The Pro-apoptotic proteins BAK and BAX: Activation and Apoptotic Pore Formation

A dissertation submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

by

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Abstract

The BCL-2 family proteins BAK and BAX are the executioners of intrinsic apoptosis. Following activation by BH3-only proteins, they undergo structural rearrangements that promote their homo-dimerization and subsequent oligomerization to permeabilize the mitochondrial outer membrane.

This thesis investigates: 1) the interactions between a non-BCL-2 family protein VDAC2 and BAK (and BAX); 2) the stepwise interactions at which a full-length BH3-only protein tBID binds to activate full-length BAK at mitochondria; 3) the interfaces between the BH3-only proteins tBID and BAK; and 4) the formation of the apoptotic pore.

Cysteine mutagenesis and linkage studies were performed to identify a novel interface between the VDAC2 loop region between β10 and 11 and the surface hydrophobic groove of BAK. Stabilization of the BAK:VDAC2 interaction impaired BAK apoptotic activity, providing the first evidence that enhancement of the BAK:VDAC2 interaction can be exploited to avoid pathological apoptosis.

I also discovered that full-length tBID is able to induce BAK conformation change. Subsequently, tBID dissociates from BAK following exposure of the BAK BH3 and BH4 motifs prior to BAK homodimerization.

Using cysteine linkage and a novel functional screening approach, I was able to show that activation of BAK involves important interactions of BH3-only proteins with both the canonical hydrophobic binding groove of BAK (α2-5) in addition to a rear site of BAK (α6), with interaction at α6
promoting an open BAK surface hydrophobic groove to receive a BH3-only protein.

Lastly, functional screening BAK beyond the hydrophobic groove, the key domain involved in the formation of homodimers, did not inhibit mitochondrial damage. This indicated that critical protein:protein interfaces in BAK self-association are limited to the α2-5 homodimerization domain. My results are consistent with the hypothesis that BAK (and likely BAX) homodimers multimerize via protein-lipid interactions to permeabilize the mitochondrial outer membrane.

Thus, this thesis reveals novel mechanisms regulating BAK (and possibly BAX) activity. These findings provide insight into the basic biology of the pro-apoptotic proteins BAK and BAX and also potentially guide the development of therapeutic agents that can enhance or inhibit apoptosis by manipulating BAK and BAX.
Declaration

This is to certify that:

1. this thesis comprises only my original work except where indicated in the Preface,
2. due acknowledgement has been made in the text to all other material used,
1. this thesis is fewer than 100,000 words in length, exclusive of tables, figures and bibliographies.

Xiang Li
Parts of this thesis have been published, or are submitted for publication in the form of papers listed below:


Other Publications arising from my work during my PhD:

**Mark Xiang Li** and Grant Dewson. “Mitochondria and apoptosis: emerging concepts”. F1000 Prime Report 2015, April 1. DOI: 10.12703/P7-42.

Delphine Merino, James R. Whittle, François Vaillant, Antonin Serrano1, Jia-Nan Gong, Goknur Giner, Ana Leticia Maragno, Maïa Chanrion, Emilie Schneider, Bhupinder Pal, **Xiang Li**, Grant Dewson, Julius Gräsel, Kevin Liu, Najoua Lalaoui, David Segal, Marco J. Herold, David C. S. Huang, Gordon K. Smyth, Olivier Geneste, Guillaume Lessene, Jane E. Visvader and Geoffrey J. Lindeman. “Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer”. Science Translational Medicine 2017, August 2. DOI: 10.1126/scitranslmed.aam7049
Preface

In accordance with the regulations of the University of Melbourne, I ackonowledge the important contributions of others in some of the work presented in Chapter 4 and 5 as listed:

**Figure 4.3 B** Co-immunoprecipitation of tBID:BAK was performed by Dr. Iris Tan.

**Figure 5.6 D and F** Immunoprecipitation of CuPhe crosslinked tBID:BAK was performed by Dr. Colin Hockings.

The remainder of this thesis comprises my original work. Overall, I assess my contribution to the work presented in this thesis as being more than 85%.
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I cannot thank you all enough.
**Abbreviations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>A1</td>
<td>BCL-2 related protein A1</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>APAF-1</td>
<td>Apoptotic protease activating factor -1</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>B4M</td>
<td>Benzophenone-4-maleimide</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL-2 associated agonist of cell death</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL-2 agonist killer</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2 antagonist X</td>
</tr>
<tr>
<td>BCL-W</td>
<td>Apoptotic regulator BCL-W</td>
</tr>
<tr>
<td>BCL-Xₗ</td>
<td>Apoptotic regulator BCL-Xₗ</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BH</td>
<td>BCL-2 homology</td>
</tr>
<tr>
<td>BID</td>
<td>BCL-2 interacting domain death agonist</td>
</tr>
<tr>
<td>BIK</td>
<td>BCL-2 interacting killer</td>
</tr>
<tr>
<td>BIM</td>
<td>BCL-2 interacting mediator of cell death</td>
</tr>
<tr>
<td>BMF</td>
<td>BCL-2 modifying factor</td>
</tr>
<tr>
<td>BMOE</td>
<td>1,6-bis-maleimidoethane</td>
</tr>
<tr>
<td>BN</td>
<td>Blue native</td>
</tr>
<tr>
<td>BOK</td>
<td>BCL-2 related ovarian killer</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domains</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>CuPhe</td>
<td>Copper (II)(1-10-phenanthroline)</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
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DED  Death effector domain
Δ   Delta
DEER Double electron-electron resonance
DISC  Death inducing signaling complex
DMEM Dulbecco’s Modified Eagles medium
DMF  Dimethylformamide
DMSO Dimethylsulfoxide
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
DTT  Dithiothreitol
EDTA Ethylene diamine tetra acetic acid
ER  Endoplasmic reticulum
FACS  Fluorescence-activated cell sorting
FADD Fas-associated death domain
FCS  Fetal calf serum
g  Gram
GFP  Green fluorescence protein
GUV  Giant unilamellar vesicles
hr  Hour
hBAK  human BAK
HEPES N-[2-hydroxy ethyl] piperazine-N’-[2-ethane sulphonic acid]
HRK  Harakiri BCL-2 interacting protein
IASD  4-Acetamido-4’-(iodoacetyl)amino)stilbene-2,2’-disulphonic acid
IP  Immunoprecipitation
IPTG  Isopropyl β-D-1-thiogalactopyranoside
KDS-BSS  Ken D Shortman’s balanced salt solution
L       Liter
LOF     Loss of function
mBAK    mouse BAK
MCL-1   Myeloid cell leukemia sequence-1
M       Molar
µg      Microgram
mg      Milligram
µl      Microliter
µM      Micromolar
mL      Milliliter
mM      Millimolar
min     Minute
MEF     Mouse embryonic fibroblast
MFN     Mitofusin
MLM     Mouse liver mitochondria
MOM     Mitochondrial outer membrane
MOMP    Mitochondrial outer membrane permeabilization
MW      Molecular weight
NEM     N-ethyl maleimide
nM      Nanomolar
NMR     Nuclear magnetic resonance
PAGE    Polyacrylamide gel electrophoresis
PBS     Phosphate buffered saline
PCR     Polymerase chain reaction
PDB     Protein data bank
PEG-MAL Polyethylene glycol maleimide
PI      Propidium iodide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>p53</td>
<td>Protein 53 kDa</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 up-regulated modular of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SEC</td>
<td>Second</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TA</td>
<td>Tail-anchored</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>tBID</td>
<td>Truncated BID</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor associated protein with death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
</tbody>
</table>
Investigation of the BAK (and BAX) apoptotic activation and the apoptotic pore formation

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Chapter 1: Introduction

1.1 Overview of programmed cell death and apoptosis

All living organisms are made of cells (except viruses), and the genomes carried within those cells. Such genomes have been refined throughout evolution by natural selection to ensure the survival of the organism for reproduction, and the successful passage of its genome. However, it has not been widely appreciated that death is also necessary for life. Although it seems counterintuitive, a set of molecular mechanisms securing cell death is also forged by evolution. Cell death, one of the most fundamental biological processes, serves a critical role in maintaining normal human physiology and is therefore of intense interest to biomedical research.

Cells can maintain survival within certain limits. When challenged, they will use every means in their armory to survive until such physiological limits have been breached. Once breached, a doomed cell often engages a set of active cell death protocol in a very tightly controlled manner to minimize the cost to the whole organism. Cell death remains an evolving field that expands with newly defined cell death pathways. These include, autophagy-dependent cell death, necroptosis, pyroptosis, ferroptosis. These other forms of cell death that are well described elsewhere (Green & Llambi, 2015), and are beyond the scope of my current PhD work and therefore will not be covered in this thesis. This introduction will focus on one of the well-established cell death pathways: Apoptosis.
1.2 Apoptosis

1.2.1 The genesis of the apoptosis field

Ancient Greek used the word “apoptosis” to describe the falling of leaves. The term apoptosis was later suggested to John Kerr and his colleagues to represent the morphological features observed during the “active, inherently programmed phenomenon” cell deletion process (Kerr, Wyllie, & Currie, 1972). This landmark paper flagged the beginning of the apoptosis research field. A decade later, Wyllie et al. established that DNA fragmentation as one of the biological hallmarks of apoptosis, providing a means for measuring this phenomenon (Wyllie, 1980). Soon after, the causal relationships between apoptosis inhibition and cancer development were established (Cleary, Smith, & Sklar, 1986; Tsujimoto & Croce, 1986; Tsujimoto, Finger, Yunis, Nowell, & Croce, 1984; Vaux, Cory, & Adams, 1988). Such discovery has been conceptually revolutionary for our understanding of the development, as well as the treatment of, cancer, thus placing apoptosis the primary focus of cell death research from early 1990s (Lockshin, 2016).

1.2.2 Apoptosis and its cellular executioners

Morphologically (Figure 1.1), a cell that is undergoing apoptosis shrinks in size and condenses its cytoplasm, its plasma membrane starts to “bleb”, the cytoskeleton collapses, its nuclear envelope ruptures and the DNA content becomes fragmented. Then the fragmented cell contents are packaged in the membrane bound “apoptotic bodies”, whose surface are altered for phagocytosis by macrophages or neighboring phagocytic cells (Kerr et al., 1972) (Figure 1.1). This disassembly of a dying cell into apoptotic bodies is a highly-regulated process and it mediates the efficient...
clearance of apoptotic materials as well as the transfer of useful biomolecules (Atkin-Smith & Poon, 2017).
Figure 1.1 Schematic illustration of morphological features during cellular apoptosis

Illustration of the events of a cell undergoing apoptosis. The dying cell becomes separated and disassembles into membrane bound apoptotic bodies before engulfment by neighboring phagocytic cells. Note the organelles such as mitochondria in the apoptotic bodies remain relatively intact. Figure modified from the landmark paper of Kerr and colleagues (Kerr et al., 1972).
Biochemically, the search for the molecular mechanism underlying the phenotypical changes observed during apoptosis was initiated when Yuan et al. made the discovery that a cysteine protease CED-3 (Cell Death protein-3) was indispensable for apoptosis in Caenorhabditis elegans (C. elegans) (Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). A family of mammalian cysteine proteases were then identified and termed “Caspases”. Caspases are endoproteases that hydrolyze peptide bonds and have a catalytic cysteine residue at their active site. Given its proteolytic nature, caspases exist in healthy cells as inactive zymogens that required activation. Caspase cleavage happens after an Asp residue in their substrates which can either result in substrate inactivation or the generation of active signaling molecules (Shi, 2002).

Caspases have been loosely grouped according to the signaling cascade in which they function. Apart from the less understood caspase-2, -10 and -14, two major caspase families are delineated in apoptosis (mammalian caspase-3, -6, -7, -8 and -9) and inflammation (caspase-1, -4, -5 and -12 in human or caspase-1, -11 and -12 in mice)(McIlwain, Berger, & Mak, 2013). The apoptotic caspases fall into either the category of initiator caspases, which are auto-activated by conformation changes and cleavage induced by upstream adapter molecules such as APAF-1 (apoptotic protease activating factor-1) or RIPK1 (receptor-interacting serine/threonine-protein kinase-1)/FADD (Fas-associated protein with death domain) following an apoptotic insult, or effector caspases, which are activated by cleavage by an upstream initiator caspase. As in Figure 1.2, Initiator caspases (caspase-8 and -9) are commonly found to possess homotypic interaction domains such as the caspase recruitment domain (CARD) or death effector domain (DED). Once activated, initiator caspases activate the effector caspases (caspase-3, caspase-6, and caspase-
7) via proteolytic cleavage (Boatright et al., 2003; Shi, 2002). Activated effector caspases then cleave a vast number of distinct cellular substrates and eventually lead to the demolition phase of apoptosis (Slee, Adrain, & Martin, 2001). One example of effector caspase mediated apoptotic feature is the DNA degradation by CAD (caspase-activated DNase). CAD is inhibited by its inhibitor iCAD (inhibitor of CAD) in healthy cells. After activation, caspase-3 cleaves iCAD and releases the nuclease CAD which fragments the DNA in apoptotic cells (Sakahira, Enari, & Nagata, 1998).
Apoptotic signals recruit adaptor proteins such as APAF-1 clustering, hence enabling their interaction with initiator caspases through homotypic interaction via the DED domain (brown). Initiator caspases become activated due to induced proximity by adaptor proteins and then proceed to cleave and activate downstream effector caspases. Figure modified from Green (Green, 2011).

The causal link between caspase activation and cell death has drawn immense interest in understanding the molecular mechanism underlying the effectors of apoptosis. This has led to the characterization of two apoptotic signaling pathways: extrinsic apoptosis and intrinsic apoptosis, detailed below and illustrated in Figure 1.3:

**Figure 1.2 Schematic of apoptotic caspase activation**

- 28 -
Figure 1.3 Illustration of the intrinsic and extrinsic apoptosis pathways

Extrinsic (FasL (Fas ligand), TNF (tumor necrosis factor)) or intrinsic (DNA damage and cytokine deprivation) apoptotic signals either activate
cell surface death receptors (FasR and TNFR1) or induce BH3-only proteins to initiate a series signaling events leading to apoptosis. As illustrated, TNFR1 signaling leads to the activation of caspase-8 that in turn activates effector caspase-3 and -7. BH3-only proteins, members of the BCL-2 family proteins, can trigger the mitochondria outer membrane permeabilization (MOMP). MOMP releases factors such as cytochrome c that can interact with APAF-1 and form the apoptosome to activate initiator caspase-9. Caspase-9 cleaves and activates effector caspase-3 and -7 for the final execution of apoptosis. Note the cross-talk between two pathways converges at the BH3-only protein BID (BH3-interacting domain death agonist), which is cleaved and activated by caspase-8. Figure adapted from Ichim et al (Ichim & Tait, 2016).
1.2.2.1 Extrinsic apoptosis pathway

Extrinsic apoptosis occurs when one cell issues the death warrant to another cell in scenarios such as the clearance of viral infection or elimination of self-reactive immune cells (Budd, 2001; Thomson, 2001). The signal initiating extrinsic apoptosis is transmitted by plasma membrane surface death receptors which belong to the tumor necrosis factor (TNF) receptor super family (Figure 1.3) (Ashkenazi & Dixit, 1998). TNF death receptors are characterized by an extracellular domain carrying a 2-6 cysteine-rich repeats and a ~80 amino acid cytoplasmic "death domain" near the C-terminus (Ashkenazi & Dixit, 1998; Nagata, 1997). The human death receptor (DR) family includes a large number of proteins, in addition to the TNF receptors (TNFR1 and TNFR2) (Loetscher et al., 1990; G. Pan, Ni, et al., 1997a), others members of the family include DR-3 (Chinnaiyan et al., 1996), DR-6 (G. Pan et al., 1998a), CD95 (also known as Fas or Apo-1) (Itoh et al., 1991) and TRAIL (TNF-related apoptosis-inducing ligand) receptors (TRAIL receptor-1/DR-4 and TRAIL receptor-2/DR-5) (G. Pan, Ni, et al., 1997a; G. Pan, ORourke, et al., 1997b).

For the extrinsic apoptosis pathway to occur, death receptors require activation by their cognate death ligands. To date, the two best characterized death receptor-ligand signaling pathway are FasL-Fas and TNFR1-TNF (Elmore, 2007): extrinsic apoptosis is initiated when the natural ligand FasL or TNF binds to the Fas or TNFR1 receptor on the cell surface and leading to death receptor trimerization (Banner et al., 1993). An important signal transducing adaptor molecule FADD (Fas-associating protein with death domain) or TRADD (TNF receptor-associated protein with death domain) are then recruited to the receptor cluster via homotypic interactions of their C-terminal death domain (DD) with the DD of the
death receptors (BOLDIN et al., 1995; HSU, XIONG, & GOEDDEL, 1995). When FADD is recruited, a death signal is propagated via the N-terminal protein-protein interaction module death effector domain (DED) that mediates the recruitment of initiator caspase-8 and the long form of the cellular FLICE (FADD-like IL-β-converting enzyme)/caspase-8 like inhibitory protein (cFLIP_L) by DED: DED interaction, thus enabling the formation of death-induced signaling complex (DISC) (Muzio et al., 1996; Scaffidi, Medema, Krammer, & Peter, 1997). Unlike FADD, TRADD does not have a DED, TNF signaling via TRADD then requires extra adaptor molecules. Instead, Hsu and colleagues show that TRADD binds directly to FADD via the death domain and uses the DED of FADD to facilitate the formation of DISC (HSU, Shu, Pan, & GOEDDEL, 1996). Although the molecular intricacies of how these protein interact remain unclear, caspase-8 is proposed to become activated via an induced-proximity model (as depicted in Figure 1.2) in which the formation of the DISC increases the local concentration of caspase-8 that encourages caspase-8 dimerization and auto-proteolytic cleavage (Medema et al., 1997; Salvesen & Dixit, 1999). Active caspase-8 then proceeds to activate effector caspases, which mediates the final demise of the cell.

1.2.2.2 Intrinsic Apoptosis

Unlike extrinsic apoptosis, the signal triggering intrinsic apoptosis is generated within the cell independent of surface receptor activation and the pathway focuses primarily on the mitochondrial outer membrane (MOM). Apoptotic intracellular signals can be induced by a variety of stimuli including deprivation of growth factors, hypoxia, free radicals and DNA-damaging radiation.
Such apoptotic signals disrupt the intricate balance between the BCL-2 family proteins, resulting in the activation of pro-apoptotic BCL-2 protein BAK and BAX, either by direct BH3-only protein activation or the neutralization of anti-apoptotic BCL-2 proteins as reviewed (Mark Xiang Li, 2015). Multimerization of activated BAK and BAX promotes mitochondrial outer membrane permeabilization, allowing the release of the intermembrane proteins including cytochrome c (Kluck, Bossy-Wetzel, Green, & Newmeyer, 1997; Liu, Kim, Yang, Jemmerson, & Wang, 1996). The release of cytochrome c then initiates the formation of the “Apoptosome” in the presence of scaffolding protein APAF-1 and dATP (deoxyadenosine triphosphate). Apoptosome then recruits and activates initiator caspase-9 that continues to activate effector caspase-3 and -7 leading to cell death (Figure 1.3) (Cain, Bratton, & Cohen, 2002; Hu, Ding, Spencer, & Núñez, 1998; H.-E. Kim, Du, Fang, & Wang, 2005; G. Pan, ORourke, & Dixit, 1998b).

Cross-talk between extrinsic and intrinsic apoptosis pathway does occur in certain cell types, in which the activation of caspase-8 activates downstream effector caspases; concomitantly, caspase-8 also cleaves the BH3-only protein BID (BH3-interacting domain death agonist) to produce the active tBID (truncated BID) (H. Li, Zhu, Xu, & Yuan, 1998), which then can directly activate BAK and BAX or bind to pro-survival proteins (Wei et al., 2000).

1.3 BCL-2 family proteins

Research interest in BCL-2 family proteins began when the human Bcl-2 gene was discovered at the junction of translocated chromosomes 18 and
14 (t14; 18) in follicular B cell lymphoma (Tsujimoto et al., 1984). It was quickly realized that the BCL-2 gene on chromosome 18 was fused with immunoglobulin heavy chain locus on chromosome 14, resulting the dysregulation of BCL-2 transcription (Tsujimoto & Croce, 1986; Tsujimoto, Cossman, Jaffe, & Croce, 1985). Unlike previously described oncogenes, Bcl-2 did not promote cell proliferation but instead it prevented cell death. This seminal discovery of apoptosis blockade was paradigm shifting for our understanding of tumor development and reshaped the landscape of cancer medical research (Vaux et al., 1988). Since then, at least 20 BCL-2 related proteins have been identified in mammalian cells based on their amino acid sequence homology (Table 1.1).

All BCL-2 proteins contain at least one of four BCL-2 homology (BH) motifs, defined by a conserved amino acid sequence (Figure 1.4 A). BH1 and BH2 motifs were first characterized in BCL-2, with Gly 145 and Trp 188 being structurally conserved in BH1 and BH2 motifs respectively (Yin, Oltvai, & Korsmeyer, 1994). The BH3 motifs was first defined in BAK (Chittenden et al., 1995). Later, sequence analysis delineated that the BH3 motif was h₁sXXh₂XXh₃sDzh₄, where X represents any amino acid, s represents a small residue, z an acidic residue and the four h residues (h₁-4) being critical hydrophobic residues that were latterly shown to interact with the hydrophobic pockets on the surface groove of pro-survival BCL-2 proteins as well as pro-apoptotic BAK and BAX (Chen et al., 2005; Day et al., 2008; Petros et al., 2001; Sattler et al., 1997). The BH4 motif, located at the N-terminus of the protein, appears to be less conserved as it was only recently re-defined based on the conserved structure-sequence Φ₁-Φ₂-X-X-Φ₃-Φ₄, where X could be any amino acid, Φ₁, Φ₂, Φ₄ are hydrophobic residues, and Φ₃ is an aromatic residue (Kvansakul et al., 2008).
The BCL-2 family is split into 3 sub-families based on their apoptotic function: BH3-only proteins, pro-survival and pro-apoptotic BCL-2 proteins. Apart from the intrinsically unstructured BH3-only proteins (except BID), both pro-survival and pro-apoptotic BCL-2 proteins share a similar globular fold composed of nine alpha helices, with a central hydrophobic core α5 helix surrounded by α2, α3 and α4 forming a protein surface hydrophobic groove that is important for interactions between the BCL-2 proteins (Figure 1.4). A transmembrane domain located in the α9 helix of most of the pro-survival and pro-apoptotic proteins, also enables the membrane insertion of these proteins(Kale, Osterlund, & Andrews, 2018; Shamas-Din, Kale, Leber, & Andrews, 2013b; Youle & Strasser, 2008).

Table 1.1 Summary of known BCL-2 like proteins

<table>
<thead>
<tr>
<th>BH3-only proteins</th>
<th>Pro-survival BCL-2 proteins</th>
<th>Pro-apoptotic BCL-2 proteins</th>
<th>Poorly characterized BCL-2 proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIM</td>
<td>BCL-2</td>
<td>BAK</td>
<td>BCL-RAMBO</td>
</tr>
<tr>
<td>BID</td>
<td>BCL-X&lt;sub&gt;L&lt;/sub&gt;</td>
<td>BAX</td>
<td>BCL2L10</td>
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<tr>
<td>BAD</td>
<td>A1/BFL-1</td>
<td>BOK</td>
<td>BCL-G</td>
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<td>BMF</td>
<td>MCL-1</td>
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<td>MULE</td>
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<td>PUMA</td>
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<td>BIK</td>
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</table>
Figure 1.4 Sequence and structural similarity among BCL-2 family proteins

(A) Members of the BCL-2 family contain at least one or more conserved BCL-2 homology (BH) motifs. All known BCL-2 family proteins
contain at least a BH3 motif (red). Multi-domain BCL-2 proteins, pro-survival and pro-apoptotic members possess all 4 BH motifs. (B) Structural similarity among pro-survival and pro-apoptotic members. Inactive BAK (PDB: 2MIT) and BAX (PDB: 1F16) have a similar fold to the pro-survival counter parts BCL-X\textsubscript{L} (PDB: 1MAZ) and MCL-1 (PDB: 1WSX). Note the surface hydrophobic groove (green) and \(\alpha_2\) (red).

Following the discovery of the BH3 motif, the first BH3-only protein identified was BIK (BCL-2-interacting killer)(Boyd et al., 1995), followed by BAD (BCL-2-associated death promoter)(E. Yang et al., 1995), BID (BH3-interacting domain death agonist) (K. Wang, Yin, Chao, Milliman, & Korsmeyer, 1996), HRK(Imaizumi et al., 1997), BIM (BCL-2-interacting mediator of cell death)(O'Connor et al., 1998), NOXA (phorbol-12-myristate-13-acetate-induced protein 1)(Oda et al., 2000), PUMA (p53-upregulated modulator of apoptosis)(Han et al., 2001) and BMF (BCL-2 modifying factor)(Puthalakath et al., 2001). Several other proteins such as p193(S. C. Tsai et al., 2000) and MAP-1(Tan et al., 2001) have also been reported to contain a BH3-like-motif, however little is known regarding their role in apoptotic signaling and therefore are not discussed here.

1.3.1.1 Central dogma: BH3 motif binding to the canonical surface hydrophobic groove

All BH3-only proteins contain only the conserved BH3 motif with 4 hydrophobic residues mentioned above that are critical for their binding to the surface groove on both pro-survival and pro-apoptotic BCL-2 proteins. Apart from BID, BH3-only proteins are intrinsically unstructured but the BH3 motif can undergo a conformation change to adopt an amphipathic \(\alpha\)
helical structure upon binding to the surface hydrophobic groove of a pro-
survival BCL-2 protein (Hinds et al., 2007). Structural studies have
provided atomic details and confirm the similarity between the binding of
a BH3 motif peptide to the surface hydrophobic groove of various pro-
survival and pro-apoptotic BCL-2 proteins BAK and BAX (Figure
1.5) (Czabotar et al., 2007; 2013; Moldoveanu et al., 2013; Petros, Fesik, &
Olejniczak, 2005). The four signature hydrophobic residues of the BH3
motif are critical for binding to the hydrophobic pockets in the groove with
an additional salt bridge observed between charged residues contributing
to the stable interaction (Czabotar et al., 2013; Sattler et al., 1997). More
recently, an additional h₀ position of hydrophobic interaction was defined
between the N-terminus of the BID BH3 motif (I82/I83) and BAX
α3/α4 (Czabotar et al., 2013). Structural information of regions beyond
BH3 motif of BH3-only proteins is limited by the difficulty of producing
full-length recombinant protein due to their unstructured nature.
Figure 1.5 Structural similarity of BH3 only protein binding to pro-survival and pro-apoptotic proteins

Structural similarity among pro-survival and pro-apoptotic members (grey) binding to BH3 peptides (red). BAK + BID (PDB: 2M5B), BAX + BID (PDB: 4BD2), BCL-X\textsubscript{L} + BAD (PDB: 1G5J) and MCL-1+ BIM (PDB: 2NL9). Note in all known structures the BH3 peptide (red) binds similarly to the canonical surface groove.
1.3.1.2 BH3-only protein BID requires a membrane environment for its activation conformational change

The only BH3-only protein that adopts a stable globular fold is BID. Composed of eight alpha helices, BID has its central hydrophobic $\alpha_6$ and $\alpha_7$ helices surrounded by remaining amphipathic helices with the BH3 motif residing on its $\alpha_3$ helix (Chou, Li, Salvesen, Yuan, & Wagner, 1999). As depicted in Figure 1.6, BID exists in its 22 kDa inactive conformation and requires cleavage by caspase-8 to generate the active P15 (15 kDa) fragment, tBID. Biochemical and structural characterization of BID reveals that the cleaved P7 (7 kDa) fragment remains associated with the P15 fragment in solution due to strong hydrophobic interactions. BID conformation change happens in the presence of membrane lipids where the P7 fragment disassociates from the P15 fragment. Potentially, with the help of mitochondrial membrane proteins such as MTCH2 (Grinberg et al., 2005; Katz et al., 2012), the P15 fragment essentially unfolds itself by embedding its helices $\alpha_4$-$\alpha_7$ in the membrane then exposes its BH3 motif containing helix $\alpha_3$ for binding to pro-survival or pro-apoptotic BCL-2 proteins (Shamas-Din et al., 2013a). This study not only elegantly illustrated the step-wise conformation change of BID during apoptosis but also highlighted the importance of a membrane environment in studying the interaction between BCL-2 proteins that have been shown consistently to undergo structural rearrangements when binding to membranes (P. K. Kim, Annis, Dlugosz, Leber, & Andrews, 2004; Peng et al., 2006; Yethon, Epand, Leber, Epand, & Andrews, 2003).
Figure 1.6 BID undergoes multiple conformation change in the presence of membrane

Caspase-8 cleaved BID has its P7 and P15 (tBID) fragments remained associated before contacting MOM. The cleaved BID translocates to MOM and loses its P7 fragment in the presence of MOM and membrane proteins such as MTCH2. Membrane bound tBID then adopts a more open conformation and exposes its BH3 motif containing helix. Figure modified from ShamasDin and colleagues (Shamas-Din et al., 2013a).
1.3.1.3 Mode of action: how BH3-only proteins activate BAK and BAX

Since the BH3 motif of BH3-only proteins is capable of binding to the surface groove of both pro-survival and pro-apoptotic BCL-2s, the mode of action of BH3-only proteins in initiating apoptosis has been debated.

After tBID was reported to activate BAK at mitochondria (Wei et al., 2000), the “direct activation model” proposed that BH3-only proteins such as tBID, BIM and perhaps PUMA function as direct activators of BAK and BAX (Letai et al., 2002). tBID, BIM and PUMA are able to bind to both pro-survival and pro-apoptotic members whereas sensitizers such as NOXA and BAD are only able to bind pro-survival proteins thus liberating the sequestered activators (H. Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002). However, there were initial difficulties with this model. Firstly, it was difficult to capture the interaction between BAK and BAX and the proposed direct activators. Unlike the pro-survival BCL-2 proteins, BH3-only proteins were argued to interact only transiently with BAK and BAX in a “hit and run” mechanism, whereby BH3-only binding to BAK and BAX would induce conformation change thus destabilizing the interaction (H. Dai et al., 2011). Secondly, the activation of BAK and BAX could still occur in Bid<sup>−/−</sup>Bim<sup>−/−</sup>, and even in Bid<sup>−/−</sup>Bim<sup>−/−</sup>Puma<sup>−/−</sup> mice (Villunger, Labi, Bouillet, Adams, & Strasser, 2011; Willis et al., 2007).

In contrast to the proposed activator and sensitizers, recent biochemical studies indicated that most BH3-only proteins (except BAD) are able to directly activate BAK and BAX with no preference (H. Dai et al., 2011; H. Du et al., 2011; Hockings et al., 2015). In addition, researchers discovered
that in contrast, BH3-only proteins were found to bind to pro-survival BCL-2 proteins with marked specificity (Chen et al., 2005). Given the binding preference of each BH3-only protein to pro-survival proteins (Figure 1.8), Chen et al demonstrated that a combination of BH3 motif peptides that neutralized all pro-survival BCL-2s was sufficient to induce cell death (Chen et al., 2005). BAK was detected in heterodimers with MCL-1 and BCL-XL in healthy MEFs and such dimers disappeared following UV induced cell death (Willis et al., 2005). Collectively, this data suggested an alternative model: the “indirect activation model” (Chen et al., 2005). In this model, BH3-only proteins acted as pro-survival protein neutralizers to displace the “primed” or activated pro-apoptotic BAK and BAX allowing them to homo-oligomerize and disrupt mitochondria integrity and promote cell death. However, the indirect activation model did not address the mechanism of BAK and BAX “pre-activation”, implying the intrinsic activation or auto-activation of BAK and BAX.

More recently, a “unified model” has been proposed incorporating aspects of these previous models (Dewson & Kluck, 2009; Leber, Lin, & Andrews, 2007; Llambi et al., 2011). The unified activation model summarizes that the interaction of pro-survival BCL-2 proteins sequester the activator BH3-only proteins as Mode 1, whilst also sequestering activated BAK and BAX as Mode 2 (Figure 1.7).
Figure 1.7 Unified mode of BAK and BAX regulation at MOM

Simplified representation of the interactions between BCL-2 proteins that control in BAK and BAX activation. 2 Modes co-exist in the unified model of how pro-survival proteins are inhibiting apoptosis: Mode 1: Pro-survival BCL-2 proteins directly bind to the activator BH3-only proteins and prevent them from activating BAK and BAX. Mode 2 argues the pro-survival BCL-2 proteins can also directly bind and inhibit activated BAK and BAX and to block their homo-oligomerization. Mode 1 and 2 are overcome during apoptosis when the pro-survival BCL-2 proteins are overwhelmed by activated BH3-only proteins and/or activated BAK and BAX.
1.3.1.4 Multiple modes of regulation of BH3-only proteins

BH3-only proteins are the key initiators of intrinsic apoptosis and therefore their activity is subject to strict regulation at multiple levels in a healthy cell. Different to pro-survival and pro-apoptotic BCL-2 proteins that are readily expressed in most cells, BH3-only proteins can be up-regulated following apoptotic insults by means of induced transcription or induced translation (Happo, Strasser, & Cory, 2012).

At steady state, transcriptional control and post-translational modifications ensure the low/non-detectable level of BH3-only proteins in different cell types. In response to apoptotic stimuli, specific transcription factors have been reported to induce the transcription of different BH3-only proteins. One such example is the well documented p53 would transcriptionally activate Puma and Noxa in response to DNA damage (Nakano & Vousden, 2001; Oda et al., 2000; J. Yu, Zhang, Hwang, Kinzler, & Vogelstein, 2001). Post-translational modifications also play a role in BH3-only protein regulation. For instance, phosphorylation of BAD abolishes its apoptotic function, which can then be rescued by dephosphorylation of the Ca$^{2+}$ sensitive phosphatases calcineurin (H. G. Wang et al., 1999; Zha, Harada, Yang, Jockel, & Korsmeyer, 1996).

1.3.2 Multiple pro-survival BCL-2 proteins in mammalian cells

To date, the mammalian pro-survival BCL-2 proteins discovered are BCL-2 (B-cell chronic lymphocytic leukemia/lymphoma-2), BCL-X$_L$ (BCL-2 like protein 1), BCL-W (BCL-2 like protein 2), MCL-1 (myeloid-cell leukemia sequence-1) and A1 (BCL-2-related protein A1) (BFL-1 in
human). As discussed above, pro-survival proteins protect cells from apoptosis by directly binding to BH3-only proteins and/or neutralizing activated BAK and BAX from permeabilizing MOM. Structural evidence supporting this key concept was not readily available until the co-crystal structures of C-terminal truncated BCL-X_L with BH3 peptides of BAK and BIM were obtained (Liu, Dai, Zhu, Marrack, & Kappler, 2003). This study clearly demonstrated the surface hydrophobic groove composed of helices 2, 3, 4 and 5 was the receiving interface for the BH3 motif (Figure 1.4 and 1.5).

One point worth noting is the greater redundancy in mammalian pro-survival BCL-2 proteins compared to simpler organisms. To date, 5 pro-survival BCL-2 members have been identified in mammals, as compared to C. elegans which only have one pro-survival BCL-2 homolog CED-9. As shown Figure 1.8, BIM BH3 has high binding affinity to all pro-survival BCL-2s, whereas other BH3-only proteins such as NOXA preferentially binds to MCL-1 and A1. A likely reason for mammals to have multiple pro-survival BCL-2 members is to enable the fine-tuning of the response to different “stress signals”, which activate different BH3-only proteins as discussed previously. This provides an evolutionary advantage (there is only one BH3-only protein homolog EGL-1 in C. elegans).
Figure 1.8 Binding preference between BH3-only proteins and their pro-survival counter parts

BIM, PUMA and BID are discovered to have high affinity for all pro-survival BCL-2 proteins whereas BAD and NOXA are more skewed for some pro-survival proteins over others. Modified from Strasser and colleagues (Strasser, Cory, & Adams, 2011).

1.3.3 The pro-apoptotic proteins BAK, BAX and BOK

_BCL-2 homologous Antagonist Killer_ (BAK) was first identified as a binding partner of the viral apoptosis inhibiting protein E1B 19K (Farrow et al., 1995), and _BCL-2-Associated X_ protein (BAX) was identified in a heterodimer with BCL-2 (Oltvai, Milliman, & Korsmeyer, 1993). Cells lacking both BAK and BAX, but not BAK or BAX alone, are completely resistant to almost all apoptotic stimuli (Wei et al., 2001; Zong, Lindsten, Ross, MacGregor, & Thompson, 2001). Mice lacking either Bak or Bax alone are largely normal (Bax<sup>-/-</sup> mice have a minor splenomegaly and the
males are infertile (Knudson, Tung, Tourtellotte, Brown, & Korsmeyer, 1995)), whereas almost all Bak\(^{-/-}\)Bax\(^{-/-}\) mice display severe developmental defects resulting in perinatal death (Lindsten et al., 2000). These findings establish the over-lapping functions of BAK and BAX in cell killing pathways, with one or the other being absolutely required for development and apoptosis.

BCL-2-related Ovarian Killer (BOK) was identified in a yeast two-hybrid screen as a binding partner for pro-survival MCL-1 and A1 (S. Y. Hsu, Kaipia, McGee, Lomeli, & Hsueh, 1997a). Recent studies identify BOK ER (Endoplasmic Reticulum) and Golgi localization, binding to the coupling domain of the inositol 1,4,5-triphosphate receptors (IP\(_3\)Rs) suggesting a role in ER stress sensing and promoting apoptosis (Carpio et al., 2015; Schulman, Wright, Kaufmann, & Wojcikiewicz, 2013). Combined loss of BOK with BAK and BAX in the hematopoietic systems suggests a slightly more severed phenotype compared to combined loss of BAK and BAX, suggesting a pro-apoptotic role for BOK (Ke et al., 2015). Bok\(^{-/-}\) mice are normal, and even Bok\(^{-/-}\)Bak\(^{-/-}\) and Bok\(^{-/-}\)Bax\(^{-/-}\) are largely normal (Ke et al., 2013; 2012). However, Bok\(^{-/-}\)Bak\(^{-/-}\)Bax\(^{-/-}\) mice exhibit more severe developmental defects and die earlier than Bak\(^{-/-}\)Bax\(^{-/-}\) mice, indicating that BOK contributes to apoptosis during development.

1.3.3.1 BAK and BAX sequence and inactive structure

Sequence comparison between human BAK and BAX was manually aligned based on conserved amino acid property as shown in Figure 1.9. Structures of both inactive BAK and BAX shared similar globular fold composed of nine helical bundles with the core α5 surrounded by amphipathic helices similar to their pro-survival homologues (Figure 1.4).
Figure 1.9 Sequence alignment between BAK and BAX

Amino acid sequence alignment between BAK and BAX based on residue structural similarity. Each helix was color coded as indicated. Similarity were ranked and shown as indicated by (*), (:) and (.), figured adapted from Prof Peter Colman.
The N-terminus of BAK and BAX consist of a partially unstructured N-segment (aa1-23 in BAK and aa1-15 in BAX) and the α1 helix (Dewson et al., 2009; Suzuki, Youle, & Tjandra, 2000). Truncation of the N-segment does not disturb protein stability and expression (Dewson et al., 2009). No function has been reported to date regarding the N-segment in both BAK and BAX. The α1 helix of BAK and BAX contains the BH4 motif, which is defined by sequence conservation sequence described earlier in this chapter. The structure of BAK and BAX reveals that α1 helix, in particular the hydrophobic residues in BH4 motif, is buried in inactive proteins and is responsible for maintaining protein stability via interaction with α2, α5 and α6 (Moldoveanu et al., 2006). Helices α1 and α2 are connected via a long unstructured loop in both BAK and BAX. The BH3 motif of BAK and BAX is located in the α2 helix, which is also buried in the inactive BAK and BAX. As previously mentioned, the signature surface hydrophobic groove formed by α2, α3, α4 and α5 is important for BH3-only protein binding. It is worth noting another shallow groove, formed by α1 and α6 on the opposite side to the α3, α4 and α5 groove. This “rear site” is argued to be an important activation site for BAX (Gavathiotis et al., 2008). This will be discussed in detail in Chapter 5. Both BAK and BAX have an α9 C-terminus containing the hydrophobic transmembrane domain (TMD) that is important for their mitochondria outer membrane (MOM) insertion and localization. The structural information regarding α9 in BAK is unknown since the hydrophobic nature of BAK α9 makes it insoluble in either aqueous or organic solvents for crystallization (Michel, 2018; Setoguchi, Otera, & Mihara, 2006). BAX is predominantly cytosolic in healthy cells and the α9 is sequestered in its surface groove (Suzuki et al., 2000). Upon apoptotic activation, BAX translocates and insert its α9 to MOM (Gahl, He, Yu, & Tjandra, 2014).
1.3.3.2 Subcellular localization of BAK and BAX

In healthy cells, BAK is predominantly integrated in the MOM, whereas BAX is predominantly detected in the cytosol (Y. T. Hsu, Wolter, & Youle, 1997b; Schinzel, Kaufmann, & Borner, 2004; Wolter et al., 1997). Although a small population of BAK and BAX have also been reported to present at ER, no definitive physiological role has been assigned (Zong et al., 2003). BAK and BAX sub-cellular localization is recently explained in a “retro-translocation” model (Figure 1.10). By examining BAK and BAX localization at the single cell level, researchers showed that in healthy cells both BAK and BAX are constitutively targeted to MOM and are both actively retro-translocated back into cytosol possibly by interactions with BCL-XL (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2015). The prevailing BAK and BAX subcellular localization is in equilibrium between the cytosolic population and those residing on the mitochondria. The difference in protein levels of BAK and BAX in the cytosol and MOM correlates with the difference in their rate of retro-translocation. Under apoptotic stress, the retro-translocation machinery is disabled, resulting BAK and BAX accumulation at MOM (Edlich et al., 2011; Schellenberg et al., 2013; Todt et al., 2015).
The proposed model of BAK and BAX retro-translocation. In steady state, both proteins are argued to exist in an equilibrium state that both proteins traffic between cytosol and mitochondria outer membrane with BAK exhibits a much lower rate of retro-translocation compared to that of BAX. Proteins such as pro-survival protein BCL-X<sub>L</sub> is involved in BAK and BAX retro-translocation machinery that will be halted upon encountering the apoptotic signaling thus resulting BAK and BAX membrane accumulation. Note the transmembrane domain (TMD) of both BAK and BAX are modeled tucked into their surface groove when localized in cytosol.

Although it is unclear about the conformation difference between membrane and cytosolic BAK, conformation change to expose the transmembrane domain (TMD) is required for cytosolic BAX to insert into the MOM. The BAX TMD, located on its C-terminal tail, is sequestered in the BAX surface hydrophobic groove when BAX resides in the cytosol (Figure 1.10)(Suzuki et al., 2000). Both BAK and BAX C-termini can target fused GFP (Green fluorescence protein) to mitochondria suggesting their classical membrane anchor property(Setoguchi et al., 2006). Based
on structural studies, the BAX tail is held in its groove with the tail residue S184 forming a hydrogen bond with the groove residue D98 (Suzuki et al., 2000). Mutagenesis studies further supported the observation that a single point mutation of BAX S184 to hydrophobic residues such as valine leads to BAX constitutively targeting to MOM (Nechushtan, Smith, Hsu, & Youle, 1999). Upon apoptotic activation, BAX is found to accumulate at MOM, likely due to the release of α9 from its groove. The detailed mechanism of tail release will be further discussed in Chapter 4.

1.4 BAK and BAX activation

1.4.1 Activation of BAK and BAX by BH3-only proteins

As mentioned above, BH3-only proteins such as BID and BIM can directly activate BAK and BAX. BAK and BAX activation requires the BH3: groove interaction (Czabotar et al., 2013; Dewson et al., 2008). In addition, a proposed “rear site” (α1 and α6) seems to be involved in BAX activation (Gavathiotis et al., 2008). The roles of the canonical hydrophobic groove and rear site in BAK and BAX activity will be discussed in Chapter 5. Here, the introduction will focus on illustrating the known structural changes of BAK and BAX during their activation. Figure 1.11 provides an overview of BAK and BAX activation to aid the following discussion.
Figure 1.11 Schematic model of BAK and BAX activation and conformation change

BAK and BAX accumulates on the MOM after encountering apoptotic signals, BH3-only proteins such as tBID can bind to the surface hydrophobic groove and induce BAK and BAX conformation change such as dissociation of the N-terminal helix (orange rod), exposure of the BH3 motif (red) and separation of the core and latch domain. Activated BAK and BAX proceed to form the symmetric BH3-in-groove homodimer that is argued to multimerize and promote MOMP.

1.4.2 α1 disassociation

In the structures of inactive BAK and BAX (Figure 1.4), the α1 helix is associated with the rest of protein, with the BH4 motif buried to facilitate the stabilization of the inactive protein. Following BH3-only protein activation, the N-terminus of BAK and BAX disassociates from the rest of the protein as measured by the exposure of N-terminal epitopes using BAK or BAX conformation-specific antibodies(Alsop et al., 2015; Griffiths et al., 1999; Y. T. Hsu & Youle, 1997; 1998; Upton, Valentijn, Zhang, & Gilmore, 2007). This N-terminal disassociation is further supported by the measurement of distance between α1 and α6 prior (~27 Å) and after (~50 Å) activation(Aluvila et al., 2014). The disassociated α1 appears to remain solvent exposed(Alsop et al., 2015; Uren et al., 2017) in active BAK and BAX, although whether it plays a role after dissociation is unclear.
1.4.3 BH3 motif exposure

In both inactive BAK and BAX structures (Figure 1.4), the BH3 motif is buried with key residues facing towards the core of the protein (Moldoveanu et al., 2006; Suzuki et al., 2000). Binding of a BH3-only protein to the surface groove induces the partial exposure of the BAK BH3 motif before α1 disassociation as measured by epitope exposure. However, full BH3 motif exposure is only completed after α1 disassociation (Alsop et al., 2015; Dewson et al., 2008). The BAX BH3 motif also becomes exposed during activation (Gavathiotis, Reyna, Davis, Bird, & Walensky, 2010). The exposed BH3 motif then inserts itself into the groove of another activated BAK or BAX monomer to form the BAK or BAX homodimer hence the BH3 motif becomes reburied (Figure 1.13) (Brouwer et al., 2014; Czabotar et al., 2013; Dewson et al., 2008; 2012).

1.4.4 Core and latch domain separation

In the quest for structural characterization of active BAK and BAX, researchers from WEHI (Walter and Eliza Hall Institute) discovered a crystal structure of BAX “domain swapped” dimer, in which the BAX α2-5 (the “core domain”) is separated from the α6-α8 (the “latch domain”) as illustrated in Figure 1.12 (Czabotar et al., 2013). BAK was subsequently shown to adopt a similar core-latch dimer (Brouwer et al., 2014). Although the formation of this “domain-swap” dimer was not thought to reflect a physiological dimer, this structure nevertheless highlighted the separation of the core domain from the latch domain being an important conformation
change during BAK and BAX activation. The functional relevance of the core-latch separation on isolated mitochondria was confirmed as disulfide tethering the α5 and α6 to prevent the “unlatching” of BAK and BAX, blocked release of cytochrome c (Brouwer et al., 2014; Czabotar et al., 2013). However, the exact timing of the core-latch separation with respect to other conformation changes remains unclear.
Figure 1.12 Structure evidence of BAK and BAX activation conformation change

Reported structures of inactive BAK (PDB: 2MIT) and BAX (PDB:1F16) monomers and the core-latch separated, active BAK (PDB: 4U2U) and BAX (PDB:4BD2) monomer. BAK and BAX α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of protein in grey.
1.4.5 Experimental BH3-only protein-independent BAK and BAX activation

The physiological activators of BAK and BAX are the BH3-only proteins. However, through the experimental study of BAK and BAX, a range of biochemical stimuli such as low pH, mild heat and antibody-specific treatment can also trigger BAK and BAX activation *in vitro* (Cartron, Oliver, Mayat, Meflah, & Vallette, 2004; Iyer et al., 2016; Pagliari et al., 2005). These BH3-only-independent stimuli were valuable tools in the studies of BAK and BAX throughout this PhD project.

1.5 Formation of the BAK and BAX homodimers

After BAK and BAX become activated, their conformation change creates new interfaces for interactions that permit their dimerization and ultimately the formation of an apoptotic pore. The formation of BAK and BAX oligomers were observed very early on in a number of studies and proven critical for MOMP (Antonsson, Montessuit, Sanchez, & Martinou, 2001; Wei et al., 2000), yet the structures of oligomerized BAK and BAX remained unknown.

The first model of BAK oligomerization was proposed to be an asymmetric “daisy chain” structure: the BH3 motif of an activated BAK or BAX monomer binds to the rear of another activated monomeric BAK or BAX, and the chain elongates to form the apoptotic pore (Reed, 2006). However, this model was challenged by biochemical studies that demonstrated that activated BAK or BAX form symmetric BH3-in-groove homodimers (Bleicken et al., 2010; Dewson et al., 2008; 2012; Subburaj et al., 2015). In these studies, BAK or BAX single cysteine variants either
with cysteine placed in the BH3 motif or the surface groove (α3 and α4) were co-expressed in BAK and BAX double knock out cells. Disulfide bond formation was induced after tBID activation of BAK or BAX and nearly all activated BAK or BAX formed only BH3-in-groove dimers. The linkage pattern contradicted the “daisy chain” model that would predict the exposed BH3 motif should not be in close proximity to surface groove residues. Also, the fact that nearly all activated monomers formed BH3-in-groove homodimers suggested the symmetry in the dimer unit as opposed to asymmetric dimers in which a ladder of different sized oligomers would be expected. The BAK and BAX symmetric homodimerization was subsequently supported by recent structural evidence of BAK and BAX core domain (α2-α5) symmetric BH3-in-groove homodimer (Figure 1.13)(Brouwer et al., 2014; Czabotar et al., 2013).
Figure 1.13 Structure evidence of BAK and BAX BH3-in-groove homodimers

Reported structures of BAK (PDB: 4U2V) and BAX (PDB:4BDU) BH3-in-groove homodimers. BAK and BAX α2 in red and surface groove α3, 4 and 5 in green. From the top view, it is clear that the BH3 motif (in red) of one activated BAK or BAX inserted into the groove of anther activated BAK or BAX. From the side view, note BAK dimer has a much more concaved membrane facing interface. Both BAK and BAX homodimers have been reported to display a membrane facing interface rich in aromatic residues (grey).
1.6 A “secondary interface” allowing apoptotic pore formation

The establishment of the BH3-in-groove symmetric homodimer raises another question: the requirement of a “secondary” interface allowing such homodimers to form higher order oligomers for MOMP.

A number of studies have been performed using cysteine linkage. Our laboratory demonstrated that BAK and BAX homodimers can be linked via their α6 helices to form higher order oligomers(Dewson et al., 2009; 2012). Others reported the possibility of the secondary interface for higher order oligomer formation via α3:α3 interface, α5:α5 interface and more recently the α9:α9 interface(Aluvila et al., 2014; Bleicken et al., 2014; Brouwer et al., 2014; Iyer et al., 2015). However, there is still no definitive evidence to confirm such putative interfaces. The biggest caveat for confirming a bona fide multimerization interface is the lack of functional evidence in mitochondria or cells. Chapter 6 will focus on functional mapping of the putative secondary interface.

1.7 Apoptotic pore formation and MOMP

1.7.1 How big does the pore need to be?

Although BAK and BAX homodimerization in MOMP has been well documented, the process of apoptotic pore formation remains poorly understood. One question in the field is the lack of agreement in terms of the size of the apoptotic pore. An early study using recombinant BAX and an artificial liposome system reported a concentration-dependent apoptotic pore expanding from ~10 to 25 Å in diameter, large enough for cytochrome
transport, comprising of 2 to 4 BAX molecules (Saito, Korsmeyer, & Schlesinger, 2000). Later, imaging based experiments and cysteine linkage studies showed that BAK and BAX form dynamic complexes ranging from dozens to hundreds of molecules (Dewson et al., 2009; Nechushtan, Smith, Lamensdorf, Yoon, & Youle, 2001; Zhou & Chang, 2008). Based on such observations, it is unlikely that BAK and BAX form a static apoptotic pore(s) during MOMP.

1.7.2 Proteinaceous pore, proteolipidic pore or a pore at all?

Further to the molecular stoichiometry of the pore being unclear, the nature of the pore also remains to be resolved. When the structure of BCL-Xₐ was resolved, the similarity in the α helical fold between BCL-Xₐ and the pore-forming domains of bacterial toxin Diptheria/colicin A was noticed (Antignani & Youle, 2006; Muchmore et al., 1996). Hence it was proposed that BAK and BAX monomers might permeabilize membranes in a similar fashion to that of such toxins by insertion of a core hair-pin structure formed by the two central amphipathic helices into membrane lipids (Choe et al., 1992; Kagan, Finkelstein, & Colombini, 1981; Lakey & Slatin, 2001). Indeed, in Myc-null cells, chemical labeling of cysteines engineered at α5 and α6 of full-length BAX indicates that both helices were buried in the MOM prior to BAX oligomerization and MOMP (Annis et al., 2005). In line with this hypothesis, synthetic BAX α5: α6 hairpin peptides independently caused membrane permeabilization (Garcia-Saez et al., 2006). However, later evidence refuted this “hairpin insertion” model. Firstly, the evidence that core-latch domain separation occurred during BAK and BAX activation is inconsistent with the existence of an α5: α6 hairpin structure. Secondly, and more importantly, biochemical and structural studies indicated that BAK and BAX mediated MOMP requires
the formation of BAK or BAX homodimer, in which the $\alpha_5$ remains associated in the core homodimer unit (Brouwer et al., 2014; Czabotar et al., 2013; Dewson et al., 2008; 2012). Lastly, membrane-impermeable IASD (4-acetamido-4′-((iodoacetyl) amino) stilbene-2,2′- disulfonic acid) labeling to map BAK or BAX membrane exposure before and after apoptotic activation discovered that neither $\alpha_5$ nor $\alpha_6$ was found to be membrane transverse, but instead both helices collapsed and only inserted shallowly on the membrane (Westphal et al., 2014).

Numerous groups have set out to determine the structure of the apoptotic pore, guided with the assumption that BAK and BAX formed an orderly packed proteinaceous channel to permeabilize MOM. Early studies focusing on the BAX central amphipathic peptide helices suggested BAX transmembrane monomers could self-associate to destabilize MOM (Annis et al., 2005). Similarly, in silico modeling based on analyzing stepwise change in membrane conductance during BAK and BAX activation also suggested a proteinaceous channel formation (Martinez-Caballero et al., 2009). In contrast to mathematical modeling, another study reported out that BAX behaved differently to proteinaceous pores on planar phospholipid membrane bilayers that it created variable changes in membrane permeability and destabilized membrane rather than forming a membrane channel with reproducible conductance levels (Basanez et al., 1999). Several biophysical studies on BAK and BAX in the presence of artificial membrane support a hypothesis that protein: lipid interaction is important for MOMP (Landeta et al., 2011; Q. Li et al., 2010; Lovell et al., 2008; Terrones et al., 2004). Hence, the prevailing model is that BAK and BAX form a proteolipidic pore. The insertion of the core domain, but not the latch domain, of BAK and BAX homodimers is thought to promote positive lipid monolayer curvature stress and increases the bending of lipid
layers (Basanez et al., 2002; Flores-Romero, Garcia-Porras, & Basanez, 2017; Terrones et al., 2004). In this model, the polar head groups of the lipids interact with the polar residues of the BAK and BAX amphipathic helices (α5) inserted to form the lining of the pore (Flores-Romero et al., 2017). A recent imaging study on minimal membrane systems further demonstrates that BAX forms a “ring-like” structure, with a discontinuous protein: protein boundary (Flores-Romero et al., 2017; Grosse et al., 2016), resembling a proteolipidic pore structure.
Figure 1.14 Schematic of the nature of proposed BAK and BAX pore

(A) The proposed proteinaceous (left) and proteolipidic (right) pore in lipid monolayer. Alpha helices (yellow) are tightly packed and transverse the lipid (left) whereas the proteolipidic (right) pore has the lipid head groups interacting with protein in forming the lumen of the pore. (B) High concentrations of local BAK/BAX homodimers on the membrane is proposed to increase the positive curvature and therefore disrupting the membrane integrity.

Another possibility raised by a recent study questions the very existence of a structured pore. The study proposed that MOMP is induced by random/disordered aggregation of BAK or BAX dimers as in-plane insertion of such dimers may result in membrane destabilization and subsequent permeabilization (Uren et al., 2017).
1.7.3 Mechanism of BAK and BAX mediated membrane permeabilization

The mechanism of BAK and BAX mediated MOMP remains unclear (Figure 1.15). After the discovery of BAK and BAX homodimerization and its critical role in MOMP, new models have been proposed regarding how the BAK and BAX homodimers permeabilize the MOM. The “in-plane” model was proposed after resolving the structure of the BAK and BAX homodimer (Brouwer et al., 2014; Czabotar et al., 2013). The α4 and α5 helices arrangement in the BAK and BAX homodimers enables the aromatic and hydrophobic residue-rich side to form a bent planar surface (Figure 1.13). This arrangement favors an “in-plane” positioning of the BAK and BAX homodimer by inserting their aromatic and hydrophobic residue side chains into membrane lipids and “pinch” the underlying lipids, thus potentially creating enough tension on the membrane surface to permeabilize the MOM (Figure 1.15).

An alternative “clamp model” was proposed by Bleicken and colleagues based on the characterization of MOMP mediated by recombinant BAX on liposomes using Double Electron-Electron Resonance (DEER) (Bleicken et al., 2014). They proposed that the α2-α5 core domain, instead of sitting “in plane” with the MOM, clamps across the MOM lipid bilayer to become the lining of the apoptotic pore (Figure 1.15). The unlatching of α5-α6 enables α6-α9 to form the “piercing” domain to anchor on the surface of the membrane with the α9 helices from the same homodimer antiparallel inserted into the membrane. How the α2-α5 core domain slides into membrane and form the clamp model remains unclear.
Figure 1.15 Schematic of proposed BAK and BAX mediated membrane permeabilization

The in-plane insertion model proposed BAK and BAX homodimer are able to sit on top of membrane with their α6 possibly shallowly embedded in the membrane or traversing membrane lipid. The clamp model proposes the BAK and BAX homodimer slides across MOM and clamped the membrane lipid as indicated, with the core dimer responsible for the formation of the lining of the apoptotic pore.

1.8 Another layer of BAK and BAX apoptotic control by non-BCL-2 proteins: the VDAC complex

As tremendous effort has been invested in understanding the apoptotic regulation of BAK and BAX, the scope of apoptosis has been concentrated on the BCL-2 family members. There has been an inadequate understanding of BAK and BAX apoptotic regulation by non-BCL-2 family proteins. In addition to the well-known tumor suppressor protein
p53 that directly transcriptionally activates PUMA and NOXA that is reviewed elsewhere (Aubrey, Kelly, Janic, Herold, & Strasser, 2018), as well as p53’s suspected role in directly activating BAX (Chipuk et al., 2004; M. Mihara et al., 2003), recent reports highlight the emerging links between other non-BCL-2 proteins and apoptosis. For example, mitochondria quality control machinery has been implicated in impacting the outcome of apoptosis. Mitochondria inside the cell form a heterogeneous network and undergo constant fission and fusion. Key mediators of mitochondria fission and fusion such as DRP-1 (dynamin related protein-1) and mitofusins (MFN-1 and MFN-2) have been shown to co-localize with BAX (Hoppins et al., 2011; Karbowski et al., 2002). Further, mitochondria fusion has been demonstrated to control the distribution of BAK and BID, hence affecting the kinetics and efficiency of MOMP (Weaver et al., 2014).

In addition to proteins regulating mitochondria fission and fusion, another family of proteins, the Voltage-dependent Anion Channel (VDAC) proteins, whose function primarily lies in metabolite transportation are implicated in apoptosis regulation by a number of studies. Their interactions with BCL-2 family proteins and impact on apoptosis outcomes make them particularly interesting in cell death research (Mark Xiang Li, 2015) and discussed below.

1.8.1 VDAC normal physiology, channel property

Voltage-dependent Anion Channel (VDAC) proteins are the most abundant proteins on the mitochondrial outer membrane and are responsible for the bulk of small metabolite transportation across the
MOM (Benz, 1985; Mannella, 1982). Three VDAC isoforms (VDAC1, 2 and 3) are found to be present in mammals with high sequence similarity and overlapping tissue distribution (Messina, Reina, Guarino, & De Pinto, 2012). The VDAC proteins are ~30 kDa, 19-stranded β-barrel transmembrane proteins with estimated channel diameter 2.5-3 nm allowing the transportation of metabolites and ions such as ATP, ADP, NADH and Ca\(^{2+}\) across the MOM (Bayrhuber et al., 2008; Colombini, Blachly-Dyson, & Forte, 1996; Hiller et al., 2008; Schredelseker et al., 2014; Ujwal et al., 2008).

In most cells studied, VDAC1 is the predominant form expressed compared to VDAC2 and 3, making it the predominant form of VDAC on MOM and therefore the best characterized isoform of the family (Messina et al., 2012). With the expansion of studies on VDAC2 and VDAC3, the different physiological function between the VDAC isoforms have been defined. VDAC2 was initially suggested to be able to supplement VDAC1 loss in mice, however differences in channel properties were later discovered that mitochondria with VDAC1 alone were much more permeable to NADH than those only expressing VDAC2 (X. Xu, Decker, Sampson, Craigen, & Colombini, 1999).

### 1.8.2 Sequence and structures of VDAC isoforms

Mammalian VDAC isoforms show approximately 75% sequence similarity (Figure 1.16), however, clear differences still exist. Two noticeable features are: 1) the high cysteine content in VDAC2 and 3 (9 in hVDAC2, 6 in hVDAC3 but only 2 in hVDAC1) and 2) the 11(human)-12(mouse) amino acids N-terminal extension in mammalian VDAC2 (De Pinto, Messina, Lane, & Lawen, 2010; Messina et al., 2012). The high cysteine content in VDAC2 and VDAC3 are believed to promote overall
protein-membrane interaction. In addition, a recent study on VDAC3 supports the role of cysteines as ROS (reactive oxygen species) sensors (Reina et al., 2016). Given that VDAC3 is the oldest VDAC isoform, followed by VDAC2 then VDAC1, it is speculated that the cysteines in VDAC2 also inherited ROS sensing ability (De Pinto, Reina, Gupta, Messina, & Mahalakshmi, 2016). However, the increased cysteine content in VDAC2 destabilizes its barrel structure (De Pinto et al., 2016). The N-terminal extension unique to VDAC2 is reported to increase the stability of the protein (Maurya & Mahalakshmi, 2014a; 2014b; 2017). Functionally, VDAC2 N-terminal extension participates in metabolite transportation as well as improved VDAC2 voltage sensing (Maurya & Mahalakshmi, 2015).
Figure 1.16 Sequence comparison between human VDAC proteins

Amino acid sequence alignment of human VDAC 1, 2 and 3 (note the VDAC proteins are arranged according to evolution order). Cysteine (C) residues are highlighted in yellow. Protein sequence alignment performed with ClustalW2.

Structural information is still lacking for mammalian VDAC2 and 3. As VDAC2 shares 91% sequence similarity, the predicted VDAC2 structure is believed to be very similar to that of VDAC1 (Schredelseker et al., 2014; Shoshan-Barmatz et al., 2010). Experimental characterization of VDAC2 remains challenging due to its poor protein solubility and stability. In 2014, the zebrafish VDAC2 (zfVDAC2) was crystalized and revealed that zfVDAC2 indeed adopts a 19-stranded β-barrel fold with the N-terminal region folded into an α helix lying inside the channel (Figure
A major difference of VDAC2 structure compared to that of VDAC1 is the loop region connecting strand 1 and 2. Also, a population of folded VDAC2 are present as dimers. However, inferring the structure of mammalian VDAC2 based on zfVDAC2 still requires caution as zfVDAC2 does not have the N-terminal extension and the high cysteine contented mentioned above.
Figure 1.17 Structure similarity between known VDAC members

(A) Mouse VDAC1 (left cyan, PDB: 3EMN) and zebra fish VDAC2 (right green, PDB: 4BUM) exhibit great structural similarity. (B) Superimposed mVDAC1 and zfVDAC2 structure in (A) to illustrate the sufficient sequence (pink) of VDAC2 to maintain BAK membrane recruitment and the minimal motif (red) discover in the loss of function mutagenesis (side view left and top view right). In pink, the sufficient segments of VDAC2 for BAK membrane recruitment (Naghdi, Várnai, & Hajnóczky, 2015). Note the VDAC2 minimal motif (red) reaches into the open loop region facing the cytoplasmic side, potentially forming a docking site for protein interaction. (C) Sequence alignment of the sufficient sequence of VDAC2 from different species and mVDAC1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>mVDAC2</td>
<td>123 SGKIKSAYKRECINLGCDVDFDAGPAIHGSAVFGYEGWLAGYQMTFDSAKSKLTRS 179</td>
</tr>
<tr>
<td>hVDAC2</td>
<td>122 SGKIKSSYKRECINLGCDVDFDAGPAIHGSAVFGYEGWLAGYQMTFDSAKSKLTRN 178</td>
</tr>
<tr>
<td>zfVDAC2</td>
<td>111 SGKVTIAYKREFVNLGCDVDFDAGPIHGAAVGYEGWLAGYQMSFDIASKMTQN 167</td>
</tr>
<tr>
<td>mVDAC1</td>
<td>111 NAKIKGYKREHINLGCDVDFDAGPSRGLVLGEGWLAGYQNFTSKSRTQ 167</td>
</tr>
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1.8.3 VDAC proteins in cell death

Since VDAC proteins are the major regulator of mitochondria metabolism, they were soon suspected to partake in cell death pathways. Initially, VDACs were speculated to form the mitochondrial permeability transition pore (PTP) complex with proteins such as cyclophilin D to regulate membrane potential and ATP production (SZABO & ZORATTI, 1993). With reported interactions with BCL-2 family members, VDACs were implicated to drive the mitochondrial membrane permeabilization via the PTP complex in response to cell death stimuli (Brenner & Grimm, 2006; Zheng et al., 2004). However, a study reported that mouse embryonic fibroblasts (MEFs) lacking Vdac1, Vdac2, Vdac3, Vdac1/3 and even Vdac1/2/3 were virtually indistinguishable from wt (wild type) in death when challenged with a range of apoptotic stresses (Baines, Kaiser, Sheiko, Craigen, & Molkentin, 2007), hence questioning the hypothesis that VDACs are directly contributing to mitochondria permeabilization.

1.8.4 VDAC2, BAK and BAX.

Biochemical characterization of VDAC1 discovered that it is able to bind to multiple BCL-2 proteins such as MCL-1 and BCL-XL (Arbel, Ben-Hail, & Shoshan-Barmatz, 2012; H. Huang et al., 2013; H. Huang, Shah, Bradbury, Li, & White, 2014). But the physiological role of VDAC proteins in regulating pro- or anti-apoptosis remains in debate. Mice lacking Vdac1 or 3 are grossly normal (Vdac 3−/− mice have a high degree of male infertility) whereas Vdac2 deletion results in embryonic lethality (Anflous, Armstrong, & Craigen, 2001; Cheng, 2003; Sampson et
al., 2001). This has brought interest in understanding the distinct physiological role of VDAC2. Cheng and colleagues first reported that VDAC2 directly interacts with BAK on mitochondria in viable cells using cross-linking and mass spectrometry. tBID could efficiently displace BAK from VDAC2 and cells devoid of VDAC2 had increased sensitivity towards apoptotic stimuli (Cheng, 2003). Hence, the dogma remains that VDAC2 is a BAK-specific inhibitor.

However, the anti-apoptotic function of VDAC2 has been challenged by studies focusing on BAX, in the absence of BAK, requires VDAC2 for its activity and to induce robust cell death (Ma et al., 2014; Yamagata et al., 2009). We and others propose that, at least for BAX, VDAC2 is acting as its mitochondrial membrane recruiter (Dewson, 2015; Ma et al., 2014; Yamagata et al., 2009). Consistently, a similar role of VDAC2 in BAK mitochondria recruitment has been demonstrated in Vdac2−/− mouse embryonic fibroblasts (MEFs) mitochondria. It was observed that there is significantly reduced level of mitochondria BAK and increased BAK level in cytosol (Ma et al., 2014; Roy, Ehrlich, Craigen, & Hajnóczky, 2009). As predicted, these mitochondria are also resistant to tBID-induced apoptosis compared to cells lacking other VDAC isoforms (Roy et al., 2009). During the course of my studies, a conserved VDAC2-specific motif (spanning mVDAC2 amino acid 168-171) was discovered to be critical for BAK membrane recruitment (Naghdi et al., 2015)(Figure 1.17 B). Hence, we propose a unified function of VDAC2 for BAK and BAX mitochondrial targeting. The detailed molecular information regarding BAK/BAX: VDAC2 interaction is still limited. Therefore, one aim of this PhD project was to investigate the interaction between VDAC2 and BAK/BAX and the functional relevance of these interactions in regulating BAK and BAX apoptotic activity.
1.9 Targeting BCL-2 proteins in Disease.

1.9.1 Unleashing BCL-2 protein-mediated cell death in cancer

Cell death is a tightly regulated process and BCL-2 family proteins are critical in governing intrinsic apoptotic death. Evading cell death has been one of the well-established hallmarks of cancer development. In addition to the t(14:18) chromosomal translocation which amplified BCL-2 in follicular lymphoma (Tsujimoto et al., 1985), dysregulation of BCL-2 family proteins is commonly found in many types of cancer that either result in enhanced pro-survival protein expression and/or compromised function of pro-apoptotic members (Cotter, 2009).

Since up-regulation of pro-survival BCL-2 proteins is commonly observed in a broad range of cancers, there has been significant interest in developing small molecule inhibitors of pro-survival BCL-2s to tackle the addiction of cancer cells to pro-survival BCL-2 proteins. The recent FDA approval of the BH3-mimetic ABT-199 (venetoclax/ Venclexta) in treating CLL (chronic lymphocytic leukaemia) is a perfect example of rational designing of cancer therapy targeting BCL-2 family proteins (Roberts et al., 2016). ABT-199 is specifically a BCL-2 antagonist, in succession of ABT-737 (antagonist of BCL-2, BCL-X<sub>L</sub> and BCL-W) and its orally available form ABT-263 (Opferman, 2016). The increased specificity of ABT-199 to BCL-2 alone overcame the on-target side effect of induced thrombocytopenia with ABT-263 (Roberts et al., 2016). ABT-199 mimics a BH3-only protein that binds to the surface hydrophobic groove of BCL-2 with very high affinity, therefore displacing a bound BH3-only protein from, or preventing a BH3-only protein binding to, the hydrophobic
groove, allowing the BH3-only protein to activate BAK and BAX to induce apoptotic cell death.

Although ABT-199 has demonstrated strong potency in treating relapsed CLL as a single agent therapy, cancers that have high expression of MCL-1 such as multiple myeloma remain resistant to ABT-199 treatment(Punnoose et al., 2016). Following the same rationale, a MCL-1 specific BH3-mimetic S63845 has been developed and has shown very promising results in pre-clinical models(Letai, 2016).

Considering the resistance of cancer cells to single agent therapy, the key to tackle different cancers and avoid drug resistance is likely the use of combination therapy. For this aim, the ability to directly activate BAK and BAX would be desirable. As small molecules that directly activate BAK and BAX can improve the potency of cancer combination therapy with existing BH3-mimetics(Gavathiotis, Reyna, Bellairs, Leshchiner, & Walensky, 2012; Reyna et al., 2017). The current development of BAK and BAX activators will be discussed in more detail in Chapter 7.

1.9.2 Restraining BCL-2 proteins mediated cell death

On the other side, developing inhibitors of apoptosis to restrain unwanted/pathological cell death are highly sought after in different forms of acute stresses such as ischaemic-reperfusion injury, neurodegenerative diseases and certain inflammatory disorders(Favaloro, Allocati, Graziano, Di Ilio, & De Laurenzi, 2012; Friedlander, 2003; Singh & Kang, 2011). However, pharmacological inhibition of the pro-apoptotic BCL-2 proteins has been less explored and proven even more challenging compared to their pro-survival counterparts.
A few small molecules have been first reported to inhibit cytochrome c release in isolated mitochondria or in cells by interacting with BAX (Bombrun et al., 2003; Hetz et al., 2005; Polster, Basanez, Young, Suzuki, & Fiskum, 2003). There was no indication on whether such compounds are able to inhibit BAK. Furthermore, such compounds were argued to be membrane modulators rather than direct inhibitors of BAK/BAX activation or oligomerization, so these are likely to have wide ranging effects on cellular physiology. More recently, two classes of molecules have been reported to either deactivate monomeric BAK (Brouwer et al., 2017) or inhibit BAK/BAX dimerization (Niu et al., 2017).

Better understanding of BAK and BAX regulation by non-BCL-2 proteins will not only enrich our understanding of BAK and BAX activity and how it goes awry in disease, but will also highlight new possibilities of pharmacological modulation of BAK and BAX to steer apoptosis towards desired directions.

1.10 Proposed work

This thesis addresses the overarching hypothesis that there exist multiple interactions governing BAK (and BAX) apoptotic activation and downstream multimerization.

Chapter 3

**Hypothesis:** Direct interaction with VDAC2 controls BAK apoptotic function.
**Aim:** To investigate the protein-protein interactions between non-BCL-2 protein VDAC2 and BAK (and BAX) and deciphering the functional consequence of altering this interaction on apoptosis.

I employed cysteine mutagenesis to characterize the BAK: VDAC2 interface by disulfide cross-linking. An obtrusive labeling approach was used to confirm the specificity of the interaction. In this chapter, a VDAC2 variant that has stabilized interaction with BAK was discovered and tested to impair BAK mediated apoptosis.

Chapter 4

**Hypothesis:** BAK activation conformation change destabilizes interaction with tBID at MOM.

**Aim:** To investigate BAK activation conformation change and its interaction with BH3-only protein tBID on isolated mitochondria.

This study used disulfide tethering combined with immunoprecipitation by BAK conformation specific antibody to understand the stepwise events during BAK conformation change induced by tBID activation on mitochondria.

Chapter 5

**Hypothesis:** There exists multiple interfaces required for BAK (and BAX) apoptotic activation.

**Aim:** To identify the interfaces required for BAK (and BAX) apoptotic activation.

I tested both unbiased and targeted chemical cross-linking to identify tBID: BAK interfaces. A functional screening method by PEG-MAL labeling was established and applied to map BAK activation sites.
Chapter 6

**Hypothesis:** There exists a secondary interface required for BAK (and BAX) homodimers to self-associate and promote MOMP.

**Aim:** To identify the secondary interface for BAK (and BAX) multimerization.

PEG-MAL labeling method was adapted to test specifically interfaces important for BAK homodimerization and downstream oligomerization.
## 2 Chapter 2 Material and methods

### 2.1 Materials used in this thesis

Table 2.1 Reagents and recipes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin 1000 X</td>
<td>100 mg/ml ampicillin in distilled water</td>
</tr>
<tr>
<td></td>
<td>1 mL aliquot</td>
</tr>
<tr>
<td></td>
<td>Stored at -20 °C</td>
</tr>
<tr>
<td>L-Asparagine 10 mM</td>
<td>Asparagine salt in MT-PBS filter-sterilized</td>
</tr>
<tr>
<td></td>
<td>10 mL aliquot</td>
</tr>
<tr>
<td></td>
<td>Stored at -20 °C</td>
</tr>
<tr>
<td></td>
<td>Used at 100 µM final.</td>
</tr>
<tr>
<td>Blue Native PAGE anode buffer</td>
<td>50 mM Bis-Tris pH 7.0</td>
</tr>
<tr>
<td>Blue Native PAGE cathode buffer</td>
<td>50 mM N-2-hydroxy-1,1-bis(hydroxymethyl)ethylglycine</td>
</tr>
<tr>
<td></td>
<td>15 mM Bis-Tris unbuffered containing 0.02% w/v Coomassie Blue G-250</td>
</tr>
<tr>
<td>Blue Native PAGE destain buffer</td>
<td>50 % (v/v) methanol</td>
</tr>
<tr>
<td></td>
<td>25 % (v/v) acetic acid</td>
</tr>
<tr>
<td></td>
<td>Made up with distilled water</td>
</tr>
<tr>
<td>Blue Native PAGE loading Dye</td>
<td>5 % w/v Coomassie Blue R-250</td>
</tr>
<tr>
<td></td>
<td>500 mM 6-aminohexanoic acid</td>
</tr>
<tr>
<td></td>
<td>100 mM Bis-Tris pH 7.0</td>
</tr>
<tr>
<td>Blue Native PAGE transfer buffer</td>
<td>Tris-glycine transfer buffer containing 20 % methanol and 0.037 % SDS</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>BMOE 10 mM</td>
<td>1 mg in 380 µL DMSO. Used at 0.5 mM.</td>
</tr>
<tr>
<td>Complete Protease inhibitor</td>
<td>1 tablet dissolved in 1 mL distilled water.</td>
</tr>
<tr>
<td>cocktail 50 X</td>
<td></td>
</tr>
<tr>
<td>Coomassie stain for Blue Native</td>
<td>2.5 µg/mL Coomassie Brilliant Blue R-250</td>
</tr>
<tr>
<td>PAGE</td>
<td>10 % (v/v) acetic acid</td>
</tr>
<tr>
<td></td>
<td>50 % (v/v) methanol</td>
</tr>
<tr>
<td></td>
<td>Stored at 4 °C</td>
</tr>
<tr>
<td>Cross-linking buffer for BMOE</td>
<td>20 mM HEPES pH7</td>
</tr>
<tr>
<td></td>
<td>50 mM KCL</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA pH8</td>
</tr>
<tr>
<td></td>
<td>2.5 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>250 mM Sucrose</td>
</tr>
<tr>
<td></td>
<td>Completed to 1 L with distilled water</td>
</tr>
<tr>
<td>CuPhe 100 mM</td>
<td>30 mM CuSO₄ in water</td>
</tr>
<tr>
<td></td>
<td>100 mM 1,10-phenanthroline in 20 % ultra pure ethanol</td>
</tr>
<tr>
<td></td>
<td>Stored at -20 °C</td>
</tr>
<tr>
<td></td>
<td>Used at 1 mM</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagle’s</td>
<td>Dulbecco’s Modified Eagle’s medium (Gibco)</td>
</tr>
<tr>
<td>medium (DMEM)</td>
<td>3.4 g/L NaHCO₃</td>
</tr>
<tr>
<td></td>
<td>107.4 mg/L Penicillin</td>
</tr>
<tr>
<td>Buffer/Reagent</td>
<td>Composition/Details</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>100 µg/mL streptomycin</td>
<td>Osmolality 310-320 nmol/kg</td>
</tr>
</tbody>
</table>
| EWB buffer | 20 mM Bis-Tris pH 7.4  
50 mM NaCl  
10 % glycerol  
10 mM DTT  
Stored at 4 °C |
| FACS buffer | KDS-BSS  
10 % FCS |
| Hygromycin | 50 mg/mL in PBS  
Used at 50 µg/mL |
| KDS-BSS | 1.2 mM MgSO₄  
7.4 mM HEPES-NaOH  
0.8 mM K₂HPO₄  
149 mM NaCl |
| LG Agar | 1 % tryptone  
0.5 % yeast extract  
0.5 % NaCl  
0.2 % D-glucose  
10 mM Tris buffer pH 7.4  
1 mM MgCl₂  
1.5 % agar |
| MELB buffer | 100 mM sucrose  
20 mM HEPES.KOH pH7.5  
100 mM KCL  
2.5 mM MgCl₂  
Made up to 100 mL with distilled water |
<table>
<thead>
<tr>
<th>Material</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-PBS (Mouse Tonic Phosphate Buffered Saline)</td>
<td>Stored at -20 °C</td>
</tr>
<tr>
<td></td>
<td>2.85 g/L Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td>0.62 g/L NaH₂PO₄·2H₂O</td>
</tr>
<tr>
<td></td>
<td>8.7 g/L NaCl</td>
</tr>
<tr>
<td></td>
<td>Osmolality 310-330 mmol/kg</td>
</tr>
<tr>
<td>NEM (N-ethyl maleimide) 1M</td>
<td>Powder dissolved in ethanol (molecular biology grade) to 1M</td>
</tr>
<tr>
<td></td>
<td>Used at 20 mM</td>
</tr>
<tr>
<td>Onyx buffer</td>
<td>20 mM Tris pH 7.8</td>
</tr>
<tr>
<td></td>
<td>135 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1.5 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>1 mM EGTA</td>
</tr>
<tr>
<td></td>
<td>10 % v/v glycerol</td>
</tr>
<tr>
<td></td>
<td>Made up to 1L with distilled water</td>
</tr>
<tr>
<td></td>
<td>Stored at 4 °C</td>
</tr>
<tr>
<td>PEG-MAL</td>
<td>10 mM stock made with distilled water</td>
</tr>
<tr>
<td></td>
<td>Used at 0.5 mM final</td>
</tr>
<tr>
<td>Permeabilization buffer</td>
<td>MELB buffer</td>
</tr>
<tr>
<td></td>
<td>1 X complete protease inhibitor cocktail</td>
</tr>
<tr>
<td></td>
<td>0.025 % Digitonin</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (PMSF) 100 mM</td>
<td>1 mg in 57.5 μL ethanol (molecular biology grade)</td>
</tr>
<tr>
<td></td>
<td>Used at 1 mM</td>
</tr>
<tr>
<td>Substance</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>500 µg/uL stock made up in water. Used at 5 µg/µL. Stored at 4 °C.</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>2 mg/mL stock in 10 mM HEPES.KOH pH 7.4. Heated for 30 min at 37 °C to</td>
</tr>
<tr>
<td></td>
<td>inactivate lipases. Stored at -80 °C.</td>
</tr>
<tr>
<td>SDS (sodium dodecyl sulfate) 20%</td>
<td>250 g SDS powder dissolved in 5L distilled water.</td>
</tr>
<tr>
<td>SDS-PAGE sample buffer 2 X</td>
<td>150 mM Tris pH 6.8. 1.2 % SDS. 30 % v/v glycerol. 0.018 mg/mL bromophenol</td>
</tr>
<tr>
<td></td>
<td>blue. 5 % v/v β-mercaptoethanol was added for reducing sample buffer.</td>
</tr>
<tr>
<td>S-broth</td>
<td>3.5 % tryptone. 2 % yeast extract. 0.5 % NaCl. 5 mM NaOH.</td>
</tr>
<tr>
<td>Solubilization buffer</td>
<td>Onyx buffer. 1 X complete protease inhibitor cockatil. 1 % w/v Digitonin.</td>
</tr>
<tr>
<td>Super Optimal broth (SOB)</td>
<td>2 % bacto tryptone. 0.5 % yeast extract. 10 mM NaCl.</td>
</tr>
</tbody>
</table>
| TAE buffer (Tris/Acetic Acid/EDTA) | 2.5 mM KCl  
5 mM NaOH  
10 mM Tris-HCl pH 7.8  
20 mM acetic acid  
1 mM EDTA pH 8 |
|-----------------------------------|--------------------------------------------------|
| Tris/Glycine running buffer       | 250 mM Tris  
1.92 M glycine  
1 % SDS |
| Tris/Glycine transfer buffer      | 25 mM Tris  
192 mM glycine  
20 % Methanol |
| Trypsin                           | 0.5 g/L trypsin (Difco)  
8 g/L NaCl  
0.4 g/L KCl  
1 g/L D-glucose  
0.58 g/L EDTA |

Table 2.2 Antibody list

<table>
<thead>
<tr>
<th>Clone/Name</th>
<th>Origin</th>
<th>Catalog#</th>
<th>Source</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAK (DF9)</td>
<td>Rabbit</td>
<td>B5897</td>
<td>Sigma</td>
<td>Recognize aa 23-38</td>
</tr>
<tr>
<td>BAK Ab-1</td>
<td>Mouse</td>
<td>AM03</td>
<td>Calbiochem</td>
<td>Recognize aa 38-45</td>
</tr>
<tr>
<td>BAK NT</td>
<td>Rabbit</td>
<td>06-536</td>
<td>Millipore</td>
<td>Recognize aa 28-31</td>
</tr>
<tr>
<td>Protein</td>
<td>Species</td>
<td>Code</td>
<td>Source</td>
<td>Clone/Cell</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>--------</td>
<td>----------------</td>
<td>------------</td>
</tr>
<tr>
<td>BAK 4B5</td>
<td>Rat</td>
<td>In-house</td>
<td>Recognize</td>
<td>aa 82-86</td>
</tr>
<tr>
<td>BAK 7D10</td>
<td>Rat</td>
<td>In-house</td>
<td>Recognize</td>
<td>aa 51-55</td>
</tr>
<tr>
<td>BAX AC4</td>
<td>Rat</td>
<td>In-house</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BID AE5</td>
<td>Rat</td>
<td>In-house</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>Mouse</td>
<td>556432</td>
<td>BD</td>
<td>Clone</td>
</tr>
<tr>
<td>HA 3F10</td>
<td>Rat</td>
<td>11867423001</td>
<td>Roche</td>
<td>Clone</td>
</tr>
<tr>
<td>HA</td>
<td>Mouse</td>
<td>901513</td>
<td>Covance</td>
<td>Clone</td>
</tr>
<tr>
<td>Flag</td>
<td>Mouse</td>
<td>F1804</td>
<td>Sigma</td>
<td>Clone</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Mouse</td>
<td>MABN504</td>
<td>Merck</td>
<td>Clone</td>
</tr>
<tr>
<td>VDAC2</td>
<td>Rabbit</td>
<td>In-house</td>
<td>Entire mouse</td>
<td>VDAC2</td>
</tr>
</tbody>
</table>
Table 2.3 Primers for mutagenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hBAK</strong></td>
<td>Forward (FWD)</td>
<td>GGAATTCCATGGCTTCGGGGCAAGGCCCAGG</td>
</tr>
<tr>
<td></td>
<td>Reverse(RV)</td>
<td>CCGCTCGAGAGGCTGATTTGAAGAATCTTTCG</td>
</tr>
<tr>
<td>FWD R156S</td>
<td></td>
<td>CTAGGCCAGGTGAAGCTTCGTGGGACTTTC</td>
</tr>
<tr>
<td>RV R156S</td>
<td></td>
<td>GAAGTCGACACGAGCTGGTACCTGACCTAG</td>
</tr>
<tr>
<td>FWD D160Y</td>
<td></td>
<td>GTGACCGCCCTGGCTGCTACTTGCTGACTGACCTAC</td>
</tr>
<tr>
<td>RV D160Y</td>
<td></td>
<td>GTGATGCAGCATGAAAGTAGACACCACGAAGCGGGTCAC</td>
</tr>
<tr>
<td><strong>mVdac2</strong></td>
<td>FWD CysΔ</td>
<td>GAAGATCTACCATGTACCACTACGAGCAGTCCCCAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACGCTGCTGAGACGAGCACGGTACC</td>
</tr>
<tr>
<td>FWD S58C</td>
<td></td>
<td>GAATTTACACATTGGCTCTGCAATACAGACA</td>
</tr>
<tr>
<td>RV S58C</td>
<td></td>
<td>CTGCTAGTAAGTGGTCAATGGGCTGGTGGTCAGG</td>
</tr>
<tr>
<td>FWD A172C</td>
<td></td>
<td>GTACAAATCCGCTTTGACAGTGCCAAGTCAAAG</td>
</tr>
<tr>
<td>RV A172C</td>
<td></td>
<td>GCTGACAAGG</td>
</tr>
<tr>
<td>FWD Q166C</td>
<td></td>
<td>GGCATTGGCTGCTGAGGTGTACGTGACCTGGTACA</td>
</tr>
<tr>
<td>RV Q166C</td>
<td></td>
<td>GTGCC</td>
</tr>
<tr>
<td>FWD M167C</td>
<td></td>
<td>GCTGGCTCTGGTGATACATTGCCACCTTGCACAG</td>
</tr>
<tr>
<td>RV M167C</td>
<td></td>
<td>CTTGGCAGTCAAGGGTGCTTGATTCACCCAGGCAAG</td>
</tr>
</tbody>
</table>

- 89 -
Table 2.4 PCR reaction reagents

<table>
<thead>
<tr>
<th>Site-directed mutagenesis PCR</th>
<th>Annealing PCR</th>
<th>Amplification PCR</th>
<th>Colony PCR</th>
<th>Sequencing PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phusion master mix</td>
<td>25 µl</td>
<td>12.5 µl</td>
<td>25 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>Primer Fwd</td>
<td>0.5 µM</td>
<td>0.5 µM</td>
<td>0.5 µM</td>
<td>400 pM</td>
</tr>
<tr>
<td>Primer Rev</td>
<td>0.5 µM</td>
<td>0.5 µM</td>
<td>0.5 µM</td>
<td>-</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10 ng</td>
<td></td>
<td>Picked colony</td>
<td>400 ng</td>
</tr>
<tr>
<td>PCR product 5’</td>
<td></td>
<td>5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR product 3’</td>
<td></td>
<td>5 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing PCR product</td>
<td></td>
<td>4 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BDT v3.1 ready mix</td>
<td></td>
<td></td>
<td></td>
<td>1 µl</td>
</tr>
<tr>
<td>5X BDT dilution buffer</td>
<td></td>
<td></td>
<td></td>
<td>3.5 µl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>to 50 µl</td>
<td>to 25 µl</td>
<td>to 50 µl</td>
<td>to 10 µl</td>
</tr>
</tbody>
</table>
Table 2.5 PCR protocols

<table>
<thead>
<tr>
<th>Step</th>
<th>Site directed mutagenesis</th>
<th>Overlap extension PCR</th>
<th>Amplification PCR</th>
<th>Colony PCR</th>
<th>Sequencing PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94 °C, 2 min</td>
<td>-</td>
<td>94 °C, 2 min</td>
<td>94 °C, 5 min</td>
<td>96 °C, 60 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94 °C, 35 sec</td>
<td>94 °C, 35 sec</td>
<td>94 °C, 35 sec</td>
<td>94 °C, 30 sec</td>
<td>96 °C, 10 sec</td>
</tr>
<tr>
<td>Annealing (cycles)</td>
<td>58 °C, 30 sec</td>
<td>65 °C, 30 sec</td>
<td>58 °C, 30 sec</td>
<td>56 °C, 30 sec</td>
<td>50 °C, 5 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C, 60 sec</td>
<td>72 °C, 60 sec</td>
<td>72 °C, 60 sec</td>
<td>72 °C, 60 sec</td>
<td>60 °C, 4 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 °C, 5 min</td>
<td>-</td>
<td>72 °C, 5 min</td>
<td>72 °C, 5 min</td>
<td>-</td>
</tr>
<tr>
<td>Cycles</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>25</td>
<td>30</td>
</tr>
</tbody>
</table>

2.2 Methods:

2.2.1 Agarose gel electrophoresis

DNA samples mixed with 10X loading dye were resolved on 1.5 % agarose gels made in 1 X TAE with ethidium bromide (0.5 µg/ml). DNA was visualized under ultra UV light using the Bio-rad Chemi Doc™ XRS + Molecular Imager®. DNA size was estimated by 1 kilo base (kb) ladder, Life Technologies. Raw files were imported into the Bio-rad Image Lab™ software for analysis.
2.2.2 Polymerase Chain Reaction (PCR)

PCR conditions, primers were listed in the table. Typical PCR reaction was carried out in 50 µl volume, with 200 ng DNA template and 15 µM of each forward and reverse primer. PCR products were resolved using gel electrophoresis described above.

2.2.3 Restriction endonuclease digestion

PCR products, plasmid DNA (1 µg) or commercial gBlock were digested with 1 unit of restriction enzyme from NEB (New England Biolabs) with compatible 10 X buffer (Cutsmart or 3.1) at a final volume of 50 µl as NEB instructions. Digestions were done at 37 °C for at least 1 hr, and digested products were analyzed by resolving on an agarose gel as described.

2.2.4 Gel Elution of DNA fragments

DNA gel blocks were exercised from Agarose gel then purified using Promega Wizard® Kit. Final elution is 50 µl in distilled water. Concentration was calculated with automated Nanodrop™ machine from ThermoFisher.

2.2.5 DNA fragment ligation

Purified DNA inserts (7 µl) and vector (1 µl), 0.8 µl T4 DNA ligase (Promega) and 1 µl 10 X ligase buffer (Promega) were set up to final volume of 10 µl reaction at room temperature for 1 hr.
2.2.6 Transformation by electroporation of bacteria competent cells

2.5 µl of ligated mixture was transformed to 50 µl of pre-ice chilled JM109 cells using pre-chilled 0.2 cm electroporation cuvette (Cell Projects) and electroporated at 2.5 kV on a Bio-rad MicroPulser™ electroporator. Mixture was then transferred to 500 µl of S broth to recover for 1hr at 37 °C. Then the cells were pelleted by centrifugation at 6000 rpm, 1 min and resuspended in 50 µl S broth to allow plating on pre-warmed LG agar with 100 µg/ml ampicillin.

2.2.7 Preparation of electro-competent bacteria

0.5 ml of a 3 ml overnight culture of JM109 cells was used to inoculate a 500 ml culture in super optimum broth (SOB). The culture was allowed to grow at 37 °C until a spectrophotometer measurement optical density (OD) of 0.8 at a wavelength of 550 nm was reached. Cells were pelleted by centrifugation at 3000 g for 10 min at 4 °C. Cell pellets were washed twice in 500 ml ice-cold wash buffer (10% glycerol dissolved in distilled water and filter-sterilized). Cells were re-suspended in wash buffer to a final volume of 2 ml before freeze in 50 µl aliquots at -80 °C.

2.2.8 Isolation of plasmid

Isolated bacteria colony or transformed bacteria was cultured in 10 mL L-broth with 100 µg/ml ampicillin for 16 hr at 37 °C in shaking incubator. Plasmid DNA was extracted using a QIAGEN QIAprep spin mini-prep kit.

2.2.9 DNA quantification

The Nanodrop ND-1000 spectrophotometer from ThermoFisher was used to quantify plasmid DNA.
2.2.10 DNA Sequencing

 Mutagenesis was confirmed by bi-directional sequencing of plasmids. Reactions of 400 ng DNA and 20 pM primer (forward or reverse) were sequenced using ABI BigDye Terminator Version 3.1 (Applied Biosystems). PCR samples were purified with magnesium sulphate and ethanol precipitation. Purified samples were sent to the Australian genome research facility (AGRF) for Sanger sequencing. Sequencing results were analyzed with Seqman softwares.

2.2.11 Generation of recombinant BID variants

 Recombinant HA-tagged BID variants and protein chimeras used in this project were generated by or under supervision of Dr Colin Hockings. The original construct was based on GST-BID (human) by Oliver von Ahsen “pGEX-4T1-BID (OvA)”. This construct replaced the caspase-8 cleavage site in BID (amino acids 57-62) with a thrombin cleavage sequence LVPRGS between GST and BID. The recombinant protein was purified by glutathione affinity, and BID proteins were eluted after thrombin cleavage. The wild type, untagged BID construct retained the caspase-8 cleavage site and was cleaved by recombinant caspase-8. The BID DNA constructs as well as the detailed purification protocols used in this thesis were detailed by Dr. Colin Hockings and the information can be found in his thesis.

2.2.12 Mammalian cell culture

2.2.12.1 Retroviral transduction into MEFs

 Retroviral constructs were transduced using the Phoenix ecotropic packaging system. On day one, Phoenix cells were seeded (1.5 x 10^6 cells per plate) in 10 cm dishes 24 hr prior to transfection in DMEM media with 8% FCS, to obtain at 70-80% confluency at the time of transfection. On
day two, desired plasmid containing the retroviral vector was transfected using Roche’s X-tremeGENE DNA transfection reagent. 9 µl X-tremeGENE was added directly to 91 µl serum-free DME and incubated for 5 min at room temperature, prior to the addition of 3 µg plasmid DNA. The mixture was incubated for 15 min before added into Phoenix cells dish and incubated at 37 °C for 24 hr. On day three, the media was replaced with 5 ml DMEM media with 8% FCS and incubated at 37 °C for 24 hr for virus production. Meanwhile, target cells (\textit{Bak}^{−/−} \textit{Bax}^{−/−} MEFs for example) were seeded (50,000 cells per well) into 6-well plates, 24 hr prior to infection with proper media. On day four, viral containing media was removed from Phoenix cells and filtered through a 45 µM filter to remove contaminating Phoenix cells. Filtered viral media was supplemented with 4 µg/ml polybrene, mixed and set at room temperature for 5 min. The filtered virus was then added to target cells in 6-well plates after replacing original culture media. Infection was carried out by spinning plates at 1500 g for 45 min at 32 °C on a pre-warmed centrifuge. Cells were incubated for 24 hr prior to selection for polyclonal populations by sorting for GFP or by chemical selection such as hygromycin (50 µg/ml).

\textbf{2.2.12.2 Maintenance of cell culture}

All MEF cell lines were in DME media with 8% FCS, 250 µM L-asparagine, and 55 µM 2-mercaptoethanol. All HCT-116 cells lines were in DME media with 8% FCS. For cells requiring selection in culture, 50 µg/ml hygromycin or 2 µg/ml puromycin was also added to culture media for 24 hr. Cells were maintained at 37 °C in a humidified 10% CO\textsubscript{2} incubator and passaged when 70-80% confluent by trypsinization.
2.2.12.3 **Cell line storage**

For storing, cells that were confluent were trypsinized from a 10 cm dish, spun down at 350 g for 5 min to remove media, and resuspended in 1000 µl freezing solution (FCS +10% DMSO) in 1.5 ml cryo-vials, pre-chilled on ice for 1 hr before stored at -80 °C for short periods, and transferred to liquid N₂ for long-term storage.

2.2.12.4 **Cell line thawing**

Frozen cryo-vials were quickly brought to room temperature then resuspended in a total volume of 10 ml with proper pre-warmed cell culture media. Cells were then pelleted by centrifugation at 300 g, 5 min, and resuspended cells were added back to 10 cm culture plates with proper media.

2.2.12.5 **Cell line sorting by GFP**

Following transduction, polyclonal populations of cells were sorted by fluorescence assisted cell sorting (FACS) based on GFP expression. Cells were harvested from a 10 cm dish by trypsinization. Trypsin was neutralized by the addition of 1ml cell culture media. Harvested cells were centrifuged at 300 g for 5 min at 4 °C, re-suspended in PBS containing 10% FCS and passed through a sterilized plugged Pasteur pipette to remove any clumped cells. Filtered cells were then sorted for the GFP-positive population on a MoFlo™ cell sorter (Beckman Coulter) at WEHI FACS facility. Sorted cells were cultured with ciprofloxacin (200 X stock) during the initial passage to prevent any contamination.

2.2.13 **Death Assay measured by PI positivity**

Analysis of BAK and BAX apoptotic function in cells was carried out as described previously (Dewson et al 2008). On day one, cells were seeded
at a density of 10,000 cells/well for untreated negative control and for treatment with cytotoxic stimulus. On day two, cell culture media was replaced with fresh media supplemented without or with 1µM/10µM etoposide, or 0.1 µM/1 µM Actinomycin D or 10 µM ABT-737 and/or 10 µM MCL-1 inhibitor and incubated for 24 hr. On day three, untreated and treated cells were harvested (including media supernatant to collect dead cells) and centrifuged at 500 g for 5 min at 4 °C. Pellets were resuspended in 200 µl KDS-BSS containing propidium iodide (PI) to a final concentration of 5 µg/µl. PI uptake was assessed by flow cytometry using BD FACscan or FACSCalibre. Raw data was gated and analyzed using Flowjo software. Quantification of data was carried out using Prism software. Error bars represented standard error of mean (SEM), as indicated in the figure legends.

### 2.2.14 Subcellular fractionation of MEFs

Cells harvested from cultured plates were washed with 1 mL PBS at 300 g for 5 min at room temperature after cell counting. Cell pellet was then permeabilized in MELB buffer with 0.025 % Digitonin (w/v) in the presence of complete protease inhibitor for 10 min on ice at the concentration of 1 x 10⁷ cells ml⁻¹. Heavy membrane fraction was then separated from cytosolic fraction by centrifugation of permeabilized sample at 13,000 g for 5 min at 4 °C.

### 2.2.15 PEG-MAL labeling of cysteine residues

PEG-MAL was prepared to 10 mM working stock fresh before experiment in MELB buffer. Targeted cysteine substrate (isolated BAK expression mitochondria fraction for example) was treated with 0.5 mM PEG-MAL
final for 30 min at room temperature before quenched with 20 mM NEM (1 M stock in ethanol).

2.2.16  Cytochrome c release assay

Mitochondria fraction was obtained as described above. The heavy membrane fraction was resuspended in MELB buffer with complete protease inhibitor at the concentration of 1 x 10^7 cells ml^{-1}. Recombinant full length, Caspase-8 cleaved tBID or HA-BID variants was added to the membrane fraction at the indicated dose to incubate for 30 min at 30 °C to activation BAK. After activation, samples were centrifuged at 13,000 g for 5 min at 4 °C to separate the supernatant (containing cytochrome c released) and the membrane fraction. Both fractions were then treated with SDS sample buffer with 5% 2-mercaptoethanol, heated at 100 °C for 5 min to prepare for SDS-PAGE.

2.2.17  Limited proteolysis of BAK by Proteinase K to assess conformational change

BAK conformational changes were assessed by limited proteolysis. Samples were incubated on ice for 10min prior to addition of Proteinase K (PK, 30 µg/ml final) and left on ice for 20 min. Proteolysis reaction was stopped by the addition of 1 mM PMSF, and samples re-suspended in reducing SDS-PAGE sample buffer. Western blotted was carried out using BAK antibody to the BH3 domain (4B5) to detect cleaved BAK.

2.2.18  Cysteine linkage by chemical cross-linking or disulphide bond formation

BAK cysteine tethering was induced by chemical cross-linking or disulfide bonding of cysteine residues (Dewson et al., 2008). Briefly, membrane
fractions were resuspended in either cross-linking buffer or MELB prior to incubation with tBID or HA-BID variants. For chemical crosslinking of cysteine residues, membrane fractions (untreated or treated with tBID) were incubated with the homobifunctional sulphydryl-reactive cross-linker 1,6-bis-maleimidoethane (BMOE, 8 Å linker, 0.5 mM final) for 30 min at room temperature before quenching by addition of reducing SDS-PAGE sample buffer, and samples immunoblotted for BAK (BAK 23-38). Alternatively, for disulfide-bond formation, samples were incubated with the redox catalyst copper (II) (1,10-phenanthroline)3 (CuPhe) at 1 mM final for 25 min on ice. 10 mM EDTA was added to chelate copper. Samples were analyzed by non-reducing SDS-PAGE.

### 2.2.19 Chemical cross-linking using photo-reactive cross-linker B4M

B4M in powder was purchased from Sigma-Aldrich was made into 10mM working stock in DMSO and stored at –20 °C. Isolated mitochondria or recombinant protein samples were labeled with 1 mM B4M in dark for 30 min at room temperature for cysteine occupation. 10 min high intensity UV light (250 nm) was introduced for photo-activation.

### 2.2.20 SDS-PAGE/Western blotting

Commercial pre-casted Tris-glycine gels (Bio-rad) (12 % or 4-20 %) were used in this project and electrophoresis was performed on the Bio-rad Mini-PROTEAN® tetra system. Protein size standard marker was also purchased from Bio-rad (see materials). Gels were run using tris-glycine based SDS-PAGE running buffer (see materials) at 200 V constant for desired amount of time (in the case of separating closely migrating bands, the dye front was run out). After running, gels were transferred onto either
nitrocellulose membranes (for cytochrome c detection) or PVDF membranes (pre-activated in methanol). Transfer was carried out in transfer buffer (see materials) at 30 V constant for 150 min. After transfer, membranes were blocked with 5 % skim milk (in TBST) for at least 30 min at room temperature. Membranes were then incubated with the desired primary antibody in TBST for 1-2 hr at room temperature after TBST pre-washing (see antibody list and dilution in materials). After primary antibody incubation, membranes were washed with 10 mL TBST for 5 min to reduce non-specific binding. Then, membranes were incubated with the appropriate secondary antibody in TBST for 1 hr. Membranes were washed twice with 10 mL TBST for 5 min, and once with 10 mL TBS. Protein bands were detected using the Millipore Luminata™ Forte western HRP substrate. Chemiluminescence was detected under a Bio-rad ChemiDocTM XRS+ Molecular Imager®. Raw files were imported into the Bio-rad Image LabTM software for analysis.

2.2.21 Blue-Native PAGE

Blue-Native (BN) PAGE was performed to assess BAK complexing with VDAC2 and BAK activation. Untreated or tBID-treated membrane fractions were centrifuged at 13,000 g for 5 min at 4 °C and solubilized in empty well buffer (EWB) containing 10mM DTT and 1% Digitonin on ice for 30 min to solubilize the membrane proteins. Samples were then centrifuged again at 13,000 g for 5 min at 4 °C, to remove pelleted insoluble debris. Supernatants were supplemented with BN-PAGE coomassie dye (see materials) and samples separated on 4-12 % Novex® Native PAGE gels using commercial BN-PAGE anode buffer and blue cathode buffer, at 150 V (8 mA per gel). The blue cathode buffer was replaced with anode buffer after about 30 min, when the dye front was about halfway through the gel, then continued gel running until dye front
reached the bottom. Gels were then transferred to PVDF membranes activated by methanol and in transfer buffer similar to standard Western blotting transfer with addition of 0.037 % SDS (see materials). After transfer, membrane was first stained with BN-PAGE coomassie stain for 5min, then de-stained in de-stain buffer (50 % methanol and 25 % acetic acid) and washed 3 times in 1XTBST buffer prior blocking (5 % skim milk in TBST) for 1 hr at room temperature. Immunoblotting was carried out with indicated antibody in the results as standard Western blotting.

2.2.22 Immunoprecipitation for detecting protein-protein interactions

All immunoprecipitation (IP) experiments were carried out using Onyx buffer (see materials) supplemented with complete protease inhibitor (CPI). Digitonin (1 %) solubilized membrane fractions or cytosolic fractions supplemented with 1% Digitonin were kept on ice throughout the experiment. Samples were normally at 250 µl volume, then first pre-cleaned with 50 µl pre-washed (3 times in Onyx+CPI and 1 time in 1% Digitonin +Onyx+CPI, 500 µl volume per wash, at 13,000 g for 1 min at 4 °C) 50% slurry of Sepharose beads (see materials) for at least 30 min at 4 °C in 1.5 mL Eppendorf tubes on a wheel roller. After pre-clean, samples were centrifuged at 13,000 g for 1 min at 4 °C to separate the beads from protein lysate. Supernatants were transferred to fresh 1.5 mL Eppendorf tubes, 50 µl of such lysates were taken as “Input” and mixed with 50 µl of 2 X SDS-PAGE sample buffer. The remaining lysate was incubated with 2 µg desired antibody for binding target protein of interest for at least 1 hr at 4 °C in 1.5 mL Eppendorf tubes on a wheel roller. Subsequently, 50 µl pre-washed (3 times in Onyx+CPI and 1 time in 1 % Digitonin +Onyx+CPI, 500 µl volume per wash, at 13,000 g for 1 min at 4 °C) 50 %
slurry of Protein G Sepharose beads (see materials) was added to
immunoprecipitate the protein: antibody complex for 1 hr at 4 °C in 1.5
mL Eppendorf tubes on a wheel roller. Then sample mixtures were
centrifuged at 13,000 g for 1 min at 4 °C to pellet the Protein G Sepharose
beads. Supernatants were removed and the beads were washed 3 times in
Onyx+CPI and 1 time in 1% Digitonin +Onyx+CPI, 500 µl volume per
wash, at 13,000 g for 1 min at 4 °C. After the last wash, pelleted Protein G
Sepharose beads were resuspended in 100 µl 2 X SDS-PAGE sample
buffer (no 2ME added in CuPhe tethering experiments), then subject to 100
°C heating for 5 min, allowing the elution of immunoprecipitated protein.
Finally, Protein G Sepharose beads were spun down at 13,000 g for 1 min
and the supernatant fraction containing immunoprecipitated protein was
transferred to fresh in 1.5 mL Eppendorf tubes and marked as “IP”.
Samples were then resolved on 12 % SDS-PAGE gel as described.
3 Chapter 3: VDAC2 in regulating BAK and BAX activity

3.1 Introduction

VDAC2 has been implicated as a critical regulator of BAK and BAX apoptotic function as discussed in Chapter 1. In a recent study, we undertook an unbiased genome-wide CRISPR/Cas9 library screen aiming to uncover novel regulators of apoptosis (bioRxiv DOI: 10.1101/266668). To identify genes contributing to BAX-dependent apoptosis, the screen was carried out on Mcl-1/Bak deficient MEFs to induce apoptosis with the BH3 mimetic ABT-737. As expected, sgRNA targeting Bax were enriched in surviving cells. In addition, multiple sgRNA targeting Vdac2 were also significantly enriched. This result, consistent with our previous study (Ma et al., 2014), provides strong genetic evidence that VDAC2 is important for BAX to mediate apoptosis. Our data has suggested that VDAC2, in addition to its role in BAX mitochondrial recruitment, is required for BAX-dependent killing.

The pro-apoptotic role of VDAC2 in BAX-dependent apoptosis presages its likely importance for the BAX close relative BAK. In line with past observations, BAK is indeed complexed with VDAC2 on MOM (Cheng, 2003; Lazarou et al., 2010; Roy et al., 2009). However, the physiological role of the BAK:VDAC2 interaction has been in debate where studies have proposed VDAC2 as an inhibitor of BAK by physical sequestration (Cheng, 2003; Lazarou et al., 2010). More recent studies indicate that VDAC2 holds a pro-apoptotic role in apoptosis by promoting BAK mitochondria targeting (Naghdi et al., 2015; Roy et al., 2009).
We and others have previously reported that both BAK and BAX associate with a VDAC2-containing, large molecular weight mitochondria membrane complex in healthy cells. However both BAK and BAX disassociate from the membrane complex following apoptotic activation (Lazarou et al., 2010; Ma et al., 2014; Roy et al., 2009). Therefore, understanding the molecular details of the BAK/BAX:VDAC2 interaction will provide a platform for designing small molecules to manipulate apoptosis. In this chapter, I will describe the biochemical and cellular experiments performed to characterize the BAK/BAX:VDAC2 interaction as well as the functional impact of altering such interaction on cell death.

3.2 Results

3.2.1 Mutation in the loop region between β10 and β11 of VDAC2 altered BAK mitochondria complex and cell death

During the course of my studies, Naghdi et al. characterized the VDAC2 motif required for BAK mitochondria recruitment (Naghdi et al., 2015). I first set out to confirm their finding that VDAC2 D170E was a minimal loss-of-function variant that impaired BAK MOM recruitment and complex formation. Indeed, wt mouse VDAC2, but not the D170E variant, rescued the high molecular weight BAK complex in Vdac2-deficient MEFs (Figure 3.2 A, lane 4 to 3 and 2).

Based on the published study (Naghdi et al., 2015) and the zfVDAC2 structure (Schredelseker et al., 2014), I reasoned residues that were near the
reported\textsuperscript{168} TFDS\textsuperscript{171} motif (red) in VDAC2 (Figure 3.1 B) might be in close proximity/interacting with BAK and possibly also BAX. To test this by cysteine guided cross-linking, a single cysteine was engineered at position A172 on VDAC2, or at S58C on the opposite of the VDAC2 protein as a negative control (Figure 3.1 B). BN-PAGE analysis revealed an interesting finding that both VDAC2 S58C and A172C, similar to wt VDAC2, could rescue the higher molecular weight complex with BAK. However, the VDAC2 A172C variant seemed to promote or stabilize the BAK complex, indicated by decreased monomeric BAK and increased complex associated BAK (Figure 3.1 A, anti-BAK, lane 6 to 3 and 5). No marked difference was observed for BAX distribution by probing the cytosolic fraction (Figure 3.1 A). Furthermore, the VDAC2 A172C: BAK complex was more resistant to disassociation induced by tBID (Figure 3.1 C). Similar to wt MEFs, ectopically re-expressed wt VDAC2 and the S85C variant could form the \(~480\text{kDa}\) complex with BAK and BAK completely disassociated and oligomerized after tBID activation. In addition, the VDAC2 A172C variant appeared to remain with BAK that the \(~480\text{kDa}\) complex was stable after even 100 nM tBID treatment (Figure 3.1 C).
Mutation in the loop region between β10 and β11 of VDAC2 altered BAK mitochondria complex and cell death

(A) BN-PAGE revealed VDAC2 A172C increased affinity for BAK but not BAX. Wild type or Vdac2−/− MEFs ectopically expressing mouse HA-tagged VDAC2 and its variants were harvested. Cytosol and membrane fractions from such MEFs were isolated for BN-PAGE analysis. Due to the lack of VDAC2 specific antibodies, the in-house polyclonal VDAC2 antibody recognized VDAC1 and 3, resulting the background band in Figure 3.1 A, lower panel of anti-VDAC2. Data representative of three independent experiments. (B) Schematic of VDAC2 variants mapped onto zebrafish VDAC2 (zfVDAC2) structure (PDB: 4BUM, green: zfVDAC2, blue: residues mutated to cysteine). 168TFDS171 motif in red. (C) Vdac2−/− MEFs ectopically expressing mouse HA-tagged VDAC2 and its variants were harvested. Membrane fractions from these MEFs were isolated to confirm the expression of the proteins by reducing SDS-PAGE (upper) and samples were also challenged with 100 nM tBID before BN-PAGE analysis (lower). Data representative of two independent experiments. (D) Vdac2−/− MEFs ectopically expressing mouse HA-tagged VDAC2 wt and A172C were harvested. Membrane fractions from such MEFs were isolated and challenged with increasing dose of tBID before reducing SDS-PAGE analysis. Data representative of two independent experiments. (E) Death assay of the VDAC2 A172C mutant. Cultured Vdac2−/− MEFs ectopically expressing mouse HA-tagged VDAC2 and its variants were treated with increasing dose of Actinomycin D (ActD) (0, 0.1, 1 µM) or Etoposide (Etop) (0, 1, 10 µM) for 24 hrs before FACS analysis of dead cells by PI exclusion. BAK, BAX and VDAC2 expression were confirmed by Western blot as indicated. Error bars represented standard error of the mean. Data representative of three independent experiments. Statistics performed using Prism software, unpaired t-tests comparing wt and A172C VDAC2 mutants treated with Etoposide or ActD.

To further interrogate the enhanced BAK:VDAC2 A172C interaction and its impact on BAK function, cytochrome c release assay on isolated mitochondria from these cells was performed in response to a titration of
tBID (Figure 3.1 D). Interestingly, the data suggested a delay in the kinetics of cytochrome c release from VDAC2 A172C expressing mitochondria compared to wt VDAC2 (Figure 3.1 D).

Next, a cell viability assay was performed to test the effect of VDAC2 A172C on cell death. Given that BAK-mediated cytochrome c release from isolated mitochondria was impaired, I hypothesized that the VDAC2 mutant would impair BAK-mediated (but not BAX-mediated) apoptosis. Vdac2−/− MEFs re-expressing VDAC2 variants were treated with increasing dose of Actinomycin D (ActD) or Etoposide (Etop) for 24 hours (Figure 3.1 E). ActD has been shown to inhibit transcription by physically binding to DNA at the transcription initiation complex and thus preventing the elongation of RNA chain (Sobell, 1985). Proteins with short half-life such as MCL-1 are then sensitive to proteasome degradation and therefore induces predominantly BAK-dependent killing (X. Du, Youle, FitzGerald, & Pastan, 2010). Etoposide induces cell death by causing breaks in DNA strands and p53-induced apoptosis with no preference for BAK or BAX (Jamil, Lam, Majd, Tsai, & Duronio, 2015; Pommier, Leo, Zhang, & Marchand, 2010), therefore served as a control in the test.

Consistent with a previous study (Baines et al., 2007), Vdac2−/− MEFs could die efficiently in response to both apoptotic stimuli (Figure 3.1 E). The VDAC2 A172C variant expressing MEFs, however, demonstrated significantly reduced cell death compared to wt VDAC2 when treated with ActD at both 0.1 µM and 1 µM concentrations, but not when treated with Etop (Figure 3.1 E), suggesting that the stabilized BAK:VDAC2 interaction inhibited BAK activity.
3.2.2 Investigating the BAK transmembrane tail interaction with VDAC2

The C-terminal transmembrane anchor of both BAK and BAX have been implicated in their interaction with VDAC2 (Lazarou et al., 2010; Ma et al., 2014). As the two critical VDAC2 residues T168 and D170 reported for BAK membrane recruitment are exposed to the cytosol, other membrane buried residues of β-strands 7-10 could contribute to interaction with BAK transmembrane anchor (Figure 1.17 B). Therefore, I was interested to test whether the BAK C-terminal transmembrane anchor specifically interacted with residues on VDAC2 β-strands 7-10, the sufficient segment reported for gain-of-function of VDAC2 (Naghdi et al., 2015).

The BAK C-terminal transmembrane anchor could interact with the VDAC2 β-strand residues that orientated towards the membrane lipid. Alternatively, given the previously mentioned VDAC2 residues T168 and D170 both had their sidechains pointing toward the lumen of the VDAC2 channel, it was possible that the BAK transmembrane tail could insert into VDAC2 lumen (Figure 3.2 A). To test these two models, two residues on VDAC2 were chosen for cysteine mutagenesis adjacent to the reported “TFDS” motif (Naghdi et al., 2015): VDAC2 Q166 and M167, with opposing direction (Q166 facing the lumen and M167 towards MOM lipid)(Figure 3.1 E). Proximity dependent cross-linking was then employed to resolve these different models. A collaborator, Dr Sweta Iyer, had previously generated and characterized a panel of BAK variants with single cysteines introduced along the BAK TM domain (Iyer et al., 2015). I selected three BAK mutants that retained their apoptotic function (Iyer et al., 2015) but were on different sides of the C-terminal transmembrane helix to test in my cross-linking experiments (Figure 3.2 B).
Figure 3.2 BAK transmembrane domain variants display distinct BAK:VDAC2 complex distribution

(A) Sequence representation of BAK transmembrane domain. BAK transmembrane domain (TMD) located on the BAK helix 9 with its amino acid sequence detailed in grey. 3 BAK variants I188C, N190C and G196C was labeled in red. (B) In silico model of BAK transmembrane domain (left) using Pymol software with relative positions of hBAK I188C, N190C and G196C (red). BN-PAGE analysis of hBAK I188C, N190C and G196C membrane complex in the presence or absence of tBID (20 nM) (right). Data representative of two independent experiments.

To test VDAC2: BAK TMD cross-linking, the BAK TMD single cysteine variants were co-expressed with either Q166C or M167C on an otherwise
Cys null VDAC2 construct in Bak/Bax/Vdac2 triple knock out (TKO) MEFs to allow linkage mapping specifically of the mutant variants (Figure 3.3 A). Oxidizing reagent CuPhe ((copper (II)(1,10-phenanthroline)3))-driven cysteine cross-linking was carried out on such variants and samples were assessed on non-reducing SDS-PAGE (Figure 3.3 B). A cross-linked BAK:VDAC2 is expected to migrate at 55 kDa on SDS-PAGE. Interestingly, both BAK I188C and N190C variant had a band migrating at ~50kDa in the presence of CuPhe, irrespective of the mutation in VDAC2, suggesting linkage to proteins other than VDAC2. Consistent with this, no cross-linked band was detected in the anti-VDAC2 immunoblots corresponding to the expected molecular weight of BAK:HA-VDAC2 complex (~60kDa, with HA-tag on VDAC2). The cross-linked band was not detected in controls that were devoid of BAK, suggesting this might be a BAK:BAK dimer. This would suggest the chosen cysteine position on BAK and/or VDAC2 might not be in proximity for cross-linking or that the BAK TMD is not directly interfacing with VDAC2.
3.2.3 PEG-MAL label of BAK surface groove specifically disrupt the BAK:VDAC2 interaction and causes BAK membrane disassociation.

As VDAC2 is important for both BAK and BAX mitochondria targeting (Ma et al., 2014; Naghdi et al., 2015), understanding the molecular interaction between VDAC2 with BAK and BAX could help us identify new therapeutic targets for modulating apoptosis. In Chapter 5, I used the PEG-MAL (polyethylene glycol maleimide) labeling approach to map interfaces that are important for BAK activity. The obstructive molecule PEG-MAL is expected to interrupt protein:protein interactions due to its large size ~5kDa. During the mapping, I observed that labeling certain BAK residues resulted in BAK disassociation (estimated ≥50% PEG-labeled BAK on SDS-PAGE) from mitochondria (Figure 3.4 B, supernatant fraction). The majority of BAK variants did not significantly disassociate from mitochondria following labeling (Figure 5.12 and Appendices Figure 9.1 A), supporting that this was specific to the labeled
position and not a non-specific effect of PEG-MAL on the membrane. Disassociated residues were confined largely to a small region of BAK surface groove (BAK α2, 3 and 4) (Figure 3.4 A, B and Appendices Figure 9, 1 A).

Based on this observation and the known involvement of VDAC2 in BAK membrane targeting, I hypothesized that PEG-MAL labeling of BAK disrupted the BAK:VDAC2 interaction. To test this hypothesis, the most strongly disassociated BAK residues such as R88 (α3) and N124 (α4-5) alongside controls from BAK N-terminus (G4) and α6 (R156) that did not significantly dissociate were tested for their ability to co-immunoprecipitate with VDAC2. To preclude labeling of cysteines in VDAC2, the BAK ΔCys, G4C, R88C, N124C and R156C mutants were co-expressed with VDAC2 ΔCys in Bak/Bax/Vdac2 knock out MEFs. SDS-PAGE revealed comparable PEG-MAL labeling efficiency across all BAK variants on membrane indicated with a ~5kDa size shift and reconfirmed that PEG-MAL labeling on R88C and N124C, but not G4C or R156C disassociated BAK from MOM (Figure 3.4 B). To test if the disassociation was due to impaired BAK:VDAC2 interaction, membrane fractions of such samples were then taken for immunoprecipitation (IP) with an antibody against the N-terminal HA-tag on VDAC2. PEG-MAL treatment did not interfere with HA IP and so HA-VDAC2 was efficiently immunoprecipitated (Figure 3.4 C). When the IP fraction was probed with antibody against BAK, both the unlabeled and PEG-MAL labeled BAK could be detected in controls (Figure 3.4 C, G4C and R156C), indicating that labeling of these positions did not impair BAK:VDAC2 interaction. In contrast, PEG-MAL labeled BAK R88C and N124C did not co-immunoprecipitate with VDAC2 (Figure 3.4 B and Appendices Figure 9, 1 B), supporting the hypothesis that PEG-MAL label on residues of the
BAK groove region specifically disrupted BAK:VDAC2 interaction (Figure 3.4 A).
Figure 3.4 BAK canonical groove mediated interaction with VDAC2

(A) Schematic representation of BAK residues disassociated upon PEG-MAL labeling. Inactive BAK (PDB: 2IMT, BAK surface hydrophobic groove α3, 4 and 5 in green and the rest helices in grey) and residues that ≥50% PEG-MAL labeled BAK disassociated from membrane (purple, V52, I80, R88, Y89 and N124) (B) Example of PEG-MAL induced BAK membrane disassociation. Mitochondria fractions of BAK single cysteine variants indicated were isolated from MEFs and incubated with 0.5 mM PEG-MAL in MELB buffer with protease inhibitor for 30 min. After quenching, samples underwent centrifugation to separate the membrane and supernatant fraction for reducing SDS-PAGE analysis. Data representative of at least two independent experiments. (C) Co-immunoprecipitation of BAK:VDAC2 after PEG-MAL treated mitochondria membrane samples. PEG-MAL treatment was applied to BAK ΔCys, G4C, R88C and R156C co-expressed with VDAC2 ΔCys in Bak/Bax/Vdac2 knock out MEFs. 1% w/v digitonin solubilized post-spin membrane samples (left panel, input) were immunoprecipitated with anti-HA antibody to IP VDAC2 (right panel, IP). Samples were analyzed by reducing SDS-PAGE. Data representative of two independent experiments.
3.2.4 Disulfide cross-linking between BAK and VDAC2

Together, the data suggested that mutation of the VDAC2 loop region encompassing A172C increased BAK membrane complex association (Figure 3.1 B) and PEG-MAL labeling on BAK groove residues R88 and N124 specifically disrupted the BAK:VDAC2 interaction (Figure 3.4 C). Hence, I aimed to test whether the VDAC2 open loop region might be interacting with BAK groove region by targeted disulfide-linkage.

As proof-of-principle, Bak/Bax/Vdac2 TKO MEFs were engineered to co-express BAKΔCys, G4C, R88C, N124C or R156C with VDAC2 A172C or S58C (negative control across the lumen of VDAC2, Figure 3.1 E). Mitochondria fractions from these cells were treated with CuPhe for cysteine cross-linking. Although BAK G4C and R156C cross-linked to other membrane proteins (almost all BAK G4C migrated at ~50 and ~40kDa after CuPhe), none of the cross-linked species co-migrated with a VDAC2 immunoreactive band (Figure 3.5 A, left and right top panel). In contrast, BAK R88C and N124C both efficiently cross-linked to a protein and migrated in between 50-70kDa (Figure 3.5 A, right top panel). This BAK immunoreactive species co-migrated with a VDAC2 immunoreactive species detectable only with VDAC2 A172C, but not S58C. (Figure 3.5 A, right lower panel), supporting specific cross-linking between R88C and N124C BAK to VDAC2 A172C.
Figure 3.5 Cysteine cross-linking supports an interface between BAK groove and VDAC2 loop region

(A) CuPhe induced cysteine cross-linking between BAK groove residue R88 and N124 to VDAC2 open loop A172. Mitochondria fraction were isolated from Bak/Bax/Vdac2 TKO MEFs expressing BAKΔCys, G4C, R88C, N124C or R156C with VDAC2 A172C or S58C. Cysteine cross-linking was introduced by 1mM CuPhe incubation then samples were analysed by non-reducing SDS-PAGE. Data were representative of two independent experiments. (B)
Structural modeling of BAK:VDAC2 interaction. BAK (PDB: 2IMT, α3, 4 and 5 in green, α1 and 6 in orange and the rest helices in grey) with VADC2 interaction modeled onto zebra fish VDAC2 structure (PDB: 4BUM, green: zfVDAC2), viewing from side and top.
3.2.5 A conserved interface between VDAC2 and BAK and BAX

Given the functional redundancy, structural homology and the ability of BAK and BAX to associate with VDAC2 at mitochondria, I hypothesised that the region of VDAC2 interacting with BAK and BAX would be conserved. In MEFs lacking BAK (and MCL-1), endogenous BAX could still form the ~440kDa complex (lane 1, Figure 3.6 A). In addition, genetic deletion of Vdac2 in those cells abolished the complex (Figure 3.6 A, lane2). Ectopically expressed, FLAG-tagged VDAC2 but not FLAG-tagged VDAC1 rescued the BAX complex, suggesting that VDAC2 is a critical component in the BAX complex. To isolate the minimal VDAC2 interface sufficient for interacting with BAX, a collaborator, Dr Mark van Delft, constructed chimeric proteins using VDAC1 as a template carrying different VDAC2 segments (encompassing β sheets 6-10), one of which was the VDAC1/2a (Figure 3.6 B) (bioRxiv DOI: 10.1101/266668). Chimeric proteins were re-expressed at comparable levels to that of wt VDAC1 and VDAC2. BN-PAGE analysis revealed that VDAC1/2a (VDAC2 aa 90-178) was sufficient in rescuing the BAX complex (Figure 3.6 C). Hence, β-strands 5-12 of VDAC2 were important for BAX interaction (Figure 3.6 D). Further functional mapping by Dr. Mark van Delft refined this interaction site to β-strands 6-10 (bioRxiv DOI: 10.1101/266668), which broadly overlapped with the reported VDAC2 region (VDAC2 aa 123-179) important for its interaction with BAK(Naghdi et al., 2015). Together, the evidence supports a conserved docking site on VDAC2 for both BAK and BAX recruitment to mitochondria. Further studies are required to refine the VDAC2: BAX interface.
Figure 3.6 A region of VDAC2 important for BAX membrane complex formation

(A) BN-PAGE revealed VDAC2 was responsible for BAX membrane complex. *Mcl-1/Bak* and *Mcl-1/Bak/Vdac2* knock out MEFs ectopically expressing mouse FLAG-tagged VDAC1 or FLAG-tagged VDAC2 were harvested and membrane fractions from these MEFs were isolated for BN-PAGE analysis. Data representative of three independent experiments. (B) Schematic of VDAC 1/2 chimera (upper, green: VDAC2, blue: VDAC1) and protein expression check. A segment of VDAC2 spanning beta sheet 5 to 12 was engineered onto VDAC1 and the chimeric VDAC1/2a expressed comparable to VDAC1 and VDAC2 in *Mcl-1/Bak/Vdac2* knock
out MEFs. Data representative of two independent experiments. (C) VDAC1/2a was sufficient to rescue the BAX membrane complex revealed by BN-PAGE analysis as described previously. (D) Schematic of VDAC1/2a mapped onto zebra fish VDAC2 structure (PDB: 4BUM, green: zfVDAC2, purple beta sheet 5-12).

3.3 Discussion

Mounting evidence implicates an important role for VDAC2 in apoptosis regulation (Maurya & Mahalakshmi, 2017; Naghdi & Hajnóczky, 2016). I propose that VDAC2 constitute a mitochondrial complex that regulates the BAK and BAX membrane recruitment hence the interaction between VDAC2 and BAK and BAX would impact the outcome of apoptosis by modulating the sensitivity of mitochondria to pro-apoptotic permeabilization.

Consistent with a published study reporting a similar VDAC2 region important for BAK function (Naghdi et al., 2015), my data revealed a novel VDAC2 A172C variant with stabilized BAK membrane complex association. Importantly, this stabilized BAK:VDAC2 interaction desensitized mitochondria permeabilization and reduced BAK-dependent cell death, providing the first genetic evidence of stabilized BAK:VDAC2 interaction leading to impaired BAK activity (Figure 3.1). This result was consistent with an earlier proposal that VDAC2 acted as a BAK apoptotic inhibitor (Cheng, 2003) and indicates that stabilizing the BAK:VDAC2 interaction might be an avenue to limit pathological cell death.

Understanding the consequence of targeting BAK:VDAC2 interaction has been a major gap in the field. My studies provide genetic evidence that
stabilizing the interaction can limit apoptosis, providing a rationale for stabilising the interface with small molecule to limit pathological apoptosis for example in ischemia-reperfusion injuries and degenerative diseases.

To target the BAK:VDAC2 interaction, first we need to resolve the molecular details. My attempts to cross-link the BAK transmembrane domain to VDAC2 were unfortunately unsuccessful. The result either indicate that the BAK TMD did not interface with VDAC2 or the chosen residues were not optimal for such cross-linking.

The discovery that PEG-MAL labeling of certain BAK residues disrupted its membrane localization led to the identification of a BAK interface, largely involving its surface groove (α3, α4-5), important for its interaction with VDAC2 (Figure 3.4). This hypothesis was further supported by cross-linking that indicated such BAK groove residue R88 and N124 were indeed in close proximity with VDAC2 A172. These biochemical data are consistent with an earlier mutagenesis study where cells expressing BAK mutated within its BH3 or BH1 domain had significantly reduced BAK:VDAC2 association (Cheng, 2003). This BAK:VDAC2 interface may explain how BH3-only protein binding to the BAK hydrophobic groove dissociate the BAK:VDAC2 complex as interactions with the BH3-only protein induce conformation change to BAK surface groove and destabilize BAK:VDAC2 interaction. Hence, I propose a refined model of the BAK:VDAC2 interface involving one end of BAK surface groove (Figure 3.5 B) with VDAC2 cytoplasmic facing loop (between β 10-11), uniting the BAK and VDAC2 interaction from both sides. With the knowledge of BAK and VDAC2 variants identified in my study, future experiments will be focused on trialing unbiased chemical cross-linking methods then combined with mass spectrometry and
conventional structural biology approaches (VDAC2 A172C will be a valuable tool for crystallization of BAK:VDAC2 complex) to uncover the full detail of the BAK:VDAC2 interaction.
Chapter 4: Dissecting the transient interaction between BH3-only protein tBID with BAK

4.1 Introduction

The “activator” BH3-only protein such as tBID can activate BAK and BAX on the mitochondria outer membrane. However, such interactions have been difficult to directly capture. Instead of forming a stable protein complex, tBID is thought to only transiently interact with BAK and BAX, in a “hit and run” model (Figure 4.2 A), before becoming disassociated from activated BAK and BAX.

As discussed above, a number of studies have provided snap shots of the conformation changes in BAK and BAX after BH3-only protein activation, yet little is known regarding the stepwise order of such changes. Moreover, it remains unclear how the BH3-only protein triggers activation and disassociates during such BAK/BAX conformation changes. Studying these transient interactions by conventional structural biology approaches remains challenging, especially in the presence of a requisite membrane. The majority of previous structural studies introduced in Chapter 1 were carried out in solution with recombinant, sometimes truncated, proteins. These results should be interpreted with caution as BCL-2 family proteins such as tBID require a membrane environment for its activity (Shamas-Din et al., 2013a).

In this chapter, I aimed to biochemically dissect the transient interaction between BH3-only proteins and BAK at mitochondria. Given the difficulty in producing BH3-only proteins recombinantly, other than BID, full-length BID was adopted throughout our study as an excellent BAK activator.
Recombinant BID was then subjected to recombinant caspase-8 cleavage \textit{in vitro} as described in Chapter 2: Materials and Methods to generate biologically active tBID, which was then added to activate BAK on isolated mitochondria.

4.2 Results

4.2.1 Tethering endogenous BAK cysteines inhibited BAK conformation change and apoptotic function

From previous work in the lab, BAK conformation change on mitochondria induced by tBID was readily observed by measuring endogenous BAK cysteine proximity. In this experiment, wt hBAK was ectopically expressed in \textit{Bak}^{+/+}Bax^{+/+} mouse embryonic fibroblasts (MEFs). The two endogenous cysteines in BAK (C14 and C166) (Figure 4.1 A) can form efficient intramolecular disulfide-link induced by exogenous oxidant CuPhe (copper phenanthroline) prior to tBID activation and therefore resulted in a fast migrating band of BAK detected (inactive BAK) below the 25 kDa marker on the non-reducing SDS-PAGE gel (Figure 4.1 B, lane 1). This suggested that the flexible N-segment (black dash line), which was lacking in the structure of inactive BAK, was positioned in close proximity to the cysteines in the BAK \(\alpha6-7\) loop on mitochondria (Figure 4.1 A). An interesting observation was the significant reduction of intramolecular disulphide-linked C14-C166 after BAK activation, supporting the separation of the two endogenous cysteines after BAK activation conformation change (Figure 4.1 B, lane 2). This was consistent with BAK \(\alpha1\) disassociation during BAK activation(Alsop et al., 2015; Griffiths et al., 1999).
Figure 4.1 BAK endogenous cysteines became distant following activation

(A) Inactive human BAK structure (PDB: 2IMT. BAK α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of the helices in grey). The flexible BAK N-segment is represented by the dash line in which the C14 could be in close proximity to C166 on BAK α6. 

(B) Membrane fractions from Bak−/−Bax−/− MEFs expressing human BAK were either treated with CuPhe alone or first activated with tBID before CuPhe treatment. Samples were run on non-reducing SDS-PAGE. Data representative of three independent experiments.
To understand the functional relevance of the separation of the two endogenous cysteines to BAK apoptotic function, I tested whether tethering the two endogenous cysteines in wt BAK using a small chemical cross-linker BMOE (bismaleimidoethane), impaired cytochrome c release in response to tBID activation compared to untreated wt BAK as well as to the single cysteine controls (BAK C14S and C166S) (Figure 4.2 B). This indicated that the separation of the N-segment from the BAK α6-7 loop was a necessary step required for BAK function. We then performed immunoprecipitation with the BAK conformation specific antibody NT (BAK N-terminal specific, Millipore) and 4B5 (BAK BH3 motif specific, WEHI in-house) in order to understand how tethering endogenous cysteines impaired BAK function (Figure 4.2 C). Consistent with known BAK conformation change N-terminal exposure and BH3 motif exposure during its activation, increased immunoprecipitation of BAK was observed in untethered wt BAK, and single cysteine controls BAK C14S and C166S after tBID treatment (Figure 4.2 C, lane 2, 6 and 10). However, this increase was inhibited in wt BAK with endogenous cysteines when tethered by BMOE. This would suggest that this separation of N-segment from the BAK α6-7 loop was required for BAK N-terminal exposure and BH3 motif exposure (Figure 4.2 A).
Figure 4.2 Tethering endogenous BAK cysteines inhibited BAK conformation change

(A) The “hit and run” model of BAK activation. Left, tBID transiently hit BAK resulting its activation. Activated BAK disassociated from tBID to continue dimerization. Right, cysteine tether of endogenous BAK cysteine prevented BAK activation. (B) Membrane fractions from Bak$^{-/-}$Bax$^{-/-}$ MEFs expressing human BAK variants were either directly challenged with tBID alone or first treated with cysteine cross-linker BMOE before tBID activation. Samples were run on reducing SDS-PAGE. Data representative of three independent experiments. (C) Membrane fractions from Bak$^{-/-}$Bax$^{-/-}$ MEFs expressing human BAK variants were either directly challenged with tBID alone or first treated with cysteine cross-linker BMOE before tBID activation. Samples were then solubilized and immunoprecipitated with BAK conformation specific antibodies (BAK NT aa 23-38 and BAK 4B5 BH3 motif specific) before subjected to reducing SDS-PAGE. Data representative of two independent experiments.
4.2.2 Tethering BAK conformation change stabilizes the tBID:BAK interaction at mitochondria

The “hit and run” model proposed that the induced BAK activation conformation change destabilized the tBID:BAK interaction. Alternatively, given that the BH3-only protein binds to the BAK groove and this groove is also involved in homo-dimerization, the BH3-only proteins could be displaced by the BAK BH3 motif from another activated BAK molecule. As tethering the endogenous cysteines prevented wt BAK activation and apoptotic function, I hypothesize that constraining BAK conformation change would stabilize tBID:BAK interaction at mitochondria (Figure 4.3 A). Dr. Iris Tan tethered the endogenous cysteines in wt BAK with oxidizing agent CuPhe to form an intramolecular disulfide bond at MOM (Figure 4.3 B). tBID was then added to the sample, followed by immunoprecipitation of BAK using an antibody (7D10) that recognize both inactive and activated BAK(Iyer et al., 2016). The BID P15 fragment (tBID) immunoprecipitated with tethered wt BAK (Figure 4.3 B, lane 4), but not with untethered wt BAK nor the single cysteine controls (C14S and C166S) (Figure 4.3 B, lane 4), supporting the idea that tethering BAK conformation change was able to stabilize tBID:BAK interaction at mitochondria.
**Figure 4.3 Tethering endogenous BAK cysteines stabilized tBID:BAK interaction**

(A) The schematic of converting “hit and run” model of BAK activation to stabilized tBID:BAK interaction. **(B)** Membrane fractions from $Bak^{−/−} Bax^{−/−}$ MEFs expressing human BAK variants were either directly challenged with tBID alone or first treated with CuPhe before tBID activation. Samples were immunoprecipitated with 7D10 antibody to capture all BAK present and subsequently run on non-reducing SDS-PAGE. Data representative of two independent experiments.
To define the steps at which tBID disassociated from BAK during BAK conformation change, we took advantage of additional double-cysteine BAK mutants that when tethered could prevent specific BAK conformation changes: BAK Y41C (α1) to A79C (α2), A28C (α1/2loop) to L163C (α6) and V142C (α5) to F150C (α6) (Figure 4.3 D) (Alsop et al., 2015). Cysteine linkage BAK Y41C:A79C prevented BH3 motif exposure, A28C:L163C tethered BAK α1 disassociation and V142C:F150C stopped BAK core-latch separation (Alsop et al., 2015). All three double-cysteine mutants when untethered still exhibited similar apoptotic activity to wt BAK (Alsop et al., 2015). However, these mutants failed to permeabilize mitochondria in response to BH3-only protein activation after induction of intramolecular disulfide tether (Figure 4.4 D, lower) (Alsop et al., 2015).

To further characterize these BAK mutants, I confirmed that the induced intramolecular disulfide bond did not alter BAK conformation by performing limited proteolysis. The Proteinase K cleavage demonstrated a similar cleavage pattern in the absence or presence of CuPhe (Figure 4.3 A). Blue Native PAGE (BN-PAGE) analysis also supported that addition of CuPhe did not change membrane BAK oligomerization status (Figure 4.3 B). Next, to determine if these cysteine tethers stabilized the tBID:BAK interaction, similar tBID:BAK co-immunoprecipitation was carried out on isolated Bak−/− Bax−/− MEF mitochondria expressing only BAK Y41C:A79C or A28C:L163C or V142C:F150C (Figure 4.3 C). tBID co-immunoprecipitated with CuPhe tethered BAK Y41C:A79C and A28C:L163C but not with BAK V142C:F150C. The results indicate that tBID remains associated with BAK post α1 disassociation (A28C:L163C) as well as α2 (BH3) exposure (Y41C:A79C), given cysteine tethering both conformation changes stabilized tBID:BAK interaction. However, that no tBID was detected in CuPhe treated BAK V142C:F150C indicates that
preventing BAK core-latch separation could not stabilize the tBID interaction, placing tBID disassociation downstream of core-latch domain separation.
Figure 4.4 Tethering BAK double cysteines variants inhibited BAK activation and stabilized interaction with tBID

(A) Membrane fractions from Bak\textsuperscript{−/−} Bax\textsuperscript{−/−} MEFs expressing human BAK double cysteine variants were either directly treated with Proteinase K alone or first treated with CuPhe to induce disulfide bond formation before Proteinase K cleavage. Samples were run on reducing SDS-PAGE gel. Data representative of two independent experiments. (B) Membrane fractions from Bak\textsuperscript{−/−} Bax\textsuperscript{−/−} MEFs expressing human BAK double cysteine variants were treated with/without CuPhe to induce disulfide bond formation before running on BN-PAGE. Data representative of two independent experiments. (C) Membrane fractions from Bak\textsuperscript{−/−} Bax\textsuperscript{−/−} MEFs expressing human BAK double cysteine variants were treated with/without CuPhe to induce disulfide bond formation before running on BN-PAGE. Data representative of two independent experiments.
solubilization and immunoprecipitation with 7D10 BAK antibody that capture all forms. Samples were run on non-reducing SDS-PAGE. Data representative of three independent experiments. (D) (Upper) Schematic representation of intramolecular disulfide bond formation between BAK Y41C (α1) to A79C (α2), A28C (α1/2loop) to L163C (α6) and V142C (α5) to F150C (α6) (PDB: 2IMT. BAK α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of protein in grey). (Lower) Membrane fractions from Bak−/−Bax−/− MEFs expressing human BAK double cysteine variants were treated with/without CuPhe to induce disulfide bond formation before activated with tBID. Cytochrome c release was detected on reducing SDS-PAGE. Data representative of two independent experiments.

4.3 Discussion

BH3-only proteins are the key initiators of apoptosis. Detailing the interactions of how BH3-only proteins activate BAK and BAX has been a major challenge in the cell death field. A number of limitations such as producing full-length recombinant BH3-only proteins and BAK and the required mitochondria membrane environment, on top of their proposed transient nature of their interaction, have increased the difficulty of characterizing their action with conventional structural approaches. Hence, BH3-only proteins induced BAK and BAX activation at mitochondria has been in a black box with only a few snapshots of their interactions available.

Understanding the interaction between BH3-only proteins with BAK and BAX is critical for the identification of new drug targets to manipulate apoptosis. In this chapter, I attempted to investigate the interactions between full-length tBID:BAK at the MOM. Consistent with the published
literature, BAK conformation change was required for its apoptotic function (Figure 4.2 B). This led me to the hypothesis that limiting BAK structural rearrangement could stabilize tBID:BAK interaction at membrane. The co-immunoprecipitation of tBID and wt BAK after tethering BAK endogenous cysteines supported this hypothesis. Next, by repurposing the previously published BAK double cysteine variants (Alsop et al., 2015), I was able to define the step at which tBID disassociates from BAK. With the cysteine tether and co-IP approach, tBID remained bound to BAK before BAK core-latch separation, placing the core-latch separation downstream of BAK α1 disassociation and/or α2 (BH3) exposure. Hence, I propose a refined schematic of BAK structural rearrangement during its activation (Figure 4.5). My data revealed that tBID disassociates from BAK after the core-latch separation, an event known to precede BAK homodimerization (Brouwer et al., 2014; Czabotar et al., 2013). This also suggests that tBID disassociation was likely due to BAK activation conformation change rather than displacement by the BH3 motif of another activated BAK.
Figure 4.5 Schematic summary of refined stepwise conformation change during tBID-induced BAK activation

tBID directly interacts and activates BAK on MOM. Binding of the tBID BH3 motif to BAK surface hydrophobic groove induces BAK conformation change where BAK BH3 motif becomes partially exposed. tBID disassociates from BAK after the full exposure of BAK BH3 motif and the disassociation of BAK α1 helix. The disassociation of tBID is followed by the BAK core-latch domain separation that permits subsequent BAK homodimerization.
5 Chapter 5: Mapping the tBID:BAK interface

5.1 Introduction

Understanding the molecular details of the interactions between BH3-only proteins and BAK/BAX will help us decipher how BH3-only proteins activate BAK/BAX whilst also devising ways to interfere with it. The canonical hydrophobic surface groove is now an established binding site on BAK/BAX for BH3-only protein interaction (Brouwer et al., 2014; Czabotar et al., 2013; Moldoveanu et al., 2013). Whether other interaction sites exist for BH3-only protein-induced BAK and BAX activation remains in debate.

As mentioned in Chapter 1, BAX was reported to have an additional “rear site” that interacts with BH3-only proteins, involving its α1 and α6 helices (Gavathiotis et al., 2008; 2010). Via a NMR approach, a stabilized α helical peptide of the BIM BH3 motif was observed to directly interact with the BAX “rear site”, distinct from its canonical hydrophobic groove which leads to BAX activation (Gavathiotis et al., 2008). The BH3 peptide interaction with the BAX rear site induced structural rearrangement of the BAX surface groove to mobilize its constrained BAX C-terminal tail for mitochondrial targeting (Gavathiotis et al., 2010). Such an observation is consistent with a previous yeast-two-hybrid study that suggested the BAX α1 helix was able to interact with the BH3 motif of PUMA (Gallenne et al., 2009). Additionally, a novel inhibition site on BAX was reported that a stabilized peptide of the BH4 motif of BCL-2 directly interacted with BAX residues from α1, α1-2 loop, α2-3 and α5-6 (Barclay et al., 2015). This interaction was able to block BH3-only protein-induced BAX activation (Barclay et al., 2015). These observations suggest the existence
of previously unrecognized BH3-only protein interaction sites on BAX in addition to the BAX surface hydrophobic groove that impact on its apoptotic activity.

However, similar observations have not been reported for BAK. This has led to the possibility that the non-canonical groove interactions are only required for cytosolic BAX membrane translocation, a prerequisite for BAX apoptotic activity. Additionally, in the course of my studies, Leshchiner et al. published that BAK, in contrast to BAX, does not possess a rear activation site (Leshchiner, Braun, Bird, & Walensky, 2013). However, their study was based on a recombinant soluble BAX-like BAK mutant. This soluble BAK mutant auto-translocates to mouse liver mitochondria, albeit its BAX-like α9 that is engineered to have reduced hydrophobicity (Leshchiner et al., 2013).

Recent studies put forward a different opinion that suggests both BAK and BAX are similarly targeting towards MOM and are both actively retro-translocated back into cytosol, thus questioning the distinct mode of actions between BAK and BAX (Edlich et al., 2011; Todt et al., 2015). Given the structural and functional similarity between BAK and BAX, I hypothesize that, similar to BAX, there are additional site(s) between BAK and BH3-only proteins that contribute to BAK apoptotic activation.
5.2 Results

5.2.1 Mapping the transient interaction of tBID with BAK at mitochondria using an unbiased photo-reactive chemical cross-linker

The initial attempt to map the tBID:BAK interactions at mitochondria used a powerful and unbiased chemical cross-linking combined with mass spectrometry analysis. This approach, as illustrated in the workflow (Figure 5.1), would potentially enable the identification of all interfaces present between a BH3-only protein tBID and BAK at MOM in an unbiased fashion. For this purpose, a zero-length, bifunctional, photo-reactive, benzophenone-4-maleimide (B4M) cross-linker was initially tested to cross-link full-length tBID to wt BAK at mitochondria. The bifunctional B4M possesses a maleimide moiety that can be efficiently conjugated to a cysteine residue (DeLeon-Rangel, Ishmukhametov, Jiang, Fillingame, & Vik, 2013; Tao, Lamkin, & Scheiner, 1985). The other arm of B4M is photo-reactive and can be activated by high intensity UV light (250 nm) allowing unbiased cross-linking of proximal molecules. Cross-linked products can then be isolated for protease digest and identified by mass spectrometry (Leitner, Faini, Stengel, & Aebersold, 2016; Rappsilber, 2011). In wt BID, there are no cysteine residues in the P15 fragment. Hence, to identify cross-linking partners to the tBID BH3 motif, tBID R84C variant was engineered for B4M conjugation (Figure 5.2 B). R84C locates adjacent to the newly discovered h$_0$ position of the tBID BH3 motif that is critical for mediating tBID BH3:BAX surface hydrophobic groove interaction as introduced in Chapter 1 (Czabotar et al., 2013). The R84C variant was tested for its ability to induce cytochrome c release and was found to be functionally comparable to wt tBID (Figure 5.2 A) and was
used for subsequent cross-linking studies. Another tBID variant tBID R84C/G94E was chosen as a negative control in the study. The G94E mutation had been previously reported to significantly impair cell death, presumably due to the glutamic acid disrupting the binding to the BAK/BAX canonical surface hydrophobic groove (Wei et al., 2000). The tBID R84C should, in theory, cross-link at least to the BAK surface hydrophobic groove and map the interactions occurring between full-length proteins at MOM.
B4M is conjugated onto single cysteine variant of full-length recombinant tBID via its thiol (SH)-reactive maleimide group. tBID-B4M will then be added to isolated mitochondria from Bak^−/− Bax^−/− MEFs re-expressing wt human BAK. High-intensity UV irradiation will be introduced during BAK activation to photo cross-link tBID. Cross-linked tBID:BAK products are then isolated and subjected to mass spectrometry analysis for residue identification.

B4M has a molecular weight (MW) of less than 300 Da, hence making it difficult to directly assess its coupling efficiency on conventional SDS-PAGE. The B4M to tBID R84C conjugation was tested indirectly via a cysteine competition assay where B4M treated full-length recombinant tBID R84C in solution prevented another cysteine reactive molecule PEG-MAL (polyethylene glycol maleimide, 5 kDa) from coupling with the same cysteine residue as shown in Figure 5.2 B. PEG-MAL alone was able to efficiently label tBID R84C and induce a detectable gel shift due to the
large MW of PEG-MAL (Figure 5.2 B, lane 2). The strong reduction of PEG labeling (lane 3, Figure 5.2 B) confirmed B4M labeling of cysteines.

Figure 5.2 Validation of tBID single cysteine variants and B4M labeling efficiency

(A) Membrane fractions from Bak−/−Bax−/− MEFs expressing human wt BAK were directly challenged with increasing doses of tBID wt or its single cysteine variant R84C and I83C. Supernatant (S) and pellet (P) fractions were separated and subject to SDS-PAGE for cytochrome c detection. Data is representative of two independent experiments. (B) Full-length recombinant tBID R84C was either labeled with 1 mM B4M in solution or 0.5 mM PEG-MAL. The B4M labeled sample was subsequently treated with 0.5 mM PEG-MAL to check cysteine occupancy by B4M. Data representative of two independent experiments.
B4M-conjugated tBID variants were added to activate wt BAK on isolated mitochondria from Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs (re-expressing hBAK) for cross-linking. Due to the transient nature of the tBID:BAK interaction, conditions to trap the interaction were first tested. From our previous studies, tBID at 20 nM was able to induce full BAK dimerization after 30 min incubation at 30 °C (Dewson et al., 2009). The first attempt was to UV irradiate isolated mitochondria at several time points after addition of tBID in order to trap the tBID:BAK interaction (Figure 5.3 A). Western blotting for BID indicated that the B4M induced the cross-linking of BID to other membrane proteins however seemingly not to BAK, as no 1X tBID:BAK (predicted MW of 39 kDa) nor BAK cross-linked to multiple tBID was detected by co-migration (Figure 5.3 B). Noticeably, the cross-linking pattern of tBID R84C closely resembled the R84C/G94E negative control. Hence, UV irradiation introduced at the specific time points downstream of BID addition failed to capture the tBID:BAK interaction on MOM in these trials.
Figure 5.3 B4M cross-linking tBID R84C to BAK at mitochondria

(A) Schematic of B4M cross-linking tBID to BAK at various time points. B4M conjugated tBID was added to activate membrane BAK and subjected to UV radiation during BAK activation to induce tBID:BAK cross-linking. (B) The fixed time points approach. Full-length recombinant tBID R84C and R84C/G94E control was labeled with 1 mM B4M in solution before adding to membrane fractions from Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs expressing human wt BAK. tBID:BAK activation was allowed for indicated time periods (5, 10, 20 and 30 min) before UV radiation (1 min).
was introduced. After UV radiation, each sample was solubilized in 1% digitonin prior to reducing SDS-PAGE analysis in duplicates. Data representative of two independent experiments. (C) The “Pulsed” cross-linking/accumulation approach. Full-length recombinant tBID R84C was labeled with/without 1 mM B4M in solution before adding to membrane fractions from Bak^-/- Bax^-/- MEFs expressing human wt BAK. Only one UV irradiation (1 min) was introduced either at the beginning (0 min, lane 5) or the end of BAK activation (30 min, lane 1,2,3,4 and 6). Lane 7 (Pulsing) was subject to pulsed (5 second) UV irradiation every minute throughout the experiment (30 min). Samples were analyzed on reducing SDS-PAGE in duplicate. Data representative of three independent experiments.

Next, UV irradiation was “pulsed” for 5 seconds every minute during the 30-min reaction at the beginning of tBID addition to accumulate any cross-linked tBID:BAK (Figure 5.3 C). This strategy originated from the hypothesis that each dose of UV irradiation might cross-link a small amount of tBID: BAK, thus pulsing UV could achieve accumulation of cross-linked tBID:BAK over time. Unfortunately, no difference in cross-linking between pulsed UV and UV 1 min (lane 6 and 7) at fixed time point (30 min after tBID:BAK incubation) was observed (Figure 5.3 C). B4M conjugated tBID R84C was largely cross-linked to other membrane proteins, with a similar banding pattern observed in lane 3 in mitochondria with or without BAK (Figure 5.3 C, lane 3, 6 and 7).
Figure 5.4 B4M cross-linking tBID I83C to BAK at mitochondria

Full-length recombinant tBID I83C and control I83C/G94E was labeled with 1 mM B4M in solution before adding to membrane fractions from Bak^-/-Bax^-/- MEFs expressing human wt BAK or not. UV irradiation (1 min) was introduced at indicated time points post BAK activation. Samples were solubilized in 1% digitonin prior to reducing SDS-PAGE analysis in duplicate. Note the I83C/G94E migrated slightly differently to its true MW weight on SDS-PAGE, similar to observed in R84C/G94E in Figure 5.3, caused by the charge mutation. Data representative of three independent experiments.
It was possible that the orientation of B4M on tBID R84C was not optimal for cross-linking. Hence, I tested another functional tBID variant I83C (Figure 5.2 A), as this was found to interact with the BAX hydrophobic groove (h0)(Czabotar et al., 2013), and was tested using B4M cross-linking to BAK at mitochondria, similar to Figure 5.3 B of tBID R84C. Shown in Figure 5.4, the altered B4M orientation indeed generated a different cross-linking pattern (Figure 5.4, lower panel blots, anti-BID). However, none of the observed higher molecular bands were tBID cross-linked to BAK as the BAK immunoblot revealed no immunoreactive cross-links, and the cross-linking pattern from the BID immunoblot was very similar to the Bak/Bax DKO control.

5.2.2 Mapping the tBID transient interaction with BAK at mitochondria using the chemical cross-linker DMTMM.

As the B4M approach failed to trap any tBID:BAK specific interaction, an alternative unbiased cross-linking coupling reagent DMTMM (4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) (Figure 5.5 A) was next tested. Based on published studies, DMTMM is designed for zero-length unbiased cross-linking between acidic and basic residues at a neutral pH (Figure 5.5 B)(Leitner et al., 2014). In contrast to B4M, DMTMM does not require the additional UV activation step(Leitner et al., 2014). This advantage of having DMTMM present at the time of tBID and BAK interaction might increase the chance of capturing any transient interactions, thus bypassing the difficulty of trapping the interaction at the
right time. One potential challenge of this approach is the limited number of primary amines present in BAK (2 lysines with 1 in the TMD tail), although carboxylic residues are more abundant (Figure 1.9).

For this study, wt tBID was added to mitochondria isolated from either Bak/Bax DKO or DKO re-expressing wt BAK to induce BAK activation. DMTMM with optimized condition for cross-linking based on a published study was added at the beginning (during, lanes 3, Figure 5.5 C) or after tBID-induced BAK activation (post, lanes 4, Figure 5.5 C)(Leitner et al., 2014). DMTMM induced potential tBID and BAK cross-linking on MOM, as indicated by an ~40 kDa band. It was tempting to speculate that the ~40 kDa band co-migrated in both immunoreactive blots to BAK and BID was the cross-linked tBID:BAK product. However, as the band persisted at the same abundance after full BAK activation, when tBID is expected to have disassociated from BAK (BAK would be almost fully dimerized after 30 min of 20 nM tBID activation(Dewson et al., 2009)), refuted this possibility. In addition, this band was also detected in Bak/Bax DKO controls.

Other commonly used chemical cross-linkers such as formaldehyde, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide + N-hydroxysuccinimide and disuccinimidyl suberate were also tested for tBID and BAK cross-linking. Unfortunately, none of these cross-linkers tested effectively trapped tBID:BAK interactions on MOM in conditions tested (Appendices Figure 9.2).
Figure 5.5 DMTMM cross-linking tBID to BAK at mitochondria

(A) Chemical structure of cross-linker DMTMM. (B) Schematic of DMTMM mediated zero length unbiased cross-linking between acidic and basic residues (C) Full-length recombinant wt tBID was added to membrane fractions from Bak⁻/⁻Bax⁻/⁻ MEFs expressing human wt BAK or not. Cross-linker DMTMM was mixed with samples either in the beginning of the reaction (During) or at the end of BAK activation (Post). Samples were run in duplicate on reducing SDS-PAGE. Data representative of three independent experiments.
5.2.3 Mapping the tBID transient interaction with BAK at mitochondria using targeted chemical cross-linking

As the unbiased cross-linking approach tested above had proven to be challenging, I attempted a more targeted approach using cysteine guided cross-linking between a single cysteine bearing tBID and a panel of BAK single cysteine mutants. This targeted approach has proven to be powerful in trapping BAX and BAX interactions at MOM (Dewson et al., 2008; 2009; 2012). Given tBID I83C and R84C were both functional in activating mitochondrial BAK, I hypothesized that the major barrier to linkage study was the transient “hit and run” nature of the tBID:BAK interaction on MOM. Hence, to preclude the downstream disassociation of tBID:BAK, a semi-cytosolic BAK variant, BAK/BAXCS, was tested in pilot experiments by our collaborator Dr Colin Hockings to validate the cross-linking approach (Figure 5.6 B, D and F). BAK/BAXCS was described as a BAK mutant with its C-terminal residues swapped with those of BAX, resulting in its semi-cytosolic localization (Ferrer, Frederick, Gulbis, Dewson, & Kluck, 2012). BAK/BAXCS was shown to be able to translocate to MOM and was functional (Ferrer et al., 2012). Importantly, similar to wt BAK on MOM, BAK/BAXCS is able to undergo certain conformation changes as indicated by the increased Ab-1 IP (BAK N-terminal exposure specific) after BH3-only protein activation in the cytosol (Figure 5.6 B), suggesting interactions with tBID can occur in the cytosol. However, BAKBAX/CS failed to form homodimers or higher order oligomers in the cytosol in response to tBID activation (Ferrer et al., 2012).
One could predict that this incomplete conformation change and failure to homo-oligomerize would lead to a stable interaction of BAK/BAXCS with tBID in solution (Figure 5.6 A). Indeed, cross-linking between the tBID BH3 motif variants and BAK/BAXCS surface hydrophobic groove variants in a pilot trial (Figure 5.6 D). Cytosolic fractions from Bak/Bax DKO MEFs re-expressing BAK/BAXCS surface hydrophobic groove single cysteine variants were treated with tBID BH3 motif single cysteine variants or wt tBID (cysteine null). The oxidizing agent CuPhe was added 15 min post BAK/BAXCS activation to induce intermolecular disulfide formation (Figure 5.6 C). As shown in Figure 5.6 D, following BAK immunoprecipitation, cross-linked tBID: BAK/BAXCS based on co-migration was readily detectable in BAK groove residue K113C and H99C samples treated with tBID R84C and I83C, but not in the cysteine null wt tBID control. Hence, this result indicated that CuPhe-induced targeted cysteine cross-linking could capture the tBID:BAK/BAXCS interaction and, consistent with the findings in Chapter 4, constraining BAK activating conformation change could stabilize its interactions with tBID.

Having established this proof-of-principal with BAK surface hydrophobic groove linkage, I tested whether linkage could occur at other sites on BAK, including the “rear site” in BAK/BAXCS. Interestingly, both tBID I83C and R84C readily cross-linked to BAK α6 residues tested R156C, D160C and H164C (Figure 5.6 E). This suggested the close proximity between the BAK/BAXCS “rear site” and tBID. In addition, the observed linkage efficiency between I83C and R84C tBID was reversed when comparing the linkage to the surface hydrophobic groove and the rear site, suggesting the nature of interactions might be different at these sites.
Figure 5.6 Cysteine guided cross-linking between BAK/BAXCS and tBID BH3 motif variants
(A) Schematic of impaired “hit and run” model of interaction between BAK/BAXCS and tBID. BAK/BAXCS might have intrinsically stable interaction with tBID due to its disabled dimerization. (B) Comparing activation conformation change of BAK/BAXCS to full-length wt BAK at mitochondria. Cytosolic fractions of Bak−/−Bax−/− MEFs re-expressing human BAK/BAXCS and membrane fractions from Bak−/−Bax−/− MEFs re-expressing human wt BAK was challenged with full-length recombinant activator tBID, tBIM and sensitizer tBAD. Samples were then immunoprecipitated with BAK antibody 7D10 to capture all BAK or conformation specific antibody Ab-1 to capture N-terminal exposed BAK. Data representative of two independent experiments. (C) Schematic of CuPhe induced cysteine cross-linking using BAK/BAXCS. Cytosolic fractions of Bak−/−Bax−/− MEFs re-expressing human BAK/BAXCS were isolated from permeabilized cells before incubated with 100 nM full-length recombinant tBID single cysteine variant for 15 min before CuPhe treatment for intramolecular disulfide formation. Samples were then immunoprecipitated with BAK 7D10 antibody and run on non-reducing SDS-PAGE. (D) tBID (wt, R84C and I83C) was tested for cross-linking to BAK/BAXCS groove variant H99C and K113C as described in (C). Note the different Westernblotting pattern of anti-BID to (E) was caused by different anti-HA antibody (Covance in (C) to Roche (E)). (E) tBID (wt, R84C and I83C) were tested for cross-linking to BAK/BAXCS α6 variant R156C, D160C and H164C as described in (C). (F) tBID (wt, R84C and R84C/G94E) were tested for cross-linking to BAK/BAXCS surface hydrophobic groove reside H99C and α6 variant H164C as described in (C). Data representative of two independent experiments.
To support the specificity of this cross-linking, I included the negative control, tBID/G94E. tBID G94E has reduced binding to BCL-2 and inducing cytochrome c release as the charge mutation would likely to oppose the hydrophobic interaction required for BAK/BAX surface hydrophobic groove binding (K. Wang et al., 1996; Wei et al., 2000). As predicted, tBID R84C/G94E did not cross-link to cytosolic BAK/BAXCS surface hydrophobic groove (lane 5 to 6), neither to the BAK/BAXCS rear site α6 (lane 8 to 9), consistent with the reported G94E loss-of-function (LOF) (Figure 5.6 F) (Wei et al., 2000).

After validating this cross-linking approach using cytosolic BAK/BAXCS, I moved on to test the targeted disulfide cross-linking of these tBID BH3 motif variants to full-length BAK on MOM as described in the work flow (Figure 5.7 A). Mitochondria fractions were isolated from Bak/Bax DKO MEFS expressing BAK single cysteine variants. tBID variants were added to the mitochondria sample to activate BAK for 15 min before the addition of CuPhe for cysteine cross-linking. In contrast to BAK/BAXCS in cytosol, neither wt tBID or tBID R84C cross-linked to any BAK variants tested but they both triggered efficient BAK dimerization (Figure 5.7 C, BAK immunoblots, ~50 kDa band). Consistent with its LOF, tBID R84C/G94E did not induce any BAK homodimerization. For example, in wt BAK, almost all BAK remained inactive and migrated as an intermolecular constrained monomer compared to tBID wt and R84C treated samples (Figure 5.7 C, BAK immunoblot lane 3 compare to lane 1 and 2). No tBID:BAK cross-linking was detected for all tBID variants tested at BAK α4/5 loop residue N124C, supporting the specificity of the linkage detected at BAK hydrophobic groove and the rear site.
In contrast to BAK/BAXCS, tBID R84C did not cross-link to the hydrophobic groove H99C or the rear site α6 R156C of full-length BAK on MOM (Figure 5.7 C). This is because that tBID already disassociated from activated BAK on MOM, indicated by the presence of BAK homodimers (Figure 5.7 C), and my data showed that tBID disassociated upstream of BAK homodimerization (Chapter 4). Surprisingly, cross-linked tBID:BAK was readily detected between tBID R84C/G94E and BAK hydrophobic groove residue H99C as well as the rear site α6 R156C (Figure 5.7 C, arrows), but not N124C. The result suggested that tBID interacts with BAK at both BAK surface hydrophobic groove and its rear site containing the α6 helix on MOM (Figure 5.7 B). This also highlighted the importance of a membrane environment to the activity of BCL-2 proteins. The tBID G94E, by examining the BID: BAK BH3-in-groove structure (Czabotar et al., 2013; Moldoveanu et al., 2013), could still retain some of its ability to interact with the BAK hydrophobic groove as the critical hydrophobic resides were still present.
Figure 5.7 Cysteine guided cross-linking tBID to BAK surface groove and rear site at mitochondria

(A) Schematic of CuPhe induced cysteine cross-linking between tBID and BAK at mitochondria. Membrane fraction of Bak−/−Bax−/− MEFs expressing human BAK was obtained from permeabilized cells before incubation with 100 nM full-length recombinant tBID single cysteine variants for 15 min before CuPhe treatment for intramolecular disulfide formation. Samples were then immunoprecipitated with BAK 7D10 antibody and run on non-reducing SDS-PAGE. (B) Model of cross-linked tBID variants to BAK surface hydrophobic groove (left) and to BAK rear site (right). Each residue as labeled. Model simulation was created with Pymol software using inactive human BAK: BID structure (PDB: 2M5B). BAK α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of protein in grey. BID BH3 motif in purple. (C) tBID variants (wt, R84C and R84C/G94E) were tested as in (A) for cross-linking to BAK groove variant H99C and α6 variant R156C compared to controls. Data representative of three independent experiments.
To validate the observed tBID:BAK cross-linking was a genuine protein: protein interaction rather than a non-specific linkage, a competition assay was performed in which mitochondria of Bak/Bax DKO re-expressing BAK H99C or R156C were pre-treated with cysteine null tBID ΔCys/G94E (unable to be cross-linked by CuPhe) at equal or in 3-fold excess compared to the later tBID R84C/G94E added (Figure 5.8 A). I predicted in the case of a genuine protein: protein interaction that tBID G94E should compete for the same interface against tBID R84C/G94E, and so reduce linkage of tBID R84C/G94E to BAK. Consistent with this hypothesis, the co-migrating bands representing the cross-linked tBID:BAK were significantly reduced with increasing concentration of tBID ΔCys/G94E at both BAK hydrophobic groove residue H99C as well as the rear site α6 R156C (Figure 5.8 B, lanes 3 to 1, lanes 6 to 4), supporting a genuine protein: protein interaction interface at both sites.
Figure 5.8 Validation of cross-linked tBID to BAK surface hydrophobic groove and α6 rear site at mitochondria

(A) Schematic of the competition assay. Membrane fractions of Bak<sup>−/−</sup> Bax<sup>−/−</sup> MEFs re-expressing human BAK single cysteine variants were isolated and first incubated with tBIDΔCys/G94E at 100 nM (1:1) or 300 nM (3:1) to the later added full-length recombinant tBID R84C/G94E. CuPhe was added for intramolecular disulfide cross-linking. Samples were immunoprecipitated with BAK 7D10 antibody and run on non-reducing SDS-PAGE. (B) tBID R84C/G94E cross-linking to BAK groove variant H99C and α6 variant R156C were detected in decrease in the presence of tBID ΔCys/G94E competition. Data representative of two independent experiments.
5.2.4 Analysis of tBID:BAK interactions on MOM by functional screening

The induced proximity of tBID and BAK at the BAK surface hydrophobic groove and the rear site by CuPhe cross-linking did not necessarily indicate a functional role in BAK activation. Thus, I developed an independent approach to functionally interrogate important interface(s) for BAK activation. As illustrated in Figure 5.9, single cysteines were engineered to cover all BAK helices. These BAK cysteine mutants were then stably re-expressed in Bak/Bax DKO MEFs and validated previously by death assay (Westphal et al., 2014). Isolated mitochondria were labeled with the obstructive, monofunctional, cysteine-reactive 5 kDa PEG-maleimide. I predicted that this labeling would interfere with protein: protein interactions. Full-length tBID was added to activate BAK on MOM and subsequent cytochrome c release was detected on SDS-PAGE. Impaired/inhibited cytochrome c release indicated that the labeled interface was important for BAK apoptotic activity.
Figure 5.9 Schematic of the PEG-MAL labeling workflow

Membrane fractions of Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs re-expressing human BAK single cysteine variants were isolated and incubated with the 5 kDa PEG-MAL (red triangle, chemical structure as indicated) for cysteine labeling. After quenching, PEG-MAL labeled BAK was then activated with full-length recombinant tBID to induce the cytochrome c release. Impaired cytochrome c release would indicate the labeled interface is critical for BAK function as it could be important for BAK activation, homodimerization or its oligomerization.
PEG-MAL was first tested to label exposed BAK cysteines efficiently on isolated mitochondria at 0.5 mM for 30 min with the labeling retarding the migration of BAK on SDS-PAGE with a corresponding ~5 kDa molecular weight increase per label (Figure 5.10 A). Shown in Figure 5.10 B and C, PEG-MAL labeling efficiency reflected the surface exposure of the cysteine that was consistent with the known structure of inactive BAK (Moldoveanu et al., 2006). For example, M71 faces towards the core of the inactive BAK and therefore the cysteine introduced at this position was resistant to PEG-MAL labeling (Figure 5.10 B and C). The PEG-MAL labeling profile of wt BAK suggested that both endogenous cysteines (C14 and C166) were accessible to the label thus the observed 2X PEG-MAL band. However, the presence of the 1X PEG-MAL labeled species (~30 kDa band) even at 10 mM PEG-MAL suggested that one of the cysteines might be partially buried in the membrane compared to the other, or that a population of wt BAK interacted with other membrane proteins and therefore masking this cysteine from labeling (Figure 5.10 A).
Figure 5.10 Establishing the PEG-MAL labeling of BAK on isolated mitochondria

(A) Time and dose response of PEG-MAL labeling on isolated mitochondria. Membrane fractions of Bak\textsuperscript{−/−}Bax\textsuperscript{−/−} MEFs re-expressing human BAK wt or Cys null control were isolated and labeled with increasing dose of PEG-MAL as indicated over 30 min (upper panel). 0.5 mM PEG-MAL labeling BAK on MOM over different time periods (lower panel). Samples were run on reducing SDS-PAGE. Data representative of three independent experiments. (B) Structural representation of inactive BAK and residue C166 and M71 as labeled. (PDB: 2IMT. BAK α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of protein in grey). (C) PEG-MAL labeling efficiency depended on residue exposure. Membrane fractions of Bak\textsuperscript{−/−}Bax\textsuperscript{−/−} MEFs re-expressing human BAK variants were isolated and labeled with PEG-MAL. Samples were run on reducing SDS-PAGE. Data representative of three independent experiments.
5.2.5 Labeling BAK surface hydrophobic groove with PEG-MAL blocked tBID-induced cytochrome c release

After optimizing the conditions for PEG-MAL labeling, an initial screen was carried out on a panel of BAK single cysteine mutants spanning BAK α2-6 helices following activation with 100 nM tBID. Among the residues tested, only PEG-MAL labeling at BAK Y89 blocked cytochrome c release (Figure 5.11 A). The screen was then expanded to incorporate 41 BAK single cysteine mutants as summarized in Figure 5.11 B. PEG-MAL labeling did not induce cytochrome c release in any of the positions tested (Figure 5.11 B and appendix). Residues that were at least 50% labeled on SDS-PAGE were classified as “labeled” in the study, and these labeled residues largely reflected the surface exposure of these positions in the structure of inactive BAK. Residues that were buried towards the core of the protein (α4 and 5) were poorly labeled (Appendices Figure 9.4)

Of the 41 residues tested, only the surface hydrophobic groove residue BAK Y89 blocked cytochrome c release after PEG-MAL labeling, consistent with the known importance of BAK surface hydrophobic groove for its activation. None of the other positions tested, including in BAK α6, impaired cytochrome c release at 100 nM tBID activation.
Figure 5.11 Functional screening of BAK single cysteine variants by PEG-MAL labeling on isolated mitochondria with high dose of tBID

(A) The initial screen of BAK single cysteine variants. Mitochondria fractions of BAK single cysteines variants were isolated as previously described and pre-labeled with/without PEG-MAL before challenged with 100 nM full-length recombinant tBID. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection. Data representative of at least two independent experiments. (B) Summary of BAK residues tested in the screening. Black triangle indicates residues that were resistant to label (<50% protein was labeled on SDS-PAGE). Blue triangle indicates residues that were labeled but did not impair cytochrome c release in response to 100 nM tBID activation. Red triangle indicates residue that blocked cytochrome c release at 100 nM tBID activation. Samples were run on reducing SDS-PAGE. Data representative of at least two independent experiments.
5.2.6 Labeling BAK α6 with PEG-MAL impairs cytochrome c release induced by limiting dose of tBID

Results of the functional screening supported the BAK surface hydrophobic groove as important for BAK activity, consistent with the targeted cross-linking data (Figure 5.7). However, it also suggested the BAK “rear site” did not have a role in BAK function despite an ability to cross-link to tBID (Figure 5.7 and 5.8). Given the linear chemical structure and flexible nature of PEG-MAL (Figure 5.9), it is possible that the PEG-MAL label might be displaced by excessive (100 nM) tBID used in the first screen. To test this, I repeated the PEG-MAL screen again on the panel of BAK single cysteine variants in response to a threshold concentration of tBID. Titration of tBID indicated that 1 nM tBID was sufficient to induce full cytochrome c release (Figure 5.12 A), thus the PEG-MAL screen was repeated at 1 nM tBID activation. As expected, PEG-MAL labeling Y89C again blocked BAK activity with 1 nM tBID (Figure 5.12 B). Consistent with the previous screen at 100 nM, labeling at most BAK positions did not impair BAK function in response to the limiting 1 nM tBID activation (Figure 5.12 D and Appendices Figure 9.5). However, labeling at several residues in the BAK α6 (R165C, D160C and H164C) impaired cytochrome c release (Figure 5.12 B and C). This finding suggests a functional role for BAK α6 in its activity, consistent with the previous targeted tBID:BAK cross-linking observations (Figure 5.7 and 5.8).

The inhibition of labeling BAK α6 was only observed at lowered concentration of tBID. This suggests the tBID-induced BAK activation was the limiting step rather than downstream BAK homodimerization because this inhibitory effect can be overcome by the addition of more tBID. Hence, I tested if labeling at multiple positions on BAK α6 would
have a more profound inhibitory effect. To this end, two cysteines at BAK α6 (D160C and H164C) were simultaneously labeled with PEG-MAL and were found to completely abolish cytochrome c release even when induced by 100 nM tBID, compared to unlabeled controls (Figure 5. 12 D).

In contrast to the endogenous cysteines in wt BAK, the D160C/H164C double cysteines were either fully (2x) labeled or completely unlabeled (Figure 5. 12 D). The endogenous cysteine C166 which resides closely to D160 in the structure of inactive BAK (Figure 5.10 B) suggests that distinct populations of BAK may exist at mitochondria with different accessibility to their α6 helix, potentially due to complexing with the VDAC2 or other mitochondrial proteins.

One interesting observation during the second PEG-MAL labeling screen with limiting dose of tBID was the lack of inhibition on other BAK surface hydrophobic groove residues tested (for example H99C, Y110C, K113C, S117C, H141C and H145C). The likely explanation is the linear flexible chemical structure of PEG-MAL might be displaced by in coming tBID. With limiting options of other commercial substitutes, I had tested dual label K113C/I114C in the BAK hydrophobic groove. Unfortunately, the K113C/I114C was not able to be stably expressed in cells (data not shown).
Figure 5.12 Functional screening of BAK single cysteine variants by PEG-MAL on isolated mitochondria with limiting dose of tBID

(A) tBID titration on wt BAK at mitochondria. Mitochondria fractions of BAK wt were isolated as previously described and activated with increasing dose of full-length recombinant tBID. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection. Data representative of two independent experiments. (B) Pre-label PEG-MAL at BAK groove but also at α6 inhibited cytochrome c release at 1 nM tBID activation. Data representative of three independent experiments. (C) Structural representation of inactive BAK and residue Y89C, R156C, D160C and H164C as labeled. (PDB: 2IMT. BAK α1 and 6 in orange, α2 in red and surface groove α3, 4 and 5 in green, the rest of protein in grey.). (D) Dual labeling of PEG-MAL at hBAK D160C/H164C completely blocked cytochrome c release even at high dose (100 nM) tBID activation. PEG-MAL label was performed as previously described. Mild heat (43°C) was used as a positive control for BAK function. BAK D160C/H164C was activated with low (5 nM) and high (100 nM) dose of tBID. (E) Updated summary of BAK residues tested in the screening. Black triangle indicates residues that were resistant to label (<50% protein was labeled).
on SDS-PAGE). Blue triangle indicates residues that were labeled but did not impair cytochrome c release in response to tBID activation. Red triangle indicates residue that blocked cytochrome c release at 100 nM tBID activation. Yellow triangle indicates residue that blocked cytochrome c release at 1 nM tBID activation. Samples were run on reducing SDS-PAGE. Data representative of at least two independent experiments.

To further test if the observed inhibitory effect of PEG-MAL labeling of the BAK rear site was limited to tBID, BAK rear site cysteine variants were PEG-MAL labeled and challenged with recombinant tBID chimeras with the BH3 motif derived from either BIM (tBIM) or PUMA (tPUMA) developed by our collaborator Dr Colin Hockings (Hockings et al., 2015). Interestingly, both tBIM and tPUMA-induced cytochrome c release were blocked by PEG-MAL labeling at the BAK rear site (BAK R156C, D160C/H164C) (Figure 5. 13), suggesting that the BAK rear site could be important for a broad class of BH3-only protein activation.
Figure 5.13 PEG-MAL pre-labeled BAK rear site cysteine variants at isolated mitochondria also impaired BIM and PUMA-induced BAK activation

Mitochondria fractions of BAK R156C (A) and D160C/H164C (B) were isolated and PEG-MAL labeled as described before. Samples were activated with 1 nM of full-length recombinant tBID, tBIM and tPUMA. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection. Data representative of two independent experiments.
5.2.7 Labeling BAK α6 with PEG-MAL impairs BAK activation.

PEG-MAL labeling could interfere with any steps BAK activity in apoptosis. These steps include BAK activation, homodimerization as well as oligomerization (Figure 5.9). To understand how PEG-MAL labeling impairs tBID-induced BAK activity, I performed blue native (BN)-PAGE analysis in combination with antibody gel-shift assay using a BAK conformation-specific antibody (aa23-32) to investigate BAK conformation change upon activation. As introduced before, the BAK N-terminal becomes exposed during activation so antibodies recognizing BAK N-terminal epitopes can bind and gel-shift the activated BAK. At steady state, BAK is detected in two distinct populations on BN-PAGE gel (Figure 5.14): ~66 kDa monomeric BAK and ~480 kDa in association with the VDAC2 complex (Lazarou et al., 2010; Ma et al., 2014). The high molecular weight VDAC2: BAK complex disassociates upon apoptotic activation and BAK homodimers are then detected ~146 kDa (lane 2 in Figure 5.14). As expected, unlabeled BAK only gel-shifted with the antibody after activation with tBID (Figure 5.14, compare lanes 4 with 5). In addition, PEG-MAL labeling of BAK ΔCys (cysteine null) did not affect BAK homodimerization (compare lane 2 and 3) or antibody gel-shift (Figure 5.14 compare lanes 5 and 6). BAK G4C was tested as a negative control, in which we could observe BAK homodimers with 2X PEG-MAL labeled (lane 3) and the PEG-MAL labeling did not impair antibody gel-shifting of activated BAK dimers (compare lane 2, 3, 4 and 5). Labeling BAK Y89C with PEG-MAL to obscure the hydrophobic groove abolished the formation of BAK homodimers, indicated by the absence of 2X PEG-MAL labeled BAK homodimers and the accumulation of 1X PEG-MAL labeled BAK monomer (lane 2 and 3). Antibody gel-shift of 1X PEG-MAL
labeled BAK Y89C monomer was impaired but not completely blocked (compare lanes 5 and 6). Accumulation of 1X PEG-MAL monomers was also detected in the rear site residues BAK R156C and H164C (lanes 3), suggesting that BAK homodimerization is inhibited by labeling the rear site. Consistent with this, antibody gel shift of PEG-MAL labeled BAK R156C and H164C monomers was also reduced, but again not completely blocked. Together, this data indicates that blocking the hydrophobic groove could impair BAK activation conformation change as well as its homodimerization, whereas labeling the BAK α6 rear site compromised BAK activation conformation change. However, 2X PEG-MAL labeled BAK homodimers of BAK R156C and H164C were also detected (Figure 5. 14 B). This observation indicates that labeling of BAK α6 did not obscure BAK homodimerization, which is consistent with the BH3-in-groove model of BAK dimer formation(Dewson et al., 2008; 2012).
Figure 5.14 BN-PAGE analysis revealed impaired BAK activation upon PEG-MAL labeling

(A) and (B) Gel shift assay of PEG-MAL labeled BAK variants at 100 nM and 1 nM tBID activation. Membrane fractions of Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs re-expressing human BAK Y89C, Cys null, G4C, R156C or H164C were isolated and labeled with PEG-MAL before activation with 100 nM (A middle) or 1 nM (A right and B) tBID. BAK antibody aa 23-38 (N-terminal specific) was added to indicated sample for gel shifting activated BAK. Samples were run on BN-PAGE after solubilization. Data representative of two independent experiments.
5.2.8 BAK surface groove transiently opens during its activation

Given the ability of PEG-MAL to label exposed but not buried residues, I used this approach to monitor BAK transformation during its activation. BAK M71C that was resistant to PEG-MAL label before activation (Figure 5.10), became exposed to label during tBID-induced activation (lane 3), consistent with the exposure of the BAK BH3 motif during BAK activation conformation change (Dewson et al., 2008). Comparing the inactive BAK monomer (Moldoveanu et al., 2006) and the BID BH3 bound BAK structures (Moldoveanu et al., 2013), an interesting feature was that the BAK surface hydrophobic groove adopted a more open conformation when occupied by a BID BH3 peptide. As a consequence, BAK α5 helix residue A130 became exposed (Figure 5.15 B, structure c and d). Previously, I found residues clustered at the core α5 helix, including A130, were poorly labeled in inactive BAK (summary Figure 5.12). BAK A130C was then tested for PEG-MAL labeling in the presence of tBID activation (Figure 5.15 A). Similar to M71C, BAK A130C became significantly labeled by PEG-MAL when added during tBID-induced activation, but was again restricted after tBID activation (Figure 5.15 A lower, compare lane 2, 3 and 4). The PEG-MAL labeling of A130C was consistent with the structural prediction that the BAK surface hydrophobic groove opens to expose the α5 helix at the base of the groove during tBID-induced activation, which then becomes less accessible after BAK BH3-in-groove homodimerization (Figure 5.15 B).
Figure 5.15 PEG-MAL labeling revealed BAK groove opening during activation

(A) PEG-MAL labeling of BAK residue M71 and A130 revealed BAK activation conformation change. Membrane fractions of Bak−/−Bax−/− MEFs expressing human BAK M71C (upper) and A130C (lower) were isolated and labeled with PEG-MAL before (Pre), during (During) and after (Post) 30 min tBID activation as indicated in the figure. Samples were run on reducing SDS-PAGE. Data representative of three independent experiments. (B) Structural comparison of A130C exposure in inactive BAK monomer (a and b) (PDB: 2IMT) and BAK: BID BH3 NMR complex (c and d) (PDB:2M5B). Inactive BAK monomer cartoon (a) and surface (b) representation, with Y89 and A130 as labeled. Note: A130 was observed to not surface exposed in (b). Surface representation of BAK: BID BH3 NMR complex (c) and with BID BH3 removed (d) revealed the exposure of A130 (red). (α1 and 6 in orange, surface groove in green, BID BH3 in purple.). (C) PEG-MAL labeling of BAK rear site residues R156C and H164C inhibited the labeling of A130C. Membrane
fractions of $Bak^{−/−}Bax^{−/−}$ MEFs expressing human BAK A130C and A130C/R156C and A130C/H164C were isolated and labeled with PEG-MAL in the presence/absence of increasing dose of tBID as indicated in the figure. Mild heat was introduced as a positive control as described before. Samples were run on reducing SDS-PAGE. Data representative of two independent experiments.
5.2.9 BID interaction with BAK α6 promotes the opening of the BAK surface hydrophobic groove

The observed opening of the BAK surface hydrophobic groove could be induced by BID BH3 binding, or it could be a prerequisite for BID BH3 binding to the BAK hydrophobic groove. I hypothesized that tBID interaction to the BAK rear site could promote BAK surface hydrophobic groove opening and allow its subsequent engagement with tBID. To test this hypothesis, I engineered BAK double cysteine variants A130C/R156C and A130C/H164C. As introduced in Chapter 1, mild heat was shown to trigger BAK activation and induce cytochrome c release independent of BH3-only protein actions (Pagliari et al., 2005). Hence, mild heat treatment was used as a positive control in this study (Figure 5.15 C, in lanes 5) to show all cysteines engineered can be labeled during heat-induced BAK activation. In BAK A130C single cysteine variant, PEG-MAL was only able to label the cysteine in the presence of tBID, not before tBID addition, consistent with previous results (Figure 5.15 C). The PEG-MAL labeling efficiency of A130C alone increased with increasing dose of tBID. In both of the BAK groove/rear site double cysteine variants (A130C/R156C and A130C/H164C), PEG-MAL labeling revealed predominantly 1X labeled BAK, consistent with the poor labeling of A130C but efficient PEG-MAL labeling to BAK R156C and H164C single cysteine variants (Figure 5.12 and 5.15).

If the BAK hydrophobic groove opening was induced by tBID binding to the canonical groove, then the A130C residue should become efficiently labeled in the presence of tBID to produce a 2X PEG-MAL labeled species, and the PEG-MAL labeled species should increase with increasing dose of tBID. However, very little 2X PEG-MAL labeled BAK A130C/R156C and
A130C/H164C were detected event at 10 nM tBID activation. This suggests that the PEG-MAL labeling on the BAK rear site prevented the opening of the surface hydrophobic groove, implicating the surface groove opening happens after tBID interaction with BAK rear site. These results further indicate that the BAK rear site as a trigger site for BAK activation that it allosterically regulates the surface hydrophobic groove opening for BH3-only proteins engagement.

5.2.10 PEG-MAL labeling BAK α6 also inhibits antibody induced BAK activation

Monoclonal 7D10 BAK antibody was discovered to induce human BAK activation by binding to the BAK α1-2 loop region thus inducing BAK conformation change and cytochrome c release (Iyer et al., 2016). Given that the critical BAK rear activation site covers regions spanning helices α1 and 6, I tested if PEG-MAL labeling on the BAK rear site could inhibit 7D10-induced BAK activation. Interestingly, PEG-MAL labeling at BAK rear site α6 R156C inhibited 7D10-induced cytochrome c release (Figure 5.16 A, upper blot). PEG-MAL labeling did not impair 7D10 binding to BAK as confirmed by efficient immunoprecipitation of PEG-MAL labeled BAK (Figure 5.16 A, lower blot). It is currently unclear how the 5 kDa PEG-MAL label on BAK α6 could impair the antibody’s function on α1. It is possible that PEG-MAL labeling on α6 could allosterically increase the stability of the rear site that in turn helps in the stabilization of α1 even in the presence of 7D10 binding.
Figure 5.16 Manipulation of BAK rear site inhibited BAK function

(A) PEG-MAL labeling of BAK rear site R156C inhibited 7D10 induced BAK activation. Membrane fractions of Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs re-expressing human BAK R156C were isolated and treated with/without PEG-MAL before 7D10 activation as indicated. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection (upper). Pellet fraction of lanes 2 and 4 in the upper figure were also solubilized and immunoprecipitated with anti-rat secondary antibody to check 7D10 binding of BAK (lower). Data representative of two independent experiments. (B) Structural representation of H43 and M60 position around the BAK rear site (PDB: 2IMT) (α1 and 6 in orange, α2 in red, surface groove in green, rest in grey.). (C) CuPhe constraining BAK α1 movement impaired BAK activation. Membrane fractions of Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs re-expressing human BAK H43C/M60C were isolated and treated without/with CuPhe to induce intramolecular disulfide activated with an increasing dose of tBID as indicated in the figure. Mild heat was introduced as a positive control as described before. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection Samples were run on non-reducing SDS-PAGE to check intramolecular disulfide formation. Data representative of two independent experiments.
5.2.11  **Constraining BAK α1 movement impairs BAK activation.**

To test the hypothesis that stabilizing the BAK rear site could allosterically dampen BAK activation, I trialed if a cysteine disulfide constrain of BAK H43C:M60C (α1 to α2) (Figure 5.16 B)(Iyer et al., 2016), could prevent BAK α1 disassociation and hence BAK activation. Oxidizing agent CuPhe was used to induce intramolecular disulfide bond between BAK H43C:M60C. Intramolecular disulfide bond formation was confirmed by the fast migration of CuPhe treated sample on non-reducing SDS-PAGE, compared to untreated BAK control (Figure 5.16 C). High dose of tBID (100 nM) and mild heat could induce efficient cytochrome c release regardless of intramolecular disulfide. This was consistent with PEG-MAL labeling could only block cytochrome c release at 1 nM tBID but not 100 nM (Figure 5.16 C). At 1 nM tBID, cytochrome c was efficiently released by the unconstrained BAK H43C:M60C variant, but greatly reduced after BAK intramolecular tether. Although not definitive, this suggests the stabilization of BAK rear site inhibits its apoptotic activity. Together with the R156C PEG label inhibition of 7D10 result, future experiments will be required to further test this hypothesis.

5.2.12  **In the search of BAK α6 loss-of-function mutants.**

A missing piece of evidence that would consolidate the rear site as a functionally important interface for BAK function was a loss-of-function (LOF) mutant at the rear site. On the contrary, a number of BAK LOF mutants have already been reported in its surface hydrophobic groove (H.
Based on the PEG-MAL labeling results, I tested if a triple serine mutation at BAK R156S/D160S/H164S was sufficient to make BAK refractory to tBID activation. hBAK R165S/D160S/H164S was stably expressed in Bak/Bax DKO MEFs. Cell death and mitochondrial cytochrome c release assays were performed on two independent clones #1 and #2 (Figure 5.17). As shown, both clones of hBAK R165S/D160S/H164S MEFs died efficiently in response to increasing dose of Actinomycin-D (ActD) and cytochrome c release was also comparable to wt BAK in response to increasing dose of tBID, indicating that hBAK R165S/D160S/H164S retained normal function.
Figure 5.17 Testing BAK rear site R156S/D160S/H164S mutant

(A) Death assay of BAK rear site triple serine mutant. Cultured Bak<sup>-/-</sup>Bax<sup>-/-</sup> MEFs re-expressing human BAK wt, R156C, H164C and independent clones of BAK R156S/D160S/H164S (clone 1 and 2) were treated with increasing dose of ActD for 24 hrs before FACS analysis of death cells by PI incorporation. BAK expression was confirmed by western blot of whole cell lysate. Error bars represented standard error of the mean. Data representative of two independent experiments. (B) Cytochrome c release assay of BAK rear site triple serine mutant. Membrane fractions of Bak<sup>-/-</sup>Bax<sup>-/-</sup> MEFs expressing human BAK wt and two clones of BAK R156S/D160S/H164S (clone 1 and 2) were treated with increasing dose of tBID. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for cytochrome c detection. Data representative of two independent experiments.
5.2.13 Potential BAK rear site mutants in cancer.

Of interest, Catalogue of somatic mutations in cancer (COSMIC) search revealed frequent recorded mutations in BAK from various cancers (Figure 5.18 A). As expected, the BAK surface hydrophobic groove harvests a cluster of mutations. However, two BAK rear site mutations BAK R156S and D160Y that coincide with the same positions in my previous PEG-MAL labeling screening caught my attention (Figure 5.18 A). To test the function of these BAK mutations, I cloned BAK R156S and D160Y and tested their function by cytochrome c release assay. Both variants induced cytochrome c release similarly to wt BAK in response to a titration of tBID (Figure 5.18 B). The results indicated the R156S or D160Y mutations on their own were unlikely to impair BAK activity. However, it is possible that cancer cells accumulate very subtle hypomorphic mutations in BAK that provide them with a selective advantage over time.
Figure 5.18 Testing BAK rear site mutations based on COSMIC entry

(A) COSMIC search result of a histogram on registered BAK single amino acid substitution mutations across BAK from a variety of cancer patients. (B) Cytochrome c release assay of BAK rear site mutants. Membrane fractions of Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs expressing human BAK wt and two BAK cancer mutants R156S and D160Y were isolated and treated with increasing doses of tBID. Supernatant (S) and membrane/pellet fractions (P) were separated for Cytochrome c detection. Data representative of three independent experiments.
5.3 Discussion

In this chapter, I aimed to tackle a major question in the field as to how BAK activation was initiated by an activator BH3-only protein such as tBID. To this end, it was important to understand the interactions between the two proteins importantly at mitochondria and to identify the interfaces required for their interaction.

The development of unbiased chemical cross-linking coupled with mass-spectrometry analysis has been instrumental in deriving structural information of protein: protein interactions and protein complexes that are not accessible by conventional structural approaches such as crystallography and NMR (Leitner et al., 2016; C. Yu & Huang, 2018). As the transit nature of tBID:BAK interaction can be challenging for crystallography and NMR analysis, chemical cross-linking guided mass-spectrometry analysis becomes an attractive option to map the interfaces between tBID and BAK. However, my attempts to test this with different cross-linking chemistries revealed certain limitations. Although both B4M and DMTMM cross-linkers induced detectable cross-linking of tBID, no linkage to BAK was observed likely due to the abundance of other membrane proteins present in this experimental system. For example, as mentioned in Chapter 1, tBID MOM insertion requires interactions with MTCH2, a mitochondria protein of 31 kDa (Grinberg et al., 2005; Shamas-Din et al., 2013a). This interaction is likely reflected in the DMTMM cross-linked species in the BID-immunoblot in Figure 5.5. Also, amongst the protein interactions occurring at mitochondria, specific tBID:BAK specific interactions might only be of low occurrence, which may therefore preclude its detection by SDS-PAGE. Secondly, the major challenge is still capturing this transient interaction. The majority of chemical cross-linking
guided mass-spectrometry analysis performed to date have been applied to stable protein:protein interactions, and have largely used recombinant proteins.

No reliable cross-linking chemistries that have been reported so far that are suitable for capturing the transient tBID:BAK interactions at MOM. For B4M, the main challenge remains the timing of UV irradiation, as altering the orientation of the B4M from tBID R84 to I83 (Figure 5.4) did not improve tBID cross-linking to BAK. Neither introducing UV light at fixed time points or the pulsing UV during BAK activation was able to capture the tBID:BAK interactions (Figure 5.3 B and C). Although the use of DMTMM avoided the need for UV irradiation, the transient interactions between tBID and BAK still could not be linked with DMTMM. Instead, both tBID and BAK are readily linked to other membrane proteins that are likely rich in acidic or basic residues such as MTCH2 and VDAC proteins (Figure 5.5 C).

Throughout these experiments with unbiased cross-linking, high background has been observed in each trial. In this case, enrichment of BAK or tBID specific interaction would be desired. With the BAK cysteine variants library established in our laboratory, cysteine guided, targeted cross-linking provided a solution for capturing the tBID:BAK interaction. As discussed in Chapter 4, limiting BAK conformation change was able to stabilize its interaction with tBID. This principle has been further supported with the experiments either using BAK/BAXCS that is defective in oligomerization (Figure 5.6) and the hypomorphic tBID G94E that fails in inducing BAK activation conformation change (Figure 5.7). In both cases, tBID cross-linking to BAK surface canonical groove was directly detected, consistent with other reports stating the importance of BH3:
groove interaction for BAK activation (Brouwer et al., 2014; Czabotar et al., 2013; Dewson et al., 2008; 2012). In addition, tBID interacting with BAK rear site residue on helix 6 was also observed. These results are the first line of evidence to suggest full-length tBID interacts with BAK at two distinct sites on mitochondria. This newly discovered BAK rear site is mapped on to helix 6, potentially consistent to the reported BAX rear pocket trigger site (α1 and α6) (Gavathiotis et al., 2008), suggesting a conserved mechanism for both BAK and BAX activation.

A concern with cysteine guided cross-linking is that the induced proximity may not necessarily imply functional importance. To complement my cross-linking approach, I developed a novel PEG-MAL labeling based functional screening approach to investigate interfaces that are important for BAK apoptotic activity. The obstructive PEG-MAL labeling is established as a versatile tool to study protein: protein interaction (Figure 5.11 and 5.12) and also to infer protein conformation change (Figure 5.14 and 5.15).

My data revealed PEG-MAL labeling at BAK rear site blocked BAK apoptotic function in response to limiting dose of tBID, but not when tBID was in excess, suggesting the rear site inhibition observed is on BAK activation rather than downstream oligomerization. BN-PAGE analysis of PEG-MAL labeling on rear site residue R156C and H164C confirmed impaired BAK activation alone (Figure 5.14). The results confirmed the functional importance of the BAK rear site for BAK activation, supporting the cysteine cross-linking mapping results.

The PEG-MAL labeling screen at high concentration of tBID activation first revealed that the BAK surface hydrophobic groove is absolutely
critical for tBID-induced BAK activation (Figure 5.11 and 5.12). Consistent with the structure of inactive BAK (Figure 5.15 C) (Moldoveanu et al., 2006), Y89 was proposed as a gatekeeper for the BAK surface groove that its side chain partially occluded the BAK surface groove (Moldoveanu et al., 2006; Pang et al., 2012). This had led the authors to propose that a necessary step in BAK activation would be to open the hydrophobic groove for BH3-only protein binding, although the mechanism for this conformation change was unknown. I now show the interactions between tBID and BAK rear site is likely the driving force for the BAK hydrophobic groove opening, implicating this rear site interaction a prerequisite step in BAK activation. My results contradict the previous Leshchiner et al. study that questioned the existence of a BAK rear site by studying the BAX-like mutant form of BAK (Leshchiner et al., 2013). Unlike cytosolic BAX that has its α9 tail tucked into its surface hydrophobic groove, the mutant BAK with a BAX-like α9 tail auto-translocates to membrane independent of BH3-only protein stimulation, suggesting a difference in protein conformation. In addition, the cross-linking of stapled BH3-only protein peptide to the mutant BAK was detected 10 min after BAK activation (also followed by a 2 hr UV irradiation to induce cross-linking), a time when efficient liposome permeabilization was observed in their system (Figure 3 C (Leshchiner et al., 2013)). This suggests detected observed cross-linking might be downstream of BAK homodimerization. Further experiments are required to explain the differences between our studies.

To further understand the interactions of tBID:BAK rear site, PEG-MAL was adapted to monitor BAK conformation change. As mentioned above in 5.2.7, a careful review of tBID BH3-in-BAK groove structure prompted the hypothesis that the rear activation site was required to induce BAK
surface hydrophobic groove opening to accommodate tBID. This was supported with PEG-MAL labeling of BAK rear site inhibiting the PEG-MAL labeling of BAK hydrophobic groove residue A130 during tBID-induced BAK activation (Figure 5.15 C). This result suggests that the BAK rear site activation is a prerequisite step prior to the BH3: canonical groove interaction, thus implying a conserved mechanism of BAK and BAX activation. The original proposal of BAX rear pocket trigger site ($\alpha_1$ and $\alpha_6$) was to allow the release of BAX TMD tail and promote its mitochondria insertion (Gavathiotis et al., 2008). Now my data would suggest both BAK and BAX rear site are inducing their surface hydrophobic groove opening for the incoming tBID activation. The BAX TMD tail release is likely a result of the groove opening or being displaced by the incoming tBID. Hence, I propose a new model of tBID-induced BAK activation at mitochondria (Figure 5.19).

In addition to blocking tBID interaction with the BAK rear site, it is also possible that PEG-MAL labeling on BAK rear site is having a secondary effect of allosterically modulating BAK activity. The evidence that PEG-MAL labeling was able to stop 7D10 antibody-induced BAK activation, in addition to BAK $\alpha_1$ constrain impaired BAK activation, suggested the possibility that allosterically stabilization BAK rear site could impair its activity (Figure 5.16). Further studies are required to investigate the biophysical properties of the BAK rear site.

The discovery of BAK rear site is important to our knowledge of BAK apoptotic activation. In addition, a few small molecules targeting BAX rear site (discussed in detail in Chapter 7) have been reported after the discovery of the BAX rear activation site. The same principle also applies to BAK.
The rear activation site of BAK provide a new interface for the design of pharmacological agents that allow us to directly inhibit or activate BAK.

Unfortunately, my attempts to characterize the COSMIC cancer BAK mutants did not reveal any loss-of-function BAK variants. However, it is important to remember that it may require more than a single substitution to dampen BAK function. This suggests that these cancer related mutations are not contributing to cancer. Cancer cells are likely to harness small hypomorphic mutations that impair BAK activity over time to gain survival advantages. More work need to be done to comprehensively analyze all mutations in BAK rear site in order to understand how these mutations affect BAK activity.
Figure 5.19 A refined model of BAK activation at mitochondria

Full-length tBID activates BAK at mitochondria by first interacting with BAK rear site to induce the opening of BAK surface hydrophobic groove. The BAK open groove is then able to accommodate an incoming tBID that will induce BAK activation conformation change as illustrated.
6 Chapter 6: Understanding the formation of the apoptotic pore

6.1 Introduction

BAK and BAX activation and dimerization are indispensable for MOMP and subsequent cytochrome c release. In previous chapters, I have discussed in detail about BAK and BAX activation and their homodimerization. Little is known in regards to the events that happen between BAK and BAX dimerization and the formation of the apoptotic pore. Therefore, the “holy grail” in the field to understand how BAK/BAX homodimers self-associate to disrupt the MOM. BAK and BAX symmetrical BH3-in-groove homodimerization has highlighted the importance of identifying a putative secondary interface for the multimerization of these BAK and BAX homodimers. Identification of this secondary interface would greatly aid in resolving the structure of the apoptotic pore mediating MOMP, and would provide a valuable target for pharmacological inhibition of cell death.

6.2 Results

6.2.1 Using heat as a BID-independent activation to characterize the BAK multimerization interface by PEG-MAL functional screen

In Chapter 5 the PEG-MAL labeling screen of important BAK interfaces was performed with tBID-induced activation. Using a BH3-only protein independent stimulus of mild heat (43 °C)(Pagliari et al., 2005), the PEG-
MAL labeling approach could be adapted to interrogate interfaces important for down-stream BAK oligomerization. Mild heat activates BAK and induces its dimerization, similarly to tBID-induced activation. Therefore, any inhibition of cytochrome c release would infer an interface important for BAK multimerization or interaction with the MOM (Figure 6.1 A). Hence, the PEG-MAL screen was repeated on the same BAK single cysteine mutants tested in Chapter 5 with 100 nM tBID or mild heat treatment. As expected, and consistent with activation by tBID, PEG-MAL labeling of BAK Y89C blocked BAK dimer formation hence inhibiting cytochrome c release induced by either tBID or heat activation, emphasizing the importance of the BAK surface groove for homodimerization (Figure 6.1 B).
Figure 6.1 Labeling the periphery of BAK homodimers did not impair BAK function

(A) Schematic of PEG-MAL screen with heat induced BAK activation, dimerization and oligomerization. Heat bypassed the requirement of an activation interface, therefore any blockade by PEG-MAL would be due to the interference of multimerization. (B) Example of the PEG-MAL screen. Membrane fractions from Bak<sup>−/−</sup>Bax<sup>−/−</sup> MEFs expressing human wt BAK or its single cysteine variants were pre-labeled with/without PEG-MAL.
PEG-MAL as previously described. Then 100 nM tBID or mild heat treatment was applied for BAK activation. Supernatant (S) and pellet (P) fractions were separated and subject to SDS-PAGE for cytochrome c detection. Data representative of two independent experiments. (C) Schematic summary of PEG-MAL screened residues on BAK homodimer (top view, left) (side view, right) (PDB: 4U2V. BAK α2 in red and surface groove α3, 4 and 5 in green, residues that labeled but showed no inhibition in blue and Y89 in purple stick).

Although our previous data suggested that BAK α6 could serve as a potential multimerization interface (Dewson et al., 2008; 2009), PEG-MAL labeling of BAK α6 single cysteine variants, as well as dual labeled BAK D160C/H164C, did not inhibit heat-induced cytochrome c release (Figure 6.1 B and Figure 5.12 D), questioning the role of BAK α6 as a functional interface required for BAK oligomerization. In fact, other than BAK Y89, PEG-MAL labeling at no positions on the BAK homodimers impaired heat-induced cytochrome c release (Figure 6.1 C and Appendices Figure 9.5). With all the positions tested mapped onto BAK homodimer structure (Figure 6.1 C), almost the entire periphery of the BAK dimer was seemingly not functionally important for oligomerization assessed by functional mapping with PEG-MAL labeling.
6.2.2 Monitoring BAK conformation change with PEG-MAL revealed the structural rearrangements post BAK dimerization

The structural snapshots of activated BAK shown in Chapter 1 are limited to truncated recombinant protein in the absence of a membrane environment. Information of activated BAK conformation on mitochondria is lacking. In Chapter 5, PEG-MAL was shown to be a useful tool to monitor protein conformation change. As we were interested in characterizing BAK conformation change during the course of its activation. BN-PAGE analysis was also carried out by comparing PEG-MAL labeling pre, during and post BAK activation (Figure 6.2). Consistent with the data in Chapter 5, labeling wt BAK prior to activation did not prevent BAK dimerization and the labeled BAK formed dimers that were predominantly 4X PEG-MAL labeled (Figure 6.2, lane 3 and 4). In lane 7, PEG-MAL labeling during BAK activation revealed similar labeling profile to pre-activation labeling. Interestingly, in lane 8, PEG-MAL labeling post-BAK activation, there was a significant reduction of the 4X PEG-MAL labeled species and an increase in less labeled species (Figure 6.2). This result suggests that either the cysteine in the BAK N-terminus (C14) or its α6 (C166) become buried after BAK dimerization in a protein:protein interface or in membrane.

BN-PAGE revealed that pre-labeling of the BAK groove residue Y89C prevented homodimerization in response to heat as well as in response to tBID. Interestingly, BAK dimerization was inhibited even when PEG-MAL was added during tBID activation (Figure 6.3, lane 7). When PEG-MAL labeling was performed post tBID activation, unlabeled BAK Y89C dimers were the predominant species, indicating that Y89C becomes re-
buried in the BAK homodimer, consistent with previous published dimer structure (Figure 6.1 and 6.2). A130C, which was poorly labeled prior to activation detected by SDS-PAGE (Figure 5.15), had an increased PEG-MAL labeled species on BN-PAGE (Figure 6.2 lane 7 compared to lane 5) consistent with its induced exposure during activation (Figure 5.15). Similar to Y89C, the post-activation PEG-MAL labeling profile of A130C was consistent with the BAK homodimer structure (Brouwer et al., 2014) that A130 was buried therefore no PEG-labeled BAK A130C dimer was observed. Interestingly, PEG-MAL labeled BAK A130C was not able to form homodimers, consistent with the BAK surface hydrophobic groove being critical for homodimerization (Figure 6.2, lane 5 and 6).
Figure 6.2 Using PEG-MAL labeling monitoring BAK activation conformation change

Membrane fractions from Bak^-/Bax^- MEFs expressing human BAK variants were isolated and either pre-treated with PEG-MAL (pre) then activated with 100nM tBID or mild heat for 30 min, or adding PEG-
MAL together with tBID (during) or after (post) BAK activation. Samples were then solubilized and ran on BN-PAGE. Data representative of two independent experiments.

6.3 Discussion

The secondary oligomerization interface for BAK/BAX homodimers to self-associate and permeabilize the MOM is the holy grail of apoptosis research. Specifically, the nature of the apoptotic pore was previously thought to be proteinaceous. However, emerging evidence has challenged this model and instead a proteolipidic pore structure has been proposed (Basanez et al., 1999; 2002; Basanez, Soane, & Hardwick, 2012; Terrones et al., 2004).

Under the assumption of a proteinaceous apoptotic pore, we had previously reported the BAK α6 as a multimerization interface for BAK homodimers based on cysteine linkage analysis (Dewson et al., 2009), in addition to other reported BAK helices such as α3, α5 and α9 (Aluvila et al., 2014; Brouwer et al., 2014; Iyer et al., 2015). No functional assessment has been performed on these proclaimed interfaces to give a definitive answer on their role in BAK apoptotic pore formation.

In this study, PEG-MAL labeling once again proved to be a versatile tool as it enabled a comprehensive screen for the functionally relevant BAK oligomerization interface(s) (Figure 6.1A). From this screen, PEG-MAL labeling BAK Y89C inhibited heat-induced cytochrome c release due to the blockade of BAK homodimerization (Figure 6.1 and 6.2), consistent
with the BAK surface hydrophobic groove being critical for dimerization as well as highlighting the requirement for BAK homodimerization for MOMP. No other residues, including the reported BAK α3, α5, α6 and α9 helices, surrounding the BAK dimer periphery impaired heat-induced cytochrome c release upon PEG-MAL labeling, questioning the existence of a stable protein:protein interface for BAK oligomerization. This observation is contradictory to the proteinaceous apoptotic pore model, in which an ordered pore formation requires stable protein:protein interaction. Hence, my results are consistent with the apoptotic pore that mediates MOMP is likely proteolipidic.

As a proof-of-concept, PEG-MAL labeling combined with BN-PAGE analysis has proven to be a power approach to monitor conformation of activated BAK on mitochondria (Figure 6.2). The result of PEG-labeling of wt BAK after activation revealed previously unrecognized conformation change: the reburial of its N-terminus and/or α6 helices due to possible protein:protein or protein:lipid interaction. To fully map the conformation of BAK during and after its activation, more BAK variants covering all its helices will need to be tested using this approach in the future. Again, PEG-MAL labeling of the BAK Y89 and A130 results highlighted the importance of BAK surface groove in its homodimerization, consistent with the BAK homodimer structure (Brouwer et al., 2014).
7 Chapter 7: Discussion

This thesis investigated the series of events during the activation of the pro-apoptotic BCL-2 protein BAK: 1) the molecular interaction of BAK with a non-BCL-2 family protein and apoptosis regulator VDAC2, 2) delineating BAK’s transient interaction with its activator BH3-only protein tBID and 3) investigating the mechanism by which BAK promotes MOMP. This chapter aims to assess the findings in this thesis, discuss their implications and suggest future experiments that can be built upon.

7.1 Targeting the VDAC2:BAK interaction to inhibit cell death

As an emerging concept in the apoptosis field, non-BCL2 apoptotic regulator proteins have been under the spotlight for their ability to impact cell fate (Mark Xiang Li, 2015). VDAC2 was initially proposed to be a BAK inhibitor by Cheng et al. (Cheng, 2003), but only recently has been shown to be necessary for BAK (Naghdi et al., 2015; Roy et al., 2009) and especially for BAX (Ma et al., 2014)(bioRxiv DOI: 10.1101/266668) mediated cell death. Hence, how VDAC2 differentially impacts the structurally and functionally similar BAK and BAX is a major question in the field.

To answer this question, we need to understand the molecular interactions between VDAC2 and BAK/BAX. In Chapter 3, my results focused on the identification and characterization of the VDAC2:BAK interface. Although the BAK TMD was previously suggested to interact with VDAC2 (Lazarou et al., 2010), cysteine disulfide linkage studies performed
on 3 BAK TMD residues I188C, N190C and G196C revealed no detectable linkage to either VDAC2 Q166C or M167C, which are adjacent to the reported critical VDAC2 motif required for BAK membrane import (Naghdi et al., 2015). My results suggest that the BAK TMD, although is important for BAK membrane import, does not interact directly with VDAC2 or alternatively, the BAK residues tested here were not optimal for linkage study (Figure 3.3). To further investigate the BAK TMD interaction with VDAC2, a panel of single cysteine variants covering BAK TMD residues 188-205 should be tested in order to conclusively rule out a role for the BAK TMD in interacting with VDAC2.

The interesting observation from the functional PEG-MAL screen (Chapter 5) that certain BAK cysteine variants disassociate from MOM upon PEG-MAL labeling leading to the mapping of a novel interface on BAK that interacts with VDAC2. Based on the residues tested, disassociated BAK residues were clustered around the BAK surface hydrophobic groove (α3, α4-5 loop, Figure 3.4 A), this observation is broadly consistent with Cheng et al. who reported that mutations in BAK BH1 and BH3 motif significantly reduced the BAK:VDAC2 complex (Cheng, 2003). I confirmed that the PEG-MAL labeling induced disassociation was due to the disruption of BAK:VDAC2 interaction by co-immunoprecipitation. Of the residues tested in my proof-of principle study, only previous disassociated BAK variants (R88 and N124), not the controls (G4 and R156), selectively lost the PEG-MAL labeled form of BAK in the VDAC2 IP, whereas the unlabeled BAK was still detected (Figure 3.4 C), supporting that PEG-MAL labeling at BAK surface hydrophobic groove specifically disrupted the BAK:VDAC2 interaction. This result is the first evidence of the region on BAK that is important for its interaction with VDAC2. The PEG-MAL labeling and BAK:VDAC2
co-immunoprecipitation will be extended to other BAK single cysteine variants to complete and confirm the mapped BAK interface.

Naghdi et al. published their study during my PhD on the identification of a VDAC2 motif required for BAK mitochondria import (Naghdi et al., 2015). This study prompted me to further characterize the interface on VDAC2 that interacts with BAK. VDAC2 A172C was engineered just after the reported $^{168}$TFDS$^{171}$ VDAC2 motif, with the aim of testing its ability to link with cysteines in BAK. Interestingly, VDAC2 A172C appeared to stabilize interaction with BAK at mitochondria, indicated by altered BAK:VDAC2 membrane complex distribution (Figure 3.1 A). The VDAC2 A172C:BAK complex was more resistant to tBID-induced BAK disassociation (Figure 3.1 B). More importantly, this stabilized VDAC2:BAK interaction seemed to impair BAKs ability in mediating MOMP (Figure 3.1 C) as well as impair BAK-mediated cell death in cells (Figure 3.1 D). My results provided the first genetic evidence that stabilized BAK:VDAC2 indeed inhibits BAK apoptotic activity, consistent with Cheng et al.’s proposal that VDAC2 is an inhibitor of BAK apoptotic function (Cheng, 2003). In addition, VDAC2 A172C has been proven to be a valuable tool in characterizing the BAK:VDAC2 interface. Cysteine disulfide linkage using VDAC2 A172C and BAK R88C/N124C yielded specific cross-linked BAK:VDAC2 on mitochondria, further supporting the close proximity of both BAK and VDAC2 interface identified in my study (Figure 3.5 A and B). A recent report of a modified BIM peptide that forms a stable complex with BAK, in contrast to the natural BIM peptide that activates BAK in the “hit and run” fashion, inhibited BAK activation by binding to BAK surface hydrophobic groove with very high affinity (Brouwer et al., 2017). In this study, binding of both the natural and the modified BIM to BAK surface hydrophobic groove disrupted the
BAK:VDAC2 complex at mitochondria, consistent with my hypothesis that BAK surface hydrophobic groove is interfacing with VDAC2 and suggesting the disruption of VDAC2 interaction by BH3-only protein binding is an early step involved in BAK activation.

Future studies will be built on these data to use VDAC2 A172C’s advantage in stabilized BAK interaction to allow crystallography to characterize the BAK:VDAC2 interaction, and perhaps enable the analysis of the BAK:VDAC2 high molecular weight membrane complex. As an alternative approach to characterize the BAK:VDAC2 interface, a collaborator Dr Christoph Grohmann has synthesised new bi-functional cysteine-photo reactive cross-linkers to allow unbiased chemical cross-linking and mass spectrometry analysis. The aim is to conjugate the cross-linker through the thiol-reactive moiety on BAK single cysteine variant R88C and in parallel VDAC2 A172C and subsequently use the UV reactive moiety of the linker to map residues in proximity in an unbiased fashion. In addition, in collaboration with Dr Mark van Delft, our data suggested that the similar region of VDAC2 (β-strands 6-10, containing the $^{168}$TFDS$^{171}$ VDAC2 motif) is also important for its interaction with BAX, indicating a conserved mechanism. BAX equivalent residues to those on BAK that disassociate from MOM upon PEG-MAL treatment will be made on a mitochondrial membrane-associated BAX variant BAX S184L to test if similar residues on BAX mediate its interaction with VDAC2.

Taken from the PEG-MAL functional mapping data (Figure 5.12), disrupting the BAK:VDAC2 interaction did not seem to majorly impact MOMP (e.g. PEG-MAL labeled R88C could still induce efficient cytochrome c release). In contrast, stabilizing the BAK:VDAC2
interaction impaired cell death (Figure 3.1). My results suggest that small molecules that can enhance or stabilize the BAK:VDAC2 interaction can become promising inhibitors for apoptosis. Such compounds would represent valuable research tool for further investigations in apoptosis, but also potential reagents to limit pathological apoptosis in cardiac ischaemic injuries and Parkinson diseases where inhibitors targeting downstream caspases have failed (O'Brien & Dixit, 2001).

7.2 Refining the stepwise BAK conformation change during BH3-only protein-induced activation

In contrast to most previous studies that have employed BH3 motif peptides, truncated or mutated BAK in solution or conjugated to artificial membrane systems, my study focused on the molecular interactions of full-length proteins resident on the mitochondria. I determined that full-length tBID and BAK could interact at mitochondria outer membrane and the resulting conformation change of BAK induces its activation as well as the disassociation of tBID (Chapter 4). Guided by our knowledge of BAK structural rearrangements, I was interested to understand the interaction between different BAK conformers with its activator tBID. With the help of intramolecular cysteine linkage, I was able to trap BAK at a specific conformation and more importantly prove that preventing BAK conformation change can stabilize its interaction with tBID on the MOM to generate snap-shots of their interaction during the course of BAK activation. These findings enable us to order the different known BAK conformation changes and generate a refined model of BAK activation as shown in Figure 4.5. My data suggest that it is the activation-induced conformation change that promotes BH3-only protein dissociation rather
than the competition by another activated BAK during BAK homooligomerization, and this dissociation of BH3-only protein happens upstream of BAK core-latch separation.

7.3 Further understanding the mechanism of BH3-only protein-induced BAK activation

In Chapter 5, I aimed to investigate the mechanism by which BH3-only proteins directly induce BAK activation on mitochondria with a range of approaches including cross-linking based protein proximity mapping and a novel functional screening method. The PEG-MAL screening method I developed is a novel and powerful approach to functionally identify interfaces required for BAK activity. The PEG-MAL labeling method has provided a simple, but versatile platform that can be coupled with different experimental stimuli to monitor protein conformation change as well as interpreting protein-protein interaction. Thus, the PEG-MAL approach is adaptable to the biochemical analysis of other membrane proteins. In my study, the PEG-MAL screening provided an important functional complement to targeted linkage analysis, revealing that obstructing the BAK canonical surface hydrophobic groove as well as the rear site of BAK α6 helix abolished cytochrome c release even in response to very high dose of tBID activation. This evidence strongly suggests the involvement of a BAK rear site composing of α6 in its activation (Figure 5.12). PEG-MAL labeling on BAK α6 also inhibited BAK activation by recombinant chimeric BIM and PUMA proteins, suggesting a conserved role of BAK α6 in BH3-only proteins-induced BAK activation (Figure 5.13). The transient, potentially low affinity interaction with BAK α6 may explain the
difficulty in resolving this interaction by conventional structural biology approaches.

To uncover the underlying mechanism of the BAK rear activation site, BN-PAGE based gel shift assay was performed on BAK groove (Y89) and α6 (R156, D160 and H164) residues. Results indicated PEG-MAL labeling at BAK groove completely abolished BAK homodimerization as well as impaired BAK activation, whereas the impact of PEG-MAL labeling on BAK α6 is limited to compromised BAK activating conformation change (Figure 5.14). Careful comparison between known BAK structures in the absence and presence of BH3 binding revealed the activation-induced BAK α5 exposure, consistent with my experimental data of increased PEG-MAL labeling of BAK α5 residue A130 during tBID activation (Figure 5.15 A and B). My hypothesis of BAK α6 rear site acting as the “on-off” switch controlling BAK surface groove opening is supported by the experiment where pre-labeling BAK α6 rear site inhibited the tBID-induced PEG-MAL labeling on BAK α5 residue A130C (Figure 5.15 C).

My discovery of the rear activation site for BAK aligns with the previously reported rear activation site on BAX(Gavathiotis et al., 2008; 2010). The BAX rear site was discovered through an NMR study with stapled-BH3 peptide and it was proposed that an allosteric switch to encourage BAX membrane translocation by releasing the self-quenched BAX tail from its surface hydrophobic groove(Gavathiotis et al., 2008; 2010). However, a report later from the same group suggests that BAK does not have a rear site, raising the possibility that the rear site interaction was unique to BAX for inducing BAX mitochondria targeting, but was unnecessary for mitochondrial resident BAK(Leshchiner et al., 2013). As discussed previously (Chapter 5), the study involved a soluble mutant BAK in
solution that might not represent BAK at the MOM. In addition, the studies on BAK and BAX retro-translocation support a conserved mechanism for BAK and BAX MOM targeting. My studies of full-length BAK expressed in cells now directly resolve the conflict regarding the existent of a BAK rear site and its important role in regulating BAK apoptotic activity. Taken together, the data supports an important role of both BAK and BAX α6 as a binding site for BH3-only proteins to trigger the activation of BAK and BAX to induce the opening of their surface hydrophobic groove for the incoming BH3-only protein activation. Further experiments need to be focused on discovering the BAK and BAX loss-of-function mutation in the rear activation site with comprehensive alanine scanning.

The implications of the discovery of the BAK and BAX rear activation site extends into the development of small molecules that can potentiating cell death. After the identification of the BAX rear site, in silico screening yielded the first gain-of-function BCL-2 protein modulator BAX activator molecule 7 (BAM7) that selectively activates BAX to induce apoptosis (Gavathiotis et al., 2012). The later optimized lead compound, BTSA1 (BAX trigger site activator 1) is a potent, selective activator of BAX rear site and is able to induce BAX-dependent killing of cancer cells in primary AML (acute myeloid leukaemia) cells and xenografts in vivo (Reyna et al., 2017). Such development supports that further characterization of the BAK rear site could provide a platform for the rational design of molecules that can directly and selectively induce BAK dependent killing.

Therapies targeting ubiquitously expressed BCL-2 proteins, in this case BAK and BAX, have not been extensively explored due to the possible side effects of killing healthy cells. However, in contrast to healthy cells,
many cancer cells are already primed for apoptosis primed state (Certo et al., 2006). The success of ABT-199/Venetoclax in the treatment of relapsed or refractory CLL provides solid support for targeting BCL-2 family proteins as a cancer therapy (Roberts et al., 2016). BTSA1 targeting BAX also suggested specificity to leukemic cells (Pritz et al., 2017). In the same vein, it is reasonable to predict small molecules that directly target BAK will be useful in a combined anti-cancer regime.

One interesting observation regarding rear site allosteric regulation in MCL-1 has been recently reported (Lee et al., 2016). A compound called MAIM1 (MCL-1 allosteric inhibitor molecule 1) was designed to covalently target an exposed cysteine C286 on the α6 helix of MCL-1 and subsequently allosterically disrupts MCL-1 canonical binding to BH3-only proteins (Lee et al., 2016). Mutagenesis of MCL-1 C286W recapitulated such a phenomenon in vitro and in cellular studies, hence opening an avenue of allosteric targeting pro-survival BCL-2 proteins at a rear site in addition to the surface groove binding with current BH3-mimetic compounds. Although data supporting the allosteric rear site regulation in other multi-domain BCL-2 proteins is lacking, I speculate that the rear site allosteric regulation may be a conserved mechanism for all BCL-2 proteins given their structural similarity.

7.4 Toward understanding the apoptotic pore formation

How BAK and BAX oligomerize and promote MOMP has been an ongoing debate. My PEG-MAL labeling screening has confirmed the functional importance of the BAK canonical groove as a critical interface
for its homodimerization. In contrast to previous reports suggesting BAK helices such as α3, 5 and 6 contributing to its oligomerization interface (Aluvila et al., 2014; Brouwer et al., 2014; Dewson et al., 2009), no other regions labeled with PEG-MAL impaired heat-induced BAK activation (Figure 6.1), question the existence of a stable protein:protein interface for BAK oligomerization.

This functional assessment of BAK multimerization interfaces has extended our understanding of the formation of the apoptotic pore. Firstly, PEG-MAL labeling inhibits BAK homodimerization at BAK surface groove (Y89) (Figure 6.2) as well as BAK mediated cytochrome c release in the absence of BAK homodimers (Figure 6.1). This supports that BAK homodimerization is required for MOMP and it also refutes the hypothesis that membrane-integrated BAK or BAX monomers are sufficient to assemble and perturb MOM integrity (Kuwana, Olson, Kiosses, Peters, & Newmeyer, 2016; X.-P. Xu et al., 2013). Secondly, Basanez et al. using patch-clamp analysis first reported that BAX created variable changes on membrane permeability, instead of forming a tightly packed proteinaceous pore with reproducible conductance levels (Basanez et al., 1999). They then proposed BAX permeabilize the MOM by promoting positive monolayer curvature stress to form lipidic-pore type structures (Basanez et al., 1999; 2002; Terrones et al., 2004). The absence of a proteinaceous pore formed by stable protein:protein interfaces is consistent with my observation of the absence of a PEG-MAL obstructed oligomerization interface between BAK homodimers, favouring that BAK apoptotic pore is also of proteolipidic. However, the limitation of my study is the lack of the mechanistic analysis of MOMP. Hence, I have not been able to differentiate if the MOMP is caused by the in-plane insertion model (Brouwer et al., 2014) or the proposed clamp model (Figure 1.
Preventing cell death can be extremely beneficial against excitotoxicity that leads to acute neuronal degeneration after stroke, traumatic brain injury and epileptic seizures (Engel, Plesnila, Prehn, & Henshall, 2011). Excitotoxicity has been demonstrated to be mediated, at least partially, by BAX (D'Orsi et al., 2012). BAK, despite its controversial role in neuronal apoptosis during development, has been found to promote neuronal cell death after stroke (Fannjiang et al., 2003). Inhibition of BAK and BAX mediated MOMP would be useful in these settings. A few small molecules have been introduced to modulate BAX channel to inhibit cytochrome c release and therefore prevent neuronal cell death (Bombrun et al., 2003; Hetz et al., 2005; Polster et al., 2003). However, the potency of such molecules is only moderate when used at micromolar concentration and no direct evidence has been shown that they act by inhibiting BAK and BAX oligomerization. Little is known about the mechanistic action of such compounds. The recent report of BAK/BAX oligomer antagonist iMAC falls along similar lines (Peixoto et al., 2017). Such events highlight the importance of understanding BAK/BAX oligomerization and its impact on rational design of anti-apoptotic inhibitors. An interesting recent study from Prof David Andrew’s group reported the discovery of a number of small molecule inhibitors of both BAK and BAX (Niu et al., 2017). Such compounds prevented neuronal death predominantly via direct inhibition of BAK and BAX dimerization (Niu et al., 2017). This study provides a first line of evidence that blocking BAK and BAX mediated MOMP can prevent pathological cell death. Additionally, recent reports also suggest small molecule inhibitors of succinate dehydrogenase somewhat impair
MOMP downstream of BAK/BAX oligomerization, previously considered the point of no return in apoptosis (X. Jiang et al., 2016; L. Li et al., 2017).

8 Concluding remarks

Results presented in this thesis have revealed the interface between a novel non-BCL-2 protein VDAC2 and BAK. They have suggested stabilizing the BAK:VDAC2 interaction can inhibit BAK apoptotic activity, provides an avenue for potential pharmacological manipulation of cell fate. This thesis also presents a novel mechanism of BAK activation via its rear site and offers insights of BAK apoptotic pore formation, both of which are of significance for direct targeting BAK apoptotic activity.
9 Appendices
Figure 9.1 Mapping the BAK:VDAC2 interface with PEG-MAL labeling
(A) Summary of the dissociated BAK residues by PEG-MAL labeling. (Lower) Mitochondria fractions of BAK single cysteine variants indicated were isolated from MEFs and incubated with 0.5 mM PEG-MAL in MELB buffer with protease inhibitor for 30 min. After quenching, samples underwent centrifugation to separate the membrane and supernatant fraction for reducing SDS-PAGE analysis. Data representative of least two independent experiments. (Upper) BAK residues tested were mapped on inactive BAK structure (PDB: 2IMT. BAK surface groove α3, 4 and 5 in green and the rest helices in grey. Residues that dissociate from the MOM are labeled red as indicated. Residues that do not dissociate from the MOM are labeled blue). (B) Co-immunoprecipitation of BAK:VDAC2 after PEG-MAL treated mitochondria membrane samples. PEG-MAL treatment was applied to BAK N124C co-expressed with VDAC2 ΔCys in Bak/Bax/Vdac2 knock out MEFs. 1% w/v digitonin solubilized post-spin membrane samples (input) were immunoprecipitated with anti-HA antibody to IP VDAC2 (IP). Samples were analyzed by reducing SDS-PAGE. Data representative of two independent experiments.
Chemical cross-linkers tested for tBID:BAK linkage. 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide + N-hydroxysuccinimide (EDC+NHS), formaldehyde (FA) and disuccinimidyl suberate (DSS) were tested together with B4M and DMTMM to cross-link equal molar recombinant mouse BAK and full-length tBID in MELB buffer. Samples were analyzed by SDS-PAGE. Data representative of two independent experiments.
### A

**Input (membrane)**

<table>
<thead>
<tr>
<th>BAK</th>
<th>wt</th>
<th>ΔCys</th>
<th>H99C</th>
<th>N124C</th>
<th>R156C</th>
</tr>
</thead>
<tbody>
<tr>
<td>tBID</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: anti-BAK</td>
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<tr>
<td>WB: anti-HA (BID)</td>
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Non-reducing SDS-PAGE

### B

**Input (membrane)**

<table>
<thead>
<tr>
<th>BAK</th>
<th>H99C</th>
<th>R156C</th>
</tr>
</thead>
<tbody>
<tr>
<td>tBID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: anti-BAK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: anti-HA (BID)</td>
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Non-reducing SDS-PAGE
Figure 9.3 Cysteine linkage of tBID to BAK surface hydrophobic groove as well as the rear site

Input fraction of the CuPhe-induced cysteine cross-linking between tBID and BAK at mitochondria (A) and the input fraction of the competition assay (B) Figure 5.8.

Figure 9.4 Summary of the Bak residues that are resistant to PEG-MAL labeling

Summary of BAK residues tested in the PEG-MAL labeling screening that were resistant to label (<50% protein was labeled on SDS-PAGE). Mitochondria fractions of BAK single cysteines variants were isolated as previously described and labeled with/without PEG-MAL. membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE. Data representative of two independent experiments. (Lower) Residues tested were mapped on inactive BAK structure (PDB: 2IMT. BAK surface groove α3, 4 and 5 in green and the rest helices in grey. Residues resistant to PEG-MAL labeling are labeled in teal).
Mitochondria fractions of BAK single cysteines variants were isolated as previously described and pre-labeled with/without PEG-MAL before challenged with 100 nM full-length recombinant tBID and mild heat (upper) or with 1 nM tBID. Supernatant (S) and membrane/pellet fractions (P) were separated and run on reducing SDS-PAGE for

**Figure 9.5 PEG-MAL screening of interfaces required for BAK apoptotic function**
cytochrome $c$ detection. Data representative of at least two independent experiments.


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