

A brewing understanding of the regulation of Bax function by Bcl-xL and Bcl-2

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Summary

Bcl-2 family members form a network of protein-protein interactions that regulate apoptosis through permeabilization of the mitochondrial outer membrane. Deciphering this intricate network requires streamlined experimental models, including the heterologous expression in yeast. This approach had previously enabled researchers to identify domains and residues that underlie the conformational changes driving the translocation, the insertion and the oligomerization of the pro-apoptotic protein Bax at the level of the mitochondrial outer membrane. Recent studies that combine experiments in yeast and in mammalian cells have shown the unexpected effect of the anti-apoptotic protein Bcl-xL on the priming of Bax. As demonstrated with the BH3-mimetic molecule ABT-737, this property of Bcl-xL, and of Bcl-2, is crucial to elaborate about how apoptosis could be reactivated in tumoral cells.

1-Introduction: Bcl-2 family members v/s BH3-containing proteins

Apoptosis is the main type of programmed cell death in animals, participating to the modeling of organs during the development, and to tissue homeostasis during the whole life. It is activated following external or internal stimuli, and leads to a non-inflammatory response, opposite to other forms of cell death, such as necrosis.

It is generally considered that there are two types of apoptotic signaling pathways:

- the exogenous pathway, when death is initiated by the binding of an external ligand to a receptor, involves the direct activation of caspases, a group of proteases responsible for the implementation of apoptotic characteristics;

- the endogenous pathway, when death is initiated “from the inside”, for example through the activation of transcription factor p53 following an alteration to DNA, involves mitochondria.

Alterations of the apoptotic program are associated with major age-related diseases, such as cancers when apoptosis is deficient, or degenerative diseases when apoptosis is over-activated. It is because of the existence of these major health issues that apoptosis is one of the most studied processes of cell biology in the last 20 years. A considerable amount of knowledge has been accumulated about the signaling pathways that regulate cell death, both under physiological and pathological situations. However, it still remains difficult to predict how these pathways modulate the molecular mechanisms underlying apoptosis, because of antagonistic effects between signaling pathways regulating cell death and survival, and the redundancy between parallel pathways. This intricacy is further increased in cell lines, where survival pathways are often over-activated while death pathways are repressed. This is why streamlined experimental models are needed to investigate the link between cellular and molecular regulations.

The group of proteins known as Bcl-2 family members are central players of apoptosis, because they form the interface between the early signaling events engaging the cells into the apoptotic process, and the later events conferring their apoptotic characteristics to the cells, leading to their elimination by the immune system (Adams and Cory, 2007, for a review). Bcl-2 family members are characterized by 4 homology domains, called BH1 to BH4. These domains are the keystones of these proteins' function and regulation, as they enable their mutual interaction within an intricate and highly regulated network.

Classically, the Bcl-2 family has been divided into 3 sub-families: anti-apoptotic proteins (*e.g.* Bcl-2, Bcl-xL), pro-apoptotic proteins (Bax, Bak, Bok), and BH3-only proteins (*e.g.* Bid, Bad, Puma, Bim) (Fig.1). It is noteworthy that, while the two first sub-families are defined on the basis of a well-identified biological function, the last one is defined by default, on the basis of a structural property.

As a matter of fact, recent phylogenetic studies have shown that BH3-domains are present in many proteins other than the Bcl-2 family (Aouacheria et al., 2013) including, for example, the mammalian protein Beclin-1 (Liang et al., 1999), or the yeast protein Ybh3/Bxi1/YNL305C (Cebulski et al., 2011; Buttner et al., 2011). However, those BH3-domains do not derive from a common ancestor, opposite to the canonical BH3 domain of pro- and anti-apoptotic Bcl-2 family members, but rather result from a convergent evolution (Aouacheria et al., 2013). These studies redefined the BH3-domain as a short interacting motif, that is thus not limited to the Bcl-2 family members (Aouacheria et al., 2015). According to these studies, BH3-containing proteins could be re-organized into three categories (Aouacheria et al., 2013) (Fig.1):

- true Bcl-2 homologs, that contain three or four BH domains, and that can be anti-apoptotic (*e.g.*

1 Bcl-2, Bcl-xL) or pro-apoptotic (Bax, Bak, Bok); Bid, which has only the BH3 domain, is
2 considered as a true Bcl-2 homolog because of its overall structural similarity with Bcl-2;

3 - BH3-only proteins that contain a canonical BH3-domain (e.g. Bad, Puma, Bim);

4 - BH3-only proteins that contain a non-canonical BH3-domain (e.g. Beclin-1, Bnip3L/Nix, yeast
5 Ybh3).
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7 These studies show that true Bcl-2 homologs are only present in animals: vertebrates (including
8 Bcl-2, Bax, and their homologs in non-mammalian vertebrates), nematode (*C.elegans* Ced-9) and
9 insects (*D.melanogaster* Buffy and DeBcl). The same observation can be done for proteins
10 containing a canonical BH3-domain, in vertebrates (including Bid, Puma, Bad) and in the nematode
11 (Egl-1). All the BH3-containing proteins found in non-animal organisms contain a divergent
12 domain, e.g. Ybh3 in the yeast *S.cerevisiae*, Rad9 in the yeast *S.pombe*, MCF in the bacterium
13 *P.luminescens*, and viral proteins such as the protein CORE from the hepatitis C virus (Aouacheria
14 et al., 2013). These proteins may have a function that is unrelated to the Bcl-2 family. For example,
15 yeast Ybh3/Bxi1/YNL305C is involved in the UPR response and might modulate calcium dynamics
16 (Cebulski et al., 2011), as shown for its bacterial homolog (Chang et al., 2014). However, like all
17 the proteins containing a divergent BH3-domain, it might be potentially able to bind to Bcl-2
18 homologs, namely Bax, particularly when it is over-expressed. Conversely, Bcl-2 homologs might
19 regulate the function of proteins containing a divergent BH3 domain, independently from apoptosis:
20 as an example, it has been proposed that the pro-autophagic function of Beclin-1 is negatively
21 regulated by the overexpression of Bcl-2 (Patingre et al., 2005), although the effect of Bcl-2 on
22 autophagy might also be indirect (Lindqvist et al., 2014).
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39 **2-Bcl-2 family, MOMP and cytochrome c**

40 Bcl-2 homologs function is essential to the regulation of mitochondrial outer membrane
41 permeabilization (MOMP), the key event irreversibly engaging the cells towards death. Following
42 MOMP, a group of particular proteases, the caspases, are activated. This activation occurs through
43 two processes: (1) procaspases are cleaved into two subunits and reorganized into active
44 heterotetramers and (2) IAPs, a family of caspases inhibitors, are inactivated.
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49 There is now a large consensus among investigators to consider that MOMP is caused by the
50 formation of a large pore in the mitochondrial outer membrane (MOM), that enables the release of
51 several proteins from the mitochondrial intermembrane space to the cytosol. Five of these proteins
52 have been identified as 'apoptogenic factors' and are described below (Fig.2):
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56 - Cytochrome c (12.5kDa) is a central soluble component of the electron transport chain; when
57 released, it binds to Apaf-1, forming a complex that catalyzes the cleavage and the activation of
58 caspase-9.
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- Smac/Diablo (25kDa) binds to IAPs, promoting their degradation, and thus preventing the inhibition of caspases.

- Omi/HtrA2 (49kDa) is a protease that inactivates IAPs.

- Endonuclease G (28kDa) participates to chromatin degradation.

- Apoptosis-Inducing Factor (AIF, 61kDa) is a redox protein that participates to the regulation of the respiratory chain. During apoptosis, it participates to chromatin condensation, but its precise function remains elusive.

It is still unclear whether these proteins, that have different sizes, are released simultaneously and through the same mechanism. Indeed, most studies are done on cultured cells with a sustained apoptotic trigger (such as the addition of an antitumoral drug). Under these conditions, apoptosis is followed by necrosis.

The initial steps of apoptosis might only involve a limited MOMP, without alteration of the inner mitochondrial membrane (see below). On the opposite, the secondary necrotic process might be associated to the activation of the large unselective channel called the mitochondrial Permeability Transition Pore (mPTP), that induces the permeabilization of the inner mitochondrial membrane (Bernardi et al., 2015, for review). This is followed by a massive mitochondrial swelling leading to the unselective rupture of the MOM, and the release of the whole intermembrane space content into the cytosol (Fig.2). Consequently, the mechanistic aspects of selective MOMP should be studied at the early stages of the apoptotic process, when mPTP is still close.

Electrophysiological studies on MOM from apoptotic cells showed the presence of a large conductance channel, that also appeared in the MOM from yeast cells expressing human Bax (Pavlov et al., 2001), or on isolated membranes incubated with Bax oligomers (Dejean et al., 2005). Interestingly, the size of this channel was sufficient to transport cytochrome c (12.5kDa), but not to transport a bigger protein, such as hemoglobin tetramer (60kDa) (Dejean et al., 2005). Electrical properties of this channel showed the existence of different conductance substates, suggesting a process of stepwise Bax oligomerization (Martinez-Caballero et al., 2010). Recent studies showed that Bax oligomers form large ring-like structures in the MOM that could be assimilated to the pore of the MOMP machinery (Große et al., 2016; Salvador-Gallego et al., 2016).

Among the apoptogenic factors, cytochrome c is one of the most ubiquitous and conserved proteins in the whole living world (Margoliash and Fitch, 1968), a feature most certainly related to its central function as a mobile electron carrier that shuttles between respiratory complexes. As such, it can serve as a universal marker for MOMP, including in model systems that do not carry out canonical apoptosis. Most specifically, the release of cytochrome c became a standard in studies regarding the function of animal Bcl-2 homologs after they are expressed in yeast (Fig.3). The expression of human Bax was shown to promote the release of cytochrome c in yeast (Manon et al., 1997) even

1 before it was shown in human cells (Jurgensmeier et al., 1998). Like in mammalian cells, several
2 death signals in yeast, such as acetic acid treatment, induce dramatic mitochondrial morphological
3 alterations and a non-selective release of intermembrane space proteins, that have homologies with
4 mammalian apoptogenic factors (Eisenberg et al., 2007; Cheng et al. 2008; Pereira et al., 2008, for
5 reviews) (Fig.2). Interestingly, Bax effects in yeast do not depend on other proteins that are released
6 following death stimuli (Kissova et al., 2006), such as acetic acid treatment, that may induce a
7 necrotic type of death depending on massive mitochondrial permeabilization resembling mPTP
8 (Pereira et al., 2010). This supports the view that, in mammalian cells, only cytochrome would be
9 released through Bax-formed channels, while other apoptogenic factors would be released through
10 other systems, such as mPTP (Kroemer et al., 2007, for review) or ceramides channels (Colombini,
11 2010, for review).

12 Due to the ease to obtain large amounts of yeast biomass, cytochrome c can be easily quantified by
13 redox spectrophotometry, and compared to non-released cytochromes, such as cytochrome b, a
14 membrane-inserted component of respiratory complex III (Fig.3). This allowed measuring the
15 activities of different Bax mutants, with more precision than western-blot quantification (Arokium
16 et al., 2004, 2007; Renault et al., 2015). Furthermore, redox spectrophotometry measures the
17 mitochondrial cytochrome c content after it has been assembled with heme in the mitochondrial
18 intermembrane space (Gonzalez and Neupert, 1990), thus limiting the quantification to cytochrome
19 c that has transited through mitochondria. On the opposite, western-blot against cytochrome c also
20 reveal the heme-free apoprotein, before it has been assembled within the mitochondria, meaning
21 that a deficient addressing might be erroneously taken for an increased release.

22 The redox spectrophotometry measurement of cytochrome c in yeast mitochondria, following the
23 heterologous expression of human Bax mutants has provided a considerable amount of data about
24 the domains, residues, and possible partners involved in Bax ability to interact with MOM and to
25 release cytochrome c. For example, the consequences of the replacements of Proline residues at
26 positions 8, 13 and 168 by smaller and more flexible Glycine/Alanine residues have pointed out the
27 role of the mobility of the N-terminal unstructured domain and of the C-terminal α -helix in Bax
28 activation (Arokium et al., 2004). Electric charge inversions at different positions (Arg9/Asp154)
29 confirmed that favoring the movement of these domains (by disrupting salt bridges), was sufficient
30 to promote the localization of Bax to the MOM (Arokium et al., 2007). Furthermore, this method
31 has allowed evidencing the involvement of the mitochondrial receptor Tom22 in the adequate
32 translocation of Bax (Bellot et al., 2007; Renault et al., 2012), and the direct activation of Bax by
33 BH3-only proteins tBid (Gonzalves et al., 2005) and Puma (Gallenne et al., 2009). This method has
34 thus proved to be an adequate measurement of the ability of Bax to permeabilize the MOM.

35 In parallel, Bax expression in yeast induces a reduction of yeast growth and a loss of viability.

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However, these phenotypes involve additional effects beyond the release of cytochrome c, and are consequently less precise reporters of Bax effects on mitochondria than the direct measurement of cytochrome c release (Kissova et al., 2006). Nevertheless, they proved their usefulness for the rapid screening of factors that potentially modulate the effects of Bax (Xu and Reed, 1998; Pan et al., 2001; Laloux et al., 2010; Ubaidillah et al., 2013).

3-Bax retrotranslocation

For long, it had been considered that Bax translocation from the cytosol to the mitochondria was a unidirectional process. This came from the observation that, in non-apoptotic cells, Bax localization is essentially diffuse in the cytosol while, after apoptosis is triggered, it is relocated to mitochondria (Wolter et al., 1997) to form membrane-inserted oligomers that are responsible for MOMP (Antonsson et al., 2000, 2001). Since this process involves major conformational changes (Suzuki et al., 2000; Peyerl et al., 2007), it was hardly conceivable that these oligomers could be disassembled, and that Bax could go back to its previously inactive state in the cytosol. However, significant observations suggested that the passage from soluble/monomeric Bax to membrane-inserted/oligomeric Bax is not a one-step process. For example, it has been shown that, during anoikis, Bax could be relocalized to mitochondria, but that the process was reversible (Owens et al., 2009). This, and other experiments, demonstrated that 'mitochondrial Bax' was not obligatorily 'membrane-inserted' Bax (Renault et al., 2013).

Structural studies have allowed designing a complex Bax mutant that has a constitutive mitochondrial localization but that cannot support the conformational change associated to the oligomerization, thus remaining incompetent for cytochrome c release. Under oxidative conditions, this mutant displays two internal disulfide bonds that constraint helices $\alpha 1$ and $\alpha 2$, and $\alpha 1$ - $\alpha 2$ loop and $\alpha 6$, therefore blocking the 'opening' of Bax structure associated to its activation (Edlich et al., 2011). When expressed in HCT-116 or HeLa cells, a GFP-tagged version of this mutant displayed a mitochondrial localization and did not trigger apoptosis, as expected. Following the photobleaching of GFP fluorescence in cell nuclei, both nuclear and cytosolic fluorescence disappeared while mitochondrial fluorescence remained. This reflected the rapid dynamics of exchange between the nucleus and the cytosol, and its absence for mitochondrial Bax. However, by following the re-appearance of the fluorescence in the cytosol, the authors showed that a fraction of mitochondrial Bax could be retrotranslocated from the mitochondria to the cytosol. Most interestingly, this retrotranslocation process was greatly accelerated when the anti-apoptotic protein Bcl-xL was over-expressed (Edlich et al., 2011). This led the authors to conclude that, in non-apoptotic cells, (1) Bax subcellular localization followed a dynamic equilibrium between mitochondria and cytosol and that (2) anti-apoptotic proteins could displace this equilibrium towards a more cytosolic localization. An

1 additional interesting observation was that a mutant of Bcl-xL deleted of the C-terminal α -helix
2 (Bcl-xL Δ C) was unable to promote Bax retrotranslocation, and further experiments demonstrated
3 that the deletion of the 4 last residues of Bcl-xL were sufficient to prevent it (Todt et al., 2013).

4 The question remained whether this process was only dependent on the intrinsic properties of
5 interaction between the two proteins, or also involved external factors (such as, for example, the
6 presence of other Bcl-2 family members). Since yeast does not contain endogenous Bcl-2 family
7 members, it is a powerful tool to answer this question. Bax was expressed alone, or co-expressed
8 with full-length Bcl-xL or truncated Bcl-xL Δ C in a yeast mutant inactivated for the major vacuolar
9 protease Pep4p (the yeast ortholog of cathepsin D), and in the presence of the general inhibitor of
10 serine-proteases PMSF. Under these conditions, following the addition of an inhibitor of protein
11 synthesis (cycloheximide), the cellular Bax content remained stable for at least 4 hours.
12 Mitochondria were then isolated to measure the evolution of Bax mitochondrial content following
13 cycloheximide addition. Since the total content was stable, any decrease of the mitochondrial
14 content would indicate retrotranslocation. When expressed alone, mitochondrial Bax levels
15 remained constant. When co-expressed with Bcl-xL, mitochondrial Bax levels were decreased by
16 50% within 2 hours. When co-expressed with Bcl-xL Δ C, mitochondrial Bax levels remained
17 constant. Similar experiments were done in mouse pro-lymphocytic cells FL5.12, with the same
18 results, showing that the experiment in yeast actually led to observations relevant to mammalian
19 physiology. These results were therefore consistent with the hypothesis that Bcl-xL (but not Bcl-
20 xL Δ C) was able to promote the retrotranslocation of Bax, when the two proteins were co-expressed
21 in yeast. This demonstrated that the retrotranslocation of Bax by Bcl-xL was only dependent on
22 molecular properties of Bax and Bcl-xL, without interference from factors present in mammalian
23 cells and absent from yeast, such as other Bcl-2 family members (Renault et al., 2015).

24 Importantly, no retrotranslocation was observed when a constitutively active mutant of Bax,
25 carrying a substitution P168A (Arokium et al., 2004; Cartron et al., 2005) was used. This supports
26 the view that, once it is inserted and oligomerized (since it is able to promote the release of
27 cytochrome c), Bax cannot be retrotranslocated.
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49 ***4-Steady-state Bax subcellular localization***

50 Intuitively, the existence of Bax retrotranslocation suggests that, in the presence of Bcl-xL, the
51 steady-state Bax localization should be more cytosolic than when Bax is expressed alone. This can
52 be correlated with the classical view of over-expressed Bcl-xL (or Bcl-2) sequestering Bax away
53 from mitochondria, thus preventing apoptosis by restraining Bax ability to reach and permeabilize
54 MOM.
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60 Opposite to this prediction, we observed that the over-expression of Bcl-xL or of Bcl-2 increased
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1 the mitochondrial localization of endogenous Bax in FL5.12 cells (Teijido and Dejean, 2010;
2 Renault et al., 2015). Strikingly, Bax mitochondrial content reached the same level as measured in
3 parental cells committed to apoptosis through IL-3 removal. Although less marked, a similar effect
4 was observed in HCT-116 cells overexpressing Bcl-xL, showing that it was not a unique property of
5 FL5.12 cells.
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7 We then investigated if this apparently paradoxical effect of Bcl-xL on Bax mitochondrial
8 localization was only dependent on the interaction between Bax and Bcl-xL or if other factors were
9 involved, by doing the experiments in yeast. We observed that Bax mitochondrial content was
10 increased when Bcl-xL was co-expressed with Bax in yeast. This last result showed that Bcl-xL-
11 mediated Bax increase at the mitochondria was a general phenomenon, that depended only on Bax
12 and Bcl-xL.
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14 Most interestingly, we observed that the co-expression of Bcl-xL Δ C induced an even higher
15 increase of Bax mitochondrial localization in FL5.12 cells, in HCT-116 cells, and in yeast (Renault
16 et al., 2015). As discussed above, truncated Bcl-xL Δ C is not able to promote Bax retrotranslocation.
17 This suggests that Bcl-xL regulates Bax mitochondrial localization through two opposed processes:
18 retrotranslocation, for which Bcl-xL Δ C is impaired, and translocation, promoted both by full-length
19 Bcl-xL and truncated Bcl-xL Δ C (Fig.4).
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31 ***5-Bax priming is enhanced in cells overexpressing Bcl-xL Δ C or Bcl-xL***

32 The immediate consequence of the increase in the steady-state level of mitochondrial Bax is a
33 potentially higher ability to initiate apoptosis. However, the presence of Bcl-xL, and the interaction
34 between the two proteins, obviously prevent Bax activation. This can be verified by the fact that
35 IL-3 removal induced a lower level of apoptosis in Bcl-xL-overexpressing FL5.12 cells, than in
36 parental cells. Here again, the inhibition only depends on the interaction between Bax and Bcl-xL
37 since, in yeast, the effect of a constitutive mitochondrial and active mutant of Bax on mitochondrial
38 permeabilization is also prevented by the co-expression of Bcl-xL.
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46 On the other hand, the overexpression of Bcl-xL Δ C does not prevent the apoptosis of FL5.12 cells
47 induced by the removal of IL-3, and does not prevent the permeabilization of yeast mitochondrial
48 membrane by a constitutively active Bax mutant. In line with these observations, co-
49 immunoprecipitation assays showed that the interaction between Bax and Bcl-xL Δ C was not
50 detectable under conditions where the one between Bax and full-length Bcl-xL was. This
51 demonstrates that the presence of the C-terminal α -helix of Bcl-xL is required for a stable
52 interaction and subsequent efficient inhibition of Bax.
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59 Furthermore, we observed that the expression of Bcl-xL Δ C stimulated Bax-driven MOMP in yeast.
60 This rather unexpected effect was dependent on the interaction between the two proteins, since it
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1 was abrogated by introducing the canonical G138A mutation in the BH1-domain of Bcl-xL Δ C
2 (Renault et al., 2015), that is known to prevent the interaction between the two proteins (Ottillie et
3 al., 1997).

4 These results showed that, although it might not be stable enough to be detectable by co-
5 immunoprecipitation, a transient interaction between Bax and Bcl-xL Δ C occurs and is sufficient to
6 increase mitochondrial Bax content, but is not sufficient to prevent Bax-induced permeabilization.
7 This was not observed with full-length Bcl-xL since its interaction with Bax, while inducing
8 translocation of the pro-apoptotic protein, was stable enough (detectable by co-
9 immunoprecipitation, as a matter of fact) to also keep it inactive.

10 Since Bcl-xL Δ C is not a natural variant of the protein, we searched for experimental conditions
11 which could reveal that full-length Bcl-xL is also able to prime Bax. Experiments on purified
12 recombinant proteins had previously shown that the binding, then release, of Bax and Bcl-xL could
13 initiate the active conformation of Bax that can be detected with the 6A7 monoclonal antibody (Hsu
14 and Youle, 1997). The release could be induced by peptides corresponding to the BH3-domain of
15 canonical BH3-only proteins, such as Puma, or by BH3-mimetic molecules such as ABT-737
16 (Gautier et al., 2011).

17 Therefore we used ABT-737 to disrupt Bax/Bcl-xL interaction both in the FL5.12 and the yeast
18 models. The addition of ABT-737 to parental FL5.12 cells did not induce apoptosis, consistently
19 with previous works (Certo et al., 2006). On the opposite, the addition of ABT-737 to FL5.12 cells
20 overexpressing Bcl-xL induced a significant level of apoptosis (Renault et al., 2015).

21 We observed that ABT-737 had no effect on the biomass of yeast cells expressing Bax alone, or Bcl-
22 xL alone, but it significantly decreased the biomass of cells co-expressing Bax and Bcl-xL.
23 Although this result might not rely only on Bax-induced MOMP, for the reasons discussed above
24 (Kissova et al., 2006), it is nevertheless in accordance with the effect of ABT-737 observed in
25 FL5.12 cells.

26 These experiments show that, both in FL5.12 cells and in yeast, the transient interaction between
27 Bax and Bcl-xL followed by the release of the interaction, promotes the activation of Bax, like
28 previously reported using pure recombinant proteins. Also, the study of yeast cells co-expressing
29 Bax, Bcl-xL (or Bcl-2) and BH3-only proteins, have suggested that the lasts could only activate Bax
30 indirectly, through the inhibition of Bcl-xL or Bcl-2 (Gerecova et al., 2013). A sequential expression
31 of Bax and Bcl-xL/Bcl-2, then BH3-only proteins, could provide a mean to study whether a given
32 BH3-only protein is able or not to promote the release of Bax under an active conformation.
33 Considering the hypothesis proposed above, this should happen with all the BH3-only proteins,
34 with small efficiency differences due to their higher or lower specificity towards Bcl-2 or Bcl-xL.

35 This has interesting consequences on the potential efficiency of BH3-mimetic molecules in anti-
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1 tumoral therapy. These molecules were designed to prevent the inhibition of apoptosis by anti-
2 apoptotic Bcl-2 family members (essentially Bcl-2, Bcl-xL, and Mcl-1), that are overexpressed in
3 most cancer cells (see *e.g.* Tang et al., 1998; Pena et al., 1999, Castilla et al., 2006). The fact that, by
4 breaking the interaction between Bax and Bcl-2/xL, not only they liberate Bax, but they further
5 activate it, renders these molecules even more desirable as anti-tumor therapy, provided that Bax is
6 still expressed and not mutated.
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10 ***6-A correlation between Bax phosphorylation and its interaction with Bcl-xL?***

11 From the discussion above, Bcl-xL is able to stimulate both Bax translocation to the MOM, and Bax
12 retrotranslocation to the cytosol. This might appear like a futile cycle, but actually results in a high
13 turnover of Bax localization and, as we have observed, a high mitochondrial content of 'primed'
14 Bax, ready to trigger apoptosis. This might make the system more reactive than if Bax was only
15 sequestered away from mitochondria, since Bax is already on target, ready to be activated after the
16 release of its interaction with Bcl-2/Bcl-xL. However, the efficiency of such a 'dynamic cycling'
17 model implies that the interaction between Bax and Bcl-xL has different properties depending if it
18 happens in the cytosol or in the MOM. This may rely on biochemical differences between cytosolic
19 and mitochondrial Bax.
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29 Bax phosphorylation on different residues has been observed by several groups (Gardai et al., 2004;
30 Linseman et al., 2004; Xin et al., 2007). Two main residues, S163 and S184, are substrates of
31 kinases, although this does not exclude that other residues could be targeted as well. S163 is
32 phosphorylated by GSK3 β . This implies that T167 should also be phosphorylated, since the
33 consensus site for GSK3 β is SxxxT_P(S_P). S184 is the target of different kinases, including AKT and
34 PKC ζ . Interestingly AKT and GSK3 β have opposite effects since the former phosphorylates and
35 inactivates the later. Furthermore, AKT is generally involved in survival signaling pathways, while
36 GSK3 β is involved in death pathways (Martini et al., 2014), making their opposite effects on Bax
37 rather predictable.
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46 The heterologous expression of phosphomimetic and non-phosphorylatable mutants of S163 and
47 S184 in yeast suggests that modifications of these residues participate to the regulation of the
48 interaction of Bax with mitochondria (Arokium et al., 2007; Simonyan et al., 2016). When present
49 alone, the mutation S163D does not have any particular phenotype, but it can activate Bax when it
50 is combined to other mutations that facilitate intramolecular movements (Arokium et al., 2007).
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55 The phosphorylation of S184 has attracted much interest because it is located in the C-terminal α -
56 helix, of which the movement is crucial to initiate Bax relocalization. It had been shown that the
57 phosphorylation of S184 retained Bax away from mitochondria, while its dephosphorylation has the
58 opposite effect (Gardai et al., 2004). This could be reproduced in yeast where the mutants S184A/V
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1 have a higher mitochondrial localization than the mutant S184D (Arokium et al., 2007; Simonyan et
2 al., 2016). However, this did not fully correlate with the ability of these mutants to induce MOMP.
3 Indeed, the mutant S184D was surprisingly more efficient than mutants S184A/V (and wild-type
4 Bax), to promote the release of cytochrome c. These results suggest that the intrinsic capacity of the
5 phosphomimetic mutant to induce MOMP was higher than that of the non-phosphorylatable mutant,
6 that was apparently in contradiction with the fact that Bax phosphorylated on S184 is less able to
7 induce apoptosis than the non-phosphorylated protein (Gardai et al., 2004). Part of the answer to
8 this paradox might be related to the fact that, in mammalian cells, Bax is not expressed alone, but in
9 the presence of, namely, anti-apoptotic proteins. Bax phosphomimetic and non-phosphorylatable
10 mutants were then co-expressed with full-length Bcl-xL or truncated Bcl-xL Δ C (Simonyan et al.,
11 2016). We observed that the mitochondrial localization of the phosphomimetic mutant S184D did
12 not differ from that of wild-type Bax, with an increase induced by Bcl-xL and a further increase
13 induced by Bcl-xL Δ C, linked to the absence of retrotranslocation, as described above. Interestingly,
14 the behavior of the non-phosphorylatable mutant S184A differed: its mitochondrial content was
15 increased by Bcl-xL, like that of wild-type Bax and of the phosphomimetic mutant S184D, but it
16 was not further increased, and was even about twice less increased, by Bcl-xL Δ C. Since the major
17 difference between full-length Bcl-xL and truncated Bcl-xL Δ C is their capacity to retrotranslocate
18 Bax, this result showed that retotranslocation did not have any effect on the mitochondrial
19 localization of the non-phosphorylatable mutant Bax-S184A (Simonyan et al., 2016). This suggests
20 that the ability of Bax to be retrotranslocated might depend on its phosphorylation, namely on S184.
21 In mammalian cells, a correlation between the phosphorylation of S184 and the retrotranslocation of
22 Bax by Bcl-xL had also been observed (Schellenberg et al., 2013): indeed, a mutant GFP-Bax-
23 S184V was retrotranslocated slower than GFP-BaxWT, paralleling the observations in yeast
24 discussed above. Also, the overexpression of AKT stimulated GFP-BaxWT retrotranslocation.
25 However, it was not mentioned whether the overexpression of AKT had or not an effect on the
26 retrotranslocation of GFP-Bax-S184V, and it cannot be yet ascertained that this effect of AKT and
27 the consequence of the phosphorylation of S184 on Bax retrotranslocation are strictly correlated. It
28 should be noted that, when Bax and AKT were co-expressed in yeast, not all the effects of AKT
29 were abolished by the substitution S184V, suggesting that part of the effect of AKT may occur
30 through the phosphorylation of an additional residue besides S184 (Simonyan et al., 2016).
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54 **7-Bcl-xL v/s Bcl-2**

55 Before Bcl-xL, the overexpression of Bcl-2 had been shown to increase the mitochondrial
56 localization of Bax in non-apoptotic FL5.12 cells (Teijido and Dejean, 2010). However, in spite of
57 this apparent similarity, the consequences of Bcl-xL or Bcl-2 overexpression on Bax conformation
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1 were clearly distinct. Both proteins induced the conformational change of the N-terminus, leading
2 to a recognition by the 6A7 antibody, and reveal the association of Bax to the MOM. But, while the
3 overexpression of Bcl-2 also favored the formation of Bax oligomers (Teijido et al., 2010), the
4 overexpression of Bcl-xL did not (Renault et al., 2013, 2015). It is yet difficult to draw any
5 definitive conclusion about the consequences of this difference. Indeed, under both conditions, Bax
6 remains inactive, being inhibited by its anti-apoptotic partner, and no MOMP was observed. But
7 these observations emphasize the view that the overexpressions of Bcl-2 and Bcl-xL are not
8 identical, considering their respective effects on Bax. This might be a crucial consideration for
9 attempting to re-activate apoptosis in Bcl-2- or Bcl-xL-overexpressing tumoral cells.

10 Recent structural data brought major advances in the knowledge of Bax organization in the outer
11 mitochondrial membrane. The interaction of Bax Δ C (deprived of its C-terminal hydrophobic α -
12 helix) with the BH3 domain of Bid favors the formation of a symmetrical Bax dimer, in which
13 helices α 5 and α 6 would first lay flat on the membrane instead of being inserted as a hairpin, and
14 then would arrange a large pore by tilting in the membrane (Czabotar et al., 2014; Westphal et al.,
15 2014). The formation of the pore as a juxtaposition of dimers has been proposed as an alternative to
16 the membrane-inserted α -helices (Bleicken et al., 2014; Subburaj et al., 2015). This model is
17 compatible with recent microscopy images showing the ring-like organization of Bax in
18 mitochondria of apoptotic cells (Salvador-Gallego et al., 2016; Große et al., 2016). It is also
19 compatible with electrophysiological data suggesting that the pore formed by Bax have different
20 incremental sizes (Martinez-Caballero et al., 2009).

21 In non-apoptotic FL5.12 cells overexpressing Bcl-2, the presence of oligomers similar in size to
22 those observed in apoptotic cells have been observed (Renault et al., 2013), while no oligomer was
23 detected in the same cells overexpressing Bcl-xL (Renault et al., 2015). By correlating these
24 observations to the structural model of Bax oligomers, one could speculate that Bcl-2 would favor
25 the formation of Bax oligomers that remain lain on the membrane, while Bcl-xL prevents the
26 formation of oligomers, maybe by stabilizing Bax dimers. In any case, the rupture of the interaction
27 between Bax and its anti-apoptotic partner would be sufficient to promote the organization of the
28 pore-forming oligomer.

29 ***8-Concluding remarks***

30 The intrinsic pathway of apoptosis, that involves mitochondria, is triggered by signals originating
31 within the cell, such as, typically, p53 expression. Nevertheless, most Bcl-2 family members are
32 expressed at a basal level, both in tissues and in cultured cells. The presence of Bcl-2 family
33 members, both in proliferating and post-mitotic cells, might not only reflect the requirement for a
34 rapid response to apoptotic stimuli, but also non-apoptotic functions of these proteins. Namely, it

1 has been shown that both Bcl-2 and Bcl-xL could regulate autophagy (Pattingre et al., 2005; Maiuri
2 et al., 2007; Priault et al., 2010; Beaumatin et al., 2016). Interestingly, the effects of both proteins on
3 autophagy are distinct, paralleling their distinct effect on Bax-regulation discussed above. It has
4 also been shown that Bax could regulate mitochondrial dynamics (Karbowski et al., 2006; Cleland
5 et al., 2011). In this short survey, we have shown that, even in non-apoptotic cells, the interactions
6 between pro- and anti-apoptotic Bcl-2 family members determine the way a cell is able to respond
7 to a potential apoptotic trigger, such as an anti-tumoral treatment. The fact that these properties are
8 conserved in yeast demonstrates that they are dependent on their intrinsic molecular features. As a
9 matter of fact, the heterologous expression in yeast continues to be an outstanding tool to study the
10 molecular mechanisms underlying the function of these proteins.
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helix is a second ART sequence and plays a role in the interaction with anti-apoptotic BCL-xL.

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Legends to Figures

Figure 1. Schematic representation of the classification of Bcl-2 family members and BH3-containing proteins.

TOP: Classical representation of the Bcl-2 family members, bases on their function (anti- v/s pro-apoptotic) and on their structure (multidomain v/s BH3-only).

BOTTOM: Phylogenetic classification based on the evolution of BH domains . True Bcl-2 homologs include multidomain proteins (anti- and pro-apoptotic) and Bid, that has a structure closely related to that of Bcl-xL (Chou et al., 1999). BH3-domains can be divided into canonical domains, that are evolutionary related to those of Bcl-2 homologs, and non-canonical domains, that might proceed from a convergent evolution (Aouacheria et al., 2013, 2015).

Figure 2. Mitochondrial proteins released during cell death in mammals and yeast.

TOP: In mammalian cells, Bax/Bak-dependent apoptosis is associated to the formation of a large pore in the MOM, that allows the release of cytochrome c. It remains however unclear if this pore also allows the release of other factors. Necrosis and late apoptosis are associated to the opening of mPTP, that induces a non-selective release of all the factors localized in the intermembrane space.

BOTTOM: In yeast, the heterologous expression of Bax is associated to the formation or the same pore as in mammals, supporting that this pore contains Bax (Pavlov et al., 2001). Other factors can be released following death signals specific to yeast, such as acetic acid treatments (Pereira et al., 2010). However, Bax effects in yeast do not depend on these factors (Kissova et al., 2006).

Figure 3. Measurement of Bax-driven release of cytochrome c from yeast mitochondria by redox spectrophotometry and immunoblotting.

Mitochondria were isolated from yeast cells expressing wild-type Bax (BaxWT, poorly active) or a constitutively mitochondrial and active mutant Bax-P168A (Arokium et al., 2004).

LEFT: Redox spectrophotometry measurements. A mitochondrial suspension (3-5mg protein/mL) was placed in the cuvettes of a double-beam spectrophotometer. The reference cuvette was oxidized with potassium ferricyanide and the sample cuvette was reduced with sodium dithionite. The difference spectra were acquired between 500 and 650nm. Cytochrome c+c1 and cytochrome b contents were calculated from the height of the peaks, as indicated, and manually corrected with the difference of the isobestic points at 540nm and 575nm. A molar extinction coefficient of 18,000 M⁻¹. cm⁻¹ for both types of cytochromes was used, and cytochrome c was calculated by considering that the stoichiometry cytochrome b/cytochrome c1 is 2.

RIGHT: Immunoblotting measurements. 100μg of mitochondrial proteins or of post-mitochondrial

1 supernatants were blotted, and immunodetected with a custom antibody against yeast cytochrome c.
2 Antibodies against Phosphoglycerate Kinase (Pgk) and subunit 2 of respiratory complex IV (Cox2)
3 were used as loading controls. The immunoblotting against Bax shows that the mutant Bax-P168A
4 is more localized in mitochondria than BaxWT (Renault et al., 2015).
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8 **Figure 4. Comparison of the effects of full-length Bcl-xL and truncated Bcl-xL Δ C on Bax**
9 **mitochondrial localization.**

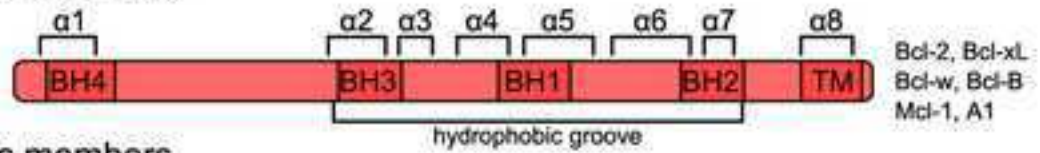
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11 LEFT: Bax is under a dynamic equilibrium between mitochondria and cytosol, owing to the
12 processes of Bcl-xL-driven translocation and retrotranslocation. Mitochondrial Bax is primed but
13 remains inactive because of the interaction with Bcl-xL. The addition of a BH3-mimetic (or the
14 expression of a BH3-only partner) enables the derepression of the interaction and the full activation
15 of Bax.
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20 RIGHT: Bcl-xL Δ C stimulates the translocation but not the retrotranslocation, leading to a higher
21 mitochondrial Bax content. Furthermore, the interaction between Bax and Bcl-xL Δ C is weak, and
22 Bax can be fully activated without the need of a BH3-mimetic.
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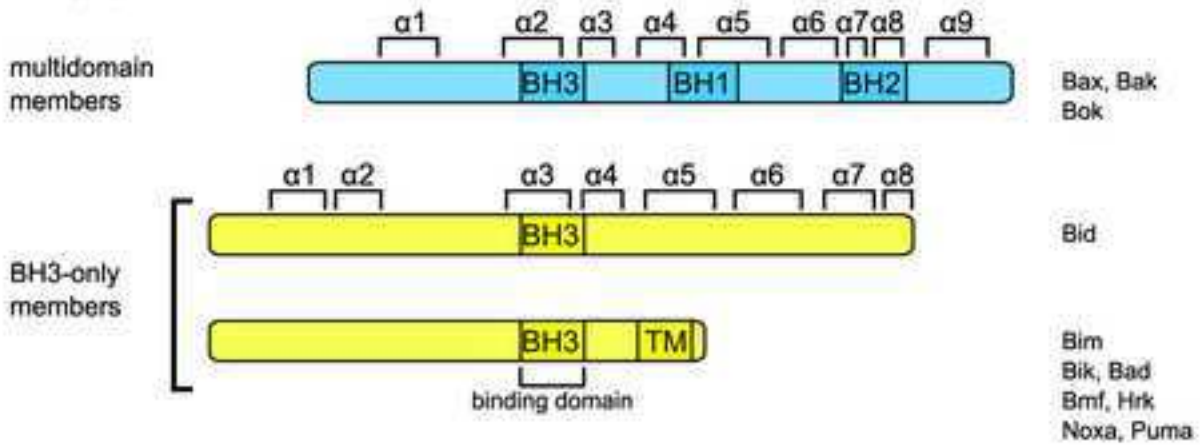
Figure 1
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Functional/structural classification

Antiapoptotic members

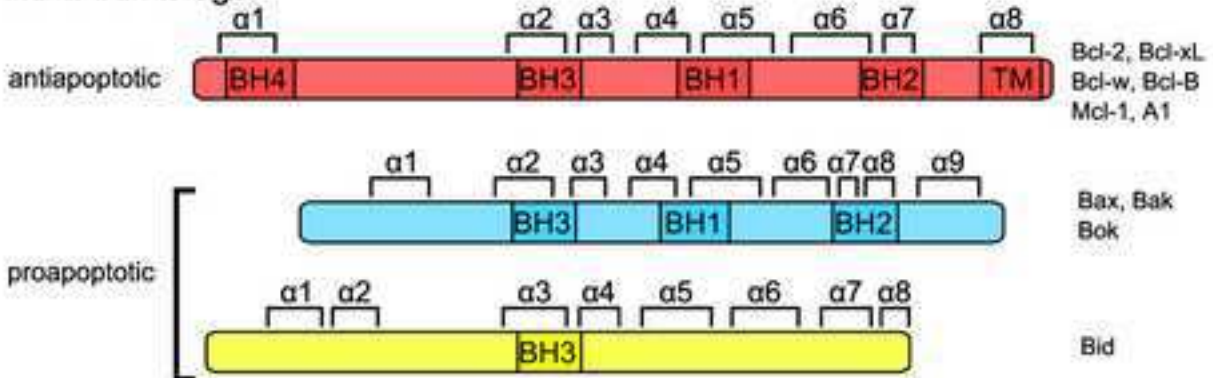


Proapoptotic members



Phylogenetic classification

Bcl-2 homologs



Canonical BH3-domain



Non-canonical BH3-domain

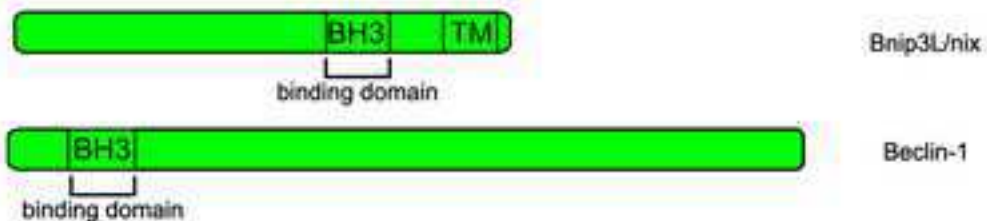
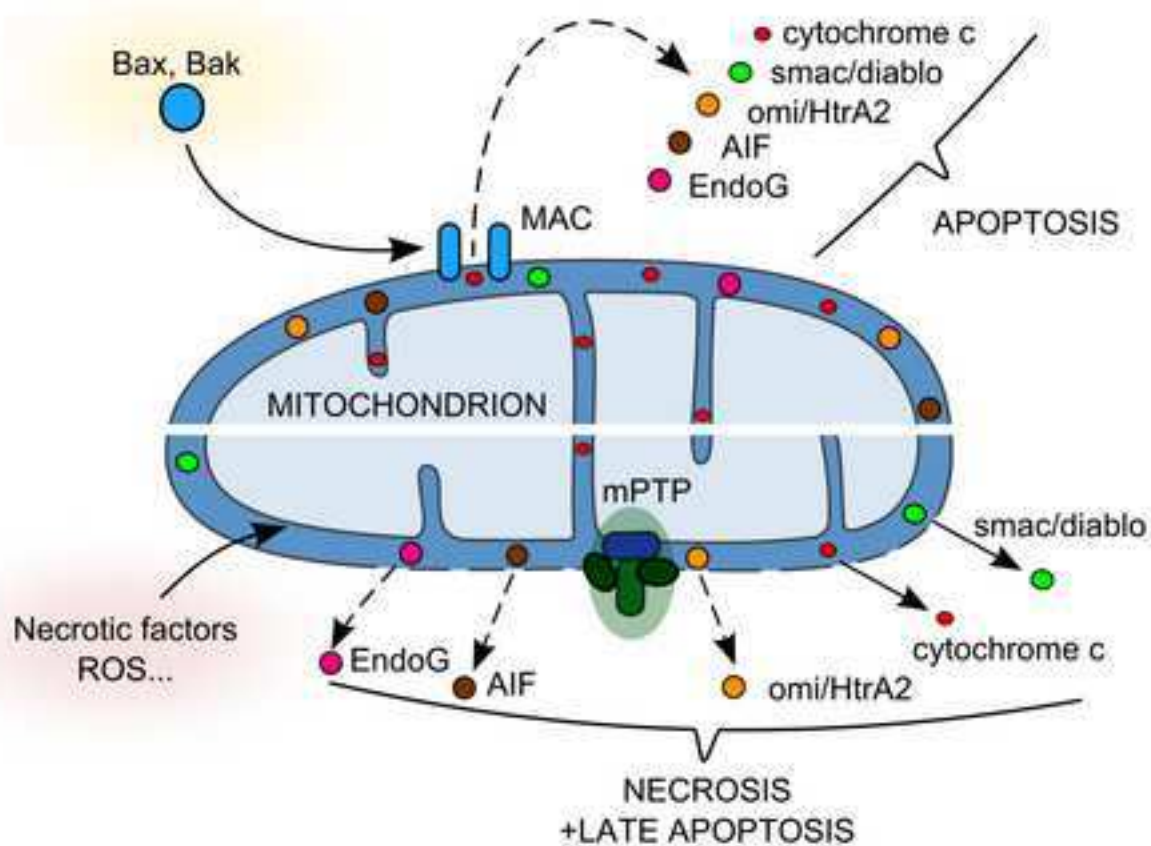
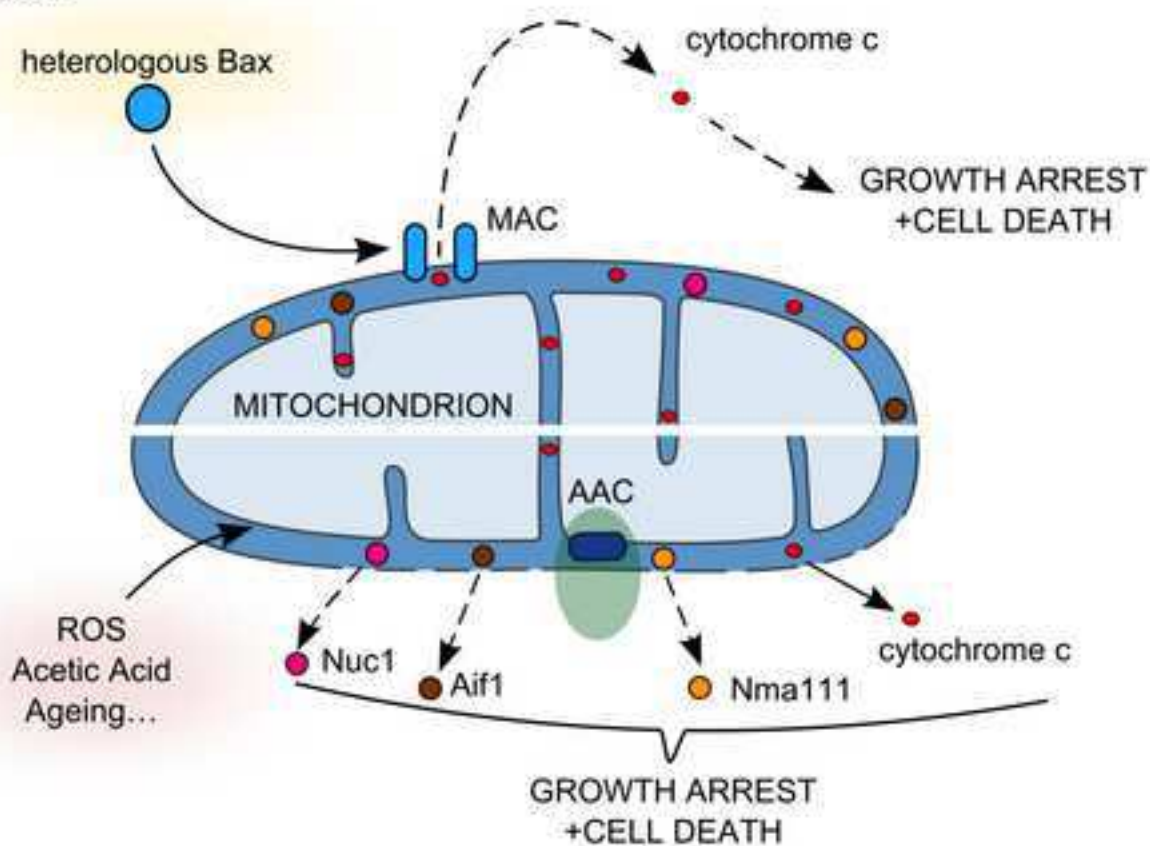


Figure 2
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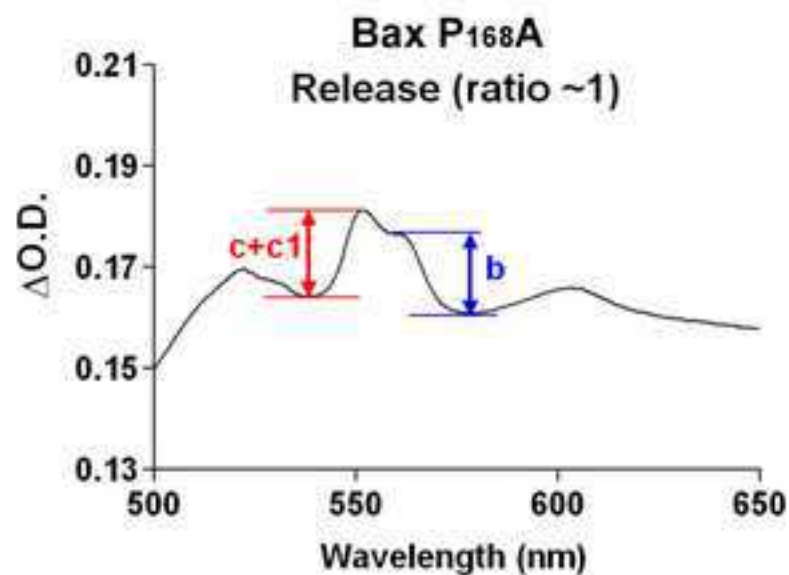
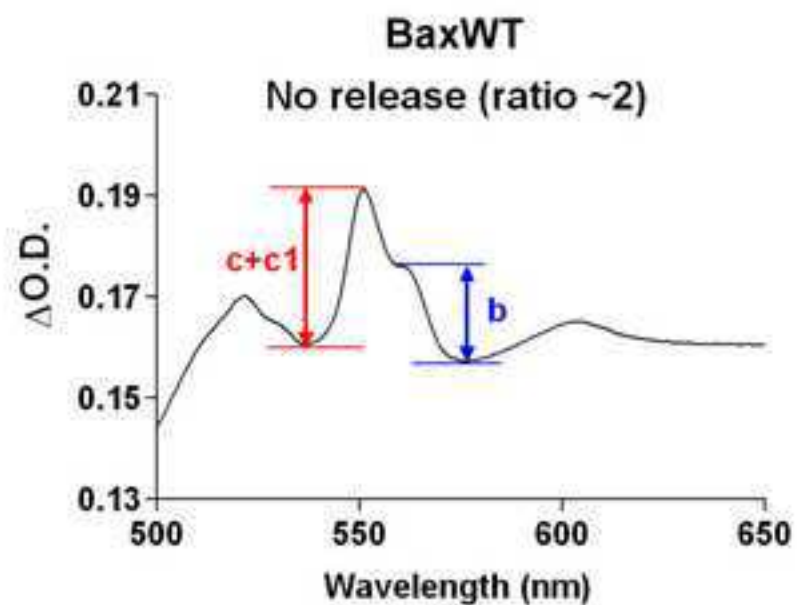
Mammals



Yeast



Redox spectrophotometry



Immunoblotting

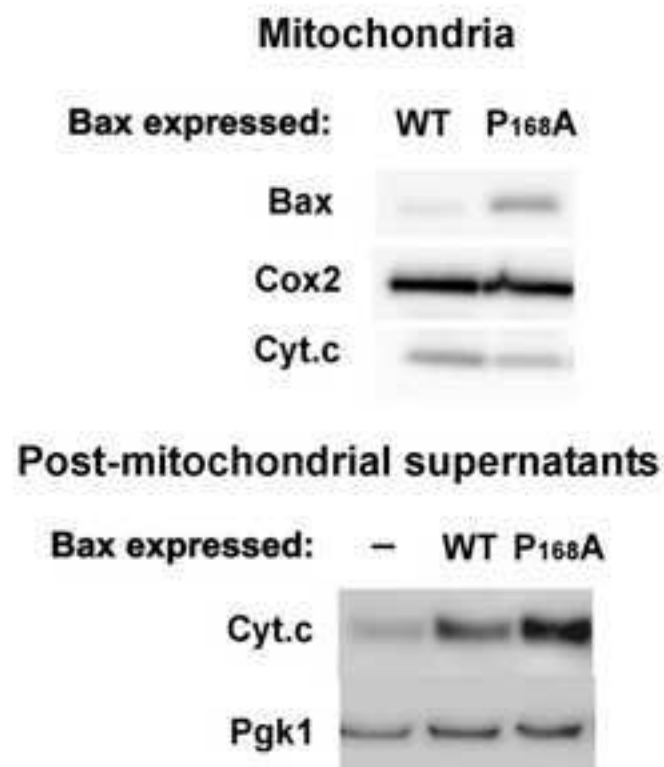
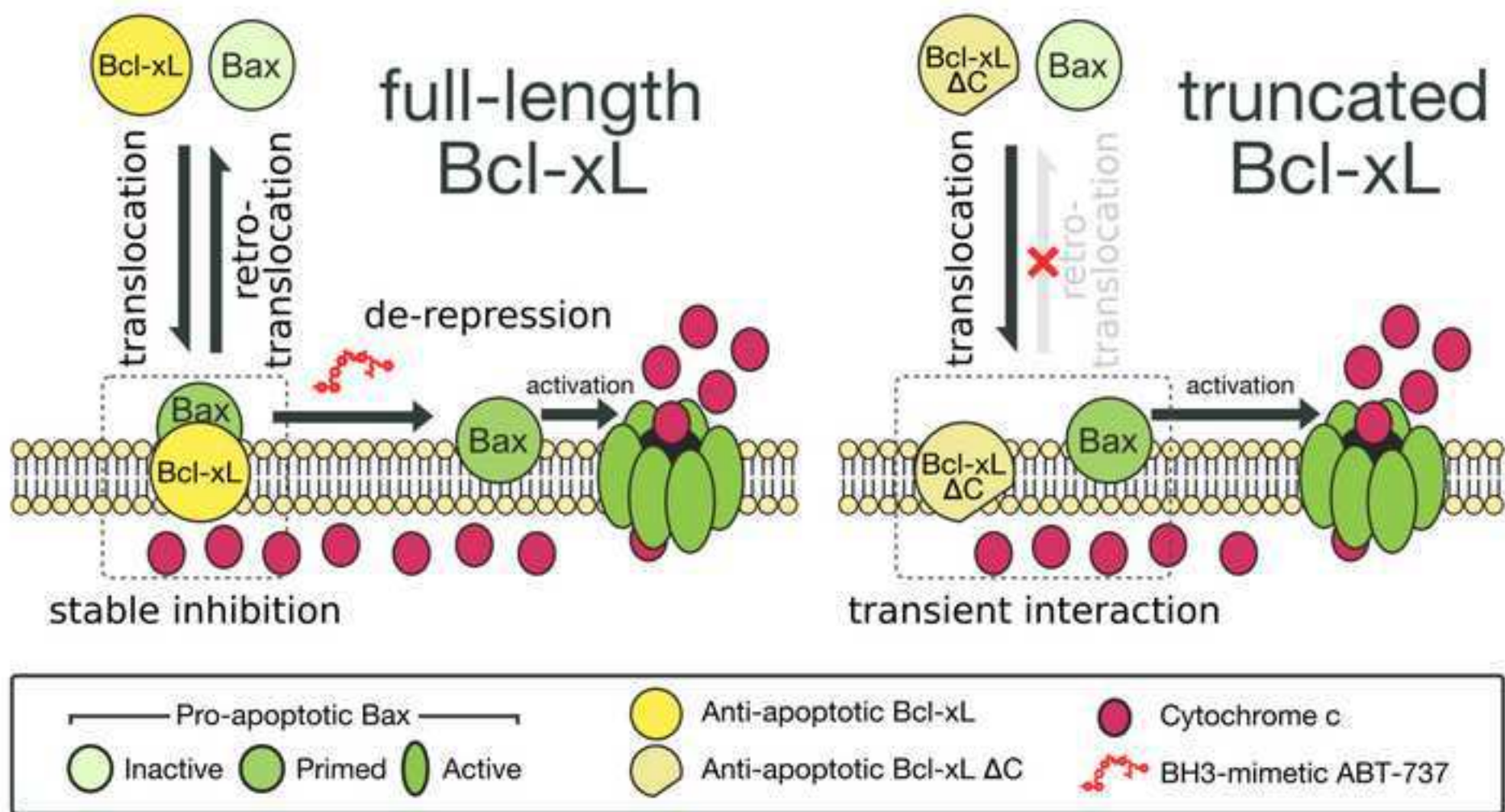


Figure 4
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A brewing understanding of the regulation of Bax function by Bcl-xL and Bcl-2

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Summary

Bcl-2 family members form a network of protein-protein interactions that regulate apoptosis through permeabilization of the mitochondrial outer membrane. Deciphering this intricate network requires streamlined experimental models, including the heterologous expression in yeast. This approach had previously enabled researchers to identify domains and residues that underlie the conformational changes driving the translocation, the insertion and the oligomerization of the pro-apoptotic protein Bax at the level of the mitochondrial outer membrane. Recent studies that combine experiments in yeast and in mammalian cells have shown the unexpected effect of the anti-apoptotic protein Bcl-xL on the priming of Bax. As demonstrated with the BH3-mimetic molecule ABT-737, this property of Bcl-xL, and of Bcl-2, is crucial to elaborate about how apoptosis could be reactivated in tumoral cells.

1-Introduction: Bcl-2 family members v/s BH3-containing proteins

Apoptosis is the main type of programmed cell death in animals, participating to the modeling of organs during the development, and to tissue homeostasis during the whole life. It is activated following external or internal stimuli, and leads to a non-inflammatory response, opposite to other forms of cell death, such as necrosis.

It is generally considered that there are two types of apoptotic signaling pathways:

- the exogenous pathway, when death is initiated by the binding of an external ligand to a receptor, involves the direct activation of caspases, a group of proteases responsible for the implementation of apoptotic characteristics;

- the endogenous pathway, when death is initiated “from the inside”, for example through the activation of transcription factor p53 following an alteration to DNA, involves mitochondria.

Alterations of the apoptotic program are associated with major age-related diseases, such as cancers when apoptosis is deficient, or degenerative diseases when apoptosis is over-activated. It is because of the existence of these major health issues that apoptosis is one of the most studied processes of cell biology in the last 20 years. A considerable amount of knowledge has been accumulated about the signaling pathways that regulate cell death, both under physiological and pathological situations. However, it still remains difficult to predict how these pathways modulate the molecular mechanisms underlying apoptosis, because of antagonistic effects between signaling pathways regulating cell death and survival, and the redundancy between parallel pathways. This intricacy is further increased in cell lines, where survival pathways are often over-activated while death pathways are repressed. This is why streamlined experimental models are needed to investigate the link between cellular and molecular regulations.

The group of proteins known as Bcl-2 family members are central players of apoptosis, because they form the interface between the early signaling events engaging the cells into the apoptotic process, and the later events conferring their apoptotic characteristics to the cells, leading to their elimination by the immune system (Adams and Cory, 2007, for a review). Bcl-2 family members are characterized by 4 homology domains, called BH1 to BH4. These domains are the keystones of these proteins' function and regulation, as they enable their mutual interaction within an intricate and highly regulated network.

Classically, the Bcl-2 family has been divided into 3 sub-families: anti-apoptotic proteins (*e.g.* Bcl-2, Bcl-xL), pro-apoptotic proteins (Bax, Bak, Bok), and BH3-only proteins (*e.g.* Bid, Bad, Puma, Bim) (Fig.1). It is noteworthy that, while the two first sub-families are defined on the basis of a well-identified biological function, the last one is defined by default, on the basis of a structural property.

As a matter of fact, recent phylogenetic studies have shown that BH3-domains are present in many proteins other than the Bcl-2 family (Aouacheria et al., 2013) including, for example, the mammalian protein Beclin-1 (Liang et al., 1999), or the yeast protein Ybh3/Bxi1/YNL305C (Cebulski et al., 2011; Buttner et al., 2011). However, those BH3-domains do not derive from a common ancestor, opposite to the canonical BH3 domain of pro- and anti-apoptotic Bcl-2 family members, but rather result from a convergent evolution (Aouacheria et al., 2013). These studies redefined the BH3-domain as a short interacting motif, that is thus not limited to the Bcl-2 family members (Aouacheria et al., 2015). According to these studies, BH3-containing proteins could be re-organized into three categories (Aouacheria et al., 2013) (Fig.1):

- true Bcl-2 homologs, that contain three or four BH domains, and that can be anti-apoptotic (*e.g.*

1 Bcl-2, Bcl-xL) or pro-apoptotic (Bax, Bak, Bok); Bid, which has only the BH3 domain, is
2 considered as a true Bcl-2 homolog because of its overall structural similarity with Bcl-2;

3 - BH3-only proteins that contain a canonical BH3-domain (*e.g.* Bad, Puma, Bim);

4 - BH3-only proteins that contain a non-canonical BH3-domain (*e.g.* Beclin-1, Bnip3L/Nix, yeast
5 Ybh3).
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7 These studies show that true Bcl-2 homologs are only present in animals: vertebrates (including
8 Bcl-2, Bax, and their homologs in non-mammalian vertebrates), nematode (*C.elegans* Ced-9) and
9 insects (*D.melanogaster* Buffy and DeBcl). The same observation can be done for proteins
10 containing a canonical BH3-domain, in vertebrates (including Bid, Puma, Bad) and in the nematode
11 (Egl-1). All the BH3-containing proteins found in non-animal organisms contain a divergent
12 domain, *e.g.* Ybh3 in the yeast *S.cerevisiae*, Rad9 in the yeast *S.pombe*, MCF in the bacterium
13 *P.luminescens*, and viral proteins such as the protein CORE from the hepatitis C virus (Aouacheria
14 et al., 2013). These proteins may have a function that is unrelated to the Bcl-2 family. For example,
15 yeast Ybh3/Bxi1/YNL305C is involved in the UPR response and might modulate calcium dynamics
16 (Cebulski et al., 2011), as shown for its bacterial homolog (Chang et al., 2014). However, like all
17 the proteins containing a divergent BH3-domain, it might be potentially able to bind to Bcl-2
18 homologs, namely Bax, particularly when it is over-expressed. Conversely, Bcl-2 homologs might
19 regulate the function of proteins containing a divergent BH3 domain, independently from apoptosis:
20 as an example, it has been proposed that the pro-autophagic function of Beclin-1 is negatively
21 regulated by the overexpression of Bcl-2 (Pattingre et al., 2005), although the effect of Bcl-2 on
22 autophagy might also be indirect (Lindqvist et al., 2014).
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39 **2-Bcl-2 family, MOMP and cytochrome c**

40 Bcl-2 homologs function is essential to the regulation of mitochondrial outer membrane
41 permeabilization (MOMP), the key event irreversibly engaging the cells towards death. Following
42 MOMP, a group of particular proteases, the caspases, are activated. This activation occurs through
43 two processes: (1) procaspases are cleaved into two subunits and reorganized into active
44 heterotetramers and (2) IAPs, a family of caspases inhibitors, are inactivated.
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49 There is now a large consensus among investigators to consider that MOMP is caused by the
50 formation of a large pore in the mitochondrial outer membrane (MOM), that enables the release of
51 several proteins from the mitochondrial intermembrane space to the cytosol. Five of these proteins
52 have been identified as 'apoptogenic factors' and are described below (Fig.2):
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56 - Cytochrome c (12.5kDa) is a central soluble component of the electron transport chain; when
57 released, it binds to Apaf-1, forming a complex that catalyzes the cleavage and the activation of
58 caspase-9.
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- Smac/Diablo (25kDa) binds to IAPs, promoting their degradation, and thus preventing the inhibition of caspases.

- Omi/HtrA2 (49kDa) is a protease that inactivates IAPs.

- Endonuclease G (28kDa) participates to chromatin degradation.

- Apoptosis-Inducing Factor (AIF, 61kDa) is a redox protein that participates to the regulation of the respiratory chain. During apoptosis, it participates to chromatin condensation, but its precise function remains elusive.

It is still unclear whether these proteins, that have different sizes, are released simultaneously and through the same mechanism. Indeed, most studies are done on cultured cells with a sustained apoptotic trigger (such as the addition of an antitumoral drug). Under these conditions, apoptosis is followed by necrosis.

The initial steps of apoptosis might only involve a limited MOMP, without alteration of the inner mitochondrial membrane (see below). On the opposite, the secondary necrotic process might be associated to the activation of the large unselective channel called the mitochondrial Permeability Transition Pore (mPTP), that induces the permeabilization of the inner mitochondrial membrane (Bernardi et al., 2015, for review). This is followed by a massive mitochondrial swelling leading to the unselective rupture of the MOM, and the release of the whole intermembrane space content into the cytosol (Fig.2). Consequently, the mechanistic aspects of selective MOMP should be studied at the early stages of the apoptotic process, when mPTP is still close.

Electrophysiological studies on MOM from apoptotic cells showed the presence of a large conductance channel, that also appeared in the MOM from yeast cells expressing human Bax (Pavlov et al., 2001), or on isolated membranes incubated with Bax oligomers (Dejean et al., 2005). Interestingly, the size of this channel was sufficient to transport cytochrome c (12.5kDa), but not to transport a bigger protein, such as hemoglobin tetramer (60kDa) (Dejean et al., 2005). Electrical properties of this channel showed the existence of different conductance substates, suggesting a process of stepwise Bax oligomerization (Martinez-Caballero et al., 2010). Recent studies showed that Bax oligomers form large ring-like structures in the MOM that could be assimilated to the pore of the MOMP machinery (Große et al., 2016; Salvador-Gallego et al., 2016).

Among the apoptogenic factors, cytochrome c is one of the most ubiquitous and conserved proteins in the whole living world (Margoliash and Fitch, 1968), a feature most certainly related to its central function as a mobile electron carrier that shuttles between respiratory complexes. As such, it can serve as a universal marker for MOMP, including in model systems that do not carry out canonical apoptosis. Most specifically, the release of cytochrome c became a standard in studies regarding the function of animal Bcl-2 homologs after they are expressed in yeast (Fig.3). The expression of human Bax was shown to promote the release of cytochrome c in yeast (Manon et al., 1997) even

1 before it was shown in human cells (Jurgensmeier et al., 1998). Like in mammalian cells, several
2 death signals in yeast, such as acetic acid treatment, induce dramatic mitochondrial morphological
3 alterations and a non-selective release of intermembrane space proteins, that have homologies with
4 mammalian apoptogenic factors (Eisenberg et al., 2007; Cheng et al. 2008; Pereira et al., 2008, for
5 reviews) (Fig.2). Interestingly, Bax effects in yeast do not depend on other proteins that are released
6 following death stimuli (Kissova et al., 2006), such as acetic acid treatment, that may induce a
7 necrotic type of death depending on massive mitochondrial permeabilization resembling mPTP
8 (Pereira et al., 2010). This supports the view that, in mammalian cells, only cytochrome would be
9 released through Bax-formed channels, while other apoptogenic factors would be released through
10 other systems, such as mPTP (Kroemer et al., 2007, for review) or ceramides channels (Colombini,
11 2010, for review).

12 Due to the ease to obtain large amounts of yeast biomass, cytochrome c can be easily quantified by
13 redox spectrophotometry, and compared to non-released cytochromes, such as cytochrome b, a
14 membrane-inserted component of respiratory complex III (Fig.3). This allowed measuring the
15 activities of different Bax mutants, with more precision than western-blots quantification (Arokium
16 et al., 2004, 2007; Renault et al., 2015). Furthermore, redox spectrophotometry measures the
17 mitochondrial cytochrome c content after it has been assembled with heme in the mitochondrial
18 intermembrane space (Gonzalez and Neupert, 1990), thus limiting the quantification to cytochrome
19 c that has transited through mitochondria. On the opposite, western-blots against cytochrome c also
20 reveal the heme-free apoprotein, before it has been assembled within the mitochondria, meaning
21 that a deficient addressing might be erroneously taken for an increased release.

22 The redox spectrophotometry measurement of cytochrome c in yeast mitochondria, following the
23 heterologous expression of human Bax mutants has provided a considerable amount of data about
24 the domains, residues, and possible partners involved in Bax ability to interact with MOM and to
25 release cytochrome c. For example, the consequences of the replacements of Proline residues at
26 positions 8, 13 and 168 by smaller and more flexible Glycine/Alanine residues have pointed out the
27 role of the mobility of the N-terminal unstructured domain and of the C-terminal α -helix in Bax
28 activation (Arokium et al., 2004). Electric charge inversions at different positions (Arg9/Asp154)
29 confirmed that favoring the movement of these domains (by disrupting salt bridges), was sufficient
30 to promote the localization of Bax to the MOM (Arokium et al., 2007). Furthermore, this method
31 has allowed evidencing the involvement of the mitochondrial receptor Tom22 in the adequate
32 translocation of Bax (Bellot et al., 2007; Renault et al., 2012), and the direct activation of Bax by
33 BH3-only proteins tBid (Gonzalves et al., 2005) and Puma (Gallenne et al., 2009). This method has
34 thus proved to be an adequate measurement of the ability of Bax to permeabilize the MOM.

35 In parallel, Bax expression in yeast induces a reduction of yeast growth and a loss of viability.

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However, these phenotypes involve additional effects beyond the release of cytochrome c, and are consequently less precise reporters of Bax effects on mitochondria than the direct measurement of cytochrome c release (Kissova et al., 2006). Nevertheless, they proved their usefulness for the rapid screening of factors that potentially modulate the effects of Bax (Xu and Reed, 1998; Pan et al., 2001; Laloux et al., 2010; Ubaidillah et al., 2013).

3-Bax retrotranslocation

For long, it had been considered that Bax translocation from the cytosol to the mitochondria was a unidirectional process. This came from the observation that, in non-apoptotic cells, Bax localization is essentially diffuse in the cytosol while, after apoptosis is triggered, it is relocated to mitochondria (Wolter et al., 1997) to form membrane-inserted oligomers that are responsible for MOMP (Antonsson et al., 2000, 2001). Since this process involves major conformational changes (Suzuki et al., 2000; Peyerl et al., 2007), it was hardly conceivable that these oligomers could be disassembled, and that Bax could go back to its previously inactive state in the cytosol. However, significant observations suggested that the passage from soluble/monomeric Bax to membrane-inserted/oligomeric Bax is not a one-step process. For example, it has been shown that, during anoikis, Bax could be relocalized to mitochondria, but that the process was reversible (Owens et al., 2009). This, and other experiments, demonstrated that 'mitochondrial Bax' was not obligatorily 'membrane-inserted' Bax (Renault et al., 2013).

Structural studies have allowed designing a complex Bax mutant that has a constitutive mitochondrial localization but that cannot support the conformational change associated to the oligomerization, thus remaining incompetent for cytochrome c release. Under oxidative conditions, this mutant displays two internal disulfide bonds that constraint helices $\alpha 1$ and $\alpha 2$, and $\alpha 1$ - $\alpha 2$ loop and $\alpha 6$, therefore blocking the 'opening' of Bax structure associated to its activation (Edlich et al., 2011). When expressed in HCT-116 or HeLa cells, a GFP-tagged version of this mutant displayed a mitochondrial localization and did not trigger apoptosis, as expected. Following the photobleaching of GFP fluorescence in cell nuclei, both nuclear and cytosolic fluorescence disappeared while mitochondrial fluorescence remained. This reflected the rapid dynamics of exchange between the nucleus and the cytosol, and its absence for mitochondrial Bax. However, by following the re-appearance of the fluorescence in the cytosol, the authors showed that a fraction of mitochondrial Bax could be retrotranslocated from the mitochondria to the cytosol. Most interestingly, this retrotranslocation process was greatly accelerated when the anti-apoptotic protein Bcl-xL was over-expressed (Edlich et al., 2011). This led the authors to conclude that, in non-apoptotic cells, (1) Bax subcellular localization followed a dynamic equilibrium between mitochondria and cytosol and that (2) anti-apoptotic proteins could displace this equilibrium towards a more cytosolic localization. An

1 additional interesting observation was that a mutant of Bcl-xL deleted of the C-terminal α -helix
2 (Bcl-xL Δ C) was unable to promote Bax retrotranslocation, and further experiments demonstrated
3 that the deletion of the 4 last residues of Bcl-xL were sufficient to prevent it (Todt et al., 2013).

4 The question remained whether this process was only dependent on the intrinsic properties of
5 interaction between the two proteins, or also involved external factors (such as, for example, the
6 presence of other Bcl-2 family members). Since yeast does not contain endogenous Bcl-2 family
7 members, it is a powerful tool to answer this question. Bax was expressed alone, or co-expressed
8 with full-length Bcl-xL or truncated Bcl-xL Δ C in a yeast mutant inactivated for the major vacuolar
9 protease Pep4p (the yeast ortholog of cathepsin D), and in the presence of the general inhibitor of
10 serine-proteases PMSF. Under these conditions, following the addition of an inhibitor of protein
11 synthesis (cycloheximide), the cellular Bax content remained stable for at least 4 hours.
12 Mitochondria were then isolated to measure the evolution of Bax mitochondrial content following
13 cycloheximide addition. Since the total content was stable, any decrease of the mitochondrial
14 content would indicate retrotranslocation. When expressed alone, mitochondrial Bax levels
15 remained constant. When co-expressed with Bcl-xL, mitochondrial Bax levels were decreased by
16 50% within 2 hours. When co-expressed with Bcl-xL Δ C, mitochondrial Bax levels remained
17 constant. Similar experiments were done in mouse pro-lymphocytic cells FL5.12, with the same
18 results, showing that the experiment in yeast actually led to observations relevant to mammalian
19 physiology. These results were therefore consistent with the hypothesis that Bcl-xL (but not Bcl-
20 xL Δ C) was able to promote the retrotranslocation of Bax, when the two proteins were co-expressed
21 in yeast. This demonstrated that the retrotranslocation of Bax by Bcl-xL was only dependent on
22 molecular properties of Bax and Bcl-xL, without interference from factors present in mammalian
23 cells and absent from yeast, such as other Bcl-2 family members (Renault et al., 2015).

24 Importantly, no retrotranslocation was observed when a constitutively active mutant of Bax,
25 carrying a substitution P168A (Arokium et al., 2004; Cartron et al., 2005) was used. This supports
26 the view that, once it is inserted and oligomerized (since it is able to promote the release of
27 cytochrome c), Bax cannot be retrotranslocated.
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49 ***4-Steady-state Bax subcellular localization***

50 Intuitively, the existence of Bax retrotranslocation suggests that, in the presence of Bcl-xL, the
51 steady-state Bax localization should be more cytosolic than when Bax is expressed alone. This can
52 be correlated with the classical view of over-expressed Bcl-xL (or Bcl-2) sequestering Bax away
53 from mitochondria, thus preventing apoptosis by restraining Bax ability to reach and permeabilize
54 MOM.
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60 Opposite to this prediction, we observed that the over-expression of Bcl-xL or of Bcl-2 increased
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1 the mitochondrial localization of endogenous Bax in FL5.12 cells (Teijido and Dejean, 2010;
2 Renault et al., 2015). Strikingly, Bax mitochondrial content reached the same level as measured in
3 parental cells committed to apoptosis through IL-3 removal. Although less marked, a similar effect
4 was observed in HCT-116 cells overexpressing Bcl-xL, showing that it was not a unique property of
5 FL5.12 cells.
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7 We then investigated if this apparently paradoxical effect of Bcl-xL on Bax mitochondrial
8 localization was only dependent on the interaction between Bax and Bcl-xL or if other factors were
9 involved, by doing the experiments in yeast. We observed that Bax mitochondrial content was
10 increased when Bcl-xL was co-expressed with Bax in yeast. This last result showed that Bcl-xL-
11 mediated Bax increase at the mitochondria was a general phenomenon, that depended only on Bax
12 and Bcl-xL.
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14 Most interestingly, we observed that the co-expression of Bcl-xL Δ C induced an even higher
15 increase of Bax mitochondrial localization in FL5.12 cells, in HCT-116 cells, and in yeast (Renault
16 et al., 2015). As discussed above, truncated Bcl-xL Δ C is not able to promote Bax retrotranslocation.
17 This suggests that Bcl-xL regulates Bax mitochondrial localization through two opposed processes:
18 retrotranslocation, for which Bcl-xL Δ C is impaired, and translocation, promoted both by full-length
19 Bcl-xL and truncated Bcl-xL Δ C (Fig.4).
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31 ***5-Bax priming is enhanced in cells overexpressing Bcl-xL Δ C or Bcl-xL***

32 The immediate consequence of the increase in the steady-state level of mitochondrial Bax is a
33 potentially higher ability to initiate apoptosis. However, the presence of Bcl-xL, and the interaction
34 between the two proteins, obviously prevent Bax activation. This can be verified by the fact that
35 IL-3 removal induced a lower level of apoptosis in Bcl-xL-overexpressing FL5.12 cells, than in
36 parental cells. Here again, the inhibition only depends on the interaction between Bax and Bcl-xL
37 since, in yeast, the effect of a constitutive mitochondrial and active mutant of Bax on mitochondrial
38 permeabilization is also prevented by the co-expression of Bcl-xL.
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46 On the other hand, the overexpression of Bcl-xL Δ C does not prevent the apoptosis of FL5.12 cells
47 induced by the removal of IL-3, and does not prevent the permeabilization of yeast mitochondrial
48 membrane by a constitutively active Bax mutant. In line with these observations, co-
49 immunoprecipitation assays showed that the interaction between Bax and Bcl-xL Δ C was not
50 detectable under conditions where the one between Bax and full-length Bcl-xL was. This
51 demonstrates that the presence of the C-terminal α -helix of Bcl-xL is required for a stable
52 interaction and subsequent efficient inhibition of Bax.
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59 Furthermore, we observed that the expression of Bcl-xL Δ C stimulated Bax-driven MOMP in yeast.
60 This rather unexpected effect was dependent on the interaction between the two proteins, since it
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1 was abrogated by introducing the canonical G138A mutation in the BH1-domain of Bcl-xL Δ C
2 (Renault et al., 2015), that is known to prevent the interaction between the two proteins (Ottillie et
3 al., 1997).

4 These results showed that, although it might not be stable enough to be detectable by co-
5 immunoprecipitation, a transient interaction between Bax and Bcl-xL Δ C occurs and is sufficient to
6 increase mitochondrial Bax content, but is not sufficient to prevent Bax-induced permeabilization.
7 This was not observed with full-length Bcl-xL since its interaction with Bax, while inducing
8 translocation of the pro-apoptotic protein, was stable enough (detectable by co-
9 immunoprecipitation, as a matter of fact) to also keep it inactive.

10 Since Bcl-xL Δ C is not a natural variant of the protein, we searched for experimental conditions
11 which could reveal that full-length Bcl-xL is also able to prime Bax. Experiments on purified
12 recombinant proteins had previously shown that the binding, then release, of Bax and Bcl-xL could
13 initiate the active conformation of Bax that can be detected with the 6A7 monoclonal antibody (Hsu
14 and Youle, 1997). The release could be induced by peptides corresponding to the BH3-domain of
15 canonical BH3-only proteins, such as Puma, or by BH3-mimetic molecules such as ABT-737
16 (Gautier et al., 2011).

17 Therefore we used ABT-737 to disrupt Bax/Bcl-xL interaction both in the FL5.12 and the yeast
18 models. The addition of ABT-737 to parental FL5.12 cells did not induce apoptosis, consistently
19 with previous works (Certo et al., 2006). On the opposite, the addition of ABT-737 to FL5.12 cells
20 overexpressing Bcl-xL induced a significant level of apoptosis (Renault et al., 2015).

21 We observed that ABT-737 had no effect on the biomass of yeast cells expressing Bax alone, or Bcl-
22 xL alone, but it significantly decreased the biomass of cells co-expressing Bax and Bcl-xL.
23 Although this result might not rely only on Bax-induced MOMP, for the reasons discussed above
24 (Kissova et al., 2006), it is nevertheless in accordance with the effect of ABT-737 observed in
25 FL5.12 cells.

26 These experiments show that, both in FL5.12 cells and in yeast, the transient interaction between
27 Bax and Bcl-xL followed by the release of the interaction, promotes the activation of Bax, like
28 previously reported using pure recombinant proteins. Also, the study of yeast cells co-expressing
29 Bax, Bcl-xL (or Bcl-2) and BH3-only proteins, have suggested that the lasts could only activate Bax
30 indirectly, through the inhibition of Bcl-xL or Bcl-2 (Gerecova et al., 2013). A sequential expression
31 of Bax and Bcl-xL/Bcl-2, then BH3-only proteins, could provide a mean to study whether a given
32 BH3-only protein is able or not to promote the release of Bax under an active conformation.
33 Considering the hypothesis proposed above, this should happen with all the BH3-only proteins,
34 with small efficiency differences due to their higher or lower specificity towards Bcl-2 or Bcl-xL.

35 This has interesting consequences on the potential efficiency of BH3-mimetic molecules in anti-
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1 tumoral therapy. These molecules were designed to prevent the inhibition of apoptosis by anti-
2 apoptotic Bcl-2 family members (essentially Bcl-2, Bcl-xL, and Mcl-1), that are overexpressed in
3 most cancer cells (see *e.g.* Tang et al., 1998; Pena et al., 1999, Castilla et al., 2006). The fact that, by
4 breaking the interaction between Bax and Bcl-2/xL, not only they liberate Bax, but they further
5 activate it, renders these molecules even more desirable as anti-tumor therapy, provided that Bax is
6 still expressed and not mutated.
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10 ***6-A correlation between Bax phosphorylation and its interaction with Bcl-xL?***

11 From the discussion above, Bcl-xL is able to stimulate both Bax translocation to the MOM, and Bax
12 retrotranslocation to the cytosol. This might appear like a futile cycle, but actually results in a high
13 turnover of Bax localization and, as we have observed, a high mitochondrial content of 'primed'
14 Bax, ready to trigger apoptosis. This might make the system more reactive than if Bax was only
15 sequestered away from mitochondria, since Bax is already on target, ready to be activated after the
16 release of its interaction with Bcl-2/Bcl-xL. However, the efficiency of such a 'dynamic cycling'
17 model implies that the interaction between Bax and Bcl-xL has different properties depending if it
18 happens in the cytosol or in the MOM. This may rely on biochemical differences between cytosolic
19 and mitochondrial Bax.
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29 Bax phosphorylation on different residues has been observed by several groups (Gardai et al., 2004;
30 Linseman et al., 2004; Xin et al., 2007). Two main residues, S163 and S184, are substrates of
31 kinases, although this does not exclude that other residues could be targeted as well. S163 is
32 phosphorylated by GSK3 β . This implies that T167 should also be phosphorylated, since the
33 consensus site for GSK3 β is SxxxT_P(S_P). S184 is the target of different kinases, including AKT and
34 PKC ζ . Interestingly AKT and GSK3 β have opposite effects since the former phosphorylates and
35 inactivates the later. Furthermore, AKT is generally involved in survival signaling pathways, while
36 GSK3 β is involved in death pathways (Martini et al., 2014), making their opposite effects on Bax
37 rather predictable.
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46 The heterologous expression of phosphomimetic and non-phosphorylatable mutants of S163 and
47 S184 in yeast suggests that modifications of these residues participate to the regulation of the
48 interaction of Bax with mitochondria (Arokium et al., 2007; Simonyan et al., 2016). When present
49 alone, the mutation S163D does not have any particular phenotype, but it can activate Bax when it
50 is combined to other mutations that facilitate intramolecular movements (Arokium et al., 2007).
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55 The phosphorylation of S184 has attracted much interest because it is located in the C-terminal α -
56 helix, of which the movement is crucial to initiate Bax relocalization. It had been shown that the
57 phosphorylation of S184 retained Bax away from mitochondria, while its dephosphorylation has the
58 opposite effect (Gardai et al., 2004). This could be reproduced in yeast where the mutants S184A/V
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1 have a higher mitochondrial localization than the mutant S184D (Arokium et al., 2007; Simonyan et
2 al., 2016). However, this did not fully correlate with the ability of these mutants to induce MOMP.
3 Indeed, the mutant S184D was surprisingly more efficient than mutants S184A/V (and wild-type
4 Bax), to promote the release of cytochrome c. These results suggest that the intrinsic capacity of the
5 phosphomimetic mutant to induce MOMP was higher than that of the non-phosphorylatable mutant,
6 that was apparently in contradiction with the fact that Bax phosphorylated on S184 is less able to
7 induce apoptosis than the non-phosphorylated protein (Gardai et al., 2004). Part of the answer to
8 this paradox might be related to the fact that, in mammalian cells, Bax is not expressed alone, but in
9 the presence of, namely, anti-apoptotic proteins. Bax phosphomimetic and non-phosphorylatable
10 mutants were then co-expressed with full-length Bcl-xL or truncated Bcl-xL Δ C (Simonyan et al.,
11 2016). We observed that the mitochondrial localization of the phosphomimetic mutant S184D did
12 not differ from that of wild-type Bax, with an increase induced by Bcl-xL and a further increase
13 induced by Bcl-xL Δ C, linked to the absence of retrotranslocation, as described above. Interestingly,
14 the behavior of the non-phosphorylatable mutant S184A differed: its mitochondrial content was
15 increased by Bcl-xL, like that of wild-type Bax and of the phosphomimetic mutant S184D, but it
16 was not further increased, and was even about twice less increased, by Bcl-xL Δ C. Since the major
17 difference between full-length Bcl-xL and truncated Bcl-xL Δ C is their capacity to retrotranslocate
18 Bax, this result showed that retotranslocation did not have any effect on the mitochondrial
19 localization of the non-phosphorylatable mutant Bax-S184A (Simonyan et al., 2016). This suggests
20 that the ability of Bax to be retrotranslocated might depend on its phosphorylation, namely on S184.
21 In mammalian cells, a correlation between the phosphorylation of S184 and the retrotranslocation of
22 Bax by Bcl-xL had also been observed (Schellenberg et al., 2013): indeed, a mutant GFP-Bax-
23 S184V was retrotranslocated slower than GFP-BaxWT, paralleling the observations in yeast
24 discussed above. Also, the overexpression of AKT stimulated GFP-BaxWT retrotranslocation.
25 However, it was not mentioned whether the overexpression of AKT had or not an effect on the
26 retrotranslocation of GFP-Bax-S184V, and it cannot be yet ascertained that this effect of AKT and
27 the consequence of the phosphorylation of S184 on Bax retrotranslocation are strictly correlated. It
28 should be noted that, when Bax and AKT were co-expressed in yeast, not all the effects of AKT
29 were abolished by the substitution S184V, suggesting that part of the effect of AKT may occur
30 through the phosphorylation of an additional residue besides S184 (Simonyan et al., 2016).
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54 **7-Bcl-xL v/s Bcl-2**

55 Before Bcl-xL, the overexpression of Bcl-2 had been shown to increase the mitochondrial
56 localization of Bax in non-apoptotic FL5.12 cells (Tejido and Dejean, 2010). However, in spite of
57 this apparent similarity, the consequences of Bcl-xL or Bcl-2 overexpression on Bax conformation
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1 were clearly distinct. Both proteins induced the conformational change of the N-terminus, leading
2 to a recognition by the 6A7 antibody, and reveal the association of Bax to the MOM. But, while the
3 overexpression of Bcl-2 also favored the formation of Bax oligomers (Teijido et al., 2010), the
4 overexpression of Bcl-xL did not (Renault et al., 2013, 2015). It is yet difficult to draw any
5 definitive conclusion about the consequences of this difference. Indeed, under both conditions, Bax
6 remains inactive, being inhibited by its anti-apoptotic partner, and no MOMP was observed. But
7 these observations emphasize the view that the overexpressions of Bcl-2 and Bcl-xL are not
8 identical, considering their respective effects on Bax. This might be a crucial consideration for
9 attempting to re-activate apoptosis in Bcl-2- or Bcl-xL-overexpressing tumoral cells.

10 Recent structural data brought major advances in the knowledge of Bax organization in the outer
11 mitochondrial membrane. The interaction of Bax Δ C (deprived of its C-terminal hydrophobic α -
12 helix) with the BH3 domain of Bid favors the formation of a symmetrical Bax dimer, in which
13 helices α 5 and α 6 would first lay flat on the membrane instead of being inserted as a hairpin, and
14 then would arrange a large pore by tilting in the membrane (Czabotar et al., 2014; Westphal et al.,
15 2014). The formation of the pore as a juxtaposition of dimers has been proposed as an alternative to
16 the membrane-inserted α -helices (Bleicken et al., 2014; Subburaj et al., 2015). This model is
17 compatible with recent microscopy images showing the ring-like organization of Bax in
18 mitochondria of apoptotic cells (Salvador-Gallego et al., 2016; Große et al., 2016). It is also
19 compatible with electrophysiological data suggesting that the pore formed by Bax have different
20 incremental sizes (Martinez-Caballero et al., 2009).

21 In non-apoptotic FL5.12 cells overexpressing Bcl-2, the presence of oligomers similar in size to
22 those observed in apoptotic cells have been observed (Renault et al., 2013), while no oligomer was
23 detected in the same cells overexpressing Bcl-xL (Renault et al., 2015). By correlating these
24 observations to the structural model of Bax oligomers, one could speculate that Bcl-2 would favor
25 the formation of Bax oligomers that remain lain on the membrane, while Bcl-xL prevents the
26 formation of oligomers, maybe by stabilizing Bax dimers. In any case, the rupture of the interaction
27 between Bax and its anti-apoptotic partner would be sufficient to promote the organization of the
28 pore-forming oligomer.

29 ***8-Concluding remarks***

30 The intrinsic pathway of apoptosis, that involves mitochondria, is triggered by signals originating
31 within the cell, such as, typically, p53 expression. Nevertheless, most Bcl-2 family members are
32 expressed at a basal level, both in tissues and in cultured cells. The presence of Bcl-2 family
33 members, both in proliferating and post-mitotic cells, might not only reflect the requirement for a
34 rapid response to apoptotic stimuli, but also non-apoptotic functions of these proteins. Namely, it
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1 has been shown that both Bcl-2 and Bcl-xL could regulate autophagy (Pattingre et al., 2005; Maiuri
2 et al., 2007; Priault et al., 2010; Beaumatin et al., 2016). Interestingly, the effects of both proteins on
3 autophagy are distinct, paralleling their distinct effect on Bax-regulation discussed above. It has
4 also been shown that Bax could regulate mitochondrial dynamics (Karbowski et al., 2006; Cleland
5 et al., 2011). In this short survey, we have shown that, even in non-apoptotic cells, the interactions
6 between pro- and anti-apoptotic Bcl-2 family members determine the way a cell is able to respond
7 to a potential apoptotic trigger, such as an anti-tumoral treatment. The fact that these properties are
8 conserved in yeast demonstrates that they are dependent on their intrinsic molecular features. As a
9 matter of fact, the heterologous expression in yeast continues to be an outstanding tool to study the
10 molecular mechanisms underlying the function of these proteins.
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18 ***Aknowledgements***

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Legends to Figures

Figure 1. Schematic representation of the classification of Bcl-2 family members and BH3-containing proteins.

TOP: Classical representation of the Bcl-2 family members, bases on their function (anti- v/s pro-apoptotic) and on their structure (multidomain v/s BH3-only).

BOTTOM: Phylogenetic classification based on the evolution of BH domains . True Bcl-2 homologs include multidomain proteins (anti- and pro-apoptotic) and Bid, that has a structure closely related to that of Bcl-xL (Chou et al., 1999). BH3-domains can be divided into canonical domains, that are evolutionary related to those of Bcl-2 homologs, and non-canonical domains, that might proceed from a convergent evolution (Aouacheria et al., 2013, 2015).

Figure 2. Mitochondrial proteins released during cell death in mammals and yeast.

TOP: In mammalian cells, Bax/Bak-dependent apoptosis is associated to the formation of a large pore in the MOM, that allows the release of cytochrome c. It remains however unclear if this pore also allows the release of other factors. Necrosis and late apoptosis are associated to the opening of mPTP, that induces a non-selective release of all the factors localized in the intermembrane space.

BOTTOM: In yeast, the heterologous expression of Bax is associated to the formation or the same pore as in mammals, supporting that this pore contains Bax (Pavlov et al., 2001). Other factors can be released following death signals specific to yeast, such as acetic acid treatments (Pereira et al., 2010). However, Bax effects in yeast do not depend on these factors (Kissova et al., 2006).

Figure 3. Measurement of Bax-driven release of cytochrome c from yeast mitochondria by redox spectrophotometry and immunoblotting.

Mitochondria were isolated from yeast cells expressing wild-type Bax (BaxWT, poorly active) or a constitutively mitochondrial and active mutant Bax-P168A (Arokium et al., 2004).

LEFT: Redox spectrophotometry measurements. A mitochondrial suspension (3-5mg protein/mL) was placed in the cuvettes of a double-beam spectrophotometer. The reference cuvette was oxidized with potassium ferricyanide and the sample cuvette was reduced with sodium dithionite. The difference spectra were acquired between 500 and 650nm. Cytochrome c+c1 and cytochrome b contents were calculated from the height of the peaks, as indicated, and manually corrected with the difference of the isobestic points at 540nm and 575nm. A molar extinction coefficient of 18,000 M⁻¹. cm⁻¹ for both types of cytochromes was used, and cytochrome c was calculated by considering that the stoichiometry cytochrome b/cytochrome c1 is 2.

RIGHT: Immunoblotting measurements. 100μg of mitochondrial proteins or of post-mitochondrial

1 supernatants were blotted, and immunodetected with a custom antibody against yeast cytochrome c.
2 Antibodies against Phosphoglycerate Kinase (Pgk) and subunit 2 of respiratory complex IV (Cox2)
3 were used as loading controls. The immunoblotting against Bax shows that the mutant Bax-P168A
4 is more localized in mitochondria than BaxWT (Renault et al., 2015).
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8 **Figure 4. Comparison of the effects of full-length Bcl-xL and truncated Bcl-xL Δ C on Bax**
9 **mitochondrial localization.**

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11 LEFT: Bax is under a dynamic equilibrium between mitochondria and cytosol, owing to the
12 processes of Bcl-xL-driven translocation and retrotranslocation. Mitochondrial Bax is primed but
13 remains inactive because of the interaction with Bcl-xL. The addition of a BH3-mimetic (or the
14 expression of a BH3-only partner) enables the derepression of the interaction and the full activation
15 of Bax.
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20 RIGHT: Bcl-xL Δ C stimulates the translocation but not the retrotranslocation, leading to a higher
21 mitochondrial Bax content. Furthermore, the interaction between Bax and Bcl-xL Δ C is weak, and
22 Bax can be fully activated without the need of a BH3-mimetic.
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