IV. It also promotes the increase in ATP synthesis. As for Bcl-2, it activates the complex IV of the respiratory chain. (B) Bcl-xL, at the MIM, interacts

with the β -subunit of the ATP synthase and activates ATP synthesis. Inspired from (Giménez-Cassina and Danial, 2015).

Mitochondrial metabolism is also involved in the determination of the fate of the haematopoietic stem cell (HSC). A kinase called ATM plays a pivotal role in the regulation of the self-renewal and quiescence of these HSCs through the regulation of reactive oxygen species (ROS) (Ito et al., 2004). The BH3-only member Bid acts as an ATM-effector and controls the state of quiescence of HSCs (Maryanovich and Gross, 2013). In fact, at the MOM, resides a receptor for t-Bid called mitochondrial carrier homolog 2 (MTCH2). The latter enables tBid to reach the mitochondria and facilitates the downstream activation of pro-apoptotic proteins (Zaltsman et al., 2010). Deletion of MTCH2 leads to the increase in OXPHOS resulting in the entry of HSCs into cell cycle. Bid negatively regulates MTCH2 that, in turn, represses the mitochondrial diameter and metabolism (**Figure 15**) (Maryanovich et al., 2015).

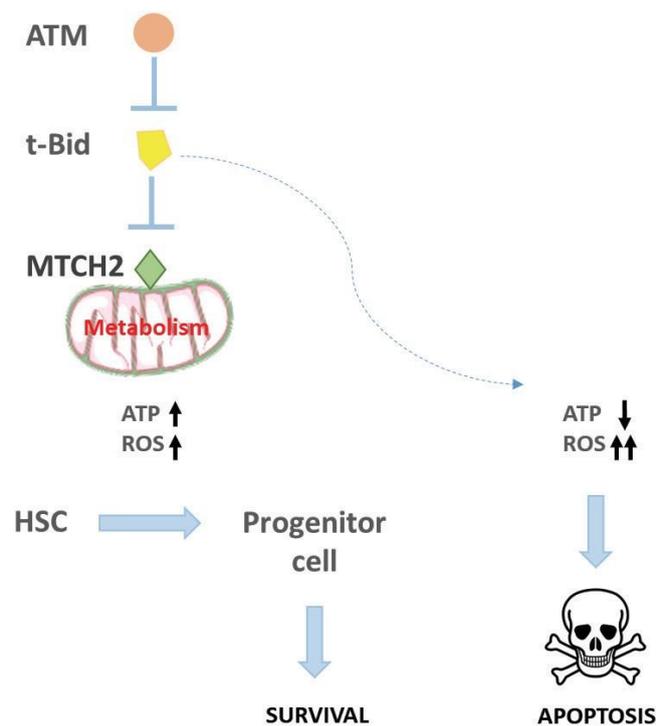


Figure 15: *The ATM-BID-MTCH2 pathway.*

Mitochondrial metabolism controls the fate of HSCs. Bid acts as an upstream negative regulator of MTCH2 that in turn, acts as a repressor of mitochondrial metabolism. On the MOM, MTCH2 targets tBid and facilitates the activation of downstream pro-apoptotic effectors leading to apoptosis. This pathway constitutes a switch between HSC survival, differentiation and apoptosis.

Furthermore, mitochondria can undergo fission and fusion mechanisms regulated by several proteins such as Mitofusin (mfn), dynamin-related protein (Drp1) and Opa1. Bcl-xL was shown to be involved in mitochondrial dynamics. In neurons, Bcl-xL augments the rates of fusion/fission and induces a longer mitochondrial morphology through its capacity to increase mitochondrial biomass. These mechanisms are critical to neuronal function and might result in severe consequences whenever compromised (Berman et al., 2009). As for Mcl-1, the rates of both splicing products induce a huge hyperfusion of the mitochondrial network in a Drp1-dependent manner (Morciano et al., 2016).

The enlarged roles of Bcl-2 members in energy metabolism provide insights into new ways these proteins affect and regulate cellular homeostasis apart from apoptosis. This is ensured due to the interactions of Bcl-2 members with binding partners mainly present at the mitochondria.

1.7.4. Cytoskeleton dynamics and migration

As we can see, the Bcl-2 squad displays diverse functions beyond apoptosis regulation. Another role of this family consists in acting on cytoskeletal dynamics and controlling migration. Taking the example of the Nr-13 zebrafish ortholog, 'Nr3' was shown to be highly expressed during the first period of embryonic development. More specifically, it was found in the yolk syncytial layer (YSL), an extra-embryonic structure resulting from the merge of the embryo's marginal cells with the yolk sac before the start of gastrulation. Our team has demonstrated the involvement of Nr3 in epiboly: the very first morphogenetic step of zebrafish embryogenesis characterized by the migration of the embryonic cells from the animal to the vegetal poles. Actually, Nr3 interacts with a Ca^{2+} channel at the ER called Inositol triphosphate receptor (IP3R) where it decreases ER- Ca^{2+} discharge. Accordingly, any loss of Nr3 results in a high cytosolic Ca^{2+} level increase occurring during epiboly activating the Ca^{2+} -dependent myosin light chain kinase (MLCK) thus leading to the hypercontraction of an actin-myosin ring. This will further result in the detachment of the embryo from the yolk sac (Bonneau et al., 2011; Popgeorgiev et al., 2011).

Moreover, Bcl-2 also plays a role in migration and invasion. The overexpression of Bcl-2 enhanced the metastatic potential and promoted cell migration/invasion, *in-vivo* and *in-vitro*, respectively (Del Bufalo et al., 1997). Then, the ectopic expression of Bcl-2 in malignant glioma cell lines prompted cell migration through an upregulation of matrix metalloproteases (MMP) specifically MMP2 and MMP9 (Wick et al., 1998). Bcl-2 might also prompt the activation of transcriptional factors such as NF- κ B, leading to the expression of genes related to migration and invasion of tumor cells by regulating Ca²⁺ homeostasis. Bcl-2 does so by either increasing ER Ca²⁺ leakage or decreasing ER Ca²⁺ re-uptake, thus raising cytosolic Ca²⁺ levels which, in its turn, participates in the activation of the transcriptional complex AP-1 involved in migration (Bonneau et al., 2013; Pahl and Baeuerle, 1996; Wang et al., 2008).

As for Bcl-xL, its expression in breast cancer cells was shown to increase metastasis. It was proved to do so by induction of cytokine resistance, enhancing cell survival in circulation along with anchorage-independent growth (Fernández et al., 2000). Four years later, this protein was shown to increase indeed metastatic potential but without affecting primary tumor growth in the mammary gland (Martin et al., 2004). In glioma cells, only long-term Bcl-xL induction leads to an increase the transforming growth factor beta 2 (TGF β), MMP2 and MMP14 expressions that in turn, enhance the invasiveness (Weiler et al., 2006). Furthermore, Bcl-xL promotes pancreatic islet tumor cell invasion. In fact, overexpression of Bcl-xL in pancreatic islet tumor cells changed the cell morphology and remodeled the actin cytoskeleton prompting cell migration and invasion. Myosin Va was presented as a new Bcl-xL binding partner necessary for its migratory effects (Du et al., 2007). Likewise, overexpression of Bcl-xL also promoted cell migration and invasion in human melanoma and glioblastoma cell lines. These cells displayed a higher tumor sphere formation ability along with high levels of stem cell markers insisting on the idea that Bcl-xL plays a major role in cancer stem cell maintenance (Trisciuglio et al., 2017).

1.7.5. Neural plasticity

Neural plasticity, also called neuroplasticity, is defined by the ability of the nervous system to modify itself, on the function and structure levels as a response to a certain injury. This process was also shown

to be involved in learning and memory. Neuroplasticity includes the extension of neurites consequently forming new synapses (von Bernhardi et al., 2017).

The Bcl-2 family is involved in the control of neuronal survival, development and injury. Bcl-2 as well as Bcl-xL expressions are important for embryonic development, more specifically, in the embryonic nervous system. Even though Bcl-2 is expressed in neuronal tissues during embryonic development, it was shown to be down-regulated in the adult central nervous system (CNS) with aging. In the peripheral nervous system (PNS) including neurons and supporting cells of sympathetic and sensory ganglia, Bcl-2 is expressed indefinitely. On the other hand, Bcl-xL is expressed in the adult CNS even with aging (González-García et al., 1995; Merry et al., 1994). In the CNS, Bcl-2 promotes the growth and regeneration of retinal severed axons in an 'anti-apoptotic-independent' manner. In fact, the same regeneration potential was inhibited with the addition of the pan caspase inhibitor ZVAD but the neuronal survival was supported (Chen et al., 1997).

As for Bcl-xL, it was demonstrated to play an important protective role in the case of naturally dying neurons in contrast to Bcl-w that is mostly important at later stages (Middleton et al., 2001). In fact, Bcl-xL has a role in synaptic transmission since the injection of the full-length protein in the presynaptic terminal led to the increase in postsynaptic response and boosted up the recovery rate from synaptic depression (Jonas et al., 2003b). Presynaptic plasticity might depend on ATP release from mitochondria and this was proven to be controlled by the Bcl-2 family (Hickman et al., 2008). Furthermore, Bcl-xL regulates mitochondrial bioenergetics, synaptic transmitter release and vesicle recycling as well as neurite growth. In fact, Bcl-xL positively regulates the mitochondrial fission protein Drp1 and triggers its GTPase activity in order to induce synapse formation through mitochondrial stimulation (Li et al., 2008). Moreover, this anti-apoptotic member interacts with the MOM Ca^{2+} channel VDAC and enhances the ATP conductance as well as Ca^{2+} release of this channel in synapses and in cancer cells (Jonas et al., 2014). It actually regulates neuronal metabolic efficiency through its interaction with F_1F_0 (Alavian et al., 2011b).

1.8. 'Indispensable existence': physiological roles of Bcl-2 partners

When we say indispensable, we mean it. The Bcl-2 gang, the killers as well as the superheroes, definitely governs the life/death switch of the cell... and the whole organism. Indeed, genetic experiments on mice put in evidence the vital roles of the Bcl-2 proteins. Here, we review some of the most important physiological aspects (**Table 2**).

1.8.1. *Pro-survival Bcl-2 proteins*

Starting with the first protein Bcl-2: Transgenic mice homozygously lacking Bcl-2 survived the whole embryonic stage but abnormalities started to show post-birth. These mice exhibited pleiotropic consequences such as increased lymphoid cell death in their thymus and spleen, defects in their small intestine, growth retardation and hair hypopigmentation in the second hair follicle cycle. They also displayed polycystic kidneys that led to their postnatal mortality (Kamada et al., 1995; Nakayama et al., 1993; Veis et al., 1993).

As for Bcl-xL deficient mice, their phenotype was merciless: they died at around embryonic day thirteen (E13-E13.5). In 1995, the team of Motoyama generated double *Bcl-x* knockout (KO) chimeric mice. Massive apoptosis was evident in the postmitotic neurons of the CNS and the hematopoietic cells of the liver. Also, the maturation of lymphocytes was abolished and the lifespan of the immature ones was reduced. They concluded that Bcl-xL is indispensable for the survival of immature cells of the CNS and hematopoietic system (Motoyama et al., 1995). Furthermore, Bcl-xL is important to regulate CNS apoptosis postnatal since as we already mentioned, Bcl-xL expression remains high in the CNS after birth. In fact, Bcl-xL is neuroprotective after birth in mice overexpressing human Bcl-xL under the control of a pan-neuronal promoter (Parsadonian et al., 1998). Due to the embryonic lethality of Bcl-xL deficient mice, researchers started to work with conditional KO. In 2000, a conditional KO of Bcl-xL was realized

with Cre-LoxP recombination system using a Cre recombinase under the control of the MMTV-LTR promoter. In these mice, Bcl-xL provided the survival of erythrocytes at the end of maturation since the conditional deficiency of this protein resulted in hemolytic anemia and a huge splenomegaly. Moreover, this increase in observed cell death in erythroid cells was Bax-independent (Wagner et al., 2000). In the mammary gland, the expression of Bcl-xL is the highest amongst the Bcl-2 family. In Bcl-xL conditional KO mice, Bcl-xL is not necessary for mammapoiesis but during the first stage of involution. In fact, at that stage, epithelial apoptosis and tissue remodeling occurred when Bcl-xL was deficient (Walton et al., 2001). Bcl-xL prompts the survival of mature platelets. In a study where Bcl-xL was targeted by a certain drug; discussed in (part 4.2.1.1.), it induced severe thrombocytopenia along with a transient thrombocytopeny that could compromise the hemostatic function of platelets (Schoenwaelder et al., 2011).

Mcl-1 deficient mice embryos faced peri-implantation lethality. In-vitro, null blastocysts could not neither hatch nor attach and displayed a maturation delay. Thus, Mcl-1 has a fundamental function in embryonic development at an earlier stage (around E3.5-4) than Bcl-xL (Rinkenberger et al., 2000). Other critical functions of Mcl-1 were assessed in conditional KO experiment. Mcl-1 is important for lymphoid development and the maintenance of mature T and B lymphocytes. Furthermore, it was later considered an essential provider of the homeostasis of hematopoietic progenitor cells (Opferman et al., 2003, 2005). Conditional KO of this anti-apoptotic member in heart led to cardiac failure and mitochondrial dysfunction (Wang et al., 2013).

The pro-survival protein Bcl-w was shown to be crucial in spermatogenesis but its deletion in mice did not affect neither embryonic development nor postnatal survival. Bcl-w $-/-$ mice were viable and healthy and females were fertile (Print et al., 1998).

Bcl-2A1 or Bfl1 deletion in mice does not compromise their embryonic development. This protein is considered an essential factor in the survival of neutrophils and mast cells since its KO resulted in their apoptosis (Xiang et al., 2001). Also, it was shown to be an essential factor in the maturation of B cells (Sochalska et al., 2016). Constitutive knockdown of all A1 isoforms protects mice from anaphylaxis and demonstrates that Bfl1 is important for the homeostasis of connective tissue mast cells (Ottina et al., 2015).

As for Nrh or Bcl2L10, its KO in mice did not produce a damageable phenotype since transgenic mice were viable and healthy. But in zebrafish models, the inactivation of this gene led to lethality in an apoptosis-independent manner (Popgeorgiev et al., 2011; Russell et al., 2002). Thus, anti-apoptotic proteins display crucial roles in mammalian development including early embryogenesis, neurogenesis and hematopoiesis (**Figure 16**) (Opferman and Kothari, 2018).

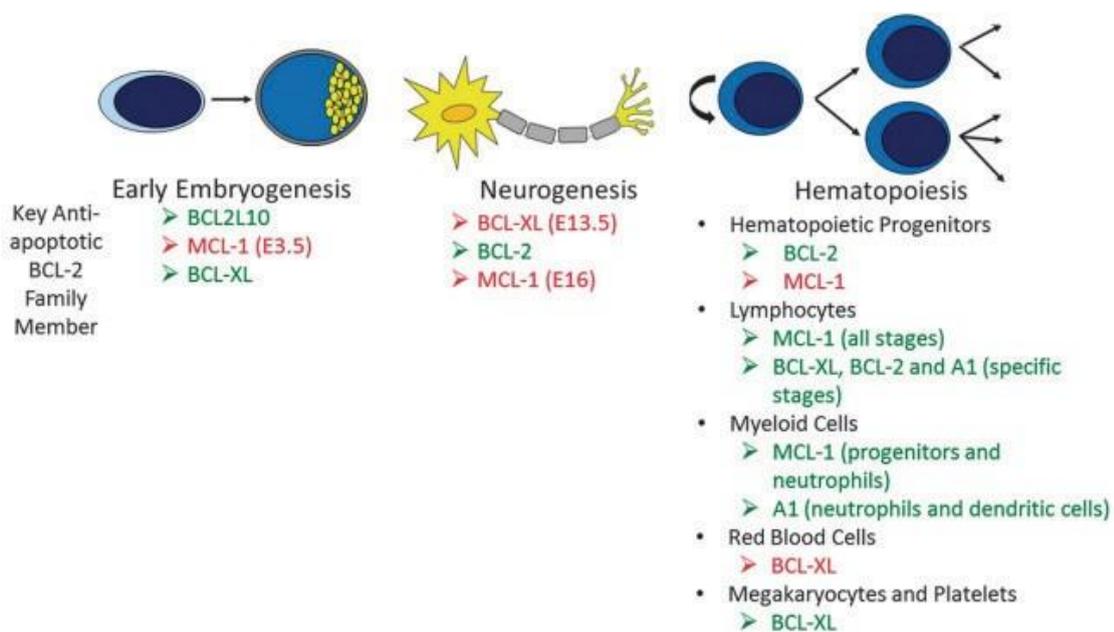


Figure 16: Anti-apoptotic proteins in embryogenesis, neurogenesis and hematopoiesis.

Graphical summary of the major roles of the anti-apoptotic members in mammalian development. Mcl-1 is crucial for early embryonic development and Bcl-xL is vital for neurogenesis. During hematopoiesis, Mcl-1 plays a major role in the survival of hematopoietic stem cells, lymphocytes and neutrophils and Bcl-xL promotes the survival of mature platelets. Red: most involved factors in each aspect of development that might include a damageable phenotype. Green: All pro-survival members playing a role in mammalian development. From (Opferman and Kothari, 2018).

1.8.2. Pro-apoptotic Bcl-2 proteins

The invalidation consequences of pro-apoptotic proteins are far less severe than those of anti-apoptotic ones. Bax deficient mice are viable but exhibit lineage-specific peculiarities. In fact, the invalidation of Bax causes lymphoid hyperplasia and male germ cell apoptosis leading to male infertility (Knudson et al., 1995). Also, Bax was shown to be a critical mediator of naturally occurring PNS and CNS cell death during embryogenesis (White et al., 1998).

As for Bak deficient mice, they were phenotypically healthy, reproductively active and did not develop age-related diseases. To note, Bax and Bak have complementary roles in the control of mammalian apoptosis and tissue homeostasis (Lindsten et al., 2000). No defects were detected in the inactivation of Bok in mice. It might have a tissue-specific restricted function but only a minimal impact *in-vivo* (Ke et al., 2012b).

The BH3-only protein-invalidation does not also cause a lethal phenotype in mice. The most severe phenotype among them is that of the loss of Bim. Actually, Bim acts as a barrier to the immune system: the removal of Bim in mice led to the accumulation of lymphoid and myeloid cells, perturbations in T cell development and autoimmune kidney disease. Thus, Bim maintains the homeostasis in the hematopoietic system (Bouillet et al., 1999). Likewise, the loss of Bim made the granulocytes resistant to cytotoxic drugs (Villunger et al., 2003b). A conditional Bim KO mouse model using Cre-LoxP recombination system with Cre expressed in the hematopoietic system validated the hematopoietic phenotypes observed in Bim KO mice in all tissues (Herold et al., 2014). Bid depletion in mice might cause fatal hepatitis due to Fas-linked hepatocellular apoptosis and hemorrhagic necrosis. So, Bid is a critical substrate for death-receptor signaling *in-vivo* (Yin et al., 1999). By gene targeting, Puma-deficient mice were generated. In a p53-dependent manner, Puma is indispensable for ionizing radiation (IR) - triggered hematopoietic and nervous cell death. It also deregulates c-Myc expression (Jeffers et al., 2003). Noxa acts in a similar way to Puma where it also contributes to the p53 response *in-vivo*. Following DNA damage, Noxa is involved in the oncogene-independent gradual apoptosis activated by p53 (Shibue et al., 2003). No major defects were detected for Bik, Hrk, Bad and Bmf (Strasser, 2005).

Bcl-2 member	KO phenotype in mice
Pro-survival / multidomain	
Mcl-1	Embryonic lethality at E3.5-4. Peri-implantation defects. Conditional KO: lethal cardiac failure.
Bcl-xL	Embryonic lethality at E13.5. Massive apoptosis of immature cells of the nervous and hematopoietic systems. Conditional KO: Involution defects.
Bcl-2	Post-natal lethality: polycystic kidneys. Lymphoid cell death. Hair hypopigmentation. Growth retardation.
Bcl-w	No lethality. Male infertility due to spermatogenesis defects.
Bcl-2A1/Bfl-1	No lethality. Neutrophils and mast cells apoptosis .
Bcl2L10/Nrh	No lethality. Embryonic lethality of Diva/Boo in zebrafish models.
Anti-survival / multidomain	
Bax	Lymphoid hyperplasia. Male infertility.
Bak	No major defects detected.
Bok	No major defects detected.
Anti-survival / BH3-only	
Bim	Accumulation of B and T cells. Perturbations in T cell development. Autoimmune kidney disease.
Bid	Fatal hepatitis due to Fas-linked hepatocellular apoptosis.
Puma	Resistance to DNA-damage induced cell death.
Noxa	Resistance to DNA-damage induced cell death.
Bid	No major defects detected.
Hrk	No major defects detected.
Bad	No major defects detected.
Bmf	No major defects detected.

Table 2: Summary of the phenotypic consequences of Bcl-2 proteins' loss in mice.

Phenotype severity ascending order: Gray < Yellow < Blue < Green.

1.9. Posttranslational modifications: *Regulating the regulators?*

Even the regulators are indeed regulated. The activity of Bcl-2 proteins is controlled by posttranslational modifications (PTM) including phosphorylation, ubiquitination, proteosomal degradation and deamidation. Accordingly, Bcl-2 members undergoing these modifications can either promote or inhibit apoptosis, depending on the cellular context. Here, we review some of the most important multidomain Bcl-2 family PTMs that affect apoptosis signaling pathways insinuating on their role in disease pathogenesis with a specific focus on the anti-apoptotic protein Bcl-xL.

1.9.1. *Phosphorylation*

Phosphorylation of pro-survival Bcl-2 proteins

Since Bcl-2 interacts with pro-apoptotic proteins in order to inhibit apoptosis, any deregulation of its PTMs might result in tremendous consequences including malignant transformation. At first, the phosphorylation of Bcl-2 in its flexible loop, located between BH3 and BH4 domains was considered crucial for its anti-apoptotic function. This exact region is conserved among Bcl-xL and Mcl-1 too. Deletion of this region will either promote or inhibit the anti-apoptotic effect of Bcl-2 in paclitaxel-mediated apoptosis (Wang et al., 1999). Bcl-2 is phosphorylated at Ser70 by protein kinase C α (PKC α) resulting in the abrogation of apoptosis in etoposide-treated myeloid cells (Deng et al., 2001a). In fact, Bcl-2 can either be phosphorylated on Ser70 alone or on other residues including Thr69 and Ser87 simultaneously. All the mentioned PTMs aid in the survival enhancement of Bcl-2 against paclitaxel-induced cell death and further stabilize this member (Deng et al., 2004).

In response to taxol, Bcl-xL is phosphorylated at Ser62 or Ser49 by Jun N-terminal Kinase (JNK) leading to the inhibition of its pro-survival function in prostate cancer cells (Basu and Haldar, 2003). These phosphorylations could also be mediated by other kinases such as Mitogen-Activated Protein Kinase (MAPK9) / JNK2 or even Polo-Like Kinase (PLK) 1/3. Moreover, Ser62 phosphorylation plays a role in S and G2 phases of the cell cycle thus slowing the entry into mitosis (Wang et al., 2012a). At the mitochondria, JNK also phosphorylates Bcl-xL on Thr47 and Thr115 residues in response to DNA damage

leading to apoptosis initiation (Kharbanda et al., 2000). Bcl-xL could be phosphorylated at other residues including Ser14 (residing in BH4 domain) by oxidative stress-activated Mst1 in cardiac myocytes destabilizing Bcl-xL-Bax binding (Del Re et al., 2014). Also, on Ser73 by cdk2 necessary to induce kidney cell death (Seng et al., 2016) and on Ser28 as shown by motif-targeted quantitative proteomics (Beaumat et al., 2017; Tsai et al., 2015). As for Bcl-xS (a pro-apoptotic *Bcl-x* transcript), the phosphorylation takes place on Ser106 and Ser228 of inhibiting its association with VDAC leading to apoptosis (Wei et al., 2009).

As for Mcl-1, it has a PEST domain upstream of its BH3 domain which is a Proline, Glutamate, Serine, Threonine rich domain associated with a shorter half-life (2–4 hours) and proteasomal degradation. Accordingly, PEST domain phosphorylation affects the stability of Mcl-1 (Domina et al., 2004). Mcl-1 could be phosphorylated at Thr92 and Thr163 maintaining its stability and pro-survival potential (Ding et al., 2008) or at S159 leading to its degradation by the E3 ubiquitin ligase SCF(FBW7) (Inuzuka et al., 2011). Other phosphorylation sites have been noted: on Ser155, Ser159 and Thr163 as glycogen synthase kinase 3 (GSK3) targets, on Ser64 where the phosphorylation enhances its pro-survival activity (Ding et al., 2007). Recent findings presented a novel degradation-independent function of Mcl-1 phosphorylation in colon cancer cells. This is mediated by histone deacetylase inhibitors (HDACi) inducing GSK3 β -dependent Mcl-1 phosphorylation without degradation or downregulation which might be pretty interesting in anti-cancer therapies developing new Mcl-1 targeting agents in order to overcome resistance (Tong et al., 2018).

Bcl-w is phosphorylated by Akt leading to a decrease in its interaction with pro-apoptotic proteins but no phosphorylation sites are identified (Garofalo et al., 2008).

Finally, there are no current data available involving the phosphorylation of neither Bcl-B/Nrh nor Bfl-1/Bcl-2A1 (**Table 3**).

Phosphorylation Pro-survival Bcl-2 member	Residue	Outcome	References
Bcl-2	Ser70	Inhibition of apoptosis	(Deng et al., 2001a)
	Thr69 + Ser87 + Ser70	Inhibition of apoptosis + Bcl-2 stability	(Deng et al., 2004)
Bcl-xL	Ser14	Destabilizes Bcl-xL/Bax binding	(Del Re et al., 2014)
	Ser49	Inhibition of pro-survival function + promoting apoptosis	(Basu and Haldar, 2003)
	Ser62	Inhibition of pro-survival function + promoting apoptosis + slowing entry into mitosis	(Basu and Haldar, 2003); (Wang et al., 2012a)
	Ser73	Kidney cell death	(Seng et al., 2016)
	Ser106 or Ser228 (of Bcl-xS)	Inhibition of Bcl-xL/VDAC interaction + promoting apoptosis	(Wei et al., 2009)
	Thr47	Inhibition of pro-survival function + promoting apoptosis	(Kharbanda et al., 2000)
	Thr115	Inhibition of pro-survival function + promoting apoptosis	(Kharbanda et al., 2000)
Mcl-1	Thr92 or Thr163	Promoting Mcl-1 stability + Pro-survival function	(Ding et al., 2008)
	Ser159	Promoting apoptosis + Increasing Mcl-1 degradation	(Inuzuka et al., 2011)
	Ser155 + Ser159 + Thr163	Promoting apoptosis + Increasing Mcl-1 degradation	(Ding et al., 2007)
	Ser64	Inhibition of apoptosis	(Ding et al., 2007)
Bcl-w	Not identified	Decrease Bcl-w/pro- apoptotic proteins interaction	(Garofalo et al., 2008)
Bcl-2A1/Bfl-1	Not identified	Not identified	Not identified
Bcl-2L10/Nrh	Not identified	Not identified	Not identified

Table 3: Major phosphorylation modifications of anti-apoptotic Bcl-2 proteins.

Phosphorylation of anti-survival Bcl-2 proteins

Pro-apoptotic members are also controlled by PTMs. Here, we cite the major phosphorylations of the multidomain pro-apoptotic proteins Bax, Bak and Bok. Phosphorylation/Dephosphorylation of all pro-apoptotic proteins and their subsequent references are displayed in **(Table 4)**.

Bax is phosphorylated by Akt at Ser184 in its C-terminal hydrophobic domain resulting in the decrease of its translocation to the mitochondria, its heterodimerization with Mcl-1, Bcl-xL and A1 and thus the inactivation of apoptosis in neutrophils (Gardai et al., 2004). Moreover, PKC-zeta was demonstrated to phosphorylate Bax at Ser184 promoting its sequestration in the cytosol and the abrogation of the cisplatin-induced apoptosis (Gao et al., 2007). More recently, the Ser184 phosphorylation was proved the most important one in affecting Bax's function. In fact, the ability of Bax and Bcl-xL to interact depends on two factors: 1- Bax phosphorylation at Ser184 and 2- Bax and Bcl-xL localizations (Garenne et al., 2016). Furthermore, this exact phosphorylation, specifically in Akt-signaling pathway, is responsible for Bax binding to BH3-only proteins in solution and prevents its insertion into the MOM (Kale et al., 2018). On the other hand, GSK3 phosphorylation of Bax at Ser163 promotes its mitochondrial localization in neurons leading to their cell death (Kim et al., 2006; Linseman et al., 2004).

In contrast, Bak needs to be dephosphorylated to initiate the activation process. In healthy cells, Bak is well phosphorylated. In case of DNA damage, Bak undergoes a dephosphorylation at Tyr108 required for its N-terminal conformational change and subsequent oligomerization (Fox et al., 2010). Another dephosphorylation site was discovered in Bak's hydrophobic groove on Ser117 that is thought to be necessary for Bak activation. Actually, this dephosphorylation allows the binding of activator BH3-only proteins to BAK inducing its activation and cytochrome c release (Azad et al., 2012). Likewise, the dephosphorylation of Bak on Tyr110 in its hydrophobic groove plays a mechanistic role in its activation (Azad and Storey, 2013). However, the most recent study of Bak activation did not imply that the dephosphorylation of Bak is required for its apoptotic function (Tran et al., 2013).

It was only recently that PTM data for Bok emerged. The latter can be phosphorylated by different kinases including protein kinase A (PKA), PKC and GSK3 α/β . More precisely, inhibition of GSK α or β led

to an increase of the expression of Bok in breast cancer cells. To date, this is the first study to show that Bok is a *bona fide* target of several kinases including GSK3 (Onyeagucha et al., 2017).

Phosphorylation Anti-survival Bcl-2 member	Residue	Outcome	References
Bax	Ser184	Inhibition of apoptosis	(Gardai et al., 2004; Gao et al., 2007; Garenne et al., 2016; Kale et al., 2018)
	Ser163	Promoting apoptosis	(Linseman et al., 2004)
	Thr167	Promoting apoptosis	(Kim et al., 2006)
Bak	Dephosphorylation at Tyr108	N-terminal conformational change + oligomerization	(Fox et al., 2010)
	Dephosphorylation at Ser117	Increase Bak/BH3-only interaction + promoting apoptosis	(Azad et al., 2012)
	Dephosphorylation at Tyr110	Promoting apoptosis	(Azad and Storey, 2013)
Bok	Not identified	Inhibition of apoptosis	(Onyeagucha et al., 2017)
Bim	Ser65 or Ser69	Targeting for proteasomal degradation	(Ley et al., 2004)
	Ser55, Ser65, Ser100	Inhibition of apoptosis	(Harada et al., 2004)
	Ser65, Thr112	Promoting apoptosis	(Putcha et al., 2003) (Hübner et al., 2008)
	Thr116	Promoting apoptosis + increase Bim/Bcl-xL interaction	(Conage-Pough et al., 2013)
	Tyr92, Tyr161	Promoting apoptosis + increase Bim/Bcl-xL interaction	(Aira et al., 2018)
Bik	Thr33, Ser35	Promoting apoptosis	(Verma et al., 2001)
	Thr124	Targeting for proteasomal degradation	(Lopez et al., 2012)
Puma	Ser10	Inhibition of apoptosis	(Fricker et al., 2010)
Noxa	Ser13	Cytosolic retention + inhibition of apoptosis	(Lowman et al., 2010; Karim et al., 2015; Rath et al., 2015)
Bid	Thr58	Inhibition of Caspase 8 cleavage + inhibition of apoptosis	(Desagher et al., 2001)
	Thr59	Inhibition of apoptosis	(Prakasam et al., 2014)
	Ser66	Promoting apoptosis when mitotic exit is delayed	(Wang et al., 2014)
Hrk	Not identified	Not identified	Not identified
Bad	Ser112, Ser136, Ser155	Inhibition of apoptosis	(Datta et al., 2000; Zha et al., 1996)
	Ser170	Inhibition of apoptosis	(Drams et al., 2002)
	Ser128	Promoting apoptosis	(Donovan et al., 2002; Konishi et al., 2002)
	Thr201	Inhibition of apoptosis	(Yu et al., 2004)
	Ser75, Ser99, Ser118	Inhibition of apoptosis	(Polzen et al., 2009; Mann et al., 2019)
Bmf	Ser74, Ser77	Promoting apoptosis	(Shao and Aplin, 2012)

Table 4: *Major phosphorylation modifications of pro-apoptotic Bcl-2 proteins.*

Yellow boxes: Multidomain pro-apoptotic proteins. Green boxes: BH3-only pro-apoptotic proteins

1.9.2. Ubiquitination and proteasomal degradation

Ubiquitination plays major roles in cellular processes such as proteasomal degradation, DNA repair and regulation of cell cycle. Accordingly, if the ubiquitination was not processed properly, the whole degradation system could be affected. In that case, protein aggregation might cause severe human diseases such as cancer. Bcl-2 proteins do not escape this addition. In general, the phosphorylation of anti-apoptotic Bcl-2 proteins seems to play a role in the prevention of their degradation. Here, we review briefly the multidomain Bcl-2 members which expression levels depend on ubiquitination leading to proteasomal degradation.

Degradation of pro-survival Bcl-2 proteins

In general, the phosphorylation of anti-apoptotic Bcl-2 proteins seems to play a role in the prevention of their degradation. The putative MAP kinase sites of Bcl-2 including Thr56, 74 and Ser 87 when phosphorylated, inhibit the degradation of this anti-apoptotic member what suggests a link between the apoptosome and the proteasome pathways (Dimmeler et al., 1999). A recent study on the degradation of Bcl-2 and apoptosis induction put in evidence the downregulation of Bcl-2 by a tumor suppresser protein called ARTS that antagonizes the X-linked inhibitor of apoptosis (XIAP). In fact, ARTS and Bcl-2 colocalize at the MOM. Upon apoptotic stimuli, ARTS recruits XIAP and forms a ternary complex with the BH3 domain of Bcl-2 permitting XIAP to enhance the ubiquitination on Lys17 followed by degradation of Bcl-2. Accordingly, XIAP plays the role of an E3 ligase in this process (Edison et al., 2017).

In addition, the expression levels of Bcl-xL were greatly decreased by proteasome-mediated degradation in the case of ultraviolet radiation B (UVB)-mediated apoptosis in basal carcinoma cells further insisting on the proteasomal degradation of this member (Park and Lee, 2009). In contrast, Parkinson disease and cancer associated protein 'DJ-1' stabilizes Bcl-xL at the mitochondria in an oxidation-dependent manner in response to UVB via inhibiting its ubiquitination and subsequent proteasomal degradation (Ren et al., 2011). Furthermore, Bcl-xL phosphorylation at Ser62 by JNK triggers its polyubiquitination thus degradation. A serine/threonine protein phosphatase 6 (PP6) opposes this action by dephosphorylating

Bcl-xL (Kajihara et al., 2014). Also, PARK2, a Ubiquitin E3 ligase, was shown to bind to and ubiquitinate Bcl-xL in order to regulate apoptosis and mitochondrial depolarization (Gong et al., 2016). Another novel factor, RNF183, a membrane-spanning RING finger protein is overexpressed in ER stress. This overexpression increases apoptosis through the interaction, polyubiquitination and subsequent degradation of Bcl-xL by RNF183 (Wu et al., 2018).

Ubiquitination affects mostly the anti-apoptotic members of the Mcl-1 subgroup including Mcl-1 itself, Bfl-1 and Bcl21L10/Nrh. In 2005, Zhong and colleagues demonstrated that Mcl-1 could be ubiquitinated on five lysine (Lys5, 40, 136, 194 and 197) residues by a novel E3 that they called MULE for Mcl-1 Ubiquitin Ligase E3. This ubiquitination is indispensable for the degradation of Mcl-1 DNA damage- induced apoptosis (Zhong et al., 2005). Another E3 ligase implicated in Mcl-1 ubiquitination is the Skp1- CUL1-F box protein SCF ^{β -TrCP} that requires Mcl-1 phosphorylation by GSK3beta to mediate recognition (Ding et al., 2007). Trim17 is yet another E3 ligase responsible for the ubiquitination of Mcl-1 necessary for neuronal apoptosis (Magiera et al., 2013). On the other hand, a deubiquitinylase (DUB) called ubiquitin-specific peptidase 9 X-linked (USP9X) regulates Mcl-1 and promotes cell survival (Schwickart et al., 2010). Moreover, a study showed that a minor fraction of Mcl-1 can still be degraded even if the ubiquitination machinery is blocked since under these conditions, turnover occurs normally by an alternative, yet unknown, ubiquitin-independent process (Stewart et al., 2010). In all cases, the degradation of Mcl-1 seems a crucial mechanism in several contexts: in the response of colon cancer cells to targeted therapeutics (Tong et al., 2017) and in the control of mitotic cell death (Clarke et al., 2018).

As for Bfl-1/Bcl-2A1, its stability and function are controlled at its C-terminus. In fact, the latter rendered Bfl-1 highly unstable by enhancing its polyubiquitination and subsequent proteasomal degradation (Herold et al., 2006). Any mutation in Bfl-1 or in the upstream signaling pathways, mainly involving Akt, leading to its ubiquitination might predispose to lymphoma (Fan et al., 2010). Recently, novel insights on the enzymes regulating Bfl-1 stability were displayed. Two E3 ubiquitin ligases regulate the fate of Bfl-1. TRIM28 binds Bfl-1 at the mitochondria and enhances its ubiquitination. On the other hand, TRIM17 blocks TRIM28 further stabilizing A1 and inhibiting its subsequent degradation. This molecular

rheostat uncovers two antagonistic functions of proteins belonging to the same family (Lionnard et al., 2019).

As mentioned previously, Bcl-w undergoes a phosphorylation mediated by Akt. This phosphorylation was shown to modulate the amount of Bcl-w. To understand the mechanism of this regulation, HeLa cells were treated with the proteasome inhibitor MG-132. This inhibition did not increase Bcl-w expression suggesting that the ubiquitin pathway is not, at least directly, involved in the Akt-mediated regulation of Bcl-w (Garofalo et al., 2008).

Nrh is also an ubiquitination victim. Ubiquilin 1 (Ubq1n) interacts with Nrh specifically and stabilizes it by prompting its mono or polyubiquitination and relocation in the cytosol (Beverly et al., 2012). The expression level as well as the anti-apoptotic activity of Nrh are directly affected by its ubiquitination. The latter takes place in a loop between the BH1 and 2 domains. Several potential ubiquitination sites have been proposed such as Lys119, 120 and 128 with the latter being the main site. These ubiquitination sites facilitate the degradation of Bcl-B that is thus an unstable protein subject to ubiquitination and degradation at steady state (van de Kooij et al., 2013).

Degradation of anti-survival multidomain Bcl-2 proteins

Starting with Bax, the first evidence on the involvement of the ubiquitin-proteasome pathway in the regulation of its expression was presented by Li and colleagues in 2000. The abrogation of the proteasome activity leads to the overexpression of Bax in the cytosol and the mitochondria and its subsequent interaction with Bcl-2 (Li and Dou, 2000). The ubiquitin ligase Parkin is shown to also ubiquitinate Bax preventing its basal and apoptotic stress-induced translocation to the mitochondria (Johnson et al., 2012). In a recent study where Bax was not ubiquitinated, Parkin inhibited Bax-mediated apoptosis. The mechanism by which it does so is still unknown (Bernardini et al., 2019).

Moreover, it was shown that E6 proteins from HPV type 5 could prompt Bak degradation through the ubiquitin proteasome pathway. This decreases the amount of Bak needed for apoptosis execution (Underbrink et al., 2008). In E6-expressing cells, Bak, through its hydrophobic groove, interacts with the

putative BH3 domain of an E3 ubiquitin ligase called HERC1 upon UV damage (Holloway et al., 2015). As mentioned before, Parkin not only interacts with Bax but also with Bak. Upon mitophagy induced by mitochondrial damage, Parkin ubiquitinates Bak at a conserved lysine residue in its hydrophobic groove. This ubiquitination abrogated Bak activity by interfering with its activation and inhibiting the formation of oligomers (Bernardini et al., 2019).

Bok stability is directly related to its binding to IP3Rs. Unbound Bok is deleterious to cell survival (Schulman et al., 2016). The stability of this protein is controlled by the ER-associated degradation pathway where Bok is ubiquitinated by the E3 ligase AMFR/gp78 and then targeted to degradation (Llambi et al., 2016b).

1.9.3. Deamidation

The last PTM that we are discussing in this literature review is deamidation consisting in the removal of an amide functional group of asparagine and glutamine and its conversion to other carboxyl groups. This process alters the structure and function of proteins. Generally, asparagine is converted to aspartic acid by a process called nonenzymatic deamidation that adds a negative charge into the protein. Bcl-xL is the most affected Bcl-2 protein by this process (Kutuk and Letai, 2008). Upon DNA damage stimuli such as etoposide or cisplatin, Bcl-xL is deaminated at Asn52 and Asn66 which constitutes a critical process for apoptosis (Deverman et al., 2002). These residues are present in the unstructured loop separating the $\alpha 1$ (BH4 domain) and $\alpha 2$ (BH3 domain) helices of Bcl-xL and are indispensable for its pro-survival role. Any mutation converting them into alanine residues increases this anti-apoptotic function. Furthermore, it was shown that tumor cells gain resistance to apoptotic stimuli just by inhibiting the deamidation of Bcl-xL. Accordingly, this inhibition is a key factor in the regulation of cancer cell death (Takehara and Takahashi, 2003). In T cell transformation, an oncogenic tyrosine kinase called p56lck abrogates DNA-damage induced Bcl-xL deamidation, hence, inhibits Bax conformational change leading to apoptosis (Zhao et al., 2004). Moreover, Rb and p53 tumor suppressor proteins regulate Bcl-xL deamidation. In fact, in normal cells, p53 activates Rb through p21WAF1/CIP1. Hypophosphorylated thus active Rb inhibits the deamidation of Bcl-xL promoting cell survival. However, upon DNA damage in tumor cells with inactive p53/Rb pathways, BH3-only proteins are active and Bcl-xL deamidation is unhindered

leading to apoptosis (**Figure 17**) (Johnstone, 2002). Bcl-xL deamidation at the mentioned residues may generate iso-Asp52 and 66. The latter inhibit the sequestration of BH3-only members Bim and Puma by Bcl-xL (Zhao et al., 2007). Likewise, this exact deamidation leads to the adenovirus protein E1A-dependent sensitization of ovarian cancer cells to cisplatin (Chang et al., 2006). In addition, BCR-ABL fusion kinase and the Janus tyrosine kinase 2 (JAK2) were demonstrated to inhibit Bcl-xL deamidation and subsequently apoptosis in primary cells from patients with chronic myeloid leukemia upon DNA damage (Zhao et al., 2008).

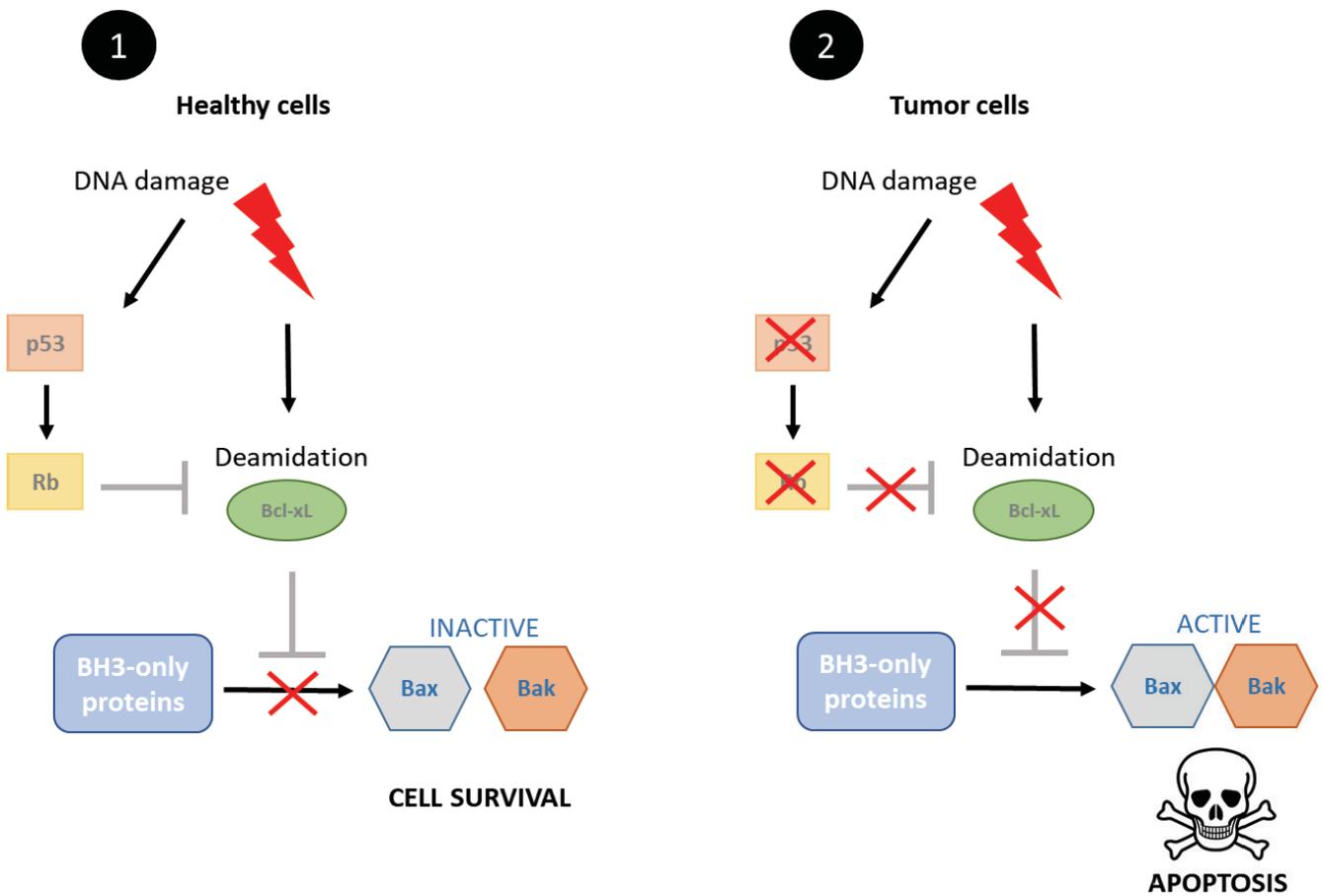


Figure 17: *Bcl-xL deamidation induced apoptosis upon DNA damage.*

In normal cells, p53 is activated upon DNA damage. The latter also induces the deamidation of Bcl-xL thus abrogating its activity to interact with BH3-only proteins. However, p53 activates Rb that, in turn, inhibits Bcl-xL

deamidation resulting in cell survival **(1)**. In almost all tumors, p53 and subsequently Rb pathways are inactive. This will abrogate the inhibition of deamidation of Bcl-xL, that will result in the activation and interaction of BH3-only proteins with Bax and Bak leading to apoptosis **(2)**.

In fact, Bcl-xL protein level is constantly controlled by deamidation process that activate a PEST sequence necessary for the targeting of this member for degradation. This degradation is mostly modulated by a pathway involving the proteolytic enzyme Calpain. Moreover, this PTM has been a conserved mechanism from the basal metazoan to us, humans. Thus, it indeed plays a crucial role in the regulation of Bcl-xL (Dho et al., 2013). Bcl-xL deamidation could also happen in the absence of DNA damage. In this case, monodeamination of Asn52 occurs in normal and transformed cells (Beaumatinet et al., 2016). Recently, several ion transporters were shown to increase the pH needed to enhance the rate of Bcl-xL deamidation upon DNA damage in specific cells. Also, Bcl-xL possesses highly conserved histidine residues close to its two deamidation sites. It was shown that the deamidation of Bcl-xL involves these residues while intramolecularly catalyzed. Plus, DNA damage stimuli enhances this catalytic activity in cancer cells (Dho et al., 2018). The different signaling pathways regulating the deamidation of Bcl-xL as well as the therapeutic applications are detailed in the review of Beaumatinet and colleagues (Beaumatinet et al., 2017).

In this first section, I have summarized an important body of the current knowledge about the Bcl-2 family of proteins including their structure, main function and regulations. As they tend to involve several cellular pathways, I will further elaborate their implications in Ca²⁺ homeostasis, the Unfolded Protein Response and cancer progression in the following parts. With a specific focus on Bcl-xL, I will introduce the research project at the end of the introduction.

2. Calcium: *key messenger... under the control of the Bcl-2 members*

2.1. *Few words about cellular calcium*

Ca^{2+} is a second messenger indispensable for the correct functioning of the cells. It impacts nearly every aspect of cellular life. The basal concentration of cytosolic free Ca^{2+} is between 50 to 100nM which turns out to be approximately 10^4 times lower than in the extracellular space that is around 1–2mM (Bronner, 2001; Clapham, 2007). Any sudden change in intracellular Ca^{2+} concentration can lead to severe consequences including cell death (Brini et al., 2013). Indeed, a huge increase in cytosolic Ca^{2+} , causing an abnormal increase in mitochondrial Ca^{2+} levels, will result in the opening of the mPTP. This opening will then cause inner mitochondrial membrane depolarization and thus, mitochondrial swelling. This could lead to necrosis, or even apoptosis by the release of IMS proteins such as cytochrome c activating the caspases (Takeuchi et al., 2015). Furthermore, mitochondria are the main source of ROS. Any excess Ca^{2+} -dependent NADPH oxidases stimulation will result in oxidative stress due to major ROS production. The latter has severe consequences mainly in the nervous system that is particularly sensitive to oxidative stress such as neuronal cell death after ischemia or Alzheimer onset (Zündorf and Reiser, 2011). In addition, mutations in Ca^{2+} channels can also be detrimental: Dysregulated Ca^{2+} ATPase called Sarco/endoplasmic reticulum calcium transport ATPase (SERCA) expression is found in several types of cancers (colon, breast and lung) which can serve as a marker of differentiation and a potential therapeutic target as well (Dang and Rao, 2016). These are few proofs of the importance of the regulation of Ca^{2+} homeostasis.

2.2. Calcium transporters: *the ones that matter!*

Ca^{2+} enters the cells through Ca^{2+} channels of the plasma membrane: Voltage gated calcium channel (VDCC), Ca^{2+} release-activated Ca^{2+} Channel (CRAC) also called store-operated calcium channel. A network of mitochondria, in proximity to the plasma membrane, is found responsible for the uptake of Ca^{2+} by the cells. Located 5–30 nm near the mitochondria, the endoplasmic/sarcoplasmic reticulum (ER/SR) is also a major cellular Ca^{2+} store (Takeuchi et al., 2015). Accordingly, a way of communication between ER/SR and mitochondria presumably exists promoting the Ca^{2+} crosstalk between them: This is done through Mitochondria-Associated Membranes (MAM) consisting in part of the ER rich in lipid biosynthetic enzyme activities that will reversibly be attached to mitochondria (Vance, 2014a). In this region, IP_3R on the ER and VDAC on the MOM are present prompting Ca^{2+} efflux from the ER and its uptake by mitochondria, respectively. Upon Ca^{2+} stores depletion, the Stim1/Orai1 complex is activated. STIM1, an EF hand protein within the ER membrane, senses this Ca^{2+} depletion and migrates to around 25nm of the plasma membrane where it multimerizes and activates the CRAC channel of which the channel forming subunit is a four-transmembrane domain plasma membrane protein called Orai1 replenishing the ER with Ca^{2+} . This process is called the store operated calcium entry or SOCE (Clapham, 2007). Another ER/SR receptor called Ryanodine Receptor (RyR) releases Ca^{2+} from the ER lumen to nearby mitochondria. Moreover, mitochondrial calcium uniporter (MCU) is found on the inner mitochondrial membrane and is responsible for the transport of Ca^{2+} to the mitochondrial matrix. MCU has been shown to be regulated by many proteins such as MICU1, MICU2 and EMRE. On the other hand, reverse Ca^{2+} movement from the mitochondria to ER/SR also takes place, preventing the depletion of ER Ca^{2+} and controlling cytosolic Ca^{2+} increase. This is mainly done by a Na^+ - Ca^{2+} exchanger called NCLX and another H^+ - Ca^{2+} exchanger whose molecular identity is still controversial, but the best candidate being Leucine-zipper-EF hand-containing transmembrane region (Letm1). NCLX is voltage dependent and electrogenic with a ($>3\text{Na}^+$ in) to (1Ca^{2+} out) stoichiometry. Once Ca^{2+} is in the cytosol, it is taken up by the ER/SR *via* SERCA replenishing the ER with Ca^{2+} . The mitochondria and ER networks constitute a spider web that constantly sequesters Ca^{2+} . This vast system ensures that Ca^{2+} stays only briefly free before being extruded or taken up by, for instance, NCLX or SERCA, respectively.

Accordingly, Ca^{2+} —inside or outside of the mitochondria— plays crucial roles in the regulation of many vital processes as mentioned in (part 1.7.) and the molecules responsible for Ca^{2+} flux in the cell i.e. all the calcium transporters, are implicated in these processes (Jiang et al., 2013; Periasamy and Kalyanasundaram, 2007; Takeuchi et al., 2015).

2.2.1. IP3 receptors

One of the most studied Ca^{2+} channels are the IP3Rs found on the ER. Accordingly, we will briefly introduce their structure, activation mechanism and mediated Ca^{2+} release.

2.2.1.1. Structure of IP3Rs

It was back in 1986, that the first known receptor of Inositol 1,4,5-triphosphate (IP3) was discovered. IP3 acts as a second messenger for many neurotransmitters, hormones and growth factors and signals the release of Ca^{2+} from organelle stores. It was first shown to induce the release of sequestered Ca^{2+} from microsomes. Based on the functioning of Inositol phosphates Ca^{2+} – releasing ability, IP3 binds to a certain receptor on the ER that was simply called Inositol triphosphate receptor IP3R or P400 as Mikoshiba's team named it (Furuichi et al., 1989; Spät et al., 1986). This receptor belongs to a family of receptors that include three IP3Rs called IP3R1, IP3R2 and IP3R3. The last two share 69% and 62% homology with IP3R1, respectively (Blondel et al., 1993; Südhof et al., 1991). Even though all IP₃R isoforms display sequence homology represented by sequence identity of around 70%, specifically within the TM regions where they share around 90% sequence identity, they exhibit significant differences in the regulatory cytoplasmic domains. These channels are ubiquitously expressed on the ER of virtually all eukaryotic cells. The entire IP3R is of a large size comprising four subunits encompassing 2700aa each. Each subtype of the receptor displays specific properties concerning its regulation by endo and exogenous ligands as well as cell type distribution. The main difference in the three subtypes falls in the cytoplasmic region *via* alternative gene splicing. Also, this diversity is provided by the homo and hetero-tetrameric assembly of the subunits into functional channels (Fedorenko et al., 2014; Foskett et al., 2007).

Due to the fact that IP3Rs constitute large molecules, the complete structure was so difficult to reveal. It was not until 2015 that the complete 3D homotetramer structure of IP3R1 was unveiled by cryo-Electron Microscopy yielding a resolution of 4.7Å (**Figure 18**) (Fan et al., 2015).

The IP3R is composed of several domains. At the N-terminus, IP3 binding occurs between aa 225–578. This fragment constitutes the IP3 Binding Domain (BD) (Yoshikawa et al., 1996). The IP3 negatively charged phosphate groups bind the BD *via* hydrogen bonds within the BD (Wilcox et al., 1997). The Suppressor Domain (SD) is the domain present at the very beginning of the N-terminus. It encompasses the aa region 1 to 225. The SD decreases the IP3 affinity to IP3R. In fact, the IP3 binding ability of IP3R is strictly affected by the attenuation of the binding affinity of the BD by the SD. IP3R3 BD–SD chimeric constructs displayed an affinity constant (K_d) of 49nM, 14 nM and 163nM for IP3R1, IP3R2 and IP3R3, respectively. On the other hand, IP3R BD alone had a K_d of only 2nM (Iwai et al., 2007). Furthermore, the SD plays a crucial role in IP3R Ca^{2+} release since any loss of this region results in IP3R1 incapable of releasing Ca^{2+} but able of binding IP3 molecules (Uchida et al., 2003). The third domain, called the IP3R Modulatory and Transducing Domain (MTD), is the central domain and constitutes the largest fragment of this channel from aa579 to 2275. Also, a deletion of the 651–1130aa fragment of the MTD impairs the Ca^{2+} release function of the IP3R (Uchida et al., 2003). This domain binds numerous regulatory proteins involved in the control of IP3R Ca^{2+} release. A small intervening lateral domain (ILD) links the MTD with the channel forming domain (CFD). From aa 2276 to 2589, the CFD is crucial for the Ca^{2+} ions transfer through the ER membrane. It contains six putative α -helical TM domains (TM1–6) with TM 5 and 6 mostly modulating tetramer assembly and TM6 forming the Ca^{2+} channel domain as displayed later by 3D structure (Fan et al., 2015; Galvan et al., 1999). The overexpression of this domain enhances Ca^{2+} leakage and ER Ca^{2+} depletion (Nakayama et al., 2004). The very last domain is the Coupling Domain (CD) present at the C-terminus and encompasses aa 2590 to 2749. This domain is located at the cytosolic side and is composed of a helical linker (LNK) region along with a C-Terminal Domain (CTD) (Fan et al., 2015). Both N and C-termini of IP3R are intracellularly composing a huge cytosolic part of the channel as predicted by membrane topology studies. The fact that the CD is close to the N-terminus BD makes it involved in the transfer of conformational energy mediated by the IP3 binding to IP3R (**Figure 18**) (Yamazaki et al., 2010).

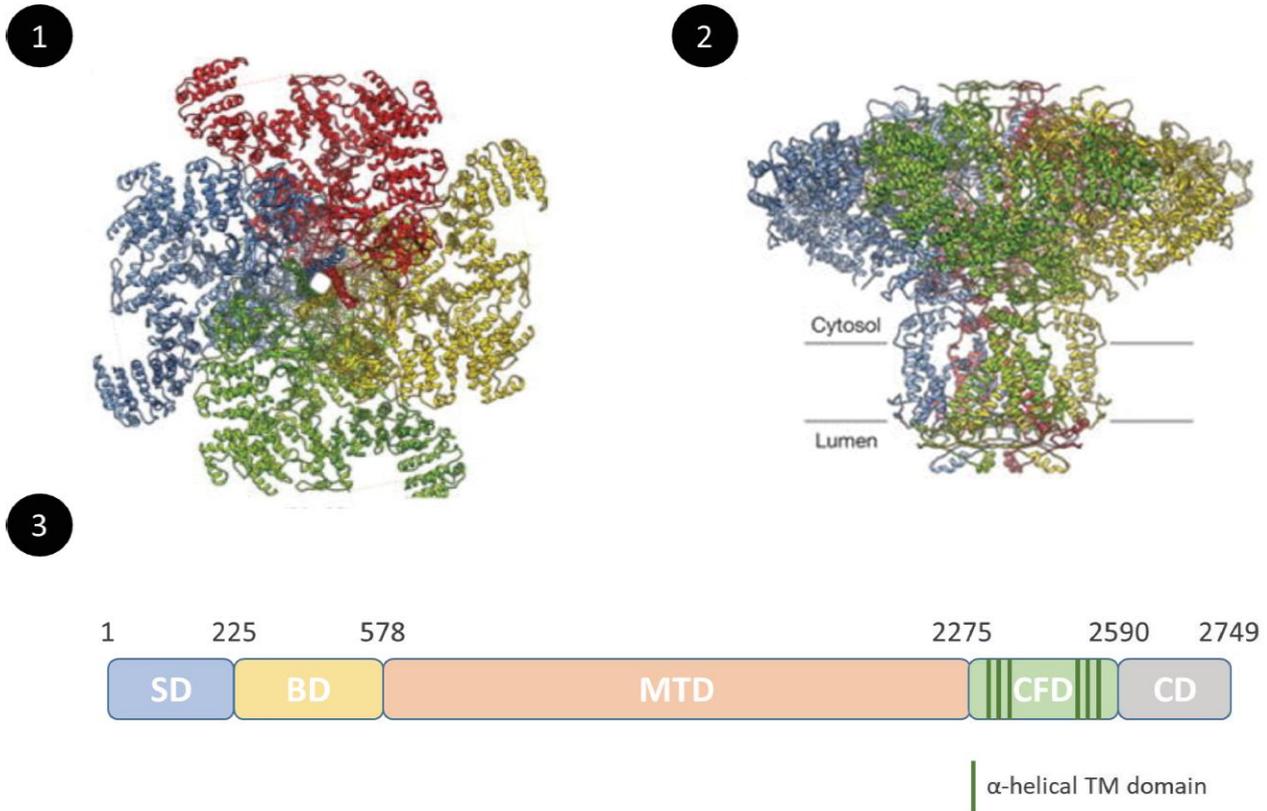


Figure 18: 3D and primary structure of IP3R1.

Top view **(1)** and side view **(2)** of the 3D structure of IP3R1 tetramer as displayed by cryo-EM. Each color represents one monomer of the IP3R tetramer. The Ca²⁺ channel is present in the very center. The side view puts in evidence the huge cytosolic domain compared to the ER TM one. Adapted from (Fan et al., 2015). In **(3)**, a schematic presentation of mouse IP3R1 primary structure displaying the discussed domains. The numbers represent the amino acids position in the protein.

2.2.1.2. IP3R channel opening and activation

At first, IP3 formation is stimulated by external stimuli including hormones and growth factors as already mentioned. These stimuli actually activate the G-protein coupled receptors (GPCRs) or the protein tyrosine kinase-linked receptors (PTKRs). The latter are coupled to different PLC isoforms. The GPCRs are coupled to PLC β isoforms, whereas the RTKs are linked to the PLC α isoforms. Once active, PLC hydrolyzes the precursor lipid phosphatidylinositol 4,5-bisphosphate (PIP2) in order to generate both plasma membrane anchored – diacylglycerol (DAG) and freely-diffused IP3 (Kadamur and Ross, 2013). IP3 main function is to bind to the IP3R in order to activate the release of Ca²⁺ from the ER (Foskett et al., 2007). Activation of the channel starts by the binding of IP3 to IP3R BD. In fact, before the opening, IP3 needs to bind to each of the four subunits. Whenever it bound the 4 monomers, IP3 induces a conformational change sensitizing the Ca²⁺-binding site. It is then that Ca²⁺ binds to this exact site opening the channel and promoting its release into the cytosol (**Figure 19**) (Alzayady et al., 2016).

Several regulators of IP3R bind to the MTD to control the release of Ca²⁺. The concentration of Ca²⁺ directly affects the IP3-IP3R Ca²⁺ pathway. It acts in two modes where it either stimulates Ca²⁺ release at low levels or inhibit when it surpasses 300nM. Indeed, this double control of the IP3R by both IP3 and Ca²⁺ is fundamental for cell signaling different functions.

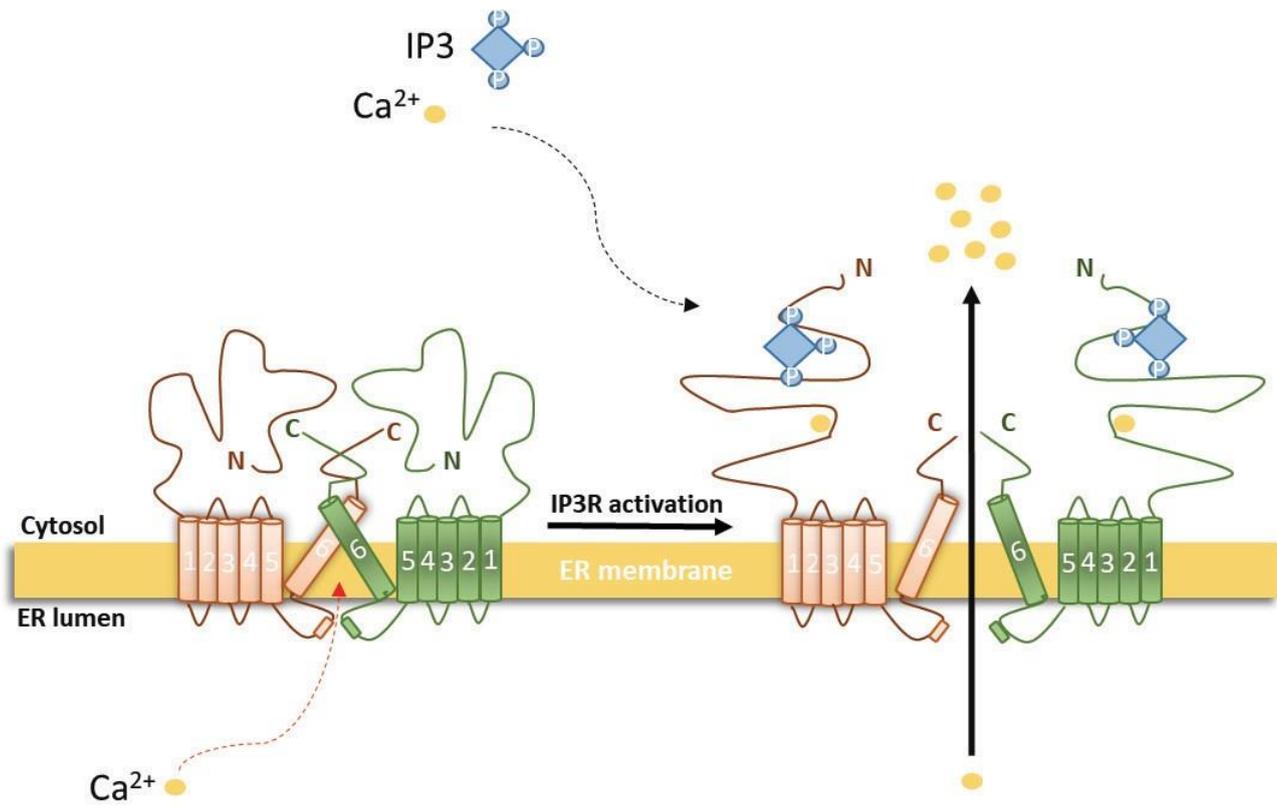


Figure 19: *IP3R activation and calcium release.*

The IP3R is composed of four monomers, two of which are displayed in this schematic representation. Six TM domains are present in the channel forming domain (CFD) along with a pore loop between TM5 and TM6. Both N and C-termini are in the cytosol. IP3 and Ca^{2+} must bind near the N-terminal region in order to induce the IP3R conformational change required for the propagation of Ca^{2+} signals down to the TM opening thus the pore. Ca^{2+} is released from the ER lumen to the cytosol where it acts as a key messenger for numerous cellular functions.

2.2.1.3. IP3R-mediated Ca²⁺ release

One feature of the IP3R signaling pathway is the release of Ca²⁺ as brief signals/transients repeated over time called Ca²⁺ oscillations (Berridge, 1990). These oscillations have been discussed in several cell types including hepatocytes (Rooney et al., 1989), pancreatic acinar cells (Futatsugi et al., 2005), oocytes (Cheek et al., 1993) and endothelial cells (Moccia et al., 2003). Another characteristic of Ca²⁺ oscillations is their stimuli intensity–dependent frequency (Berridge and Galione, 1988). This frequency coding controls many cellular functions such as liver metabolism and differential gene expression. The latter is more efficiently initiated by Ca²⁺ spikes rather than steady level Ca²⁺ concentration (Li et al., 1998b).

How are these Ca²⁺ oscillations generated?

This is still a matter of debate. However, they might be driven by complex feedback interactions between IP3 and Ca²⁺ (Gaspers et al., 2014; Politi et al., 2006). In general, Ca²⁺ spikes induce a modest increase in cytosolic Ca²⁺ levels that could reach 600nM along with a limited spatial spread of less than 6µm in a transient opening of the IP3R (less than 1s) (Niggli, 1999; Thomas et al., 2000). Furthermore, these oscillations can propagate from one site to its neighbor where they activate it. This type of propagation is called saltatoric spreading the Ca²⁺ signals intracellularly (Berridge et al., 2000). Few years later, Foskett and colleagues put in evidence a mechanism by which Ca²⁺ oscillations propagate leading to a coordinated opening of neighboring channels that could belong to the IP3R family or another ER Ca²⁺ – sensitive channel, the RyR. It was called Ca²⁺ – induced Ca²⁺ – release or CICR. Accordingly, Ca²⁺ signals are spread all over the cell (Foskett et al., 2007). The MAMs facilitate the propagation of Ca²⁺ spike in the intracellular space (Vance, 2014b).

2.3. Bcl-2 proteins *in action*

Bcl-2 proteins and Ca²⁺ signaling are in a complicated, even complex, relationship. It has been more than 20 years that the link between the two was established. Indeed, Bcl-2 members affect intracellular Ca²⁺ fluxes and dynamics. Despite their major role at the mitochondria, numerous members of this family are found at the ER where they execute non-canonical roles involving intracellular Ca²⁺ regulation. Some of these functions are discussed in (part 1.7.) and are much more detailed in the review of our team (Bonneau et al., 2013). Here, we highlight the regulation of Ca²⁺ signaling by the Bcl-2 gang whether at the ER, or at the mitochondria.

2.3.1. *At the ER*

Some of the Bcl-2 clan members reside at the ER membrane where they interact with Ca²⁺ transporters mainly IP3R and RyR. Many of the interactions and their consequences remain a subject for an open debate since a lot of controversial works had highlighted opposite outcomes for the same interaction.

2.3.1.1. Control over IP3R gates

It was back in 1993 that the very first implication of Bcl-2 in Ca²⁺ signaling was demonstrated. In fact, upon IL-3 depletion, IL-3-dependent hematopoietic cells undergo apoptosis due to a Ca²⁺ transport from the ER to the mitochondria. By overexpressing Bcl-2, Ca²⁺ transfer along with subsequent apoptosis were blocked. It was shown that Bcl-2 negatively affects Ca²⁺ release from the ER (Baffy et al., 1993; Lam et al., 1994). Years later, a concrete interaction of Bcl-2 and IP3R was highlighted. Bcl-2 interacts with a stretch of 20 aa (1389–1408) corresponding to a region in the MTD of the IP3R1 (Rong et al., 2008). Actually, the BH4 domain of Bcl-2 is the one involved in the binding to IP3R MTD. Upon this interaction, Bcl-2 inhibits IP3R channel opening, ER Ca²⁺ release and subsequent apoptosis initiation. Interestingly, Bcl-2 BH4 loss or inhibition transforms the protein into a pro-apoptotic member (Rong et al., 2009).

Furthermore, Bcl-xL is another pro-survival protein that interacts with IP3R. The consequences of this interaction are a bit more complicated than those of Bcl-2. In fact, Bcl-xL binds to the C-terminus CD of IP3R in quiescent cells sensitizing the channel to low concentration of IP3, prompting Ca²⁺ oscillations,

increasing thus ER Ca²⁺ release and affecting mitochondrial energetics (White et al., 2005). Also, a redundancy was shown between IP3R isoforms in their capacity to sensitize cells to apoptotic stimuli by interacting with Bcl-xL responsible for regulating their activities what results in an anti-apoptotic response (Li et al., 2007). In contrast to Bcl-2, Bcl-xL BH4 domain is not responsible for this interaction. Even though only one aa residue (Lys17 in Bcl-2 instead of Asp11 in Bcl-xL) is different between Bcl-2 and Bcl-xL BH4 domains, these two do not affect IP3R-mediated Ca²⁺ release in the same manner. The BH4 domain of Bcl-xL was not able to bind the MTD of IP3R nor inhibit IP3-induced Ca²⁺ release (IICR) in both intact and permeabilized cells. Mutations reversing both aa residues in the BH4 domain of both proteins only abrogated the IP3R inhibition function of Bcl-2 (Monaco et al., 2012). Actually, the team of Foskett presented for the first time the major player in this Bcl-xL interaction with IP3R: the hydrophobic cleft. In fact, Bcl-xL induces a biphasic regulation of IP3R. This means that Bcl-xL might activate or inhibit channel gating depending on the binding of Bcl-xL to two BH3 resembling domain helices in the IP3R C-terminus or to only one of them present in the CD, respectively. Moreover, any disruption of these interactions severely affects cell survival by sensitizing them to apoptosis (Yang et al., 2016). In general, this team has shed light on BH3-like domains in IP3R that seem to attract Mcl-1. The latter binds to the C-terminus of all three mammalian IP3R isoforms where it sensibilizes IP3R to low concentrations of IP3. Bcl-2 was shown to share this effect but to a lesser extent (Eckenrode et al., 2010). A specific form of the oncogene K-Ras, K-Ras4b, when phosphorylated, associates with the C-terminus of IP3R in a Bcl-xL-dependent way and blocks pro-survival Ca²⁺ oscillations mediated by Bcl-xL (Sung et al., 2013). Furthermore, following prolonged ER stress induction, Bcl-xL translocates to the MAMs where it interacts with IP3R3 in order to maintain mitochondrial Ca²⁺ uptake and enhances the mitochondrial and cellular bioenergetics in periods of stress (Williams et al., 2016).

Another anti-apoptotic Bcl-2 member involved in the regulation of IP3R is Bcl2L10/Nrh. Both Nrh and its zebrafish ortholog Nrz bind IP3R1 with their BH4 domains. However, these two are an exception for other pro-survival proteins in the way that they bind the BD of IP3R and not the MTD nor its C-terminus. In fact, by binding to the IP3R BD, Nrh negatively affects ER Ca²⁺ release thus enhancing its pro-survival activity (**Figure 20**) (Nougarede et al., 2018). Also, Nrh activity is affected by an IP3R BD molecule called IRBIT. Both proteins inhibit the activation of this channel. However, upon apoptotic stress, IRBIT is dephosphorylated what makes it an inhibitor for Nrh. Furthermore, it promotes ER-mitochondria contact

through MAMs in order to facilitate ER-Ca²⁺ release required for mitochondrial apoptosis (Bonneau et al., 2016). Our team has elaborated the binding of Nrz to the BD of IP3R and showed that Nrz competitively binds to the ligand BD of IP3R1 inhibiting thus IP3 binding and subsequent channel activation (Bonneau et al., 2014).

As for the pro-apoptotic members, apart from Bok, no concrete interaction with IP3R was noted. In fact, Bax/Bak double KO mouse embryonic fibroblasts (MEF) displayed a decrease in ER Ca²⁺ concentration leading to a reduced flux of Ca²⁺ from the ER to the mitochondria upon ER stress inducers. However, expression of recombinant Bax in these cells restored ER Ca²⁺ levels in contrast to the expression of mitochondria-targeted Bax that did not affect Ca²⁺ signaling. Hence, the expression of only ER-targeted Bax/Bak might enhance the ER Ca²⁺ fluxes (Oakes et al., 2005; Scorrano et al., 2003).

Bok on the other hand, physically interacts with IP3R1 and IP3R2 and to a lesser extent IP3R3. Its BH4 domain actually binds the region encompassing amino acids 1895–1903 of IP3R1 CD near the proteolysis regulatory sites. Interestingly, Bok does not affect the Ca²⁺ release function of the channel but regulates its expression and protects it from proteolysis (Schulman et al., 2013). In fact, unbound Bok is ubiquitinated and proteasome-degraded but IP3R-bound one is stabilized. Apparently, unbound Bok is considered deleterious to cell survival and its fate literally depends on its interaction with IP3R (Schulman et al., 2016).

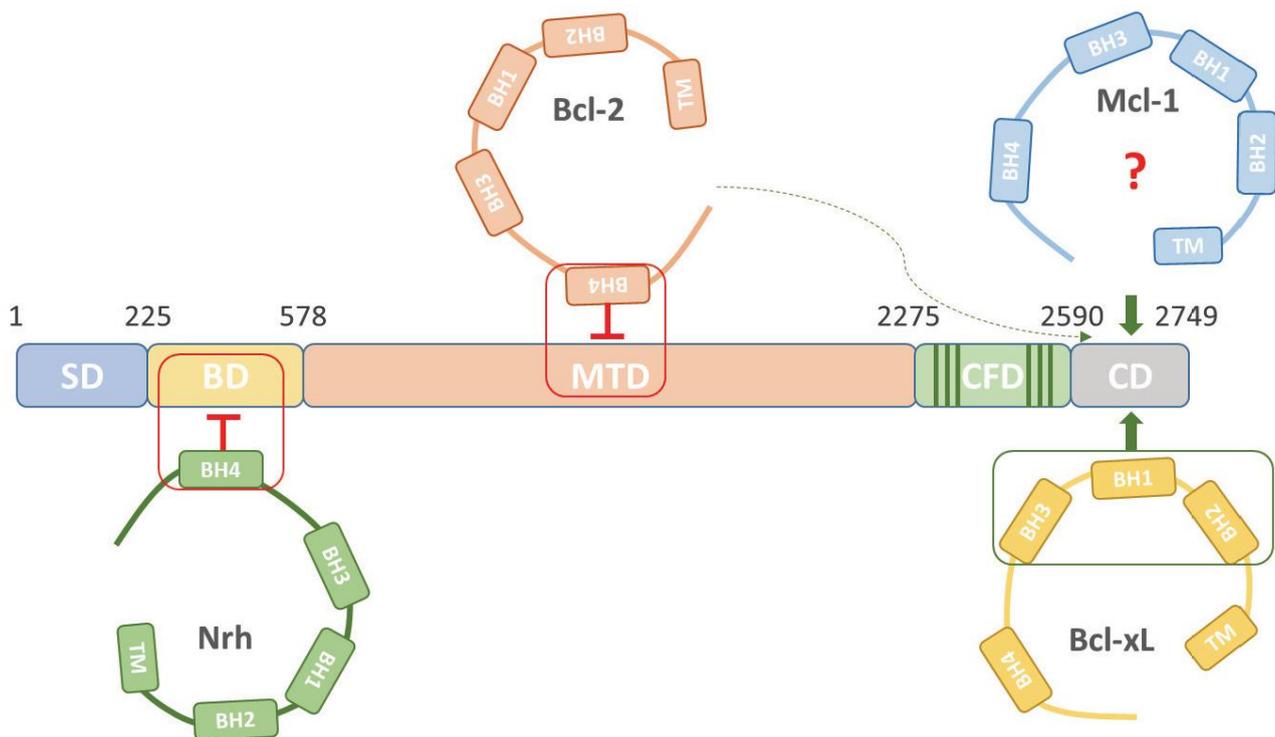


Figure 20: Anti-apoptotic proteins interactions with IP3R domains.

Via their BH4, Nrh and Bcl-2 negatively interact with the BD and MTD of IP3R, respectively. As for Mcl-1 and Bcl-xL, they positively interact with the CD. In fact, through its hydrophobic cleft, Bcl-xL and Mcl-1 (via an unknown domain) sensitize the channel to low concentrations of IP3. Bcl-2 might also share this effect to a lesser extent.

2.3.1.2. Shutting RyR gates

Along their regulation of IP3Rs, Bcl-2 proteins regulate the Ryanodine Receptors. This family of receptors is composed of three isoforms all responsible for the release of Ca^{2+} from the (SR/ER). They actually form homotetramers with a large cytosolic region along with a TM domain. Ca^{2+} and other proteins such as Bcl-2 and Bcl-xL trigger the opening of these ion channels in which mutations might result in severe disorders or cardiac arrhythmias (Van Petegem, 2012). These receptors are considered a major player in the CICR since Ca^{2+} ions promote their opening to a longer extent than the IP3Rs (Hymel et al., 1988).

Bcl-2 also binds RyR. In fact, a conserved sequence in all RyRs has a strong similarity with Bcl-2 IP3R binding site. Similarly, the BH4 domain of Bcl-2, and not its hydrophobic cleft, abrogates RyR – Ca²⁺ release since pharmacological inhibition of the BH3 binding pocket had no impact on ER – Ca²⁺ fluxes. As for Bcl-xL, it also interacts with RyR, specifically RyR3 but the site of interaction is still unknown. The consequence of the binding of the BH4 domain of Bcl-xL to the RyR3 abrogated ER – Ca²⁺ release. Point mutations in this domain replacing Lys87 resulted in the inability of Bcl-xL to regulate the RyR3 (Vervliet et al., 2015). Recent data show for the first time a therapeutic significance of the BH4 domain function of both Bcl-2 and Bcl-xL that inhibit pathological RyR-triggered Ca²⁺ release and associated necrosis in the case of biliary acute pancreatitis (Vervliet et al., 2018).

2.3.2. At the mitochondria

Mitochondria are the organelles that buffer cytosolic Ca²⁺ what will prompt mitochondrial integrity and ATP production. Accordingly, Ca²⁺ is a key activator of the TCA cycle. However, the excess of it might result in tremendous consequences such as mPTP opening, mitochondria bursting and cytochrome c release. On the MOM, mitochondria take up Ca²⁺ through the already mentioned VDAC. Once in the IMS Ca²⁺ passes through the MIM-resident MCU into the matrix. Most of the Bcl-2 family regulation occurs on the MOM *via* its control over VDAC.

The VDAC family consists of three isoforms that are integrated in the MOM. Bcl-xL was demonstrated to interact with VDAC1 even though this interaction is still controversial. Through its BH4 domain, Bcl-xL binds VDAC and closes it further inhibiting the mitochondrial Ca²⁺ uptake when purified VDAC is inserted in the liposomes. In fact, the authors suggested that Bax and Bak enhance the release of cytochrome c from VDAC and that Bcl-xL, by binding to this channel, prevents this passage (Shimizu et al., 1999, 2001). This was rapidly contested as another study had shown that Bcl-xL indeed allows metabolite exchange across the MOM but without inducing the release of cytochrome c from the IMS. Moreover, Bcl-xL maintains VDAC in an open conformation facilitating this metabolite exchange upon apoptotic stimulation (Vander Heiden et al., 2001). This was supported by other studies where VDAC1 and 3 KO in cells could not protect from cytochrome c release and its consequences (Baines et al., 2007; Cheng et al., 2003). In *Bcl-x* KO MEFs, mitochondria take up less Ca²⁺ after being triggered by ATP or Ca²⁺ itself.

However, whenever they were complemented by the mitochondrial form of Bcl-xL, they re-gain their mitochondrial Ca²⁺ uptake function *via* VDAC restoring thus apoptosis resistance (Eno et al., 2012). Also, the same team has already shown that Bcl-xL is vital for Ca²⁺ uptake via its interaction with VDAC1 and 3 (Huang et al., 2013). In contrast, the BH4 domain of Bcl-xL (and not Bcl-2) binds to VDAC1 and inhibits mitochondrial Ca²⁺ uptake thus abrogating apoptosis (Monaco et al., 2015). Accordingly, Bcl-xL interaction with VDAC is still a matter of debate. Yet, this contradiction could be explained by the fact that Bcl-xL allows the normal functioning of the mitochondria via mitochondrial Ca²⁺ uptake but inhibits it when the excess of Ca²⁺ signals could possibly lead to apoptosis. As for the binding site on VDAC, it was shown that the N-terminal region along with two internal sequences bind in a specific manner to Bcl-xL (Arbel et al., 2012). In another study, Bcl-xL was shown to interact also with VDAC2 in a complex involved in the inhibition of autophagy (Yuan et al., 2015).

Bcl-2 also binds VDAC1 since its pro-survival activity was affected by VDAC1-N-terminal peptides (Arbel and Shoshan-Barmatz, 2010). Since the BH4 of Bcl-2 is not the one responsible for this action (Monaco et al., 2015), further studies are needed to elaborate the interaction of Bcl-2 and VDAC.

As for Mcl-1, it interacts with VDAC1 and 3 but very weakly with VDAC2. This binding results in the metabolites influx into the mitochondria and the formation of ROS promoting thus lung cancer cell migration. Plus, VDAC1 appears to have a higher affinity to Mcl-1 binding than to Bcl-xL (**Figure 21**) (Huang et al., 2014).

Some pro-apoptotic members also interact with VDAC. Bax, for instance, increases the VDAC Ca²⁺ pore only in the presence of t-Bid (Banerjee and Ghosh, 2004). In contrast, Bid and not Bax induces the closure of VDAC1 decreasing metabolite exchange between mitochondria and the cytosol and resulting in mitochondrial dysfunction (Rostovtseva et al., 2004). As for Bak, it interacts with VDAC2 at a conserved domain in its C-terminus. This interaction seems indispensable for mitochondrial Bak-recruitment and subsequent t-Bid apoptosis initiation. Bim has the capacity to activate VDAC by directly interacting with it thus, inducing apoptotic changes in yeast mitochondria along with lysis of erythrocytes (Sugiyama et al., 2002). Further studies are needed to elaborate the exact function of each Bcl-2 proteins, especially the pro-apoptotic ones, in the regulation of VDAC-Ca²⁺ signaling.

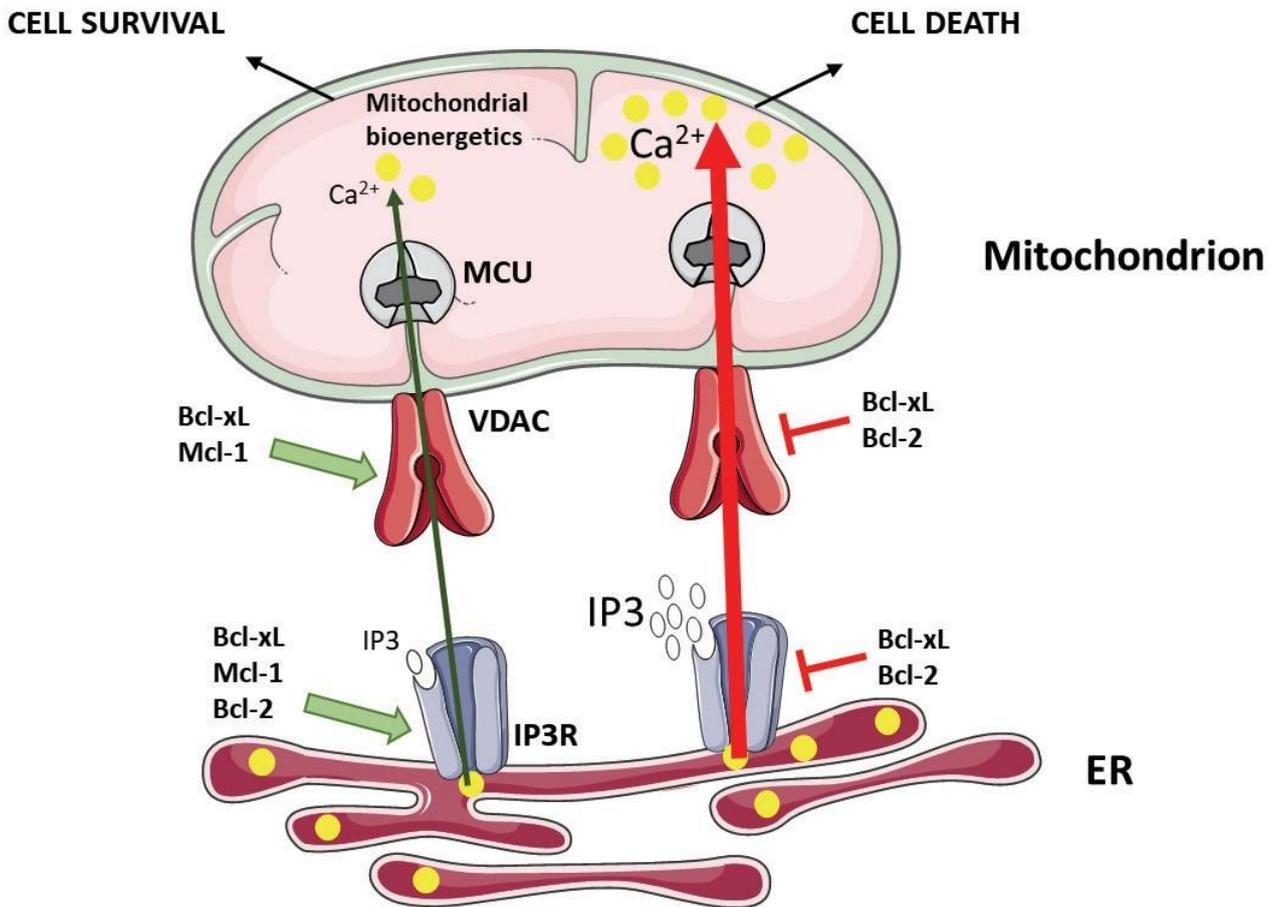


Figure 21: Modulation of ER to mitochondria Ca^{2+} transfer by some of the pro-survival Bcl-2 members.

Intracellular Ca^{2+} fluxes are crucial for the control of cell fate. A constant Ca^{2+} mitochondrial influx is a must for maintaining mitochondrial bioenergetics (green arrow). However, an excess Ca^{2+} uptake might provoke MOMP resulting in cell death (red arrow). Accordingly, pro-survival Bcl-2 proteins regulate these fluxes. Bcl-xL, Mcl-1 and to a lesser extent Bcl-2, sensitize the IP3R to low concentrations of IP3 allowing Ca^{2+} transfer to the mitochondria where Bcl-xL and Mcl-1 increase Ca^{2+} uptake required for the TCA cycle fueling and cell survival. During pro-death signaling where increased concentrations of IP3 might result in excessive Ca^{2+} uptake by the mitochondria prompting apoptosis, Bcl-xL and Mcl-1 might inhibit both IP3R and VDAC in order to limit Ca^{2+} abundance in the mitochondria.

3. The Endoplasmic Reticulum: *beyond folding and storing*

As shown until now, each organelle in the cell undergoes specific functions. The mitochondria and the ER are interconnected through the MAMs what allows Ca^{2+} transfer required for cellular pathways. The ER was visualized for the first time in fibroblast-like chicken cells by electron microscopy. It was then described as a 'delicate lace-work extending throughout the cytoplasm' due to the fact that it appears as a network of elongated tubules spreading in the cytosol surrounded by a membrane. The latter is responsible for the transfer of molecules to the cytosol (Palade and Porter, 1954; Porter et al., 1945). Here, we review the structure and principal functions of the ER and its regulation by the Bcl-2 family of proteins.

3.1. Structure of the ER

The ER can be either Rough (RER) if ribosomes are present on the membrane or Smooth (SER) if the ribosomes are absent. Both types can either be connected or totally separated organelles (Borgese et al., 2006). The general structure of the ER is a network of membranes called cisternae that are held together by the cytoskeleton. The phospholipid membrane engulfs the cisternal space also called lumen to separate it from the cytosol. The ER spreads in the cytosol interacting with several other compartments. As mentioned before, the ER can be arranged to form contact sites with the mitochondria called MAMs which play a major role in intracellular Ca^{2+} signaling (Hayashi et al., 2009). It might also be in contact with the plasma membrane (PM) through STIM1 and CRAC needed for SOCE. Several proteins stabilize the ER-PM contacts such as vesicle-trafficking protein (SEC22b) (Daste et al., 2015). This organelle might interact with the endosomes that are membrane-bound compartment originating from trans-Golgi. There, the ER contributes to cholesterol maintenance (Wilhelm et al., 2017). Furthermore, the ER was shown to interact with the endolysosomal system suggesting it might play a role in autophagy. Either alone or interconnected to other compartments, the ER performs its variable functions depending on the cell needs as discussed in the next parts.

3.2. Main functions of the ER

The ER is considered a protein synthesis factory where it folds and stores proteins. It also stores Ca^{2+} and regulates its intracellular fluxes and contributes in the synthesis/storage of lipids. Moreover, it plays a role in glucose metabolism. All of these roles validate the importance of this organelle responsible for the coordination of bioenergetics and metabolic pathways, hence, cell fate.

Protein folding and quality control

The ER plays a major role in protein synthesis, folding, maturation, quality control and degradation. Not only this, but it also ensures their delivery to the wanted site. The ER constitutes an environment filled with chaperones and foldases responsible for the folding, assembly and PTMs of nearly 30% of all proteins that are co-translationally ER-targeted (Braakman and Bulleid, 2011). Of the processing modifications, we mention signal sequence cleavage, N-linked glycosylation and proline isomerization resulting in conformational changes required for protein proper folding (Braakman and Bulleid, 2011). In the case of misfolded proteins, they might be detrimental to the cell. Accordingly, they need to be regulated. In fact, protein misfolding is a natural phenomenon that might be triggered by several factors discussed in the next part. However, the ER has developed ways to overcome protein misfolding also known as 'quality control' mechanisms. If the protein is terminally misfolded, in other terms, could never be correctly folded, the ER is able to get rid of it through an elimination process called ER associated degradation (ERAD) targeting it for ubiquitin-proteasomal degradation (Kamhi-Nesher et al., 2001; Meusser et al., 2005).

Lipid synthesis

The ER executes other functions involved in lipid and membrane formation and fat accumulation required for energy storage. In general, lipid synthesis takes place around the membrane and at the contact sites with other organelles where lipid vesicles could be exported regularly. Accordingly, this organelle changes its membrane structure according to the cellular lipid composition. It also ensures

cholesterol homeostasis *via* cholesterol sensors (Brown and Goldstein, 1999). Moreover, many enzymes involved in the synthesis of sterols, sphingolipids and phospholipids reside in the ER. The exact synthesis of the cited lipids occurs at the ER membrane (Fagone and Jackowski, 2009). Also, sphingolipid synthesis might occur in the MAMs (Patwardhan et al., 2016).

ER export

The ER, through the secretory pathway, also works as a delivery system for newly synthesized proteins and lipids. Any deficiency in the secretion might result in detrimental consequences in the ER. Of the initial vesicles crucial to the constant anabolic export are the ER coat protein II (COPII) complex consisting in forming the vesicle budding from the ER (Barlowe et al., 1994). In addition, other non-vesicular transport systems might occur such as lipid droplet accumulation of large lipoprotein cargo. Interestingly, the ER is shown to be involved in peroxisome biogenesis through a sorting process involving peroxisomal membrane proteins (PMPs) and pre-peroxisomal vesicular carriers (ppVs) that fuse afterwards to form peroxisomes (Dimitrov et al., 2013).

Ca²⁺ homeostasis

Intracellular Ca²⁺ fluxes regulate vital processes in the cell. As discussed in (part 2), Ca²⁺ is a key messenger that plays crucial roles in intracellular pathways involving gene expression, protein synthesis, cell proliferation, apoptosis and so on. The ER is the main hub for intracellular Ca²⁺ thereby controlling Ca²⁺ homeostasis. In order to regulate these fluxes, the ER employs several mechanisms. First, SERCA pump allows the active transport of Ca²⁺ from the cytosol to the ER lumen. Second, ER Ca²⁺ channels and transporters providing the export of Ca²⁺ into the cytosol. Finally, a number of ER- Ca²⁺ chaperones are ready to sequester free luminal Ca²⁺ such as calreticulin, Grp94, BiP and protein disulfide isomerase (Coe and Michalak, 2009). Based on the cell needs, the interconnection between the ER and the mitochondria or the ER and the PM facilitates the occurrence of all three mechanisms. Consequently, the ER is considered a key organelle supervising the whole cellular metabolism (**Figure 22**).

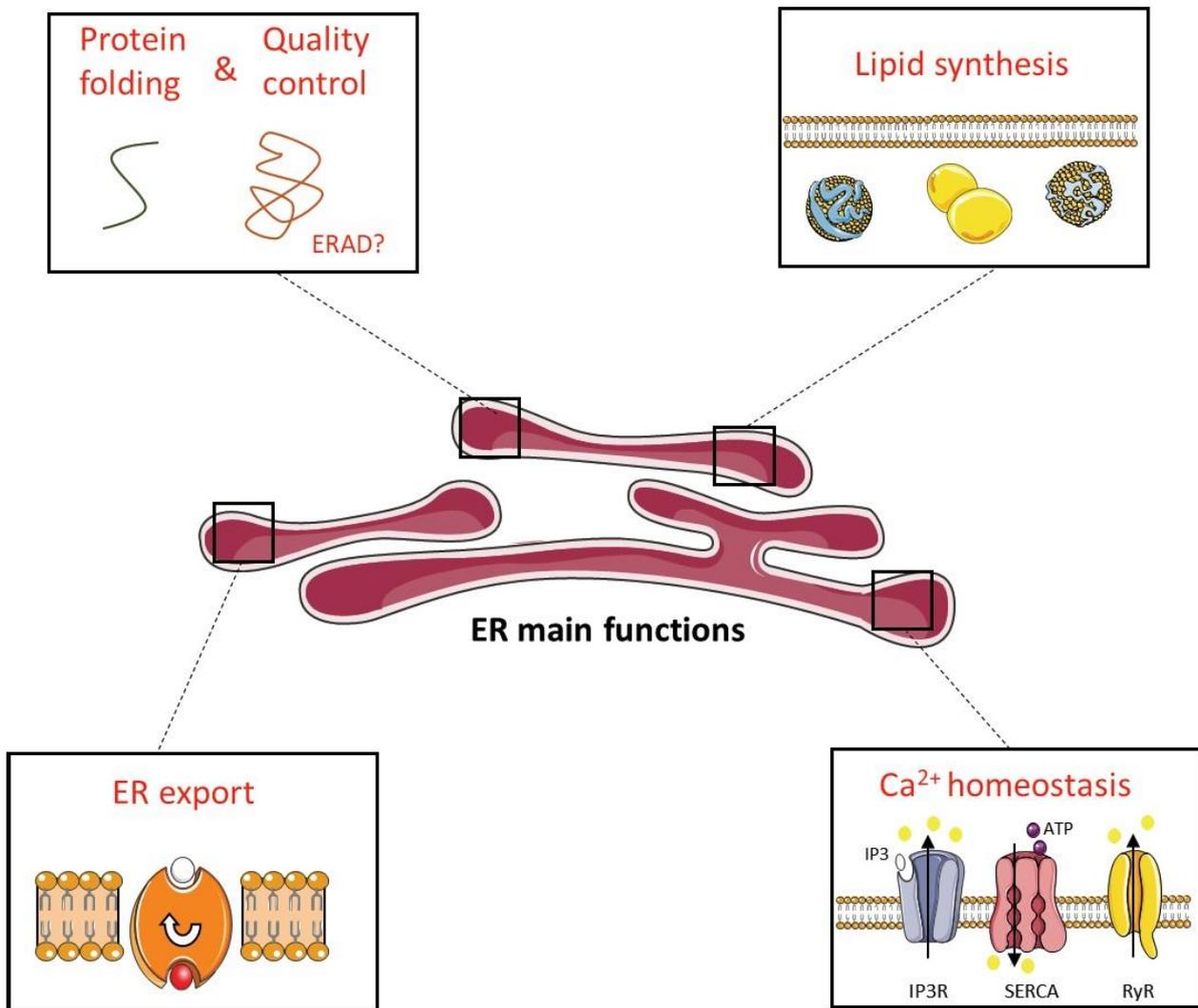


Figure 22: Main functions of the ER.

Apart from its famous role in protein synthesis, the ER performs several other ones. Of which, lipid synthesis, Ca²⁺ export through vesicle budding and quality control for terminally misfolded proteins. Perturbations at the level of the ER functions might result in tremendous consequences discussed in the next part.

3.3. ER stress

The circumstances that perturb ER homeostasis generate a cellular condition often called 'ER stress'. Many perturbations might activate a cellular response that involves several adaptive pathways required to overcome this stress and re-maintains ER homeostasis (Hetz, 2012).

3.3.1. Causes of ER stress

Intrinsic and extrinsic perturbations might occur resulting in the activation of response pathways that are dependent on the nature of the perturbing agent/condition and the intensity as well as duration of the stress. Here, we list the main factors contributing to ER stress.

3.3.1.1. Intrinsic perturbations

Many diseases involve cell autonomous mechanisms resulting in ER stress of which cancer and diabetes. Genetic instability and frequent mutations are considered to be hallmarks of cancer and might lead to a continuous activation of ER stress response pathways resulting in cell growth, proliferation and cancer progression (Hanahan and Weinberg, 2011). Accordingly, cancer cells depend on high protein production to grow what affects ER functions. In cancer, the enormous load of mutations might lead itself to constitutive ER stress. For instance, the extremely elevated numbers of mutated proteins in melanoma that have the highest mutation amount are a prime source of intrinsic ER stress. In this case, ER stress might result in senescence that plays a major role in chemoresistance (Pluquet et al., 2015). Furthermore, ER stress could also be linked to diabetes. In fact, pancreatic β cells possess a highly developed ER in order to control insulin production and export in response to higher blood glucose levels. In the diabetic Akita mouse, the C96Y insulin variant results in the impairing of its own biogenesis and its accumulation in the ER causing serious ER stress. Accordingly, the ER could not handle the mutation-mediated stress causing the death of pancreatic β cells and the onset of type 1 diabetes (Harding and Ron, 2002; Oyadomari et al., 2002).

3.3.1.2. Extrinsic perturbations

Tumor microenvironment stress

Increased rate of proliferation in tumor cells empties their microenvironment of necessary nutrients and oxygen resulting in cellular stress that manifests as hypoxia, acidosis and starvation. All of the cited lead to ER stress possibly disturbing protein and lipid synthesis. Moreover, glucose starvation undermines glycosylation prompting ER stress (Giampietri et al., 2015).

Exposure to ER stress inducers

Molecules used to induce *in-vitro* ER stress have been identified. They include Tunicamycin that targets the N-linked glycosylation of the protein thus blocking protein folding and transit through the ER (Schultz and Oroszlan, 1979). In contrast, dithiothreitol abrogates the formation of protein disulfide bonds (Cleland, 1964). As for ER to Golgi apparatus trafficking, Brefeldin A causes the resorption of Golgi membrane to the ER and inhibits protein secretion (Liu et al., 1992). Other molecules target the active influx of ER Ca^{2+} by inhibiting SERCA such as Thapsigargin and cyclopiazonic acid. Hence, they decrease ER Ca^{2+} levels and debilitate the ER ability to properly fold proteins (Pilot et al., 2007; Thastrup et al., 1990).

On the other hand, other molecules are found to alleviate ER stress. 4-phenylbutyric acid (4-PBA) decreases the accumulation of misfolded proteins (Malo et al., 2013). Moreover, an endogenous bile acid capable of diminishing ER stress in islet cells called Tauroursodeoxycholic acid (TUDCA) is the taurine conjugate to a FDA-approved drug called UDCA for primary biliary cirrhosis (Lee et al., 2010). The mechanisms by which these molecules and proteostasis regulators work are still unknown.

Temperature

Another ER stress-causing perturbation involves changes in temperature. In fact, body temperature maintenance is vital for all metazoan species. Any change driving it far from 36–37°C, can lead to protein denaturation and accumulation (Lepock, 2005). Mildly increased temperatures might lead to ‘thermotolerance’ in mammalian cells that consists in the overexpression of numerous heat shock proteins and ER stress markers (Bettaieb and Averill–Bates, 2005; Liu et al., 2012). As for mild hypothermia, it was shown to cause ER stress in human pluripotent cells that are normally activated to protect against relentless stress in a process known as ER hormesis (Liu et al., 2013; Mollereau, 2015).

Excessive production of ROS

ROS are a major source of cellular stress. Accordingly, when their production trespasses the antioxidant ability of the cell, ER stress occurs (Zeeshan et al., 2016). During ER stress, functional mitochondrial respiration and biogenesis might be able to reduce ROS production in order to allow cell survival (Knupp et al., 2019) knowing that ROS are generated in the ER and mitochondria. In fact, the ER constitutes an oxidizing platform facilitating disulfide bond formation leading to around 25% of cellular ROS production (Malhotra and Kaufman, 2007). Several signaling pathways link the ER to ROS production that involve glutathione (GSH)/glutathione disulfide, Ca²⁺ and ER oxidoreductin 1 (ERO1) (Cao and Kaufman, 2014).

Whether directly or indirectly, all of the cited perturbations play a role in ER stress induction leading to diseases onset and resistance to known treatments.

3.3.2. Adaptation to ER stress: Unfolded Protein Response (UPR)

As a response to ER stress, cells initiate a cellular signaling pathway termed the unfolded protein response or UPR. The aim of the latter is to help these cells overcome ER stress by decreasing protein synthesis, getting rid of misfolded proteins and aid the ER in correctly folding others. This response is generally regulated by three dominant sensors: Inositol requiring enzyme 1 (IRE1), protein kinase RNA-activated (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). An ER chaperone called heat shock protein (hsp) A5 (or glucose-regulated protein 78 (GRP78) or binding immunoglobulin protein (BiP)) usually inactivates these sensors by binding to their ER luminal domains (Bertolotti et al., 2000; Shen et al., 2002). It is one of the most highly expressed ER resident chaperones that is targeted to the ER through an N-terminal cleaved signal peptide and a C-terminal ER retention motif (KDEL). Upon ER stress induction, BiP is engaged by the piled up misfolded proteins thus liberating the sensors. Furthermore, a new chaperone Hsp47 was shown to free BiP from the luminal domain of IRE1 thus facilitating IRE1 oligomerization (Sepulveda et al., 2018). Accordingly, BiP-free IRE1 and PERK either homodimerize or oligomerize then undergo an autophosphorylation in order to activate downstream signaling pathways (Bertolotti et al., 2000). On the other hand, when BiP dissociates from ATF6, the latter translocates to the Golgi apparatus *via* an unmasked ER export signal (Shen et al., 2002; Ye et al., 2000). Consequently, it is clear that BiP negatively regulates the UPR even though BiP-independent pathways have also been elaborated. Here, we review the three main sensor pathways of the UPR (**Figure 23**).

3.3.2.1. IRE1 pathway

Two human isoforms of IRE1 (α and β) exist and share an important sequence homology of around 32% (Meusser et al., 2005) with a ubiquitous presence of IRE1 α known as IRE1 hereafter. Upon BiP dissociation from IRE1, the latter oligomerizes and activates its cytosolic kinase domain. Face-to-face, the oligomers trans-autophosphorylate (Ali et al., 2011). Specific serine residues in the kinase activation domain (Ser724, 726 and 729) are indispensable for the activation of the RNase domain and for the recruitment of tumour necrosis factor receptor-associated factor 2 (TRAF2) and JNK (Prischi et al., 2014;

Urano et al., 2000). Once active, the IRE1 cytosolic RNase domain cleaves dual stem loops of the X-box binding protein 1 (XBP1) mRNA (Sidrauski and Walter, 1997; Yoshida et al., 2001). In fact, IRE1 catalyses a 26 nucleotide intron splicing from human XBP1 mRNA resulting in a spliced isoform termed XBP1s. The latter is a leucine zipper transcription factor needed for the activation of gene transcription (Liou et al., 1990). Accordingly, the unspliced XBP1 (XBP1u) is not capable of this since it lacks a transactivation domain (Yoshida et al., 2001). Furthermore, XBP1u negatively controls the transcriptional potential of XBP1s and prompts its own mRNA translocation to the ER membrane by partially translating its N-terminal domain (Chalmers et al., 2017; He et al., 2010).

Once cleaved, XBP1s initiates the transcription of numerous targets such as chaperones, foldases and ERAD pathway components in the aim of diminishing ER stress (Iwakoshi et al., 2003). In addition, XBP1s was shown to be involved in the regulation of several metabolic pathways including lipid biosynthesis (So et al., 2012) and glucose metabolism (Zhou et al., 2011). XBP1s interacts *via* its transactivation domain with RNA polymerase II but the exact mechanisms through which it initiates gene transcription are still unknown. It also interacts with several transcription factors such as AP1 (Ono et al., 1991), Oestrogen Receptor α (ER α) (Ding et al., 2003), forkhead box O1 (Fox O1) (Zhou et al., 2011), ATF6 (Reinke et al., 2013), cAMP response element-binding protein (CREB) (Reinke et al., 2013) and hypoxia inducible factor 1 α (HIF1 α) (Chen et al., 2014). Other targets of the RNase activity of IRE1 include substrates with a consensus sequence (CUGCAG) in a mechanism conserved in eukaryotes called regulated IRE1-dependent decay (RIDD) (Hollien et al., 2009; Oikawa et al., 2010). It is interesting to note that IRE1 is able to degrade its own mRNA (Tirasophon et al., 2000). In fact, RIDD is a homeostatic process utilized by the ER to reduce its protein load via degrading mRNAs (Hollien and Weissman, 2006). This basal activity is progressively enhanced depending on the severity and duration of ER stress (Maurel et al., 2014). Moreover, IRE1 β seems to translationally repress *via* another mechanism involving mRNA degradation through the 28S ribosome (Iwawaki et al., 2001). Finally, separating IRE1 RIDD activity and IRE1-mediated XBP1 splicing activity seems to be a hard job especially *in-vivo* (**Figure 23**).

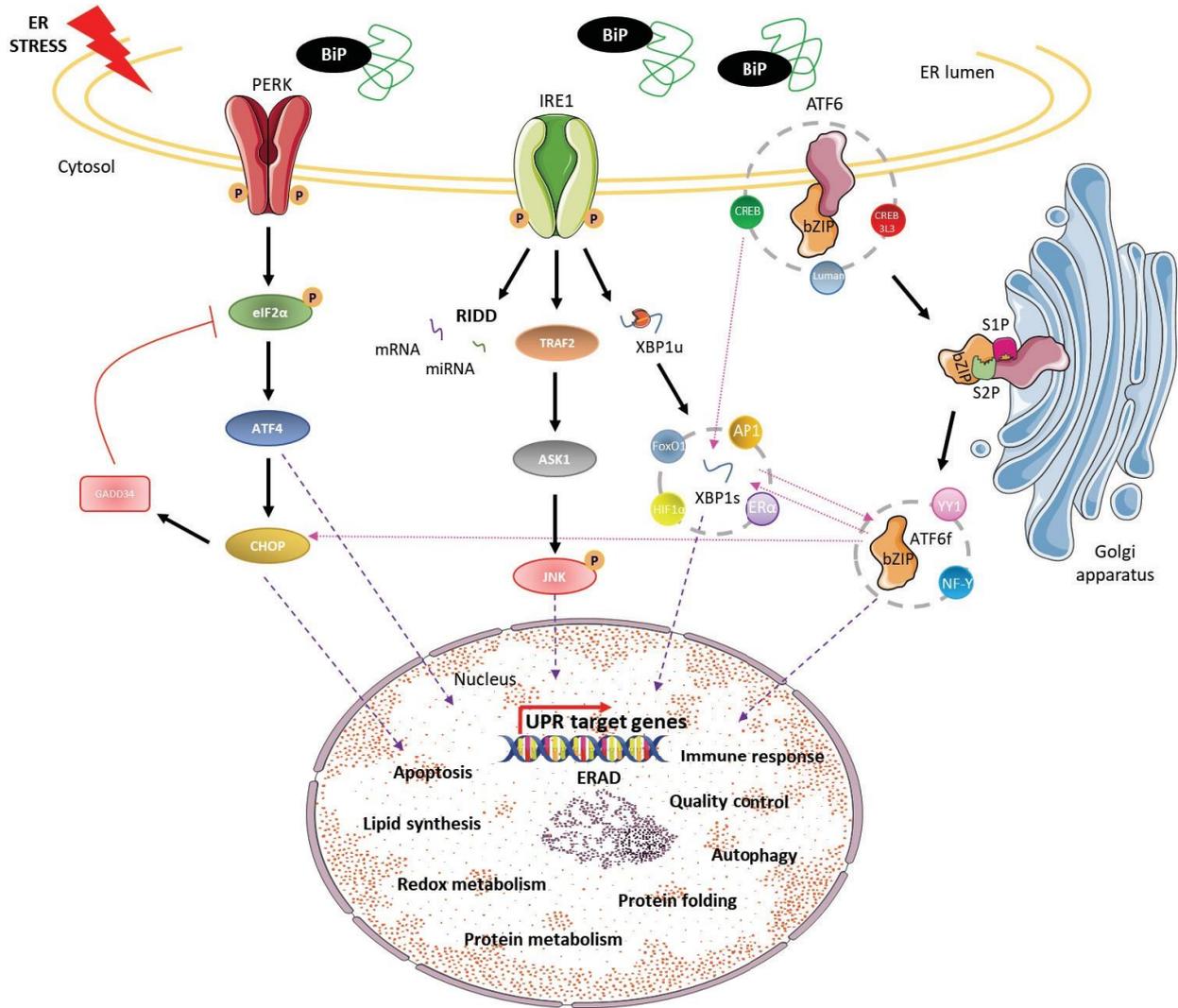


Figure 23: The UPR signaling pathways activated by ER stress.

Upon ER stress, misfolded proteins accumulate in the ER and engage the BiP liberating them from the three UPR sensors: PERK, IRE1 and ATF6. These stress sensors initiate the signaling cascades *via* the production/activation of several transcription factors such as ATF4, XBP1s and ATF6f along with associated processes (RIDD, ERAD) to reduce the misfolded ER protein load. The UPR activates transcription of genes depending on the translational demand in order to re-establish ER homeostasis and prompt cell survival. However, in case of unresolved stress, pro-death mechanisms are activated resulting in cell death.

3.3.2.2. PERK pathway

PERK is a serine/threonine kinase ubiquitously expressed initially discovered in rat pancreatic islets. It was shown to phosphorylate the eukaryotic initiation factor 2 α (eIF2 α) on serine 51 and is constituted of an ER luminal and a cytosolic kinase domain pretty much like IRE1 (Harding et al., 1999; Shi et al., 1998). Also, the dissociation of BiP from the luminal domain causes PERK oligomerization followed by an activating autophosphorylation leading to eIF2 α phosphorylation (McQuiston and Diehl, 2017). The latter is a part of the eIF2 heterotrimer that controls the initial step of protein synthesis by enhancing the interaction of tRNA to 40S ribosomal subunits (Adams et al., 1975; Lloyd et al., 1980). Furthermore, eIF2 α phosphorylation blocks the translation machinery by inhibiting eIF2 β involved in protein synthesis. Accordingly, this results in the decrease of ER protein load during ER stress (Harding et al., 2000a; Rowlands et al., 1988). On the other hand, many transcripts are more effectively translated during PERK-mediated repression of translation. For instance, activating transcription factor 4 (ATF4) is generally inefficiently translated from the protein-coding AUG. However, in the case of the decrease of upstream open reading frames translation, the initiation of translation is shifted towards AUG increasing thus the amount of efficiently translated ATF4 (Harding et al., 2000b; Vallejo et al., 1993). ATF4 interacts with a specific site in the promoter of CAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) or (GADD153) and leads to its expression (Fawcett et al., 1999; Harding et al., 2000b). After that, both CHOP and ATF4 activate the transcription of genes tangled with protein synthesis and UPR. However, this represents a double-edge sword in the meaning that ATF4/CHOP-enhanced protein synthesis might result in ATP depletion, oxidative stress and eventually cell death (Han et al., 2013; Hiramatsu et al., 2014).

Moreover, the phosphorylated form of eIF2 α (p-eIF2 α) increases directly CHOP and UPR proteins translation (Barbosa et al., 2013). For instance, all three ATF4, CHOP and p-eIF2 α positively regulate growth arrest and DNA-damage-inducible 34 (GADD34) (Fornace et al., 1989; Marciniak et al., 2004). The latter interacts with type 1 protein serine/threonine phosphatase (PP1) leading to the dephosphorylation of eIF2 α and establishing a negative feedback loop needed to re-activate the translation machinery (Connor et al., 2001; Novoa et al., 2001).

In fact, the interplay between translational arrest caused by p-eIF2a and ATF4 mediated expression of adaptive genes (aa transport, metabolism, oxidative stress protection, protein homeostasis and autophagy) leads to the same outcome: decreasing protein load on ER lumen and helping the cell cope with ER stress (Quirós et al., 2017). Prolonged stress induces a pro-death response basically regulated by the phosphorylation status of eIF2a (Rajesh et al., 2015). Consequently, PTMs are shown once again to be crucial for the function of proteins especially under stress conditions (**Figure 23**).

3.3.2.3. ATF6 pathway

ATF6 is a transcription factor belonging to the leucine zipper protein family. It has two homologs: ATF6a and ATF6 β (Ernst et al., 1987; Zhu et al., 1997). Once activated in the ER, ATF6 is transported to the Golgi through interaction with COPII where it is processed by site 1 protease (S1P) and S2P thus releasing its cytosolic domain fragment (ATF6f). The latter has several domains of which one containing a nuclear localization signal (NLS) driving the protein to the nucleus where it activates the transcription of UPR genes, ERAD components and also XBP1 (Shen et al., 2002; Yamamoto et al., 2007). ATF6 β might look very similar to ATF6a but it acts as its inhibitor by heterodimerizing with it (Harding et al., 2000b).

Furthermore, ATF6 controls the expression of several basic-Leucine Zippers (bZIPs) including CREB (Vallejo et al., 1993), CREB3L3 and XBP1 (Montibeller and de Belleruche, 2018) and transcription factors like serum response factor (Zhu et al., 1997), yin yang (YY1) (Palam et al., 2011) and general transcription factor Y (NF-Y) complex (Hiramatsu et al., 2014). Actually, ATF6 plays the role of a mediator of IRE1 and PERK signaling pathways as it increases the expression of XBP1 along with CHOP in order to boost the UPR (Fornace et al., 1988, 1989). ER-resident bZIPs, other than ATF6, are also involved in the UPR like Luman, CREB and CREB3L3 and many others reviewed in (Asada et al., 2011) (**Figure 23**).

3.3.3. ER stress + Bcl-2 proteins = Complicated relationship?

When ER stress reaches a dead end, the Bcl-2 gang intrudes to save the situation... or worsen it. Chronic ER stress results in a pro-death response switch. Many kinds of cell death might occur in this case including apoptosis, necroptosis and autophagy. Here, we focus on apoptosis (**Figure 24**).

Both intrinsic and extrinsic apoptotic pathways might be activated following prolonged ER stress. Accordingly, caspases are activated in both cases. In the case of ER stress-induced apoptosis, BH3-only proteins are majorly concerned especially Puma, Noxa and Bim (Kim et al., 2009a). In fact, BH3-only deficient cells resist to ER-stress mediated apoptosis (Gorman et al., 2012). As ER stress results in gene transcription, BH3-only members are also upregulated under these conditions leading to Cytochrome c release. All three branches of the UPR are involved. For instance, CHOP, after its activation by PERK pathway, regulates apoptosis during ER stress. In fact, CHOP induces the expression of the phosphatase GADD34 needed to reverse the translation inhibition practiced by p-eIF2a permitting the apoptosis-related genes transcription as mentioned in (part 3.3.2.2.)(Novoa et al., 2001). Bim and Puma are transcriptionally upregulated by CHOP that also inhibits the transcription of other anti-apoptotic members such as Mcl-1 (Gomez-Bougie et al., 2016).

Other than CHOP, p53 plays a role in the expression of BH3-only proteins in the case of ER stress even though this needs further elaboration (Li et al., 2006).

Furthermore, IRE1-XBP1s signaling pathway normally leads to cell survival however, when active IRE1 binds to TRAF2, apoptosis signal-regulating kinase 1 (ASK1) is activated along with its downstream targets p38 MAPK and JNK. The latter, when phosphorylated, might upregulate Bid and Bim and inhibit anti-apoptotic Bcl-2, Bcl-xL and Mcl-1 (Deng et al., 2001b; Kim et al., 2009b). As for p38 MAPK, it actively phosphorylates CHOP leading to the upregulation of Bim prompting apoptosis (Puthalakath et al., 2007).

Furthermore, a new role of non-coding RNAs called microRNAs (miRNAs) in the initiation of apoptosis is established during ER stress. In fact, ATF4 induces the expression of miRNA29a responsible for the downregulation of Mcl-1 further stimulating apoptosis (Nolan et al., 2016). Likewise, IRE1, by

downregulating miRNA7, increases the stability of RNF183 responsible for the ubiquitination and subsequent degradation of Bcl-xL, as discussed in (part 1.9.2.). Consequently, apoptosis occurs following prolonged ER stress-enhanced RNF183 expression (Wu et al., 2018). In fact, the degradation of several inhibitory miRNAs *via* the IRE1a RIDD activity results in the activation of caspase 2, which in turn cleaves and activates Bid initiating the mitochondrial pathway of apoptosis (Upton et al., 2008). Under prolonged ER stress, IRE1a might be turned off in some cellular systems, decreasing the pro-survival effect of XBP1 and upregulating the death receptor 5 (DR5) that engages caspase 8 and enhances Bid-dependent apoptosis (Lu et al., 2014). Always in the IRE1 pathway, Bax and Bak seem to form a protein complex with the cytosolic domain of IRE1a *via* their BH1 and 3 domains thus stabilizing its active conformation (Hetz et al., 2006). Moreover, Bok was shown to be involved in ER stress. This BH3-only protein is naturally degraded through ERAD, but under ER stress, it is stabilized and it promotes MOMP (Carpio et al., 2015b; Llambi et al., 2016b).

Our team has put in evidence a new function of Bcl2L10/Nrh in UPR. This protein abrogates UPR initiation by inhibiting the PERK pathway. Furthermore, this study shows for the first time the regulation of a Bcl-2 homolog of the UPR upstream of the mitochondria through its interaction and inhibition of IP3R-mediated Ca^{2+} release (Nougarede et al., 2018). Moreover, only one study puts in evidence a direct role of Bcl-xL in ER stress resistance. During *in-vitro* plasma cell differentiation, the UPR is activated. Following this, changes in protein expression occur such as decreased Bcl-2 and Mcl-1 expression and increased expression of Bim. Upon Bcl-xL induction, these changes were reversed. Knockdown (KD) of Bcl-xL results in a CHOP-dependent cell death suggesting that Bcl-xL might protect from CHOP-dependent apoptosis during plasma cell differentiation but this needs further elaboration (Gaudette et al., 2014). As presented, the exact contribution of Bcl-2 proteins to ER stress resistance/induction is still elusive and requires more studies.

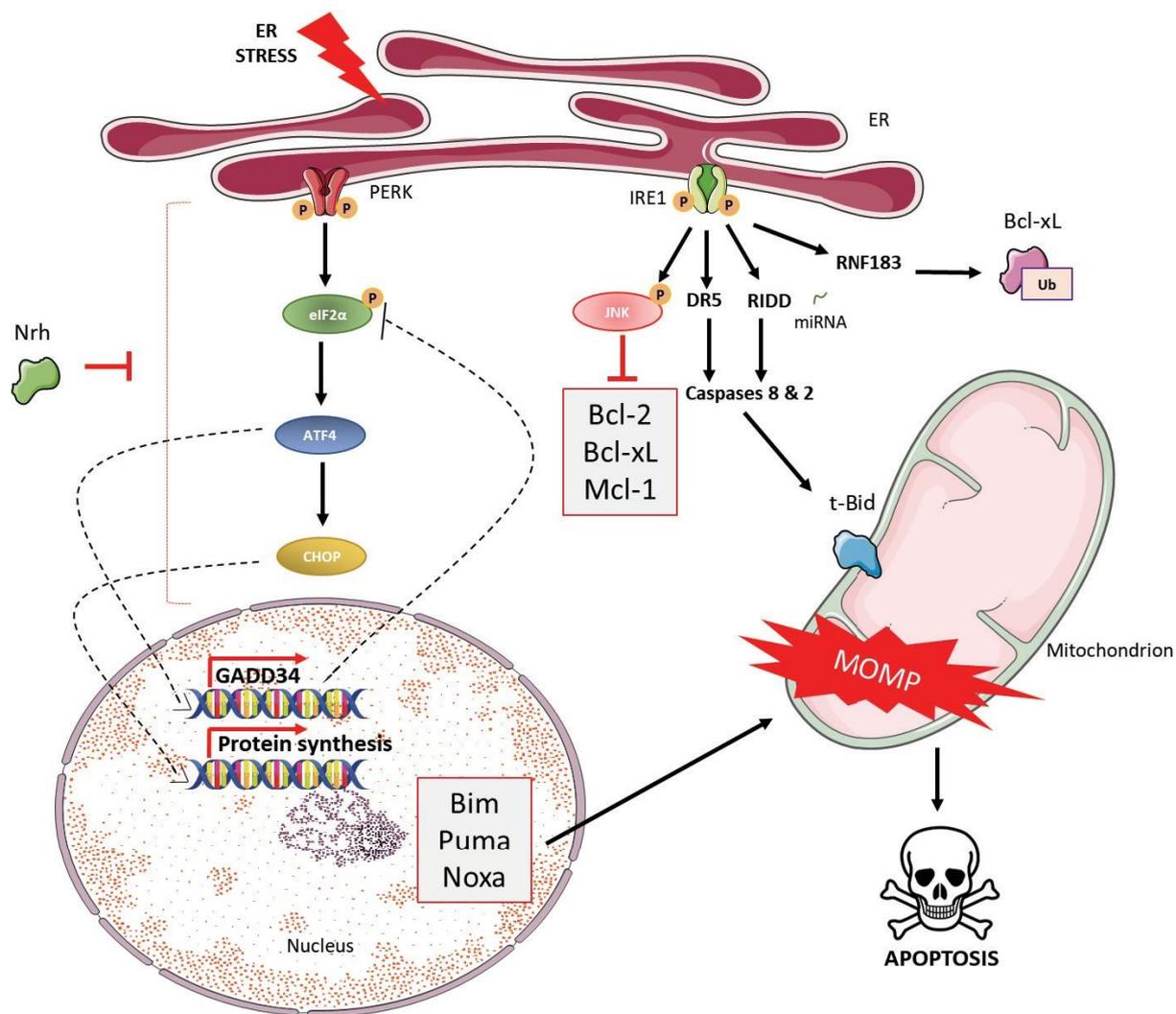


Figure 24: The Bcl-2 family involvement in ER stress-activated UPR.

Under prolonged ER stress, the UPR activates the apoptotic machinery. The activation of IRE1 leads to several ER-resident miRNA degradation during RIDD leading to the activation of caspase 2. In turn, the latter cleaves and activates t-Bid initiating the mitochondrial pathway of apoptosis. Also, RIDD downregulates miRNA7 stabilizing thus RNF183 responsible for Bcl-xL ubiquitination and proteasomal degradation. Under prolonged ER stress, IRE1 might be turned off leading to the upregulation of DR5 thus activating caspase 8 enhancing t-Bid mediated apoptosis. Phosphorylated and activated JNK inhibits Bcl-2, Bcl-xL and Mcl-1. The activation of PERK leads to the phosphorylation of eIF2a inducing global protein arrest. Under these conditions, ATF4 is selectively translated and along with CHOP, transcriptionally activates several BH3-only proteins that engage the mitochondria. Furthermore, the ATF4/CHOP complex resumes protein synthesis through the upregulation of GADD34 phosphatase causing cell death. All of these events result in MOMP. Finally, Nrh might be able to inhibit PERK pathway promoting cell survival.

3.3.4. ER stress in physiology and disease

ER stress pathways have been elaborated and intensely studied in cellular interactions that make up normal physiology. Therefore, experimental model organisms have been used to further study ER stress signaling in both physiology and disease. ER stress and subsequent UPR signaling were demonstrated critical in normal development and embryology, cell growth and differentiation, glucose, lipid and aa metabolism (Almanza et al., 2019). On the other hand, ER stress is a major contributor to diseases onset and it was shown to be involved in hypoxia, cancer and chemotherapy (Koumenis, 2006). An oncogenic role of ER stress was established after it generated pre-oncogenic cells that led to hepatocellular carcinoma under high-fat-diet-induced inflammatory conditions (Nakagawa et al., 2014). ER stress is involved in neurodegeneration like in the case of *Retinitis pigmentosa* retinal degeneration (Ryoo et al., 2007) or Alzheimer's disease (AD) (Unterberger et al., 2006). ER stress and UPR pathways are also involved in viral infections that might include human cytomegalovirus (HCMV) infection (Isler et al., 2005), hepatitis C virus (HCV) infection (Tardif et al., 2004), hepatitis B virus (HBV) infection (Huang et al., 2005), vesicular stomatitis virus (VSV) infection (Baltzis et al., 2004) and many others. Also, they are implicated in metabolism diseases as in the case of diabetes mellitus cardiovascular disease (Blackwood et al., 2019), nonalcoholic fatty-acid liver disease (Wang et al., 2012b) and nonalcoholic steatosis (Rutkowski et al., 2008; Zhang et al., 2011). The cited diseases are minor compared to all the consequences that ER stress might cause. Accordingly, numerous small molecules have been reported to modulate – either activate or inhibit – the UPR. Interestingly, these molecules have had beneficial effects in diverse human diseases. The understanding of how these molecules interact with the active sites and modulate the function of all UPR arms will profoundly impact on the structure-based drug discovery of novel UPR modulators (Almanza et al., 2019).

4. Implication of the Bcl-2 family of proteins in cancer

We have investigated in previous sections the importance of the Bcl-2 family in the regulation of cell survival/cell death switch. Like Yin Yan, cell death and survival are interconnected and interdependent. Complementary as they be, both phenomena contribute to the normal functioning of the body. Therefore, Bcl-2 family disruptions in any way might result in severe complications compromising homeostasis. Going back to their discovery, Bcl-2 was first identified in a B-cell lymphoma case suggesting a very direct implication in cancer. Here, we focus on the contribution of the Bcl-2 clan in cancer and the treatments proposed to target it.

4.1. Bcl-2 and cancer: *no pain, no gain*

4.1.1. *Altered expression of Bcl-2 proteins in cancer*

Enhanced pro-survival Bcl-2 members' expression

Apoptosis evasion plays a major role in oncogenic transformation by enhancing tumor growth and proliferation, survival during metastasis and chemotherapy resistance. Accordingly, it is with no surprise that anti-apoptotic Bcl-2 proteins expression is upregulated in several cancers. This process might be executed under different aspects including chromosomal translocation as in the case of *Bcl2* t(12;18) translocation, gene amplification, enhanced translation and protein stability.

Even though the first implication of Bcl-2 proteins was through a chromosomal translocation, it was shown that this aspect is not very common among Bcl-2 proteins and it is not entirely oncogenic since it was found in peripheral blood lymphocytes of healthy people (Liu et al., 1994) and was demonstrated only weakly tumorigenic in mice (Strasser et al., 1993). Another more common form of Bcl-2-mediated oncogenic transformation is increased gene copy number. It was mainly modeled in the hematopoietic system where upregulation of *bcl2*, *bclx* and *mcl1* might give a clue about future lymphoma development. One of the most frequent chromosomal gains are those of the *mcl1* and *bclx* amplifications in a 26-tumor type case study. These amplifications were duplicated in around 40% of the studied

specimens and occur mainly in the breast and lung cancer, interestingly (Beroukhim et al., 2010). It is clearer now that *mcl1* amplification prevails that of the other pro-survival members in numerous solid cancers as analyzed via cBioportal (**Figure 25**). Logically, mutations/deletions of this category of proteins is a rare event in cancer (Cerami et al., 2012). It is crystal clear that pro-survival Bcl-2 proteins cannot execute the 'job' by themselves and promote cancer progression by their own. Thus, co-amplifications of proto-oncogenes such as *myc* with *mcl1* or even *bclx* have been reported in cancer and *myc* with *bcl2* in cell culture and in B-cell lymphoma mice model (Campbell et al., 2010; Strasser et al., 1990).

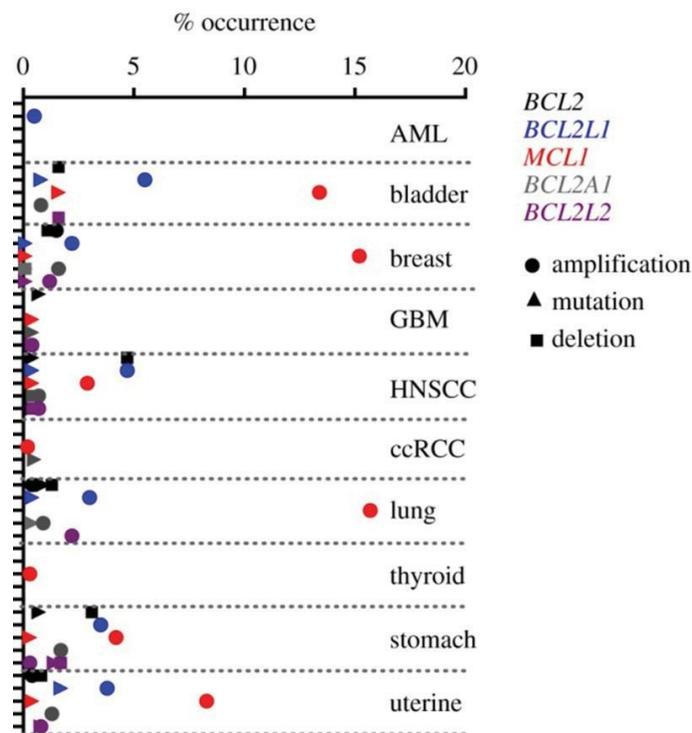


Figure 25: Frequency of genomic alterations of anti-apoptotic Bcl-2 members in solid cancers.

Frequency of amplification, mutation and deletion (dot, triangle, square, respectively) of *Bcl2*, *Bcl2L1* (*Bclx*), *Mcl1*, *Bcl2A1*, *Bcl2L2* (*Bclw*) (as shown in colors) in several types of cancer as analyzed by cBioportal in The Cancer Genome Atlas (TCGA) studies. AML: acute myeloid leukaemia; GBM: glioblastoma; HNSCC: head and neck squamous cell carcinoma; ccRCC: kidney renal clear cell carcinoma. From (Campbell and Tait, 2018).

Furthermore, increased translation/protein synthesis due to an increased transcription of certain anti-apoptotic genes enhances their expression in cancers. For instance, the proto-oncogene *c-ABL* increases Mcl-1 transcription and translation levels *via* STAT3/NF- κ B pathways in chronic lymphocytic leukaemia (CLL) (Allen et al., 2011). This was replicated by the BCR-ABL oncoprotein in a mouse model of B-cell acute lymphoblastic leukaemia (B-ALL) (Koss et al., 2013). Bcl-xL is also affected by increased protein translation in a study where they also proved that Mcl-1 acts as a downstream mediator to the oncogenic translation initiation factor eIF2e (Wendel et al., 2007). Furthermore, mutations causing the loss of the ubiquitin ligase FBW7 required to ubiquitinate and subsequently degrade Mcl-1 lead to Mcl-1 levels increase involved in tumorigenesis and also chemotherapy resistance (Gasca et al., 2016) which constitute a new way to upregulate Bcl-2 pro-survival members other than genetic alterations.

It is interesting to note that the increased levels of these proteins do not entirely establish marked apoptotic resistance. For example, high Bcl-2 levels sensitize cells to ABT-737 drug-induced apoptosis that actually targets Bcl-2/Bcl-xL/Bcl-W *via* the clemency of harbored Bim from Bim/Bcl-2 complexes in lymphoid and leukemic cells (Mérino et al., 2012). These cells are called primed for death and the measurements of mitochondrial priming threshold is a good way to predict their response to chemotherapy (Certo et al., 2006; Lopez and Tait, 2015; Ni Chonghaile et al., 2011).

Acting beyond tumorigenesis, Mcl-1 upregulated levels when diagnosed, associates with breast cancer poor prognosis (Campbell et al., 2010). Moreover, its amplification is important in chemotherapy-resistant breast cancer what suggests that it could be a form of an innate or acquired resistance (Balko et al., 2014). Mcl-1 seems to be the exception in this case as other anti-apoptotic proteins (such as Bcl-2) increased levels are usually associated with good prognosis in breast cancer (Dawson et al., 2010; Lipponen et al., 1995). In another study, Bcl-xL levels were increased in the presence of a certain p53/double minute 2 protein (MDM2) inhibitor HDM201 that normally prevents p53 protein degradation indicating that Bcl-xL plays a role in treatment-resistance (Chapeau et al., 2017).

Reduced anti-survival Bcl-2 members' expression

Whoever talks about increasing pro-survival protein levels, also talks about reducing those of anti-survival during tumorigenesis. Therefore, anti-apoptotic Bcl-2 proteins could act as a target in cancer.

For what concerns BH3-only proteins, only Bad KO mice were predisposed to late apparent lymphoma (Labi et al., 2006; Ranger et al., 2003). One might think of redundancy between BH3-only proteins that complements their functional loss. Several BH3-only protein KO, as in the case of Bim and Bmf KO mice, enhanced B lymphadenopathy associated with autoimmune glomerulonephritis but that appeared only in aged mice (Labi et al., 2014). Accordingly, deficiency in BH3-only proteins is not entirely oncogenic but when complemented with upregulated Myc for instance, drastically enhances lymphoma development. In this case, mutated Bim further enhances Myc effect since Bim is considered a suppressor of Myc-mediated B-cell leukemia (Egle et al., 2004). Also, deletion of the *Bbc3* gene that encodes PUMA or PUMA promoter methylation was shown in several cancers especially Myc-induced B-cell lymphomagenesis (Beroukhi et al., 2010; Garrison et al., 2008).

As for Bax and Bak, all four alleles should be mutated/deleted to abrogate their pro-apoptotic function. In a natural way, Bax and Bak levels decrease in mammals with age. However, total loss of Bax and Bak has not been totally elucidated in cancer (Sarosiek et al., 2017). Furthermore, the subcellular localization of the effector proteins plays a major role in cancer prognosis as in the case of mitochondrial Bax that was shown to improve apoptosis susceptibility and prognosis in patients suffering from AML. When KO in mice, both proteins do not induce tumor suppression but when KO individually, mice die perinatally (Knudson et al., 1995; Lindsten et al., 2000). Moreover, conditional *Bax/Bak* deletion in the haematopoietic system of mice induces lethal autoimmune diseases that might actually hide tumor development (Mason et al., 2013). As for Bax/Bak deficiency in mice brain and testicles, it results in tumor formation (Katz et al., 2013). Furthermore, the other overall less-famous effector protein Bok is actually more famous in cancer. Its deficiency is a frequent event in numerous types of cancers (Beroukhi et al., 2010). This protein was shown to be downregulated in non-small cell lung cancer (NSCLC) and upregulated Bok expression is correlated with good prognosis in patients with lymphnodes where Bok seems to antagonize cell migration and epithelial to mesenchymal transition (EMT) (Moravcikova et al., 2017). In an interesting manner, the loss of Bok abrogated the response to ER stress and its subsequent activation of Bim and Puma suggesting that Bok plays a tumor prompting role upstream of MOMP thus promoting chemical-induced hepatocarcinogenesis in mice. It was also shown in this case that Bok promoted tumor growth even though this needs further elaboration (Rabachini et al., 2018).

A summary of some Bcl-2 members genetic alterations is schematically represented in Delbridge and colleagues' review on the 30-year knowledge about the Bcl-2 family (Delbridge et al., 2016) (**Figure 26**).

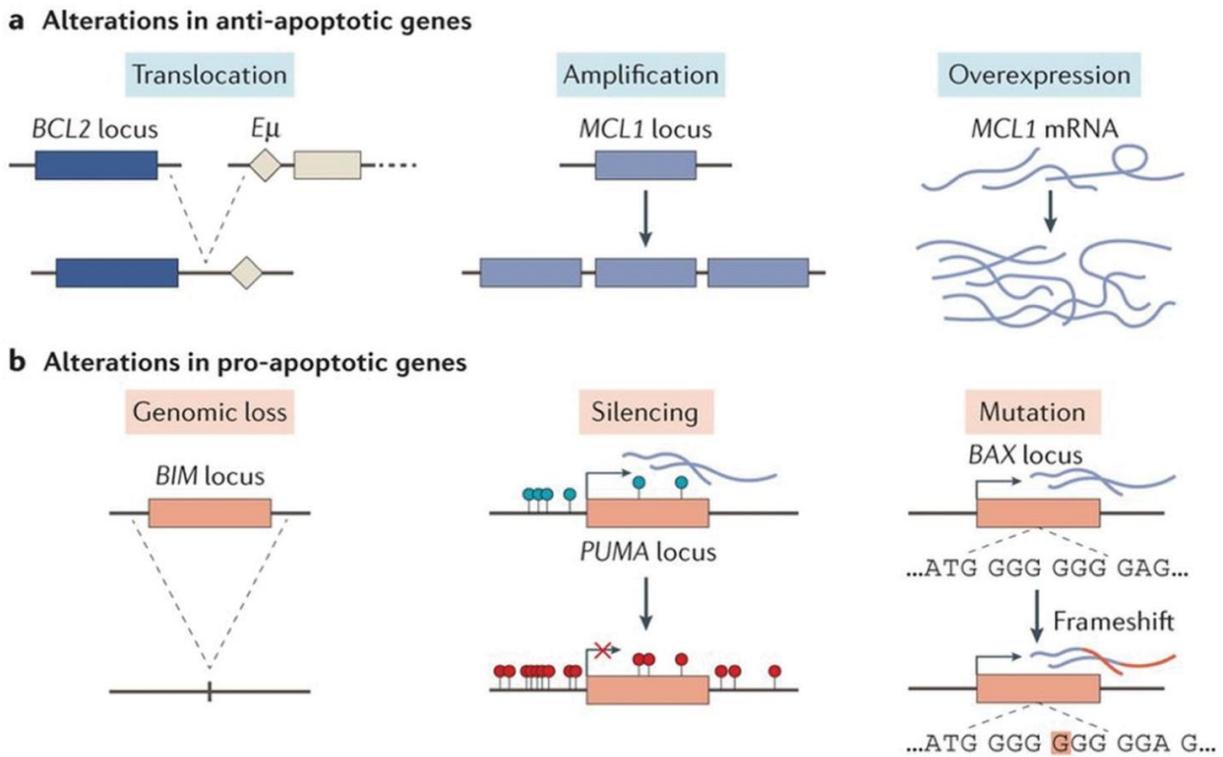


Figure 26: Some genetic alterations of both anti- and pro-apoptotic Bcl-2 members in cancer.

Several alterations affect the genes of the Bcl-2 family in certain types of cancer. **(a)** Chromosome translocation, gene copy number amplification and overexpression might increase the levels of pro-survival Bcl-2 proteins. **(b)** As for the loss of anti-survival members, it could be provoked by genomic deletion, gene silencing due to hypermethylation as in the case of Puma or a frameshift mutation. From (Delbridge et al., 2016).

4.2. Targeting the gang: BH3-mimetics and others

The resistance to therapy established by certain anti-apoptotic proteins suggested that targeting these members on the protein or gene level might aid in cancer regression. It is then that the idea of BH3-mimetics came out.

4.2.1. Introducing the 'fake-BH3' molecules

The equilibrium set by Bcl-2 members can be altered in favor of clinical use in cancer therapy. For instance, DNA-damage inducing drugs like etoposide might be able to switch on p53 TS transcription factor. Puma and Noxa are two target of the latter but even with their-upregulation, the increased levels of anti-apoptotic members still promote cell survival. Therefore, comes the idea that what matters most is the neutralization of these anti-apoptotic proteins.

In the last decade, researchers cogitated and conceived a range of pro-survival proteins' inhibitors able to interact with the hydrophobic cleft of these proteins in the same way that BH3-only proteins engage this region. These molecules were called BH3-mimetics thanks to the fact that they imitate on the structure and function levels the BH3 domain of BH3-only proteins. Accordingly, they are able to antagonize anti-apoptotic members and activate pro-apoptotic ones. Although, another subclass is able to convert anti-apoptotic proteins into ant-survival ones.

Here, we review the well-known BH3-mimetics and some other molecules/peptides.

4.2.1.1. Targeting Bcl-2 members

Pan-pro-survival Bcl-2 inhibitors (BH3-mimetics)

The very first BH3 mimetic with a large range of targets was ABT-737 developed *via* NMR-based fragment screening. This small molecule imitates the BH3 domain of Bad thus binds Bcl-2, Bcl-xL and Bcl-w with high affinity of less than 1 μ M. Doing this, it expels BH3-only proteins and promotes their liberation to initiate apoptosis in cell lines and reduces the growth of tumors in some xenograft models (Oltersdorf et

al., 2005). A derivation of this molecule is the orally available drug ABT-263 also called Navitoclax (Tse et al., 2008). This drug is allowed for clinical testing but side effects emerged soon after. A dose-limiting thrombocytopenia occurred due to its inhibition of Bcl-xL on which platelets depend for their survival (Mason et al., 2007; Wilson et al., 2010). However, some preclinical models have encouraged the use of this compound in clinical trials as a combination therapy in mostly solid tumors. For instance, combining both cited molecules with a specific cytotoxic drug increased Bim expression (Chen et al., 2011a). On the other hand, combining therapies might enhance chemotherapy toxicity, thus, the need for more targeted strategies (**Figure 27**) (Juin et al., 2013).

Other synthetic molecules that inhibit pro-survival Bcl-2 proteins include Obatoclax (GX15-O70) that also binds their hydrophobic cleft (Nguyen et al., 2007) and the glossypol synthetic isomers (AT-101, TW-37 and BI-97C1) that target more specifically Bcl-2, Bcl-xL and Mcl-1 (Mohammad et al., 2007; Wei et al., 2010). Obatoclax and AT-101 compounds have a potent activity *in-vitro* but have a limited anti-tumoral one in clinical trials where they are used in combination with other chemotherapies (Hwang et al., 2010; Stein et al., 2016). The last two, TW-37 and BI-97C1 show a more promising result *in-vitro* but have not been tested clinically yet. However, the preclinical data of TW-37, in particular, are even more promising (Dash et al., 2011; Lei et al., 2017).

Specific pro-survival Bcl-2 inhibitors (BH3-mimetics)

Due to the emerging requirement of inhibiting specifically an individual Bcl-2 member, other small molecules were displayed. The success of this strategy was directly presented with the Bcl-2 specific BH3-mimetic ABT-199 also known as Venetoclax that spares platelets (Souers et al., 2013). The latter has gotten FDA status to be used in CLL without the need for an additional drug leading to a prominent complete response in around 20% of the patients (Roberts et al., 2016). When used in combination with rituximab, the percentage is even increased to 51% (Seymour et al., 2017). Other promising but less efficient effects were displayed in non-hodgkin lymphomas (Davids et al., 2017) and AML (Konopleva et al., 2016) also in combination therapy with bortezomib and dexamethasone in multiple myeloma (MM) (Moreau et al., 2017). Venetoclax seems to work best in combination therapy where it was proven

efficient in preclinical models of breast cancer (Vaillant et al., 2013) and is currently in clinical trials of combination therapy with tamoxifen that are going in a rather promising direction (Lok et al., 2019).

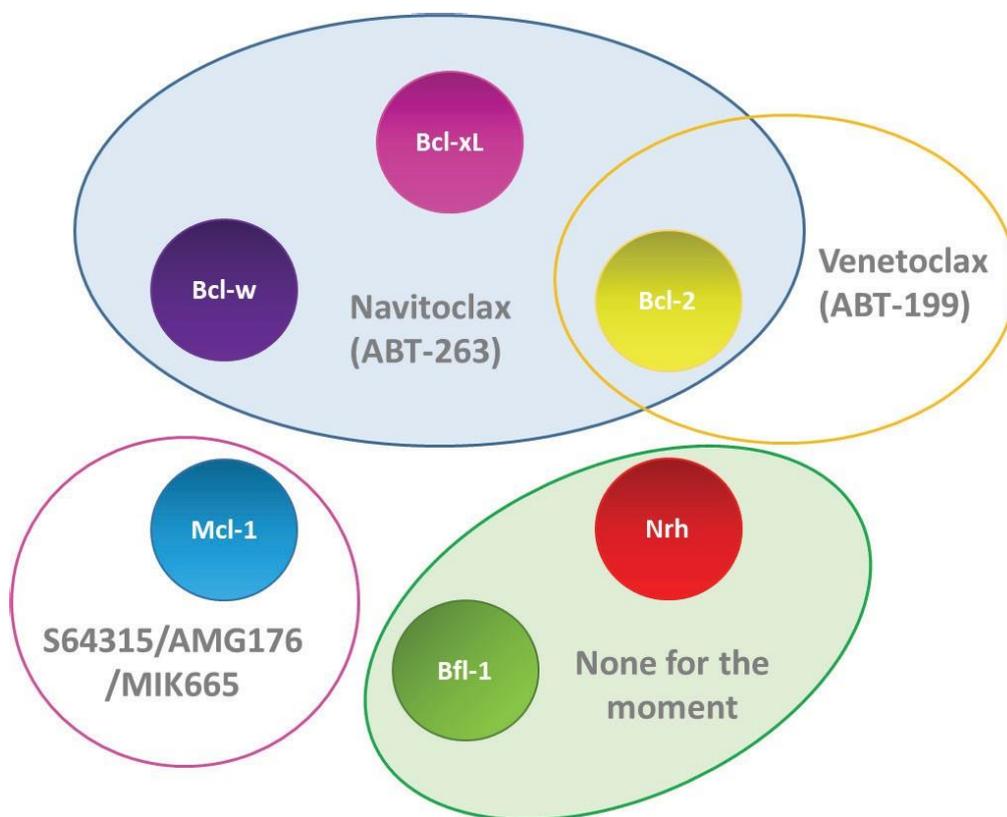


Figure 27: Selective BH3-mimetics under clinical trials.

BH3-mimetics have passed a long way in research and are now in specific phases of clinical trials/use. This graphical representation summarizes the drugs targeting (Bcl-xL, Bcl-2 and Bcl-w), Bcl-2 alone and Mcl-1 alone. Combinational therapies are more common nowadays as in the case of Mcl-1. No drugs have passed to clinical studies concerning Bfl-1 and Nrh/Bcl-2L10.

As for Bcl-xL, its most potent selective inhibitors are WEHI-539 (Lessene et al., 2013) and A-1155463 (Tao et al., 2014) and its orally available related compound A-1331852. These molecules are mostly helpful for research due to the compromising effect of Bcl-xL targeting on platelets' life span.

Mcl-1 has long been associated with chemotherapy resistance as discussed in (part 4.1.1). Accordingly, researchers have developed drugs that targeted it specifically. Many inhibitors were created but were not specific enough and exhibited high off-targets levels (Varadarajan et al., 2013). Most of these

inhibitors are classified as Noxa-like molecules due to their homology with the BH3 domain of Noxa. Since then, new molecules have emerged and tested *in-vitro* and *in-vivo*. Of which, we cite: the potent inhibitor A1210477 that restricts the growth of several cancer cell lines (Leverson et al., 2015); UMI-77 that abrogated pancreatic and breast cancer cell line growth (Abulwerdi et al., 2014; Campbell et al., 2018); S63845 that has been effective in leukemia and lymphoma models (Kotschy et al., 2016). Furthermore, Mcl-1 inhibitors in combination therapy (S64315/MIK665/AMG176) are currently in phase 1 trials in hematopoietic cancers (**Figure 27**).

In a recent study, Henz and colleagues show that specific BH3-mimetics cause apoptosis in several hematological malignancies that is correlated with severe mitochondrial perturbations. They assessed the activity of ABT-199, A-1331852 and S63845 that results in mitochondrial swelling and toxicities what confirms the importance of Bcl-2, Bcl-xL and Mcl-1 in protecting mitochondrial integrity (Henz et al., 2019).

Anti-survival Bcl-2 activators

Another way to use targeted therapy is by developing drugs that activate pro-apoptotic members in the sole aim of killing tumor cells. Therefore, a recent study has put in evidence a Bax activator called BTS1 that had displayed anti-tumor potential in AML xenografts without toxicity side effects (Reyna et al., 2017). This molecule binds with high affinity to the N-terminal activation site of Bax and promotes its conformational change leading to its mitochondrial translocation and mediated apoptotic pathway (**Figure 28**). This pharmacophore model was inspired by early reported models of BIM BH3 helix called Stabilized Alpha-Helix of Bcl-2 domain (SAHB) and BAM7 compound that bind to the N-terminal triggersite of Bax (Gavathiotis et al., 2008, 2012).

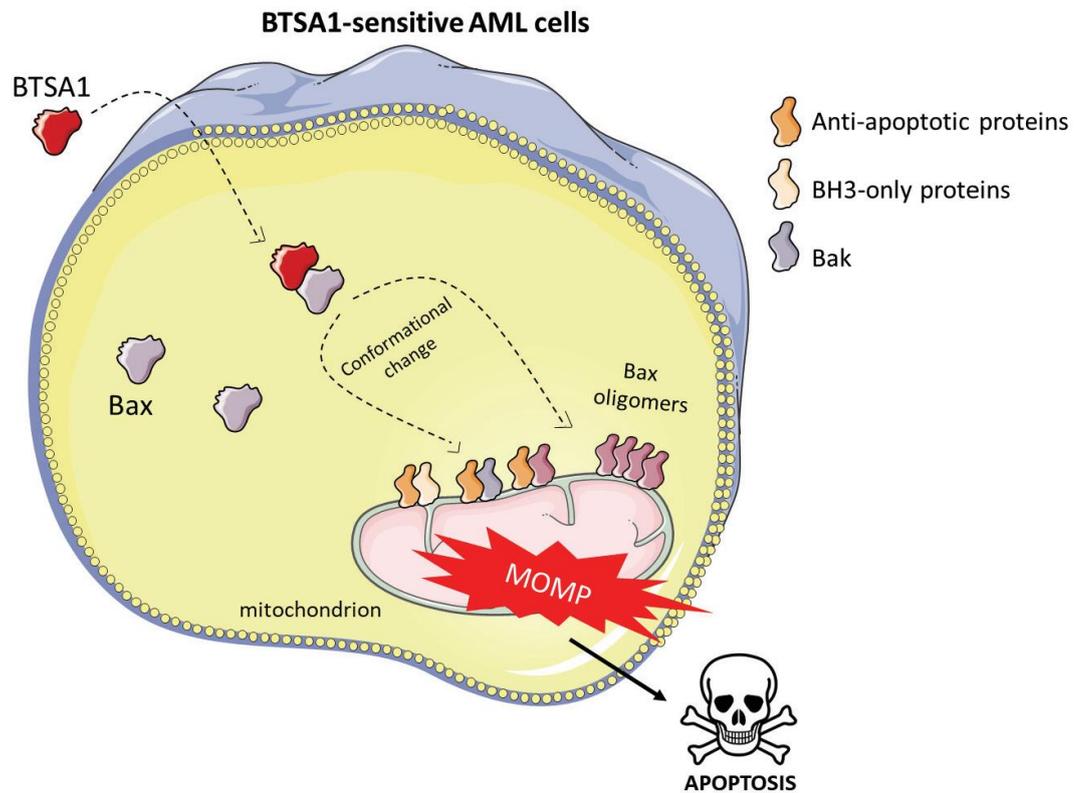


Figure 28: Proposed model for BTSA1 activity in AML cells.

BTSA1 binds to the N-terminal trigger site in soluble cytosolic Bax, initiating conformational changes leading to its mitochondrial translocation where it oligomerizes and promotes mitochondrial apoptosis in AML cells. Inspired by (Reyna et al., 2017).

4.2.1.2. Efficiency of BH3-mimetics

Despite of the fame and reputation of BH3-mimetics as new tools for targeting cancer cells, their efficiency is always questioned. For instance, resistance to Bcl-2 and Bcl-2/Bcl-xL targeting BH3-mimetics has been proven. Also, ABT-737 in-vitro treatment led to an increase in Mcl-1 levels that was later shown to promote resistance to this exact drug even *in-vivo* (van Delft et al., 2006; Konopleva et al., 2006; Williams et al., 2017). Furthermore, only Bcl-2 overexpressing lymphoid and leukemic cells were sensitive to ABT-737 treatment. In contrast, the overexpression of Bcl-xL and Bcl-w conferred a potent resistance even with the upregulation of Bim. This looked suspicious in a way that ABT-737 should be

able to target all three cited anti-apoptotic proteins with the same affinity, which is not the case (Mérino et al., 2012). It seems that the expression levels of proteins play a major role in determining their response to ABT-mimetics. Likewise, overexpressed Bcl-xL confers resistance to WEHI-539 treatment. Moreover, Bcl-xL interaction partners and its subcellular localization extremely affect its sensitivity to WEHI-539. In fact, for Bcl-xL to efficiently sequester Puma and Bim, it needs to be bound to membranes. Accordingly, increasing its shuttling rates to the cytosol, enhances its sensitivity to BH3-mimetics. However, WEHI-539 seems less efficient in disrupting Puma/Bcl-xL or Bim/Bcl-xL interactions partly explaining its incomplete response in certain cells (Pécot et al., 2016).

Moreover, Bcl-2 proteins do not only interact with each other. One should not forget that other interactors are present and perform multiple cellular functions that might result in cell survival or death. Accordingly, by targeting Bcl-2 proteins, we are targeting a whole cellular system that goes beyond apoptosis. For instance, Bcl-xL interacts with Beclin1 as already mentioned. This interaction is rigorously dependent on Bcl-2 BH3 receptor domain and upon BH3-mimetics administration against Bcl-xL, the cell is most likely driven into autophagy or DNA repair mechanisms. Accordingly, the latter consequences might affect the clinical efficiency of BH3-mimetics (Maiuri et al., 2007). Moreover, BH3-mimetics are not conceived in a way that maintains cell homeostasis at completely all levels. Ca^{2+} homeostasis could be extremely affected by BH3-mimetics addition. Interactions between Bcl-2 anti-apoptotic proteins and IP3R for example or VDAC are not targeted by BH3-mimetics, on the contrary, by targeting Bcl-2 proteins that regulate these channels, the latter will be no longer controlled. Hence, tremendous consequences might result maybe in the favor of tumor cell survival (Juin et al., 2013).

4.2.1.3. Therapeutic approaches

In this aspect, other molecules have been proposed to target protein interactions instead of protein domains. An inhibiting peptide, which sequence is appropriated to Bcl-2 binding site on IP3R, acts in competition with IP3R in order to bind Bcl-2 thus enhancing calcium-mediated apoptosis (Rong et al., 2008). The structure of the peptide was improved by a TAT fusion and called BIRD-2 (Bcl-2 IP3 Receptor Disruptor-2). Actually, it binds to the BH4 domain abrogating Bcl-2/IP3R interaction promoting Ca^{2+} -mediated apoptosis in CLL, MM, and follicular lymphoma cells, including cells resistant to ABT-263 and

ABT-199. Plus, combination therapies involving BIRD-2 and some BH3-mimetics enhanced even more apoptosis in these cells (Lavik et al., 2015). Furthermore, a new molecule that targets the BH4 domain of Bcl-2 required for its anti-apoptotic activity has been proposed. BDA366 binds to the BH4 domain with high affinity, inducing a conformational change that enables its pro-survival potential and converts Bcl-2 into a killer protein. Combination treatments have shown strong synergy against lung cancer *in-vivo*. By targeting this domain on Bcl-2, the whole Bcl-2/IP3R interaction is also targeted thus making this molecule hit two major signaling pathways impinging on Ca²⁺ signaling (Han et al., 2015). A recent study showed that BIRD-2 can defeat cisplatin resistance, thus re-sensitizing ovarian cancer cells towards cisplatin. Accordingly, BIRD-2 can be used to get rid of cancer cells, even though it is not directly killing the cells itself (Kerkhofs et al., 2019; Xie et al., 2018).

All of those data insist on the importance of the targeting strategies that will be conceived in order to minimize as much as possible the off-targets and increase the specificity and selectivity of the drugs.

5. Bcl-xL, where does it stand in the Bcl-2 cluster?

Based on the literature review presented here, from the importance of the Bcl-2 family in the regulation of apoptosis to the regulation of Ca²⁺ fluxes and its involvement in the UPR to finally its implications in diseases, in particular, cancers, there is no doubt in saying that the existence of this gang is crucial and indispensable for the proper functioning of the body. This dynamic family is integrated in numerous cellular signaling pathways that any disruption of one member might result in serious consequences. Throughout the past 35 years, plentiful studies have been conducted on each and every member of the Bcl-2 clan in order to comprehend their exact roles in normal and malignant development. Nonetheless, non-canonical functions of Bcl-2 proteins have emerged to even complicate the understanding of their existence. It seems that their roles are subcellular localization-dependent where they interact and activate/repress limitless signaling pathways. Of the anti-apoptotic proteins, we chose to focus on the pro-survival Bcl-xL. Here, we briefly recall the involvement of Bcl-xL in normal and malignant development and introduce my study project.

5.1. Bcl-xL involvement in normal and cancer development

Bcl-xL is firstly identified as a Bcl-2 anti-apoptotic protein lying in the mitochondrial membrane where it protects cells from apoptosis induction (Shamas-Din et al., 2013b). The *Bcl-x* gene is composed of three exons where exon 2 is a target for an alternative splicing (AS) event that results in two isoforms that actually act in an antagonistic way in the regulation of cell death: Bcl-xL (long isoform) is capable of repressing Bax and Bak actions and abrogating apoptosis, and Bcl-xS (short isoform) is pro-apoptotic (**Figure 29**) (Boise et al., 1993b). In fact, Bcl-xS can directly bind to and inhibit Bcl-xL and Bcl-2 proteins by creating heterodimers, resulting in the release of Bak (Plötz et al., 2012). As for the protein itself, Bcl-xL is a 233 aa protein with BH1-4 domains along with a TM domain. In contrast, Bcl-xS lacks a segment of 63 aa constituting both BH1 and 2 domains that are essential for the anti-apoptotic activity. Hence, the latter is inhibited in Bcl-xS. The AS of *Bcl-x* is a very critical event and needs to be regulated in order to determine whether the cell is sensitive or resistant to apoptosis. Bcl-xL is the most abundant form of the protein and is widely expressed mainly in the brain, kidneys and most importantly in the adult thymus (Boise et al., 1993b). It protects from cell death in different cited mechanisms. Consequently,

overexpressing Bcl-xL might result in enhanced cell survival (Yip and Reed, 2008), of which pancreatic islets β -cells (Carrington et al., 2009). More extremely, this overexpression might lead to cancer progression as in the case of breast (España et al., 2004) and urothelial (Yoshimine et al., 2013) cancers. Also, it facilitates the resistance to chemotherapy (Amundson et al., 2000). Accordingly, one might think that overexpressing Bcl-xS might induce exactly the opposite effect by favoring apoptosis in both pancreatic (Barbour et al., 2015) and cancer (Mercatante et al., 2001) cells.

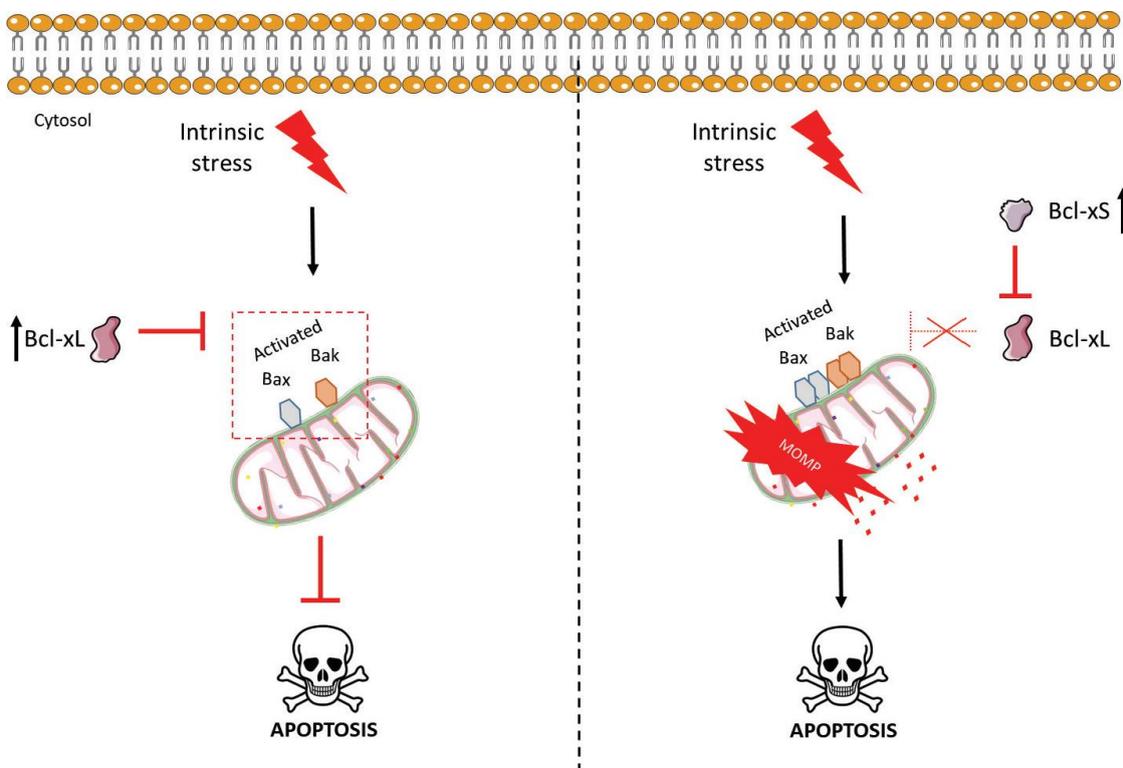


Figure 29: Signaling of Bcl-xL and Bcl-xS in the mitochondrial pathway of apoptosis.

Upon activation of the mitochondrial pathway of apoptosis by an internal stress, the anti-apoptotic Bcl-xL abrogates the activity of Bax/Bak thus preventing MOMP and subsequent apoptosis. In contrast, Bcl-xS inhibits Bcl-xL leading to the release of activated Bax and Bak executioners of MOMP and the necessary downstream events leading to cell death. Thus, Bcl-xL is anti-apoptotic and Bcl-xS is pro-apoptotic.

Before elaborating the wicked role of Bcl-xL in disease progression, we must first discuss its role in development. In fact, Bcl-xL has been shown to play vital roles in embryogenesis and nervous and hematopoietic systems development.

During early embryogenesis, Bcl-xL levels drastically increase from the two-cell stage and remain this way until the formation of the late blastocyst (Metcalf et al., 2004).

At around E10-11, Bcl-2 expression decreases after the formation of the neural tube and Bcl-xL expression increases and remain elevated throughout the neuronal ontogeny until it reaches its peak in differentiating cells (Abe-Dohmae et al., 1993; Merry et al., 1994). KO of *Bcl2l1*, the gene encoding Bcl-xL, leads to mouse embryonic mortality at E13.5 due to massive apoptosis in the immature cells of the nervous and hematopoietic systems. Interestingly, Bcl-xL induces the death of post-mitotic immature neurons of CNS suggesting its importance in immature cells survival during developmental stages (Motoyama et al., 1995). On the other hand, conditional ablation of this gene in a certain range of CNS neurons by Cre-LoxP system does not end in mice lethality but it decreased the number of catecholaminergic neurons indicating the importance once again of Bcl-xL in the proper development of catecholaminergic cells. Also, these cells did not produce striatal dopamine and had a decreased total brain mass (Savitt et al., 2005). Moreover, Bcl-xL plays a neuroprotective role during ischemic brain injury (Cao et al., 2002). The deficiency of Bcl-xL in the brain has serious effects even though the exact signaling regulating its expression in this organ are not clearly elaborated.

Furthermore, Bcl-xL plays a major role in the hematopoietic system. Bcl-xL was shown to regulate and induce the survival of double-positive (CD4+ CD8+) thymocytes. In fact, *Bcl-x-KO* thymocytes are more sensitive to apoptosis. However, germline ablation of its gene does not lead to deficiency in HSCs (Ma et al., 1995). In red blood cells, Bcl-xL expressions are upregulated during the megakaryocyte differentiation. In fact, mature erythrocytes have a short life-span and are regulated by the action of erythropoietin (EPO) that actually induces the expression of Bcl-xL (Sanz et al., 2001). These results are associated with the study mentioned where genetic KO of *Bcl2l1* leads to embryonic lethality due to a deficiency in mature red blood cells (Motoyama et al., 1995). In a similar manner, conditional KO of this gene in the hematopoietic system leads to an extreme anemia and profound splenomegaly caused in part by the reticulocytes cell death (Wagner et al., 2000). Bcl-xL is a major factor in the regulation of

megakaryocyte and platelets. Thrombopoietin (TPO) induces the differentiation of megakaryocytes and prompts their survival. TPO also increases the levels of Bcl-xL (Kirito et al., 2002). Accordingly, the loss of this anti-apoptotic protein drastically decreased the number of mice mature megakaryocytes (Debrincat et al., 2012). Furthermore, the idea of apoptosis in platelets was so unreal due to their lack of nuclei, however, these organelles possess a number of Bcl-2 family and display apoptotic features. This observation was confirmed by the use of the BH3 mimetic ABT-737 that targets Bcl-2, Bcl-xL and Bcl-w in mice. Upon treatment, they suffered from a transient thrombocytopenia (Oltersdorf et al., 2005).

This drug, and its derivative Navitoclax, induce platelet apoptosis by abrogating the activity of Bcl-xL and promoting Bak-dependent apoptosis (Mason et al., 2007; Zhang et al., 2007). Either genetic or pharmacological, the loss of Bcl-xL clearly affects platelet survival (**Figure 30**).

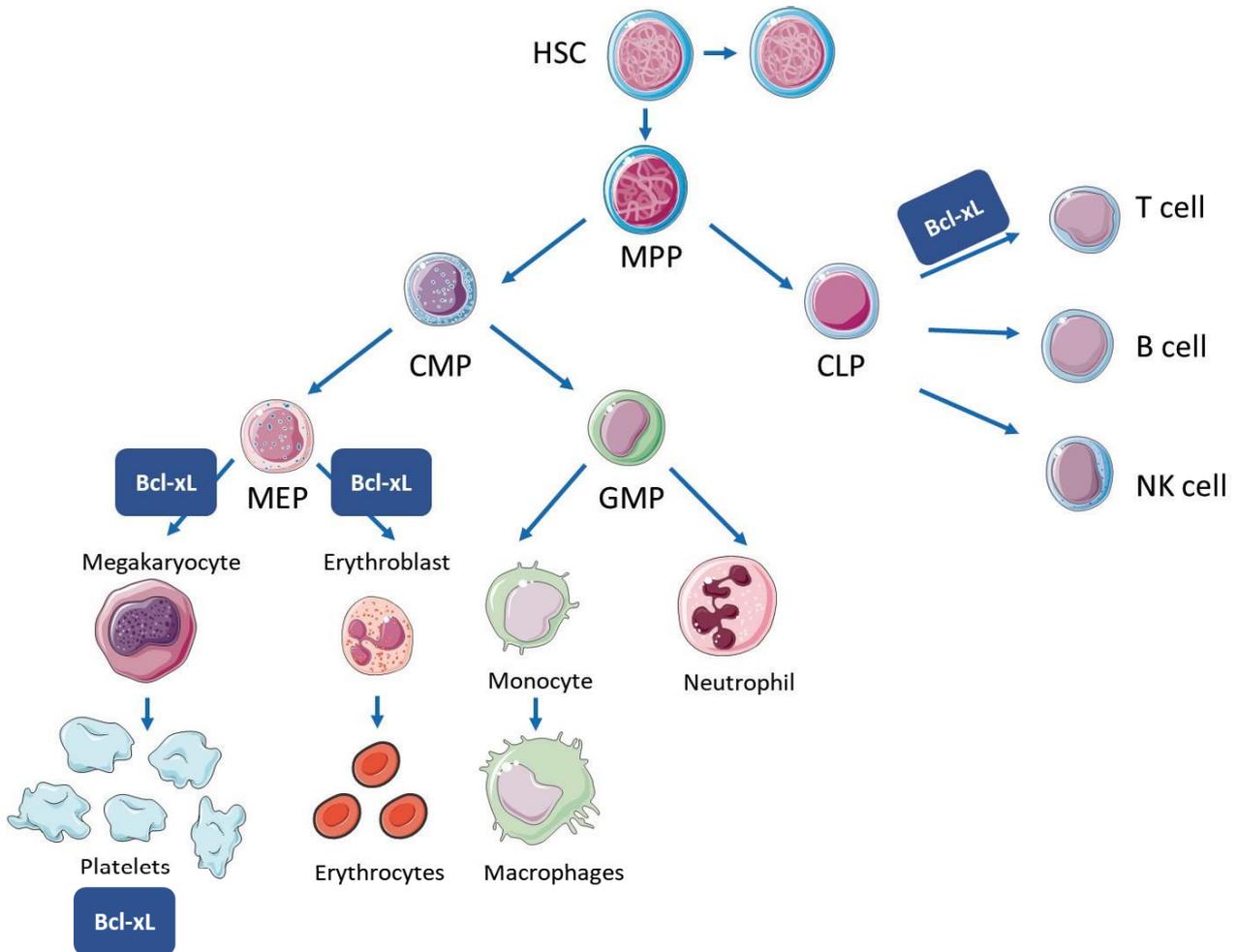


Figure 30: *Bcl-xL* involvement in hematopoiesis.

A HSC is capable of self-renewal and can generate multi-potent progenitors (MPPs). The latter gives rise to either common myeloid progenitor (CMP) or common lymphocyte progenitor (CLP). CMPs generate megakaryocyte-erythroid progenitor (MEP) and granulocyte-monocyte progenitor (GMP). The MEPs generate the megakaryocytes that further produce the platelets along with the erythroblasts that give rise to erythrocytes. The GMP give rise to monocytes that become macrophages and neutrophils. CLPs generate T and B lymphocytes and natural killer (NK) cells. *Bcl-xL* has been shown to be required for the differentiation of T cells, for the differentiation of megakaryocytes and erythroblasts and for platelet survival. Inspired by (Opferman and Kothari, 2018).

We have shown the most important implications of Bcl-xL in development. In the following part, we will briefly discuss its involvement in cancer progression.

Cancer cells might avoid cell death by disrupting the expressions of genes involved in apoptosis regulation such as *Bcl2l1* (Fernald and Kurokawa, 2013). Upregulated Bcl-xL levels have been seen in numerous cancers and have been associated with chemotherapy resistance. Of the human cancers involved, we cite: hepatocellular carcinoma (Watanabe et al., 2002), renal carcinoma (Gobé et al., 2002), pancreatic cancer (Ghaneh et al., 2002) and prostate cancer (Lebedeva et al., 2000).

A growing body of evidence supports the idea that Bcl-xL performs its pro-oncogenic activities by not only abrogating apoptosis but also favoring *non-cliché* triggers of cancer progression. Indeed, abrogating apoptosis is an old-fashioned concept compared to the induction of cell migration, invasion and metastasis. Bcl-xL was shown to play a role in the invasion of human malignant glioma cells (Weiler et al., 2006) and colorectal cancer cells (Koehler et al., 2013). This member actually facilitates the migration and metastasis without affecting tumor growth in breast cancer independently from its anti-apoptotic potential (Choi et al., 2016; Martin et al., 2004). In contrast, Bcl-xL enhanced tumor formation and growth when overexpressed and decreased tumor invasiveness when KO but without influencing apoptosis nor tumor growth (Choi et al., 2016; Hager et al., 2009).

There is an involvement of Bcl-xL in EMT upon ionizing radiation that induced its accumulation promoting cancer cell invasion and EMT-correlated markers in lung cancer cells (Ho et al., 2010). This is promoted by the nuclear localization of the protein. In human colorectal cells, the KD of *Bcl-x* by Adenovirus-mediated RNA interference inhibited their proliferation and colony formation. Also, it cardinally suppressed their migration and invasion further insisting on this non-canonical function of Bcl-xL (Yang et al., 2011).

Moreover, Bcl-xL is shown to promote tumor angiogenesis *via* the NFκB/interleukin 8 (CXCL8) axis in several cell lines like glioblastoma and melanoma (Gabellini et al., 2008, 2018; Giorgini et al., 2007). In fact, both Bcl-2 and Bcl-xL are important factors in the regulation of the unidirectional crosstalk between tumor and neovascular endothelial cells (Karl et al., 2007).

Other data have shed the light on the involvement of Bcl-xL in cancer stem cell (CSC) nature of several tumors. In fact, the survival of lung (Zeuner et al., 2014) and colon (Colak et al., 2014) tumors primarily depends on Bcl-xL. A particular involvement in senescence of Bcl-xL was also reported where its activation is a critical feature through which senescent cells resist to apoptosis. Targeting Bcl-xL *in-vitro* and *in-vivo* induced apoptosis in senescent cells (Yosef et al., 2016).

Consequently, we chose to study this protein based on its vital involvement in mammalian development and in cancer progression as shown in this brief overview.

5.2. Aim of the project

As discussed throughout my bibliography review, non-canonical roles of the Bcl-2 family have emerged and novel functions and interactions correlate this family to numerous cellular signaling pathways. Bcl-xL is a ubiquitously expressed protein that has been shown to be vital in embryonic development (Motoyama et al., 1995). Soon enough, it was considered a marker of tumor progression in invasive breast cancers (Olopade et al., 1997). This member was shown to be upregulated in cancer where it not only inhibits apoptosis but also promotes proliferation, migration and invasion.

Being strictly mitochondrial at first, Bcl-xL was later shown to localize at the ER where it performs other subcellular functions (Minn et al., 1997; Tagami et al., 2000). Afterwards, numerous data highlighted the involvement of Bcl-xL in Ca^{2+} homeostasis. A recent study in our team had shed the light on the exclusive functions of Bcl-xL in specific subcellular compartments. Both KD and KO of Bcl-xL in mammary cancer cells resulted in a reduced migration of these cells in an individual and non-directional manner. However, the complementation with Bcl-xL targeted to the mitochondria, and not the ER, rescued the migration phenotype. It appeared that Bcl-xL at the mitochondria interacts with VDAC1 and promotes the migration *via* the intracellular Ca^{2+} fluxes independently from its role in apoptosis prevention (Bessou et al., under review). In a study conducted by Eno and colleagues, Bcl-xL was shown to perform various functions depending on its subcellular localization. They generated *Bclx*-KO MEFs that were vulnerable to apoptotic stimuli. When transfected with mitochondria-targeted Bcl-xL, these MEFs were able to regain their anti-apoptotic function in contrast to those transfected with ER-targeted Bcl-xL that were sensitive to apoptotic stimuli. However, only ER-targeted Bcl-xL MEFs were capable of restoring Ca^{2+} homeostasis. These data demonstrate that Bcl-xL is only anti-apoptotic when present at the mitochondria and it impinges on Ca^{2+} homeostasis when present at the ER where it has no involvement in apoptosis regulation (Eno et al., 2012). Indeed, at the ER, Bcl-xL interacts with Ca^{2+} transporters even though some interactions' consequences are still debatable as mentioned in (part 2).

Compiling the research data along with the results we got in the team, we had two main questions in mind:

- 1- What is the exact role of Bcl-xL at the ER?
- 2- What is the contribution of the ER and mitochondrial pools of Bcl-xL in mammalian development?

To answer these questions, our team has generated genetically modified mice that express Bcl-xL exclusively at the ER or at the mitochondria by Cre-LoxP system. The first thing we did is to check the expression of Bcl-xL in the mice (in several organs and in MEFs extracted from the mice).

My study involved the complete characterization of isolated MEFs from these genetically modified mice. The subcellular localization of Bcl-xL was verified by three different techniques in the targeted MEFs compared to the WT MEFs. Afterwards, we assessed the protective capacity of MEF with cytotoxic drugs and ER stress inducers. Interestingly, we have demonstrated that ER-targeted Bcl-xL is able to confer resistance against ER stress. Accordingly, we thought that this resistance might involve Ca^{2+} regulation. To assess this, we performed several Ca^{2+} detection assays in order to see whether Bcl-xL at the ER acts differently from that on the mitochondria when ER stressed. Indeed, it does where it basically decreases the release of Ca^{2+} from IP3R. We then thought to assess the Unfolded Protein Response in these MEFs upon ER stress. In an interesting manner, Bcl-xL at the ER was shown to decrease the UPR markers' expression compared to WT and mitochondria-targeted Bcl-xL.

This study puts in evidence a moonlighting function of Bcl-xL at the ER where it is able to decrease IP3R-mediated Ca^{2+} release upon ER stress thus attenuating the UPR and protecting the cell from apoptosis.

RESULTS

ARTICLE 1

Unmasking a novel role of the anti-apoptotic member Bcl-xL at the Endoplasmic Reticulum

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In preparation for submission

Abstract

Apoptosis plays a role in cell homeostasis in both normal development and disease. Bcl-xL, a member of the Bcl-2 family of proteins, regulates the intrinsic pathway of apoptosis. It is overexpressed in several cancers. This member has a dual subcellular localization and is found at the mitochondria as well as the endoplasmic reticulum (ER). However, the subsequent function of its ER localization is poorly explored. In order to decipher the functional contributions of the mitochondrial and reticular pools of Bcl-xL, we generated genetically modified mice expressing exclusively Bcl-xL at the ER referred to as ER-xL or mitochondria referred to as Mt-xL. Subcellular localization experiments on mice embryonic fibroblasts (MEFs) validated the exclusive Bcl-xL localization in these mice. By performing cell death assays, we showed that ER-xL MEFs show increased vulnerability to apoptotic stimuli but are more resistant to ER stress. Furthermore, ER-xL MEFs demonstrated a reduced expression of the Unfolded Protein Response (UPR) markers upon ER stress and displayed reduced Inositol triphosphate receptor (IP3R)-mediated calcium release. We have shown for the first time that upon ER stress, Bcl-xL at the ER negatively regulates IP3R inhibiting ER calcium depletion necessary for UPR initiation and subsequently apoptosis. Overall, this work reveals a moonlighting function of Bcl-xL at the ER, apart from its cliché regulation of apoptosis.

Introduction

Bcl-xL is a member of the famous Bcl-2 family of proteins ¹. This family decides the fate of cells by regulating apoptosis. It is composed of numerous proteins that are divided based on their Bcl-2 homology (BH) structure and function. Bcl-xL and Bcl-2 itself belong to the multidomain anti-apoptotic members whose sole aim is to prevent apoptosis. On the other hand, other multidomain proteins exist (Bax, Bak and Bok) but promote cell death. The last subgroup regroups the BH3-only proteins (Bad, Bid, Puma...) that only possess the BH3 domain as their name implies and are anti-survival ². Proapoptotic proteins induce mitochondrial outer membrane permeabilization (MOMP), a necessary checkpoint upstream of the activation of the caspase cascade resulting in apoptosis ³.

Based on their crucial function of regulating cell death, dysregulations on the gene or protein levels of these proteins might have detrimental outcomes such as cancer progression. Indeed, Bcl-xL is upregulated in hepatocellular and renal carcinoma ^{4,5} and pancreatic cancer ⁶. In breast cancer, Bcl-xL increased levels enhanced metastatic potential without affecting tumor growth in the mammary gland independently from its anti-apoptotic potential ^{7,8}. Furthermore, Bcl-xL was shown to play a role in the invasion of human malignant glioma ⁹ and colorectal cancer cells ¹⁰. Consequently, Bcl-xL plays a vital role in cancer progression.

Throughout the years, non-canonical functions of this family were unmasked and involved intracellular calcium (Ca^{2+}) fluxes ¹¹. Besides their mitochondrial localization, most of the Bcl-2 proteins translocate to other subcellular compartments where they perform diverse functions such as cell cycle regulation ¹². The endoplasmic reticulum (ER) constitutes a welcoming hub for some of the Bcl-2 proteins where they interact with ER-localized Ca^{2+} channels and impinge on Ca^{2+} homeostasis regulation. For instance, Bcl-2, *via* its BH4 domain, interacts with the modulatory and transducing domain (MTD) of the inositol-3-phosphate receptor 1 (IP3R1) thus inhibiting ER Ca^{2+} discharge ¹³. In contrast, Bcl-xL interacts with the coupling domain (CD) of IP3R and sensitizes the channel to low concentration of IP3. Hence, this interaction induces ER Ca^{2+} release and promotes Ca^{2+} oscillations required for mitochondrial energetics ¹⁴. Numerous studies have elaborated the role of Bcl-xL at the mitochondria but so little have studied its contribution on the ER. Mouse embryonic fibroblasts (MEFs) in which Bcl-xL is exclusively targeted to the ER were sensitive to apoptotic stimuli but in an interesting manner, were capable of restoring Ca^{2+} homeostasis, even though the underlying mechanisms are not elaborated ¹⁵. Therefore, Bcl-xL performs completely distinct roles at each organelle. It is important to note that the mice knockout (KO) of the *bclx* gene encoding Bcl-xL resulted in embryonic lethality at embryonic day 13.5 insisting on the indispensable role this protein plays not only in cancer progression but also in embryonic development ¹⁶. The question that remained unanswered is: what is beyond regulating ER Ca^{2+} fluxes in mammalian development and cancer progression?

To address this matter, we generated genetically modified mice by Cre-LoxP recombination system in which Bcl-xL is targeted exclusively to the ER or to the mitochondria. Both mice survive the embryonic stage suggesting that the ER localization of the protein is indeed crucial for embryonic development.

After extracting MEFs, we verified the subcellular localization along with the expression of Bcl-xL. Upon cytotoxic drug treatment, MEFs with Bcl-xL targeted to the ER were more prone to cell death inducers but interestingly resisted ER stress stimuli. We hypothesized that Bcl-xL might confer this resistance through its influence on IP3R-Ca²⁺ release. Indeed, by several intracellular and intra-organelle Ca²⁺ measurements, we show that ER-targeted Bcl-xL decreases IP3R Ca²⁺ release during ER stress. In an interesting manner, the ER localization of the protein reduced the Unfolded Protein Response (UPR) markers' expression in ER stress conditions compared to wildtype (WT) and mitochondria-targeted Bcl-xL. Altogether, our data shed the light on a moonlighting function of Bcl-xL at the ER where it reduces the UPR through the reduction of IP3R-Ca²⁺ release upon ER stress.

Results

Bcl-xL possesses a double subcellular localization and protects from ER stress

Knowing that Bcl-xL modulates cellular responses to pro-apoptotic stimuli, we wanted to verify this regulation first in MEFs and second, in our system. To do so, MEFs deficient in Bcl-xL expression *bclx*-Knockout (KO) were first verified for their lack of Bcl-xL (Figure 1A) and treated, along with WT MEFs, with cytotoxic drugs such as the protein kinase C (PKC) inhibitor staurosporine and the topoisomerase inhibitor etoposide (Figure 1B). Representative curves of induced cell death-labelled cells are shown in (Supplementary Figure 1A,B). As expected, KO MEFs were more vulnerable to apoptotic inducers than WT MEFs. Likewise, KO MEFs were sensitive to the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) inhibitor thapsigargin suggesting a role of the protein in ER stress response (Figure 1C) with the representative curves shown in (Supplementary Figure 1C). In order to verify whether the cell death observed is apoptosis-dependent, we treated WT and KO MEFs with staurosporine (250nM for 6hrs) or thapsigargin (4µM for 24hrs) and we checked for cleaved caspase 3 staining, marker of apoptosis. As shown in (Figure 1D), the cell death increase in these MEFs is caspase-dependent and was significantly induced in KO compared to WT MEFs further confirming the results in (Figure 1B,C). Representative images of the cleaved caspase 3 staining are presented in (Supplementary Figure 1D). Furthermore, we investigated the influence of Bcl-xL on the UPR, a conserved ER stress feedback in response to ER stress inducing agents such as thapsigargin. In an interesting manner, eIF2a expression and eIF2a

phosphorylation were increased even at the start point at t=0 hour (hr) further insisting on the major regulation of Bcl-xL in situations of ER stress. Moreover, CHOP and ATF4 levels were gradually and significantly enhanced in KO MEFs upon the treatment constituting as well major events in the UPR¹⁷ (Figure 1E). Establishing that Bcl-xL also modulates ER stress response, we wanted to verify the subcellular localization of this protein in MEFs. Subcellular fractionation experiments on WT MEFs put in evidence the major localization of Bcl-xL at the mitochondria followed by the cytosol and to a lesser but great extent, the ER (Figure 1F). In fact, Bcl-xL is predominantly localized on the mitochondrial outer membrane (MOM) targeted by its C-terminal signal sequence¹⁸. Previous data have shown diverse functions of Bcl-xL at each subcellular compartment¹⁵. However, the underlying mechanisms promoting these functions are still not elaborated, at least not at the ER.

Bcl-xL is localized at the ER in ER-xL mice and the mitochondria in Mt-xL mice

In order to decipher the exact functional contribution of the ER and mitochondrial pools of Bcl-xL, we have generated recombinant mice by Cre-LoxP system in which Bcl-xL is exclusively at the ER (ER-xL) or at the mitochondria (Mt-xL). An engineered vector containing the WT Bcl-xL exon 3 and Cytochrome B (CB5) or ActA mutant sequences that target the proteins to the ER or mitochondria, respectively, was inserted to replace the WT Bcl-xL exon 3 in floxed mice. Cre-recombination induced WT exon 3 excision and CB5 or ActA mutant expression. Accordingly, three protein products are possible: WT Bcl-xL with intact transmembrane (TM) domain, ER-xL with CB5 targeting sequence and Mt-xL with ActA targeting sequence (Figure 2A).

Once ER-xL and Mt-xL mice were generated, we verified Bcl-xL expression at the gene level (Supplementary Figure 2A) where PCR results exposed the difference in size of DNA products of WT, heterozygous (HET) (WT allele, mutant allele), ER-xL and Mt-xL mice. Next, we sought to verify Bcl-xL expression on the protein level in tissues of the lung, liver and brain of WT, ER-xL and Mt-xL mice (Figure 2B). Modifying the TM domain of the protein, both CB5 and ActA sequences result in a larger Bcl-xL protein sequence of 445 and 442 amino acids (aa), respectively. Likewise, Bcl-xL protein expression was certified in MEFs derived from WT, ER-xL and Mt-xL mice embryos at embryonic day 13 (Figure 2C). MEFs were immortalized by serial passaging and used in all experiments thereafter.

Once the protein expression demonstrated, we aimed at verifying the subcellular localization of Bcl-xL in immortalized MEFs. To do so, we have performed immunofluorescence experiments on all three types of MEFs transfected with ER-targeted GFP. As shown in (Figure 2D), Bcl-xL is mainly at the ER in ER-xL MEFs, at the mitochondria in Mt-xL MEFs and co-localized at both organelles in WT MEFs as indicated by the profile plots of fluorescence intensity. The subcellular localization of Bcl-xL was also confirmed by ER-xL and Mt-xL MEFs subcellular fractionation technique as displayed in (Figure 2E) and by ImmunoGold labelling of Bcl-xL in (Supplementary Figure 2B,C).

Bcl-xL at the ER, and not at the mitochondria, confers resistance to ER stress

Upon the verification of the expression and localization of Bcl-xL, we wanted to challenge the anti-apoptotic potential of the protein by treating the MEFs with staurosporine. Accordingly, cleaved caspase 3 assay on fixed MEFs after staurosporine treatment for 6hrs put in evidence the vulnerability of ER-xL MEFs compared to WT and Mt-xL treated MEFs. Interestingly, the control ER-xL MEFs displayed slightly more cleaved caspase 3 compared to the other control MEFs indicating that the exclusive ER localization itself of the protein stresses the cells (Figure 3A). Representative images of caspase 3 cleavage are displayed in (Supplementary Figure 3A). These data were confirmed by the obviously greater cleavage of PARP indicating apoptosis upon staurosporine treatment in ER-xL compared to WT MEFs (Figure 3B). Cell cycle measurements with propidium iodide (PI) displayed the SubG1 population as highest in ER-xL MEFs further confirming the cell death vulnerability of these MEFs (Supplementary Figure 3B). Altogether, these data highlight the importance of the mitochondrial localization of Bcl-xL in apoptosis prevention.

To analyze the role of Bcl-xL under ER stress conditions, MEFs were treated with thapsigargin or protein folding blocker tunicamycin. Interestingly, ER-xL MEFs significantly resisted ER stress induction compared to other MEFs as shown in (Figure 3C) with the corresponding curves in (Supplementary Figure 3C,D). Cleaved caspase 3 immunofluorescence assay on MEFs treated with thapsigargin for 24hrs also confirmed the resistance of ER-xL MEFs (Figure 4D). Representative images of the staining are displayed in (Supplementary Figure 3E). Moreover, PARP cleavage was shown to be more pronounced in WT and Mt-xL MEFs compared to ER-xL MEFs upon 24hrs of thapsigargin treatment. Interestingly, the basal level

of expression of PARP in the latter cells was lower indicating a greater resistance to apoptosis (Figure 3E). Therefore, the ER localization of Bcl-xL, and not the mitochondrial one, is responsible for the protection against ER stress.

ER-localized Bcl-xL impairs UPR initiation under ER stress conditions

To further understand how ER-localized Bcl-xL protects from ER stress, we checked the influence of this localization on the UPR as already demonstrated with KO MEFs (Figure 1E). Upon thapsigargin treatment for 24hrs, ER-xL MEFs displayed reduced PERK phosphorylation compared to WT and Mt-xL cells (Figure 4A). This phosphorylation is indeed the first step of the PERK arm pathway activation in the UPR¹⁹. Moreover, Mt-xL MEFs exhibited much higher ATF4 expression levels, gradually increased phosphorylation of eIF2a and CHOP compared to ER-xL MEFs and WT MEFs (Figure 4B).

Then, we ought to check whether the UPR response is modified also on the gene level. ATF3, a downstream effector of ATF4, induces the transcription of CHOP and GADD34²⁰. The latter is needed to guide protein phosphatase 1 (PP1) to dephosphorylate eIF2a. As for ATF5, it is a downstream effector of phosphorylated eIF2a that delays translation initiation upon ER stress²¹. Also, the IRE1 arm of the UPR results in xbp1 splicing activating the transcription of genes involved in cell death²². Consequently, the ER localization of Bcl-xL decreases the mRNA expression of all cited markers in ER stress situations further confirming the protein-level correlated results (Figure 4C).

Altogether, we have found new evidence on ER stress reduction by a non-mitochondrial localized Bcl-xL.

ER-xL blocks IP3R-Ca²⁺ release

In order to understand how Bcl-xL acts on the UPR, we hypothesized that this influence could be through ER-Ca²⁺ regulation. Indeed, Bcl-xL physically interacts with IP3R on the ER through its hydrophobic cleft (BH2, BH1 and BH3) and sensitizes it to low concentrations of IP3^{14,23,24}. This protein also binds to and inhibits Ryanodine Receptor 3 (RyR)-mediated Ca²⁺ release²⁵. Accordingly, Bcl-xL is a major modulator of Ca²⁺ fluxes at the ER. To confirm the physical interaction of Bcl-xL with IP3R in our MEFs, a Proximity

Ligation Assay (PLA) was performed on WT and KO MEFs (Figure 5A,B). Indeed, Bcl-xL interacts with IP3R in WT MEFs.

Once proven, our aim was to elaborate the exact effect of Bcl-xL on IP3R-Ca²⁺ discharge. By blocking SERCA with thapsigargin, ER-Ca²⁺ passive leakage was detected in MEFs transfected with the fluorescent ER-targeted Ca²⁺ indicator R-CEPIA1er. As shown, ER-xL MEFs were able to release more Ca²⁺ to the cytosol than WT and Mt-xL ones (Figure 5C,D).

To decipher if the increase in ER-Ca²⁺ release is Bcl-xL/IP3R dependent, we activated IP3R by m-3M3FBS Phospholipase C activator enhancing the production of IP3. Strikingly, IP3R-Ca²⁺ release in ER-xL MEFs was significantly slower compared to the WT. In contrast, Mt-xL MEFs were able to enhance this release upon IP3R activation (Figure 5E,F). Two hypothesis were elaborated: Either less Ca²⁺ is present in the ER of ER-xL MEFs or Bcl-xL inhibits IP3R. To examine this, ⁴⁵Ca²⁺ measurements were conducted in permeabilized MEFs. In fact, Ca²⁺ release was detected after loading the cells with ⁴⁵Ca²⁺ with the addition of the Ca²⁺ ionophore A23187. The latter will sequester Ca²⁺ ions that will then be subtracted from the stored ⁴⁵Ca²⁺. Accordingly, IP3R-Ca²⁺ release will be determined as the rate of the decrease of the ER ⁴⁵Ca²⁺ storage *via* the addition of IP3²⁶⁻²⁸. As shown in (Figure 5G), ER-xL MEFs displayed lower ⁴⁵Ca²⁺ discharge from IP3R upon increased concentrations of IP3. Altogether, our data show that Bcl-xL reduces IP3R-Ca²⁺ release.

We also checked the store operated calcium entry (SOCE) that is a crucial mechanism promoting the entry of Ca²⁺ ions to the ER in response to ER-Ca²⁺ depletion^{29,30}. After emptying ER-Ca²⁺ stores by thapsigargin, CaCl₂ was added to analyze the SOCE. As a consequence, SOCE was markedly increased in ER-xL MEFs, even though, it was also enhanced in Mt-xL MEFs compared to the WT (Supplementary figure 4A,B). As for cytosolic Ca²⁺ levels after the ionophore ionomycin injection, they were highest in WT MEFs compared to the others (Supplementary figure 4C,D).

ER-xL decreases the UPR by inhibiting IP3R-Ca²⁺ depletion upon ER stress induction

Our results put in evidence, first, the protection conferred by ER-localized Bcl-xL against ER stress and second, the inhibition of IP3R-Ca²⁺ by the same ER-localized Bcl-xL. We hypothesized that a link between

both observations is present. In order to verify this hypothesis, we inhibited the IP3R by 2-Aminoethoxydiphenyl-borate (2-APB) ^{31,32} and treated the MEFs with thapsigargin for 24hrs. Interestingly, a significant reduction in UPR markers' expression was observed in both WT and Mt-xL MEFs in a resembling way to ER-xL MEFs protecting from ER stress (Figure 6A). It is worth to note that ATF4 and CHOP levels were significantly decreased in Mt-xL in this case compared to previous results without IP3R inhibition; recall (Figure 4B). To further confirm this result, we used Xestospongin C, another IP3R inhibitor ^{33,34}. Indeed, by inhibiting IP3R, the UPR markers' expression is once again decreased upon thapsigargin treatment (Figure 6B). It is important to add that these IP3R inhibitors do not inhibit specifically and solely IP3R. Actually, 2-APB also performs inhibitory effects on ion channels like transient receptor potential (TRP) family ^{35,36}, potassium channels ³⁷, and volume-regulated anion channels³⁸. Likewise, Xestospongin C also inhibits SERCA ^{39,40}. Consequently, these inhibitors could not be considered as potent selective blockers of IP3Rs. However, our results were clearly replicated with the use of both inhibitors suggesting that the shared effect could be that of the IP3R inhibition.

Finally, we propose a model for Bcl-xL action on the ER (Figure 6C). Upon ER stress, Bcl-xL – through its inhibition of IP3R-Ca²⁺ release – reduces the UPR resulting in the abrogation of the cell death response. This protein was shown to restore Ca²⁺ homeostasis¹⁵ but our data highlight the downstream consequences putting in evidence the indirect anti-apoptotic role of Bcl-xL at the ER.

Discussion

Despite the direct role of Bcl-2 proteins in regulating apoptosis, non-canonical functions emerged throughout the years ¹¹. Bcl-2 members perform these various roles mainly through intracellular calcium fluxes regulation ^{39,41}. Although mitochondria are considered the major hub of Bcl-2 proteins, many can also localize to the ER where they might exert opposing effects on ER-mediated calcium handling ⁴². Bcl-xL is majorly targeted to the MOM through its TM flanked by two basic aa ¹⁸. However, this protein can localize to the ER where it interacts and affects ER calcium content ^{14,43}.

As any anti-apoptotic protein, Bcl-xL overexpression promoting cancer progression is not to be underestimated in numerous types of cancers ⁸⁻¹⁰. However, targeting Bcl-xL by the orally available BH3

mimetic ABT-263 induced a dose-limiting thrombocytopenia due to the inhibition of this protein on which platelets depend for their survival^{44,45}. Thus understanding the multiple cellular and molecular mechanisms that drive these subcellular localization-mediated regulations is an important step towards designing effective targeting therapies against anti-apoptotic members. Here, we specifically interrogated mechanisms beyond the localization of Bcl-xL at the ER and its involvement in ER calcium regulation. *Bcl-x* KO MEFs' vulnerability to cytotoxic drugs replicated previous data insisting on MOMP prevention role of Bcl-xL². Furthermore, *Bcl-x* KO enhanced the UPR suggesting a function of Bcl-xL in ER stress resistance affecting the UPR.

By generating ER and mitochondria-targeted Bcl-xL modified mice, we aimed at understanding how each organelle affects Bcl-xL functions. Staurosporine significantly enhanced cell death in ER-xL MEFs further confirming the cell death protection potential of mitochondrial Bcl-xL¹⁵. Interestingly, ER-xL MEFs were resistant to ER stress inducing agents thapsigargin and tunicamycin. Reduced UPR markers expression further confirmed this resistance. In fact, Bcl-xL was shown to protect from ER stress through the intermediary of the BH3-only protein Bim. By sequestering Bim at the mitochondria, Bcl-xL reduces CHOP-induced apoptosis during plasma cell differentiation⁴⁶. Also, Bcl-xL on the MOM was shown to inhibit the translocation of Bim to the ER upon ER stress thereby abrogating caspase 12-mediated apoptosis in murine myoblast cells⁴⁷. In contrast, our data highlight the involvement of the ER localization of Bcl-xL, and not the mitochondrial one, in ER stress reduction.

At the ER, Bcl-xL interacts with the coupling domain of IP3R where it promotes ER-to-mitochondria calcium signaling by sensitizing IP3R to basal levels of IP3¹⁴. Prolonged activation of IP3R by m-3M3FBS and Ca⁴⁵ flux assays both showed that Bcl-xL at the ER reduces IP3R-mediated calcium discharge. This phenomenon might act as a survival defense mechanism in case of ER stress. In fact, ER calcium stores depletion is a critical trigger of UPR initiation. Based on the duration and severity of the stress, unfolded proteins accumulate in ER lumen. As a consequence, ER-stress sensors (PERK, IRE1 and ATF6) are activated, setting in motion early adaptive responses that prompt survival or late responses provoking apoptosis under conditions of severe or prolonged ER stress⁴⁸. Considering a link between the inhibitory action of Bcl-xL on IP3R and the resistance towards ER stress demonstrated only in ER-xL MEFs, we hypothesized that the inhibition of IP3R will reduce UPR markers expression in WT and Mt-xL MEFs.

Indeed, by doing so, ER stress response was reduced in the cited MEFs. Interestingly, ATF4 levels were further dropped even in ER-xL MEFs suggesting that not only Bcl-xL at the ER reduces UPR initiation but also this reduction is IP3R inhibition-dependent.

Altogether, we propose a novel mode of action of the anti-apoptotic member Bcl-xL at the ER. Upon ER stress induction, Bcl-xL negatively regulates IP3R-mediated calcium release preventing ER calcium depletion. Accordingly, this action averts UPR initiation that might lead to apoptosis execution further promoting cell survival.

Materials and methods

Cell lines and drugs. Mouse Embryonic Fibroblasts (WT, ER-xL and Mt-xL) were extracted from mice embryos at Embryonic day 13 (E13) and cultured along with *Bcl-x KO* MEFs (from Li laboratory) and HeLa cells under standard cell culture conditions (37°C, 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) high glucose medium (Gibco) supplemented with 10% Fetal Bovine Serum (FBS ; Sigma Aldrich), 100U/mL penicillin and 100µg/mL streptomycin (Gibco). Several cytotoxic drugs, Ca²⁺ ionophores, channel inhibitors were used: Staurosporine (Sigma Aldrich), Etoposide (Sigma-Aldrich), Thapsigargin (Enzo Life Sciences), Tunicamycin (Sigma Aldrich), Ionomycin (Enzo Life Sciences), 2-APB (Tocris), Xestospongine C (Tocris), m-3M3FBS (Sigma Aldrich). Z-VAD-FMK (Enzo Life Sciences) was used as a pan caspase inhibitor.

Cell death detection. For cell death detection experiments, MEFs were analyzed using Incucyte ZOOM live-cell imaging system (Essen Bioscience). In brief, cell death was assessed using SYTOX Green nucleic acid stain at 250nM concentration (ThermoFisher Scientific) plus the addition of several cell death inducers: Staurosporine 1µM, Etoposide 10µM, Thapsigargin 1µM, Tunicamycin 2µg/mL. Images were automatically acquired in phase and green fluorescent channels every one hour for 72 hours (hrs) at 4X magnification. Data were processed using a dedicated algorithm (Essen Bioscience). Cell proliferation was estimated using the phase-contrast images of the controls of the same experiments.

Immunofluorescence. MEFs were seeded on a glass coverslip in 12-well plates at around 40% of confluence and transfected with ER-targeted GFP. After cell attachment, the medium was discarded and the cells were incubated with mitochondria-staining dye (MitoTracker Red CMXRos, Life technologies) for 20min at 37°C then fixed with 4% Paraformaldehyde with 0.3% Triton X100. MEFs were washed 3 times with 0.1% Triton-PBS then incubated for 20 minutes (min) with blocking buffer (0.1% Triton X100, 3% BSA in PBS). Cells were subsequently incubated for 1hr with primary antibodies and 1hr with Alexa fluor 563 coupled secondary antibodies. Cells were washed 3 times after the incubation with both antibodies. Coverslips were mounted with a Dako mounting medium. Bcl-xL antibody (Cell Signaling #2764; 1/200) was used to detect endogenous Bcl-xL. For apoptosis assay after 250nM staurosporine treatment for 6hrs or 4µM thapsigargin treatment for 24hrs, the same procedure was performed without transfecting the MEFs. Cleaved caspase 3 (Cell Signaling #9661, 1/1000) antibody was used to assess cell death followed by Alexa fluor 563 coupled secondary antibodies. Nuclei were detected with Hoechst 33342 dye (Invitrogen #H3570) at 10µg/mL. Images were acquired using a Zeiss 780 confocal microscope.

Antibodies used for Western Blot. The following antibodies were used: Vinculin (Santa Cruz #sc-55465; 1/2000), Purified Mouse Anti-ATP Synthase β - F0F1 (BD Transduction Laboratories #612518; 1/2000), Calnexin (Cell signaling #2679; 1/1000), α -tubulin (Santa Cruz #sc-32293; 1/1000), Actin (Sigma-Aldrich #A2066; 1/500), PERK (Cell signaling #3192; 1/1000), phospho-PERK (Cell signaling #3179; 1/1000), eIF2a (Cell Signaling #2103, 1/1000), phospho-eIF2a (Cell Signaling #3398, 1/1000), ATF4 (Cell Signaling #11815, 1/1000), CHOP (Cell Signaling #5554, 1/1000), Bcl-xL (Cell Signaling #2764, 1/1000), PARP (abcam #ab6079, 1/500), Cleaved caspase 3 (Cell Signaling #9661, 1/1000), VDAC1 (abcam #ab14734, 1/1000), TOM20 (Santa Cruz #sc-11415, 1/500), GRP75 (Santa Cruz #sc-133137, 1/1000), HA (Sigma Aldrich #H6908, 1/1000), Flag M2 (Sigma Aldrich #F3165, 1/1000). HRP-conjugated goat anti-mouse and goat anti-rabbit antibodies (DAKO) were used as secondary antibodies. Immunoblotting was performed according to standard procedures. For kinetic immunoblotting, MEFs were treated with the indicated drug and were harvested at each indicated time point where they were lysed and their protein content was measured.

Subcellular fractionation. MEFs were harvested by trypsinisation and homogenized in MB buffer (210mM mannitol, 70mM sucrose, 1mM EDTA and 10mM HEPES (pH 7.4) containing protease inhibitors) by shearing (around 20 times) with a 1mL syringe and a 26G 5/8 needle. Homogenization was visually confirmed under the microscope. All steps were carried out at 4°C. Cell extracts were centrifuged 3 times (10min at 1,500g) to remove the nuclear fraction (pellet). Supernatants were then centrifuged 3 times (10min at 10,600 g). After the third round of centrifugation, crude mitochondria pellet was carefully collected with a micropipette, suspended in MB buffer and a Percoll medium (225mM mannitol, 25mMHEPES (pH 7.4), 1mMEGTA and 30% Percol (vol/vol)) and centrifuged for 30min at 95000g separating the MAMs and the mitochondria. The supernatant was centrifuged for 1hr at 100,000g. After this last round of centrifugation, the obtained microsome pellet was resuspended in RIPA buffer. The purity of obtained fractions was assessed by Western blot.

Intracellular calcium measurements. For ER-Ca²⁺ measurements, MEFs were transfected with 2µg RCEPIA 1er probe 48hrs before starting the experiment. Cells were seeded at 40% confluence in 8 well Nunc™ Lab-Tek™ II Chamber Slide and incubated with EM buffer (121mM NaCl; 5.4mM KCl, 2.6mM MgCl₂ hexahydrate, 6mM NaHCO₃, 5mM D-Glucose, 25mM HEPES pH 7.3). 10µM thapsigargin injections were done while quantifying the fluorescence with a Zeiss LSM 780 confocal microscope.

SOCE measurements were performed on MEFs incubated for 1hr with 5µM Fluoforte dye, and washed with BBS containing 0.1mM EGTA. After 20 seconds (s) of baseline fluorescence reading, thapsigargin 10µM was injected followed by 2mM CaCl₂ once the fluorescence drops back to baseline.

For cytosolic Ca²⁺ measurements, MEFs were seeded onto a 96-well plate one day before the experiment. Cells were then loaded with 5µM Fluoforte dye (Enzo Life Sciences) in EM buffer for 45min at 37°C followed by 2 washes with EM buffer. Fluorescence was assessed using the ClarioSTAR plate reader, following 15µM Ionomycin injections (Enzo Life Sciences).

For IP3R-induced Ca²⁺ release, transfected MEFs with RCEPIA 1er probe were injected with 25 µM m-3M3FBS (Sigma-Aldrich), direct activator of Phospholipase C. The Fluorescence was assessed using

Zeiss LSM 780 confocal microscope. The speed of Ca^{2+} release was calculated by measuring the slope of the increase of fluorescence upon injection.

ImmunoGold labelling. Subcellular localization of endogenous Bcl-xL in WT, ER-xL, Mt-xL and Bcl-x KO MEFs was performed by Bcl-xL ImmunoGold labeling and Transmission Electron Microscopy (TEM) imaging. Uranyl acetate staining was used to allow for subcellular structure visualization in fixed cell sections. Images were collected using a JEOL 1400JEM transmission electron microscope and Zeiss LSM780 confocal microscope.

Proximity Ligation Assay (PLA). MEFs were grown on coverslips and fixed with methanol for 2 min. After two PBS washes, MEFs were saturated with the blocking solution followed by a 1 hr at 37°C incubation with the primary antibodies: Bcl-xL (Cell Signaling #2764, 1/1000) and IP3R C-terminal (abcam #ab190239, 1/1000). PLA secondary antibodies probes were added and incubated for 1hr at 37°C after 3 PBS washes. Ligation and amplification (100 min at 37°C) steps followed. Cells were mounted with Duolink II Mounting medium containing DAPI and images were acquired using the fluorescence microscope.

$^{45}\text{Ca}^{2+}$ assay. $^{45}\text{Ca}^{2+}$ flux analysis was performed as previously described^{27,28}. After permeabilizing the MEFs, IP3R-mediated Ca^{2+} release was assessed through the addition of increasing concentrations of IP3. Steady state $^{45}\text{Ca}^{2+}$ were used to load intracellular non-mitochondrial Ca^{2+} stores where the release of $^{45}\text{Ca}^{2+}$ from the plasma membrane was assessed at specific time intervals. 1.5 μM maximal IP3 concentration was used. MEFs were loaded with $^{45}\text{Ca}^{2+}$ in the presence of the Ca^{2+} ionophore A23187 in order to determine the passively bound Ca^{2+} value that will be subtracted to calculate exclusively the amount of releasable Ca^{2+} from the ER.

Cell cycle FACS analysis. FACS analysis with Propidium Iodide (PI) (abcam) was carried out by flow cytometry (BD FACSCanto™ II, BD Bioscience) according to manufacturer's recommended protocol.

Briefly, MEFs were seeded and grown overnight onto a 12-well plate. Afterwards, they were trypsinized and harvested. The obtained cell pellet was washed with PBS and fixed with cold ethanol for 30min at 4°C. After 2 PBS washes, MEFs were treated with 50 µl of a 100 µg/ml stock of RNase to ensure that only DNA is stained. 200 µL of PI (from 50 µg/ml stock solution) was then added and the cell cycle was analyzed by flow cytometry (BD FACSCanto™ II, BD Bioscience) using untreated cells as negative control for gating. A total of 10000 events were recorded during the experiments (n=3). The percentage of each cell population was analyzed using BD FACSDIVA 8.0.1 software (BD Bioscience, USA).

Genotyping by PCR. PCR-based analysis was used to genotype mice. Each reaction mixture consisted of deoxynucleoside triphosphates, MgCl₂, a forward primer specific for Bcl-xL exon 3: EXE-F (5'-GGAAAGGCCAGGAGCGCCTTC-3'), a reverse complementary primer specific for Bcl-xL exon 3: XTAG-R (5'-CCCAACCCTGTGATAGGGCAAG-3'), Taq polymerase, specific buffer and genomic DNA. Conditions for PCR were as follow: (1) 5min at 98°C; (2) 35 cycles, with 1 cycle consisting of 30s at 94°C, 30s at 59°C, and 40s at 72°C and (3) an elongation period of 5min at 72°C. Amplified products were analyzed by electrophoresis in 2% agarose gels and bands were visualized by Gel-Doc BIO-RAD imaging system.

RT qPCR. Total RNA was isolated from MEFs following treatment with 4µM thapsiragin over 24hrs with the NucleoSpin RNA plus from MACHEREY-NAGEL, following the manufacturer's instructions. For the reverse transcription, 1µg was used for cDNA synthesis using a Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (SuperScript II from ThermoFisher Scientific) and random primers. One mL of RT reaction was used for the PCR following the protocol: 1 min 94uC and 20 cycles of amplification by 1 min 94uC, 1 min 55uC, 1 min 72uC, and finishing with 5 min at 72uC. Amplified products were visualised in 1% agarose with ethidium bromide. The following primers were used:

Statistical analyses. Student t-tests were performed when comparing two means and ANOVA was used for comparison of three means or more. A p-value equal to or under 0.05 was considered statistically significant.

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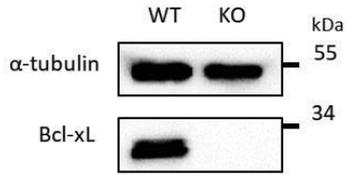
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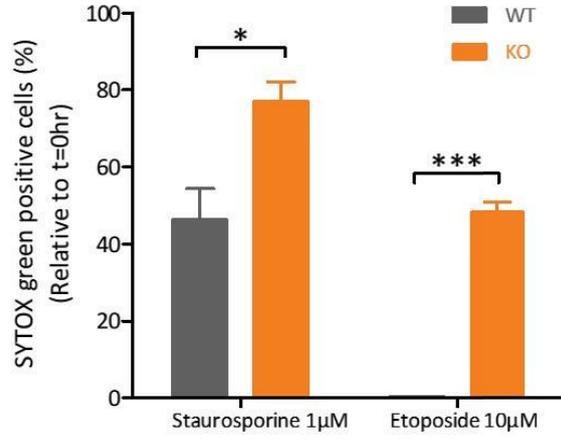
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Figure1

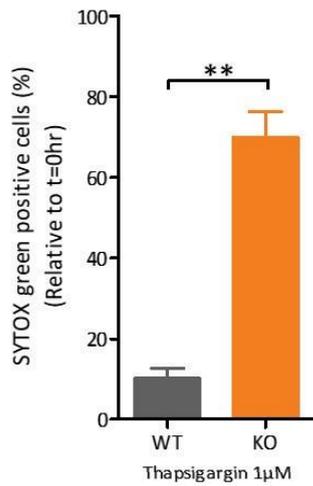
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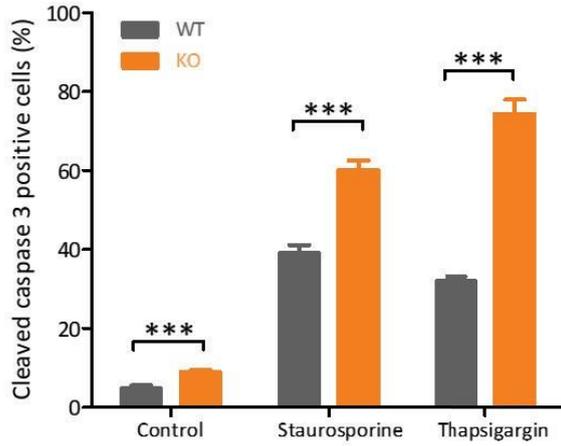
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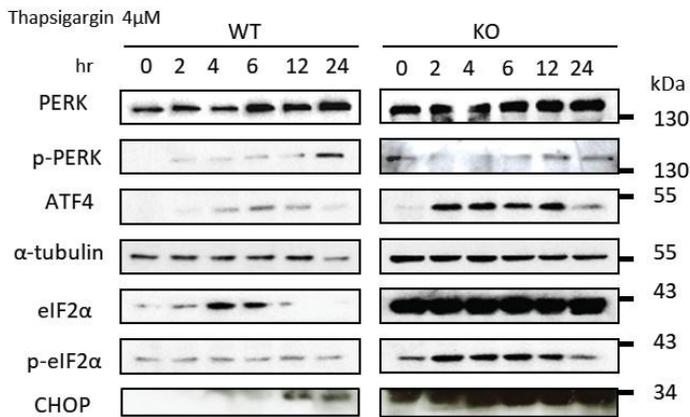
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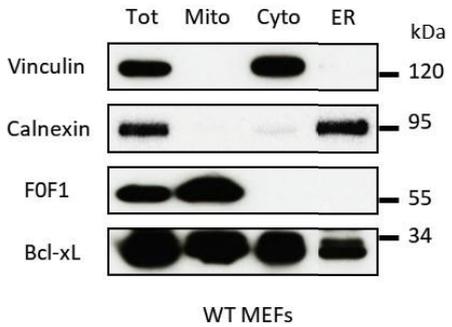
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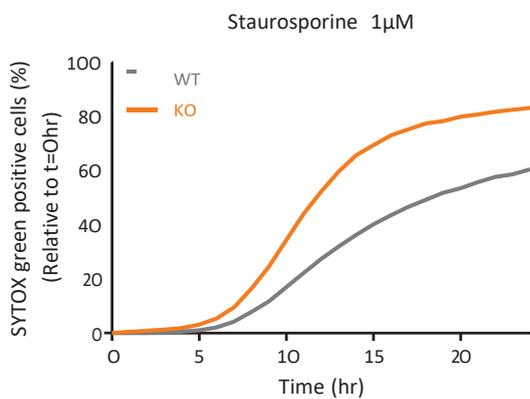


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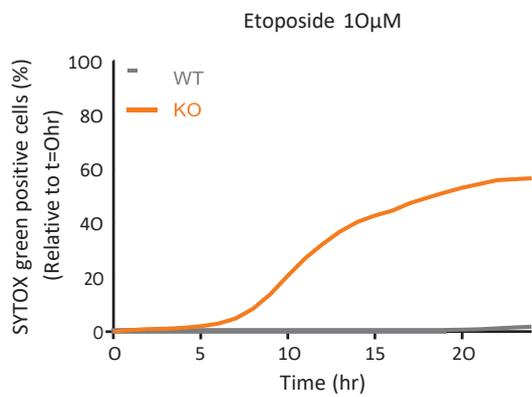


Supplementary Figure 1

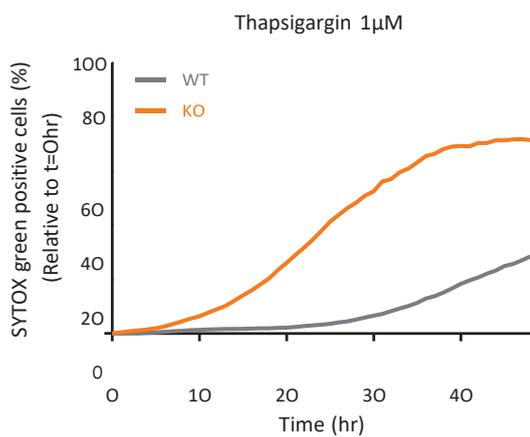
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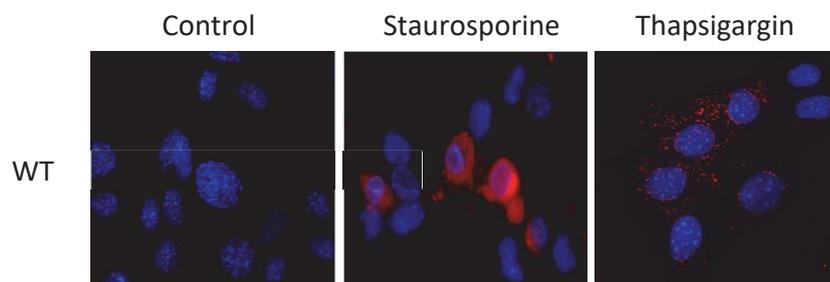
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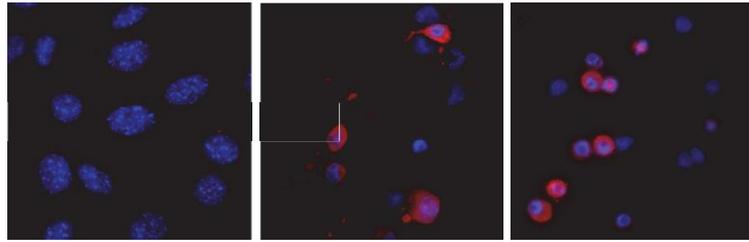
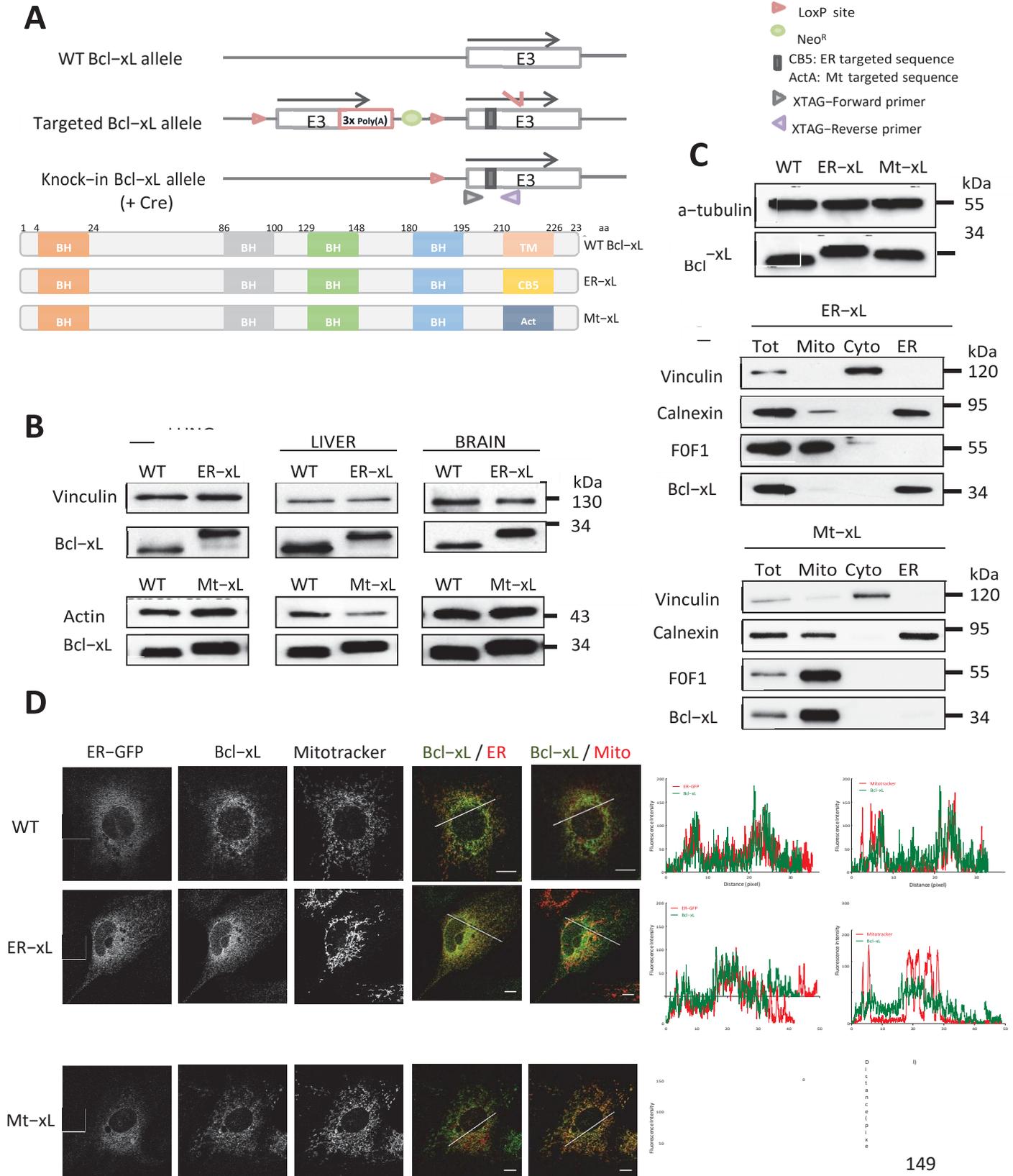
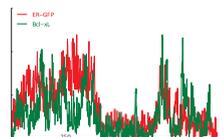
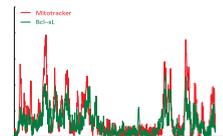


Figure 2





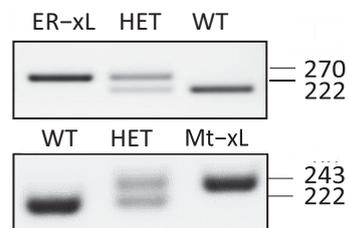
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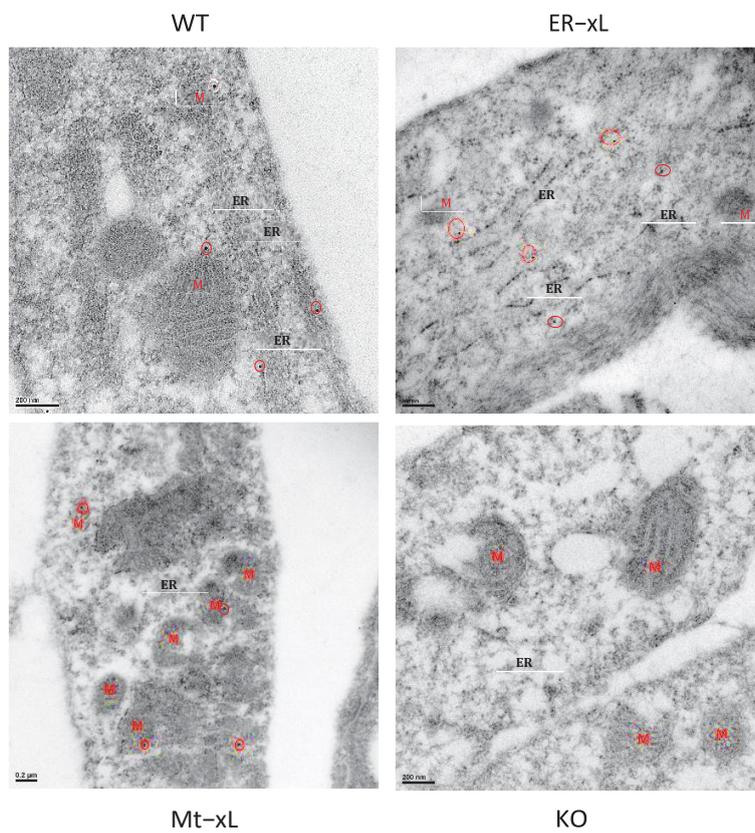
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Supplementary Figure 2

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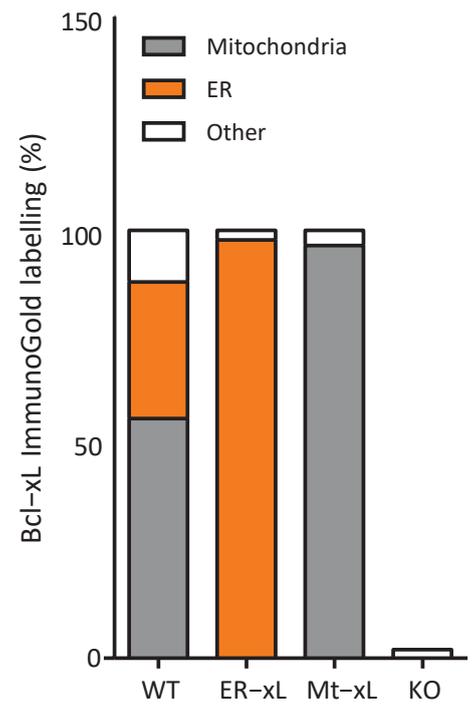
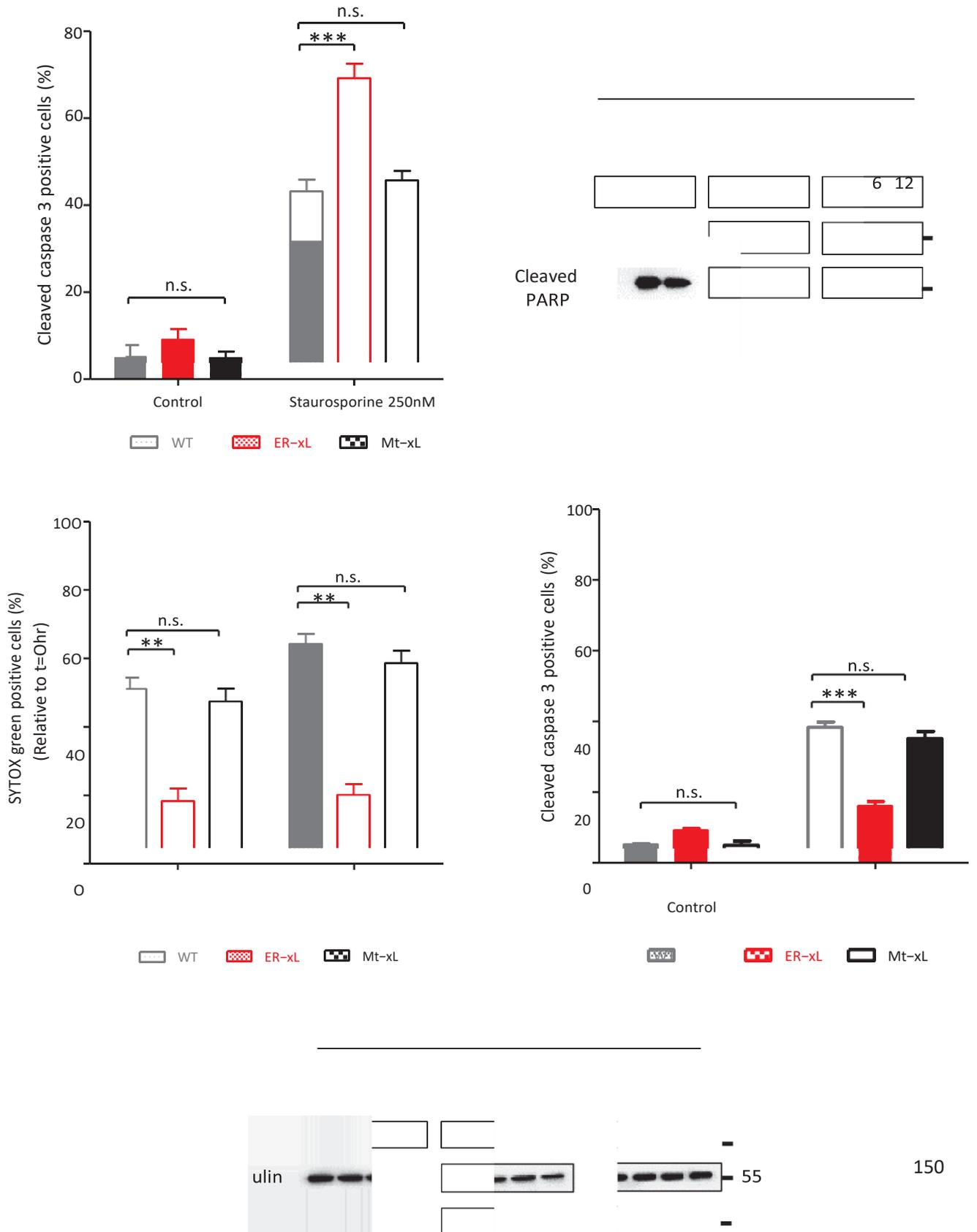


Figure 3



Supplementary Figure 3

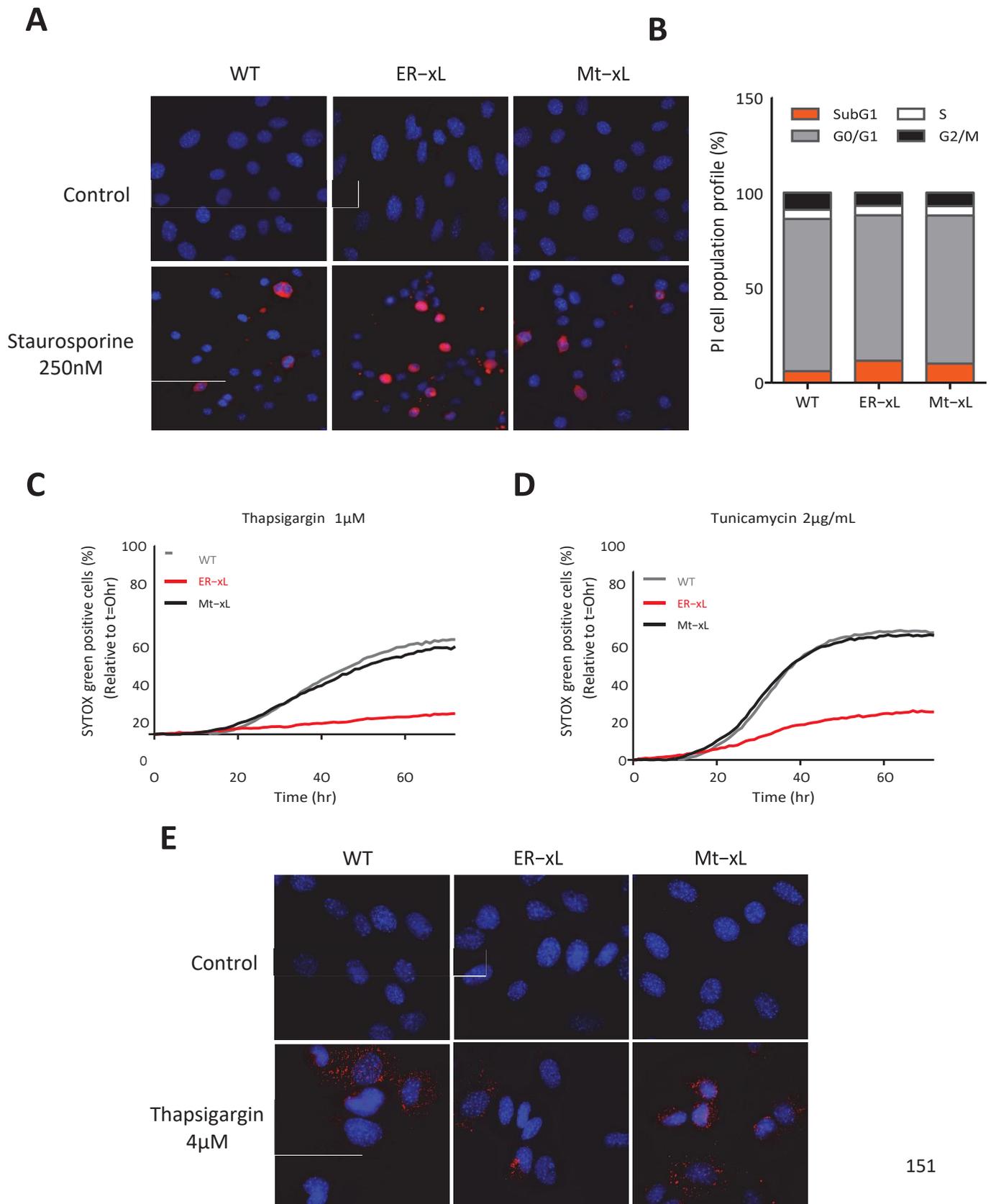
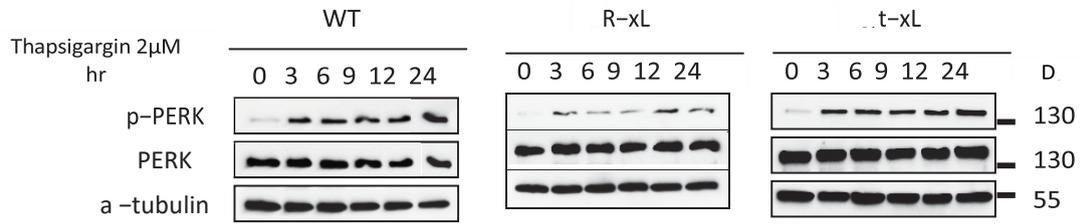
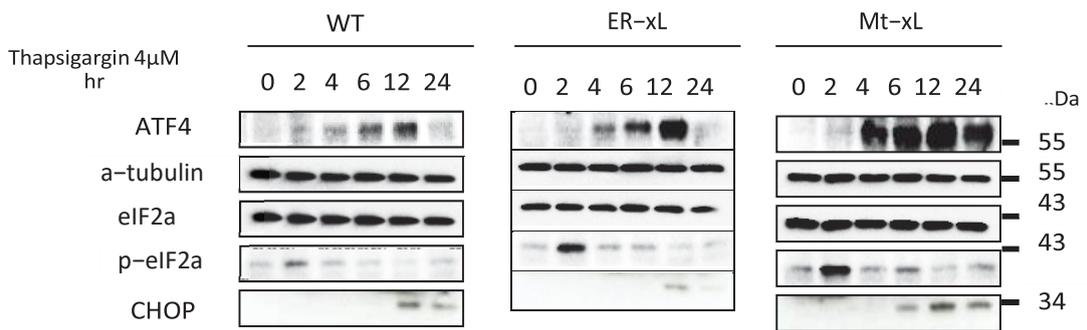


Figure 4

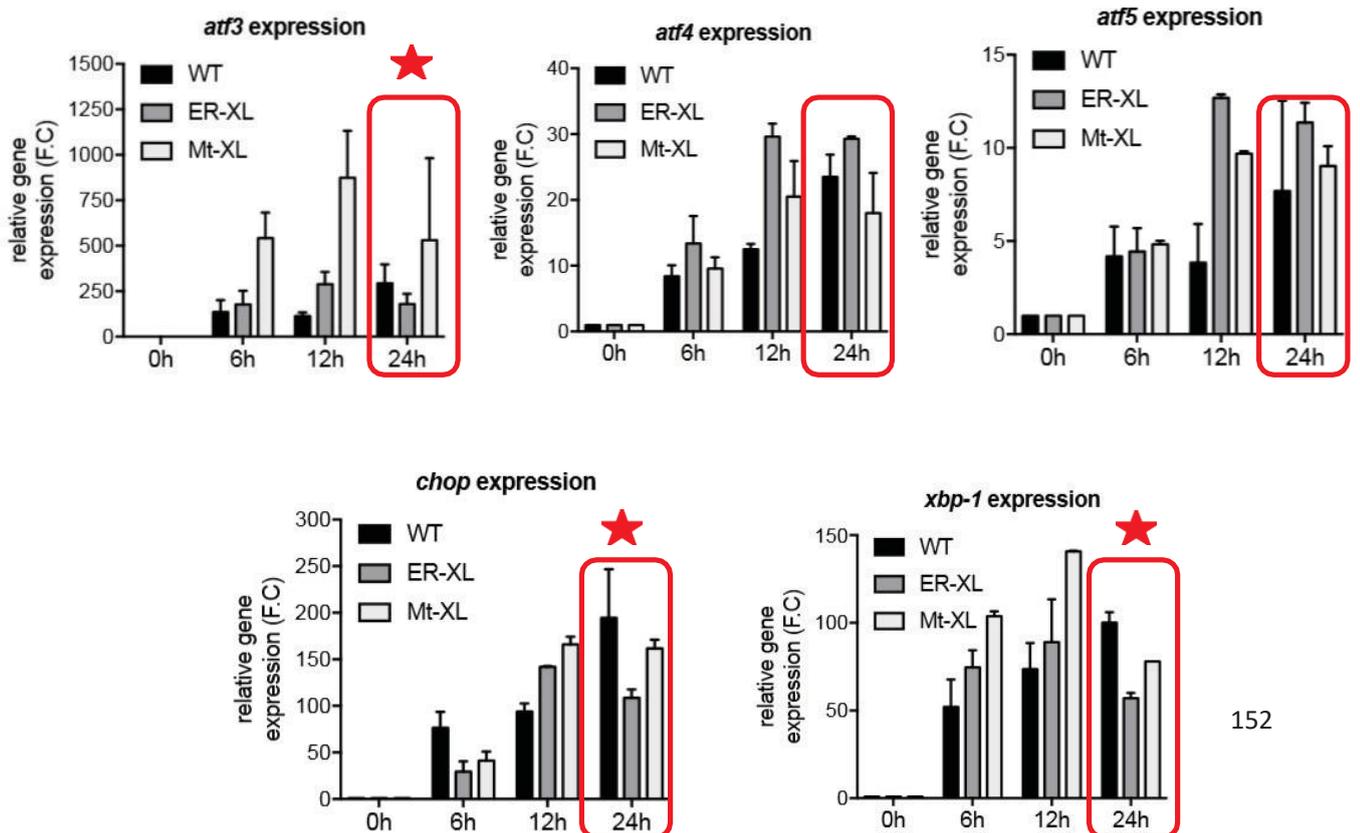
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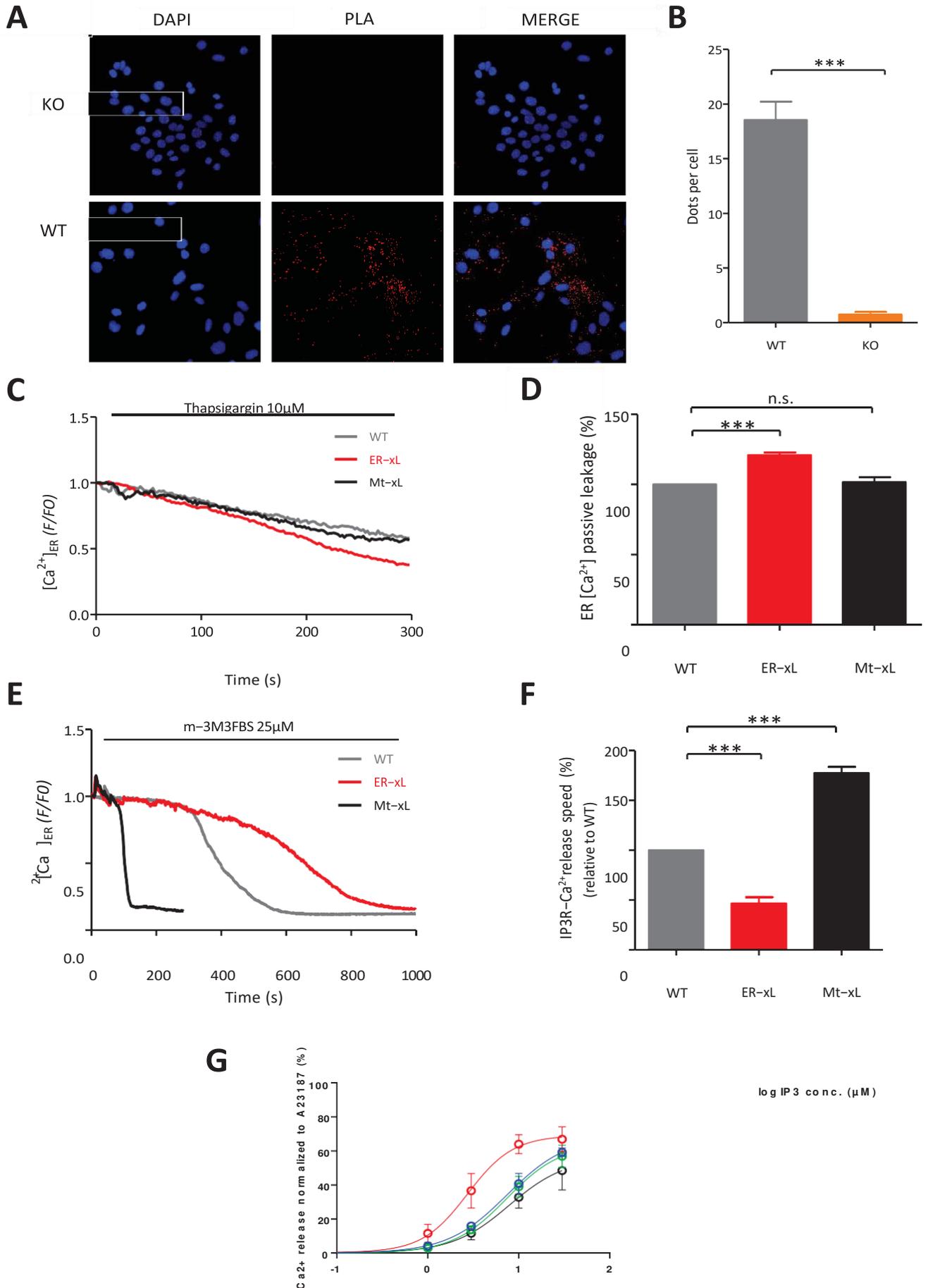
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C



Supplementary Figure 5



- ⊖ WT
- ⊖ Mt-xL
- ⊖ WT
- ⊖ ER-xL

- ME Fwt
- ME F KIR
- ME Fwt
- ME F KIM

Supplementary Figure 4

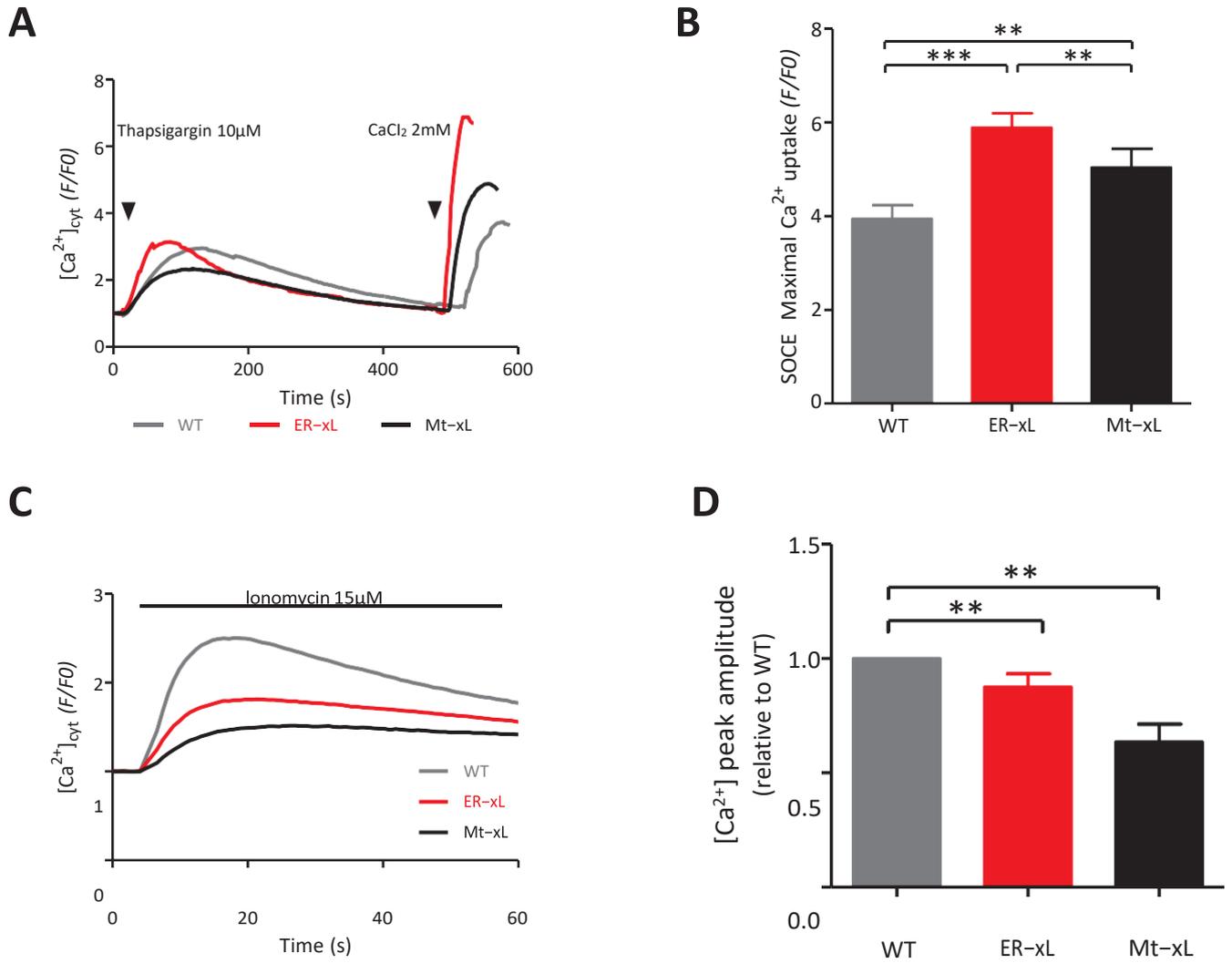


Figure 6

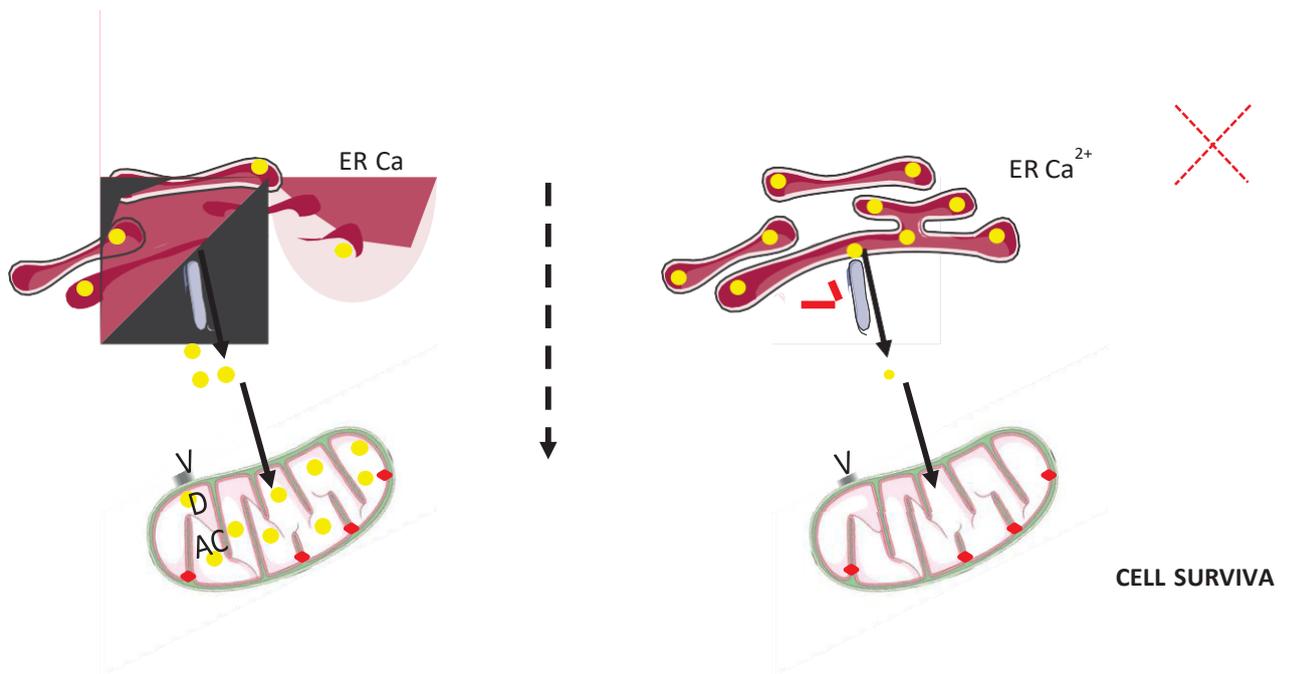
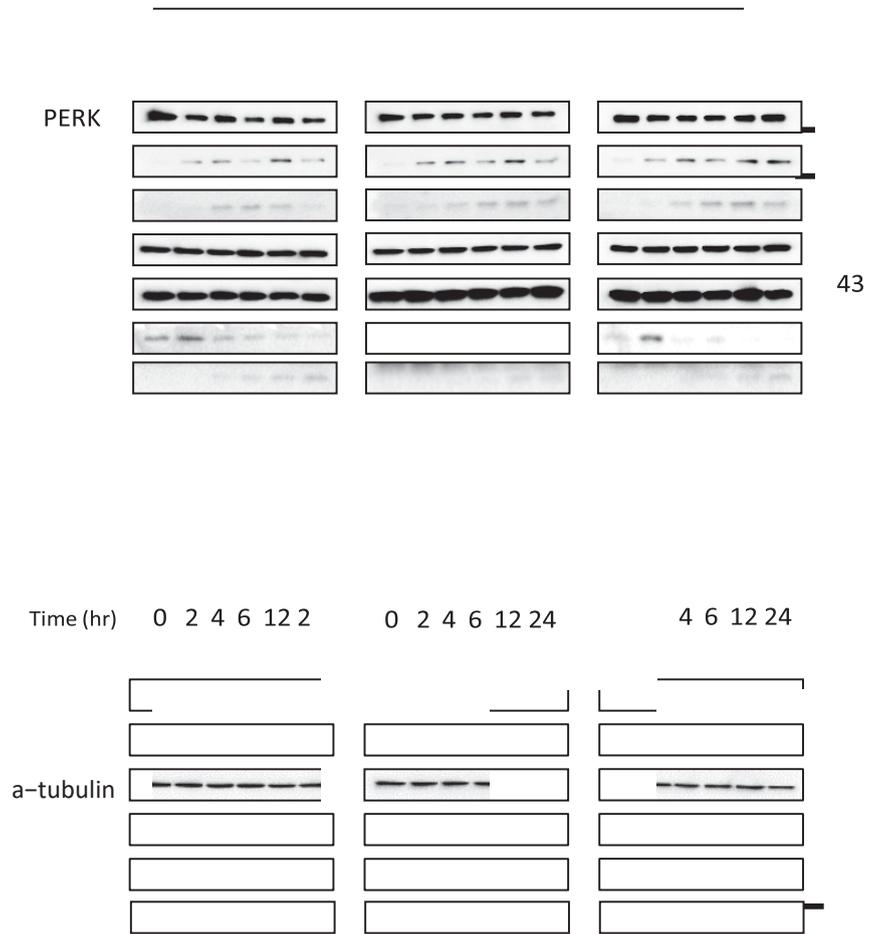


Figure legends

Figure 1. Bcl-xL possesses double subcellular localization and protects from ER stress.

(A) Western blot detecting Bcl-xL endogenous levels in WT and verification of *Bcl-x* KO in *Bcl-x* KO MEFs. α -tubulin is used as a loading control. (B) Cell death quantification (% of Sytox GREEN marked cells) in WT and *Bcl-x* KO MEFs treated with 1 μ M staurosporine or 10 μ M etoposide for 24 hrs (mean \pm SD; n=3; *, p<0.05, ***, p<0.001). Results are displayed at t=15hrs. (C) Cell death quantification (% of Sytox GREEN marked cells) in WT and *Bcl-x* KO MEFs treated with 1 μ M thapsigargin for 36 hrs (mean \pm SD; n=3; **, P<0.01). Results are displayed at t=32hrs. (D) Quantification of Cleaved Caspase 3 stained WT and KO MEFs after treatment with 250nM staurosporine for 6hrs or 4 μ M thapsigargin for 24hrs. 5 images of an average of 30 cells each were taken per cell type per experiment (n=3). Control: treated with DMSO (mean \pm SD; n=3; ***, p<0.001). (E) Kinetics of UPR markers in the presence of 4 μ M thapsigargin for 24hrs in WT and *Bcl-x* KO MEFs. α -tubulin was used as a loading control. (F) The localization of endogenous Bcl-xL in WT MEFs was assessed by subcellular fractionation resulting in four fractions: (Tot) whole-cell lysates; (Mito) mitochondria; (Cyto) cytosol and ER followed by western blot where vinculin is used as a cytosol marker, calnexin as an ER marker and FOF1 ATPase as a mitochondrial maker.

Supplementary Figure 1. Cytotoxic and ER-stress mediated drug treatment on WT, ER-xL and Mt-xL MEFs

Representative curves of cell death quantification (% of Sytox GREEN marked cells) in WT and *Bcl-x* KO MEFs treated with staurosporine 1 μ M (A) or Etoposide 10 μ M (B) for 24 hrs or with thapsigargin 1 μ M (C) for 48 hrs. SYTOX Green is used to detect dead cells. (D) Representative images of cleaved caspase 3 stained WT and KO MEFs after staurosporine and thapsigargin treatment for 6hrs and 24hrs, respectively. Blue fluorescence: Nuclei. Red fluorescence: cleaved caspase 3.

Figure 2. Generation and verification of ER-xL and Mt-xL mice.

(A) The mice genotyping strategy was done with a Cre-LoxP recombination system where an engineered vector containing the WT Bcl-xL exon 3 (E3) and CB5 or ActA mutant sequences targeting Bcl-L either to the ER or mitochondria, respectively, was inserted to replace the WT Bcl-xL E3 in floxed mice. Cre-recombination induced WT exon 3 excision and CB5 or ActA mutant expression. Accordingly, three protein products are possible: WT Bcl-xL with intact TM domain, ER-xL with CB5 targeting sequence and Mt-xL with ActA targeting sequence. Neomycin is used as a inducer of vector resistance (Neo^R). Two primers were used: XTAG-Forward and XTAG-Reverse. Poly(A): Poly(A) tail. (B) Western blot detecting Bcl-xL endogenous levels in cells extracted from the lung, liver and brain of WT, ER-xL and Mt-xL mice. Vinculin and actin were used as loading controls. (C) Western blot detecting Bcl-xL endogenous levels in WT, ER-xL and Mt-xL MEFs extracted at E13. α -tubulin is used as a loading control. (D) Bcl-xL subcellular localization detection in WT, ER-xL and Mt-xL MEFs by Immunofluorescence. Co-localization was assessed using pCS2+GFP-CB5 (ER-GFP) transfection and Mitotracker staining for ER and mitochondrial localization, respectively. Profile plots of fluorescence signals detecting the intensity of fluorescence along the white segments on merged images were quantified by ImageJ software and shown to the right. (E) Bcl-xL endogenous expression in ER-xL (Top panel) and Mt-xL (lower panel) MEFs post-subcellular fractionation was detected by western blot. Vinculin is used as a cytosol marker, calnexin as an ER marker and F0F1 ATPase as a mitochondrial maker. (Tot) whole-cell lysates; (Mito) mitochondria; (Cyto) cytosol.

Supplementary Figure 2. Genotyping and localization of Bcl-xL

(A) PCR results confirming the genotype of the genetically modified mice: WT for Bcl-xL^(WT/WT); HET for Bcl-xL^(WT/ActA) or ^(WT/CB5); ER-xL for Bcl-xL^(CB5/CB5) and Mt-xL for Bcl-xL^(ActA/ActA). Bp: base pair. HET: Heterozygous. (B) Representative images for Bcl-xL ImmunoGold labeling in WT, ER-xL, Mt-xL and *Bcl-x* KO MEFs. Red circle: Gold particle; M: mitochondria; ER: Endoplasmic Reticulum. Scale bar: 200 nm. (C) Quantification of Bcl-xL ImmunoGold labelling in WT, ER-xL, Mt-xL and *Bcl-x* KO MEFs (n=15 cells per cell type).

Figure 3. ER-xL MEFs resist ER stress-mediated cell death.

(A) Quantification of Cleaved Caspase 3 stained WT, ER-xL and Mt-xL MEFs after treatment with 250nM staurosporine for 6 hrs. 5 images of an average of 30 cells each were taken per cell type per experiment (n=3). Control: treated with DMSO (mean \pm SD; n=3; ***, p<0.001; n.s., non-significant, p>0.05). (B) Kinetics of PARP cleavage in WT, ER-xL and Mt-xL MEFs following 1 μ M staurosporine treatment over 12 hours. α -tubulin was used as a loading control. (C) Cell death quantification (% of Sytox GREEN marked cells) in WT, ER-xL and Mt-xL MEFs treated with 1 μ M thapsigargin or 2 μ g/mL tunicamycin for 72 hrs (mean \pm SD; n=3; n.s., non-significant, p>0.05; **, P<0.01). Results are displayed at t=72hrs. (D) Quantification of Cleaved Caspase 3 stained WT and KO MEFs after treatment with 4 μ M thapsigargin for 24hrs. 5 images of an average of 30 cells each were taken per cell type per experiment (n=3). Control: treated with DMSO (mean \pm SD; n=3; n.s., non-significant, p>0.05; ***, p<0.001). (E) Kinetics of PARP cleavage in WT, ER-xL and Mt-xL MEFs following 4 μ M thapsigargin treatment over 24 hours. α -tubulin was used as a loading control.

Supplementary Figure 3. Bcl-xL at the ER protects from ER stress induced cell death

(A) Representative images of cleaved caspase 3 stained WT, ER-xL and Mt-xL MEFs after staurosporine treatment for 6hrs. Blue fluorescence: Nuclei. Red fluorescence: cleaved caspase 3. (B) Percentages of DNA content in each cell cycle phase are shown for each cell type after FACS analysis with Propidium Iodide (PI) in WT, ER-xL and Mt-xL MEFs (n=3). Representative curves of cell death quantification (% of Sytox GREEN marked cells) in WT, Er-xL and Mt-xL MEFs treated thapsigargin 1 μ M (C) and tunicamycin 2 μ g/mL for 72 hrs (D). SYTOX Green is used to detect dead cells. (E) Representative images of cleaved caspase 3 stained WT, ER-xL and Mt-xL MEFs after thapsigargin treatment for 24hrs. Blue fluorescence: Nuclei. Red fluorescence: cleaved caspase 3.

Figure 4. Bcl-xL at the ER reduces the UPR.

Kinetics of UPR markers in the presence of 2 μ M (A) and 4 μ M (B) thapsigargin over 24hrs in WT, ER-xL and Mt-xL MEFs. α -tubulin was used as a loading control. (C) RT-PCR assay was used to analyze the mRNA levels of the UPR markers xbp-1s, CHOP, ATF-3, ATF-4 and ATF-5 in WT, ER-xL and Mt-xL MEFs after 4 μ M thapsigargin treatment over 24 hours.

Figure 5. Bcl-xL at the ER interacts with and blocks IP3R.

(A) Representative images showing the endogenous interactions between IP3R – C terminus and Bcl-xL in WT and *Bcl-x* KO MEFs after Proximity ligation assay (PLA). DAPI was used to stain nuclei. (B) Quantification of PLA experiments (mean \pm SD; n=3; ***, p< 0.001). (C) Representative curves of ER passive calcium leakage in WT, ER-xL and Mt-xL MEFs transfected with CEPIA-1er after 10 μ M thapsigargin injection. (D) Quantification of the slope coefficient of ER passive calcium leakage in MEFs (mean \pm SD; n=3; ***, p< 0.001; n.s., non-significant). (E) Representative curves of ER calcium release in WT, ER-xL and Mt-xL MEFs transfected with CEPIA-1er after 25 μ M m-3M3FBS (PLC activator) injection. (F) Quantification of the slope coefficient of ER calcium release after PLC activation in MEFs (mean \pm SD; n=3; ***, p< 0.001). (G) Representative curves of ER calcium release normalized to A23187 maximal ionophore release response after ⁴⁵Ca²⁺ flux analysis. IP3-induced calcium release in Mt-xL MEFs is clearly sensitized compared to ER-xL MEFs when compared to WT MEFs.

Supplementary Figure 4. Bcl-xL interacts with IP3R and affects intracellular Ca²⁺ fluxes.

(A) Representative curves of Store Operated Calcium Entry (SOCE) assessed with 5 μ M Fluoforte after draining the ER from calcium by 10 μ M thapsigargin injection followed by 2mM CaCl₂ injection in WT, ER-xL and Mt-xL MEFs. (B) The ratio of fluorescence indicating the maximal calcium uptake after CaCl₂ injection is shown (mean \pm SD; n=3; **, p<0.01; ***, p< 0.001). (C) Representative curves of cytosolic calcium release assessed with 5 μ M Fluoforte after 15 μ M Ionomycin injection in WT, ER-xL and Mt-xL

MEFs using a plate reader. (D) The ratio of fluorescence indicating $[Ca^{2+}]$ peak amplitude relative to WT is shown (mean \pm SD; n=3; **, p<0.01).

Figure 6. Bcl-xL at the ER reduces the UPR by inhibiting IP3R upon ER stress induction.

(A) Kinetics of UPR markers after 4 μ M thapsigargin and 100 μ M 2-APB treatment over 24 hours in WT, ER-xL and Mt-xL MEFs. 2-APB is considered as IP3R inhibitor. α -tubulin was used as a loading control. (B) Kinetics of UPR markers after 4 μ M thapsigargin and 1 μ M Xestospongine C treatment over 24 hours in WT, ER-xL and Mt-xL MEFs. Xestospongine C is an IP3R and SERCA inhibitor. α -tubulin was used as a loading control. (C) Proposed model for the role of Bcl-xL at the ER. ER stress might deplete ER Ca^{2+} stocks resulting in: 1- mitochondrial apoptosis initiation through mPTP opening and 2- the UPR activation leading to the transcription of genes promoting apoptosis. Hence, the outcome is cell death. Under these conditions, Bcl-xL at the ER interacts with and inhibits the IP3R abrogating ER Ca^{2+} depletion required for apoptosis induction. Consequently, Bcl-xL at the ER performs a new indirect anti-apoptotic function upon ER stress.

'Are Bcl-2 proteins' functions conserved throughout metazoan evolution?'

Introduction to ARTICLE 2

As indicated throughout this manuscript, Bcl-2 proteins are majorly known for their regulation of apoptosis. We have stated the timeline of the cloning of the human Bcl-2 homologs since the cloning of Bcl-2 in 1986; refer to (Figure 3). However, various Bcl-2 related proteins in vertebrate species like zebrafish and invertebrate like hydra, drosophila and nematodes have been discovered. On the structure level, they possess one to four BH domains with a TM domain. Another shared feature is their involvement in mitochondrial cell death regulation. In an interesting fashion, these proteins are present at various organelles and several are shown to interfere with intracellular Ca^{2+} fluxes. For instance, Nrz – the zebrafish ortholog of human Nrh – plays a crucial role in the progression of zebrafish epiboly. On the molecular level, it interacts with IP3R at the ER and decreases ER- Ca^{2+} discharge. As demonstrated in Article 1, Bcl-xL also negatively regulates IP3R-mediated Ca^{2+} release which plays a major part in ER stress resistance and UPR reduction. So the question left unanswered is: are Bcl-2 proteins' functions conserved throughout metazoan evolution?

To answer this, we targeted the most basal branch of metazoans called placozoans. One of the few representatives of this phylum is *Trichoplax adhaerens*. We were able to identify and characterize novel Bcl-2-related proteins that we called trBcl-2Like (trBcl-2L). Four trBcl-2L proteins share BH and TM domain homology, as well as secondary structures, with Bcl-2 proteins. On the structure level, two proteins (trBcl-2L1 and trBcl-2L2) clustered with the anti-apoptotic group and the two others (trBax and trBak) with the pro-apoptotic one displaying resemblance to human Bax and Bak, respectively. Interestingly, trBak acts more like a BH3-only sensitizer protein where it binds to and inhibits anti-apoptotic trBcl-2L1 and L2. TrBax is a genuine Bax-like pro-apoptotic protein capable of inducing MOMP in *bax/bak* deficient cells. These proteins were shown to be localized at both the mitochondria and the ER. At the latter, trBcl-2L1 is able to increase IP3R-mediated Ca^{2+} release upon histamine stimulation suggesting a conserved evolutionary mechanism also englobing the regulation of intracellular Ca^{2+} fluxes.

One important feature on the gene level is a shared BH2 splitting intron within all four *trbcl2*-like genes. Interestingly, this intron has been found not only in placozoan *bcl2*-related genes, but also in approximately all metazoan ones further suggesting a common genetic ancestry between all related Bcl-2 proteins. What is also interesting about this family is the fact that one common gene might be able to influence several functional traits resulting in normal and abnormal cellular developments. In fact, the regulation of cell death, as well as Ca^{2+} homeostasis, by structurally similar proteins from way different origins suggests a conservation of vital functions from the most ancestral form of multicellularity. Therefore, concerning Bcl-2 proteins known as apoptosis regulators, our data put in debate their initial famous function. Another question comes to our mind:

Which came first, the chicken or the egg?

ARTICLE 2

Pleiotropy of Bcl-2 family proteins is an ancient trait in the metazoan evolution

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Condensed title: TrBcl-2-like proteins have multiple functions

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ABSTRACT

In the animal kingdom, proteins of the Bcl-2 family are widely recognized as regulators of mitochondrial outer membrane permeabilization (MOMP), leading to apoptotic cell death. These proteins were recently also shown to control IP₃-dependent calcium fluxes at the level of the endoplasmic reticulum (ER). However, the origin and evolution of these pleiotropic functions remain elusive. Here, we molecularly characterized the four members of the Bcl-2 family (trBcl-2L1 to -2L4) in the most primitivemetazoan, namely *Trichoplax adhaerens*. Primary structure and phylogenetic analyses demonstrated that all four trBcl-2 homologs belong to the multidomain Bcl-2 group and present a conserved C-terminus transmembrane (TM) domain. TrBcl-2L1 and trBcl-2L2 are highly divergent proteins clustering with the anti-apoptotic Bcl-2 members, whereas trBcl-2L3 and trBcl-2L4 were homologous to the pro-apoptotic Bax (trBax) and Bak (trBak). Interestingly, at the functional level, trBak operates as a BH3 only sensitizer repressing the anti-apoptotic activities of trBcl-2L1 and trBcl-2L2, whereas trBax leads to MOMP, similarly to the well-known indirect model of Bax activation.

Finally, we found that trBcl-2L1 and trBcl-2L2 had a dual ER and mitochondrial subcellular localization. By generating two TM domain mutants we demonstrated that trBcl-2L1 targeted to the ER was able to control IP₃-dependent calcium fluxes, whereas Mito-trBcl-2L1 represses trBax-dependent MOMP, suggesting that Bcl-2 pleiotropy is an ancient trait conserved throughout evolution.

INTRODUCTION

Apoptosis represents one of the hallmarks of multicellularity in the animal kingdom (Zmasek and Godzik, 2013). This process shapes the body during the embryonic development, maintains tissue homeostasis and eliminates undesirable or potentially harmful cells from the organism (Fuchs and Steller, 2015). Proteins belonging to the Bcl-2 family represent one of the main regulators of apoptosis. They control a key step in the induction of apoptosis called the mitochondrial outer membrane permeabilization (MOMP) (Kale et al., 2018). Initially discovered in chromosomal translocations in follicular lymphomas, Bcl-2 homologs have now been found in almost all metazoans. Indeed, genetic screening of cell death

mutants performed in nematodes and subsequent studies in drosophila, zebrafish and hydra uncovered a number of Bcl-2-related proteins. (Hengartner et al., 1992; Kratz et al., 2006; Lasi et al., 2010; Quinn et al., 2003). These proteins contain one to four B-cell homology domains, as well as a hydrophobic C-terminus transmembrane domain (Singh et al., 2019). Although the control of apoptosis differs substantially between roundworms, insects and mammals, seminal experiments conducted in these models reinforced the dogma that Bcl-2 family of proteins were evolutionary selected to control the survival of the cell at the level of the mitochondria. However, a number of Bcl-2-related proteins are localized not only to the MOM but are also found at the endoplasmic reticulum (ER). Indeed, Bcl-2 and related members bind to the ER membranes and control intracellular calcium fluxes through direct interactions with the calcium channel inositol 1,4,5-trisphosphate receptor (IP₃R) (Li et al., 2007; Popgeorgiev et al., 2011; Rong et al., 2008). Calcium represents a key second messenger implicated in a broad range of processes including cell cycle, cell metabolism, cell migration and apoptosis, and Bcl-2 family members have also been linked to the control of some of these processes through the control of calcium fluxes (Bonneau et al., 2013).

Recently we showed that two Bcl-2 homologs named Nrz and Bcl-wav were able to orchestrate the first morphogenic movements, during early zebrafish embryonic development independently from their roles in apoptosis. Nrz exerts this non-canonical function at the ER by directly regulating IP₃R permeability, whereas Bcl-wav binds to VDAC channel at the MOM and enhances mitochondrial calcium uptake (Popgeorgiev et al., 2011; Prudent et al., 2013) (Bonneau et al., 2014). We demonstrated that these molecular interactions control actomyosin contractility and dynamics in the developing embryo. Thus, the assumption that Bcl-2 family proteins appeared and were selected throughout metazoan evolution as sole MOMP regulators is now challenged (Prudent et al., 2015).

Metazoans appeared during the late Precambrian period around 780 million years ago (Degan Shu, 2014). Placozoans represent one of the most basal branching metazoans with *Trichoplax adhaerens* being one of the three living representatives of this phylum (Osigus et al., 2019). It possesses the simplest animal morphology and is composed of only six cell types (Smith et al., 2014). It does not undergo embryonic development but rather divides by scission. Recently, the genome of *Trichoplax adhaerens* was sequenced (Srivastava et al., 2008).

Here we describe the identification and the molecular characterization of four *Trichoplax adhaerens* Bcl-2-like (trBcl-2L1 to -2L4) proteins. Phylogenetic analyses demonstrated that *Trichoplax* possesses two anti-apoptotic Bcl-2 homologs (trBcl-2L1 and -2L2) and two members clustering with the pro-apoptotic subgroup (trBcl-2L3 and -2L4). We demonstrated that trBcl-2L3 is a genuine Bax-like protein, whereas trBcl-2L4 is a Bak homolog. Interestingly, trBcl-2L3 is a pro-apoptotic protein capable of inducing MOMP in *bax/bak*-deficient baby mouse kidney (BMK) cells whereas trBcl-2L4 acts as BH3-only sensitizer repressing anti-apoptotic activities of trBcl-2L1 and trBcl-2L2.

Finally, we showed that trBcl-2L1 and trBcl-2L2 possess dual subcellular localizations at the ER and the mitochondria, and demonstrate that trBcl-2L1 controls IP₃R-dependent calcium fluxes. Overall, these results suggest that Bcl-2 proteins may have emerged as multifunctional cell factors capable of controlling both MOMP and calcium fluxes.

RESULTS

In silico identification and characterization of Bcl-2-like proteins

We performed extensive tBlastN screening of the genome of the *Trichoplax adhaerens* Grell-BS-1999 strain publically available on the NCBI. Using the full length human Bcl-2 protein as well as individual BH and TM domains as a query we identified four protein encoding sequences potentially corresponding to Bcl-2 homologs. We named them *Trichoplax adhaerens* Bcl-2-like proteins or trBcl-2l (Figure 1A). We further confirmed the expression and the sequence of the four *trbcl2*-like genes in *Trichoplax adhaerens* by performing reverse transcriptase PCR using specific primers targeting the exon #1 of each gene (Figure S1A). Sequences were deposited into the NCBI databank under the following accession numbers KU500586.1, KU500587.1, KU500588.1, and KU500589.1 corresponding to the trBcl-2L1, trBcl-2L2, trBcl-2L3 and trBcl-2L4, respectively. Multiple alignments coupled with secondary structure prediction showed that all four sequences presented the typical multidomain BH organization, the TM domain located in the C-terminus end, as well as all alpha helices secondary structures (Figure 1A, Figure S1B). Interestingly, analysis of the primary structure of trBcl-2L3 also highlighted a unique S/T rich region between alpha helix 1 and 2. This region was predicted as a mucin-like domain which is found in secreted

proteins. Of note, during the course of our survey we were not able to detect BH3-only encoding genes in the *T. adhaerens* genome.

In effect, trBcl-2 homologs cluster in two groups at both genomic and protein levels. For instance, all four trBcl-2-like genes possess a unique BH2 splitting intron. However, this intron was relatively long for *trbcl2l1* and *trbcl2l2* with 1991 nt and 1725 nt, respectively whereas it was significantly shorter in *trbcl2l3* and *trbcl2l4* with 463 nt and 280 nt, respectively. Furthermore, trBcl-2L1 and trBcl-2L2 proteins had similar molecular weights and isoelectric points (pI): 24766 and 24282 Da and pI of 8.65 and 8.85, respectively, whereas trBcl-2L3 and trBcl-2L4 were slightly bigger, 27382 and 27944 Da with lower pI 6.96 and 6.45 respectively. Finally, using local alignment matrices and neighbor joining phylogenetic tree generation, we showed that trBcl-2L1 and trBcl-2L2 were able to cluster with the anti-apoptotic subgroup of the Bcl-2 family, whereas trBcl-2L3 and trBcl-2L4 clustered with the pro-apoptotic subgroup (Figure 1B&C). TrBcl-2L1 and -2L2 homologs were highly divergent, whereas trBcl-2L3 and trBcl-2L4 clustered well with Bax and Bak homologs, respectively. We hereafter named these proteins trBax and trBak, respectively.

Role of trBcl-2 proteins in the control of MOMP

To gain further mechanical insight into the molecular activity of these trBcl-2-like proteins, we performed apoptosis assay in HeLa cells by analyzing the activation of Caspase 3 using a specific antibody. The transient expression of trBcl-2L1 ($2.2\% \pm 1.3\%$) and trBcl-2L2 ($2.4\% \pm 1.2\%$) for 24 h did not lead to a significant increase in Caspase 3 activation compared to control cells expressing the ER-targeted EGFP ($6.3\% \pm 5.2$). Conversely, the expression of trBax ($89.5\% \pm 4.5$) and trBak ($88.7\% \pm 4.5$) led a massive Caspase 3 activation (Figure 2A&S2A), close to that observed with the zebrafish orthologue of Bax (zBax) ($87.0\% \pm 4.7$), suggesting that these two Trichoplax proteins are genuine Bcl-2 pro-apoptotic members (Figure 2A&S2A). We further confirmed these results using zebrafish embryos as a model. Indeed, during zebrafish development, embryos are resistant to apoptotic stimuli prior to the gastrulation stage which starts approximately at 6 hours post fertilization (hpf). The forced expression of Bcl-2 pro-apoptotic proteins has been shown to induce apoptosis in these embryos prior to this stage (Kratz et al., 2006; Popgeorgiev et al., 2011). We thus injected at the one cell stage *in vitro* transcribed mRNA encoding for

each Bcl-2 homolog. Expression of trBcl-2L1 and -2L2 did increase embryo mortality. However, the expression of trBax and trBak led to embryo death, reaching almost 100% at 6 hpf (Figure 2B). Interestingly, co-expression of trBcl-2L1 and -2L2 in combination with the trBax and trBak proteins led to the complete inhibition of their pro-apoptotic activity. These results suggest that trBax and trBak are pro-apoptotic Bcl-2 members, the activities of which may be antagonized by trBcl-2L1 and -2L2 proteins probably through direct interactions. To test this hypothesis, we performed fluorescence resonance energy transfer (FRET)-based interaction analyses between an EGFP donor and an Alexa568 acceptor in BMK DKO cells. Indeed, we expressed EGFP-tagged trBax and trBak in combination with Flag-tagged trBcl-2L1 and trBcl-2L2 and performed immunofluorescence using anti-Flag and secondary antibodies coupled with Alexa568 fluorophore. Interactions between trBcl-2-like proteins were estimated by measuring the dequenching of EGFP emission induced by Alexa568 photobleaching. As seen on Figure 2C, FRET efficacy between EGFPtrBax/Flag-trBcl-2L1 ($5.84\% \pm 0.84\%$) and EGFPtrBak/Flag-trBcl-2L2 ($5.45\% \pm 0.65\%$) was similar to control cells expressing EGFP fused to human Bax and to Flag-tagged human Bcl-2 protein ($4.92\% \pm 0.78\%$). Interestingly, FRET efficacy between EGFPtrBak/Flag-trBcl-2L1 ($11.18\% \pm 1.34\%$) was close to the positive control a NeonGreen-Flag-Nrh ($11.20\% \pm 0.93\%$) protein, whereas EGFPtrBax and Flag-trBcl-2L2 interacted only weakly ($1.97\% \pm 0.96\%$) (Figure 2C&S2B).

We next evaluated the capacity of trBax to induce apoptosis independently of the presence of endogenous Bax and Bak by performing a series of apoptosis assays using baby mouse kidney cells knocked out for endogenous *bax* and *bak* (BMK DKO cells) (Figures S2C). When expressed in these cells, EGFP tagged trBax rapidly translocated to the mitochondria where it induced mitochondrial fragmentation, Δg loss and Cytochrome C release (Figures S2D-H). Furthermore, the majority of the trBax expressing cells were Caspase 3-positive ($86.8\% \pm 3.1\%$) in comparison to control cells ($0.5\% \pm 0.45\%$), demonstrating that trBax is a *bona fide* Bax-like protein (Figure 2D). Of note, trBcl-2L1 ($24.8\% \pm 3.8\%$) and to lesser extent trBcl-2L2 ($54.9\% \pm 3.1\%$) were able to counteract the pro-apoptotic activity of trBax (Figure 2D).

However, inhibition of trBax pro-apoptotic activity was not restricted to Trichoplax Bcl-2 members. Indeed, when human Bcl-2 homologs were expressed in combination with trBax we observed a significant decrease in the number of Caspase 3-positive cells (Figure 2E&S2I). This was however not the case for all human anti-apoptotic Bcl-2 proteins. For instance, Mcl-1, Bfl-1, Nrh and Bcl-w inhibited trBax

in a similar manner as trBcl-2L1, while Bcl-2 and Bcl-xL had no effect on its pro-apoptotic activity, suggesting an evolutionary conservation in the interaction between the hydrophobic BH3 domain-binding groove of anti-apoptotic Bcl-2 proteins and the BH3 domain of trBax.

Next, we evaluated the effect of trBak expression on BMK DKO apoptotic cell death. Although trBak was highly expressed in BMK DKO cells, it did not result in Cytochrome C release or significant Caspase 3 activation showing that the pro-apoptotic activity of trBak is dependent on the presence of endogenous Bax or Bak (Figure 2D&S2C-E, S2G). Interestingly, trBak co-expression inhibited the anti-apoptotic activity of trBcl-2L1 and trBcl-2L2 over trBax, without increasing its pro-apoptotic potential suggesting that this protein actually acts as a BH3-only sensitizer (Figure 2D&2F).

Subcellular localization of trBcl-2 proteins

In vertebrates, members of the Bcl-2 family can be localized both at the level of the mitochondria and ER. In order to test whether this feature was also present in Trichoplax Bcl-2 homologs, we transiently expressed N-terminus Flag-tagged trBcl-2-like proteins in Cos-7 cells. We stained mitochondria and ER membranes with the mitochondrial dye Mitotracker Deep red and with ER-targeted EGFP, respectively. Under these conditions we were able to detect trBcl-2L1 and trBcl-2L2 in mitochondrial and ER membranes (Figure 3A). Of note, cells expressing these two proteins presented hyper fused perinuclear mitochondria. In the case of trBcl-2L2, mitochondria also presented a decreased membrane potential, detected by the decreased fluorescence of the Mitotracker Deep red dye. In addition, intracellular trBcl-2L2 localization was more dispersed than trBcl-2L1, suggesting that this protein could be also cytosolic. When we expressed trBax and trBak in the presence of the pan Caspase inhibitor zVAD-FMK we detected these proteins majorly at the level of the mitochondria (Figure 3A). These observations were confirmed by performing subcellular fractionation on BMK DKO cells followed by immunoblotting. Indeed, trBcl-2L1 was found to be membrane bound whereas a fraction of trBcl-2L2 was found in the cytosol (Figure 3B). Bax localization to MOM represents a dynamic process of translocation and retrotranslocation which depends on internal cues most importantly the $\alpha 9$ hydrophobic helix containing the TM domain but also

the a1 helix and a proline residue located after the BH2 domain (Cartron et al., 2005; Edlich et al., 2011; Schinzel et al., 2004). It has been shown that the point mutation of this residue at position 168 into alanine actually impairs the translocation of human Bax (hBax) (Schinzel et al., 2004; Simonyan et al., 2017). Interestingly, trBax WT is a natural alanine mutant (alanine at position 212) which possesses a1 and a9 hydrophobic helices as well as a unique S/T rich region between a helix 1 and 2, predicted as a mucin-like domain, found in secreted proteins. In order to understand how these residues regulate trBax localization at the MOM we generated two site-directed mutants: P212A and N(8)T in which we replaced the alanine residue with proline and the eight threonine residues by asparagine residues, respectively. We also generated three trBax deletion mutants, lacking the a1 ($\Delta a1$), a9 ($\Delta a9$) or both a1 and a9 ($\Delta a1\Delta a9$) helices (Figure S3A). Expression of EGFP-tagged WT trBax rapidly resulted in its translocation to the mitochondria in comparison with hBax in which a significant fraction remained cytosolic suggesting that trBax does not possess the same translocation-retrotranslocation dynamics as human Bax does (Figure S3B).

When we expressed trBax mutants we detected that only proteins lacking a9 helix have cytosolic localization. In addition P212A and N(8)T mutations did not affect the mitochondrial distribution of trBax. This mitochondrial localization was tightly correlated with the capacity of trBax to induce Caspase 3 activation. Indeed, trBax mutants lacking the TM domain had reduced capacities to induce cell death in BMK DKO cells (Figure S3C).

The importance of the TM domain for the subcellular localization of the other trBcl-2 proteins was further demonstrated. To this end we generated mutants lacking the last hydrophobic alpha helix (ΔTM) predicted *in silico*. When expressed in Cos-7 cells the trBcl-2L1 ΔTM , L2 ΔTM and trBak ΔTM mainly displayed a cytosolic distribution (Figure S3D).

TrBcl-2L1 has a dual activity at the mitochondrion and ER

Since two members of the Bcl-2 family in Trichoplax have a dual ER/mitochondria subcellular localization, we hypothesized that these proteins may control intracellular calcium fluxes at the level of the ER. We focused on trBcl-2L1 which possesses a clear ER and mitochondrial localization without proteolytic

cleavage. To test the activity of trBcl-2L1 at the ER we expressed this protein in combination with the ER targeted Ca^{2+} biosensor R-CEPIA1er and subject the cells to IP_3 -dependent calcium release using histamine. In comparison with control cells (0.60 ± 0.051) which were transfected with the empty vector, trBcl-2L1-expressing cells had a lower level of intraluminal calcium (0.37 ± 0.082) following histamine treatment, suggesting that trBcl-2L1 is able to increase the permeability of the IP_3R calcium channel (Figure 4A-C). This observation was further confirmed at the level of the cytosol and the mitochondria where trBcl-2L1 expression induces concomitant increase in cytosolic (1.58 ± 0.02) and matrix Ca^{2+} concentrations (1.60 ± 0.02) compared to control cells (1.35 ± 0.04) (1.47 ± 0.034) (Figure S4A-D). In order to test whether this regulation depends on the ER localization of trBcl-2L1, we generated two single subcellular mutants by replacing its endogenous TM domain with the TM domain of the ER-based Cytochrome B5 protein or with the TM domain of the MOM-localized ActA protein (Figure S4E-H). Expression of trBcl-2L1CB5 but not trBcl-2L1ActA was able to sustain higher IP_3R dependent calcium release demonstrating that indeed this molecular function of trBcl-2L1 depends on its ER-localization (Figure 4D&E). Interestingly, when we tested the capacities of each trBcl-2L1 mutant to repress the pro-apoptotic activity of trBax we observed the opposite result (Figure 4F); TrBcl-2L1ActaA was able to repress trBax-induced apoptosis, whereas trBcl-2L1CB5 had no effect.

Altogether these results suggest that at the molecular level trBcl-2 proteins possess distinct functions in the control of the MOMP or in the regulation of intracellular calcium fluxes which are predetermined by their subcellular localization (Figure 4G).

DISCUSSION

During the course of our research we identified and molecularly characterized four Bcl-2 homologs encoded by the genome of *Trichoplax adhaerens*. All four genes presented a single conserved BH2 splitting intron, which is located specifically after the TGG codon encoding a tryptophan residue. This specific intron is not restricted to placozoans but has been found in almost all *bcl2* homologous genes in multicellular animals (Aouacheria et al., 2005). This observation suggests that Bcl-2 multidomain members arose from a common *bcl2* ancestral gene which already possessed this BH2 splitting intron. However, it remains unclear whether this ancestral gene had pro- and/or anti-apoptotic functions or even functions unrelated to the regulation of MOMP. Mining the *Trichoplax* genome did not allow us to

detect a gene encoding a BH3-only protein. One possibility is that *Trichoplax* encodes for a highly divergent BH3-only protein which currently remains elusive. Alternatively, it is possible that trBak, although structurally related to multidomain Bcl-2 proteins, plays the role of a functional BH3-only protein. Indeed, when expressed in BMK DKO cells, trBak failed to induce Cytochrome C release and Caspase 3 activation in comparison with trBax which promoted MOMP independently of the endogenous Bax and Bak status. Furthermore, trBak binds to both anti-apoptotic trBcl-2L1 and -2L2. This suggests that trBak protein may function as a BH3 only sensitizer that repress heterodimers formation between anti-apoptotic trBcl-2 proteins and trBax which ultimately results in trBax oligomerization. Indeed, our results show that trBax is a *bona fide* pro-apoptotic Bax like protein that is majorly localized to the MOM, where induces MOMP and cytochrome C release in cells lacking endogenous Bax and Bak. Interestingly, unlike its human ortholog which dynamically navigates between the cytosol and the MOM through translocation and retrotranslocation, trBax seems to directly target the mitochondrion. This mitochondrial localization depends on the C-terminus TM domain. Indeed, trBax mutants lacking this hydrophobic helix mainly displayed a cytosolic distribution and a decreased pro-apoptotic activity. Interestingly, deletion of both $\alpha 1$ and $\alpha 9$ helices completely abolished the capacity of trBax to induce Caspase 3 activation, suggesting that the hydrophobic $\alpha 1$ helix may also contribute to trBax localization to the MOM.

TrBax protein also possesses a serine/threonine rich sequence located between the $\alpha 1$ and $\alpha 2$ helices. *In silico* prediction identified this region as a mucin-like domain. Mucins are secreted proteins subjected to O-linked glycosylation at these serine/threonine residues at the level of the Golgi apparatus. Although mutation of these residues into asparagine residues did not affect trBax subcellular localization, it is tempting to speculate that *in vivo* trBax might function as an excreted cellular toxin. Thus it would be of interest to detect the subcellular localization of trBax and determine its glycosylation profile in *Trichoplax*. Our current results strongly suggest that in *Trichoplax*, trBcl-2 proteins may contribute to control apoptosis through the regulation of MOMP. Indeed, analysis of the *Trichoplax* genome revealed the presence of key components of the mitochondrial pathway including Apaf-1, Cytochrome C as well as initiator and effector caspases (Kamm et al., 2019; Srivastava et al., 2008). However, whether apoptosis occurs in *Trichoplax in vivo* remains unclear. Research performed on this model using nutlin-3 a cis-

imidazoline analog which inhibits the interaction between Mdm2 and p53 or roscovitine a cyclin-dependent kinase inhibitor were shown to induce TUNEL-positive cells, indicating that at least in stress conditions apoptosis may operate in placozoans (von der Chevallerie et al., 2014). Placozoans are small animals that lack embryonic development and reproduce mainly asexually through fission or budding. They are commonly found in coastal zones in tropical and subtropical waters. Thus, it is tempting to speculate that these animals may commonly be exposed to various stresses including UV radiation, or mechanical and chemical stresses, suggesting that apoptosis may play an important role in the maintenance of homeostasis.

We finally demonstrated that the anti-apoptotic *Trichoplax* Bcl-2 members possess double ER and mitochondrial subcellular localization, indicating that they may act at the level of the ER especially in the control of the intracellular calcium levels. This was actually the case for trBcl-2L1, which is able to increase the permeability of IP₃R stimulated by histamine. Interestingly, this function required the sole ER localization of trBcl-2L1, whereas the mitochondrial localization is required for inhibiting trBax MOMP. These results demonstrate that at the molecular level members of the Bcl-2 family are actually multifunctional and that the interaction between Bcl-2 family proteins and calcium related channels such as IP₃R appeared early in the evolution of metazoans. Future research should be performed on *Trichoplax* animals in order to analyze the implications of these two functions *in vivo*.

MATERIALS AND METHODS

TrBcl-2 ORF cloning and expression analysis The open reading frames of the four Bcl-2 homologs in *Trichoplax* were cloned using cDNA from the *Trichoplax adhaerens* Grell clone (kindly provided from Bernd Schierwater lab) using the following primers:

trBcl-2L1-F 5'ATGTCTCCTGAACGCGAAAAAATGG
trBcl-2L1-R 5'TCACCATCTTTCCCACACTGCAAGCG
trBcl-2L2-F 5'ATGACATCTTCCTTCGATGAGGCATC
trBcl-2L2-R 5'TTATCTATTAACAGAAAGTATGCACAAGTACC

trBcl-2L3-F 5'ATGGCCGATTTACGTATGTA
trBcl-2L3-R 5'TTAGCTACTGTTATATCGTTGCCATGTG
trBcl-2L4-F 5'ATGTCTAGCACTATTACAATAGCTGAATCGC
trBcl-2L4-R 5'TTATTCTCTAAAATAGCCTGAAAGCCATAGG

The PCR amplicons obtained were cloned into the pJET1.2/blunt cloning vector (Thermo Fisher Scientific) and sent for sequencing. Verified sequences were deposited in an NCBI databank with the following Acc. Numbers KU500586.1 (trBcl-2L1), KU500587.1 (trBcl-2L2), KU500588.1 (trBcl-2L3), KU500589.1 (trBcl-2L4).

For expression analysis internal primers targeting exon 1 of each *bcl2* homolog were used for PCR amplification with the following sequences:

Int-trBcl-2L1-F 5'TCCGTAATGGCTGCAACTGG
Int-trBcl-2L1-R 5'CTCACGTA
Int-trBcl-2L2-F 5'GCTTACCCAACGTTTGTGGG
Int-trBcl-2L2-R 5'TGTTGCTTCGATCCAGGTAG
Int-trBcl-2L3-F 5'CCACCACTACTCAGTCATGTCC
Int-trBcl-2L3-R 5'TGTTGGTGACGTCGGTTCAA
Int-trBcl-2L4-F 5'CTATCGGCAAATCCGGCTCA
Int-trBcl-2L4-R 5'TGCGCTAAATGGCGTCCTAT

Flag-tagged sequences were ordered using G-blocks synthesis (IDTDNA) and were cloned into the pCS2+ expression vector between BamHI/XhoI. TrBax sequence was codon optimized (trBaxopt) for expression in eukaryote cell system. These sequences were used for PCR amplification. HA-tagged trBcl-2-like and 3xFlag-tagged sequences were cloned into the pCS2+ expression vector between BamHI/XhoI and EcoRI/XhoI restriction sites respectively. TrBak, trBax and associated mutants were cloned in pEGFP-C1 vector using BglII/EcoRI restriction sites using the following primers:

HA-trBcl-2L1-F
5'ATATGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGTCTCCTGAACGCGAAAAAATGG
HA-trBcl-2L1-R
5'ATATCTCGAGTCACCATCTTTCCCACTGCAA

HA-trBcl-2L2-F

5'ATATGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGACATCTTCCTTCGATGAGGCATC

HA-trBcl-2L2-R

5'ATATCTCGAGTTATCTATTAACAGAAAGTATGCACAAGTACC

HA-trBax-F

5'ATATGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGGCCGATTCACGTATGTA

HA-trBax-R

5'ATATCTCGAGTTAGCTACTGTTATATCGTTGCCATGTG

HA-trBak-F

5'ATATGGATCCATGTACCCATACGATGTTCCAGATTACGCTATGTCTAGCACTATTACAATAGC

HA-trBak-R

5'ATATCTCGAGTTATTCTCTAAAATAGCCTGAAAGCCATAGG

3xFlag-trBax-F

ATATGAATTCGCCGATTCACGTATGTA

3xFlag-trBax-R

ATATCTCGAGTTAGCTACTGTTATATCG

EGFP-deltaa1-trBaxopt-F

GTGTAGATCTAAGTCCGTGACTTCCACACCAACGA

EGFP-trBaxopt-R

ATATGAATTCCTAGCTGCTGTTGTAGCGCTGC

EGFP-deltaTM-trBaxopt-R

ATATGAATTCCTAGCTGCTGTTGTAGCGCTGC

P212A-F

TATATACAGCAACCGGACTGGAAGAACAAT

P212-R

ATTTGTTCTTCCAGTCCGGTTGCTGTATATA

Cell lines and transfection HeLa, Cos-7, BMK WT or DKO cell lines were grown under standard cell culture conditions (37°C, 5% CO₂) in Dulbecco's modified Eagle's medium (DMEM) high glucose medium

(Gibco) supplemented with 10% FBS (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cell transfection was performed using X-tremeGENE™ HP DNA Transfection Reagent (Roche) according to the manufacturer's protocol. Six hours post transfection cell medium was removed and cells were incubated for 24–48 h with new medium.

Subcellular fractionation All steps were carried out at 4°C. Transfected BMK DKO cells placed in two 15 cm in diameter plates were washed twice in PBS and added to 1,5 mL of ice cold MB buffer (210mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.5) containing protease inhibitors). Cells were disrupted by shearing with a dounce homogenizer for 20 to 30 times. The disrupted cells were then centrifuged twice at 600 g for 5min to eliminate cell nuclei and centrifuged at 7,000 g to pelled crude mitochondria. Pellet was washed two times with fresh MB buffer and centrifuged at 10,000 g. Mitochondria were resuspended in an appropriate volume of MB buffer for further analyses. The supernatant was centrifuged at 100,000 g for 1 h, and the pellet containing the microsomal fraction was resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0,1% SDS, 50 mM Tris-HCl (pH 7.4), containing protease inhibitors). Fifteen µg of each subcellular fraction and 50 µg of the total fraction were analyzed by western blot.

Western blot analysis and antibodies Western blots were performed as previously described (Arnaud et al., 2006). The following antibodies were used: F₁F₀ ATPase (BD #612518; dilution: 1/1,000), Vinculin (Santa Cruz #sc-55465; dilution: 1/2000), Calnexin (Cell Signaling Technology # 2679; dilution: 1/1000), Flag M2 (Sigma # F3165; dilution: 1/1,000), HA (Ozyme # BLE901501; dilution: 1/1,000), Cytochrome C (BD Bioscience # 556433; dilution: 1/1000). HRP-conjugated goat anti-mouse, rabbit anti-mouse and goat anti-rabbit secondary antibodies (DAKO) were used as secondary antibodies. Western blot analysis was performed according to standard procedures.

Immunofluorescence staining, FRET measurements, cytochrome C release and apoptosis detection Cells were placed on a glass coverslip in 12-well plates at 50% confluence and left to attach overnight. Cells were then transfected with trBcl-2-like encoding vectors in combination or pCS2+EGFP-CytB5 (ER-localized EGFP) for ER staining. For trBax and trBak subcellular localization zVAD-FMK was added 6 h

post-transfection in order to delay cell detachment. For direct mitochondrial visualization cells were incubated with Mitotracker™ deep red dye (1/10,000) 20 min at 37°C. Cells were then washed once with medium for 20 min and then fixed with a 4% paraformaldehyde solution for 20 min followed by 3 washes with PBS. Following cell permeabilization using PBS Triton X-100 0.1% (PBT) plus BSA 3%, cells were incubated with primary antibodies Flag M2 (Sigma # F3165; dilution: 1/2,500), Cytochrome C (BD Bioscience # 556433; dilution: 1/1000) or Tom20 (Santa Cruz # sc-11415; dilution: 1/500). Goat anti-mouse or goat anti-rabbit IgG Alexa Fluor 488 (green fluorescent) or 568 (red fluorescent) were used as secondary antibodies (Molecular Probes) and used at 1/1,000 dilution for 1 h. Nuclei were visualized using Hoechst 33342 dye (Invitrogen #H3570) at 10 µg/mL.

For FRET-based interaction analyses BMK DKO cells were transfected with Flag-tagged trBcl-2L1 and L2 in combination with EGFP-tagged trBax and trBak. Twenty-four hours later cells were fixed and immunostained using mouse anti-Flag M2 antibody coupled anti-mouse Alexa568 secondary antibody. FRET was measured as previously described (Rong et al., 2008) Briefly EGFP fluorescent intensities were measured before and after photobleaching Alexa568 fluorophore. FRET efficacy was calculated using the following formula $(1 - \frac{EGFP_{pre}}{EGFP_{post}}) * 100$

For apoptosis detection HeLa and BMK DKO cells transiently transfected with trBcl-2like proteins for 24 hours. Cells were fixed and permeabilized as stated above and incubated with anti-activated Caspase 3 antibody (BD Bioscience # 559565; dilution: 1/500). Pyknotic nuclei were labeled using Hoechst 33342 dye. Cells were observed under a fluorescent microscope and the percentage of Caspase 3- and Hoechst-positive cells was determined.

Ca²⁺ measurements ER calcium releasing capacities in BMK DKO stably expressing trBcl-2L1 or empty vector were measured using the ER probe R-CEPIA1er recombinant protein. Cells were transfected with pCMV R-CEPIA1er (Addgene #58216). Twenty-four hours later, cells were seeded onto 96-well plates and incubated overnight. Cells were washed with Ca²⁺-free balanced salt solution (BSS) [121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 6 mM NaHCO₃, 5.5 mM D-Glucose, 25 mM Hepes (pH 7.3)] and incubated with 180 µL of BSS buffer. Raw fluorescence values were collected every 0.21 sec for 2 min at 28°C using

a Clariostar microplate fluorescence reader (BMG Labtech). After 5 sec of basal line measurement, 100 μ M histamine (Sigma) was injected.

Cytosolic Ca^{2+} levels were detected in HeLa cells using Fluoforte dye (Enzo Life Sciences). Cells seeded in 96-well plates were incubated with 5 μ M Fluoforte for 1 hr at 37°C, in a Ca^{2+} -free BSS. Raw fluorescence values were collected every 0.21 sec for 2 min at 28°C using the Clariostar microplate reader. Mitochondrial calcium measurements were performed using Rhod-2 AM chemical dye or CEPIA2mt recombinant protein. Rhod-2 detection was performed as previously described for Fluoforte with the following modifications. Rhod-2 AM dye was incubated at 2.5 μ M with 0.2% of pluronic acid (Molecular probes) for 20 min at 37°C in BBS- Ca^{2+} (BBS supplemented with 2 mM of Ca^{2+}), and washed for 20 min with BBS- Ca^{2+} at room temperature. Raw fluorescence values were collected every 0.21 sec for 2 min at room temperature using the Clariostar microplate reader.

For CEPIA2mt detection, BMK DKO cells were co-transfected with pCS2+Flag-trBcl-2L4 and pCMV CEPIA2mt (Addgene #58218) and seeded at 40% in 8 well Nunc™ Lab-Tek™ II Chamber Slide. Twenty-four hours later, cells were washed with BBS- Ca^{2+} . After 10 sec of baseline, 100 μ M of histamine was added. Raw fluorescence values were collected every 0.21 sec for 2 min at room temperature using the Clariostar microplate reader.

Zebrafish experiments Zebrafish (crosses between AB and TU strains) were raised and maintained at 28.5°C according to standard procedures (Westerfield, 1995). Embryos were collected after fertilization and injected at the one-cell stage with *in vitro* transcribed mRNA encoding trBcl-2L1 to -2L4 proteins (100 pg total mRNA/ embryo) using mMACHINE SP6 Transcription Kit (Thermo Fischer). Embryo death was monitored at regular intervals for 6 h post-fertilization.

Statistical analysis Error bars displayed on graphs represent the means \pm S.E.M. of three independent experiments. Statistical significance was analyzed using the Student t-test. $P < 0.05$ was considered significant.

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AUTHOR CONTRIBUTIONS

N.P performed all the experiments except for the following: L.J performed the cloning and the characterization of the trBcl-2L1 mutants. NP, T.T.M.N and N.R. performed the subcellular localization experiments. R.G., performed the cloning and expression of the codon optimized trBax sequence and deltaTM mutants as well as the cytosolic and mitochondrial calcium measurements. S.M., performed the yeast experiments; H.O. performed Trichoplax DNA/RNA extractions. N.P., H.O., B.S., R.R. and GG participate in the study design and writing the manuscript.

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FIGURE LEGENDS

Figure 1. Sequence and expression analysis of Bcl-2 homologs found in *Trichoplax adhaerens*.

(A) Schematic representation of the primary structures of four multidomain *Trichoplax adhaerens* Bcl-2-like (trBcl-2L1 to -2L4) proteins. The positions of conserved BH1-BH4 domains and of the C-terminus transmembrane domain (TM) as well as the locations of the predicted alpha helices are indicated with colored boxes and orange cylinders respectively. The white triangles represent the position of the conserved BH2 splitting intron. The hydrophobic profile for each protein is presented below. An amino acid scale bar is presented above. **(B)** *In silico* hybridization matrix representing the alignment of four trBcl-2-like proteins versus Bcl-2 related multidomain proteins found in *Danio rerio* (zebrafish = z), *Gallus gallus* (chicken = c), *Xenopus tropicalis* (xenopus = x), and *Homo sapiens* (human = h). Colored boxes refer to the total alignment score (blue; poor alignment, red; high alignment (> 60)). trBcl-2L1 and trBcl-2L2 appear to cluster with anti-apoptotic members, whereas trBcl-2L3 and trBcl-2L4 with the pro-apoptotic Bax and Bak, respectively. **(C)** Phylogenetic tree using the neighbor joining clustering method. TrBcl-2L1 and trBcl-2L2 cluster with the Bcl-2-like subgroup whereas trBcl-2L3 and trBcl-2L4 cluster with Bax and Bak orthologs, respectively.

Figure 2. Control of MOMP by trBcl-2-like proteins.

(A) Quantification of the Caspase 3 activation in HeLa cells transiently expressing trBcl-2-like proteins (mean \pm SEM; three independent experiments). **(B)** Graph presenting the effect of the trBcl-2-like protein expression on the mortality of zebrafish embryos. In contrast to trBcl-2L1 and trBcl-2L2 which had no effect mRNA injection (100 ng/ μ L) into one cell stage embryos of trBax and trBak induced early mortality during gastrulation (mean \pm SEM; three independent experiments). **(C)** Graph showing the results from FRET-based interaction analyses between an EGFP donor and an Alexa568 acceptor. BMK DKO cells expressing EGFP-tagged trBax and trBak in combination with Flag-tagged trBcl-2L1 and trBcl-2L2 were fixed and labeled using anti-Flag and secondary anti-IgG mouse antibodies coupled with Alexa568. Interactions between trBcl-2-like proteins were estimated by measuring the dequenching of EGFP emission induced by Alexa568 photobleaching. **(D)** Histogram showing the effect of trBcl-2-like protein expression on Caspase 3 activation in BMK double *bax* $-/-$, *bak* $-/-$ knock out (DKO) cells. In comparison to HeLa cells, trBak protein is unable to induce Caspase 3 activation in BMK DKO cells (mean \pm SEM; three

independent experiments). **(E)** Histogram showing the effect of the expression of human Bcl-2 homologs on the pro-apoptotic activity of trBax protein in BMK DKO cells. Proteins belonging to the Mcl-1 subgroup as well as Bcl-w antagonize trBax pro-apoptotic activity, while Bcl-2 and Bcl-xL have no effect. (mean \pm SEM; three independent experiments). **(F)** Histogram showing the effect of the expression of trBak protein on the pro-apoptotic activity of trBax in BMK DKO cells. Cells were transfected with increasing amounts of trBax expressing vector 50, 100 and 200 ng in the presence of constant amount of pCS2+ empty vector or pCS2+trBak. TrBak coexpression did not lead to increase of the percentage of Caspase 3-positive cells compared to trBax expressing vector plus empty vector (mean \pm SEM; three independent experiments)

Figure 3. Subcellular localization of trBcl-2-like proteins.

(A) Representative confocal images of Cos-7 cells expressing trBcl-2-like proteins. Subcellular localization of each protein was analyzed using ER-targeted EGFP for staining ER membranes, the Mitotracker™ deep red dye for labeling mitochondria, and the anti-Flag antibody for the detection of Flag tagged Bcl-2-like proteins. Merged channels between Flag and ER-GFP and between Flag and Mitochondria were presented on the right. White boxes represent the enlarged area of each merged image. Scale bars: 20 μ m. **(B)** Subcellular fractionation of BMK DKO cells expressing Flag-tagged trBcl-2-like proteins. TrBcl-2L1 and trBcl-2L2 proteins were found in crude mitochondrial and microsomal fractions. Full length trBcl-2L2 was also detected in the cytosol fraction. TrBax and trBak were found mostly in the crude mitochondria fraction. Anti F₁F₀ ATPase, Calnexin and Vinculin antibodies were used as mitochondrial, ER and cytosol markers, respectively.

Figure 4. Dual role of the trBcl-2L1 protein at the ER and mitochondrion.

(A) Representative images of HeLa cells expressing ER targeted R-CEPIA1er biosensor in combination with pCS2+ empty vector or pCS2+ vector encoding trBcl-2L1. Cells were treated with histamine (100 μ M) to induce Ca²⁺ depletion in the ER. **(B)** Traces showing the relative changes in R-CEPIA1er fluorescence intensities normalized against the baseline value (F/F₀ ratio). Histamine (100 μ M) stimulation induces ER Ca²⁺ depletion. Compared to control cells, trBcl-2L1-expressing cells possess higher ER releasing capacities. **(C)** Histogram depicting the maximum ER release (F_{max}-F_{min} difference)

of HeLa cells expressing trBcl-2L1 or transfected with empty vector. (mean±S.E.M; n = 3 independent experiments). ** $P < 0.01$. **(D)** Representative curves showing the relative changes in R-CEPIA1er fluorescence intensities normalized against baseline values (F/F_0 ratio) following histamine (100 μ M) treatment. ER-targeted trBcl-2L1 and WT trBcl-2L1 have similar releasing capacities, whereas Mito-targeted trBcl-2L1 resembles cells transfected with empty vector. **(E)** Histogram depicting the maximum ER release ($F_{max}-F_{min}$ difference) of HeLa cells expressing WT trBcl-2L1 or single localization mutants (mean \pm S.E.M; n = 3 independent experiments). * $P < 0.05$. N.S. not significant. **(F)** Quantification of the Caspase 3 activation in HeLa cells expressing trBax alone or in combination with trBcl-2L1 WT protein or dedicated single localization mutants (mean \pm S.E.M; n = 3 independent experiments). *** $P < 0.001$. N.S. not significant. (mean \pm SEM; three independent experiments). **(G)** Schematic representation of the model of action of trBcl-2 like proteins. Anti-apoptotic trBcl-2L1 and L2 proteins are able to locate to the mitochondria and the ER. At the level of the mitochondria trBcl-2L1 and L2 bind and inhibit trBax pro-apoptotic activity whereas trBak acts as repressor of their anti-apoptotic activity allowing trBax monomers to accumulate and form pores at the MOM. Independently of this function, trBcl-2L1 is also able to attach to the ER membrane where it controls IP3R dependent calcium release.

Supplementary Figure S1. Expression and analysis of trBcl-2-like protein primary structures.

(A) RT-PCR on RNA extracted from whole *Trichoplax* animals. *TrBcl2like* genes expression was detected using specific exon internal primers. DNA negative (- control) as well as genomic DNA were used as negative controls. L1 primers amplifying plasmid DNA encoding trbcl2l1 ORF was used as a positive PCR control. **(B)** ClustalW alignments of trBcl-2-like proteins with characterized Bcl-2 homologs in multiple species. Sequences corresponding to the BH homology and TM domains were highlighted with colored boxes.

Supplementary Figure S2. Control of MOMP by TrBcl-2-like proteins.

(A) Representative fluorescence images of the effect of trBcl-2-like protein expression on Caspase 3 activation in HeLa cells. Cells were stained with anti-activated Caspase 3 antibody and counterstained with a nuclear dye H333342. mCherry and the zebrafish ortholog of Bax (zBax) were used as negative and positive controls, respectively. Scale bar: 100 μ m. **(B)** Representative confocal images demonstrating the

changes in EGFP (acceptor) fluorescence following Alexafluor 568 (donor) photobleaching. BMK cells expressing different combinations of EGFP-tagged trBax and Bak and Flag tagged trBcl-2L1 and trBcl-2L2 were fixed and immunostained with anti-Flag primary and anti-mouse IgG secondary antibodies linked with the Alexa568 fluorophore. Subtraction between EGFP fluorescence before and after photobleaching are shown in the right-hand column. NeonGreen-Flag-Nrh protein was used as positive control. Scale bar: 10 μ m. **(C)** Immunoblot showing the content of endogenous Bax and Bak proteins in BMK WT versus BMK double *bax*^{-/-}, *bak*^{-/-} knock out cells. F₁F₀ ATPase was used as a loading control. **(D)** Immunoblot presenting the results of Cytochrome C release assay performed in BMK DKO cells. Cells transfected with empty vector or vector expressing trBax or trBak were subjected to Digitonin treatment (25 μ g/mL) for 5 min. Cells were then separated by centrifugation in a pellet (P) fraction containing mitochondrial membranes, and supernatant (S) containing soluble proteins. The two fractions were immunostained with anti-Cytochrome C and anti-F₁F₀ ATPase antibodies. **(E)** Representative confocal images showing the subcellular distribution of Cytochrome C in EGFP-, EGFPtrBax- and EGFPtrBak-expressing BMK DKO cells. In control EGFPtrBak cells, Cytochrome C has a mitochondrial localization and becomes cytosolic following trBax induced MOMP. Scale bar: 20 μ m. **(F)** Representative confocal images showing the changes in morphology of EGFPtrBax-expressing BMK cells. Following translation trBax was rapidly translocated to the mitochondria where it led to rapid cell shrinkage and cell blebbing. Scale bar: 10 μ m. **(G)** Western blot (50 μ g total proteins) showing the expression of trBcl-2-like proteins in BMK DKO cells. Asterisks highlight the bands corresponding to predicted molecular weights. Loading 50 μ g of total protein extract and the use of zVAD-FMK (20 μ M) was not sufficient to detect trBax. **(H)** Western blot (100 μ g total proteins) of protein extracts obtained from detached cells and adherent cells expressing Flag-tagged trBax or transfected with empty vector (-). TrBax protein was highly enriched in the detached cells where it was detected as a multiprotein complex (black arrowhead) resistant to Laemmli treatment at 37°C but became monomeric (red arrowhead) once the temperature was raised to 95°C for 5 min. **(I)** Western blot analysis of the expression of Flag-tagged human Bcl-2 homologs in BMK cells. The Vinculin protein was used as loading control.

Supplementary Figure S3. Subcellular localization of trBax dedicated mutants.

(A) Schematic representation of the primary structures of trBax WT and associated mutants. The positions of conserved BH1–BH4 domains and of the C-terminus transmembrane domain (TM) as well as the locations of the predicted alpha helices are indicated with colored boxes and brown cylinders, respectively. **(B)** Representative confocal images showing the subcellular localization of EGFP-tagged trBax WT protein and associated mutants. Mitochondria were labeled using anti-Tom20 antibody. Merged channels between EGFP and Tom20 were presented on the right. Scale bar: 20 μm . **(C)** Histogram showing the effect of expression of trBax WT and associated mutants on Caspase 3 activation in BMK DKO cells. Deletion of the TM domain strongly impairs the pro-apoptotic activity of trBax (mean \pm SEM; three independent experiments). **(D)** Representative confocal images showing the subcellular localization of HA-tagged trBcl-2-like ΔTM mutants. Mitochondria were labeled using anti-Tom20 antibody. HA fusion proteins were detected using anti-HA antibody. Merged channels between HA and Tom20 were presented on the right. Scale bar: 20 μm .

Supplementary Figure S4. Effect of trBcl-2L1 expression on cytosolic and mitochondrial calcium fluxes

(A) Representative curve of cytosolic Ca^{2+} rise following ER- Ca^{2+} release induced by histamine (100 μM) stimulation. TrBcl-2L1 expression led to an increase in the ER-dependent cytosolic Ca^{2+} rise. Fluoforte fluorescence intensities were normalized against baseline values (F/F_0 ratio). **(B)** Histogram depicting the maximum Ca^{2+} release in the cytosol (F_{max}/F_0 ratio) of HeLa cells following histamine (100 μM) treatment, from (E). (mean \pm S.E.M; $n = 3$ independent experiments). *** $P < 0.001$. **(C)** Representative curves of the relative changes in the mitochondrial Rhod-2AM fluorescence intensity normalized to the baseline (F/F_0 ratio) reflecting the mitochondrial Ca^{2+} rise following histamine (100 μM) stimulation in HeLa cells expressing or not trBcl-2L1 protein. **(D)** Histogram depicting the maximum mitochondrial Ca^{2+} increase (F_{max}/F_0 ratio) following histamine (100 μM) treatment relative to control. (mean \pm S.E.M; three independent experiments). ** $P < 0.01$. **(E)** Schematic diagram presenting the construction of trBcl-2L1 mutants with single subcellular localization. BH and TM domains are presented with colored boxes. **(F)** Western blot showing the expression of HA-tagged trBcl-2L1 mutants with single subcellular localization in HeLa cells. **(G)** Subcellular fractionation of HeLa cells expressing HA-tagged trBcl-2L1ActA and CB5 mutants. Anti F_1F_0 ATPase, Calnexin and Vinculin antibodies were used as mitochondrial, ER and cytosol

markers, respectively. **(H)** Representative confocal images of HeLa cells expressing trBcl-2L1 mutants with single subcellular localization. Subcellular localization of each protein was analyzed using anti-KDEL antibody for the staining of ER membranes anti-Tom20 antibody for labeling mitochondria anti-HA antibody for detection of HA tagged Bcl-2-like proteins. Merged channels between HA and KDEL and between HA and mitochondria are presented on the right. Scale bar: 10 μ m

FIGURES

Figure 1

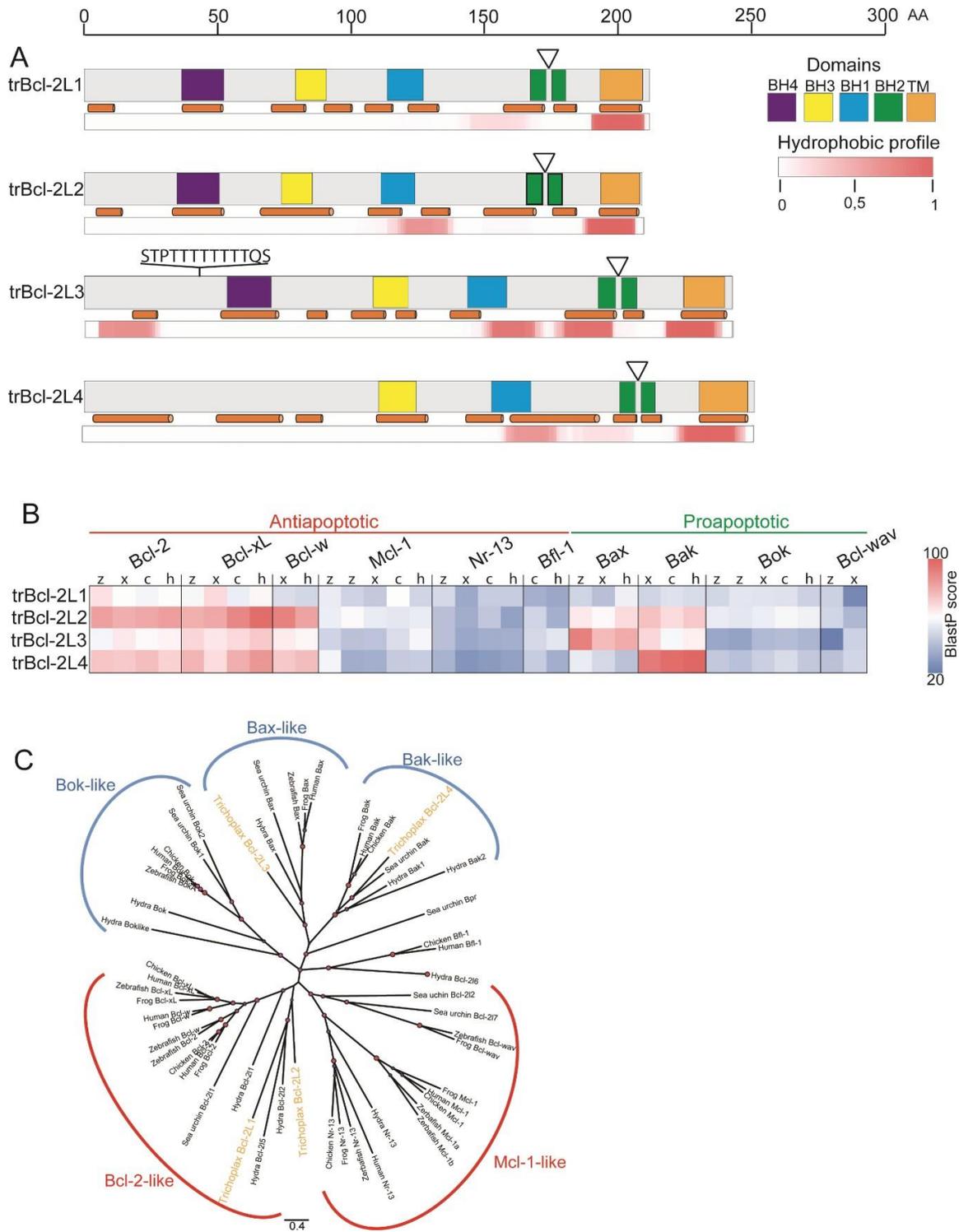


Figure 2

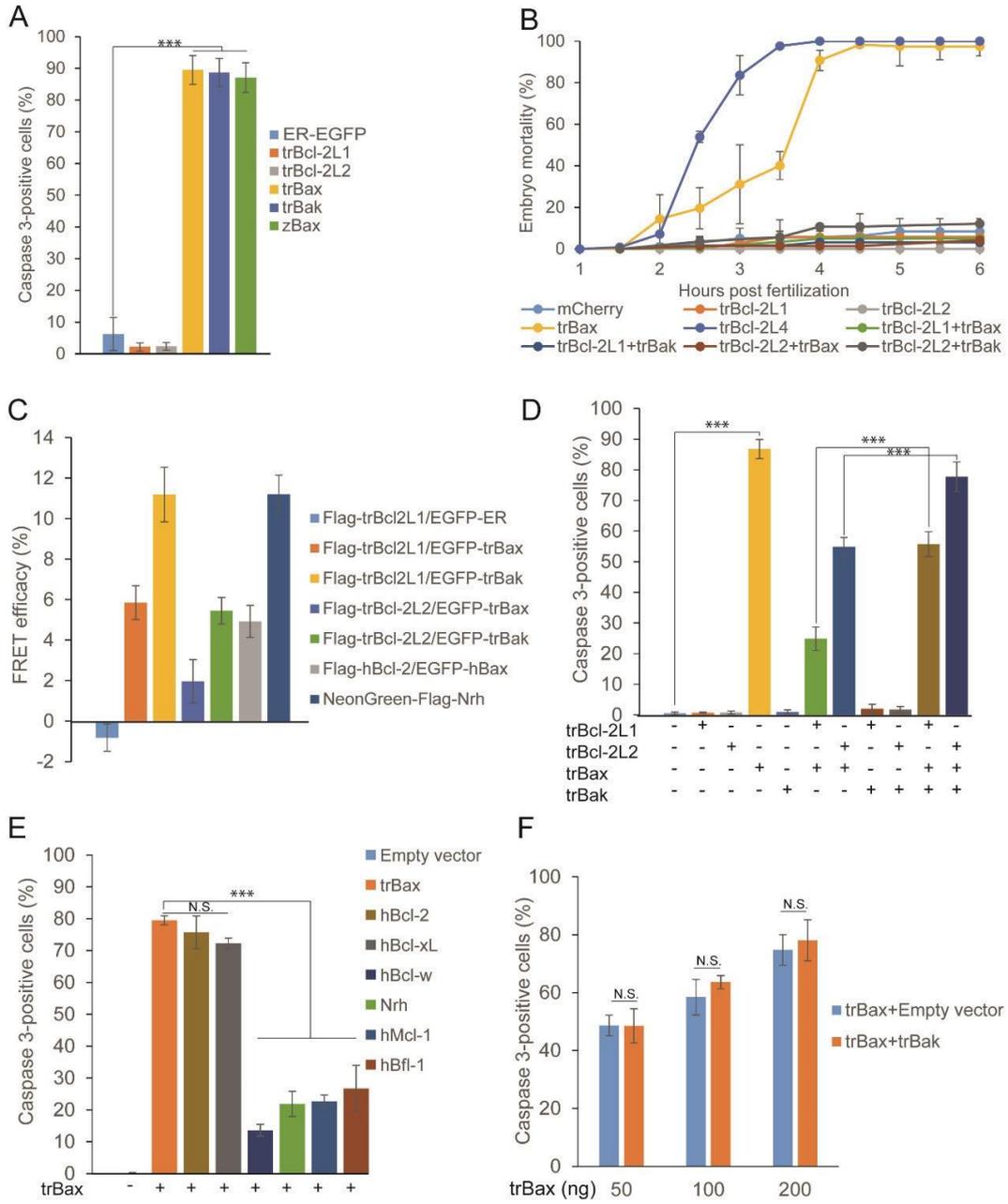


Figure 3

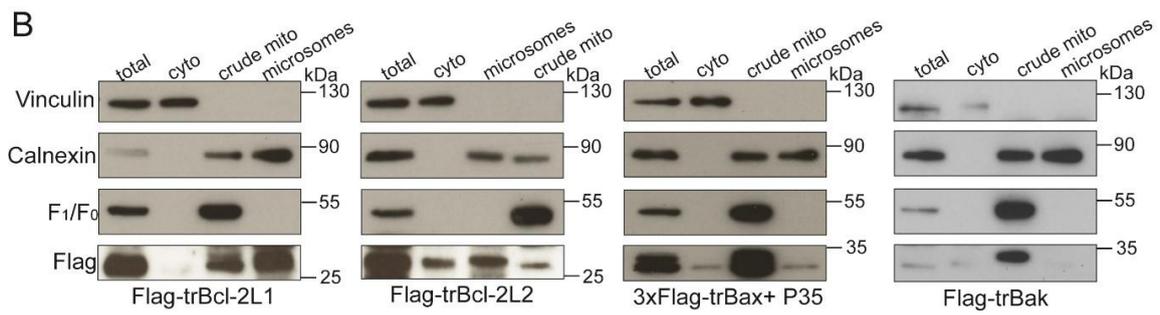
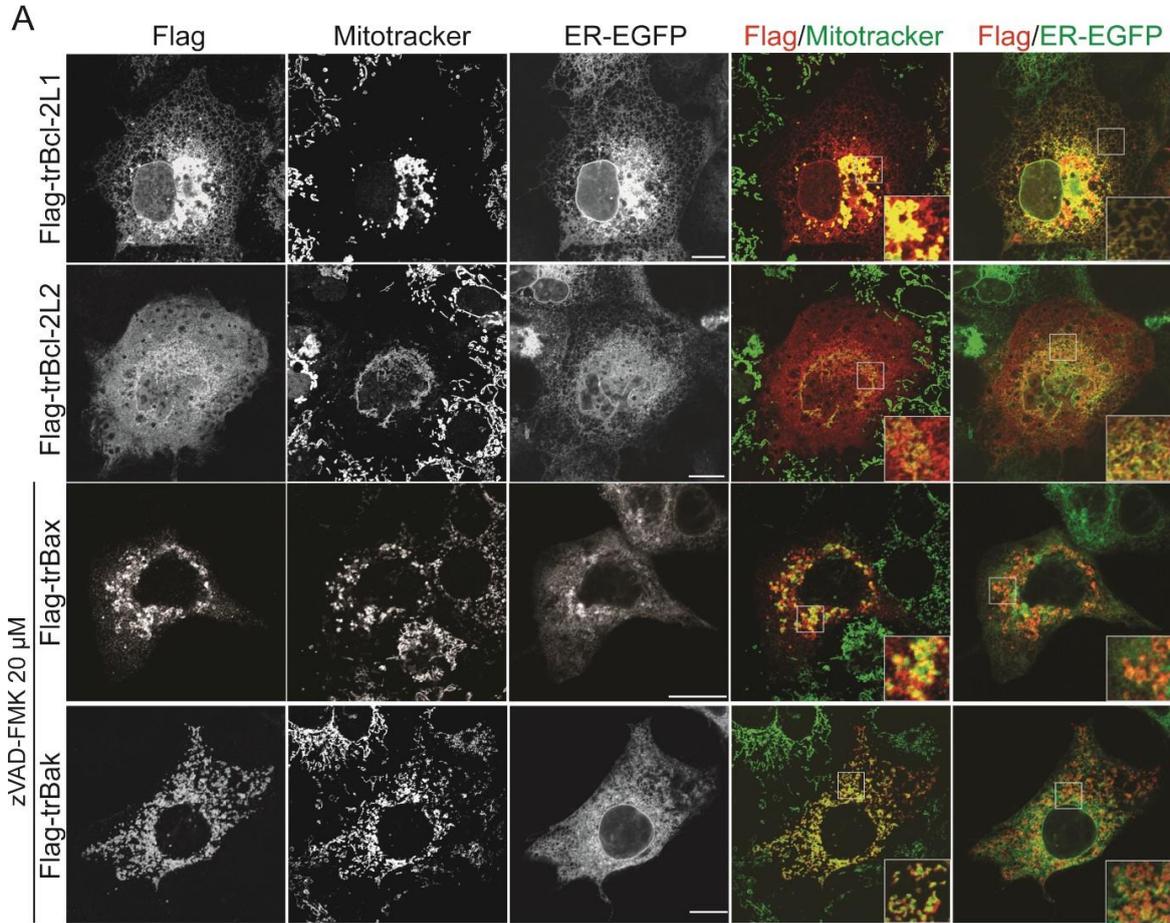


Figure 4

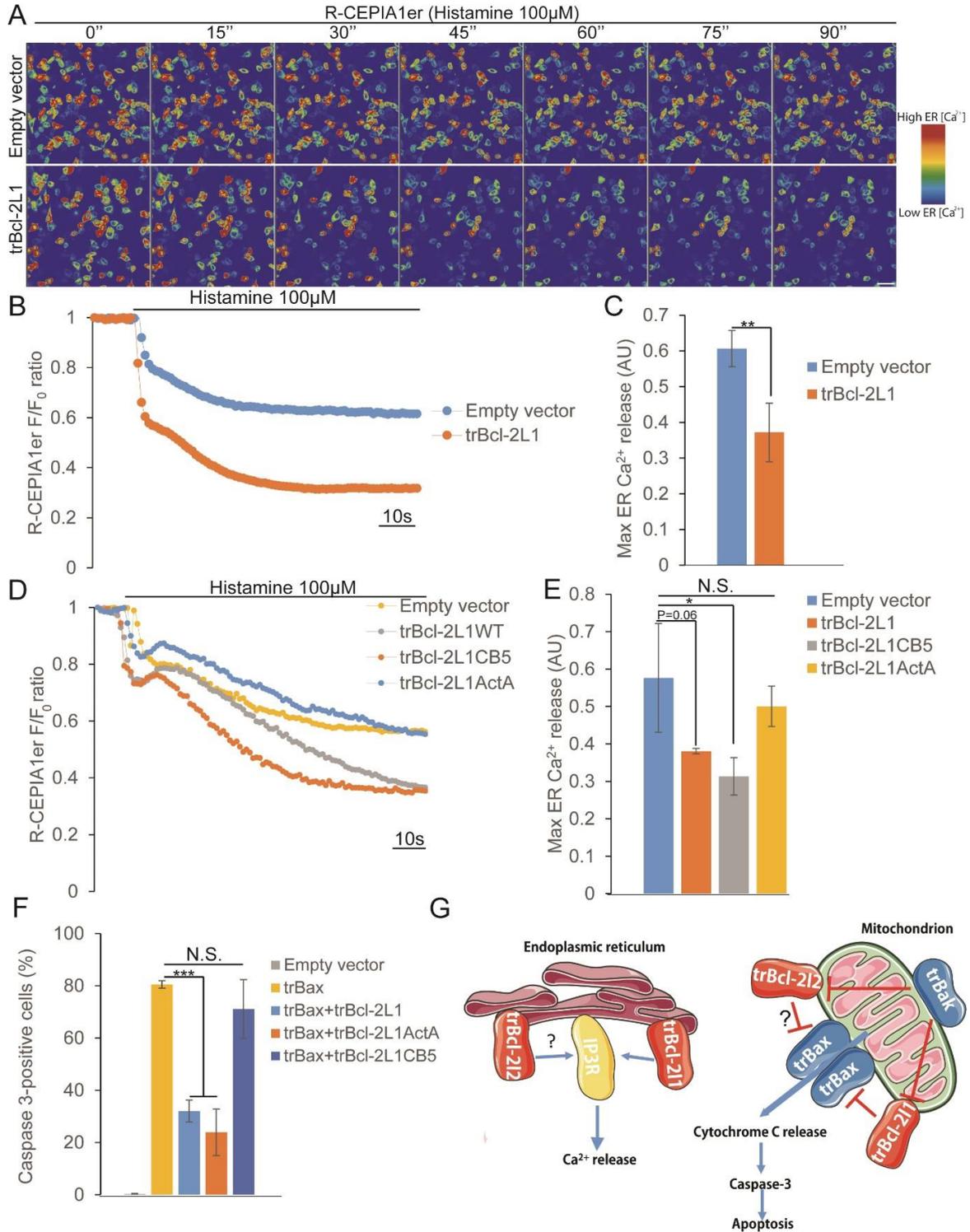


Figure S2

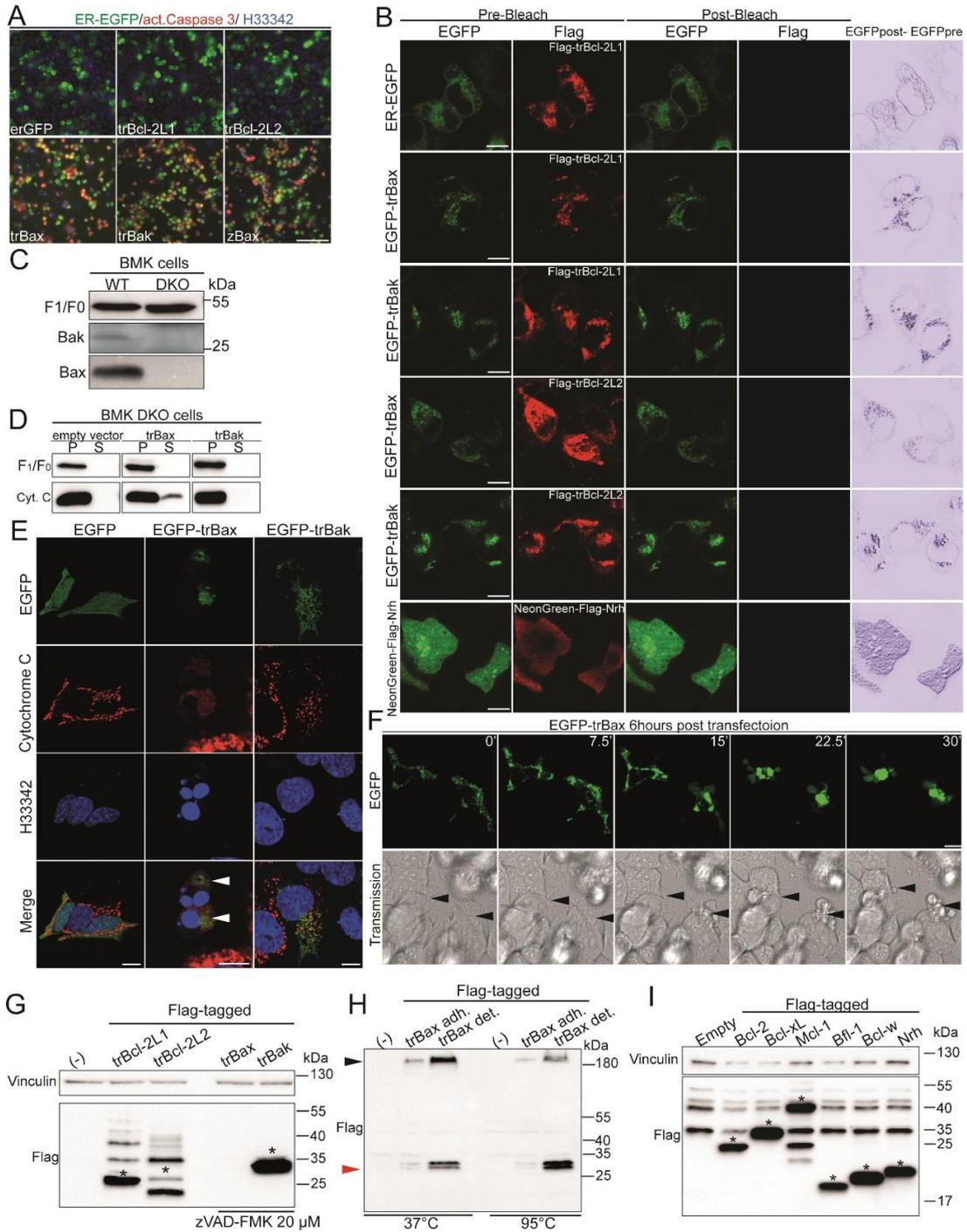


Figure S3

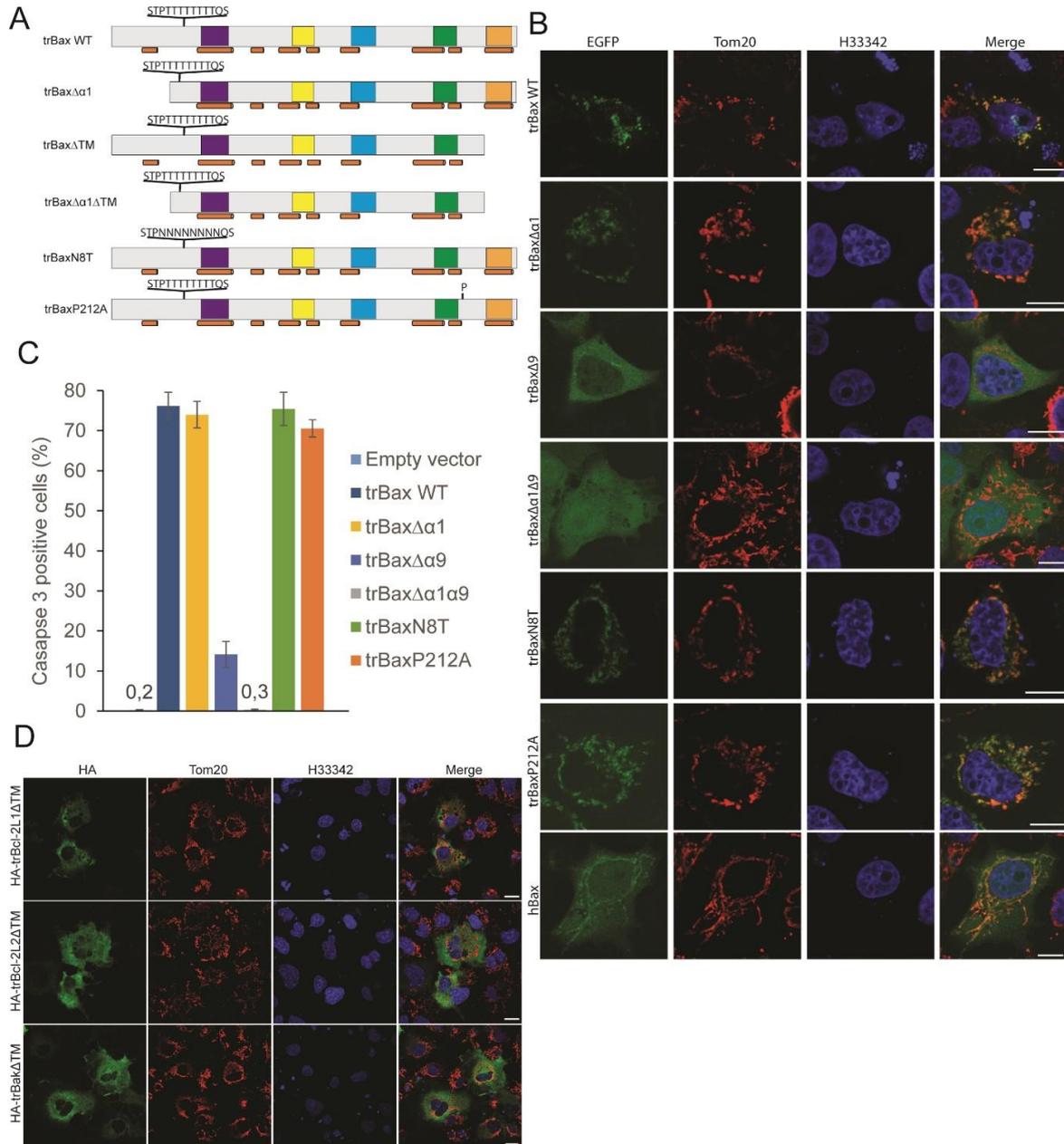
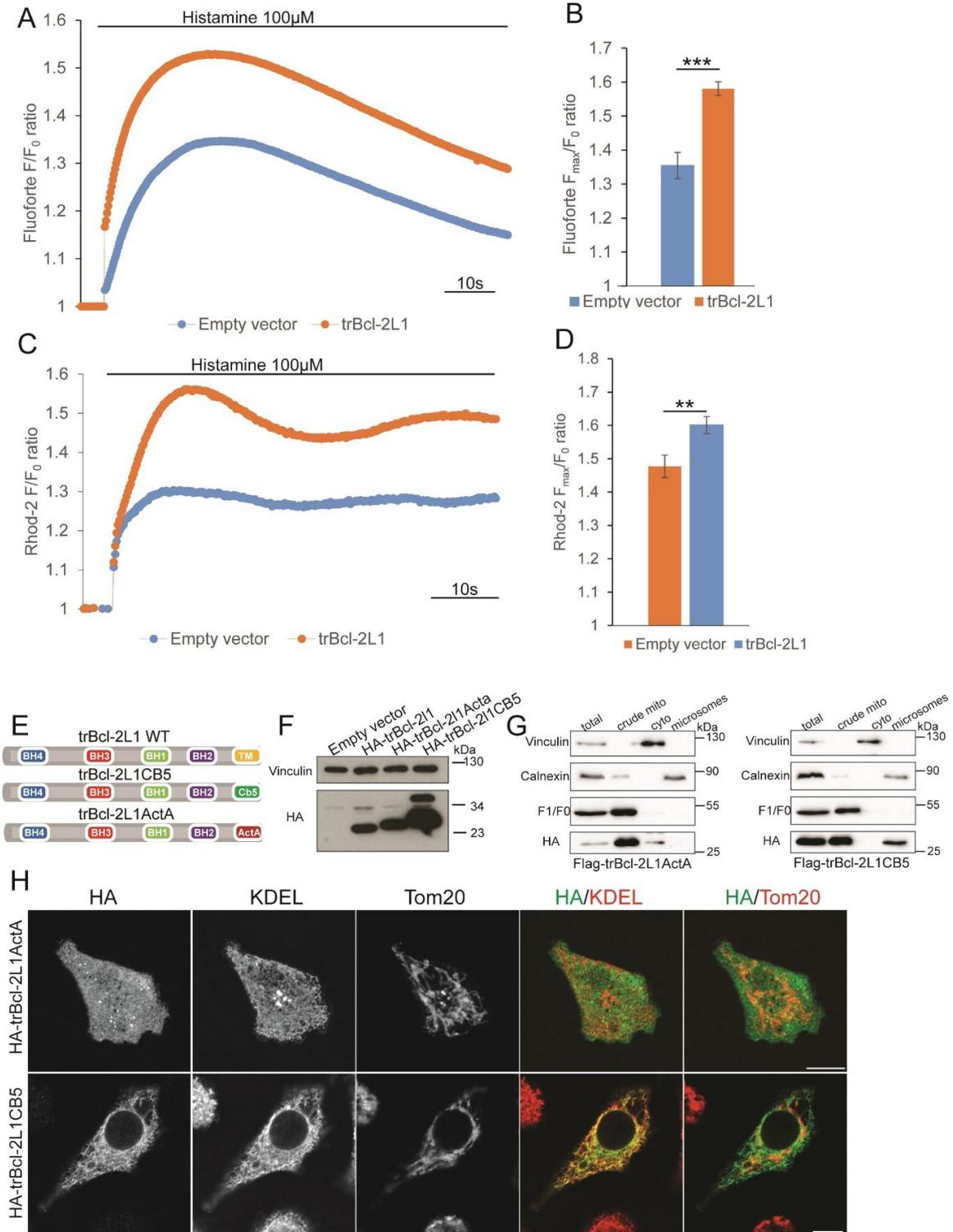


Figure S4



DISCUSSION

Following a great deal of interest in the apoptotic regulatory role of Bcl-2 proteins at the level of the mitochondria, several studies emerged and re-focused their attention on numerous cellular and molecular non-canonical involvements of this protein family (Galluzzi et al., 2008, 2012). Throughout the years, efforts have been put into the discovery and characterization of Bcl-2 proteins functional aspects independent from their control over cell death. Indeed, accumulating evidence proved the contribution of several Bcl-2 members to the execution of diverse cellular functions *via* their wide subcellular localization and dynamics. Accordingly, different members were also localized outside the mitochondria such as Bcl-2, Bax, Bak and Bcl-xL (Annis et al., 2001; Kaufmann et al., 2003; Scorrano et al., 2003; Thomenius et al., 2003). In particular, the ER was in the spotlight acting as a critical hub for Bcl-2 proteins. It has been more than twenty years that the first studies about the intracellular Ca²⁺ homeostasis modulation of Bcl-2 proteins at the ER came out (Baffy et al., 1993; Lam et al., 1994; Magnelli et al., 1994). Since then, various data on the interactions between Bcl-2 members and Ca²⁺ channels were elaborated and were sometimes controversial.

Our team is interested in non-canonical functions of this protein family, especially those involving Ca²⁺ homeostasis. We were able to prove several interactions and subsequent effects such as IP3R1/Nrz interaction controlling zebrafish cytoskeleton dynamics (Popgeorgiev et al., 2011), VDAC/Bcl-wav interaction driving blastomeric migration in zebrafish (Prudent et al., 2013b), IP3R1/Nrh interaction inhibiting ER-Ca²⁺ release and subsequent apoptosis (Nougarede et al., 2018) and VDAC/Bcl-xL interaction modulating the activity of the channel and regulating cell migration (Bessou et al., under reviewing). During my PhD, we were interested in deciphering the role of Bcl-xL at the ER knowing that this member interacts with IP3R. Also, we were curious about the original function of Bcl-2 proteins based on all non-canonical roles we, and so many other teams, have discovered.

A- A novel role for Bcl-xL at the ER

Bcl-xL protects from ER stress

It is no surprise that *bclx* KO MEFs were sensitive to apoptotic stimuli. Bcl-xL is an anti-apoptotic protein responsible for the retro-translocation of Bax and Bak thus inhibiting their MOM oligomerization and subsequent MOMP (Edlich et al., 2011; Todt et al., 2015). Interestingly, KO MEFs were also vulnerable to ER stress-mediated cell death. Knowing that ER stress might result in increased ER Ca²⁺ leakage, this might lead to Ca²⁺-mediated MOMP (Bernardi, 2013). The absence of Bcl-xL will definitely enhance MOMP in KO MEFs where there is no longer sequestration of Bax and Bak nor Bcl-xL-modulation of VDAC Ca²⁺ uptake activity. We can also predict that WT MEFs might be more and more vulnerable if ER stress is not resolved. In fact, under prolonged ER stress, Bcl-xL itself could be degraded. One hypothesis might involve the transmembrane E3 ligase RNF183 that is overexpressed in ER stress conditions. This ligase interacts with Bcl-xL promoting its polyubiquitination and subsequent degradation (Wu et al., 2018).

When checking the kinetic measurements of UPR markers upon ER stress induction by thapsigargin, we noticed that KO MEFs were already stressed at t=0hr. Basal levels of CHOP, phospho-PERK and phospho-eIF2a clearly put in evidence the unstable state these MEFs are in. Interestingly, eIF2a is highly expressed in KO MEFs. In fact, the latter is a part of the eIF2 heterotrimer that controls the initial step of protein synthesis by enhancing the interaction of tRNA to 40S ribosomal subunits (Adams et al., 1975; Lloyd et al., 1980). So instead of reducing ER protein load, KO MEFs are promoting more protein synthesis creating a positive feedback loop where more ER stress leads to more protein synthesis that, in its turn, further enhances ER stress. In these MEFs, the major contributing factor to ER stress is eIF2a. Accordingly, the aim of phospho-eIF2a is to induce translational arrest. Along with CHOP and ATF4 mediating the expression of adaptive genes, these eIF2a downstream effectors try to reduce ER protein load (Quirós et al., 2017). Thus, Bcl-xL presence in the cell plays a role in ER stress resistance. Knowing that the ER and mitochondria are in close proximity (around 10–50nm (Giacomello and Pellegrini, 2016)) and based on the data covering ER stress in WT and KO MEFs, we verified the subcellular localization of Bcl-xL in WT MEFs. Interestingly, the subcellular fractionation resulted in an important expression of Bcl-

xL not only at the mitochondria, but also at the ER. Accordingly, we decided to elaborate the role of Bcl-xL at the ER hypothesizing its involvement in ER stress resistance.

Bcl-xL at the ER confers resistance to ER stress

To test this hypothesis, we generated ER-xL and Mt-xL mice. First of all, we verified the expression of Bcl-xL in cells extracted from different organs and MEFs extracted from embryos. The difference in size of both CB5 and ActA sequences were put in evidence in Western Blot results. For the subcellular localization, we have used three different techniques. First, we performed subcellular fractionation on all three cell lines. We tried to get the purest mitochondria we could have even though technical difficulties might affect mitochondrial purity. In order to confirm the results, we transfected the cells with ER-targeted GFP and stained the mitochondria with Mitotracker. With the fluorescence intensity profile plots we got, we were able to verify the ER localization of Bcl-xL in ER-xL MEFs, the mitochondrial one in Mt-xL MEFs and both localizations in WT MEFs as previously demonstrated. ImmunoGold labelling using Bcl-xL antibody further confirmed the results.

Second of all, we treated the MEFs with the cytotoxic drug staurosporine (inhibitor of PKC). We observed significant vulnerability of ER-xL MEFs compared to WT and ER-xL ones. This result confirms the known indispensable anti-apoptotic role of Bcl-xL at the mitochondria (Eno et al., 2012). We were curious about the anchorage of Bcl-xL at the MOM in WT and Mt-xL MEFs. In order to verify it, we extracted mitochondria from these MEFs by subcellular fractionation and treated them with sodium carbonate (Na_2CO_3) converting membranes into membrane sheets only retaining integral membrane proteins (Kim et al., 2015). As shown in (Annex 1A), Bcl-xL in both WT and Mt-xL mitochondria is well integrated in the MOM compared to TOM20 that was released upon the treatment. These results could justify the fact that both WT and Mt-xL MEFs had approximately the same response to staurosporine. Furthermore, we noticed a slightly, but insignificantly, higher cell death % in ER-xL untreated MEFs compared to the other control MEFs suggesting a basal intracellular stress also confirmed by FACS assessment of the SubG1 population.

This slight increase might be due to a serious stress. We hypothesized that ER-xL MEFs might be more prone to ROS production upon cytotoxic stress especially that Bcl-xL has been shown to exert a constant degree of control on various facets of bioenergetic metabolism (Michels et al., 2013). To verify this, we treated the MEFs with hydrogen peroxide (H_2O_2) and checked for ROS production with confocal microscopy. As shown in (Annex 1B), ROS production in all three cell lines was similar suggesting that either another type of cellular stress is present or the absence of Bcl-xL at the mitochondria itself is enough to stress the MEFs.

Upon the treatment with ER stress inducers thapsigargin and tunicamycin, ER-xL MEFs were resistant to ER stress-mediated cell death. Not only PARP cleavage is the least pronounced in ER-xL MEFs, but also the basal levels of PARP are downregulated in these cells. This finding itself answered to the initial question we were asking: Bcl-xL at the ER, and not at the mitochondria, protects from ER stress.

To further understand how this protein is conferring this protection, we treated the cells with thapsigargin and checked for UPR markers expression. Starting with PERK the very first kinase in the PERK arm of the UPR, it was less phosphorylated in ER-xL MEFs. This indicates that PERK is not completely liberated from GRP78 also demonstrating that the accumulation of unfolded or misfolded proteins in the lumen of the ER is not yet complete (Bertolotti et al., 2000). Lower expression of eIF2a in ER-xL MEFs compared to the others is an indicator of the regulation of protein synthesis in these cells. Interestingly, in all MEFs, a peak of phosphorylation of eIF2a is observed two hours after the treatment that is significantly reduced thereafter. In fact, p-eIF2a, ATF4 and CHOP positively regulate the expression of GADD34 (Fornace et al., 1989; Marciniak et al., 2004). Once active, GADD34 interacts with the phosphatase PP1 responsible for the dephosphorylation of phospho-eIF2a (Connor et al., 2001; Novoa et al., 2001). Accordingly, the expression of phospho-eIF2a ($t=2hrs$) and the increasing levels of ATF4 and CHOP will result in the dephosphorylation of phospho-eIF2a ($t>2hrs$).

On the RNA level, the expressions of ATF3 and CHOP were lower in ER-xL MEFs also confirming protein results. Furthermore, less xbp1 cleavage in ER-xL cells indicate a lower activation of IRE1 and ATF6 arms of the UPR (Liou et al., 1990). Interestingly, ATF4 mRNA levels are high but not significantly different in all three cell types. In fact, two upstream Open Reading Frames (uORFs) are located in the 5'-leader of

ATF4 mRNA. Phospho-eIF2a delays ribosomal initiation once uORF1 is translated. This facilitates inhibitory uORF2 bypass thus increasing the translation of *ATF4* further increasing the levels of ATF4 proteins and subsequent target ones (Vattem and Wek, 2004). Briefly, both transcription and translation regulations control ATF4-mediated response enhancing ATF4 protein levels upon ER stress inductions.

Bcl-xL at the ER negatively regulates IP3R-Ca²⁺ discharge

The interaction of Bcl-xL and IP3R was already highlighted in previous studies. The hydrophobic cleft of this protein interacts with the CD of IP3R sensitizing the latter to low levels of IP3 (White et al., 2005; Yang et al., 2016). We wanted to verify this interaction in our model. PLA on WT MEFs suggested an interaction between IP3R and Bcl-xL. Indeed, the PLA enables the detection of proteins, that might be interacting, in a proximity theoretically ranging from 0 to 40 nm (Debaize et al., 2017).

Assessing the passive Ca²⁺ leakage by inhibiting SERCA with thapsigargin, ER-xL MEFs were able to passively leak more Ca²⁺ (of around 20%). In fact, the ER Ca²⁺ stores are balanced between SERCA activity actively taking up Ca²⁺ into the ER and the passive leak of Ca²⁺ from the ER. One hypothesis about the higher Ca²⁺ leakage capacity of ER-xL MEFs is a higher activity of the ribosome-translocon complex in these cells which is known to mediate ER passive discharge of Ca²⁺ (Coppenolle et al., 2004). Interestingly, when assessing the SOCE, ER-xL MEFs have the highest SOCE. This is not surprising since ER calcium leakage (preferentially occurring *via* the translocon) activates the store-operated current evoked by thapsigargin treatment (Flourakis et al., 2006). Also, cytosolic Ca²⁺ measurements complete SOCE data in a way that ER-xL and Mt-xL MEFs have less cytosolic Ca²⁺ content due to the fact that their ER take up more Ca²⁺.

We were interested in knowing whether Ca²⁺ leakage is also affected by IP3R. By activating phospholipase C (PLC), IP3 is more produced favoring the activation of IP3R. Surprisingly, ER-xL MEFs discharge Ca²⁺ at a significantly slower rate than WT and Mt-xL ones. This results raised the hypothesis that Bcl-xL might negatively regulate IP3R-mediated Ca²⁺ release. In fact, Bcl-xL is known to induce a biphasic regulation of IP3R. In other terms, this protein might either activate or inhibit channel gating depending on the binding of Bcl-xL to either two domain helices at the CD of IP3R or only one,

respectively (Yang et al., 2016). Accordingly, in our MEFs, we suggest that Bcl-xL might only bind to one BH3 resembling domain helix in IP3R C-terminus in ER-xL MEFs thus inhibiting the channel. To see if our hypothesis stands still, we performed Calcium-45 flux assay on permeabilized MEFs loaded with ⁴⁵Ca with the addition of the ionophore A23187 sequestering intracellular Ca²⁺ (Missiaen et al., 1992, 1996). Increasing the concentrations of IP3, we were able to verify IP3R-mediated Ca²⁺ discharge. ER-xL MEFs were able to release the least ⁴⁵Ca *via* IP3R confirming our hypothesis that Bcl-xL at the ER negatively regulates IP3R-mediated Ca²⁺ release.

Bcl-xL at the ER decreases the UPR by reducing IP3R-mediated Ca²⁺ release upon ER stress induction

ER Ca²⁺ depletion is a serious cellular stress and a trigger for the UPR (Mekahli et al., 2011). We hypothesized that Bcl-xL at the ER confers resistance to ER stress and reduces the UPR *via* its negative regulation on IP3R-mediated Ca²⁺ release. After inhibiting IP3R first with 2-Aminoethoxydiphenyl-borate (2-APB) (Hagenston et al., 2009; Xiao et al., 2010) and second with Xestospongine C (Gafni et al., 1997; Wilcox et al., 1998), we treated the MEFs with thapsigargin for 24hrs and checked UPR markers' expression. In an interesting fashion, the UPR was drastically reduced in WT and Mt-xL MEFs that both displayed a similar response to that of ER-xL ones. Importantly, they showed lower phosphorylation of PERK, and less expression of both ATF4 and CHOP, major influencers of the UPR outcome. Interestingly, ER-xL MEFs displayed an observable reduction of ATF4 levels compared to the previous results indicating that indeed, the total inhibition of IP3R might prevent the initiation of UPR and reduce ER stress. To note, both 2-APB and Xestospongine C could not be considered specific potent IP3R inhibitors. 2-APB also has inhibitory effects on transient receptor potential (TRP) family (Ma et al., 2001; Xu et al., 2005), potassium channels (Ma et al., 2010), and volume-regulated anion channels (Lemonnier et al., 2004). As for Xestospongine C, it also inhibits SERCA (Castonguay and Robitaille, 2002; De Smet et al., 1999). However, the replication of our results and their effect on the UPR might indicate that the influences of these drugs might converge in IP3R inhibition further confirming our work hypothesis.

As discussed in this part, Bcl-xL has been shown to perform yet another non-canonical function by regulating IP3R-mediated Ca²⁺ release preventing ER Ca²⁺ depletion and reducing the UPR thus conferring resistance to ER stress. Like other Bcl-2 members, Bcl-xL seems to exert several functions at

critical hubs in order to regulate diverse cellular aspects involving the main functional triangle: adaptation to cellular stress, homeostatic regulation of cell metabolism and cell death control. In this context, we might speculate that Bcl-2 proteins and their homologs might have originated as controllers of cell death-unrelated functions and acquired, later in evolution, the ability to regulate cell fate. This is what drove us to go back 'evolution-wise' and verify the expression and functions of Bcl-2 related proteins in a basal branching multicellular animal *Trichoplax adhaerens*.

B- Functional diversity of the Bcl-2 proteins dates back in metazoan evolution

In 1883, the placozoa phylum was discovered. Then, one well-studied species was identified: *Trichoplax adhaerens* (Eitel et al., 2013). The latter is a small ciliated marine animal with six cell types displaying the most simple and basic morphology of a free-living multicellular animal (Jakob et al., 2004; Smith et al., 2014). This makes *Trichoplax adhaerens* an important candidate of the representation of the basic and ancestral model of metazoan evolution (Schierwater, 2005). In 2008, its genome was sequenced (Srivastava et al., 2008b). Hence, we were interested in discovering Bcl-2-related genes/proteins in this basal branching metazoan and their subsequent functions.

Identification and characterization of trBcl-2Like proteins

After *in-silico* analysis of *Trichoplax adhaerens* genome, we were able to find four encoding protein sequences proved to be Bcl-2 homologs that we called trBcl-2Like. All four share a typical multidomain Bcl-2 protein structural organization: several BH domain, TM domain and alpha helices secondary structures. No BH3-only encoding gene sequences were found in *Trichoplax adhaerens* genome suggesting that BH3-only proteins are the product of advanced evolution, or that BH3-only proteins are pretty well divergent in this species. On the gene level, all four homologs possess a common BH2 splitting intron that is also present in *bcl2*-related genes of a wide range of metazoans (Aouacheria et al., 2005b). This finding suggests that *bcl2* genes might originate from one single ancestral gene comprising this intron and have diverged during the course of evolution. We were then able to show that trBcl-2L1 and trBcl-2L2, despite being highly divergent, clustered with the anti-apoptotic members and trBcl-2L3 and trBcl-2L4 pretty much resembled Bax and Bak, respectively, thus calling them trBax and trBak. The idea

that two of the four Bcl-2 related proteins clustered with anti- and pro-apoptotic groups indicate an ancestral cell death linked function, yet to be elaborated.

Indeed, trBax and trBak displayed a high Caspase 3 activation compared to the first two homologs confirming their role as pro-apoptotic proteins. This result was also confirmed by zebrafish enhanced mortality after the injection of only *trbax* and *trbak* mRNAs reaching 100% only six hours post fertilization. While trBcl-2L1 and trBcl-2L2 were able to counteract the activities of trBax and trBak, we were able to show by fluorescence resonance energy transfer (FRET) analysis that trBak interacts with both trBcl-2L1 and trBcl-2L2 and trBax mainly interacts with trBcl-2L1 having only a weak interaction with trBcl-2L2. Evaluating the capacity of so-called pro-apoptotic members to induce apoptosis, Bax was shown to translocate to the mitochondria and act as genuine pro-apoptotic protein in a way it led to MOMP. Interestingly, few human anti-apoptotic proteins were able to inhibit the pro-apoptotic activity of trBax. This suggests a conserved interaction between the BH3 domain of trBax and the hydrophobic groove of these human Bcl-2 proteins. As for trBak, it was shown to act more like a BH3-only sensitizer where it cannot induce MOMP on its own but inhibits trBcl-2L1 and trBcl-2L2 inhibitory activity on trBax. This finding is quite interesting in a way we can predict how BH3-only protein function started to emerge in multidomain Bcl-2-related protein.

trBcl-2Like proteins might have a dual subcellular localization and interfere with Ca²⁺ homeostasis

Subcellular localization analyses have demonstrated a preference of mitochondrial and ER membranes for trBcl-2L1 and a more diffuse localization of trBcl-2L2 where it can also be found in the cytosol. trBax and trBak were both mainly mitochondrial with a slight presence in the microsomes. These data confirm the dynamics of this protein family that might constitute a conserved trait during evolution. For instance, human Bax is a quite dynamic protein that is constantly retro-translocated from the mitochondria to the cytosol (Edlich et al., 2011; Todt et al., 2013). A Bax mutant with alanine at aa position 168 impairs the retro-translocation process (Schinzel et al., 2004b; Simonyan et al., 2017). Interestingly, trBax is a natural mutant having this point mutation what allowed it to be well integrated at the mitochondria. We can assume that being a well mitochondrial integral protein is one of the reasons that trBax transfection is highly cytotoxic. Furthermore, the TM domain of trBcl-2Like proteins is indispensable for the membrane localization of these proteins and its deletion might cause their cytosolic diffuse distribution. For trBax,

the TM is critical for its MOMP activity. In an interesting fashion, this protein possesses a serine/threonine rich sequence located between the $\alpha 1$ and $\alpha 2$ helices that constitutes a mucin-like domain. This suggests that trBax could act as a secreted protein. Human Bax does not possess this sequence suggesting that trBax might function as a secreted cellular toxin in this organism probably through a cellular defense mechanism required for the protection from MOMP.

Since trBcl-2L1 and trBcl-2L2 also reside at the ER, we speculated that they might affect ER Ca^{2+} homeostasis. Indeed, trBcl-2L1, only at the ER, enhances IP3R-mediated Ca^{2+} release. In an interesting manner, this protein also bound human IP3R suggesting that Bcl-2 proteins-IP3R interaction is conserved through metazoan evolution. When targeted to the mitochondria, this protein performs its anti-apoptotic role and does not interfere with Ca^{2+} fluxes further indicating that Bcl-2 functions are subcellular localization-dependent.

Although these data do not answer the question whether the initial ancestral function of Bcl-2 proteins is indeed cell death regulation, they give new clues about the involvement of Bcl-2 proteins in non-canonical roles covering Ca^{2+} homeostasis. This study is another proof on the importance of the subcellular localization and dynamics of the Bcl-2 family in their interactions and subsequent functions.

CONCLUSION AND PERSPECTIVES

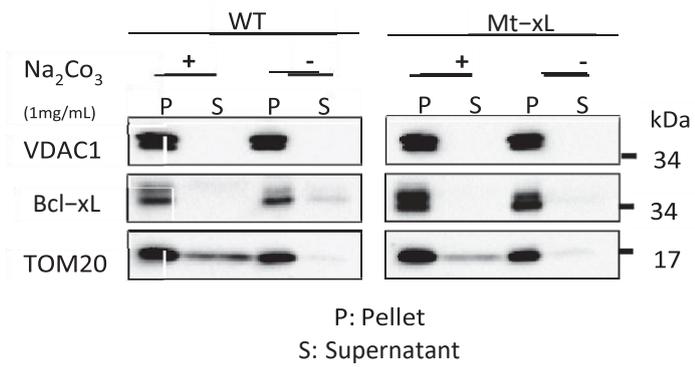
During my PhD, I was interested in the subcellular localization, essentially the ER localization, of Bcl-2 proteins. I was able to prove a functionality of the subcellular localization of Bcl-xL at the ER. Indeed, this protein was shown to protect from ER stress at the reticular level. In contrast to previous data demonstrating an activation interaction between IP3R and Bcl-xL, I was able to prove a negative regulation of Bcl-xL on IP3R-mediated Ca^{2+} discharge. Upon ER stress, Bcl-xL binds to and inhibits IP3R activity preventing ER Ca^{2+} depletion required for the initiation of the UPR. Thus, UPR markers expression is reduced and cell survival is maintained. In this perspective, Bcl-xL indirectly performs an anti-apoptotic role. Always in this respect, I was able to participate in the identification and characterization of trBcl2Like proteins. Four Bcl-2 homologs were found in the simple and basal metazoan *Trichoplax adhaerens*. We were able to identify two anti-apoptotic acting proteins (trBcl-2L1 and trBcl2-L2) and two pro-apoptotic ones (trBax and trBak). The anti-apoptotic members inhibit trBax and trBak. trBax acts as a *bona fide* Bax protein inducing MOMP and trBak acts as a BH3-only sensitizer. Having a dual mitochondrial and ER subcellular localization, trBcl-2L1 positively regulates IP3R-mediated Ca^{2+} release indicating the importance of Bcl-2 proteins' localization at the ER and its influence on Ca^{2+} homeostasis. The lessons learnt from the basal branching placozoan demonstrate that the pleiotropy of Bcl-2 proteins is indeed a conserved trait in metazoan evolution.

In order to further elaborate the functional contribution of Bcl-xL at the ER, several propositions could take place. For the *in-vitro* studies, it would be interesting to check the levels GADD34 in thapsigargin-treated MEFs in order to further understand the mechanism of phosphorylation/dephosphorylation of eIF2a especially in ER-xL MEFs. To further prove the IP3R inhibition-UPR reduction effect of Bcl-xL, we can also block IP3R with an IP3 sponge retaining the ability to bind and titrate cellular IP3 or make IP3R-KO WT, ER-xL and Mt-xL MEFs then treat them with thapsigargin. We might also check BH3-only proteins' expression after ER stress induction especially Bim known to be upregulated through the UPR. We can confirm the ER resistance we proved *in-vivo* by intraperitoneal injection of thapsigargin/tunicamycin in WT, ER-xL and Mt-xL mice in order to analyze their livers and compare liver steatosis. Also, we can extract proteins and RNA from collected hepatocytes and verify UPR markers expression. Another interesting

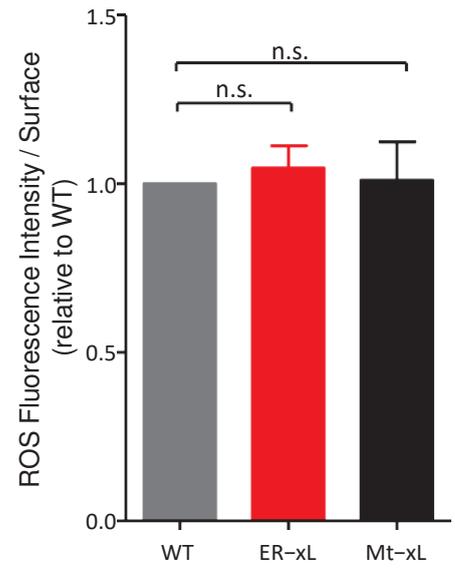
proposition is providing mice with a high fat diet (Achard and Laybutt, 2012). Indeed, if ER stress mediated by this high fat diet is not resolved, severe UPR signaling leads to insulin resistance and metabolic dysregulation that could be analyzed. As for Bcl-2 pleiotropy study, we can first elaborate the role of trBcl-2L2 on the ER with Ca^{2+} assays and cell death analyses in order to verify if its acts like trBcl-2L1. As demonstrated with Bcl-xL, we can assess the role of trBcl-2L1 and trBcl-2L2 under ER stress conditions by transfecting HeLa cells for example with these proteins and treat them with ER-stress inducers. It would be interesting to check *in-vivo* whether trBax indeed acts as a secreted toxin by injecting this protein into *Trichoplax adhaerens*.

ANNEX I

A



B



ANNEX II



Subcellular Localization and Dynamics of the Bcl-2 Family of Proteins

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Bcl-2 family proteins are recognized as major regulators of the mitochondrial pathway of apoptosis. They control the mitochondrial outer membrane permeabilization (MOMP) by directly localizing to this organelle. Further investigations demonstrated that Bcl-2 related proteins are also found in other intracellular compartments such as the endoplasmic reticulum, the Golgi apparatus, the nucleus and the peroxisomes. At the level of these organelles, Bcl-2 family proteins not only regulate MOMP in a remote fashion but also participate in major cellular processes including calcium homeostasis, cell cycle control and cell migration. With the advances of live cell imaging techniques and the generation of fluorescent recombinant proteins, it became clear that the distribution of Bcl-2 proteins inside the cell is a dynamic process which is profoundly affected by changes in the cellular microenvironment. Here, we describe the current knowledge related to the subcellular distribution of the Bcl-2 family of proteins and further emphasize on the emerging concept that this highly dynamic process is critical for cell fate determination.

Keywords: Bcl-2 family, subcellular localization, mitochondria, endoplasmic reticulum, nucleus

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INTRODUCTION

Intracellular compartmentalization is a fundamental feature of eukaryotic cells. It allows the physical segregation and simultaneous execution of distinct biochemical processes within the same cell. In fact, proper subcellular targeting and distribution are critical for the function of intracellular proteins. B-cell lymphoma 2 (Bcl-2) family of proteins are acknowledged as major regulators of the intrinsic pathway of the programmed cell death type 1 commonly known as apoptosis. Bcl-2, the founding member of the family, was discovered more than 30 years ago in the course of a study on the t(14;18) genomic translocation. Translocations between chromosome 18 (q21) and chromosome 14 (q32) are frequently observed in human B-cell follicular lymphomas and can result in the relocation of the *bcl2* open reading frame downstream of the enhancer promoter region of the *igh* heavy chain immunoglobulin gene (Tsujiimoto et al., 1984, 1985). This translocation results in upregulation of *bcl2* gene expression (Cleary et al., 1986). Previous observations on other B-cell lymphomas such as Burkitt's lymphoma or mantle cell lymphoma, in which similar translocations led to the overexpression of oncogenes such as *c-myc* or *cyclin d1*, suggested that *bcl2* was another oncogene inducing uncontrolled proliferation. However, soon after this discovery, Vaux and colleagues demonstrated that Bcl-2 could sustain cell survival of lymphoid cells in absence of Interleukin-3, establishing Bcl-2 as the founding member of a new class of oncoproteins that

LIST OF ABBREVIATIONS

2-APB: 2- Aminoethoxydiphenyl-borate	CARD: Caspase Recruitment Domain
3NP: 3-nitropropionic acid	CB5: Cytochrome B
4PBA: 4-phenylbutyric acid	ccRCC: kidney renal clear cell carcinoma
Å: Ångström	CD: Coupling Domain
Aa: Amino acid	CDK: Cyclin-Dependent Kinase
AD: Alzheimer's disease	Ced9: Cell death abnormal-9
ADP: Adenosine biphosphate	CFD: Channel Forming Domain
AIF: Apoptotic-Inducing Factor	CFD: channel forming domain
AML: acute myeloid leukaemia	CHOP: CAAT/enhancer-binding protein (C/EBP) homologous protein
AMP: Adenosine MonoPhosphate	CICR: Ca ²⁺ - induced Ca ²⁺ - release
ANT: Adenine Nucleotide Transporter	CICR: Calcium-Induced Calcium Release
Apaf-1: Apoptosis protease-activating factor-1	CLL: chronic lymphocytic leukaemia
ASK1: apoptosis signal-regulating kinase 1	CLL: Chronic Lymphocytic Leukemia
ATF: activating transcription factor	CNS: central nervous system
ATG: Autophagy-related genes	COPII: coat protein II
ATM: ataxia talenlectesia mutated	COX: cytochrome c oxidase
ATP: Adenosine Triphosphate	CRAC: Ca ²⁺ release-activated Ca ²⁺ Channel
Bcl-2: B-Cell Lymphoma 2	CRD: Cystein rich domain
Bcl-xL: B-Cell Lymphoma-Extra Large	CREB: cAMP response element-binding protein
Bcl-xS: B-Cell Lymphoma-Extra Short	CSC: cancer stem cell
BD: Binding Domain	CTD: C-Terminal Domain
BH: Bcl-2 Homology	Da: Dalton
BIRD-2: Bcl-2 IP3 Receptor Disruptor-2	DAG: diacylglycerol
BMK: baby mouse kidney	DAG: DiAcylGlycerol
BSS: Balanced Salt Solution	DD: Death Domain

DED: Death Effector Domain

Diablo: Direct Inhibitor of Apoptosis–Binding protein with LOW pI

DISC: Death Inducing Signaling Complex

DKO: Double KO

DNA: DeoxyriboNucleic Acid

DR: Death receptor

DRAM1: DNA damage–regulated autophagy modulator 1

Drp1: dynamin–related protein

DUB: deubiquitinylase

eIF2: eukaryotic initiation factor 2

EM: Electron Microscopy

EMT: epithelial to mesenchymal transition

EndoG: Endonuclease G

EPO: erythropoietin

ER: Endoplasmic Reticulum

ERAD: ER associated degradation

ERO1: ER oxidoreductin 1

ERa: Oestrogen Receptor a

ETC: electron transport chain

FADD: FAS–associated Death Domain

FAK: Focal Adhesion Kinase

FAO: Fatty Acid Oxidation

FLIP: Fluorescence Loss In Photobleaching

FOX O1: forkhead box O1

FRET: fluorescence resonance energy transfer

GADD34: DNA–damageinducible 34

GBM: glioblastoma

GFP: green fluorescent protein

GK: glucokinase

GPCR: G–protein coupled receptor

GRP78: glucose–regulated protein 78

GSH: glutathione

GSK–3: Glycogen Synthase Kinase

GTP: Guanosine triphosphate

HC/BV: hepatitis B/C virus

HCMV: human cytomegalovirus

HDACi: Histone deacetylase inhibitors

HIF1a: hypoxia inducible factor 1 a

HNSCC: head and neck squamous cell carcinoma

HSC: haematopoietic stem cell

IAPs: inhibitors of apoptosis

IICR: IP3–induced Ca²⁺ release

IL: Interleukin

ILD: intervening lateral domain

iMOMP: Incomplete MOMP

IMS: Intermembrane Space

IP3: Inositol 1,4,5–triphosphate

IP3R: Inositol 1,4,5–triphosphate Receptor

IRE1: Inositol requiring enzyme 1

JAK2: Janus tyrosine kinase 2

JNK: Jun N–terminal Kinase

Kd: Affinity Constant

KD: Knockdown

KO: Knockout

LABC: locally advanced breast cancer
 LNK: linker domain
 MA: Membrane anchor
 MAMs: Mitochondria–Associated Membranes
 MAPK: Mitogen–Activated Protein Kinases
 MCU: mitochondrial calcium uniporter
 MDM2: double minute 2 protein
 MEF: Mouse Embryonic Fibroblast
 mfn: Mitofusin
 MIM: Mitochondrial inner membrane
 miRNA: microRNA
 MLCK: myosin light chain kinase
 MM: multiple myeloma
 MMP: matrix metalloproteases
 MOM: Mitochondrial outer membrane
 MOMP: Mitochondrial Outer Membrane Permeabilization
 mPTP: mitochondrial Permeability Transition Pore
 mRNA: messenger RNA
 MTCH2: mitochondrial carrier homolog 2
 MTD: Modulatory and Transducing Domain
 MULE: Mcl–1 Ubiquitin Ligase E3
 NFAT: nuclear factor of activated T cells
 NFkB: Nuclear Factor kB
 NLS: nuclear localization signal
 NMR: Nuclear Magnetic Resonance
 NOM: Nuclear outer membrane
 NSCLC: non–small cell lung cancer
 OXPPOS: oxidative phosphorylation
 PCD: Programmed Cell Death
 PCNA: Proliferating Cell Nuclear Antigen
 PCR: Polymerase Chain Reaction
 PERK: protein kinase RNA–activated (PKR)–like ER kinase
 PEST: Proline, Glutamate, Serine Threonine rich domain
 PIP2: phosphatidylinositol 4,5–bisphosphate
 PKA: Protein Kinase A
 PKC: Protein Kinase C
 PLA: Proximity Ligation Assay
 PLC: PhosphoLipase C
 PLK: Polo–Like Kinase
 PM: Plasma membrane
 PMPs: peroxisomal membrane proteins
 PNS: peripheral nervous system
 Pp: protein phosphatase
 Pp1: type 1 protein serine/threonine phosphatase
 PPP: pentose phosphate pathway
 ppVs: pre–peroxisomal vesicular carriers
 PTKRs: protein tyrosine kinase–linked receptors
 PTM: posttranslational modification
 qPCR: quantitative Polymerase Chain Reaction
 RER: Rough ER
 RIDD: regulated IRE1–dependent decay
 RNA: RiboNucleic Acid
 ROS: Reactive Oxygen Species

RyR: Ryanodine Receptor

S1/2P: site 1/2 protease

SAHB: Stabilized Alpha-Helix of Bcl-2 domain

SD: Suppressor Domain

SER: Smooth ER

SERCA: Sarco/endoplasmic reticulum calcium transport ATPase

siRNA: short interfering RNA

SMAC: Second mitochondria-derived activator of caspase

SOCE: store operated calcium entry

SR: sarcoplasmic reticulum

SUFU: Suppressor of fused

T. adhaerens: *Trichoplax adhaerens*

TCA: Transport Chain Activity

TCA: Tricarboxylic Acid Cycle

TCR: T-Cell Receptor

TGF β : Transforming growth factor beta

TM: Transmembrane

TMR: TetraMethylRhodamine

TNF: Tumor Necrosis Factor

TNFR: TNF Receptor

TNF-a: Tumor Necrosis Factor a

TPO: Thrombopoietin

TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain

TRAF6: TNFR-Associated Factor 6

TRAIL: TNF-Related Apoptosis Ligand

trBcl-2L : trBcl-2Like

TRP: transient receptor potential

TS: Tumor Suppressor

TUDCA: Tauroursodeoxycholic acid

Ubq1n: Ubiquilin 1

UPR: Unfolded Protein Response

USP9X: ubiquitin-specific peptidase 9 X-linked

UVB: ultraviolet radiation B

VDAC: Voltage-Dependent Anion Channel

VDCC: Voltage gated calcium channel

VSV: vesicular stomatitis virus

XBP1: X-box binding protein 1

XIAP: X-linked inhibitor of apoptosis

YSL: yolk syncytial layer

YSL: Yolk Syncytial Layer

YY1: yin yang

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