EXPLORING THE PRO-APOPTOTIC FUNCTION OF BIM

EXPLORING THE PRO-APOPTOTIC FUNCTION OF BIM

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Lay Abstract

Apoptosis is a type of programmed cell death which plays a fundamental role in maintaining homeostasis in multi-cellular organisms. The Bcl-2 family has been identified as the central players in regulation of apoptosis. It consists of anti-apoptotic proteins (e.g Bcl-XL) and pro-apoptotic proteins which are further classified as BH3 proteins (e.g Bim, Bid) and effector proteins (e.g Bax, Bak). Here we showed that Bim is constitutively active *in vitro* and preferably activates Bax, distinguishing itself from Bid which preferably activate Bak. We showed that the C-terminus membrane binding domain (MBD) of Bim plays a crucial role in reguating binding and activation of Bax, as well as providing extra binding support to Bcl-XL, independent of its membrane binding feature.

Abstract

Apoptosis is a type of programmed cell death which plays a fundamental role in maintaining homeostasis in multi-cellular organisms. The Bcl-2 family has been identified as the central players in regulation of apoptosis. It consists of anti-apoptotic proteins (e.g Bcl-XL) and pro-apoptotic proteins which are further classified as BH3 proteins (e.g Bim, Bid) and effector proteins (e.g Bax, Bak). BH3-proteins regulate apoptosis by activating the pro-apoptotic proteins Bax and Bak to permeabilize mitochondria, and/or by inhibiting anti-apoptotic proteins such as Bcl-XL and Bcl-2.

In this study, we employed fluorescence spectroscopy and functional assays with full-length Bim and showed that major Bim isoforms have similar activities *in vitro*. Bim preferably activates Bax over Bak while Bid preferably activates Bak. Bim displayed a unique binding to Bcl-XL so that the Bim-Bcl-XL complex is resistant to BH3-mimic drug ABT-263 treatment while Bid does not. A Bcl-XL enhancer BH3 TCTP was also shown to interact with Bim-Bcl-XL and Bid-Bcl-XL complex differently, where a single mutation abolished its enhancement of Bid-Bcl-XL but not Bim-Bcl-XL.

Closer investigation of the dual apoptotic functions of the BH3-protein Bim revealed that the C-terminal membrane binding domain (MBD) is unexpectedly also involved both in binding of Bim to Bax in solution and in activating Bax. Multiple mutations in this domain reduced or abolished binding to membranes but did not affect binding to Bax or correlate with Bax activation. Deletion eliminated binding to and activation of Bax, but not binding to or inhibition of Bcl-XL. Thus MBD mediates binding to both membranes and Bax separately. On the other hand, although the MBD is not the determining factor for interactions with Bcl-XL, our data demonstrates Bim MBD also plays a major role in binding to Bcl-XL. The C-terminal MBD was shown to be contributing to the ABT-263 resistance of Bim-Bcl-XL complex by directly interacting with Bcl-XL. We discovered additional interactions between the MBD of Bim and both Bcl-XL and Bcl-2. Our data suggested a novel topology and mechanism for the Bim-MBD that positions the central hydrophobic residues of the MBD appropriately for binding to Bcl-XL.

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Introduction

CHAPTER I: Regulating cell death at, on, and in membranes.

Preface

The work presented in this chapter has been previously published as a review in:

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Contribution of authors:

X.C. wrote the entire review and prepared all the figures and tables, with the exception that K.J. prepared section 4. LB and DWA edited the review and directed the layout of the sections.

Objective of the review:

To explain how the Bcl-2 family regulates apoptosis and discuss how membrane binding affects the structure and function of each of the three categories of Bcl-2 proteins (pro-apoptotic, pore-forming, and anti-apoptotic). The recognition of the potential important role of Bim membrane binding to its function inspired my study on the membrane binding domain (MBD) of Bim in the following chapters.

Highlights:

- The Bcl-2 family of proteins plays a critical role in apoptosis by regulating Mitochondria Outer Membrane Permeabilization (MOMP) via a series of protein–protein and protein–membrane interactions.
- Members in the Bcl-2 family are divided into three classes:
  - The pore-forming multi-BH domain proteins (e.g. Bax, Bak) permeablize the membrane to cause MOMP.
  - The BH3 proteins either directly activate Bax/Bak (e.g. tBid, Bim) or indirectly promote the process by binding to anti-apoptotic proteins.
  - Anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL and Mcl-1) bind and inhibit BH3 proteins or Bax/Bak via mutual sequestration.
- Bcl-2 family members were demonstrated to adopt a different conformation after interaction with membranes. Most of these interactions were observed to be saturable and reversible.
- Targeting of BH3 proteins to membranes, specifically Bim, is considered as a crucial step in regulating apoptosis.

1. Introduction: Mitochondria, Endoplasmic Reticulum and Apoptosis

The homeostasis of multi-cellular organisms is maintained by highly regulated mechanisms that ensure cells multiply in the correct context and are removed by programmed cell death when they pose a threat to the organism or are no longer needed. Apoptosis is a common type of programmed cell death which plays a fundamental role in eradicating old, excess, or dysfunctional cells (Kerr et al, 1972). Dysregulated apoptosis is at the heart of the pathophysiology of a wide variety of diseases: insufficient apoptosis leads to cancer and autoimmunity while hyperactive apoptosis is associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's (Ramírez Chamond R., 1999).

Apoptosis can be triggered from signals both extrinsic and intrinsic to the cell (Kerr et al, 1972). The extrinsic pathway is activated by extracellular death ligands, like tumour necrosis factor-α (TNF-α) binding their cognate death receptors; the intrinsic pathway is initiated by a wide variety of intracellular stressors (DNA damage, premature mitotic arrest, the unfolded protein response, etc.) that ultimately lead to the mechanical permeablization of the Mitochondrial Outer Membrane (MOM) (Chang et al, 1998; Chen & Goeddel, 2002; Ferraro & Cecconi, 2007; Merksamer & Papa, 2010; Meyn, 1997). Disruption of the MOM results in the release of apoptogenic factors such as cytochrome *c* and SMAC from the inter-membrane space (IMS) to the cytosol (Tait & Green, 2010; Wang, 2001), which in turn activates the downstream "executioner" caspases that proteolytically cleave a wide variety of intracellular substrates thereby disrupting

cellular physiology. Cleavage of a subset of these caspase targets in a co-ordinated fashion produces the phenotypic changes characteristic of an apoptotic cell (Chipuk et al, 2006).

The Bcl-2 family of proteins regulate both intrinsic and extrinsic pathways but are most intimately involved with the intrinsic pathway where they regulate Mitochondria Outer Membrane Permeablization (MOMP) via a series of protein-protein and protein-membrane interactions (Leber et al, 2007; Leber et al, 2010b). Members in the Bcl-2 family are defined by the presence of one or more of the four conserved motifs known as Bcl-2 homology (BH) regions (termed BH1-4) (Chittenden et al, 1995; Yin et al, 1994). The function of individual family members is largely determined by the specific combination of BH regions. The anti-apoptotic proteins (e.g. Bcl-2, Bcl-w, Bcl-XL, Mcl-1 and A1) contain all four BH regions whereas the pro-apoptotic proteins are comprised of the pore-forming multi-BH domain proteins (e.g. Bax, Bak, Bok) that contain BH regions 1-3 and a modified version of region 4 and the BH3 proteins (e.g. Bid, Bim, Bad, Bmf, Bik, Puma, Noxa, Hrk, Blk, Nip3, bNip3, Mule etc.) that are structurally distant from each other, sharing only the BH3 region (Youle & Strasser, 2008). During intrinsic apoptosis, BH3 proteins cause Bax/Bak to oligomerize within and permeabilize the MOM, whereas the anti-apoptotic family members inhibit this process at multiple steps (Gavathiotis et al, 2010b; Wei et al, 2001).

Aside from the well-founded focus on mitochondrial dysfunction in apoptosis, other intracellular organelles are also involved with regulating cell death. The

endoplasmic reticulum (ER) is a large and continuous membranous network that extends throughout the cytoplasm that functions primarily in protein production and transportation (Lavoie & Paiement, 2008), calcium (Ca^{2+}) homeostasis, lipid and membrane biosynthesis and serves as both donor membrane and the initiating locus for autophagy. The disruption of ER function has been implicated in the pathophysiology of many cell-death related diseases (Kim et al, 2006; Martinez et al, 2010; Minamino & Kitakaze, 2010; Szegezdi et al, 2006). The ER is both functionally and physically linked to mitochondria with implications for mitochondrial apoptosis(Kornmann, 2013). The existence of ER Mitochondria-Associated Membranes (MAMs) was first detected by electron microscopy in 1960s (Robertson, 1960) and velocity sedimentation experiments where ER components co-purified with mitochondria (Vance, 1990). Furthermore MAM may be involved in regulating mitochondrial dynamics such as fission and fusion (Lackner, 2013; Vannuvel et al, 2013).

There are also distinct signals that regulate cell survival that uniquely arise from the ER. For example, ER stress will trigger a signalling pathway called Unfolded Protein Response (UPR) which, aside from its primary function of slowing down protein translation and enhancing protein folding, can also promote cell death via both mitochondria-dependent and -independent pathways (Hollien, 2013; Kim et al, 2006; Martinez et al, 2010; Szegezdi et al, 2006; Vannuvel et al, 2013). Several Bcl-2 family proteins are located at the ER membrane where they regulate cell death and autophagy such as Bcl-2, Bik, and Beclin-1 (Germain et al,

2002; Lithgow et al, 1994; Sinha & Levine, 2008). The death signals transmitted from the ER to mitochondria include both transcriptional up-regulation, and post-translational modification and activation of BH3 family members such as Bim, Puma and Noxa (Ghosh et al, 2012; Puthalakath et al, 2007a; Verfaillie et al, 2013) as well as regulation of Ca^{2+} efflux via Bap31 and inositol-3-phosphate receptors (Wang et al, 2011). Calcium efflux from the ER to the cytosol can result in an accumulation of Ca^{2+} in the mitochondrial matrix which triggers the opening of the mitochondrial Permeablization Transition Pore (PTP) resulting in the permeablization of the MOM and subsequent cell death (Berridge, 2002; Denton et al, 1988; Wang et al, 2011).

Given the important roles mitochondria and ER play in cell survival and that Bcl-2 family proteins are targeted to these membranes, we will review the development of explanatory models for how these proteins regulate apoptosis emphasizing recent findings about how membrane binding modulates the structure and function of these proteins, and in turn how the Bcl-2 family regulates the dynamics and the integrity of these organelles.

2. Models of the mechanism of action of the Bcl-2 family

2.1 Initial Models: Rheostat, Direct Activation and De-repression

As the number of Bcl-2 family members increased beyond the original founding member, Bcl-2, the models explaining the functional significance of binding interactions increased as well (Figure 1). The discovery of the second Bcl-2

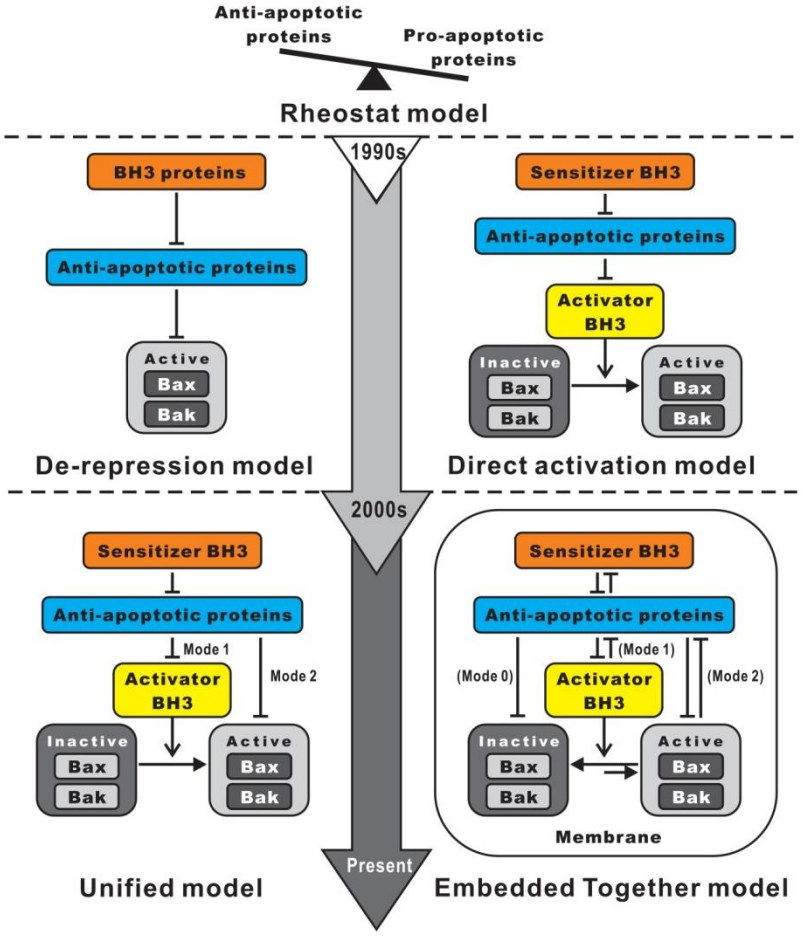

Figure1: Models illustrating mechanisms for the regulation of MOMP by Bcl-2 proteins

The earliest “Rheostat” model proposes that cell fate is determined by the ratio between pro- and anti-apoptotic proteins. Later models such as the De-repression and Direct Activation models hold different views on the role of BH3 proteins and anti-apoptotic proteins. The Embedded Together model and the newly proposed Unified model both agree that Bax/Bak need to be activated by BH3 proteins and that anti-apoptotic proteins inhibit MOMP through multiple interaction partners. The Unified model differs from the Embedded Together model chiefly by formalizing the relationship between mitochondrial dynamics and apoptotic regulation. However, the Embedded Together model distinguishes itself from other models by recognizing that: 1) The membrane is an active participant in the interactions between Bcl-2 family proteins. 2) Binding of anti-apoptotic proteins to BH3 or activated Bax/Bak leads to mutual sequestration. 3) MOMP is controlled by multiple reversible equilibria.

family protein, Bax, in 1993, generated the first “Rheostat” model of Bcl-2 regulation that proposed that the numeric ratio between pro- and anti-apoptotic proteins determines cell fate (Oltvai et al, 1993; Yang et al, 1995). Thus excess Bax compared to Bcl-2 would elicit apoptosis via activation of caspases, but how this occurred was not known (Oltvai et al, 1993; Yang et al, 1995). The subsequent identification of other pro-apoptotic family members, the BH3 proteins, that (with the exception of Bid (Kvansakul et al, 2008)) were structurally different from Bax by containing only the BH3 region elicited two competing models (De-repression and Direct Activation) that addressed the question of how Bax is activated, and how Bcl-2 prevents this.

The De-repression model postulates that Bax (and its closely related “cousin” Bak) are constitutively active and therefore need continuous binding to anti-apoptotic proteins to repress their activity. In this case BH3 proteins serve solely to displace Bax/Bak from anti-apoptotic proteins (Chen et al, 2005). An important facet of this model is that the combinatorial specificity of BH3 proteins for other anti-apoptotic proteins discovered after the identification of Bcl-2 allows for the fine-tuning of cell death regulation (Chen et al, 2005). For example Bid, Bim and Puma interact with all of the anti-apoptotic members; Bad only binds to Bcl-2, Bcl-XL, and Bcl-w; and NOXA specifically engages Mcl-1 and A1 (Chen et al, 2005). Thus the model proposes that different sets of BH3 proteins, activated by specific stress signals, will neutralize the corresponding subsets of anti-apoptotic proteins to liberate the Bax/Bak.

In contrast to the De-repression model, the Direct Activation model states that Bax/Bak need to be activated to oligomerize and form pores in the MOM. The BH3 proteins perform this function in two distinct ways which divides them into functional sub-groups (Letai et al, 2002; Terradillos et al, 2002). First, Bax/Bak can be activated by direct binding of “activator” BH3 proteins such as tBid (the truncated and active form of Bid), Bim and Puma (Gallenne et al, 2009; Letai et al, 2002; Schulman et al, 2013). Anti-apoptotic proteins such as Bcl-XL sequester the activators from Bax to prevent MOMP and cell death. This creates a scenario whereby “sensitizer” BH3 proteins such as Bad and Noxa displace BH3 activators from anti-apoptotic proteins, and thus activate Bax/Bak indirectly.

Therefore the De-repression and Direct Activation models propose different binding partners and functions for both the BH3 and anti-apoptotic family members. Both models cite evidence from a variety of assays to support their unique features, including co-immunoprecipitation assays in transfected cells, binding of peptides to truncated proteins in vitro, and gene knockout experiments in mice. However, certain inconsistencies persist: the De-repression model does not explain that in all cells, particularly growing ones, only a small fraction of Bax/Bak can be co-immunoprecipitated with anti-apoptotic proteins, in contrast to the claim that all Bax/Bak needs to be neutralized for survival (Hsu & Youle, 1997; Sedlak et al, 1995; Vogel et al, 2012). The direct-activation model needed to be modified to acknowledge that anti-apoptotic proteins can also directly inhibit the auto-activation of Bax/Bak (Ruffolo & Shore, 2003; Tan et al, 2006). Both models

propose that the interactions between the proteins are unidirectional rather than reversible equilibria.

2.2 The active role of membranes in apoptosis regulation and the Embedded Together model

To reconcile these differences concerning the proposed functions and binding partners of Bcl-2 family members an active role of membranes needs to be considered. This was not directly included in earlier models partly due to certain experimental limitations. For example, assays such as co-immunoprecipitations and immunoblotting from cell lysates require detergents to solubilize membranes. The detergents used in these experiments can affect the binding interactions between Bcl-2 family proteins: Triton X-100 artifactually promotes interactions between Bcl-2 family proteins (such as Bax and Bcl-2 or Bcl-XL), while CHAPS disrupts interactions between Bcl-2 members (Lovell et al, 2008). Furthermore, in vitro experiments, that do not require detergents, have classically used deletion mutants or peptides of Bcl-2 family members lacking the putative trans-membrane (TM) region due to difficulties in purifying the full length proteins (Chen et al, 2005). In this circumstance, any effect of membrane binding on protein-protein interactions that might occur in vivo would be lost.

More recent evidence indicates that this is an important concern for Bcl-2 family members in all three functional groups. For example, Bax undergoes a series of conformation changes during activation when it transforms from a cytoplasmic or

loosely membrane bound protein to a protein with helices 5, 6 & 9 integrated in the membrane before it oligomerizes with other Bax monomers to form a pore in the MOM, an event not observed with solution based assays (Annis et al, 2005). Targeting of tBid to the membrane also causes a conformation change that allows it to both activate Bax (Shamas-Din et al, 2013a), and facilitate the membrane insertion of cytoplasmic Bcl-XL (Garcia-Saez et al, 2009). These tBid membrane interactions are sensitive to membrane lipid composition (Lutter et al, 2000) and catalyzed by the non-Bcl-2 family protein Mtch2 (Shamas-Din et al, 2013a; Zaltsman et al, 2010). Furthermore recent reports suggest other novel roles for non-Bcl-2 family mitochondrial membrane proteins and lipids in the regulation of MOMP. For example, a GTPase of the dynamin superfamily named Drp1 was found to enhance the pore forming activity of Bax (Montessuit et al, 2010). It has also been reported that tBid-Bax interaction on the membrane induces redistribution of membrane lipids (Terrones et al, 2004).

Based on data obtained using recombinant full-length proteins and in vitro model systems free of detergents to assay Bcl-2 family protein-membrane binding and membrane permeabilization, the “Embedded Together” model was proposed to explain the regulation of MOMP in apoptosis (Leber et al, 2007; Leber et al, 2010b; Shamas-Din et al, 2011). In addition to the interactions of some family members seen in solution (i.e. in the cytoplasm of cells), Bcl-2 family members were proposed and later demonstrated to adopt a different conformation after interaction with membranes. Most of these interactions were observed to be

saturable and reversible. Thus the Embedded Together model incorporates protein-membrane and protein-protein interactions of Bcl-2 family proteins that are tightly regulated by multiple parallel equilibria. A focal point of the model describing the irreversible step in apoptosis, posits that the activation of Bax/Bak at the membrane is the rate-limiting step in MOMP, in part because it involves distinct stepwise but reversible conformational changes leading to formation of the oligomeric pore. Most activator BH3 proteins such as Bim, tBid and Puma can bind to membranes spontaneously where they recruit Bax (as well as anti-apoptotic proteins) to the membrane, initiating a series of conformational changes of Bax on the membrane leading to oligomerization of Bax. In the case of Bak, which is constitutively bound to the membrane, the first conformation change that leads to tight binding to membranes is not required. The model also suggests the involvement of accessory proteins such as Mtch2 and posits roles for post-translational modifications of the proteins (11,12). Recent descriptions of the model made explicit that the molecular interactions are not one way. Thus binding of membrane bound anti-apoptotic proteins to activator BH3 proteins doesn't just lead to inhibition of the activator protein but, in this scenario the activator protein behaves like a sensitizer in that it inhibits the anti-apoptotic protein. This kind of inhibition was called mutual sequestration (Shamas-Din et al, 2013b). Furthermore, the anti-apoptotic proteins also bind to activated Bax/Bak on the membrane with high affinity, leading to mutual sequestration of this pair.

Sensitizer BH3 proteins such as Bad and Noxa have little binding affinity for Bax/Bak but can engage anti-apoptotic proteins with combinatorial specificity (e.g. Bad binding to Bcl-XL and Bcl-2, Noxa binding to Mcl-1, etc.), a scenario previously included in the De-repression model (Chen et al, 2005) but here complex formation inhibits both proteins. For example, when the sensitizer BH3 protein Bad recruits Bcl-XL into membranes the function of both proteins is inhibited via mutual sequestration (Jeong et al, 2004). Competition for binding from sensitizer BH3 proteins can disrupt the sequestration complex formed by activator BH3 and anti-apoptotic proteins, as well as displace activated Bax/Bak from anti-apoptotic proteins, so that they are sensitizers for both mechanisms proposed by the De-repression and Direct Activation models (Leber et al, 2007; Leber et al, 2010b; Shamas-Din et al, 2011). The dual nature of the effect of these binding interactions makes the description of proteins as ‘activators’ or ‘sensitizers’ artificial and cumbersome.

A critical and previously under-appreciated feature of the Embedded Together model is that the target membrane plays an active role in mediating the interactions between Bcl-2 family proteins rather than being a “passive recipient” in the process. Thus MOMP is controlled by multiple reversible equilibria including protein-protein and protein-membrane interactions that initiate these conformational changes. For example, interaction between full length tBid and Bax is detected only when membranes are present (Lovell et al, 2008). Precisely measuring the rates and dissociation constants for these individual interactions

will be required to permit the prediction of the direction and extent of a specific interaction when multiple interacting partners are present, as would occur in vivo.

The Embedded Together model also diverges from its predecessors by stating that anti-apoptotic proteins can be 'activated' in the sense that anti-apoptotic proteins undergo conformational changes that enhance or change their function in response to being recruited to the membrane by pro-apoptotic family members. The regulation of function by mutual sequestration proposes that the difference between activator and inhibitor is quantitative rather than qualitative as the balance is determined by the local concentrations of anti-apoptotic proteins and their binding partners. In support of this, Bcl-2 inserts helices 5 and 6 into membranes upon direct binding of BH3-activators, a conformation change critical for Bcl-2 mediated inhibition of Bax oligomerization (Dlugosz et al, 2006; Peng et al, 2006). This conformational change thus activates Bcl-2 even though in the absence of other changes the net effect of binding is inhibition of both the BH3-activator and Bcl-2 due to mutual sequestration. However, because binding is reversible and governed by an equilibrium binding constant the model predicts that additional interactions can release the activated Bcl-2 from the BH3-activator.

Finally the equilibrium binding underpinning the model suggests that another molecular mechanism by which membrane-bound anti-apoptotic proteins inhibit Bax/Bak is by functioning as a dominant negative Bax/Bak by capping the activated Bax/Bak monomer/oligomer and preventing it from further oligomerization. In keeping with the theme elaborated above, this is a form of

mutual sequestration that arises because the anti-apoptotic proteins bind to one of the oligomerization surfaces of Bax/Bak but the anti-apoptotic proteins cannot further oligomerize. This function for Bcl-XL was demonstrated by the observation that Bcl-XL inhibited Bax-activation induced by tBid mt1 (a tBid mutant that no longer binds to Bcl-XL) in liposomes and mitochondria (Billen et al, 2008) and in live cells using FLIP (fluorescence loss in photobleaching) and FRAP (fluorescence recovery after photobleaching) (Edlich et al, 2011). Recently a new pro-survival mechanism was found for Bcl-XL in which it retro-translocates peripherally membrane-bound Bax to the cytosol (Billen et al, 2008; Edlich et al, 2011; Todt et al, 2013). This novel mechanism named MODE 0 will be discussed in detail in the following section.

2.3 Unified model

Another model has recently been proposed to account for how the anti-apoptotic proteins function by inhibiting both BH3 activators and Bax/Bak. This model also relates these two different modes of inhibition to the role of mitochondrial dynamics in apoptosis regulation (Llambi et al, 2011). In this model, sequestration of activators by the anti-apoptotic proteins is defined as MODE 1 and the inhibition of Bax/Bak by anti-apoptotic proteins is called MODE 2. While inhibition through either mode prevents MOMP, MODE 2 is considered more potent. In addition MODE 2 prevents mitochondrial fusion and promotes mitochondrial fragmentation.

By introducing the terms MODE 1 and MODE 2 the unified model more explicitly states the two ways anti-apoptotic proteins can inhibit apoptosis previously incorporated in the Embedded Together model. In this model, MODE 1 is equivalent to the inhibition of BH3 proteins and anti-apoptotic proteins by mutual sequestration, while MODE 2 reflects the dominant negative function of the anti-apoptotic proteins as inhibitors of Bax/Bak. The Embedded Together model predicts that both events can occur simultaneously and are controlled by both the relative abundance and the relative binding affinities of the Bcl-2 family members expressed in that particular cell. Thus unlike the Unified model, the Embedded Together model proposes that the dominance of MODE 1 vs MODE 2 depends on the extent to which the proteins are bound to membranes and the predominance of one mode over the other can change dynamically depending on both the amount of protein and post-translational modifications of the Bcl-2 proteins, such as phosphorylation, that affect binding to other family members and the membrane. The complexity of the inter-relationship of all of these events contributes to apoptosis occurring in a cell autonomous manner.

3. Importance of membranes: changes in conformation of Bcl-2 family proteins during apoptosis

A critical feature of the Embedded Together model is that for many Bcl-2 family members, membrane binding induces conformational changes that modify function (Leber et al, 2007; Leber et al, 2010b; Shamas-Din et al, 2011). Even for those family members like Bcl-2 and Bak which are constitutively integrated in the

membrane, interacting with other membrane-bound binding partners causes distinct conformations that have functional consequences (Dlugosz et al, 2006; Leshchiner et al, 2013). In this section we will review the details of these processes.

3.1 The role of specific membrane components in MOMP regulation

An initial hint of the importance of membranes in Bcl-2 family regulation came from reports of the importance of cardiolipin in regulating tBid-Bax mediated liposome permeabilization (Schlame et al, 2000). Cardiolipin (CL) is a mitochondria-specific membrane lipid synthesized within and localized predominantly to the inner mitochondrial membrane. However, a minor fraction of CL is located in the MOM (Schlame et al, 2000). This small pool of surface-exposed CL appears to impact the pro-apoptotic function of Bid and Bax (Kuwana et al, 2002; Terrones et al, 2004) as removing CL from MOM-like liposomes abrogated pore formation by Bid and Bax in vitro (Terrones et al, 2004). In addition, CL binds to a truncated Bak mutant in liposomes and increases its sensitivity to Bid activation and subsequent membrane permeabilization (Landeta et al, 2011). Using high enough concentrations (μM range) of Bid BH3 peptides can also induce Bax pore formation in nanodisc lipid bilayers lacking CL (Xu et al, 2013). The elimination of CL in yeast expressing human Bax also has no effect on Bax insertion into the MOM or induction of MOMP (Iverson et al, 2004). These reports indicate that CL is not absolutely

required for Bax-dependent MOM permeablization, but does not leave out the possibility of CL enhancing the activity or membrane binding of BH3 activators. It has been reported that CL promotes Bid translocation to liposomes, MOM and mitochondrial contact sites (Garcia-Saez et al, 2009; Lutter et al, 2000; Lutter et al, 2001). CL also promotes mitochondrial localization of caspase 8, providing a platform in cells for activation of Bid via cleavage by caspase 8 (Gonzalvez et al, 2008; Jalmar et al, 2013). The BH3 sensitizer protein Bad also contains a lipid binding region that favours negatively charged lipids like cardiolipin (Hekman et al, 2006). Whether or not membrane binding by the anti-apoptotic proteins is affected by CL is currently unknown.

Cholesterol is the predominant component that controls the fluidity of membranes in mammalian cells. Although mitochondria only contain a restricted pool of cholesterol (3~5% of the total cellular cholesterol) (Montero et al, 2010), it plays a major role during apoptosis where it inhibits Bax-mediated MOMP by lowering membrane fluidity (Lucken-Ardjomande et al, 2008). Correspondingly, enhanced mitochondrial cholesterol in cancer cells increases resistance to chemotherapy and Bax mediated MOMP, whereas lowering the cholesterol content of the cells induces p53 dependent activation of Bax (Lee et al, 2010; Montero et al, 2008). The mechanism for this negative regulation of Bax by cholesterol is still poorly understood, although some evidence suggests high concentrations of cholesterol hinders the insertion of Bax into the membrane (Christenson et al, 2008) or the activation of Bax in the membrane (Lucken-Ardjomande et al, 2008).

The link between the sphingolipid ceramide and programmed cell death was discovered in 1993 when cells treated with C2-ceramide, a cell permeable ceramide analog, underwent apoptosis (Obeid et al, 1993). Additionally, cell death by C2-ceramide was shown to be Bax dependent (von Haefen et al, 2002). Ceramide has been shown to act synergistically with Bax to promote MOMP (Ganesan et al, 2010). Thus it is well established that C2-ceramide can regulate apoptosis. However, cellular ceramide is primarily generated by sphingomyelin hydrolysis via sphingomyelinase (SMase) or by *de novo* synthesis via Ceramide synthase (CerS) (Zhang & Saghatelian, 2013). Increasing the amount of ceramide at the mitochondria by SMases or CerSs has been reported to promote Bax translocation to the MOM, subsequent MOMP and apoptosis, by a process that can be prevented by Bcl-2 or inhbitors of the aforementioned enzymes (Birbes et al, 2001; Chalfant et al, 2002; Chipuk et al, 2012; Dbaibo et al, 2001; Lee et al, 2011). Nevertheless the molecular mechanism by which ceramide promotes apoptosis is still a mystery. It has been demonstrated that ceramide can form channels large enough to release IMS proteins from mitochondria (Colombini, 2010; Elrick et al, 2006; Siskind et al, 2002) but it is not clear how this function could be regulated by Bax. Additionally a downstream metabolite of ceramide, hexadecenal, was shown to directly bind Bax and promote Bax activation and pore formation in large unilamellar vesicles, even when these vesicles lacked CL (Chipuk et al, 2012). Clearly the field would benefit from a

systematic examination of the quantitative effects of various lipids on the regulation of MOMP.

3.2 Bax/ Bak change conformation in membranes

Bax and Bak are the effectors of MOMP that insert into the MOM and induce membrane permeabilization allowing the release of contents from the IMS that activate downstream effectors of apoptosis, such as cytochrome *c* and SMAC (Kluck et al, 1997; Liu et al, 1996). Both Bax and Bak contain 9 alpha-helices. The crystal structures of Bax and Bak suggest that they share a similar structure with each other and with their anti-apoptotic counterparts (Muchmore et al, 1996; Suzuki et al, 2000; Wang et al, 2009). Helices 5 and 6 are in the center of the soluble version of the protein and are surrounded by amphipathic helices, that shield them from water and form a hydrophobic groove on the surface of Bax and Bak (Suzuki et al, 2000; Wang et al, 2009). Notably, a similar hydrophobic groove is present in anti-apoptotic proteins and is the binding site for the BH3 region of pro-apoptotic Bcl-2 family members (Czabotar et al, 2007; Liu et al, 2003; Petros et al, 2000; Sattler et al, 1997). Based on this observation, it is likely that the hydrophobic groove plays a similar role in Bax/Bak. Indeed, a Bid BH3 peptide has been shown to bind to this site in Bax and Bak by NMR/X-Ray Crystallography (Czabotar et al, 2013; Moldoveanu et al, 2013). Both Bax and Bak contain a carboxyl-terminal putative trans-membrane (TM) domain in helix 9 which targets the proteins to the membrane (Czabotar et al, 2013; Moldoveanu et

al, 2006; Schinzel et al, 2004). Soluble monomeric Bax, which is not active, is stable in the cytoplasm because the helix 9 TM domain binds in *cis* to the hydrophobic groove (Hsu et al, 1997b; Wolter et al, 1997); activation of Bax by binding to a BH3 protein causes a conformational change that displaces helix 9 such that it then inserts into membranes (Annis et al, 2005).

In the Embedded Together model, displacement of helix 9 from the hydrophobic groove is one of several distinct steps in Bax activation, but not the first (Figure 2) (Leber et al, 2007; Leber et al, 2010b; Shamas-Din et al, 2011). The initial step is transient binding of Bax to the membrane without insertion of the TM into membranes; as with all the other steps, the cytoplasmic and peripherally-membrane-attached forms of Bax are in equilibrium. In growing cells, without active BH3 proteins present, the equilibrium is shifted toward the cytoplasmic form but some Bax is peripherally attached to the mitochondria membrane even in the absence of apoptotic stimuli (Desagher et al, 1999; Edlich et al, 2011; Gilmore et al, 2000; Valentijn et al, 2008). Significantly, peripheral binding of Bax to liposomes leads to the exposure of an amino-terminal epitope recognized by the monoclonal antibody 6A7, which suggests a structural change that possibly facilitates Bax binding to membrane-bound BH3 proteins that leads to further conformational changes in Bax (Yethon et al, 2003). Studies using FLIP and FRAP reveal that Bax cycles on and off mitochondria in growing cells spontaneously (Schellenberg et al, 2013) by a process that may be facilitated by an anti-apoptotic protein like Bcl-XL (Edlich et al, 2011; Todt et al, 2013). Thus

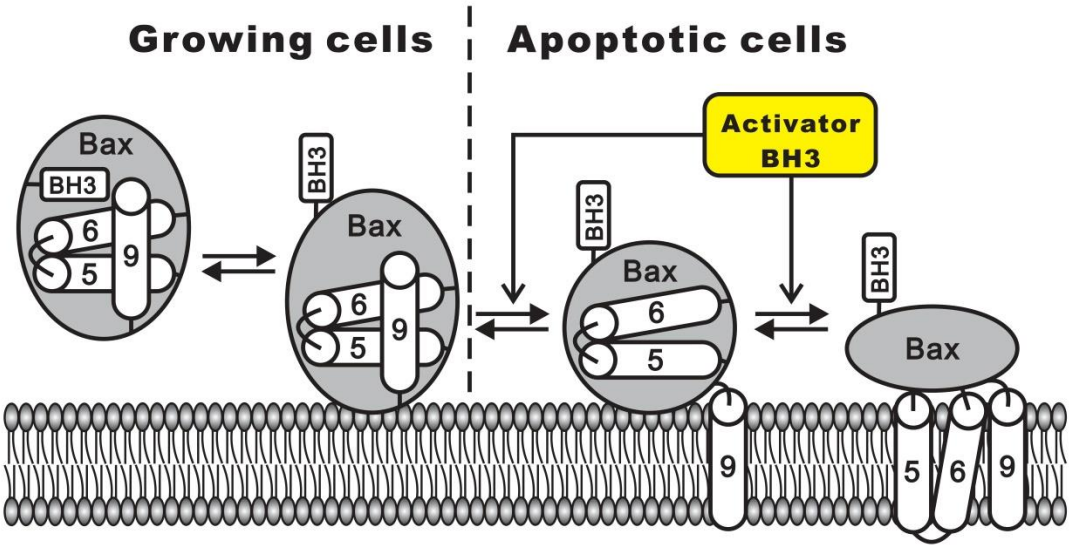

Figure2: Bax undergoes a step-wise activation mechanism that is tightly controlled by multiple equilibria

In growing cells, in the absence of apoptotic signals, cytoplasmic and peripheraly-bound Bax are in an equilibrium that greatly favours the cytoplasmic form. Upon activation by various physical factors and/or activator BH3 proteins, Bax undergoes further conformational changes involving unfolding of the protein and the insertion of helices 5,6 and 9 into the membrane. Here Bax inserts helix 9 before inserting helices 5 and 6 into the membrane.. This activated form of Bax then goes on to recruit more cytoplasmic Bax that oligomerizes and eventually permeabilizes the MOM.

the concentration of Bax at the membrane is kept low until an activator binds to Bax, triggering a conformational change that increases the affinity of Bax for the membrane shifting the equilibrium towards a membrane-bound state and effectively ‘capturing’ Bax within the membrane.

In contrast, Bak is constitutively inserted in the MOM via its helix 9 TM region, leaving the hydrophobic groove exposed, and bypassing the membrane targeting step(s) seen with Bax (Ferrer et al, 2012; Griffiths et al, 1999). The high affinity of Bak for membrane insertion may be due to the increased hydrophobicity of the Bak TM region compared to that of Bax, which favors binding to membranes rather than to the hydrophobic groove in *cis* (Ferrer et al, 2012). Consistent with this notion, mutations in the Bak TM that make its amino acid composition similar to Bax results in binding of the mutated helix 9 to the hydrophobic groove and, like Bax, shifts the Bak binding equilibrium to favor localization in the cytoplasm (Ferrer et al, 2012; Leshchiner et al, 2013).

Bax and Bak can also be activated by various physical factors such as changes in pH (Cartron et al, 2004) and heat (Pagliari et al, 2005) but in vitro these conditions are more extreme than found in cells. Exposure to MOM components like certain lipids that have been shown to activate Bax in vitro may be more physiologically relevant (Chipuk et al, 2012). However, the most studied mode of activation is through activator BH3 proteins binding Bax/Bak (Gavathiotis et al, 2008; Kim et al, 2009; Kuwana et al, 2002; Lovell et al, 2008). The interaction between BH3 proteins and Bax/Bak has been described as a "hit and run"

mechanism as the interaction is transient (Wei et al, 2000). After binding to activator BH3 proteins, Bax/Bak undergo major conformational changes resulting in a complete structural rearrangement of the proteins including the amino-terminal region, BH3 region, and helices 5,6 and 9 (the latter helix only in Bax) (Annis et al, 2005; Cartron et al, 2005; Kim et al, 2009). One or more of these conformation changes may reduce the affinity of Bax for BH3 proteins resulting in the observed ‘hit-and-run’ phenomenon. Recently a crystal structure of Bax (lacking helix 9) and BH3 peptides revealed that Bid BH3 and Bax BH3 bind to the canonical hydrophobic groove, resulting in a partial displacement of helix 2 (Czabotar et al, 2013). This moves the helix 2/3 side of the Bax groove away from the bound BH3 peptide and further "opens up" the groove, which might weaken the contact between BH3 peptide and Bax (Czabotar et al, 2013). This may be the structural basis of the transient nature of BH3 protein binding to Bax. The BH3 domain located in helix 2 exposed after activation may facilitate the homo-oligomerization of Bax/Bak by reducing the affinity for BH3 proteins and/or increasing the affinity for Bax/Bak and the anti-apoptotic proteins (Dewson et al, 2008; Kim et al, 2009; Zha et al, 1996a).

Bax spontaneously inserts into the membrane when helix 9 is displaced from the hydrophobic groove (Czabotar et al, 2013; Gavathiotis et al, 2008; Kim et al, 2009). However, displacement of helix 9 also releases helices 5 and 6 from the hydrophobic core and they become tightly associated with the membrane, possibly in a hairpin fashion. In support of this schema, IASD labeling assays

suggest that Bax inserts helices 5,6 and 9 and Bak inserts helix 5 and 6 into the into the MOM after activation (Annis et al, 2005; Oh et al, 2010). Insertion of the hairpin, comprised of helix 5 and 6, and the intervening sequence into the MOM has been implicated in the pore-forming function of Bax, based on structural similarity to bacterial toxins such as Colicin A and Diphtheria toxin (Muchmore et al, 1996; Wang & London, 2009). Another mechanism for dissociation of helix 5 and 6 from the Bax/Bak core was suggested by recent crystallography studies and termed “core/latch dissociation". In this process, the hairpin temporarily opens up, allowing helices 6-8 (the "latch") and helices 1-5 (the "core") to dissociate from each other (Czabotar et al, 2013). Cross-linking of the cysteines between helices 5 and 6 inhibits Bax pro-apoptotic function, consistent with the functional requirement for dissociation (Czabotar et al, 2013). By measuring these reactions simultaneously, it was revealed that at least for tBid and Bax they occur as an ordered series of events (Lovell et al, 2008), although to date all of these measurements have been based on Bax activation by tBid therefore, the process may vary depending on the activator.

By using stabilized (“stapled”) peptides from the BH3 region of the activator Bim a novel "rear pocket" interaction site in Bax was discovered. The rear pocket is located on the opposite side of Bax from the canonical BH3 hydrophobic groove and involves helices 1 and 6 (Gavathiotis et al, 2008). Binding of the Bim BH3 to this site results in displacement of the helix 1, 2 loop which may facilitate the dispatch of helix 9 from the hydrophobic groove, suggesting an additional

activation mechanism of Bax by BH3 proteins (Gavathiotis et al, 2010a). However it is still unclear whether these stapled peptides faithfully recapitulate binding of the BH3 domain in full-length proteins, and whether this dimeric interaction measured in a solution-based assay also occurs on membranes, as would happen in vivo. An equivalent 'rear pocket' binding site has not yet been identified in Bak. However in Bak the helix 9 TM is constitutively inserted in the membrane, which by exposing the canonical groove on an ongoing basis may render rear-pocket activation irrelevant for Bak.

3.3 Bax/Bak Pore formation on the MOM

Several models have been proposed describing the composition of the Bax/Bak pore (Figure 3). Early models suggested that Bax and Bak function by modifying existing proteinaceous channels. For example, Bax may increase the permeability of the MOM by regulating VDAC1 channels (Godlewski et al, 2002). However since Bax and Bak can activate, oligomerize and permeabilize MOM-like liposomes without any other membrane proteins, other models postulated that oligomerized Bax/Bak are the sole components of the pore. This has also been demonstrated in more physiologic membrane assays where Bax progressively forms a 5.5-6 nm sized pore composed of 9–12 monomers in mitochondria (Martinez-Caballero et al, 2009).

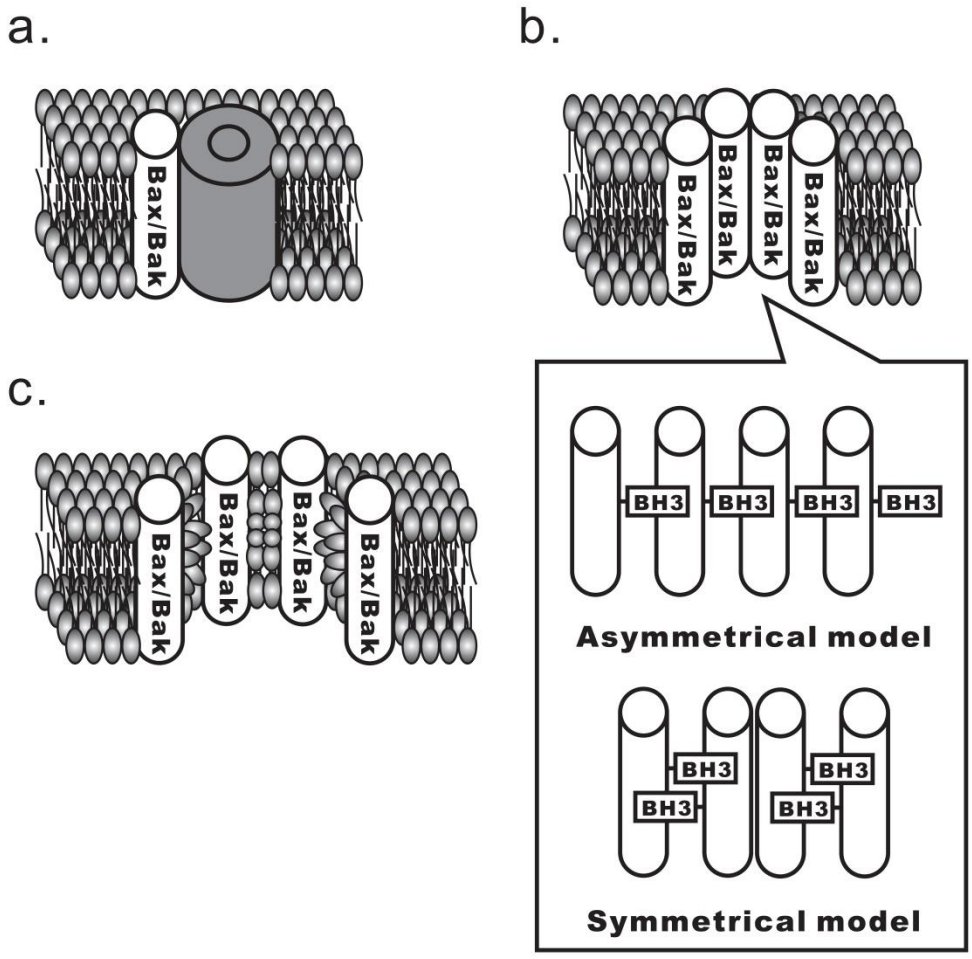

Figure3: Potential mechanisms of Bax/Bak mediated membrane permeabilization

a. Early models postulate that Bax/Bak regulate existing channels located within the MOM such as VDAC1. b. Other theories suggest that oligomerized Bax/Bak are the sole components of the pore. Two models, the symmetrical and asymmetrical models, propose potential explanations for the mechanism of Bax/Bak oligomerization. c. Recent evidence has suggested that lipids can regulate Bax/Bak pore formation, indicating the possible existence of a protein-lipidic pore.

How are these oligomers assembled? It is clear that the BH3 region in helix 2 of Bax/Bak is essential for homodimerization (Dewson et al, 2008; Kim et al, 2009; Zha et al, 1996a). Beyond this there are two interpretations for how this sequence (and potentially other binding sites) mediates oligomerization: The symmetrical model and the asymmetrical model. In the symmetrical model, Bax/Bak form dimers in which the BH3 regions bind reciprocally to the front hydrophobic groove of each other (Dewson et al, 2008). This resultant dimer can then bind to other dimers via the rear pocket allowing oligomers to grow in multiples of two. In support of this, a Bax "BH3-in-groove homodimer" crystal structure was obtained showing that a Bax BH3 binds to the front groove of BaxΔC (Czabotar et al, 2013). Recent work using the site-directed spin labeling method of electron paramagnetic resonance (EPR) spectroscopy and chemical cross-linking have indicated that Bak disengages helices 1 and 6 and dimerizes with helices 2-5. This unit is structurally homologous to the Bax "BH3-in-groove homodimer". This method also identified a novel -interface involving the carboxyl-termini of helices 3 and 5 (Aluvila et al, 2013; Czabotar et al, 2013). Extrapolation from the structure of the dimer indicated that Bak forms a lipidic pore with 4-6 homo-dimers aligned on the edge of the pore (Aluvila et al, 2013).

The asymmetrical model was proposed based on the discovery of the rear pocket in Bax; in this case, binding of a BH3 activator to the rear pocket is the initiator of a conformational change that permits the BH3 region of Bax to bind to the rear pocket of another Bax monomer, exposing the BH3 region of the latter,

propagating elongation of the oligomer (Reed, 2006). Consistent with this model, the NMR structure of a stapled Bax BH3 peptide/Bax complex showed that the BH3 peptide bound the rear pocket of Bax (Gavathiotis et al, 2010a). Recent modeling studies also propose that Bak oligomerizes to generate a pore-forming octamer in a similar manner, although a definitive rear pocket in Bak has not been discovered (Pang et al, 2012). Though these two mechanisms identify slightly different BH3 region interaction sites, both agree that the BH3 region and the hydrophobic pockets are important for Bax/Bak oligomerization.

Whatever the exact alignment of Bax/Bak monomers in the oligomer, recent evidence indicates that lipids regulate pore formation. For example, lipids with either positive or negative intrinsic curvature modify Bax-mediated liposome permeabilization (Basanez et al, 2002; Garcia-Saez et al, 2006; Terrones et al, 2004). Reciprocally, Bax binding at very low concentrations can destabilize lipid bilayers, suggesting the pore may contain both a protein and a lipid component (Basanez et al, 1999). A structural model has been proposed in which Bax/Bak cooperate with mitochondrial lipids to form a toroidal protein-lipidic pore in which the two leaflets of the bilayer fuse to each other (Terrones et al, 2004). Mechanistically this may be mediated by Bax/Bak rearrangement of lipids within the MOM that helps to stabilize the pore (Terrones et al, 2004). Kinetic studies have been interpreted to suggest that Bax oligomerization is not the rate-limiting step of MOMP, indicating the possibility of lipid involvement in pore formation (Kushnareva et al, 2012). Additionally, experiments using an isolated helix 5

peptide of Bax support the formation of toroidal protein-lipidic pore (Qian et al, 2008).

Indeed the two views about pore composition may be complementary rather than mutually exclusive as there may be transitions between a proteinaceous pore and a protein-lipidic pore which depend on the equilibria of binding between lipids and Bax/Bak monomers and oligomers. For example, membrane permeabilization may start with a small proteinaceous pore. As the oligomer grows in size, the curvature tension of the membrane increases causing the intrinsically curved lipids to be re-arranged and participate in enlargement of the pore.. The balance may be determined by local Bax/Bak concentration, and lipid composition and curvature. This model would be consistent with a Bax/Bak conformational change, which occurs on the membrane, being the rate-limiting step in MOMP (Lovell et al, 2008). The published data suggests that Bax/Bak activation and oligomerization are the crucial decision steps in the commitment to the execution phase of apoptosis, and that lipids not only regulate this critical step but contribute to Bax/Bak pore structure and stability.

Bok, the third mammalian pro-apoptotic multi-domain protein, has been less intensively investigated. Bok was found to be highly expressed in germ tissues and may be associated with placental pathologies (Hsu et al, 1997a; Ray et al, 2010), however it was also found to be expressed in a variety of cell types (Ke et al, 2012). Similar to Bax, it has been reported that Bok translocates from the cytoplasm to mitochondria during apoptosis (Gao et al, 2005) although it was

recently found that both overexpressed and endogenous Bok mainly localizes to the membranes of the ER and Golgi apparatus (Echeverry et al, 2013). Bok displays a high sequence homology to Bax/Bak, and as such it was predicted to function similarly by causing MOMP but recent evidence suggests that this may not be the case. Overexpression of Bok failed to kill Bax/Bak double knockout cells treated with apoptotic stimuli suggesting that Bok may not act in a similar manner to that of Bax and Bak (Echeverry et al, 2013). Additionally this study showed that Bok induced apoptosis was largely Bax/Bak dependent, indicating that Bok acts upstream of Bax/Bak (Echeverry et al, 2013).

3.4 Targeting of BH3 proteins to membranes as a crucial step in regulating apoptosis: overview and context

After activation, BH3 proteins translocate to the MOM and/or the ER where they bind to pore-forming or anti-apoptotic proteins. Sequence analysis suggests that most BH3 proteins (Bid, Bim, Bmf, Bik and Puma) have a carboxyl-terminal tail-anchor region (Wilfling et al, 2012). Other than Bik, which is constitutively localized to the ER, the listed BH3 proteins are found at the MOM only after activation (Wilfling et al, 2012). In some cases the specific tail-anchor sequences are sufficient to determine the sub-cellular localization of proteins. For examples, the predicted tail-anchor regions of Bim and Puma are sufficient to target and insert GFP fusion proteins into the MOM (Wilfling et al, 2012). Other BH3 proteins

such Bad and Beclin-1 contain other types of hydrophobic sequences important for subcellular membrane localization (Hekman et al, 2006; Sinha & Levine, 2008) while Noxa contains a conserved mitochondrial targeting sequence found in non BH3 proteins at its carboxyl-terminus (Seo et al, 2003).

There are clear functional consequences of appropriate membrane targeting as BH3 peptides, derived from the sequence of specific BH3 proteins, bind to the appropriate Bcl2-family members but with markedly decreased affinity compared to the full-length proteins (Sarosiek et al, 2013). Moreover many full-length BH3 proteins such as Bim, Bmf and Bad lack a definitive structure in solution, but nevertheless show binding preferences in vivo implying that they attain a specific conformation when associated with the membrane (Hinds et al, 2007). The Embedded Together model would predict that these intrinsically unstructured proteins may adopt distinct conformations on the membrane which promote their function. We will discuss how these features are relevant for specific BH3 activator and sensitizer proteins.

3.4.1 BH3 proteins and membranes: Bim, Bid and Bad

Bim is an important mediator of apoptosis initiated by many types of intracellular stress including DNA damage and the ER-associated unfolded protein response (Concannon et al, 2010; Puthalakath et al, 2007b; Soo et al, 2012). Three major isoforms of Bim are formed by alternative splicing of mRNA: BimEL, BimL, and BimS (Hinds et al, 2006). BimEL and BimL are present in many different tissues

and cell types (O'Reilly et al, 2000) while BimS is almost never present in normal cells and has been detected only in HEK 293 cells (Putcha et al, 2001). Thus, BimEL and BimL are likely the most relevant isoforms mediating an apoptotic response for most cell types.

Although in vitro studies of protein function using full length proteins are lacking, structural studies using Bim BH3 peptides indicate that Bim binds to the hydrophobic groove of anti-apoptotic proteins (Czabotar et al, 2007; Liu et al, 2003). It seems likely that binding anti-apoptotic proteins causes the Bim BH3 region to adopt a defined alpha helical structure. As described above, NMR data with stapled peptides suggest that the Bim BH3 region binds directly to the Bax rear pocket to provoke Bax activation. It remains to be determined if the Bim BH3 region also binds the hydrophobic 'front pocket' on Bax and, if so, what the relative affinities are for the two pockets. However, a carboxyl-terminal deletion mutant of Bim lacking the membrane anchoring domain (BimΔC) failed activate Bax in vitro; this interaction can be partially restored if BimΔC is brought to the membrane by other mechanisms (e.g. by histidine-tag-Ni2+ chelating or Tom5 complex targeting sequence) (Terrones et al, 2008). Recent studies claim that Bim spontaneously inserts into the MOM via its putative tail-anchor, and that binding to the MOM is crucial for activating Bax (Weber et al, 2007). Both of these results are consistent with Bim folding into a defined structure when it targets to membranes. However, it is unlikely that the mechanism is closely related to that of authentic tail-anchor proteins as the putative tail-anchor

sequence of Bim contains charged residues which are normally incompatible with tail-anchor mediated insertion into the membrane. Thus, while the carboxyl-terminal 'hydrophobic region' of Bim is necessary and sufficient for binding to MOM (Wilfling et al, 2012) it is unlikely that it mediates membrane binding by a conventional tail-anchor insertion mechanism.

Because Bim binding to membranes occurs spontaneously and the unmodified protein is constitutively active there must be a control mechanism to prevent from Bim constantly provoking apoptosis. BimS appears to be regulated transcriptionally while BimL and BimEL are sequestered to the cytoskeleton via a short peptide motif (DKSTQTP) encoded by exon 4 that binds to dynein Light Chain LC1 (Puthalakath et al, 1999). The c-Jun NH2-terminal kinase (JNK), which is activated upon apoptosis stimuli such as cell stress, phosphorylates Bim at 2-3 conserved sites on and near the LC1 binding motif, specifically at Thr-56 and at least one of the adjacent serine residues, Ser-44 and/or Ser-58 (sequence positions refer to human BimL). Phosphorylation in this motif in BimL and BimEL causes the release of Bim from the motor complexes resulting in spontaneous relocalization to the MOM (Geissler et al, 2013; Lei, 2003). Thus phosphorylation can regulate BimL and BimEL activity in cells. Consistent with the complex regulation of apoptosis by many intracellular signalling pathways, Bim is also phosphorylated on serine-87 through PIP3/Akt pathway, although this has the opposite consequence to JNK phosphorylation as it leads to ubiquitination and degradation of the protein (Qi et al, 2006). The absence of the dynein binding

motif and the associated regulatory phosphorylation sites in BimS probably accounts for the extremely potent apoptotic activity of this isoform(Lei, 2003).

Bid is another activator BH3 protein that binds to and activates Bax in membranes (Lovell et al, 2008). It was first reported in 2000 that Bid induces the oligomerization and insertion of Bax into the MOM, inspiring much of the subsequent work on Bid using reconstituted systems (Eskes et al, 2000). The BH3 region of Bid also binds to Bak as demonstrated by a crystal structure of the dimer (Moldoveanu et al, 2013). Unlike other BH3 proteins, in solution Bid has a distinct 3D-structure that is homologous to multi-BH domain family members like Bax and Bcl-XL. Functionally Bid serves as a link between the extrinsic pathway and the intrinsic pathways: at the onset of extrinsic apoptosis, Bid targets to membranes once it is cleaved into cBid by active caspase 8 which itself requires prior activation via death-receptor mediated cleavage. During the intrinsic pathway of apoptosis there are other mechanisms by which either caspase 8 is activated or Bid is cleaved. However in this case, cleavage of Bid generally occurs after MOMP and appears to provide amplification of the death signal. The cleaved version of Bid (cBid) consists of two fragments: an amino-terminal p7 fragment and a carboxyl-terminal p15 fragment (tBid). Using liposomes or isolated mitochondria with fluorescently labeled proteins, our lab has shown that membrane targeting of cBid is sufficient to cause the dissociation of the p7 fragment from tBid (Shamas-Din et al, 2013a). Thereafter a series of conformational changes ensue in which tBid first unfolds at the membrane such

that α-helices 4, 5 and 8 interact with the membrane followed by insertion of α-helices 6 and 7 into the membrane (Shamas-Din et al, 2013a). This conformational change on the membrane constitutes the rate-limiting step for cBid activation. Interestingly the MOM protein Mtch2 facilitates tBid binding to membranes (Zaltsman et al, 2010) and accelerates the conformational change of tBid thereby activating Bax (Shamas-Din et al, 2013a). However, Mtch2 is not essential for the cBid-mediated Bax permeablization of all membranes, as liposomes or proteo-liposomes containing cardiolipin but lacking Mtch2 can be permeablized by cBid and Bax(Schafer et al, 2009; Shamas-Din et al, 2013a). Numerous studies have indicated that the spontaneous membrane binding of tBid causes the migration of soluble Bax and Bcl-XL to membranes (Billen et al, 2008; Garcia-Saez et al, 2009; Lovell et al, 2008; Qi et al, 2006). Recent studies have also shown that in many situations tBid preferably activates Bak while Bim preferably activates Bax (Sarosiek et al, 2013).

The best characterized sensitizer BH3 protein is Bad. Two lipid binding regions have been identified in Bad, one of which confers binding to cholesterol and the other to negatively charged lipids (Hekman et al, 2006). Survival signals trigger the PI3K/Akt pathway and the phosphorylation of Bad at three conserved serine residues: S112, S136 and S155. Upon phosphorylation, Bad is sequestered by 14-3-3 chaperone proteins (Harada et al, 1999; Zha et al, 1996b). The phosphorylated Bad-14-3-3 complex has high affinity for cholesterol rich lipid membranes but a low affinity for the MOM, thus phosphorylation of Bad at these

sites prevents the interaction of Bad with anti-apoptotic proteins (Hekman et al, 2006). When dephosphorylated, Bad localizes to the negatively charged MOM and exerts its pro-apoptotic effects by binding to anti-apoptotic proteins (Hekman et al, 2006). As mentioned above, while Bad binding inhibits Bcl-XL, it also activates Bcl-xl by causing the recruitment of Bcl-XL to the MOM because Bad binding to Bcl-XL is reversible. Thus, the outcome of mutual sequestration depends on the expression level, localization and post-translational modifications of Bad. Taken together these observations suggest that differential interactions with membranes and BH3 proteins govern structural changes that modify their function.

3.5 Anti-apoptotic proteins: Multiple mechanisms to inhibit MOMP

Anti-apoptotic Bcl-2 family members are important guardians of cell survival (Oltvai et al, 1993). Bcl-XL, Mcl-1 and Bcl-w are located in the cytoplasm in growing cells and localize to the mitochondria during apoptosis (Hsu et al, 1997b; Nijhawan et al, 2003; Wilson-Annan et al, 2003). In contrast, Bcl-2 constitutively binds to both the MOM and the ER membrane (Lithgow et al, 1994; Nguyen et al, 1993) but undergoes a conformational change, after binding membranes, essential for it efficiently inhibit Bax mediated MOMP (Dlugosz et al, 2006).

Detailed studies on the structure and function of some anti-apoptotic proteins are difficult for various technical reasons: it is challenging to purify recombinant Bcl-2 due to its marked tendency to aggregate in vitro while Mcl-1 is notoriously

unstable both in vivo and in vitro. However, once purification of full length Bcl-XL (i.e. with the carboxyl-terminal targeting sequence intact) was possible, useful information concerning the mechanism(s) of apoptosis inhibition and how this is modified by binding membranes started to become available.

Despite opposite effects on apoptosis, there is a striking resemblance between Bcl-XL and Bax in terms of their structure and behavior. Both proteins contain hydrophobic grooves for binding BH3 regions of other proteins, and a carboxyl-terminal hydrophobic TM tail bound to this region in *cis* (Liu et al, 2003; Sattler et al, 1997). Both Bcl-XL and Bax remain mainly cytoplasmic or peripherally bound to membranes in growing cells and only bind tightly to the membrane after activation, by shifting the equilibrium to favor the membrane bound form (Hsu et al, 1997b). Bcl-XL inhibits Bax/Bak activation and oligomerization on the MOM by several non-exclusive mechanisms (Billen et al, 2008). Behaving as a dominant-negative Bax, Bcl-XL binds to BH3 proteins (MODE 1) or activated Bax/Bak (MODE 2). Both MODEs 1 and 2 lead to the membrane localization of Bcl-XL and mutual sequestration of Bcl-XL and its binding partners (Billen et al, 2008). In both cases binding between Bcl-XL with its partners involves the interaction of the hydrophobic groove of Bcl-XL with the BH3 region of either Bax/Bak or BH3 proteins(Liu et al, 2003; Sattler et al, 1997). Recently studies have suggested that there are other sites outside of the BH3 region of Bim that mediate binding to Bcl-XL, as mutations that abrogate the binding of a Bim BH3 peptide to Bcl-XL did

not prevent binding of a similarly mutated full-length Bim to Bcl-XL in vivo (Aranovich et al, 2012).

It is now recognized that in addition to directly engaging and sequestering pro-apoptotic proteins, Bcl-XL also actively transports peripherally membrane-associated Bax to the cytoplasm (Billen et al, 2008; Edlich et al, 2011; Todt et al, 2013). This mechanism has been dubbed MODE 0 to complement the previously recognized MODEs 1 and 2 (Westphal et al, 2013). The mechanism of retro-translocation of Bax is still unclear, but there are hints that direct interaction is involved as mutations have been identified in the Bax BH3 domain, the Bcl-XL hydrophobic groove (Edlich et al, 2011) and the BH4 region of Bcl-XL that inhibit this process (Billen et al, 2008). A possible explanation for MODE 0 inhibition, consistent with the Embedded Together model, would involve MODE 0 acting principally on peripherally bound Bax. This form of Bax undergoes a limited conformational change in vitro, involving the exposure of an amino-terminal epitope recognized by the monoclonal 6A7 antibody with possible changes in the BH3 region (Yethon et al, 2003). An exposed site on Bax may interact transiently with either peripherally or integrally bound Bcl-XL, shifting the conformational equilibrium in Bax towards the cytoplasmic 6A7 negative form thereby reducing the affinity of the complex for the membrane and allowing Bax to retro-translocate back to the cytoplasm. The difference between Bax inactivation by anti-apoptotic proteins via MODE 0 and MODE 2 is the membrane status of Bax (Llambi et al, 2011; Yethon et al, 2003). MODE 0 inhibition occurs in the absence of a BH3

activator, where Bcl-XL binds peripherally membrane bound Bax resulting in dissociation of both from the membrane. Reciprocally, MODE2 inhibition occurs when a BH3 activator promotes Bax conformation changes on the membrane and shifts the equilibrium towards membrane insertion. Then membrane-inserted Bax will recruit Bcl-XL from the cytoplasm where mutual sequestration results in MODE 2 inhibition. Thus, control of Bax subcellular localization clearly serves as a distinct mechanism governing survival in healthy cells. Other reports indicate that Bax can retro-translocate independent of anti-apoptotic proteins in mouse mammary epithelial cells (Schellenberg et al, 2013). However, in this study Bax was fully inserted into the MOM rather than peripherally bound to the membrane, indicating yet another poorly understood mechanism that regulates Bax localization (and hence function) in some cells.

Recently Mcl-1 has become a better recognized target for cancer treatment as it mediates chemotherapy resistance in many cancer cell lines (Glaser et al, 2012; Wei et al, 2006; Wuilleme-Toumi et al, 2005; Xiang et al, 2010). Not surprisingly, Mcl-1 is over-expressed in many cancers and cancer cell lines (Beroukhim et al, 2010; Glaser et al, 2012; Wei et al, 2006; Wuilleme-Toumi et al, 2005; Xiang et al, 2010). A special role of Mcl-1 for cell survival is suggested by experiments in which genetic deletion of Mcl-1 induces cell death even when other anti-apoptotic proteins are present in these cells (Glaser et al, 2012; Xiang et al, 2010). Among the anti-apoptotic proteins, Mcl-1 possesses several unique structural and functional features including a long and intrinsically unstructured amino-terminal

region. The region contains interacting sites that target the protein for ubiquitin-dependent (Inuzuka et al, 2011; Schwickart et al, 2010; Warr et al, 2005; Zhong et al, 2005) and ubiquitin-independent (Stewart et al, 2010) degradation via a multi-step mechanism that is partly responsible for the extremely short half-life of Mcl-1 (Nijhawan et al, 2003). When expressed in cells approximately 80 percent of the exogenous Mcl-1 is located in the cytoplasm of growing cells (Germain & Duronio, 2007). Partial proteolysis of the amino-terminus of Mcl-1 on mitochondria gives rise to three different species: 40kD (full-length), 38kD, and 36kD (Perciavalle et al, 2012). Full-length 40kD Mcl-1 is tightly bound to the MOM and resists alkaline extraction; 38kD Mcl-1 is enriched at the MOM but has a lower binding affinity to membranes. Both 40kD and 38kD Mcl-1 fulfill their pro-survival function by mutually engaging specific pro-apoptotic proteins such as Bim (Perciavalle et al, 2012). The binding mechanism may be the same as other anti-apoptotic proteins like Bcl-XL, as the crystal structure of the stable core fragment of Mcl-1 in complex with Bim BH3 and Noxa BH3 shows great similarity with the Bcl-XL-BH3 complex (Czabotar et al, 2007; Liu et al, 2010; Liu et al, 2003; Sattler et al, 1997).

The amino-terminal region of Mcl-1 has been shown to result in a fraction of the mitochondrial bound Mcl-1 inserting into the MOM with an opposite orientation. In this situation the amino-terminus of Mcl-1 enters the mitochondria matrix where it undergoes further cleavage to generate the 36kD form. This novel matrix localized form of Mcl-1 regulates mitochondrial fission/fusion that, as noted above,

modulates apoptotic function (Perciavalle et al, 2012). In the next section we will examine the regulation of these reciprocal processes by the Bcl-2 family in more detail.

4. Mitochondria Dynamics and Apoptosis

Mitochondria are highly dynamic organelles with major morphological differences seen between cell types. These differences in morphology are dictated by cytoskeletal transport and the relative rates of fission and fusion. By shifting the balance of these rates the morphology can range from a long filamentous network to highly fragmented uniformly shaped mitochondria.

Mitochondrial fission and fusion is regulated by large self-assembling dynamin-related GTPases. Fusion of the MOM is mediated by the partially redundant, membrane anchored mitofusins Mfn1 and Mfn2 which can form homotypic or heterotypic oligomers on the MOM (Chen et al, 2003; Rojo et al, 2002). When cells lack either Mfn1 or Mfn2, the mitochondria become highly fragmented resulting in a phenotype that can be rescued by overexpression of either Mfn1 or Mfn2 (Chen et al, 2003). Fusion of the mitochondrial inner membrane (MIM) is mediated by Opa1 which is located in the IMS anchored to the MIM within cristae and cristae junctions (Griparic et al, 2004; Olichon et al, 2002). Fission of mitochondria is carried out by a single protein Drp1 that is predominately cytoplasmic and localizes to sites of mitochondria fragmentation (Frank et al, 2001; Labrousse et al, 1999; Smirnova et al, 1998); a process which may be

facilitated by ER-Mitochondria contact sites (discussed below) (Friedman et al, 2011).

Mitochondrial fission and fusion are imperative for cells to respond to metabolic and environmental stress and play a prominent role in apoptosis (Youle & van der Bliek, 2012). In the majority of healthy cells mitochondria exist as a filamentous network and, upon transformation, cellular stress or apoptosis, undergo increased fission (Cereghetti & Scorrano, 2006). Generally, cells with highly fused networks of mitochondria are more resistant to apoptosis compared to cells with fragmented mitochondrial morphology. The overexpression of a dominant negative Drp1 results in decreased mitochondrial fission and impairs both MOMP and apoptotic cell death (Frank et al, 2001). Furthermore, overexpression of Opa1 results in increased fusion and protection from apoptosis by preventing MOMP (Frezza et al, 2006). The mechanism of Opa1 protection may be due in part to control of cristae remodelling, which is independent of its role in mitochondrial fusion, whereby it prevents the redistribution and release of cytochrome *c* stored in cristae by forming tight cristae junctions (Frezza et al, 2006; Scorrano et al, 2002). Conversely, knockdown of Opa1 by siRNA results in fragmented mitochondria with unstructured cristae and spontaneous apoptosis that can be reduced by overexpression of Bcl-2, which presumably prevents Bax/Bak mediated MOMP (Olichon et al, 2003). Additionally, cells overexpressing Drp1 have highly fragmented mitochondria that are more sensitive to staurosporine induced apoptosis (Szabadkai et al, 2004). The exact mechanisms

that regulate the interplay between mitochondrial dynamics and apoptosis are currently unclear however accumulating evidence suggests that the Bcl-2 family proteins can regulate fission and fusion.

During apoptosis Bax colocalizes to mitochondrial foci containing Drp1 where subsequent mitochondrial fission occurs, and Bax remains associated at both tips of the newly formed mitochondria (Karbowski et al, 2002; Nechushtan et al, 2001). Interestingly, Bax was found to also colocalize with Mfn2, a promoter of mitochondrial fusion, at the same fission sites (Karbowski et al, 2002). To corroborate this finding, a soluble monomeric conformationally restrained Bax, which cannot insert into the MOM and thus does not promote MOMP, was found to associate with Mfn2 in healthy cells and promote mitochondrial fusion. This fusion was impaired by the addition of staurosporine or tBid (Hoppins et al, 2011). Furthermore, during apoptosis Drp1 becomes stably associated with the MOM in a Bax/Bak dependent manner that occurs before MOMP (Wasiak et al, 2007). The BH3 protein cBid plays a role in mitochondrial fission since addition of cBid to isolated mitochondria causes the disassembly of OPA1 complexes resulting in remodelled cristae that allow the mobilization and efficient release of cytochrome *c* from cristae during MOMP (Frezza et al, 2006; Yamaguchi et al, 2008). Cristae remodelling has also been observed for the BH3-only proteins BimS and Bnip3 (Landes et al, 2010; Yamaguchi et al, 2008). cBid mediated cristae remodelling transpires in a Bax/Bak dependent manner and can occur in the absence of MOMP (Yamaguchi et al, 2008). Exactly how cBid, a cytosolic protein targeted to

the OMM, results in the disassembly of Opa1 complexes located at the IMM, is currently unknown. There must be unidentified proteins responsible for transmitting a signal from the OMM to the IMM in order for Opa1 disassembly to occur. Typically cBid and Bax have similar effects within the cell, promoting apoptosis, so it is interesting to note that the addition of cBid has an opposing effect, promoting fission, to that of Bax, promoting fusion, in the aforementioned study (Hoppins et al, 2011). The opposite effect observed is likely because a mutant Bax was used that is soluble and cannot insert into the MOM due to engineered disulphide bonds that prevent Bax conformation changes. These studies suggest that in growing cells cytoplasmic Bax works to promote fusion and upon the induction of apoptosis Bax inserts in and oligomerizes within the MOM where it can then promote the binding of Drp1 to the MOM thereby increasing fission leading to MOMP and mitochondrial fragmentation. It is to be noted that fission is not necessarily required for apoptosis to occur. When Drp1 was knocked down via RNAi, cytochrome c release was impaired however other apoptogenic IMS proteins such as Smac/DIABLO and Omi/Htra2 were released and the cells still underwent apoptosis(Estaquier & Arnoult, 2007; Parone et al, 2006). This is in contrast to the studies that suggest fission is required for Bax/Bak mediated apoptosis (Frank et al, 2001; Lee et al, 2004). All four studies suggest that mitochondrial fission is required for efficient cytochrome c release, potentially via cristae remodelling, and that the discrepancies between the

studies may be explained by differences in cell types that require complete release of IMS localized apoptogenic factors in order to induce apoptosis.

The mechanism of how anti-apoptotic Bcl-2 family proteins regulate mitochondrial dynamics is less clear. As described above, post-translational proteolytic cleavage of Mcl-1 results in a truncated species that is tethered to the MIM, and is required for proper cristae structure and mitochondrial fusion (Perciavalle et al, 2012). Furthermore, Bcl-XL can stimulate mitochondrial fusion in vitro and in growing cells (Berman et al, 2009; Hoppins et al, 2011; Sheridan et al, 2008). However, in response to apoptotic stimuli ectopically expressed Bcl-XL or Bcl-2 did not prevent Bax/Bak dependent mitochondrial fission but did prevent cytochrome *c* release suggesting that MOMP can be uncoupled from mitochondrial fragmentation (Sheridan et al, 2008). It is unknown whether or not Bcl-XL or Bcl-2 affects mitochondrial dynamics by directly binding to fission or fusion machinery, or indirectly by sequestration of Bax/Bak and BH3-only proteins.

5.1 Cell death and the Endoplasmic Reticulum

The Bcl-2 family proteins regulate cell death at both the mitochondria and ER. This is evident by the fact that all three classes of the Bcl-2 family proteins target to the ER where they regulate both apoptosis and autophagy. Autophagy is primarily a cell survival mechanism activated by many stressors, most notably

cellular starvation, and is mediated by the accumulation of autophagosomes that degrade and recycle intracellular contents such as damaged organelles and proteins (Leber et al, 2010a). Excessive autophagy results in cell death through degradation of the Golgi complex, the ER and finally the nucleus (Mukhopadhyay et al, 2014). One important link between autophagy and apoptosis is through the dual-regulation of both processes via Bcl-2 and Bcl-XL. The BH3-only protein Beclin-1 has a central role in autophagy where it promotes autophagosome nucleation(Oberstein et al, 2007). Bcl-2 localized to the ER can bind to and sequester Beclin-1 resulting in decreased autophagic cell death (Pattingre et al, 2005). Furthermore cells expressing a Beclin-1 mutant which cannot bind Bcl-2 have increased levels of autophagic cell death suggesting that the anti-apoptotic Bcl-2 family proteins help regulate autophagy by keeping Beclin-1 activity in check (Pattingre et al, 2005). The BH3 protein Bad is able to stimulate autophagy by disrupting the interaction between Bcl-2/Bcl-XL and Beclin-1, providing a link between autophagy and apoptosis (Maiuri et al, 2007). Bim may represent another potential link between autophagic and apoptotic cell death processes as at least one report suggests that Bim can inhibit autophagy by recruiting Beclin1 to microtubules (Luo et al, 2012). Nutrient starvation results in Bim phosphorylation by JNK causing Bim to release Beclin-1 and dissociate from microtubules, this results in the simultaneous induction of autophagy via Beclin-1 and apoptosis by Bim (Luo et al, 2012).

Another way cell death can arise from the ER is the initiation of the unfolded protein response (UPR). Generally UPR results from the accumulation of misfolded proteins within the ER as a result of a loss of ER homeostasis by physiological stress such as hypoxia and oxidative stress (Logue et al, 2013). If UPR cannot reduce the amount of unfolded proteins within the ER lumen, thus relieving ER stress, then apoptosis is triggered (Ron & Walter, 2007). ER stress is sensed by at least three ER-localized proteins, ATF6, IRE1α and PERK that are maintained in an inactive form in growing cells by binding to the chaperone Grp78. Increased unfolded proteins within the ER lumen results in the dissociation of Grp78 from ATF6, IRE1 α and PERK where they trigger UPR (Bertolotti et al, 2000). ATF6 and PERK activation during UPR results in upregulation of the transcription factor CHOP which upregulates the BH3 proteins Bim and Puma, in a p53 independent manner, and downregulates Bcl-2 resulting in apoptosis (Galehdar et al, 2010; Ghosh et al, 2012; McCullough et al, 2001; Okada et al, 2002). Moreover, overexpression of Bcl-2 or Bcl-XL protects cells from death triggered by ER stress (Murakami et al, 2007). It is unclear whether or not protection of apoptosis by Bcl-2 and Bcl-XL is mediated through prevention of MOMP at the mitochondria or by an ER specific cell death mechanism. However, there is some data that suggests that overexpression of Bcl-2 specifically targeted to the ER prevents cell death even after cytochrome *c* has been released in response to ER stress (Annis et al, 2001; Hacki et al, 2000). This implies that there are cell death pathways mediated through the ER by the Bcl-2

family proteins since MOMP, the point of no return in apoptosis, has already occurred within these cells. Additionally, some forms of cell death can be mediated at the ER that are not related to ER stress. Several agonists that induce cell-stress result in the post-translational modification of E-cadherin, preventing it's trafficking from the ER to the plasma membrane, exacerbating apoptosis via the initiation of anoikis (Geng et al, 2012). Clearly there is significant crosstalk between the ER and Mitochondria allowing for various forms of cell stress to be sensed and dealt with via apoptosis. This crosstalk may be facilitated, in part, by regions of the ER and Mitochondria that are in close proximity.

5.2 Integrating the organelles: Mitochondrial Associated Membranes

The Mitochondria and ER have specific regions within the cell that are tightly associated termed "mitochondrial associated membranes" (MAMs) which may link cell death processes mediated by both the ER and mitochondria (Grimm, 2012). The ER has a very interconnected tubular structure and, much like mitochondria, the morphology of the ER is highly dynamic with tubules constantly undergoing fission and fusion (Pendin et al, 2011). It has even been suggested that specialized tubules of the ER wrap around and constrict the mitochondria facilitating mitochondrial fission (Friedman et al, 2011). Drp1 stably associates with mitochondria localized to these ER-mitochondria contact sites suggesting that the ER marks sites for mitochondrial division. Additionally, the formation of

constriction sites was found to be independent of the mitochondrial fission machinery but may depend on Mfn2 homotypic complexes between the ER and MOM (de Brito & Scorrano, 2008; Friedman et al, 2011). Furthermore, as a possible link between ER stress and mitochondrial dynamics and apoptosis, a recent study has identified that Mfn2 is phosphorylated by JNK during cellular stress. Phosphorylation increases ubiquitination and proteasomal degradation of Mfn2 leading to a decrease in the rate of fusion and thus extensive fragmentation of the mitochondrial network and cell death (Leboucher et al, 2012). Additionally, autophagy is dependent on Mfn2 stabilization of MAMs since depletion of Mfn2 diminishes MAMs and severely compromises autophagy (Hailey et al, 2010). The enrichment of Mfn2 at MAMs, paired with the information that Bax colocalizes and potentially interacts with MFN2 suggests that MOMP is regulated at MAMs by Bcl-2 family proteins..

6. Conclusion

With the basic interaction network of Bcl-2 family members established, we are now at a stage where growing knowledge is yielding practical results. The discovery that some cancer cells are “addicted” to one or more anti-apoptotic proteins for survival provides a way to categorize malignancies for target-specific treatment (Davids & Letai, 2012). Small molecules mimicking the BH3 regions of Bad (e.g. ABT-737, ABT-263) bind to and inhibit Bcl-XL and Bcl-2 with high affinity (Oltersdorf et al, 2005) and have shown notable benefits in lymphoid

malignancies as monotherapy or in combination with other chemotherapies (Ackler et al, 2012; Roberts et al, 2012). A recent derivative ABT-199 that selectively targets Bcl-2 but not Bcl-XL has reduced thrombocytopenia (Souers et al, 2013) and is starting phase III clinical trials. We propose that membrane bound Bcl-2 family proteins may serve as a more precise target for drug screening. Our reasoning is that the Bcl-2 family proteins adopt distinct conformations at, on and in intracellular membranes altering their affinities for one another and potentially exposing new binding sites for targeted therapies.

Initially, regulation of the Bcl-2 family was thought of as a rheostat where the relative levels of pro- and anti-apoptotic proteins determines the fate of a cell. At present, it is clearly apparent that regulation of apoptosis is much more complicated. Whether the cell lives or dies is governed by a set of complex equilibria not only between the protein-protein interactions of the Bcl-2 family but also by their interaction with membranes. It is evident that intracellular membranes play an active role in apoptosis as the Bcl-2 family proteins undergo substantial conformational changes upon binding membranes that modifies their affinity for other Bcl-2 family proteins and other binding partners. Even more complexity is added via the localization of Bcl-2 family proteins at different intracellular membranes, where the Bcl-2 family proteins seem to regulate more than just MOMP. It is obvious that there is still much to learn about the Bcl-2 family proteins. It will be exciting to see additional studies that answer the mechanistic details of how Bcl-2 family proteins are regulated by each other, by

their localization and by membranes, helping us to fully understand cell death regulation and aiding in the development of novel disease therapies.

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Introduction of Bim

BH3 protein Bim (Bcl-2-interacting mediator of cell death) was originally identified as a Bcl-2-interacting protein by screening a bacteriophage lambda cDNA expression library constructed from a mouse thymic lymphoma (O'Connor et al, 1998). Bim has been reported to have essential functions in embryogenesis, the control of hematopoietic cell death, and the fate of anergic B cells, and also acts as a barrier against autoimmunity (Bouillet et al, 2000; Oliver et al, 2006). Its ablation in mice results in increased numbers of lymphocytes, plasma cells, and myeloid cells, and facilitates the development of fatal autoimmune glomerulonephritis (Strasser et al, 2000). Bim has also been shown to mediate apoptosis initiated by intracellular stressors in various cell types (Concannon et al, 2010; Mahajan et al, 2014; Puthalakath et al, 2007).

Three major isoforms of Bim result from alternative mRNA splicing: BimEL, BimL, and BimS (O'Connor et al, 1998). All three isoforms include the BH3-domain required for binding other Bcl-2 family proteins and a predicted C-terminus membrane binding domain (MBD) (Weber et al, 2007; Wilfling et al, 2012). BimL and BimEL are sequestered in the cytoskeleton via interactions between a short peptide motif (DKSTQTP) and dynein light-chain (DLC) (Puthalakath et al, 1999). The c-Jun NH2-terminal kinase (JNK), which is activated upon apoptosis stimuli such as cell stress, is shown to phosphorylate Bim at 2-3 conserved sites on and near the LC1 binding motif, specifically Thr-56 and at least one of the adjacent serine residues Ser-44 and/or Ser-58 (sequence positions refer to human BimL). The phosphorylation in this motif inactivates its

dynein binding function and consequently causes the release of Bim from the motor complexes and spontaneous targeting to the mitochondria outer membrane (MOM) (Lei, 2003). Consistent with the complex regulation of apoptosis by many intracellular signalling pathways, the BimEL isoform has been found to be further regulated by phosphorylation in its EL-specific domain: Phosphorylation on Ser-69 through ERK/MAPK pathway and Ser-87 through PIP3/Akt pathway (sequence positions refer to human BimEL) has the opposite consequences to JNK phosphorylation as it leads to ubiquitination and degradation of the protein (Luciano et al, 2003; Qi et al, 2006). Surprisingly, it was found that phosphorylation on Ser-59 on BimEL through the ERK/MAPK pathway facilitates Bim binding to the dynein in a complex containing BimEL, LC8 and Beclin-1, which protect cells from anoikis (Buchheit et al, 2015). It was unknown if similar regulation mechanism exists for BimL due to the lack of EL-specific domain. BimS is regulated transcriptionally and the absence of a DLC binding motif which includes negative regulatory phosphorylation sites in BimS probably accounts for the more potent activity in cells (Lei, 2003).

Evidences have shown that the predicted MBD of Bim is crucial to its membrane targeting as well as function (Weber et al, 2007). MBD of Bim by itself is sufficient to determine the sub-cellular localization of proteins, as the MBD sequence target and insert GFP fusion proteins into the MOM (Wilfling et al, 2012). One article has claimed that Bim may translocate to ER in C2C12 cells upon ER stress but the evidence was compromised by the possibility of

contamination of the tested ER membrane by mitochondria portion (Morishima et al, 2004). Sequence analysis indicates that this MBD potentially forms an amphipathic α-helix distinct from conventional tail anchors as it contains two arginine residues (R130&134) on the same side of the helix.

The cross-species sequence alignment (Figure 1) reveals that the sequence of mammalian Bim was highly conserved (>85% identity between human and dog/mouse/rat). Bim in platypus/reptile/amphibian share >52% identity in sequence with its mammalian counterpart, with highly conserved BH3 domain and MBD. The zebrafish Bim has an overall 26% identity and 35% similarity with its human counterpart while retaining 100% conservation of the BH3 domain (Jette et al, 2008). The sequence of MBD in zebrafish Bim was very different from those of mammalian Bim, however it still retains the amphipathic feature and at least one Arg. The sequence (or structural) conservation of BH3 and MBD domain across species implies a essential role of the duo in the function of Bim. The lack of a DLC binding motif in zebrafish Bim suggests that the regulation via Bim binding to microtubules may be a mechanism adopted later in evolution. It's worth noting that all species analyzed shows conservation of the (human) serine 69 despite surrounding areas of divergent sequence, which implicates a conserved regulation of Bim via phosphorylation.

Despite the importance in apoptosis, the role of the Bim in apoptosis remains unclear. Due to the difficulty expressing and purifying Bim, most *in vitro* experiments to date have been conducted with peptides or deletion mutants

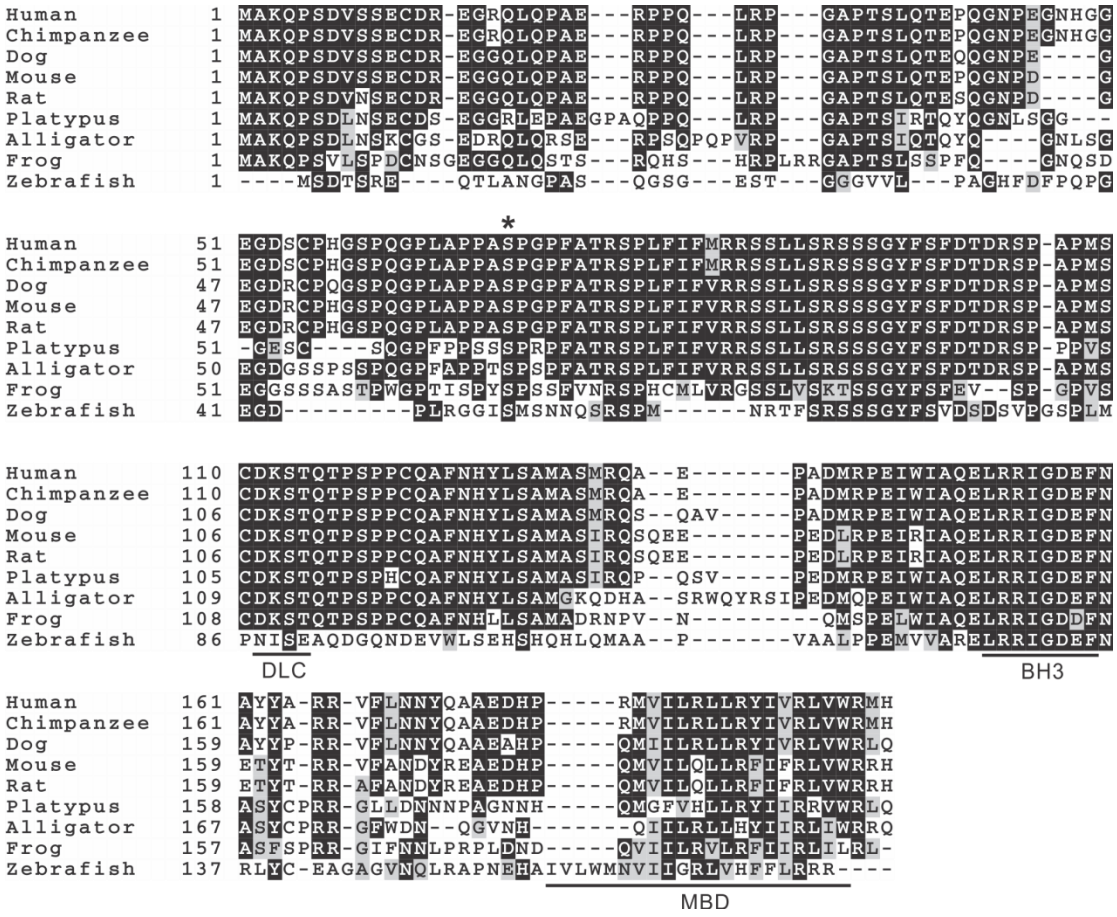

Figure 1: Alignment of Bim (EL) proteins across species

The Bim (EL) genes from multiple vertebrate species aligned using ClustalW and Boxshade from the EMBnet server. The BH3 domain is highly conserved across species. The mammalian dynein light-chain (DLC) binding site was conserved in most species except for Zebrafish. Membrane binding domain (MBD) sequence is conserved in mammalian/reptile/amphibia, while zebrafish Bim MBD share structural but not sequence similarity with its mammalian/reptile/amphibia counterpart. * indicates the position of human serine 69 which is conserved in all species analyzed.

Sequences used in alignment: Human (GI: 2895496); Chimpanzee (GI: 410353661); Dog (GI: 545527813); Mouse (GI: 2895500); Rat (GI: 25282467); Platypus (GI: 620941533); Alligator (GI: 564227369); Frog (GI: 73853860); Zebrafish (GI: 209418514)

lacking the MBD. Bim has been reported to have particularly important sensitizer function as it binds to all known anti-apoptotic proteins leading to inhibition by mutual sequestration (Chen et al, 2005). Structure studies with Bim BH3 peptides indicate that Bim binds to the hydrophobic groove of anti-apoptotic proteins with an alpha helical Bim BH3 domain (Liu et al, 2003). On the other hand, evidence of Bim as activator has been limited and sometimes contradicting each other. For example, NMR data with stapled peptides suggest that Bim BH3 binds directly to the Bax rear pocket leading to activation (Gavathiotis et al, 2008). Bim BH3 peptide stablized by a neutual aptamer scaffold was also shown to bind to Bax in cells (Stadler et al, 2014). However, Bim mutants lacking the MBD (Bim∆MBD) failed in activating Bax to induce membrane permeabilization (Weber et al, 2007), a function that is partially restored if Bim∆MBD is brought to the membrane by other mechanisms (Terrones et al, 2008). Such data do provide some insights on the function of Bim but have to be interpreted with caution as peptides or MBD deletion mutants do not reflect the function of full-length proteins. Assessing binding interactions between Bim and Bax in cells via co-immunoprecipitation and immunoblotting may not resolve the issue of how Bim functions, because these methods give inconsistent results that are prone to detergent artifacts (Hsu & Youle, 1997; Lovell et al, 2008). Indeed, immunoblotting from cell lysates give contradicting results on whether Bim binds Bax (Merino et al, 2009; Willis et al, 2007). Thus, how Bim regulates MOMP remains to be elucidated and a thorough study with full-length Bim protein is required to reveal its function.

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Methods

CHAPTER II: Examining the molecular mechanism of bcl-2 family proteins at membranes by fluorescence spectroscopy.

Preface

The work presented in this chapter has been previously published as a method paper in:

Kale J, Chi X, Leber B, Andrews D (2014) Examining the molecular mechanism of bcl-2 family proteins at membranes by fluorescence spectroscopy. *Methods Enzymol* **544:** 1-23

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Contribution of authors:

X.C. provided data for and wrote the section 3, K.J. prepared the rest of the sections. LB and DWA edited the paper and directed the layout of the sections.

Objective of the manuscript:

To explain the methods and methodological approaches used to study the function of Bcl-2 family proteins. These methods were used for the experiments reported in this thesis. Although the example data for some experiments in this chapter was provided by JK I have made extensive use of all of these assays.

Highlights:

- The ANTS/DPX release assay can be used to dissect exactly how the different classes of Bcl-2 family proteins affect permeabilization of the OMM.
- Fluorescence resonance energy transfer (FRET) can be used to detect direct binding of protein to protein.
- NBD fluorescence change and iodide quenching assay can be used to determine the topology of proteins within membrane.

1. INTRODUCTION

The Bcl-2 family of proteins regulates permeabilization of the outer mitochondrial membrane (OMM). In most cell types once the OMM is permeabilized the cell is committed to undergoing programmed cell death (Budd et al, 2000). The sequence of events leading to permeabilization of the OMM begins with pro-death signals triggering post-translational modifications of activator BH3-only proteins, such as the cleavage of Bid to cBid, that target them to the OMM where they bind to and activate the pore-forming proteins Bax and Bak. Activation of Bax and Bak results in their oligomerization within the OMM followed by permeabilization of the OMM and release of intermembrane space proteins such at cytochrome c and SMAC that act in downstream apoptotic pathways, ending in cellular apoptosis (Shamas-Din et al, 2013b). The anti-apoptotic proteins, such as Bcl-2 and Bcl-X_L inhibit apoptosis by binding to and sequestering both BH3-only activators and Bax/Bak (Bogner et al, 2010).

Significant research has been focused on determining the structure of Bcl-2 family proteins. X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy have revealed that the Bcl-2 family proteins share a highly conserved core structure (Petros et al, 2004). These studies have provided insight into how the Bcl-2 family proteins bind to each other and suggest how they may interact with membranes. However, the current relatively static structures for the Bcl-2 family are for proteins without the lipid bilayer required for functional interactions of several of the Bcl-2 family proteins (Leber et al, 2007).

Determining the structures of proteins within a membrane mimetic environment using X-ray crystallography and NMR spectroscopy is particularly difficult. These techniques require a large amount of protein in a sample environment that does not mimic that of the cell and typically includes detergents that can alter the functions of the Bcl-2 family proteins (Hsu & Youle, 1997). As an example, unlike native Bax, detergent treated Bax can cause permeabilization of the OMM when added to isolated mitochondria (Antonsson et al, 2000); can form oligomers that can be cross-linked in the absence of membranes (Zhang et al, 2010) and has undergone a conformational change that is a pre-requisite for Bax activation (Yethon et al, 2003). Additionally, the zwitterionic detergent CHAPS can prevent the authentic interaction of Bax and tBid (Lovell et al, 2008), further reinforcing the need to study the Bcl-2 family proteins in the absence of detergents.

Fluorescence based techniques are well suited to study protein dynamics at membranes under physiological conditions in the absence of detergents (Kale et al, 2012). Fluorescence spectroscopy allows observation of protein:protein and protein:membrane binding dynamics in real-time while gathering information about the kinetics and affinities of these interactions that cannot be measured using typical structural techniques due to complications from the membrane (Perez-Lara et al, 2012; Satsoura et al, 2012). Additionally, by using an environment sensitive probe such as NBD, it is possible to determine the environment of specific residues as they undergo conformational changes within membranes (Malhotra et al, 2013; Shamas-Din et al, 2013a). Initial fluorescence

based studies of the Bcl-2 family proteins have used a simple *in vitro* system to study the dynamic interactions that occur at, on and within membranes.

2. AN *IN VITRO* FLUORESCENCE-BASED LIPOSOME SYSTEM

The molecular function of Bcl-2 family proteins is to permeabilize membranes. Therefore, to study the function of these proteins, a biochemical system is required that includes a phospholipid bilayer that separates two distinct aqueous compartments mimicking that of the cytoplasm and the interior of cellular organelles. We and others (Bleicken et al, 2010; Landeta et al, 2011; Ren et al, 2010; Shamas-Din et al, 2013a) have used different variations of liposome or proteoliposome based systems to study the core mechanism of Bcl-2 family protein regulation of membrane permeabilization. All of these systems lack the detergents typically required for biochemical and structural studies of membrane proteins. For our studies fluorescently labeled purified full-length recombinant proteins and artificial membranes in the form of liposomes with a composition mimicking that of the OMM are used. This system is free of any other complicating factors such as unknown binding partners that may be present at the OMM or within the cytoplasm.

To use fluorescence to study proteins at membranes it is essential to make judicious choices of fluorophore, type of measurement and instrument. Fluorescence measurements require excitation of the fluorophore with a specific wavelength of light and then recording the emission from the fluorophore.

Excitation of the fluorophore is accomplished by illuminating the sample with a set wavelength of light. The system must not contain components or contaminants that absorb light of the same wavelength as the fluorophore or they will absorb the excitation light. Upon excitation, after some period of time, termed the fluorescence lifetime (typically 1-10 nanoseconds), the fluorophore returns to the ground electronic state via emission of a photon at a lower energy, and thus longer wavelength, than the illuminating (excitation) light. Therefore, the system must also be free of molecules that absorb the emitted light because fluorescence is of much lower intensity than the excitation light. Molecules such as quenchers that provoke non-radiative decay of the fluorophore must also be avoided as they change the fluorescence properties of the dyes. If these conditions are met, changes in both fluorescence lifetime and emission intensity can provide specific information about the underlying biochemical properties of the protein the fluorophore is attached to (Lakowicz, 2006).

2.1 Site specific protein labeling

The fluorescence based techniques we use to study the Bcl-2 family proteins require purified recombinant proteins labeled with a fluorophore at a specific location. There are two main options for labeling proteins, thiol or primary amine labeling. Cysteine residues are less abundant than lysines in most protein sequences thus we most frequently create single cysteine mutants to label the protein as this approach minimizes the number of mutations in the protein. There

is a full spectrum of fluorescent probes available for purchase, which have different spectral properties, that can be can be ordered with attached thiol reactive moieties such as iodoacetamide or maleimide derivatives. Dyes must be chosen that are compatible not only with your protein of choice but also with the system and equipment available.

In the methods reported below, the proteins were labeled with the low molecular weight fluorescent probes DAC (*N - (7 - Dimethylamino - 4 - methylcoumarin - 3 - yl) maleimide*) and NBD (N,N'-Dimethyl-N-(Iodoacetyl)-N'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl) Ethylenediamine). The small size of these dyes is a distinct advantage as they rarely perturb protein function however, measurements of NBD fluorescence require a sensitive instrument as the quantum yield (ratio of photons emitted to photons absorbed) is low. Moreover, excitation of DAC requires an ultraviolet light source and both the excitation and emission of this dye overlap endogenous fluorophores in cells typically limiting its use to liposome based systems. Many brighter (higher quantum yields and extinction coefficients) fluorescent dyes have molecular weights above 1kDa, and in our experience these larger dyes frequently change the function of the protein they are attached to.

Initially it is best to follow the labeling protocol included by the manufacturer when labeling your protein of interest, however it is often necessary to deviate from these conditions to get labeling that is both specific and efficient. Briefly, the protein and labeling reaction are both in a HEPES or PBS based buffer at pH 7.0-

7.5. This pH range allows the cysteines to be most-reactive while decreasing the reactivity of primary amines. A 10-20x molar excess of dye is added to the sample tube and the labeling reaction is rotated at room temperature for approximately 2 hours in the dark. A reducing agent (5 mM DTT) is then added to quench the reaction and free dye is removed via gel filtration or affinity chromatography (providing the recombinant protein contains an affinity tag). The protein is then dialyzed into a storage buffer to remove any remaining free dye and the protein is aliquoted and stored for later use. The efficiency of labeling is generally determined by absorbance spectroscopy as outlined in the protocol supplied by the manufacturer.

Single cysteine mutants of the purified recombinant protein need to be assayed functionally before and after labeling to determine if the mutation or the addition of the dye alters protein function. Ideally we begin using mutants where one of the endogenous cysteines is present to minimize the amount of mutations introduced into the protein. If the protein does not contain any cysteines, choosing which residue to mutate to cysteine for efficient labeling and proper protein function is largely serendipitous. Typically if the structure is known, one begins using solvent exposed residues, since those located in hydrophobic regions are difficult to label. Algorithms used to predict solvent exposure or antigenicity (antigenic sites tend to be both structured and solvent exposed) can often be useful in selecting a location if the structure of your protein is unknown.

2.2 Production of mitochondria-like liposomes

Large unilamellar vesicles (LUVs) are liposomes that have one lipid bilayer with a mean diameter of 120-140 nm (Hope et al, 1985). OMM-like LUVs have been established as a valid biochemical model for membrane permeablization by Bcl-2 family members (Kuwana et al, 2002). These liposomes are assembled from lipids in fixed molar ratio similar to that of the OMM, based upon lipid composition studies from solvent extracted Xenopus mitochondria (Kuwana et al, 2002). Such liposome-based systems allow the analysis of Bcl-2 family proteins in a simple context while preserving their authentic functions. It is possible to more directly explore Bcl-2 family function in this kind of system because the protein and lipid components are well defined and tractable, unlike isolated mitochondria or proteoliposomes prepared from membranes.

2.2.1 – Preparing lipid films into liposomes

1. Chloroform solublized lipids are added to a glass test tube to make a lipid mixture of a defined composition (Table 1) to a total of 1 mg lipid mass. The chloroform is evaporated off with nitrogen gas while rotating the tube to ensure an even distribution of lipids on the wall and then put under vacuum for 2 hours at room temperature to remove any remaining chloroform. The dry lipid film is then either used immediately or can be stored for up to 2 weeks at -20°C. To reduce lipid oxidation by atmospheric oxygen during storage it is advisable to layer nitrogen or argon gas on top of the lipid film and seal the tube with parafilm.

Table 1- Mitochondria-like lipid film composition

Name	Company	Catalog #	Molar percent (%)	Molecular weight (g/mol)	Amount needed for 1 mg lipid film (mg)
"PC" - L-α-phosphatidylcholine (Egg, Chicken)	Avanti	840051C	48	770.123	0.4596
"PE" - L-α-phosphatidylethanolamine (Egg, Chicken)	Avanti	841118C	28	726.076	0.2528
"PI" - L-α-phosphatidylinositol (Liver, Bovine)	Avanti	840042C	10	902.133	0.1122
"DOPS" -1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine	Avanti	840035C	10	810.025	0.1007
"TOCL"- 1,1',2,2'-tetra-(9Z-octadecenoyl) cardiolipin	Avanti	710335C	4	1501.959	0.0747

2. The dry 1 mg lipid film is hydrated with 1 mL of assay buffer (10mM HEPES, 200mM KCl, 5mM $MgCl_2$, 0.2mM EDTA, pH7) . The lipids become suspended and spontaneously form lipid bilayer vesicles due to the association of the hydrophobic tails, forming the center of the bilayer, and the grouping of the hydrophilic heads of the phospholipids, forming the edges of the bilayer. However, these vesicles are multilamellar as they contain more than one lipid bilayer and their size distribution is not homogeneous. To generate unilamellar liposomes the lipid mixture is subjected to 8-10 freeze/thaw cycles by alternately placing the sample vial in liquid nitrogen and a warm water bath (Hope et al, 1985). The unilamellar liposomes are extruded eleven times through a filter with 0.1μm pore size to produce liposomes of a uniform size, at a final concentration of 1 mg/mL lipid.

3. MEMBRANE PERMEABILIZATION ASSAY

The Bcl-2 family proteins play a pivotal role in regulating apoptosis by controlling the permeabilization of the OMM through the activation of Bax/Bak. Thus a membrane permeablization assay is one crucial functional assay for the Bcl-2 family proteins. To assay liposome permeabilization the liposomes are encapsulated with a polyanionic fluorophore, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), and cationic quencher, p-xylene-bis-pyridinium bromide

(DPX). Due to the high local concentration of DPX, ANTS fluorescence is quenched when liposomes are still intact. Recombinant Bax and/or other Bcl-2 family proteins and/or reagents are added to the system in order to assay permeabilization. As the liposomes permeabilize, ANTS and DPX are released from the liposomes, greatly decreasing the local concentration of the quencher resulting in a gain of ANTS fluorescence. The kinetics and extent of membrane permeabilization can reveal crucial information for studying relationships between Bcl-2 family members and how they regulate membrane permeabilization.

3.1 ANTS/DPX release assay

1. A dry 1 mg lipid film is hydrated with 1 mL of assay buffer with the addition of ANTS (12.5 mM) and DPX (45mM). The lipid suspension is vortexed until the ANTS and DPX dissolve, and liposomes are created as above via 10 freeze thaw cycles and extrusion through a 0.1µm pore size membrane

2. Excess ANTS and DPX are removed by applying the extruded liposomes onto a CL2B size-exclusion column (10 mL bed volume), that separate the ANTS/DPX encapsulated liposomes from the free ANTS/DPX in solution (Billen et al, 2008; Yethon et al, 2003). Fractions (1 mL each) are collected in glass tubes and the liposome containing fractions (typically fractions 3 and 4) are identified by an increase in cloudiness of the sample which occurs due to light scattering by the liposomes. The two liposome containing fractions are combined resulting in a final ANTS/DPX liposome concentration of approximately 0.5 mg/ mL lipid. These

liposomes can now be used to test the regulation of membrane permeabilization by the Bcl-2 family proteins

3. The assay is set up in a low protein binding 96 well plate and in each well to be measured, 8 µL of ANTS/DPX liposomes are added to 92 µL of assay buffer. Background measurements (F_0) are recorded at 30°C using a fluorescence plate reader (Tecan M1000 pro) set to excite the sample at 355 nm (5 nm bandwidth) and collect emission at 520 nm (12 nm bandwidth).

4. Proteins are added to the desired concentrations and combinations in each well and fluorescence emission of ANTS (F) is recorded every minute for 3 hours at 30°C. Any increase in fluorescence emission is directly related to membrane permeabilization.

5. To normalize the data, 100% ANTS release is determined by the addition of Triton to each well at a final concentration of 0.2% (w/v) causing permeabilization of all liposomes and ANTS fluorescence is measured (F_{100}). This results in a slight over-estimation of the intensity of 100 percent release due to the dye becoming trapped in detergent micelles. Nevertheless, the release percentage generally does not take this into account and is calculated as follows:

$$\text{ANTS Release (\%)} = \frac{\mathrm{F} - \mathrm{F_0}}{\mathrm{F_{100}} - \mathrm{F_0}} \times 100\%$$

The ANTS/DPX release assay can be used to dissect exactly how the different classes of Bcl-2 family proteins affect permeabilization of the OMM. When cBid (20 nM), Bax (100 nM) or Bcl-X_L (40 nM) are added individually to liposomes they do not cause membrane permeabilization (Figure 1.1). Incubation of liposomes with cBid and Bax results in membrane permeabilization due to cBid binding to membranes causing separation of the two fragments of cBid and the larger of the two (tBid) activating Bax. Bcl-X_L inhibits this process by binding to and inhibiting both tBid and Bax (Billen et al, 2008; Lovell et al, 2008). Obviously, other techniques are needed to discern exactly how these interactions occur (see FRET section below), however this assay allows the functional consequence of the addition of any number of various combinations of Bcl-2 family members or small molecule effectors of the proteins to be determined. Furthermore, it provides information on how changes in relative concentrations between the proteins can change the extent of permeabilization or how alterations in the parameters of the assay affect membrane permeabilization. For example, it is possible quantify how changes in liposome composition affect Bcl-2 family proteins functions to permeabilize membranes or test specific mutations that may inhibit/activate the protein of interest. Additionally, the kinetics of pore formation can be studied allowing the comparison of kinetics for Bax mediated membrane permeabilization in response to various BH3-only activators (Figure 1.2).

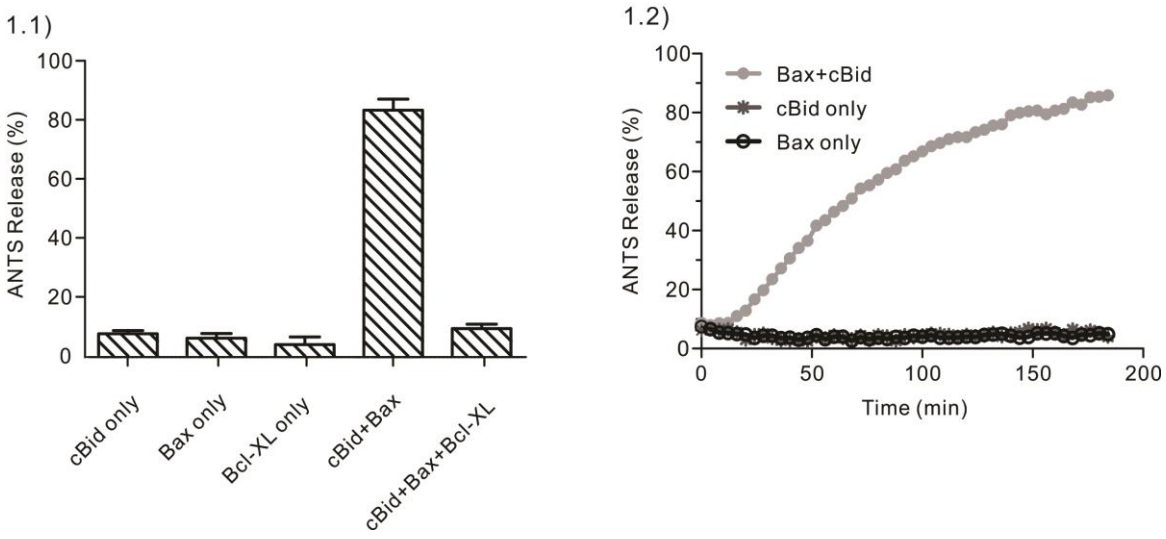

Figure 1

1.1) Endpoint values of ANTS assay with 100 nM Bax, 20 nM cBid, 40nM Bcl-XL or both, or with 100 nM Bax, 20 nM tBid, and 40nM Bcl-XL. (n=3) 1.2) Liposomes encapsulated with ANTS and DPX were incubated with 100 nM Bax, 20 nM cBid, or both. Membrane permeabilization was assayed by an increase of ANTS fluorescence.

4. FLUORESCENCE RESONANCE ENERGY TRANSFER

Here Fluorescence Resonance Energy Transfer (FRET) will be used to detect binding between cBid and Bax, and Bax oligomerization. FRET is possible between fluorophores when the emission spectra of one fluorescent molecule, termed the donor, overlaps the excitation spectra of another fluorophore, the acceptor. When a donor fluorophore is excited by light, an electron moves to a higher energy state and, in the presence of an acceptor, the energy is transferred non-radiatively to the acceptor fluorophore via dipole-dipole interactions between the two probes. This transfer of energy results in a decrease of the donor emission, and it is the change in the light emitted by the donor that we will track to measure FRET between two proteins.

One of the main advantages of FRET is that it requires both the donor and acceptor fluorophores to be in close proximity for the required dipole coupling to occur. As a result FRET efficiency decreases to the 6^{th} power of distance according to the formula for FRET efficiency (E) at a fixed donor acceptor distance:

$$E = \frac{R_0^6}{R_0^6 + r^6}$$

Where R_0 is the Förster distance, the distance between a donor acceptor pair at which a 50% FRET efficiency is observed, and *r* is the distance between the

donor and acceptor. The distance dependence of FRET is illustrated in Figure 2.1 where FRET efficiency is calculated for distances between a donor and acceptor pair with an R_0 of 50 Å (Lakowicz, 2006). For this dye pair, FRET will only be detected if the distance between the two fluorophores is 70Å or less. Typical R_0 values for a donor and acceptor pair are between 30-60Å, similar to the size of proteins, thus if FRET between donor and acceptor labeled proteins is detected then they are binding.

4.1 Detecting the interaction between two proteins using FRET

As mentioned in the introduction, the BH3-only protein cBid targets to, and embeds within the OMM where it recruits and activates cytosolic Bax (Leber et al, 2007; Lovell et al, 2008). Active membrane bound Bax oligomerizes within the OMM and resulting in membrane permeabilization. Here, we are using DAC and NBD as the donor and acceptor molecules respectively. We will be using FRET to detect 1) the binding between cBid and Bax and 2) the binding between Bax molecules during oligomerization.

1. Liposomes are made as above (section 2.2.1) resulting in liposomes at a concentration of 1 mg/mL lipid

2. The fluorimeter (Photon Technology International) is set to record the fluorescence of DAC (380 nm excitation, 2nm slit width; 460 nm emission, 10 nm slit width) with stirring for 1 hour at 37°C. Either 200 µL of liposomes and 800 µL of assay buffer, or as a control, 1 mL of assay buffer is added to a quartz cuvette

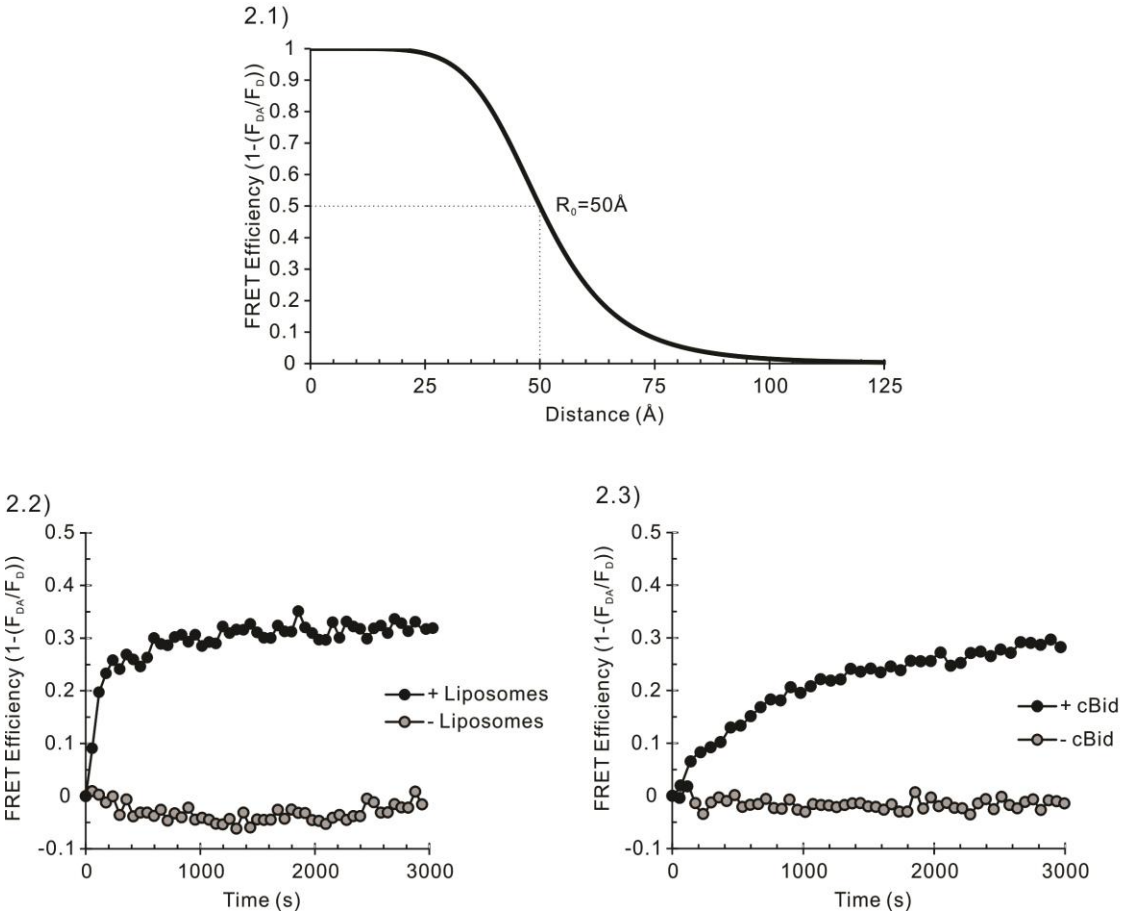

Figure 2

2.1) FRET efficiency as a function of distance between a dye pair with a theoretical Förster distance of 50 Å. 2.2) FRET between cBid-DAC (20 nM) and Bax-NBD (100 nM) in the presence (black circles) and absence (grey circles) of liposomes (0.2 mg/mL). 2.3) FRET between Bax-DAC (20 nM) and Bax-NBD (100 nM) in samples containing liposomes (0.2 mg/mL) with (black circles) or without (grey circles) 20 nM cBid

and the signal is read until it remains stable (approximately 5 minutes). Two reactions are required to detect FRET. One that contains both the donor and acceptor labeled proteins and a control that contains the donor labeled protein and unlabeled acceptor protein. This control accounts for any changes in the donor protein that occur due to binding interactions, conformational changes or environment changes that may affect the spectral properties of the donor dye.

3. The donor labeled protein is added to the cuvette at a concentration of 20 nM and DAC fluorescence is read until the signal is stable. At this point the acceptor protein that is either labeled with NBD or unlabeled is added to the system at a concentration of 100 nM. It is important to keep the amount of acceptor higher (5-10 x) than that of the donor. This allows for the donor protein to be saturated by the acceptor ensuring that every donor dye has an acceptor for FRET to occur.

4. The DAC signal is recorded for 1 hour at 37°C. FRET efficiency (E) is measured by comparing the relative intensity of the donor in the presence of labeled (F_{DA}) and unlabeled (F_D) acceptor and is calculated by:

$$E = 1 - \frac{F_{DA}}{F_D}$$

Figure 2 illustrates two binding interactions between the Bcl-2 family proteins. Donor (DAC) labeled cBid (20 nM) is incubated with acceptor (NBD) labeled Bax (100 nM), and only in the presence of liposomes do the two proteins

interact (Figure 2.2). This underlines the point that many functional interactions of the Bcl-2 family proteins only occur in the presence of a lipid bilayer. Additionally, the activator protein cBid is required for Bax to oligomerize, since FRET between donor (DAC) and acceptor (NBD) labeled Bax is only observed when cBid is added to the system (Figure 2.3). Since we can observe the interactions of two proteins in real-time, kinetics of the reactions can be determined. Indeed, it is clear from the data shown that the cBid-Bax interaction occurs faster than Bax oligomerization suggesting that cBid first binds to and activates Bax followed by Bax oligomerization. Additionally, it is possible to generate a binding curve where an affinity for the interaction can be determined as was done for the binding between cBid and Bax (Lovell, et al., 2008). To do this multiple FRET measurements are obtained by titrating the amount of acceptor while keeping the donor concentration fixed.

5. TRACKING THE CONFORMATION CHANGES OF A PROTEIN

NBD is an environment sensitive low-molecular weight fluorescent dye that has been used to track environment changes of specific residues of proteins (Dattelbaum et al, 2005; Lin et al, 2011b) . The emission intensity and fluorescence lifetime increases and the emission peak of NBD blueshifts from 570nm, in an aqueous environment, to 530 nm when it is in a hydrophobic environment due to a decrease in fluorescence quenching by water (Crowley et al, 1993). The small size of NBD allows for the specific labeling of single cysteine

mutants of proteins, with less potential perturbation of wild-type function. Importantly, NBD is uncharged but has sufficient polar characteristics that it remains stable in both polar and non-polar environments such that it is less likely than other environment sensitive dyes to change the membrane binding characteristics and/or conformation of the protein being studied (Shepard et al, 1998). These properties of NBD make it particularly useful to study membrane binding proteins such as Bax and cBid that transition from the aqueous environment and embed into a membrane bilayer (Lovell et al, 2008; Shamas-Din et al, 2013a).

5.1 NBD-emission assay

Real-time changes in the fluorescence of NBD can be measured to determine whether and when specific regions of Bax (labeled with NBD) insert into the membrane during the activation of Bax. It is known from chemical labeling studies that Bax inserts helices 5, 6 and 9 into the membrane (Annis et al, 2005). By labeling Bax at residue 175 (helix 9) it is possible to track the conformational change of Bax as it transitions from a soluble monomer to membrane embedded oligomer.

1. The fluorimeter is set to record NBD fluorescence (475 nm excitation, 2nm slit width; 530 nm emission, 10 nm slit width) and, as in the FRET experiment above, 200 µL of 1 mg/mL liposomes are added to 800 µL of assay buffer in a quartz cuvette. Background signal (Bg) is recorded with stirring until stable at 37°C.

2. NBD labeled Bax (100 nM) is added to the cuvette. Since Bax does not insert into membranes in the absence of an activator (Hsu & Youle, 1998), Bax-NBD can be incubated with liposomes and an initial fluorescence value can be recorded (F_0) . Alternatively, the very first point upon addition of the protein can be used as the F_0 value if the protein insert into lipids too rapidly. This approach is useful for proteins that are unstable in the assay solution such as cBid which spontaneously targets to membranes (Shamas-Din et al, 2013a). In the absence of membranes cBid has sufficient exposed hydrophobicity that it tends to aggregate and to stick to the walls of the cuvette.

3. In our example the change in emission over time (ΔF) of the dye labeled Bax is collected once an activator, cBid, is added. Fluorescence intensity plateaus after the protein comes to equilibrium (1 hour endpoint). By calculating the F/F_0 value one can track the relative change in emission intensity of the labeled residue in real-time:

$$\Delta F = \frac{F - Bg}{F_0 - Bg}$$

Both residues 3 and 175 of Bax transition to a more hydrophobic environment as indicated (Figure 3.1) by the relative change in emission (ΔF). Since the environment change of the residue can be tracked over time, kinetics of membrane binding can be measured. Tracking the kinetics of the environment changes of various residues as a protein undergoes a conformational change

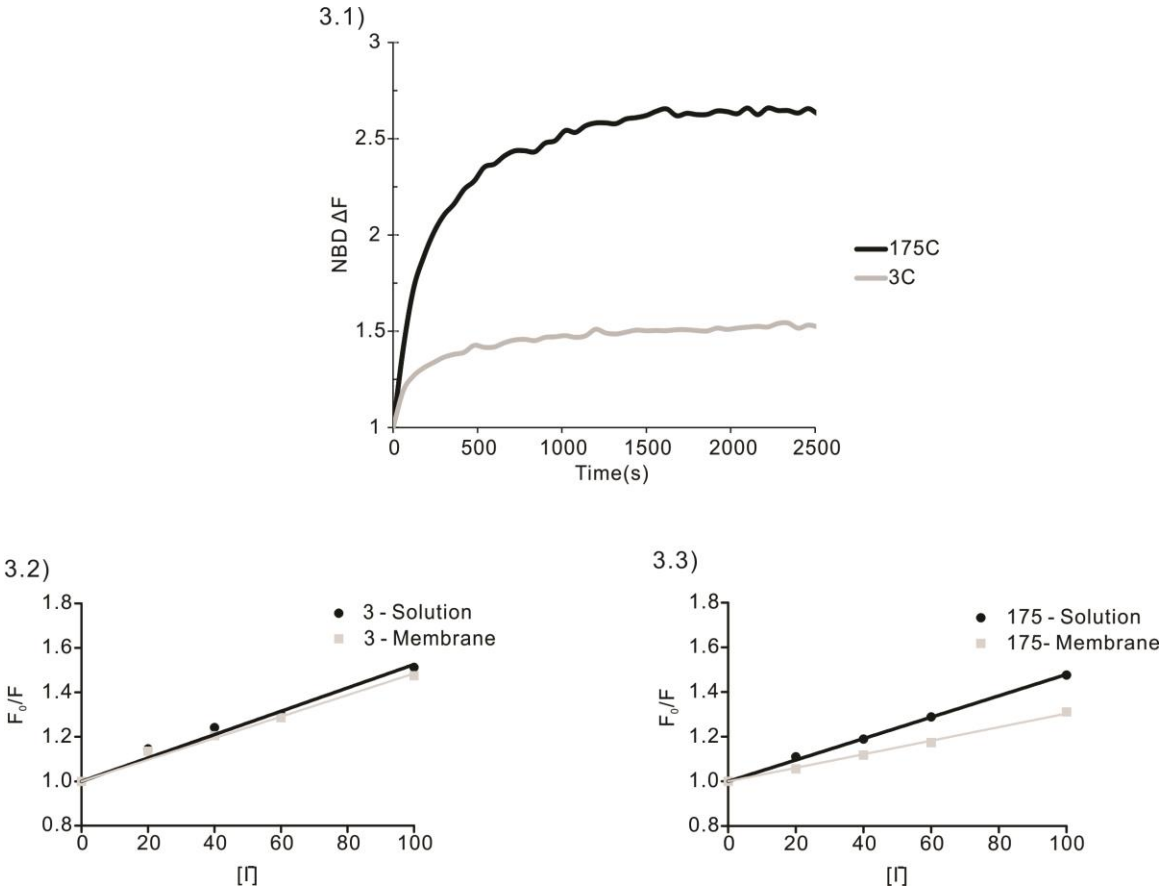

Figure 3

3.1) NBD emission change for Bax 175C-NBD (100 nM) and Bax 3C-NBD (100 nM) upon addition of cBid (20 nM) in the presence of liposomes (0.2 mg/mL) 3.2&3.3) Iodide quenching data of 100 nM Bax 3C-NBD (3.2) or 175C-NBD (3.3) in solution (black) or in the presence of liposomes (0.2 mg/mL) and cBid (20 nM) (grey).

could be used to order specific structural changes of the protein (Shamas-Din et al, 2013a). Here, the carboxyl terminus of Bax (residue 175) seems to have slower kinetics compared to that of the amino terminus of Bax (residue 3) suggesting that Bax undergoes a conformational change at residue 3 before that of 175. Additionally, residue 175 has a larger change in NBD emission suggesting that it is moving to a more hydrophobic environment. This paired with the quenching data discussed below, suggests that residue 175 of Bax inserts into the phospholipid bilayer. As this residue is part of a larger hydrophobic sequence believed to span the bilayer the kinetics for this residue likely represent insertion of the Bax carboxyl-terminal tail into membranes. This is in accordance with data that shows that residue 175C is embedded within the membrane (Annis et al, 2005). By using various activators of Bax or mutations known to perturb Bax function we could see if these changes affect the extent of or rate at which Bax helix 9 inserts into phospholipid bilayers.

6. DETERMINING THE TOPOLOGY OF PROTEINS WITHIN MEMBRANES

Fluorescence quenching by heavy atoms such as iodide can be used to determine how exposed a fluorescently tagged residue is to the solvent. This is due to collisional quenching that occurs when I^- collides with an excited fluorophore, resulting in a loss of energy back to ground state without emission of a photon. Typically collisional quenching requires direct molecular interaction with

the fluorophore such that the distance of quenching is <2Å giving a very high resolution to detect solvent accessibility (Lakowicz, 2006).

Since I^- quenches NBD fluorescence (Crowley et al, 1993; Lin et al, 2011a), this technique would be advantageous to look at the difference of residue solvent accessibility between soluble monomeric Bax, in the absence of activator, and membrane bound oligomeric Bax, in the presence of an activator.

6.1 Iodide quenching of NBD labeled Bax

1. As in the method for NBD emission change, the fluorimeter is set to record NBD fluorescence, and 200 µL of 1 mg/mL liposomes are added to 800 µL of assay buffer in a quartz cuvette. Background signal is read with stirring until stable at 37°C.

2. NBD labeled Bax (100 nM) and cBid (20 nM) are added to cuvettes containing either liposomes (200 µL liposomes, 800 µL assay buffer) for quenching of membrane bound Bax, or assay buffer only (1 mL assay buffer), for quenching of solution Bax. NBD emission (F_0) is recorded after incubation of the sample at 37°C for one hour.

3. Multiple quenching reactions are set up where aliquots of Potassium Iodide (2M, supplemented with 2 mM sodium thiosulfate to prevent oxidation) and Potassium Chloride (2M) stock solutions are added to each sample so that the total ion concentration, and thus ionic strength, in samples is the same (typically

100 mM) (Table 2). The NBD emission for each concentration of KI is determined (F) and collisional quenching is calculated by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + K_{sv}[Q]$$

Where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity at a specific quencher concentration [Q] and K_{sv} is the Stern-Volmer quenching constant.

Table 2- Iodide quenching data for Bax 175C

KI (mM)	KCl (mM)	Solution* (F0/F)	Membrane* (F0/F)
0	100	1	1
20	80	1.110372	1.056134
40	60	1.189457	1.117945
60	40	1.288603	1.173357
100	0	1.476333	1.311353

*Values for solution and membrane quenching are plotted in figure 3.3

As the concentration of I^- increases, so does the extent of quenching as determined by the Stern-Volmer equation, allowing the titration curve to be fit with a line where the slope is the Stern-Volmer constant (K_{sv}). The smaller the Stern-Volmer constant the more protected a residue is from the solvent. Quenching can then be used to compare the change in exposure of Bax residues to solvent upon the addition of an activator. Correlating with the NBD emission changes, residue 3 shows no change in protection from quenching upon the addition of cBid (Figure 3.2), whereas residue 175C of Bax becomes more protected from quenching, in agreement with this region of Bax inserting into the bilayer (Figure 3.3) (Annis et al, 2005).

7. CONCLUSION

Here, four techniques have been highlighted to show how membrane proteins can be studied by fluorescence spectroscopy. The high sensitivity of fluorescence based assays along with the ability to probe the dynamics of protein:protein and protein:membrane interactions in real-time lends itself well to study a complex regulatory system such as the Bcl-2 family of proteins. The techniques described here have been adapted to study many different aspects of apoptosis regulation in vitro and in live cells and can be applied to study other biological systems (Kale et al, 2012).

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Study Rationale and Thesis Outline

Due to the difficulty expressing and purifying full-length Bim, most *in vitro* experiments to date have been conducted with peptides or deletion mutants lacking the MBD, which may affect the affinities of these interactions (Chen et al, 2005). Assessing binding interactions between Bim and Bax in cells via co-immunoprecipitation and immunoblotting may not resolve the issue of how Bim functions, because these methods give inconsistent results that are prone to detergent artifacts (Hsu & Youle, 1997; Lovell et al, 2008). Thus the role of the Bim in membrane binding, sensitizer and activator functions remains unclear.

To answer those questions, I first successfully purified recombinant full-length murine BimL and BimS and assayed them in an *in vitro* MOM-like liposome system where individual interactions can be measured and their effects quantified without interference from other cellular component. By performing membrane permeabilization assay and protein-protein Förster resonance energy transfer (FRET) with labeled proteins in the liposome system, I confirmed that both Bim major isoforms: BimL and BimS have similar activity in terms of binding and activating Bax as well as binding anti-apoptotic protein Bcl-XL. Next, to compare Bim with well-established BH3 activator Bid, I carried out membrane permeabilization assay in mouse liver mitochondria by measuring cytochrome c release with Bim or Bid protein. By using mitochondria isolated either from WT mice or Bak -/- mice, I set up conditions where Bim or Bid only activate either Bax or Bak, and compare their activity as EC50 from BH3 protein titration curves. To eliminate the possible interference of mitochondrial protein such as Mtch2, same

comparisons have been carried out with BH3 peptides and/or in the liposome system. The sensitizer function of Bim and Bid was quantified with the affinity to Bcl-XL. To achieve this, I generated binding curves by performing BH3 protein-Bcl-XL FRET in the liposome system. Previous work has revealed that Bad-BH3 mimetic drug ABT-263 (and its precursor ABT-737) could not displace Bim from Bcl-XL binding (Aranovich et al, 2012; Liu et al, 2012). Thus multiple Bim-Bcl-XL or Bid-Bcl-XL binding curves were generated under different concentrations of ABT-263. The impact of ABT-263 were observed with the shift of dissociation constant (Kd) from the binding curves. I have also tested the function of the recently discovered anti-apoptotic BH3 protein TCTP by showing its capability of enhancing the Bcl-XL function of inhibiting Bim and Bid mediated Bax activation. This was done by setting up specific conditions in the liposome permeabilization assay where TCTP can promote Bcl-XL inhibiting Bim, Bid (and Bax) respectively. A reported mutant TCTP R21A that significantly reduced its Bcl-XL binding was test as a control.

By sequence analysis, Bim contains a very unusual C-terminal membrane binding domain (MBD) in that it includes two positive charged Arg residues yet is considered crucial for binding Bim to membranes (Weber et al, 2007; Wilfling et al, 2012). To quantify the functional importance of the MBD, I created multiple mutations on the domain including a mutant deleting the entire MBD sequence (BimΔMBD). I assess binding of these mutants to lipid membranes, to Bax and Bcl-XL by FRET individually and report the affinity in the form of Kd. The

sensitizer function of Bim∆MBD was further examined in the form of disrupting Bid-Bcl-XL and Bax-Bcl-XL FRET, and restoring Bid-Bax mediated membrane permeabilization which was inhibited by Bcl-XL in liposome system and at mitochondria.

To examine the interaction between Bim and Bcl-XL and the functional consequence of Bim inhibition of Bcl-XL in cells where other factors or protein modifications may modulate these effects, Elizabeth Osterlund from our lab measured binding interactions in MCF-7 breast cancer cells between mCerulean3 fused to Bcl-XL (Cer-Bcl-XL) that are stably expressed in cell and transiently transfected N-terminal Venus-fused Bim (V-Bim) mutants by Fluorescence Lifetime Imaging Microscopy based Förster Resonance Energy Transfer (FLIM-FRET) (Aranovich et al, 2012). It was difficult to measure Bim-Bax interactions with FLIM-FRET, as exogenous expression of V-Bim and Cer-Bax proved to be too toxic for cells. Thus I measure the cell death induced by transient transfected Bim mutants as an indirect approach test how Bim mutants activate Bax or Bak in cells, I transiently expressed N-terminal Venus-fused Bim mutants in WT BMK cells, WT HCT116 cells and WT MEF cells. Cell death was quantified apoptosis by AnnexinV staining.

Lastly, to better understand the role of MBD of Bim in binding to Bcl-XL, I introduced mutations on MBD and observed the effect of ABT-263 on Bim -Bcl-XL complex to screen for the crucial residues that are important for the extra binding. To achieve this, I observed Bim-Bcl-XL complex by FRET with specific

concentration of labeled Bim MBD mutants and Bcl-XL so that 100% Bim is binding to Bcl-XL. ABT-263 is then titrated in to generate dose response curve for ABT-263 as the decrease of the Bim-Bcl-XL FRET is propotional to the displacement of Bim from Bcl-XL. I also looked into the conformation of MBD on the membrane and how interaction with Bcl-XL would affect it. This is achieved through introducing single-cysteine residues across the MBD of Bim labeled with environment sensitive dye NBD individually and monitored for its hydrophobicity and protection against aqueous quencher iodide upon binding to membrane and Bcl-XL.

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Manuscript

CHAPTER III: Purification of Bim and Characterization of functional differences between Bim and cBid.

Preface

Some of the work presented in this chapter has been previously published in:

1. Sarosiek KA, **Chi X**, Bachman JA, Sims JJ, Montero J, Patel L, Flanagan A, Andrews DW, Sorger P, Letai A (2013) BID Preferentially Activates BAK while BIM Preferentially Activates BAX, Affecting Chemotherapy Response. *Mol Cell* **51:** 751-765
2. Thebault S, Agez M, **Chi X**, Stojko J, Cura V, Telerman SB, Maillet L, Gautier F, Billas-Massobrio I, Birck C, Troffer-Charlier N, Karafin T, Honore J, Senff-Ribeiro A, Montessuit S, Johnson CM, Juin P, Cianferani S, Martinou JC, Andrews DW, Amson R, Telerman A, Cavarelli J (2016) TCTP contains a BH3-like domain, which instead of inhibiting, activates Bcl-xL. *Sci Rep* **6:** 19725

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Objective of the chapter:

To purify recombinant protein of Bim, and to characterize the functional difference between Bim and the other well-studied activator Bid.

Highlights:

- Major Bim isoforms, BimL and BimS, have similar activities when examined in *in vitro* system.
- Bim preferentially activates Bax, and Bid preferentially activates Bak.
- Unlike Bid, Bim binding to Bcl-XL is resistant to Bcl-XL inhibitor ABT-263.
- TCTP, a novel anti-apoptotic BH3 protein, depends on its residue R21 for enhancing Bid (and Bax) inhibition by Bcl-XL, but not for Bim inhibition by Bcl-XL.

Introduction

Multi-cellular organisms maintains the homeostasis via apoptosis by eliminating the old, unhealthy or redundant cells (Kerr et al, 1972). Consequently, deregulation of apoptosis has been found to be related to the onset of many diseases (Ramírez Chamond R., 1999). Bcl-2 family proteins play a crucial role in regulating apoptosis by controlling permeabilization of the mitochondrial outer membrane (MOM). Disruption of MOM results in the release of apoptogenic factors such as cytochrome c and SMAC from the inter-membrane space (Tait & Green, 2010; Wang, 2001), which is followed by the initiation of downstream executioner caspases and eventually leads to cell death (Chipuk et al, 2006).

Members of the Bcl-2 family contain at least one of four conserved motifs known as the Bcl-2 homology (BH) regions, termed BH1, BH2, BH3, and BH4. The Bcl-2 family consists of both anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins such as Bcl-2, Bcl-XL and Mcl-1 contain all four BH regions. The pro-apoptotic proteins can be further divided into multi-BH domain proteins such as Bax, Bak, and BH3 proteins such as Bid, Bim, Puma, Bad and Noxa, based on the presence of one or more BH regions (Youle & Strasser, 2008). Some BH3 proteins like Bim, Bid are termed "activator" as they cause Bax or Bak to oligomerize on and permeablize the MOM. Anti-apoptotic proteins inhibit the process by binding to both activator BH3 and activated Bax, resulting in mutual sequestration. BH3 proteins also possess "sensitizer" function where excess amount of BH3 proteins may inhibit anti-apoptotic proteins, releasing the

sequestered activator BH3 and activated Bax. The equilibrium of interactions in-between Bcl-2 family members and membranes regulate the permeablization of MOM (Leber et al, 2007; Leber et al, 2010; Shamas-Din et al, 2011). Interestingly, a novel BH3 protein named Translationally Controlled Tumor Protein (TCTP) have been recently identified, which was described as a pro-survival protein by potentiating both Mcl-1 and Bcl-XL and antagonizing the P53 tumor suppressor (Amson et al, 2012; Liu et al, 2005; Yang et al, 2005; Zhang et al, 2002). This marks the first anti-apoptotic BH3 protein identified and suggests a new pathway of regulation of MOM permeabilization (MOMP).

BH3 protein Bim (Bcl-2-interacting mediator of cell death) was originally identified as a Bcl-2-interacting protein by screening a bacteriophage lambda cDNA expression library constructed from a mouse thymic lymphoma (O'Connor et al, 1998). Of the pro-apoptotic BH3 proteins, both Bim and Bid were recognized as BH3 activators that can directly mediate Bax and Bak activation on the membrane and bind to anti-apoptotic proteins leading to mutual sequestration. Although Bim and Bid have been considered to be functionally redundant with regard to Bax and Bak activation, several differences in function and physiological roles have emerged for these two proteins. Bim and Bid have non-overlapping physiological roles in maintenance of homeostasis and distinct expression patterns. Bim, but not Bid, has been linked to cell death following growth factor withdrawal (Biswas & Greene, 2002) and to the apoptosis that is required for proper regulation of the immune system (Bouillet et al, 1999). In

contrast, Bid, but not Bim, is cleaved and activated by caspases 8 and 10 (generating its truncated active form tBid) to trigger cell death in response to activation of cell surface death receptors by Fas, TNF, and TRAIL (Li et al, 1998; Luo et al, 1998). Bid has also been implicated in apoptosis following treatment with DNA damaging agents (Kamer et al, 2005; Zinkel et al, 2005). These functional differences are generally attributed to differences in stress-induced regulation of Bid and Bim rather than to any difference in activation of Bax or Bak.

The role of Bid has been relatively well characterized: Recently it has been shown that membrane targeting of caspase-cleaved Bid (cBid) causes the dissociation of the p7 fragment from tBid, and a series of conformational changes ensue thereafter. The conversion between these conformations on the membrane act as the rate-limiting step for tBid activation (Shamas-Din et al, 2013). In contrast, studies to characterize Bim using full length protein have been difficult due to its intrinsically unstructured and highly hydrophobic nature (Hinds et al, 2006). As a result, *in vitro* experiments have classically used deletion mutants or peptides of Bim which may not reflect the true nature of the full length protein. The study of Bim is further complicated by the existence of several isoforms of Bim, which have different activities in cells (Hinds et al, 2006). Thus, the function of Bim remains to be elucidated.

In this study, we successfully purified full-length recombinant proteins of Bim in large quantity and compared Bim and tBid for their activator and sensitizer function. We show that although both Bim and tBid can activate both Bax and

Bak, they exhibit differential activation preferences: Bim preferentially activates Bax, and tBid preferentially activates Bak. Both preferences were observed consistently using either recombinant proteins or BH3 domain peptides. Furthermore, we showed that Bim and tBid bind to Bcl-XL with similar affinity. Suprisingly, unlike tBid, Bim binding to Bcl-XL is resistant to the interference by Bad-mimicking Bcl-XL inhibitor ABT-263, suggesting that Bim may bind to Bcl-XL with additional sites (this part will be further explored in Chapter3). We have also characterized the first anti-apoptotic BH3 protein TCTP by showing its capability of enhancing the Bcl-XL function of inhibiting Bim and tBid mediated Bax activation. We identified mutation (R21A) in TCTP that specifically nullify its function on Bcl-XL inhibiting tBid but not Bim, which further indicates possible different binding topologies on Bcl-XL between Bim and tBid.

Results

Purification of Bim

Three major isoforms generated by alternative splicing of mRNA, BimEL, BimL, and BimS(Hinds et al, 2006). All three isoforms contain a N-terminal sequence, a BH3 domain, and a predicted membrane binding domain (MBD). BimL differ from BimS by including a Dynein light chain binding domain (DLB) which is involved in Bim post-translational regulation in cells (Fig 1A). BimEL contains an extra sequence in between the N-terminal domain and the DLB. To obtain pure recombinant Bim protein in large quantity, codon-optimized DNA

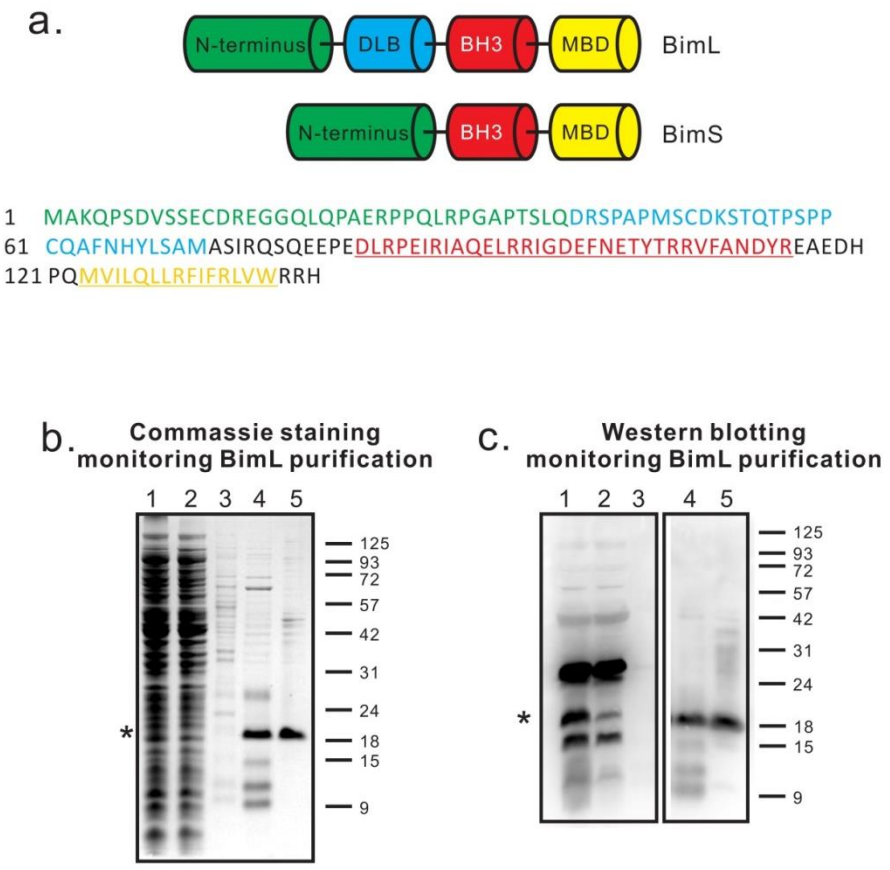

Fig 1: Purification of Bim

- A Stick diagram of the BimL and BimS isoform depicting the various domains along with the sequence of the protein (upper) with the amino acid (aa) sequence of BimL (lower). The amino acid sequences of the BH3 domain and predicted MBD are underlined. Each region in the aa sequence was shown in the same color shown in stick diagramcolored.
- B Commassie Blue stained SDS-PAGE gel monitoring the Bim purification protocol. Samples at important steps of the purification of Bim were collected as follows: 1. Bacteria lysates; 2. Nickel column flow; 3. Nickel column wash; 4. Nickel column elution; 5. HPPS column elution. The protein is indicated by * on the left and molecular weight (kD) is labeled on the right.
- C Western blot (IB: Bim) monitoring the Bim purification protocol. Samples at important steps of the purification of Bim were collected the same way as Fig 1B. The protein is indicated by * on the left and molecular weight (kD) is labeled on the right.

encoding BimL/S isoform were cloned into pBluescript II KS(+) vector where a His-tag-TEV sequence was genetically fused to the N-terminus of Bim sequences. BimL isoform is expressed as a 18.4 KDa protein with a >95% purity (Fig 1B, 1C). BimS isoform is expressed as a 16.2 KDa protein with the same purification protocol as BimL. Expression of the EL isoform with the same approach was attempted but the yield and purity were too poor (data not shown).

Bim isoforms function similarly in vitro

In live cells, BimS was reported to be the most potent among the three isoforms (Lei, 2003). It was reported that BimEL and BimL undergo the regulation by phosphorylation in the DLB region (Geissler et al, 2013; Lei, 2003) The absence of the dynein binding motif and the associated regulatory phosphorylation sites in BimS probably accounts for the extremely potent apoptotic activity of this isoform(Lei, 2003). However quantative comparison between Bim isoforms *in vitro* was lacking, so it was still unclear whether the activity difference between isoforms in cell is due to a combination of the post-translational modification and intrinsic affinity difference or is solely an effect of the former one. To investigate this in a rigorous fashion, we first examined the function of the recombinant proteins of the two Bim isoform with purified mitochondria or in *in vitro* liposome systems where individual interactions can be measured without the interference of other cellular components. To quantify the activator function we examined mitochondrial membrane permeabilization by incubating the Bim isoforms with recombinant Bax in the presence of Bax/Bak DKO BMK mitochondria containing

mCherry-fused SMAC in the inter-membrane space. The permeabilization of MOM was measured by the release of SMAC-mCherry from pellet portion when fractionated by centrifugation. Consistent with our previous observation (Sarosiek et al, 2013), BimL and BimS induced Bax-mediated membrane permeabilization similarly at low nM concentration (EC50≈0.5nM) (Fig 2A). The same results were also seen using a well-established MOM-like liposome permeabilization assay, where the effects of mitochondrial proteins were further removed (Kale et al, 2014) (Fig 2B).

To directly measure the binding interactions that mediate the activator function and sensitizer function, we calculated the dissociation constants (Kd) by Förster resonance energy transfer (FRET) between Bim and Bax or Bcl-XL in the presence of liposomes (Fig 2C, 2D). The results indicate that BimL and BimS bind to Bax (Kd≈30nM) or Bcl-XL (Kd≈1nM) with same affinities. Thus, both isoforms function similarly *in vitro*. BimL is present in many different tissues and cell types (O'Reilly et al, 2000) while BimS is almost never present in normal cells and has been only recently detected in HEK 293 cells (Putcha et al, 2001). Thus, BimL is likely the more relevant isoforms mediating an apoptotic response for most cell types. The following studies were conducted with murine BimL isoform if not specifically indicated.

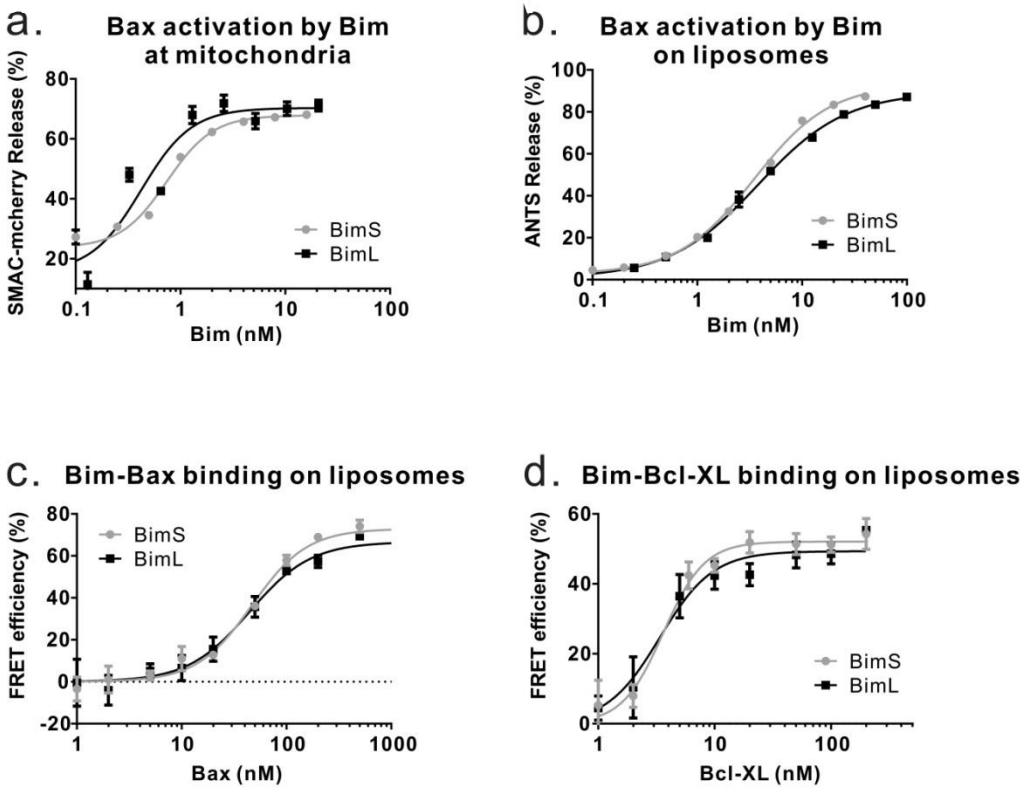

Fig 2: Bim isoforms function similarly in vitro

- A Bax activation by Bim assessed by measuring Smac-mCherry release from mitochondria after incubation of Bax (50 nM) with mitochondria isolated from SMAC-mCherry expressing DKO BMK cells and the indicated amounts of BimL or BimS. Data are mean ± SD (n=3).
- B Bax activation by Bim assessed by measuring ANTS-DPX release from liposomes after incubation of Bax (100nM) with liposome encapsulated ANTS-DPX and the indicated amounts of BimL or BimS. Data are mean ± SD (n=3).
- C Bim binding to Bax measured by FRET in samples containing MOM-like liposomes. Bim-Bax FRET was measured with 10nM Alexa568-labeled BimL or BimS and the indicated amounts of Alexa647-labeled Bax. Data are mean ± SD (n=3).
- D Bim binding to Bcl-XL measured by FRET in samples containing MOM-like liposomes. Bim-Bcl-XL FRET was measured with 10nM Alexa568-labeled BimL or BimS and the indicated amounts of Alexa647-labeled Bcl-XL. Data are mean ± SD (n=3).

Bid preferably activates Bak while Bim preferably activates Bax

It had previously observed that the Bid BH3 peptide was more effective than the Bim BH3 peptide at inducing cytochrome release in mouse liver mitochondria, which contain detectable levels of Bak, but not Bax (Letai et al, 2002). We therefore hypothesized that Bim and Bid may not be fully redundant in their ability to activate Bax and Bak. We therefore tested our hypothesis with our characterized recombinant proteins, using cytochrome c release from mitochondria as an assay endpoint. Isolation of heavy membranes from mouse livers yields mitochondria that contain Bak but not Bax (Letai et al, 2002). It has previously been shown that these Bak-containing mitochondria release cytochrome c more readily in response to Bid peptide than Bim (Letai et al, 2002), but the activity of full-length proapoptotic proteins has not been explored in this setting. We found that tBid was more efficient at inducing cytochrome c release than Bim protein across a range of concentrations (Fig 3A), consistent with our previous data. Next, we isolated liver mitochondria that lack detectable Bax or Bak from Bak−/− mice (Hsu & Youle, 1997) and added exogenous, recombinant Bax followed by tBid or Bim. Mitochondria in this assay released cytochrome c at much lower concentrations of Bim than mitochondria from WT mice, but tBid protein was still more efficient than Bim (Fig 3B). Mtch2 has recently been identified as a powerful facilitator of tBid recruitment to the mitochondrial membrane of liver cells, where it activates Bax or Bak (Shamas-Din et al, 2013), which may explain why tBid was a more efficient activator of Bax in this model.

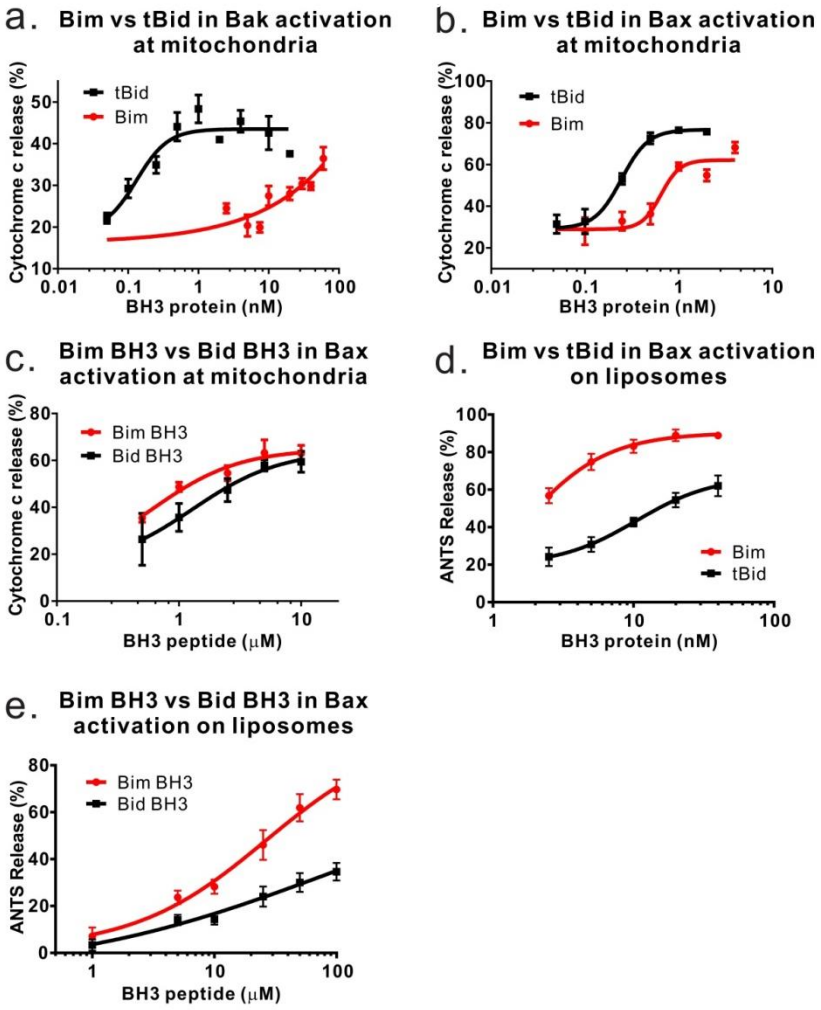

Fig 3: Bid preferably activates Bak while Bim preferably activates Bax

- A Heavy membranes, including mitochondria, were isolated from WT mouse livers that contain Bak, but not Bax. Mitochondria were treated with the indicated concentrations of tBid and Bim protein, and cytochrome c release was measured. Data are mean ± SD (n=3).
- B Heavy membranes, including mitochondria, were isolated from Bak−/− mouse livers that do not contain Bax or Bak. Mitochondria were then incubated in the presence of recombinant Bax and indicated concentrations of tBid or Bim proteins, and cytochrome c release was measured. Data are mean ± SD (n=3).
- C Heavy membranes, including mitochondria, were isolated from Bak−/− mouse livers that do not contain Bax or Bak. Mitochondria were then incubated in the presence of recombinant Bax and indicated concentrations of Bid BH3 or Bim

BH3 peptides, and cytochrome c release was measured. Data are mean ± SD (n=3).

- D ANTS release from MOM-like liposomes was monitored in the presence of recombinant Bax and indicated concentrations of tBid or Bim. Addition of only Bax or Bim/tBid alone at highest doses shown yielded a background ANTS release of less than 5%. Data are mean ± SD (n=3).
- E ANTS release from MOM-like liposomes was monitored in the presence of recombinant Bax and indicated concentrations of Bid BH3 or Bim BH3 peptides. Data are mean ± SD (n=3).

To test this, we treated these Bax-containing mitochondria with BH3 peptides instead of full-length proteins since Bid BH3 lacks the Mtch2 binding domain (Katz et al, 2012). Consistent with our previous data, we observed that Bim BH3 was more efficient as an activator of Bax than Bid BH3 (Fig 3C). Finally, we used the aforementioned liposome-based system to assay Bax activation without the effect of other organellar proteins, once again observing that Bim BH3 and Bim are more efficient activators of recombinant Bax than the Bid peptide or protein (Fig 3D, 3E).

Bim but not Bid binding to Bcl-XL is resistant to ABT-263 treatment

It is commonly accepted that BH3-proteins engage in the hydrophobic binding pockets of anti-apoptotic proteins through a conserved BH3 domain (Chen et al, 2005). ABT-263, a drug designed to mimic the BH3 domain of Bad, is an inhibitor of both Bcl-XL and Bcl-2. ABT-263 binds to the soluble domains of Bcl-XL and Bcl-2 with nano-molar affinity and displaces BH3 peptides derived from different BH3-proteins *in vitro* (Tse et al, 2008). BH3 profiling data indicate that Bim and Bid are apparently functionally redundant as sensitizers as both are able to bind and inactivate all five of the major anti-apoptotic proteins (Bcl-2, Bcl-w, Mcl-1, Bfl-1, and Bcl-XL) (Certo et al, 2006). However, previously our lab have observed a discrepancy between this data and the activities of the drugs in live cells with Fluorescent Lifetime Imaging Microscopy (FLIM)-FRET studies in live cells demonstrated that ABT-263 displace Bad and tBid but not Bim from binding to Bcl-XL and Bcl-2 (Aranovich et al, 2012; Liu et al, 2012). Thus, we assess the

affinities of tBid to Bcl-XL by FRET in the liposome system with the titration of ABT-263 (Fig 4A) and compared with that of Bim (Fig 4B). Results indicate that both tBid and Bim bind to Bcl-XL with similar affinities on membrane (Kd<1nM). Coinciding with our observations in live cells, the binding of tBid to Bcl-XL was much more sensitive to ABT-263 (Kd increased to >20nM with 2μM ABT-263) compared to Bim (Kd<7nM with 2μM ABT-263). It should be noted that Bim binds to Bcl-XL with higher affinity (Kd≈2nM) than tBid (Kd≈27nM) in the absence of lipid membranes, and same and even more distinct responses to ABT-263 were observed (Fig 4C, 4D). These results indicate that other than the BH3 domain, possible extra Bcl-XL binding sites on Bim may be contributing the resistence to ABT-263 by Bim-Bcl-XL complex.

Specific residue of TCTP is crucial for promoting Bcl-XL binding to tBid but not Bim

TCTP, an important regulator of the tumor reversion program (Amson et al, 2013; Telerman & Amson, 2009), have been recently identified as a member of BH3 protein. Although TCTP was shown to promote cell survival via potentiating Bcl-XL (Yang et al, 2005), it remains unknown how TCTP regulates Bcl-XL. Given the importance in cancer of the anti-apoptotic proteins of the Bcl2 family (Juin et al, 2013) of which Bcl-XL is a member and the focus on therapies that inhibit Bcl-XL, it becomes relevant to provide an understanding of any positive regulators of Bcl-XL. By using extensive biochemical and biophysical studies, It was shown that TCTP forms heterotetrameric complexes with Bcl-XL via crucial

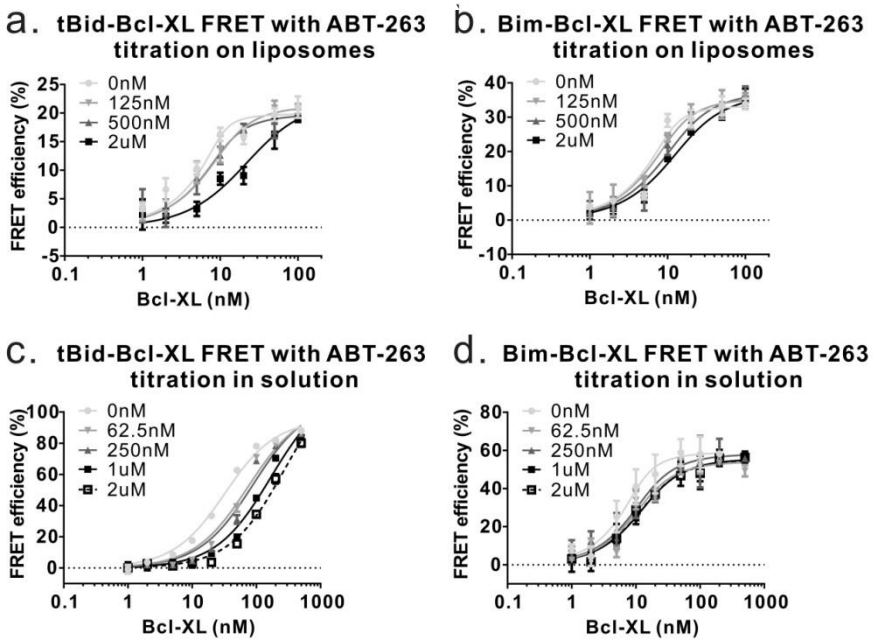

Fig 4: Bim but not Bid binding to Bcl-XL is resistant to ABT-263 treatment

- A tBid binding to Bcl-XL under the treatment of ABT-263 measured by FRET in samples containing MOM-like liposomes. At each indicated amount of ABT-263, tBid-Bcl-XL FRET was measured with 20nM Alexa568-labeled tBid and the indicated amounts of Alexa647-labeled Bcl-XL. Data are mean ± SD (n=3).
- B Bim binding to Bcl-XL under the treatment of ABT-263 measured by FRET in samples containing MOM-like liposomes. At each indicated amount of ABT-263, Bim-Bcl-XL FRET was measured with 20nM Alexa568-labeled Bim and the indicated amounts of Alexa647-labeled Bcl-XL. Data are mean ± SD (n=3).
- C tBid binding to Bcl-XL under the treatment of ABT-263 measured by FRET in solution. At each indicated amount of ABT-263, tBid-Bcl-XL FRET was measured with 20nM Alexa568-labeled tBid and the indicated amounts of Alexa647-labeled Bcl-XL. Data are mean ± SD (n=3).
- D Bim binding to Bcl-XL under the treatment of ABT-263 measured by FRET in solution. At each indicated amount of ABT-263, Bim-Bcl-XL FRET was measured with 20nM Alexa568-labeled Bim and the indicated amounts of Alexa647-labeled Bcl-XL. Data are mean ± SD (n=3).

interactions between the N-Terminal region of TCTP (TCTP11-31) and the BH3 binding groove of Bcl-XL (Thebault et al, 2016). The same group also found a mutation R21A in TCTP that abrogated the interaction between TCTP and Bcl-XL.

Based on this observation, we hypothesize that TCTP binding directly to Bcl-XL modifies Bcl-XL function, and we test this theory by assaying the impact of TCTP in the liposomes permeabilization system (Fig 5). We set up individual assay conditions where Bcl-XL inhibits permeabilization by binding tBid (Fig 5A, 5B) or Bax (by using tBidmt1, a mutant which does not bind to Bcl-XL (Billen et al, 2008), Fig 5C, 5D) or Bim (Fig 5E, 5F), thereby preventing Bax oligomerization on membranes. Recombinant TCTP enhanced Bcl-XL inhibition of liposome permeabilization under all three conditions in a dose-dependent manner (Fig 5A, 5C, 5E). TCTP R21A mutant protein no longer enhanced Bcl-XL activity for tBid and Bax inhibition (Fig 5B, D). Suprisingly, TCTP R21A still remain as functional as WT for enhancing Bcl-XL inhibiting Bim. In control experiments designed to determine if TCTP affected the activity of Bax directly, TCTP had no effect in reactions not containing Bcl-XL (Fig EV5).

Discussion

BH3 proteins regulate apoptosis via the direct activation of the mitochondrial pore-forming proteins Bax and Bak (activator) and/or binding with anti-apoptotic proteins for duo-inhibition (sensitizer function). Our studies have quantatively examined the activator and sensitizer function of major isoforms of Bim, and

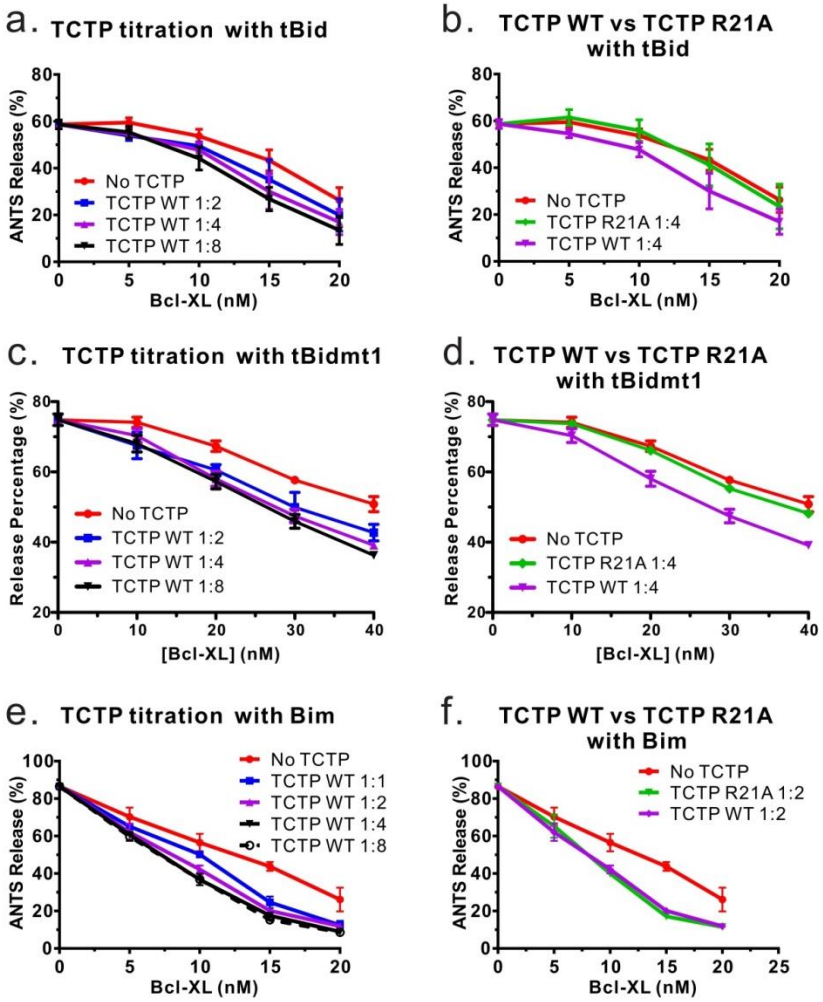

Fig 5: Specific residue of TCTP is crucial for promoting Bcl-XL binding to tBid and Bax, but not Bim

in vitro reconstitution assay on liposome permeabilization. Increasing concentrations of Bcl-XL were incubated in different molecular ratios [Bcl-XL:TCTP] with TCTP WT (5A, 5C, 5E) or with a fixed amount of TCTP R21A (B, D, F) at pH 9 for 45 min at 37°C, as specified. The treated Bcl-XL was then added to reactions containing ANTS-DPX liposomes, 100nM Bax and 20nM of either tBid (5A, 5B) or tBid mt1(5C, 5D) or Bim (5E, 5F) at pH 7 and liposome permeabilization was quantified by ANTS release after 5h incubation at 37°C. Data are mean ± SD (n=3).

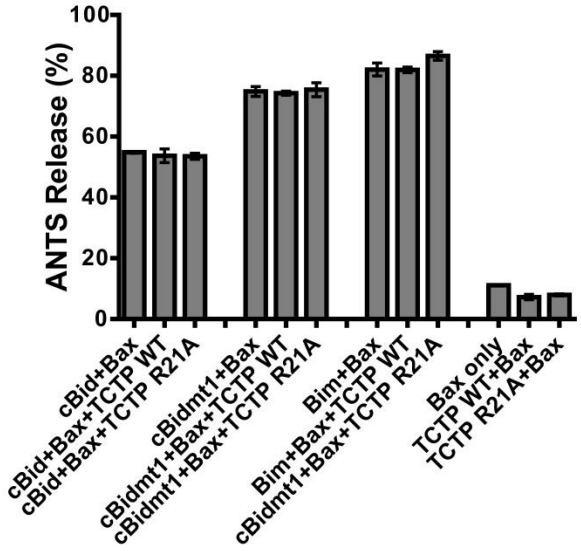

Fig EV5: TCTP had no measureable effect on the function of any of the proteins other than Bcl-XL.

ANTS release from MOM-like liposomes was monitored after incubation with 320nM TCTP (WT or R21A), 100nM Bax and 20nM of tBid or tBidmt1 or Bim as indicated. Data are mean ± SD (n=3).

showing that their activities are similar *in vitro* (Fig 2). Our finding suggests that in cells Bim isoforms are possibly functionally redundant and mainly being regulated via post-translation modification.

Bim and Bid have been considered to be functionally redundant with respect to their capacity of activating Bax and Bak and sharing similar binding profiles for the anti-apoptotic members of the Bcl-2 family (Certo et al, 2006). Here we showed that Bid preferentially activates Bak while Bim preferentially activates Bax in both mouse mitodchondria system and in the (Fig 3). The preference of Bid for Bak was generally stronger than Bim for Bax. Notably, these preferences were observed with unstructured BH3 domain peptides as well as full-length proteins, which suggest that the preferences are predominantly based on the amino acid sequences of the two proteins. In addition, other factors such as Mtch2 may also influence the selectivity we observed by modulating the activation process and thus provide another means by which to control these effectors in certain cell types (Katz et al, 2012; Shamas-Din et al, 2013). Furthermore, the preferential activation of Bax and Bak by Bim and Bid can potentially be used to explain previous reports of either Bax or Bak dependence for apoptosis induced by various insults abound. For example, the apoptosis induced by c-Myc activation was shown to be entirely dependent on Bax but not Bak (Dansen et al, 2006). This could be explained by the fact that c-Myc-induced apoptosis is triggered via upregulation of Bim (Egle et al, 2004). Similarly, Bak-deficient glioblastoma cells were reported to be completely resistant to Fas-

mediated cell death (Cartron et al, 2003) and correspondingly Fas ligand elicits death receptor signaling and leads to caspase 8 activation and subsequent Bid cleavage and activation (Li et al, 1998).

On the other hand, we have demonstrated that Bim, but not tBid, is resistant to Bcl-XL inhibitor ABT-263 that competitively inhibit Bcl-XL as a BH3 mimic, coinciding with previous in cell observations (Aranovich et al, 2012; Liu et al, 2012). These data suggest that Bim differ from Bid by binding to Bcl-XL with potential extra binding sites that contribute to the resistance to BH3 mimics. The finding of the unique resistance of Bim to ABT-263 opens our eyes to a new different level of regulation may be present in cells and potentially may become novel target for target-specific small molecules. This subject was further investigated in Chapter V of the thesis.

Lastly, we reported biochemical evidence that underlie the positive regulation of Bcl-XL by TCTP (Fig 5). This established the first anti-apoptotic BH3 protein in Bcl-2 family. We have also identified mutation R21A in TCTP that abolished the enhancing function for cBid-Bcl-XL and Bax-Bcl-XL interaction, but not for Bim-Bcl-XL interaction. We hypothesize that TCTP functions by expose the hydrophobic groove of Bcl-XL but is displaced easily when other BH3 protein or Bax is present due to a relative low affinity. Although R21A mutation in TCTP reduced its affinity to Bcl-XL enough to not being able to enhance the engagement tBid or Bax, it may still retain transient interaction with the

hydrophobic groove which can be captured by Bim, which binds to Bcl-XL with additional sites other than BH3 domain.

To conclude, our results characterized the difference in function of two most notorious BH3 proteins, Bid and Bim. These findings could be that guiding chemotherapy decisions based on alterations in the main BH3 protein player in certain cells. Future studies may identify additional modes of regulation and provide a more complex and nuanced picture of how cell fate is determined by these critical Bcl-2 family proteins.

Materials and methods

Protein Purification

WT and single cysteine mutants of Bax, Bcl-XL, and tBid were purified as described previously (Kale et al, 2014). tBidmt1 was purified with the same protocol used for tBid (Kale et al, 2014).

For BimWT and single cysteine mutants of Bim, the cDNA encoding full-length wild-type murine BimL/BimS was introduced into pBluescript II KS(+) vector (Stratagene, Santa Clara, CA). Sequences encoding a polyhistidine tag followed by a TEV protease recognition site (MHHHHHHGGSGGTGGSENLYFQGT) were added to create an in frame fusion to the N-terminus of Bim.

The recombinant proteins were expressed in Arabinose Induced (AI) Escherichia coli strain (Life Tech, Carlsbad, CA). E. coli cells were growing at 37 degree to OD 0.7~0.8 then induced with 0.2% Arabinose for 3~4 hr at 30 degree. Havested cells were resuspended in Lysis Buffer (20mM Tris pH8.0, 20mM NaCl, 1% CHAPS, 5mM imidazole, 20% Glycerol) then lysed by mechanical disruption with a French press. The cell lysate was run on a Nickel-NTA column (Qiagen, Valencia, CA, 300uL bed-volume per liter of culture) to bind the recombinant His-tag fused proteins. The column was then washed with 20 bed-volume of Wash Buffer (20mM Tris pH8.0, 50mM NaCl, 0.5% CHAPS, 10mM imidazole, 20% Glycerol), and Elution Buffer (20mM HEPES pH7.2, 10mM NaCl, 0.3% CHAPS, 300mM imidazole, 20% Glycerol) was applied to elute the proteins. This eluate was then adjusted to 150 mM NaCl and applied to 600uL bed-volume of High Performance Phenyl Sepharose (HPPS) column pre-equilibrated with HPPS Binding Buffer (20mM HEPES pH7.2, 100mM NaCl, 0.3% CHAPS, 20% Glycerol). The column was washed with 3-4mL of HPPS Binding Buffer. Bim was eluted with HPPS elution buffer (20mM HEPES pH7.2, 0.3% CHAPS, 20% Glycerol) and dialyzed against Dialysis Buffer (10mM HEPES pH7.0, 1mM NaCl, 20% Glycerol), then flash-frozen and stored at -80 °C.

Protein labeling

Single cysteine mutants of Bax, Bcl-XL, tBid and Bad were labeled with the indicated maleimide-linked fluorescent dyes as described previously (Kale et al, 2014; Lovell et al, 2008). Single cysteine mutants of Bim were labeled with the

same protocol as tBid with the exception that the labeling buffer also contained 4M urea.

Membrane permeabilization

Membrane permeabilization assays with liposomes encapsulating ANTS and DPX were performed as described previously (Kale et al, 2014). To measure permeabilization of BMK mitochondria, the indicated amounts of proteins were incubated with mitochondria purified from DKO BMK cells expressing mCherry fluorescent protein fused to the SMAC import peptide responsible for localization in the inter-membrane space. After incubation for 45 min at 37^{o} C. Samples were centrifuged at 20000g for 10min to separate the pellet and supernatant fractions. Membrane permeabilization was calculated by: $\mathrm{Permeabilization}\ (\%) = [\frac{\mathrm{F_{supernatant}}}{(\mathrm{F_{supernatant}}+\mathrm{F_{pellet}})}] * 100\%$, where F is the intensity of mCherry fluorescence in the fraction (Shamas-Din et al, 2013). For mouse liver mitochondria, cytochrome *c* release was measured by Western blot and calculated as described previously (Sarosiek et al, 2013).

Protein-protein binding

For tBid-Bcl-XL FRET, single cysteine mutants of tBid (126C) were purified and labeled with Alexa 568-maleimide. The single cysteine mutant of Bcl-XL (152C) was purified and labeled with Alexa 647-maleimide. Alexa568 labeled tBid were incubated with either Alexa647-labeled or unlabeled Bcl-XL along with the

indicated concentrations of BimWT or BimΔMBD. The intensity of Alexa568 fluorescence with unlabeled or Alexa647-labeled Bcl-XL was measured as $F_{unlabeled}$ or $F_{labeled}$ respectively. FRET efficiency was calculated by: $\text{FRET efficiency}\ (\%) = \ [1 - \frac{F_{labeled}}{F_{unlabeled}}] * 100\%$ as described previously (Shamas-Din et al, 2013). The data was fit to a binding model as described below. Lines of best fit were calculated using least squares in Graphpad Prism.

For Bim-Bax or Bim-Bcl-XL FRET, single cysteine mutants of Bax (Bax126C) and Bcl-XL (Bcl-XL152C) were purified and labeled with Alexa647-maleimide then titrated into incubations containing Alexa568 labeled Bim proteins. FRET efficiency was determined as published for tBid-Bcl-XL (Lovell et al, 2008). Binding was measured after incubation at 37^{o} C for 1h unless specified otherwise. Lines of best fit were calculated using least squares in Graphpad Prism.

Dissociation constants (Kd) were measured in solution with and without added liposomes. Curves were fit to an advanced function taking into account the concentration of acceptor ([A]) change when [A] is close to Kd:

$$F = (F_{max})\left(\frac{([D] + [A] + K_d) - \sqrt{([D] + [A] + K_d)^2 - 4[D][A]}}{2[D]}\right)$$

[D] is the concentration of donor, F indicates the FRET efficiency with the concentration of acceptor as [A], F_{max} is the maximum FRET efficiency in the curve. The maximum FRET efficiency is a function of the relative positions, dipole orientations and mobility of the dyes as well as the affinity of binding. Therefore,

comparisons were based on Kd's estimated from the data rather than maximum

FRET efficiencies obtained.

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CHAPTER IV: The C-terminus of Bim is required for activation of Bax not inhibition of Bcl-XL.

Preface

The work presented in this chapter has been submitted as a research article to EMBO J:

Chi X, Qian L, Osterlund E, Leber B, Andrews DW, The C-terminus of Bim is required for activation of Bax not inhibition of Bcl-XL.

Contribution of authors:

DWA and BL directed the project. X.C. carried out the experiments other than FLIM-FRET and analyzed the data. Q.L. and E.O. carried out the FLIM-FRET experiments and analyzed the data. All authors discussed experimental design, the interpretation of results and contributed to writing the manuscript.

Objective of the chapter:

To explore the unique effect of the membrane binding region (MBD) of Bim on its pro-apoptotic function.

Highlights:

- The C-terminal membrane binding domain (MBD) of Bim plays a critical role in both binding of Bim to Bax and activation of Bax. Unlike the only other well studied BH3-protein Bid, regulation of Bax is independent of the membrane binding function of the C-terminal domain of Bim.
- We report quantitative analyses of the functional effects of multiple mutations in this domain that reduced or abolished binding to membranes without affecting binding to Bax thereby separating these functions. Point mutations in this domain of Bim were also identified that disabled Bax activation.
- Deletion of the domain abolished all physiologically relevant interactions of Bim with membranes and Bax but did not prevent Bim binding to and inhibiting of Bcl-XL.

Abstract

BH3-proteins regulate apoptosis by activating the pro-apoptotic proteins Bax and Bak to permeabilize mitochondria, and/or by inhibiting anti-apoptotic proteins such as Bcl-XL and Bcl-2. Both functions have been ascribed to direct binding interactions between the BH3-sequences and the BH3-peptide binding pockets on the proteins regulated. Closer investigation of the dual apoptotic functions of the BH3-protein Bim revealed that the C-terminal membrane binding domain is unexpectedly also involved both in binding of Bim to Bax in solution and in activating Bax. Multiple mutations in this domain reduced or abolished binding to membranes but did not affect binding to Bax or correlate with Bax activation. Deletion eliminated binding to and activation of Bax, but not binding to or inhibition of Bcl-XL. Thus the C-terminal domain of Bim mediates binding to both membranes and Bax separately, but is not essential for interactions with Bcl-XL.

Introduction

Apoptosis is a highly conserved form of programmed cell death that can be triggered by extrinsic or intrinsic signals. It plays a fundamental role in maintaining homeostasis by eliminating old, excessive or dysfunctional cells in multi-cellular organisms (Kerr et al, 1972). Defective regulation of apoptosis has been found in many diseases (Ramírez Chamond R., 1999) and is considered one of the hallmarks of cancer pathogenesis (Hanahan & Weinberg, 2011).

Bcl-2 family proteins play a decisive role in intrinsic apoptosis by regulating the integrity of the mitochondrial outer membrane (MOM). Permeabilization of the MOM results in the release of factors into the cytoplasm that activate the executioner caspases that mediate cell death (Chipuk et al, 2006). The Bcl-2 family consists of anti-apoptotic proteins including Bcl-XL and Bcl-2, pro-apoptotic proteins including Bax and Bak which permeabilize the MOM directly and contain multiple domains homologous to Bcl-2 (BH-domains), and a group of regulatory proteins including Bim, Bid, Puma, Hrk, Bad and Noxa that contain a single Bcl-2 homology domain (BH3-proteins). These regulatory proteins include proteins that bind to and activate Bax or Bak and those that function by releasing both sequestered BH3-proteins and activated Bax from anti-apoptotic proteins like Bcl-XL (Chi et al, 2014).

The biochemical basis for the difference between BH3 proteins has been attributed entirely to differences in affinities of the BH3-domain for the BH3-

peptide binding sites on multi-domain pro-and anti-apoptotic proteins. However, static affinities and variations in expression levels only permit coarse regulation of cell death. One way finer control over apoptosis is manifest is via membrane binding interactions that significantly alter the equilibrium of many Bcl-2 family interactions. For example, at physiologically relevant concentrations only membrane bound Bid activates Bax (Lovell et al, 2008; Shamas-Din et al, 2013a).

The BH3-protein Bim is an important mediator of apoptosis initiated by many intracellular stressors (Concannon et al, 2010; Mahajan et al, 2014; Puthalakath et al, 2007). Bim has a particularly important function as a regulator of multi-domain anti-apoptotic proteins as it binds and thereby inhibits by mutual sequestration all known anti-apoptotic proteins (Chen et al, 2005; Shamas-Din et al, 2013b). Data *in vitro* and in cells strongly suggest that Bim also functions to directly activate the membrane permeabilizing function of Bax and Bak (Sarosiek et al, 2013). However, Bim lacking the C-terminal hydrophobic membrane binding domain (MBD) failed to activate Bax to induce membrane permeabilization leading to the conclusion that like Bid, Bim binding to membranes is important for activation of Bax (Terrones et al, 2008). Due to the difficulty in expressing and purifying full-length Bim, most *in vitro* experiments have been conducted with peptides or deletion mutants lacking the MBD, which may have different affinities for the relevant interactions (Chen et al, 2005). Assessing binding interactions between Bim and Bax in cells via co-immunoprecipitation and immunoblotting may not resolve the issue of how Bim functions, because these methods give

inconsistent results that are prone to detergent artifacts (Hsu & Youle, 1997; Lovell et al, 2008). Thus despite the putative importance of membrane binding, the role(s) of the Bim MBD in Bim binding to; membranes, anti-apoptotic and pro-apoptotic proteins remain unclear.

Here we show that sequences within the Bim MBD required for Bim binding to membranes are not required for it to bind to Bax in solution or on membranes. Moreover, unlike Bid, activation of Bax by Bim does not require Bim to bind to membranes. In contrast, the MBD augments but is not required for Bim to bind to and inhibit Bcl-XL. We show that the unusual sequence of the Bim MBD controls its dual pro-apoptotic functions.

Results

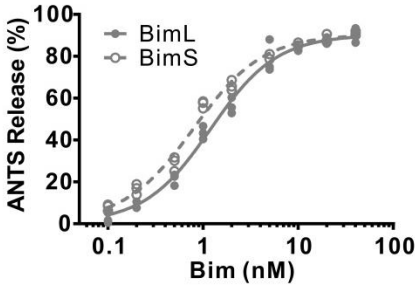
The C-terminus of Bim binds membranes and mediates Bim binding to Bax

Three major isoforms of Bim result from alternative mRNA splicing: BimEL, BimL, and BimS (O'Connor et al, 1998). All three isoforms include the BH3-domain required for binding other Bcl-2 family proteins and a C-terminal membrane binding domain (MBD). BimS is regulated transcriptionally while BimL and BimEL share an LC1 motif that when phosphorylated inhibits the proteins by sequestering them on the cytoskeleton by binding them to dynein Light Chain

LC1 (Lei, 2003). The absence of a LC1 binding motif in BimS probably accounts for the more potent activity of this isoform in cells (Lei, 2003).

To investigate the molecular mechanisms for the apoptotic function of Bim without interference from other cellular components, we purified recombinant murine BimL and BimS and assayed them in an *in vitro* liposome system where individual interactions can be measured and their effects quantified. To measure activation of Bax by Bim, we incubated the different Bim isoforms with recombinant Bax and liposomes with a lipid composition similar to MOM encapsulating a dye/quencher pair ANTS (8-Aminonaphthalene-1,3,6-Trisulfonic Acid, Disodium Salt) and DPX (*p*-Xylene-Bis-Pyridinium Bromide). Bim-mediated membrane permeabilization results in an increase in fluorescence due to the release of the encapsulated dye/quencher pair (Kale et al, 2014). In this assay system both BimL and BimS activated Bax equivalently as expected (Appendix Figure S1). BimS is regulated transcriptionally and is rarely present in healthy cells while BimL and BimEL are present in most tissue types (O'Reilly et al, 2000). Consequently, unless specified otherwise we used the BimL isoform (referred to here as BimWT) in our experiments.

By sequence analysis, the C-terminal MBD shared by all Bim isoforms is unusual in that it includes two positive charged Arg residues yet is considered crucial for binding Bim to membranes (Weber et al, 2007; Wilfling et al, 2012). To quantify the functional importance of the MBD we created a mutant, BimΔMBD, truncated after P121 to delete the entire MBD sequence. To assess binding to



Appendix Figure S1: Activation of Bax mediated membrane permeabilization by BimS and BimL are equivalent

Bax activation was assessed by measuring ANTS-DPX release from liposomes after incubation with 100nM Bax with the indicated amounts of BimS (open symbols) or BimL (solid symbols). Data from independent experiments are shown as individual points (n=3), some points are not visible due to overlap.

lipid membranes directly we measured Förster resonance energy transfer (FRET) between fluorophore-labeled recombinant Bim and BimΔMBD with DiD labeled liposomes (Fig 1A). We also quantified Bim binding to mitochondrial outer membranes with mitochondria isolated from $BAK^{-/-}$ mouse liver (Fig EV1A), which lack Bax and Bak (Shamas-Din et al, 2013a). In both cases, BimWT spontaneously bound to the membranes, while stable binding of BimΔMBD to membranes was undetectable.

To assess if the MBD alone is sufficient to target Bim to mitochondria in cells, constructs encoding the yellow fluorescent protein Venus fused to either BimWT (V-BimWT) or the Bim MBD (P121 to H140) (Venus-MBD) were transiently expressed in $BAX^{-/-}$ $BAK^{-/-}$ (DKO) baby mouse kidney (BMK) cells. Mitochondrial localization of these constructs was measured using Pearson correlation coefficients for co-localization of Venus and Mitotracker Red (Li et al, 2004). Both proteins co-localized with Mitotracker Red similarly, but to a lesser extent than the mitochondrial targeting control Venus-ActA (Zhu et al, 1996). As expected, when Venus was expressed alone as a cytoplasmic control it showed minimal co-localization with Mitotracker Red (Fig EV1B). This suggests that in live cells the MBD is sufficient to localize a significant fraction of Bim to mitochondria consistent with our data in vitro and previous reports (Weber et al, 2007; Wilfling et al, 2012).

Deletion of the MBD in Bim has been reported to prevent its apoptotic activity both *in vitro* and *in vivo* (Weber et al, 2007). To determine the molecular

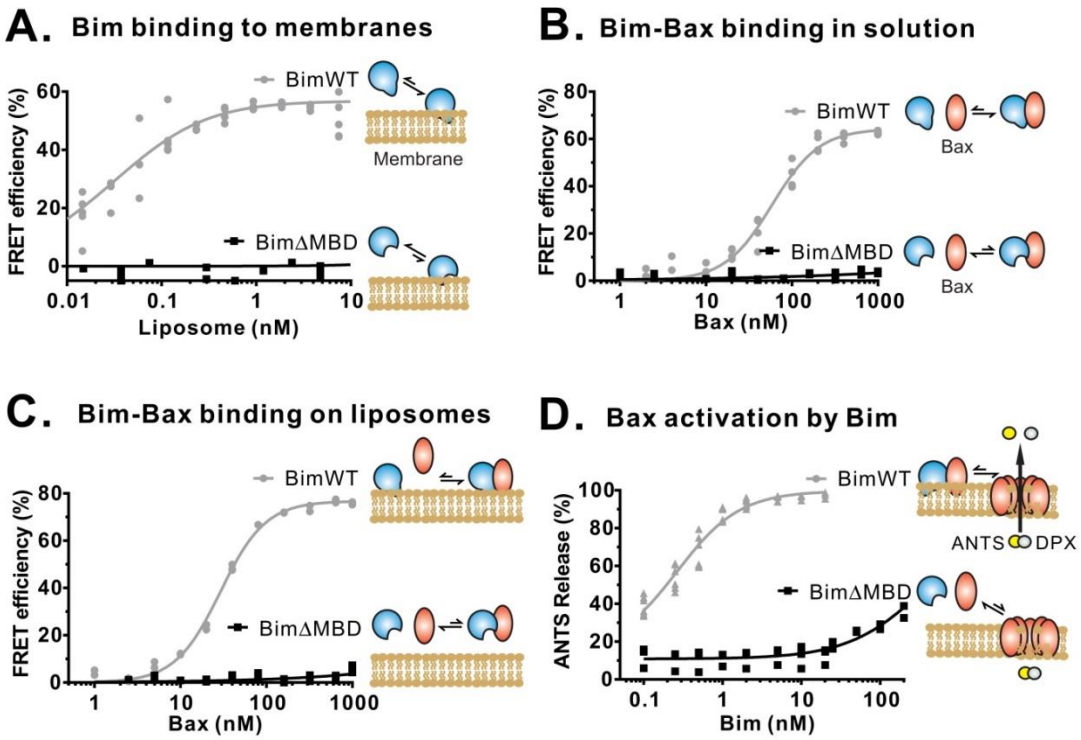


Fig 1: The C-terminus of Bim binds membranes and mediates Bim binding to Bax

Data from independent experiments are shown as individual points (n=3), some points overlap and therefore are not visible. In figures, cartoons indicate the binding interactions being measured. Equilibria symbols indicate the predicted balance of complexes for Bim (blue), Bax (red) and membrane (tan) at physiologic (low nM) concentrations.

- A Bim binding to MOM-like liposomes assessed by measuring FRET between 20nM Alexa568-labeled BimWT or BimΔMBD and the indicated amounts of DiD labeled liposomes.
- B Bim binding to Bax in solution measured by FRET. Bim-Bax FRET was measured with 4nM Alexa568-labeled BimWT or BimΔMBD and the indicated amounts of Alexa647-labeled Bax.
- C Bim binding to Bax measured by FRET in samples containing MOM-like liposomes. Bim-Bax FRET was measured with 4nM Alexa568-labeled BimWT or BimΔMBD and the indicated amounts of Alexa647-labeled Bax.
- D Bax activation by Bim assessed by measuring ANTS-DPX release from liposomes after incubation of Bax (100nM) with liposome encapsulated ANTS-DPX and the indicated amounts of BimWT or BimΔMBD.

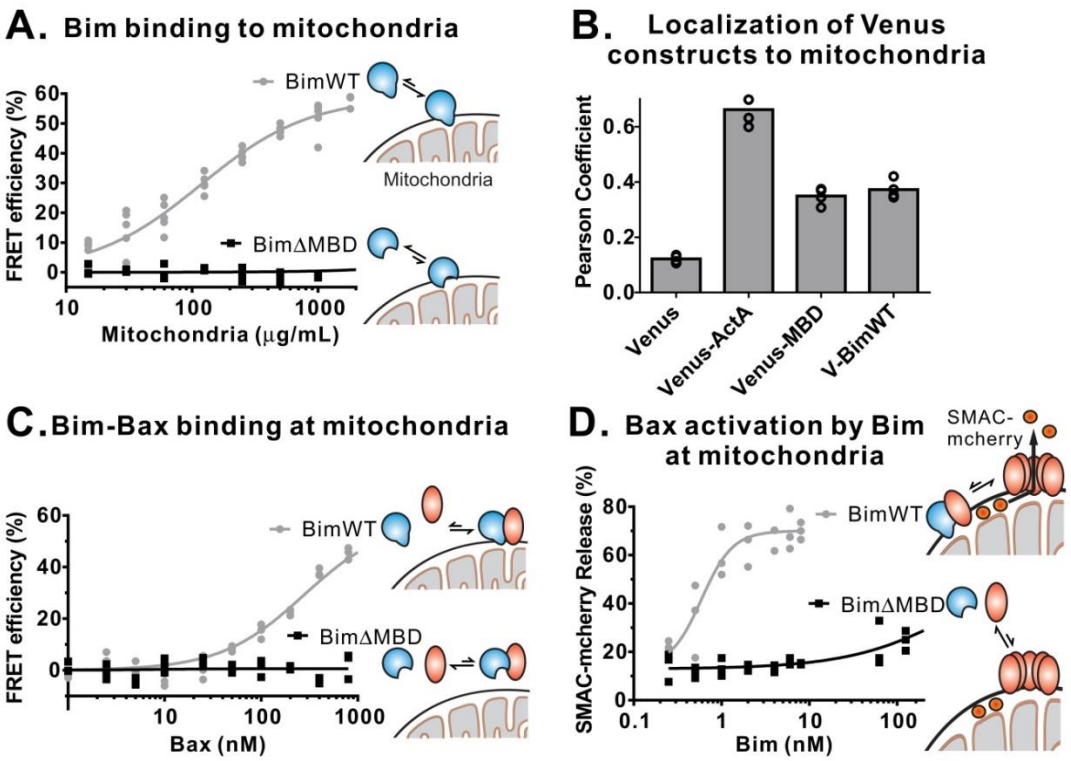


Fig EV1: Comparison of Bim mutants on mitochondria

Data from independent experiments are shown as individual points, some of which overlap and are therefore not visible. Similar to Fig 1, cartoons indicate the binding interactions being measured.

- A The MBD of Bim is necessary for the protein to bind to mitochondria. Binding of 4nM Alexa568-labeled BimWT (n=5) or BimΔMBD (n=3) to the indicated amounts of DiD labeled mouse liver mitochondria lacking Bax and Bak was assessed by measuring FRET.
- B The MBD of Bim is sufficient to target Venus to mitochondria in live cells. N-terminal-Venus-fused BimWT (V-BimWT) and Venus-fused to the Bim MBD (Venus-MBD) were transiently expressed in $Bax^{-/-}$ $Bak^{-/-}$ (DKO) BMK cells (n=4). Expression of Venus and Venus-ActA were used as negative and positive controls, respectively. After 24 hrs mitochondria were visualized in the cells by staining with Mitotracker Red. Micrographs recorded for Venus and Mitotracker channels using an Opera automated microscope were analyzed for co-localization using Pearson correlation coefficients calculated for the Venus and Mitotracker channels using a routine in the Opera software (Acapella V2.0, PerkinElmer).

- C Deletion of the MBD inhibits Bim binding to Bax at mitochondria. Bim binding to Bax was measured by FRET in samples containing mouse liver mitochondria, 4nM Alexa568-labeled BimWT (grey) or BimΔMBD (black) and the indicated amounts of Alexa647-labeled Bax (n=3). These binding curves can be compared directly within this experiment but not directly with the curves measured for proteins with liposomes (Fig 1C) as mitochondria contain unknown amounts of other potential binding partners for both Bim and Bax that will compete with the interaction between them. Thus while the addition of mitochondria reduced the binding affinity of BimWT for Bax (from ~60 nM in solution to ~275nM) the decrease was not enough to eliminate activation of Bax (Fig EV1D) at physiologic (low nM) concentrations of the proteins.
- D Deletion of the MBD inhibits Bim activation of Bax permeabilization of mitochondria. Permeabilization of the outer mitochondrial membrane by Bax (50 nM) in response to activation by the indicated amounts of Bim and BimΔMBD was assessed by measuring SMAC-mCherry release from mitochondria isolated from SMAC-mCherry expressing DKO BMK cells (n=3).

mechanism involved we assessed the impact of the Bim MBD on the interaction of Bim and Bax by measuring FRET between the proteins. Unexpectedly and unlike the BH3-only protein tBid (Lovell et al, 2008), BimWT bound to Bax even in the absence of a membrane, while BimΔMBD had no relevant Bax binding function (Fig 1B). In the presence of liposomes (Fig 1C) or mitochondria (Fig EV1C) BimWT continued to bind to Bax but the addition of membranes did not restore Bax-binding by BimΔMBD. Together this data suggests that the Bim MBD may be involved in Bax binding to Bim independent of its role in mediating Bim binding to membranes.

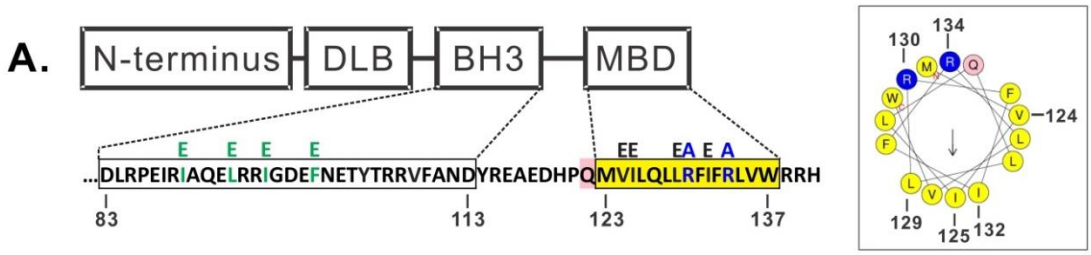
To determine if Bax binding by the MBD is productive and contributes to the activation of Bax to permeabilize membranes, we compared BimWT and BimΔMBD in both Bax-mediated liposome (Fig 1D) and mitochondrial outer membrane (MOM) permeabilization (Fig EV1D). Consistent with our previous observations (Sarosiek et al, 2013), low nM concentrations of BimWT induced Bax-mediated membrane permeabilization in both systems, while BimΔMBD did not activate Bax until it exceeded physiological concentrations and reached the μM range.

Specific residues in the Bim MBD mediate membrane and Bax binding

Our functional and binding assays showing that BimWT but not BimΔMBD bound Bax in solution (Fig 1A-D) provided strong but indirect evidence that the Bim MBD has dual regulatory functions: membrane binding, and Bax

binding/activation. To elucidate the relationship between the two functions and identify residues responsible for the respective interactions we undertook a mutagenesis approach. Sequence analysis predicts that the Bim MBD forms an amphipathic α-helix distinct from the transmembrane helices of conventional tail anchors. It contains two arginine residues (R130&134) predicted to be on the same hydrophilic side of the helix and hydrophobic residues (e.g. I125, L129, I132) on the other side (Fig 2A). To determine the functional importance of these residues, multiple Bim MBD mutants were tested including: BimMBD2A in which R130 and R134 were mutated to alanine and a series of single hydrophobic residue substitutions by glutamate (V124E, I125E, L129E, and I132E) (Fig 2A). As an inactive control we used the previously published BimBH3-4E in which four hydrophobic residues in the BH3 domain were replaced with glutamate (Chen et al, 2005).

To examine the effects of the MBD mutations, binding was measured between the Bim constructs and: liposomes (Appendix Figure S2A), Bax in solution (Appendix Figure S2B), and Bax in the presence of liposomes (Appendix Figure S2C). Bax activation by Bim mutants was also measured by liposome membrane permeabilization (Appendix Figure S2D). The maximum FRET efficiency is a function of the relative positions, dipole orientations and mobility of the dyes as well as the affinity of binding. Therefore, comparisons were based on dissociation constants (Kd) (Fig 2B) estimated from the data (Appendix Figure S2), rather than FRET efficiency. To compare binding interactions to Bim



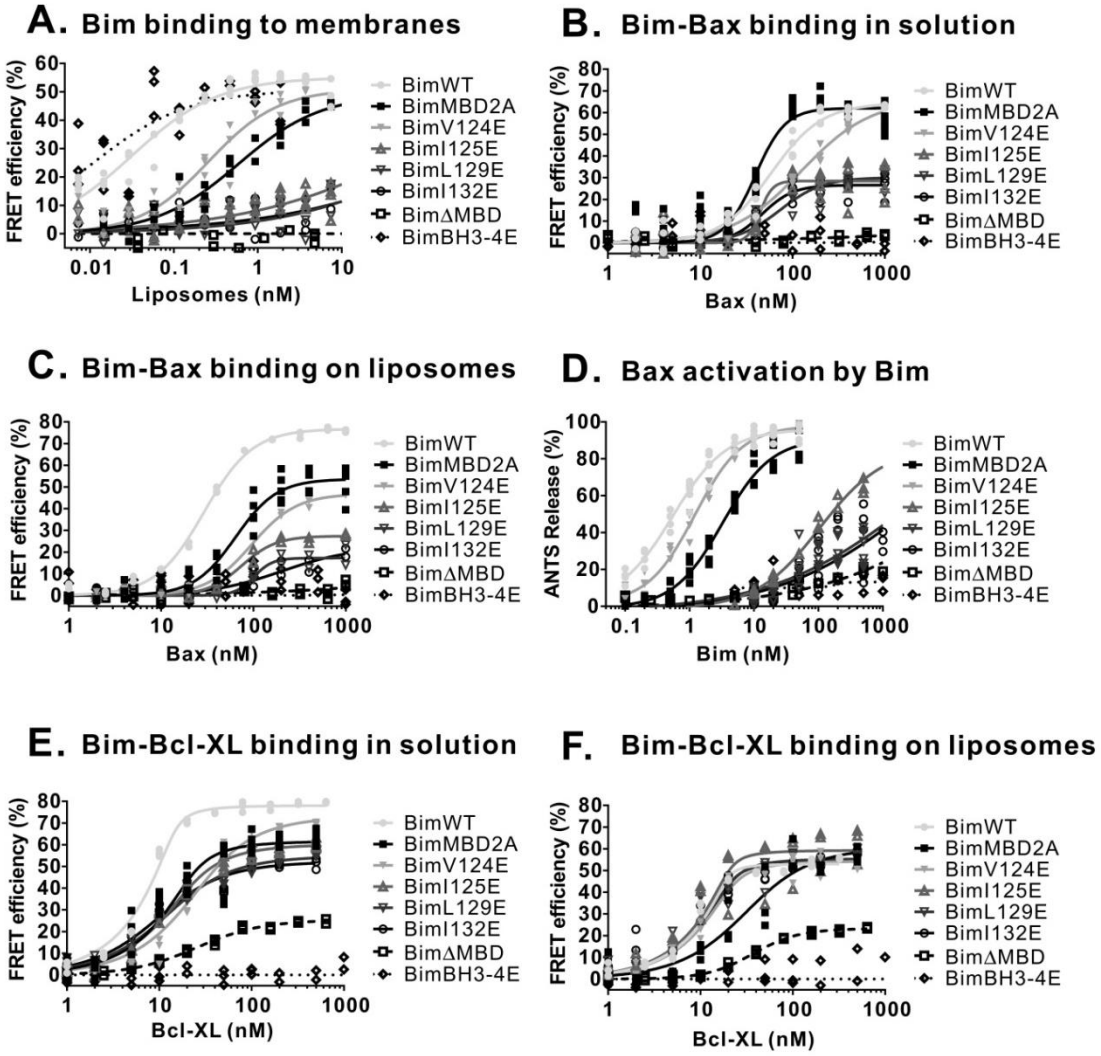
B.

High affinity — Low affinity

		BimWT	BimMBD2A	BimV124E	BimI125E	BimL129E	BimI132E	BimΔMBD	BimBH3-4E
Kd of Bim-liposome (pM)		31±4	588±181	260±55	>>1000	>>1000	>>1000	>>1000	11±5
Kd of Bim-Bax (nM)	In solution	59±7	40±3	96±10	47±10	73±12	47±5	>>1000	>>1000
	On membrane	29±1	64±7	95±9	74±9	101±14	202±128	>>1000	>>1000
EC50 of ANTS release (nM)		0.5±0.1	3±0.3	1.2±0.1	105±14	>1000	>1000	>1000	>>1000
Kd of Bim-Bcl-XL (nM)	In solution	1±1	3±1	3±1	3±1	2±1	1±1	16±2	>>1000
	On membrane	1±1	20±7	2±1	3±3	5±3	2±1	35±5	>>1000

Fig 2: Specific residues in the Bim MBD mediate membrane and Bax binding and Bax activation

- A Stick diagram of the BimL isoform depicting the various domains along with the sequence of the carboxyl half of the protein and the mutations studied here. The amino acid sequences of the BH3 domain and predicted MBD are boxed. Four essential hydrophobic residues in BH3 domain are colored green. Two potential important positive charged residues in the MBD are colored blue. The mutation generated for each individual residue is labeled on top of the original sequence. A predicted alpha helix structure generated via HeliQuest (http://heliquest.ipmc.cnrs.fr) is shown on the right, indicating the unique amphipathic feature of the MBD. The arrow shows the polarity direction from hydrophilic to hydrophobic. Residues are colored as in the linear sequence.
- B Binding of Bim mutants to Bax and Bcl-XL expressed as dissociation constants (Kd) measured from data in Appendix Figure S2A-C, E-F for each binary interaction. Activation of Bax (EC50) measured from ANTS-DPX assays in Appendix Figure S2D. Values are mean ± SEM (n=3). The table was color-coded in a heat map fashion as follows: red 0-40; light red 40-80; light pink 80-120; white 120-500; light blue 500-1000; Dark blue >1000 nM except for binding to liposomes which is in pM. The Kds for 'on membrane' measurements are apparent values since diffusion for the protein fraction bound to membranes is in two dimensions while for the fraction of protein in solution diffusion is in three dimensions and several of the binary interactions take place in both locations. Apparent Kd values may also be affected by competing interactions with membranes.



Appendix Figure S2: Quantitative analyses of Bim and Bim mutants binding to membranes, Bax and Bcl-XL

Data from independent experiments are shown as individual points (n=3), some points are not visible due to overlap. The mutants analyzed are indicated to the right of the graphs.

- A Mutations in the MBD affect Bim binding to membranes. Bim to membrane FRET was measured using 20nM Alexa568-labeled Bim mutants and the indicated concentrations of DiD labeled liposomes.
- B-C Mutations in the MBD affect Bim binding to Bax. Binding b) in solution and c) with 2.9nM MOM-like liposomes was measured using FRET between 20nM Alexa568-labeled Bim mutants and the indicated concentrations of Alexa647-labeled Bax.

- D Mutations in the MBD affect Bim mediated activation of Bax. Bax activation was measured by membrane permeabilization of ANTS/DPX liposomes with 100nM Bax and the indicated concentrations of Bim mutants.
- E-F Point mutations in the Bim MBD do not affect binding to Bcl-XL. FRET was measured e) in solution or f) with 2.9nM MOM-like liposomes for 20nM Alexa568-labeled Bim mutants and the indicated concentrations of Alexa647-labeled Bcl-XL.

To permit accurate estimation of the binding constants presented in Fig. 2, data of Appendix Figure S2B-F was collected to saturation for all mutants (for some curves 1600nM or 3200nM concentrations were required). For presentation purposes all curves were truncated at 1000 nM.

mediated Bax activation, we also calculated the EC_{50} for Bim mutants from the membrane permeabilization assay (Fig 2B).

Mutation of individual hydrophobic residues on the hydrophobic side of the Bim MBD (BimI125E, BimL129E or BimI132E) abolished binding to membranes. In contrast, mutations on the other side of the helix including BimV124E and BimMBD2A still bound membranes albeit with substantially decreased affinity (Fig 2B). When N-terminal Venus-fused Bim MBD mutant constructs were expressed in DKO BMK cells, V-BimMBD2A, V-BimI125E, V-BimL129E, V-BimI132E had diffuse localizations similar to the distribution of Venus used as a cytoplasmic control. In contrast, the localization of V-BimWT and V-BimBH3-4E resembled the mitochondrial localized control Venus-ActA (Fig EV2) as predicted by the *in vitro* data (Fig 1).

Despite the drastic changes in affinity for membranes among Bim MBD mutants, the affinities of the mutants for binding to Bax were similar in solution and did not change greatly when membranes were present (Fig 2B). This data further confirmed that regulating binding to membranes and binding to Bax are independent functions of the Bim MBD. In the case of BimI125E, a mutant that activates Bax to permeabilize liposomes, the initial interaction with Bax must occur in solution as neither protein spontaneously bound to membranes (Fig 2B, Appendix Figure S2A).

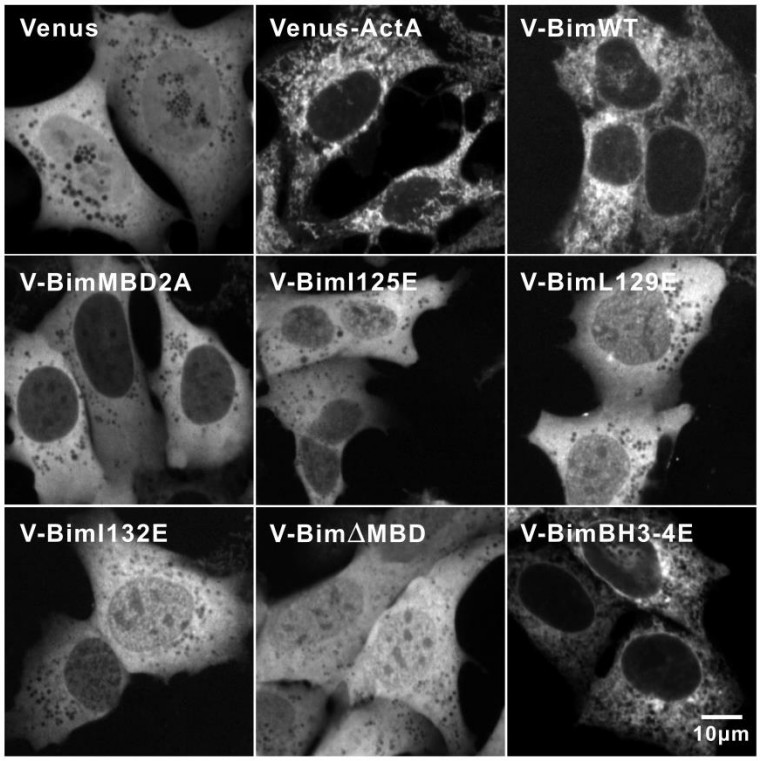


Fig EV2: Localization of Bim mutants in cells

$Bax^{-/-}$ $Bak^{-/-}$ (DKO) Baby Mouse Kidney (BMK) cells transiently transfected with constructs expressing Venus or N-terminal-Venus-fused Bim constructs: V-BimWT, V-BimMBD2A, V-BimΔMBD, V-BimI125E, V-BimL129E, V-BimI132E and V-BimBH3-4E. Cells were imaged using an automated confocal microscope and compared to the mitochondrial protein Venus-ActA and the cytoplasmic protein Venus used as controls. Representative images were selected based on visual assessment.

To our surprise there was not a good correlation between Bim binding to membranes and Bax activation. For example, while BimWT bound to membranes with a Kd of 31 pM, BimMBD2A and BimL125E bound to membranes only poorly (Kds of ~600 and >1000 pM, respectively) yet both triggered Bax mediated membrane permeabilization. In contrast, BimL129E and BimI132E did not bind to membranes or trigger membrane permeabilization even though they bound to Bax (Fig 2B).

Although no specific residues within the MBD were identified that inhibit Bim binding to Bax without disrupting Bim binding to membranes, it is possible that the MBD may provide structural support to position the BH3 domain or that it contributes to an extra binding site in Bim for Bax. Consistent with these possibilities, BimL129E and BimI132E, two Bim mutants that did not bind membranes, retained reasonable affinities for Bax (Kds ~100-200nM) but were unable to activate it.

As expected, the BimBH3-4E mutant, in which the BH3 domain is dysfunctional, no longer binds to or activates Bax even though its MBD is still intact and binds membranes. This result suggests that the Bim MBD provides a secondary role in Bax binding and activation, rather than an independent high affinity binding site (Fig 2B).

<u>The MBD is not required for Bim to inhibit Bcl-XL</u>

As the Bim MBD is essential for Bim to mediate Bax activation we investigated the importance of the Bim MBD for binding to and inhibiting Bcl-XL. Binding between Bim MBD mutants and Bcl-XL measured via FRET (Appendix Figure S2E-F) resulted in calculated Kds for the interactions that were generally similar (1-3 nM) to that obtained for binding to Bim WT (Fig 2B). As expected, the negative control, BimBH3-4E, did not bind Bcl-XL. Surprisingly, all of the mutants that were unable to activate Bax including BimΔMBD retained high affinity binding to Bcl-XL (Fig 2B, Appendix Figure S2E-F, Fig EV3A). Indeed when measured in the same way, BimΔMBD bound to Bcl-XL in solution and with membranes (Kds of 16 and 35 nM, respectively) values similar to those of the well characterized Bcl-XL inhibitory protein Bad (~30nM in solution and ~60nM with liposomes). These experiments suggest that Bim mediated inhibition of Bcl-XL does not require the Bim MBD or Bim binding to membranes.

Bim binding to Bcl-XL promotes apoptosis by releasing from Bcl-XL either an activator BH3-protein (Mode1) or activated Bax or Bak (Mode2) (Llambi et al, 2011). In a direct comparison in the ANTS/DPX liposome assay system BimΔMBD was almost as effective as the well-established Bcl-XL inhibitory BH3 protein Bad at functionally reversing Bcl-XL mediated inhibition of cBid or Bax (Fig 3A-B). Furthermore, both BimWT and BimΔMBD inhibited Bcl-XL binding to cBid and Bax in a concentration dependent manner (Fig EV3B-C) confirming that BimΔMBD retained both Mode 1 and Mode 2 inhibitory activity. Although BimΔMBD inhibited Bcl-XL binding to cBid and Bax less effectively than BimWT it

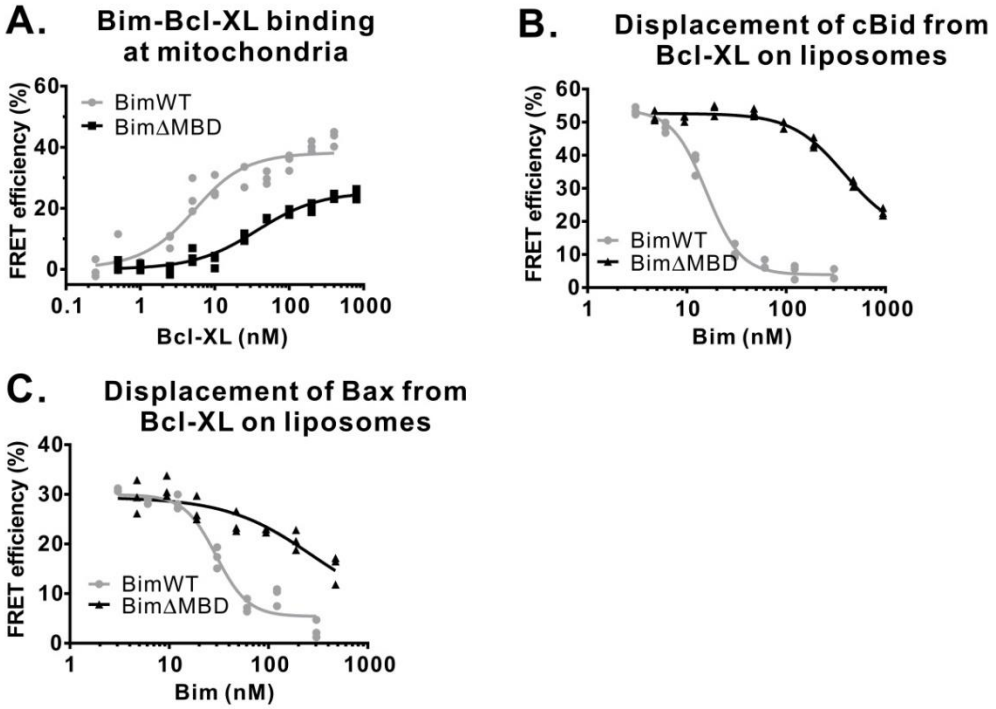


Fig EV3: The MBD improves but is not required for Bim to bind to Bcl-XL at mitochondria and to displace cBid and Bax from Bcl-XL on liposomes

- A Data from independent experiments are shown as individual points (n=3), some points are not visible due to overlap. a) BimWT and BimΔMBD bind to Bcl-XL at mitochondria. Binding of 4nM Alexa568-labeled BimWT or BimΔMBD with the indicated amounts of Alexa647-labeled Bcl-XL was measured by FRET in samples containing mouse liver mitochondria. The apparent Kd values for binding to Bcl-XL by BimWT (3±1nM) and of BimΔMBD (35±5nM) at mitochondria were very similar to those obtained on liposomes, 1±1nM and 35±5nM (Fig 2B), respectively.
- B To measure displacement of cBid from Bcl-XL, complexes of Alexa568-labeled cBid (4nM) and Alexa647-labeled Bcl-XL (10nM) were formed in incubations containing 2.9 nM liposomes. Control FRET measurements indicated that at those concentrations binding of cBid by Bcl-XL was saturated and that all of the proteins other than BimΔMBD were bound to liposome membranes. Displacement of cBid from Bcl-XL was measured by loss of FRET between Alexa568-labeled cBid and Alexa647-labeled Bcl-XL upon addition of the indicated concentrations of BimWT or BimΔMBD. The data suggest that while cBid and Bim bind to Bcl-XL relatively similarly (~16 nM Bim was required to displace half of 4nM cBid from 10 nM Bcl-XL) BimΔMBD does not compete

efficiently with cBid for binding to Bcl-XL (~ 390 nM BimΔMBD was required to displace half of 4nM cBid from 10 nM Bcl-XL).

C Bim mediated displacement of Bax from Bcl-XL was measured as in b) except that complexes between Bcl-XL and Bax were assembled from Alexa568-labeled Bax (10nM), a Bid mutant that activates Bax but does not bind Bcl-XL (10nM cBidmt1), Alexa647-labeled Bcl-XL (30nM), 2.9nM liposomes and the indicated concentrations of BimWT or BimΔMBD were added to displace Bax from Bcl-XL. Because in these reactions 30 nM Bcl-XL was required to bind 10 nM Bax saturably as opposed to the 10 nM Bcl-XL used in b) the concentrations of BimWT and BimΔMBD needed to displace Bax are higher than for displacement of cBid. The curves are further complicated because the displaced Bax can also bind to BimWT and BimΔMBD. Thus while this data demonstrate that both BimWT and BimΔMBD displace Bax from Bcl-XL the curves are difficult to interpret in terms of quantitative absolute binding constants.

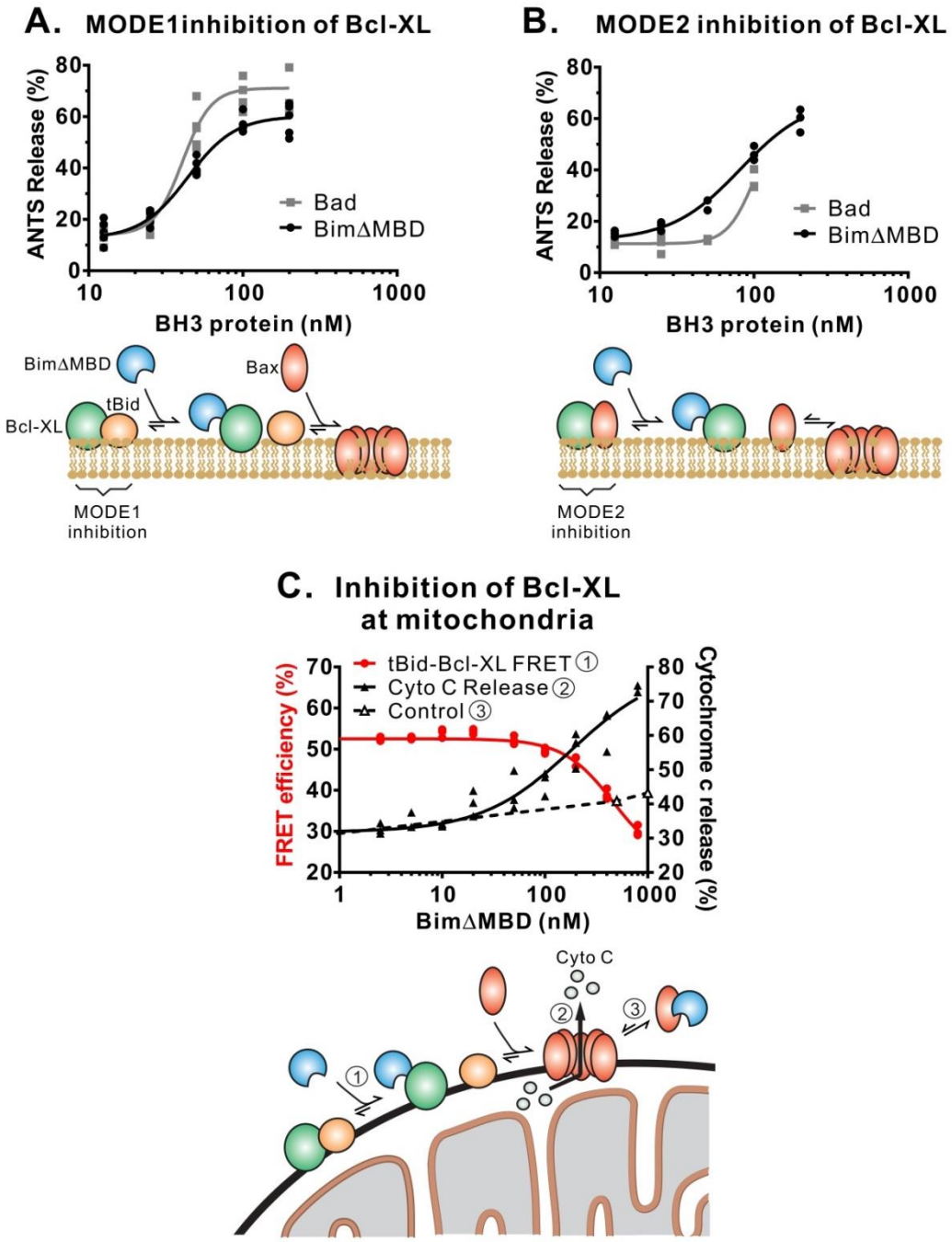


Fig 3: The MBD is not required for Bim to inhibit Bcl-XL

Data from independent experiments are shown as individual points (n=3), some points are not visible due to overlap. Cartoons indicate the interactions being measured, Bim (blue), Bax (red), tBid (orange), Bcl-XL (green), membranes (tan).

- A BimΔMBD and Bad release cBid from Bcl-XL. 100nM Bax, 20nM cBid and 40nM Bcl-XL were incubated with the indicated amounts of the positive control Bad or BimΔMBD and liposomes encapsulating ANTS-DPX. Liposome permeabilization was assessed after incubation at 37^{o}C for 3 hrs by measuring the increase in fluorescence due to ANTS-DPX release.
- B BimΔMBD and Bad release Bax from Bcl-XL. 100nM Bax, 20nM cBidmt1 (which activates Bax but does not bind Bcl-XL) and 40nM Bcl-XL were incubated with the indicated amounts of the positive control Bad or BimΔMBD and liposomes encapsulating ANTS-DPX. Liposome permeabilization was assessed after incubation at 37^{o}C for 3 hrs by measuring the increase in fluorescence due to ANTS-DPX release. Solubility issues limited the amount of Bad that could be added to these assays.
- C Inhibition of Bcl-XL function in mitochondria by BimΔMBD.. BimΔMBD mediated displacement of cBid from Bcl-XL was measured by FRET (red line). Samples contained 4nM Alexa568-labeled cBid, 10nM Alexa647-labeled Bcl-XL and the indicated concentration of BimΔMBD and mitochondria lacking Bax and Bak. Following FRET measurements, 25nM Bax was added and after 1h incubation mitochondria were pelleted by centrifugation. MOMP was assessed by quantifying cytochrome *c* in the pellet and supernatant fractions by western blotting (solid black line). The control curve (dashed line) represents cytochrome *c* release from mitochondria incubated with 25nM Bax and BimΔMBD. Numbers in circles relate the data to the cartoon.

was still much more effective than previous reports using BH3 peptides (Sarosiek et al, 2013). Finally, in mitochondria, Bim∆MBD disrupted the interaction between cBid and Bcl-XL as measured by FRET (Fig 3C, red) resulting in Bax activation and MOMP as measured by cytochrome c release (Fig 3C, black). This activity is due to inhibition of Bcl-XL function, as the same concentration of Bim∆MBD (used as a control) did not directly activate sufficient Bax to mediate MOMP (Fig 3C, dashed).

Deletion of the MBD does not prevent the functional interaction between Bim and Bcl-XL in live cells

To examine the interaction between Bim and Bcl-XL and the functional consequence of Bim inhibition of Bcl-XL in cells where other factors or protein modifications may modulate these effects, we measured binding interactions between the proteins in MCF-7 breast cancer cells by Fluorescence Lifetime Imaging Microscopy based Förster Resonance Energy Transfer (FLIM-FRET) (Aranovich et al, 2012). MCF-7 cells stably expressing mCerulean3 fused to Bcl-XL (Cer-Bcl-XL) were transiently transfected with constructs expressing N-terminal Venus-fused Bim constructs: V-BimWT, V-Bim∆MBD, V-BimBH3-4E. FRET was measured by a decrease in the fluorescence lifetime of the Cer-Bcl-XL donor and in intensity-weighted FLIM images is depicted by a color shift from blue to red. Binding to Cer-Bcl-XL was detected for both V-BimWT and V-Bim∆MBD, as shown by a second peak with a shorter lifetime in the histogram of the lifetimes recorded for individual pixels. As expected, binding was not detected

for the negative control V-BimBH3-4E (Fig 4A). Relative pseudo-dissociation constants (Kd_r values) were obtained by plotting FRET efficiency for each mitochondrial ROI of the 100 image datasets against the corresponding acceptor:donor intensity ratios (Liu et al, 2012). By this measure there was no detectable difference between V-BimWT and V-Bim∆MBD binding to Cer-Bcl-XL (Fig 4B). Exogenous expression of Cer-Bcl-XL and the V-Bim constructs is likely to result in cellular concentrations higher than the <1nM absolute Kd measured *in vitro*; hence if the binding constants for the proteins expressed in cells are also in the low nM range, FLIM-FRET is not expected to detect affinity differences for these Bim constructs because the expression levels of the proteins are higher than the Kds. In contrast and as expected, the negative control BimBH3-4E shows a linear increase in FRET efficiency with acceptor:donor ratio characteristic of random collisions between donor and acceptor proteins rather than an authentic binding interaction (Liu et al, 2012). Thus similar to our *in vitro* experiments both BimWT and Bim∆MBD bound to Bcl-XL in live cells. Moreover common morphological features of cell death, such as cell rounding are evident for both Bim constructs but not BimBH3-4E (Fig 4A) suggesting that the Bcl-XL inhibitory activity of Bim∆MBD is sufficient to kill these cells.

We were unable to measure Bim-Bax interactions with FLIM-FRET, as exogenous expression of V-Bim and Cer-Bax proved to be too toxic for cells. Therefore, we transiently expressed N-terminal Venus-fused Bim mutants in WT and DKO BMK cells and quantified apoptosis by AnnexinV staining (Fig 4C). As

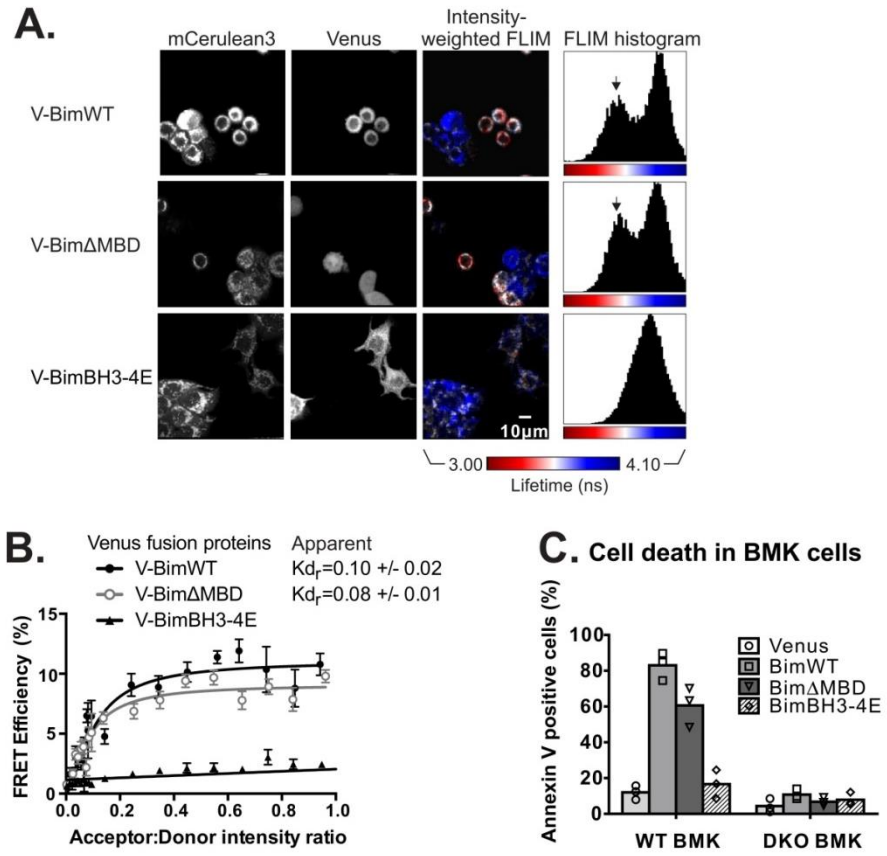


Fig 4: Deletion of the MBD does not prevent the functional interaction between Bim and Bcl-XL in cells

- A Binding of Bim and Bim mutants to Bcl-XL in live cells measured by FLIM FRET. MCF-7 cells stably expressing mCerulean3-Bcl-XL (MCF7-mC3-Bcl-XL cells) were transiently transfected with plasmids encoding Venus or N-terminal-Venus-fused BimWT, BimΔMBD or BimBH3-4E (as indicated to the left). 24 hours later the cells were imaged by automated time correlated single photon counting. Donor (mCerulean3) intensity images show stable expression of mC3-Bcl-XL while acceptor (Venus) intensity images indicate the localization of the acceptor fusion protein in transiently transfected cells. Intensity-weighted FLIM images in a continuous pseudo-color scale from 3.0-4.1 ns display shorter lifetimes (FRET) as red and longer as blue. FLIM histograms depict the number of pixels for each lifetime, and FRET is indicated by the distance between the shorter lifetime peak (arrows) and the main lifetime peak from untransfected cells in the same images.
- B Binding curves for N-terminal-Venus-fused BimWT, BimΔMBD and BimBH3-4E binding to mC3-Bcl-XL. Each curve represents FLIM-FRET data acquired from 100 images for each Bim construct (as indicated) expressed in MCF7-mC3-

Bcl-XL cells. Data are mean ± SEM. Relative pseudo Kd values (Kd_r) calculated from the curves (right) in arbitrary intensity ratio units.

- C Wild type (WT) or $Bax^{-/-}$ $Bak^{-/-}$ (DKO) Baby Mouse Kidney (BMK) cells transiently transfected with constructs expressing Venus or N-terminal-Venus-fused BimWT, BimΔMBD or BimBH3-4E, were analyzed for cell death 24 hours after transfection by measuring AnnexinV staining from Venus-positive (transfected) cells, imaged using an automated confocal microscope. The Y axis indicates the percentage of Venus positive cells that are Annexin V positive. Data from independent experiments are shown as individual points, some points are not visible due to overlap. (>400 cells/construct were analyzed in each experiment, n=3)

expected, V-BimWT induced significant apoptosis, while Venus alone and V-BimBH3-4E did not. V-BimΔMBD killed BMK cells only slightly less efficiently than BimWT ($p=0.0024$, calculated using Student's t test with a Bonferroni correction). Similar results were obtained in MEFs while in HCT-116 cells induction of cell death was equivalent for V-BimWT and V-BimΔMBD (Fig EV4). The results were not biased by V-Bim expression level as frequency analysis showed similar Venus intensity distributions for V-BimWT and V-BimΔMBD (Fig EV5). Thus, although the expression level varied widely between cells within each population there were similar numbers of cells expressing high and low amounts of the different fusion proteins such that there was no correlation between expression level and cell killing by the different proteins (Fig EV5). In additional control experiments, none of the Bim constructs killed DKO BMK, DKO MEF or DKO HCT-116 cells, confirming that the cell death induced by the mutants is dependent on Bax and/or Bak.

Discussion

Our results demonstrate that the C-terminal MBD of Bim is required for Bim to bind to and activate Bax. Deletion of the MBD reduces Bim binding to Bax to levels undetectable with the approximately physiological concentrations analyzed here. Instead the EC50 for Bax activation by Bim mutants without the MBD is in the M range, similar to that of a Bim BH3 peptide (Sarosiek et al, 2013). Thus, at physiologic concentrations the Bim MBD is required for Bim to activate Bax.

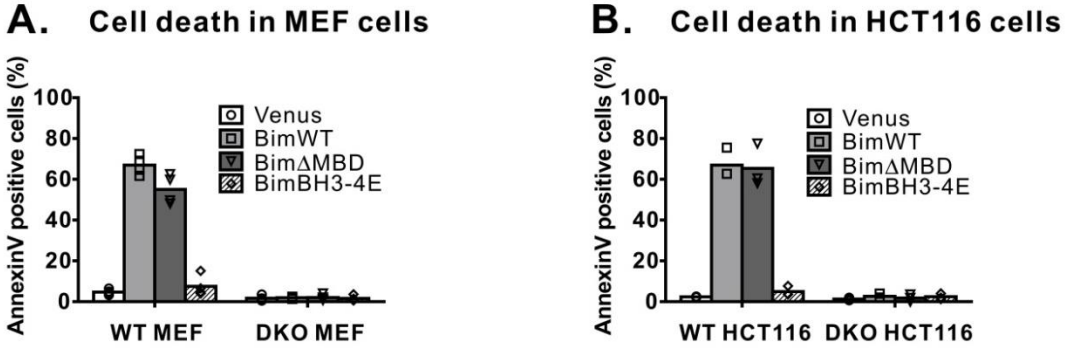


Fig EV4: Bim binding to Bcl-XL is sufficient to induce Bax/Bak dependent apoptosis in MEF and HCT116 cells

Deletion of the MBD does not prevent Bim from inducing Bax/Bak dependent apoptosis in MEF (Fig EV4A) and HCT116 (Fig EV4B) cells. In the names of the fusion proteins V- indicates Venus fused to the N-terminus of BimWT, BimΔMBD and BimBH3-4E. The fusion proteins were expressed in the indicated cell lines by transient transfection of the encoding plasmids. Compared to the wild-type protein (V-BimWT) killing of WT MEF's by V-BimΔMBD was slightly impaired in WT MEF ($p=0.0007$, calculated using Student's t test with a Bonferroni correction) and not-impaired in WT HCT-116 cells ($p>0.99$, calculated using Student's t test with a Bonferroni correction). As expected, expression of the control proteins Venus and the non-binding Bim mutant V-BimBH3-4E did not kill either cell line. Cell death was due to activation of Bax or Bak in the wild-type cells as none of the proteins killed DKO cells (DKO MEF, DKO HCT-116) that do not express either Bax or Bak. Cell death was measured 24hr after transfection as percentage of transfected (Venus-positive) cells that are AnnexinV positive. Data from independent experiments are shown as individual points, some points are not visible due to overlap. >400 cells/construct were analyzed in each experiment, n=4 for Fig EV4A and n=3 for Fig EV4B.

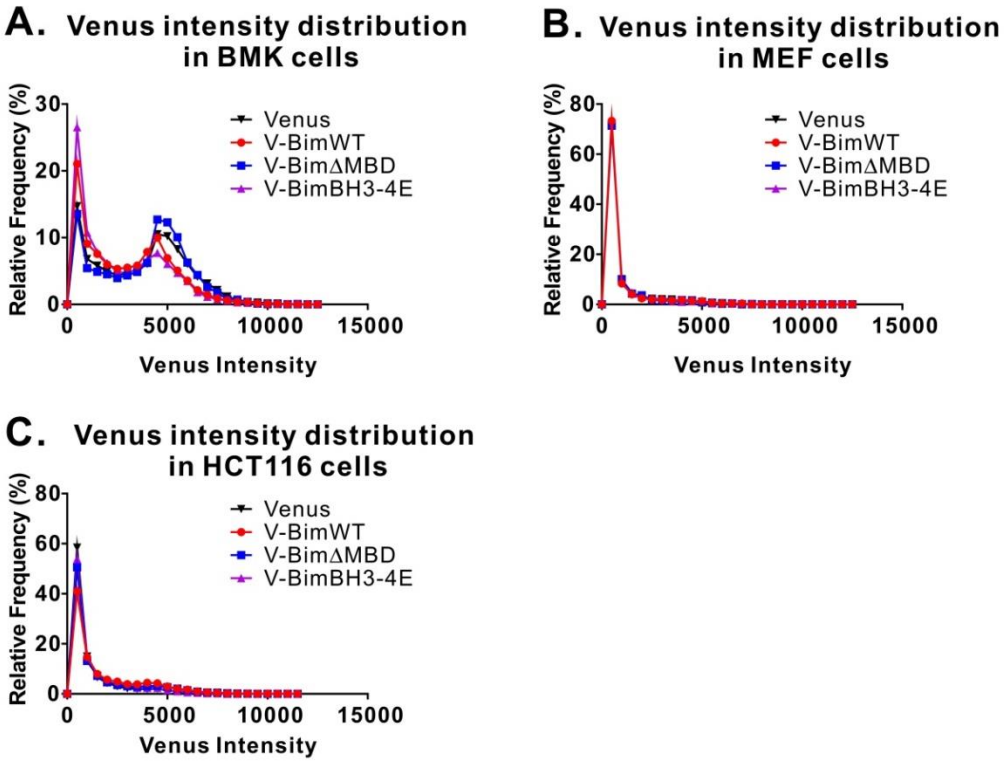


Fig EV5: Bim fusion proteins were expressed equivalently within each cell line.

Control experiments verified that although expressed differently between cell lines the different Venus fusion proteins were expressed equivalently within each cell line and expression level did not correlate with cell killing. For each cell line the relative amount of each of the fusion proteins was estimated by measuring the fluorescence intensity for individual cells. The number of cells expressing a specific intensity level (in bins of 500 intensity units) was plotted for each of the mutants. Cell numbers in each bin were divided by the total number of Venus positive cells to calculate the relative frequency (% of the total Venus positive cell population).

- A Venus intensity distribution of the Venus-positive cells in BMK cell death assays (Fig 4C). For unknown reasons there was a pronounced bimodal distribution of expression levels in BMK cells but no correlation between Venus intensity per cell and cell killing.
- B Venus intensity distribution for Venus-positive cells in MEF cell death assays (Fig EV4A).
- C Venus intensity distribution for Venus-positive cells in HCT116 cell death assays (Fig EV4B).

This domain of Bim also mediates binding to membranes (Fig 1A, Fig EV1A-B) as expected (Weber et al, 2007). However, Bim does not have to bind membranes to bind to Bax as shown for BimI125E, BimL129E and BimI132E (Fig 2B). In this regard, Bim differs significantly from cBid which binds to Bax only after cBid binds to membranes (Lovell et al, 2008; Shamas-Din et al, 2013a). This result suggests that different therapeutic strategies may be required to inhibit Bim and Bid mediated apoptosis. Our results also strongly suggest a role for specific residues within the Bim MBD in activation of Bax. Comparison of the data for Bim I125E with BimL129E and BimI132E reveals that while BimI125E does not bind membranes but activates Bax, BimL129E and BimI132E bind to Bax with and without membranes but are unable to activate it. Taken together these results suggest that Bim residues L129 and I132 contribute directly to Bim mediated Bax activation.

Unlike binding to Bax we found no critical role for the Bim MBD in Bim binding to Bcl-XL. FRET studies with purified proteins revealed that even deleting the entire region had only a small effect on the affinity as both BimWT and Bim∆MBD bound to Bcl-XL with nM-range Kds. Deletion of the MBD therefore eliminated Bim mediated activation of Bax with only minimal effect on Bim mediated inhibition of Bcl-XL and presumably also the other multi-domain anti-apoptotic proteins known to be inhibited by Bim including Bcl-2, Mcl-1 and A1. Consistent with Bim∆MBD inhibiting Bcl-XL and presumably other multi-domain

pro-apoptotic proteins, we detected Bcl-XL binding to Bim∆MBD by FLIM-FRET in live MCF7 cells (Fig 4B).

The apoptotic activity of Bim is likely mediated by a combination of Bax-activation and inhibition of anti-apoptotic proteins. By measuring the affinities for the different interactions (Fig 2B) and combining this information with the known approximately nM physiological concentrations of the proteins it was possible to create schema illustrating protein flow and the unexpected dual effects of the Bim MBD on regulating apoptosis (Fig 5). Flow in the schema is indicated by the different lengths of the equilibria arrows to illustrate the consequences of the various dissociation constants in Fig 2. Thus, even though Bim L129E binds both Bax and Bcl-XL it is unable to activate Bax thus the functional interaction is with Bcl-XL. The binding measurements in Fig 2 allow prediction of the outcome of more subtle differences in interactions for Bim and the Bim mutants. For example, even though BimL125E activates Bax the concentration required is around 100nM (close to the maximum seen in cells) while the dissociation constant for Bcl-XL (3nM) is such that in cells BimL125E would also normally bind and inhibit Bcl-XL rather than activate Bax (Fig 2).

Our results further suggest that Bim∆MBD is a unique reagent that may permit inhibition of all of the multi-domain anti-apoptotic proteins without activating the multi-domain pro-apoptotic proteins, a combination of functions not matched by other Bcl-2 family proteins or small molecules. This allowed us to demonstrate that the inhibition of anti-apoptotic proteins by Bim is sufficient to kill

BMK, MEF and HCT-116 cells in a manner dependent on Bax or Bak (Fig 4C, Fig EV4). It also suggests a reinterpretation of previous data claiming that Bim∆MBD is non-functional based on data in HEK293 cells as instead indicating that in HEK293 cells inhibition of multi-domain anti-apoptotic proteins is not sufficient to cause apoptosis (Weber et al, 2007). This suggests that unlike HEK293 cells, the cells lines that we examined are 'primed' by binding of an anti-apoptotic protein to either active Bax and Bak or an activator BH3 protein (Certo et al, 2006). Thus, Bim∆MBD can be used to analyze the primed state of tumor cells and may prove useful for identifying patients likely to respond to BH3-mimetic drugs.

While membrane binding mediated by the MBD is not prerequisite for interaction with Bax, binding to membranes increases subsequent Bax activation possibly through facilitating Bax conformational changes on the membrane (Fig 2B). Thus, it is likely that in cells expressing endogenous Bim that binding to membranes contributes to the efficiency with which the protein kills cells. However, this advantage was lost with expression of exogenous protein (Fig 4).

Substituting the two charged arginine residues in the center of the MBD with the more hydrophobic amino acid alanine disrupted rather than enhanced binding to membranes suggesting that the mechanism by which the MBD binds Bim to membranes differs from typical tail-anchored membrane proteins. Combined with our identification of residues predicted to be on the opposite side of the helix (I125, L129, I132) as important for the membrane binding function of MBD (Fig 2B, Appendix Figure S2A) our data suggests that the MBD binds to the surface of

the membrane as an amphipathic helix. We speculate that in this configuration the positively charged residues are important for interactions with negative phospholipid head groups on membranes while the hydrophobic residues may form a binding surface for MBD to interact with the hydrophobic chains in the lipid.

Our data suggests a model in which the unusual MBD of Bim acts as a switch between activation of Bax/Bak and inhibition of Bcl-XL/Bcl-2/Mcl-1 (Fig 5). The molecular mechanism of this modulation is a combination of both binding to membranes by Bim that recruits Bax to membranes, and of the Bax binding activity of the Bim MBD that independent of membrane binding increases the affinity of the Bim-Bax interaction and contributes to the activation of the pore forming activity of Bax.

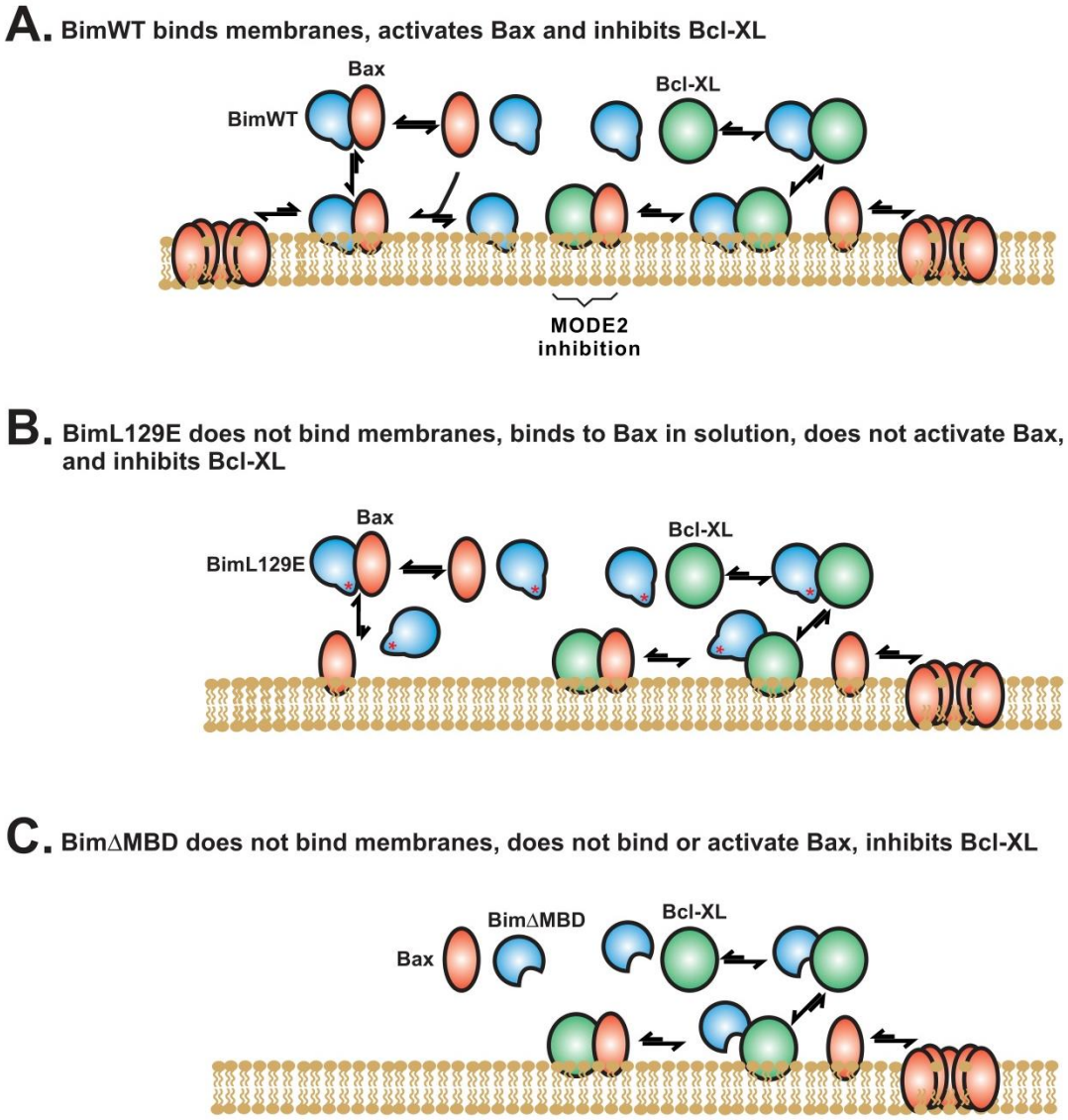


Fig 5: Pro-apoptotic activities of Bim controlled by the MBD

Direction of flow indicated by lengths of the equilibria arrows is based on the Kds measured for the binding interactions (Fig 2B), the approximate cellular concentrations of the various proteins and activity assays with liposomes and mitochondria. Interactions between BimWT, BimL129E, BimΔMBD (blue), Bax (red) and Bcl-XL (green) are shown in the cytoplasm and on the membrane (tan). The affinities for the interactions indicate that in cytoplasm binding to Bcl-XL is favored over binding to Bax. However, the subcellular concentrations of proteins are close to the Kd's, thus for the wild-type proteins both activities occur in cells and the local concentration differences on membranes determines which function predominates. Comparison of BimL129E (shown) BimI125E and BimΔMBD shows that the MBD, not membrane binding, controls the

interaction between Bax and Bim. Bcl-XL inhibits apoptosis through both MODE1 (inhibiting BH3 proteins) and MODE2 (inhibiting activated Bax). For simplicity only MODE2 is shown, regulation of MODE 1 is expected to be analogous.

- A BimWT binds membranes and can activate Bax and inhibit Bcl-XL.
- B BimL129E has no detectable membrane binding activity, binds to but does not activate Bax yet retains full Bcl-XL inhibitory activity. BimL129E binding to Bax is not reduced enough to account for the complete loss in Bax activation and membrane permeabilization. Thus BimL129 is involved in binding to and activating Bax.

C Deletion of the Bim MBD eliminates the interaction between Bim and Bax at physiologically relevant concentrations demonstrating that the MBD is required for Bim to activate Bax. The MBD is not required for binding to and inhibiting Bcl-XL as Bim∆MBD binds sufficiently tightly to Bcl-XL to release both pro-apoptotic BH3-proteins and Bax.

Materials and methods

Protein Purification

WT and single cysteine mutants of Bax, Bcl-XL, and cBid were purified as described previously (Kale et al, 2014). cBidmt1 was purified with the same protocol used for cBid (Kale et al, 2014).

For BimWT and single cysteine mutants of Bim, the cDNA encoding full-length wild-type murine BimL was introduced into pBluescript II KS(+) vector (Stratagene, Santa Clara, CA). Sequences encoding a polyhistidine tag followed by a TEV protease recognition site (MHHHHHHGGSGGTGGSENLYFQGT) were added to create an in frame fusion to the N-terminus of Bim.

BimMBD2A was created by mutating R130 and R134 in BimWT to alanine. Bim∆MBD was constructed by introducing a stop codon (TAA) after position P121 to delete the entire MBD. BimV124E, BimI125E, BimL129E and BimI132E were generated by mutating the corresponding hydrophobic residues to glutamate. As a negative control for live cell experiments BimBH3-4E was constructed by glutamate substitution of I146, L150, I153, and F157 in the BH3 domain (Chen et al, 2005).

The recombinant proteins were expressed in Arabinose Induced (AI) Escherichia coli strain (Life Tech, Carlsbad, CA). E. coli cells were lysed by mechanical disruption with a French press. The cell lysate was run on a Nickel-NTA column

(Qiagen, Valencia, CA) to bind the recombinant His-tag fused proteins and a buffer containing 300mM imidazole was applied to elute the proteins. This eluate was then adjusted to 150 mM NaCl and applied to a High Performance Phenyl Sepharose (HPPS) column. Bim was eluted with a no salt buffer and dialyzed against a buffer containing 10mM HEPES pH7.0, 20% Glycerol, then flash-frozen and stored at -80 °C.

Protein labeling

Single cysteine mutants of Bax, Bcl-XL, cBid and Bad were labeled with the indicated maleimide-linked fluorescent dyes as described previously (Kale et al, 2014; Lovell et al, 2008). Single cysteine mutants of Bim were labeled with the same protocol as cBid with the exception that the labeling buffer also contained 4M urea.

Bim binding to membranes

Liposomes (100 nm diameter) with a lipid composition resembling MOM were prepared as described previously (Kale et al, 2014). Mouse liver mitochondria were isolated from $Bak^{-/-}$ mice and therefore lack both Bax and Bak. Liposomes and mitochondria labeled with DiD (Life Tech, Carlsbad, CA) (0.5% for liposomes and 2% for mitochondria, % mass ratios) were used for labeling mitochondria and liposomes, respectively as described previously (Shamas-Din et al, 2013a). The single-cysteine mutant of Bim, BimQ41C, was labeled with Alexa568-maleimide. The labeled proteins were incubated with the indicated

amount of either unlabeled or DiD-labeled mitochondria or liposomes at 37^{o} C for 1h. Intensities of Alexa568 fluorescence were measured in both experiments as $F_{unlabeled}$ and $F_{labeled}$ respectively. FRET, indicating protein-membrane interaction, was observed by the decrease of Alexa568 fluorescence when Bim bound to DiD labeled membranes compared to unlabeled membranes. FRET efficiency was calculated by: $\text{FRET efficiency } (\%) = \left[1 - \frac{F_{labeled}}{F_{unlabeled}}\right] * 100\%$ as described previously (Shamas-Din et al, 2013a). The data was fit to a binding model as described below. Lines of best fit were calculated using least squares in Graphpad Prism.

Membrane permeabilization

Membrane permeabilization assays with liposomes encapsulating ANTS and DPX were performed as described previously (Kale et al, 2014). To measure permeabilization of BMK mitochondria, the indicated amounts of proteins were incubated with mitochondria purified from DKO BMK cells expressing mCherry fluorescent protein fused to the SMAC import peptide responsible for localization in the inter-membrane space. After incubation for 45 min at 37^{o} C. Samples were centrifuged at 20000g for 10min to separate the pellet and supernatant fractions. Membrane permeabilization was calculated by: $\text{Permeabilization } (\%) = \left[\frac{F_{supernatant}}{(F_{supernatant}+F_{pellet})}\right] * 100\%$, where F is the intensity of mCherry fluorescence in the fraction (Shamas-Din et al, 2013a). For mouse liver mitochondria, cytochrome

c release was measured by Western blot and calculated as described previously (Sarosiek et al, 2013).

Protein-protein binding

For cBid-Bcl-XL and Bax-Bcl-XL FRET, single cysteine mutants of cBid (126C) and Bax (126C) were purified and labeled with Alexa 568-maleimide. The single cysteine mutant of Bcl-XL (152C) was purified and labeled with Alexa 647-maleimide. Alexa568 labeled cBid or Bax were incubated with either Alexa647-labeled or unlabeled Bcl-XL along with the indicated concentrations of BimWT or Bim∆MBD. In the case of Alexa568 Bax, cBidmt1, a mutant of cBid that does not interact with Bcl-XL (Billen et al, 2008), was added to activate Bax. The intensity of Alexa568 fluorescence with unlabeled or Alexa647-labeled Bcl-XL was measured as $F_{unlabeled}$ or $F_{labeled}$ respectively. FRET was calculated as above.

For Bim-Bax or Bim-Bcl-XL FRET, single cysteine mutants of Bax (Bax126C) and Bcl-XL (Bcl-XL152C) were purified and labeled with Alexa647-maleimide then titrated into incubations containing Alexa568 labeled Bim proteins. FRET efficiency was determined as published for cBid-Bcl-XL (Lovell et al, 2008). Binding was measured after incubation at 37^{o} C for 1h unless specified otherwise. Lines of best fit were calculated using least squares in Graphpad Prism.

Dissociation constants (Kd) were measured in solution with and without added liposomes. Curves were fit to an advanced function taking into account the concentration of acceptor ([A]) change when [A] is close to Kd:

$$F = (F_{max})\left(\frac{([D] + [A] + K_d) - \sqrt{([D] + [A] + K_d)^2 - 4[D][A]}}{2[D]}\right)$$

[D] is the concentration of donor, F indicates the FRET efficiency with the concentration of acceptor as [A], F_{max} is the maximum FRET efficiency in the curve. The maximum FRET efficiency is a function of the relative positions, dipole orientations and mobility of the dyes as well as the affinity of binding. Therefore, comparisons were based on Kd's estimated from the data rather than maximum FRET efficiencies obtained.

The mCerulean3 (donor) to Venus (acceptor) FRET system we employed has been validated previously in live cells (Markwardt et al, 2011). To measure FRET in live cells time correlated single photon counting FLIM measurements (~100 images for each binding pair) were made on an ISS Alba microscope. Regions of interest (ROI) within each image were identified using a MATLAB script and the corresponding intensities and lifetime data were binned according to the acceptor:donor intensity ratio (Aranovich et al, 2012). For each bin, % FRET efficiency +/- SEM was calculated and these measurements were used for curve-fitting using one-site specific binding equation with a Hill slope in GraphPad Prism.

Cell death assay for Venus-Bim constructs

BMK, MEF and HCT116 cells were maintained at 37^{o}C (5% v/v CO_2) in dMEM complete [dMEM, 10% Fetal Bovine Serum, 1% essential AA (Gibco,

Grand Island, NY)]. Cells were seeded in Aurora 384-well plates (1000 cells/well for BMK and MEF, 2000 cells/well for HCT116). One day later cells were transfected using FugeneHD (Promega, Madison, WI) with plasmids encoding Venus, or Venus-fused BimWT, BimΔMBD, BimBH3-4E in an EGFP-C3 backbone. Cell culture medium (dMEM complete) was added to each reaction (50µl/0.05ug DNA) and the whole mix added to each well (50µl/well) of a pre-aspirated 384-well plate of cells. After 24 hours, cells were stained with Draq5 and Rhodamine-labeled AnnexinV and image acquisition was performed using the Opera High Content Screening System (Perkin Elmer, Woodbridge, ON) with a 20x air objective. Untransfected cells and cells treated with 1µg/mL staurosporine were used as negative and positive controls for AnnexinV staining. Cells were identified automatically using software as described previously(Shamas-Din et al, 2013a). Intensity features were extracted using a script (dwalab.ca) written for Acapella high content imaging and analysis software (Perkin Elmer, Woodbridge, ON). Cells were scored as Venus or Annexin V positive if the Venus or Annexin V intensity was above a threshold. This threshold was defined as the average intensity plus two standard deviations for the Venus or Annexin V channels in images of non-transfected cells. Cell death ascribed to the Venus-Bim fusion proteins was quantified as the percentage of Venus positive cells that were also Annexin V positive. To show that the Venus expression level on a cell by cell basis across all cells in different transfections was similar over the entire population of cells, frequency histograms were

generated for Venus-positive cells using bins in intervals of 500 intensity units. These distributions were used to verify that Annexin V positivity reflected the activity of the mutants rather than alterations in expression levels of the mutants. The number of cells in each intensity bin was divided by the total number of Venus positive cells in that transfection to calculate the relative frequency (% of the total Venus positive cell population). The graph was generated in GraphPad Prism. While the distribution of expression levels for individual cells varied between cell lines they were similar within one cell line for the different mutants expressed. Thus, the apoptotic activity of the mutants can be compared quantitatively for each cell line.

Imaging and Colocalization analysis for Venus-Bim constructs

Venus-fused BimWT, BimMBD2A, Bim∆MBD, BimI125E, BimL129E, BimI132E, BimBH3-4E and Venus-fused to the Bim MBD (Venus-MBD) were expressed by transient transfection in DKO BMK cells as described above. DKO BMK cells were used to prevent Bim from killing the cells. After 24hrs cells were stained with Draq5 and Mitotracker Red and images were acquired using an Opera automated microscope. Venus-ActA, (located at mitochondria) and Venus alone (located in cytoplasm and nucleus) were used as localization controls. Images of individual cells were identified by cell segmentation of Draq5 images. For Venus, V-BimWT, Venus-MBD and Venus-ActA, co-localization of Venus and Mitotracker Red signals were assessed within individual cells for the cytoplasmic region using Pearson Correlation Coefficients.

Tissue Culture for FLIM-FRET

Human breast cancer MCF-7 cells stably expressing mCerulean3-Bcl-XL (MCF7-mC3-BclXL cells) were maintained at 37^{o}C (5% v/v CO_2) in αMEM complete [αMEM (Invitrogen, Carlsbad, CA), 10% Fetal Bovine Serum (HyClone, Logan, UT), 1% PenStrep (cat. No 15140, Gibco, Grand Island, NY), 400µg/ml G418]. Transfections were performed as described by the manufacturer using the Fugene HD transfection reagent (Cat No. 231A, Promega, Madison, WI). For FLIM ~10 000 MCF7-mC3-BclXL cells/well (8 well chamber slide from Thermo Scientific) were seeded in 300µl αMEM complete (no G418 antibiotic). One day later cells were transfected with plasmids encoding Venus, or one of the Venus-Bim fusion proteins (V-BimWT, V-BimΔMBD or V-BimBH3-4E).

FLIM-FRET-data collection and analysis

FLIM data was collected on an automated ISS Alba system 18-26 hours after transfection. Fresh media (250µl) was added to samples before imaging. Four frames at 0.1ms/pixel dwell time were acquired for each image at 60x magnification and 25^{o}C. For each transfection, 100 images were collected automatically in a position series. Lifetime data was processed with a MATLAB script developed in house: ROIs were chosen based on a similar acceptor:donor ratio across the region, then the average lifetime across that ROI was calculated by averaging all of the decay curves for the pixels within the ROI. The data was analyzed using Microsoft Excel and a Channel2 (mCerulean3) cutoff chosen

visually using untransfected cells was applied to all datasets to minimize the impact of autofluorescence. The data was binned by acceptor:donor ratio (bin size: 0-0.01, 0.01-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.5-1.0, 1.0-1.5, 1.5-2.0, 2.0-2.5). For each bin, the average % FRET efficiency and standard error were calculated. % FRET efficiency$= (1 - \frac{\text{Average lifetime of bin}}{\text{Average untransfected lifetime}}) * 100$. The maximum FRET efficiency is a function of the relative positions, dipole orientations and mobility of the FPs as well as the affinity of binding. Therefore, comparisons were based on relative Kd's (Kd_r) estimated from the data using GraphPad Prism and calculated using an equation for one site specific binding with Hill slope (Liu et al, 2012; Osterlund et al, 2015).

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CHAPTER V: Bim double-bolt locks to Bcl-XL

Preface

The work presented in this chapter has been submitted as part of a research article to Nature Structural & Molecular Biology:

Qian L, Chi X, Leber B, Andrews DW, Bim double-bolt locks to Bcl-XL and Bcl-2.

Contribution of authors:

X.C. prepared all recombinant BimL mutants and carried out all of the liposome based experiments. Q.L. prepared all the mammalian expressing plasmids, customized the ImageJ script for FLIM-image analysis, planned and performed all live-cell based experiments including FLIM-FRET, co-localization analysis and functional assays . D.W.A. and B.L. directed the project. All authors contributed to interpreting the results, writing the manuscript and generating the model.

Objective of the chapter:

This chapter serves as a follow-up on the unexpected ABT-263 resistance of Bim-Bcl-XL complex discovered in Chapter III. Combined with live-cell based FLIM-FRET experiements (carried out by Q.L.), I utilized the well-established *in vitro* fluorescene technics in previous chapters to explore the dual role of MBD of Bim in binding to mitochondrial membranes and to Bcl-XL and identity the specific residues that are crucial to this interaction.

Highlights:

- ABT-263 displaces Bad and tBid but not Bim from Bcl-XL.
- The Bim-BH3 and the Bim-MBD contribute to the resistance of Bcl-XL:Bim complexes to ABT-263.
- The Bim-MBD improves Bim binding to Bcl-XL independent of its role in binding Bim to membranes.
- The Bim-MBD interacts with Bcl-XL through hydrophobic interactions, specifically at residue L185 (sequence positions refer to human BimEL, corresponding to L129 in BimL isoform).

Introduction

Tumor initiation, progression and resistance to chemotherapy rely on cancer cell survival by bypassing apoptosis (Kirkin et al, 2004). Bcl-2 family proteins regulate intrinsic apoptosis and mitochondrial integrity through direct physical interactions between the pro-apoptotic proteins Bax and Bak, the anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1 and the BH3-proteins Bad, Bid and Bim (Qian Liu, 2014; Youle & Strasser, 2008). Cancer cells are often primed for cell death as upregulated BH3-proteins are sequestered by anti-apoptotic proteins. Releasing specific sequestered BH3-proteins from Bcl-XL and Bcl-2 in primed cells may be a way to more selectively kill cancer cells than broadly inhibiting anti-apoptotitc proteins (Certo et al, 2006; Del Gaizo Moore et al, 2007; Goldsmith et al, 2006; Ni Chonghaile & Letai, 2008).

It is commonly accepted that BH3-proteins engage in the hydrophobic binding pockets of anti-apoptotic proteins through a conserved BH3 domain (Chen et al, 2005). ABT-263 (an orally available analogue of ABT-737), designed to mimic the BH3 domain of Bad, is an inhibitor of both Bcl-XL and Bcl-2. ABT-263 displays nanomolar affinities for the soluble domains of Bcl-XL and Bcl-2, and *in vitro* displaces from them BH3-peptides derived from different BH3-proteins (Tse et al, 2008). However a discrepancy between this data and the activities of the drugs in live cells was observed independently from two groups. In one, increased expression of Bcl-XL in human non-hodgkin lymphomas led to resistance to ABT-737 despite enhanced Bim expression (Merino et al, 2012). In

the other Fluorescent Lifetime Imaging Microscopy (FLIM) – Forster Resonance Energy Transfer (FRET) studies in live cells demonstrated that ABT-263 and ABT-737 displace full-length Bad and tBid (an activated form of Bid protein) but not the major isoforms of Bim (BimEL, BimL and BimS) from binding to Bcl-XL and Bcl-2 (Aranovich et al, 2012; Liu et al, 2012). Among the Bcl-2 family, Bim is the only BH3-protein that binds all anti-apoptotic proteins with high affinities and can directly activate Bax and Bak to initiate apoptosis (Chen et al, 2005; Kuwana et al, 2005; Letai et al, 2002). Therefore the unexpected lack of activity for BH3-mimetics on displacement of Bim could result in unexpected activities for BH3-mimetic drugs in patients including a lack of efficacy in some cancer cells.

Most of our understanding about the specificity and selectivity of BH3-proteins in binding anti-apoptotic proteins and Bax/Bak was derived using truncated proteins or peptides in solution due to the difficulty in employing full-length Bcl-2 proteins in a physiologically relevant environment. However, the conformational changes that occur in the Bcl-2 family proteins when they bind to membranes cause dramatic changes in their interacting partners and relative affinities (Leber et al, 2007; Leber et al, 2010; Lovell et al, 2008). This might explain the discrepancy between the efficiency of ABT-263 to displace BH3-protein fragments in solution versus full-length proteins in live cells (Liu et al, 2013).

Here we used FLIM-FRET to identify the major domains and specific residues in Bim that are responsible for binding to Bcl-XL and that confer

resistance of the complexes to ABT-263. FLIM-FRET enables direct measurement of full-length protein:protein interactions in their physiological environments and quantification of the inhibition of these interactions by mutagenesis or drug treatment (Aranovich et al, 2012; Kale et al, 2012; Liu et al, 2012).

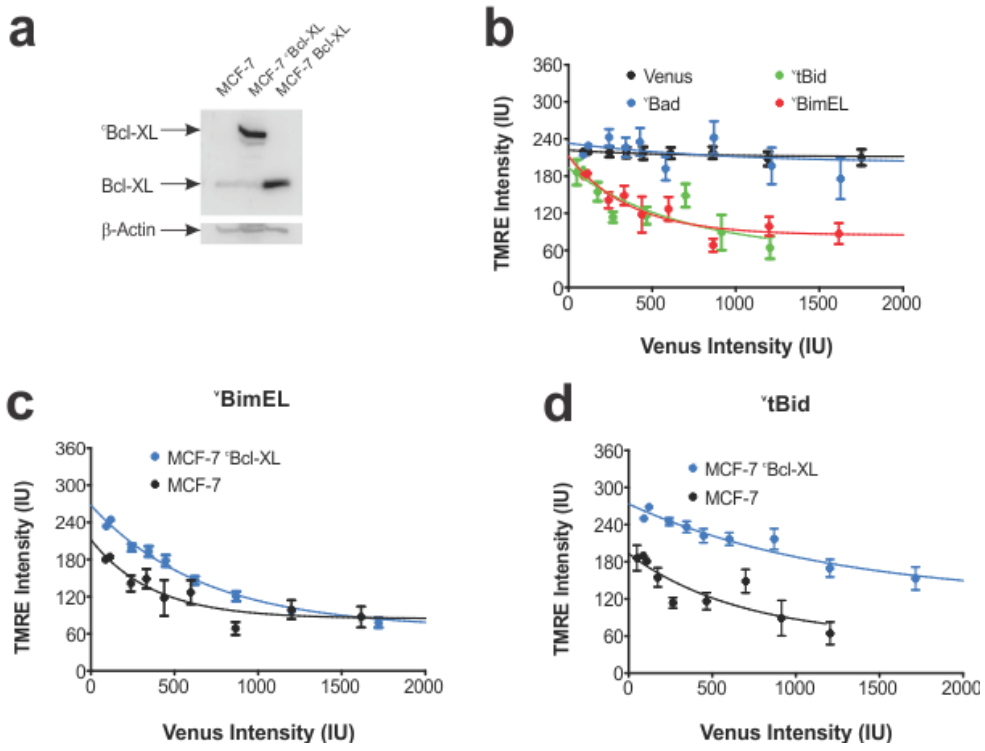
In addition to identifying the relative importance of specific residues within the BH3 domain that defines the binding specificity of Bim, we discovered additional interactions between the C-terminal membrane-binding domain (MBD) of Bim and Bcl-XL. Unexpectedly our data demonstrates a dual role for the Bim-MBD in binding to mitochondrial membranes and to Bcl-XL. Our data suggested a novel topology and mechanism for the Bim-MBD that positions the central hydrophobic residues of the MBD appropriately for binding to Bcl-XL.

Results

ABT-263 displaces Bad and tBid but not Bim from Bcl-XL.

Previously we measured protein:protein interactions in live cells using quantitative FLIM-FRET between proteins genetically fused to the yellow fluorescent protein tag Venus and the red tag mCherry or the cyan tag mCerulean and Venus respectively using a laborious manual method (Aranovich et al, 2012; Kale et al, 2012; Liu et al, 2012; Mergenthaler et al, 2012). The availability of brighter cyan fluorescence proteins such as mCerulean3 (mCer3) and new instrumentation capable of automated time correlated single photon

counting (TCSPC) from two fluorescence proteins simultaneously enabled development of an automated practical FLIM-FRET method to make comparative studies of drug responses by mutant Bcl-2 family proteins. Throughout this paper, fluorescent-tag-fused proteins are labeled by a superscripted c for mCerulean3-fused and a superscripted v for Venus-fused. The letters are in front of the protein names for amino-terminal fusion, and after for the carboxyl-terminal fusion. In our studies ^{v}BH3-proteins were expressed by transient transfection in a MCF-7 cell line stably over-expressing ^{c}Bcl-XL (supplementary Fig. 1a). Transient expression of ^{v}BH3-proteins ensured that within the population of cells there was a large enough range of expression levels to allow collection of the data needed to generate binding curves (Aranovich et al, 2012; Kale et al, 2012; Liu et al, 2012). Despite being fused to fluorescent proteins, ^{c}Bcl-XL and the ^{v}BH3-proteins ^{v}tBid, ^{v}Bim and ^{v}Bad retained their expected anti- or pro-apoptotic properties, respectively (supplementary Fig. 1b-d). To generate quantitative binding curves TCSPC data were collected for ^{c}Bcl-XL (FRET donor) and ^{v}BH3-proteins (FRET acceptor), and regions of interest (ROIs) within cells were automatically identified based on mCer3 signal intensity (Fig. 1a). For each ROI the TCSPC data was used to determine the average fluorescence lifetime of the mCer3 donor and the fluorescence intensities of both the donor (mCer3) and the acceptor (Venus). All of the raw data were binned according to FRET acceptor to donor ratio (Fig. 1b), averaged, and fitted to a binding curve with 95% confidence intervals calculated (Fig. 1d-f).



Supplementary Figure 1: Exogenously expressed fluorescence protein fusions to Bim, Bid, Bcl-XL are functional in MCF-7 cells

(a) Immunoblotting of lysates from MCF-7 cells (lane 1) and MCF-7 cells expressing exogenous ^{c}Bcl-XL (lane 2) or Bcl-XL (Lane 3) with an antibody to Bcl-XL suggests that the exogenous proteins are at least 20-fold over-expressed compared to endogenous Bcl-XL. The same blot was probed for β-actin as a loading control.

(b) Transient expression of exogenous ^{v}tBid (green) and ^{v}BimEL (red) but not ^{v}Bad (blue) nor the control protein Venus (black) kills MCF-7 cells. Images of individual cells were assessed for Venus intensity as an estimate of relative expression of the fusion proteins and TMRE intensity as fraction of mitochondria with an intact inner membrane transmembrane potential. Loss of mitochondrial transmembrane potential that correlates with expression of Venus intensity demonstrates that the ^{v}tBid and ^{v}BimEL fusion proteins retain pro-apoptotic activity. Data are mean ± SEM ($n \geq 20$).

(c-d) The fusion protein ^{c}Bcl-XL protects MCF-7 cells from apoptosis induced by (c) ^{v}BimEL or (d) ^{v}tBid. Cell viability was assessed by staining with TMRE 24 hours after transient transfection of MCF-7 cells (black) or MCF-7 cells overexpressing ^{c}Bcl-XL (blue) with plasmids encoding ^{v}BimEL or ^{v}tBid as indicated above the panels. Venus and TMRE intensities were analyzed for individual cells as in (b).

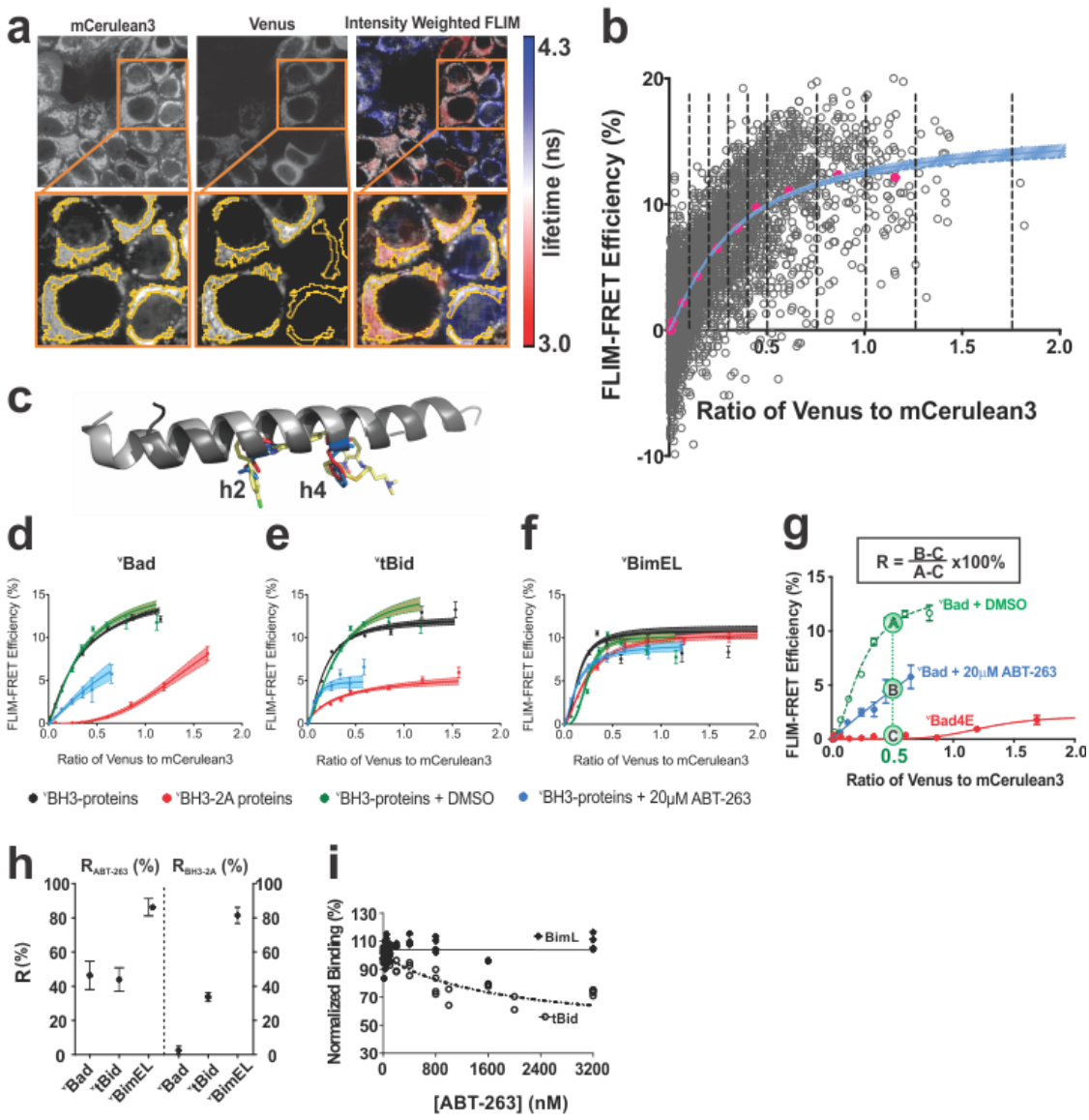


Figure 1: ABT-263 does not displace Bim from binding to Bcl-XL

(a) Live cell images including mCerulean3 channel, Venus channel and intensity weighted FLIM images from FLIM-FRET measurements for the interaction between ^{c}Bcl-XL and ^{v}Bad. A higher magnification view is shown below with Domains Of Interest (ROIs) for binding curve fitting indicated. Fluorescence lifetime images are presented in a continuous pseudocolor scale ranging from 3 to 4.2 ns. Intensity weighted fluorescence lifetime images were generated using ImageJ and both the contrast and gamma of the intensity image were adjusted so that the mitochondrial area can be easily visualized. Intensity weighted images are used only as an interpretive guide for the histograms and were not used for any of the calculations reported.

(b) Binding curve fitting after binning. Data from more than 8,500 ROIs were binned into groups according to the ratio of Venus to mCerulean3 intensities, and the FLIM-FRET efficiency values (red) were plotted versus the ratio of Venus to mCerulean3 intensity. The data were fit to a binding curve for the interaction between ^{c}Bcl-XL and ^{v}Bad (blue) with the 95% confidence interval shown.

(c) Structural alignment of ABT-263 with BH3 peptides from the complexes: Bcl-XL:Bim-BH3 (1PQ1, blue), Bcl-XL:Bad-BH3 (2BZW, pink), and Bcl-XL:ABT-263 (4QNQ, yellow). ABT-263 and side chains of the key hydrophobic residues (h2 and h4) in the BH3 peptides are shown.

(d-f) ABT-263 can displace ^{v}Bad, ^{v}tBid but not ^{v}BimEL from binding ^{c}Bcl-XL in MCF-7 cells. Binding curves for ^{c}Bcl-XL and ^{v}BH3-proteins ^{v}Bad (d), or ^{v}tBid (e), or ^{v}BimEL (f) in the absence (black) or presence of DMSO (green), 20 μM ABT-263 (blue), or BH3-2A mutation in the BH3 protein (red). Individual points are the average FLIM-FRET efficiencies in corresponding bins (n ranges from 20 to 3,000 in each bin combined from 3 independent experiments), the error bars indicated standard error of the mean, and the dotted shadowed area represents the 95% confidence interval for each binding curve. Blue lines are truncated because at higher ratios of Venus to cerulean3 there is sufficient free BH3 protein to kill the cells.

(g) Calculation of $R_{ABT\text{-}263}$ for ^{c}Bcl-XL:^{v}BH3-protein interactions. To maximize the dynamic range of the assay and quantify the impact of ABT-263 on the ^{v}BH3-proteins binding to ^{c}Bcl-XL, we interpolated the FLIM-FRET efficiency from the fitted binding curves at an intensity ratio of Venus to mCerulean3 of 0.5 (point A, B and C). The signal remaining after the addition of ABT-263 expressed as a percentage of the signal prior to addition of the drug is defined as $\mathbf{R_{ABT\text{-}}263}$. The non-binding mutant ^{v}Bad4E, in which h1, h2, h3 and h4 in the BH3 domain were all mutated to glutamic acid, served as a control for FRET due to random collisions (point C).

(h) $R_{ABT\text{-}263}$ and $R_{BH3\text{-}2A}$ for ^{c}Bcl-XL:^{v}BH3-proteins. Data are mean ± 95% confidence intervals from FLIM-FRET binding curves shown in Fig. 1d-f.

(i) ABT-263 displaces tBid but not Bim from Bcl-XL in vitro. $R_{ABT\text{-}263}$ for Bcl-XL:tBid and Bcl-XL:Bim quantified for purified full-length proteins incubated with liposomes. Data from independent experiments are shown as individual points, some points are not visible due to overlap (n=3).

Studies with recombinant truncated proteins and peptides suggest that BH3-proteins engage the hydrophobic pocket of anti-apoptotic proteins through four conserved hydrophobic residues (h1-h4) within the BH3 domain (Chen et al, 2005). ABT-263 prevents BH3-protein binding by competing for the same binding site on Bcl-XL by specifically mimicking the binding of residues h2 and h4 in the BH3 domain (Fig. 1c) (Tse et al, 2008). To determine the importance of these contacts for BH3-proteins binding to Bcl-XL in live cells, we expressed wild-type and mutant ^{v}BH3-proteins with alanine mutations in the h2 and h4 positions (BH3-2A) and measured their binding to ^{c}Bcl-XL. As expected, BH3-2A mutations in ^{v}Bad and ^{v}tBid disrupted binding to ^{c}Bcl-XL. However the BH3-2A mutant of ^{v}BimEL bound to ^{c}Bcl-XL similarly to wild-type ^{v}BimEL (Fig. 1d-f, compare black and red), suggesting interactions with other residues contribute to binding Bim to Bcl-XL. Consistent with this result ABT-263 displaced both ^{v}Bad and ^{v}tBid but not ^{v}BimEL from ^{c}Bcl-XL (Fig. 1 d-f, compare green and blue). Therefore, for ^{v}tBid and ^{v}Bad it was not possible to measure binding to ^{c}Bcl-XL at ratios of Venus to mCer3 intensities greater than ~ 0.7 in the presence of ABT-263 because the released ^{v}BH3-proteins killed the cells.

To maximize the dynamic range of the assay and quantify the impact of ABT-263 on the ^{v}BH3-proteins binding to ^{c}Bcl-XL, we interpolated the FLIM-FRET efficiency from the fitted binding curves at intensity ratios of Venus to mCer3 of 0.5 and 0.25 respectively, to account for differences in the expression of ^{c}Bcl-XL and in the cell lines. The fraction of the FRET signal remaining in the

presence of ABT-263 expressed as a percentage is defined here as **Resistance** to displacement by ABT-263 ($\mathbf{R_{ABT\text{-}263}}$, Fig. 1g). The effects of mutations on the binding of BH3-proteins can be calculated similarly and thereby quantitatively compared to the effect of drugs and other mutations using Resistance as a common measure. For the BH3-2A mutation this value is reported as $\mathbf{R_{BH3\text{-}2A}}$. In all cases the FRET signal from the non-binding mutant ^{v}Bad4E, in which h1, h2, h3 and h4 in the BH3 domain were mutated to glutamic acid, served as a control for FRET due to random collisions and was subtracted as background (Fig. 1g).

Plotting $R_{ABT\text{-}263}$ for different interactions indicated that >80% of ^{c}Bcl-XL:^{v}BimEL complexes were resistant to the addition of ABT-263. In contrast, ^{c}Bcl-XL:^{v}Bad and ^{c}Bcl-XL:^{v}tBid complexes were less than 50% resistant to the drug (Fig. 1h-left black). The $R_{BH3\text{-}2A}$ values also reflect the minimal effect of BH3-2A mutations on ^{c}Bcl-XL:^{v}BimEL complexes ($R_{BH3\text{-}2A}$ ~ 80%) compared to ^{c}Bcl-XL:^{v}Bad ($R_{BH3\text{-}2A}$ ~ 2%) and ^{c}Bcl-XL:^{v}tBid ($R_{BH3\text{-}2A}$ ~30%) complexes (Fig. 1h).

To confirm that the stability of the complexes we measured in live cells was due to direct interactions between the proteins and not due to interactions with other proteins or post-translational modifications of the proteins, FRET was measured for donor-fluorophore-labeled recombinant BimL or tBid with acceptor-fluorophore-labeled Bcl-XL in a cell free liposome based system. In this assay the BH3-proteins were added at amounts predetermined to result in complete binding of BimL or tBid to Bcl-XL. ABT-263 was then titrated into the samples to compete with the BH3-proteins (Fig. 1i). As expected, concentrations of ABT-263 that

displaced tBid from Bcl-XL (IC50 ~ 400nM) had little effect on the interaction between Bcl-XL and BimL (stable at > 3μM). Thus direct binding of Bim to Bcl-XL is more resistant to ABT-263 than binding of tBid to Bcl-XL.

The Bim-BH3 and the Bim-MBD contribute to the resistance of Bcl-XL:Bim complexes to ABT-263.

It is commonly accepted that the only binding interaction between Bim and anti-apoptotic proteins is via the BH3 domain of Bim (Bim-BH3) binding in the hydrophobic groove formed by the BH3-1-2 domains of the anti-apoptotic proteins(Liu et al, 2010; Sattler et al, 1997). However, this conclusion is based on measurements made with truncated proteins and/or peptides.

Unlike what was reported based on studies of partial proteins in vitro, in live cells the binding of full-length versions of all three Bim isoforms (BimEL, BimL and BimS) to Bcl-XL is resistant to ABT-263(Liu et al, 2012). Therefore, the N-terminal, BH3, and C-terminal MBD domains shared by these isoforms must contain the sequences that confer ABT-263-resistant binding. To identify these sequences mutants harboring deletions of these domains in ^{V}BimEL and mutants in which the BH3 domains were exchanged between BimEL and Bad (Fig. 2a) were assayed by FLIM-FRET for resistance to ABT-263 (supplementary Fig. 2a-f). Truncation of the N-terminus of ^{V}BimEL had little impact on the measured $R_{ABT\text{-}263}$ value, but swapping the BH3 domain in Bad for that in BimEL increased while the

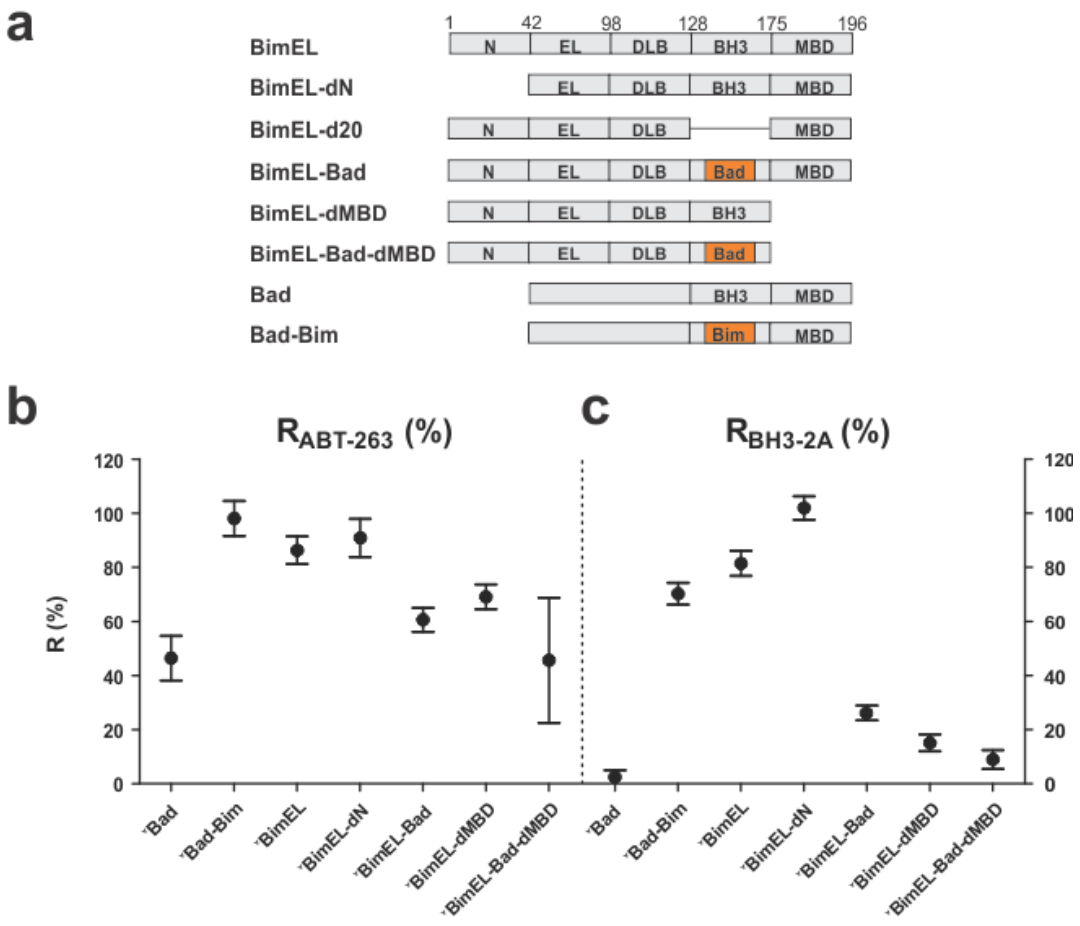
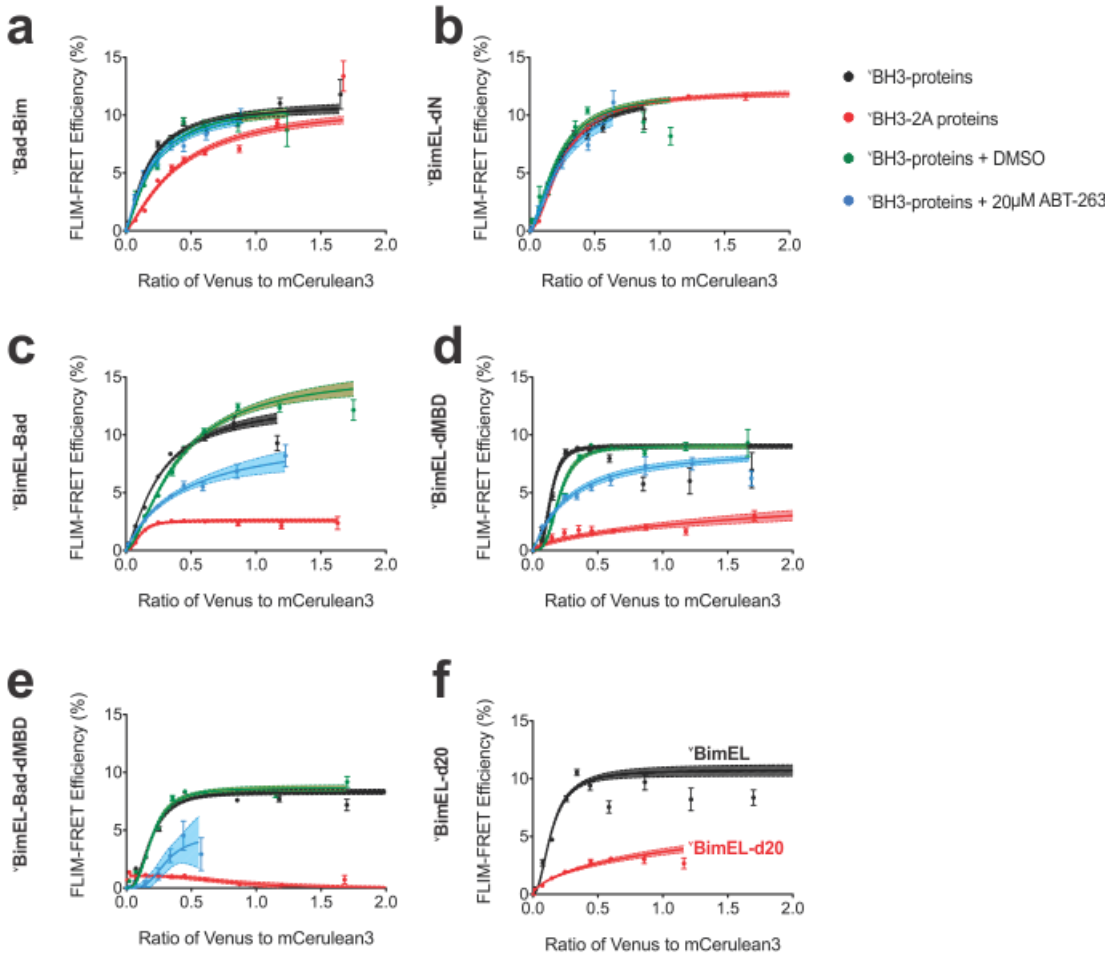


Figure 2: The resistance of ^{c}Bcl-XL:^{v}BimEL :^{v}BimEL complexes to ABT-263 is dependent on both the Bim-BH3 and the Bim-MBD

(a) Diagram for BimEL or mutant constructs with Venus fused to the N-termini of the proteins.

(b) $R_{ABT-263}$ for ^{c}Bcl-XL:^{v}BH3-protein complexes. Data are mean ± 95% confidence intervals calculated from FLIM-FRET binding curves shown in supplementary Fig. 2a-f (green and blue).

(c) R_{BH3-2A} of ^{c}Bcl-XL:^{v}BH3-protein complexes. Data are mean ± 95% confidence intervals calculated from FLIM-FRET binding curves shown in supplementary Fig. 2a-f (black and red).



Supplementary Figure 2: Binding curves demonstrating that exchanging the BH3 region for that from Bad or altering the Bim-MBD impairs Bim binding to Bcl-XL

FLIM-FRET binding curves for the proteins indicated to the left of each row and ^{c}Bcl-XL in MCF-7 cells illustrate the extent of binding for untreated or DMSO treated controls (black and green, respectively), cells treated with 20 μM ABT-263 (blue) or cells expressing BH3-2A mutant BH3-proteins (red). Data points in FLIM-FRET binding curves correspond to the average FLIM-FRET efficiency for binned data (20 ≤cells/bin≤3,000). Data are mean ± SEM; Shadowed area represents 95% confidence interval for each binding curve.

converse decreased $R_{ABT-263}$ for binding of the chimeric proteins to ^{c}Bcl-XL (Fig. 2b). Unexpectedly, deletion of the Bim-MBD also resulted in a significant decrease in $R_{ABT-263}$ for ^{c}Bcl-XL:^{v}BimEL complexes (Fig. 2b). Combining the two mutations by replacing the Bim-BH3 domain with Bad-BH3 and deleting the Bim-MBD reduced $R_{ABT-263}$ to the similar value as ^{v}Bad has, indicating roles for both the Bim-MBD and the Bim-BH3 in binding anti-apoptotic proteins (Fig. 2b).

To determine the importance of interactions via the two domains in a different way R_{BH3-2A} was measured for the interactions between similar mutants and ^{c}Bcl-XL in live cells (Fig. 2c). As expected, in the control experiment the 2A mutation abolished the interaction between ^{v}Bad and the anti-apoptotic proteins but had minimal effect on ^{v}BimEL binding. Moreover, replacing the Bad-BH3 domain with the Bim-BH3 domain harboring the BH3-2A mutation increased the affinity of Bad binding to anti-apoptotic proteins (increased R_{BH3-2A}). Thus, additional residues within the Bim-BH3 sequence contribute to Bim binding to anti-apoptotic proteins. However, replacing the Bim-BH3 domain with the Bad-BH3 domain did not reduce R_{BH3-2A} to zero as it does for ^{v}Bad (compare ^{v}Bad with ^{v}BimEL-Bad) suggesting that domains of Bim outside the BH3 domain also contribute to binding to anti-apoptotic proteins. Consistent with the additional binding interaction being provided by the Bim-MBD domain as suggested by the $R_{ABT-263}$ results in Fig. 2b, deleting the Bim-MBD dramatically impaired binding of BimEL BH3-2A mutants to Bcl-XL (^{v}BimEL-dMBD, R_{BH3-2A} <20%). Together these results demonstrate that sequences in the BH3 domain and the MBD of Bim are

involved in binding to Bcl-XL and necessary and sufficient to confer resistance of complexes of these proteins to ABT-263.

The Bim-MBD improves Bim binding to Bcl-XL independent of its role in binding Bim to membranes.

The C-terminal 20 amino acids of Bim constitute a membrane binding domain as deletion of this domain abrogated binding to membranes (O'Connor et al, 1998). We therefore hypothesized that the membrane binding function of this domain might impact the resistance of Bcl-XL:Bim complexes to either ABT-263 treatment or BH3-2A mutations by increasing the local concentration of the proteins on mitochondria. However, the sequence of the Bim-MBD is very different from the hydrophobic C-terminal MBDs of other Bcl-2 family proteins such as Bcl-2, Bcl-XL, Bax and Bik. Instead of an uninterrupted hydrophobic sequence of more than 15 residues the Bim-MBD includes multiple positively charged Arg residues (Fig. 3a).

For this reason, we examined the importance of sequences within the Bim-MBD domain for ABT-263 resistant binding of ^{V}BimEL to ^{C}Bcl-XL. A series of mutants were generated in which hydrophobic (I181, L185, I188 and V192) and hydrophilic (R186 and R190) residues were substituted with charged amino acids (Asp/Glu) or Ala (MBD-2A), respectively (Fig. 3a). The impact of these mutations on both the subcellular localization of ^{V}BimEL and the resistance to ABT-263

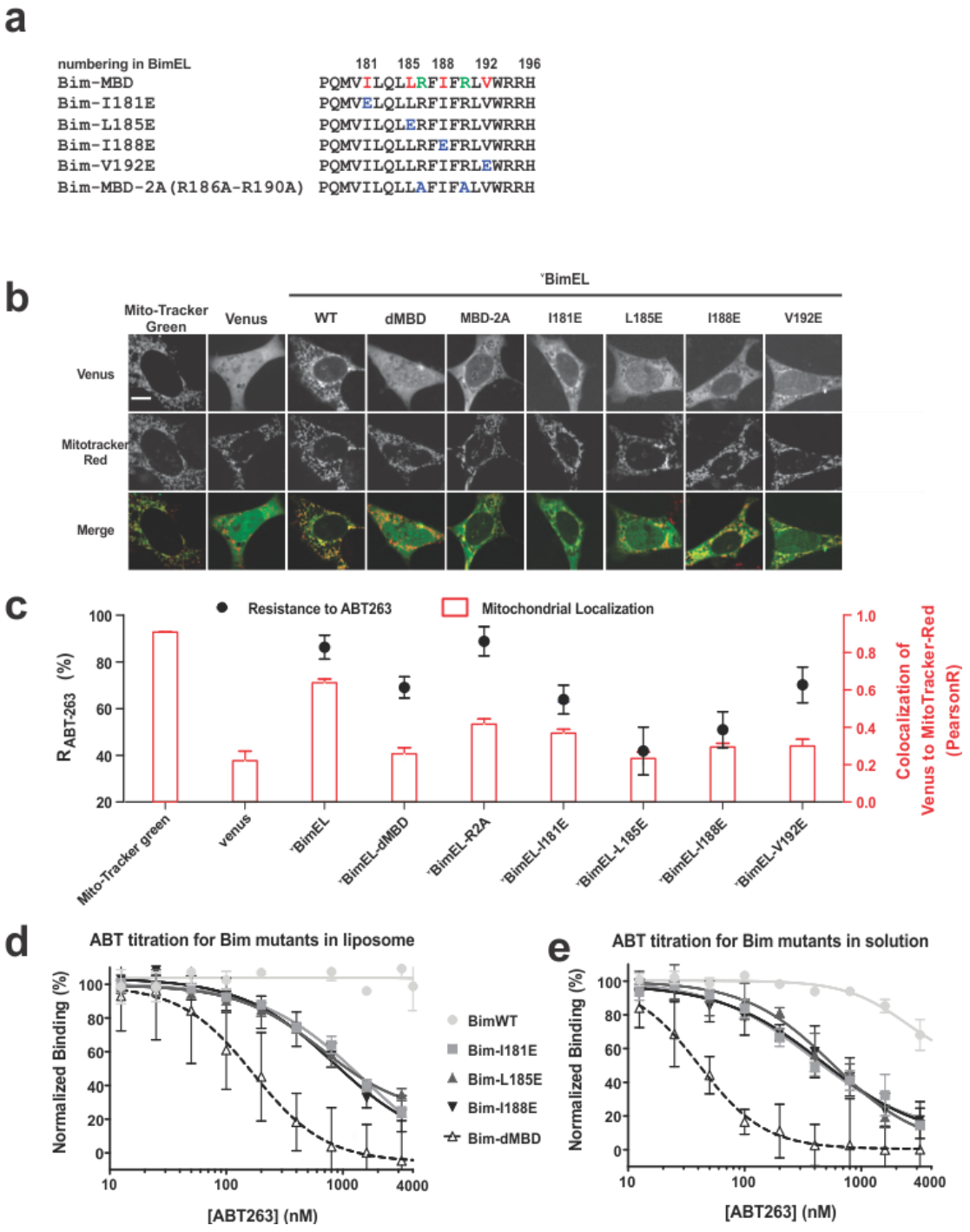


Figure 3: Binding of the Bim-MBD to Bcl-XL is independent of binding to membranes

(a) Sequence alignment for the Bim-MBD and mutants. Hydrophobic residues, blue; hydrophilic residues, green; numbering is for BimEL.

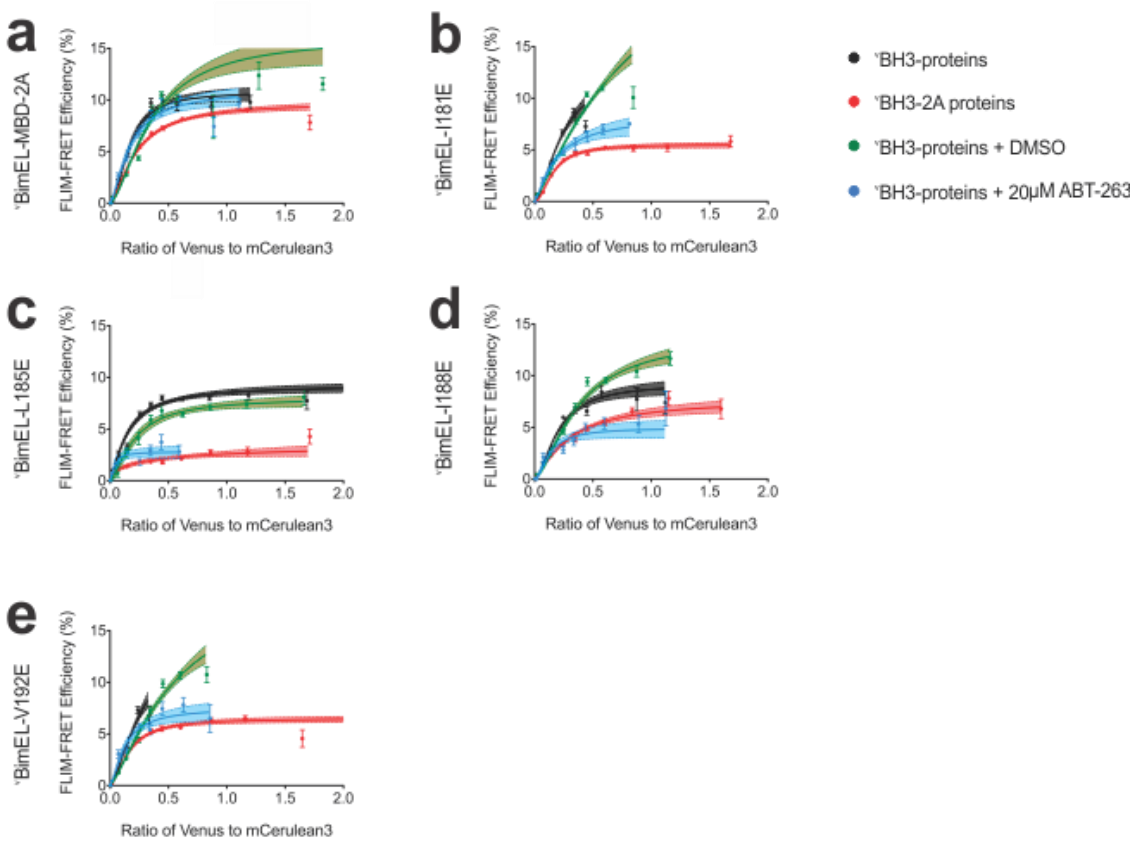
(b) Representative images showing sub-cellular localization of ^{v}BimEL-mutants compared to mitotracker. The scale bar represents 10 μm.

(c) Cellular localization and $R_{ABT-263}$ for ^{v}BimEL-mutants bound to ^{c}Bcl-XL. Co-localization (Red bars) were assessed using Pearson Correlation Coefficients for Venus and MitoTracker-Red signals. Data are mean ± SEM (n>30). $R_{ABT-263}$ of ^{c}Bcl-XL:^{v}BimEL-mutant complexes are shown as black dots. Data are mean ± 95% confidence intervals calculated from FLIM-FRET binding curves shown in supplementary Fig. 3a-l. Lack of correlation between localization and $R_{ABT-263}$ for ^{v}Bim-MBD-2A and and ^{v}Bim-V192E demonstrate that the two functions of the Bim-MBD are independent.

(d-e) Bcl-XL:BimL binding is resistant to ABT-263 in the absence of binding to membranes. Bcl-XL:BimL binding in the presence of different concentrations of ABT-263 quantified *in vitro* using purified full-length proteins with (d) and without (e) liposomes. Data from independent experiments are shown as individual points, some points are not visible due to overlap (n=3).

treatment of ^{v}BimEL binding ^{c}Bcl-XL were quantified. Surprisingly although the mitochondrial localization of all ^{v}BimEL mutants including deletion of the entire domain (vBimEL-dMBD) was relatively poor (Fig. 3b-c), the mutants displayed different binding properties to ^{c}Bcl-XL (Fig. 3c and supplementary Fig. 3a-e). For example similar to ^{v}BimEL, the MBD-2A and V192E mutants bound to ^{c}Bcl-XL in an ABT-263 resistant fashion (high $R_{ABT-263}$). In contrast, ^{v}BimEL mutants L185E and I188E were impaired with a ~ 40-50% decrease in $R_{ABT-263}$ for binding to ^{c}Bcl-XL. These results suggest that localization to mitochondria and binding to anti-apoptotic proteins by the Bim-MBD are independent functions.

To assess the effect of the Bim-MBD binding to membranes on the interaction between Bim and Bcl-XL directly we measured the interaction using purified proteins and liposome membranes. Due to the difficulty in purifying BimEL, for these experiments binding was measured by FRET for BimL and BimL-mutants to Bcl-XL in incubations with and without liposome membranes. In this assay displacement from Bcl-XL of BimL or mutants thereof (I181E, L185E and I188E and dMBD, numbered according to the corresponding position in the BimEL sequence) was measured for different doses of ABT-263. Coinciding with our observations in live cells, the binding of BimL-dMBD to Bcl-XL was much more sensitive to ABT-263 (IC50 ≈ 200 nM) compared to BimL (IC50>>4μM). As expected from the measurements in live cells (Fig. 3b-c), mutation of I181, L185 and L188 in the MBD domain each resulted in increased sensitivity to displacement by ABT-263 although to a smaller extent than deletion of the entire



Supplementary Figure 3: FLIM-FRET data for Bim-MBD mutants binding to Bcl-XL

FLIM-FRET data for ^{c}Bcl-XL binding to the ^{v}BimEL mutants indicated to the left of the panels. Untreated and DMSO treated controls, black and green, respectively. Cells treated with 20 μM ABT-263, blue. Data points in FLIM-FRET binding curves correspond to the average FLIM-FRET efficiency for binned data (20 ≤cells/bin≤3,000). Data are mean ± SEM; Shadowed area represents 95% confidence interval for each binding curve.

domain (Fig. 3d). In the absence of membranes, BimL binding to Bcl-XL was more easily displaced by ABT-263 (compare Fig. 3d with 3e). Nevertheless, substitution of BimL I181, L185 or I188 with a negatively charged amino-acid further decreased resistance of Bcl-XL:BimL complexes in solution to ABT-263 (Fig. 3e). Thus these residues in the Bim-MBD domain augment binding of BimL to Bcl-XL directly and independent of Bim binding to membranes.

The Bim-MBD interacts with Bcl-XL through hydrophobic interactions.

Our observation on the direct interaction between Bim-MBD and Bcl-XL suggests that current assumptions that the Bim-MBD inserts into lipid bilayers as a transmembrane helix similar to what has been reported for other tail-anchors (Petros et al, 2004) may not be correct. As an initial test of this hypothesis we expressed a version of BimEL with Venus fused to the C-terminus (BimEL^{V}) to prevent the Bim-MBD from adopting a transmembrane topology. Moreover, distance constraints mean that this protein can only undergo FRET with Bcl-XL if the Venus protein is located on the cytoplasmic side of the membrane. Unexpectedly the fusion of Venus to the C-terminus of BimEL moderated but did not abolish targeting of BimEL to mitochondria (Fig. 4a and 4c bars) and did not impair ABT-263 resistant binding to ^{c}Bcl-XL (Fig. 4b and 4c dots). This result not only confirms the two independent functions of Bim-MBD, but also suggests that

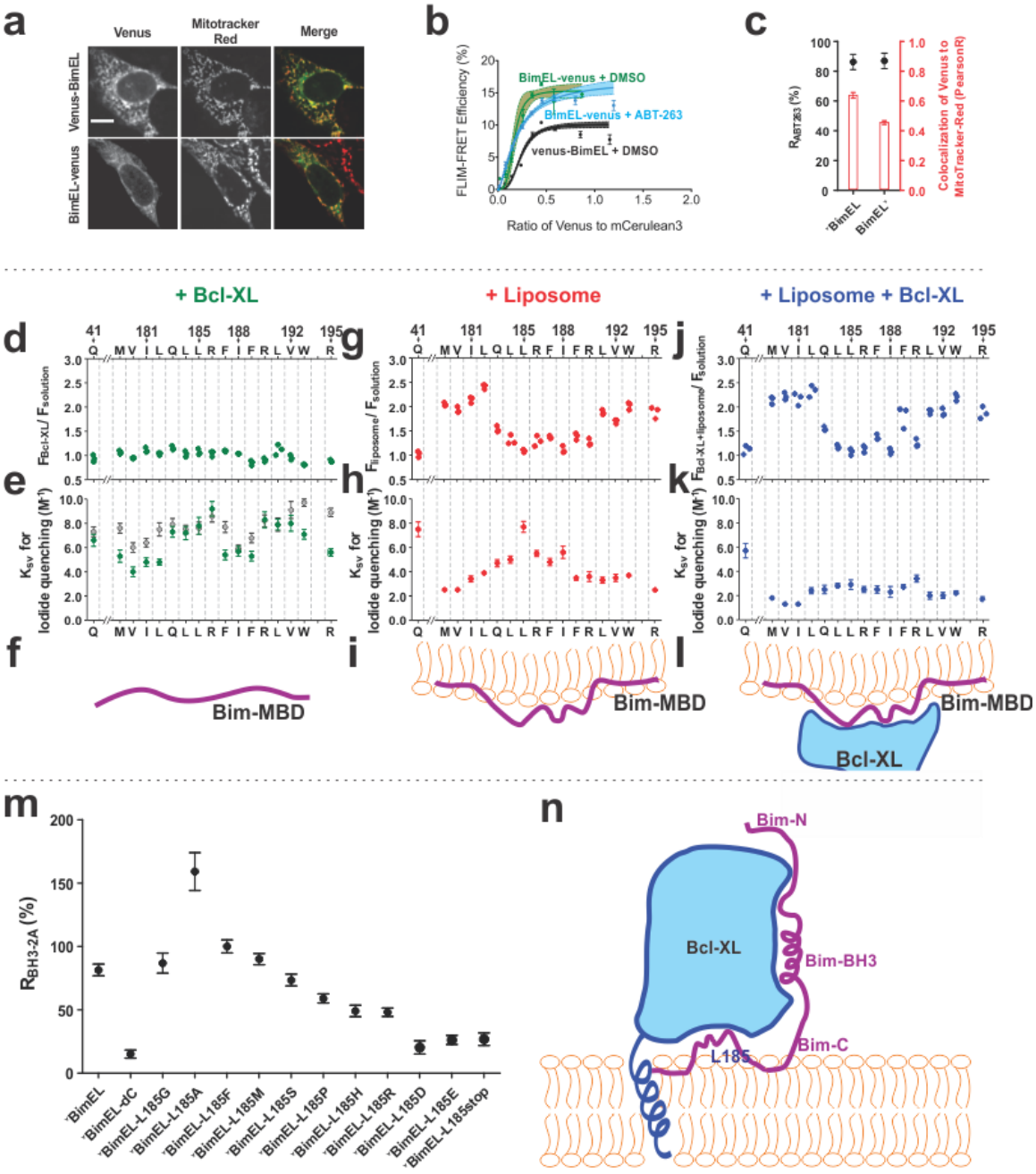


Figure 4: The Bim-MBD binds to membranes with a non-conventional topography that enables concomitant binding to Bcl-XL

(a) Representative images for cellular localization of Venus-BimEL and BimEL-Venus.

(b) FLIM-FRET binding curves for the interactions between ^{c}Bcl-XL and BimEL^{v} in the absence (green) or presence of ABT-263 (blue). Data from ROIs were binned into groups (n ranges from 20 to 3,000 in each bin combined from 3 independent

experiments) according to the ratio of Venus to mCerulean3 intensities, and the FLIM-FRET efficiencies calculated for each bin. FLIM-FRET efficiency values (individual points +/- SEM) were plotted versus the ratio of Venus to mCerulean3 intensity. The data were fit to a binding curve for the interaction with the 95% confidence interval shown.

(c) Comparison between ^{v}BimEL and BimEL^{v} in sub-cellular localization and $R_{ABT-263}$ when bound to Bcl-XL. Analysis of cells expressing ^{v}BimEL or BimEL^{v} for colocalization of Venus and MitoTracker-Red signals (PearsonR, red). Error bars, SEM, n >30. $R_{ABT-263}$ of ^{c}Bcl-XL:^{v}BimEL or ^{c}Bcl-XL:BimEL^{v} complexes, black dots. Data are mean ± 95% confidence intervals calculated from FLIM-FRET binding curves shown in Fig. 4b.

(d-l) Interaction of the Bim-MBD with liposomes and Bcl-XL using purified recombinant BimL protein. The amino-acids and positions indicate the locations in Bim of introduced single-cysteines used for labeling with NBD. Positions are numbered as in BimEL to facilitate comparisons between figures. Top row, (d, g, j) NBD fluorescence changes in response to the addition of (d) Bcl-XL (green), (g) liposomes (red), (j) Bcl-XL and liposomes (blue). Larger numbers indicate increased hydrophobicity of the environment of the NBD. Lower row, (e, h, k) iodide quenching constants (K_{sv}) for the same mutants and binding partners as above (quenching data, supplementary Fig. 4a-d). Larger K_{sv} values indicate increased exposure to iodide. Data are mean ± 95% confidence intervals. Illustrations (f, i, l) of possible interactions for the Bim-MBD based on the data above each.

(m) The effect of amino-acid substitutions at position185 of BimEL on binding to Bcl-XL. R_{BH3-2A} of ^{c}Bcl-XL:^{v}BimEL complexes were derived from binding curves (supplementary Fig. 4e-n). Data are mean ± 95% confidence intervals.

(n) Model of the double bolt locked Bcl-XL:Bim:membrane complex. The BH3 domain of Bim engages the hydrophobic groove of Bcl-XL. The Bim-MBD adopts a conformation in which the two ends associate with the membrane (lipid bilayer in orange) and the central domain binds to Bcl-XL. The C-terminus of Bcl-XL also interacts with the membrane adopting a helical transmembrane conformation (Yao et al, 2015).

when bound to membranes the residues of the Bim-MBD that bind ^{c}Bcl-XL, for example L185, are on the cytoplasmic side.

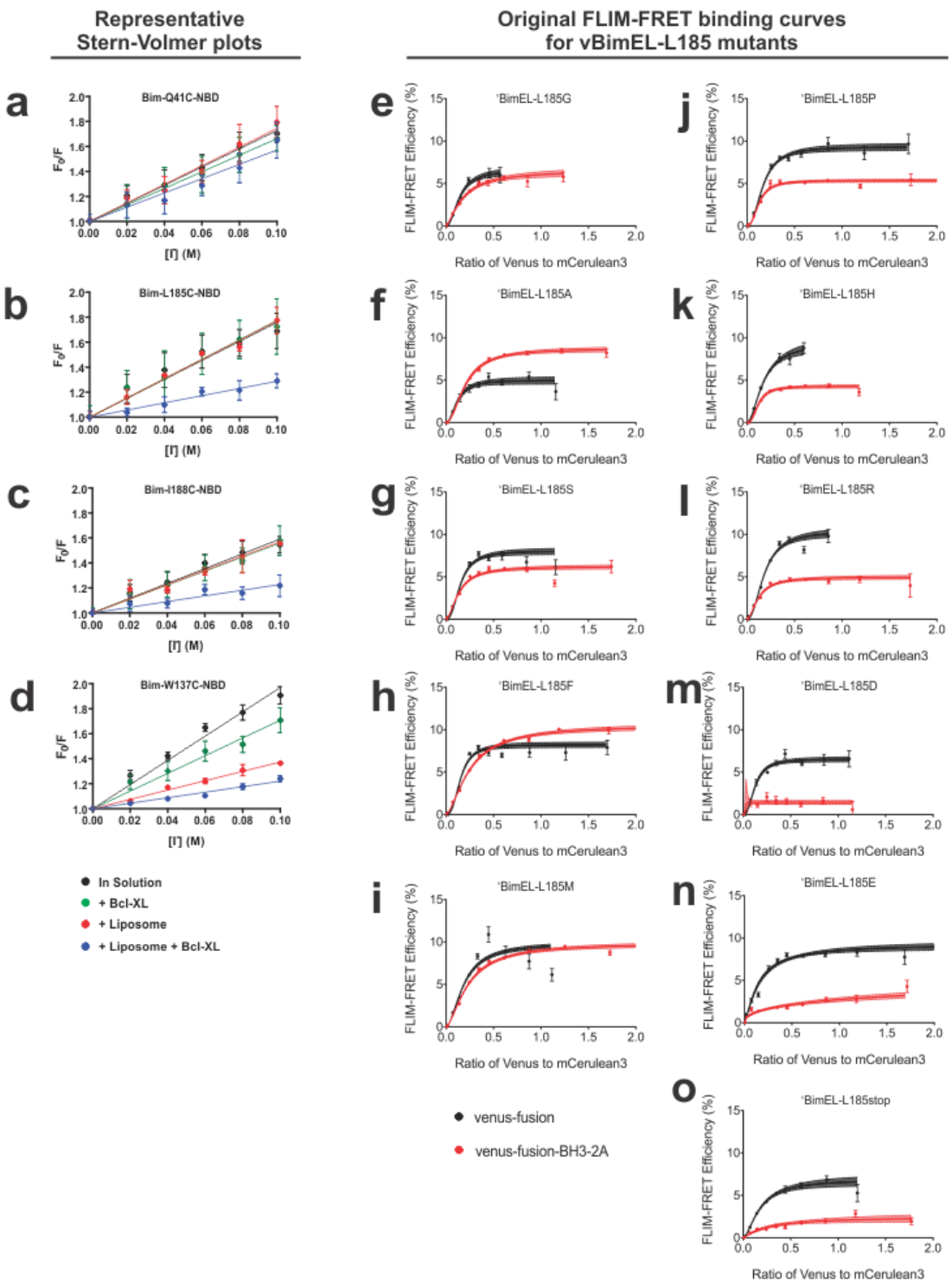
To examine the topography of the Bim-MBD in solution and membrane bound states, we used a well-established procedure in which an environment sensitive fluorescent dye N,N0-dimethyl-N-(Iodoacetyl)-N0-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine (NBD) was attached to recombinant BimL at specific sites and the environment of the dye was measured by fluorescence spectroscopy(Kale et al, 2014). To this end, a series of mutants were generated in which individual residues across the Bim-MBD were replaced with cysteine to enable NBD-labeling. As a solvent exposed control, a cysteine was located at position 41 of Bim. The fluorescence intensities of these mutants were recorded in the absence or presence of liposomes, Bcl-XL, and/or the aqueous quencher iodide(Johnson, 2005).

The fluorescence of NBD increases when the dye inserts into a lipid bilayer or becomes deeply buried in the interior of a protein. When Bim bound to Bcl-XL in solution there were no significant changes in hydrophobicity at any of the positions of the probe suggesting that during complex formation these residues do not become sufficiently buried to be protected from water (Fig. 4d and 4f). In contrast, when BimL bound to liposomes the NBD fluorescence increased at positions 179-182 and 191-195 suggesting that these two domains anchor the MBD in the lipid bilayer (Fig. 4g and 4i). Complex formation with Bcl-XL on the membrane did not change the pattern of hydrophobicity increases

markedly, suggesting that complex formation does not change the way the Bim-MBD interacts with membranes substantially (Fig. 4j and 4l).

To further probe the interaction between the Bim-MBD, the membrane and Bcl-XL, iodide quenching was used to identify residues protected by protein-protein interactions in addition to those protected by binding to membranes. Due to the relatively large size of iodide, residues involved in protein-protein interactions are often protected but unlike residues interacting with lipids the NBD fluorescence does not increase substantially (Supplementary Fig. 4a-d) (Johnson, 2005).

When Bim was incubated together with Bcl-XL in solution the NBD probes in the Bim-MBD were not protected from iodide (Fig. 4e), suggesting a relatively low affinity interaction between the Bim-MBD and Bcl-XL in solution (Fig. 4f). As expected, when bound to membranes the residues at both ends of the Bim-MBD that interact with the membrane were protected from iodide (Fig 4 g-h, compare residues 179-182 and 191-195). However, residues in the middle of the sequence surrounding residue 185 exhibited variable levels of protection from iodide with the NBD located at position 185 as accessible to iodide as the soluble control residue 41 (Fig. 4h). This result indicates that when Bim binds to membranes the domain between the two membrane binding sites in the Bim-MBD remains accessible on the surface of the membrane (Fig. 4i). In contrast, when Bcl-XL was added to samples with liposomes the entire Bim-MBD was



Supplementary Figure 4: Bim-MBD adopts a non-conventional conformation when interacting with Bcl-XL through hydrophobic interactions

(a-d) Representative Stern-Volmer quenching plots for specific residues in Bim. The fluorescence of BimL mutants labeled on the single cysteine with NBD (indicated at the top of each panel) was recorded before (F0) and after (F) incubation with the indicated concentration of idodide. F0/F was plotted against iodide concentration to generate Stern-Volmer quenching plots. Data are mean ± STDEV (n >3). Stern-Volmer constants (K_{sv}) (calculated as the slope of the line of linear best fit) for Bim in solution (black) were compared to values for K_{sv} for incubations containing Bim and Bcl-XL (green), Bim and liposomes (red) and Bim, liposomes and Bcl-XL (blue). The plots selected show the aqueous control Bim Q41C; a residue protected primarily by interaction with the liposome membrane independent from Bcl-XL, Bim W193C; and two residues protected only when both liposomes and Bcl-XL were added, Bim-L185C and Bim-I188C (numbering was increased to account for the 56 extra residues in BimEL to facillitate comparison with other figures).

(e-o) FLIM-FRET binding curves for ^{v}BimEL (black) and ^{v}BimEL-BH3-2A (red) with the indicated amino acid substitutions for L185 indicate that non-charged amino acids are preferred for binding to ^{c}Bcl-XL. Substitutions that negatively impact ^{v}BimEL-BH3-2A binding to ^{c}Bcl-XL result in red curves with lower FLIM-FRET efficiencies than the ones obtained for ^{v}BimEL (black). The substitution L185A increased ^{v}BimEL-BH3-2A binding back to approximately the same as ^{v}BimEL WT (red line above black curve). Substitutions with other uncharged amino acids had little impact however, substitution with charged residues dramatically reduced binding of ^{v}Bim-BH3-2A to ^{c}Bcl-XL on membranes (compare panels e-i with j-o). Data are mean ± SEM; Shadowed area represents 95% confidence interval for each binding curve.

protected from iodide (Fig. 4k) strongly suggesting that on membranes Bcl-XL binds to the central domain of the Bim-MBD (Fig. 4l).

As position L185 was shown to have the largest effect on binding of BimEL to ^{c}Bcl-XL (largest decrease in $R_{ABT-263}$, Fig. 3c), and is central to the domain that was protected from iodide when BimL bound to Bcl-XL we made a series of mutations at this location for ^{v}BimEL and ^{v}BimEL-BH3-2A. The impact of the amino acid substitutions on R_{BH3-2A} for ^{v}BimEL binding to ^{c}Bcl-XL was analyzed in live cells using quantitative FLIM-FRET (Fig. 4m). In general exchanging L185 with other amino acids with hydrophobic or polar non-charged side-chains (G, F, M, and S) did not affect the response of ^{c}Bcl-XL:^{v}BimEL complex to BH3-2A mutations significantly, except for the L185A mutant that displayed increased binding affinity (increased R_{BH3-2A}). For Bim-L185P, R_{BH3-2A} decreased by about 20%, possibly due to a change in the structure or rigidity of the Bim-MBD. Replacing L185 with hydrophilic residues (H, R, D or E) decreased R_{BH3-2A}, by ~30 – 60% comparable to truncation of the MBD at position 185 (L185stop) or deletion of the entire MBD domain (^{v}BimEL-dMBD). When assayed for $R_{ABT-263}$, binding of ^{v}BimEL-L185E to ^{c}Bcl-XL was less resistant to ABT-263 than ^{v}BimEL-dMBD (Fig. 4c) suggesting that a charge at this position actively interferes with binding. Taken together these results strongly suggest that L185 interacts with Bcl-XL through hydrophobic interactions.

Overall our data suggest a revised view of the Bcl-XL:Bim interaction by including the contribution of the Bim-MBD to binding with Bcl-XL (Fig. 4n).

Because Bim is bound to Bcl-XL by two different domains, the Bim-BH3 and Bim-MBD, the proteins are effectively double-bolt locked together.

Discussion

Using FLIM-FRET and full-length proteins in live cells together with biochemical assays, purified full-length proteins and liposomes, we discovered that the binding interactions between Bcl-XL and Bim are more extensive and result in a much higher affinity interaction than measured previously using peptides or protein fragments(Chen et al, 2005). Our results demonstrate that compared to Bad, the increased binding affinity of Bim for Bcl-XL is due to the combined effect of additional interactions with both the Bim-BH3 domain and the central hydrophobic domain of the Bim-MBD (encompassing residues 184-190). Together the two binding sites confer resistance to the inhibitors ABT-263 and to mutation of Bim-BH3 residues at h2 and h4 that we confirmed to be the major anti-apoptotic protein binding residues for both tBid and Bad (Fig. 1d-f).

We identified a novel interaction between the Bim-MBD domain and Bcl-XL that greatly increased the affinity of the interaction between Bim and Bcl-XL resulting in binding that was resistant to ABT-263 and mutation of h2 and h4 in the BH3 domain of Bim (Fig. 2b-c). Moreover, the Bim-MBD directly participates in interactions with Bcl-XL (Fig. 3b-e). Thus unlike conventional lock and key models, the interaction of Bim with Bcl-XL is more reminiscent of a double bolt lock with binding mediated by two distant interacting surfaces working together.

Even though the affinity of the MBD domain alone for Bcl-XL is low, double-bolt lock binding results in a very stable interaction. This mechanism also explains how Bim differs from Bad and tBid, which can be displaced by ABT-263 when bound to Bcl-XL. Unlike what was concluded from cell free assays using partial proteins, a double bolt lock model suggests that in cells displacement of Bim from anti-apoptotic proteins will require novel strategies targeting both interacting sites.

Our revaluation of the Bim-MBD also challenges the conventional belief that the C-terminal MBD sequence only functions to bring and keep Bim at the outer mitochondrial membrane (O'Connor et al, 1998; Terrones et al, 2008). Indeed our data suggest that the Bim-MBD does not adopt a transmembrane topology (Fig. 4a-l) as hypothesized for the C-terminal domains of other Bcl-2 family proteins(Petros et al, 2004). Instead our data is most consistent with the MBD being pinned to the membrane by two hydrophobic sequences at either end while the middle is exposed to the cytoplasmic environment (Fig. 4g-i). We speculate that this topology allows the central domain of the Bim-MBD to bind to the membrane surface electrostatically via two Arg residues (Fig. 3b-c and supplementary Fig. 3a). This hypothesis is consistent with the observation that substitution of these residues with Ala decreased localization of the protein to membranes. Binding of this domain via hydrophobic residues at each end and electrostatically via argenine side chains is an arrangement that may facilitate Bim binding to Bcl-XL through hydrophobic interactions including residue L185 (Fig. 4m). This insight suggests the Bim-MBD functions as an entirely new type of

membrane binding domain. Moreover, the nature of the interactions with Bcl-XL and the observation that in healthy cells Bim is not bound to anti-apoptotic proteins suggest it may be possible to generate a pair of small molecules that selectively kill cancer cells by efficiently releasing Bim from Bcl-XL.

Materials and methods

Constructs and compounds for assays in live cells

Plasmids encoding the mCerulean3, or Venus fluorescence proteins in place of the EGFP coding domain in pEGFP-C1 (Clontech) were kind gifts from Mark A. Rizzo (University of Maryland) and Ray Truant (McMaster University) respectively. To generate plasmids encoding the fusion proteins the required coding domains were amplified by PCR and inserted into the plasmids encoding the appropriate fluorescence protein. All of the plasmids included coding sequences for a GGS linker sequence between the coding domains except for ^{v}Bad (linker sequence, SGLRSRGG) and BimEL^{v} (linker sequence, SRGGGPVAT). All the swapping and site-directed mutants were obtained through PCR-based mutagenesis using oligonucleotides from Integrated DNA Technologies and Phusion DNA polymerase from New England Biolabs. ABT-263 was purchased from Selleckchem.

Cells, culture conditions and transfections

The MCF-7 human breast cancer cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% of fetal bovine serum (HyClone) and non-essential amino acids (NEAA, Invitrogen). Transfections were performed using Fugene HD reagent according to the manufacturer's standard protocol (Promega). MCF-7 cell clones stably transfected with vectors encoding mCerulean3, ^{c}Bcl-XL were selected in DMEM supplemented with 10% fetal bovine serum, non-essential amino acids (NEAA,

Invitrogen) and 500 μg/ml neomycin. After 3 weeks colonies were isolated and cultured as above. Clones that stably express mCerulean3 or ^{C}Bcl-XL were selected and then cultured with 50 μg/mL Blasticidin S. For microscopy, cells were seeded in 384-well imaging plates (PerkinElmer) and cultured as above for 24 hours prior to transient transfection. Cells treated with ABT-263 were incubated at 37°C for 18-24h in a fresh media containing 20μM drug before imaging unless indicated otherwise.

Fluorescence lifetime measurements

Steady-state fluorescence images and fluorescence lifetime images were both acquired using a custom-built Alba confocal microscope from ISS, which measures fluorescence lifetime by time correlated single photon counting (TCSPC). Channels of mCerulean3 and Venus were acquired simultaneously using 445 nm and 514 nm pulse interleaved excitation (PIE) through a 442/512/561 multiband filter and emission split by a 520nm-longpass filter was collected through 459-499nm and 528-555nm bandpass filters, respectively. All images were acquired as 256 pixels x 256 pixels at 25°C using a 60× 1.3NA PlanApo water immersion objective and laser powers set to minimize photobleaching and photon pileup during acquisition with 0.1ms per pixel dwell time and 5-frame repeat. TCSPC-data were processed using VistaVision software (ISS) and the average lifetime for each pixel was obtained by fitting FLIM pixels binned to ensure a total photon count >1000 for each set of binned

pixels analyzed. The lifetime and photon count for each pixel in both channels were exported for additional analysis using ImageJ.

To generate binding curves, the ROIs containing mitochondria were selected, and the corresponding intensities in both mCerulean3 and Venus channels and the average lifetime for mCerulean3 were extracted from the original TCSPC data using a customized ImageJ script. ROIs were automatically discarded from the analysis if the mCerulean3-BclXL signal was too low (and therefore noisy), close to saturated or the Venus signal was close to saturated. The ratio of Venus to mCerulean3 was calculated using the intensities of each channel. FLIM-FRET efficiency (E%) was calculated for each ROI as: $E\%=(1-\tau i/\tau 0)\times 100\%$, where τi is the mean lifetime for that ROI and $\tau 0$ is the average lifetime of all ROIs not expressing detectable Venus. FLIM-FRET efficiency was then distributed into bins of the same size according to Venus:mCerulean3 intensity ratio (bin size 0.1 for ratios lower than 0.5, 0.25 for ratios between 0.5 and 1.25, 0.5 for ratios higher than 1.25) and plotted (± se) against Venus:mCerulean3 ratio. The binding curves were fitted using GraphPad Prism version 5.0d for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com) with the function: $E\%=Emax\times(I_{Venus}\div I_{mCerulean3})^h/[Kd^h+(I_{Venus}\div I_{mCerulean3})^h]$. Emax is the maximum FLIM-FRET efficiency corresponding to saturation of donor binding sites by an acceptor; $I_{mCerulean3}$ and I_{Venus} are intensities of mCerulean3 and Venus, respectively; Kd is the relative equilibrium dissociation constant; h is the Hill slope.

Cell viability and membrane potential measurements

BMK-WT Cells transiently expressing Venus-tagged constructs of interest were stained with 5 µM DRAQ 5 together with 10 nM tetramethylrhodamine (TMRE, Life Technologies) in complete DMEM at 37°C for 30 minutes. All images were then recorded in 673 pixels x 509 pixels format at 25°C using a 20× 0.45 NA air objective in an automated OPERA confocal microscope. DRAQ 5, Venus, and TMRE were excited with cw-lasers at 635 nm, 488 nm, and 561 nm, respectively. Imaging conditions including laser excitation power, exposure time and camera gain were identical for all samples. Images were segmented using Acapella based on the DRAQ 5 channel that gives the margins of both nucleus and cytoplasm. Intensity features were extracted using a script (dwalab.ca) written for Acapella high content imaging and analysis software (Perkin Elmer, Woodbridge, ON).

Cellular localization analysis

BMK-DKO Cells transiently expressing Venus-tagged constructs of interest were stained with 5nM DRAQ 5 together with 500 nM MitoTracker-Red (Life Technologies) in complete DMEM at 37°C for 30 minutes. All images were then recorded in 673 pixels x 509 pixels resolution at 25°C using a 40× 0.9 NA water immersed objective in an automated OPERA confocal microscope. DRAQ 5, Venus, and MitoTracker Red were excited with cw lasers at 635 nm, 488 nm, and 561 nm, respectively. Imaging conditions including laser excitation power,

exposure time and camera gain were identical for all samples. Images were segmented using Acepella based on the DRAQ 5 channel that gives the margins of both nucleus and cytoplasm. The Pearson coefficient R between Venus channel and MitoTracker Red channel for each cell in which Venus intensities were between 700 and 1500 were further extracted using Acapella and analyzed in Microsoft Excel.

Recombinant protein purification and labeling

WT and single cysteine mutants of Bcl-XL and tBid were purified as described previously(Kale et al, 2014). For Bim, the cDNA encoding full-length wild-type murine BimL was introduced into pBluescript II KS(+) vector (Stratagene, Santa Clara CA). Sequences encoding a polyhistidine tag followed by a TEV protease recognition site (MHHHHHHGGSGGTGGSENLYFQGT) were added to create an in-frame fusion to the N-terminus of BimL. Bim-dMBD was constructed by introducing a stop codon (TAA) after position P121 to delete the entire Bim-MBD. Single cysteine mutagenesis was introduced through PCR-based mutagenesis using oligonucleotides from Integrated DNA Technologies and Phusion DNA polymerase from New England Biolabs.

The recombinant proteins were expressed in Arabinose Induced (AI) Escherichia coli strain (Life Tech, Carlsbad, CA). E. coli cells were lysed by mechanical disruption with a French press. The cell lysate was run on a Nickel-NTA column (Qiagen, Valencia CA) to bind the recombinant His-tag fused

proteins and after washing a buffer containing 300mM imidazole was applied to elute the proteins. This elution was then adjusted to 150 mM NaCl and applied to a High Performance Phenyl Sepharose (HPPS) column. Bim was eluted with a no salt buffer and dialyzed against a buffer containing 10mM HEPES pH7.0, 20% Glycerol, then flash-frozen and stored at -80 °C.

Single cysteine mutants of Bcl-XL and tBid were labeled with the indicated maleimide-linked fluorescent dyes as described previously (Kale et al, 2014; Lovell et al, 2008). Single cysteine mutants of BimL were labeled with the same protocol as tBid with the exception that the labeling buffer also contained 4M urea.

FRET measurements of interactions between recombinant proteins

Single cysteine mutants of BimL (41C) and tBid (126C) were purified and labeled with Alexa 568-maleimide. A single cysteine mutant of Bcl-XL (152C) was purified and labeled with Alexa 647-maleimide. Alexa568 labeled BimL or tBid was incubated with either Alexa647-labeled or unlabeled Bcl-XL along with the indicated concentrations of ABT-263. The intensity of Alexa568 fluorescence with unlabeled or Alexa647-labeled Bcl-XL was measured as $F_{unlabeled}$ or $F_{labeled}$ respectively. FRET, indicating protein-protein interaction, was quantified using the decrease of Alexa568 fluorescence when BimL or tBid bound to Alexa647-labeled Bcl-XL compared to unlabeled Bcl-XL. FRET efficiency was calculated as: FRET efficiency (%)= $(1-F_{labeled}/F_{unlabeled})*100\%$ as described previously for cBid-Bcl-XL. Binding was measured after incubation at 37°C for 1h.

To compare the inhibitory effect of ABT-263 on different Bim mutants or tBid binding to Bcl-XL, ABT-263 was titrated into a solution containing a fixed concentration of Bim or tBid and Bcl-XL. The concentrations were chosen so that FRET efficiency (F) is close to the maximum (F_{max}) in the absence of ABT-263. Inhibition of Bim/tBid binding to Bcl-XL by ABT-263 is observed by the decrease of FRET efficiency relative to Fmax (Normalized binding=F/F_{max}). Data was fitted to an inhibitor dose response equation with a Hill slope: $F/F_{max}=((100*X^H)/(IC50^H+X^H))$, Where X indicates the concentration of ABT-263, IC50 is the concentration of ABT-263 that gives 50% of the normalized binding. H (HillSlope) describes the steepness of the family of curves. Lines of best fit were calculated using least squares in Graphpad Prism.

NBD fluorescence assay and iodide quenching

The single cysteine mutants of Bim were purified and labeled with NBD (N,N0-dimethyl-N-(Iodoacetyl)-N0-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine; Molecular Probes, Cat. #: D-2004). The NBD fluorescence assay and iodide quenching of NBD-labeled Bim mutants was performed as described previously for Bax(Kale et al, 2014), with the exception that no cBid was used and that the assays were performed in 100ul volume in low protein binding 96-well plates on a fluorescence plate reader (Tecan M1000). Specifically, 20nM NBD-labeled Bim alone or 20nM Bim plus 50nM of Bcl-XL were incubated with 0.2mg/mL (final lipid concentration) of liposomes. For the “In solution” control,

20nM NBD-labeled Bim was incubated with an equal volume of assay buffer instead of liposomes.

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CHAPTER VI: Concluding Remarks

The BH3 protein Bim is an important mediator of apoptosis initiated by many intracellular stressors (Concannon et al, 2010; Mahajan et al, 2014; Puthalakath et al, 2007). Although most studies have examined the function of Bim *in vivo*, extensive characterization of the function of full length Bim *in vitro*, in comparison to Bid, has been limited due to its intrinsic unstructured properties and highly hydrophobic nature.

The thesis aimed to overcome the challenges faced by other previous attempts. The work presented in this thesis highlights the molecular mechanism by which Bim promotes cell death. We showed that the three predominant Bim isoforms have comparable activities in triggering Bax mediated membrane permeabilization *in vitro*. Bim distinguishes itself from the well characterized Bid by preferably activating Bax over Bak and binding to Bcl-XL with additional binding sites which contribute to its resistance to BH3-mimic drug ABT-263. We have identified the C-terminal MBD of Bim, which has been considered as the domain required to binding of Bim to the membrane, as the crucial domain that regulates Bim binding to and activating Bax. We also found that MBD contributed to the ABT-263 resistance of Bim-Bcl-XL complex by directly interacting with Bcl-XL, but is not the determining factor for Bim-Bcl-XL interaction.

<u>Bim isoforms function with the same activity *in vitro*</u>

Our *in vitro* studies have demonstrated that the major Bim isoforms (BimL and BimS) were constitutively active and promote Bax mediated membrane

permeabilization. Therefore, various mechanisms of regulation of Bim activity exist in cells to prevent apoptosis. Indeed BimL and BimEL are known to be regulated via binding to dynein (Puthalakath et al, 1999) and phosphorylation at positions Thr-56, Ser-44 and/or Ser-58 (sequence positions refer to human BimL) in cells (Lei, 2003). The BimEL isoform has been found to be further regulated by phosphorylation in its EL-specific domain, which targets the protein to degradation (Luciano et al, 2003; Qi et al, 2006).

From what we've known so far, two questions will be interesting to pursue in the future: i) what is the role of dynein binding in regulating the activity of Bim? ii) Does the phosphorylation of Bim change its binding affinity?

The activity of BimEL and BimL, but not BimS, have been reported to be regulated by binding to the dynein light chain in normal cells. It is unknown that whether this interaction serves solely to retain Bim in the cytosol, or whether it triggers a conformational change of Bim rendering it unable to associate with its binding partners at the membrane. Our data with transient transfected Venus-fused BimL (V-BimL) suggests that even minimally detectable amounts of V-BimL induce apoptosis in multiple cell lines, which suggests against regulation of Bim via dynein binding. However, it may be possible that the dyneins in cells were already occupied by the endogenous Bim and overexpressed V-Bim, even minimally detectable under the opera microscope, was enough to saturate the remaining dyneins and kill the cells. Due to the complexities of conducting studies *ex vivo,* examining the effect of purified dynein on Bim function in the more

simplified *in vitro* liposome system would be a better initial approach to tackle this question.

The Ser/Thr-to-Glu mutation is shown to be capable of mimicking the effect of phosphorylation by introducing negative charges (Huang & Erikson, 1994), the pseudo-phosphorylated mutation S44E, T56E, S58E can be introduced respectively or in different combinations in BimL to compare the activity of the pseudo-phosphorylated BimL to the wildtype counterpart. It will be really interesting if any of the pseudo-phosphorylation post an either positive or negative effect on the interaction, as it will suggest the phosphorylation effect on the function of Bim more than just affecting its localization or degradation. Other EL-specific phosphorylation sites can be test in the same manner in the future when purified recombinant BimEL is available.

Bim preferably activates Bax and Bid preferably activate Bak

Although Bim and Bid are often considered functionally redundant activators, the two proteins indeed hold distinct differences; Bim is constitutively active and its activity is negatively regulated by cyto-skeleton binding (Puthalakath et al, 1999), phosphorylation (Paterson et al, 2012) and degradation (Weber et al, 2016), whereas Bid shares structural and functional features with other multi-domain Bcl-2 family proteins (Billen et al, 2008) and its pro-apoptotic function requires activation via cleavage of caspase-8 (Li et al, 1998) and a subsequent conformational changes on the membrane (Shamas-Din et al, 2013).

Thus, Bim and Bid may have non-overlapping physiological roles in maintenance of homeostasis as they are regulated via different signal pathways. Indeed, Bim has been shown to play a more important role than Bid in regulating apoptosis in the hematopoietic system. Mice with a genetic ablation of Bim and mice with an acute loss of Bim via a conditional deletion display similar hematopoietic abnormalities such as accumulation of lymphoid and myeloid cells as well as symptoms such as acute autoimmunity (Bouillet et al, 1999; Herold et al, 2014). Such phenomenon can be explained by that Bim's function as the crucial regulator required for eliminating autoreactive B- and T-lymphocytes via apoptosis (Bouillet et al, 2002; Enders et al, 2003). Interestingly, Bax knockout mice exhibit a similar hyperplasia of B cells while Bak knockout mice do not (Knudson et al, 1995; Lindsten et al, 2000). It is possible that the preference of Bim for Bax is the result of co-evolution as both proteins play crucial roles in tightly regulating the hematopoietic system. On the other hand, the preference of Bid activating Bak was also supported by the fact that $BAK^{-/-}$ but not $BAX^{-/-}$ mice can be protected from BID-mediated, FasL-induced hepatocyte apoptosis (Hikita et al, 2011).

<u>The potential use of BimΔMBD as a universal sensitizer to characterize primed cells and to study the regulatory role of sensitizer function *in vivo*</u>

Cancer cells have been shown to evade death by blocking the mitochondrial apoptotic pathway via mutations or overexpression of Bcl-2 family proteins, making it a critical feature that distinguishes cancer cells from normal

cells (Slavov & Dawson, 2009). Recently, it has been proposed that some cancer cells are “primed” to the presence of high levels of anti-apoptotic proteins for tumor maintenance and survival independent of treatment (Certo et al, 2006; Ni Chonghaile & Letai, 2008) a phenomenon that is not observed in normal cells. Priming can be mediated through one or a combination of different anti-apoptotic proteins. For example, chronic lymphocytic leukemia (CLL) cells are primed to Bcl-2 expression (Del Gaizo Moore et al, 2007) while Mcl-1 is often over expressed in breast tumors (Ding et al, 2007). In primed cells with anti-apoptotic proteins highly over-expressed such that all BH3-activators and activated Bax/Bak are sequestered, BH3 mimetics derived from either sensitizers or activators are predicted to selectively kill cancer cells with little harm to the normal cells in which the all the pro-apoptotic proteins are not sequestered and readily available to withhold activated BH3 activator proteins.

A novel technique called BH3 profiling exploits the binding affinity differences of BH3-proteins to the anti-apoptotic proteins and can detect the type of block a cancer cell utilizes to escape apoptosis (Letai, 2008). In BH3 profiling mitochondria isolated from cancer cell lines or patient samples are treated with either Bad or Noxa BH3-peptide/protein (that only target Bcl-XL/Bcl-2 or Mcl-1, respectively), those peptides/proteins that elicit MOMP in vitro can predict the cells’ responses to BH3-mimetics or chemotherapy (Brunelle et al, 2009; Davids et al, 2012; Ni Chonghaile & Letai, 2008; Vo et al, 2012).

Our work showed that Bim lacking its C-terminus membrane region (BimΔMBD) lost binding to Bax and were unable to activate Bax, but still retain its anti-apoptotic binding function. BimΔMBD is established as a universal sensitizer that binds and mutual-sequesters all known anti-apoptotic protein, including Mcl-1 and Bcl-XL. Thus unlike Bad or Noxa that only inhibit certain anti-apoptotic proteins, BimΔMBD can be used as a better indicator for initial diagnosis of the primed state of tumor cells and gives a clear “Yes” or “No” answer of whether the patient may respond to BH3-mimetic drugs. Specifically, in the BH3 profiling assay adding only one protein BimΔMBD would achieve the same, if no better, effect as conventional combination of Bad and Noxa for the inhibition of all anti-apoptotic proteins. A further tailoring on the treatment can be then achieved by using individual BH3 peptides or proteins to determine functionally the dependence of specific cancer cells on particular Bcl-2 family proteins.

In addition, the fact that BimΔMBD lacked its activator function but still retained sensitizer function makes it a great tool to observe the effect of sensitizer function of Bim in homeostasis and development. For example, since Bim has been shown to be crucial in regulating the apoptosis in the hematopoietic system and loss of Bim leads to an accumulation of lymphoid and myeloid cells (Bouillet et al, 1999), it would be intriguing to knock-in BimΔMBD in the Bim deficient mice and observe if it restores the function. This would reveal if Bim fulfills its regulatory role in hematopoietic system through activator and/or sensitizer function.

The membrane topology of MBD of Bim and its interaction with Bcl-XL and Bax

Our work highlights the Bim-MBD as an entirely new type of membrane binding domain. Instead of adopting a trans-membrane topology like conventional tail anchors, our studies indicate that the MBD of Bim is pinned to the membrane by two hydrophobic sequences at either end while the middle is exposed to the cytoplasmic environment. This topology allows the central region of the Bim-MBD to bind to the membrane surface electrostatically via two Arg residues. Our lab previously has reported both tBid and Bax could migrate between membranes as the result of reversible membrane binding (Shamas-Din et al, 2014). The unique membrane binding pattern of Bim by its MBD may facilitate the migration between membrane as well as mobility on the membrane of Bim as it would face less resistance from lipids when diffusing compared to a conventional trans-membrane topology, thus give Bim a potential advantage on the kinetics of pore formation as it move away from an existing pore to initiate a new pore at other locations with faster speed. This hypothesis that the unique MBD affect the mobility of Bim on the membrane can be tested with the same experiment setting as previously reported (Shamas-Din et al, 2014), as well as on a planar membrane system using fluorophore-labeled proteins and measures the diffusion time of membrane bound BimWT vs tBid, Bax or Bim MBD mutants that may alter its insertion mechanism (e.g. BimMBD2A in which the two positive charged Arg residues were mutated to alanine) (Shivakumar et al, 2014).

Our data suggests that Bim MBD provides a secondary role in Bax binding and activation and Bcl-XL binding, rather than an independent high affinity binding site. However, the discovery of MBD directly involved in binding to Bax or Bcl-XL challenges the conventional belief that the C-terminal MBD sequence only functions to bring and keep Bim at the outer mitochondrial membrane (O'Connor et al, 1998; Terrones et al, 2008). This study has revealed an entirely new binding interaction required for efficient activation of Bax by Bim and Bcl-XL binding by Bim, thus provides potential targets for small compounds to regulate apoptosis and kill cancer cells.

The role of TCTP in potentiating Bcl-XL provides novel view on the function of BH3 proteins

In studying Bim binding to Bcl-XL, we came across with one unique BH3 protein member:TCTP. Unlike other BH3 proteins, TCTP forms a hetero-tetramer with Bcl-XL by interacting with the hydrophobic groove of Bcl-XL with its non-canonical but functional BH3 domain (Thebault et al, 2016). Combined with data from Dr. Amson's group (Thebault et al, 2016), our results demonstrate that TCTP activates the anti-apoptotic function of Bcl-XL. We speculate that activation is achieved by virtue of the low affinity of the interaction as replacing the H1 domain of TCTP BH3 increases the affinity of the interaction and abolishes the activity of TCTP (Thebault et al, 2016). Perhaps, the BH3-TCTP competes with Bcl-XL H9 for the BH3-binding pocket on Bcl-XL, partially activating Bcl-XL. It would not be surprising if other proteins with non-canonical BH3 peptides

potentiating the anti-apoptotic effect of specific Bcl2 family members were identified. That BH3-proteins can either increase or decrease the activity of Bcl-XL increases the complexity of the system but also highlights the potential for identification of new therapeutic targets which would not only "hit" the anti-apoptotic proteins but also positive BH3-like regulators.

One plausible approach for further investigate the role of TCTP is to purify and label the recombinant protein of TCTP and directly measure its binding affinity to Bcl-XL. If a complex is observed, we can titrate in activator BH3 proteins to generate displacement curve and use calculated models based on the Kd measured for individual interaction to determine if the Bcl-XL enhancement function of TCTP works as allosteric effect or direct displacement.

In conclusion, my thesis studies showed that Bim spontaneously binds to the membrane via its MBD and preferentially activates Bax. On the membrane, Bim adopted a unique conformation such that the MBD is pinned to the membrane by two hydrophobic sequences at either end while the middle is exposed to the cytoplasmic environment. This middle portion of MBD, specifically residues L129 (sequence positions refer to mouse BimL), play a crucial role in Bax activation as well as provide extra binding support to Bcl-XL (which contributes to the ABT-263 resistance) independent of regulating membrane binding of Bim. Mutation L129E and deletion of MBD deprived Bim of its membrane binding and Bax activation but not Bcl-XL binding function, though the extra binding sites to Bcl-XL is lost (Fig 1). Thus, my study provide new and surprising insights into the mechanisms

used to regulate programmed cell death with direct implications for targeting this family of proteins therapeutically.

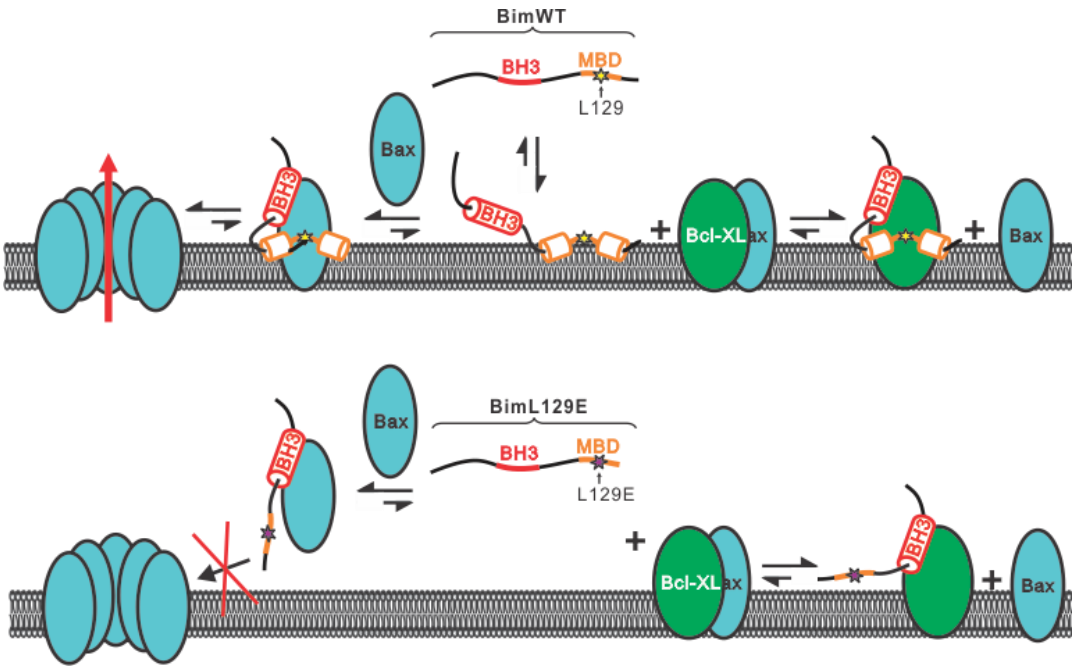

Fig 1. The unique MBD of Bim regulates its apoptotic function

Fig 1. The unique MBD of Bim regulates its apoptotic function. Interactions between BimWT, BimL129E, Bax (Bax) and Bcl-XL (green) are shown in the cytoplasm and on the membrane. The BH3 domain was highlighted with red and MBD with orange. Crucial residue L129 was labeled as yellow star on the MBD domain and mutation L129E was labeled as purple star. MOM permeabilization by Bax pores was shown by the red arrow going through the pore. Direction of flow indicated by lengths of the equilibria arrows is based on the Kds measured for the binding interactions. Bcl-XL inhibits apoptosis through both MODE1 (inhibiting BH3 proteins) and MODE2 (inhibiting activated Bax). For simplicity only MODE2 is shown, regulation of MODE 1 is expected to be analogous.

(upper part) BimWT binds membranes with a unique conformation that the MBD is pinned to the membrane by two hydrophobic sequences at either end while the middle portion containing residue L129 is exposed to the cytoplasmic environment. Residue L129 was crucial in activation Bax independent of membrane binding as well as providing extra binding support for Bim binding to Bcl-XL.

(lower part) Mutation L129E in Bim MBD disrupted not only the membrane binding but also the Bax activation function of Bim at physiologically relevant concentrations, even though Bim129E binds to Bax with similar affinity as BimWT. The MBD is not required for binding to and inhibiting Bcl-XL as BimL129E binds sufficiently tightly to Bcl-XL to release both pro-apoptotic BH3-proteins and Bax. Deletion of the Bim MBD (BimΔMBD) has the same effect as L129E mutation except that the interaction between Bim and Bax was completed eliminated. For simplicity only BimL129E was shown as a representative.

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